CompCytogen 9(1): 1–15 (2015)
doi: 10.3897/CompCytogen.v9i1.8731
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Laser microdissection-based analysis of the Y sex chromosome of the Antarctic fish *Chionodraco hamatus* (Notothenioidei, Channichthyidae)

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Academic editor: *N. Shapoval* | Received 10 October 2014 | Accepted 9 December 2014 | Published 5 February 2015

http://zoobank.org/9E6EEA88-A980-4C49-BAC9-3A79F69E804D

Citation: Cocca E, Petraccioli A, Morescalchi MA, Odierna G, Capriglione T (2015) Laser microdissection-based analysis of the Y sex chromosome of the Antarctic fish *Chionodraco hamatus* (Notothenioidei, Channichthyidae). Comparative Cytogenetics 9(1): 1–15. doi: 10.3897/CompCytogen.v9i1.8731

Abstract

Microdissection, DOP-PCR amplification and microcloning were used to study the large Y chromosome of *Chionodraco hamatus*, an Antarctic fish belonging to the Notothenioidei, the dominant component of the Southern Ocean fauna. The species has evolved a multiple sex chromosome system with digametic males showing an X1YX2 karyotype and females an X1X1X2X2 karyotype. Fluorescence in situ hybridization, performed with a painting probe made from microdissected Y chromosomes, allowed a deeper insight on the chromosomal rearrangement, which underpinned the fusion event that generated the Y. Then, we used a DNA library established by microdissection and microcloning of the whole Y chromosome of *Ch. hamatus* for searching sex-linked sequences. One clone provided preliminary information on the presence on the Y chromosome of the CHD1 gene homologue, which is sex-linked in birds but in no other vertebrates. Several clones from the Y-chromosome mini-library contained microsatellites and transposable elements, one of which mapped to the q arm putative fusion region of the Y chromosome. The findings confirm that interspersed repetitive sequences might have fostered chromosome rearrangements and the emergence of the Y chromosome in *Ch. hamatus*. Detection of the CHD1 gene in the Y sex-determining region could be a classical example of convergent evolution in action.

Keywords

Sex chromosomes, Antarctic fish, CHD1 gene

Introduction

The chromosome microdissection and microcloning technique (Almeida-Toledo et al. 2000) can be used for a number of applications, including generation of chromosome painting probes and construction of genetic linkage as well as physical maps. First applied to *Drosophila melanogaster* (Meigen, 1830) polytene chromosomes and later employed to obtain whole or partial human chromosome paints (Jauch et al. 1992, Stanyon et al. 1995, Bigoni et al. 1997, Morescalchi et al. 1997, Yang et al. 2006), chromosome microdissection is considered a suitable bridge between cytogenetics and molecular genetics (Zhou and Hu 2007). Chromosome microdissection has recently been applied to lower vertebrates to generate painting probes for phylogenetic studies (Diniz et al. 2008, Giovannotti et al. 2009, Machado et al. 2011, Pokorná et al. 2011, Trifonov et al. 2011, Kawagoshi et al. 2012). In cold-blooded vertebrates its application may be hampered by difficulties in identifying the chromosomes clearly in the karyotype of the species, where the heteromorphic sex chromosome is often the one most easily recognizable (Almeida-Toledo et al. 2000, Odierna et al. 2004, Kuprijanova et al. 2005, Cioffi et al. 2012). Sex chromosomes have originated independently many times in both animals and plants from ordinary autosomes (Arnemann et al. 1986, Almeida-Toledo et al. 2000). Their evolution is characterized by a loss of gene function on the non-recombining Y chromosome, as seen in many taxa (Badenhorst et al. 2012, Böhne et al. 2014).

While sex-determining genes have been identified and mapped on the heteromorphic sex chromosomes of mammals and birds (Handley et al. 2004, Lahn and Page 2004), data on their linkage and localization in cold-blooded vertebrates are only just beginning to be reported.

Fish sex chromosomes often appear undifferentiated, sharing a similar morphology and a comparable gene composition (Charlesworth 2004, Charlesworth et al. 2005). The sex-determining region (SDR) has been identified in several teleost species (Geschwend et al. 2012, Liew et al. 2012) and the first likely sex-determining gene was found in medaka, *Oryzias latipes* (Temminck & Schlegel, 1846), in which a copy of the Dmrt1 gene, DmY, maps on the putative Y sex chromosome. Some correspondence has been found, in other fish as well as for the congeneric species (Matsuda 2005, Kondo et al. 2009), while novel candidates, all coding for members of the TGF-beta signalling pathway, have recently been identified in different teleostean species (Kikuchi and Hamaguchi 2013, Bohne et al. 2014).

Some fishes present multiple sex-chromosome systems, in which the initial stage of differentiation seems to be independent of heterochromatin accumulation in chromosomes and to be related to chromosome rearrangement, whereas simple sex-chromosome systems show progressive heterochromatin accumulation (Almeida-Toledo et al. 2000, Cioffi et al. 2012).

The variety of sex-chromosome systems and the broad range of sex-determination mechanisms typical of fishes make it an interesting group in which to explore sex-chromosome evolution. The present study was designed to seek correlations between

cytologically recognizable fish X and Y chromosomes and to establish whether they are related to different genetic make-up or simply to fusion events, perhaps trigged by differential amplification of repetitive DNA fractions.

To shed light on the genome composition of a teleost sex chromosome, laser microdissection and pressure catapulting (LMPC) was used to isolate the Y chromosome from standard mitotic plates of the Antarctic icefish *Chionodraco hamatus* (Lönnberg, 1905) belonging to the Notothenioidei, the dominant endemic component of the Southern Ocean fauna. The physiological and molecular adaptations that have enabled their evolution have made this Antarctic clade unique among vertebrates. The karyotype of most Notothenioidei shows 2n = 48 mostly acrocentric chromosomes, which is considered basic for Perciformes, and nucleolus organizer regions (NORs) localized on the telomeres of a single chromosome pair (Morescalchi et al. 1996, Pisano and Ghigliotti 2009). Heteromorphic sex chromosomes have been found in species belonging to three notothenioid families (Morescalchi et al. 1992, Caputo et al. 2002). In such species males have a large Y chromosome, resulting from a centric or tandem fusion event, and a consequent decrease in chromosome number to 2n = 47. This feature is shared by *Ch. hamatus,* the model selected for this study. *Ch. hamatus* females have 2n=48 chromosomes with a small metacentric pair, a submetacentric pair, 20 acrocentric pairs and a $X_1X_1X_2X_2$, multiple sex chromosome system, where X_1 is submetacentric and X_2 is acrocentric; males of *Ch. hamatus* present 2n=47 with the same basal chromosome set plus the X1X2Y sex chromosome system, where the Y is the largest submetacentric element, likely, derived from X1-X2 tandem fusion (Morescalchi et al. 1992).

Ch. hamatus Y chromosomes were isolated and amplified by degenerate oligonucleotide primed PCR (DOP-PCR) as described by Telenius et al. (1992). The DNA fragments generated were (i) labelled with biotin, hybridized *in situ* to metaphase spreads, and (ii) used to construct a Y-chromosome mini-library that was subsequently screened for Y-chromosome DNA sequences.

Methods

Y chromosome microdissection

Ch. hamatus specimens were collected near Mario Zucchelli Station, on the coast of Terra Nova Bay (74°42'S, 164°07'E), during Italian Antarctic expeditions in 2000 and 2005. They were killed by severing the spinal cord according to animal welfare guidelines.

Mitotic chromosome plates were prepared from a *Ch. hamatus* male as described in Morescalchi et al. (1992). The chromosome suspension was dropped onto slides covered with a polyethylene naphthalate membrane, and Y chromosomes were microdissected using a PALM MicroBeam system (Carl Zeiss, Italy, http://www.zeiss.it/). All procedures were as described by Schermelleh et al. 1999.

Y chromosomes (20/slide) were outlined and excised using a low-energy laser beam and catapulted by a single laser pulse directly into the cap of an Eppendorf tube, to minimize contamination.

DOP-PCR

DOP-PCR reactions were carried out without chromosome pre-treatment (Telenius et al. 1992). The DOP primer sequence 5'-CCGACTCGAGNNNNNNATGTGG-3' was used to conduct a PCR reaction with 1 × ThermoSequenase reaction buffer, 40 μM dNTPs, 2 μM DOP primer, and 10 U ThermoSequenase enzyme.

The first amplification was carried out by RAMP-PCR: 94 °C for 5 min; 12 cycles at low stringency (94 °C for 90 s, 32 °C for 2 min, increasing by 0.2 °C/s to 72 °C, then 72 °C for 2 min), followed by 35 cycles at high stringency (94 °C for 90 s, 52 °C for 90 s and 72 °C for 90 s). 10 μl of the DOP-PCR product was run on 1% agarose gel.

Fluorescence *In Situ* Hybridization (FISH)

FISH with the Y probe

The species specificity of the probe was checked by dot-blot hybridization under stringent conditions using digoxigenin-labelled Y chromosome material as a probe on human and fish total genomic DNA spotted on a nitrocellulose membrane. Dot-blot analysis confirmed the absence of contamination, showing a strong hybridization signal only to *Ch. hamatus* genomic DNA (data not shown).

The DOP-PCR-amplified chromosome material was re-amplified and labelled with 16-dUTP-biotin (Roche). The PCR mix comprised 1 x *Taq* polymerase buffer (2 mM $MgCl_2$), 40 μM dATP, dGTP and dCTP, 28 μM dTTP, 12 μM 16-dUTP biotin, 2 μM DOP primer, and 0.05 U/μl *Taq* polymerase under the following conditions: (1×) 94 °C for 5 min; (35×) 90 °C for 1 min and 30 s, 52 °C for 1 min and 30 s, 72 °C for 1 min and 30 s, and (1×) 72 °C for 5 min. The FISH procedure was performed under high stringency conditions (50% formamide, 2 × SSC, 10% dextran sulphate). Metaphase spreads for FISH were prepared on ethanol-cleaned slides, air-dried and incubated overnight at 37 °C. Chromosomes were denatured in 70% deionized formamide 2 × SSC at 72 °C for 7 min and dehydrated in an ethanol series.

A DNA mixture of approximately 400 ng of the chromosome painting probe and 10 μg of the Cot-1 fraction of *Ch. hamatus* (Yan et al. 2010) was prepared, ethanol precipitated, vacuum dried, and dissolved in 10 μl of hybridization mixture containing 50% deionized formamide, 10% dextran sulphate, and 2 × SSC. After denaturation (10 min at 75 °C) the probe was dropped onto a slide and spread over the hybridization area using a 22 × 22 mm glass coverslip. The edges of the coverslip were sealed with rubber cement and slides were incubated for 3 days at 37 °C in a humid chamber.

Post-hybridization washes were in 0.4 × SSC/0.1% Triton X-100 for 5 min at 42 °C and in 2 × SSC/0.1% Triton X-100 for 15 min at room temperature. Signal detection was accomplished using AlexaFluor-Streptavidin 488 (Molecular Probes) according to Cocca et al. (2011). Chromosomes were counterstained with propidium iodide in Vectashield mounting medium (Vector), analysed using a fluorescence microscope (Zeiss Axioskop) equipped with a CCD camera (Hamamatsu Chilled), and processed with the Isis FISH imaging system (v. 3.04, Metasystems).

Mini-library construction

DOP-PCR fragments were purified with the NucleoSpin Extract II Kit (Macherey-Nagel). Three µl aliquots were cloned into the TOPO TA Cloning Kit (Invitrogen Life Technologies). Transformation and clone analysis were conducted according to the manufacturer's instructions. Recombinant clones were isolated and PCR amplification was used to establish insert presence and size. Sequencing was performed by Primm Biotech (Milano, Italy). The nucleotide sequences, ranging from 200 to 800 bp, were used for homology searches in four databases (National Center for Biotechnology Information, NCBI: http://www.ncbi.nlm.nih.gov; DNA DataBank of Japan, DDBJ: http://www.ddbj.nig.ac.jp; Nucleotide EST Database; and RepBase: http://www.girinst.org/repbase) using BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov), FASTA (http://www.ebi.ac.uk/Tools/sss/fasta), and RepeatMasker (http://www.repeatmasker.org) sequence alignment programs.

Results

The results of FISH, performed on metaphase plates of *Ch. hamatus* males and females using the newly generated Y-chromosome paint, are shown in Fig. 1. In males (Fig.1a) a strong hybridization signal was detected along the Y chromosome. On other chromosomes the signal was faint up to the subtelomeric regions. A single medium-sized acrocentric chromosome showed a clear pericentromeric signal. On female metaphase plates (Fig.1b), the hybridization signal was intense and confined to the pericentromeric and interstitial regions of two medium-sized acrocentrics. Thirty sequences were recovered by cloning with clear information obtained only for four of them.

The identification of syntenic regions may have been hampered by sequence length and by the lack of whole sequenced genomes of Antarctic fishes. Most clones were found to contain redundant repetitive sequences; moreover, several clones did not show significant homologies to any genomic or translated sequence contained in the GenBank database, suggesting that they may correspond to untranslated or non-conserved regions of genes already known from other species.

FASTX analysis of clone C11 (251 bp) against library 'B170.0 vertebrates' yielded the highest similarity score for fish genome fragments. Interestingly, its uninterrupt-

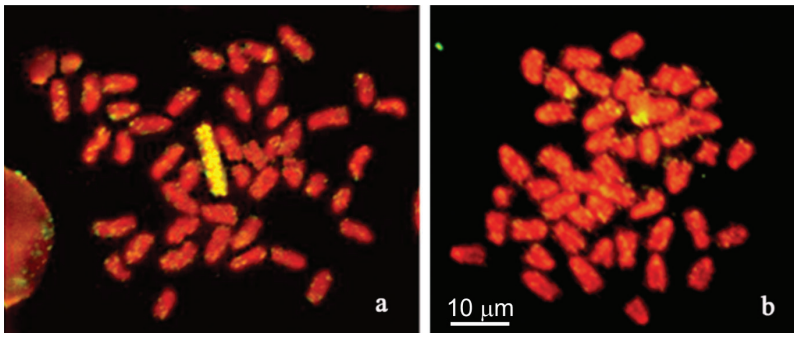

Figure 1. Mitotic chromosomes of *Ch. hamatus.* FISH with the Y chromosome paint on *Ch. hamatus* **a** male (2n = 47 and X1X2Y sex-chromosome system) and **b** female (2n = 48) metaphase spreads.

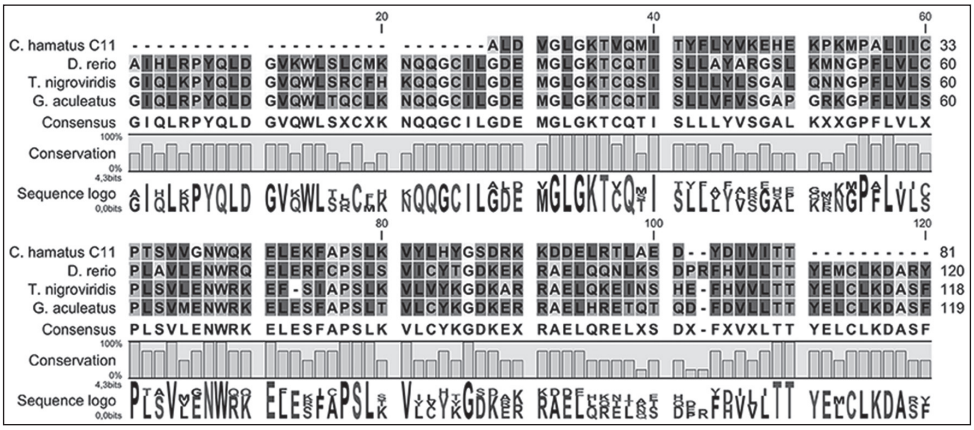

Figure 2. Alignment of the N-terminal portion of a Chromodomain-helicase-DNA-binding protein 1-like (CHD1) with the homologous CHD1 sequences of fish: *Danio rerio*, *Tetraodon nigroviridis*, and *Gasterosteus aculeatus*.

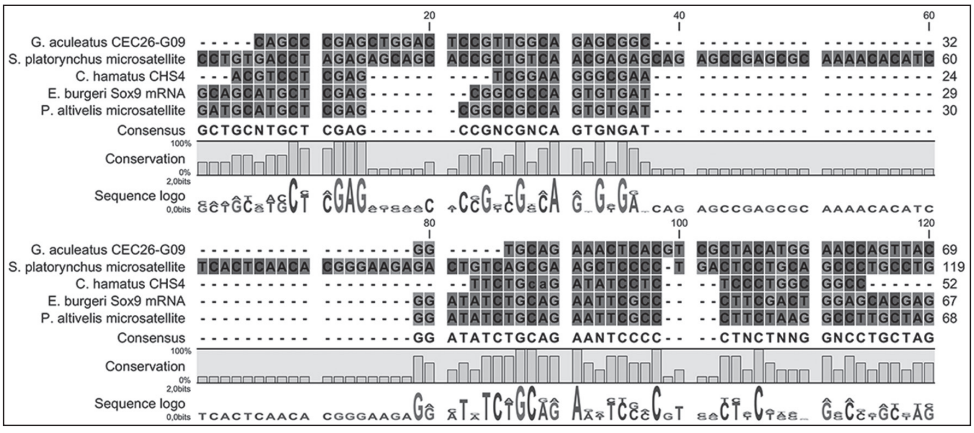

Figure 3. Repetitive sequence of clone CHS4 aligned with several loci of *Plecoglossus altivelis*, *Gasterosteus aculeatus*, *Scaphirhynchus platorynchus* and *Eptatretus burgeri*. Notably, in *E. burgeri* the sequence is found in Sox9.

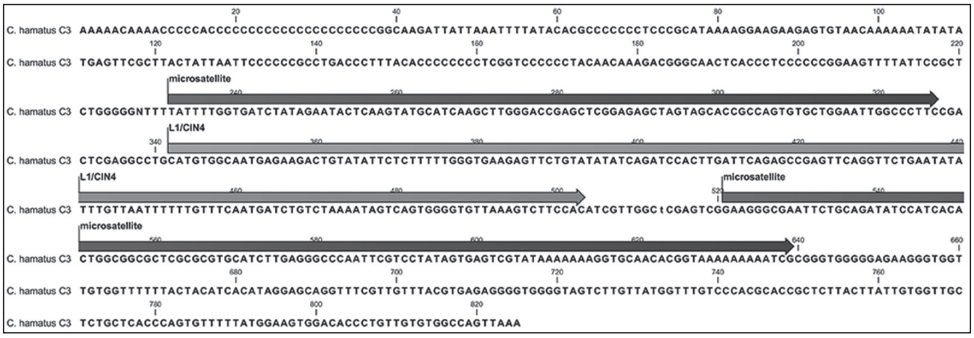

Figure 4. Structure of clone C3: the partial LINE (position 341–504 bp) is bordered by microsatellite regions both upstream (232–325 bp) and downstream (520–640 bp).

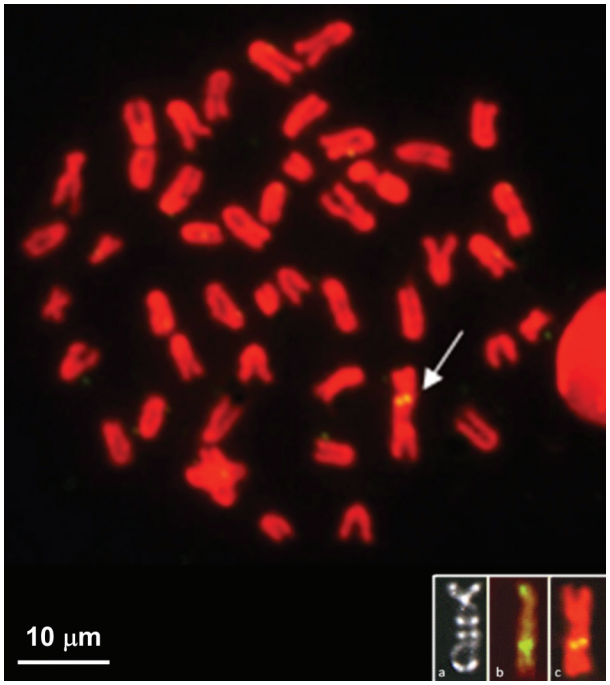

Figure 5. *In situ* hybridization of the C3 probe (CIN1/L4) to the *Ch. hamatus* male karyotype: the probe clearly maps interstitially on the q arm of the Y chromosome. Insert: **a** C-banded and chromomycin-stained Y chromosome of *Ch. hamatus* and **b** FISH performed using the Tc1-like DNA transposon as probe (modified from fig. 5 of Capriglione et al. 2002) compared to **c** C3 hybridized Y chromosome.

ed amino acid translation was found to be homologous to an N-terminal portion of a Chromodomain-helicase-DNA-binding protein 1-like (CHD1) (The European nucleotide archive: at http://www.ebi.ac.uk/ena/data/view/LM999957), a conserved protein with a putative role in chromatin architecture (Fig. 2).

RepeatMasker analysis allowed identification in clone CHS4 of a microsatellite strand whose sequence is conserved in other teleost species. FASTA and BLAST analysis

showed that our sequence aligned with several loci in the genome of *Plecoglossus altivelis* (Temminck & Schlegel, 1846), *Gasterosteus aculeatus* (Linnaeus, 1758), *Scaphirhynchus platorynchus* (Rafinesque, 1820), and *Eptatretus burgeri* (Girard, 1855). Notably, in *E. burgeri* the sequence is found in Sox9 mRNA for Sry-box protein 9 (Ota et al. 2007) (Fig. 3). Moreover, a similar sequence is found in the ATP-binding cassette of *Trematomus newnesi* (Boulenger, 1902), another notothenioid species.

In two other clones, C3 (825 bp) and C5 (244 bp), putative ancient LINE (Long Interspersed Element) fragments (non-LTR retrotransposon of the L1/CIN4 family) were found.

In clone C3 the partial LINE (position 342–503 bp) is bordered by repetitive regions both upstream (232–327 bp) and downstream (521–639 bp) (Fig. 4). FISH disclosed that the clone clearly mapped interstitially on the q arm of the Y chromosome (Fig. 5).

Discussion

Chromosome microdissection provides valuable chromosome information on lower vertebrate, fish, amphibian, and reptilian genomes, in which chromosome identification is hampered by difficulties in obtaining specific chromosome markers, and detection of syntenic regions is prevented by the lack of sequenced genomes.

Ch. hamatus Y-chromosome paint hybridized strongly to a single acrocentric chromosome pair, perhaps X2X2, in the female karyotype. The signal localization on the pericentromeric region suggests that, at least in this species, sequences, which are still found on the Y chromosome, might have been accumulated on the female X_2X_2 pair after sex chromosome emergence.

Because the heteromorphic Y is probably the result of a tandem fusion of autosomes and morphologically undifferentiated sex chromosomes, this event may have been followed by a reduction in recombination and by formation of new linkage groups between genes that were originally found on different chromosomes, including sexually antagonistic ones (Cioffi et al. 2012, Yano et al. 2014).

The interstitial chromomycin-positive heterochromatic region of the q arm, corresponding to the region involved in the tandem fusion event (Morescalchi et al. 1992), seems to be a good candidate for the breakpoint of rearrangement, also due to the presence of a selective, preferential accumulation of repetitive sequences and transposable elements.

In a previous paper we mapped a mobile element belonging to the Tc1/mariner DNA transposon family on the q arm of the Y chromosome (Capriglione et al. 2002). The L1/CIN4 LINE isolated from the present Y-specific mini-library is also located interstitially on the long arm of the Y chromosome and might be part of this segment. These multiple ancient L1 lineages, which are occasionally found in the majority of teleost genomes, might represent the ancestral state of vertebrate L1 and are often still active (Furano et al. 2004). These elements are known to provide the machinery for retro-duplication, promoting the trafficking of particular genes on and off the sex chromosomes (Geschwend et al. 2012). Their location suggested that these mobile

elements and, perhaps, other as yet unidentified elements, may have constituted a hotspot for chromosome rearrangement and Y shaping, playing a role in the induction of chromosome rearrangement and the appearance of the Y chromosome in *C. hamatus*. This Y-specific repetitive sequence could also be involved in Y chromosome differentiation in *C. hamatus*, since differential accumulation of repeats, i.e. microsatellites, is one of the earliest stages of sex-chromosome differentiation (Jones and Singh 1985, Arnemann et al. 1986, Schlötterer 2000, Kubat et al. 2008, Pokornà et al. 2011).

Repetitive sequences preserved in teleost species have helped to identify conserved genetic synteny and measure the degree and patterns of sex-chromosome differentiation in comparative genomic studies of fish (Nanda et al. 1990, Brunelli et al. 2008, Cioffi et al. 2011, Shikano et al. 2011).

The microsatellite sequence isolated from the *C. hamatus* Y chromosome in our study appears to be conserved. In addition, it aligned with several loci in the genome of ancient and modern fishes, including *T. newnesi*, an Antarctic notothenioid.

The presence of Y chromosome-specific microsatellites is well established in literature, mainly in mammals and the variability of their loci has been widely exploited to generate haplotypes for phylogenetic and forensic analysis (Goldstein et al. 1995, Sun et al. 2009). In lower vertebrates this correlation is less clear, even though the accumulation of Bkm repeats containing tandem arrays of GATA tetranucleotides on the degenerated W chromosomes of advanced snakes is one of the most extensively studied events of sex chromosome differentiation.

In fish, comparative genomic studies have shown that some of these microsatellite sequences, preserved in different teleost species, can help to examine conserved genetic sinteny, to clarify phylogeny as well as to measure the degree and patterns of sex chromosome differentiation (Brunelli et al. 2008, Cioffi et al. 2011, Yano et al. 2014).

Finally, although the present data do not allow any hypothesis to be advanced, it is intriguing that in *E. burgeri* this sequence is found in Sox9 mRNA for Sry-box protein 9, which together with steroidogenic factor 1 regulates transcription of the anti-Muellerian hormone (AMH) gene (Ota et al. 2007).

With regard to the partial *CHD1* gene sequences found in one of our Y clones, two *CHD1* gene homologues have been described in birds, which are characterized by ZW female heterogamety. In birds these homologues are associated with the heteromorphic sex-chromosome pair, one being W-linked and the other Z-linked (Fridolfsson and Hellegren 2000). However, *CHD1* genes have not been found to be sex-linked in eutherian mammals or in other organisms.

Even though the SDR has been identified in some teleost species (Froschauer et al. 2002, Volff and Schartl 2002, Volff et al. 2003), little is known about the loss and gain of fish genes involved in the molecular dynamics of the sex-specific region of sex chromosomes. The mechanisms controlling sex determination and differentiation in lower vertebrates remain unclear, since different genes seem to be involved, depending on species. For this reason it is difficult to say whether the partial *CHD1* homologue isolated in the *Ch. hamatus* Y mini-library might belong to the Y chromosome regions linked to sex differentiation. Further sequencing studies are needed to establish this.

Conclusions

The data seem to confirm that sex chromosomes arose independently in distant taxa several times during fish radiation (Matsubara et al. 2006, Charlesworth and Mank 2010). In *Ch. hamatus*, interspersed repetitive sequences and transposable elements seem to have played a pivotal role in promoting the first steps of chromosome rearrangement and the origin of the Y chromosome. In contrast, the present data do not allow ascribing the presence of the *CHD1* gene, related to the sex chromosomes only in avian genomes, to the Y-chromosome SDR. Of course, this finding may merely be the result of convergent evolution. The repetitive sequences isolated in this study may be a starting point to sequence the entire male-specific region of *Ch. hamatus*, and further research may provide additional data on Y-chromosome gene linkage and genome organization.

Acknowledgements

This work was supported by the Italian M. U. R. S. T. (Ministry of the University and Scientific and Technological Research)-PRIN 2005.

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CompCytogen 9(1): 17–29 (2015)
doi: 10.3897/CompCytogen.v9i1.8488
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Cytogenetic study of heptapterids (Teleostei, Siluriformes) with particular respect to the *Nemuroglanis* subclade

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Academic editor: *R. Noleto* | Received 25 August 2014 | Accepted 7 November 2014 | Published 5 February 2015

http://zoobank.org/6C02C43E-8F05-45E1-87EA-5F676F348AF2

Citation: Kantek DLZ, Peres WAM, Moreira-Filho O (2015) Cytogenetic study of heptapterids (Teleostei, Siluriformes) with particular respect to the *Nemuroglanis* subclade. Comparative Cytogenetics 9(1): 17–29. doi: 10.3897/CompCytogen.v9i1.8488

Abstract

The catfish family Heptapteridae (order Siluriformes) is endemic to the Neotropics and is one of the most common of the fish families in small bodies of water. Although over 200 species have been identified in this family, very few have been characterized cytogenetically. Here, we analyze the chromosome genomes of four species of Heptapteridae: *Cetopsorhamdia iheringi* (Schubart & Gomes, 1959), 2n = 58, comprising 28 metacentric (m) + 26 submetacentric (sm) + 4 subtelomeric (st) chromosomes; *Pimelodella vittata* (Lütken, 1874), 2n = 46, comprising 16m + 22sm + 8st; *Rhamdia* prope *quelen* (Quoy & Gaimard, 1824), 2n = 58 comprising 26m + 16sm + 14st + 2 acrocentric; and *Rhamdiopsis* prope *microcephala* (Lütken, 1874), 2n = 56, comprising 12m + 30sm + 14st. The nucleolus organizer regions (NORs) were located in a single chromosome pair in all species. The two species that belonged to the subclade *Nemuroglanis*, *C. iheringi* and *R.* prope *quelen*, had a diploid chromosome number of 58 and an interstitial NOR adjacent to a C^+ block located on one of the larger chromosome pairs in the complement. Our results from conventional cytogenetic techniques in combination with FISH using 18S and 5S rDNA probes corroborated the taxonomical hypothesis for the formation of the *Nemuroglanis* subclade.

Keywords

Siluriformes, Heptapteridae, chromosomes, 5S and 18S rDNA, cytotaxonomy

Introduction

In recent years, various classification changes have led to the current taxonomic status of the catfish family Heptapteridae. Lundberg et al. (1991a, b) suggested the division of the family Pimelodidae into the subfamilies Pimelodinae, Pseudopimelodinae, and Rhamdiinae. Subsequently, on the basis of phylogenetic studies in the Siluriformes, Pinna (1998) elevated the subfamily Rhamdiinae to the level of a family, Rhamdiidae. Bockmann and Guazzelli (2003) later established the family Heptapteridae instead of Rhamdiidae; this family includes 24 genera and 189 valid species (Ferraris 2007) of small fish, commonly known as "bagres" or "mandis". These fish are characterized by a long adipose fin, three pairs of barbels, an elongated body, and a grayish body color. They are endemic to the Neotropics and have a wide distribution in the water courses of Central and South America, with many species distributed in areas of ichthyological endemism. They live on the bottom of small and medium rivers, at low to medium depths, and are usually solitary with nocturnal habits (Bockmann and Guazelli 2003).

Subclades of Rhamdiinae (= Heptapteridae) have been identified in phylogenetic analyses of morphological data (Ferraris 1988, Lundberg et al. 1991a, Bockmann 1994): *Rhamdia* (Bleeker, 1858) and *Pimelodella* (Eigenmann & Eigenmann, 1888) are assigned to a basal group; while *Cetopsorhamdia* (Eigenmann & Fisher, 1916) and *Rhamdiopsis* (Haseman, 1911) have been placed in the *Nemuroglanis* subclade.

The diploid chromosome number in the Heptapteridae varies from 2n = 42 in *Imparfinis hollandi* (Haseman, 1911) (Margarido and Moreira-Filho 2008) to 2n = 58 in many other species. The latter chromosome number is the most frequent and is also considered a plesiomorphic character (Swarça et al. 2007, Borba et al. 2011). The karyotypes of heptapterid species comprise mainly metacentric and submetacentric chromosomes (see below) suggesting that pericentric inversions were more frequent than centric fissions in the evolution of the family. Nucleolus organizer regions (NORs) are usually present on one chromosome pair, and may be terminal or interstitial. These data suggest that extensive chromosomal rearrangements were involved in speciation within this group (Swarca et al. 2007). The reduction in diploid number may have been produced by successive chromosome fusions with deletions and inversions, such as those responsible for NOR position variation among species. B chromosomes are present in some species and are considered to be of recent origin, and without phylogenetic implications (Borba et al. 2011).

The presence of an interstitial NOR, which is usually located on the largest chromosome pair of the complement and adjacent to a C^+ block, and the predominance of 2n = 58, are all cytogenetic characters strongly associated with the *Nemuroglanis* subclade (Kantek et al. 2009).

As there have been relatively few cytogenetic studies in the Heptapteridae, and because of the need to obtain further data to substantiate proposals on the cytotaxonomy of the family (Borba et al. 2011), the present study was undertaken to provide the first analysis, to our knowledge, of the karyotype of *Pimelodella vittata* (Lütken, 1874). We also used various cytological methods to analyze three other heptapterid species and compared the new data with those previously published to examine the cytotaxonomy of this family.

Material and methods

Specimens of four heptapterid species were collected from the Minhocas stream (S20°31'55.2", W046°02'42.1"), a tributary of the Piumhi river (Minas Gerais state): nine (seven males and two females) *Cetopsorhamdia iheringi* (Schubart & Gomes, 1959) (MNRJ 31477); six (five males and one female) *P. vittata* (MNRJ 29330); 10 (five males, four females and one of an undetermined sex) *Rhamdia* prope *quelen* (Quoy & Gaimard, 1824) (MNRJ 29329, MNRJ 29326); and 18 (eight males, seven females and three of undetermined sex) of *Rhamdiopsis* prope *microcephala* (Lütken, 1874) (MNRJ 29325).

Mitotic metaphase preparations were made as described by Bertollo et al. (1978). Chromosome morphologies were assigned using the arm size ratio criteria proposed by Levan et al. (1964). Heterochromatin was identified by C-banding (Sumner 1972) and NORs were detected by silver nitrate staining (Howell and Black 1980). Metaphase preparations analyzed after conventional staining (Giemsa) were also subjected to C-banding, allowing the assemblage of sequential karyotypes.

The 18S and 5S rDNA sites on the chromosomes were located by the fluorescence *in situ* hybridization (FISH) technique (Pinkel et al. 1986), with a stringency of 77%, using probes obtained from *Prochilodus argenteus* (Agassis, 1829) (Hatanaka and Galetti Jr 2004) and *Leporinus elongatus* (Valenciennes, 1850) (Martins and Galetti Jr 2001), respectively. The two probes were labeled with 14-dATP-biotin through nick translation in accordance with the manufacturer's instructions (Bionick Labelling System, Invitrogen). Chromosomes were counterstained with DAPI (0.2 mg/ml) and analyzed using an Olympus BX50 epifluorescence microscope. Image-Pro Plus software (Media Cybernetics) was used for image capture.

Results

Cetopsorhamdia iheringi

Cells from all *C. iheringi* specimens had 2n = 58 and a karyotypic formula of 28 metacentric (m), 26 submetacentric (sm) and 4 subtelocentric (st) chromosomes (Fig. 1a), with no evidence of heteromorphic sex chromosomes.

Silver staining showed that the NOR was located interstitially on the short arm of pair 1, and formed a secondary constriction (Fig. 1a box). Constitutive heterochromatin was present in the pericentromeric regions of several chromosome pairs (Fig. 1b) in addition to visible C^+ blocks in the NOR-bearing pair (Fig. 1a, b).

FISH with the 18S rDNA probe confirmed that the NOR was located interstitially on the short arm of pair 1 (Fig. 4a). FISH using the 5S ribosomal probe revealed the existence of a large number of these sequences on the NOR-bearing chromosomes, covering a large part of the chromosomes above and below the 18S ribosomal sites. There was synteny between the 18S and 5S rDNAs (Fig. 4b, c).

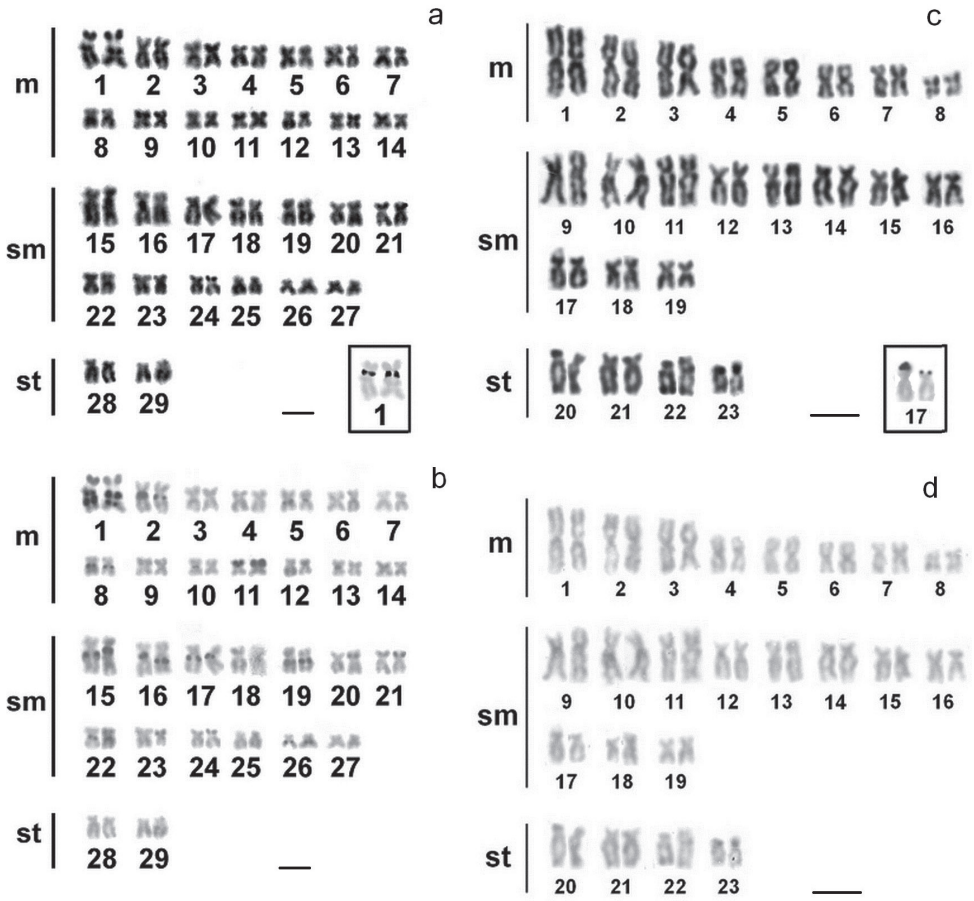

Figure 1. Karyotypes of *Cetopsorhamdia iheringi* (**a, b**) and *Pimelodella vittata* (**c, d**) after sequential Giemsa staining (**a, c**), C- banding (**b, d**) and Ag-NOR staining (boxes). Bar = 10 μm.

Pimelodella vittata

All cells from *P. vittata* specimens had 2n = 46 and a karyotypic formula of 16m, 22sm and 8st chromosomes (Fig. 1c), with no evidence of heteromorphic sex chromosomes.

Silver staining located the NORs to the terminal region of the short arm of pair 17, where they formed a secondary constriction (Fig. 1c box). It was possible to see weak C^+ bands close to the centromeres in some chromosomes (Fig. 1d).

FISH using the 18S rDNA probe confirmed the NOR location (Fig. 4d). Only one 5S rDNA locus was present in *P. vittata* in the terminal region of a submetacentric/subtelocentric chromosome pair (Fig. 4e). The 18S and 5S rDNA loci were not on the same pair of chromosomes (Fig. 4e, f).

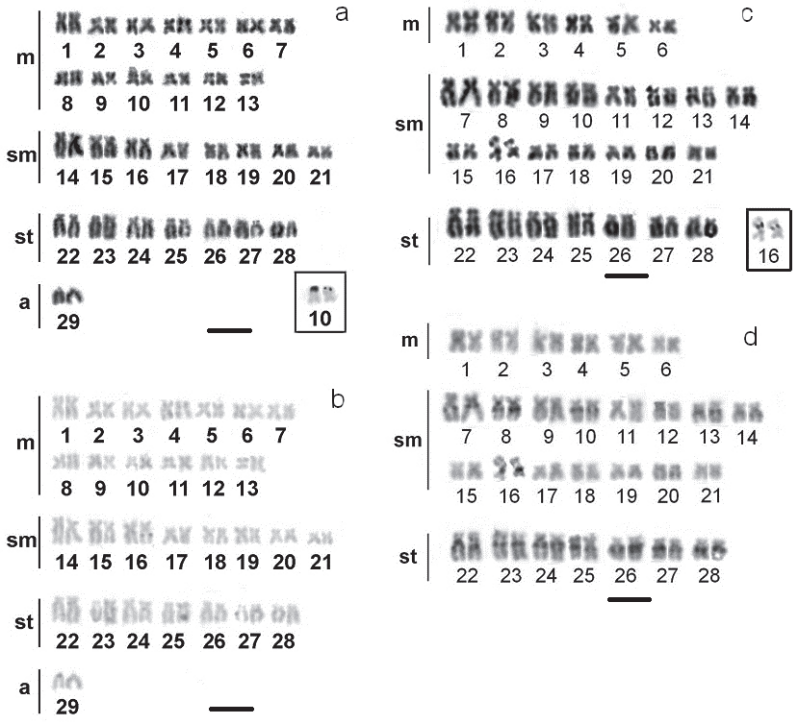

Figure 2. Karyotypes of *Rhamdia* prope *quelen* (**a, b**) and *Rhamdiopsis* prope *microcephala* (**c, d**) after sequential Giemsa staining (**a, c**), C- banding (**b, d**) and Ag-NOR staining (boxes). Bar = 10 μm.

Rhamdia prope quelen

Cells from all specimens, apart from one, had 2n = 58 and a karyotypic formula of 26m, 16sm, 14st and 2 acrocentric chromosomes (Fig. 2a), with no evidence of heteromorphic sex chromosomes. One triploid specimen with 3n = 87 was found (Fig. 3).

Silver staining indicated the NOR was located in the terminal region of chromosome pair 10, where it formed a secondary constriction (Fig. 2a box). The chromosomes did not show any heterochromatic segments (Fig. 2b).

FISH using the 18S rDNA probe hybridized to the same region as the Ag-NOR (Fig. 5a, c). Only one 5S rDNA locus was identified; this was located at an interstitial position on a submetacentric chromosome pair (Fig. 5b, d).

Rhamdiopsis prope microcephala

Cells from all specimens had 2n = 56 and a karyotypic formula of 12m, 30sm and 14st chromosomes (Fig. 2c), with no evidence of heteromorphic sex chromosomes.

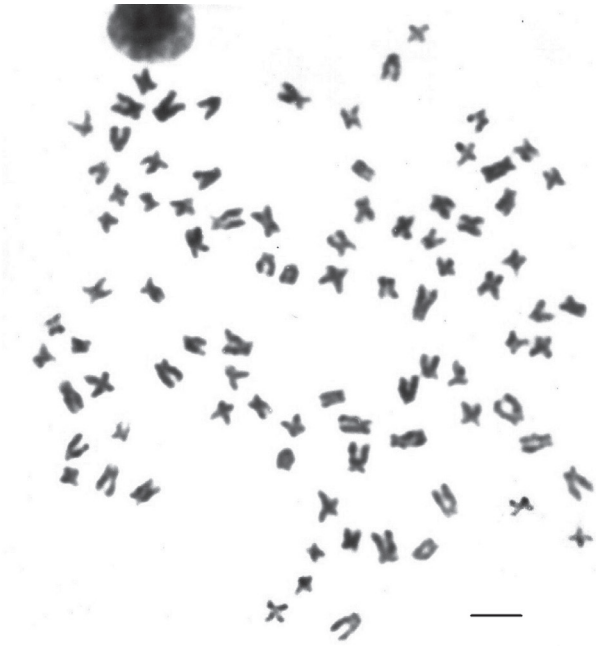

Figure 3. Metaphase of the triploid specimen of *Rhamdia* prope *quelen*. Bar = 10 μm.

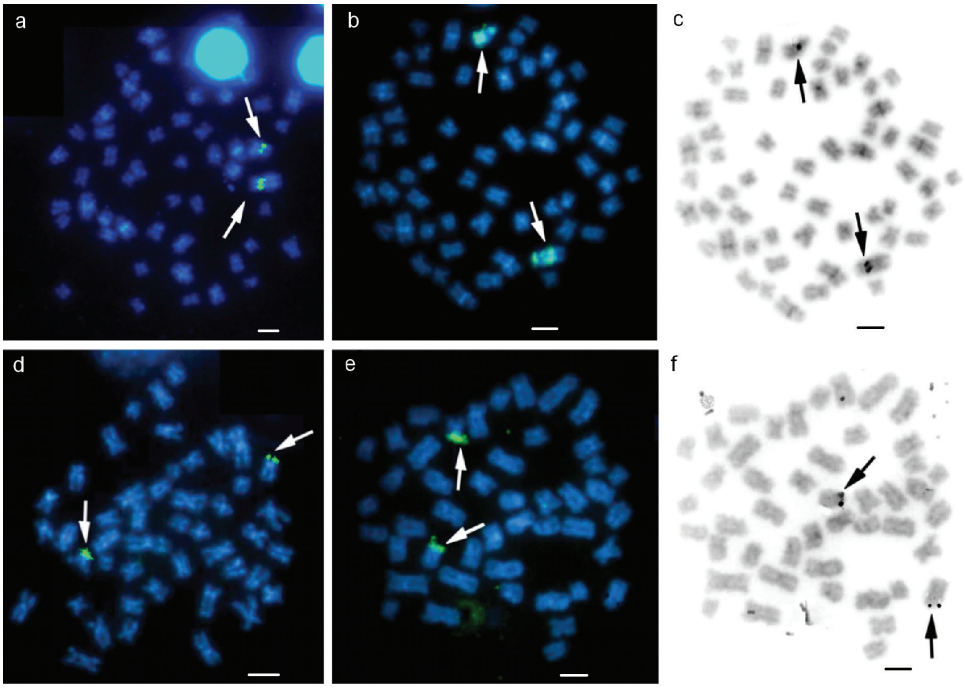

Figure 4. Metaphases of *Cetopsorhamdia iheringi* (**a, b, c**) and *Pimelodella vittata* (**d, e, f**) subjected to fluorescence *in situ* hybridization (FISH) with an 18S rDNA probe (**a, d**) and 5S rDNA (**b, e**). The metaphases shown after Ag-NOR staining (**c, f**) are the same as those used for 5S FISH. Bar =10 μm.

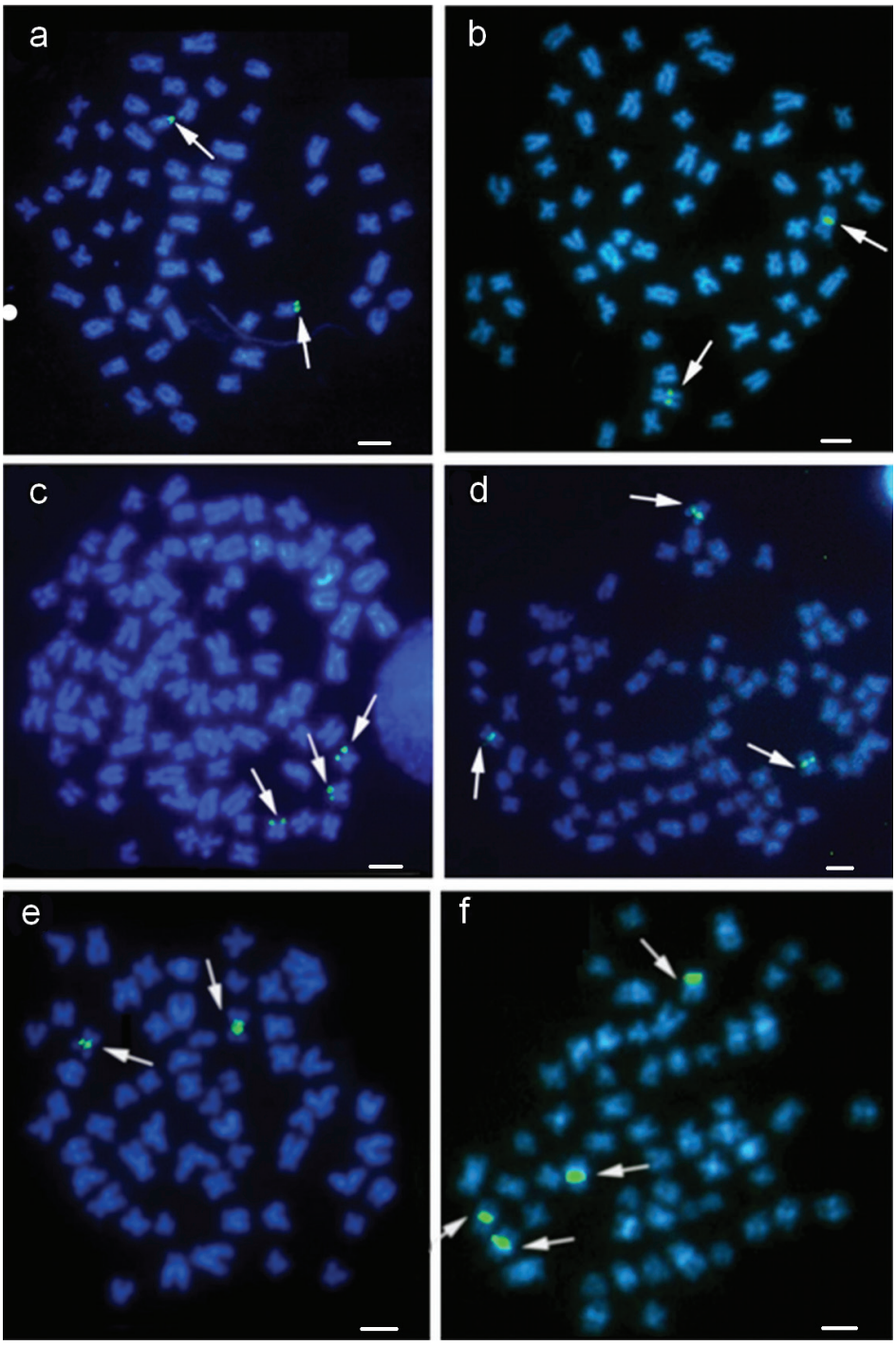

Figure 5. Metaphases of *Rhamdia* prope *quelen* (**a, b, c, d**) and *Rhamdiopsis* prope *microcephala* (**e, f**) subjected to fluorescence *in situ* hybridization with an 18S rDNA probe (**a, c, e**) and 5S rDNA (**b, d, f**). Metaphases c and d belong to the triploid specimen. Bar =10 μm.

Silver staining indicated the NOR was located in an interstitial region of chromosome pair 16, where it formed a secondary constriction (Fig. 2c box). Constitutive heterochromatin was present in the pericentromeric regions of several chromosome pairs (Fig. 2d).

FISH using the 18S rDNA probe hybridized to the same region as the Ag-NOR (Fig. 5e). Two 5S rDNA loci were identified at a terminal position on a submetacentric/subtelocentric chromosome pair (Fig. 5f).

Discussion

The diploid chromosome number of 58 in *C. iheringi* and *R.* prope *quelen* is the most common karyotype number in the family Heptapteridae (Fenocchio and Bertollo 1990, Vissotto et al. 1999, Vissotto et al. 2001, Stolf et al. 2004, Kantek et al. 2009, Borba et al. 2011). The karyotype of 2n = 46 observed here in *P. vittata* is the same as reported for some other *Pimelodella* spp. (Dias and Foresti 1993, Vasconcelos and Martins-Santos 2000, Garcia and Almeida-Toledo 2010), *P. avanhandavae* (Eigenmann, 1917) (Vissoto et al. 1999), *P. meeki* (Eigenmann, 1910) (Vidotto et al. 2004, Garcia and Almeida-Toledo 2010, Borba et al. 2011, Gouveia et al. 2012), *P. boschmai* (Van der Stigchal, 1964) (Garcia and de Almeida-Toledo 2010) and *P. gracilis* (Valenciennes, 1836) (Garcia and de Almeida-Toledo 2010). Other *Pimelodella* species have different diploid chromosome numbers (Vasconcelos and Martins-Santos 2000, Swarça et al. 2003, Garcia and de Almeida-Toledo 2010).

The identification of a triploid specimen (3n = 87) in *R.* prope *quelen* is not unusual; indeed, three other cases have already been reported for *Rhamdia* (Swarça et al. 2007, Tsuda et al. 2010). The fertilization of a non-reduced (diploid) gamete by a reduced (haploid) gamete, such as an ovule (2n) by a sperm (n), is the most probable origin of these specimens (Morelli et al. 1983, Kantek et al. 2007).

The *Nemuroglanis* subclade is characterized by the presence of an interstitial NOR adjacent to a C^+ block and the predominance of 2n = 58; these characteristics are present in the analyzed species from the genus *Cetopsorhamdia* (Vissoto et al. 1999 and present study), *Taunayia bifaciata* (Eigenmann & Norris, 1900) (Borba et al. 2011) and in five species of the genus *Imparfinis* (Eigenmann & Norris, 1900) (Kantek et al. 2009, Borba et al. 2011, Gouveia et al. 2012). If 2n = 58 is a plesiomorphic trait of the Heptapteridae family (Borba et al. 2011), then the reduction to 2n = 56 might indicate synapomorphy, grouping *I.* prope *piperatus* (Vissoto et al. 2001, Fenocchio et al. 2003), *R.* prope *microcephala* (present study) and *R. microcephala* (Lütken, 1874) (Fonseca et al. 2003). The hypothesis is supported by the presence of an interstitial NOR located on chromosomes that are not metacentric and not the largest in the karyotype of these species. The species *Phenacorhamdia tenebrosa* (Schubart, 1964), which belongs to the *Nemuroglanis* subclade, also has 2n = 58 (Borba et al. 2011), but no interstitial NOR. Since 2n = 58 is considered the basal number for Heptapteridae (Fenocchio et al. 2003, Borba et al. 2011), and the species *Imparfinis borodini* (Mees

& Cala, 1989) (Vissoto et al. 1999), *I. hollandi* (Margarido and Moreira-Filho 2008) and *Heptapterus mustelinus* (Valenciennes, 1835) (Yano and Margarido 2012) have a reduced diploid number (2n = 52, 2n = 42 and 2n = 54, respectively), it is possible that Robertsonian translocations were responsible for the karyotypic changes.

The C-banding and Ag-NOR patterns of *Rhamdia* and *Pimelodella* species (Swarça et al. 2007, Borba et al. 2011, Gouveia et al. 2012) are distinctly different from most taxa of the *Nemuroglanis* subclade that have been analyzed. The existence of cytogenetic characteristics that separate the recognized groups of Heptapteridae was initially proposed by Fenocchio et al. (2003). Thus, for example, the interstitial C^+ band pattern is a more common feature of species of the *Nemuroglanis* subclade, such as *C. iheringi* and *R.* prope *microcephala* (Fig. 1b, 2d, respectively). Other species of the family Heptapteridae that do not belong to this subclade, such as *P. vittata* (Fig. 1d) and *R.* prope *quelen* (Fig. 2b), have different patterns of heterochromatin distribution (Swarça et al. 2007, Garcia et al. 2010, Garcia and Almeida-Toledo 2010). However, as the majority of heptapterid species have not been studied cytogenetically studied, then it is difficult to elaborate broader proposals.

Another cytogenetic characteristic that may be diagnostic of the *Nemuroglanis* subclade is the synteny between 18S and 5S rDNA. Up until now, only *Imparfinis schubarti* (Gomes, 1956) (Kantek et al. 2009) and *C. iheringi* (present study) have been found to show this characteristic. Other genera in the Heptapteridae that do not belong to the *Nemuroglanis* subclade, such as *Pimelodella* and *Rhamdia* (Garcia et al. 2003, Garcia et al. 2010, present study), do not show this synteny. However, as *R.* prope *microcephala* did not have this characteristic, then the association between 5S and 18S rDNA might be a synapomorphy, shared by the group of species in the *Nemuroglanis* subclade that have a 2n = 58 karyotype and possess interstitial NORs on the largest chromosome pair of the complement.

The 5S ribosomal gene consists of multiple copies of a highly conserved 150 base pair sequence, separated by highly variable non-transcribed spacers (Williams and Strobeck 1985). These variable sequences, which were caused by insertions/deletions, mini-repetitions and pseudogenes, are useful for evolutionary studies and serve as population markers for many organisms, including plants (Zanke et al. 1995), mammals (Suzuki et al. 1994) and fishes (Martins et al. 2002). Variations in these spacers have also been detected in some neotropical fishes, such as *Leporinus* (Martins and Galetti Jr 2001) and *Brycon* (Wasko et al. 2001). A comparison of the outcome of analysis of *I. schubarti* (Kantek et al. 2009) and *C. iheringi* indicates that despite the relative evolutionary proximity of the species (both belong to the *Nemuroglanis* clade), and the likely localization of these sequences to homeologous chromosomes, there is nevertheless considerable differences in the signals obtained with the 5S rDNA probe. The large 5S rDNA blocks on *C. iheringi* chromosomes presumably originated through duplication of the 5S rDNA of an ancestral species close to these taxa. Other species of heptapterids considered more basal in the family, such as species belonging to the genera *Rhamdia* and *Pimelodella*, have only small 5S rDNA signals; this suggests that the presence of the large 5S rDNA block in *C. iheringi* is an apomorphic character. Based on the supposed homogeneity among 5S rDNA repeats,

several studies have proposed that 5S rDNA is subject to concerted evolution (Arnheim 1983), where duplicated gene family members evolve as a single unit that undergoes a high degree of homogenization (as a unit in concert) (Pinhal et al. 2011).

Prior to this study, variability in the number and location of 5S ribosomal genes has been reported among Siluriformes (Kavalko et al. 2004) except for *Rhamdia* (Garcia et al. 2010). The analyses here confirm the variability observed by other authors, and also the conservation of 5S rDNA in *Rhamdia*.

Until now, only the genera *Imparfinis*, *Cetopsorhamdia*, *Heptapterus*, *Phenacorhamdia*, *Rhamdiopsis*, *Pimelodella*, *Rhamdia*, and *Taunayia* had been cytogenetically analyzed; these represent only eight of the 24 genera in the family Heptapteridae (Yano and Margarido 2012). The first five belong to the subclade *Nemuroglanis*. More studies involving this family may assist in the elucidation of cytotaxonomy and chromosome evolution in this family.

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CompCytogen 9(1): 31–50 (2015)
doi: 10.3897/CompCytogen.v9i1.8535
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

High chromosomal variation in wild horn fly *Haematobia irritans* (Linnaeus) (Diptera, Muscidae) populations

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Academic editor: *V. Gokhman* | Received 1 September 2014 | Accepted 4 December 2014 | Published 5 February 2015

http://zoobank.org/9E9D5E84-2782-4D37-9909-960BCDD0A118

Citation: Forneris NS, Otero G, Pereyra A, Repetto G, Rabossi A, Quesada-Allué LA, Basso AL (2015) High chromosomal variation in wild horn fly *Haematobia irritans* (Linnaeus) (Diptera, Muscidae) populations. Comparative Cytogenetics 9(1): 31–50. doi: 10.3897/CompCytogen.v9i1.8535

Abstract

The horn fly, *Haematobia irritans* is an obligate haematophagous cosmopolitan insect pest. The first reports of attacks on livestock by *H. irritans* in Argentina and Uruguay occurred in 1991, and since 1993 it is considered an economically important pest. Knowledge on the genetic characteristics of the horn fly increases our understanding of the phenotypes resistant to insecticides that repeatedly develop in these insects. The karyotype of *H. irritans*, as previously described using flies from an inbred colony, shows a chromosome complement of 2n=10 without heterochromosomes (sex chromosomes). In this study, we analyze for the first time the chromosome structure and variation of four wild populations of *H. irritans* recently established in the Southern Cone of South America, collected in Argentina and Uruguay. In these wild type populations, we confirmed and characterized the previously published "standard" karyotype of 2n=10 without sex chromosomes; however, surprisingly a supernumerary element, called B-chromosome, was found in about half of mitotic preparations. The existence of statistically significant karyotypic diversity was demonstrated through the application of orcein staining, C-banding and H-banding. This study represents the first discovery and characterization of horn fly karyotypes with 2n=11 (2n=10+B). All spermatocytes analyzed showed 5 chromosome bivalents, and therefore, 2n=10 without an extra chro-

mosome. Study of mitotic divisions showed that some chromosomal rearrangements affecting karyotype structure are maintained as polymorphisms, and multiple correspondence analyses demonstrated that genetic variation was not associated with geographic distribution. Because it was never observed during male meiosis, we hypothesize that B-chromosome is preferentially transmitted by females and that it might be related to sex determination.

Keywords

Karyotypes, genetic variability, population structure, B-chromosome, H-banding, evolution, chromosomal rearrangements

Introduction

The economic importance of the horn fly, *Haematobia irritans* (Linnaeus, 1758) (Diptera: Muscidae) is based on its role as an obligate bloodsucking ectoparasite that plagues cattle around the world (Palmer and Bay 1981, Williams et al. 1985, Valério and Guimarães 1983). First reported in Argentina and Uruguay in 1991 (Luzuriaga et al. 1991, Carballo and Martínez 1991), it has been considered an economically important species in both countries, that can be found from the tropical North (22°02.22'S) to the temperate South (36°18.55'S) and between longitudes 64°33.67'W and 56°54'W (Anziani et al. 1993). Despite its economic importance, no information on the rate of eventual new invasions of this fly is available. In Argentina, the region called "Humid Pampa" has the most favourable conditions for the expansion of this insect and several generations per year, frequently produce sudden, extensive and damaging infestations of cattle (Bulman et al. 1999, Mancebo et al. 2001). Moreover, many local populations from Central and North Argentina demonstrated resistance to fenvalerate and pyrethroids (Torres et al. 1996, Sheppard and Torres 1998, Guglielmone et al. 2001), indicating significant changes in the descendants of the original invading stock that could make them even more invasive and destructive in the future. Insecticide resistance is one of the best examples of rapid microevolution found in nature (Silva et al. 2012) and has strong economical implications. Given the widespread presence of this pest in South Brazil and Paraguay (Sheppard and Torres 1998), and the prevalent cattle trade among these neighbouring countries, the horn fly population in Argentina is likely to be subjected to gene flow.

LaChance (1964) and Avancini and Weinzierl (1994) reported that *H. irritans* showed a karyotype of 2n=10 composed of five pairs of chromosomes without a heteromorphic pair of sex chromosomes. As an insect source, all these researchers examined the same laboratory colony maintained at the USDA Livestock Insects Research Laboratory which, in turn, was derived from flies collected in the field at Kerrville, Texas (USA) and reared at least from 1962 on. Apart from *H. irritans*, the only cyclorrhaphan Diptera having 2n=10 without a heteromorphic pair of chromosomes is *Muscina stabulans* (Fallen, 1817) (Parise et al. 1996, 2007). To our knowledge, no detailed cytological studies of wild horn flies are available.

Here we for the first time analyze wild populations of *H. irritans* which have recently established in the Southern Cone of South America and exhibit considerable

karyotypic diversity. The only related study is the analysis of genotypic variability in three horn fly populations from Brazil, Colombia and Dominican Republic, which was assessed by random amplification of polymorphic DNA (Gatto Brito et al. 2008). In addition, the mitochondrial genome of *H. irritans* was studied by Oliveira et al. (2008).

Materials and methods

Ethics statement

This study was carried out on private lands, with kind permission from the landowners. For data regarding permissions for field collecting, please contact the corresponding author. Furthermore, we previously confirmed that no official permission was required for this field work, as it did not involve any endangered or protected species.

Collection of adult horn flies and eggs

Sampling of adults from different populations of *H. irritans* feeding on cattle was performed from 2004 to 2010 on private lands at the following main stations in Argentina: Ferreyra in Córdoba Province (31°56.67'S; 61°01.0'W); Trancas in Tucumán Province (26°21.66'S; 65°03.0'W); and Bolivar in Buenos Aires Province (35°50.0'S; 64°01.0'W). Sampling of adult *H. irritans* from Uruguay was performed in Palmitas (33°25.0'S; 58°07.0'W). Under normal weather, populations of *H. irritans* develop in these countries from early spring (October) to the beginning of fall (late March) (Guglielmone et al. 2001, 2002, Tarelli 2004), with the average degree of infestation depending on the climate. Samples showing high mortality when transferred from the field to the laboratory were discarded.

Adults were collected on livestock with a sweep net and transferred using positive phototropism to cages with rags soaked with 0.05% sodium citrate-added bovine blood as food source (Filiberti et al. 2009). Females were allowed to oviposit on pieces of cloth impregnated with 8.5 g/l NaCl solution for 12±1 h at 30 °C. After hatching, first-instar larvae were transferred to batches of urine-free bovine feces for feeding. Larval development took place in an environmental chamber at 29±1 °C. The life cycle of *H. irritans* was partially described elsewhere (Basso et al. 2011).

Cytology

Mitosis and meiosis were studied in neuroblasts of sub-esophageal ganglia of third-instar larvae and in spermatocytes of pharate and freshly eclosed adults respectively. Unfortunately, it was logistically impossible to simultaneously dissect brains and gonads in the same larvae.

Preparation of ganglia

Ganglia were dissected in a drop of insect Ringer's solution (Hayes 1953) and transferred first to 1% sodium citrate solution for 10–15 minutes, and then to methanol/acetic acid (3:1) for 40 seconds. After that, using a slide previously soaked in chilled methanol, each ganglion was transferred into a drop of freshly prepared and chilled 60% glacial acetic acid. The ganglion was then disaggregated with a pair of needles and a micropipette to disperse cells. After that, the slide was placed three times on a hot plate (75 °C) for 3 sec, air-dried, dehydrated, and stored at -20 °C before use.

Preparation of testes

Testes of pharate adult males (120 h after puparium formation) and eclosed adults up to 36 h after emergence were used (Basso et al. 2011). The tissues were fixed and stained in a drop of lacto-acetic 2% orcein solution, covered with a cover slip and squashed 10 minutes later. Preparations with mitotic and meiotic metaphases were then sealed and stored at 6 °C before use.

Chromosome banding and idiograms

Mitotic chromosome spreads from cerebral ganglia were C-banded using $Ba(OH)_2$ treatment at 27–29 °C for 7 min and stained with 5% Giemsa Gurr (Merck, Germany) solution in phosphate buffer having pH=6.8 (Basso et al. 1995). H-banding was carried as described by Gatti et al (1976). The cytological preparations, described above, were re-hydrated with phosphate buffer having pH=7 (0.15 M NaCl, 0.03 M KCl and 0.01 M Na_2HPO_4) for 5 min. The slides were stained with 0.5 μg/ml Hoechst 33258 diluted in phosphate buffer during 10 min and then briefly rinsed with deionized water and air-dried. Mounting was performed in McIlvaine buffer with pH=7 (0.16 M dibasic sodium phosphate, 0.04 M sodium citrate). Preparations were kept in the dark during 24 hours before examination under a Zeiss Axioplan fluorescence microscope. Images were recorded with an Olympus DP72 digital camera, time exposure being manually adjusted. The relative chromosome length and centromere index were calculated after measurements taken both on preparations of ganglia and testes. The idiograms were drawn based on these measurements. Data were obtained from at least 10 metaphase plates per chromosome spread. Circa 1000 insects were dissected to obtain 287 individuals with good quality metaphases.

Statistical analysis

Multiple Correspondence Analysis (MCA) is a method of factorial analysis that transforms a set of categorical or qualitative variables into a small number of orthogonal

variables (Le Roux and Rouanet 2004). We grouped the individuals by seven karyotypic formulae. The variables that characterized the individuals were: the karyotypes classified into seven groups, the chromosome number (2n=10 or 2n=11), and the presence of satellites on each chromosome of the haploid karyotype (satellite [s1, s2, s3, s5] or absence of satellite). These 14 variables were used as active ones, while the geographical locations, i.e. Bolivar, Palmitas, Trancas and Ferreyra, were included as illustrative variables (Escofier and Pagés 1992, Lebart et al. 1995, 1996). Published data from Texas (LaChance 1964, Avancini and Weinzierl 1994) were also used in the analysis as illustrative individuals (n=24), i.e. as unique previous data to make comparisons. With the factorial axes from MCA, a hierarchical classification by Ward was imposed (Johnson and Wichern 1992). Data were processed using SAS software (2009) and SPAD3 (Lebart et al. 1996).

Results

Chromosomes of four wild horn fly populations from Argentina and Uruguay were studied by cytological analysis of mitotic and meiotic metaphases. Brains of 232 third-instar larvae from three locations (Ferreyra, n=134; Bolivar, n=25; Palmitas, n=73) and testes of 55 individuals from three locations (Ferreyra, n=16; Trancas, n= 14; Palmitas, n=25) were analyzed.

Brain cell preparations from 72 to 96-hour third-instar larvae with empty guts showed mitotic pro-metaphases, metaphases and anaphases. Somatic pairing was observed in metaphase plates as reported for other dipterans (Stevens 1908, Metz 1916, Joyce et al. 2012). We distinguished two types of chromosome numbers within the studied samples: 2n=10 and 2n=11. All spermatocytes analyzed showed only 5 chromosome bivalents, i.e. 2n=10. No extra chromosome was found. Preparations from ovaries did not allow for chromosomal analysis since only interphase nuclei at the karyosome stage were present.

Karyotype I: reference karyotype

Most specimens (n=150) were analyzed from Ferreyra. The most frequent chromosome number from this location was 2n=10, both on preparations from brains and testes. We arbitrarily defined the corresponding chromosome set as the reference karyotype (Karyotype I).

Karyotype I showed two metacentric chromosomes (pairs 2 and 5) and three submetacentric chromosomes (pairs 1, 3 and 4) (Fig. 1a–f and Table 1). Chromosome 2 had a distinctive secondary constriction (Fig. 1a–b) which is the location of the nucleolar organizer (not shown), and chromosome 3 carried a satellite (Fig. 1a–b and Table 1). Sex chromosomes were not identified since no heterochromosomes (XY) could be distinguished from the autosomes. In male meiosis, a satellite on chromosome 3 was clearly observed in some spermatocytes (Fig. 1e–f).

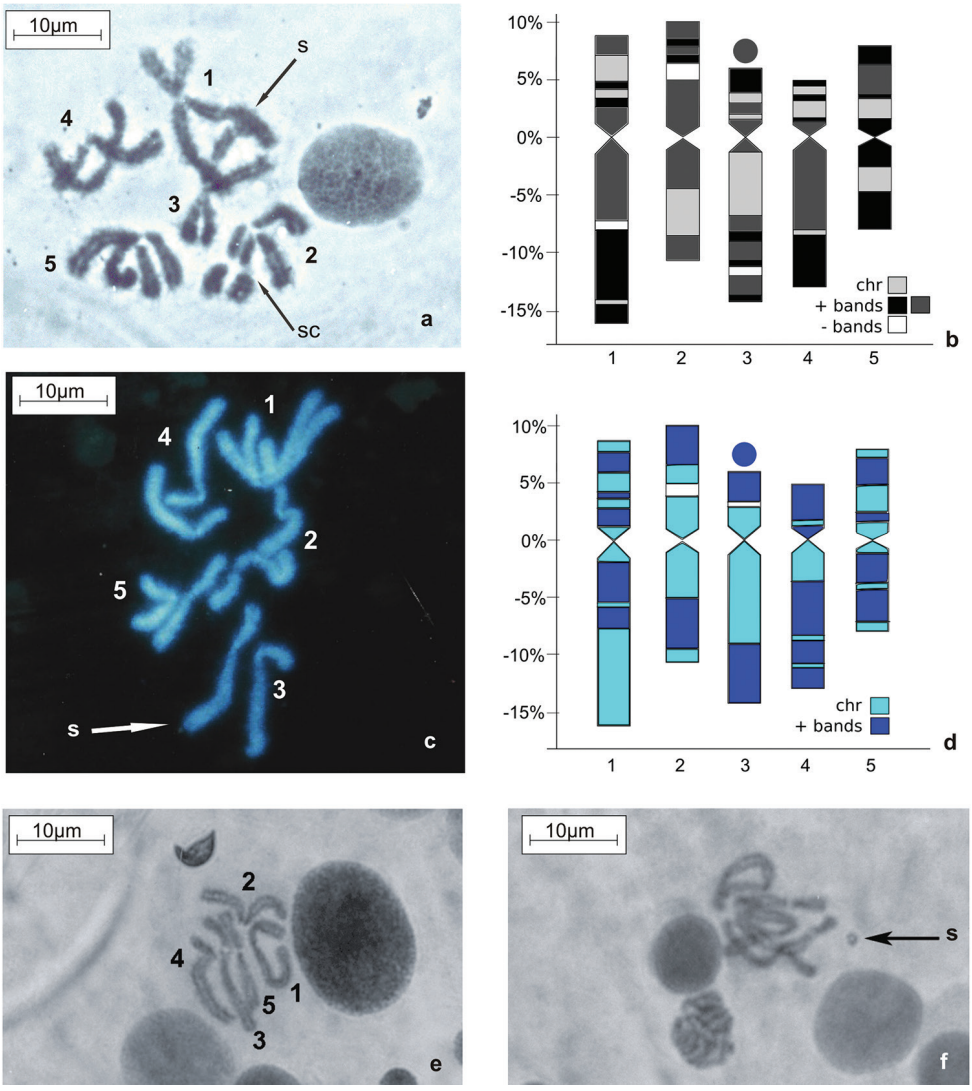

Figure 1. Reference karyotype of *H. irritans* with 2n=10. **a** Giemsa stained C-banded mitotic metaphase from larval brain; arrow indicates satellite on chromosome 3, sc: secondary constriction **b** Idiogram of the C-banded haploid set **c** Hoechst 33258 stained H-banded mitotic metaphase from larval brain **d** Idiogram of the H-banded set **e–f** Orcein stained meiotic metaphases II from testis; both figures were observed in the same individual. **a**, **c**, **f** arrow indicates satellite on chromosome 3. sc: secondary constriction on chromosome 2.

C-banding of karyotype I allowed for detection of satellites (Fig. 1a). The idiogram of C-banded haploid karyotype I is shown on Fig. 1b. Chromosome 1 carried a wide C-block on the long arm and one distal band on the short arm. Chromosome 2 showed a conspicuous C-negative band on one arm that marked a secondary constriction. The long arm of chromosome 3 showed three narrow distal marks and a secondary constric-

Table 1. Frequency distribution of karyotypes within populations of *H. irritans* from Argentina and Uruguay. sat: Chromosome carrying satellite; (a) grouping of karyotype formulae in the Multiple Correspondence Analysis.

A. Frequent karyotypes	Ferreyra n=150	Bolivar n=25	Trancas n=14	Palmitas n=98	(a)
Chromosome number 2n=10					
2M + 3SM					
Karyotype I (reference karyotype) 2M (2, 5) + 3SM (1, 3, 4); 3 sat	0.40	0.24	0	0.44	1
Karyotype II 1 sat	0.08	0	0.57	0.01	1
Karyotype III 2, 5 sat	0	0.24	0	0	1
Karyotype IV 3SM (2,3,4); 2 sat	0.02	0	0	0	2
1M + 4SM					
Karyotype V (inversion) 1M (2) + 4SM (1, 3, 4, 5); 1 sat	0.02	0	0.43	0.02	3
Chromosome number 2n = 11					
2M + 3SM + B					
Karyotype VI (translocation 1-5) 2M (1, 2) + 3SM (3, 4, 5) + B; 3 sat	0.32	0	0	0.43	4
1M + 4M/SM + B					
Karyotype VII (inversion) 1M (2) + 4M/SM (1, 3, 4, 5) + B; 2 sat	0.007	0.40	0	0	5
B. Rare karyotypes	Ferreyra n=150	Bolivar n=25	Trancas n=14	Palmitas n=98	
Chromosome number 2n=10					
5SM					
Karyotype VIII 5 SM (1, 2, 3, 4, 5)	0	0	0	0.01	6
1M/SM up to 5M/SM, chromosomal polymorphisms with complex rearrangements					
Karyotype IX (translocation 2-4) 1M (5) + 3SM (1, 3, 4) + 1M/SM (2); 2 sat	0.013	0	0	0	6
Karyotype X (translocation 2-3) 1M (5) + 2SM (1, 4)+ 2M/SM (2, 3); 2 sat	0.047	0	0	0	6
Karyotype XI 1M (2) + 2SM (3, 4) + 2M/SM (1, 5); 1, 5 sat	0	0	0	0.01	6
Karyotype XII 1M (2) + 2SM (3, 4) + 2M/SM (1, 5); 2, 5 sat	0.007	0	0	0.01	6
Karyotype XIII (inversion on chromosome 4) 2SM (1, 3) + 3M/SM (2, 4, 5)	0.013	0	0	0.02	6
Karyotype XIV (inversions) 1M (5) + 1SM (2) + 3M/SM (1, 3, 4)	0.007	0	0	0,01	6
Karyotype XV (translocations) 1SM (4) + 4M/SM (1, 2, 3, 5); 2 sat	0.02	0.04	0	0	6
Karyotype XVI 5 M/SM (1, 2, 3, 4, 5)	0.006	0	0	0.01	6
Chromosome number 2n = 11					
Mosaic specimens carrying nuclei with free or attached B chromosomes: formulae with heteromorphic pairs: SM ≠ SM+B					
Karyotype XVII (inversion on chromosome 4) 2M (2, 5) + 2SM (1, 3) + [1SM+B] (4); 2, 3 sat	0.02	0.04	0	0.01	7

Karyotype XVIII (inversion on chromosome 3) 1M(2) + 2SM(1, 4) + 1M/SM (5) + [1SM+B] (3); 2 sat	0.007	0	0	0.01	7
Karyotype XIX (inversions on chromosomes 3, 4) 2M (2, 5) + 1SM(1)+ [2SM+B] (3, 4); 2 sat	0.013	0.04	0	0.01	7
Total	1	1	1	1	

tion followed by a conspicuous satellite (Fig. 1b). Chromosome 4 carried a wide C-block nearly occupying the entire long arm. Chromosome 5 showed a narrow interstitial positive band within a distinct C-banded arm while the other arm revealed no bands. All chromosomes showed pericentromeric C-bands at least in one arm (Fig. 1a–b).

H-banded karyotype I is documented on Fig. 1c. The idiogram of the H-banded haploid set is shown on Fig. 1d. Prominent H-bands were observed in all the chromosomes, and apart from chromosome 4, H-bands did not mark the centromeres. A secondary constriction on chromosome 2 was marked by a gap, i.e. a negative H-band. Chromosome 3 carried positive H-bands on the satellite and the distal regions of both arms. Chromosomes 4 and 5 had large H-bands; chromosome 4 carried a centromeric H-band on the short arm (Fig. 1c–d).

Chromosomal rearrangements and heteromorphisms

Using karyotype I as the reference one, somatic chromosomal polymorphisms affecting chromosome number and/or morphology were detected. Cytological preparations with 2n=11 but without a heteromorphic chromosome pair were selected. This karyotype is formed due to presence of an acrocentric chromosome carrying a small C-positive short arm (Fig. 2a, b, c1, c2, d and Table 1). A summary of variants is represented in the composite C-banding idiogram (Fig. 2b). Chromosome 2 always carried a secondary constriction in all individuals from different populations (see below). Additionally, C- and H-banding revealed variation in the number and position of the bands and in the presence of satellites on chromosomes 1, 2, and 5; no satellite was found on chromosome 4 (n=287) (Fig. 2c1, e, g and 3a). Major changes in C-heterochromatin distribution affected pairs 1, 2 and 5 (Fig. 1b and 2b). The polymorphic long arm of chromosome 3 showed a curved shape on one homologue, lack of pairing and a change in the position of secondary constriction (Fig. 2b, e, e2). A variant of chromosome 4 showed change in C-heterochromatin distribution on the long arm: one of the homologs exhibited the darkest band on the long inverted arm, leading to lack of pairing (Fig. 1b, 2b, e). In the same specimen, a chromosomal bridge (2e1) as well as another metaphase plate (Fig. 2e2) with polymorphic pairs 3 and 4, add further evidence for these rearrangements, as they could arise from an inversion. A submetacentric variant of chromosome 5 was revealed by C-banding (Fig. 2a, b and e). The extra small chromosome was positively H-banded (Fig. 2d). Unpaired chromosomal segments were recognized in two metaphases of the same specimen (Fig. 2f1, f2), indicating banding heteromorphisms and structural polymorphisms.

Frequent and rare karyotypes

We identified 19 main chromosomal profiles in Argentina and Uruguay (Table 1), through differences in the chromosome banding (heteromorphisms), chromosome rearrangements (polymorphisms) and chromosome number (Fig. 2a, c–g and 3a–d); frequency distribution of these profiles within each population was calculated. These profiles were classified in seven chromosomal formulae (Table 1). Table 1A groups the most frequent chromosomal formulae (≥0.150) with the exception of formula 1M + 4SM (see below), whereas rare karyotypes are shown in Table 1B.

Karyotypes composed of two metacentric and three submetacentric chromosomes (formula 2M + 3SM) are represented by variants I, II, III (Table 1A, column "a" Group 1) and IV (Table 1A, column "a" Group 2). Karyotype II is similar to karyotype I but with a satellite on chromosome 1. Karyotype III showed satellites on chromosomes 2 and 5. Karyotype IV has undergone a significant change that gave rise to metacentric chromosome 1 and submetacentric chromosome 2 (Fig. 3b1 and b2) and thus we used it in our Multiple Correspondence Analysis (Table 1, column "a" Group 2). Karyotype V has one metacentric (chromosome 2) and four submetacentric chromosomes, i.e. 1M + 4SM (Table 1, Group 3); chromosome 1 carries a satellite (Fig. 2g). This karyotype is dominant in Trancas population.

Karyotypes with 2n=11 (2n=10+B) and formula 2M + 3SM + B correspond to karyotype VI (Fig. 2a) (Table 1A, Group 4),whereas those with formula 1M + 4M/SM + B carrying four polymorphic pairs formed by one metacentric and one submetacentric chromosome correspond to karyotype VII (Fig. 2c1 and c2) (Table 1A, column "a" Group 5). Karyotype VI appeared to be dominant in our samples from Ferreyra and Palmitas, whereas karyotype VII was dominant in Bolivar.

Rare karyotypes (Table 1B) were not found in the Trancas population. Formula 5SM corresponds to karyotype VIII (2n=10), composed of five pairs of submetacentric chromosomes (not shown). Only one specimen of that kind was found in the Palmitas population (Table 1B column "a" Group 6). The sixth formula, 1M/SM to 5M/SM (2n=10) includes eight very scarce karyotypes (IX to XVI) from Ferreyra and Palmitas, carrying 1 to 5 polymorphic pairs composed of one metacentric and one submetacentric chromosome (Table 1B, Group 6). C- and H- bandings provide strong evidence for complex rearrangements along with lack of somatic chromosomal pairing (Fig. 2d, 2f1, 2f2, 3b1, 3b2, 3c1, 3c2). Rare karyotypes XVII, XVIII and XIX with formula SM≠SM + B (Table 1B, column "a" Group 7) include mosaic insects carrying nuclei with 2n=10 and 2n=11 and having polymorphic pairs of chromosomes (Fig. 3a); the extra chromosome is free or attached to one of the homologs of pair 3 or pair 4 (Table 1B, Group 7) (Fig. 2e, e1, e2).

Population structure

Within the Ferreyra population, karyotype I (2n=10) and karyotype VI (2n=11) were present in 40% and 32% of the insects respectively (Table 1). Due to the fact that the

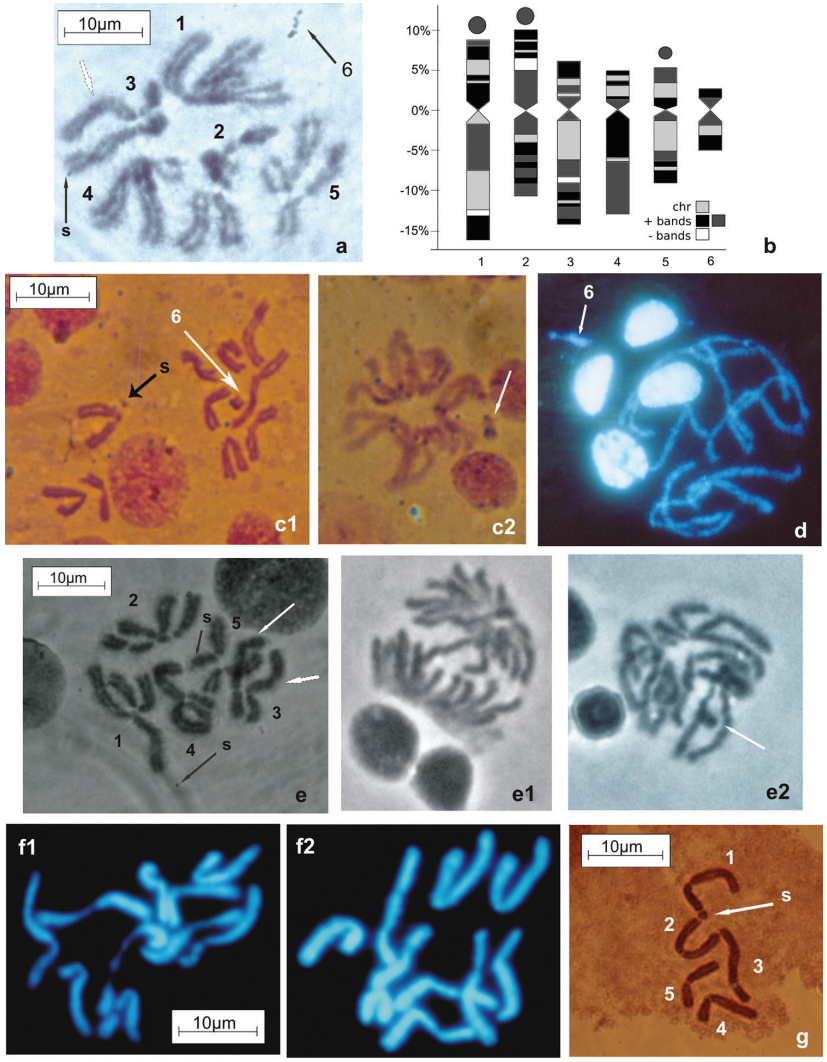

Figure 2. Chromosome variants in *H. irritans*. **a–f** mitotic plates from larval brain cells. **a** Giemsa C-banded metaphase plate with 2n=11 and karyotype VI; black arrow indicates B-chromosome, white arrow indicates relocation of secondary constriction on the curved long arm of one homologue of pair 3, "s" indicates satellite on chromosome 3 **b** Composite C-banding idiogram showing some chromosome variants found in different specimens **c** Giemsa C-banded metaphases with 2n=11 found in the same larva with karyotype VII; white arrow indicates B-chromosome, black arrow indicates satellite on metacentric chromosome 2 **d** Hoechst 33258 stained prometaphase with 2n=11 carrying complex rearrangements; white arrow indicates B-chromosome **e** Giemsa stained C-banded metaphase with 2n=10 and heteromorphic pairs 3 and 4; black arrows indicate satellites on chromosomes 1 and 5, white arrows indicate both long arms of pair 3 with one of them carrying an attached B-chromosome **e1** anaphase with a bridge **e2** metaphase from the same specimen as in **e** and **e1**; white arrow indicates rearrangement in one of the homologues of pair 3, black arrow on pair 4 **f** Hoechst 33258 stained metaphases with 2n=10 and karyotype XV carrying complex rearrangements **g** Orcein stained meiotic metaphase II from testis with karyotype V; white arrow indicates satellite on chromosome 1.

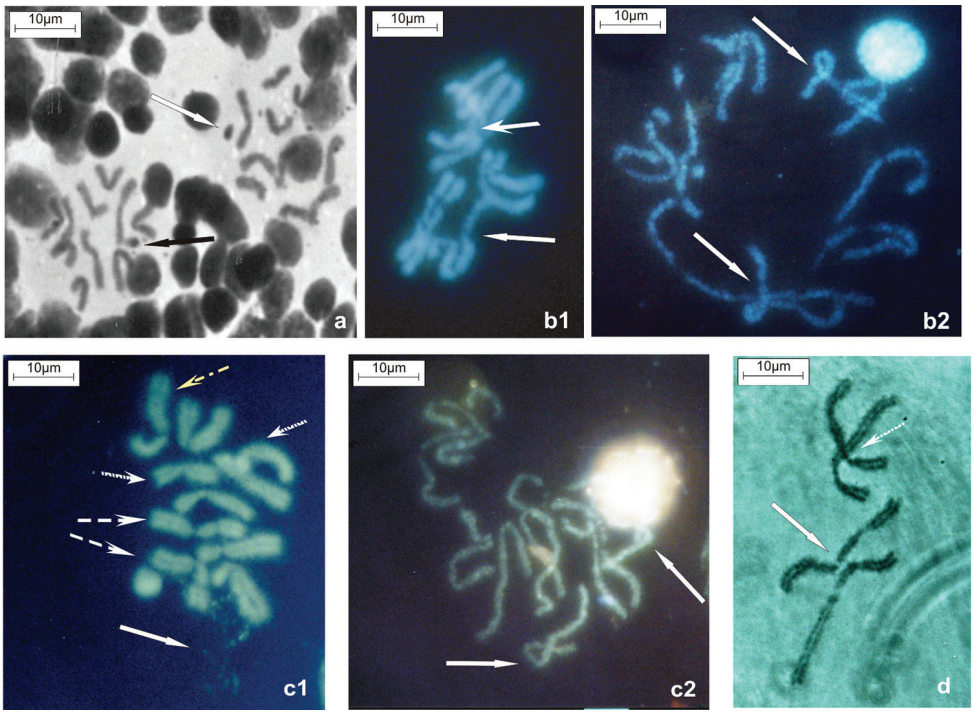

Figure 3. Chromosome variants in *H. irritans*. Mitotic plates from larval brain cells. **a** Two C-banded metaphases with karyotype XVIII, one with 2n=11 (2n=10+B) and the other with 2n=10 with free and attached B-chromosomes (white and black arrows respectively) **b1** and **b2** Hoechst 33258 stained metaphase and prometaphase of the same specimen with karyotype IV; arrows indicate rearranged pairs 1 and 2 **c** Hoechst 33258 stained metaphase and prometaphase **c1** heteromorphisms of pairs 1 (dashed yellow arrow), 4 and 5 (dotted arrows) and the nucleolus close to chromosome 2 (dashed white arrow), expression of ribosomal DNA located on secondary constriction on chromosome 2 (white arrow) **c2** H-banded karyotype with heteromorphisms; arrows indicate rearrangements on prometaphase chromosomes **d** C-banded incomplete metaphase with chromosomal pairs 1 and 3 (dotted arrow); white arrow indicates pair 1 carrying heterozygous inversion.

sample size of this population was large (n=150), we detected specimens with 16 different karyotypes (Table 1). Insects with 2n=10 and chromosome variations represented 23.3% within this population; those included karyotypes II, IV (Fig. 3b1, b2 and Table 1) and all the series of karyotypes from 1M/SM up to 5M/SM (group 6). All the karyotypes with 2n=11 (Table 1, groups 4, 5 and 7) were present in 36.7% of Ferreyra specimens. However, the extra chromosome was never observed in preparations from the testes.

Within the Bolivar population, 52% flies had 2n=10. The frequency of karyotype I was 24%. Interestingly, karyotype III with the same frequency and satellites on chromosome 2 was unique to this population (Table 1). Additionally, karyotype VII with 2n=11 (Fig. 2a, c1, c2, d, 3a and Table 1) was present in 40% of the insects within Bolivar sample.

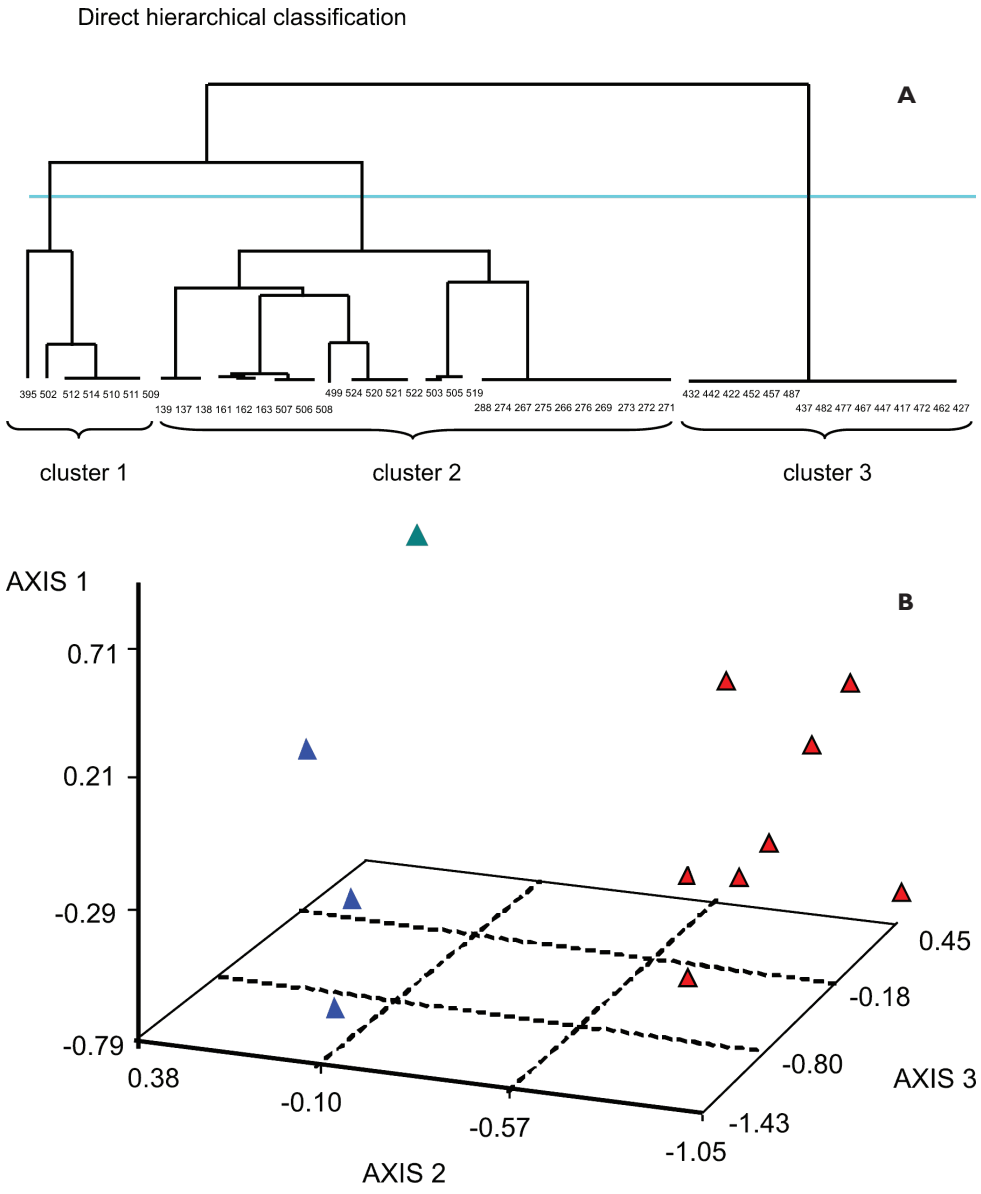

Figure 4. Clustering and spatial distribution of *H. irritans*. **a** The most significant discrimination is obtained by the first three axes: karyotypes, satellites and zygotic number of chromosomes, and therefore the three clusters are the most representative. Texas individuals (LaChance 1964, Avancini and Wienzierl 1994) were also included **b** Spatial distribution of individuals; clusters 1 to 3 and Texas individuals are shown in blue, red, green and black respectively.

In the Trancas population, only a small sample was studied on the basis of male meiosis. All the insects had karyotype II (2M + 3SM) or karyotype V (1M + 4SM) (Fig. 2g and Table 1). Both karyotypes showed a satellite on chromosome 1, but they

differed in the morphology of chromosome 5, which appeared to be metacentric in karyotype II but submetacentric in karyotype V.

In the Palmitas population, karyotypes I (2n=10) (44%) and VI (2n=11) (43%) were dominant. Although 56% insects carried rearrangements, flies having 2n=11 prevailed (46%; Table 1). Karyotypes II, V, VIII, IX, X, XII and XIV also had structural rearrangements (10%). This was the only population where an individual carrying karyotype VIII with five submetacentric pairs (formula 5SM) was found. Karyotype II was uncommon in Palmitas but frequent in Trancas (Table 1).

MCA produced 14 axes from 14 nominal variables (see Materials and Methods). From these, we have chosen seven factors that explained 92.25% of the whole variation. Graphical displays constructed from cluster analysis obtained from those factors, were used to summarize proximities between the specimens and to show associations between the categorical variables. The dendrogram (Fig. 4a) showed the hierarchical classification, where the greatest discrimination was obtained by the first three factors: karyotypes, satellites and zygotic chromosome number. Therefore the three clusters that grouped similar individuals were the most representative. As expected, the factorial analysis has clearly shown that localities were not a discriminating factor.

Cluster 1 (n=141) is characterized by individuals from groups 1 and 3 (Table 1A, column "a"), with 2n=10, and satellites on chromosome 1 (s1), or on chromosome 2, 3 or 5. This cluster grouped all Trancas individuals, 50% of Ferreyra individuals and 47% of Palmitas individuals. Neither specimens from groups 2, 4, 5, 6 and 7 nor those with 2n=11 were found in this cluster (Table 1A, column "a").

Cluster 2 (n=56) is characterized by all individuals of groups 2, 5, 6 and 7 (Table 1, column "a"); those with 2n=10 predominated. All insects with satellites on chromosomes 2 or 5 as well as those without satellites were found in this cluster. There were very few individuals of group 1 (10.71%) and no individuals of group 4 (Table 1, column "a"). Cluster 2 grouped 76%, 10% and 18% of Bolivar, Palmitas and Ferreyra individuals respectively.

Cluster 3 (n=90) is characterized by individuals with 2n=11, of group 4 and with a satellite on chromosome 3; it is composed by 32% of Ferreyra individuals and 43% of Palmitas individuals (Table 1A, column "a").

The specimens were represented in three-dimensional graphical displays constructed using the first three factorial axes (Fig. 4b). Data on the flies from Texas (LaChance 1964, Avancini and Weinzierl 1994) are also given (in black); the spatial distribution of all individuals (Fig. 4b) showed them as a part of cluster 2 (in red). The clusters well differ from each other; all individuals in cluster 3 have identical karyotypes.

Discussion

This study is the first chromosomal analysis of recently established wild populations of the horn fly, originally introduced in Brazil in 1983, in the Southern Cone of South

America. We believe that the karyotype study provides a basic tool for understanding the population dynamics. Unfortunately, this research is limited by the lack of a full-cycle laboratory rearing technique of the horn fly.

Chromosomal rearrangements

We here document chromosomal rearrangements that affect the chromosome number and morphology. We also confirm that the main chromosome number in *H. irritans* is 2n=2x=10. For the first time we show the existence of an extra chromosome in three populations, resulting in the presence of chromosome sets with 2n=11 (2n=10+B).

Our study showed that the main chromosomal formula for the karyotypes found in Ferreyra, Palmitas, Trancas and Bolivar was 2M + 3SM. Variation found in the populations from Ferreyra, Palmitas and Bolivar (Table 1), involved some complex chromosomal rearrangements such as those in karyotypes VIII to XVI (Table 1B) and mosaic specimens such as those with karyotypes XVII to XIX carrying nuclei with 10 and 11 chromosomes. Within the best-analyzed populations, i.e. Ferreyra and Palmitas, individuals with 5M/SM or 5SM were found within all clusters (Fig. 4a, b). This karyotype diversity was previously reported neither by LaChance (1964) nor by Avancini and Weinzierl (1994). LaChance (1964) studied chromosome sets of the horn fly on the preparations from larval brain cells (n=18) or testes (n=11) and described a unique karyotype of *H. irritans* with 2n=10 composed by five pairs of autosomes (4M + 1 SM) without a distinct secondary constriction. Thirty years later, Avancini and Weinzierl (1994) described individuals from the same colony, but with only one karyotype 2n=10, composed by five pairs of autosomes (3M +2 SM) based on the analysis of "ten adult testis cells in meiotic metaphase" or "a few larval brain cells"; those authors found that submetacentric chromosome 3 seemed to carry the nucleolar organizer in the pericentromeric region. However, we found that chromosome 2 carried a secondary constriction that denotes the location of the nucleolar organizer (not shown) and can reveal a satellite during the cell cycle. Mobility of the satellites between different chromosomes can be explained either by transposition or fragmentation of the nucleolar organizer.

Origin and transmission of the small extra chromosome

Extra chromosomes were found within karyotypes from Ferreyra, Bolivar and Palmitas, but we could not determine whether it was the same B-chromosome in all cases. A B-chromosome might be derived from a fragmented autosome or a sex chromosome, i.e. a centric fragment derived from amplification of the paracentromeric region (Fig. 2b–e, 3a, Table 1). It could originate by inversions that require at least two breaks on the same chromosome.The small chromosome could arise by a pericentric inver-

sion taking place close to one inessential chromosomal end (3b1, 3b2, 3d). When two crossing-overs involving three chromatids take place, i.e. one within the inverted region and the other within the proximal region, a bridge gives origin to small chromosomes (Fig. 2b, e, e1, e2).

Lampe et al. (1998, 2001) reported the presence of transposons in *H. irritans*, and *HIMAR1* is one of the only two known active mariner elements (Lampe et al. 1998). Heterochromatic B-chromosomes, usually revealed by C-banding, typically originate from satellite blocks of repeated DNA sequences which vary in type, kind of repeat, and copy number (Franks et al. 1996), as a result of unequal crossing-over and reduced recombination (Bigot et al. 1990, Charlesworth and Sniegowski 1994). Camacho et al. (2000) proposed that B-chromosomes might be amalgamations of transposable DNA. As indicated in Table 1, rearrangements affecting chromosomes 3 or 4 were associated with B-chromosomes (Fig. 2e, e2; autosome 3, Fig. 3a). Analyses of C-banding (Fig. 1b, 2a, b, c1, c2, e, e1, e2) and H-banding patterns in brain larval cells (Fig. 1c, d, 2d–f1, f2) at the population level, suggested that the extra small chromosomes might originate through breaks within chromosome 3 or chromosome 4.

Population frequencies of B-chromosomes result from a balance between their transmission rates and their effects on host fitness (Basso and Lifschitz 1995, Camacho et al. 2000). Our observations suggest that a free B-chromosome is only transmitted via female meiosis. Differential transmission of the B-chromosome was cytogenetically studied in *Ceratitis capitata* (Wiedemann, 1824) (Basso and Lifschitz 1995), where 63 progenies from reciprocal crossings were studied and the B-chromosome observed in both sexes, in somatic cells (cerebral ganglia tissue) as a free chromosome, or terminally attached to the long arm of the X-chromosome, appearing as a larger X. Males transmit both free Bs and large X-chromosomes to their progeny. Females of *C. capitata* transmit the large Xs to their progeny, at a higher rate than the standard Xs, suggesting either differential fate of the oocytes or a preferential co-orientation during the first meiotic division. A similar phenomenon that involved preferential survival of spermatozoa bearing Bs was also described in this species by Manso and Lifschitz (1986).

In our studies, the extra chromosomes might be associated with sex determination as the frequencies of individuals with 2n=11 in Ferreyra, Bolivar and Palmitas were 0.367, 0.48 and 0.46 respectively. The extra chromosome was observed in around 50% of larval brains and was not observed in preparations from testes, further suggesting that the B-chromosome is restricted to females. We hypothesize that this B-chromosome is preferentially transmitted by females, and that it might be related to sex determination because it was never observed during male meiosis. Sex chromosomes were previously proposed as ancestors of the Bs (Hewitt 1973). At the parsimonious hypothesis, homomorphic chromosomes may have sex determination factor like in *Aedes aegypti* (Hall et al. 2014). In spite of the difficulties to demonstrate B-chromosomes in gonad tissues, we believe that extra chromosomes behave in a similar way that those reported in *C. capitata* (Basso and Lifschitz 1995), because they are transmitted from parents to the offspring.

Rearrangements, hybridization and polymorphisms

Although a direct investigation of demographic structure is difficult in natural conditions (Roderick 1996), study of the genetic structure of wild horn fly populations that is affected by mutations, migration or gene flow, selection and genetic drift, is a basic tool to understand population dynamics and insecticide resistance that must be complemented by DNA sequencing studies.

MCA generated three clusters: cluster 1 grouped specimens with 2n=10 from all four populations; cluster 2 grouped rare karyotypes with 2n=10 and 2n=11 as well as insects from Texas (Fig. 4a–b); cluster 3 grouped insects with 2n=11 from Ferreyra and Palmitas. Since karyotypes from Texas were respectively reported 50 and 20 years ago (LaChance 1964, Avancini and Weinzierl 1994), loss of chromosome variants was probably due to inbreeding, thus both karyotypes were grouped in cluster 2. We did not find the karyotypes from Kerrville within our populations. However, other less frequent chromosome sets carrying heteromorphic M/SM pairs, grouped in the same cluster together with Texas karyotypes. Moreover, it is significant that the correspondence analysis revealed that localities did not discriminate between populations, apart from the chromosome number and karyotype structure (Fig. 4a–b). Since up to 1200 generations of the horn fly during the last 50 years have occurred, metacentric chromosomes that were reported by LaChance (1964) and Avancini and Weinzierl (1994), may have been transformed into submetacentric ones. Inversions which reduced interchromosomal recombination and also maintained the polymorphism, are likely to have been responsible for this significant change. On the other hand, hybridization produced further recombination between the different variants.

Abundant karyotypes were analyzed in two locations, Ferreyra in Argentina and Palmitas in Uruguay. Resilience of genomes to massive introgression through hybridization, can allow for rapid adaptive response to anthropogenic selection (Clarkson et al. 2014). Thus, our results explain repeated appearance of resistant phenotypes in *H. irritans*.

Conclusions

We confirmed the chromosome set with 2n=10 as the reference karyotype in wild populations of *H. irritans* invading the Southern Cone of South America. Karyotypic variants were characterized for the first time; half of these variants were 2n=11 due to the presence of a B-chromosome.

The B-chromosome was observed only in mitotic divisions, mainly as a free acrocentric chromosome.

Horn fly control will highly benefit from genetic studies focusing on the understanding of sex determining mechanisms, which are necessary to design appropriate control strategies, as related to the adaptation of these insects to control measures such as insecticides.

Acknowledgments

We thank Dr. C. Argaraña and Dr. A. Filiberti for providing the Ferreyra specimens. Funding for this project was provided by the ANPCYT-PICT 2003-351, CONICET (Argentina), and the University of Buenos Aires (UBA).

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CompCytogen 9(1): 51–65 (2015)
doi: 10.3897/CompCytogen.v9i1.9002
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Basic cytogenetics and physical mapping of ribosomal genes in four *Astyanax* species (Characiformes, Characidae) collected in Middle Paraná River, Iguassu National Park: considerations on taxonomy and systematics of the genus

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Academic editor: *I. Kuznetcova* | Received 20 November 2014 | Accepted 14 January 2015 | Published 9 February 2015

http://zoobank.org/88C25B43-804F-4729-90D2-962BC067024B

Citation: Paiz LM, Baumgärtner L, da Graça WJ, Margarido VP (2015) Basic cytogenetics and physical mapping of ribosomal genes in four *Astyanax* species (Characiformes, Characidae) collected in Middle Paraná River, Iguassu National Park: considerations on taxonomy and systematics of the genus. Comparative Cytogenetics 9(1): 51–65. doi: 10.3897/CompCytogen.v9i1.9002

Abstract

Karyotypes and chromosomal characteristics of both minor and major rDNAs in four fish species known popularly as "lambaris", namely *Astyanax abramis* (Jenyns, 1842), *Astyanax asuncionensis* Géry, 1972, *Astyanax correntinus* (Holmberg, 1891) and *Astyanax* sp. collected from downstream of the Iguassu Falls (Middle Paraná River basin), preservation area of the Iguassu National Park, were analyzed by conventional and molecular protocols. *A. abramis* had diploid chromosome number 2n=50 (4*m*+30*sm*+8*st*+8*a*) and single AgNORs (pair 22), *A. asuncionensis* had 2n=50 (8*m*+24*sm*+6*st*+12*a*) and single AgNORs (pair 20), *Astyanax* sp. had 2n=50 (4*m*+26*sm*+8*st*+12*a*) and single AgNORs (pair 25), and *A. correntinus* had 2n=36 (12*m*+16*sm*+2*st*+6*a*) and multiple AgNORs (pairs 12, 15, 16, 17). FISH with 18S rDNA showed a single site for *A. abramis*, *A. asuncionensis* and *Astyanax* sp. and multiple for *A. correntinus* (14 sites). FISH with 5S rDNA showed single 5S-bearing loci chromosome pair only for *A. asuncionensis* and multiple for *A.*

abramis (four sites), *A. correntinus* (five sites) and *Astyanax* sp. (four sites). Distinct distribution patterns of heterochromatin were observed for karyotypes of all species, with the exception of the first acrocentric chromosome pair characterized by centromeric, interstitial-proximal and telomeric blocks of heterochromatin on the long arm, which may represent homeology between karyotypes of *A. abramis* and *A. asuncionensis*. Our study showed species-specific characteristics which can serve in diagnosis and differentiation between *A. abramis* and *A. asuncionensis*, considered cryptic species, as well as strengthening the occurrence of a species of *Astyanax* not yet described taxonomically. In addition, the data obtained from first cytogenetic studies in *A. correntinus* suggest a high similarity with *A. schubarti* Britski, 1964, suggesting that these species may belong to the same morphological group and that can be phylogenetically related.

Keywords
Fish cytogenetics, chromosome banding, rDNA-FISH, karyotype differentiation, rDNA sites multiplication

Introduction

Characiformes are considered one of the most diversified groups in the world freshwater ichthyofauna, comprising 18 families with 270 genera and more than 1,700 species (Nelson 2006). This diversity is recognized mainly in the Neotropical region, which is home to around 1,000 species in Brazilian hydrographic systems alone (Buckup et al. 2007).

Among the families that comprise Characiformes, four are in the African continent (Alestidae, Citharinidae, Distichodontidae and Hepsetidae) and 14 are in Neotropical regions (Acestrorhynchidae, Anostomidae, Characidae, Chilodontidae, Crenuchidae, Ctenolucidae, Curimatidae, Cynodontidae, Erythrinidae, Gasteropelecidae, Hemiodontidae, Lebiasinidae, Parodontidae and Prochilodontidae) (Nelson 2006); however, some authors recognize Serrasalmidae as valid in Characiformes (Jégu et al. 2003, Calcagnotto et al. 2005, Ortí et al. 2008). Recently, Oliveira et al. (2011) proposed a study by rearranging the phylogenetic relationships in the order, suggesting a new definition for Characidae, based on analysis of sequences of 2 mitochondrial genes and 3 nuclear genes, obtained from 166 genera distributed in 18 acknowledged families, and another 56 genera were considered as *incertae sedis*. This study raises the subfamilies Bryconinae, Iguanodectinae and Triportheinae to families Bryconidae, Iguanodectidae and Thiportheidae, respectively. Albert et al. (2011) added Chalceidae (comprised only by *Chalceus*), resulting in a new classification for Characiformes composed of 23 families (including Serrasalmidae, above mentioned).

Astyanax Baird & Girard, 1854 known popularly as "lambaris", includes around 140 valid species and probably many not yet discovered and/or awaiting formal description (Froese and Pauly 2014). Being a genus with the highest species count in Characidae distributed in Central and South America, in Brazilian basins *Astyanax* comprises around 50 valid species (Buckup et al. 2007). The major inconsistencies shown in the family occur in *incertae sedis*, in which the taxonomic construction is a catch-all assemblage and includes several distinct lineages with absence of proven monophyly (Lima et al. 2003, Mirande 2010). Belonging to this group, *Astyanax* was first allocated in Tetragonopterinae; however, systematic reviews in the subfam-

ily have listed all genera (except for Tetragonopterus) as *incertae sedis* (Lima et al. 2003). Recently, Mirande (2009) proposed, with phylogenetic contributions, a new relationship among genera *incertae sedis*, resulting in a phylogenetic lineage within the Characidae named "clade *Astyanax*".

Ichthyofaunal researches in river systems of southern Brazil were carried out particularly in systems that comprise the Upper Paraná River basin (Langeani et al. 2007, Graça and Pavanelli 2007) and the Iguassu River basin (Ingenito et al. 2004, Bifi et al. 2006, Baumgartner et al. 2012). In the hydrographic system of Paraná-Paraguay basin there were 110 species identified, eight being represented by *Astyanax* (Neris et al. 2010). *Astyanax altiparanae* Garutti & Britski, 2000 was described for the Upper Paraná River basin and *A. asuncionensis* Géry, 1972 for the Middle-Lower Paraná River and Paraguay River basins (Lima et al. 2003). In this same region, *A. abramis* (Jenyns, 1842) is a sister-group of *A. asuncionensis*, and may be considered highly cryptic by presenting similar morphological characteristics (Britski et al. 2007). *A. correntinus* (Holmberg, 1891) and *A. pellegrini* Eigenmann, 1907 are grouped in "clade *Astyanax*" next to *A. asuncionensis* and *A. abramis* (Mirande 2009). Despite the proximity in the clade, *A. correntinus* does not morphologically fit in any of the artificial groups recognized in *Astyanax*, otherwise occurring in complexes *A. bimaculatus* (Jenyns, 1842), *A. fasciatus* (Cuvier, 1819) and *A. scabripinnis* (Jenyns, 1842).

Astyanax comprises interesting species for cytogenetic studies, with different evolutionary models that show from maintenance of a preserved chromosomal condition to derived karyotype characteristics, used as important tools in the differentiation and identification of species (Moreira-Filho and Bertollo 1991, Vicari et al. 2008, Ferreira-Neto et al. 2009, Peres et al. 2008). Available cytogenetic data reveal diploid chromosome numbers within *Astyanax* that vary from 2n = 36 in *A. schubarti* Britski, 1964 (Morelli et al. 1983) to 2n = 50, as observed *A. scabripinnis*, *A. fasciatus*, *A. altiparanae* and *A. jacuhiensis* (Cope, 1894) (Souza and Moreira-Filho 1995, Artoni et al. 2006, Ferreira-Neto et al. 2009, Pacheco et al. 2010). Martinez et al. (2012) carried out a review of twenty populations of *A. altiparanae*, observing intraspecific differences in the karyotypes, and the number and position of the nucleolus organizing regions (NORs). Likewise, for chromosomes of *A. fasciatus* (Pazza et al. 2006, Medrado et al. 2008) and *A. scabripinnis* (Mantovani et al. 2004, Santos and Morelli 2006), interpopulation differences mainly associated with heterochromatin distribution patterns were observed.

The physical mapping of genes 5S rDNA and 18S rDNA has also been used to characterize different populations in the species of *A. scabripinnis* complex (Souza et al. 2001, Mantovani et al. 2005, Fernandes and Martins-Santos 2006, Peres et al. 2008), *A. altiparanae* complex (Almeida-Toledo et al. 2002, Fernandes and Martins-Santos 2006), *A. fasciatus* complex (Ferreira-Neto et al. 2012) and *A. jacuhiensis* (Pacheco et al. 2010). These data documented highly variable NORs phenotype diversity in the representatives of the genus (Almeida-Toledo et al. 2002, Mantovani et al. 2005, Fernandes and Martins-Santos 2006, Peres et al. 2008).

The aim of the present study was to characterize using the conventional and molecular cytogenetic techniques, the karyotypes and chromosomal characteristics of rDNA

in the species *A. abramis*, *A. asuncionensis*, *Astyanax* sp. and *A. correntinus*, collected downstream from the Iguassu River Falls (middle Parana River), to contribute to the taxonomy of one of the major component of Neotropical Characidae fish diversity.

Methods

The specimens analyzed were deposited in Coleção Ictiológica do Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura - (NUP), Universidade Estadual de Maringá: nine specimens of *A. abramis* (four males and five females, NUP 14581), 25 specimens of *A. asuncionensis* (13 males and 12 females, NUP 14584), 25 specimens of *A. correntinus* (11 males and 14 females, NUP 14582) and one specimen of *Astyanax* sp. (female, NUP 14583), in the Iguassu River, in the stretch with around 25 km between downstream of the Iguassu Falls and its mouth on the Paraná River, Middle Parana River basin, located in the preservation area of the Iguassu National Park (25°38'18.72"S; 54°28'4.74"W).

All the specimens were anesthetized and sacrificed by an overdose of clove oil (Griffiths 2000). The chromosome preparations were obtained from anterior kidney cells by means of the techniques by Bertollo et al. (1978) and Foresti et al. (1993) using 0.02% colchicine treatment for 40 or 30 minutes, respectively. Thirty metaphases spreads from each fish were analyzed and ten of the best mitotic metaphases were used to measure karyotypes.

The AgNORs were revealed by silver impregnation according to Howell and Black (1980) and C-banding followed Sumner (1972), with modifications suggested by Lui et al. (2012).

The physical mapping of 5S rDNA and 18S rDNA loci was carried out by fluorescence *in situ* hybridization (FISH) according to Pinkel et al. (1986) and modifications suggested by Margarido and Moreira-Filho (2008), using probes obtained from *Leporinus elongatus* (Martins and Galetti 1999) and from *Prochilodus argenteus* (Hatanaka and Galetti 2004) DNAs, respectively. The hybridization was performed under high stringency condition (77%). Probes were labeled by nick translation with digoxigenin-11-dUTP (5S rDNA) and biotin-16-dUTP (18S rDNA) (Roche®). The detection of signals was performed with antidigoxigenin-rhodamine (Roche®) for probe of 5S rDNA and amplified avidin-FITC with biotinylated anti-avidin (Sigma-Aldrich) for probe of 18S rDNA, the chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 50 µg/mL).

The metaphases were photographed using a BX 61 epifluorescence microscope, coupled with Olympus DP 71 digital camera with the Olympus DP Controller software 3.2.1.276. The chromosomes were classified and organized in accordance with Levan et al. (1964) in metacentric (*m*), submetacentric (*sm*), subtelocentric (*st*) and acrocentric (*a*). The fundamental number (FN) was calculated considering *m*, *sm* and *st* chromosomes as having two arms, and *a* chromosomes as having only one chromosome arm.

Results

Astyanax abramis

The 2n was 50 chromosomes (4*m*+30*sm*+8*st*+8*a*, FN=92) for males and females (Fig. 1a). A single pair of NORs was located in terminal position on the p arm of chromosome pair 22 (Fig. 1a, in box). C-banding showed centromeric heterochromatin blocks in pairs 7, 14 and 21, pericentromeric on the q arm of pairs 22 and 24, telomerics on the p and q arms in pair 22, and coincident with the NORs (Fig. 1b). The FISH revealed multiple sites of 5S rDNA in centromeric position in the *sm* pair 7 and the *sm* pair 20, and a single site of 18S rDNA in terminal position on the p arm of the *a* pair 22 (Fig. 2a).

Astyanax asuncionensis

The 2n was 50 chromosomes (8*m*+24*sm*+6*st*+12*a*, FN=88) for males and females (Fig. 1c). A single pair of NORs was located in a terminal position on the p arm of chromosome pair 20 (Fig. 1c, in box). C-banding showed centromeric heterochromatin blocks in pairs 2, 3 and 20, pericentromeric on the p arm of pair 8, on the q arm of pairs 9, 13 and 14, telomerics on the q arm of pair 8, on the p and q arms in pair 20, and coincident with the NORs (Fig. 1d). FISH revealed a single site of 5S rDNA in centromeric position in the *sm* pair 9, and a single site of 18S rDNA in terminal position on the p arm of the *a* pair 20 (Fig. 2b).

Astyanax correntinus

The 2n was 36 chromosomes (4*m*+26*sm*+8*st*+12*a*, FN=66) for males and females (Fig. 1e). Multiple AgNORs bearing pairs were located in terminal position on the p arm of chromosome pair 12, on the p and q arms in pair 17, on the q arm of one chromosome from pair 15 and on the q arm of one chromosome from pair 16 (Fig. 1e, in box). C-banding showed centromeric heterochromatin blocks in pairs 1, 2, 4, 7, 9, 10, 12 and 14, telomeric on the q arm of pair 17, on the p arm of pair 18, on the p and q arms in pair 16, and coincident with the NORs (Fig. 1f). FISH revealed multiple sites of 5S rDNA in centromeric position in the *m* pairs 2 and 4, and in one chromosome from the *sm* pair 12. Multiple sites of 18S rDNA were observed in terminal position on the p arm of the *sm* pair 12, the *a* pairs 16, 17 and 18, and on the q arm of the *sm* pair 9 and *st* pair 15, and on the q arm of one chromosome from the *m* pair 4 and the *a* pairs 16 and 17 (Fig. 2c).

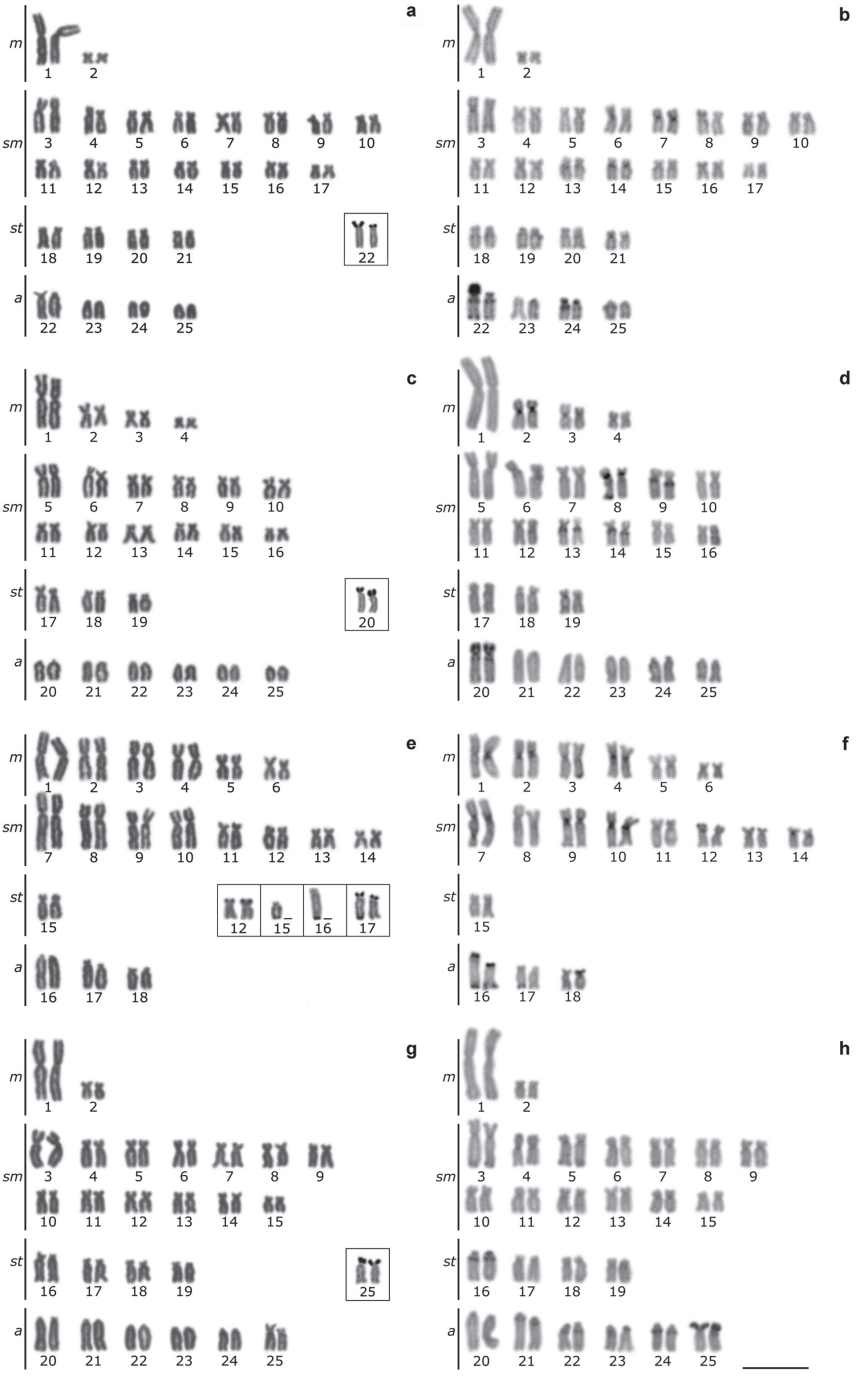

Figure 1. Karyotypes arranged from Giemsa-stained chromosomes of: **a** *A. abramis* **c** *A. asuncionensis* **e** *A. correntinus* **g** *A.* sp.; and from C-banded chromosomes of: **b** *A. abramis* **d** *A. asuncionensis* **f** *A. correntinus* **h** *Astyanax* sp. The AgNORs bearing chromosomes are framed. Bar = 10 μm.

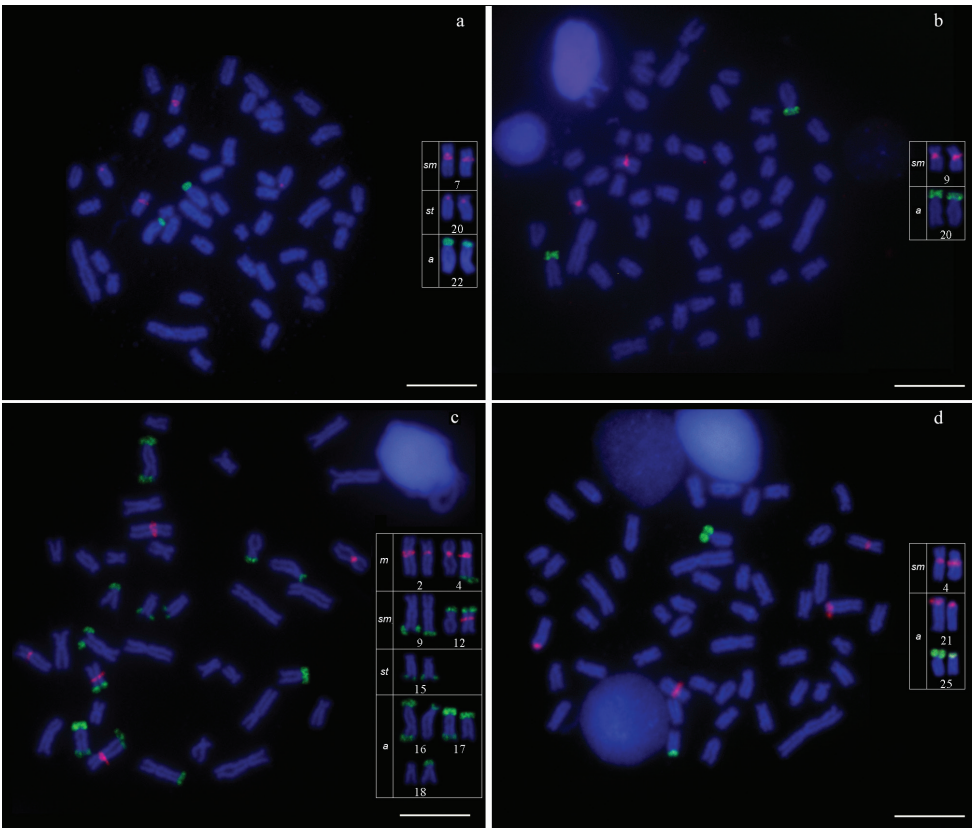

Figure 2. Metaphases chromosomes spreads after FISH with 5S rDNA probe (red) and 18S rDNA probe (green) of: **a** *A. abramis* **b** *A. asuncionensis* **c** *A. correntinus* **d** *Astyanax* sp. The 5S rDNA and 18S rDNA bearing chromosomes are framed. Bar = 10 μm.

Astyanax sp.

The 2n was 50 chromosomes (12*m*+16*sm*+2*st*+6*a*, FN=88) (Fig. 1g). A single pair of AgNORs was located in terminal position on the p arm of chromosome pair 25 (Fig. 1g, in box). C-banding showed pericentromeric heterochromatin blocks on the q arm of pairs 4, 16, 21, 22 and 24, telomeric on the q arm of pair 5, and coincident with the NORs (Fig. 1h). FISH revealed multiple sites of 5S rDNA in centromeric position in the *sm* pair 4 and the *a* pair 21, and single site of 18S rDNA in terminal position on the p arm of the *a* pair 25 (Fig. 2d).

Table 1 presents a comparison of the cytogenetical data (2n, karyotype formula, AgNORs, C-banding, 18S rDNA and 5S rDNA) obtained for the *Astyanax* species analyzed in the present study.

Table 1. Summary of the cytogenetical data for the *Astyanax* species analyzed in the present study.

Species	*A. abramis*	*A. asuncionensis*	*A. correntinus*	*Astyanax* sp.
2n	50	50	36	50
Karyotype formula	4*m*+30*sm*+8*st*+8*a*	8*m*+24*sm*+6*st*+12*a*	4*m*+26*sm*+8*st*+12*a*	12*m*+16*sm*+2*st*+6*a*
AgNORs	Single: - pair 22, *a*, tel, p arm	Single: - pair 20, *a*, tel, p arm	Multiple: - pair 12, *sm*, tel, p arm - pair 15, *st*, tel, q arm - pair 16, *a*, tel, q arm - pair 17, *a*, bitel	Single: - pair 25, *a*, tel, p arm
Heterochromatin (C-banding)	Centromeric, pericentromeric and telomeric	Centromeric, pericentromeric and telomeric	Centromeric and telomeric	Pericentromeric and telomeric
18S rDNA	Single: - pair 22, *a*, tel, p arm	Single: - pair 20, *a*, tel, p arm	Multiple: - pair 4, *m*, tel, q arm - pair 9, *sm*, tel, q arm - pair 12, *sm*, tel, p arm - pair 15, *st*, tel, q arm - pair 16, *a*, tel/bitel - pair 17, *a*, tel/bitel - pair 18, *a*, tel, p arm	Single: - pair 25, *a*, tel, p arm
5S rDNA	Single: - pair 7, *sm*, cent	Single: - pair 9, *sm*, cent	Multiple: - pair 2, *m*, cent - pair 4, *m*, cent - pair 12, *sm*, cent	Multiple: - pair 4, *sm*, cent - pair 21, *a*, tel

m: metacentric; *sm*: submetacentric; *st*: subtelocentric; *a*: acrocentric; cent: centromeric; tel: telomeric; bitel: bitelomeric; p: short arm; q: long arm.

Discussion

Diploid numbers and karyotype formulae

Although the present study revealed the same diploid chromosome number (2n= 50) for *A. abramis*, *A. asuncionensis* and *Astyanax* sp., with karyotypes dominated by bi-armed chromosomes, karyotypes differed among these three species, and can be used as a species-specific cytogenetic profile (Fig. 1a, c, g). Similar results were also found in other species of *Astyanax* (Oliveira et al. 1988, Daniel-Silva and Almeida-Toledo 2001, Kavalco et al. 2003), including the presence of the first large-sized *m* chromosome pair, these characteristics being assigned to an ancestral condition in genus (e.g. Portella et al. 1988, Ferreira-Neto et al. 2009, Kavalco et al. 2009, among others). Different from the species that possess these ancestral conditions in the genus, *A. correntinus* has 2n = 36 and a karyotype containing eight large *m-sm* chromosome pairs (Fig. 1e). These findings are similar to that found in *A. schubarti* by Morelli et al. (1983) and Almeida-Toledo et al. (2002) that shares with *A. correntinus* a low 2n originating from chromosome fusions, presence of large meta-submetacentric chromosomal pairs, in addition to low number of subtelo-acrocentric chromosomes. In addition, the external appearance of *A. correntinus* and *A. schubarti* shows similarly high body, a horizontal silver band on

the side of the body and a large amount of non-branching rays in anal fin in relation to other *Astyanax* species. Based on the cytogenetic results and morphological similarities it is possible to hypothesize that the two species may be phylogenetically closely related.

Nucleolus organizing regions and 18S rDNA

The number and position of NORs (Ag-impregnation and 18S rDNA-FISH), i.e. NOR phenotypes, observed in karyotypes of *Astyanax* species under study were conserved for the three species with 2n=50, with presence of a single site always located on the p arm in terminal position of *a* chromosome pair in *A. abramis*, *A. asuncionensis* and *Astyanax* sp. (Fig. 2a, b, d). *A. abramis* and *A. asuncionensis* are part of the *A. bimaculatus* complex, which is diagnosed by showing an oval humeral spot and caudal peduncle blotch, extending to the end of middle caudal rays. Similar results were observed in populations of *A. altiparanae*, which is also part of the *A. bimaculatus* complex (Domingues et al. 2007, Peres et al. 2008, Pacheco et al. 2011), although for *A. altiparanae* intraspecific variations were also observed when different populations were compared (Fernandes and Martins-Santos 2006, Ferreira-Neto et al. 2009). However, in chromosomes of *A. correntinus* multiple NORs were observed both by Ag- impregnation (Fig. 1e, seven sites) and by 18S rDNA-FISH (Fig. 2c, 14 sites), with some pairs presenting these sites in only one of the homologous chromosomes. Multiple NORs were also observed in karyotype of *A. schubarti* with of four ribosomal sites observed through 18 rDNA-FISH (Almeida-Toledo et al. 2002). In *A. scabripinnis*, up to 16 chromosomes bearing these ribosomal genes were observed (Ferro et al. 2001, Mantovani et al. 2005), showing the high degree of number variability observed for the genus.

5S rDNA

As to the 5S rDNA-FISH, simple sites were observed in karyotype of *A. asuncionensis* located in centromeric position (Fig. 2b), similar to that found in different populations of *A. altiparanae*, although they differ in location (interstitial-proximal position, Ferreira-Neto et al. 2009, Pacheco et al. 2011). Multiple sites were observed in karyotypes of *A. abramis* (Fig. 2a, four sites), *A. correntinus* (Fig. 2c, five sites) and *Astyanax* sp. (Fig. 2d, four sites), in centromeric position for all 5S rDNA-bearing chromosomes. Our findings indicate thus interspecific differences, which can be used as a diagnostic tool for their differentiation, because they are morphologically diagnosed only by differences in the number of perforated scales on the lateral line – up to 40 in *A. asuncionensis* and 42 or more in *A. abramis* (Britski et al. 2007). Likewise, *A. correntinus* shows a greater number of chromosomes bearing sites of 5S rDNA compared to that observed in *A. schubarti* (four sites) (Almeida-Toledo et al. 2002). In addition to *A. correntinus* showing a high number of chromosomes bearing sites of 5S rDNA, it was observed synteny of 5S and 18S sites in one of the chromosomes from pairs 4 and 12 (Fig. 2c). Mantovani

et al. (2005) also observed synteny in a population of *A. scabripinnis* for these ribosomal genes, being a characteristic considered derived for *Astyanax* in terms of genomic organization and chromosomal evolution. Therefore, despite the distribution of 5S rDNA sites being considered conserved for some groups of fish, the results observed show variation regarding the number and location of these ribosomal genes in *Astyanax*.

Distribution pattern of heterochromatin

With regards to the distribution pattern of heterochromatin, although low amount in *A. abramis*, *A. asuncionensis* and *Astyanax* sp., it was found mainly in centromeric and interstitial-proximal position, in addition to NORs associated (Fig. 1b, d, h). These results were also observed in other phylogenetically close species of the genus, as in *A. altiparanae* (Domingues et al. 2007, Ferreira-Neto et al. 2009) and *A. jacuhiensis* (Pacheco et al. 2010). Still, in karyotypes *A. abramis* and *A. asuncionensis*, the first pair of *a* chromosomes, with the NORs on the p arm, both share the same pattern of bands: centromeric heterochromatin, interstitial-proximal heterochromatin on the q arm, and telomeric heterochromatin on the q arm, and this pair may represent homeological chromosomes (Fig. 1b, d). In *A. correntinus*, centromeric heterochromatins were observed in most *m-sm* chromosome pairs (Fig. 1f), being this pattern similar to that observed in *A. schubarti* (Daniel-Silva and Almeida-Toledo 2001). Differently from *A. abramis*, *A. asuncionensis* and *A. correntinus*, *Astyanax* sp. showed a particular pattern, with the presence of five *st-a* chromosome pairs carrying heterochromatin in interstitial-proximal position on the q arm, in addition to some *sm* chromosome pairs bearing heterochromatin both in centromeric and telomeric positions on the q arm (Fig. 1h). According to our morphological observation, *Astyanax* sp. is part of the *A. scabripinnis* complex defined by Bertaco and Malabarba (2001), but does not fit into any taxonomically described species in this complex. The cytogenetic data corroborate this hypothesis; therefore, we believe that this is a new species, and that a greater number of specimens are required to confirm it.

Conclusions

The present study shows species-specific cytogenetic markers which can serve in diagnosis and differentiation between *A. abramis* and *A. asuncionensis*, considered cryptic species (deep body; presence of a well defined, black, horizontal humeral spot; absence of maxillary tooth; and the presence of circuli in posterior field of scales), as well as strengthening the occurrence of a species of *Astyanax* not yet described taxonomically (elongated body; absence of a well defined, black, horizontal humeral spot; presence of one maxillary tooth; and the absence of circuli in posterior field of scales). In addition, the data obtained from first cytogenetic studies in *A. correntinus* suggest a high similarity

with *A. schubarti*, suggesting that these species may belong to the same morphological group (deep body; absence of a well defined, black, horizontal humeral spot; presence of one maxillary tooth; and the absence of circuli in posterior field of scales; broad silvery lateral band) and that can be phylogenetically related. Further studies, including another species of the "clade *Astyanax*" and molecular analyses of mitochondrial genes sequences, may confirm these hypotheses.

Acknowledgements

The authors are grateful to Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) for authorizing the capture of the fishes (License number: SISBIO 10522-1). The authors thank to Universidade Estadual do Oeste do Paraná (UNIOESTE), Parque Nacional do Iguaçu, Macuco Safari, Grupo de Pesquisa em Tecnologia de Produção e Conservação de Recursos Pesqueiros e Hídricos (GETECH) and to the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (NUPÉLIA) for logistical support. This study was financed by CAPES (Coordenadoria de Aperfeiçoamento de Ensino Superior), Fundação Araucária (Fundação Araucária de Apoio e Desenvolvimento Científico e Tecnológico do Estado do Paraná) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

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CompCytogen 9(1): 67–78 (2015)
doi: 10.3897/CompCytogen.v9i1.8804
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Cytogenetic analysis of two locariid species (Teleostei, Siluriformes) from Iguatemi River (Parana River drainage) in Brazil

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Academic editor: *N. Bogutskaya* | Received 22 October 2014 | Accepted 30 December 2014 | Published 10 March 2015

http://zoobank.org/C8F32234-AC30-429C-AC21-CA35FE03E366

Citation: Fernandes CA, Alves DS, Guterres AR, Martins-Santos IC (2015) Cytogenetic analysis of two locariid species (Teleostei, Siluriformes) from Iguatemi River (Parana River drainage) in Brazil. Comparative Cytogenetics 9(1): 67–78. doi: 10.3897/CompCytogen.v9i1.8804

Abstract

Fishes of the Loricariidae family, known as "cascudos", constitute an endemic group in Neotropical freshwaters. In this study, were cytogenetically examined two species of Loricariidae (*Pterygoplichthys anisitsi* Eigenmann & Kennedy, 1903 and *Farlowella amazonum* (Günther, 1864) belonging to Hypostominae and Loricariinae subfamilies respectively) from Iguatemi River. Our study provide the first description regarding C-band and fluorochromic analysis in *F. amazonum*. In *Farlowella amazonum*, diploid number was 58 chromosomes, with single Ag-NOR and heterochromatic blocks in centromeric regions of some chromosomes and large subtelomeric blocks were evidenced on the long arm of the pair 27, being this region CMA_3^+/$DAPI^-$. The *Pterygoplichthys anisitsi* showed diploid number equal 52 chromosomes, with single Ag-NOR and heterochromatic blocks in centromeric and telomeric regions of some chromosomes and conspicuous large telomeric blocks on the long arm of the pair 10, being this region CMA_3^+/$DAPI^-$. The results show that karyotype formula is nonconservative in *P. anisitsi* and *F. amazonum*.

Keywords

Pterygoplichthini, Chromomicin A_3, DAPI, Chromosomal evolution, Fishes

Introduction

Fishes of the Loricariidae family, known as "cascudos", constitute an endemic group in Neotropical freshwaters and are morphologically characterised by the body covered by several rows of plates and a ventral mouth with lips forming a sucker (Graça and Pavanelli 2007). Currently, this family includes 887 valid species in seven subfamilies: Hypoptopomatinae, Loricariinae, Hypostominae, Neoplecostominae, Lithogeninae, Delturinae, and Ancistrinae (Eschmeyer and Fong 2014). In earlier phylogenetic studies, Ancistrinae (as a tribe Ancistrini) was considered as a tribe in the family Hypostominae along with Hypostomini, Rhinelepini, Pterygoplichthini, and Corymbophanini (Armbruster 2004). According to the latter author, the Pterygoplichthini is composed by genera and species groups: *Pterygoplichthys* Gill, 1858, *Hemiancistrus annectens* group, being that *Liposarcus* Günther, 1864, and *Glyptoperichthys* Weber, 1991 are recognized as synonyms of *Pterygoplichthys*.

Available cytogenetic data for Hypostominae subfamily show that the diploid number ranges from 2n = 34 in *Ancistrus cuiabae* Knaack, 1999 (Mariotto et al. 2009) and *Anscistrus* sp. *purus* INPA-25625 (Oliveira et al. 2009) to 2n = 84 in *Hypostomus* sp. 2 (Cereali et al. 2008). The tribe Hypostomini is one of the most studied from the cytogenetic point of view, with wide variation in chromosome number ranging from 2n = 54 in *Hypostomus plecostomus* (Linnaeus, 1758) (Muramoto et al. 1968, cited by Artoni and Bertollo 2001) to 2n = 80 in *Hypostomus* sp. E (Artoni and Bertollo 1996). On the other hand, for tribe Pterygoplichthini cytogenetic studies are scarce, which all species presenting a diploid number of 52 chromosomes, as observed in *Pterygoplichthys joselimaianus* (Weber, 1991) (Oliveira et al. 2006), *Pterygoplichthys anisitsi* Eigenmann & Kennedy, 1903 (cited as *Liposarcus anisitsi* – Alves et al. 2006), *Pterygoplichthys multiradiatus* (Hancock, 1828) (cited as *Liposarcus multiradiatus* – Alves et al. 2006) and *Pterygoplichthys gibbiceps* (Kner, 1854) (cited as *Glyptoperichthys gibbiceps* – Alves et al. 2006).

According to Alves et al. (2003), in Loricariinae subfamily, only some genera as: *Harttia* Steindachner, 1877, *Loricaria* Linnaeus, 1758, *Loricariichthys* Bleeker, 1862, *Rineloricaria* Bleeker, 1862, and *Sturisoma* Swainson, 1838 were analyzed cytogenetically, presenting diploid number ranging of 2n = 36 in *Rineloricaria latirostris* (Boulenger, 1900) (Giuliano-Caetano 1998) to 2n = 74 in *Sturisoma* cf. *nigrirostrum* Fowler, 1940 (Artoni and Bertollo 2001). Specifically, in the genus *Farlowella* Eigenmann & Eigenmann, 1889 (Loricariinae) cytogenetic studies are rare, and restricted to *F. amazonum* (Günther, 1864), which has showed a diploid number of 58 chromosomes (Fernandes et al. 2012).

In the present study, we carried out cytogenetic analyses in *Pterygoplichthys anisitsi* Eigenmann & Kennedy, 1903 and *Farlowella amazonum* (Günther, 1864). Besides Giemsa, we used C-band, Ag-NOR, CMA_3 and DAPI techniques to evaluate cytogenetically the species. Our results provide the first description of C-band and analysis with fluorochromes in *F. amazonum* and these results were used to discuss some aspects of the chromosome evolution in the Hypostominae and Loricariinae subfamilies.

Materials and methods

Four (2 males and 2 females) specimens of *Farlowella amazonum*, from Dourado stream and four (2 males and 2 females) specimens of *Pterygoplichthys anisitsi*, from Água Boa stream were analyzed. Dourado (23°51'04,9"S and 54°25'13,9"W) and Água Boa (23°50'16,65"S and 54°20'55,54"W) streams are tributaries of right bank of the Iguatemi River, Mato Grosso do Sul State, Brazil (Fig. 1).

The fishes were identified and deposited in the State University of Mato Grosso do Sul, Mundo Novo. The experiments followed ethical conducts, and before evisceration process, the fishes were anesthetized by an overdose of clove oil (Griffiths 2000). Metaphasic chromosomes were obtained from anterior kidney cells using the air-drying technique (Bertollo et al. 1978). Analysis of the C-positive heterochromatin (C-bands) followed the basic procedure of Sumner (1972), with some minor adaptations. The NORs were detected by means of silver nitrate staining (Ag-NORs), according to Howell and Black (1980). Regions rich in GC and AT were detected by fluorochromes chromomycin A_3 (CMA_3) and DAPI (4'6-diamidino-2-phenylindole) respectively, according to procedure proposed by Schmid (1980).

About 30 metaphases were analyzed for each specimen and those with better chromosome morphology were used in the karyotype analysis. The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a) according to their arm ratio (Levan et al. 1964). For the determination of the fundamental number (FN), or number of chromosome arms, the m, sm and st chromosomes were considered as bearing two arms and the acrocentric chromosomes only one arm.

Results

Pterygoplichthys anisitsi presented a modal diploid number of 52 chromosomes in males and females, distributed in 14m+26sm+8st+4a, with a FN of 100 in both sexes (Fig. 2a). The Ag-NORs were located in a subtelomeric position on the long arm of the acrocentric pair 9, coinciding with a secondary constriction (Fig. 2a). Heterochromatic blocks evident at telomeric regions were observed in the pairs 17, 25 and 26. Also, evident bitelomeric markings were present in the pairs 10 and 15, and conspicuous large telomeric blocks were present on the long arm of the pair 10 and interstitial blocks were evidenced on the long arm pair 9, adjacent to the Ag-NOR region (Fig. 2b). CMA_3 staining produced fluorescent signals in the telomeric regions of some chromosomes, fluorescent signals bitelomeric in the pairs 9, 10 being conspicuous the fluorescent signals on the long arm of the pair 10 (Fig. 3a). DAPI staining proved adjacent markings to $CMA3^+$ and fluorescent signals in the telomeric regions of some chromosomes, indicating that those regions are rich in AT (Fig. 3b). In addition, DAPI staining revealed pale regions corresponding to telomeric regions on the long arm of the pair 10, coinciding with CMA_3^+ region, confirming that those regions are poor in AT.

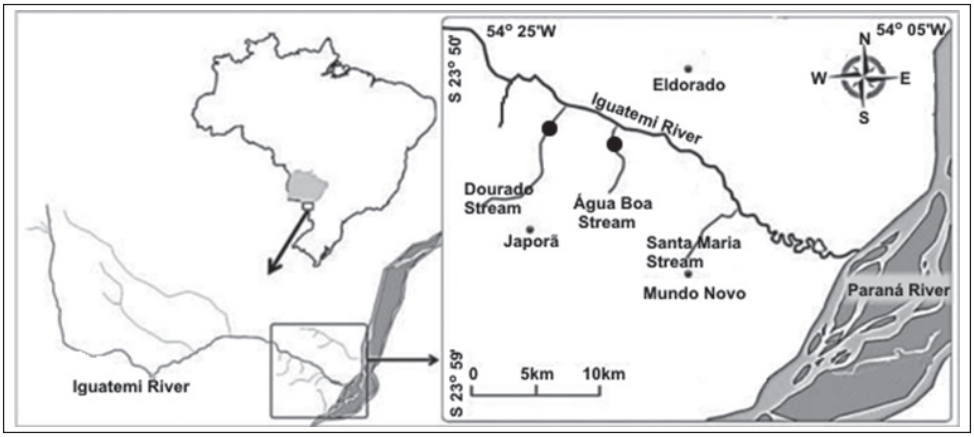

Figure 1. Localization of the Água Boa and Dourado streams from Iguatemi River Basin where specimens were captured. Dark circles indicate the sampled points.

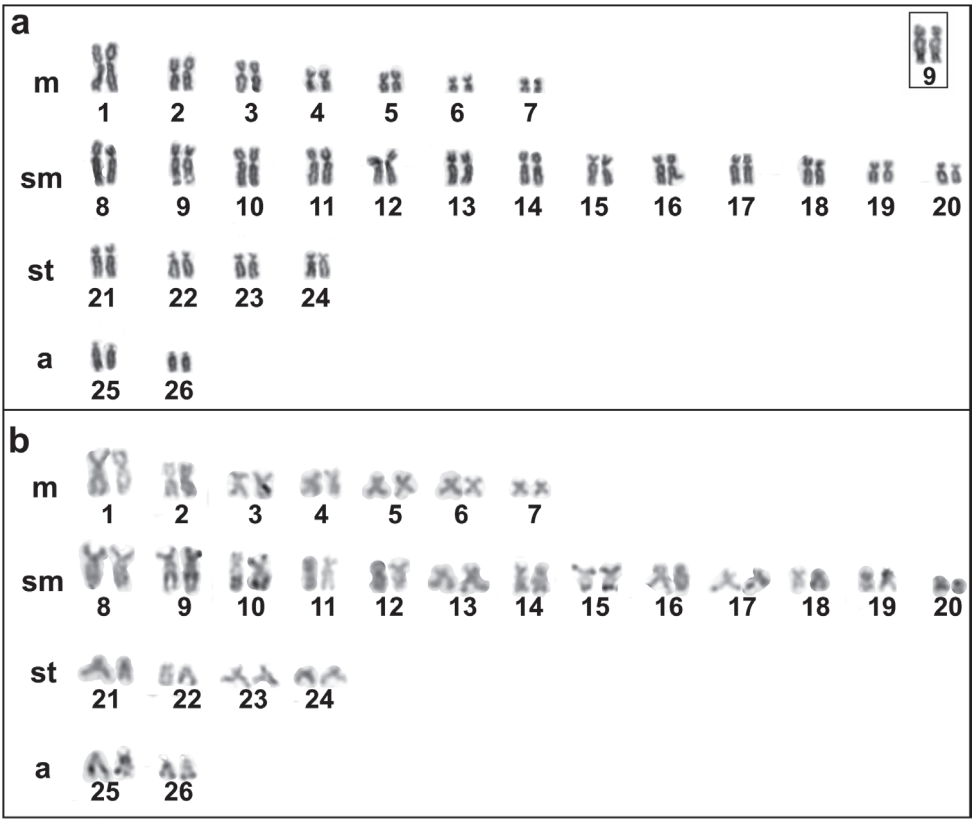

Figure 2. Karyotypes stained with Giemsa (**a**) and C-banding (**b**) of *Pterygoplichthys anisitsi* from Água Boa stream. Box: pair 9, bearing the NOR.

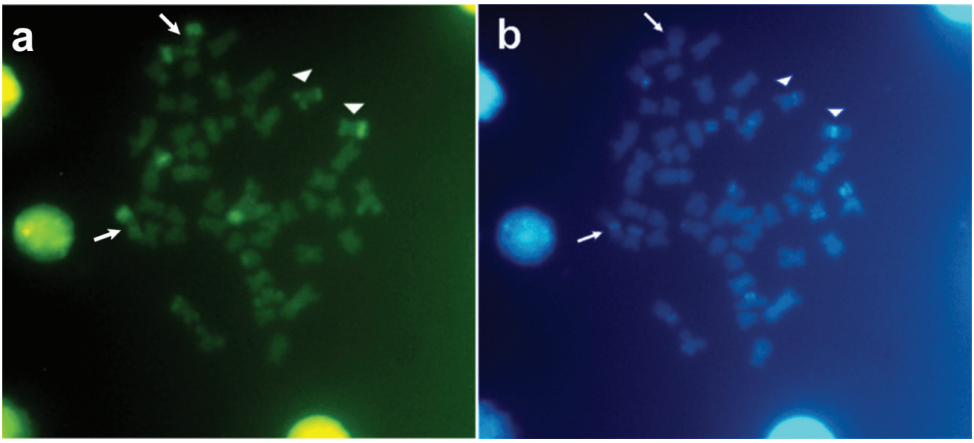

Figure 3. Metaphases of *Pterygoplichthys anisitsi* stained with (**a**) Chromomycin A_3 and (**b**) DAPI. Arrows indicate pair 10 and arrows head indicate pair 9 (NOR-bearing).

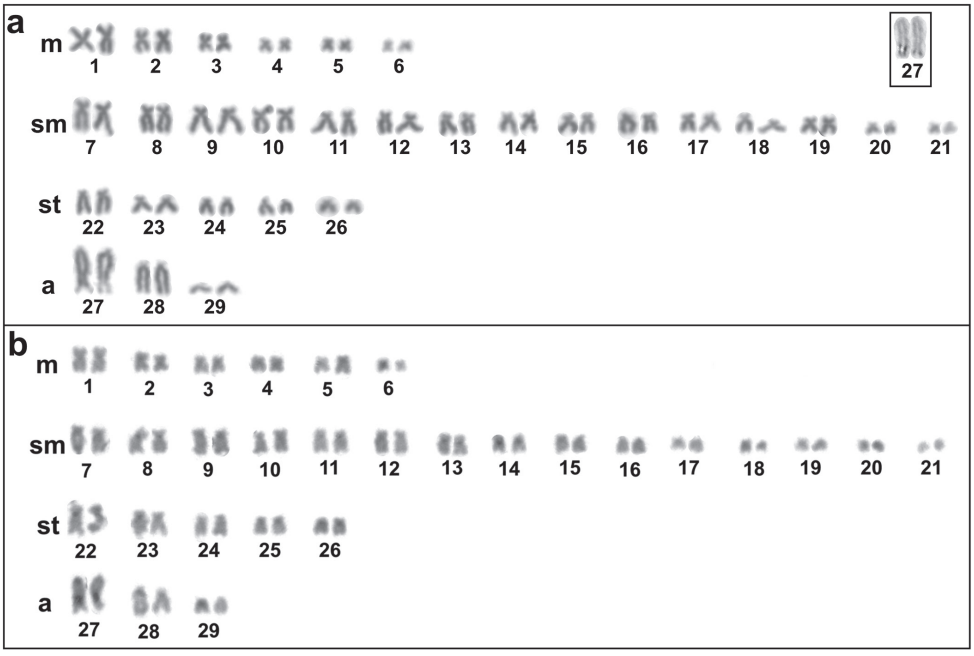

Figure 4. Karyotypes stained with Giemsa (**a**) and C-banding (**b**) of *Farlowella amazonum* from Dourado stream. Box: pair 27, bearing the NOR.

Farlowella amazonum presented a modal diploid number of 58 chromosomes in males and females, distributed in 12m+30sm+10st+6a, with a FN of 110 in both sexes (Fig. 4a). The Ag-NORs were located in a telomeric position on the long arm of the pair 27, coinciding with a secondary constriction and with size heteromorphism (Fig. 4a). Heterochromatic blocks at centromeric regions were observed in the pairs

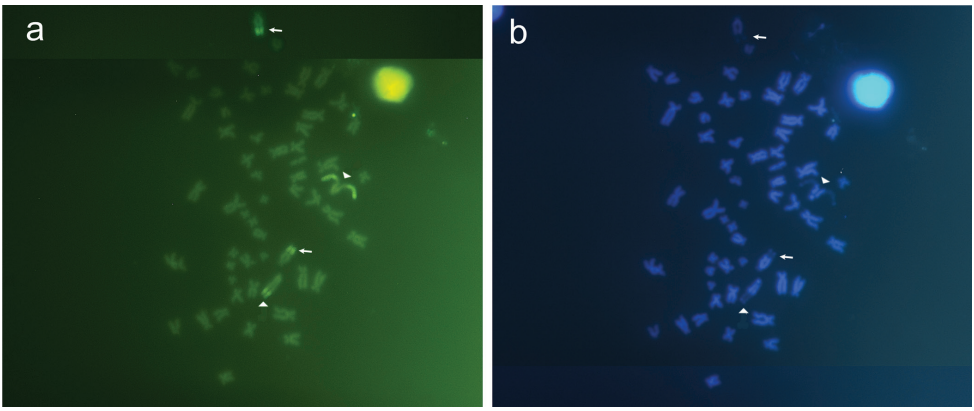

Figure 5. Metaphases of *Farlowella amazonum* stained with (**a**) Chromomycin A_3 and (**b**) DAPI. Arrows indicate pair 28 and arrows head indicate pair 27 (NOR-bearing).

8, 11, 13, 14, 22, 23 and 27 and large subtelomeric blocks were evidenced on the long arm of the pair 27, adjacent to the NOR region (Fig. 4b). CMA_3 staining produced fluorescent signals on the long arm of the pair 27, corresponding to Ag-NOR region with size heteromorphism. This staining also evidenced fluorescent signals on the end portion of the long arm of the pair 28, indicating that these regions are rich in GC (Fig. 4a). DAPI staining revealed only pale regions corresponding to the CMA_3 marked regions, confirming that those regions are poor in AT (Fig. 4b).

Discussion

Diploid chromosome numbers ranged from 2n = 34 to 2n = 84 in Hypostominae (Cereali et al. 2008; Mariotto et al. 2009). However, when different tribes of Hypostominae are separately analyzed, it is possible to notice that this variation is not widespread among them. If Pterygoplichthini is concerned, the diploid number of 52 chromosomes found in *P. anisitsi* is also observed in *Pterygoplichthys joselimaianus* from Lago Quatro Bocas (Oliveira et al. 2006), *P. anisitsi* from Miranda and Tietê Rivers (Alves et al. 2006), *P. multiradiatus* from Orinoco River (Alves et al. 2006), and *P. gibbiceps* from Orinoco River (Alves et al. 2006). In spite of this trend towards conservatism in relationship to diploid number, differences in karyotype constitution in Pterygoplichthini, especially regarding the number of acrocentric chromosomes are observed. *Pterygoplichthys anisitsi* presented four acrocentric chromosomes, as well as, populations of *P. anisitsi* from Tietê River (Alves et al. 2006) and Preto River (Artoni et al. 1999), while *P. anisitsi* from Miranda River presented sixteen acrocentric chromosomes (Alves et al. 2006). On the other hand, *P. joselimaianus* (Oliveira et al. 2006), *P. multiradiatus* (Alves et al. 2006) and *P. gibbiceps* (Alves et al. 2006) do not present acrocentric chromosomes. Thus, all these populations of *Pterygoplichthys* studied differ in their karyotypic formulae, with intra- and interspecific variations, suggesting the oc-

currence of chromosome rearrangements, such as pericentric inversions, that can alter the morphology of the chromosomes without changing the diploid number.

The intraspecific variation in *P. anisitsi* may be explained by the distribution of species already analyzed. Thus, *P. anisitsi* populations of the Iguatemi (present study), Tietê and Preto Rivers (Alves et al. 2006) that belong to the same watershed (Upper Paraná River Basin) showed no differences in the number of acrocentric chromosomes, except the *P. anisitsi* population of the Miranda River (Alves et al. 2006) that belongs to another basin (Paraguai River basin) showed a number of acrocentric chromosomes different. Therefore, the geographical isolation of *P. anisitsi* populations (Paraná River Basin and the Paraguai River Basin) may have facilitated the establishment of karyotypic variation. The *P. multiradiatus* and *P. gibbiceps* populations (Alves et al. 2006) are also geographically isolated (Orinoco River basin) of the *P. anisitsi* populations. Thus, the lack of gene flow between them could favor the establishment of distinctive changes in each sample, putatively resulting in a speciation process.

With respect to nucleolar organizer regions, the present study detected two active NORs in *P. anisitsi*. Others species previously analyzed of tribe Pterygoplichthini as *P. joselimaianus* (Oliveira et al. 2006), *P. anisitsi* (Artoni et al. 1999, Alves et al. 2006), *P. multiradiatus* (Alves et al. 2006; Alves et al. 2012) and *P. gibbiceps* (Alves et al. 2006) also present this same pattern, with subterminal markings, but with differences in location (long or short arm) and type of NOR-bearing chromosome. According to Oliveira and Gosztonyi (2000) the condition of single Ag-NORs in subterminal location is the possible basal condition for the Siluriformes. Thus, in Pterygoplichthini all species analyzed presented single Ag-NORs in subterminal location, suggesting the maintenance of this basal condition. The presence of single Ag-NORs also is described in Ancistrini (Alves et al. 2006; Mariotto et al. 2011; Cardoso et al. 2013), which is coincident with Ag-NORs results for Pterygoplichthini. Furthermore, diploid number of 52 chromosomes is predominant in Ancistrini (Kavalco et al. 2005, Alves et al. 2006). This statement reinforces the sister-group relationship between Pterygoplichthini and Ancistrini hypothesized by Armbruster (2004).

In *P. anisitsi*, the present study revealed that Ag-NOR is compositionally GC-rich and heterochromatin blocks adjacent NOR region are compositionally AT-rich (Fig. 6a). In *Hypostomus* sp. B heterochromatin blocks adjacent NOR regions also are compositionally AT-rich (Artoni and Bertollo 1999). In addition, heterochromatin blocks visualized by C-banding correspond to majority of the chromosomes marked with CMA_3, suggesting that these constitutive heterochromatins are compositionally GC-rich. This can be clearly observed in the pair 10, which was C-band$^+$, CMA3$^+$ and DAPI$^-$. The DAPI standing produced fluorescent signals adjacent to CMA3$^+$ markings, and also in the telomeric regions of some chromosomes, probably in those with heterochromatic blocks CMA3$^-$, showing that these regions are compositionally AT-rich. In Pterygoplichthini, there are few studies focused on constitutive heterochromatin, which are restricted to *P. anisitsi* from Preto River (Artoni et al. 1999), confirming the pattern of compositionally GC-rich regions. The constitutive heterochromatin GC-rich are commonly found in Loricariidae (Artoni et al. 1999; Artoni and Bertollo

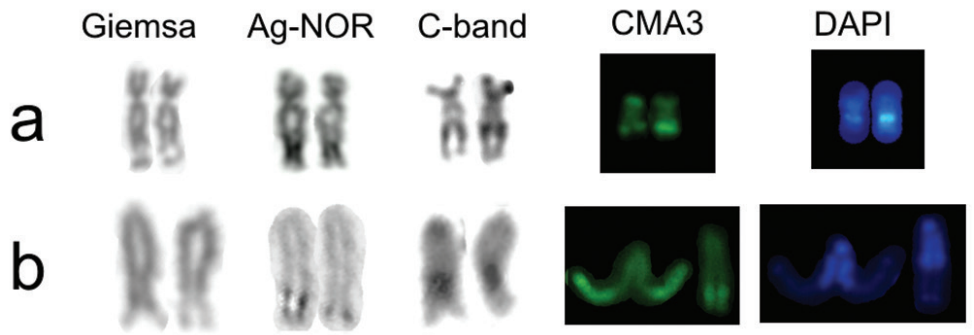

Figure 6. NOR-bearing chromosomes of *Pterygoplichthys anisitsi* (upper row – **a**) and *Farlowella amazonum* (lower row – **b**).

1999; Kavalco et al. 2004; Rubert et al. 2008). However, constitutive heterochromatin AT-rich as described for *P. anisitsi* is rare event among fishes, being reported mainly among some Hypostominae species (Artoni and Bertollo 1999).

With 239 species, Loricariinae is second largest subfamily of Loricariidae in species number (Eschmeyer and Fong 2014). Despite this high species number, cytogenetic descriptions are restricted to the karyotypes of few genera. *Farlowella amazonum* analyzed in this study had a diploid number of 58 chromosomes, which is coincident with previously described diploid number of a population from Água Boa stream (Fernandes et al. 2012), but with minor karyotype differences, mainly regarding to the number of metacentric and submetacentric chromosomes. The population analyzed here presented 12 metacentric and 30 submetacentric chromosomes, while the population previously described by Fernandes et al. (2012) presented six metacentric and 38 submetacentric chromosomes. Although the two streams are tributaries of the Iguatemi River, which could facilitate gene flow between the two populations of *F. amazonum* and conservation of micro- and macrostructure karyotypic, it is possible that this is not happening, since the differences in numbers metacentric and submetacentric chromosomes between the two populations is significant. Therefore, probably the isolation two populations *F. amazonum* may have facilitated the establishment of karyotypic changes. In this species, the occurrence of chromosome rearrangements, such as pericentric inversions, that can alter the morphology of the chromosomes without changing the diploid number probably occurred in the karyotypic evolution of the group. This inference emphasizes the importance of develop more studies focused on cytogenetic of genus *Farlowella* in order to clear such question.

In *F. amazonum*, were detected single Ag-NORs in a telomeric position on the long arm of the pair 27, corresponding to the same location described in *F. amazonum* from Água Boa stream (Fernandes et al. 2012). Others species previously analyzed of Loricariinae as *Harttia kronei* Miranda Ribeiro, 1908, *Rineloricaria kronei* (Miranda Ribeiro, 1911), *Rineloricaria cadeae* (Hensel, 1868), *Rineloricaria* n. sp., *Harttia lori-*

cariformis Steindachner, 1877 (Alves et al. 2003) and *Harttia punctata* Rapp Py-Daniel & Oliveira, 2001 (Blanco et al. 2014) also presented two NOR-bearing chromosomes, but located on the short arm, except the *H. loricariformis* and *H. punctata* that presented NOR interstitial on the long arm.

The CMA_3/DAPI results for *F. amazonum*, which is the first description of literature, showed that Ag-NOR is compositionally GC-rich (Fig. 6b). In *Rineloricaria cadeae*, *Rineloricaria strigilata* (Hensel, 1868) and *Rineloricaria pentamaculata* Langeani & de Araújo, 1994 the Ag-NOR also showed compositionally GC-rich (Maia et al. 2010). On the other hand, *Harttia loricariformis* that presented C-bands conspicuous in the NOR-bearing did not present positive CMA_3 or DAPI staining heterochromatin (Kavalco et al. 2004). NOR-size heteromorphism among homologues detected in *F. amazonum* by Ag-NOR and confirmed by CMA_3/DAPI staining, is a common event in fish. According to Phillips et al. (1989), CMA_3 analysis coupled to silver nitrate has been useful in detecting polymorphism of these sites. The NOR-size heteromorphism may be explained by transposition events or unequal crossing-over in this region.

Regarding to C-band pattern, also inedited for *F. amazonum*, the results revealed weak centromeric markings in some chromosomes and large subtelomeric blocks on the long arm of the pair 27, adjacent to the NOR region. These large heterochromatic blocks showed correlation with CMA_3 markings, suggesting that these constitutive heterochromatins are compositionally GC-rich (Fig. 6b).

The interstitial position heterochromatic blocks adjacent to the NOR region in *F. amazonum* and *P.anisitsi* may indicate that heterochromatin dispersive processes, as proposed by (Schweizer and Loidl 1987), are common to the subfamilies Hypostominae and Loricariinae, that revealed to be independent of the heterochromatin compositional, AT-rich in *P. anisitsi* and GC-rich in *F. amazonum*.

According to Kavalco et al. (2005) in Loricariidae, the diploid number of 54 chromosomes seems to be a plesiomorphic condition. The cytogenetic data obtained in present study for *P. anisitsi*, as well as, those described in the literature for species of Pterygoplichthini (Artoni et al. 1999, Alves et al. 2006, Oliveira et al. 2006), show that all have 2n = 52 chromosomes. The presence of 2n = 52 chromosomes in Pterygoplichthini is probably an apomorphic characteristic, suggesting the reduction in the diploid number in the ancestor of this tribe. The diploid number of 58 chromosomes in *F. amazonum* also is probably an apomorphic characteristic, suggesting the increase in the diploid number in the ancestor in the *Farlowella*. According to Kavalco et al. (2005) in the subfamily Loricariinae, both centric fusion, centric fissions and pericentric inversions arise as common karyotypic rearrangements.

Acknowledgements

The authors thank the Brazilian agency Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT) for financial support.

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CompCytogen 9(1): 79–87 (2015)
doi: 10.3897/CompCytogen.v9i1.8587
http://compcytogen.pensoft.net

SHORT COMMUNICATIONS

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

B chromosomes in the species *Prochilodus argenteus* (Characiformes, Prochilodontidae): morphologicalidentity and dispersion

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Academic editor: *G. Furgala-Selezniow* | Received 15 September 2014 | Accepted 28 January 2015 | Published 20 March 2015

http://zoobank.org/83103179-4367-42A9-8621-ACA7043AA67F

Citation: Penitente M, Foresti F, Porto-Foresti F (2015) B chromosomes in the species *Prochilodus argenteus* (Characiformes, Prochilodontidae): morphological identity and dispersion. Comparative Cytogenetics 9(1): 79–87. doi: 10.3897/CompCytogen.v9i1.8587

Abstract

B chromosomes have attracted the attention of Neotropical fish cytogeneticists in recent years, both for their remarkable occurrence in this group and also because of the interest in studies of the genetic structure and role played in the genome of these organisms. The aim of this study was to report the first occurrence of supernumerary chromosomes in *Prochilodus argenteus* (Agassiz, 1829), this being the fifth carrier species among thirteen within the genus *Prochilodus* (Agassiz, 1829). The extra elements identified in this species are small sized heterochromatic chromosomes characterized by a low mitotic instability index, being very similar to other supernumerary chromosomes described in the species of the genus *Prochilodus*. Morphology, structure and dispersion of the supernumerary genomic elements which occur in species of this genus are discussed aiming to better understand aspects involved the origin of supernumerary chromosomes and the differentiation process and relationships among species of this family.

Keywords

Curimbatá, cytogenetic, neotropical fish, supernumerary chromosomes

Introduction

Among the Characiformes fish, representatives of the family Prochilodontidae are distinguished by their abundance in the environments in which they occur, by their wide distribution in South America and the high migratory capacity displayed by the species of this group of organisms (Castro 1990, Sivasundar et al. 2001, Turner et al. 2004). According to Castro and Vari (2004) the family Prochilodontidae consists of three genera: *Prochilodus* (Agassiz, 1829), *Semaprochilodus* (Fowler, 1941), and *Ichthyoelephas* (Posada Arango, 1909), which include thirteen, six and two species, respectively. This family can be easily distinguished from other Characiformes families through a distinct set of morphological characters (Castro 1990), and fishes of the genus *Prochilodus* can be highlighted as a pioneer group in studies of B chromosomes among Neotropical fishes.

Besides the conservative karyotype formulae found among representatives of the genus *Prochilodus*, variation in the chromosome number can be observed among species due to the occurrence of supernumerary chromosomes. Among seven species of this genus, the occurrence of B chromosomes has been identified and described in four species, present in *Prochilodus lineatus* Valenciennes, 1836 (Pauls and Bertollo 1983), *Prochilodus nigricans* (Agassiz, 1829) (Pauls and Bertollo 1990, Venere et al. 1999), *Prochilodus brevis* (Steindachner, 1874) (Pauls and Bertollo 1990) and *Prochilodus mariae* Eigenmann, 1922 (Oliveira et al. 2003), but not occurring in cells of *Prochilodus argenteus* (Agassiz, 1829), *Prochilodus costatus* (Valenciennes, 1850) and *Prochilodus vimboides* (Kner, 1859), as described by Pauls and Bertollo (1990).

In this paper we describe for the first time the occurrence of supernumerary chromosomes in *P. argenteus*, identifying its similarity with the extra genomic elements characteristic of this group. The description of the occurrence of supernumerary chromosomes in this species, besides addressing structural and morphological aspects, has also broadened the knowledge of aspects involved in the differentiation, evolution and relationships among species of the genus *Prochilodus*.

Material and methods

In this study 29 individuals of the species *P. argenteus* (Table 1) resulting from crosses performed in the fish farm Projeto Peixe, Cravinhos – SP, Brazil, involving matrices derived from natural population of the São Francisco River, captured near Três Marias – MG, Brazil, were analyzed. The procedures for collection, maintenance and analysis of the fish were performed in accordance with the international protocols on animal experimentation followed by the Universidade Estadual Paulista. Morphometric and meristic data were taken following Castro and Vari (2004) and specimens were deposited at Laboratório de Biologia e Genética de Peixes fish collection, Universidade Estadual Paulista, Botucatu, SP. Vouchers of the used material are described in Table 1.

Table 1. B chromosome frequency and Mitotic Instability index (MI) of somatic cells in *P. argenteus*.

Specimen identification	Number of B per cell				MB	N	MI
	0B	1B	2B	3B			
4170	30	-	-	-	0B	30	0.000
4172	1	12	-	-	1B	13	0.157
4173	30	-	-	-	0B	30	0.000
4175	30	-	-	-	0B	30	0.000
4176	3	27	-	-	1B	30	0.200
4177	1	29	-	-	1B	30	0.034
4178	-	1	29	-	2B	30	0.036
4180	5	25	-	-	1B	30	0.337
4181	-	30	-	-	1B	30	0.000
4182	-	2	28	-	2B	30	0.065
4183	2	12	-	-	1B	14	0.294
4184	-	15	-	-	1B	15	0.000
4188	30	-	-	-	0B	30	0.000
4189	30	-	-	-	0B	30	0.000
4190	1	15	-	-	1B	16	0.133
4250	4	26	-	-	1B	30	0.274
4251	30	-	-	-	0B	30	0.000
4252	-	14	-	-	1B	14	0.000
4253	30	-	-	-	0B	30	0.000
4254	1	11	-	-	1B	12	0.173
4256	-	2	15	-	2B	17	0.111
4257	25	-	-	-	0B	25	0.000
4258	-	1	13	-	2B	14	0.072
4259	30	-	-	-	0B	30	0.000
4260	2	28	-	-	1B	30	0.136
4261	-	-	4	26	3B	30	0.082
4262	3	15	-	-	1B	18	0.337
4263	30	-	-	-	0B	30	0.000
4264	1	13	-	-	1B	14	0.152
						X_{MI}	0,136

(MB) modal number of B chromosomes, (N) number of metaphases analyzed, (MI) Mitotic Instability index, (X_{MI}) average MI among individuals with supernumerary chromosomes.

Chromosome preparations involved previous use of mitosis stimulation (Lozano et al. 1988, Oliveira et al. 1988) followed by cell suspension preparations using kidney tissue fragments of the individuals, according to the protocol proposed by Foresti et al. (1981). The karyotype organization was performed according to the method of Levan et al. (1964), using images processed by Adobe Photoshop CS5 program. To quantify the Mitotic Instability index (MI) the method of Pardo et al. (1995) was used. Active

nucleolar regions (NOR) in metaphase chromosomes were identified by using silver nitrate staining (Howell and Black 1980) and the detection of constitutive heterochromatin (C-banding) was performed according to Sumner (1972).

Chromosomal mapping of ribosomal genes was performed with the technique of fluorescent *in situ* hybridization (FISH) according to Pinkel et al. (1986) using 5S and 18S rDNA probes obtained by PCR (Polymerase Chain Reaction) from genomic DNA of *P. argenteus*. Primers A (5_-TACGCCCGATCTCG TCCGATC-3_) and B (5_-CAGGCTGGTATGGCCGTAAGC-3_) (Pendás et al. 1994) were used to obtain the 5S probe, and NS1 (5_-GTAGTCATATGCTTGTCTC-3_) and NS8 (5_-TCCGCAGGTTCACCTACGGA-3_) according White et al. (1990), to obtain the 18S rDNA probe. The 5S probe was labeled with biotin-dUTP and the 18S probe was labeled with Digoxigenin-dUTP (Roche) by PCR, according to the manufacturer's instructions. The preparations were stained with DAPI (4-6-diamidino-2-phenylindole) and examined in a fluorescence light microscope (BX 50, Olympus) equipped with an Olympus Q-color 5 digital camera. The photomicrographs were obtained using Q-Capture Pro 5.1.1.14 software.

Results and discussion

Cytogenetic analysis performed on specimens of *Prochilodus argenteus* revealed the diploid number of 2n = 54 and fundamental number of 108 for this species, with a karyotype composed of meta and submetacentric chromosome types (Figure 1). No morphological differentiation between males and females was detected, confirming data published by Pauls and Bertollo (1983, 1990), Hatanaka and Galleti Jr. (2004) and, more recently by Voltolin et al. (2013). This karyotype identity, also present in other species already described is a conserved feature in the genus *Prochilodus*, and it may be identified also among the components of related groups (Galetti Jr. et al. 1994, Arai 2011). Among the 29 individuals analyzed the presence of up to three B chromosomes was observed, with a modal number in metaphases analyzed between zero and one B chromosome (Table 1, Figure 2), being that 14 individuals presented one B chromosome, four presented two B chromosomes and a single specimen carried three B chromosomes. Ten individuals were not carriers of supernumerary chromosomes in their cells in this sample.

One of the first descriptions of B chromosomes in Neotropical fish refers to additional chromosomes found in *P. lineatus* by Pauls and Bertollo (1983). Since then this species has been one of the most widely used in studies on the origin, inheritance and maintenance of these chromosomes in fish. Moreover, the occurrence of supernumerary chromosomes was also described in other species within the genus *Prochilodus* (Pauls and Bertollo 1990, Venere et al. 1999, Oliveira et al. 2003), adding to this list the species *P. argenteus* taken in this work. The number of studied species thus highlights this genus as one of the most representative and significant among Neotropical fishes concerning with the occurrence and study of these extra elements.

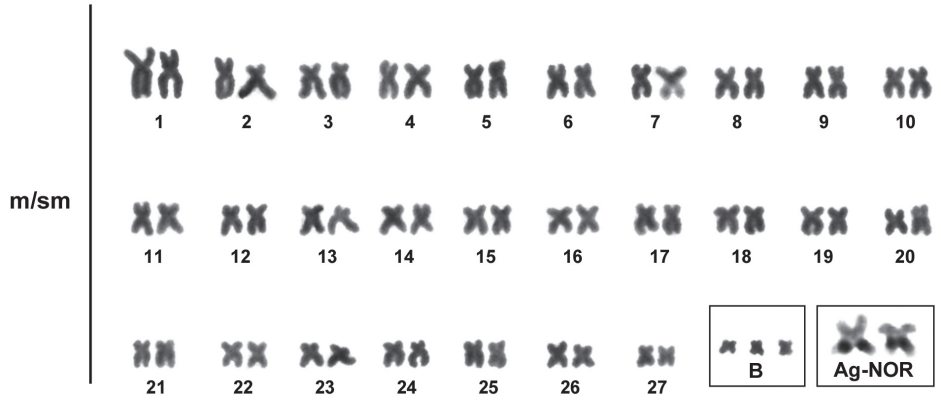

Figure 1. Karyotype of *P. argenteus* (2n=54 chromosomes). In the inset, three B chromosomes and the NOR bearing pair.

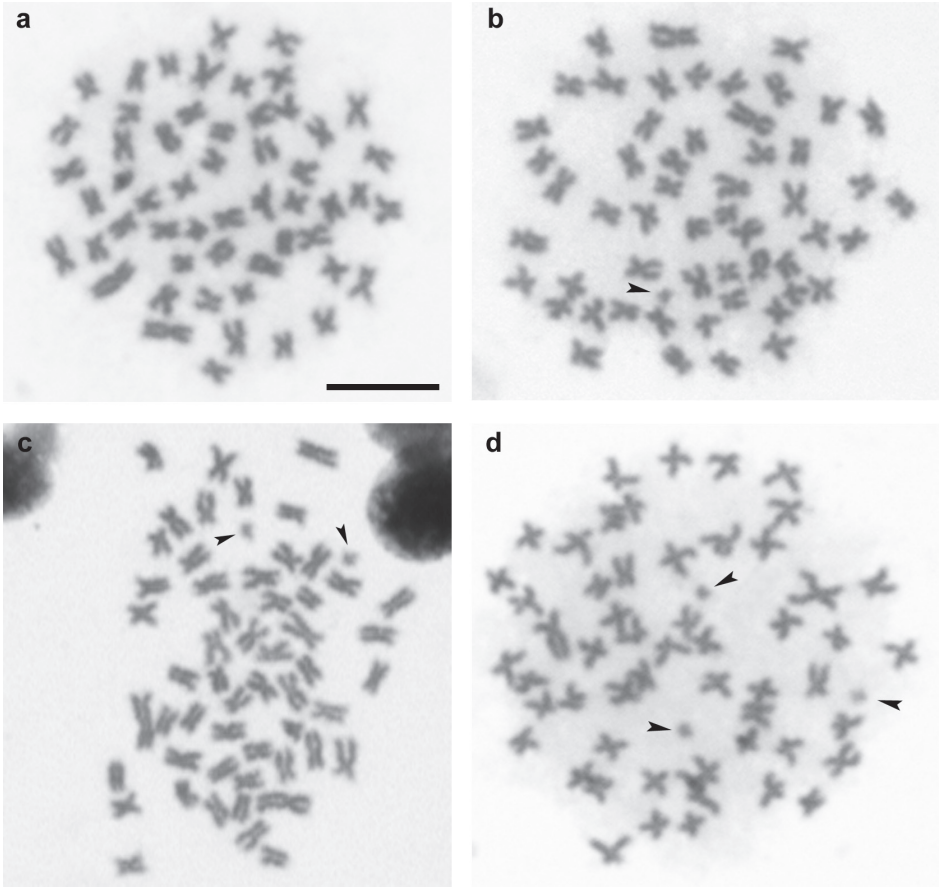

Figure 2. Metaphase plates of *P. argenteus* showing a cell of individual without B chromosomes (**a**); in individual presenting one B chromosome (**b**); cell presenting two B chromosomes (**c**); and cell presenting tree B chromosomes indicated by arrows (**d**). Bar = 10 μm.

The supernumerary genomic elements found in this species can be easily identified in metaphasic cells due to their small size when compared with the chromosomes of the standard complement, usually heterochromatic, but presenting diverse and complex patterns of heterochromatin distribution (Figure 3). Such microchromosomes generally representing the metacentric type revealed no NOR mark after silver nitrate staining. Similarly, analysis of *in situ* hybridization using 5S and 18S rDNA probes did not reveal the presence of these genes in the supernumerary chromosomes (Figure 4). Visible signs of hybridization were observed in sinteny in one pair of chromosomes in the normal complement, as previously reported by Hatanaka and Galleti Jr. (2004) and Voltolin et al. (2013).

An interesting feature of B chromosomes present in the carrier species of this genus is the identification as completely or partially heterochromatic microchromosomes, which exhibit expressive inter and intra-individual number variation (Table 1). A higher frequency of variation is found in *P. lineatus*, which includes individuals without B chromosomes as well as others with up to nine supernumerary elements (Voltolin et al. 2011). In other carrier species the frequency of occurrence is less wide-ranging, with zero to three in *P. mariae* (Oliveira et al. 2003) and from zero to two in *P. brevis* and *P. nigricans* (Pauls and Bertollo 1990, Venere et al. 1999). It can be considered that the particularity of these genomic elements that do not follow the Mendelian segregation laws in meiosis could determine their independent nature, providing different ways of influence to their accumulation or disappearance during the evolutionary process.

The occurrence of homoplasy could be considered to explain the origin of morphologically similar genomic elements in species of the same biological group. Thus, the existence of specific chromosomes bearing structural elements capable of originating extra chromosomes in an ancestral form could act in an independent way and give rise to supernumerary chromosomes found today in some of these species. However, it can be also considered the idea of a common origin of these elements in all species of the genus from an ancestral carrier, followed by the loss in some species during the diversification process.

The rate of mitotic instability (MI) calculated from individuals with supernumerary revealed a mean value of 0.136 (Table 1). In studying the variation of MI in *P. lineatus*, Cavallaro et al. (2000) observed a decrease of 0.486 to 0.004 among individuals between the years 1979 to 1989, suggesting the occurrence of a stabilizing trend in the population studied. Comparison of the above results with the mean value of MI found in *P. argenteus* in the present study (MI = 0.136) permits the inference that the low values of instability in the sample analyzed in this work presents could be an indicative that the species is developing the stabilization of B chromosomes in somatic cells in the course of next generations.

The great similarity found in morphology, size, heterochromatic nature and frequency of B chromosomes of *P. argenteus* with those of other species in the genus *Prochilodus*, described as microchromosomes and, in general, totally heterochromatic

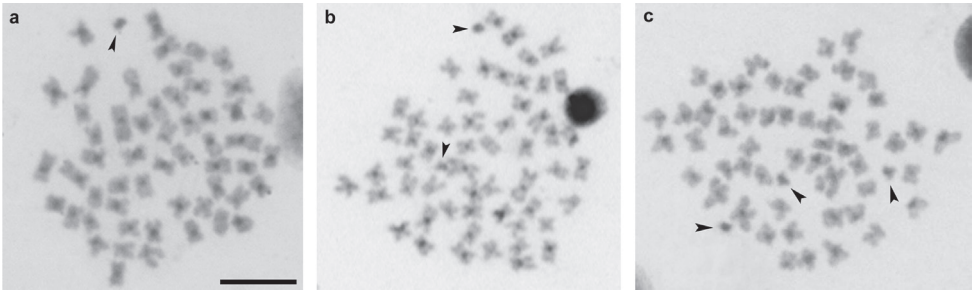

Figure 3. C-banding in metaphases plates of *P. argenteus* showing the distribution of heterochromatin in B chromosomes. Different cells showing one B chromosome (**a**); two B chromosomes (**b**); and tree B chromosomes indicated by arrows (**c**). Bar = 10 µm.

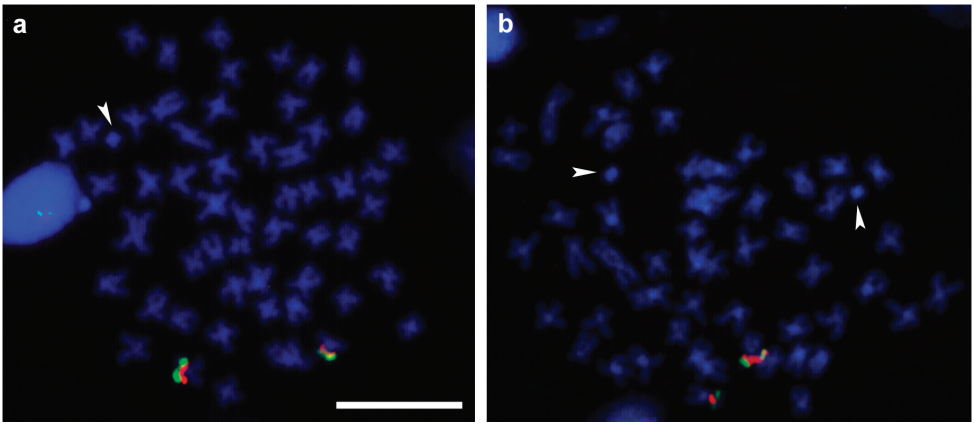

Figure 4. Fluorescent *in situ* hybridization using probes of 5S (green) and 18S (red) rDNA in metaphasic cells of *P. argenteus*, carrying one B chromosome (**a**) and two B chromosomes (**b**), indicated by arrows. FISH marked segments are visualized only on the NOR bearing chromosomes. Bar = 10 µm.

(Pauls and Bertollo 1983, 1990, Venere et al. 1999, Oliveira et al. 2003, Voltolin et al. 2013) can identify patterns of origin and dispersion of these genomic elements in this group of organisms. The morphological and structural analyses of these extra elements in the genus *Prochilodus* could suggest a possible common mechanism of origin which would manifest independently in the species of this genus, and then would follow their own paths of differentiation and evolution.

Acknowledgements

The authors are grateful to Suely Marlene Rodrigues from Projeto Peixe fish farm for providing fish samples used in the present work. Funds supporting this study were provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

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CompCytogen 9(1): 89–102 (2015)
doi: 10.3897/CompCytogen.v9i1.4611
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Cytogenetic analyses using C-banding and DAPI/CMA3 staining of four populations of the maize weevil *Sitophilus zeamais* Motschulsky, 1855 (Coleoptera, Curculionidae)

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Academic editor: *C. Nokkala* | Received 29 January 2015 | Accepted 13 March 2015 | Published 31 March 2015

http://zoobank.org/B42387BA-DAEE-4D6B-9A93-B449E6A1A45B

Citation: Silva AA, Braga LS, Guedes RNC, Tavares MG (2015) Cytogenetic analyses using C-banding and DAPI/CMA3 staining of four populations of the maize weevil *Sitophilus zeamais* Motschulsky, 1855 (Coleoptera, Curculionidae). Comparative Cytogenetics 9(1): 89–102. doi: 10.3897/CompCytogen.v9i1.4611

Abstract

Cytogenetic data avalaible for the maize weevil *Sitophilus zeamais* Motschulsky, 1855 (Coleoptera: Curculionidae), one of the most destructive pests of stored cereal grains, are controversial. Earlier studies focused on single populations and emphasized chromosome number and sex determination system. In this paper, the karyotypes of four populations of *S. zeamais* were characterized by conventional staining, C-banding and sequential staining with the fluorochromes chromomycin-A_3/4-6-diamidino-2-phenylindole (CMA_3/DAPI). The analyses of metaphases obtained from the cerebral ganglia of last instar larvae and the testes of adults showed that the species had 2n = 22 chromosomes, with 10 autosomal pairs and a sex chromosome pair (XX in females and Xy_p in males). Chromosome number, however, ranged from 2n = 22 to 26 due to the presence of 0–4 supernumerary chromosomes in individuals from the populations of Viçosa, Unai and Porto Alegre. With the exception of the Y chromosome, which was dot-like, all other chromosomes of this species were metacentric, including the supernumeraries. The heterochromatin was present in the centromeric regions of all autosomes and in the centromere of the X chromosome. The B chromosomes were partially or totally heterochromatic, and the Y chromosome was euchromatic. The heterochromatic regions were labeled with C-banding and DAPI, which showed that they were rich in AT base pairs.

Keywords

Heterochromatin, B chromosomes, C-banding, karyotype, fluorochromes

Introduction

Cytogenetic analyses are traditionally powerful tools for species characterization, identification and recognition of cryptic species, and the establishment of phylogenetic relationships and the evolutionary history of a species (Holecová et al. 2002, Rozek et al. 2004, Lachowska et al. 2004, 2006, 2008, 2009, Angus et al. 2011). Although such studies typically focus on single populations of different species, karyotype differences do exist among populations and may be potentially important for their divergence (e.g., Hsiao and Hsiao 1984).

Insect pest species present interesting cytogenetic challenges with potential practical consequences. Grain weevils (Coleoptera: Curculionidae) are a good example and include three important pest species of stored cereal grains (the granary weevil *Sitophilus granarius* (Linnaeus, 1875), the maize weevil *S. zeamais* Motschulsky, 1855, and the rice weevil *S. oryzae* (Linnaeus, 1763)), in addition to the tamarind weevil *S. linearis* (Herbst, 1797). These species belong to a 14 species genus of suspected Eurasian origin but with a current cosmopolitan distribution (Delobel and Grenier 1993). Grain weevils are also frequently found in archeological sites and provide important information on the human history of past urban environments, the origins of grain and likely dispersal routes, and routes of grain trade, in addition to the history of storage (Levinson and Levinson 1994, Kenway and Carrott 2006, Smith and Kenward 2011). Curiously, the evolutionary history of the grain weevils remains a matter of debate, with few cytogenetic studies and conflicting results.

The first cytogenetic analysis of a *Sitophilus* species described the karyotype of *S. granarius* with 12 pairs of chromosomes and the meioformula 5 + XX (Inkmann 1933, cited in Smith and Virkki 1978). Subsequently, a series of studies reviewed by Smith and Virkki (1978) found that the chromosome number for *S. oryzae* varied from 11 and 12 to 22, and the meioformulae were 5 + XX, 5 + X:XX, 10 + Xy or 10 + neoXY:XX. Furthermore, Barrion et al. (1988) reported that *S. oryzae* had 2n = 19 and a meioformula of n = 9 + XO, whereas both Zhi-Yua et al. (1989) and Moraes et al. (2003) described a karyotype with 22 chromosomes (2n = 20 + Xy). Takenouchi (1958, cited in Smith and Virkki 1978) also described the karyotype of *S. sasakii* (Takahashi, 1928) as containing 22 chromosomes (n = 10 + Xy).

For *S. zeamais*, the object of study of this work, Smith and Brower (1974) reported the presence of 22 chromosomes and a sex determination system of the neoXY type, plus the presence of 3–6 supernumerary chromosomes. Barrion et al. (1988), however, found 20 chromosomes (2n = 18 + XY), while Zhi-Yua et al. (1989) related the presence of 2n=22 chromosomes. More recently, Moraes et al. (2003) confirmed the occurrence of 22 chromosomes (2n = 20 + Xy) and the presence of 1 to 4 supernumerary chromosomes in this species.

However, these analyses were performed with meiotic chromosomes, obtained primarily through a squash of adult weevil testes because of the difficulty of obtaining mitotic metaphasic chromosomes (Petitpierre 1996), and focused on describing chromosome numbers and sex determination systems. Currently, however, more refined

cytogenetic techniques, such as C-banding and base-specific fluorochrome staining, are used for cytogenetic characterization of different species of Curculionidae (Hsiao and Hsiao 1984, Holecová et al. 1997, 2002, 2013, Lachowska and Holecová 2000, Rozek et al. 2004, Lachowska et al. 2004, 2006, 2009). These techniques provide a better characterization of the karyotypes and reveal differences in the amount and location of heterochromatic regions between closely related species, as well as the AT and GC base pair constitution of these regions. Thus, these techniques may also be used to understand the karyotypic evolution of this group.

The current cytogenetic techniques indicated above show that most Curculionidae have a small amount of heterochromatin, located primarily in a centromeric/pericentromeric position (Holecová et al. 2002, Rozek et al. 2004, Lachowska et al. 2004, 2005). Some species, however, exhibited additional bands in the interstitial and/or in the telomeric regions, as was the case of *Acalles fallax* Boheman, 1844 and *A. echinatus* (Germar, 1824) (Lachowska et al. 2009). Additionally, with the use of fluorochromes, it was possible to show that the heterochromatin of most Curculionidae was AT rich (the C-bands coinciding with $DAPI^+$ bands) (Lachowska 2008, Lachowska et al. 2008). Conversely, the CMA_3^+ bands were rarely found in the species of this family (Holecová et al. 2013).

Thus, because of the discrepancies with the karyotype of *S. zeamais* and the difficulties of working with meiotic chromosomes, the present work aimed to adapt methodologies to obtain mitotic chromosomes from cerebral ganglia cells of *S. zeamais* to characterize the karyotype of this species. We also analyzed four different populations of this species to verify the consistency of results and the existence of inter-population variations. It was also expected that this technique would be used in future studies as an easy, rapid and inexpensive method for the identification and separation of species of this genus. Additionally, we intended to develop a more detailed map of the location and composition of the heterochromatic regions in the genome of *S. zeamais*, with the use of C- and fluorochrome-banding techniques.

Materials and methods

Biological material

The larvae from four populations of *Sitophilus zeamais*, representing the occupation and migration route of this species in Brazil, were used. Because of the widespread distribution of this species in Brazil, populations from the north, south and center of the country were used. From the north of Brazil, a population from Cruzeiro do Sul (07°37'52"S and 72°40'12"W), a municipality located in the Acre State, was used. To represent the expansion of this species into more central regions, two populations with opposite locations in Minas Gerais State were used, one from Unai, in the northwest (16°21'27"S and 46°54'22"W), and the other from Viçosa, in the Zona da Mata Mineira (20°45'14"S and 42°52'55"W). From the south of Brazil, a population from Porto Alegre was selected (30°01'59"S and 51°13'48"W).

The populations were placed in glass containers (1.5 L) containing grains of maize and were stored in an environmentally controlled rearing room (25 ± 2 °C, 70 ± 10% relative humidity and a photoperiod of 12:12 h L:D). At the beginning of the analyses, the populations had been in culture for 6 months, 4 years, 1 and 6 months for the north, two central, and southern populations, respectively.

From preliminary tests, the last larval instar was determined to be the optimal stage for extraction of cerebral ganglia and preparation of slides because of the high number of cells in metaphase. As this stage developed inside the maize grain, the collection of larvae was preceded by inspection of the grain with X-ray equipment coupled to a 14-bit digital camera (Faxitron X-Ray Corp., Wheeling radiography equipment, IL, USA).

Cytogenetic analyses

a) Cerebral ganglia analyses

The cerebral ganglia of individuals of the last larval stage were processed according to Imai et al. (1988) after incubation in a hypotonic solution of colchicine (1% sodium citrate plus 0.005% colchicine) for 1 h 45 min. After 24 h, the slides were stained with 4% Giemsa in Sörensen`s phosphate buffer pH 6.8, for 12 min. On average, 35 individuals of each population were analyzed.

The C-banding technique was performed according to Lachowska et al. (2005), with modifications at the time of the HCl treatment (0,3M, for 4 min) and the $Ba(OH)_2$ incubation (3 min). The fluorochrome staining with DAPI/CMA_3 was performed according to Schweizer (1980), with modifications related to the order of use of fluorochromes and the processing times (DAPI was used first for 30 min, followed by the CMA_3 for 1 h). The use of distamycin was also omitted.

b) Gonadal analyses

To verify the behavior of the sex chromosomes and consequently confirm the sex determination system of the species, the analyses of the testes were performed according to Dias et al. (2012). Males were identified by morphology of the rostrum, which was smaller, thicker and more punctuated than the female rostrum (Khan and Musgrave 1968).

Chromosomal Analyses

On average, 10 metaphases per slide were evaluated with an Olympus BX60 microscope coupled to an image capturing system (Image-Pro Plus™, Version 6.3, Media Cybernetics®, 2009). The slides stained with fluorochromes (CMA_3/DAPI) were analyzed with an epifluorescence light microscope using excitation filters WB (λ = 330–385 nm) and WU (λ = 450–480 nm) under oil immersion at 100× magnification. The chromosomes were classified according to Levan et al. (1964), and the karyotypes were mounted by pairing chromosomes in decreasing order of size.

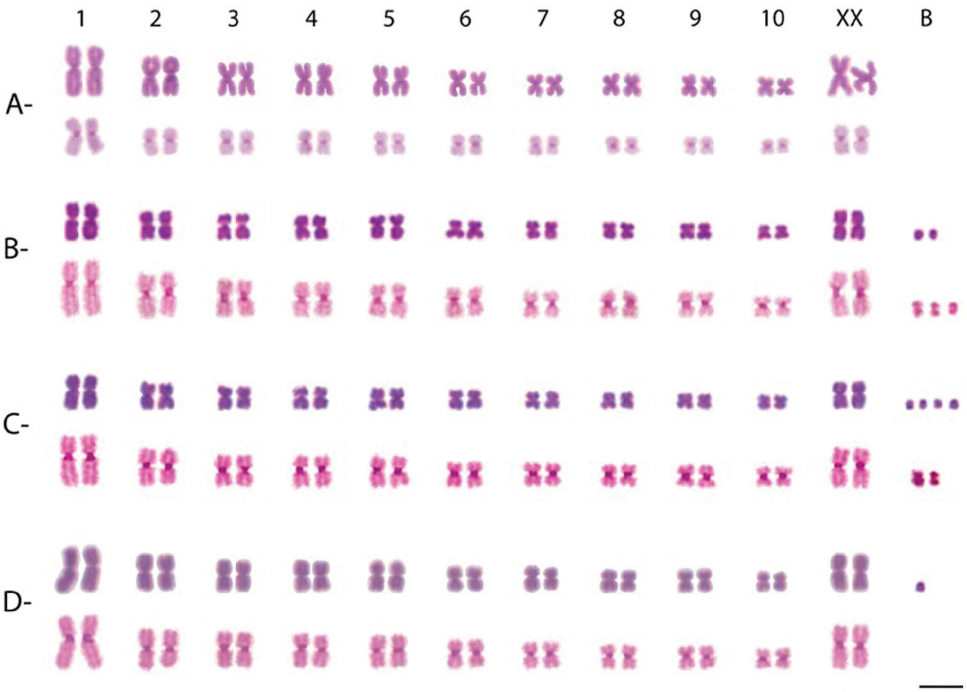

Figure 1. Karyotypes of females of *S. zeamais*. Populations from Cruzeiro do Sul (**A**), Unai (**B**), Porto Alegre (**C**), and Viçosa (**D**). Chromosomes show Giemsa staining and C-banding. B chromosomes are found in different populations. Bar = 10 μm.

Results

The analyses of the cerebral ganglia showed that all populations of *Sitophilus zeamais* exhibited a karyotype with 20 autosomes and a pair of sex chromosomes, i.e., 2n = 22 chromosomes (Fig. 1). The autosomal chromosomes in the four populations exhibited metacentric morphology and a gradual reduction in size. The X chromosome of this species was also metacentric and relatively large, with an intermediate size compared to the first and second pair of autosomes (Fig. 1). The Y chromosome presented a dot-like morphology and was the smallest chromosome of the karyotype (Fig. 2). The sexual pair was identified with comparisons between male and female metaphases.

The analyses of the gonadal cells confirmed the chromosome number of this species, i.e., eleven chromosomal pairs. Additionally, the analyses showed the association of the "parachute" type between the sex chromosomes in metaphase I cells from male insects. Therefore, the meioformulae, n = 10 + XX and n = 10 + Xyp, were observed in females and males of *S. zeamais*, respectively.

All individuals in the population from Cruzeiro do Sul (AC) had 22 chromosomes, whereas the chromosome numbers ranged from 22 to 26 in the populations from Viçosa (MG), Unai (MG) and Porto Alegre (RS). These numerical changes occurred because of the presence of 0–4 B chromosomes (Table 1), which were found in

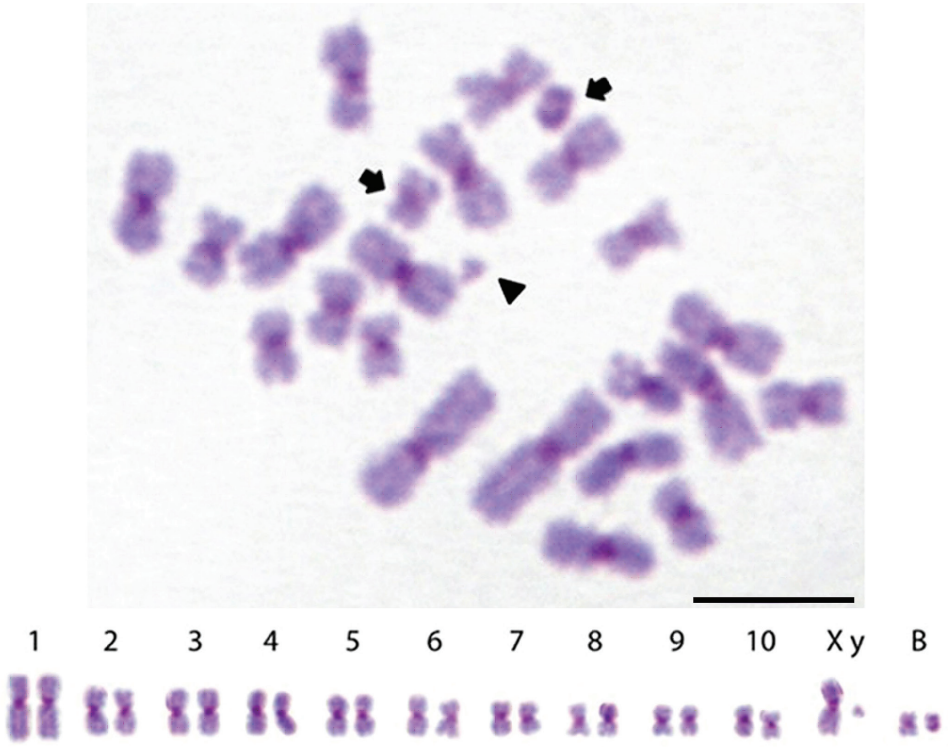

Figure 2. Metaphases and karyotypes of males of *S. zeamais* (2n = 22 + 2BS) from Porto Alegre. Chromosomes show C-banding. The arrows indicate the two types of B chromosomes, and the arrowhead indicates the Y chromosome. Bar = 10 μm.

cells of the same individual, in individuals of the same population and/or in individuals of different populations. These B chromosomes, in general, were larger than the Y chromosome and were easily distinguishable from the latter and from the autosomes and did not pair with each other or with the normal complement chromosomes.

Analyses of the less condensed metaphases revealed the presence of two types of B chromosomes. The type I B chromosomes were completely heterochromatic, and therefore, it was not possible to clearly define its morphology. In type II B chromosomes, the heterochromatic block was restricted to the centromeric region, which allowed recognition of their metacentric morphology (Fig. 2). The type I B chromosomes were found in both females and males and were present in all three populations, whereas the type II B chromosomes were found exclusively in the males from the Porto Alegre and Viçosa populations (Table 1).

All autosomes and the X chromosome presented small heterochromatic blocks in the centromeric region after C-banding in the four analyzed populations (Fig. 1). However, the Y chromosome of *S. zeamais* had no positive C-bands, i.e., it was entirely euchromatic. The four populations also showed DAPI$^+$ bands in the centromeric region of all autosomes and the X chromosome, which coincided with the heterochro-

Table 1. Frequency and types of B chromosomes found in the different populations of *Sitophilus zeamais*.

Population	Frequency of B Chromosomes (%)	Type of B Chromosome	N° slides analyzed
Cruzeiro do Sul	0%	-	35 females 2 males
Unaí	27,1% with 0 B 26,2% with 1B 24,8% with 2 Bs 21,9% with 3 Bs 0% with 4 Bs	Only type I	35 females
Porto Alegre	34,1% with 0 B 16,2% with 1 B 25,6% with 2 Bs 12,9% with 3 Bs 11,2% with 4 Bs	- Type I Type I Type I and II Type I Type I and II Type I	13 females 8 females 7 females 3 males 6 females 1 male 4 females
Viçosa	34,8% with 0 B 22,4% with 1 B 17,7% with 2 Bs 6,6% with 3 Bs 18,5% with 4 Bs	- Type I Type I Type I and II Type I Type I Type I and II	15 females 9 females 6 females 2 males 2 females 5 females 1 male

matic regions revealed with C-banding. The B chromosomes were partially or completely stained by this fluorocrome (Fig. 3A and B). No positive staining for CMA_3 was identified in the examined populations (Fig. 3C and D).

Discussion

The karyotypes described for the four populations of *S. zeamais* (2n = 22 chromosomes, with metacentric morphology) corroborated data for more than 42% of the 600 species of Curculionidae analyzed cytogenetically and most likely represented the ancestral karyotype of Curculionidae (Smith and Virkki 1978, Lachowska et al. 1998, 2006, 2008, Holecová et al. 2002, 2013, Rozek et al. 2009). Thus, the numerical difference found in the comparison with Barion et al. (1988), who described the karyotype of *S. zeamais* as having 2n = 20 chromosomes, as well as the differences related to the different species of *Sitophilus*, are due to intrinsic characteristics of the different techniques used.

The testes-squashing technique, commonly used in studies of Curculionidae (Rozek et al. 2004), did not allow a clear definition of chromosome morphology or an exact count of chromosomal pairs due to superposition. However, the quality of metaphases

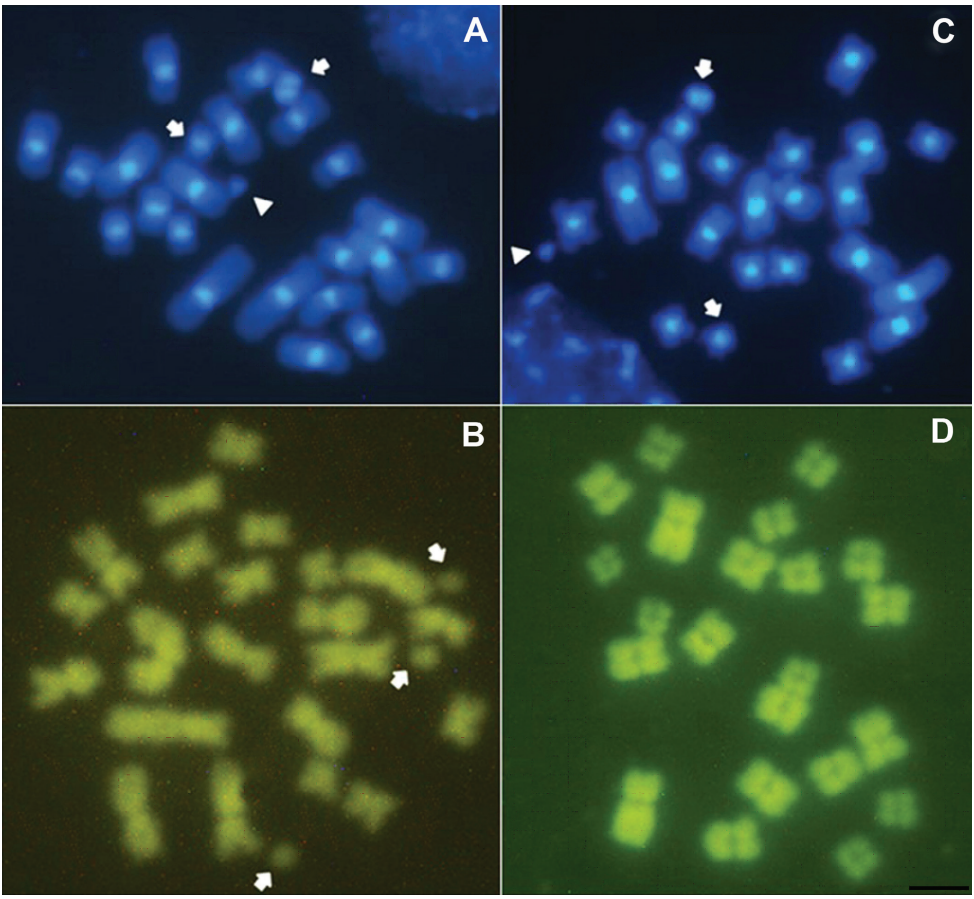

Figure 3. Metaphases of *S. zeamais* males (**A** and **C**) and females (**B** and **D**) stained with DAPI and CMA_3. The arrows indicate the B chromosome, and the arrowhead indicates the Y chromosome. Bar = 10 μm.

obtained in the present study with the cerebral ganglia dissociation shows clearly that this technique was effective to obtain metaphase cells with an adequate degree of condensation. Thus, this technique facilitated the determination of chromosome morphology, as well as the pairing of homologous chromosomes, and helped to overcome the difficulties encountered for the chromosome characterization of these insects. Therefore, it is also expected that with this technique it may be possible to detect chromosome differences related to size, morphology (e.g., metacentric vs. submetacentric) and the presence of secondary constrictions that facilitate distinction of closely related species.

Three of the four populations of *S. zeamais* analyzed exhibited variations in chromosome numbers due to the presence of 0–4 B chromosomes. Only in the population that originated from northern Brazil (Cruzeiro do Sul) were these chromosomes not detected. Therefore, these chromosomes apparently appeared in different populations during the expansion in the country, by different mechanisms, as they were also found in the samples analyzed by Moraes et al. (2003).

The presence of supernumerary chromosomes in Curculionidae species, however, is rare. Among more than 600 species karyotyped, only seven showed the presence of these chromosomes, *Gelus californicus* (LeConte, 1876) (Ennis 1972), *Sitophilus zeamais* (Smith and Brower 1974, Moraes et al. 2003), *Anthonomus grandis* (Boheman, 1843) (Nort et al. 1981), *Astychus* sp., *Phytoscaphus inductus* (Boheman, 1843) (Dey 1989), *Barypeithes pellucidus* Boheman 1834 (Holecová et al. 2005) and *Otiorhynchus atroapterus* (De Geer, 1775) (Holecová et al. 2013). Similar to the present study, the majority of these studies reported variations in the number of B chromosomes detected in cells, which reinforced their nonMendelian inheritance.

One difference, however, was that the B chromosomes identified in the species listed above were tiny and were similar in size to that of the Y chromosome (dot-like), whereas those identified in *S. zeamais*, though also small compared with other chromosomes in the karyotype, were clearly larger than the Y chromosome. This difference in size and the partially heterochromatic B chromosomes of *S. zeamais* evidenciated that these type II B chromosomes had a metacentric morphology, which also helped to differentiate them from the other B chromosomes previously identified in Curculionidae. Additionally, we found that *S. zeamais* females had only type I B chromosomes, whereas the males had both types. Previously, euchromatic B chromosomes were observed in only two other species of Curculionidae, *Barypeithes pellucidus* (Holecová et al. 2005) and *Otiorhynchus atroapterus* (Holecová et al. 2013). The B chromosomes were often heterochromatic in other species. Therefore, in general, the B chromosomes of *S. zeamais* possessed the same characteristics of B chromosomes of most organisms and were heterochromatic, smaller than the chromosomes of the A complement, and with a nonMendelian distribution.

Analyses of the gonadal cells of *S. zeamais* showed the "parachute" association between the X chromosome (which was relatively large) and the Y chromosome (which was the smallest chromosome of the karyotype, with a dot-like appearance). Consequently the sex determining mechanism was of the Xy_p type. In contrast, previous analyses defined the sex determination mechanism of *S. zeamais* as neoXY (Smith and Brower 1974). However, the results of the present study, particularly the small size of the Y chromosome, which did not show any evidence of fusion or translocation between sex chromosomes and autosomes, were in accordance with the system proposed here, which was also considered the ancestral one for this group (Smith and Virkki 1978, Lachowska et al. 1998, 2006, 2008, Lachowska and Holecová 2000, Rozek et al. 2009, Goll 2012, Holecová et al. 2013).

The C-banding patterns observed, as well as the absence of positive bands in the Y chromosome, corroborated literature data for most Curculionidae, as well as for several other insect species (Juan and Petitpierre 1989, Imai 1991, Rozek 1998, Almeida et al. 2000, Proença et al. 2002, Holecová et al. 2002, 2008, 2013, Rozek et al. 2004, Zacaro et al. 2004, Lachowska et al. 2004, 2005, 2006, 2008, 2009, Schneider et al. 2007, Lachowska 2008, Kajtoch et al. 2009). However, Curculionidae species with a large heterochromatic block on the genomes have been found (Holecová et al. 2002, Lachowska et al. 2004, 2009, Rozek et al. 2004).

Another variable aspect of the Curculionidae, when considering the heterochromatin, is the banding pattern of the Y chromosome. In many species, including *S. zeamais*, this chromosome is euchromatic; in others, such as *Centricnemus leucogrammus* Germar, 1824, *Acalles fallax*, *A. petryszaki* Dieckmann, 1982, *Otiorhynchus atroapterus* and *O. bisulcatus* (Fabricius, 1781), the presence of a completely heterochromatic Y chromosome was observed (Lachowska et al. 2006, 2009, Holecová et al. 2013). In contrast, the Y chromosome was described as with some heterochromatic regions (Lachowska et al. 2004, 2005) in *Acalles echinatus*, *Barypheites chevrolati* (Boheman, 1843), *B. formaneki* (Fremuth, 1971) and *B. mollicomus* (Ahrens, 1812). Thus, future studies that involve a larger number of species could demonstrate that the amount and type and location of heterochromatin regions in the chromosomes (pericentromeric, subtelomeric or intercalary) could be useful to characterize species, as well as to establish the evolutionary relationships between them (Holecová et al. 2002, 2013, Lachowska et al. 2004, 2005).

The sequential C-banding and DAPI staining performed in our study indicated that the centromeric regions of most chromosomes of *S. zeamais* were AT-rich. This result was enhanced because no CMA_3 positive bands were identified in any of the analyzed populations. Moreover, the DAPI positive bands were often found in the same regions that were stained by C-bands in Curculionidae, which confirmed the high AT content in the heterochromatin of these insects (Lachowska 2008, Lachowska et al. 2008, Holecová et al. 2013). Lachowska (2008), Lachowska et al. (2008) and Holecová et al. (2013), for example, observed $DAPI^+$ bands (pericentromeric or centromeric) on the karyotypes of six *Otiorhynchus* species, one of *Cirrohynchus* Frivaldszky, 1892, one of *Dodicastichus* (Gyllenhal, 1834), five of *Barypeithes* and two of *Strophosoma* Billberg, 1820. However, only in *Barypeithes interpositus* (Roubal, 1920), *B. formaneki* (Lachowska 2008) and in *Otiorhynchus morio* (Lachowska et al. 2008) was possible to verify a weak CMA_3^+ band in a single autosomal pair, possibly coincident with the Nucleolus Organizer Region (NOR). According to these authors, the absence of positive signs in the other analyzed species could represent a small number of rDNA genes in the genomes. Thus, *Otiorhynchus* s. str. *bisulcatus* seemed to be an exception when comparing the fluorescent banding patterns of Curculionidae because this species showed, at the same time, positive marks for DAPI and CMA_3 in several chromosomes. According to Holecová et al. (2013), this pattern indicated that the heterochromatin of this species consisted of repeats rich in AT and GC base pairs.

In conclusion, our cytogenetic analyses showed that the methodologies employed were effective for the characterization of the *S. zeamais* karyotype and could be further used for comparing karyotypes of other species of this genus. The karyotype of *S. zeamais* possesses features common to most species of Curculionidae, and B chromosomes are found in different populations of this species.

Acknowledgments

The authors are grateful to the Brazilian agencies FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais), CNPq (Conselho Nacional para o Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and UFV (Universidade Federal de Viçosa) for financial support. We are also grateful to Dr. Silvia G. Pompolo and Dr. Denilce M. Lopes from the General Biology Department of Federal University of Viçosa for their technical assistance and valuable suggestions during the development of this work.

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CompCytogen 9(1): 103–118 (2015)
doi: 10.3897/CompCytogen.v9i1.8671
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Comparative study of mitotic chromosomes in two blowflies, *Lucilia sericata* and *L. cluvia* (Diptera, Calliphoridae), by C- and G-like banding patterns and rRNA loci, and implications for karyotype evolution

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Academic editor: *P. Michailova* | Received 29 September 2014 | Accepted 22 December 2014 | Published 31 March 2015

http://zoobank.org/CF4F21F2-181A-4B0C-B175-313AA08E2E9B

Citation: Chirino MG, Rossi LF, Bressa MJ, Luaces JP, Merani MS (2015) Comparative study of mitotic chromosomes in two blowflies, *Lucilia sericata* and *L. cluvia* (Diptera, Calliphoridae), by C- and G-like banding patterns and rRNA loci, and implications for karyotype evolution. Comparative Cytogenetics 9(1): 103–118. doi: 10.3897/CompCytogen.v9i1.8671

Abstract

The karyotypes of *Lucilia cluvia* (Walker, 1849) and *L. sericata* (Meigen, 1826) from Argentina were characterized using conventional staining and the C- and G-like banding techniques. Besides, nucleolus organizer regions (NORs) were detected by fluorescent *in situ* hybridization (FISH) and silver staining technique. The chromosome complement of these species comprises five pairs of autosomes and a pair of sex chromosomes (XX/XY, female/male). The autosomes of both species have the same size and morphology, as well as C- and G-like banding patterns. The X and Y chromosomes of *L. cluvia* are subtelocentric and easily identified due to their very small size. In *L. sericata*, the X chromosome is metacentric and the largest of the complement, showing a secondary constriction in its short arm, whereas the Y is submetacentric and smaller than the X. The C-banding patterns reflect differences in chromatin structure and composition between the subtelocentric X and Y chromosomes of *L. cluvia* and the biarmed sex chromosomes of *L. sericata*. These differences in the sex chromosomes may be due to distinct amounts of constitutive heterochromatin. In *L. cluvia*, the NORs are placed at one end of the long-X and of the long-Y chromosome

arms, whereas one of the NORs is disposed in the secondary constriction of the short-X chromosome arm and the other on the long-Y chromosome arm in *L. sericata*. Although the G-like banding technique does not yield G-bands like those in mammalian chromosomes, it shows a high degree chromosomal homology in both species because each pair of autosomes was correctly paired. This chromosome similarity suggests the absence of autosomal rearrangements during karyotype evolution in the two species studied.

Keywords
Blowflies, karyotype evolution, sex chromosomes, heterochromatin, G-like banding pattern, rDNA-FISH

Introduction

The dipteran family Calliphoridae (blowflies) includes several common synanthropic forms, most of which have saprophagous habits. Some blowflies are considered a serious public health problem since certain species can cause myiasis in humans and domestic animals. Other blowflies are of great medical, veterinary, and forensic importance (Hanski and Kuusela 1980, Kuusela and Hanski 1982, Greenberg 1991, Martínez Sánchez et al. 2000, Centeno et al. 2002, 2004, Agrawal et al. 2010, Davydov 2011). Calliphorids are recognized as the first wave of faunal succession on human cadavers (Nuorteva 1977, Smith 1986) and, therefore, are the primary and most accurate indicators of the time of death (Centeno et al. 2002, Ames and Turner 2003). The larvae of certain facultative parasites are used in maggot therapy to treat infected chronic wounds in humans and vertebrates by allowing the removal of necrotic tissue, which induces the formation of granular tissue and the growth of healthy skin (Sherman 2002, Horobin et al. 2005, Parnés and Lagan 2007, Davydov 2011).

Within Calliphoridae, *Lucilia cluvia* (Walker, 1849) and *L. sericata* (Meigen, 1826) are among the most abundant exploiters of carcasses and faeces as food sources, oviposition sites, and sites for larval development, being their biological development very important in the field of forensic science. *Lucilia sericata* is one of the first insects to arrive at a corpse and their immature flies are used to estimate the minimum portion of the *post-mortem* interval, known as *PMI* (Hanski and Kuusela 1980, Kuusela and Hanski 1982, Martínez Sánchez et al. 2000, Centeno et al. 2002, Rueda et al. 2010). The larvae of *L. sericata*, *L. cluvia*, and *L. illustris* (Meigen, 1826) are the most suitable and effective facultative parasites used in human wound treatment of injuries that conventional treatments fail to heal (Sherman and Wyle 1996, Bani-Ardalani 2005, Zapata et al. 2008, Rueda et al. 2010, Thyssen et al. 2013). Uncertain or incorrect taxonomic identification of maggot and/or imaginal stages could have unpredictable consequences for the implementation of larvae of different blowfly species in the larval therapy. Therefore, cytogenetic studies have significant value because they allow differentiating between related species that are cryptic and/or morphologically similar, particularly in their larval stages, and provide information and useful diagnostic characters at the species level.

In Calliphoridae, cytogenetic data are scarce and refer almost exclusively to the karyotype, C-banding and/or the C value of a very few species (Boyes and van Brink

1965, Boyes and Shewell 1975, Bedo 1980, 1991, Parise-Maltempi and Avancini 2001, El-Bassiony 2006, Ullerich and Schöttke 2006). This family shows a remarkably uniform karyotype (2n = 12), generally comprising five pairs of large euchromatic autosomes and a pair of heteromorphic sex chromosomes. Previous reports have revealed a great deal of similarity among autosomes and variation in size and morphology of the sex chromosomes from species to species (Boyes and Shewell 1975, Bedo 1980, Ullerich and Schöttke 2006, Agrawal et al. 2010). Thirty-two species of *Lucilia* were taxonomically described (Stevens and Wall 1996, Whitworth 2010, 2014), but only seven species were studied. These are *L. sericata* from Africa and Germany, *L. illustris* and *L. caesar* (Linnaeus, 1758) from Germany and Japan, *L. porphyrina* Walker, 1856 and *L. ampullacea* Villeneuve, 1922 from Japan, *L. eximia* (Wiedemann, 1819) from Brazil, and *L. cuprina* (Wiedemann, 1830) from Australia (Boyes and Shewell 1975, El-Bassiony 2006, Ullerich and Schöttke 2006, Agrawal et al. 2010). In *Lucilia* species, the autosomes are less variable and very seldom appear to be heterochromatic as compared to sex chromosomes, which show a considerable interspecific variation in size and shape. *Lucilia illustris* has a relatively long X chromosome whereas *L. ampullacea* and *L. caesar* have short heteromorphic sex chromosomes (Boyes and Shewell 1975, Bedo 1991, Ullerich and Schöttke 2006, Agrawal et al. 2010).

In the present work, we examined and compared the karyotype of *L. cluvia* and *L. sericata* from Argentina. We analysed the constitutive heterochromatin content and distribution, and identified the nucleolus organizer regions (NORs) in female and male mitotic chromosomes of these species by means C-banding and fluorescent *in situ* hybridization (FISH) with 18S rDNA heterologous probes, respectively. In order to confirm the accurate detection of ribosomal genes location, we also applied the silver impregnation for staining NORs on mitotic chromosomes that were transcriptionally active during the preceding interphase. We also identify the chromosome pairs in both species by means of G-like banding. Finally, we discuss our cytogenetic results and compare them with those previously described.

Materials and methods

Fly sources

Lucilia cluvia and *L. sericata* occurring in grasslands, shrubs and forest habitats in open areas near Buenos Aires City (34°36'14"S and 58°22'54"W), Argentina, were collected using beef meat as baits between January and May 2014. For mitotic analysis, chromosome bandings and fluorescent *in situ* hybridization (FISH) technique, 7 females of *L. cluvia* and 20 females of *L. sericata* were collected. Flies were identified using Mariluis and Schnack key (2002). After identification, the females were transferred into a cage for oviposition at 22 ± 2 °C, and 60 ± 5% relative humidity. The rearing cages were supervised daily and flies were allowed to develop into third-instar (L3) larvae. The number of egg-clusters oviposited by each female was ranked between 200–250 eggs.

Chromosome preparations

Mitotic chromosomes were obtained from the neural ganglia of L3 larvae. At least 100 females and males (20–30 larvae for each sample) of each species were analysed. Larvae were injected with 0.01 ml of colchicine (0.1 mg/ml) and dissected under a stereomicroscope after 45 min treatment. Brains were dissected using fine forceps, dispersed in 3 ml of KCl 0.075 M for 15 min at 25 °C, centrifuged at 600 rpm for 10 min, and fixed in methanol:acetic acid (3:1) overnight. The cell suspension was dropped onto clean slides using air-drying technique (Rothfels and Siminovitch 1957, Chirino et al. 2014). For karyotype analysis and chromosome length measurements, chromosome preparations were made from brains of untreated larvae with colchicine.

Chromosome bandings

C-banding was performed according to Sumner (1972) with modifications: slides were treated with 0.2 N HCl for 20 min at room temperature, 5% saturated solution of $Ba(OH)_2$ at 50 °C for 1–2 min and 2X SSC at 60 °C for 60 min; slides were then stained with 3% Giemsa solution at pH 6.8 for 20–30 min. G-like banding was performed following the method of Wang and Fedoroff (1972) with modifications: within 96 h after air drying, slides were incubated in phosphate buffered saline (PBS) for 10 min and in 0.1%, 0.05% and 0.025% trypsin for 1–6 min; slides were then air-dried and stained with 1% Giemsa solution at pH 6.8 for 5–10 min.

Detection of the nucleolus organizer regions (NORs) on mitotic chromosomes was done following the silver staining method of Howell and Black (1980) with slight modifications. Onto the slides, 30 µl of 1% aqueous gelatine solution with 0.5% formic acid and 20 µl drops of silver nitrate at 50% were dropped. The slides were covered with coverglass and incubated at 45 °C for 2–10 min until the silver staining mixture became yellowish. The slides were washed with distilled water, air-dried, and examined immediately under microscope.

Fluorescence in situ hybridization

Unlabelled 18S ribosomal DNA (rDNA) probes, derived from genomic DNA of the true bug, *Dysdercus albofasciatus* Berg, 1878 (Heteroptera: Pyrrhocoridae), were obtained and labelled with biotin 14-dATP by nick translation using a BioNick Labeling System (Invitrogen, Life Technologies Inc., San Diego, CA, USA) as described in Fuková et al. (2005) and Bressa et al. (2009). FISH with a biotinylated 18S rDNA probe was performed following the procedure described in Bressa et al. (2009). Hybridization signals were detected with Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs. Inc., West Grove, PA, USA).

Microscopy, photographs and image processing

Fifty mitotic metaphases of females and males per individual of *L. cluvia* and *L. sericata* were analysed to determine the karyotype of each species. Ten metaphases of each species (*L. cluvia* and *L. sericata*) were used to perform each species idiogram. Lengths of chromosomes were calculated and expressed as percentage of the female haploid set. Measurements were also performed on five banded karyotypes of each species to avoid errors in chromosome identification. At least 40 G-like banded, 20 C-banded, 20 rDNA-FISH, and 20 Ag-NOR cells for each gender and species were examined and photographed. Idiograms illustrating the G-like banding patterns were obtained.

Results

Chromosome complement

The female and male karyotypes and C-banding pattern of *L. sericata* were already reported (El-Bassiony 2006, Ullerich and Schöttke 2006). However, we examined in detail different karyotype features of this species, such as chromosome banding patterns, percentage of heterochromatin and number and location of NORs. As a result, we made a comparison study of the mitotic karyotypes of both *Lucilia* species.

Table 1. Comparison of the relative lengths of chromosomes of *Lucilia cluvia* and *L. sericata* in % of the haploid set (mean ± SE).

Chromosome pair	TL †	Short arm (p)	Long arm (q)	AI (classification) ‡
Lucilia cluvia				
1	23.24 ± 1.95	10.11 ± 0.67	12.48 ± 0.91	0.81 ± 0.05 (M)
2	18.63 ± 1.10	7.97 ± 0.66	9.22 ± 0.69	0.87 ± 0.09 (M)
3	18.35 ± 0.55	5.93 ± 0.53	10.93 ± 0.38	0.54 ± 0.06 (SM)
4	17.43 ± 2.07	6.16 ± 0.50	10.28 ± 1.91	0.62 ± 0.14 (M)
5	16.51 ± 0.44	7.36 ± 0.24	8.47 ± 0.28	0.87 ± 0.05 (M)
X	5.84 ± 0.86	1.26 ± 0.21	3.82 ± 0.74	0.35 ± 0.12 (ST)
Y	4.77 ± 0.28	1.07 ± 0.18	3.46 ± 0.26	0.31 ± 0.07 (ST)
Lucilia sericata				
1	19.44 ± 0.24	8.71 ± 0.29	9.59 ± 0.40	0.91 ± 0.07 (M)
2	17.55 ± 0.89	7.18 ± 1.04	9.13 ± 0.82	0.80 ± 0.19 (M)
3	15.18 ± 0.34	4.82 ± 0.78	8.80 ± 0.49	0.55 ± 0.12 (SM)
4	14.77 ± 0.32	5.34 ± 0.60	8.60 ± 0.48	0.58 ± 0.09 (M)
5	11.95 ± 0.40	5.67 ± 0.28	6.64 ± 0.37	0.78 ± 0.09 (M)
X	19.97 ± 1.35	8.75 ± 0.31	10.77 ± 0.97	0.82 ± 0.05 (M)
Y	13.70 ± 2.20	4.75 ± 1.39	8.15 ± 0.50	0.58 ± 0.14 (SM)

† TL = total length.

‡ AI = arm index; M = metacentric; SM = submetacentric; ST = subtelocentric.

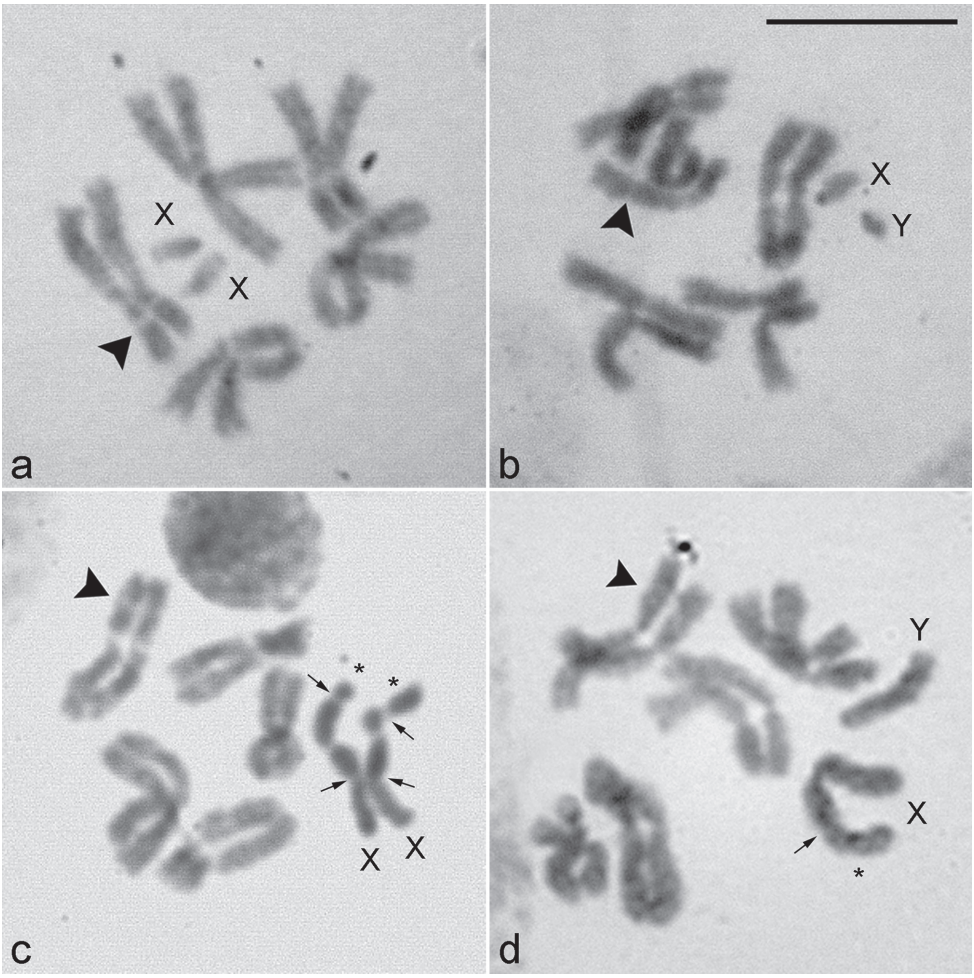

Figure 1. Female and male karyotypes of *L. cluvia* (**a–b**) and *L. sericata* (**c–d**), 2n = 10 + XX/XY, stained with 3% Giemsa. X, Y = sex chromosomes. Arrowheads show the secondary constriction in chromosome 2. Arrows show the secondary constriction in the X chromosomes. Asterisks indicate the satellite. Bar = 10 μm.

The diploid chromosome complements of *L. cluvia* and *L. sericata* are 2n = 12, consisting of five large biarmed autosomal pairs and one sex chromosome pair (XX/XY, female/male; Fig. 1). In both species, *L. cluvia* (Fig. 1a–b) and *L. sericata* (Fig. 1c–d), the autosomes show a very close somatic pairing, the sex chromosomes tend to not pair with each other or stay unpaired, and all the chromosomes are similar in morphology and size, except for the sex chromosomes (Table 1). In female and male somatic metaphases of *L. cluvia* (Fig. 1a–b) and *L. sericata* (Fig. 1c–d), pairs 1, 2, 4 and 5 are metacentric chromosomes, whereas pair 3 comprises two submetacentric chromosomes. Pair 2 possesses a secondary constriction in the short arm.

In mitotic metaphases of *L. cluvia*, the X and Y chromosomes are subtelocentric and are easily identified among the remaining five pairs of autosomes due to their very small

size (Fig. 1a–b; Table 1), being the Y chromosome slightly smaller than the X chromosome (Fig. 1b). On the other hand, the X chromosome of *L. sericata* is metacentric and the longest of the diploid set, representing 20.0% of the haploid set. The Y sex chromosome is submetacentric, smaller than the X chromosome and represents 13.7% of the set (Fig. 1c–d). In female of this species, both X chromosomes present a secondary constriction in their short arms and a satellite at terminal position (Fig. 1c; Table 1). Both X chromosomes can be distinguished due to the different size of their satellites, leading to a significant increase in their size (Table 1).

Chromosome bandings

The C-banding pattern of autosomes is mainly limited to a single narrow band at the centromeric region in each of five pairs in both species of *Lucilia* (Fig. 2). Besides, two interstitial bands are observed on each short arm of chromosomes 2 and 3. In the former, the C-band is associated with a secondary constriction (Fig. 2). Both species show differences in the C-banding pattern in the X and Y sex chromosomes. In *L. cluvia*, the X chromosome has a small C-positive band located in the proximal pericentromeric region of its long arm, whereas the Y chromosome is euchromatic (Fig. 2a–b, e). In *L. sericata*, the satellite and the proximal region of the short X-chromosome arm are heterochromatic (Fig. 2c–e), except the secondary constriction where a single nucleolus is located and observed as a negative heteropyknotic body (Fig. 2d–e). The long X-chromosome and the long Y-chromosome arms are almost heterochromatic, except the distal regions (Fig. 2c–e). From these results, we found significant differences in the content and distribution of constitutive heterochromatin in *L. cluvia* and *L. sericata*. In the former, the constitutive heterochromatin is mainly located on the chromosome X (3.1% of the total chromosome length) and the autosomal pair 3 (1.8%). In the latter, most of constitutive heterochromatin is found on the X (15.4% of its total length) and to a lesser extent on the Y (8.4%) and pair 3 (2.4%).

The homology between the karyotypes of *L. cluvia* and *L. sericata* is illustrated in Figure 3. In all mitotic chromosome preparations, G-like bands are very evident and always present on homologous chromosomes from *L. cluvia* and *L. sericata*. These bands dispersed along the chromosomes are useful for idiogram reconstruction (Fig. 3a–b). The distribution of G-like banding pattern was homologated in both species of *Lucilia*, and the chromosomal homology in their karyotypes was observed since each pair of autosomes was correctly paired (Fig. 3a, b). The distribution of G-like bands in the X sex chromosomes of *L. cluvia* was coincident to that observed in the short arm of the X chromosomes of *L. sericata* (Fig. 3c).

Localization of rDNA by FISH and Ag-NOR banding

In preparations of mitotic metaphases from both sexes of *L. cluvia* and *L. sericata*, FISH experiments with the 18S rDNA probe show two clusters of rDNA genes, one

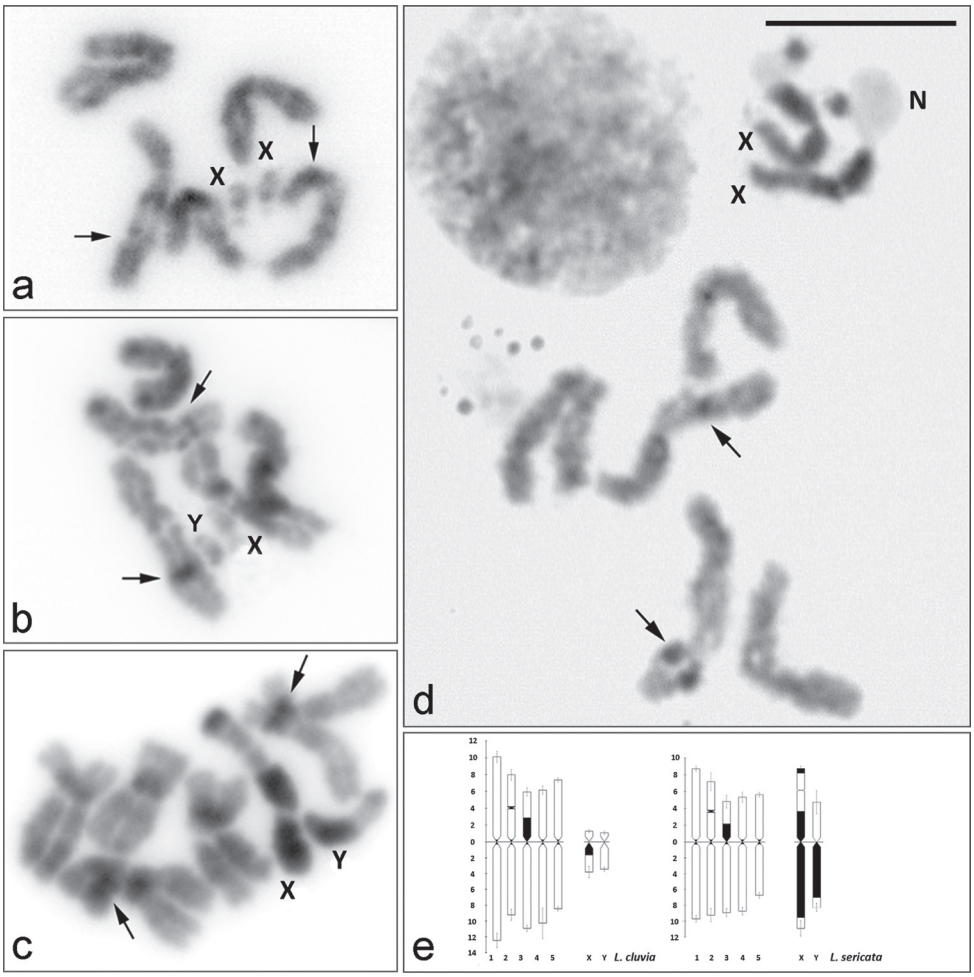

Figure 2. C-banding on female and male mitotic chromosomes of *L. cluvia* (**a–b**) and *L. sericata* (**c–d**), stained with 3% Giemsa, and C-banded idiograms of autosomes and sex chromosomes of *L. cluvia* and *L. sericata* (**e**). X, Y = sex chromosomes. N = nucleolus. Arrows indicate C-positive heterochromatin bands at the secondary constriction in chromosome 2 and at interstitial position in chromosome 3. Bar = 10 μm.

of them located on the X chromosome and the other on the Y chromosome (Fig. 4). In females of *L. cluvia*, a single cluster of hybridization signals is regularly observed at terminal region of long arm of each X chromosome (Fig. 4a). In males, the hybridization signals are observed both at the end of the long-X chromosome arm as at the terminal position on the long-Y chromosome arm (Fig. 4b). In females of *L. sericata*, the rDNA probe displays strong hybridization signals in the secondary constriction of the short arm of both X chromosomes (Fig. 4c). In male metaphase complements, the hybridization signals are clustered in the secondary constriction of the short X-chromosome arm and in the proximal region of the large arm of the Y chromosome (Fig. 4d). In

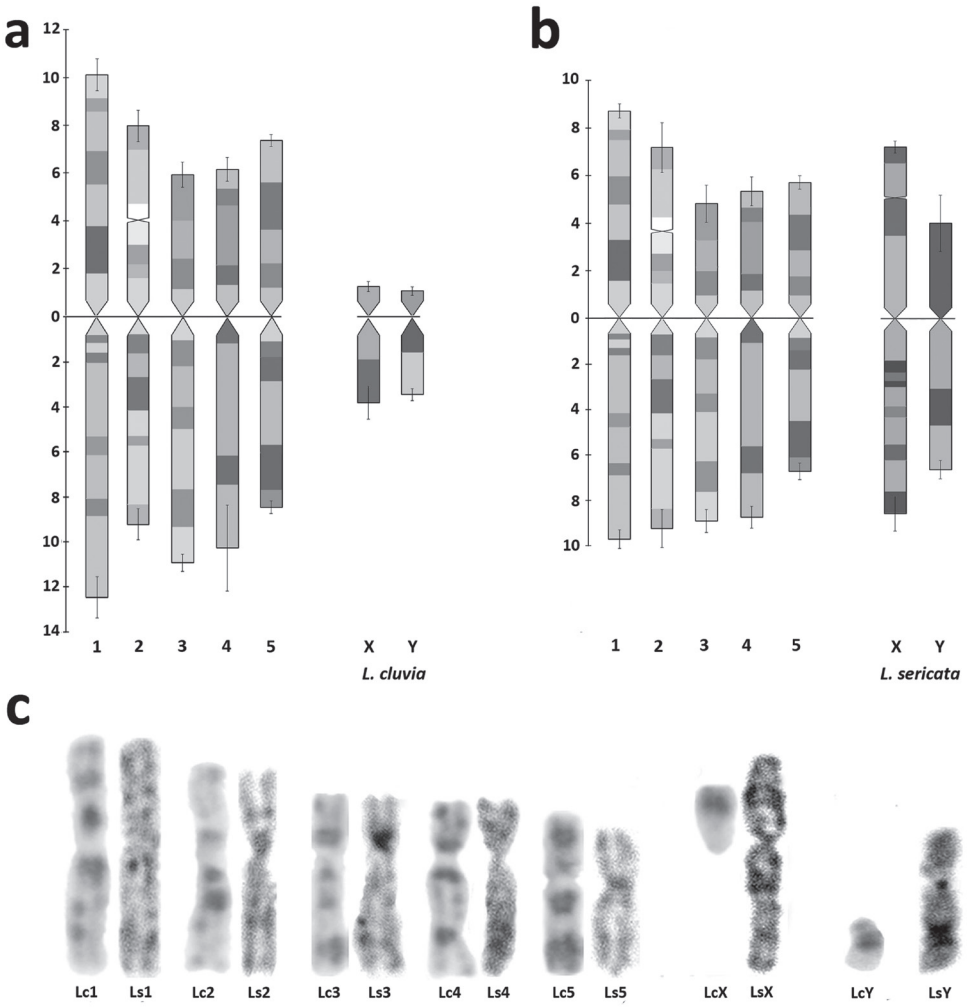

Figure 3. Pattern of G-like bands in the ideogram of *L. cluvia* (**a**) and *L. sericata* (**b**), and G-like banding homology between chromosomes of *L. cluvia* (Lc) and *L. sericata* (Ls) (**c**) revealing a high degree of conservation in G-like banding patterns between homologous chromosomes.

most male metaphases of *L. sericata*, the intensity of the hybridization signals differs between the two sex chromosomes, with the Y chromosome showing stronger and larger clusters of signals (Fig. 4d).

A single nucleolus is present on both X and Y sex chromosomes of *L. sericata* (Fig. 4e–f). At female mitotic metaphases, positive Ag-NORs are observed in the secondary constriction of the short arm of both X chromosomes (Fig. 4e). Male mitotic metaphases show positive Ag-NORs in the secondary constriction of the short X-chromosome arm and in the large arm of the Y chromosome (Fig. 4f).

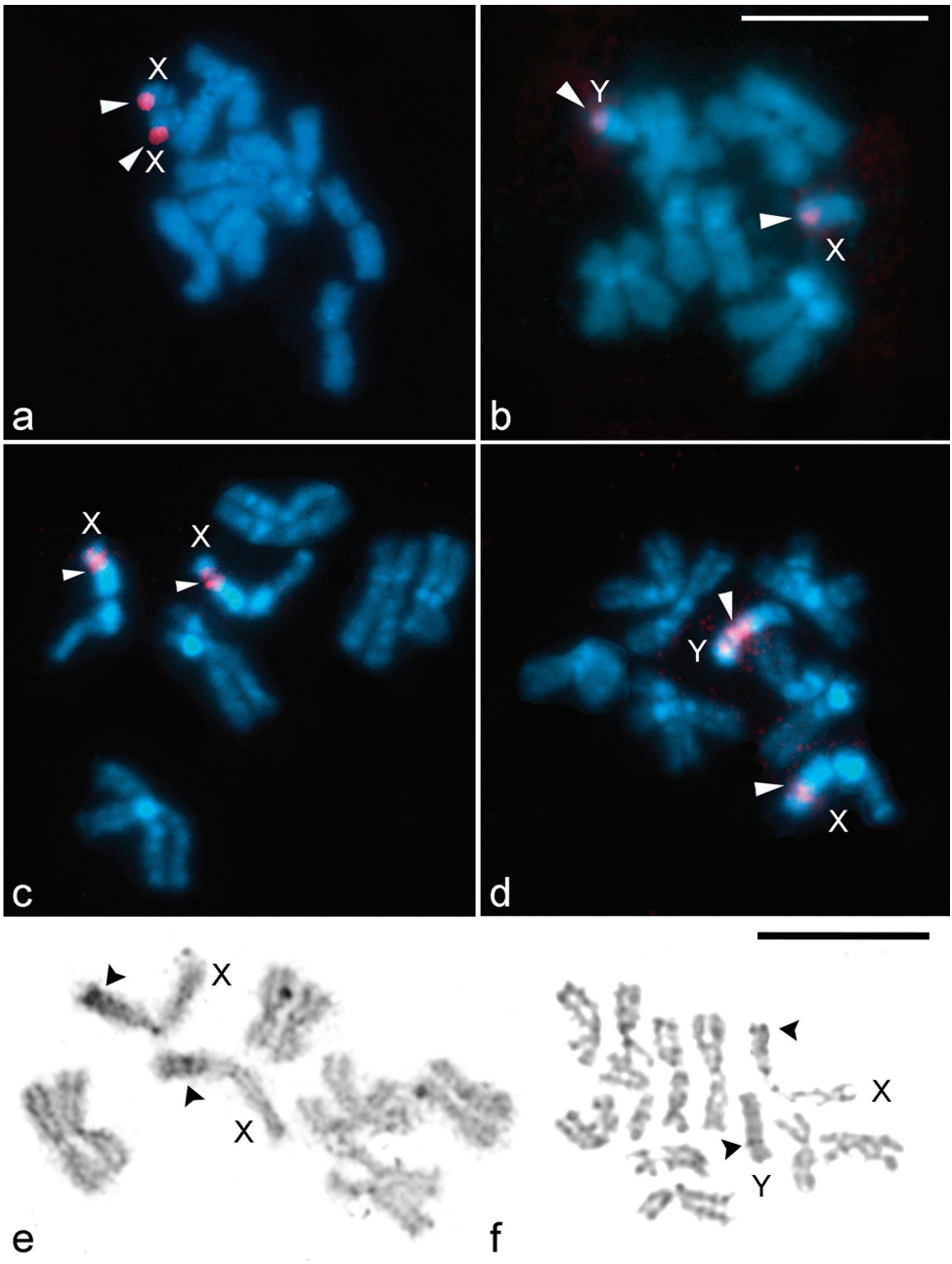

Figure 4. Location of rDNA genes and Ag-NOR sites on female and male mitotic chromosomes of *L. cluvia* (**a–b**) and *L. sericata* (**c–d**, **e–f**) using FISH with 18S rDNA probes (red signals, arrowheads) and silver impregnation technique. Chromosomes were counterstained with DAPI (blue). X, Y = sex chromosomes. Arrowheads indicate hybridization signals (**a–d**) and Ag-NOR sites (**e–f**) in both sex chromosomes. Bar = 10 μm.

Discussion

The karyotypes of the blowflies *Lucilia cluvia* and *L. sericata* examined in the present study agree fairly well with the earlier findings known from other *Lucilia* species and members of Calliphoridae. The species of this family show remarkable karyotypic uniformity with 2n = 12, comprising five pairs of large or medium-sized meta/submetacentric autosomes and a heteromorphic XX/XY sex chromosome pair (female/male) (Boyes and Shewell 1975, Azeredo-Espin and Pavan 1983, Parise-Maltempi and Avancini 2001, Ullerich and Shöttke 2006, Agrawal et al. 2010, Holecová et al. 2012).

Our analysis of mitotic chromosomes based on conventional staining and C- and G-like bandings revealed a homology in the five pairs of autosomes and a noticeable sex chromosome variation with respect to morphology, size and heterochromatin content in metaphase karyotypes of *L. cluvia* and *L. sericata*. The autosomes of Calliphoridae reveal a great deal of stability as compared to the sex chromosomes, which show variation in shape and size from one species to another (Boyes and Shewell 1975, Azeredo-Espin and Pavan 1983, Parise-Maltempi and Avancini 2001, Ullerich and Shöttke 2006, Agrawal et al. 2010, Holecová et al. 2012). Moreover, the autosomes in *L. cluvia* and *L. sericata* exhibited a very close somatic pairing (i.e. side-by-side pairing), a characteristic feature of chromosome complement of all the dipterans where the homologous chromosomes tend to lie next to one another. Consequently, the diploid complements give the appearance of a haploid set (Agrawal et al. 2010). Nonetheless, the sex chromosomes XX in females and XY in males did not show such intimate somatic pairing and tended to lie separately (Boyes and Shewell 1975, Ullerich and Shöttke 2006, Agrawal et al. 2010, Holecová et al. 2012, this study).

In *L. cluvia* and *L. sericata*, some characteristics of the karyotype and C-banding described herein resemble those previously reported and those of closely related species (Bedo 1980, El-Bassiony 2006, Ullerich and Shöttke 2006): i) the autosome pairs of both species decrease gradually in size and present small centromeric C-positive bands, and ii) the X chromosome of *L. sericata*, which is larger than autosome pair 1, has in its short arm a satellite and a subterminal secondary constriction where a single nucleolus is located. However, we observed the presence of interstitial C-positive heterochromatic bands in autosome pairs 2 and 3, and significant differences in morphology and C-banding pattern of the Y chromosome. In the Argentine population of *L. sericata*, the short arm of the Y submetacentric chromosome was found to be completely euchromatic and only the long Y-chromosome arm was mainly C-positive, whereas the Y telocentric chromosome from the African population is entirely C-banded (Ullerich and Shöttke 2006). A clear distinction between the populations from Argentina and Africa could be established due to the amount and distribution of constitutive heterochromatin in autosomes and in the Y sex chromosome. The data presented herein reveal a substantial polytypic variation in *L. sericata* and indicate that this chromosome polytypism might be due to the difference in gain of constitutive heterochromatin in the genome of both geographically isolated populations. Further studies are needed to

clarify the relationship between heterochromatin content and the geographical, ecological or environmental characteristics of the species under study.

Lucilia cluvia and *L. sericata* showed a high degree of similarity since homology of each autosome pair was established throughout G-like banding patterns, suggesting the absence of chromosome rearrangements in autosomes of both species and maybe within the genus *Lucilia* during karyotype evolution. Hence, the autosome pairs of *L. cluvia* and *L. sericata* were homologated by size and morphology, as well as by C- and G-like banding patterns. Considering the strong similarity of autosomes in Diptera calyptrate muscoid (Foster et al. 1980) together with the results here presented, we may infer that the autosomes of Calliphoridae retain a high degree of structural integrity and morphological stability.

The most remarkable interspecific dissimilarity of the *Lucilia* species herein studied is related to the X and Y sex chromosomes that show considerable variability in size, shape, and chromosome organization. Our results show that in *L. cluvia*, the X and Y chromosomes are subtelocentric and the smallest of the complement, with the proximal pericentromeric region of the long-X chromosome arm heterochromatic and the Y chromosome euchromatic. In contrast, the X chromosome is metacentric and the longest of the complement and the Y chromosome is a medium-sized submetacentric in *L. sericata*, being both of them mainly heterochromatic. In closely related species, the genome-size differences may be wholly explained by differential amounts of non-coding DNA (i.e. transposable elements, satellite DNAs, simple sequence repeats) (Gregory and Hebert 1999, Graur and Li 2000, Petrov 2001), and caused by diverse mechanisms such as duplications, deletions, genome mutations, activity of transposable elements, and amplification, accumulation or elimination of heterochromatin (Petrov 2001). Most segments of constitutive heterochromatin on eukaryotic chromosomes contain high concentrations of highly repeated (satellite) DNA and vary in composition and in length within and among species (Sumner 2003). Several dipteran species present different degrees of heterochromatinization in their sex chromosomes (Boyes and Shewell 1975, Bedo 1980, 1991, Baimai 1998, Parise-Maltempi and Avancini 2000, 2001, 2007, El-Bassiony 2006, Ullerich and Shöttke 2006, Agrawal et al. 2010, Holecová et al. 2012). Boyes and van Brink (1965) showed a tendency for the X chromosome, and to a lesser degree the Y, to accumulate heterochromatin in several subfamilies of calyptrate Diptera. Based on our findings, the interspecific size differences in the sex chromosomes of these *Lucilia* species could be due to differential amounts of constitutive heterochromatin, resulting from the amplification of pre-existing heterochromatin and/or the loss and/or gain of new heterochromatin.

In the present study, FISH experiments using 18S rDNA heterologous probes revealed two rDNA clusters in *L. cluvia* and *L. sericata*, one located in the X and the other one in the Y chromosome. The accurate detection of ribosomal genes on both sex chromosomes in *L. sericata* was confirmed by means of silver impregnation. The determination of the number and location of the NORs makes them essential cytological markers for the study of karyotype structure and chromosome evolution since the rDNA genes are noticeably conserved among dipteran species. In most species studied, the NORs are located in the sex chromosomes (Bedo and Howells 1987, Bedo 1992, Willhoeft and

Franz 1996, Willhoeft 1997). Considering the previous cytogenetic reports together with the mitotic karyotype, C-banding pattern and rDNA-FISH results here presented, we propose that in *L. sericata* the X and Y sex chromosomes accumulated a sufficient number of repetitive DNA sequences, leading to an increase in chromosome size.

Among these blowflies, there are some cryptic or isomorphic species, which cause great taxonomic problems because of their similarity in external morphology of maggot and/or imago stages. The results presented here showed that the C- and G-like bands, and rDNA loci may be considered as essential cytological markers to compare karyotypes of phylogenetically related species and, also, of sibling species. Besides, the use of these approaches may also contribute to the analysis of changes in karyotype related to the evolutionary process and to a better understanding of taxonomic relationships.

Acknowledgments

We thank Dr. N. D. Centeno for the grateful taxonomic determination of the specimens (Laboratorio de Entomología Aplicada y Forense, Universidad Nacional de Quilmes, Argentina). This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) (PICT 1198) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (PIP 0204) for Merani M.S., and CONICET (PIP 0281) and University of Buenos Aires (UBA) (UBACyT W917) for Bressa M.J.

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CompCytogen 9(1): 119–132 (2015)
doi: 10.3897/CompCytogen.v9i1.9012
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Heterochromatin distribution and comparative karyo-morphological studies in *Vigna umbellata* Thunberg, 1969 and *V. aconitifolia* Jacquin, 1969 (Fabaceae) accessions

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Academic editor: *L. Peruzzi* | Received 24 November 2014 | Accepted 21 January 2015 | Published 31 March 2015

http://zoobank.org/E63C4064-560F-41FB-941C-B8220BA5C3CE

Citation: Shamurailatpam A, Madhavan L, Yadav SR, Bhat KV, Rao SR (2015) Heterochromatin distribution and comparative karyo-morphological studies in *Vigna umbellata* Thunberg, 1969 and *V. aconitifolia* Jacquin, 1969 (Fabaceae) accessions. Comparative Cytogenetics 9(1): 119–132. doi: 10.3897/CompCytogen.v9i1.9012

Abstract

Chromosome studies along with heterochromatin distribution pattern analysis have been carried out in two domesticated species of *Vigna* Savi, 1824 which grow in contrasting geo-climatic conditions of India: *Vigna umbellata* Thunberg, 1969, a legume well acclimatized to subtropical hilly regions of North-east India and *V. aconitifolia* Jacquin, 1969, a species of arid and semi-arid regions in desert plains of Western India. Karyo-morphological studies in both species reveal $2n = 22$ chromosomes without any evidence of numerical variation and the overall karyotype symmetry in chromosome morphology suggest that the diversification at intraspecific level in genus *Vigna* has occurred through structural alteration of chromosomes, rather than numerical changes. Heterochromatin distribution as revealed by fluorochrome binding pattern using CMA_3 and DAPI, confirms the occurrence of relatively more GC content in *V. aconitifolia* as compared to *V. umbellata*. However, AT content was found to be comparatively higher in *V. umbellata* which perhaps play a role in species interrelationships.

Keywords

Asymmetry index, C-heterochromatin, Fabaceae, karyotype, NOR-chromosomes, *Vigna*

Introduction

The pantropical genus *Vigna* Savi, 1824 (Fabaceae) includes 104 described species (Lewis et al. 2005). Among its subgenera, only *Ceratotropis* Marechal, 1978 is known for its rich species diversity in Asia (Verdcourt 1970, Marechal et al. 1978, Tateishi 1996). Tomooka et al. (2002) recognized 21 species in the subgenus *Ceratotropis*, out of which six species are domesticated: azuki bean (*V. angularis* Willdenow, 1969), mung bean (*V. radiata* Linnaeus, 1954), black gram (*V. mungo* Linnaeus, 1956), rice bean (*V. umbellata* Thunberg, 1969), moth bean (*V. aconitifolia* Jacquin, 1969) and creole bean (*V. reflexo-pilosa* var. *glabra* Marechal, 1911). The genetic resources and diversity in cultivated and wild forms of subgenus *Ceratotropis* occurring in Indian subcontinent are extremely rich and interesting (Bisht et al. 2005). The domesticated *V. aconitifolia* is confined only to the tropical region of India, while *V. umbellata* is widely domesticated across the South-east Asia. The origin of *V. umbellata* is considered to be Indo-China region and also to a certain extent from South-east Asia (Marechal et al. 1978, Baudoin and Marechal 1988).

The structure and morphology of the chromosomes are of vital importance when studying the origin, evolution and classification of taxa (Yang et al. 2005) as well as distance or relatedness among diverse genomes (Stace 2000, Kumar and Rao 2002). Quite a few number of reports dealing with such studies are available for *Vigna* species (Rao and Chandel 1991, Rao and Raina 2004, Shamurailatpam et al. 2012).

Chromosome location and characterization of C-heterochromatin by fluorescence staining procedures which preferentially stain GC-rich DNA and DAPI, which localised AT-rich regions has been successfully applied in a large number of Fabaceae taxa including *Cicer arietinum* Linnaeus, 1753 (Galasso et al. 1996a); *Phaseolus calcaratus* Roxburgh, 1832 (Zheng et al. 1991); *Sesbania tetraptera* Hochstetter, 1871 (Forni-Martins et al. 1994, Forni-Martins and Guerra 1999); *Vicia faba* Linnaeus, 1753 (Greilhuber 1975); *Vigna ambacensis* Welwitsch, 1978 (Galasso et al. 1996b).

A certain degree of chromosomal variation at inter-specific level of the genus *Vigna* has been documented using cytogenetic approaches by earlier workers (Rao and Chandel 1991, Shamurailatpam et al. 2012). Hence, it will be quite significant to see the extent of variation among the domesticated species of *Vigna* (*Ceratotropis*). *V. umbellata* is a species domesticated extensively in the subtropical hilly and moist regions of North-east India. On the other hand, *V. aconitifolia* has been adapted to the arid and semi-arid region of tropical Western plain of India. Analysis of karyo-morphological details in *V. umbellata* and *V. aconitifolia*, adapted to extremely contrasting environmental conditions, may ultimately help us to define their chromosome variation. Meaningful propagation programs can be developed from such information.

Materials and methods

Karyo-morphological studies were undertaken in ten accessions each of *V. umbellata* and *V. aconitifolia*. The germplasm has been obtained from Indian Council of Agricultural Research (ICAR), Baranapi, Meghalaya and also from National Bureau of Plant Genetic Resources (NBPGR), New Delhi. Actively growing root tips of about 1–2 cm long were excised from germinating seeds on moist filter paper in Petri dishes at 25 ± 2 °C, pre-treated with 0.025% colchicine (Himedia) for 3 h at room temperature (20 ± 2 °C). The root tips after pre-treatment were fixed in freshly prepared ethanol-acetic acid (v/v, 3:1) and subsequently stored at 4 °C until required. For slide preparation, the root tips were washed twice in distilled water, hydrolyzed in 1N HCl at 60 °C for 8 min and stained in Feulgen stain (leuco-basic fuchsin) for 45 min. The stained root tips were thoroughly washed and subsequently squashed in 1% acetocarmine. The microphotographs of the metaphase plates were taken from both temporary and permanent preparations. At least 10–15 clear preparations of chromosome complements of each species were analyzed. Photo-idiograms were prepared from photomicrographs by cutting out individual chromosome and arranging them in descending order of their length and matching on the basis of morphology, the chromosomes were resolved into 11pairs. The standard method of chromosome classification given by Battaglia (1955) classification of metacentric / median (V), submetacentric/ submedian (L), subtelocentric (J) and telocentric (I) based on the arm ratio of 1:1, >1:1<1.3, >1:3<1:0 and 1:0 respectively was employed for comparison. The degree of asymmetry was estimated by means of the parameters proposed by Peruzzi and Eroğlu (2013): Coefficient of Variation of Chromosome Length (CV_{CL}) and Mean Centromeric Asymmetry (M_{CA}).

For heterochromatin characterization, root-tips were digested in 2% cellulase and 20% pectinase solution for 180 min at 37°C. Meristems were washed in distilled water, squashed in a drop of 45% acetic acid, and frozen in liquid nitrogen. The slides were stained with DAPI (2 µg/ml): glycerol (1:1, v/v) solution to allow selection of the best plates. Subsequently, they were destained in ethanol: glacial acetic acid (3:1, v/v) for 30 min and transferred to absolute ethanol for 1 h, both at room temperature. Slides were air-dried and aged for 3 days at room temperature. The slides were stained with CMA_3 (0.5 mg/ml, 1 h) and DAPI (2 µg/ml, 30 min), mounted in McIlvaine's buffer (pH 7.0): glycerol (1:1, v/v), and stored for 3 days (Schweizer and Ambros 1994). Slides were analyzed under Leica DM 4000 B microscope and photographs were carried out with different filter combinations using Leica CCD camera.

Results

The somatic chromosome number of all the accessions had consistently $2n = 2x = 22$ (Fig. 1). The chromosome complements were resolved into 11 pairs which formed a graded series from longest to shortest within the idiograms. A noticeable difference in

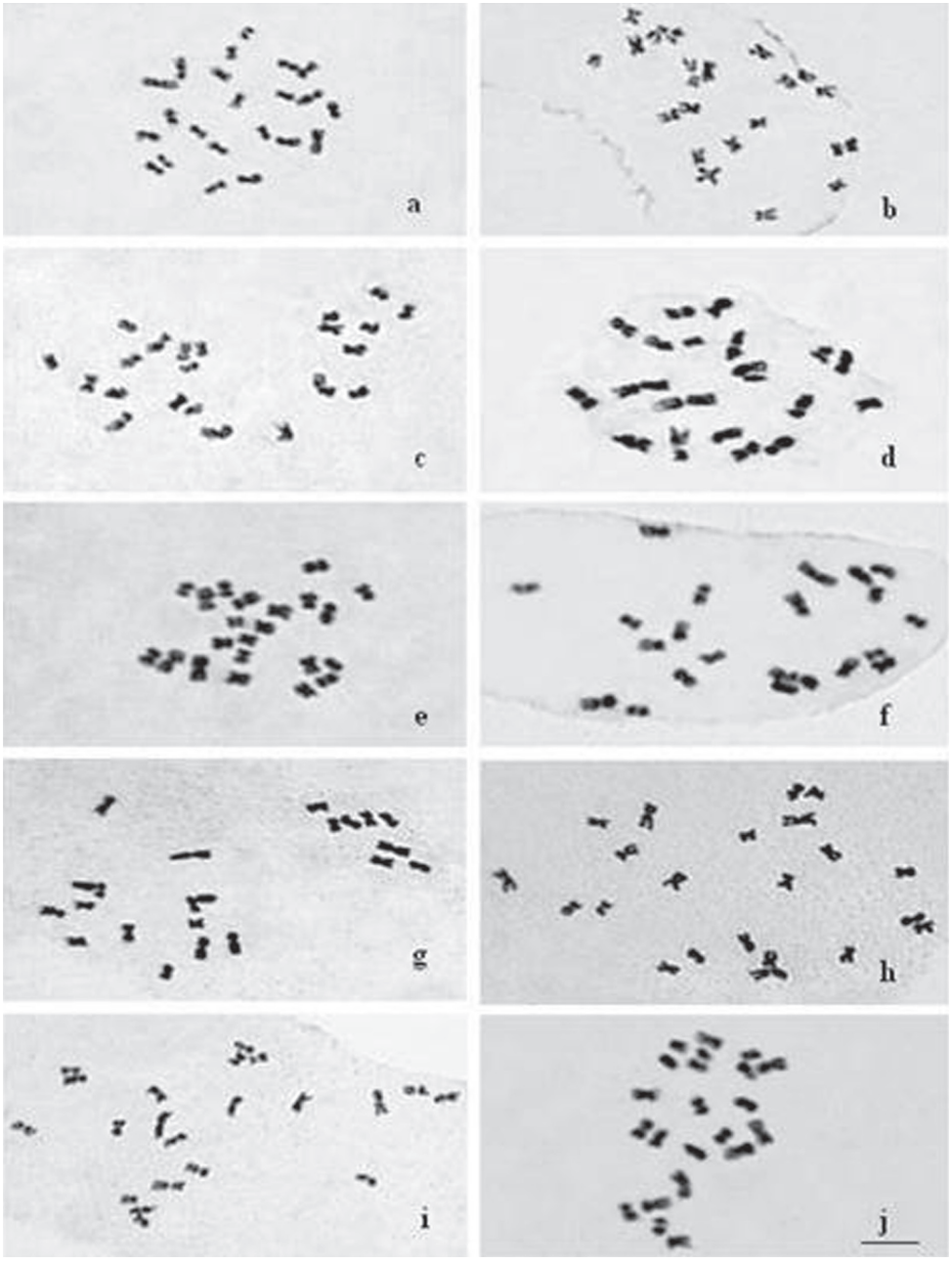

Figure 1. Mitotic complements of 10 accessions of *V. umbellata*. **a–j: a** BKSB 205 **b** TRB 160 **c** RBS 35 **d** IC 551699 **e** BKSB 192 **f** RBS 53 **g** IC 55440 **h** IC 176563 **i** EC 97882 **j** BKSB 194. Bar = 5 μm.

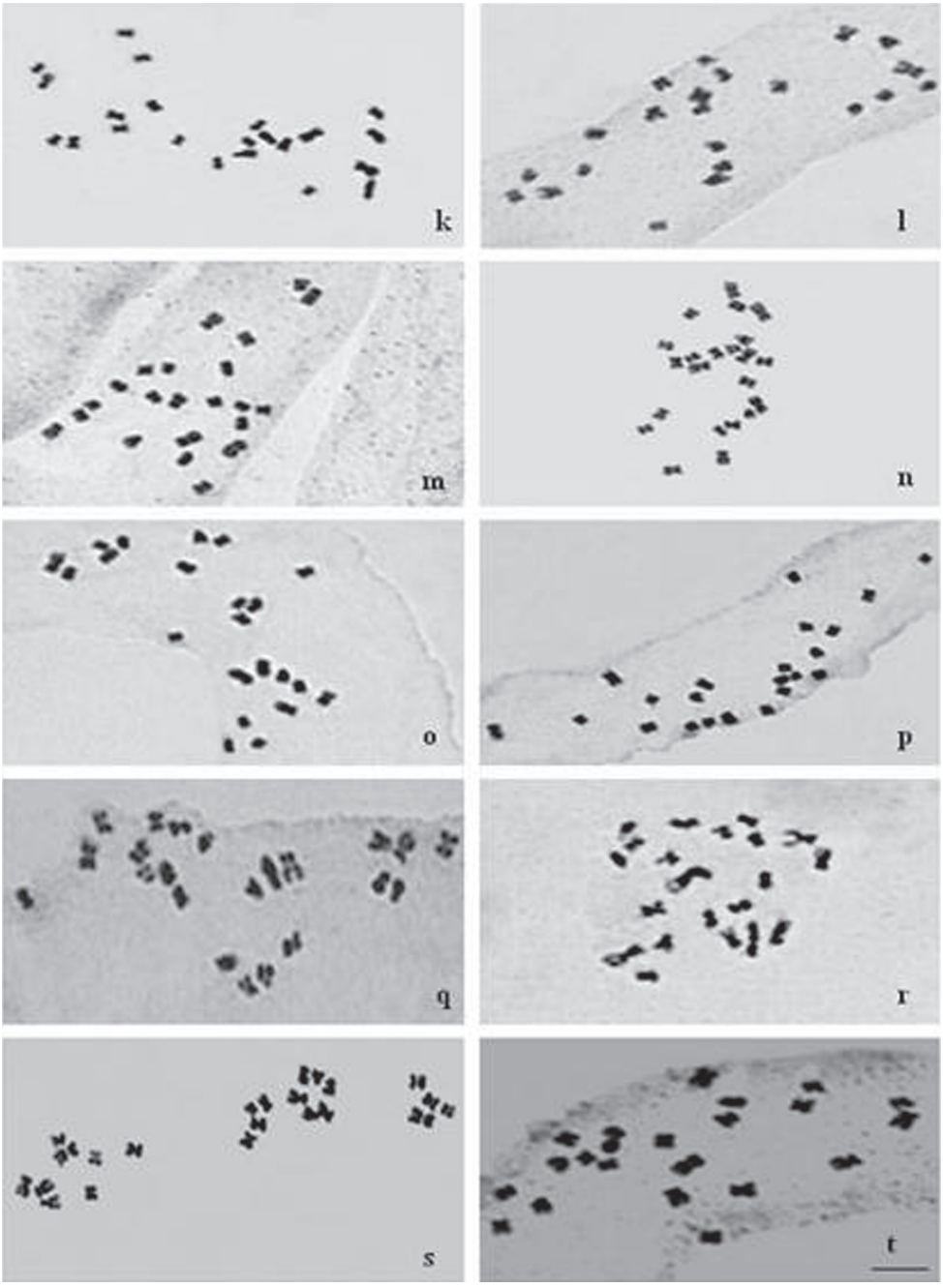

Figure 1. Continued. **k–t: k** IC 36157 **l** VDV 6175 **m** IC 472147 **n** RM 040 **o** IC 39809 **p** IC 285159 **q** IC 36592 **r** IC 472173 s IC 39713 **t** IC 36562. Bar = 5 μm.

Table 1. Karyomorphology and arm ratio in studied taxa of *Vigna*.

Sl. no.	Species	Accessions no.	2n	Chromosome arm length (L/S ratio)											Ratio of longest and shortest chromosome	Karyotype formula
				I	II	III	IV	V	VI	VII	VIII	IX	X	XI		
1	*V. umbellata*	BKSB 205	22	1.12 1.14	1.8	1.5	1.5	1.25	1	1.3	1.3	1.3	1.3	1.3	2.4	2V + 20L
2	*V. umbellata*	TRB 160	22	1.2 1.5	1.1 1.25	1.75	1.37	1	1	1	1.3	1.3	1	1	2.2	12V + 10L
3	*V. umbellata*	RBS 35	22	1.2	1.6	1	1	1	1	1	1.15	1.3	1.3	1	2.8	12V + 10L
4	*V. umbellata*	IC 551699	22	1 1	1.3	1.7	1.15	1.57	1.75	1.1	1	1	1	1	4.0	12V + 10L
5	*V. umbellata*	BKSB 192	22	1	1	1	1.25	1.12	1	1	1	1.3	1	1	2.0	16V + 6L
6	*V. umbellata*	RBS 53	22	1.2	1.1	1.25	1.25	1	1	1	1	1.3	1.3	1	2.3	12V + 10L
7	*V. umbellata*	IC 55440	22	1.57 1.42	1.2	1.35	1.12	1.12	1.12	1	1	1	1.15	1.3	3.0	6V + 16L
8	*V. umbellata*	IC 176563	22	1.14 1.28	1.6	1.1	1.25	1.6	1.3	1	1	1.6	1.3	1	2.6	8V + 14L
9	*V. umbellata*	EC 97882	22	1.7	1.47	1.2	1.37	1.2	1	1	1.65	1.3	1.3	1	2.3	8V + 14L
10	*V. umbellata*	BKSB 194	22	1	1	1	1.3	1.3	1.3	1.3	1.3	1	1	1	3.0	12V + 10L
11	*V. aconitifolia*	IC 36157	22	1.33	1.9	2	1.5	1.1 1.5	1.5	1.5	1.5	1	1	1	1.75	6V + 16L
12	*V. aconitifolia*	VDV 6175	22	1.5 1.66	2.5	1.33	2	1.5	1.5	1.5	1.5	1.25	1	1	2.5	4V + 16L + 2J
13	*V. aconitifolia*	IC 472147	22	2	2.5	2.5 2	1	1	1	1.5	1.75	1.5	1	1	2.25	10V + 12L
14	*V. aconitifolia*	RM 040	22	1.5	1.66	3 2	1.5	2	2	1.75	1.25	1.5	1.5	1	2.5	2V + 18L + 2J
15	*V. aconitifolia*	IC 39809	22	1.57	2	1.66	1.33	2	1.16	1	1.25	1.25	1.5	1	3.0	4V + 18L
16	*V. aconitifolia*	IC 285159	22	1.33	2	1.5	1.5	1	1.5	1.25	1	1	1	1	3.5	10V + 12L
17	*V. aconitifolia*	IC 36592	22	1.75 1.6	1.62	1	1	1.66	1	1	1.33	1.33	1	2	2.16	10V + 12L
18	*V. aconitifolia*	IC 472173	22	2.4 1.8	1.83 2	1.86	1.25	1.12	1	1	1	1	1	1	4.25	14V + 8L
19	*V. aconitifolia*	IC 39713	22	2	2.5	1	2	1	1.5	1.5	1.5	1.5	1.5	1	2.25	6V + 16L
20	*V. aconitifolia*	IC 36562	22	1.58	3	1.49	1.33	1	1	1	1.5	1.5	1.5	1	2.5	8V + 14L

length between the longest and the shortest chromosomes within the complement was recorded (Table 1). The longest chromosome of the haploid complement was almost 2.5 times longer than the shortest one in *V. aconitifolia* accessions, while it was 2 times longer than the shortest one in *V. umbellata* accessions. Further investigated accessions belonging to *V. umbellata* and *V. aconitifolia* had metacentric, submetacentric and subtelocentric chromosomes in their respective chromosome complements. Submetacentric chromosomes outnumbered the metacentric ones in *V. aconitifolia* accessions while metacentric chromosomes outnumbered the submetacentric chromosomes in the case of *V. umbellata* accessions.

Various accessions of these species have shown distinctive variation in the karyotype with respect to number of metacentric and submetacentric chromosomes (Fig. 2). Subtelocentric chromosomes were found in *V. aconitifolia* but not in *V. umbellata* accessions. Heteromorphic chromosome and nucleolar chromosomes are recorded in the accessions of both *V. umbellata* and *V. aconitifolia*.

Telocentric chromosomes were absent in both the taxa studied. Heteromorphic chromosomes were observed in some of the *V. umbellata* accessions: BKSB 205 (1st pair, Fig. 2a), TRB 160 (1st and 2nd pair, Fig. 2b), IC 551699 (1st pair, Fig. 2d), IC55440 (1st pair, Fig. 2g) and IC 176563 (1st pair, Fig. 2h). In *V. aconitifolia* heteromorphic chromosomes were found in IC 36157 (5th pair, Fig. 2k), VDV 6175 (1st pair, Fig. 2l), IC 472147 (3rd pair, Fig. 2m), RM040 (3rd pair, Fig. 2n), IC 36592 (1st pair, Fig. 2q) and IC 472173 (1st and 2nd pair, Fig. 2r) accessions. Nucleolar Organizing Regions (NORs), as a secondary constriction/satellites, were observed in *V. umbellata* accessions RBS 35 (1st pair, Fig. 2c), IC 551699 (2nd pair, Fig. 2d), and EC 97882 (3rd and 4th pair, Fig. 2i). *Vigna umbellata* was characterized by the presence of both metacentric and submetacentric chromosomes and two *V. aconitifolia* accessions (VDV 6175 and RM 040) were characterized by the presence of distinct subtelocentric chromosome, though their position differed in karyotype. The remaining accessions were devoid of any subtelocentric chromosome.

According to the scatter plot obtained by CV_{CL} vs. M_{CA}, BKSB 192 (*V. umbellata*) and EC 97882 (*V. umbellata*) showed the lowest (2.81) and highest (55.07) M_{CA} respectively (Fig. 4). Furthermore IC 285159 (*V. aconitifolia*) and RM 040 (*V. aconitifolia*) showed lowest (10.8) and highest (22.88) M_{CA} values. In *V. umbellata* TRB 160 and IC 551699 exhibited lowest (19.59) and highest (37.4) CV_{CL} values. Among *V. aconitifolia* accessions IC 36157 and IC 472173 had shown lowest (19.92) and highest (42.64) CV_{CL} values.

A comparative account of heterochromatin distribution pattern within the chromosome complements in *V. umbellata* and *V. aconitifolia* has been summarized in Table 3 and the data have been illustrated in Fig. 3. The CMA_3^+ and $DAPI^+$ binding sites were found either in terminal or in interstitial regions, in both the taxa studied. *V. umbellata* had more of $DAPI^+$ sites 3.1(± 1.9) in the interstitial region of the chromosomes and the terminal binding sites were 1.8(± 0.6). The number of chromosomes showing different CMA^+ and $DAPI^+$ sites also ranged from 2–7 in this

Figure 2. Photo-idiograms of **a–j** 10 accessions of *V. umbellata* **a** BKSB 205 **b** TRB 160 **c** RBS 35 **d** IC 551699 **e** BKSB 192, **f** RBS 53 **g** IC 55440 **h** IC 176563 **i** EC 97882 **j** BKSB 194 **k–t** 10 accessions of *V. aconitifolia* **k** IC 36157 **l** VDV 6175 **m** IC 472147 **n** RM 040 **o** IC 39809 **p** IC 285159 **q** IC 36592 **r** IC 472173 **s** IC 39713 **t** IC 36562. Heteromorphic groups marked above the short arm and nucleolar groups are marked below the long arm.

Table 2. Karyotype formulae and characteristics in the studied taxa of *Vigna*. SC the shortest chromosome length; LC the longest chromosome length; CL mean length of chromosome; CI mean centromeric index; SD standard deviation; CV_{CL} component expressing the relative variation in chromosome length; M_{CA} mean centromeric asymmetry.

Sl. no.	Accessions no.	2n	Range SC-LC (µm)	Ratio LC/SC	CL (µm) Mean (± SD)	CI Mean (± SD)	CV_{CL}	M_{CA}
1	BKSB 205	22	17-7	2.4	9.13 (± 2.76)	42.87 (± 3.47)	30.25	14.30
2	TRB 160	22	11-5	2.2	7.95(± 1.55)	45.22 (± 4.65)	19.59	9.09
3	RBS 35	22	17-6	2.8	9.18(± 2.95)	46.93 (± 3.85)	32.19	6.18
4	IC 551699	22	24-6	4	11.5(± 4.3)	45.59 (± 5.03)	37.4	9.06
5	BKSB 192	22	12-6	2	8.45(± 1.78)	48.57 (± 2.49)	21.16	2.81
6	RBS 53	22	14-6	2.3	8.40(± 1.74)	47.09 (± 2.91)	20.72	5.78
7	IC 55440	22	18-6	3	9.31(± 2.99)	46.55 (± 2.92)	32.13	6.49
8	IC 176563	22	16-6	2.6	9.18(± 2.66)	44.29 (± 4.65)	29.02	11.37
9	EC 97882	22	14-6	2.3	9.18(± 2.27)	44.94 (± 3.94)	24.82	55.07
10	BKSB 194	22	18-4	3	7.09(± 1.62)	46.75 (± 3.55)	22.86	6.49
11	IC 36157	22	7-4	1.75	5.22 (± 1.04)	42.44 (± 6.07)	19.92	15.32
12	VDV 6175	22	10-4	2.5	5.68 (± 1.54)	40.76 (± 7.06)	27.25	18.55
13	IC 472147	22	9-4	2.25	5.81 (± 1.36)	41.61 (± 8.54)	23.54	16.51
14	RM 040	22	10-4	2.5	6.09 (± 1.67)	38.78 (± 6.45)	27.52	22.88
15	IC 39809	22	12-4	3	6.72 (± 2.02)	42.04 (± 6.17)	30.12	16.22
16	IC 285159	22	7-2	3.5	4.68 (± 1.25)	43.63 (± 5.72)	26.85	10.8
17	IC 36592	22	11-6	2.16	7.95 (± 1.60)	43.89 (± 6.32)	20.22	12.21
18	IC 472173	22	17-4	4.25	8.59 (± 3.66)	44.99 (± 7.29)	42.64	10.14
19	IC 39713	22	9-4	2.25	5.72 (± 1.28)	40.47 (± 6.85)	22.44	19.05
20	IC 36562	22	10-4	2.5	6.22 (± 1.47)	43.34 (± 8.09)	23.68	15.13

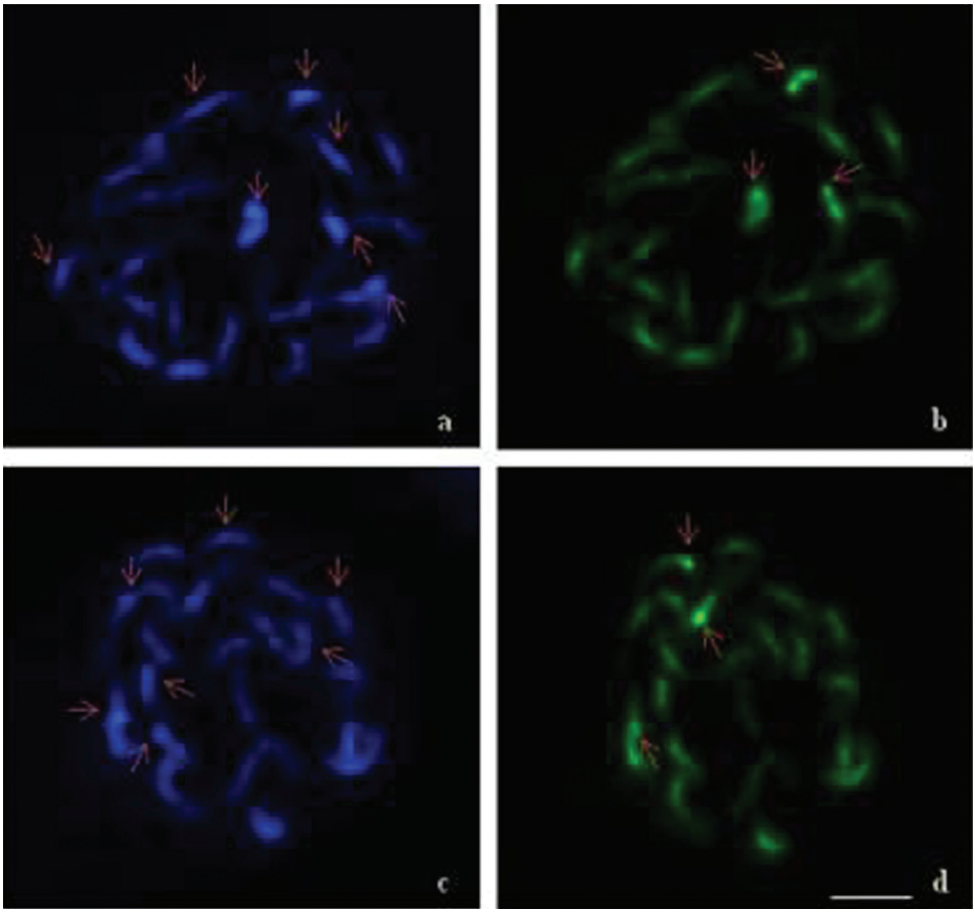

Figure 3. Differentially stained mitotic chromosomes complements **a–b** *V. umbellata* **c–d** *V. aconitifolia*. Arrows indicate CMA⁺ and DAPI⁺ sites. Scale bar = 5 μm in all the figures.

Table 3. Distribution of CMA⁺ and DAPI⁺ sites in the chromosomes of *Vigna* species.

Species	Mean± SD of CMA⁺ sites in chromosomes		Mean± SD of DAPI⁺ sites in chromosomes		Range of CMA⁺ sites Terminal	Range of DAPI⁺ sites Interstitial
	Terminal	Interstitial	Terminal	Interstitial		
V. umbellata	1.7 ± 0.8	2.1 ± 0.8	1.8 ± 0.6	3.1 ± 1.9	1.7 ± 0.8	2.1 ± 0.8
V. aconitifolia	2.9 ± 1.3	2 ± 1.2	2.7 ± 0.7	2.3 ± 0.8	2.9 ± 1.3	2 ± 1.2

species. On the other hand, in *V. aconitifolia* the heterochromatin block comprised more of CMA⁺ binding sites 2.9(± 1.3), which were found in the terminal region of the chromosomes while 2(± 1.2) binding sites were interstitial in position. The number of chromosomes showing CMA⁺ sites ranged from 3–7, while those showing the DAPI⁺ sites ranged from 3–8.

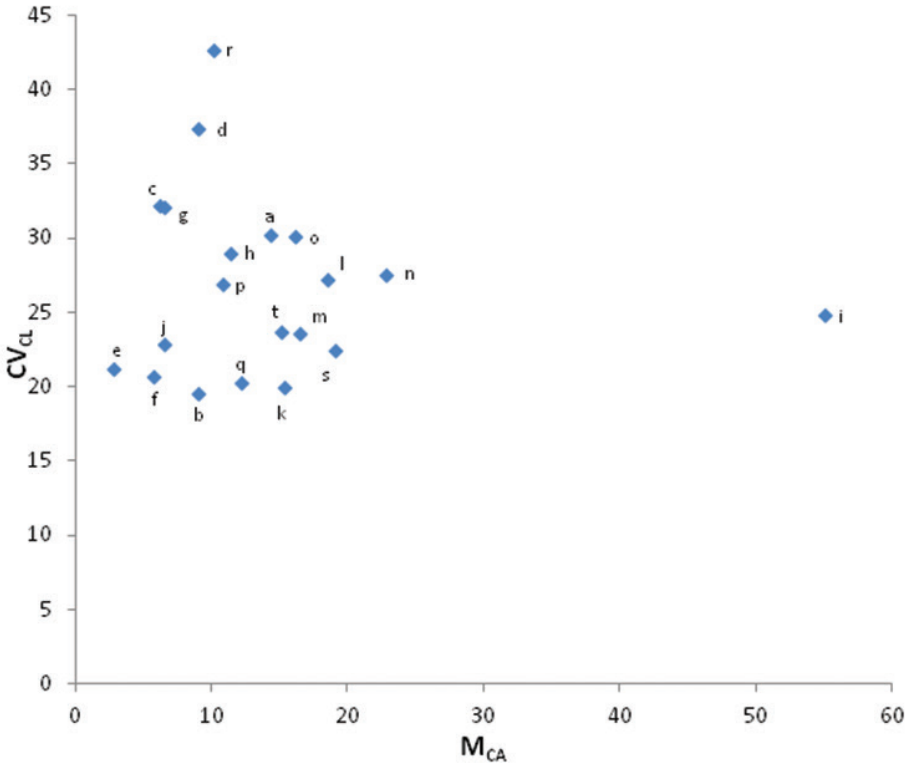

Figure 4. Scatter plot based on the karyotype parameters M_{CA} (x axis) vs. CV_{CL} (y axis) **a** BKSB 192 **b** RBS 53 **c** RBS 35 **d** BKSB 194 **e** IC 55440 **f** IC 551699 **g** TRB 160 **h** IC 472173 **i** IC 285159 **j** IC 176563 **k** IC 36592 **l** BKSB 205 **m** IC 36562 **n** IC 36157 **o** IC 39809 **p** IC 472147 **q** VDV 6175 **r** IC 39713 **s** RM 040 **t** EC 97882.

Discussion

The present data, combined with the chromosome counts available from the literature confirm the somatic chromosome number of $2n = 22$ for both species, *V. umbellata* and *V. aconitifolia*. Such observation received support from reports of Singh and Roy (1970), Rao and Chandel (1991), Rao and Raina (2004), Shamurailatpam et al. (2012). The presence of subtelocentric chromosomes in *V. aconitifolia* accessions is in agreement with the earlier report of Sinha and Roy (1979).

All the accessions of *V. umbellata* and *V. aconitifolia* have shown no deviation in somatic chromosome numbers and overall karyotype appearance. However, *V. umbellata* had a higher degree of karyotype asymmetry as compared to *V. aconitifolia*, suggesting structural rearrangements in karyotypes. Hence, the observed karyotype variation is likely to have originated by structural changes in chromosomes vs. duplication, deletions, interchanges and inversions (Stebbins 1971, Rao and Chandel 1991). Thus, structural alteration of the chromosomes involving centric fusion and centromere repositioning might have influenced the speciation in genus *Vigna*.

Due to the very small size of chromosomes accompanied by technical difficulties, the nucleolus organisers among the chromosome complements could not be clearly resolved. Other cytogenetic techniques such as silver staining and fluorescence *in situ* hybridization (FISH) can be useful in detecting NOR-loci on chromosomes.

The DAPI$^+$ binding sites in chromosomes, which are indicative of AT-rich region, were recorded in the interstitial regions of chromosomes in *V. umbellata*. However CMA$^+$ sites, found mostly in *V. aconitifolia* chromosomes, suggest that the heterochromatin blocks were rich in GC base composition at terminal regions of chromosomes. The higher distribution of AT- and GC- repetitive sequence in heterochromatin blocks is probably reflecting the processes of divergent evolution of repetitive sequences, in heterochromatin regions of *Vigna* species (Shamurailatpam et al. 2014).

In the course of evolution, most of the heterochromatin regions tend to increase (Ikeda 1988), this phenomenon is also observed in *Vigna* (Shamurailatpam et al. 2015). Certain genera such as *Vicia*, *Phaseolus*, *Sesbania*, *Cicer* and *Vigna* (Greilhuber 1975, Zheng et al. 1991, Forni-Martins et al. 1994, Galasso et al. 1996a, b, Forni-Martins and Guerra 1999) showed a heterochromatin-rich chromosome configuration, that might have been involved in diversification of this genus. *Vigna umbellata*, which is domesticated extensively in the sub tropical hilly and moist regions of North-east India, had its heterochromatin blocks rich in AT content with fewer GC base pairs. On the contrary, more GC content in heterochromatin blocks was observed in *V. aconitifolia*, which is acclimatized to the arid and semi-arid region of tropical Western plains of India, helping the species to overcome adverse climatic conditions of Indian desert. Our observations in this regard constitute a first attempt to probe the role of heterochromatin distribution pattern, if any, in species differentiation of plant groups.

Acknowledgements

The present work is supported by a grant from the World Bank funded by Indian Council of Agriculture Research (ICAR) project received through National Agriculture Innovation Program (NAIP). We thank the Head, Department of Biotechnology and Bioinformatics, North Eastern Hill University (NEHU), Shillong for providing facilities. Sincere thanks are also due to members of the Plant Biotechnology Laboratory for their help. The generous support for germplasm of *V. umbellata* extended by Dr. A. Pattanayak, Division of Biotechnology, ICAR complex for North Eastern Hill Region, Barapani, Meghalaya is duly acknowledged.

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CompCytogen 9(1): 133–144 (2015)
doi: 10.3897/CompCytogen.v9i1.4463
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Further karyosystematic studies of the *Boreonectes griseostriatus* (De Geer) group of sibling species (Coleoptera, Dytiscidae)–characterisation of *B. emmerichi* (Falkenström, 1936) and additional European data

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Academic editor: *Natalia Golub* | Received 5 January 2015 | Accepted 26 February 2015 | Published 31 March 2015

http://zoobank.org/B0383F0E-BC18-4F8C-BBE2-0CA199846159

Citation: Angus RB, Angus EM, Jia F, Chen Z-n, Zhang Y (2015) Further karyosystematic studies of the *Boreonectes griseostriatus* (De Geer) group of sibling species (Coleoptera, Dytiscidae)–characterisation of *B. emmerichi* (Falkenström, 1936) and additional European data. Comparative Cytogenetics 9(1): 133–144. doi: 10.3897/CompCytogen.v9i1.4463

Abstract

A lectotype is designated for the Tibetan species *Deronectes emmerichi* Falkenström, 1936 (Currently *Boreonectes emmerichi* (Falkenström)), and its habitus, as well as the median lobe and parameres of its aedeagus, are figured along with additional comparative material. Material of *B. emmerichi* from Sikkim (BMNH) represents the first record of a *Boreonectes* Angus, 2010 species from India. The karyotype of *B. emmerichi* is described as having 26 pairs of autosomes plus sex chromosomes which are X0 (♂), XX (♀). The karyotype is most like that of *B. macedonicus* (Géuorguiev, 1959), but with slight differences. Additional chromosomal information is given for *B. griseostriatus griseostriatus* (De Geer, 1774) in the French Alps, *B. g. strandi* (Brinck, 1943) on the Kola Peninsula, *B. multilineatus* (Falkenström, 1922) in the Pyrenees and *B. ibericus* (Dutton & Angus, 2007) in the Spanish Picos de Europa.

Keywords
Coleoptera, Dytiscidae, Karyotype, C-banding, species complex, Tibet, lectotype, Sikkim, first record from India

Introduction

The group of species related to *Boreonectes griseostriatus* (De Geer, 1774) presents serious taxonomic problems. Helliesen (1890), working in Norway, recognised an inland montane stripy form, which he regarded as true *griseostriatus*, and a coastal, more blotchy form, which he named *maritimus* sp. n. Falkenström (1922) showed that De Geer's *griseostriatus* in fact referred to the coastal form, and described the inland form as *Deronectes multilineatus* Falkenström, 1922. Many subsequent authors rejected the notion that more than one species was involved. Thus Zaitzev (1953) in the Israeli English translation (1972) noted that "This species (*B. griseostriatus*) varies markedly in many characters; all attempts to establish subspecies and varieties are unjustified, because almost all varieties are connected by transitions." However, chromosome analysis by Angus began to show that several species were involved and Dutton and Angus (2007) demonstrated the existence of seven chromosomally distinct species, all difficult or even impossible to recognise on external morphology. Further investigations by Angus (2008, 2009, 2010a, b) gave more information, culminating in the erection of a new genus, *Boreonectes* Angus, 2010, for these and some related species. However, there remain a number of described Palaearctic taxa for which there are no chromosomal data. Prominent among these is *B. emmerichi* (Falkenström, 1936), described from Sichuan and regarded as widespread in Tibet (Nilsson and Hájek 2015).

A beetle-collecting trip to the Tibetan Plateau in June 2013 (Angus 2013) enabled the senior author to obtain living material of *B. emmerichi*. Laboratory facilities in Qinghai Normal University enabled preparation of slides with dividing cells for karyotype investigation. The opportunity is also taken to include additional data on European species.

Material and methods

The species studied are listed in Table 1 and the localities are shown in Figs 4, 7–9. The museums housing material discussed here are the Natural History Museum, London (BMNH), the Naturhistoriska Riksmuseet, Stockholm (NRMS), the Museum of Biology, Sun Yat-sen University, Guangzhou (SYSU) and the Barcelona branch of the Spanish Institute for Evolutionary Biology (IBEB). The methods of chromosome preparation, C-banding and photography are as given by Dutton and Angus (2007), with the treatment times in colchicine and ½-isotonic KCl each 12 minutes. With the Chinese material (*B. emmerichi*) C-banding was attempted in London, several weeks after initial preparation of the slides. This was moderately successful, though not as

Table 1. Material used for chromosome analysis.

Species	Locality	No. of specimens analysed	Location of specimens
B. emmerichi	CHINA, Qinghai Province, Gangca, 1 km SE of Gangca Dasi. (Fig. 4)	2 ♂♂, 1♀ (Whole beetle illustrated.)	BMNH
	CHINA, Qinghai Province, ca 20 km W of Maduo. (Fig. 4)	3♂♂, 2♀♀ (Whole beetles illustrated.)	BMNH
	CHINA, Xizang Autonomous Region, Sejilashan. (Fig. 4)	(Whole beetle illustrated.)	SYSU, BMNH
	CHINA, Xizang Autonomous Region, Nam Tso. (Fig. 4)	(Whole beetle illustrated.)	IBEB
	INDIA, Sikkim, Lachen. (Fig. 4)	(Whole beetle illustrated.)	BMNH
B. g. griseostriatus	FRANCE, Savoie, S of Lac du Mont Cenis. (Fig. 7)	2 ♂♂, 2♀♀	BMNH
B. g. strandi	RUSSIA, Kola Peninsula, near Teriberka. Leg. P. Petrov. (Fig. 8)	5 ♂♂, 4 ♀♀	BMNH
B. multilineatus	FRANCE, Hautes-Pyrénées, Lac d'Anapéou. Leg. F. Bameul. (Fig. 9)	5 ♂♂	BMNH
B. ibericus	SPAIN, Cantabria, Lagos de Lloroza. (Fig. 9)	1 ♂	BMNH

good as that obtained with 48-hour-old slides. Specimens from which chromosomes were obtained are housed in the BMNH. Habitus photographs of whole beetles (apart from the Nam Tso specimen, for which Ignacio Ribera sent the picture) were taken with a Leica M125 stereomicroscope + Canon EOS 550D digital camera, and the parameres with a Zeiss Axioskop + Canon EOS 450D digital camera, in the Sackler Bioimaging Laboratory of the Natural History Museum. Both were stacked using Helicon Focus software. The scanning electron micrographs are of uncoated specimens. Those shown in Fig. 2a–f were taken in the Electron Microscope Unit of the Natural History Museum, using a Zeiss Leo 1455VP SEM in low vacuum mode and backscattered electrons. The one in Fig. 2g was taken in the Biomedical Imaging Unit of Southampton General Hospital, using an FEI Quanta 200 SEM in low vacuum mode, using secondary electrons to image.

Results and discussion

B. emmerichi (Falkenström, 1936: 88).

Falkenström described *B. emmerichi* (as *Deronectes emmerichi*) from 15 specimens, including five males, from the Kangding area of Sichuan, on the eastern edge of the Tibetan Plateau. Seven specimens are listed with the data "China, Szechuan, Mukue-Tatsienlu" and eight "China, Szechuan, Tatsienlu Tjiji (Urwald Rodungen)". Tatsienlu is the former name of Kangding and Urwald Rodungen (a German term) are

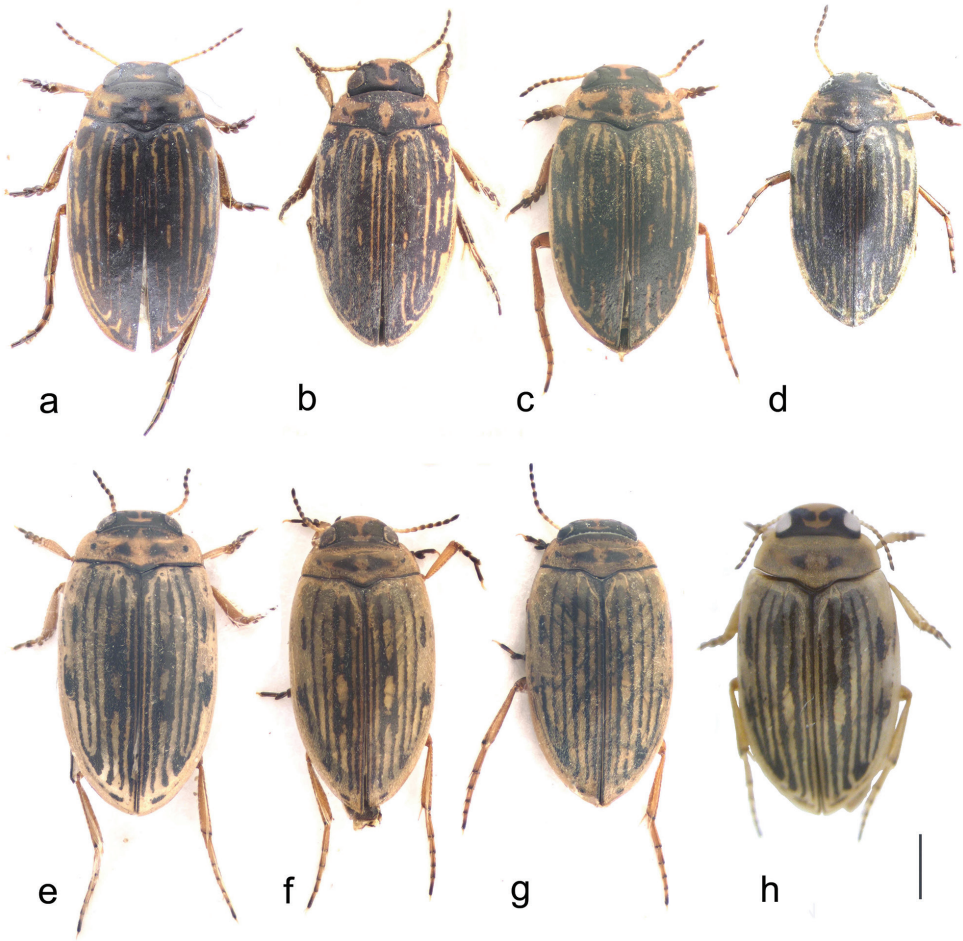

Figure 1. Habitus photographs of *Boreonectes emmerichi*. **a** lectotype ♂ **b** paralectotype ♀ **c** dark ♂, Sejilashan, Xizang **d** small dark ♂; Lachen, Sikkim **e** ♀, Gangca Dasi **f** ♂, Maduo (aedeagal median lobe: Fig. 2c) **g** ♂, Maduo (aedeagal median lobe: Fig. 2b) **h** ♀, Nam Tso. Scale = 1 mm.

clearings in primary forest. Three syntypes, an intact male labelled as Holotypus, a dissected male without the genitalia and a female labelled as Allotypus, are housed in the Falkenström collection (NRMS) and there is a further female syntype in London (BMNH). We do not know the whereabouts of the other specimens listed by Falkenström. The female with the Allotypus label has a data label "China, Szechuan, Mukue-Tatsienlu" but all the others have the labels "China, Szechuan, Tatsienlu Tjiji (Urwald Rodungen)". The intact male is here designated lectotype, so the type locality is fixed as Tatsienlu Tjiji, 29°59.906'N, 101°57.492'E. The remaining specimens are paralectotypes. We have dissected this male, and the median lobe (penis) is shown in Fig. 2a, left paramere in Fig. 3a and habitus in Fig. 1a. As noted by Falkenström, this is a rather dark species, with the black markings very heavy. All the specimens are

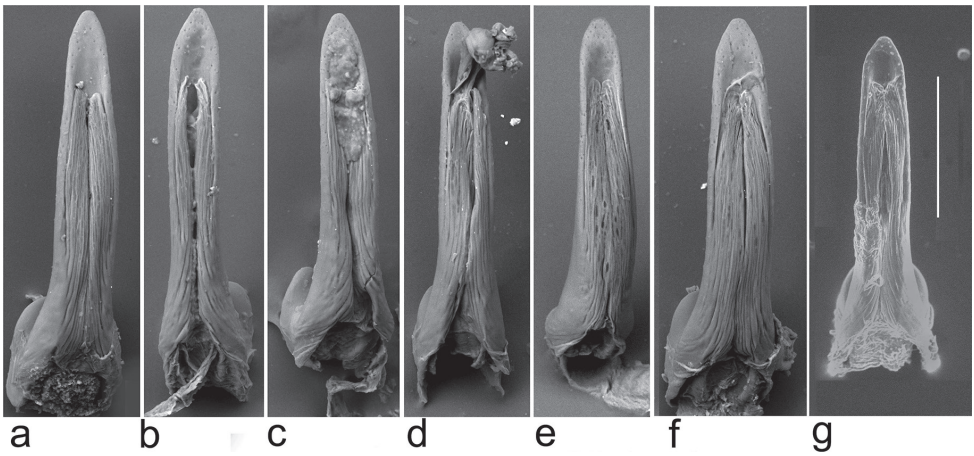

Figure 2. Scanning electron micrographs of aedeagal median lobes (ventral view). **a** *B. emmerichi*, lectotype **b–d** *B. emmerichi*, Maduo **d** with a partially extruded spermatophore **e**, **f** *B. emmerichi*, Gangca Dasi **g** *B. macedonicus*, Karanikoličko Jezero, Macedonia; Scale = 0.2 mm.

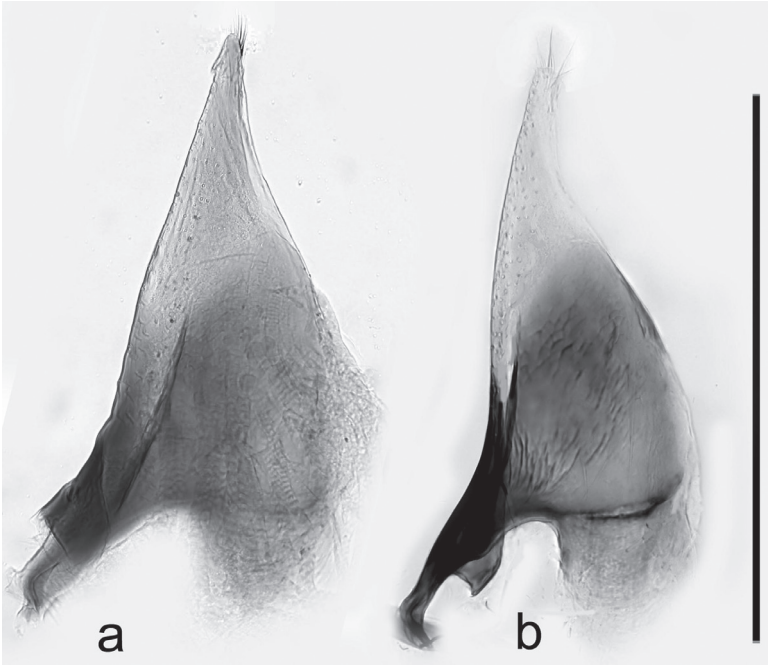

Figure 3. *Boreonectes emmerichi*, left parameres. **a** lectotype **b** Gangca Dasi. Scale = 0.5 mm.

similarly dark, and the London female is shown in Fig. 1b. This heavy dark pattern is matched by material collected by Fenglong Jia in swampy pools among dense bushes on Sejilashan Mountain near Namcha Barwa in SE Xizang (Jia et al. 2012) (Fig. 1c). It seems likely that this dark pattern is associated with wooded or bushy habitats–the

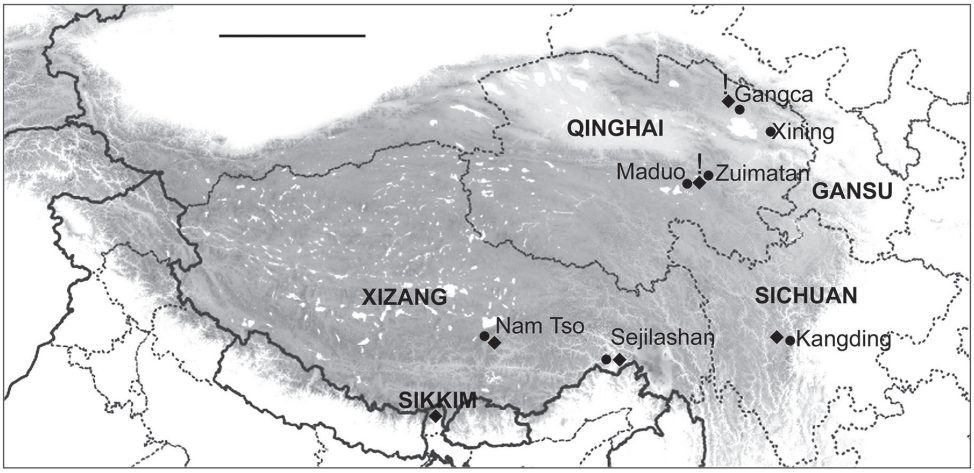

Figure 4. Map of the Tibetan Plateau showing *B. emmerichi* localities (♦). Localities from which specimens giving chromosome preparations were obtained indicated by exclamation mark (!). Scale line = 500 km.

type localities are clearly in a wooded zone. Material from Sikkim (BMNH) is also dark, and rather small (Fig. 1d). This material, 10 specimens with the data "Sikkim. Tangu. 11500 ft. 26.iv.1924. Maj. R. W. G. Hingston" represents the first known occurrence of a *Boreonectes* species in India. The locality Tangu is given by Google earth as Lachen, with an altitude of 2749 m. To attain an altitude of 11500 ft (3505 m) it is necessary to travel about 10 km further north along the road to Tibet (Gurudongmar Road). One specimen is further labelled "In a mountain pool" and one female is carrying a spermatophore of the *Nebrioporus* pattern (Shirt and Angus 1992).

Material from more open areas of the Tibetan Plateau, from Gangca in the north to Nam Tso in the south, has the dark markings less extensive, especially on the pronotum (Fig. 1e–h). There is some variation in the size and shape of the median lobe. The lectotype has the median lobe elongate and slightly longer than in some material (Fig. 2a), but material taken in shallow pools in the riverine flatlands about 20 km E of Maduo includes specimens with larger more elongate median lobes (Fig. 1b) as well as shorter relatively broader ones (Fig. 1c, d) while material from Gangca Dasi shows some variation in median lobe width (Fig. 1e, f). This material appears to be chromosomally uniform, as would be expected if all the specimens belong to the same species, *B. emmerichi*. The median lobe of *B. macedonicus*, which has a similar karyotype to *B. emmerichi*, is shown in Fig. 2g. It is noticeably smaller than that of *B. emmerichi.* Fig. 3a shows the left paramere of the *B. emmerichi* lectotype, while a Gangca Dasi specimen is shown in Fig. 3b.

Further support for the view that all the Tibetan material discussed here belongs to the same species is given by unpublished preliminary DNA data supplied by Ignacio Ribera. The mitochondrial gene CO1 is very similar in material from Gangca, Maduo and Nam Tso (S Tibet 21.VII.10, S Namtso lake 4750m, banks, 30°37'03"N, 90°43'30"E, leg. Joachim Schmidt) with slight differences (less than 1.6% overall)

which correspond with geographical distance between the populations (Fig. 4), and show a considerably larger separation from any other *Boreonectes* species for which there are molecular data.

Mitotic chromosomes, arranged as karyotypes, are shown in Fig. 5m–r, and meiotic chromosomes in Fig. 6. There are 26 pairs of autosomes and the sex chromosomes are X0 (♂), XX (♀). This karyotype closely resembles that of *B. macedonicus* (Fig. 5s), but autosome 12 appears slightly more evenly metacentric and autosome 26 appears more or less the same size as pairs 24 and 25, as against clearly smaller than these pairs in *B. macedonicus*. The X chromosome is shown as an unpaired submetacentric in the C-banded first metaphase of meiosis shown in Fig. 6a. In the mitotic karyotypes the X chromosome appears about the same size as autosome 1 (longer than autosome 1 in *B. macedonicus*) but in second metaphase of meiosis (Fig. 6c) it appears longer. These are difficult karyotypes to work with. The chromosomes appear very condensed in the few successful preparations obtained, and the C-banding is not very good. Nevertheless, these karyotypes are sufficient to show that this Tibetan material has its own characteristic karyotype, and the differences in the relative lengths of autosomes 24, 25 and 26 when compared with those of *B. macedonicus* are sufficient to demonstrate that there has been translocation of material between different autosomes, indicating that these are indeed different species.

B. griseostriatus griseostriatus (De Geer, 1774)

Mitotic chromosomes of a Mt Cenis specimen, arranged as a karyotype are shown in Fig. 5c (plain, Giemsa stained) and d (C-banded), while Fig. 5a, b shows Swedish material for comparison. Although two chromosomes are missing from the Mt Cenis karyotype (positions marked with asterisks (*) in the figure), the forms of the remaining chromosomes make it clear that this is indeed *B. griseostriatus*. The localities in the western Alps from which *Boreonectes* populations yielding karyotypes have been obtained are shown in Fig. 7. The presence of *B. griseostriatus* in the Mt Cenis area suggests that the range of *B. alpestris* (Dutton & Angus, 2007) (nearest locality: Italy, Colle del Nivolet in the Gran Paradiso (Angus 2010b)) may not extend into France.

B. griseostriatus strandi (Brinck, 1943)

Mitotic chromosomes of a Teriberka specimen, arranged as karyotypes, are shown in Fig. 5e, g (plain, Giemsa stained) and Fig. 5f, h (C-banded). The karyotype shown in Fig. 5g, h lacks one replicate of autosome 29 but is included as the form of the chromosomes is particularly clear. As expected of *B. g. strandi*, the chromosomes show no differences from those of typical *B. griseostriatus* (Fig. 5a–d). The distinguishing feature of *B. g. strandi* is its size. Angus (2009) gave the size range

Figure 5. Mitotic chromosomes of *Boreonectes* spp., arranged as karyotypes. **a–d** *B. griseostriatus* **a, b** Sweden (from Dutton, Angus (2007)) **c, d** Mt Cenis **e–h** *B. g. strandi*, Teriberka **i–l** *B. multilineatus* **i, j** Scotland (from Dutton, Angus (2007)) **k, l** Lac d'Anapéou **m–r** *B. emmerichi* **m, n** from mid-gut, Maduo **o–r** from testis, Gangca Dasi **s** *B. macedonicus*, Crno Ezero, Macedonia (from Angus (2008)) **t, u** *B. ibericus* **t** Peña Lara (from Dutton, Angus (2007)) **u** Lagos de Lloroza. **b, d, f, h, j, l, n, p, r** C-banded, the rest plain, Giemsa-stained. Missing chromosomes indicated by asterisks (*). Scale line = 5 μm.

of Norwegian *strandi* as 4.6–5.2 mm (♂), 4.6–5.3 mm (♀) and Brinck gave the size range of *strandi* (both sexes) as 4.8–5.5 mm as against 4.0–4.8 mm for normal *griseostriatus*. The size range of the Teriberka material is 5.0–5.1 mm (♂), 4.9–5.2 mm (♀), clearly *strandi*.

The localities from which populations of *B. g. strandi* yielding karyotypes have been obtained are shown in Fig. 8. The most easterly published record of *B. g. strandi* is from near Murmansk (Brinck 1943, Lindberg 1930), about 70 km west of Teriberka.

It should be noted that Nilsson and Hájek (2015) list *strandi* as a straight synonym of *griseostriatus*. Here we follow Brinck (1943) in regarding it as a subspecies in view of its larger size and restricted geographical distribution.

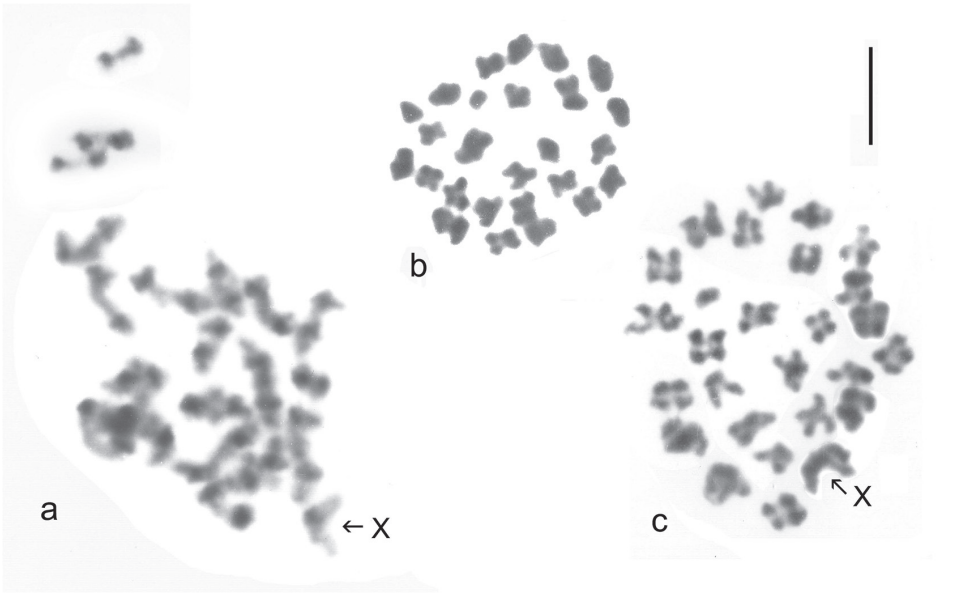

Figure 6. Meiotic chromosomes of *B. emmerichi* from Gangca Dasi. **a** metaphase I, C-banded **b**, **c** metaphase II plain, Giemsa-stained **b** male-determining nucleus with 26 chromosomes **c** female-determining nucleus with 27 chromosomes including X. Scale line = 5 μm.

B. multilineatus (Falkenström, 1922)

Mitotic chromosomes of a specimen from the Lac d'Anapéou, arranged as karyotypes, are shown in Fig. 5k (plain, Giemsa stained) and Fig. 5l (C-banded). The arrangement of the chromosomes is the same as in Scottish *B. multilineatus* (Fig. 5i, j). The Pyrenean localities for *B. multilineatus* are shown in Fig. 9. Lac d'Anapéou is only about 25 km west of the previous locality from which it was recorded in the Pyrenees, Lac d'Oncet (Angus 2010b) but is on a different spur of the Pyrenees. Both of these localities are on the French side of the west-central Pyrenees. At the moment *B. ibericus* has not been found in the Pyrenees, but it may be expected to occur there as its range extends to the Alpes Maritimes (Dutton and Angus 2007).

B. ibericus (Dutton & Angus, 2007)

An unbanded, Giemsa stained karyotype from a Lagos de Lloroza specimen is shown in Fig. 5u, while Fig. 5t shows one from a Peña Lara specimen. The Lloroza specimen is homozygous for the long (fused) form of autosome 1 and thus lacks any free autosome 24, while the Peña Lara specimen is heterozygous for the fusion and has one replicate of autosome 24. The Lagos de Lloroza (Fig. 9) are about 70 km further east than the Puerto de los Señales locality from where Dutton and Angus (2007) recorded *B. ibericus*, thus extending its known northern Spanish range a little closer to the Pyrenees.

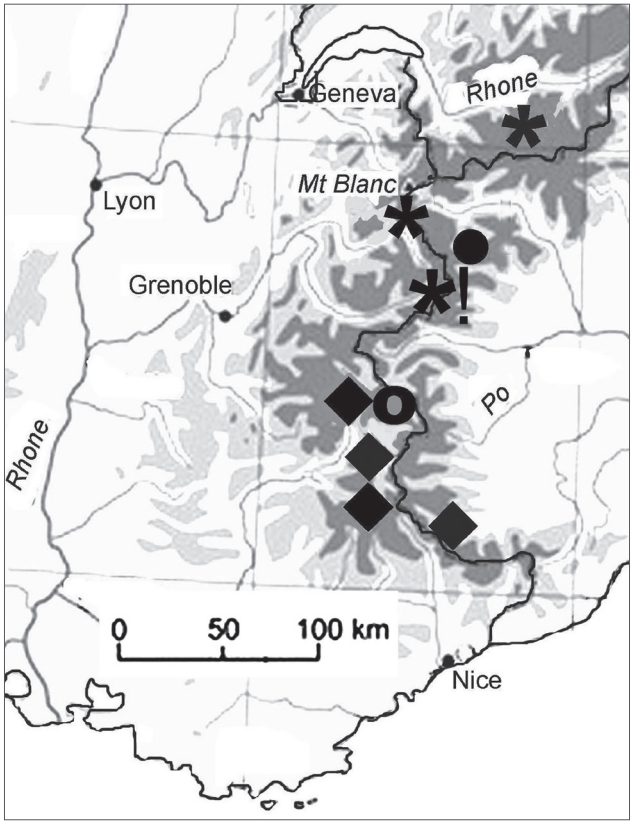

Figure 7. Map of the western Alps showing the sources of populations of *B. griseostriatus* and relatives from which chromosome data were obtained. Symbols: * *griseostriatus* ● *alpestris* ♦ *ibericus* o *inexpectatus*. New data indicated by exclamation mark (!).

Figure 8. Map showing the localities of *B. g. strandi* populations from which chromosome data were obtained (*****). New record indicated by exclamation mark (!).

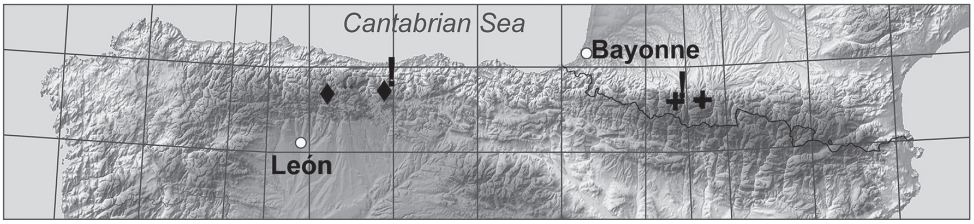

Figure 9. Map showing the localities of *B. multilineatus* populations in the Pyrenees (**+**), and of *B. ibericus* populations in the Picos de Europa area (♦), from which chromosome data were obtained. New data indicated by exclamation mark (!).

Acknowledgements

This work has been supported by research facilities in the Natural History Museum, London, the School of Biological Sciences of Royal Holloway University of London, the Biology and Geography School of Qinghai Normal University in Xining, the Institute of Entomology of the Life Science School of Sun Yat-sen University, Guangzhou and the Biomedical Imaging Unit of Southampton General Hospital, all of whom we thank for their support. We also thank Ignacio Ribera for permission to use his unpublished DNA data on *B. emmerichi* and related species, Franck Bameul and Pyotr Petrov for collecting Pyrenean *B. multilineatus* and Kola Peninsula *B. griseostriatus strandi*, and Tomasz Goral for the SEM pictures taken in the Natural History Museum, London. We thank Martin Fikácek for the map of the Tibetan Plateau used as the template for Fig. 4, Lucia Labrada and Carlos Luque for the map used as a template for Fig. 9, and Hans Fery for his detailed and very helpful referee's comments on the taxonomic part of this paper.

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