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**PROGRAMA DE PÓS-GRADUAÇÃO EM  
CIÊNCIAS BIOLÓGICAS (ZOOLOGIA)**

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**EVOLUÇÃO EM CYCLORAMPHIDAE (ANURA):  
DIVERSIDADE E ESPECIAÇÃO NA MATA ATLÂNTICA BRASILEIRA**

**FÁBIO PERIN DE SÁ**

Tese apresentada ao Instituto de  
Biociências do Campus de Rio Claro,  
Universidade Estadual Paulista, como  
parte dos requisitos para obtenção do  
título de Doutor em Ciências Biológicas  
(Área de Concentração: Zoologia).

**NOVEMBRO - 2019**

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**Orientador:** Dr. Célio Fernando Baptista Haddad

**Coorientadora:** Dra. Kelly Raquel Zamudio

**Rio Claro  
2019**

S111e Sá, Fábio Perin de  
Evolução em Cycloramphidae: Diversidade e especiação na  
Mata Atlântica brasileira / Fábio Perin de Sá. -- Rio Claro, 2019  
186 f. : il., tabs., fotos, mapas

Tese (doutorado) - Universidade Estadual Paulista (Unesp),  
Instituto de Biociências, Rio Claro  
Orientador: Célio Fernando Baptista Haddad  
Coorientadora: Kelly Raquel Zamudio

1. Evolução. 2. Seleção natural. 3. Filogenia. 4. Hibridação.  
5. Anfíbio. I. Título.

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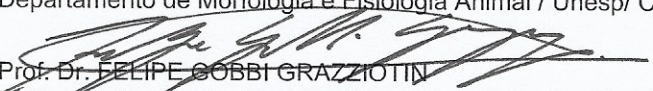
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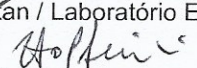
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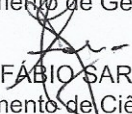
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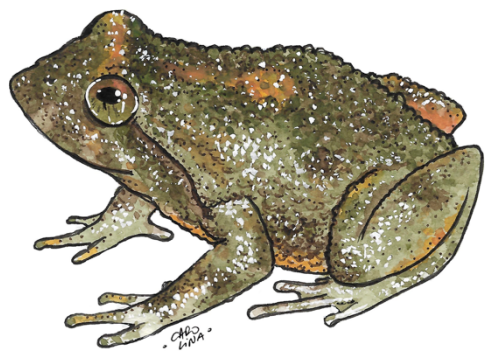
  
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Rio Claro, 08 de novembro de 2019



## **AGRADECIMENTOS**

É imensa a alegria ao chegar nesta etapa de minha jornada. É fácil reconhecer que sozinho não teria conseguido percorrer todo o caminho. Ao escrever neste momento sinto a gratidão pelos momentos e aprendizados, mas sobretudo às pessoas que fizeram meu caminhar muitas vezes mais leve e prazeroso e em muitas outras vezes o fizeram possível. O texto que deixo pode ser lido como um sincero abraço de obrigado.

O professor Célio Haddad e a professora Kelly Zamudio são pessoas inspiradoras, a oportunidade de os ter tido como orientadores ao longo destes anos traz uma grande satisfação. Sou grato por todas as oportunidades que me proporcionaram e por confiarem em mim. Sempre me impulsionando um pouco mais adiante, agradeço aos dois pela compreensão, pelas risadas e pelas discussões sobre ciência e sobre a vida. Sempre acolhedores, é uma alegria os reconhecer como amigos.

Agradeço à minha família, Luzia Perin, Diogo Perin, Rafael Queiroz e Suélen Figueiredo, pois é imensurável o apoio e o amor que recebi de vocês para que esta tese hoje possa estar aqui consolidada. Obrigado por acreditarem em mim, por toda a compreensão e por sempre estarem prontos para conversar. Agradeço também ao meu pai, Silvio de Sá (em memória).

Sou também muito grato a dois amigos que tive o privilégio de reconhecer ao longo do doutorado, Thais Condez e Leo Malagoli. Pela Mata Atlântica, laboratórios e pela vida, a caminhada teve muito mais cores com a companhia de vocês. Quanta alegria e risadas!

Agradeço aos queridos colegas de laboratório da UNESP e de CORNELL pelas contribuições e pela companhia e afeto no dia-a-dia ao longo destes anos: Juliane Monteiro, Caitlin McDonald, Ariadne Sabbag, Délio Baêta, Ana Paula Motta, Maria Akopyan, Megan Barkdull, Carla Lopes, Priscila Lemes, Nicholas Polato, Amanda Santiago, Anyelet Aguilar, Cinnamon Mittan, Bianca Berneck, Marcus Thadeu Santos, Luciana Fusinato, Boris Blotto, Mariana Lyra, Lucas Bandeira, Pedro Taucce, João Giovanelli, Natália Sales, Tereza Thomé, Thiago Carvalho, Ana Carolina Calijorne, Paulo Pinheiro, Kaleb Gatto, Rafael Consolmagno, Olívia Araújo, Danilo Delgado, Francisco Brusquetti e Jordan Garcia. À Miranda Gray, à Nadya

Pupin e ao Steve Bogdanowicz meu especial obrigado pela amizade e pela atenção à tudo que foi preciso pelos laboratórios.

Proporcionando camadas intermináveis de conhecimento, a importância dos parques naturais e dos museus é imensurável. Assim, agradeço aos parques nos quais coletei e aos museus que visitei e, estendendo aos gestores e funcionários, agradeço pelo zelo e profissionalismo. Particularmente agradeço aos curadores e técnicos dos museus que gentilmente me receberam: Taran Grant, José Pombal Junior, Felipe Toledo, Rayna Bell, Sergio Potsch, Manoela Cardoso e Addison Wynn. Agradeço a todos que direta ou indiretamente contribuíram com minhas coletas de dados. Agradeço aos funcionários, aos pós-doutores e aos professores da pós graduação da UNESP de Rio Claro.

Agradeço também a todos os colaboradores que muito contribuíram com os estudos desta tese, em particular: Vanessa Verdade, Miguel Rodrigues, Cinthia Brasileiro, Erika Santana, Gustavo de Gaspari e Pavitra Muralidhar. Agradeço aos professores Harry Greene e Patrícia Morellato pelas conversas e amizade. Sou também grato pela oportunidade que tive em orientar a Andréa de Lourenço, o Caio Pompeu e a Clara Silva, levo comigo aprendizado, amizade e crescimento.

Finalmente, agradeço a todos os anuros, estes animais incríveis que tanto têm me ensinado sobre os processos naturais.

O imprescindível financiamento desta tese foi concedido principalmente pela Fundação de Amparo à Pesquisa no Estado de São Paulo (FAPESP) por meio de bolsa de estudo (#2014/24972-4). O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001. Os estágios no exterior (#2016/06876-3 e #2018/17993-6) assim como a participação em cursos e congressos também foram financiados pelas agencias de fomento citadas.

Muito obrigado!!

**“A alegria não chega apenas no encontro do achado,  
mas faz parte do processo da busca.  
E ensinar e aprender não pode dar-se fora da procura, fora da boniteza e da  
alegria.”**

Paulo Freire

## RESUMO

As forças de seleção natural e sexual atuam sobre os indivíduos e podem os levar ao isolamento reprodutivo. O acúmulo de modificações evolutivas pode levar ao surgimento de novas entidades. Espécie é unidade basal em biologia; assim, com sua exuberante biodiversidade, a Mata Atlântica é uma floresta neotropical fundamental para estudos de processos evolutivos em anfíbios anuros. Reunindo os gêneros *Cycloramphus*, *Thoropa* e *Zachaenus*, a família Cycloramphidae agrupa espécies de anuros saxícolas e terrestres, todas endêmicas da Mata Atlântica brasileira. Pelos seus hábitos especializados de vida e reprodução, os cicloramfídeos formam um grupo atrativo para estudos evolutivos. Na presente tese são apresentados quatro capítulos que abordam três escalas distintas da evolução: a diversificação entre espécies, populações e indivíduos. São abordados: (1) a filogenia molecular multilocus de *Cycloramphus-Zachaenus*, demonstrando que a evolução da terrestrialidade no clado se correlaciona com o dimorfismo sexual em tamanho; (2) a diversificação fenotípica associada a diversificações genéticas em populações de *C. boraceiensis* e *C. dubius*, demonstrando que estas duas espécies-irmãs saxícolas apresentam zona de contato e hibridação; (3) o sistema de acasalamento promíscuo e as relações sociais em *C. boraceiensis*, demonstrando que tamanho das fêmeas e dos machos e parentesco genético influenciam a formação dos casais; e (4) o sistema de acasalamento poligínico com fidelidade na espécie saxícola *Thoropa taophora*, estendendo o sistema harêmico para todos os tetrápodes.

**análises comparativas, anfíbios, *Cycloramphus*, ddRADseq, dimorfismo sexual, discordância mitonuclear, evolução correlacionada, filogenia molecular multilocus, hibridação, microssatélites, parentesco genético, reconstrução ancestral, seleção sexual, sistema de acasalamento, sucesso reprodutivo, tamanho do corpo, *Thoropa*, *Zachaenus*, zona de contato.**

## ABSTRACT

Natural and sexual selection forces act on individuals and may lead to reproductive isolation. The increase in evolutionary modifications may lead to the emergence of new entities. Species is the basal unity in biology; thus, with its exuberant biodiversity, the Atlantic rainforest is a fundamental Neotropical forest for studies of evolutionary processes in anuran amphibians. Comprising the genera *Cycloramphus*, *Thoropa*, and *Zachaenus*, the family Cycloramphidae gathers saxicolous and terrestrial anuran species, all endemic to the Brazilian Atlantic rainforest. Because of their specialized life and reproductive habits, cycloramphids are an attractive group for evolutionary studies. In the present thesis we show four chapters that address three distinct scales of evolution: the diversification among species, populations, and individuals. We goaled: (1) the multilocus molecular phylogeny of *Cycloramphus-Zachaenus*, demonstrating that the evolution of terrestriality in the clade is correlated with the sexual size dimorphism; (2) the phenotypic diversification associated with the genetic diversifications in populations of *C. boraceiensis* and *C. dubius*, demonstrating that these two saxicolous sister species show a contact zone and hybridization; (3) the promiscuous mating system and the social relations in *C. boraceiensis*, demonstrating that female and male sizes, and mate relatedness influence pair formation; and (4) the polygynic mating system with fidelity in the saxicolous species *Thoropa taophora*, extending the harem system for all tetrapods.

**amphibians, ancestral reconstruction, body size, comparative analyses, contact zone, correlated evolution, *Cycloramphus*, ddRADseq, hybridization, mate relatedness, mating system, microsatellite, mitonuclear discordance, multilocus molecular phylogeny, reproductive success, sexual dimorphism, sexual selection, *Thoropa*, *Zachaenus*.**

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## **INTRODUÇÃO GERAL**

### **PROCESSOS EVOLUTIVOS: DIVERSIFICAÇÃO E ESPECIAÇÃO**

Apesar dos diversos conceitos, muitas vezes até mesmo parcialmente incompatíveis, espécie ainda constitui uma unidade fundamental na biologia (Mayr 1982, Claridge et al. 1997, Jackson et al. 2017). O ponto principal do conceito de espécie está vinculado à sua delimitação, norteando a resolução das fronteiras e da quantidade das entidades consideradas existentes (de Queiroz 2007). No entanto, devido a discordância sobre os critérios para reconhecimento das entidades, a delimitação das espécies é uma das questões mais desafiadoras da filogenética e da genética de populações (Coyne & Orr 2004). Em essência, a interrupção do fluxo gênico é o que proporciona a conclusão da formação de novas espécies e, desta forma, de Queiroz (2007) propõe um conceito unificado de espécie, retendo apenas o elemento comum de todas as propostas anteriores: espécies são linhagens metapopulacionais que estão sob os mesmos e independentes processos evolutivos.

Em organismos relacionados, o quão o fluxo gênico será responsável pela variação genética entre populações distintas está fortemente associado aos diversos processos mutacionais, distribuições geográficas, histórias naturais e regimes de seleção envolvidos (Endler 1973, Slatkin 1987, Barraclough & Vogler 2000, Servedio & Boughman 2017). Particularmente nos processos relacionados à adaptação e especiação locais, a seleção sexual desempenha diversas e complexas funções, podendo não só promover como também em alguns casos inibir tais processos (Emlen & Oring 1977, Andersson 1994, Servedio & Boughman 2017, Cooney et al. 2017). Existe um amplo conjunto de evidências indicando que a seleção sexual de fato contribui para os processos de especiação; entretanto, hoje se compreende que a seleção sexual tem atuação apenas em conjunto com a seleção natural (Servedio & Boughman 2017).

### **DIVERSIDADE E ENDEMISMO DOS ANUROS NA MATA ATLÂNTICA**

As florestas neotropicais do domínio da Mata Atlântica são excelentes locais de estudo para a compreensão dos processos de diversificação e especiação, pois

abrigam uma das biodiversidades mais vastas do mundo (Morellato & Haddad 2000, Corlett & Primack 2005, Carnaval et al. 2009). Particularmente com relação aos anfíbios anuros, a alta diversidade e o elevado índice de endemismos são marcadamente observáveis em áreas montanhosas da Mata Atlântica. Este fato se deve à forte associação destas áreas com uma elevada variedade climática e ambiental (variações em níveis de umidade, variações em temperatura e topografias acidentadas; Heyer & Maxson 1983, Silva et al. 2012, Vasconcelos et al. 2019). Esta variedade em características climáticas e ambientais proporciona inúmeros microhabitats adequados ao desenvolvimento dos anuros.

Quando comparados aos outros tetrápodes, os anuros apresentam baixa capacidade de dispersão, o que reflete em elevada filopatria e evidentes estruturações filogeográficas (Beebee 1996, Zeisset e Beebee 2008, Brunes et al. 2015, Rodríguez et al. 2015). A variedade em microhabitats na Mata Atlântica, somada à filopatria dos anuros, gera condições que exigem adaptações extremas à sobrevivência, que, por sua vez, podem levar ao surgimento de endemismos dentro de uma rede natural de barreiras ao fluxo gênico (Carnaval et al. 2009, Vasconcelos et al. 2014, Vasconcelos et al. 2019). Estas interações animal-ambiente são perceptíveis pela exuberante diversidade em espécies de anuros na Mata Atlântica, apresentando variadas morfologias, biologies e comportamentos (Haddad & Prado 2005, Haddad et al. 2013).

## **FAMÍLIA CYCLORAMPHIDAE (ANURA)**

Cycloramphidae é uma família de anuros que ocorre exclusivamente na Mata Atlântica do Brasil, sendo composta pelos gêneros *Cycloramphus*, *Thoropa* e *Zachaenus* (Frost et al. 2006, Grant et al. 2006, Fouquet et al. 2013, Jetz & Pyron 2018). No entanto, o monofiletismo dos gêneros vigentes ainda não está totalmente demonstrado (Heyer 1983, Heyer & Maxson 1983, Verdade 2005, Sabbag et al. 2018). Os cicloramfídeos são encontrados em regiões montanhosas das formações de Mata Atlântica, ocorrendo desde o estado do Rio Grande do Sul até a porção sul do estado da Bahia.

Apesar do pouco conhecimento acerca da história natural de tais espécies, dois agrupamentos ecomorfológicos são distinguíveis para *Cycloramphus*, sendo um grupo terrestre e o outro saxícola (Verdade 2005). As espécies de *Zachaenus* se assemelham aos *Cycloramphus* terrestres (Lutz 1947, Heyer & Crombie 1979, McDiarmid & Altig 1999, Verdade 2005, Brasileiro et al. 2007, Zocca et al. 2014). Com membros curtos e ausência de membranas interdigitais, os cicloramfídeos terrestres são espécies que vivem associadas à serapilheira de áreas florestadas ou ao solo de campos de altitude, com algumas delas apresentando hábitos semifossoriais. A reprodução ocorre no próprio ambiente terrestre. Os ovos terrestres são grandes e despigmentados, sendo depositados em cavidades úmidas, embaixo de troncos ou em tocas. Há cuidado com a prole envolvendo um ou ambos os parentais. Os girinos terrestres eclodem bastante desenvolvidos e são endotróficos. Por sua vez, as espécies de *Thoropa* se assemelham aos *Cycloramphus* saxícolas (Bokermann 1965, Coccoft & Heyer 1988, Haddad & Sazima 1989, Giaretta & Facure 2003, Giaretta & Facure 2004, Verdade 2005). Com o corpo achatado e membranas interdigitais desenvolvidas, os *Cycloramphus* saxícolas são espécies que vivem associadas a riachos encachoeirados de áreas florestadas ou campos de altitude. A reprodução ocorre no próprio ambiente saxícola. Os ovos terrestres são também grandes e despigmentados, sendo depositados sobre rochas emergentes nos riachos ou em fendas úmidas de paredões rochosos nas proximidades de cachoeiras. Há cuidado paternal, com os machos altamente territoriais defendendo ativamente as desovas. Os girinos semiterrestres eclodem em estágios iniciais de desenvolvimento e são exotróficos.

## OBJETIVOS

Na presente tese são apresentados quatro capítulos de estudos de processos evolutivos de diversificação e especiação em espécies dos gêneros *Cycloramphus*, *Thoropa* e *Zachaenus* (família Cycloramphidae), anuros endêmicos da Mata Atlântica do Brasil. Com o uso de ferramentas moleculares e incluindo dados morfológicos, biológicos, ecológicos e comportamentais, são abordadas três escalas distintas da evolução: a diversificação entre espécies, entre populações e entre indivíduos. Especificamente, os capítulos abordam:

- I. A diversificação entre espécies: por meio da construção da filogenia molecular multilocus do clado *Cycloramphus-Zachaenus* e, incluindo métodos de reconstrução de estados ancestrais e análises comparativas, é demonstrado como a evolução da terrestrialidade nos cicloramfídeos se correlaciona com o dimorfismo sexual em tamanho.
- II. A diversificação entre populações: por meio do estudo de variações fenotípicas (padrões morfológicos dorsais) associadas a variações genômicas (SNPs; sequenciamento de próxima geração) e a variações de um fragmento de gene mitocondrial (16S), é demonstrado como se caracteriza a zona de contato entre as linhagens das espécies-irmãs saxícolas *Cycloramphus boraceiensis* e *C. dubius*.
- III. A diversificação entre indivíduos: por meio de genotipagem de microssatélites e análises genéticas de parentesco, é demonstrado como se caracteriza o sistema de acasalamento em *Cycloramphus boraceiensis*, evidenciando as relações entre tamanho do corpo, parentesco genético, escolha de parceiros e sucesso reprodutivo.
- IV. A diversificação entre indivíduos: por meio de caracterização larval e de registros comportamentais do ritual de corte, é demonstrado como se caracteriza o sistema de acasalamento em *Thoropa taophora*, evidenciando a fidelidade entre parceiros e apresentando um novo sistema de acasalamento para anfíbios, o qual já era conhecido para todos os demais tetrápodes.

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CAPÍTULO I

**Male-male competition and repeated evolution of terrestrial breeding in  
Atlantic Coastal Forest frogs**  
*(manuscrito aceito na revista Evolution)*

# Male-male competition and repeated evolution of terrestrial breeding in Atlantic Coastal Forest frogs

Running title: TERRESTRIAL BREEDING IN FROGS

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**AUTHOR CONTRIBUTIONS:** F.P.S., K.Z., and C.F.B.H. conceived the project and designed the analyses. K.Z., C.F.B.H., M.T.R., V.K.V., and M.T.C.T. collected field samples. M.M.G. collected molecular sequences. F.P.S. collected phenotypic data and performed comparative analyses. F.P.S., K.Z., and C.F.B.H. wrote the manuscript with contributions from all authors.

**ACKNOWLEDGMENTS:** We thank W.R. Heyer, B. Hedges, M. Solé, I.R. Dias, H.R. da Silva, B.V.M. Berneck, and P.P.G. Taucce for tissue samples; T.H. Condez for help with comparative analyses; T. Grant, J.P. Pombal Jr., L.F. Toledo, R.C. Bell, and S.P. Carvalho-e-Silva for specimen loans; C.M. Cozzi for drawings; and M.W. Cardoso and R.C. Gonzalez for technical support. This research was supported by grants from São Paulo Research Foundation (FAPESP; #2014/24972-4, #2016/06876-3, and #2018/17993-6 to F.P.S., #2008/50928-1, #2013/50741-7, and #2014/50342-8 to C.F.B.H., #2001/05470-8 to V.K.V., and #2003/10335-8 to M.T.R.), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Finance Code 001; to F.P.S.), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; to C.F.B.H., M.T.R., and K.Z.). Field research and collection permits were issued by the appropriate Brazilian agencies.

**CONFLICT OF INTEREST STATEMENT:** The authors declare no competing interests.

**DATA ACCESSIBILITY STATEMENT:** All data are available in the text and associated files.

Terrestrial breeding is a derived condition in frogs, with multiple transitions from an aquatic ancestor. Shifts in reproductive mode are typically associated with morphological adaptations, with repeated transitions imposing similar selective pressures. We examine the diversification of reproductive modes and body size in the Neotropical frog genera *Cycloramphus* and *Zachaenus*, both endemic to the Atlantic rainforest of Brazil. Species in this clade either breed in rocky streams (saxicolous) or in terrestrial environments, allowing us to investigate reproductive habitat shifts. We constructed a multilocus molecular phylogeny and inferred evolutionary histories of reproductive habitats, body sizes, and sexual size dimorphism (SSD). The common ancestor was small, saxicolous, and had low SSD. Terrestrial breeding evolved independently three times and we found a significant association between reproductive habitat and SSD, with shifts to terrestrial breeding evolving in correlation with decreases in relative male body size, but not female body size. Terrestrial breeding increases the availability of breeding sites and results in concealment of amplexus, egg-laying, and parental care, therefore reducing male-male competition at all stages of reproduction. We infer that correlated evolution of terrestrial reproduction and small males is due to release from intense male-male competition that is typical of exposed saxicolous breeding.

**KEY WORDS:** correlated evolution, *Cycloramphus*, reproductive mode, sexual selection, sexual size dimorphism, *Zachaenus*.

Diversification involving shifts in habitat use is often associated with phenotypic changes that optimize performance in new environments (Fleagle and Mittermeier 1980; Glor et al. 2003; Streelman and Danley 2003; Bell et al. 2010). Invasion of new

habitats imposes new selective pressures, often requires adaptive evolution, and thus plays a fundamental role in shaping diversity (Losos et al. 1997). Similar environments impose similar selective pressures, which can drive adaptive convergence (McCracken et al. 1999; Ben-Moshe et al. 2001; Webster and Dawson 2004). Phenotypic convergences due to independent shifts towards similar habitats are especially common when species are members of the same monophyletic group (Schluter 2000; Losos 2011).

Tetrapods have evolved recurrent shifts between aquatic and terrestrial breeding habitats throughout their evolutionary history (Vermeij and Dudley 2000; Sander 2012; Kelley and Pyenson 2015). Invasion of new habitats for reproduction can be advantageous in that it opens new competition-free environments for offspring, and in some cases can trigger higher rates of evolutionary diversification (Wagner 2001; Sahney et al. 2010; Crump 2015). Living amphibians have diversified in almost all habitats on the planet (Wells 2010), with multiple independent shifts between aquatic and terrestrial breeding modes (Chippindale et al. 2004; Wei et al. 2009; San Mauro et al. 2014). For frogs, these evolutionary transitions are primarily associated with egg deposition sites and tadpole development sites (Haddad and Prado 2005; Zamudio et al. 2016); however, adults also experience new selective pressures as they move to terrestrial habitats for breeding. The prevailing hypothesis for frogs is that the selective advantage of escaping aquatic predators during early life stages, coupled with suitable climatic conditions (humidity and temperature), promotes the evolution of terrestrial breeding (Goin and Goin 1962; Wells 2010; Gomez-Mestre et al. 2012). An alternative hypothesis posits that terrestrial breeding decreases male-male competition that is typical of high-density aquatic breeding habitats (Zamudio et al. 2016). Terrestrial breeding reduces the need for costly behaviors associated with

male defense of territories and females, and also reduces the potential for fitness loss due to polyandry that can occur in breeding aggregations where males directly compete for females (Zamudio et al. 2016).

Across all frogs, terrestrial breeding is a derived condition from an aquatic-breeding ancestor (Gao and Wang 2001; Wells 2010; Gomez-Mestre et al. 2012). Trends toward terrestrial breeding include increased independence from waterbodies for courting and breeding adults, for egg deposition, and for larval development (Duellman and Trueb 1994). The African *Phrynobatrachus* species (Zimkus et al. 2012), the Afro-Asian rhacophorids (Meegaskumbura et al. 2015), and the afrobatrachians (Portik and Blackburn 2016) all have aquatic basal lineages shifting toward more derived terrestrially-reproducing species. In contrast, the South American *Pleurodema* and Leptodactylidae have evolved terrestrial breeding, but with reversals to more aquatic breeding modes (Heyer 1969; Faivovich et al. 2012; Pereira et al. 2015). Clearly, the overall trend toward terrestrial breeding across all anurans hides substantial diversity in the transitions between aquatic and terrestrial breeding in different frog lineages (Gomez-Mestre et al. 2012; Zamudio et al. 2016). The South American species in the family Cycloramphidae have two reproductive modes. Saxicolous species in this clade breed and lay eggs in rock or root crevices at the margins of fast-flowing streams, commonly next to waterfalls (Giaretta and Cardoso 1995; Giaretta and Facure 2003). Males of saxicolous species are highly territorial; they mate within their territories and guard egg clutches that develop into semiterrestrial exotrophic tadpoles (Haddad and Sazima 1989; Giaretta and Cardoso 1995; Giaretta and Facure 2003; Verdade 2005). In contrast, terrestrial species mate and lay eggs under trunks or inside constructed burrows in leaf-litter of forest floors. Therefore, mating and oviposition for terrestrial species is concealed, and males and

females tend the eggs, which hatch into terrestrial endotrophic tadpoles that complete development on land (Heyer and Crombie 1979; McDiarmid and Altig 1999; Verdade 2005; Brasileiro et al. 2007; Zocca et al. 2014).

Previous phylogenetic studies have found that the 30 species in the genera *Cycloramphus* and *Zachaenus* are monophyletic (family Cycloramphidae; Verdade 2005; Fouquet et al. 2013; Faivovich et al. 2014; Grant et al. 2017). As in other clades, cycloramphid species with different reproductive modes have different body shapes and sizes (Verdade 2005; Blackburn et al. 2013), yet we do not know whether the specific selective forces acting on these traits are primarily driven by natural selection imposed by new environments, or sexual selection on one or both sexes. Shifts in reproductive habitat include changes in environmental conditions, which might select directly for differences in body size. For example, the increased risk of desiccation could select for larger body sizes for water conservation (Nevo 1973; Gouveia and Correia 2016; Gouveia et al. 2019) or locomotion and exploitation of leaf-litter habitats could select for smaller body sizes (Lehr and Coloma 2008; Lehr and Catenazzi 2009; Kraus 2010; Kraus 2011). These selective pressures should be equal for males and females, thus we predict that if these mechanisms are at work, size will vary equally in both sexes. In contrast, intraspecific social interactions also potentially act as strong selective forces, shaping behaviors and morphology (West-Eberhard 1983). Males of saxicolous species benefit from large sizes due to the need to defend resources and exclude competing males in exposed environments (Lutz 1947; Giaretta and Cardoso 1995; Giaretta and Facure 2003). Terrestrially breeding male frogs will likely experience less male-male competition, primarily because breeding sites in leaf-litter are broadly available, and terrestrial breeding conceals mating behaviors, amplexus, and egg-laying (Verdade 2005; Zamudio et al. 2016).

Thus, if sexual selection is indeed the primary driving force in species that adopt new reproductive habitats, we expect sex-specific selection resulting in different patterns of change between males and females during transitions to terrestrial breeding.

Here, we reconstructed the evolutionary history of reproductive mode, male and female body size, and sexual size dimorphism in the *Cycloramphus-Zachaenus* frog clade. We inferred a multilocus molecular phylogeny and used it to map reproductive and body size traits. We used comparative analyses to test hypotheses about the evolutionary forces behind this reproductive diversification, and explored the potential natural and sexual selection drivers for the evolution of terrestriality. Specifically, we tested three hypotheses: (1) terrestriality evolved only once in this clade, as indicated by an earlier morphological phylogeny (Verdade 2005); (2) males and females vary in overall sizes according to reproductive habitats due to natural selection imposed by environmental factors (Nevo 1973; Lehr and Coloma 2008; Lehr and Catenazzi 2009; Kraus 2010; Kraus 2011; Gouveia and Correia 2016; Gouveia et al. 2019); and (3) males decrease in size in terrestrial species because shifts to terrestriality impose lower sexual selection for large body sizes due to lower levels of male-male competition (Zamudio et al. 2016).

## *Materials and Methods*

### **FOCAL TAXA AND SAMPLING**

We gathered 86 tissue samples, including one to seven representatives of 25 of the 28 known *Cycloramphus* species and both of the *Zachaenus* species, totaling 90% of the currently recognized diversity in the clade. The completeness of our dataset is possible because of samples collected during expeditions in Brazil in the 1970s and 1980s; among them are samples from type localities and surroundings for a number

of species that are currently declined or disappeared (Eterovick et al. 2005). Our genetic matrix only lacks *Cycloramphus catarinensis*, *C. diringshofeni*, and *C. duseni* for which tissues are unavailable in collections (Andersson 1914; Bokermann 1957; Heyer 1983; Lingnau et al. 2008). Based on previous studies (Frost et al. 2006; Grant et al. 2006; Fouquet et al. 2013; Jetz and Pyron 2018; Sabbag et al. 2018) we selected as outgroups *Thoropa lutzi* and *T. taophora* (from the sister genus of the *Cycloramphus-Zachaenus* clade) and one species each from the genera *Crossodactylus*, *Hylodes*, and *Megaelosia* (family Hylodidae). We rooted our trees on *Paratelmatobius poecilogaster* (family Leptodactylidae). To ensure accurate taxonomic assignments for all species in our tree, whenever possible we selected representatives from type localities or their vicinity and checked external morphology of all vouchers (Table S1). Field research was approved by the committee on animal care use at Universidade Estadual Paulista, Rio Claro, Brazil.

## MOLECULAR PROCEDURES

We extracted whole genomic DNA from ethanol-preserved tissues using a DNeasy Blood & Tissue kit (Qiagen Inc., Valencia, CA) and following the manufacturer's protocol. Using published primers (Table 1), we PCR-amplified and sequenced three mitochondrial (*16S* [593 base pairs, bp], *Cytochrome-b* [635 bp], *ND1* [1401 bp]) and four nuclear ( *$\alpha$ -fibrinogen* [531 bp], *Cmyc* [410 bp],  *$\beta$ -crystallin* [475 bp], and  *$\beta$ -fibrinogen* [520 bp]) gene fragments. All PCRs were carried out in a final volume of 25  $\mu$ L, each containing 1-10 ng DNA template, 1X PCR Buffer (with 1.5-3 mM  $MgCl_2$ ), 1X BSA, 0.4  $\mu$ M of forward and reverse primers, 0.76 mM dNTPs, and 0.625 units of Taq polymerase (Roche Diagnostics, Indianapolis, IN). Amplification conditions included an initial denaturation at 94°C for 5 min, followed by 35-40 cycles

of denaturation at 94°C for 1 min, annealing at primer-specific temperatures from 48-60°C for 1 min (Table 1), extension at 72°C for 1 min, followed by a final extension at 72°C for 5-10 min. For all *α-fibrinogen* PCR amplicons (which have indels), and for any weak amplicons of *Cytochrome-b*, *ND1*, *Cmyc*, *β-crystallin*, and *β-fibrinogen*, we cloned PCR products using the TOPO TA cloning kit with PCR 2.1-TOPO TA vector (Life Technologies, Camarillo, CA). We picked and amplified up to as many as ten positive clones per amplicon using universal M13 primers and the same PCR conditions as above, but with an annealing time of 45 s (Table 1). We purified all final PCR amplicons using a cocktail of 1X SAP buffer, SAP enzyme (1 unit; Life Technologies), and exonuclease I (10 units; Life Technologies). We sequenced the purified PCR products in both directions using BigDye terminator cycle sequencing (Applied Biosystems, Foster City, CA) on an ABI 3730xl Genetic Analyzer (Applied Biosystems).

## PHYLOGENETIC ANALYSES

We assembled all forward and reverse DNA sequences using Sequencher version 5 (Gene Codes Corporation, Ann Arbor, MI) and aligned them using Clustal W implemented in Mega version 7 (Kumar et al. 2016). We coded all single polymorphic sites in heterozygous individuals with IUPAC ambiguity codes, and translated coding sequences to ensure reading frame conservation. We phased haplotypes via the probabilistic Bayesian algorithm PHASE (Stephens et al. 2001; Stephens and Donnelly 2003), implemented in DnaSP version 5 (Librado and Rozas 2009). We ran PHASE five times for each nuclear gene using distinct random seeds to check for consistency of haplotype reconstructions across runs. All specimens included in our

phylogenetic analyses along with GenBank sequence accession numbers are listed in Table S1.

We determined the best partitions and the best models for each partition subset using the Akaike information criterion (AIC) in PartitionFinder version 2 (Lanfear et al. 2017). To check for stability of tree topologies, we performed maximum likelihood analyses in RAxML version 8 (Stamatakis 2014) testing distinct partitioning search algorithms (*greedy* and *rcluster*) and compared analyses obtained with only concatenated mitochondrial genes, with each nuclear gene separately, and with the final dataset combining all mitochondrial and nuclear genes. We inferred our final topology of species-level phylogenetic relationships within *Cycloramphus-Zachaenus* with Bayesian analysis and all genes concatenated. We used MrBayes version 3.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003; Ronquist et al. 2012) to perform two parallel runs with four chains each, adopting the Markov Chain Monte Carlo approach (MCMC). We ran analyses for 10,000,000 generations with sampling every 1000 generations, resulting in 10,000 trees. We evaluated runs to ensure that all parameters had reached stationarity (average standard deviation of split frequencies = 0.005585; average PSFR = 1.000; maximum PSFR = 1.013). We discarded the first 25% of generations as burn-in and calculated a 50% majority rule consensus tree (Holder et al. 2008; Lamsdell et al. 2017) from the combined remaining trees from both runs. We checked for the SD of split frequencies between two runs (<0.01) and the effective sample size in Tracer version 1.5 (Drummond and Rambaut 2007). We edited the tree using FigTree version 1.4 (Rambaut 2014).

## PRUNING THE PHYLOGENETIC TREE

For phylogenetic signal, ancestral reconstruction, and correlated evolution analyses, we kept *Thoropa taophora*, but pruned all other outgroups from the final Bayesian tree. To select terminals retained in the pruned tree, we used currently recognized taxa. We selected one terminal per species or deeply-divergent species lineages. When species included well-supported divergent lineages that were geographically separated (potentially indicative of cryptic or undescribed species), we retained independent lineages and collected morphological data specifically for those intraspecific lineages. In one particular case of a paraphyletic species (*Cycloramphus eleutherodactylus* relative to *C. faustoi*) we opted to keep *C. faustoi* as a sister taxon of one lineage of *C. eleutherodactylus* because it is an insular species, and thus may show interesting shifts in body size. We used the pruned phylogeny as the starting point for all downstream analyses.

## ECOLOGICAL AND MORPHOLOGICAL TRAITS

To complete our phenotypic matrix, we supplemented our own collections of cycloramphid species in the field with data from museum collections. We visited six institutions: Célio F. B. Haddad amphibian collection, Universidade Estadual Paulista, Rio Claro, São Paulo (CFBH); Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro (MNRJ); Museu de Zoologia da Universidade de São Paulo, São Paulo (MZUSP); Museu de Zoologia da Universidade Estadual de Campinas “Adão José Cardoso”, Campinas, São Paulo (ZUEC); Departamento de Zoologia, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro (ZUFRJ); and National Museum of Natural History, Smithsonian Institute, Washington D.C. (USNM). We measured the majority of cycloramphid material currently available in scientific collections. Our phenotypic matrix included data from a total of 960

specimens from 40 *Cycloramphus-Zachaenus* and one outgroup species (*Thoropa taophora*). The only species missing in our morphological dataset was *Zachaenus carvalhoi*, for which we obtained body size measurements from the literature (Neto et al. 2016). For each specimen, we measured snout-vent length (SVL) of males and females, defined as the distance between the tip of the snout and the cloaca. We determined sex by checking for external inguinal macroglands present only in adult males and oocytes that are often visible through the skin in adult females. Because all species in the *Cycloramphus-Zachaenus* clade have female-biased sexual size dimorphism (SSD), we used a size dimorphism index that was the ratio of female to male body size (SDI: [female SVL/male SVL]-1; Lovich and Gibbons 1992). Our final phenotypic data matrix included reproductive habitat use, male and female body sizes, and SDI (Table S2).

## ASSESSMENT OF PHYLOGENETIC SIGNAL

We investigated the extent to which our traits show phylogenetic signal in R version 3.3 (R Core Team 2016). We estimated phylogenetic signal by testing whether our data exhibit a significant tendency for related species to resemble each other under predictions of the Brownian motion model (Blomberg et al. 2003). For reproductive habitat (discrete trait: saxicolous or terrestrial), we used the R function *phylo.signal.disc* developed by E. L. Rezende ([www.github.com/juliema/publications](http://www.github.com/juliema/publications)), which corresponds to the “fixed tree, character randomly reshuffled” model (Maddison and Slatkin 1991). This algorithm estimates the minimum number of trait state transitions at each node assuming maximum parsimony, and compares those with median distributions from a randomized tip dataset. Observed transitions that are significantly less than the randomized median indicate significant phylogenetic signal in the data. For male and female body sizes, and SDI (continuous traits), we

estimated Pagel's lambda (Pagel 1999) and Blomberg's K (Blomberg et al. 2003) in PHYTOOLS (function *phylosig*; Ives et al. 2007; Revell 2012). Lambda varies from 0, indicating no phylogenetic signal, to 1, indicating strong phylogenetic signal. To evaluate whether lambda was significantly different than 0, we used a likelihood ratio test (LRT) using the R package NLME (functions *gls* and *pchisq*; Posten and Hallahan 1989; Pagel 1997; Pinheiro and Bates 2000; Freckleton et al. 2002). Values of Blomberg's K close to 1 suggest an evolutionary process under the Brownian motion model of evolution, indicating that trait similarity is proportional to divergence. A value of  $K > 1$  indicates that traits are more similar and  $K < 1$  indicates that traits are more different than expected by phylogenetic distance under a Brownian motion model.

## ANCESTRAL STATE RECONSTRUCTIONS

To test hypotheses about the evolution of terrestriality in the *Cycloramphus-Zachaenus* clade we reconstructed ancestral states for reproductive habitat (saxicolous or terrestrial), male and female body sizes, and SDI on our phylogeny.

For reproductive habitat, we first used the R package PHYTOOLS to reconstruct marginal ancestral states (functions *rerootingMethod* and *fitMk*; Paradis et al. 2004; Revell 2012), which consists of finding the maximum likelihood estimate at each node and integrating it over all other nodes (Yang et al. 1995). We ran this discrete state reconstruction under three distinct models: equal rates (ER), symmetrical rates (SYM), and all rates different model (ARD). Using LRT and AIC, ancestral reconstructions did not differ significantly between the three models (similar values), thus we selected ER as the simplest model for our data. Using stochastic character mapping (Bollback 2006), we estimated ancestral reproductive habitat states generating the probabilities of each node with 1000 iterations on our tree from the

Bayesian phylogenetic reconstruction (function *make.simmap*; Huelsenbeck et al. 2003; Paradis et al. 2004; Bollback 2006; Revell 2012). To account for phylogenetic uncertainty, we repeated this final analysis step across 500 randomly selected trees from the posterior distribution.

For continuous traits (body sizes and SDI), we used different solutions for missing data. First, we removed the tips with missing morphological values from our tree (Portik and Blackburn 2016; Sauquet et al. 2017). Second, we kept our original tree and used the ancestral value for each missing taxon, assuming parsimonious change. Both analyses resulted in similar estimates, therefore, we report results of reconstructed ancestral states in the R package PHYTOOLS (function *anc.Bayes*; Revell 2012), removing tips with missing values, running an MCMC with 1,000,000 generations, and jointly finding the set of states at internal nodes. The first 200,000 generations were discarded as burn-in. For final character mapping of ancestral states on the phylogeny (model *fastAnc* implemented in function *contMap*; Felsenstein 1985; Revell 2012; Revell 2013) we inferred tips with missing values, so that all taxa or lineages analyzed were represented.

## PHYLOGENETIC COMPARATIVE ANALYSES

To test the hypotheses that body size traits in the *Cycloramphus-Zachaenus* clade are associated with saxicolous or terrestrial adult habitat use and to understand the directionality of evolution in these traits, we conducted phylogenetic comparative analyses. We implemented BayesTraits version 3 to document correlated evolutionary transitions of discrete traits (Pagel 1994, 1999; Pagel et al. 2004; Pagel and Meade 2006). Because this analysis only tests for correlated evolution in pairs of dichotomous traits, we transformed continuous traits into categorical classes (Sauquet et al. 2017) using the midpoint for each body size or SDI range across all

species. The midpoint of an interval is the lowest range plus the highest range divided by two. We classified species as “small” (when below the midpoint value for all tips) or “large” (when above the midpoint value for all tips). Likewise, we classified each SDI trait as “low” or “high” with a break at the midpoint for SDI values across all species. The thresholds for size class categories were 42.4 mm for species SVL; 44.1 mm for female SVL; 39.3 mm for male SVL; and 0.17 for SDI. We kept our original tree and for each missing taxon we used the ancestral value. For each pair of binary traits, there are four possible states and eight rate parameters ( $q_{ij}$ ) that quantify the rate of change in traits, assuming that only a single change in one trait may occur instantaneously. The  $q_{ij}$  statistic represents the divergence between the observed and expected association of states  $i$  and  $j$ . The expected association is the product of the marginal probabilities of finding these states ( $i$  and  $j$ ) at the same phylogenetic node (Huelsenbeck et al. 2003).

To control for phylogenetic uncertainty, we assessed transitions on 800 randomly selected trees from the Bayesian phylogeny. To test alternative evolutionary scenarios and describe how binary traits coevolved, we used reversible-jump MCMC (Pagel and Meade 2006), with independent, dependent, and covarion models (Tuffley and Steel 1998). For each model, we ran two independent chains of 1,000,000 generations, sampling every 1000 generations. For calculating marginal likelihoods, we used stepping-stone sampling with 100 samples and 1000 iterations per sample. We assessed convergence and verified that effective sample sizes (ESS) for all parameters were greater than 200 in Tracer version 1.5 (Drummond and Rambaut 2007). We also determined that acceptance rate values fell between 20-40%, which is expected under convergence. We compared marginal likelihoods to obtain the relative performance of the distinct models via Bayes factors (BF), using

BF > 2 as positive evidence and BF > 5 as strong evidence, following the manual for BayesTraits version 3. For traits with evidence for the dependent models of evolution, we discarded the first 10,000 generations as burn-in and calculated mean rates of transition among trait states. We calculated the z-scores of trait transitions, expressed as percentage, from the proportion of occurrences of a value of zero estimated for relevant transition parameters (Pagel and Meade 2006). High z-scores indicate unlikely paths for evolutionarily correlated trait pairs.

First, we tested whether transitions between saxicolous and terrestrial breeding were correlated with changes in overall species body size (pooling females and males). We coded each species or lineage as “saxicolous” or “terrestrial” and transformed SVLs to the binary states of “small” or “large”. Second, we repeated the test above separating each species or lineage by sex, separately coding females and males for each trait. When separated by sex, we considered phylogenies with all individuals included (inferring values for missing data). In the sex-specific analyses, we tested whether transition rates between small and large body sizes differed between saxicolous and terrestrial species, or vice versa, and whether those correlations were similar for both sexes. Finally, we coded each species or lineage as “saxicolous” or “terrestrial” and with low or high SDI to assess whether transitions in reproductive habitat were correlated with changes in sexual size dimorphism.

## *Results*

### **PHYLOGENETIC RELATIONSHIPS**

The concatenated multilocus matrix included all mitochondrial (*16S*, *Cytochrome-b*, and *ND1*) and nuclear genes (*α-fibrinogen*, *Cmyc*, *β-crystallin*, and *β-fibrinogen*). PartitionFinder selected 18 partitions across all mitochondrial and nuclear genes

(Table S3). Our combined Bayesian tree recovered a monophyletic *Cycloramphus-Zachaenus* clade with high support for the majority of nodes. Our topology indicated that *Cycloramphus* is paraphyletic and includes the genus *Zachaenus*, and thus *Zachaenus* should not be a separate genus (Fig. 1). We retain the original generic names, but taxonomic revision of this group is clearly necessary. Phylogenetic analyses of mitochondrial and nuclear genes independently yielded highly congruent topologies (Figs. S1-S5).

### ASSESSMENT OF PHYLOGENETIC SIGNAL

Using our Bayesian phylogeny, excluding tips lacking trait data, we confirmed phylogenetic signal for most of our traits with high significance, indicating that it is necessary to consider phylogenetic relationships in our trait analyses (Revell 2010). We also performed the same analyses using the phylogenetic tree with inferred missing tip values and the results did not vary, thus we report results here using the fullest tree possible for each trait. For reproductive habitats, the observed transitions were significantly lower ( $= 3$ ) than the randomized median transitions ( $= 14$ ), indicating strong phylogenetic signal. For our continuous traits, we obtained values as follows: for overall species SVL,  $\lambda = 0.99$ , LRT = 0.44, and  $K = 2.14$ ; for female SVL,  $\lambda = 0.99$ , LRT = 0.24, and  $K = 1.07$ ; for male SVL,  $\lambda = 0.99$ , LRT = 1, and  $K = 3.21$ ; and for SDI,  $\lambda = 0.96$ , LRT = 1, and  $K = 1.62$ . All  $\lambda$  values suggest strong phylogenetic signal, but LRTs indicate that female SVL and overall species SVL follow a Brownian model of evolution. Blomberg's  $K$  results were  $> 1$  for all traits, indicating that closely-related species and lineages are more similar than expected for all continuous traits, but male SVL had the largest  $K$  (3.21) and female SVL the smallest (1.07).

## ANCESTRAL RECONSTRUCTIONS OF SPECIES HABITATS AND TRAITS

Our results show that reproductive modes are clustered, with terrestrial species grouping in several distinct clades that are derived from two saxicolous ancestral monophyletic groups. The reconstructed ancestor for the clade was saxicolous with overall relatively small body size (estimated overall species SVL = 31.23 mm), and very little difference between male and female body sizes (estimated female SVL = 43.39 mm, estimated male SVL = 43.71 mm, estimated SDI = 0.095). From the saxicolous *Cycloramphus* ancestor, terrestriality evolved independently several times (Fig. 2) and male and female body sizes and SSD have increased and decreased in distinct lineages (Figs. 3, 4, and 5).

## EVOLUTIONARY CORRELATIONS BETWEEN SPECIES HABITATS AND TRAITS

We tested for correlated evolution in reproductive habitats and body sizes across all species using our phylogeny. For the three analyses of correlated trait evolution, we considered models that showed positive BF values, and at least one transition rate with a z-score < 0.10, indicating that fewer than 10% of the iterations from the MCMC were assigned to zero for at least one transition rate. We found positive support for the dependent evolutionary model for all three body size traits (mean overall species, male, and female sizes) and for SDI with respect to reproductive mode (Table 2).

The results from dependent models of correlated evolution between overall species size (Fig. 6A), female size (Fig. 6B), and male size (Fig. 6C) showed different correlated transition rates with reproductive habitat. The pattern was the same for overall mean species size and female size. Significant transitions between body size and reproductive habitat only occurred in saxicolous species, and the transitions for those correlated phenotypes were approximately equal in both directions. For male body size, in addition to significantly higher transition rates in saxicolous species, we

found an asymmetrically significant transition rate between terrestrial reproductive habitats and small size (Fig. 6C). These results indicate that in saxicolous species changes in male and female body sizes occur symmetrically, although the rates of change in males seem to be higher than in females (Fig. 6B, C). In contrast, in terrestrial species only changes in male size are significant, and evolved asymmetrically, toward smaller males. Finally, in the analyses of SDI and reproductive habitat (Fig. 6D), we found significant evolutionary correlations for SDI and reproductive habitat across saxicolous and terrestrial species in the phylogeny. From the ancestral state (saxicolous and with low SDI), we found significant transitions in the evolutionary progression of these two correlated traits. Saxicolous species vary significantly in SDI, with a much higher transition rate toward lower SDI (males increasing in size and becoming closer to females in size). High SDI (when males are much smaller than females) is significantly correlated with transitions to terrestrial habitats ( $q_{24}$ ). Terrestrial species vary significantly in SDI with equal rates in both directions (low and high SDI). Combined, the analyses of body size and SDI show that SDI changes in a correlated manner with reproductive habitat, but that it is changes in male size that are primarily contributing to differences in sexual dimorphism.

## *Discussion*

### **REPEATED EVOLUTION OF TERRESTRIALITY IN CYCLORAMPHIDS**

Our phylogenetic reconstruction recovered several independent shifts to terrestrial breeding in the *Cycloramphus-Zachaenus* clade. This result rejects the hypothesis that terrestriality evolved once and indicates that phenotypic similarities among distinct terrestrial species groups are the result of adaptive convergence (Verdade

2005). Novel environments contain both niche availability (which allows for persistence of invading populations) and niche discordance (which generates diversified selection) and thus provide ecological opportunity (*sensu* Wellborn and Langerhans 2015) for diversifying species and lineages. The rocky fast-flowing streams within forested habitats that are recovered as the ancestral habitat for cycloramphids are surrounded by thick and highly humid leaf-litter on the forest floors, which is an abundant and available niche with ideal conditions for terrestrial egg deposition (Lutz 1947; Zocca et al. 2014). Our data show that this ecological opportunity has repeatably stimulated divergence, with the invasion of terrestrial habitats resulting in coordinated changes in phenotypes (Losos et al. 1997; McCracken et al. 1999; Ben-Moshe et al. 2001; Webster and Dawson 2004; Wellborn and Langerhans 2015). These repeated habitat invasions allow us to explore the mode of selection on the species in each of those invasions and whether those targets were the same in each case.

### **SIZE DIMORPHISM AND HABITAT TRANSITIONS IN CYCLORAMPHIDS**

For cycloramphids, overall species body sizes are not clustered by reproductive mode, as both saxicolous and terrestrial species show high variances in male and female body sizes (Table S2; Figs. 3 and 4). This result rejects the hypothesis that both males and females vary in overall sizes according to reproductive habitats purely due to natural selection on both sexes imposed by environmental factors. The alternative explanation for changes in SSD with reproductive mode is that the sexes are under independent selection for body sizes, and that the selective mechanisms are sex-specific. All species in the clade show female-biased SSD. The predominant explanation for female-biased SSD in frogs is strong fecundity selection with larger

females having fitness advantages due to larger clutch sizes (Shine 1979; Lüddecke 2002; Pupin et al. 2010). This selective advantage likely underlies the consistently larger sizes in cycloramphid females (Table S2). In contrast, we found more evolutionary change in males than in females with transitions to novel reproductive habitats, specifically, males become smaller with transitions to terrestrial breeding. These different changes in male and female body sizes result in consistent changes in SSD, with the largest differences in body sizes between the sexes always associated with terrestrial habitats (Fig. 5). Our analyses of correlated evolution confirm that evolutionary patterns for males and females are distinct. First, saxicolous males transition between small and large sizes in both directions at approximately doubled rates (compared to saxicolous females, Fig. 6B, C). Second, we found that terrestriality is correlated with smaller body sizes in males, but not females. Lastly, correlations between SSD and reproductive mode show that shifts from saxicolous toward terrestrial niches are associated with increases in SSD, primarily due to decreases in male sizes in terrestrially breeding species. Combined, trait mapping, phylogenetic signal, and correlated trait evolution analyses validate our hypothesis that males are the sex that primarily changes size with the shifts in reproductive modes, indicating that males are under stronger selection during habitat transitions. Given the biology of our focal species, we infer that sexual selection, specifically male-male competition, is driving changes in male body sizes and hence SSD in *Cycloramphus* and *Zachaenus*.

## BREEDING RESOURCES AND MALE-MALE COMPETITION IN CYCLORAMPHIDS

Body size is an important trait for intrasexual competition among male frogs, with larger individuals commonly having fitness advantages due to enhanced capacity for territorial defense and in some cases protection of offspring (Wilbur et al. 1978; Bateson 1983; Bourne et al. 2001; Ospina-L. et al. 2017). Typically, limited availability of breeding resources and the degree to which they are monopolizable, result in mating systems that rely on male competition and higher degrees of intrasexual aggression (Emlen and Oring 1977; Howard 1978; Vonk and Shakelford 2017). Thus, sex-specific responses to the characteristics of the habitat can promote sexually dimorphic traits, with distinct reproductive roles yielding distinct selective regimes for each sex (Fairbairn 1997; Blanckenhorn 2000; Fairbairn et al. 2007).

Data on behavior and natural history for *Cycloramphus* and *Zachaenus* are difficult to obtain, especially for the terrestrial species that are cryptic and conceal mating behaviors in nests (Verdade 2005; Lingnau et al. 2008; Weber et al. 2011; Nunes-de-Almeida et al. 2016). Overall, we know that saxicolous males are highly territorial, discouraging intruders by emitting aggressive calls, and repelling conspecific males that invade their breeding sites by biting and pushing them away (Lutz 1947; Giaretta and Cardoso 1995; Giaretta and Facure 2003). In some saxicolous species, males guard clutches (Giaretta and Cardoso 1995; Giaretta and Facure 2003). Terrestrially-breeding males also show aggressive behaviors toward conspecifics and allospecifics (Neto et al. 2016), and we know that in some terrestrial species both males and females guard their clutches against intruders (Heyer and Crombie 1979; Verdade 2005; Brasileiro et al. 2007; Zocca et al. 2014). However,

territorial and guarding behaviors between saxicolous and terrestrial *cycloramphids* are very different, primarily due to the degree of exposure of their breeding sites.

Saxicolous *Cycloramphus* have eggs, larvae, and mating behaviors that are exposed to conspecific male competitors (Giaretta and Cardoso 1995; Giaretta and Facure 2003; Verdade 2005; Maia-Carneiro et al. 2012), a condition that was likely shared by their common ancestor. Saxicolous environments are not only more exposed but offer limited suitable egg-laying sites in splash zones in the vicinity of waterfalls (Haddad and Sazima 1989; Giaretta and Cardoso 1995; Lima et al. 2010; Silva and Ouverney 2012). This resource limitation leads to intense competition for egg-laying sites and presumably favors larger males that have an advantage in antagonistic interactions. In the saxicolous genus *Thoropa*, the sister taxon of the *Cycloramphus* clade (Frost et al. 2006; Grant et al. 2006; Fouquet et al. 2013; Jetz and Pyron 2018; Sabbag et al. 2018), the intensity of male-male competition has been well characterized. *Thoropa taophora* has a polygynous mating system associated with scarce breeding seeps and high levels of male-male competition (Muralidhar et al. 2014), and monopolist males exhibit paternal care of eggs and protect them against cannibalism by conspecific males (Giaretta and Facure 2004; Consolmagno et al. 2016). They discourage intruders using many of the same behaviors known for saxicolous *Cycloramphus* and use spines on their thumbs in male-male agonistic interactions (Bokermann 1965; Giaretta and Facure 2004; Muralidhar et al. 2014). In cases of extreme limitation in breeding resources, we expect intense selection for larger males, and consequently a decrease in dimorphism. Moreover, elevated levels of aggression in species where males defend territories often also selects for sexual dimorphism in weaponry (Emlen 2008), which is observed in males of all *Thoropa* species as enlarged forearms and thumb spines

(Bokermann 1965; Caramaschi and Sazima 1984; Cocroft and Heyer 1988; Feio et al. 2006). Similar weaponry is present in males and females of *Cycloramphus ohausi* (Heyer 1983), a species that has intermediate female-biased SSD and is the first species to diverge in the *Cycloramphus* clade. Jaw adductor musculature is also more developed in males and females of the terrestrial *Cycloramphus bolitoglossus* species group (Heyer 1983; Verdade and Rodrigues 2003) and might be associated to aggressive behaviors such as biting. Unfortunately, we lack natural history information for *C. ohausi* and *C. bolitoglossus* species, and specifically how these spines and jaw adductor musculature enhance male and female fitness. Spines are absent and jaw adductor musculature are less developed in males or females of all other cycloramphids, but variation in male-male competition associated with limited breeding resources has clearly been present over much of their evolutionary history.

Terrestrial *Cycloramphus* and *Zachaenus* lay eggs under trunks or inside constructed burrows in the leaf-litter on forest floors. They hatch endotrophic terrestrial tadpoles in advanced stages that use yolk to complete the development inside the nest; thus, terrestrial species have eggs, larvae, and mating behaviors that are concealed from conspecifics (Lutz 1947; Heyer and Crombie 1979; McDiarmid and Altig 1999; Verdade 2005; Brasileiro et al. 2007; Zocca et al. 2014). The leaf-litter environment not only allows concealment but also offers abundant suitable egg-laying sites in humid forest floors. The broad availability of breeding sites likely reduces competition and agonistic male-male interactions among terrestrial cycloramphids.

## **SEXUAL SELECTION AND SEXUAL DIMORPHISM ACROSS FROGS**

Sexual size dimorphism is a composite character resulting from different selective pressures on the sexes (Carranza 2009). Many factors can affect male and female body sizes, making the analysis of sexual size dimorphism across larger taxonomic groups difficult (Fleagle and Mittermeier 1980; Fairbairn 1997; Blanckenhorn 2000; Fairbairn et al. 2007; Ospina-L. et al. 2017; Gouveia et al. 2019). Our data confirm some of the predictions from broader studies of SSD across all frogs and also provide information about the environmental and behavioral contexts that might explain the high variance and size-dependence in selection on male and female sizes across mating systems (Nali et al. 2014). Across all frogs, the main selective pressure driving the predominance of female-biased SSD is female-fecundity (Shine 1979; Lüddecke 2002; Pupin et al. 2010), although the correlation between female size and SSD is most evident for species with smaller overall body size (Nali et al. 2014). Across all frogs, male size and SSD are also correlated, such that species with larger males show less-pronounced SSD, with males becoming closer to female sizes (Nali et al. 2014). However, the relationship between male size and SSD holds for both territorial and nonterritorial species, demonstrating that selection on territorial males does not contribute disproportionately to SSD, and that lineage-specific ecology and behavior could be masking the role of male size in studies of SSD evolution at larger phylogenetic scales (Nali et al. 2014). The lack of evidence for male-male competition as an important driver of SSD across all frogs is perhaps not surprising because male frogs have evolved a number of size-independent strategies that contribute to fitness. These include forearm thickness for male displacement, time at reproductive site, energy expenditure in calling activity, early sexual maturity, and alternative mating tactics (Howard and Kluge 1985;

Haddad 1991; Howard et al. 1994; Prado and Haddad 2003; Vieites et al. 2004; Byrne 2008; Zhang and Lu 2013). Our analysis of the *Cycloramphus* clade allowed us to focus on social dynamics during breeding, which in our system are influenced by reproductive habitat. Analyses of a single clade with repeated reproductive transitions allowed us to examine how interactions during breeding impose selective mechanisms that differ between the sexes.

The importance of social dynamics during breeding have been highlighted in other taxa. Dale et al. (2007) argued that social mating system is a key life-history variable that predicts size allometry and SSD in birds because of differential selection on males and females. Likewise, habitat types have also influenced the evolution of male body size and SSD in turtles, in which terrestrial males are proportionally larger than aquatic ones (Ceballos et al. 2013), likely because of territoriality and increased male-male interactions in terrestrial species. In *Cycloramphus*, the clear distinction between territorial defense in saxicolous males and lack thereof in terrestrial ones permits a lineage-specific comparison of species that repeatedly and consistently differ in ecology and behavior, and underscores the importance of habitat transitions for differential selection on males and females.

The long-standing hypothesis for transitions to terrestrial breeding in frogs is the ‘predator-avoidance’ hypothesis, which posits that the selective advantage of moving eggs and/or larvae out of waterbodies drives the evolution of more terrestrial reproductive modes, especially in tropical regions with adequate temperature and humidity conditions for the development of anamniote eggs on land (Goin and Goin 1962; Wells 2010; Gomez-Mestre et al. 2012). Our analyses indicate that predator avoidance may not be the only driver of the highly diverse reproductive modes observed in tropical frogs (Haddad and Prado 2005; Verdade 2005; Zamudio et al.

2016). Although avoidance of aquatic predators may be important in some cases, in lineages with high degrees of male-male competition and high costs of territoriality, an equally important selective force may be avoidance of those costs. The multiple transitions to terrestrial reproductive modes observed in frogs, and in amphibians more generally, likely also involve aspects of the social context in which breeding occurs, and the potential fitness benefits of avoiding the costs associated with intraspecific competition.

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**Table 1. Mitochondrial and nuclear genes sequenced in this study with primer names, primer sequences, and specific PCR annealing temperatures.**

| Gene                | Primer name  | Sequence (5'–3')             | Annealing | Reference                    |
|---------------------|--------------|------------------------------|-----------|------------------------------|
| 16S                 | 16Sa-L       | CGCCTGTTTATCAAAAACAT         | 53°C      | Palumbi et al. (1991)        |
|                     | 16Sb-H       | CCGGTCTGAACTCAGATCACGT       |           | Palumbi et al. (1991)        |
| <i>Cytochrome-b</i> | MVZ15        | GAAC TAATGGCC CACACWWTACGNAA | 48°C      | Moritz et al. (1992)         |
|                     | MVZ16        | AAATAGGAARTATCAYTCTGGTTTRAT  |           | Moritz et al. (1992)         |
| <i>ND1</i>          | 16S-frog     | TTACCCTRGGGATAACAGCGCAA      | 60°C      | Wiens et al. (2005)          |
|                     | 16Sf (L2507) | CCCCGCCTGTTTACCAAAAA         |           | Macey et al. (2001)          |
|                     | ND1-int-ACA  | ACGTGATCTGAGTTCAGACCG        |           | Robertson and Zamudio (2009) |
|                     | tMet-frog    | TTGGGGTATGGGCCCAAAAGCT       |           | Wiens et al. (2005)          |
| <i>α-fibrinogen</i> | MVZ47        | AGTGAAAGATACAGTCACAGTGCTAGG  | 49-59°C   | Bell et al. (2011)           |
|                     | MVZ48        | GGAGGATATCAGCACAGTCTAAAAAG   |           | Bell et al. (2011)           |
| <i>Cmyc</i>         | cmyc1U       | GAGGACATCTGGAARAARTT         | 57°C      | Crawford (2003)              |
|                     | Cmyc-ex2d R  | TCATTCAATGGGTAAGGGAAGACC     |           | Wiens et al. (2005)          |
| <i>β-crystallin</i> | Cryb1Ls      | CGCCTGATGTCTTTCCGCC          | 55°C      | Dolman and Phillips (2004)   |
|                     | Cryb2Ls      | CCAATGAAGTTCTCTTTCTCAA       |           | Dolman and Phillips (2004)   |
| <i>β-fibrinogen</i> | Fib-b17U     | GGAGAAAACAGGACAATGACAATTCAC  | 53°C      | Prychitko and Moore (1997)   |
|                     | Fib-b17L     | TCCCCAGTAGTATCTGCCATTAGGGTT  |           | Prychitko and Moore (1997)   |
| <i>M13</i>          | M13F         | GTA AACGACGGCCAG             | 54°C      | Messing (1983)               |
|                     | M13R         | CAGGAAACAGCTATGAC            |           | Messing (1983)               |

**Table 2. Transition rate models and marginal likelihoods (estimated in BayesTraits and expressed on a natural log scale) were used to select models with stronger support for trait correlations based on Bayes factors.**

| Traits correlation, model        | Independent | Dependent | Covarian |
|----------------------------------|-------------|-----------|----------|
| Reproductive mode vs. SVL        | -42.859     | -41.741†  | -40.046  |
| Reproductive mode vs. female SVL | -42.892     | -41.529†  | -39.952  |
| Reproductive mode vs. male SVL   | -43.943     | -42.859†  | -41.177  |
| Reproductive mode vs. SDI        | -40.708     | -37.130‡  | -36.114  |

SVL = snout-vent length. †Best models for trait correlations, with positive evidence based on Bayes factors ( $BF > 2$ ). ‡Best model for trait correlations, with strong evidence based on Bayes factors ( $BF > 5$ ).

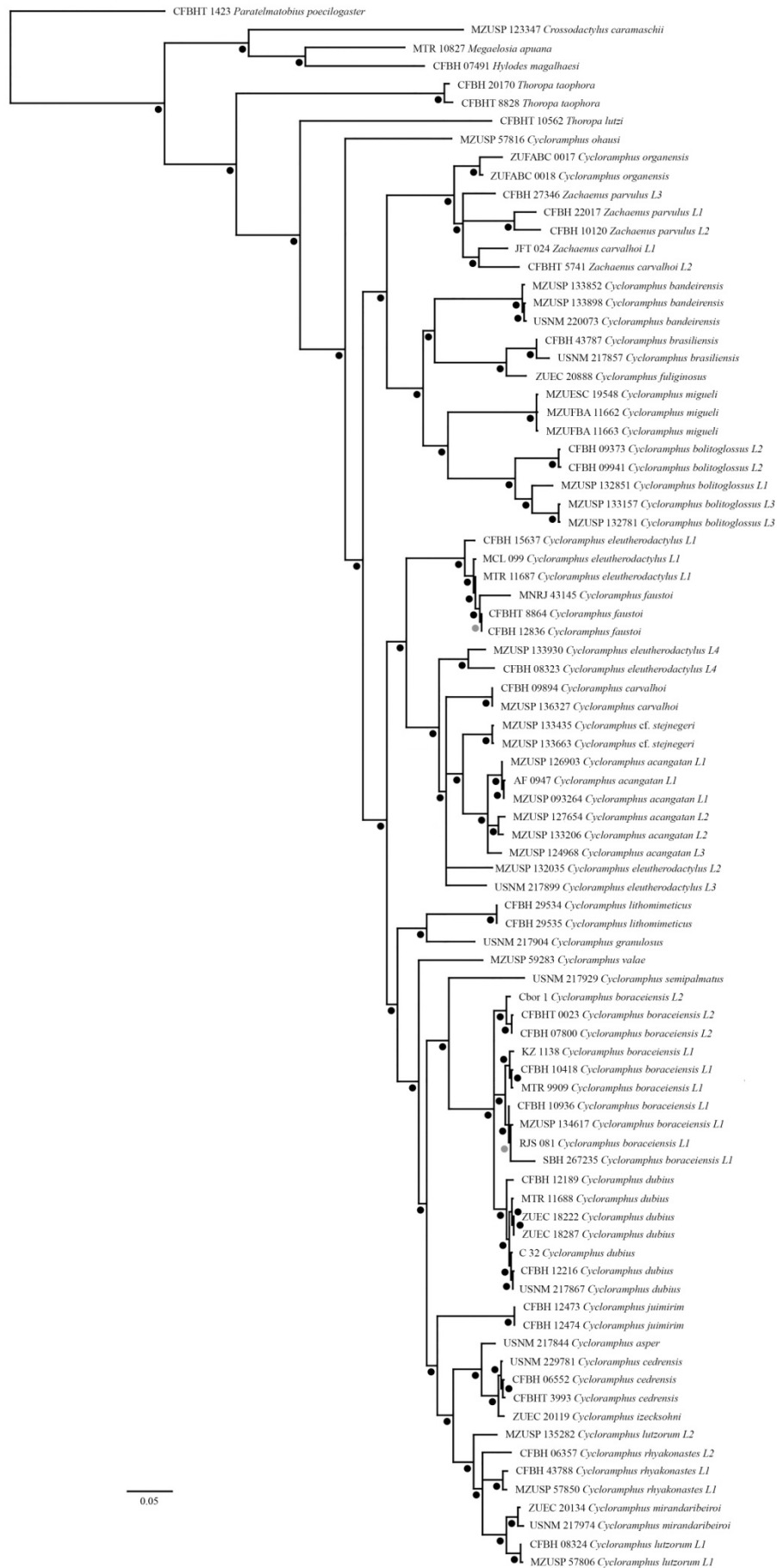


**Figure 1. Bayesian multilocus-based phylogeny for 46 species or lineages of *Cycloramphus* and *Zachaenus* (40 study species and six outgroup species). *L1*, *L2*, *L3*, and *L4* indicate deeply-divergent species lineages. Dots next to each node indicate Bayesian posterior probabilities (PP), with black dots indicating clades supported with PP > 90 and with gray dots indicating clades recovered with PP > 70. Nodes with PP < 50 are collapsed. This phylogeny was pruned to include named species and deeply divergent lineages for downstream comparative analyses.**

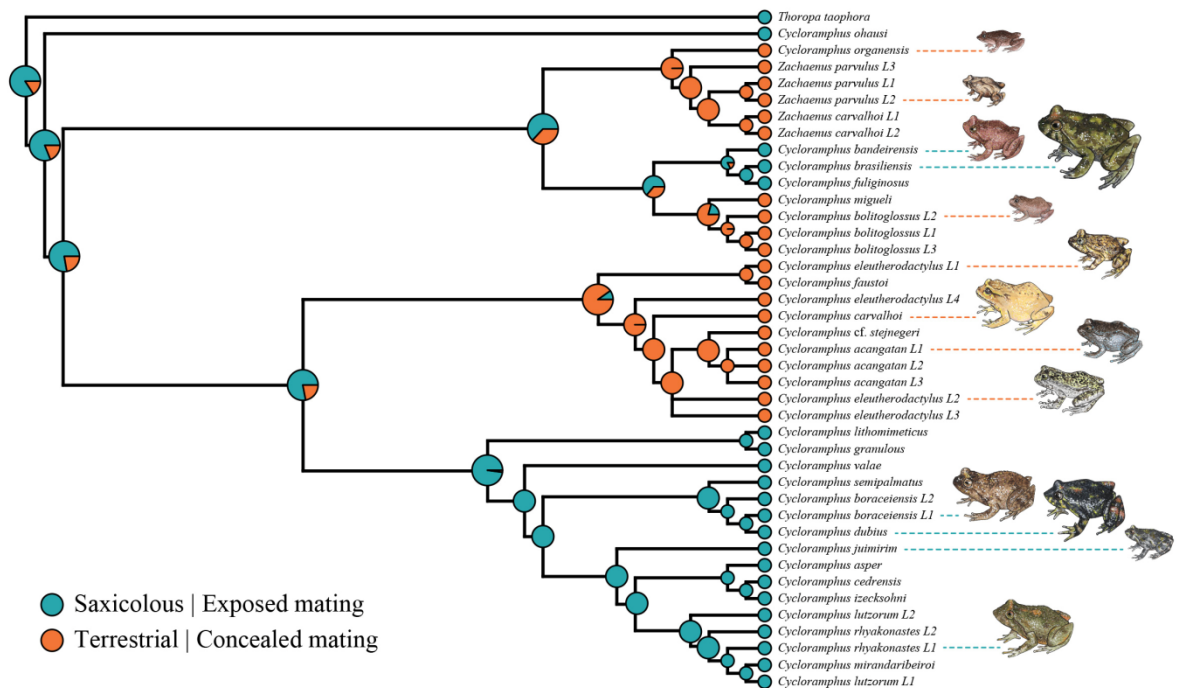


**Figure 2. Reconstructed evolution of reproductive habitat in *Cycloramphus-Zachaenus* frogs. Saxicolous habitats are associated with exposed mating and terrestrial habitats are associated with concealed mating. L1, L2, L3, and L4 indicate deeply-divergent species lineages. This phylogeny was used as a starting point for all downstream comparative analyses.**

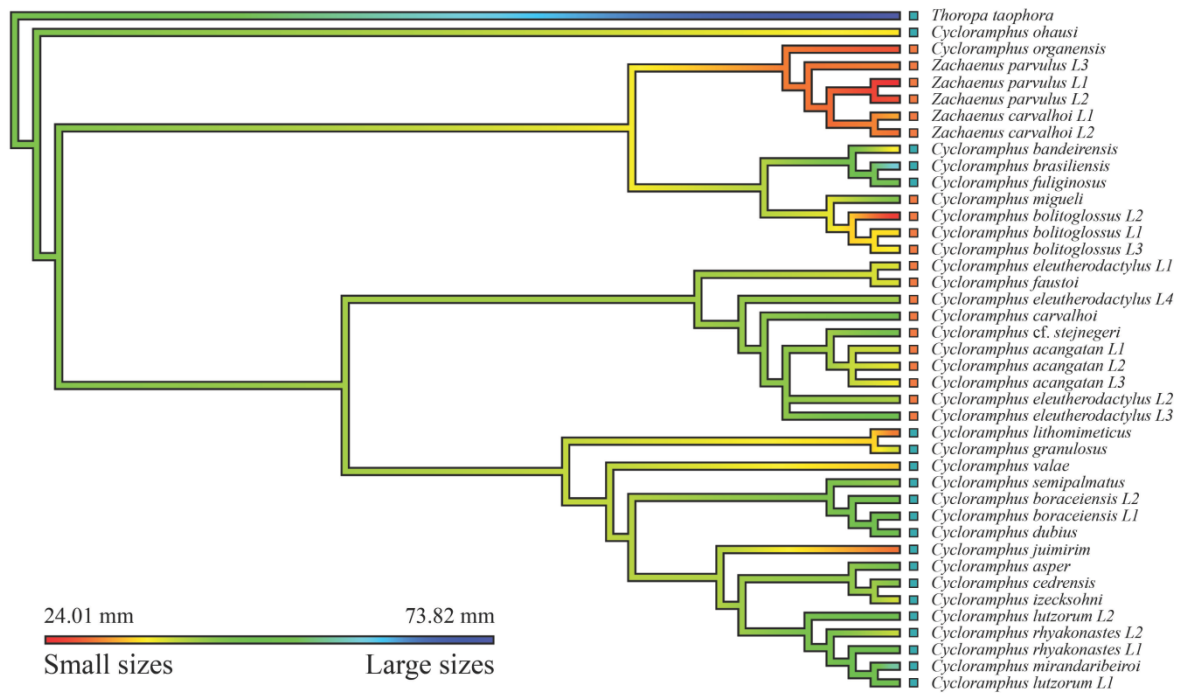


**Figure 3. Reconstructed evolution of overall species body sizes in *Cycloramphus-Zachaenus* frogs.** Blue squares and orange squares indicate saxicolous habitats (exposed mating) and terrestrial habitats (concealed mating), respectively. L1, L2, L3, and L4 indicate deeply-divergent species lineages.

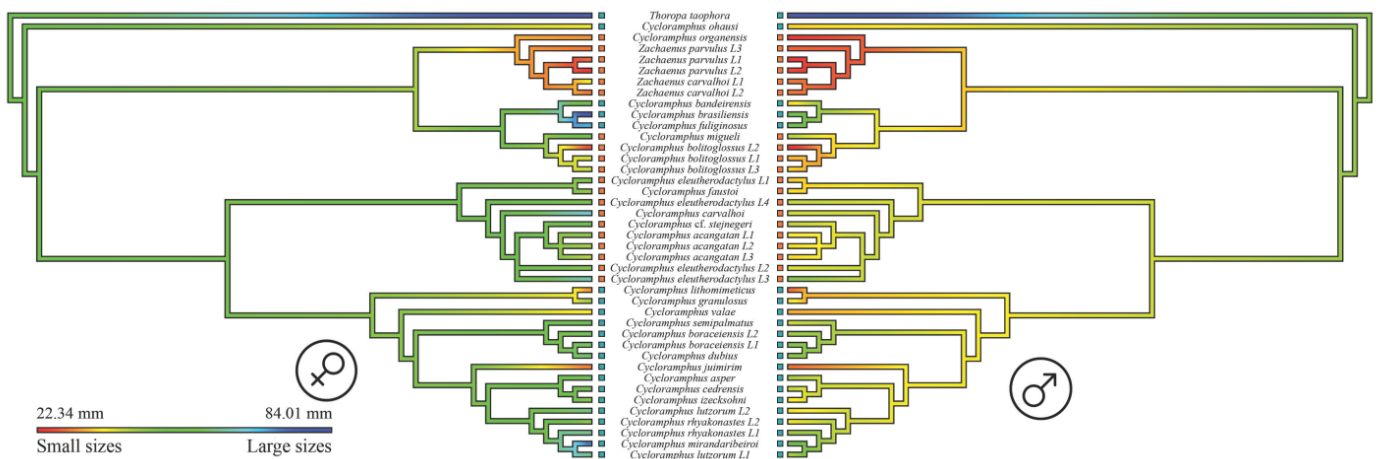


**Figure 4. Reconstructed evolution of female (♀) and male (♂) body sizes in *Cycloramphus-Zachaenus* frogs. Blue squares and orange squares indicate saxicolous habitats (exposed mating) and terrestrial habitats (concealed mating), respectively. L1, L2, L3, and L4 indicate deeply-divergent species lineages.**

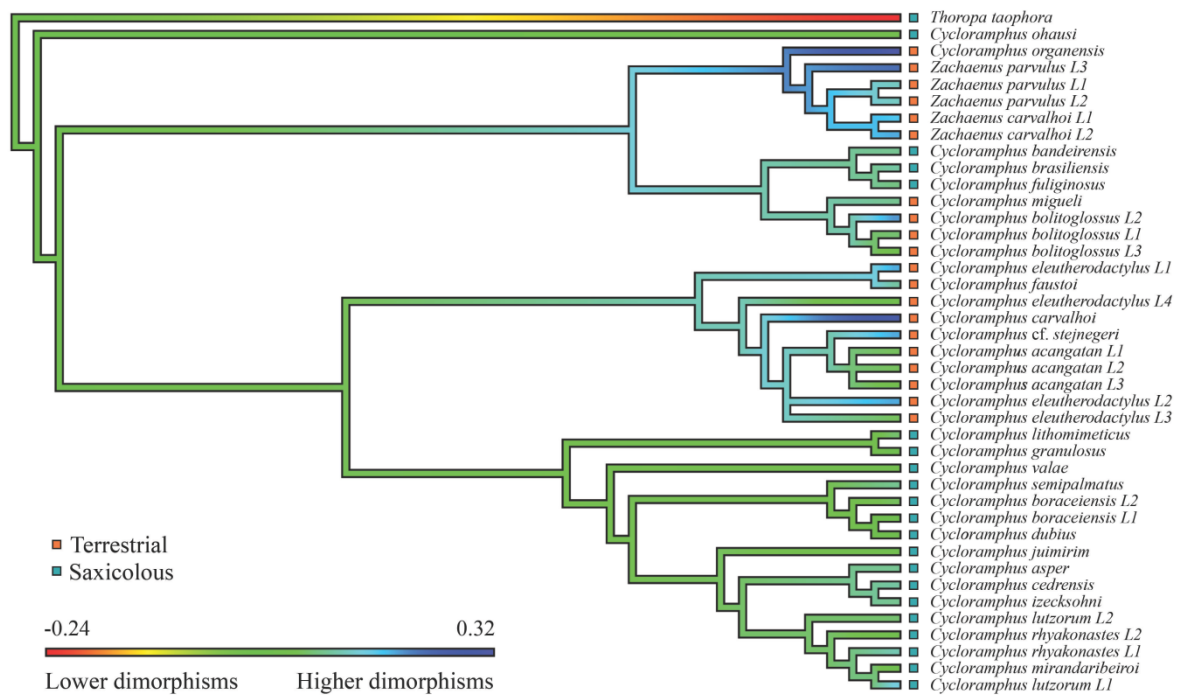


**Figure 5. Reconstructed evolution of sexual size dimorphism (SSD) in *Cycloramphus-Zachaenus* frogs on the pruned tree. Blue squares and orange squares indicate saxicolous habitats (exposed mating) and terrestrial habitats (concealed mating), respectively. SSD was quantified by the sexual dimorphism index (SDI) with lower and higher indices indicating lower and higher divergences in sizes between conspecific males and females. L1, L2, L3, and L4 indicate deeply-divergent species lineages.**

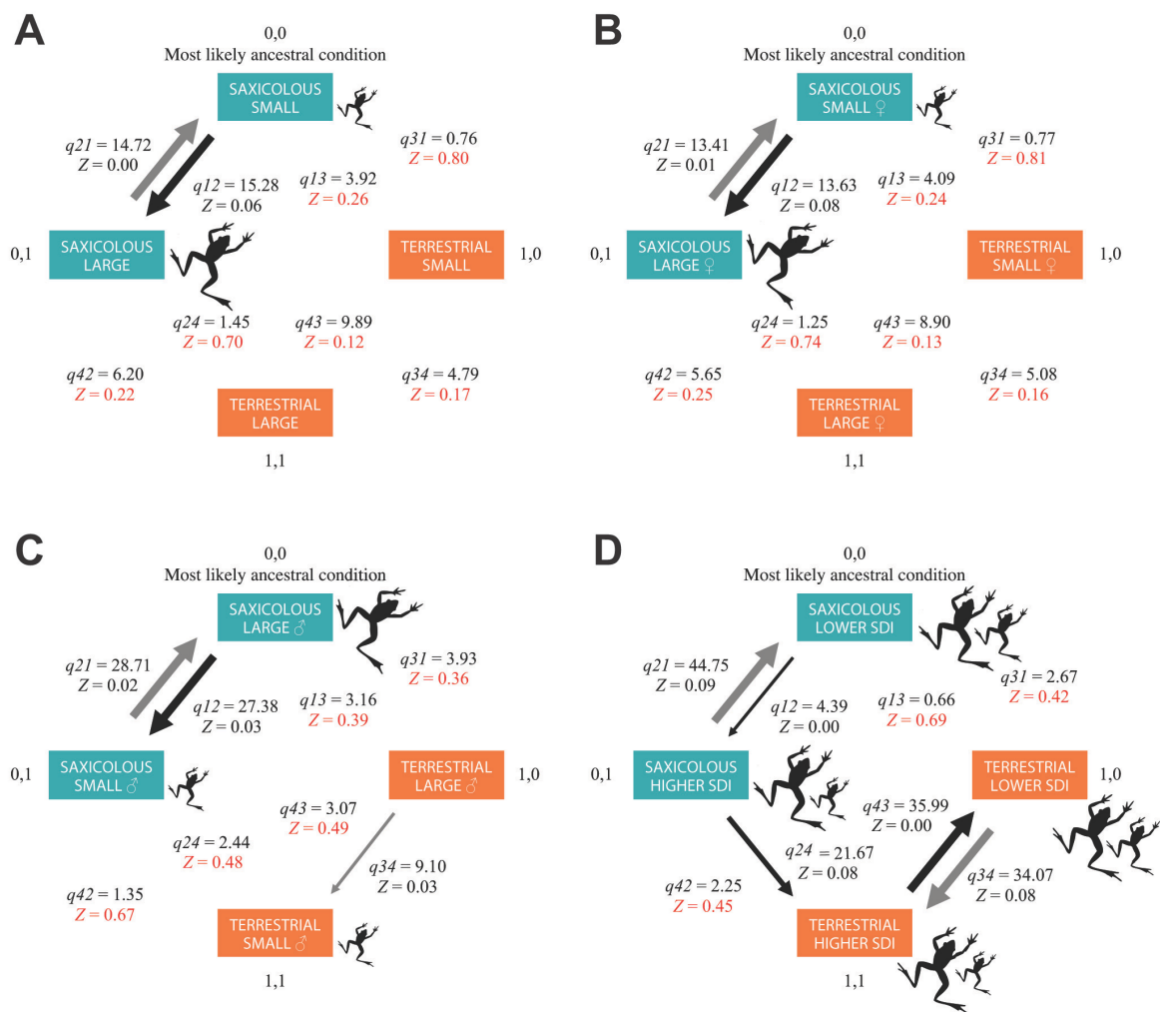


**Figure 6. Evolutionary transitions across *Cycloramphus* and *Zachaenus* frog species. BayesTraits transitions for species reproductive habitats (saxicolous or terrestrial) and (A) overall species body sizes, (B) female body sizes, (C) male body sizes, and (D) sexual size dimorphism (SSD). SSD was quantified by the sexual dimorphism index (SDI), with lower and higher indices indicating lower and higher divergences in sizes between conspecific males and females. Arrow thickness is proportional to each transition rate between trait states. For each transition, we reported a  $q$  value, representing the mean transition parameter, and a  $Z$  value, the proportion of iterations that were assigned to zero (i.e., unlikely transitions). Low  $Z$  values indicate more likely evolutionary transitions. Transitions are considered possible when  $Z < 0.10$  (10% of the iterations are assigned to zero) or when the nonsignificant transition parameter ( $q$  value) is higher than that of the lowest parameter that yielded a significant transition.  $Z$  values for non-significant transitions are shown in red.**

# **Male-male competition and repeated evolution of terrestrial breeding in Atlantic Coastal Forest frogs**

*Supporting Information*

**Table S1. Specimens included in our phylogenetic analyses along with GenBank sequence accession numbers.**

| Species                              | Voucher number | Tissue number | Field number         | Type         | Locality                                                                  | GenBank sequence accession number |              |          |                      |          |                     |                     |
|--------------------------------------|----------------|---------------|----------------------|--------------|---------------------------------------------------------------------------|-----------------------------------|--------------|----------|----------------------|----------|---------------------|---------------------|
|                                      |                |               |                      |              |                                                                           | 16S                               | Cytochrome-b | ND1      | $\alpha$ -fibrinogen | Cmyc     | $\beta$ -crystallin | $\beta$ -fibrinogen |
| <i>Cycloramphus acangatan L1</i>     | MZUSP 093264   | -             | AF 0344   MTR 00344  | Topotype     | Parque Estadual Intervales, Ribeirão Grande, SP, Brazil                   | MN265152                          | -            | MN382570 | MN382409             | MN266325 | MN274440            | MN382489            |
| <i>Cycloramphus acangatan L1</i>     | -              | -             | AF 0947              | Paratopotype | Parque Estadual Intervales, Ribeirão Grande, SP, Brazil                   | MN265153                          | -            | MN382571 | MN382410             | MN266326 | MN274441            | MN382490            |
| <i>Cycloramphus acangatan L1</i>     | MZUSP 126903   | -             | MTR 07126            | Paratopotype | Parque Estadual Intervales, Ribeirão Grande, SP, Brazil                   | MN265154                          | MN266403     | MN382572 | MN382411             | MN266327 | MN274442            | MN382491            |
| <i>Cycloramphus acangatan L2</i>     | MZUSP 127654   | -             | AF 1302   ITH 0477   | Paratype     | Juquitiba, SP, Brazil                                                     | MN265155                          | MN266404     | MN382573 | MN382412             | MN266328 | MN274443            | MN382492            |
| <i>Cycloramphus acangatan L2</i>     | MZUSP 133206   | -             | AF 1569   MTR 10438  | -            | Reserva Biológica de Paranapiacaba, Santo André, SP, Brazil               | MN265156                          | MN266405     | MN382574 | MN382413             | MN266329 | MN274444            | MN382493            |
| <i>Cycloramphus acangatan L3</i>     | MZUSP 124968   | -             | ITH 0342             | Paratype     | Piedade, SP, Brazil                                                       | MN265157                          | MN266406     | MN382575 | MN382414             | MN266330 | MN274445            | MN382494            |
| <i>Cycloramphus asper</i>            | USNM 217844    | -             | RH 7832   SBH 267232 | Topotype     | between Queçaba and São Bonifácio, SC, Brazil                             | MN265158                          | MN266407     | MN382576 | MN382415             | MN266331 | MN274446            | MN382495            |
| <i>Cycloramphus bandeirensis</i>     | MZUSP 133852   | -             | MTR 10812            | Topotype     | Pico da Bandeira, Parque Nacional do Caparaó, Ibitirama, ES, Brazil       | MN265159                          | MN266408     | MN382577 | MN382416             | MN266332 | MN274447            | MN382496            |
| <i>Cycloramphus bandeirensis</i>     | MZUSP 133898   | -             | MTR 10853            | Topotype     | Pico da Bandeira, Parque Nacional do Caparaó, Ibitirama, ES, Brazil       | MN265160                          | MN266409     | MN382578 | MN382417             | MN266333 | MN274448            | MN382497            |
| <i>Cycloramphus bandeirensis</i>     | USNM 220073    | -             | RH 8076   SBH 267233 | Paratopotype | Pico da Bandeira, Parque Nacional do Caparaó, Ibitirama, ES, Brazil       | MN265161                          | MN266410     | MN382579 | MN382418             | MN266334 | MN274449            | MN382498            |
| <i>Cycloramphus bolitoglossus L1</i> | MZUSP 132851   | -             | MVS 106              | -            | Garuva, SC, Brazil                                                        | MN265162                          | MN266411     | MN382580 | MN382419             | MN266335 | MN274450            | MN382499            |
| <i>Cycloramphus bolitoglossus L2</i> | CFBH 09373     | CFBHT 2093    | -                    | -            | Anitápolis, SC, Brazil                                                    | MN265163                          | MN266412     | MN382581 | -                    | MN266336 | MN274451            | MN382500            |
| <i>Cycloramphus bolitoglossus L2</i> | CFBH 09941     | CFBHT 2886    | -                    | -            | Rio da Prata, Anitápolis, SC, Brazil                                      | MN265164                          | MN266413     | MN382582 | MN382420             | MN266337 | MN274452            | MN382501            |
| <i>Cycloramphus bolitoglossus L3</i> | MZUSP 132781   | -             | -                    | -            | São José dos Pinhais, PR, Brazil                                          | MN265165                          | MN266414     | MN382583 | MN382421             | MN266338 | MN274453            | MN382502            |
| <i>Cycloramphus bolitoglossus L3</i> | MZUSP 133157   | -             | Cbol 1               | -            | Serro e Gemido, São José dos Pinhais, PR, Brazil                          | MN265166                          | MN266415     | MN382584 | MN382422             | MN266339 | MN274454            | MN382503            |
| <i>Cycloramphus boraceiensis L1</i>  | MZUSP 134617   | -             | AF 0772   MTR 09494  | -            | Barra do Una, São Sebastião, SP, Brazil                                   | MN265167                          | MN266416     | MN382585 | MN382423             | MN266340 | MN274455            | MN382504            |
| <i>Cycloramphus boraceiensis L1</i>  | -              | -             | AF 1861   MTR 09909  | -            | Ilhabela, SP, Brazil                                                      | MN265168                          | MN266417     | MN382587 | MN382424             | MN266341 | MN274456            | MN382505            |
| <i>Cycloramphus boraceiensis L1</i>  | -              | -             | KZ 1138              | -            | Rio Perequê, Ilhabela, SP, Brazil                                         | MN265169                          | MN266418     | MN382586 | MN382425             | MN266342 | -                   | MN382506            |
| <i>Cycloramphus boraceiensis L1</i>  | -              | -             | RJS 081              | -            | Parque Estadual da Serra do Mar, São Sebastião, SP, Brazil                | MN265170                          | MN266419     | MN382588 | MN382426             | MN266343 | MN274457            | MN382507            |
| <i>Cycloramphus boraceiensis L1</i>  | -              | -             | SBH 267235           | Paratopotype | Estação Biológica de Boracéia, Salesópolis, SP, Brazil                    | MN265171                          | -            | -        | -                    | -        | MN274458            | -                   |
| <i>Cycloramphus boraceiensis L1</i>  | CFBH 10418     | CFBHT 2649    | RJS 138              | -            | Trilha do Veloso, Ilhabela, SP, Brazil                                    | MN265172                          | MN266420     | MN382589 | MN382427             | MN266344 | MN274459            | MN382508            |
| <i>Cycloramphus boraceiensis L1</i>  | CFBH 10936     | CFBHT 9727    | CAB 156              | -            | Barra do Una, São Sebastião, SP, Brazil                                   | MN265173                          | MN266421     | MN382590 | MN382428             | MN266345 | MN274460            | MN382509            |
| <i>Cycloramphus boraceiensis L2</i>  | -              | -             | Cbor 1               | -            | Instituto Oceanográfico da Universidade de São Paulo, Ubatuba, SP, Brazil | MN265174                          | MN266422     | MN382591 | MN382429             | MN266346 | MN274461            | MN382510            |
| <i>Cycloramphus boraceiensis L2</i>  | -              | CFBHT 0023    | -                    | -            | Parque Estadual da Serra do Mar, Picinguaba, Ubatuba, SP, Brazil          | MN265175                          | MN266423     | MN382592 | MN382430             | MN266347 | MN274462            | MN382511            |

|                                          |              |             |                      |              |                                                                          |          |          |          |          |          |          |          |
|------------------------------------------|--------------|-------------|----------------------|--------------|--------------------------------------------------------------------------|----------|----------|----------|----------|----------|----------|----------|
| <i>Cycloramphus boraceiensis</i> L2      | CFBH 07800   | -           | KZ 1082              | -            | 4 km from the São Paulo State border, Paraty, RJ, Brazil                 | MN265176 | MN266424 | MN382593 | MN382431 | MN266348 | MN274463 | MN382512 |
| <i>Cycloramphus brasiliensis</i>         | CFBH 43787   | CFBHT 22665 | PPGT 633             | -            | Parque Nacional da Serra dos Órgãos, Teresópolis, RJ, Brazil             | MN265177 | MN266425 | MN382594 | MN382432 | MN266349 | MN274464 | MN382513 |
| <i>Cycloramphus brasiliensis</i>         | USNM 217857  | -           | RH 5908   SBH 267234 | -            | Vale da Revolta, 5 km NE Alto do Soberbo, Teresópolis, RJ, Brazil        | MN265178 | MN266426 | MN382595 | -        | -        | -        | MN382514 |
| <i>Cycloramphus carvalhoi</i>            | CFBH 09894   | CFBHT 2311  | -                    | -            | Campos do Jordão, SP, Brazil                                             | MN265179 | MN266427 | MN382596 | MN382433 | MN266350 | MN274465 | MN382515 |
| <i>Cycloramphus carvalhoi</i>            | MZUSP 136327 | -           | AF 1599   MTR 11031  | -            | Campos do Jordão, SP, Brazil                                             | MN265180 | MN266428 | MN382597 | MN382434 | MN266351 | MN274466 | MN382516 |
| <i>Cycloramphus cedrensis</i>            | -            | CFBHT 3993  | KZ 1430              | -            | Rota das Cachoeiras, Corupá, SC, Brazil                                  | MN265181 | MN266429 | MN382598 | MN382435 | MN266352 | MN274467 | MN382517 |
| <i>Cycloramphus cedrensis</i>            | CFBH 06552   | CFBHT 238   | -                    | -            | Corupá, SC, Brazil                                                       | MN265182 | MN266430 | MN382599 | MN382436 | MN266353 | MN274468 | MN382518 |
| <i>Cycloramphus cedrensis</i>            | USNM 229781  | -           | RH 7970   SBH 267245 | Paratopotype | Rio dos Cedros, SC, Brazil                                               | MN265183 | MN266431 | MN382600 | MN382437 | -        | MN274469 | MN382519 |
| <i>Cycloramphus dubius</i>               | -            | -           | AF 1747   MTR 11688  | -            | Estação Biológica de Boracéia, Salesópolis, SP, Brazil                   | MN265184 | MN266432 | MN382601 | MN382438 | MN266354 | MN274470 | MN382520 |
| <i>Cycloramphus dubius</i>               | -            | -           | C 32                 | -            | Parque Estadual da Serra do Mar, Itutinga-Pilões, Cubatão, SP, Brazil    | MN265185 | MN266433 | MN382602 | MN382439 | MN266355 | MN274471 | MN382521 |
| <i>Cycloramphus dubius</i>               | CFBH 12189   | -           | CAB 624              | -            | Parque Estadual da Serra do Mar, Itariru, Peruibe, SP, Brazil            | MN265186 | MN266434 | MN382603 | MN382440 | MN266356 | MN274472 | MN382522 |
| <i>Cycloramphus dubius</i>               | CFBH 12216   | -           | CAB 695              | -            | Parque Estadual da Serra do Mar, Curucutu, Itanhaém, SP, Brazil          | MN265187 | MN266435 | MN382604 | MN382441 | MN266357 | MN274473 | MN382523 |
| <i>Cycloramphus dubius</i>               | ZUEC 18222   | -           | 0220                 | -            | Route SP-098, Biritiba-Mirim, SP, Brazil                                 | MN265188 | -        | MN382605 | MN382442 | MN266358 | MN274474 | MN382524 |
| <i>Cycloramphus dubius</i>               | ZUEC 18287   | -           | 0263                 | -            | Bertioga, SP, Brazil                                                     | MN265189 | -        | MN382606 | MN382443 | MN266359 | MN274475 | MN382525 |
| <i>Cycloramphus dubius</i>               | USNM 217867  | -           | RH 7787   SBH 267236 | Topotype     | Estrada Velha São Paulo-Santos, km 47, Cubatão, SP, Brazil               | MN265190 | MN266436 | MN382607 | MN382444 | MN266360 | MN274476 | MN382526 |
| <i>Cycloramphus eleutherodactylus</i> L1 | -            | -           | AF 1746   MTR 11687  | -            | Estação Biológica de Boracéia, Salesópolis, SP, Brazil                   | MN265191 | MN266437 | MN382608 | MN382445 | MN266361 | MN274477 | MN382527 |
| <i>Cycloramphus eleutherodactylus</i> L1 | -            | -           | MCL 099              | -            | Estação Biológica de Boracéia, Salesópolis, SP, Brazil                   | MN265192 | MN266438 | MN382609 | MN382446 | MN266362 | MN274478 | MN382528 |
| <i>Cycloramphus eleutherodactylus</i> L1 | CFBH 15637   | -           | KZ 1176              | -            | Road to Ponta Sul, Ilhabela, SP, Brazil                                  | MN265193 | MN266439 | MN382610 | MN382447 | MN266363 | -        | MN382529 |
| <i>Cycloramphus eleutherodactylus</i> L2 | MZUSP 132035 | -           | EEB 0264             | -            | Estação Ecológica de Bananal, SP, Brazil                                 | MN265194 | MN266440 | MN382611 | MN382448 | MN266364 | MN274479 | MN382530 |
| <i>Cycloramphus eleutherodactylus</i> L3 | USNM 217899  | -           | RH 6681   SBH 267238 | -            | Represa do Guinle, 3 km NE Alto do Soberbo, Teresópolis, RJ, Brazil      | MN265195 | MN266441 | MN382612 | MN382449 | MN266365 | MN274480 | MN382531 |
| <i>Cycloramphus eleutherodactylus</i> L4 | CFBH 08323   | CFBHT 1551  | -                    | -            | Parque Estadual Turístico do Alto Ribeira, Santana, Iporanga, SP, Brazil | MN265196 | MN266442 | MN382613 | MN382450 | MN266366 | MN274481 | MN382532 |
| <i>Cycloramphus eleutherodactylus</i> L4 | MZUSP 133930 | -           | AF 1075   MTR 09324  | -            | Parque Estadual Intervales, Ribeirão Grande, SP, Brazil                  | MN265197 | MN266443 | MN382614 | MN382451 | MN266367 | MN274482 | MN382533 |
| <i>Cycloramphus faustoi</i>              | -            | CFBHT 8864  | CLP 483              | Topotype     | Saco do Funil, Ilha dos Alcatrazes, São Sebastião, SP, Brazil            | MN265198 | MN266444 | MN382615 | MN382452 | MN266368 | MN274483 | MN382534 |
| <i>Cycloramphus faustoi</i>              | CFBH 12836   | CFBHT 8873  | IQ 100               | Paratopotype | Saco do Funil, Ilha dos Alcatrazes, São Sebastião, SP, Brazil            | MN265199 | MN266445 | MN382616 | MN382453 | MN266369 | MN274484 | MN382535 |
| <i>Cycloramphus faustoi</i>              | MNRJ 43145   | CFBHT 8928  | CAB 924              | Paratopotype | Saco do Funil, Ilha dos Alcatrazes, São Sebastião, SP, Brazil            | MN265200 | MN266446 | MN382617 | MN382454 | MN266370 | MN274485 | MN382536 |
| <i>Cycloramphus fuliginosus</i>          | ZUEC 20888   | -           | 1569                 | -            | Barra do Sana, Macaé, RJ, Brazil                                         | MN265201 | -        | MN382618 | MN382455 | -        | MN274486 | -        |
| <i>Cycloramphus granulosus</i>           | USNM 217904  | -           | RH 5052   SBH 267240 | Topotype     | Fazenda do Veado, Serra da Bocaina, São José do Barreiro, SP, Brazil     | MN265202 | MN266447 | MN382619 | MN382456 | MN266371 | MN274487 | MN382537 |
| <i>Cycloramphus izecksohni</i>           | ZUEC 20119   | -           | 0777                 | -            | Pomerode, SC, Brazil                                                     | MN265203 | -        | MN382620 | MN382457 | MN266372 | MN274488 | MN382538 |

|                                     |              |             |                      |              |                                                                              |          |          |          |          |          |          |          |
|-------------------------------------|--------------|-------------|----------------------|--------------|------------------------------------------------------------------------------|----------|----------|----------|----------|----------|----------|----------|
| <i>Cycloramphus juimirim</i>        | CFBH 12473   | CFBHT 3996  | KZ 1604              | Topotype     | Trilha do Imperador, Estação Ecológica de Juréia, Iguape, SP, Brazil         | MN265204 | MN266448 | MN382621 | MN382458 | MN266373 | MN274489 | MN382539 |
| <i>Cycloramphus juimirim</i>        | CFBH 12474   | CFBHT 3997  | KZ 1605              | Topotype     | Trilha do Imperador, Estação Ecológica de Juréia, Iguape, SP, Brazil         | MN265205 | MN266449 | MN382622 | MN382459 | MN266374 | MN274490 | MN382540 |
| <i>Cycloramphus lithomimeticus</i>  | CFBH 29534   | CFBHT14623  | HS 01                | Topotype     | Cachoeira de Itinguçu, Coroa Grande, Itaguaí, RJ, Brazil                     | MN265206 | MN266450 | MN382623 | MN382460 | MN266375 | MN274491 | MN382541 |
| <i>Cycloramphus lithomimeticus</i>  | CFBH 29535   | CFBHT14624  | HS 02                | Topotype     | Cachoeira de Itinguçu, Coroa Grande, Itaguaí, RJ, Brazil                     | MN265207 | MN266451 | MN382624 | MN382461 | MN266376 | MN274492 | MN382542 |
| <i>Cycloramphus lutzorum</i> L1     | CFBH 08324   | CFBHT 1550  | -                    | Topotype     | Parque Estadual Turístico do Alto Ribeira, Santana, Iporanga, SP, Brazil     | MN265208 | MN266452 | MN382625 | MN382462 | MN266377 | MN274493 | MN382543 |
| <i>Cycloramphus lutzorum</i> L1     | MZUSP 57806  | -           | RH 7791   SBH 267241 | Paratopotype | Road to Apiaí, 11 km W Iporanga, SP, Brazil                                  | MN265209 | MN266453 | MN382626 | MN382463 | MN266378 | MN274494 | MN382544 |
| <i>Cycloramphus lutzorum</i> L2     | MZUSP 135282 | -           | MVS 210              | -            | Rio Sagrado, Morretes, PR, Brazil                                            | MN265210 | MN266454 | MN382627 | MN382464 | MN266379 | MN274495 | MN382545 |
| <i>Cycloramphus migueli</i>         | MZUESC 19548 | -           | ID 480               | -            | Serra do Timbó, Amargosa, BA, Brazil                                         | MN265211 | MN266455 | MN382628 | MN382465 | MN266380 | MN274496 | MN382546 |
| <i>Cycloramphus migueli</i>         | MZUFBA 11662 | -           | PEU 530              | -            | Ubaíra, BA, Brazil                                                           | MN265212 | MN266456 | MN382629 | MN382466 | MN266381 | MN274497 | MN382547 |
| <i>Cycloramphus migueli</i>         | MZUFBA 11663 | -           | PEU 535              | -            | Ubaíra, BA, Brazil                                                           | MN265213 | MN266457 | MN382630 | MN382467 | MN266382 | MN274498 | MN382548 |
| <i>Cycloramphus mirandaribeiroi</i> | ZUEC 20134   | -           | 0723                 | Topotype     | Route PR-410 to Curitiba, Recanto Mãe Catira, Morretes, PR, Brazil           | MN265214 | -        | -        | MN382468 | MN266383 | MN274499 | MN382549 |
| <i>Cycloramphus mirandaribeiroi</i> | USNM 217974  | -           | RH 7644   SBH 267239 | Paratopotype | Route PR-410 to Curitiba, 15 km W São João da Graciosa, Morretes, PR, Brazil | MN265215 | -        | MN382631 | MN382469 | -        | MN274500 | MN382550 |
| <i>Cycloramphus ohausi</i>          | MZUSP 57816  | -           | RH 5895   SBH 267242 | Topotype     | Vale da Revolta, 5 km NE Alto do Soberbo, Teresópolis, RJ, Brazil            | MN265216 | MN266458 | MN382632 | MN382470 | MN266384 | MN274501 | MN382551 |
| <i>Cycloramphus organensis</i>      | ZUFABC 0017  | -           | VV 01                | Topotype     | Parque Nacional da Serra dos Órgãos, Petrópolis, RJ, Brazil                  | MN265217 | -        | -        | MN382471 | MN266385 | MN274502 | MN382552 |
| <i>Cycloramphus organensis</i>      | ZUFABC 0018  | -           | VV 02                | Topotype     | Parque Nacional da Serra dos Órgãos, Petrópolis, RJ, Brazil                  | MN265218 | MN266459 | MN382633 | MN382472 | MN266386 | MN274503 | MN382553 |
| <i>Cycloramphus rhyakonastes</i> L1 | CFBH 43788   | CFBHT 22666 | BB 514               | Topotype     | Parque Estadual Pico do Marumbi, Morretes, PR, Brazil                        | MN265219 | -        | MN382634 | MN382473 | MN266387 | MN274504 | MN382554 |
| <i>Cycloramphus rhyakonastes</i> L1 | MZUSP 57850  | -           | RH 7693   SBH 267237 | Paratopotype | Route PR-410 to Curitiba, 12 km W São João da Graciosa, Morretes, PR, Brazil | MN265220 | -        | MN382635 | MN382474 | MN266388 | MN274505 | MN382555 |
| <i>Cycloramphus rhyakonastes</i> L2 | CFBH 06357   | CFBHT 0456  | -                    | -            | Parque Ilha do Cardoso, Cananéia, SP, Brazil                                 | MN265221 | MN266460 | MN382636 | MN382475 | MN266389 | MN274506 | MN382556 |
| <i>Cycloramphus semipalmatus</i>    | USNM 217929  | -           | RH 7789   SBH 267243 | Topotype     | Estrada Velha São Paulo-Santos, Cubatão, SP, Brazil                          | MN265222 | MN266461 | MN382637 | -        | -        | MN274507 | MN382557 |
| <i>Cycloramphus cf. stejnegeri</i>  | MZUSP 133435 | -           | EEB 1714             | -            | Estação Ecológica de Bananal, SP, Brazil                                     | MN265223 | MN266462 | MN382638 | MN382476 | MN266390 | MN274508 | MN382558 |
| <i>Cycloramphus cf. stejnegeri</i>  | MZUSP 133663 | -           | EEB 0471             | -            | Estação Ecológica de Bananal, SP, Brazil                                     | MN265224 | MN266463 | MN382639 | MN382477 | MN266391 | MN274509 | MN382559 |
| <i>Cycloramphus valae</i>           | MZUSP 59283  | -           | RH 8186   SBH 267244 | -            | Road to Bom Jesus, 10 km W from Timbó do Sul, SC, Brazil                     | MN265225 | MN266464 | MN382640 | -        | -        | -        | MN382560 |
| <i>Zachaeus carvalhoi</i> L1        | -            | -           | JFT 024              | -            | Cariacica, ES, Brazil                                                        | MN265226 | MN266465 | MN382641 | MN382478 | MN266392 | MN274510 | MN382561 |
| <i>Zachaeus carvalhoi</i> L2        | -            | CFBHT 5741  | MTR 12613            | -            | Córrego do Calçado, Santa Marta, Ibitirama, ES, Brazil                       | MN265227 | MN266466 | MN382642 | MN382479 | MN266393 | MN274511 | MN382562 |
| <i>Zachaeus parvulus</i> L1         | CFBH 22017   | CFBHT 12623 | BB 139               | Topotype     | Parque Nacional da Serra dos Órgãos, Teresópolis, RJ, Brazil                 | MN265228 | MN266467 | MN382643 | MN382480 | MN266394 | MN274512 | MN382563 |
| <i>Zachaeus parvulus</i> L2         | CFBH 10120   | CFBHT 2427  | -                    | Topotype     | Petrópolis, RJ, Brazil                                                       | MN265229 | MN266468 | -        | -        | -        | MN274513 | MN382564 |
| <i>Zachaeus parvulus</i> L3         | CFBH 27346   | CFBHT 13005 | MTCT 0264            | -            | Santa Maria Madalena (Parque Estadual do Desengano), RJ, Brazil              | MN265230 | MN266469 | MN382644 | MN382481 | MN266395 | MN274514 | MN382565 |
| <i>Thoropa lutzi</i>                | -            | CFBHT 10562 | -                    | -            | Alegre, ES, Brazil                                                           | MN265231 | -        | MN382645 | MN382482 | MN266396 | MN274515 | MN382566 |

|                                      |              |            |           |   |                                                                                     |          |          |          |          |          |          |          |
|--------------------------------------|--------------|------------|-----------|---|-------------------------------------------------------------------------------------|----------|----------|----------|----------|----------|----------|----------|
| <i>Thoropa taophora</i>              | CFBH 20170   | CFBHT 8735 | CAB 1873  | - | Ilha dos Gatos, São Sebastião, SP, Brazil                                           | MN265232 | MN266470 | MN382646 | MN382483 | MN266397 | MN274516 | -        |
| <i>Thoropa taophora</i>              | -            | CFBHT 8828 | CAB 59    | - | Ilha das Couves, Ubatuba, SP, Brazil                                                | MN265233 | MN266471 | MN382647 | MN382484 | MN266398 | MN274517 | -        |
| <i>Crossodactylus caramaschii</i>    | MZUSP 123347 | -          | ITH 0275  | - | Piedade, SP, Brazil                                                                 | MN265234 | MN266472 | MN382648 | MN382485 | MN266399 | -        | MN382567 |
| <i>Hylodes magalhaesi</i>            | CFBH 07491   | CFBHT 1217 | -         | - | Monte Verde, Camanducaia, MG, Brazil                                                | MN265235 | MN266473 | MN382649 | MN382486 | MN266400 | MN274518 | -        |
| <i>Megaelosia apuana</i>             | -            | -          | MTR 10827 | - | Parque Nacional do Caparaó, Ibitirama, ES, Brazil                                   | MN265236 | MN266474 | MN382650 | MN382487 | MN266401 | MN274519 | MN382568 |
| <i>Paratelmatobius poecilogaster</i> | -            | CFBHT 1423 | -         | - | Parque Estadual da Serra do Mar, Santa Virgínia, São Luiz do Paraitinga, SP, Brazil | MN265237 | MN266475 | MN382651 | MN382488 | MN266402 | -        | MN382569 |

**Table S2. Summary of phenotypic data matrix including female and male body sizes, sexual size dimorphism, reproductive habitat use, and sampled localities grouped by species and lineage.**

| Species                               | Sex    | <i>n</i> | Mean SVL $\pm$ SD (range) mm   | Overall mean SVL mm | SDI  | Ecology     | Sampled localities                                                                                                                                   |
|---------------------------------------|--------|----------|--------------------------------|---------------------|------|-------------|------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Cycloramphus acangatan</i> L1†     | Female | 11       | 39.82 $\pm$ 4.7 (33.22–48.76)  | 38.21               | 0.09 | Terrestrial | Guapiara and Ribeirão Grande (Parque Estadual Intervales), and São Miguel Arcanjo (Parque Estadual Carlos Botelho), SP, Brazil                       |
|                                       | Male   | 12       | 36.60 $\pm$ 3.98 (31.69–43.68) |                     |      |             |                                                                                                                                                      |
| <i>Cycloramphus acangatan</i> L2†     | Female | 10       | 38.44 $\pm$ 4.17 (32.28–43.79) | 36.87               | 0.09 | Terrestrial | Cotia and Ibiúna (Reserva do Morro Grande), Embu, Juquitiba, Santo André (Reserva Biológica de Paranapiacaba), and São Bernardo do Campo, SP, Brazil |
|                                       | Male   | 10       | 35.29 $\pm$ 2.19 (32.57–39.01) |                     |      |             |                                                                                                                                                      |
| <i>Cycloramphus acangatan</i> L3†     | Female | 11       | 37.28 $\pm$ 5.54 (31.77–47.95) | 36.14               | 0.07 | Terrestrial | Piedade and Pilar do Sul, SP, Brazil                                                                                                                 |
|                                       | Male   | 22       | 35.00 $\pm$ 2.89 (30.15–39.86) |                     |      |             |                                                                                                                                                      |
| <i>Cycloramphus asper</i> †           | Female | 5        | 47.74 $\pm$ 2.59 (44.97–50.68) | 45.21               | 0.12 | Saxicolous  | between Queçaba and São Bonifácio, SC, Brazil                                                                                                        |
|                                       | Male   | 4        | 42.69 $\pm$ 3.85 (37.06–45.66) |                     |      |             |                                                                                                                                                      |
| <i>Cycloramphus bandeirensis</i> †    | Female | 0        | -                              | 35.19               | -    | Saxicolous  | Ibitirama (Pico da Bandeira, Parque Nacional do Caparaó), ES, Brazil                                                                                 |
|                                       | Male   | 7        | 35.19 $\pm$ 2.05 (32.13–37.10) |                     |      |             |                                                                                                                                                      |
| <i>Cycloramphus bolitoglossus</i> L1† | Female | 2        | 36.03 $\pm$ 0.13 (35.94–36.12) | 34.32               | 0.10 | Terrestrial | Corupá, Garuva, and Joinville, SC, Brazil                                                                                                            |
|                                       | Male   | 3        | 32.61 $\pm$ 3.00 (29.16–34.63) |                     |      |             |                                                                                                                                                      |
| <i>Cycloramphus bolitoglossus</i> L2  | Female | 13       | 27.97 $\pm$ 1.64 (24.09–29.86) | 25.41               | 0.22 | Terrestrial | Anitápolis, SC, Brazil                                                                                                                               |
|                                       | Male   | 2        | 22.86 $\pm$ 0.28 (22.66–23.05) |                     |      |             |                                                                                                                                                      |
| <i>Cycloramphus bolitoglossus</i> L3  | Female | 4        | 36.61 $\pm$ 2.39 (33.09–38.33) | 35.54               | 0.06 | Terrestrial | Bocaiúva do Sul (Volta Grande) and São José dos Pinhais, PR, Brazil                                                                                  |
|                                       | Male   | 4        | 34.47 $\pm$ 2.17 (33.08–37.67) |                     |      |             |                                                                                                                                                      |
| <i>Cycloramphus boraceiensis</i> L1†  | Female | 50       | 47.98 $\pm$ 6.08 (36.71–58.97) | 47.24               | 0.03 | Saxicolous  | Ilhabela, Salesópolis (Estação Biológica de Boracéia), and São Sebastião (Barra do Una and Parque Estadual da Serra do Mar), SP, Brazil              |
|                                       | Male   | 44       | 46.50 $\pm$ 4.75 (36.78–58.02) |                     |      |             |                                                                                                                                                      |
| <i>Cycloramphus boraceiensis</i> L2   | Female | 64       | 47.68 $\pm$ 5.81 (35.65–59.09) | 45.66               | 0.09 | Saxicolous  | Caraguatatuba and Ubatuba (including Parque Estadual da Serra do Mar), SP, and Paraty (including Parque Nacional da Serra da Bocaina), RJ, Brazil    |
|                                       | Male   | 64       | 43.63 $\pm$ 4.42 (35.19–56.87) |                     |      |             |                                                                                                                                                      |
| <i>Cycloramphus brasiliensis</i>      | Female | 16       | 62.51 $\pm$ 7.12 (48.39–71.81) | 59.36               | 0.11 | Saxicolous  | Guapimirim, Petrópolis, and Teresópolis (including Alto do Soberbo and Parque Nacional da Serra dos Órgãos), and Silva Jardim, RJ, Brazil            |
|                                       | Male   | 18       | 56.21 $\pm$ 5.36 (47.07–66.27) |                     |      |             |                                                                                                                                                      |
| <i>Cycloramphus carvalhoi</i>         | Female | 1        | 52.03                          | 45.67               | 0.32 | Terrestrial | Campos do Jordão, SP, Brazil                                                                                                                         |
|                                       | Male   | 2        | 39.32 $\pm$ 1.75 (38.08–40.55) |                     |      |             |                                                                                                                                                      |

|                                           |        |    |                            |       |      |             |                                                                                                                                                                                     |
|-------------------------------------------|--------|----|----------------------------|-------|------|-------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Cycloramphus cedrensis</i> †           | Female | 2  | 45.91 ± 2.16 (44.38–47.44) | 43.19 | 0.13 | Saxicolous  | Rio dos Cedros, SC, Brazil                                                                                                                                                          |
|                                           | Male   | 5  | 40.46 ± 1.73 (38.96–42.54) |       |      |             |                                                                                                                                                                                     |
| <i>Cycloramphus dubius</i> †              | Female | 84 | 48.17 ± 6.16 (36.78–62.94) | 47.03 | 0.05 | Saxicolous  | Bertioga, Biritiba-Mirim, Cubatão (including Estrada Velha São Paulo-Santos, km 40-47), Peruipe (Parque Estadual da Serra do Mar), Santos, Santo André, and São Vicente, SP, Brazil |
|                                           | Male   | 49 | 45.90 ± 4.06 (36.75–57.65) |       |      |             |                                                                                                                                                                                     |
| <i>Cycloramphus eleutherodactylus</i> L1† | Female | 4  | 41.06 ± 2.12 (38.13–42.75) | 37.48 | 0.21 | Terrestrial | Ilhabela and Santo André (Reserva Biológica de Paranapiacaba), SP, Brazil                                                                                                           |
|                                           | Male   | 7  | 33.90 ± 2.08 (30.85–36.97) |       |      |             |                                                                                                                                                                                     |
| <i>Cycloramphus eleutherodactylus</i> L2  | Female | 7  | 40.37 ± 1.52 (38.26–43.12) | 40.37 | -    | Terrestrial | Bananal (Estação Ecológica de Bananal), SP, Brazil                                                                                                                                  |
|                                           | Male   | 0  | -                          |       |      |             |                                                                                                                                                                                     |
| <i>Cycloramphus eleutherodactylus</i> L3  | Female | 10 | 50.69 ± 5.40 (40.83–60.30) | 48.53 | 0.09 | Terrestrial | Teresópolis (Represa do Guinle, 3 km NE Alto do Soberbo, and Parque Nacional da Serra dos Órgãos), RJ, Brazil                                                                       |
|                                           | Male   | 10 | 46.38 ± 4.16 (38.70–52.64) |       |      |             |                                                                                                                                                                                     |
| <i>Cycloramphus eleutherodactylus</i> L4  | Female | 8  | 41.42 ± 4.49 (35.75–47.30) | 41.00 | 0.02 | Terrestrial | Apiaí and Iporanga, SP, Brazil                                                                                                                                                      |
|                                           | Male   | 9  | 40.59 ± 5.71 (34.20–50.31) |       |      |             |                                                                                                                                                                                     |
| <i>Cycloramphus fausto</i> †              | Female | 4  | 39.7 ± 5.78 (31.25–44.34)  | 37.44 | 0.13 | Terrestrial | São Sebastião (Saco do Funil, Ilha dos Alcatrazes), SP, Brazil                                                                                                                      |
|                                           | Male   | 5  | 35.18 ± 2.62 (31.21–37.88) |       |      |             |                                                                                                                                                                                     |
| <i>Cycloramphus fuliginosus</i> †         | Female | 23 | 55.59 ± 5.20 (45.40–64.54) | 52.37 | 0.13 | Saxicolous  | Macaé (Barra do Sana) and Rio de Janeiro (Guanabara, Realengo, and Tijuca), RJ, Brazil                                                                                              |
|                                           | Male   | 23 | 49.15 ± 4.00 (40.32–55.30) |       |      |             |                                                                                                                                                                                     |
| <i>Cycloramphus granulosus</i> †          | Female | 13 | 38.54 ± 3.24 (31.89–42.20) | 37.83 | 0.04 | Saxicolous  | São José do Barreiro (Campo de Fruticultura and Fazenda do Veado, Serra da Bocaina), SP, Brazil                                                                                     |
|                                           | Male   | 14 | 37.11 ± 2.96 (30.99–40.35) |       |      |             |                                                                                                                                                                                     |
| <i>Cycloramphus izecksohni</i> †          | Female | 14 | 39.98 ± 4.71 (31.38–48.92) | 37.91 | 0.12 | Saxicolous  | Corupá, Joinville (including Pirabeiraba), and Pomerode, SC, Brazil                                                                                                                 |
|                                           | Male   | 11 | 35.84 ± 3.12 (31.18–39.90) |       |      |             |                                                                                                                                                                                     |
| <i>Cycloramphus juimirim</i> †            | Female | 11 | 29.85 ± 5.28 (23.62–37.92) | 29.01 | 0.06 | Saxicolous  | Iguape (Estação Ecológica de Juréia), SP, Brazil                                                                                                                                    |
|                                           | Male   | 17 | 28.18 ± 3.13 (23.55–36.34) |       |      |             |                                                                                                                                                                                     |
| <i>Cycloramphus lithomimeticus</i> †      | Female | 4  | 30.95 ± 0.84 (29.79–31.80) | 29.48 | 0.10 | Saxicolous  | Itaguaí (Cachoeira de Itinguçu, Coroa Grande), RJ, Brazil                                                                                                                           |
|                                           | Male   | 13 | 28.02 ± 1.62 (25.36–31.14) |       |      |             |                                                                                                                                                                                     |
| <i>Cycloramphus lutzorum</i> L1†          | Female | 8  | 49.42 ± 6.33 (39.58–56.58) | 45.84 | 0.17 | Saxicolous  | Apiaí, Eldorado, and Iporanga (including Parque Estadual Turístico do Alto Ribeira, Santana, and road to Apiaí, 11 km W Iporanga), SP, Brazil                                       |
|                                           | Male   | 19 | 42.27 ± 2.31 (39.42–48.57) |       |      |             |                                                                                                                                                                                     |
| <i>Cycloramphus lutzorum</i> L2           | Female | 1  | 48.87                      | 48.87 | -    | Saxicolous  | Morretes, PR, Brazil                                                                                                                                                                |
|                                           | Male   | 0  | -                          |       |      |             |                                                                                                                                                                                     |

|                                           |        |    |                             |       |      |             |                                                                                                                           |
|-------------------------------------------|--------|----|-----------------------------|-------|------|-------------|---------------------------------------------------------------------------------------------------------------------------|
| <i>Cycloramphus miguelti</i> †            | Female | 2  | 46.08 ± 8.27 (40.23–51.93)  | 43.69 | 0.12 | Terrestrial | São José (Fazenda Unacau) and Uruçuca (Parque Estadual da Serra do Conduru), BA, Brazil                                   |
|                                           | Male   | 1  | 41.30                       |       |      |             |                                                                                                                           |
| <i>Cycloramphus mirandaribeiroi</i> †     | Female | 5  | 57.70 ± 9.89 (46.64–67.56)  | 56.58 | 0.04 | Saxicolous  | Morretes (Route PR-410 to Curitiba, 6-16 km W São João da Graciosa), PR, Brazil                                           |
|                                           | Male   | 8  | 55.46 ± 6.29 (44.66–64.02)  |       |      |             |                                                                                                                           |
| <i>Cycloramphus ohausi</i> †              | Female | 19 | 34.96 ± 2.11 (31.88–38.69)  | 34.85 | 0.01 | Saxicolous  | Petrópolis and Teresópolis (Parque Nacional da Serra dos Órgãos), RJ, Brazil                                              |
|                                           | Male   | 14 | 34.75 ± 3.13 (29.47–40.40)  |       |      |             |                                                                                                                           |
| <i>Cycloramphus organensis</i> †          | Female | 3  | 30.92 ± 0.87 (29.94–31.59)  | 27.13 | 0.32 | Terrestrial | Petrópolis (Parque Nacional da Serra dos Órgãos), RJ, Brazil                                                              |
|                                           | Male   | 6  | 23.34 ± 1.80 (21.50–25.88)  |       |      |             |                                                                                                                           |
| <i>Cycloramphus rhyakonastes</i> L1†      | Female | 12 | 49.81 ± 3.75 (41.41–53.99)  | 46.80 | 0.14 | Saxicolous  | Morretes (São João da Graciosa), PR, Brazil                                                                               |
|                                           | Male   | 13 | 43.79 ± 3.21 (37.44–48.71)  |       |      |             |                                                                                                                           |
| <i>Cycloramphus rhyakonastes</i> L2       | Female | 1  | 39.57                       | 38.50 | 0.06 | Saxicolous  | Cananéia (Ilha do Cardoso), SP, Brazil                                                                                    |
|                                           | Male   | 1  | 37.43                       |       |      |             |                                                                                                                           |
| <i>Cycloramphus semipalmatus</i> †        | Female | 20 | 44.73 ± 2.82 (37.53–48.67)  | 42.27 | 0.12 | Saxicolous  | Cubatão and Santo André (Reserva Biológica de Paranapiacaba), and Salesópolis (Estação Biológica de Boracéia), SP, Brazil |
|                                           | Male   | 24 | 39.81 ± 2.39 (34.33–46.57)  |       |      |             |                                                                                                                           |
| <i>Cycloramphus</i> cf. <i>stejnegeri</i> | Female | 5  | 45.59 ± 3.30 (40.53–48.38)  | 45.59 | -    | Terrestrial | Bananal (Estação Ecológica de Bananal), SP, Brazil                                                                        |
|                                           | Male   | 0  | -                           |       |      |             |                                                                                                                           |
| <i>Cycloramphus valae</i> †               | Female | 10 | 34.17 ± 2.59 (30.50–37.97)  | 33.12 | 0.07 | Saxicolous  | Bom Jardim da Serra, Praia Grande, and Timbé do Sul, SC, Brazil                                                           |
|                                           | Male   | 11 | 32.08 ± 1.21 (30.75–35.06)  |       |      |             |                                                                                                                           |
| <i>Zachaenus carvalhoi</i> L1†            | Female | 1  | 34.41                       | 31.63 | 0.19 | Terrestrial | Cariacica and Santa Teresa, ES, Brazil                                                                                    |
|                                           | Male   | 6  | 28.85 ± 1.56 (26.35–30.83)  |       |      |             |                                                                                                                           |
| <i>Zachaenus carvalhoi</i> L2             | Female | 0  | -                           | 29.00 | -    | Terrestrial | Ibitirama, ES, Brazil                                                                                                     |
|                                           | Male   | 2  | 29.00 ± 0.80 (28.42–29.61)‡ |       |      |             |                                                                                                                           |
| <i>Zachaenus parvulus</i> L1†             | Female | 1  | 28.84                       | 26.97 | 0.15 | Terrestrial | Teresópolis (Alto do Soberbo, Parque Nacional da Serra dos Órgãos), RJ, Brazil                                            |
|                                           | Male   | 1  | 25.10                       |       |      |             |                                                                                                                           |
| <i>Zachaenus parvulus</i> L2†             | Female | 2  | 25.68 ± 0.97 (24.99–26.36)  | 24.01 | 0.15 | Terrestrial | Petrópolis, RJ, Brazil                                                                                                    |
|                                           | Male   | 1  | 22.34                       |       |      |             |                                                                                                                           |
| <i>Zachaenus parvulus</i> L3              | Female | 2  | 29.75 ± 0.06 (29.70–29.79)  | 29.75 | -    | Terrestrial | Santa Maria Madalena (Parque Estadual do Desengano), RJ, Brazil                                                           |
|                                           | Male   | 0  | -                           |       |      |             |                                                                                                                           |

|                         |        |    |                             |       |       |            |                                                                          |
|-------------------------|--------|----|-----------------------------|-------|-------|------------|--------------------------------------------------------------------------|
| <i>Thoropa taophora</i> | Female | 25 | 63.64 ± 12.64 (45.30–92.64) | 73.82 | -0.24 | Saxicolous | São Sebastião (Ilha dos Gatos) and Ubatuba (Ilha das Couves), SP, Brazil |
|                         | Male   | 10 | 84.01 ± 7.70 (67.52–91.30)  |       |       |            |                                                                          |

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*L1*, *L2*, *L3*, and *L4* indicate deeply-divergent species lineages. SVL = snout-vent length. SSD was quantified by the sexual dimorphism index (SDI) with lower and higher indices indicating lower and higher divergences in sizes between conspecific males and females. †Holotype, paratypes, or topotypes. ‡Measurement obtained from the literature (Neto et al. 2016).

**Table S3. Models selected for the optimal partitioning strategy obtained across all mitochondrial and nuclear genes.**

| Partition | Genes                                                                                                                                                                  | Models  |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------|
| 1         | 16S                                                                                                                                                                    | GTR+I+G |
| 2         | <i>Cytochrome-b</i> cp1                                                                                                                                                | SYM+I+G |
| 3         | <i>Cytochrome-b</i> cp2, <i>ND1</i> cp2                                                                                                                                | HKY+I+G |
| 4         | <i>Cytochrome-b</i> cp3                                                                                                                                                | GTR+I+G |
| 5         | <i>tRNA<sub>leu</sub></i> ( <i>ND1</i> )                                                                                                                               | GTR+I+G |
| 6         | <i>ND1</i> cp1                                                                                                                                                         | GTR+I+G |
| 7         | <i>ND1</i> cp3                                                                                                                                                         | GTR+I+G |
| 8         | <i>tRNA<sub>ile</sub></i> ( <i>ND1</i> )                                                                                                                               | SYM+G   |
| 9         | <i>tRNA<sub>gln</sub></i> ( <i>ND1</i> )                                                                                                                               | HKY+G   |
| 10        | <i>α-fibrinogen</i>                                                                                                                                                    | GTR+G   |
| 11        | <i>Cmyc</i> cp1, <i>Cmyc</i> cp2                                                                                                                                       | HKY+I   |
| 12        | <i>Cmyc</i> cp3                                                                                                                                                        | SYM+G   |
| 13        | <i>mRNAa</i> cp1 ( <i>β-crystallin</i> ), <i>mRNAa</i> cp2 ( <i>β-crystallin</i> ), <i>mRNAb</i> cp1 ( <i>β-crystallin</i> )                                           | JC      |
| 14        | <i>mRNAa</i> cp3 ( <i>β-crystallin</i> ), <i>mRNAb</i> cp2 ( <i>β-crystallin</i> ), <i>mRNAb</i> cp3 ( <i>β-crystallin</i> )                                           | F81     |
| 15        | <i>β-crystallin</i>                                                                                                                                                    | HKY+G   |
| 16        | <i>mRNAa</i> cp1 ( <i>β-fibrinogen</i> )                                                                                                                               | JC      |
| 17        | <i>mRNAa</i> cp2 ( <i>β-fibrinogen</i> ), <i>mRNAb</i> cp1 ( <i>β-fibrinogen</i> ), <i>mRNAb</i> cp2 ( <i>β-fibrinogen</i> ), <i>mRNAb</i> cp3 ( <i>β-fibrinogen</i> ) | GTR+G   |
| 18        | <i>mRNAa</i> cp3 ( <i>β-fibrinogen</i> ), <i>β-fibrinogen</i>                                                                                                          | HKY     |

cp abbreviates codon position.

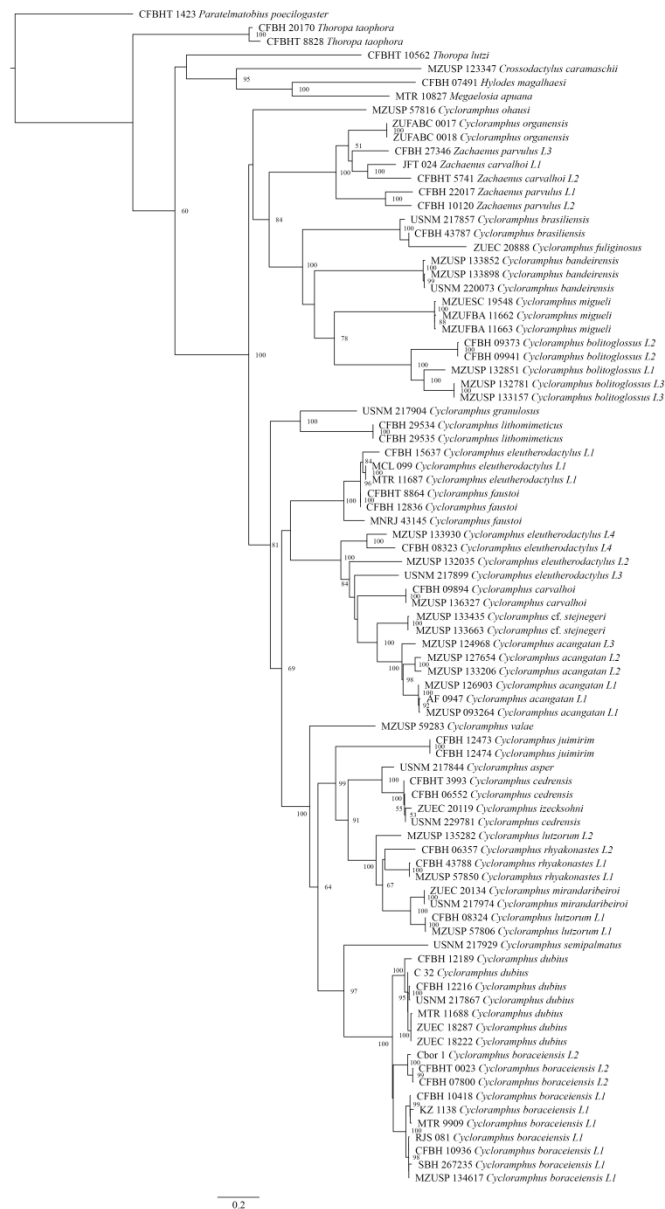
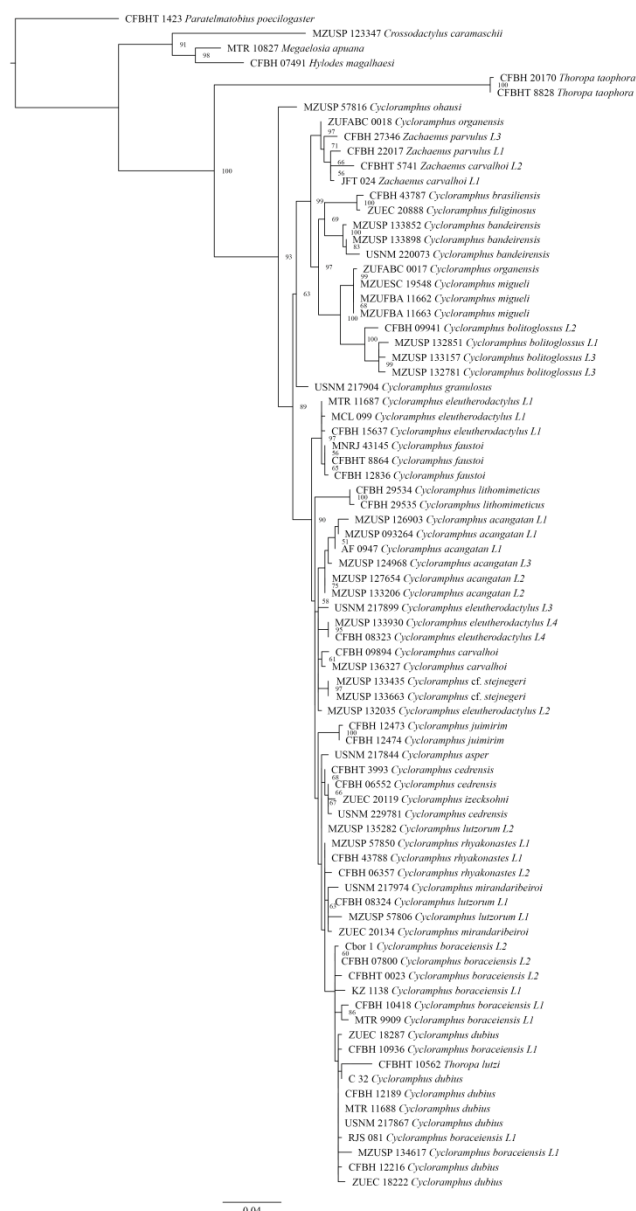


**Figure S1. *Cycloramphus* clade maximum likelihood tree reconstructed with concatenated mitochondrial genes (16S, *Cytochrome-b*, and *ND1*). We found distinct partition schemes from the search algorithms tested (*greedy* and *r-cluster*) but both resulting trees present the same topology and bootstrap support values. *L1*, *L2*, *L3*, and *L4* indicate deeply-divergent species lineages. The numbers next to each node indicate bootstrap supports. We report subclades supported above threshold (bootstrap > 70) and subclades recovered but supported below threshold (bootstrap > 50). Bootstrap ≤ 50 are not reported.**



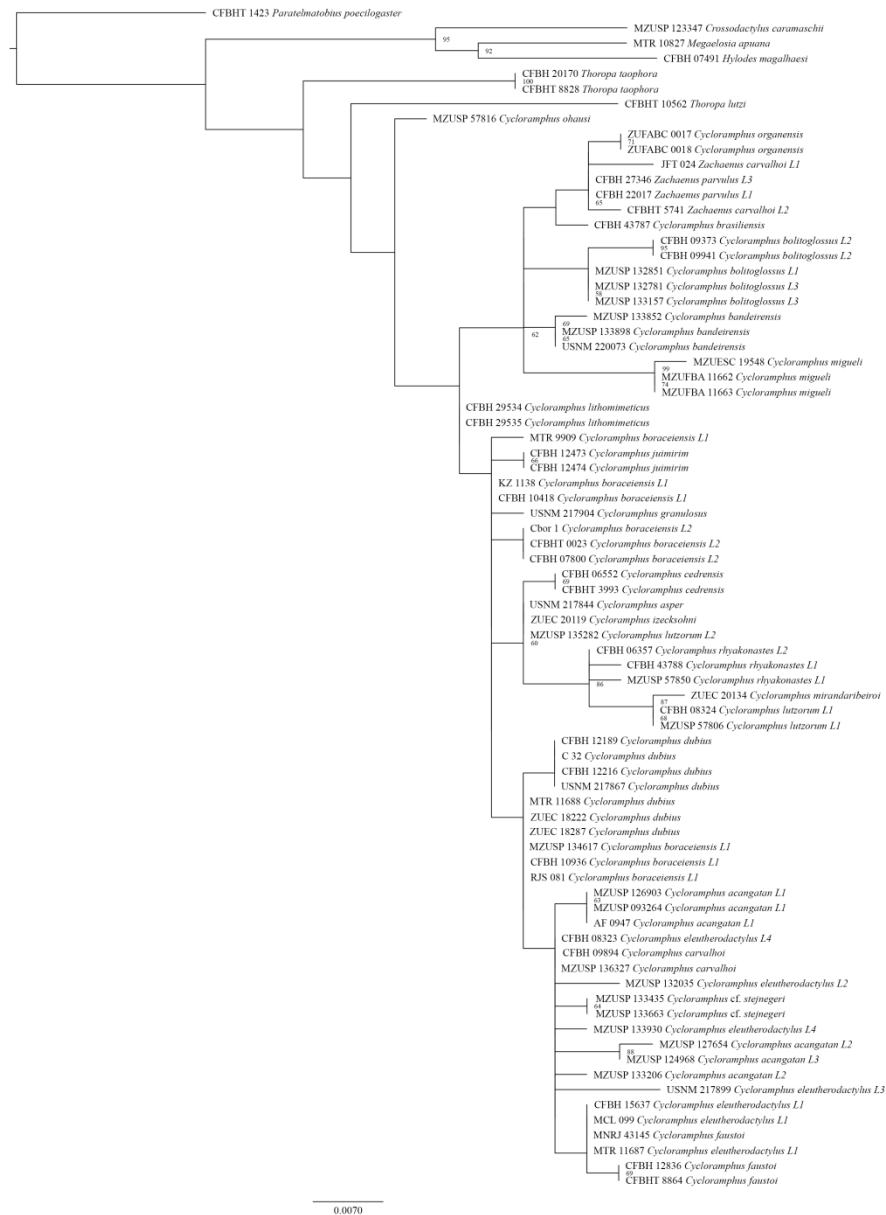


**Figure S3. *Cycloramphus* clade maximum likelihood tree reconstructed with the nuclear gene *Cmyc*. We found the same partition scheme for both search algorithms tested, *greedy* and *rcluster*. L1, L2, L3, and L4 indicate deeply-divergent species lineages. The numbers next to each node indicate bootstrap supports. We report subclades supported above threshold (bootstrap > 70) and subclades recovered but supported below threshold (bootstrap > 50). Bootstrap ≤ 50 are not reported.**

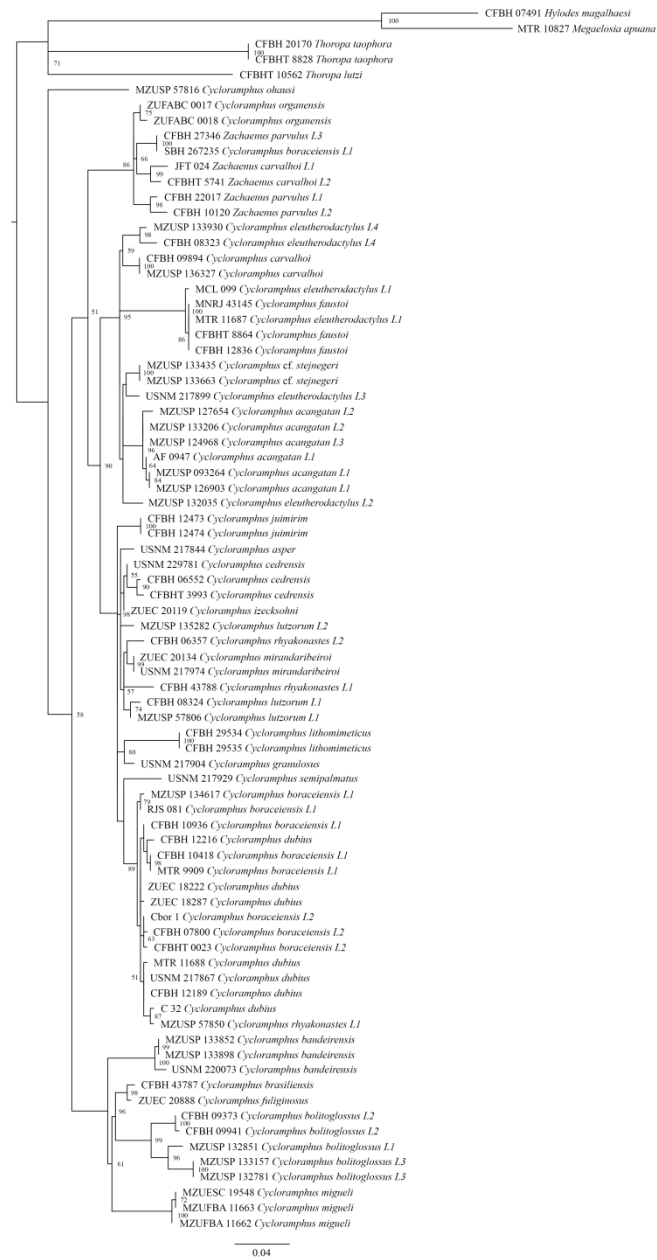


**Figure S4. *Cycloramphus* clade maximum likelihood tree reconstructed with the nuclear gene  $\beta$ -crystallin. We found the same partition scheme for both search algorithms tested, *greedy* and *rcluster*. L1, L2, L3, and L4 indicate deeply-divergent species lineages. The numbers next to each node indicate bootstrap supports. We report subclades supported above threshold (bootstrap > 70) and subclades recovered but supported below threshold (bootstrap > 50). Bootstrap  $\leq$  50 are not reported.**

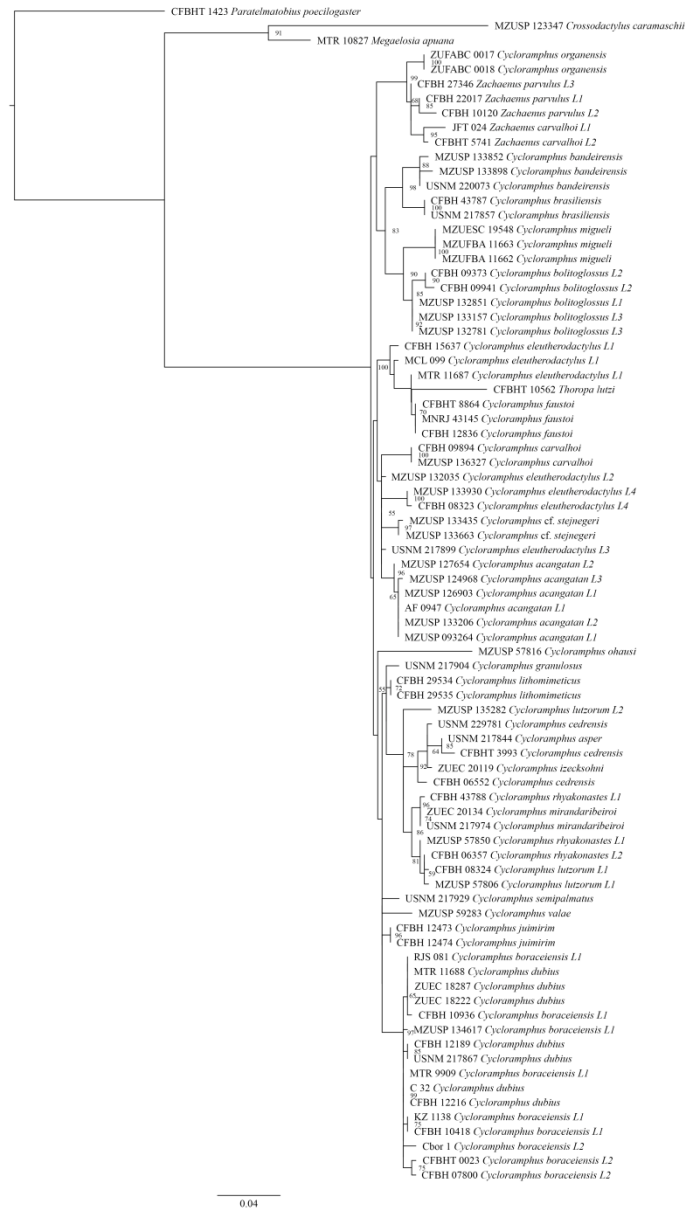


**Figure S5. *Cycloramphus* clade maximum likelihood tree reconstructed with the nuclear gene  $\beta$ -fibrinogen. We found the same partition scheme for both search algorithms tested, *greedy* and *rcluster*. L1, L2, L3, and L4 indicate deeply-divergent species lineages. The numbers next to each node indicate bootstrap supports. We report subclades supported above threshold (bootstrap > 70) and subclades recovered but supported below threshold (bootstrap > 50). Bootstrap  $\leq$  50 are not reported.**

## CAPÍTULO II

### **Asymmetry in genetic and phenotypic introgression in a frog hybrid zone from the Atlantic rainforest**

# Asymmetry in genetic and phenotypic introgression in a frog hybrid zone from the Atlantic rainforest

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## Funding information

São Paulo Research Foundation (FAPESP), Grant Numbers: #2013/50741-7, #2014/24972-4, #2014/50342-8, #2016/06876-3, and #2018/17993-6

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Finance Code: 001

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Grant Number: 306623/2018-8

## Abstract

Discordant mitochondrial and nuclear genetic patterns are common and often coincide with variability in phenotypes among animals. Particularly relevant in hybrid zones, mitonuclear discordance is expected mainly because the mitochondrial genome is haploid and primarily uniparentally inherited, whereas nuclear loci and morphological characters are presumably evolving at slower rates. Overall, discordant genetic and phenotypic differentiation can reveal evolutionary divergence and the processes that maintain boundaries among sister species or lineages. Here we characterize patterns of genetic and phenotypic variation in a transect across a hybrid zone between *Cycloramphus boraceiensis* and *C. dubius*, two sister species

of frogs endemic to the Atlantic rainforest of Brazil. We examined genomic-scale nuclear diversification using double-digest restriction site-associated DNA sequencing (ddRADseq) across 17 populations along a transect encompassing the two parental forms and a contact zone between them. We compare the distribution of nuclear variability with that of a mitochondrial locus (*16S*) and a morphological trait that is diagnostic for the species (the type and extent of dorsal tubercles). Our data indicate that this secondary contact zone currently has ongoing hybridization, with mitonuclear discordance and asymmetrical introgression of nuclear alleles. The distribution of dorsal morphological patterns follows the distribution of genomic variation (nuclear data). From the three data types, we infer that female-biased dispersal combined with allospecific intrasexual competition driven by size is promoting the asymmetry in introgression. This hybrid zone occurs in a highly diverse and historically dynamic region, with known contractions and expansions of species ranges. Secondary contacts affect distribution of genetic and phenotypic variation, contributing to the origin and maintenance of diversification in the Atlantic rainforest.

## KEYWORDS

anuran, body size, contact zone, *Cycloramphus boraceiensis*, *Cycloramphus dubius*, ddRADseq, mitonuclear discordance, sexual selection

## 1 | INTRODUCTION

Animal species commonly show geographic variation associated with speciation processes (Coyne & Orr, 2004; Endler, 1977; Lee, Podos & Sung, 2019). Even in recently diverged new species, microhabitat or topographic variation often isolates lineages, facilitating diversification and speciation (Devitt, Stuart & Moritz, 2011; Garcia-R, Gonzalez-Orozco & Trewick, 2019). Sometimes, divergent species that subsequently come into secondary contact will interbreed. Mutations gradually

accumulated over both species in allopatry will potentially act as premating barriers for interbreeding when their populations are once again in sympatry (Noor, 1999; Pfennig & Rice, 2014; Seddon, 2005). In cases where hybrid offspring occur in zones of secondary contact, negative epistatic interactions will work as postmating barriers, typically reinforcing the distinction among species through unfit hybrids, a consequence of parental genomic incompatibility or reduced ecological competitive fitness (Coyne & Orr, 2004; Orr, 1995; Sequeira et al., 2005; Yoshida et al., 2019). Conversely, hybridization and subsequent backcrossing (introgressive hybridization) can sometimes provide a source of adaptive genetic variation. Introgressive hybridization is known across a wide range of animal taxa, and it can incorporate novel genetic variation in introgressed population (Anderson, 1948; Grant & Grant, 1994; Lewontin & Birch, 1966; Todesco et al., 2016), sometimes forming stable hybrid zones (Brelsford & Irwin, 2009; Endler, 1977; Mallet, 1986) with hybrids having superiority over parental individuals (Moore, 1977; Todesco et al., 2016). The outcome of interactions between species across secondary contact zones is highly variable and complex, and understanding these variable outcomes informs on mechanisms leading to diversification and speciation.

Ruled by pre- and post-zygotic isolation mechanisms (Moran et al., 2019; Sasa, Chippindale & Johnson, 1998; Wirtz, 1999), interactions can be uneven across animal populations experiencing secondary contact, often resulting in asymmetric patterns of admixture in different characters (Devitt, Stuart & Moritz, 2011; Johnson, White, Phillips & Zamudio, 2015; Pfennig & Simovich, 2002; Veen et al., 2001). For genetic introgression, distinct selective constraints, such as coding or noncoding loci, effective population sizes, and whether introgression occurs in autosomal or organellar DNA, can lead to genealogical discordances (Maddison, 1997; Singhal &

Moritz, 2012). Particularly relevant in hybrid zones, discordance between organellar and nuclear loci have been identified in diverse taxa (Edwards, 2009; Toews & Brelsford, 2012). This discordance is expected because the mitochondrial genome is haploid and commonly uniparentally inherited. Additionally, discordant mitochondrial and nuclear genetic patterns are common and often coincide with variability in phenotypes (Toews & Brelsford, 2012). The study of hybrid zones can potentially reveal the selective, behavioral, and demographic mechanisms acting on populations, such as differential selection (Cheviron & Brumeld, 2009), sex-biased dispersal (Crochet et al., 2003), mechanisms underlying mate choice (Wirtz, 1999), or historical spatial expansion or contraction, and drift (Currat, Ruedi, Petit & Excoffier, 2008).

Amphibians are typically phylopatric (Wells, 2010; Zeisset & Beebee, 2008) and species in the Atlantic rainforest are highly diverse and show high levels of endemism due to specialized microhabitat use (Heyer & Maxson, 1983; Silva et al., 2012; Vasconcelos et al., 2019); nonetheless, hybridization between species does occur (Haddad, Pombal, & Batistic, 1994; Mable, Alexandrou & Taylor, 2011), providing a natural laboratory for diversification studies. Historically dynamic regions often show hybrid zones with known contractions and expansions of species ranges in a highly diverse biome (Amaral et al., 2018; Carnaval et al., 2014; Thomé, Zamudio, Haddad & Alexandrino, 2014). Here, we characterized patterns of genetic and phenotypic variation along a hybrid zone between *Cycloramphus boraceiensis* and *C. dubius*, two sister species of frogs endemic to the Atlantic rainforest in southeastern Brazil (de Sá et al., in press). Occurring in the states of São Paulo and Rio de Janeiro, both frog species are saxicolous, living and breeding in crevices formed by rocks or roots at the margins of fast-flowing streams, commonly next to

waterfalls (Giaretta & Cardoso, 1995; Giaretta & Facure, 2003). Males are territorial and guard their egg clutches (Giaretta & Cardoso, 1995; Giaretta & Facure, 2003). Females might also show strong intrasexual competition and choose mates based on territorial and/or phenotypic traits, such as breeding site and/or male body size (Richardson et al., 2010; Roithmair, 1994; Wells, 2010). Despite being morphologically diagnosable, *C. boraceiensis* and *C. dubius* are very similar. *Cycloramphus dubius* lacks distinct dorsal white tubercles that are present in *C. boraceiensis* (Heyer, 1983). Furthermore, both species have very similar karyotypes ( $2n = 26$ ; Beçak et al., 1970; Noletto et al., 2011; Silva, Haddad & Kasahara, 2001) and have been found in the wild with partially sympatric distributions, thus providing an excellent system for studies of population genetics and gene flow in recently diverged species.

Here, we explore nuclear genetic diversification with genomic-scale data, comparing it to the distribution of a mitochondrial locus and dorsal patterns along the secondary contact zone, thus examining the outcome of hybridization in the *C. boraceiensis* and *C. dubius* system. Testing for concordance among nuDNA, mtDNA, and phenotypes, we infer the processes that have governed introgression in different character types. Understanding how secondary contact affects the distribution of genetic and phenotypic diversity, and in turn how those processes contribute to the origin and maintenance of lineages is critically important for our understanding of diversification and speciation processes in the Atlantic rainforest.

## 2 | MATERIAL AND METHODS

### 2.1 | Sample collection

We sampled the saxicolous species *Cycloramphus boraceiensis* and *C. dubius* across their distributions. These two species are generally parapatric and are known to be sister taxa (de Sá et al., in press). From 2011–2016 we collected frogs and tadpoles in the field by hand at night. Sampling across natural habitats in the coastal Atlantic rainforest of southeastern Brazil and supplementing our own collection with data from museum collections, we gathered information from 17 populations of *C. boraceiensis* and *C. dubius* ( $N = 708$  individuals; Table 1 and Figure 1). We included body size measurements of adults and dorsal patterns species diagnosis for a total of 614 voucher specimens and obtained 302 tissue samples from those populations for our genetic analyses (Table 1). Focusing on the contact zone, we sampled outward from there to include most of the areas in which *C. boraceiensis* and *C. dubius* occur, only lacking frogs from the extreme north of the *C. boraceiensis* distribution (municipality of Angra dos Reis and its island, Ilha Grande; Bittencourt-Silva & Silva, 2013). We also included vouchers and tissues from the type localities of both species.

At sampling localities we euthanized frogs and tadpoles with anesthetic overdose (5% lidocaine) and preserved tissues from tadpoles and adults and subadults (livers, fingertips, toe tips, or thigh muscles) in 100% ethanol. All vouchers and tissues are accessioned in the Célio F. B. Haddad amphibian collection (CFBH), Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Rio Claro, São Paulo, Brazil.

## 2.2 | Phenotypic data

At each sample site, we captured individuals and collected data on body size (snout–vent length, SVL) and dorsal pattern. We complemented our phenotypic dataset measuring specimens available in four institutions in Brazil: CFBH amphibian collection, UNESP (CFBH); Museu de Zoologia da Universidade de São Paulo (MZUSP), São Paulo; Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ), Rio de Janeiro; and Museu de Zoologia da Universidade Estadual de Campinas “Adão José Cardoso” (ZUEC), Campinas, São Paulo. We implemented the scoring system designed by Heyer (1983) to categorize *Cycloramphus* species based on dorsal pattern variation. We categorized individuals into one of two dorsal pattern types: uniform and white-tubercled (Figure 2A). We also discovered a novel combination of the two basic dorsal pattern types, which we categorized as “intermediate”. We calculated the proportions of the three dorsal types at each sampling site and compared average male and female body size (SVL) among species using a t-test in R v. 3.5.2 (R Core Team, 2018).

## 2.3 | Mitochondrial DNA sequencing and analysis

We isolated total genomic DNA from tissues using a DNeasy Blood & Tissue Kit (Qiagen, CA, USA) following manufacturer’s protocol. Using polymerase chain reaction (PCR), we amplified and sequenced the mitochondrial gene fragment 16S ribosomal DNA (mtDNA, 16S; 593 base pairs, bps) using primers 16Sa-L (5’-CGCCTGTTTATCAAAAACAT) and 16Sb-H (5’-CCGGTCTGAACTCAGATCACGT) (Palumbi et al., 1991). We carried out PCRs in a final volume of 25  $\mu$ L, each containing 1–10 ng DNA template, 1X PCR Buffer (with 1.5–3 mM  $MgCl_2$ ), 1X BSA, 0.4  $\mu$ M of forward and reverse primers, 0.76 mM dNTPs, and 0.625 units of Taq

polymerase (Roche, IN, USA). Amplification conditions included an initial denaturation at 94°C for 5 min, followed by 35-40 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min, followed by a final extension at 72°C for 5-10 min. We purified final PCR products using a cocktail of 1X SAP buffer, SAP enzyme (1 unit; Life Technologies), and exonuclease I (10 units; Life Technologies). We sequenced purified PCR products in both directions using BigDye terminator sequencing chemistry (Applied Biosystems, CA, USA) on an ABI automated 3730xl Genetic Analyzer (Applied Biosystems).

We conducted a statistical parsimony analysis using the program TCS v. 1.21 (Clement, Posada & Crandall, 2000) to generate a haplotype network. The connection limit was set at 95% and gaps in sequences were read as missing data. The haplotype network was visualized using tcsBU (dos Santos et al., 2016).

## **2.4 | Nuclear DNA sequencing**

Following methods in Peterson et al. (2012), we generated doubled digest restriction-site associated DNA sequence libraries (ddRADseq) and collected genome-wide SNP data to characterize nuclear genetic diversification along our hybrid transect (nuDNA). We included 56 uniquely barcoded/indexed individuals in each library, reaching an optimal level of multiplexing (based on a pilot library and the genome size of frogs). We combined digestion and adapter ligation steps, comprising 100 ng genomic DNA (10.5  $\mu$ l at 10 ng  $\mu$ l<sup>-1</sup>), 15 units of both SbfI-HF and MspI (NEB, MA, USA; 0.75  $\mu$ l each), 300 units of T4 DNA ligase (NEB, 0.75  $\mu$ l), 3  $\mu$ l of 10 × CutSmart ligase buffer (NEB), 1.0  $\mu$ l each of a uniquely barcoded SbfI-P1 adapter (one of eight) and MspI-P2 adapter (Integrated DNA Technologies, IA, USA; 0.25  $\mu$ M each), 3  $\mu$ l of 10 mM ATP (NEB), and 9.25  $\mu$ l of ddH<sub>2</sub>O for a total reaction volume of 30  $\mu$ l. We

incubated the restriction-ligation mixture at 37°C for 30 min, and then at 20°C for 1 h. Afterwards, we pooled 20  $\mu$ l from each of the eight barcoded samples. We PCR-amplified each pool using 20 ng P1/P2 adapted DNA template (10  $\mu$ l at 2 ng  $\mu$ l<sup>-1</sup>), 12.5  $\mu$ l 2X Phusion PCR Master Mix (2X, NEB), and 1.25  $\mu$ l each of forward index primer and a uniquely indexed reverse primer (one of seven; 5  $\mu$ M each, Integrated DNA Technologies). We pooled PCRs across index groups and size-selected them to retain fragments from 300 to 1,000 bps. Each library was single-end sequenced to 100 bps on one lane of the Illumina HI-SEQ 2,500 at the Cornell University Genomics Facility.

## 2.5 | Quality filtering and SNP calling

Reads were processed in STACKS v. 1.48 (Catchen et al., 2013) with the module *process\_radtags* to discard low-quality reads and reads with ambiguous barcodes or RAD cut sites. The remaining reads were demultiplexed to produce *fastq* files for each individual. Next, we used the module *denovo\_map* to merge stacks (i.e., identical set of reads) into loci within individuals and to build a catalog of loci across individuals. We set the minimum depth of coverage required to create a stack (*-m* option) and the maximum number of pairwise differences allowed between any two stacks within a locus (*-M* option) to three reads. We allowed a maximum of one mismatch when building the catalog of loci across individuals (*-n* option). Variants were called using the default SNP model with a genotype likelihood ratio test critical value ( $\alpha$ ) of 0.05. Using the populations module, we filtered SNPs to retain those present in at least two sampling sites ( $p = 2$ ) and present in 50% of individuals ( $r = 0.5$ ). Following genotyping, we further filtered our data to remove sequence reads

with low depth of coverage (<2X per individual) and/or a minor allele frequency less than 0.5%.

## **2.6 | Population genomic analyses: PCAs, structure, and hybrid ancestry**

We conducted a PCA on the centered, unscaled genotype matrix to visualize and infer patterns of population structure once with the full dataset, and again after excluding the insular population (site 12; IBEL). We further quantified population structure and examined genetic admixture using Entropy (Gompert et al., 2014), a Bayesian method similar to the admixture model implemented in STRUCTURE (Pritchard, Stephens & Donnelly, 2000) that deals with uncertainty in genotypes and estimates the proportion of ancestry of a set of individuals from contributing populations. Next, we used the *adegenet* R package v. 2.1.1 to identify the best-fitting number of clusters given Bayesian Information Criterion (BIC) values for K ranging from one to twelve. This optimal clustering solution was then used to perform a Discriminant Analysis of Principal Components (DAPC), a multivariate analysis that minimizes within-group variance while maximizing among-group variance (Jombart, Devillard & Balloux, 2010). To determine the ancestry of the putative hybrids, we assigned two source populations and calculated the fraction of loci that combine ancestry from these two parental populations in Entropy (interpopulation ancestry, denoted  $Q_{12}$ ; Gompert & Buerkle, 2016). The isolated insular population (site 12; IBEL) was excluded from the analysis, and we estimated hybrid ancestry between *C. dubius* and the two lineages of *C. boraceiensis*.

## 2.7 | Ethics statement

This study followed protocols approved by Ethics Committee on Animal Use of the Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil. Field research, collections, and export permits were authorized by the appropriate Brazilian agencies.

## 3 | RESULTS

### 3.1 | Dorsal pattern and body size

Populations varied in the proportions of the three dorsal types (Figure 3A). Sites 1-3 exhibit the uniform dorsal pattern typical of *C. dubius*, and sites 10, 11, 14-17 all exhibit the white-tubercled dorsal pattern typical of *C. boraceiensis*. Sites 4-7 primarily include the uniform pattern, but also include intermediate morphs at proportions of 9-20%. Sites 8 and 9 include all three morphs, with  $\geq 50\%$  of individuals exhibiting the intermediate phenotype. Individuals from sites 12 and 13 mostly exhibit the white-tubercled dorsal pattern, with a small proportion of intermediate morphs ( $\leq 3\%$ ). Whereas species of *Cycloramphus* exhibit no sexual dimorphism in dorsal pattern, we found that males of *C. dubius* are larger than males of *C. boraceiensis* (Figure 2B), but females are the same size (Figure 2C).

### 3.2 | Haplotype network

We identified a total of 42 haplotypes, including 10 haplotypes of the *C. dubius* lineage and 32 of the *C. boraceiensis* lineage (Figure 4). Haplotypes of *C. dubius* and *C. boraceiensis* differ by a minimum of 10 mutational steps. The 32 haplotypes of *C. boraceiensis* consist of 19 haplotypes present in sites 10-12 (lineage *boral*, Figure 4) that differ by a minimum of five mutational steps from the 13 haplotypes in sites 13-

17 (lineage *borall*, Figure 4). The haplotype network also shows one of the *boral* haplotypes shared with *C. dubius* at a sampling locality (site 10). The *boral* lineage is more distantly related to the *C. dubius* haplotypes compared to the *borall* lineage (Figure 4) but is geographically adjacent to the distribution of *C. dubius* (Figure 1).

### 3.3 | Genetic structure and admixture

Our final genetic data set included 5,972 SNPs with an average of 153x depth of coverage per individual per SNP ( $\pm 51$  SD) and 14.5% of missing data. Geographic distribution of populations (Figure 1) is mirrored by the genetic clustering found in the PCA (Figure 5A). Specifically, PC1, which explains 20.2% of the variance in data, separates populations from north to south, including a separate cluster of individuals from sites 10 and 11 (SALE and SSEB). Individuals from the isolated insular population (site 12, IBEL) are separated along PC2, which further explains 15% of the variance, and accounts for additional separation of sites 10 and 11 from the remaining clusters in the north and south. With the isolated insular population removed, the second PCA plot (Figure 5B) clearly shows three clusters with many admixed, intermediate genotypes. High levels of population structure and admixture were also evident in the admixture analysis performed in Entropy, indicating that a large fraction of the variation could be accounted for by four populations ( $K = 4$ , Figure 3B), corresponding to the four clusters revealed by the PCA analysis. Further partitioning the data into six populations ( $K = 6$ , Figure 3C) revealed additional structure by distinguishing the sampling sites at the extreme ends of our sampling transect. Based on  $K$  values evaluated using BIC scores, DAPC identified  $K = 10$  as the optimal number of clusters found within the data. However, visual inspection of  $K$

values ranging from 7-10 reveal no additional population clusters, instead highlighting the extent of admixture among populations.

### 3.4 | Hybrid ancestry

The combination of admixture proportion ( $q$ ) and interpopulation ancestry ( $Q_{12}$ ) (Figure 6) summarizes the genomic composition of hybrids at the contact zone. We generated three triangle plots of hybrid ancestry, one with each value of  $K$  from  $K = 4$  represented on the x-axis, excluding the isolated deme at site 12, which shows little evidence of admixture with neighboring demes. Thus, our triangle plots represent hybrids between *dubius* (sites 1-9) and *boral* (sites 10 and 11) (Figure 6A), *boral* and *borall* (sites 13-17) (Figure 6B), and *dubius* and *borall* (Figure 6C). We found evidence of a few  $F_1$  hybrids between *boral* and *borall/dubius*, which are intermediate in their hybrid ancestry but heterozygous at most of the loci (points at top of the triangles; Figure 6A and B). We find little evidence of late generation hybrids, which have intermediate levels of genome-wide ancestry, but lower interpopulation ancestry, and fall within the center of the triangle. The majority of hybrids are backcrossed individuals between *dubius* and *boral* and between *dubius* and *borall*, as indicated by the points that fall toward the left and right sides of the triangles. We find evidence of more backcrossed hybrids with a pure *dubius* parent than any other deme. Finally, individuals that fall along the x-axis (intermediate admixture proportions, with no inter-population ancestry) are interpreted as showing a pattern of geographic isolation by distance.

## 4 | DISCUSSION

### 4.1 | Population structure and genetic diversity

We investigated the phenotypic and genetic structure among populations of sister frog species that are endemic to the Atlantic rainforest in southeastern Brazil, *Cycloramphus boraceiensis* and *C. dubius*. These sister species have high levels of similarity in morphology, behaviors, and ecological niches (Giaretta & Cardoso, 1995; Giaretta & Facure, 2003; Heyer, 1983), but, even with sympatry, our mitochondrial data confirm that they belong to independent haplogroups (Figure 4). Further, we found that *C. boraceiensis* has higher intraspecific genetic differentiation than *C. dubius*. MtDNA and nuDNA structure for *C. dubius* are homogeneous across populations (sites 1-10), recovering a single cluster for the species (Figures 3B and 4). Conversely, *C. boraceiensis* populations (sites 10-17) are split along their geographic distribution into three main nuDNA clusters: central, insular, and northern genetic clusters (Figure 3B). The insular mtDNA haplotypes are positioned within the central cluster (*boraI* haplogroup; Figure 4). Previous studies have shown that this is typical of a very recent isolation, likely with island *C. boraceiensis* populations isolated during sea-level oscillation from the late Pleistocene (Grazziotin, Monzel, Echeverrigaray & Bonatto, 2006). Commonly when mainland populations have geographic structure and are very large, and/or when island isolation happened recently, the small population isolated on the island shows paraphyly of mainland alleles (Avice, 2000; Funk & Omland, 2003; Johnson, Adler & Cherry, 2000; Moritz, 1994). The four recovered clusters are further supported by the ddRADseq-based PCAs (Figure 5).

The distribution of both species along the Brazilian coastal Atlantic rainforest is markedly constrained by the escarpments of the Serra do Mar mountain chain. High

gradient streams and waterfalls are crucial microhabitats for *C. boraceiensis* and *C. dubius*. These specialized and endemic saxicolous frogs live in rocky habitats next to fast-flowing streams and deposit their terrestrial eggs in crevices that are precisely positioned in the splash-zones of waterfalls, likely because these areas have optimal conditions for embryonic development (Giaretta & Cardoso, 1995; Giaretta & Facure, 2003). Both studied species are clearly mirroring the hydrographic basins in the studied Brazilian coast, with *C. boraceiensis* located in the North Coast basin and *C. dubius* in the Santos Lowlands basin. Furthermore, the geographic distribution of these basins might also at some level explain population diversification within *C. boraceiensis*, because site 11 (SSEB) shows uplands extending in the direction of site 12 (IBEL; Campanha, Ens & Ponçano, 1994). These uplands are also likely maintaining natural barriers between central and northern *C. boraceiensis* genetic clusters.

## 4.2 | Hybrid zone and allospecific intrasexual competition

We examined the polymorphism in dorsal patterns for *C. boraceiensis* and *C. dubius*, and detected more diversity than has been previously described (Heyer, 1983). Besides the uniform morphotype from pure *C. dubius* individuals and the white-tubercled morphotype from pure *C. boraceiensis* individuals, we also found populations showing an array of intermediate morphs. With the exception of only a few individuals with intermediate morphs (site 12: two individuals from 70 sampled; site 13: one individual from 41 sampled), the three *C. boraceiensis* genetic clusters are monomorphic (white-tubercled morph; Figure 3A), whereas the single *C. dubius* genetic cluster is polymorphic (uniform, intermediate, and white-tubercled morphs; Figure 3A). Sites 1-3 have only monomorphic *C. dubius* populations (uniform morph),

whereas sites 4-7 have a small proportion of intermediate morphs (9-20%). Intermediate morphs are predominant at sites 8 and 9 ( $\geq 50\%$ ). Overall, genetically assigned *C. dubius* individuals express the three morphotypes: uniform at sites 1-9; intermediate at sites 4-9; and white-tubercled at sites 8-10. Therefore, we confirm the existence of an asymmetric hybrid zone, showing a well-defined north-south cline introgression of *C. boraceiensis* nuDNA structure mirroring the dorsal morph variation in the direction of *C. dubius* populations, with the sites 8-10 fully composed of hybrid individuals (Figure 3A and B). The ddRADseq-based PCAs and triangle plots of hybrid ancestry further confirm this unidirectional introgression (Figures 5 and 6). Furthermore, site 10 is the only sampled population with *C. boraceiensis* and *C. dubius* individuals sharing mitochondrial haplotypes, thus showing mitonuclear discordance in the system that we studied. *Cycloramphus dubius* cluster shows a large degree of north-south dorsal pattern and nuDNA variation, but little mitochondrial diversity. Only a few haplotype variants radiate from common haplotypes shared by the majority of individuals across the different populations (Figure 4). Differences in the adaptive landscapes for nuDNA and mtDNA and/or demographic asymmetries can create discordant mitonuclear patterns and distributions; female-biased dispersal or disparities in female abundance among hybridizing groups are some factors that facilitate dispersal of mtDNA in discordance with the dispersal of nuDNA (Funk & Omland, 2003).

The introgression of northern *C. boraceiensis* cluster in direction of *C. dubius* cluster occurs but northern *C. boraceiensis* and *C. dubius* clusters currently have with disjunct geographic distributions (i.e., allopatric populations), thus indicating historically ancestral genetic retention. In contrast, the introgression of central *C. boraceiensis* cluster in direction of *C. dubius* cluster is consistent, and these clusters

currently overlap in geographic distributions (i.e., populations partially sympatric), thus indicating a secondary contact with hybridization. Our findings clearly suggest that pre-zygotic isolation mechanisms are failing to prevent hybridization between central *C. boraceiensis* and *C. dubius*. A likely scenario for this observed pattern is that topography allowed the secondary contact between central *C. boraceiensis* and *C. dubius* clusters. Individuals from the central *C. boraceiensis* cluster were likely dispersing at some level from site 11 to the south direction, whereas individuals of *C. dubius* were likely more intensively expanding from sites 3-8 to the north direction (according the nuDNA introgression; Figure 3B), then establishing a secondary contact zone at sites 8-10, which in turn is promoting the dorsal pattern diversity observed. The diversity detected in dorsal patterns is unlikely associated with any sexual selection mechanism, but rather than that might show distinct expressed phenotypes, with intermediate morphs mostly indicating F<sub>1</sub> and backcrossed hybrids.

With very similar phenotypes and admixture of dorsal pattern morphologies, our findings show that we can distinguish the studied species by male sizes. Notably the females of both species have the same size, but *C. boraceiensis* males are smaller (Figure 2B and C). Among anurans, sexual selection is an evolutionary factor that can explain cline distribution of hybrids, and it is interesting that we found introgression of nuDNA in the direction of the larger males. Both *C. boraceiensis* and *C. dubius* have the same reproductive strategy and behaviors: males are highly aggressive, defending territories and guarding eggs (Giaretta & Cardoso, 1995; Giaretta & Facure, 2003), whereas females likely compete intrasexually and choose mates (Richardson et al., 2010; Roithmair, 1994; Wells, 2010). Mainly female-biased dispersal added to allospecific intrasexual competition driven by sizes is promoting the hybrid zone found. As females are relatively less phylopatric than males, because

males demand energy maintaining territories; thus, the chances of a female meet an allospecific male is higher than the opposite. Whether any allospecific female-female contest takes place, both *C. boraceiensis* and *C. dubius* females have equal advantages because they are indistinct on body size. However, the larger *C. dubius* males are favoring the selection and directing the hybrid introgression. Larger *C. dubius* males are acting as supernormal stimulus for both conspecific and allospecific females, whereas the smaller *C. boraceiensis* males are acting as subnormal stimulus for females, thus (Wirtz, 1999). Therefore, mate choices performed by females have a crucial role in *C. boraceiensis* and *C. dubius* divergence.

#### ACKNOWLEDGMENTS

We thank W.R. Heyer and B. Hedges for contributing tissue samples of *Cycloramphus*; L.R. Malagoli, T.H. Condez, and R.L. Romero for help with fieldwork; T. Grant, J.P. Pombal Jr., and L.F. Toledo for specimen loans and laboratory facilities; and M.M. Gray and N.C. Pupin for technical support. This research was financed by grants from São Paulo Research Foundation (FAPESP; #2014/24972-4, #2016/06876-3, and #2018/17993-6 to F.P.S., and #2008/50928-1, #2013/50741-7, and #2014/50342-8 to C.F.B.H.), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Finance Code 001; to F.P.S.), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; to C.F.B.H. and K.Z.).

#### AUTHOR CONTRIBUTIONS

F.P.S., C.F.B.H., and K.Z. conceived the project. F.P.S. and E.M.S. collected field samples. F.P.S. collected all molecular and phenotypic data. M.A. performed

analyses. F.P.S., M.A., and K.Z. wrote the manuscript with input from E.M.S. and C.F.B.H.

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**TABLE 1** Sampled populations of *Cycloramphus boraceiensis* and *C. dubius* from southeastern Brazil. Gathering exact locality and final sample sizes for the three data categories included in the analyses.

| Site | Acronym | Population                    | SNP (n) | 16S (n) | dorsal pattern (n) | Location               |
|------|---------|-------------------------------|---------|---------|--------------------|------------------------|
| 1    | PTOL    | Pedro de Toledo, SP           | 10      | 10      | 13                 | 24°15'4"S, 47°13'28"W  |
| 2    | PERU    | Peruíbe, SP                   | 16      | 16      | 14                 | 24°10'53"S, 47°0'42"W  |
| 3    | ITAN    | Itanhaém, SP                  | 6       | 6       | 1                  | 23°59'49"S, 46°44'30"W |
| 4    | SVIC    | São Vicente, SP               | 2       | 2       | 5                  | 23°56'46"S, 46°29'28"W |
| 5    | SANT    | Santos, SP                    | 22      | 32      | 36                 | 23°52'46"S, 46°27'41"W |
| 6    | SPSA    | Road São Paulo-Santos, SP     | 1       | 1       | 43                 | 23°51'52"S, 46°27'44"W |
| 7    | CUBA    | Cubatão, SP                   | 20      | 25      | 32                 | 23°48'44"S, 46°21'17"W |
| 8    | BERT    | Bertioga, SP                  | 15      | 17      | 28                 | 23°46'39"S, 46°7'3"W   |
| 9    | BIRI    | Biritiba-Mirim, SP            | 2       | 2       | 6                  | 23°42'29"S, 46°2'54"W  |
| 10   | SALE    | Salesópolis, SP               | 2       | 2       | 17                 | 23°37'51"S, 45°52'11"W |
| 11   | SSEB    | São Sebastião, SP             | 69      | 78      | 96                 | 23°42'58"S, 45°45'17"W |
| 12   | IBEL    | Ilhabela, SP                  | 35      | 36      | 70                 | 23°52'30"S, 45°25'36"W |
| 13   | CARA    | Caraguatatuba, SP             | 25      | 25      | 41                 | 23°35'10"S, 45°25'5"W  |
| 14   | UBAT    | South Ubatuba, SP             | 2       | 2       | 78                 | 23°24'57"S, 45°6'59"W  |
| 15   | PICI    | Picinguaba, North Ubatuba, SP | 23      | 24      | 72                 | 23°21'53"S, 44°47'29"W |
| 16   | PACU    | Road Paraty-Cunha, RJ         | 5       | 5       | 9                  | 23°12'28"S, 44°49'27"W |
| 17   | PARA    | Paraty, RJ                    | 19      | 19      | 53                 | 23°5'11"S, 44°43'30"W  |

*Note:* SP = São Paulo State and RJ = Rio de Janeiro State. See Figure 1 for site location on map.

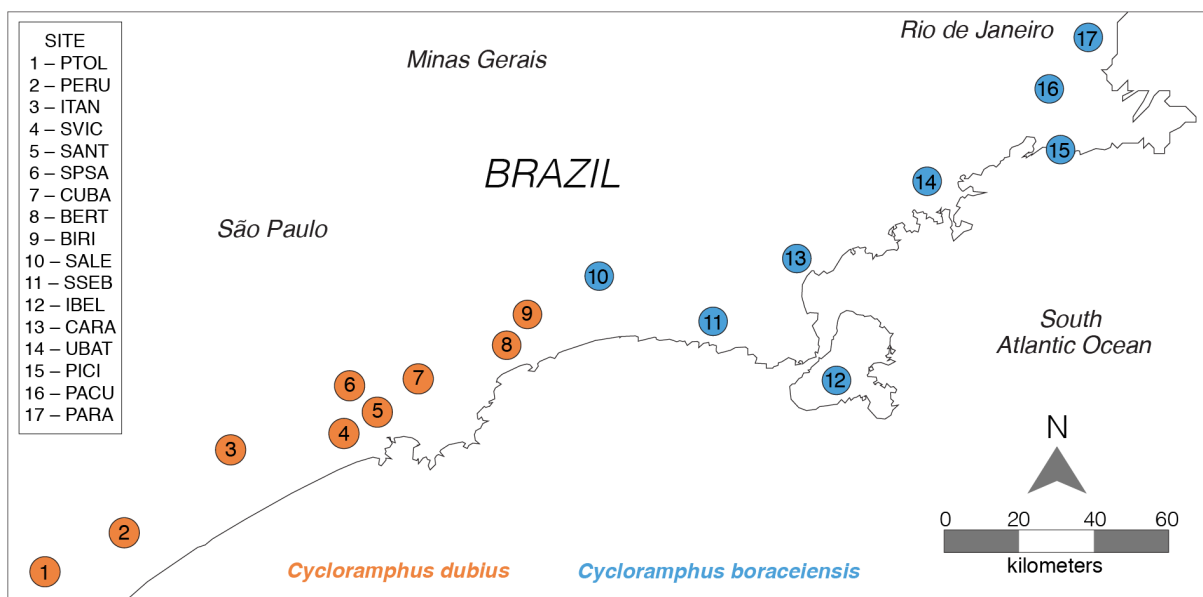


**FIGURE 1.** Population sampling of *Cycloramphus boraceiensis* and *C. dubius* in São Paulo and Rio de Janeiro states in southeastern Brazil. Circles indicate population localities and are colored based on species distributions (Heyer, 1983).

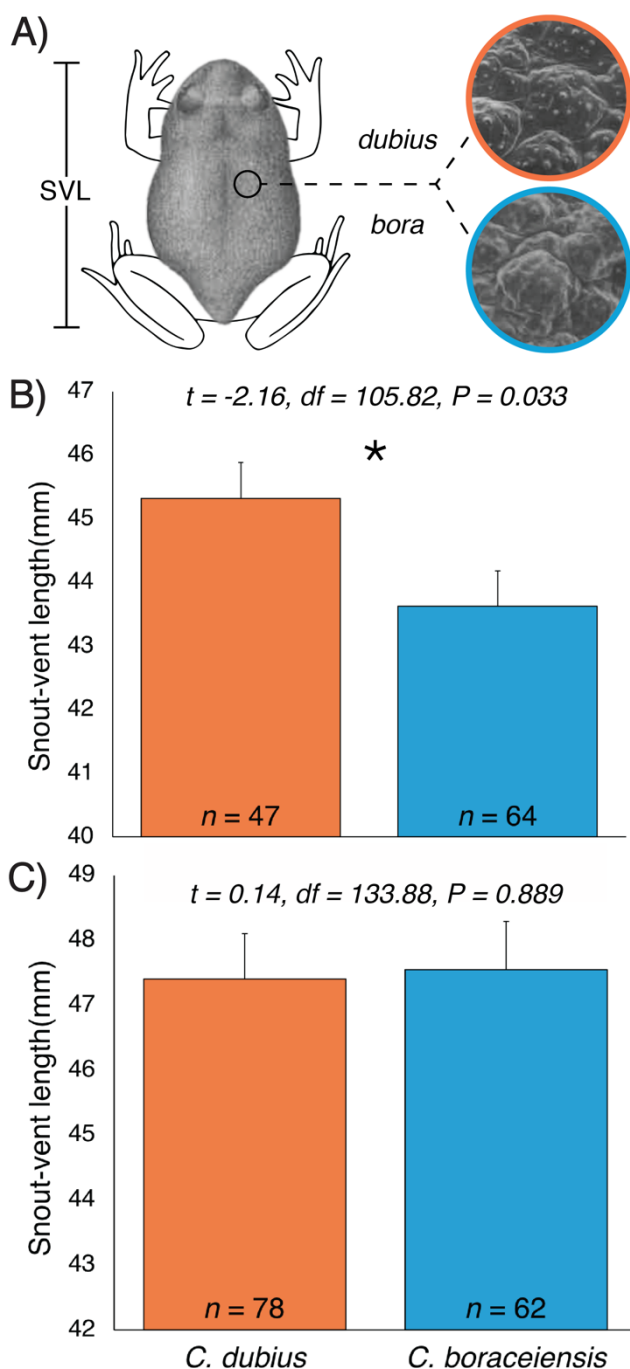


**FIGURE 2.** Dorsal pattern and male body size differ between *Cycloramphus boraceiensis* and *C. dubius*. (A) *Cycloramphus boraceiensis* exhibits a dorsal texture with white-tipped tubercles (bottom) whereas *C. dubius* lacks white-tipped tubercles (top). (B) Males of *C. dubius* are larger than males of *C. boraceiensis*, but (C) females are the same size.

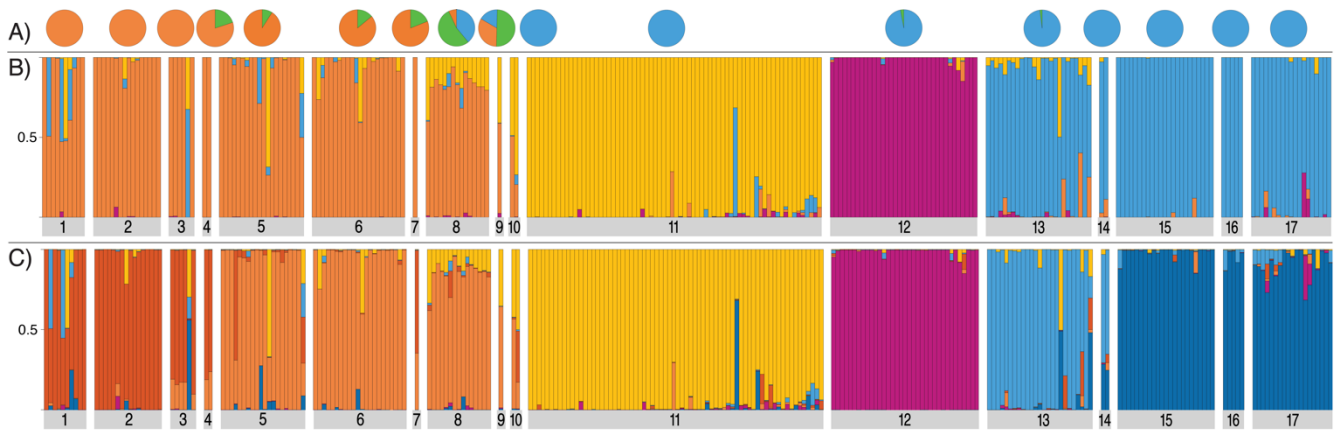


**FIGURE 3.** (A) Pie charts show proportions of the three dorsal types at each sampling site, where orange representing the uniform pattern typical of *C. dubius*, green representing the intermediate pattern, and blue representing the white-tubercled pattern typical of *C. boraceiensis*. Bar plot of admixture proportions for  $K = 4$  (B) and  $K = 6$  (C). Each bar corresponds to an individual, and each colored segment depicts the proportion of an individual's genome inherited from one of the inferred source populations. White vertical lines separate sampling sites.

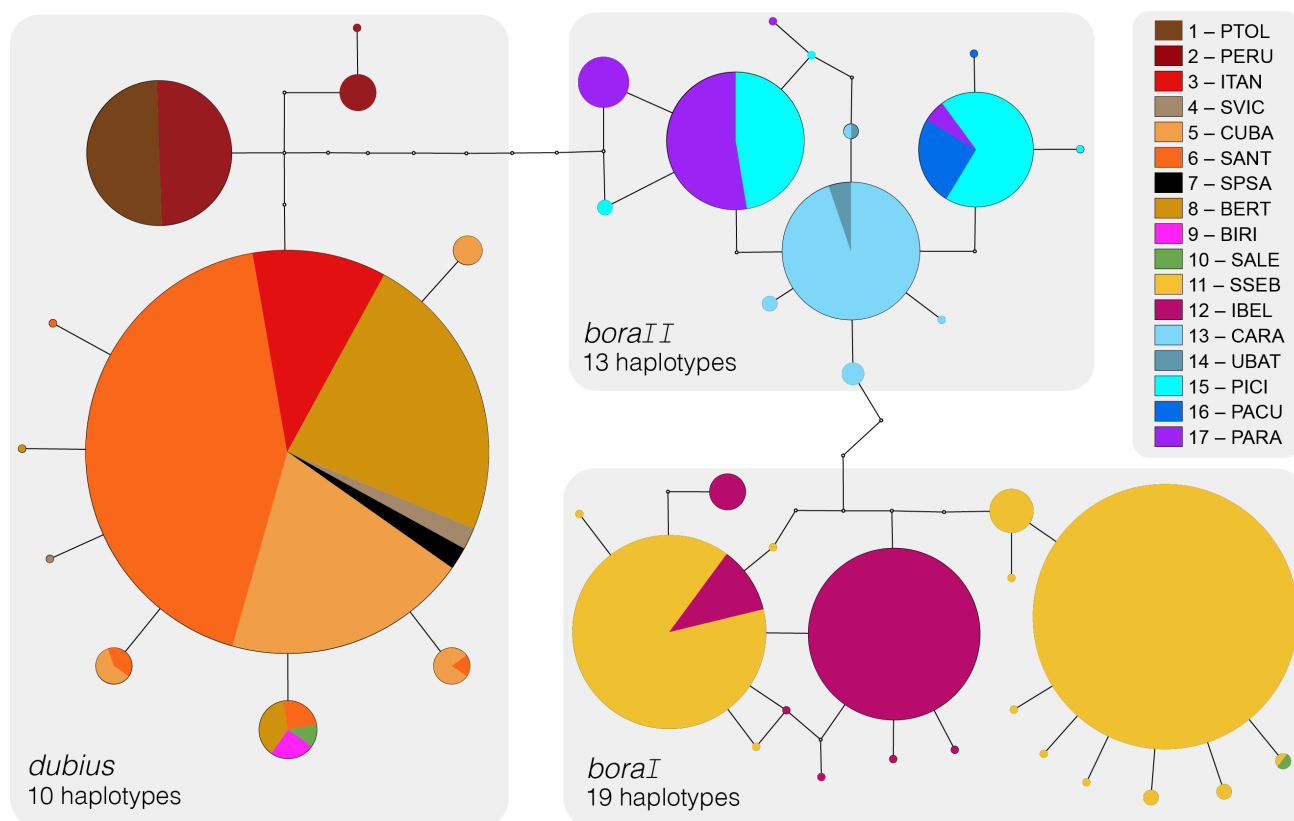


**FIGURE 4.** TCS haplotype network based on the alignment of 615 bp of the mitochondrial gene 16S, showing the relationships among recorded haplotypes. Small open circles correspond to missing (or hypothetical) haplotypes and each line between two haplotypes represents one mutational step. Circle sizes depict frequency of haplotypes. Light grey boxes encompass the three main haplogroups.

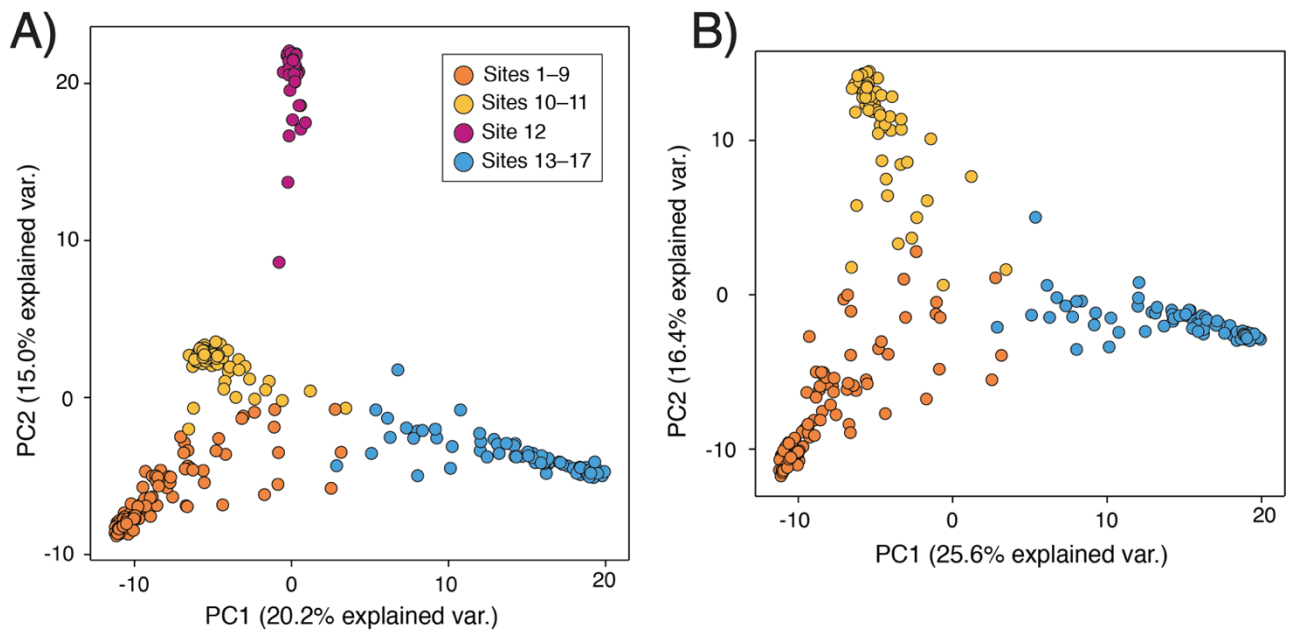


**FIGURE 5.** Summary of population genetic structure based on principal component axis one (PC1) and axis two (PC2) for (A) the full dataset, and (B) with site 12 removed. These axes combined explain over a third of the variation in genotype estimates for 5,972 variable nucleotides. Points denote individuals and are colored based on majority membership to one of four demes (Figure 3B).

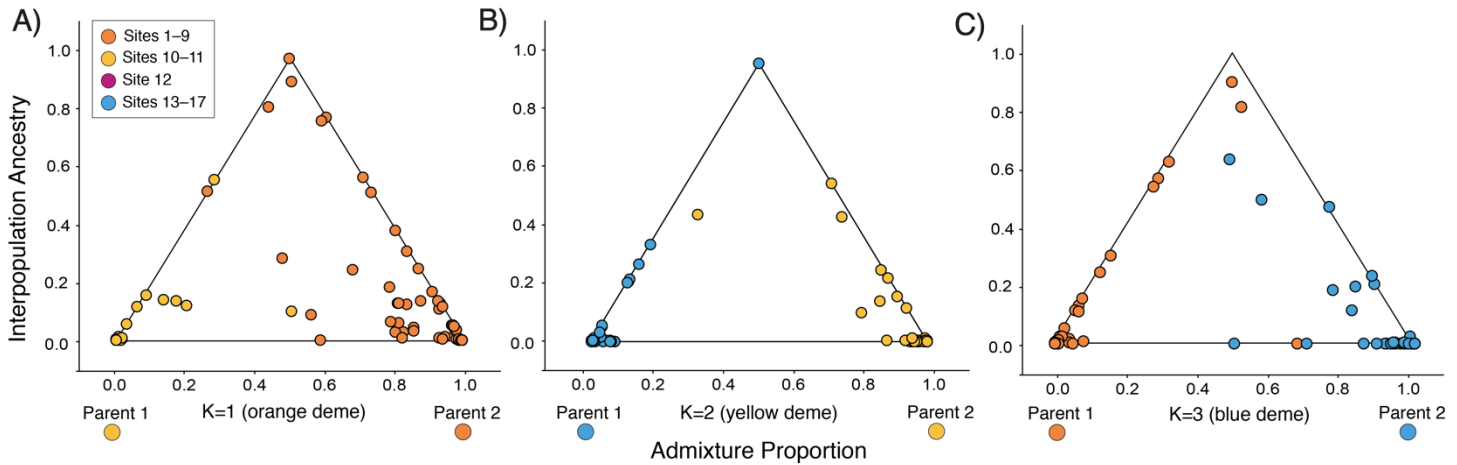


**FIGURE 6.** Triangle plots of hybrid ancestry showing the relationship between admixture proportion ( $q$ ) and the fraction of loci at which individuals have ancestry from one of two parental populations ( $Q_{12}$ ). Admixture proportions (x-axes) are based on the three demes resolved by  $K = 4$  (Figure 3B), excluding the isolated pink deme. Points denote individuals and are colored based on majority membership to one of four demes (Figure 3B).  $F_1$  hybrids are intermediate in their hybrid ancestry but heterozygous at most of the loci, and therefore fall toward the top of the triangles. Similarly,  $F_2$  hybrids have a similar genome-wide level of ancestry, but lower interpopulation ancestry, and fall within the center of the triangle. Backcrossed individuals fall toward the left and right sides of the triangle. Individuals that fall along the x-axis (intermediate admixture proportions, with no inter-population ancestry) are interpreted as geographic isolation by distance.

## CAPÍTULO III

**Body size and relatedness shape a promiscuous mating system in a territorial Neotropical frog**

# Body size and relatedness shape a promiscuous mating system in a territorial Neotropical frog

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## Funding information

São Paulo Research Foundation (FAPESP), Grant Numbers: #2013/50741-7, #2014/24972-4, #2014/50342-8, #2016/06876-3, and #2018/17993-6

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Finance Code: 001

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Grant Number: 306623/2018-8

## Abstract

Sexual selection via female choice acts on males to reinforce traits that increase chances of being chosen as mates and of successfully competing for access to females. However, in some cases, males also choose mates and those choices can be crucial components of evolution by sexual selection. In anurans both female and male body sizes are potentially important traits associated with intrasexual competition, and thus could play important roles in sexual selection. Here, we use parentage analyses to characterize the genetic mating system of the Neotropical saxicolous frog *Cycloramphus boraceiensis*, to determine female and male traits

associated with reproductive success. Males of this species defend territories and provide parental care by guarding terrestrial clutches at edges of fast-flowing streams. Our parentage analyses revealed a promiscuous system, with both females and males having sequential mates. We found occasional rearing of mates, but no evidence of mate fidelity. Larger females have reproductive advantages, and are likely selected by males due to increased fecundity. Whereas for larger males the advantages of larger body size are not consistent, likely varying according to distinct habitats and population conditions. Mating was not assortative by size in our population, however, we found that the average genetic relatedness of mated pairs was significantly greater than expected if mating were random in the population. With both females and males competing for resources and mates, our findings show that this promiscuous system with male parental care has a nonrandom organization, but mainly female body size and mate relatedness shape the social structure in *C. boraceiensis*.

## KEYWORDS

anuran, *Cycloramphus boraceiensis*, intrasexual competition, mate choice, mating system, microsatellites, sexual selection, territoriality

## 1 | INTRODUCTION

Darwin (1871) first noted that animal mate choices are typically not random and, since his observation, studies across the tree of life have characterized mating systems, elucidating the behaviors and phenotypic traits that underlie mate choices. These sexually-selected behaviors and traits include those involved in signaling dominance, such as large body sizes that provide advantages in aggressive interactions, and territorial behaviors that provide access to mates (Andersson, 1994). The current focus of studies in sexual selection has typically been on female

mate choice, which maintains and reinforces the male traits that increase their chances of being chosen as mates and compete for access to females (Andersson, 1994; Bateman, 1948; Darwin, 1871). However, there are reproductive costs for males that can also lead them to choosy behaviors, selecting females according to some advantageous trait (Bonduriansky, 2001; Flatt & Heyland, 2011; Swierk & Langkilde, 2019) and, thus promoting female-female competition. Similar to female mate choice based on male qualities (Campbell, 1972; Emlen & Oring, 1977; Mayr, 1963), males often evaluate female traits and gain fitness benefits when mating with preferred partners (Byrne & Rice, 2006; Chen, Salcedo & Sun, 2012; Johnstone, Reynolds & Deutsch, 1996). Broad evidence shows that females compete for mating resources or partners with intrasexual competition among females also influencing trait evolution and mating system dynamics (Cain & Ketterson, 2012; Cain & Rosvall, 2014; Comendant, Sinervo, Svensson & Wingfield, 2003; Rosvall, 2011).

Male anurans typically call from territories they defend, attracting females that choose larger males that defend higher-quality sites (Andersson & Simmons, 2006; Howard, 1978). Larger males have a competitive advantage in territorial and female defense due to their higher performance in male-male combat and displacement of other males amplexing females (Berven, 1981; Howard & Kluge, 1985), which prevents interference in breeding aggregations. Fertility of females is almost always positively correlated with female body size (Nali, Zamudio, Haddad & Prado, 2014; Wells, 2010). Therefore, if the number of females within a breeding territory is limited, or if males can only breed with a limited number of mates at a time, males can also benefit by being choosy, defending and mating preferentially with larger females that will increase their reproductive success. In species where both males and females

are competing for the largest partners they can access, sexual selection might result in size-based assortative mating.

Particular conditions are required for sexual selection to shape the evolution of male mate choice in anuran mating systems. First, costs for males (such as parental care or territorial limitation) have to be high enough to make male choosy behaviors beneficial, increasing the likelihood that males gain fitness by selecting high-quality females. Second, females also need to exercise mate choice behaviors, such that body traits or site qualities enhance female acquisition probability. When both males and females select partners based on size, size-assortative mating will also be beneficial because reproductive success depends not only on the total number of eggs produced but on the number of eggs successfully fertilized by the male. In animals with external fertilization, greater distances between female to male cloaca is inversely related to fertilization success (Davies & Halliday, 1977; Robertson, 1990). With females choosing males of nearly equivalent body size, size-assortative mating is also a pattern often detected in anurans (Bateson, 1983; Heatwole & Sullivan, 1995; Robertson, 1990). Finally, kinship-based mate choice falls along a continuum: on one end, mate choice may be active, with individuals avoiding or preferring to mate with related individuals among available potential mates; alternatively, natal migration might constrain mate choice, with the pool of potential mates determined by the individuals who are present in the population (Keller & Arcese, 1998; Pusey & Wolf, 1996; Wiley & Poston, 1996). The level of mate relatedness will influence fitness and the maintenance of genetic variation, and can lead to inbreeding or outbreeding depression (Keller & Waller, 2002; Lynch, 1991; Sugg, Chesson, Dobson & Hoogland, 1996).

To test for the relative roles of female and male-choice in a territorial frog, we characterized the mating system of *Cycloramphus boraceiensis*, a Neotropical saxicolous species endemic to the Atlantic rainforest of Brazil. By combining genetic parentage and phenotypic data for two populations of this species, we explored mechanisms leading to pair-formation in *C. boraceiensis* and the determinants of reproductive fitness for both females and males. Our focal species has a prolonged breeding season, living and breeding under conditions with high potential for polygamy (Emlen & Oring, 1977). Females deposit terrestrial eggs on rocky surfaces next to waterfalls of fast-flowing streams, selecting optimal microhabitat conditions for embryonic development (Giaretta & Cardoso, 1995; Giaretta & Facure, 2003; Verdade, 2005). Males defend monopolizable territories where they call to attract females and guard their terrestrial clutches, thus exhibiting high intrasexual competition and male-male agonistic interactions (Hartmann, Hartmann, Prado & Garcia, 2003; Verdade, 2005).

For *C. boraceiensis*, female fitness depends on the successful oviposition of eggs in a high-quality male territory, whereas male fitness depends on the maintenance of those territories and protection of clutches (Giaretta & Facure, 2003; Haddad & Prado, 2005; Hartmann, Hartmann, Prado & Garcia, 2003; Verdade, 2005). Larger, more fecund females that select larger males with the best territories and paternal care will increase their reproductive success. Likewise, larger males with high-quality territories, and clutch-guarding ability should select larger females, maximizing clutch sizes and fertilization success. Because males likely have site fidelity and individuals are likely philopatric, we also would expect kin-avoidance during pair formations to increase genetic variability.

Here, we hypothesized that: (1) larger females have advantages in intrasexual competition, not only producing more eggs but displacing smaller females and mating with larger males; (2) larger males have advantages in intrasexual competition, defending and monopolizing breeding sites, guarding clutches, displacing smaller males, and selecting larger females to increase their fitness; (3) if female and male choices are in fact assortative according to size, and the relative distance between cloacae affects the fertilization success of pairs during amplexus, we expect a pattern of size-matched pairs; and (4) given that mate relatedness has consequences for fitness and for the maintenance of genetic variation, we expect a negative correlation between genetic relatedness and mating probability, which would decrease the potential for inbreeding depression.

## **2 | MATERIALS AND METHODS**

### **2.1 | Study species**

*Cycloramphus boraceiensis* (family Cycloramphidae) is a Neotropical saxicolous frog endemic to the Atlantic rainforest in Brazil (Heyer, 1983; Verdade, 2005). This nocturnal species has a prolonged breeding season, living and mating in rocky outcrops next to fast-flowing streams (Haddad & Prado, 2005; Hartmann, 2004; Heyer, Rand, Cruz, Peixoto & Nelson, 1990; Verdade, 2005). Males of saxicolous *Cycloramphus* species are typically highly aggressive, and they defend territories and monopolize breeding sites (de Sá et al., in press; Hartmann, Hartmann, Prado & Garcia, 2003). Preferred breeding sites are crevices or roots on rock outcrops next to waterfalls (Giaretta & Cardoso, 1995; Giaretta & Facure, 2003; Haddad & Prado, 2005; Verdade, 2005), where males care for and defend their terrestrial egg clutches during all embryonic stages prior to hatching (Giaretta & Cardoso, 1995; Giaretta &

Facure, 2003; Haddad & Prado, 2005; Verdade, 2005). Tadpoles are semi-terrestrial and live in the same habitats as the adults until metamorphosis (Giaretta & Facure, 2003; Haddad & Prado, 2005).

## 2.2 | Study population

We studied two populations of *C. boraceiensis* in the Parque Estadual da Serra do Mar: Núcleo Caraguatatuba in the municipality of Caraguatatuba, and Núcleo Picinguaba in the municipality of Ubatuba, State of São Paulo, Brazil. These locations are montane, with ombrophilous dense forest and a tropical humid climate, with elevated rainfall year-round (Sanchez, Pedroni, Leitão-Filho & Cesar, 1999). In Caraguatatuba, we focused on an area of approximately 0.85 km<sup>2</sup> (23°35'23.3"S, 45°25'24.5"W), which includes a network of six fast-flowing streams. In Picinguaba, we focused on a total area of approximately 0.65 km<sup>2</sup>, composed of two stream networks that are two kilometers apart from each other: the first with two fast-flowing streams (23°21'39.2"S, 44°47'11.5"W); the second was a large waterfall and pool, locally known as Cachoeira da Escada (23°21'19.8"S, 44°46'10.3"W).

## 2.3 | Field methods

At our study sites, *C. boraceiensis* peak breeding activity occurs between September and May (Hartmann, 2004). In Caraguatatuba, we conducted fieldwork between May and August 2017, and between November 2017 and July 2018, totaling 100 days in the field. In Picinguaba, we conducted fieldwork between November 2017 and March 2018, totaling 20 days in the field. We collected tissue samples from all frogs and clutches encountered at each site. In Caraguatatuba we captured 133 adults (56 females and 77 males) and 61 immatures (30 subadults, 17 juveniles, and 14

tadpoles). In Picinguaba we captured 29 adults (11 females and 18 males), 74 immatures (11 subadults, 46 juveniles, and 17 tadpoles), and three distinct clutches containing a total of 180 eggs in the initial stages of embryonic development (average  $\pm$  SD = 60 eggs  $\pm$  10.58). The attending males of these clutches are included in the adults sampled.

We collected tissue samples from adult frogs (finger- or toe-clip) for molecular analysis. Adult frogs were immediately released at the location of capture. A subset of adults was collected and, in this case, liver or thigh muscle tissue was removed for molecular analysis. All other life stages were euthanized by anesthetic overdose (lidocaine 5%) and preserved in their entirety. All samples were preserved in 100% ethanol for downstream molecular analyses.

We sexed adult frogs based on the presence of the inguinal macrogland, a sexually dimorphic trait found only in males. When possible, calling activity in males or the presence of oocytes visible through the relatively transparent skin in the inguinal region of reproductive females, complemented sex identification. We recorded the following phenotypic data for each adult: snout-vent length (SVL, measured with a digital caliper to the nearest  $\pm$  0.1 mm); body mass (measured with a field scale to the nearest  $\pm$  1.0 g).

## **2.4 | DNA extraction and microsatellite genotyping**

We isolated genomic DNA using a HotSHOT extraction protocol (Montero-Pau, Gómez & Muñoz, 2008) with 50  $\mu$ L of 25 mM NaOH and 50  $\mu$ L of 40 mM Tris-HCl for a final volume of 100  $\mu$ L. We then sequenced genotypes of 50 variable microsatellite loci for all samples using a protocol optimized for high-throughput amplicons. We performed two multiplex PCRs, each amplifying 25 loci in 10  $\mu$ L reaction volumes

containing 5  $\mu$ L of Qiagen multiplex PCR kit 2x master mix, 2.5  $\mu$ L of water, 1  $\mu$ L of primer mix, and 1.5  $\mu$ L of DNA template from each sample. PCRs consisted of an initial denaturation step of 95 °C for 15 min; 35 cycles of 94 °C for 30 s, 56 °C for 90 s, and 72 °C for 90 s; and a hold at 15 °C. We diluted 2  $\mu$ L of this PCR product from each sample with an equal volume of water. The dilute PCR products were used as the templates for a second PCR, which added Illumina Nextera dual barcode sequences. The thermocycler protocol was as follows: 92 °C for 2 min; six cycles of 94 °C for 30 s, 62 °C for 1 min, and 68 °C for 1 min; and 68 °C for 10 min. We combined 3  $\mu$ L of each barcoded sample to produce two pools, corresponding to the two sets of multiplexed primers. We cleaned 200  $\mu$ L of each pooled PCR product with 140  $\mu$ L of Ampure XP, then resuspended DNA in 100  $\mu$ L of 0.5x AE buffer. We quantified both libraries fluorometrically using a Qubit DNA broad-range assay kit and a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies). We sequenced both barcoded samples on an Illumina MiSeq (v2 kit, 2 x 250bp) at the Biotechnology Resource Center at Cornell University (Ithaca, NY). We demultiplexed the resulting barcoded sequences by using bcl2fastq Conversion Software 2.96 v2.18 (Illumina, Inc.).

## **2.5 | Parentage analyses**

We estimated population allele frequencies and preliminarily explored parentage assignments using CERVUS v3.0.7 (Kalinowski, Taper & Marshall, 2006; Marshall, Slate, Kruuk & Pemberton, 1998). CERVUS uses a pairwise likelihood comparison approach, generating locus-by-locus likelihood scores for each potential parent for each offspring, then assigning parentage to the parent with the highest LOD score (obtained by taking the natural log of the likelihood ratio). Estimates of parentage

from CERVUS were used to infer the number of mates per female and per male, the probability of an actual father and mother being included among the sampled individuals, and parent-offspring relationships (based on assignments made at the 95% confidence threshold). These estimates were then used as parameters for more stringent parentage assignment and inference of unsampled parental genotypes in the program COLONY v2.0.6.5 (Jones & Wang, 2010). COLONY uses a maximum likelihood method to assign sibship and parentage jointly over a pedigree. We applied the full-likelihood method as the criterion for assessing plausible pedigree configurations. Based on our CERVUS results, we provided population allele frequencies, selected polygamous mating without inbreeding for both sexes, and provided a list of known parent-offspring relationships (to lead assignments made in COLONY). We assumed a complexity prior for our COLONY runs; this reduces false sibship assignments by discouraging the formation of unnecessarily complex pedigrees. We set the combined error and mutation rate at 0.05, ensuring flexibility in parentage assignments (as suggested in the documentation for COLONY; see also Hammond, Bourke & Bruford, 2001, Muralidhar, de Sá, Haddad & Zamudio, 2014). COLONY typically returns similar parental genotypes and sibship reconstructions over a large range of error rates (Wang, 2004). The dropout rate (number of missing genotypes) for each locus was calculated as the number of missing genotypes relative to the total number of samples genotyped at each population. For each of the two populations, Caraguatatuba and Picinguaba, we ran COLONY using a single chain at the maximum precision and the longest run time provided by the program.

## 2.6 | Reproductive success and assortative mating analyses

Body condition was estimated as the log-transformed body mass divided by log-transformed SVL (Speakman, 2001). We used Shapiro-Wilk's tests to assess if body size distributions of females and males (SVL, mass, and body condition) deviated from normality. To test whether larger individuals have reproductive advantage, we used unpaired one-tailed Student's t-test, assuming equal variance and with significance threshold of  $p = 0.05$ . For data that were not normally distributed, we used unpaired one-tailed Mann Whitney's test. We compared SVL, body mass, and body condition between assigned mothers and unassigned females from all sites and assigned fathers and unassigned males from all sites. To assess the degree of assortative mating, we calculated the Pearson correlation between mates' SVL, body mass, and body condition, based on all mating relationships between sampled individuals identified by COLONY, considering positive or negative Pearson values  $< 0.3$  as negligible correlations, between 0.3 and 0.5 as weak correlations, between 0.5 and 0.7 as moderated correlations, and  $> 0.7$  as strong correlations. For data that did not follow normality, we used Spearman correlations.

## 2.7 | Mate relatedness analyses

We used the package RELATED (Pew, Muir, Wang & Frasier, 2015) in R v3.3 (R Core Team, 2016) to measure relatedness for all parents, sampled and inferred. For the inferred parents, we used genotypes inferred by COLONY, retaining only inferred alleles with likelihood scores greater than 0.8 in our analysis; alleles given a lower score were treated as missing data. We chose to calculate Wang's relatedness estimator (W; Wang, 2002), which corrects for variable information content across multiple highly polymorphic loci and is robust to sampling bias in a reference

population (Blouin, 2003; Wang, 2002, 2006). We calculated the average pairwise  $W$  for mated pairs from each population, Caraguatatuba ( $N = 50$ ) and Picinguaba ( $N = 48$ ). To assess whether mated pairs are less related to one another than expected if mate choice was random within each population, we also calculated 10,000 times the average pairwise  $W$  among 50 (for Caraguatatuba) and 48 (for Picinguaba) pairs of parental genotypes, randomly sampled from all possible opposite-sex pairs at each study population. We then compared the average pairwise  $W$  for actual mated pairs with this distribution of bootstrapped means to test for departure from random mating.

## **2.8 | Ethics statement**

This study followed protocols approved by Ethics Committee on Animal Use of the Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil. Field research, collections, and export permits were authorized by the appropriate Brazilian agencies.

## **3 | RESULTS**

### **3.1 | Population sizes and sex ratios**

We sampled a total of three clutches and their respective guardian males, totaling 180 eggs, but not all of the eggs were fertilized (first clutch: 59 embryos and 9 non-fertilized eggs; second clutch: 35 embryos and 13 non-fertilized eggs; third clutch: 61 embryos and 3 non-fertilized eggs). We also sampled 135 offspring individuals (31 tadpoles, 63 juveniles, and 41 subadults) and 162 adult frogs in Caraguatatuba and Picinguaba. Adults at both populations displayed a slightly male-biased adult sex ratio (Caraguatatuba, 58% male: 56 females, 77 males; Picinguaba, 62% male: 11

females, 18 males), consistent with the fact that operational sex ratios in anuran mating systems are typically skewed towards males (Elmberg, 1987; Green, 2013).

### **3.2 | Genotype clustering and characterization of the mating system**

DNA extraction and genotyping were successful for 74.7% of all fertilized eggs (embryos; 115/154), 86.7% of all other offspring (tadpoles, juveniles, and subadults; 117/135), 86.3% of adult males (82/95), and 89.5% of adult females sampled (60/67). Using our assignment criteria in COLONY, we clustered each offspring with its mother and father, and with its full- and half-siblings, determining genotypes for all sampled and inferred parents but only accepting clusters with probabilities >0.95. We recovered 34 clusters from Caraguatatuba (excluding four clusters supported below 0.95 level) and 13 clusters from Picinguaba (excluding two clusters supported below 0.95 level). Recovered clusters are represented in Figures 1 and 2. Across all adults sampled, 26.9% of females (18/67) were assigned as mothers and 27.4% of males (26/95) were assigned as fathers to at least one of the sampled offspring. For the offspring sampled, 74.7% of embryos (115/154), 67.7% of tadpoles (21/31), 68.3% of juveniles (43/63), 80.5% of subadults (33/41) were assigned to parents. All parental genotypes included (sampled and inferred) had high likelihood values and were based on a large number of loci, yielding reliable results in the downstream calculations of relatedness.

In Caraguatatuba, 86.8% (33/38) of mated females sired offspring with a single male and 13.2% (5/38) sired with two males. In Picinguaba, 64% (16/25) of mated females sired offspring with a single male, 28% (7/25) sired with two males, and 8% (2/25) sired with three males. Thus, *Cycloramphus* females are polyandrous. In Caraguatatuba, 89.7% (35/39) of mated males sired offspring with a single female

and 10.3% (4/39) sired with two females. In Picinguaba, 66.7% (16/24) of mated males sired offspring with a single female, 16.7% (4/24) sired with two females, and 16.7% (4/24) sired with three females. Thus, *Cycloramphus* males are polygynous.

In Caraguatatuba, both females and males mate with one or two individuals (females:  $1.13 \pm 0.34$  mates,  $N = 38$ ; males:  $1.10 \pm 0.31$  mates,  $N = 39$ ), with no statistical difference in number of mates between sexes (unpaired one-tailed Mann Whitney's test:  $p = 0.4168$ ). In Picinguaba, both females and males mate with one, two, or three individuals (females:  $1.44 \pm 0.65$  mates,  $N = 25$ ; males:  $1.5 \pm 0.78$ ,  $N = 24$ ), also with no statistical difference in number of mates between sexes (unpaired one-tailed Mann Whitney's test:  $p = 0.488$ ). Overall females and males have the same number of mates (females:  $1.25 \pm 0.51$  mates, range = 1–3 mates,  $N = 63$ ; males:  $1.25 \pm 0.57$  mates, range = 1–3 mates,  $N = 63$ ), resulting in a promiscuous mating system. The three clutches sampled were full-sibships, thus despite multiple mating, only one female and one male fertilize each clutch, with no evidence of multiple paternity or maternity within clutches.

We observed only occasional recurrent pair formation ( $N = 6$ ) with a 3-month  $\pm 1.26$  (range = 1–5-months) interval between remated clutches. In Caraguatatuba, two pairs remated with a 4-month interval  $\pm 1.41$  (range = 3–5-months); in Picinguaba, four pairs remated with a 2.5-month interval  $\pm 1.00$  (range = 1–3-months). Our findings show that females and males mate with several individuals, with no evidence of pair exclusivity in both populations, and thus mate fidelity is unlikely.

### 3.3 | Fitness and assortative mating

In Caraguatatuba, the average adult female body length and weight were  $46.2 \text{ mm} \pm 7.04$  (range = 35.2–57.1 mm) and  $12 \text{ g} \pm 5.53$  (range = 4.2–26.4 g), and the average

adult male body length and weight were  $42.6 \text{ mm} \pm 4.43$  (range = 33.9–54.5 mm) and  $9.8 \text{ g} \pm 3.28$  (range = 4.7–20.1 g). In Picinguaba, the average adult female body length and weight were  $52.6 \text{ mm} \pm 4.51$  (range = 44–60.4 mm) and  $15 \text{ g} \pm 3.18$  (range = 10.3–20.3 g), and the average adult male body length and weight were  $47.3 \text{ mm} \pm 4.45$  (range = 41–58.9 mm) and  $11.1 \text{ g} \pm 2.04$  (range = 8–15.8 g).

In Caraguatatuba, females assigned as mothers to at least one offspring (SVL =  $48.38 \text{ mm} \pm 6.80$ , range = 36.41–57.06 mm,  $N = 12$ ; weight =  $13.92 \text{ g} \pm 5.99$ , range = 5.78–26.40 g,  $N = 12$ ; body condition =  $0.66 \pm 0.09$ , range = 0.49–0.82,  $N = 12$ ) are larger than unassigned females (SVL =  $45.54 \text{ mm} \pm 7.06$ , range = 35.24–56.58 mm,  $N = 42$ ; weight =  $11.44 \text{ g} \pm 5.35$ , range 4.23–21.69 g,  $N = 44$ ; body condition =  $0.61 \pm 0.10$ , range = 0.40–0.78,  $N = 42$ ). These differences are statistically significant (unpaired one-tailed Mann Whitney's test: SVL,  $p = 0.0197$ ; weight,  $p = 0.0119$ ; unpaired one-tailed Student's  $t$  test: body condition,  $p = 0.0122$ ). Males assigned as fathers (SVL =  $41.97 \text{ mm} \pm 5.10$ , range = 33.87–51.59 mm,  $N = 18$ ; weight =  $10.07 \text{ g} \pm 4.13$ , range = 5.17–20.12 g,  $N = 18$ ; body condition =  $0.60 \pm 0.09$ , range = 0.46–0.76,  $N = 18$ ) are smaller than unassigned males (SVL =  $42.78 \text{ mm} \pm 4.24$ , range = 34.16–54.50 mm,  $N = 59$ ; weight =  $9.72 \text{ g} \pm 3.01$ , range = 4.71–19.10 g,  $N = 58$ ; body condition =  $0.59 \pm 0.07$ , range = 0.43–0.75,  $N = 58$ ), but assigned males are heavier and have higher body condition. However, these differences are not statistically significant (unpaired one-tailed Student's  $t$  test: SVL,  $p = 0.2487$ ; body condition,  $p = 0.4224$ ; unpaired one-tailed Mann Whitney's test: weight,  $p = 0.5$ ). Correlations among sampled mates ( $N = 10$ ) are all negligible (Spearman: SVL = -0.16; Pearson: body weight = 0.11; body condition = 0.01), suggesting non-assortative mating in Caraguatatuba.

In Picinguaba, females assigned as mothers to at least one sampled offspring (SVL = 53.72 mm  $\pm$  5.85, range = 44.04–60.35 mm,  $N$  = 6; weight = 16.96 g  $\pm$  2.67, range = 13.50–20.25 g,  $N$  = 6; body condition = 0.71  $\pm$  0.04, range = 0.66–0.76,  $N$  = 6) are larger than unassigned females (SVL = 51.31 mm  $\pm$  2.04, range = 49.19–54.58 mm,  $N$  = 5; weight = 12.70 g  $\pm$  1.99, range 10.25–15.75 g,  $N$  = 5; body condition = 0.64  $\pm$  0.03, range = 0.60–0.69,  $N$  = 5). Females assigned as mothers were significantly heavier and had higher body condition than unassigned females (unpaired one-tailed Student's  $t$  test: SVL,  $p$  = 0.2039; weight,  $p$  = 0.0082; body condition,  $p$  = 0.0088). Males assigned as fathers (SVL = 49.41 mm  $\pm$  1.82, range = 47.74–52.38 mm,  $N$  = 8; weight = 12.47 g  $\pm$  1.66, range = 10.50–15.75 g,  $N$  = 8; body condition = 0.64  $\pm$  0.03, range = 0.61–0.70,  $N$  = 8) are larger than unassigned males (SVL = 45.63 mm  $\pm$  5.27, range = 41.02–58.89 mm,  $N$  = 10; weight = 9.94 g  $\pm$  1.59, range = 8.00–13.00 g,  $N$  = 10; body condition = 0.60  $\pm$  0.03, range = 0.55–0.63,  $N$  = 10). These differences are statistically significant (unpaired one-tailed Mann Whitney's test: SVL,  $p$  = 0.0116; unpaired one-tailed Student's  $t$  test: weight,  $p$  = 0.0023; body condition,  $p$  = 0.0013). Correlations among sampled mates ( $N$  = 4) are negligible for SVL but moderate for body weight and body condition (Pearson: SVL = -0.06; body weight = 0.53, body condition = 0.67). Therefore, mating in Picinguaba is positively assortative for body weight and body condition.

Females who are larger, heavier, and have better body condition have higher fitness in both populations, therefore we infer that these traits provide reproductive advantages over smaller females. The patterns for males are less consistent, with larger males having reproductive advantages over smaller males only in Picinguaba. Our findings suggest that both females and males likely have strong intrasexual competition, but reproductive success across males might have higher levels of

variation according to distinct habitats and population conditions. Overall, we found that *Cycloramphus boraceiensis* does not mate assortatively according to these three body size traits. We found strong selection for large female size, but less evidence of higher fitness for large males.

### 3.4 | Mate relatedness

Within each population, Caraguatatuba and Picinguaba, we compared the average pairwise  $W$  (Wang's relatedness estimator) for mated pairs (mated pairs in Caraguatatuba:  $W = 0.52$ ; mated pairs in Picinguaba:  $W = 0.21$ ) to populations of opposite-sex adult pairs randomly resampled from all possible opposite-sex pairs (Figure 3). For both populations, we found that the average relatedness of mated pairs was significantly outside the distribution of mean relatedness values of randomly resampled pairs. Therefore, *C. boraceiensis* females and males are mating with more related individuals than would be expected if pair formations were random.

## 4 | DISCUSSION

### 4.1 | Mating system characterization

*Cycloramphus boraceiensis* is structured in a promiscuous mating system, with both females and territorial males having equal numbers of multiple sequential mates (1–3 mates per individual). We found no evidence of multiple parents within clutches, as would be expected if males are defending exclusive breeding sites from competing males. However, even with only a few occasional re-pairing of mates, we also did not find any evidence for mating fidelity. Thus, distinct females occasionally mate with the same male and at the same breeding site, but not at the same time.

Likely high levels of male-male competition and territoriality in *C. boraceiensis* (Hartmann, Hartmann, Prado & Garcia, 2003; Verdade, 2005) promote high certainty of paternity, which likely evolved along with male parental care in the species (Ah-King, Kvarnemo & Tullberg, 2005; Clutton-Brock, 1991; Royle, Smiseth & Kölliker, 2012). Saxicolous habitats can be hostile for anamniotic eggs due to high risks of desiccation and exposure to predators, diseases, and parasites, therefore egg guarding and attendance by males is likely critical for offspring survival in *C. boraceiensis* (Bickford, 2004; Clutton-Brock, 1991; Poo & Bickford, 2013; Royle, Smiseth & Kölliker, 2012). Overall, when the cost of losing mating opportunities offsets the importance of paternal care for offspring survival, and when the direct costs of multiple mating by females offset the direct and indirect benefits, both paternal care and female multiple mating might evolve concurrently (Ihara, 2002). With individuals facing a hostile environment for clutches, promiscuity system might have evolved in *C. boraceiensis* as a consequence of direct and indirect benefits associated with sequential polygamy on offspring survival. The temporal and spatial spreading of eggs due to the sequential distribution of paternal care decreases the risk of reproductive failure associated with low-fertility, insufficient care, or less-suitable sites for embryo development, thus increasing offspring survival (Byrne & Keogh, 2009; McLeod & Marshall, 2009).

## **4.2 | Fitness and non-assortative mating**

Although large variance in adult female and male body sizes, and evidence of differential reproductive success across body sizes, our three first hypotheses associating fitness and phenotypes are only partially supported by our findings. Overall, our results show that the fitness benefits of large body size are primarily in

females and mating is not size-assortative. Summarizing: larger females might be stronger and have advantages during intrasexual competition by producing more eggs and likely displacing smaller females, thus having higher fitness but not soliciting larger males; larger males might also be stronger and have some advantages during intrasexual competition by defending and monopolizing optimal breeding sites, guarding clutches, and displacing smaller males, thus having higher fitness, but because females do not discriminate against smaller males these advantages might disappear according to distinct habitats and population conditions; and mating is non-assortative because females choose mates not based on male phenotypic traits.

According to sexual selection theory, males will defend reproductive resources only when they are monopolizable and scarce, thus controlling access to mates (Emlen & Oring, 1977). The degree of intrasexual competition for resources will depend on the availability of the resources and the degree to which they are monopolizable (Emlen & Oring, 1977). Males of *Cycloramphus boraceiensis* vigorously defend their territory and guard their terrestrial clutches, thus investing high levels of energy in territorial maintenance and parental care (Hartmann, Hartmann, Prado & Garcia, 2003; Verdade, 2005). Despite the abundant and continuous distribution of fast-flowing streams surrounded by rocky habitats in the studied areas, the specialized and endemic saxicolous frog studied deposits its terrestrial eggs in crevices that are precisely positioned in the splash-zones of waterfalls, likely because these are optimal microhabitats for embryonic development (Giaretta & Facure, 2003; Verdade, 2005). Therefore, optimal breeding conditions that are critical for offspring survival may be monopolizable by males. These desirable microhabitat conditions along with the crucial paternal egg guarding and

attendance facilitate intrasexual contest and male territoriality. Also, because variance in intrasexual reproductive success might rely on breeding site quality, the quality of the territory might also be a factor in female mate choice.

Larger *C. boraceiensis* females had significantly higher fitness over smaller females, but the same pattern was not consistent for larger males across populations. Thus, although there may be some benefit for large males in terms of breeding site defense, the strength of selection on large sizes for males is less pronounced than for females. Typically, anuran body sizes are positively correlated with mate acquisition in males and with fecundity in females (Cadeddu & Castellano, 2012; Gerhardt & Huber, 2002; Nali, Zamudio, Haddad & Prado, 2014; Tejedo, 1992; Wells, 2010). However, particular reproductive costs for males can also lead them to choosy behaviors, selecting females according to some advantageous trait (Bonduriansky, 2001; Flatt & Heyland, 2011; Swierk & Langkilde, 2019). Anyways, the operational sex ratio in our studied populations was skewed towards males, thus if any mate choice has place here it would be performed by females rather than males. Larger *C. boraceiensis* females are likely more fecund, but they also may have more opportunities to mate with higher-quality males through intense intrasexual competition. There is broad documentation on females also influencing trait evolution and mating system dynamics, with evidences on females competing for mating resources or partners (Cain & Ketterson, 2012; Cain & Rosvall, 2014; Comendant, Sinervo, Svensson & Wingfield, 2003; Rosvall, 2011). In *C. boraceiensis*, males are highly competitive maintaining optimal breeding sites and call from territories they defend to attract females, but they are not necessarily choosy during mate choices; whereas females are most likely also highly competitive accessing males (consequently, accessing breeding sites), attracted by calls of

males, larger females can displace smaller females, but likely they do not necessarily solicit only larger mates. It is possible that not female and male mate choices but intrasexual competition is primarily mediating the difference in the strength of selection on large sizes for females and males, which results in non-assortative mating relative to the body size.

Because for some systems low-quality individuals may prefer low-quality mates, reducing risks associated with intrasexual competition (Härdling & Kokko, 2005), it could be possible that all size-classes of *C. boraceiensis* individuals had similar chances to breed, with larger females mating with larger males and smaller females mating with smaller males. However, not only larger females are consistently having higher fitness, but they are also mating with both large and small males. Actually, non-assortative mating is a plausible cause for the variable number of non-fertilized eggs that we found within all sampled clutches (13.2%, 27.1%, and 4.7% of unfertilized eggs in each clutch), likely with distances between female and male cloacae inversely proportional to fertilization rates (Davis & Halliday, 1977; Robertson, 1990). The probable reason why this disadvantage in fertilization does not lead to assortative mating in *C. boraceiensis* is because the advantages of female size in fecundity and intrasexual competition for accessing males likely override the costs. Because size-assortative mating is widely detected across animal taxa (e.g., Pack et al., 2012; Vreys & Michiels, 1997), it is commonly accepted that both females and males get fitness advantages when choosing their mates (Byers & Waits, 2006; Lancaster, Hipsley & Sinervo, 2009). However, other studies also document absence of size-assortative mating in anuran populations (Briggs, 2008; Olson, Blaustein & O'Hara, 1986; Ritke & Semlitsch, 1991; Sullivan, 1987). In the North American wood frog *Rana sylvatica*, for example, when males mate with

preferred females they produce fewer and lower-quality offspring (lower viability and growth) versus when they mate with nonpreferred females (Swierk & Langkilde, 2019). In fact, neither occurrence nor absence of size-assortative mating in anuran populations has yet a coherent biological explanation (Wells, 2010).

*Thoropa*, the saxicolous sister genus of *Cycloramphus*, is very similar in terms of its reproductive natural history (Giaretta & Facure, 2004; Frost et al., 2006; Fouquet et al., 2013; Jetz & Pyron, 2018). Both *Thoropa* and *Cycloramphus* saxicolous species breed in fast-flowing streams where males defend and monopolize breeding sites. Also using parentage analyses, Muralidhar, de Sá, Haddad & Zamudio (2014) demonstrated that *T. taophora* has a polygynous mating system, with a monopolist male repeatedly mating with two females and suggesting group fidelity over a prolonged breeding season. That system, however, occurred only in wild populations with extreme scarcity of breeding sites. In populations where breeding sites were abundant and more homogenously distributed, *Thoropa* adopted a promiscuous mating system, confirming that resource availability and population sizes result in different social structures (Emlen & Oring, 1977). The promiscuous mating system found in our study for *C. boraceiensis* is likely due in part to an abundant and continuous distribution of fast-flowing streams surrounded by rocky habitats in the studied areas, making several sequential mates available. It is worth to highlight that natural scarcity of microhabitat resources in portions of the forest along with differences in *C. boraceiensis* population sizes will most likely result in different mating system structures.

### 4.3 | Mate relatedness

Inbreeding can cause negative effects on offspring, such as fixation of harmful alleles in small populations, decrease of heterozygosity, and expression of deleterious recessive phenotypes (Oh & Badyaev, 2006; Pusey & Wolf, 1996; Ralls, Ballou & Templeton, 1998). In *Cycloramphus boraceiensis*, as we found that females have sequential mates and are not choosy when selecting mates, we would expect to find a high genetic diversity for mated pairs, with direct benefits for the species. Thus, kin avoidance during mate choices was expected as a good adaptive strategy for *C. boraceiensis*. Surprisingly this was not observed, and we actually found the opposite, refuting our fourth hypothesis. Although mating in *C. boraceiensis* is non-assortative by sizes, the average relatedness of mated couples is significantly greater than the relatedness of random sampled pairs. Typically, there are two explanations on how behaviors affect mate choices: (1) the natal dispersal determines the mating options, with the movement from the birth site to the breeding site setting the potential mates available to be chosen as mates; (2) females and males actively choose mates, with avoidance or preference of related individuals as mates (Keller & Arcese, 1998; Pusey & Wolf 1996; Van de Castele & Matthysen, 2006; Wiley & Poston, 1996). Evolution of inbreeding avoidance can be associated with sex-biased dispersal, where one sex leaves the natal site, thus decreasing chances of inbreeding for both sexes (Van de Castele & Matthysen, 2006). This mechanism seems to be not existent for *C. boraceiensis*, with our findings suggesting that both sexes are in some level philopatric. When the natal dispersal mechanism is not existent, evolutionary forces might select individuals, leading to the establishment of active choice with avoidance of related mates based on relatedness cues, such as spatial information, familiarity, or recognition alleles (Blouin & Blouin, 1988; Pusey & Wolf, 1996). For *C.*

*boraceiensis*, it seems that not only both females and males do not disperse, but they also do not have any other mechanism to avoid related mates. Studying *Thoropa taophora*, Muralidhar, de Sá, Haddad & Zamudio (2014) observed that females sharing the same male showed lower relatedness than population average. They attributed this pattern mainly to the fact that the species has elevated larval cannibalism. In *C. boraceiensis* cannibalism is not known, indicating that selection for kin avoidance might be absent.

Inbreeding can be not totally disadvantageous, with costs depending on the particularities of each species or population. Lack of evidence for inbreeding depression (Keane, Creel & Waser, 1996; Koenig & Dickinson, 2004), exclusion of deleterious alleles (Thornhill, 1993), and maintenance of beneficial coadapted gene complexes (Bateson, 1983) are some factors that have been observed in association with inbreeding tolerance. Besides, when alternative mating is possible for both partners and when the benefits from kin preference outweighs the costs from inbreeding depression (Dawkins, 1979; Kokko & Ots, 2006), inbreeding is recognized for increasing inclusive fitness (Bengtsson, 1978; Blum & Blum, 1979; Lehmann & Perrin, 2003). Also, according to the differential allocation hypothesis, mate relatedness affects parental investment, with parents modulating their investment based on the quality of their partners and offspring (Burley, 1986, 1988; Sheldon, 2000). Based on both inclusive fitness and differential allocation hypothesis, kin preference in *C. boraceiensis* pairs can be evolutionarily strengthening the paternal care behavior because eggs are carrying genetic materials very close to their parents. Furthermore, the increase in parental care by an individual that mate with a relative might guarantee the survival of any less-fit young, compensating any inbreeding effect (Margulis, 1997). Therefore, in particular circumstances mate

relatedness can be tolerated or even preferred (e.g., Cohen & Dearborn, 2004; Kleven, Jacobsen, Robertson & Lifjeld, 2005; Thünken, Bakker, Baldauf & Kullmann, 2007), so absence of mechanisms to avoid inbreeding are not always a consequence of the restriction on breeding opportunities or of the incapacity for relatedness recognition (Kokko & Ots, 2006).

## ACKNOWLEDGEMENTS

We thank Marcio Martins for suggesting study areas; Clara H. S. Silva, Nadya C. Pupin, and Steve M. Bogdanowicz for support with field and laboratory work. This research was funded by grants from São Paulo Research Foundation (FAPESP; #2014/24972-4, #2016/06876-3, and #2018/17993-6 to F.P.S., and #2013/50741-7 and #2014/50342-8 to C.F.B.H.), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Finance Code 001; to F.P.S.), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; to C.F.B.H. and K.Z.).

## AUTHOR CONTRIBUTIONS

F.P.S., C.F.B.H., and K.Z. conceived the study. F.P.S., G.G.G., and C.A.B. collected field data. F.P.S. and M.B. collected microsatellite genotypes and performed all analyses. F.P.S., M.B., K.Z., and C.F.B.H. wrote the manuscript with input from G.G.G. and C.A.B.

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**FIGURE 1.** Mating network of *Cycloramphus boraceiensis* in Caraguatatuba, São Paulo, Southeastern Brazil. Unlikely clusters are not included. Females (F) assigned as mothers are labeled with red circles and males (M) assigned as fathers are labeled with green circles. Inferred maternal and paternal genotypes are indicated by iF and iM, respectively.

**FIGURE 2.** Mating network of *Cycloramphus boraceiensis* in Picinguaba, Ubatuba, São Paulo, Southeastern Brazil. Unlikely clusters are not included. Females (F) assigned as mothers are labeled with red circles and males (M) assigned fathers are labeled with green circles. Inferred maternal and paternal genotypes are indicated by iF and iM, respectively.

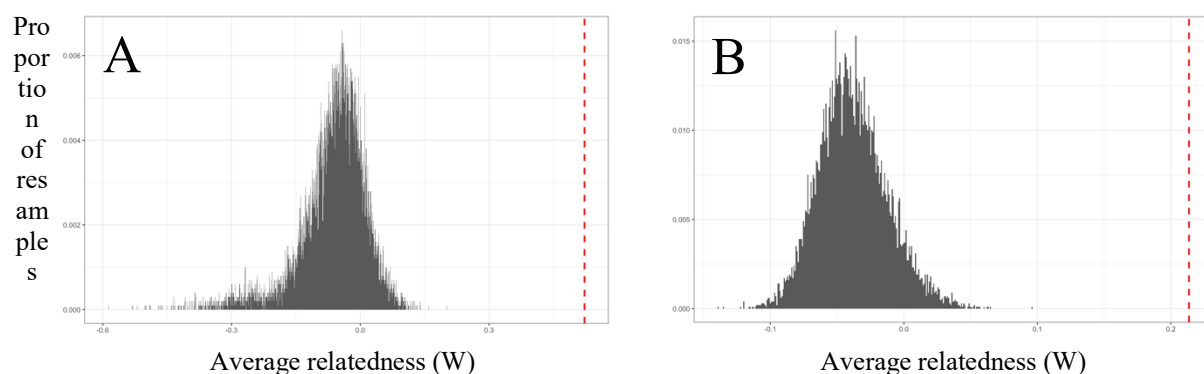


**FIGURE 3.** Distribution of the average relatedness for pairs of *Cycloramphus boraceiensis* adults randomly sampled from all possible opposite-sex pairs. Based on Wang's relatedness estimator (W), average relatedness was calculated separately for each sampled population, (A) Caraguatatuba and (B) Picinguaba, Ubatuba, both localities from State of São Paulo, Southeastern Brazil. Red dashed lines indicate the average relatedness of actual mated pairs in each studied population.

## CAPÍTULO IV

**Unexpected reproductive fidelity in a polygynous frog**

*(manuscrito sob revisão na revista Science Advances)*

**Title****Unexpected reproductive fidelity in a polygynous frog****Short title****An unknown mating system in amphibians****Authors**

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**Abstract**

Polygynous mating systems with group fidelity are a common animal organization, typically consisting of multiple females in a mated group with a single male for an extended period of time (also referred to as harem polygyny). Single-male polygyny with reproductive fidelity occurs in invertebrates, bony fishes, and some tetrapods, such as lizards, mammals, and birds. In amphibians, despite high diversity, this mating system is unknown. Combining data on larval development, molecular paternity assignment, and *in situ* behavioral observations, we reveal high fidelity during a prolonged breeding season in a Neotropical polygynous frog. Monopolist males dominate scarce breeding sites, guarding offspring and mating exclusively with multiple females. This system likely evolved in response to intense competition for breeding sites and intrasexual competition for mates.

## Introduction

Animal social organizations are characterized by mating systems with different levels of cohesiveness, along a continuum from polygamy by both sexes (promiscuous mating) to monogamy (1, 2). Polygamy is the evolutionarily ancestral condition in vertebrates, with monogamy evolving in many clades, including amphibians (3, 4). The evolution of monogamy is typically driven by the need for parental investment, and reinforced by establishment of strong mate bonds (1, 2, 5). The most common social organization among vertebrates is polygyny, with males maximizing their fitness by mating with several females (2, 5). Across polygynous species, variable levels of mate fidelity and/or bonding have evolved (1–4, 6). In cases of extreme resource limitation, polygynous males often defend mates or resources, or increase parental investment to guarantee offspring survival, leading to evolution of mate bonding and sometimes parental care (1, 2). This general pattern is not present in frogs, where resource or female defense are common, but polygyny does not involve mate fidelity (7–10) as in other animal groups (11–15). Therefore, frogs, even those with prolonged breeding seasons, have decoupled resource or female defense from any form of fidelity or pair bonding, making them an exception among tetrapods (16–18).

Here, combining data on larval development, molecular paternity assignment, and *in situ* behavioral observations, we reveal unexpected high fidelity in the saxicolous frog *Thoropa taophora* (family Cycloramphidae). Endemic to the Brazilian Atlantic rainforest, this frog has a peculiar semi-terrestrial tadpole that feeds and develops until metamorphosis in freshwater seeps flowing on the surfaces of outcrops and rocky shores (19, 20). During the night, males are markedly aggressive and defend seeps as breeding territories for egg deposition by females (19).

Throughout the ten-month breeding season (19, 21), males defend their territories against potential predators, including conspecifics, which can cannibalize eggs and tadpoles (19). We found that monopolist males mate with two genetically unrelated females (a dominant and a secondary) recurrently and exclusively during the prolonged breeding season, and therefore this species represents the first known case of single-male polygyny with reproductive fidelity in amphibians.

## Results

### Territorial defense and mating behaviors

To examine the possibility of pair-bonding in this polygynous species we video-recorded individual interactions among males and females under natural conditions, and described specific interaction types and their frequencies (hereafter indicated by  $n$ ; table S1). Monopolist males remain close to their eggs and tadpoles, protecting them and occasionally patrolling territorial boundaries. They discourage intruders by emitting aggressive calls ( $n = 807$ ), sometimes also sparsely adding advertisement calls ( $n = 103$ ), and by displaying body-raising postures ( $n = 105$ ). Monopolist males use vigorous jump-attacks ( $n = 148$ ), kicks ( $n = 16$ ), and embraces that press keratinized spines on their thumbs against the intruder's body ( $n = 9$ ) to repel conspecific males that invade their territory or begin to cannibalize eggs (movies S1 and S2).

We also video-captured mating behaviors which, combined with parentage studies, confirm that *Thoropa taophora* is polygynous, with one monopolist male mating with two females, simultaneously sharing the same breeding seep for both females' egg clutches (22, 23) over a prolonged breeding season with multiple reproductive bouts. Monopolist males interact in courtship with two or three females

at the same time, with distinct types and levels of interactions between male and each female, indicating a hierarchy among females (table S1, Fig. 1, and movies S3 and S4). In a typical mating interaction, a dominant female mates with the male ( $n = 3$ ) and emits female reciprocal calls (24) in response to courtship behaviors ( $n = 10$ ). A secondary female, and sometimes a third peripheral female, are mostly motionless during mating activities of the dominant female. Our field videos revealed three behavioral tactics in *T. taophora* courtship: (I) a female approaches the male, turns her back toward him, and positions herself under him, thus stimulating amplexus (this behavior was only observed for dominant females,  $n = 35$ ); (II) a female moves within the territory, the male approaches the female (observed for dominant,  $n = 110$ , secondary,  $n = 19$ , and peripheral females,  $n = 13$ ), and sometimes amplexes her (amplexus only observed for dominant females,  $n = 5$ ); or (III) a female begins to cannibalize eggs, the male immediately stops the behavior by either chasing her away (observed for dominant,  $n = 14$ , secondary,  $n = 3$ , and peripheral females,  $n = 2$ ), by briefly embracing her (observed for secondary,  $n = 2$ , and peripheral females,  $n = 2$ ), or by effectively amplexing her (only observed for dominant females,  $n = 2$ ). These behavioral tactics show that female-female competition is acting within the mated group, with clear social ranks and different fitness outcomes for females.

Males respond differently to behaviors by females with different ranks. Amplexus by the monopolist male precludes further cannibalism by the dominant female and results in new eggs (deposited by the monopolist male and the dominant female). When a secondary or a peripheral female cannibalizes eggs, the monopolist male immediately approaches and briefly embraces the female, which stops cannibalism. This embracing behavior with secondary and peripheral females does not resemble amplexus and does not result in mating, but interrupts female cannibalism of eggs. In

our video recordings, we never observed mating with secondary or peripheral females; however, molecular parentage analyses of offspring in seeps confirm that secondary females also mate and deposit eggs that are fertilized by the monopolist male (23).

### **Tadpole cohorts and reproductive fidelity**

We paired parentage analyses (23) with tadpole cohort analyses to measure the reproductive success of dominant, secondary, and peripheral females in seeps. Reconstructed parental genotypes for tadpoles collected at seven breeding seeps indicate a polygynous mating system in *Thoropa taophora*, with larval half-sibships within each seep coming from a father and two mothers (23). Based on the parentage data, we separated tadpoles per mother from each site sampled and show that the two females contribute unevenly to the total number of tadpoles. Each one of the seven breeding seeps analyzed included 56% – 97% (mean =  $86 \pm 14.8\%$ ) of tadpoles from a dominant female and 3% – 44% of tadpoles (mean =  $14 \pm 14.8\%$ ) from a secondary female (Table 1 and table S2). Tadpoles sampled from each one of 14 mothers (two mothers per breeding seep) varied in size from  $4.3 \pm 1.6$  mm to  $10.4 \pm 1.7$  mm and ranged in developmental Gosner stages (25) from  $27 \pm 3$  to  $39 \pm 2$  ( $n = 563$ ; table S2). This range of developmental stages includes individuals recently hatched to stages immediately preceding metamorphosis (Table 1), and indicates that the tadpoles belong to different cohorts, hatched from eggs laid at different times by the same mating pair. Overall, developmental stages of tadpoles assigned to the two females overlap entirely, indicating that the dominant and secondary females mate multiply with the same male several times during the breeding season. We define this multiple mating by mated pairs as reproductive fidelity (Fig. 2; 1, 7, 16–

18). We found no evidence of females or males mating in more than one breeding seep, even when neighboring seeps were only a few meters apart. Therefore, the genetic parentage and tadpole cohort analyses corroborate our field behavior observations of male monopoly of breeding seeps, and repeated mating with the same two females.

## Discussion

Our findings extend female-defense polygynous mating systems with reproductive fidelity (harem polygyny; 1, 16–18) to all tetrapod groups and raise some interesting questions about the contexts that favor fidelity over promiscuous mating systems. The scarcity of breeding sites, and their discrete and monopolisable nature, promote intense competition among males for sites and among females for access to those sites. Males reach higher reproductive success not only by monopolizing scarce breeding resources, but by maintaining females, and aggressively excluding all other conspecific intruder males from their territories (1, 2, 5). The fitness benefits of breeding site monopoly must be extremely high, explaining male investment in intense, and likely costly, aggressive behaviors (1, 5). High male intrasexual aggression can also select for sexual dimorphism in weaponry that females lack (26). In *T. taophora* this is reflected in the enlarged male forearms and keratinized thumb spines (19, 27) we observed being used in male-male agonistic interactions.

When critical resources are unevenly distributed, a female mating with an already paired male at a superior-quality breeding site will most likely have equal or higher reproductive success than a female mating with an unpaired male at a poorer-quality site, thus promoting polygyny (1, 5). Females within each single-male group also exhibited a social rank, with dominant females having higher reproductive success

(28). The cost of polygyny in this system is the loss of eggs due to cannibalism by females in the group. We do not currently know whether females preferentially eat the eggs of others, but we previously showed that females sharing a seep are less-related to each other than expected given levels of kinship in the overall population (23). We hypothesize that the low female-female relatedness in the polygynous group offsets the potential cost of cannibalism of closely related tadpoles. Low relatedness between reproductive *T. taophora* females in each breeding seep also results in more diverse offspring for the monopolist male, compensating for inbreeding effects inherent in polygyny (5). Our behavioral observations complement earlier parentage analyses and show that cannibalism of eggs by females is in fact part of the mating repertoire and can elicit male amplexus, thus promoting complex costs and benefits in *T. taophora*. Cannibalism within the same group is unfavorable for the monopolist males but advantageous for females, who acquire food energy, reduce competition from embryos produced by the other females, increase resource availability for their own embryos (especially if females can distinguish their own eggs), and finally gain new fertilized eggs through new mating opportunities.

The constraints imposed by limited breeding sites, coupled with the risks to offspring survival, have imposed strong sexual selection on *T. taophora* males and females, leading to a diversity of mating behaviors that were previously unknown. In amphibians, the existence of long-term fidelity between mated individuals, and the behaviors that maintain it, has been studied primarily in monogamous species (3, 4) where participation of both parents is required for successful reproduction. We propose that reproductive fidelity in frogs may be more common than previously thought, and among polygynous frogs, will most likely occur in reproductively

specialized species that depend on specific breeding habitats that are not ubiquitous in the environment.

## **Materials and Methods**

### **Focal populations and sampling**

We studied the mating system of *Thoropa taophora* at two different Atlantic rainforest sites in the state of São Paulo, Brazil. Both study sites were coastal rocky outcrops, with few freshwater seeps of small dimensions (1 to 4 m<sup>2</sup>,  $n = 17$ ) which are the ideal breeding sites for this species. All field research was approved by the Committee on Animal Care Ethics at Universidade Estadual Paulista, Rio Claro.

In January 2004, we sampled *Thoropa taophora* larvae on a 664 m long rock shore outcrop at Itaguá beach (23°27' S, 45°03' W), Municipality of Ubatuba, São Paulo state, Brazil. We randomly collected tadpoles from seven freshwater seeps (ranging from 52 to 154 larvae per seep; mean =  $85.4 \pm 37.6$ ,  $n = 598$ ) and stored them in 95% ethanol. Freshwater seeps were separated by 10 to 95 m (mean =  $44 \pm 39.07$  m) of unsuitable dry rock surfaces, which prevents larval migration from their birthplaces. Using a stereomicroscope, we examined all collected tadpoles, classifying them into developmental stages (25). Because tadpoles from Gosner stage 42 onwards have four developed limbs and can migrate from one seep to another, we only selected individuals ranging between Gosner stages 25 and 41. Using a digital caliper, we measured total body lengths (including tail) of all tadpoles to the nearest millimeter. All data on tadpole developmental stages and body sizes, as well as their original breeding seep, are listed in table S2. To measure the strength of the correlation between developmental stages and body sizes for tadpoles we applied the non-parametric Spearman rank correlation coefficient using

R package stats (function `cor.test`; 29, 30). As expected, body size of tadpoles in *T. taophora* is strongly correlated with developmental stage ( $r = 0.965$ ,  $P = 2.2 \cdot 10^{-16}$ ,  $n = 553$ ; fig. S1). The variance in body sizes and developmental stages among sibs can be used to infer whether full-sib tadpoles belong to the same clutch, or clutches laid at different time points. This is corroborated by developmental timelines for closely related species (31, 32) that indicate that plasticity alone is insufficient to explain the range of *T. taophora* tadpole body sizes within each breeding seep.

Between October 2012 and February 2013, we carried out behavioral studies of *Thoropa taophora* on a 600 m<sup>2</sup> rocky shore outcrop in a nearby population at Toque-Toque Grande beach, Municipality of São Sebastião (23°50' S, 45°31' W), São Paulo state, Brazil. Over a period of five months, we spent 53 nights in the field, totaling 138 hours of direct observations under natural conditions and 59 hours and 45 minutes of video recordings under natural conditions at 10 freshwater seeps. We observed territorial defense and courtship behaviors and counted the number of each behavior in the video footage (table S1) using focal animal and all occurrence sampling methods (33, 34). We performed video recordings at night using a Sony DCRSR85 camera in nightshot mode. Between 18:00h and 06:00h, we placed the camera next to male territories to record behaviors without the presence of researchers whenever possible. Altogether we observed 17 events of males agonistically interacting (from 55 hours and 38 minutes of videos) and two reproductive events (entailing courtship, amplexus, and oviposition; from 4 hours and 7 minutes of videos), with the presence of up to three females simultaneously at the same reproductive site. From these videos we characterized types and quantified frequencies of behaviors performed by males and females before and during courtship in polygynous groups (table S1).

## Parentage analyses

In a previous study, we genotyped *Thoropa taophora* tadpoles for parentage analyses using microsatellites (23). We reconstructed parental genotypes and showed that tadpoles from each of the same seven breeding seeps are always half-siblings, sharing a single father and one of two genetically unrelated mothers (23). At the time we hypothesized that females might visit the same breeding seep at different times to oviposit partial clutches, and pointed out the necessity of direct behavioral observations to determine behaviors that might promote potential female breeding site fidelity. In this study we grouped all *T. taophora* sampled tadpoles by genetic kinship, and examined the temporal spread of tadpoles (according to Gosner developmental stages) within each breeding seep. Reinforced by *in situ* behavioral observations, our study reveals aspects of mating system dynamics not previously known for amphibians.

## Supplementary Materials

Fig. S1. Significant positive correlation between body size and developmental stage in *Thoropa taophora* larvae ( $r = 0.965$ ,  $P < 0.001$ ,  $n = 553$ ).

Table S1. Guarding, territorial, and courtship behaviors performed by *Thoropa taophora* adults. Table S2. Body sizes and developmental stages for all *Thoropa taophora* larvae assigned to dominant and secondary females at each of the breeding sites sampled.

Movies S1 and S2. Monopolist male aggressively defends his breeding seep territory.

Movies S3 and S4. *Thoropa taophora* is the first amphibian with a documented single-male polygynous mating system with reproductive fidelity.

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**Acknowledgments:** We thank A.M. Haddad for collection of larvae and G. Machado for help with behavioral observations. **Funding:** Our work was supported by grants from the São Paulo Research Foundation (FAPESP; #2012/00205-9, #2014/24972-4, #2016/06876-3, and #2018/17993-6 to FPS, and #2008/50928-1, #2012/17220-0, and #2013/50741-7 to C.F.B.H.), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; to C.F.B.H., R.C.C., and C.A.B.), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES - Finance Code 001; to F.P.S., R.C.C., and C.A.B.), and National Science Foundation (NSF; to K.Z.). **Author contributions:** F.P.S., K.Z., and C.F.B.H. conceived the project and designed the analyses; C.F.B.H. collected field samples for paternity analyses; F.P.S. and C.F.B.H. analyzed variation in larval development; F.P.S. and P.M. genotyped larvae and performed parentage analyses; R.C.C. and C.A.B. recorded field behavioral data; F.P.S. and R.C.C. analyzed behavioral data; F.P.S., R.C.C., C.F.B.H., and K.Z. wrote the manuscript with contributions from all authors. **Competing interests:**

Authors declare no competing interests. **Data and materials availability:** All data are available in the main text or the supplementary materials.

## Figures and Tables

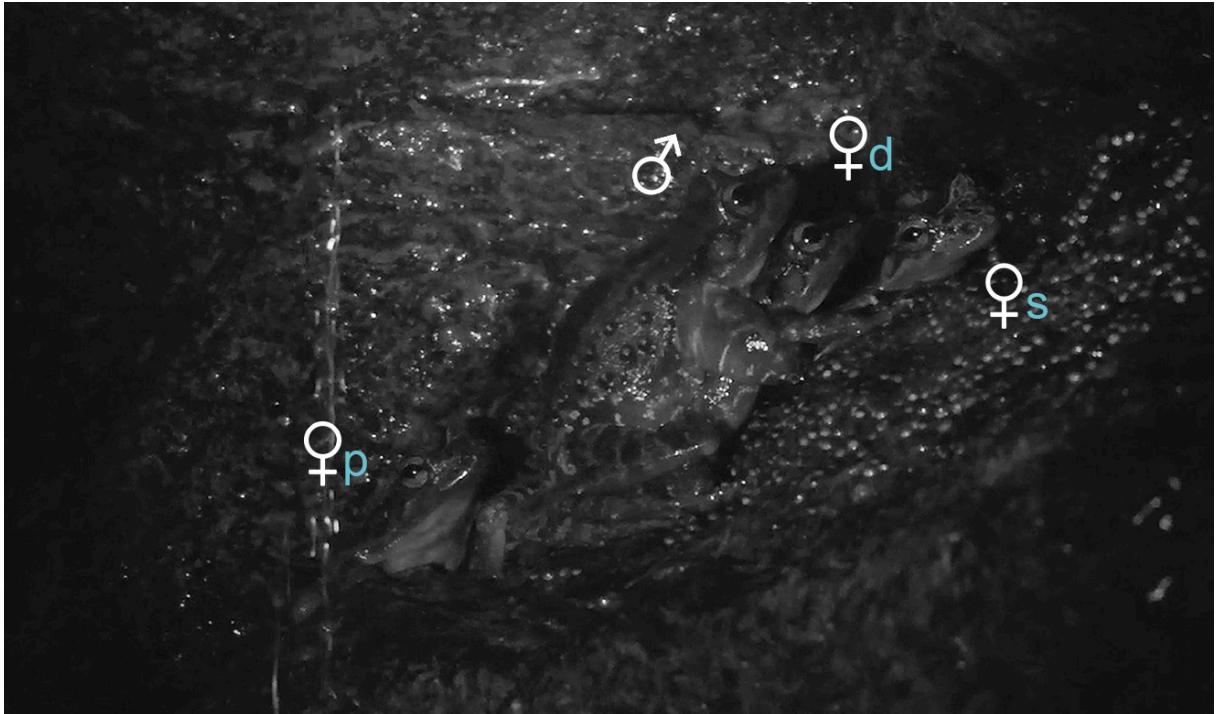


**Fig. 1. Mating in *Thoropa taophora* occurs in single-male polygynous groups with reproductive fidelity, a system well-known among amniote tetrapods but only now recognized in amphibians. Males (♂; note enlarged male forearm, a sexually dimorphic trait) monopolize scarce breeding seeps necessary for egg deposition and larval development. In this photo the male is in his territory with eggs adhered to the humid rock surface, and amplexing the dominant female (♀<sub>d</sub>). A secondary female (♀<sub>s</sub>) and a peripheral female (♀<sub>p</sub>) are near the amplexing pair. Municipality of São Sebastião, São Paulo, Brazil.**

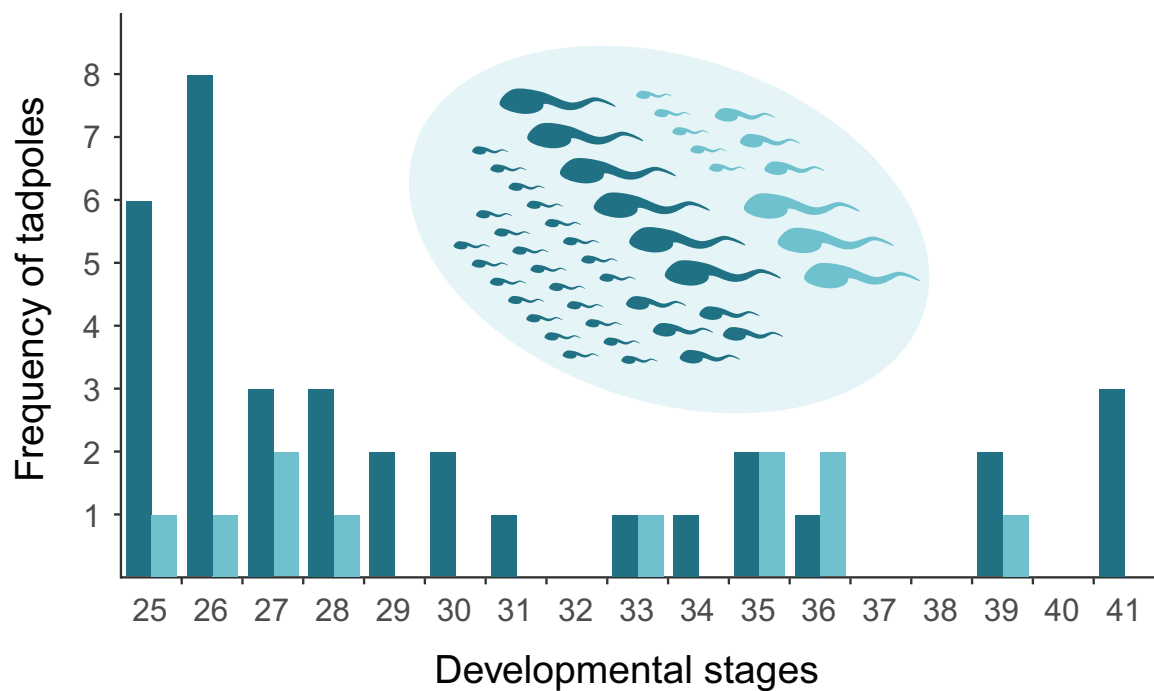


**Fig. 2. Larval developmental stages within seven breeding seeps show recurrent mating by two females with the same monopolist male throughout the breeding season.** Frequencies of larvae mothered by two females (dominant and secondary) from one of the seeps sampled. Post-embryonic tadpoles ranged from developmental stage 25 (immediately post-eclosion) through stage 41 (immediately preceding emergence of all four limbs). Tadpoles of the dominant female are in darker color ( $n = 35$ ) and of the secondary female in lighter color ( $n = 11$ ). Inset scheme shows the proportion of offspring sampled from each mother. The same pattern was found in all breeding seeps sampled (Table 1).

**Table 1. *Thoropa taophora* tadpole body sizes and developmental stages for offspring of dominant and secondary females at each breeding seep analyzed in the parentage study.**

| Females ( <i>n</i> = 14) |           | Tadpoles ( <i>n</i> = 563) |                 |         |                                                                                   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|--------------------------|-----------|----------------------------|-----------------|---------|-----------------------------------------------------------------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
|                          |           | <i>n</i>                   | Body sizes (mm) |         | Gosner developmental stages from recently hatched (25) to near metamorphosis (41) |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                          |           |                            | Smallest        | Largest | 25                                                                                | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 |
| 1 <sup>st</sup> Seep     | Dominant  | 29                         | 5.5             | 8.8     |                                                                                   |    |    |    |    |    |    | ✓  | ✓  | ✓  | ✓  | ✓  |    |    |    |    |    |
|                          | Secondary | 23                         | 3.4             | 7.1     | ✓                                                                                 |    | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  |    |    |    |    |    |    |    |
| 2 <sup>nd</sup> Seep     | Dominant  | 94                         | 3.3             | 11.8    | ✓                                                                                 | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  |    |
|                          | Secondary | 7                          | 3.5             | 7.6     | ✓                                                                                 |    |    | ✓  |    |    |    | ✓  | ✓  | ✓  |    |    |    |    |    |    |    |
| 3 <sup>rd</sup> Seep     | Dominant  | 55                         | 3               | 11.8    | ✓                                                                                 | ✓  | ✓  | ✓  | ✓  |    | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  |    |
|                          | Secondary | 6                          | 7.5             | 11.6    |                                                                                   |    |    |    |    |    |    |    | ✓  |    |    |    |    | ✓  | ✓  | ✓  |    |
| 4 <sup>th</sup> Seep     | Dominant  | 59                         | 3.2             | 11.6    | ✓                                                                                 | ✓  | ✓  | ✓  |    | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  |    | ✓  |    |
|                          | Secondary | 6                          | 5.8             | 10.6    |                                                                                   |    |    |    |    | ✓  |    | ✓  |    |    |    |    | ✓  |    |    | ✓  |    |
| 5 <sup>th</sup> Seep     | Dominant  | 37                         | 3.3             | 10.8    | ✓                                                                                 | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  |    |    | ✓  |    | ✓  |    |
|                          | Secondary | 12                         | 3.8             | 9.9     | ✓                                                                                 | ✓  | ✓  | ✓  |    |    |    | ✓  |    | ✓  | ✓  |    |    | ✓  |    |    |    |
| 6 <sup>th</sup> Seep     | Dominant  | 145                        | 3.5             | 12.7    | ✓                                                                                 | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  |    |
|                          | Secondary | 5                          | 4.8             | 10.9    |                                                                                   |    | ✓  |    |    |    |    | ✓  |    |    |    |    |    | ✓  | ✓  |    |    |
| 7 <sup>th</sup> Seep     | Dominant  | 81                         | 3.4             | 9.9     | ✓                                                                                 | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  |    |    | ✓  | ✓  |    |    | ✓  | ✓  |    |    |
|                          | Secondary | 4                          | 7.2             | 9.2     |                                                                                   |    |    |    |    | ✓  | ✓  |    | ✓  |    |    |    |    | ✓  |    |    |    |

## Supplementary Materials

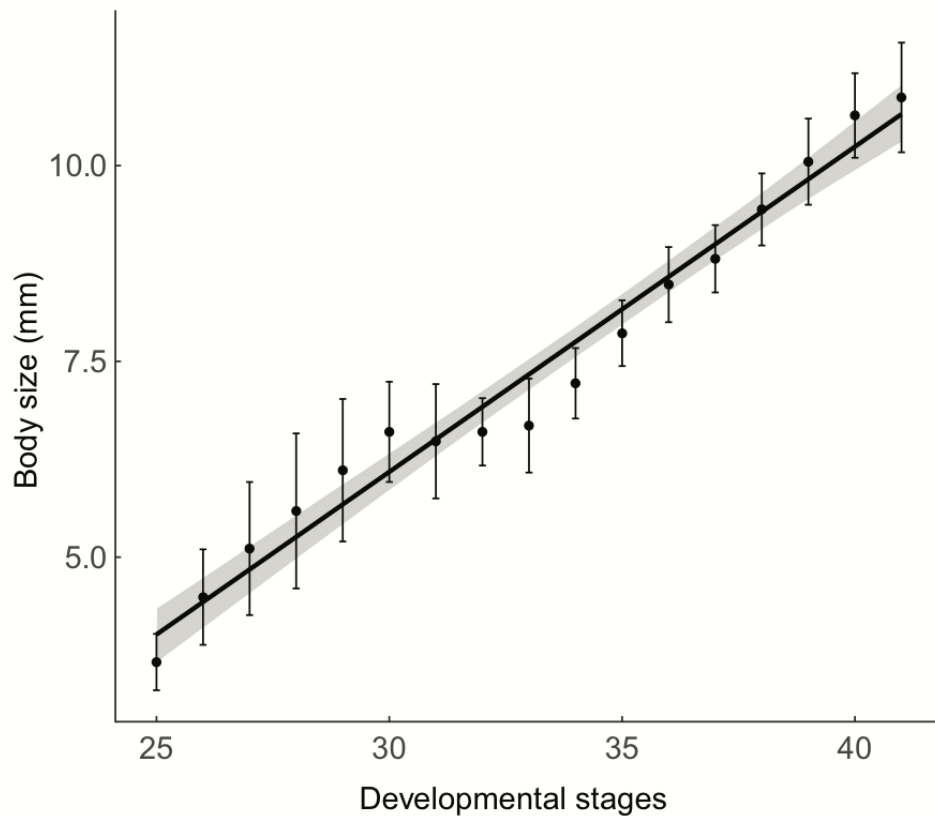


**Fig. S1. Significant positive correlation between body size and developmental stage in *Thoropa taophora* larvae ( $r = 0.965$ ,  $P < 0.001$ ,  $n = 553$ ).** Tadpoles mothered by each female in the breeding seep vary in larval development from recently hatched to near metamorphosis, thus indicating they belong to different clutches of the same parents, a result of reproductive fidelity over multiple mating bouts. Confidence intervals (95%) are shown in grey. Black dots reflect average of body sizes and bars reflect  $\pm$  standard error values.

**Table S1. Guarding, territorial, and courtship behaviors performed by *Thoropa taophora* adults.** Calls, visual displays, and attacks performed by monopolist males when protecting offspring and territories, and female calls and mating behaviors performed during courtship by monopolist males and dominant, secondary, and peripheral females. From October 2012 to February 2013, we video-captured data in the Municipality of São Sebastião, São Paulo, Brazil.

| Guarding, territorial, and courtship behaviors | Monopolist male ( <i>n</i> = 13) | Dominant female ( <i>n</i> = 2) | Secondary female ( <i>n</i> = 2) | Peripheral female ( <i>n</i> = 2) |
|------------------------------------------------|----------------------------------|---------------------------------|----------------------------------|-----------------------------------|
| Aggressive call                                | <i>n</i> = 807                   | -                               | -                                | -                                 |
| Advertisement call                             | <i>n</i> = 103                   | -                               | -                                | -                                 |
| Body-raising posture                           | <i>n</i> = 105                   | -                               | -                                | -                                 |
| Jump-attack                                    | <i>n</i> = 148                   | -                               | -                                | -                                 |
| Kick                                           | <i>n</i> = 16                    | -                               | -                                | -                                 |
| Embrace pressing thumb thorns                  | <i>n</i> = 9                     | -                               | -                                | -                                 |
| Reciprocal call                                | -                                | <i>n</i> = 10                   | <i>n</i> = 0                     | <i>n</i> = 0                      |
| Mating strategy (I)                            | -                                | <i>n</i> = 35                   | <i>n</i> = 0                     | <i>n</i> = 0                      |
| Amplexus from strategy (I)                     | -                                | <i>n</i> = 35                   | <i>n</i> = 0                     | <i>n</i> = 0                      |
| Mating strategy (II)                           | -                                | <i>n</i> = 110                  | <i>n</i> = 19                    | <i>n</i> = 13                     |
| Amplexus from strategy (II)                    | -                                | <i>n</i> = 5                    | <i>n</i> = 0                     | <i>n</i> = 0                      |
| Mating strategy (III)                          | -                                | <i>n</i> = 16                   | <i>n</i> = 5                     | <i>n</i> = 5                      |
| Chase from strategy (III)                      | -                                | <i>n</i> = 14                   | <i>n</i> = 3                     | <i>n</i> = 3                      |
| Embrace from strategy (III)                    | -                                | <i>n</i> = 0                    | <i>n</i> = 2                     | <i>n</i> = 2                      |
| Amplexus from strategy (III)                   | -                                | <i>n</i> = 2                    | <i>n</i> = 0                     | <i>n</i> = 0                      |

**Table S2. Body sizes and developmental stages for all *Thoropa taophora* larvae assigned to dominant and secondary females at each of the breeding sites sampled.** All body sizes (mm) and developmental stages of tadpoles sampled from 14 females (two per breeding seep) in the Municipality of Ubatuba, São Paulo, Brazil. Larval development of each offspring assigned to each female ranged from individuals recently hatched (stage 25) to others about to metamorphose (stage 41), indicating reproductive fidelity over multiple mating bouts.



## **Movies S1 to S4**

**Movies S1 and S2. Monopolist male aggressively defends his breeding seep territory.** Monopolist males discourage intruders by emitting aggressive calls, sometimes also sparsely adding advertisement calls, and by displaying body-raising postures. They repel other conspecific males that invade a territory or that begin to cannibalize eggs with vigorous jump-attacks, kicks, and embraces that press keratinized spines on their thumbs against the intruder's body.

**Movies S3 and S4. *Thoropa taophora* is the first amphibian with a documented single-male polygynous mating system with reproductive fidelity.** Monopolist males interact in courtship with two or three females at the same time, with distinct types and levels of interactions between male and each female, indicating a hierarchy among females. Females cannibalize eggs, triggering amplexus by the monopolist male.

## CONCLUSÕES GERAIS

- i. O ancestral comum do clado *Cycloramphus-Zachaenus* foi provavelmente pequeno, saxícola e com baixo dimorfismo sexual em tamanho. A partir desse ancestral, a terrestrialidade evoluiu independentemente três vezes. Ao longo da evolução há uma associação significativa entre o habitat reprodutivo e o dimorfismo sexual em tamanho. As passagens para a reprodução terrestre evoluíram de modo correlacionado com diminuições no tamanho de corpo dos machos, mas não no tamanho de corpo das fêmeas. A reprodução terrestre aumenta a disponibilidade de sítios reprodutivos, além de resultar em uma maior ocultação do amplexo, da oviposição e do cuidado parental, reduzindo assim a competição intrasexual dos machos em todos os estágios reprodutivos. É inferido que a evolução da reprodução terrestre correlacionada com o tamanho menor dos machos é decorrente de uma atenuação na intensa competição macho-macho, que é típica na reprodução saxícola (por ser mais exposta).
- ii. Na zona de contato secundária das espécies-irmãs saxícolas *Cycloramphus boraceiensis* e *C. dubius* está ocorrendo hibridação entre linhagens, havendo discordância mitonuclear e introgressão assimétrica de alelos nucleares. A distribuição do padrão morfológico dorsal das linhagens segue aproximadamente a distribuição das variações genômicas (dados nucleares). É inferido que principalmente uma dispersão maior das fêmeas em relação aos machos somada à competição intrasexual interespecífica (de fêmeas e de machos) está promovendo as discordâncias genéticas e fenotípica encontradas. Esta zona híbrida ocorre precisamente em uma região altamente diversificada e historicamente dinâmica, com contrações e expansões conhecidas para uma ampla gama de espécies. Contatos secundários afetam a distribuição das variações genéticas e fenotípicas, contribuindo para a origem e manutenção da diversificação na Mata Atlântica.

- iii. A espécie *Cycloramphus boraceiensis* apresenta um sistema de acasalamento promíscuo, com ambos fêmeas e machos tendo parceiros sequenciais. Ocasionalmente há formações recorrentes de pares, mas os dados de fidelidade são inconclusivos. As fêmeas maiores têm vantagens reprodutivas. As vantagens reprodutivas dos machos maiores variam de acordo com as distintas condições ambientais e populacionais. Na formação dos casais não há correlação de tamanhos entre fêmeas e machos, porém os casais formados são geneticamente mais relacionados do que a média populacional. Com ambos fêmeas e machos competindo por recursos, os resultados sugerem que o parentesco genético influencia a estrutura social em *C. boraceiensis*. Este sistema promíscuo com cuidado parental dos machos é organizado de modo não aleatório, provavelmente envolvendo competição intrasexual marcante tanto para os machos como para as fêmeas.
  
- iv. Ao longo de estações reprodutivas prolongadas, a espécie *Thoropa taophora* apresenta sistema de acasalamento poligínico com macho único e alta fidelidade intragrupo, o que também é referido como harém. O macho monopolizador domina o sítio reprodutivo que é escasso, guardando a prole e reproduzindo exclusivamente com duas fêmeas. Este sistema provavelmente evoluiu em resposta à intensa competição por sítios reprodutivos e à intensa competição intrasexual por parceiros. Este sistema de acasalamento não era conhecido para anfíbios e é aqui estendido para todos os tetrápodes.