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LUANA RODRIGUES LIMA

DIVERSIDADE CRÍPTICA EM *PIPA CARVALHOI*? UMA ABORDAGEM INTEGRADA

MACEIÓ - AL
2019

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Dissertação apresentada ao Programa de Pós-Graduação em Diversidade Biológica e Conservação nos Trópicos, Instituto de Ciências Biológicas e da Saúde. Universidade Federal de Alagoas, como requisito para obtenção do título de Mestre em CIÊNCIAS BIOLÓGICAS, área de Biodiversidade.

Orientador: Professora Dra. Tamí Mott

Co-orientador: Daniel Pacheco Bruschi

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RESUMO

Os anuros são um dos grupos com maior diversidade críptica. Para descobrir essa diversidade, a taxonomia integrativa baseada em diferentes fontes de evidências tem sido empregada. *Pipa carvalhoi* é uma espécie interessante a ser estudada integrando diferentes conjuntos de dados. Ela tem uma distribuição disjunta e baixa capacidade de dispersão, sugerindo que a diferenciação destas linhagens pode ter ocorrido. Neste estudo utilizamos a taxonomia integrativa para acessar a diversidade de *Pipa carvalhoi*, perguntando se as linhagens independentes estão em processo de diferenciação, ou já se tornaram espécies diferenciadas. Para testar essa hipótese, dados morfológicos (girinos e adultos), moleculares (16S rRNA) e citogenéticos foram analisados. Três clados bem sustentados foram recuperados dentro de *Pipa carvalhoi*. A estrutura dos clados está de acordo com o sistema de bacias hidrográficas: o clado I ocorre na bacia do Atlântico Nordeste, o clado II nas bacias dos rios São Francisco e Atlântico Leste e o clado III na bacia do Atlântico Sudeste. A análise de bPTP recuperou três candidatas a nova espécies, congruentes com os clados recuperados na Inferência Bayesiana e Máxima Verossimilhança. As distâncias genéticas entre os três clados de *P. carvalhoi* foram maiores que o valor esperado para a divergência interespecífica recuperada usando LocalMinima. Os dados citogenéticos não diferiram entre os clados. A morfologia dos adultos das diferentes localidades foi semelhante, enquanto os caracteres morfométricos dos girinos revelaram agrupamentos morfológicos com correspondência parcial com a estrutura genética. Nossos resultados mostraram uma estruturação genética que indica que *P. carvalhoi* é na verdade três espécies crípticas, aparentemente relacionadas às bacias hidrográficas.

Palavras chave: Pipidae, taxonomia, hipótese do desacoplamento adaptativo, girino, Anura.

ABSTRACT

Anurans are one of the groups with the highest cryptic diversity. To uncover this diversity, the integrative taxonomy based on different sources of evidences has been employed. *Pipa carvalhoi* is an interesting species to be studied integrating diferent datasets. It has a disjoint distribution and low dispersal capacity suggesting that differentiation can be already occurred. In this study we use integrative taxonomy to assess *Pipa carvalhoi* diversity, asking whether independent lineages are in the process of differentiation, or have already become differentiated species. To test this hypothesis, morphological (tadpoles and adults), molecular (16S rRNA) and karyological data were analyzed. Three well-supported clades were recovered within *Pipa carvalhoi*. The structure of clades agree with drainage system: clade I occurs at the Northeastern Atlantic river basin, clade II at the San Francisco and East Atlantic river basins, and clade III at the Southeastern Atlantic river basin. The bPTP analysis recovered three candidate species, congruent with the clades recovered in the Bayesian Inference and Maximum Likelihood. Genetic distances among three clades of *P. carvalhoi* were higher that the expected value for interspecific divergence recovered using LocalMinima. Cytogenetic data did not differ among clades. The morphology of adults from different localities were similar, whereas the morphometric characters of tadpoles revealed morphological clusters with partial match with the genetic structure. Our results showed a strong genetic structure that indicates that *P. carvalhoi* is actually three cryptic species, apparently related to the hydrographic basins.

Key words: Pipidae, taxonomy, adaptive decoupling hypothesis, tadpole, Anura.

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1. APRESENTAÇÃO

As espécies são unidades fundamentais da Biologia, sendo sua delimitação de extrema importância para diversas disciplinas (DE QUEIROZ, 2007). O conceito de espécie pode variar entre diferentes linhas de pensamentos, sendo crescente o número de estudos que discutem sobre o seu conceito e métodos para delimitá-las (ANDERSSON, 1990; DE QUEIROZ, 2007; HAUSDORF, 2011; KANTA; PLANGKLANG; SUBSINGHA, 2014; SIMPSON, 1951; SITES; MARSHALL, 2004). Um conceito que vem sendo utilizado é o conceito geral de linhagem onde uma espécie é vista como uma linhagem, ou seja, uma sequência temporal/espacial de populações de ancestrais e descendentes (DE QUEIROZ, 1999; 2007).

Atualmente, mais de 1,2 milhões de espécies eucariotas são conhecidas (MORA et al., 2011), sendo estas descritas utilizando principalmente dados morfológicos (PANTE; CHOELINCK; UILLANDRE, 2014). Entretanto, muitos pesquisadores têm sugerido que há um número ainda muito maior de espécies não conhecidas podendo ser reveladas utilizando novas abordagens (FOUQUET et al., 2007; FUNK; CAMINER; RON, 2012; PRITI et al., 2016; YOUNGER et al., 2018).

A integração de diferentes fontes de informação é atualmente reconhecida como a abordagem mais eficiente para investigar questões taxonômicas, uma vez que busca representar a complexidade biológica baseada em linhas de evidências independentes e complementares. A Taxonomia Integrativa foi formalmente sugerida por Dayrat (2005), como uma ciência que visa delimitar e descrever as espécies integrando diferentes linhas de evidências. Para guiar a tomada de decisão, duas estratégias podem ser consideradas: 1) taxonomia integrativa por congruência, no qual deve haver congruência entre duas ou mais fontes de evidências (PADIAL et al., 2010); e 2) taxonomia integrativa por acumulação, no qual a divergência em uma ou mais fontes de evidências, que constituam um carácter taxonômico, pode definir uma nova espécie (PADIAL et al., 2010). A primeira estratégia é a mais utilizada pois é pouco provável que um padrão de congruência entre linhas de evidências independentes venham a surgir por acaso na natureza, diminuindo assim a possibilidade de designar espécies incorretamente (PADIAL et al., 2010).

Desvendar e descrever as espécies crípticas é de extrema importância, pois o conhecimento sobre o *status* de conservação de um táxon e informação sobre as regiões com altos níveis de riqueza e endemismo subsidiam as ações prioritárias para conservação (FUNK; CAMINER; RON, 2012). Para minimizar essa problemática, a taxonomia integrativa vem auxiliando na delimitação de espécies em diversos grupos, se mostrando especialmente importante em grupos que apresentam diversidade críptica (espécie distintas, porém, morfologicamente indistinguíveis), como é caso dos anfíbios anuros. Estudos recentes têm revelado que a diversidade desse grupo têm sido subestimada (FOUQUET et al., 2007; FUNK; CAMINER; RON, 2011; STUART; INGER; VORIS, 2006), e muitas espécies podem estar sendo extintas antes mesmo de serem conhecidas (BARNOSKY et al., 2011)

Na taxonomia de anuros, a morfologia de adultos vem sendo complementada com a morfologia interna e externa de girinos (BARRASSO et al., 2012; DUARTE et al., 2017; HAAS, 1995; LARSON, 2002, 2005), estudos cariotípicos caracterizando o número e morfologia de cromossomos e a Região Organizadora de Nucléolos NORs) (Suryadnaya, 2014; Andrade et al., 2016; Pansonato et al., 2016) assim como estudos moleculares (VACHER et al., 2017; WALKER; LYRA; HADDAD, 2018). Esta integração de abordagens vem auxiliando estudos taxonômicos.

Uma espécie de anuro que o emprego da taxonomia integrativa (dados moleculares, citogenéticos e morfológicos) pode auxiliar na revelação de espécies crípticas é *Pipa carvalhoi*. Essa espécie aquática é endêmica do Brasil e apresenta distribuição disjunta. Uma área de ocorrência se estende do Ceará até Sergipe e a outra, distante cerca de 600 Km, inclui o Sul da Bahia até o Espírito Santo (Frost, 2019). Ademais, é uma espécie aquática restrita à corpos d'água de quatro diferentes bacias hidrográficas, Atlântico Nordeste, Atlântico Leste, São Francisco e Atlântico Sudeste, podendo se dispersar apenas nos períodos de chuvas torrenciais, sugerindo que a espécie apresenta baixa capacidade de dispersão (TRUEB; CANNATELLA, 1986) podendo estar diferenciando nas diferentes áreas de ocorrência.

REFERÊNCIAS

ANDERSSON, L. The driving force: species concepts and ecology. **Taxon**, v. 39, n. 3, p. 375–382, 1990.

BARNOSKY, A. D. et al. Has the Earth's sixth mass extinction already arrived? **Nature**, v. 471, n. 7336, p. 51–57, 2011.

BARRASSO, D. A. et al. External morphology, chondrocranium, cranial muscles, and buccopharyngeal features of tadpoles of *Pleurodema thaul* (Anura: Leiuperidae): a comparison with *P. bufonium*. **Herpetologica**, v. 68, p. 48–59, 2012.

CARVALHO, M. A. et al. Dynamics of chromosomal evolution in the genus hypsiboas (Anura: Hylidae). **Genetics and Molecular Research**, v. 13, n. 3, p. 7826–7838, 2014.

DAYRAT, B. Towards integrative taxonomy. **Biological Jounal of the Linnean Society**, v. 85, p. 407–415, 2005.

DE QUEIROZ, K. The general lineage concept of species and the defining properties of the species category. 1999.

DE QUEIROZ, K. Species concepts and species delimitation. **Systematic biology**, v. 56, n. 6, p. 879–886, 2007.

DUARTE, V. R. G. et al. The tadpole of *Scinax skuki* (Anura: Hylidae) from the type locality, with a description of its larval skeleton. **Studies on Neotropical Fauna and Environment**, v. 52, n. 3, p. 204–215, 2 set. 2017.

FOUQUET, A. et al. Revealing cryptic diversity using molecular phylogenetics and phylogeography in frogs of the *Scinax ruber* and *Rhinella margaritifera* species groups. **Molecular Phylogenetics and Evolution**, v. 43, n. 2, p. 567–582, 2007.

FUNK, W. C.; CAMINER, M.; RON, S. R. High levels of cryptic species diversity uncovered in Amazonian frogs. **Proc. R. Soc. B.**, v. 279, n. 1734, p. 1806–1814, 2011.

FUNK, W. C.; CAMINER, M.; RON, S. R. High levels of cryptic species diversity uncovered in Amazonian frogs. **Proceedings. Biological sciences/ The Royal Society**, v. 279, n. 1734, p. 1806–14, 2012.

HAAS, A. Cranial features of dendrobatid larvae (Amphibia: Anura: Dendrobatidae). **Journal of morphology**, v. 224, p. 241–264, 1995.

HAUSDORF, B. Progress toward a general species concept. **Evolution**, v. 64, n. 4, p. 923–931, 2011.

KANTA, S.; PLANGKLANG, B.; SUBSINGHA, W. World's largest Science, Technology & Medicine Open Access book publisher c. **Energy Procedia**, v. 56, p. 604–609, 2014.

LARSON, P. M. Chondrocranial development in larval *Rana sylvatica* (Anura: Ranidae): Morphometric analysis of cranial allometry and ontogenetic shape change. **Journal of Morphology**, v. 252, n. 2, p. 131–144, 2002.

LARSON, P. M. Ontogeny, phylogeny, and morphology in anuran larvae: Morphometric analysis of cranial development and evolution in *Rana* tadpoles (Anura: Ranidae). **Journal of Morphology**, v. 264, n. 1, p. 34–52, 2005.

MEZZASALMA, M. et al. Karyological analyses of *Pseudhymenochirus merlini* and *Hymenochirus boettgeri* provide new insights into the chromosome evolution in the anuran family Pipidae. **Zoologischer Anzeiger - A Journal of Comparative Zoology**, v. 258, p. 47–53, 2015.

MORA, C. et al. How many species are there on earth and in the ocean? **PLos Biology**, v. 9, n. 8, p. e1001127, 2011.

PADIAL, J. M. et al. The integrative future of taxonomy. **Frontiers in Zoology**, v. 7, n. 1, p. 16, 2010.

PANTE, E. P.; CHOELINCK, C. S.; UILLANDRE, N. P. From integrative taxonomy to species description : one step beyond. **Systematic Biology**, v. 64, n. 1, p. 152–160, 2014.

PRITI, H. et al. Integrative Taxonomic approach for describing a new cryptic species of bush frog (Raorchestes: Anura: Rhacophoridae) from the Western Ghats, India. **Plos One**, v. 11, n. 3, p. e0149382, 2016.

SIMPSON, G. G. **The species concept.** v. 5, n. 4, p. 285-298, 1951.

SITES, J. W.; MARSHALL, J. C. Operational criteria for delimiting species. **Annual Review of Ecology, Evolution, and Systematics**, v. 35, n. 1, p. 199–227, 2004.

STUART, B. L.; INGER, R. F.; VORIS, H. K. High level of cryptic species diversity revealed by sympatric lineages of Southeast Asian forest frogs. **Biology**, v. 1887, p. 470–474, 2006.

SURYADNAYA, N. N. Comparative analysis of karyotypes of two cryptic species of Pelobatid frogs (Amphibia, Anura) of Ukraine. **Vestnik zoologii**, v. 48, n. 6, p. 511–520, 2014.

TRUEB, L.; CANNATELLA, D. C. Systematics, morphology, and phylogeny of genus *Pipa* (Anura: Pipidae). **Herpetologica**, v. 42, n. 4, p. 412–449, 1986.

VACHER, J. P. et al. Cryptic diversity in Amazonian frogs: Integrative taxonomy of the genus *Anomaloglossus* (Amphibia: Anura: Aromobatidae) reveals a unique case of diversification within the Guiana Shield. **Molecular Phylogenetics and Evolution**, v. 112, p. 158–173, 2017.

WALKER, M.; LYRA, M. L.; HADDAD, C. F. B. Molecular phylogenetics and evolution phylogenetic relationships and cryptic species diversity in the brazilian egg- brooding

tree frog , genus *Fritziana* Mello-Leitão 1937 (Anura : Hemiphractidae). **Molecular Phylogenetics and Evolution**, v. 123, p. 59–72, 2018.

YOUNGER, J. L. et al. Molecular phylogenetics and evolution hidden diversity of forest birds in Madagascar revealed using integrative taxonomy. **Molecular Phylogenetics and Evolution**, v. 124, p. 16–26, 2018.

2 REVISÃO DE LITERATURA

2.1 Espécies crípticas

Conceituar espécies é um dos maiores desafios para os sistematas e taxonomistas, e isso gerou um pluralismo de conceitos (CARSTENS et al., 2013; DE QUEIROZ, 2007; HAUSDORF, 2011; WILEY, 1978). Mayden (1997) listou 24 conceitos, muitos desses incompatíveis entre si. De Queiroz (2007) sugeriu um conceito geral de espécie, no qual, espécies seriam linhagens de metapopulações que evoluem separadamente, não necessariamente diferindo fenotipicamente, nem sendo reprodutivamente isoladas ou ecologicamente divergentes, pois esses atributos podem ou não ser adquiridos durante a especiação. Porém, esse conceito ainda causa confusão na questão da delimitação de espécie, por não haver um atributo particular que possa universalmente delimitá-las (DE QUEIROZ, 2007; FIŠER; ROBINSON; MALARD, 2018). Há diversas evidências de que especiação pode ser conduzida por mecanismos completamente diferentes, podendo levar a espécies crípticas (FIŠER; ROBINSON; MALARD, 2018). Essas, por sua vez, são duas ou mais espécies morfologicamente similares que erroneamente foram classificadas como uma única espécie (BICKFORD et al., 2007).

Três possíveis mecanismos podem causar semelhanças morfológicas entre espécies distintas: 1) divergência recente, no qual não teria havido tempo o suficiente para se diferenciarem morfologicamente; 2) conservantismo de nicho, no qual a divergência morfológica de espécies descendentes é limitada pela seleção; e 3) convergência morfológica, em que a semelhança morfológica pode evoluir de forma independente entre espécies não relacionadas como consequência de pressões seletivas semelhantes (Trontelj et al., 2009; Bravo and Brumfield, 2014).

Fišer e colaboradores (2018) testaram as três hipóteses supracitadas usando 439 espécies crípticas de Amphipoda como modelo. Para testar a hipótese da divergência recente utilizaram a idade estimada dos nós das árvores filogenéticas. A hipótese do conservatismo de nicho não foi diretamente testada, porém, os autores analisaram na literatura complexos de espécies irmãs ecologicamente especializadas em micronichos. Para testar a hipótese da convergência evolutiva foi quantificada a proporção de espécies crípticas que não apresentavam relações próximas. Eles encontraram que apenas 11% das espécies crípticas de Amphipoda

evoluíram recentemente, o que não sustentaria a hipótese da divergência recente, porém destacaram a possibilidade de viés de metodologia (diferentes métodos de datação são utilizados por diferentes autores e para diferentes conjuntos de dados), para estimar a idade das espécies. Apesar do conservatismo de nicho filogenético não ter sido explicitamente testado, os autores encontraram diversas espécies crípticas irmãs ecologicamente especializadas que apoiaria essa hipótese. Ademais, mais de 25% das espécies analisadas apresentaram indícios de convergência evolutiva. Assim como os Amphipoda, diversos outros grupos apresentam grande número de espécies crípticas podendo estas serem geradas por diferentes mecanismos (Vacher et al., 2017; Milián-García et al., 2018; Stiffler et al., 2018).

Um grupo que tem se destacado no número de espécies crípticas são os anfíbios, no qual estudos moleculares frequentemente revelam diversidades muito subestimadas (CAMARGO; DE SÁ; HEYER, 2006; FOUQUET et al., 2007; FUNK; CAMINER; RON, 2011; PRITI et al., 2016; VACHER et al., 2017). Funk et al. (2011) estimaram o número de espécies crípticas em anuros amazônicos dos gêneros *Engystomops* e *Boana* com base em dados moleculares (genes mitocondriais e nucleares), morfológicos e bioacústicos. Para o gênero *Engystomops* o que eram consideradas duas espécies revelaram ser na realidade, cinco a sete espécies. E duas espécies reconhecidas de *Boana*, representam de fato seis a nove espécies, sugerindo haver um número extremamente subestimado de espécies de anuros na Amazônia. Peloso et al. (2018) apresentaram uma hipótese filogenética do grupo *Boana semilineata* utilizando dois marcadores mitocondriais (16S e COI). Neste estudo os autores encontram seis linhagens candidatas a novas espécies, no qual uma foi descrita no artigo (PELOSO et al., 2018)

A presença de diversidade críptica nos anfíbios é particularmente preocupante pois este grupo taxonômico apresenta acentuado declínio mundialmente (40% das espécies já apresentam algum declínio populacional [IUCN, 2018]) e muitas das espécies crípticas podem ser extintas mesmo antes de serem conhecidas pela Ciência (BARNOSKY et al., 2011).

Desvendar e descrever as espécies crípticas são de extrema importância, pois o conhecimento sobre o *status* de conservação de um táxon e informação sobre as regiões com altos níveis de riqueza e endemismo subsidiam as ações prioritárias

para conservação (FUNK; CAMINER; RON, 2012). Para minimizar essa problemática, muitos taxonomistas têm adotado o uso da taxonomia integrativa *sensu* Dayrat (2005), que visa a integração de diferentes fontes de evidências para caracterizar e delimitar espécies, proporcionando uma maior confiabilidade nas decisões taxonômicas e auxiliando a desvendar espécies crípticas (FERRÃO et al., 2016; LUQUE-MONTES et al., 2018; RODRÍGUEZ et al., 2017; VACHER et al., 2017a, 2017b).

2.2 Taxonomia integrativa

A taxonomia foi tradicionalmente baseada em dados morfológicos, porém, o uso exclusivo dessa abordagem pode apresentar limitações, pois analisa apenas umas das facetas da evolução, podendo ignorar espécies crípticas e/ou recém divergentes (DAYRAT, 2005). Apesar de aproximadamente 1,2 milhões de espécies atuais de eucariotas já serem descritas (MORA et al., 2011), há um número ainda muito maior de espécies não conhecidas (BICKFORD et al., 2007; FOUQUET et al., 2007; FUNK; CAMINER; RON, 2012; YOUNGER et al., 2018). Com isso, novas fontes de evidências, como por exemplo, dados moleculares, ecológicos, bioacústicos começaram a ser utilizadas em revisões taxonômicas. Esta abordagem integrada foi nomeada de taxonomia integrativa, sendo definida como a ciência que visa delimitar as unidades taxonômicas através de uma abordagem multidisciplinar (DAYRAT, 2005).

Diferenças nos resultados obtidos através do uso de diferentes fontes de evidências geravam discordâncias quanto o uso da abordagem integrativa para delimitação de espécies (PADIAL et al., 2010; VALDECASAS; WILLIAMS; WHEELER, 2008). Padial (2010), visando contornar esta problemática, sugeriu formalmente duas estratégias para guiar as decisões taxonômicas através da abordagem integrativa: integração por congruência e integração por acumulação.

A taxonomia integrativa por congruência segue o pressuposto de que há a necessidade da convergência de duas ou mais fontes de evidências independentes para diferenciar espécies. Essa estratégia é bem aceita, pois é improvável que unidades evolutivas distintas apresentem um padrão de coerência arbitrariamente entre si (PADIAL et al., 2010). Entretanto, uma das principais limitações desta

estratégia reside na possibilidade de subestimar espécies, visto que o processo de especiação nem sempre é acompanhado por mudanças nos estados dos caracteres em todos os níveis (PADIAL et al., 2010). A taxonomia integrativa por acumulação baseia-se no pressuposto de que as divergências presentes em qualquer linha de evidência taxonômica podem sugerir a existência de uma espécie (PADIAL et al., 2010). Essa estratégia é capaz de detectar mais facilmente espécies que divergiram recentemente, porém, pode superestimar a diversidade de espécies (PADIAL et al., 2010).

2.3 Taxonomia integrativa em anuros

Na taxonomia de anfíbios anuros, o maior desafio é a grande quantidade de diversidade críptica (FOUQUET et al., 2007; FUNK; CAMINER; RON, 2012; STUART; INGER; VORIS, 2006; VACHER et al., 2017a), com isso, a taxonomia integrativa tem sido amplamente utilizada para auxiliar na caracterização essa diversidade (FERRÃO et al., 2016; LUQUE-MONTES et al., 2018; VACHER et al., 2017). As abordagens bioacústicas, morfológica (incluindo ambas fases de vida, larval e adulta), molecular, citogenética e biogeográfica vem sendo utilizadas na taxonomia de anuros. Neste estudo, vamos nos deter as quatro últimas abordagens.

2.3.1 Abordagem morfológica

Historicamente, os anuros foram descritos com base principalmente na morfologia externa dos adultos (CAIN, 1954). Entretanto, o uso exclusivo dessa abordagem subestimou a diversidade atual, sendo este um dos grupos que apresenta maior descrição de novas espécies (HANKEN, 1999). Ademais, o ciclo de vida complexo dos anuros, no qual a maioria das espécies passa por uma fase larval aquática (girino) completamente diferente da sua forma adulta terrestre, limitou o conhecimento de uma espécie a sua fase adulta, dificultando a identificação dos girinos (CARNEIRO; MAGALHÃES; JUNCA, 2004; WOGEL et al., 2000). Com isso, surgiu o interesse pelo conhecimento da morfologia dos girinos (MCDIARMID; ALTIG, 1999; WOGEL et al., 2000).

Lataste (1879) foi o primeiro pesquisador a sugerir que os caracteres larvais teriam importância para a taxonomia dos anuros. Mais tarde, Orton (1953) dividiu os girinos em quatro grupos com base em caracteres larvais, como estrutura da câmera opercular, posição do espiráculo e estrutura do disco oral. O tipo larval I foi caracterizado pela presença de duas aberturas no espiráculo e bordas labiais estreitas no disco oral. Este tipo larval, considerado o mais simples, é encontrado nos girinos das famílias Pipidae e Rinophrynidae. Os tipos larvais II e III incluem girinos com apenas uma abertura do espiráculo na região ventral do corpo, no tipo II há apenas uma abertura oral com presença de abas dérmicas, enquanto que no tipo III as larvas já apresentam estruturas orais queratinizadas, ambos os grupos também alocam famílias consideradas menos derivadas. Já o tipo IV, incluía os grupos mais derivados e com características mais complexas, apresentando disco oral queratinizado e abertura do espiráculo única e do lado esquerdo. Porém, alguns autores não aceitaram essa proposta (GRIFFITHS, 1963; GRIFFITHIS; CARVALHO, 1965; SOKOL, 1975), argumentando que este estudo analisou poucos caracteres larvais, e que carcateres anatômicos internos não foram analisados (STARRET, 1973). Atualmente, os caracteres larvais tem revelado ser informativos em alguns grupos de anuros até mesmo a nível específico (HAAS, 2003; HERO, 1990; NASCIMENTO; SKUK, 2006). A morfologia dos girinos tem sido essencial até mesmo para identificar espécies crípticas cujo adulto apresenta morfologia indistinguível (BARRIO-AMORÓS et al., 2006; DUELLMAN; DE SÁ, 1988; SUAREZ-MAYORGA; LYNCH, 1999).

Além da morfologia externa dos girinos, a morfologia interna também tem se mostrado informativa pra estudos taxonômicos (HAAS, 1995, 1997, 2003, LARSON, 2002, 2005; LARSON; DE SÁ, 1998). O condrocrânio é um estojo cartilaginoso que protege o encéfalo e sustenta os órgãos dos sentidos e o aparato mandibular. O aparato hiobranquial é uma estrutura também cartilaginosa, articulada ventralmente ao condrocrânio e é responsável pela sustentação das brânquias e do aparato filtrador (MCDIARMID, R. W.; ALTIG, 1999).

Estudo dessas estruturas é relativamente recente, e um dos primeiros grupos de anuros que teve o condrocrânio e aparato hiobranquial descritos foi o gênero *Pipa* da família Pipidae (SOKOL, 1977). Esse se caracteriza por ser largo e plano e há

apenas uma placa simples, a placa suprarrostral, no local onde se encontra a *cornua trabeculae* e a cartilagem suprarostral nos girinos de todas as outras famílias não Pipidae (DE SÁ; SWART, 1999; SOKOL, 1977). Apesar de ter sido um grupo estudado pioneiramente, o condrocrânio e aparato hiobranquial de apenas cinco de 41 espécies de Pipidae apresentam descrição: *Hymenochirus boettgeri* (DE SÁ; SWART, 1999), *Xenopus laevis* (SCHMIDT; SCHUFF; OLSSON, 2011; TRUEB; HANKEN, 1992), *X. tropicalis* (SOKOL, 1977), *Pipa pipa* (TRUEB; PÚGENER; MAGLIA, 2000) e *P. carvalhoi* (SOKOL, 1977).

2.3.2 Abordagem molecular

Estudo taxonômicos e sistemáticos de anuros utilizando a abordagem molecular são recentes, tendo início na década de 90 (GRAYBEL, 1993; HASS; DUNSKI; MAXSON, 1995; HAY et al., 1994; RICHARDS; MOORE, 1996), e se tornando amplamente utilizado a partir de 2000 (PYRON; WIENS, 2011; FAIVOVICH et al., 2005; SCHÄUBLE; MORITZ; SLADE, 2000; VENCES et al., 2000).

Com o advento dos estudos moleculares, o número de espécies novas de anfíbios tem aumentando significativamente (GLAW; KÖHLER, 1998; KÖHLER et al., 2005; PELOSO et al., 2018; STUART; INGER; VORIS, 2006). Stuart et al. (2006) analisaram duas espécies de anuros do sudeste da Ásia utilizando genes mitocondriais, e recuperaram um complexo com pelo menos 14 espécies ainda não descritas. Isso também é recorrente para as espécies de anuros da região Neotropical. Fouquet et al. (2007) analisaram 60 espécies da Guiana utilizando o gene mitocondrial 16S rRNA e encontraram 129 espécies candidatas a novas espécies, mais do que o dobro das espécies atualmente conhecidas. Estudos como esses reforçam a importância da inclusão de dados moleculares nos estudos taxonômicos, visto o grande número de espécies crípticas em anuros.

2.3.3 Abordagem citogenética

Estudos citogenéticos têm se mostrado eficientes como ferramentas adicionais para delimitação de espécies em diversos grupos, tanto em plantas, como em animais (Abucarma and Martins-Santos, 2001; Schutzman, Vovides, and Dehgan, 2016). São comuns variações estruturais, como os rearranjos cromossômicos que resultam em

inversões (DUARTE et al., 2010; LOURENÇO; RECCO-PIMENTEL; CARDOSO, 1998) e translocações (CARVALHO et al., 2014). Tais variações podem indicar isolamento entre as entidades taxonômicas e, portanto, levadas em consideração na tomada de decisões taxonômicas. Para anfíbios anuros, isso não é diferente, no qual diferenciações morfológicas e numéricas dos cromossomos podem ser encontradas mesmo em espécies muito próximas e que apresentem morfologia externa semelhante (BORKIN et al., 2001; CUEVAS, 2008; MEDEIROS; ROSSA-FERES; RECCO-PIMENTEL, 2003; SURYADNAYA, 2014). As espécies *Pelobates vespertinus* e *P. fuscus* apresentam morfologia externa semelhantes, porém podem ser diferenciadas pela morfologia de seus cromossomos. Ambas espécies apresentam 2n=26 cromossomos, mas se diferenciam pela posição do centrômero nos cromossomos pares 10 e 11. Em *P. fuscus* o par 10 é metacêntrico e o par 11 é submetacêntrico, enquanto que em *P. vespertinus* o par 10 é submetacêntrico e o 11 é metacêntrico (SURYADNAYA, 2014). Outro exemplo do uso de abordagem citogenética na resolução de questões taxonômicas de anuros pode ser observado no caso da remoção da sinonímia de *Pseudopaludicula ameghini* de *P. mystacalis*. Essas duas espécies, consideradas a mesma entidade taxonômica por suas semelhanças morfológicas representam duas espécies distintas, portadoras inclusive de números cromossômicos distintos (2n=20 e 2n=16, respectivamente) (FÁVERO et al., 2011). Esse mesmo tipo de dado foi utilizado na reavaliação em outro par de espécies crípticas*, P. temetzi* e *P. falcepis* (2n=20 e 2n=22, respectivamente) (FÁVERO et al., 2011).

Na família Pipidae, o número cromossômico no gênero *Xenopus* é uma importante evidência evolutiva, utilizada para sugestões de adequações taxonômicas em níveis intragenéricos (na definição da separação dos gêneros de *Xenopus* e *Silurana*) e intraespecíficos (na identificação de novas espécies, em sua maioria poliplóides naturais). Dentro do gênero *Silurana*, é possível reconhecer dois clados onde a principal sinapomorfia atribuída é o número cromossômico: um clado reúne as espécies derivadas de um cariótipo ancestral 2n=20, retendo a condição plesiomórfica em Pipidae (considerado gênero *Silurana* por EVANS et al. (2004) e todas as espécies poliplóides com cariótipos derivados desse. O segundo clado, considerando o gênero *Xenopus*, reúne espécies originadas a partir de um cariótipo ancestral de 2n=18. Este cariótipo representa a base para evento de

alopoliploidização gerando espécies portadoras de 2n=36 cromossomos e/ou portadoras de número cromossômicos múltiplos desse cariótipo (36, 72 ou 108 cromossomos, Evans et al., 2004, 2015; Chain and Evans, 2006; Evans and Evans, 2008; Uno et al., 2013).

Mesmo em casos onde os cariótipos são extremamente uniformes em número e morfologia similares dos cromossomos, outras características cromossômicas podem ser utilizadas para a distinção entre os cariótipos. Por exemplo, o número e a ghut67yji8localização das regiões organizadoras de nucléolo (NOR) entre populações de *Pristimantis fenestratus* (2n=34) de três localidades brasileiras (Borba e Manaus, estado do Amazonas e Rio Claro, estado do Acre) foram utilizadas como critérios para sugerir a necessidade de revisão da taxonômica nessa entidade taxonômica. A NOR foi localizada nos pares 5 e 7 nos exemplares de Rio Claro, no par 10 nos indivíduos de Manaus, e no par 1 nos de Borba (SIQUEIRA et al., 2009).

2.3.4 Abordagem biogeográfica

A presença e distribuição das espécies são determinadas por fatores históricos, bióticos e abióticos, que não ocorrem de maneira aleatória (BROWN; LOMOLINO, 2006). Com isso, uma importante abordagem utilizada em estudos taxonômicos é a biogeográfica.

Os anuros são, em sua maioria, terrestres, porém dependentes de água para sua respiração e reprodução sendo algumas espécies completamente aquáticas (POUGH; JANIS; HEISER, 2008). A distribuição das espécies de anuros é limitada pelas suas restrições fisiológicas (fácil dessecamento da pele e ovos, larvas aquáticas, etc.) e por sua dispersão limitada (VASCONCELOS et al., 2014).

Estudo com anuros realizado na Mata Atlântica mostrou que os padrões de distribuição nessa região são congruentes com as distintas ecorregiões identificadas. Os autores mostraram que o clima, a topografia e a vegetação são importantes características para explicar a distribuição das espécies (VASCONCELOS et al., 2014). Para peixes de água doce foi observado que bacias hidrográficas frequentemente limitam a dispersão de espécies (ABELL et al., 2008).

Este mesmo padrão pode ser encontrado em espécies de anuros aquáticas e semiaquáticas, que são limitadas a corpos d'água.

Com o objetivo de explicar o padrão de distribuição das espécies e a especiação, diversas hipóteses biogeográficas foram levantadas e vêm sendo testadas. Uma das principais hipóteses é a dos refúgios, que condiciona os padrões de distribuição das espécies as mudanças climáticas do Pleistoceno. Estes eventos cíclicos fizeram com que as florestas tropicais se contraíssem em refúgios separados por florestas secas e savanas, sendo esse isolamento responsável por proporcionar especiação (HAFFER, 1969; MORITZ et al., 2000). Outra hipótese é baseada na interrupção de conexão entre populações através de barreiras (por exemplo um rio), impedindo o fluxo gênico. As populações de cada lado da barreia gradualmente divergem para formar espécies separadas (MORITZ et al., 2000). Uma terceira hipótese é o modelo de gradiente, no qual fortes gradientes ambientais (por exemplo, um habitat) propiciam divergência adaptativa e especiação. Espera-se que isso resulte em espécies irmãs adaptadas a ambientes distintos e adjacentes (ENDLER, 1982; ERWIN, 1991; MORITZ et al., 2000). Estas hipóteses vêm sendo testadas também para anuros, e os resultados vem mostrando que diferentes processos explicam a especiação em diferentes grupos de anuros (CARNAVAL; BATES, 2007; VASCONCELOS; RODRÍGUEZ; HAWKINS, 2011; GASCON; LOUGHEED; BOGART, 1998; NIELSON; LOHMAN; SULLIVAN, 2001).

2.4 A família Pipidae Gray, 1825 e o gênero *Pipa* Laurenti, 1768

Pipidae é uma família de anuros aquáticos e semiaquáticos que apresenta características peculiares, tais como retenção de linha lateral até a fase adulta, alimentação por sucção, ausência de tímpano e membranas interdigitais (TRUEB; CANNATELLA, 1986). Atualmente essa família é composta por 41 espécies, agrupadas em quatro gêneros: três restritos a África (*Hymenochirus*, *Pseudhymenochirus* e *Xenopus*) e um endêmico as Américas do Sul e Central (*Pipa*).

Duas características são diagnósticas de *Pipa*: as pontas dos dedos dos membros anteriores apresentam formação de lobos e o seu modo reprodutivo, no qual, após a fecundação, o macho leva os ovos até as bolsas formadas no dorso da fêmea, nas quais o desenvolvimento ocorre até a fase de girino ou de um metamorfo completo

(CANEDO; GARCIA, 2006; DUNN, 1948; TRUEB; CANNATELLA, 1986). Dentre as sete espécies de *Pipa* (*P. aspera*, *P. myersi*, *P. parva*, *P. pipa*, *P. arrabali*, *P. snethlageae* e *P. carvalhoi*), quatro destas (*P. aspera*, *P. arrabali*, *P. pipa* e *P. snethlageae*) liberam ovos grandes que permanecem nas bolsas do dorso da fêmea até o completo desenvolvimento (GARDA; BIAVATI; COSTA, 2006; TRUEB; CANNATELLA, 1986). Em contraste, *P. myersi*, *P. parva* e *P. carvalhoi* liberam ovos pequenos (GARDA; BIAVATI; COSTA, 2006) que eclodem das bolsas do dorso da fêmea em girinos e completam o desenvolvimento em corpos d'água (FERNANDES et al., 2011; TRUEB; CANNATELLA, 1986). As espécies nas quais todo o desenvolvimento embrionário ocorre no dorso da fêmea formam um grupo monofilético, assim como as que apresentam desenvolvimento direto, segundo as filogenias com base em caracteres morfológicos e comportamentais já realizadas (CANNATELLA; TRUEB, 1988; TRUEB; CANNATELLA, 1986). Somente três espécies e *Pipa* têm o cariótipo descrito. *Pipa pipa* com 2n = 22 (MORESCALCHI; GARGIULO; OLMO, 1970), *P. parva*, 2n = 30 (MORESCALCHI, 1968) e *Pipa carvalhoi* apresenta 2n = 20 (MEZZASALMA et al., 2015). Das três espécies de *Pipa* que passam pela fase larval, apenas *P. parva* e *P. carvalhoi* apresentam a morfologia externa das larvas descritas (SOKOL, 1977).

Pipa carvalhoi é endêmica do Brasil, sendo encontrada em corpos d'água situados em áreas de Mata Atlântica e de Caatinga, além de sua distribuição abranger quatro bacias hidrográficas, Atlântico Nordeste, Atlântico Leste, São Franscisco e Atlântico Nordeste. Apresenta distribuição disjunta, sendo uma área estendendo do Ceará até Sergipe e a outra do Sul da Bahia até o Espírito Santo (FROST, 2019). O holótipo de *P. carvalhoi* não era conhecido, porém, o exame dos síntipos depositados na coleção do Museu Nacional do Rio de Janeiro (MNRJ) ajudou a desvendar quem é o exemplar tipo, visto que uma etiqueta manuscrita por Miranda foi encontrada indicando qual o espécime era o tipo. Este espécime, o lectótipo, foi coletado no município de Poção, no estado de Pernambuco, sendo portanto, a localidade-tipo da espécie (CARAMASCHI, 1989). Os parátipos são de quatro localidades em Pernambuco (Garanhuns, Pesqueira, Rio Branco e Capueira).

Pipa carvalhoi apresenta corpo robusto e longo, as pernas são consideravelmente longas. A cabeça, em vista dorsal, é larga e triangular e o focinho é pontudo, porém,

com terminação arredondada. Toda a superfície do corpo é coberta por tubérculos, sendo mais concentrado na região dorsal. Os tubérculos são menores e mais amplamente distribuídos na região dos ombros e quase não visíveis na cabeça. O sistema de linha lateral da espécie é bem desenvolvido, no qual uma série de estruturas é distribuída desde a região subrostral até a margem anterior do olho (DUELLMAN; TRUEB, 1994).

Pipa carvalhoi pode ser diferenciada das outras espécies do gênero pela combinação dos seguintes caracteres: 1) crânio mais longo do que largo 2) dentes premaxilares e maxilares numerosos bem desenvolvidos e em forma de presa; 3) ponta dos dedos apresentando quatro lobos distais de igual tamanho; 4) tubérculo metatarsal interno presente e pouco desenvolvido; 5) pontas queratinizadas sobre os dedos do pé I-III; 6) lábio superior formando bolso no ângulo da mandíbula e; 7) desenvolvimento com presença de estágio larval (TRUEB; CANNATELLA, 1986). Além das características morfológicas, *P. carvalhoi* também pode ser diferenciada pela sua distribuição, sendo a única que ocorre na Mata Atlântica e Caatinga (FROST, 2019).

REFERÊNCIAS

ABELL, R. et al. Freshwater Ecoregions of the World: A new map of biogeographic units for freshwater biodiversity conservation. **BioScience**, v. 58, n. 5, p. 403–414, 2008.

ABUCARMA, M.; MARTINS-SANTOS, I. C. Karyotype and B chromosome of *Rhamdia* species (Pisces, Pimelodidae) endemic in the river Iguacu basin. **Cytologia**, v. 66, p. 299–306, 2001.

ALEXANDER PYRON, R.; WIENS, J. J. A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. **Molecular Phylogenetics and Evolution**, v. 61, n. 2, p. 543–583, 2011.

BARNOSKY, A. D. et al. Has the Earth's sixth mass extinction already arrived? **Nature**, v. 471, n. 7336, p. 51–57, 2011.

BARRIO-AMORÓS, C. L. et al. *Hyla vigilans* Solano, 1971, a second species for the genus *Scarthyla*, redescription and distribution in Venezuela and Colombia. **Zootaxa**, v. 1349, p. 1–18, 2006.

BICKFORD, D. et al. Cryptic species as a window on diversity and conservation. **Trends in Ecology and Evolution**, v. 22, n. 3, p. 148–155, 2007.

BORKIN, L. J. et al. Cryptic speciation in *Pelobates fuscus* (Anura, Pelobatidae): evidence from DNA ow cytometry. **Amphibia-Reptilia**, v. 22, n. 4, p. 387–396, 2001.

BRAVO, G. A.; JR, J. V. R.; BRUMFIELD, R. T. Adaptive processes drive ecomorphological convergent evolution in antwrens (Thamnophilidae). **Evolution**, v. 68, n. 10, p. 2757–2774, 2014.

BROWN, James H.; LOMOLINO, Mark V. Biogeografia. In: Biogeografia. 2006.

CAIN, Arthur J.; SHEPPARD, Philip M. Natural selection in Cepaea. *Genetics*, v. 39, n. 1, p. 89, 1954.

CAMARGO, A.; DE SÁ, R. O.; HEYER, W. R. Phylogenetic analyses of mtDNA sequences reveal three cryptic lineages in the widespread neotropical frog *Leptodactylus fuscus* (Schneider, 1799) (Anura, Leptodactylidae). **Biological Jounal of the Linnean Society**, v. 87, n. 1978, p. 325–341, 2006.

CANEDO, C.; GARCIA, J. P. Diet of *Pipa carvalhoi* (Amphibia , Pipidae) is not influenced by female parental care. **Herpetological Review**, v. 37, n. 1, p. 95–96, 2006.

CANNATELLA, D. C.; TRUEB, L. Evolution of pipoid frogs: intergeneric relationships of the aquatic frog family Pipidae (Anura). **Zoological Journal of the Linnean Society**, v. 94, p. 1–38, 1988.

CARAMASCHI, U. Notes on the type specimens of *Pipa carvalhoi* (Miranda-Ribeiro, 1937) (Anura, Pipidae). **Journal of herpetology**, v. 23, n. 2, p. 192–193, 1989.

CARNAVAL, A. C.; BATES, J. M. Amphibian DNA shows marked genetic structure and tracks Pleistocene climate change in northeastern Brazil. **Evolution**, v. 61, n. 12, p. 2942–2957, 2007.

CARNEIRO, M. C. L.; MAGALHÃES, P. S.; JUNCA, F. A. Descrição do girino e vocalização de *Scinax pachychrus* (Miranda-Ribeiro, 1937) (Amphibia, Anura, Hylidae). **Arquivos do Museu Nacional**, v. 62, n. 3, p. 241–246, 2004.

CARSTENS, B. C. et al. How to fail at species delimitation. **Molecular Ecology**, v. 22, n. 17, p. 4369–4383, 2013.

CARVALHO, M. A. et al. Dynamics of chromosomal evolution in the genus *Hypsiboas* (Anura: Hylidae). **Genetics and Molecular Research**, v. 13, n. 3, p. 7826–7838, 2014.

CHAIN, F. J. J.; EVANS, B. J. Multiple mechanisms promote the retained expression of gene duplicates in the tetraploid frog *Xenopus laevis*. **PLoS Genet**, v. 2, n. 4, p. e56, 2006.

CUEVAS, C. C. A new species of the genus *Alsode*s (Anura: Neobatrachia) from the Nothofagus forest, Coastal Range, Southern Chile, identified by its karyotype. **Zootaxa**, v. 1771, p. 43–53, 2008.

DA SILVEIRA VASCONCELOS, T.; RODRÍGUEZ, M. Á.; HAWKINS, B. A. Biogeographic distribution patterns of south american amphibians: A regionalization based on cluster analysis. **Natureza e Conservação**, v. 9, n. 1, p. 67–72, 2011.

DAYRAT, B. Towards integrative taxonomy. **Biological Jounal of the Linnean Society**, v. 85, p. 407–415, 2005.

DE QUEIROZ, K. Species concepts and species delimitation. **Systematic biology**, v. 56, n. 6, p. 879–886, 2007.

DE SÁ, R. O.; SWART, C. C. Development of the suprarostral plate of pipoid frogs. **Journal of Morfology**, n. 240, p. 143–153, 1999.

DUARTE, T. C. et al. Chromosome analysis in *Pseudopaludicola* (Anura, Leiuperidae), with description of sex chromosomes XX/XY in P. saltica. **Hereditas**, v. 147, n. 2, p. 43–52, 2010.

DUELLMAN, W. E.; DE SÁ, R. O. A new genus and species of South American hylid frog with a highly modified tadpole. **Tropical Zoology**, v. 1, n. 1, p. 117–136, 1988.

DUELLMAN, W. E.; TRUEB, T. **Biology of Amphibia**. 2ª edition ed. The Johns Hopkins University Press, 1994.

DUNN, E. R. American Frogs of the family Pipidae. **American Museum Novitates**,

n. 1384, 1948.

ENDLER, J. A. Problems in distinguishing historical from ecological factors in biogeography. **Integrative and Comparative Biology**, v. 22, n. 2, p. 441–452, 1982.

ERWIN, T. L. An Evolutionary Basis for Conservation Strategies. **Science**, v. 253, n. 5021, p. 750–752, 1991.

EVANS, B.; EVANS, B. J. Genome evolution and speciation genetics of clawed frogs (*Xenopus* and *Silurana*). **Frontiers in Bioscience**, v. 13, p. 4687–4706, 2008.

EVANS, B. J. et al. A mitochondrial DNA phylogeny of African clawed frogs : phylogeography and implications for polyploid evolution. **Molecular phylogenetics and evolution**, v. 33, n. 1, p. 197–213, 2004.

EVANS, B. J. et al. Genetics , morphology , advertisement calls , and historical records distinguish six new polyploid species of african clawed frog (*Xenopus*, Pipidae) from West and Central Africa. **PLoS ONE**, v. 10, n. 12, p. e0142823, 2015.

FAIVOVICH, J. et al. Systematic review of the frog family Hylidae, with special reference to Hylinae: phylogenetic analysis and taxonomic revision. **Bulletin of the American Museum of Natural History**, v. 294, p. 1–240, 2005.

FÁVERO, E. R. et al. Intrageneric karyotypic variation in *Pseudopaludicola* (Anura: Leiuperidae) and its taxonomic relatedness. **Zoological studies**, v. 50, n. 6, p. 826–836, 2011.

FERNANDES, T. L. et al. Carrying Progeny on the Back : Reproduction in the Brazilian aquatic frog *Pipa carvalhoi*. **South American Journal of Herpetology**, v. 6, n. 3, p. 161–176, 2011.

FERRÃO, M. et al. High species richness of *Scinax* treefrogs (Hylidae) in a threatened amazonian landscape revealed by an integrative approach. **PLoS ONE**, v. 11, n. 11, p. 1–16, 2016.

FIŠER, C.; ROBINSON, C. T.; MALARD, F. Cryptic species as a window into the paradigm shift of the species concept. **Molecular Ecology**, v. 27, n. 3, p. 613–635, 2018.

FOUQUET, A. et al. Underestimation of species richness in neotropical frogs revealed by mtDNA analyses. **PLoS ONE**, v. 2, n. 10, p. e1109, 2007a.

FOUQUET, A. et al. Revealing cryptic diversity using molecular phylogenetics and phylogeography in frogs of the *Scinax ruber* and Rhinella margaritifera species groups. **Molecular Phylogenetics and Evolution**, v. 43, n. 2, p. 567–582, maio 2007b.

FUNK, W. C.; CAMINER, M.; RON, S. R. High levels of cryptic species diversity uncovered in Amazonian frogs. **Proc. R. Soc. B.**, v. 279, n. 1734, p. 1806–1814, 2011.

FUNK, W. C.; CAMINER, M.; RON, S. R. High levels of cryptic species diversity uncovered in Amazonian frogs. **Proceedings. Biological sciences/ The Royal Society**, v. 279, n. 1734, p. 1806–14, 2012.

GARDA, A. A.; BIAVATI, G. M.; COSTA, G. C. Sexual dimorphism, female fertility, and diet of P*ipa arrabali* (Anura, Pipidae) in Serra do Cachimbo, Pará, Brazil. **South American Journal of Herpetology**, v. 1, n. 1, p. 20–24, 2006.

GASCON, C.; LOUGHEED, S. C.; BOGART, J. P. Patterns of genetic population differentiation in four species of amazonian frogs : a test of the Riverine Barrier Hypothesis. **Biotropica**, v. 30, n. 1, p. 104–119, 1998.

GLAW, F.; KÖHLER, J. Amphibian species diversity exceeds that of mammals. **Herpetological Review**, v. 29, n. 1, p. 11–12, 1998.

GRAYBEL, A. phylogeny Bufo. **Molecular Phylogenetics and Evolution**, v. 2, n. 3, p. 256–296, 1993.

GRIFFITHS, I. The phylogeny of the Salientia. **Biological Reviews of the Cambridge Philosophical Society**, v. 38 p. 241-292, 1963.

GRIFFITHS, I.; CARVALHO, A.L. On the validity of employing larval characters as major phyletic indices in Amphibia, Salientia. **Revista Brasileira de Biologia**, v. 25 p.115-121, 1965.

HAAS, A. Cranial features of dendrobatid larvae (Amphibia: Anura: Dendrobatidae). **Journal of morphology**, v. 224, n. December, p. 241–264, 1995.

HAAS, A. The larval hyobranchial apparatus of discoglossoid frogs : its structure and bearing on the systematics of the Anura (Amphibia : Anura). **Zoological Systematics and Evolutionary Research**, v. 35, p. 179–197, 1997.

HAAS, A. Cladistics phylogeny of frogs as inferred from primarily larval characters (Amphibia : Anura). **Cladistics**, v. 19, p. 23–89, 2003.

HAFFER, J. Speciation in Amazonian Forest birds. **Science**, v. 165, n. 3889, p. 131–137, 1969.

HANKEN, James. Why are there so many new amphibian species when amphibians are declining? **Trends in Ecology & Evolution**, v. 14, n. 1, p. 7-8, 1999.

HASS, C. A.; DUNSKI, J. F.; MAXSON, L. R. Divergent lineages within the *Bufo margaritifera* complex (Amphibia: Anura; Bufonidae) revealed by albumin immunology. **Biotropica**, p. 238–249, 1995.

HAUSDORF, B. Progress toward a general species concept. **Evolution**, v. 64, n. 4, p. 923–931, 2011.

HAY, J. M. et al. Phylogenetic relationships of amphibian families inferred from DNA Sequences of mitochondrial 12s and 16s ribosomal RNA genes. **Molecular Biology**

And Evolution, v. 12, n. 5, p. 928–937, 1994.

HERO, J. M. . An illustrated key to tadpoles occurring in the Central Amazon rainforest, Manaus, Amazonas, Brazil. **Amazoniana Limnologia Et Oecologia Regionalis Systemae Fluminis Amazonas**, v. 11, n. 2, p. 201–262, 1990.

KÖHLER, J. et al. New Amphibians and Global Conservation: A Boost in species discoveries in a highly endangered vertebrate group. **BioScience**, v. 55, n. 8, p. 693, 2005.

LARSON, P. M. Chondrocranial development in larval *Rana sylvatica* (Anura: Ranidae): Morphometric analysis of cranial allometry and ontogenetic shape change. **Journal of Morphology**, v. 252, n. 2, p. 131–144, 2002.

LARSON, P. M. Ontogeny, phylogeny, and morphology in anuran larvae: Morphometric analysis of cranial development and evolution in *Rana* tadpoles (Anura: Ranidae). **Journal of Morphology**, v. 264, n. 1, p. 34–52, 2005.

LARSON, P. M.; DE SÁ, R. Chondrocranial morphology of *Leptodactylus* larvae (Leptodactylidae: Leptodactylinae): Its utility in phylogenetic reconstruction. **Journal of Morphology**, v. 238, n. 3, p. 287–305, 1998.

LIRIOMYZA, G.; AGROMYZIDAE, D.; JEM, J. M. Cryptic diversity hidden within the Leafminer. **Genes**, v. 9, n. 11, p. 554, 2018.

LOURENÇO, L. B.; RECCO-PIMENTEL, S. M.; CARDOSO, A. J. Polymorphism of the nucleolus organizer regions (NORs) in *Physalaemus petersi* (Amphibia, Anura, Leptodactylidae) detected by silver staining and fluorescence in situ hybridization. **Chromosome Research**, v. 6, n. 8, p. 621–628, 1998.

LUQUE-MONTES, I. et al. An integrative assessment of the taxonomic status of putative hybrid leopard frogs (Anura : Ranidae) from the Chortís Highlands of Central America, with description of a new species An integrative assessment of the taxonomic status of putative hybrid l. **Systematics and Biodiversity**, p. 1–17, 2018.

MCDIARMID, R. W.; ALTIG, R. **Tadpoles: the biology of anuran larvae**. Chicago: University of Chicago Press, 1999.

MEDEIROS, L. R.; ROSSA-FERES, D. C.; RECCO-PIMENTEL, S. M. Chromosomal differentiation of *Hyla nana* and *Hyla sanborni* (Anura, Hylidae) with a description of NOR polymorphism in H. nana. **Journal of Heredity**, v. 94, n. 2, p. 149–154, 2003.

MEZZASALMA, M. et al. Karyological analyses of *Pseudhymenochirus merlini* and *Hymenochirus boettgeri* provide new insights into the chromosome evolution in the anuran family Pipidae. **Zoologischer Anzeiger - A Journal of Comparative Zoology**, v. 258, p. 47–53, 2015.

MILIÁN-GARCÍA, Y. et al. Genetic evidence supports a distinct lineage of American crocodile (Crocodylus acutus) in the Greater Antilles. **PeerJ**, v. 6, p. e5836, 2018.

MORA, C. et al. How many species are there on earth and in the ocean? **PLoS Biology**, v. 9, n. 8, p. e1001127, 2011.

MORESCALCHI, A. Hypotheses on the phylogeny of the Salientia, based on karyological data none. **Experientia**, v. 24, n. 9, p. 964–966, 1968.

MORESCALCHI, A.; GARGIULO, G.; OLMO, E. Notes on the chromosomes of some Amphibia. **Journal of Herpetology**, v. 4, n. 1/2, p. 77–79, 1970.

MORITZ, C. et al. Diversification of Rainforest Faunas: An integrated molecular approach. **Annual Review of Ecology and Systematics**, v. 31, p. 533–563, 2000.

NASCIMENTO, F. A. C. DO; SKUK, G. O. O girino de *Chiasmocleis alagoanus* Cruz, Caramaschi & Freire, 1999 (Anura: Microhylidae). **Biota Neotropica**, v. 6, n. 3, p. 1–5, 2006.

NIELSON, M.; LOHMAN, K.; SULLIVAN, J. Phylogeography of the tailed frog (Ascaphus truei): implications for the biogeography of the Pacific Northwest. **Evolution**, v. 55, n. 1, p. 147–160, 2001.

ORTON, G. L. The Systematics of Vertebrate Larvae. **Systematic Zoology**, v. 6, p. 76–86, 1953.

PADIAL, J. M. et al. The integrative future of taxonomy. **Frontiers in Zoology**, v. 7, n. 1, p. 16, 2010.

PELOSO, P. L. V. et al. Phylogeny of map tree frogs, *Boana semilineata* species group, with a new Amazonian species (Anura: Hylidae). **South American Journal of Herpetology**, v. 13, n. 2, p. 150–169, 2018.

POUGH, F. H.; JANIS, C. M.; HEISER, J. B. **A Vida dos Vertebrados**. 4 edition ed. São Paulo- SP: Atheneu Editora São Paulo, 2008.

PRITI, H. et al. Integrative taxonomic approach for describing a new cryptic Species of bush frog (Raorchestes: Anura: Rhacophoridae) from the Western Ghats, India. **Plos One**, v. 11, n. 3, p. e0149382, 2 mar. 2016.

RICHARDS, C. M.; MOORE, W. S. A phylogeny for the African treefrog family Hyperoliidae based on mitochondrial rDNA. **Molecular Phylogenetics and Evolution**, v. 5, n. 3, p. 522–532, 1996.

RODRÍGUEZ, A. et al. Cryptic within cryptic: Genetics, morphometrics, and bioacoustics delimitate a new species of Eleutherodactylus (Anura: Eleutherodactylidae) from Eastern Cuba. **Zootaxa**, v. 4221, n. 5, p. 501–522, 2017.

SCHÄUBLE, C. S.; MORITZ, C.; SLADE, R. W. A molecular phylogeny for the frog genus *Limnodynastes* (Anura: Myobatrachidae). **Molecular Phylogenetics and Evolution**, v. 16, n. 3, p. 379–391, 2000.

SCHMIDT, J.; SCHUFF, M.; OLSSON, L. A role for FoxN3 in the development of

cranial cartilages and muscles in *Xenopus laevis* (Amphibia: Anura: Pipidae) with special emphasis on the novel rostral cartilages. **Journal of Anatomy**, v. 218, n. 2, p. 226–242, fev. 2011.

SCHUTZMAN, B.; VOVIDES, A. P.; DEHGAN, B. Two new species of *Zamia* (Zamiaceae, Cycadales) from southern Mexico. **Botanical Gazette**, v. 149, n. 3, p. 347–360, 2016.

SIQUEIRA, S. et al. Unusual intra-individual karyotypical variation and evidence of cryptic species in Amazonian populations of *Pristimantis* (Anura, Terrarana). **Hereditas**, v. 146, p. 141–151, 2009.

SOKOL, O. M. The free swimming Pipa larvae, with a review of pipid larvae and pipid phylogeny (Anura: Pipidae). **Journal of Morphology**, v. 154, n. 3, p. 357–425, 1977.

STIFFLER, L. L. et al. Quantitative acoustic differentiation of cryptic species illustrated with King and Clapper rails. **Ecology and Evolution**, n. October, p. 1–11, 2018.

STUART, B. L.; INGER, R. F.; VORIS, H. K. High level of cryptic species diversity revealed by sympatric lineages of Southeast Asian forest frogs. **Biology**, v. 1887, n. June, p. 470–474, 2006.

SUAREZ-MAYORGA, Á. M.; LYNCH, J. D. Redescription of the tadpole of *Hyla vigilans* (Anura: Hylidae) and notes about possible taxonomic relationships. **Caribbean Journal of Science**, v. 37, n. 1–2, p. 116–119, 1999.

SURYADNAYA, N. N. Comparative analysis of karyotypes of two cryptic species of Pelobatid frogs (Amphibia, Anura) of Ukraine. **Vestnik zoologii**, v. 48, n. 6, p. 511–520, 2014.

TRONTELJ, P. et al. A molecular test for cryptic diversity in ground water : how large are the ranges of macro-stygobionts? **Freshwater Biology**, v. 54, n. 4, p. 727–744, 2009.

TRUEB, L.; CANNATELLA, D. C. Systematics, morphology, and phylogeny of genus *Pipa* (Anura: Pipidae). **Herpetologica**, v. 42, n. 4, p. 412–449, 1986.

TRUEB, L.; HANKEN, J. Skeletal development in *Xenopus laevis* (Anura: Pipidae). **Journal of Morphology**, v. 214, n. 1, p. 1–41, 1992.

TRUEB, L.; PÚGENER, L. A.; MAGLIA, A. M. Ontogeny of the bizarre: An osteological description of *Pipa pipa* (Anura: Pipidae), with an account of skeletal development in the species. **Journal of Morphology**, v. 243, n. 1, p. 75–104, 2000.

UNO, Y. et al. Homoeologous chromosomes of *Xenopus laevis* are highly conserved after whole-genome duplication. **Nature**, v. 111, n. 5, p. 430–436, 2013.

VACHER, J. et al. Cryptic diversity in Amazonian frogs: Integrative taxonomy of the genus *Anomaloglossus* (Amphibia: Anura: Aromobatidae) reveals a unique case of

diversification within the Guiana Shield. **Molecular Phylogenetics and Evolution**, v. 112, p. 158–173, 2017a.

VACHER, J. P. et al. Cryptic diversity in Amazonian frogs: Integrative taxonomy of the genus *Anomaloglossus* (Amphibia: Anura: Aromobatidae) reveals a unique case of diversification within the Guiana Shield. **Molecular Phylogenetics and Evolution**, v. 112, p. 158–173, 2017b.

VALDECASAS, A. G.; WILLIAMS, D.; WHEELER, Q. D. 'Integrative taxonomy' then and now: a response to Dayrat (2005). **Biological Jounal of the Linnean Society**, v. 93, n. 1, p. 211–216, 2008.

VASCONCELOS, T. S. et al. Biogeographic Distribution Patterns and Their Correlates in the Diverse Frog Fauna of the Atlantic Forest Hotspot. **PLoS ONE**, v. 9, n. 8, p. e104130, 2014.

VENCES, M. et al. Phylogeny and classification of poison frogs (Amphibia: Dendrobatidae), based on mitochondrial 16S and 12S ribosomal RNA gene sequences. **Molecular Phylogenetics and Evolution**, v. 15, n. 1, p. 34–40, 2000.

WILEY, E. O. The Evolutionary Species Concept Reconsidered. **Systematic Zoology**, v. 27, n. 1, p. 17, 1978.

WOGEL, H. et al. Girinos de cinco espécies de anuros do Sudeste do Brasil (Amphibia: Hylidae, Leptodactylidae, Microhylidae). **Boletim do Museu Nacional**, n. 427, p. 1–16, 2000.

Garrick, R. C., K. E. Newton, and R. J. Worthington. Cryptic diversity in the southern Appalachian Mountains : genetic data reveal that the red centipede , *Scolopocryptops sexspinosus* , is a species complex. **Journal of Insect Conservation**, v. 22, n. 5–6, p. 799–805, 2018.

YOUNGER, J. L. et al. Molecular phylogenetics and evolution hidden diversity of forest birds in Madagascar revealed using integrative taxonomy. **Molecular Phylogenetics and Evolution**, v. 124, p. 16–26, 2018.

3 ARTIGO: Cryptic diversity in *Pipa carvalhoi*? An integrative approach

Amphibia-reptilia

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Article

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Abstract

Anurans are one of the groups with the highest cryptic diversity. To uncover this diversity, the integrative taxonomy based on different sources of evidences has been employed. *Pipa carvalhoi* is an interesting species to be studied integrating diferent datasets. It has a disjoint distribution and low dispersal capacity suggesting that differentiation can be already occurred. In this study we use integrative taxonomy to access *Pipa carvalhoi* diversity, asking whether independent lineages are in the process of differentiation, or have already become differentiated species. To test this hypothesis, morphological (tadpoles and adults), molecular (16S rRNA) and karyological data were analyzed. Three well-supported clades were recovered within *Pipa carvalhoi*. The structure of clades agree with drainage system: clade I occurs at the Northeastern Atlantic river basin, clade II at the San Francisco and East Atlantic river basins, and clade III at the Southeastern Atlantic river basin. The bPTP analysis recovered three candidate species, congruent with the clades recovered in the Bayesian Inference and Maximum Likelihood. Genetic distances among three clades of *P. carvalhoi* were higher that the expected value for interspecific divergence recovered using LocalMinima. Cytogenetic data did not differ among clades. The morphology of adults from different localities were similar, whereas the morphometric characters of tadpoles revealed morphological clusters with partial match with the genetic structure. Our results showed a strong genetic structure that indicates that *P. carvalhoi* is actually three cryptic species, apparently related to the hydrographic basins.

Key words: Pipidae, taxonomy, adaptive decoupling hypothesis, tadpole, Anura.

Introduction

Delimiting species is unquestionably important because species remains a main unit of biodiversity in many disciplines such as biogeography, ecology and macroevolution (Sites and Marshall, 2004). Generally, a new species does not arise in a quick and punctual event, but rather through a slow process of population differentiation (De Queiroz, 2007; Sukumaran and Knowles, 2017). This makes the work of taxonomists challenging because some sources of evidence may not detect this process, while others may point to a speciation event that has not yet been completed, leading to overestimated diversity (Edwards, Knowles, and Edwards, 2014; Reudenstein et al., 2017; Folk et al., 2018). Moreover, De Queiroz (1998) emphasize the importance of distinguishing between what a species is and the criteria used for its delimitation (e.g. the standards used to decide whether certain lineages are to be considered distinct species).

Integrative taxonomy sensu Dayrat (2005) is a discipline that characterizes species based in different sources of evidences, helping in the detection of lineages that may have completed the process of speciation and thus providing greater reliability to taxonomic decisions (Schlick-Steiner et al., 2010; Orrico et al., 2017; Walker, Lyra, and Haddad, 2018; Younger et al., 2018). Padial et al. (2010) suggested two criteria for integrating the information used to guide taxonomic decisions: (i) congruence or (ii) accumulation. Integrative taxonomy by congruence follows the presupposed that there is a need for the convergence of two or more independent sources of evidence in order to differentiate species. However, because the process of speciation is not always accompanied by changes in character states at all levels (Adams et al., 2009), this strategy may result in underestimating the number of species in a given system. On the other hand, integrative taxonomy by accumulation is based on the premise that differences detected in any line of evidence may support the existence of a species (De Queiroz, 2007). This strategy is able to detect recently emerged species more easily, however, under the risk of overestimating diversity as lineages that are still in the process of speciation can be wrongly elevated to the species status (Padial et al., 2010).

Nonetheless, integrative taxonomy has been helping taxonomists in delimiting species in several groups, being especially important in those that present cryptic

diversity, as is the case of anuran amphibians. For this group, lines of evidence that measure lineage divergence at the genetic level has proven specially useful, with molecular studies often revealing underestimated diversity (Funk, Caminer, and Ron, 2012; Vacher et al., 2017; Walker, Lyra, and Haddad, 2018). Because amphibians worldwide are facing declines, many species may become extinct before being described (Funk, Caminer, and Ron, 2012), urging reassessment of their taxonomic status so that conservation measures can be taken.

Pipa carvalhoi is endemic to Brazil and presents a disjoint distribution that remains unexplained; its range comprises an area extending from the state of Ceará to the state of Sergipe, and another area about 600 Km southward extending from the southermost part of the state of Bahia to the state of Espírito Santo (Frost, 2019, figure 1). These two areas of occurrence are only in partial agreement with the local river basins; the Atlântico Nordeste, São Franciso, Atlântico Leste and Atlântico Sudeste river basins (sensu IBGE 2000, Figure 1). As demonstrated for fish, river basins constitute ecoregions where organisms of similar lifestyles may show shared patterns of species distribution and associated ecological and evolutionary processes (Abell et al., 2008). The limits of river basins may represent important barriers for the dispersal of the species therein, both freshwater aquatic species, but also species capable of some over land dispersal, although at different levels (Abell et al., 2008). Thus, it is possible that both the diversification and the distribution of *P. carvalhoi* are influenced by the drainage system at some extent.

In this study we use integrative taxonomy to access lineage diversity in *Pipa carvalhoi*, asking whether independent lineages are in the process of differentiation, or have already become differentiated species, being therefore candidates to new species awaiting formal description. To test this hypothesis, we analyzed morphological, molecular and karyological characters of *P. carvalhoi*, addressing two questions i) if the genetic diversity is structured into groups corresponding to the two disjoint areas of occurrence for the species or, alternatively, according to the drainage system, and ii) if there is agreement of the genetic structure with other lines of evidence such as karyotypic, and/or morphological variation of tadpoles and adults.

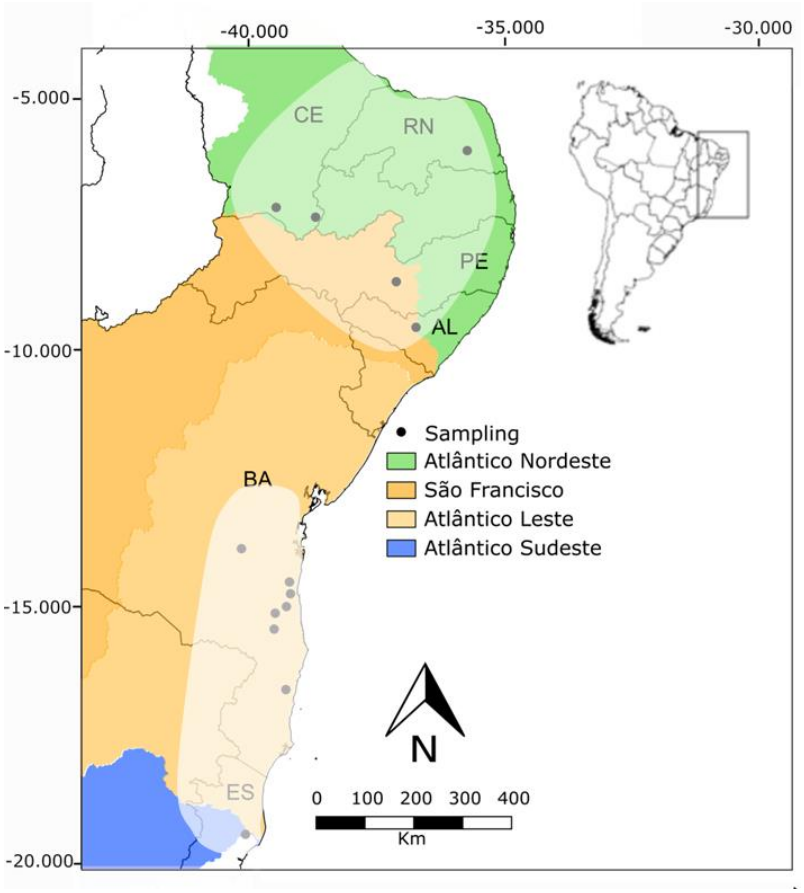

Figure 1. Map showing areas of occurrence for *Pipa carvalhoi*, main river basins in the region (indicated by colors), and localities sampled for this study (black circles). CE = Ceará, RN = Rio Grande do Norte, PE = Pernambuco, AL = Alagoas, BA = Bahia, and ES = Espírito Santo. (Fonte: Autor).

Material and Methods

Sampling

Fieldwork including collecting of *Pipa carvalhoi* was carried out in four Brazilian states: Pernambuco (PE): Buíque municipality, Ceará (CE): Crato municipality, Rio Grande do Norte (RN): Nova Cruz municipality and Espírito Santo (ES): Santa Teresa municipality, comprising the two areas of occurrence and the Atlântico Nordeste, São Francisco, Atlântico Leste and Atlântico Sudeste river basins, between September 2017 and March of 2018. Tadpoles were euthanized with lidocaine 2% soon after collection, while adults underwent the cytology protocol before euthanasia (see Cytogenetics bellow). For molecular analysis, complementary samples of *P. carvalhoi*, *P. pipa*, *P. arrabali* e *Xenopus laevis* were obtained from

collections or from Genbank. A list with all material analyzed is in the Supplementary Material (S1-4)

Molecular protocols and analyses

Total genomic DNA was extracted using Phenol-Chloroform (Sambrook, 2001). The fragment of mitochondrial gene 16S rRNA was amplified by Polymerase Chain Reaction (PCR) using the primers 16Ssar: 5'CGCCTGTTTATCAAAAACAT3' and 16Ssbr: 5'CCGGTCTGAACTCAGATCACGT3' (Palumbi et al., 2002). The PCR consisted of 35 cycles of denaturation at 94° C for 45 seconds, annealing at 55° C for 1 minute and extension at 72° C for 1 minute. The amplicons were purified with isopropanol and sequenced unidirectionally with Big Dye terminator chemistry. Eletropherograms were checked in the Bioedit software v 7.2.5 (Hall, 2011).

To test the monophyly of *P. carvalhoi* and to verify its genetic structure, we included as external group samples of *P. pipa*, *P. arrabali* and *P. parva* and performed a phylogenetic reconstruction rooting in *Rhinophrynus dorsalis* following Frost et al. (2006). Sequences obtained in this study and in Genbank were aligned in the Mafft software (Katoh and Standley, 2013). The choice of the best evolutionary model was performed in PartitionFinder 2.1.1 (Lanfear et al., 2012) through the Bayesian Information Criterion (BIC). For the phylogenetic reconstruction we first used Bayesian Inference (BI) as implemented in the MrBayes 3.2 software (Ronquist et al., 2012), considering the posterior probability of clades as a measure of their support. The analysis consisted of two independent runs of 10 million generations each, with sampling at each 1.000 gererations. The first 25% of the trees were discarded as burn-in. All sites with gaps were not considered from the analyses. We also conducted a maximum likelihood (ML) reconstruction in the RAxML HPC2 v. 8.2.8 software (Stamatakis, 2014), implemented in the CIPRES Science Gateway (Miller et al., 2010), evaluating clade support with 1,000 rapid bootstrap replicates. To delimit the number of species, a Bayesian implementation of the model Poisson Tree Process (bPTP) was used (Zhang et al., 2013).

To evaluate genetic variation among clades, we calculated Kimura-2-Parameters (K2P) distances in the Mega X software (Kumar et al., 2018). To estimate the most likely threshold for this distance we used the localMinima function of the Spider

package (Brown et al., 2012) in the R platform version 3.5.2 (https://www.r-project.org/). To verify the correlation between genetic and geographic distance we performed a Mantel test on the R platform using the Vegan package (Oksamen et al., 2013).

Cytogenetics

To access karyotype variation, five to 20 specimens from each sampled locality were analyzed. Initially the animals were treated with colchicine 2% and euthanized after four hours following the Herpetological Animal Care and Use Committee (HACC) of American Society of Ichthyologists and Herpetologists. To obtain cell suspensions, we used the protocol proposed by King and Rofe (1976) and Schmid (1978). The chromosomes were stained with Giemsa 10% for the determination of karyotypes. For the detection of nucleolus organizer region (NORs) we silver stained preparations using the Ag–NOR method according to Howell and Black (1980). The chromosomes were classified according to the values proposed by Green and Sessions (1991).

Morphology

We measured morphological variation in adults and tadpoles. For the morphology of adults, 109 specimens were examined for six discrete characters following Dunn (1948) and Trueb and Cannatella (1986): body shape, presence of eyelid and eardrum, shape of the digital extremities of the anterior limbs, metatarsal tubercle and dermal changes around the mouth. Seventeen morphometric characters were also included, eight of which followed Herrel and Bonneaud (2012): snout-vent length, lengths of the tarsus, of the thigh, of the tibia, of the foot, of the arm, of the forearm and head width; five followed Kok and Kalamandeen (2008): head length, length of the snout, snout width, eye diameter, interorbital distance, and three additional characters are proposed here: width of the body under the arms, width of the body under inguinal region e distance of the arms to the legs. Specimens from both sexes were considered together in analyses.

For the larval morphological variation, we examined 64 tadpoles between stages of development 50 to 58 for external morphology following Nieuwkoop and Faber

(1956), and forty-two tadpoles between the stages 48-59 (preferably stage 54 due to a higher frequency in our sample) for internal morphology. For the larval external morphology, 15 discrete characters were analyzed following the terminology adopted by McDiarmid and Altig (1999): Body shape in dorsal and lateral views, snout in dorsal and lateral views, eyes position, position and shape of the nostrils, position of the nostril in relation to the snout and eyes, position of the opening of the spiracle, position of the cloacal tube and its opening, ventral and dorsal fin height, oral disc position and presence of flagellum. We also analyzed 24 morphometric characters, six proposed by McDiarmid and Altig (1999): total body and tail lengths, maximum tail height, height and width of the caudal muscles, 13 proposed by Lavilla and Scrocchi (1986): body width at eye and nostril levels, maximum body height and width, width of the oral opening, extranarinal and extraorbital distances, diameter of the eyes and nostrils, distance from the eyes to the nostrils and distance from the snout to the nostrils, two suggested by Grosjean (2005): dorsal and ventral fin heights, and three additional characters proposed in this study: length of intestinal region, width of the opening of the spiracle and cloacal tube length. In order to visualize the chondrocranium and the hiobranquail apparatus, the tadpoles were clear and stained following the protocol suggested by Dingerkus and Uhler (1977). After this process, these structures were photographed using stereomicroscope Leica S8 coupled with a camera Leica LAS EZ. The nomenclature followed Larson and De Sá (1998) and Sokol (1977).

For both adults and tadpoles, we evaluated the presence of morphological groupings through Principal Component Analysis (PCA) with linear regression residuals to remove the size effect (adults and tadpoles separately) and the possible variation between stages of development (tadpoles).

Results

Molecular

The molecular matrix consisted of 51 sequences with 300 base pairs, 30 of which belong to *Pipa carvalhoi*. The most appropriate evolutionary model according to the bayes information criterion (BIC) was the SYM+G. *Pipa carvalhoi* was recovered as a well-supported monophyletic group showing high statistical support (posterior

probability = 1.0, bootstrap = 93). Its genetic diversity was distributed into three well-supported clades (clades I, II e III – Figure 1) whose relationships showed high support according to BI and ML. Clade I included individuals collected in the states of Ceará (Crato and Mauriti) and Rio Grande do Norte (Serra Caiada). Clade I was recovered as sister to clade III, which presents individuals from the southermost distribution of the species in the state of Espírito Santo (Linhares). Clade II included individuals from the states of Alagoas (Igaci), Bahia (Porto Seguro, Buerarema, Ilhéus, Jequié, Uruçuca and Camacan) and Pernambuco (Buíque), being recovered as sister group of clade I + clade III. The distribution of clades showed no coherence with the disjoint areas of occurrence of *P. carvalhoi*, but matched the structure of the drainage system with clade I occurring at the Atlântico Nordeste river basin, clade II at the São Francisco and Atlântico Leste river basins, and clade III at the Atlântico Sudeste river basin (Figure 2).

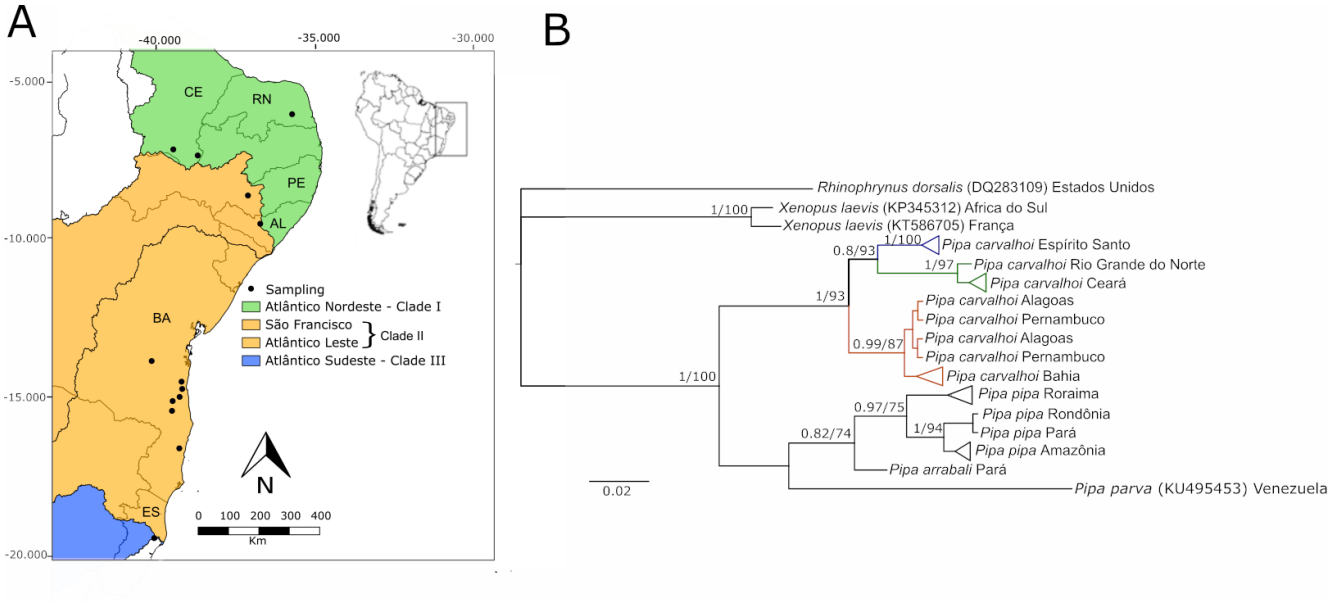

Figure 2. A, Map showing areas of occurrence for *Pipa carvalhoi*, main river basins in the region (indicated by colors), and localities sampled for this study (black circles). CE = Ceará, RN = Rio Grande do Norte, PE = Pernambuco, AL = Alagoas, BA = Bahia, and ES = Espírito Santo. B, Phylogram of *Pipa carvalhoi* obtained through Bayesian Inference and Maximum Likelihood using the evolutionary model SYM+G and a 300 bp fragment of the 16S rRNA mitochondrial gene. Values in the nodes are posterior probabilities/bootstrap support. (Fonte: Autor).

The bPTP analysis recovered three candidate species within *P. carvalhoi*, congruent with the clades recovered in the BI e ML. The clade I was recovered by bPTP as a possible species with posterior probability 0.63, the clade II presented support of 0.78 and clade III with support 0.94. The analysis also recovered three species for *P. pipa*, one composed of specimens from the state of Amazonas with support 0.77, another by specimens from the states of Rondônia and Pará presenting support 0.78 and a third consisting of specimens from the state of Roraima with support 0.94. The chain MCMC converged, which supports the reliability of the results.

Genetic distances (K2P) ranged from 5.7% to 7% between the clades of *Pipa carvalhoi* (Table 1). In the results obtained with the localMinima function, the first depression in the distance density curve, which represents the most probable value of the transition between intra and interspecific divergences was 3.18 % (Figure 3). The Mantel test revealed a significant correlation between genetic distance and geographic distance (r = 0.51, p = 0.001).

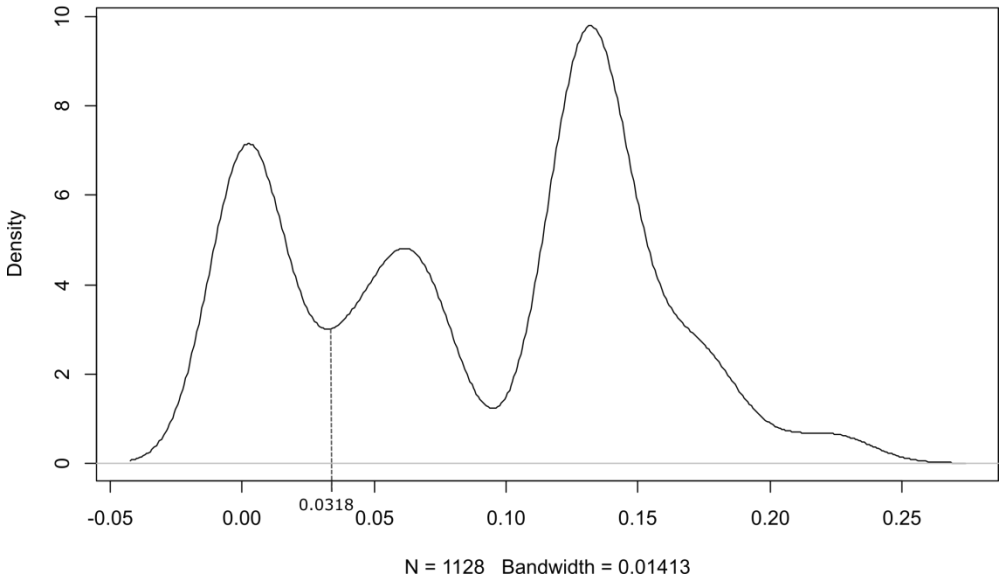

Figure 3. Density curve of genetic distances of species of the genus *Pipa* obtained through of the LocalMinima through alignment with 300 bp fragment of the 16S rRNA mitochondrial gene: The first depression in the curve corresponds to 3.18%. (Fonte: Autor)

Table 1. Genetic distances among clades of *Pipa* using a 300 base pair fragment of the 16S rRNA mitochondrial gene and Kimura-2-parameters evolutionary model. Values correspond to intraspecific distances in *Pipa carvalhoi* and *Pipa pipa*. *Pipa pipa* (1) = specimens from the state of Roraima; *Pipa pipa* (2) = specimens from the states of Rondônia and Pará; *Pipa pipa* (3) = specimen from the state of Amazonas. Percentage values. NA = non applicable, because a single sequence was available. All localities are in Brazil.

Specie/Clade	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1. Pipa carvalhoi (Clado I)	0.4							
2. Pipa carvalhoi (Clado II)	7.0	0.2						
3. Pipa carvalhoi (Clado III)	5.9	5.7	0.2					
4. Pipa pipa (1)	17.0	12.6	13.1	0.0				
5. Pipa pipa (2)	17.5	13.9	14.4	4.2	0.0			
6. Pipa pipa (3)	17.0	13.5	14.8	3.5	1.3	0.0		
7. Pipa arrabali	18.0	14.4	14.0	5.7	6.5	5.7	NA	
8. Pipa parva	19.6	22.5	21.9	19.8	17.9	17.4	15.4	NA

Interclade karyotype comparison reveals conservative chromosomal features as diploid number, morphology and NOR position (Figure 4). The karyotype of the analyzed populations of *Pipa carvalhoi* was composed by three pairs of metacentric chromosomes (pairs 1, 4 e 8), two submetacentric (pairs 2 e 7), three subtelocentric (pairs 3, 5 e 6) and two telocentric (pairs 9 e 10). Only one chromosome NOR bearing was detected, located in the subtelocentric region of the long arm of the pair 9.

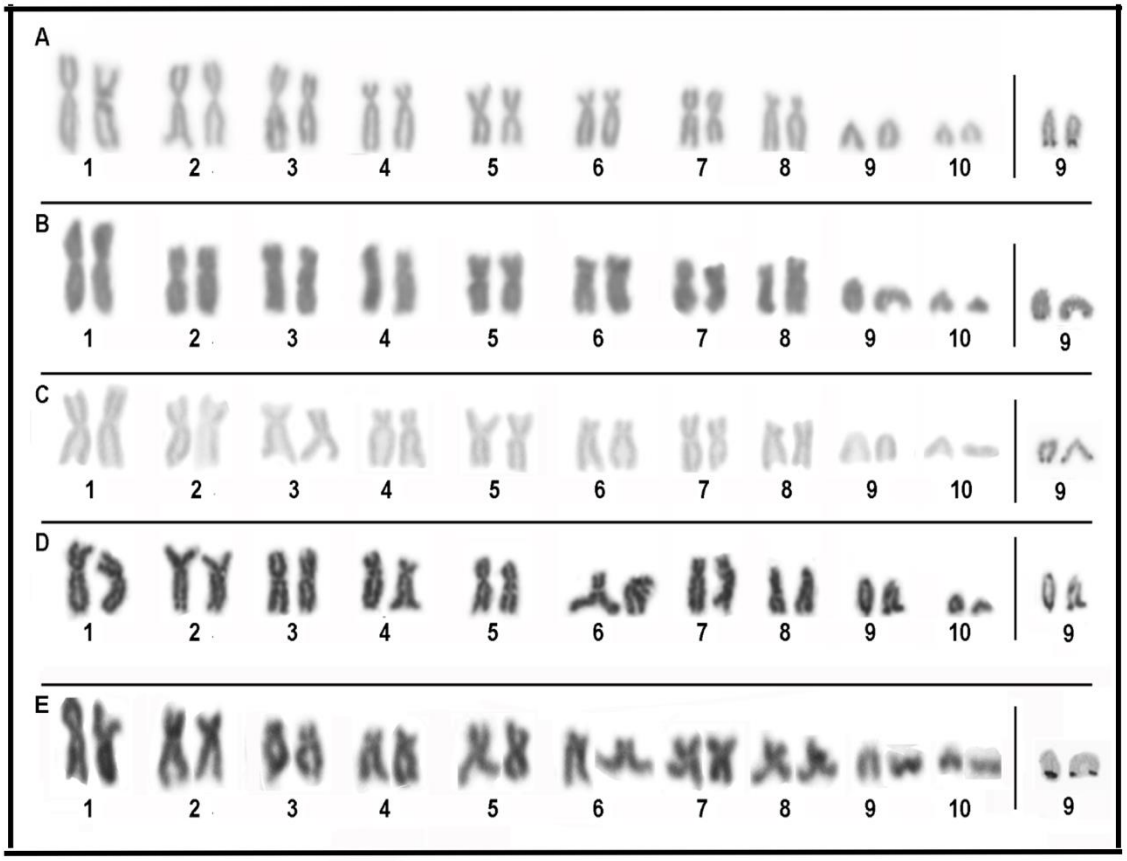

Figure 4. Karyotype of the *Pipa carvalhoi* submitted to conventional Giemsa staining. In (A) specimens from Crato, Ceará State, (B) Nova Cruz, Rio Grande do Norte State, (C) Buíque, Pernambuco State, (D) Santa Teresa, Espírito Santo State and (E) Buerarema, Bahia State. Highlighted (at the right), NOR-bearing chromosomes submitted to the silver impregnation method (Ag-NOR). (Fonte: Autor).

Morphological

Adults from different populations did not exhibit variation in discrete characters (Supplementary Material 5). All specimens had a body flattened dorso-ventrally, small head, absence of eyelid and eardrum, and tongue completely attached to the floor of the mouth. The upper lip overlaps the lower lip at the mouth's corner, forming a pocket at the angle of the jaw. The distal region of each digit of the anterior limbs presented four symmetrically organized lobes. A metatarsal tubercle in the hind limbs and keratinized tips on toes I-III similar to claws were found.

In the PCA of the morphometric data of adults, the two major components cumulatively accounted for 36% of the total variance, PC1 being responsible for 24%

and PC2 for 12%. Clusters comprising samples from each clade overlapped completely, showing no apparent ordering within the morphometric space (Figure 5).

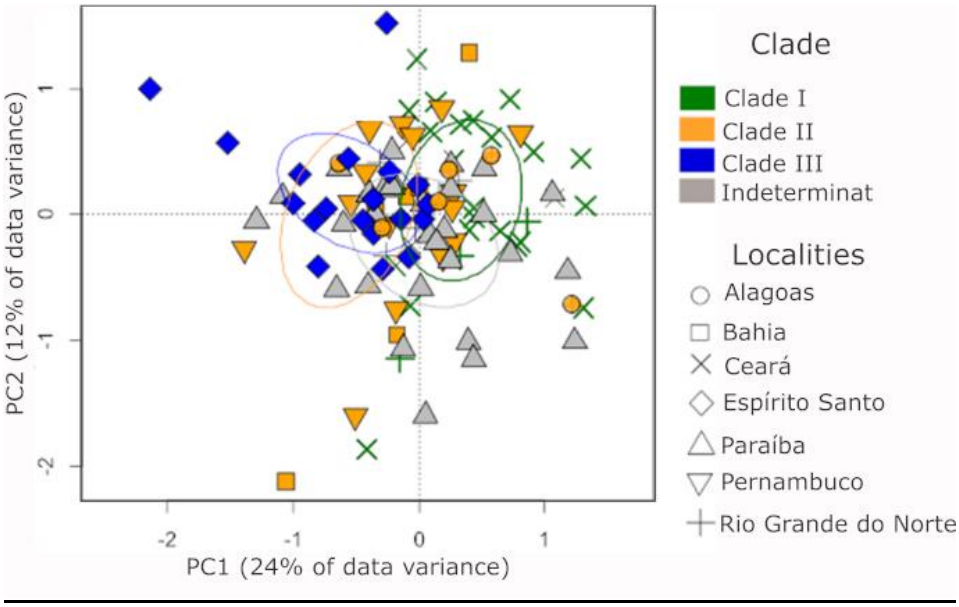

Figure 5. Principal Component Analysis (PCA) based on residual morphometric data of adults of *Pipa carvalhoi.* PC1 explains 24% and PC2 12% of the total variance. The colors and shapes represent the clades and localities, respectively. The clade named as "indeterminate" consists of the specimens of Paraíba that were not included in the molecular approach and its phylogenetic placement is unknown. (Fonte: Autor).

The tadpoles of the analyzed localities were also morphologically similar. The shape of the body was differentiated in the cephalic and intestinal regions. The cephalic region is oval, slightly square in dorsal view. The intestinal region is circular and presents the peritoneal membrane dark and visible in lateral and ventral views. Body is triangular in lateral view. Absence of keratinized structures in the oral apparatus, being composed only of a previously located oral opening. Snout is wide in dorsal view. The nostrils are dorsal and elliptical, closer to the snout than to the eyes. The eyes are lateral. The spiracle has two ventral openings. The cloacal tube is ventral, as well as its opening. The insertion of the dorsal fin is in the tail. Dorsal fin is low, while the ventral fin is high. Tadpoles present a flagellum at caudal termination, being lost in specimens fixed. The only discrete variation was on the body and snout of the tadpoles from Bahia. They present the body more elongated and snout narrower in dorsal view than those from all other locations.

The values of the morphometric variables are presented in the Supplementary Materials 6 e 7. In the PCA, the first two main components (PC1 e PC2) explained

cumulatively 52% of the total variance, being PC1 responsible for 31% and PC2 for 21%. Morphological clusters were partially concordant with the genetic structure (Figure 6). The tadpoles of clade III formed a cohesive group separated from the tadpoles from clade II. These gathered into a more distributed group in the morphometric space, with substructure according to the geographical distribution. This group overlapped with tadpoles from the state of Paraíba, for which no sequences are available. Unfortunately, no tadpoles were available for clade I.

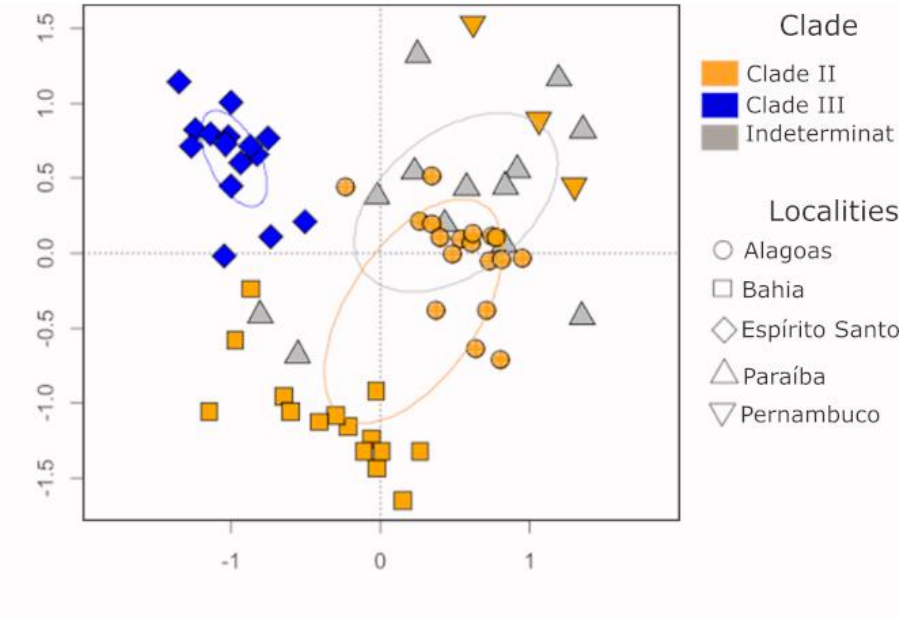

Figure 6. Principal Component Analysis (PCA) based on residual morphometric data of tadpoles of *Pipa carvalhoi.* PC 1 explains 31% and PC2 21% of the total data variance. The colors and shapes represent the clades and states, respectively. The clade named as "undetermined" refers to specimens from the state of Paraíba that was not included in the molecular analyses. (Fonte: Autor)

We detected no variation in chondrocranium features among populations of *Pipa carvalhoi* exception to two features: the lateral alae of the suprarrostral plate and the ventrolateral process of the palatoquadrate. Specimens from Espírito Santo have a smaller lateral alae when compared to specimens from all other localities. Moreover, specimens from Espírito Santo have the ventrolateral process of the palatoquadrate a long, and a practically straight bar, where all other specimens from all other localities has a almost horizontal bar with curves. For all other characters of chondrocranium, all populations were similar. Chondrocranium is rectangular in dorsal view, longer than wider, and depressed in lateral view, widest at ventrolateral process of palatoquadrate. Palatoquadrate is thin. The suprarrostral cartilage was

modified on a semilunar suprarostral plate with short triangular lateral alae. The quadratoethmoidal process is fused to the distal region of the suprarrosternal plate, delimiting the choanas. The muscular process quadrate is rectangular and reduced. Cornua trabeculae are absent and the ethmoidal region of the larvae consists of a single horizontal plate, the planum internasale. Each orbital cartilage contains two large forame, its basal portions end anterior to the otic capsule. The otic plate is thick. Parotic crest protrudes horizontally from the lateral walls of the otic capsules. Confluent with the parotic crista there is a large optical plate that fuses completely to the patatoquadrate.

The ascending process of palatoquadrate fuses with the dorsal end of the pila antotica and the larval otic process. Meckel's cartilage is wider in the extremities, previously it is fused with the infrarrostral cartilage, this is short and present "U" shape.

The hybranchial apparatus is formed by large ceratohyals, triangular plates with rounded angles and deeply emarginate posterior edges, ending next to the branchial baskets. The ceratobranchialis present lateral projections, similar to a "net" and are directly connected to the posteromedial processes of the ceratohyals. The copula II and anterior is absent. The hypobranchial plate is narrow. There are four ceratobranchials on each side. Spicules are absent.

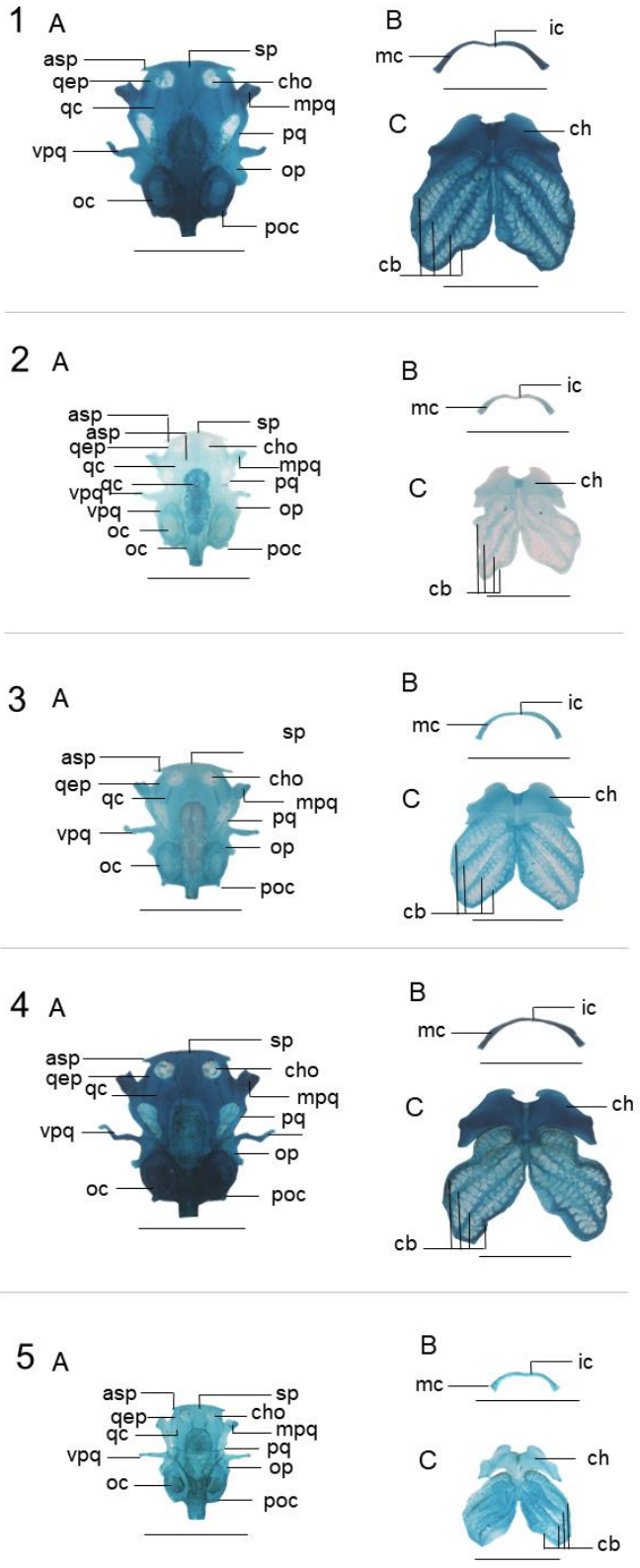

Figure 7: Chondrocranium and hyiobranquial apparatus of *Pipa carvalhoi* tadpoles in the developmental stage 54. Tadpoles were collected in 1- Alagoas; 2- Bahia; 3- Paraíba; 4- Pernambuco and 5- Espírito Santo. A. Chondrocranium in dorsal view; B. Meckel's and

infrarostral cartilages; C. Hyiobranquial apparatus. Asp = alae of suprarostral plate, cb = ceratobranchials, ch = ceratohyal, cho = choana, ic = infrarrostral cartilage, mc = meckel's cartilage, mpq = muscular process quadrate, oc = otic capsule, op = optic plate, poc = posterolateral process of otic crest, pq = palatoquadrate, qc = quadratocranial commissure, qep = quadratoethmoidal process, sp = suprarrostral plate, vpq = ventrolateral process of the palatoquadrate. (Fonte: Autor)

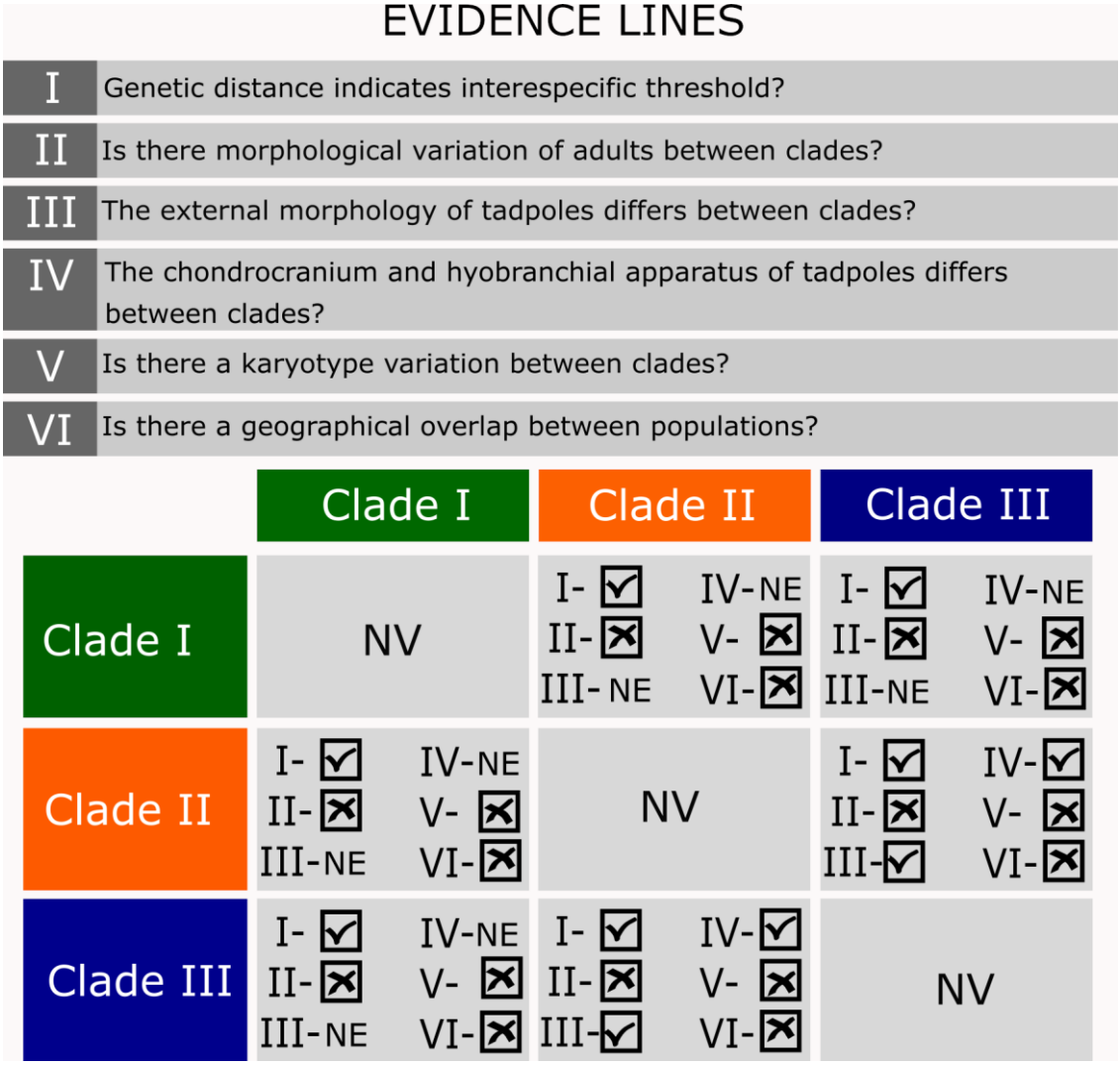

Figure 8: Pairwise comparison among results of the genotypic and phenotypic evidences evaluated to three clades recovered to *Pipa carvalhoi* samples. NV represent absence of intrapopulation variation, NE= no evaluated. (Fonte: Autor)

Discussion

Molecular variation

Molecular analysis of the mitochondrial fragment of the 16S rRNA gene supports *Pipa carvalhoi* as a monophyletic species. However, our sampling did not include all species in the genus and our results are pending more inclusive sampling. A

phylogenetic analysis based in osteological characters and considering all *Pipa* species showed *P. carvalhoi* as the sister species to the clade comprising *P. aspera* + [*P. arrabali* + [*P. snethlageae* + *P. pipa*]] (Trueb and Massemin, 2001). Sampling from hypotheses based on molecular data are very incomplete for *Pipa* species, with the most recent including only three species and recovering *P. carvalhoi* as sister to *P. parva* + *P. pipa* (Jetz and Pyron, 2018). Due to sampling, these hypotheses are hard to compare but the relative position of *P. parva* seems to differ between them.

Within *Pipa carvalhoi*, genetic structure was distributed into three deeply divergent clades, which were identified in the Bayesian PTP analysis as three distinct species with support from 0.63 to 0.94. Although outside the scope of this study, a similar result was found for *P. pipa* with the bPTP analysis recovering three species as well. The threshold estimated by LocalMinima was 3.18%, a much lower value than genetic distances found between clades ‘I and II’ and ‘I and III’ of *Pipa carvalhoi.* In fact, the genetic distance of 7% between the clades I and II overcomes the divergence between *P. pipa* e *P. arrabali* (5.7% – 5.9%). These analyses reinforce the idea that genetic diversity in *Pipa* species is highly underestimated.

Deep genetic structure was also found in aquatic and semiaquatic anurans of the genus *Pseudis* based on fragments of three mitochondrial genes (12S, tRNAval and 16S). The phylogenetic analysis recovered two lineages of *P. bolbodactyla* and three lineages in *P. paradoxa*. *Pseudis bolbodactyla* is distributed in the São Francisco, Paraná e Tocantis basins, which are all connected, while *P. paradoxa* presents a disjoint distribution, occurring in the São Francisco, Amazon and Mearim River basins (Garda and Cannatella, 2007). Posteriorly, morphological data was used to characterize these species and found strike variation in coloration distinguishing *P. bolbodactyla* and *P. paradoxa* throughout its distribution, being particularly extensive in *P. paradoxa* (Garda, Santana, and São-Pedro, 2010). These authors also highlighted the limitations of using only mitochondrial genes, with matrilineal inheritance and suggest the incorporation of nuclear genes (Garda and Cannatella, 2007), a limitation that is also shared with our study.

Cytogenetics

All individuals of *Pipa carvalhoi* collected in different localities displayed the same diploid number, 2n=20 chromosomes with identical karyotypes. *Pipa pipa* and *P. arrabali* share the same chromosome number (2n=22), however karyotypes of these species are morphologically distinguishable (Morescalchi, 1968; Morescalchi et al., 1970, DPB personal observation). The diploid number 2n=20 is considered ancestral in the Pipidae family, being present also in the *Pseudhymenochirus merlini* and *Hymenochirus boettgeri* species (Mezzasalma et al., 2015). In this sense, it is not surprising that lineages of *P. carvalhoi* present a conserved karyotype.

Morphological variation

The morphology of adults of *Pipa carvalhoi* did not show variation among clades. Nevertheless, the PCA of morphometric characters of tadpoles recovered specimens from clade III as isolated from others. The clade II form a substructure related to their geographical distribution, in which, there are subgroups formed by each state that compose the clade. *Pipa carvalhoi* is semiaquatic in the adult phase, but adults and larvae present striking differences in their ecological niches. Adults are active at night, hidding under the substrate during the day, emerging sporadically to breathe (Trueb and Cannatella, 1986). In contrast, the tadpoles are active during the day, exploring in the water column and can be easily seen near the surface (LL personal observation). Adults are carnivores, feed on arthropod larvae, insects, adult diptera and even coespecific tadpoles (Canedo and Garcia, 2006), while the tadpoles present only an oral opening with absence of keratinized pieces, indicating a filtering habit (McDiarmid and Altig, 1999).

Larvae and adults may undergo different environmental pressures, causing divergence between larvae and convergence between adults, or the opposite (Cruz, 1982; Duellman, Trueb, 1994; McDiarmid; Altig, 1999), leading conflicts in the interpretation of evolutionary pathways (Lande, 1992; Sherratt et al., 2017). This is more evident in species with complex life cycles (Ebenman, 1992; Bonett et al., 2018). Distinct evolutionary patterns at different phases of life gave rise to the “adaptive decoupling hypothesis”, which assumes that the different stages of life are relatively free to evolve independently (Ebenman, 1992; Moran, 1994). To test this, Sherratt et al. (2017) analyzed morphometric and geometric data of larvae and adults of 166 species of Australian anurans and observed high levels of homoplasy in

morphological characters of the tadpoles, resulting in low phylogenetic signal whereas in adults, morphological homoplasy was considered moderate.

Larval morphology have shown efficiency in the taxonomy of anurans (Haas, 2003; Barrasso et al., 2012). The species *Scarthyla vigilans* and *S. goinorum*, for example, are indistinguishable morphologically when adults, being differentiated only by the vocalization and the morphology of tadpoles (Duellman and De Sá, 1988; Suarez-Mayorga and Lynch, 1999; Barrio-Amorós et al., 2006). Thus, considering both phases of the anuran lifecycle in taxonomic assessments is desirable, especially considering that the morphological phylogenetic signal in the different phases can vary according to the species. In addition, the analysis of adult morphology alone may be inefficient in to detect cryptic species (Funk, Caminer, and Ron, 2012; Vacher et al., 2017; Walker, Lyra, and Haddad, 2018). Thus, *P. carvalhoi* tadpoles appear to present a higher phylogenetic signal than adults, being also potentially interesting for studies on the adaptive decoupling hypothesis.

Biogeography

The genetic structure of *Pipa carvalhoi* recovered in this study does not match the two areas of occurrence of the species, with the clade II occurring in both areas. Nevertheless, structure seems to be related with the drainage system. Genetic distance between clades I and II is greater than between the clades I and III, which are geographically more distant. It is possible that this lower genetic divergence between clades I and III may be related to past paleodrenages that connected basins currently isolated (Thomaz, Malabarba, and Knowles, 2017; Thomaz and Knowles, 2018), in which the basins Atlântico Leste, Nordeste and Atlântico Sudeste could be connected in the past.

This structure based on the geographical basins corroborates the hypothesis proposed by Trueb and Canatella (1986) that the origins of river basins constitute important biogeographic events for the speciation of the genus *Pipa.* The authors suggested that the ancestor of the genus was widely distributed throughout South America. This ancestor was later splitted into two species, one of which was restricted to the northwest of South America and another occupied the drainages of the Orinoco, and Amazônia and also the southeast region of Brazil. The most

northerly species was divided into two areas, the basin of Maracaibo in Venezuela (*Pipa parva*) and northwest Colombia and Panama (*Pipa myersi*). The species of the Orinoco region, Amazônia and southeastern Brazil suffered a vicariant event that resulted in *Pipa carvalhoi,* which was isolated along the lowlands of the east coast of Brazil, and one species in the drainage regions of Orinoco and Amazônica basin. Vicariant events in this latter species resulted in *Pipa arrabali* and in the ancestor of the species *P. pipa* and *P. snethlageae*.

Final considerations

Variations can be detected more easily at the molecular level than in other sources of evidence (Stuart, Inger, and Voris, 2006; Fouquet et al., 2007; Walker, Lyra, and Haddad, 2018). However, a great challenge is to detect whether genetic structure correspond to isolated lineages that have endured the speciation process or simply correspond to within species population structure (Sukumaran and Knowles, 2017). As shown by De Queiroz (2007), after a point of cleavage in one species to form two new ones, lineages may experience a long time accumulating new attributes, before being recognizable as different species, the so-called "gray zone". Therefore, in this phase, there is great disagreement between the different criteria used to delimit species because some sources of evidence may be able to detect this process, while others may not yet. Thereby, genetic divergences found in *P. carvalhoi*, together with the conserved karyotype and few morphological variations suggest that *P. carvalhoi* three lineages that may be in this “gray zone”.

In this study, we analyzed morphological, molecular and karyological data of *Pipa carvalhoi*, asking if genetic diversity in this species is structured in two groups corresponding to the disjoint areas of occurrence, and if there is agreement of the genetic structure with the karyotype and morphological variation of tadpoles and adults. Our results pointed to a deep genetic structure, apparently more related to hydrographic basins. We did not find karyotype and morphological variation in adults, but there is variation in tadpole morphology that is in agreement with our molecular results. Thus, taxonomic reassessment may be necessary for taxonomy to reflect evolutionary history in this species. Furthermore, the difference in the morphological signal of tadpoles and adults suggests the adaptive decoupling between these two phases of life mediated by different selective pressures. Together, the results

presented here indicate that *P. carvalhoi* presents three lineages evolving independently and an interesting species to trace evolutionary rates of tadpoles and adults in a comparative framework.

The status of *Pipa carvalhoi* was least concern according to IUCN assessment (www.iucnredlist.org). However, based on the results of this study, a reassessment of the status of conservation of each one of the three evolutionary lineages recovered is needed. Especially, the lineage III, which seems to occur only in the state of Espírito Santo.

REFERÊNCIAS

Abell, R., Thieme, M.L., Revenga, C., Bryer, M., Kottelat, M., Bogutskaya, N., Coad, B., Mandrak, N., Balderas, S.C., Bussing, W., Stiassny, M.L.J., Skelton, P., Allen, G.R., Unmack, P., Naseka, A., Ng, R., Sindorf, N., Robertson, J., Armijo, E., Higgins, J. V., Heibel, T.J., Wikramanayake, E., Olson, D., López, H.L., Reis, R.E., Lundberg, J.G., Sabaj Pérez, M.H., Petry, P. (2008): Freshwater ecoregions of the world: a new map of biogeographic units for freshwater biodiversity conservation. Bioscience **58**: 403–414.

Adams, D.C., Berns, C.M., Kozak, K.H., Wiens, J.J. (2009): Are rates of species diversification correlated with rates of morphological evolution? Proc. R. Soc. B Biol. Sci. **276**: 2729–2738.

Barrasso, D.A., Alcade, L., Martinazzo, L.B., Basso, N.G. (2012): External morphology, chondrocranium , cranial muscles , and buccopharyngeal features of tadpoles of *Pleurodema thaul* (Anura: Leiuperidae): a comparison with *P. bufonium*. Herpetologica **68**: 48–59.

Barrio-Amorós, C.L., Pascual, A.D. de, Jairo, J., Infante, E., Chacón, A. (2006): *Hyla vigilans* Solano, 1971, a second species for the genus *Scarthyla*, redescription and distribution in Venezuela and Colombia. Zootaxa **1349**: 1–18.

Bonett, R.M., Phillips, J.G., Ledbetter, N.M., Martin, S.D., Lehman, L. (2018): Rapid phenotypic evolution following shifts in life cycle complexity. Proc. R. Soc. B Biol. Sci. **285**: 20172304.

Brown, S.D.J., Collins, R.A., Boyer, S., Lefort, M.C., Malumbres-Olarte, J., Vink, C.J., Cruickshank, R.H. (2012): Spider: An R package for the analysis of species identity and evolution, with particular reference to DNA barcoding. Mol. Ecol. Resour. **12**: 562–565.

Canedo, C., Garcia, J.P. (2006): Diet of *Pipa carvalhoi* (Amphibia, Pipidae) is not Influenced by Female Parental Care. Herpetol. Rev. **37**: 95–96.

Cruz, C.A.G. (1982): Conceituação de grupos de espécies de *Phyllomedusinae* brasileiras com base em caracteres larvários (Amphibia, Anura, Hylidae). Arq. Da Univ. Fed. Rural Do Rio Janeiro **5**: 147–171.

Dayrat, B. (2005): Towards integrative taxonomy. Biol. Jounal Linn. Soc. **85**: 407–415.

Dingerkus, G., Uhler, L.D. (1977): Enzyme clearing of alcian blue stained whole small vertebrates for demonstration of cartilage. Stain Technol. **52**: 229–232.

Duellman, W.E., Sá, R.O. De (1988): A new genus and species of south american hylid frog with a highly modified tadpole. Trop. Zool. **1**: 117–136.

Duellman, W.E., Trueb, T. (1994): Biology of Amphibia. The Johns Hopkins University Press.

Dunn, E.R. (1948): American frogs of the family Pipidae. Am. Museum Novit.

Ebenman, B. (1992): Evolution in organisms that change their niches during the life cycle. Am. Nat. **139**: 990–1021.

Edwards, D.L., Knowles, L.L., Edwards, D.L. (2014): Species detection and individual assignment in species delimitation : can integrative data increase efficacy ? Proc. R. Soc. B Biol. Sci. **281**: 20132765.

Folk, R.A., Ginori, J.C., Soltis, D.E., Floden, A.J. (2018): Integrative identification of incipient lineages in Heuchera longiflora (Saxifragaceae). Bot. J. Linn. Soc. 1–19.

Fouquet, A., Gilles, A., Vences, M., Marty, C., Blanc, M., Gemmell, N.J. (2007): Underestimation of species richness in neotropical frogs revealed by mtDNA analyses. PLoS One **2**: e1109.

Frost, D.R., Grant, T., Faivovich, J., Bain, R.H., Haas, A., Haddad, C.F.B., Sá, R.O. De, Channing, A., Wilkinson, M., Donnellan, S.C., Raxworthy, C.J., Campbell, J. a., Blotto, B.L., Moler, P., Drewes, R.C., Nussbaum, R. a., Lynch, J.D., Green, D.M., Wheeler, W.C. (2006): The amphibian tree of life. Bull. Am. Museum Nat. Hist. **297**: 1–291.

Frost, Darrel R. (2019): Amphibian Species of the World: an Online Reference. Version 6.0. Electronic Database accessible at http://research.amnh.org/herpetology/amphibia/index.html. American Museum of Natural History, New York, USA.

Funk, W.C., Caminer, M., Ron, S.R. (2012): High levels of cryptic species diversity uncovered in Amazonian frogs. Proc. Biol. Sci. **279**: 1806–14.

Garda, A., Santana, D.J., São-Pedro, V. de A. (2010): Taxonomic characterization of Pseudae, geograph distribition, external morphology, and morphometry. Zootaxa **2666**: 1–28.

Garda, A.A., Cannatella, D.C. (2007): Phylogeny and biogeography of paradoxical frogs (Anura, Hylidae, Pseudae) inferred from 12S and 16S mitochondrial DNA. Mol. Phylogenet. Evol. **44**: 104–114.

Green, D.M.-, Sessions, S.K.- (1991): Amphibian cytogenetics and evolution.

Grosjean S. 2005. The choice of external morphological characters and developmental stages for tadpole-based anuran taxonomy, a case study in *Rana* (Sylvirana) nigrovittata (Blyth, 1855) (Amphibia, Anura, Ranidae). Contrib Zool. **74**:61–76.

Haas, A. (2003): Cladistics phylogeny of frogs as inferred from primarily larval characters (Amphibia : Anura). Cladistics **19**: 23–89.

Hall, T. (2011) BioEdit: An important software for molecular biology. GERF Bull Biosci **2**: 60–61.

Herrel, A., Bonneaud, C. (2012): Trade-offs between burst performance and maximal exertion capacity in a wild amphibian, *Xenopus tropicalis*. J. Exp. Biol. **215**: 3106–3111.

Howell, W. T., Black, D. A. (1980). Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia, **36**: 8, 1014-1015.

Jetz, W., Pyron, R.A. (2018): The interplay of past diversification and evolutionary isolation with present imperilment across the amphibian tree of life. Nat. Ecol. Evol. **2**: 850–858.

Katoh, K., Standley, D.M. (2013): MAFFT Multiple Sequence Alignment Software Version 7 : Improvements in Performance and Usability Article Fast Track. Mol. Biol. Evol. **30**: 772–780.

King, Max; Rofe, R. (1976): Karyotypic variation in the australian gekko *Phyllodactylus marmoratus* (Gray) (Gekkonidae: Reptilia). Chromosoma **87**: 75–87.

Kok, P.J.R., Kalamandeen, M. (2008): Introduction to the taxonomy of the amphibians of Kaieteur National Park, Guyana.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K. (2018): MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. **35**: 1547–1549.

Lande, R. (1992): A quantitative genetic theory of life history evolution. Ecology **63**: 607–615.

Lanfear, R., Calcott, B., Ho, S.Y.W. (2012): Guindon PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses Mol. Biol. Evol. **29**: 1695-1701.

Larson, P.M., Sá, R. De (1998): Chondrocranial morphology of *Leptodactylus larvae* (Leptodactylidae: Leptodactylinae): Its utility in phylogenetic reconstruction. J. Morphol. **238**: 287–305.

McDiarmid, R. W.; Altig, R. (1999): Tadpoles: the biology of anuran larvae. Chicago, University of Chicago Press.

Mezzasalma, M., Glaw, F., Odierna, G., Petraccioli, A., Guarino, F.M. (2015): Karyological analyses of *Pseudhymenochirus merlini* and *Hymenochirus boettgeri* provide new insights into the chromosome evolution in the anuran family Pipidae. Zool. Anzeiger - A J. Comp. Zool. **258**: 47–53.

Miller, M.A., Pfeiffer, W., Schwartz, T. (2010): Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Gateway computing environments workshop (GCE). Ieee, 1-8.

Moran, N.A. (1994): Adaptation and constraint in the complex life cycles of animals.

Annu. Rev. Ecol. Syst. **25**: 573–600.

Morescalchi, A. (1968): Hypotheses on the phylogeny of the Salientia, based on karyological data none. Experientia **24**: 964–966.

Morescalchi, A., Gargiulo, G., Olmo, E. (1970): Notes on the chromosomes of some Amphibia. J. Herpetol. **4**: 77–79.

Nieuwkoop PD, Faber J. (1956): Normal table of *Xenopus laevis* (Daudin). A systematical and chronological survey of the devel-opment from fertilized egg till the end of metamorphosis. Am-sterdam: North Holland. 252.

Oksamen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H. (2013): Package 'vegan'. Community Ecol. Packag. Version **2**:.

Orrico, V.G.D., Nunes, I., Mattedi, C., Fouquet, A., Lemos, A.W., Rivera-Correa, M., Lyra, M.L., Loebmann, D., Pimenta, B.V.S., Caramaschi, U., Rodrigues, M.T., Haddad, C.F.B. (2017): Integrative taxonomy supports the existence of two distinct species within Hypsiboas crepitans (Anura: Hylidae). Salamandra **53**: 99–113.

Padial, J.M., Miralles, A., la Riva, I. De, Vences, M. (2010): The integrative future of taxonomy. Front. Zool. **7**: 16.

Queiroz, K. De (1999): The general lineage concept of species, species chteria, and the process of speciation and terminological recommendations.

Queiroz, K. De (2007): Species concepts and species delimitation. Syst. Biol. **56**: 879–886.

Freudenstein, J. V., Broe, M. B., Folk, R. A., Sinn, B. T. (2017): Biodiversity and the species concept — lineages are not enough. Syst. Biol. **66**: 644–656.

Ronquist, F., Teslenko, M., Mark, P. Van Der, Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P. (2012): Mrbayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. **61**: 539–542.

Sambrook, J., Russell, D.W. (2001): Extraction, purification, and anlysis of mRNA from eukaryotic cells. Molecular cloning 7-1.

Schlick-Steiner, B.C., Steiner, F.M., Seifert, B., Stauffer, C., Christian, E., Crozier, R.H. (2010): Integrative Taxonomy: A Multisource approach to exploring biodiversity. Annu. Rev. Entomol. **55**: 421–438.

Schmid, M. (1978): Chromosome banding in amphibia. chromosoma **66**: 361–388.

Sherratt, E., Vidal-García, M., Anstis, M., Keogh, J.S. (2017): Adult frogs and tadpoles have different macroevolutionary patterns across the Australian continent. Nat. Ecol. Evol. **1**: 1385–1391.

Sites, J.W., Marshall, J.C. (2004): Operational criteria for delimiting species. Annu. Rev. Ecol. Evol. Syst. **35**: 199–227.

Sokol, O.M. (1977): The free swimming *Pipa* larvae, with a review of pipid larvae and pipid phylogeny (Anura : Pipidae). J. Morphol. **154**: 357–425.

Stamatakis, A. (2014): RAxML version 8 : a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics **30**: 1312–1313.

Stuart, B.L., Inger, R.F., Voris, H.K. (2006): High level of cryptic species diversity revealed by sympatric lineages of southeast Asian forest frogs. Biology (Basel). **1887**: 470–474.

Suarez-Mayorga, Á.M., Lynch, J.D. (1999): Redescription of the tadpole of *Hyla vigilans* (Anura : Hylidae) and notes about possible taxonomic relationships. Caribb. J. Sci. **37**: 116–119.

Sukumaran, J., Knowles, L.L. (2017): Multispecies coalescent delimits structure , not species. PNAS **114**: 1607–1612.

Thomaz, A.T., Knowles, L.L. (2018): Flowing into the unknown: inferred paleodrainages for studying the ichthyofauna of Brazilian coastal rivers. Neotrop. Ichthyol. **16**: 1–13.

Thomaz, A.T., Malabarba, L.R., Knowles, L.L. (2017): Genomic signatures of paleodrainages in a freshwater fish along the southeastern coast of Brazil: Genetic structure reflects past riverine properties. Heredity (Edinb). **119**: 287–294.

Trueb, L., Cannatella, D.C. (1986): Systematics, Morphology, and Phylogeny of Genus Pipa (Anura: Pipidae). Herpetologica **42**: 412–449.

Trueb, L., Massemin, D. (2001): The osteology and relationships of *Pipa aspera* (Amphibia: Anura: Pipidae), with notes on its natural history in French Guiana. Amphibia-Reptilia **22**: 33–54.

Vacher, J., Kok, P.J.R., Rodrigues, M.T., Dias, J., Lorenzini, A., Martinez, Q., Fallet, M., Courtois, E.A., Blanc, M., Gaucher, P., Dewynter, M., Jairam, R., Ouboter, P., Thébaud, C., Fouquet, A. (2017): Cryptic diversity in Amazonian frogs: Integrative taxonomy of the genus *Anomaloglossus* (Amphibia: Anura: Aromobatidae) reveals a unique case of diversification within the Guiana Shield. Mol. Phylogenet. Evol. **112**: 158–173.

Walker, M., Lyra, M.L., Haddad, C.F.B. (2018): Molecular phylogenetics and evolution phylogenetic relationships and cryptic species diversity in the brazilian egg-brooding tree frog , genus Fritziana Mello-Leitão 1937 (Anura : Hemiphractidae). Mol. Phylogenet. Evol. **123**: 59–72.

Younger, J.L., Strozier, L., Maddox, J.D., Nyári, Á.S., Bon, M.T., Raherilalao, M.J., Goodman, S.M., Reddy, S. (2018): Molecular phylogenetics and evolution hidden diversity of forest birds in Madagascar revealed using integrative taxonomy. Mol.

Phylogenet. Evol. **124**: 16–26.

Zhang, J., Kapli, P., Pavlidis, P., Stamatakis, A. (2013): A general species delimitation method with applications to phylogenetic placements. Bioinformatics **29**: 2869–2876.

4 CONCLUSÕES

Nossos resultados apontam para uma estrutura genética profunda em *Pipa carvalhoi*, que apresenta relação com as bacias hidrográficas.

Não encontramos variação cariotípica e morfológica nos adultos, porém existe variação na morfologia dos girinos que é concordante com nossos resultados moleculares. Essa diferença no sinal morfológico de girinos e adultos sugere o desacoplamento adaptativo entre estas duas fases da vida mediada por pressões seletivas diferentes.

Em conjunto, os resultados aqui apresentados indicam que *P. carvalhoi* apresenta três linhagens evoluindo independentemente.

5. APÊNDICES

Amphibia-Reptilia

Cryptic diversity in *Pipa carvalhoi*? An integrative approach

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Supplementary Material – tabela S1

Table S1. Tissue samples of *Pipa carvalhoi* included in this study. Acronyms represent: AAGARDA = Adrian Garda, CHUFPB = Coleção Herpetológica da Universidade Federal da Paraíba, MTR = Miguel Trefaut Rodrigues, MUFAL = Museu de História Natural da Universidade Federal de Alagoas, UEFS = Universidade Federal de Feira de Santana. All samples were collected in Brazil.

Museum number or Field number	Species	Locality, state
AAGARDA1175	*Pipa carvalhoi*	Serra Caiada/RN
AAGARDA2691	*Pipa carvalhoi*	Crato/CE
UEFS1098	*Pipa carvalhoi*	Mauriti/CE
UEFS1099	*Pipa carvalhoi*	Mauriti/CE
UEFS1122	*Pipa carvalhoi*	Mauriti/CE
AAGARDA4	*Pipa carvalhoi*	Araçuaí/MG
AAGARDA7994	*Pipa carvalhoi*	Buíque/PE
VC53	*Pipa carvalhoi*	Buíque/PE
MTR15988	*Pipa carvalhoi*	Camacan/BA
MTR16082	*Pipa carvalhoi*	Camacan/BA
MTR5933	*Pipa carvalhoi*	Jussari/BA
CHUFPB2225	*Pipa carvalhoi*	Porto Seguro/BA
CHUFPB2297	*Pipa carvalhoi*	Porto Seguro/BA
CHUFPB3973	*Pipa carvalhoi*	Buerarema/BA
CHUFPB3974	*Pipa carvalhoi*	Buararema/BA
CHUFPB3975	*Pipa carvalhoi*	Buararema/BA

CHUFPB3976	*Pipa carvalhoi*	Buararema/BA
CHUFPB3977	*Pipa carvalhoi*	Buararema/BA
CHUFPB4248	*Pipa carvalhoi*	Una/Ilhéus/BA
CHUFPB12862	*Pipa carvalhoi*	Jequié/BA
CHUFPB16157	*Pipa carvalhoi*	Uruçuca/BA
CHUFPB16158	*Pipa carvalhoi*	Uruçuca/BA
CHUFPB16159	*Pipa carvalhoi*	Uruçuca/BA
CHUFPB16160	*Pipa carvalhoi*	Uruçuca/BA
CHUFPB 16161	*Pipa carvalhoi*	Uruçuca/BA
02_PC_ES	*Pipa carvalhoi*	Linhares/ES
03_PC_ES	*Pipa carvalhoi*	Linhares/ES
MTR12367	*Pipa carvalhoi*	Linhares/ES
MUFAL12485 (AL386)	*Pipa carvalhoi*	Maceió/AL
MUFAL12485 (AL387)	*Pipa carvalhoi*	Maceió/AL
MTR13267	*Pipa pipa*	Maruim/AM
MTR13268	*Pipa pipa*	Maruim/AM
MTR13269	*Pipa pipa*	Maruim/AM
MTR13287	*Pipa pipa*	Maruim/AM
MTR13288	*Pipa pipa*	Maruim/AM
HT907	*Pipa pipa*	Caracaraí/RR
HT908	*Pipa pipa*	Caracaraí/RR
HT940	*Pipa pipa*	Caracaraí/RR
HT989	*Pipa pipa*	Caracaraí/RR
HT1002	*Pipa pipa*	Caracaraí/RR

HT1017	*Pipa pipa*	Caracaraí/RR
HT1035	*Pipa pipa*	Caracaraí/RR
HT1037	*Pipa pipa*	Caracaraí/RR
HT1042	*Pipa pipa*	Caracaraí/RR
HT3258	*Pipa pipa*	Careiro/AM
HT4278	*Pipa pipa*	Morrinho/PA
HT324	*Pipa arrabali*	Juruá/AM

Supplementary Material – table S2

Table S2. Individuals of *Pipa carvalhoi* included in the cytogenetic analyses. Museum number or Field number, locality, state, geographic coordinates, collection date. All localities are in Brazil.

Field Number	Locality	State	Geographic Coordinates	Collection Date
LABI98-103	Nova Cruz	Rio Grande do Norte	6° 27' 51"S, 35° 25' 40"O	08 December, 2017
LABI135-149	Crato	Ceará	7°21’55.80”S, 39°26’26.66”O	09 September, 2017
LABI106-124	Buíque	Pernambuco	8° 37' 23"S, 37° 09' 21"O	29 January, 2018
LABI233-237	Santa Teresa	Espírito Santo	40° 35' 25"S, 19° 55' 34"O	02 March, 2018

Supplementary Material – table S3

Table S3. Adult specimens of *Pipa carvalhoi* analyzed in this study. Museum or field numbers, locality, state. All specimens are from Brazil. Acronyms: MUFAL= Natural History Museum of Federal University of Alagoas, CHUFPB = Coleção Herpetológica da Universidade Federal da Paraíba, LABI = Laboratório de Biologia Integrativa, MBML = Museu Biológco Melo Leitão e MUFAL = Museu de história Natural da Universidade Federal de Alagoas.

Museum number or Field number	Locality	State
MUFAL11444	Maribondo	Alagoas
MUFAL11447	Maribondo	Alagoas
MUFAL11453-11455	Maribondo	Alagoas
MUFAL13657	Igaci	Alagoas
MZUFS 1362-1367	Ibicuí	Bahia
LABI125-149	Crato	Ceará
MBML307	Santa Teresa	Espírito Santo
MBML309-313,315-320, 322-323	Baixo Guandu	Espírito Santo
LABI233-237	Santa Teresa	Espírito Santo
CHUFPB2384, 2387-2388, 2391-2395, 2403, 2407, 2426, 2429, 2431, 2434, 2436-2437	Boa Vista	Paraíba
CHUFPB6216	Areia	Paraíba
CHUFPB9014	São João do Cariri	Paraíba

CHUFPB9298-9304	Araruna	Paraíba
CHUFPB9635-9636, 9641, 9643	Nascente	Pernambuco
CHUFPB9648-9849, 9654	Serra Talhada	Pernambuco
AA8001	Buíque	Pernambuco
AA8006	Buíque	Pernambuco
AA8019	Buíque	Pernambuco
AA8031	Buíque	Pernambuco
AA1174-1175	Serra Caiada	Rio Grande do Norte
LABI98-103	Nova Cruz	Rio Grande do Norte

Supplementary Material – table S4

Table S4. Tadpoles of *Pipa carvalhoi* analyzed in this study. Museum and field numbers, locality, state. Acronyms: MBML = Museu Biológco Melo Leitão, MUFAL = Museu de história Natural da Universidade Federal de Alagoas, MZFS-DAR = Museu de Zoologia da Universidade Federal de Sergipe – Divisão Anfíbios e Répteis.

Museum number or Field number	Locality	State
MUFAL 12485 (GAL01-18)	Igaci	Alagoas
MZFS-DAR379 (GBA01-15)	Ibicuí	Bahia
MBML323 (GES01-15)	Santa Teresa	Espírito Santo
GPB01-13	São José da Lagoa Tapada	Paraíba
GPE01-03	Buíque	Pernambuco

Supplementary Material – table S5

Table S5. Morfometric data of adult specimens of *Pipa carvalhoi* nested by clade. Measurements in mm. N = number of specimens analyzed. Maximum and minimum values followed by mean ± standard deviation presented in parentheses.

Clade	One (N=38)	Two (N=27)	Three (N= 14)	Indeterminate (N=27)
Snout-vent length	65.70-33.78 (54.24±9.41)	63.28-43.63 (50.51±5.16)	57.43-25.39 (42.40±11.15)	70.04-40.23 (53.75±7.38)
Head length	20.30-9.18 (16.06±3.36)	17.77-11.43 (15.50±1.54)	19.72-6.48 (11.20±3.54)	20.13-11.19 (15.66±2.14)
Head width	17.38-10.16 (13.83±2.25)	20.03-11.28 (16.19±2.07)	16.60-6.56 (12.03±3.26)	21.45-12.13 (17.16±2.30)
Lengths of the tarsus	19.20-7.29 (13.81±2.67)	15.04-10.10 (12.76±1.15)	14.58-6.14 (10.25±2.47)	15.54-10.61 (13.14±1.34)
Lengths of the thigh	30.32-13.60 (23.81±4.41)	27.50-17.44 (22.19±2.51)	25.44-10.55 (17.24±4.58)	30.88-18.23 (23.79±3.05)
Lengths of the tibia	27.49-13.75 (22.65±3.94)	23.90-18.11 (20.71±1.80)	23.80-10.07 (17.24±4.42)	27.53-16.15 (22.52±2.79)
Lengths of the foot	27.89-12.93 (21.52±3.90)	25.16-16.51 (21.05±2.66)	24.06-9.75 (17.93±4.53)	30.87-18.08 (23.06±2.93)
Lengths of the arm	11.89-6.38 (9.44±1.55)	9.68-6.14 (8.05±1.07)	10.94-4.41 (7.72±2.18)	11.72-6.52 (8.71±1.24)
Lengths of the forearm	14.80-6.00 (11.76±2.60)	13.40-8.30 (10.36±1.16)	8.43-4.23 (6.56±1.44)	13.39-7.94 (10.97±1.47)
Snout width	4.49-2.07 (3.51±0.60)	4.88-2.60 (3.81±0.57)	4.09-1.63 (3.06±0.75)	4.81-2.65 (3.72±0.50)
Eye diameter	3.61-1.87 (2.97±0.39)	3.33-2.25 (2.76±0.35)	2.91-1.90 (2.32±0.36)	3.97-2.54 (3.05±0.34)
Interorbital distance	10.20-4.89 (7.99±1.38)	8.15-5.98 (7.14±0.57)	8.23-4.20 (6.18±1.40)	10.79-5.82 (7.76±1.16)
Lenght of the snout	9.33-3.05 (5.80±1.27)	6.12-4.05 (4.89±0.50)	5.85-2.95 (4.15±0.86)	7.34-4.20 (5.51±0.86)
Head width eyes level	16.50-7.67 (12.80±2.38)	17.81-6.91 (11.74±2.26)	11.95-5.73 (8.76±2.11)	14.68-8.20 (11.83±11.56)

width of the body under the arms	27.87-12.92 (21.53±3.98)	25.57-16.80 (20.31±2.80)	24.67-9.09 (16.71±4.94)	32.48-16.29 (21.90±3.55)
width of the body under inguinal region	27.72-12.03 (18.64±3.38)	26.71-10.73 (18.22±3.88)	23.55-9.78 (17.00±4.85)	28.14-14.04 (20.43±3.77)
distance of the arms to the legs	28.52-12.60 (21.05±5.15)	25.26-12.50 (18.02±3.35)	25.08-7.74 (17.27±5.49	28.07-13.97 (19.25±3.56)

Supplementary Material – table S6

Table S6. Morphometry of *Pipa carvalhoi* tadpoles grouped by clade and stage of development, respectively. Stage of development following Nieuwkoop and Faber (1956). Measurements in mm. N = number of specimens analyzed. Maximum and minimum values followed by mean ± standard deviation presented in parentheses.

Clade	Two								Three			
Stage of development	51 (N=1)	52 (N=4)	53 (N=3)	54 (N=7)	55 (N=)	56 (N=6)	57 (N=4)	58 (N=7)	52 (N=1)	54 (N=4)	55 (N=8)	56 (N=2)
Total length	24.66	34.06-28.07(30.75 ±2.54)	33.84-29.33(31.29 ±2.31)	33.9-29.65(31.63 ±1.74)	43.88-27.91(35.19±8.07)	49.35-30.93(36.42±7.03)	50.25-34.2(42.68 ±7.12)	51.86-44.36(47.32±2.45)	24.65	32.58-27.08 (28.99 ±2.55)	39.80-21.61 (32.96± 5.20)	41.70-36.87 (39.29±3.42)
total body length	9.02	12.49-10.03(11.20 ±0.93)	10.80-9.78(10.42 ±0.56)	12.25-10.4(11.32 ±0.70)	14.82-9.66(12.76±2.73)	15.49-10.73(12.68±1.82)	16.86-10.48(14.73±2.87)	18.35-16.01(16.80±0.74)	7.69	9.30-8.35 (8.72± 0.41)	10.53-8.96 (9.97±0.57)	11.97-11.30 (11.64±0.47)
Stomach length	4.15	5.5-4.29(4.96±	4.98-4.25(4.72±	7.47-4.13(5.68±	6.65-4.09(5.37	7.25-4.70(5.84	9.29-4.79(7.49±	11.07-7.40(9.19	4.19	4.89-4.05 (4.54±	5.29-4.33 (4.89±0.	5.54-5.11 (5.33±0.30

		0.49)	0.41)	1.09)	±1.28)	±1.13)	1.94)	±1.18)		0.35)	33)	)
Tail length	18.95	27.61-22.13(23.95±2.16)	27.1-22.04(24.89±2.59)	28.17-21.38(24.97±2.32)	36.92-21.8(29.12±7.57)	41.98-24.91(30.04±6.87)	42.26-27.82(35.84±6.88)	41.40-36.68(38.77±1.84)	20.40	27.12-21.58 (23.54±2.49)	30.63-27.40 (29.09±1.33)	33.72-30.73 (32.23±2.11
body width at eye level	5.76	7.67-6.54(7.09±0.51)	7.8-5.71(6.937±1.09)	7.64-6.07(6.74±0.52)	10.40-6.74(8.03±2.04)	10.86-6.61(7.98±1.47)	10.86-6.89(8.40±1.71)	8.80-7.93(8.38±0.32)	4.62	5.69-5.04 (5.43±0.31)	6.85-5.74 (6.28±0.33)	6.94-6.88 (6.91±0.04)
body width at nostril level	2.94	3.98-2.74(3.21±0.50)	3.3-2.56(3.00±0.39)	4.43-2.78(3.32±0.52)	6.74-3.63(4.773±1.71)	6.52-3.03(3.84±1.33)	7.00-2.86(4.30±1.84)	5.17-3.21(3.99±0.64)	3.31	3.86-2.85 (3.38±0.49)	3.90-3.11 (3.57±0.30)	3.96-3.95 (3.96±0.01)
maximum body height	4.28	5.78-3.68(5.05±0.84)	5.38-3.46(4.66±1.04)	6.43-4.59(5.65±0.67)	7.46-5.07(6.11±1.22)	7.19-5.53(6.39±0.80)	9.56-5.89(7.81±1.60)	10.00-7.63(9.19±0.78)	4.36	4.85-4.10 (4.51±0.32)	5.96-5.22 (5.59±0.26)	7.07-6.34 (6.71±0.52)

maximum body width	5.86	7.36-6.27(6.92±0.42)	7.70-5.32(6.65±1.21)	7.60-5.53(6.74±0.72)	10.40-6.92(8.20±1.90)	10.86-6.98(8.12±1.48)	10.82-6.95(8.53±1.64)	9.80-8.58(9.27±0.41)	4.62	5.66-5.03 (5.35±0.28)	6.85-5.64 (6.12±0.40)	6.51-6.45 (6.48±0.04)
Maximum tail height	5.10	6.5-5.1(5.90±0.56)	7.6-6.5(7.13±0.56)	8.4-6.5(7.42±0.77)	10.40-7.20(8.40±1.74)	9.50-7.40(8.28±0.72)	11.01-7.40(9.42±1.58)	11.50-9.40(10.18±0.77)	5.50	6.09-4.92 (5.46±0.51)	7.57-6.49 (7.02±0.39)	8.45-7.86 (8.16±0.42)
Caudal muscles height	3.10	4.5-3.1(3.60±0.53)	4.8-3.1(4.03±0.86)	4.5-3(3.62±0.50)	5.00-3.5(4.43±0.81)	5.40-3.60(4.20±0.64)	6.20-3.40(5.25±1.26)	6.50-5.00(6.01±0.50)	2.03	2.64-2.03 (2.28±0.26)	3.05-2.46 (2.79±0.22)	3.44-3.05 (3.25±0.27)
Caudal muscles width	2.40	3-2.7(2.84±0.11)	2.9-2.3(2.66±0.321)	3.5-2.7(3.01±0.31)	4.50-2.90(3.63±0.80)	4.40-3.10(3.483±0.47)	5.40-3.00(4.50±1.06)	5.90-4.50(5.32±0.43)	1.87	2.26-1.97 (2.04±0.15)	2.65-2.16 (2.42±0.17)	2.95-2.46 (2.70±0.35)
Width of the oral opening	4.00	5.7-4.6(4.96±0.48)	6-3.2(4.63±1.40)	5.50-4.00(4.85±0.50)	6.50-4.20(5.23±1.16)	6.90-4.50(5.23±0.88)	7.00-5.40(5.82±0.78)	6.50-5.00(5.62±0.48)	2.75	3.44-3.05 (3.22±	3.93-3.34 (3.70±0.	4.13-3.93 (4.03±0.14)

										0.17)	21)	
Extranarinal distances	2.10	2.5-2.2(2.36±0.11)	2.5-1.9(2.16±0.30)	2.50-2.10(2.211±0.14)	2.20-2.10(2.16±0.05)	2.90-1.90(2.26±0.35)	2.70-2.00(2.25±0.31)	2.70-1.90(2.32±0.28)	1.77	1.97-1.77 (1.84±0.09)	2.16-1.87 (2.04±0.10)	2.26-2.16 (2.21±0.07)
Extraorbital distance	7.20	8.3-7.3(7.94±0.43)	8.4-6.8(7.73±0.83)	9.00-6.90(8.12±0.75)	11.20-7.90(9.03±1.87)	11.80-8.10(9.00±1.38)	12.30-7.80(9.87±1.86)	10.90-9.70(10.18±0.43)	5.31	6.19-5.70 (6.02±0.23)	7.27-6.19 (6.75±0.35)	7.47-7.27 (7.37±0.14)
Eyes diameter	1.20	1.4-1.2(1.32±0.083)	1.20-0.90(1.10±0.173)	1.30-1.00(1.20±0.11)	1.60-1.201.36±0.20)	1.50-0.70(1.18±0.27)	1.70-1.20(1.40±0.21)	1.60-1.40(1.47±0.07)	1.08	1.28-1.08 (1.18±0.80)	1.38-1.18 (1.25±0.07)	1.47-1.38 (1.43±0.07)
Nostril diameter	0.60	0.70-0.70(0.70±0.00)	0.70-0.60(0.63±0.05)	0.70-0.60(0.62±0.04)	0.80-0.40(0.63±0.20)	0.80-0.60(0.73±0.08)	0.80-0.60(0.70±0.11)	1.00-0.70(0.81±0.10)	0.59	0.59-0.49 (0.57±0.05)	0.61-0.59 (0.59±0.01)	0.69-0.59 (0.64±0.07)
Distance from the	1.20	1.30-1.00(1.08±	1.20-0.80(1.03±	1.30-0.80(1.02±	1.80-1.00(1.36	1.30-1.00(1.15	1.80-1.00(1.45±	2.10-1.80(1.88	0.88	1.08-0.98	1.47-1.08	1.38-1.08 (1.23±0.21

eyes to the nostrils		0.13)	0.20)	0.17)	±0.40)	±0.10)	0.33)	±0.12)		(1.01±0.5)	(1.22±0.17)	)
Intranarial distance	0.90	1-0.9(0.96±0.05)	10-0.80(0.93±0.11)	1.00-0.60(0.85±0.15)	1.00-0.90(0.96±0.05)	1.00-0.80(0.91±0.07)	1.20-0.70(0.90±0.21)	1.20-0.80(0.97±0.12)	0.79	0.79-0.69 (0.76±0.5)	0.88-0.69 (0.82±0.07)	0.88-0.88 (0.88±0.00)
Intraorbital distance	4.90	6.50-5.20(5.92±0.49)	6.30-5.00(5.70±0.65)	6.90-5.00(6.04±0.69)	8.70-6.00(6.90±1.55)	9.20-6.00(6.81±1.20)	9.80-6.70(7.62±1.45)	7.70-7.00(7.27±0.29)	3.93	4.42-3.93 (4.25±0.23)	5.50-4.52 (4.95±0.31)	5.70-4.92 (5.31±0.56)
Width of the opening of the spiracle	1.40	1.70-1.30(1.50±0.19)	1.6-0.9(1.26±0.35)	1.80-1.00(1.42±0.26)	1.80-1.40(1.60±0.19)	1.80-1.40(1.56±0.16)	1.60-1.30(1.47±0.15)	2.00-1.70(1.81±0.12)	1.08	1.38-0.98 (1.20±0.17)	1.47-1.08 (1.29±0.13)	1.53-1.28 (1.40±0.18)
Distance from the snout to the	0.60	0.70-0.50(0.62±0.083)	0.60-0.40(0.50±0.10)	0.60-0.50(0.55±0.05)	0.90-0.40(0.60±0.26)	0.70-0.50(0.61±0.07)	0.90-0.50(0.62±0.18)	0.90-0.70(0.74±0.07)	0.39	0.49-0.49 (0.49±	0.59-0.39 (0.48±0.	0.59-0.49 (0.54±0.07)

nostril										0)	06)	
Cloacal tube length	2.00	3.2-1.7(2.46±0.55)	3.70-2.10(3.13±0.89)	4.20-1.80(3.10±0.82)	3.70-1.60(2.96±1.18)	4.80-3.50(4.15±0.45)	4.50-1.30(3.40±1.43)	4.50-3.50(3.97±0.35)	2.56	3.64-2.46 (3.10±0.51)	4.42-2.95 (3.9±0.49)	4.33-4.23 (4.28±0.07)
Dorsal fin heights	0.70	0.80-0.50(0.62±0.13)	1.00-0.60(0.80±0.19)	1.20-0.30(0.77±0.30)	1.10-0.50(0.90±0.34)	1.20-0.80(1.033±0.19)	1.30-0.90(1.02±0.18)	1.20-0.50(0.84±0.23)	1.08	0.79-0.49 (0.64±0.13)	0.88-0.69 (0.84±0.07)	0.98-0.88 (0.93±0.07)
Ventral fin heights	1.90	3.70-2.10(2.74±0.62)	3.80-1.50(2.93±1.25)	4.60-1.30(3.24±1.29)	4.50-1.40(3.40±1.73)	5.40-1.20(3.75±1.54)	5.70-2.50(3.92±1.35)	4.20-2.00(3.17±0.73)	3.34	3.83-2.95 (3.42±0.36)	4.42-3.64 (4.05±0.26)	4.62-4.52 (4.57±0.07)

Supplementary Material – table S7

Table 7. Morphometry of *Pipa carvalhoi* tadpoles from Paraíba State. This locality did not have representatives in the molecular approach and consequently it could not be assigned in a clade. Stage of development following Nieuwkoop and Faber, (1956). Measurements in mm. N = number of specimens analyzed. Maximum and minimum values followed by mean ± standard deviation presented in parentheses.

Clade	Undetermined				
Stage of development	50 (N=2)	53 (N=3)	54 (N=5)	55 (N=1)	57 (N=3)
Total length	16.56-16.06 (16.31±0.35)	30.69-10.09 (23.30±11.46)	34.18-10.79 (26.96±9.51)	37.80	41.48-37.80 (39.99±1.94)
Total body length	5.60-5.24 (5.42±0.25)	10.04-9.50 (9.70±0.29)	10.92-9.71 (10.51±0.49)	12.52	13.37-12.52 (12.90±0.43)
Stomach length	2.23-1.98 (2.10±0.17)	3.97-3.37(3.71±0.30)	4.72-4.17 (4.48±0.24)	4.67	5.21-4.67 (4.99±0.28)
Tail length	15.50-12.87(14.18±1.85)	25.66-22.34(23.82±1.68)	28.84-19.13 (24.22±3.80)	30.54	36.15-30.54 (33.14±2.82)
Body width at eye level	4.09-3.65 (3.87±0.31)	7.10-6.72 (6.87±0.20)	7.65-7.20 (7.44±0.20)	8.90	9.70-8.90 (9.25±0.40)
Body width at nostril level	2.42-2.41 (2.41±0.01)	4.81-4.13 (4.46±0.34)	6.10-4.11 (4.97±0.71)	5.70	6.36-5.60 (5.88±0.41)
Maximum body height	2.70-2.66 (2.68±0.02)	4.36-3.88 (4.17±0.25)	4.88-4.00(4.59±0.35)	6.00	6.67-5.91 (6.19±0.41)
Maximum body width	4.18-3.85 (4.01±0.23)	6.92-6.69(6.83±0.12)	7.38-4.99(6.75±1.01)	9.07	10.14-6.13 (8.44±2.07)

Maximum tail height	13.50-4.00 (8.75±6.71)	6.00-4.60 (5.43±0.73)	6.70-6.00 (6.38±0.35)	7.50	9.50-7.40 (8.13±1.18)
Caudal muscles height	2.80-1.80 (2.30±0.71)	3.10-3.00 (3.03±0.05)	3.50-3.20 (3.32±0.10)	3.80	4.00-3.80 (3.86±0.11)
Caudal muscles width	1.30-1.20 (1.25±0.07)	2.90-2.20 (2.53±0.35)	3.00-2.60 (2.80±0.15)	3.20	3.50-3.20 (3.33±0.15)
Width of the oral opening	2.20-2.00 (2.10±0.14)	4.80-4.00 (4.53±0.46)	5.40-4.70 (5.00±0.29)	5.60	5.80-5.60 (5.70±0.10)
Extranarinal distances	1.50-1.40 (1.45±0.07)	2.30-2.10 (2.20±0.09)	2.50-2.30 (2.36±0.09)	2.60	2.60-2.30 (2.46±0.15)
Extraorbital distance	4.40-4.10 (4.25±0.21)	7.80-7.30 (7.60±0.26)	8.40-7.80 (8.00±0.25)	9.60	10.40-9.10 (9.70±0.65)
Eyes diameter	0.90-0.90 (0.90±0.00)	1.30-1.20 (1.23±0.05)	1.40-1.20 (1.32±0.08)	1.40	1.60-1.40 (1.50±0.10)
Nostril diameter	0.40-0.40 (0.40±0.00)	0.70-0.60 (0.63±0.05)	0.70-0.60 (0.68±0.04)	0.80	0.80-0.80 (0.80±1.35)
Distance from the eyes to the nostrils	0.60-0.50 (0.55±0.07)	1.10-1.00 (1.06±0.05)	1.50-1.10 (1.22±0.16)	1.70	1.70-1.30 (1.53±0.20)
Intranarianl distance	0.60-0.50 (0.55±0.07)	0.90-0.80 (0.86±0.05)	1.00-0.90 (0.94±0.05)	1.00	1.00-0.90 (0.93±0.06)
Intraorbital distance	3.10-2.90 (3.00±0.14)	5.90-5.70 (5.80±0.10)	6.20-5.70 (6.02±0.20)	7.50	8.20-7.20 (7.63±0.51)
Width of the opening of the spiracle	0.90-0.80 (0.85±0.07)	1.60-1.50 (1.53±0.05)	2.40-1.60 (2.02±0.31)	2.50	2.50-2.30 (2.43±0.11)
Distance from the	0.40-0.30	0.60-0.40	0.60-0.50	0.80	0.80-0.70

snout to the nostril	(0.35±0.07)	(0.50±0.10)	(0.58±0.04)		(0.76±0.06)
Cloacal tube length	1.80-1.70 (1.75±0.07)	2.60-1.20 (1.96±0.70)	3.20-2.30 (2.72±0.40)	3.60	4.50-3.60 (4.10±0.45)
Dorsal fin heights	0.50-0.50 (0.50±0.00)	0.60-0.60 (0.60±0.00)	0.80-0.50 (0.66±0.13)	1.00	1.00-1.00 (1.00±0.00)
Ventral fin heights	2.00-1.80 (1.90±0.14)	2.50-1.30 (2.10±0.69)	2.60-2.00 (2.30.24)	2.30	3.30-2.30 (2.70±0.53)