An Acad Bras Cienc (2021) 93(2): e20190426 DOI 10.1590/0001-3765202120190426
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

ECOSYSTEMS

Sex chromosomes in the Vizcacheras' White-lipped frog, *Leptodactylus bufonius* (Anura, Leptodactylidae)

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Abstract: Cytogenetic analyses were performed on specimens of *Leptodactylus bufonius* from different localities in Argentina. Mitotic chromosomes were studied with Giemsa and differential staining techniques (Ag-NOR, C-banding, and CMA_3/DAPI) and fluorescence *in situ* hybridization with the 18S DNAr probe. All specimens showed karyotypes with 2n = 2x = 22 and FN = 44. Secondary constrictions were present in the long arm of chromosome pair 8, coincident with Ag-NOR and hybridization signals of the 18S DNAr probe. The C-banding technique evidenced an important amount of heterochromatin with a sex-linked pericentromeric band in the short arm of chromosome pair 4. This heterochromatic band was heteromorphic in males but present in both homologues of females, and it was CMA_3 positive (DAPI negative) at fluorescence staining. The occurrence of heteromorphic XY sex chromosomes in *L. bufonius* is the second known case in *Leptodactylus* and the fifth within the speciose family Leptodactylidae.

Key words: C-banding, cytogenetics, heterochromatin, heteromorphism, XY.

INTRODUCTION

The diverse and complex ways of sex determination among different vertebrate taxa constitute an interesting research field that is currently focused on revealing genes and mechanisms involved, as well as chromosome locations (Bull 1983, Valenzuela 2008). To date, this is a poorly studied topic in Anura, and those species with already identified sex chromosomes are just a small proportion as compared with other vertebrate orders (Miura 2017). Besides, the characteristics of such phenomena and associated evolutionary histories are barely known (Schmid 1983, Schmid et al. 2010, Uno et al. 2015).

However, an extraordinary variety of sex chromosome systems were described in anurans (Odierna et al. 2007, Nascimento et al. 2010, Schmid et al. 2010, Saba & Tripathi 2014, Patawang et al. 2014, Gazoni et al. 2018, Sangpakdee et al. 2017). While most species show simple heteromorphic chromosomes (XY, ZW), there are also complex mechanisms, as W0 founded in *Leiopelma hochstetteri* (Green 1988) or the multiple sex chromosomes observed in *Leptodactylus pentadactylu*s (Gazoni et al. 2018). Other extreme examples are *Xenopus tropicalis* (Roco et al. 2015) and *Glandirana rugosa* (Miura & Ogata 2013), in which different sex-determining systems coexist: XY, ZW, and non-differentiated sex chromosomes. Moreover, sex chromosome polymorphisms were also observed in *Gastrotheca pseustes*, with two different Y-chromosome morphs (Schmid et al. 1990). The impressive variation of sex

determination systems in anurans, also reported at different morphological differentiation stages, makes this group an excellent target for studying sex chromosomes origin and evolution in vertebrates.

Most sex chromosomes already identified in anuran amphibians are microscopically indistinguishable (homomorphic) when they are studied with conventional staining techniques (Hillis & Green 1990, Schmid 1983, Schmid & Steinlein 2001, Eggert 2004). Because of this, some researchers employed traditional cytogenetic markers such as C-bands, Ag-NOR, replication banding patterns, and DNA base-specific fluorochromes (see Schmid et al. 2010 for a review). More recently, modern molecular cytogenetic techniques like comparative genomic hybridization and chromosome mapping of repetitive sequences with fluorescent *in situ* hybridization have been used (Abramyan et al. 2009, Vittorazzi et al. 2014, Gatto et al. 2016, 2018, 2019).

Heterogametic Y or W chromosomes that are morphologically identical to their counterparts (X and Z, respectively) would indicate that they still did not develop supramolecular evident differences at the chromosomal level, representing a primitive stage of evolution of the sex chromosomes (Schmid 1990, Schartl et al. 2016). It has been suggested that this feature could be related to the dynamics of sex-determining genes when they fail to be anchored to a specific chromosome, determining a continuous process of replacement called "turnover of sex-determining genes and sex chromosomes" (Schartl 2004, Sarre et al. 2011, Miura 2017). Furthermore, other processes could be responsible for the prevalence of homomorphic sex chromosomes in anurans like the "fountain of youth" hypothesis (Perrin 2009), in which occasional sex reversion events may occur (XY females or ZW males), enhancing the recombination in sex-specific regions and preventing the accumulation of deleterious mutations.

The family Leptodactylidae currently comprises 231 species (Frost 2021), distributed in three subfamilies: Leptodactylinae, Leiuperinae, and Paratelmatobiinae. Within the first, the Neotropical genus *Leptodactylus* is a natural group of 82 currently recognized species (Frost 2021), clustered in the *L. fuscus*, *L. latrans*, *L. melanonotus*, and *L. pentadactylus* groups (Heyer 1969). *Leptodactylus bufonius* of the *L. fuscus* group is one of the most abundant species in the semi-arid environments of the American Gran Chaco, and surrounding areas in northern Argentina, Paraguay, southeastern Bolivia, and the states of Mato Grosso and Mato Grosso do Sul in central Brazil (Heyer 1978, Schalk & Leavitt 2017, Brusquetti et al. 2019). Like other species of the *L. fuscus* group, *L. bufonius* presents burrowing habits, and males build subterranean chambers in moist soil near streams or ponds for the incubating foam nests produced during amplexus (Gallardo 1964, Maxson & Heyer 1988, Ponssa 2008, Faggioni et al. 2017).

There is a large amount of information about chromosome data in *Leptodactylus* that covers near half of its 82 recognized species, with a widespread characteristic karyotype of 2n = 2x = 22 and bi-armed chromosomes (FN = 44) (Brum-Zorrilla & Sáez 1968, Bogart 1974, Heyer & Diment 1974, Silva et al. 2000, 2004, 2006, Amaro-Ghiraldi et al. 2004, 2006, Arruda & Morielle-Versute 2008, de Oliveira et al. 2012, Gazoni et al. 2018, 2021, de Oliveira et al. 2013, Coelho et al. 2016, Gonzalez et al. 2016). To date, there is a single report of chromosomic sex determination in these frogs, described in *L. pentadactylus*, with a mechanism involving multiple sex chromosomes (Gazoni et al. 2018).

Cytogenetic studies in *Leptodactylus bufonius* are mainly restricted to the description

of its diploid number, based only on few specimens of a small portion of its geographic distribution in Argentina (Barbieri 1950, Brum-Zorrilla & Sáez 1968, Bogart 1974, Heyer & Diment 1974). In the present study, we make a thorough characterization of cytogenetics in this species with the aid of different banding techniques in specimens from several localities in Argentina. We also describe a new case of heteromorphic sex chromosomes in Anura, which constitutes the second known in the genus *Leptodactylus*.

MATERIALS AND METHODS

We analyzed 41 specimens of *Leptodactylus bufonius* (21 males, 20 females; Fig. 1, details in Appendix). Mitotic metaphases were obtained from cell suspensions of bone marrow and intestinal epithelium, using the protocol described by Schmid et al. (2010). Slides were conventionally stained with 10% phosphate-buffered Giemsa (pH 6.8). The nucleolar organizer regions (NORs) were detected by Ag-NOR staining according to Howell & Black (1980) and by fluorescent *in situ* hybridization (FISH) with the ribosomal 18S biotinylated probe (Pinkel et al. 1986). Location of heterochromatin was determined using standard C-banding technique (Sumner 1972), and the composition of heterochromatic AT-rich and GC-rich bands was evidenced, respectively, with the fluorochromes DAPI (4', 6-diamidino-2-phenylindole) and CMA_3 (Chromomycin A3) (Schweizer 1976). Karyotypes were arranged in decreasing size, according to the nomenclature proposed by Green & Sessions (1991, 2007). Assessment of chromosome size and morphology was performed with DRAWID v0.26 software (Kirov et al. 2017).

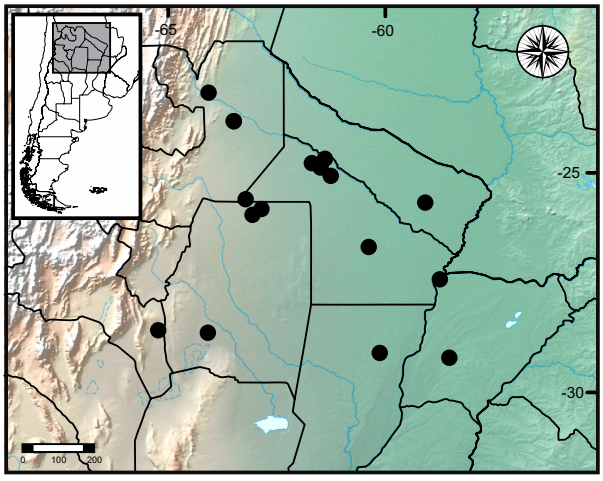

Figure 1. Sampling localities of *Leptodactylus bufonius* analyzed.

RESULTS

The karyotype of *Leptodactylus bufonius* is composed of 11 pairs of bi-armed chromosomes (2n = 2x = 22; FN = 44), arranged in seven pairs of large and medium-sized chromosomes and four small ones. Pairs 1, 2, 5, 6, 8–11 are metacentric, pair 3 is submetacentric, whereas pairs 4 and 7 are subtelocentric (Fig. 2; Table I). NOR sites were identified in the interstitial region of the long arm of both homologues of pair 8 (Fig. 3), according to silver staining and hybridization signals of the 18S DNAr after FISH experiments, in coincidence with secondary constrictions.

The C-banding pattern revealed heterochromatin in the centromeric region of all chromosomes, with a large number of additional heterochromatic bands (Fig. 2b and d). Interstitial C-bands were observed on the short arm of pairs 1, 2, 5, 6, 8, and 9, and on the long arm of pairs 3, 5–9, with a conspicuous heterochromatic band on both arms of pair 6. Pericentromeric C-bands were detected on the short arm of pair 7 and the long arm of pairs 4 and 10.

Chromosome pair 4 showed a pericentromeric C-band on the long arm of both homologues, although a sex-biased heteromorphism was observed for the presence

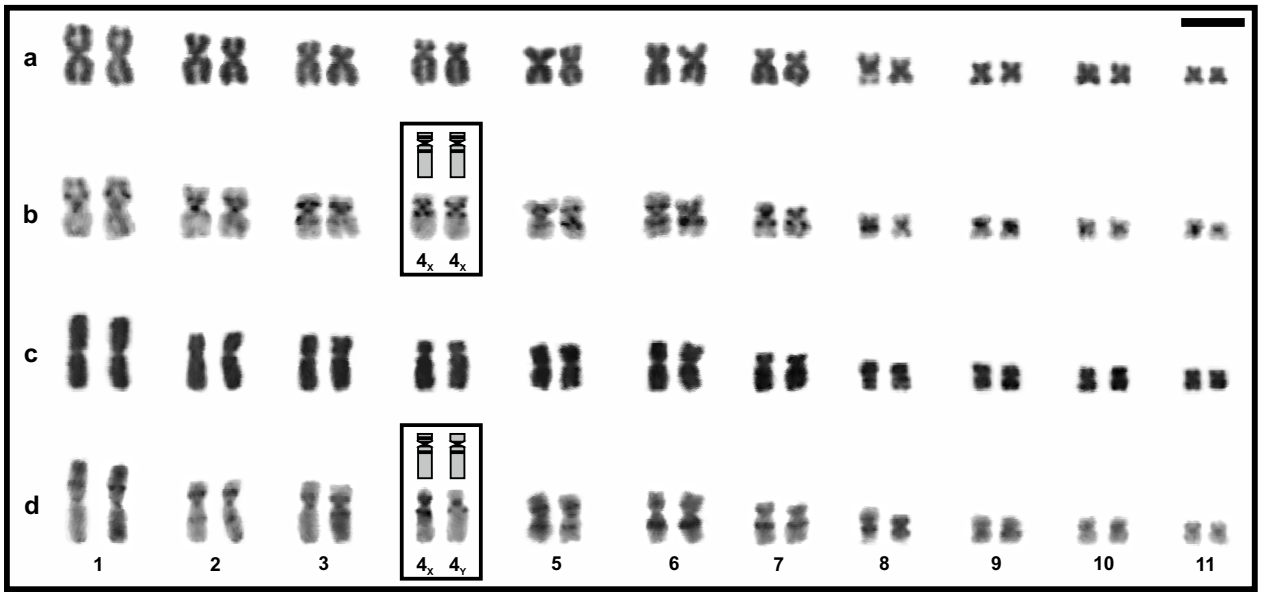

Figure 2. Karyotypes of *Leptodactylus bufonius*. Female. Conventional staining (a) and C-banding (b). Male. Conventional staining (c) and C-banding (d). Sex chromosomes (pairs 4) are shown in boxes. Bar = 5 µm.

of a pericentromeric C-band on the short arm of this pair. Over 25 specimens analyzed (14 males, 11 females), this C-band was present on both homologues in females but only in one of the homologues in males (Fig. 2, in boxes; Fig. 4).

Additionally, three other polymorphic variations in the C-banding pattern were detected in individuals of both sexes and from different localities, without defined geographical patterns (Fig. 5). First, a polymorphism for the presence of an interstitial C-band was identified in chromosome pair 2. This condition was detected in homozygosis in a male (LGE 12949; Fig. 5a) and in heterozygosis in three males (LGE 12163, LGE 12948, and LGE 13439; Fig. 5b) and two females (LGE 12046 and LGE 13437). The remaining specimens did not present interstitial bands in this pair (homozygous condition, without bands; Fig. 5c). A second polymorphism was detected for the presence of an additional interstitial C-band on the short arm of pair 6, detected in homozygosis in four males (LGE 10085, LGE 12163, LGE 12949 and LGE 13439, Fig. 5d) and two females (LGE 9264 and LGE 10084). Finally, a polymorphism consisted of the presence of a pericentromeric band on the longs arms of pair 7, detected only in homozygosis in three males (LGE 12944, LGE 13264, and LGE 13391; Fig. 5f) and three females (LGE 8098, LGE 9264, and LGE 10084) of the sample.

The CMA_3/DAPI fluorochromes staining evidenced CMA_3 positive (DAPI negative) heterochromatin in the interstitial region of the short arms of pairs 1–6 and 8 (including the heteromorphic band in pair 4), in the long arms of pairs 3 and 5, 7–8, and in the pericentromeric region of pairs 4 (Fig. 6). The conspicuous heterochromatic band observed on the short arm of pair 6 was characterized as CMA_3 negative and DAPI negative. A CMA_3 positive (DAPI negative) bright fluorescent band was detected in the interstitial position of pair 8, in coincidence with secondary constrictions (Fig. 3b and c). Similarly, the heteromorphic band observed on pair 4 was characterized as CMA_3 positive (DAPI negative). Although the CMA_3 bands were conspicuous, the

Table I. Chromosome morphology in *Leptodactylus bufonius*. Abbreviations: % set = percentage of total complement; CI = centromeric index; SD = standard deviation; M = metacentric; SM = submetacentric; ST = subtelocentric.

Chromosome number	1	2	3	4x	4y	5	6	7	8	9	10	11
% set	15.74	12.25	10.98	5.17	4.99	10.47	10.09	8.51	6.55	5.6	5.13	5.59
CI ± SD	0.478 ± 0.02	0.402 ± 0.04	0.369 ± 0.05	0.239 ± 0.02	0.236 ± 0.02	0.429 ± 0.04	0.429 ± 0.04	0.258 ± 0.04	0.425 ± 0.05	0.433 ± 0.04	0.441 ± 0.04	0.419 ± 0.04
Type	M	M	SM	ST	ST	M	M	ST	M	M	M	M

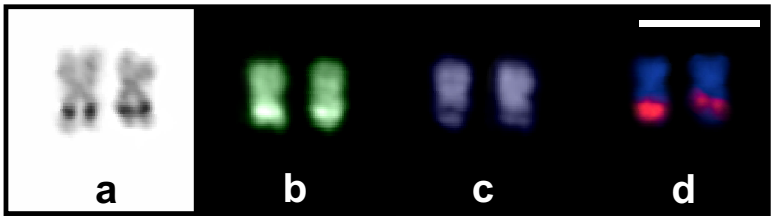

Figure 3. NOR-bearing chromosomes pair in *Leptodactylus bufonius* (pair 8), characterized by Ag-NOR (a), CMA_3 (b), DAPI (c), 18s DNAr probe (d). Bar = 5 µm.

Figure 4. Ideogram and sex chromosomes (pairs 4) from five females (a) and five males (b) of *Leptodactylus bufonius*, after C-banding.

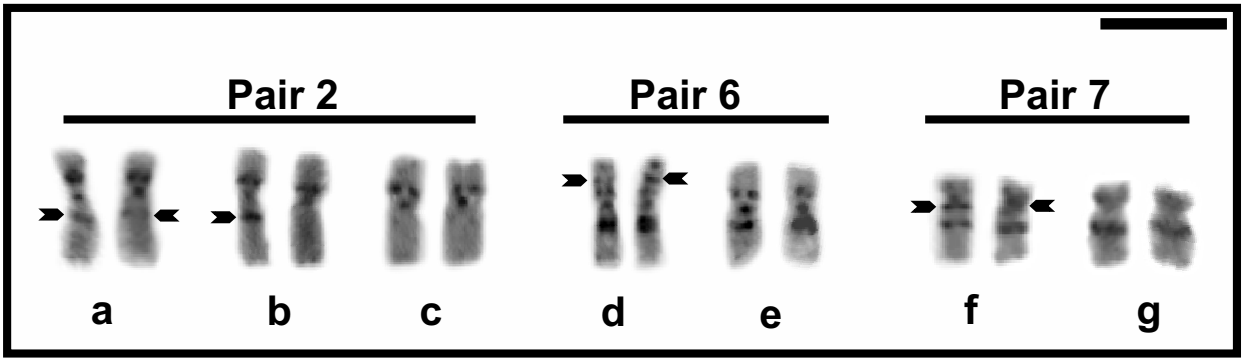

Figure 5. Chromosome polymorphisms for the presence of additional C-bands in *Leptodactylus bufonius*. Polymorphic C-bands in homozygosis (a, d, and f), heterozygosis (b), and chromosomes without additional bands (c, e, and g). Bar = 5 µm.

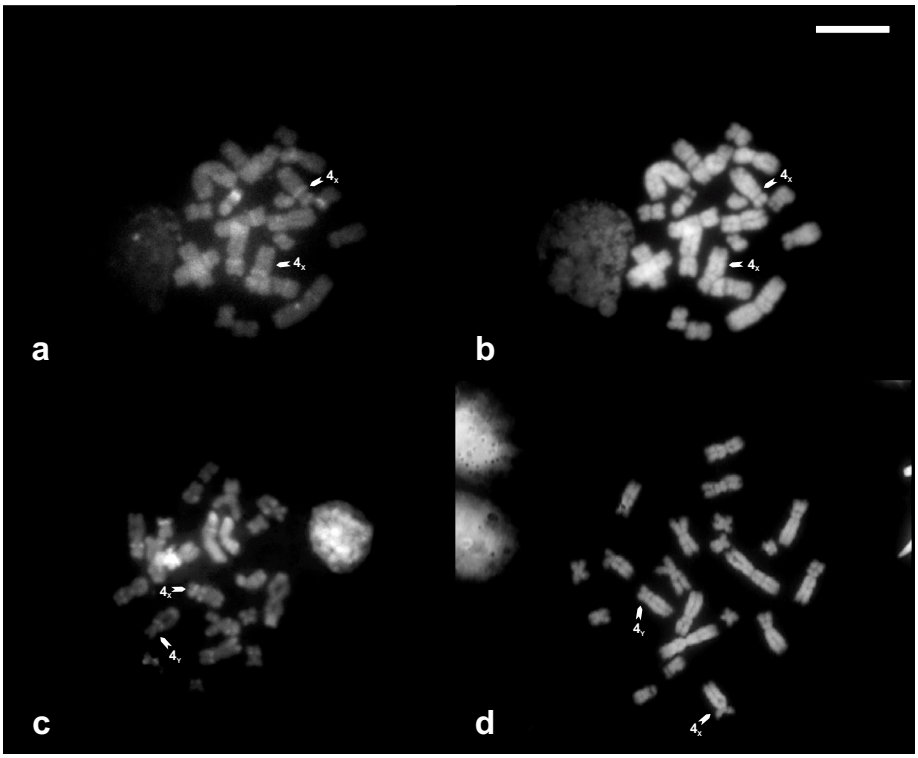

Figure 6. CMA_3/DAPI banding patterns in *Leptodactylus bufonius*. Mitotic metaphases of a female (a, b) and male (c, d), stained with CMA_3 and DAPI fluorochromes (left and right, respectively). Sex chromosomes are indicated with arrows. Bar = 5 µm.

negative DAPI bands depended on the state of condensation of the chromosomes.

DISCUSSION

The 2n = 2x = 22 and FN = 44 reported here for *Leptodactylus bufonius* agrees with previous data (Barbieri 1950, Brum-Zorrilla & Sáez 1968, Bogart 1974, Heyer & Diment 1974), and the morphology of chromosomes is in concordance with that reported by Bogart (1974, Fig. 2). The 2n = 2x = 22 is the widespread chromosome number in Leptodactylidae (Tomatis et al. 2009, Targueta et al. 2010, Gazoni et al. 2012, Vittorazzi et al. 2014, Lourenço et al. 2015, Cardozo et al. 2016 and cites therein), and could be considered the plesiomorphic condition for *Leptodactylus*. In this genus, 34 species have been cytogenetically analyzed (Brum-Zorrilla & Sáez 1968, Bogart 1974, Heyer & Diment 1974, Silva et al. 2000, 2004, 2006, Amaro-Ghiraldi et al. 2004, 2006, Arruda & Morielle-Versute 2008, de Oliveira et al. 2012, Gazoni et al. 2012, 2018, 2021, de Oliveira et al. 2013, Coelho et al. 2015, Gonzalez et al. 2016). Only two of them have a different basic chromosome number: *L. brevipes* (2n = 2x = 20; Gazoni et al. 2012, 2021) and *L. silvanimbus* (2n = 2x = 24; Amaro-Ghiraldi et al. 2006), which would represent autapomorphic character states.

The information involving differential staining techniques in *Leptodactylus bufonius* is provided herein for the first time. NORs in the pair 8 of this species are in a similar position than most of the *Leptodactylus* already studied and is considered the plesiomorphic state in the genus (Silva et al. 2000, Amaro-Ghilardi et al. 2004, 2006, Arruda & Morielle-Versute 2008, de Oliveira et al. 2012, Gazoni et al. 2012, Coelho et al. 2016). The few known exceptions were observed in *L. mystacinus* (pair 4 or 8; Amaro-Ghilardi et al. 2006, Silva et al. 2006), *L. natalensis* (pair 7; Gazoni et al. 2021), *L. petersii*

(pair 4; Amaro-Ghilardi et al. 2006, Gazoni et al. 2012, 2021), *L. rhodomystax* (pair 3; Gazoni et al. 2012), and *L. brevipes* (pair 4; Gazoni et al. 2012, 2021).

The C-banding in *Leptodactylus bufonius* seems not to be typical of *Leptodactylus*, where karyotypes of the species usually bear poor amounts of heterochromatin (Silva et al. 2000, 2006, Amaro-Ghilardi et al. 2004). Variation in the distribution of heterochromatic bands other than centromeric has only been reported for *L. latrans* (Silva et al. 2000, Amaro-Ghilardi et al. 2004), *L. fuscus* (Silva et al. 2000), and *L. petersii* (Coelho et al. 2016).

Furthermore, the C-banding technique evidenced the presence of a chromosome sex-determination system of the type XY in chromosome pair 4. In all analyzed females, this pair was indistinguishable regarding C-bands, while males presented a pericentromeric C-band in the short arm of only one of the homologues. Similar heteromorphisms in terms of the amount of heterochromatin were described for *Gastrotheca walkeri* and *G. ovifera*, and species of the genus *Eupsophus*, in which the Y chromosome lacks heterochromatin and differs in size with its homologue (Iturra & Veloso 1981, Cuevas & Formas 1996, Schmid et al. 2002).

According to several cytogenetic and molecular studies about the evolution of sex chromosomes, the morphological differentiation of heteromorphic sex chromosomes may initiate with heterochromatin accumulation (Ray-Chaudhuri et al. 1971, Jones 1984, Schmid & Steinlein 2001). Therefore, *Leptodactylus bufonius* and other above-mentioned anuran species do not fit in this evolutionary model, as the Y or W chromosomes do not present large amounts of heterochromatin. To explain this, Schmid et al. (2002) suggested that the presence of a smaller amount of heterochromatin in chromosome Y compared to the chromosome X could be due to deletion rather than accumulation. They further suggest that the heterochromatinization of the Y or W chromosome is possibly not the only evolutionary way that originates the morphological differentiation of sex chromosomes. There are some examples in anurans that may confirm this assumption. For instance, in *Rana japonica,* there is a single block of heterochromatin in the pericentromeric region of the long arm of the X chromosome that is absent in the Y chromosome (Miura 1994), and in *Eupsophus migueli,* the Y chromosome does not show heterochromatic regions at all (Iturra & Veloso 1989). Similarly, in the North American teiid lizard *Aspidoscelis tigris* there is less pericentromeric heterochromatin in the Y than the X chromosome (Bull 1978).

Among Leptodactylidae, there are only a few reported cases of sex chromosomes. In the *Engystomops petersi* species complex (as *Physalaemus petersi* in Lourenço et al. 1998, 1999, and *Engystomops petersi* and *E. freibergi* in Targueta et al. 2010), different types of heteromorphic XY chromosomes were detected. In this example, it is noteworthy that in some specimens from Acre, Brazil, the X chromosome contained interstitial heterochromatic segments, absent in the Y chromosomes, while in other individuals from the same population, a terminal NOR in the long arm of the Y chromosome can be observed. Furthermore, some individuals of this species from Puyo, Ecuador, only presented XY chromosomes with different morphology. In *Physalaemus ephippifer*, ZW chromosomes were identified by an additional segment, which comprises a distal NOR and an adjacent terminal C-band in the short arm of the W chromosome (Nascimento et al. 2010). Heteromorphic XY chromosomes were also described for *Pseudopaludicola saltica* (Duarte et al. 2010). In *Leptodactylus*, sex chromosomes were confirmed only for *L.*

pentadactylus (Gazoni et al. 2018), which presents the largest number of sex chromosomes found among vertebrates. Males of this species show a chromosome ring consisting of 12 elements, resulting from multiple translocation events. Barale et al. (1990) also reported an XY system for *L. macrosternum (*as *L. chaquensis)* and proposed a sex heteromorphism regarding a pericentromeric C-band in the first chromosome pair. However, these results differ from those obtained by Gazoni et al. (2012), who ruled out the occurrence of sex chromosome differentiation in this species.

A recent phylogenetic analysis of *Leptodactylus* recovered *L. bufonius* as the sister taxon of *L. troglodytes, L. cupreus, and L. mystacinus + L. apepyta*, in a clade that is sister of all remaining species of the *L. fuscus* group (de Sá et al. 2014, Schneider et al. 2019). In this group, the C-banding pattern is known only for *L. mystacinus*, *L. gracilis*, *L. plaumanni*, *L. fuscus*, and *L. notoaktites* (Silva et al. 2000, 2004, 2006, Arruda & Morielle-Versute 2008, de Oliveira et al. 2013), in which sexual chromosomes have never been detected. The large number of *Leptodactylus* species of which C-banding patterns are unknown does not allow us to determine whether the presence of sex chromosomes in *L. bufonius* corresponds to an autapomorphy or if it is a more extended condition in the *L. fuscus* group or the entire genus.

In our study case, the use of conventional banding techniques was sufficient to detect an XY sex chromosome system in *Leptodactylus bufonius*. However, a more exhaustive characterization is necessary with more resolutive techniques like fluorescent *in situ* hybridization or comparative genome hybridization.

Acknowledgments

The authors thank to the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and ANPCyT PICTs 2013-404, 2015-0813, 2015-0820, 2015-2381 and 2017-2437 for financial support. We also thank Claudio Borteiro for important observations in a first draft of the of the manuscript and Andrea Dallagnol, Mónica Soliz, Alberto Taffarel, Jimena Grosso, Grisel Navarro, Francisco Brusquetti, Orlando Escalante, Ana Sofía Duport Bru, Federico Marangoni, Julián Lescano, Emiliano Galli, María Laura Ponssa, María José Tulli, Claudio Borteiro, Daiana Paola Ferraro, Francisco Kolenc, Erica Kubish, Carina Nieto, Marcelo Tedros their help in the fieldwork and provision specimens.

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How to cite
SCHNEIDER RG, FERRO JM, REINKO IN, BOERIS JM, CARDOZO DE & BALDO D. 2021. Sex chromosomes in the Vizcacheras' White-lipped frog, *Leptodactylus bufonius* (Anura, Leptodactylidae). An Acad Bras Cienc 93: e20190426. DOI 10.1590/0001-3765202120190426.

Manuscript received on April 9, 2019;
accepted for publication on June 13, 2019

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