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ORIGINAL ARTICLE

# More sex chromosomes than autosomes in the Amazonian frog *Leptodactylus pentadactylus*

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## Abstract

Heteromorphic sex chromosomes are common in eukaryotes and largely ubiquitous in birds and mammals. The largest number of multiple sex chromosomes in vertebrates known today is found in the monotreme platypus (*Ornithorhynchus anatinus*, $2n = 52$) which exhibits precisely 10 sex chromosomes. Interestingly, fish, amphibians, and reptiles have sex determination mechanisms that do or do not involve morphologically differentiated sex chromosomes. Relatively few amphibian species carry heteromorphic sex chromosomes, and when present, they are frequently represented by only one pair, either XX:XY or ZZ:ZW types. Here, in contrast, with several evidences, from classical and molecular cytogenetic analyses, we found 12 sex chromosomes in a Brazilian population of the smoky jungle frog, designated as *Leptodactylus pentadactylus* Laurenti, 1768 (Leptodactylinae), which has a karyotype with $2n = 22$ chromosomes. Males exhibited an astonishing stable ring-shaped meiotic chain composed of six X and six Y chromosomes. The number of sex chromosomes is larger than the number of autosomes found, and these data represent the largest number of multiple sex chromosomes ever found among vertebrate species. Additionally, sequence and karyotype variation data suggest that this species may represent a complex of species, in which the chromosomal rearrangements may possibly have played an important role in the evolution process.

**Keywords** Meiotic multivalents · Multiple sex chromosomes · Translocations · Amphibia · Leptodactylidae

## Introduction

The evolution of sex chromosomes has occurred independently numerous times in several eukaryotic lineages. Although their origins are independent, sex chromosomes across lineages display several common features and represent a fascinating example of evolutionary convergence. Among these common features, the restriction of recombination, repetitive DNA accumulation, and gene loss in the Y or W chromosomes may have led to chromosome differentiation and the possible establishment of morphologically distinct (heteromorphic) elements (Bachtrog et al. 2014; Graves 2016; Wright et al. 2016). In vertebrates, heteromorphic sex chromosomes involved in sex determination are common in mammals (X0, XY, or multiple XY chromosomes) and birds (ZW). In contrast, morphologically differentiated sex chromosomes are less common among fish, amphibians, and reptiles but can involve XX:XY and ZZ:ZW sex systems (Ellegren 2011; Wright et al. 2016; Bachtrog et al. 2014; Graves 2016).

The widespread occurrence of homomorphic sex chromosomes observed in amphibians and fish is explained by two major hypotheses. The high-turnover hypothesis suggests that the chromosomes have insufficient time to degenerate because mutations affecting the sex-determining pathway appear regularly, causing changes to the ancestral sex chromosome (Schartl 2004; Volff et al. 2007; Sarre et al. 2011). This hypothesis has been supported by several studies in amphibians (Hotz et al. 1997; Miura 2007; Malone and Fontenot 2008; Stöck et al. 2011a; Stöck et al. 2013a) and fish (Tanaka et al. 2007; Cnaani et al. 2008; Ross et al. 2009). According to the "fountain of youth" hypothesis (Perrin 2009), the

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homomorphy of sex chromosomes is maintained by eventual recombination events. Although X-Y recombination is prevented in males, it can occur in XY females during occasional sex reversal events given that recombination patterns are expected to rely on phenotype rather than genotypic sex (Perrin 2009). The fountain of youth hypothesis has been supported in studies on amphibians (Stöck et al. 2011b; Guerrero et al. 2012; Stöck et al. 2013b).

Although most amphibians present homomorphic sex chromosomes, heteromorphic conditions have been reported in a few species, which primarily exhibit simple ZZ:ZW or XX:XY sex chromosome systems (Schmid et al. 2010). Cases of multiple sex chromosomes in amphibians have been sporadically reported. For example, in the anurans *Strabomantis biporcatus* (formerly *Eleutherodactylus maussi*) (Schmid et al. 1992) and *Pristimantis riveroi* (Schmid et al. 2003), an X1X1X2X2♀:X1X2Y♂ sex chromosome system evolved through centric fusion involving the original Y and a large autosome. Recently, an X1Y1X2Y2 system was documented in males of one population of *Rana temporaria* from Sweden (Rodrigues et al. 2016). With the exception of birds, in which only the simple ZW system has been observed to date, multiple sex chromosome systems have been well-documented in several species of amniotes, mostly mammals (Graves 2016; Pokorná et al. 2014). The most intriguing and unusual multiple sex chromosome meiotic chains among vertebrates have been observed in the monotremes short-beaked echidna (*Tachyglossus aculeatus*) and platypus (*Ornithorhynchus anatinus*), in which males present 9 and 10 sex chromosomes, respectively (Murtagh 1977; Grützner et al. 2004; Gruetzner et al. 2006; Rens et al. 2007).

Commonly called the smoky jungle frog, *Leptodactylus pentadactylus* (Laurenti, 1768) is a species distributed in the Amazon basin, occurring in Colombia, Peru, Bolivia, Brazil, Guyana, Suriname, and French Guyana (Frost 2017). In a cytogenetic study on the Neotropical genus *Leptodactylus*, a meiotic ring with 12 chromosomes, besides five bivalents, was observed in one male specimen assigned to this species (Gazoni et al. 2012). This structure was the result of multiple reciprocal translocations confirmed by comparing replication bands, mainly for the largest chromosomes of the *L. pentadactylus* specimen (Gazoni et al. 2012). Cells with $2n = 22$ and FN = 44, which are characteristic of most of *Leptodactylus* species, were observed during mitotic metaphases; however, not all chromosomes were paired (Gazoni et al. 2012). Nevertheless, the meiotic chain was apparently resolved during meiosis with alternate segregation, allowing the formation of balanced gametes (Gazoni et al. 2012). As these findings were based on the analysis of only one male, it was not possible to infer whether this chain is well-established in the population or the species or whether it might represent a sex chromosome chain. Karyotypes with $2n = 22$ and FN = 44 have been described for individuals identified as *L. pentadactylus* collected from other locations in Brazil, Ecuador, and Peru (Brum-Zorilla and Saez 1968; Bogart 1974; Heyer and Diment 1974; Amaro-Ghilardi et al. 2004; Coelho et al. 2016) without the occurrence of spontaneous translocations (see comments in Gazoni et al. 2012).

The presence of sex chromosome chains is rare and has been observed in some plants, invertebrates, and (as noted above) the monotremes platypus and short-beaked echidna, being their formation due to fusions or translocations involving autosomes (neo-sex chromosomes) (Ellegren 2011; Rens et al. 2007; Šíchová et al. 2015; Šíchová et al. 2016). Meiotic multivalents with more than four chromosomes have been observed in a few anurans (Lourenço et al. 2000; Siqueira et al. 2004; Campos et al. 2008) and fish (Gross et al. 2009); however, in these cases, the translocations considered responsible for the terminal chromosome associations are present in both sexes. An alternative hypothesis for some of these chromosome associations involves non-chiasmatic ectopic pairing due to heterochromatic telomeric sequences (Schmid et al. 2010). Nevertheless, heteromorphisms suggesting translocations are present in most of these cases. In all these organisms, the chains are not well-fixed in the studied populations, and the number of elements forming the chains is labile.

Here, we present a chromosomal analysis of 13 specimens (seven males and six females) of *L. pentadactylus* from Paranaíta, Mato Grosso State, Brazil, using classical and molecular cytogenetic approaches to test the hypothesis of the presence of a sex-related chromosomal chain in this population. In addition, we included a genetic diversity analysis using mitochondrial and nuclear markers to explore the variation within this population.

## Material and methods

Classical and molecular cytogenetic analyses were performed on six females and seven males of *L. pentadactylus* collected in the Brazilian southern Amazon in the municipality of Paranaíta, Mato Grosso State, between 2012 and 2014 (Table S1 – Electronic supplementary material). The analyzed specimens were deposited in the Célio F. B. Haddad amphibian collection (CFBH), Departamento de Zoologia, Instituto de Biociências, UNESP, Rio Claro, São Paulo, Brazil. All specimens were collected under permission provided by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio-SISBIO authorization number 30202-2).

### Chromosome preparations

Deep sedation euthanasia by dermal absorption of lidocaine hydrochloride 5% was in accordance with the Ethical Committee in Animal Use (CEUA – permission number 027/2011), UNESP, Rio Claro, Brazil. Direct cytological

preparations were obtained from the bone marrow, liver, intestine, and testis after a 4-h in vivo colchicine treatment (Baldissera Jr. et al. 1993; Schmid 1978).

### Conventional and molecular cytogenetic analyses

Conventional analysis was performed using Giemsa staining in all animals, and chromosome preparations of males and females were submitted to fluorescence in situ hybridization (FISH) experiments, according to Pinkel et al. (1986), using specific probes, such as biotinylated $GATA_{(8)}$ microsatellite (Sigma, St. Louis, MO, USA), telomeric repeat $TTAGGG_{(n)}$ (Telomere PNA FISH Kit/FITC – DakoCytomation, Glostrup, Copenhagen, Denmark), and major rDNA HM123 (Meunier-Rotival et al. 1979). Comparative genomic hybridization (CGH) was performed according to Abramyan et al. (2009) with the following modifications: 300 ng of female and male genomic DNAs labeled with Biotin-16-dUTP and Anti-Digoxigenin (Roche Diagnostics, Indianapolis, IN, USA) through nick translation, respectively, were co-precipitated with female unlabeled digested DNA (100–500 bp). Then, 3 μL of probe was added to the mixture for hybridization; this process was performed separately for male and female chromosome preparations. The probes labeled with biotin were detected with streptavidin-Alexa Fluor®-488 (green spectrum – Invitrogen, San Diego, CA, USA), and digoxigenin-labeled DNA was detected using anti-digoxigenin-rhodamine (red spectrum – Roche Diagnostics, Indianapolis, IN, USA). Images were captured using the software DP Controller and a cooled camera (DP71) connected to a fluorescence microscope Olympus BX51 (Olympus Corporation, Shinjuku, Tokyo, Japan). Slight uniform adjustments in contrast were performed using DP Manager Software. Figures were organized using the Corel X6 Suit program (Corel Corporation, Ottawa, ON, Canada).

### DNA sequences analyses

We extracted whole genomic DNA from ethanol-preserved tissues (liver or muscle) using either the ammonium acetate precipitation method (Maniatis et al. 1982) or QIA Quick DNeasy kits (Qiagen Inc., Hilden, North Rhine-Westphalia, Germany) following the manufacturer's guidelines. We amplified and sequenced two mitochondrial (*16S ribosomal RNA gene* (16S), *cytochrome oxidase c subunit* I (COI)) and two nuclear (*brain-derived neurotrophic factor* gene (BDNF), *cellular myelocytomatosis* gene (C-myc)) gene fragments for all analyzed specimens. DNA fragments were amplified by standard PCR technique using the primers listed in Table S2 – Electronic supplementary material (Crawford 2003; Lyra et al. 2016; Palumbi et al. 1991; Van der Meijden et al. 2007; Wiens et al. 2005). PCR products were purified via enzymatic reaction and sequenced with the BigDye™ terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, CA, USA) in an automatic Sequencer 3730XL by Macrogen Inc., Seoul, South Korea. Sequences were verified and direct and reverse sequences were converted to a single contig using Geneious Pro software V6.1.5 (Biomatters Ltda). The sequences were deposited in GenBank (Table S1 – Electronic supplementary material). Alignments were conducted in the online version of MAFFT v6 (Katoh 2013), and sequences from each gene fragment were converted into unique haplotypes using the *collapser* function of Fabox software (Villesen 2007). We inferred haplotype networks for each gene fragment using TCS v1.21 (Clement et al. 2000).

To better understand the relationship between the samples analyzed here and other populations of *L. pentadactylus*, we estimated maximum-likelihood phylogenetic tree using the16S sequences. We included all 16S sequences of *L. pentadactylus* available in GenBank along with some closed related species (see Fig. 4 for accession numbers). The phylogenetic inference was done in PhyML 3.0 using Smart Model Selection option (Guindon et al. 2010; Lefort et al. 2017). We also estimated uncorrected p-distances between populations and species using MEGA v.6 (Tamura et al. 2013).

## Results

### Conventional Giemsa-staining cytogenetic analyses

The karyotype of the analyzed specimens presented $2n = 22$ and FN = 44 (Fig. 1a, b). Some of the chromosomes were not paired in males, whereas all seven females had 11 pairs of homologous chromosomes. Six X and six Y chromosomes were identified in the karyotypes of males, whereas 12 X chromosomes where present in the females. The analyses of meiotic cells in males, i.e., cells in diakinesis/metaphase I, revealed translocated chromosomes forming a ring-shaped chromosomal chain in all males analyzed. These translocations were recognized previously by comparison of replication banding patterns in the study of Gazoni et al. (2012). This closed chain harbored 12 elements that corresponded to X1Y1X2Y2X3Y3X4Y4X5Y5X6Y6, and five typical bivalents are present (Fig. 1c). Chromosomes 1, X3 (formerly chromosome 5), 3, 4, 5, X5 (formerly chromosome 10), and X6 (formerly chromosome 11) were identified as metacentric; chromosomes 2, X1 (formerly chromosome 3), and X4 (formerly chromosome 7) were identified as submetacentric; and chromosomes X2 (formerly chromosomes 4) were identified as subtelocentric (Fig. 1a). All seven males analyzed in this work presented a karyotype with at least one chromosome homologous to each chromosome pair of females. However, the male karyotype presented six translocated chromosomes that characterized the Y chromosomes, three of which were

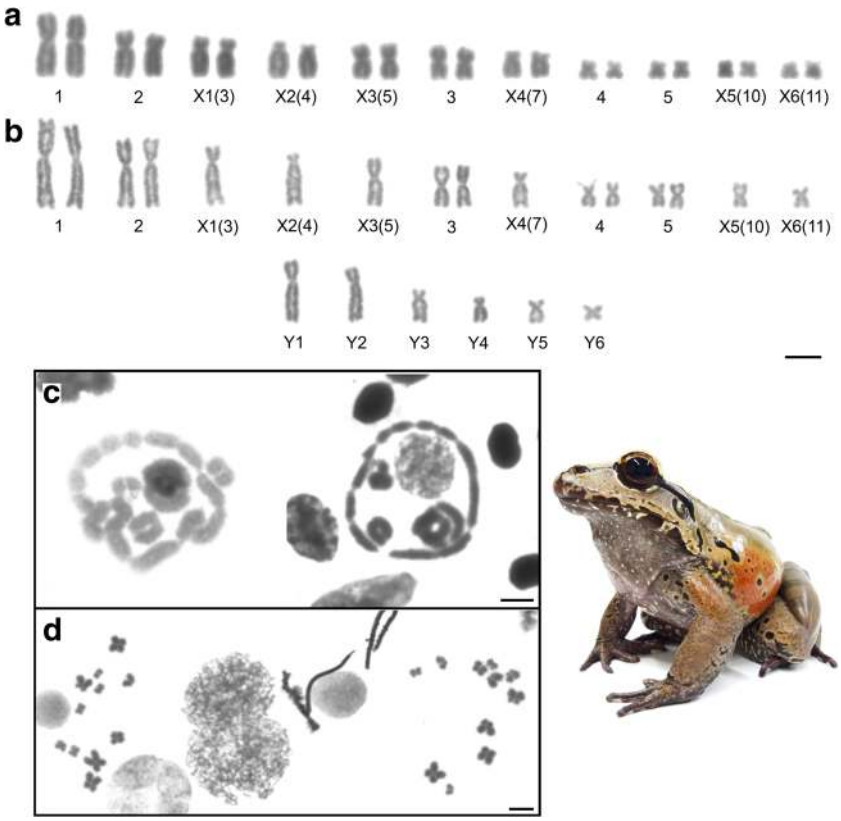


**Fig. 1** Giemsa stained chromosomes of *L. pentadactylus* from Paranaíta, MT, Brazil. Numbers in parentheses refer to the presumed ancestral chromosomes, compared with *L. pentadactylus* previously studied from other locations, as well as with other species of *Leptodactylus* phylogenetically close to *L. pentadactylus* (all of them without heteromorphisms or sex chromosomes recognized). **a** Karyotype of female. **b** Karyotype of male. **c** Meiotic (diakinesis) chromosomes of two males showing the same ordering of chromosomes in their ring-shaped chains composed of 12 sex chromosomes. **d** Two meiotic (metaphase II) constitutions in male *L. pentadactylus*: autosomes plus Y chromosomes (left) and autosomes plus X chromosomes (right). Scale bar corresponds to 5 μm

conspicuously unpaired (Fig. 1b). All meiotic cells from the eight males studied presented the same chromosomal constitution and ordering by elements size in the multivalent ring (Fig. 1c). Metaphases II had two constitutions in all analyzed males, in which six non-rearranged chromosomes (X chromosomes) or six rearranged chromosomes (Y chromosomes) were found in addition to five autosomes (Fig. 1d).

## Molecular cytogenetic analyses

Fluorescence in situ hybridization (FISH) using $GATA_{(8)}$ microsatellite as probes revealed signal exclusively in the pericentromeric region of two small chromosomes in mitotic metaphases of both sexes (Fig. 2a, b). In males, these chromosomes corresponded to a heteromorphic pair (X5 and Y5) participating in the meiotic ring (Fig. 2c), and each chromosome was identified in two spermatocyte constitutions (Fig. 2d). The telomeric motifs were exclusively mapped to the canonical telomeric regions, and intrachromosomal telomeric sequences (ITS) were not observed in females (Fig. 2f) and males (Fig. 2e), including those translocated Y chromosomes. The chromosomes belonging to meiotic chain are associated by terminal regions, i.e., telomere-telomere contact. The HM123 rDNA (18S + 28S) probe hybridized in the terminal regions of the short arms of chromosomes 8 in males and females, which form one of the bivalents in male meiosis, out of the chromosome chain (Fig. 2f (inset)). The CGH experiments did not reveal differential hybridization signal intensity between males and females using male and female genomic DNAs as co-hybridized probes. Faint signals for the two probes were observed along the entire euchromatin, and stronger signals were observed in centromeric heterochromatin and terminal regions (Figs. 3 and 4).

## Sequence analyses

We found very low genetic variability among samples for both mitochondrial and nuclear markers (Fig. S1 – Electronic supplementary material), supporting that all individuals belong to the same population. Phylogenetic inferences revealed two clades for *L. pentadactylus*, one including all samples from Paranaíta, MT, and the other including samples from Peru, Brazil (Pará State), and French Guiana. The uncorrected p-distance between samples from Paranaíta and others *L. pentadactylus* ranged between 3.4 and 4.9%. The distances between clades for 16S fragment (sensu Vieites et al. 2009) might suggest that *L. pentadactylus* is a complex of species that deserves further taxonomic investigations.

# Discussion

An established chromosomal chain with 12 chromosomes in *Leptodactylus pentadactylus* presented in this study revealed

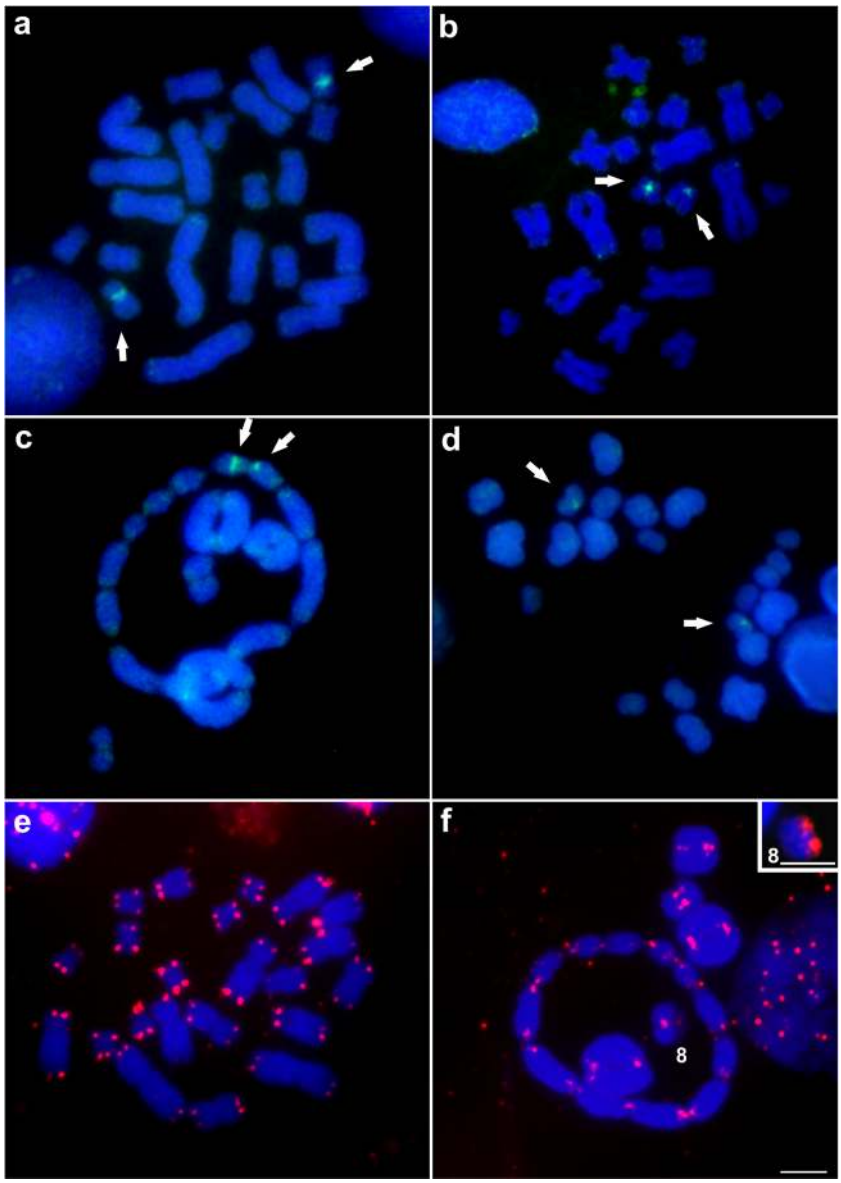


**Fig. 2** FISH using three different repetitive DNA sequences in chromosomes of *L. pentadactylus* from Paranaíta, MT. **a** $GATA_{(n)}$ repeats accumulation in one pair of X chromosomes (homomorphic) in female. **b** $GATA_{(n)}$ microsatellite repeats accumulation in an heteromorphic chromosome pair (one X and one Y chromosome) in male. **c** Diakinesis from male showing the association of the chromosome pair with $GATA_{(n)}$ accumulation in chain. **d** Metaphases II from male showing segregation of the X and Y chromosomes in distinct spermatocyte karyotypes. **e** Telomeric $TTAGGG_{(n)}$ repeats distribution in mitotic chromosomes of female. **f** Telomeric mapping in male diakinetic chromosomes of male; the inset shows rDNA hybridization in the bivalent composed by chromosomes 8. Scale bar corresponds to 5 μm

an unprecedentedly high number of sex chromosomes in vertebrates. The species previously holding this record was the platypus (*Ornithorhynchus anatinus*), with 10 XY chromosomes arranged in an open chain during diakinesis. Additionally, the relative number of sex chromosomes to autosomes is higher in *L. pentadactylus* than in the platypus. The platypus has 10 sex chromosomes in a karyotype of $2n = 52$ chromosomes (Grützner et al. 2004; Gruetzner et al. 2006), whereas *L. pentadactylus* has greater than 50% of its chromosomes represented by sex chromosomes (12 in a karyotype of $2n = 22$ chromosomes). As previously demonstrated for a single *L. pentadactylus* male (Gazoni et al. 2012), the FISH-mapped telomere sequences did not identify ITSs (intrachromosomal telomeric sequences), in both male and female mitotic metaphases. This occurs despite the translocations that occurred in the karyotype, and terminal telomere signals confirmed the presence of 12 chromosomes in the meiotic ring.

The translocation steps that occurred in the Y chromosomes might have led to a reduction of recombination events with the presumably non-translocated X chromosomes, as commonly occurring in evolving sex chromosomes (Wright et al. 2016; Bachtrog et al. 2014; Graves 2016). In *L. pentadactylus*, it is expected that a minimum of seven translocated chromosomal segments have occurred among the Y chromosomes, with the presence of at least two rearranged regions in one of the Y chromosomes, to form the closed chain, as demonstrated by Gazoni et al. (2012).

The factors that allowed *L. pentadactylus* to accumulate these numerous translocations remain unclear. Like well documented in other derived sex systems, the chromosomes incorporated in the multivalent chain in males are potentially

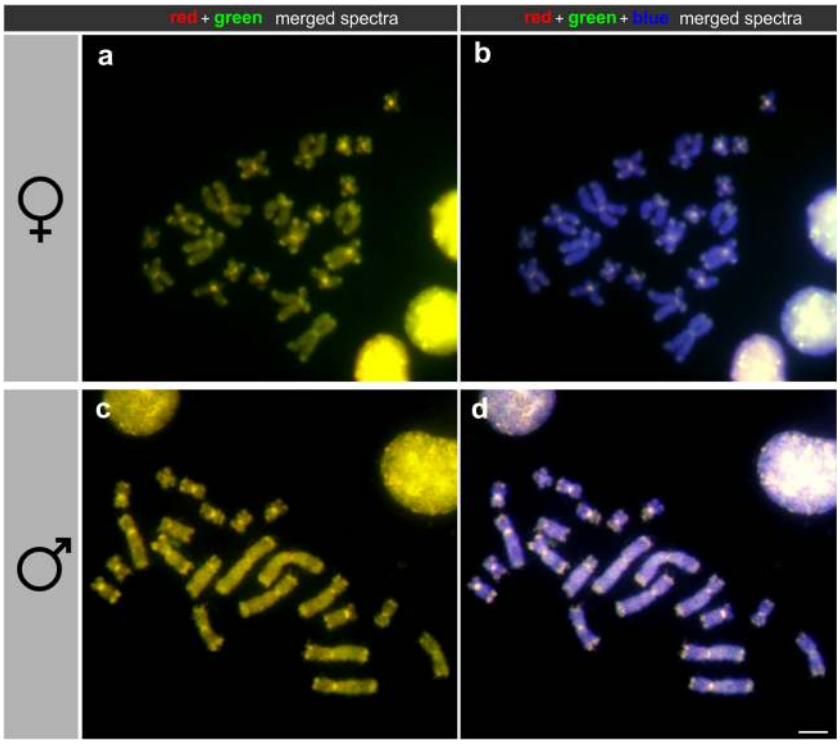


**Fig. 3** Comparative genome hybridization (CGH) in female and male of *L. pentadactylus*, showing no particular chromosome regions for both sexes. Red (male genome DNA—anti-digoxigenin-rhodamin detected) and green (female genome DNA—streptavidin-Alexa Fluor-488 detected) spectrum merged in female (**a**) and male (**c**), and red, green, and blue (DAPI staining) spectrum merged: female (**b**) and male (**d**). Scale bar corresponds to 5 μm

less recombinant, a fact that could optimize or increase particular antagonist genes, thereby providing advantages to the heterogametic sex or to the species, as discussed in Pokorná et al. (2014), although it deserves more experimental analysis, for example, using antibodies to study the synaptonemic complex.

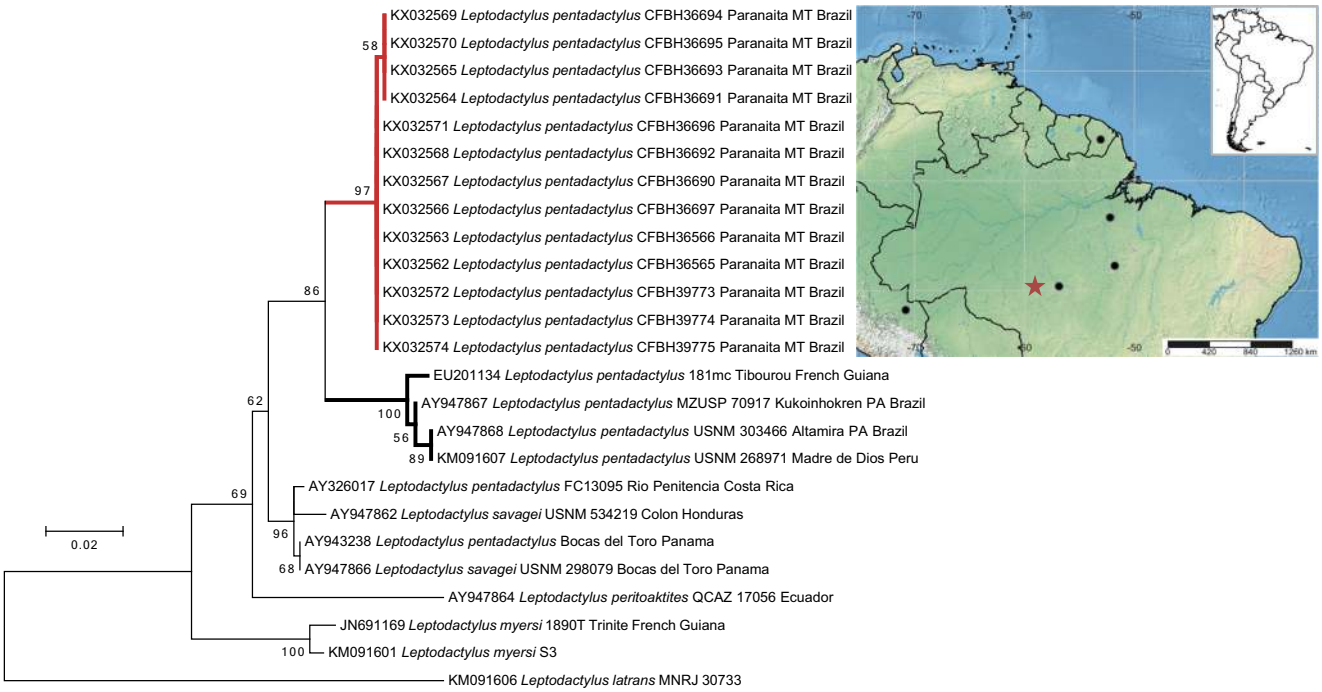


**Fig. 4** Maximum-likelihood tree obtained for *Leptodactylus pentadactylus* and closely related species and geographical distribution of samples. Red star indicates *L. pentadactylus* from Paranaíta, MT, Brazil. Black dot indicates other samples of *L. pentadactylus* available in GenBank. GenBank accession numbers are shown in each terminal. There are two sequences of *L. pentadactylus* from Panamá and Costa Rica, respectively. These sequences are probably misidentified and might be *Leptodactylus savagei* according geographical location and genetic diversity

The stable chromosome constitutions found for each sex of *L. pentadactylus* demonstrate that the sex-linked chromosomes are fixed in the analyzed population. Additionally, the balanced proportion of males and females (7♂ and 6♀) collected randomly in the wild may suggest no interference of the translocations in the sex ratio. Microsatellite motifs are common in the Y/W chromosomes in diverse sauropsid taxa, suggesting that these repetitive motifs are commonly present in sauropsid genomes at low copy numbers and that amplifications of these motifs might have a functional role in composing the constitutive heterochromatin of sex chromosomes (Matsubara et al. 2016). These authors proposed that the amplification of microsatellite repeats is tightly associated with the differentiation and heterochromatinization of Y/W chromosomes in sauropsids and other taxa. GATA and AGAT repeat motifs, which are considered as the same repeat motif because they have the same hybridization patterns (Matsubara et al. 2016), were amplified and hybridized to sex chromosomes of *Mus musculus* (Mammalia, Muridae) (Singh et al. 1994), the turtle species *Chelodina longicollis* (Chelidae), and the squamate species *Notechis scutatus* (Elapidae), *Bassiana duperreyi* (Scincidae), *Aprasia parapulchella* (Pygopodidae), and four *Anolis* species (Polychrotidae) (O'Meally et al. 2010; Matsubara et al. 2013; Gamble et al. 2014; Rovatsos et al. 2015; Matsubara et al. 2016). However, no data regarding the accumulation of microsatellites on the chromosomes of anurans are presently available.

The $GATA_{(n)}$ microsatellite accumulation in one chromosome pair that participates in the meiotic sex-ring suggests the initial differentiation of at least one chromosome pair in the meiotic sex chain in *L. pentadactylus*. This marker provided additional evidence for the heteromorphic condition of one small pair, with the presence of fluorescence signals pericentromerically in one clearly heteromorphic chromosome pair (submetacentric X, and subtelocentric Y – Fig. 2b) in males and in a homomorphic pair (submetacentrics XX – Fig. 2a) in females.

The same pattern of this marker observed in the chromosome pair participating in the sex chromosome chain, as well as the presence of the rDNA exclusively observed in the chromosomes 8 of both sexes, forming a bivalent outside the chain in males (as reported previously for a male specimen – Gazoni et al. 2012), reinforces that the chromosomes participating in the chain are the same in all analyzed males and that the chromosome rings are well-established in this species.

CGH data on amphibian chromosomes were only reported to *Xenopus laevis* and *Xenopus tropicalis* (Uno et al. 2008), the cane toad *Rhinella marina* (as *Bufo marinus*) (Abramyan et al. 2009), and *Pseudis tocantins* (Gatto et al. 2016). In the *Xenopus* study, males and females exhibited the same hybridization signals as observed here for *L. pentadactylus*; no detectable sex-specific signals were observed, even between the known homomorphic sex chromosomes. In *Rhinella marina*, specific signals were observed in one chromosome 7 in females, thereby confirming a ZZ/ZW sex chromosome system involving the NOR-bearing chromosomes. In *Pseudis tocantins*, CGH experiments suggested that different heterocromatic bands in the Z and W chromosomes may be due to the presence of different types of sequences or also due to variation in the number of sequences present in both the chromosomes.

Our CGH analyses suggest that the absence of sex-specific signals could be due to absence, insignificant loss or gain, or due to non-differentiation of DNA content of the sex chromosomes in *L. pentadactylus*. Although the technique presents limitations to detect minor molecular alterations, this may further suggest an absent or initial stage of content differentiation of this multiple sex chromosome system, with no evident molecular differentiation between the heteromorphic sex chromosomes.

*Leptodactylus pentadactylus* is a widespread species and the karyotypes found in the males specimens from Paranaíta, MT, Brazil, are distinct compared with those found from other populations in the Amazon basin previously karyotyped (Brum-Zorilla and Saez 1968; Bogart 1974; Heyer and Diment 1974; Coelho et al. 2016). These authors described indistinct chromosomal constitutions for both males and females, with karyotypes very similar to the described for females in this study. An exception was the karyotype of one specimen of *L. pentadactylus* collected in the municipality of Claudia (east of state of Mato Grosso) and analyzed by Amaro-Ghilardi et al. (2004), who found rearrangements which, at that time, were attributed to have originated after in vitro fibroblast culture procedure. Unfortunately, the authors could not determine the sex or visualize meiotic chromosomes of the specimen, a small juvenile (Gazoni et al. 2012). This could be a priority area to study adult specimens, as the Claudia population may present the same karyotype features as the specimens from Paranaíta, in addition to a possible closer phylogenetic relationship.

We also found high genetic distances between Paranaíta population and other *L. pentadactylus* populations distributed in the Amazon basin, suggesting that it may represent a complex of species. These data together suggest that detailed studies of geographic variation in morphology, cytogenetic, and molecular data, including specimens from Suriname, the type location for *L. pentadactylus*, are important to shed light on the taxonomic status of these confusing taxa. More importantly, this will lead to a better understanding of the distribution and evolution of this multiple sex chromosome system.

For now, we suggest that the karyotype differences present in this potential complex of species could possibly have an important role in their evolution process, where the rearrangements found in *L. pentadactylus* from Paranaíta, MT, may be responsible for partial or even complete reproductive isolation of this population.

New approaches, particularly those involving sex-related DNA sequences identification and mapping, synaptonemal complex analyses, and chromosome painting with chromosome-specific probes, are strongly interesting in the *L. pentadactylus* species group. Undoubtedly, this special case of multiple sex chromosomes makes *L. pentadactylus* an interesting organism for studies aiming to improve knowledge on the origin, evolution, and biological significance of this special genome organization.

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**Author contributions** T. G., P. P. P. M., and C. F. B. H. designed the study. T. G. performed the cytogenetic analyses. D. C. C. M. assisted on microsatellite and CGH experiments. M. L. L. performed DNA sequencing and similarity analyses. T. G. and H. N. collected the specimens. T. G. and P. P. P. M. wrote the main manuscript, and all authors contributed to improve it. All authors approved the final version of the manuscript.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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