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Male meiosis, heterochromatin characterization and chromosomal location of rDNA in *Microtomus lunifer* (Berg, 1900) (Hemiptera: Reduviidae: Hammacerinae)

María Georgina Poggio, María José Bressa, Alba Graciela Papeschi

Laboratorio de Citogenética y Evolución, Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. Int. Güiraldes 2160, C1428EGA, Ciudad Universitaria, Ciudad Autónoma de Buenos Aires, Argentina.

Corresponding author: *María Georgina Poggio* (mgpoggio@ege.fcen.uba.ar)

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Abstract

In the present work, we analysed the male meiosis, the content and distribution of heterochromatin and the number and location of nucleolus organizing regions in *Microtomus lunifer* (Berg, 1900) by means of standard technique, C- and fluorescent bandings, and fluorescent *in situ* hybridization with an 18S rDNA probe. This species is the second one cytogenetically analysed within the Hammacerinae. Its male diploid chromosome number is 31 (2n=28+X_1X_2Y), including a minute pair of m-chromosomes. The diploid autosomal number and the presence of m-chromosomes are similar to those reported in *M. conspicillaris* (Drury, 1782) (2n=28+XY). However, *M. lunifer* has a multiple sex chromosome system X_1X_2Y (male) that could have originated by fragmentation of the ancestral X chromosome. Taking into account that *M. conspicillaris* and *M. lunifer* are the only two species within Reduviidae that possess m-chromosomes, the presence of this pair could be a synapomorphy for the species of this genus. C- and fluorescent bandings showed that the amount of heterochromatin in *M. lunifer* was small, and only a small CMA_3 bright band was observed in the largest autosomal pair at one terminal region. FISH with the 18S rDNA probe demonstrated that ribosomal genes were terminally placed on the largest autosomal pair. Our present results led us to propose that the location of rDNA genes could be associated with variants of the sex chromosome systems in relation with a kind of the sex chromosome systems within this family. Furthermore, the terminal location of NOR in the largest autosomal pair allowed us to use it as a chromosome marker and, thus, to infer that the kinetic activity of both ends is not a random process, and there is an inversion of this activity.

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Keywords
Hemiptera, Reduviidae, Hammacerinae, meiosis, m-chromosomes, evolutionary trends, rDNA-FISH

Introduction

Reduviidae is the largest family of predaceous land Hemiptera and includes about 6500 species and subspecies in 930 genera and 22 subfamilies. These insects are abundant, occur worldwide, and are voracious predators (thus their name, "assassin bugs") (Coscarón 1998, Ambrose 1999, Schaefer and Panizzi 2000).

All hemipteran species possess holokinetic chromosomes, i.e. chromosomes without primary constrictions and, hence, without localized centromeres. This order is unique in that the autosomes, m-chromosomes and sex chromosomes have different meiotic behaviours. During mitosis microtubules attach to the entire length of sister chromatids, and at anaphase they segregate parallel to each other and perpendicular to the polar spindle (holokinetic behaviour) (Schrader 1935, Hughes-Schrader and Schrader 1961, White 1973). However, several reports provide evidence that kinetic activity during meiosis is restricted to the chromosome ends where no kinetochore structures are observed, and the chromosomes can be regarded as telokinetic (Motzko and Ruthmann 1984). Both chromosome ends can show kinetic activity in such a way that the chromosome end which was inactive at the first meiotic division become active during the second one (Camacho et al. 1985, Nokkala 1985, Pérez et al. 1997, Cattani et al. 2004, Viera et al. 2009).

As a rule, autosomal bivalents are chiasmatic, whereas sex chromosomes and m-chromosomes are achiasmatic (Ueshima 1979, Manna 1984, Papeschi and Mola 1990, González-García et al. 1996, Suja et al. 2000, Viera et al. 2009). In general, the autosomal bivalents show a single chiasma terminally located (rod bivalents) and orientate at metaphase I with their long axes parallel to the polar axis. During both meiotic anaphases only their ends are able to show kinetic activity leading the chromosome/chromatid segregation to opposite poles (pre-reductional division) (Ueshima 1979, Camacho et al. 1985, Pérez et al. 1997, Viera et al. 2009). Conversely, bivalents with two terminal chiasmata (ring bivalents) orientate with their long axes parallel to equatorial plate and two different behaviours have been described: i) one chiasma releases first, and then one pair of terminal regions becomes free to attach to the spindle and an axial orientation is finally achieved, or ii) alternative sites of kinetic activity become functional (Mola and Papeschi 1993, Papeschi et al. 2003, Viera et al. 2009). On the other hand, the sex chromosomes are achiasmatic and behave as univalents during meiosis I. Most sex chromosomes segregate their chromatids equationally at anaphase I and reductionally at anaphase II (post-reductional division) (Ueshima 1979, Manna 1984, Papeschi and Mola 1990, González-García et al. 1996, Suja et al. 2000, Viera et al. 2009). Finally, the m-chromosomes are generally of the small size, and are usually unpaired and thus achiasmatic during early prophase I. However, previous reports in

Coreidae describe the occurrence of regular synapsis of the m-chromosomes (Toscani et al. 2008). At late diakinesis they come close each other, and at metaphase I they are always associated end-to-end, i.e. touch-and-go pairing, forming a pseudobivalent which orientate axially. The first meiotic division is reductional and the second one is equational for the m-chromosomes (Wilson 1909a, Papeschi and Bressa 2006).

Apart from the general characteristics of hemipteran species previously described, the Reduviidae are characterized by a modal diploid number of autosomes of 20 with a range between 10 and 34, and both simple and multiple sex chromosome systems (XY/XX, X0/XX, and X_nY/X_nX_n; male/female) (Ueshima 1979, Manna 1984, Poggio et al. 2007a). Cytogenetic data are currently available for about 152 species belonging to 11 subfamilies; 79 of them belong to Triatominae, 33 to Harpactorinae, 12 to Stenopodainae, and 10 to Peiratinae. The remaining species are evenly distributed among seven other subfamilies: Reduviinae (7 species), Ectrychodiinae (3), Emesinae (3), Phymatinae (2), Bactrodinae (1), Hammacerinae (1), and Saicinae (1) (Poggio et al. 2007a, Kaur et al. 2009, Panzera et al. 2010). Within Hammacerinae, only *Microtomus conspicillaris* (Drury 1782) has been cytogenetically analysed. Its diploid chromosome number is 2n=30 with a sex chromosome system XY/XX and a pair of minute chromosomes denoted as m-chromosomes (Piza 1957).

Furthemore, cytogenetic data for species belonging to Reduviidae point to the presence of C-heterochromatin at terminal regions on a few or all autosomal pairs, and/or on one of the sex chromosomes, whereas the other one is completely heterochromatic (Poggio et al. 2006, Panzera et al. 2010). However, in Triatominae inter- and intraspecific differences in the position, quantity and meiotic behaviour of constitutive heterochromatin have revealed considerable cytogenetic variability (Panzera et al. 2010).

So far, the location of nucleolus organizing regions (NORs) has been analysed in only 14 reduviid species by Ag-NOR, fluorescent banding and/or fluorescent *in situ* hybridization (FISH) with ribosomal DNA (rDNA) probes (18S, 26S or 45S). These results show that in Reduviidae the NOR can be located either at terminal position on one autosomal pair, or on the sex chromosomes. The presence of NORs in both X and Y chromosomes was reported in two species belonging to two different subfamilies (Harpactorinae and Triatominae) (Morielle-Souza and Azeredo-Oliveira 2007, Poggio et al. 2008), and NORs on one autosomal pair plus on one sex chromosome was found in four species, three of them belonging to Triatominae (Morielle-Souza and Azeredo-Oliveira 2007, Bardella et al. 2008, Panzera et al. 2008) and one to Harpactorinae (Poggio et al. 2007b). Of all species analysed, in only two species belonging to Triatominae the NOR regions co-localized with CMA_3 bright bands (Severi-Aguiar et al. 2006, Morielle-Souza and Azeredo-Oliveira 2007).

In the present work, we analysed in detail the male meiosis of *Microtomus lunifer* (Berg, 1900) (Hammacerinae) to verify the presence of a pair of m-chromosomes, the content and distribution of heterochromatin by C- and fluorescent bandings, and examined the number and location of NORs by FISH. Lastly, the position of a NOR at the terminal region of the largest autosomal pair allowed us to use it as a chromosome marker and to describe its behaviour during both meiotic divisions.

Material and methods

Insects

We used three males of *Microtomus lunifer* from Pampa del Indio, Chaco province (coll. 2008).

Chromosome preparations

All the analysed specimens were brought alive to the laboratory. The male gonads were dissected in physiological solution. Afterwards, one of the testes was fixed for 15–30 min in freshly prepared Carnoy fixative (ethanol: chloroform: acetic acid, 6:3:1), and was kept at 4ºC in 70% ethanol for meiotic studies. Slides were prepared by the squash technique in a drop of 2% iron acetic haematoxylin following conventional procedures. For C- and fluorescent bandings, and FISH techniques, spread chromosome preparations were made from the other testis as described in Traut (1976). Then the preparations were dehydrated in an ethanol series (70%, 80%, and 96%, 30 sec. each) and stored at –20ºC until use.

C- and Fluorescent bandings

C- and fluorescent bandings were then applied to spread chromosome preparations to reveal heterochromatin and its base composition. C-banding was performed according to Papeschi (1988). The slides pre-treated for C-banding were stained with 4',6-diamidino-2-phenylindole (DAPI; Fluka BioChemika, Sigma Aldrich Production GmbH, Buchs, Switzerland) for a better resolution of C-bands.

Fluorescent banding with AT-specific DAPI and GC-specific chromomycin A_3 (CMA_3; Fluka BioChemika) was carried out as follows: after removal from freezer, the slides were placed immediately into cold 70% ethanol for 2 min. Then, they were transferred through 80% and 100% ethanol, 30 sec each, and air-dried. The slides were submerged in a coplin jar with methanol for two hours. Once dried, they were rinsed with Mc Ilvaine´s buffer pH 7 (0.1 M citric acid, 0.2 M Na_2HPO_4, in distilled water). Each chromosome preparation was dyed with 75 µl of DAPI solution (0.01 mg/ml, in Mc Ilvaine's buffer), covered with 24x50 mm transparency cover slides, and kept at room temperature (RT) for 20 min in darkness in a moist chamber. Afterwards, the preparations were rinsed three times using distilled water, Mc Ilvaine's buffer and distilled water. Then, the slides were dyed with 50 µl of CMA_3 solution (0.6 mg/ml, in Mc Ilvaine´s buffer), covered with 24x50 mm transparency cover slide, and incubated at RT for 1 hour in dark in a moist chamber. After this period, the preparations were rinsed again with distilled water, Mc Ilvaine's buffer and distilled water, and then let them air-dried. The slides were mounted in Antifade based on DABCO (Sigma Al-

drich; for composition see Traut (1999)), and covered with 24x40 mm cover glass. The cover glass was sealed with rubber cement, and the slides were stored at 37°C in dark in a moist chamber three days.

Fluorescent *in situ* hybridization with 18S rDNA probes

Unlabelled 18S rDNA probes were generated by polymerase chain reaction (PCR) using universal arthropod primers: forward 5'-CCTGAGAAACGGCTACCACATC-3' and reverse 5'-GAGTCTCGTTCGTTATCGGA-3' (Whiting 2002). Total genomic DNA of *Dysdercus albofasciatus* Berg, 1978 obtained by standard phenol-chloroform-isoamylalcohol extraction, was used as a template. PCR was done following the procedure described in Fuková et al. (2005). The PCR product showed a single band of about 1000 bp on a 1% agarose gel. The band was cut out from the gel, and the DNA was extracted using a QIAquick Gel Extraction Kit (Quiagen GmbH, Hilden, Germany). The 18S rDNA fragment was re-amplified by PCR and then labelled with biotin-14-dATP by nick translation using a BioNick Labeling System (Invitrogen, Life Technologies Inc., San Diego, CA, USA). FISH with biotinylated 18S rDNA probe was performed essentially following the procedure in Sahara et al. (1999) with several modifications described in Fuková et al. (2005) and in Bressa et al. (2009).

Analysis of sites of kinetic activity

The location of NOR regions in the largest autosomal pair of *M. lunifer* allowed us to analyse the behaviour of the terminal regions which were kinetically active. The number of cells at metaphase I and metaphase II, in which the kinetically active terminal regions of this autosomal pair were associated to the NOR (Figs 5d, g) or not (Figs 5e, f), were counted. The hypotheses described below were tested using a Chi-squared goodness of fit test.

H_{01}: the kinetic activity of both ends (with/without NOR) at both meiotic divisions is a random process.

H_{02}: the chromosome end that is active during the first meiotic division becomes inactive during the second one and vice versa.

Microscopy and image processing

Preparations were observed in a Leica DMLB microscope equipped with a Leica DFC350 FX CCD camera and Leica IM50 software, version 4.0 (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). Black-and-white images of chromosomes were recorded separately for each fluorescent dye. Images were pseudocoloured (light blue for DAPI, green for CMA_3, red for Cy3) and processed with an appropriate software.

Results

Male chromosome complement and meiosis

Microtomus lunifer possesses a male diploid chromosome number of 31, and its complement comprises 14 autosomal bivalents and a multiple sex chromosome system X_1X_2Y (Fig. 1). In spermatogonial prometaphase, the sex chromosomes and an autosomal pair are easily recognized because of their small size, whereas the rest of the autosomes cannot be distinguished due to their similar size. An association between a nucleolus and an autosomal pair is observed (Fig. 2a).

At early pachytene, it is not possible to individualize each autosomal bivalent. However, the three sex chromosomes are positively heteropycnotic and lie close to each other forming a pseudo-trivalent. At late pachytene the bivalents continue their condensation, and the sex chromosomes become isopycnotic (Fig. 2b). From diplotene onwards, 13 autosomal bivalents, two univalents and three sex chromosomes are clearly distinguished in some cells (Fig. 2c, f), whereas in other ones 14 autosomal bivalents and three sex chromosomes are also observed (Fig. 2d, e). It can be noticeably seen that the sex chromosomes differ slightly in size (Fig. 2d–f). At metaphase I, the sex univalents lie at the periphery of the ring formed by the autosomal bivalents, and their different size is evident (Fig. 2g, h). At this stage, the smallest chromosome pair does not show any defined position and can be found either being part of the ring (Fig. 2g) or at its centre (Fig. 2h). This smallest pair can be observed migrating precociously in some cells (33 out of 100 cells) (Fig. 2h). At anaphase I, autosomal bivalents divide reductionally, while the sex chromosomes segregate equationally. Therefore, at telophase I two nuclei with 17 chromosomes each ($14A+X_1X_2Y$) are observed. Second meiotic division follows immediately after telophase I without an interkinesis stage. At metaphase II, the autosomes dispose at the equatorial plane forming a ring, and in the centre of it the sex chromosomes form a pseudo-trivalent (Fig. 2i). The Y chromosome is orientated towards the spindle pole opposite to that of X_1 and X_2. At anaphase II, 15 chromosomes migrate to one of the poles ($14A+Y$) and 16 to the opposite one ($14A+X_1X_2$) (Fig. 2j).

There is usually only one chiasma on each autosomal bivalent, which can be terminally or, less frequently, subterminally located, although they can show two chiasmata (Fig. 2d, g). Cells with two ring bivalents are seldom observed, while those with

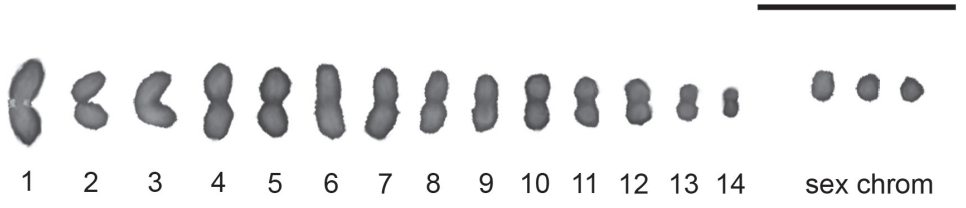

Figure 1. Male meiotic karyotype of *Microtomus lunifer*. Chromosomes are counterstained with DAPI; the largest autosomal pair is recognized by the presence of the rDNA hybridization signals. Bar = 10 μm

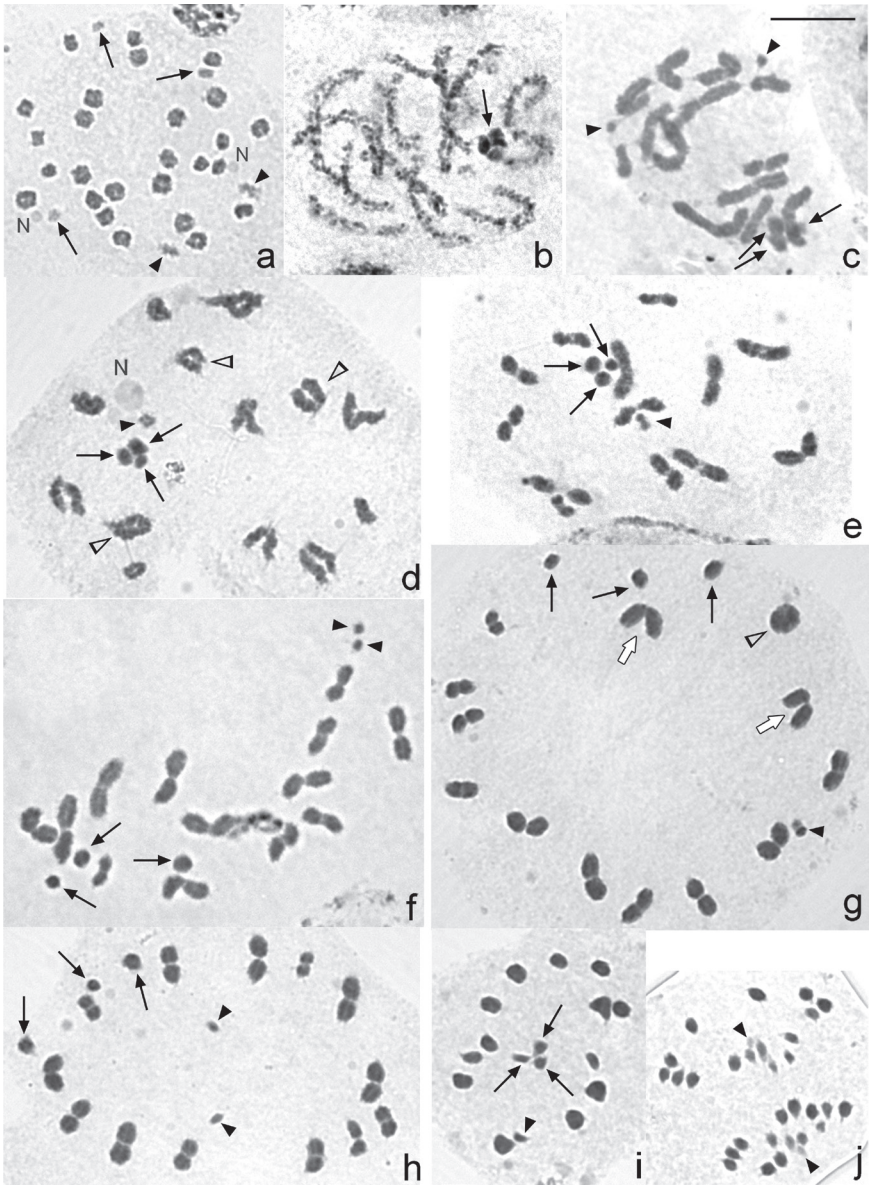

Figure 2a–j. Male meiosis in *Microtomus lunifer*. **a** Spermatogonial prometaphase **b** Late pachytene **c** Diplotene. The smallest chromosome pair is observed as two univalents (black arrowheads) **d** Diplotene. The smallest chromosome pair is as a pseudo-bivalent (black arrowhead) **e** Diakinesis. The smallest chromosome pair is as a pseudo-bivalent (black arrowhead) **f** Diakinesis. The smallest chromosome pair is observed as two univalents (black arrowheads) **g** Metaphase I. The smallest chromosome pair (black arrowhead) formed a pseudo-bivalent and is placed in the bivalent autosomal ring **h** Metaphase I. The smallest chromosome pair lies in the centre of the ring and migrates precociously (black arrowheads) **i** Metaphase II **j** Anaphase II. Black arrows: sex chromosomes. Black arrowheads: smallest chromosome pair. White arrowheads: autosomal bivalents with two chiasmata. White arrows: V-shaped bivalents. N: nucleolus. Chromosomes are stained with 2% iron acetic haematoxylin. Bar = 10 μm

three ring bivalents are even rarer (Fig. 2d). In this species three kinds of bivalents are observed: rod (Fig. 2c–h), ring (Fig. 2d, g) and V-shaped (Fig. 2g) bivalents from diplotene to metaphase I. Mean chiasma frequency in cells at diakinesis-metaphase I is 14.76, being 15 (38.7%, 93 analysed cells) and 14 (68.7%, 99 analysed cells) the most frequent number of chiasmata at diakinesis and at metaphase I, respectively.

C- and Fluorescent bandings

The amount of heterochromatin in *M. lunifer* is small: very small C-positive dots (from 10 to 20) are detected in cells at leptotene-zygotene. At this stage, the sex chromosomes are observed as completely C-positive (Fig. 3a). However, this C-banding pattern can no longer be detected from diplotene onwards (Fig. 3b). All meiotic chromosomes show uniform staining with DAPI (Fig. 3c, e) and CMA_3 fluorochromes (Fig. 3d, f), except for the largest autosomal bivalent. A small CMA_3 bright band is observed at one of the terminal regions of the largest autosomal pair (Fig. 3d, f). Besides, the smallest pair of chromosomes is both DAPI and CMA_3 dull.

Location of rDNA

In *M. lunifer*, FISH experiments with 18S rDNA probes reveal a single cluster placed at one terminal region of the largest autosomal pair (Fig. 4a–g). In spermatogonial metaphases, it is clearly observed that the hybridization signals are at terminal regions of both sister chromatids of both homologous chromosomes (Fig. 4a). From diplotene onwards, the hybridization signals are detected at one terminal region of the largest autosomal bivalent (Fig. 4b, c). However, both at metaphase I and metaphase II the NOR-autosomal pair shows two different orientations depending on the location of the hybridization signals: the ends with the NOR oriented to the poles (Fig. 4d, g) or the ends without NOR oriented to the poles (Fig. 4e, f).

Meiotic behaviour and kinetic activity of the NOR-bivalent

In *M. lunifer*, FISH experiments provide a reliable chromosome marker in the NOR-autosome pair to analyse its meiotic behaviour during both meiotic divisions (Figs 4, 5). The presence of a single cluster of rDNA at only one of the ends of each homologous chromosome of the NOR-bivalent allows us to distinguish whether both ends (carrying the NOR or not) take part in the kinetic behaviour of this autosomal pair. At metaphase I, this NOR-bivalent is axially oriented and shows two types of configuration: either the chromosome ends bearing the hybridization signals (Figs 4d, 5a) or the ends that do not bear them are directed towards the poles (Figs 4e, 5b). At metaphase II, the sister chromatids reach an axial orientation and present the same two arrange-

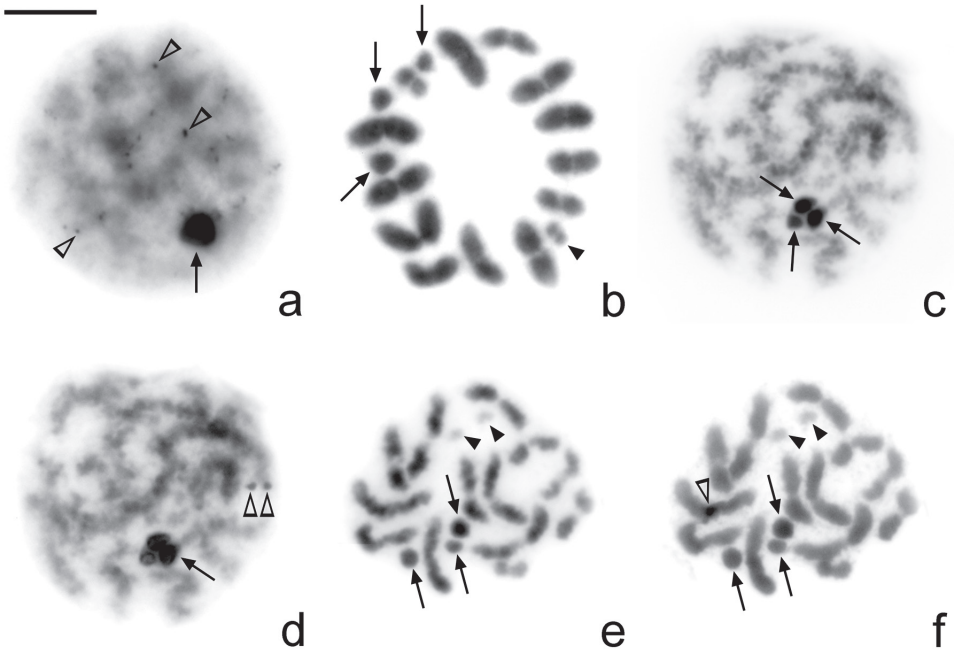

Figure 3a–f. *Microtomus lunifer*. **a–b** C-banding and **c–f** Fluorescent banding: **c, e** DAPI and **d, f** CMA_3. **a** Leptotene-zygotene. Very small C-positive dots can be observed in the autosomal chromatin; sex chromosomes are C-positive **b** Metaphase I. No C-positive bands can be detected **c–d** Pachytene **e–f** Diakinesis. No DAPI (**c, e**) and neither CMA_3-positive bands (**d, f**) can be detected, except for a small CMA_3 bright band in one of the terminal regions of the largest autosomal pair. Arrows: sex chromosomes. Black arrowheads: smallest autosomal pair. White arrowheads: positive dots/bands. Bar = 10 μm

ments: either the chromatid ends carrying the hybridization signals (Figs 4g, 5c) or the other ends that do not carry those (Figs 4f, 5d) are oriented towards the poles.

To test whether the kinetic activity of both ends is a random process at metaphase I and metaphase II, the configurations of the NOR-autosomal bivalent in three individuals were scored. The results demonstrate that at metaphase I the kinetic activity of this NOR-bivalent is restricted to the chromosome ends that do not carry the hybridization signals in 67% of the analysed cells (216 out of 322), whereas in the remaining 33% the kinetic activity occurs at the ends that carry the hybridization signals (Table 1). At metaphase II, however, the kinetic activity is located at the chromatid ends bearing the NOR in 76% of the cells (117 out of 154), and in the remaining cells, at the chromatid ends without it (24% of the cells). Comparing the frequencies of configurations of this NOR-autosome pair between cells at metaphase I and metaphase II, we can observe similar frequencies of cells in which the kinetic activity at metaphase I is restricted to the chromosome ends not carrying the NOR and cells at metaphase II where the kinetic activity is located at the chromatid ends bearing the NOR, and vice versa. Statistical analysis corroborates that: i) the kinetic activity of both ends is not a

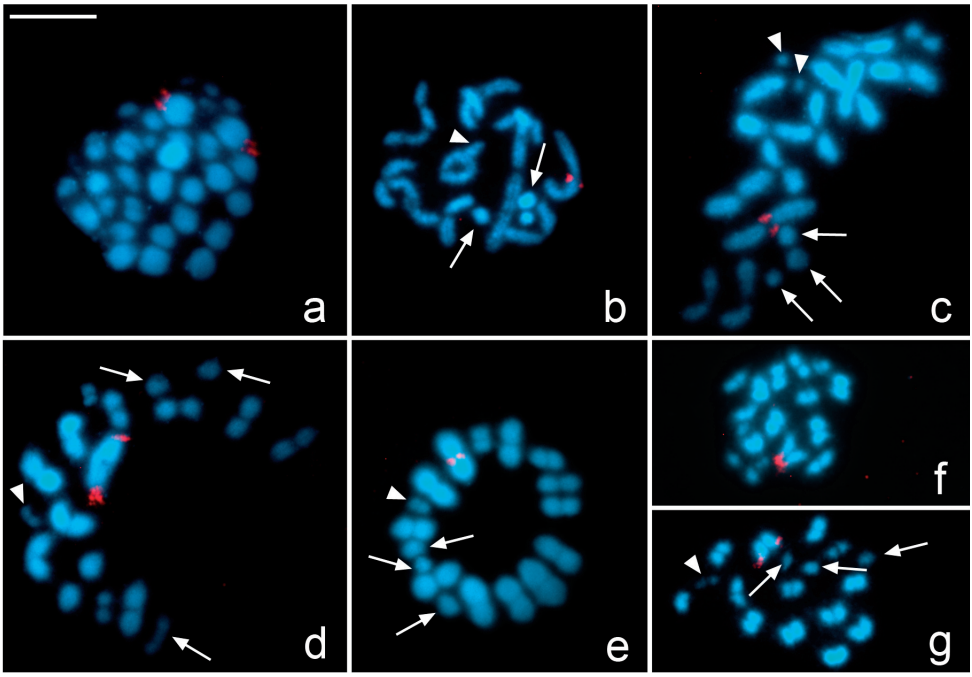

Figure 4a–g. *Microtomus lunifer*. Fluorescent *in situ* hybridization with an 18S rDNA probe. **a** Spermatogonial prometaphase **b** Diplotene **c** Diakinesis **d–e** Metaphase I **f–g** Metaphase II. Hybridization signals in red. Chromosomes are counterstained with DAPI (blue). Arrows: sex chromosomes. White arrowheads: smallest autosomal pair. Bar = 10 μm

random process at metaphase I and metaphase II ($X^2_{(specimen\ 1,\ meta\ I)}$=19.5; $X^2_{(specimen\ 1,\ meta\ II)}$=23.5; $X^2_{(specimen\ 2,\ meta\ I)}$=7.71; $X^2_{(specimen\ 2,\ meta\ II)}$=7.11; $X^2_{(specimen\ 3,\ meta\ I)}$=13.16; $X^2_{(specimen\ 3,\ meta\ II)}$=13.53 > $CV_{(L=1;\ \alpha=0.05)}$=3.84), and ii) the ends that are active during the first meiotic division become inactive during the second one ($X^2_{(specimen\ 1)}$=3.49; $X^2_{(specimen\ 2)}$=8.82x10^{-4}; $X^2_{(specimen\ 3)}$=2.38 < $CV_{(L=1;\alpha=0.05)}$=3.84).

Discussion

Diploid chromosome number, sex chromosome system and chiasma frequency

So far, the cytogenetic analysis of 153 species from Reduviidae reveals a chromosome diploid number that varies from 10 to 34, with both simple and multiple sex chromosome systems (XY/XX, X0/XX, and X_nY/X_nX_n) (Ueshima 1979, Manna 1984, Poggio et al. 2007a, Kaur et al. 2009, Panzera et al. 2010). Within Hammacerinae, *Microtomus lunifer* constitutes the second species cytogenetically analysed, and its diploid autosomal number and the presence of a minute pair of autosomes agree with the previous report in *M. conspicillaris* (2n=30=28+XY/XX) (Piza 1957).

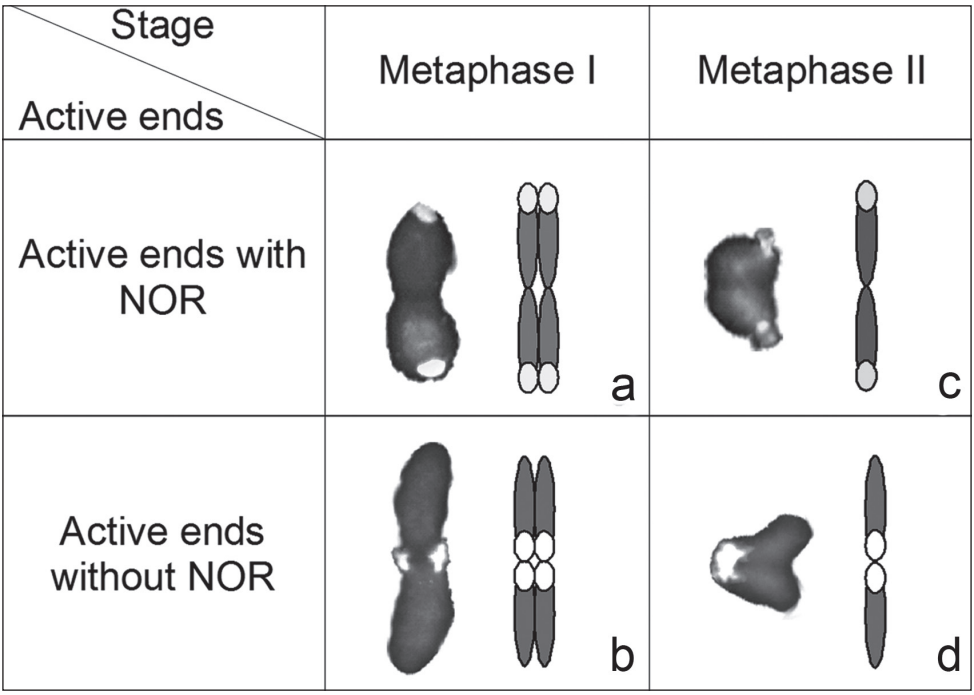

Figure 5a–d. Photos (left) and diagrams (right) illustrating two alternative orientations of the autosomal pair with the NOR: **a–b** at metaphase I and **c–d** metaphase II in *Microtomus lunifer.* Chromosomes: grey; rDNA clusters: white.

It deserves attention that, even though *M. lunifer* possesses the same diploid autosomal number as *M. conspicillaris*, both species differ in their sex chromosome system; the former has a multiple sex chromosome system X_1X_2Y (male) whereas the latter presents a simple sex chromosome system XY (male). The most common sex chromosome system in Hemiptera is the simple system XY/XX (male/female). Nevertheless, the other simple system X0/XX, multiple systems (X_n0, X_nY, XY_n, X_nY_n) and neo-systems are also reported (Ueshima 1979, Manna 1984, Jacobs 2003, 2004, Papeschi and Bressa 2006, Bressa et al. 2009). In many examples it was described differences in sex chromosomes systems within a genus, and even among species (Pfaler-Collander 1941, Manna 1984, Papeschi 1994, 1996, Bressa et al. 2003). Notwithstanding the multiple sex chromosome systems are not as common as the simple systems in Hemiptera, the former are especially frequent in Nepidae, Cimicidae and Reduviidae (Ueshima 1979, Poggio et al. 2007a).

It is generally accepted that multiple systems in Hemiptera are the result of fragmentation(s) of the X and/or Y chromosome(s) of an ancestral simple sex chromosome system (Heizer 1950, Hughes-Schrader and Schrader 1961, Ueshima 1979, Manna 1984, Papeschi 1996, Papeschi and Bressa 2006). The holokinetic nature of the hemipteran chromosomes and the achiasmatic behaviour of sex chromosomes during

Table 1. Frequencies of cells at metaphase I and metaphase II showing the kinetic activity restricted to the NOR or not NOR ends of the largest autosomal pair.

Specimen	Frequency	Metaphase I		Metaphase II	
		configuration a	configuration b	configuration c	configuration d
1	F_1*	24	66	40	7
	f_1*	0.27	0.73	0.85	0.15
2	F_2*	12	30	26	10
	f_2*	0.29	0.71	0.72	0.28
3	F_3*	70	120	51	20
	f_3*	0.37	0.63	0.72	0.28

*, F_n = absolute frequency of specimen n; f_n = absolute frequency of specimen n

male meiosis are the main facts that support this hypothesis and may account for the variability (Ueshima 1979, Manna 1984, Thomas 1987). In most cases of multiple systems the increase in the number of sex chromosomes is not accompanied by a reduction in the number of autosomes. In most cases, the multiple systems were originated by fragmentation, except in three species of *Acantocephala* (=*Metapodius*) (Laporte, 1833) (Coreidae) (Wilson 1909b) and in *Cimex lectularius* (Linnaeus, 1758) (Cimicidae) (Darlington 1939, Slack 1939, Ueshima 1979, Grozeva et al. 2010) in which the multiple sex chromosome systems could have originated by a non-disjunction. In the present work, the size comparison of the three sex chromosomes of *M. lunifer* with the X and Y chromosomes of *M. conspicillaris*, where the Y is slightly smaller than the X, reveals that the relative size of the X_1 plus the X_2 does not differ significantly from the size of the single X. Hence, it is suggested that the original X was fragmented into two unequal chromosomes, one larger (X_1) than the other (X_2). In *M. lunifer*, the male meiotic behaviour of the sex chromosomes was regular, and the new X_1 and X_2 should have been repaired the broken ends to ensure their stability due to the fragmentation. This hypothesis is also based on that the two X chromosomes of *M. lunifer* are slightly different in size, making it unlikely that this system has arisen by aneuploidy (non-disjunction). Thus, *M. conspicillaris* has the ancestral simple sex chromosome system (XY), and the multiple system X_1X_2Y of *M. lunifer* might have originated by fragmentation of the ancestral X chromosome.

In Hemiptera, autosomal bivalents are chiasmatic (except in a few families, such as Nabidae, Miridae, Cimicidae; see Nokkala and Grozeva (2000), Poggio et al. (2009)) and present as a rule only one chiasma per bivalent (Ueshima 1979, Manna 1984). Nevertheless, the presence of two terminal chiasmata in large autosomal bivalents has been increasingly reported lately (Camacho et al. 1985, Mola and Papeschi 1993, Bressa et al. 2001, Jacobs and Liebenberg 2001, Rebagliati et al. 2001, Bressa et al. 2002, Jacobs and Groeneveld 2002, Papeschi et al. 2003, Rebagliati et al. 2003, Rebagliati and Mola 2010). On the basis of the meiotic chromosomes behave as telokinetic the ring-shaped bivalents should have some mechanism/s to ensure

their attachment to the spindle fibres. Previous reports proposed that both terminal regions and secondary constrictions, or sites beside them, are able of attaching to spindle fibres and of developing kinetic activity (Camacho et al. 1985, Mola and Papeschi 1993, Papeschi et al. 2003). The analysis of chiasma frequency in *M. lunifer* shows that there are from one to three autosomal bivalents with two chiasmata at diakinesis-metaphase I, which increases the expected frequency of 14 to 14.76 (with a range from 14 to 17). Besides, the mean chiasma frequency is higher in diakinesis than in metaphase I. This difference between the two stages may be due to a decrease in the number of autosomal bivalents with two chiasmata that it is consistent with the presence of V-shaped bivalents in metaphase I. Thus, one of the two chiasmata releases firstly, one pair of terminal regions becomes free to attach to the spindle, and the bivalent finally adopts a rod shape.

Evolutionary trends and presence of m-chromosomes

The basal position of Hammacerinae was earlier proposed by Clayton (1990) based on the fact that it is the only subfamily that retained some plesiomorphic characters of the closest sister groups to Reduviidae. All current analyses based on both morphological characters and mitochondrial and nuclear ribosomal genes support the position of Hammacerinae as a sister group of all remaining subfamilies of this family (Weirauch 2008, Weirauch and Munro 2009). Taking into account the cytogenetic characteristics of reduviids, Poggio et al. (2007a) proposed that the ancestral autosomal diploid number for Cimicidae and Reduviidae should be 28, and the evolutionary trends within reduviids should have involved a reduction in autosomal number through fusions and an increase in the number of sex chromosomes through fragmentations (multiple systems). Our present results together with the previous study in *M. conspicillaris* lead us to suggest that Hammacerinae presents the ancestral autosomal diploid number proposed for Reduviidae. Hence, the cytogenetic results support the cladistic analysis of reduviids based on morphological and molecular characters (Weirauch 2008, Weirauch and Munro 2009).

A particular feature of both *Microtomus* species is the presence of a minute chromosome pair with a different meiotic behaviour from that of autosomes and sex chromosomes, the so-called m-chromosomes. Most reports on the behaviour of the m-chromosomes described them as asynaptic and achiasmatic throughout early meiotic prophase after conventional staining squashed spermatocytes. At diakinesis they approach each other, and at metaphase I they are always associated end-to-end (touch-and-go pairing) forming a pseudo-bivalent that segregates reductionally at anaphase I. However, minor modifications of this typical male meiotic behaviour are found among different taxa, particularly with regard to the size, the pycnotic cycle, the meiotic behaviour, and the arrangement at both metaphases I and II (Wilson 1905a, b, 1909a, b, 1911, Nokkala 1986, Suja et al. 2000, Bressa et al. 2005, Toscani et al. 2008). An exception to the lack of synapsis and chiasmata in the m-chromosomes has

been described in *Coreus marginatus* (Linnaeus, 1758) (Coreidae). In this coreid bug, some male meiotic cells showed a small synaptonemal complex corresponding to the m-chromosome pair that later appears as a chiasmatic bivalent in diplotene (Nokkala 1986, Suja et al. 2000).

From diplotene onwards, the smallest chromosome pair of *M. lunifer* appeared not only as structures resembling true bivalents (Figs 2d, e; 4b) (Nokkala 1986), but also as two univalents (Figs 2c, f; 3e-f; 4c) (Ueshima 1979, Manna 1984, Papeschi and Bressa 2006). Since this minute chromosome pair could not be recognized until diplotene, it is not possible to assure whether they are asynaptic/achiasmatic or desynaptic. At metaphase I, the m-chromosomes were always observed as a pseudo-bivalent not only lying in the centre of the ring of autosomal bivalents but also forming part of it. Even though the m-chromosomes migrated precociously, this pair of chromosomes as well as the autosomes segregated reductionally during anaphase I.

Taking into account the meiotic behaviour of the m-chromosomes in *C. marginatus* and the presence of m-chromosome pair in *M. conspicillaris* our results allow us to suggest that the minute chromosome pair of *M. lunifer* could be considered a pair of m-chromosomes.

Up to now, *M. conspicillaris* and *M. lunifer* are the only two species within Reduviidae that possess a pair of m-chromosomes; thus, the presence of this pair could be a synapomorphy for the species of *Microtomus* Illiger, 1807.

C- and fluorescent bandings

In Hemiptera early reports on C-positive heterochromatin showed that C-bands are terminally located in some or all the chromosomes. However, interstitial C-positive bands are described in a few species and some of them correspond to secondary constrictions and NORs (Camacho et al. 1985, Panzera et al. 1995, Grozeva and Nokkala 2001, Ituarte and Papeschi 2004, Bressa et al. 2005, Franco et al. 2006, Bressa et al. 2008). The meiotic karyotype of *M. lunifer* is almost devoid of heterochromatin, except for a few dots only detectable at early meiotic prophase in the autosomal chromatin mass.

The use of fluorescent DNA-binding dyes with different specificities allows a better characterization of heterochromatic regions in terms of their relative enrichment with AT or GC base pairs. Most reports referring to heterochromatin characterization on hemipteran species describe C-bands as DAPI bright and CMA_3 dull. The presence of a CMA_3 bright band was detected in a few species at interstitial or terminal position, either on autosomes or sex chromosomes, and they are generally associated to NORs (González-García et al. 1996, Papeschi et al. 2001, Papeschi et al. 2003, Rebagliati et al. 2003, Cattani et al. 2004, Grozeva et al. 2004, Papeschi and Bressa 2006). In *M. lunifer*, our results of fluorescent bandings show the presence of a small CMA_3 bright band in one of the terminal regions of the largest autosomal pair. This CMA_3 bright band could represent an NOR (see below).

Location of rDNA

In Reduviidae the location of NORs was analysed in only 14 species belonging to the subfamilies Harpactorinae (2 species) and Triatominae (12 species) by Ag-NOR, fluorescent banding and/or FISH with rDNA probes (18S, 26S or 45S). The present paper brings the first information about the number and chromosomal location of ribosomal gene clusters in Hammacerinae. Using rDNA-FISH we show here that *M. lunifer* has an rDNA cluster, which is located at one terminal region of the largest autosomal pair.

In *M. lunifer* the NOR is associated with a small CMA_3 bright band. The results of the fluorescent banding and FISH in this species agree with those described for *Rhodnius pallescens* Barber, 1932 (Morielle-Souza and Azeredo-Oliveira 2007), and *T. vitticeps* (Stål, 1859) (Severi-Aguiar et al. 2006), in which the NOR regions co-localized with a CMA_3 positive band and, therefore, the repeating unit of ribosomal is G+C-rich.

Taking into account the data on the number and location of rDNA clusters along with the type of sex chromosome systems in Reduviidae, we can observe different patterns of rDNA distribution. The NOR is generally located at terminal position on the X chromosome, or on both X and Y chromosomes in the species that have XY sex chromosome system. On the other hand, in most cases the NOR is placed at terminal position on an autosomal pair in the species with multiple sex chromosome systems (X_nY). Providing that the ancestral male karyotype of Reduviidae had 2n=30=28+XY, the NOR would have been at a terminal region of the sex chromosomes. Thus, a single pair of NOR-autosomes in species with multiple sex systems (X_nY) might be due to the ability of NOR to change its number and position (Arnheim et al. 1980, Schubert and Wobus 1985, Zhang and Sang 1999, Shishido et al. 2000, Roy et al. 2005, Datson and Murray 2006, Schubert 2007, Cabrero and Camacho 2008, Bressa et al. 2009, Nguyen et al. 2010).

Meiotic behaviour and kinetic activity of the NOR-bivalent

In two species of Coreidae, *Carlisis wahlbergi* Stål, 1858 and *Camptischium clavipes* (Fabricius, 1803), most crossovers occurred in the distal half of the NOR-bivalent (Fossey and Liebenberg 1995, Cattani et al. 2004). The authors suggested that the NOR could act as a crossover repellent since it could be a hindrance for a recombination event. On the contrary, in the NOR-autosomal bivalent of *M. lunifer*, as well as in the previously analysed species *Nezara viridula* (Linnaeus, 1758) (Pentatomidae) (Camacho et al. 1985), the chiasmata can be formed more frequently at the terminal region near the NOR than in the other one (without NOR). Thus, we could propose that the presence of a NOR does not interfere with the meiotic recombination in this species.

In *M. lunifer* the location of the NOR at one chromosome end was used as a chromosome marker that allowed us to discern both ends, determine whether both

terminal regions could be kinetically active and analyse the behaviour of autosomes during both meiotic divisions. The hypotheses which were tested are the followings: i) the kinetic activity of both ends at both meiotic divisions is a random process, and ii) those regions that were active during anaphase I become inactive during anaphase II and vice versa. From our results it can be concluded that both terminal regions are able to develop kinetic activity at first and second meiotic divisions, but the election of the kinetic end is not a random process. In addition, those chromosome ends that show kinetic activity in the first meiotic division are inactive in the second one, and vice versa.

The identification of the factor/s and the mechanism/s involved in the restriction of the kinetic activity to only one chromosome/chromatid end in holokinetic chromosomes of Hemiptera remains unsolved. The presented results here together with previous papers (Pérez et al. 1997, 2000, Viera et al. 2009) allow us to suggest that the euchromatic chromosome ends could present kinetic activity more frequently than the other ends composed of repetitive DNA sequences, i.e. blocks of heterochromatin or ribosomal genes, at metaphase I, and vice versa at metaphase II. However, it will be necessary to analyse more hemipteran species to elucidate the factor/s and the mechanism/s that influence the determination of those ends kinetically active in their holokinetic chromosomes.

In summary, the analysis of meiosis, the determination of the distribution, number and location of heterochromatin blocks and rDNA loci could be useful for the taxonomic identification of species, the analysis of karyotype evolution, and for a better knowledge of chromosome structure and organization.

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RESEARCH ARTICLE

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Karyotypes of *Chironomus* Meigen (Diptera: Chironomidae) species from Africa

Wolfgang F. Wülker[1], I.I. Kiknadze[2], A.G. Istomina[2]

1 *Zur Bitzenmatte 9, D79249 Merzhausen, Germany* **2** *Institute of Cytology and Genetics, Russian Academy of Sciences, Siberian Branch, Novosibirsk, 630090, Lavrentiev pr. 10, Russia*

Corresponding author: *W.F. Wülker* (Wolfgang.Wuelker@biologie.uni-freiburg.de)

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Abstract

The karyotypes of six African *Chironomus* species (*Ch. alluaudi* Kieffer, 1913, *Ch. transvaalensis* Kieffer, 1923, *Ch*. sp. Nakuru, *Ch. formosipennis* Kieffer, 1908, *Ch.* prope *pulcher* Wiedemann, 1830, *Ch.* sp. Kisumu) were investigated; four of these karyotypes were described for the first time (*Ch.* sp. Nakuru, *Ch. formosipennis*, *Ch*. prope *pulcher*, *Ch*. sp. Kisumu). Of the six *Chironomus* karyotypes, three had "pseudothummi" cytocomplex chromosome arms combinations AE CD BF G (*Ch. alluaudi*, *Ch. transvaalensis*, *Ch.* sp. Nakuru), two had "thummi" cytocomplex arms combinations AB CD EF G (*Ch. formosipennis*, *Ch.* prope *pulcher*), and one had "parathummi" arm combinations AC BF DE G (*Ch.* sp. Kisumu). Thus, three of the ten main cytocomplexes known were detected in Africa. Detailed photomaps of all chromosome arms, with the exception of arms B and G, were prepared for the karyotypes of *Ch. alluaudi, Ch. transvaalensis, Ch*. sp. Nakuru*, Ch.* prope *pulcher;* the karyotypes of *Ch. formosipennis, Ch.* sp. Kisumu could only be fragmentarily mapped.

Endemic African banding sequences were characteristic for most of the chromosomal arms in all species studied. However, basic sequences, which can be present in different *Chironomus* species on different continents (Wülker, 1980; Kiknadze et al., 2008), were also detected also in several African species *(Ch. alluaudi, Ch*. sp. Nakuru*,* and *Ch. formosipennis*). The banding sequences of African species studied allow discussion of the derivation of modern banding patterns from hypothetical species, living before separation of cytocomplexes and continents.

Keywords

Chironomus, karyotype, banding sequences, chromosomal polymorphism, chromosomal evolution

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Introduction

As shown by cytogenetic analysis of chromosomal evolution, the divergence of animal karyotypes during speciation was mainly mediated by para- and pericentric inversions, altering the gene orders in linkage groups (Dobzhansky 1970, White 1977, King 1993, Zdobnov et al. 2002). The other types of chromosomal rearrangements (translocations, fusions, duplications) play an additional role in rearrangements of the linear structure of genome. Alteration of the gene orders in chromosomes during evolution can be visualized in Diptera, which possess polytene chromosomes with distinct banding sequences. The bands of polytene chromosomes, which form species-specific banding sequences, are considered as genetic markers to analyze divergence patterns of the linear genome structure during evolution. The use of the number of chromosomal breakpoints as a divergence measure provided establishment of phylogenetic relationships between species (Kiknadze et al. 2008). Species of the genus *Chironomus* have four giant chromosomes with seven chromosome arms (A-G). Based on the different combination of the arms, caused by whole-arm translocations, the *Chironomus* species are grouped into several cytocomplexes (Keyl 1962, Wülker 1980). Cytocomplex is not a taxonomic term. It includes the species with definite chromosome arms combinations, but not similar morphologically. Comparison of banding sequences between species from different cytocomplexes have shown that karyotypes can include species-specific sequences and so called basic sequences, common to more than one cytocomplex and in more than one continent. Such basic sequences were probably present before the separation of species and cytocomplexes (Keyl 1962, Wülker 1980).

By global analysis of banding sequences in Eurasia, North and South America, Australia, we have traced banding sequence changes during *Chironomus* species divergence and continent dispersal (Martin et al. 1974, Wülker 1980, Wülker et al, 1989, Kiknadze at al. 2003, 2008). It was shown that in Eurasia, North America, and Australia, banding sequence pools of many species were represented mainly by endemic continent-specific sequences. However, basic sequences, common for different continents were also found in karyotypes of some species in addition to the endemic sequences. Such basic sequences were noted also in two African *Chironomus* species (*Ch. alluaudi,* and *Ch*. sp. Nakuru) (Martin 1979, Wülker 1980). It was of interest to study how often such basic sequences can be found among African species. However, the data on *Chironomus* karyotypes in Africa are very scanty despite there being much information on the morphology of African chironomids. Wülker (1980) has presented photographs of seven chromosome arms of *Ch. alluaudi*; Martin (1979) has quoted the arm F banding sequence of *Ch. transvaalensis*; Wülker et al. (1989) included the banding sequences of *Ch. transvaalensis* arms A, and F in their list of *Chironomus* sequences, and have shown the position of *Ch. alluaudi* and *Ch. transvaalensis* on the phylogenetic tree.

This paper contains full descriptions of the karyotypes of six African *Chironomus* species (*Ch. alluaudi, Ch. transvaalensis, Ch*. sp. Nakuru*, Ch. formosipennis, Ch.* prope *pulcher*, *Ch.* sp. Kisumu). Among them four karyotypes are described for the first time

(*Ch.* sp. Nakuru*, Ch. formosipennis, Ch.* prope *pulcher, Ch.* sp. Kisumu). Detailed photomaps of arms A, C, D, E, and F are presented for *Ch. alluaudi, Ch. transvaalensis,* and *Ch.* sp. Nakuru. The chromosome arms could be mapped only partly for *Ch. formosipennis, Ch*. prope *pulcher* and *Ch.* sp. Kisumu.

The presence of further basic banding sequences in the karyotypes of African *Chironomus* species was discovered, along with endemic continent-specific (Ethiopian region) sequences.

Evolutionary divergence of "thummi" and "pseudothummi" cytocomplexes is discussed.

Material and methods

Forth instar larvae of African *Chironomus* species were used for karyotype study. 35 years ago, one of us (W.W.) had the opportunity to visit Kenya (22.12.1975–16.01.1976). From a base at the house of relatives in Nairobi, he went with family (wife and 3 sons) to collect chironomids to the west to Lake Nakuru and Lake Victoria, to the north to Abrader Mount Ca. 3000 m above N.N., and to the southeast to Tsavo National park, Mombasa and vicinity. Other material was contributed by colleagues: Mount Elgon and Lake Naivasha (Peter N. Cox), Mount Kenya, near 4350 m (scientific excursion of University Erlangen, Germany, under Prof. Dr. Rüppell), Zigi River, Tanzania (Dr. J. Grunewald). The list of collection sites of *Chironomus* larvae is presented in Table 1. We have not identified species *Ch.* sp. Nakuru and *Ch*. sp Kisumu, but the study of the banding sequences of their karyotypes was very important for purpose of our paper.

Larvae were fixed in ethanol-glacial acetic acid (3:1). The technique of chromosome preparation was as usual (Keyl, 1962). The identification of chromosome banding sequences follows by Keyl (1962) for arms A, E, and F, and by Dévai et. al., (1989) for arms C and D.

To trace the relationship of African *Chironomus* banding sequences with sequences from other continents, we compared them with known basic sequences; if basic sequences for some of species were unknown, we compared them with *Chironomus piger* standard (ST).

We have pointed to previous literature on morphological characteristics of species studied at the beginning of each species description. Most part of the material (larvae, pupa, adults and karyotype slides) is now deposited in Zoologische Staatssammlungen in Münich (Germany).

Equipment of the Center of Microscopy Analysis of Biological Objects of SB RAS in the Institute of Cytology and Genetics (Novosibirsk) was used in accomplishment of this work: microscope "Axiokop" 2 Plus, CCD camera AxioCam HRc, software package AxioVision 4 (Zeiss, Germany).

Table 1. Collection sites and number of specimens of African *Chironomus* species.

Species	Collection sites	Collection date	Collector	Number of specimens
Chironomus alluaudi	Kenya: drinking troughs brooks and pools at Endebess/Mt. Elgon, mountain lakes W of Nakuru, Aberdare mountains up to 3300 m, ponds at MtKenya 4350 m, near Limuru (north of Nairobi), Athi-river south of Nairobi, Amboseli-park	29.12.75 03.01.76 12.01.76 13.01.76 10.01.76	P. N. Cox, W.Wülker. G. Rüppell W. d'Oleire-Oltmanns,H. Koehler W. Wülker	120
Chironomus transvaalensis	Kenya: pool east Lake Victoria, north and south Athi-river near Nairobi, west of Mombasa, Tansania: Kikuwi-river	27.12.75 13.01.76	W. Wülker J. Grunewald	115
Chironomus sp. Nakuru	Kenya: brook south east Lake Nakuru	26.12.75	W. Wülker	9
Chironomus formosipennis	Kenya: Lake Naivasha, Tansania: Zigi-river, running waters		P. N. Cox, J. Grunewald	15
Chironomus prope *pulcher*	Kenya: two pools in short distance, River Athi south of Nairobi	13.01.76	W. Wülker	6
Chironomus sp. Kisumu	Kenya: flat pools 10 and 22 km east Lake Victoria, together with *Ch. transvaalensis,* brook in Amboseli-park (Kilaguni)	27.12.75 10.01.76	W. Wülker	7

Results

***Chironomus alluaudi* Kieffer, 1913**
http://species-id.net/wiki/Chironomus_alluaudi

Previous reports: Kieffer 1913, imago.
Freeman 1957, imago.
Wülker 1980, photo of arms A-G
Wülker et al. 1989, phylogenetic position
Kiknadze et al. 2004, list of banding sequences of arms A, C, D, E, and F

Karyotype (Fig. 1a). Haploid number n=4, arm combination AE CD BF G ("pseudothummi" cytocomplex), centromere bands not heterochromatinized, nucleolus in arm G (terminal), at least 3 Balbiani Rings (BRs) on arm G, inversion polymorphism in arms C and G.

Banding sequences (Fig. 1b-g)

Arm A (Fig. 1b) has the sequence all A1 identical with the main sequence of arm A found in many *Chironomus* species (*Ch. holomelas* Keyl, 1961, *Ch. melanescens* Keyl, 1961, etc.) and it is considered a cosmopolitan basic sequence (holA1).

Figure 1a. Karyotype of *Chironomus alluaudi*. In this and all other Figures: **allA1.1**, **allE1.1** etc. – symbols of arm and homozygous genotypic combinations **N** – nucleolus **BR** – Balbiani ring, arrows show centromeric bands, brackets near chromosome arms show inversions.

allA1 1a-2c 10a-12c 3i-2d 9e-4a 13a-19f

Arm E (Fig. 1, c, 7, b) has the sequence allE1 identical with *Chironomus piger* ST (cosmopolitan basic sequence).

allE1 1a - 13g

Arm C (Fig. 1, d) has two sequences, allC1 and allC2, differing by a simple inversion. The sequence allC1 differs greatly from the basic sequence in arm C; therefore we have compared it with *Chironomus piger* ST: differing by seven inversion steps from pigST:

allC1 1a-2g 11c-10a 16a-17a 6h-2h 11d-15e 9f-7a 17b-22g
allC2 1a-2g 11c-10a 16a-17a 15e-11d 2h-6h 9f-7a 17b-22g
hyp 5 1a-2g 11d-15e 17a-16a 10a-11c 7a-9f 6h-2h 17b-22g
hyp 2+3+4 1a-2g 11d-15e 16a-17a 10a-11c 7a-9f 6h-2h 17b-22g
hyp 1 1a-2g 17a-16a 15e-11d 11c-10a 9f-7a 6h-2h 17b-22g
pigST 1a-2g 2h-6h 7a-9f 10a-11c 11d-15e 16a-17a 17b-22g

Arm D (Fig. 1, e) has single sequence allD1 differing by one inversion step from pigST:

allD1 1a-9e 19h-10a 20a-24g
pigST 1a-9e 10a-19h 20a-24g

Arm B (Fig. 1, a) not mapped, monomorphic. The common BR is not developed.

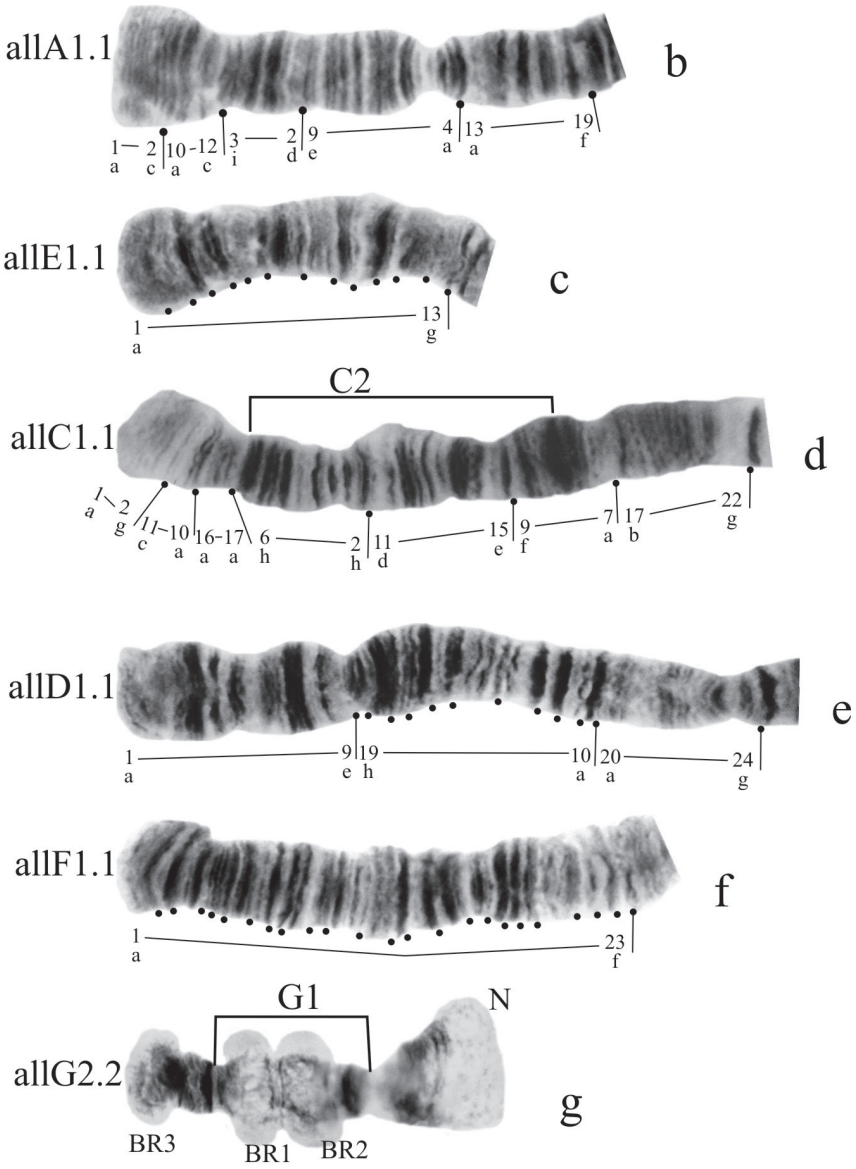

Figure 1b-g. Homozygous banding sequences of *Chironomus alluaudi* in arms A, E, C, D, F and G. The designations are the same as in Fig. 1.

Arm F (Fig. 1, f) has the sequence allF1, identical with pigST (cosmopolitan basic sequence).

allF1 1a–23f

Arm G (Figs 1, a, g) not mapped, has two sequences allG1 and allG2 differing by one simple inversion in the central part of arm G, including two of the Balbiani rings.

In total, the banding sequence pool of *Ch. alluaudi* contains 9 sequences. Six of them endemic for Africa (Ethiopian sequences), three of them (allA1, allE1, allF1) belong to the category of cosmopolitan basic sequences. *Ch. alluaudi* can be considered as a *Chironomus* species with a primitive karyotype (Wülker, 1980, 2010).

Larva: "thummi-type" (no tubuli laterales) on abdominal segment VII). Mentum with high lateral tooth, median tooth as in other *Chironomus* species, pectin epipharyngis about 11 teeth, antenna black with 4 segments, paralabial plates about 40 striae.

Distribution: different places in Africa (Freeman, 1957), Kenya (leg. Wülker, Jan. 1976). Dunking troughs brooks and pools at Endebess/Mt Elgon (N. Cox leg.); mountain lakes W of Nakuru, Aberdare mountains up to 3300m, ponds Mt. Kenya 4350m (Oltmanns leg.) near Limuru (north of Nairobi), Athi river south of Nairobi, Amboseli-park (Wülker leg.)

***Chironomus transvaalensis* Kieffer, 1923**

http://species-id.net/wiki/Chironomus_transvaalensis

Previous reports: Kieffer 1923, imago.
Mc Lachlan 1969, 1971: larva and pupa.
Freeman 1957, imago.
Martin 1979, banding sequence of chromosome arm F.
Wülker, Dévai and Dévai 1989, banding sequences of arms A, E, and F, phylogenetic position of species.

Karyotype (Fig. 2, a). Haploid number n=4, arm combination AE CD BF G ("pseudothummi" cytocomplex), centromeric bands not heterochromatinized, nucleolus in arm C, inversion polymorphism in arms C and G.

Banding sequences (Fig. 2, b-f).

Arm A (Fig. 2, b) has the sequence trvA1, differing by only one inversion step from the basic sequence holA1.

trvA1 1a-2c 10a-12c 3i-c 5a-9e 2d-3b 4d-a 13a-19f
holA1 1a-2c 10a-12c 3i-c 3b-2d 9e-5a 4d-a 13a-19f

Arm E (Fig. 2, c) has the banding sequence trvE1, differing only by one step from basic sequence aciE1 (*Ch. acidophilus* Keyl, 1960 etc.)

trvE1 1a-2b 5a-10b 3e-2c 4h-3f 10c-13g
aciE1 1a-3e 10b-5a 4h-3f 10c-13g

Arm C (Fig. 2, d, j) has two banding sequences, trvC1 and trvC2, differing by one simple inversion (Fig. 2, j). The sequence trvC1 is formed by four inversion steps from a basic sequence, (lonC1), found in several *Chironomus* species (*Ch. longistylus* Goetghebuer, 1921, *Ch. anthracinus* Zetterstedt, 1860 etc.).

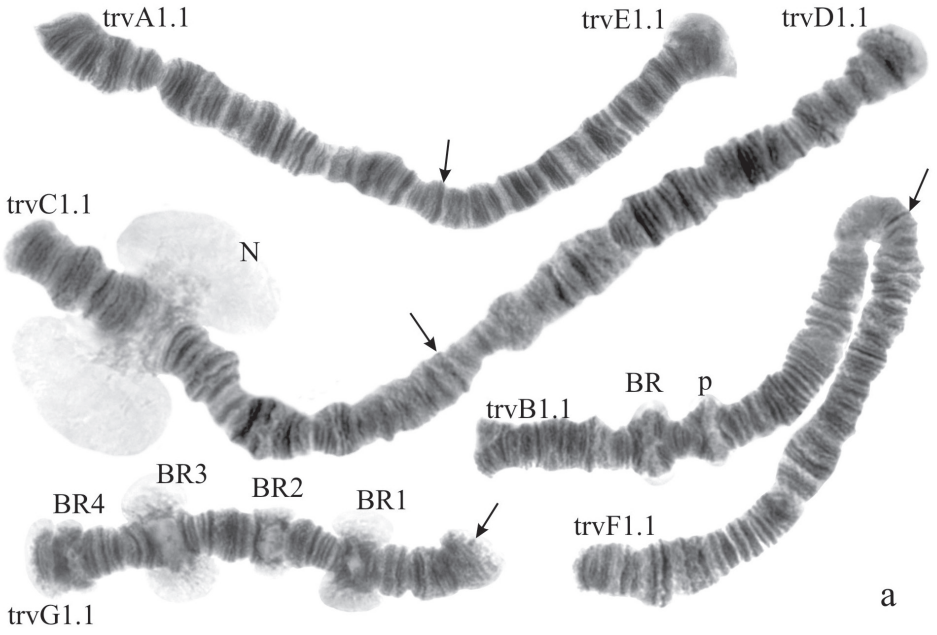

Figure 2a. Karyotype of *Chironomus transvaalensis*. **p** – puff and the designations are the same as in Fig. 1

trvC1 1a-2e 11d-12d 2f-6b 13a-15e 8a-11c 6gh 17a-16a 7d-a 6f-c 17b-22g
hyp 3 1a-2e 11d-12d 6b-2f 13a-15e 8a-11c 6gh 17a-16a 7d-a 6f-c 17b-22g
hyp 2 1a-2e 2f-6b 12d-11d 13a-15e 8a-11c 6gh 17a-16a 7d-a 6f-c 17b-22g
hyp 1 1a-2e 2f-6b 11d-12d 13a-15e 8a-11c 6gh 17a-16a 7d-a 6f-c 17b-22g
lonC1 1a-2e 2f-6b 11c-8a 15e-13a 12d-11d 6gh 17a-16a 7d-a 6f-c 17b-22g

Arm D (Fig. 2, e) has the sequence trvD1 differing from pigST by four inversion steps.
trvD1 1a-2h 4b-7e 12d-7f 19h-13a 4a-3a 20a-24g
hyp 2 1a-2h 4b-7e 7f-12d 13a-19h 4a-3a 20a-24g
hyp 1 1a-2h 19h-13a 12d-7f 7e-4b 4a-3a 20a-24g
pigST 1a-2h 3a-4a 4b-7e 7f-12d 13a-19h 20a-24g

Arm B (Fig. 2, a) not mapped, monomorphic. BR is well developed.

Arm F (Fig. 2, f) has the banding sequence trvF1 differing from cosmopolitan basic pigST by three inversion steps.
trvF1 1a-2a 10d-3f 14f-16g 14e-11a 2b-3e 17a-23f
hyp 2 1a-2a 10d-3f 16g-14f 14e-11a 2b-3e 17a-23f
hyp 1 1a-2a 10d-3f 3e-2b 11a-14e 14f-16g 17a-23f
pigST 1a-2a 2b-3e 3f-10d 11a-14e 14f-16g 17a-23f

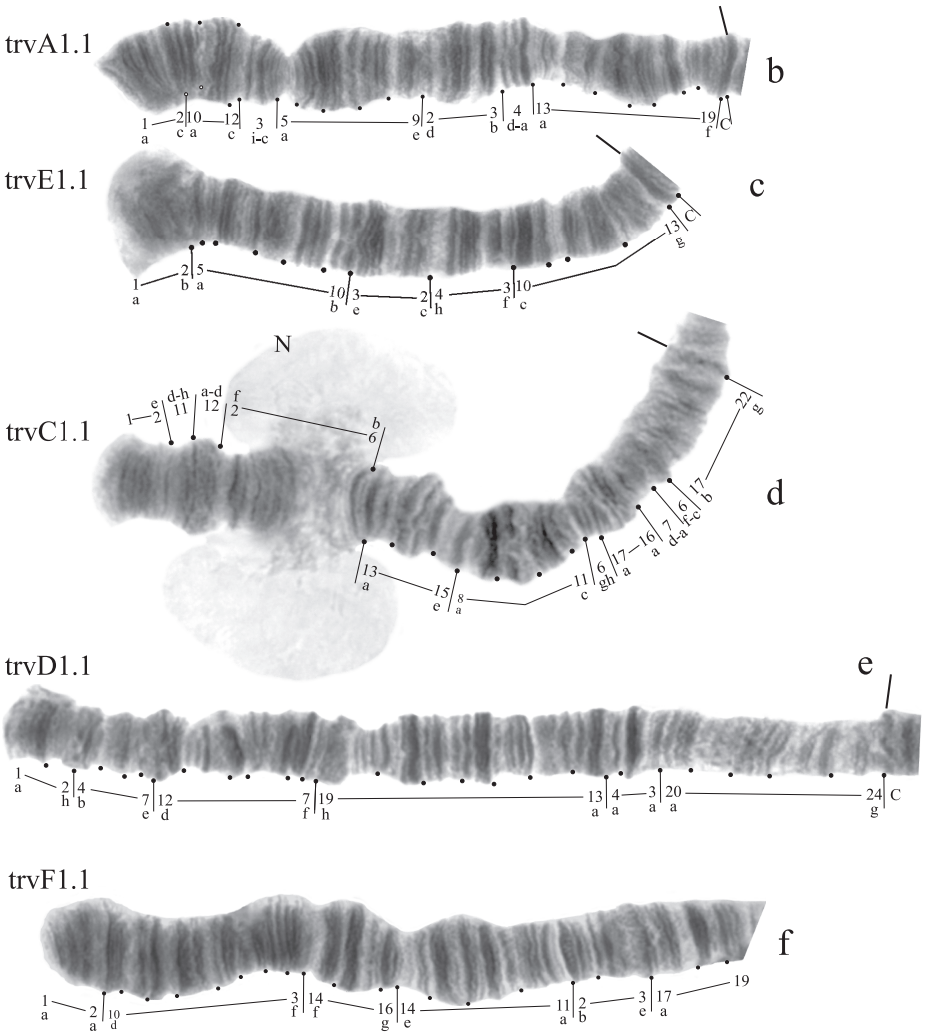

Figure 2b-f. Homozygous banding sequences of *Chironomus transvaalensis* in arms A, E, C, D and F.

Arm G (Fig. 2, g-i) has three banding sequences, trvG1, trvG2, and trvG3. The sequence trvG2 differs from trvG1 by a short inversion in the region BR1 (Fig. 2, h); the sequence trvG3 – by long inversion of central part of arm G (Fig. 2, i). Both last sequences were found as heterozygotes. There are four Balbiani rings.

In total, the banding sequence pool of *Ch. transvaalensis* contains 10 sequences, all of them are Ethiopian endemic sequences.

Larva: tubuli laterales at abdominal segment VII. Other characters - Mc Lachlan, 1969.

Distribution: various places in Africa, Freeman (1957); Blantyre Malawi (Mc Lachlan), Wülker, 1957: pool east Lake Victoria, Kikuwi-river, Tanzania (J. Grunewald), Pretoria South Africa, Israel (Martin, personal communication).

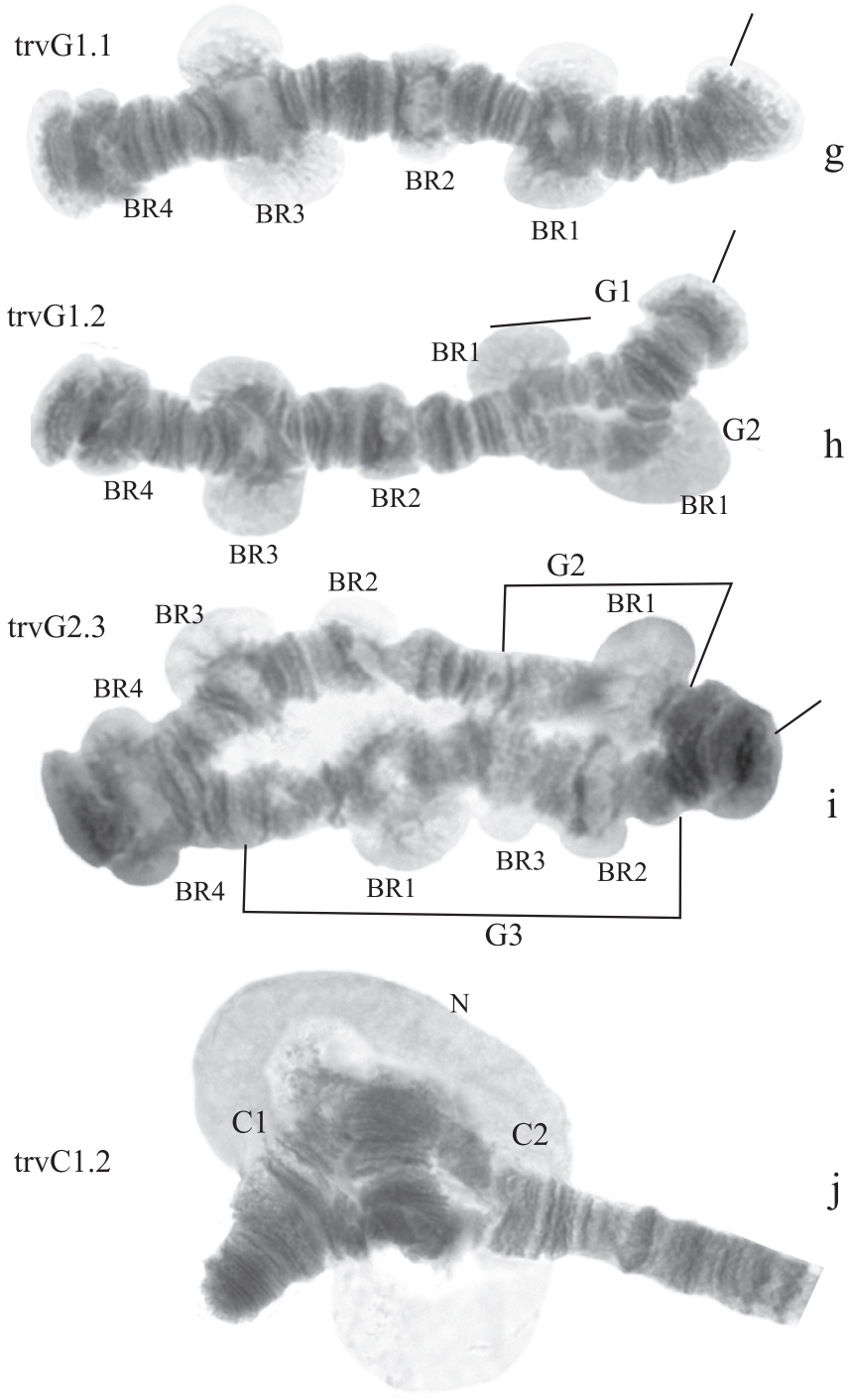

Figure 2g-j. Homozygous and heterozygous banding sequences of *Chironomus transvaalensis* in arm G (**g–i**) and heterozygous inversion in arm C (**j**). Brackets above arms indicate the localization of inversions. The designations are the same as in Fig. 1.

Chironomus sp. Nakuru

Previous report: Wülker, 1980, banding pattern of arms A, E, and F. This species was not identified as well as *Ch*. sp. Kisumu because there was no additional possibility to collect larvae for rearing. However, the study of *Ch*. sp. Nakuru karyotype was very important for comparative analysis of Ethiopian *Chironomus* banding sequences with *Chironomus* sequences of the other continents.

Karyotype (Fig. 3, a). Haploid number n=4, arm combination AE CD BF G ("pseudothummi" cytocomplex), centromeric bands not heterochromatinized, nucleoli on arms F and G, Balbiani rings on arms G, B, and A. Chromosomal polymorphism was not recorded.

Banding sequences (Fig 3, b-f).

Arm A (Fig. 3, b) has the banding sequence nakA1 identical with cosmopolitan basic sequence found in many species (*Ch. holomelas, Ch. melanescens*, etc.)

nakA1 1a-2c 10a-12c 3i-2d 9e-4a 13a-19f

Arm E (Fig. 3, c) has banding sequence nakE1 differing by two inversion steps from the cosmopolitan basic sequence lonE1 (*Ch. longistylus, Ch. anthracinus* etc.).

nakE1 1a-3e 12g-a 5a-10b 4h-3f 10c-11d 13a-g
hyp1 1a-3e 12g-a 11d-10c 3f-4h 10b-5a 13a-g
lonE1 1a-3e 5a-10b 4h-3f 10c-11d 12a-g 13a-g

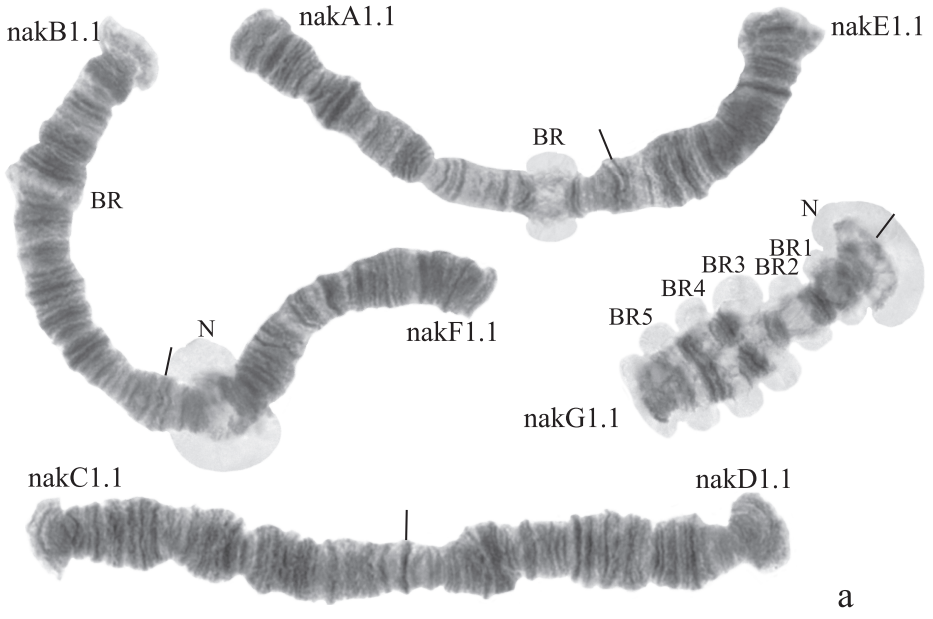

Figure 3a. Karyotype of *Chironomus* sp. Nakuru. The designations are the same as in Fig. 2a.

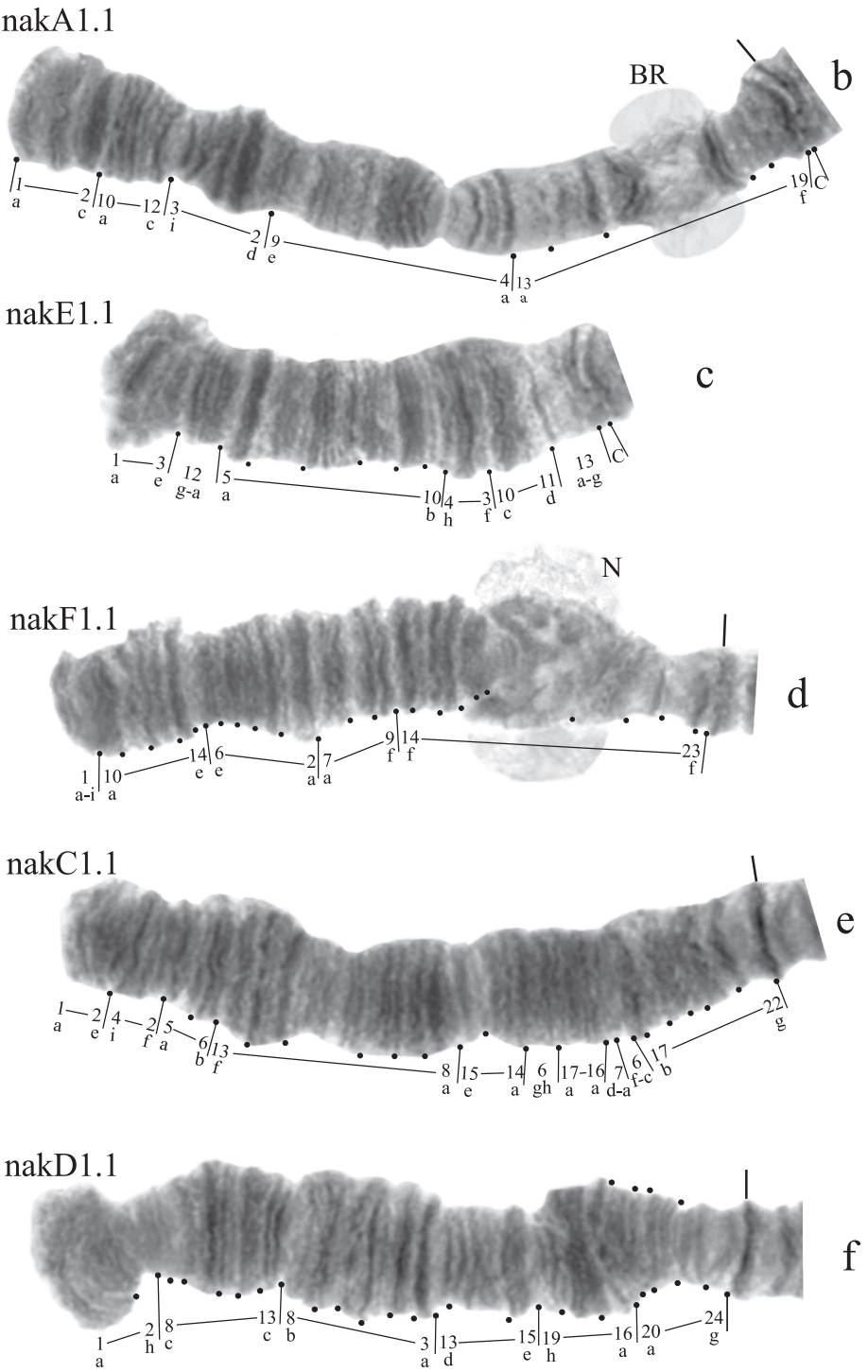

Figure 3b-f. Homozygous banding sequences of *Chironomus* sp. Nakuru in arms A, E, F, C and D. The designations are the same as in Fig. 1.

Arm C (Fig. 3, e) has the sequence nakC1 differing by four inversion steps from basic pattern lonC1 (*Ch. longistylus, Ch. anthracinus*, etc.) and by seven inversion steps from *Chironomus piger* ST (Fig. 7, b).

nakC1 1a-2e 4i-2f 5a-6b 13f-11d 11c-8a 15e-14a 6gh 17a-16a 7d-a 6f-c 17b-22g
hyp1+2 1a-2e 4i-2f 5a-6b 11d-13f 14a-15e 8a-11c 6gh 17a-16a 7d-a 6f-c 17b-22g
lonC1 1a-2e 2f-4i 5a-6b 11c-8a 15e-14a 13f-11d 6gh 17a-16a 7d-a 6f-c 17b-22g

Arm D (Fig. 3, f) has the banding sequence nakD1 differing from pigST by three inversion steps.

nakD1 1a-2h 8c-13c 8b-3a 13d-15e 19h-16a 20a-24g
hyp 1 1a-2h 13c-8c 8b-3a 13d-15c 16a-19h 20a-24g
pigST 1a-2h 3a-8b 8c-13c 13d-15c 16a-19h 20a-24g

Arm B (Fig. 3, a) not mapped, monomorphic. The common BR is not developed.

Arm F (Fig. 3, d) has the banding sequence nakF1 formed by four inversion steps from pigST.

nakF1 1a-i 10a-14e 6e-2a 7a-9f 14f-23f
hyp 3 1a-i 10a-14e 2a-6e 7a-9f 14f-23f
hyp 2 1a-i 10a-14e 9f-2a 14f-23f
hyp 1 1a-i 14e-10a 9f-2a 14f-23f
pigST 1a-i 2a-9f 10a-14e 14f-23f

The arm F of *Chironomus* sp. Nakuru has a nucleolus in region 17–19.

Arm G (Fig. 3, a) has the banding sequence nakG1. It differs from the most of *Chironomus* species arm G by numerous Balbiani rings. It is possible to suggest that some of them can be nucleoli. But it is often impossible to differentiate nucleoli and Balbiani rings without electron microscopy or in situ hybridization.

In total, seven banding sequences are found in sequence pool of *Ch*. sp. Nakuru, six chromosomal arms have Ethiopian endemic sequences, and one arm (A) a cosmopolitan basic sequence.

Larva: long tubuli laterales at abdominal segment VII, extremely long antenna, gula light, no dark stripe on clypeus.

Distribution: brook to SE of Lake Nakuru, Kenya

***Chironomus formosipennis* Kieffer, 1908**

http://species-id.net/wiki/Chironomus_formosipennis

Previous reports: Kieffer 1908, imago.
Freeman 1957, imago.
Dejoux 1970, imago.

Dejoux 1970, pupa.
Dejoux 1970, larva.

Karyotype (Fig. 4). Haploid number n=4, arm combinations AB CD EF G ("thummi" cytocomplex), centromeric bands not heterochromatinized, nucleoli in arms A and G, Balbiani ring in arm G. Chromosomal polymorphism was not recorded.

Banding sequence was determined only in arm E. The sequence frmE1 was identical with the cosmopolitan basic pattern, aprE1 (as in *C. aprilinus* Meigen, 1818)

frmE1 1a-3e 10b-3f 10c-13g

Larva: long tubuli laterales at abdominal segment VII. Other characters - Dejoux, 1970.

Distribution: Lake Naivasha, Kenya, Zigi-river, Tanzania, running waters.

Chironomus prope *pulcher* Wiedemann, 1830

Previous reports: Wiedemann 1830, imago.
Freeman 1957, imago.
Dejoux 1968, imago, pupa, larva.

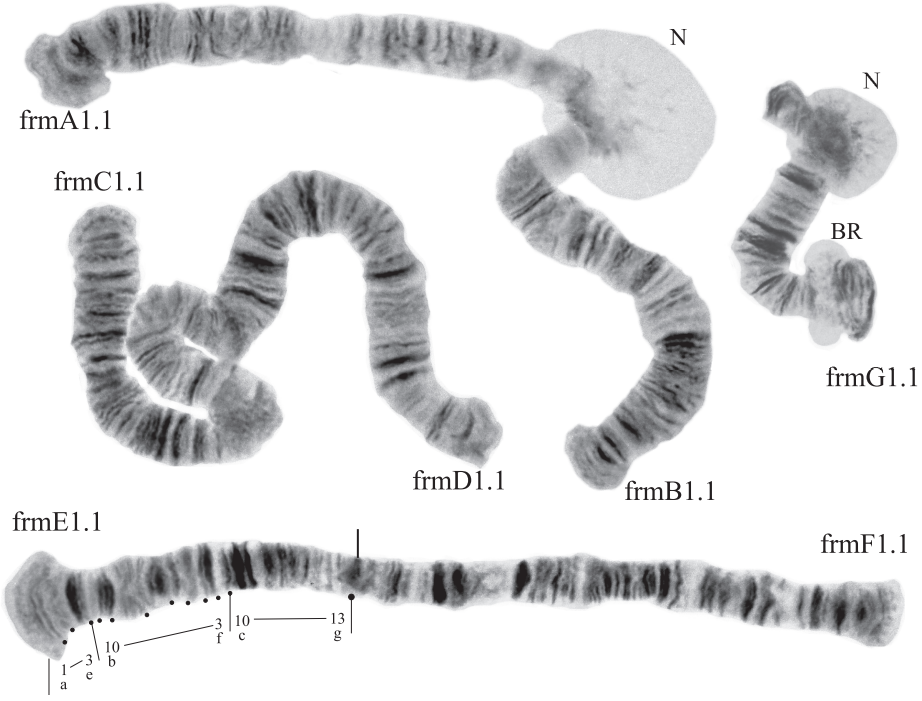

Figure 4. Karyotype of *Chironomus formosipennis*. The designations are the same as in Fig. 1.

The association to this species is based on one male adult from the collecting sites of the larvae.

Karyotype (Fig. 5, a). Haploid number n=3, arm combination AB CD FEG (modified "thummi" cytocomplex), centromeric bands not heterochromatinized, nucleolus in arm F (at the very telomeric end) and nucleolus-like bodies at the ends of arms A, B, E; Balbiani rings are in arms G and B. Chromosomal polymorphism in arm C (Fig. 5, a).

Banding sequences (Fig. 5, a, b-e).

Arm A (Fig. 5, b) has the banding sequence pulA1, formed by four inversions from pigST

pulA1 1a-3i 8g-6a 16d-17h 11e-9a 4ab 5e-4c 16c-12a 18a-19f
hyp 3+4 1a-3i 8g-6a 16d-17h 11e-9a 4abcd-5e 16c-12a 18a-19f
hyp 1+2 1a-3i 8g-6a 5e-4a 9a-11e 17h-16d 16c-12a 18a-19f
pigST 1a-3i 4a-5e 6a-8g 9a-11e 12a-17h 18a-19f

Arm B (Fig. 5, a) not mapped, monomorphic. It has a sequence pulB1. The common BR is well developed.

Arm C (Fig. 5, a) not mapped. It has two banding sequences pulC1 and pulC2 differing by a simple inversion, which involved practically the whole central part of arm C.

Arm D (Fig. 5, c) has the sequence pulD1, formed by five inversion steps from pigST

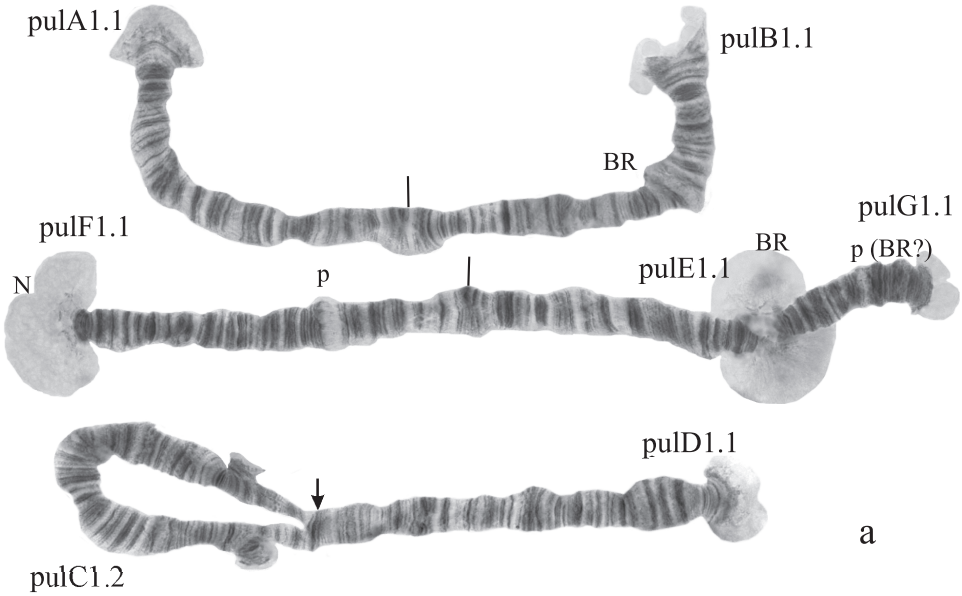

Figure 5a. Karyotype of *Chironomus* prope *pulcher.* The designations are the same as in Fig. 1.

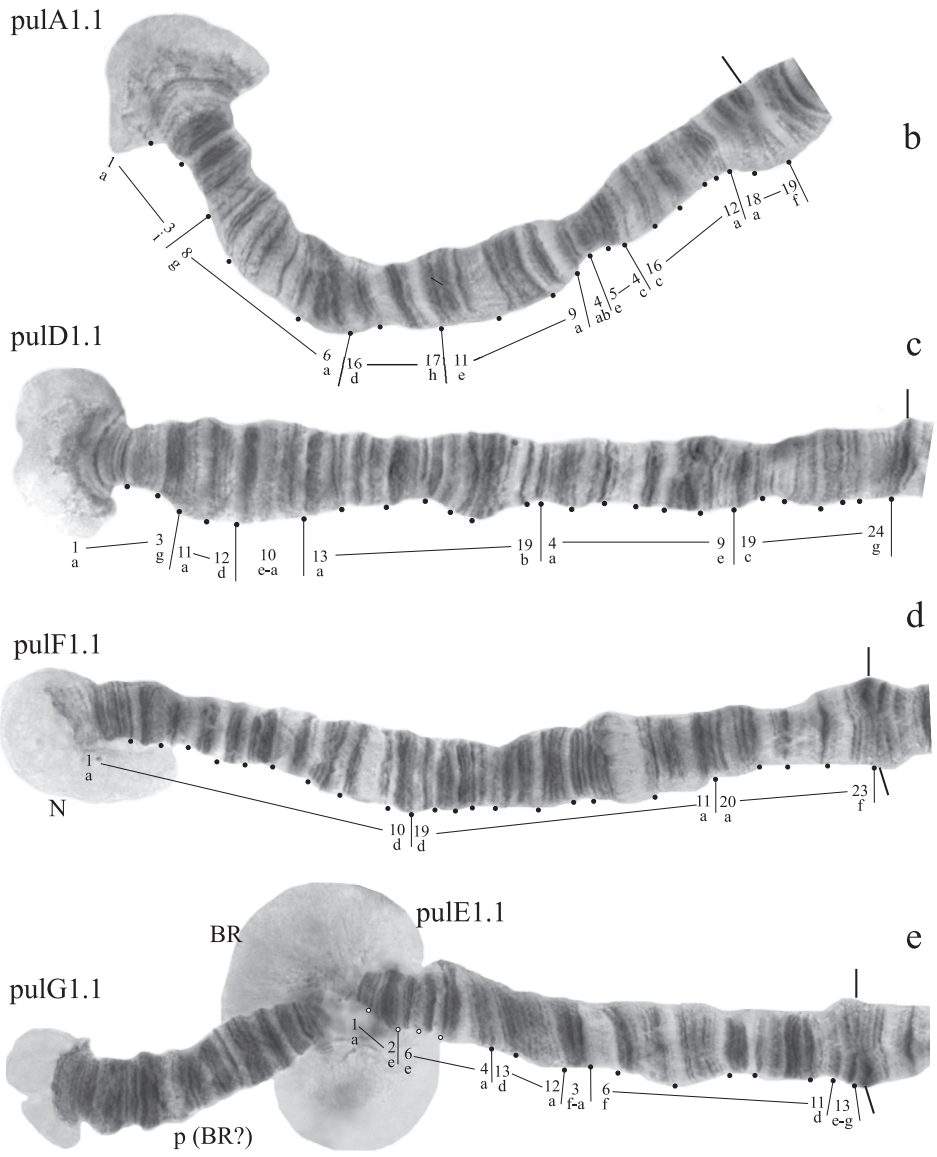

Figure 5b-e. Homozygous banding sequences of *Chironomus* prope *pulcher* in arms A, D, E, F, and G.

pulD1	1a-3g 11a-12d 10e-a 13a-19b 4a-9e 19c-24g
hyp 4	1a-3g 10a-e 12d-11a 13a-19b 4a-9e 19c-24g
hyp 2+3	1a-3g 10a-e 11a-12d 13a-19b 4a-9e 19c-24g
hyp 1	1a-3g 19b-13a 12d-11a 10e-a 9e-4a 19c-24g
pigST	1a-3g 4a-9e 10a-e 11a-12d 13a-19b 19c-24g

Arm E (Fig. 5, e) has the banding sequence pulE1, formed by three inversion steps from pigST.

pulE1 1a-2e 6e-4a 13d-12a 3f-a 6f-11d 13e-g
hyp 2 1a-2e 6e-4a 13d-12a 11d-6f 3a-f 13e-g
hyp 1 1a-2e 6e-4a 3f-a 6f-11d 12a-13d 13e-g
pigST 1a-2e 3a-f 4a-6e 6f-11d 12a13d 13e-g

Arm F (Fig. 5, d) has the sequence pulF1, formed by one simple inversion from pigST.

pulF1 1a-10d 19d-11a 20a-23f
pigST 1a-10d 11a-19d 20a-23f

The characteristic of arm F in *Ch.* prope *pulcher* is the presence of the nucleolus at the telomeric end, which is a rare event among *Chironomus* species.

Arm G (Fig. 5, e) is joined with arm E. There is large Balbiani ring near the site of fusion, and a small Balbiani ring or puff in the center of arm G. A small nucleolus is possibly developed at the telomeric end of arm G.

In total, eight banding sequences were recorded in the *Ch.* prope *pulcher* banding sequence pool. All of them are endemic for Ethiopia. There are no basic sequences.

Larva: long tubuli laterales on abdominal segment VII. Other characters - Dejoux, 1968.

Distribution: two pools within a short distance, River Athi south of Nairobi, Kenya.

Chironomus sp. Kisumu

Karyotype (Fig. 6, a). Haploid number n=4, arm combination AC BF DE G ("parathummi" cytocomplex), centromeric bands not heterochromatinized, nucleoli in arms E and G, Balbiani rings in arms B and G. Chromosomal polymorphism was not recorded.

Banding sequences (Fig. 6, b-f)

Arm A (Fig. 6, b) has the sequence kisA1, formed by 3 inversion steps from pigST.

kisA1 1a-k 19d-16b 10a-16a 2a-9e 19ef
hyp 1 1a-k 19d-16b 16a-10a 9e-2a 19ef
pigST 1a-k 2a-9e 10a-16a 16b-19d 19ef

Arm C (Fig. 6, c) has the sequence kisC1, formed by 8 inversion steps from pigST.

kisC1 1a-2e 5d-6f 22e-17a 2f-5c 16h-14a 11f-13f 6g-11e 22fg
hyp 4+7 1a-2e 5d-6f 22e-17a 2f-5c 6g-11e 11f-13f 14a-16h 22fg
hyp 3 1a-2e 5d-6f 22e-17a 16h-14a 13f-11f 11e-6g 5e-2f 22fg
hyp 2 1a-2e 5d-6f 6g-11e 11f-13f 14a-16h 17a-22e 5e-2f 22fg

Figure 6a. Karyotype of *Chironomus* sp. Kisumu.

hyp 1 1a-2e 22e-17a 16h-14a 13f-11f 11e-6g 6f-5d 5e-2f 22fg
pigST 1a-2e 2f-5c 5d-6f 6g-11e 11f-13f 14a-16h 17a-22e 22fg

Arm D (Fig.6, d) has the sequence kisD1, formed by 6 inversion steps from *Chironomus piger* ST:

kisD1 1a-e 3g-1f 18c-16a 14c-15e 23d-18d 8d-4a 14b-9a 23e-24g
hyp 4+5 1a-e 3g-1f 18c-16a 15e-14c 23d-18d 8d-4a 14b-9a 23e-24g
hyp 3 1a-e 3g-1f 14c-15e 16a-18c 18d-23d 8d-4a 14b-9a 23e-24g
hyp 1+2 1a-e 3g-1f 4a-8d 23d-18d 18c-16a 15e-14c 14b-9a 23e-24g
pigST 1a-e 1f-3g 4a-8d 9a-14b 14c-15e 16a-18c 18d-23d 23-24g

Arm E (Fig. 6, e) has the sequence kisE1, formed by two inversion steps from *Chironomus piger* ST

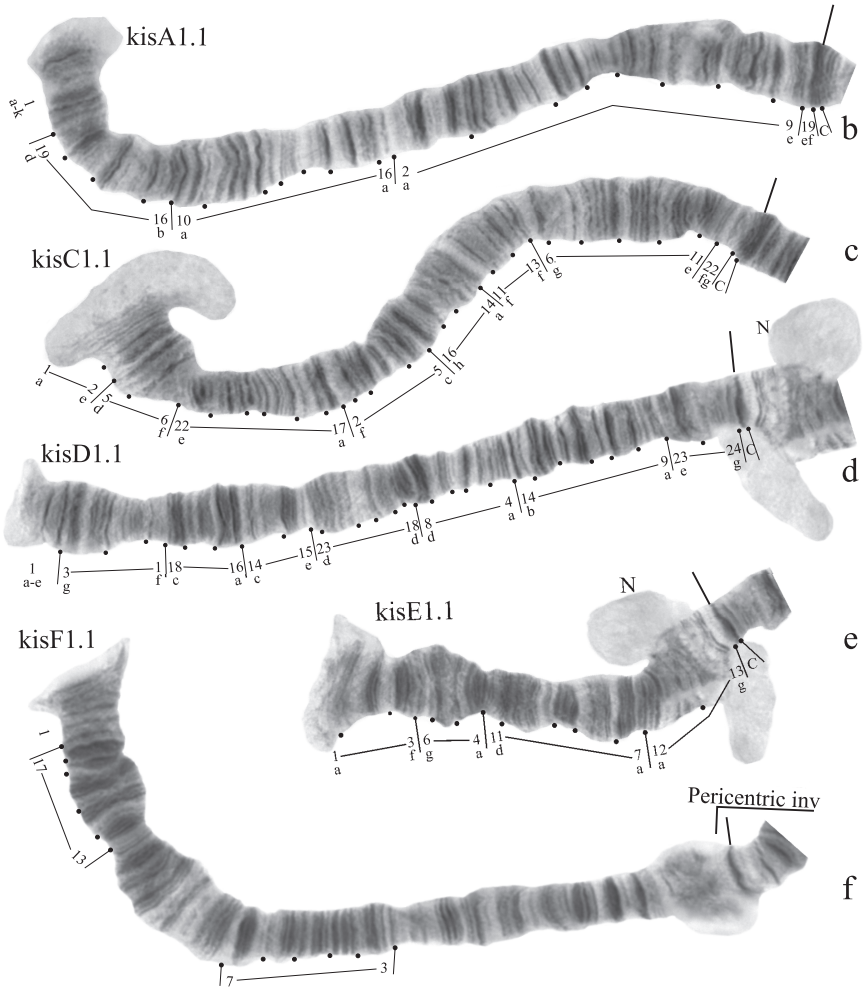

Figure 6b-f. Homozygous banding sequences of *Chironomus* sp. Kisumu in arms A, C. D, E and F.

kisE1 1a-3f 6g-4a 11d-7a 12a-13g
pigST 1a-3f 4a-6g 7a-11d 12a-13g

Presence of a nucleolus in region 13 in arm E is a great characteristic of the *Ch*. sp. Kisumu karyotype.

Arm B (Fig. 6, a) not mapped. It has one sequence – kisB1. The common BR is well developed.

Arm F (Fig. 6, f) has the sequence kisF1. It was mapped only fragmentarily because of complex inversions in comparison with *Chironomus piger* ST. The presence of a large Balbiani ring situated just near the centromeric band is a characteristic of arm F in the *Ch*. sp. Kisumu karyotype. There is pericentric inversion in the chromosome BF (Fig. 6, a, f).

Arm G (Fig. 6, a) is longer than usual in *Chironomus* species. There is a nucleolus and four Balbiani Rings on arm G. One of Balbiani Rings, noted by the black dot in Fig. 6, a, was developed only in some cells of the salivary gland cells.

In total, seven Ethiopian endemic banding sequences are found in the sequence pool of *Ch*. sp. Kisumu. All these sequences differ from *Ch. parathummi* Keyl, 1961 sequences.

Larva: long tubuli laterales on abdominal segment VII.

Distribution: near Victoria lake, Kenya.

Discussion

Karyotypes of six African *Chironomus* species were studied. Four of these karyotypes were described for the first time (*Ch.* sp. Nakuru*, Ch. formosipennis, Ch.* prope *pulcher, Ch.* sp. Kisumu). Detailed photomaps of arms A, C, D, E, and F were presented, also for the first time, for *Ch*. *alluaudi*, *Ch. transvaalensis*, and *Ch.* sp. Nakuru.

Among the species studied, three species (*Ch. transvaalensis*, *Ch*. prope *pulcher*, *Ch*. sp. Kisumu) have only endemic Ethiopian banding sequences in their karyotypes, while cosmopolitan basic banding sequences were discovered in the karyotypes of the other species, along with endemic sequences (*Ch. alluaudi, Ch.* sp. Nakuru*, Ch. formosipennis*). The presence of these basic sequences indicates a relationship of African *Chironomus* species to *Chironomus* species from other continents before their separation (Kiknadze et al. 2008).

The results on African species are relevant the problem whether or not the chromosome arm combination of the "thummi" cytocomplex is rare in Southern continents. At the moment, one species in South America (*Chironomus* sp. Las Brisas, Wülker, Morath, 1989), one species in India (*Ch. javanus* Kieffer, 1924), and two species in Australia (*Ch. javanus, Ch. queenslandicus* Martin, 2005) are known to have this "thummi" cytocomplex chromosome arm combination (Martin 2010, Martin, pers. comm. and this paper).

Earlier it was demonstrated (Wülker 1980), that the presence of basic sequences in arms A, E, F of some *Chironomus* species of the "thummi" and "pseudothummi" cytocomplexes supports an idea that the basic sequences existed in hypothetical stem species before the separation of the complexes. The results of this paper contribute to the understanding of chromosome arms C and D in phylogeny in both cytocomplexes, in addition to data on arms A, E and F published earlier (Wülker 1980, 2010, Kiknadze et al. 2008).

Keyl (1962) established the hypothesis, that "the hypothetical species, which crossed the border between "thummi" and "pseudothummi" cytocomplexes" had most probably three banding patterns in arm E (in Keyl's terms): standard as *Ch. piger* Strenzke, 1959, pattern as *Ch. aprilinus* Meigen, 1838 and others, pattern as *Ch. aberratus* Keyl,1961 and others. We can ask, whether these three patterns are known today in both cytocomplexes. This is indeed so (Fig. 7, a) with the exception of the fact that

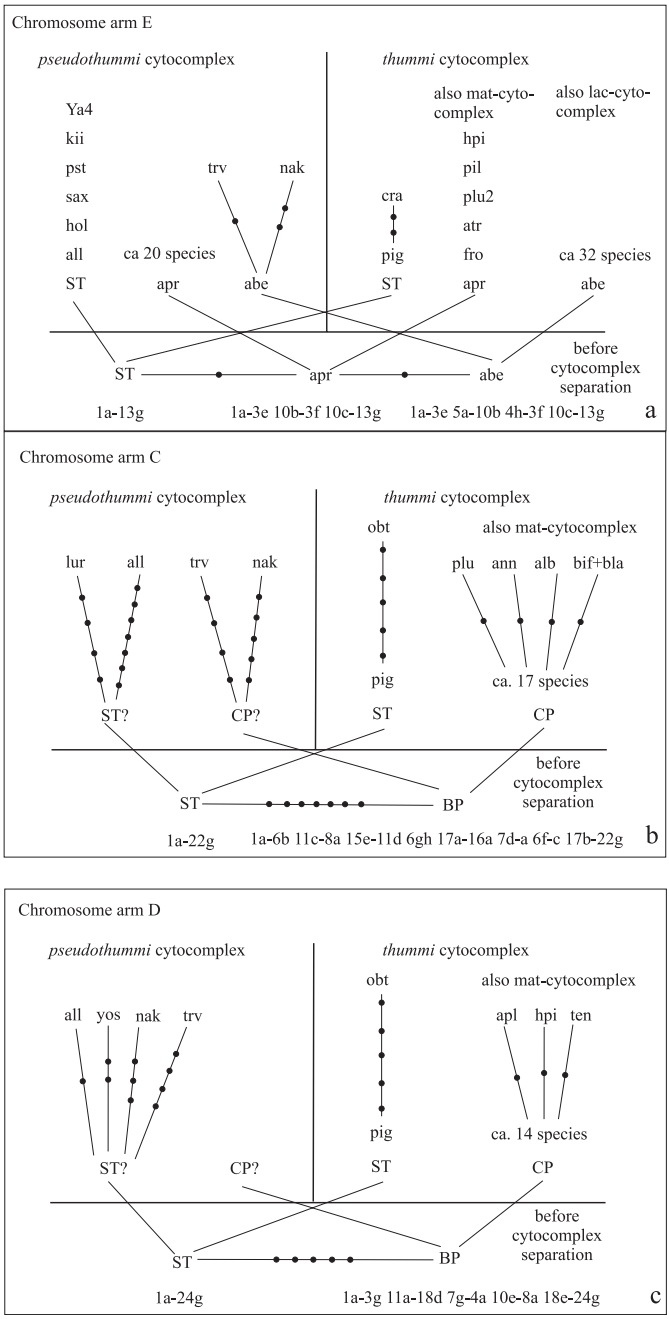

Figure 7a-c. Relations of recent species and hypothetical "basic" species before separation of the cytocomplexes in arm E (**a**), arm C (**b**), and arm D (**c**). The data of Keyl (1962) and Kiknadze (unpublished) were also used. **Dots** – inversion steps between banding sequences; **ST** – piger standard after Keyl (1962) and Dévai et al. (1989). **all** – *alluaudi*, **atr** – *atrella*, **apr** – *aprilinus*, **abe** – *aberratus*, **cra** – *crassicaudatus*, **fro** – *frommeri*, **hol** – *holomelas*, **hpi** – *heteropilicornis*, **kii** – *kiiensis*, **pil** – *pilicornis*, **plu** – *plumosus*, **pst** – *pseudothummi*, **sax** – saxatilis, **trv** – *transvaalensis*.

the pattern of *Ch. aberratus* itself is not known in the "pseudothummi" cytocomplex, but there are the sequences trvE1 and nakE1 which differ only by 1–2 inversions from abeE (Fig. 7, a).

In arms C and D, an accumulation of species with the identical sequences was previously observed only in the "thummi" cytocomplex (Wülker, 2010). With the data of this paper we can propose that chromosome arms C and D had also two patterns before separation of the cytocomplexes (*Chironomus piger* ST sequence sensu Keyl, 1962 and basic pattern sensu Wülker, 1980, 2010). Fig. 7, b shows that pattern ST and basic themselves are not found in "pseudothummi" cytocomplex (question marks in Fig. 7, b), but there are several species, which have banding patterns differing only by a few inversions from ST and basic. The African species (*Ch. alluaudi, Ch. transvaalensis, Ch*. sp. Nakuru, *Ch. formosipennis*) play an important role in the development of the arm C and D phylogeny. Fig. 7, c demonstrates that there are ST and basic patterns in the 'thummi' cytocomplex, but only patterns close to ST were found in the "pseudothummi" cytocomplex: allD1 only by one, yosD1 by two, nakD1 by three, and trvD1 by four inversions from ST.

A great peculiarity of some African *Chironomus* karyotypes is the presence of large numbers of functionally active chromosome sites, especially Balbiani rings. For example 5 BRs were found in *Ch. transvaalensis* (Figs 2, a, 2, g-i), 6 BRs in *Ch.* sp. Nakuru (Fig. 3, a). Most *Chironomus* species have two or three visible BRs since e.g. many species have the gene for BR4 but do not express it, and the number seen may also vary with developmental stage.

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RESEARCH ARTICLE

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Patterns of rDNA chromosomal localization in Palearctic *Cephalota* and *Cylindera* (Coleoptera: Carabidae: Cicindelini) with different numbers of X-chromosomes

Sonia J. R. Proença[1], Artur R. M. Serrano[1], José Serrano[2], José Galián[2]

1 *Centro de Biologia Ambiental /Departamento de Biologia Animal/, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, Bloco C2 - 3º Piso, 1700 Lisboa, Portugal* **2** *Departamento de Zoología y Antropología Física, Campus de Espinardo, Universidad de Murcia, 30100 Murcia , Spain*

Corresponding author: *José Galián* (jgalian@um.es)

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Abstract

The ribosomal clusters of six Paleartic taxa belonging to the tiger beetle genera *Cephalota* Dokhtourow, 1883 and *Cylindera* Westwood, 1831, with multiple sex chromosomes (XXY, XXXY and XXXXY) have been localised on mitotic and meiotic cells by fluorescence *in situ* hybridization (FISH), using a PCR-amplified 18S rDNA fragment as a probe. Four patterns of rDNA localization in these tiger beetles were found: 1. Two clusters located in one autosomal pair; 2. Two clusters located in one autosomal pair and one in an X chromosome; 3. Three clusters located in three heterosomes (XXY); 4. Two clusters located in one autosomal pair and two in the heterosomes (one of the Xs and the Y). These results illustrate that ribosomal cistrons have changed their number and localization during the evolution of these genera, showing a dynamic rather than a conservative pattern. These changes in rDNA localization are uncoupled with changes in the number of autosomes and/or heterosomes. A mechanism that involves transposable elements that carry ribosomal cistrons appears to be the most plausible explanation for these dynamics that involve jumping from one location in the genome to another, in some cases leaving copies in the original location.

Keywords

Cephalota, Cylindera, Cicindelini, Coleoptera, FISH, ribosomal genes, chromosome evolution

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Introduction

Often closely related species differ in their karyotypes, both in terms of changes in chromosome number and morphology and/or localization of genes in chromosomes. Whether these changes have played a significant role as isolation mechanisms in speciation (White 1978; King 1993), or have been an accompanying consequence of this isolation (Futuyma and Mayer 1980; Coyne and Orr 2004) has generated some debate among cytogeneticists. It is accepted that chromosomal rearrangements reduce gene flow between populations (Spirito 1998) by reducing fitness of chromosomally heterozygous individuals, thus acting as an effective isolation mechanism (revision in King 1993), or by reducing recombination rates and extending the effects of linked isolation genes (Rieseberg 2001; Livinsgtone and Rieseberg 2004). Tiger beetles, in particular members of the subtribe Cicindelina, are suitable taxa to explore the link between chromosomal rearrangements and great species diversity. Cytogenetics is still a poorly developed discipline in tiger beetles, with very few cicindelid species karyotyped (Serrano and Galián 1998; Galián and Hudson 1999; Proença et al. 1999; Galián et al. 2002, 2007; Proença et al. 2002a, b, 2005; Zacaro et al. 2004), not exceeding about 4% of more than 2415 described taxa (Wiesner 1992; Lorentz 2005; Pearson and Cassola 2005). Within the more recent tribe Cicindelini the generalized karyotype is made up of nine to eleven autosomal pairs of decreasing size (Galián et al. 1990), plus a sex chromosome mechanism of the X_nY type, where n varies between 2 and 4 that forms a non-chiasmatic multivalent connected by telomeric proteins during meiosis (Giers 1977). The sex multivalent segregates all X chromosomes to one pole and the Y-chromosome to the other during first meiotic division. Multiple sex chromosomes have been found in other taxa of the tribes Cicindelini and Collyrini (Galián et al. 1990, 2002, 2007). Single systems representing secondary loss of both X- and Y-chromosomes have been described in *Cylindera germanica* (XY/XX) (Giers 1977), *Cylindera paludosa* (X0/XX) (Serrano et al. 1986), *Odontocheila confusa* (XY/XX) and *O. nodicornis* (X0/XX) (Proença et al. 2002a). On the other hand, single systems (XY/XX and X0/XX) have been considered as an ancestral state in the morphologically more primitive lineages, namely the tribes Megacephalini (Serrano et al. 1986; Galián and Hudson 1999; Proença et al. 2002b), Mantichorini and Omini (Galián et al. 2002).

Characterization of the number and distribution of ribosomal DNA (rDNA) genes using fluorescence *in situ* hybridization (FISH) provides landmarks for the construction of physical maps in comparative genomics, and is useful for phylogenetic and evolutionary studies. Galián et al. (1995) initiated molecular cytogenetic studies on tiger beetles by reporting the localization of these highly repetitive and conserved rDNA clusters in some Palearctic species of the genus *Cicindela*. Galian et al. (2002) showed that more primitive lineages (Manticorini, Omini and Megacephalini) have a high number of rDNA loci, located exclusively in the autosomes (three and four pairs), whereas more advanced lineages (tribes Collyrini and Cicindelini) show a lower number of rDNA loci but with a variety of localization patterns. According to overall evidence these loci may be found on the autosomes (one autosomal pair), on the heterosomes (one of the X chromosomes and the Y) or in both types of chromosomes (one autosomal pair plus heterosomal copies lo-

cated on one of the X chromosome) (Galián et al. 1995; Galián and Hudson 1999; Galián et al. 2002; Proença et al. 2002a, b; Proença and Galián 2003; Zacaro et al. 2004). This dynamism was exemplified by the occurrence of frequent changes of the rDNA loci between autosomes and sex chromosomes in North American species of *Cicindela* and related taxa (*Cylindera* and *Cicindelidia* among others) (Galián et al. 2007).

These interspecific differences are mirrored, as expected, by intraspecific differences in particular species of tiger beetles. A population study regarding the number and localization of rDNA clusters in *Cicindela (Calomera) littoralis* and *Lophyra flexuosa* showed that both species were polymorphic for these traits as a single population of each species had an rDNA localization different from all the other populations (Proença and Galián 2003). These polymorphisms provide the basis for the fixation of local chromosomal variants giving rise to local chromosomal races and the subsequent formation of karyotypic barriers to gene flow, and eventually to differentiated phylogenetic entities.

In this paper we apply fluorescence *in situ* hybridization with a 18S ribosomal probe to six Paleartic taxa of the genera *Cephalota* and *Cylindera* with different sex chromosome systems (XO, XXY, XXXY, XXXXY), some of which were previously studied cytogenetically by Galián et al. (1995) only using silver staining. This last method may underestimate the actual number of chromosomes carrying rDNA genes as non active or seldom active NORs remain unnoticed. We shall also discuss the putative evolutionary implications of the patterns of localization and number of rDNA loci.

Material and methods

Material

Individuals belonging to the six species studied were collected in the localities listed in Table 1. Males and females were analysed in all species, although in *Cephalota dessertico-loides*, *C. circumdata* and *Cylindera paludosa*, only males provided interpretable plates.

The specimens were identified by the authors and are deposited in the collection of the Department of Animal Biology, University of Lisbon, and in the Department of Zoology and Physical Anhropology, University of Murcia.

Chromosome preparations

Karyological analyses were carried out on gonads dissected from beetles anaesthetised with ethyl-acetate. Testes and ovaries were given a hypotonic treatment in distilled water and fixed using fresh ethanol-acetic acid solution (3:1) for 1 h, with several changes of the fixative solution during the next day and were kept at –20 ºC until studied. Squashes were made on a slide in 70% acetic acid and coverslips were removed after freezing in liquid nitrogen. The slides containing well spread mitotic and meiotic figures were aged for at least 3 days in a 37 ºC incubator.

In situ hybridization

FISH was performed as previously described (Galián et al. 1999; Sánchez-Gea et al. 2000; Galian et al. 2007) with minor modifications. The ribosomal probe was obtained by amplification of an 18S rDNA fragment as described in De la Rúa et al. (1996). Briefly, chromosome spreads were pre-treated with DNase-free RNase in 2× SSC for 1 h at 37 ºC, followed by treatment with 0.005% pepsin in 10 mM HCl for 10 min. After digestion the chromosomes were fixed with fresh paraformaldehyde in NaOH 0.1 N, dehydrated in a graded ethanol series and air dried. The hybridization mixture containing 50% deionized formamide, 2× SSC, 50 mM sodium phosphate (pH = 7.0), 10% dextran sulphate and 4 ng/ml of labelled probe was denatured by boiling for 3 min and placed on ice. The slides were heated on an 80 ºC hot plate for 5 min. A 30 ml aliquot of the denatured hybridization mixture was placed over the denatured slides and covered with a 22 × 22 mm coverslip. The slides were then transferred to a humid chamber at 80 ºC, and the temperature was allowed to drop slowly to 37 ºC for hybridization overnight. After hybridization coverslips were carefully removed and the slides were then given a stringent wash for 3× 5 min in 50% formamide, 2× SSC at 37 ºC. Sites of probe hybridization were detected with avidin-fluorescein isothiocyanate (FITC). The signal was amplified twice using goat anti-avidin-biotin. Slides were counterstained with propidium iodide and mounted with antifade solution to prevent the fluorescence fading away. Slides were examined with a Leitz photomicroscope and photographed with Imation colour film 100 ASA.

Silver staining

Active NORs were detected with silver according to the Howell and Black (1980) technique, with slight modifications. Two solutions were prepared, one colloidal developer containing 0.2 g powdered gelatine in 10 ml distilled water and 0,1 ml of formic acid and a solution of 50% $AgNO_3$, centrifuged at 13000 g for 5 min to separate the silver previously precipitated and kept in the dark. One part of the colloidal developer and two parts of the silver solution were placed on the slides, mixed, covered with a coverslip and incubated at 70 ºC on a hot plate until the solution has turned a deep golden-brown colour. The slides were rinsed thoroughly in distilled water, counterstained with 5% Giemsa in phosphate buffer pH 6.8, washed and air-dried.

Results

Detailed karyotypes of the six species investigated have been reported previously (Serrano et al. 1986; Serrano and Collares-Pereira 1989, 1992; Collares-Pereira and Serra-

no 1990; Galián et al. 1990) and were used for comparisons. Male and female mitotic metaphases and first and second male meiotic cells were analysed and compared to identify homology among labelled chromosomes. The karyotypes of *Cephalota hispanica*, *C. maura* and *Cylindera trisignata* are represented in Figure 1 to illustrate the three types of multiple sex chromosome systems. The number of rDNA carrying chromosomes varies from 2 to 4 (Table 1) and they are restricted to the autosomes, to the heterosomes, or are found in both types of chromosomes.

The rDNA probe hybridizes to the third autosomal pair in male and female mitotic figures of *Cephalota hispanica* (Fig. 2a, b) and the signal is distantly located. Meiotic figures confirm hybridization on the third autosomal bivalent (Fig. 2c, d). Second metaphase plates have one signal each, $9+X_1X_2$ and 9+Y (Fig. 2e, f).

Cephalota maura shows four signals in female mitotic metaphases (Fig. 2g), in two small and two medium-sized chromosomes. Male mitotic plates show signals in two small and one medium-sized element (Fig. 2h). In male diakinesis two fluorescent signals are seen in one small autosomal bivalent and one signal in the sex vesicle (Fig. 2i, j), most likely in one of the Xs. This is confirmed by the observation of second metaphase plates that are of two types, with 12 elements ($9+X_1X_2X_3$, Fig. 2k, l) and 2 signals, and with 10 elements (9+Y, Fig. 2m, n) and one signal.

Male spermatogonial mitosis of *Cephalota deserticoloides* shows signals in four small chromosomes (Fig. 3a, b). Meiotic plates show two hybridization sites in the sex vesicle and one additional site in one autosomal bivalent (Fig. 3 c, d). A similar pattern is shown by *Cephalota circumdata*, which has signals in 4 chromosomes in male mitosis (Fig. 3e, f). First metaphase plates of this species have one autosomal bivalent and two heterosomes labelled (Fig. 3g).

A different situation is present in *Cylindera trisignata*, where three labelled chromosomes are observed in male mitosis. These chromosomes may correspond to 3 of the 5 heterosomes as they are of different (from medium to small) size. Female mitotic plates show four signals in one small and one medium-sized pairs (Fig. 3h, i). Male meiotic figures confirm this interpretation and show fluorescent signal in three of the five elements of the sex vesicle (Fig. 3j). This is further confirmed in second metaphase plates where two types are observed. Two signals are present in cells with $9+X_1X_2X_3X_4$ (Fig. 3k, l) and one signal is present in cells with 9+Y (Fig. 3m, n).

Cylindera paludosa is the only species studied without multiple sex chromosomes (males are n= 7+XO). Observations here made in individuals from Salinas de Pinilla agree with the description of the karyotype (Galián et al. 1990) in individuals from Albatera, and the autosomal localization of the rDNA sites obtained from individuals from Hellin (Galián et al. 1995).

Silver staining was performed for *C. hispanica*, *C. circumdata* and *C. trisignata* to locate active NORs in interphase nuclei. *C. hispanica* and *C. circumdata* showed silver precipitates outside the condensed sex vesicle in interphase nuclei (Fig. 4 a, b), and *C. trisignata* showed silver precipitates inside the sex vesicle (Fig. 4c).

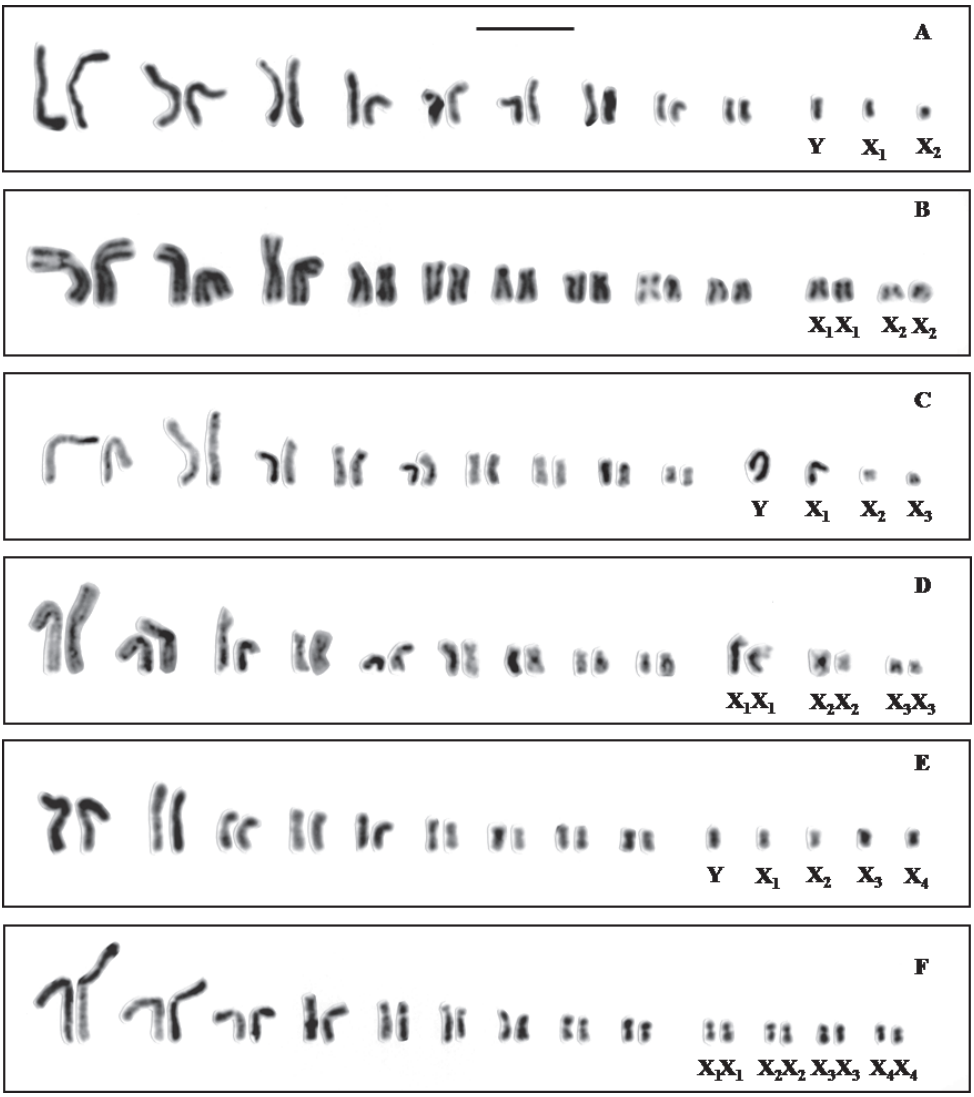

Figure 1a–f. Standard karyotypes of **a** *C. hispanica* male **b** *C. hispanica* female **c** *C. maura* male **d** *C. maura* female **e** *C. trisignata* male and **f** *C. trisignata* female. Bar = 5μm.

Discussion

Detection of rDNA sites by fluorescence *in situ* hybridization

The results for *Cylindera paludosa* corroborate previous findings (Galián et al. 1995) as one fluorescent signal was found in one pair of autosomes. *Cephalota maura* and *C. deserticoloides* have rDNA sites in the sex chromatin, as determined in early meiotic stages,

Table 1. Localities, male meioformula and pattern of rDNA localization for sampled species of tiger beetles.

Species	Localities	Meioformula	Pattern of rDNA localisation
Cephalota (Cassolaia) maura (Linnaeus 1758)	Castro Marim (Portugal)	9 + XXXY	(3 signals) Autosomes (2), Heterosome (X)
Cephalota (Cephalota) hispanica (Gory 1833)	Castro Marim (Portugal)	9 + XXY	(2 signals) Autosomes
Cephalota (Taenidia) circumdata imperialis (Klug 1834)	Salinas de Pinilla (Spain)	9 + XXXY	(4 signals) Autosomes (2), Heterosome (XY)
Cephalota (Taenidia) deserticoloides (Codina 1931)	Albatera (Spain)	9 + XXXY	(4 signals) Autosomes (2), Heterosome (XY)
Cylindera (Cylindera) paludosa (Dufour 1820)	Salinas de Pinilla (Spain)	7 + X0	(2 signals) Autosomes
Cylindera (Eugrapha) trisignata (Dejean 1822)	Carrapateira (Portugal)	9 + XXXXY	(3 signals) Heterosomes (XXY)

which confirms previous findings with silver staining (Galián et al. 1995). Analysis of first metaphase figures indicates that whereas *C. deserticoloides* has rDNA genes in the Y chromosome and in one of the Xs, *C. maura* has only copies in one of the Xs. Moreover, fluorescent signals were found in two additional sites of one autosomal pair in both species, not detected previously with silver staining (Galián et al. 1995). On the other hand, in *Cephalota circumdata*, silver staining only detected the heterosomal copies of the rDNA genes, being the autosomal copies detected only by FISH. In *Cephalota hispanica*, with copies only in the autosomes and in *Cylindera trisignata*, with copies only in the heterosomes, silver staining and FISH gave the same results. It is therefore confirmed that the silver staining technique does not detect the actual number of chromosomes carrying NORs in tiger beetles. This is probably due to the existence of rDNA clusters that are inactive through most part of the cell cycle, remaining unnoticed. Further studies on the activity of NORs on more populations of these species are needed to assess the pattern of activity, if any, of these apparently "silent" rDNA clusters.

Patterns of rDNA localization

Four patterns of rDNA localization were found in the tiger beetles species analysed in this paper. These patterns are: i) One cluster located in each member of an autosomal pair (two signals); ii) Two clusters located in an autosomal pair and one in an X chromosome (three signals); iii) Three clusters located in three of the heterosomes XXY (three signals); and iv) One cluster located in each member of an autosomal pair and in two of the heterosomes, apparently one of the Xs and the Y (four signals). The last two patterns are described for the first time, and are added to the four previously described in the genus *Cicindela* and related taxa by Galián et al. (1995), Galián and

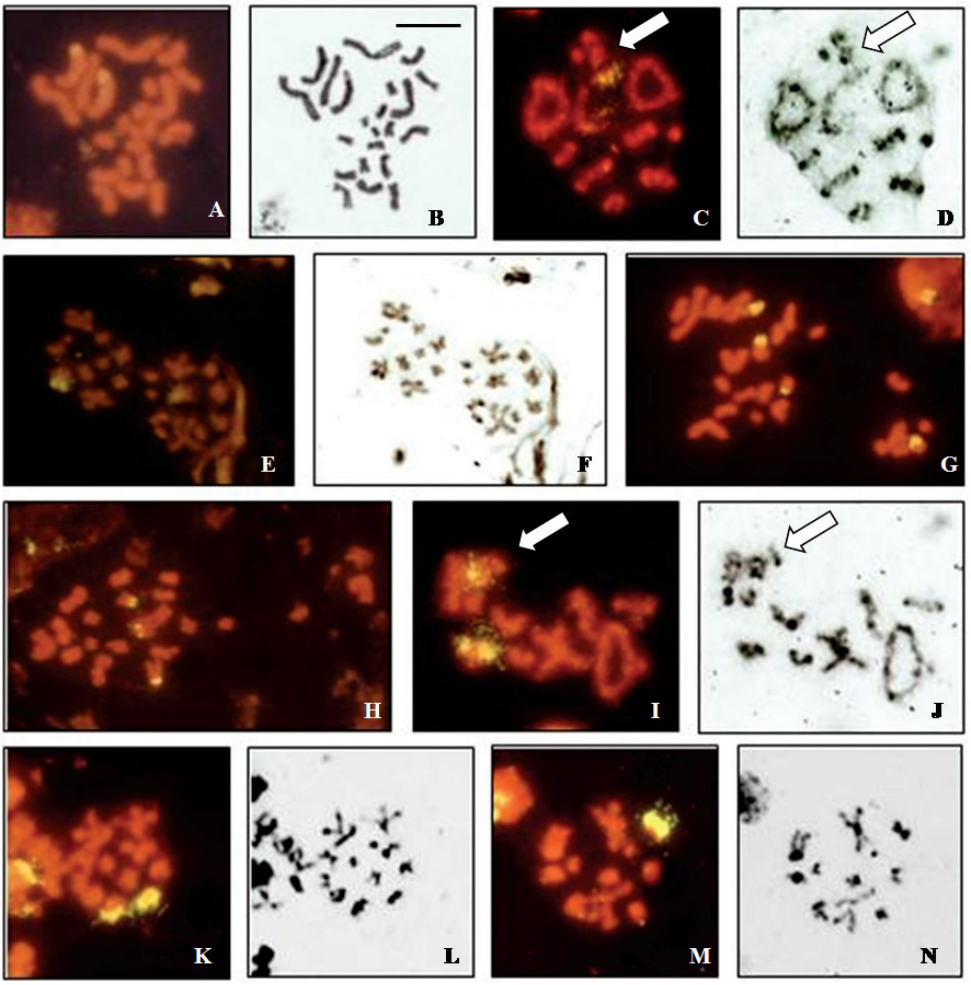

Figure 2a–n. Localization of rDNA clusters in *Cicindela*, revealed by *in situ* hybridization of the PCR amplified ribosomal probe to squashed mitotic and meiotic chromosomes. The phase contrast image of some cells is figured to the right of the fluorescence image. **a, b** *Cicindela hispanica*, female mitotic metaphase **c, d** *C. hispanica*, male metaphase I plate; n=9+X_1X_2Y, **e, f** *C. hispanica*, male metaphase II plates, n = 9+Y and n = 9+X_1X_2, **g** *C. maura*, female mitotic metaphase **h** *C. maura*, male mitotic metaphase **i, j** *C. maura*, male metaphase I plate; n=9+$X_1X_2X_3Y$ **k, l** *C. maura*, male metaphase II plate (n=9+ $X_1X_2X_3$) **m, n** *C. maura*, metaphase II plate (n=9+Y). Arrows indicate the sex chromatin. Bar = 5μm.

Hudson (1999), Proença and Galián (2003) and Galián et al. (2007). The new patterns suggest that the number and distribution of particular housekeeping genes such as the ribosomal cistrons, have undergone many changes during the radiation of the tribe Cicindelini. This dynamic pattern contrast with the relative numerical stability of the number of autosomes found in Palearctic members of the tribe (most species have nine pairs, except for *Cylindera paludosa*). Likewise, changes in rDNA loci are probably not coupled with changes in the number of heterosomes, as shown by the two patterns

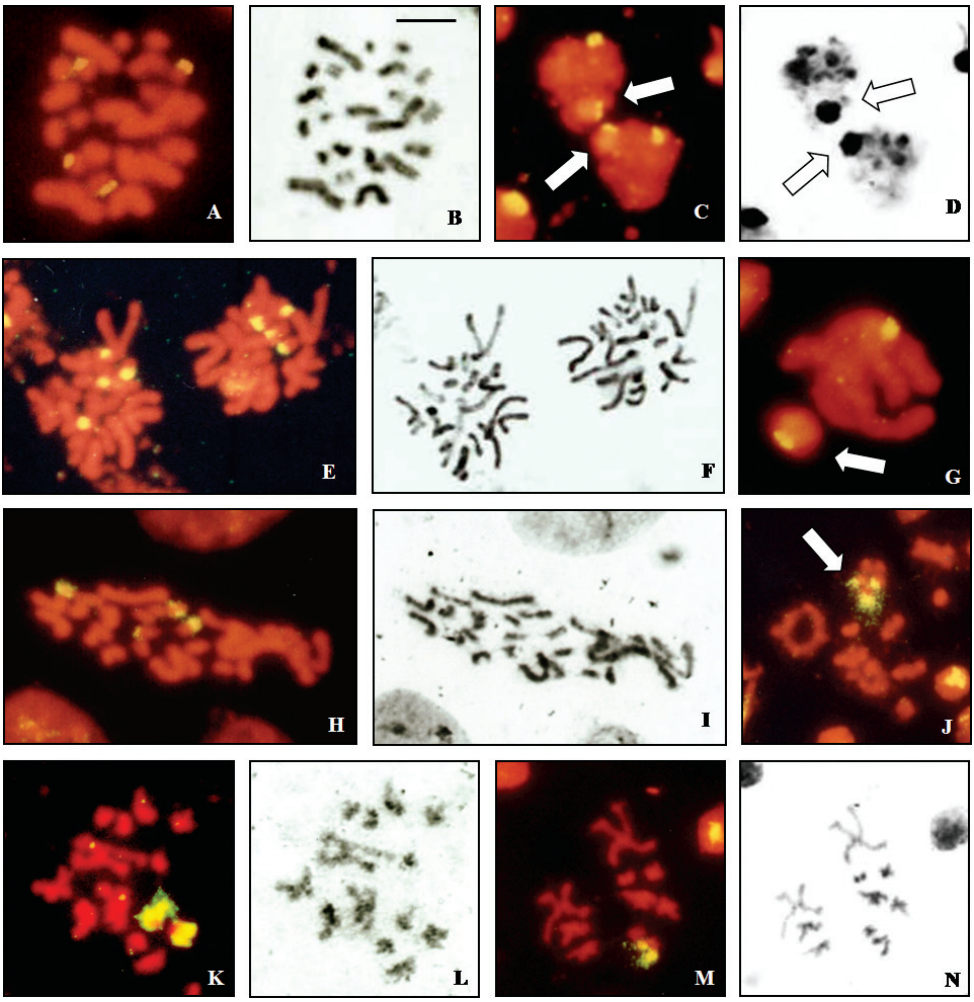

Figure 3a–n. Localization of rDNA clusters in *Cicindela*, revealed by *in situ* hybridization of the PCR amplified ribosomal probe to squashed mitotic and meiotic chromosomes. The phase contrast image of some cells is figured to the right of the fluorescence image. **a, b** *C. deserticoloides*, male mitotic metaphase **c, d** *C. deserticoloides*, zygotene nuclei; n=9+$X_1X_2X_3Y$, **e, f** *C. circumdata*, male mitotic metaphase **g** *C. circumdata*, zygotene nuclei; n=9+$X_1X_2X_3Y$ **h, i** *C. trisignata*, female mitotic metaphase **j** *C. trisignata*, metaphase I plate, n=9+$X_1X_2X_3X_4Y$ **k, l** *C. trisignata*, male metaphase II plate (n=9+ $X_1X_2X_3X_4$) **m, n** *C. trisignata*, male metaphase II plate (n=9+Y). Arrows indicate the sex chromatin. Bar = 5 μm.

found in the populations of *Cicindela littoralis* (Proença and Galián 2003). The frequent movements of ribosomal genes from the autosomes to the multiple heterosomes (or vice-versa) during the evolution of Nearctic and Palearctic species of Cicindelini (Galian et al. 2007, this paper) is described for the first time in insects and is worth analysing in other Coleoptera and insects of other orders to test whether it is a more widespread pattern. It has been suggested that these rearrangements could be one of the causes of the great species diversity in this tribe (Galián et al. 2007). A possible

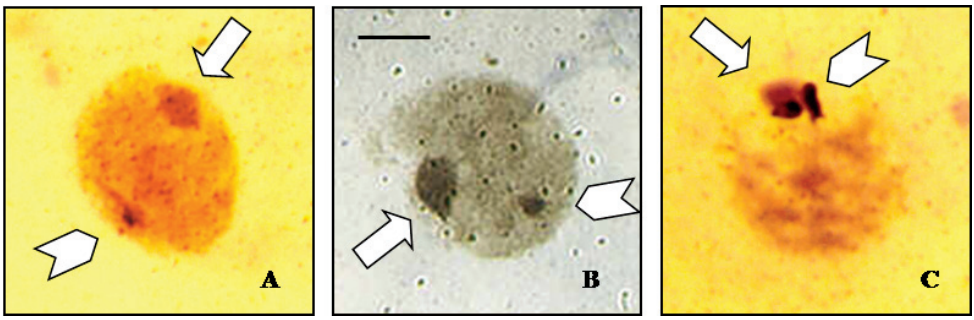

Figure 4a–c. Silver staining of meiotic nuclei, showing nucleolar activity on the autosomes of **a** *C. hispanica*, and **b** *C. circumdata*, and on the sex chromosomes of **c** *C. trisignata*. Arrows indicate the sex chromatin and arrowheads point the nucleolar active sites. Bar = 5 μm.

origin of these changes is the occurrence of transposable elements that jump from one part of the genome to another within and between chromosomes (leaving or not copies on the original site) with special preference for the rDNA sites, as reported by Granger et al. (2004) for *Caenorhabditis elegans* through insertional mutagenesis experiments. Mariner-*like* transposable elements were successfully amplified in *C. maura* (Proença and Galian preliminary results).

Species of the more primitive lineages such as *Amblycheila*, *Megacephala* and *Manticho*ra have from two to four autosomal pairs carrying ribosomal genes (Galián and Hudson 1999; Galián et al. 2002; Proença et al. 2005). This fact suggests that within the Palearctic species of the tribe Cicindelini rDNA genes have jumped from the autosomes to the heterosomes, either leaving copies in the autosomes, as in *Cicindela litorallis* (Proença and Galián, 2003), *Cephalota maura*, *C. deserticoloides*, and *C. circumdata* (this paper), or not as in *Myriochila melancholica* (Galián et al. 1995). The same hypothesis was also put forward for the Nearctic species of *Cicindela* subgenus *Cicindelidia* (Galian et al. 2007), as *C. flohri, C. nebuligera, C. obsoleta, C. rufiventris* and *C. sedecimpunctata* have the rDNA loci on one autosomal pair whereas *C. aterrima, C. nigrocoerulea, C. ocellata, C. roseiventris* and *C. rugatilis* retain a copy on one autosomal pair and bear additional copies on one (X) or two (XY) heterosomes. More data on different tiger beetle lineages would test this hypothetical scenario. Whatever the nature of the mechanisms causing the marked variation of rDNA localization among species of Cicindelini, it is clear that these are also operating within some species, as inferred from the polymorphism found in *Lophyra flexuosa* and *Cicindela littoralis* (Proença and Galián 2003).

The results in *Cylindera trisignata*, ($X_1X_2X_3X_4Y$) give some clues about the origin of the fourth X. This species has three heterosomes with ribosomal genes. This fact may support the hypothesis of a mechanism of X dissociation rather than the incorporation of autosomal segments into the multiple sex chromosomes system as the origin of the 4X condition. This last hypothesis was proposed by Collares-Pereira and Serrano (1990), Guénin (1952) and Dasgupta (1967) to explain the increase in the number of heterosomes. The fact that the heterosomes with the fluorescent signals are the Y, the

X_1 and likely the very small X_4, provides experimental support to the X-dissociation hypothesis.

Acknowledgements

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Cytogenetic characterization of three Balistoidea fish species from the Atlantic with inferences on chromosomal evolution in the families Monacanthidae and Balistidae

Lorena Corina Bezerra de Lima, Pablo Ariel Martinez, Wagner Franco Molina

Department of Cell Biology and Genetics, Centro de Biociências, Universidade Federal do io Grande do Norte, Campus Universitário, 59078 – 970 Natal, RN, Brazil

Corresponding author: *Wagner Franco Molina* (molinawf@pq.cnpq.br)

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Abstract

The Tetraodontiformes are the most derived group of teleostean fish. Among other apomorphies, they are characterized by a high degree of fusions or significant bone loss in the head and body. In the early phylogenetic proposals presented for this order, the families Balistidae and Monacanthidae have been unanimously considered to be closely related. Although they have moderate species diversity, they are scarcely known in cytogenetic aspect and chromosomal pattern comparisons between these groups have yet to be established. The species *Cantherhines macrocerus* (Hollard,1853), *C. pullus* (Ranzani, 1842) (Monacanthidae) and *Melichthys niger* (Bloch, 1786) (Balistidae) were cytogenetically analyzed using conventional (Ag-impregnation, C-banding, CMA_3- and DAPI-fluorescence) and molecular (FISH with an *18S rDNA* probe) cytogenetic protocols. The karyotypes of all three species were very similar possessing diploid chromosome numbers 2n = 40 and composed exclusively of acrocentric chromosomes. Single NOR-bearing pair as well as positive heterochromatic blocks at pericentromeric regions were identified in the karyotypes of the three species studied. NOR-bearing sites were positively labeled after Ag-impregnation, C-banding, CMA_3-fluorescence and FISH with an *18S rDNA* probe but were negative after DAPI-fluorescence. Such remarkable shared conspicuous chromosomal characters corroborate either close phylogenetic relationship of these families, previously established by morphological and molecular data, or rather conservative nature of karyotype differentiation processes. The later hypothesis, however, appears less probable due to centric or *in tandem* fusions documented for another Balistoidea species.

Keywords

Balistoidea, fish cytogenetics, karyotype evolution, Tetraodontiformes

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Introduction

The order Tetraodontiformes, which stands out among marine fish for its marked diversity, is composed of approximately 430 species distributed in nine families (Nelson, 2006). This group is the most recent branch of Neoteleostean radiation, representing a post-Perciformes lineage (Elmerot et al., 2002) and although it is generally recognized as a monophyletic group, the relationships between its families and genera have yet to be defined (Holcroft, 2004).

Among the Tetraodontiformes, the superfamily Balistoidea (leatherjackets) includes the families Balistidae (triggerfish) and Monacanthidae (filefish), with a fossil record that dates back to the Early Eocene and probably to the Late Cretaceous (Frickhinger, 1995; Santini, Tyler, 2003). Based on morphological similarities (e.g., osteological and myological characters), molecular studies using RAG1 gene sequences and DNA content data (Winterbottom, 1974; Brainerd et al., 2001; Holcroft, 2004, 2005), and more recently analysis of complete mitochondrial genomes (mitogenome) (Yamanoue et al., 2008), members of families Balistidae and Monacanthidae are considered monophyletic sister groups.

To date nearly 60 species of Tetraodontiformes have been cytogenetically studied (Sá-Gabriel, Molina, 2005). Cytogenetic analyses have been carried out in 15 Balistidae species, most from the Pacific region, and only few from the Western Atlantic. A total of ten Monacanthidae species are karyotyped, being one from the Brazilian coast.

In this work, we revise cytogenetic data for *Melichthys niger* (Bloch, 1786) (Balistidae) and describe the karyotype and other chromosomal characteristics for *Cantherhines macrocerus* (Hollard, 1853) and *C. pullus* (Ranzani, 1842) (Monacanthidae), to compare the chromosomal patterns of the families Balistidae and Monacanthidae, estimating their divergence level.

Material and methods

We analyzed 42 specimens of *Melichthys niger* and 14 of *Cantherhines macrocerus*, collected in the Saint Peter and Saint Paul's Archipelago (00º55'15" N, 029º20'60" W), 1010 km from the Brazilian northeastern coast and about 1.824 km) from the African coast, and two specimens of *Cantherhines pullus* collected in the coastal region of Salvador (12º 58'S, 38º 31'W), Bahia state, northeastern Brazil (Fig. 1).

The specimens were subjected to mitotic stimulation as proposed by Molina (2001) for a period of 24-48 hours. Mitotic chromosomes were obtained following the protocol developed by Gold et al. (1990). Cell suspensions obtained from fragments of the anterior portion of specimens' kidney were spread onto slides with a film of water heated at 60ºC. The active nucleolus organizer regions (NOR) were identified by silver nitrate staining as described by Howell and Black (1980), while the heterochromatic regions were visualized by C-banding (Sumner, 1972). The FISH technique (according to Pinkel et al., 1986) was performed using an *18S rDNA* probe from *Prochilodus*

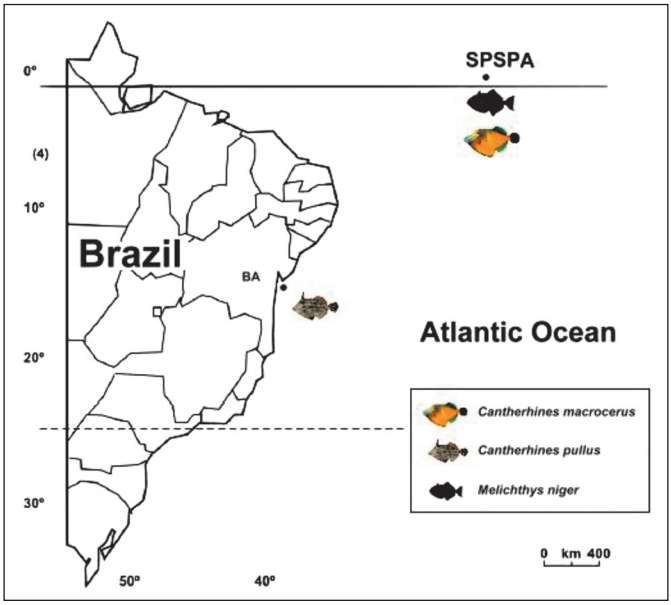

Figure 1. Map showing the geographic collection points of the species *Melichthys niger*, *Cantherhines macrocerus* and *C. pullus*. SPSPA – Saint Peter and Saint Paul's Archipelago; BA – Bahia state.

argenteus, Agassiz, 1829 (Hatanaka, Galetti, 2004), labeled with biotin-14-dATP by nick translation according to the manufacturer's instructions (BioNick Labeling System; Invitrogen, Carlsbad, CA, U.S.A.). The hybridization signal was detected by the streptavidin-fluorescein isothiocyanate conjugate. Sequential staining with AT- specific 4'-6-diamidino-2-phenylindole (DAPI) and GC-specific Chromomycin A_3 (CMA_3) fluorochromes was performed as described by Schweizer (1980). The metaphases were photographed by a DP70 digital image capture system coupled to an Olympus BX50 epifluorescence microscope. About thirty metaphases of each specimen were analyzed to determine the modal number of mitotic chromosomes and the karyotype. The chromosomes were classified according to centromere position and organized into decreasing size order, as proposed by Levan et al. (1964).

Results

The specimens of *Melichthys niger* showed 2n=40 chromosomes and a karyotype consisting of 20 pairs of acrocentric (a) chromosomes (NF=40) (Fig. 2a). The presence of a conspicuous secondary constriction was observed in the interstitial position on the long arm of the chromosome pair No. 2, corresponding to the nucleolus organizer regions (NORs), identified by Ag-NOR sites and by *in situ* hybridization with an *18S rDNA* ribosomal probes (Fig. 2, upper boxes). The heterochromatic blocks were reduced in size and dispersed in the pericentromeric regions in most of the chromo-

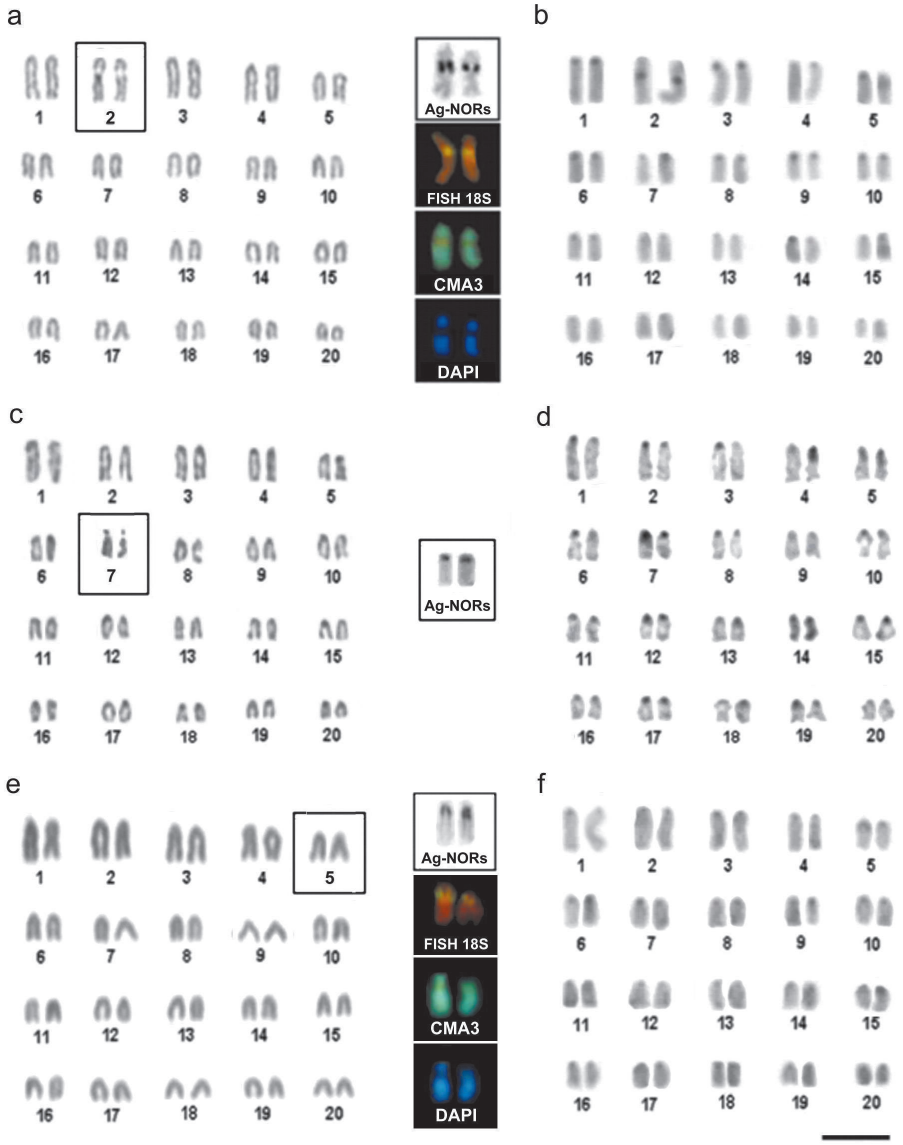

Figure 2. Karyotypes of *Melichthys niger* (**a, b**), *Cantherhines macrocerus* (**c, d**) and *Cantherhines pullus* (**e, f**), arranged from Giemsa stained (**a, c, e**) and C-banded chromosomes (**b, d, f**). In the center highlighted are the NOR-bearing pairs of analyzed species (2nd, 7th and 5th, respectively) after Ag-NOR staining, *in situ* hybridization with an *18S rDNA* probe, CMA_3 and DAPI fluorescence. Bar = 5μm.

some pairs (Fig. 2b). The NORs were heterochromatic and CMA_3 positive and DAPI negative (Fig. 2, upper boxes).

The specimens of *Cantherhines macrocerus* (Monacanthidae) had 2n=40 chromosomes and karyotype composed of a acrocentric chromosomes (Fig. 2c). Ag-NOR

sites were located in the pair No. 7 in interstitial region, near the centromere (Figure 2, middle box). C-banding revealed heterochromatic blocks distributed in the pericentromeric region in most of the chromosome pairs (Figure 2d) and more intensively stained on the secondary constriction of the NOR-bearing pair. In this species, experiments using FISH probes and fluorochrome staining were unsuccessful.

The specimens of *C. pullus* showed 2n=40 chromosomes and karyotype composed entirely of acrocentric chromosomes (Fig. 2e). The NORs were identified at the pericentromeric position of pair No. 5, as revealed by Ag-NOR-staining and FISH with an *18S rDNA* probes (Fig. 2, lower box). The heterochromatic regions were distributed in centrometric and pericentromeric positions in most of the chromosomes. The NORs sites were heterochromatic (Fig. 2f), and CMA positive and DAPI negative (Fig. 2, lower box). None of the karyotypes displayed sex-related chromosome heteromorphism.

Discussion

Six out of the ten karyotyped species in the family Monacanthidae have diploid numbers ranging from 2n = 33/34 to 36 chromosomes. Such low 2n numbers have been a noticeable characteristic for Monacanthidae species. The present data for *C. macrocerus* and *C. pullus* increase the range of the highest 2n for representatives of this family. Surveys involving a larger number of genera may confirm a possible basal karyotype with 40 chromosomes for this family, showing on average lower diploid values than those of the Balistidae. Based on chromosomal number, *C. macrocerus* and *C. pullus* would be placed in the family Balistidae.

The karyotype of the individuals of *C. macrocerus* from the Saint Peter and Saint Paul's Archipelago was similar to those described for specimens from the coast of Rio de Janeiro (Pauls, 1993), even though these populations were 2000 km apart. It is not clear whether the common karyotype found in *C. macrocerus* populations is maintained due to gene flow by the transport of their pelagic larvae by ocean currents, as has been commonly identified for a number of reef species (Rocha, 2003; Feitoza et al., 2005), or if they were isolated too recently to accumulate observable chromosomal differences.

In this study, the data obtained for *Melichthys niger* corroborate results described for the species earlier (Sá-Gabriel, Molina, 2005). However, the use of a larger number of individuals of this species, observation of lower chromatid condensation and the use of complementary cytogenetic protocols, such as CMA_3/DAPI fluorochromes and FISH with an *18S rDNA* probes, allow more precise localization of the NOR-bearing pair to the 2nd largest pair of the karyotype.

The chromosomal characteristics observed in *C. macrocerus, C. pullus* and *M. niger*, like the presence of single NOR and the pericentromeric heterochromatin blocks as reported in other Tetraodontiformes (Grützner et al., 1999; Mandrioli, 2000; Fischer et al., 2000), Perciformes (Caputo et al., 2001; Molina, 2007), Mugiliformes (Nirchio et al., 2009), Beryciformes (Bacurau, Molina, 2004), corroborate the hypothesis that

these are ancestral characteristics for each of these clades and therefore not exclusive to the families Monacanthidae and Balistidae.

In the karyotypes of species under our study, as well as in other representatives of Balistidae and Monacanthidae (Sá-Gabriel, Molina, 2004), extra-pericentromeric heterochromatic regions were present only when adjacent to or associated with the major ribosomal sites. Given that the constitutive heterochromatin blocks in fish chromosomes are often associated with karyotype diversification (Mantovani et al., 2000; Molina, Bacurau, 2006), it is possible that these regions present preferentially in pericentromeric position in chromosomes of Balistoidea species and they are involved in the karyotypic differentiation of this group. The presence of heterochromatin associated with NORs in adjacent or interspersed regions (Pendás et al., 1993), as shown in the NOR-bearing pair in *M. niger*, may contribute to the occurrence of structural rearrangements involving NOR-bearing pairs (Vicari et al., 2003). Indeed, chromosome fusions have been commonly identified as the main mechanism of the karyotype diversification within this clade (Kitayama, Ojima, 1984). Although a physical mapping of telomeric sequences is not yet available, the lower 2n in both Balistidae and Monacanthidae corroborate that centric and/or *in tandem* fusion events, followed by pericentric inversions, seem to have been important mechanisms in the karyotype differentiation of these post-Perciformes group.

Repetitive sequences and transposition elements are closely related to the heterochromatic regions, and although account for less than 10% of the genome of the Tetraodontiformes studied (Brenner et al., 1993), they may be effective promoters of chromosomal breaks, deletions, inversions and amplifications (Lim, Simmons, 1994; Fischer et al., 2000). Knowledge regarding aspects of the Balistoidea genome is still limited, although it is known that, like Tetraodontidae, this clade is composed of species with compact genomes that evolved independently (Brainerd et al., 2001). The Tetraodontidae genome has been extensively studied (Crolius et al., 2000), revealing that the small amount of repetitive sequences is located in the centromeres and arms of a chromosomes. This physical disposition seems to be present in the chromosomes of Balistoidea and could help to explain why centromere and telomere regions would be prone to ocurrence of *in tandem* or centric fusions rearrangements. In addition to the aforementioned data, a large number of acrocentric chromosomes and FN near or slightly higher than the diploid chromosome values were observed. These characteristics are not common in other families of the same order, such as the Tetraodontidae (Sá-Gabriel, Molina, 2005), where pericentric inversions seem to have occurred more frequently.

Among the Perciformes, the presence of single interstitial NORs is common, representing an ancestral condition (Affonso et al., 2001). Similarly, the presence of heterochromatin restricted to the centromeric regions is also a typical cytogenetic trait of this group (Molina, Galetti, 2002). The location and frequency of NORs sites and heterochromatic regions in *C. macrocerus*, *C. pullus* and *M. niger* are consistent with the general pattern found in Perciformes species, demonstrating the apparent conservativeness of these characters.

The set of cytogenetic characters already available in Monacanthidae and Balistidae species indicate a greater karyotypic similarities and common tendencies of karyotype evolution with other groups of the order Tetraodontiformes, corroborating previous analyses based on morphological and molecular data (Winterbottom, 1974; Brainerd et al., 2001; Holcroft, 2005; Yamanoue et al., 2008).

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