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TATIANA FEITOSA QUIRINO

ASPECTO DA HISTÓRIA NATURAL E ANÁLISE GENÉTICA DE DUAS ESPÉCIES DA FAMÍLIA ODONTOPHRYNIDAE DO NORDESTE BRASILEIRO

FORTALEZA
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Tese apresentada ao Programa de Pós-Graduação em Sistemática, Uso e Conservação da Biodiversidade; da Universidade Federal do Ceará (UFC); como parte para obtenção do título de Doutor em Sistemática e Taxonomia. Área de pesquisa Taxonomia, Sistemática e Evolução Biológica.

Orientador: Prof. Dr. Robson Waldemar Ávila / UFC.

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BANCA EXAMINADORA

____

Prof. Dr. Robson Waldemar Ávila (Orientador)
Universidade Federal do Ceará (UFC)

____

Prof. Dr. Geraldo Jorge Barbosa Moura
Universidade Federal Rural de Pernambuco (UFRPE)

____

Prof. Dr. Samuel Cardoso Ribeiro
Universidade Federal do Cariri (UFCA)

____

Prof. Dr. Drausio Honório Morais
Universidade Federal de Uberlândia (UFU)

____

Profa. Dra. Débora Praciano de Castro
Universidade Federal do Oeste do Pará (UFOPA)

____

Profa. Dra. Juliana Araripe
Universidade Federal do Pará (UFPA)

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Nomeamos espécies, catalogamos mundos. Mas hoje o mais árduo ainda é nomear a nós mesmas como legítimas, competentes e dignas, em um espaço que por tantas vezes nos nega a voz.

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RESUMO

A região Neotropical possui a maior abundância de anfíbios anuros do mundo com 8.729 espécies descritas atualmente, destas 1.144 tem registro para o Brasil. Os estudos que envolvem ecologia de populações ajudam a entender a abundância e os processos de distribuição de espécies, suas relações com características ambientais, interações e proximidades filogenéticas. A similaridade morfológica de algumas populações animais dificulta a sua identificação, causando confusão entre a sua real distribuição, principalmente quando existe variação geográfica entre as populações. O nosso objetivo foi descrever a estrutura genética, analisar a variação morfométrica, bioacústica e os parâmetros ecológicos em populações de *Proceratophrys renalis* e *Odontophrynus carvalhoi* em áreas do Nordeste do Brasil. Até o momento, estas espécies foram registradas em brejos-de-altitude, regiões dentro do domínio das Caatingas que representam fragmentos isolados de Floresta Atlântica e Amazônia, no qual servem como refúgios para fauna e flora relictuais, o que pode caracterizar estas regiões como importantes centros de endemismo. Desta forma, este trabalho tem como objetivo principal revisar a taxonomia e a estruturação haplotipica de populações de *P. renalis* e *O. carvalhoi* presentes nos brejos-de-altitude do Ceará e ao longo da sua distribuição no Nordeste. Para tanto, sequencias do gene mitocondrial 16S rDNA foram obtidas do tecido muscular e posteriormente sequenciadas e analisadas, assim como sequenciamentos depositados no GenBank. Caracteres morfométricos e meristicos analisados através de uma amostragem de espécimes depositados em coleções herpetológicas que contenham materiais representativos do grupo. O canto dos espécimes foi obtido através de gravações durante o período reprodutivo e através de cantos disponíveis na Fonoteca Neotropical Jacques Vielliard (FNJV) da Coleção Audiovisual do Museu de Diversidade Biológica da Unicamp, que foram analisado através de espectogramas e ocilogramas. Foi observado também a morfologia e característica dos girinos. Para os parâmetros ecológicos: foram considerados a composição de helmintos e descritores parasitológicos de uma população de *O. carvalhoi* e o comportamento reprodutivo entre machos de *P. aff renalis*.

Palavras-chave: taxonomia; anuros; molecular; bioacústica.

ABSTRACT

The Neotropical region harbors the highest abundance of anuran amphibians in the world, with 8,729 species currently described, of which 1,144 are recorded for Brazil. Studies involving population ecology help to understand species abundance and distribution processes, their relationships with environmental characteristics, interactions, and phylogenetic proximities. The morphological similarity of some animal populations complicates their identification, causing confusion regarding their actual distribution, particularly when there is geographic variation among populations. Our objective was to describe the genetic structure, analyze morphometric, bioacoustic variation, and ecological parameters in populations of Proceratophrys renalis and Odontophrynus carvalhoi in areas of Northeast Brazil. So far, these species have been recorded in high-altitude swamps, regions within the Caatinga domain representing isolated fragments of Atlantic Forest and Amazonia, which serve as refuges for relict fauna and flora, potentially characterizing these regions as important centers of endemism. Thus, this work aims to primarily revise the taxonomy and haplotypic structuring of populations of P. renalis and O. carvalhoi present in high-altitude swamps in Ceará and along their distribution in the Northeast. To this end, sequences of the mitochondrial gene 16S rDNA were obtained from muscle tissue and subsequently sequenced and analyzed, as well as sequences deposited in GenBank. Morphometric and meristic characters were analyzed through a sampling of specimens deposited in herpetological collections containing representative materials of the group. Specimens' calls were obtained through recordings during the reproductive period and using calls available in the Neotropical Sound Archive Jacques Vielliard (FNJV) of the Audiovisual Collection of the Museum of Biological Diversity of Unicamp, which were analyzed through spectrograms and oscillograms. The morphology and characteristics of tadpoles were also observed. For ecological parameters, the composition of helminths and parasitological descriptors of an O. carvalhoi population, and the reproductive behavior among P. aff renalis males, were considered.

Keywords: taxonomy; anurans; molecular; bioacoustics.

SUMÁRIO

1 INTRODUÇÃO GERAL

O Brasil detém atualmente mais de 1.144 espécies de anfíbios anuros (Segalla et al., 2021). Destas, cerca de 40 ocorrem nos chamados Brejos de Altitude (Moura, 2010), que são áreas de exceção existentes na depressão sertaneja, consideradas verdadeiras "ilhas" de floresta úmida na Caatinga com formação associada principalmente a processos históricos ocorridos no Pleistoceno (Andrade-Lima, 1982). Além disso, os "Brejos de altitude" podem ser considerados um elo importante que evidencia retração e expansão das florestas atlânticas e amazônicas no passado, incluindo a presença de espécies típicas e filogeograficamente próximas a esses ambientes (Borges-Nojosa e Caramaschi, 2003; Fouquet et al., 2012). Sendo também responsáveis por abrigar uma rica e peculiar herpetofauna da região nordeste (Carvalho-e-Silva et al., 2015; Freitas et al., 2019). Estes fragmentos de florestas úmidas estão localizados a 600 metros de altitude ou em maiores elevações, que persistem em áreas montanhosas no nordeste do Brasil, abrigando várias espécies típicas de florestas tropicais, as quais são incapazes de habitar as regiões áridas da Caatinga adjacente.

As primeiras publicações sobre anfíbios da Caatinga, registraram inicialmente 48 espécies (Rodrigues, 2003), e mais recente abriga 56 espécies de anfíbios (Albuquerque et al., 2012). Porém, novas espécies típicas da Caatinga são continuamente descritas (Pombal-Jr et al., 2012; Magalhães et al., 2014), incluindo espécies da chamada "zona de exceção climática", que estão localizadas em áreas montanhosas e que abrigam relictos (espécies encontradas em certas áreas isoladas, remanescentes de fauna outrora amplamente distribuída) de Mata Atlântica e Amazônia (enclaves mésicos), que são os Brejos de altitude (Napoli et al., 2011; Teixeira-Jr et al., 2012; Roberto et al., 2014), o que elevou esse número para 98 espécies descritas atualmente, distribuídas em 12 famílias (Garda et al., 2017, 2018).

Dentre essas espécies, 20 foram consideradas endêmicas, 14 das quais restritas aos enclaves mésicos (12 na Chapada Diamantina e quatro nos brejos de altitude do Ceará). Somente quatro espécies endêmicas estiveram associadas aos ambientes de baixada caracterizados pela típica vegetação xerofítica da Caatinga. Outras 13 espécies tiveram distribuições quase que exclusivas para o bioma Caatinga, ocorrendo apenas pontualmente fora dele, em suas margens, e poderiam ser consideradas endêmicas sob critério menos rigoroso (Garda et al., 2017, 2018).

Estudos recentes sobre pesquisas moleculares revelaram os padrões e processos que diversificam a fauna de anfíbios da América do Sul (Amaro et al., 2013), mas estudos focados apenas em espécies típicas da Caatinga ainda são raros (Thomé et al., 2016; Oliveira et al., 2015; Werneck et al., 2015; São-Pedro et al., 2014). É importante ressaltar que elucidar as questões taxonômicas é parte primordial na busca por respostas às questões ecológicas e evolutivas (Bortolus, 2008; Padial et al., 2010). Neste contexto, no intuito de reconhecer as espécies, considerando-as como linhagens independentes (de Queiroz, 2007), e ao mesmo tempo inferir os processos responsáveis pela origem e manutenção da biodiversidade, o uso de abordagens integrativas é cada vez mais exigido e recomendado, uma vez que diversas fontes de caracteres podem resolver o status taxonômico e evolutivo dos organismos.

Os anfíbios anuros são utilizados como modelos para se estudar processo de adaptações que envolvem fatores genéticos e fenotípicos, que ajustam constantemente os indivíduos e as populações às condições ambientais imediatas (Stebbins, 1974). Eles fornecem exemplos sobre como os animais evoluíram de um ambiente predominantemente aquático para uma vida terrestre, embora ainda dependam da água para o desenvolvimento das larvas e para evitar dessecamentos (Heyer, 1969; 1970; 1976). As adaptações são manifestadas através de comportamentos que refletem mecanismos fisiológicos de regulação relacionados à reprodução, oviposição, cuidado parental, alimentação, períodos de atividade e utilização dos micro-habitats disponíveis (Duellman e Trueb, 1994; Pombal Jr. e Haddad, 2005).

Dessa forma, essa tese está organizada em quatro capítulos, a seguir:

CAPÍTULO 1 – Taxonomic revision of *Proceratophrys renalis* (Miranda-Ribeiro, 1920) (Anura, Odontophrynidae) with the description of a new species.

CAPÍTULO 2 – Comportamento territorial de *Proceratophrys aff. renalis* (Miranda-Ribeiro, 1920) na Serra de Maranguape, Ceará.

CAPÍTULO 3 – Helminths Infecting the Carvalho's Escuerzo Odontophrynus carvalhoi from the Brazilian State of Ceará.

CAPÍTULO 4 – Population Structure and Genetic Diversity in *Odontophrynus carvalhoi* Savage and Cei, 1965 (Anura: Odontophrynidae) from five states in the Brazilian Northeast.

Capitulo 1

Taxonomic revision of *Proceratophrys renalis* (Miranda-Ribeiro, 1920) (Anura, Odontophrynidae) with the description of a new species

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Corresponding author name and email: Tatiana F. Quirino – tata_tatifeitosa@hotmail.com

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High taxon name (i.e. taxon section in Zootaxa website) and number of new taxa described in the paper: Amphibia, one

Taxonomic revision of *Proceratophrys renalis* (Miranda-Ribeiro, 1920) (Anura, Odontophrynidae) with the description of a new species

TATIANA FEITOSA QUIRINO[1,4], IGOR JOVENTINO ROBERTO[2], ANTONIO RAFAEL LIMA RAMOS[1], RENATA PEREZ[1], ETIELLE BARROSO DE ANDRADE[3] & ROBSON WALDEMAR ÁVILA[1]

1 Núcleo Regional de Ofiologia, Centro de Ciências, Campus do Pici, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil.
2 Universidade Estadual do Ceará, Faculdade de Educação, Ciências e Letras de Iguatu, Iguatu, Ceará, Brazil.
3 Grupo de Pesquisa em Biodiversidade de Biotecnologia do Centro-Norte Piauiense, Instituto Federal de Educação, Ciência e Tecnologia do Piauí, Pedro II, Piauí, Brazil.

[4] CORRESPONDENCE*:* e-mail, tata_tatifeitosa@hotmail.com

RRH: QUIRINO *ET AL*—NEW SPECIES OF *PROCERATOPHRYS*

Abstract

Proceratophrys renalis is a frog species widely distributed in Atlantic Forest in northeastern Brazil, with additional records in ecotone áreas with Cerrado in the state of Minas Gerais. Herein, we performed a taxonomic review based on morphological, morphometric, molecular analysis, bioacoustic parameters, and tadpoles analysis along species distribution. The results revealed considerable variation among populations, and we conclude that populations north to São Francisco River have sufficient differences to be considered as new species. *Proceratophrys* sp. nov., a having a long and single palpebral appendage and rostral appendage absent is diagnosed by snout-vent length 15.46 – 68.1 mm in males and 22.8 – 61.9 mm in females, snout rounded in dorsal view, obtuse spatulate in lateral view, absence of large tubercles near the buccal commissure, symmetrical dorsal crest forming a continuous line, with a mid dorsal constriction and advertisement call with dominant frequency of 315 – 991 Hz and 24 – 47 pulses per note.

Key Words: Amphibia; Neotropical; Systematics; Taxonomy.

Introduction

The family *Odontophrynidae* Lynch, 1969 comprises 56 anuran species included in three genera (Pyron & Wiens 2011): One species of *Macrogenioglottus* Carvalho, 1946, two species of *Odontophrynus* Reinhardt and Lütken, 1862, 12 species, and *Proceratophrys* Miranda-Ribeiro, 1920, 43 species. *Proceratophrys* is the most specious genus in the family and is widely distributed in Brazil, Paraguay and Argentina (Pyron & Wiens 2011; Segalla *et al.* 2019; Mângia *et al.* 2020; Frost 2023).

Based in morphological similarity, species of *Proceratophrys* have been allocated in four phenetic groups (Prado & Pombal 2008; Ângulo & Reichle 2008; Cruz & Napoli 2010), no corroborated by molecular, with the exception of *P. rondonae* Prado and Pombal, 2008, *P. schirchi* (Miranda-Ribeiro 1937), and *P. minuta* Napoli, Cruz, Abreu and Del Grande, 2011, which are not associated to any group. Species with palpebral appendages are arranged in two groups: *Proceratophrys appendiculata* and *P. boiei* (Izecksohn *et al.* 1999; Prado & Pombal 2008). The *Proceratophrys appendiculata* complex (Izecksohn *et al.* 1999; Cruz & Napoli 2010) has a triangular rostral appendage and comprises *P. appendiculata* (Günther 1873); *P. belzebul* Dias, Amaro, Carvalho-e-Silva, and Rodrigues, 2013a; *P. gladius* Mângia, Santana, Cruz, and Feio, 2014; *P. itamari* Mângia, Santana, Cruz, and Feio, 2014; *P. izecksohni* Dias, Amaro, Carvalho-e-Silva, and Rodrigues, 2013a; *P. laticeps* Izecksohn and Peixoto, 1981; *P. mantiqueira* Mângia, Santana, Cruz, and Feio, 2014; *P. melanopogon* (Miranda-Ribeiro, 1926); *P. moehringi* Weygoldt and Peixoto, 1985; *P. phyllostomus* Izecksohn, Cruz and Peixoto, 1999; *P. pombali* Mângia, Santana, Cruz, and Feio, 2014; *P. sanctaritae* Cruz and Napoli, 2010; *P. subguttata* Izecksohn, Cruz, and Peixoto, 1999; and *P. tupinamba* Prado and Pombal, 2008. Species in *Proceratophrys boiei* complex lacks the rostral appendage and includes *P. boiei* (Wied-Neuwied 1824); *P. paviotii* Cruz, Prado, and Izecksohn, 2005; and *P. renalis* (Miranda-Ribeiro 1920).

Proceratophrys renalis was described as *Ceratophrys renalis* by Miranda-Ribeiro (1920) and later placed in the synonymy of *Proceratophrys boiei* by Bokermann (1966) with the corroboration of Izeckson and Peixoto (1981). Posteriorly, Prado and Pombal Jr. (2008) revalidated *Proceratophrys renalis*, indicating the lectotype and the type-locality as Itabuna municipality, Bahia state, Northeastern Brazil. Currently, *Proceratophrys renalis* occurs in the Atlantic Forest from southern Bahia to Paraíba state, in Cerrado areas along the Jequitinhonha basin in Minas Gerais state, in addition to relictual populations in forest enclaves in Caatinga Domain in the Paraíba, Pernambuco and Ceará states (Prado & Pombal Jr. 2008; Mângia *et al.* 2012). Recently, we collected individuals from multiple populations across its distribution, which exhibit marked differences in acoustic and morphological features, particularly between populations north and south of the São Francisco River. Thus, herein we use an integrative approach to revise the status of populations known as *Proceratophrys renalis* and describe a new species.

Material and Methods

Material examined

A total of 221 specimens were analysed, 123 for the new candidate species, from populations north of the São Francisco River and 98 for morphological comparisons. This specimens are housed at the following collections: Museu de Biologia Professor Mello Leitão (MBML-Anfíbios) from Instituto Nacional da Mata Atlântica – INMA, Santa Teresa municipality, Espírito Santo state; Coleção de Anfíbios do Museu de Zoologia (ZUEC-AMP) from Universidade Estadual de Campinas – UNICAMP, Campinas municipality, São Paulo state; Coleção Herpetológica da Universidade Federal da Paraíba (CHUFPB), João Pessoa municipality, Paraíba state; Coleção Herpetológica da Universidade Federal do Pernambuco (CHUFPE) and Coleção Herpetológica da Universidade Rural do Pernambuco (CHUFRPE), Recife municipality, Pernambuco state; Coleção Herpetológica do Museu de História Natural da Universidade Federal de Alagoas (MHNUFAL), Maceió municipality, Alagoas state; Coleção Herpetológica do Museu de Zoologia da Universidade Estadual de Santa Cruz (MZUESC), Ilhéus municipality, Bahia state; Coleção de Anfíbios da Universidade Federal do Mato Grosso (UFMT-A), João Pessoa municipality, Paraíba state; Coleção Herpetológica da Universidade Regional do Cariri (URCA-H) and Coleção Herpetológica do Núcleo Regional de Ofiologia da Universidade Federal do Ceará (CHUFC-A), Fortaleza municipality, Ceará state (Appendix).

Molecular analysis

We extracted DNA from tissue samples of 13 specimens using DNeasy kit (Qiagen, Inc.), and obtained partial sequence of 16S rDNA gene (576 base pairs) by polymerase chain reaction (PCR) following protocol described in Ávila *et al.* (2018). We amplified and sequenced the 16S rRNA gene using the primers 16SAR and 16SBR developed by Palumbi *et al.* (1991), then the PCR products were purified with ExoSAP (ThermoFisher), and sent to ACTGene Molecular Analysis, Porto Alegre, Brazil.

For phylogenetic analysis, we included molecular data from 31 species, including tje outgroup, available on Genbank (Table 1). As outgroup we follow the phylogenetic hypothesis of Mângia *et al.* (2022) and include specimens of *Thoropa miliaris* (Spix 1824) and the closely related genera *Odontophrynus* and *Macrogenioglotus* (Table 1). Vouchers of moleculares of Proceratophrys renalis will be deposited in Genbank, under acceptance.

All sequences (N = 58) were visualised and aligned in Geneious R9 (Biomatters) with MAFFT Multiple Alignment plug-in (version 1.3, Biomatters Ltd, Katoh 2013), following the algorithm G-INS-I with standard definitions (gap opening = 1.53, gap extension = 0.123). Bayesian inference analysis was performed using MrBayes 3.2 (Ronquist *et al.* 2012) with the GTR+I+G model of substitution selected via Akaike information criterion as implemented in JModeltest 2.1.7 (Darriba *et al.* 2012). The analysis was performed with 100 million Markov Chain Monte Carlo (MCMC), sampled every 1000 steps and the first 10% of steps were discarded as burn-in. The stationary of the posterior distributions for all model parameters, including medians and 95% Highest Posterior Density intervals (HPD) of the nodes was checked on Tracer v1.7 software (Rambaut *et al.* 2018). For observation and edition of consensus tree, we used the program Figtree v.1.4.3 (Rambaut 2016). Uncorrected *p*-distance was calculated in MEGA 7 (Tamura *et al.* 2013) with 1000 bootstrap.

Table 1. - Accession numbers in GenBank for 16SrDNA sequences used in the phylogenetic analysis.

Species	Museum voucher	Locality	GenBank No.	Reference
Proceratophrys sp. nov.	TEC8173 (URCA-H15709)	Serra de Maranguape, Maranguape, CE		Present study
Proceratophrys sp. nov.	TEC8171(CHUFCA9957)	Serra de Maranguape, Maranguape, CE		Present study
Proceratophrys sp. nov.	TEC8172(CHUFCA9958)	Serra de Maranguape, Maranguape, CE		Present study
Proceratophrys sp. nov.	CHUFCA9956	Serra de Maranguape, Maranguape, CE		Present study
Proceratophrys sp. nov.	URCA-G184(URCA-H4093)	REBIO Pedra Talhada, Quebrangulo, AL		Present study
Proceratophrys sp. nov.	TEC8143(CHUFCA9674)	Refúgio de Vida Silvestre Mata do Siriji, São Vicente Ferrer, PE		Present study
Proceratophrys sp. nov.	TEC8142(CHUFCA9671)	Refúgio de Vida Silvestre Mata do Siriji, São Vicente Ferrer, PE		Present study
Proceratophrys sp. nov.	URCA-G196(URCA-H4138)	RPPN Pedra D'antas, Lagoa dos Gatos, PE		Present study
Proceratophrys sp. nov.	ZUFRJ8665	Brejo dos Cavalos, Caruaru, PE	JN814584	Amaro *et al*. 2011
Proceratophrys sp. nov.	ZUFRJ8682	Brejo da Madre de Deus, PE	FJ685700	Amaro *et al.* 2009
Proceratophrys appendiculata	MNRJ53936	Teresópolis, RJ	KF214151	Dias *et al.* 2013
Proceratophrys appendiculata	MNRJ37291	Petrópolis, RJ	KF214152	Dias *et al.* 2013
Proceratophrys appendiculata	MNRJ54759	Cachoeiras de Macacu, RJ	KF214153	Dias *et al.* 2013
Proceratophrys ararype	CHUFPE156	Crato, CE	KX858852	Mangia *et al.* 2018
Proceratophrys avelinoi	DB1246	Misiones, Argentina	FJ685691	Amaro *et al.* 2009
Proceratophrys belzebul	MTR9456	Barra do Una, São Sebastião, SP	KF214154	Dias *et al.* 2013
Proceratophrys belzebul	CFBH8062	São Luís do Paraitinga, SP	KF214155	Dias *et al.* 2013
Proceratophrys belzebul	28	Picinguaba, Ubatuba, SP	KF214156	Dias *et al.* 2013
Proceratophrys bigibbosa	DB2313	Misiones, Argentina	FJ685692	Amaro *et al.* 2009
Proceratophrys boiei	AF1587	São Paulo, São Paulo	FJ685693	Amaro *et al.* 2009
Proceratophrys boiei	AF2139	Parque Nacional do Caparão, Santa Marta, Ibitirama, ES	JN814629	Amaro *et al.* 2011
Proceratophrys boiei	CBFH6714	Camanducaia, MG	JN814638	Amaro *et al.* 2011
Proceratophrys boiei	CH2325	Cristina, MG	JN814645	Amaro *et al.* 2011
Proceratophrys boiei	CFBH10318	Treviso, SC	JN814649	Amaro *et al.* 2011
Proceratophrys boiei	CFBHT13078	Parque Estadual dos três picos, Nova Friburgo, RJ	KU495464	Lyra *et al.* 2017
Proceratophrys boiei	CFBHT12631	Trilha da Pedra do Sino, Teresópolis, RJ	KU495465	Lyra *et al.* 2017
Proceratophrys brauni	CFBHT11463	Campos Novos, SC	KU495472	Lyra *et al.* 2017
Proceratophrys concavitympanum	ARI1193	Aripuanã, MT	KX858855	Mangia *et al.* 2018
Proceratophrys cristiceps	AF887	PARNA, Serra das Confusões, PI	FJ685695	Amaro *et al.* 2009
Proceratophrys cururu	FSFL580	Cardeal Mota, MG	FJ685696	Amaro *et al.* 2009
Proceratophrys cururu	MTR ALCX175P74	PARNA Serra do Cipó, Conceição de Mato Dentro, MG	KU495477	Lyra *et al.* 2017
Proceratophrys cururu	MTR ALCX125P23	PARNA Serra do Cipó, Conceição de Mato Dentro, MG	KU495478	Lyra *et al.* 2017

Proceratophrys goyana	AF1188	Petrolina de Goiás, GO	FJ685697	Lyra *et al.* 2017
Proceratophrys itamari	MZUSP135186	Campos do Jordão, SP	KF214147	Dias *et al.* 2013
Proceratophrys izecksohni	MNRJ64584	Paraty, RJ	KF214157	Dias *et al.* 2013
Proceratophrys laticeps	AF1900	Reserva Natural Vale do Rio Doce, Linhares, ES	FJ685698	Amaro *et al.* 2009
Proceratophrys mantiqueira	MZUFV10139	Araponga, MG	KF214143	Dias *et al.* 2013
Proceratophrys melanopogon	TG3295	Serra da Bocaina, São José do Barreiro, SP	KF214149	Dias *et al.* 2013
Proceratophrys minuta	MZUSP146499 (AF2308)	Miguel Calmon, BA	JX982965	Teixeira *et al.* 2012
Proceratophrys moratoi	CFBH6515	Itirapina, São Paulo	FJ685689	Amaro *et al.* 2009
Proceratophrys pombali	AF1988	Bertioga, SP	KF214144	Dias *et al.* 2013
Proceratophrys redacta	MTR22579	Morro do Chapéu, BA	JX982967	Teixeira *et al.* 2012
Proceratophrys renalis	CFBH17943	Ilhéus, BA		Present study
Proceratophrys renalis	UESC1931	Igrapiuna, BA		Present study
Proceratophrys renalis	UFBA507	Igrapiuna, BA		Present study
Proceratophrys renalis	TUESC714	Uruçuca, BA		Present study
Proceratophrys renalis	UESC1509	Itamaraju, BA		Present study
Proceratophrys salvatori	RAB3149	PARNA Chapada dos Veadeiros, Alto Paraíso de Goiás, GO	MT196399	Magalhães *et al.* 2020
Proceratophrys schirchi	371	São Lourenço, Santa Teresa, ES	FJ685701	Amaro *et al.* 2009
Proceratophrys tupinamba	MNRJ54541	Ilha Grande, RJ	KF214158	Dias *et al.* 2013
Proceratophrys tupinamba	MTR15449	Ilha Grande, RJ	KF214159	Dias *et al.* 2013
Proceratophrys velhochico	UFMG6224 (FSFL4837)	APA Boqueirão da Onça, Campo Formoso, BA	MT537176	Mangia *et al.* 2020
Macrogenioglottus alipioi	AF919	Serra do Teimoso, Jussari, BA	FJ685684	Amaro *et al.* 2009
Macrogenioglottus alipioi	AF1607	Caucaia do Alto, Cotia, SP	FJ685685	Amaro *et al.* 2009
Odontophrynus americanus	AF665	Poços de Caldas, MG	FJ685686	Amaro *et al.* 2009
Odontophrynus carvalhoi	JC1224	Mucugê, BA	FJ685687	Amaro *et al.* 2009
Odontophrynus cultripes	FSFL875	Varginha, MG	FJ685688	Amaro *et al.* 2009
Thoropa miliaris	AF1434	Santos, SP	FJ685682	Amaro *et al.* 2009

Morphometric characters

Twenty-eight morphometric measurements were taken with digital calipers (0.1 mm precision) following Heyer *et al.* (1990), Prado and Pombal Jr. (2008), Ávila *et al.* (2010), Watters *et al.* (2016). The following measurements were taken: snout-vent length (SVL), head length (HL), head width (HW), eye diameter (ED), tympanum diameter (TD), interorbital distance (IOD), eye-nostril distance (END), internarinal distance (IND), upper eyelid width (UEW), arm length (AL), forearm length (FAL), hand length (HAL), thigh length (THL), tibia length (TL), tarsal length (TAL), foot length (FL), length of fourth toe (T4L), length of third finger (F3L), outer medial metacarpal tubercle width (MMTW), outer median metacarpal tubercle length (MMTL), outer distal metacarpal tubercle width (DMTW), outer distal metacarpal tubercle length (MMTL), inner metacarpal tubercle width (IMTW), inner metacarpal tubercle length (IMTL), length of webb in largest toe (WL), distance for the interocular crest to the tip of snout (DICS), body width (BW), body length (BL) and body height (BH).

Terminology for head shape follows Heyer *et al.* (1990) and webbing formulae follow Savage & Heyer (1967) as modified by Myers & Duellman (1982). Sex was determined by examination of gular colour and presence of vocal slits in males and its absence in females.

To test differences between populations recognized by molecular data we performed a Discriminant Function Analysis (DFA) in morphometric variables. Further, a non-parametric multivariate analysis of variance with permutation (perMANOVA) using the DFA scores was performed to check if the groups were correctly classified.

Acoustic analysis

We analyse 52 calls from nine individuals of *Proceratophrys renalis*: 23 calls (5 specimens) from Maranguape municipality in the Ceará state; four calls (one specimen) from Igrapiúna municipality and three calls (one specimen) from Santa Terezinha, both in the Bahia state; 22 calls (two specimens) from São Vicente Ferrer in the Pernambuco state. Individuals from Maranguape were recorded in 21 March 2007 by Igor Joventino Roberto (two specimens, unvouchered), using a Sony TCM 5000 EV tape recorder coupled to a Sennheiser ME 66 directional microphone and 08 February 2022 by Tatiana Feitosa Quirino (three specimens, CHUFC-A 10163, 10164, and 10165) using a

TASCAM DR-40 digital recorder with a Yoga unidirectional microphone, positioned at approximately 10-150 cm from the target male, recorder set at a 44.1 kHz sample rate and 16 bits resolution. The calls from Santa Terezinha municipality are available in Fonoteca Neotropical Jacques Vielliard (FNJV 33781) and were recorded by Adrian Garda on 26 September 2013. The calls from Igrapiúna municipality were recorded at Reserva Ecológica Michelin by Iuri Ribeiro Dias on 27 March 2010 with Marantz PMD660 digital recorder.

Calls were analysed using Raven Pro 1.6.1, 64-bit version (Cornell Lab of Ornithology, 2011) with the following settings: window type = Hanning, window size = 256 samples, 3 dB filter bandwidth = 248 Hz, overlap = 89.8% (locked), DFT size = 1,024 samples (locked), Hop size (0.590 ms) and a grid spacing (spectral resolution) = 43.1 Hz. Figures were generated using Seewave v.2.0.2 package (Sueur *et al*. 2008) on the R platform (version 3.2.3; R Development Core Team 2015). Seewave settings were as follows: Hanning window, 90% overlap, and 512 points resolution (FFT). Call terminology follows Köhler *et al.* (2017), following the note centered approach.

The following temporal parameters were measured from the waveform: call duration (CD), number of pulses per call (NP), Delta Frequency (ΔF), Peak Frequency (PF), minimum frequency (MF) and maximum frequency (MFx). Pulse repetition rate were calculated per second (PR). Dominant frequency (peak of frequency of note; Hz) was obtained from spectrograms. Vouchers of call records of *Proceratophrys renalis* will be deposited in Fonoteca Neotropical Jacques Vielliard (FNJV), under acceptance.

To test the differences between the groups recognized through molecular analysis, we conducted a Principal Component Analysis (PCA) using the call variables. All statistical analysis were performed in R version 4.1.1 (R Core Team 2020) environment in R Studio interface (R Studio Team 2020).

Tadpoles analysis

Description of tadpoles is based on three individuals collected at Serra de Maranguape at stages 33-35 of Gosner (1960). Terminology and measurements follow Altig and McDiarmid (1999). The following morphometric variables were taken: total length (TL); body length (BL); body height (BH); body width (BW); tail height (TH); tail length (TLE); tail muscle height (TMH); tail muscle width (TMW); dorsal fin height (DFH); ventral fin height (VFH); Snout-nostril distance (SND); internarial distance

(IND); eye-nostril distance (END); eye diameter (ED); interocular distance (IOD); oral disc width (ODW); spiracle length (SL); anal tube length (ATL). Morphological measurements of the TL, BL, BH, BW, and TH were taken with a digital calliper (0.1 mm precision) under stereomicroscope. The remaining measurements were taken using an Opton digital camera attached to a stereomicroscope and analysed with the TCapture software.

Results

Our phylogenetic analysis results show that *Proceratophrys* is monophyletic, however the relationships between the species are not well resolved and weakly supported. The phylogenetic analysis showed that *Proceratophrys renalis* is paraphyletic, with a clade including specimens from Bahia state sister of *Proceratophrys laticeps* + a clade north to São Francisco River, with low support (Fig. 1). Our analysis showed an unresolved relationship with low levels of posterior probability (posterior probability = 26) between the clade of *P. renalis* + *P. laticeps* + *P. cururu* and other clade with *P. boiei* + *P. goyana*. Within *P. renalis*, populations distributed north to the São Francisco river Alagoas, Ceará, and Pernambuco states are more closely related to *P. laticeps* than southern populations in the Bahia state (Fig. 2). These two clades are highly supported (posterior probability = 100).

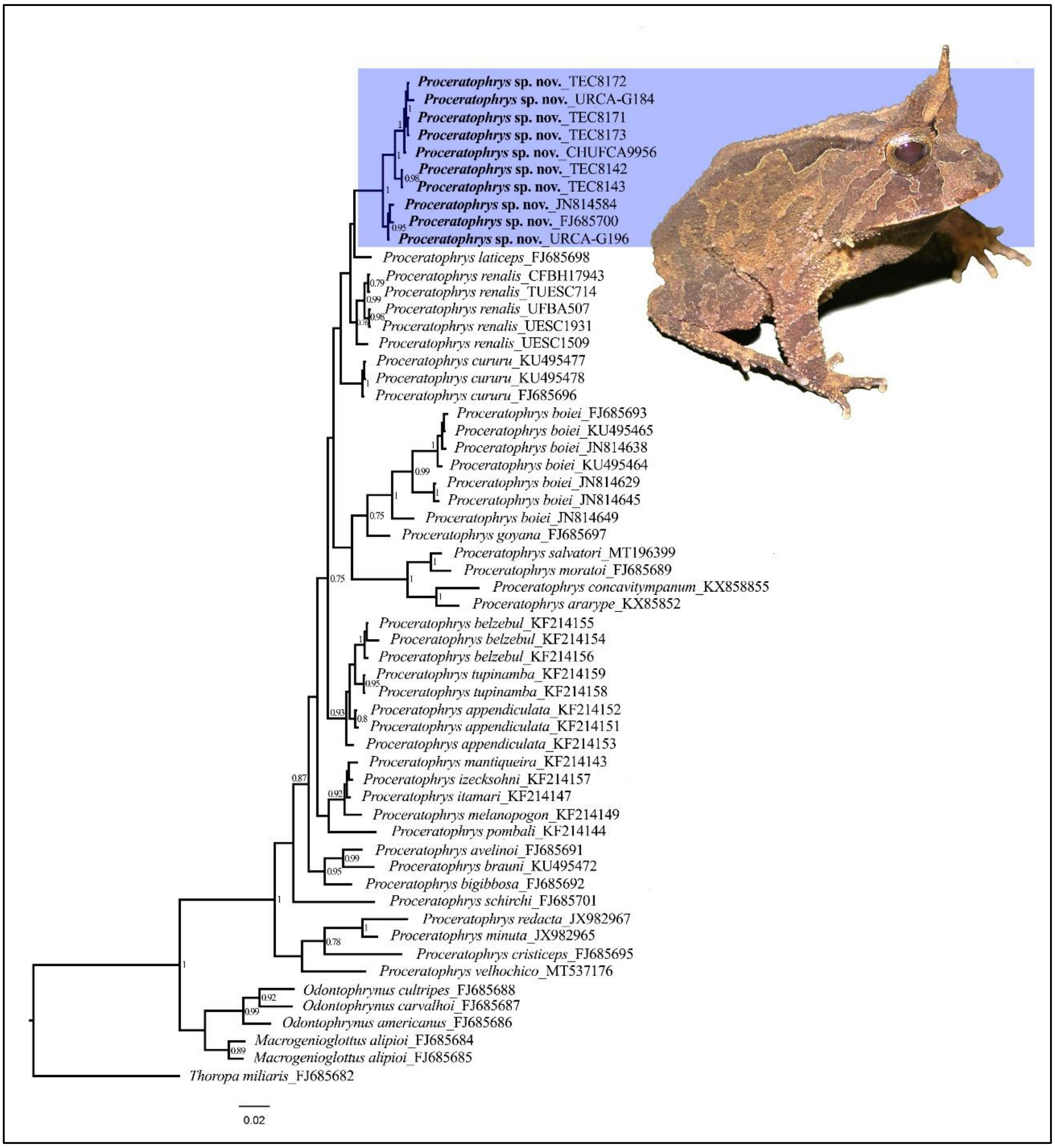

FIG. 1 - Maximum clade credibility 16S rRNA mitochondrial gene tree as inferred from a Bayesian analysis in BEAST. Scale indicates rate of base substitutions per site. The blue portion indicates the phylogenetic position of *Proceratophrys* sp. nov. Sequences provided by us are highlighted in bold.

The uncorrected *p*-distance within *Proceratophrys* varied between 3.7% to 9.2%. The *p*-distance of *Proceratophrys renalis* varied between 3.7% from *P. laticeps*, 3.8% from *P. cururu*, and 9.2% from *P. ararype* and *P. concavitympanum* (Table 2). Genetic distance between specimens of *P. renalis* varied from 0% to 4.2%, indicating that populations from Bahia state (*p*-distance 0-1.3%), including individuals from Ilhéus municipality, distant 26 Km from the municipality of Itabuna, the type locality, are

Table 2. - Uncorrected p-distance (below diagonal) and standard deviation (above diagonal) between species of *Proceratophrys*. Molecular distances are based on the 576bp fragment of 16S rDNA.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)
(1) *P.* sp.																														
(2) *P. renalis*	0.04																													
(3) *P. laticeps*	0.04	0.02																												
(4) *P. cururu*	0.04	0.03	0.03																											
(5) *P. tupinambá*	0.04	0.03	0.03	0.04																										
(6) *P. apendiculata*	0.05	0.03	0.03	0.04	0.01																									
(7) *P. goyana*	0.05	0.04	0.04	0.04	0.04	0.04																								
(8) *P. izecksohni*	0.05	0.03	0.03	0.03	0.03	0.04	0.05																							
(9) *P. itamari*	0.05	0.03	0.03	0.03	0.03	0.03	0.05	0.00																						
(10) *P. mantiqueira*	0.05	0.04	0.03	0.04	0.04	0.04	0.05	0.01	0.01																					
(11) *P. belzebul*	0.05	0.04	0.04	0.04	0.01	0.01	0.05	0.04	0.04	0.04																				
(12) *P. melanopogon*	0.06	0.04	0.04	0.04	0.04	0.04	0.06	0.01	0.01	0.02	0.05																			
(13) *P. boieie*	0.06	0.05	0.04	0.06	0.05	0.05	0.05	0.06	0.05	0.06	0.06	0.06																		
(14) *P. avelinoi*	0.06	0.05	0.05	0.05	0.04	0.05	0.05	0.04	0.04	0.05	0.05	0.05	0.07																	
(15) *P. pombali*	0.06	0.05	0.05	0.05	0.05	0.05	0.05	0.04	0.04	0.04	0.06	0.04	0.06	0.06																
(16) *P. bigibbosa*	0.06	0.04	0.04	0.04	0.04	0.04	0.04	0.03	0.04	0.04	0.05	0.04	0.06	0.04	0.06															

(17) *P. brauni*	0.06	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.05	0.08	0.03	0.06	0.04														
(18) *P. schirchi*	0.08	0.07	0.06	0.07	0.07	0.06	0.07	0.06	0.06	0.06	0.07	0.07	0.08	0.06	0.07	0.07	0.07													
(19) *P. cristiceps*	0.08	0.08	0.07	0.08	0.07	0.07	0.09	0.07	0.07	0.07	0.08	0.08	0.09	0.07	0.08	0.08	0.09	0.07												
(20) *P. minuta*	0.08	0.07	0.07	0.07	0.06	0.06	0.07	0.06	0.07	0.07	0.08	0.07	0.08	0.07	0.08	0.06	0.07	0.08	0.06											
(21) *P. velhochico*	0.09	0.08	0.07	0.08	0.07	0.08	0.07	0.07	0.07	0.08	0.09	0.08	0.08	0.07	0.08	0.07	0.07	0.07	0.08	0.07										
(22) *P. redacta*	0.09	0.08	0.07	0.08	0.07	0.07	0.08	0.07	0.07	0.07	0.08	0.08	0.09	0.08	0.09	0.06	0.08	0.09	0.08	0.04	0.07									
(23) *P. concavitympanum*	0.09	0.08	0.07	0.08	0.07	0.07	0.07	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.10	0.10	0.09	0.09	0.10								
(24) *P. ararype*	0.09	0.08	0.08	0.09	0.07	0.06	0.07	0.08	0.08	0.07	0.07	0.08	0.08	0.08	0.09	0.09	0.08	0.10	0.10	0.10	0.09	0.10	0.05							
(25) *M. alipioi*	0.11	0.10	0.09	0.09	0.10	0.10	0.09	0.09	0.09	0.09	0.10	0.10	0.09	0.09	0.10	0.09	0.10	0.09	0.10	0.11	0.09	0.11	0.11	0.12						
(26) *O. moratoi*	0.07	0.07	0.06	0.07	0.06	0.05	0.06	0.06	0.06	0.06	0.06	0.07	0.07	0.06	0.07	0.07	0.07	0.07	0.08	0.09	0.08	0.10	0.07	0.05	0.11					
(27) *O. Salvatori*	0.08	0.07	0.06	0.07	0.06	0.06	0.06	0.06	0.06	0.06	0.07	0.07	0.07	0.06	0.07	0.07	0.06	0.08	0.08	0.09	0.09	0.10	0.05	0.05	0.10	0.02				
(28) *O. americanus*	0.10	0.9	0.08	0.09	0.09	0.09	0.09	0.08	0.08	0.08	0.10	0.09	0.10	0.09	0.10	0.09	0.10	0.09	0.10	0.11	0.08	0.10	0.11	0.11	0.06	0.10	0.10			
(29) *O. carvalhoi*	0.11	0.10	0.09	0.10	0.10	0.10	0.10	0.09	0.09	0.09	0.11	0.09	0.10	0.09	0.10	0.09	0.10	0.10	0.11	0.12	0.10	0.11	0.12	0.12	0.06	0.11	0.10	0.05		
(30) *O. cultripes*	0.12	0.11	0.10	0.11	0.11	0.11	0.10	0.10	0.10	0.10	0.11	0.10	0.11	0.10	0.11	0.10	0.10	0.10	0.11	0.12	0.10	0.11	0.12	0.12	0.07	0.11	0.11	0.04	0.04	
(31) outgroup	0.17	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.15	0.17	0.16	0.17	0.15	0.15	0.16	0.15	0.14	0.14	0.16	0.15	0.16	0.15	0.16	0.13	0.16	0.15	0.14	0.13	0.14

different from populations north to São Francisco River (*p*-distance 2.9-4.2%) in Alagoas, Ceará, and Pernambuco states. Populations north to the São Francisco River varied between 0-2.1%.

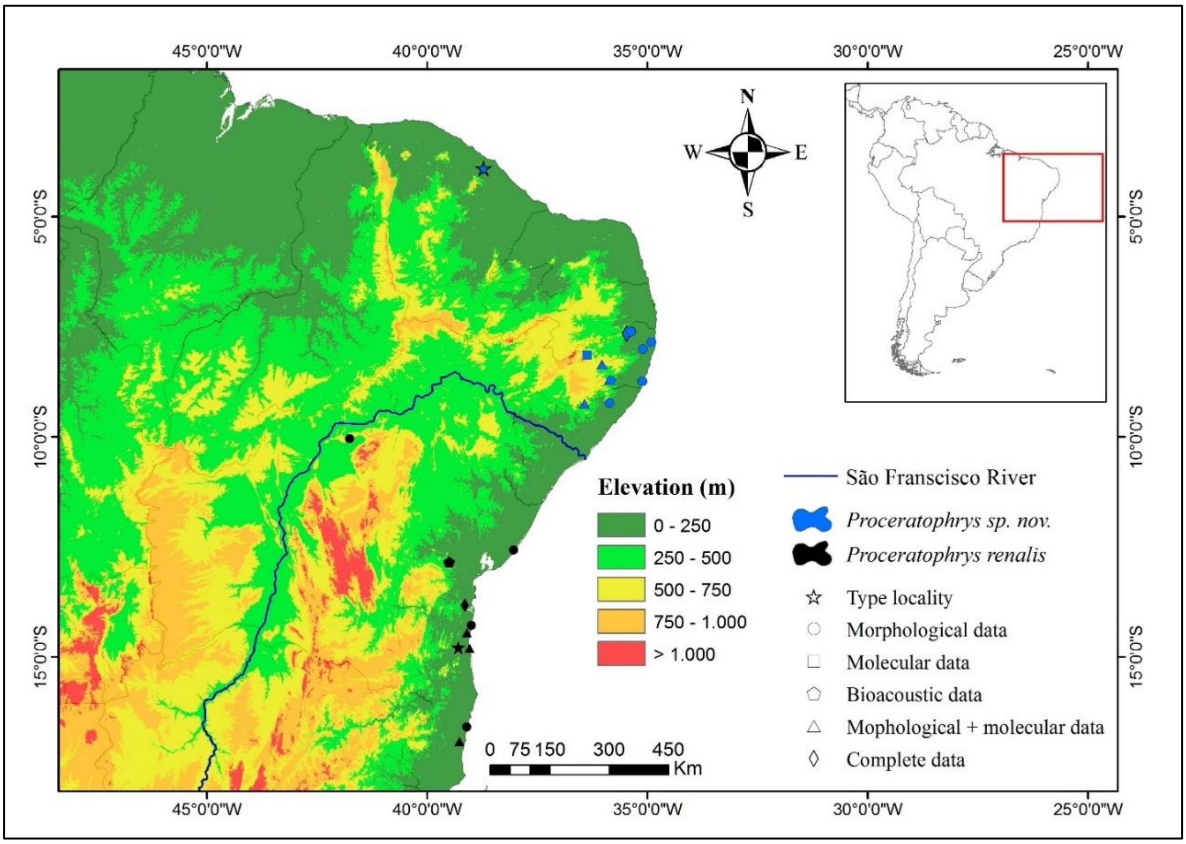

FIG. 2 - Geographic distribution of *Proceratophrys renalis* (black) and *Proceratophrys* sp. nov. (blue). The stars represent the type locality for the two species, and for *Proceratophrys* sp. nov., it includes all analyzed data (molecular, morphological, and bioacoustic).

Considering the distinction of two groups within *P. renalis* in the phylogenetic analysis, the DFA showed low overlap in morphological variables for males and no overlap in females between southern and northern populations (Fig. 3A, B). For males, variables that contributed most for group distinction were HAL, FAL and THL, and the differences were significant (F = 95.263, P<0.001). For females, differences was also significant (F = 1584.9, P<0.001) and the variables that contributed most were BW, THL and HL. The classification matrix for the northern and southern samples of *Proceratophrys renalis* indicated a high level of accuracy of identification. For southern populations, males were 97.2% and females 100% correctly identified, whereas for the southern populations males were 75% and females 100% correctly identified in 75%.

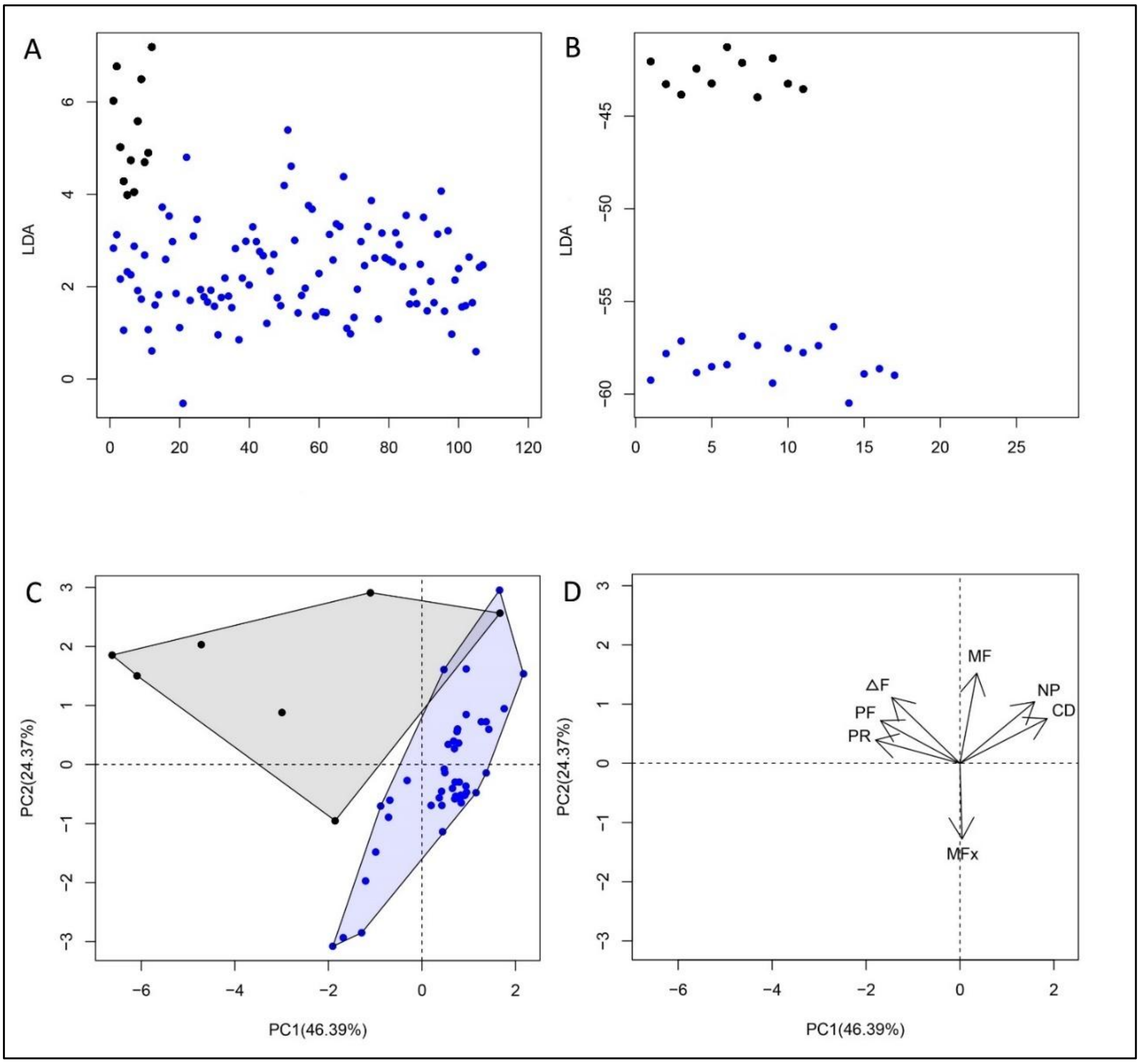

FIG. 3 - Discriminant function analysis with morphometric data of males (A) and females (B) and principal component analysis with acoustic data showing the data variation (C) and high vectors (D), obtained in the analysis between populations of *Proceratophrys* sp. nov. (blue) and *P. renalis* (black).

The PCA showed that acoustic parameters of southern and northern populations of *P. renalis* do not overlap (Fig. 3C), with the first two components accounting for about 61% of the total variation. The variables that contributed most were call duration, number of pulses and delta frequency (Fig. 3D).

Given that different independent lines of evidence (genetic, morphological and acoustic) supported the distinction of southern and northern populations of *Proceratophrys renalis*, thus we will describe a new species distributed north to São Francisco River and provide call description and comments on *P. renalis*, now restricted to Bahia state.

Proceratophrys renalis
(Fig. 4C, D, F, H, J, L)

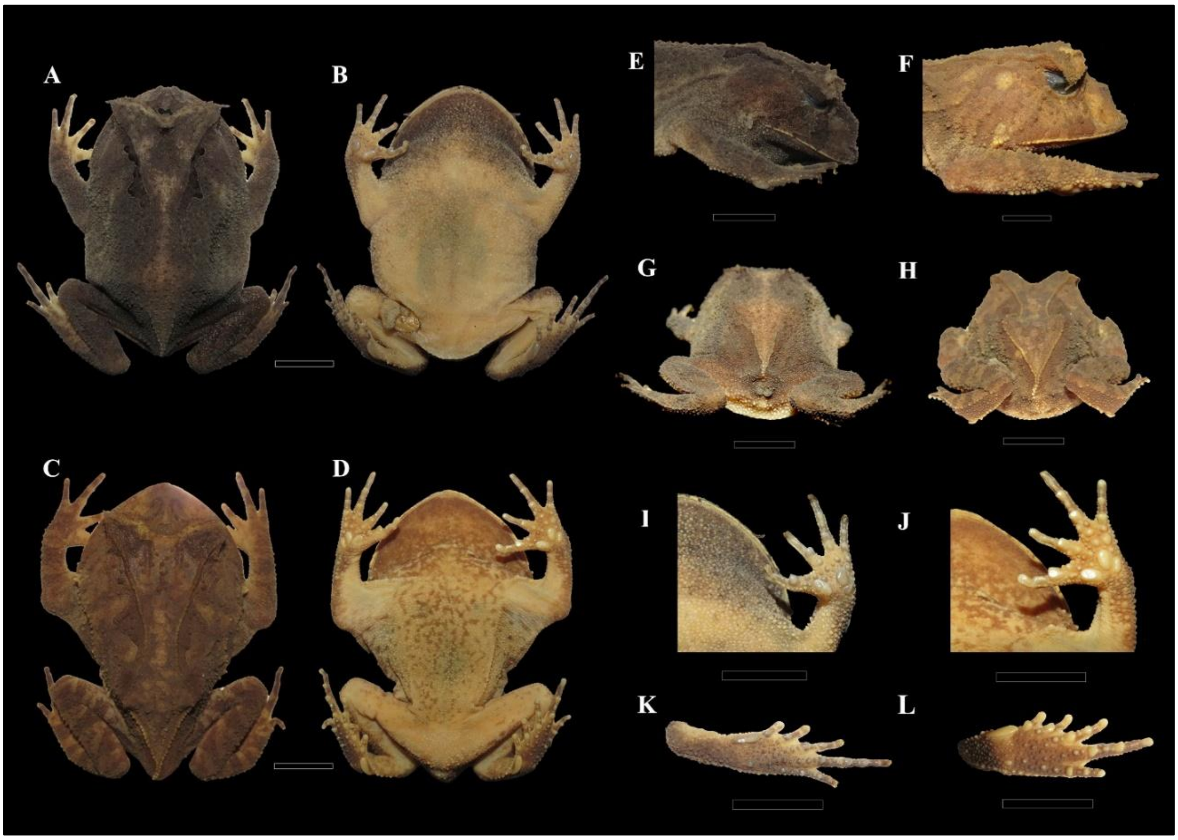

FIG. 4 - *Proceratophrys* sp. nov.: A) view of dorsal, B) ventral, E) lateral of head, G) posterior of the body, I) hand and K) foot. *Proceratophrys renalis*: C) view of dorsal, D) ventral, F) lateral of head, H) posterior of the body, J) hand and L) foot.

Prado and Pombal (2008) provided a detailed redescription of the lectotype; also, they presented comparisons between similar species and osteological descriptions.

Diagnosis (Based on Prado & Pombal 2008 and examined specimens). *Proceratophrys renalis* is diagnosed by the following combination of characters: (1) SVL ranging from

30.8–58.2 mm in males and 21–53 mm in females; (2) palpebral appendage long, single; (3) rostral appendage absent; (4) snout rounded in dorsal view, obtuse spatulate in lateral view; (5) canthal crest developed; (6) presence of large tubercle near the buccal commissure; (7) symmetrical dorsal crest forming a continuous line, with a mid dorsal constriction; (8) dominant frequency of 708.8 ± 217.3 Hz; (9) 24–42 pulses per note.

Advertisement call (Based on Santana *et al.* 2011 and examined specimens). The advertisement call of *Proceratophrys renalis* consists of a multipulsed note (Fig. 5B) with a duration of 0.41 ± 0.16 seconds (0.26–0.72 s), emitted sporadically with 31.3 ± 7.7 pulses/note (24–42 pulses/note). Low frequency of 400.0 ± 80.5 Hz (258.6–496.1 Hz), high frequency of 1108.4 ± 140.8 Hz (1006.3–1396.6 Hz), dominant frequency of 708.8 ± 217.3 Hz (513.0–1138.0 Hz), and a peak frequency of 709.8 ± 50.1 Hz (656.0–750.0 Hz).

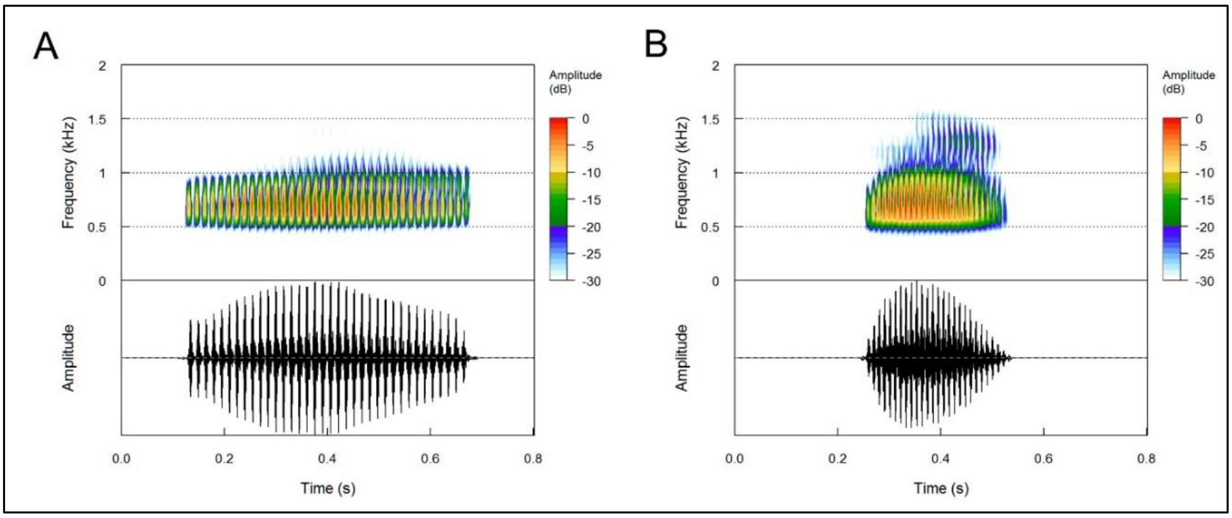

FIG. 5 - Spectrogram and oscillogram of a single call: A) *Proceratophrys* sp. nov. recorded from Maranguape municipality in the Ceará state. B) *Proceratophrys renalis* from Igrapiúna municipality in the Bahia state.

Tadpoles. Not known.

Geographic distribution. Proceratophrys renalis is distributed mostly in the Atlantic Forest in the Brazilian states of Bahia and Sergipe to transitional areas between Cerrado and Caatinga in the state of Minas Gerais (Mângia *et al.* 2012) and in Boqueirão da Onça National Park at Caatinga in Bahia state.

Proceratophrys sp. nov.

(Fig. 4A, B, E, G, I, K; 6; 7)

Proceratophrys boiei Carnaval (2002)

Proceratophrys renalis Prado and Pombal (2008) in part

Proceratophrys renalis Nascimento *et al.* (2010)

Proceratophrys renalis Santana *et al.* (2011)

Proceratophrys renalis Mângia *et al.* (2012)

Proceratophrys renalis Peixoto *et al.* (2013)

Proceratophrys renalis Dubeux *et al.* (2019)

Proceratophrys renalis Dubeux *et al.* (2020)

Holotype. CHUFC-A 10159 (adult male) collected at Maranguape municipality, Ceará state, Brazil (3º54’16.14” S and 38º42’56.80” W; 626 m), on February 08, 2022 by Tatiana Feitosa Quirino, Antonio Rafael Lima Ramos, and Atilas Rodrigues de Sousa (Fig. 6A).

FIG. 6 - Variation in coloration of *Proceratophrys* sp. nov. recorded from: A, B) Maranguape municipality in the Ceará state, C, D, E, F) São Vicente Ferrer municipality, in the Pernambuco state.

Paratypes. Seventy-one adult males and fourteen adult females: **Ceará state:** CHUFC-A 3127, December 26, 1997, CHUFC-A 3463, June 01, 2003, CHUFC-A 3567, July 20, 2003, and CHUFC-A 3549, June 19, 2003, CHUFC-A 5631, June 27, 2010, by Daniel Cassiano Lima and collaborators, CHUFC-A 3818, April 16, 2005 by Diva Maria Borges-Nojosa, Paulo Cascon, and Júlio César L. Melo, CHUFC-A 7025, May 18, 2015, CHUFC-A 7048, 8319, May 19, 2015 by Déborah Praciano, Marcela Portela, and Diva Maria Borges-Nojosa, CHUFC-A 9885-94, February 26 until March 13, 2020 by Cícero Ricardo de Oliveira, CHUFC-A 9955-59, April 19-27, 2019, CHUFC A 9960, May 01, 2019 by Kássio Castro Araújo, and Dalilange Batista-Oliveira, CHUFC-A 10160-70, and 10174-76, February 08-19, 2022 by Tatiana Feitosa Quirino and collaborators, all adult males collected at Maranguape municipality; CHUFC-A 7029, 7042, 7053, May 14-15, 2015 by Déborah Praciano, Marcela Portela, and Diva Maria Borges-Nojosa, all adult females collected at Maranguape municipality; **Pernambuco state:** CHUFC-A 9671-74, July 21, 2021 by Robson Waldemar Ávila and, Igor Joventino Roberto, all adult males collected at São Vicente Ferrer municipality; URCA-H 547, April 03, 2011, URCA-H 4138-39, August 07, 2012, URCA-H 5112, March 13, 2013, all adult males and URCA-H 4142, adult females, August 07, 2012, collected by Igor Joventino Roberto and collaborators at Lagoa dos Gatos municipality; URCA-H 6218, adult male, July 08, 2013, collected by Igor Joventino Roberto and collaborators at Jaqueira municipality; MHNUFAL 8235, adult male, February 29, 2012 collected by G. Skuk, MHNUFAL 10663, adult male, February 29, 2012, collected by Lima M. G. and collaborators, and MHNUFAL 8234, adult female, February 29, 2012 by G. Skuk, at Cabo de Santo Agostinho municipality; MHNUFAL 8253-54, adult males, April, 2002 by Freire E. M. X. and collaborators at collected at Bezerros municipality; **Alagoas state:** URCA-H 6247, adult male, July 11, 2012 and URCA-H 4093, adult female, August 13, 2012, collected by Igor Joventino Roberto and collaborators at Quebrangulo municipality; MHNUFAL 2245-46, July 27, 2002, MHNUFAL 2522, July 08, 2003, MHNUFAL 2540, May 29, 2003, all adult males collected by Lima, M. G. and collaborators, at Muricí municipality; MHNUFAL 3467-68, September 25, 2004, MHNUFAL 4282, May 21, 2005, MHNUFAL 5135, 5139, 5141, 5186, August 20, 2005, MHNUFAL 5149, 5151, August 27, 2005, MHNUFAL 5728-29, October 22, 2005, MHNUFAL 8241, September 30, 2005, collected by Nascimento F. A. C. and collaborators, MHNUFAL 4987, 5249, 5234, August 13, 2005, MHNUFAL 8236, 8238, October 02, 2005, MHNUFAL 8239-40, September 17, 2005, collected by Silva U. G. and collaborators, MHNUFAL 5028, August 13, 2005, MHNUFAL 5099, 5102, August 20, 2005, MHNUFAL 5209-10, September 10, 2005, MHNUFAL 5283, 5288, 8239-40, September 17, 2005, MHNUFAL 5639, October 29, 2005, by Sena G. A. B. and collaborators, all adult males from Maceió municipality; MHNUFAL 6437, April 11, 2006, MHNUFAL 5142, August 20, 2005, MHNUFAL 4248, May 01, 2005, MHNUFAL 3021, July 17, 2004, by Nascimento F. A. C. and collaborators, MHNUFAL 5478, June 04, 2005, by Sena G. A. B. and collaborators, MHNUFAL 4633, August 02, 2005, MHNUFAL 4962, August 06, 2005, by Silva U. G., all adult females collected at Maceió municipality; MHNUFAL 7311, 7314, adult males, September 12, 2008, collected by Silva B. V. M. and Silva F. O. L. at Passo de Camaragibe municipality; MHNUFAL 7394, adult male, February 28, 2009, collected by Silva B. V. M. and Silva F. O. L. at Rio Largo municipality; MHNUFAL 8247-48, March 22, 1996, by Freire E. M. X. and collaborators, MHNUFAL 12422, May 29, 2016 by Santos W. F. all adult males collected at Muricí municipality; MHNUFAL 11157, adult female, July, 2013 by dos Santos A., collected at Flexeira municipality.

Referred specimens:

Pernambuco state: MHNUFAL 5020, collected at Cabo de Santo Agostinho Municipality; CHUFPE A 0767-68, adult males, collected at Vicência municipality; CHUFPE A 1007, 1019, 1023-24, 1026-27, 1029-30, adult males and CHUFPE A 1022, adult female, collected at Timbauba municipality; CHUFPE A 1415-16, CHUFRPE 2013-15, adult males, collected at Igarassu municipality; CHUFPE A 1583, adult male, collected at Caruaru municipality; CHUFRPE 315, adult male, CHUFRPE 2805, adult female, collected at São Lourenço da Mata municipality; CHUFRPE 657, adult female, collected at Tamandaré municipality; CHUFRPE 2491-92, 2494, adult males, collected at Gurjaú municipality; CHUFRPE 5450, 5459, 5469, 5474-76, adult males, collected at São Vicente Ferrer municipality; **Alagoas state:** CHUFRPE 195, MHNUFAL 5540, adult males, collected at Maceió municipality.

Etymology.

Diagnosis. Proceratophrys sp. nov. is diagnosed by the following combination of characters: (1) SVL ranging from 15.46–68.1 mm in males and 22.8–61.9 mm in females;

(2) palpebral appendage long, single; (3) rostral appendage absent; (4) snout rounded in dorsal view, obtuse spatulate in lateral view; (5) canthal crest developed; (6) absence of large tubercles near the buccal commissure; (7) symmetrical dorsal crest forming a continuous line, with a mid dorsal constriction; (8) dominant frequency of 315–991 Hz; (9) 24–47 pulses per note.

Comparison. From the 43 known species of *Proceratophrys*, the new species can be readily distinguished by the presence of palpebral appendages from 25 species (palpebral appendage absent in *P. ararype*, *P. avelinoi*, *P. bagnoi*, *P. bigibbosa*, *P. branti*, *P. brauni*, *P. carranca*, *P. concavitympanum*, *P. cristiceps*, *P. cururu*, *P. dibernardoi*, *P. goyana*, *P. huntingtoni*, *P. kaingang*, *P. korekore*, *P. minuta*, *P. moratoi*, *P. palustris*, *P. redacta*, *P. rotundipalpebra*, *P. salvatori*, *P. schirchi*, *P. strussmannae*, *P. velhochico*, and *P. vielliardi*). Also, *Proceratophrys* sp. nov. differs from *P. avelinoi*, *P. bigibbosa*, *P. brauni*, *P. kaingang* and *P. palustris*, by lacking postocular swellings (present in these species).

From the species with palpebral appendages, the new species can be distinguished from *P. appendiculata*, *P. belzebul*, *P. gladius*, *P. itamari*, *P. izecksohni*, *P. laticeps*, *P. mantiqueira*, *P. melanopogon*, *P. moheringi*, *P. phyllostomus*, *P. pombali*, *P. sanctaritae*, *P. subguttata* and *P. tupinamba* by lacking a rostral appendage (present in those species).

Proceratophrys sp. nov. is more similar to *P. boiei*, *P. paviotii*, *P. renalis*, and *P. rondonae*. The new species, however, differs from *P. rondonae* by the unicuspidate palpebral appendage (multi-cuspidate in *P. rondonae*). From *P. boiei*, the new species is differentiated by frontoparietal crest less developed and tubercles less developed in buccal commissure, lower call duration with 0.34-0.68 s (0.70-0.80 s in *P. boiei*; Heyer *et al.* 1990), 24-47 pulses (30-35 in *P. boiei*; Heyer *et al.* 1990), and lower dominant frequency with 315-991 Hz (350-1350 Hz in *P. boiei*; Heyer *et al.* 1990). From *P. paviotii*, the new species differ by the large tubercle on buccal comissure absent (present), rounded snout (subelliptical in *P. paviotti*), call duration 0.34-0.68 s (0.35-0.43 s in *P. paviotti*; Cruz *et al.* 2005), 24-47 pulses (26-32 in *P. paviotti*; Cruz *et al.* 2005), and lower dominant frequency with 315-991 Hz (660-1228 Hz in *P. paviotti*; Cruz *et al.* 2005). Finally, from *P. renalis*, the most similar species, the new species differs by the large tubercle on buccal commissure absent (present), 24-47 pulses (24-42 in *P. renalis*), lower dominant frequency 315-991 Hz (513-1138 Hz in *P. renalis*).

Description of the holotype. Adult male, 47.7 mm SVL, head wider than long, length of head 33% of SVL; snout rounded in dorsal view, obtuse spatulate in lateral view; nares elliptical, slightly prominent, internarial distance 64% of eye-nostril distance; canthal crest present, marked, preocular crest present, loreal region concave; eye directed anterolaterally, small, its size equal to eye-nostril distance; palpebral appendage unique, triangular, broad at the base with spike-like projection, base of eyelid densely covered by tubercles of different sizes; tympanum indistinct, its diameter 42% of eye diameter; interocular crest markedly curved; vocal sac not expanded externally; vomerine teeth in two groups lying between choanae; tongue cordiform, free posteriorly; vocal slits large; frontoparietal crest poorly developed; small tubercles present at the angle of jaw; arm and forearm robust, dorsolateral surfaces of forearms with three to four rows of warts; lateral margins of ventral surface of forearm with a row of warts; hand robust, finger lengths IV < II < I < III; webbing absent; inner metacarpal tubercle large, elliptical; outer metacarpal tubercle divided in two parts, the internal oval and the external elliptical; scarce small rounded supernumerary tubercles; subarticular tubercles large, rounded; legs robust, thigh length longer than tibia length, twice the tarsal length, foot length almost equal than tigh length, toe lengths I < II < V <III < IV; webbing formula I 1–2 II 1–3 III 2–4 IV 4–2 V; inner metatarsal tubercle large, spatulated; outer metatarsal tubercle small, oval; numerous small rounded supernumerary tubercles; subarticular tubercles large, nearly rounded, and grooved anteriorly and posteriorly. Dorsal surface rough, with conical tubercles of different size, more concentrated on the dorso-lateral region and members; symmetrical dorsal crests of small tubercles poorly marked, joining the edge of palpebral appendage, extending to join above sacrum, presenting a constriction on mid dorsum; ventral surfaces, except hands and feet, uniformly covered by numerous small, rounded warts.

Measurement of the holotype (mm). SVL 47.7, HL 15.6, HW 24.1, ED 5.0, TD 2.1, IOD 10.7, END 5.0, IND 3.2, UEW 9.8, AL 10.5, FAL 11.6, HAL 12.8, THL 18.9, TL 16.3, TAL 9.9, FL 18.2, T4L 9.1, F3L 7.5, MMTW 1.4, MMTL 1.8, DMTW 0.9, MMTL 2.0, IMTW 1.2, IMTL 2.5, WL 1.5, DICS 10.6, BW 25.1, BL 23.9 and BH 18.1.

Colour in life of the holotype. Dorsal background light brown, with arms, flanks and legs grey. Area delimited by the ocular-dorsal ridge of warts dark brown, with two dark brown blotches on each side. Two brown bands from the eye to the upper lip. Two transverse dark-brown bars on arms and three transverse dark-brown bars on legs. Throat dark brown, belly and ventral surfaces of arms and legs light brown.

Colour in preservative. Same coloration, except brown surfaces became grey in preservative.

Advertisement call. The advertisement call of *Proceratophrys* sp. nov. was already described by Santana *et al*. (2011) under the name *P. renalis*. The following description is based on 45 calls from seven specimens recorded at Maranguape and São Vicente Ferrer municipalities. The advertisement call of *Proceratophrys* sp. nov. consists of a multipulsed note (Fig. 5A) with a duration of 0.52 ± 0.08 seconds (0.34–0.68), emitted sporadically with 34.6 ± 5.1 pulses/note (24–47). Low frequency of 394.6 ± 76.4 Hz (198.3–578.9), high frequency of 1080.2 ± 75.0 Hz (894.7–1190.1), dominant frequency of 691.7 ± 117.7 Hz (315.8–991.7), and a peak frequency of 706.0 ± 37.7 Hz (656.0–775.0).

Variation among paratypes. Males and females are almost the same size and descriptive measurements can be found in table 3. Specimens are congruent with respect to morphological characters. Weak symmetrical dorsal crest appears in 27% of the specimens, including the holotype. Dorsal coloration varies to cream to dark brown, even reddish brown in few specimens, and ventral coloration is light cream with scattered dark brown punctuations, except from one individual (CHUFRPE 2805) that have immaculate belly, one individual (CHUFPE-A 1023) which presented arms with punctuations and seven specimens which have punctuations restricted to gular region.

Table 3. - Measurements (mm) of males and females in the type series of *Proceratophrys* sp. nov. including the holotype and species of *Proceratophrys renalis*. Means ± standard deviation; ranges into parentheses. * significant difference.

Measurements	***Proceratophrys* sp. nov Males (*n* = 107)**	***Proceratophrys* sp. nov Female (*n* = 17)**	***P. renalis* Males (*n* = 6)**	***P. renalis* Female (*n* = 7)**
SVL	49.62 ± 0.89 (68.12–15.46)	45.24 ± 3.31 (61.86–22.77)	44.86 ± 3.94 (58.17–30.83)	31.98 ± 3.87 (53.15–21.04)
HL	18.74 ± 0.44 (28.21–6.54)	18.34 ± 1.4 (25.3–7.79)	18.83 ± 1.74 (24.29–11.87)	12.85 ± 1.77 (23.52–8.05)
HW	24.66 ± 0.42 (33.68–7.18)	22.11 ± 1.6 * (30.67–10.7)	21.99 ± 2.27 (28.29–13.55)	14.78 ± 2.15 (27.25–9.22)
ED	4.81 ± 0.1 (6.99–1.73)	4.61 ± 0.33 (6.35–2.23)	4.52 ± 0.31 (5.38–3.37)	3.83 ± 0.29 (5.52–2.82)
TD	1.95 ± 0.05 (3.49–0.43)	1.9 ± 0.12 (2.89–0.79)	1.8 ± 0.24 (2.6–0.89)	1.21 ± 0.24 (2.72–0.67)
IOD	9.32 ± 0.18 (11.97–1.64)	8.84 ± 0.62 * (12.47–3.72)	8.61 ± 0.83 (11.09–5.75)	6.19 ± 0.71 (10.41–4.2)
END	5.05 ± 0.09 (6.52–1.3)	4.63 ± 0.27 * (6.76–2.96)	4.5 ± 036 (5.3–3.1)	3.24 ± 0.3 (5.1–2.62)
IND	2.65 ± 0.05 (3.86–0.85)	2.36 ± 0.17 (3.26–1.05)	2.49 ± 0.27 (3.55–1.76)	1.85 ± 0.22 (3.02–1.2)
UEW	10.15 ± 0.18 (13.6–3.29)	9.35 ± 0.59 * (11.96–4.73)	8.5 ± 1.21 (12.19–4.47)	4.99 ± 1.09 (11.97–2.93)
AL	7.82 ± 0.21	6.36 ± 0.41	6.32 ± 0.33	4.78 ±0.44

	(13.0–3.45)	(10.68–3.65)	(7.9–5.5)	(6.36–3.0)
FAL	12.21 ± 0.2 (15.7–3.65)	10.96 ± 0.73 * (14.92–5.8)	10.72 ± 1.22 (14.28–6.14)	6.82 ± 1.25 (14.52–4.0)
HAL	13.25 ± 0.21 (17.46–4.3)	12.03 ± 0.78 (16.11–6.59)	11.7 ± 0.8 (14.31–8.96)	8.89 ± 0.89 (13.7–6.31)
THL	19.46 ± 0.33 (26.05–6.62)	17.56 ± 1.17 * (23.29–9.19)	16.66 ± 1.39 (21.12–11.63)	12.1 ± 1.26 (19.15–8.22)
TL	17.64 ± 0.28 (23.77–5.75)	16.05 ± 1.06 * (22.09–8.73)	15.71 ± 1.34 (19.74–10.83)	11.27 ± 1.36 (18.92–7.12)
TaL	8.4 ± 0.16 (12.4–1.98)	7.11 ± 0.39 (10.45–4.51)	7.31 ± 0.51 (8.75–5.37)	6.01 ± 0.53 (8.61–4.39)
FL	18.92 ± 0.32 (25.48–6.23)	17.63 ± 1.15 (23.83–9.69)	17.47 ±1.34 (21.29–12.66)	13.31 ± 1.27 (19.67–9.0)
T4L	9.9 ± 0.18 (14.05–3.03)	9.06 ± 0.62 (11.87–4.54)	9.2 ± 0.87 (11.47–6.14)	6.6 ± 0.7 (10.43–4.57)
F3L	7.86 ± 0.15 (10.75–2.4)	7.14 ± 0.48 (9.49–3.04)	6.93 ± 0.58 (9.06–4.95)	5.09 ± 0.61 (8.55–3.11)
MMTW	1.19 ± 0.03 (2.92–0.22)	0.99 ± 0.1 (1.62–0.26)	1.26 ± 0.17 (1.87–0.68)	0.86 ± 0.16 (1.83–0.52)
MMTL	1.98. ± 0.05 (3.1–0.32)	1.83 ± 0.19 (31.13–2.84)	1.77 ± 0.23 (2.39–0.79)	1.17 ± 0.22 (2.52–0.68)
DMTW	1.1 ± 0.03 (2.09–0.31)	0.97 ± 0.07 (1.4–0.45)	1.05 ± 0.1 (1.31–0.68)	0.83 ± 0.1 (1.32–0.52)
DMTL	1.96 ± 0.04	1.8 ± 0.15	1.65 ± 0.19	1.22 ± 0.19

	(3.08–0.66)	(2.81–0.53)	(2.19–1.02)	(2.34–0.72)
IMTW	1.26 ± 0.03	1.17 ± 0.09	1.38 ± 0.12	0.84 ± 0.12
	(1.89–0.42)	(1.65–0.55)	(1.77–0.95)	(1.54–0.48)
IMTL	2.26 ± 0.04	2.01 ± 0.16 *	2.09 ± 0.12	1.32 ± 0.2
	(3.22–0.7)	(2.85–0.5)	(2.36–1.6)	(2.42–0.67)
WL	1.44 ± 0.04	1.29 ± 0.1	1.28 ± 0.2	0.9 ± 0.14
	(2.44–0.48)	(2.01–0.69)	(2.02–0.67)	(1.7–0.55)
DICS	9.45 ± 0.17	8.83 ± 0.6 *	8.26 ± 0.96	5.23 ± 0.84
	(13.17–3.01)	(13.45–4.71)	(10.19–4.91)	(10.49–3.81)
BW	23.74 ± 0.61	21.21 ± 2.18	18.97 ± 2.63	15.88 ± 2.39
	(36.45–6.32)	(42.33–8.68)	(26.72–10.86)	(26.71–7.24)
BL	19.7 ± 0.49	17.51 ± 1.51	17.17 ± 1.98	11.94 ± 1.75
	(30.98–5.59)	(26.99–8.43)	(22.84–9.97)	(21.65–6.65)
BH	14.2 ± 0.33	12.6 ± 1.05	11.72 ± 1.15	9.49 ± 1.14
	(22.9–3.7)	(19.8–5.39)	(15.79–7.47)	(13.79–4.93)

Tadpoles. The tadpole of *Proceratophrys* sp. nov. was already described by Nascimento *et al*. (2010) under the name *P. renalis*. The following description is based on three samples from Maranguape, Ceará in stages 33-35. Body wider than high, depressed, oval in dorsal view, ovoid-elongated in lateral view; snout oval in dorsal and lateral view, positioned dorsally with nostril ovoid; eyes positioned dorsally, laterally directed; spiracle sinistral, positioned laterally, posterodorsally directed, inner wall small, free from body; intestinal tube circularly coiled, switchback point located at center of abdominal region. Vent tube dextral, with free end, at the level of the inferior margin of the ventral fin, with the dorsal membrane shorter than the ventral one; oral disk keratinized, emarginate dorsally and ventrally, surrounded by marginal papillae which are interrupted at the anterior labium; tooth row formula 2(2)/3(1); upper jaw serrated, longer than lower jaw; fin of medium height with rounded shape, slightly convex posteriorly; It has a homogeneous colour, with marbled fins.

Geographic distribution and natural history. The new species is known in Atlantic Forest north to São Francisco River, in Brazilian states of Alagoas and Pernambuco and also in the rainforest enclave (= Brejo de Altitude) at Serra de Maranguape in Ceará state. The species was recorded in three Protected Areas: the Refúgio da Vida Silvestre Matas do Siriji, a state protected area in São Vicente Ferrer municipality in Pernambuco state, Área de Proteção Ambiental Serra de Maranguape, a state protected area at Maranguape municipality, Ceará state and Reserva Biológica de Pedra Talhada (Fig. 7), a federal protected area at Quebrangulo municipality, Alagoas state. At Maranguape, males were recorded in calling activity from February-March, the peak of the rainy season, from 50 cm to streams and also floating in water. In the Atlantic forest, males were observed calling near (1m to 30 cm) streams in July, also the peak of the rainy season.

FIG. 7 - Live specimens of *Proceratophrys* sp. nov. from Reserva Biológica de Pedra Talhada, a federal protected area at Quebrangulo municipality.

Discussion

Anuran taxonomy in Neotropics has experienced a recent burst in descriptions of new species, one of the highest rates of new species description among terrestrial vertebrates (Vasconcelos *et al.* 2019). For *Proceratophrys*, 56% (24 species) of the known species have been described in the last 15 years (Frost 2023), besides the reallocation of two former *Odontophrynus* to *P. moratoi* and *P. salvatori* (Amaro *et al.* 2009). This high species description rate suggests that the richness of the genus is underestimated (Teixeira-Jr. *et al.* 2012). In fact, other studies detected possibly new species (Mângia *et al.* 2012; Santana *et al.* 2021).

In the present study, the tree topology shows a relationship between the new species, *P. laticeps* and *P. renalis*, although with low support. This relationship, along with closest proximity with *P. cururu* have been demonstrated in other studies (Santana *et al.* 2021; Mangia *et al*. 2022). Carnaval (2002) found a high level of genetic divergence in populations of *Proceratoprys* sp. nov. (as *P. boiei*) in Atlantic Forest, suggesting that fragmentation events prior to early Pleistocene may have prevented genetic flow. Several studies have pointed that the São Francisco River seems to act as a barrier for gene flow (Recoder & Rodrigues 2020; Thomé *et al*. 2021), separating populations on the north and south margins and thus promoting speciation in many animal groups, such as lizards (Werneck *et al.* 2015), mammals (Pontes *et al*. 2013), birds (Dickens *et al.* 2021), and anurans (Andrade *et al.* 2020; Oliveira *et al.* 2021).

In northeastern Brazil, several new endemic frog species restricted north to São Francisco River have been described over the last years: such as *Adelophryne nordestina*, *Dendropsophus tapacurensis*, and *Sphaenorhynchus cammaeus* (Lourenço-de-Morais *et al.* 2021; Oliveira *et al.* 2021; Roberto *et al.* 2017). Those species occurs mainly in two regions and harbours a high diversity of amphibians with several threatened species (Roberto *et al.* 2017; Dubeux *et al*. 2020; Freitas *et al.* 2019): the Pernambuco Endemism Center (PEC) and Highland marshes (=Brejos de Altitude). Besides the high diversity, these regions are considered extremely threatened due to anthropogenic activities (Ribeiro *et al.* 2009), which brings concerns regarding conservation (Tabarelli & Silva 2003).

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APPENDIX

Specimens examined

***Proceratophrys ararype*.**—BRAZIL: Ceará: Crato: URCA-H 15579-98; Missão Velha: URCA-H 15599-611.

***Proceratophrys appendiculata*.**—BRAZIL: Rio de Janeiro: Teresópolis: ZUEC-AMP 4126. São Paulo: São José dos Campos: ZUEC-AMP 24717, 24723.

***Proceratophrys belzebul*.**—BRAZIL: São Paulo: São Luiz do Paraitinga: ZUEC-AMP 20494-95, 21925.

***Proceratophrys boiei*.**—BRAZIL: Rio de Janeiro: Teresópolis: CHUFC-A 2136, Espírito Santo: Santa Teresa: MBML 8763; Marilândia: MBML 2549. Santa Catarina: São Bento do Sul: ZUEC-AMP 5280. São Paulo: São Luiz do Paraitinga: ZUEC-AMP 19289; São Francisco Xavier: ZUEC-AMP 24844.

***Proceratophrys* sp. nov..**—BRAZIL: Alagoas: Flexeiras: MUFAL 11157; Maceió: CHUFRPE 195, MUFAL 3021, 3467-68, 4248, 4282, 4633, 4962, 4987, 5020 5028, 5099, 5102, 5135, 5139, 5141-42, 5149, 5151, 5186, 5209-10, 5234, 5249, 5283, 5288, 5478, 5540, 5639, 5728-29, 6437, 8236, 8238-41; Murici: ZUEC-AMP 23428, MUFAL 2445-46, 2522, 2540, 8247-48, 8253-54, 12422; Passo de Camaragibe: MUFAL 7211, 7314; Rio Largo: MUFAL 7394. Pernambuco: Bezerros: MUFAL 8234-35; Cabo de Santo Agostinho: MUFAL 10663; Caruaru: CHUFPE A-1583. Igarassu: CHUFRPE 2013-15, CHUFPE 1415-16; Gurjaú: CHUFRPE 2491-92, 2494; São Lourenço da Mata: CHUFRPE 315, 2805; São Vicente Ferrer: CHUFRPE 5450, 5459, 5469, 5474-76; Tamandaré: CHUFRPE 657; Timbauba: CHUFPE A-1007, 1019, 1022-24, 1026-27, 1029-30; Vicência: CHUFPE A-0767-68.

***Proceratophrys concavitympanum*.**—BRAZIL: Pará: Curionópolis: URCA-H 2107, Marabá: CHUFC-A 10678. Mato Grosso: Aripuanã: UFMT 11697, 11699; Colniza: UFMT 6808. Juína: UFMT 6996, 7825.

***Proceratophrys cristiceps*.**—BRAZIL: Ceará: Aiuaba: URCA-H 7366, 7385, 7393, 7396, 7408, 7416, 7418. Baturité: CHUFC-A 3722. Crateús: URCA-H 4744. Pacajus: CHUFC-A 4562. Paracuru: URCA-H 5773-74. Pentecoste: CHUFC-A 5001, 5018-19, 5193. São Gonçalo do Amarante: URCA-H 5669, 5775, 5860. Pernambuco: Betânia: CHUFC-A 3331.Exu: URCA-H 1462-63; Ouricuri: URCA-H 2988-89. Piauí: Ilha Grande: CHUFC-A 10504-06. Rio Grande do Norte: João Câmara: URCA-H 422, 427, 483-85, 487-88, 493, 498, 501.

***Proceratophrys cururu*.**—BRAZIL: Minas Gerais: Jaboticatubas: ZUEC-AMP 1948.

***Proceratophrys gladius*.**—BRAZIL: São Paulo: São José do Barreiro: CHUFC-A 2091.

***Proceratophrys goyana*.**—BRAZIL: Tocantins: Arraias: CHUFC-A 9291-92.

***Proceratophrys huntingtoni*.**—BRAZIL: Mato Grosso: Chapada dos Guimarães: UFMT 1745-49, 11133-35.

***Proceratophrys korekore*.**—BRAZIL: Mato Grosso: Apiacás: UFMT 7906. Paranaíta: UFMT 7534, 7963, 9882, 9990, 10038, 10041, 10046, 10054, 10067, 10109;

***Proceratophrys laticeps*.**—BRAZIL: Bahia: Camacan: MBML 7204. Espírito Santo: Aracruz: MBML 11882, 11562; Santa Teresa: MBML 6412.

***Proceratophrys mantiqueira*.**—BRAZIL: Rio de Janeiro: Itatiaia: ZUEC-AMP 13352. Minas Gerais: Itamonte: ZUEC-AMP 21559.

***Proceratophrys melanopogon*.**—BRAZIL: São Paulo: São José do Barreiro: ZUEC-AMP 6807.

***Proceratophrys moratoi*.**—BRAZIL: São Paulo: Botucatu: ZUEC 7031-33.

***Proceratophrys renalis*.**—BRAZIL: Bahia: Igrapiúna: MZUESC 15969-70; Ilhéus: MZUESC 6832-33, 7219-20, 10849-50; Itacaré: ZUEC-AMP 16637-39; Itamaraju: MBML 8324; Mata de São João: MUFAL: 12692-96; Trancoso: MZUESC 12977; Vitória da Conquista: MZUESC 3428-29.

***Proceratophrys strussmannae*.**—BRAZIL: Mato Grosso: Jauru: UFMT 5859, 6659, 7869, 7872, 7874, 7876, 7878, 7880, 7882, 7885, 7886, 8319, 8320, 8377–8380.

Capitulo 2

Comportamento territorial de *Proceratophrys aff. renalis* (Miranda-Ribeiro, 1920) na Serra de Maranguape, Ceará

Comportamento territorial de *Proceratophrys aff. renalis* (Miranda-Ribeiro, 1920) na Serra de Maranguape, Ceará

Introdução

As vocalizações acústicas entre anfíbios, são sinais sonoros emitidas para transmitir informações em diferentes contextos como: competição pelo local de vocalização (Arak, 1983), atração de parceiros para a reprodução (Brenowitz e Rose, 1999; Alonso e Rodríguez, 2003) e para manter espaço entre os machos no mesmo espaço/território (Brenowitz, 1989; Bastos e Haddad, 2002; Gerhardt, 2002). Assim, o canto agonístico pode ser definido como um tipo de canto emitido como resposta a perturbações causadas como a presença de outro indivíduo da mesma espécie, para delimitar um território ou para predadores potenciais (Zank et.al 2008).

Sugere-se que o canto agonístico esteja relacionado também a cantos agressivos (Zank et.al 2008), que são cantos mais longos e apresentam um som característico (Delgado 2010). Inserido neste contexto, comportamentos territoriais [comportamentos agressivos, como comunicação visual, vocalização e combate físico (Heyer et al., 1990; Ryan, 2001; Hödl e Amézquita, 2001; Wogel et al., 2004a)] podem ser encontrados em espécies que competem por recursos (comida, abrigo, parceiros e sítios reprodutivos) dentro de uma área específica que é defendida (Maher e Lott, 1995; Pröhl, 2005). Ao excluir competidores potenciais desta área, os indivíduos territoriais garantem acesso prioritário aos recursos essenciais para sua sobrevivência e reprodução (Wells, 1977; Kaufmann, 1983; Maher e Lott, 1995).

O desfecho das interações sociais que determinam o domínio de uma dada área pode ser influenciado por diversos fatores, tais como tamanho (Howard, 1978; Davies e Halliday, 1978; Dyson e Passmore, 1992), condição física (Bastos e Haddad, 2002; Wogel et al., 2004a) e tempo de residência dos competidores (Crump, 1988; Given, 1988; Pombal et al., 1994; Wogel et al., 2004a). Parâmetros como comprimento rostro-cloacal (CRC) e massa corporal, também podem ser avaliados durante a disputa, para analisar a habilidade de luta de seu oponente e a sua probabilidade de ser bem sucedido (Marden and Waage, 1990; Vieira and Peixoto 2013). Desta forma, indivíduos mais fracos e menores podem evitar entrar em disputas com indivíduos mais fortes e maiores, reduzindo assim o gasto energético durante a disputa (Parker, 1974; Howard, 1978; Given, 1988).

Em anfíbios anuros, comportamentos territorialistas foram relatados para diversas famílias, como Aromobatidae, Dendrobatidae, Hylodidae, Leptodactylidae, Odontophrynidae (Wells, 1980a, b; Duellman e Trueb, 1986; Haddad e Giaretta, 1999; Wogel et al., 2004b; Pröhl, 2005; Narvaes e Rodrigues, 2005; Hartmann et al., 2005; Hartmann et al., 2006; Valdez e Maneyro, 2016). Neste contexto, a exclusão competitiva pode ser realizada através de comportamentos agressivos, como comunicação visual, vocalização e combate físico (Heyer et al., 1990; Ryan, 2001; Hödl e Amézquita, 2001; Wogel et al., 2004a). Tais comportamentos estão associados principalmente a cenários reprodutivos (Mathis et al., 1995), especialmente em espécies que apresentam reprodução prolongada, cujos sítios reprodutivos podem estar disponíveis durante todo o ano (Narvaes, 1997; Duellman e Trueb, 1994), No entanto, também há relatos para espécies de reprodução explosiva (Brasileiro et al., 2020).

Os sinais acústicos podem ter diferentes funções, sendo utilizadas na atração de fêmeas, nas disputas por territórios e interações agressivas entre machos, atuando diretamente na organização social (Gerhardt, 1994). Nos anuros, a vocalização é um mecanismo de comunicação eficiente e tem como função primária advertir a presença de um indivíduo a outros da mesma espécie (Duellman e Trueb, 1986), bem como corte e territorialidade (Lea, 2000). Neste último caso, alguns autores sugerem que parâmetros espectrais e temporais no canto (e.g. frequência dominante, taxa de vocalização) podem influenciar o sucesso em disputas agonísticas entre machos (Wells 1988; Márquez et. al. 2001; Reichert e Gerhardt 2011; Bastos et al. 2011; Reichert e Gerhardt 2013). No entanto, a influência de alguns parâmetros (e.g. duração da chamada e número de pulsos) no sucesso em disputas envolvendo contato físico ainda é pouco conhecida (Burmeister et al. 2002; Wells 2007; Dyson et al. 2013).

O comportamento social dos anuros geralmente está focado na competição entre machos, em função de obter a fêmea para o acasalamento. Este comportamento pode acarretar em interações acústicas, exibições de posturas e, em alguns momentos, até pode ocasionar agressões físicas entre machos (Wells, 1977; Cardoso e Haddad, 1984; Martins et al, 1988; Pombal et al, 1994; Bastos e Haddad, 1995; Haddad e Giaretta, 1999). Contudo, os combates físicos podem ser bastante onerosos para os indivíduos, e em muitas vezes não são vantajosos, pois aumenta a vulnerabilidade para um ataque de predador (Robertson, 1986; Martins et al., 1998).

Segundo Wells (1977) a territorialidade ocorre em anuros que tem a reprodução prolongada, com chegada assincrônica de machos e fêmeas no coro. Entretanto, neste estudo foi observado confronto físico dos machos, não correspondendo ao proposto por Wells (1977), vez que houve disputa por território. As brigas de anuros podem progredir por níveis de agressão e avançar desde cantos de anúncio emitidos em antifonia entre vizinhos, até a emissão de cantos agressivos. Ademais, apresentam a exibição de sinais visuais (em espécies que realizam esse comportamento) e podem culminar em brigas físicas (Wogel et al., 2004; Reichert e Gerhardt, 2011).

Sendo assim, o presente trabalho tem como objetivo analisar o comportamento territorialista entre machos da espécie *Proceratophrys aff. renalis* (Miranda-Ribeiro, 1920), descrevendo as interações e emissões acústicas realizadas pelos indivíduos durante o comportamento agonístico.

Material e Métodos

Área e espécies de estudo

As espécies de *Proceratophrys* são animais de difícil visualização em função da camuflagem e comportamentos associados (Sazima, 1978; Izecksohn e Peixoto, 1996). A reprodução da maioria das espécies é concentrada na estação úmida e quente (Weygoldt e Peixoto, 1985; Giaretta e Sazima, 1993; Kwet e Faivovich, 2001; Giaretta e Facure, 2008), aparentemente associada à ocorrência de chuvas fortes (Izecksohn e Peixoto, 1996; Kwet e Faivovich, 2001).

Sua vocalizam se dá principalmente à noite (Giaretta e Sazima, 1993; Kwet e Faivovich, 2001; Giaretta e Facure, 2008), porém, podem vocalizar durante o dia depois de chuvas fortes (Giaretta e Sazima, 1993; Izecksohn e Peixoto, 1996; Bernarde e Anjos, 1999; Kwet e Faivovich, 2001). A maioria das informações sobre comportamento reprodutivo são notas em trabalhos taxonômicos e, até o momento, nenhum trabalho detalhou a biologia reprodutiva de uma espécie de *Proceratophrys* (Martins e Giaretta, 2008).

Proceratophrys renalis (Miranda-Ribeiro, 1920), espécie descrita para a os brejos de Altitude, como a Serra de Maranguape, município de Maranguape, Ceará, distribuída ao Norte do Rio São Francisco, na Mata Atlântica da Paraíba ao sul do Estado da Bahia e no interior do Brasil, em áreas de Brejos de Altitude.

Foram realizadas gravações de quatro indivíduos, sendo uma interação entre machos (CHUFC A 10.159-161; 10.163). As observações foram realizadas no dia 8 de fevereiro de 2022 entre às 18 e 19 horas em um trecho de mata com um córrego na Serra de Maranguape (3º54'16.14" S e 38º42'56.80" W; 626 m). A Serra compreende os municípios de Maranguape e Caucaia dentro da Região Metropolitana de Fortaleza (RMF), ao norte do Ceará, a uma distância de aproximadamente 30 km da capital, possuindo uma área de 318,69 km² e seu ponto culminante é o Pico da Rajada com altitude que chegam até 920 metros (Souza e Oliveira, 2008). A temperatura média da região varia entre 26 e 28°C, o clima é Tropical Quente Úmido, e a precipitação média anual é de 1378,9 mm, com chuvas concentradas de janeiro a maio (IPEC, 2017).

Análise das emissões acústicas

A vocalização dos indivíduos foi realizada a cerca de 1 metro de distância, com um gravador portátil Digital Tascam DR40X com microfones unidirecionais acoplados, com frequência de 96 kHz e 24 bits de resolução e posteriormente salva em arquivo wave.

A gravação foi analisada usando o software Raven Pro 1.6.4 (Cornell Lab of Ornithology, 2011), com as seguintes configurações: tipo de janela = Hanning, tamanho da janela = 256 amostras, largura de banda do filtro de 3 dB = 248 Hz, sobreposição = 89,8% (bloqueado), tamanho DFT = 1.024233 amostras (bloqueado), tamanho do salto (0,590 ms) e espaçamento de grade (resolução espectral) = 43,1 Hz. As imagens sonoras foram geradas usando o pacote Seewave v.2.0.2 (Sueur et al. 2008) na plataforma R (versão 3.2.3; R Development Core Team 2015). As configurações do Seewave foram as seguintes: janela Hanning, sobreposição de 90% e resolução de 512 pontos (FFT). A terminologia da chamada segue Köhler et al. (2017), seguindo a abordagem centrada em notas.

A terminologia para parâmetros acústicos está de acordo com Köhler et al., 2017. Os seguintes parâmetros temporais foram medidos a partir da estrutura da onda: duração do canto (DC); intervalos do canto (IC); frequência mínima (FM); frequência máxima (FMx); frequência dominante (FD); pico de frequência (PF); número de pulsos (NP) e a taxa de repetição de pulso (PR). As taxas de repetição de pulsos foram calculadas por segundos.

Para visualizar possíveis diferenças entre os cantos, conduzimos uma Análise de Componentes Principais (PCA) utilizando as variáveis acústicas obtidas. Aplicamos o Teste t de Student para testar a existência de diferenças entre os cantos de anúncio e cantos agonísticos para todas as variáveis acústicas. Todas as análises estatísticas foram realizadas no ambiente R versão 4.1.1 (R Core Team 2020) na interface R Studio (R Studio Team 2020).

Consideramos confrontos físicos como interações agonísticas envolvendo contato físico direto entre rivais (Reichert e Gerhardt, 2011). E ao final da disputa agonística, os indivíduos foram capturados manualmente e medidos quanto à massa através de dinamômetros de precisão (Pesola) e as medidas morfométricas do comprimento rostro-cloacal (CRC), Largura do Corpo (LCor), Altura do Corpo (ACor) e Comprimento do Corpo (CCor) foram aferidas com paquímetro de precisão 0,01 mm.

Resultados

Os indivíduos machos foram observados em atividade de vocalização durante o período chuvoso, após dias de precipitação constante. O registro se deu no início da noite (aproximadamente 17:40 hs), no interior da mata, ao longo de um pequeno riacho temporário, pedregoso e de correnteza leve. Os indivíduos estavam em cima das pedras, próximo à água, na margem do riacho.

Analisamos uma disputa agonística entre dois machos de *P. aff renalis* (CHUFCA 10.160, CRC = 54.19 mm, m = 14.6g e 10.161, CRC = 52.39 mm, m = 10.5g) e comparamos com dois cantos de anúncio que também foram gravados na mesma noite. A disputa territorial teve duração de cerca de 3 min e 35s, iniciando com um canto de anúncio após a percepção de outro indivíduo que estava em silêncio. Os indivíduos se aproximaram, havendo a intensificação do canto do macho (dominante), e posteriormente iniciou-se um confronto físico. O macho dominante conseguiu derrubar e virar o outro de barriga para cima, ficando por cima do mesmo (Fig. 1). Esse confronto visual foi acompanhado de sinais acústicos emitidos por ambos.

O canto gravado foi avaliado em canto que se deu antes do confronto físico e canto durante o confronto físico, e após isso comparado com os parâmetros descritos para essa espécie.

O canto de anúncio de *P. renalis* é multipulsionado, com duração de 0.41 ± 0.16 segundos (0.26 – 0.72 s), emitido esporádicamente 31.3 ± 7.7 pulsos/note (24 – 42 pulsos/note). A frequência mínima de 400,0 ± 80,5 Hz (258,6 – 496,1 Hz), a frequência máxima de 1108,4 ± 140,8 Hz (1006,3 – 1396,6 Hz), frequência dominante de 708,8 ±

217,3 Hz (513,0–1138,0 Hz) e o pico de frequência de 709,8 ± 50,1 Hz (656,0–750,0324 Hz) (Santana et al., 2011).

Figura 1. Confronto físico entre indivíduos da espécie de *Proceratophrys aff renalis* na serra de Maranguape, Ceará.

O canto de anúncio que antecedeu a disputa teve duração de cerca de 1 min e 45s, enquanto a duração durante o confronto físico teve 1 min e 40s. A estrutura acústica do canto agonístico é composto por notas multipulsionadas e mais frequente que o canto de anúncio, não apresentando estrutura harmônica (Fig. 2). Este canto possui duração de 0.43 ± 0.04 segundos (0.37 - 0.49 s), emitindo 29 ± 2.58 pulsos/nota (25 - 33 pulsos/nota). A frequência mínima de 360.1 ± 12.82 Hz (335.6 – 374.3 Hz), a frequência máxima de 1156 ± 53.1 Hz (1084 – 1278 Hz), frequência dominante de 796.4 ± 61.46 Hz (722.8 – 942.2 Hz) e o pico de frequência de 689.1 Hz (Tabela 1). O pico de frequência manteve-se constante durante o tempo de observação.

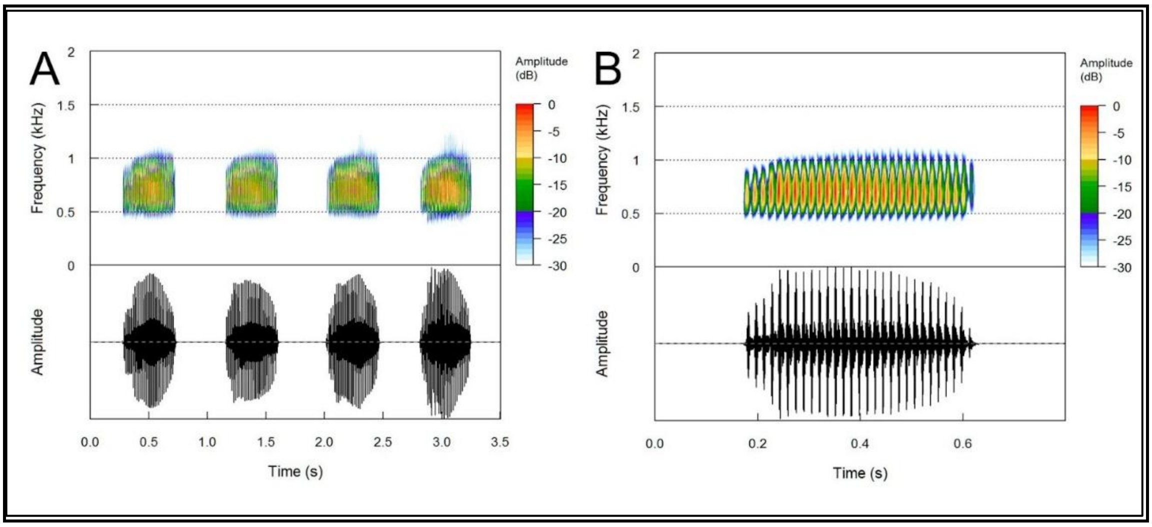

Figura 2. Representação do sonograma e espectograma do canto agonístico de *Proceratophrys aff renalis*. A) Frequência de repetição de vários cantos. B) único canto.

Tabela 1. Comparativo entre os parâmetros acústicos entre os cantos de anúncio e agonístico de *Proceratophrys aff. renalis* da Serra de Maranguape, Ceará. Médias ± desvio padrão; intervalos entre parênteses. * O valor manteve-se constante.

	Canto agonístico (n=10)	Canto de anúncio (n=22)	Teste estatístico
Low Frequency (Hz)	360.1 ± 12.82 (335.6 – 374.3)	397.7 ± 40.3 (350.0 – 481.2)	***P<0.05***
High Frequency (Hz)	1156.0 ± 53.1 (1084 - 1278)	1103.7 ± 55.56 (962.5 - 1181.2)	***P < 0.05***
Delta Frequency (Hz)	796.4 ± 61.46 (722.8 - 942.2)	713.9 ± 59.44 (568.8 - 787.5)	***P < 0.05***
Peak Frequency (Hz)	689.1*	696.9±25.34 (689.1 - 775.2)	*P > 0.05*
Pulses	29 ± 2.58 (25 - 33)	35.18 ± 1.81 (31 - 39)	***P < 0.05***
Call Duration (s)	0.43 ± 0.04 (0.37 - 0.49)	0.54 ± 0.03 (0.48 - 0.6)	***P < 0.05***
Pulse Rate (nº pulse/s)	66.54 ± 0.57 (65.68 - 67.35)	65.27 ± 0.73 (64.19 - 66.77)	***P < 0.05***
Interval Between Calls (n=6)	0.48 ± 0.13 (0.35 - 0.72)		

A PCA mostrou que os parâmetros acústicos entre os diferentes cantos emitidos pelos espécimes de *Proceratophrys aff. renalis* não se sobrepõem (Fig. 3A), com os dois primeiros componentes, respondendo por cerca de 66.5% da variação total. As variáveis que mais contribuíram foram: duração do canto, número de pulsos e a frequência (Fig. 3B).

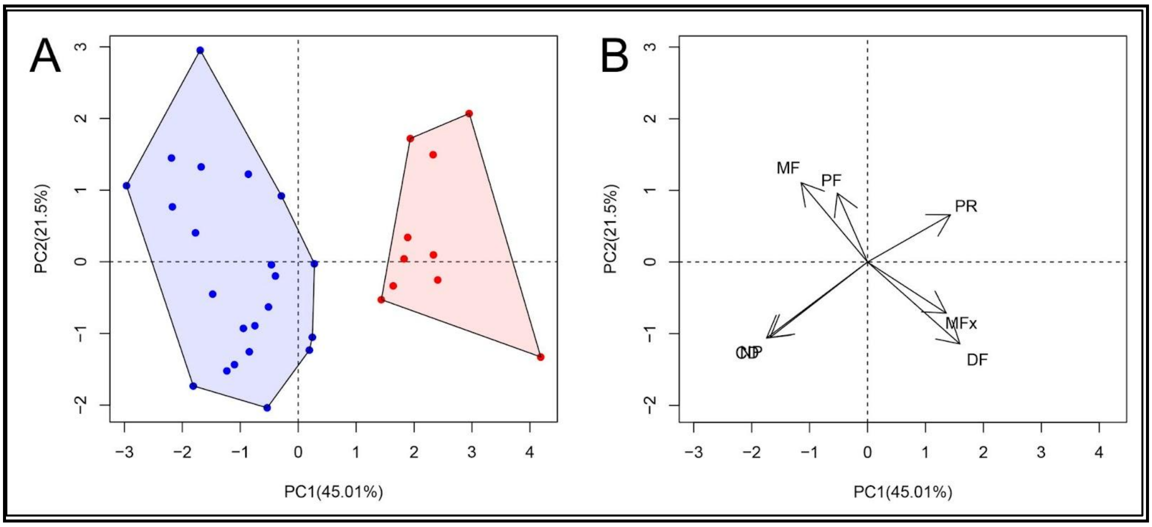

Figura 3. Análise de componentes principais com dados acústicos mostrando a variação dos dados (A) e vetores elevados (B), obtidos na análise entre os cantos de anúncio (azul) e agonístico (vermelho) de *Proceratophrys aff. renalis*.

Discussão

Aqui, descrevemos o canto agressivo, com confronto físico entre dois machos de *Proceratophrys aff. renalis.* Estes, são geralmente diferentes dos cantos de anúncios apresentando um aumento dos parâmetros temporais do canto, tais como, o número de notas e taxa de repetição, já os parâmetros espectrais, como frequência dominante podem variar, porém com menor frequência (Morais et al., 2012; Reichert e Gerhardt, 2013). De fato, o investimento em alterar esses parâmetros temporais normalmente aumenta com a redução da distância entre machos rivais ou em resposta à emissão de cantos por coespecíficos (Bastos e Haddad, 1995; Guimarães e Bastos, 2003; Toledo e Haddad, 2005; Osiejuk e Jakubowska, 2017).

Ainda não há comprovação que afirme se os machos de anuros escolhem seus sítios de vocalização por características acústicas, mas certamente características físicas estão envolvidas na propagação e direção do som (Wells and Schwartz, 1982). De acordo com Wells (1988), as fêmes preferem altas taxas de vocalização, portanto, espera-se que os machos aumentem a taxa de vocalização em resposta ao aumento da densidade masculina (ver Wagner, 1989). Conforme sugerido por Wagner (1989), esse comportamento permite que os machos aumentem sua atratividade para as fêmeas. Os machos da espécie de *Proceratophrys aff. renalis*, vocalizam em locais de mata fechada, entretanto em lugares sem obstáculos próximos ao sítio de vocalização, o qual facilita e evita interrupções na propagação e direção do som, além de não prejudicar os sinais visuais.

Durante as interações territoriais, as vocalizações são importantes para evitar lesões ou mortes que poderiam ser causadas pelos combates (Martins et al, 1998; Bastos e Haddad, 2002). Portanto, pode-se concluir que as disputas agonísticas entre machos de *P. aff. renalis* incluem interações acústicas e lutas físicas, com mudanças nos parâmetros de duração de canto, número de pulsos e frequência. Essa análise das propriedades temporais do canto de anfíbios favorece o desempenho da importância comportamental social dos indivíduos, ampliando a compreensão das interações entre os mesmos.

Capitulo 3

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Helminths Infecting the Carvalho's Escuerzo *Odontophrynus carvalhoi* from the Brazilian State of Ceará

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Tatiana Feitosa Quirino[1],*; Dalilange Batista-Oliveira [2]; Matheus Calixto Saldanha[3] & Robson Waldemar Ávila[1,2]

[1] Graduate Course in Systematics, Use, and Conservation of Biodiversity, Departament of Biology, Pici Campus, Federal University of Ceará, Fortaleza-CE Zip Code 60440-900, Brazil.

[2] Graduate Course of Ecology and Natural Resources, Department of Biology, Pici Campus, Federal University of Ceará, Fortaleza – CE Zip Code 60440-900, Brazil.

[3] Graduate Course Biology, Departament of Biology, Pici Campus, Federal University of Ceará, Fortaleza-CE Zip Code 60440-900, Brazil.

*** Corresponding author: tata_tatifeitosa@hotmail.com**

Tatiana Feitosa Quirino: https://orcid.org/0000-0001-8184-8705

Dalilange Batista-Oliveira: https://orcid.org/0000-0002-4140-6643

Matheus Calixto Saldanha: https://orcid.org/0009-0005-8884-7015

Robson Waldemar-Ávila: https://orcid.org/0000-0003-3641-8321

Abstract

The family Odontophrynidae comprises 40 anuran species widely distributed in South America, ranging from Brazil to Argentina and Paraguay. *Odontophrynus carvalhoi* Savage and Cei, 1965, a medium-sized species with terrestrial habits and explosive reproduction, is one of the representatives of this family. Despite its extensive distribution, data regarding its natural history are limited, with published information primarily focused on activity patterns, diet, and defensive behavior. In this study, we present data on the composition and infection patterns of endoparasites associated with *O. carvalhoi* in a relictual forest in the Brazilian state of Ceará. Th e parasite community comprised 11 species, with *Aplectana hylambatis* (76.67%) and *Oswaldocruzia mazzai* (79.49%) being the most prevalent. Additionally, we report new occurrences of parasite species for *O. carvalhoi*, contributing to the understanding of the parasitic fauna in the Neotropical region. These findings underscore the importance of parasitological research for a comprehensive understanding of the ecology and biology of this amphibian species.

Keywords: Parasites; Amphibian; Odontophrynidae; Relictual forest.

Resumen

La familia Odontophrynidae comprende 40 especies de anuros ampliamente distribuidas en América del Sur, desde Brasil hasta Argentina y Paraguay. *Odontophrynus carvalhoi* Savage & Cei, 1965, especie de tamaño mediano, hábitos terrestres y reproducción explosiva, es uno de los representantes de esta familia. A pesar de su extensa distribución, los datos sobre su historia natural son limitados, y la información publicada se centra principalmente en patrones de actividad,dieta y comportamiento defensivo. En este estudio, presentamos datos sobre la composición y patrones de infección de endoparásitos asociados con *O. carvalhoi* en un bosque relicto en el estado brasileño de Ceará. La comunidad de parásitos estuvo compuesta por 11 especies, siendo *Aplectana hylambatis* (76,67%) y *Oswaldocruzia mazzai* (79,49%) las más prevalentes. Además, reportamos nuevas ocurrencias de especies de parásitos para *O. carvalhoi*, contribuyendo al conocimiento de la fauna parasitaria en la región Neotropical. Estos hallazgos subrayan la importancia de la investigación parasitológica para una comprensión integral de la ecología y biología de esta especie de anfibio.

Palabras clave: Parásitos; Anfibios; Odontophrynidae; Bosque relicto.

Resumo

A família Odontophrynidae compreende 40 espécies de anfíbios amplamente distribuídas na América do Sul, abrangendo desde o Brasil até a Argentina e o Paraguai. *Odontophrynus carvalhoi* Savage e Cei, 1965, uma espécie de porte médio com hábitos terrestres e reprodução explosiva, é uma das representantes dessa família. Apesar de sua distribuição extensiva, dados sobre sua história natural são limitados, com informações publicadas concentradas principalmente em padrões de atividade, dieta e comportamento defensivo. Neste estudo, apresentamos dados sobre a composição e padrões de infecção de endoparasitas associados a *O. carvalhoi* em uma floresta relictual no estado brasileiro do Ceará. A comunidade de parasitas incluiu 11 espécies, sendo *Aplectana hylambatis* (76,67%) e *Oswaldocruzia mazzai* (79,49%) as mais prevalentes. Adicionalmente, relatamos novas ocorrências de espécies de parasitas para *O. carvalhoi*, contribuindo para a compreensão da fauna parasitária na região Neotropical. Essas descobertas destacam a importância da pesquisa parasitológica para uma compreensão abrangente da ecologia e biologia dessa espécie de anfíbio.

Palavras-chave: Helmintos; Anfíbio; Odontophrynidae; Floresta relictual.

Introduction

The family Odontophrynidae is composed by 40 anuran species widely distributed in South America, from Brazil to Argentina and Paraguay (Segalla et al., 2019; Mângia et al., 2020; Frost, 2021). The genus *Odontophrynus* Reinhardt & Lütken, 1862, currently have 12 species (Frost et al., 2017) belonging to three phenetic groups (Savage & Cei, 1965; Caramaschi, 1996; Caramaschi & Napoli, 2012): *O. americanus*, *O. cultripes* and *O. occidentalis*. Besides, *O. salvatori* actually is not allocated to any group (Amaro et al., 2009).

Odontophrynus carvalhoi, belongs to O. cultripes group, was described by Savage & Cei, 1965, from a single specimen collected in Poção municipality, state of Pernambuco, Brazil. Its distribution encompasses the Jequitinhonha River valley, in Minas Gerais state, to northeastern Goiás state and Ceará state in the north, in phytophisiognomies of Atlantic Forest, Cerrado and Caatinga Biomes (Juncá, 2006; Lisboa et al., 2010; Caramaschi & Napoli, 2012; Dias et al., 2014; Santos et al., 2017).

It is a medium sized species, with terrestrial habits and explosive reproduction (Lynch, 1971; Freitas & Silva, 2004; Caramaschi & Napoli, 2012). Tadpoles are laid in small streams inside forested habitats (Santos et al., 2017). Despite its huge distribution, data on natural history of *O. carvalhoi* are scant, with published information about activity patterns and diet (Brito et al., 2012) and defensive behavior (Bezerra et al., 2010; Borges-Nojosa et al., 2016). Until now, there is no information regarding endoparasites infecting *O. carvalhoi*, and several studies pointed out the importance of knowledge of parasites given the role of these organisms in ecosystem regulation. In particular, the diversity of helminths associated with amphibians is considered rich and diversified, although it is a hidden diversity and therefore undersampled (Poulin, 2014; Campião et al., 2014).

Herein, we present data on composition and infection patterns of endoparasites associated with *O. carvalhoi* from a relictual forest in the Brazilian state of Ceará.

Material and Methods

This study was carried out in the municipality of Guaramiranga, located at the Baturité massif, northern Ceará state, Brazil. Its mountainous relief, dissected forming small valleys, covered by rainforest vegetation, is classified as a remnant of the Atlantic Forest. Altitude. The annual rainfall has an average of 1737.5 mm/year, with a rainy season from January to May. Its average temperature ranges from 24º to 26ºC (Vale & Soares, 2006; IPECE, 2017).

Field work was taken in three sampling points: Guaramiranga farm (04º15'54,91" S; 38º56'00,24" W), Álvaro farm (04º17'17,29" S; 38º57'00,05" W), Riacho Fundo farm (04º15'42,86" S; 38º55'07,57" W), Parque das Trilhas (04º16'13,63" S; 38º56'19,10" W), Vale das Nuvens (4º16'05,10" S; 38º54'54,96" W) and Pernambuquinho farm (04º12'19,73" S; 38º 57'37,70" W) . We used specimens collected from 1994 to 2022, that are deposited in the Herpetological Collection of the Universidade Federal do Ceará (CHUFC-A 2.918, 3.738-39, 3.740-41, 3.769-73, 3.799, 3.864, 3.875-76, 9.961-70, 10.275-81, 10.283-10.288, 10.999), and Herpetological Collection of the Universidade Regional do Cariri (URCA-H 16.041), Ceará state, Brazil.

Thirty-nine individuals of *O. carvalhoi* were collected by hand through active and auditory searches (Bernarde, 2012). The specimens were placed in separate containers and euthanized by a lethal injection of lidocaine (CFMV, 2013), fixed with 10% formalin according to Calleffo (2002). Morphometric measurements of hosts were taken with a digital caliper Mitutoyo® (precision 0.01 mm).

The specimens were necropsied with a midventral incision and all organs and coelomic cavity were searched for helminths. Parasites found were preserved in 70% ethanol, fixed according Amato et al. (1991) and Andrade (2000), and mounted in temporary slides for identification. For identification, we follow Yamaguti (1961), Sprent (1978), Vicente et al. (1991), Anderson (2000) and Gibbons (2010), as well recent descriptions. Slides were analyzed under microscope with computerized image analysis system. Voucher helminths were deposited at Coleção Parasitológica of the Universidade Federal do Ceará. Parasitological descriptors (prevalence, mean intensity of infection and abundance) were calculated according to the specifications of Bush et al. (1997).

To evaluate the impact of host size on infection intensity, we employed linear regression. Differences in the prevalence and intensity of infection between genders were assessed using the chi-square method. The analyses were conducted on the R platform, "Commander R" package (R CORE TEAM, 2022, version 4.2.0) (R Foundation, 2017).

Ethic aspects: This study was approved by the Ethics Committee on Animal Use of the Federal University of Ceará (CEUA-UFC) under the protocol 6314010321.

Results

We examined 39 specimens of *O. carvalhoi*, being 16 males and 23 females, which are infected with at least one endoparasite species (overall prevalence 84,97%). We found 5.681 helminths, with mean intensity of infection 153.54 ± 32.38. Parasite community was composed by 11 species: Aplectana hylambatis (Baylis, 1927), *Oswaldocruzia mazzai* Travassos, 1935, *Cosmocerca brasiliense* Travassos, 1925, *Gorgoderina parvicava* Travassos, 1922, *Physaloptera* sp., *Raillietnema* sp., *Rhabdias* sp., *Strongyloides* sp., *Parapharyngodon* sp., *Oxyascaris* sp. and *Ochoterenella* sp., besides Cosmocercidae larvae. The most prevalent taxa were *A. hylambatis* (76.67%) and *O. mazzai* (79.49%), being the first one with the higher abundance (102.49) and mean intensity of infection (121.12). *G. parvicava*, *Strongyloides* sp. and *Parapharyngodon* sp. have the lower prevalence (2.56%) (Table 1).

Table 1. Hosts, number of helminths (NH), mean abundance (MA), mean intensity of infection (MII) with standard error (SE), intensity of infection amplitude (IIA) and infection site (IS) of helminths associated with *Odontophrynus carvalhoi*.

Helminth	NH	MA±SE	MII±SE	IIA	IS
Aplectana hylambatis	3692	102.49±24.57	121.12±27.87	1-725	B/E/IG/ID
Oswaldocruzia mazzai	618	79.49±15.85	19.94±9.94	1-312	E/IG/ID
Cosmocerca brasiliense	11	0.28±0.2	5.5±0.5	5-6	ID
Gorgoderina parvicava	5	0.13±0.13	5	5	B
Physaloptera sp.	219	5.62±1.92	15.64±4.28	1-52	E/IG/ID
Oxyascaris sp.	2	0.05±0.04	1	1	IG/ID

Helminth	NH	MA±SE	MII±SE	IIA	IS
Raillietnema sp.	273	7.0±6.4	91.0±79.25	1-249	B/ID
Rhabdias sp.	139	3.56±0.83	6.04±1.15	1-21	P/F/E
Strongyloides sp.	2	0.05±0.05	2	2	ID
Parapharyngodon sp.	1	0.03±0.03	1	1	IG
Ochoterenella sp.	414	10.62±5.34	18.82±9.17	1-205	CAV/B/F/C/ID

Mean richness was 3.41 ± 0.23 helminths/hosts, and the higher richness (S=6) was found in a single host. There was an influence of host size on mean intensity of infection ($r=0.48$; $p<0.05$; $n=39$). As for the influence of size on the average intensity of infection between the sexes, we can observe that were significant for males and females ($r=0.56$; $p<0.05$; $n=16$, $r=0.47$; $p<0.05$; $n=23$, respectively).

Discussion

Helminths community associated with amphibians are characterized by generalist species (Aho, 1990). Low host specificity, combined with the general lack of studies with Neotropical amphibian species, are often the main causes of new host records (Campião et al., 2015; Oliveira et al., 2019). In Brazil, species of Odontophrynidae still need further studies to understand what are the host specificities and patterns of infection and abundance (Campião et al., 2014).

Species of Aplectana are usually found infecting the large intestine of reptiles and amphibians, have a direct life cycle and actively infect their hosts (Travassos, 1931; Anderson, 2000; Campião et al., 2014; Lins et al., 2017). This genus has been already reported in four odontophrynids: *Proceratophrys tupinamba* Prado and Pombal, 2008 and *P. boiei* (Wied-Neuwied, 1824) infected by *A. delirae* Fabio, 1971 (Boquimpani-Freitas et al., 2001; Klaion et al., 2011), and P. cristiceps (Silva et al., 2019; Sampaio et al., 2020) and *Odontophrynus americanus* Duméril & Bibron, 1841, infected by *A. membranosa* (Lent & Freitas, 1948).

Aplectana hylambatis is a generalist species that parasitizes a wide range of hosts. In the Neotropical region it has been observed in different countries and families of anurans such as Bufonidae, Leptodactylidae, Ceratophryidae e Microhylidae from Peru

(Bursey et al., 2001); Bufonidae, Mycrohylidae, Leiuperidae, Leptodactylidae and Hylidae from Paraguay (Masi Pallares & Maciel, 1974; Baker & Vaucher, 1986); Bufonidae from Uruguay (Lent & Freitas, 1948); Bufonidae, Leptodactylidae and Leiuperidae from Argentina (Gutiérrez, 1945; Sueldo & Ramírez, 1976; Ramírez et al., 1979; Baker, 1980; González & Hamann, 2006, 2010). In Brazil, it was reported infecting Hylidae, Microhylidae and Leptodactylidae at São Paulo and Mato Grosso do Sul states (Campião et al., 2016; Aguiar et al., 2021).

Cosmocerca Diensing, 1861, is a genus that pursues a wide geographic distribution and is commonly found as parasites of amphibians (Navarro et al., 1988). *Cosmocerca brasiliense* can infect amphibian hosts by ingestion of an infective larvae (L3) or penetration through the skin (Goldberg at al., 2002a). After infection, larvae migrate to the host intestine, reaching sexual maturity. This species has been recorded parasitizing many hosts in South America, such as Ecuador (Dyer & Altig, 1976; Mcallister et al., 2010a), Guiana (Mcallister et al., 2010b) and Peru (Bursey et al., 2001). In Brazil, it was reported by Travassos (1925); Vicente et al. (1991); Boquimpani-Freitas et al. (2001); Martins & De Fabio (2005); Goldberg et al. (2007); Santos et al. (2013), (2016); Aguiar et al. (2014); Klaion (2011); Campião et al. (2014); Oliveira et al. (2022).

Oswaldocruzia mazzai infect several anurans (e.g., Campião et al., 2014; Teles et al., 2015; Alcantara et al., 2018; Oliveira et al., 2019), but we reported for the first time the infection of this nematode in the genus *Odontophrynus*. The great host diversity recorded for *O. mazzai* is related to the direct life cycle and the simple mode of transmission that can occur by ingestion of eggs or larval penetration of the host’s skin (Anderson, 2000).

The genus Gorgoderina Looss, 1902, includes 57 species, all of them described as parasites of the bladder of anurans and salamanders. In Brazil, seven species of this genus are known. *G. parvicava* is well distributed in Neotropical region and has already been reported as parasites of several anurans in Brazil, such as *Leptodactylus chaquensis*, Cei, 1950, L. labyrinthicus, (Spix, 1824), *L. latrans* (= L. ocellatus) (Steffen, 1815), *L. pentadactylus*, (Laurenti, 1768), *Rhinella crucifer*, (Wied-Neuwied, 1821), *R. diptycha*, (Cope, 1862), *R. icterica*, (Spix, 1824), *R. marina*, (Linnaeus, 1758), *Pseudis paradoxa* (Linnaeus, 1758), and *Pristimantis relictus* Roberto et al., 2022, (Alcantara et al., 2022; Oliveira et al., 2022).

Nematodes of the genus *Physaloptera* have a worldwide distribution and have been recorded in several terrestrial vertebrates, including felines (Ogassawara, 1986), rodents (Tung et al., 2009), lizards (Da Silva et al., 2008, Cabral et al., 2018) and anurans (Da Graça et al., 2017). In amphibians, this parasite is usually found in the larval stage, making it difficult to identify at the species level, and it is suggestive that these amphibians are not definitive hosts. Although there is not enough data on their life cycle, nematodes of this genus are known to utilize insects during their intermediate phase (Anderson, 2000). Additionally, the acquisition of *Physaloptera* by anuran hosts occurs through the ingestion of infected insects, mainly Orthoptera (Klaion et al., 2011).

Raillietnema sp. is known to have a direct life cycle and transmission that occurs via ingestion or penetration of larvae through the skin (Anderson, 2000). Studies have reported the presence of this parasite infecting *P. aridus* (Teles et al., 2017), as well as infecting lizards. The species *R. spectans* (Bursey et al., 1998), are the most frequent in anurans (Vicente et al., 1991; Teles et al., 2015).

The specific identification of the *Rhabdias* found here was not possible, because there is high morphological similarity, which makes molecular data useful in species recognition (Müller et al., 2018). Nematodes of this genus are lung parasites, commonly infecting amphibians and reptiles, by direct transmission and infection occurs by active penetration into the skin of hosts (Langford & Janovy, 2009; Kuzmin et al., 2015).

Although the life cycle of *Strongyloides* is not known, it can be considered a direct or indirect cycle, the first being the most common (Santos et al., 2010). Infection occurs on land through skin penetration or ingestion of infected prey (Mati & Melo, 2014; Sulieman et al., 2015). Although there are several records of this nematode infecting amphibian species (Campião et al., 2014; Sulieman et al., 2015; Mascarenhas et al., 2021), this is the second record of the genus *Strongyloides* acting as a parasite for species of the Odontophrynidae Family and the first for *O. carvalhoi*

Parapharyngodon Chatterji, 1933, is a genus of parasitic nematodes with monoxenic cycle (Anderson, 2000). These have already been registered in amphibian species (Pereira et al., 2017), being commonly found in reptiles (Avila & Silva, 2010).

Ochoterenella is reported from South and Central America, with several species known only from females and larvae. The genus is remarkably diverse in bufonids such as *Rhinella marina* (Linnaeus, 1758) (Travassos, 1929; Caballero, 1944; Bain & Prod'Hon, 1974; Bain et al., 1979; Esslinger 1986, 1987, 1988a, b, 1989), while only two of 15 species have been described for Leptodactylidae with few records for hylids (Lima et al., 2012).

Capitulo 4

Population Structure and Genetic Diversity in *Odontophrynus carvalhoi* Savage and Cei, 1965 (Anura: Odontophrynidae) from five states in the Brazilian Northeast

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POPULATION STRUCTURE AND GENETIC DIVERSITY IN *Odontophrynus carvalhoi* Savage and Cei, 1965 (ANURA: ODONTOPHRYNIDAE) FROM FIVE STATES IN THE BRAZILIAN NORTHEAST

Tatiana Feitosa Quirino[1], Mariny Oliveira Arruda[2], Renata Perez[3], Robson Waldemar Ávila[3]

[1] Programa de Pós-Graduação em Sistemática, Uso e Conservação da Biodiversidade, Universidade Federal do Ceará, Campus do Pici, Avenida Humberto Monte, Fortaleza, CE, 60440-900, Brazil. tata_tatifeitosa@hotmail.com.

[2] Programa de Pós-Graduação Ecologia e Recurso Natural, Universidade Federal do Ceará, Campus do Pici, Avenida Humberto Monte, Fortaleza, CE, 60440-900, Brazil.

[3] Núcleo Regional de Ofiologia, Centro de Ciências, Universidade Federal do Ceará, Campus do Pici, Fortaleza, Ceará, Brazil.

ORCID
Quirino, T. F. 0000-0001-8184-8705
Arruda, M. O. 0000-0001-7491-8584
Perez, R. 0000-0002-8710-4309
Ávila, R. W. 0000-0003-3641-8321

Headings: Genetic and Structure in *Odontophrynus carvalhoi*

Abstract

Studies on the conservation of genetic resources of species aim to quantify their diversity and understand its magnitude, nature, and distribution among and within populations. To ensure the maintenance of biodiversity, it is widely recognized that high levels of genetic diversity are essential. In this context, the objective of this work was to characterize the population structure and genetic diversity of *Odontophrynus carvalhoi* through the analysis of the mitochondrial molecular marker (16S). We used genetic sequences for the species from different locations, including the Brejos de Altitude, Planalto da Borborema, Mata Atlântica, and Chapada Diamantina, for the analysis of diversity indices and population structure, as well as for the construction of the phylogenetic tree. The results indicated that *Odontophrynus carvalhoi* does not present a distinct lineage, with populations from the Planalto da Borborema and the Mata Atlântica in the same group, sharing haplotypes, and demonstrating absence of population structure. Although the genetic difference between populations is small, the Brejos de Altitude and the Chapada Diamantina exhibit greater genetic distance, revealing population structure with a unique population stock. On the other hand, populations sharing the same haplotypes suggest gene flow between them.

Keywords

Population genetics; Genetic diversity indices; Amphibians; Northeast.

Introduction

One of the premises of population genetics predicts that dispersal decreases with increasing geographical distance, so that gene flow is generally lower between geographically distant populations, causing a pattern of isolation by distance (Wright 1943). In this sense, historically isolated populations are the main candidates for studies on the origin of genetic and phenotypic differences, as they would have been potentially exposed to long-term genetic drift or divergent selection pressures, without the influences of gene flow homogenization (Avise 2000; Nosil et al. 2009; Kaefer et al. 2013).

Long-term isolation can result in speciation, and although the resulting groups may contain morphologically similar individuals, they may not recognize each other reproductively (Schluter 2009). Thus, studies on the conservation of genetic resources of species have been of great importance, as they are increasingly used for quantifying genetic diversity and understanding its magnitude, nature, and distribution among and within populations (Perez 2008).

The mitochondrial DNA (mtDNA) control region, which is responsible for all mitochondrial regulation and transcription, has high mutation rates and is widely used in intraspecific studies of various biological groups (Stewart and Barker 1994; Starkey et al. 2003). The advantages of using these markers include the fact that prior knowledge of DNA sequences of the target species is not necessary (Wolfe 2005), which facilitates their application in species that have not been studied before. The produced fragments have high reproducibility, high polymorphism, and require little laboratory equipment infrastructure for experiment execution (Zietkiewicz et al. 1994; Bornet and Branchard 2001; Casu et al. 2011). In addition to providing a dynamic approach to studying genomic variations, with potential applications in population molecular genetics, taxonomy, genome mapping, as well as tracking somatic mutations (Zietkiewicz et al. 1994).

The mitochondrial gene 16S ribosomal, corresponding to a highly conserved region, is widely used in understanding phylogenetic and phylogeographic relationships. Many studies integrate information from this gene (Austin 2002; Ron et al. 2006; Faivovich et al. 2010; Vieira 2010). According to Vences et al. (2005), the 16S rRNA gene can be considered a standard marker for phylogeny reconstruction in amphibians. Besides amphibians, it has been applied in phylogenetic analyses of different animal groups, such as fish (Wang et al., 2001), reptiles (Van Der Kuyl et al. 2002), birds (Dimcheff et al. 2002), and mollusks (Barucca et al. 2004). Vences et al. (2005) provided evidence that the mitochondrial 16S rRNA gene can be considered the universal DNA barcoding for amphibians, even more efficient than COI (Cytochrome c oxidase subunit I) in identifying this group of animals.

Anuran amphibians are identified as a group of high cryptic diversity in tropical regions due to the strong phenotypic conservatism among species in contrast to the strong structuring and high genetic differentiation observed among populations (Zeisset and Beebee 2008; Vieites et al. 2009; Kaefer et al. 2013).

The genus *Odontophrynus* Reinhardt & Lütken, 1862 consists of soil-burrowing anurans with medium size and nocturnal activity (Caramaschi and Napoli 2012; Frost 2021). The phylogenetic position of this genus and its species has been discussed for over a decade (Pyron and Wiens 2011; Martino et al. 2019; Magalhães et al. 2020). However, despite discussions, the genus could be phenotypically diagnosed by the synapomorphy of foot musculature, with differences such as the absence of roughness on the dorsal surface of the toes and the presence of tubercles on the tenar surfaces (Amaro et al. 2009; Blotto et al. 2017). Currently, the genus includes twelve species arranged in two groups: the *Odontophrynus americanus* and *O. cultripes* groups, in addition to *O. occidentalis* (Berg 1896), a species not associated with any group (Martino et al. 2019; Rosset et al. 2021; Moroti 2022).

Odontophrynus carvalhoi, belonging to the *O. cultripes* group, was described by Savage and Cei (1965), and its current distribution includes from the Jequitinhonha River Valley (south) in the state of Minas Gerais, through the northeast of the state of Goiás, to the state of Ceará (north), encompassing the biomes of the Atlantic Forest, Cerrado, and Caatinga (Feio and Caramaschi 1995; Juncá 2006; Lisboa et al. 2010; Loebmann and Haddad 2010; Caramaschi and Napoli 2012; Dias et al. 2014; Santos et al. 2017).

This species has a terrestrial habit, easily camouflaging in the environment, with numerous glands scattered on the dorsum, evident parotoids, and an internally modified metatarsal tubercle in the shape of a "spade," a characteristic that allows this species to bury itself, avoiding desiccation (Lynch 1971; Freitas and Silva 2004; Caramaschi and Napoli 2012). It exhibits explosive reproduction, with egg laying likely occurring in lentic environments, in the mud at the bottom of water bodies, and its tadpoles are found in small streams (Bastos et al. 2003).

To ensure the maintenance of biodiversity, it is known that high levels of genetic diversity are necessary. Therefore, the aim of this work was to characterize the population structure and genetic diversity of *Odontophrynus carvalhoi* through the analysis of the mitochondrial molecular marker (16S). The results obtained can be used to guide future proposals for the management and conservation of the species.

Methods

Phylogenetic Analysis:

The phylogenetic analysis included 22 species, with the outgroup consisting of *Thoropa miliaris*. Nine sequences were newly generated, and 13 sequences were available on GenBank. All sequences were utilized for constructing the phylogenetic tree. For the genetic structure analysis of the *Odontophrynus carvalhoi* population, 12 sequences of 16S rRNA (536 bp) were used. The newly generated sequences represented locations in Alagoas (Pedra Talhada), Pernambuco (Brejo dos Cavalos and Poção, the species' type locality), and Ceará (Maciço de Baturité). Additionally, sequences from GenBank (accession numbers: FJ685687, OM243020, OM243021) from Bahia (Mucugê) and Paraíba (Pocinhos) were included. These sequences were from six locations across five different states in Northeast Brazil.

Methods

Study Area:

The Caatinga biome has a complex physiognomy, primarily characterized by xerophytic vegetation (Cole 1960; Ab'Saber 1998), although it also contains enclaves of tropical forest and Cerrado elements (in elevated areas called "brejos") (Ab'Saber 1974, 1977; Velloso et al. 2002; Guedes et al. 2020). Due to its heterogeneity, the Caatinga is divided into nine ecoregions (Silva et al. 2017). This study utilized samples from four ecoregions: Borborema Plateau (Poção and Pocinhos), Atlantic Forest (Pedra Talhada and Brejo dos Cavalos), Chapada Diamantina Complex (Mucugê), and Altitude Brejo (Guaramiranga).

The Borborema Plateau extends across the states of Rio Grande do Norte, Paraíba, Pernambuco, and Alagoas (Mendonça-Diniz et al. 2015). Pocinhos is located in the physiographic zone of the Paraiban Agreste, while Poção is in the Pernambuco Agreste, both within the geomorphological unit of the Plateau. The vegetation is characterized by Subcaducifolious and Caducifolious Forests typical of agreste áreas (IBGE 2023). The plateau consists of ancient massifs, called arched crystalline nuclei, with altitudes ranging from 400m to 600m, surrounded by peripheral depressions like the Sertão and São Francisco depressions (Ab'Saber 1953; Barros, Monteiro and Cestaro 2018).

The Atlantic Forest has been considered one of the world's 25 hotspots due to its high species diversity, high endemism, and the degree of threat to the ecosystem. The Pedra Talhada Biological Reserve, located between Alagoas and Pernambuco, in transition with the Caatinga, is one of the significant remnants of this forest. The João de Vasconcelos Sobrinho Ecological Park in Brejo dos Cavalos, Caruaru, preserves a lush forest with significant diversity and water sources for public supply (CPRH 1994).

Mucugê, situated in the semi-arid region, is located in the Chapada da Diamantina (Brasil 2021). This mountainous area is characterized by high endemism, making it a biodiversity conservation hotspot (Giulietti and Pirani 1988; Prance 1994). It is considered the main mountainous massif of the Caatinga, featuring vegetation typical of Cerrado, rupestrian fields, and semideciduous seasonal forest (Giulietti et al. 2004; Rocha et al. 2005; Queiroz et al. 2005). The altitude ranges from 500 to 2033 m, and the climate is mild, with temperatures between 16°C and 22°C, providing more humid areas and resources (Giulietti et al. 2004; Rocha et al. 2005; Queiroz et al. 2005).

The EnvironmentalProtection Area of Maciço do Baturité (APA-Serra de Baturité) constitutes an area characterized by climatic differentiation known as "Brejos de Altitude" (Moura 2010). These areas are exceptional zones within the Sertão depression, considered true "islands" of humid forest in the Caatinga, associated mainly with historical processes that occurred in the Pleistocene (Andrade-Lima 1982). Furthermore, the "Brejos de Altitude" can be considered an important link that highlights the retraction and expansion of Atlantic and Amazon forests in the past, including the presence of typical species closely related to these environments, both phylogenetically and geographically (Borges-Nojosa and Caramaschi 2003; Fouquet et al. 2012). They are also responsible for harboring a rich and distinctive herpetofauna in the northeastern region (Carvalho-e-Silva et al. 2015; Freitas et al. 2019).

DNA Extraction, Amplification, and Sequencing:

DNA extraction was carried out from tissue samples of muscle or liver from specimens preserved in 70% ethanol, sourced from the Tissue Collection of the Regional Center of Ophiology (UFC) at the Federal University of Ceará (UFC). Extractions were performed using the Promega Wizard® DNA purification kit, following the manufacturer's instructions. The 16S region of the mitochondrial genome was amplified using primers described by Palumbi (Palumbi et al. 1991).

The final volume of the polymerase chain reaction (PCR) reaction was 15 µl and included: 2.0 µl of dNTP (100 mM), 1.5 µl of 10×PCR Buffer (200 mM Tris–HCl, pH 8.4, 500 mM KCl), 2.3 µl of MgCl2 (50 mM), 1.5 µl of each primer (50 ng/µl), 0.5 µl of Taq polymerase (5 U/µl, Ludwig), 1 µl of DNA (approximately 50 ng/µl), and 4.7 µl of ultrapure water to complete the final volume. The DNA amplification protocol included initial denaturation at 92°C for 1 min, followed by 35 cycles of 92°C for 1 min, 50°C for 40s, 72°C for 1 min 30s, and a final extension at 72°C for 5 min. PCR products were visualized by 1% agarose gel electrophoresis, purified using EXO-SAP, and sequenced using the BigDye fluorescent kit on an AB-3500 sequencer (Applied Biosystems).

Phylogenetic Analysis, Diversity, and Population Structure:

The sequences were analyzed and edited using the Geneious v.7.0 software (Kearse et al. 2012), and multiple sequence alignment was performed using Maffit v6 (Katoh et al. 2002). Bayesian inference, conducted in the Mr. Bayes software (Ronquist and Huelsenbeck, 2003), generated phylogenetic trees with 100 million generations. The Figtree v.1.4.3 program (Rambaut 2016) was utilized for the observation and editing of the consensus tree. A linear correlation test using the Tamura-Nei correlation coefficient was conducted to assess the distance between groups. Haplotype diversity (h) and nucleotide diversity (π) indices, as well as the number of polymorphic sites, were generated using the DnaSP v.5.1 program (Librado and Rozas 2010). Haplotype networks and their analyses were developed using the Haploviewer v.4.2 program (Barrett et al. 2005). To investigate the genetic variability within populations, a Molecular Variance Analysis (AMOVA) was performed in Arlequin v3.5.12. In the same program, the degrees of genetic distinction between species populations were evaluated through pairwise FST tests (Weir and Hill 2002; Excoffier and Lischer 2010).

Result

The phylogenetic tree, based on Bayesian and maximum parsimony analyses, revealed that *Odontophrynus carvalhoi* is rooted in a clade composed exclusively of *Odontophrynus* species (Fig 1). These species are from various locations, including Alagoas (Rebio Pedra Talhada, Quebrangulo), Pernambuco (Brejo dos Cavalos and Poção), Ceará (Maciço de Baturité), Bahia (Mucugê), and Paraíba (Pocinhos). The results indicate that the species does not form a distinct lineage, and the group from Planalto da Borborema and Mata Atlântica is rooted within the same group.

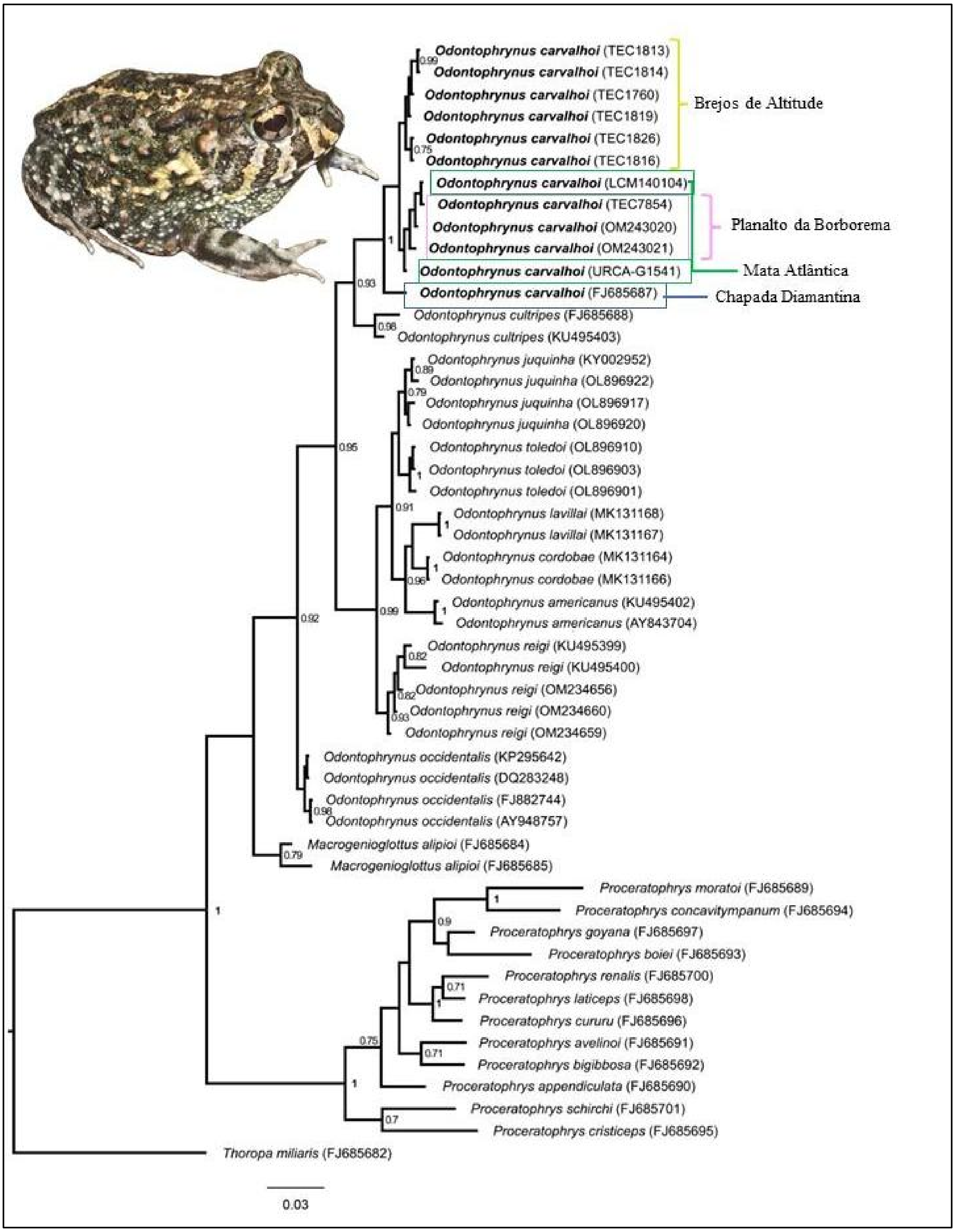

Fig. 1 Bayesian phylogenetic analysis of the 16S gene for the genus *Odontophrynus*. The external group consists of *Thoropa miliaris*. The colors used in the tree represent the groups corresponding to the colors used in the haplotype network.

Population structure analysis was conducted using 536 base pairs of the mitochondrial 16S rRNA gene from 12 specimens, revealing 14 variable sites and 5 parsimony-informative sites. Genetic distances between groups were calculated for *Odontophrynus carvalhoi* populations sampled from different ecoregions. The greatest genetic distance was observed between the Brejo de Altitude and Chapada Diamantina groups (0.02687), approximately 2.7% (Table 1).

Table 1. Genetic diversity between *Odontophrynus carvalhoi* populations.

	(1)	(2)	(3)	(4)
(1) Brejo de Altitude	-			
(2) Mata Atlântica	0,005642	-		
(3) Planalto da Borborema	0,006589	0,000937	-	
(4) Chapada Diamantina	0,026874	0,022921	0,023901	-

The analysis conducted in DNASP revealed the presence of six haplotypes for the species, indicating high haplotype diversity (h = 0.864) and low nucleotide diversity (π = 0.00795) (Table 2). It is noteworthy that the Analysis of Molecular Variance (AMOVA) and the pairwise FST test for the mitochondrial marker 16S showed that the genetic diversity within the group was higher among populations (67.52%) than within them (32.48%) (Table 3).

Table 2. Indices of genetic diversity recorded in Odontophrynus carvalhoi for the mitochondrial marker 16S

Locality	*N*	*S*	*H*	*h*	π
Brejo de Altitude	6	3	4	0,867	0,00611
Planalto da Borborema	3	1	2	0,09877	0,0019
Mata Atlântica	2	1	2	1	0,00192
Chapada da Diamantina	1	0	1	0	0
Total	12	14	6	0,864	0,00795

Number of individuals analyzed (N), number of polymorphic sites (S), number of haplotypes (H), haplotype diversity (h), nucleotide diversity (π).

Table 3. Molecular variance analysis for *Odontophrynus carvalhoi* using 16S mitochondrial markers. Assessed for genetic diversity within and between populations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	3	11.250	1.21277 Va	67.52*
Within populations	8	4.667	0.58333 Vb	32.48
Total	11	15.917	1.79610	

Degree of freedom (d.f.), **p*-value significativo (p= 0.00098)

The haplotype network indicated that the population of Brejos de Altitude (Serra de Baturité) is composed of three different haplotypes not shared with other studied areas. On the other hand, two individuals from Planalto da Borborema share the same haplotypes as the Mata Atlântica population, except for one individual from the municipality of Pocinhos, which has a different haplotype. The population of Chapada Diamantina is characterized by a haplotype that has differentiated from the populations of Planalto da Borborema and Mata Atlântica by eight mutational events that modified its allelic state. Thus, population structure is observed for the populations of Brejos de Altitude and Chapada Diamantina (Fig. 2).

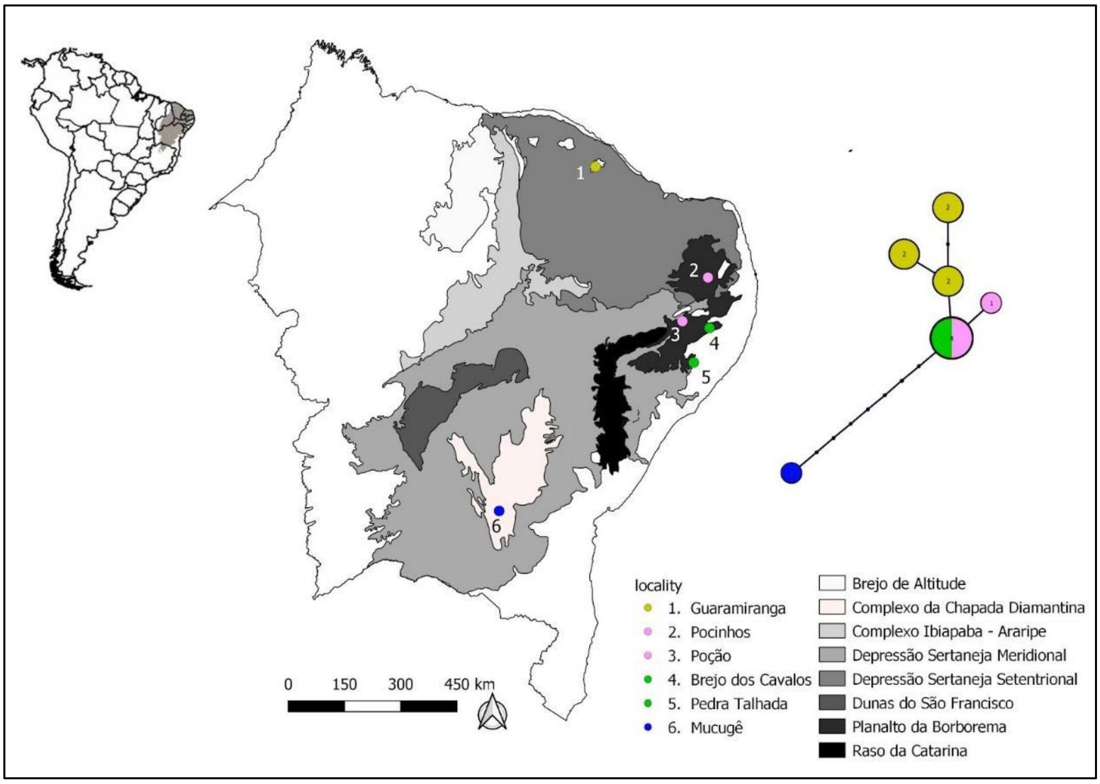

Fig. 2 a) Distribution of *Odontophrynus carvalhoi* sampled for this study in diferente ecoregions of the Brazilian Northeast. b) 16S haplotype network describing among the four diferente haplogropups. The colors used in the map to represent each group correspond to the colors used in the haplotype network. The points represent mutational Steps between haplotypes.

Discussion

Population structure is a well-documented phenomenon in amphibian literature (Elmer et al. 2007; Zeisset and Beebee 2008; Kaefer et al. 2013), revealing that populations often exhibit distributions where estimates of gene flow between them are nearly nonexistent. This research is the first to report genetic structuring of *Odontophrynus carvalhoi* populations, although our results should be interpreted with caution as they are based on the analysis of a single molecular marker.

With the data obtained from the genetic distance between groups, we found that *Odontophrynus carvalhoi* populations did not differ significantly when comparing distances between specimens (Magalhães et al. 2020). Vences et al. (2005), observed differences where levels of genetic divergence in lineages, populations, and sister species in temperate areas are consistently below the 3% threshold suggested for the 16S rDNA gene (Fromhage et al. 2003; Veith et al. 2003). As a result, the authors believe that this 3% threshold can be a useful tool for documenting the biodiversity of tropical toads in various situations.

One explanation for this low value may be due to the time required for genetic differentiation to occur between populations, as the 16S marker is a conserved marker with a low mutation rate (Vences et al. 2005; Faivovich et al. 2010). Compared to other markers, such as nuclear markers, it is widely used to analyze phylogenetic and phylogeographic relationships (Austin 2002; Ron et al. 2006; Vieira 2010).

The high haplotypic diversity and low nucleotide diversity for the mitochondrial 16S gene suggest that the population consists of a large number of closely related haplotypes, which can be expected after a recent demographic event, either a recent population expansion or contraction (Nyakaana et al. 2008; Gonçalves et al. 2009). This can be evidenced in the literature for other vertebrate groups (Grazziotin et al. 2006; Pellegrino et al. 2005; Bell et al. 2012), as well as invertebrates (Francisco 2016), where the low genetic diversity found would be due to recolonization of areas after a population bottleneck, and the low genetic diversities observed today would reflect the population expansion after this bottleneck.

Given this observed diversity, the presence of two groups in Chapada Diamantina and Brejos de Altitude with population structure was noteworthy. Thus, it is expected that with ongoing isolation and resulting long-term genetic drift, separation into new species will occur. Thus, it is important to highlight the presence of endemism in isolated locations (Pinheiro et al. 2014). Brejos de Altitude is known to have endemic amphibian species and harbors a representative biodiversity of anurans that occur in the state (Roberto and Loebbman 2016). Similarly, Chapada Diamantina is characterized by hosting species endemic to the region (Silva and Casteleti 2005).

While the other two, Planalto da Borborema and Mata Atlântica, still show haplotype sharing. In the latter case, the geographical proximity between locations may facilitate movement of individuals between populations, allowing gene exchange, as found for populations of Phyllopezus pollicaris in the Diagonal Seca and for two species of orchids distributed along the Northeastern inselbergs (Werneck et al. 2012; Pinheiro et al. 2014). Thus, the absence of geographical barriers in locations contributed to the lack of limitation on gene flow (Werneck et al. 2012; Pinheiro et al. 2014). Therefore, it would be relevant for future research to explore other more variable mitochondrial and nuclear markers for a better analysis of population structure and the evolutionary history of the species (Faivovich et al. 2010; Casu et al. 2011).

From the data obtained through the AMOVA analysis, it was observed that the greatest variation was between populations. Therefore, we affirm that this change indicates that these populations may be genetically distant. The survival of natural populations in the face of environmental changes depends on adaptations that are directly related to diversity (Frankham et al. 2008). According to Frankham et al. (2008), the greater the genetic variability within a population, the greater the chances of species perpetuation.

Arruda et al. (2017) analyzed populations of the species Proceratophrys moratoi in the Tietê River region, observing significant differences in genetic diversity between southern and northern-central populations, emphasizing the significant role of rivers (Tietê, Grande, and Paranaíba) as barriers to gene flow.

This study highlights the importance of molecular identification methods and genetic structure analysis for accurate estimates of biodiversity. In this perspective, according to Gamble et al. (2012), the identification of divergent evolutionary lineages within a species has important conservation implications, contributing to increased biodiversity levels in a region and a more accurate distribution of recently described species within the species complex.

Conclusion

Our results indicate that the species *Odontophrynus carvalhoi* does not present a distinct lineage, the populations distributed in the Planalto da Borborema and the Mata Atlântica root in the same group, presenting the sharing of the same haplotypes, revealing gene sharing, thus showing absence of population structure.

The populations of the Brejos de Altitude and the Chapada Diamantina present greater genetic distance, revealing population structure for both with the presence of a unique population stock.

Finally, for obtaining more specific results regarding genetic diversity indices, population structure, and demographic history for the species, it would be relevant to continue studies using more variable mitochondrial and nuclear markers, as well as morphological and bioacoustic analyses.

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Author contributions

TFQ, RWA, and RP: conceived and designed this study. TFQ, and RWA: conducted fieldwork to obtain tissue samples. MOA, and TFQ: conducted laboratory work, MOA, TFQ, and RP: analyzed the data, and TFQ: prepared the map and figures. TFQ: wrote the manuscript with significant input from all co-authors. All authors reviewed the manuscript and approved the submitted version.

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Declarations

Conflicts of interest

The authors declare no commercial or financial conflicts of interest.

Ethics approval

The samples were collected under SISBIO (ICMBio) license 29613, and fieldwork followed protocols and guidelines by ICMBio of the Ministry of Environment of Brazil.

6 CONSIDERAÇÕES FINAIS

Variações morfológica, genéticas, acústica, assim como os recentes estudos sobre condrocrânio de girinos, nos fornecem dados entre populações ao longo de um gradiente geográfico, e estes pode ocorrer em maior ou menor grau e, geralmente, quanto mais distantes estiverem as populações, ou isoladas em vales ou áreas montanhosas, maiores serão as diferenças que podemos evidenciar. Atualmente, estudos sobre sistemática e filogenética são cada vez mais importantes para auxiliar no processo de descobertas de novas espécies, ajudando a conhecer a real biodiversidade existente, pricipalmente em áreas isoladas como brejos-de-altitudes.

Este trabalho contribuiu para reduzir a lacuna de conhecimento em torno da real diversidade existente na família Odontophrynidae através da descrição de uma nova espécie de *Proceratophrys* para os brejos cearenses, bem como, a descrição comportamental na época reprodutiva. Assim, aumentado a riqueza de espécies e o número de anfíbios endêmicos dessas regiões.

Já para a espécies de *Odontophynus carvalhoi* podemos estabelecer uma estrutura populacional genética, revelando o compartilhamento de genes entre espécies geograficamente próximas, realizamos também uma ampliação sobre os helmintos que infectam uma população de um brejo-de-altitude. Desta forma, fornecemos mais elementos que corroboram a importância da conservação dos brejos inseridos na Caatinga.

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