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Differential repetitive DNA composition in the centromeric region of chromosomes of Amazonian lizard species in the family Teiidae

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Abstract

Differences in heterochromatin distribution patterns and its composition were observed in Amazonian teiid species. Studies have shown repetitive DNA harbors heterochromatic blocks which are located in centromeric and telomeric regions in *Ameiva ameiva* (Linnaeus, 1758), *Kentropyx calcarata* (Spix, 1825), *Kentropyx pelviceps* (Cope, 1868), and *Tupinambis teguixin* (Linnaeus, 1758). In *Cnemidophorus* sp.1, repetitive DNA has multiple signals along all chromosomes. The aim of this study was to characterize moderately and highly repetitive DNA sequences by $C_o t$1-DNA from *Ameiva ameiva* and *Cnemidophorus* sp.1 genomes through cloning and DNA sequencing, as well as mapping them chromosomally to better understand its organization and genome dynamics. The results of sequencing of DNA libraries obtained by $C_o t$1-DNA showed that different microsatellites, transposons, retrotransposons, and some gene families also comprise the fraction of repetitive DNA in the teiid species. FISH using $C_o t$1-DNA probes isolated from both *Ameiva ameiva* and *Cnemidophorus* sp.1 showed these sequences mainly located in heterochromatic centromeric, and telomeric regions in *Ameiva ameiva*, *Kentropyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin* chromosomes, indicating they play structural and functional roles in the genome of these species. In *Cnemidophorus* sp.1, $C_o t$1-DNA probe isolated from *Ameiva ameiva* had multiple interstitial signals on chromosomes, whereas mapping of $C_o t$1-DNA isolated from the *Ameiva ameiva*

and *Cnemidophorus* sp.1 highlighted centromeric regions of some chromosomes. Thus, the data obtained showed that many repetitive DNA classes are part of the genome of *Ameiva ameiva*, *Cnemidophorus* sp.1, *Kentroyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin*, and these sequences are shared among the analyzed teiid species, but they were not always allocated at the same chromosome position.

Keywords
centromere, $C_o t$1-DNA, FISH, heterochromatin, telomere

Introduction

Teiidae are a Neotropical lizard family characterized by karyotype diversity with a diploid number ranging from 34 to 52 chromosomes, as well as differences in heterochromatin composition and distribution patterns (Carvalho et al. 2015a, b). Amazonian teiid species exhibit considerable heterochromatic blocks located in centromeric and terminal regions of most chromosomes in *Ameiva ameiva* (Linnaeus, 1758), *Cnemidophorus* sp.1, *Kentropyx calcarata* (Spix, 1825) and *Kentropyx pelviceps* (Cope, 1868), whereas *Tupinambis teguixin* (Linnaeus, 1758) has few heterochromatic blocks in the centromeric regions of macrochromosomes, indicating differential heterochromatin distribution among teiid species (Carvalho et al. 2015a, b).

These heterochromatin blocks usually contain repetitive DNA, such as ribosomal DNA 5S, telomeric sequences, tropomyosin 1 genes, and the retrotransposons *Rex* 1 and *SINE* (Carvalho et al. 2015b). These repetitive elements have been mapped in chromosomes of *Ameiva ameiva*, *Kentropyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin.* They were mainly located in heterochromatic centromeric and telomeric regions, and appeared to act on the structural organization of the centromere and/or telomere (Carvalho et al. 2015b). However, the composition of the heterochromatic fraction in the genome of *Ameiva ameiva*, *Kentropyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin* was not restricted to ribosomal DNA 5S sequences, telomeric sequences, tropomyosin 1 gene, and retrotransposons *Rex* 1 and *SINE* because some chromosomes have heterochromatic blocks that are not hybridization signals of these repetitive elements (Carvalho et al. 2015a, b). In *Cnemidophorus* sp.1 the pattern of organization of these sequences is different from other teiids, presenting multiple signals along all chromosomes, with compartmentalized blocks mainly in interstitial chromosome regions (Carvalho et al. 2015b). This indicates differential composition of the centromeric region of this species.

Repetitive DNA may be isolated by various strategies, among them $C_o t$1-DNA is used to isolate total fraction of moderately and highly repetitive DNA sequences in the genome (Vicari et al. 2010). $C_o t$1-DNA is based on DNA re-association kinetics, where genome repetitive fractions tend to rapidly reanneal after total genomic DNA denaturation. Thus, average renaturation time of a particular sequence depends on the number of copies found in the genome and in time almost all DNA of a denatured sample will reassociate (Zwick et al. 1997, Ferreira and Martins 2008). Repetitive

DNA libraries of various species have identified sequences of microsatellites, satellites, ribosomal DNA, and transposable elements (transposons and retrotransposons) in the genome repetitive fraction (Hřibová et al. 2007, Zhang et al. 2012, Terencio et al. 2015). This DNA library enriched with moderate and highly repetitive sequences ($C_o t$1-DNA) may be mapped in chromosomes and sequenced, which helps in analysis and has aided in understanding the dynamic and genomic organization, in terms of the repetitive fraction, as well as in evolutionary processes (Ferreira and Martins 2008, Szinay et al. 2010, Yu et al. 2013).

The aim of this study was to characterize sequences of moderately and highly repetitive DNA sequences in *Ameiva ameiva* and *Cnemidophorus* sp.1 genomes. These teiid species have a large amount of heterochromatin that is organized differentially. Libraries enriched with repetitive DNA were cloned, sequenced, used as probes, and chromosomally mapped in *Ameiva ameiva*, *Cnemidophorus* sp.1, *Kentropyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin*. Furthermore, they assisted in the understanding of genomic sequence organization and dynamics in karyotypes of these Amazonian teiid species.

Material and methods

Samples of *Ameiva ameiva*, *Cnemidophorus* sp.1, *Kentropyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin* were collected in Amazonas State, Brazil, in different locations (Table 1). All of the collections were conducted with permission from the Brazilian Environmental Protection Agency (ICMBio/SISBIO 41825-1) (Table 1).

The animals were euthanized after capture in the field with a lethal dose of the anesthetic sodium thiopental to avoid being deprived of food or water. This research was approved by the Ethics Committee for Animal Experimentation of the Fundação Universidade do Amazonas/Universidade Federal do Amazonas (UFAM) (number 041/2013). No endangered or protected species were used in this research. The animals underwent cytogenetic procedures, were fixed with 10% formaldehyde (injected in the coelom and digestive tract), and preserved in 70% alcohol. Voucher specimens were deposited in the Herpetological Collection of the Instituto Nacional de Pesquisas da Amazônia (INPA H31712, 33213, 34791, 34841, 35018). All samples were identified by the researcher Dr. Federico Arias.

Mitotic chromosomes were obtained from bone marrow cell suspensions *in vitro* using colchicine (Ford and Hamerton 1956). Because the larger heterochromatic regions in *Ameiva ameiva* and *Cnemidophorus* sp.1 represent divergence in the physical chromosomal mapping of different classes of repetitive DNA compared to other teiids, these two species were used to obtain a genomic library enriched with moderately and highly repetitive DNA, following the renaturation kinetics technique $C_o t$1-DNA (Zwick et al. 1997, Ferreira and Martins 2008). Genomic DNA samples from *Ameiva ameiva* and *Cnemidophorus* sp.1 (50 μl of 100–500 ng/μl of DNA in 0.3 M NaCl) were autoclaved (120°C) for 5 min to obtain fragments between 100 to 2000 bases pairs.

Table 1. Species of the Teinae and Tupinambinae subfamilies: collection sites, number and the analyzed animals and voucher specimens (lots) are listed. AM: Amazonas.

Subfamily	Species	Collection sites	Number and sex the analyzed animals	Voucher specimens (lots)
Teiinae	*Ameiva ameiva*	São Sebastião do Uatumã, AM Santa Isabel do Rio Negro, AM Tapauá, AM São Sebastião de Cuieiras, AM Reserva Adolpho Ducke, AM	30 (thirteen males; thirteen females; four without sex identification)	INPA H33213
	Cnemidophorus sp.1	Manaus, AM	13 (five males; eight females)	INPA H35018
	Kentropyx calcarata	São Sebastião do Uatumã, AM São Sebastião de Cuieiras, AM	7 (three males; four females)	INPA H31712
	Kentropyx pelviceps	Tapauá, AM	3 (three females)	INPA H34841
Tupinambinae	*Tupinambis teguixin*	São Sebastião do Uatumã, AM Tapauá, AM Reserva Adolpho Ducke, AM	5 (four females; one without sex identification)	INPA H34791

Then, samples were denatured at 95°C for 10 min, placed on ice for 10 seconds, and subsequently heated to reannealment at 65°C for 5 minutes. Thereafter, samples were incubated at 37°C for 8 minutes with a unit of S1 nuclease enzyme, whose function is to digest single-stranded DNA. Repetitive fraction of these samples was recovered by freezing in liquid nitrogen and subsequent DNA extraction using phenol-chloroform. The resulting DNA fragments were cloned and sequenced. C_0t1-DNA fragments of *Ameiva ameiva* and *Cnemidophorus* sp.1 were ligated into the plasmid vector pMOS-Blue blunt ended (GE Healthcare). Clones were sequenced in an automated ABI 3130 DNA sequencer XL (Applied Biosystem). The alignment of sequences was performed using the Clustal W tool (Thompson et al. 1994) included in the 7.0 BioEdit program (Hall 1999). The generated clones were submitted to BLASTN to detect similarity with public domain sequences contained in the NCBI database (http://www.ncbi.nlm.nih.gov), as well as in the Repbase database (Jurka et al. 2005) from the *Genetic Information Research Institute* (Giri) (http://www.girinst.org/repbase/), using the software CENSOR (Kohany et al. 2006).

C_0t1-DNA libraries were tagged using digoxigenin-11-dUTP for nick translation reaction, according to manufacturer's instructions (Dig-Nick Translation mix Roche). Anti-digoxigenin rhodamine (Roche) was used for signal detection. C_0t1-DNA libraries from *Ameiva ameiva* tagged using digoxigenin-11-dUTP were hybridized with chromosomes of the species. Further, homologous hybridizations were performed with Cot1-DNA libraries in *Cnemidophorus* sp.1. Probes obtained from C_0t1-DNA of *Ameiva ameiva* and C_0t1-DNA of *Cnemidophorus* sp.1 were also hybridized with chromosomes of other analyzed teiid species. FISH was performed under 77% stringency (2.5 ng/probe, 50% formamide, 10% dextran sulfate, and 2× SSC at 37°C for 18 h) (Pinkel et al. 1986). Chromosomes were counter stained with DAPI (2 mg/ml) in VectaShield mounting medium (Vector).

Chromosomes were analyzed using an epifluorescence microscope (Leica DFC 3000G). Metaphase stages were photographed; the karyotypes were loaded in Adobe Photoshop CS4 software and measured using Image J software. Afterward, the karyotypes were organized following the karyotype formula in karyotypes from *Ameiva ameiva*, *Kentropyx calcarata and Kentropyx pelviceps* were classified as gradual series of acrocentric chromosomes; those of *Cnemidophorus* sp.1 as biarmed, uniarmed, and microchromosomes; and those of *Tupinambis teguixin* as macro and microchromosomes.

Results

A total of 40 *Ameiva ameiva* Cot1-DNA clones were sequenced wherein 12 sequences corresponded 8 to microsatellites, 1 to transposons, 1 to retrotransposons, and 1 genes, all having high similarity with repetitive DNA deposited in public DNA banks (Table 2). For *Cnemidophorus* sp.1, 30 C_0t1-DNA clones sequenced wherein 8 sequences corresponded 6 to microsatellites, 1 to transposons and 1 genes, all also being highly similar to the repetitive DNAs deposited in public DNA banks (Table 3).

Table 2. Repetitive sequences obtained fraction $C_o t$1-DNA *Ameiva ameiva* with deposited sequences in the NCBI databases and GIRI.

Clone	Homology	Similarity	Identity
AA 1	DNA Transposons	Tc1-like de *Labeo rohita* (GenBank AY083617.1)	100%
AA 2	Microsatellite	*Betula platyphylla* var. *japonica* (GenBank AB084484.1)	100%
AA 3	Gene	TAP2 mRNA de *Oryzias latipes* (GenBank AB033382.1)	100%
AA 4	Microsatellite	*Coffea canephora* (GenBank EU526584.1)	100%
AA 5	Microsatellite	*Salmo salar* (GenBank Y11457.1)	96%
AA 6	Microsatellite	*Serranus cabrilla* (GenBank AM049431.1)	95%
AA 7	Microsatellite	*Hypericum perforatum* (GenBank FR732510.1)	93%
AA 8	Microsatellite	*Apteronemobius asahinai* (GenBank AB621739.1)	100%
AA 9	Non-LTR Retrotransposons	CR 1 (RepBase/GIRI*)	88%
AA 10	Microsatellite	*Bos taurus* (GenBank AF271953.1)	81%
AA 11	Microsatellite	*Colias behrii* (GenBank FN552755.1)	100%
AA 12	DNA Transposons	Tc1/mariner (RepBase/GIRI*)	80%

Table 3. Repetitive sequences obtained fraction $C_o t$1-DNA *Cnemidophorus* sp.1 with deposited sequences in the NCBI databases and GIRI.

Clone	Homology	Similarity	Identity
Cn 1	Gene	TAP2 mRNA de *Oryzias latipes* (GenBank AB033382.1)	100%
Cn 2	Microsatellite	*Betula platyphylla* var. *japonica* (GenBank AB084484.1)	100%
Cn 3	DNA Transposons	Tc1-like de *Labeo rohita* (GenBank AY083617.1)	100%
Cn 4	Microsatellite	*Colias behrii* (GenBank FN552755.1)	93%
Cn 5	Microsatellite	*Glaucosoma hebraicum* (GeneNank FJ409080.1)	97%
Cn 6	Microsatellite	*Colias behrii* (GenBank FN552755.1)	99%
Cn 7	Microsatellite	*Apteronemobius asahinai* (GenBank AB621739.1)	90%
Cn 8	Microsatellite	*Salmo salar* (GenBank Y11457.1)	96%

By using the homologous probe of $C_o t$1-DNA, *Ameiva ameiva* hybridization signals were located in the centromeric region/short arm of pairs 1 to 18, except pairs 9, 16, and 17, which showed interstitial signals (Figures 1a). When homologous hybridization of $C_o t$1-DNA *Cnemidophorus* sp.1 was performed with chromosomes of the species itself, signals were observed in the centromeric region/short arm of certain chromosomes, whereas the majority of chromosomes showed no signals in this chromosome region.

Hybrization using the heterologous probe of $C_o t$1-DNA obtained from *Ameiva ameiva* in *Cnemidophorus* sp.1 presented multiple signals along all chromosomes, with compartmentalized blocks mainly in interstitial regions (Figure 1b). In *Kentropyx calcarata* and *Kentropyx pelviceps*, signals were located at the centromeric region in the majority of chromosomes, with some pairs having terminal and interstitial signals (Figures 1c and 1d, respectively) and *Tupinambis teguixin* presenting signals in the

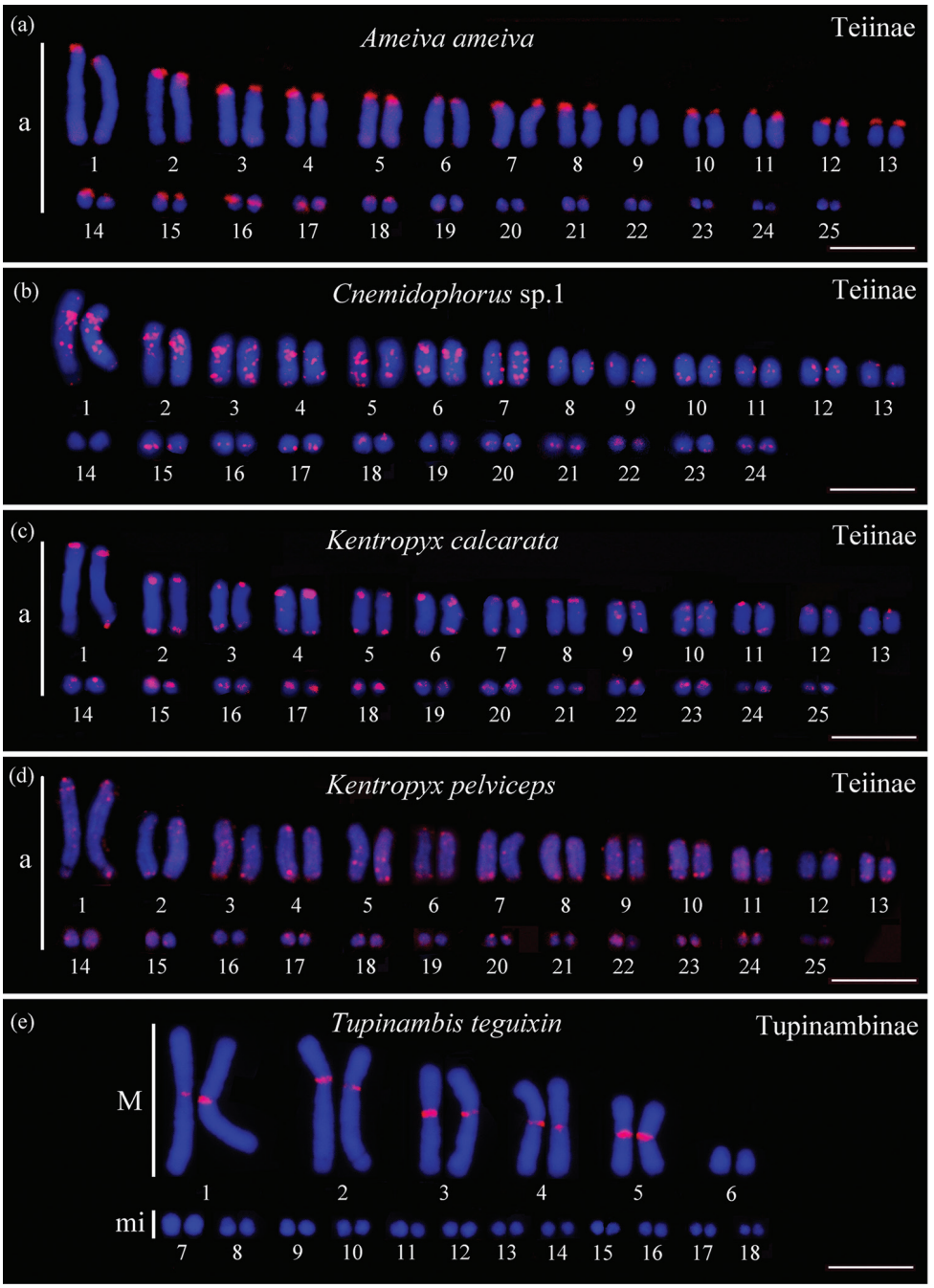

Figure 1. Karyotypes with *Ameiva ameiva* C$_o$*t*1-DNA probe hybridized (signal red). **a** *A. ameiva* **b** *Cnemidophorus* sp.1 **c** *Kentropyx calcarata* **d** *Kentropyx pelviceps* and **e** *Tupinambis teguixin*. The chromosomes were counterstained with DAPI. **a** = gradual series of acrocentric chromosomes. **m** = Macrochromosome, **mi** = microchromosome. Scale bar: 10 μm.

centromeric region in pairs 1, 2, 3, 4, and 5 (Figures 1e). Similar signal patterns were observed in heterologous hybridization of C$_o$*t*1-DNA obtained from *Cnemidophorus* sp.1 in chromosomes of *Ameiva ameiva*, *Kentropyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin*; however, the signals were more tenuous.

Discussion

Several classes of repetitive DNA are included in the genome of Amazonian teiid species, such as ribosomal DNA 5S, telomeric sequences, tropomyosin 1 genes, and retrotransposons *Rex* 1 and *SINE*. Most of these repetitive DNA sequences are allocated to heterochromatin regions, in addition to acting structurally in the centromeric and telomeric organization for *Ameiva ameiva*, *Cnemidophorus* sp.1, *Kentropyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin* (Carvalho et al. 2015b). In addition to these functions, heterochromatin can have other activities in the genome. It can act in chromosome segregation, nuclear organization, mitosis regulation in cell cycle progression, cell proliferation, gene expression regulation, and may affect the process of gene recombination (Grewal and Jia 2007, Skipper 2007, Buhler 2009, Bloom 2014).

However, heterochromatin of teiids is not limited to ribosomal DNA 5S, telomeric sequences, tropomyosin 1 gene, retrotransposons *Rex* 1 and *SINE*, and presents a complex composition with various repetitive DNA. In libraries obtained by C$_o$*t*1-DNA sequencing, it was evidenced that different microsatellites, transposons, retrotransposons, some gene families and other type of sequences (e.g. satellite DNAs) are also present in this fraction of moderately and highly repetitive DNA. They were allocated preferentially to the centromeric and telomeric regions of *Ameiva ameiva*, *Kentropyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin.* These sequences are also present in *Cnemidophorus* sp.1; however, they were present in euchromatic regions, similar to the pattern observed for telomeric sequences, tropomyosin 1 genes, and retrotransposons *Rex* 1 and *SINE*.

The sequences of repetitive DNA that were more abundant in moderately and highly repetitive fractions of the genome obtained using C$_o$*t*1-DNA in *Ameiva ameiva* and *Cnemidophorus* sp.1 were microsatellites, which were homologous with sequences of other organisms deposited in public databases, including plants, fish, mammals, bird and insect (Tables 2 and 3). However, the differences in microsatellites isolated from different species are noteworthy. Microsatellite accumulation may be associated with differentiation of sex chromosomes in some species of Sauropsida, because of high suppression of recombination, degeneration, and heterochromatinization (Pokorná et al. 2011, Gamble et al. 2014, Matsubara et al. 2015), but none of the species analyzed in this study had differentiated sex chromosomes; males and females had identical chromosomal constitution.

Microsatellite or simple sequence repeats (SSRs) are short sequences organized in long segments made up of tandem repeat units and are found in coding or non-coding regions in diverse species genomes (de Oliveira et al. 2015). In genetic analysis, they

are considered good molecular markers due to its high abundance in the genome, co-dominant inheritance, multi-allelic nature, good reproducibility, and has been used in studies of population genetics, phylogeny, linkage maps, and relationships (Qi et al. 2015, Čížková et al. 2015)

SSRs have functional roles in the genome, such as gene regulation, replication in transcription, protein function, and genome organization (Qi et al. 2015). These SSRs are generally located in centromeric regions and chromosomes ends of various organisms, such as those observed in *Ameiva ameiva*, *Kentropyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin* in this study, which corroborates the results of other cytogenomic studies involving satellites/microsatellite DNA from some species of lizards (Lacertidae, Scincidae and Varanidae) and snakes (Colubridae, Pythonidae, and Viperidae). These studies showed that the SSRs are located in the heterochromatin region, specifically in the centromeric, pericentromeric, and/or telomeric regions in chromosomes, suggesting differences in the compositions of these regions in the Squamata genome (Singh et al. 1976, Grechko et al. 2005, Chaiprasertsri et al. 2013, Giovannotti et al. 2013, 2014, Matsubara et al. 2015).

In addition to microsatellites, about 50% of sequences obtained by $C_o t$1-DNA from two species, exhibited similarities to transposable elements (transposons and retrotransposons). One important characteristic of these transposable elements is the transposition mechanism; retrotransposons transpose via an intermediate from RNA and transposons move up the genome through DNA copies that may be contributing to diversity and plasticity of the genome during evolution (Kordis 2009, Kojima et al. 2015). Sequences presented similarity ranging from 80% to 100% with Tc1-like and Tc1/mariner DNA transposons. The Tc1 transposon is found in many eukaryotic genomes and moves into and/or across genomes. It is an element that has defective copies that have been carried across several mutations causing genetic element inactivation in the genome (Ivics and Izsvák 2015, Tellier et al. 2015). Tc1 are not highlighted in the active form in vertebrate genomes (Dornan et al. 2015, Ivics and Izsvák 2015, Tellier et al. 2015).

An retrotransposon, non-LTR retrotransposon CR1 (Chicken Repeat 1) was identified only in the *Ameiva ameiva* genome; however, this does not indicate that it was not present in the genome of *Cnemidophorus* sp.1, because it simply may not have been identified in this study. Retroelement CR1 is a LINE family that is widely distributed in various organisms, including vertebrates (birds, reptiles, and fish) and invertebrates (Thompson et al. 2009). CR1 contains a 5'UTR region, two reading frames (ORFs 1 and 2), and a terminal region 3'UTR. Terminal region 3'UTR have small repeats (microsatellites) relatively conserved within each of the seven CR1 groups (groups A to G) (Suh 2015). The CR1 groups are the only transposable elements that were active during the evolution of birds and reptiles, and thus, have been widely used as phylogenetic markers, in species identification, and for understanding genome evolution within Amniota (Suh et al. 2014, Suh 2015)

Repetitive DNA rDNA 5s, tropomyosin 1 genes, and retroelements *Rex* 1 and *SINE* (Carvalho et al. 2015b), have also been mapped on chromosomes of teiid species

analyzed in this study, nevertheless they were not highlighted in sequencing of moderately and highly repetitive DNA obtained by the $C_o t$1-DNA technique. $C_o t$1-DNA is product of DNA genomic concentration (C_o), renaturation time in seconds (t), and a constant that depends on buffer cation concentration (Britten and Kohne 1968, Britten et al. 1974). Still, the results of isolated sequences by this technique may be different due to differences in DNA fragmentation (which occur randomly) or cloning processes which may explain failure to obtain repetitive DNAs 5S rDNA tropomyosin 1 genes and retroelements Rex 1 and SINE that also comprise the repetitive fraction of the five analyzed teiid species.

Some clones presented similarity with part of the gene TAP-2 (transporter associated with antigen processing). TAP is encoded by class I major histocompatibility complex (MHC) genes and are responsible for the transport of antigen peptides from the cytoplasm to endoplasmic reticulum (Zhao et al. 2006, Murata et al. 2009). This carrier is comprised of TAP-1 and TAP-2 subunits and is essential in antigen processing and highly conserved among various eukaryotic species (Zhao et al. 2006, Murata et al. 2009). Some genes may be associated with repetitive DNA in different species genomes and may involve various functions, such as genome stability, gene expression regulation, chromatin formation, and as miRNAs (Roberts et al. 2014, Liang et al. 2015). This association of genes with repetitive DNA has been found in some species of lizards, fish, and mammals (Valente et al. 2011, Pokorná et al. 2011, Terencio et al. 2015), and is also evident for analyzed species in this study.

The physical chromosomal map of homologous probes and/or heterologous of $C_o t$1-DNA showing signals associated with heterochromatic regions in centromeric and telomeric regions in chromosomes of *Ameiva ameiva*, *Kentropyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin*; and corroborated partially the standard heterochromatic (Carvalho et al. 2015a) the analyzed species. In addition, interstitial signals are also located in interstitial regions of the smaller pairs of chromosomes of *Ameiva ameiva*, *Kentropyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin*, which confirm the presence of these sequences in this region. However, if we compare the location of these moderately and highly repetitive DNA obtained from $C_o t$1-DNA with other repetitive DNA already mapped to the same location (Carvalho et al. 2015b), the pattern of signals are similar, being allocated to heterochromatic regions in centromeric and telomeric regions of chromosomes of these teiid species. Although various classes of repetitive DNA can be located in the same chromosomal region from different species, the number of copies of each element may be different (Chaiprasertsri et al. 2013).

In *Cnemidophorus* sp.1, hybridization of moderately and highly repetitive sequences obtained from $C_o t$1-DNA from *Ameiva ameiva* presented multiple signals along chromosomes with compartmentalized blocks in interstitial regions, which probably are located in euchromatic regions since they are not located in centromeric and telomeric heterochromatic regions of chromosomes (Carvalho et al. 2015a, b). This pattern is similar to the distribution pattern of other repetitive DNA (Carvalho et al. 2015b), indicating that the pattern of chromosomal organization of repetitive elements is different

from the other analyzed teiid species. Moreover, repetitive DNA located in euchromatic regions may significantly influence regulatory regions a gene expression because distribution of repetitive elements in the genome, especially the high association to genes with metabolic function, might be the result of a positive selection during evolution and imply practical roles of these elements in gene functions (Wang et al. 2007, Liang et al. 2015). Studies with $C_o t$1-DNA demonstrated that although mouse and human genomes have similar families of repetitive elements, primary sequences differ significantly among genomes, the human $C_o t$1-DNA do not hybridize to chromosomes in mice and the same happens with human (Hall et al. 2014). Furthermore, $C_o t$1-DNA was hybridized on all euchromatic regions of chromosomes (Hall et al. 2014).

On the other hand, FISH using $C_o t$1-DNA homologous probe revealed signals mainly in centromeric regions of chromosomes of *Cnemidophorus* sp.1. Thus, heterochromatic centromeric fraction of *Cnemidophorus* sp.1 seems to be composed of microsatellites and transposable elements obtained by $C_o t$1-DNA of the specie itself and elucidated by its sequencing, being different of the sequences obtained by $C_o t$1-DNA of *Ameiva ameiva*, despite belonging to same categories. Yet $C_o t$1-DNA contains a variety of different sequences, including the satellite DNA which are among the major component of centromeric heterochromatin, conversely to TE or microsatellite which are interspersed along chromosomes. Other repetitive elements may be present in the heterochromatic fraction of this species, which have not been detected by $C_o t$1-DNA (Carvalho et al. 2015b). Further, in the centromeric region, other repetitive DNA centromere specifics (Rosic et al. 2014) may be present that may be allocated in both macrochromosomes and microchromosomes, since they share the same repetitive DNA (Giovannotti et al. 2014, Matsubara et al. 2015, this study).

Although its function and multiprotein components are conserved among organisms, these repetitive DNA are extremely divergent regarding its structure, organization, dynamics, and propagation mechanisms, influencing the diversification and evolution of centromeres (Plohl et al. 2014). Therefore, it is clear that several mechanisms may operate in the diversification of these regions, such as unequal crossing-over, gene conversion, changes mediated by transposable elements, and slippage of DNA polymerase replication, resulting in differential composition of heterochromatin in closely related species, among different chromosomes in the same species, or in species-specific chromosomes (Chaiprasertsri et al. 2013, Aldrup-MacDonald and Sullivan 2014, He et al. 2015, Gao et al. 2015).

Conclusion

This study contributes to understanding the heterochromatic fraction composition and structure and organization of repetitive DNA of teiid genomes and indicates that the different classes of moderately and highly repetitive DNA are part of *Ameiva ameiva*, *Cnemidophorus* sp.1, *Kentropyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin* genome. This means that these sequences are shared among the analyzed teiid species, although not

always allocated on the same chromosome region. Nevertheless, the physical mapping of repetitive DNA revealed similarity among the species *Ameiva ameiva*, *Kentropyx calcarata*, *Kentropyx pelviceps*, and *Tupinambis teguixin* and showed that the centromeric fraction of *Cnemidophorus* sp.1 is different from that of the other analyzed species.

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Organization of some repetitive DNAs and B chromosomes in the grasshopper *Eumastusia koebelei koebelei* (Rehn, 1909) (Orthoptera, Acrididae, Leptysminae)

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Abstract

B chromosomes occur in approximately 15% of eukaryotes and are usually heterochromatic and rich in repetitive DNAs. Here we describe characteristics of a B chromosome in the grasshopper *Eumastusia koebelei koebelei* (Rehn, 1909) through classical cytogenetic methods and mapping of some repetitive DNAs, including multigene families, telomeric repeats and a DNA fraction enriched with repetitive DNAs obtained from DOP-PCR. *Eumastusia k. koebelei* presented 2n=23, X0 and, in one individual, two copies of the same variant of a B chromosome were noticed, which are associated during meiosis. The C-positive blocks were located in the pericentromeric regions of the standard complement and along the entire length of the B chromosomes. Some G+C-rich heterochromatic blocks were noticed, including conspicuous blocks in the B chromosomes. The mapping of 18S rDNA and U2 snDNA revealed only autosomal clusters, and the telomeric probe hybridized in terminal regions. Finally, the DOP-PCR probe obtained from an individual without a B chromosome revealed signals in the heterochromatic regions, including the entire length of the B chromosome. The possible intraspecific origin of the B chromosomes, due to the shared pool of repetitive DNAs between the A and B chromosomes and the possible consequences of their association are discussed.

Keywords

Cytogenetics, DOP-PCR, FISH, multigene family, Orthoptera

Introduction

The grasshoppers of the subfamily Leptysminae (Orthoptera, Acrididae) are divided into two tribes, Leptysmini and Tetrataeniini, comprising 75 species distributed exclusively in the Neotropical region (Amedegnato 1974, Carbonell 1977, Roberts and Carbonell 1982). The genus *Eumastusia* (Rehn, 1909) belongs to Tetrataeniini, with one species and two subspecies recognized, *E. koebelei koebelei* (Rehn, 1909) and *E. k. chapadendis* Roberts & Carbonell, 1980. For Leptysminae, few chromosomal data are available and, as in other Acrididae grasshoppers, most species exhibit the basic karyotype 2n=23, X0♂ with acrotelocentric chromosomes (Mesa et al. 1982, Loreto and de Souza 2000, Rocha et al. 2004). However, derived karyotypes arising from diploid number reduction were reported in *Stenopola pallida* (Bruner, 1906), *Leptysma argentina* Bruner, 1906 and *Tetrataenia surinama* (Linnaeus, 1764) (Mesa et al. 1982, Bidau and Hasson 1984). Additionally, B chromosomes have been reported in some species (Bidau and Hasson 1984, Confalonieri and Bidau 1986, Rocha et al. 2004), but no studies using molecular cytogenetic approaches have been conducted to elucidate the origin and evolution of these chromosomes.

B chromosomes are present in approximately 15% of eukaryote species and although discovered in 1907, they remain a mystery regarding their origin and evolution in most species (Houben et al. 2014). They are dispensable elements, largely known for their selfish nature as genomic parasites with patterns of non-Mendelian inheritance and a tendency to accumulate (Camacho 2005, Houben et al. 2014). These elements may arise from chromosomes of the carrier species or as a result of interspecific hybridization (Camacho et al. 2000), and they have their own evolutionary fate in different species and types of B chromosome (Banaei-Moghaddam et al. 2015). In some species, iso B chromosomes, formed by two identical arms, were described, which usually arise from centromere misdivision of telo- or acrocentric B chromosomes (see for example Grieco and Bidau 2000, Marques et al. 2012, Valente et al. 2014).

The accumulation of repetitive DNAs as an evolutionary process has been frequently reported for B chromosomes (Camacho 2005, Houben et al. 2014, Banaei-Moghaddam et al. 2015). These repetitive DNAs have been informative for understanding chromosomal and genomic evolution among grasshoppers (Cabrero and Camacho 2008, Cabrero et al. 2009, Cabral-de-Mello et al. 2011a, Anjos et al. 2015, Camacho et al. 2015, Palacios-Gimenez et al. 2015), as well as the possible evolutionary history of B chromosomes (Teruel et al. 2010, Oliveira et al. 2010, Bueno et al. 2013). To contribute to the understanding of chromosomal diversification, B chromosome evolution and patterns of repetitive DNA organization in Leptysminae, a poorly studied group, we analyzed the chromosomes of the species *Eumastusia koebelei koebelei* (Acrididae, Leptysminae). The analyses were performed through conventional and differential chromosome staining and through fluorescent *in situ* hybridization (FISH) using distinct probes, such as 18S rDNA, the TTAGG telomeric motif, U2 snDNA and a repetitive DNA fraction obtained by degenerate oligonucleotide-primed PCR (DOP-PCR).

Material and methods

Ten adult males of *E. k. koebelei* were collected in Serrolândia/Pernambuco, Brazil. The testes were fixed in Carnoy's solution (3:1 absolute ethanol:acetic acid) and stored at -20°C until use. For chromosomal preparations, the tissues were macerated in a drop of 50% acetic acid and the slides were dried using a hot plate at 40–45°C. All individuals were studied using conventional staining with 5% Giemsa to describe the general karyotype structure. C-banding was performed according to Sumner (1972) and fluorochrome staining (CMA_3/DA/DAPI) was performed according to Schweizer et al. (1983).

The 18S ribosomal DNA (rDNA) sequence and the U2 snDNA were obtained through polymerase chain reaction (PCR) from the genomes of *Dichotomius semisquamosus* (Curtis, 1845) (Coleoptera, Scarabaeidae) and *Abracris flavolineata* (De Geer, 1773) (Orthoptera, Acrididae), respectively, using primers described by Cabral-de-Mello et al. (2010) and Bueno et al. (2013). Telomeric probes were obtained by PCR using the complementary primers $(TTAGG)_5$ and $(CCTAA)_5$, following the protocol proposed by Ijdo et al. (1991). Genomic amplification preferential for the repetitive DNAs was performed through DOP-PCR using as template the DNA from an individual without B chromosomes (Telenius et al. 1992). The DOP primer (5' CCG ACT CGA GNN NNN NAT GTG G3') was used following the specifications described by Mazzuchelli and Martins (2009).

The 18S rDNA probe and DOP-PCR product were labeled using biotin-14-dATP through nick translation (Invitrogen, San Diego, CA, USA), while the telomeric probe and U2 snDNA were labeled through PCR with digoxigenin-11-dUTP (Roche, Mannheim, Germany). Fluorescent *in situ* hybridization (FISH) was performed according to the protocol proposed by Pinkel et al. (1986) with modifications described by Cabral-de-Mello et al. (2010). Single or double-color FISH was performed with the distinct probes and at least 200 ng of each probe was used. Probes labeled with biotin-14-dATP were detected using streptavidin-Alexa Fluor 488 (Invitrogen), and probes labeled with digoxigenin-11-dUTP were detected using anti-digoxigenin-Rhodamine (Roche). All preparations were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted in Vectashield (Vector, Burlingame, CA, USA). Chromosomes and signals were observed using an Olympus BX61 epifluorescence microscope equipped with appropriate filters. Photographs were recorded with a DP70 cooled digital camera. The images were merged and optimized for brightness and contrast with Adobe Photoshop CS2.

Results and discussion

The karyotype of *E. k. koebelei* is in accordance with previous descriptions (Mesa and Fontanetti 1983), corresponding to the modal karyotype for grasshoppers (Hewitt 1979, Mesa et al. 1982), which consists of 23 acrotelocentric chromosomes and the

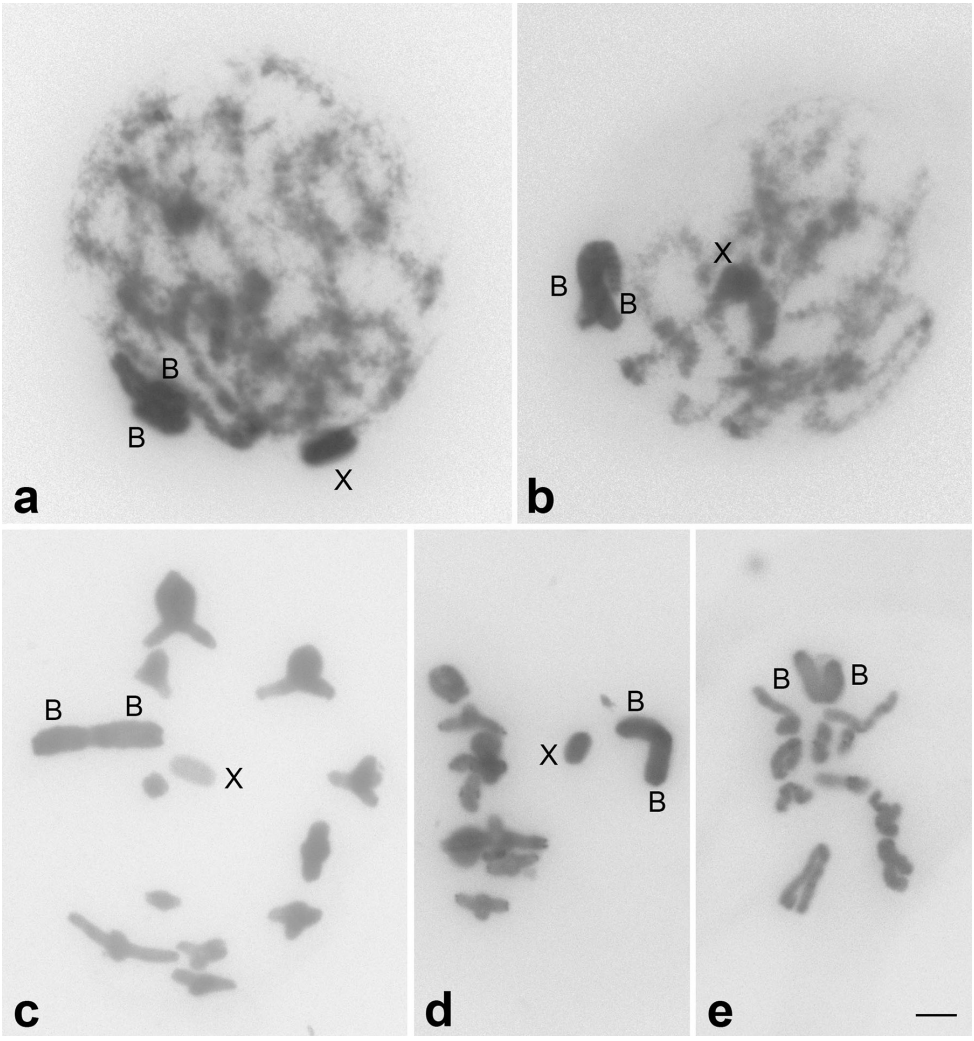

Figure 1. Conventional staining with Giemsa in meiotic cells of *E. k. koebelei* harboring B chromosomes. **a** zygotene **b** early pachytene **c** metaphase I **d** anaphase I **e** metaphase II. B chromosomes are associated side by side in initial meiosis (**a, b**) and by centromere in other cells (**c–e**). These chromosomes are also segregated to the same pole in **d** and maintained together in metaphase II (**e**). X and B chromosomes are indicated. Bar: 5 μm.

X0 sex-determining system in males (Figure 1). This chromosomal pattern is also frequent in Leptysminae, occurring in 20 of 22 species studied (Mesa et al. 1982, Bidau and Hasson 1984, Confalonieri and Bidau 1986, Loreto and de Souza 2000, Rocha et al. 2004). Among the ten analyzed individuals, one carried two acrocentric B chromosomes, which showed differential or similar condensation between them, depending on the cell analyzed (Figure 1). For the other Leptysminae, distinct variants of B chromosomes were previously observed in *Stenopola dorsalis* (Thunberg, 1827) (Rocha

et al. 2004), *Cylindrotettix obscurus* (Thunberg, 1827) and *C. santarosae* Roberts, 1975 (Confalonieri and Bidau 1986). Throughout meiosis, the two B chromosomes were associated, including metaphase II (Figure 1). From initial meiosis to pachytene, the B chromosomes were associated side by side, apparently linked by the centromere (Figure 1a, b). After diplotene, these elements remained connected by centromeres (Figure 1c–e), appearing as a single large biarmed chromosome under conventional analysis. These two B chromosomes segregate to the same pole during anaphase I (Figure 1d). This association suggests similarity between the two B chromosomes and that they could be two copies of the same B variant. Moreover, this association could influence the inheritance of these extra chromosomes, increasing the possibility of their segregation to the same anaphase pole, causing accumulation of these elements. In other grasshoppers, there are examples of acrocentric B chromosomes that are not associated throughout meiosis, such as in *Rhammatocerus brasiliensis* (Bruner, 1904) (Loreto et al. 2008).

C-banding revealed pericentromeric C-positive heterochromatic blocks in the A complement (Figure 2a), with the blocks in pairs 1, 2, 4-7, 9-11 and X chromosome being G+C-rich, while the rest of the heterochromatin was neutral for CMA_3 or DAPI fluorochromes. The blocks in pairs 4 and 7 occurred in only one of the homologues. In pairs 3 and 5, terminal CMA_3^+ blocks were also noticed, being heteromorphic for pair 3 (Figure 2b). This pattern of C-positive pericentromeric blocks associated with CMA_3^+ heterochromatic blocks and/or heterochromatin without base specificity (A+T or G+C) observed for the A chromosomes of *E. k. koebelei* is similar to other Leptysminae species, such as *Cornops aquaticum* (Bruner, 1906), *Stenopola dorsalis*, *Stenacris xanthochlora* (Marschall, 1836), *Tucayaca parvula* Roberts, 1977 and *Belosacris coccineipes* (Bruner, 1906), as well as in other species of Acrididae (Hewitt 1979, King and John 1980, Loreto and de Souza 2000, Rocha et al. 2004). In the two B chromosomes, the heterochromatin was distributed along their entire length (Figure 2d), and in the pericentromeric region a remarkable CMA^{3+} block was noticed. This CMA_3^+ area appeared as a conspicuous block in metaphase I while in initial meiosis (pachytene), due to less condensation, dispersed dots were always observed side by side (Figure 2e, f) due to the association of the two B chromosomes. The shared CMA_3^+ block in both B chromosomes reinforces their similarity, and we could speculate that a G+C-rich repetitive DNA, such as satellite DNA, could be present in the centromere of these B chromosomes, facilitating their constant association. This situation could cause a centromeric division failure that could favor the occurrence of whole-arm translocations leading to the formation of an isochromosome, proposed as a hypothesis for B isochromosome origin in the grasshopper *Metaleptea brevicornis adspersa* (Johannson, 1763) (Grieco and Bidau 2000).

Another argument favoring the notion of repetitive DNA enrichment in the C-positive regions was confirmed through the use of the DOP-PCR fraction as a probe, which revealed strong signals in these areas (Figure 2c). This is also valid for the B chromosomes, which were completely labeled (Figure 2g). The enrichment of distinct classes of repetitive DNAs in B chromosomes is a common pattern and these sequences could be involved with B chromosome differentiation and evolution (Houben et al. 2014, Banaei-Moghaddam et al. 2015). Considering that the DOP-PCR probe was

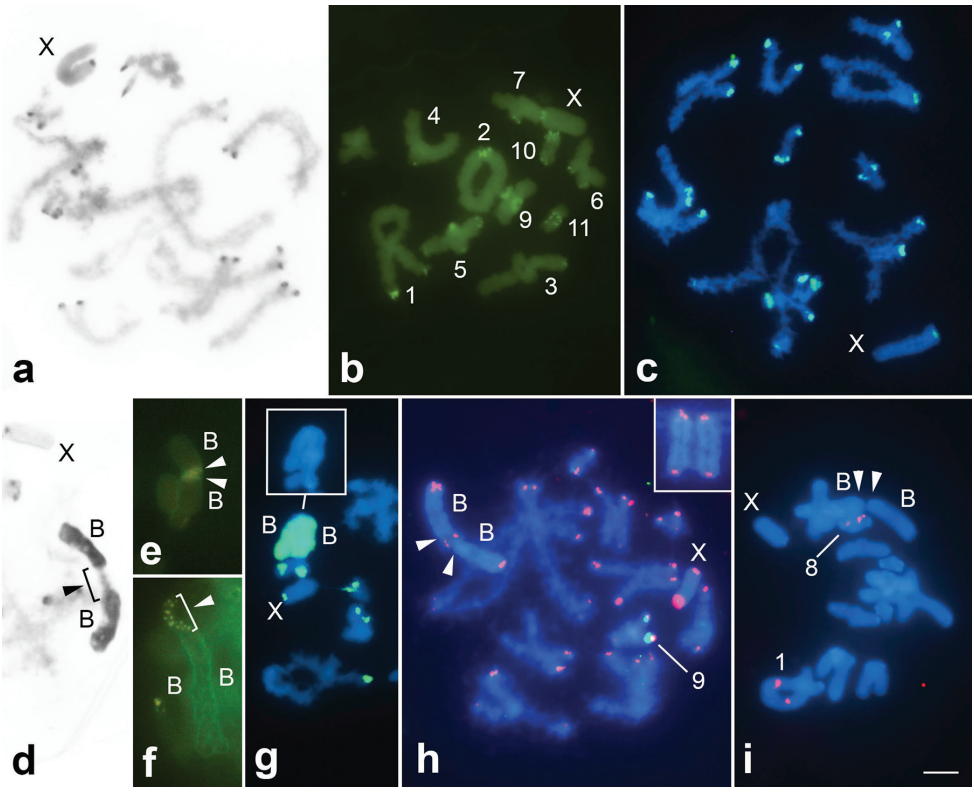

Figure 2. C-banding (**a, d**), CMA_3 staining (**b, e, f**) and FISH using as probes DOP-PCR (**c, g**), 18S rDNA-green and TTAGG-red (**h**) and U2 snDNA (**i**) in meiotic cells of *E. k. koebelei* with B chromosomes (**d–i**) and without them (**a–c**). **a, h** late pachytene **b** early diakinesis **c, d, g** diplotene **e, i** metaphase I **f** zygotene. Images **d–g** partially highlight B chromosomes. X, B and other chromosomes harboring specific signals are indicated; arrowheads point to the centromeres of B chromosomes. Inserts in **g**, **h** highlight B chromosomes. Bar: 5 μm.

obtained from an individual without B chromosomes, the repetitive DNA amplified using this approach is from the A genome. The hybridization signals in the B chromosomes indicate that this element shares repetitive sequences with the A complement, suggesting an intraspecific origin for the B chromosome. An intraspecific origin for B chromosomes was also suggested for other grasshoppers using distinct chromosomal markers, such as *Abracris flavolineata* (Menezes-de-Carvalho et al. 2015) and *Locusta migratoria* (Linnaeus, 1758) (Teruel et al. 2010), as well as other animal groups. Our result is similar to reports for the beetle *Dichotomius geminatus* (Arrow, 1913) using as probe the C_0t-1 DNA fraction that also isolates repetitive DNAs, such as the DOP-PCR, indicating the sharing of sequences between the B chromosome and the A complement (Cabral-de-Mello et al. 2011b). Although we suggest an intraspecific origin for the B chromosome in *E. k. koebelei*, it is impossible to determine if this event is related either to autosomes or the X chromosome, because both presented signals

for the DOP-PCR probe and CMA_3^+ blocks. It is also impossible to define the specific type of shared sequence, as the DOP-PCR probe is anonymous.

FISH with the telomeric probe revealed terminal signals in all chromosomes, including the B chromosome (Figure 2i). This result was expected considering that the karyotype of *E. k. koebelei* does not experienced gross chromosomal rearrangements observed in other Leptysminae, such as *Stenopola pallida*, *Tetrataenia surinama* and *Leptysma argentina*, bearing in mind the ancestral karyotype for grasshoppers (Mesa et al. 1982, Bidau and Hasson 1984). For the B chromosome, this probe confirmed that one individual harbored two B chromosomes (Figure 2i, insert) instead of one large biarmed chromosome, as suggested by conventional analysis. The mapping of multigene families revealed one pair of clusters on the same bivalent for 18S rDNA, proximally in pair 9. For U2 snDNA, four clusters on two bivalents were noticed, with interstitial placement in pair 1 and 8 in decreasing order of size (Figure 2h, i). This multigene family and the U1 snDNA located in pair 3 (Anjos et al. 2015) were not observed in the B chromosomes (Figure 2 h, i). It is more parsimonious to consider that chromosomes 1, 3, 8 and 9 were not involved in the origin of B chromosomes, but it could not be completely ruled out. Alternatively, these sequences could be lost during B chromosome differentiation, or the origin of the B chromosome did not involve the regions containing these sequences.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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RESEARCH ARTICLE

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Cytogenetic data on six leafcutter ants of the genus *Acromyrmex* Mayr, 1865 (Hymenoptera, Formicidae, Myrmicinae): insights into chromosome evolution and taxonomic implications

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Abstract

Cytogenetic data for the genus *Acromyrmex* Mayr, 1865 are available, to date, for a few species from Brazil and Uruguay, which have uniform chromosome numbers (2n = 38). The recent cytogenetic data of *Acromyrmex striatus* (Roger, 1863), including its banding patterns, showed a distinct karyotype (2n = 22), similar to earlier studied *Atta* Fabricius, 1804 species. Karyological data are still scarce for the leafcutter ants and many gaps are still present for a proper understanding of this group. Therefore, this study aimed at increasing cytogenetic knowledge of the genus through the characterization of other six species: *Acromyrmex balzani* (Emery, 1890), *A. coronatus* Fabricius, 1804, *A. disciger* (Mayr, 1887), *A. echinatior* (Forel, 1899), *A. niger* (Smith, 1858) and *A. rugosus* (Smith, 1858), all of which were collected in Minas Gerais – Brazil, except for *A. echinatior* which was collected in Barro Colorado – Panama. The number

and morphology of the chromosomes were studied and the following banding techniques were applied: C-banding, fluorochromes CMA_3 and DAPI, as well as the detection of 45S rDNA using FISH technique. All the six species had the same chromosome number observed for already studied species, i.e. 2n = 38. *A. balzani* had a different karyotype compared with other species mainly due to the first metacentric pair. The heterochromatin distribution also showed interspecific variation. Nevertheless, all the studied species had a pair of bands in the short arm of the first subtelocentric pair. The fluorochrome CMA_3 visualized bands in the short arm of the first subtelocentric pair for all the six species, while *A. rugosus* and *A. niger* also demonstrated in the other chromosomes. The AT-rich regions with differential staining using DAPI were not observed. 45S ribosomal genes were identified by FISH in the short arm of the first subtelocentric pair in *A. coronatus*, *A. disciger* and *A. niger*. The uniform chromosome number in the genus *Acromyrmex* (2n = 38) suggests that *A. striatus* (2n = 22) should be transferred to a new genus. Other aspects of the chromosome evolution in ants are also discussed.

Keywords
Chromosome evolution, karyotype, fungus-growing ants, biodiversity, heterochromatin, FISH

Introduction

Fungus-growing ants belong to the *Atta*-genus group (Ward et al. 2015) corresponding to the tribe Attini in the traditional sense. Leafcutter ants comprise a particular group of fungus-growing ants which are referred to as dominant herbivores of the Neotropics (Hölldobler and Wilson 1990). They include the genera *Acromyrmex* Mayr, 1865 and *Atta* Fabricius, 1804 and are exclusively found in the New World, primarily in the Neotropical region (Mayhé-Nunes and Jaffé 1998) and are considered the most derived group of ants arising about 8-12 million years ago (Schultz and Brady 2008, Mehdiabadi and Schultz 2010).

The genus *Acromyrmex* contains 33 described species (or more than 60 taxa if all subspecies and variations are included) (Bolton 2014, Rabeling et al. 2015). They are distributed from California (USA) to Patagonia (Argentina), excluding Chile. Most Brazilian species are widely distributed, although some of them have more restricted distribution (Gonçalves 1961, Mayhé-Nunes 1991, Delabie et al. 2011).

The genus *Acromyrmex* has been subdivided into two subgenera, *Acromyrmex* and *Moellerius* Forel, 1893 (Emery 1913), based on morphological traits. A phylogenetic study based on the morphological traits of this genus showed that the two subgenera formed distinct groups, of which *Moellerius* was considered the most derived (Mayhé-Nunes 1991). However, recent phylogenetic molecular studies of the genus *Acromyrmex*, including five species of the subgenus *Moellerius*, subdivided *Acromyrmex* species into distinct clusters (Cristiano et al. 2013). Only two of them, *Acromyrmex balzani* (Emery, 1890) and *Acromyrmex landolti* (Forel, 1885), were placed in the same group, suggesting that *Acromyrmex* and *Moellerius* could not be monophyletic (Cristiano et al. 2013); similar results were observed by Sumner et al. (2004). These data suggest that the two subgenera *Acromyrmex* and *Moellerius* do not represent natural groups.

Leafcutter ants are one of the most studied groups of fungus-growing ants (Mayhé-Nunes 1991), both in terms of biology and geographic distribution. Their status as agricultural pests has contributed to their knowledge, although taxonomic limits of different species are sometimes unclear (Delabie et al. 2011, Bacci et al. 2009). Under these circumstances, the so-called "integrative taxonomy" can produce more consistent results by complementing data obtained by different techniques (Schlick-Steiner et al. 2010). Nowadays, ant cytogenetics is a rapidly developing research field (Delabie et al. 2012). Cytogenetic data on fungus-growing ants with information for at least the chromosome number and morphology are available at present for 38 taxa (reviewed in Barros et al. 2011, Cristiano et al. 2013, Barros et al. 2013, 2014a, 2014b, Cardoso et al. 2014, Barros et al. 2015), corresponding to about 10% of described species (Brandão et al. 2011). In some ant genera, e.g. in *Mycetarotes* Emery, 1913 and *Cyphomyrmex* Mayr, 1862, chromosome numbers are variable at the species level (reviewed in Barros et al. 2011). However, species within the genera *Atta* (Barros et al. 2014a, Fadini and Pompolo 1996, Murakami et al. 1998, Barros et al. 2015) and *Acromyrmex* (Fadini and Pompolo 1996, Goñi et al. 1983) have the same chromosome numbers, 2n = 22 and 2n = 38, respectively, and similar chromosome morphology.

Cytogenetic data on the leafcutter ants are scarce. Namely, these data are available for five *Atta* species (Barros et al. 2014a, Fadini and Pompolo 1996, Murakami et al. 1998, Barros et al. 2015). In these species, 2n = 22 and a karyotypic formula of 2n = 18m+2sm+2st were found. Similar banding patterns were also observed in different species (Barros et al. 2014a, Murakami et al. 1998, Barros et al. 2015) which belonged to the three of four species groups defined on the basis of molecular data (Bacci et al. 2009). Cytogenetic data on *Acromyrmex* are also restricted but available for some taxa collected in Brazil: *A.* (*A.*) *crassispinus* (Forel, 1909); *A.* (*A.*) *subterraneus molestans* Santschi, 1925; *A.* (*A.*) *subterraneus subterraneus* Forel, 1893 (Fadini and Pompolo 1996); and in Uruguay: *A.* (*A.*) *ambiguus* Emery, 1888; *A.* (*A.*) *hispidus* Santschi, 1925; and *A.* (*Moellerius*) *heyeri* (Forel, 1899) (Goñi et al. 1983). All the species had the same chromosome number, 2n = 38. However, *Acromyrmex* (*Moellerius*) *striatus* (Roger, 1863) has recently shown 2n = 22, with a karyotypic formula of 2n = 20m+2sm (Cristiano et al. 2013), the same chromosome number found in all *Atta* species studied to date (Barros et al. 2014a, Fadini and Pompolo 1996, Murakami et al. 1998, Barros et al. 2015). Since *A. striatus* belongs to the well-supported clade which is quite distinct from other members of the genus *Acromyrmex*, it is suggested that this species is a sister group of all other leafcutter ants, which split before the divergence between *Acromyrmex* and *Atta* (Cristiano et al. 2013). Despite the same chromosome number, karyotypes of *A. striatus* and *Atta* species differ in morphology of two chromosome pairs as well as in their banding patterns (Cristiano et al. 2013, Barros et al. 2014a). The aim of the present study is therefore to describe chromosome sets of six species of the genus *Acromyrmex* to update our knowledge of karyotype evolution of leafcutter ants and Neotropical Formicidae in general.

Material and methods

Six cytogenetically studied *Acromyrmex* species were collected between August 2008 and March 2010 in the state of Minas Gerais – Brazil, except for *Acromyrmex echinatior* (Forel, 1899) which was collected in Panama (Table 1). Metaphases were obtained according to Imai et al. (1988) using larval ganglia or testes of freshly defecated larvae. To study chromosome morphology, metaphases were analyzed using conventional 4% Giemsa staining. The karyotypes were composed by arranging chromosomes according to their size and chromosome arm ratio (r) (Levan et al. 1964). Ten best metaphases per species with a similar degree of condensation were measured. Karyotypes were composed using the Corel Photopaint X3® software. The colonies and individuals analyzed are listed in Table 1. For the banding techniques, 4 to 10 individuals per species were used: C-banding for heterochromatin detection was performed according to Sumner (1972) with minor adaptations suggested by Barros et al. (2013); sequential fluorochrome staining with CMA_3/DA/DAPI (Schweizer 1980) was done to reveal specific GC- and AT-rich regions. To detect nucleolus organizer regions (NORs) in three species, 2 to 4 individuals of each species were studied using fluorescence *in situ* hybridization (FISH) with the 45S rDNA probe isolated from *Arabidopsis thaliana* (Moscone et al. 1996). The metaphases were observed and photographed using an Olympus® BX 60 microscope attached to a Q Color 3 Olympus® image capture system. For fluorochrome analysis, filters WB (450-480 nm) and WU (330-385 nm) were used for studying CMA_3 and DAPI staining, respectively, as well as Leica microscope DMRA2 filter Y3 (545/30 nm), attached to a D Leica IM50 Version 5 Release 190 software was used for FISH analysis.

Adult ant specimens were identified by J.H.C. Delabie and deposited in the ant collection at the Laboratório de Mirmecologia do Centro de Pesquisas do Cacau (CPDC/Brazil).

Results

All studied species had the same diploid chromosome number, 2n = 38 (Table 1, Fig. 1); males of *Acromyrmex coronatus* Fabricius, 1804 showed n = 19 (Table 1).

Chromosome measurements revealed morphological differences between similar karyotypes (Table 1). Several chromosome pairs were easily recognizable among different species, i.e. the first metacentric pair, the two largest subtelocentric pairs and the largest (or unique in some species) acrocentric pair (Fig. 1). In the karyotype of *A. balzani*, the first metacentric pair was larger than in any other studied species (Fig. 1). The size of the first metacentric and the first subtelocentric chromosome pairs were similar in all species (Fig. 1b–f), again except for *A. balzani* (Fig. 1a). No geographical intraspecific differences between the karyotypes were found (Table 1).

Table 1. *Acromyrmex* spp. cytogenetically studied in this paper. Locality, sample size (number of colonies/individuals stained with Giemsa), diploid (2n) and haploid (n) chromosome number and karyotypic formula.

***Acromyrmex* species**	**Locality (coordinates)**	**Colony – Individuals**	**2n (n)**	**Karyotypic formula**
A.(*Moellerius*) *balzani* (Emery, 1890)	Viçosa – MG – Brasil (20°45'S; 42°51'W)	3 – 12	38	2n = 12m + 10sm + 14st + 2a
A.(*Moellerius*) *balzani* (Emery, 1890)	Paraopeba – MG – Brazil (19º 17'S; 44º 29'W)	2 – 15	38	2n = 12m + 10sm + 14st + 2a
A.(*A.*) *coronatus* Fabricius, 1804	São Tiago – MG – Brazil (20°54'S; 44°30'W)	1 – 10	38 (19)	2n = 12m + 8sm + 16st + 2a
A.(*A.*) *coronatus* Fabricius, 1804	Paraopeba – MG – Brazil (19º17'S; 44º29'W)	5 – 20	38	2n = 12m + 8sm + 16st + 2a
A. (*A.*) *disciger* (Mayr, 1887)	Santos Dumont – MG – Brazil (21°27'S; 43°32'W)	2 – 15	38	2n = 10m + 12sm + 14st + 2a
A. (*A.*) *niger* (Smith, F. 1858)	Viçosa – MG – Brazil (20°45'S; 42°51'W)	3 – 21	38	2n = 12m + 14sm + 10st + 2a
A. (*A.*) *rugosus* (Smith, F. 1858)	Florestal – MG – Brazil (19°52'S; 44°24'W)	1 – 6	38	2n = 16m + 12sm + 8st + 2a
A. (*A.*) *rugosus* (Smith, F. 1858)	Paraopeba – MG – Brazil (19º 17'S; 44º 29'W)	5 – 22	38	2n = 16m + 12sm + 8st + 2a
A. (*A.*) *echinatior* (Forel, 1899)	Barro Colorado – Panama (9°9'N; 79°50'W)	2 – 10	38	2n = 8m + 6sm + 14st + 10a

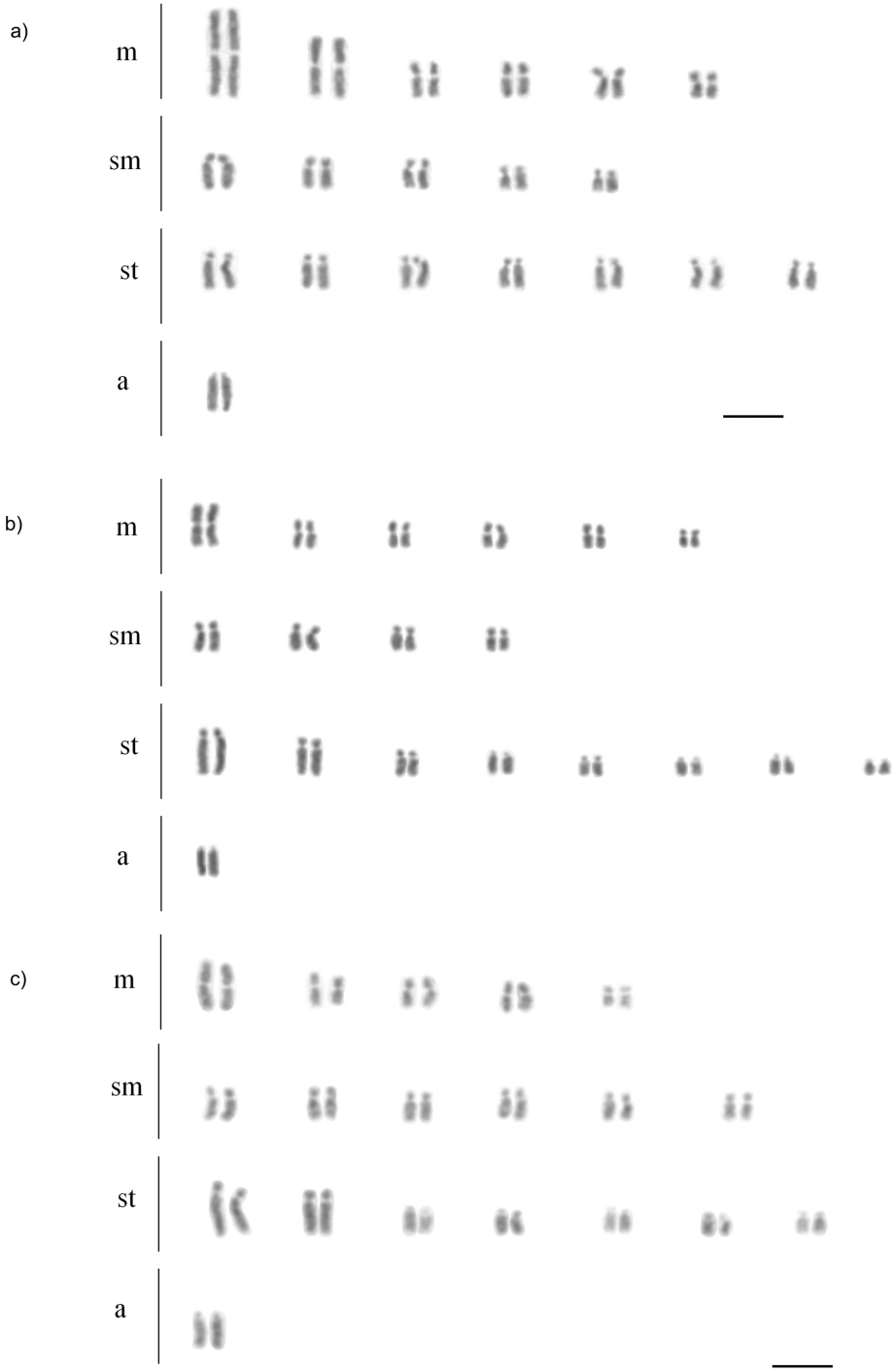

Figure 1. Karyotype of *Acromyrmex* species. **a** *A. balzani* **b** *A. coronatus* **c** *A. disciger* **d** *A. rugosus* **e** *A. niger* **f** *A. echinatior*. All species have 2n = 38. Bar = 5 μm.

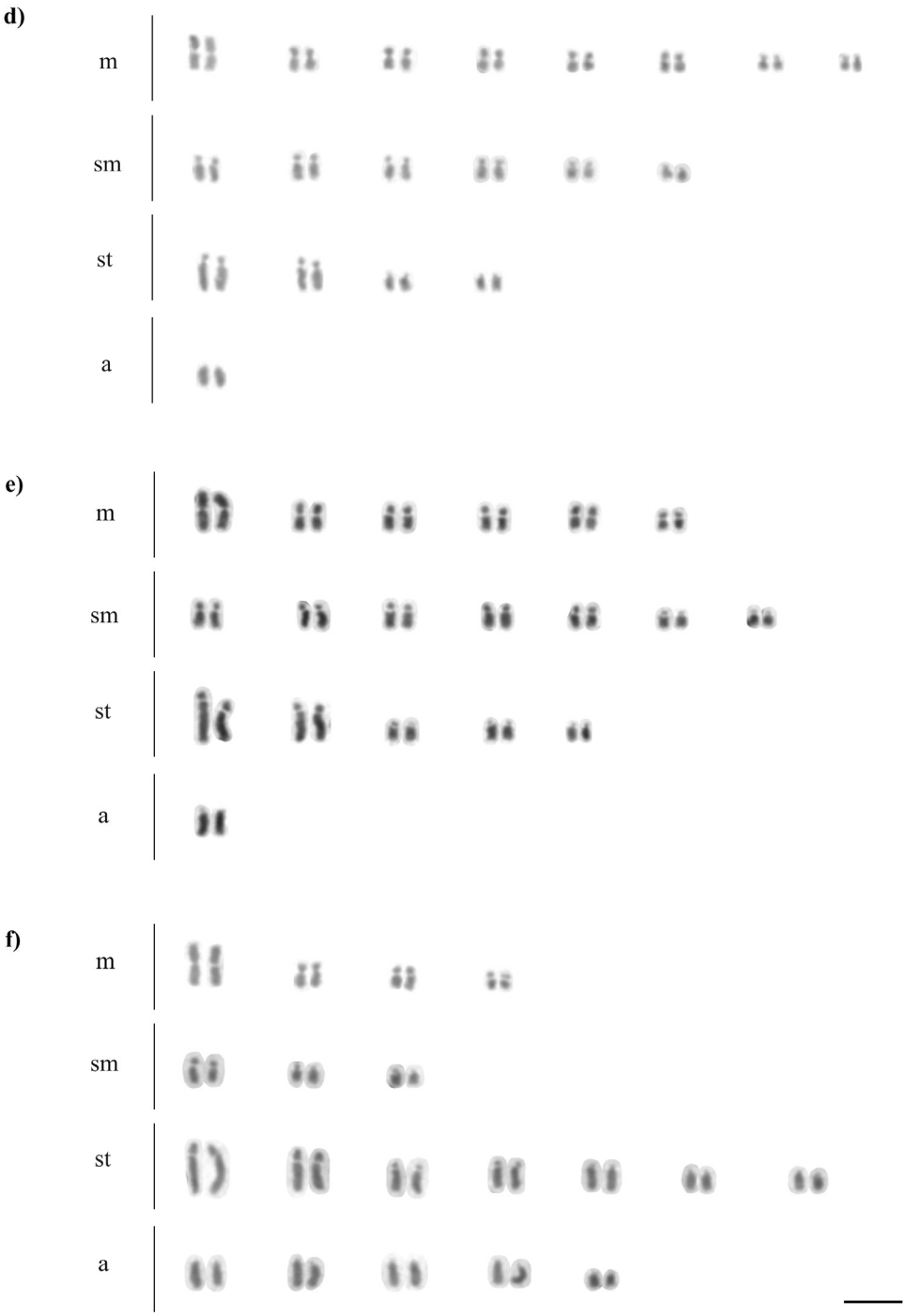

Figure 1. Continue.

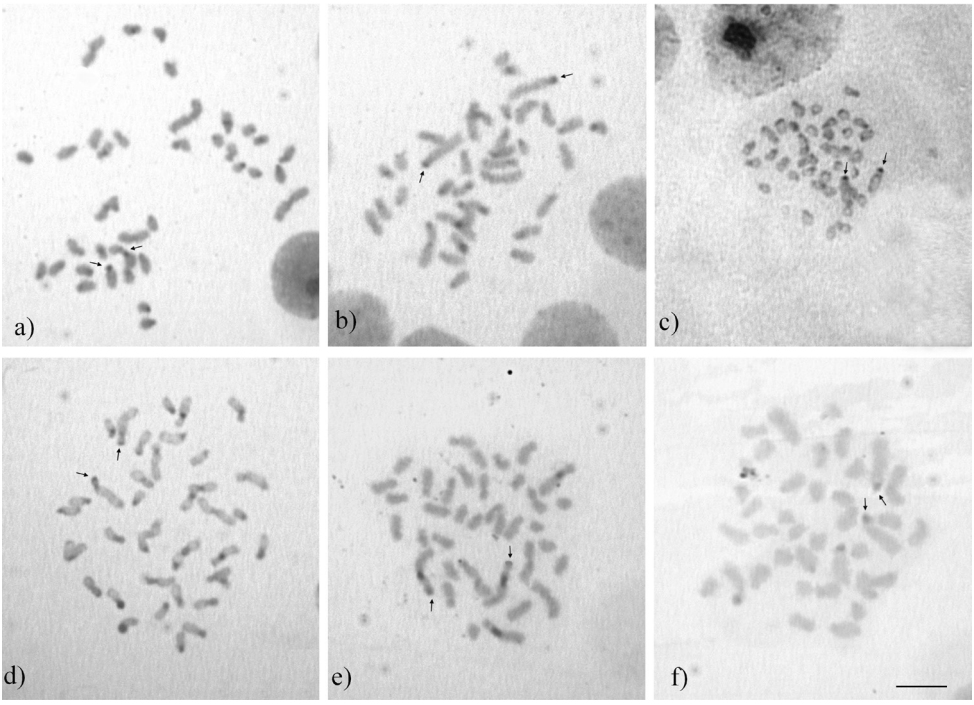

Figure 2. C-banded metaphases of *Acromyrmex* species. **a** *A. balzani* **b** *A. coronatus* **c** *A. disciger* **d** *A. rugosus* **e** *A. niger* **f** *A. echinatior*. Arrows indicate C-bands in the largest subtelocentric chromosome pair. Bar = 5 μm.

The C-banding results of *A. balzani*, *A. coronatus*, *A. disciger* (Mayr, 1887) and *A. rugosus* (Smith, 1858) indicated bands in some chromosomes: in the short arms of the submetacentric and subtelocentric and also in the centromeric regions of the metacentric chromosomes (Fig. 2). The largest subtelocentric pair (denominated as ST1) showed bands in all the species. *A. disciger* (Fig. 2c), *A. rugosus* (Fig. 2d) and *A. coronatus* (Fig. 2b) had bands in the telomeric region of the short arm. In *A. balzani* (Fig. 2a) and *A. echinatior* (Fig. 2f) karyotypes, the heterochromatic bands were observed in the short arm of ST1 pair. *A. niger* (Smith, 1858) (Fig. 2e) showed bands in the telomeric region of the short arm of the ST1 pair and also in the long arm. Moreover, *A. rugosus* and *A. niger* had additional bands in the pericentromeric region and in the long arm region of the second subtelocentric pair, respectively.

The short arms of the ST1 pair revealed differences in the banding patterns among the species with fluorochrome CMA_3. *A. disciger* (Fig. 3c) and *A. coronatus* (Fig. 3b) in the telomeric regions. *A. balzani* (Fig; 3a) in the short arms; and *A. echinatior* in the interstitial region (Fig. 3f). However, *A. niger* (Fig. 3e) and *A. rugosus* (Fig. 3d), besides the telomeric regions of the ST1 pair, also showed bands in additional chromosomes. *A. niger* (Fig. 3e) had additional bands in the long arm of the ST1 pair and in the long arm of the second larger subtelocentric pair. *A. rugosus* (Fig. 3d) showed small bands in the telomeric regions of ST1 and in at least three other chromosomes.

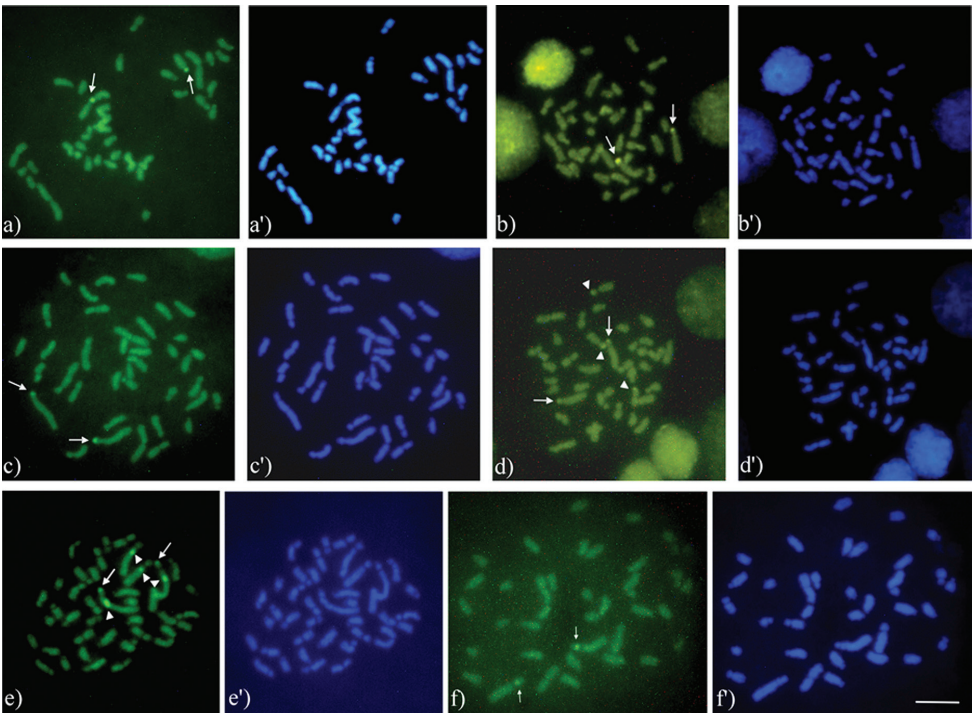

Figure 3. Metaphases of *Acromyrmex* species stained with CMA_3 and DAPI, respectively. **a** *A. balzani* **b** *A. coronatus* **c** *A. disciger* **d** *A. rugosus* **e** *A. niger* **f** *A. echinatior*. Arrows indicate CMA_3-positive bands in the largest subtelocentric pair. Arrowheads indicate additional CMA_3-positive bands in *A. niger* and *A. rugosus*. Bar = 5 μm.

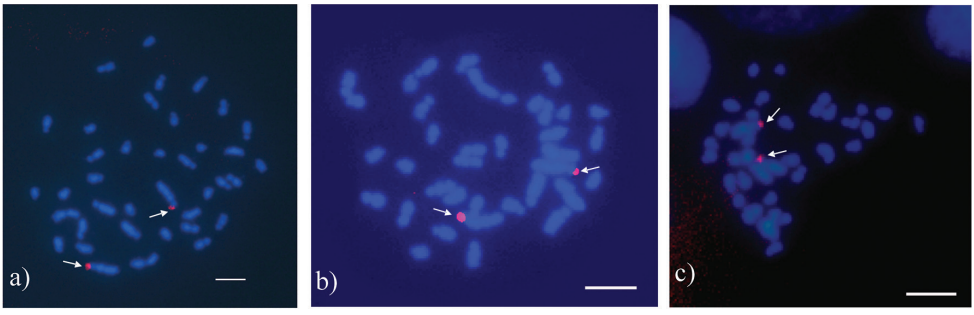

Figure 4. Metaphases of *Acromyrmex* species. FISH with 45S rDNA probe. **a** *A. disciger* **b** *A. coronatus* **c** *A. niger*. Arrows indicate hybridization signals in the subtelocentric pair. Bar = 5 μm.

DAPI-positive bright bands which could correspond to AT-rich regions were not revealed (Fig. 3a–f). Instead, DAPI-negative regions which co-localized with CMA_3-positive bands were visualized. FISH with the 45S rDNA probe visualized telomeric bands in ST1 pairs of *A. coronatus*, *A. disciger* and *A. niger* (Fig. 4).

Discussion

Karyotypes of *Acromyrmex* species observed in this study can be distinguished only on the basis of chromosomal measurements. Differential heterochromatin growth is therefore responsible for small but robust differences in chromosomal morphology, and these differences could not be observed using classification proposed by Imai (1991). This classification is based on heterochromatin distribution, and it does not reflect differences in the chromosome size among similar karyotypes. However, some chromosomes had the chromosome arm ratio (r) within the limits of the classification (submetacentric or subtelocentric). In the ants *A. coronatus* and *A. niger* the chromosomes were classified according to the greater ratio (r), i.e., as subtelocentrics.

The largest metacentric pair of *A. balzani* strongly differs in size from that of other species probably due to complex chromosomal rearrangements that need to be further investigated. This can be explained by the fact that *A. balzani* forms a separate clade together with *A. landolti* according to the molecular phylogeny presented by Cristiano et al. (2013).

The six studied species showed heterochromatic segments on the short arms of ST1 pair. It was observed that these GC-rich heterochromatic regions correspond to NOR which is, in turn, confirmed by FISH with the 45S rDNA probe in the chromosomes of *A. coronatus*, *A. disciger* and *A. niger*. In the latter species, this technique revealed a single NOR, although additional multiple CMA_3-positive bands also were observed. This means that these additional bands are not related to the ribosomal genes. NORs are generally GC-rich and CMA_3-positive in different organisms (Reed and Phillips 1995). However, CMA_3-positive regions are not always rDNA clusters (Sumner 1990), as was observed in *A. niger*. Multiple CMA_3-positive bands and a single NOR revealed by FISH were observed in the fungus-growing ant *Mycocepurus goeldii* (Forel, 1893) (Barros et al. 2010, 2012); however, ribosomal gene mapping studies of Formicidae of the Neotropical region using FISH are scarce.

The nonspecific banding pattern of DAPI staining revealed in the present work is similar to those observed for other fungus-growing ants such as *M. goeldii* (Barros et al. 2010), *A. striatus* (Cristiano et al. 2013), *Trachymyrmex fuscus* Emery, 1934 (Barros et al. 2014) and *Atta* species (Barros et al. 2014, 2015).

Up to now, 12 *Acromyrmex* species (plus the only subspecies) are cytogenetically studied. All of them show 2n = 38, including both subgenera *Acromyrmex* and *Moellerius* (Fadini and Pompolo 1996, Goñi et al. 1983). However, *A. striatus* with 2n = 22 differs from other already known species (Cristiano et al. 2013). The latter chromosome number is also characteristic of all *Atta* species. Both *Acromyrmex* and *Atta* are considered the most derived genera of fungus-growing ants (Schultz and Brady 2008, Mehdiabadi and Schultz 2009). Since differences in chromosomal morphology and banding patterns can be observed within *Acromyrmex*, it differs in this respect from *Atta*.

Patterns of heterochromatin distribution on short arms of some submetacentric and subtelocentric chromosomes of *Acromyrmex* species suggest that centric fissions which contributed to the origin of the derived karyotype with 2n = 38, probably occurred in the karyotype of the most recent common ancestor of this group. Moreover,

recent molecular phylogenetic reconstruction by Cristiano et al. (2013) also suggests that *A. striatus* is a sister group to the remaining leafcutter ants. The above-mentioned fissions were followed by heterochromatin growth which played an important role in maintaining telomeric stability according to the minimum interaction theory proposed by Imai et al. (1994). Differential heterochromatin growth in *Acromyrmex* is responsible for interspecific variation in the size of heterochromatic blocks. However, different species of this genus retain the same chromosome number (2n = 38), except for *A. striatus* with 2n = 22 (Cristiano et al. 2013).

Besides data on the chromosome numbers, multiple GC-rich segments were observed in the fungus-growing ants *M. goeldii* (Barros et al. 2010), *Sericomyrmex* sp. (Barros et al. unpublished data), *T. fuscus* (Barros et al. 2014b) and *A. striatus* (Cristiano et al. 2013). Nevertheless, single CMA_3-positive bands were found in the short arms of ST1 pairs in all *Acromyrmex* spp. and in the fourth chromosome pair of *Atta* species. Multiple GC-rich segments observed in *A. niger* do not represent NORs, and therefore are probably derived.

Cytogenetic data permitted the differentiation among four of the six *Acromyrmex* species studied. *A. balzani*, included in the *Moellerius* subgenus, showed the largest metacentric chromosome pair with lower size compared with the other species. *A. echinatior*, besides the higher quantity of acrocentric chromosomes, also had interstitial bands in the ST1 pair for the fluorochrome CMA_3, differing from the other species, which suggest the possibility of inversion. *A. niger* showed multiple CMA_3-positive bands: in the telomeric regions of the short arms of the ST1 pair, in the pericentromeric regions of the long arm of the ST1 pair and in the second largest subtelocentric pair. *A. rugosus* had a greater proportion of metacentric chromosomes compared with the other *Acromyrmex* and also showed small bands in the telomeric regions of at least three other chromosomes. *A. coronatus* and *A. disciger* could only be cytogenetically differentiated from the other species by slight differences in the morphology that are probably due to the differential growth of heterochromatin on the short arms of the chromosomes.

Five of the six *Acromyrmex* species studied in this paper were collected in a particular area in the South East of South America. However, *A. echinatior* was collected in Central America, which is more than 5,000 km from the main study area. Moreover, another three species analyzed by Goñi et al. (1983) were collected in Uruguay, expanding the knowledge of South American *Acromyrmex*. The chromosome number is uniform among different species of *Acromyrmex* (sensu stricto), although chromosome morphology and banding patterns of these ants allow the identification of some species via their karyotypes. However, karyotype structure of *A. striatus* suggests that it belongs to a different lineage and therefore, according to sequences obtained from nuclear genes, this species does not belong to the "true" *Acromyrmex* lineage but to the sister group to the remaining leafcutter ants (Cristiano et al. 2013). A new genus therefore could be erected due to the karyotypic features of *Acromyrmex* which are further supported by combining the already published cytogenetic and molecular data (Cristiano et al. 2013) together with the additional karyological information. In this case, cytogenetics shows its importance as an additional tool in integrative taxonomy.

Conserved chromosome numbers were found in certain ant genera, as in *Pogonomyrmex* Mayr, 1868 in which 13 of 15 studied species had the same chromosome number. The two other species were transferred to another subgenus *Ephebomyrmex* Wheeler, 1902 (Taber et al. 1988). *Camponotus* (*Myrmothrix*) spp. also presented uniform chromosome number (Mariano et al. 2003), as did the other members of the genera *Pheidole* Westwood, 1839, *Lasius* Fabricius, 1804 and *Iridomyrmex* Mayr, 1862 (reviewed in Lorite and Palomeque 2010). Other animal groups, such as most birds and different genera of insects of the order Lepidoptera also demonstrated conservatism in respect to the chromosome number (White 1973), as did some bee genera, such as *Melipona* Illiger, 1806 (reviewed in Rocha et al. 2007) and *Partamona* Schwarz, 1939 (reviewed in Martins et al. 2009).

Our data confirmed uniformity of the chromosome number (2n = 38) in the studied *Acromyrmex* species. However, chromosomal rearrangements such as heterochromatin growth are likely to be responsible for karyotypic differentiation in this ant group. Location of rDNA clusters of other leafcutter ants (especially *A. striatus*) also needs to be determined using molecular cytogenetic techniques (FISH). Moreover, cytogenetic studies of other members of fungus-growing ants, e.g. of the genus *Trachymyrmex* Forel, 1893 which represents the sister group to leafcutter ants, will be important for better understanding of chromosomal evolution of this group and Neotropical Formicidae in general.

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RESEARCH ARTICLE

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ZZ/ZW sex chromosome system in the endangered fish *Lignobrycon myersi* Miranda-Ribeiro, 1956 (Teleostei, Characiformes, Triportheidae)

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Abstract

Lignobrycon myersi is an endemic fish species from a few coastal rivers in northeastern Brazil. Based on molecular evidence, *L. myersi* and genera *Triportheus* Cope, 1872, *Agoniates* Müller & Troschel, 1845, *Clupeacharax* Pearson, 1924 and *Engraulisoma* Castro, 1981 were placed in the family Triportheidae. In the present work, we report the first cytogenetic data for *L. myersi* to test the hypothesis that *Lignobrycon* and *Triportheus* are closely related. Studied specimens presented 2n=52 with 28 metacentric (m), 18 submetacentric (sm) and six subtelocentric (st) chromosomes for males and 27 m, 19 sm and 6 st for females, characterizing a ZZ/ZW sex chromosome system. The Z chromosome corresponds to the largest chromosome in karyotype while the W is about 50% smaller than the Z and largely heterochromatic. Terminal nucleolus organizer regions, GC-rich sites and 18S rDNA signals were detected on pair 14. However, additional 18S rDNA sites were observed in the W chromosome. The 5S rDNA was mainly detected on long arms of pair 7. The apparent synapomorphic chromosomal traits of *Triportheus* and *L. myersi* reinforce their close phylogenetic relationship, suggesting that the ZZ/ZW chromosome system in both genera has arisen before cladogenic events.

Keywords

Evolution, female heterogamety, rDNA, sex determination, *Triportheus*

Introduction

Lignobrycon myersi Miranda-Ribeiro, 1956 is a small characin fish (about 11 cm in length) characterized by a compressed body with keeled coracoids, adapted to swim near the surface. The type-locality of *L. myersi* is located in the Almada river basin, a costal drainage in Bahia (Castro and Vari 1990). Nearly 10 years later, this species was also collected in the nearby Contas river basin in Bahia (Castro and Jucá-Chagas 2008). Because of its narrow geographic range, associated with intensive environmental degradation (deforestation, pollution and impoundment), *L. myersi* is currently listed in the IUCN Red List of Threatened Species of Brazil (Castro and Jucá-Chagas 2008).

Based on external morphology and osteological evidence, *L. myersi* has been regarded as the only living sister-group of the elongate hatchetfish *Triportheus* Cope, 1872, composing the subfamily Triportheinae within Characidae (Malabarba 1998). Nonetheless, phylogenetic studies using DNA sequences of two mitochondrial and three nuclear genes revealed that this monophyletic group should be expanded and elevated to a family status (Triportheidae), including the following genera of tetras or freshwater sardines: *Agoniates* Müller & Troschel, 1845, *Clupeacharax* Pearson, 1924, *Engraulisoma* Castro, 1981, *Triportheus* and *Lignobrycon* (Oliveira et al. 2011).

Interestingly, *Triportheus* is one of the few fish groups in which sex chromosomes have probably appeared prior to the adaptive radiation of this genus (Artoni and Bertollo 2002). Thus, all species of *Triportheus* studied so far share a 2n = 52 and a ZZ/ZW sex chromosome system in which the W is remarkably smaller than Z chromosomes and usually carries 18S rDNA cistrons (Artoni and Bertollo 2002, Diniz et al. 2008a, 2009, Marquioni et al. 2013). Only *Triportheus venezuelensis* Malabarba, 2004 is differentiated by presenting nucleolus organizer regions (NORs) on Z chromosomes (Nirchio et al. 2007) (Table 1). This trend combined to the close relationship between *Lignobrycon* and *Triportheus* revealed by morphological and molecular analyses is appealing to cytogenetic studies in *L. myersi*.

Therefore, the present work reports the first cytogenetic characterization in *L. myersi* in order to understand the evolution of sex chromosomes within Triportheidae, particularly in relation to *Triportheus* species.

Material and methods

Fourteen specimens of *L. myersi* (4 males and 10 females) were collected in their type-locality in Braço (14°40'52"S/39°14'39"W) and Almada (14°39'35"S/39°13'24"W) Rivers, both belonging to the Almada River basin in the state of Bahia, northeastern Brazil (Fig. 1). Voucher specimens of *L. myersi* were deposited under the code MBML 6400 in the fish collection of the Biology Museum Prof. Mello Leitão.

Metaphase chromosomes were obtained from anterior kidney cells as described by Netto et al. (2007), without mitotic stimulation. Chromosome spreads were stained with 5% Giemsa in phosphate buffer for karyotyping. Heterochromatin segments

Table 1. Cytogenetic data in Triportheidae (species marked with "*" show synteny of 18S and 5S rNA).

Species	2n	Sex system	18S rDNA	5S rDNA	Reference
Lignobrycon myersi	52	ZZ/ZW	1 pair/W	2-4 pairs	present study
Triportheus albus	52	ZZ/ZW	1 pair/W	1 pair	Diniz et al. (2009); Marquioni et al. (2013)
*T. angulatus**	52	ZZ/ZW	2 pairs/Z/W	1 pair	Marquioni et al. (2013)
*T. auritus**	52	ZZ/ZW	2 pairs/W	5 pairs	Marquioni et al. (2013)
T. culter	52	ZZ/ZW	1 pair/W	-	Falcão (1988)
T. guentheri	52	ZZ/ZW	1 pair/W	1 pair	Diniz et al. (2009); Bertollo and Cavallaro (1992)
*T. nematurus**	52	ZZ/ZW	1 pair/W	1 pair	Diniz et al. (2008a); Marquioni et al. (2013)
*T. signatus**	52	ZZ/ZW	1 pair/W	1 pair	Diniz et al. (2009); Marquioni et al. (2013)
*T. trifurcatus**	52	ZZ/ZW	2 pairs/W	1 pair	Marquioni et al. (2013)
T. venezuelensis	52	ZZ/ZW	1 pair/Z	-	Nirchio et al. (2007)

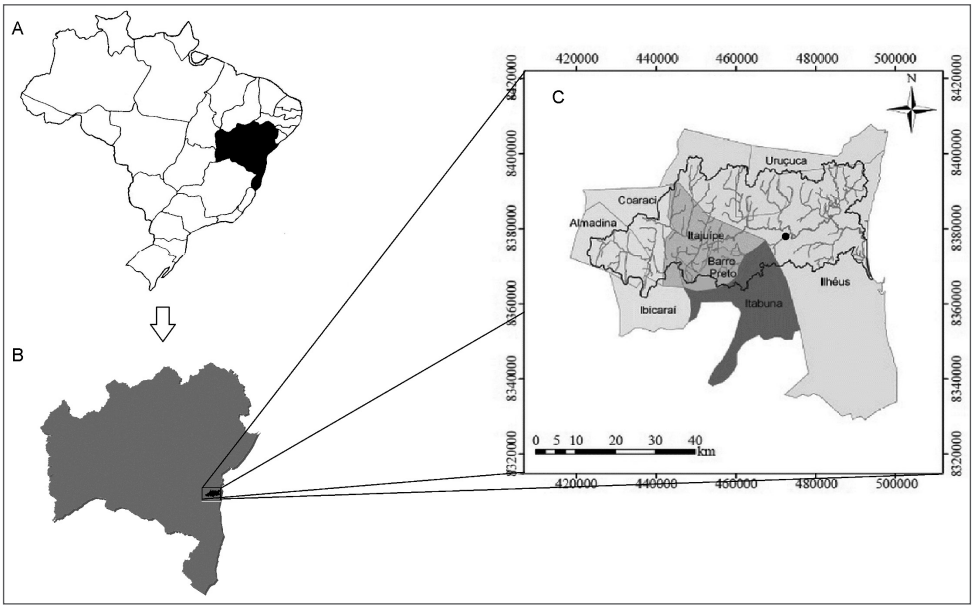

Figure 1. Map of Brazil (**a**), highlighting the state of Bahia (**b**) and collection site of *Lignobrycon myersi* in the Almada river basin (**c**).

were visualized by C-banding (Sumner 1972) and active nucleolar organizer regions (Ag-NORs) were detected by silver nitrate staining (Howell and Black 1980). The GC- and AT-rich sites were identified by chromomycin A_3 (CMA_3) and 4,6-diamidino-2-phenylindole (DAPI), respectively (Schmid 1980).

The fluorescence *in situ* hybridization (FISH) was performed to map simultaneously 18S and 5S rDNA on chromosomes of *L. myersi* according to Pinkel et al. (1986), with slight modifications and high stringency hybridization conditions (77%). The 18S rDNA probe was obtained from DNA of the red-eyed tetra *Moenkhausia sanctafilomenae* Steindachner, 1907 as described by Hatanaka and Galetti (2004),

labeled with biotin-16-dUTP via nick translation using the BioNick Labeling System kit (Invitrogen) and signals were detected using avidin-fluorescein isothiocyanate (FITC) conjugate (Sigma). The 5S rDNA probe was obtained from DNA of the headstander *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti 1999), labeled with digoxigenin-11-dUTP via nick translation using Dig-Nick Translation Mix kit (Roche), and detected with anti-digoxigenin-rhodamine antibodies (Roche). Chromosomes were counterstained using DAPI (0.2 mg/mL) in Vectashield Mounting Medium (Vector) and slides were stored in a dark chamber up to analysis.

Metaphases were photographed using an Olympus BX-51 epifluorescence microscope equipped with digital camera and the software Image-Pro Plus® v. 6.2. The chromosomes were classified according to their morphology as proposed by Levan et al. (1964). The chromosomal pairs were arranged in karyotypes by decreasing size of chromosomes, as usually presented in cytogenetic reports of *Triportheus* (e.g. Diniz et al. 2008a).

Results

Both males and females of *L. myersi* shared a modal diploid number of 2n = 52. The chromosomal pairs of males were homomorphic (Fig. 2a), being composed of 28 metacentric (pairs 1, 2, 10, 12, 15, 18–26), 18 submetacentric (pairs 3, 4, 6–9, 11, 14, 16) and six subtelocentric (pairs 5, 13, 17) chromosomes. In turn, females were differentiated by the presence of a single metacentric chromosome equivalent to pair 1, besides a small submetacentric chromosome, absent in males (Fig. 2c). Therefore, *L. myersi* is characterized by the occurrence of differentiated sex chromosomes of ZZ/ZW type, being the Z chromosomes equivalent to the first and largest chromosomal pair.

The heterochromatin segments were distributed in small amounts over pericentromeric and terminal regions of some chromosomal pairs (Fig. 2b). The small submetacentric W chromosome was mostly heterochromatic with euchromatin restricted to terminal region of short arms (Fig. 2d).

The silver staining revealed a single NOR-bearing submetacentric pair (14^{th}) with heteromorphic marks at terminal regions on long arms in both sexes (Fig. 2e, i). Similarly, GC-rich sites (CMA_3^+ and $DAPI^-$) were coincident with Ag-NORs (Fig. 2f, j) and also characterized by size heteromorphism since fluorescent signals were occasionally absent in one of the homologues (Fig. 2j).

The FISH with 18S rDNA probe confirmed the presence of NORs on pair 14 as well as the size differences between clusters in homologous chromosomes (Fig. 2g, k), as verified by silver nitrate and CMA_3 staining before. In addition, 18S rDNA sequences were also detected at interstitial region of the W chromosome (Fig. 2k).

The 5S rDNA cistrons were located at a terminal position on the long arms of a subtelocentric chromosomal pair (7^{th}) in both sexes (Fig. 2h, l). Male specimens were further characterized by an additional 5S rDNA signal on short arms of subtelocentric chromosomes from pair 15 (Fig. 2h). However, it is not possible to state if these additional sequences are male-specific because of the reduced sampling (four specimens) in FISH experiments.

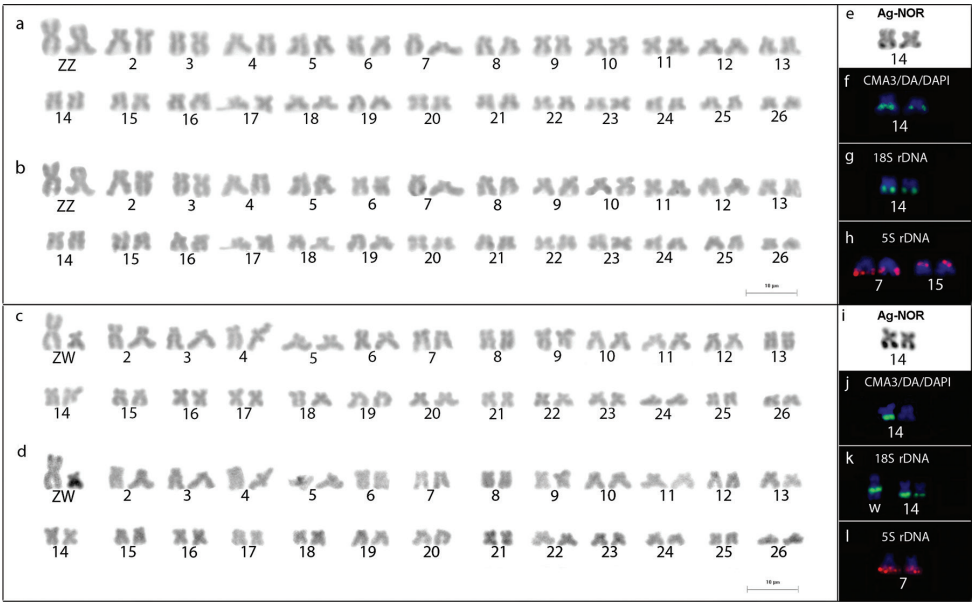

Figure 2. Karyotypes of male (**a, b**) and female (**c, d**) *Lignobrycon myersi* after Giemsa staining (**a, c**) and C-banding (**b, d**), bearing ZZ (1st pair) and ZW sex chromosomes, respectively. On the right, the Ag-NOR bearing chromosomes (**e, i**), GC-rich region (CMA_3^+/$DAPI^-$) (**f, j**), 18S rDNA (**g, k**) and 5S rDNA (**h, l**) in males (**e–h**) and females (**i–l**).

Discussion

In spite of advances in cytogenetic studies of tropical ichthyofauna over the last decades, chromosomal reports about native fish populations from hydrographic basins in northeastern South America are recent and scarce (Bitencourt et al. 2012, Almeida et al. 2013, Nascimento et al. 2014, Medrado et al. 2015).

The karyotypic macrostructure of *L. myersi* is similar to that reported in *Triportheus* in as much as both genera share 2n = 52 biarmed chromosomes and a differentiated ZZ/ZW sex chromosome system (Table 1). Even though ZZ/ZW sex chromosomes are relatively frequent in neotropical fishes, they have evolved independently in most lineages (Cioffi et al. 2012). *Triportheus* was regarded as the only exception in which the presence of heteromorphic sex chromosomes could be considered an apomorphic trait based on some peculiar features (Artoni et al. 2001, Artoni and Bertollo 2002, Diniz et al. 2009 among others), which are now also identified in *L. myersi* for the first time.

Namely, the Z chromosome of *L. myersi* corresponds to the largest metacentric chromosome of the karyotype, a feature also observed in *Triportheus* (Artoni et al. 2001, Artoni and Bertollo 2002, Nirchio et al. 2007, Diniz et al. 2008a). Even though the W chromosome in *Triportheus* species is invariably smaller than the Z chromosome, a comparative analysis of the relative length of the W chromosome in relation to the Z chromosome (WRL) revealed three trends in this genus, as follows: (1) species

with WRL higher than 60%, (2) species with WRL ranging from 40 to 60%, and (3) species with WRL below 40% (Diniz et al. 2008a). Using the same parameters, the W chromosome of *L. myersi* is about 50% smaller than Z, being classified as a medium-sized W chromosome as reported in *T. nematurus* Kner, 1858, *T.* prope. *signatus*, and *T. guentheri* Garman, 1890 (Falcão 1988, Sánchez and Jorge 1999, Artoni et al. 2001, Diniz et al. 2008a).

It should be pointed out that *T. guentheri* occurs in the São Francisco river basin (Reis et al. 2003). This basin shares a common evolutionary history with coastal rivers in Bahia, being isolated from each other by Espinhaço Range (Chaves et al. 2015). Therefore, the presence of a medium-sized W chromosome (see Diniz et al. 2008a, 2008b) might be a basal feature in Triportheidae. The similarity in sex chromosome structure and adjacent geographic range suggest a close phylogenetic relationship between *L. myersi* and *T. guentheri*, which remains to be investigated.

Another trait that reinforces the conserved structure of sex chromosomes in *Triportheus* is the presence of 18S rDNA on the W chromosomes of all species (Artoni and Bertollo 2002) but *T. venezuelensis* (Table 1). Moreover, the 18S rDNA on the Z chromosome of *T. venezuelensis* was not stained by silver nitrate suggesting that it is an inactive rDNA cistron (Nirchio et al. 2007).

In turn, *L. myersi* was characterized by a single pair of Ag-NORs located at terminal regions of pair 14. Single NORs are widespread in several fish taxa (Gornung et al. 2013), but rarely found in Characidae (e.g. Medrado et al. 2015), thereby providing additional support to the removal of *L. myersi* and *Triportheus* from this family (Oliveira et al. 2011). The location of NORs in autosomes allowed differentiating *L. myersi* and *Triportheus* species, since they are differentially located on long and short arms, respectively. However, this distinctive position of 18S rDNA cistrons might either be a result of actual chromosomal rearrangements (transpositions or inversions) or a technical artifact related to differences in condensation of chromosomes or biased measurements by each author.

On the other hand, the FISH with 18S rDNA probes showed that, similarly to other *Triportheus* species, *L. myersi* also bears NORs on the W chromosome, even though they were inactive in studied samples (i.e. undetected by silver nitrate staining) (Fig. 2k). This result strengthens that the origin of differentiated sex chromosomes has taken place before the diversification in Triportheidae, instead of being restricted to the origin of *Triportheus* (Diniz et al. 2009). Putatively, during the evolutionary history of *L. myersi*, the 18S rDNA sequences may have partially degenerated and thus inactivated (see Wilson and Makova 2009) while remaining functional in *Triportheus*, thus detectable by silver nitrate staining. To confirm this suggestion, a larger number of individuals should be cytogenetically analyzed for Ag-NORs at different periods, since this apparent inactivation can either be a transitory cell state or a polymorphic condition.

Large amounts of heterochromatin are a common feature of W and Y chromosomes in animals (Wilson and Makova 2009, Livernois et al. 2012), being clearly observed in *L. myersi* and several species of *Triportheus* (e.g., Artoni and Bertollo 2002, Diniz et al. 2008a, 2008b, Cioffi et al. 2012). Thus, the heterochromatinization of

W chromosomes seems to be associated with degeneration followed by chromosomal reduction during evolution of sex chromosomes (Bertollo and Cavallaro 1992, Diniz et al. 2008b). Indeed, Z and W chromosomes of species in early stages of sex chromosome differentiation, such as ratite birds (ostrich, emu and allies), are similar in both size and content of heterochromatin/euchromatin (Livernois et al. 2012) even though the relationship between age and sex chromosome degeneration is currently under debate (Bachtrog et al. 2014).

In spite of sharing a similar C-banding pattern, the base composition of repetitive DNA within heterochromatin segments of W chromosomes in *Triportheus* and *L. myersi* seems more variable. While the GC-rich heterochromatic regions (CMA_3^+) in *L. myersi* were interspersed to Ag-NORs only, as reported in some species of *Triportheus* (Artoni & Bertollo, 2002), conspicuous CMA_3^+ signals were reported in both autosomal NORs and W chromosomes of other species like *T. nematurus* (Diniz et al. 2008a). In fact, the GC-rich blocks in *L. myersi* were so reduced that no fluorescent signal was detected in homologues of some metaphase spreads (Fig. 2j).

The most divergent chromosomal trait observed in *L. myersi* and other triportheids refers to the distribution of 5S rDNA sites, thereby demonstrating the evolutionary dynamics of this class of ribosomal genes and their potential to cytotaxonomy (Affonso and Galetti 2005, Molina et al. 2012). Most *Triportheus* species analyzed so far share syntenic 18S and 5S rDNA cistrons (Table 1), regarded as an ancestral trait for this genus (Diniz et al. 2008a; Marquioni et al. 2013). The non-synteny of both rDNA classes in *L. myersi* (Fig. 2g-h, k-l) supports this inference, suggesting that transposition of 18S rDNA cistrons to adjacent position of 5S rDNA cistrons or vice-versa has taken place after the differentiation of *Lignobrycon* and *Triportheus*. Moreover, *Triportheus* species usually present 5S rDNA on short arms of a single sm pair (Marquioni et al. 2013), while *L. myersi* was characterized by conspicuous signals on long arms of pair 7 in both sexes and on short arms of a second pair in male samples.

Therefore, the location of 5S rDNA sites in *L. myersi* should represent an autopomorphic trait, even though the numerical polymorphism in 5S rDNA signals should be further investigated. On the other hand, the lack of synteny between 18S and 5S rRNA genes has been also reported in *T. guentheri* from São Francisco river basin, reinforcing the putative evolutionary relationship between this species and *L. myersi*, as abovementioned.

In conclusion, the cytogenetic results agree with morphological (Malabarba 1998) and molecular evidence (Oliveira et al. 2011) by revealing a series of synapomorphies between *Lignobrycon myersi* and *Triportheus* that reinforce their close evolutionary relationship. Moreover, present results suggest that ZZ/ZW sex chromosomes have evolved in the basal Triportheidae lineage, including other taxa than *Triportheus*. In this sense, further cytogenetic studies in other genera allocated in Triportheidae (*Agoniates*, *Clupeacharax* and *Engraulisoma*) by Oliveira et al. (2011) are strongly encouraged. Similarly, chromosomal analyses in other populations of *L. myersi* (e.g. Contas River) can be useful to evaluate interpopulation differences or the existence of cryptic forms that should be prioritized for conservation.

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RESEARCH ARTICLE

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Meiotic behavior and H3K4m distribution in B chromosomes of *Characidium gomesi* (Characiformes, Crenuchidae)

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Abstract

Characidium gomesi Travasso, 1956 specimens from the Pardo River have up to four heterochromatic supernumerary chromosomes, derived from the sex chromosomes. To access the meiotic behavior and distribution of an active chromatin marker, males and females of *C. gomesi* with two or three B chromosomes were analyzed. Mitotic chromosomes were characterized using C-banding and FISH with B chromosome probes. Meiocytes were subjected to immunofluorescence-FISH assay using anti-SYCP3, anti-H3K4m, and B chromosomes probes. Molecular homology of supernumeraries was confirmed by FISH and by its bivalent conformation in individuals with two of these chromosomes. In individuals with three Bs, these elements formed a bivalent and a univalent. Supernumerary and sex chromosomes exhibited H3K4m signals during pachytene contrasting with their heterochromatic and asynaptic nature, which suggest a more structural role than functional of this histone modification. The implications of this result are discussed in light of the homology, meiotic nuclear organization, and meiotic silencing of unsynapsed chomatin.

Keywords

Immunodetection, Chromosome painting, SYCP3, synaptonemal complex

Introduction

B chromosomes are genomic structures that are extra to the standard genome set, apparently inert and found throughout animals, plants and fungi (Houben et al. 2014). Although aspects relating to the origin, evolution and function of these chromosomes remain unknown, in some cases they may be derived from autosomes or sex chromosomes (Teruel et al. 2010, Bueno et al. 2013, Pansonato-Alves et al. 2014) or from interspecific crosses (Schartl et al. 1995, McAllister and Werren 1997, Perfectti and Werren 2001). B chromosomes are usually composed of repetitive sequences of DNA, not carrying essential genes (Camacho. 2005, Houben et al. 2014). However, essential gene sequences have been found in some B chromosomes (Banaei-Moghaddam et al. 2013, Ruiz-Estevez et al. 2013, Trifonov et al. 2013), and their presence can generates some phenotypic effects (Houben et al. 2014). During meiosis, B chromosomes show no recombination with A chromosomes, implying a very different evolutionary path (Houben et al. 2014). In addition, B chromosomes are not always found in pairs and do not segregate in a predictable manner during meiosis, which leads to a non-Mendelian segregation in some cases. Even when found in pairs, the transmission rates of B chromosomes are often lower than 0.5 because they are unstable during mitosis and/or meiosis. However, some B chromosomes may show transmission rates higher than 0.5, ensuring their accumulation, an important feature of parasites B chromosomes (Camacho 2000).

The analysis of chromosome pairing during meiosis is a branch of cytogenetic in plants and animals, showing interesting aspects about the chromosome synapsis in the early stages of meiosis (Wallace and Wallace 2003, Basheva et al. 2014, Sanchez-Moran and Armstrong 2014). In particular, the meiotic behavior of B chromosomes has been investigated across different organisms to evaluate aspects of homology, segregation, importance in gamete viability, and transmission to offspring (Fontana and Vickery 1973, Camacho et al. 1980, Jones 1991, Aquino et al. 2013). In *Mazama americana* (Erxleben, 1777), B chromosomes behave as homologous bivalent forms even though they may also occur as univalents, showing erratic behavior responsible for non-Mendelian segregation patterns (Aquino et al. 2013). In fishes bearing four B chromosomes, both the tetravalent conformation and the presence of two bivalents has been witnessed also suggesting homology between these chromosomes. (Pauls and Bertollo 1983, Dias et al. 1998). Meiotic chromosome behavior involves a complex dynamics of chromatin modification playing an essential role in chromosome function and gene regulation (Manterola et al. 2009, Vaskova et al. 2010). In fact, methylation, acetylation, and phosphorylation of the histone nucleosomal core are involved in chromatin assembly, and linked with active and silent transcriptional states during meiosis. For example, the histone H3 methylated at serine 4 (H3K4m) is a epigenetic modification of chomatin that has been linked to gene activation in model organisms, such as mammals (Santos-Rosa et al. 2002, Bernstein et al. 2002, Pokholok et al. 2005, Koina et al. 2009). This modification has been associated to genetic families transcription of

barley B chromosomes (Carchilan et al. 2007) and also with X active chromosome in the X-chromosome inactivation process of mammals (Koina et al. 2009). Thus, this histone modification is a good active chromatin marker to analyze B chromosome behavior during meiosis.

The genus *Characidium* Reinhardt, 1867 comprises a group of interesting fish for cytogenetic and molecular studies because of the ZZ/ZW sex determination system (Maistro et al. 1998, Centofante et al. 2001, 2003, Maistro et al. 2004, Noleto et al. 2009), and the occurrence of supernumerary chromosomes (Maistro et al. 1998, Pansonato-Alves et al. 2010, 2011a, 2011b). B chromosomes have been found in *Characidium* cf. *zebra* Eigenmann, 1909 (Miyazawa and Galetti Jr 1994, Venere et al. 1999), *Characidium oiticicai* Travassos, 1967, *Characidium pterostictum* Gomes, 1947 (Pansonato-Alves et al. 2010) and *C. gomesi* (Pansonato-Alves et al. 2011a), in different levels of heterochromatinization (Pansonato-Alves et al. 2010, 2011b). Individuals of *C. gomesi* from the Pardo River posses up to four clearly acrocentric B chromosomes of large size, entirely heterochromatic and originating from the sex chromosomes (Pansonato-Alves et al. 2014). Therefore, supernumerary chromosomes of *C. gomesi* represent an interesting model for the study of origin and evolution of these genomic elements in fishes, including chromosome behavior during meiosis, a process less explored in this group of vertebrates.

In this study, we addressed the meiotic behaviour of B chromosomes in specimens of *C. gomesi* by means of molecular cytogenetics and immunodetection techniques. B chromosome paint probes and antibodies against synaptonemal complex protein 3 (SYCP3) (Lammers et al. 1994) and against methylated histone H3 at lysine 4 (H3K4) (Pokholok et al. 2005, Godmann et al. 2007, van der Heijden et al. 2007, Kouzarides 2007) were used.

Materials and methods

Chromosome preparation

Nine males individuals and seven females of *C. gomesi* collected in the Rio Pardo basin of the Rio Paranapanema, Botucatu, São Paulo, Brazil (22°59'25"S and 48°25'40"W) were analyzed (Table 1). The animals were collected in accordance with Brazilian environmental laws of permission to collect issued by MMA / IBAMA / SISBIO, number 3245. The collection procedures, maintenance and analysis of the animals were performed in accordance with international regulations of animal experiments, followed by the Universidade Estadual Paulista (CEEAA / IBB / UNESP protocol number 304). The animals were anesthetized, dissected and mitotic chromosome preparations were obtained following the protocol described by Foresti et al. (1981). The C-banding was performed in mitotic cells according to Sumner (1972). Gonads were removed and processed for SC visualization according to the technique described by Araya-Jaime et al. (2015).

Table 1. Individuals of *C. gomesi* analysed F: female, M: male

Ind. nº	Sex	Nº of cells with 0-3 Bs			
		0	1	2	3
75231	M	4	15	3	
75232	M	4	15	5	
75233	F	25			
75234	M	23			
75236	F	7	8	4	
75237	M	8	11	8	
75238	M	3	8	15	
75239	M	8	2	9	
75240	M	1	6	10	
75241	F	2	4	11	12
75242	M	20			
75250	F	6	2	6	17
75251	F	15			
75252	F	5	6	7	
75253	M	17			
75254	F	3	11	17	

B chromosome painting in mitotic cells

The B chromosome probe was produced as described by Pansonato-Alves et al. (2014) and hybridized in mitotic cells following the procedure described by Pinkel et al. (1986), under high stringency conditions (200 ng probe, 50% formamide, 10% dextran sulfate, 2xSSC at 37 °C overnight).

Immunodetection and FISH in meiotic cells

Meiotic preparations were immersed for 20 min in 0.01 M citrate buffer preheated to 90 °C. Then, they were incubated in a humidified chamber at 37 °C for 1 h as described by Araya-Jaime et al. (2015). Incubation solutions consisted of rabbit antibodies against SYCP3 of medaka fish (Iwai et al. 2006) diluted 1: 100 in PBS, and mouse antibody against the H3K4m histone of rabbit (Abcam, ab8895) diluted 1: 100 in PBS. After washing in PBS, the slides were subjected to a second incubation in a humid chamber with anti-rabbit donkey IgG conjugated with isothiocyanate fluorescein (FITC; Jackson Immuno Research Laboratories) for detecting SYCP3 and anti-mouse goat IgG conjugated with Texas Red (Invitrogen, Cat No. a-31553) for detecting H3K4m, both diluted 1: 100 in PBS for 1 h at room temperature.

Meiotic preparations were hybridized with the B chromosome probe in accordance with the protocol from Pinkel et al. (1986), adapted by Araya-Jaime et al. (2015) after immunodetection to not affect SYCP3 and H3K4m signals. Because FISH can

darken the immunodetection signal, SYCP3 and H3K4m images were captured before the hybridization procedure. Furthermore, the slides were exposed to light before the hybridization to lose the H3K4m signal so that it is not confused with the signals from the B chromosome probe, as this probe was detected with anti-digoxigenin-rhodamine.

Slides were stained with DAPI and mounted with an antifade solution (Vetashield). The images were digitally captured using Image Pro Plus 6.0 software (Media Cybernetics) with appropriate filters of the epifluorescence microscope (Olympus BX61) equipped with an Olympus DP70 camera. Final composition of the images was performed with the application of Adobe Photoshop CS6 image editor software, using image and uniform size scales.

Results

The *C. gomesi* specimens showed 2n=50 chromosomes with ZZ/ZW sex chromosomes. In addition, we found individuals with 2–3 acrocentric B chromosomes, with intraindividual number variation (Fig. 1a, Table 1).

All chromosomes of standard complement showed positive C-bands in the pericentromeric region. The W and B chromosomes showed completely dark C-banding, while the Z chromosome showed a large pericentromeric C-band (Figure 1a). Individuals with three supernumerary chromosomes evidenced variation in the amount of heterochromatin between these elements was observed. Chromosome painting with the B chromosome probe demonstrated homology between the B and W chromosomes, as well as with the pericentromeric region of the Z chromosome (Figure 1b).

Twenty-five bivalents corresponding to the A chromosomes were identified by immunodetection with SYCP3 on cells in the pachytene stage of all individuals. Males and females bearing two B chromosomes exhibited cells with 25 and 26 bivalents (Figure 2a). Additionally, two females with three B chromosomes showed 25 and 26 bivalents, plus an univalent in 50% of the cells (Figure 2b, easily recognized by the bright green signal of SYCP3 and positive hybridization with the B chromosome probe), and in some cells, the subterminal regions of the heteromorphic sex chromosomes showed no synapse during the pachytene. H3K4m immunodetection in pachytene presented a dot-like labeling pattern on autosomes, sex and B chromosomes (Figure 3a). Chromo-

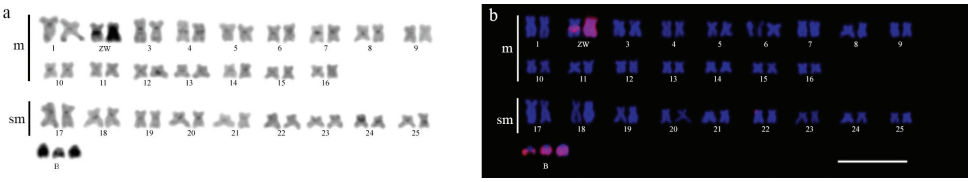

Figure 1. Mitotic karyotype of female *C. gomesi* with three B chromosomes. **a** C- banding. Note heterochromatin pattern on B and sex chromosomes **b** Chromosome painting using a B chromosome probe and contrasted with DAPI. Notice probe hybridization on B and sex chromosomes. Bar = 10 μm.

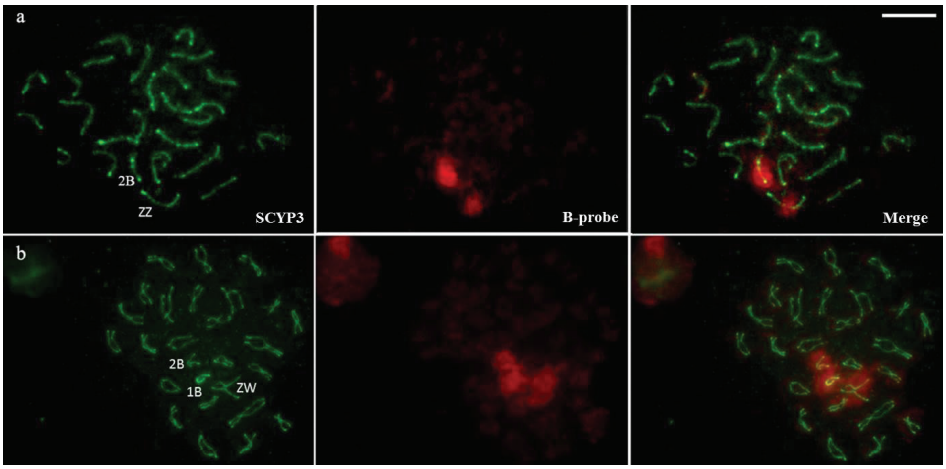

Figure 2. Synaptonemal complex of *C. gomesi* after immunodetection with anti-SCYP3 (green) and chromosome painting with B chromosome probe (red). **a** Male pachytene bearing two B chromosomes. Note the positive hybridization of the B paint probe on the bivalents of the B and Z chromosomes **b** Female diplotene bearing three B chromosomes. Note the presence of a B univalent and the ZW bivalent corresponding to the Z and W chromosomes, all positive for the B probe (right). Bar = 10 μm.

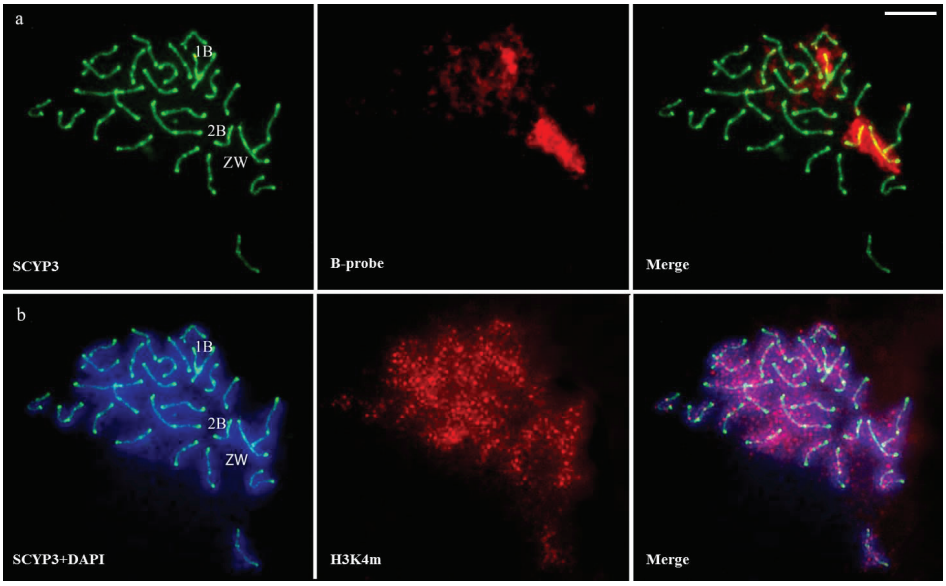

Figure 3. Meiotic cell of *C. gomesi* in pachytene stage with three supernumerary chromosomes. **a** Synaptonemal complex protein identified anti- SYCP3 (green) and B chromosome probe (red). Note the positive hybridization of B paint probe on two bivalents and one univalent (ZW, 2B and 1B, respectively) **b** Synaptonemal complex protein identified with anti-SYCP3 counterstained with DAPI (blue), evidencing ZW bivalent and the meiotic formations of B chromosomes; chromatin regions associated with transcriptional activities identified with anti-H3K4m (red) and DAPI consterstaining (blue) evidencing the ZW bivalent and the meiotic formations of B chromosomes. Bar = 10 μm.

some painting with the B chromosome probe showed hybridization signals similar to that of mitotic cells (Figure 1b), which corroborates the identification of sex chromosomes (Figures 3b, ZW) and of B chromosomes (Figures 3b, 2B, 1B).

Discussion

The species of the *Characidium* genus have intriguing cytogenetic features because both sex and supernumerary chromosomes can be found in the same individual (Miyazawa and Galetti Jr. 1994, Vicari et al. 2008, Pansonato-Alves et al. 2010, 2011a). Moreover, in some species, such as *C. gomesi*, individuals carry totally heterochromatic B chromosomes (Figure 1), with molecular homology to the sex chromosomes (Pansonato-Alves et al. 2014 and this work). Results of meiotic analysis also demonstrated a perfectly paired bivalent between the two B chromosomes while meiocytes with three supernumerary chromosomes resulted in one bivalent and one univalent of B chromosomes (Figure 2).

Despite sequence homology between B and sex chromosomes in *C. gomesi* (Figures 1b, 2, and 3a; see also Pansonato-Alves et al. 2014), the meiotic figures analyzed revealed that the supernumerary chromosomes do not form a multivalent chromosome (Figures 2b and 2a). This recombination restriction with potential donor chromosomes was observed in other organisms and can be considered a starting point in the process of independent evolution of the extra chromosomes (Houben et al. 2014). However, the bivalent conformation of the B chromosomes (Figures 2 and 3) and the results of chromosome painting in mitotic cells (Figure 1b) reinforce the idea of similarity in the supernumerary elements of *C. gomesi*. The homology between B chromosomes has been observed in meiotic cells of other fish species, such as *Prochilodus lineatus* (Valenciennes, 1837) (Pauls and Bertollo 1983, Dias et al. 1998, Portela-Castro et al. 2001) and in mammals, such as the American red fox *Vulpes fulvus* (Desmarest, 1820) (Switonski et al. 1987) and the Korean field mouse *Apodemus peninsulae* (Thomas, 1907) (Kolomiets et al. 1988). The formation of a multivalent involving B chromosomes was initially suggested by Pauls and Bertollo (1983) in *P. lineatus* and later confirmed by Dias et al. (1998). They noted that some meiotic cells of individuals carrying four B chromosomes would either reveal a tetravalent or two bivalents involving the B chromosomes suggesting homology between these chromosomes.

The chromosome pairing seen in meiosis has been interpreted as homology (Ramsey and Schemske 2002) and observed as occurring also between B chromosomes of *C. gomesi*. However, such a pairing may occur in a variety of contexts (McKee 2004). In *Drosophila melanogaster* Meigen, 1830 (McKim and Hayashi-Hagihara 1998) and the roundworm *Caenorhabditis elegans* (Maupas, 1900) (Dernburg et al. 1998), these synapses can occur even in the absence of homologous recognition. Conversely, in the F1 of allotetraploid plants, for example, pairing between homologous chromosomes as well as between homeologous chromosomes occur, which are described as partially homologous chromosomes (reviewed in Ramsey and Schemske 2002). As previously

described, the chromosome pairing seems to not always indicate complete homology between chromosomes. Nevertheless, the synapse between two B chromosomes of *C. gomesi* is considered a crucial condition for chiasmus formation. This formation is mainly composed of physical connections between homologous chromosomes that ensure correct segregation during the first meiotic division (Tsubouchi and Roeder 2003). Furthermore, meiotic recombination caused by the occurrence of crossing-over (Zhang et al. 2014) could be generating a homogenization of the sequences present on both chromosomes; however, a confirmation of late recombination nodules in the B bivalent is required to indicate exchange of genetic material and support this phenomenon.

B chromosomes were detected by hybridization with a probe made from a single supernumerary chromosome (Figures 1b, 2, and 3). However, despite chromosome painting suggested that all supernumerary were composed of similar types of repetitive DNA, one of them did not synapse with the other during pachytene (Figure 2). Even without conclusive evidence about the actual identity of the unsynapsed B chromosomes, the observation of less heterochromatin in one of the supernumerary chromosomes (Figure 1a) could be indicative of the interference of differential heterochromatin composition on the pairing process.

The existence of mechanisms limiting the multivalent pairing between homologous chromosomes was proposed to occur in autopolyploid plants, among which the presence of genetic factors can have a significant effect (see reviewed in Ramsey and Schenske 2002). Therefore, when the B chromosomes carry specific genetic elements, such as the Ph1 gene for example, the pairing of homologous chromosomes may be prevented (Dover and Riley 1972, Kousaka and Endo 2012). When meiosis begins, chromosomes must meet and pair with their counterpart. In this process, the chromosomes are aligned and brought closer, followed by homology recognition and synapsis (McKee 2004, Scherthan et al. 1996, Scherthan 2001). However, this process of meeting between the counterparts during the first meiotic prophase remains largely unknown (Pawlowski and Cande 2005, Da Ines et al. 2014). Thus, the presence of B chromosomes in bivalent and univalent conformation in meiocytes of *C. gomesi* appears to depend not only on the similarity of their sequences but also on their meeting and alignment in the meiotic nucleus.

The lack of pairing of one of the B chromosomes in *C. gomesi* (Figure 2b and 3) was previously observed in other eukaryotic organisms and also linked to the silencing of genes present in these chromosomal regions. In the fungus *Neurospora crassa* Shear & B.O. Dodge, 1927 (Shiu and Metzenberg 2002) and mice (Turner et al. 2005), chromosomal regions or entire chromosomes not pairing with their counterpart suffer inactivation and induce silencing during meiotic prophase. This process is called meiotic silencing by unpaired DNA (MSUC) and is believed to protect the genome against the invasion of transposable elements (Shiu and Metzenberg 2002). Such silencing was also observed in small unsynapsed regions of trivalents, resulting from multiple Robertsonian translocations (Manterola et al. 2009) and of sex chromosomes, a process known as meiotic sex chromosome inactivation (MSCI). According

to these observations, it is expected that the univalent B chromosome of *C. gomesi* as well as the unsynapsed region of the sex chromosomes may become inactivated during meiosis since this is a general process observed in other organisms. Studies focusing on transcriptional repression of the B univalent are interesting because they try to confirm this hypothesis.

In mammals, the levels of histone H3 mono-, di- and tri-methylation at lysine 4 is highly dynamic during the development of germ cells. In addition to the possible role of H3K4 methylation in chromatin reorganization, a connection to gene activation has been suggested (Bernstein et al. 2002, Santos-Rosa et al. 2002, Pokholok et al. 2005, Godmann et al. 2007, van der Heijden et al. 2007, Koina et al. 2009). In this study, the presence of histone H3K4m in B chromosomes of *C. gomesi* (Figure 3b) disagrees with the heterochromatic nature of these genomic elements (Figure 1b) since this epigenetic marker shows preferential association with euchromatic regions (Godmann et al. 2007, Koina et al. 2009). This incongruence has been noted before in the B chromosomes of barley which simultaneously present subterminal heterochromatic regions enriched with tri-methylated histone H3K4 and repetitive gene families (D1100 and E3900) that are transcriptionally active (Carchilan et al. 2007).

On the other hand, because the lack of synapsis is strongly related to the meiotic silencing by the unpaired DNA process (MSUC) in several organisms (Turner et al. 2005, Burgoyne et al. 2009, Manterola et al. 2009), the presence of H3K4m on the B univalent of *C. gomesi* may not necessarily be related to transcription. It is noteworthy that the silencing of telomeres (Nislow et al. 1997, Singer et al. 1998) and of rDNA (Briggs et al. 2001, Bryk et al. 2002) is also related to processes involving histone H3 methylation at lysine 4. Therefore, it can be assumed that the presence of this epigenetic marker in the chromosomes of pachytene stage cells of *C. gomesi* may be involved in some of the above processes, in addition to its possible role in the reorganization of chromatin during meiosis.

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Chromosome comparison of 17 species / sub-species of African Goliathini (Coleoptera, Scarabaeidae, Cetoniinae)

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Abstract

The mitotic karyotypes of 17 species of African Goliathini (Cetoniinae) are described using various chromosome banding techniques. All but one are composed of 20 chromosomes, mostly metacentric, forming a karyotype assumed to be close to that of the Polyphaga ancestor. The most derived karyotypes are those of *Goliathus goliatus* Drury, 1770, with eight pairs of acrocentrics and *Chlorocana africana* Drury, 1773, with only14 chromosomes. In species of the genera *Cyprolais* Burmeister, 1842*, Megalorhina* Westwood, 1847*, Stephanocrate*s Kolbe, 1894 and *Stephanorrhina* Burmeister, 1842, large additions of variable heterochromatin are observed on both some particular autosomes and the X chromosome. Species of the genera *Eudicella* White, 1839 and *Dicronorrhina* Burmeister, 1842 share the same sub-metacentric X. Although each species possesses its own karyotype, it remains impossible to propose robust phylogenetic relationships on the basis of chromosome data only.

Keywords

Cetoniinae, Goliathini, Coleoptera, chromosome banding, comparison

Introduction

Cetoniinae, a large sub-family of Scarabaeidae (Coleoptera), is composed of about 3200 species grouped into ten tribes. Goliathini is one of the large tribes of this sub-family, with about 410 identified species, almost exclusively distributed in Asia and Africa. Data on their chromosome constitution are very scarce, with only three Asian species studied, *Rhomborrhina unicolor* Motschulsky, 1861 and *R. polita* Waterhouse, 1873 (Yadav et al. 1979), and *Jumnos ruckeri* Saunders, 1839 (Macaisne et al. 2006). Chromosome data are not much richer for the whole Cetoniinae sub-family, with only 28 species studied (Smith and Virkki 1978, Yadav et al. 1979, Macaisne et al. 2006, Dutrillaux et al. 2008). We also reported the chromosome formulae and NOR (Nucleolus Organizer Region) location of 14 additional species of Cetoniinae and described the karyotype of *Goliathus goliatus* Drury, 1770 (Dutrillaux and Dutrillaux 2012). All but four species had a 20,XY mitotic karyotype formula, and a Xyp meiotic sex chromosome formula in the males. In the literature, when chromosome morphology is provided, it appears that almost all autosomes are meta- or sub-metacentric, the X chromosome is generally acrocentric, with variable amounts of heterochromatin on its short arm and the Y is punctiform. These characteristics are shared with most Scarabaeoidea species studied (Yadav et al. 1979, Wilson and Angus 2004, 2005, Dutrillaux et al. 2011), which suggests that their karyotypes have not undergone drastic changes during evolution.

At first glance, this apparent karyotype homogeneity might indicate that chromosome rearrangements rarely occurred during the multiple speciation events having originated more than 30,000 Scarabaeoidea and 3200 Cetoniinae species. However, cautious comparisons, after chromosome banding and NOR localisation, revealed small differences in morphology, indicating that rearrangements, principally intra-chromosomal changes, have occurred (Wilson and Angus 2004, 2005, Dutrillaux et al. 2007, 2008). They are just difficult to detect because chromosome sizes are generally gradually decreasing and morphologies not so different, except when acrocentrics are formed. In addition, heterochromatin amounts may vary, independently of euchromatin rearrangements, which may cause variations of chromosome size and morphology not related to structural rearrangements *sensu stricto* and lead to misinterpretations.

Here, we report mitotic and meiotic chromosome data of 17 species of African species belonging to Goliathini. Their chromosomes are compared with the use of various staining techniques. Each species possesses its own karyotype. Most inter-specific differences seem to be the consequence of inversions and heterochromatin variations. With the exception of two species, *G. goliatus* and *C. africana* Drury, 1773, all species conserved a karyotype composed of 20 chromosomes, principally sub-metacentric, thus not deeply different from that of many other Scarabaeidae.

Material and methods

All but one species studied here were obtained by breeding developed by amateur entomologists from whom we obtained larvae. We pursued the breeding until imagine stage using oak leaf-mould. Diagnoses were performed according to Sagai and Nakai (1998). Young male imagines were anaesthetized with ethyl acetate and dissected for extracting their gonads. Testes were dropped and dilacerated in O.88 M KCl where they remained for 15 min (metaphase stages studies) or 7h (pachytene stage analysis). The cell in suspension were transferred into either O.55 M KCl or diluted fetal calf serum (1/3 serum: 2/3 distilled water) for 15 min., fixed and spread as described by Dutrillaux et al. (2010). Giemsa staining and C-banding were successfully applied in all species and silver (NOR) staining in meiotic cells of all but two species. For some species, we used light Giemsa (LG = low Giemsa stain concentration and short staining time) to improve chromatid differentiation and obtain a kind of G-banding on pro-metaphases from spermatogonia or gonocytes. We also occasionally used cells from the mid gut, according to Angus (1982). Only the karyotypes of *Dicronorrhina derbyana oberthuri* Deyrolle, 1876 and Goliathus goliatus have been described before, but we reported formulae and NOR localization for all these species (Dutrillaux and Dutrillaux 2012). We classified their chromosomes by decreasing size, as usual, but making abstraction of heterochromatin, present in large and variable amounts in some species, and followed ISCN (1985) chromosome nomenclature.

Results and discussion

Brief description of the male karyotypes

Amaurodes passerini Westwood, 1844 (Fig. 1): mitotic formula: 20,XY; meioformula: 9+Xyp. - Chromosome morphology: pair no 1 metacentric; pairs no 2–7 sub-metacentric; pairs no 8 and 9 and X acrocentric; Y punctiform.

- C-banding: fairly large juxta-centromeric C-band on pairs 1-8, smaller on pair 9 and X, absent on the Y. Presence of a faint C-band at the telomeric region of the chromosome 4p arm (4pter).
- After LG staining, a banding differentiates all chromosome pairs (Fig. 18).
- Silver staining: at pachynema of meiotic prophase, nucleoli are always next to the sex bivalent; at metaphase I, strong staining of the space between the X and Y. NOR location: Xp (p=short arm, according to ISCN, 1985)).

Chlorocala africana Drury, 1773 (Fig. 2): mitotic formula: 14,XY; meioformula: 6+Xyp.

- Chromosome morphology: pairs N°1-4 metacentric; pairs N° 5 and 6 acrocentric; X acrocentric with two frequent gaps and Y punctiform.
- C-banding: quite discrete at all centromeric regions, and also at intercalary regions of chromosomes 1–4.

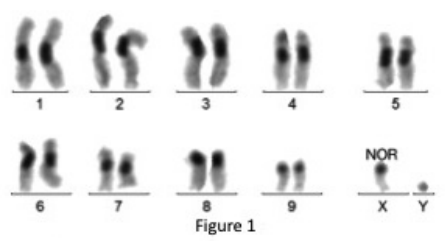


Figure 1

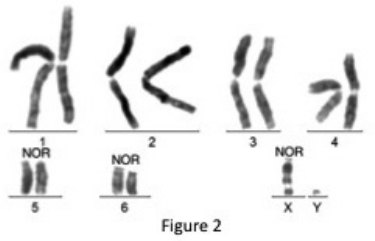


Figure 2

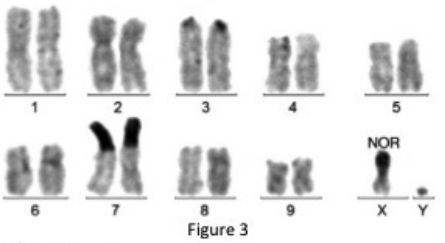


Figure 3

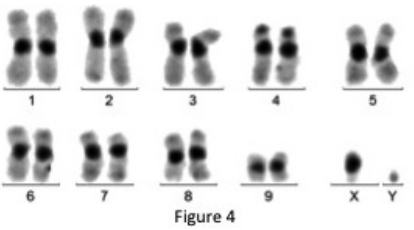


Figure 4

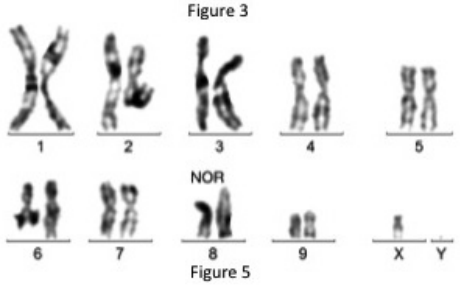


Figure 5

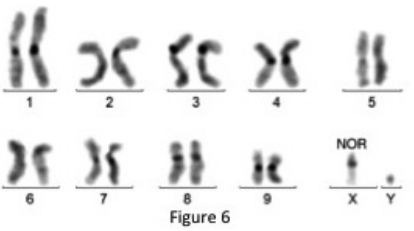


Figure 6

Figures 1–6. **1** C-banded karyotype of *Amaurodes passerini* **2** Giemsa stained karyotype of *Chlorocana africana* **3** C-banded karyotype of *Cyprolais hornimani*, with large heterochromatic fragments on chromosomes 7 and X **4** C-banded karyotype of *Dicronorrhina derbyana derbyana* **5** Giemsa stained karyotype of *Dicronorrhina micans* **6** C-banded karyotype of *Eudicella aethiopica*.

- Silver staining: present at mitotic metaphase on short arms of acrocentrics (N° 5, 6 and X). At pachynema, the sex bivalent is intensely stained, as well as the centromeric regions of bivalents 5 and 6. Nucleoli are associated with the sex bivalent and bivalent 6 short arm. At metaphase I, there is an intense staining of the space between the X and Y. NOR location: Xp, 5p, 6p.

Cyprolais hornimani Bates, 1877 (Fig. 3): mitotic formula: 20,XY; meioformula: 9+Xyp.

- Chromosome morphology: all the autosomes but N° 3 appear to be meta- or submetacentric after Giemsa staining. The X is sub-metacentric and the Y punctiform.
- C-banding: very faint or absent on most autosomes. Only the acrocentric N° 3 is clearly C-banded at centromeric region. Large additional heterochromatic segments are present at 7p terminal region and on Xp.
- Silver staining: intense on the Xp arm and on the Y at mitotic metaphase. At pachynema, nucleoli are alongside the sex bivalent. The heterochromatic region of bivalent 7 is frequently close or at contact with the sex bivalent. At metaphase I, intense staining of the space between the X and Y. NOR location: Xp, Y?

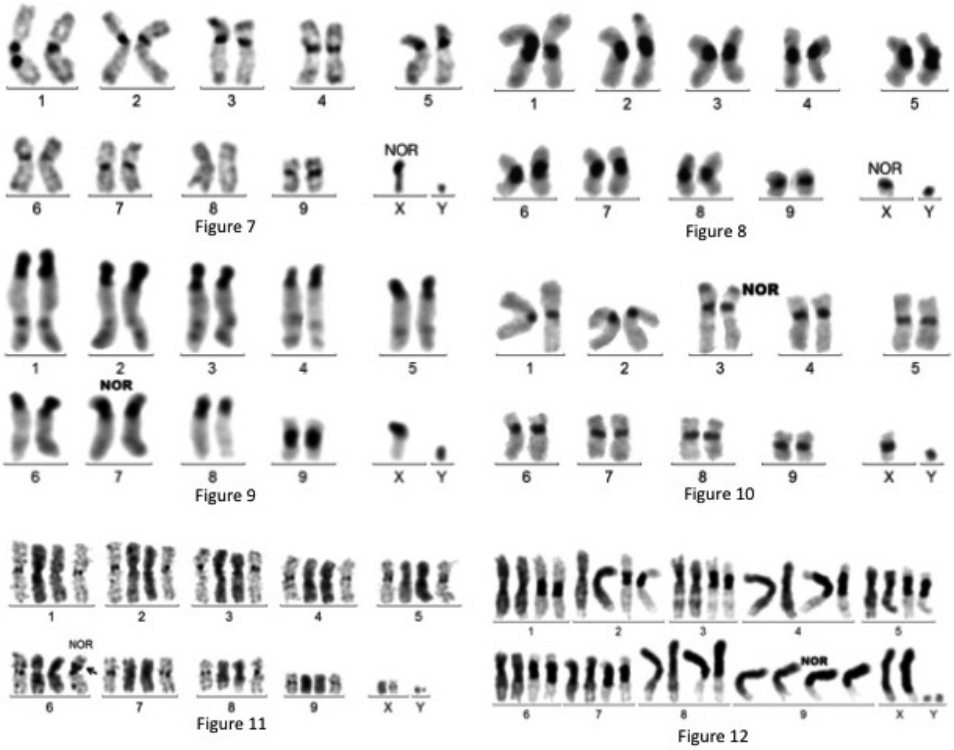
Dicronorrhina derbyana derbyana Westwood, 1843 (Fig. 4) and ***D. d. oberthuri*** Deyrolle, 1876: mitotic karyotype formula 20,XY; meioformula : 9+Xyp. One male *D. d. oberthuri* with a disomy Y was reported (Dutrillaux and Dutrillaux 2011b). Besides this particularity, no difference was noticed between the two subspecies.
- Chromosome morphology: all the autosomes are meta- or sub-metacentric; the X is metacentric and the Y punctiform.
- C-banding: large juxta-centromeric C-bands on centromeric regions of all chromosomes, including the X and Y, with only slight variations. Discreet C-bands are present at terminal or sub-terminal regions of the 4p arm, and occasionally other chromosome arms.
- LG staining: a discreet banding differentiates all chromosome pairs.
- NOR staining: at pachynema, nucleoli are located alongside the short arm of a small bivalent, N° 7 or 8.

Dicronorrhina micans Drury, 1773 (Fig. 5): mitotic formula: 20,XY; meioformula: 9+Xyp.
- Chromosome morphology: all autosomes but pair N° 8 are meta- or sub-metacentric. Pair N° 8 is acrocentric, the X is sub-metacentric and the Y punctiform.
- C-banding: large juxta-centromeric bands in pairs N° 1-7, smaller in pairs N° 8 and 9 and sex chromosomes.
- Silver staining: at pachynema, nucleoli are located on the short arm of bivalent N° 8. NOR location: 8p arm

Eudicella aethiopica Müller, 1941 (fig. 6): mitotic formula: 20,XY; meioformula: 9+Xyp.
- Chromosome morphology: all autosomes are meta- or sub-metacentric. The X is sub-metacentric and the Y punctiform.
- C-banding: fairly large juxta-centromeric C-bands on pairs N° 1, 3, 4 and 9, small on pairs 2, 7, 8 and X, and very small on pairs N° 5 and 6 and Y.
- Silver staining: intense on the Xp arm and the Y at mitotic metaphase; presence of nucleoli in association with the intensely stained X component of the sex bivalent at pachynema; and intense at the X and Y junction of the Xyp bivalent at metaphase I. NOR location: Xp arm.

Eudicella gralli Buquet, 1836 (Fig. 7): mitotic formula: 20,XY; meioformula 9+Xyp.
- Chromosome morphology: all autosomes meta- or sub-metacentric; X acrocentric or sub-metacentric (inter-individual variation?), Y almost punctiform.
- C-banding: large C-bands at all juxta-centromeric regions, except for pair N° 8 and chromosome Y. Frequent small C-band on chromosome 3pter. The Xp arm may be either C-banded (acrocentric form) or not (sub-metacentric form).
- Silver staining: as for *E. aethiopica*: Xp arm.

Eudicella smithi MacLeay, 1838 (Fig. 8): mitotic formula: 20,XY; meioformula: 9+Xyp.



Figures 7–12. 7 C-banded karyotype of *Eudicella gralli* **8** C-banded karyotype of *Eudicella smithi.* NOR on Xp arm **9** C-banded karyotype of *Goliathus goliathus*. NOR on 7 p arm **10** C-banded karyotype of *Mecynorrhina polyphemus confluens*. NOR on 3p arm **11** Giemsa stained (center) and C-banded karyotype of *Mecynorrhina torquata*. NOR on 6p arm **12** Giemsa stained (left) and C-banded (right) karyotype of *Megalorrhina harrisi*. The short arms of chromosomes 8, 9 and X are entirely heterochromatic. NOR on 9p arm.

- Chromosome morphology: all autosomes and the X meta- or sub-metacentric and the Y punctiform.
- C-banding: large and variable juxta-centromeric C-bands on pairs N° 1–8, smaller on pair N° 9 and sex chromosomes. A dispensable C- band exists on chromosome 2pter.
- NOR staining: as for *E. aethiopica*: Xp arm.

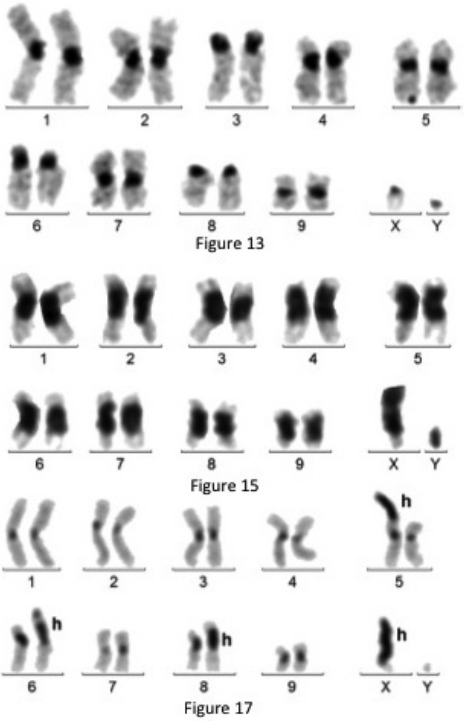
Goliathus goliatus Drury, 1770 (Fig. 9): mitotic formula: 20,XY; meioformula 9+Xyp. The C-banded karyotype was reported in Dutrillaux and Dutrillaux (2012).
- Chromosome morphology: the X chromosome and all autosomes but pair no 9 are acrocentric. The Y is punctiform.
- C-banding: intense C-bands are present at the centromere regions of most chromosomes. Faint C-bands are also distally located on the long arms of chromosomes1 to 5.

- LG staining: all chromosome pairs could be identified.
- NOR staining: At pachynema, the sex bivalent is intensely stained, and nucleoli are associated with the short arm of an acrocentric, presumably bivalent 7. At metaphase I, the space between the X and Y is intensely stained. NOR location: 7p arm.

Mecynorrhina polyphemus confluens Fabricius, 1781 (Fig. 10): mitotic formula: 20,XY; meioformula: 9+Xyp.
- Chromosome morphology: all autosomes meta- or sub-metacentric; X metacentric, Y punctiform.
- C-banding: fairly intense at all juxta-centromeric regions of all autosomes and sex chromosomes; presence of a C-band on 3pter.
- LG staining: all chromosome pairs could be identified (Fig. 18).
- Silver staining: at pachynema, nucleolus are alongside the terminal region of bivalent 3; intense staining of the sex bivalent in the space between X and Y at metaphase I. NOR location: 3p arm.

Mecynorrhina torquata Drury, 1782 (Fig. 11): mitotic formula: 20,XY; meioformula: 9+Xyp.
- Chromosome morphology: all autosomes meta- or sub-metacentric; X metacentric, Y small.
- C-banding: moderately large C-bands at juxta-centromeric regions of pairs N° 1–4 and 6–8, small on pairs 5 and 9, and almost inexistent on sex chromosomes; presence of a small C-band on 3pter.
- LG staining: identification of all chromosome pairs.
- Silver staining: intense at the proximal region of chromosome 6p arm at mitotic metaphase and on the Xyp bivalent at metaphase I. NOR location: 6p arm.

Megalorrhina harrisi Westwood, 1847 (Fig. 12): mitotic formula: 20,XY; meioformula 9+Xyp. The parachute like sex bivalent is unusually large, with the presence of heterochromatin, opposite to the Y, which is associated with the euchromatic part of the X.
- Chromosome morphology: all the autosomes and the X appear to be meta- or sub-metacentric after Giemsa staining, and the Y is quite small.
- C-banding: large or very large juxta-centromeric C-bands on all chromosomes but the Y. On pairs N° 8 and 9 and the X, one arm is entirely heterochromatic. Thus, these chromosomes must be regarded as acrocentric, although they look metacentric. Large variations of heterochromatin lead to a marked polymorphism. For instance, the size of the X may vary by twofold among individuals.
- LG staining: identification of all chromosome pairs (Fig. 18)
- Silver staining: at pachynema, the sex bivalent is intensely stained, and nucleoli are at contact with the short arm of bivalent 9. At metaphase I, the space between X and Y is stained, as usual in Xyp bivalents. NOR location: 9 p arm



Figures 13–17. 13 C-banded karyotype of *Plaesiorrhinella watkinsiana* **14** C-banded karyotype of *Rhamphorrhina bertoloni*. Heterochromatic Xp arm **15** C-banded karyotype of *Stephanocrates preussi*. Heterochromatic Xp arm **16** Giemsa stained karoytype of *Stephanorrhina guttata* **17** C-banded karyotype of *Stephanorrhina princeps*. H : heterochromatin.

Plaesiorrhinella watkinsiana Lewis, 1879 (Fig. 13): mitotic formula: 20,XY; meioformula: 9+Xyp.

- Chromosome morphology: pairs N° 1, 2, 4, 5, 7 and 9 meta- or sub-metacentric, pairs N° 3, 6 and 8 acrocentric. The X is acrocentric and the Y punctiform.
- C-banding: fairly intense at all juxta-centromeric regions of all autosomes and faint on sex chromosomes.
- LG staining: identification of all chromosome pairs.
- Silver staining: at pachynema, nucleoli are associated with the centromeric region of the acrocentric bivalent 6. NOR location: 6p arm.

Rhamphorrhina bertolonii Lucas, 1879 (Fig. 14): mitotic formula: 20,XY; meioformula: 9+Xyp.

- Chromosome morphology: all autosomes are metacentric or sub-metacentric, the X is a large sub-metacentric, and the Y is a small metacentric.
- C-banding: limited to centromeric regions on autosomes, it stains most of the X and Y.
- LG staining: identification of all chromosome pairs (Fig. 18).

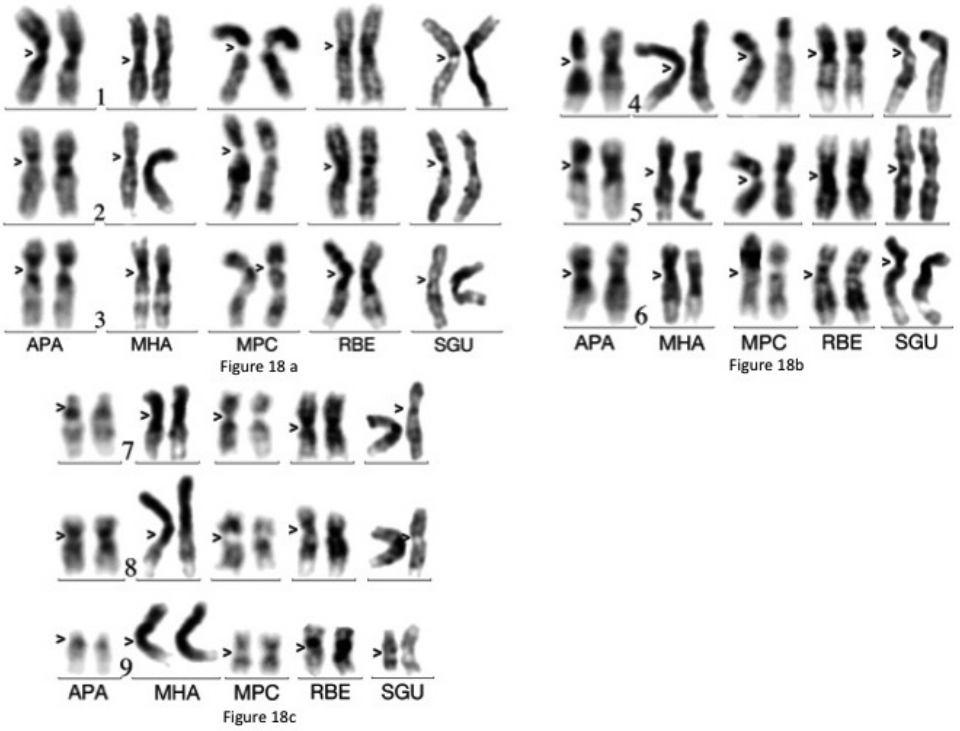


Figure 18. Comparison of autosomes from gonocytes of 5 species after Giemsa light staining: *A. passerini* (APA), *M. harrisi* (MHA), *M. polyphemus confluens* (MPC), *R. bertoloni* (RBE) and *S. guttata* (SGU). **a** Chromosomes 1–3 **b** chromosomes 4–6 **c** chromosomes 7–9. Centromeres are indicated by arrow heads.

- NOR staining: at pachynema, nucleoli are located alongside the sex bivalent, which is unusually large, due to the presence of a large heterochromatic fragment on the X chromosome. This heterochromatin prevents to accurately locate both centromere and NOR on this chromosome.

Stephanocrates preussi Kolbe, 1892 (Fig. 15): mitotic formula: 20,XY; meioformula: 9+Xyp.

- Chromosome morphology: all the autosomes are meta- or sub-metacentric. The X is unusually large and sub-metacentric; the Y is acrocentric.
- C-banding: very large C-bands at all juxta-centromeric regions of all chromosomes, representing 30-40% of their whole length. The large size of the X is principally related to the presence of heterochromatin. X and Y form a large parachute bivalent at metaphase I.
- Silver staining: at pachynema, nucleoli are recurrently located near the centromere region of a large metacentric, which could not be identified. At metaphase I, the large parachute sex bivalent is deeply stained between the X and the Y. NOR location: autosomal.

Stephanorrhina guttata Olivier, 1789 (Fig. 16): mitotic formula: 20,XY; meioformula: 9+Xyp
- Chromosome morphology: all the autosomes and the X appear to be metacentric or sub-metacentric (chromosomes 1, 3, 6 and 8) or sub-metacentric after Giemsa staining, and the Y is quite small.
- C-banding: in addition to non-remarkable juxta-centromeric C-bands, presence of a small C-band on the terminal region of the 5p arm. The small Xp arm is heterochromatic and the Y remains unstained.
- LG staining: identification of all chromosome pairs (Fig. 18).
- Silver staining: nucleoli remain alongside the sex bivalent at pachynema and the space between X and Y is intensely stained at metaphase I. NOR location: probably on the heterochromatic short arm of the X.

Stephanorrhina princeps Oberthür, 1880 (Fig. 17): mitotic formula: 20,XY; meioformula: 9+Xyp.
- Chromosome morphology: all the autosomes and the X appear to be metacentric or sub-metacentric after Giemsa staining, and the Y is quite small.
- C-banding: in addition to juxta-centromeric heterochromatin on all chromosomes, large additional and polymorphic heterochromatic segments occur on chromosomes 5,6, 8 and the X.
- LG staining: identification of all chromosome pairs.

Chromosome comparison and evolution

The karyotypes of all species except *C. africana* are composed of 20 chromosomes, a number observed in most Cetoniinae, Dynastinae and Melolonthinae (Smith and Virkki 1978, Dutrillaux et al. 2007, 2008, 2011, Gianoulis et al. 2011) and other Scarabaeidae species studied so far (Angus et al. 2007, Bione et al. 2005, Dutrillaux and Dutrillaux 2013, Silva et al. 2009, Moura et al. 2003, Smith and Virkki 1978, Wilson and Angus 2004, 2005, Yadav et al. 1979). As proposed by several of these authors, it is very likely that this number is that of their common ancestral karyotype. Thus, the karyotype of *C. africana*, with 14 chromosomes is highly derived. It possesses three pairs of very large chromosomes, which probably originated by translocation (fusion) of ancestral chromosomes.

The 16 other karyotypes comprise nine pairs of autosomes of gradually decreasing size. Their similar sizes among the different karyotypes may have the following interpretations:

– neither translocations nor other inter-chromosomal exchanges occurred during evolution/speciation processes;
– exchanges occurred, but involved very small fragments, hard to detect;
– exchanges involved large fragments of similar sizes, preserving chromosome size.

Exchanges of very small fragments are unlikely. They are harmful because they lead to deleterious, but viable chromosomal imbalances, in progeny of heterozygote translocation carriers, as shown in human pathology. Thus, they should have been strongly counter-selected during evolution. Exchanges of large fragments may exist, but it would be very unlikely that they systematically involved fragments of similar size. Thus, the more likely interpretation is that speciation and evolution processes have occurred with few or without inter-chromosomal rearrangements in this tribe of beetles. Then, either chromosome rearrangements rarely occurred, or they were mostly of the intra-chromosomal type such as inversions. This last interpretation is, by far, the most likely (Dutrillaux and Dutrillaux 2009). Unfortunately, the occurrence of inversions is not easy to detect in poorly banded chromosomes, as those of beetles, except when they drastically modify their morphology, as changing a meta-/sub-metacentric into an acrocentric, or *vice versa*. In beetles, most autosomes are metacentric or sub-metacentric. Unless there is a strong selective constraint against acrocentrics, it is likely that ancestral karyotypes were already composed of meta- and sub-metacentrics. Starting from such a karyotype, the presence of acrocentrics would sign the occurrence of inversions. Acrocentrics are observed in six species. As regards their numbers, the karyotype of *G. goliatus*, with eight acrocentric pairs, is by far the most derived. The five other karyotypes exhibit one to three acrocentric pairs. Most chromosomes involved are small. Although some recurrences exist, principally for chromosome 8, which is acrocentric in *A. passerini, D. micans, G. goliathus, M. harrisi* and *P. watkinsiana*, this does not allow us to propose a phylogenetic scheme based on the sequence of autosome inversions.

The X chromosome exhibits four different morphologies: acrocentric, sub-metacentric, metacentric and more or less sub-metacentric, with one euchromatic and another heterochromatic arm (C-banded). The two former morphologies were observed in species of Cetoniini, Dynastinae and Melolonthinae (Dutrillaux et al. 2007, 2008, Gianoulis et al. 2011) but it was proposed that the acrocentric was likely to be the ancestral form. Thus, the five species with a sub-metacentric X (two *Dicronorrhina* and three *Eudicella* species) might have a same derivative X, which may constitute an argument to put together the two genera. The two species with a metacentric X belong to the same genus *Mecynorrhina*.

Large amounts of heterochromatin are present in the X chromosome of six species belonging to five genera (*Cyprolais, Rhamphorrina, Megalorrhina, Stephanocrates* and *Stephanorrhina*). This is also an argument to put together these five genera. Finally, the presumably ancestral acrocentric X is conserved in 4 species: *A. passerini* and *P. watkinsiana* and 2 with highly rearranged autosomes, *G. goliatus* and *C. africana*.

As in many other Scarabaeidae (Dutrillaux and Dutrillaux 2012), the NOR is frequently located on the X. In species with autosomal NORs, the NOR is located on different autosomes, which suggests that different events displaced it from the ancestral position on the X chromosome.

On the whole, it remains very difficult to propose undisputable phylogenetic relationships by using these classical cytogenetic data. The detected rearrangements are too few,

and the species studied probably represent a too small and heterogenous sample of the sub-family. If inversions are in cause, it is not certain that molecular cytogenetics (FISH) would significantly improve the results, at least by the use of chromosome painting.

Finally, an improvement could come from chromosome banding, but a major problem remains: the difficulty for inducing a consistent banding of euchromatin. Beetle chromosomes are apparently not composed of large heterogeneous DNA fragments, as ALU and LINE sequences, associated to R- and G-banding in mammalian chromosomes (Bickmore and Craig 1997). We tried to use various techniques of DNA denaturation (Dutrillaux and Lejeune 1971), without success. We also tried enzymatic digestions, which inconsistently gave some results (Dutrillaux et al. 1971, Seabright 1971). To our knowledge, euchromatin banding of mitotic chromosomes was rarely induced in beetles (Angus 1982), which illustrates a technical difficulty. In this study, we tried to take advantage of the variations of chromosome compaction from cell type to cell type, and in a given cell type, with cell differentiation level. Drastic changes of chromatid compaction occur during early gametogenesis, in relation with variations of DNA methylation, around the birth period of the mouse (Coffigny et al. 1999, Bernardino-Sgherri et al. 2002). The particular aspects of chromosomes, described in mouse gonocytes, are occasionally observed in immature gonads of beetles. In particular, some germ cells, probably gonocytes, exhibit a poor banding that appropriate Giemsa staining and computer driven contrast adjustments can improve. This allowed us to perform more accurate comparisons for five species (Fig. 18), and show the occurrence of inversions among sub-metacentric chromosomes. These inversions group *A. passerini* and *M. harrisi* on the one hand and *R. bertoloni* and *S. guttata* on the other hand. *M. polyphemus* appears to have an intermediary position. This confirms that apparently similar karyotypes can differ by cryptic chromosome rearrangements, as shown in many mammalian species, but not in beetles.

Conclusion

All the karyotypes of the 17 studied species differ from each other in some respects by inversions, heterochromatin variations and translocations. As expected, congeneric species possess more similar karyotypes than species from different genera, but it remains impossible to propose a phylogeny based on chromosome changes. It is noteworthy that *G. goliathus*, which has some remarkable phenotypic characters, such as large size, hairy thorax, cephalic horns in the male, has a most derived karyotype, with 8/9 inverted autosomes. It would be tempting to consider that a relationship, even indirect, exists between the accumulation of chromosome rearrangements and that of gene mutations determining phenotype changes. However, *C. africana*, which has a non-remarkable morphology among Cetoniinae, but a highly rearranged karyotype, confirms that it would be hazardous to propose such correlation.

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SHORT COMMUNICATION

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Simultaneous visualization of different genomes (J, JSt and St) in a *Thinopyrum intermedium* × *Thinopyrum ponticum* synthetic hybrid (Poaceae) and in its parental species by multicolour genomic in situ hybridization (mcGISH)

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Abstract

Multicolour genomic *in situ* hybridization (mcGISH) using total genomic DNA probes from *Thinopyrum bessarabicum* (Săvulescu & Rayss, 1923) Á. Löve, 1984 (genome J^b or E^b, $2n = 14$), and *Pseudoroegneria spicata* (Pursh, 1814) Á. Löve, 1980 (genome St, $2n = 14$) was used to characterize the mitotic metaphase chromosomes of a synthetic hybrid of *Thinopyrum intermedium* (Host, 1805) Barkworth & D.R. Dewey, 1985 and *Thinopyrum ponticum* (Podpěra, 1902) Z.-W. Liu et R.-C.Wang, 1993 named „Agropyron glael" and produced by N.V. Tsitsin in the former Soviet Union. The mcGISH pattern of this synthetic hybrid was compared to its parental wheatgrass species. Hexaploid *Th. intermedium* contained 19 J, 9 J^{St} and 14 St chromosomes. The three analysed *Th. ponticum* accessions had different chromosome compositions: 43 J + 27 J^{St} (PI531737), 40 J + 30 J^{St} (VIR-44486) and 38 J + 32 J^{St} (D-3494). The synthetic hybrid carried 18 J, 28 J^{St} and 8 St chromosomes, including one pair of J-St translocation and/or decreased fluorescent intensity, resulting in unique hybridization patterns. Wheat line Mv9kr1 was crossed with the *Thinopyrum intermedium* × *Thinopyrum ponticum* synthetic hybrid in Hungary in order to transfer its advantageous agronomic traits (leaf rust and yellow rust resistance) into wheat. The chromosome composition of a wheat/A.glael F_1 hybrid was 21 wheat + 28 wheatgrass (11 J + 14 J^{St}+ 3 S). In the present study, mcGISH involving the simultaneous use of St and J genomic DNA as probes provided information about the type of *Thinopyrum* chromosomes in a *Th. intermedium*/*Th. ponticum* synthetic hybrid called A. glael.

Keywords
multicolour GISH, *Thinopyrum intermedium*, *Thinopyrum ponticum*, Agropyron glael, J, J^{st}, St genomes

Introduction

N.V. Tsitsin produced a synthetic hybrid in the former Soviet Union by crossing *Thinopyrum intermedium* (Host, 1805) Barkworth & D.R. Dewey, 1985 (=*Agropyron glaucum* Roemer & Schultes, 1817, 2n=6x=42) with *Thinopyrum ponticum* (Podpěra, 1902) Z.-W.Liu & R.-C.Wang, 1993 (=*Agropyron elongatum* Host ex P. Beauvois, 1812, 2n=10x=70) (Tsitsin 1954). The hybrid plants were named "Agropyron glael" (A. glael, 2n=8x=56, Tsitsin 1979), from an abbreviation of "glaucum" and "elongatum". This name (A. glael) will be used hereafter in this article. A number of A. glael plants were maintained in Martonvásár (Hungary) thanks to cooperation between the Hungarian Academy of Sciences and the Moscow Research Institute of Agriculture -"Nemchinovka" in the 1960's. The hybrid plants had 56 chromosomes.

Both wheatgrass species are long been known to have superior resistance to various diseases (Wang 2011). They can be crossed with wheat, making them a potential source of gene pool for wheat improvement. In 2001, wheat line Mv9kr1 was crossed with A. glael in Hungary in order to transfer its advantageous agronomic traits (leaf rust and yellow rust resistance) into wheat (Molnár-Láng et al. 2012).

Polyploid *Thinopyrum* (Á. Löve, 1980) species contain genomes similar to the J (E^b, J^b) genome of the diploid *Th. bessarabicum* (Săvulescu & Rayss, 1923) Á. Löve, 1984 (2n=2x=14) (Östergen 1940) or the E (E^e, J^e) genome of *Th. elongatum* (Host, 1802) D.R. Dewey, 1984 (2n=2x=14) (Cauderon and Saigne 1961), which are closely related (Ceoloni et al. 2014), and sometimes also contain a third genome (S or St) from *Pseudoroegneria spicata* (Pursh, 1814) Á. Löve, 1980 (2n=2x=14). The S genome of *Pseudoroegneria* (Nevski, 1934) genus was renamed to St in order to discriminate from the S genome of Sitopsis section of *Aegilops* Linnaeus, 1753 species (Wang et al. 1995). Ceoloni (2014) also mentioned this genome as St/S. *Th. intermedium* has been described using various genome formulas, including E^eE^bSt (Wang and Zhang 1996), E^1E^2St (Zhang et al. 1996) and JJ^sS (Chen et al. 1998). Wang et al. (2011) mentioned J^s as E^{St} (J^{St}). J^{St} symbol will be used hereafter to describe this special chromosome type of *Th. intermedium*. Kishii et al. (2005) and Mahelka et al. (2011) revealed new aspects of its genomic composition, suggesting the possible presence of a *Dasypyrum* (Cosson & Durieu de Maisonneuve, 1855) T. Durand, 1888 (V) genome. Recently Wang et al. (2015) published genotypic data obtained using EST-SSR primers derived from the putative progenitor diploid species *Ps. spicata*, *Th. bessarabicum* and *Th. elongatum*, which indicated that the V genome was not one of the three genomes in intermediate wheatgrass. They proposed the $J^{vs}J^rSt$ genome designation, where J^{vs} and J^r represented ancestral genomes of the present-day J^b of *Th. bessarabicum* and J^e of *Th. elongatum*, J^{vs} being the more ancient. The change of J^s to J^{vs} is based on the study of Mahelka et al. (2011) and Deng et al. (2013), as all 14 chromosomes of J^s showed GISH/FISH

hybridization signals from V-genome probes [*Dasypyrum villosum* (Linnaeus, 1753) P. Candargy, 1901], but only 8 to 11 of the 14 chromosomes have the centromeric region being hybridized by the St genome probe (Chen et al. 1998, Zhang et al. 1996, Kishii et al. 2005, Tang et al. 2011, Deng et al. 2013). FISH analysis using pMD232-500 as probe (originating from *Secale cereale* Linnaeus, 1753 cv. Kustro) indicated that the 14 J chromosomes of *Th. intermedium* bear FISH signals. According to their findings the J genome is changed to J^r.The genome constitution of *Th. ponticum* was described using the $JJJJ^sJ^s$ (Chen et al. 1998) and $E^eE^bE^xStSt$ (Li and Zhang 2002) formulas.

Genomic *in situ* hybridization (GISH) or multicolour genomic *in situ* hybridization (mcGISH) offered new opportunities for testing genome relationships in plants (Bennett et al. 1991), for describing hybrid character (Keller et al. 1996), for visualizing genomes simultaneously (Mukai et al. 1993), and for studying genome organization and evolution (Chen et al. 1994, Mahelka et al. 2011).

Multicolour genomic *in situ* hybridization was used in the present study for the simultaneous visualization of the J and St genomic DNA of A. glael and their parental wheatgrass species (*Th. intermedium*, *Th. ponticum*) and to describe the chromosome composition of these materials. As previously published papers had different findings and the authors proposed different genome formulas in *Th. intermedium*, difficulties in identification of the different genomes were expected in our study. As *Th. ponticum* chromosomes belonged to two different genomes (J and J^{St}), precise detection and identification of them was probable despite of the high chromosome number. There were no former molecular cytogenetic data about the A. glael, but the presence of all the three different chromosome types (J, J^{St}, St) of the two parental wheatgrass species was hoped-for.

Methods

Thinopyrum intermedium, *Th. ponticum*, their synthetic hybrid A.glael, and the wheat/A. glael F_1 hybrid were analysed cytogenetically (Table 1). Seeds of A.glael, wheat/A.glael F_1 hybrid, *Th. intermedium*, and *Th. ponticum* were germinated, after which mitotic metaphase chromosome spreads were prepared according to Lukaszewski et al. (2004). McGISH was performed in order to simultaneously visualize the different wheatgrass chromosomes in *Th. intermedium*, *Th. ponticum*, A.glael, and in the Mv9kr1/A. glael F_1 hybrid. J (E^b) genomic DNA from *Th. bessarabicum* labelled with biotin-11-dUTP (Roche Diagnostics, Mannheim, Germany) and St genomic DNA from *Ps. spicata* labelled with digoxigenin-11-dUTP were produced using the random primed labelling protocol. The hybridization mixture contained 100 ng each of the labelled probes/slide, dissolved in a 15 µl mixture of 100% formamide, 20×SSC and 10% dextran-sulphate at a ratio of 5:1:4, and 3000 ng *Triticum aestivum* (Linnaeus, 1753) DNA (genotype Mv9kr1, BBAADD) as a block when needed. Hybridization was performed at 42°C overnight. Streptavidin-FITC (Roche) and Anti-Digoxigenin-Rhodamine (Roche) dissolved in TNB (Tris-NaCl-blocking buffer) were used in the detection phase.The

Table 1. Species and genotypes analysed in the present study.

Genotype	Accession number	Genebank	Geographic origin
Thinopyrum intermedium	PI565004	USDA ARS GRIN	Russia
Thinopyrum ponticum	PI 636523	USDA ARS GRIN	Argentina
Th. ponticum	PI531737	USDA ARS GRIN	Argentina
Th. ponticum	PI 547313	USDA ARS GRIN	Russia
Th. intermedium × *Th. ponticum* synthetic hybrid: Agropyron glael	glael-8/2008	Martonvásár Cereal Genebank	Russia
Mv9kr1 × A. glael F_1 hybrid	112705	Martonvásár Cereal Genebank	Hungary

slides were screened using a Zeiss Axioskop-2 fluorescence microscope equipped with filter sets appropriate for DAPI (Zeiss Filterset 01), and for the simultaneous detection of FITC and Rhodamine (Zeiss filter set 24). Images were captured with a Spot CCD camera (Diagnostic Instruments) and processed with Image Pro Plus software (Media Cybernetics).

Results

Thinopyrum intermedium

McGISH, performed using J and St genomic DNA probes, simultaneously discriminated three different genomes in the segmental autoallohexaploid *Th. intermedium* (Fig. 1a–b). Among the 42 chromosomes, 14 fluoresced bright red along their whole length, showing the presence of the St genome. The St probe gave also a hybridization signal in the centromeric region of 9 chromosomes, where the other parts hybridized with the J genome, resulting in two-coloured chromosomes with a bow-tie shape. These J^{St}-type chromosomes differed to those of the J, where the chromosomes hybridized with the J genomic DNA probe over the entire length with no centromeric St signals. The intensity of the green fluorescence signal was not uniform, the J^{St} chromosomes being fainter than J. In the telomeric segment of some J and J^{St} chromosomes a weak St genomic hybridization signal was detected, although in a few other chromosomes this fragment was unlabelled. One satellited chromosome was observed where the NOR region was hybridized to St genomic DNA. The analysed accession (No. PI565004) contained 19 J, 9 J^{St} and 14 St chromosomes.

Thinopyrum ponticum

The analysed *Th. ponticum* contained 70 chromosomes and two groups could be distinguished based on their mcGISH pattern (Fig. 2). Bright green fluorescence signals

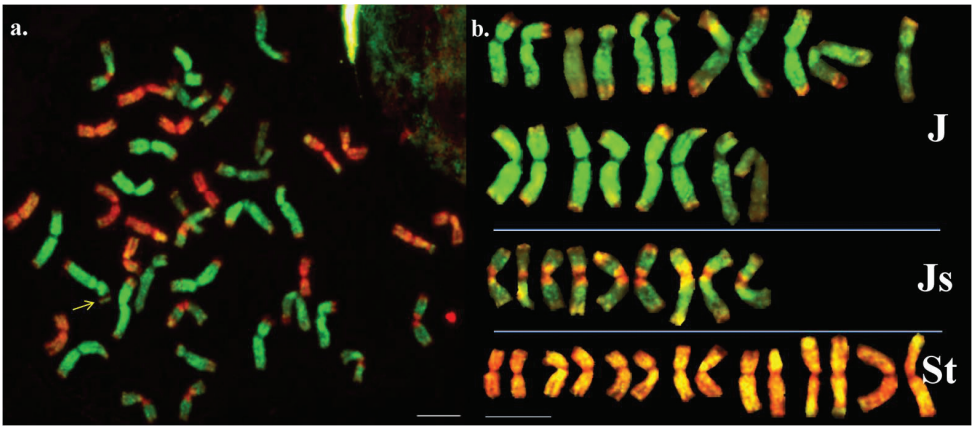

Figure 1. Results of multicolour genomic *in situ* hybridization on *Thinopyrum intermedium.* **a** Karyotype of a complete cell using *Thinopyrum bessarabicum* (J, green) and *Pseudoroegneria spicata* (St, red) genomic DNA as probes. Chromosome with satellite is indicated with arrow **b** Karyogram of *Thinopyrum intermedium* chromosomes. Top row: J chromosomes; middle row: J^{St} chromosomes with the St pericentromeric region; bottom row: St chromosomes. Bar = 10 μm.

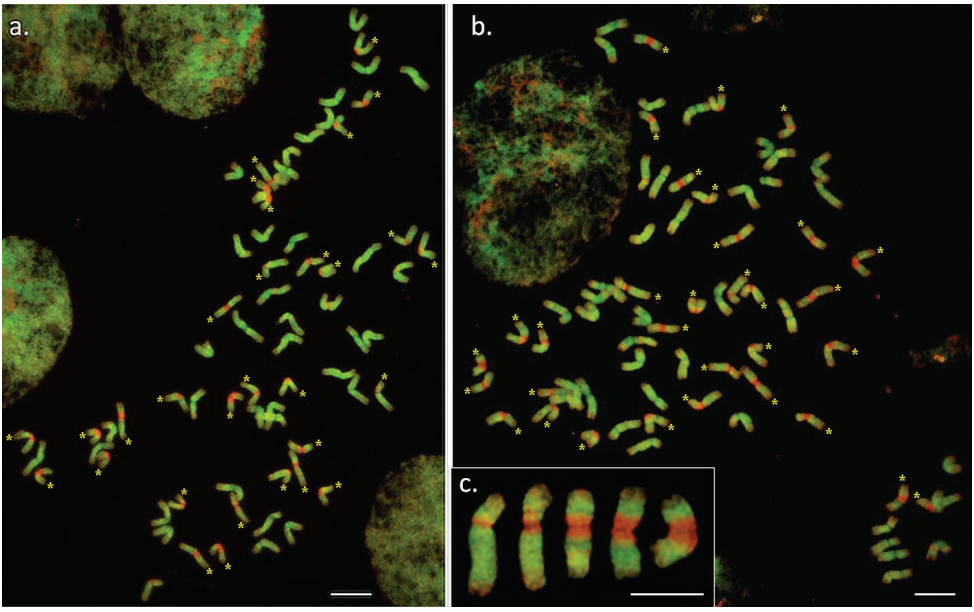

Figure 2. Multicolour genomic *in situ* hybridization on *Thinopyrum ponticum.* **a** Karyotype of *Th. ponticum* (accession VIR-44486) carrying 40 J and 30 J^{St}chromosomes, using *Thinopyrum bessarabicum* (J, green) and *Pseudoroegneria spicata* (St, red) genomic DNA as probes **b** 38 J and 32 J^{St}chromosomes identified in *Th. ponticum* (accession D-3494) **c** J^{S} chromosomes with different lengths of St DNA in the centromeric region. J^{St} chromosomes were marked with asterisks. Bar = 10 μm.

marked the J chromosomes, while those with St (red) pericentromeric regions belonged to the J^{St} genome. The three analysed accessions showed different chromosome compositions: 43 J + 27 J^{St} (PI531737), 40 J + 30 J^{St} (PI 547313, Fig. 2a) and 38 J + 32 J^{St} (PI636523, Fig. 2b). The length of the St segment in the J^{St} chromosome varied (Fig. 2c). Each J^{St} chromosome showed a short section of St hybridization close to the centromere, while others fluoresced bright red on almost 1/3 of the chromosomes in the centromeric-pericentromeric regions. There was variation in the intensity of the green fluorescence signal, J^{St} chromosomes being fainter than J. The telomeric region of most of the chromosomes did not hybridize with the J or St genomic DNA probes and remained unlabelled.

Thinopyrum intermedium × *Th. ponticum synthetic hybrid*: A. glael

McGISH made it possible to discriminate three different groups of A. glael chromosomes (Fig. 3a). The designation of the A. glael chromosomes was J, J^{St} and St, as the synthetic hybrid contains chromosomes from both *Th. intermedium* and *Th. ponticum*. Digoxigenin-labelled St genomic DNA hybridized to four pairs of submetacentric chromosomes, which were thus identified belonging to the St genome. One pair of chromosomes was mainly red, but an St-J translocation was detected in the long arm (marked with yellow arrowheads). Nine pair of chromosomes with only green fluorescence signals were identified as J genome, though three pairs showed lower fluorescence intensity, while the others were bright. The remaining fourteen pairs had various lengths of St genomic hybridization in the pericentromeric region, showing the presence of the J^{St} genome.

Wheat/A. glael F_1 hybrid

Chromosome counting detected 49 chromosomes in the wheat/A. glael F_1 hybrid (21 wheat + 28 wheatgrass), 28 of which hybridized with the J and/or St genomes during mcGISH, discriminating the wheatgrass chromosomes from the unlabelled wheat (Fig. 3b). Only three chromosomes hybridized with the St genomic DNA over their entire length. Eleven chromosomes had no red fluorescence signal in the centromeric region, and were thus identified as J. Two of them had only very weak J signals, with stronger St hybridization in the telomeric region. The remaining 14 chromosomes had various lengths of St genomic hybridization in the pericentromeric region, showing the presence of the J^{St} genome. Some of the J and J^{St} chromosomes had a weak St genomic hybridization signal in the telomeric region. Some of these chromosomes carried several J-St, J^{St}-St, translocations and/or decreased fluorescent intensity was observed in the pericentromeric and telomeric regions, resulting in unique hybridization patterns (marked with asterisks in Fig. 3c). The chromosome composition of the F_1 hybrid was 21 wheat + 11 J + 14 J^{St}+ 3 S.

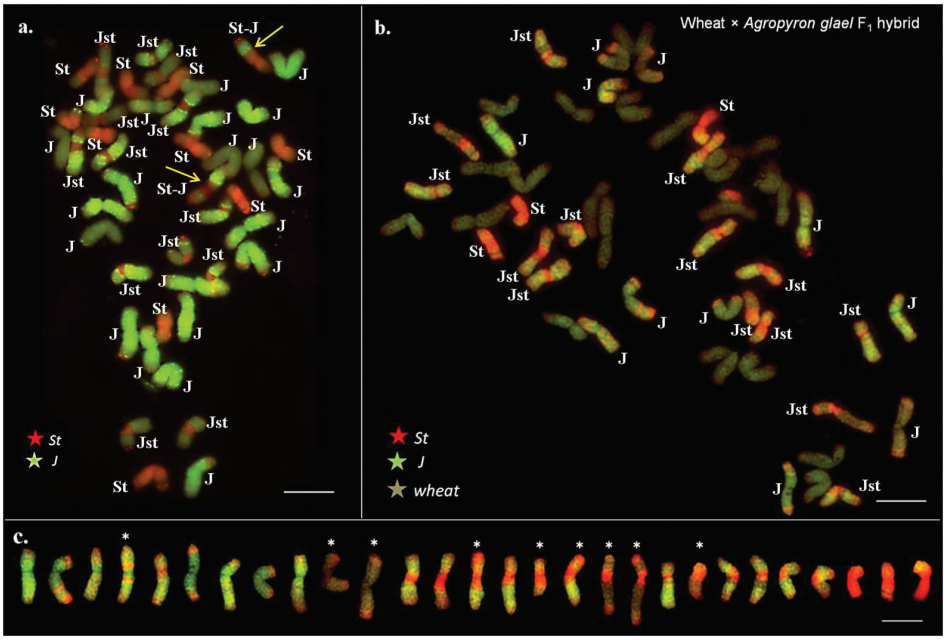

Figure 3. Multicolor genomic *in situ* hybridization pattern of Agropyron glael and the wheat (Mv9kr1 genotype)/A. glael F1 hybrid. **a** Karyotype of a partial cell of A. glael using *Thinopyrum bessarabicum* (J, green) and *Pseudoroegneria spicata* (St, red) DNA probes. Translocation between J and St chromosomes were marked with arrows **b** Karyotype of a complete cell of wheat (Mv9kr1 genotype)/A. glael F_1 hybrid using *Th. bessarabicum* (J, green) and *Ps. spicata* (St, red) genomic DNA simultaneously as probes and wheat genomic DNA as block simultaneously **c** Karyogram of A.glael chromosomes present in the wheat/A.glael F_1 hybrid. Nine A. glael chromosomes with hybridization patterns different to their parental species are marked with asterisks. Bar = 10 μm.

Discussion

GISH or mcGISH, a modification of fluorescence *in situ* hybrization, has been used to characterize genomes and chromosomes in polyploid *Thinopyrum* species (Chen et al. 1998, Tang et al. 2000, Li and Zhang 2002, Mahelka et al. 2011). In the present study, mcGISH involving the simultaneous use of St and J genomic DNA as probes provided information about the number and type of *Thinopyrum* chromosomes and demonstrated the presence of intergenomic (J-St) chromosome rearrangements in A. glael.

Chen et al. (1998) used GISH with one labelled genomic DNA probe and one nonlabelled blocking genomic DNA during the characterization of these wheatgrass species. They proposed the symbol J^S to represent J chromosomes with St repeated sequences and GISH signals around the centromeric regions. This chromosome type was the same which has been described in this study using two labelled genomic DNA probes. The use of mcGISH enabled the J and J^{St} genomes of *Th. ponticum* and the J, J^{St} and St genomes of *Th. intermedium* to be precisely discriminated using J and St labelled genomic DNA simultaneously.

As the number of J and J^{St} chromosomes was usually odd [19 J + 9 J^{St} in *Th. intermedium* and 43 J + 27 J^{St} (PI531737) in *Th. ponticum*], it is possible that J-J^{St} chromosome pairing can occur in meiosis, as reported by Chen et al. (2001). Most of the wheatgrass chromosomes were typical *Thinopyrum* chromosomes in A. glael and in the wheat/A. glael F_1 hybrid, while others showed notable differences when the mcGISH patterns were compared to those of *Th. ponticum* and *Th. intermedium*: decreased fluorescence intensity, J-St translocations in the telomeric region of J^{St} chromosomes, and unlabelled chromosome parts in all types of chromosomes. Chen et al. (2001) reported a high frequency of chromosome pairing between J-J^{St}, J-St and J^{St}-St chromosomes, as the result of which genetic exchange is possible between these genomes. Several minor J-St and J^{St}-St translocations were observed in A. glael and the wheat/A.glael F_1 hybrid. These translocations may have occurred during the formation of the synthetic hybrids. As the J-J^{St}-St chromosomes paired at high frequency, it may be that A. glael is not only a hybrid of the two wheatgrass species, but that the genetic composition has changed or been enriched with DNA sequences from other species during the long maintenance period (decades), as wheatgrass species are open-pollinating and very polymorphic. This could explain the presence of different hybridization patterns between the wheatgrass chromosomes in A. glael and the wheat/A.glael F_1 hybrid.

Several types of genome composition and chromosome numbers have been reported for *Th. intermedium* (Chen et al. 1998, Tang et al. 2000, Da Yong et al. 2004). Chen et al. (1998) detect 41 chromosomes (18 J, 10 J^{St}, 13 St) in a line derived from Portugal (PI249145), 49 chromosomes (18 J, 10 J^{St}, 21 St) in a French genotype. Most of the analyzed accessions carried 42 chromosomes, but the number of each chromosome type was various: 20 J + 8 J^{St} + 14 St in 'Chef', 'Clarke' (USA), 18 J + 10 J^{St} + 14 St in PI317406 (Afghanistan), and 21 J + 7 J^{St} + 14 St in PI547333 (China) (Chen et al. 1998). Tang et al. (2000) analyzed a Chinese accession and identified 21 J + 7 J^{St} + 14 St chromosomes. Da Yong et al. (2004) could detect 28 J + J^{St} and 14 St chromosomes in PI 469214 (USA), PI 578698 (Russia), and Z1141 (Canada). In this study we could detect 42 chromosomes including 19 J, 9 J^{St} and 14 St. According to other findings and our results, when the chromosome number was not 42, the number of St chromosomes was derived. The number of J + J^{St} chromosomes was always 28. Mahelka et al. (2011) detected 42 chromosomes in different *Th. intermedium* accessions, and 14 of which hybridized with *Dasypyrum villosum* genomic DNA, and also carried St genomic DNA hybridization signal in the pericentromeric region. Mahelka et al. (2011) concluded that the genomic heterogeneity of intermediate wheatgrass was higher than had been assumed, making this species more interesting as a source of desirable agronomic traits.

Nucleolar dominance, an epigenetic phenomenon in which one parental set of ribosomal RNA (rRNA) genes is silenced in an interspecific hybrid or during allopolyploidization, first reported in the 1930s (Navashin 1934). Only ribosomal RNA genes inherited from one parent are transcribed (Pikaard 2000), and the nucleolus organiser regions (NORs), the sites of rRNA genes from the other parent(s) are suppressed. The phenomenon was observed in several interspecific hybrids (Gautam et al. 2014), including wheat/*Thinopyrum elongatum* addition lines (Linc et al. 2012). *Thinopyrum intermedium* and *Thinopyrum ponticum* are allopolyploid species, thus nucleolar dominance

can probably be observed in them, and especially in their synthetic hybrid (A.glael). Loss of secondary constrictions can be observed during allopolyploidization or the formation of interspecific hybrids, which can be studied using rDNA probes by FISH. It was not part of this study, but it is planned in the future. Allopolyploidy can induce rapid genome evolution, and can cause genomic shock. The nature of this phenomenon were investigated (Matsuoka 2011). Its manifestation icludes chromosomal rearrangement, the gain and loss of chromosome segments, gene repression and activation, subfunctionalization, transposon activation, and changes in the epigenome (Wang et al. 2014). Multicolour GISH is a powerful technique to detect interspecific and intergeneric chromosome rearrangement. According to our mcGISH results, we observed minor chromosomal rearrangement, St-J translocations in nine chromosomes of A.glael in the wheat/ A.glael F1 hybrid, which chromosome patterns couldn't observed in *Thinopyrum* parental species. The reduction of number of St chromosomes were also detected.

As A. glael contains chromosomes from the two most valuable *Thinopyrum* species, changes in its genome could result in new invaluable genetic material, especially for wheat breeding.

Conclusions

In the present study, mcGISH involving the simultaneous use of St and J genomic DNA as probes provided information about the genome composition and the type of *Thinopyrum* chromosomes in a *Th. intermedium*/*Th. ponticum* synthetic hybrid called A. glael.

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RESEARCH ARTICLE

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Rearrangement hotspots in the sex chromosome of the Palearctic black fly *Simulium bergi* (Diptera, Simuliidae)

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Abstract

An extreme example of nonrandom rearrangements, especially inversion breaks, is described in the polytene chromosomes of the black fly *Simulium bergi* Rubtsov, 1956 from Armenia and Turkey. A total of 48 rearrangements was discovered, relative to the standard banding sequence for the subgenus *Simulium* Latreille, 1802. One rearrangement, an inversion (*IIS-C*) in the short arm of the second chromosome, was fixed. Six (12.5%) of the rearrangements were autosomal polymorphisms, and the remaining 41 (85.4%) were sex linked. More than 40 X- and Y-linked rearrangements, predominantly inversions, were clustered in the long arm of the second chromosome (IIL), representing about 15% of the total complement. The pattern conforms to a nonrandom model of chromosome breakage, perhaps associated with an underlying molecular mechanism.

Keywords

Caucasus Mountains, nonrandom breakage, polytene chromosomes, sex linkage

Introduction

The sex chromosomes of the Simuliidae have commanded attention because of their suggested role in driving speciation (Procunier 1989, Rothfels 1989, Conflitti et al. 2015), a role also noted in other groups, such as the Drosophilidae (Presgraves 2008). Although cytologically undifferentiated sex chromosomes (X_0Y_0) are frequent in the Simuliidae, the X and Y of most species in the family are identified by sex-linked rearrangements easily visible in the polytene complement, most commonly inversions, but also heterobands, nucleolus organizer expression, and other phenomena (Post 1982, Procunier 1982a).

Unlike the separate heteromorphic X and Y chromosomes of organisms such as *Anopheles* Meigen, 1818 mosquitoes and *Drosophila* Fallén, 1823, any one of the three chromosomes (I, II, or III) functions as both the X and the Y chromosome in the Simuliidae. The simuliid sex-chromosome system, therefore, is more similar to that in the closely related family Chironomidae in which the sex chromosomes are generally undifferentiated, although males are sometimes distinguished by rearrangements, such as inversions, that mark the Y chromosome (Martin 1962, Newman 1977). Often, the linkage of rearrangements to sex in the Simuliidae is not complete, resulting in partial sex linkage (Rothfels 1980). Within species of the Simuliidae, sex-chromosome polymorphism is common but nearly always confined to a single chromosome (Adler et al. 2010). These sex-linked structural phenomena are typically paracentric in the long arm (L) or short arm (S), but also can be pericentric (Bedo 1977). Different sex chromosomes (e.g., I versus II) typically signal the presence of separate species (Bedo 1975, Brockhouse 1985). Sex-linked chromosomal rearrangements that produce heterozygosity suppress crossing over and the accompanying risk of breaking up adaptive complexes of sex-determination genes (Rothfels 1980, Post 1982).

Inversions often build on one another to produce elaborate sex chromosomes in a particular arm of the Simuliidae (Rothfels 1980). Even in species or groups of species in which the sex chromosomes are cytologically undifferentiated, the autosomal and fixed inversions tend to be concentrated in a few arms (e.g., Post et al. 2007, Adler et al. 2015).

Simulium (*Simulium*) *bergi* Rubtsov, 1956, a black fly in the *S. venustum* species group (Adler and Crosskey 2016), was described from the Lesser Caucasus of southern Georgia (Rubtsov 1956) and later discovered in Armenia (Terteryan 1968). It was long considered a Caucasian endemic (Chubareva and Petrova 2008) until its discovery in Ankara Province of Turkey (Crosskey and Zwick 2007). *Simulium bergi*, nonetheless, remains a geographically restricted, little-collected species; before our study, it had been recorded from only three sites.

General features of the karyotype of *S. bergi* from Armenia have been provided, such as the lengths of the polytene chromosomes (Chubareva and Petrova 1979). Photographs of the metaphase and the entire polytene complements and the polytene centromere regions also have been presented (Chubareva et al. 2003, Chubareva and Petrova 2008). We use the polytene chromosomes to explore the evolutionary relationships and cytogenetic structure of *S. bergi* at two sites: one in the Armenian Caucasus

and one in eastern Turkey just beyond the western margin of the Lesser Caucasus. In particular, we examine the unique sex-chromosome system of *S. bergi*, which involves a large number of X- and Y-linked rearrangements in a restricted region of the polytene complement, to argue in favor of a nonrandom model of chromosomal reorganization.

Material and methods

Larvae (penultimate and ultimate instars) were collected with forceps primarily from trailing vegetation in one stream each in Armenia and Turkey (Table 1) and fixed in 1:3 acetic ethanol (modified Carnoy's solution). Our Armenian sample of 8 larvae was collected about 38 km south of the type locality of *S. bergi* in Tambovka, Akhalkalaki District, Georgia. Our Turkish sample of 30 larvae was collected about 105 km southwest of the type locality. The two sampling sites were less than 90 km apart. Larvae were identified morphologically using the keys and descriptions of Rubtsov (1956) and Terteryan (1968); identifications were confirmed chromosomally using the photomap of the complement presented by Chubareva and Petrova (2008).

The posterior portion of each larval abdomen was removed and processed for Feulgen staining, following procedures of Rothfels and Dunbar (1953), but using 5N HCl at room temperature (Charalambous et al. 1996). One gonad and both salivary glands containing nuclei with the stained polytene chromosomes were dissected out with fine needles, placed in a drop of 50% acetic acid, and spread under a coverslip, with thumb pressure. Larval gender was determined by gonadal shape—slender and elongated in females and rounded in males—and confirmed cytologically by absence (females) or presence (males) of meiotic clusters.

The chromosomal banding sequences of all stained larvae were compared with maps of the standard reference sequence for the subgenus *Simulium*. For this comparison, we used the standard maps of Rothfels et al. (1978) for the IS, IL, IIL, and IIIS arms and of Adler et al. (2016) for the IIS and IIIL arms. Newly discovered inversions were numbered in order of discovery, following the last numbered inversion of Rothfels et al. (1978) for IS and IIIL and of Huang et al. (2011) for IIL. Heterobands (hb)—thickened bands (with enhanced DNA content) relative to the corresponding bands in the standard sequence—and thicker blocks of condensed chromatin, or heterochromatin (hc), not ascribable to a visible band were named for the section in which they occurred. Fixed rearrangements are italicized; all other rearrangements are not. Only chromosome

Table 1. Collection sites for larvae of *Simulium bergi* used in chromosomal analyses.

Country	Location	Latitude Longitude	Elevation (m asl)	Collection Date	Females: Males
ARMENIA	Shirak Province, Saragyugh	41°08.51'N, 43°50.05'E	ca. 2150	14 June 2002	5:3
TURKEY	Kars Province, Bogatepe	40°48.37'N, 42°53.37'E	ca. 2200	07 June 2015	13:17

arms with polymorphic rearrangements are shown in our figures (i.e., IS, IIL, and IIIL); other arms are identical to the sequences for the *Simulium venustum* group, including the fixed sequence in IIS, previously presented by Rothfels et al. (1978) and Chubareva and Petrova (2008). Thus, chromosomes IS, IIL, and IIIL of larvae collected in Turkey were photographed under oil immersion on an Olympus BX40 compound microscope. Chromosomal maps were constructed by scanning photographic negatives, with a 9000F Mark II CanoScan, and importing the images into Adobe® PhotoShop® Elements 8. All chromosomal rearrangements then were marked on the photographic maps to indicate their precise locations and breakpoints.

We identified the sex chromosome of *S. bergi*, based on a preponderance of one or more chromosomal rearrangements in one sex. We then followed the banding sequence of the homologue with the sex-linked rearrangement(s) to determine if additional rearrangements were on the same (cis) or a different (trans) homologue. In the heterogametic sex—the male (XY)—determination of linkage was complicated by heterozygosity. Thus, twisting and overlapping homologues sometimes could not be followed adequately to determine if two rearrangements were cis or trans; in these cases, X or Y linkage could not be resolved. Because females were the homogametic sex (XX), we inferred that any rearrangement in the IIL arm of females was X linked.

Stained and unstained portions of two Armenian larvae (and two pupae) and some larvae from Turkey were transferred to 80% ethanol and deposited in the Clemson University Arthropod Collection, South Carolina, USA, along with all photographic negatives of chromosomes. The majority of Armenian and Turkish larvae were placed in the Department of Parasitology, Erciyes University, Turkey, for future molecular analysis.

Results

General features. The banding sequences of all 38 larvae (18 females, 20 males) of *S. bergi* were analyzed completely. The general features of the polytene complement conformed to the photograph by Chubareva and Petrova (2008). All larvae had the typical $n = 3$ haploid complement, with submetacentric chromosomes, and the lengths expressed as I > II ≈ III. Homologues were tightly paired (Figs 1–3). A chromocenter and supernumerary chromosomes were absent. Ectopic pairing of centromeres occurred in 0–20% of the nuclei of each larva. Centromere bands were diffuse and within expanded regions; the CI region (Fig. 1) was the most expanded, followed by the CII and then the CIII regions. The nucleolar organizer was in the standard subgeneric position in the base of IIIL at the junction of sections 87 and 88.

Fixed inversions. The fundamental banding sequence common to all larvae was derived from the standard subgeneric sequence by a single fixed inversion, *IIS-C* (*sensu* Rothfels et al. 1978), which reversed the ring of Balbiani and the "bulges" marker. The

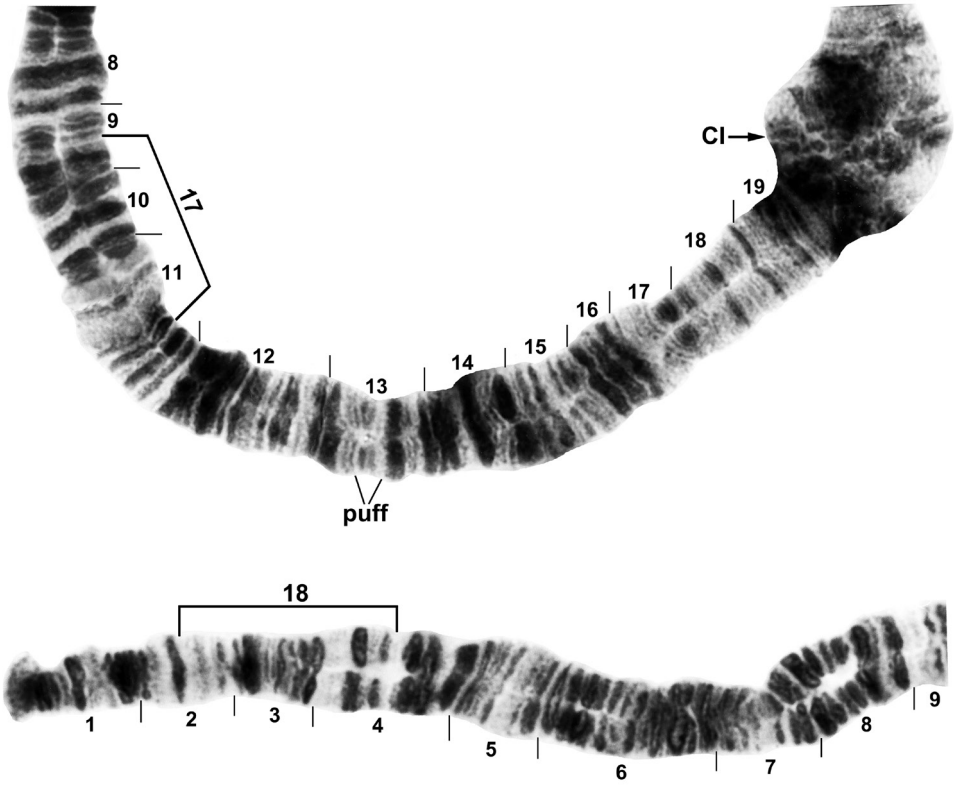

Figure 1. Chromosome arm IS of *Simulium bergi* (male larva), representing the *Simulium* subgeneric standard. Limits of two autosomal inversions and a puff are indicated with brackets on the standard sequence. CI = centromere of chromosome I.

IIS sequence, therefore, was identical to that in figure 11 of Rothfels et al. (1978) and figure 210 of Chubareva and Petrova (2008).

Autosomal polymorphisms. Six autosomal polymorphisms were discovered (Table 2): IS-17, IS-18, IIIL-10, one puffed band in IS (Figs 1, 3), and two heterobands in IL. All six of these rearrangements occurred only once each and only in the heterozygous condition, except IIIL-10 (Fig. 3), which was found in 29% of all larvae, including homozygously in one Armenian larva. IIIL-10, was shared between Armenian and Turkish populations, although its frequency was significantly greater in Armenia ($\chi^2 = 11.9$, df = 1, $p = 0.001$).

Sex chromosomes. IIL was inferred as the sex arm in the Turkish population (Table 3), based on inversion IIL-22 (Figs 2, 4A), which appeared exclusively in the heterozygous condition in 76.5% of the 17 males and in none of the 13 females; no IIL-22 homozygotes were found. We tentatively consider IIL as the sex arm in the Armenian population where 1 of the 3 Armenian males had IIL-22, although a larger

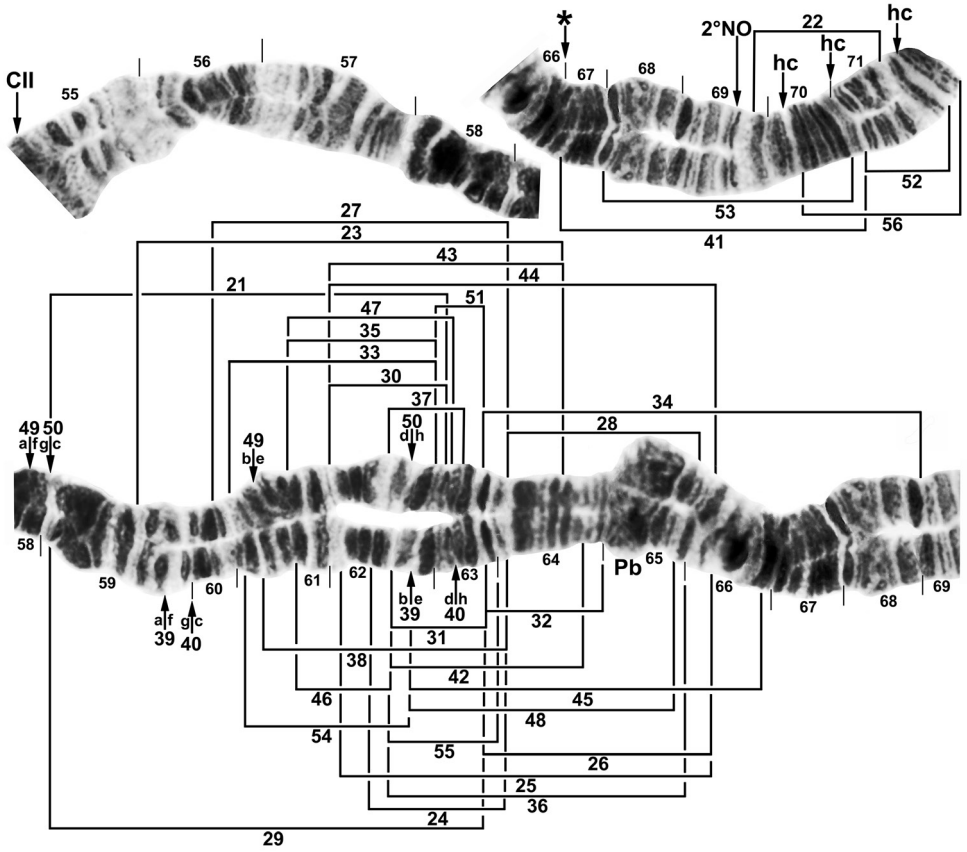

Figure 2. Composite map of chromosome arm IIL of *Simulium bergi* (female larva), representing the *Simulium* subgeneric standard. Breakpoints of sex-linked inversions are indicated with brackets or arrows. Ordering the two independent sets of chromosome fragments indicated by the letters "a" through "h" will produce the inverted sequence for IIL-39,40 and IIL-49,50. CII = centromere of chromosome II, hc = insertion point for heterochromatic block, Pb = parabalbiani, 2°NO = location of secondary nucleolar organizer, * = insertion point for 7 additional bands (only when IIL-41 is present).

Table 2. Frequency of homologues with autosomal rearrangements in two populations of *Simulium bergi*.

	ARMENIA	TURKEY
Larvae (*n*)	8	30
Homologues (n)	16	60
IS-17†	0.00	0.02
IS-18	0.06	0.00
IS-puff(13)	0.06	0.00
IL-hb26	0.06	0.00
IL-hb(telomere)	0.00	0.02
IIIL-10	0.44	0.08

† Left column represents autosomal rearrangements, all of which are indicated on Figs 1, 3; frequencies are based on a maximum of 1.00.

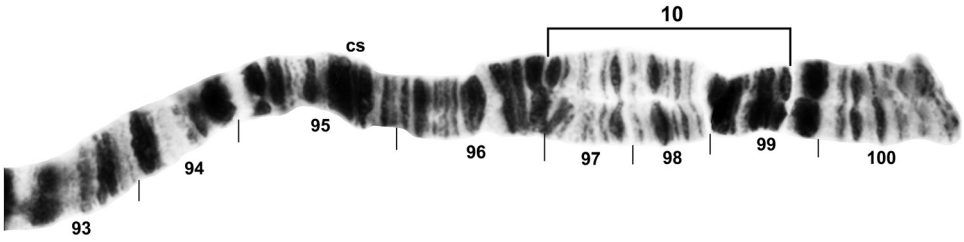

Figure 3. Distal half of chromosome IIIL of *Simulium bergi* (female larva), representing the *Simulium* subgeneric standard. Limits of autosomal inversion IIIL-10 are indicated with a bracket on the standard sequence; cs = cup and saucer marker.

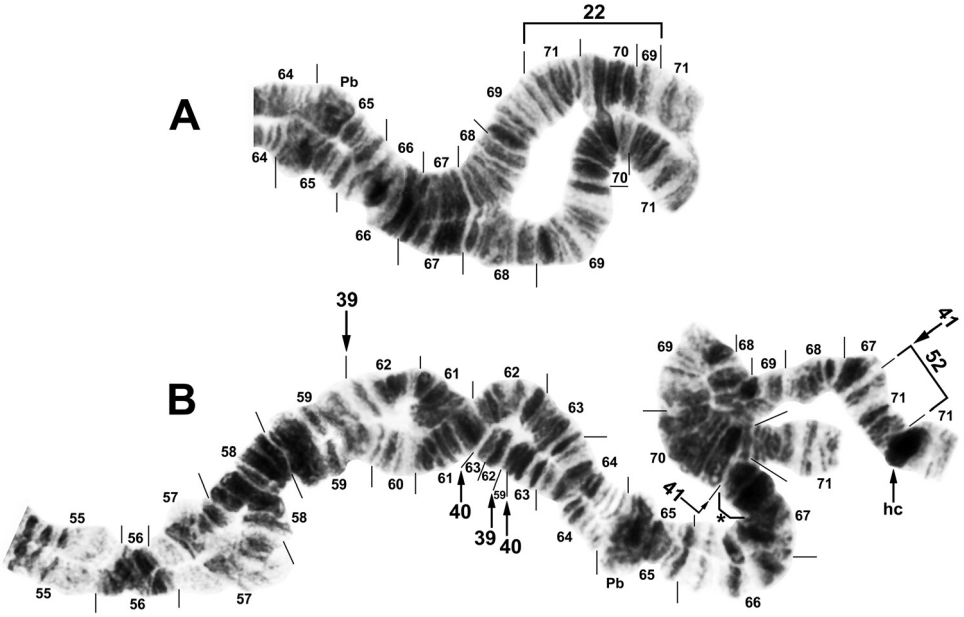

Figure 4. Chromosome arm IIL of male larvae of *Simulium bergi*. **A** Distal half of chromosome, showing heterozygous expression of the common Y-linked inversion IIL-22 **B** Complex sex-chromosome configuration, showing one homologue with IIL-39,40 and the other with IIL-41,52+hc71+7 extra bands; hc = heterochromatic block, Pb = parabalbiani, * = 7 additional bands inserted in one homologue.

sample is needed to test the hypothesis of Y linkage. Our combined sample of 38 larvae included 41 rearrangements in IIL (Fig. 2). All 38 larvae were heterozygous for at least one rearrangement in IIL. By following the homologue with IIL-22, we established that this inversion was on the same homologue as four other inversions (IIL-23, IIL-34, IIL-43, and IIL-56); these inversions, therefore, also were linked to the Y chromosome. More than 20 rearrangements were linked to the X chromosome. An X chromosome (X_0) with no rearrangements occurred in both Armenia and Turkey. Of 41 rearrangements in IIL, 16 (all in males) could not be determined as linked

Table 3. Number of larvae with each sex-linked rearrangement in two populations of *Simulium bergi*.

	Armenia	Turkey	X or Y Linked[†]
Females: Males	5:3	13:17	–
IIL-21[‡]		1	
IIL-22	1	13	Y
IIL-23	1	3	X, Y[§]
IIL-24		2	
IIL-25		2	X
IIL-26		2	X
IIL-27		1	
IIL-28		3	
IIL-29		2	X
IIL-30		4	X
IIL-31		2	
IIL-32		1	
IIL-33		1	X
IIL-34		2	Y
IIL-35		1	X
IIL-36		1	X
IIL-37		1	X
IIL-38		1	
IIL-39		2	
IIL-40		1	
IIL-41		1	
IIL-42		1	X
IIL-43		3	X, Y
IIL-44	3	1	X
IIL-45		1	
IIL-46		1	
IIL-47		1	
IIL-48		1	
IIL-49		1	X
IIL-50		1	X
IIL hc71		1	
IIL extra bands[|]		1	
IIL-51		1	X
IIL-52	1		Y
IIL-53	1		X
IIL-54	1		X
IIL-55	1		X
IIL-56	2		Y
IIL hc70	1		X

	Armenia	Turkey	X or Y Linked[†]
IIL hc70/71	1		X
IIL 2ºNO	1		X

[†] An empty cell indicates that X or Y linkage of the rearrangement could not be determined.
[‡] Left column represents sex-linked chromosomal rearrangements in the IIL arm, all of which are indicated on Figs 2, 4; frequencies are based on a maximum of 1.00.
[§] IIL-23 was in cis conformation with IIL-22 in Armenia, where we tentatively consider IIL-22 to be Y linked, based on only 3 males available for study. IIL-23 was associated with the X chromosome in Turkey.
[|] Seven extra bands appeared in one homologue at the junction of sections 66/70 created by IIL-41; the same homologue had IIL-52 and IIL hc71, and the opposite homologue had IIL-39,40 (Fig. 4B).

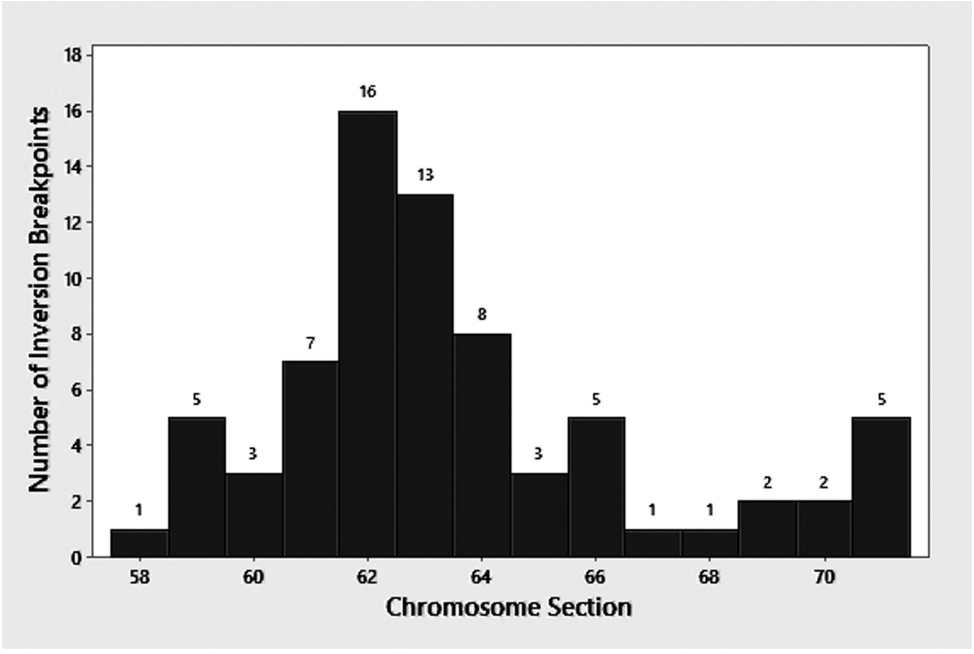

Figure 5. Distribution of breakpoints of 36 sex-linked inversions in the IIL arm of *Simulium bergi*. Breakpoints are plotted according to section number of the standard banding sequence for the subgenus *Simulium*. Breakpoints falling at the junction of two sections are tallied for the distalmost of the two sections.

to either the X or the Y, including one larva with the most complex set of rearrangements (IIL-39,40 on one homologue and IIL-41,52+hc71+7 extra bands on the other homologue; Fig. 4B). Four IIL rearrangements (IIL-22, IIL-23, IIL-30, and IIL-44) were shared between Armenia and Turkey. The concentration of 36 inversions in IIL involved some pairs, such as IIL-35 and IIL-47, that differed by only one or two visible bands. The distribution of breakpoints for the sex-linked inversions followed a bimodal distribution, with a nearly normally distributed central cluster and a smaller subterminal cluster (Fig. 5).

Discussion

Chromosomal insights into taxonomy. We consider our Armenian and Turkish populations of *S. bergi* conspecific, based on shared chromosomal characters, *viz.*, the entire fixed banding sequence, autosomal polymorphism IIIL-10, and four sex-linked inversions. Rearrangements unique to Armenia or Turkey probably reflect, in large part, small sample sizes. We would not expect restricted gene flow, given the flight capabilities of simuliids (Adler et al. 2005) and the short distance (< ca. 100 km), availability of appropriate breeding habitats, and similar elevations between our two sampling sites and between either of our two sampling sites and the type locality in southern Georgia. These three sites also are in the same ecoregion, the Eastern Anatolian Montane Steppe (World Wildlife Fund 2015). We, therefore, suggest that our populations are conspecific with the type specimen. Conspecificity with more distant populations is unknown. The only insight comes from a photograph of the total polytene complement of *S. bergi* collected from the Argichi River about 165 km southeast of our Armenian site (Chubareva and Petrova 2008). It shows the standard subgeneric banding sequence in chromosomes I and III and the *C* sequence in IIS. IIL is standard, although sections 68–69 (= sections 26–27 on the map of Chubareva and Petrova 2008) appear knotted and might have either an extra block of heterochromatin expressed heterozygously or a small inversion that cannot be interpreted.

The presence of *IIS-C* chromosomally confirms the original (Rubtsov 1956) morphological placement of *S. bergi* in the *S. venustum* species group. Of the 18 nominal species in the *S. venustum* group analyzed chromosomally (Adler and Crosskey 2015), *S. bergi* is the least differentiated—only one inversion (*IIS-C*) removed from the subgeneric standard. However, one other analyzed member of the group, *S. paramorsitans* Rubtsov, 1956, also has a fixed banding sequence (Adler et al. 1999) identical to that of *S. bergi*. The two species are, therefore, homosequential (*sensu* Carson et al. 1967); that is, they have the same fixed chromosomal banding sequence but differ morphologically, especially in their larval head patterns, as shown by Rubtsov (1956). Although their fixed sequences are identical, *S. paramorsitans* and *S. bergi* are at opposite extremes in the differentiation of their sex chromosomes: undifferentiated in the former (Adler unpublished) and highly diverse in the latter. Other Palearctic members of the *S. venustum* group, such as *S. longipalpe* Beltyukova, 1955 (formerly *S. curvistylus* Rubtsov, 1957), *S. morsitans* Edwards, 1915, *S. posticatum* Meigen, 1838, and *S. rubtzovi* Smart, 1945 (Adler et al. 1999), show only slight fixed chromosomal differentiation from *Simulium bergi*. The absence of any shared chromosomal rearrangement, other than *IIS-C*, with other members of the *S. venustum* group precludes determination of the species most closely related to *S. bergi*.

Chromosomal fragility in the sex arm. *Simulium bergi* represents the most extreme known case of sex-chromosome differentiation in the Simuliidae, with 41 sex-linked rearrangements discovered on IIL (i.e., the sex arm) among 38 larvae. IIL distal to section 58, thus, is an area of rearrangement hotspots, particularly in the central region of the arm. This area of fragility includes sets of mimic inversions, two or more sequence reversals that resemble one another, differing by as little as one band (Rothfels 1989).

Widespread species can exhibit a significant cumulative degree of sex-chromosome polymorphism over their entire geographical distribution, with inversions often replacing one another across the distribution (e.g., *S. vittatum*, Zetterstedt 1838; Rothfels 1980). Typically, however, the number of sex-linked rearrangements is more limited within a population, often to a single example, linked either to the X or the Y (Adler et al. 2016). Yet, examples of multiple sex-linked rearrangements in a single population are not uncommon. Two cytoforms ('A' and 'D'), probably cryptic species, of *S. colombaschense* (Scopoli, 1780), each exhibits sex-chromosome polymorphism within a population at one river site; 'A' has 4 X-linked and 1 Y-linked inversions at a single site, and 'D' has 4 X-linked and 4 Y-linked inversions (Adler et al. 2016). *Simulium conundrum* Adler, Currie & Wood, 2004 (formerly *S. tuberosum* 'FGH') at a single site in Newfoundland has 4 Y-linked inversions but no X-linked inversions (McCreadie et al. 1995). In contrast, the number of rearrangements, per geographic site, linked to sex in *S. bergi* is extraordinary. A single Y-linked inversion (IIL-22), nonetheless, forms the backbone of the sex-chromosome system, occurring in 76% of Turkish and 33% of Armenian male larvae. The X chromosome is indiscriminate in its sex-linked rearrangements; none of the 20 or more rearrangements is represented in more than 4 (22%) of the 18 total female larvae.

In species with multiple rearrangements linked to sex, the sex-differential region tends to become progressively enlarged (Rothfels 1980). In the *S. ochraceum* species complex from Central America, for example, 2 X chromosomes and 6 Y chromosomes are found in populations of *S. ochraceum* 'A'. Sex linkage in this species involves not only inversions along the entire IIL arm, but also a supernumerary band polymorphism (Hirai et. al. 1994). By contrast, *Cnephia dacotensis* (Dyar & Shannon, 1927) is one of the few species that shows sex-differential regions spanning the centromere region, and is unique in that the sex chromosomes involve only polymorphic bands in chromosome I (Procunier 1975, 1982b). *Simulium bergi* shows a concentration of inversions between sections 60 and 65, with additional, albeit fewer, inversions breaking beyond this segment.

At least two inversions, IIL-23 and IIL-43, were linked predominantly to the X, but in one larva each, they were linked to the Y. Sex exceptions in the Simuliidae are frequent (e.g., Rothfels & Featherston 1981). They have been considered ancestral relicts, the result of crossing over, or products of transposable element excision (Rothfels 1980, Brockhouse 1985, Bedo 1989).

The concentration of inversions in particular areas of the macrogenome, such as the IIL arm of *S. bergi*, emphasizes that inversions are not random. Inversion concentrations have been known and easily visualized for decades in the polytene chromosomes of dipterans (e.g., Novitski 1946, Rothfels and Fairlie 1957) including the Simuliidae (Landau 1962). Yet, a random model was invoked for many years to explain two-break chromosomal rearrangements, such as inversions, at least in mammalian systems (Nadeau and Taylor 1984). Nonrandom models to explain inversion clusters have been proposed, such as the stress-in-pairing model (Rothfels and Fairlie 1957) and fragile breakage model (Bailey et al. 2004), the latter based on mammalian chromosomes that do not offer the

polytene benefit of detailed visualization. Repair of breaks depends on multiple factors, such as chromosome position (Lee et al. 2016). Breakage in chironomid midges has been hypothesized to occur preferentially in areas of the complement containing repetitive blocks of DNA (Bovero et al. 2002). In *Anopheles* mosquitoes, the X chromosome, which has accumulated inversions about three times more rapidly than have the autosomes, is associated with high densities of transposable elements and satellites, suggesting a possible mechanism for the origin of inversions (Sharakhov et al. 2016). Transposable elements have been invoked to explain the presence of multiple sex-determining regions in species of the Simuliidae, although direct evidence is still wanting and alternative models have been considered (Procunier 1982b, Bedo 1984, Brockhouse 1985). In support of a non-random model is the association of high G+C regions with areas of high-frequency breakage (Webber and Ponting 2005). Also relevant to general genome organization is the finding that AT-rich (heterochromatic) polytene bands of the *Simulium vittatum* complex are randomly dispersed throughout the complement (Procunier and Smith 1993).

We draw attention not only to clustered inversions in the distal three-quarters of the IIL sex arm, but also to the clustering of other rearrangements. Five structural phenomena, other than inversions, such as heterochromatic blocks, are found in the sex arm (15% of the polytene complement) compared with three in the autosomal portion (85% of the complement). If the pattern is consistent, it suggests that models accounting for increased inversion frequency also should accommodate the increased frequency of nonbreak-type rearrangements, such as the addition of heterochromatin. The functions of heterochromatin are varied and include suppression of recombination (Grewal and Jia 2007), potentially contributing to the maintenance of blocks of genes related to sex determination.

What is not known is whether a particular area of the complement—the IIL arm of *S. bergi*, for example—is more susceptible to breakage or if the breaks that occur are more likely to persist through subsequent generations, or if both phenomena play a role. Also unknown is whether a visualized break shared by two or more inversions is equivalent at the level of the base pairs. This question can be addressed through molecular characterization of the distal and proximal breakpoint sequences (Sharakhov et al. 2006). *Simulium bergi* offers an exceptional case for exploring the molecular nature of chromosomal breaks and other rearrangements related to the sex chromosomes, while affording a physical map of the phenomenon.

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RESEARCH ARTICLE

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Chromosomal analysis of *Physalaemus kroyeri* and *Physalaemus cicada* (Anura, Leptodactylidae)

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Abstract

All the species of *Physalaemus* Fitzinger, 1826 karyotyped up until now have been classified as 2n = 22. The species of the *P. cuvieri* group analyzed by C-banding present a block of heterochromatin in the interstitial region of the short arm of pair 5. *Physalaemus cicada* Bokermann, 1966 has been considered to be a member of the *P. cuvieri* species group, although its interspecific phylogenetic relationships remain unknown. The PcP190 satellite DNA has been mapped on the chromosomes of most of the species of the *P. cuvieri* group. For two species, *P. cicada* and *P. kroyeri* (Reinhardt & Lütken, 1862), however, only the chromosome number and morphology are known. Given this, the objective of the present study was to analyze the chromosomes of *P. cicada* and *P. kroyeri*, primarily by C-banding and PcP190 mapping. The results indicate that *P. kroyeri* and *P. cicada* have similar karyotypes, which were typical of *Physalaemus*. In both species, the NORs are located on the long arm of pair 8, and the C-banding indicated that, among other features, *P. kroyeri* has the interstitial band on chromosome 5, which is however absent in *P. cicada*. Even so, a number of telomeric bands were observed in *P. cicada*. The mapping of the PcP190 satellite DNA highlighted areas of the centromeric region of the chromosomes of pair 1 in both species, although in *P. kroyeri*, heteromorphism was also observed in pair 3. The cytogenetic evidence does not support the inclusion of *P. cicada* in the *P. cuvieri* group. In the case of *P. kroyeri*, the interstitial band on pair 5 is consistent with the existence of a cytogenetic synapomorphy in the *P. cuvieri* species group.

Keywords
NOR, C Banding, PcP190 satDNA

Introduction

The family Leptodactylidae is made up of three subfamilies, the Leptodactylinae, Paratelmatobiinae and Leiuperinae (Pyron and Wiens 2011, Fouquet et al. 2013, Frost 2016). The Leiuperinae include five genera, *Edalorhina* Jiménez De La Espada, 1870, *Engystomops* Jiménez De La Espada, 1870, *Physalaemus* Fitzinger, 1826, *Pleurodema* Tschudi, 1838 and *Pseudopaludicola* Miranda-Ribeiro, 1926 (Frost 2016), of which *Physalaemus* is the most diverse, with 47 species (Frost 2016). Based on the phenetic analysis of morphological data, Nascimento et al. (2005) recognized seven groups of *Physalaemus* species, the *P. cuvieri*, *P. signifer*, *P. albifrons*, *P. deimaticus*, *P. gracilis*, *P. henselii* and *P. olfersii* groups. However, an alternative approach to the phylogeny of these species, based on the analysis of mitochondrial and nuclear data, produced a new proposal, formed by two major clades, *P. signifer* and *P. cuvieri*. The *P. cuvieri* clade encompasses the *P. cuvieri*, *P. biligonigerus*, *P. henselii*, *P. gracilis* and *P. olfersii* species groups, as well as the species *P. aguirrei* Bokermann, 1966 and *P. cicada* Bokermann, 1966, whose interspecific relationships remain unclear (Lourenço et al. 2015). The *P. cuvieri* group is the largest of the *P. cuvieri* clade, formed by nine described species, *P. cuvieri* Fitzinger, 1826, *P. albonotatus* (Steindachner, 1864), *P. centralis* Bokermann, 1962, *P. cuqui* Lobo, 1993*, P. ephippifer* (Steindachner, 1864)*, P. erikae* Cruz & Pimenta, 2004*, P. fischeri* Boulenger, 1890*, P. kroyeri* (Reinhardt & Lütken, 1862) and *P. albifrons* (Spix, 1824). In the analysis of Nascimento et al. (2005), *P. cicada* was included in the *P. cuvieri* group, although the phylogenetic analyses of Lourenço et al. (2015) did not confirm this asssignment.

All the *Physalaemus* species karyotyped up until the present time show 2n = 22 (Beçak et al. 1970, Denaro 1972, De Lucca et al. 1974, Silva et al. 1999, Silva et al. 2000, Amaral et al. 2000, Lourenço et al. 2006, Ananias et al. 2007, Tomatis et al. 2009, Milani et al. 2010, Nascimento et al. 2010, Provete et al. 2012, Vittorazzi et al. 2014b). The species of the *P. cuvieri* group studied by C-banding (Silva et al. 1999, Quinderé et al. 2009, Nascimento et al. 2010, Vittorazzi et al. 2014b) all present a block of interstitial heterochromatin in the metacentric chromosome 5, which is a potential cytogenetic marker of the *P. cuvieri* group (Vittorazzi et al. 2014b, Lourenço et al. 2015).The chromosomal location of the PcP190 satellite DNA is known for *P. cuvieri*, *P. centralis*, *P. albonotatus*, *P. albifrons* and *P. ephippifer* (Vittorazzi et al. 2011, Vittorazzi et al. 2014a).

For *Physalaemus cicada* and *P. kroyeri*, the available cytogenetic data are restricted to the chromosome number and morphology (De Lucca et al. 1974). Given this, the objective of the present study was to evaluate the chromosomal features of these two species, in particular the presence of an interstitial heterochromatic band on chromosome 5, which is recognized as a chromosomal synapomorphy in the *P. cuvieri* group (Vittorazzi et al. 2014b, Lourenço et al. 2015).

Material and methods

Animals

All the individuals belonging to two species included in our analyses were deposited in the Museum of Zoology "Professor Adão José Cardoso" of the Universidade Estadual de Campinas (ZUEC). The sample of *Physalaemus kroyeri* consisted of 13 individuals (Males: ZUEC 17480-17484, 17486-17490, 17492 and 17493; Juveniles: 17485) from the municipality of Ilhéus, in Bahia, Brazil (14°47'46.65"S/ 39°10'19.94"W). For *P. cicada*, one male specimen (ZUEC 17914) was obtained from Limoeiro, in Pernambuco, Brazil (7°53'31.90"S/35°27'57.41"W) and 13 specimens (Males: ZUEC 20407-2410, 20415, 20419-20422; Females: 20413 and 20417; Juveniles: 20411, 20418) from Poço Redondo in Sergipe, Brazil (9°41'13.14"S/ 37°41'14.95"W).

The animals were collected with permission of the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA/SISBIO – Process number 10678–2, 20336–1 and 33133–1). For the subsequent techniques, all samples were extracted from euthanized specimens using anesthetic application to the skin (5% Lidocaine) to minimize animal suffering, according to recommendations of the Herpetological Animal Care and Use Committee (HACC) of the American Society of Ichthyologists and Herpetologists (available in http://www.asih.org), and approved by SISBIO/Institute Chico Mendes de Conservação da Biodiversidade as a condition for the concession license.

Chromosome preparation and staining

The metaphases were obtained from intestinal cells of the specimens treated with 2% colchicine for at least 4 hours (following Schmid et al. 2010, or adapted from King and Rofe 1976). The chromosomes were stained with Giemsa (10%) and then C-banded (King 1980). The slides were then processed using the Ag-NOR method (Howell and Black 1980) or stained with DAPI (0.5 μg/mL) or mithramycin (0.5 mg/mL). Chromosomal morphometrics were obtained using the MICROMEASURE v3.3 software (Reeves and Tear 2000) and the classification was based on the criteria of Green and Sessions (1991).

Extraction, isolation, cloning and sequencing of the DNA

The genomic DNA of *Physalaemus kroyeri* and *P. cicada* was extracted from samples macerated in TNES buffer (50 mM Tris pH 7.5; 400 mM NaCl; 20 mM EDTA; and 0.5% SDS), following Medeiros et al. (2013). Samples of the genomic DNA of *P. cicada* and *P. kroyeri* were submitted to a PCR using the primers P190F (AGA CTG GCT GGG AAT CCC AG) and P190R (AGC TGC TGC GAT CTG ACA AGG)

(Vittorazzi et al. 2011) for the isolation of the PcP190 satellite DNA. The resulting sequences were purified and ligated to the pGEM-T Easy vector (Promega, Madison, Wisconsin, USA). The recombinant vectors were used to transform *E. coli* bacteria of the JM109 lineage using a TransformAid™ Bacterial Transformation kit (Fermentas, Burlington, Ontario, Canada), following the maker's recommendations. The procedures for the selection of the recombinant clones and the extraction of the plasmidial DNA were those proposed by Sambrook et al. (1989).

To sequence the fragments, samples of the amplified PCR products were treated with a BigDye Terminator kit (Applied Biosystems, Foster City, California, USA). After precipitation and drying, the products of this reaction were resuspended in loading dye (1:5 Blue-Dextran-EDTA/Formamide), denatured for 3 minutes at 94°C and analyzed in an ABI 3730XL automatic sequencer.

All the cloned fragments were sequenced, although for the comparative analyses, only the complete PcP190 sequences were used. It is important to note that the partial units were not noticeably different in their composition from the complete sequences.

Fluorescent *in situ* Hybridization (FISH)

The labeling of the isolated PcP190 satellite DNA probes used in this analysis was based on PCR amplification in the presence of *Digoxigenin*-11-*dUTP* with a DIG Probe Synthesis PCR (Roche, Pensberg, Bavaria, Germany). The probes were mixed with salmon DNA (1 ng/μL of probe) and precipitated with ethanol. All the resulting DNA was dissolved in a hybridization buffer at pH 7 composed of deionized formamide (50%), 2x SSC, phosphate buffer (40 mM), Denhardt's solution, SDS (1%) and dextran sulfate (10%).

The hybridization method used was that described by Viegas-Péquignot (1992), with adaptations for the detection of the *Digoxigenin*-11-*dUTP*, which was based on the anti-digoxigenin antibody conjugated with rhodamine (Roche, Pensberg, Bavaria, Germany).

Results

Physalaemus kroyeri

The diploid number of *Physalaemus kroyeri* is 2n = 22, with metacentric pairs 1, 2, 5, 6, 9, and 11, submetacentric pairs 4, 7, 8 and 10, and pair 3 being subtelocentric (Figure 1a; Table 1). A secondary constriction was observed on the long arm of pair 8 (Figure 1a), coinciding with the NOR. In the specimens ZUEC 17480, ZUEC 17481 and ZUEC 17483, the NOR was heteromorphic in size (Figure 1b).

Areas of heterochromatin were detected in the centromeric regions of all the chromosomes, in the pericentromeric region of the long arm of the chromosomes of pair

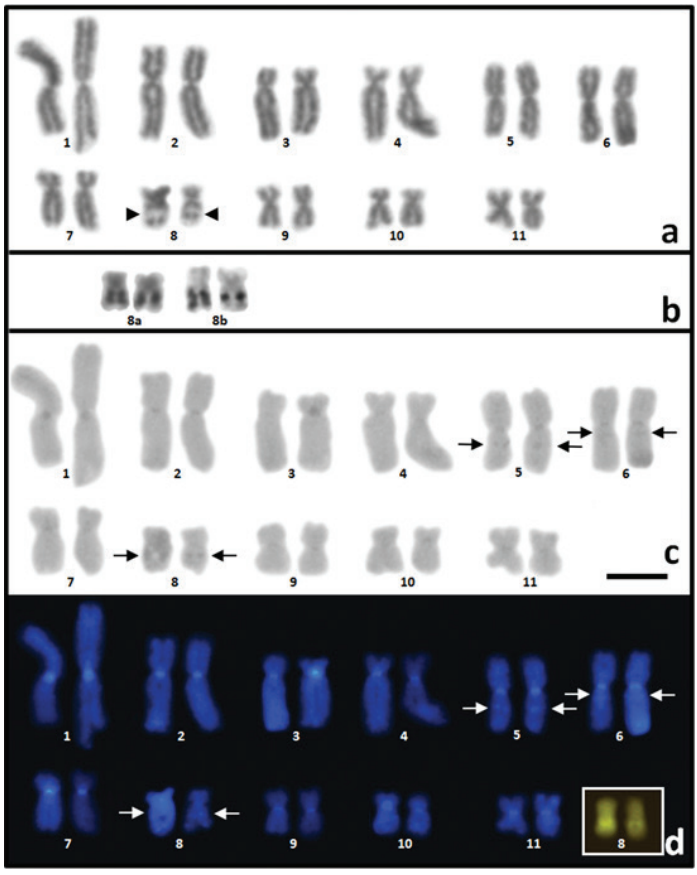

Figure 1. a Karyotype of *Physalaemus kroyeri* stained with Giemsa. The arrowhead indicates the secondary constriction **b** Pair 8 showing NOR detected by the Ag-NOR method, in the homozygote (8a) and heterozygote (8b) forms **c** C-banding stained with Giemsa and **d** C-banding stained with DAPI. Highlighted in (**d**), pair 8 stained with mithramycin. In **c** and **d**, the arrows indicate the interstitial heterochromatic bands. Scale bar: 5 μm.

Table 1. Morphometry of the karyotypes of *Physalaemus kroyeri* and *Physalaemus cicada*. NC: number of the chromosome; CI: centromeric index; AR: arm ratio; CC: chromosomal classification (Green and Session 1991). A total of 10 karyotypes were analyzed in each species.

P. kroyeri											
NC	1	2	3	4	5	6	7	8	9	10	11
CI	0.45	0.39	0.24	0.26	0.47	0.44	0.32	0.32	0.42	0.34	0.42
AR	1.15	1.5	3.11	2.8	1.11	1.22	2.09	2.01	1.33	1.94	1.34
CC	M	M	ST	SM	M	M	SM	SM	M	SM	M
P. cicada											
NC	1	2	3	4	5	6	7	8	9	10	11
CI	0.45	0.39	0.23	0.27	0.44	0.43	0.3	0.29	0.42	0.37	0.4
AR	1.19	1.51	3.13	2.8	1.23	1.27	2.28	2.43	1.37	1.62	1.48
CC	M	M	ST	SM	M	M	SM	SM	M	M	M

6, adjacent to the NOR of the chromosomes of pair 8, and interstitially on one of the arms of the metacentric chromosomes of pair 5 (Figure 1c,d). While pair 5 is metacentric, the interstitial C band is located on the arm that appears to be slightly larger. It was also possible to observe a positive mithramycin band together with the NOR (Figure 1d).

Physalaemus cicada

Physalaemus cicada has a diploid number of 2n = 22, with metacentric pairs 1, 2, 5, 6, 9, 10 and 11, submetacentric pairs 4, 7 and 8, and one subtelocentric pair, pair 3 (Figure 2a; Table 1). A large secondary constriction can be observed on the long arm of pair 8, together with the NOR (Figure 2a, inset).

Regions of constitutive heterochromatin were detected in the centromeres of all the chromosomes, in the proximal region of the long arm of the chromosomes of pair 2, in the pericentromeric region of the long arm of the chromosomes of pair 4, in the telomeric regions of both arms of the chromosomes of pairs 1, 2, 5, 6 and 7, and a similar pattern, but restricted to the long arms of pairs 3, 4, 9, 10 and 11 (Figure 2b,c). A large block of heterochromatin can be observed on the long arm of pair 8 (Figure 2b), coinciding with the NOR which was also strongly stained by mithramycin in C-banded metaphases (Figure 2c – inset).When C-banding was followed by DAPI staining, all the centromeric and telomeric C-bands were revealed as well as a band adjacent to the NOR (Figure 2).

PcP190 satellite DNA

After cloning, sequencing, and the search for similar sequences using the BLASTn tool in GenBank, it was possible to conclude that the sequences obtained with the primers P190F and P190R belong to the PcP190 satellite DNA family, which was first identified in *Physalaemus cuvieri* (Vittorazzi et al. 2011).

It was possible to clone three fragments of the PcP190 satellite DNA of *Physalaemus kroyeri*, all of which contain a complete repeat unit of this satellite DNA, of 190 bps (Figure 3). The mean similarity between these fragments was 95%, and when compared with the PcP190 sequences of *P. cuvieri* (Vittorazzi et al. 2011), the similarity was 93%. Five complete sequences of the PcP190 were obtained from *P. cicada*, of which, one was 189 bps in length, two were 192 bps long, and two were 200 bps. These differences in the size of the *P. cicada* result from a polymorphic region of 20 bps, characterized by substitutions and indels (Figure 3). The mean similarity of the *P. cicada* sequences was 88%, decreasing to 78% in comparison with *P. cuvieri* (Vittorazzi et al. 2011). The sequences obtained for *P. kroyeri* and *P. cicada* were 79% similar, on average.

In the karyotype of *Physalaemus kroyeri*, the PcP190 satellite DNA was detected in the centromeric region of pair 1. In two of the three individuals analyzed, in addition,

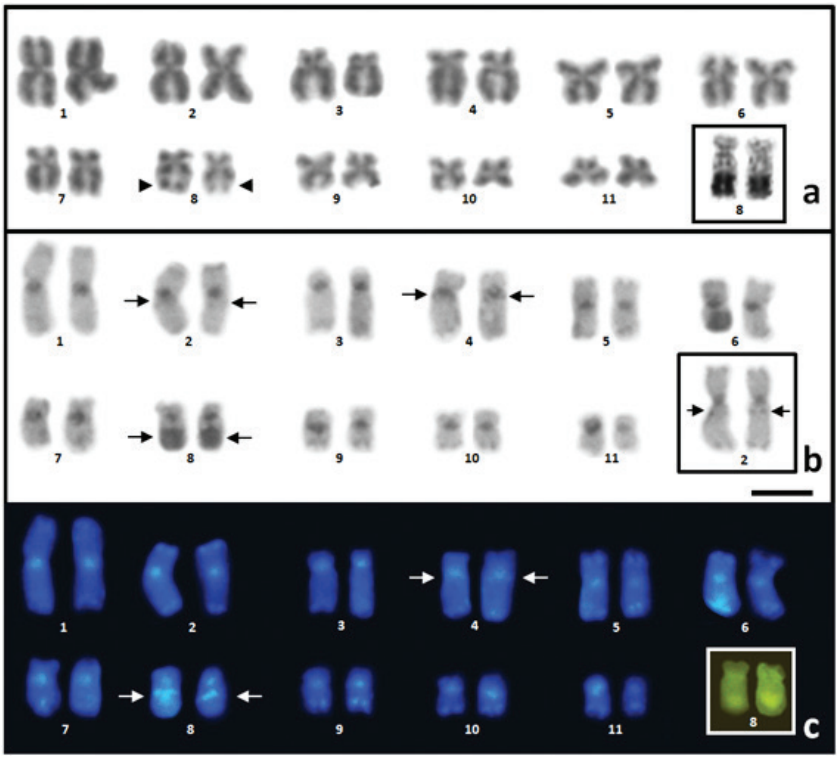

Figure 2. a Karyotype of *Physalaemus cicada* stained with Giemsa. The arrowhead indicates the secondary constriction in pair 8, highlighting the NOR in pair 8 **b** C-banding of the karyotype, highlighting the proximal C band in pair 2 **c** C-banding followed by DAPI staining, highlighting pair 8 stained with mithramycin. In **b** and **c**, the arrows indicate the interstitial and pericentromeric heterochromatic bands. Scale bar: 5 μm.

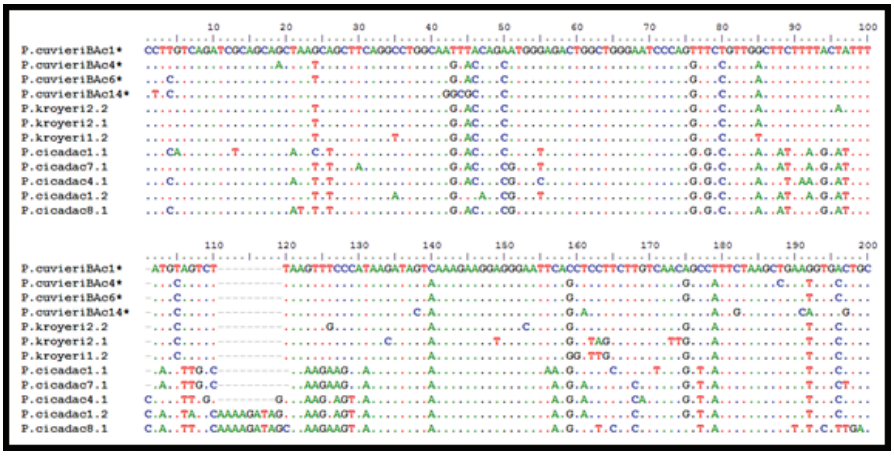

Figure 3. Alignment of the PcP190 satellite DNA sequences of the species *Physalaemus kroyeri*, *Physalaemus cicada* and *P. cuvieri* available in GenBank* (JF281121, JF281117, JF281109 and JF281124).

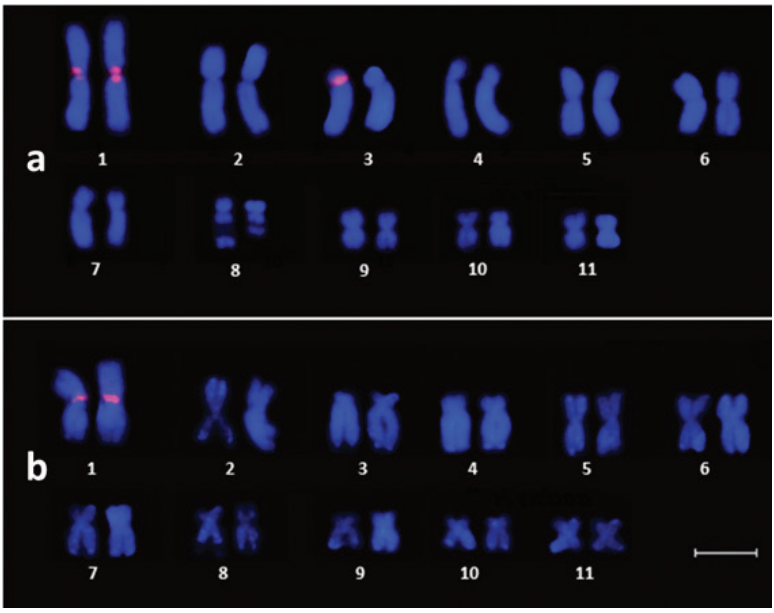

Figure 4. Karyotype of **a** *Physalaemus kroyeri* and **b** *Physalaemus cicada* hybridized with PcP190 satellite DNA probes. Note the signs of hybridization of the probe in the centromeric region of pair 1 in (**a**) and (**b**), and in one of the chromosomes of pair 3 in (**a**). Scale bar: 5 μm.

the PcP190 was also detected in the centromeric region of one of the chromosomes of pair 3 (Figure 4a). In *P. cicada*, the PcP190 was detected in the centromeric region of pair 1, in the individuals from both Limoeiro and Poço Redondo (Figure 4b).

Discussion

The number and morphology of the chromosomes observed in the karyotypes of *Physalaemus kroyeri* and *P. cicada* were the same as those found by De Lucca et al. (1974). The fundamental number (FN) of these karyotypes is 44, which is characteristic of most of the *Physalaemus* species for which cytogenetic data are available, such as *P. cuvieri* (Beçak et al. 1970, Silva et al. 1999, Quinderé et al. 2009), *P. soaresi* Izecksohn, 1965 (De Lucca et al. 1974), *P. marmoratus* (Reinhardt & Lütken, 1862) (Beçak et al. 1970, Amaral et al. 2000), *P. biligonigerus* (Cope, 1861) (Amaral et al. 2000, Silva et al. 2000), *P. henselii* (Peters, 1872), *P. riograndensis* Milstead, 1960 (Tomatis et al. 2009), *P. olfersii* (Lichtenstein & Martens, 1856) (De Lucca et al. 1974; Silva et al. 2000, Milani et al. 2011), *P. ephippifer* (Nascimento et al. 2010), *P. barrioi* Bokermann, 1967 (Provete et al. 2012), *P. albifrons*, *P. centralis* (Denaro 1972, Vittorazzi et al. 2014b), *P. albonotatus*, *P. cuqui*, and *P. santafecinus* Barrio, 1965 (Vittorazzi et al. 2014b). However, the species of the *P. signifer* clade and *P. fernandezae* (Muller, 1926)

(part of the *P. henselii* group of the *P. cuvieri* clade) (see Lourenço et al. 2015) have FN=42, due to the presence of a telocentric pair classified as pair 11.

Comparing the karyotypes of *Physalaemus kroyeri* and *P. cicada* with one another and the karyotypes described for other *Physalaemus* species, it is possible to infer homologies in the first seven pairs of chromosomes. This is because the morphology of pairs 1–7 is highly similar in the karyotypes analyzed, despite some differences in size (e.g., *P. albonotatus* in Vittorazzi et al. 2014b). Even so, it is possible that some of these inferences are erroneous, given that some pairs of chromosomes (pairs 3 and 4, for example, and 5 and 6) are very similar. On the other hand, the recognition of homologies in pairs 8 to 11 is hampered by the fact that these chromosomes are all very small and similar in morphology, except for the telocentric chromosomes classified as pair 11 in the species of the *P. signifer* clade and *P. fernandezae* (see Lourenço et al. 2015 and references therein).

In the karyotype of *Physalaemus kroyeri*, the NOR is located interstitially on the long arm of the chromosomes of pair 8, a situation also observed in *P. albifrons* (Vittorazzi et al. 2014b), which are sister species inferred by Lourenço et al. (2015), and in some populations of *P. cuvieri* (Quinderé et al. 2009). The results of the present study permit the differentiation of the karyotypes of *P. albifrons* and *P. kroyeri* by the presence of interstitial bands of heterochromatin on the long arms of pairs 6 and 8 in *P. kroyeri*, which are absent in *P. albifrons* (Vittorazzi et al. 2014b), and an interstitial band on the short arm of pair 8 in *P. albifrons*, which was absent in *P. kroyeri*. One other difference between the two species can be observed in pair 1, in which PcP190 satellite DNA is present in *P. kroyeri*, but not in *P. albifrons* (Vittorazzi et al. 2014a).

The interstitial C band in the metacentric pair 5 is present in all the species of the *Physalaemus cuvieri* group karyotyped up until now, which Vittorazzi et al. (2014b) proposed as a potential cytogenetic marker for the *P. cuvieri* group, confirmed by the phylogenetic analysis of Lourenço et al. (2015). The results of the present study also indicate that the marker is present in *P. kroyeri*, another species of the *P. cuvieri* group.

While the chromosome pair 5 of *Physalaemus kroyeri* is classified morphologically as metacentric, the arm on which the interstitial band is located is slightly larger, which calls into question the 5p position of this band in the other species of the *P. cuvieri* group. This difference may have resulted from some structural modification of the chromosome, such as a pericentric inversion, amplification of part of this arm, or a deletion on the opposite arm. Whatever the case, the difference in the position of this interstitial band does not alter its status as a chromosomal synapomorphy in the *P. cuvieri* group.

The absence of this interstitial band of heterochromatin on chromosome 5 in the karyotype of *Physalaemus cicada*, keeps the interpecific relationships of this species in doubt. While *P. cicada* has been considered to be a member of the *P. cuvieri* group, based on its morphological similarities (Lynch 1970, Nascimento et al. 2005), Lourenço et al. (2015) found no support for this arrangement in their phylogenetic analyses.

PcP190 satellite DNA

It was possible to recognize PcP190 satellite DNA in both *Physalaemus kroyeri* and *P. cicada*, as found in a number of other *Physalaemus* species, such as *P. cuvieri*, *P. centralis*, *P. albonotatus*, *P. albifrons*, *P. ephippifer*, *P. marmoratus* and *P. nattereri* (Steindachner, 1863), as well as members of other leptodactylid genera, such as *Pleurodema diplolister* (Peters, 1870), *Leptodactylus latrans* (Steffen, 1815) and *Crossodactylus gaudichaudii* Duméril & Bibron, 1841 (Vittorazzi et al. 2014a) and in the hylid genus Pseudis (Gatto et al. 2016). This sequence is well conserved, and appears to have an ancient origin in the anurans (Vittorazzi et al. 2014a, Gatto et al. 2016).

In *Physalaemus cicada*, both the sequences and the location of the PcP190 in the karyotype provide interesting insights into the comparison of this species with those of the *P. cuvieri* group. On average, the PcP190 of the species of this group are 90% similar to one another (Vittorazzi et al. 2014a), although this falls to 78% in the comparison with *P. cicada*. The chromosomal mapping of these sequences in *P. cicada* is also distinct from that of the *P. cuvieri* group, given the lack of a PcP190 site in pair 3, which is characteristic of all the species of the *P. cuvieri* group analyzed to date (Vittorazzi et al. 2011, 2014a). These differences may reflect a more distant phylogenetic relationship between *P. cicada* and the species of the *P. cuvieri* group. However, we must consider that given family of satellite DNA may present a different number of repetitions, even in closely-related species, given that the evolutionary dynamics of these sequences favors their continuous amplification and deletion in the genome. This is covered in the original proposal for a DNA satellite library (Fry and Salser 1977, Meštrović et al. 1998), which indicated that different families of satellite DNA coexist in a genome, but that new families may arise continually through the restructuring of the distribution and quantity of the older sequences.

Conclusion

The interstitial heterochromatic band on the metacentric chromosome 5, considered to be a cytogenetic synapomorphy of the *Physalaemus cuvieri* species group was found in *P. kroyeri*. In contrast, this marker was absent in *P. cicada*, which did not support the inclusion of *P. cicada* in the *P. cuvieri* species group.

Contribution of the authors

SEV developed the study, collected *P. kroyeri* and *P. cicada*, ran the analyses and drafted the manuscript. MS collected *P. kroyeri* and RGF collected *P. cicada*, both these authors revised the manuscript. SMRP and LBL developed and coordinated the study and revised the manuscript.

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