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Screening and chromosome localization of two cotton BAC clones

Xinglei Cui[1], Fang Liu[1], Yuling Liu[1], Zhongli Zhou[1], Chunying Wang[1], Yanyan Zhao[1], Fei Meng[1], Xingxing Wang[1], Xiaoyan Cai[1], Yuhong Wang[1], Renhai Peng[1,2], Kunbo Wang[1]

1 *State Key Laboratory of Cotton Biology (China)/Institute of Cotton Research of Chinese Academy of Agricultural Science, Anyang, Henan, 455000,China* **2** *Anyang Institute of Technology, Anyang, Henan, 455000, China*

Corresponding authors: *Renhai Peng* (aydxprh@163.com); *Kunbo Wang* (wkbcri@163.com)

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Abstract

Two bacterial artificial chromosome (BAC) clones (350B21 and 299N22) of Pima 90-53 cotton [*Gossypium barbadense* Linnaeus, 1753 (2n=4x=52)] were screened from a BAC library using SSR markers. Strong hybridization signals were detected at terminal regions of all A genome (sub-genome) chromosomes, but were almost absent in D genome (sub-genome) chromosomes with BAC clone 350B21 as the probe. The results indicate that specific sequences, which only exist at the terminal parts of A genome (sub-genome) chromosomes with a huge repeat number, may be contained in BAC clone 350B21. When utilizing FISH with the BAC clone 299N22 as probe, a pair of obvious signals was detected on chromosome 13 of D genome (sub-genome), while strong dispersed signals were detected on all A genome (sub-genome) chromosomes. The results showed that peculiar repetitive sequence, which was distributed throughout all A genome (sub-genome) chromosomes, may exist in BAC clone 299N22. The absence of the repetitive sequences, which exist in the two BAC clones, in D genome may account for the genome-size variation between A and D genomes. In addition, the microcolinearity analysis of the clone 299N22 and its homologous region on *G. raimondii* Ulbrich, 1932 chromosome 13 ($D_5$13) indicated that the clone 299N22 might come from A sub-genome of sea island cotton (*G. barbadense*), and a huge number of small deletions, illegitimate recombination, translocation and rearrangements may have occurred during the genus evolution. The two BAC clones studied here can be used as cytological markers but will be also be helpful to research in cotton genome evolution and comparative genomics.

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Keywords
Cotton, BAC, FISH, cytological marker, microcolinearity

Introduction

Cotton (*Gossypium* Linnaeus, 1753) provides an excellent model system for studies on polyploidization, genomic organization, and genome-size variation (Wang et al. 2010). The genus of cotton is known to be cultivated in over 100 countries and has been classified into eight diploid (2n=2x=26) genomic groups: A, B, C, D, E, F, G, K, and one allotetraploid (2n=4x=52) genomic group: AD (Percival et al. 1999). Approximately 5 MYA (million years ago) A and D genome diploids diverged, then later became reunited with allopolyploid formation 1–2 MYA (Cronn et al. 2002; Senchina et al. 2003). The latest research shows that the genome size of an A genome species is larger than that of a D genome species (Wang et al. 2012; Li et al. 2014). Many influential factors, such as polyploidization (Wendel 2000), transposable element amplification (Bennetzen 2002; Kidwell et al. 2002; Piegu et al. 2006), tandem repeat expansion (Ellegren et al. 2002; Morgante et al. 2002), gene duplication (Zhang 2003), organellar transfer to the nucleus (Shahmuradov et al. 2003), and intron size expansion (Deutsch et al. 1999; Vinogradov et al. 1999) are thought to be collectively responsible for the genome-size variation (Grover et al. 2007). Accumulation of different transposable elements classes among different genomes was thought to be the most important reason (Hawkins et al. 2006). The studies on genome-size differences between A and D genomes will help in understanding cotton evolution as well as facilitating genetic improvement of cotton.

The introduction of fluorescence *in situ* hybridization (FISH), involving hybridization of labeled DNA probes to cytological targets, such as metaphase chromosomes, interphase nuclei, and extended DNA fibers, marked the beginning of a new era for studies on chromosome structure and function. Modern methodologies and modifications, such as the development of probes from specificity for highly repeated sequences to single-copy sequence (Desel et al. 2001; Zhu et al. 1999), and from single-colored probes to multiple-colored probes (Tang et al. 2009), have all been designed to optimize the probe detection sensitivity. Nowadays, FISH is a versatile and accurate tool for chromosome localization of sequences (Gomez et al. 1997), cytogenetic map construction (Sun et al. 2013; Han et al. 2011; Cui et al. 2015), genome structure study (Zhao et al. 2011; Wang et al. 2001a), genome evolution (Wu et al. 2013), and comparative genomics study (Gan et al. 2013).

Eukaryotic genomes, with rare exceptions, are replete with interspersed repetitive DNAs, of which most are transposable elements (Feschotte 2008). Large-scale DNA sequencing has revealed that genome size is highly correlated with transposable element content (Oliver et al. 2013). The genomes of *G. arboretum* Linnaeus, 1753 and *G. raimondii* Ulbrich, 1932 have been sequenced and assembled, the comparison

between the two genomes showed the transposable elements, especially LTR, activities substantially contributed to the twofold genome-size variation (Wang et al. 2012; Li et al. 2014). In this study, two BAC clones with genome-specific repetitive sequences (350B21 and 299N22) were localized and microcolinearity of BAC clone 299N22 and its homologous region on chromosome $D_5$13 was analyzed.

Material and methods

Materials

The plant materials were obtained from National Wild Cotton Nursery in Hainan Island, China, sponsored by the Institute of Cotton Research of Chinese Academy of Agricultural Sciences (CRI-CAAS). They are also conserved in the greenhouse at CRI-CAAS' headquarter in Anyang City, Henan Province, China.

Chromosome-specific BAC clones (Wang et al. 2007) used to identify the individual chromosomes were kindly provided by Prof. Tianzhen Zhang (Nanjing Agricultural University, China).

The *G. raimondii* genome sequence was downloaded from the sequenced genome of land plants in Phytozome (http://www.phytozome.net). The *G. arboreum* genome sequence was downloaded from Cotton Genome Project (CGP: http://cgp.genomics.org.cn).

Screening of BAC library

Pima 90–53 (*G. barbadense*) BAC library screened in this paper was kindly provided by Prof. Zhiying Ma (Hebei Agricultural University, China). The simple sequence repeat (SSR) markers were selected from 3 genetic maps (Table 1) (Nguyen et al. 2004; Zhao et al. 2012; Han et al. 2006) and used to screen the BAC library. To facilitate PCR screening, a rapid method of screening BAC libraries was used to obtain positive BAC clones (Cheng et al. 2012). First, one-dimensional pools (plate pools)

Table 1. SSR markers and their genetic maps.

SSR marker	Genetic map of cotton
NAU1215	Han et al. (2006) Theor Appl Genet
CIR342	Han et al. (2006) Theor Appl Genet
NAU1023	Han et al. (2006) Theor Appl Genet
NAU1201	Han et al. (2006) Theor Appl Genet
NAU3022	Zhao et al. (2012) BMC Gnomics
NAU3384	Zhao et al. (2012) BMC Gnomics
NAU5100	Zhao et al. (2012) BMC Gnomics
CIR096	Nguyen et al. (2004) Theor Appl Genet

were made; 384 clones were pooled together on a same plate. Then, bacterial colony PCR was used to screen one-dimensional pools. Secondly, two-dimensional pools (line pools) were made and used to screen, in each of which 24 clones in a same line were pooled together. Thirdly, each clone was screened for the target DNA. Bacterial colony PCR was carried out with 1μL of Bacterial colony template in the presence of 0.5 μL of dNTPs (10mM), 0.5 U Taq DNA polymerase, 1.0 μL 10×Reaction buffer and 0.5 μL of each primer, for a final volume of 10 μL. Following initial denaturation at 95 °C for 3 min, 30 cycles of 94 °C for 45 s, annealing temperature for 45 s and 72 °C for 1 min was performed. PCR products were separated by 0.8% polyacrylamide gel electrophoresis.

DNA probe preparation

The BAC clone DNA was isolated using a standard alkaline extraction (Sambrook et al. 2002). The chromosome-specific BAC clones were labeled with digoxigenin-dUTP via nick translation, whereas the screened BAC clones were labeled with biotin-dUTP via nick translation, according to the instructions of the manufacturer (Roche Diagnostics, USA).

Chromosome preparation and FISH

Mitotic chromosome preparation and FISH procedures were conducted using a modified protocol (Wang et al. 2001b). Biotin-labeled and digoxigenin-labeled probes were detected by avidin-fluorescein (green) and anti-digoxigenin-rhodamine (red) (Roche Diagnostics, USA), respectively. Chromosomes were counterstained by 4’,6-diamidino-2-phenylindole (DAPI) in antifade VECTASHIELD solutions (Vector Laboratories, Burlingame, CA). The concentration of block DNA (genomic DNA) was 200 times that of the chromosome-specific BAC DNA. The hybridization signals were observed using a fluorescence microscope (Leica MRA2) with a charge-coupled device (CCD) camera. Final image adjustments were performed using Adobe Photoshop CS3 software.

BAC clone sequencing and microcolinearity analysis

Both BAC clone 350B21 and 299N22 were outsourced to a biological company for sequencing. The sequences of BAC clones were used as query sequences to search for its homologous regions using BLASTN algorithms against A_2 genome and D_5 genome. Microcolinearity analysis of homologous regions was achieved using software CIRCOS.

Results

Identification and selection of cotton BAC clones

A total of 192 plate pools (73728 BAC clones, nearly covering *G. barbadense* genome 3 times) were constructed and screened using bacterial colony PCR. Nineteen positive BAC clones were identified (Table 2) and selected to be probes for FISH. Seventeen clones, which showed ambiguous FISH signals or no FISH signal on *G. barbadense* mitotic metaphase chromosomes, were discarded. BAC clones 350B21 and 299N22, which showed obvious characteristic signals on *G. barbadense* mitotic metaphase chromosomes, were selected for further study.

Localization of BAC 350B21

Obvious signals were detected on terminal parts of all *G. barbadense* Linnaeus, 1753 (A_2D_2, 2n=4x=52) A sub-genome chromosomes with BAC clone 350B21 as probe. And signals were alike when using four other tetraploid species [*G. hirsutum* Linnaeus, 1753 (A_1D_1, 2n=4x=52), *G. tomentosum* Nuttall ex Seemann, 1865 (A_3D_3, 2n=4x=52), *G. mustelinum* Miers ex Watt, 1907 (A_4D_4, 2n=4x=52), *G. darwinii* Watt, 1907 (A_5D_5, 2n=4x=52)] mitotic metaphase chromosomes as target DNAs. Then, mitotic metaphase chromosomes of two A genome species [*G. arboretum* (A_1, 2n=2x=26), *G. herbaceum* Linnaeus, 1753 (A_2, 2n=2x=26)] were used as target DNAs and obvious signals were detected at terminal parts of all the chromosomes. On the contrast, no obvious signal, except two pair of weak signals, was detected on chromosomes of two D genome cotton species [*G. thurberi* Todaro, 1878 (D_1, 2n=2x=26) and *G. raimondii* (D_5, 2n=2x=26)]. The signals were alike between A genomes and A sub-genomes as well as D genomes and D sub-genomes (Fig. 1).

Table 2. Screened clones of Pima 90-53 BAC library.

SSR markers	Screened clones from BAC library
NAU1215	300N10
CIR342	268E2; 268K2
NAU1023	311A4; 311A11
NAU1201	299N22; 323O3; 317K24; 185N14
NAU3022	30A18; 106P24
NAU3384	328L13
NAU5100	389J15; 376M12; 311M1
CIR096	399A22; 162G3; 350B21;342O11

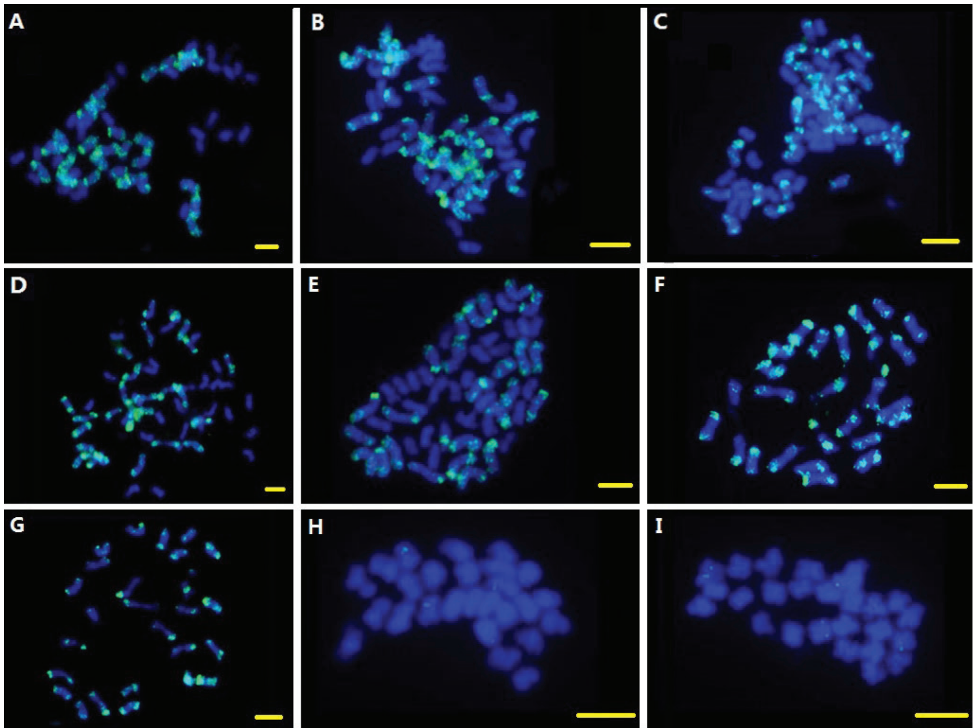

Figure 1. The FISH images of BAC clone 350B21(green) hybridized to mid-mitotic chromosomes in different *Gossypium* species, Bar: 5 µm. **A** *G. hirsutum* (A_1D_1, 2n=4x=52) **B** *G. barbadense* (A_2D_2, 2n=4x=52); **C** *G. tomentosum* (A_3D_3, 2n=4x=52) **D** *G. mustelinum* (A_4D_4, 2n=4x=52) **E** *G. darwinii* (A_5D_5, 2n=4x=52) **F** *G. arboretum* (A_1, 2n=2x=26) **G** *G. herbaceum* (A_2, 2n=2x=26) **H** *G. thurberi* (D_1, 2n=2x=26) **I** *G. raimondii* (D_5, 2n=2x=26).

Localization of BAC 299N22

Obvious disperse signals were detected on all A sub-genome chromosomes of tetraploid species [*G. hirsutum* (A_1D_1, 2n=4x=52), *G. barbadense* (A_2D_2, 2n=4x=52), *G. tomentosum* (A_3D_3, 2n=4x=52), *G. mustelinum* (A_4D_4, 2n=4x=52), *G. darwinii* (A_5D_5, 2n=4x=52)] with BAC clone 299N22 as probe. When mitotic metaphase chromosomes of two A genome species [*G. arboretum* (A_1, 2n=2x=26), *G. herbaceum* (A_2, 2n=2x=26)] were used as target DNAs, obvious signals were detected on all the chromosomes, while only a pair of obvious signals was detected on chromosome 13 of two D genome cotton species [*G. thurberi* (D_1, 2n=2x=26) and *G. raimondii* (D_5, 2n=2x=26)]. The relative position of FISH signals on chromosome $D_5$13 was measured to be about 62.4FL (FL: the percentage of the distance from the FISH site to the end of the short arm relative to the total length of the chromosome) after measuring more than 10 cells with clear chromosome spreads (Fig. 2).

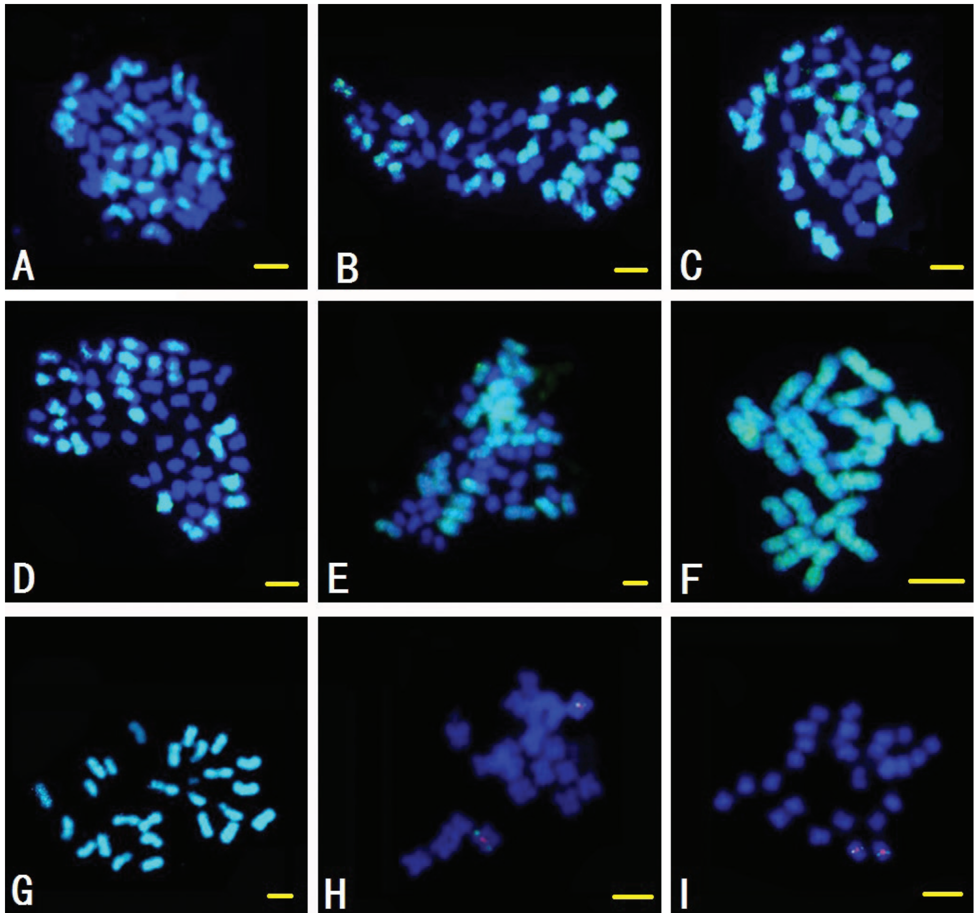

Figure 2. The FISH images of BAC clone 299N22 (green) hybridized to mid-mitotic chromosomes different *Gossypium* species, Bar: 5 µm. **A** *G. hirsutum* (A_1D_1, 2n=4x=52) **B** *G. barbadense* (A_2D_2, 2n=4x=52) **C** *G. tomentosum* (A_3D_3, 2n=4x=52) **D** *G. mustelinum* (A_4D_4, 2n=4x=52) **E** *G. darwinii* (A_5D_5, 2n=4x=52) **F** *G. arboretum* (A_1, 2n=2x=26); **G** *G. herbaceum* (A_2, 2n=2x=26) **H** *G. thurberi* (D_1, 2n=2x=26) **I** *G. raimondii* (D_5, 2n=2x=26). Red: the signal of chromosome-specific BAC clone for chromosome $D_1$13, $D_5$13.

Analysis of BAC clones sequences and microcolinearity

Sequencing of BAC clone 350B21 failed, as too many simple repeat sequences existed in the BAC clone. A new lineage-specific LTR family, which accounted for about 35% of A_2 genome while being absent in D_5 genome, was identified analyzing the sequence of BAC 299N22. The sequence of BAC clone 299N22 was used as query sequence to search for its homologous regions using BLASTN algorithms against A_2 genome (*G. arboretum*) and D_5 (*G. raimondii*) genome, respectively. When A_2 genome was used as a database, multiple dispersedly distributed hits on all chromosomes of A_2 genome were obtained (Fig. 3A), so the homologous region of BAC 299N22 in A_2 genome was

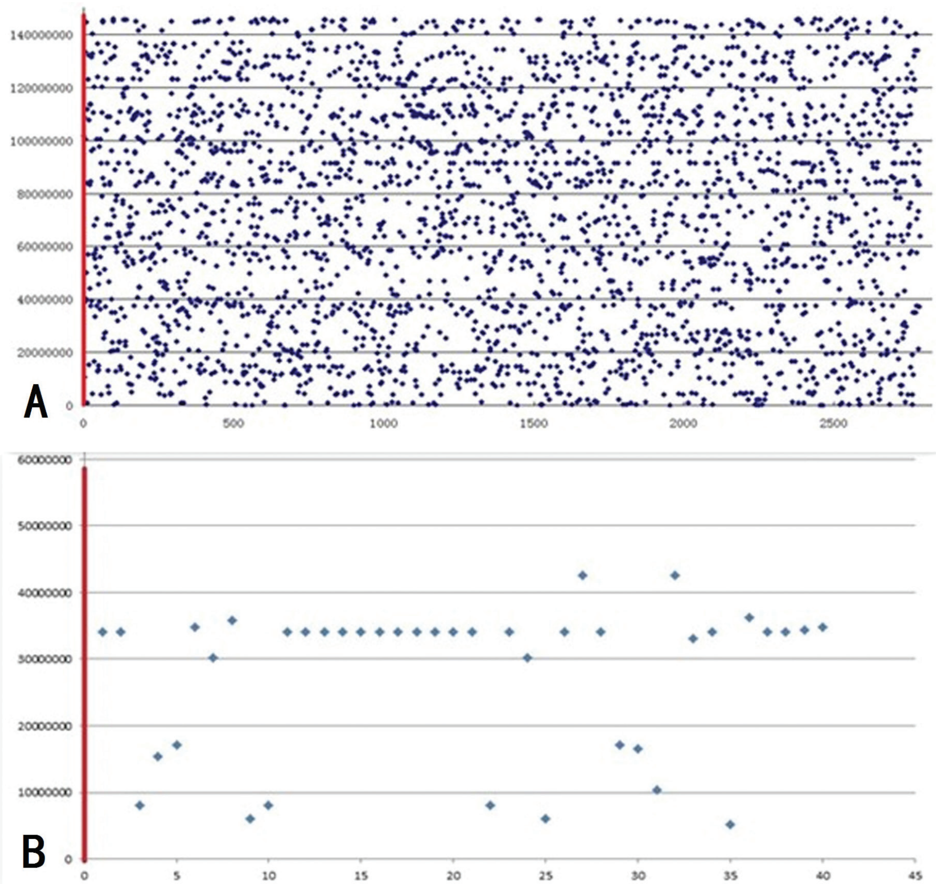

Figure 3. The distribution of BAC 299N22 clone on chromosomes. X-coordinate indicates the length of chromosome, y-coordinate indicates the hits of sequence alignment of BAC clone 299N22 and chromosomes. **A** the result of BLASN, with chromosome $A_2$02 as database **B** the result of BLASTN, with chromosome $D_5$13 as database.

not identified. When D_5 genome was used as a database, similar sequences were only detected in chromosome 13, and the density was obviously higher at the region of 34067000bp—34098000bp (58.41% of chromosome $D_5$13, the position was almost the same as FISH result) than that of other regions of $D_5$13 chromosome. When the E value was set lower, the hits were only found in that region (Fig. 3B). Therefore, the 31kb region on chromosome $D_5$13 was thought to be the homologous region of BAC 299N22. Using the CIRCOS software analysis, the microcolinearity of BAC clone 299N22 and its homologous region on chromosome $D_5$13 proved to be poor. The orders of the highly conserved fragments showed discrepancies, even the orientations of some highly conserved fragments were different. According to cotton SSR primer sequence information on NCBI and *G. raimondii* genome annotation information, 4 SSR markers, NAU1201, NAU1141, HAU3220, and MON_CGR5697, and 2 genes

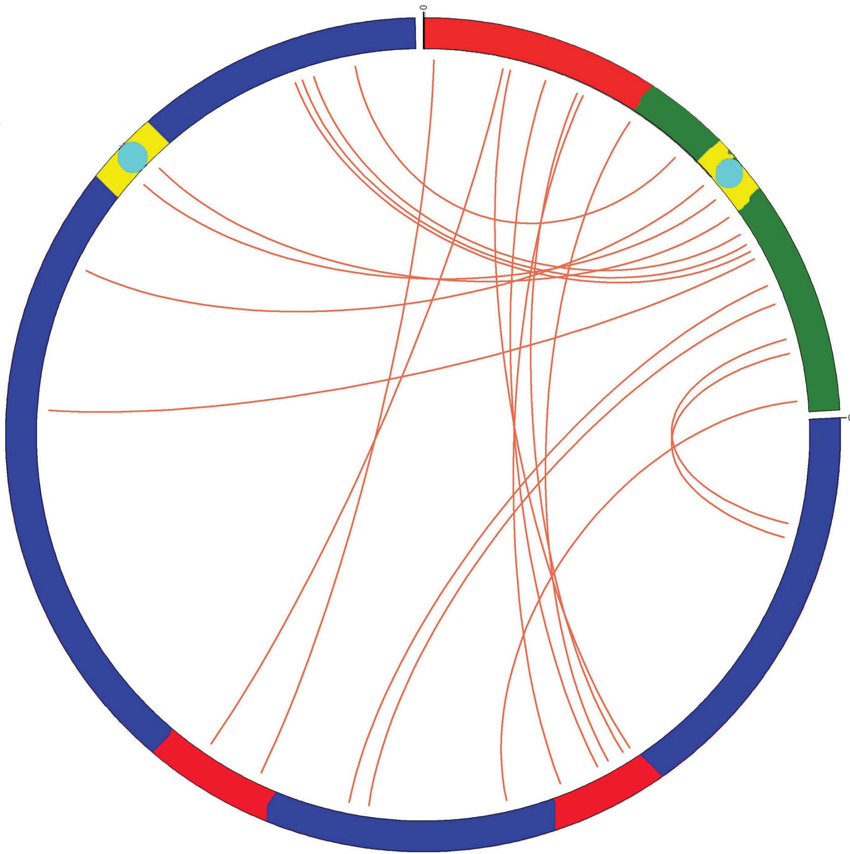

Figure 4. Microcolinearity of the BAC 299N22 and its homologous fragment on chromosome $D_5$13 of *G. raimondii*. Lower-left is BAC 299N22, top-right is its homologous fragment on chromosome $D_5$13 of *G. raimondii*. Red: gene 013G130100, yellow: 013G130200, light blue: 4 SSR markers.

013G130100 and 013G130200, were contained in the pair of homologous regions (Fig. 4). However, the distance between the two genes in BAC clone 299N22 was much longer than that on $D_5$13, and gene 013G130100 in BAC clone 299N22 was divided into two segments. The results also showed special sequences, which do not exist in D genome, repeated a huge number of times in A genome, exist in BAC 299N22.

Discussion

New cytological markers

Chromosome identification is the foundation of research on plant genetics, evolution and genomics. Conventional individual chromosome identification is mainly based on

analyzing chromosomal relative lengths and arm ratios, and, as a result, is very difficult and inaccurate when identifying chromosomes small and similar. Therefore finding suitable molecular cytogenetic markers becomes very necessary for the unambiguous identification of individual chromosomes. FISH is a reliable cytological technique for chromosome identification, and has been adapted successfully to identify the chromosomes for many plant species, including rice (Cheng et al. 2001), potato (Dong et al. 2000), sorghum (Kim et al. 2005) and so on. A set of chromosome-specific BAC clones for *G. hirsutum* chromosomes identification has been developed and was applied successfully in many cotton species (Wang et al. 2007; Wang et al. 2008; Gan et al. 2011; Gan et al. 2012). In this study, BAC clone 299N22 could be a new cytological marker for chromosome 13 of D genome (sub-genome), and its cytogenetic position was measured to be approximately 62.4 FL. As BAC clone 299N22 showed well-distributed repetitive signals on all A genome (sub-genome) chromosomes, it also could be used as a cytological marker for identifying A genome (sub-genome) chromosomes. BAC clone 350B21, which showed repetitive signals at the terminal regions of all A genome (sub-genome) chromosomes could be used as a cytological marker for identifying or labeling terminal regions of all A genome (sub-genome) chromosomes. The addition of these new cytological markers will facilitate the study of cotton genomics and evolution.

Cotton A genome (sub-genome) has unique repetitive sequences

Repetitive DNA sequences form a large portion of the genomes of eukaryotes, indicating a major contributor to variation in genome size among organisms of similar complexity (Charlesworth et al. 1994). The genus *Gossypium*, which provides a facile system for investigating the genomic organization and evolution, also has a high content of repetitive sequences in its genome. Different types of repeat sequences accounted for as much as 68.5% of the *G. arboreum* genome and approximately 57% of the *G. raimondii* genome, respectively. And most of the repetitive sequences are long terminal repeat (LTR) retrotransposons (Wang et al. 2012; Li et al. 2014).

When using BAC clone 350B21 as a probe, strong signals were detected at the terminal parts of all chromosomes of A genome (sub-genome), while being absent on D genome (sub-genome) chromosomes. The results may indicate that special repetitive sequences in BAC clone 350B21 have a bias of insertion sites at terminal parts of A genome (sub-genome) chromosomes. Another kind of repetitive sequence exists in BAC clone 299N22 showed well-distributed dispersed signals on all A genome (sub-genome) chromosomes. These unique repetitive sequences may be the major reason for the genome-size difference between A genome and D genome.

A new LTR family, which accounts for about 35% of A_2 genome while almost being absent in D_5 genome, was identified analyzing the sequence of BAC clone 299N22. The LTR family was inserted randomly along each chromosome in *G. arboretum* genome, and was different from any reported repetitive sequences in cotton

(Hawkins et al. 2006). As the LTR family accounts for so much of A genome, it should be different from any sequence reported by Zhao et al (1995) and Hanson et al (1998). The identification of the new LTR family will facilitate understanding of the differences between the two genomes. Sequencing of BAC clone 350B21 failed as too many simple repeat sequences existed in the BAC clone. This indicates that the terminal regions of A genome (sub-genome) chromosomes may be replete with simple repeat sequences. Their absence in D genome (sub-genome) indicates that they may appear after the divergence of A, D genomes and contributed to the genome-size difference between the two genomes. The similarity of signals in D genome and D sub-genome suggests that the repetitive sequences in the two BAC clones may not occur colonization after polyploidization event, this indicate they may turned to be silent before the polyploidization event.

Many factors contributed to genome-size evolution.

Many factors are thought to be responsible for the genome-size variation. The analysis of *AdhA* and *CesA* regions of different cotton genomes indicated that many forces operated collectively among genomic regions to reflect genome-size evolution (Grover et al. 2007). The microcolinearity analysis, comparative analysis of homologous sequences from different genomes, is a method of comparative genomics research for studying and speculating upon the relationships between genomes and evolution patterns. In the present study, the homologous region of BAC clone 299N22 on chromosome $D_5$13 was obtained using bioinformatics analysis. As the sequence of BAC clone 299N22 is much longer than its homologous region on chromosome $D_5$13, BAC clone 299N22 was thought to be from A sub-genome of *G. barbadense*. Microcolinearity analysis of the homologous regions showed that the orders of the most highly conserved fragments were different, even the orientations of some highly conserved fragments was different, which may indicate that a large number of translocations, inversions, and segmental rearrangements occurred during evolution. The analysis showed the length of gene parts appeared similar between the homologous regions, while gene-free regions were not. This may provide a hint that the evolution between gene islands or in gene-free regions may be the main reason for the genome-size variations, as previously reported (Grover 2004). The repetitive sequences which were distributed dispersedly on A genome chromosomes were located at the non-genetic regions, and this may indicate that the difference in non-genetic regions may be attributed to the accumulation of repetitive sequences.

Conclusions

In recent years, many achievements, such as in the study of cytogenetic map construction, genome evolution, and comparative genomics, have been obtained by using

BAC-FISH. The repetitive sequences in the two BAC clones showed distribution bias and may be an important reason for the genome-size variation. Analysis of the repetitive sequences will be helpful in the studies on cotton genome evolution and comparative genomics.

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Karyotype and genome size in *Euterpe* Mart. (Arecaceae) species

Ludmila Cristina Oliveira[1], Maria do Socorro Padilha de Oliveira[2], Lisete Chamma Davide[2], Giovana Augusta Torres[1]

1 *Universidade Federal de Lavras, Campus Universitário, Caixa Postal 3037, CEP 37200-000, Lavras-MG, Brasil* **2** *Embrapa Amazônia Oriental, Trav. Dr. Enéas Pinheiro, s/n°, Bairro Marco, CEP 66095-100, Caixa Postal 48, Belém-PA, Brasil*

Corresponding author: *Giovana Augusta Torres* (gatorres@dbi.ufla.br)

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Abstract

Euterpe (Martius, 1823), a genus from Central and South America, has species with high economic importance in Brazil, because of their palm heart and fruits, known as açaí berries. Breeding programs have been conducted to increase yield and establish cultivation systems to replace the extraction of wild material. These programs need basic information about the genome of these species to better explore the available genetic variability. The aim of this study was to compare *E. edulis* (Martius, 1824), *E. oleracea* (Martius, 1824) and *E. precatoria* (Martius, 1842), with regard to karyotype, type of interphase nucleus and nuclear DNA amount. Metaphase chromosomes and interphase nuclei from root tip meristematic cells were obtained by the squashing technique and solid stained for microscope analysis. The DNA amount was estimated by flow cytometry. There were previous reports on the chromosome number of *E. edulis* and *E. oleracea,* but chromosome morphology of these two species and the whole karyotype of *E. precatoria* are reported for the first time. The species have 2n=36, a number considered as a pleisomorphic feature in Arecoideae since the modern species, according to floral morphology, have the lowest chromosome number (2n=28 and 2n=30). The three *Euterpe* species also have the same type of interphase nuclei, classified as semi-reticulate. The species differed on karyotypic formulas, on localization of secondary constriction and genome size. The data suggest that the main forces driving *Euterpe* karyotype evolution were structural rearrangements, such as inversions and translocations that alter chromosome morphology, and either deletion or amplification that led to changes in chromosome size.

Keywords

C-value, interphase nucleus, chromosomal evolution, flow cytometry, Açaí palm

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Introduction

Euterpe (Martius, 1823) (Arecaceae-Arecoideae), is composed of seven species distributed from Central to South America (Henderson 1995). In Brazil, *Euterpe edulis* (Martius, 1824), *Euterpe oleracea* (Martius, 1824) and *Euterpe precatoria* (Martius, 1842) are considered the most important species of the genus due to their wide distribution and economic importance of their fruits and palm hearts, obtained mainly by extractive activity in Brazil (Castro 1992). The high commercial value of their products, especially of the açaí palm (*E. oleracea*), has encouraged the development of genetic improvement programs to produce cultivars with higher yield and better quality of fruits and palm heart. In addition to the economic value, cultivation instead of extraction of wild material should favor the conservation of those species, which is urgent in the case of *E. edulis* since it is a threatened species.

Cytogenetic data are critical for germplasm manipulation for such programs, especially when the use of interspecific hybrids is considered as a strategy to increase the variability and to incorporate alleles of interest (Bovi 1987). However, only the chromosome number of *E. oleracea* and *E. edulis* (2n=36) was reported in Môro et al. (1999), and there is no information on chromosome morphology. There are also no data regarding the interphase nucleus for the genus *Euterpe*. Röser (1994) studied 56 taxa belonging to six subfamilies of Arecaceae and found highly differentiated interphase nuclei, ranging from reticulate and semi-reticulated to an intermediate stage between semi-reticulate and areticulate.

Determination of genome size in plants has been recognized as a significant parameter for genomic characterization and may assist in evolutionary studies (Knight and Beaulieu 2008), genetic improvement (Doležel 1997), systematics and molecular and cellular biology (Bennet and Leitch 1995). Röser et al. (1997) used Feulgen densitometry to assess nuclear DNA amount in 83 species of palm trees, belonging to 53 genera. They observed a C-value range between 0.97 and 13.91 pg, a variation of approximately 14.3 times in genome size. *Euterpe precatoria,* was the single species analyzed, showing 5.31 pg (1C).

Therefore, the aims of this study were to compare karyotype, interphase nucleus pattern and genome size of *E. edulis*, *E. oleracea* and *E. precatoria* and discuss the karyotypic evolution within the genus.

Material and methods

Genetic material

The Açaí Palm Germplasm Bank (Banco de Germoplasma de Açaizeiro - BAG-Açaí), from Embrapa Amazônia Oriental in Belém-PA, Brazil, provided seeds from five specimens of *E. oleracea* and *E. precatoria*. The company Infrater Engenharia LTDA, headquartered in Ipatinga-MG, donated seeds from five specimens of *E. edulis*.

Karyotype analysis

Roots originating from germinated seeds were pre-treated with 2 mM 8-hydroxyquinoline for 7 h at 4 °C. Slides were prepared by the squashing technique following cell wall digestion with pectinase/cellulase (100/200U) solution at 37 °C for 1.5 h. Staining was performed with 1% propionic carmine for the analysis of the mitotic metaphases and 5% Giemsa for the evaluation of the interphase nuclei. The images were acquired in a bright-field microscope (Leica DMLS) equipped with a digital camera (Nikon Digital Sight DS-Fi1).

The short and long arms (SA and LA, respectively) of chromosomes were measured using the IMAGE TOOL 3.00 program from UTHSCA (University of Texas Health Science Center in San Antonio). The mean lengths of SA and LA of each chromosome were obtained from measurement of five different metaphases from each species and were used to prepare the ideograms. The chromosome total length (TL = SA + LA), the haploid complement total length (HCTL = $\sum$Lti), the centromeric index (CI=[SA/(SA+LA)]×100) were calculated. The chromosomes were classified based on their centromere position according to Guerra (1986). The karyotype asymmetry was calculated according to Romero Zarco (1986).

Flow cytometry

The nuclear DNA amount was estimated by flow cytometry using leaf tissue from three specimens per species. Each sample contained 20-30 mg of young leaves of the target species mixed with young leaves of *Vicia faba* L. cv. Inovec the internal reference standard with 1C=13.33 pg (Johnston et al. 1999). The samples were ground on a Petri dish with 1 mL of ice-cold Marie buffer (Doležel 1997). The final nuclear suspension was mixed with 25 µL of propidium iodide (1 mg/mL). At least 10.000 nuclei per sample were analyzed in a FacsCalibur cytometer (Becton Dickinson). Histograms were acquired using CELL QUEST PROGRAM (Becton, Dickinson and Company, San Jose, CA, USA) and analyzed using the WINMDI 2.8 software (2009).

Statistical analysis

The HCTL and nuclear DNA amount data were submitted to analysis of variance and the means compared by the Tukey's test at 5% probability using the SISVAR statistical program.

Results

E. edulis, *E. oleracea* and *E. precatoria* have the same chromosome number (2n=36), similar chromosome sizes and differ regarding chromosome morphology (Fig. 1a–f). Chromosome total length decreases gradually (Fig. 1b, d, f), ranging from 4.1 to 1.29

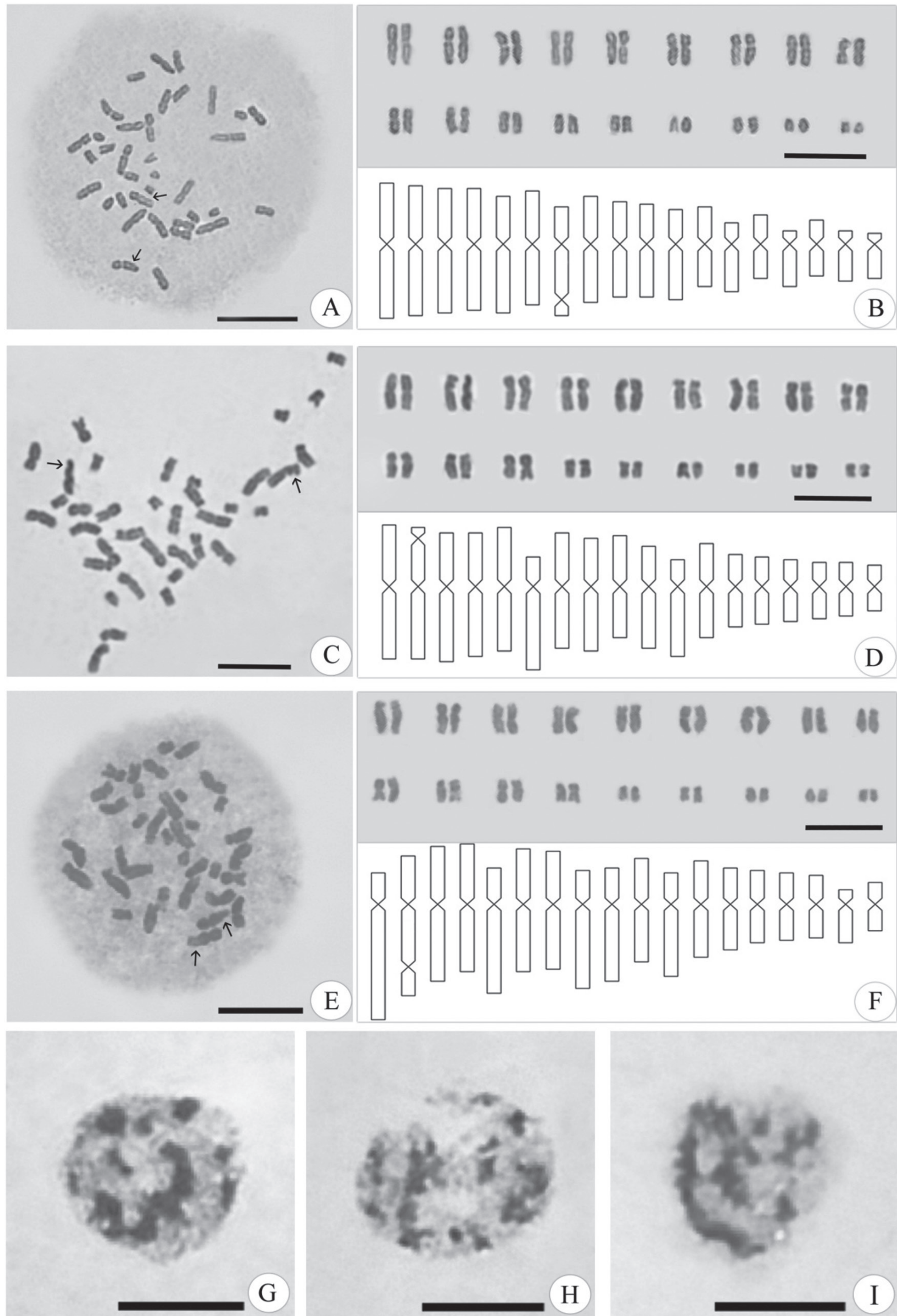

Figure 1. Mitotic metaphases, karyograms and idiogram of *Euterpe* species with 2n=36 chromosomes. *Euterpe edulis* (**A–B**), *E. oleracea* (**C-D**) and *E. precatoria* (**E–F**). Arrows indicate secondary constrictions. Semi-reticulate interphase nuclei of *E. edulis* (**G**), *E. oleracea* (**H**) and *E. precatoria* (**I**). Bar: 10 µm.

Table 1. C-value, haploid complement total length (HCTL), karyotype formula and asymmetry indexes (Romero Zarco 1986) of *Euterpe* species.

Species	C-value (pg)	HCTL (μm)	Karyotype formula	A1	A2
E. edulis	4.09 a	49.60a	12M + 3SM + 3A	0.327	0.329
E. oleracea	4.22 a	51.30a	14M + 4SM	0.259	0.327
E. precatoria	4.71 b	59.39b	11M + 6SM + 1A	0.346	0.315

Means followed by the same letter do not differ statistically by the Tukey's test at 5% probability.

in *E. edulis*; 4.08 to 1.39 in *E. oleracea* and 4.7 to 1.5 in *E. precatoria*. Variation in chromosome size within the karyotype is very similar among the species as pointed by A2 index (Table 1). The species differ mainly in chromosome morphology and genome size. As indicated by the karyotype formula and A1 index (Table 1), *E. oleracea* karyotype is the most divergent one, being more symmetric than the two others.

The chromosome pairs from 1 to 12 of *E. edulis* and *E. oleracea* are quite similar morphologically, and eight have the same classification, seven metacentric and one submetacentric. The same pairs are quite different in *E. precatoria*, which has the highest number of submetacentric chromosomes and one acrocentric pair, the largest and only pair of chromosomes with that morphology (Fig. 1b,d,f).

The chromosome pairs from 13 to 18, except 17, are all metacentric in *E. oleracea* and *E. precatoria*. The same pairs are different in *E. edulis*, with two pairs of acrocentric chromosomes (15 and 18) and one submetacentric chromosome (13). Chromosome pair 17 is the only one in the complements that differs regarding centromere position in all three species; it is metacentric in *E. oleracea*, submetacentric in *E. precatoria* and acrocentric in *E. edulis* (Fig. 1b, d, f).

One pair of chromosomes bears one secondary constriction in all three species. It is located on the long arm of pair seven (submetacentric) in *E. edulis*, in the short arm of pair two (metacentric) in *E. oleracea* and on the long arm of pair two (submetacentric) in *E. precatoria* (Fig. 1b, d, f).

E. precatoria showed HCTL and DNA content significantly higher than that of *E. edulis* and *E. oleracea* (Table 1). The mean coefficient of variation (CV) of flow cytometry data was 0.52%, which demonstrates the reliability of DNA amount estimation, since only CVs up to 2% indicate high quality analysis (Marie and Brow 1993). The genome size is estimated in 4Gb, 4.13Gb and 4.61Gb for *E. edulis*, *E. oleracea* and *E. precatoria*, using the coversion rate of 1pg = 978Mb.

Interphase nuclei were quite similar, classified as semi-reticulate due the formation of strongly pigmented chromatin structures with irregular contours (Fig. 1g, h, i).

Discussion

The chromosome number of *E. edulis* and *E. oleracea*, 2n=36, was also reported by Môro et al. (1999), while for *E. precatoria*, also 2n=36, this is the first report. Considering that

E. microcarpa also has 2n=36 (Röser 1994), *Euterpe* shows high stability in chromosome number. In Arecoideae, 2n=36 is the highest number found, but also the most rare, being characteristic of New world species. It is considered a pleisomorphic karyological feature, since the modern species, considering floral morphology, have the lowest chromosome number (2n=30 and 2n=28). The hypothesis is that starting from 2n=36 (basic number x=18) different and independent reduced dysploid series diverged not only in Arecoideae (2n=28 to 2n=36), but also in Coryphoideae (2n=28 to 2n=36) and Calamoideae (2n=26 to 2n=36) (Röser 1994).

The analyzed karyotypes showed differences in centromere and secondary constriction position. The chromosomes may differ in terms of centromere position, according to Stebbins (1971), through pericentric inversions or uneven translocations, rearrangements that substantially contribute to the increase of karyotype asymmetry. Our results for karyotype asymmetry measure (Tab 1) revealed that the karyotypes are quite similar for chromosome size and differ for chromosome morphology. Along with stability in chromosome number, 2n=36 for all *Euterpe* species studied, these data indicate that the rearrangements may be responsible for karyotype variation among the three *Euterpe* species studied.

Most karyotype studies on palm trees do not include data on the number and location of secondary constrictions. The study performed by Röser (1993) describes the karyotypes of 13 species belonging to 13 different genera of the Coryphoideae subfamily, describing the presence of secondary constrictions in 10 of them. The author found a single pair of chromosomes bearing secondary constriction in eight species: *Livistona chinensis* Brown, 1810, *Pritchardia thurstonii* Mueller & Drude, 1887, *Brahea edulis* Wendland ex Watson, 1876, *Copernicia macroglossa* Wendland, 1907, *Washingtonia robusta* Wendland, 1883, *Sabal minor* (Jacquin, 1805), *Bismarckia nobilis* Hildebrandt & Wendland, 1881 and *Phoenix canariensis* Chabaud, 1882. The other studied species showed two pairs or no pairs of chromosomes with secondary constriction.

The evolutionary direction of karyological changes was shown to be from reticulate to areticulate interphase nuclei when comparing the systematic classification of some Arecaceae subfamilies, mainly based on plant morphological characteristics, with the characterization based on the interphase nuclei and karyotypes (Röser 1994). Therefore, it is possible to infer that, regarding the organization of the interphase nucleus, the three *Euterpe* species have an intermediate level of evolution within the family.

Nuclear DNA quantification, when combined with interphase nucleus characterization and karyological data, may enable differentiation because it allows for the detection of small differences in the DNA amount between species. Those differences make it possible to infer chromosome rearrangements that may be too small to affect the physical structure of the chromosomes. Furthermore, according to Schifino-Wittmann (2001), data on the nuclear DNA amounts of species assist in the management of large germplasm collections and the control of ploidy levels in progenies generated by crosses.

Röser, Johnson and Hanson (1997) reported, through Feulgen densitometry, 5.31 pg of nuclear DNA (1C) in *E. precatoria*, 0.6 pg higher than the value reported in this study. According to Schifino-Wittmann (2001), both the nuclear genome plasticity

and certain aspects of the methodologies applied must be considered when the DNA amounts assessed by different authors are divergent. Regarding the methodology, the flow cytometry estimates using propidium iodide (PI) has shown to be highly correlated with Feulgen densitometry ones. However, despite of being a well stablished method for DNA quantification, Feulgen densitometry has some critical points in the procedure that can affect its precision (Doležel et al. 1998), which can explain the difference between our estimate and the one in the literature.

The comparison among the three species with respect to the nuclear DNA amount and total length of the haploid complement showed that *E. precatoria* has a larger genome than *E. edulis* and *E. oleracea*. Considering that they showed similar inner variation in chromosome size, the difference in DNA amount can be better explained by increase or decrease of size, by amplification or deletion, respectively, involving most of chromosomes.

Differences in genome size and chromosome morphology among the three *Euterpe* species revealed that structural rearrangements were the main force driving karyotype evolution in the genus. Higher resolution techniques, like chromosome banding and molecular hybridization (FISH) should be used to unravel the mechanisms involved.

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RESEARCH ARTICLE

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A chromosomal analysis of three species of *Timarcha* (Coleoptera, Chrysomelidae, Chrysomelinae)

Eduard Petitpierre[1]

1 *Dept. Biologia, Universitat de les Illes Balears, 07122 Palma de Mallorca, Spain*

Corresponding author: *Eduard Petitpierre* (eduard.petitpierre@uib.es)

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Abstract

The karyotypes of three species of *Timarcha* Latreille, 1829 have been analysed. *T.* (*Metallotimarcha*) *metallica* (Laicharting, 1781), has 18 + Xy_p male meioformula and 2n = 38 chromosomes, similar to those found in the two species of subgenus *Americanotimarcha* Jolivet, 1948, in agreement with morphological and molecular phylogenetic grounds. *T.* (*Timarcha*) *carmelenae* Petitpierre, 2013 displays 9 + Xy_p and 2n = 20 chromosomes as in morphologically related Andalusian species, whereas *T.* (*Timarcha*) *parvicollis* ssp. *seidlitzi* Kraatz, 1879 shows 11 + Xyp and 2n = 24 chromosomes, clearly differing from the previous species. These results are discussed in order to get an insight into the main trends of the chromosomal evolution in *Timarcha*.

Keywords

Coleoptera, Chrysomelidae, Chrysomelinae, karyotypes, *Timarcha*, evolution

Introduction

The highly speciose genus *Timarcha* Latreille, 1829 comprises more than three hundred described taxa, almost all from the Palaearctic (Gómez-Zurita 2008, Kippenberg 2010, Warchalowski 2010), and is relatively well-known from chromosomal standpoints because 42 taxa have been surveyed to date and their range of diploid numbers goes from 2n = 18 to 2n = 44 (Gómez-Zurita et al. 2004, Petitpierre 2011).

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Herein, we report the chromosome numbers, male sex-chromosome systems, and main features of their karyotypes of *T.* (*Metallotimarcha*) *metallica* (Laicharting, 1781), *T.* (*Timarcha*) *carmelenae* Petitpierre, 2013 and *T.* (*Timarcha*) *parvicollis* ssp. *seidlitzi* Kraatz, 1879 to enlarge the cytogenetic analysis of the genus and discuss the most relevant trends of its chromosomal evolution.

Material and methods

The three checked species and their geographical origins are given in Table 1. The chromosome analyses were only performed on male living individuals brought to our laboratory in Palma de Mallorca (Spain), where they were killed with ethyl acetate. The cytogenetic data were obtained by testis dissection of male adult specimens which were fixed in 45% acetic acid , later on teased into small pieces for five minutes, squashed under a coverslip, immediately frozen in liquid nitrogen to remove the coverslip, and finally treated using conventional Giemsa staining procedures. Most examined cells were at meiotic metaphase I, providing the male meioformulae, thus the number of autosomal bivalents plus the male sex-chromosome systems. Finally, we took micrographs by a ZEISS AXIOPHOT or a ZEISS AXIOSKOP photomicroscope, and subsequently enlarged them for printing.

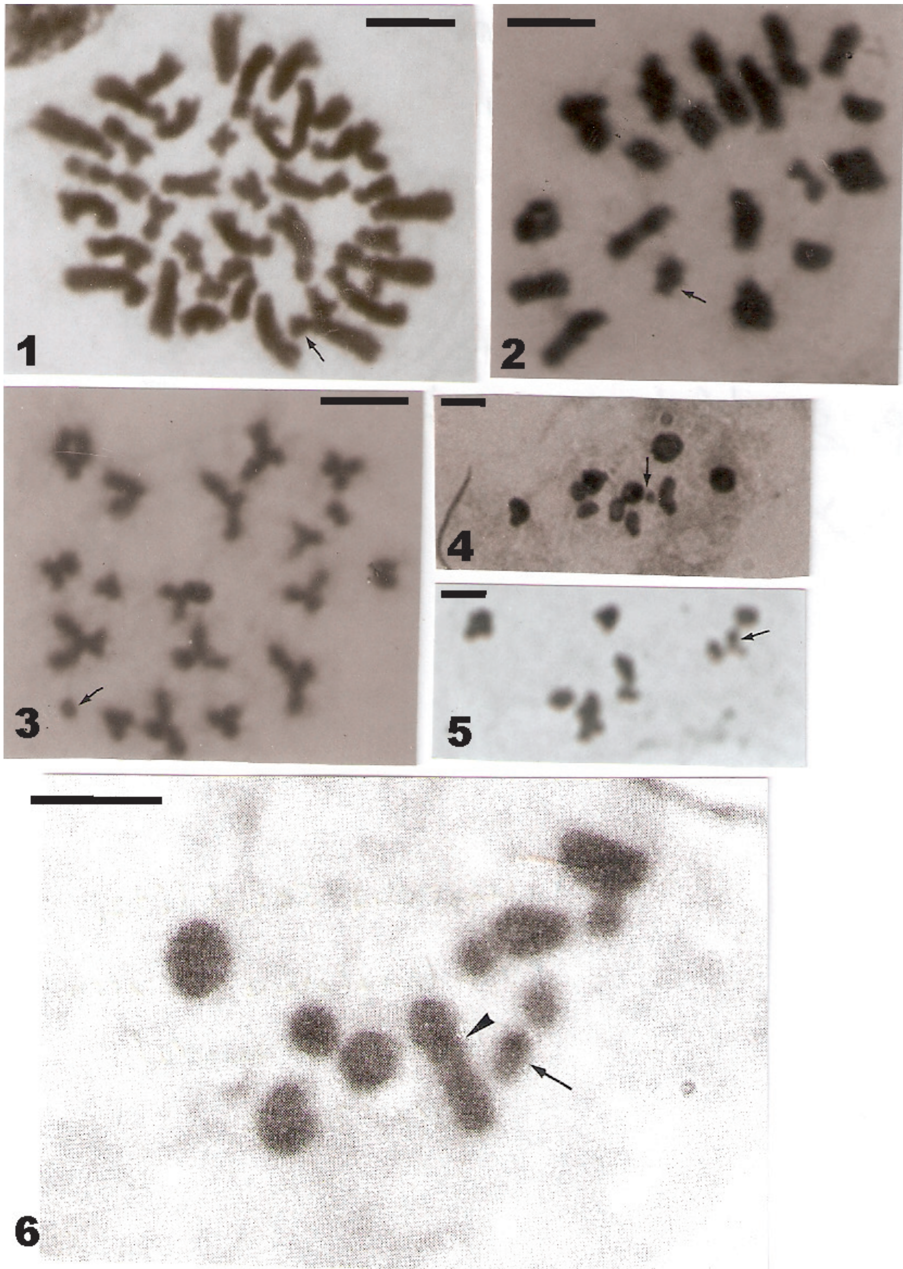
Results

Timarcha (*Metallotimarcha*) *metallica* (Laicharting, 1781)

Two males of this species have displayed 2n= 38 chromosomes and an 18 + Xy_p male meioformula, with a "parachute" Xy_p sex-chromosome system (Fig. 2). Its karyotype is composed of nine medium size and nine small autosome pairs plus a submetacentric X-chromosome of medium size and a tiny y-chromosome. Four of the medium size autosome pairs were acrocentrics and the remaining meta- or submetacentrics, and three of the small ones were acrocentrics and the other metacentrics, as shown by spermatogonial mitotic metaphases (Fig. 1) and meiotic metaphases II (Fig. 3). Thus, the fundamental number (FN) of chromosomal arms is 50.

Table 1. Chromosomally analysed species of *Timarcha* and their geographical sources. FR=France, SP=Spain.

T. metallica (Laicharting, 1781)	Deville: Bois de Waibes, Ardennes (FR)
T. carmelenae Petitpierre, 2013	P.N. Sierra de Castril: Sierra Seca, Granada (SP)
	‘’ La Sagra: collado de las Víboras, Granada (SP)
T. parvicollis seidlitzi Kraatz, 1879	Sierra Tejeda: La Maroma, Granada (SP)



Figures 1–6. 1–3 *T. metallica*: **1** spermatogonial mitotic metaphase with 2n = 38 chromosomes, the y-chromosome is arrowed **2** meiotic metaphase I with 18 + Xy_p meioformula, the Xy_p is arrowed **3** meiotic metaphase II with n = 19 chromosomes **4–5** *T. carmelenae*: meiotic metaphases I from Sierra de Castril (**4**) and La Sagra (**5**) individuals, with 9 + Xy_p meioformula, the Xy_p are arrowed **6** *T. parvicollis* ssp. *seidlitzi*: meiotic metaphase I with 11 + Xy_p meioformula, the Xy_p is arrowed and two partly overlapped autosomal bivalents are arrowheaded. Bar: 5 µm.

***Timarcha* (*Timarcha*) *carmelenae* Petitpierre, 2013**

One male individual from Sierra Seca and another from La Sagra provided meiotic metaphases I of 9 + Xy_p, again with a "parachute" Xy_p sex-chromosome system, that is 2n = 20(Xy_p) chromosomes, and showing two autosomal bivalents a bit larger than the others (Figs 4 and 5).

***Timarcha* (*Timarcha*) *parvicollis* ssp. *seidlitzi* Kraatz, 1879**

The only checked male individual provided meiotic metaphase I with an 11 + Xy_p meioformula, having also a "parachute" Xy_p sex-chromosome system, thus 2n = 24(Xy_p), where five autosomal bivalents are larger than the remaining six ones (Fig. 6).

Discussion

The diploid number of 2n = 38 chromosomes shown in *Timarcha* (*Metallotimarcha*) *metallica* should correct a previous miscounting report of 2n = 20 chromosomes (Petitpierre 1982). The high chromosome number found in this species is not displayed by any other *Timarcha* from the Palaearctic (subgenus *Timarcha* s.str.), whose range of numbers goes from 2n = 18 to 2n = 30 (Gómez-Zurita et al. 2004, Petitpierre 2011). However, high chromosome numbers are characteristic of the two species of the subgenus *Americanotimarcha* Jolivet, 1948, e.i., *T. intricata* Halderman, 1854 with 2n = 44 (Petitpierre and Jolivet 1976) and *T. cerdo* Stal, 1860 with 2n = 38 (Jolivet and Petitpierre 1992). These high chromosome numbers are in agreement with the similar morphological traits, the male genitalia and the molecular phylogenetic resemblances between the subgenera *Metallotimarcha* Motschulsky, 1860 and *Americanotimarcha* (Jolivet 1948, Iablokoff-Khnzorian 1966, Gómez-Zurita et al. 2000, Gómez-Zurita et al. 2004, Jolivet et al. 2013). Although the species of both subgenera show some plesiomorphic features, such an incomplete fusion of elytra, weak sexual dimorphism, aedeagus with a long tegmen cap, and a basal position in the molecular phylogenetic tree, their high chromosome numbers can not be considered as an ancestral character. First, because 2n = 20(Xy_p) is assumed to be the plesiomorphic and most frequent karyotype condition for Coleoptera of the suborder Polyphaga (Smith and Virkki 1978, Angus et al. 2007). Besides, this is the most common karyotype in the genus *Timarcha* where more than a half of the 42 surveyed taxa show 2n = 20(Xy_p) (Petitpierre 2011). And third, the karyotypes of both *T. metallica* and *T. intricata* share a quite high number of acrocentric autosome pairs, seven and fourteen respectively, which is an indication of their derived origin by multiple centric fissions or chromosomal dissociations from meta- or submetacentric chromosomes. Therefore, we assume that a hypothetic karyotype of 2n = 20(Xy_p) chromosomes, mostly composed of metacentrics or submetacentrics, would have been the plesiomorphous state for the genus, from which all the taxa of the three present subgenera, *Americanotimarcha*, *Metallotimarcha* and *Timarcha* s.str. may have radiated.

The karyotype of *T.* (*T.*) *carmelenae* with 2n = 20(Xy_p), with two larger autosomal bivalents and the remaining gradually decreasing, is similar to those of *T.* (*T.*) *intermedia* Herrich-Schäffer, 1838, and *T.* (*T.*) *lugens* Rosenhauer, 1856 (Petitpierre 1970, 1976). These three species share close morphological resemblances and a feeding on Brassicaceae plants, *Hormathophylla spinosa* (L.) Küpfer, 1974 for both *T.* (*T.*) *carmelenae* and *T.* (*T.*) *lugens* (González-Megías and Gómez 2001, Petitpierre and Daccordi 2013) and *Carrichtera annua* (L.) DeCandolle, 1821 for *T.* (*T.*) *intermedia* (Petitpierre 1971, Jolivet and Petitpierre 1973), in contrast with the prevalent trophism on plants of Rubiaceae and/or Plantaginaceae reported for almost all the other taxa of the subgenus *Timarcha* s.str. (Jolivet and Petitpierre 1973).

T. (*T.*) *parvicollis* ssp. *seidlitzi* shows a karyotype of 11 + Xy_p male meioformula, thus 2n = 24(Xy_p) chromosomes, which separates it strikingly from the related Andalusian species with 2n = 20(Xy_p) such as *T.* (*T.*) *insparsa* Rosenhauer, 1856, *T.* (*T.*) *marginicollis* Rosenhauer, 1856, *T.* (*T.*) *intermedia*, *T.* (*T.*) *lugens* Rosenhauer, 1856 and *T.* (*T.*) *carmelenae*, sharing a bifid mesosternum and elytra covered with spare and fine puncturation.

Another species of *Timarcha* with 2n = 24 chromosomes, *T.* (*T.*) *pratensis* (Duftschmid, 1825) (Petitpierre 1976), from Central and Eastern Europe, and Northern Italy, belongs to a very different group without any close interrelationship with *T.* (*T.*) *parvicollis* (Bechyné 1948, Warchalowski 2003).

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Chromosomal evidence of species status and evolutionary relationships of the black fly *Prosimulium petrosum* (Diptera, Simuliidae) in Armenia

Sergey Vlasov[1], Maria Harutyunova[2], Karine Harutyunova[2], Peter H. Adler[3]

1 *Department of General Biology and Bioecology, Moscow State Regional University, Radio St. 10A, Moscow 105005, Russia* **2** *Institute of Molecular Biology, Yerevan, Armenia* **3** *Department of Agricultural and Environmental Sciences, Clemson University, Clemson, SC 29634-0310, U.S.A.*

Corresponding author: *Sergey Vlasov* (sv.vlasov@mgou.ru)

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Abstract

The karyotype of Armenian populations of the black fly *Prosimulium petrosum* Rubtsov, 1955 was characterized and compared with that of all other chromosomally known Palearctic members of the *Prosimulium hirtipes* group. Analysis of the polytene chromosomes established that *Prosimulium petrosum* is most closely related to European populations of *Prosimulium latimucro* (Enderlein, 1925) with which it shares an identical fixed chromosomal banding sequence. Its validity as a species, separate from *Prosimulium latimucro*, is based on its unique sex-differential sections in the expanded centromere region of chromosome I, in agreement with the unique structural configuration of the hypostomal teeth of its larvae. *Prosimulium petrosum* and *Prosimulium latimucro*, therefore, are homosequential species, demonstrating the value of a combined chromosomal and morphological approach in determining species status.

Keywords

Black flies, chromosomal inversions, homosequential species, polytene chromosomes, sex chromosomes

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Introduction

Chromosomal rearrangements have long been considered a driving force in speciation in certain groups of organisms, based on a wealth of evidence, much of it indirect (White 1978, Rothfels 1989, Nevo 2012). If the chromosomes have played an integral role in the speciation process, individual species might be expected to carry unique signatures in their karyotype. In the dipteran family Simuliidae, the vast majority of species are chromosomally distinct from one another, even when they cannot be distinguished reliably by morphological criteria (Adler et al. 2010). Detailed banding sequences of the polytene chromosomes in the larval salivary glands of Simuliidae, consequently, have facilitated the discovery of cryptic species, provided insights into population structure and evolutionary relationships, and positioned the Simuliidae at the forefront of knowledge about the genetics of natural populations of insects (Adler and Crosskey 2015a).

The *Prosimulium hirtipes* group is a widespread Holarctic clade of the Simuliidae, consisting of 25 species in the Palearctic Region (Adler and Crosskey 2015b, Adler and Şirin 2015). Twelve of these species have been examined chromosomally, though to various degrees of precision (reviewed by Adler and Crosskey 2015a). Four species of the group occur in Armenia: *Prosimulium frontatum* Terteryan, 1956, *Prosimulium petrosum* Rubtsov, 1955, *Prosimulium rachiliense* Djafarov, 1954, and *Prosimulium tomosvaryi* (Enderlein, 1921) (Adler and Crosskey 2015b). Analyses of the polytene chromosomes of *P. frontatum*, *P. rachiliense*, and *P. tomosvaryi* have revealed cryptic biodiversity and provided hypotheses of their phylogenetic relationships (Adler and Şirin 2014). Comparative chromosomal studies of Armenian populations of *P. petrosum*, however, are lacking, although general karyotypic features, putatively of this species, have been presented for Bulgarian populations (Ralcheva and Dryanovska 1973, Ralcheva 1974, Chubareva and Petrova 2003).

Prosimulium petrosum was described from larvae and pupae collected on 26 May 1952 in Azerbaijan; the holotype larva is from River Agsu above Lake Göygöl (= Geigel) (Rubtsov 1955). Adults attributed to this species were described from Azizbekov (= Vayk) in Armenia (Rubtsov 1956). Terteryan (1968), however, suggested that the descriptions of the Armenian adults represent *P. pronevitshae* Rubtsov, 1955, now a synonym of *P. rachiliense* (Crosskey and Zwick 2007, Adler and Şirin 2014). The pupal gill figured by Rubtsov (1956), based on Azerbaijanian material, has a branching formula of (2+2+2+2)+(2+2)+(2+2), whereas that by Terteryan (1968), based on Armenian material, has a formula of (3+3+2)+(2+2)+(2+2).

Given the lack of chromosomal information for bona fide material of *P. petrosum*, we conducted a comparative band-by-band analysis of *P. petrosum* to characterize its karyotype and illuminate its taxonomic status and evolutionary relationships. In particular, we were interested in determining if *P. petrosum* is a species distinct from the morphologically similar European species, *P. latimucro* (Enderlein, 1925), or if they are conspecific.

Methods

Larvae were collected from three streams, up to about 210 km apart, in April and May in northern and southern Armenia (Table 1). The material was fixed in a 3:1 mixture of ethanol and glacial acetic acid. Pupae and adults were not collected, but 13 mature larvae with well-developed gill histoblasts were obtained. Larvae were identified morphologically as *P. petrosum*, based on structural characters (Rubtsov 1956, Terteryan 1968)—the apex of the median hypostomal tooth of our material was posterior to the apices of the lateral teeth, and the 16 filaments of the pupal gill were arranged on three, widely splayed primary trunks, with a branching formula of (3+3+2)+(2+2)+(2+2) or (3+3+2)+(2+1+1)+(2+2).

Polytene chromosomes from the larval salivary glands were stained using the lacto-aceto orcein method (Bedo 1975), which also stained gonadal tissue. Preparations were spread by squashing chromosomes on a microscope slide. Larval gender was determined by the form of the gonads: rounded in males and elongated in females. Representative chromosomal arms and selected rearrangements were photographed under oil immersion (Figs 1–4). Composite digital images from different focal planes were made with Helicon Focus 5.3 and further processed with Adobe Photoshop CS6. The banding sequences of all six chromosomal arms were compared with the standard maps of the *P. hirtipes* group (Basrur 1962) and with maps of various species in the *P. hirtipes* group (Basrur 1959, Adler and Belqat 2001, Adler and Şirin 2014).

Fixed inversions (i.e., homozygous in all larvae) are italicized in the text and underlined on our maps; floating inversions (i.e., polymorphisms) are not italicized or underlined. Inversions identical to those identified in previous studies (i.e., *IIS-6*, *IIS-7*, *IIL-9*, and *IIL-10*) were given the same numbers assigned by Basrur (1959). Newly discovered inversions were numbered to follow the last number assigned to inversions in other species of the *P. hirtipes* group currently under study and as yet unpublished. Chromosomal terminology, including terms for landmarks, follows that of Basrur (1959, 1962).

Three morphological preparations of mature larvae were deposited in the Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia. Additional morphological preparations and chromosomal photographs were deposited in the Moscow State Regional University, Moscow, Russia.

Table 1. Collection data for larvae of *Prosimulium petrosum* in Armenia.

Site	Location	Latitude Longitude	Altitude (m asl)	Date	Larvae analyzed males:females
1	Armenia, Gegarkunik Province, Ddmashen[†]	40°34'N 44°49'E	ca. 1900	21 April 2010	3:5
2	Armenia, Sjunik Province, Mogralzani-Vardanidzor, Megraget River	39°00.40'N 46°12.45'E	ca. 1265	04 May 2011	0:1
3	Armenia, Sjunik Province, Megrinsky pass	39°06.30'N 46°10.47'E	ca. 2375	04 May 2011	13:41

[†] Exact location in Ddmashen area is unknown.

Results

Karyotype. In total, 64 larvae were analyzed. One larva from Site 1 chromosomally matched the banding sequence of *P. rachiliense* cytoform 'A' (*sensu* Adler and Şirin 2014). The other 63 larvae (16 males, 47 females) were assigned to *P. petrosum*. All larvae had a diploid number of 2n = 6, with tightly paired homologues (Fig. 1).

The chromosomes were submetacentric (Fig. 1). Chromosome I (sections 1–44) was the longest, with the two arms (IS and IL) subequal in length, followed by chromosome II (sections 45–74) with the long arm (IIL) slightly longer than the short arm (IIS). Chromosome III (sections 75–100) was the shortest, with the long arm (IIIL) approximately 35% longer than the short arm (IIIS). The centromere regions of chromosomes I and II were transformed (CI_t, CII_t; *sensu* Basrur 1959), producing an expanded, flocculent area from section 19 through section 21 (CI_t) and from the middle of section 57 through section 58 (CII_t) (Figs 1–3). The centromere region of chromosome III was not expanded (Figs 1, 4).

A single, primary nucleolus organizer was in the standard position for the *P. hirtipes* group, that is, in the base of IL (Fig. 2). Landmarks that remained in the standard

Figure 1. Total polytene chromosomal complement of *Prosimulium petrosum*. Total polytene chromosomal complement of female larva of *Prosimulium petrosum*, showing the diploid condition of 2n = 6, with tightly paired homologues.

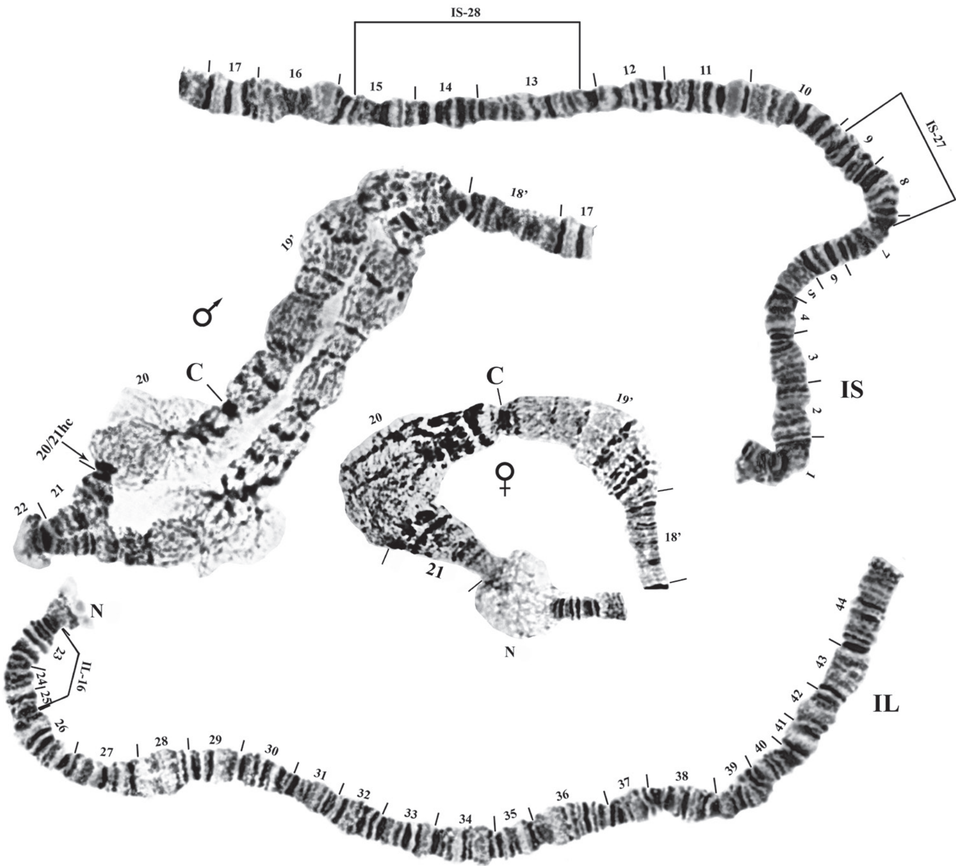

Figure 2. Chromosome I of *Prosimulium petrosum*, with male and female transformed centromere regions (CI_t). Breakpoints of autosomal heterozygous inversions are indicated by brackets. C: centromere, NO: nucleolar organizer, 20/21hc: heterochromatic band.

positions for the *P. hirtipes* group included the single Balbiani ring in the base of IIS (Fig. 3), "blister" in IIIS (Fig. 4), and "shield" and "triad" in IIIL (Fig. 4). A chromocenter was lacking, and supernumerary (B) chromosomes were absent.

Fixed (interspecific) inversions. The banding sequence of chromosome arms IS, IL, IIIS, and IIIL was identical with the standard banding sequence established by Basrur (1959, 1962) for the *P. hirtipes* group. Chromosome II, however, had four fixed inversions relative to the standard sequence—two overlapping inversions in the short arm, *IIS-6* and *IIS-7,* and two tandem inversions in the long arm, *IIL-9* and *IIL-10* (Fig. 3). The four inversions involved 73% and 58% of the sections of IIS and IIL, respectively. *IIL-9* moved the "group of 5" marker more centrally and *IIL-10* reversed the polarity of the parabalbiani.

Autosomal (intraspecific) polymorphisms. Fifteen autosomal polymorphisms were detected; all were present in the heterozygous state only. These autosomal rear-

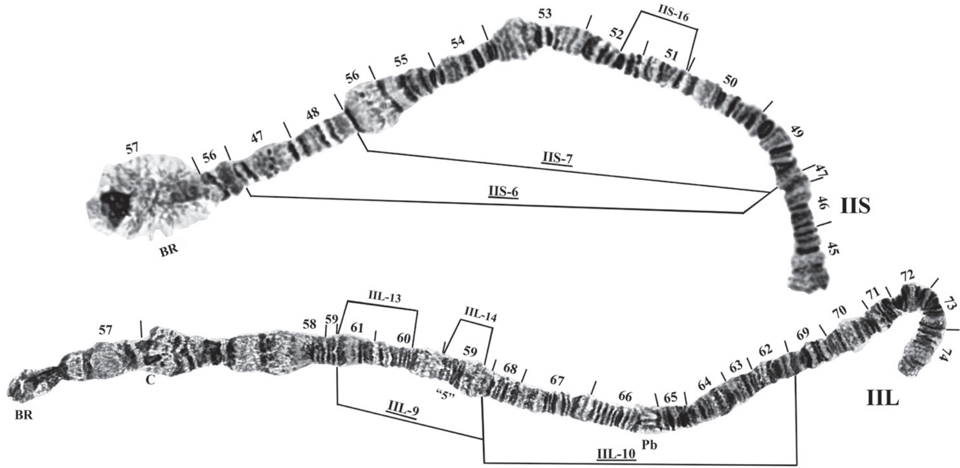

Figure 3. Chromosome II of *Prosimulium petrosum*. Relative to the standard sequence, fixed inversions *IIS-6*, *IIS-7*, *IIL-9*, and *IIL-10* are present. Breakpoints of autosomal inversions are indicated by brackets above the chromosomes. BR: Balbiani ring, C: centromere, Pb: parabalbiani, "5": group of 5 marker.

Table 2. Frequency of homologues with autosomal inversions and other rearrangements (band deletions, duplications, and heterobands) in three Armenian populations of *Prosimulium petrosum*.

Collection site	1	2	3	Armenia†
Larvae (*n*)	8	1	54	63
Chromosomal homologues (*n*)*	16	2	108	126
IS-27	0.063		0.037	0.040
IS-28	0.063			0.008
IL-16	0.063		0.009	0.016
IIS-16			0.009	0.008
IIL-13			0.009	0.008
IIL-14			0.028	0.024
IIIL-32			0.019	0.016
IIIL-33			0.028	0.024
IIIL-dif‡			0.028	0.024
100dlT‡			0.046	0.040
90hb			0.019	0.016
87dp			0.009	0.008
Mean number of heterozygous inversions/larva§				0.333
Mean number of all heterozygous chromosomal rearrangements/larva§				0.460

† All three collection sites combined.

* Frequencies of rearrangements were calculated on the basis of the number of homologues.

‡ Three larvae, which had IIIL-dif (= IIIL-34, IIIL-35+IIIL-36+IIIL-37), also carried heterozygous deletion $100dl_T$, the frequency of which is accounted for separately; two additional larvae had heterozygous deletion $100dl_T$ in the absence of IIIL-dif.

§ IIIL-dif was treated as a single inversion for the purpose of presenting means.

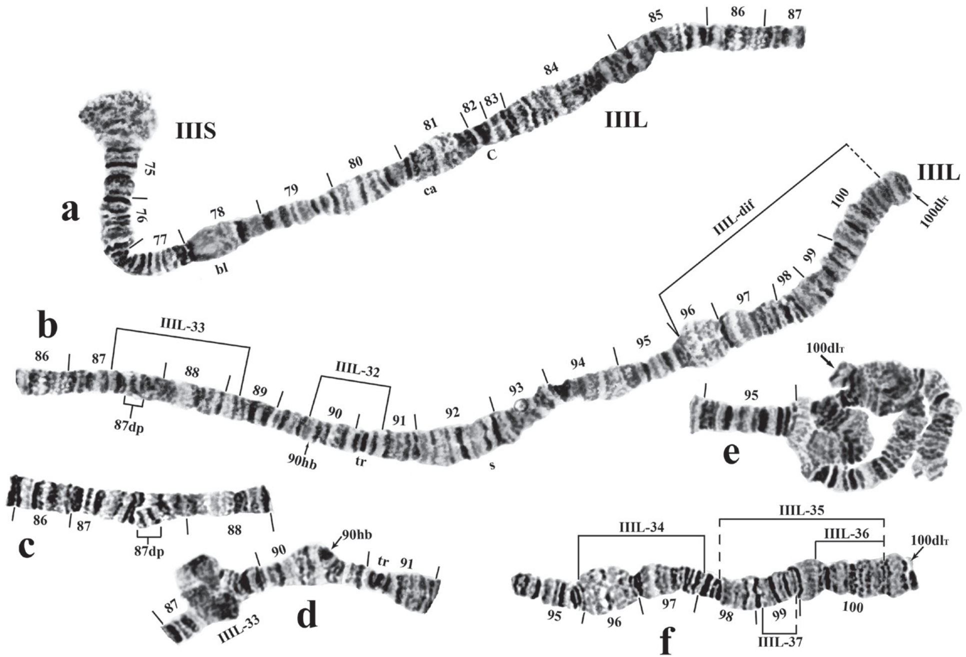

Figure 4. Chromosome III of *Prosimulium petrosum*. **a, b** chromosome III of *Prosimulium petrosum*. Breakpoints of autosomal inversions and location of 2 additional bands (87dp) are indicated by brackets. IIIL-dif is an inversion complex, hypothesized to consist of four inversions. Arrows indicate locations of 90hb and 100dlT **c** heterozygous band duplication 87dp **d** heteroband 90hb and heterozygous inversion IIIL-33 **e** complex set of heterozygous inversions, collectively referred to as IIIL-dif; arrow shows deletion 100dlT in telomere of one homologue. f - heterozygous deletion 100dlT; breakpoints of individual inversions IIIL-34, IIIL-35+IIIL-36+IIIL-37, which comprise complex inversion IIIL-dif, are indicated by brackets; dashed line designates approximate limits of inversions. C: centromere, bl: blister, ca: capsule, s: shield, tr: triad.

rangements included 12 inversions, one heteroband (90hb), a duplication of bands (87dp), and a telomeric deletion (100dlT) (Table 2, Figs 2, 3, 4). Four inversions in sections 96–100 of IIIL formed a complex set of loops collectively referred to as IIIL-dif (Fig. 4, e). The proposed breakpoints of these four inversions are shown in Fig. 4, f. All autosomal polymorphisms were expressed in low frequency (< 0.065; Table 2).

Sex-differential region. All 16 males had a heterochromatic band (20/21hc) at the junction of sections 20 and 21 and lacked conjugation in the CI_t region, typically from section 20 through the beginning of section 21 (Fig. 2), although one male was unpaired from the beginning of section 19 to the beginning of section 21; no inversion could be discerned in the unpaired region. Females lacked the heterochromatic band and exhibited complete pairing of homologues in the CI_t region. Thus, the expanded centromere region of chromosome I was the sex-differential segment, with males X_0Y_1 and females X_0X_0. In two males, ectopic pairing of CI_t and CII_t occurred in some nuclei.

Discussion

Our chromosomal analysis requires taxonomic context, especially a reasonable assignment of the correct species name. The larvae from our three sites in Armenia are chromosomally cohesive. Based on gill structure, they conform to previous Armenian collections (Terteryan 1968), rather than to Azerbaijanian material (Rubtsov 1956), of *P. petrosum*. Based on hypostomal structure, they precisely match the Azerbaijanian (holotype) (Rubtsov 1956). Our Armenian collections and the type locality of *P. petrosum* in Azerbaijan are 140–160 km apart, and all are in the same ecoregion—the Caucasus Mixed Forests Ecoregion (World Wildlife Federation 2015). Although a slight difference in the branching pattern of the gill between Armenian and Azerbaijanian samples possibly indicates the presence of cryptic species, we attribute the difference to intraspecific variation, which is common, especially on the dorsal trunk, in members of the *P. hirtipes* group (Stloukalova 2004). Similarly, although we found mature larvae 1.0–1.5 months earlier (end of April–beginning of May) than did Terteryan (1968), the seasonal difference could be attributable to altitude or perhaps climatic variation among years. Given the minimal geographic distance, dispersal ability of simuliids (Adler et al. 2005), identical ecoregion, and morphological similarity, we conclude that our Armenian populations are conspecific with the holotype.

Although our material corresponds with the type (Caucasican) concept of *P. petrosum* (Rubtsov 1955), a larger question is whether *P. petrosum* is a unique species or conspecific with *P. latimucro*, as suggested by Adler and Crosskey (2015b), based on morphological similarity. Yankovsky (2003) suggested that the projection of the median hypostomal tooth anterior to the lateral teeth and the second-order branching (i.e., 3+3+2) of the upper gill trunk distinguish the larva of *P. latimucro* from that of *P. petrosum*. Accordingly, our samples correspond with *P. petrosum*, based on the hypostomal teeth, and with *P. latimucro*, based on the branching of the gill.

What do the banding sequences of the polytene chromosomes reveal about possible conspecificity of *P. petrosum* and *P. latimucro* and their evolutionary relationships? The Armenian population of *P. petrosum* shares *IIS-6,7* and *IIL-9* with *P. latimucro*, *P. rufipes* (Meigen, 1830), and *P.* "aff. 3" of Basrur (1959), and fixation of *IIL-10* with *P. rufipes*, *P.* "aff. 3", and Moroccan *P. latimucro* (Adler and Belqat 2001, Adler and Şirin 2014). In European populations of *P. latimucro*, inversion IIL-10 is absent or polymorphic. *Prosimulium petrosum* differs from *P. rufipes*, *P.* "aff. 3", and Moroccan *P. latimucro* by lacking *IS-18*, *IIS-8*, and *IIL-11*, respectively. *Prosimulium petrosum* does not share any autosomal polymorphisms with any studied member of the *P. hirtipes* group. We conclude that European populations of *P. latimucro* are most closely related to *P. petrosum*.

Males and females of *P. petrosum* consistently differ in the expression of their CI_t region, indicating the general location of the sex-determining locus. The sex chromosomes of the Simuliidae often are associated with rearrangements, such as inversions and heterobands, although the X and Y also can be microscopically undifferentiated (X_0Y_0) (Rothfels 1980, Post 1982). Any of the three chromosomes (I, II, or III) can

function as the sex chromosome. Identical, differentiated sex chromosomes are rarely shared between species (Rothfels and Freeman 1983, Adler et al. 2015). Thus, the sex chromosomes can be useful in species discovery and identification (Rothfels 1989).

Lack of pairing of homologues in the CI_t region, observed in males of *P. petrosum*, also is found in at least some populations of other Palearctic members of the *P. hirtipes* group, such as *P. hirtipes* (Fries, 1824), *P. latimucro*, and *P.* "aff. 3", and often serves as the basis for further elaboration of the Y chromosome, such as the addition of sex-linked inversions and heterobands (Basrur 1959, Adler unpublished). The heterochromatic band 20/21hc of *P. petrosum* also appears on the Y chromosome of various members of the *P. hirtipes* group, including Moroccan populations of *P. rufipes* and *P. latimucro* and some European populations of *P. latimucro* and *P.* "aff. 3", often with various repatternings of banding in the CI_t region (Adler and Belqat 2001, Adler unpublished). No conspicuous repatterning was observed in the CI_t region of *P. petrosum*. We do not know if the unpaired CI_t condition and heterochromatic band 20/21hc are identical across populations and species, and if so, if their shared nature reflects common ancestry, introgression, or independent origins. Species differences in other members of the *P. hirtipes* group, such as those in eastern North America often are based on minor differences in the centromere region, especially of CIII (Rothfels and Freeman 1977).

A Y chromosome based on an unpaired CI_t region, coupled with 20/21hc, without an associated inversion or band repatterning, uniquely characterizes *P. petrosum*. The allopatric nature of *P. petrosum* and *P. latimucro*, however, presents a challenge for evaluating reproductive isolation; the nearest chromosomally analyzed populations of *P. petrosum* and *P. latimucro* are more than 1,500 km apart. Our analysis of the photographs by Ralcheva (1974) of putative *P. petrosum* from Bulgaria suggests that IS and IL are standard, *IIS-6,7* and *IIL-9* are present, and IIL-10 is absent; the sex chromosomes and larval morphology were not mentioned. Based on available evidence, *P. petrosum* of Ralcheva (1974), therefore, is probably *P. latimucro*, and most closely resembles populations in the Swiss Alps (as *P. inflatum* "aff. 1" of Basrur 1959). The chromosomal characteristics of all other analyzed populations identified as *P. petrosum* and *P. latimucro* are entirely congruent with the respective configurations of the hypostomal teeth. Thus, we argue that *P. petrosum* is a distinct species on the basis of unique chromosomal features corroborated by distinct hypostomal features. *Prosimulium petrosum* and European *P. latimucro*, therefore, are homosequential species—they have the same fixed banding sequence but differ morphologically, a phenomenon first discovered in *Drosophila* (Carson et al. 1967). Although not common in the Simuliidae, previous examples of homosequential species include several members of the *Simulium vernum* group (Hunter 1987, Adler et al. 2004, Seitz and Adler 2015).

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RESEARCH ARTICLE

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Sex chromosome diversity in Armenian toad grasshoppers (Orthoptera, Acridoidea, Pamphagidae)

Alexander G. Bugrov[1,2], Ilyas E. Jetybayev[1,3], Gayane H. Karagyan[4], Nicolay B. Rubtsov[3]

1 *Institute of Systematics and Ecology of Animals, Russian Academy of Sciences, Siberian Branch, Frunze St. 11, 630091 Novosibirsk, Russia* **2** *Novosibirsk State University, Pirogov St., 2, 630090 Novosibirsk, Russia* **3** *Institute of Cytology and Genetics, Russian Academy of Sciences, Siberian Branch, Pr. Lavrentjeva, 630090 Novosibirsk, Russia* **4** *Scientific Center of Zoology and Hydroecology NAS RA, P. Sevak 7, 0014 Yerevan, Armenia*

Corresponding author: *Alexander G. Bugrov* (bugrov@fen.nsu.ru; bugrov04@yahoo.co.uk)

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Abstract

Although previous cytogenetic analysis of Pamphagidae grasshoppers pointed to considerable karyotype uniformity among most of the species in the family, our study of species from Armenia has discovered other, previously unknown karyotypes, differing from the standard for Pamphagidae mainly in having unusual sets of sex chromosomes. *Asiotmethis turritus* (Fischer von Waldheim, 1833), *Paranocaracris rubripes* (Fischer von Waldheim, 1846), and *Nocaracris cyanipes* (Fischer von Waldheim, 1846) were found to have the karyotype 2n♂=16+neo-XY and 2n♀=16+neo-XX, the neo-X chromosome being the result of centromeric fusion of an ancient acrocentric X chromosome and a large acrocentric autosome. The karyotype of *Paranothrotes opacus* (Brunner von Wattenwyl, 1882) was found to be 2n♂=14+X_1X_2Y and 2n♀=14+$X_1X_1X_2X_2$, the result of an additional chromosome rearrangement involving translocation of the neo-Y and another large autosome. Furthermore, evolution of the sex chromosomes in these species has involved different variants of heterochromatinization and miniaturization of the neo-Y. The karyotype of *Eremopeza festiva* (Saussure, 1884), in turn, appeared to have the standard sex determination system described earlier for Pamphagidae grasshoppers, 2n♂=18+X0 and 2n♀=18+XX, but all the chromosomes of this species were found to have small second C-positive arms. Using fluorescent *in situ* hybridization (FISH) with 18S rDNA and telomeric $(TTAGG)_n$ DNA repeats to yield new data on the structural organization of chromosomes in the species studied, we found that for most of them, clusters of repeats homologous to 18S rDNA localize on two, three or four pairs of autosomes and on the X. In *E. festiva*, however, FISH with labelled 18S rDNA painted C-positive regions of all autosomes and the X chromosome; clusters

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of telomeric repeats localized primarily on the ends of the chromosome arms. Overall, we conclude that the different stages of neo-Y degradation revealed in the Pamphagidae species studied make the family a very promising and useful model for studying sex chromosome evolution.

Keywords

Pamphagidae grasshoppers, karyotype, autosome, neo sex chromosome evolution, neo-X, neo-Y chromosomes, FISH analysis, rDNA and telomeric repeats

Introduction

About 600 species of Pamphagidae, or “toad grasshoppers” as they are commonly known, are distributed in the desert and mountainous landscapes of Africa, Central Asia, and the Western and Eastern Mediterranean (Uvarov 1966, Massa 2013), including some species found in Armenia (Avakian 1968). To date, the family Pamphagidae remains one of most poorly karyotyped groups among the grasshoppers. One reason for this lies in the very low densities of the toad grasshopper populations. Until recently, exceptional karyotypic conservatism was considered a prominent feature of this family. Diploid sets of chromosomes in these grasshoppers consisted of 19 (♂) and 20 (♀) acrocentric chromosomes; the described sex determination were XO♂/XX♀ (White 1973, Hewitt 1979, Camacho et al. 1981, Santos et al. 1983, Cabrero et al. 1985, Fu Peng 1989, Mansueto and Vitturi 1989, Vitturi et al. 1993, Warchałowska-Śliwa et al. 1994).

However, karyotyping of some Pamphagidae species from Central Asia and Bulgaria forced researchers to reconsider this notion of a uniform karyotype structure within the family. Reciprocal translocation between an ancient acrocentric X chromosome and one of the acrocentric autosomes was discovered to have taken place in some pamphagid species (Bugrov 1986, Bugrov and Warchałowska-Śliwa 1997, Bugrov and Grozeva 1998, Li et al. 2005), leading to the formation of a large, biarmed neo-X chromosome. Following the nomenclature of White (1940), the arm of the neo-X chromosome derived from the ancient acrocentric X chromosome is denoted as an XL arm, the autosome part of the neo-X as an XR arm. In males, the autosome homologous to the XR arm is referred to as the neo-Y chromosome.

This article reports the results of our comparative analysis of the karyotypes of Armenian Pamphagidae grasshoppers and our study of the structural organization of their chromosomes, including the distribution of clusters of telomeric repeats and repetitive DNA homologous to 18S rDNA in the chromosomes of the species studied.

Material and methods

Material collection and karyotype analyses

Males of *Eremopeza festiva* (Saussure, 1884) (n=19), *Asiotmethis turritus* (Fischer von Waldheim, 1833) (subfamily Thrinchinae) (n=5); *Nocaracris cyanipes* (Fischer von

Waldheim, 1846) (n=9), *Paranocaracris rubripes* (Fischer von Waldheim, 1846) (n=6), and *Paranothrotes opacus* (Brunner von Wattenwyl, 1882) (n=3) (subfamily Pamphaginae, tribe Nocarodeini) were collected in the early summer season of 2013 in mountain and desert landscapes in Armenia.

After 0.1% colchicine solution was injected into males for 1.5–2.0 hours, the tests testes were dissected and placed into 0.1% solution of sodium citrate for 15 minutes, fixed in 3:1 ethanol:glacial acetic acid for 15 minutes and kept in 70% ethanol. Air-dried chromosome preparations were made by the standard squashing technique (Darlington and La Cour 1976).

Three females of *Paranocaracris rubripes* were kept in cages with moisturized sand to obtain egg pods. Each portion of eggs was stored in a separate Petri dish with moist sand and placed in an incubator. After 15–20 days of incubation at room temperature, the eggs were placed into a solution of 0.05% colchicine in 0.9% sodium citrate and the tops of the nonmicropylar ends were removed. They were then incubated at 30 °C for 1.5–2 h. The eggs were fixed in 3:1 ethanol:acetic acid for 15 minutes and embryos were dissected out of the eggs and transferred into distilled water for 15–20 min at room temperature. Air-dried preparations were made on pre-cleaned slides by macerating the embryos in a drop of 60% acetic acid.

C-banding of the chromosome preparations was performed according to Sumner's protocol (1972). Slides were treated with 0.2 N HCL for 15–20 min, then incubated in the saturated solution of $Ba(OH)_2$ at 61 °C for 3–5 min, rinsed in water, and placed into 2xSSC at 61 °C for 60 min. After being rinsed in water the slides were stained with 2% Giemsa solution.

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) on mitotic and meiotic chromosomes was carried out as described in previous studies (Rubtsov et al. 2000, Rubtsov et al. 2002). Clusters of repetitive DNA homologous to 18S rDNA in metaphase chromosomes were visualized by FISH with a fragment of human 18S rDNA (Malygin 1992) labelled with digoxygenin or biotin. DNA labelling was performed as described in Rubtsov et al. (2000). The DNA probe for detection of telomeric repeats $(TTAGG)_n$ in metaphase chromosomes was generated with non-template PCR (Ijdo et al. 1991) with 5′-TAACCTAACCTAACCTAACC-3′ and 5′-TTAGGTTAGGTTAGGTTAGG-3′ primers according to standard protocol (Sahara et al. 1999).

Visualization of hybridized DNA labelled with digoxygenin or biotin was performed with antidigoxigenin sheep antibodies conjugated with Rhodamine or with avidin-FITC conjugates, respectively.

Chromosome counterstaining was preformed after FISH with 4′,6-diamidino-2-phenylindole (DAPI) using Vectashield antifade containing 200ng/ml DAPI. Vectashield antifade was put under glass cover, which was then sealed with rubber cement.

Microscope analysis

Microscopic analysis was carried out at the Center for Microscopy of Biological Subjects (Institute of Cytology and Genetics, Novosibirsk, Russia). Chromosomes were studied with an AxioImager M1 (Zeiss) fluorescence microscope equipped with filter sets #49, #46HE, #43HE (Zeiss) and a ProgRes MF (MetaSystems) CCD camera. The ISIS5 software package (MetaSystems GmbH, Germany) was used for image capture and analysis.

Chromosome nomenclature

The nomenclature suggested for Pamphagidae grasshoppers (Camacho et al. 1981) was used in the description of chromosomes and karyotypes.

Results

Karyotype description of *Eremopeza festiva* (Saussure, 1884). The karyotype of *E. festiva* consisted of biarmed chromosomes, 2n♂=19, (18AA+X). The autosomes fell into three groups based on size: 4 long (L_1–L_4), 4 medium (M_5–M_8), and 1 short (S_9). The size of the X chromosome is approximately similar to the L_2 pair. Large chromosomes and the largest medium-sized pair (L_1-L_4 and M_5) are subacrocentric. The M_6, M_7, M_8, S_9 pairs and the X chromosome are submetacentrics. Pericentric C-blocks appeared in all chromosomes of the complement except M_7. The size of the C-blocks varied for different chromosome pairs. In the smallest chromosomes of the set (M_8 and S_9), distal C-positive blocks were observed on their long arms (Fig. 1a). The short chromosome arms in the autosomes are C-negative. In the prophase of meiosis, the long autosomes form 2–3 chiasmata, the medium-sized ones form 1–2, and the short autosome forms only one chiasma (Fig. 1b).

The telomeric DNA probe hybridized on the termini of all chromosomes. FISH signals of the telomeric DNA probe differed in size and intensity on chromosome arms ($L_{1,}$ $L_{3,}$ $M_{7,}$ $M_{8,}$ X), between homologous chromosomes (L_1, $L_{3,}$ M_7) (Fig. 1c, d). Besides telomeric location, polymorphic interstitial telomeric sequences (ITSs) were discovered in pericentric regions of L_3, L_4, M_6, S_9 and the X chromosome (arrows on Fig. 1e).

FISH with 18S rDNA probe returned a signal for the C-positive regions on almost all autosomes and the X chromosome. The intensity of hybridization signal varied from very intense in L_1, L_4, M_6, M_8, S_9, to very weak in L_3 and M_7 chromosome pairs. (Fig. 1c, d). The FISH signal was also observed in the C-negative short arms of L_1, M_5, M_6, M_8, and S_9 chromosomes (Fig. 1c, d).

Karyotype description of *Asiotmethis turritus* (Fischer von Waldheim, 1833). The karyotype of *A. turritus* consisted of 18 chromosomes (2n♂=18; 16AA+neo-X+neo-Y). Autosomes were acrocentric and fell into three groups accord-

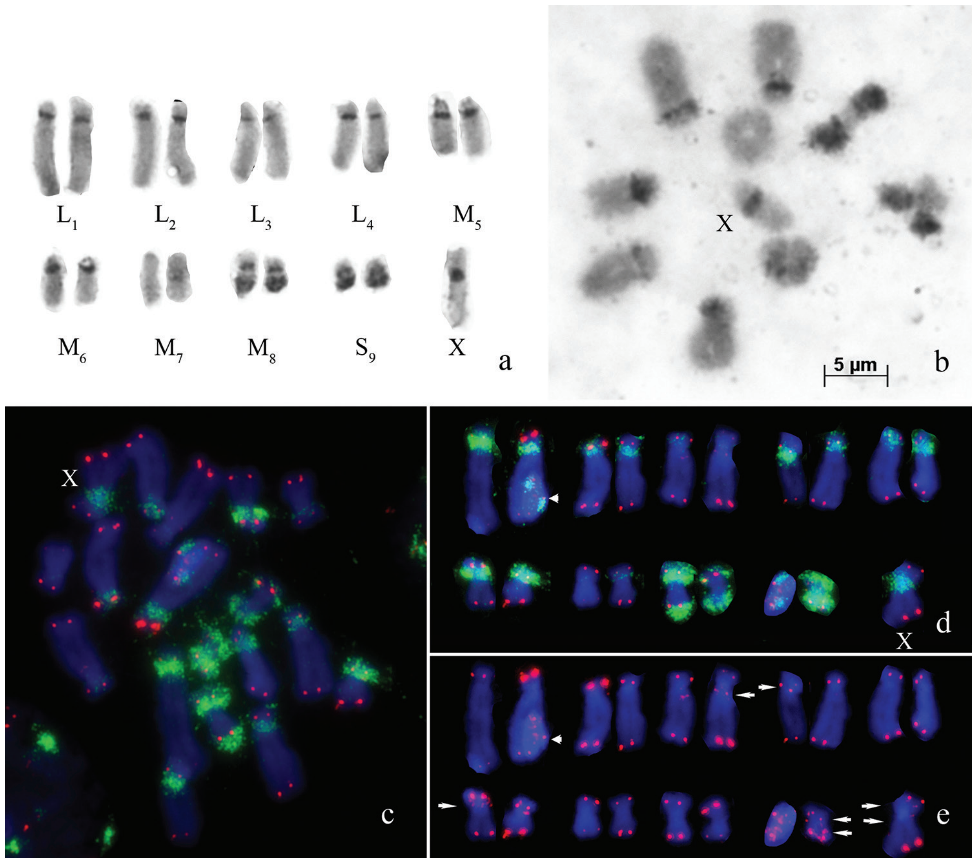

Figure 1. *Eremopeza festiva* – **a** C-banded spermatogonial metaphase chromosomes **b** C-banded meiotic metaphase I **c, d** Fluorescence *in situ* hybridization (FISH) with 18S rDNA probe (green) and a telomeric repeat probe (red) with – spermatogonial metaphase chromosomes, arrowhead indicate chromosome S_9 overlapping on chromosome L_2 **e** Fluorescence *in situ* hybridization (FISH) with telomeric repeats (red) on the same chromosome plate without green channel, arrows indicate interstitial telomeric sites. Bar: 5 µm.

ing to their size: 3 large (L_1–L_3), 4 medium (M_4–M_7), and 1 small (S_8) chromosome pair. The neo-X chromosome was submetacentric and the largest chromosome in the karyotype. The neo-Y chromosome was a large acrocentric, in size equal to the XR arm of the neo-X (Fig. 2a, b). Partial pairing in the prophase of meiosis and the formation of two chiasmata between the neo-Y and the XR indicated homology of the neo-Y and XR distal regions (Fig. 2c, d). C-heterochromatic blocks of different sizes are located in the pericentric regions of all autosomes (Fig. 2a ,b, c). The Y-chromosome has a small pericentric C-band and several interstitial C-bands in its proximal part (Fig. 2b, c).

The telomeric DNA probe hybridized on the termini of all chromosomes (Fig. 2d). ITSs were not observed on *A. turritus* chromosomes.

FISH with 18S rDNA probe returned a signal on the C-positive pericentric region of three pairs of large autosomes. At least on one of them, the cluster of sequences

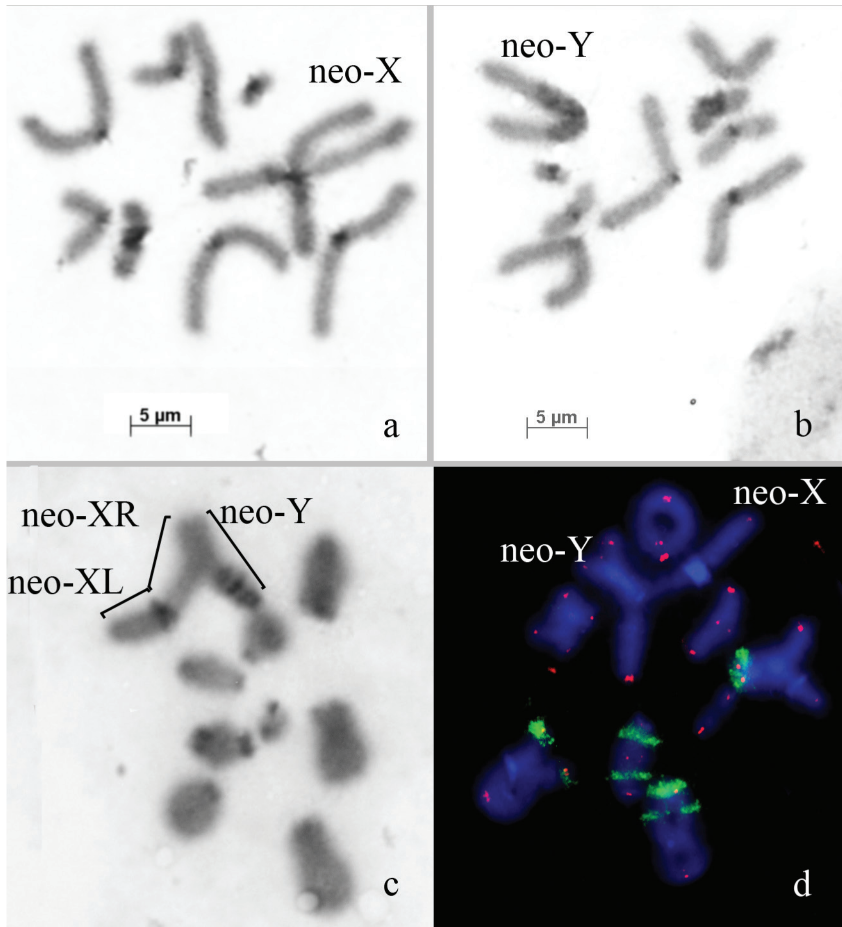

Figure 2. *Asiotmethis turritus* – **a** C-banded anaphase I chromosome with the neo-X chromosome **b** C-banded anaphase I chromosomes with the neo-Y chromosome **c** metaphase I chromosomes with C-band **d** FISH of 18S rDNA probe (green) and a telomeric repeat probe (red) with metaphase I chromosomes. Bar: 5 µm.

homologous to 18S rDNA was polymorphic in size (Fig. 2d). Interstitial clusters of rDNA were revealed in the proximal part of the L_1 chromosome and in the middle of the M_6 chromosome. Both interstitial clusters of rDNA were localized in C-negative regions (Fig. 2d).

Karyotype description of *Paranocaracris rubripes* (Fischer von Waldheim, 1846). The karyotype of *P. rubripes* consisted of 18 chromosomes (2n♂=18; 16AA+neo-X+neo-Y). Autosomes were acrocentric and fell into three groups according to size: 3 large (L_1–L_3), 4 medium (M_4–M_7), and 1 small (S_8) chromosome pair. The neo-X chromosome was here again submetacentric and the largest chromosome in the karyotype. The neo-Y was acrocentric, distinctly smaller in size than the XR arm

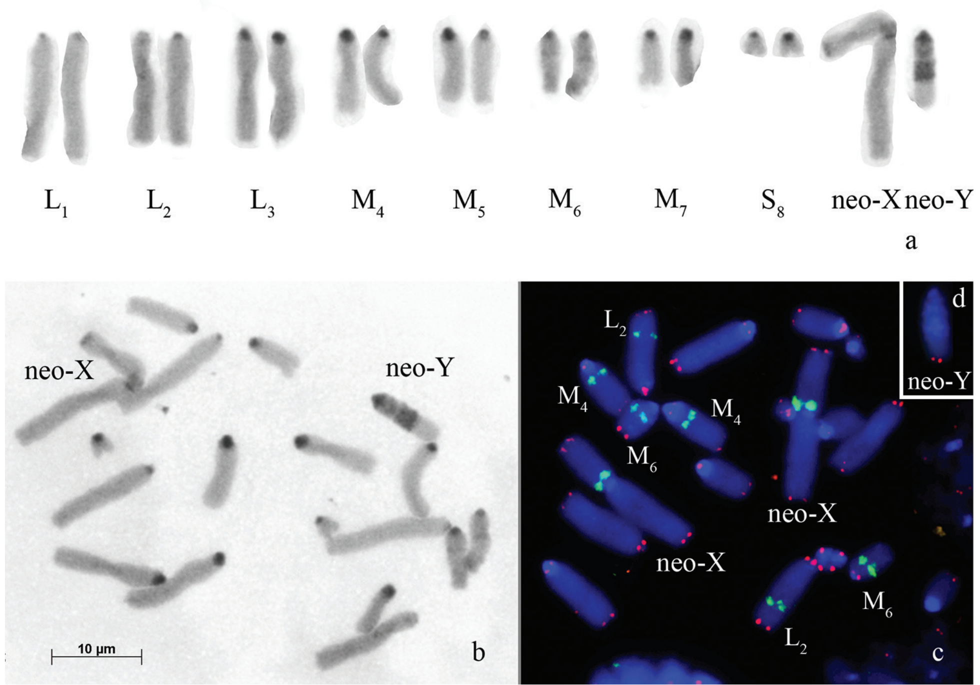

Figure 3. *Paranocaracris rubripes* – **a, b** C-banded mitotic chromosomes from embryos **c, d** FISH with 18S rDNA probe (green) and a telomeric repeat probe (red) on embryos mitotic metaphase chromosomes **c** female metaphase with two metacentric neo-X chromosomes **d** neo-Y chromosome. Bar: 10 µm.

of the neo-X (Fig. 3a, b). C-heterochromatic blocks of different sizes are located in the pericentric regions of all autosomes. Interstitial C-positive blocks were observed in the proximal region of the M_6 chromosome pair, and the proximal region of the XL arm, but stained less intensively than the pericentromeric C-blocks. A minute telomeric C-positive block was observed in the XL arm. Part of the neo-Y chromosome was strongly heterochromatinized. In the neo-Y chromosome one small and two medium-sized interstitial blocks were observed in its proximal half, in addition to a medium-sized pericentric C-positive block (Fig. 3a, b).

The telomeric DNA probe hybridized on the termini of all chromosomes (Fig. 3c). ITSs were not observed on *P. rubripes* chromosomes.

Clusters of 18S rDNA repeats were localized interstitially in three pairs of autosomes (a distal cluster on the L_2, a proximal cluster on the M_4 and a proximal cluster on the M_6 chromosomes) and in the neo-X-chromosome. In the neo-X chromosome and M_6 autosome, clusters of 18S rDNA repeats were localized in interstitial C-positive blocks (Fig. 3c).

Karyotype description of *Nocaracris cyanipes* (Fischer von Waldheim, 1846). The karyotype of *N. cyanipes* consisted of 18 chromosomes (2n♂=18; 16AA+neo-X+neo-Y).

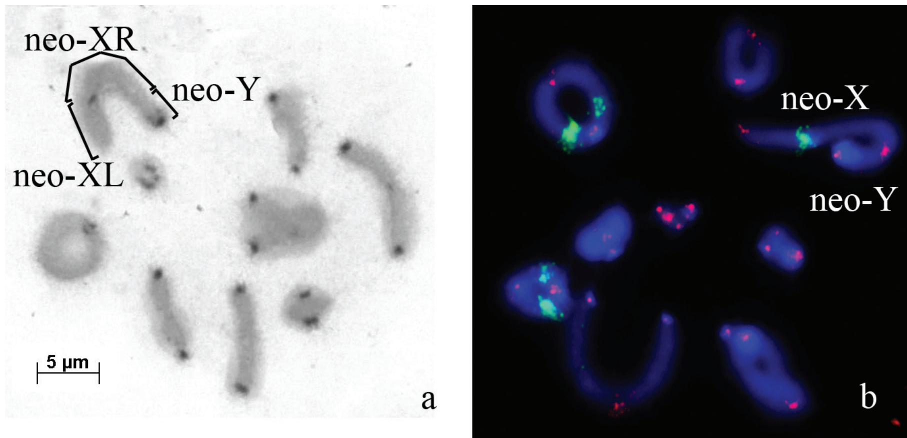

Figure 4. *Nocaracris cyanipes* – **a** C-banded meiotic metaphase I chromosomes **b** FISH with 18S rDNA probe (green) and a telomeric repeat probe (red) of meiotic metaphase I chromosomes. Bar: 5 µm.

The karyotype and C-banding patterns of samples from the Armenian population proved to be similar to an earlier studied population from the North Caucasus (Bugrov and Warchałowska-Śliwa 1997). Autosomes were acrocentric and fell into three groups according to size: 3 large (L_1–L_3), 4 medium (M_4–M_7), and 1 small (S_8) chromosome pair. The neo-X chromosome was again submetacentric and the largest chromosome in the karyotype. The neo-Y was acrocentric, distinctly smaller in size than the XR arm of the neo-X. C-heterochromatic blocks of medium size were located in the pericentric regions of all autosomes. An interstitial C-positive block was observed in the proximal region of the M_6 chromosome pair, but it stained less intensively than the pericentromeric C-blocks. A small telomeric C-positive block was observed in the S_8 chromosome. A minute telomeric C-positive block was observed in the XL arm, whereas the thin C-positive block on the M_6 chromosomes described in samples from North Caucasian population was not found in the Armenian samples. Part of the neo-Y chromosome was strongly heterochromatinized. In the neo-Y chromosome, in addition to a pericentric C-block, a medium-sized interstitial block was observed in its proximal part. At meiotic prophase, large bivalents form two chiasmata, medium bivalents form 1 or 2 chiasmata, while small bivalents form only one chiasma. The neo-XY bivalent forms only one distal chiasma between the XR arm of the neo-X and the neo-Y (Fig. 4a, b).

The telomeric DNA probe hybridized on the termini of all chromosomes (Fig. 4b). ITSs were not observed on *N. cyanipes* chromosomes (Fig. 4b).

Clusters of 18S rDNA repeats were localized in two pairs of autosomes and in the neo-X chromosome. In the neo-X chromosome this cluster was localized in the proximal region of the XL arm. The autosomes carry clusters of rDNA in the interstitial regions of the L_3 and M_6 chromosomes (Fig. 4b).

Karyotype description of *Paranothrotes opacus* (Brunner von Wattenwyl, 1882). The male karyotype of *P. opacus* consisted of 17 chromosomes (2n♂=14+X_1X_2Y.) Autosomes were acrocentric and fell into three groups according to size: 2 large (L_1–L_2),

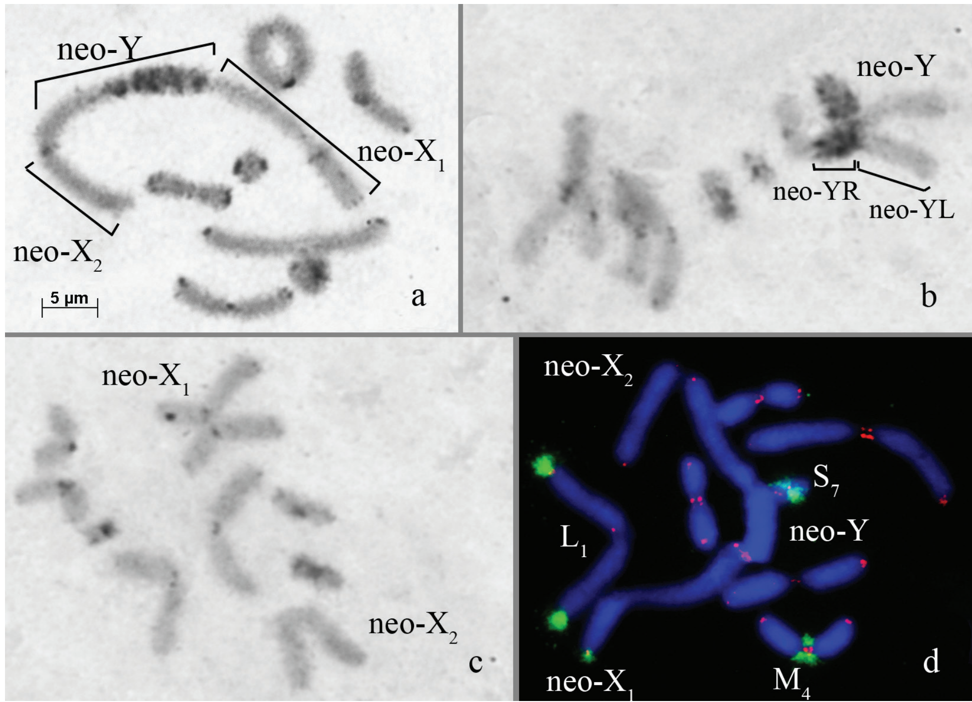

Figure 5. *Paranothrotes opacus* – **a** C-banded diakinesis chromosomes with the neo-X_1, neo-X_2 and neo-Y chromosomes **b** C-banded anaphase I chromosomes with the neo-Y chromosome **c** C-banded anaphase I chromosomes with the neo-Y chromosome **d** FISH of 18S rDNA probe (green) and a telomeric repeat probe (red) with diakinesis chromosomes. Bar: 5 µm.

4 medium (M_3– M_6), and 1 small (S_7) chromosome pair. The neo-X_1 and the neo-Y chromosomes were submetacentric and the largest chromosomes in the karyotype. The neo-X_2 chromosome was one of the largest acrocentric chromosomes. Small-sized C-heterochromatic blocks were located in the pericentric regions of all autosomes and in the neo-X_1. Minute terminal C-positive blocks were located on the L_1, L_2, M_3, M_5, M_6, S_7 chromosomes and both arms of the neo-X_1 chromosome. An interstitial C-band was located in the proximal part of the X_1R arm. The short arm (YL) of the Y-chromosome is strongly heterochromatinized with multiple small C-negative bands (Fig 5a, b).

The telomeric DNA probe hybridized on the termini of all chromosomes (Fig. 5d). ITSs were not observed on *P. opacus* chromosomes.

Clusters of 18S rDNA repeats were localized in the telomeric regions of the L_1, M_4, S_7 chromosomes and the X_1R arm of the X_1 chromosome (Fig. 5d).

Discussion

Comparative cytogenetic analysis of all the Armenian Pamphagidae grasshopper species studied showed karyotypes unusual for the family. The chromosome number previously

considered standard for Pamphagidae grasshoppers (19 for males and 20 for females; (XO♂/XX♀) was found in only in one of the species studied, *E. festiva*. Nevertheless, the chromosome morphology in this species appeared to be different from the standard karyotype. In contrast to the acrocentric chromosomes of earlier studied species, all chromosomes in *E. festiva* were biarmed with short second arms. The formation of a biarmed chromosome from an initially acrocentric chromosome without changing the chromosome number in the karyotype is usually considered a result of pericentric inversions (White 1973, Hewitt 1979). Chromosome reorganization of this type has been described in many taxa of grasshoppers (Hewitt 1979), but in Pamphagidae similar chromosome evolution has only been discovered in *Melanotmethis fuscipennis*, with three pairs of autosomes and the X chromosome showing a short euchromatic arm (Bugrov and Warchałowska-Śliwa 1997). It is impossible to precisely identify the mechanism of such short arm formation, which could potentially be the result of chromosome region inversion or of centromere transposition by neocentromere formation. The ITSs discovered in pericentric C-blocks of four pairs of autosomes and the X could be considered an argument in favour of an explanation involving chromosome region inversion. We suppose that DNA amplification took part in the formation of at least some of the short arms. Repeats homologous to 18S rDNA enriched some of the short arms. DNA amplification involving rDNA also took place in the formation of C-positive blocks in the distal part of the long arms of the M_8 and S_9 chromosomes. It is should be noted that usually rDNA amplification or enrichment of some chromosome regions by rDNA leads to its heterochromatization (Bugrov et al. 2003, Jetybayev et al. 2012). We observed this phenomenon in the distal part of the long arms of the M_8 and S_9 chromosomes but short arms of the chromosomes remained C-negative. Their DNA content is now a question of special interest and can be revealed by sequencing the microdissected material of the short arms (Bugrov et al. 2007).

In three of the species studied (*A. turritus*, *N. cyanipes*, *P. rubripes*), the autosomes looked like standard autosomes for Pamphagidae grasshoppers except those autosomes involved in translocation with the X chromosomes. In all three of these species, the chromosome number was 2n=18 in both males and females and the karyotype included sex chromosomes untypical for grasshoppers: neo-XY♂/neo-XX♀. Furthermore, whereas the autosomes showed conservatism we observed intensive evolution in the sex chromosomes. Obviously, the first step of neo-X and neo-Y formation was a translocation between an ancient acrocentric X chromosome and a large acrocentric autosome. In *A. turritus* we observed the next step of sex chromosome evolution: the neo-Y acquired small interstitial C-positive blocks in its proximal region. As a result, we observed heteromorphization of initial homologues elements, namely XR arms of the neo-X and neo-Y chromosomes. In prophase I of meiosis the XR and neo-Y were similar in length, conjugated with their distal C-negative regions, and formed a bivalent, usually with two chiasmata. The proximal region of the neo-Y chromosome, which was enriched with repeated sequences, was not involved in pairing and recombination (Fig. 2). In *N. cyanipes* and *P. rubripes*, on the other hand, we probably observed the neo-Y chromosome after yet another next step of evolution: the neo-Y chromosome in these species was significantly shorter in comparison with the XR and showed a significantly

larger heterochromatic part than was observed in the neo-Y chromosome of *A. turritus*. Regardless of the form of autosomal bivalents observed in the species studied, XR and the neo-Y chromosome in *N. cyanipes*, *P. rubripes* were associated only in the distal regions. A similar type of pairing was previously reported for species of the Nocarodeini tribe (Bugrov and Warchałowska-Śliwa 1997, Bugrov and Grozeva 1998).

In *P. opacus* we discovered a new chromosome sex determination system for Pamphagidae grasshoppers (neo-X_1X_2Y♂/neo-$X_1X_1X_2X_2$♀), which reflects the most advanced stage of sex chromosome evolution in the Nocarodeini tribe. This form of sex determination was the result of an additional reciprocal translocation involving the medium C-positive neo-Y-chromosome and a large autosome. As such, this chromosome reorganization might be considered the next step of sex chromosome evolution in comparison with *A. turritus*, *N. cyanipes*, *P. rubripes*, and species belong to the tribe Nocarodeini tribe. This led to the transformation of a typical Nocarodeini acrocentric neo-Y chromosome into a submetacentric neo-Y-chromosome. In this species, the unpaired acrocentric autosome becomes another heterosome – a neo-X_2 chromosome. This finding emphasizes once again the promising prospects for studying the Pamphagidae karyotype as a research model of sex chromosome evolution.

FISH using 18S rDNA with chromosomes of Pamphagidae grasshoppers showed that the clusters of rDNA differ in size and location among the species studied. In *E. festiva* they were found in the pericentromeric C-positive regions of all chromosomes, but could be different in size even on homologous chromosomes. In other species, rDNA clusters are usually localized in the pericentromeric, intercalary or telomeric region of two, three or four pairs of chromosomes, including the neo-X chromosome. It should be emphasized that in *A. turritus* one pair of autosomes showed double rDNA clusters. Three clusters of rDNA on one chromosome were previously reported for *Pamphagus ortolaniae* (Vitturi et al. 2008). Multiple localizations of rDNA clusters in a chromosome is a very rare type of rDNA cluster distribution (Cabrero and Camacho 2008, Jetybayev et al. 2012, Palacios-Gimenez et al. 2013).

In most of the species studied, the clusters of telomeric repeats were located in chromosome termini. However, some chromosomes of *E. festiva* showed ITSs (Fig. 1e). One of the possible sources of ITSs might be pericentric inversions, which led to the formation of biarmed chromosomes. However, ITSs were not revealed in the regions of chromosome fusion leading to neo-X, chromosome of *A. turritus*, *N. cyanipes*, *P. rubripes* and neo-X_1, neo-Y chromosomes in *P. opacus*. These findings suggest that translocation of the X-chromosome and autosome was accompanied with the loss of small regions containing telomeric repeats.

Conclusion

The karyotype of Pamphagidae grasshoppers was once considered to be among the very conservative (Camacho et al. 1981). However, we have found Armenian Pamphagidae grasshopper species to be characterized by intensive karyotypic evolution.

Overall, two different types of karyotype reorganization were found to be evidenced in Armenian Pamphagidae grasshoppers. In *E. festiva*, evolutionary chromosome rearrangements have led to a karyotype consisting of exclusively biarmed chromosomes with numerous C-positive regions enriched with repeats homologous to rDNA. We suppose that one of the important evolutionary processes that led to the formation of *E. festiva*'s modern biarmed karyotype involved the massive amplification of repetitive DNA.

The karyotypes of *A. turritus*, *N. cyanipes* and *P. rubripes*, in turn, were formed as a result of translocations involving the X chromosome and one of the autosomes. Furthermore, neo sex chromosomes showed additional evolutionary changes, namely, the formation of C-positive regions and the loss of euchromatic regions. Comparison of the sex chromosomes in these species revealed different stages of Y chromosome evolution.

The classical model of sex chromosome evolution postulates that sex chromosome degradation takes place due to suppression of recombination between parts of the sex chromosomes; in evolution the region of one of the sex chromosomes accumulates repetitive sequences and loses euchromatic gene-rich material. These processes lead to heterochromatinization and shrinking of one of the sex chromosome (Muller 1914, White 1973, Charlesworth et al. 2005, Traut et al. 2008). The neo-Y chromosomes of Pamphagidae grasshoppers have gone through the typical Y chromosome evolutionary stages, which could be identified in the species studied. Initial accumulation of C-positive blocks was observed in the neo-Y chromosome of *A. turittus,* whereas the neo-Y chromosome of *N. cyanipes* and *P. rubripes* showed an advanced stage of sex chromosome evolution associated with the loss of the part of C-negative regions. In *P. opacus*, the neo-Y chromosome have gone through a more complex rearrangement: a partial heterochromatization and fusion with an autosome, generating a complex system of sex chromosomes, (neo-X_1X_2Y♂/neo-$X_1X_1X_2X_2$♀).

Taken together, the different sets of sex chromosomes in the Armenian Pamphaginae species studied provide evidence that the Pamphagidae family offers an excellent model for studying the mechanisms of sex chromosome evolution. Its advantage lies in the availability of different stages of sex chromosome evolution, ranging from an initial XO♂/XX♀ sex determination system to a newly arisen neo-XY♂/XX♀ system, an advanced neo-XY♂/XX♀ system with significantly degraded neo-Y chromosome and even a very complicated neo-X_1X_2Y♂/ neo-$X_1X_1X_2X_2$♀ sex determination system.

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RESEARCH ARTICLE

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Identification of homogeneously staining regions by G-banding and chromosome microdissection, and FISH marker selection using human Alu sequence primers in a scleractinian coral *Coelastrea aspera* Verrill, 1866 (Cnidaria)

Takahiro Taguchi[1], Satoshi Kubota[1], Takuma Mezaki[2], Erika Tagami[3], Satoko Sekida[4], Shu Nakachi[2], Kazuo Okuda[4], Akira Tominaga[1,3]

1 *Division of Human Health and Medical Science, Graduate School of Kuroshio Science, Kochi University, Nankoku, Kochi 783-8505, Japan* **2** *Kuroshio Biological Research Foundation, Otsuki, Hata County, Kochi 788-0333, Japan* **3** *Department of Molecular & Cellular Biology, Kochi Medical School, Kochi University* **4** *Division of Marine Bioresources, Graduate School of Kuroshio Science, Kochi University, 2-5-1 Akebono-cho, Kochi 780-8520, Japan*

Corresponding author: *Takahiro Taguchi* (ttaguchi@kochi-u.ac.jp)

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Abstract

Karyotype analysis was performed on the scleractinian coral *Coelastrea aspera* Verrill, 1866, commonly found along temperate coasts in Japan (30–35°N) and in coastal waters in the Indian and Pacific oceans. G-banding of *C. aspera* was successfully performed, although the banding pattern was not as clear as that in mammals. The karyogram clearly revealed that this coral had a homogeneously staining region (hsr) in chromosome 11. This hsr consisted of ribosomal RNA (rRNA) related genes, which was demonstrated by fluorescence in situ hybridization (FISH) with probes generated using 28S ribosomal DNA (rDNA) primers and those generated through chromosome microdissection. In addition, we conducted silver-stained nucleolus organizer region (Ag-NOR) analysis and found Ag depositions in the interphase nuclei but not on rRNA gene loci and hsr(s) in the mitotic stage. The hsr of this coral was observed in approximately 50% of the metaphase spreads analyzed. This may explain the diversity of coral rDNA based on the molecular study of sequence analysis. Furthermore, it was discovered that human telomere and Alu repeated

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sequences were present in this *C. aspera*. Probes derived from human Alu sequences are expected to play an important role in the classification of corals. Overall, our data can be of great value in discriminating among scleractinian coral species and understanding their genetics, including chromosomal evolution.

Keywords

Coral, karyotype, hsr, FISH, chromosome microdissection, Alu repeats

Introduction

Species of the genus *Coelastrea* Verrill, 1866 belong to the family Merulinidae (Huang et al. 2014) and form massive colonies, usually spherical or elongated, with well-developed pale lobes. *Coelastrea aspera* Verrill, 1866 used in the present study is found in the Red Sea and Gulf of Aden, the southwest and northern Indian Ocean, the central Indo-Pacific, Australia, Southeast Asia, Japan and the East China Sea, the West Pacific, and the Central Pacific (DeVantier et al. 2014).

In scleractinian corals, the available chromosomal data, including their karyotypes and gene loci on chromosomes, has been limited, although many studies of other aspects of their biology such as ecology and physiology have been reported. Chromosomal evolution in corals occurs in closely related taxa within and at the species level (Kenyon 1997). The establishment of each karyotype among many coral species will help promote genetic research and coral genome projects (Shinzato et al. 2011).

In humans, a karyotype is usually established using the G-banding pattern shown on chromosomes (Seabright 1973). However, it has been difficult to get G-banding patterns in invertebrates, including scleractinian corals. Their G-banding pattern may facilitate the establishment of their karyotypes. Thus far, we have reported the karyotypes of two scleractinian corals belonging to the genera *Acropora* and *Echinophyllia* by molecular cytogenetic techniques (Taguchi et al. 2013, 2014). We found a characteristic chromosome with a homogeneously staining region (hsr)-like structure consisting of ribosomal RNA (rRNA) genes that were demonstrated by fluorescence in situ hybridization (FISH) in both corals (Taguchi et al. 2013, 2014). An hsr was originally found in mammalian cells as the result of a huge number of gene amplifications through drug-selection using methotrexate (Biedler and Spengler 1976). An hsr is a chromosomal segment(s) with various lengths and uniform staining intensity after G banding and sometimes found in mammalian cancer cells. This type of aberration has been known as an amplification of oncogenes (Takaoka et al. 2012). It is important to investigate if the presence of this hsr-like structure is a common phenomenon in coral chromosomes. Furthermore, there may be a possibility that the hsr-like structure involves not only rDNA but other specific sequences.

Obtaining FISH markers is not only profitable in helping to establish karyotypes but also in comparing syntenic relations among coral species. In our previous study (Taguchi et al. 2013), we selected a probe that was obtained from polymerase chain reaction (PCR) using the primers of human satellite DNA sequences and demonstrated some specific signals on the chromosomes of the coral *Echinophyllia aspera*, i.e., the fact that coral

DNA shared common sequences with humans, such as the telomere consensus sequence (TTAGGG)n (Zielke and Bodnar 2010) and satellite III, which contains the consensus sequence (TTCCA)n (Taguchi et al. 2013). These findings prompted us to screen other repeated DNAs from corals, which may share sequences with those of humans.

In this study, we analyzed *C. aspera* chromosomes using not only a conventional G-banding method but also molecular cytogenetic techniques. We successfully identified the hsr of *C. aspera* chromosome 11 using trypsin-treated G-banding, followed by karyotyping. Following that, we conducted chromosome microdissection (CMD) to regenerate DNA sequences consisting of the hsr to see if this involved not only rDNA but also some other specific sequences. Then, we applied CMD technique. Microdissected DNA was amplified by PCR and used as a painting probe of hsr. Moreover, to see the nature of the rRNA gene on the chromosomes of *C. aspera*, we performed silver-stained nucleolus organizer region (Ag-NOR) analysis (Bloom and Goodpasture 1976, Trerè et al. 2000) and found that interphase nuclei appeared as black dots combined with silver grains, but not on rRNA gene loci including hsrs on metaphase chromosomes. Furthermore, we revealed by FISH analysis that sequences homologous to human Alu repeats are found in *C. aspera* chromosomes and we showed that these DNA markers for FISH may become good tools for classifying corals.

Materials and methods

Coral collection

C. aspera is hermaphroditic; that is during spawning, eggs and sperm packed together into discreet bundles are released from the mouths of fertile polyps. The gametes of *C. aspera* were collected at Nishidomari (32°46'N; 132°43'E), in Kochi Prefecture, Japan (Fig. 1). The release of gamete bundles was observed between 8:45 pm and 9:15 pm on July 29, 2013; these bundles were collected in the field using plastic cups placed over part of the colonies during spawning. After collection, egg-sperm bundles were broken apart, and then eggs and fertilized eggs were rinsed using 0.2 μm filtered seawater (ADVANTEC cartridge filter; Advantec Toyo corp., Tokyo, Japan) to remove external contaminants.

Chromosome preparations and G-banding

We have previously reported the method for making coral chromosome preparations (Taguchi et al. 2013, 2014). In brief, 9–12 h after artificial fertilization, the embryos were treated with 0.005% (v/v) colchicine (Sigma, St. Lous, MO, USA) followed by a hypotonic solution to spread the chromosomes and then fixed with a fresh mixture of absolute methanol and glacial acetic acid (3:1). Fixed embryos were soaked in diethyl ether overnight to remove intracellular lipids and returned to the fixative. Twenty to fifty embryos were cut into several pieces for which a fine needle was used to tear them apart into their

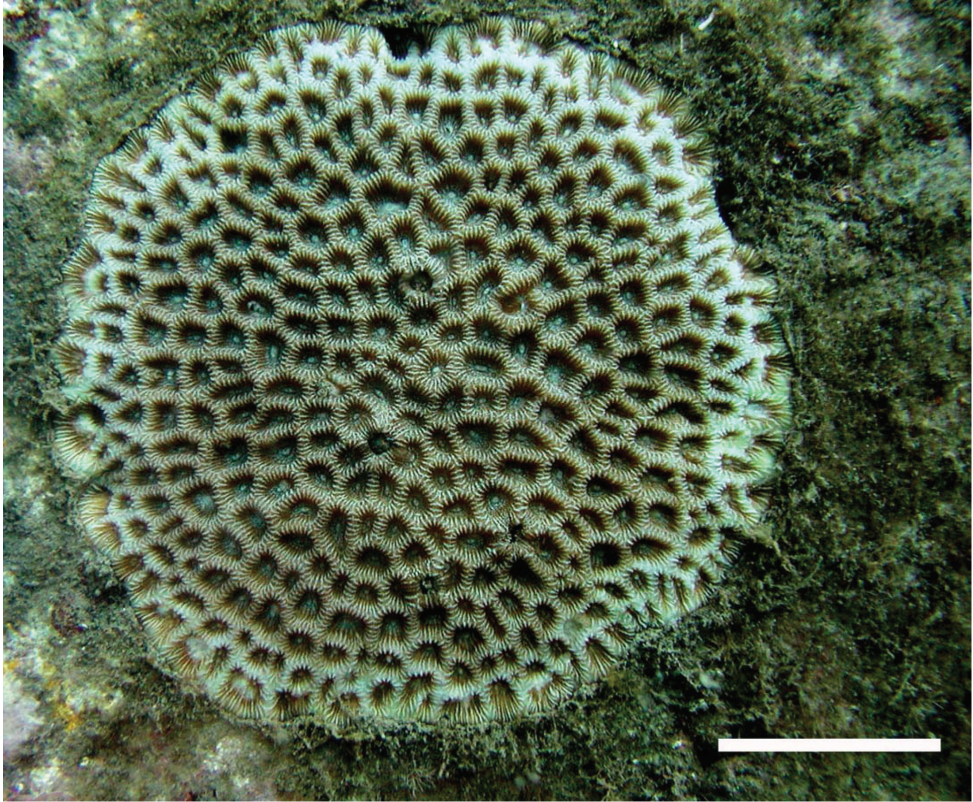

Figure 1. Appearance of *C. aspera* in the sea. Scale bar: 3 cm.

constituent cells. Suspensions containing these small pieces of embryos were transferred into a 1.5 mL tube filled with fixative. The tube was centrifuged at 2000 × *g* for 2 min and then the pellet was re-suspended in 0.5 ml of fresh fixative. One drop containing fragments of embryos and isolated cells was placed on a clean slide and then air-dried to spread the chromosomes. For G-banding, air-dried slides with metaphase spreads were treated with 0.025% trypsin solution for 1 min, and then stained with Giemsa solution (Wako, Osaka, Japan) that was diluted to 5% with 0.06 M phosphate buffer (pH 6.8) before use. Human metaphase cells were harvested from male normal lymphocytes, and slides were prepared according to standard methods (Taguchi et al. 1993).

Ag-NOR staining

Metaphase spreads on a slide were stained with silver solution (50% $AgNO_3$ solution at 37 °C for 1–5 h), which binds to the nucleolus organizing regions (NOR), i.e., the secondary constrictions (stalks) of acrocentric chromosomes in the case of humans (Bloom and Goodpasture 1976).

DNA extraction

Coral DNA from *C. aspera* embryos (approximately 200–300) or sperm (approximately 0.1 mL) was extracted using a Wizard genomic DNA purification kit purchased from Promega corporation (Madison, WI, USA) according to the manufacturer's instructions.

Chromosome microdissection of an hsr portion of chromosome 11

Five hsrs were scraped using a chromosome microdissection (CMD) technique as previously described (Taguchi et al. 2003). In brief, a glass needle with a diameter of about 2 µm was prepared from a glass capillary (GD-1, Narishige, Tokyo, Japan) using a pipette puller, PC-10 (Narishige). CMD was then performed using an inverted microscope (Olympus, Tokyo, Japan) with a glass needle attached to a mechanical micromanipulator, Eppendorf 5171 (Hamburg, Germany). Five microdissected hsr fragments were placed in a 0.5 mL tube, and then a 20 µL aliquot of digestion buffer containing 0.5 mg/mL proteinase K in 100 mmol/L Tris-HCl (pH 8.0) was added. Degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) was used to universally amplify the microdissected hsr DNA in a thermal cycler, WK-0518 (Wako, Osaka, Japan). The procedure used was essentially the same as that of Weber et al. (1998). PCR was conducted in a final volume of 50 µL containing 4 µL 25 mmol/L $MgCl_2$, 5 µL 10× PCR buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl, pH 8.0), 2 µL 5 mmol/L deoxynucleotide triphosphate, 5 µL 17 mmol/L primer 6 MW (5′-CCGACTCGAGNNNNNNATGTGG-3′, with N = A, C, G, and T), 0.5 µL of (2.5 U) Taq polymerase (Takara, Japan), and 20 µL of microdissected DNA in 100 mmol/L Tris-HCl, pH 8.0. The PCR conditions used were as follows: 10 min at 93 °C, followed by 10 cycles, each of 1 min duration at 94 °C, 1.3 min at 30 °C, 3 min transition at 30–72 °C, and 3 min extension at 72 °C; subsequently, 35 cycles, each of 1 min duration at 94 °C, 1 min at 62 °C, 3 min at 72 °C, and an additional 1 s/cycle to the extension step, and a final extension of 10 min.

Generation of probes of rRNA genes and human Alu DNA sequences

The probes for the rRNA genes and Alu-derived DNAs were obtained from PCR products using the primers described by Chen et al. (2000) and Watson et al. (1992), respectively. The gene encoding nuclear partial 28S rDNA for Acropora and human Alu-derived DNAs was amplified from *C. aspera* embryo DNA by a polymerase chain reaction (PCR), using 28s rDNA forward 5′-GGCGACCCGCTGAATTCAAGCAT-AT-3′ and reverse 5′-GCTTTGGGCTGCAGTCCCAAGCAACCCACTC-3′ primers (Chen et al. 2000). For the human Alu DNA sequence, the forward 5′-AACGTCACTCGGCTCTA-3′ and the reverse 5′- TTGCAGTGAGCCGAGAT -3′ primers were used (Watson et al. 1992). PCR was performed using a thermal cycler

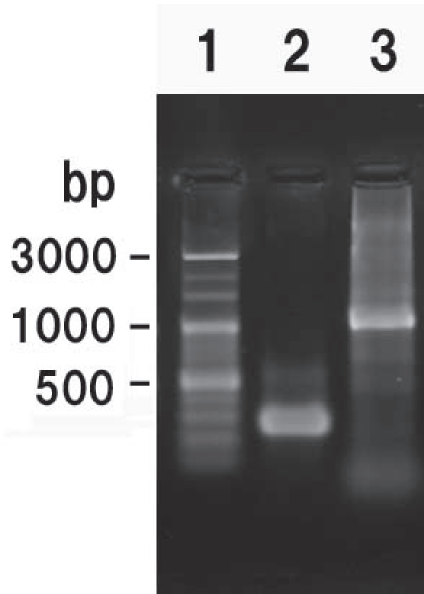

Figure 2. Electrophoresis of PCR products from the partial rDNA and the sequence generated by human Alu primers. **1** 100bp marker **2** 28S rDNA **3** Alu.

(WK-0518, Wako, Osaka, Japan). The PCR reaction conditions for 28S rDNA were as follows: pre-heating for 2 min at 95 °C, followed by 4 cycles at 94 °C (30 s), 60 °C (1 min) and 68 °C (3.5 min), and 30 cycles at 94 °C (30 s), 56 °C (30 s), and 72 °C (1 min), with a final extension at 72 °C for 10 min. The PCR reaction conditions for human Alu DNA was as follows: pre-heating for 2 min at 98 °C, followed by 30 cycles at 98 °C (10 s), 55 °C (30 s), and 72 °C (1 min). Figure 2 shows the electrophoresis image of PCR product obtained by the 28S rDNA and human Alu primers. The amplified bands on the gel (200–300 bp band for 28S rDNA; 1000 bp band for Alu; Fig. 2) were cut out and purified using the QIAquick PCR purification kit (Quiagen) according to the manufacturer's instructions.

FISH

A human digoxigenin-labeled telomere probe was purchased from Appligene Oncor (Lifescreen, Watford, UK). Random prime labeling of the probe DNA from PCR products was performed with fluorescein-12-dUTP (F-dUTP) or cyanine-3-dUTP (Cy3-dUTP) in accordance with the kit protocol (Invitrogen, Tokyo, Japan). FISH was conducted as previously reported (Takaoka et al. 2012), with slight modifications. In brief, the metaphase preparations were denatured in 70% formamide/2× saline-sodium citrate at 73 °C for 3 min. Then 1 μl of probe was mixed with 10 μl of hybridization solution (H7782, Sigma, Japan) and denatured at 80 °C for 10 min. Hybridiza-

tion of the probes was performed at 37 °C in the CO_2 incubator for 12–15 h, followed by post-hybridization washes, DAPI (4',6-diamidino-2-phenylindole) counterstaining and visualization of the probes under a fluorescence microscope (Olympus BX50).

Image acquisition and processing

The slides were examined with an Olympus BX-50 fluorescence microscope. Images of suitable metaphase spreads and interphase nuclei were acquired on an Olympus DP70 microscope workstation equipped with a cooled charge-coupled device and FISH analysis software. The miller units used for each fluorescence light (FITC, Cy-3 and DAPI) were U-NIBA, U-MWU, and U-MWIB (Olympus), respectively.

Results

The diploid karyotype in *C. aspera*

A survey of 50 metaphase plates of *C. aspera* identified a complement of 28 chromosomes. Then we tried to make a conventional trypsin G-banding (Fig. 3A) and eight metaphase cells were karyotyped. Consequently, an hsr was identified in the long arm of chromosome 11 (Fig. 3B). During the survey of G-banded metaphase spreads, 31 metaphase spreads with an hsr were found in 60 cells examined (about 50%). A tentative karyogram was shown in Figure 3B. In this karyogram, chromosomes were arranged in decreasing order of chromosome length from 1 to 14. While chromosome 11 was easily identified due to the hsr and the location of its rRNA genes, other chromosomes were not so easy to distinguish precisely because of the poor G-banding pattern and similarities in chromosome length and centromere location. The karyogram consisted of thirteen submetacentric (pairs 1-13) and one metacentric (pairs 14) chromosomes (Fig. 3B).

FISH mapping of rRNA genes and human telomere sequences, and Ag-NOR staining

Localizations of the rRNA genes by FISH are shown in Figure 4. The rRNA gene loci are mapped on one pair of homologous chromosome 11. Figure 4 shows the extra-large yellowish-green domain (left) with an hsr as well as small signals (right) in the metaphase spreads. The rate of metaphase spreads with an hsr was more than 50% (31/60). FISH analysis revealed that the human telomeric probe (TTAGGG)n hybridized intensely to the telomeres (red fluorescence) of all *C. aspera* chromosomes, suggesting that the telomere sequences of this coral may be identical to those of humans (Fig. 4). Ag-NOR staining showed five to eight dark dots on interphase nuclei, but no dot seemed to be

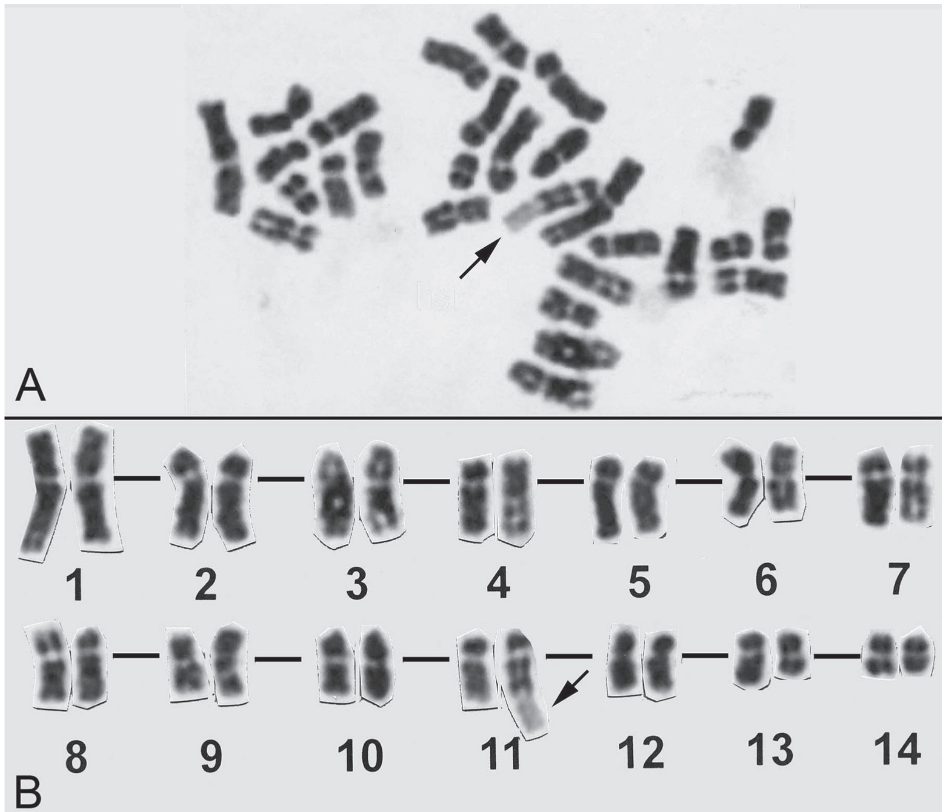

Figure 3. A G-banded metaphase spread (**A**) and its karyogram (**B**). Both stained with Giemsa. Arrows indicate hsrs.

seen on any chromosomes of *C. aspera* (Fig. 5A). The human metaphase spread, which was positive, was stained with $AgNO_3$ at the same time as a control (Fig. 5B, C).

CMD and FISH

Five hsr portions were scraped from five chromosome 11 by a glass needle (Fig. 6C, D). DOP-PCR products from these five microdissected chromosomes were observed as smear electrophoretic bands of DNA with a range from 200 to 900 bp on a 2% agarose gel, with most fragments being concentrated between around 400 and 600 bp. Screening with direct PCR probes by FISH demonstrated that the product had a high specificity not only on the hsr region of chromosome 11 but also on the terminal region of another chromosome (probably chromosome 2, Fig. 6A; arrows). FISH signals were also detected in an interphase nucleus. This microdissected DNA mainly consists of rDNA and may also contain some other sequences.

Figure 4. A dual color FISH image of *C. aspera* obtained by a probe generated by PCR using rRNA gene primers for 28S (green) and human telomere probe (red). Arrowheads indicate rRNA genes loci on one homologous chromosome 11 (one has an hsr with a long and large green signal). Scale bar: 5 µm.

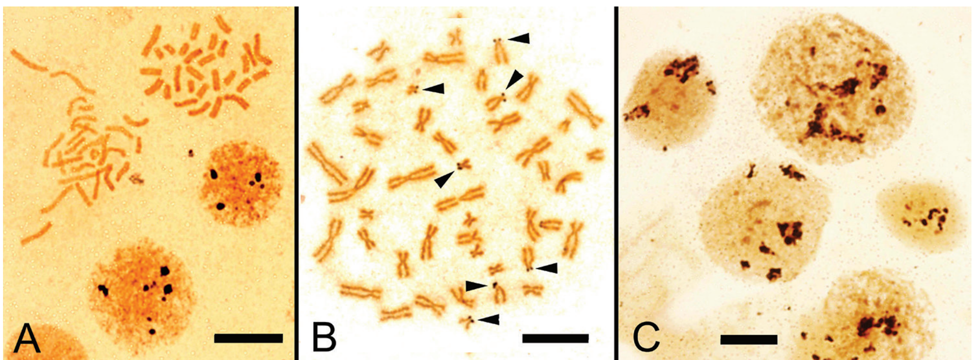

Figure 5. Ag-NOR staining. **A** Interphase nuclei and metaphases of *C. aspera*. Note that five to seven dark domains on interphase nuclei were seen but did not seem to appear on two metaphases **B** The human metaphase spread with black dots on the seven acrocentric chromosomes (arrowheads) **C** Human interphase nuclei. Scale bar: 10 µm.

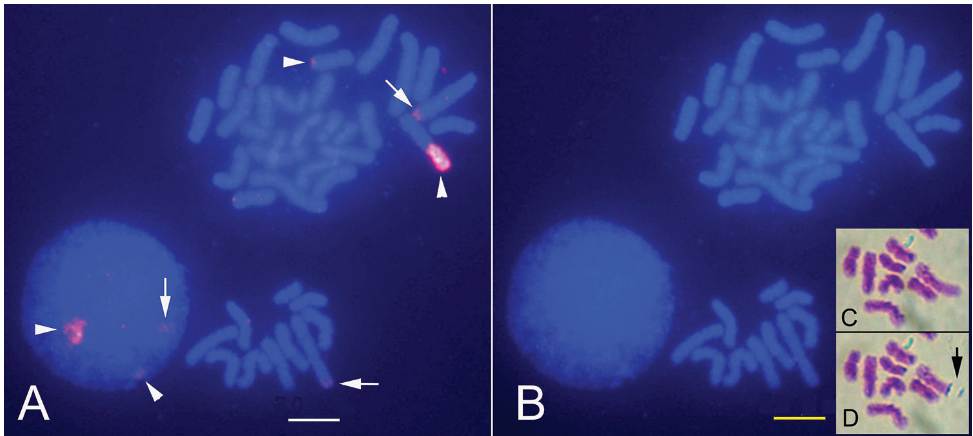

Figure 6. FISH signal by the probe from the microdissection-generated DNA probe of *C. aspera*'s hsr. **A** Metaphase cells with a distinct signal on the hsr (an arrowhead). Note that the hsr-derived probe hybridized not only with rRNA gene (arrowheads) but also with terminal ends of other chromosomes (arrows) in both full (2n=28, upper) and partial (lower) metaphases **B** The same cells were stained with DAPI. Chromosome microdissection of the hsr in chromosome 11 **C** Before microdissection of the hsr **D** After scraping of the hsr (an arrow). Scale bar: 5 µm.

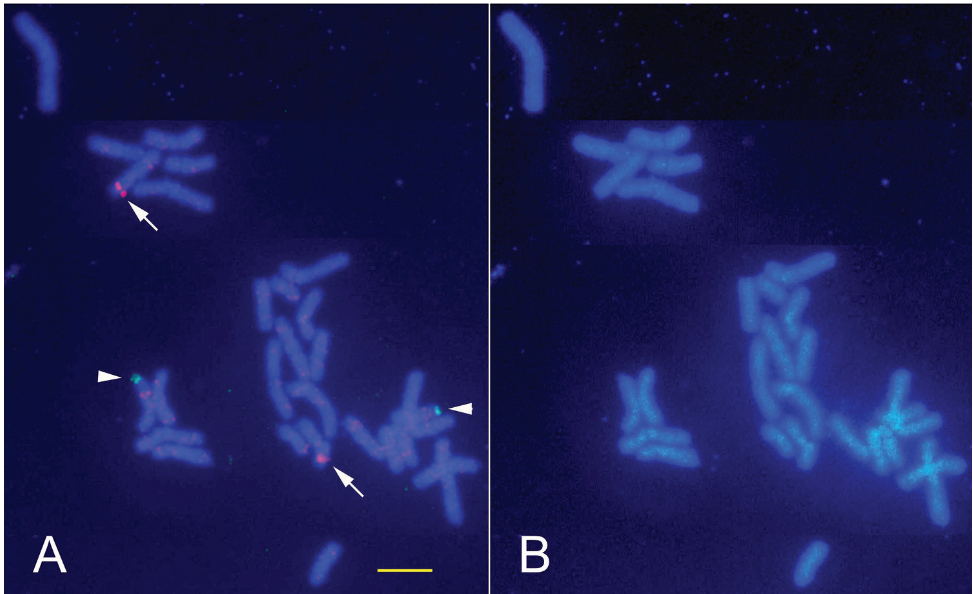

Figure 7. Dual FISH image with human Alu-derived and rRNA gene probes. **A** Red FISH signals by human Alu-repetitious DNA-derived probe were seen on two homologous chromosomes (arrowheads). Green signals by 28S rRNA genes (arrows). In this cell, no hsr was seen **B** The same chromosomes stained with DAPI. Scale bar: 5 µm.

Detection of human Alu sequence by FISH

Figure 7A shows four red signals on the FISH image of *C. aspera* obtained by the probe generated from the human Alu sequence primers. However, there were many scattered small faint red signals, suggesting that other Alu sequences were present. Two distinct reddish signals are seen on each telomeric region of two chromosomes of *C. aspera* (Fig. 7A).

Discussion

There are more than 800 species of scleractinian corals in the world (Veron 2000). There are discrepancies between conventional taxonomy and molecular data (Fukami et al. 2004). To promote understanding of coral genetics, we have been trying to accumulate molecular cytogenetic data for scleractinian corals. So far, we have published two molecular cytogenetic reports with FISH using the scleractinian corals, *Acropora solitaryensis* (Acroporidae) and *E. aspera* (Pectiniidae) (Taguchi et al. 2013, 2014), which are both distributed on the western sea coast of Japan (Wallace 1999). The present study is the third molecular cytogenetic report using *C. aspera* belonging to the third family Merulinidae.

We carried out G-banding to establish the *C. aspera* karyotype using embryos. It is difficult in general to obtain a high quality G-banding in invertebrate chromosomes due to the relatively small chromosome sizes and insufficient digestion by trypsin. In this coral, we could produce a G-banding pattern on chromosomes by trypsin-treatment, although it was not fully satisfactory (Fig. 3). By G-banding, we could arrange each chromosome in decreasing order of its length and identify chromosome 11 with an hsr, the presence of which we have already predicted in a previous study through FISH analysis in two corals (*Acropora* and *Echinophyllia*). Visualization of rRNA (28S) gene and telomere sequences by FISH mapping revealed that the rDNA gene locus was on the terminal end of a long arm of chromosome 11, and the telomere sequences were quite similar to humans (TTAGGG)n because we used a human telomere probe as suggested by molecular data (Zielke and Bodnar 2010) and our previous studies (Taguchi et al. 2013, 2014). An hsr was composed of rRNA genes because FISH signals of the rDNA probe were highlighted. It is of interest that large signals seen in the elongated chromosome 11 were confirmed, by G-banding, to be on an hsr which is sometimes seen in human cancer cells as a resultant of oncogene amplification (Takaoka et al. 2012). An hsr was originally found in mammalian cells as the result of a huge number of gene amplifications through drug-selection using methotrexate (Biedler and Spengler 1976). Based on our recent studies of three scleractinian coral species, including *C. aspera*, an hsr found in the coral chromosomes seemed to be a common and consistent feature in scleractinian corals (Taguchi et al. 2013, 2014). We also observed an hsr-like structure in the two other corals, *Micromussa amakusensis* Veron, 1990 and *Trachyphyllia geoffroyi audouin* Audouin, 1826 (data not shown).

To verify the nature of an hsr composer of rDNA cytogenetically, we tried to visualize NOR proteins using Ag-NOR staining (Bloom and Goodpasture 1976). When we performed Ag-NOR analysis with *C. aspera*, human metaphase spreads were stained at the same time as a control (about seven pairs of dots appeared on the chromosome stalks of acrocentric chromosomes and several domains seen on human interphase nuclei; Fig. 5B, C). Consequently, five to eight dark dots on the interphase nuclei of the coral were stained with Ag-NORs, whereas no dot seemed to be present on any chromosomes, including hsrs (Fig. 5A). Ag depositions produced by Ag-NOR appear on Ag-NOR related proteins (Trerè et al. 2000). Ag-NOR related proteins remain at metaphase in humans but not in *C. aspera* (Fig. 5). The fact that no Ag deposition was observed on rRNA gene loci of the coral metaphase chromosomes suggests that Ag-NOR related proteins did not remain on the coral chromosomes, unlike in humans and, as far as known, in all other studied plants and animals (Rodionov 1999).

To elucidate if the hsr of *C. aspera* is composed only of rDNA or includes some other sequences together with rDNA, we carried out CMD, followed by FISH. We successfully regenerated probes made from the microdissected DNA by DOP-PCR. The FISH experiment confirmed that these probes hybridized on rRNA gene loci and on the terminal end of another chromosome. This suggested that the hsr consists not only of rRNA genes but also some other repeated sequences.

In the previous study (Taguchi et al. 2013), we obtained a probe by PCR using the primers of human satellite III sequences (Fowler et al. 1988) and it gave some specific FISH signals on the chromosomes of the coral *E. aspera*. We also revealed that this probe from coral DNA used satellite III primers strongly hybridized to the human chromosome 9 centromere. This indicated that scleractinian coral DNA shared common sequences to not only the human telomere sequence (TTAGGG)n (Zielke and Bodnar 2010) but also human satellite III repetitive sequences. In this study, we confirmed the presence of another human-related Alu-repeated sequence in *C. aspera*, based on the fact that the Alu-derived probes gave FISH signals to one or two specific portions of some chromosomes. The Alu-repeat family is one of several families consisting of repetitive elements in the human genome. An Alu element is a short stretch of DNA originally characterized by the action of the Alu (*Arthrobacter luteus*) restriction endonuclease (Schmid and Deininger 1975). Alu elements are about 300 base pairs long and are therefore classified as short interspersed elements (SINEs) among the class of repetitive DNA elements. Different kinds of Alu elements occur in large numbers of primates and comprise 11% of the human genome (Kriegs et al. 2007). They have wide-ranging influences on gene expression (Häsler and Strub 2006). We also observed that human satellite DNAs tended to hybridize to the rDNA of *C. aspera* (data not shown in the results). These suggest that some of the repetitive sequences may be conserved both in corals and humans, as reported in our previous study of *E. aspera* (Taguchi et al. 2013). Highly repetitive DNA sequences may become a good tool for identifying phylogenetic positions (Koga et al. 2012). The human-derived probes from satellite III and Alu that we used, would be useful for the detection of specific locations on chromosomes to conduct more precise karyotyping and for criteria classifying coral species.

In conclusion, we have successfully performed chromosome analysis by using conventional banding and molecular cytogenetic techniques, such as G-banding, CMD and FISH. The telomere sequences and rRNA genes could be mapped on the *C. aspera* chromosomes. Our research suggests that the telomere sequence of *C. aspera* may be identical to the human telomere sequence. We demonstrated through G-banding that *C. aspera* has an hsr on chromosome 11. CMD-FISH revealed that the elongated segment of chromosome 11 was an hsr and was occupied by rDNAs and some other sequences. Metaphase spreads with an hsr were seen in more than 50% of cells observed. These new findings would lead to surveys of other scleractinian coral species to find clues in solving difficulties in taxonomy.

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Evolutionary trends in the family Curimatidae (Characiformes): inferences from chromosome banding

Tatiane Ramos Sampaio[1], Larissa Bettin Pires[1], Natália Bortolazzi Venturelli[1], Mariana Campaner Usso[1], Renata da Rosa[1], Ana Lúcia Dias[1]

1 *Departamento de Biologia Geral, CCB, Universidade Estadual de Londrina, P.O Box 6001, Londrina, Paraná CEP 86051-970, Brazil*

Corresponding author: *Ana Lúcia Dias* (anadias@uel.br)

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Abstract

The family Curimatidae is a fish group usually considered chromosomally conserved in their diploid number. However, some studies show small changes in the karyotype microstructure, and the presence of B chromosomes, indicating a chromosomal diversification within the group, even if structural changes in the karyotypes are not visible. Few studies associate this trait with an evolutionary pattern within the family. This study aimed to characterize the karyotype, nucleolus organizer regions (NORs), and heterochromatin distribution of six species of Curimatidae of the genera *Cyphocharax* Fowler, 1906 and *Steindachnerina* Fowler, 1906: *C. voga* (Hensel, 1870), *C. spilotus* (Vari, 1987), *C. saladensis* (Meinken, 1933), *C. modestus* (Fernández-Yépez, 1948), *S. biornata* (Braga et Azpelicueta, 1987) and *S. insculpta* (Fernández-Yépez, 1948) and contribute data to a better understanding of the mechanisms involved in the chromosomal evolution of this group of fish. All specimens had 2n=54, m-sm, and B microchromosomes. Five species exhibited single NORs, except for *S. biornata*, which showed a multiple pattern of ribosomal sites. NORs were chromomycin A_3 positive (CMA_3^+) and 4'-6-diamino-2-phenylindole ($DAPI^-$) negative, exhibiting differences in the pair and chromosomal location of each individual of the species. FISH with 5S rDNA probe revealed sites in the pericentrometic position of a pair of chromosomes of five species. However, another site was detected on a metacentric chromosome of *C. spilotus.* Heterochromatin distributed both in the pericentromeric and some terminal regions was revealed to be CMA_3^+/$DAPI^-$. These data associated with the previously existing ones confirm that, although Curimatidae have a very conservative karyotype macrostructure, NORs and heterochromatin variability are caused by mechanisms of chromosome alterations, such as translocations and/or inversions, leading to the evolution and diversification of this group of fish.

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Keywords
Fluorochromes, heterochromatin, karyotype evolution, pisces, rDNA

Introduction

Cytogenetic studies in Neotropical fish reveal great chromosome diversity with both intra- and interspecific karyotype variability. Within the order Characiformes, there are two distinct trends: groups that show a significant difference in diploid number and/or karyotype formulae and karyotypically homogeneous groups (Galetti et al. 1994). Given these trends, the family Curimatidae belongs to the second group. Of the 101 described species (Netto-Ferreira et al. 2011), 38 have been cytogenetically assessed. The studies revealed that 32 of latter exhibited a diploid number (2n) of 54 chromosomes and a fundamental number (FN) equal to 108 (Sampaio et al. 2011).

Small changes in the karyotype microstructure involving the nucleolus organizer regions (NORs) and heterochromatin distribution pattern occur as a result of chromosomal evolution. Such alterations can be regarded as relevant cytogenetic markers. Consequently, despite being considered conserved, some species of this group present exceptions to the observed regularity, allowing inferences about the evolutionary pathways within the family (Galetti Jr. et al. 1994; Galetti Jr. 1998).

Another feature considered a chromosomal diversification within Curimatidae is the presence of B chromosomes in some species (Vênere et al. 2008). This chromosome, also called supernumerary or accessory, may exhibit either a similar morphology to that of the chromosomes of the A complement, or one that is to a clearly distinct. The number of Bs may vary among the different cells of the same individual in species that possess them. This variation may be ascribable to an anaphasic delay, with the removal of B from some cells or tissues, or to meiotic nondisjunction, when both chromatids migrate to the same pole (Camacho et al. 2000). Hitherto, B chromosomes have been described in seven species of Curimatidae of different populations: *Cyphocharax gouldingi* Vari, 1992, *C. modestus* (Fernández-Yépez, 1948), *C. saladensis* (Meinken, 1933), *C. spilotus* (Vari, 1987), *C. voga* (Hensel, 1870), *Steindachnerina biornata* (Braga & Azpelicueta, 1987) and *S. insculpta* (Fernández-Yépez, 1948) (Sampaio et al. 2011; Vênere et al. 2008).

Although a number of cytogenetic studies show conservation of the diploid number (2n=54) in the family Curimatidae, divergence of nucleolus organizer regions and C-banding was observed. Nevertheless, few studies correlate the cytogenetic characteristics to the evolutionary trends within the family. Thus, this study aimed to characterize the karyotype, nucleolus organizer regions (NORs), and heterochromatin distribution of six species of Curimatidae of the genera *Cyphocharax* Fowler, 1906 and *Steindachnerina* Fowler, 1906, as well as contribute to a better understanding of the mechanisms underlying the chromosomal evolution of this interesting group of fish.

Materials and methods

Collection sites

Six species of the family Curimatidae were analysed: *Cyphocharax voga*, *C. spilotus*, *C. saladensis*, *C. modestus*, *Steindachnerina biornata* and *S. insculpta*, collected from the Laguna dos Patos Hydrographic System/RS, Tramandaí River basin/RS, and Paranapanema River basin/SP/PR (Table 1). Voucher specimens are catalogued in the Zoology Museum of the Universidade Estadual de Londrina, Paraná, under catalog numbers: MZUEL 1374 - *Cyphocharax modestus*; MZUEL 5058 - *C. saladensis*; MZUEL 5106 - *C. spilotus*; MZUEL 5105 - *C. voga*; MZUEL 5059 - *Steindachnerina biornata*; MZUEL 1042 - *S. insculpta*.

Table 1. Species, collection sites and hydrographic basins.

Species	Number of individuals	Collection sites	Hydrographic basin
Cyphocharax modestus	5♀, 6♂	Três Bocas stream, Londrina, PR, Brazil S 23°17'12.9" W 51°13'58.2"	Paranapanema river
Cyphocharax saladensis	1♀, 9♂	Agronomic Experiment Station of UFRGS's Dam, Eldorado do Sul, RS, Brazil S 30°05'33.7" W 51°40'40.0"	Laguna dos Patos hydrographic system
Cyphocharax spilotus	2♀, 2♂	Capivara stream, Barra do Ribeiro, RS, Brazil S 30°17'34.0" W 51°19'21.2"	
	1♂	Gasômetro, Porto Alegre, RS, Brazil S 30°02'06.3" W 51°14'29.12"	
Cyphocharax voga	1♀, 1♂	Saco da Alemoa river, Eldorado do Sul, RS, Brazil S 29°59'15.6" W 51°14'24.1"	
	3♀, 9♂	Capivara stream, Barra do Ribeiro, RS, Brazil S 30°17'34.0" W 51°19'21.2"	
	1♀, 3♂	Gasômetro, Porto Alegre, RS, Brazil S 30°02'06.3" W 51°14'29.12"	
	5♂	Barros lagoon, Osório, RS, Brazil S 29°56'30.0" W 50°19'32.0"	
	3♀, 4♂	Quadros lagoon – Barra do João Pedro, Maquiné, RS, Brazil S 29°46'21.2" W 50°05'08.0"	Tramandaí river
Steindachnerina biornata	1♀, 1♂	Forquetinha river, Canudos do Vale, RS, Brazil S 29°24'22.4" W 52°03'19.2"	Laguna dos Patos hydrographic system
Steindachnerina insculpta	3♀, 2♂	Três Bocas stream, Londrina, PR, Brazil S 23°17'12.9" W 51°13'58.2"	Paranapanema river
	2♂	Pavão stream, Sertanópolis, PR, Brazil	
	6♀, 12♂	Jacutinga river, Londrina, PR, Brazil S 23°23'6.6" W 51°04'35.8"	
	3♀, 7♂	Água dos Patos river, Iepê, SP, Brazil S 23°12'23.3" W 50°56'49.1"	
Total of individuals:	93		

Conventional staining

Mitosis was stimulated by injecting animals with a yeast suspension (Lee and Elder 1980). Mitotic chromosomes were obtained by direct preparation, removing the anterior kidney, with hypotonic treatment, methanol:acetic acid fixation and air-drying (Bertollo et al. 1978). Lastly, the chromosomes were stained with 5% Giemsa in phosphate buffer (pH 6.8), and classified as metacentric (m) and submetacentric (sm) (Levan et al. 1964).

Chromosome Banding

The distribution of heterochromatin was analyzed by C-banding (Sumner 1972). Silver nitrate staining of the active nucleolus organizer regions (AgNOR) was performed according to Howel and Black (1980). The GC and AT-rich bands were detected using Chromomycin A3 (CMA3) and 4',6-diamidino-2-phenylindole (DAPI), respectively, according to Schweizer (1980).

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) followed the methods described by Pinkel et al. (1986) with an 18S rDNA probe obtained from *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka and Galetti Jr. 2004). The 18S rDNA probe was labeled with biotin-14-dATP (Roche Applied Science) by nick translation and the 5S rDNA probe from *Leporinus elongatus* Linnaeus, 1758 (Martins and Galetti Jr. 2001) was labeled with digoxigenin 11-dUTP (Roche Applied Science) by PCR. The hybridization signal was detected using avidin-FITC (fluorescein isothiocyanate) (Life Technologies) for the 18S rDNA probe and anti-digoxigenin-rhodamine (Roche Applied Science) for the 5S rDNA probe. The chromosomes were counterstained with propidium iodide or DAPI, respectively. All the images were acquired with a Leica DM 4500 B microscope equipped with a DFC 300FX camera and Leica IM50 4.0 software and optimized for best constrast and brightness with Adobe Photoshop CS6 software.

Results

All species analyzed showed 54 meta-submetacentric chromosomes (m-sm) and fundamental number (FN) equal to 108. All populations presented individuals with B microchromosomes of a dot type in all somatic cells (Figs 1, 2). Terminal secondary constrictions occurred in *Cyphocharax voga* and *Steindachnerina biornata*, on the long arm of pairs 5 and 3, respectively (Figs 2a, b, box), and in the interstitial position of *Cyphocharax spilotus*, on the short arm of the second pair (Fig. 1c, box).

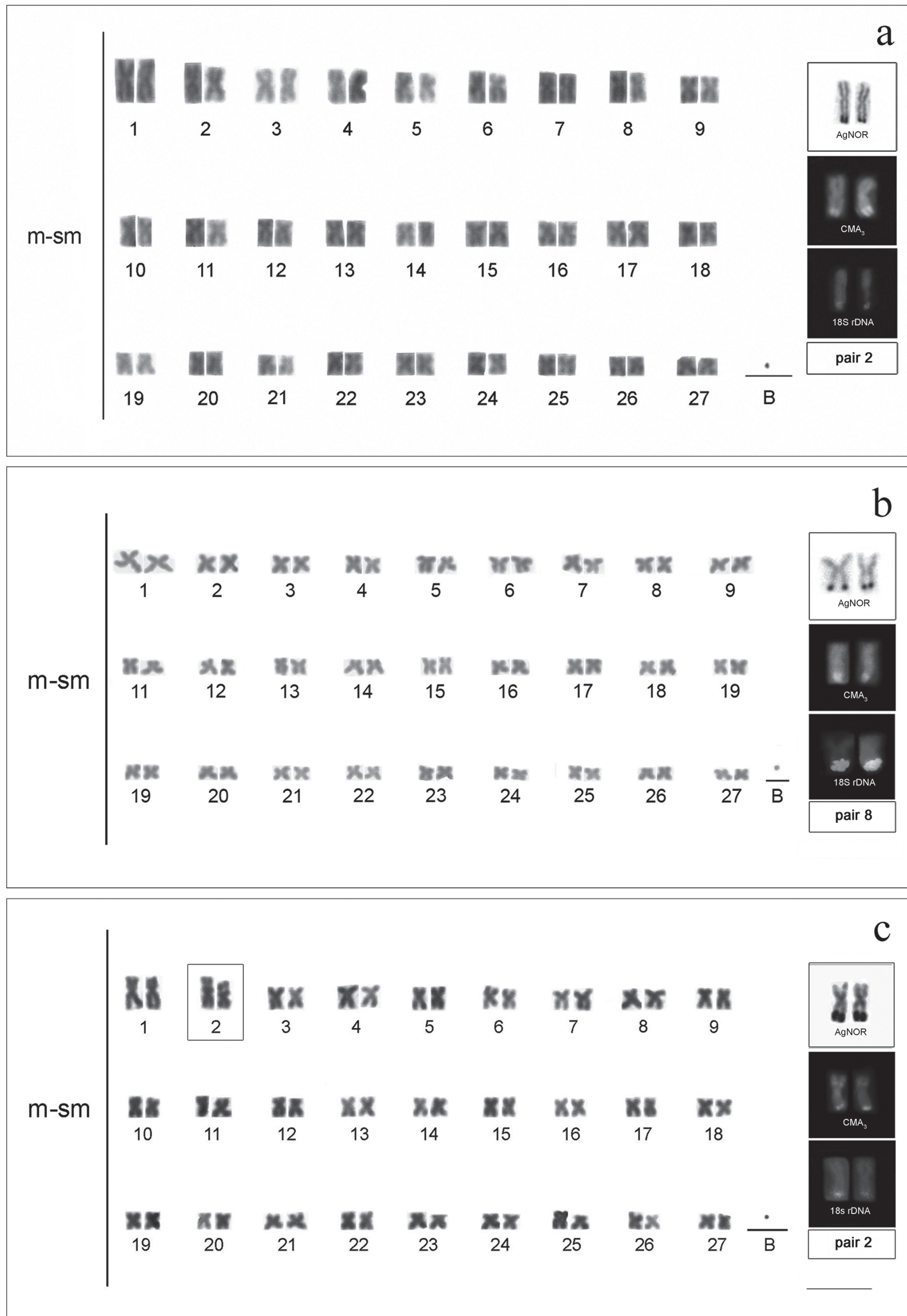

Figure 1. Karyotypes with B microchromosome of: **a** *Cyphocharax modestus* **b** *Cyphocharax saladensis* **c** *Cyphocharax spilotus,* showing AgNORs, CMA_3 and 18S rDNA sites of each species. Note the secondary constrictions in square box (**c**). Bar: 5 µm.

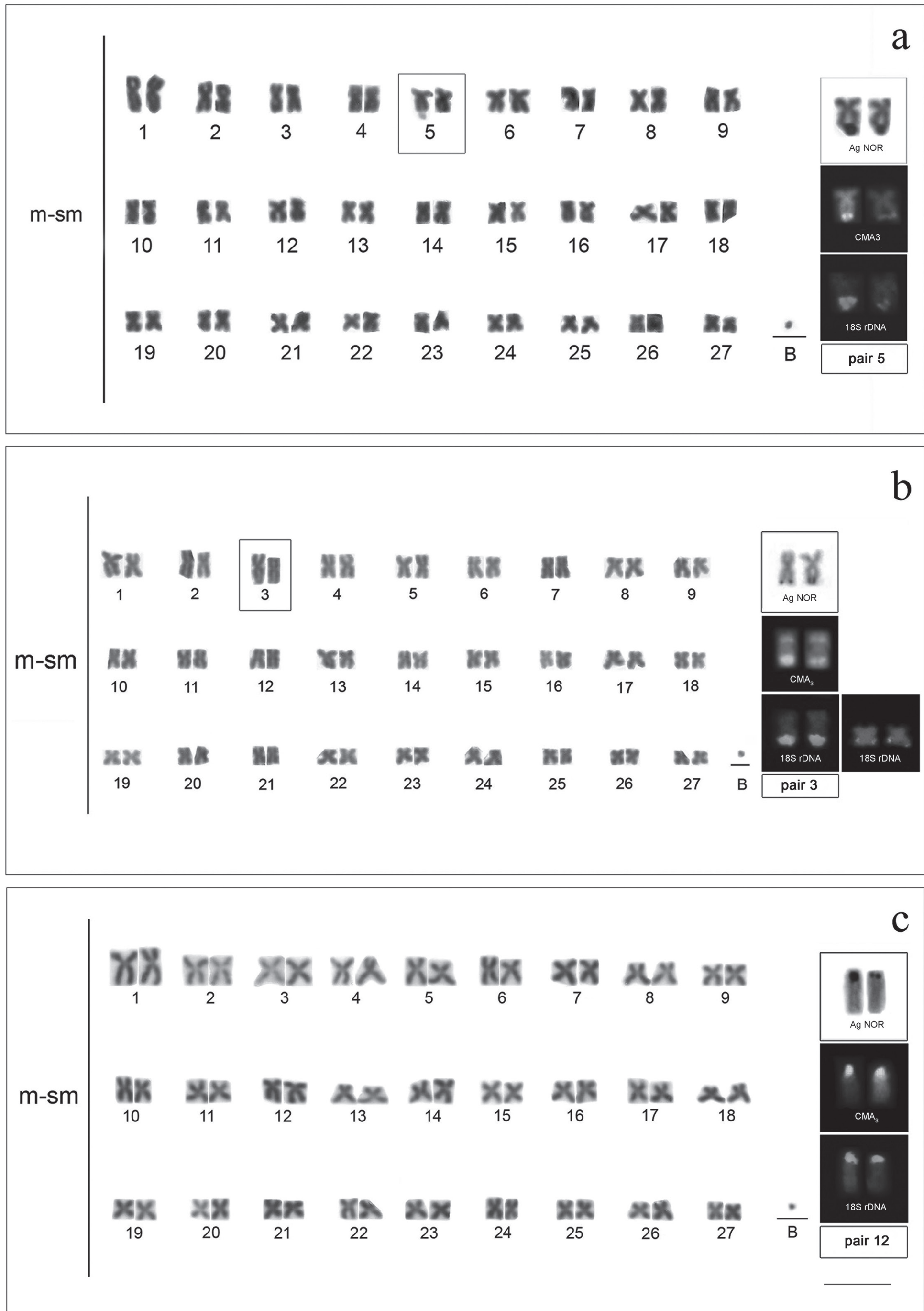

Figure 2. Karyotypes with B microchromosome of: **a** *Cyphocharax voga* **b** *Steindachnerina biornata* **c** *Steindachnerina insculpta*, showing AgNORs, CMA_3 and 18S rDNA sites of each species. Note the secondary constrictions in square box (**a**, **b**). Bar: 5 µm

Table 2. Chromosome pairs and positions of the nucleolus organizer regions (AgNORs).

Species	AgNOR pair	AgNOR position on chromosome	Secondary constriction
Cyphocharax modestus	02	Terminal/long arm	------
Cyphocharax saladensis	08	Terminal/long arm	------
Cyphocharax spilotus	02	Terminal/long arm	Interstitial/short arm
Cyphocharax voga	05	Terminal/long arm	Terminal/long arm
Steindachnerina biornata	03	Terminal/long arm	Terminal/long arm
Steindachnerina insculpta	12	Terminal/short arm	------

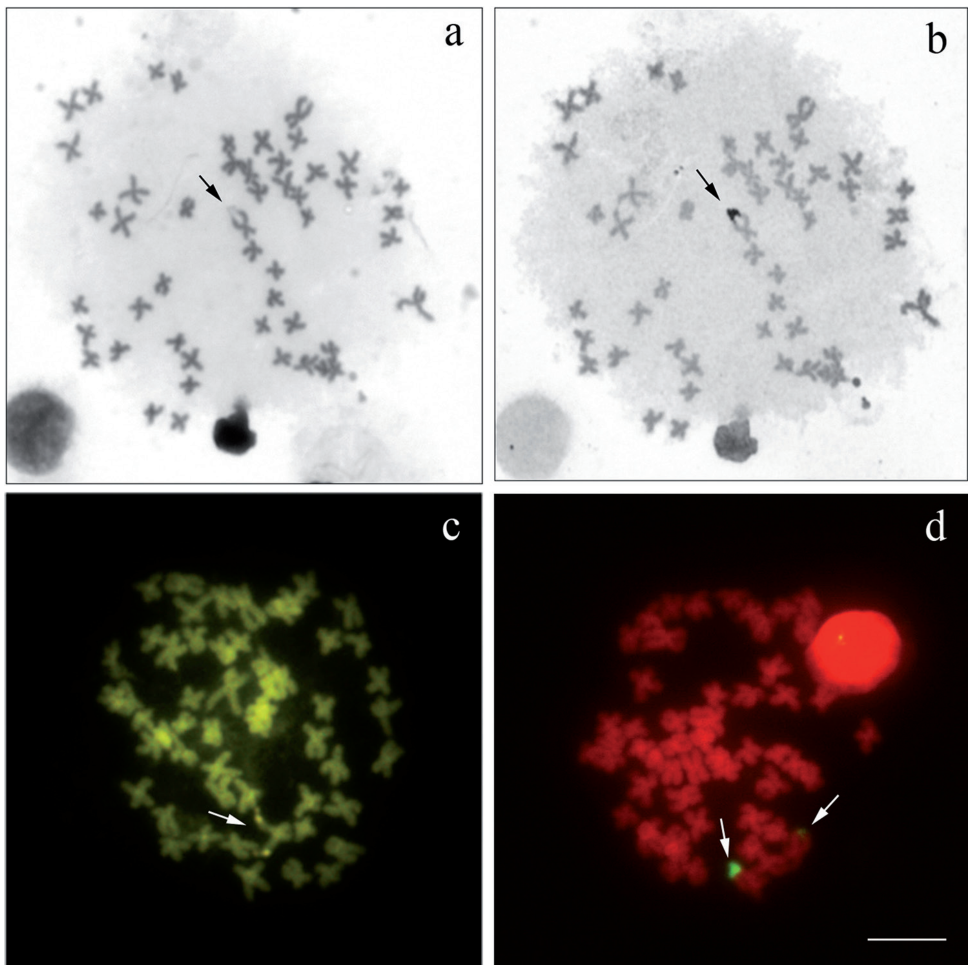

Figure 3. Metaphases of *Cyphocharax voga* (Barros lagoon/RS): **a** Giemsa **b** AgNOR (sequential) **c** CMA_3 **d** 18S rDNA FISH. The arrows indicate the chromosome carrying the secondary constriction and AgNOR. Bar: 5 µm.

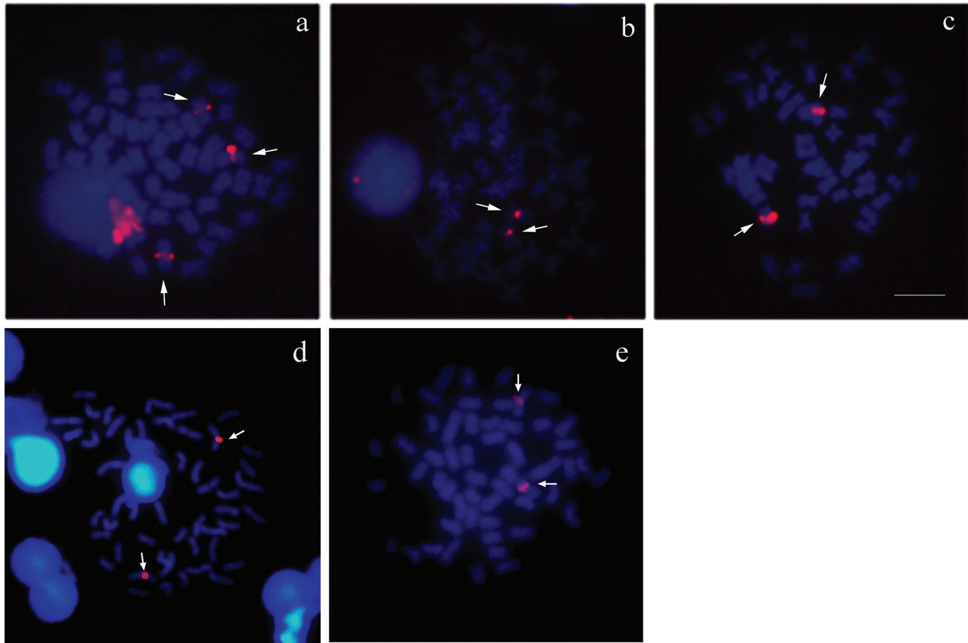

Figure 4. 5S rDNA FISH of: **a** *Cyphocharax spilotus* **b** *Cyphocharax voga* **c** *Steindachnerina insculpta* **d** *Cyphocharax modestus* **e** *Cyphocharax saladensis*. Note in (**a**) the presence of a small chromosome of *C. spilotus* with 5S rDNA sites (arrowhead). Bar: 5 µm.

One AgNOR was observed in the terminal region of a pair of chromosomes in all species (Figs 1, 2, box). Table 2 shows the pair and the position of this region in each species. The secondary constriction was coincident with the AgNOR in *C. voga* (pair 5) and *S. biornata* (pair 3) (Figs 2a, b, box). In *C. spilotus*, the AgNOR was located in the terminal position on the long arm of pair 2, and was not coincident with the interstitial constriction on the short arm of this same pair (Fig. 1c, box).

The AgNORs in the species *Cyphocharax modestus*, *C. saladensis*, *C. spilotus*, *C. voga*, and *Steindachnerina insculpta* were confirmed by fluorescence *in situ* hybridization (FISH) using an 18S rDNA probe (Figs 1, 2, box). *Steindachnerina biornata* presented a small pair of metacentric chromosomes with 18S ribosomal sites in the terminal region of the long arm, besides the pair impregnated with silver (Fig. 2b, box). Staining with CMA_3 fluorochromes revealed fluorescent signals in the terminal region of a chromosome pair corresponding to the AgNORs in all species (Figs 1, 2, box).

Two individuals of *Cyphocharax voga* collected in the Lagoa dos Barros/RS showed a block corresponding to the AgNOR and the CMA_3 fluorochrome on the secondary constriction of a chromosome. FISH revealed two chromosomes with terminal 18S rDNA sites. One of the sites was larger than the other, revealing heteromorphism of this region (Fig. 3).

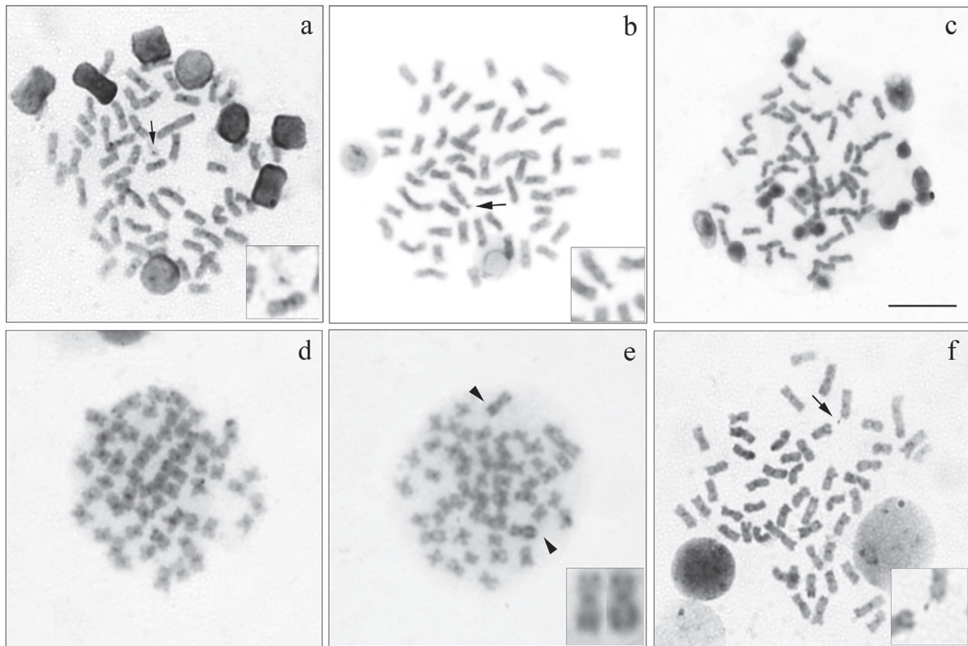

Figure 5. Metaphases with C-banding of: **a** *Cyphocharax modestus* **b** *Cyphocharax saladensis* **c** *Cyphocharax spilotus* **d** *Cyphocharax voga* **e** *Steindachnerina biornata* **f** *Steindachnerina insculpta.* Arrows and square box in (**a**), (**b**) and (**f**) highlight the heterochromatic B microchromosome. Note in (**e**) the pair of *S. biornata* with terminal heterochromatic regions on the long and short arm. Bar: 5 µm.

FISH with a 5S rDNA probe revealed sites in the pericentromeric position of a pair of metacentric chromosomes of five species: *Cyphocharax spilotus*, *Cyphocharax voga*, *Steindachnerina insculpta*, *Cyphocharax modestus* and *Cyphocharax saladensis*. Furthermore, another site was detected on a smaller metacentric chromosome of *Cyphocharax spilotus* (Fig. 4). These regions did not coincide with the 18S rDNA site. In *Steindachnerina biornata*, we could not obtain favorable results with the 5S rDNA probe.

Heterochromatin in Curimatidae species was preferentially observed in the pericentromeric and some terminal regions (Fig. 5). After fluorochrome staining, all heterochromatic regions proved CMA_3^+ (Figure 6). *Steindachnerina biornata* exhibited heterochromatin in the two terminal regions of the NOR-bearing pair, namely one block on the long arm and a discrete marking on the short arm. After CMA_3 fluorochrome staining, these areas became fluorescent (Figs 5e, 6e).

Microchromosome B proved to be heterochromatic in *Cyphocharax modestus, C. saladensis*, and *Steindachnerina insculpta* (Figures 5a, b, f box, respectively). Its visualization with C-banding was not possible in the other species. Only in *C. saladensis*, the heterochromatic fluorescent B chromosome was observed after staining with CMA_3 fluorochrome (Figure 6b).

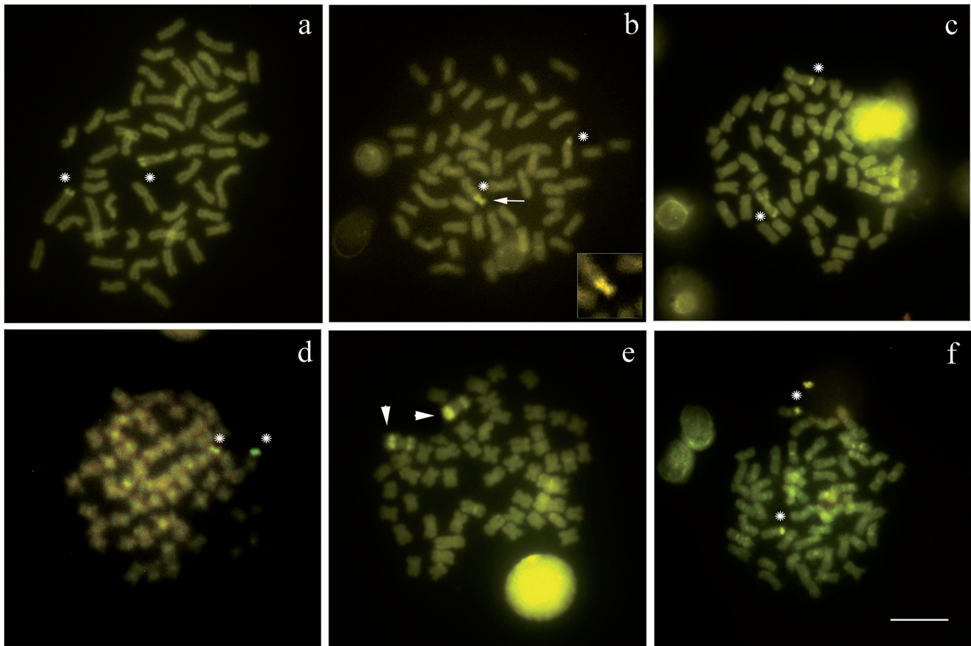

Figure 6. Metaphases with C-banding staining with CMA_3 of: (**a**) *Cyphocharax modestus*; (**b**) *Cyphocharax saladensis*; (**c**) *Cyphocharax spilotus*; (**d**) *Cyphocharax voga*; (**e**) *Steindachnerina biornata*; (f) *Steindachnerina insculpta.* The (*) indicates the NOR pairs. Note in (**b**) the heterochromatic CMA_3^+ B microchromosome of *C. saladensis* (arrow and square box) and in (**e**) the heterochromatic pair of *S. biornata* (arrowhead). Bar: 5 µm.

Discussion

This study showed the first chromosome banding data for populations of Curimatidae of the Lagoa dos Patos and Tramandaí River basins, in the state of Rio Grande do Sul, as well as the first data on the species *Cyphocharax saladensis* and *Steindachnerina biornata*. All species maintained the pattern, presenting 2n = 54 m-sm. The model proposed by Feldberg et al. (1992), corroborates that this is an ancestral karyotype of Curimatidae and that variations of this condition represent derived characters. Considering Feldberg's assertions, it is possible to affirm that concerning the karyotype macrostructure, the Curimatidae species studied herein have basal karyotypes. The presence of basal karyotypes is common in this group. However, Brassesco et al. (2004), found variations in the diploid number of *Cyphocharax platanus* (Günther, 1880), which showed a 2n = 58 and karyotype formula of 52m-sm+6st and *Potamorhina squamoralevis* (Braga & Azpelicueta, 1983), which had 2n = 102 and 14m-sm +88a. These data indicate that the chromosomal evolution in some species of Curimatidae is followed by alterations as centric fissions and inversions in the karyotype macrostructure (Feldberg et al. 1993; Brassesco et al. 2004).

Sampaio et al. (2011) analyzed the mitotic and meiotic behavior of B microchromosomes in the species assessed herein, corroborating that this is an important cytogenetic characteristic in this group of fish. Currently, the occurrence of these B chromosomes has been reported in seven species of Curimatidae from different populations, corresponding to 18.42% of the total studied species (Sampaio et al. 2011). Although considered a remarkable feature in the Curimatidae family, only 2 of the 8 genera analyzed, i.e., *Cyphocharax* and *Steindachnerina*, have presented this type of chromosome thus far (Table 3).

Besides the presence of B chromosomes, another striking feature of the Curimatidae species are the nucleolus organizer regions. Previous works have described the AgNORs of *Cyphocharax spilotus* and *Steindachnerina insculpta* on other pairs besides those observed here (Table 3), showing an interpopulation variability in the location of AgNORs among Curimatidae. These fish occur in different ecosystems of the Neotropical region, and isolated populations can be established under different environmental conditions, enabling an increase in the frequency of certain variations (Brassesco et al. 2004; Vari 2003). These variations may be ascribable to rearrangements of the chromosomal microstructure, such as translocations and/or inversions (Venere and Galetti Jr. 1989; De Rosa et al. 2007).

All studied populations of *Cyphocharax modestus* presented the AgNOR on pair 2. The populations of *C. voga* presented the AgNOR mainly on pair 5 (Table 3), indicating that these sites can be considered important species-specific cytogenetic markers (Venere et al. 2008; De Rosa et al. 2007).

In many fish groups, including Curimatidae, there is a high correlation between AgNORs and secondary constriction (Feldberg et al. 1992; Teribele et al. 2008; Gouveia et al. 2013). However, the presence of secondary constriction without rDNA sequences, as in *Cyphocharax spilotus*, is a characteristic rarely observed in fish. But this can occur due to the existence of pseudo-NORs, appearing decondensed and stained with silver nitrate, being transcriptionally inactive (Prieto and McStay 2008).

The results of FISH in *Steindachnerina biornata* showed another species with multiple NOR patterns among Curimatidae. The above method revealed an unusual feature, which was observed only in *Curimata inornata* Vari, 1989, *Cyphocharax nagelii* (Steindachner, 1881), *Steindachnerina amazonica* (Steindachner, 1911), and *S. gracilis* Vari & Vari, 1989 (Venere et al. 2008). As shown in Table 3, most studies with NORs have utilized only silver nitrate, which may explain the small number of species with multiple sites in this group of fish.

The existing literature presents scarce data on fluorochrome staining in the family Curimatidae, with reports only in *Cyphocharax modestus* and *Steindachnerina insculpta* (De Rosa et al. 2007; Teribele et al. 2008; Martins et al. 1996) and the results are coincident with those observed in this study, indicating that NORs are rich in GC base pairs.

NOR heteromorphism in the homologous chromosomes of two individuals of *Cyphocharax voga* from the population of the Lagoa dos Barros/RS may be attributable

Table 3. Chromosome studies in the family Curimatidae. *2n*, diploid number; *FN*, fundamental number; *m*, metacentric; *sm*, submetacentric; *st*, subtelocentric; *a*, acrocentric; *B*, supernumerary chromosome; *term.*, terminal; *peric.*, pericentromeric; *centr.*, centromeric; *inters.*, interstitial.

Species	Locality	2n	Karyotypic formula	FN	AgNOR pair	Position	Number of cistrons 18S rDNA	Number/position of cistrons 5S rDNA	C banding	Reference
Curimata cyprinoides	Negro and Solimões river/AM	54	44m + 10sm	108	3	term. long arm	-	-	-	3
	Araguaia river/MT	54	44m + 10sm	108	7	term. long arm	-	-	-	16
Curimata inornata	Negro and Solimões river/AM	54	40m + 14sm	108	21	inters. short arm	-	-	-	3
	Araguaia river/MT	54	40m + 14sm	108	3, 22	term. long arm	-	-	Peric./term.	16
Curimata kneri	Negro and Solimões river/AM	54	40m + 14sm	108	27	term. short arm	-	-	-	3
Curimata ocellata	Negro and Solimões river/AM	56	40m + 16sm	112	26	inters. short arm	-	-	-	3
Curimata vittata	Negro and Solimões river/AM	54	42m + 12sm	108	9	term. long arm	-	-	-	3
Curimatella alburna	Negro and Solimões river/AM	54	46m + 8sm	108	14	term. long arm	-	-	-	3
Curimatella dorsalis	Miranda river/MS	54	46m + 8sm	108	13	term. short arm	-	-	Peric.	7
	Paraná river/AR	54	54m/sm	108	2	term. long arm	-	-	Centr./term.	11
Curimatella imaculata	Araguaia river/GO	54	46m + 8sm	108	24	inters. long arm	-	-	Peric.	16
Curimatella lepidura	São Francisco river/SP	54	54m/sm	108	9	term. short arm	-	-	-	2
Curimatella meyeri	Negro and Solimões river/AM	54	46m + 8sm	108	9	term. long arm	-	-	-	3
Curimatopsis myersi	Miranda river/MS	46	42m + 4sm	92	-	-	-	-	-	7
Cyphocharax gilbert	Paraibuna river/SP	54	44m + 10sm	108	2	term. short arm	-	-	Peric./term.	16
Cyphocharax cf. *gillii*	Bento Gomes river/MT	54	54m/sm	108	1	inters. long arm	-	-	-	2
Cyphocharax gouldingi	Araguaia river/GO	54	54m + B	108	2	term. long arm	-	-	Peric.	16
Cyphocharax modestus	Tiête river/SP	54	54m/sm/B	108	-	term. long arm	-	-	Centr./term.	1
	Águas de São Pedro/SP	54	54m/sm	108	2	term. long arm	-	-	-	2
	Três Bocas stream/PR	54	54m/sm + B	108	2	term. long arm	2	-	Peric./term.	6, 13, 15, 18, 19
	Mogi-Guaçu river/SP	54	54m/sm + B	108	-	-	-	-	Peric.	8
	Taquari river/PR	54	54m/sm + B	108	2	term. long arm	2	-	Peric./term.	13, 15
	Tibagi river/PR	54	54m/sm	108	2	term. long arm	2	-	-	15
	Água da Floresta river/PR	54	54m/sm	108	2	term. long arm	2	-	-	15
	Paranapanema river/SP	54	54m/sm + B	108	2	term. long arm	2	4/peric. short arm	Centr./term.	12, 14, 17

Species	Locality	2n	Karyotypic formula	FN	AgNOR pair	Position	Number of cistrons 18S rDNA	Number/position of cistrons 5S rDNA	C banding	Reference
	Tietê river/SP	54	54m/sm	108	2	term. long arm	2	4/peric. short arm	Centr./term.	12, 14, 17
Cyphocharax nagelii	Mogi-Guaçu river/SP	54	54m/sm	108	25	term. short arm	-	-	-	2
	Mogi-Guaçu river/SP	54	46m + 8sm	108	1, 2, 6, 11, 21	term. long / short arm	-	-	Peric./term.	16
Cyphocharax platanus	Paraná river/AR	58	52m/sm + 6st	116	5	term. short arm	-	-	Centr.	11
	Pirá-Pytá stream/ AR	58	48m + 4 sm + 6st	116	6	term. short arm	-	-	Peric./term.	16
Cyphocharax cf. *spilurus*	Madeira river/RO	54	54m/sm	108	10	term. long arm	-	-	-	2
Cyphocharax spilotus	Paraná river/AR	54	54m/sm + B	108	1	inters. long arm	-	-	Centr./term.	10, 11
	Capivara stream/RS	54	54m/sm + B	108	2	term. long arm	2	-	Peric./term.	18, 19
	Gasômetro/RS	54	54m/sm + B	108	2	term. long arm	2	3/peric. short arm	Peric./term.	18, 19
Cyphocharax vanderi	Preto river/SP	54	54m/sm	108	6	term. long arm	-	-	-	2
Cyphocharax voga	Bolacha stream/RS	54	54m/sm	108	6	term. long arm	-	-	-	2
	Paraná river/AR	54	54m/sm	108	-	term. long arm	-	-	Inters./term.	11
	Saco da Alemoa river/RS	54	54m/sm + B	108	5	term. long arm	2	-	Peric./term.	18, 19
	Capivara stream/RS	54	54m/sm + B	108	5	term. long arm	2	-	Peric./term.	18, 19
	Gasômetro/RS	54	54m/sm + B	108	5	term. long arm	2	-	Peric./term.	18, 19
	Barros lagoon/RS	54	54m/sm + B	108	5	term. long arm	2	2/peric. short arm	Peric./term.	18, 19
	Quadros lagoon/RS	54	54m/sm + B	108	5	term. long arm	2	-	Peric./term.	18, 19
Cyphocharax saladensis	A.E.S UFRGS dam/RS	54	54m/sm + B	108	8	term. long arm	2	2/peric. short arm	Peric./term.	18, 19
Potamorhina altamazonica	Negro and Solimões river/AM	102	2m + 2sm + 98a	106	5	term. long arm	-	-	Peric./inters/ term.	4
Potamorhina latior	Negro and Solimões river/AM	56	52m + 2sm + 2st	112	25	term. long arm	-	-	Peric./term.	4
Potamorhina pristigaster	Negro and Solimões river/AM	54	42m + 12sm	108	25	term. short arm	-	-	Peric.	4
Potamorhina squamoralevis	Paraná river/AR	102	14m/sm + 88a	116	-	term. long arm	-	-	Centr.	11
Psectrogaster amazonica	Araguaia river/MT	54	44m + 10sm	108	17	term. short arm	-	-	Peric.	16

Species	Locality	2n	Karyotypic formula	FN	AgNOR pair	Position	Number of cistrons 18S rDNA	Number/position of cistrons 5S rDNA	C banding	Reference
Psectrogaster curviventris	Miranda river/MS	54	42m + 12sm	108	20	term. short arm	-	-	Peric.	7
	Paraná river/AR	54	54m/sm	108	-	inters. long arm	-	-	Centr./term.	11
Psectrogaster rutiloides	Negro and Solimões river/AM	54	42m + 12sm	108	9	term. long arm	-	-	-	3
Steindachnerina amazonica	Araguaia river/GO	54	42m + 12sm	108	2, 23	term. long arm	-	-	Peric./term.	16
Steindachnerina biornata	Forquetinha river/RS	54	54m/sm + B	108	3	term. long arm	4	-	Peric./term.	18, 19
Steindachnerina brevipinna	Miranda river/MS	54	48m + 6sm	108	17	term. short arm	-	-	Centr./term.	7
	Paraná river/AR	54	54m/sm	108	15	term. long arm	-	-	Centr./ inters./term.	11
Steindachnerina conspersa	Paraguai river/MS	54	54m/sm	108	2	inters. long arm	-	-	-	2
	Paraná river/AR	54	54m/sm	108	2	term. long arm	-	-	Centr./ inters/term.	11
Steindachnerina elegans	São Francisco river/SP	54	54m/sm	108	25	term. short arm	-	-	-	2
Steindachnerina gracilis	Araguaia river/MT	54	38m + 16sm	108	-	term. long arm	-	-	Peric.	16
Steindachnerina cf. *guentheri*	São Francisco river/AC	54	54m/sm	108	24	term. short arm	-	-	Peric./inters/ term.	9
Steindachnerina insculpta	Mogi-Guaçu river/SP	54	54m/sm	108	25	term. short arm	-	-	-	2
	Passa-Cinco river/SP	54	54m/sm	108	25	term. short arm	-	-	-	2
	Paranapanema river/SP	54	54m/sm + B	108	-	-	-	-	Peric.	5
	Reserva Jurumirim/SP	54	54m/sm + B	108	-	-	-	-	Peric.	5
	Paranapanema river/SP	54	54m/sm	108	7	term. short arm	2	2/peric. short arm	Centr./term.	12, 14, 17
	Tietê river/SP	54	54m/sm	108	7	term. short arm	2	2/peric. short arm	Centr./term.	12, 14, 17
	Três Bocas stream/PR	54	54m/sm + B	108	7	term. short arm	2	-	Peric./term.	13, 15
	Taquari river/PR	54	54m/sm	108	7	term. short arm	2	-	Peric./term.	13, 15
	Tibagi river/PR	54	54m/sm	108	7	term. short arm	2	-	Peric./term.	13, 15
	Água da Floresta river/PR	54	54m/sm	108	7	term. short arm	2	-	Peric./term.	13, 15
	Cachoeira de Emas/SP	54	54m/sm	108	22	term. short arm	-	-	Peric./term.	16
	Água dos Patos river/SP	54	54m/sm + B	108	12	term. short arm	2	-	Peric./term.	18, 19

Species	Locality	2n	Karyotypic formula	FN	AgNOR pair	Position	Number of cistrons 18S rDNA	Number/position of cistrons 5S rDNA	C banding	Reference
	Três Bocas streams/PR	54	54m/sm + B	108	12	term. short arm	2	2/peric. short arm	Peric./term.	18, 19
	Pavão stream/PR	54	54m/sm + B	108	12	term. short arm	2	-	Peric./term.	18, 19
	Jacutinga river/PR	54	54m/sm + B	108	12	term. short arm	2	-	Peric./term.	18, 19
Steindachnerina leucisca	Negro and Solimões river/AM	54	48m + 6sm	108	15	term. short arm	-	-	-	3

1. Venere and Galetti (1985); **2.** Venere and Galetti (1989); **3.** Feldberg et al. (1992); **4.** Feldberg et al. (1993); **5.** Oliveira and Foresti (1993); **6.** Martins et al. (1996); **7.** Navarrete and Júlio-Jr. (1997); **8.** Venere et al. (1999); **9.** Carvalho et al. (2001); **10.** Fenocchio et al. (2003); **11.** Brassesco et al. (2004); **12.** De Rosa et al. (2006); **13.** Gravena et al. (2007); **14.** De Rosa et al. (2007); **15.** Teribele et al. (2008); **16.** Venere et al. (2008); **17.** De Rosa et al. (2008); **18.** Sampaio et al. (2011); **19.** present paper.

to unequal crossing over, where the small site may have become inactive, or could not be detected by silver nitrate or CMA_3 because of their size. Teribele et al. (2008), obtained similar results in an individual of *Cyphocharax modestus* collected in the Taquari River/PR.

FISH with the 5S rDNA probe revealed results coincident with those found by Da Rosa et al. (2006) in studies on the *C. modestus* and *S. insculpta,* which also showed ribosomal sites in the pericentromeric region of a chromosome pair, suggesting the existence of homology between these species. These authors observed smaller signals on a second pair of chromosomes in *C. modestus,* similar to the small 5S rDNA site found on the single metacentric chromosome in *C. spilotus*.

To explain the presence of larger and smaller 5S rDNA sites, De Rosa et al. (2006), compared Curimatidae with other families comprising species with the same behavior sequences, such as *Leporinus* Agassiz, 1829 and *Schizodon* Agassiz, 1829 (Anostomidae), *Parodon* Valenciennes, 1850 (Parodontidae) and *Prochilodus argenteus* Spix & Agassiz, 1829 (Prochilodontidae). These families, along with Curimatidae, form a monophyletic group based on morphological characteristics showing that their 5S rDNA clusters have possibly been preserved from significant changes during the evolution.

C-banding analyses did not allow us to characterize and differentiate among the species and/or genera analyzed in this study. However, Venere et al. (2008) observed a pronounced diversification in the distribution and amount of heterochromatin in some species of Curimatidae, differentiating between the genera *Steindachnerina* and *Cyphocharax,* indicating the heterochromatin characterization in chromosomes of each group.

The difference in the amount of heterochromatin in Curimatidae reflects the interpopulation variability occurring within this family. It is believed that the amount of heterochromatin can play a significant role in the chromosome evolution in this fish group. As previously mentioned, Curimatidae can be established in isolated populations under different environmental conditions. Such conditions may enable increased variations in the distribution of heterochromatin.

CMA_3 fluorochrome staining revealed fluorescent signals in the heterochromatic regions of many chromosomes of the complement, showing that heterochromatin in these species consists mostly of GC base pairs. A chromosomal pair detected in *Steindachnerina biornata* can be considered a species-specific marker, since we evidenced heterochromatin in the two terminal regions of the NOR-bearing pair, i.e., a block on the long arm associated with the NOR and a more discreet marking on the short arm. The NOR adjacent to the heterochromatic blocks may facilitate chromosome breakage, leading to structural rearrangements in these regions (Moreira-Filho et al. 1984).

In *Cyphocharax modestus, C. saladensis,* and *Steindachnerina insculpta,* the B microchromosome presented itself entirely heterochromatic, indicating the total absence of gene activity, as in other studied populations of *C. modestus* (Gravena et al. 2007; Venere et al. 1999) and *S. insculpta* (Gravena et al. 2007). The heterochromatic B chromosome of *C. saladensis* proved CMA_3^+, therefore, rich in GC base pairs.

Two hypotheses have been proposed for the origin of B chromosomes in Curimatidae (Martins et al. 1996). The first suggests a common B chromosome ancestor,

which may have arisen in the ancestors of the family, and eliminated from the present species that do not have B-chromosome. The second proposes that B chromosomes may have had a recent and independent origin, resulting in closely related species, or even in the same species, with differences in the pattern and composition of heterochromatin. The second hypothesis seems to be more viable.

In conclusion, these data associated with the previously existing studies for the group, show that, although Curimatidae have a very conservative karyotype macrostructure, the interpopulation variation in NOR locations and distribution of heterochromatin are caused by important mechanisms of chromosome alterations, such as translocations and/or inversions, leading to the evolution and diversification of this group of fish.

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RESEARCH ARTICLE

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The contribution of cytogenetics and flow cytometry for understanding the karyotype evolution in three *Dorstenia* (Linnaeus, 1753) species (Moraceae)

Paulo Marcos Amaral-Silva[1], Wellington Ronildo Clarindo[1], Tatiana Tavares Carrijo[2], Carlos Roberto Carvalho[3], Milene Miranda Praça-Fontes[1]

1 *Laboratório de Citogenética, Departamento de Biologia, Centro de Ciências Agrárias, Universidade Federal do Espírito Santo. CEP: 29.500-000 Alegre – ES, Brazil* **2** *Laboratório de Botânica, Departamento de Biologia, Centro de Ciências Agrárias, Universidade Federal do Espírito Santo. CEP: 29.500-000 Alegre – ES, Brazil* **3** *Laboratório de Citogenética e Citometria, Departamento de Biologia Geral, Centro de Ciências Biológicas e da Saúde, Universidade Federal de Viçosa. CEP: 36.570-000 Viçosa – MG, Brazil*

Corresponding author: *Milene Miranda Praça-Fontes* (milenemiranda@yahoo.com.br)

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Abstract

Chromosome morphometry and nuclear DNA content are useful data for cytotaxonomy and for understanding the evolutionary history of different taxa. However, the chromosome number is the only karyotype aspect reported for the species of *Dorstenia* so far. In this study, the nuclear genome size of *Dorstenia arifolia* (Lamarck, 1786), *Dorstenia bonijesu* (Carauta & C. Valente, 1983) and *Dorstenia elata* (Hooker, 1840) was evaluated and their karyotype morphometry accomplished, with the aim of verifying the potential of those parameters to understand evolutionary issues. Mean nuclear 2C value ranged from 2C = 3.49 picograms (pg) for *D. elata* to 2C = 5.47 pg for *D. arifolia*, a variation of ± 1.98 pg. Even though showing a marked difference in 2C value, the three species exhibited the same 2n = 32. Corroborating the flow cytometry data, differences in chromosome morphology were found among the karyotypes of the species investigated. Based on this and the only phylogeny proposed for *Dorstenia* thus far, structural rearrangements are related to the karyotype variations among the three species. Besides, the karyological analysis suggests a polyploid origin of the *Dorstenia* species studied here.

Keywords

Cytogenetics, euploidy, karyotype, flow cytometry, chromosome structure

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Introduction

The pantropical genus *Dorstenia* Linnaeus, 1753 comprises about 105 species distributed in Asia, Africa and Neotropical regions (Carauta 1978, Berg 2001). Thirty-nine species occur in Brazil (Romaniuc-Neto et al. 2015), representing the sections *Dorstenia* Linnaeus, 1753, *Emigdioa* Carauta, 1976 and *Lecanium* Fischer & Meyer, 1846. Carauta (1978) and Berg (2001) have contributed much of the knowledge about the morphology of Neotropical species, while the phylogenetic reconstructions using molecular data (Misiewicz and Zerega 2012) consolidated the hypothesis about the genus' monophyly. Despite the potential information of cytogenetic data for taxonomic (Stace 2000) and phylogenetic studies (Melo and Guerra 2003), this marker has been little explored in previous works on *Dorstenia*.

According to the current knowledge, the basic chromosome number in African species of *Dorstenia* is x = 12 and x = 13, while in American species x = 14, 15 and 16 (Berg 2001) are found. Fagerlind (1944) reported *Dorstenia mannii* Hooker f., 1871 as a tetraploid (2n = 48). In addition, intraspecific variation in chromosome number have been reported for subspecies of *Dorstenia psilurus* Welwitsch, 1869 (2n = 26 and 2n = 40) (Misiewicz and Zerega 2012) and *Dorstenia elata* Hooker, 1840 2n = 26 or 32 (Krause 1931, Hoen 1983, unpublished data). These data indicate that the evolution of the karyotype in *Dorstenia* involved euploidy and aneuploidy events. However, it is still necessary to invest efforts in confirming and understanding the chromosome changes reported for some species in previous studies. Considering that chromosome alterations are a significant mechanism of diversification and speciation in Angiosperms (Stace 2000, Peer et al. 2009, Weiss-Schneeweiss et al. 2013), the investigation of this aspect in *Dorstenia* can bring light to the knowledge of speciation processes in the genus.

Cytogenetic approaches, which regard the chromosome number, morphometric measurements and karyotype analysis, contribute to the understanding of evolutionary processes in plants (Shan et al. 2003). The knowledge of these aspects in related species helps to elucidate issues related to diversification of a taxonomic group (Clarindo et al. 2012). Morphometric analysis of the chromosomes is a way to determine the karyotype changes that occurred throughout evolution, the processes that led to the diversification, and the direction taken by evolution (Gao et al. 2012).

Numeric and structural chromosome rearrangements have been reported as triggers of karyotype changes in several plant taxa. Therefore, nuclear genome size variation occurs between phylogenetically related species due to these alterations (Bonifácio et al. 2012, Raskina et al. 2008). For this reason, nuclear DNA content measurement has been increasingly employed in systematic approaches using flow cytometry (FCM). In addition to its practicality and reproducibility, FCM is useful to reveal differences between taxa (Stace 2000), especially in groups of species that exhibit conserved chromosome number (Mabuchi et al. 2005).

The cytogenetic and FCM approaches in *Dorstenia* could provide relevant information to sections and species taxonomy, as well as contribute to understanding the evolutionary history of the genus. The main goal of this study was therefore to meas-

ure the nuclear 2C value, determine the chromosome number and characterize the karyotype of Neotropical species of *Dorstenia*: *D. arifolia* Lamarck, 1786, *D. bonijesu* Carauta & C. Valente, 1983 and *D. elata* Hooker, 1840.

Material and methods

Plant samples – Specimens of *D. elata*, *D. bonijesu* and *D. arifolia* (Fig. 1 a, c, e, respectively) were collected in an Atlantic Rainforest remnant located in the city of Castelo – ES, Brazil. Voucher specimens were included in the herbarium VIES: T.T. Carrijo 1516 (*D. arifolia*); T.T. Carrijo 1682 (*D. bonijesu*) and T.T. Carrijo 1618 (*D. elata*).

FCM – Nuclear suspensions were obtained by chopping (Galbraith et al. 1983) of leaf fragments (1 cm^2) excised from each *Dorstenia* species (sample) and *Solanum lycopersicum* Linnaeus, 1753 (internal standard, 2C = 2.00 picograms – Praça-Fontes et al. 2011). Samples and standard leaf fragments were simultaneously chopped with razor blade in a Petri dish containing 0.5 ml OTTO-I nuclear extraction buffer (Otto 1990) supplemented with 2 mM dithiothreitol and 50 µg ml^{-1} RNAse (Praça-Fontes et al. 2011). Afterwards, 0.5 ml of the OTTO-I buffer was added, the suspensions were filtered through 30-µm nylon mesh, placed into microtube and centrifuged at 100 × *g* for 5 min. The precipitate was resuspended in 100 µl OTTO-I buffer and incubated for 10 min (Praça-Fontes et al. 2011). The nuclei suspensions were stained with 1.5 ml of OTTO-I:OTTO-II (1:2 – Otto 1990, Loureiro et al. 2006) solution supplemented with 2 mM dithiothreitol, 50 µg ml^{-1} propidium iodide and 50 µg ml^{-1} RNAse (Praça-Fontes et al. 2011). The nucleus suspensions were kept in the dark for 30 min, then filtered through 20-µm nylon mesh. The samples were analyzed in a flow cytometer Partec PAS II/III (Partec GmbH, Germany). Histograms were analyzed with the Partec Flow Max software tools to measure nuclear DNA content. The genome size of the *Dorstenia* species was calculated according to the formula:

$$2C_D = \left(\frac{C1}{C2}\right) . 2C_S$$

wherein:

$2C_D$: value of 2C DNA content (pg) of each *Dorstenia* species,
C1: average G_0/G_1 peak channel of the *Dorstenia* species,
C2: average G_0/G_1 peak channel of *S. lycopersicum*,
$2C_S$: value of 2C DNA content of *S. lycopersicum* (2.00 pg).

Cytogenetics – Stems with length of approximately 15 cm exhibiting one leaf pair were excised and disinfected with 1% $NaOCl_2$ solution for 15 min. These propagules were maintained in hydroponic system for rooting. The system was oxygenated by a compressor coupled to a plastic hose, which was immersed in H_2O. The roots were treated with 3 µM or 4 µM amiprophos-methyl (APM) for 16 h or 18 h at 4 °C. The roots were washed in dH_2O for 20 min, fixed in methanol:acetic acid solution (3:1)

and stored at -20 °C (Carvalho et al. 2007). After 24 h, the roots were washed in dH_2O and macerated in pectinase solution 1:40, 1:45, 1:50, 1:55 or 1:60 (enzyme:dH_2O) for 1 h 45 min or 2 h at 34 °C. The material was washed in dH_2O, fixed again and kept at -20 °C until use. Using the macerated root meristems, slides were prepared by cell dissociation and air-drying techniques, then placed onto a hot plate at 50 °C (Carvalho et al. 2007). The slides were stained with 5% Giemsa (Merck®) for 8 min, washed twice in dH_2O, and air-dried. All slides were analyzed under a Nikon Eclipse Ci-S microscope (Nikon). The capture of metaphase images was performed using 100× objective and a CCD camera (Nikon Evolution™) coupled to a Nikon 80i microscope (Nikon).

Morphometric analysis – The chromosomes of three *Dorstenia* species were characterized as to the total length, length of the long and short arms, and classes. The chromosome class was determined as proposed by Levan et al. (1964) and reviewed by Guerra (1986): r = length of the long arm / length of the short arm. The asymmetry of the karyotype was evaluated using the method proposed by Zarco (1986), using the formulae:

$$A_2 = \frac{s}{\bar{X}}$$

where A_2 = interchromosomal asymmetry index, s = standard deviation, and $\bar{X}$ = average length of the chromosomes.

Results

Flow cytometry measurements

The nuclear suspensions resulted in histograms with G_0/G_1 peaks showing coefficient of variation below 3.45%. Thus, the isolation and staining procedures provided suspensions containing isolated, intact and stoichiometrically stained nuclei. Using the histograms, the mean 2C nuclear DNA content of *Dorstenia* species was measured for the first time. The values were 2C = 3.49 pg ± 0.0035 (1C = 1.71 bp × 10^9) for *D. elata*; 2C = 4.05 pg ± 0.014 (1C = 1.98 bp × 10^9) for *D. bonijesu*; and 2C = 5.47 pg ± 0.002 (1C = 2.67 bp × 10^9) for *D. arifolia* (Fig. 1 b, d, f). The mean value for *D. arifolia* was 36.20% higher than for *D. elata*, and 26.00% greater than for *D. bonijesu*. Besides, *D. bonijesu* showed a DNA content 13.83% higher than that of *D. elata*. Based on these values, an interspecific variation of the nuclear genome size was identified between the species.

Cytogenetics

The rooting of vegetative propagules occurred after 40 days in hydroponic system. The disinfection of the propagules contributed for relatively rapid rooting due to absence of contamination. Owing to the lack of cytogenetic studies for the genus *Dorstenia*, we tested different treatments with microtubule inhibitor as well as distinct procedures of

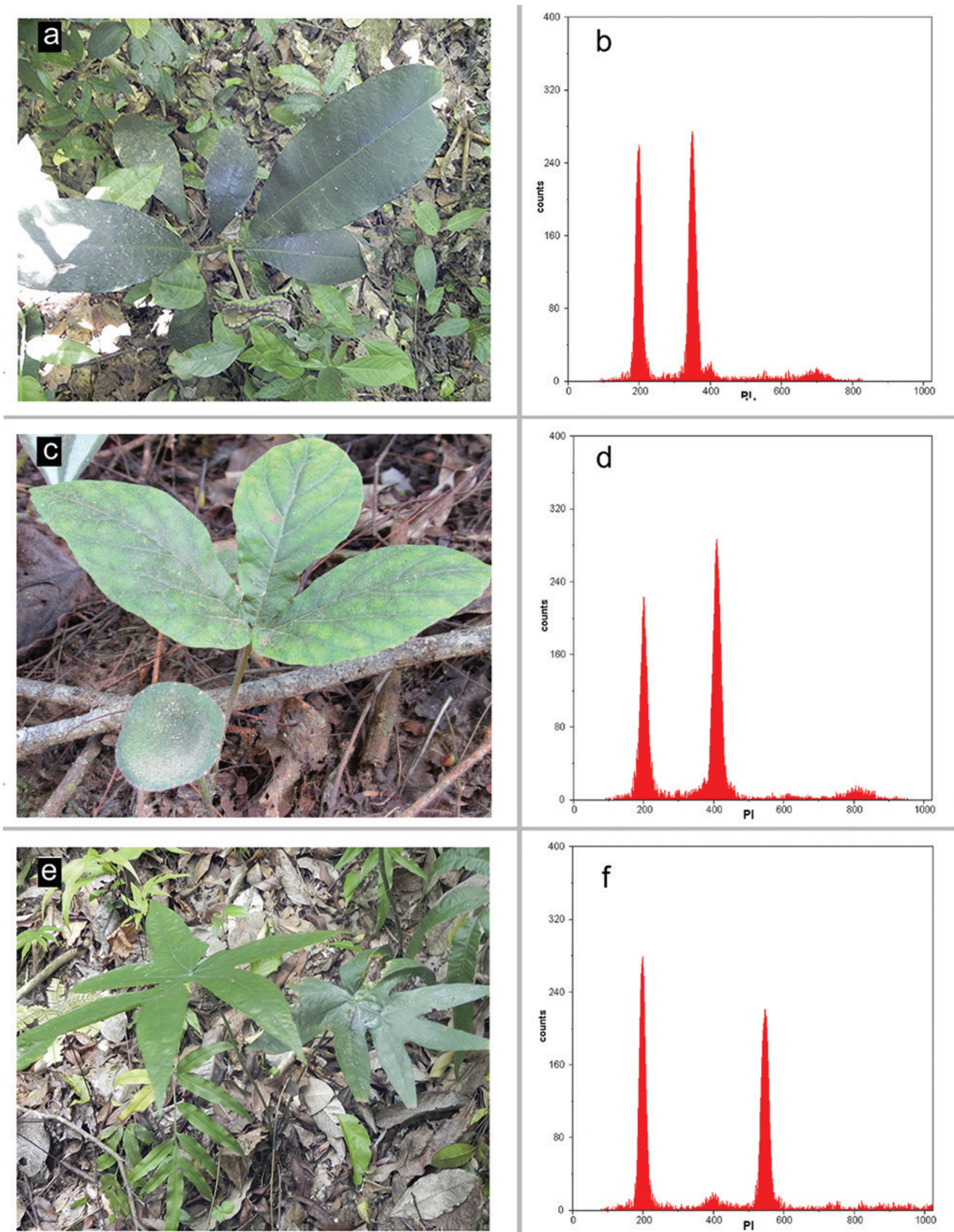

Figure 1. Representative adult plants of *D. elata* (**a**), *D. bonijesu* (**c**) and *D. arifolia* (**e**) of the Atlantic Rainforest remnant located in the Castelo city (ES, Brazil). FCM histograms showing G_0/G_1 peaks generated by nuclei suspensions of *S. lycopersicum* (internal standard, channel 200, 2C = 2.00 pg), and of *D. elata* (**b** channel 349, 2C = 3.49 pg), *D. bonijesu* (**d** channel 405, 2C = 4.05 pg) and *D. arifolia* (**f** channel 547, 2C = 5.47 pg).

enzymatic maceration. Root meristems treated with 4 μM APM for 16 h at 4 °C and macerated in 1:60 pectinase solution for 1 h 45 min at 34 °C resulted in metaphases adequate for karyotype characterization of the *Dorstenia* species (Fig. 2).

The differences chromatin compaction levels verified among the metaphases (Fig. 2 a, b) of the species allowed morphometric analysis (Table 1) and karyogram assembly (Fig. 2). All *Dorstenia* species showed a conserved number of 32 chromosomes.

Morphometric analysis of the chromosomes

The mean values for the sum of total length as well as short- and long-arm length differed among the species (Table 1). *D. arifolia* showed the highest total, short- and long-arm length in relation to other species, corroborating the FCM data. *D. bonijesu*, which exhibited an intermediate mean 2C value, presented the lowest total chromosome and short-arm length. In comparison to *D. elata*, *D. bonijesu* displayed a greater mean value for the long arm (Table 1). The A2 index also varied between the species: *D. bonijesu* exhibited the most asymmetrical karyotype, with $A_2 = 0.16$, followed by *D. arifolia* ($A_2 = 0.14$) and *D. elata* ($A_2 = 0.13$).

Based on the morphometric data, the chromosome class was determined and the differences between the karyotypes for the three species were endorsed. *D. elata* showed twelve metacentric chromosome pairs (1, 2, 4, 5, 7, 8, 9, 10, 13, 14, 15 and 16), two submetacentric (3 and 6) and two acrocentric pair (11 and 12) (Fig. 2 a, b). *D. bonijesu* exhibited four metacentric chromosome pairs (1, 2, 3 and 15), ten submetacentric (4, 5, 6, 8, 9, 10, 11, 12, 13 and 14) and two acrocentric ones (7 and 16) (Fig. 2c). *D. arifolia* presented four metacentric chromosome pairs (1, 2, 5 and 10) and twelve submetacentric ones (3, 4, 6, 7, 8, 9, 11, 12, 13, 14, 15 and 16) (Fig. 2d).

Discussion

Despite exhibiting the same number of chromosomes (2n = 32), the species of *Dorstenia* studied here show distinct mean nuclear 2C values. The interspecific DNA content variation indicates that the karyotypes differ between the species (Gitaí et al. 2014). Considering that the *Dorstenia* species have the same chromosome number, the karyotype divergences are probably associated to chromosome structure.

The morphometric analysis revealed karyomorphological differences in the sum of the mean values for total chromosome length, and the short arm and long arm (Table 1). Besides, some chromosomes presented distinct classes among the species, such as in chromosomes 7 and 16, which are submetacentric in *D. arifolia*, acrocentric in *D. bonijesu* and metacentric in *D. elata* (Fig. 2, Table 1). In view of this, structural chromosomal rearrangements have occurred during the evolutionary history of the group. This is supported by the interchromosomal asymmetry index (A_2, Zarco 1986), which also varied between the three *Dorstenia* species, despite the predominance of meta-

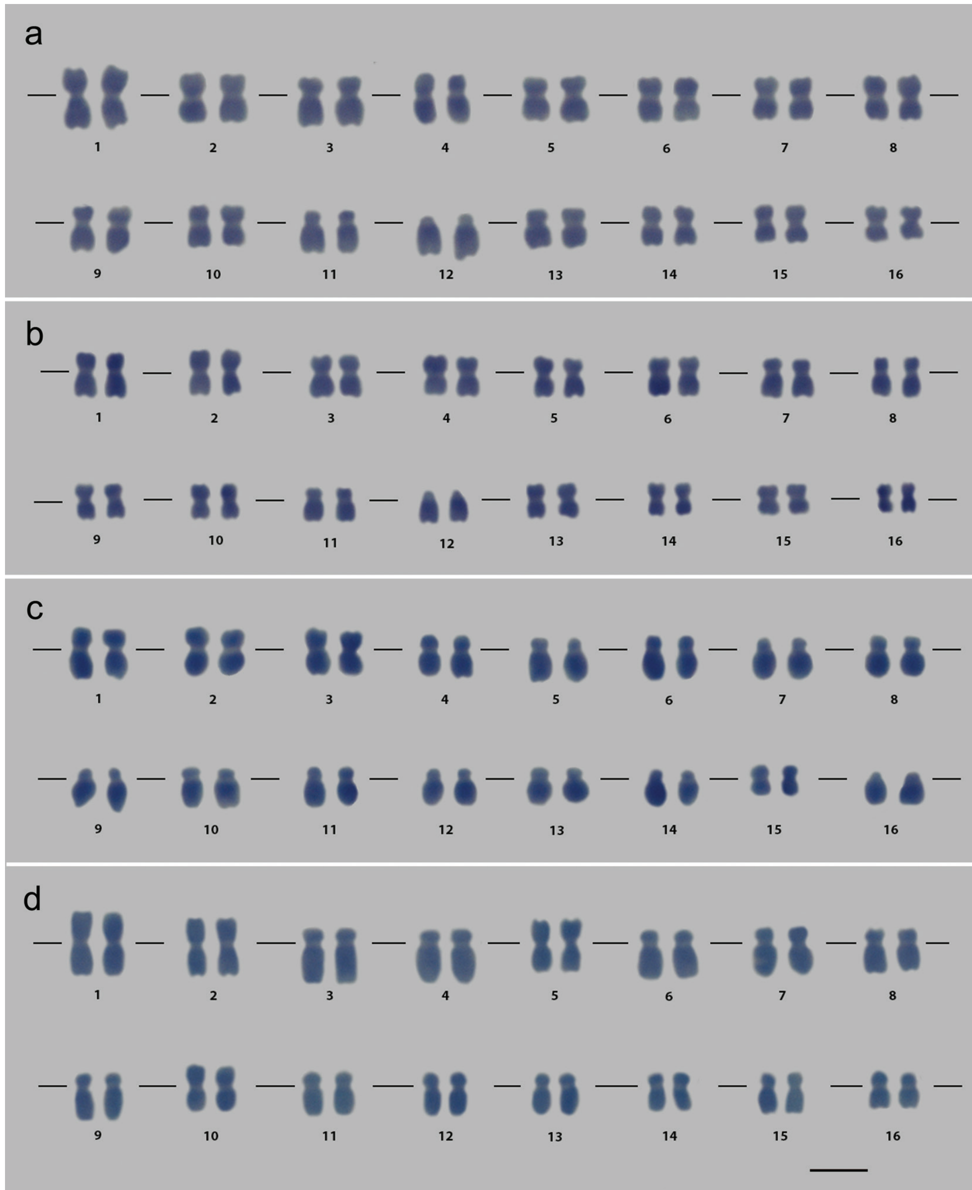

Figure 2. Karyograms assembled from mitotic chromosomes of *D. elata* (**a**, **b**), *D. bonijesu* (**c**) and *D. arifolia* (**d**). Note the distinct chromatin compact level (**a**, **b**) showed by the mitotic chromosomes of *D. elata*. **a**, **b** *D. elata* showed twelve metacentric chromosome pairs (1, 2, 4, 5, 7, 8, 9, 10, 13, 14, 15 and 16), two submetacentric (3 and 6) and two acrocentric pair (11 and 12) **c** *D. bonijesu* exhibited four metacentric chromosome pairs (1, 2, 3 and 15), ten submetacentric (4, 5, 6, 8, 9, 10, 11, 12, 13 and 14) and two acrocentric ones (7 and 16) **d** *D. arifolia* presented four metacentric chromosome pairs (1, 2, 5 and 10) and twelve submetacentric ones (3, 4, 6, 7, 8, 9, 11, 12, 13, 14, 15 and 16). Bar: 5 μm.

Table 1. Morphometry of the metaphasic chromosomes of *D. elata* (2C = 3.49 pg, 2n = 32), *D. bonijesu* (2C = 4.05 pg, 2n = 32) and *D. arifolia* (2C = 5.47 pg, 2n = 32).

Chrom.	*D. elata*						*D. bonijesu*						*D. arifolia*					
	Total (µm)	Arms		r	Class	Size (%)	Total (µm)	Arms		r	Class	Size (%)	Total (µm)	Arms		r	Class	Size (%)
		Short	Long					Short	Long					Short	Long			
1	4.72	2.09	2.63	1.26	M	8.01	4.50	1.95	2.55	1.31	M	7.97	5.39	2.57	2.82	1.10	M	7.93
2	4.27	2.00	2.27	1.14	M	7.25	4.27	1.90	2.37	1.25	M	7.56	5.21	2.45	2.76	1.13	M	7.67
3	4.21	1.66	2.55	1.54	SM	7.15	4.04	1.81	2.23	1.23	M	7.15	4.87	1.48	3.39	2.29	SM	7.17
4	3.87	1.78	2.09	1.17	M	6.57	4.00	1.45	2.55	1.76	SM	7.08	4.81	1.39	3.42	2.46	SM	7.08
5	3.84	1.75	2.09	1.19	M	6.52	3.86	1.27	2.59	2.04	SM	6.83	4.69	2.27	2.42	1.07	M	6.90
6	3.81	1.48	2.33	1.57	SM	6.47	3.81	1.45	2.36	1.63	SM	6.75	4.36	1.42	2.94	2.07	SM	6.42
7	3.81	1.69	2.12	1.25	M	6.47	3.72	0.90	2.82	3.13	A	6.59	4.33	1.39	2.94	2.12	SM	6.37
8	3.72	1.57	2.15	1.37	M	6.32	3.59	1.22	2.37	1.94	SM	6.36	4.18	1.45	2.73	1.88	SM	6.15
9	3.66	1.54	2.12	1.38	M	6.21	3.59	1.22	2.37	1.94	SM	6.36	4.18	1.27	2.91	2.29	SM	6.15
10	3.59	1.75	1.84	1.05	M	6.10	3.54	1.00	2.54	2.54	SM	6.27	3.93	1.81	2.12	1.17	M	5.78
11	3.54	0.71	2.83	3.99	A	6.01	3.40	0.95	2.45	2.58	SM	6.02	3.93	1.36	2.57	1.89	SM	5.78
12	3.48	0.62	2.86	4.61	A	5.91	3.04	0.86	2.18	2.53	SM	5.38	3.84	1.39	2.45	1.76	SM	5.65
13	3.30	1.33	1.97	1.48	M	5.60	2.95	0.95	2.00	2.11	SM	5.22	3.78	1.21	2.57	2.12	SM	5.56
14	3.24	1.42	1.82	1.28	M	5.50	2.95	0.81	2.14	2.64	SM	5.22	3.63	1.18	2.45	2.08	SM	5.34
15	3.03	1.30	1.73	1.33	M	5.14	2.63	1.09	1.54	1.41	M	4.66	3.51	1.24	2.27	1.83	SM	5.16
16	2.81	1.36	1.45	1.07	M	4.77	2.59	0.59	2.00	3.39	A	4.59	3.33	1.21	2.12	1.75	SM	4.90
Sum	56.09	24.36	34.54	-	-	100.00	53.89	19.42	37.06	-	-	100.00	64.64	25.09	42.88	-	-	100.00

Chrom – chromosome; r – arm ratio (long/short); Size – % size in relation to sum of the mean values of total length; M – metacentric; SM – submetacentric; A – acrocentric; Sum – sum of the mean values.

centric and submetacentric chromosomes. Therefore, the FCM and cytogenetic data indicate that structural chromosome changes have occurred throughout the evolution of these *Dorstenia* species.

According to the more recent phylogeny for the genus, *D. arifolia* occupies a basal position in comparison to *D. elata* (Misiewicz and Zerega 2012). Given that *D. arifolia* has mean nuclear 2C value and total genome length (Fig. 1 and 2, Table 1) greater than *D. elata*, the structural chromosome alterations seem to promote loss of DNA sequences. Thus, deletions can be involved in the karyotype changes in *Dorstenia*.

Based on the morphometric analysis, groups of morphologically identical chromosome pairs were found for each *Dorstenia* species: 3–4, 6–7, 11–12 and 13–14 in *D. arifolia*; 5–6, 11–12 and 13–14 in *D. bonijesu*; and 7–8 and 14–15 in *D. elata* (Fig. 2, Table 1). Regarding this karyotype aspect, polyploidization events have occurred during the evolution of these species. As the three species produces reduced reproductive cells (x = 16), the disploidy can also explain the evolutionary scenario of the *Dorstenia* karyotype. The polyploid origin of plant species has been shown by classical cytogenetics, such as for the genera *Psidium* Linnaeus, 1753 (Souza et al. 2015), *Claytonia* Linnaeus, 1753 (McIntyre 2012) and *Cardamine* Linnaeus, 1753 (Marhold et al. 2010). From the assembly of accurate karyograms using chromosomes with different levels of chromatin compaction, the type of polyploidy was also evidenced, with autopolyploidy being found in *Glycine max* (Linnaeus, 1753) Merrill, 1917 (Clarindo et al. 2007) and *Zephyranthes* Herbert, 1821 (Felix et al. 2011), and allopolyploidy in *Paullinia cupana* Kunth, 1821 (Freitas et al. 2007) and *Triticum aestivum* Linnaeus, 1753 (Kamel 2006). Polyploidy has played a key role in plant evolution, with estimates maintaining euploidy in the ascendancy of all angiosperms (Abbott et al. 2013, Soltis et al. 2014). In addition, 15% of speciation events in this taxon are directly involved with polyploidization (Abbott et al. 2013).

Conclusion

The nuclear 2C value and karyogram indicate changes covering chromosome number and structure that occurred during the karyotype evolution of *D. arifolia*, *D. bonijesu* and *D. elata*. The combination of FCM and classical cytogenetics revealed differences among the *Dorstenia* species that can be exploited in phylogenetic approaches, as the results support the current knowledge on the phylogeny of *Dorstenia*.

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SHORT COMMUNICATION

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Karyotype of three *Lonchophylla* species (Chiroptera, Phyllostomidae) from Southeastern Brazil

Brunna Almeida[1,2], Roberto Leonan Morim Novaes[3], Marcia Aguieiras[1], Renan de França Souza[4], Carlos Eduardo Lustosa Esbérard[5], Lena Geise[1]

1 *Universidade do Estado do Rio de Janeiro, Departamento de Zoologia, Rua São Francisco Xavier 524, 20550-013, Maracanã, Rio de Janeiro, RJ, Brazil* **2** *Museu Nacional do Rio de Janeiro, Departamento de Vertebrados, Quinta da Boa Vista s/n, 20940-040, São Cristóvão, Rio de Janeiro, RJ, Brazil* **3** *Fundação Oswaldo Cruz, Campus Fiocruz da Mata Atlântica, Estrada Rodrigues Caldas 3400, 22713375, Taquara, Rio de Janeiro, RJ, Brazil* **4** *Universidade do Estado do Rio de Janeiro, Departamento de Ecologia, Rua São Francisco Xavier 524, 20550-013, Maracanã, Rio de Janeiro, RJ, Brazil* **5** *Universidade Federal Rural do Rio de Janeiro, Instituto de Biologia, Km 47 da antiga estrada Rio-São Paulo, 23851-970, Seropédica, RJ, Brazil*

Corresponding author: *Brunna Almeida* (brunninhaas@gmail.com)

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Abstract

Lonchophylla Thomas, 1903 is a Neotropical bat genus that comprises 12 species, with little cytogenetic information available. Here we present the description of the karyotype of three species collected in Southeastern Brazil. *Lonchophylla bokermanni* Sazima, Vizotto & Taddei, 1978, *Lonchophylla dekeyseri* Taddei, Vizotto & Sazima, 1983, and *Lonchophylla peracchii* Dias, Moratelli & Esberard, 2013 showed the same diploid number 2n = 28 and the same autosomal fundamental number FNa = 50, in both *L. bokermanni* and *L. peracchii*. We observed that the karyotypes were also cytogenetically similar when we compared the studied species with other species within the same genus. It is therefore not possible to differentiate the species using only karyotypes with conventional staining. However, this information increases the knowledge of the genus and can be one more important character for a better phylogenetic comprehension of this taxon.

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Keywords
Karyology, chromosomes, Bokermann's Nectar Bat, Atlantic Forest, Cerrado, Endangered species, Lonchophyllinae, range extension

Introduction

In recent years, new species and a genus of the subfamily Lonchophyllinae were described: *Lonchophylla peracchii* Dias, Moratelli & Esberard, 2013, *L. inexpectata* Moratelli & Dias, 2015, and *Hsunycteris* Parlos, Timm, Swier, Zeballos & Baker, 2014 (Dias et al. 2013, Parlos et al. 2014, Moratelli and Dias 2015). For the description of bat species, morphological and morphometric characteristics are usually employed, but the use of other tools such as cytogenetic analysis can provide essential information for evolutionary relationships of bats (Varella-Garcia and Taddei 1989, Garcia and Pêssoa 2010), as already seen for rodents, for example (Romanenko and Volobouev 2012). Although there are few cytogenetic data for Lonchophyllinae, they were nevertheless informative for systematic rearrangements of this taxon (see Parlos et al. 2014).

In Brazil, there are records for five species of this genus: *Lonchophylla bokermanni* Sazima, Vizotto & Taddei, 1978, *Lonchophylla dekeyseri* Taddei, Vizotto & Sazima, 1983, *Lonchophylla inexpectata*, *Lonchophylla mordax* Thomas, 1903 and the new species, *Lonchophylla peracchii* mentioned above. There are karyotype data available until now for the two congeneric taxa from outside the country, *Lonchophylla robusta* Miller, 1912 and *Lonchophylla concava* Goldman, 1914 (Parlos et al. 2014), but no cytogenetic data were available for Brazilian species. Therefore, this study is the first to describe the karyotype of *L. bokermanni*, *L. dekeyseri* and *L. peracchii*.

Material and methods

Five individuals of *Lonchophylla* were collected and four were karyotyped: one adult female (MN79997) and one adult male of *L. bokermanni* (MN81467), one adult female of *L. dekeyseri* (MN80002) and one adult male of *L. peracchii* (MN81468).

L. bokermanni was captured in Fazenda Santa Cruz, Diamantina municipality (18°16'11"S; 43°23'04"W, 1.129 m a.s.l), in the Vale do Jequitinhonha, Minas Gerais State (Figure 1). The locality has a Cerrado vegetation classified as arboreal savanna with enclaves of deciduous forest (IBGE 2012). Sampling occurred in March 2011 using 13 to 15 mist-net (9 × 3 m, 35 mm mesh), which remained open in the first six hours after the sunset for six consecutive nights.

L. dekeyseri was captured in Fazenda Ilha, Itinga municipality (16°38'05"S; 41°50'54"W, 240 m a.s.l), Vale do Jequitinhonha, Minas Gerais State (Figure 1). The locality is in the Cerrado, with vegetation classified as open savanna in transition with Dry Forest (IBGE 2012). The sampling procedures were performed in March 2012,

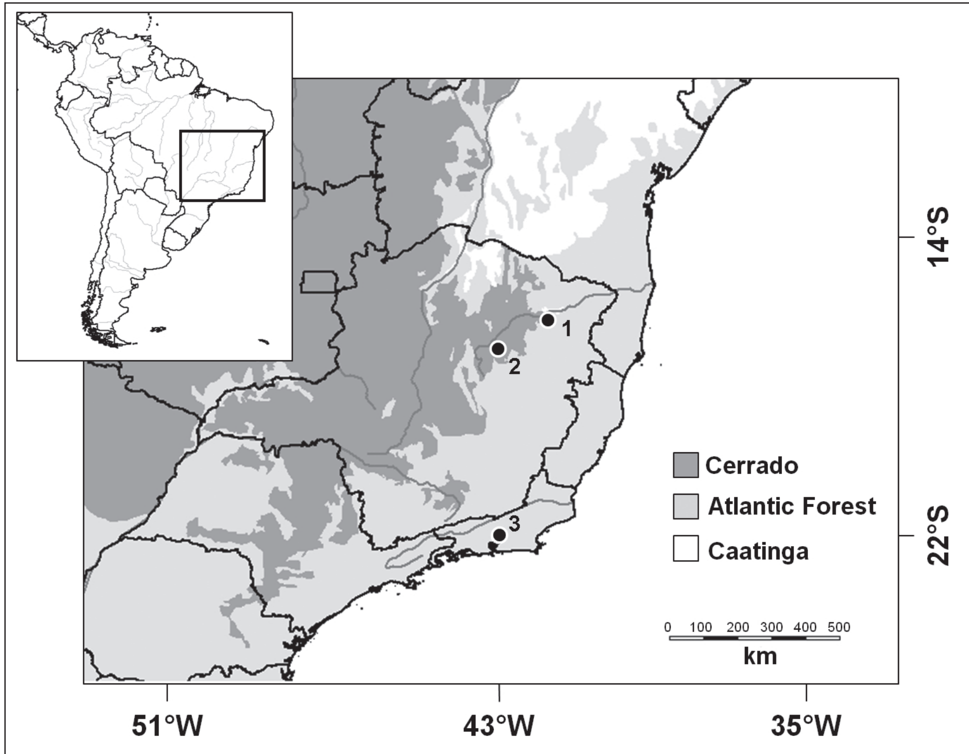

Figure 1. Localities of *Lonchophylla* species records: **1** Itinga, Minas Gerais **2** Dimantina, Minas Gerais **3** Magé, Rio de Janeiro.

using 12 to 16 mist-nets (9 × 3 m, 35 mm mesh) that remained open in the first six hours after the sunset for seven consecutive nights.

L. peracchii was captured in Reserva Particular do Patrimônio Natural El Nagual, Magé municipality (22°32'55"S; 43°03'20"W, 197 m a.s.l), Rio de Janeiro State (Figure 1). The locality is in the Atlantic Forest, with vegetation classified as Ombrophilous Dense Forest (IBGE 2012). Sampling occurred in August 2012 using two mist-nets (12 × 3 m, 30 mm mesh) that remained open throughout night period (± 12 hours) for two consecutive nights.

Chromosomes in metaphases were obtained through *in vitro* bone marrow culture grown in Dulbecco´s MEM with 10% fetal bovine serum and colchicine for 2 hours, following by an incubation in KCl 0.075M solution at 37 °C by 30 minutes, centrifuged, fixed in Carnoy solution (methanol: acetic acid, 3:1). The fixation step was repeated three times. Preparation was done by dropping one drop by distance onto clean microscope slides and air-dried. Conventional staining with Giemsa 5% was used to observe diploid number (2n) and Fundamental Number of autosomal arms (FNa) and chromosome morphology variation. This analysis was carried out using an

optic photomicroscope (Nikon Eclipse 50i), in a 1,000 increase – lenses of 100 plus 10 ocular lenses.

Captures were authorized by IBAMA (1785/89-IBAMA) and SISBIO (4156/95-46 in the Vale of Jequitinhonha and 3893-1/28717 in Magé).

Results and discussion

All three species showed the same diploid number 2n = 28 and an autosomal fundamental number FNa = 50 was observed (Figure 2). The autosomal complement of males *L. bokermanni* and *L. peracchii* consists of 12 pairs of meta/submetacentrics varying from large to small, and a pair of small acrocentric chromosomes (FNa = 50). Two size classes of autosomal chromosomes can be observed – the eight first are all large chromosomes, and in the second row (Figure 2A–C), with smaller ones, including four metacentric and the smallest chromosome of the karyotype, the only acrocentric ones. The X chromosome is a medium sized metacentric and the Y is a minute acrocentric, smaller than the last pair of autosomal complement. Similarly, the karyotype of *L. dekeyseri* can be characterized but the identification of the sex chromosome pair was impossible in the sole collected specimen which was a female.

Karyotype comparison is considered as an important tool to establish phylogenetic relationships and as a taxonomic tool to confirm some species identities (Baker 1970, Silva et al. 2006, Urdampilleta et al. 2013). However, the resolution power of the cytogenetic method is not the same for all groups. Sometimes, it is necessary to analyze as many as possible the species' karyotypes (Garcia and Pessoa 2010). In bats, the few available published karyotype data from South America (Moratelli and Morielle-Versute 2007) make it difficult to propose, using such kind of information, new or different taxonomic arrangements and a better comprehension of the systematics of Neotropical bats (Garcia and Pessoa 2010).

Three new karyotypes here described for *L. bokermanni*, *L. dekeyseri* and *L. peracchii* are similar to those known for *L. robusta* (Baker 1973, Baker 1979) and *L. concava* (Parlos et al. 2014). A species currently allocated to the genus *Hsunycteris* and previously described as *L. thomasi*, presents different karyotype compositions: 2n = 30, FNa = 34; 2n = 32, FNa = 34, 38 and 40; 2n = 36, FNa = 48. Additionally, this species also presented an increased number of acrocentric chromosomes, whereas in other *Lonchophylla* species, only a pair of small acrocentric is observed (Pair 13 in Figure 2) (Parlos et al. 2014).

The karyotype conservatism in Microchiroptera has been observed in other studies (Varella-Garcia et al. 1989, Sousa and Araújo 1990) which well corroborate with our results. Even if species distinction is not evident for representatives of the *Lonchophylla* genus through the conventional chromosome characteristics, the generic separation of *Lonchophylla* – *Hsunycteris* is supported by their different karyotypes.

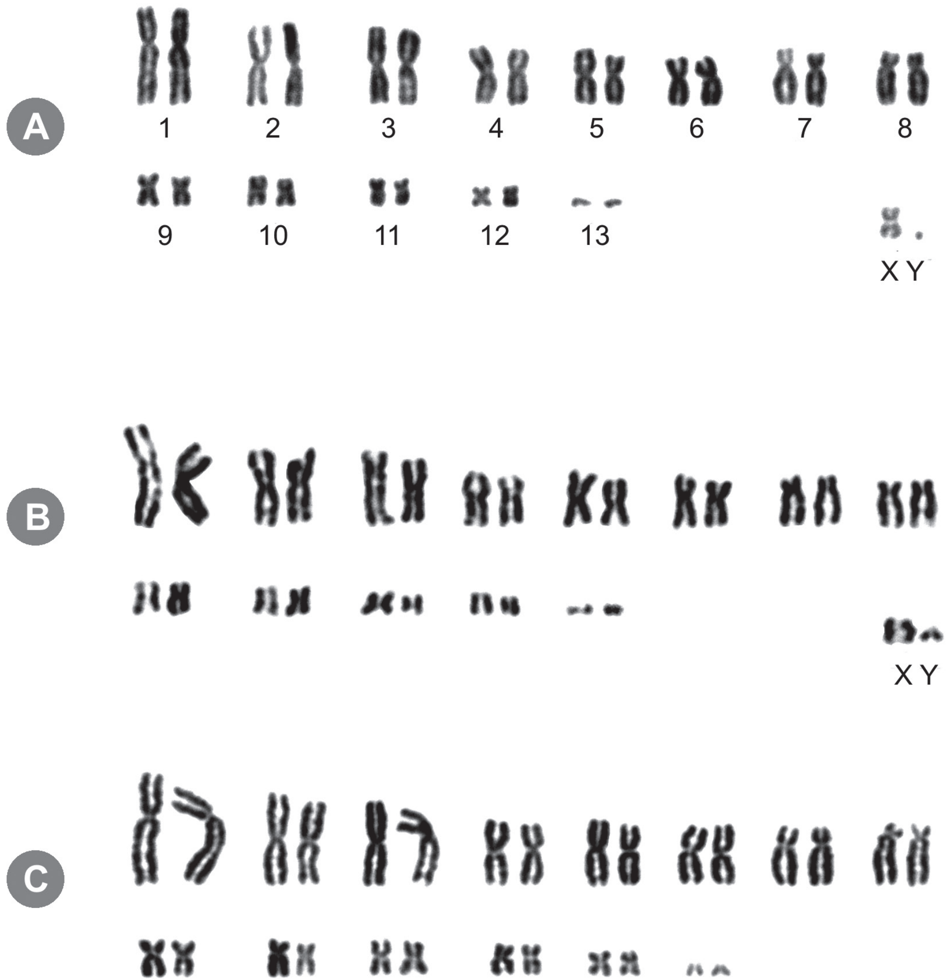

Figure 2. Giemsa-stained karyotypes of **A** *Lonchophylla bokermanni* 2n = 28, FNa = 50 (male, MN81467) **B** *Lonchophylla peracchii* 2n = 28, FNa = 50 (male, MN81468) and **C** *Lonchophylla dekeyseri* 2n = 28 (female, MN80002).

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SHORT COMMUNICATIONS

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Refinement of the karyological aspects of *Psidium guineense* (Swartz, 1788): a comparison with *Psidium guajava* (Linnaeus, 1753)

Anelise Machado Marques[1], Amélia Carlos Tuler[2], Carlos Roberto Carvalho[3], Tatiana Tavares Carrijo[4], Marcia Flores da Silva Ferreira[5], Wellington Ronildo Clarindo[1]

1 *Laboratório de Citogenética, Departamento de Biologia, Centro de Ciências Agrárias, Universidade Federal do Espírito Santo, CEP: 29.500-000 Alegre – ES, Brazil* **2** *Escola Nacional de Botânica Tropical, Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, CEP: 22.460-036 Rio de Janeiro – RJ, Brazil* **3** *Laboratório de Citogenética e Citometria, Departamento de Biologia Geral, Centro de Ciências Biológicas e da Saúde, Universidade Federal de Viçosa, CEP: 36.570-000 Viçosa – MG, Brazil* **4** *Laboratório de Botânica, Departamento de Biologia, Centro de Ciências Agrárias, Universidade Federal do Espírito Santo, CEP: 29.500-000 Alegre – ES, Brazil* **5** *Laboratório de Genética e Melhoramento Vegetal, Departamento de Biologia, Centro de Ciências Agrárias, Universidade Federal do Espírito Santo, CEP: 29.500-000 Alegre – ES, Brazil*

Corresponding author: *Wellington Ronildo Clarindo* (welbiologo@gmail.com)

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Abstract

Euploidy plays an important role in the evolution and diversification of *Psidium* Linnaeus, 1753. However, few data about the nuclear DNA content, chromosome characterization (morphometry and class) and molecular markers have been reported for this genus. In this context, the present study aims to shed light on the genome of *Psidium guineense* Swartz, 1788, comparing it with *Psidium guajava* Linnaeus, 1753. Using flow cytometry, the nuclear 2C value of *P. guineense* was 2C = 1.85 picograms (pg), and the karyotype showed 2n = 4x = 44 chromosomes. Thus, *P. guineense* has four chromosome sets, in accordance with the basic chromosome number of *Psidium* (x = 11). In addition, karyomorphometric analysis revealed morphologically identical chromosome groups in the karyotype of *P. guineense*. The high transferability of microsatellites (98.6%) further corroborates with phylogenetic relationship between *P. guajava* and *P. guineense*. Based on the data regarding nuclear genome size, karyotype morphometry and molecular markers of *P. guineense* and *P. guajava* (2C = 0.95 pg, 2n = 2x = 22 chromosomes), *P. guineense* is a tetraploid species. These data reveal the role of euploidy in the diversification of the genus *Psidium*.

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Keywords
Psidium, polyploidy, karyotype evolution, cytogenetic, flow cytometry, SSR markers

Introduction

Psidium Linnaeus, 1753 is a genus of Myrtaceae that comprises about 92 species (Govaerts et al. 2013), predominantly distributed in the Neotropics. The species of this genus differ from those belonging to other Myrtaceae genera by seeds with bony testa, cochlear embryo with small cotyledons and large hypocotyl (Landrum and Kawasaki 1997). Brazil is a relevant center of *Psidium* species diversity, comprising approximately 60 taxa widely distributed in different biomes (Sobral et al. 2014). The genus is economically important (Rai et al. 2010), with *Psidium guajava* Linnaeus, 1753, *Psidium cattleyanum* Sabine, 1821 and *Psidium guineense* Swartz, 1788 being the most relevant commercial species for fruit production and/or source of compounds in the pharmaceutical industry. Of these taxa, *P. cattleianum* (Costa and Forni-Martins 2007, Costa et al. 2008, Souza et al. 2015) and *P. guajava* (Costa and Forni-Martins 2007, Coser et al. 2012) are the best-known species with regard to cytogenetic features.

Karyotypic characterization has been applied to better understand the changes that occur during genome evolution (Éder-Silva et al. 2007). Based on previous cytogenetic studies, euploidy has led to diversification in *Psidium* (Briggs and Walters 1997). In fact, a series of euploid organisms, such as diploid (2n = 22), tetraploid (2n = 44), hexaploid (2n = 66) and octoploid (2n = 88) species (Atchison 1947, Costa and Forni-Martins 2006a, 2006b, 2007), derived from the basic x = 11 chromosome number (Atchison 1947, Costa et al. 2008), has been reported for the genus. Nevertheless, the relationship among species that arose from euploidy events is still poorly understood in *Psidium*.

According to current knowledge, few *Psidium* species are diploid (2n = 22), such as *Psidium chinense* Loudon, 1830 (Naitani and Srivastava 1965), *Psidium friedrichsthalianum* Niedenzu, 1893 and *P. guajava*, which is the only diploid species whose karyotype has been characterized (Coser et al. 2012). Considering that the genus *Psidium* shows polyploid species (2n = 44–88 chromosomes), the allo- and/or autopolyploidization in diploid species of this genus can be related to the occurrence of polyploidy. Thus, the chromosome number and karyotype characterization of the polyploid species represents the basis to understand the origin and diversification in *Psidium*.

Euploid species are key models for evolution because they provide evidence of the polyploidization event that promoted diversification and speciation. Considering that, this study aimed to refine the knowledge about karyological aspects of *Psidium guineense*. Besides, a comparison was performed with the diploid species (2x = 22) *P. guajava*, because this species is the only of the *Psidium* genus characterized from flow cytometry (FCM), cytogenetic (Coser et al. 2012) and molecular markers (Risterucci et al. 2005, Guavamap 2008, Nogueira et al. 2015).

Material and methods

Psidium guajava fruits were obtained from 50 plants growing in orchards located in different regions of the Brazil. *Psidium guineense* fruits were obtained from indigenous populations occurring in Atlantic Forest remnants located in the Municipalities of Alegre (four individuals), Itapemirim (three individuals), Santa Teresa (seven individuals), and Conceição da Barra (six individuals), all located in Espírito Santo state. The sampling was done between 2012 and 2014.

FCM and molecular analyses were conducted with the same 50 individuals of *P. guajava* and 20 of *P. guineense*. Due to FCM results, karyotype characterization was performed using seeds obtained from ten distinct plants of the two species. *Solanum lycopersicum* Linnaeus, 1753, 'Stupické' (reference standard for FCM, 2C = 2.00 picograms – pg; Praça-Fontes et al. 2011) seeds were supplied by Dr. Jaroslav Doležel (Experimental Institute of Botany – Czech Republic).

2C nuclear measurement

Leaves were collected from *S. lycopersicum* (standard), *P. guajava* and *P. guineense* (samples). Nuclei suspensions were obtained from leaf fragments of the standard and of each sample, according to a previously described protocol (Otto 1990, Coser et al. 2012). These suspensions were analyzed in a Partec PAS® flow cytometer (Partec® GmbH, Munster – Germany) equipped with a laser source (488 nm). Nuclei-emitted propidium iodide fluorescence was collected by an RG 610-nm band-pass filter. The equipment was calibrated for linearity and aligned with microbeads and standard solutions according to the manufacturer's recommendations. FloMax® software (Partec®) was used for the data analysis. Six independent replicates were performed for each individual, with over 10,000 nuclei analyzed per replicate. The mean 2C values of *P. guajava* and *P. guineense* were calculated by dividing the mean channel of the G_0/G_1 fluorescence peak for the reference standard by the mean channel of the G_0/G_1 peak for each sample.

Karyotype characterization

Seeds of *P. guineense* and *P. guajava* were germinated in Petri dishes containing distilled water (dH_2O) at 30 °C. The roots showing 1.0–2.0 cm in length were treated for a period of 4, 15 or 19 h with the microtubule-inhibiting agents amiprophos-methyl (APM, Nihon Bayer Agrochem K. K.®) or oryzalin (ORY, Sigma®) at a final concentration of 4 μM. Subsequently, the roots were washed with dH_2O for 20 min, then fixed in fresh methanol:acetic acid (Merck®) solution (3:1). The fixative was changed three times, and the roots were stored at -20 °C for 24 h. The roots were washed and incubated for

2:00, 2:15 or 2:30 h at 34 °C in pectinase solution (Sigma®, E6287) at ratios of 1:8, 1:10, 1:12 or 1:15 (enzyme:water). Next, the roots were washed for 10 min in dH_2O, fixed once more, and stored at -20 °C (Coser et al. 2012). Slides were prepared using the techniques of root meristem dissociation and air-drying (Carvalho et al. 2007). The slides were analyzed and the chromosome images were captured with a Media Cybernetics® Evolution™ charge-coupled device (CCD) video camera mounted on a Nikon 80i microscope (Nikon – Japan).

Molecular analysis

The genomic DNA was extracted from young leaves according to Doyle and Doyle (1990). The integrity and concentration of the DNA samples were verified using a Nanodrop™ 2000. Amplification reactions were performed using 142 simple sequence repeat (SSR) markers (Suppl. material 1) designed for *P. guajava* (Risterucci et al. 2005, Guavamap 2008). Each amplification reaction consisted of 15 µL of solution containing: 60 ng DNA, 0.3 µM of each primer, 1.5 U Taq polymerase DNA (Phoneutria), 1.7 µM $MgCl_2$ and 0.2 µM dNTPs. The following program was used: denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at temperature (T_a) of 50 °C or 55 °C for 1 min, and extension at 72 °C for 8 min. The reactions were performed in a Veriti® 96-Well Thermal Cycler ABI. The amplification products were separated using 6% polyacrylamide gel electrophoresis, stained with ethidium bromide, and photographed using a photo-documentation system (ChemiDoc XRS + System – Bio-Rad™). For confirmation, up to three independent replications were performed.

Results and discussion

The FCM protocol, using isolation buffer for 10 min and staining buffer for 30 min, provided peaks relative to G_0/G_1 nuclei with coefficient of variation (CV) lower than 3.46%, and thus high resolution. This result indicates that the suspensions contained sufficient number of intact, isolated and stoichiometrically stained nuclei.

Based upon the large number of plant samples of distinct genotypes evaluated in this study, the mean nuclear 2C value is 0.95 pg for all *P. guajava* plants (Fig. 1a) and 1.85 pg for all *P. guineense* pants (Fig. 1b). The 2C values of *P. guajava* and *P. guineense* are small compared with those of most angiosperms, according to reference values defined by Bennett and Leitch (2011). Similarly, low 2C DNA content values were also found in some Myrtaceae, such as the genus *Eucalyptus* L'Hér. 1789, which varies from 0.80 to 1.50 pg.

Psidium guajava was one of the first Myrtaceae species for which the nuclear genome size was measured using Feulgen microdensitometry. With this method, mean values of 2C = 0.66 pg (Bennett and Smith 1976) and 2C = 1.24 pg (Ohri 2002) were

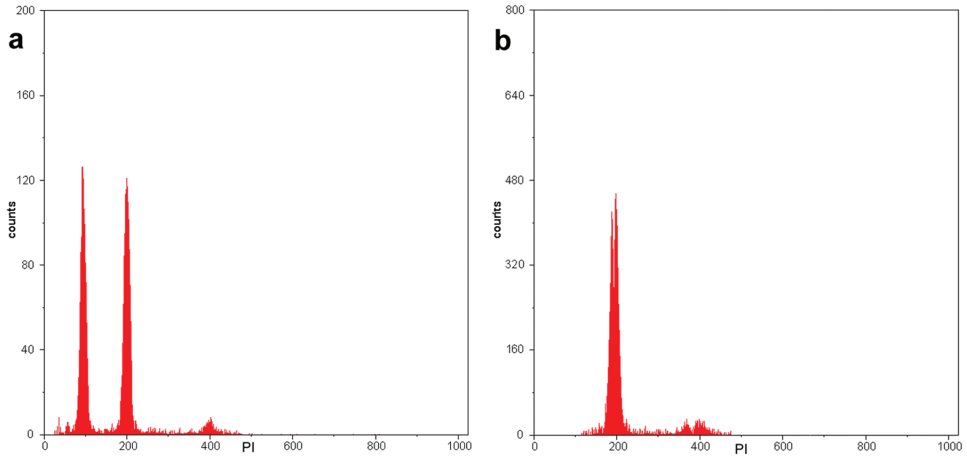

Figure 1. Representative histograms obtained from FCM analysis of nuclear suspensions stained with propidium iodide. **A** G_0/G_1 nuclei peak of the sample *P. guajava* (2C = 0.95 pg), positioned in channel 95, and the standard *S. lycopersicum* (2C = 2.00 pg) in channel 200 **B** G_0/G_1 nuclei peak of the sample *P. guineense* (2C = 1.85 pg), positioned in channel 185, and the standard *S. lycopersicum* (2C = 2.00 pg) in channel 200.

obtained. Nuclear DNA content has also been measured for *P. guajava* using FCM, and the mean values were 2C = 0.507 pg ('White'), 2C = 0.551 pg ('Red', Costa et al. 2008), 2C = 0.95 pg (28 genotypes, Coser et al. 2012), 2C = 0.99 pg ('Paluma') and 2C = 1.02 pg ('Purple', Souza et al. 2015). In the present study, the 2C value for *P. guineense* was 2C = 1.85 pg, approximately twice that observed in *P. guajava* (2C = 0.95 pg). The 2C value of *P. guineense* has also been measured as 2C = 2.02 pg (Souza et al. 2015).

The distinct 2C values observed for *P. guineense* and *P. guajava* may be related to the different techniques, plant standards, nuclear isolation and staining procedures used. More inconsistent values of DNA content were found by Costa et al. (2008), who used *Arabidopsis thaliana* Linnaeus, 1753, 'Columbia' (2C = 0.32 pg) as reference standard. The leaf of this species exhibits endopolyploidy (2C, 4C, 8C…) (Yotoko et al. 2011); thus, it is necessary to correctly check the reference G_0/G_1 peak to measure the 2C value of the sample based on the 2C nuclei of this standard.

Based on DNA content, the occurrence of karyotype modifications that increased the genome size may have played a role in the origin of *P. guineense*. To confirm this hypothesis, karyotypic characterization was accomplished for *P. guineense* and *P. guajava*. The root tips that were treated with 4 μM APM for 15 h and macerated in 1:10 pectinase solution for 2 h provided the most adequate metaphases for morphometric analysis. Metaphases were chosen based on relevant characteristics: well-spread chromosomes with well-defined constriction, without chromatin deformations and cytoplasmic background noise. These features allowed accurate chromosome counting, morphometric characterization and assembly of the karyograms (Fig. 2, Table 1).

Table 1. Morphometric data and classification of the chromosomes of *P. guajava* and *P. guineense*. The mean values of total length, short and long arms were measured using at least 10 metaphases of each species.

P. guajava						*P. guineense*					
Chrom[a]	Total[b]	Short arm[b]	Long arm[b]	r[c]	Class[d]	Chrom[a]	Total[b]	Short arm[b]	Long arm[b]	r[c]	Class[d]
1	2.03	0.73	1.30	1.78	SM	1–2	1.82	0.63	1.20	1.91	SM
2	1.60	0.52	1.08	2.08	SM	3–4	1.60	0.60	1.00	1.67	SM
3	1.57	0.64	0.93	1.45	M	5–6	1.53	0.57	0.96	1.70	SM
4	1.52	0.73	0.79	1.08	M	7–8	1.44	0.55	0.89	1.62	SM
5	1.47	0.58	0.89	1.53	SM	9–10	1.30	0.50	0.80	1.60	SM
6	1.42	0.53	0.89	1.68	SM	11–12	1.25	0.58	0.68	1.17	M
7	1.37	0.53	0.84	1.58	SM	13–14	1.20	0.48	0.73	1.53	SM
8	1.15	0.56	0.59	1.05	M	15–16	1.17	0.43	0.74	1.70	SM
9	1.12	0.45	0.67	1.49	M	17–18	1.06	0.30	0.76	2.53	SM
10	1.05	0.50	0.55	1.10	M	19–20	0.93	0.25	0.68	2.73	SM
11	0.85	0.27	0.58	2.15	SM	21–22	0.66	0.20	0.46	2.29	SM
Total[e]	15.15						13.96				

[a]Chrom – chromosome of *P. guajava* and chromosome groups of *P. guineense*; [b]Length in µm; [c]Measured by arm ratio – long/short; [d]Class: M – metacentric and SM – submetacentric; [e]Total value based on basic chromosome number X = 11.

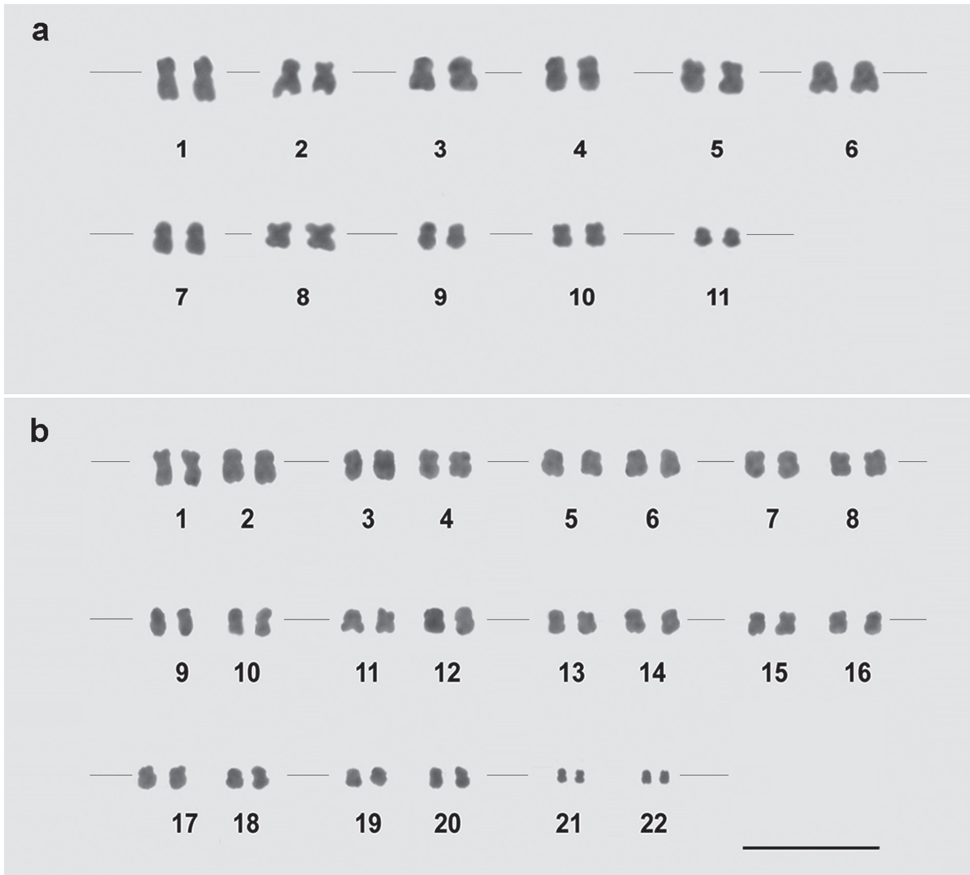

Figure 2. A *P. guajava* karyogram showing 2n = 2x = 22 chromosomes, being five metacentric (3, 4, 8, 9, 10) and six submetacentric pairs (1, 2, 5, 6, 7, 11) **B** *P. guineense* karyogram showing 2n = 4x = 44 chromosomes, with two metacentric (11, 12) and twenty submetacentric pairs (1–10, 13–22). Note groups of morphologically identical chromosomes, such as 1 and 2, 3 and 4, 21 and 22. Bar = 5 μm.

The chromosome number of *P. guajava* and of *P. guineense* were accurately determined here as 2n = 2x = 22 and 2n = 4x = 44, respectively (Fig. 2, Table 1). Thus, no intraspecific karyotype variations were identified for all *P. guajava* and *P. guineense* plants. Differently, other studies have reported cytotypes for *P. guineense* (Srivastava 1977) and mainly for *P. guajava* (Kumar and Ranade 1952, Majumder and Mukherjee 1972, Srivastava 1977, Costa and Forni-Martins 2006a, Éder-Silva et al. 2007, Souza et al. 2015), indicating the occurrence of an intraspecific chromosome variation related to euploidy and aneuploidy. During all period of the experiments (2012–2014), none plant exhibiting somatic chromosome number variation was recorded for both *Psidium* species.

Psidium guajava exhibited metacentric (pairs 3, 4, 8, 9, 10) and submetacentric chromosomes (pairs 1, 2, 5, 6, 7, 11). This species had relatively small and morphologically similar chromosomes, two of which (1 and 11) were distinguished by their

very distinct total length. Paredes et al. (2006) reported variation in the morphometric classification of the chromosomes of some *P. guajava* genotypes, relating seven metacentric, two submetacentric and two acrocentric chromosome pairs. However, the same authors reported eight metacentric, one submetacentric and two acrocentric chromosome pairs in other genotypes. Coser et al. (2012) studied for the first time the morphometric characterization of *P. guajava* using enzymatic cellular dissociation of the roots and air-drying of the slides. The authors observed that, independently of genotype, *P. guajava* has 2n = 2x = 22 chromosomes with five metacentric (3, 4, 8, 9, 10) and six submetacentric pairs (1, 2, 5, 6, 7, 11).

As observed for *P. guajava*, the karyotype of *P. guineense* also showed only metacentric (11, 12) and submetacentric (1–10, 13–22) chromosomes (Table 1). Previous cytogenetic approaches revealed a karyotype for *P. guineense* of 2n = 4x = 44 chromosomes (Chakraborti et al. 2010). Besides metacentric and submetacentric chromosomes, Chakraborti et al. (2010) also reported an acrocentric one, as well as a chromosome pair distinguished by a secondary constriction for *P. guineense*. The two latter features were not found in the present work.

The karyomorphometric analysis also revealed groups of morphologically identical chromosomes in *P. guineense*: 1–2, 3–4, 5–6, 7–8, 9–10, 11–12, 13–14, 15–16, 17–18, 19–20 and 21–22 (Table 1). Therefore, the cytogenetic procedures discriminated 11 chromosome groups, equivalent to the basic chromosome number of the genus *Psidium*. Based on total size and class, the previous study performed by Chakraborti et al. (2010) identified only four chromosome groups (A, B, C and D) for *P. guineense*.

Considering the basic chromosome number of *Psidium* (x = 11) (Atchison 1947, Costa et al. 2008), the cytogenetic data suggest the origin of *P. guineense* from a polyploidization event. Therefore, the cytogenetic data confirm the FCM results in which the mean DNA contents of *P. guajava* (2C = 0.95 pg) and *P. guineense* (2C = 1.85 pg) indicate the polyploidy origin of the latter species. Polyploid species have been reported for *Psidium* (Atchison 1947, Andrade and Forni-Martins 1998, Costa and Forni-Martins 2006a, 2006b, 2007, Costa et al. 2008), as tetraploid (2n = 44, *Psidium acutangulum* Candolle, 1828, *P. cattleyanum* Sabine, *Psidium grandifolium* Candolle, 1828, *Psidium friedrichsthalianum* and *P. guineense*), hexaploid (2n = 66, *P. cattleyanum*) and octoploid (2n = 88, *P. cattleyanum*) plants.

From meiotic analysis in *P. guineense*, Chakraborti et al. (2010) related the occurrence of 22 bivalents and, consequently, of a Mendelian segregation in anaphases. These facts and cytogenetic data found here suggest that *P. guineense* is a true allopolyploid. A true allopolyploid is a hybrid formed through reproductive cells of species with different karyotypes (Stebbins 1947). Due of this, homologous chromosomes paring in meiosis, enabling the establishment only of bivalents and the formation of viable reproductive cells. Therefore, the reproductive behavior of the true allopolyploids is like a diploid species, allowing the maintenance of the ploidy level during the generations, as observed for *P. guineense* (Chakraborti et al. 2010).

The variation in chromosome number seen in the genus *Psidium* can promote genetic isolation and possibly create barriers to gene flow (Stace 1991), leading to specia-

tion (Briggs and Walters 1997). Polyploidy is considered one of the main mechanisms of evolution in plants (Soltis et al. 2003). Auto- or allopolyploids may exhibit genetic and phenotypic alterations compared with their ancestral species (Soltis and Soltis 1999, Mable 2003). These changes can be observed in the first generation after polyploidization or hybridization, and also along the evolutionary history of the polyploid, leading to increased diversity (Soltis and Soltis 1999, Soltis et al. 2009, Weiss-Schneeweiss et al. 2013).

Among the 142 SSR markers, 140 were amplified in *P. guineense*, representing 98.6% of transferability. The high amplification rate (98.6%) found for the *P. guajava* SSR primers in *P. guineense* showed that the annealing regions are conserved in both species, revealing the high similarity between them. This result also evidenced that these DNA sequences of *P. guineense* are very similar in relation to *P. guajava*, since values of cross-amplification of approximately 73% have been reported for species of the same genus (Barbará et al. 2007). According to Barbará et al. (2007) and Nogueira et al. (2015), the transferability rate of the SSR is higher among species phylogenetically related due to conservation of the sequences between them. Due this fact, SSR markers have been used to compare the similarity level between the genome of distinct species, allowing to analyze the phylogenetic relationship (Buschiazzo and Gemmell 2010, Meglécz et al. 2012, Nogueira et al. 2015). As well as for SSR markers, *P. guajava* and *P. guineense* exhibit strong morphological similarity between them. This fact makes it laborious to identify these species at specific level. Based on this fact, in this study, *P. guajava* and *P. guineense* were distinguished from leaf (number of veins, hairiness scattered over the abaxial leaf and adaxial) and floral (apiculus) structures.

Of the 140 primers, 117 were chosen to determine the total number of alleles, which varied from 170 for *P. guineense* to 148 for *P. guajava* (Suppl. material 1). The occurrence of three and four alleles in *P. guineense* for 9.6% of the primers in comparison to 3.4% in *P. guajava* (2x = 22) corroborates the polyploid origin of *P. guineense* (4x = 44) evidenced by nuclear DNA content and karyotype. Besides that, the molecular data reveal the occurrence of some duplicated sequences, such as the 316 and 422 SSR loci (Suppl. material 1), which showed three allele forms in both species. Based on these results, SSR markers can be considered an important complementary tool to study the genome evolution in *Psidium*, as is already the case for investigating the genome of vertebrates (Buschiazzo and Gemmell 2010).

This study points to the tetraploidy origin of *P. guineense*. These results reveal the importance of combining cytogenetic and molecular markers for a better understanding of how euploid events have influenced the speciation process in angiosperms.

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Supplementary material 1

SSR locus, annealing temperature (TA), motif, and allele number amplified in the genome of *P. guineense* and *P. guajava*.

Authors: Anelise Machado Marques, Amélia Carlos Tuler, Carlos Roberto Carvalho, Tatiana Tavares Carrijo, Marcia Flores da Silva Ferreira, Wellington Ronildo Clarindo

Data type: Word document.

Explanation note: The Supplementary material 1 summarizes the SSR locus, annealing temperature (TA), motif, and allele number amplified in the genome of *P. guineense* and *P. guajava*.

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RESEARCH ARTICLE

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Chromosomal variation in Argentine populations of *Akodon montensis* Thomas, 1913 (Rodentia, Cricetidae, Sigmodontinae)

Matías Maximiliano Malleret[1], Carolina Alicia Labaroni[1], Gabriela Verónica García[2], Juan Martín Ferro[1], Dardo Andrea Martí[1], Cecilia Lanzone[1]

1 *Laboratorio de Genética Evolutiva (LGE) FCEQyN, IBS (CONICET-UNaM), Nodo Posadas, Félix de Azara 1552, CP3300, Posadas, Misiones, Argentina* **2** *IBS (CONICET-UNaM), Nodo Iguazú, Bertoni 85, CP3370, Puerto Iguazú, Misiones, Argentina*

Corresponding author: *Carolina Alicia Labaroni* (carolinalabaroni@gmail.com)

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Abstract

The genus *Akodon* Meyen, 1833 is one of the most species-rich among sigmodontine rodents and has great chromosome variability. *Akodon montensis* has a relatively broad distribution in South America, and Argentine populations are located in the southernmost region of its range. Brazilian populations have important chromosomal variability, but cytogenetic data from Argentina are scarce. We performed a chromosome characterization of natural populations of *A. montensis* using conventional staining, C-banding, Ag-NORs and base-specific fluorochromes. A total of 31 specimens from five localities of Misiones Province, in Argentina, were analyzed. The 2n=24 chromosomes was the most frequently observed karyotype. However, five individuals presented 25 chromosomes due to a supernumerary B-chromosome; and one individual had 2n=26 due to one B plus a trisomy for chromosome 11. Additionally, two XY females and two variants of the X chromosomes were found. C-positive centromeric bands occurred in all chromosomes; additional C-bands were observed in some autosomes, the X, Y and B chromosomes. Ag-NORs were observed in five autosomes, and the B chromosome was frequently marked. Fluorochrome banding was similar among karyotypes of the analyzed populations. Comparisons of cytogenetic data among populations of Argentina and Brazil showed the presence of high intraspecific variability in *A. montensis* and some differences among regions.

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Keywords
Rodents, karyotype variability, chromosome banding, heterochromatin, Ag-NORs

Introduction

The genus *Akodon* Meyen, 1983, with about 41 species, is considered one of the most species-rich group within the subfamily Sigmodontinae. Its species are widely distributed in South America and inhabit a variety of habitats, among subtropical and tropical moist forest as well as desert regions (Musser and Carleton 2005). From a taxonomic point of view, the genus includes morphologically very similar species, and cytogenetic data is valuable for identifying them, such as *Akodon cursor* (Winge, 1887) and *A. montensis* (Yonenaga-Yassuda et al. 1975; Barros et al. 2009).This genus has high karyotypic variability, with chromosome numbers varying from 2n=46 (FN=46) in *A. serrensis* Thomas, 1902 to 2n=10 (FN=14) in *A.* sp. (Barros et al. 2009). In different species, several chromosome variations were described, including pericentric inversions and Robertsonian translocations in autosomes, modifications of sex chromosomes and the presence of B chromosomes (Silva and Yonenaga-Yassuda 1998; Fernández-Donoso et al. 2001; Bianchi 2002).

Akodon montensis is an abundant species distributed in Argentina, Brazil and Paraguay, and has a great chromosomal variability (Kasahara and Yonenaga-Yassuda 1982; Musser and Carleton 2005). Previous cytogenetic analysis demonstrated that the standard chromosome complement of *A. montensis* is 2n=24 (FN=42), with both X and Y chromosomes acrocentric (Yonenaga-Yassuda et al. 1975; Kasahara and Yonenaga-Yassuda 1982; Liascovich and Reig 1989). However, for animals from Brazil, Kasahara and Yonenaga-Yassuda (1982) described a morphological variation for the X chromosome, which was present in both sexes. In populations of Brazil XY fertile females were detected using specific DNA probes from the Y chromosome (Fagundes et al. 2000). Additionally the presence of supernumerary or B chromosomes was reported for specimens from Brazil (Yonenaga-Yassuda et al. 1975; Kasahara and Yonenaga-Yassuda 1982; Di-Nizo et al. 2014). Cytogenetic data on natural populations of *A. montensis* in Argentina are scarce. Liascovich and Reig (1989) studied four specimens from the Provincial Park "Islas Malvinas", in Misiones Province; all specimens had no variations in the standard complement.

In order to contribute to the knowledge of karyotypic variability in *A. montensis* we analyzed specimens from different localities of Misiones Province, Argentina, which is a part of the southernmost area of the range (Pardiñas and Teta 2006).

Material and methods

A total of 31 specimens of *Akodon montensis* (18 females and 13 males) were collected from five localities of Misiones Province, Argentina (Fig. 1). Vouchers were deposited

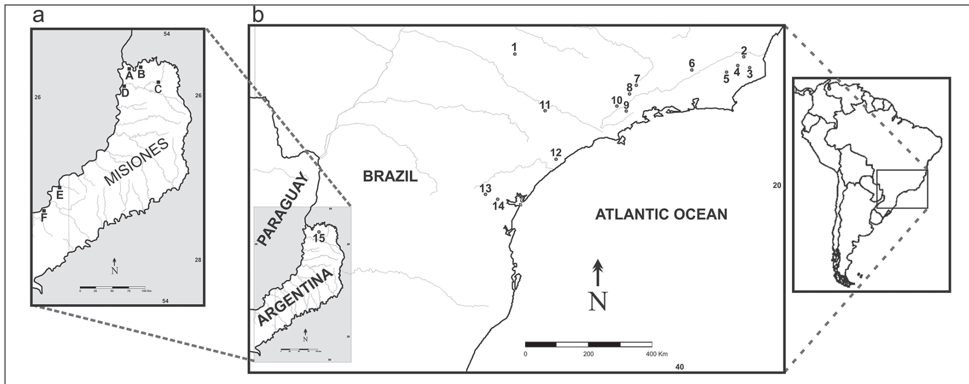

Figure 1. Map indicating **a** collection sites of *Akodon montensis* in the province of Misiones, Argentina analyzed in this work: **A** and **B** Iguazú **C** Parque Provincial Urugua-í **D** Puerto Esperanza **E** San Ignacio **F** Candelaria **b** different localities in Brazil and Argentina where *A. montensis* has been studied previously at cytogenetic level; Brazil: **1** Boracéia **2** Sumidouro **3** Nova Friburgo **4** Teresópolis **5** Petrópolis **6** S. J. do Barreiro **7** Taubaté **8** Caçapava **9** Salesópolis **10** Guararema **11** Itapetininga **12** Iguape **13** Quatro Barras **14** Tres barras; Argentina: **15** Misiones, Urugua-í.

in the biological collection of the Instituto de Biología Subtropical (IBS-CONICET-UNaM). Chromosome preparations were obtained from bone marrow and testes (Ford and Hamerton 1956; Evans et al. 1964). Ten metaphase spreads were counted for each specimen, except in the individual with trisomy in which we counted 30. Conventional staining was performed with Giemsa (10%) to construct karyotypes. The distribution of constitutive heterochromatin (C-bands) was determined according to Sumner (1972) method. In order to identify chromosome homology and characterize sequences rich in AT and GC base pairs, the staining with the fluorochromes DAPI (4,6-diamidino-2-phenylindole) and CMA_3 (Chromomicine A_3) respectively, were conducted according to Schweizer´s method (1976, 1980). Ag-NORs staining was performed with the technique proposed by Howell and Black (1980) to detect active nucleolus organizer regions (NORs). In order to test if NORs carried by the B chromosome have any effect on the activation of autosomal NORs we made Student´s tests using INFOSTAT software.

Results

All individuals of *Akodon montensis* had an autosome complement composed of nine pairs of large to medium size metacentric chromosomes, and two small-sized pairs, one acrocentric and one metacentric. The sex chromosome pair is XX/XY (Fig. 2).

Twenty-five individuals (fourteen females and eleven males) presented a karyotype with 2n=24 and FN=42 (Fig. 2a). Five specimens (four females and one male) had 25 chromosomes in all analyzed cells due to the presence of a small submetacentric B chromosome (Fig. 2c). The supernumerary chromosome was found in the five locali-

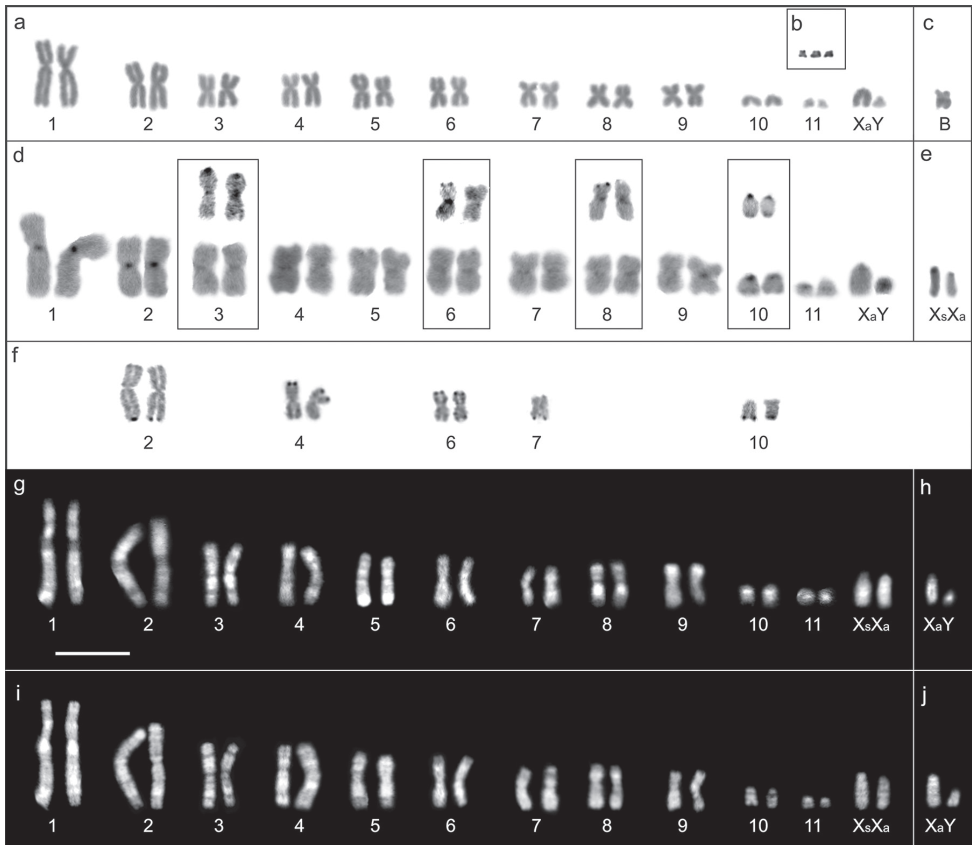

Figure 2. Mitotic chromosomes of *A. montensis*: **a** Giemsa stained karyotype of a male with 2n=24; FN=42 **b** the trisomy for pair 11 **c** Giemsa stained B chromosome **d** C-banded karyotype of a male, in the boxes pairs with telomeric C-bands are showed **e** C-band pattern of Xs-Xa sex chromosomes **f** Ag-NORs bearing chromosomes **g, i** karyotypes of a female with DAPI/ CMA_3 fluorochrome staining respectively **h, j** DAPI/CMA_3 fluorochrome pattern of sex chromosomes of a male. Bar = 10 μm.

ties, representing 19.35% of the total sample. Only one male had 26 chromosomes in all analyzed cells (N=30) due to one B and to a trisomy for pair 11 (Fig. 2b, Table 1).

The Y chromosome was small acrocentric. The X was a medium-sized chromosome and showed two morphological variants: acrocentric (Xa) observed on both sexes, and subtelocentric (Xs) detected only for females (Figs 2, 3). From eighteen females, nine (56.25%) were homozygous for Xa (Fig. 3a), six (37.50%) were heterozygous (Fig. 3b), and one (6.25%) showed both Xs chromosomes (Fig. 3c). Additionally, two females apparently were heterogametic with XY chromosomes, the one from Iguazú had the Xs (Fig. 3d), and the other from Candelaria had Xa chromosome (Table 1).

Positive C-band (C+) were found in the pericentromeric region of pairs 1 to 11, and at the telomeres of pairs 3, 6, 8 and 10 (Fig. 2d). Acrocentric and subtelocentric variants of X chromosome had positive C-bands in the pericentromeric regions (Fig. 2d–e). Additionally, the subtelocentric X chromosome presented a large positive

Table 1. Sampling localities of *Akodon montensis* analyzed in this work. Geographical coordinates, N=number of individuals indicating females (F) and males (M), 2n=chromosome number, sex chromosomes morphology for the X (Xa=acrocentric, Xs=subtelocentric, the number of individuals with each genotype are indicated in bracket), and frequency of B chromosome in each locality (F_B).

Locality (Lat/Long)	N	2n		Sex Chromosome types	F_B
		24	25 (24+B)		
Iguazú (25°42.08'S; 54°20.68'W)	10F	8	2	XaXa(7)	0.13
				XsXa (2)	
				XsY (1)**	
	6M	6	-	XaY (6)	
San Ignacio (27°16.88'S; 55°34.72'W)	2F	1	1	XsXa (1) XaXa (1)	0.25
	2M	2	-	XaY (2)	
Puerto Esperanza (25°59.23'S; 54°38.85'W)	4F	3	1	XsXa (3) XsXs (1)	0.25
Urugua-í (25°51.33'S; 54°10.02'W)	1F	1	-	XaXa (1)	0.20
	4M	3	1	XaY (4)	
Candelaria (27°22.79'S; 55°38.54'W)	1F	1	-	XaY (1)**	0.50
	1M	-	1*	XaY (1)	
Total	31	25	6	-	0.19

*an individual with one B and trisomy for pair 11
**the heterogametic females

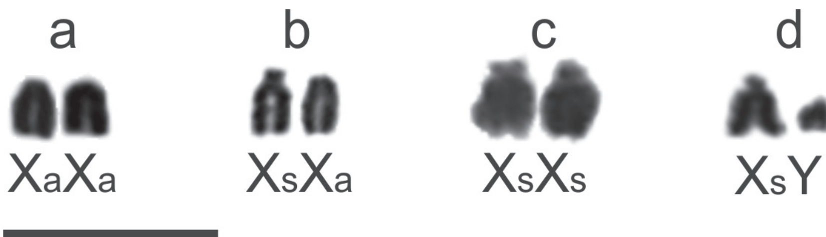

Figure 3. Variants of sex chromosomes in the females of *A. montensis* with Giemsa staining: **a** homozygous acrocentrics **b** acrocentric and subtelocentric **c** homozygous subtelocentrics **d** heterogametic sex chromosomes. Bar = 10 µm.

C-band, which covered its short arm (Fig. 2e). The Y chromosome was completely heterochromatic (Fig. 2d). The B chromosome showed two C+ bands, one was interstitial and the other pericentromeric (Fig. 4b).

Ag-NORs were evident in the distal position of pairs 2, 4, 6, 7 and 10 (Fig. 2f). However, the number of positive signals varied between two and seven in different cells (See Suppl. material 1). Pair 10 was active in most (92/100) analyzed cells. Additionally, the B chromosome frequently was stained (28/36 cells) in one or both telomeric ends (Fig. 4c). The total number of positive Ag-NORs was different between cells with B (four specimens, 36 cells, mean 5.639, SD=1.76) and without the B chromosome (ten specimens, 64 cells, mean 4.328, SD=1.07; T-test=-4.637, df=98,

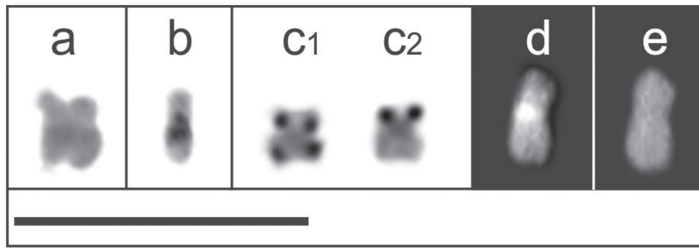

Figure 4. B chromosome of *A. montensis*: **a** Giemsa staining **b** C-banding, pericentromeric and interstitial C-bands **c** silver nitrate staining with Ag-NORs in both telomeric ends (**C1**) and single in the end of the short arm (**C2**) **d, e** DAPI/ CMA_3 fluorochrome stained respectively. Bar = 10 µm.

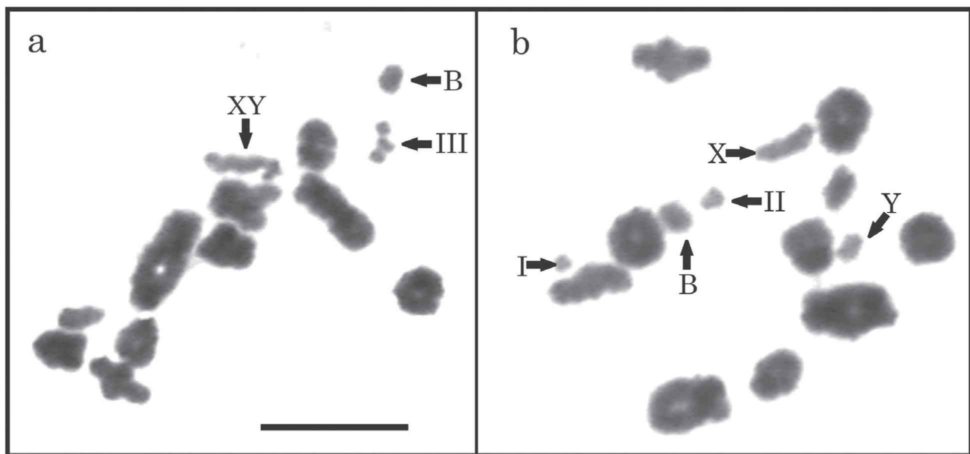

Figure 5. Diakinesis cells of an individual with trisomy and one B chromosome: **a** 10 autosomal bivalents, plus a trivalent (III) of chromosome 11, the sex pair XY and the supernumerary chromosome as univalent (B) **b** Note the presence of pair 11 as one bivalent (II) plus a univalent (I) and X chromosome dissociated of Y. Bar = 10 µm.

$p<0.001$). The exclusion of the supernumerary chromosome from the analysis resulted in no statistically significant difference in the number of active NORs in autosomes in cells with (mean 4.194, SD=1.348) and without the B chromosome (T-test=0.545; df=98; p=0.587).

The banding pattern with DAPI/CMA_3 was similar in all specimens and varied among chromosomes (Fig. 2g–j). The pericentromeric regions of different autosomes had a heterogeneous pattern of DAPI/CMA_3 staining, which were negative, positive or neutral depending of the considered pair (Fig. 2g, i). In sex chromosomes, the pericentromeric regions of Xa and Xs were neutral with both fluorochromes, while the short arm of the Xs was DAPI negative/CMA_3 positive (Fig. 2g–h). The Y chromosome showed a small interstitial DAPI positive band, being telomeres CMA_3 positive and the centromere CMA_3 neutral (Fig. 2h, j). The B chromosome showed a DAPI positive/CMA_3 neutral band in the pericentromeric region (Fig. 4d–e).

Meiotic cells of a male with 2n=24 showed 11 autosomal bivalents during diakinesis and one sex bivalent, which was recognized by its differential pyknosis, size and shape. From 30 studied cells in the specimen with trisomy with a B chromosome (2n=26), the three chromosomes 11 were observed either a trivalent (14/30) or as one bivalent plus a univalent (16/30) (Fig. 5). In addition, we observed a cell in which the X and Y chromosome were dissociated (Fig. 5b).

Discussion

The studied populations of *Akodon montensis* from Brazil showed high chromosome variability (Kasahara and Yonenaga-Yassuda 1982; Fagundes et al. 2000). However in Argentina, with a low sample size, no karyotype variation had been detected previously (Liascovich and Reig 1989). In this work we found the same variability described in Brazil, which involve the presence of a B chromosome, X chromosomes variants and seeming XY females.

Constitutive heterochromatin (CH) is in mammals, and particularly in rodents, an important source of karyotype variability (Graphodatsky et al. 2011). *A. montensis* has small positive C-bands in the pericentromeric regions of all chromosomes (Kasahara and Yonenaga-Yassuda 1982; this work), which is common in *Akodon* species, and in rodents in general (Ortiz et al. 1998; Lisanti et al. 2001; Ventura et al. 2006; Lanzone et al. 2011; Labaroni et al. 2014).

Patterns of fluorescent bands DAPI/CMA_3 are comparable to G- and R-banding respectively (Veyrunes et al. 2007). Our results of DAPI staining showed high homology among karyotypes of specimens from Argentina and those for Brazil studied with G-banding method (Fagundes and Yonenaga-Yassuda 1998; Silva and Yonenaga-Yassuda 2004), which indicates a high conservation in the standard karyotype of this abundant and widely distributed species.

The XX/XY sex chromosome system is the most common among mammals, being males heterogametic and females homogametic. However, certain species depart from this pattern (Graphodatsky et al. 2011). In our sample two females presented heteromorphic sex chromosomes (XY). In *A. montensis* from Brazil the occurrence of XY female was confirmed with molecular cytogenetic techniques (Fagundes et al. 2000). In *Akodon*, some species contain a large proportion of XY fertile females (Hoekstra and Edwards 2000; Bianchi 2002). Even though, in *A. montensis* this condition has a relative low frequency (Fagundes et al. 2000; this work).

In Brazil and Argentina, two morphologies for the X chromosome were observed: acrocentric and subtelocentric (Kasahara and Yonenaga-Yassuda 1982; Fagundes et al. 2000; Di-Nizo et al. 2014; this work). This polymorphism has three possible combinations in females: homozygous acrocentric (XaXa) and subtelocentric (XsXs), and heterozygous (XaXs). The XsXs found in one female is reported for the first time. Females with XaXa were the most frequent in specimens studied here (56.25%) and in Brazil (75%) (Kasahara and Yonenaga-Yassuda 1982). Additionally, XY females with differ-

ent types of X chromosomes were detected in both countries (Fagundes et al. 2000; this work). In males, we observed only the Xa; but in Brazilian populations males with both X types were found (Kasahara and Yonenaga-Yossuda 1982). Thus, the data suggest differences in the frequencies of X chromosome variants among populations, but larger sample sizes are needed to validate these observations.

Sex chromosomes of several rodents showed variation in the amount and distribution of heterochromatin (Patton and Sherwood 1983). In this work both Xa and Xs presented CH in the pericentromeric regions. Additionally, the short arms of Xs had positive C-bands. However, the data from different localities of Brazil are controversial. Some authors detected the same pattern described here (Fagundes et al. 2000); but in another study the short arm of Xs did not show CH (Kasahara and Yonenaga-Yassuda 1982). The Y chromosome of *A. montensis* from Argentina was completely heterochromatic. The same pattern was observed in several mammals, and particularly in individuals of *A. montensis* from Brazil (Kasahara and Yonenaga-Yassuda 1982; Waters et al. 2007). Although, Fagundes et al. (2000) described a non heterochromatic Y chromosome for *A. montensis*.

B chromosomes (Bs) appear as supernumerary elements to the standard chromosome complement and are highly variables (Silva and Yonenaga 2004; Vujošević and Blagojević 2004; Ventura et al. 2015). The B of *A. montensis* studied here had identical morphology to those detected in Brazil (Yonenaga-Yassuda et al. 1975; Kasahara and Yonenaga-Yassuda 1982; Yonenaga-Yassuda et al. 1992; Fagundes et al. 2000; Silva and Yonenaga-Yassuda 2004). However, the described C- and G-banding patterns varied in different studies. Some authors described the B chromosome as slightly heterochromatic and uniformly G-banded (Kasahara and Yonenaga-Yassuda 1982; Silva and Yonenaga-Yassuda 2004); while others reported it as almost heterochromatic with conspicuous pericentromeric C-bands (Kasahara 2009). The B studied here had two heterochromatic bands (pericentromeric and interstitial), which were partially DAPI positive/CMA_3 neutral. CH patterns on Bs have been extensively studied in some species of rodents, in which most often appear as almost completely heterochromatic. Additionally, in some cases Bs vary within and among populations, as in *Perognathus baileyi* (Merriam, 1894) and *Nectomys squamipes* Brants, 1827 (Silva and Yonenaga-Yassuda 2004; Vujošević and Blagojević 2004). In *A. montensis*, the described patterns suggest that different polymorphisms for B chromosomes may be coexisting in this species.

In *A. montensis* the B chromosome showed NORs at the end of both arms, which are also coincident with the location of rDNA detected by fluorescent *in situ* hybridization (Kasahara 2009). The presence of Ag-NORs in Bs has been described in other rodent species such as *Sooretamys angouya* (Fischer, 1814) and *Apodemus peninsulae* (Thomas, 1907) (Silva and Yonenaga-Yassuda 2004; Vujošević and Blagojević 2004). In *A. montensis* from Brazil was detected a low frequency of Bs with NOR activity, where only one of four analyzed individuals presented Ag-NOR marks (Yonenaga Yassuda et al. 1992). In this work B-chromosome had Ag-NOR marks in one or both ends in high frequency, which lead to a higher average of active NORs in the cells. These observations support the hypothesis that different B chromosomes can be present in *A. montensis*.

Variation in the frequency of B chromosomes is common among populations (Silva and Yonenaga Yassuda 2004; Vujošević and Blagojević 2004; Ventura et al. 2015). In *A. montensis* the frequency of individuals with Bs appears to vary among localities, but several populations were studied with low sample size. In this study the total frequency of individuals with a B chromosome was 19%. Compiled data from Brazil (N=346) calculated a total frequency of 28.13% for individuals with 1 B, 2.27% with two Bs, and 0.28% with unstable Bs that formed a mosaic of 1B-2Bs (Silva and Yonenaga-Yassuda 2004). In this work individuals with more than one B were not identified. This chromosome was stable in mitoses and meioses, since no evidence for accumulation or elimination were detected.

Finally, in the present paper we report for the first time a trisomy of chromosome 11 in a single individual. In *A. cursor* also were observed an individual with trisomy for chromosome 7 (Fagundes et al. 1998). No phenotypic malformations were detected in both cases. However the frequency of trisomies in natural populations and the biological consequences of this condition have not been investigated yet.

In conclusion, chromosome data for *Akodon montensis* showed high variability in all studied populations throughout its geographic range. However, additional data are needed to understand the dynamic of the multiple chromosome polymorphism observed in this species of sigmodontine rodents.

Acknowledgements

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Supplementary material 1

Pattern of Ag-NOR´s distribution in *Akodon montensis* from Argentina analyzed in this work.

Authors: Matías Maximiliano Malleret, Carolina Alicia Labaroni, Gabriela Verónica García, Juan Martín Ferro, Dardo Andrea Martí, Cecilia Lanzone

Data type: Chromosome pairs with positive signals after silver staining.

Explanation note: 1 HOM = only one homologue was marked, 2 HOM = both homologues were marked. 2 Telo = both ends of the B chromosome were marked; 1 Telo = only one end of the B chromosome was marked, being “p” when the short arm was marked and “q” when the long arm was marked. Individuals from 1 to 10 had no supernumerary chromosome and from 11 to 14 had the B chromosome. Total = total number of Ag-NOR marks in each cell; Total without B = total number of Ag-NOR marks excluding that of the B chromosome.

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RESEARCH ARTICLE

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Cytogenetic characterization and B chromosome diversity in direct-developing frogs of the genus *Oreobates* (Brachycephaloidea, Craugastoridae)

Juan Martín Ferro[1], Alberto Taffarel[1], Darío Cardozo[1], Jimena Grosso[2], María Pía Puig[3], Pablo Suárez[4], Mauricio Sebastián Akmentins[5], Diego Baldo[1]

1 *Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical (CONICET-UNaM), Facultad de Ciencias Exactas Químicas y Naturales, Universidad Nacional de Misiones; Félix de Azara 1552, CPA N3300LQF Posadas, Argentina* **2** *Fundación Miguel Lillo, Instituto de Herpetología; Miguel Lillo 251, CP 4000, San Miguel de Tucumán, Tucumán* **3** *Universidad Nacional de Salta (UNSa), Avenida Bolivia 5150, Salta, Argentina* **4** *Laboratório de Citogenética, Instituto de Ciências Biológicas, Universidade Federal do Pará, Tv. Augusto Correia 1, CEP 66075-900, Belém, Pará, Brazil* **5** *Centro de Investigaciones y Transferencia de Jujuy (CIT-JUJUY), CONICET-UNJu, Av. Bolivia 1711 (4600), San Salvador de Jujuy, Argentina*

Corresponding author: *Juan Martín Ferro* (ferrojm@gmail.com)

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Abstract

Oreobates Jiménez de la Espada, 1872 is a large group of South American frogs with terrestrial reproduction and direct development, located in the superfamily Brachycephaloidea. About 260 brachycephaloidean species have been cytogenetically studied so far, at least with standard techniques. However, this information represents fewer than 17% species of the family Craugastoridae Hedges, Duellman & Heinicke, 2008, where the genus *Oreobates* is included. In the present work, using a diversity of standard and molecular techniques, we describe the karyotype of *O. barituensis* Vaira & Ferrari, 2008, *O. berdemenos* Pereyra, Cardozo, Baldo & Baldo, 2014 and *O. discoidalis* (Peracca, 1895), from northwestern Argentina. The three species analyzed showed a diploid karyotype with 2n = 22 biarmed chromosomes, fundamental number (FN) = 44, nucleolus organizer regions (NORs) located pericentromerically on pair 7, and a centromeric and pericentromeric C-banding pattern. We observed variations in the chromosome number in *O. barituensis* due the presence of two morphs of B chromosomes, one medium-sized telocentric (B_T) and another subtelocentric and smaller (B_{st}). Both B chromosomes are mitotically

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stable and were recorded in all somatic and germinal cells analyzed. The B_T chromosome occurred at a maximum of one per individual (2n = 22+B_T), and the other one was observed single (2n = 22 + B_{st}) or as a pair in two doses (2n = 22 + 2B_T). We additionally observed other supernumerary chromosomes in the three species analyzed, all of them euchromatic, small, dot-shaped and with instability during mitoses, showing a frequency of occurrence below 50% in studied specimens. The occurrence of polymorphic and spontaneous chromosomal rearrangements and supernumerary chromosomes is a recurrent feature reported in frogs with terrestrial habits (Brachycephaloidea and Hemiphractidae Peters, 1862), which suggests that Brachycephaloidea may be a promising group for studying the origin and maintenance of B chromosomes in anurans.

Keywords

Cytogenetics, accessory elements, ribosomal DNA, Anura

Introduction

Superfamily Brachycephaloidea includes a large group of frogs with terrestrial reproduction and direct development, with more than a thousand species assigned to three families: Brachycephalidae Günther, 1858, Craugastoridae, and Eleutherodactylidae Lutz, 1954 (Frost 2015). From a cytogenetic perspective, about 26% of brachycephaloidean species were studied at least with conventional staining techniques, including 13% of Brachycephalidae (7 spp.), 17% of Craugastoridae (128 spp.) and 57% of Eleutherodactylidae (120 spp.) (Campos and Kasahara 2006, Schmid et al. 2010, Díaz et al. 2012, Kaiser et al. 2015). Brachycephaloidea presents an important karyotypic diversity, with diploid numbers (2n) ranging from 16 to 38, an unusually high frequency of spontaneous and polymorphic Robertsonian rearrangements, B chromosomes, and the occurrence of spontaneous somatic supernumerary chromosomes which do not form polymorphisms (Schmid et al. 2010).

Within Craugastoridae (subfamily Holoadeninae Hedges, Duellman & Heinicke, 2008), *Oreobates* Jiménez de la Espada, 1872 is a South American genus with 23 species that occurs on the lower slopes of the Andes from the upper Amazon basin in southern Colombia to northern Argentina, reaching eastwards some areas in western Brazil (Frost 2015). In Argentina, the genus is represented by three species, *O. berdemenos* Pereyra, Cardozo, Baldo & Baldo, 2014, which is phylogenetically related to *O. lundbergi* and characterized by an incomplete discoidal fold (Pereyra et al. 2014); and the cryptic species, *O. barituensis* Vaira & Ferrari, 2008 and *O. discoidalis* (Peracca, 1895), recovered as sister species in the most inclusive phylogenetic analyses, both with a complete discoidal fold (Padial et al. 2014, Pereyra et al. 2014). When compared to other Brachycephaloidea genera, the cytogenetic information available for *Oreobates* is extremely scarce and only two species were studied to date, *O. discoidalis* (Brum-Zorilla and Sáez 1968 [as *Eleutherodactylus*], Schmid et al. 2010), and *O. crepitans* (Bokermann, 1965) (Siqueira et al. 2009, as [*Pristimantis*]). Both taxa share the same chromosome formulae (2n = 22, FN = 44) and similar chromosomal morphology. In the latter the heterochromatin pattern and nucleolus organizer regions were described

by C- banding and silver staining (Ag-NORs) respectively, whereas the karyotype of *O. discoidalis* was only studied with standard staining techniques (Giemsa).

B chromosomes (Bs) are dispensable extra chromosomes in the standard karyotype (As) present in many taxa, and usually lack phenotypic effects on their hosts. Their prevalence in animal populations is highly variable, being one of the main causes of chromosomal polymorphism in eukaryotes (Jones and Rees 1982, Beukeboom 1994, Camacho et al. 2000). Until now, the presence of B chromosomes in Brachycephaloidea has been described in three species, *Craugastor* sp., *Eleutherodactylus gundlachi* Schmidt, 1920 and *Oreobates discoidalis*. However, another sort of chromosomal variation due to supernumeraries is frequently observed. Although they are associated with spontaneous chromosomal aberrations, their main difference with Bs lies in the fact that they are not polymorphic (Schmid et al. 2010).

In order to complement the karyotypic information available for *Oreobates*, in the present work we studied three species (*O. barituensis*, *O. berdemenos,* and *O. discoidalis*), from several localities in northwestern Argentina. Chromosome morphology, heterochromatin distribution and composition, and location of nucleolar organizer regions are described. We discuss and evaluate the apparent chromosomal homogeneity observed for the genus *Oreobates,* in contrast to the variability reported by the presence of supernumerary chromosomes.

Material and methods

We studied 64 specimens of both sexes of *O. barituensis* (N = 40), *O. berdemenos* (N = 14), and *O. discoidalis* (N = 10). Chromosome preparations were obtained from bone marrow, gut epithelium and testes in males (Schmid et al. 2010). Animals were euthanized with 5% lidocaine and fixed in 4% formalin. Vouchers were preserved in 70% ethanol and stored in the herpetological collections of Fundación Miguel Lillo, Tucumán, Argentina (FML, and provisional field numbers MSA), and Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical, Posadas, Misiones, Argentina (LGE, and provisional field numbers MSA). The complete list of specimens analyzed, collection sites, sex, and voucher numbers are detailed in the Supplementary file 1 online. The diploid number (2n) and Fundamental Number (FN) were obtained after counting at least ten cells per specimen. Mitotic and meiotic preparations were stained with a phosphate-buffered Giemsa solution (10%). Heterochromatic regions were identified by C-banding (Sumner 1972). Silver-staining (Howell and Black 1980) and fluorescent in situ hybridization (FISH) with a ribosomal 18S biotinylated probe (Pinkel et al. 1986) were carried out to evidence active nucleolar organizer regions Ag-NORs and the presence of repetitive rDNA, respectively. To study the nucleotide composition of heterochromatin (prevalence of repetitive sequences AT and CG), we used the fluorochromes base specific DAPI (4’, 6-diamidino-2-phenylindole) and CMA_3 (Chromomycin A_3), following Schweizer (1976). Karyotypes were arranged in decreasing size, according

the nomenclature of Green and Sessions (1991, 2007). The relative length and centromeric index (CI) of chromosomes were obtained from mitotic metaphases using the software Micromeasure 3.3 (Reeves and Tear 2000).

The advertisement call of *O. discoidalis* remains unknown, and those described by Ferrari and Vaira (2008) and Akmentins (2011) correspond to *O. berdemenos* (Pereyra et al. 2014). To avoid misidentification of specimens, collections of *O. discoidalis* were made near the type locality (San Miguel de Tucumán, Argentina), whereas individuals from Jujuy and Salta Provinces were considered as *O. barituensis,* based on their advertisement calls as described by Vaira and Ferrari (2008) and Akmentins (2011). Specimens of *O. berdemenos* were collected at the type locality of the species (Abra Colorada, Jujuy province, Argentina), and Nogalar de los Toldos (Salta province, Argentina). The identity of *O. berdemenos* specimens was confirmed by morphological and/or acoustic characters (Supplementary file 1 online).

Results

Oreobates barituensis, *O. berdemenos*, and *O. discoidalis* shared diploid karyotypes with 22 bi-armed chromosomes (2n = 22; FN = 44). Pairs 1, 2, 5, 6, 8–11 were metacentric, while 3, 4 and 7 submetacentric (Fig. 1; Table 1). In all specimens analyzed of the three species, the C-banding technique showed a high predominance of centromeric and pericentromeric heterochromatin, as well as an interstitial band on the long arm pairs 6 (Fig. 1D–F). However, this band varied in its staining intensity or even was absent in some metaphases of a given slide. DAPI/CMA_3 fluorochromes staining, evidence DAPI positive marks at centromeric and pericentromeric regions on almost all chromosomes (CMA_3 negative). In the three species, CMA_3 positive marks (DAPI negative) were evident only on pericentromeric position over pair 7, coincident with secondary constriction sites (Fig. 2), Ag-NORs (Fig. 3A–C) and with the hybridization signals of 18S rDNA probe after FISH experiments (Fig. 3D–F).

We found variations in the chromosome number in eight specimens of *Oreobates barituensis* as a consequence of two different B chromosomes (Fig. 1G–J; 3G–J; 4). Both supernumeraries were present in every somatic cell analyzed (mitotically stable). One of them, was a telocentric large-sized chromosome, arbitrarily named herein B_T, which reached a maximum of one per cell (2n = 22 + B_T). It was observed in three specimens from Peña Alta (LGE 6203, 4784–5) and two from Normenta (MSA 176, 180). C–banding revealed that this chromosome has a significant amount of heterochromatin in the whole arm, mainly visible in the centromeric region (Fig. 1I). The heterochromatin was DAPI positive/CMA_3 negative with an interstitial mark DAPI negative/CMA_3 positive (Fig. 3I, J). The B_T chromosome showed terminal active Ag-NOR sites after silver staining (Fig. 3G). In situ hybridization with the ribosomal 18S probe showed a terminal mark on B_T matching with Ag-NORs, but also brightly interstitial rDNA heterochromatin associated with negative Ag-NORs (Fig. 3H). The other supernumerary was a subtelocentric small chromosome, named as B_{st}, found in

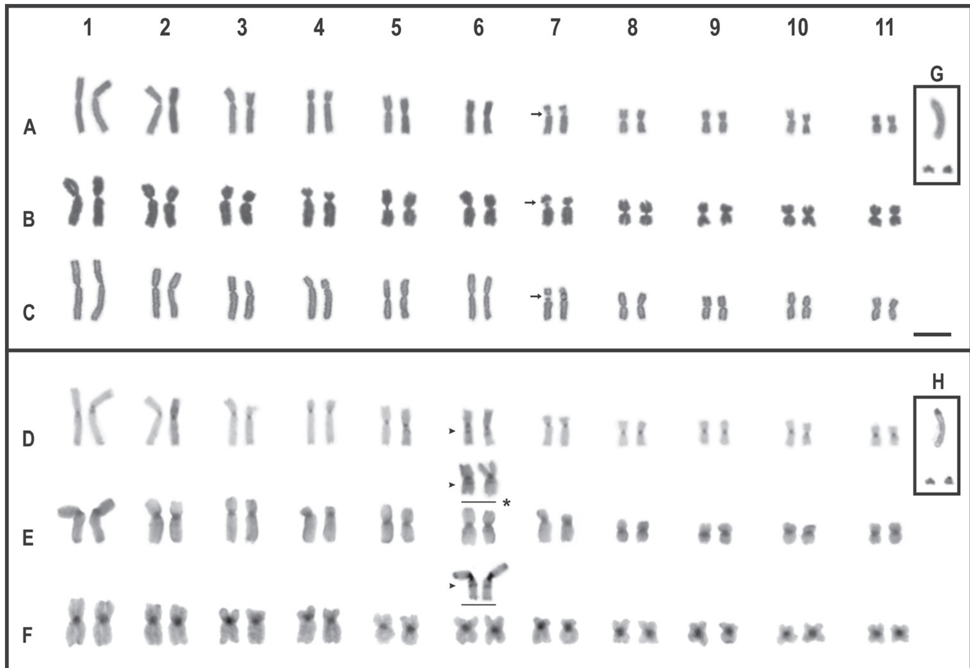

Figure 1. Karyotypes of *Oreobates barituensis* (**A, D**), *O. berdemenos* (**B, E**), *O. discoidalis* (**C, F**), the large telocentric B_T and the small subtelocentric B_{st} supernumerary chromosomes in *O. barituensis*(Boxes **G** and **H**). Giemsa stained (**A–C, G**), C- banding (**D–F, H**). The insets (*) shows interstitial C-bands. Arrowheads point to C positive bands. Bar = 10 µm.

the three specimens analyzed from Tiraxi (MSA 168, 195; LGE 9652). While a single individual carried only one B (2n = 22 + B_{st}), the others brought it in a double dose (2n = 22 + $2B_{st}$). After C-banding, this small sized B_{st} stained darker than A chromosomes, with a conspicuous darker heterochromatic centromere (Fig. 1J), positive for DAPI (Fig. 4A–C).

In addition, eight specimens of *O. barituensis* (LGE 4785; 6202; MSA 127–8, 161, 164, 174, 177), four *O. berdemenos* (FML 24626, MSA 138, 142–3), and one *O. discoidalis* (FML 24513), showed variations in the chromosome number attributable to dot-shaped and mitotically unstable supernumerary chromosomes, named here B_d. These elements share a similar shape with no evident primary constrictions, and no more than one per metaphase was observed (i.e. 2n = 22; 22 + B_d), with an occurrence below 50% per individual. C-banding and DAPI staining showed mostly a euchromatic nature of these elements (Fig. 4E).

The Meiosis I analyses on males of *O. barituensis* with supernumerary chromosomes B_T and B_{st} evidenced the presence of 11 bivalents, corresponding to the A standard complement in addition to Bs. The B_T chromosome occurred as a single univalent with not differentiable pyknosis from other chromosomes (Fig. 5A). On the other hand, metaphases I from specimens with B_{st} showed a clearer staining than As (Fig.

Table 1. Chromosome morphology in the three species of *Oreobates*. Chromosome types according to Green and Sessions (1991), Centromeric Index (CI), metacentric (m: 0.500 to 0.375); submetacentric (sm: 0.374 to 0.250); SD = Standard Deviation.

Chromosome number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Oreobates barituensis											
%Set	15%	12%	11%	11%	10%	9%	8%	7%	6%	6%	6%
CI ± SD	0.43 ± 0.02	0.38 ± 0.03	0.37 ± 0.05	0.28 ± 0.06	0.45 ± 0.02	0.43 ± 0.03	0.34 ± 0.04	0.46 ± 0.02	0.46 ± 0.02	0.45 ± 0.03	0.47 ± 0.03
Type	**m**	**m**	**sm**	**sm**	**m**	**m**	**sm**	**m**	**m**	**m**	**m**
Oreobates berdemenos											
%Set	15%	12%	11%	11%	10%	9%	8%	7%	6%	6%	6%
CI ± SD	0.42 ± 0.02	0.38 ± 0.02	0.35 ± 0.05	0.25 ± 0.04	0.44 ± 0.02	0.45 ± 0.02	0.33 ± 0.04	0.45 ± 0.05	0.47 ± 0.02	0.45 ± 0.03	0.46 ± 0.03
Type	**m**	**m**	**sm**	**sm**	**m**	**m**	**sm**	**m**	**m**	**m**	**m**
Oreobates discoidalis											
%Set	15%	12%	11%	10%	10%	9%	8%	7%	6%	6%	6%
CI ± SD	0.44 ± 0.02	0.39 ± 0.02	0.37 ± 0.04	0.29 ± 0.04	0.45 ± 0.02	0.44 ± 0.03	0.27 ± 0.05	0.46 ± 0.02	0.46 ± 0.03	0.43 ± 0.05	0.48 ± 0.01
Type	**m**	**m**	**sm**	**sm**	**m**	**m**	**sm**	**m**	**m**	**m**	**m**

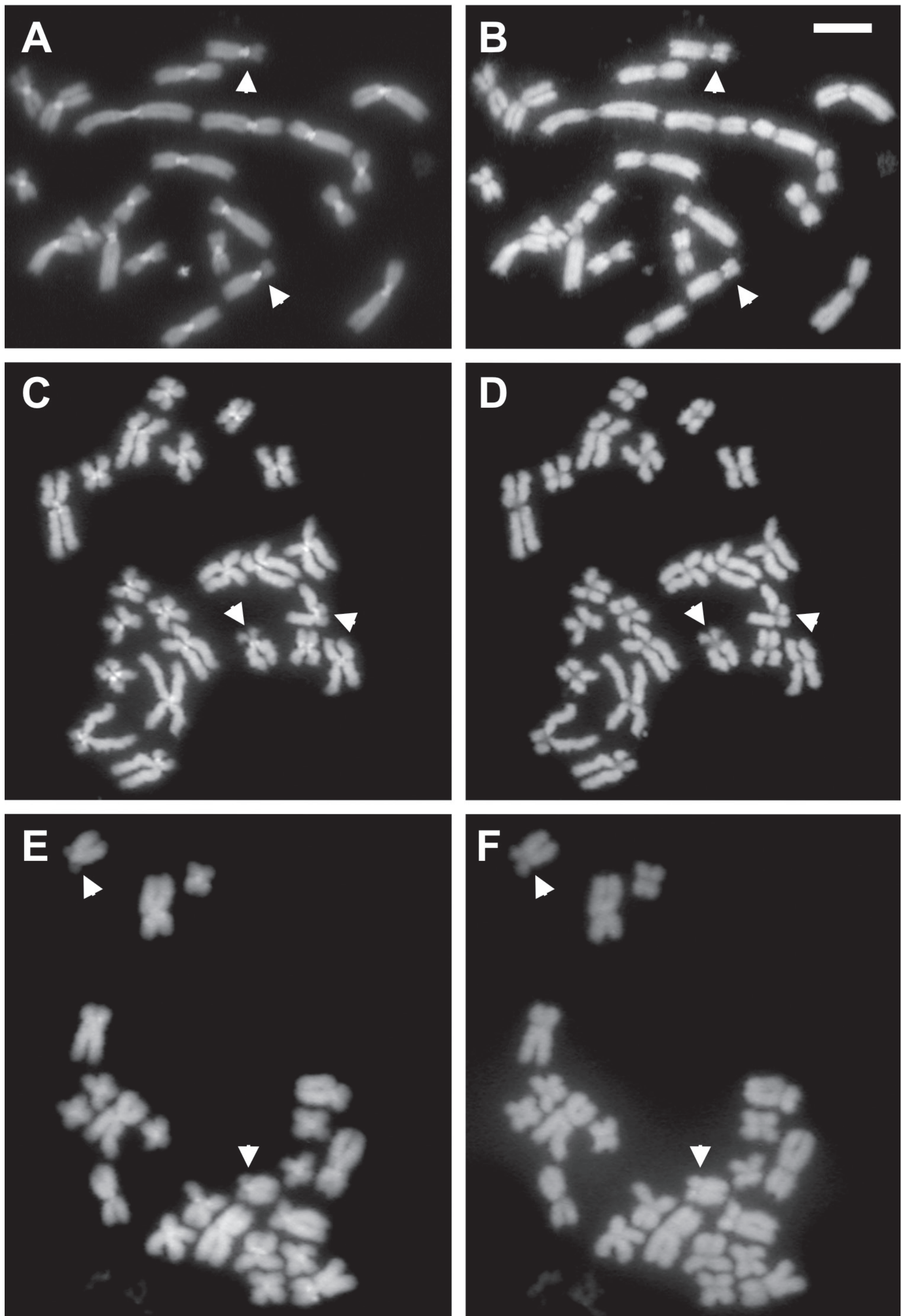

Figure 2. Mitotic metaphases of *O. barituensis* (**A, B**), *O. berdemenos* (**C, D**) and *O. discoidalis* (**E, F**) stained with DAPI (**A, C, E**) and CMA_3 (**B, D, F**), arrowheads point pairs 7. Bar = 10 µm.

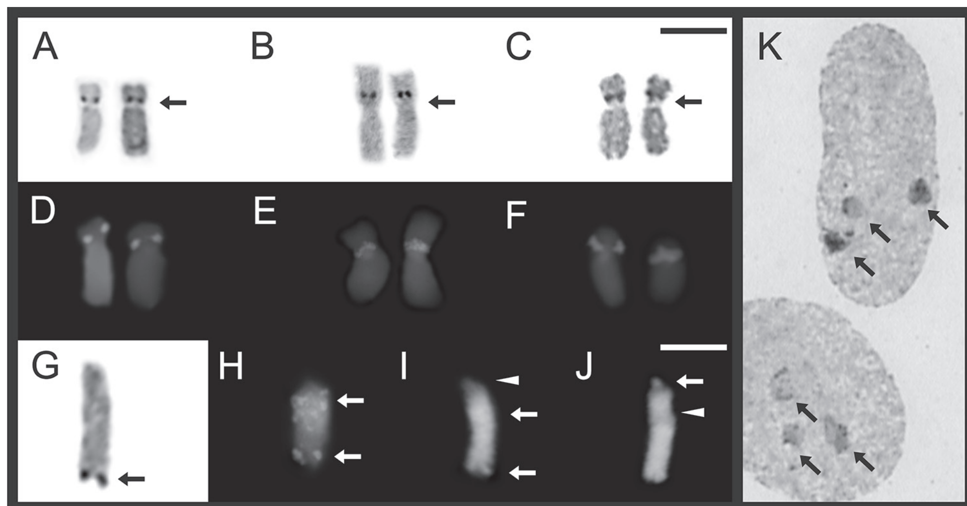

Figure 3. NORs bearing chromosome pairs (**A–C**) and rDNA (**D–F**), in *Oreobates barituensis* (**A, D**), *O. berdemenos* (**B, E**) and *O. discoidalis* (**C, F**). B_T chromosome in *O. barituensis* showing positive NORs (**G**), active and inactive rDNA (**H**), CMA_3 (**I**) and DAPI (**J**). Two interphase cells with three active NORs after silver staining (**K**). Black arrows indicate Ag-NORs, white arrows and arrowheads shows positive and negative fluorescent marks, respectively. Bars = 10 µm.

5B). These elements were always observed as an univalent, and in those individuals carrying two Bs, both were detected as univalents (Fig. 5B) or even paired as a bivalent (Fig. 5C). In the latter case, the association between both B_{st} was euchromatic rather than heterochromatic (Fig. 5C). Meiosis of these elements from individuals carrying B_d supernumeraries could not be studied due to poor quality of preparations.

Discussion

The genus *Oreobates* is composed of 23 species (Frost 2015), of which four of them were cytogenetically studied: *O. barituensis* and *O. berdemenos* (this work), *O. discoidalis* (Brum-Zorrilla and Sáez 1968, Schmid 2010, this work), and *O. crepitans* (Siqueira et al. 2009). *Oreobates* species share a similar morphology of chromosomes, as well as C-banding patterns and the location of NORs. The interstitial heterochromatic band observed in the long arm of pair 6 on specimens of the three species of *Oreobates* studied here, was not previously reported for *O. crepitans* by Siqueira et al. (2009). However, with the use of similar procedures than these authors for the C-banding protocol, the band was not detectable or was variably marked; suggesting that condensation of chromosomes may play an important role in its detection.

Within the subfamily Holoadeninae, the chromosomes of only 8 of 119 recognized species were studied (Schmid et al. 2010, and references therein). The 2n = 22 with all biarmed chromosome pairs (FN = 44) shared by *Oreobates* species are also

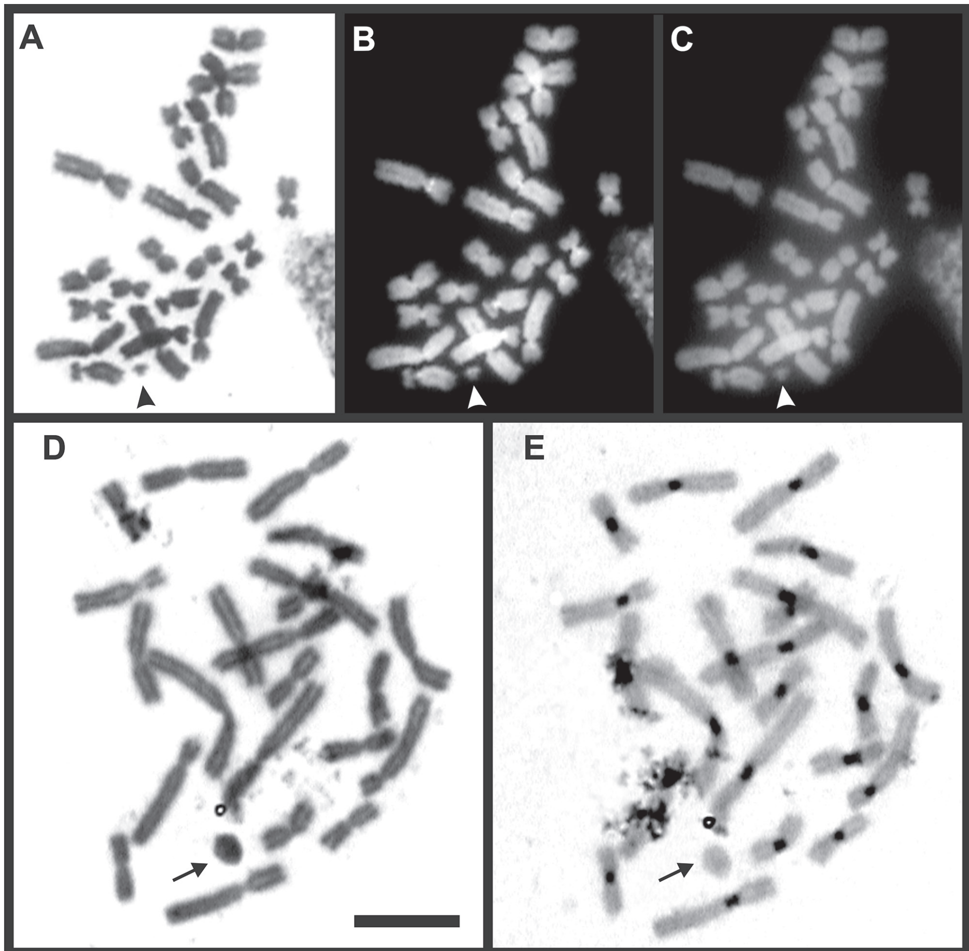

Figure 4. The small B_{st} chromosome (arrowhead) in *O. barituensis* with conventional staining (**A**), DAPI (**B**) and CMA_3 (**C**). B_d chromosome (arrow) in *O. berdemenos* evidenced after Giemsa (**D**) and C-banding (**E**). Bar = 10 µm.

present in *Phrynopus barthlenae* Lehr & Aguilar, 2002. However, *Barycholos ternetzi* (Miranda-Ribeiro, 1937) shows a 2n = 22; FN = 38, *Euparkerella brasiliensis* (Parker, 1926) 2n = 20; FN = 40, and *Holoaden bradei* Lutz, 1958 2n = 18; FN = 36 (Schmid et al. 2010). Although, *Lynchius* Hedges, Duellman & Heinicke, 2008, *Oreobates* and *Phrynopus* Peters, 1873, were recurrently recovered as related groups in several phylogenetic hypotheses (Hedges et al. 2008, Pyron and Wiens 2010, Padial et al. 2014), the scarcity of data do not allow a clear understanding of chromosome character distribution among Holoadeninae (complete absence of data for *Bryophryne*, *Hypodactylus*, *Niceforonia*, *Noblella*, and *Psychrophrynella*). However, the available karyological information suggests that a whole-biarmed karyotype of 22 chromosomes (FN = 44) is shared by species of the clade comprising *Lynchius*, *Oreobates*, and *Phrynopus*.

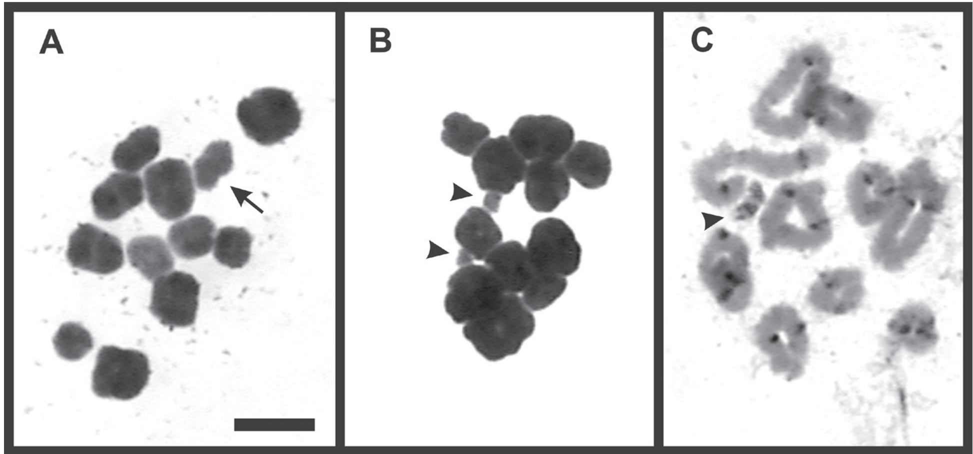

Figure 5. Meiosis I in specimens of *O. barituensis*. Metaphase I with conventional staining showing Bs as univalent: one B_T (**A**) and two B_{st} with negative pyknosis (**B**). Diakinesis after C- banding with a bivalent B_{st} (**C**). Arrow and arrowheads indicate the B_T and B_{st} respectively.

B chromosomes are widespread among eukaryotes (Beukeboom 1994, Camacho 2005), and to date have been formally reported in 20 anuran species (Green 2004 and references therein, Lanzone et al. 2008, Schmid et al. 2010 and references therein, Milani et al. 2011, Hernández-Guzmán et al. 2011, Suárez et al. 2013, Mezzasalma et al. 2015). Schmid et al. (2010) analyzed with conventional solid staining the chromosomes of 11 specimens of *O. discoidalis*, collected by J.P. Bogart in 1969 in northwestern Argentina (Aguas Negras, Jujuy). These authors found two different telocentric B chromosomes, both showing a mosaic in the chromosome number between cells of a same specimen (mitotically unstable), occurring at most once per cell. One of those Bs was smaller, similar in size to the pair 11 of the A complement, whereas the other one was larger than pair 5, resembling the B_T described in the present work for *O. barituensis*. As the specimens studied by Schmid et al (2010) were collected in late 1960s, with *Oreobates discoidalis* (= *Eleutherodactylus discoidalis*) being then the unique species recognized in Argentina, we cannot ascertain whether B_T chromosome were present in *O. barituensis* (this paper) and *O. discoidalis* (Schmid et al. 2010) or only in *O. barituensis*. However, an intriguing question is the lack of mitotic instability observed by us on this element as compared with previous reports. At present, the most accepted models for long-term evolution of B chromosomes states that the cytological behavior of Bs polymorphisms can change over time. A selfish (or parasitic) B may develop into neutral (or near-neutral) through a stabilization process induced by the standard genome of the host species (Camacho et al. 1997, Zurita et al. 1998, Camacho et al. 2000), or alternatively by regularizing their own pairing during meiosis (Araújo et al. 2001, 2002). While the mitotic instability of B chromosomes can be interpreted as a mechanism for accumulation, this phenomenon hinders an organism (or a population) to fit to an optimal number of these elements, and unless it favors the transmission of

Bs on germinal cells (i.e. by premeiotic accumulation) it would play a negative role in their long-term persistence (Nur 1963, 1969). Cavallaro et al. (2000) demonstrated that mitotic instability may take part as a possible mechanism for Bs to increase their frequency and thus invade populations. The authors observed over a ten year period a significant rise in the frequency of a B chromosome in the fish *Prochilodus lineatus* (Valenciennes, 1837). This was correlated with a decrease in the mitotic instability of the chromosome (i.e. Mitotic stabilization), suggesting that the population studied was likely under the last phase of B chromosome invasion. A possible explanation for the discrepancies observed between the present work and that of Schmid et al. (2010) concerning the mitotic behavior of the B_T in *O. barituensis* is that the instability of this element was abolished over time in the studied population (almost 50 years).

NOR-bearing B chromosomes were reported in 27 species of plants and 25 of animals [three of them anurans: *Eleutherodactylus gundlachi*, *Gastrotheca espeletia* Duellman & Hillis, 1987, and *Spea hammondii* (Baird, 1859)], showed to be supernumerary chromosomes carrying rDNA detected by silver staining and/or FISH with a rDNA probe (Green 1990, Jones 1995, Jones and Díez 2004, Silva and Yonenaga-Yassuda 2004, Camacho 2005, Cabrero and Camacho 2008, Acosta and Moscone 2010, Schmid et al. 2010, Ruiz-Estévez et al. 2013, Silva et al. 2014). The B_T chromosome in *O. barituensis* displayed active NORs in mitoses as well as in interphase nuclei, but also has interstitial heterochromatin associated with inactive 18S rDNA. A tempting target for further studies to test the origin of this B chromosome are the pair of A chromosomes carrying NORs (pair 7). It must be considered that B chromosomes may suffer a further degeneration after their origin, thus becoming heterochromatic and loosing homology with their precursors (Green 1990, Camacho et al. 2000), in addition to the observed mobility nature of rDNA sequences by transposition between non-homologous chromosomes. These facts prevent us from hypothesizing about the possible origin of this element, as B chromosomes would have acquired rDNA subsequent to their formation (Houben et al. 2013, and references therein).

Two other types of supernumerary chromosomes observed in *O. barituensis* were a small subtelocentric and heterochromatic B_{st} that occurs in a high prevalence in the locality of Tiraxi, and the euchromatic dot-like B_d, which is mitotically unstable. Interestingly, specimens of the two other species of *Oreobates* analyzed herein also showed supernumeraries B_d of similar size, and smaller than the smallest pair of the A complement. B chromosomes, which lack a functional centromere would be lost by drift (Camacho et al. 1997). The low transmission efficiency of these elements observed in somatic cells (lower than 50% per individual) due to mitotic instability would impede them to survive as true B chromosomes, unless their transmission ability would be increased in the gametes by acquiring a functional centromere. It is remarkable that the other small supernumerary elements described as B_{st} differed from those B_d by the presence of a conspicuous centromere, and because they were recorded in almost all somatic and germinal cells.

Finally, the available cytogenetic data points to the Brachycephaloidea as an extremely diverse group, with 2n ranging from 16 to 38, and FN = 26–52 (De Weese

1975, Bogart 1991, Bogart and Hedges 1995, Campos and Kasahara 2006, Green and Sessions 2007, Schmid et al. 2010). However, the occurrence of species bearing B chromosomes is not higher than in other anurans groups. Under this scenario, Schmid et al. (2010, 2012) reported the occurrence of "spontaneous somatic supernumerary marker chromosomes" as a common feature observed amongst Brachycephaloidea, at first described in the discoglossid frog *Hoplobatrachus tigerinus* (Daudin, 1802) by Yadav (1973). These elements are considered to be originated as a consequence of spontaneous chromosomal rearrangements. Like B chromosomes, are variable in composition and structure of chromatin, morphology, and behavior during mitosis, denoting thus an heterogeneous chromosome type found in more than 50 species of brachycephaloids frogs (Schmid et al. 2010, 2012). This fact leads to the issue that there might be B chromosomes or nascent Bs undetected among them, pointing to Brachycephaloidea as an interesting group for studying the origin and evolution of B chromosomes in Anurans.

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Supplementary material 1

Specimens of *Oreobates* examined

Authors: Juan Martín Ferro, Alberto Taffarel, Darío Cardozo, Jimena Grosso, María Pía Puig, Pablo Suárez, Mauricio Sebastián Akmentins, Diego Baldo

Data type: specimens data

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RESEARCH ARTICLE

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Chromosomal mapping of tandem repeats in the Yesso Scallop, *Patinopecten yessoensis* (Jay, 1857), utilizing fluorescence in situ hybridization

Xuan Li[1], Zujing Yang[1], Huan Liao[1], Zhengrui Zhang[1], Xiaoting Huang[1], Zhenmin Bao[1]

1 *Key Laboratory of Marine Genetics and Breeding (Ocean University of China), Ministry of Education, Qingdao 266003, China*

Corresponding author: *Huang Xiaoting* (xthuang@ouc.edu.cn)

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Abstract

Construction of cytogenetic maps can provide important information for chromosome identification, chromosome evolution and genomic research. However, it hasn't been conducted in many scallop species yet. In the present study, we attempted to map 12 fosmid clones containing tandem repeats by fluorescence *in situ* hybridization (FISH) in the Yesso scallop *Patinopecten yessoensis* (Jay, 1857). The results showed 6 fosmid clones were successfully mapped and distributed in 6 different pairs of chromosomes. Three clones were respectively assigned to a pair of metacentric chromosomes, a pair of submetacentric chromosomes and a pair of telocentric chromosomes and the remaining 3 clones showed their loci on three different pairs of subtelocentric chromosomes by co-hybridization. In summary, totally 8 pairs of chromosomes of the Yesso scallop were identified by 6 fosmid clones and two rDNA probes. Furthermore, 6 tandem repeats of 5 clones were sequenced and could be developed as chromosome specific markers for the Yesso scallop. The successful localization of fosmid clones will undoubtedly facilitate the integration of linkage groups with cytogenetic map and genomic research for the Yesso scallop.

Keywords

Scallop, fosmid, FISH, tandem repeats, chromosome identification

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Introduction

Chromosome characterization and identification are the very first step to genomic analysis. Construction of cytogenetic maps may enable several types of cytogenetic studies such as chromosomal rearrangements, chromosomal assignment of genes, chromosome identification and others (Wang et al. 2005, Zhang et al. 2007, 2008, Zhao et al. 2015).

The family Pectinidae, with approximately 300 extant species, is widely distributed in world oceans (Waller 2006). Till now, cytogenetic analyses have been performed in only 17 species (Odierna et al. 2006). The chromosome numbers of Pectinidae range from 26 to 38, and the published karyotypes showed that their chromosomes were similar in both size and morphology (Odierna et al. 2006, Leitão and Chaves 2008, Hu et al. 2013). In recent years, chromosome banding techniques have been applied in several scallop species for attempting to achieve chromosome identification. However, most of them failed because the stable chromosome banding patterns were difficult to obtain. NOR-banding (Nucleolus Organizer Region-) has been analyzed in 8 scallop species and only one or two pairs of chromosome can be identified (Insua et al. 1998, 2006, Pauls and Affonso 2000, Gajardo et al. 2002, López-Piñón et al. 2005, Huang et al. 2006, 2007a, Odierna et al. 2006). C-banding and fluorescence banding could illustrate the heterochromatin regions on chromosomes. Nevertheless, having been conducted in 8 kinds of scallops, these two approaches are considered not suitable for chromosome identification as well, mainly due to the huge individual differences (Insua et al. 1998, Pauls and Affonso 2000, Gajardo et al. 2002, López-Piñón et al. 2005, Odierna et al. 2006, Huang et al. 2007b, Huan et al. 2010). RE-banding (Restriction Enzyme-) was only reported in *Adamussium colbecki* (Smith, 1902) (Odierna et al. 2006). Yet, it shared a quite similar outcome to C-banding. As a result, the different banding results showed that those methods could neither offer a high enough number of bands nor provide the uniform outcome of different individuals, needed for chromosome identification.

Fluorescence *in situ* hybridization (FISH) can directly show visual images of hybridization loci, therefore, is a powerful tool to define cytogenetic location (Bartlett 2004). Because of its great advantages in specific sequences mapping, FISH has been widely used for locating repetitive sequences in scallops (Insua et al. 1998, 2006, Wang and Guo 2004, López-Piñón et al. 2005, Odierna et al. 2006, Huang et al. 2007a, 2007b, Zhang et al. 2007). There is no doubt that with repetitive sequences such as rDNA and histone genes successfully being mapped to chromosomes, it has made a progress in distinguishing chromosomes. Whereas such probes are still limited for chromosome identification uses based on the fact only a small number of chromosomes could be recognized.

Large insert clones like bacterial artificial chromosome (BAC), fosmid, P1 and so on have already been tested and proven its practicability for FISH localization (Wang et al. 2005, Zhang et al. 2008, Feng et al. 2014, Zhao et al. 2015). And it has been proven to be a viable approach to map genetic loci in bivalve. For instance, in the Zhikong scallop *Chlamys farreri* (Jones et Preston, 1904), fosmid clones showed the high efficiency of FISH mapping and 8 pairs of chromosomes were successfully distin-

guished (Zhang et al. 2008). More splendidly, based on microsatellite linkage map, an integrated genetic cytogenetic map of the Zhikong scallop was constructed utilizing BAC clones and 17 pairs of chromosomes were identified which helped genomic assembly of this species (Feng et al. 2014).

The Yesso scallop, *Patinopecten yessoensis* (Jay, 1857), is a cold water bivalve and is naturally distributed along the coastline of northern Japan, the Far East of Russia and the northern Korean Peninsula (Waller and Shumway 1991). It is a species of great economic importance in China and Japan. The production has exceeded 200k tons in 2010 (FAO website; http://www.fao.org/fishery/culturedspecies/Patinopecten_yessoensis). Some genetic researches, such as gene expression analysis, development of SSRs and construction of a linkage map, have been conducted on the Yesso scallop (Liu et al. 2009, Wang et al. 2009, Li et al. 2015). Former cytogenetic studies of the Yesso scallop showed it possessed the haploid number (n=19) and a karyotype formula of 3m+5sm+8st+3t (Zhang et al. 2007). In addition, histone H3 gene loci and rDNA loci were located by FISH and were used to discuss the karyotypic evolution in Pectinidae (Huang et al. 2007b, Zhang et al. 2007). Moreover, vertebrate telomere sequence has been used for FISH localization as well (Huang et al. 2007b). Previous studies surely contributed to the work of chromosome identification of *P. yessoensis*. Yet, more information provided by specific chromosomal markers is still needed for further cytogenetic study. Recently, a fosmid library including 122, 880 clones of *P. yessoensis* has been constructed in our lab. This library provides enough probes for us to construct a cytogenetic map for the Yesso scallop.

In the present study, to develop chromosome specific markers for chromosome mapping, we selected 12 fosmid clones containing tandem repeats. These anchored fosmid clones were labeled as FISH probes to hybridize to chromosomes of Yesso scallop. We showed the first time that fosmid clones with long tandem repeats inside can be mapped to *P. yessoensis* and succeeded in chromosome identification which would be helpful for cytogenetic research in Pectinidae.

Methods

Chromosome preparation

Trochophore larvae of *P. yessoensis* were obtained and handled referring to previous study (Huang et al. 2007b). Chromosome spreads were obtained by dissociating fixed larvae in 50% acetic acid and dropping the cellular suspension onto slides heated to 56°C.

Selection of fosmid clones and probe labeling

P. yessoensis genome sequencing data (BioProject number PRJNA259405) were subjected to tandem repeat sequences searches using TANDEM REPEATS FINDER (TRF) software (Benson 1999). In addition, the restriction fragments of two-dimensionally

pooled fosmid clones were sequenced and generated sequence tags. These sequence tags are assigned to individual fosmid clones according to the method in Oeveren et al. (2011). TRF results were then cross checked with those sequence tags and 12 mono-clones including tandem repeats were selected for probe labeling. Detailed information on tandem repeats is provided in Table 1.

Plasmid DNA from fosmid clones, with an average insert size of 30-45 kb, was extracted by standard laboratory method (Sambrook and Russell 1989) and labeled with digoxingenin-11-dUTP or biotin-16-dUTP using Dig- or Biotin-Nick Translation Mix (Roche) following the manufacturer's instruction. Labeled probes were purified by SanPrep PCR products purify kit (Sangon Biotech) and then resolved at a concentration of 5-10 ng/μl in a hybridization solution of 2×SSC, 50% deionized formamide and 10% dextran sulphate.

FISH and Co-hybridization

FISH experiments were performed following methods previously published (Huang et al. 2007b). DNA of chromosomes was denatured in a mixture containing with 70% formamide and 2×SSC at 76°C for 2 min 30 sec, dehydrated with a series of pre-cool ethanol (70%, 90%, 100%; 5 min each) and air-dried. Hybridization mix was denatured at 90°C for 5 min and cooled rapidly. After incubating with hybridization mix for 16h at 37°C in a moist chamber, slides were washed once in 50% formamide and 2×SSC for 5 min, three times in 2×SSC at 37°C (for 5 min each). Signal detection was performed using anti-digoxigenin-rhodamine (Roche) and fluorescein avidin DOS (Vector). Slides were counterstained with DAPI (4', 6-diamidino-2-phenylindole) in antifade solution (Vector). Microscopic analysis and capture of chromosome images were carried out using a Leica DM4000B microscope equipped with an epifluorescence system and the appropriate filter sets for fluorescein, rhodamine and DAPI as well as CCD camera. The signals were collected and processed with FISH software (Leica CW4000 CytoFISH Version Y 1.3.1). In each image, in order to show the relative size of chromosomes possessing positive signals, the biggest metacentric chromosome, which could be easily distinguished from the others, was particularly selected as a reference to make comparison with so that the relations between FISH results of different probes in different metaphases can be determined.

Also, co-hybridization was conducted when signals of two different probes were located in the similar chromosomes. The protocol follows the same procedure of regular hybridization. And the hybridization mix with a total volume of 30μl contained 5–10ng/μl of each probe, 50% formamide, 10% dextran sulphate and 2×SSC.

Tandem repeat sequencing and sequence analysis

Based on tandem repeat sequences scanning from *P. yessoensis* genome sequencing data, we designed six pairs of primers via PRIMER5 software (Lalitha 2000) to am-

Table 1. Primers used for tandem repeats amplification and amplification conditions.

Clone name	Tandem repeats ID	Period size	Copy number	Primer	Primer sequence(5'-3')	Annealing temperature	Extending time
PF114G13	PY_TR0611036	44	243.9	F-PF114G13 R-PF114G13	GCAAGAACATTTGTCTGCTGA GCGGACTAGGAAAGAGTGATAA	56°C	11min
PF117C11	PY_TR0191169	38	268.5	F-PF117C11 R-PF117C11	ATTAGGCACCGTTGAACAGG GGTATGGCGAGAAGACAGGAT	57.5°C	10min30s
PF9J1	PY_TR0084577	34	269.6	F-PF9J1 R-PF9J1	CATCTAATCACATTCTTACGCACC CTTCACAAGCAGGCAAATCATA	58.5°C	10min
PF105M7	PY_TR0226699	114	81.7	F-PF105M7 R- PF105M7	TGGGATTTGAGTCACGATTT ACAATGGGAACTAGGGATCAT	55°C	10min
PF126O24	PY_TR0180504	20	493.8	F-PF126O24 R- PF126O24	GAACTGAGGCGACATAGACATAG GGAAATAACTTCCCAGAACTGA	56°C	10min
PF115K10	PY_TR0380838	37	289.7	F- PF115K10 R- PF115K10	TCTATTGACAGGGCTACATTTG AACTTGGAAAGAAAGGGGAA	55°C	11min

Table 2. Hybridization and BLASTN results of the mapped 6 clones.

Clone name	Chromosome type*	Location of signals	Accession no.*	Identities
PF114G13	st	Telomeric region of 9q	F: KU041535 R: KU041536	93% 96%
PF117C11	sm	Centromeric region of 6q	F:KU041538	95%
PF9J1	t	Telomeric region of 18q	F: KU041532 R: KU041533	97% 96%
PF105M7	m	Telomeric region 2q	F: KU041534	96%
PF126O24	st	Middle region of 12q	N/A	
PF115K10	st	Centromeric region of 10q	R: KU041537	97%

*m: metacentric, sm: submetacentric, st: subtelocentric, t: telocentric; F: forward sequence, R: reverse sequence

plify the total length of tandem repeats contained in the 6 fosmid clones that we successfully located by FISH. PCR procedures were conducted following the manufacturer's instruction of Platinum Taq DNA Polymerase High Fidelity (Invitrogen). Cycling conditions were as follows: 2 min at 94°C (denaturation); 30 cycles of 15s at 94°C, 30s at annealing temperature, and 1min/kb at 68°C for extending. Detailed information about the primers can be found in Table 1. The products were purified with SanPrep PCR products purify kit (Sangon Biotech) for double end-DNA sequencing by ABI3730. Sequences were subjected to sequence similarity searches using BLASTN. All sequences were deposited into Genbank with the accession number listed in Table 2.

Results

FISH signal and distribution

In this study, 12 fosmid clones were selected for FISH localization and at least 30 metaphases were examined for each probe. Among them were six fosmid clones successfully located on the chromosomes. The remaining 6 clones did not produce any signals, therefore, could not be mapped. Paired and specific signals were observed in the analyzed metaphases and their stability was were proved by repeating FISH procedure more than once. Of the six clones that could be located on the chromosomes with unique loci, clone PF105M7 was hybridized to the telomeric region of the long arm of a pair of metacentric chromosomes (Fig. 1a), clone PF117C11 was the only one with signals mapped to the centromere region of a submetacentric chromosome pair (Fig. 1b), clone PF9J1 was hybridized to the telomeric region of the long arm of a pair of telocentric chromosomes (Fig. 1c).

Three further clones, PF114G13, PF126O24, PF115K10, were mapped to 3 different pairs of subtelocentric chromosomes. Clones PF114G13 (Fig. 1d) was assigned to the telomeric region of the long arms. Clone PF126O24 (Fig. 1e) showed signals

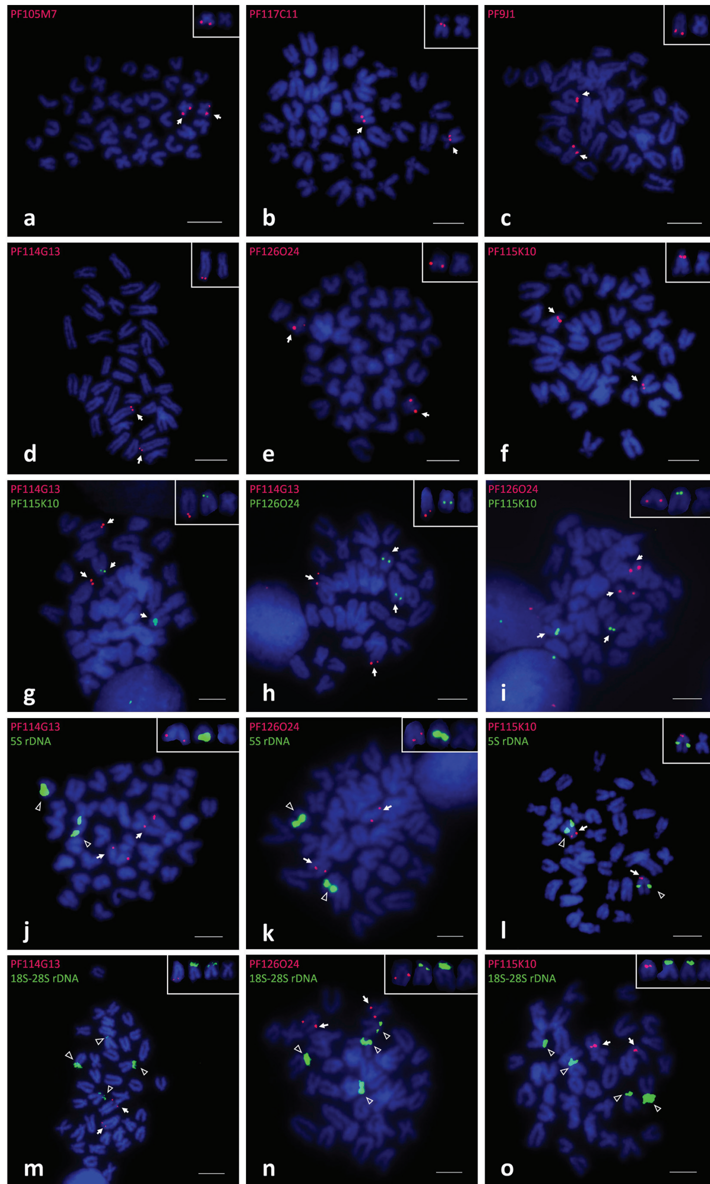

Figure 1. FISH results of fosmid clones on mitotic metaphase chromosomes of *P. yessoensis*. **a–f**: Mapping of clone PF105M7(**a**), clone PF117C11(**b**), clone PF9J1(**c**), clone PF114G13(**d**), clone PF126O24(**e**) and clone PF115K10(**f**) **g–i** Co-hybridization of clone PF114G13 & PF115K10(**g**), clone PF114G13 & 126O24(**h**) and clone PF126O24 & 115K10(**i**) **j–l** Result of co-hybridization of 3 clones and 5S rDNA sequence, i.e. PF114G13&5S rDNA (**j**), PF126O24&5S rDNA (**k**), PF115K10&5S rDNA (**l**) **m–o** Co-hybridization of 3 clones and 18S-28S rDNA, clone PF114G13 & 18S-28S rDNA (**m**), clone PF126O24 & 18S-28S rDNA(**n**), clone PF115K10 & 18S-28S rDNA (**o**). The insert figure at the top right corner for each of the probes correspond to one chromosomal location showing the labeled chromosomes adjacent to the biggest metacentric chromosome. The arrows indicate positive signals of the clones and the open triangles indicate positive signals of 5S rDNA and 18S-28S rDNA. Scale bars: 10 µm

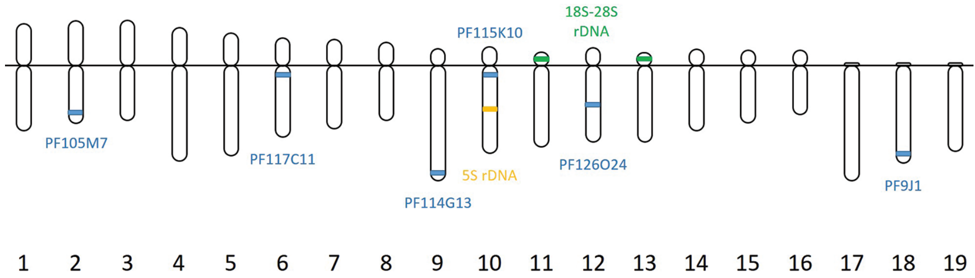

Figure 2. Chromosome ideograms of *P. yessoensis* showing chromosome assignment of 6 fosmid clones and rDNA. Chromosomes numbering is based on chromosome type and relative length. The blue blocks represent the loci of the 6 clones that have been confirmed by co-hybridization. The orange block represents the loci of 5S rDNA. The green blocks represent the loci of 18S-28S rDNA.

on the middle region of the long arms. And as shown in Fig. 1f, clone PF115K10 was mapped to a position quite near the centromere region.

The loci of clone PF105M7, PF117C11 and PF9J1 can be easily distinguished due to the significant differences observed from morphological character of chromosome pairs which they were mapped to. As for the remaining three clones, although locus position diversity was shown, because of similar chromosomal shape and size it was difficult to achieve chromosome separation only according to morphological character. Therefore, co-hybridization of these 3 clones was conducted to confirm their chromosome assignments. As shown in Fig. 1g–h, after co-hybridization, the results confirmed that PF114G13 was located on a different pair of chromosomes with PF115K10 (Fig. 1g) and PF126O24 (Fig. 1h). The co-hybridization result of clone PF126O24 and clone PF115K10 (Fig. 1i) clearly revealed these two clones were mapped to two individual pairs of chromosomes as well.

Further, we co-hybridized 5S rDNA and 18S-28S rDNA with clone PF114G13, PF115K10 and PF126O24 because they were all located on subtelocentric chromosomes. The results of co-hybridization between 5S rDNA and those 3 fosmid clones were displayed in Fig. 1j–l. Clone PF114G13 and PF126O24 showed different chromosome assignment with 5S rDNA (Fig. 1j–k). But clone PF115K10 was mapped on the same pair of chromosomes with 5S rDNA. And Fig. 1m-o demonstrated those 3 fosmid clones were located on the different chromosomes which contained 18S-28S rDNA.

The available data could be used for construction of the karyotypic ideogram of *P. yessoensis* indicating FISH mapping of the 6 clones and rDNA (Fig. 2). In summary, using these 6 fosmid clones, 6 of 19 chromosomes of Yesso scallop can be identified.

PCR and tandem repeats sequencing results

The tandem repeats from the 6 mapped fosmid clones were amplified and the sizes of products varying from about 9 to 11kb. The length of those products was identical

with TRF results. The PCR products of PF114G13 and PF9J1 were successfully sequenced from both ends. And the products of PF105M7, PF115K10 and PF117C11 were successfully sequenced from the single ends. A BLASTN analysis of the 7 sequences against the *P. yessoensis* genome sequencing data showed significant sequence matches as we expected and confirmed the existence of tandem repeats (Table 2). The sequencing result of clone PF126O24 was not matched with the tandem repeats sequence but identical to the upstream sequence of the tandem repeats with 96% match percentage, which was caused by the far position between primers and tandem repeats.

Discussion

Chromosome mapping is an essential step in understanding the genome organization. But together with the small differences in chromosome size and morphology in *P. yessoensis* and most molluscs, it still remains a challenge for unequivocal identification of each chromosome pairs. Karyotyping and DAPI-banding have been applied to gain more knowledge about chromosomes of *P. yessoensis* (Komaru and Wada 1985, Huang et al. 2007b), but the results were proven to be less useful for chromosome identification. FISH is a powerful tool which can significantly contribute to this target. However, only histone H3 gene, rDNA and vertebrate telomeric sequence have been mapped to *P. yessoensis* chromosomes so far (Huang et al. 2007b, Zhang et al. 2007). Vertebrate telomeric sequences were located on the telomeric region of all chromosomes and not suitable for chromosome identification (Huang et al. 2007b). Possessing specific chromosome loci, histone H3 gene and rDNA can be used to identify only one or two pairs of chromosomes (Huang et al. 2007b, Zhang et al. 2007).

Large insert clones like BAC, P1 and fosmid have been already successfully applied in bivalve to reach the goal of chromosome mapping (Wang et al. 2005, Zhang et al. 2008, Feng et al. 2014). In the eastern oyster *Crassostrea virginica* (Gmelin, 1791), 9 of 21 P1 clones, with average size of 75 kb, have been tested and mapped to specific chromosomes (Wang et al. 2005). What is more, fosmid clones which carry a smaller insert size ranging from 30 to 45 kb were used for FISH of *C. farreri* and showed a success rate of 42% (Zhang et al. 2008). In this study, 6 of 12 fosmid clones were successfully assigned to specific chromosomes, indicating a success rate of 50%, which is slightly higher than that in *C. virginica* or *C. farreri*. Although we shared the close success rate of hybridization, end-sequence information and hybridization results reported in *C. farreri* indicating that fosmid clones containing tandem repeats tended to show multiple signals on chromosomes (Zhang et al. 2008), the multiple signals meant that they were considered not suitable for chromosome mapping. But in our study, we first mapped tandem repeats contained in fosmid clones as unique sequence probes to specific chromosomes in *P. yessoensis* with positive result that 6 of 12 clones successfully mapped to the chromosomes (50%). Moreover, species-specific C_0t-1 DNA was widely applied during mapping of large-insert clones in order to eliminate nonspecific hybridization in the two researches mentioned above (Wang et al. 2005, Zhang et al. 2008). However,

in this study, fosmid clones contained long-size tandem repeats were tested and mapped to specific chromosomes without applying C_0t-1 DNA. As C_0t-1 DNA of *P. yessoensis* is not necessary during FISH, this indicated that long tandem repeats have great potential to be used for developing unique chromosomes markers in the Yesso scallop.

The tandem repeats sequence we chose for FISH mapping are all mini-satellite DNA which represent about 96% a large portion of tandem repeats in genome of *P. yessoensis*. Proving its potential for FISH mapping in this study, mini-satellite could be considered as a kind of ideal marker for construction of a cytogenetic map. The tandem repeats from the 6 mapped fosmid clones were amplified and sequenced from the both end. Eight end sequences were generated and the rest did not produce high-quality sequences, therefore, they are not presented here. BLASTN analysis of the 8 sequences against the genome sequence of the Yesso scallop showed significant sequence match with the target sequence which demonstrated the accuracy of whole genome profiling (WGP) method we used for decoding fosmid clones. BLASTN analysis of 7 sequences was conducted against nucleotide collection database on NCBI as well. The results showed that no significant similarity was found for five of them except sequence KU041533 and sequence KU041532 which both came from sequencing results of clone PF9J1. These two sequences were matched to the microsatellite sequence (CFJD036) of *C. farreri* with similarities of 56/63 and 57/63 respectively.

FISH analysis was widely used to establish the relationships between linkage groups and chromosomes in many eukaryotic species such as cucumber and the Zhikong scallop (Ren et al. 2009, Feng et al. 2014). Integrating genetic and cytogenetic maps would be very useful in modifying linkage groups, facilitating whole genome assembly or even detecting chromosome variation in some cases. In the present study, with chromosome assignment of DNA probes of 6 fosmid clones contained tandem repeats as probes, we identified 6 pairs of chromosomes of the Yesso scallop by FISH. In previous study, clusters of 5S rDNA and 18S-28S rDNA were localized on 3 different pairs of subtelocentric chromosomes of the Yesso scallop (Huang et al. 2007b). Therefore, we obtained 18S-28S rDNA and 5S rDNA probes by PCR amplification, labeled with biotin-16-dUTP and applied as a control for the positional relation between fosmid clones and ribosomal DNA. After co-hybridization, 8 of 19 pairs of chromosomes can be distinguished from the others in *P. yessoensis*. With more fosmid clones successfully localized on chromosomes, it will undoubtedly facilitate construction of cytogenetic maps, assignment of linkage groups and genome assembly for the Yesso scallop.

Conclusion

In the present study, we identified 6 pairs of chromosomes in the Yesso scallop by FISH using 6 fosmid clones contained tandem repeats as probes. Furthermore, along with mapping of 5S and 18S-28S rDNA, 8 of the 19 chromosome pairs were unequivocally identified. Although the FISH data presented here could not distinguish all chromo-

somes, these results represent the first step in the development of chromosome specific markers in the Yesso scallop. Ideally, it would be better to have 2 to 4 FISH probes per chromosome arm. Some additional researches are in progress in order to develop more chromosome markers to increase chromosome coverage by localizing repetitive sequences, functional genes and markers from genetic linkage map, etc.

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RESEARCH ARTICLE

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Karyological investigations and new chromosome number reports in *Bellevalia* Lapeyrouse, 1808 and *Muscari* Miller, 1758 (Asparagaceae) from Algeria

Nadjat Azizi[1], Rachid Amirouche[1], Nabila Amirouche[1]

1 *University of Sciences and Technology Houari Boumediene, Faculty of Biological Sciences, LBPO lab., Team: Biosystematics, Genetic and Evolution. USTHB, PO box 32 El-Alia, Bab-Ezzouar, 16110 Algiers, Algeria*

Corresponding author: *Nabila Amirouche* (namirouche@usthb.dz; namirouche@hotmail.com)

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Abstract

Karyological investigations were carried out on four species of *Bellevalia* Lapeyrouse, 1808 and *Muscari* Miller, 1758 (Asparagaceae) sampled in contrasting bioclimatic conditions of Algeria. The endemic *B. mauritanica* Pomel, 1874 was found to have a tetraploid cytotype 2n = 4x = 16 and an octoploid 2n = 8x = 32 which is a new report. The chromosome number 2n = 2x = 18 in *M. comosum* (Linnaeus, 1753) Miller, 1768 and *M. maritimum* Desfontaines, 1798 was in conformity with earlier reports. The latter species reveals a lesser bimodality of the karyotype. Within *M. neglectum* Gussone ex Tenore, 1842 pentaploid (2n = 5x = 45), hexaploid (2n = 6x = 54) and very rare octoploid cytotype (2n = 8x = 72) have been reported in Algeria. Principal component analysis performed on basis of karyotype parameters, showed a segregation of the different cytotypes. This study provides new karyological information, which is discussed in a taxonomic context.

Keywords

Algeria, *Bellevalia*, *Muscari*, chromosome number, karyotype, polyploidy

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Introduction

The Hyacinthaceae is one of the most important families of Asparagales, containing about 70 genera and 700-1000 species (Speta 1998, Pfosser and Speta 1999, Ali et al. 2012). Currently, they are included in the expanded Asparagaceae *sensu* APGIII (2009) as subfamily Scilloideae comprising four tribes Hyacintheae, Ornithogaleae, Urgineeae and Oziroëeae (Chase et al. 2009). Except Oziroëeae, they show a disjunct distribution pattern between the Mediterranean area, north-west Africa, western Asia and sub-Saharan Africa (Sanmartin et al. 2010, Ali et al. 2012, Pfosser et al. 2012). Hyacintheae is undoubtedly the most significant tribe, according to the number of species. Many of them present interesting patterns for examining karyological evolution and polyploidy in relation with their geographical distribution (Speta 1998, Stedje 2001, Hamouche et al. 2010, Goldblatt et al. 2012, Weiss-Schneeweiss and Schneeweiss 2013). Actually, many new chromosome records have lead to description of new species and/or change in taxonomic and nomenclatural status. That is the case of *Bellevalia* Lapeyrouse, 1808 and *Muscari* Miller, 1758.

These genera display similarities in many morphological traits, particularly concerning the floral bud stage and mature fruits. On the basis of morphological criteria, they have been traditionally linked together (Garbari and Greuter 1970) and their close relationship was supported by molecular phylogeny, placing them in the same clade (Pfosser and Speta 1999). Moreover, the geographical range of both genera covers the same areas from the western Mediterranean region (Morocco, Algeria) eastwards throughout Europe and southwestern Asia (Johnson 2003, Nersesian 2001, Bareka et al. 2008, Jafari et al. 2008, Jafari 2012a, 2012b, Borzatti Von Loewenstern et al. 2013, Demirci et al. 2013). However, from the karyological point of view, *Bellevalia* and *Muscari*, differ significantly from each other. The genus *Bellevalia* has a low basic chromosome number x = 4 with large chromosomes and several ploidy levels from 2x to 8x (Speta 1998, Johnson 2003, Yaylaci et al. 2009), while the genus *Muscari* is characterized by the base chromosome number x = 9, with more bimodal karyotype (Garbari 1984, Bentzer and Ellmer 1975, Ruiz Rejón and Oliver 1981).

Within the genus *Bellevalia*, endemic species have been recently discovered, mainly in Anatolia. Some of these new described species are diploids (2n = 2x = 8), such as *B. leucantha* K. Persson, 2006, *B. malatyaensis* Uzunhisarcikli & Duman, 2013 and *B. koyuncui* Karabacak & Yildirim, 2015 (Persson 2006, Uzunhisarcikli et al. 2013, Karabacak et al. 2015). Polyploid species such as *B. pseudolongipes* Karabacak & Yildirim, 2014 (Karabacak et al. 2014), *B. clusiana* Grisebach, 1844 (Yaylaci et al. 2009) and *B. edirnensis* N.Özhatay & Mathew, 1991 (Özhatay et al. 1991b) were identified as triploid, tetraploid and hexaploid respectively. Recently, a new hexaploid species, *B. juliana* Bareka, Turland & Kamari, 2015 (Bareka et al. 2015) was found in Greece. In Tunisia, two tetraploid endemic species were described, *B. galitensis* Bocchieri & Mossa, 1991 and *B. dolichophylla* Brullo & Minissale, 1997 (Bocchieri and Mossa 1991, Brullo and Minissale 1997). New populations of these species were recently recorded by Troìa et al. (2014). According to Brullo et al. (2009), the Tunisian species show a

close relationship with *B. pelagica* C.Brullo, Brullo & Pasta, 2009 also tetraploid, and endemic to Lampione islet (Sicily). Cytogenetic studies (Bareka et al. 2008, 2012) and phylogenetic analysis (Borzatti Von Loewenstern et al. 2013), performed on populations occurring in Greece and Italy respectively, highlighted the diversity in *Bellevalia* and raised questions about the taxonomic relationships and the origin of polyploids.

The situation in the genus *Muscari* is more complex both taxonomically and karyologically. Within this genus, four groups were traditionally recognized, alternatively considered as sections, subgenera or as separate genera (Maire 1958; Garbari and Greuter 1970; Davis and Stuart 1980; Speta 1998; Jafari and Maassoumi 2011): *Leopoldia* Parlatore, 1845, *Muscarimia* Kosteletzky ex A.S. Losina-Losinskaja, 1935, *Pseudomuscari* Garbari & Greuter, 1970 and *Muscari* Miller, 1754 (= *Botryanthus* Kunth, 1843). Species belonging to the subgenus *Leopoldia* are principally diploid although few triploid and tetraploid cytotypes have been quoted (Ruiz Rejón et al. 1985; Nersesian 2001). Species of this group, were also discovered mainly in Iran such as *L. ghouschtchiensis* Jafari & Maassoumi, 2011, *L. tabriziana* Jafari, 2012 and *L. tijtijensis* Jafari, 2012 (Jafari and Maassoumi 2011, Jafari 2012a, 2012b). In Turkey, a new endemic species, *Muscari erdalii* N.Özhatay & S.Demirci, 2013 (Demirci et al. 2013) was identified. However, within the subgenus *Muscari*, the occurrence of polyploidy is higher, particularly among the polymorphic complex *M. neglectum* Gussone ex Tenore, 1842. Populations occurring in Greece and Turkey display a ploidy series ranging from 2x to 8x (Karlén 1984, Garbari 2003). In the Iberian Peninsula, populations of *M. neglectum*, reported as tetraploid, pentaploid and hexaploid, were treated by Suárez-Santiago et al. (2007) as separate species according to their ploidy level.

Despite its biogeographical position in the south-western Mediterranean area, Algeria suffers from an obvious lack of cytotaxonomic data (Amirouche and Misset 2009). This is why it is necessary to start our research by karyological investigations. According to the ancient floras of Algeria (Desfontaines 1798-1799, Battandier and Trabut 1895-1902, Maire 1958, Quézel and Santa 1962), *Bellevalia* and *Muscari* comprise four and five species respectively. This paper is part of an ongoing program on Asparagales in Algeria and aims to complete chromosomal counts, karyotypes knowledge and geographical distribution of the polyploidy. It focuses on the endemic *B. mauritanica* Pomel, 1874, and *M. comosum* (Linnaeus, 1753) Miller, 1768, *M. maritimum* Desfontaines, 1798 and *M. neglectum* Gussone ex Tenore, 1842.

Material and methods

Sampling and taxonomic determinations

Populations used in this study were sampled from March to May 2010–2012 in various ecogeographic areas of Northern Algeria (Table 1). In each site, 5–10 bulbs were collected and cultivated in the experimental garden of Houari Boumediene University of Sciences and Technology (Algiers). Taxonomic determinations were made based on

Table 1. Origin of the studied species and geographical information of the sampling sites.

Taxon *	Locality/site	Biogeo. Sect.	Lat.	Long.	Alt.
Bellevalia mauritanica Pomel	Constantine, Tiddis	C1	36°29'N	06°30'E	546
	Mostaganem, Stidia	O1	35°47'N	00°05'W	35
	Miliana, Ain Torki	A1	36°20'N	02°18'E	715
	Algiers, Ouled Fayet	A1	36°44'N	02°57'E	186
Muscari comosum (L.) Miller	Tipaza, Ain Taghourait	A1	36°35'N	02°37'E	219
	Chlef, Ténès	A1	36°19'N	01°14'E	210
	Tizi Ouzou, Zekri	K1	36°46'N	04°34'E	800
Muscari maritimum Desfontaines	Djelfa, Guelt es Stel	H1	35°09'N	03°01'E	907
Muscari neglectum Gussone ex Tenore	Constantine, Ain El Bey	C1	36°18'N	06°36'E	750
	Constantine, Tiddis	C1	36°29'N	06°30'E	546
	Sétif, Djemila	C1	36°12'N	04°22'E	459
	Tlemcen, Mansourah	O3	34°51'N	01°18'W	1038

* Nomenclature according to Maire (1958), Dobignard and Chatelain (2013), Govaerts (2015).
Biogeographical sectors are from Quézel and Santa (1962): A: Algiers, C: Constantine, K: Kabylie, O: Oran, H: Hodna (High Plains).
Lat. Latitude, Long. Longitude, Alt. Altitude in meters.

several North-Africa and Mediterranean Floras: Desfontaines (1798–1799), Battandier and Trabut (1895–1902), Maire (1958), Quézel and Santa (1962) and Davis and Stuart (1980). The specialized taxonomic and nomenclatural websites, the *African Plant Database* (Dobignard and Chatelain 2010–2013) and the *World Check List of Selected Plants* (Govaerts 2015) were also consulted.

Chromosome preparations

Mitotic preparations were performed on young root-tips obtained from potted plants. The chromosome observations were performed using the standard Feulgen technique for staining tissues (Jahier et al. 1992), with little modifications. Root-tips were pre-

treated in 8-hydroxyquinoline (0.002%) or in 0.25 % aqueous colchicine for 5 hours at room temperature, then fixed in Carnoy fixative solution (3 : 1 (v/v) ethanol : acetic acid) at 4°C for at least 48 hours. Hydrolysis was made in 1N HCl for 7–9 min at 60°C before staining with the usual Schiff reagent. Root-tips were squashed in a drop of 45% acetic acid. The observations were made using a Carl Zeiss Axiostar-Plus microscope equipped with a Canon digital camera.

Karyotype and idiogram constructions

Measurements for karyotype and idiogram constructions were based on at least five well-spread metaphase plates of different individuals. The arrangement of homologous pairs was made using MICROMEASURE Software version 3.3 (Reeves 2001). Chromosomes are described according to the nomenclature of Levan et al. (1964) based on the chromosomal arm ratio (r = long arm/short arm) and the centromeric index (CI % = short arm/long arm + short arm × 100): metacentric (m), submetacentric (sm), subtelocentric (st) and telocentric (t). Karyotype asymmetry indices were estimated following the proposal of Peruzzi and Eroğlu (2013). The intrachromosomal asymmetry index is represented by the mean centromeric asymmetry $M_{CA} = A \times 100$, where A is the average ratio of long arm-short arm/long arm + short arm, according to Watanabe et al. (1999). The interchromosomal asymmetry index is the coefficient of variation of chromosome length $CV_{CL} = A_2 \times 100$ (Paszko 2006) where A_2 is the standard deviation of chromosome length/mean chromosome length (Romero Zarco 1986). The coefficient of variation of the centromeric index $CV_{CI} = S_{CI}/X_{CI} \times 100$ is the ratio between the standard deviation S_{CI} and the mean centromeric index X_{CI} (Paszko 2006).

Multivariate analysis

In order to compare the karyotypes of the studied species, a Principal Component Analysis (PCA) was performed using STATISTICA Software version 6. Analysis was based on six fundamental karyological parameters as proposed by Peruzzi and Altinordu (2014): chromosome number (2n), chromosome base number (x), total haploid chromosome length (THL), mean centromeric asymmetry M_{CA}, coefficient of variation of chromosome length CV_{CL} and coefficient of variation of the centromeric index CV_{CI}.

Results

Chromosome numbers, ploidy level and karyotype characteristics of the four studied species of *Bellevalia* and *Muscari* occurring in Algeria are summarized in Tables 2–3. Representative metaphases and the idiograms are shown in Figs 1–2.

Table 2. Characteristics of karyotype structure in cytotypes of *Bellevalia* and *Muscari.*

Taxon/ Cytotype/Pop.	MCL (μm) ± SD	CLR (μm)	THL (μm) ± SD	M_{CA}	CV_{CL}	CV_{CI}
B. mauritanica 4x (Tiddis)	11.63 ± 0.70	07.00–17.10	093.05 ± 04.63	32.23	32.81	33.05
B. mauritanica 4x (Stidia)	14.23 ± 0.84	10.05–20.47	113.86 ± 06.06	35.43	28.23	34.80
B. mauritanica 8x Ouled Fayet, Ain Torki	10.71 ± 0.70	06.05–18.05	171.40 ± 08.84	42.07	35.27	42.37
M. comosum 2x Tipaza, Ténès, Zekri	03.68 ± 0.39	01.94–10.49	033.51 ± 03.22	19.97	73.8	29.55
M. maritimum 2x Guelt es Stel	05.29 ± 0.27	02.37–09.38	047.64 ± 01.53	47.19	36.97	28.09
M. neglectum 5x Ain El Bey	03.17 ± 0.25	01.99–04.73	072.96 ± 05.53	15.65	23.97	4.78
M. neglectum 6x Tiddis	03.33 ± 0.10	01.80–05.39	089.96 ± 02.2	17.94	25.94	6.61
M. neglectum 8x Djemila	03.42 ± 0.36	01.96–05.35	123.24 ± 12.72	14.86	26.18	5.84

M_{CL}: mean chromosomal length, CLR: chromosome length range, THL: total haploid length, M_{CA}: mean centromeric asymmetry (Peruzzi and Eroğlu 2013), CV_{CL}: coefficient of variation of chromosome length, CV_{CI}: coefficient of variation of centromeric index (Paszko 2006).

Table 3. Chromosome number, ploidy and karyotype formula in the studied species of *Bellevalia* and *Muscari*.

Taxon	Populations	Ploidy	2*n*	Karyotype formula
B. mauritanica	Tiddis	4x	16	4m + 4st + 8sm
	Stidia	4x	16	4m-sat + 4st + 8sm
	Ouled Fayet, Ain Torki	8x	32	8m + 8st + 16sm
M. comosum	Tipaza, Ténès, Zekri	2x	18	2t + (1m + 1sm) + 14m
M. maritimum	Guelt es Stel	2x	18	6st-sat + 6sm-sat + 6m
M. neglectum	Ain El Bey, Mansourah	5x	45	45m
	Tiddis	6x	54	54m
	Djemila	8x	72	72m

Bellevalia mauritanica Pomel, 1874

Mitotic observations showed tetraploid and octaploid cytotypes with base number x = 4. The tetraploid cytotypes 2n = 4x = 16 (Fig. 1A–B; 2A–B) was found in two populations from two contrasted biogeographical sectors. Plants from biogeographical sector of Constantine (Tiddis) grow on clayey-marly soil. Their chromosomes show a total haploid length THL = 93.05 μm with mean length per chromosome (CLR) ranging from 7.00 to 17.1 μm (Table 3). The karyotype consists of 4m + 4st + 8sm. Specimens

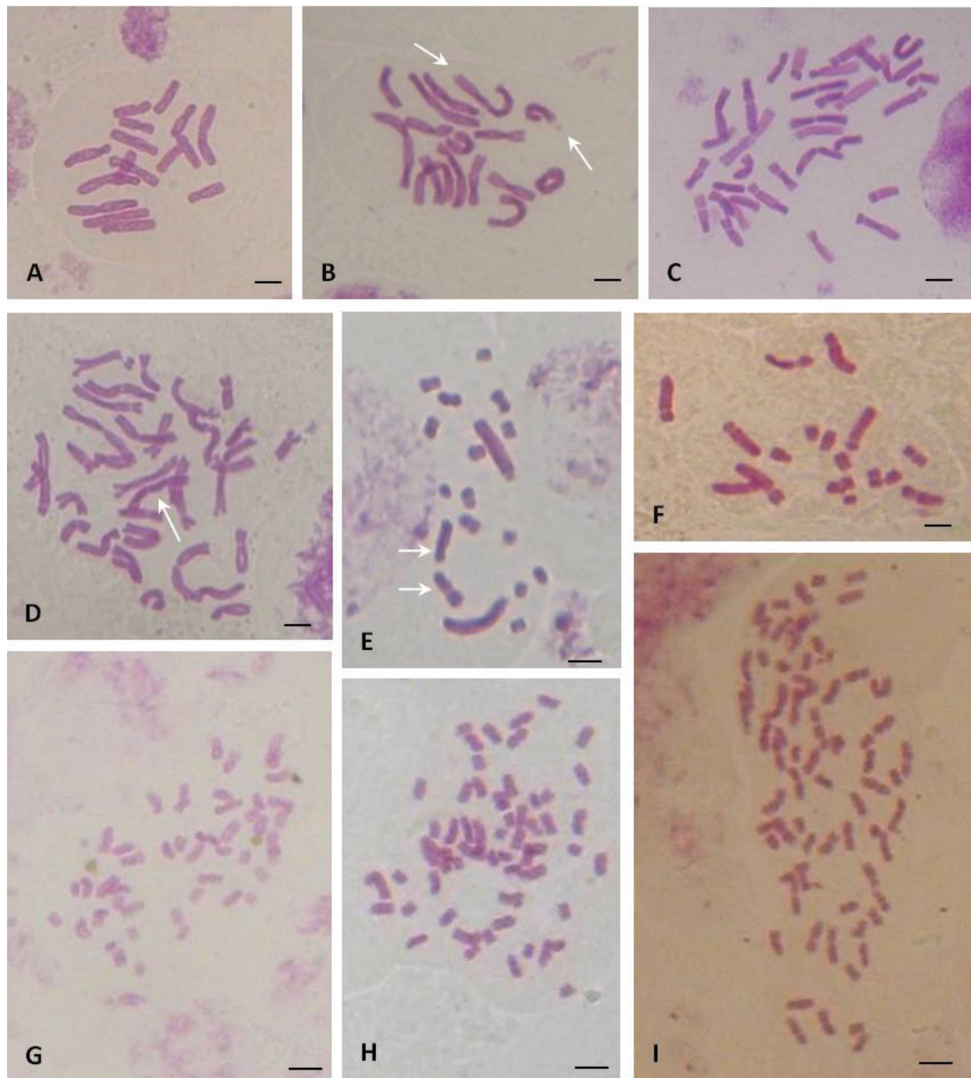

Figure 1. Mitotic metaphases of *Bellevalia* and *Muscari* from Algeria. **A–D** *B. mauritanica*: A 2n = 16 (Tiddis) **B** 2n = 16 (Stidia) arrows indicate satellites **C** 2n = 32 (Ouled Fayet) **D** 2n = 32 (Ain Torki) arrow indicates a supernumerary chromosome **E** *M. comosum* 2n = 18 (arrows: 2st polymorphic pair) **F** *M. maritimum* 2n = 18 (Guelt es stel) **G–I** *M. neglectum*: **G** 2n = 45 (Ain El Bey) **H** 2n = 54 (Tiddis) **I** 2n = 72 (Djemila). Scale bars = 5 µm.

from the biogeographical sector of Oran (Stidia) occurring on coastal sand dunes are distinguished by much larger chromosomes. The mean length per chromosome (CLR) is 10.05-20.47 µm and THL = 113.86 µm (Table 3) with a karyotype formula 4m-sat + 4st + 8sm. This karyotype is distinguished by two terminal satellites on the first largest metacentric pair (Fig. 2B). Except the occurrence of the satellites, the structure

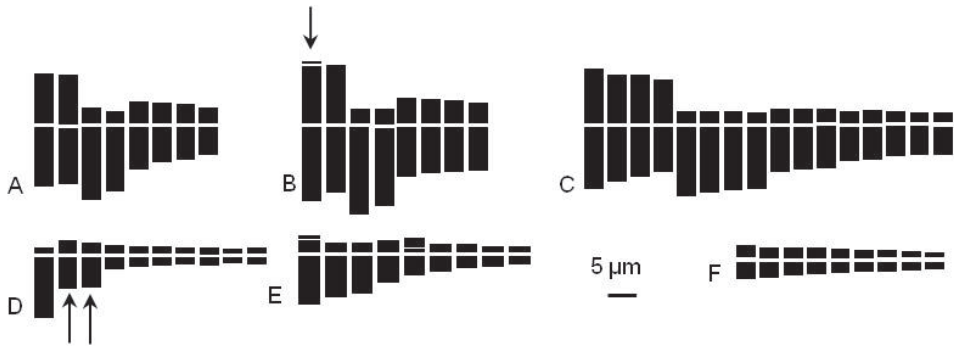

Figure 2. Idiograms of the four studied species of *Bellevalia* and *Muscari*. **A** *B. mauritanica* 4x (Tiddis) **B** *B. mauritanica* 4x (Stidia) arrow indicates satellite **C** *B. mauritanica* 8x **D** *M. comosum* 2x (Arrows indicate 2st polymorphic pair) **E** *M. maritimum* 2x **F** *M. neglectum*: symbolized haploid set for 5x, 6x and 8x.

of the two karyotypes is similar regarding the centromeric asymmetry values and the coefficient of variation (Table 3).

The octoploid cytotype 2n = 8x = 32 (Fig. 1C, 2C) was found in two populations from Ouled Fayet and AinTorki of the biogeographical sector of Algiers. This cytotype is characterized by a larger THL 171.40 µm and CLR values more extensive (6.05-18.05 µm). The karyotype formula is quite similar to that of the tetraploids. One submetacentric supernumerary chromosome was occasionally observed in octoploid individuals (Fig. 1C). The centromeric asymmetry indices (M_{CA}) of tetraploid and octaploid cytotype are rather different while the coefficients of variation (CV_{CL}) are much closer.

Muscari comosum (Linnaeus, 1753) Miller, 1768

This species is widespread in the north of Algeria. Examined populations were diploids with 2n = 18 chromosomes and a base number x = 9 (Fig. 1E). The mean length per chromosome is comprised between 1.94 µm to 10.49 µm and total length THL = 33.51 µm (Table 3). The karyotype is distinguished by two large pairs of chromosomes and seven other pairs much smaller. The first pair is telocentric; the second pair constituted by one metacentric and one submetacentric chromosome is polymorph due to structural heterozygosity (Figs 1E, 2D). All the remaining small chromosomes are metacentric. The karyotype formula is 2t + (1m + 1sm) + 14m. The values of the centromeric asymmetry (M_{CA}) and the coefficient of variation (CV_{CL}) are 73.8 and 19.97 respectively.

Muscari maritimum Desfontaines, 1798

M. maritimum is less common. The studied population lives on the sand dunes in the steppe high plains of the Saharan border (Guelt es Stel). It is also diploid with 2n = 18

(Fig. 1F). The mean length of chromosomes is between 2.37 µm and 9.38 µm with a THL = 47.64 µm (Table 3). The karyotype is characterized by 6st-sat + 6sm-sat + 6m (Fig. 2) showing two satellites: terminal on the first subtelocentric pair, and intercalary on the fifth submetacentric pair. Compared to *M. comosum*, *M. maritimum* have a less asymmetrical karyotype reflected in a low value of its centromeric asymmetry index (M_{CA}).

Muscari neglectum **Gussone ex Tenore, 1842**

In this species, three cytotypes were observed: pentaploid 2n = 5x = 45, hexaploid 2n = 6x = 54 and octaploid 2n = 8x = 72 (Figs 1G–I). All cytotypes were encountered in the eastern biogeographical sector of Constantine (Ain El Bey, Tiddis and Djemila). The western population of Mansourah (Tlemcen) is pentaploid (Tables 1–2). Compared to the previous species, chromosomes are markedly small with mean lengths between 1.80 µm and 5.39 µm and no significant difference among the three karyotypes (Table 3). This species is characterized by a rather symmetrical karyotype comprising only metacentric chromosomes (Figs 1–3, Table 3) with total length depending on the ploidy level. The centromeric asymmetry indices (M_{CA}) and the coefficients of variation are also similar.

Karyotype relationship

In order to estimate the karyological relationship among the studied taxa, a principal component analysis (PCA) was carried out on the 8 populations, each representing different species and/or cytotypes (Fig. 3). The pattern of correlation loadings of the

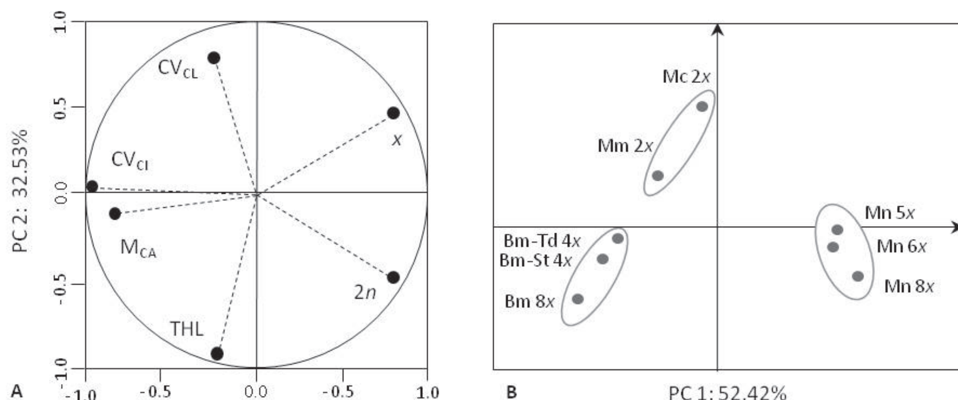

Figure 3. Principal Component Analysis of the eight cytotypes of *Bellevalia* and *Muscari*. **A** Correlation loadings of the six karyotype variables with PC1 and PC2 (abbreviations in Table 3) **B** Corresponding projection of the eight cytotypes: Bm *Bellevalia mauritanica* (Tiddis), *B. mauritanica* (Stidia), Mc *Muscari comosum,* Mm *M. maritimum,* Mn *M. neglectum*.

variables (Fig. 3A) highlights the major role of PC1 and PC2. Cumulative variance explained by these two first components approaches to 85% of the total information. The formation of PC1 was due to intrachromosomal asymmetry parameter $M_{CA,}$ the coefficient of variation of the centromeric index CV_{CI} (negative values) and to chromosome numbers 2n and x (positive values) which have a discriminant power > 0.79 (data not shown). PC2 is well described by the variables THL and CV_{CL} (inversely correlated) and, in least degree, once again, by the chromosomal numbers 2n and x (Fig. 3A). As expected, the projection of the taxa on the first two axes confirms the divergence between the cytotypes representing the genus *Muscari* from those of genus *Bellevalia* (Fig. 3B).

Cytotypes of *Bellevalia mauritanica* constitute a clearly distinct group, in which the two tetraploid cytotypes (from Stidia and Tiddis) shows close relationship. The octoploid cytotype (2n = 8x = 32) can be discreetly distinguished probably because of a higher value of the total haploid length (THL).

The karyotypes of the studied species of *Muscari* constitute two other clusters significantly different from each other (Fig. 3B): the first cluster is limited to the positive values of PC1 and involves all the 5x, 6x and 8x cytotypes of *M. neglectum*; the second cluster, showing positive values of PC2, relates to diploid karyotypes of *M. comosum* and *M. maritimum*. This distribution matches the different affiliation of the species to the two subgenera *Botryanthus* and *Leopoldia* respectively. The diploid species belonging to subgenus *Leopoldia* e.g. *M. maritimum* [= *Leopoldia maritima* (Desfontaines, 1798) Parlatore, 1845] and *M. comosum* [= *L. comosa* (Linnaeus, 1753) Parlatore, 1847] are well separated due to different asymmetry chromosomal indices M_{CA}, CV_{CI} and CV_{CL}. Within the *M. neglectum* group the three ploidy levels did not show any significant differentiation.

Discussion

Chromosome number and polyploidy in genus *Bellevalia*

The studied populations of *Bellevalia mauritanica* display two ploidy levels, tetraploid (2n = 4x = 16) and octoploid (2n = 8x = 32). This species was previously known as exclusively tetraploid besides twelve other species of the genus (Brullo et al. 2009, Bareka et al. 2012).

Usually, in the genus *Bellevalia*, the karyotypes show satellites on either the first, the second or the third pair of chromosomes (Bothmer and Wendelbo 1981, Bareka et al. 2008, 2012). Our tetraploid *B. mauritanica* from Stidia shows a similar chromosome arrangement and bears one pair of satellites on the first metacentric pair.

The octoploid level is reported here in *B. mauritanica* for the first time The polyploidy is quite abundant in *Bellevalia*, 2x, 3x, 4x, 6x and 8x levels have already been reported (Musano and Maggini 1976, Özhatay et al. 1991a, Özhatay and Johnson 1996, Johnson 2003, Yaylaci et al. 2009, Bareka et al. 2012, Karabacak et al. 2014). The

octoploid level is rare and it seems known in two species only, *B. longistyla* (Miscz.) Grossheim, 1928 (Özhatay and Johnson 1996, Johnson 2003) and *B. olivieri* (Baker) Wendelbo, 1985 (Bareka et al. 2012, 2015). Singular populations with 2n = 4x = 32 have been also quoted for *B. glauca* (Lindley) Kunth, 1843 and *B. sarmatica* (Pallas ex Miscz.) Woronow, 1927 (Bothmer and Wendelbo 1981).

In some octoploid cytotypes of *B. mauritanica*, we observed one large and metacentric supernumerary chromosome, similar to all the other homologues. It seems to be a very interesting case of aneuploidy, which has not yet been reported, to our knowledge, in genus *Bellevalia* (P. Bareka pers. comm.). Only B chromosomes were sometimes observed in diploids such as *B. saviczii* Woronow, 1927 with 2n = 8 + 1B (Gettner, 2005) and *B. koyuncui* Karabacak & Yildirim, 2015 with 2n = 8 + 2B (Karabacak et al. 2015). The occurrence of aneuploidy in a polyploid context, associated with vegetative reproduction, may indicate chromosomal changes in process providing evolutionary potential, as presumed for B-chromosomes (Johnson 2003, Weiss-Schneeweiss and Schneeweiss 2013, Bareka et al. 2015). The absence of structural differentiation and the total length of the octoploid complement, nearly twice that of the tetraploid (171.4 *versus* 93.05 µm), argue for an autopolyploidy event. Bareka et al. (2012) already concluded that autopolyploidy was the principal mechanism of polyploidization among populations occurring in Greece belonging to *B. edirnensis* hexaploid, *B. hyacinthoides* triploid and *B. ciliata* tetraploid.

Chromosome number and polyploidy in genus *Muscari*

Karyological results on *M. comosum* and *M. maritimum* agree with previous findings on the subgenus *Leopoldia* in which species are mostly diploids (Ruiz Rejón et al. 1985, Nersesian 2001, Jafari and Maassoumi 2011, Jafari 2012a, 2012b).

All the examined specimens of *M. comosum* have 2n = 2x = 18 with a markedly asymmetric karyotype consisting of 2 pairs of large chromosomes and 7 pairs of small and metacentric chromosomes. Slight variations were observed in the first pair of chromosomes, sometimes viewed as telocentric (Ruiz Rejón et al. 1981, Kostovic-Vranjes 1999) or as subtelocentric (Cuñado et al. 2000, Jafari et al. 2008). Similarly, the second pair is polymorphic with submetacentric and subtelocentric chromosomes (Ruiz Rejón et al. 1985, 1990, Cuñado et al. 2000, Kostovic-Vranjes 1999).

Concerning *M. maritimum*, the chromosome number 2n = 18 was previously quoted by Garbari and Di Martino (1972) for specimens with unspecified origin. It is also quoted by Troìa et al. (2014) for one Tunisian population at the Cap Bon. However, in our knowledge, the karyotype structure of *M. maritimum* is reported here for the first time. It would be related to that of *M. gussonei* (Parlatore) Todaro, 1872, an endemic species to Sicily (Garbari and Di Martino 1972, Davis and Stuart 1980). The karyotype of this species consists of 10 large and 8 small chromosomes (Ruiz Rejón et al. 1985). However, karyotype of Algerian specimens collected in the Saharan border is distinguishable in having satellites located on the 1st and the 5th large chromosome pair.

Muscari neglectum belongs to the subgenus *Botryanthus* which contrasts considerably with the precedent by the occurrence of ploidy series of 2x, 3x, 4x, 5x and 6x levels (Davis and Stuart 1980, Karlén 1984, Ruiz Rejón et al. 1985, Garbari 2003, Suárez-Santiago et al. 2007). Previous chromosomal counts for *M. neglectum* indicate several numbers: 2n = 18, 36, 44, 54, 55, 63 and 72 (Karlén 1984). The three ploidy levels (5x, 6x, 8x) observed in Algeria confirm the extent of polyploidy in this complex. However, no diploids or tetraploids were detected in our country. In contrast, the presence of octaploid plants is significant because the 8x level was extremely rare and only few individuals having 2n = 72 were previously quoted in a population from the northern Greece (Karlén 1984). So far, only tetraploid, pentaploid and hexaploid populations of this taxon have been observed in the western Mediterranean area, precisely in the Iberian Peninsula (Ruiz Rejón et al. 1985, Suárez-Santiago et al. 2007). This is what justifies the statement generally accepted that the diploids occur only in Greece and Turkey (Karlén 1984, Garbari 2003).

Taxonomical remarks

Morphologically, both 4x from Tiddis and 8x from Ouled Fayet and Ain Torki, are similar and belong to the endemic *B. mauritanica* precisely to var. *eu-mauritanica* Maire & Weiller, 1958. This variety is known with a geographic distribution from Central and NE Algeria throughout Tunisia and Cyrenaica. A second variety, *B. mauritanica* var. *tunetana* Battandier, 1911 is restricted to Tunisia. Concerning, the 4x population from Stidia (NW Algeria), the karyotype is distinguished by large chromosomes and satellites on the first chromosomal pair. This population of *Bellevalia* cf. *mauritanica* grows on sandy soil and differ from the type in some variable features as small scape, perigone campanulate-oblong, tepals white to sky-blue and style white. In regard to these characters and its restricted location in the NW Algeria, specimens from Stidia may be attributed to *B. dubia* var. *variabilis* (Freyn) Maire, 1941 as quoted previously (Maire 1958, Quézel and Santa 1962). However, the recent phylogenetic studies by Borzatti Von Loewenstern et al. (2013), demonstrated that *B. dubia* is diploid and narrow endemic to Sicily. Therefore, the taxonomic status of 4x samples from Stidia, considered here as *B.* cf. *mauritanica*, needs to be re-evaluated.

Within, *M. neglectum* group, undoubtedly the most complex within the genus *Muscari*, different authors recognize several distinct taxa based on their ploidy level. For example, Suárez-Santiago et al. (2007) on the basis of ITS sequences, argue that the pentaploid and the hexaploid Iberian populations, represent two different species, *M. olivetorum* Blanca, M. Ruiz Rejón & V.N. Suárez-Santiago and *M. baeticum* Blanca, M. Ruiz Rejón et V.N. Suárez-Santiago well separated from the tetraploid *M. neglectum s. str*. It is worth mentioning that *M. atlanticum* Boissier & Reuter, 1852 is the only one diploid occurring in the southern Spain and northwest of Algeria, notably at Tlemcen (Ruiz Rejón et al. 1985, Suárez-Santiago et al. 2007). Likewise, taxonomic and nomenclatural question may be raised following Maire (1958), Dobignard and

Chatelain (2010-2013) and Govaerts (2015) who considered *M. atlanticum* as a synonym of *M. neglectum*. Our new reports for Tlemcen region (Mansourah) indicates only a pentaploid number 2n = 5x = 45.

In conclusion, our results contribute to a better knowledge of Hyacinthaceae in Algeria. Beside the earlier chromosomal counts, new chromosomes numbers were ascertained from Algerian populations. That is the cases of the new reports of octoploid cytotypes in *Bellevalia mauritanica* and *Muscari neglectum*. All karyological data are illustrative and reflect the east-west pattern of polyploidy at the Mediterranean scale. Further studies are needed to reconsider the taxonomic status and the evolutionary relationships of diploid and polyploid taxa in North Africa.

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RESEARCH ARTICLE

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A chromosomal analysis of eleven species of Gyrinidae (Coleoptera)

Robert B. Angus[1], Teresa C. Holloway[2]

1 *Division of Life Sciences (Insects), The Natural History Museum, Cromwell Road, London SW7 5BD, UK*
2 *School of Biological Sciences, Royal Holloway University of London, Egham Hill, Egham, Surrey TW20 0EX, UK*

Corresponding author: *Robert B. Angus* (r.angus@royalholloway.ac.uk)

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Abstract

Karyotypes are presented for 10 species of *Gyrinus* Geoffroy, 1762: *G. minutus* Fabricius, 1798, *G. caspius* Ménétriés, 1832, *G. paykulli* Ochs, 1927, *G. distinctus* Aubé, 1836 var. *fairmairei* Régimbart, 1883, *G. marinus* Gyllenhal, 1808, *G. natator* (Linnaeus, 1758), *G. opacus* Sahlberg, 1819, *G. substriatus* Stephens, 1869, *G. suffriani* Scriba, 1855, *G. urinator* Illiger, 1807 and for *Orectochilus villosus* (Müller, 1776) (Coleoptera: Gyrinidae). The 10 *Gyrinus* species have karyotypes comprising 13 pairs of autosomes plus sex chromosomes which are X0 (♂), XX (♀), with the X chromosomes the longest in the nucleus. *O. villosus* has 16 pairs of autosomes plus X0, XX sex chromosomes. The data obtained by Saxod and Tetart (1967) and Tetart and Saxod (1968) for five of the *Gyrinus* species are compared with our results. Saxod and Tetart considered the X chromosome to be the smallest in the nucleus in all cases, and this is considered to result from confusion arising from uneven condensation of some of the chromosomes. Small differences between the chromosomes of different *Gyrinus* species have been detected, but not between Greenland and Swedish populations of *G. opacus*, nor between typical *G. distinctus* from France and *G. distinctus* var. *fairmairei* from Kuwait.

Keywords

Coleoptera, Gyrinidae, *Gyrinus*, *Orectochilus*, chromosomes, karyotypes, C-banding

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Introduction

The Gyrinidae appear to be the first coleopteran family to be subjected to chromosomal analysis using air-drying of inflated cells on glass slides (Saxod and Tetart 1967, Tetart and Saxod 1968). The first account of an acetic acid dissociation, air-drying technique for use on insect cells was by Crozier (1968). He used hypotonic saline to inflate living cells prior to fixation, whereas Tetart (1969) began by dissecting out gonads in an isotonic saline solution and placing small pieces of tissue on slides and there fixing them briefly, with a fixative comprising absolute alcohol (70%) and glacial acetic acid (30%). After a minute or so the fixative is poured off and replaced with a swelling solution comprising 40% absolute alcohol, 30% glacial acetic acid and 30% distilled water. This causes tissue swelling and cell dissociation, monitored under a microscope. Additional swelling solution may be added, and mechanical dissociation of the tissue may be needed. The slide is then dried over a spirit lamp and fresh fixative is added. Treatment with hydrochloric acid (concentration and time not given) prevents cytoplasmic staining, and the chromosomes are then stained with Giemsa.

This technique is clearly different from Crozier's hypotonic inflation of living cells, which is the basis for all subsequent air-drying techniques used on insects, but has produced some very good clear chromosome spreads for use in karyotype preparation, not least in five species of *Gyrinus* Geoffroy, 1762.

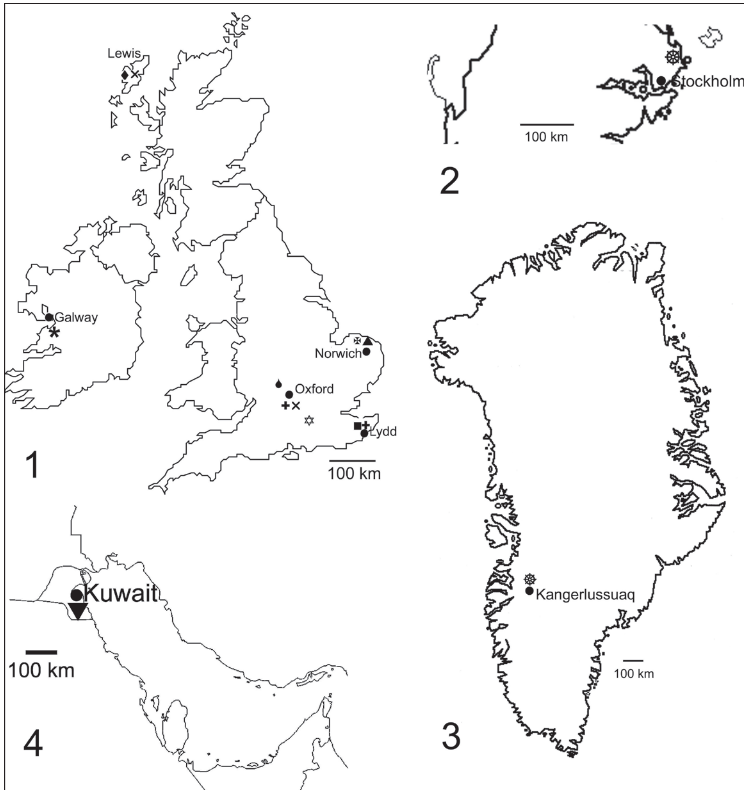
This background information provided a framework to assess the chromosomes obtained from a sample of living *G. opacus* C.R. Sahlberg, 1819, from Greenland, sent by B.O. Svensson in 1995. The first obvious result of the analysis was the discovery that *G. opacus* has an X chromosome much larger than was reported by Saxod and Tetart in any of the five species they studied. Further work by Teresa Holloway in 2006, as a final-year undergraduate project at Royal Holloway, University of London, forms the basis of this paper and shows that Saxod and Tetart were in fact mistaken in their belief that the X chromosome of *Gyrinus* species was the smallest in the nucleus, though they were correct in stating that the karyotypes comprised 13 pairs of autosomes and an X0 sex chromosome system.

Material and methods

Details of the material analysed, including the geographical source, number and sex of the specimens are given in Table 1. The localities from which the material was obtained are shown in Figs 1–4. Living adults from Greenland, Sweden and Kuwait were kept in covered aquaria and were given living adult fruit-flies (*Drosophila*), thrown down on to the surface film of the water, as food. Chromosome preparations were made from mid-gut, testis and ovaries, using the methods described by Dutton and Angus (2007). The methods of C-banding and photography, and assemblage of karyotypes were also as given by Dutton and Angus. Relative Chromosome Lengths (RCL, the length of each chromosome expressed as a percentage of the total haploid autosome length (i.e. not counting the sex chromosomes) in the nucleus) are given as approximate values, without any statistical analysis–the data are insufficient for statistical testing.

Table 1. Material used for chromosome analysis.

Species	Locality	Map	Specimens analysed
Gyrinus minutus Fabricius, 1798	SCOTLAND: Isle of Lewis	Fig 1, ♦	1 ♂
G. caspius Ménétriés, 1832	ENGLAND: Kent, Lydd	Fig 1, ■	1 ♂
G. paykulli Ochs, 1927	ENGLAND: Norfolk, Catfield Fen	Fig 1, ✠	1 ♂
G. distinctus Aubé, 1836 var. *fairmairei* Régimbart, 1883	KUWAIT: Ras Az Zawr district.	Fig 4, ▼	1 ♂
G. marinus Gyllenhal, 1808	ENGLAND: Kent, Lydd;	Fig 1, +	1 ♂
	Oxfordshire, Kennington	Fig 1, +	1 ♂
G. natator (Linnaeus, 1758)	IRELAND: Galway, Lough Briskeen	Fig 1, ★	1 ♂
G. opacus Sahlberg, 1819	SWEDEN: Upland, Vädö	Fig 2, ❆	2♂♂, 1♀
	GREENLAND: Kangerlussuaq	Fig 3, ❆	2 ♂♂, 1♀
G. substriatus Stephens, 1869	ENGLAND: Oxfordshire, Kennington	Fig 1, ×	2 ♂♂
	SCOTLAND: Isle of Lewis	Fig 1, ×	1 ♂
G. suffriani Scriba, 1855	ENGLAND: Norfolk, Catfield Fen	Fig 1, ▲	1 ♂
G. urinator Illiger, 1807	ENGLAND: Surrey, Tilford	Fig 1, ✡	4 ♂♂, 2 ♀♀
Orectochilus villosus (Müller, 1776)	ENGLAND: Oxfordshire, Stonesfield, River Evenlode	Fig 1, 🌢	1 ♂, 1 ♀



Figures 1–4. Maps showing the localities of the material studied. **1** British Isles for *G. minutus*, *G. caspius*, *G. paykulli*, *G. marinus*, *G. natator*, *G. substriatus*, *G. suffriani*, *G. urinator* and *O. villosus* **2** Stockholm area of Sweden for *G. opacus* **3** Greenland for *G. opacus* **4** Kuwait for *G. distinctus fairmairei*. For symbols see Table 1.

Results

Gyrinus

The karyotypes of all 10 species included here are broadly similar, with 2n = 26 + X0 (♂), and 26 + XX (♀). The autosomes are mainly either metacentric or submetacentric, and their RCLs range from about 11 to about 6. The X chromosome is metacentric and the largest in the nucleus, with RCL normally ranging from about 12–16. C-banding, where known, is confined to the centromere regions.

Subgenus *Gyrinulus* Zaitzev, 1907

G. minutus Fabricius, 1798

Published information: none. Mitotic chromosomes, arranged as a karyotype, are shown in Fig. 5a (plain, Giemsa-stained). Autosome pair 1 has a RCL of about 14.5, and the RCLs decrease fairly evenly along the karyotype to about 5.5 in pairs 7–13. Pair 10 has an obvious secondary constriction. Most of the autosomes are metacentric to submetacentric, with pairs 9 and 12 approaching subacrocentric. The X chromosome, clearly the longest in the nucleus, has a RCL of about 20 and is metacentric. This is the longest X chromosome encountered in the present study. Meiotic chromosomes (first division, diakinesis) are shown in Fig. 6a (plain, Giemsa-stained) and b (C-banded). The single X chromosome is clearly recognisable, as are the centromeric C-bands on all the chromosomes. Although the unpaired X chromosome appears less condensed than the autosomal bivalents, its length is in good agreement with that shown in the karyotype obtained from mitotic chromosomes (Fig. 5a). One autosomal bivalent is missing from this preparation.

Figure 5. Mitotic chromosomes of Gyrinidae, arranged as karyotypes. **a** *G. minutus*, ♂, Isle of Lewis, testis, plain (Giemsa stained) **b** *G. caspius*, ♂, Lydd, mid-gut, plain **c–e** *G. paykulli*, ♂, Catfield Fen, mid-gut **c** plain **d** partially C-banded, still showing chromosome morphology, **e** the same nucleus fully C-banded, much chromosome morphology lost **f, g** *G. distinctus fairmairei*, ♂, Kuwait, testis, plain **h, i** *G. marinus*, ♂, Lydd, testis **h** plain **i** the same nucleus C-banded **j**, **k** *G. opacus*, Sweden, mid-gut, plain **j** ♂ **k** ♀ **l**, **m** *G. opacus*, Greenland, plain **l** ♂, mid-gut **m** ♀, ovary **n**, **o** *G. natator*, ♂, Lough Briskeen, mid-gut **n** plain, **o** the same nucleus C-banded **p–s** *G. substriatus*, ♂, testis, plain **p**, **q** Kennington **r, s** Isle of Lewis **t, u** *G. suffriani*, ♂, Catfield Fen, mid-gut, Giemsa stained **t** plain, **u** with spontaneous C-type banding **v**, **w** *G. urinator*, ♂, Tilford, testis **v** plain **w** the same nucleus C-banded **x**, **y** *Orectochilus villosus*, ♂, Stonesfield, mid-gut **x** plain, **y** the same nucleus C-banded. The scale line to the right of the autosome rows of **u, v** represents 5 µm. The vertical lines on the left-hand side link karyotypes of the same species.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 X

a
b
c
d
e
f
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l
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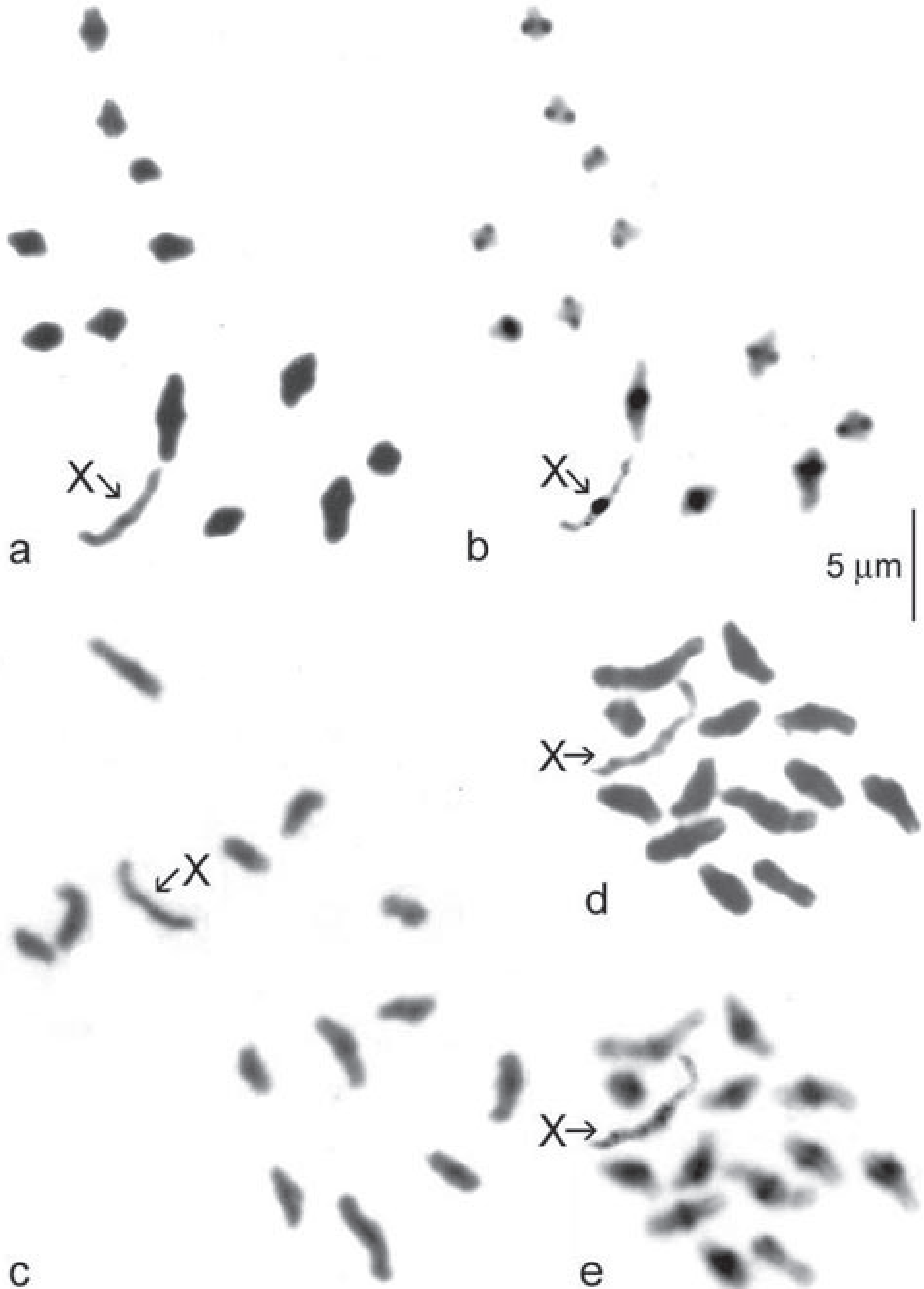


Figure 6. Meiosis, first metaphase from testis. **a, b** *G. minutus*, **a** plain (Giemsa stained) **b** the same nucleus C-banded **c** *G. substriatus*, Isle of Lewis, plain (Giemsa stained) **d, e** *G. urinator* **d** plain (Giemsa stained), **e** the same nucleus C-banded. The scale line represents 5 μm.

Subgenus *Gyrinus* s. str.

G. caspius Ménétriés, 1832

Published information: 2n = 26 + X0 (♂), karyotype: Saxod and Tetart, 1967. Mitotic chromosomes, arranged as a karyotype, are shown in Fig. 5b (plain, Giemsa-stained) The RCLs of the autosomes range from about 11.5 to about 5.5. Most are metacentric to submetacentric, with pair 3 subacrocentric. Pairs 4 and 9 have a secondary constriction in the short arm. The X chromosome is the longest in the nucleus, RCL about 13.5, and is metacentric. This contradicts Saxod and Tetart who claimed it was the shortest.

G. paykulli Ochs, 1927

Published information: n = 13 + X (♂): Saxod and Tetart, 1967; 2n = 26 + X0 (♂), karyotype: Tetart and Saxod 1968. Mitotic chromosomes, arranged as a karyotype, are shown in Fig. 5c (plain, Giemsa-stained), d (partially C-banded, morphology of the chromosomes still clear) and e (the same nucleus as d, fully C-banded but the morphology of some chromosomes lost). All the chromosomes are metacentric with the RCLs of the autosomes decreasing fairly evenly from about 10–6. The X chromosome, RCL about 11.5, is the longest in the nucleus, not the shortest as claimed by Tetart and Saxod. The centromeric C-bands are fairly large, especially on the X chromosome.

G. distinctus Aubé, 1836 var. *fairmairei* Régimbart, 1883

Published information: none for var. *fairmairei* but for French *G. distinctus* 2n = 26 + X0 (♂), karyotype: Saxod and Tetart 1967. Mitotic chromosomes, arranged as karyotypes, are shown in Fig. 5f, g. The RCLs of the autosomes range from about 10.5–5.5, with a fairly even decrease along the karyotype. Pair 10 appears more or less subacrocentric, but the others are all more or less metacentric. The X chromosome, RCL about 14, is clearly the longest in the nucleus.

G. marinus Gyllenhal, 1808

Published information: none. 2n = 26 + X0 (♂). Mitotic chromosomes, arranged as karyotypes, are shown in Fig. 5h (plain, Giemsa-stained) and i (C-banded). The RCLs of the autosome range from about 13.5–6, with fairly sharp decreases between pair1 and pairs 2 and 3 (RCLs about 9.5) and between 3 and pair 4 (RCL about 7.7), then a smaller and more gradual decrease to pair 13. All the autosomes are metacentric except for pairs 11 and 12, which are subacrocentric. The X chromosome, RCL about 16, is clearly the longest in the nucleus and is submetacentric. All the chromosomes have small centromeric C-bands.

G. opacus C.R. Sahlberg, 1819

Published information: none. 2n = 26 +X0 (♂), XX (♀). Mitotic chromosomes, arranged as karyotypes, are shown in Fig. 5j–m. The RCLs of the autosomes range from about 10–6.5, with a fairly even decrease along the karyotype. All are metacentric, with some variation in centromere position, and pair 2 has a secondary constriction in the long arm. The X chromosome, RCL about 12, is the longest in the nucleus and is metacentric. The mid-gut preparations show the chromatids narrow and well separated except at the centromere while the one from ovary (Fig. 5m) shows the chromatids wider and closer together. There is no detectable difference between Swedish and Greenland material.

G. natator (Linnaeus, 1758)

Published information: none. 2n = 26 + X0 (♂). Mitotic chromosomes, arranged as karyotypes, are shown in Fig. 5n (plain, Giemsa-stained) and o (C-banded). The RCLs of the autosomes range from about 10.5–5.5, with a fairly even decrease in length along the karyotype. All the autosomes are metacentric, with pairs 2, 6, 8 and 10 approaching submetacentric. The metacentric X chromosome, RCL about 12.5, is the longest in the nucleus. All the chromosomes have moderate centromeric C-bands.

G. substriatus Stephens, 1869

Published information: 2n–26 + X0 (♂): Saxod and Tetart 1967; Tetart and Saxod 1968; karyotype: Tetart and Saxod 1968. Mitotic chromosomes, arranged as karyotypes, are shown in Fig. 5p–s. The RCLs of the autosomes range from about 11–5.5, with a fairly even decrease along the karyotype. Pairs 1, 2, 5, 8 and 13 are evenly metacentric, pairs 3, 4, 6 and 7 are either subacrocentric or on the border with submetacentric, and pairs 9–11 are submetacentric. The X chromosome, RCL about 12, appears to be the longest in the nucleus and is metacentric with a secondary constriction on the long arm, this not always distinct. Diakinesis of first division of meiosis (Fig. 6c) shows the unpaired X chromosome slightly longer than the longest autosomal bivalent. This contradicts Tetard and Saxod who list it as the shortest in the nucleus.

G. suffriani Scriba, 1855

Published information: 2n = 26 + X0 (♂): Saxod and Tetart 1967. Mitotic chromosomes, Giemsa-stained and arranged as karyotypes, are shown in Fig. 5t, u. The

chromosomes shown in Fig. 5t appear uniformly stained, but those in Fig. 5u show spontaneous C-type banding. These bands appear very large and heavy and may be more extensive than the true C-bands. The RCLs of the autosomes range from about 11–6, with an even decrease from pair 1 to pair 4 (RCL about 7.5), then pairs 5–12 all have the RCL about 7 and pair 13 has it about 6. All except the submetacentric pair 2 are metacentric, and pair 4 has a secondary constriction in its short arm. The metacentric X chromosome, RCL about 13, is the longest in the nucleus, not the shortest as claimed by Saxod and Tetart.

G. urinator Illiger, 1807

Published information: none. 2n = 26 + X0 (♂), XX (♀). Mitotic chromosomes, arranged as karyotypes, are shown in Fig. 5v (plain. Giemsa-stained) and w (C-banded). The RCLs of the autosomes range from about 10.5–6, with an even decrease along the karyotype. Pairs 1–5, 9–11, and 13 are metacentric (though one replicate of pair 13 appears very small and may have a deletion), pairs 6–8 are submetacentric and pair 12 appears acrocentric. The metacentric X chromosome, RCL about 12, is the largest in the nucleus. C-banding shows discrete centromeric C-bands on autosome pairs 1–4, while pairs 5 –11, and 13 appear very extensively heterochromatic. The acrocentric autosome pair 12, and the X chromosome, appear to lack C-bands. Diakinesis of first division of meiosis is shown in Fig. 6d (plain, Giemsa-stained) and e (C-banded). This confirms the discrete centromeric C-bands on the larger autosomes and the much heavier ones on the shorter autosomes. Autosome 12 is shown to have a small terminal C-band and the long unpaired X chromosome no C-band at all.

Orectochilus Dejean, 1833

O. villosus (Müller, 1776)

Published information: none. 2n = 32 + X0 (♂), XX (♀). Mitotic chromosomes, arranged as karyotypes, are shown in Fig. 5x (plain, Giemsa-stained) and y (C-banded). The karyotype comprises 3 pairs of long autosomes (RCLs about 14–12) metacentric with completely heterochromatic long arms, 1 pair of shorter ones, RCL about 9.5, with a centromeric C-band and a further band on the distal part of the long arm, 2 smaller pairs, RCLs about 7 and 6, with fairly heavy centromeric C-bands, and 10 pairs of shorter autosomes, RCLs 5–3, with small centromeric C-bands. The X chromosome, RCL about 12, is the only long chromosome with a discrete centromeric C-band but otherwise euchromatic. Two C-banded incomplete nuclei obtained from a female both show two such chromosomes, confirming that this is the X chromosome.

Discussion

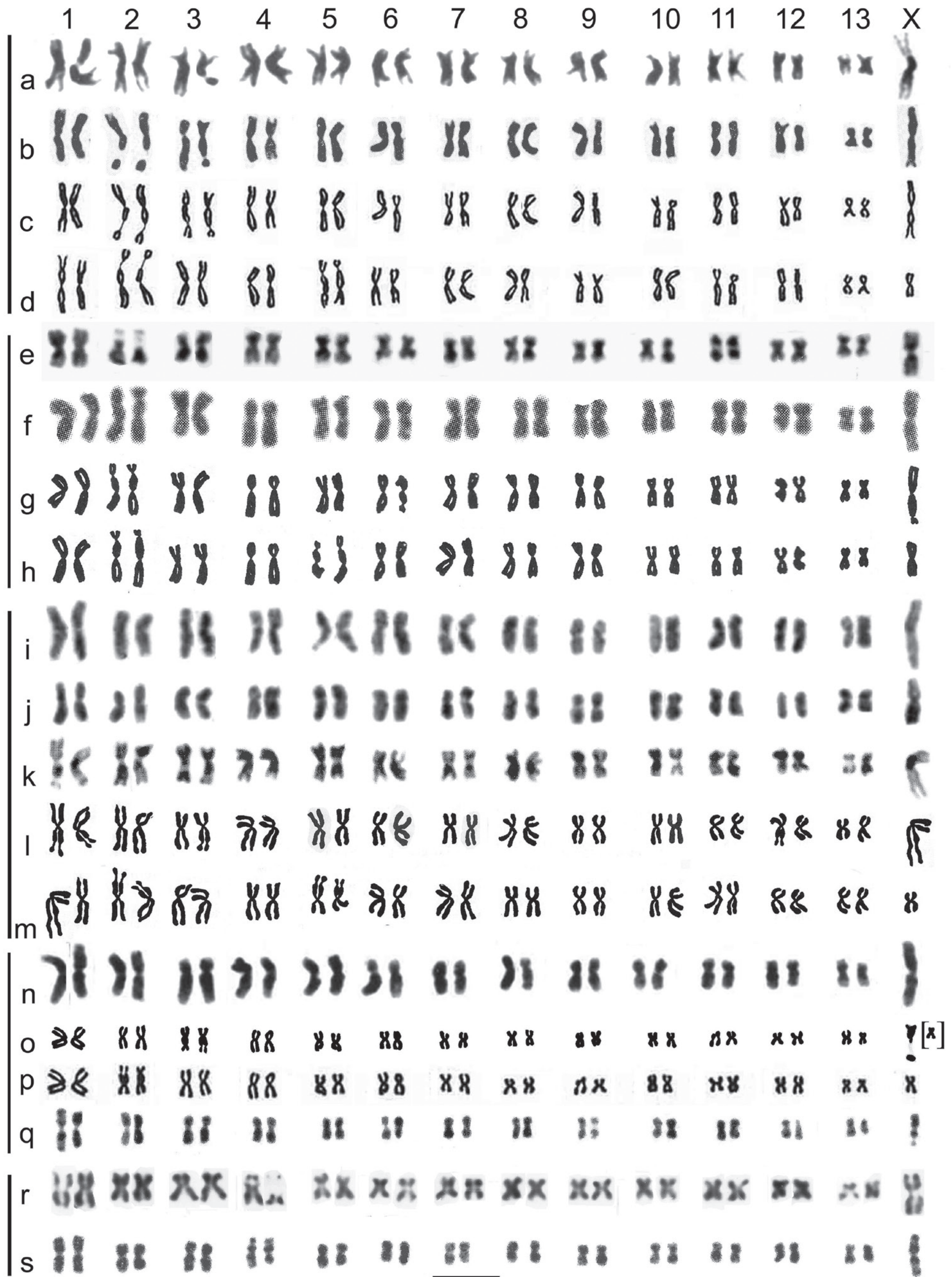
Comparison of the data presented here with those of Saxod and Tetart

Saxod and Tetart (1967, 1968) used only testis as a source of chromosome preparations. Their papers give photographs of chromosomes of five *Gyrinus* species. They presented karyotypes of four of these, and in these cases prepared drawings of the chromosomes *in situ* from the relevant photographs. These drawings were then used to pair up the chromosomes to prepare karyotypes. Before giving a species by species comparison of the data, one general point should be noted: Saxod and Tetart's illustrations consistently show secondary constrictions on various chromosomes which are more numerous and more distinct than in our material. This appears to be associated with the spreading of partially fixed chromosomes in their technique as against living material in ours.

G. caspius. Fig. 7a shows the karyotype presented here while Saxod and Tetart's photograph and drawing are shown arranged in accordance with our karyotype in Fig. 7b, c and their drawing according to their arrangement is shown in Fig. 7d. The karyotypes shown in Fig. 7b, c show an obvious mismatch in pair 12, also present, though less marked, in Fig. 7a, while the arrangement in Fig. 7d shows mismatches in pairs 1, 4 and 11. The cause of these apparent mismatches is either different degrees of condensation between the replicates of a chromosome pair, if the pairing is correct, or a mispairing if it is not. Different degrees of condensation between the two replicates of a chromosome pair are a frequent occurrence which has to be contended with when assembling karyotypes. In the present case the appearance of the X chromosome in Fig. 7a is strikingly different from the two replicates of autosome pair 1 and is sufficient to show that this attribution is correct, especially as it is consistent with data obtained from other species where the unpaired X chromosome is shown in diakinesis of first division of meiosis.

G. paykulli. Fig. 7e shows the karyotype presented here, while Tetart and Saxod's material is shown in Fig. 7f (photograph) and g (drawing), arranged according to our

Figure 7. Mitotic chromosomes of *Gyrinus* spp, arranged as karyotypes, to compare the present results with those of Saxod and Tetart (1967) and Tetart and Saxod (1968). **a–d** *G. caspius*, **a** present material (Fig. 5b) **b** Saxod, Tetart, photograph (Plate 1B) **c** idem, drawing (Fig. 2), arranged as Fig. 5b, d the same drawing as arranged by Saxod, Tetart **e–h** *G. paykulli*, **e** present material (Fig. 5c) **f** Tetart, Saxod (1968), photograph (Plate1A) **g** idem, drawing, (Fig. 1), arranged as **e, h** the same drawing as arranged by Tetart, Saxod **i, j** *G. distinctus fairmairei*, present material (Fig. 5f, g) **k–m** *G. distinctus distinctus* from Saxod, Tetart (1967) **k** photograph (Plate 1A) **l** idem, drawing (Fig. 1), arranged as the present *G. d. fairmairei* (**i, j**) **m** the same drawing as arranged by Saxod, Tetart **n–q** *G. substriatus* **n** present material (Fig. 5q) **o** drawing by Tetart, Saxod (Fig. 2) but with the X chromosome taken from their photograph (idem, Plate 1C), arranged as present material (**n**) **p** the same drawing as arranged by Tetart, Saxod. The partial X chromosome is placed as the right-hand replicate of chromosome 13 **q** karyotype prepared from Saxod, Tetart (Plate 1D) **r, s** *G. suffriani* **r** present material (Fig. 5t) **s** karyotype prepared from Saxod, Tetart, 1967 (Plate 1C). The horizontal scale-line represents 5 μm. The vertical lines on the left hand side link karyotypes of the same species.


1 2 3 4 5 6 7 8 9 10 11 12 13 X
a
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interpretation, and the drawing according to their interpretation is shown as Fig. 7h. In this case our interpretation of Tetart and Saxod's pictures shows minor mismatches in pairs 1 and 9, while their arrangement shows more serious mismatches in pairs 2 and 5, a different minor mismatch in pair 1 and the same minor mismatch in pair 9. Saxod and Tetart (1967) figure diakinesis of first division of meiosis, and this photograph is shown in Fig. 8c. The unpaired X chromosome is clearly visible and about as long as the longest bivalents. This agrees with our interpretation.

G. distinctus. Fig. 7i, j shows the karyotype presented here for var. *fairmairei*, while Saxod and Tetart's material is shown to the same arrangement if Fig. 7k (photograph) and l (drawing), with the drawing as arranged by Saxod and Tetart shown in Fig. 7m. Our arrangement shows no serious mismatches between replicates of a chromosome pair, but minor mismatches in pairs 12 and 13, while Saxod and Tetart's arrangement shows serious mismatches in pairs 1 and 2, but no mismatches in pairs 12 and 13. The other thing to note is that the detailed sequence of chromosome dimensions obtained from our material agrees very closely with those obtained when Saxod and Tetart's data are arranged in the same way. These data give no support to placing var. *fairmairei* as a species separate from *G. distinctus*.

G. substriatus. Fig. 7n shows the karyotype presented here, while Fig. 7o shows Tetart and Saxod's drawing arranged in the same way, and Fig. 7p shows the drawing as arranged by Tetart and Saxod. Finally, Fig. 7q shows a karyotype assembled according to our arrangement, from the photograph given by Saxod and Tetart. This species apparently gave Saxod and Tetart some difficulty. They chose not to assemble a karyotype from their 1967 photograph, perhaps because they thought that one of the chromosomes, lying at some distance from the others, might not belong to the same nucleus. The 1968 photograph they used is shown as their Plate 1 C and the drawing prepared from it as Fig. 2. The chromosomes as shown in the photograph are too condensed and clumped to allow assembly of a karyotype, but the drawing seems adequate. However, the photograph shows the X chromosome (as here interpreted) with a very large secondary constriction but in the drawing the distal part of the chromosome, beyond the constriction, is omitted. The chromosomes are all very condensed and neither arrangement of their material shows any obvious mismatches. However, *G. substriatus* is a species for which we have a preparation showing the unpaired X chromosome at diakinesis in the first division of meiosis. This leaves no doubt that this is a large chromosome. Tetart and Saxod (1968, Plate 1 E) show a first meiotic metaphase for this species. The chromosomes are very condensed and not clear, but what appears to be the X chromosome is as long as the longest autosomal bivalents. The karyotype shown in Fig. 7q shows the same secondary constriction in the X chromosome as that shown in Fig. 7o, p, but shows the X chromosome relatively shorter than in our material (Fig. 7n), though this is less apparent when the more condensed (shorter chromosomes) Isle of Lewis material (Fig. 5r, s) is considered.. There is also an obvious mismatch in pair 1. It seems likely that the apparent difference in X chromosome length between our material and Saxod and Tetart's results from the

more condensed chromosomes in their material, with the mismatch in pair 1 in Fig. 7q resulting from uneven condensation.

G. suffriani. The karyotype presented from our material is shown in Fig. 7r and one assembled from the photograph given by Saxod and Tetart (1967) is shown in Fig. 7s. The two agree very closely. Saxod and Tetart did not give a karyotype for *G. suffriani*, only the chromosome number. The X chromosome is the longest in both Figures and shows a secondary constriction in the long arm of Saxod and Tetart's material, comparable with that in *G. substriatus*. The secondary constriction in the short arm of chromosome 2 is apparent in both our material and that of Saxod and Tetart.

Interspecies differences

The data presented here show that the 10 species of *Gyrinus* discussed here all have broadly similar karyotypes, with 13 pairs of autosomes X0 sex chromosomes, and the X chromosome the longest in the nucleus. There are often small differences between the chromosomes of different species. Thus the relative length of the X chromosome of *G. minutus* is about 1.5 times that of the longest autosome, a bigger difference than in any of the other species studied.

There are two species-groups among the studied species. *G. caspius* and *paykulli* are conspicuously elongate parallel-sided beetles, though with very different aedeagi. Unfortunately the chromosomal material presented here is inadequate to show interspecies differences. The second group, *G. natator*, *substriatus* and *suffriani*, does show some interspecies differences. In *G. natator* the more or less submetacentric chromosome 2 is clearly longer than metacentric chromosome 3, while in *G. substriatus* metacentric chromosome 2 is longer than submetacentric 3, though only slightly so. The X chromosome is only slightly longer than chromosome 1 in these species. In *G. suffriani* the X chromosome is more clearly longer than chromosome 1, chromosome 4 has a distinct secondary constriction in its short arm, and there is more marked decrease in length between pairs 4 and 5.

There are two cases where conspecific material from widely separated localities shows no chromosomal difference. This is true of Swedish and Greenland *G. opacus*, with the Greenland material belonging to the form which lacks elytral reticulation, and of Kuwaiti and French *G. distinctus*, with the Kuwaiti material belonging to var. *fairmairei* with a yellow underside as against the largely black underside of the French material.

Gyrinus urinator is the only species with acrocentric chromosomes–chromosome 12.

The chromosomes of *O. villosus* agree with those of *Gyrinus* spp. in having an X0 sex chromosome system and a relatively long X chromosome. However, they differ in having 16 pairs of autosomes as against 13 in *Gyrinus*, and in having the three longest pairs with heterochromatic long arms, then a fairly long pair with a largely heterochromatic longer arm, and the remaining pairs short to very short with small centromeric C-bands, ranging from metacentric to subacrocentric.

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