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1916 • 2021

An Acad Bras Cienc (2021) 93(4): e20201650 DOI 10.1590/0001-3765202120201650
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

ANIMAL SCIENCE

Cytogenetic approaches provide evidence of a conserved diploid number and cytological differences between *Proceratophrys* species (Anura: Odontophrynidae)

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Abstract: Taxonomic and cytogenetic aspects of *Proceratophrys* have not been thoroughly clarified in the literature; thus, the objective of the present study was to provide unprecedent karyotype data regarding *P. schirchi*, *P. laticeps* and *P. melanopogon*. Additionally, the karyotype of *P. boiei* (2n = 22) and its ZZ / ZW sex chromosome system was analyzed for different populations of southeastern and southern Brazil. All *Proceratophrys* species have a diploid number of 2n = 22 chromosomes. In *P. schirchi*, a strong C-band was found in the long arm in one of the homologues of the pair 8 in female metaphasic cells, denoting that this chromosome pair could represent the heteromorphic sex chromosome in a ZZ / ZW sex system. Despite the conserved number of chromosomes, there are considerable chromosomal differences in *P. melanopogon* and *P. boiei* (southern Brazil), strongly differentiating them cytogenetically from other species of the genus. Moreover, with the confirmation of chromosomal heteromorphism related to sexual differentiation in *P. boiei* and the possible description of this system in *P. schirchi*, the *Proceratophrys* genus can be regarded as an interesting group for evolutionary studies and sex chromosome differentiation in anurans.

Key words: Amphibia, C-banding, Chromosome evolution, Nucleolar organizing regions, Sex chromosomes.

INTRODUCTION

Proceratophrys is the most representative genus of the Odontophrynidae, currently comprising 41 species of small to medium-sized frogs. They are distributed from eastern to southern Brazil; northeastern Argentina and Paraguay; possibly extending into Bolivia adjacent to the Brazilian border (Frost 2021). However, the number of *Proceratophrys* species is probably underestimated, as indicated by the constant discovery and description of new entities (Prado & Pombal 2008, Martins & Giaretta 2011, 2013, Cruz et al. 2012, Brandão et al. 2013, Dias et al. 2013, Godinho et al. 2013, Mângia et al. 2014, 2018, 2020).

Based on morphological similarities, some species of *Proceratophrys* Miranda-Ribeiro, 1920 genus have been grouped together into different species complexes, whereas others, due to the presence of peculiar characteristics, do not belong to any group (Prado & Pombal 2008, Amaro et al. 2009, Cruz et al. 2012, Teixeira Jr. et al. 2012, Dias et al. 2013, Mângia et al. 2018, 2020).

The *P. boiei* complex includes species that have a single long eyelid appendix without triangular rostral appendix. It includes the species *P. boiei* (Wied-Neuwied, 1824), *P. paviotii* Cruz, Prado and Izecksohn, 2005, and *P. renalis* (Miranda-Ribeiro, 1920). Species of the *P. appendiculata* complex also have single and long eyelid appendix; however, unlike the *P. boiei* group, they present a triangular rostral appendix. It includes the species *P. appendiculata* (Günther, 1873), *P. belzebul* Dias, Amaro, Carvahlo-e-Silva, and Rodrigues, 2013, *P. gladius* Mângia, Santana, Cruz, and Feio, 2014, *P. itamari* Mângia, Santana, Cruz, and Feio, 2014, *P. izecksohni* Dias, Amaro, Carvahlo-e-Silva, and Rodrigues, 2013, *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 and *P. subguttata* Izecksohn, Cruz and Peixoto, 1999.

The species *P. minuta* Napoli, Cruz, Abreu and Del Grande, 2011, *P. redacta* Teixeira, Amaro, Recoder, Vechio and Rodrigues, 2012, *P. rondonae* Prado and Pombal, 2008 and *P. schirchi* (Miranda-Ribeiro, 1937) are not associated with any of these complexes due to peculiar characteristics that do not fit in any of them (Prado & Pombal 2008, Napoli et al. 2011, Teixeira et al. 2012, Mângia et al. 2014, Frost 2021).

Recently, Mângia et al. (2020) proposed a change in the species number for the *Proceratophrys cristiceps* group. The authors reviewed the taxonomic status of *Proceratophrys* populations in the Caatinga biome based on morphological, morphometric, acoustic and multilocal genetic data, contributing to taxonomic and nomenclatural stability when describing new taxa and synonymization of other species. Without evidence supporting *P. aridus* and *P. caramaschii* as distinct species, they were characterized as junior synonyms of *P. cristiceps* (Mângia et al. 2020).

On the other hand, Magalhães et al. (2020) considered *Odontophrynus salvatori* Caramaschi, 1996 as belonging to the genus *Proceratophrys* and included this species in the *P. cristiceps* group, reinforcing the importance of several lines of evidence to avoid taxonomic instability. In this sense, cytogenetic data can provide relevant information about the chromosomal structure of taxon populations.

Cytogenetic data for *Proceratophrys* are restricted to the species *P. boiei* and *P. appendiculata* having a diploid number of 2n = 22 chromosomes (King 1990, Kuramoto 1990, Ananias et al. 2007, Amaro et al. 2012). However, *P. boiei* cytogenetics studies are slightly advanced in comparison to the other species. C-banding, Ag-NOR and molecular mapping of telomeric sequences were performed in the species, revealing different heterochromatin content and NOR positions between Brazilian southern and southeastern populations. Moreover, the occurrence of a ZZ / ZW sex system for this species (Ananias et al. 2007, Amaro et al. 2012, Da Silva et al. 2020) was reported. Recently, high-throughput analyzes were performed for *P. boiei* through genomic sequencing and bioinformatic analyzes, resulting in a large number of repetitive sequences isolated from its genome. Especially satellite DNAs sequences were deemed involved in heterochromatin formation and maintenance in the species (Da Silva et al. 2020).

Proceratophrys melanopogon species is distributed along Serra do Mar range in the southern, central, and northern portions of Rio de Janeiro state and in the east of São Paulo state, Brazil (Frost 2021), and is ecologically restricted to high altitude areas (Mângia et al. 2010). *Proceratophrys schirchi* and *P. laticeps* occurs in southeastern Bahia, Espírito Santo, northeastern Minas Gerais, and Rio de Janeiro (Frost 2021). *Proceratophrys boiei* occurs in southern Espírito Santo, southern Minas Gerais, in the western region of Rio de Janeiro, southern São Paulo, and from eastern Paraná to eastern Santa Catarina (Frost 2021).

Thus, considering the scarcity of cytogenetic studies on *Proceratophrys* in general and the increased number of newly-described species for the genus, the present study compared for the first time the chromosomes of *P. melanopogon* and *P. laticeps* (from *Proceratophrys appendiculata* complex), and *P. schirchi* (no defined group), increasing the number of karyotyped *Proceratophys* species reported in the literature. In addition, the *P. boiei* (*Proceratophrys boiei* group) karyotype was reevaluated, bringing new information about its chromosomal characteristics. Comparisons between already described karyotyped species were also carried out, providing relevant contributions to the understanding of chromosome evolution regarding this anuran group.

Abbreviations

NOR – Nucleolar Organizer Region;

Ag-NOR – Silver-positive NOR;

ICMBio – Chico Mendes Institute of Biodiversity Conservation;

CEUA – Ethics Committee on Animal Use;

FAPESP – Sao Paulo Research Foundation;

CAPES – Higher Education Improvement Coordination;

CNPq – National Council for Scientific and Technological Development.

MATERIALS AND METHODS

The individuals analyzed were collected in the wild under collection licenses issued by the Chico Mendes Institute for Biodiversity Conservation (ICMBio) protocol Nos. 59449 and 60972 (see Table I and Figure 1 for collection locations). The euthanasia of the specimens was performed under the consent and approval of the Ethics Committee on Animal Use - CEUA (permission 004752/2017), Biosciences Institute, UNESP, Rio Claro, SP, Brazil. The animals were deposited in the Célio F. B. Haddad (CFBH) amphibian collection, housed in the Department of Biodiversity, Biosciences Institute, UNESP, Rio Claro, SP, Brazil, except for the *P. boiei* species from the south of Brazil.

Metaphasic chromosomes were obtained from intestinal epithelial cells according to the protocol established by Schmid (1978), and the bone marrow and liver were collected according to Baldissera et al. (1993). The material was stained with 10% Giemsa solution, the nucleolus organizing regions (NORs) were identified through silver impregnation (Howell & Black 1980) and constitutive heterochromatin was detected using the C-banding technique according to Sumner (1972). Giemsa-stained metaphases were photographed using an Olympus BX61 microscope and black-and-white images were recorded using a DP71 cooled digital camera. The chromosomes were ordered in decreasing size and their morphology was determined based on the metacentric, submetacentric, and subtelocentric parameters (Guerra 1986). The metaphase images were optimized in brightness and contrast using Adobe Photoshop CS6 program and arranged using Corel Draw X7 software.

RESULTS

The diploid number found for the species *P. schirchi* and *P. laticeps* was 2n = 22 chromosomes in mitotic cells for both sexes. The karyotype of the analyzed specimens of *P. schirchi* and *P. laticeps* consisted of large- and medium-sized pairs of metacentric chromosomes (1, 6, 7, 8, 9, 10,

Table I. **Species of *Proceratophrys* groups used in this study, with localizations of collected and number of species utilized. M: male; F: female.**

Species	Sex	Collecting Locations (Brazil)
***Proceratophrys appendiculata* group**		
P. laticeps	1 M	Santa Teresa, Espírito Santo
P. melanopogon	1 M	Mogi das Cruzes, São Paulo
***Proceratophrys boiei* group**		
P. boiei	9 M 1 F	Mogi das Cruzes, São Paulo
	1 M 5 F	Camanducaia, Minas Gerais
	1 F	Tijucas do Sul, Paraná
	5 M 2 F	Morretes, Paraná
	2 M 2 F	São José dos Pinhais, Paraná
No group defined		
P. schirchi	1 M 1 F	Santa Teresa, Espírito Santo

and 11), submetacentric chromosomes (2, 3, and 5) and a subtelocentric chromosome (4) (Fig. 2a and b). In both species, a secondary constriction was found in the short arm of chromosome pair 8, adjacent to the centromere, coincident with the NOR (Fig. 2a and b). Heterochromatin blocks were detected by C-banding in the centromeric region of *P. schirchi* (Fig. 3c) and *P. laticeps* (Fig. 3b) chromosomes; however, in female chromosomes of *P. schirchi*, a strong C-band block on the long arm of one of the chromosomes in pair 8 was observed, indicating that this pair of chromosomes could represent a sex determination system of the ZZ / ZW type for *P. schirchi* (Fig. 3c, highlighted arrows).

The diploid number found in *P. melanopogon* was 2n = 22 in mitotic cells, and the karyotype consisted of large- and medium-sized pairs of metacentric chromosomes (1, 6, 7, 8, 9, 10, and 11), submetacentric chromosomes (2, 3, and 5), and a subtelocentric chromosome (4) (Fig. 2c). Unlike the other two species, in *P. melanopogon* a secondary constriction was found in the short arm of pair 4, where NOR is also located (Fig. 2c). Heterochromatin blocks were limited to the centromeric region of the chromosomes (Fig. 3a).

The *P. boiei* karyotype of the population of the Camanducaia, State of Minas Gerais, Mogi das Cruzes, state of São Paulo, and Tijucas do Sul, São José dos Pinhais, and Morretes, State

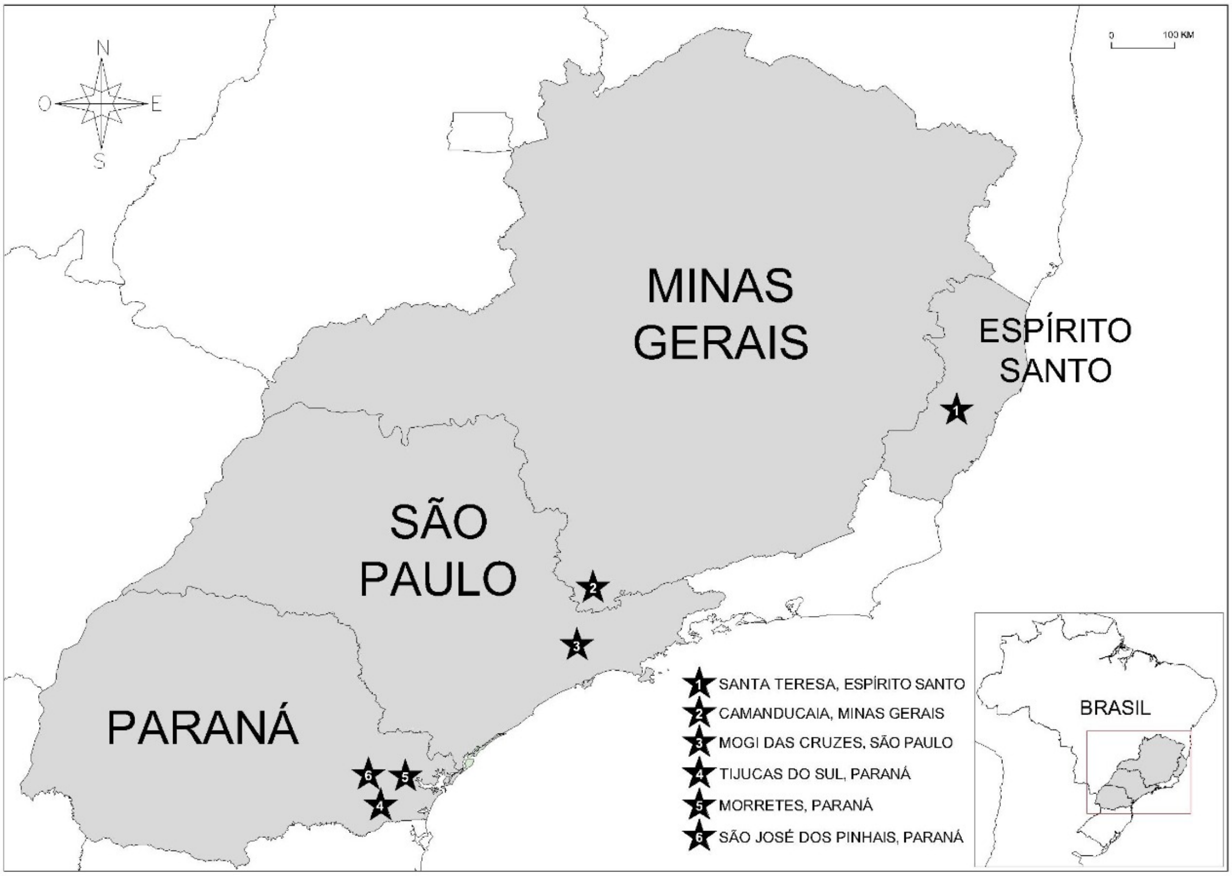

Figure 1. The collecting localities of the specimens analyzed in the present study.

of Paraná also consisted of large- and medium-sized pairs of metacentric chromosomes (1, 6, 7, 8, 9, 10, and 11), submetacentric chromosomes (2, 3, and 5), and a subtelocentric chromosome (4) in both sexes (Fig. 2d and e). However, the NOR was located in the short arm of pair 8 submetacentric in an interstitial position for localities of the Southeast (Camanducaia and Mogi das Cruzes) (Fig. 2d). Differently, in the Southern localities (Tijucas do Sul, São José dos Pinhais, and Morretes) the NOR was located in the short arm of pair 4 subtelocentric in an interstitial position (Fig. 2e).

A large number of constitutive heterochromatins in the centromeric and pericentromeric regions of all the chromosomes of *P. boiei* for the localities of Southeastern Brazil was observed. In addition, the results revealed the presence of a chromosome completely heterochromatic in only one of the homologues of pair 1 in females, described as being a sex-related chromosomal heteromorphism (Fig. 3e and f). In the populations of southern Brazil, blocks of constitutive heterochromatin restricted to the centromeric region of all the chromosomes in both sexes were identified, without evidence of chromosomal heteromorphism related to sex differentiation (Fig. 3g and h).

DISCUSSION

Conserved Diploid Number

All the species analyzed in the present study have a diploid number of 2n = 22 chromosomes, corroborating the data already described cytogenetically for *P. boiei* and *P. appendiculata*

Figure 2. **Karyotypes of *Proceratophrys* species with Giemsa-stained. a) *P. schirchi* (Santa Teresa, state of Espírito Santo). b) *P. laticeps* (Santa Teresa, state of Espírito Santo). c) *P. melanopogon* (Mogi das Cruzes, state of São Paulo). d) *P. boiei* (Camanducaia, state of Minas Gerais and Mogi das Cruzes, state of São Paulo). e) *P. boiei* (Tijucas do Sul, São José dos Pinhais, and Morretes, state of Paraná). Boxes refers to Nuclear Organizer Region impregnated with Silver Nitrate.**

(King 1990, Ananias et al. 2007, Amaro et al. 2012). Chromosome number has already been considered as a primary difference between taxa in systematic studies of anuran amphibians, in which closely related species have more similar karyotypes than relatively distinct species (King 1990). It was possible to highlight a highly conserved karyotype, with a similar karyotype formula and differences only regarding specific interpopulation chromosome banding in *P. boiei*, without changes in the diploid number.

It is important to note that some widely distributed anuran species, like *P. boiei*, usually present morphological, bioacoustics, and genetic variations along their geographical distributions, which suggests that more than one taxonomic entity is being treated under one specific name. The fact that different *P. boiei* populations have significant cytogenetic differences was previously reported by Amaro et al. (2012) and, in the present study, with the analysis of other *P. boiei* populations, we reinforce the idea that there is a species complex under the name of *P. boiei* and, therefore a thorough taxonomic revision for the genus is required.

Constitutive Heterochromatin and Heteromorphic Sex Chromosomes

The presence of heteromorphic sex chromosomes is a rare feature in anurans, and the difference between the chromosome of the sex pair is often only recognized after the use of differential staining techniques, such as C-banding, to show heterochromatic regions. To date, only 4% of the cytogenetically known species have sex chromosomes with some degree of heteromorphism (Schartl et al. 2016). Remarkably, these few species show a prevalence of larger Y and W chromosomes with various levels of heterochromatin accumulation (Schmid et al. 2010, Schartl et al. 2016). Nevertheless, species with either XX / XY or ZZ / ZW sex systems, and even multiple sex chromosomes have been found, presenting a Y or W chromosome with an accumulation of heterochromatin (Schmid et al. 2003, 2012, Busin et al. 2008, Nascimento et al. 2010, Schartl et al.

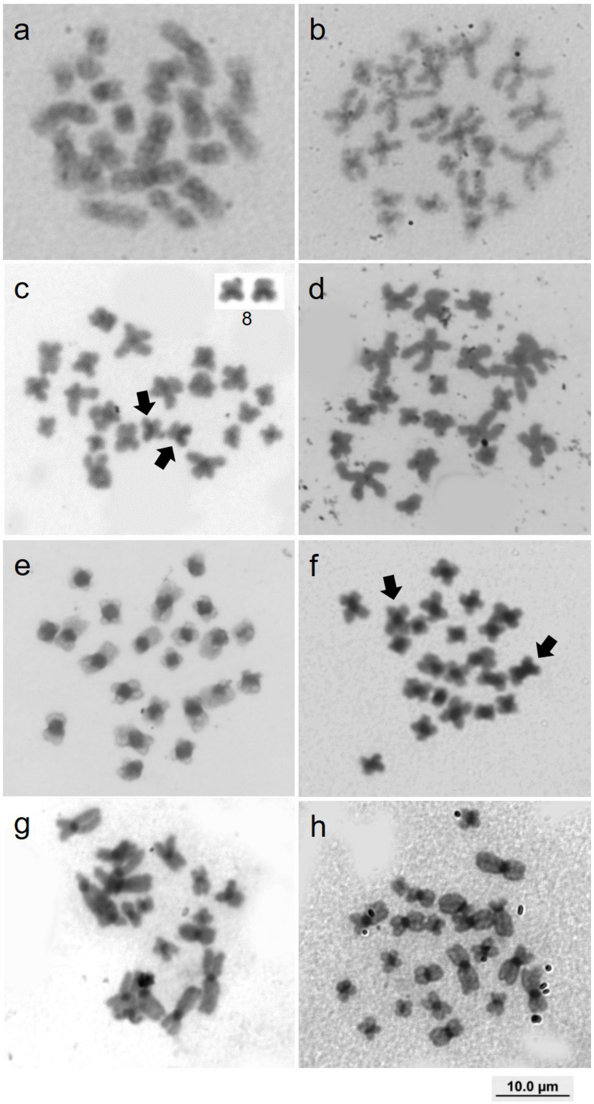

Figure 3. **C-banding in *Proceratophrys* species. a) *P. melanopogon* (Mogi das Cruzes, state of São Paulo). b) *P. laticeps* (Santa Teresa, state of Espírito Santo). c) *P. schirchi* (Santa Teresa, state of Espírito Santo) female, and d) *P. schirchi* male. e) and f) *P. boiei* (Female and Male, respectively) (Camanducaia, state of Minas Gerias and Mogi das Cruzes, state of São Paulo). g) and h) *P. boiei* (Female and Male, respectively) (Tijucas do Sul, São José dos Pinhais, and Morretes, state of Paraná). The box in c) and the arrows in f) indicate the heteromorphic sex chromosomes in *P. schirchi* (pair 8) and *P. boiei* (pair 1), respectively.**

2016, Gatto et al. 2016, Gazoni et al. 2018, Da Silva et al. 2020).

Interpopulation analysis of *P. boiei* specimens from the southeastern and northeastern of Brazil revealed unusual heterochromatic patterns between populations (Ananias et al. 2007). Populations of *P. boiei* of the southeast have large centromeric and pericentromeric heterochromatic blocks, while samples from the northeast did not present these features. In addition, females from São Paulo State (southeast) have a large heterochromatic block in one of the homologous of the chromosomal pair 1, while males have heterochromatin restricted to the centromeric region in both chromosomes. This unusual heterochromatic arrangement led Ananias et al. (2007) to propose a ZZ / ZW sex system for *P. boiei*, the first occurrence registered within Odontophrynidae. The same chromosomal heteromorphism was detected in individuals of other locations within the geographical range of the state of São Paulo, the ZZ / ZW sex chromosome system seems to be generalized in *P. boiei* (Amaro et al. 2012, Da Silva et al. 2020).

This karyotypic composition for *P. boiei* was confirmed in the present study for the sample of Mogi das Cruzes (São Paulo state) and Camanducaia (Minas Gerais state), in which the chromosomal set of the analyzed specimens are also heteromorphic in females, further highlighting the generalization of chromosomal heteromorphism related to sex in *P. boiei*. However, for populations of *P. boiei* from southern Brazil analyzed in this work, a completely different pattern from that observed in southeastern populations was found especially regarding the size and number of heterochromatic blocks and the presence of chromosomal heteromorphism related to sexual differentiation.

In the analyzed *P. boiei* individuals, the positive C-band regions were limited to the centromeric region and none showed heteromorphic chromosomes. This pattern has already been described by Amaro et al. (2012);

however, for populations from the south of São Paulo (southeastern Brazil) and one from Santa Catarina (southern Brazil). In this work, samples of *P. boiei* from southern populations in Brazil were analyzed and the results revealed the same distribution pattern of constitutive heterochromatin, restricted to the centromeric region, suggesting that this feature is consolidated in all *P. boiei* samples from the southern region.

Intraspecific variation in C-band pattern can be found, for example, among distinct populations of the same species (Amaro-Ghilardi et al. 2004, Silva et al. 2004), as in *P. boiei* (Amaro et al. 2012). For the *Proceratophrys* species analyzed in the present study, there were no interstitial and terminal markings, except for the unusual pattern already described for *P. boiei* and, in this sense, C-bands can be potentially used to identify species in this genus, and more studies based in this technique must be performed within *Proceratophrys*.

The results provide the first description of C-banding in *P. schirchi*, *P. laticeps*, and *P. melanopogon*, which showed C-positive bands predominantly located as discrete blocks in the centromeric region of the chromosomes. However, differently from *P. laticeps* and *P. melanopogon*, the *P. shirchi* female analyzed here presents a homologous chromosome with a large heterochromatic block throughout the long arm (pair 8), which not present in male chromosomes, leading us to think of the presence of ZZ / ZW sex system in this species. Sex-related heteromorphism following C-banding treatment has been reported in anurans (Ananias et al. 2007, Busin et al. 2008, Nascimento et al. 2010) and it is interesting to note that in some cases of sexual differentiation in amphibians, the W chromosome may be completely or partially heterochromatic. The putative distinct ZZ / ZW sex system in *P. schirchi*, reinforces the idea that heterochromatinization is a process that precedes the morphological differentiation of W and Y chromosomes, and that the stages of morphological differentiation of sex chromosomes in anuran species is important not only for amphibian cytogenetics but also for understanding the evolution of vertebrate sex chromosomes as a whole (Ananias et al. 2007).

Nucleolar organizer regions (NORs)

Despite the conserved diploid number in *Proceratophrys*, a difference in chromosomal formula was observed in *P. melanopogon*. An atypical secondary constriction was found in the interstitial position on the short arm of pair 4, differing from *P. schirchi*, *P. laticeps*, and *P. boiei*, whose constriction is in pair 8. Coincidentally, in *P. melanopogon* the NOR was located next to the secondary constriction found in this species. However, *P. boiei* (populations from southeastern Brazil) also showed NOR located in pair 8; however, but for the southern populations, it was present in pair 4, revealing that the different locations of NORs in this species may further contribute to characterize populations and provide relevant information for taxonomic studies. Ananias et al. (2007) found heteromorphic NORs in chromosomal pair 8 in two *P. boiei* specimens from Mata de São João, State of Bahia, Brazil, a fact that reinforces this location and position of NORs in *P. schirchi* and *P. laticeps*. However, we found a different position in *P. melanopogon* and *P. boiei* (Southern populations) in the present work, despite the fact that these are closely related species.

The NOR could appear either in the short arms, in the long arms, in the terminal, distal, interstitial, or proximal positions. However, it may occur in other chromosomal pairs, as observed for *P. melanopogon* and *P. boiei* (southern populations), where the NOR was presented in pair 4. This result suggests that micro rearrangements such as deletion/duplication of chromosomal segments or inversions may have occurred independently in this species. In this sense, a case of multiple NOR was reported for *P. boiei* from a population of Iperó, São Paulo, where an individual showed a standard NOR for the species in pair 8 and another NOR in pair 4 (Amaro et al. 2012), further reinforcing that chromosomal rearrangements have been occurring independently of the *Proceratophrys* species.

The results provided by the present study increase the number of species with known karyotypes in *Proceratophrys* and include the first karyotype description for *P. schirchi*, *P. laticeps*, and *P. melanopogon*, contributing to a better understanding of chromosomal evolution and providing input for future taxonomic and cytogenetic studies in this group of anurans. Although there is a conserved pattern related to the number of chromosomes, there are considerable chromosomal differences in *P. melanopogon* and *P. boiei* (southern Brazilian populations), substantially differentiating them from the other species. Moreover, with the confirmation of chromosomal heteromorphism related to sexual differentiation in *P. boiei* and the possible description of this system in *P. schirchi*, the *Proceratophrys* genus could be considered an interesting group for studies on the evolution and differentiation of sexual chromosomes.

Acknowledgments

The authors are grateful to Prof. Dr. Sanae Kasahara (*in memorian*) for providing part of cytogenetic material used in this work. We are also grateful to colleagues Hideki Narimatsu and Paulo Sérgio Pereira dos Santos for their help in specimen collection, to Prof. Dr. Daniel Pacheco Bruschi for kindly preparing cytogenetic material from *P. boiei* of South populations, to MSc. Carolina Crepaldi for the help in revising the manuscript and to Adalberto Mota Silva de Oliveira for his help with making the species location map. This research was supported by Sao Paulo Research Foundation (FAPESP #2017/00195_7). Studentship was granted by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Financing code 001. CFBH is grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the research fellowship (306623/2018-8), and FAPESP, for the financial support (#2013/50741-7). The authors declare no competing interests.

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How to cite

SILVA MJ, SANTOS MD, GAZONI T, CHOLAK LR, HADDAD CFB & PARISE-MALTEMPI PP. 2021. Cytogenetic approaches provide evidence of a conserved diploid number and cytological differences between Proceratophrys species (Anura: Odontophrynidae). An Acad Bras Cienc 93: e20201650. DOI 10.1590/0001-3765202120201650.

Manuscript received on October 19, 2020;
accepted for publication on February 17, 2021

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MJS analyzed the data and wrote the manuscript. MJS, MDS, TG and LRC conducted the classic cytogenetic analyses. MJS, TG, LRC and CFBH collected the specimens. CFBH identified the specimens. PPPM coordinated the research and revised the manuscript. All authors corrected, revised and discussed the data.