**Original Article**

Cytogenetic and Genome Research

Cytogenet Genome Res 2009;125:292–305
DOI: 10.1159/000235936

Accepted after revision: June 30, 2009
by M. Schmid

# Sex Chromosome Evolution in Cotton Stainers of the Genus *Dysdercus* (Heteroptera: Pyrrhocoridae)

**© Free Author Copy – for personal use only**

ANY DISTRIBUTION OF THIS ARTICLE WITHOUT WRITTEN CONSENT FROM S. KARGER AG, BASEL IS A VIOLATION OF THE COPYRIGHT.

Written permission to distribute the PDF will be granted against payment of a permission fee, which is based on the number of accesses required. Please contact permission@karger.ch

M.J. Bressa[a] A.G. Papeschi[a] M. Vítková[b, c] S. Kubíčková[d] I. Fuková[c, e] M.I. Pigozzi[f] F. Marec[b, c]

[a]Laboratory of Cytogenetics and Evolution, Faculty of Exact and Natural Sciences, University of Buenos Aires, Buenos Aires, Argentina; [b]Faculty of Science, University of South Bohemia, and [c]Institute of Entomology, Biology Centre ASCR, České Budějovice, [d]Veterinary Research Institute, Brno, Czech Republic; [e]Centre for Ecology and Conservation, University of Exeter, Cornwall Campus, Penryn, UK; [f]Institute for Research in Reproduction, Faculty of Medicine, University of Buenos Aires, Buenos Aires, Argentina

**Key Words**

Chromosome painting · Evolution · GISH · NOR · Ribosomal DNA · Sex chromosomes · Synaptonemal complex · Zoo-FISH

**Abstract**

The neo-X and neo-Y sex chromosomes of *Dysdercus albofasciatus* represent a unique model for the study of early stages of sex chromosome evolution since they retained the ability to pair and recombine, in contrast to sex chromosomes in most Heteroptera. Here we examined structure, molecular differentiation, and meiotic behaviour of the *D. albofasciatus* neo-sex chromosomes. Two related species with the ancestral X0 system, *D. chaquensis* and *D. ruficollis*, were used for a comparison. In *D. albofasciatus*, 2 nucleolar organizer regions (NORs) were identified on the neo-X chromosome using fluorescence in situ hybridization (FISH) with an rDNA probe, whereas a single NOR was found on an autosomal pair in the other 2 species. Genomic in situ hybridization (GISH) differentiated a part of the original X in the neo-X chromosome but not the neo-Y chromosome. The same segment of the neo-X chromosome was identified by Zoo-FISH with a chromosome painting probe derived from the X chromosome of *D. ruficollis,* indicating that this part is conserved between the species. Immunostaining against the cohesin subunit SMC3 revealed that only terminal regions of the *D. albofasciatus* neo-Xneo-Y bivalent pair and form a synaptonemal complex, which is in keeping with the occurrence of terminal chiasmata, whereas the interstitial region forms a large loop indicating the absence of homology. These results support the hypothesis that the neo-X chromosome evolved by insertion of the original X chromosome into 1 NOR-bearing autosome in an ancestor carrying the X0 system. As a consequence, the homologue of this NOR-autosome became the neo-Y chromosome. A subsequent inversion followed by transposition of the NOR located on the neo-Y onto the neo-X chromosome resulted in the present neo-sex chromosome system in *D. albofasciatus*.

Copyright © 2009 S. Karger AG, Basel

It is generally accepted that differentiated sex chromosomes evolved from a pair of autosomes after 1 member of the pair acquired a sex-determining function. An important step in the evolution of sex chromosomes is thought to be the restriction of recombination. This key event is a necessary prerequisite for their molecular and morphological differentiation resulting in the progressive degeneration of the chromosome that is present only

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2009 S. Karger AG, Basel
1424–8581/09/1254–0292$26.00/0

Accessible online at:
www.karger.com/cgr

María José Bressa, Laboratorio de Citogenética y Evolución
Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Güiraldes y Costanera Norte
Ciudad Universitaria C1428EHA, Ciudad Autónoma de Buenos Aires (Argentina)
Tel. +54 11 4576 3300, Fax +54 11 4576 3384, E-Mail mjbressa@ege.fcen.uba.ar

in the heterogametic sex, i.e. the Y in systems with male heterogamety or the W in systems with female heterogamety [Traut, 1999; Ayling and Griffin, 2002; Charlesworth et al., 2005; Steinemann and Steinemann, 2005; Bachtrog, 2006]. This hypothesis is based on observations in different groups of organisms, with species presenting the so-called 'primitive forms' of sex-chromosome differentiation in which the X and Y (or the W and Z) are hardly distinguished from each other, and species carrying highly evolved systems with remarkably heteromorphic sex chromosomes [Traut et al., 1999; Vyskot and Hobza, 2004; Steinemann and Steinemann, 2005].

In insects, the majority of data on the evolution of sex chromosomes were obtained in flies (Diptera) and moths (Lepidoptera) [e.g., Steinemann and Steinemann, 1998; Traut, 1999; Traut et al., 2007; Flores et al., 2008]. In the 2 groups, meiosis is achiasmatic in the heterogametic sex as documented in *Drosophila melanogaster* and several moth species [reviewed by Marec, 1996]. Consequently, the Y or W chromosomes, even those of recent origin, are non-recombining from the beginning [Traut, 1999]. Insects of the order Heteroptera (true bugs) represent another interesting model group to study sex chromosome evolution. In heteropterans, meiosis is generally chiasmatic in both sexes but the sex chromosomes are asynaptic and achiasmatic in males. Most sex-chromosome systems described so far in Heteroptera are either simple systems, XY/XX (71.4%) and X0/XX (14.7%), or multiple systems ($X_nY/X_nX_n$, $X_n0/X_nX_n$, and $XY_n/XX$; 13.5% in total) that probably originated through fragmentation of the ancestral X or Y chromosomes of simple systems, respectively [Papeschi and Bressa, 2006]. From about 1,600 species cytogenetically analyzed, only 7 species and subspecies (0.4%) have neo-sex chromosomes of complex origin. These are (male karyotypes given): *Lethocerus indicum* Lep. et Servielle with 2n = 24 + neo-Xneo-Y and *Lethocerus* sp. Mayr with 2n = 2 + neo-Xneo-Y (Belostomatidae), *Rhytidolomia senilis* (Say) with 2n = 4 + neo-Xneo-Y (Pentatomidae), *Dundocoris nodulicarinus novenus* Jacobs with 2n = 6 + neo-$XY_1Y_2$ and *D. n. septeni* Jacobs with 2n = 4 + neo-$XY_1$neo-$Y_2$ (Aradidae), *Hebrus pusillus* Fallén 2n = 22 + neo-XneoY (Hebridae), and *Dysdercus albofasciatus* Berg 2n = 10 + neo-Xneo-Y (Pyrrhocoridae) [Chickering, 1927; Chickering and Bacorn, 1933; Schrader, 1940; Jande, 1959; Bressa et al., 1999; Nokkala and Nokkala, 1999; Jacobs, 2004]. In all of them, with the exception of *D. albofasciatus*, the ancestral sex chromosome system was XY/XX [reviewed in Papeschi and Bressa, 2006].

Heteroptera have holokinetic chromosomes, i.e. chromosomes without localized centromeres, which makes identification of individual pairs very difficult, mainly due to the absence of a primary constriction but also due to the lack of convenient banding techniques [Bedo, 1984; Gokhman and Kuznetsova, 2006]. The only chromosomes that can be constantly identified are those carrying nucleolus organizer regions (NORs) and the sex chromosomes in males [Ueshima, 1979; Manna, 1984; Papeschi and Bressa, 2006].

Heteropteran sex chromosomes are discernible from early stages of male meiosis I and up to diakinesis as positively heteropycnotic, highly condensed 'chromatin bodies' [Henking, 1891; Ueshima, 1979]. The number of heteropycnotic chromatin bodies depends on the sex chromosome system, and the maximum number of chromatin bodies depends on the number of sex chromosomes [Franco et al., 2006]. This positive heteropycnosis suggests that the sex chromosomes are heterochromatic; the constitutive or facultative nature of the X and Y heterochromatin largely depends on the family, the genus and also the species [Messthaler and Traut, 1975; Papeschi, 1988, 1991; Rebagliati et al., 2003]. After pachytene, the nucleus increases its size and acquires an interphase appearance, because bivalents decondense completely while the sex chromosomes remain condensed. In the first meiotic division, autosomal bivalents segregate reductionally, but the sex chromosomes behave as univalents. At anaphase I, the sex chromosomes divide equationally, and the resulting single chromatids, either in simple or multiple systems with X and Y chromosomes, associate in the second meiotic division by the so-called 'touch-and-go pairing' and segregate reductionally at anaphase II [Ueshima, 1979; Manna, 1984; Papeschi and Bressa, 2006].

Cotton stainers of the genus *Dysdercus* (Pyrrhocoridae), particularly the New World species, show a considerable variability in the sex chromosome constitution and also in diploid chromosome numbers. In 6 Neotropical species, 4 different karyotypes were found: 2n = 14 + X0 in *D. honestus* Bloete, 2n = 14 + $X_1X_2$0 in *D. peruvianus* Guérin Méneville, 2n = 12 + X0 in *D. chaquensis* Freiberg, *D. ruficollis* Linnaeus, and *D. imitator* Bloete, and 2n = 10 + neo-Xneo-Y in *D. albofasciatus* Berg [Piza, 1947a, 1947b, 1951; Mola and Papeschi, 1997; Bressa et al., 1999, 2003]. In *D. albofasciatus*, it was proposed that the neo-X chromosome evolved by insertion of the ancestral X chromosome into an autosome followed by a large inversion, which included a part of the ancestral X chromosome. Then the autosome homologue became a neo-Y chromosome. This hypothesis was supported by (i) reduced chromosome number by 1 pair, (ii) 2 heteropycnotic chromatin bodies in the diffuse stage, indicating

separation of the ancestral X chromosome into 2 segments, (iii) occurrence of 1 and less frequently 2 terminal chiasmata in the neo-sex chromosome bivalent, and (iv) reductional segregation of the neo-XneoY bivalent at anaphase I. Thus, the *D. albofasciatus* neo-sex chromosomes displayed meiotic behaviour similar to autosomes, except a few spermatocytes, in which the neo-X and neo-Y each formed a univalent that divided post-reductionally as typical for sex chromosomes in most Heteroptera [Bressa et al., 1999]. This particular system with chiasmatic neo-sex chromosomes in males raises a question whether molecular differentiation has occurred in the non-recombining segments of the neo-X and neo-Y.

With this in mind, we used several approaches to perform a detailed comparison of sex chromosomes in 3 species of the genus *Dysdercus*, namely *D. chaquensis* and *D. ruficollis* with the original X0 system, and *D. albofasciatus* with the derived neo-Xneo-Y system. Using fluorescence in situ hybridization (FISH) with the 18S rDNA probe we localized the nucleolus organizer region (NOR) and showed that the ancestral NOR-autosome pair played the key role in the evolution of neo-sex chromosomes in *D. albofasciatus*. We also examined the structure of neo-sex chromosomes by means of C-banding and fluorescent banding, genomic in situ hybridization (GISH), and comparative chromosome painting (the so-called Zoo-FISH) using an X-chromosome painting probe derived from laser-microdissected X-chromosomes of *D. ruficollis*. Finally, we studied synaptic behaviour of the neo-X and neo-Y chromosomes and formation of the neo-Xneo-Y bivalent in *D. albofasciatus* on spread preparations of synaptonemal complexes using immunostaining against the cohesin subunit SMC3 (structural maintenance of chromosomes 3 protein). Results obtained enabled us to propose a scenario of the sex chromosome evolution in the genus *Dysdercus*.

## Materials and Methods

*Insects*

We used adult males of *Dysdercus albofasciatus* from Pereyra Iraola Park, Buenos Aires province, *D. chaquensis* from Cayastá Park, Santa Fe province, and *D. ruficollis* from El Palmar National Park, Entre Ríos province (all in Argentina). All specimens were collected in 2006.

*Chromosome Preparations*

For FISH techniques, spread chromosome preparations were made from testes of adult males. Gonads were dissected in a physiological solution for *Ephestia* [Glaser, 1917 cited in Lockwood, 1961], swollen for 10 min in a hypotonic solution (0.075 M KCl), and then fixed for 15–30 min in freshly prepared Carnoy fixative (ethanol:chloroform:acetic acid, 6:3:1). Cells were dissociated in a drop of 60% acetic acid with the help of tungsten needles and spread on the slide using a heating plate at 45°C as described in Traut [1976]. Then the preparations were dehydrated in an ethanol series (70, 80, and 96%, 30 s each) and stored at –20°C until further use.

For preparations of *D. ruficollis* meiotic chromosomes for laser microdissection of the X chromosome, we modified a protocol described in Fuková et al. [2007] as follows. Male gonads were dissected out, hypotonized, and fixed as described above. Then they were transferred into a drop of 60% acetic acid on a glass slide coated with a polyethylene naphthalate membrane (P.A.L.M. GmbH, Bernried, Germany) and spread at 45°C using a heating plate. Shortly before spreading, the membrane was treated with ultraviolet light for 30 min to prevent DNA contamination. Preparations were passed through an ethanol series (see above), air-dried, and stained with 5% Giemsa for 5 min.

*C-Banding and Fluorescent Banding*

C-banding was performed according to Papeschi [1988]. Fluorescent banding with the GC-specific chromomycin $A_3$ ($CMA_3$; Fluka BioChemika, Sigma Aldrich Production GmbH, Buchs, Switzerland) and AT-specific 4′,6-diamidino-2-phenylindole (DAPI; Fluka BioChemika) was performed according to Rebagliati et al. [2003]. The slides pretreated for C-banding were stained with DAPI for a better resolution of C-bands. Total numbers of cells and individuals examined were the following: *D. albofasciatus* (C-banding, 32 cells/2 males; fluorescent banding, 28 cells/2 males), *D. chaquensis* (C-banding, 25 cells/2 males; fluorescent banding, 20 cells/2 males), and *D. ruficollis* (C-banding, 23 cells/2 males; fluorescent banding, 27 cells/2 males).

*FISH with 18S rDNA Probes*

Unlabeled 18S ribosomal DNA (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 *D. albofasciatus*, 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 1,000 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 (Qiagen GmbH, Hilden, Germany). The 18S rDNA fragment was re-amplified by PCR and then labeled with biotin-14-dATP by nick translation using a BioNick Labeling System (Invitrogen, Life Technologies Inc., San Diego, Calif., 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]. Briefly, denaturation of chromosomes was done at 72°C for 3 min 30 s in 70% deionized formamide in 2× SSC. The probe cocktail for 1 slide (10 μl; 50% deionized formamide, 10% dextran sulfate in 2× SSC) contained 30–50 ng of the labeled probe and 25 μg of sonicated salmon sperm DNA (Sigma-Aldrich, St. Louis, Mo., USA). Hybridization was carried out overnight. Hybridization signals were detected with Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs. Inc., West Grove, Pa., USA), followed by 1 round of amplification with biotinylated anti-streptavidin (Vector Laboratories, Burlingame, Calif., USA) and Cy-3-conjugated streptavi-

din. The preparations were counterstained with 0.5 μg/ml DAPI in PBS buffer containing 1% Triton X-100 and mounted either in antifade based on DABCO (Sigma Aldrich; for composition see Traut et al. [1999]) or in Vectashield Mounting Medium (Vector Laboratories). Total numbers of cells and individuals examined in rDNA-FISH experiments were as follows: *D. albofasciatus* (120 cells/8 males), *D. chaquensis* (22 cells/2 males), and *D. ruficollis* (58 cells/5 males).

*Genomic in situ Hybridization*

Genomic DNAs were isolated separately from female and male adults by standard phenol-chloroform extraction. Labeling was done using a Nick Translation Mix (Roche Diagnostics GmbH, Mannheim, Germany). Female DNAs were labeled with Cy3-dUTP (red) (GE Healthcare Life Sciences, Little Chalfont, UK), male DNAs with Alexa Fluor 488-dUTP (green) (Molecular Probes Inc., Eugene, Oreg., USA). Unlabeled genomic DNAs, used as a species-specific competitor, were sonicated using a Sonoplus HD 2070 (Bandelin Electric, Berlin, Germany), with 2 cycles of 5 pulses at 70% power, 10 s each.

GISH was carried out essentially following the procedure of Traut et al. [1999] for comparative genomic hybridization (CGH) with slight modifications described in Vítková et al. [2007]. Denaturation of chromosomes was done at 68°C for 3 min 30 s in 70% deionized formamide in 2× SSC. One probe cocktail for each slide (10 μl; 50% deionized formamide, 10% dextran sulfate in 2× SSC) contained 500 ng of labeled female genomic DNA of *D. chaquensis*, 5.85 μg of unlabeled sonicated female genomic DNA of *D. albofasciatus*, and 25 μg of sonicated salmon sperm DNA. The other probe was composed of 410 ng of labeled male genomic DNA of *D. ruficollis*, 4.8 μg of unlabeled sonicated female genomic DNA of *D. albofasciatus*, and 25 μg of sonicated salmon sperm DNA. Hybridization was carried out for 3 days at 37°C. Stringent wash, counterstaining, and mounting were done as described in Fuková et al. (2005). GISH experiments were done on chromosome preparations made from *D. albofasciatus* males. A total of 156 cells from 7 individuals were inspected.

*Laser Microdissection of X Chromosomes and Preparation of X-Chromosome Painting Probe*

For laser microdissection of the *D. ruficollis* X chromosome, we used only well-spread mitotic spermatogonial metaphases and meiotic metaphase I spermatocytes. X chromosomes were identified in an inverted microscope according to their size (X is the smallest element in metaphase I) and microdissected with the help of a P.A.L.M. MicroLaser System (P.A.L.M. GmbH) as described in Kubickova et al. [2002].

DNA of microdissected samples, each sample containing 10 X chromosomes, was used as a template for DOP-PCR amplification in a T-personal thermocycler (Biometra, Göttingen, Germany) with degenerate primers (5′-CCGACTCGAGNNNNNNATGTGG-3′; VBC-Genomics GmbH, Vienna, Austria). The original DOP-PCR product was labeled with biotin-14-dATP (Invitrogen) by another round of DOP-PCR and used as a probe for FISH. DOP-PCR conditions were the same as described in Fuková et al. [2007].

*Zoo-FISH with the X-Chromosome Painting Probe*

In Zoo-FISH experiments, the biotinylated X-chromosome painting probe derived from microdissected X chromosomes of *D. ruficollis* was first hybridized to chromosome preparations of the same species to test specificity of the probe and only then cross-hybridized to chromosome preparations of *D. albofasciatus* males. The procedure was the same as for FISH with the rDNA probe (see above). For 1 slide, the probe cocktail contained about 30 ng of the biotinylated X-chromosome painting probe, 300 ng of unlabeled sonicated female genomic DNA from the same species as the chromosomes, and 25 μg of sonicated salmon sperm DNA. In Zoo-FISH on *D. albofasciatus* chromosomes, a total of 61 cells from 2 individuals were inspected.

*Immunostaining and Synaptonemal Complex Spreads*

Testes were dissected and minced gently in a 190-mM sucrose solution at pH 8.0. The cell suspension was dropped onto clean slides previously covered with a thin layer of 1% formaldehyde fixative, freshly made from paraformaldehyde, containing 0.05% Triton X-100. After fixation for 10 min in a humid chamber, the spreads were dried on a warm surface at 37°C and then briefly rinsed in 0.4% Kodak Photo-Flo. Immunostaining was performed as previously described [Pigozzi and Solari, 2003] using a polyclonal antibody against the cohesin component SMC3 (Millipore, Chemicon, Temecula, Calif., USA) to label meiotic axes of chromosomes (i.e., lateral elements of synaptonemal complexes), followed by detection with a goat-anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC) (Jackson ImmunoRes. Labs. Inc.). Slides were mounted in Vectashield containing DAPI as a counterstain. A total of 100 cells from 5 males of *D. albofasciatus* were analyzed.

*Microscopy and Image Processing*

The preparations were observed in epifluorescence microscopes Zeiss Axioplan 2 (Carl Zeiss Jena, Germany) equipped with an F-View CCD camera and AnalySIS software, version 3.2 (Soft Imaging System GmbH, Münster, Germany) and Leica DMLB 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, red for Cy3, and green for Alexa Fluor 488 and FITC) and processed with Adobe Photoshop, version 7.0.

## Results

Meiotic karyotypes based on metaphase I bivalents of the 3 species under study are shown in figure 1. In *Dysdercus albofasciatus* (2n = 10 + neo-Xneo-Y), the sex bivalent is heteromorphic and the largest of the complement, and 1 autosomal bivalent is noticeably larger than the other 4 (fig. 1a). Karyotypes of *D. chaquensis* and *D. ruficollis* males resemble each other with 6 autosomal bivalents of similar size and a single X chromosome (2n = 12 + X0). In *D. chaquensis*, the X is larger than the 5th and 6th bivalent, while in *D. ruficollis* it is the smallest element of the metaphase I complement (fig. 1b, c).

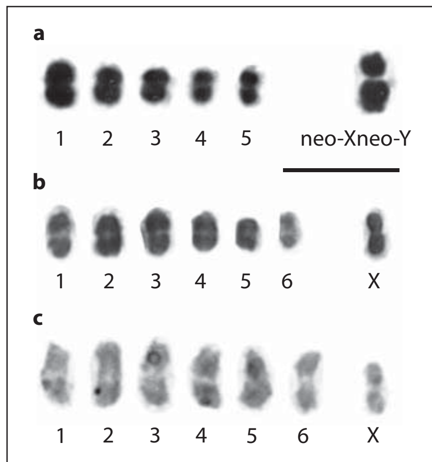



**Fig. 1.** Meiotic karyotypes of *Dysdercus albofasciatus* (**a**), *D. chaquensis* (**b**), and *D. ruficollis* (**c**). Chromosomes are stained with acetic haematoxylin. Bar = 10 μm.

*Localization of rDNA*

In *D. albofasciatus*, FISH experiments with the 18S rDNA probe showed 2 clusters of rDNA genes, both located on the neo-X chromosome (fig. 2a–g). One hybridization signal was regularly observed near 1 end and the other in a medial position of this chromosome, whereas no hybridization signal was found in the medium-sized neo-Y chromosome in spermatogonial metaphases (fig. 2a). In the diffuse stage, the neo-X chromosome showed 2 compact DAPI-bright bodies of different size (the so-called chromatin bodies), positively heteropycnotic and probably corresponding to 2 segments of the ancestral X chromosome. The probe identified 2 rDNA clusters always associated with the larger chromatin body (fig. 2b). From early diplotene onwards, 5 autosomal bivalents and the neo-Xneo-Y bivalent were distinguished. The neo-Xneo-Y bivalent displayed 2 DAPI-bright chromatin bodies, the larger one near the end of the bivalent and the smaller one in a middle position. Two clusters of hybridization signals were associated with the larger body (fig. 2c). At diakinesis-metaphase I, hybridization signals were observed in both chromatids of the neo-X. In some cells, the neo-Y chromosome seemed to be detached from the neo-X (fig. 2d), and in a few cells the neo-X and the neo-Y were present as univalents. It was shown earlier that during anaphase I the neo-Xneo-Y bivalent regularly segregates like autosomes, i.e. reductionally with the neo-X to 1 pole and the neo-Y to the other pole of the spindle, with only a few exceptions [Bressa et al., 1999]. In accordance with the previous findings, the majority of metaphase II complements in this study displayed either the neo-X chromosome with rDNA signals in both chromatids (fig. 2f) or the neo-Y chromosome without hybridization signals (not shown). Nevertheless, we also observed a few metaphase II plates, in which a neo-X chromatid labeled with the rDNA probe was associated with a neo-Y chromatid forming a pseudobivalent (fig. 2g). This configuration obviously resulted from equational division of the neo-X and neo-Y univalents at anaphase I. Thus, the present results with the rDNA probe as a marker of the neo-X chromosome confirmed previous findings [Bressa et al., 1999].

In rDNA-FISH preparations from testes of *D. chaquensis*, we failed to find mitotic metaphases. Nevertheless, hybridization signals in various meiotic cells suggested that this species has a single cluster of rRNA genes located at 1 terminal region of an autosomal pair (fig. 2h–k). At the diffuse stage, the X chromosome remained condensed forming a conspicuous DAPI-bright chromatin body without hybridization signals. We regularly observed a dispersed cluster of weak hybridization signals embedded in the decondensed mass of autosomal chromatin (fig. 2h). At diakinesis, strong hybridization signals were observed at both ends of 1 autosomal bivalent (fig. 2i). At metaphase I, the autosomal bivalents were often arranged in a ring configuration, while the X-chromosome univalent lay outside the group. One autosomal bivalent showed strong hybridization signals at 1 terminal region of each homologue (fig. 2j). In this species, as it is characteristic for Heteroptera, autosomes divided pre-reductionally, but the X chromosome segregated chromatids at anaphase I. Accordingly, each metaphase II complement showed 6 autosomes, grouped together in an arrangement similar to that observed in metaphase I, with a single chromatid of the X chromosome laying apart. The probe hybridized to 1 end of both sister chromatids of 1 autosome (fig. 2k).

In mitotic metaphases of *D. ruficollis*, 2 homologous chromosomes each showed a cluster of hybridization signals at 1 end (fig. 2l). Accordingly, a single cluster of strong signals was observed in an autosomal bivalent at pachytene, while no signal was seen on the positively heteropycnotic X-chromatin body (fig. 2m). Also at the diffuse stage, the X chromosome showed no hybridization with the rDNA probe, and hybridization signals were dispersed in autosomal chromatin (fig. 2n). At diakinesis-metaphase I, 1 autosomal bivalent showed hybridization signals at both ends (fig. 2o). At metaphase II, hybridization signals were observed at terminal positions on each sister chromatid of 1 autosome (fig. 2p).

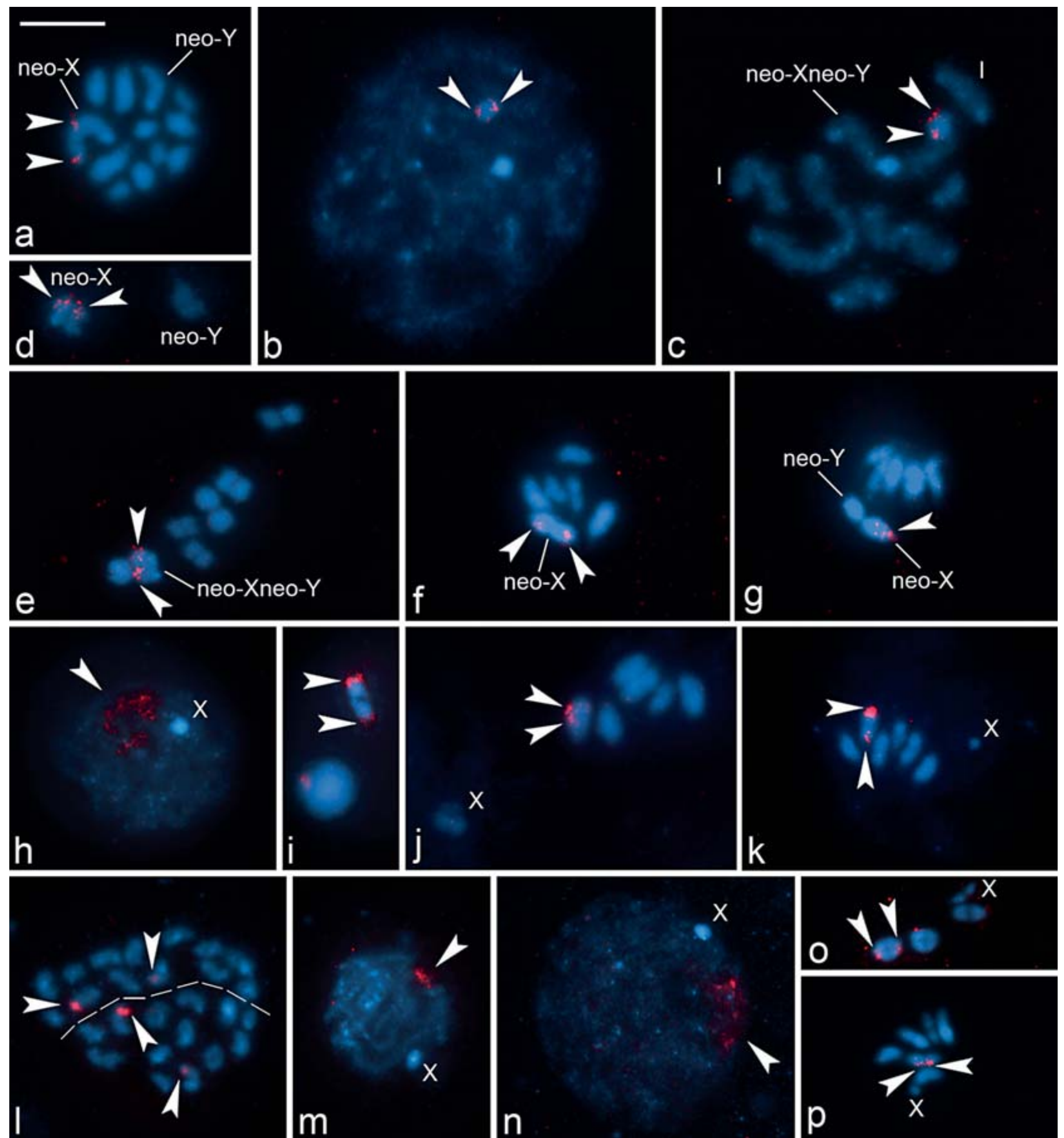



**Fig. 2.** Localization of rDNA in chromosomes of *Dysdercus albofasciatus* (**a–g**), *D. chaquensis* (**h–k**), and *D. ruficollis* (**l–p**) by FISH with 18S rDNA probes (red signals, arrowheads). Chromosomes were counterstained with DAPI (blue). **a** Spermatogonial metaphase (2n = 10 + neo-Xneo-Y) showing 2 hybridization signals on the neo-X chromosome but none on the neo-Y chromosome. **b** Diffuse stage; note the 2 heterochromatic X-bodies of different size with 2 clusters of hybridization signals on the larger X-chromatin body. **c** Early diplotene; note the neo-Xneo-Y bivalent with 2 heterochromatic X-bodies and 2 clusters of hybridization signals, both associated with the larger X-chromatin body; 1 autosomal pair is present as univalents (I). **d** A detail of the neo-Xneo-Y bivalent at diakinesis with 2 hybridization signals on each sister chromatid of the neo-X. **e** Metaphase I showing 5 autosomal bivalents and the neo-Xneo-Y sex bivalent, with strong signals of rDNA probe on each sister chromatid of the neo-X but not in the neo-Y chromosome. **f**, **g** Metaphases II with the neo-X (**f**) and with the neo-Xneo-Y pseudobivalent (pII) (**g**); note the hybridization signals in pII only on the neo-X chromatid. **h** Diffuse stage with heterochromatic X-chromatin body, showing hybridization signals dispersed in autosomal chromatin. **i** The autosomal NOR bivalent at metaphase I with hybridization signals at both ends. **j** Metaphase I (n = 6 + X); note the hybridization signals on both homologues of the autosomal NOR bivalent. **k** Metaphase II; note the hybridization signals on both sister chromatids of the NOR-autosome. **l** Two spermatogonial metaphases (white line separates probable chromosome sets, each with 2n = 12 + X0) and each showing 2 autosomes with terminal hybridization signals. **m** Pachytene showing a heterochromatic X-chromatin body and a cluster of strong hybridization signals at the terminal segment of an autosomal bivalent. **n** Diffuse stage with a conspicuous X-chromatin body and a cluster of dispersed hybridization signals in autosomal chromatin. **o** Incomplete metaphase I complement; note the NOR-autosome bivalent with hybridization signals at both ends. **p** Metaphase II (n = 6 + X); note the hybridization signals on each chromatid of the NOR autosome. Bar = 10 μm.

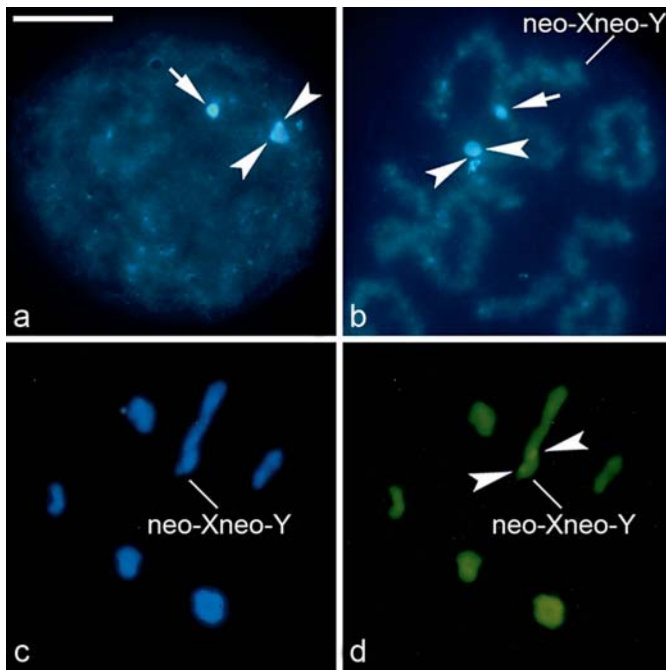



**Fig. 3.** C-banding followed by staining with DAPI (**a**, **b**), DAPI banding (**c**), and $CMA_3$ banding (**d**) in meiotic chromosomes of *Dysdercus albofasciatus* males. **a** Diffuse stage; note the completely C-positive smaller X-chromatin body (arrow) and 2 C-positive spots (arrowheads) on the larger X-chromatin body. **b** Early diplotene; note the neo-Xneo-Y bivalent with 2 heterochromatic X-bodies, the smaller one completely C-positive (arrow) and the larger one with 2 C-positive spots (arrowheads). **c**, **d** The same diakinesis without any DAPI-bright bands (**c**) and with 2 $CMA_3$-bright bands on the larger X-chromatin body (arrowheads) (**d**). Bar = 10 μm.

*Comparison of C-Banding and Fluorescent Banding Patterns*

In meiotic prophase I nuclei of *D. albofasciatus*, the smaller chromatin body was completely C-positive and the larger body showed 2 C-positive spots (fig. 3a, b). Whereas no positive C-bands were observed in *D. chaquensis* and *D. ruficollis*, suggesting that the X chromosomes of both species are devoid of constitutive heterochromatin (not shown).

Meiotic chromosomes of *D. albofasciatus* showed uniform staining with DAPI and $CMA_3$ fluorochromes, except for the sex chromosome bivalent in prophase I. From early prophase I stages until diplotene the smaller chromatin body was brighter after DAPI banding in comparison with the larger chromatin body and autosomes (not shown). However, the differential staining with DAPI was not seen in the diakinesis/metaphase I neo-Xneo-Y bivalents (fig. 3c). After $CMA_3$ banding, a pair of $CMA_3$-bright bands was observed in the larger chromatin body of the neo-Xneo-Y bivalent during the whole prophase I stage (fig. 3d). At early meiotic prophase of both *D. chaquensis* and *D. ruficollis*, the X chromosome was clearly distinguished from the autosomes as a DAPI-bright body. From diakinesis onwards all chromosomes were stained homogenously without any DAPI- or $CMA_3$-bright bands (not shown).

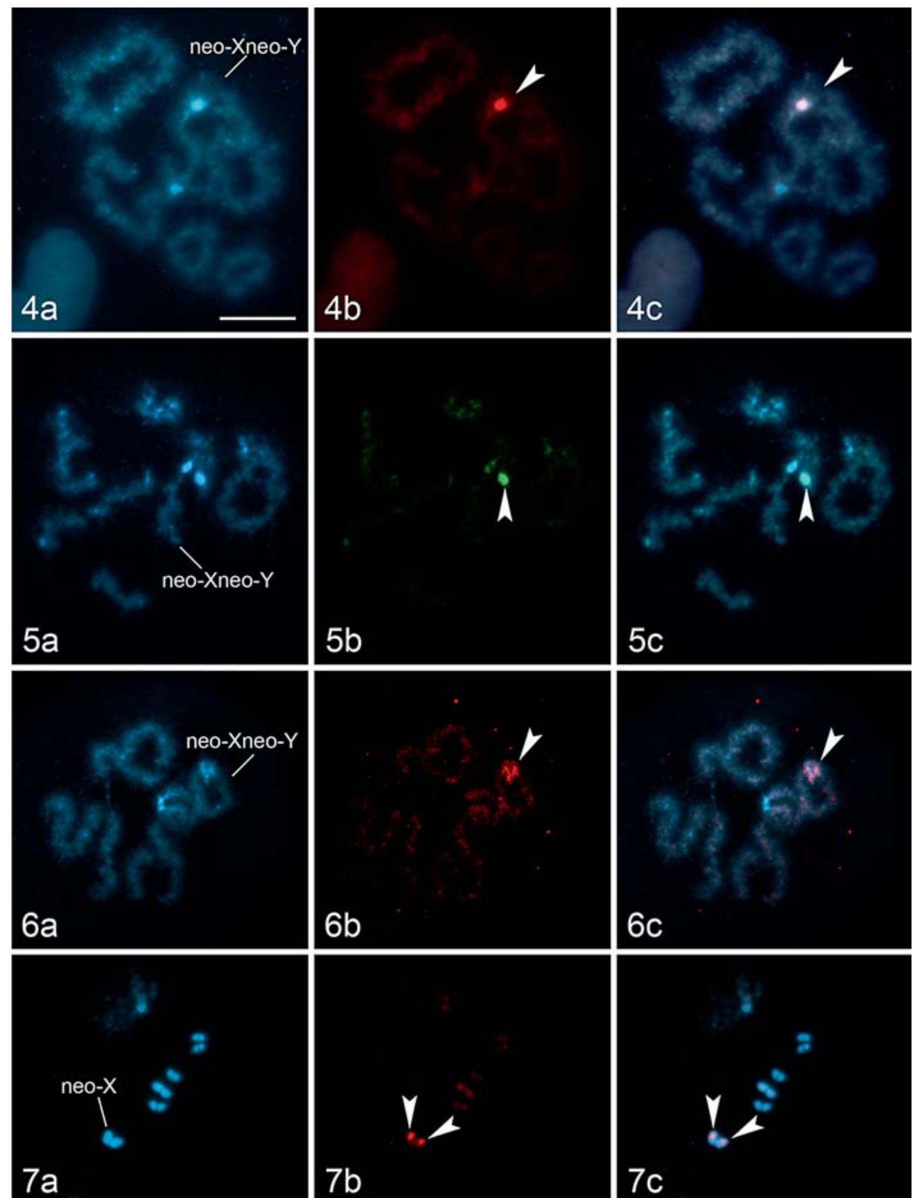
*Molecular Differentiation of Sex Chromosomes in* Dysdercus albofasciatus

In a preliminary GISH experiment, we hybridized labeled male genomic probe of *D. albofasciatus* with an excess of unlabeled female genomic DNA of the same species to chromosome preparations of *D. albofasciatus* males. The male probe highlighted both X-chromatin bodies (particularly the larger X-chromatin body), i.e. heterochromatic parts of the neo-X chromosome, but failed to differentiate the neo-Y chromosome (not shown). Similar results were obtained in a reciprocal hybridization experiment using labeled female genomic probe and unlabeled male genomic probe (not shown).

In GISH experiments between the *Dysdercus* species, both the female genomic probe from *D. chaquensis* (fig. 4a–c) and the male genomic probe from *D. ruficollis* (fig. 5a–c) provided similar hybridization patterns on meiotic chromosomes of *D. albofasciatus* males. In diplotene complements, the genomic probes displayed strong binding to the larger chromatin body of the neo-X chromosome (cf. figs. 4b and 5b with 4c and 5c, respectively) irrespective of the female or male origin of the probes. No other parts of the neo-Xneo-Y bivalent or of autosomal bivalents were highlighted with the probes. The strong binding of the probes only to the larger X-chromatin body suggests that this part of the *D. albofasciatus* neo-X chromosome is composed of highly repetitive DNA, which is also present in the genomes of *D. chaquensis* and *D. ruficollis*.

*Zoo-FISH with the X-Chromosome Painting Probe*

In a preliminary experiment, the X-chromosome probe derived from microdissected X chromosomes of *D. ruficollis* stained the single X-chromatin body in early prophase I nuclei and the entire X chromosome in later meiotic stages on male preparations of *D. ruficollis*, confirming thus its specificity (not shown). For comparative chromosome painting (Zoo-FISH), we cross-hybridized the *D. ruficollis* X-chromosome probe onto meiotic chromosomes of *D. albofasciatus* males. Similar to genomic probes after the GISH experiments between species, the




**Figs. 4–7.** GISH (**4a–c** and **5a–c**) and Zoo-FISH (**6a–c** and **7a–c**) in male meiotic chromosomes of *Dysdercus albofasciatus*. Chromosomes were counterstained with DAPI (blue), female-derived genomic probe of *D. chaquensis* was labeled with Cy3 (red) and male-derived genomic probe of *D. ruficollis* with Alexa Fluor 488 (green), and the biotinylated *D. ruficollis* X-chromosome painting probe was detected by Cy3-conjugated streptavidin (red). Figures labeled **a** show DAPI images, figures labeled **b** show hybridization signals of the probes, and figures labeled **c** show merged images. **4a–c** Diplotene showing the neo-Xneo-Y bivalent with strong binding of the *D. chaquensis* female genomic probe to the larger X-chromatin body. **5a–c** Diplotene showing the neo-Xneo-Y bivalent with strong binding of the *D. ruficollis* male genomic probe to the larger X-chromatin body. **6a–c** Diplotene showing the neo-Xneo-Y bivalent with strong binding of the X-chromosome painting probe to the larger X-chromatin body. **7a–c** Metaphase II showing strong binding of the X-chromosome painting probe to each chromatid of the neo-X. Arrowheads indicate hybridization signals. Bar = 10 μm.

X-painting probe displayed strong hybridization signals on the larger chromatin body of the neo-X chromosome but not on the smaller X-chromatin body or other parts of the bivalent, as clearly observed in diplotene nuclei (fig. 6a–c). At metaphase II, the X-painting probe identified the neo-X chromosome by strong binding to both sister chromatids but stained only less than a half of their chromatin mass (fig. 7b, c). The results suggest that the neo-X chromosome segment of *D. albofasciatus*, responsible for the formation of the larger X-chromatin body, retained extensive DNA sequence similarity to the X chromosome of *D. ruficollis*.

*Meiotic Pairing of the neo-Xneo-Y in* Dysdercus albofasciatus

We used immunolocalization of SMC3 in spermatocytes at prophase I and found that the neo-X and neo-Y chromosomes form axial elements similarly to autosomes (fig. 8a, b). During early pachytene, autosomal bivalents were synapsed or formed almost complete synaptonemal complexes (SCs), whereas both the neo-X and neo-Y sex chromosomes showed delayed pairing with their axial elements close to each other but not synapsed (fig. 8a). Later in pachytene, when the autosomal bivalents were fully synapsed, the neo-X and neo-Y chromosomes axes

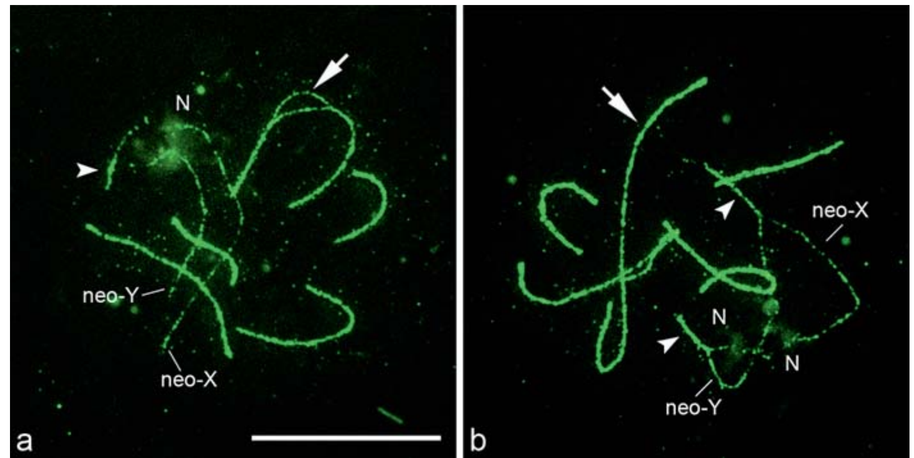



**Fig. 8.** Immunostaining of meiotic axes in pachytene spermatocytes of *Dysdercus albofasciatus*. **a** Early pachytene immunostained with antibody against SMC3 (green) showing 5 synapsed autosomal bivalents except an interstitial segment of the largest autosomal bivalent (see a loop indicated by arrow), and 2 partially aligned axes corresponding to the unpaired sex chromosomes. The longer axis represents the neo-X and the shorter one the neo-Y. **b** Pachytene complement showing 5 fully formed autosomal SCs including the largest autosomal bivalent (arrow) and a neo-Xneo-Y bivalent with synapsed terminal segments (arrowheads) but asynapsed axes in a large middle region. N, nucleoli. Bar = 10 μm.

showed partial synapsis at their terminal segments. A large interstitial region of the neo-Xneo-Y bivalent remained unpaired indicating the absence of homology. We did not observe any nuclei with completely synapsed neo-X and neo-Y. In the asynaptic region, the axial elements appeared thinner and sometimes discontinuous, forming a large loop (fig. 8b). In addition, 1 or 2 faintly stained clouds were seen associated with the neo-X axial element. According to our experience with SC immunostaining, these clouds probably represent nucleoli that often appeared unspecifically labeled on preparations made by the spreading procedure (fig. 8a, b). In this type of spread, DAPI images were not useful for localizing the position of the X-chromatin bodies, because the use of detergent turned the chromatin homogenous and did not allow the identification of condensed segments in chromosomes.

## Discussion

Structural differences between X and Y or W and Z range from obvious at first sight (heteromorphic sex chromosomes) to microscopically undetectable (homomorphic sex chromosomes). However, even homomorphic sex chromosomes are not molecularly identical. Spreading of simple sequence repeats in the Y or W chromosomes, invasion of transposable elements, late-replicating segments, and blocks of heterochromatin are apparent forms of differentiation at the molecular level [Schempp and Schmid, 1981; Haaf and Schmid, 1984; Jones and Singh, 1985; Iturra and Veloso, 1989; Nanda et al., 1992; Steinemann and Steinemann, 1992; Traut, 1994]. The molecular differentiation is possible provided that a non-recombining region in the sex chromosomes has been established. Once crossing-over has been suppressed, it becomes inevitable that the X and Y (or Z and W) chromosomes evolve separately in the non-recombining segment.

In heteropteran males, the sex chromosomes in $X_nY_n$ systems (with n varying from 1 to 6) are asynaptic and achiasmatic, behave as univalents, and divide equationally during the first meiotic division [Papeschi and Bressa, 2006]. This means that the basic requirement for the molecular differentiation of X and Y chromosomes, i.e. the absence of recombination in the heterogametic sex, is fulfilled. However, the neo-Xneo-Y sex chromosomes, which originated by rearrangements with autosomes, such as in *Lethocerus* sp., *L. indicus*, *Hebrus pulsillus*, and *Dysdercus albofasciatus*, begin as a chiasmatic pair that forms a sex-chromosome bivalent and segregates reductionally at anaphase I [Chickering, 1927; Chickering and Bacorn, 1933; Bressa et al., 1999; Nokkala and Nokkala, 1999]. These neo-Xneo-Y systems thus represent unique models for the study of early stages of sex chromosome differentiation.

*Structure of Neo-Sex Chromosomes in* Dysdercus albofasciatus

In the present work, we examined the structure of the neo-X and neo-Y chromosomes of *D. albofasciatus* (2n = 10 + neo-Xneo-Y) in comparison with the ancestral X0 sex-chromosome system in *D. chaquensis* (2n = 12 + X0) and *D. ruficollis* (2n = 12 + X0). Previous cytogenetic studies indicated association of the nucleolar material with the larger X-chromatin body in meiotic prophase of *D. albofasciatus* and with the X chromosome in *D. chaquensis*, while it appeared associated with an autosomal bivalent in *D. ruficollis* [Bressa et al., 1999, 2002, 2003]. In this study, rDNA-FISH revealed 2 rDNA clusters on the neo-X chromosome of *D. albofasciatus*, located in flanking regions of the neo-X chromosome segment forming the larger X-chromatin body, while the only rDNA cluster in *D. chaquensis* and *D. ruficollis* was located in the terminal region of 1 autosomal pair. Thus, the rDNA-FISH results in *D. albofasciatus* and *D. ruficollis* corroborated the previous observations after conventional staining. On the contrary, the results ruled out location of the NOR on the X chromosome in *D. chaquensis*. Most likely, the previously reported association of the nucleolar material with the X-chromatin body during the diffuse stage was a nonspecific association, which could occur as a result of the nucleolus dissociation from the NOR bivalent in late meiotic prophase I [Bressa et al., 2003; Cattani and Papeschi, 2004].

In *D. chaquensis* and *D. ruficollis*, no C- or fluorescent bands were observed, indicating the absence of constitutive heterochromatin. This also holds for the single X-chromatin body in meiotic prophase nuclei. Hence, the X-chromatin body in these species is obviously composed of facultative heterochromatin as typical for many other heteropteran species with simple sex chromosome systems, XY and X0 [Panzera et al., 1995; Rebagliati et al., 2003; Cattani et al., 2004; Bressa et al., 2005]. However, our results of C-banding in *D. albofasciatus* suggest that the smaller C-positive X-chromatin body is composed of constitutive heterochromatin, whereas the larger X-chromatin body consists of facultative heterochromatin except for 2 small C-positive regions. Furthermore, fluorescent banding revealed 2 $CMA_3$-positive bands on the larger X-chromatin body, indicating the presence of CG-rich sequences. Since CG-rich constitutive heretochromatin often occurs in the NOR regions [Papeschi et al., 2003; Rebagliati et al., 2003; Cattani et al., 2004; Cattani and Papeschi, 2004; Papeschi and Bressa, 2006; Criniti et al., 2009], we suppose that these 2 bands could be associated with 2 rDNA clusters revealed by rDNA-FISH. On the other hand, the lack of heterochromatin in the neo-Y chromosome provides further support for its recent autosomal origin.

*Meiotic Pairing of Neo-Sex Chromosomes in* Dysdercus albofasciatus

Immunolocalization of SMC3 in *D. albofasciatus* revealed the regular formation of meiotic axes in the autosomes as well as in the neo-X and neo-Y chromosomes during meiotic prophase I, along with a delayed pairing of the neo-Xneo-Y bivalent in comparison with autosomal bivalents. In the neo-Xneo-Y bivalent, only terminal regions were fully synapsed, while the interstitial region formed a large loop probably due to the lack of homology. Similar asynaptic loops occur in early meiotic prophase of organisms heterozygous for inversions [e.g. Moses et al., 1982; reviewed in Marec, 1996]. In previous reports, the synaptic behaviour of heteropteran sex chromosomes during male meiosis was studied by electron microscopy and silver staining of meiotic axes. The sex chromosomes of several species with different sex chromosome systems (X0, XY, $X_1X_2$0, and $X_1X_2$Y) failed to show the presence of regular axial elements (AEs) during the first meiotic prophase, either in spreads or in sections [Ruthmann and Dahlberg, 1976; Solari, 1979; Suja et al., 2000; Pigozzi and Solari, 2003; Toscani et al., 2008]. These results led to the hypothesis that the lack of formation of AEs along the heteropteran sex chromosomes is somehow related to their equational division at anaphase I (post-reductional behaviour) [Solari, 1979; Suja et al., 2000; Pigozzi and Solari, 2003; Toscani et al., 2008]. The lack of regular AEs seems to be accompanied by the absence of continuous cohesin axes in the heteropteran sex chromosomes. It results from a recent finding that the meiotic cohesion protein REC8 localizes along the autosomes in *Triatoma* species (Reduviidae), but it is absent in the sex chromosomes [Pigozzi and Solari, 2003]. Moreover, the cohesin component SMC3 forms regular AEs along the autosomal bivalents in *Graphosoma italicum* (Pentatomidae) and *Holhymenia rubiginosa* (Coreidae), but it is present only as dots or threads in their respective sex chromosomes [Toscani et al., 2008]. Thus, it seems likely that the condensation and cohesion of the sex chromosomes of heteropterans showing post-reductional behaviour is set by rules different from that of autosomes, both in simple and multiple systems [Suja et al., 2000; Pigozzi and Solari, 2003]. By contrast, we showed here that the labelling with the cohesin component SMC3 is comparatively much more regular in *D. albofasciatus* sex chromosomes, since their AEs have an autosome-like appearance and even synapse at

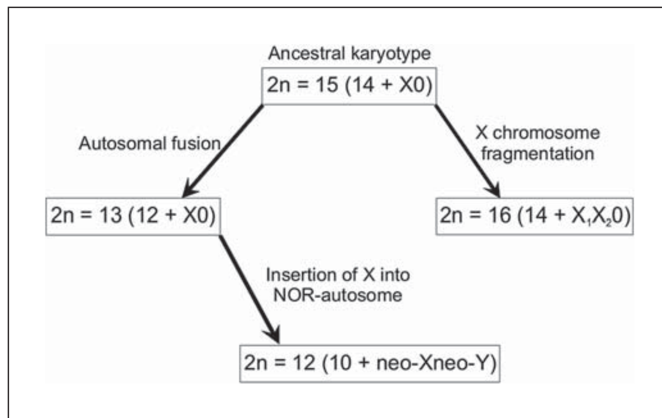



**Fig. 9.** A hypothetical scheme of karyotype evolution in American species of the genus *Dysdercus*. See text for details.

terminal regions, where chiasmata occur in diakinesis [Bressa et al., 1999]. The presence of continuous AEs along the neo-X and neo-Y together with the pre-reductional behaviour of the neo-Xneo-Y bivalent in *D. albofasciatus* lead us to suggest that the autosomal chromatin organization and autosomal SMC3 pattern in the autosomal segments of the neo-X extended to the segments of X-chromosome origin, i.e. to both X-chromatin bodies. Thus, the condensation and cohesion mechanisms of the autosomal chromosomes are preserved in this neo-sex chromosome system.

*Sex Chromosome Evolution in* Dysdercus

Published data on karyotype evolution in American species of the genus *Dysdercus* [Piza, 1947a, 1947b; Mola and Papeschi, 1997; Bressa et al., 2003] along with the present results of rDNA-FISH support the hypothesis that the ancestral male karyotype had 2n = 15 chromosomes with 7 pairs of autosomes including a single pair of NOR-autosomes and the X-chromosome univalent as found, for example, in *D. honestus* (fig. 9) [Piza, 1951; Ray-Chaudhuri and Manna, 1952; Ruthmann and Dahlberg, 1976]. An autosomal fusion between 2 non-homologous autosomes led to a reduction in the diploid number to 2n = 13, while keeping both the ancestral NOR-autosome pair and the X univalent, a situation represented by *D. chaquensis*, *D. imitator*, and *D. ruficollis* [Bressa et al., 2002; this study]. In the next step, an insertion of the whole X chromosome into the NOR-autosome resulted in the neo-sex-chromosome system found in *D. albofasciatus* (for details, see later) and brought about the further reduction in the diploid chromosome number to 2n = 12. On the other hand, a fragmentation of the single X chromosome in the ancestral karyotype resulted in multiple X chromosomes, $X_1$ and $X_2$, and led to a karyotype with 2n = 16 chromosomes as represented by *D. peruvianus* [Bressa et al., 2003].

In *D. albofasciatus*, results of GISH and Zoo-FISH suggested a significant sequence homology of the part of the neo-X chromosome forming the large X-chromatin body in meiotic prophase I, with the X chromosome of *D. chaquensis* and *D. ruficollis*. On the basis of this finding and the above discussed results of rDNA-FISH, and taking into consideration the scheme elaborated by Bressa et al. [1999] we propose the following scenario of the evolution of the neo-Xneo-Y sex-chromosome system in *D. albofasciatus* (fig. 10a–d). In the ancestor of *D. albofasciatus*, the original X chromosome (fig. 10a) was inserted into the NOR-autosome next to the rDNA cluster. This NOR-autosome thus became a neo-X chromosome and the homologous NOR-autosome became a neo-Y chromosome (fig. 10b). In the second step, a large inversion involving most of the autosomal part of neo-X and a small segment of the ancestral X (segment 1 in fig. 10c) occurred. The splitting of the original X into 2 segments of different sizes, (segment 1 and segments 2 + 3 in fig. 10c) is supported by the occurrence of 2 X-chromatin bodies in the male meiotic prophase I, 1 smaller and 1 larger (see figs. 2c, 4a and 5a). As a result of the inversion, crossing-over was restricted to the homologous terminal regions of the neo-Xneo-Y sex bivalent, which are of different sizes (cf. segments AB/ab and F/f in fig. 10c). This is consistent with the occurrence of 1 or less frequently 2 terminal chiasmata in the neo-Xneo-Y bivalent. The absence of recombination in the large central part of the neo-sex chromosomes is further corroborated by synaptic behaviour of the neo-Xneo-Y. Hence, we suggest that the SC-free interstitial region of the neo-Xneo-Y bivalent corresponds to the inversion plus 2 segments of the original X chromosome.

In the last step of evolution of the neo-Xneo-Y of *D. albofasciatus*, the NOR located on the neo-Y chromosome (originally NOR-autosome in the ancestral karyotype) transposed into the neo-X, next to the boundary between the larger X-chromatin body and the autosomal segment (fig. 10d). The lack of data does not allow us to speculate about a mechanism for this transposition. Nevertheless, the potential of NORs to change their position is well known [Schubert and Wobus, 1985], and a number of reports support the hypothesis of interchromosomal mobility of NOR regions [e.g. Arnheim et al., 1980; Roy et al., 2005). However, it is not

yet clear whether transposition mediated by mobile elements, or chromosomal rearrangements promoted by ectopic recombination (i.e. homologous recombination between repetitive sequences dispersed throughout genome) are responsible for this mobility [Schubert, 2007].

Repetitive sequences are thought to play a significant role in the evolution of Y or W sex chromosomes [Charlesworth et al., 2005; Steinemann and Steinemann, 2005; Kejnovsky et al., 2009]. Specially, early sex chromosome differentiation appears to be initiated by the accumulation of simple repeated sequences adjacent to coding regions for sex determination [Nanda et al., 1992; Volff et al., 2007; Bergero and Charlesworth, 2009]. However, GISH experiments performed in this study failed to identify the neo-Y chromosome of *D. albofasciatus*. As found in several lepidopteran species, GISH detects mainly evolutionary 'old' parts of the W chromosome that are composed of heterochromatin and consist largely of repetitive sequences [Yoshido et al., 2005]. Therefore, our GISH results suggest that the nonrecombining (asynaptic) part of the neo-Y has not yet accumulated a sufficient amount of repetitive sequences to be differentiated from the corresponding part of the neo-X chromosome. Instead of differentiating the neo-Y chromosome, genomic probes from *D. chaquensis* and *D. ruficollis* identified the larger X-chromatin body (segments 2 and 3 in fig. 10c) of the neo-X chromosome. The larger X-chromatin body was also identified by Zoo-FISH with the X-chromosome painting probe derived from *D. ruficollis*. Taken together these results suggest that (i) the larger X-chromatin body is composed of highly repetitive sequences and (ii) these sequences are well conserved between the 2 species. On the other hand, the smaller X-chromatin body (segment 1 in fig. 10c), which was not identified either by genomic probes from *D. chaquensis* and *D. ruficollis* or by Zoo-FISH, acquired a different sequence composition after it was separated from the larger body by inversion. Alternatively, the smaller X-chromatin body could represent an ancestral genetic material that had been lost in *D. chaquensis* and *D. ruficollis* after the divergence of the 3 species. We also cannot exclude an option that the smaller X-body represents a new *D. albofasciatus*-specific chromosome region. Nevertheless, the fact that the inverted segment of the neo-X chromosome between the 2 X-bodies and the corresponding part of the neo-Y chromosome remain euchromatic in spite of the absence of recombination suggests an early stage of molecular differentiation.

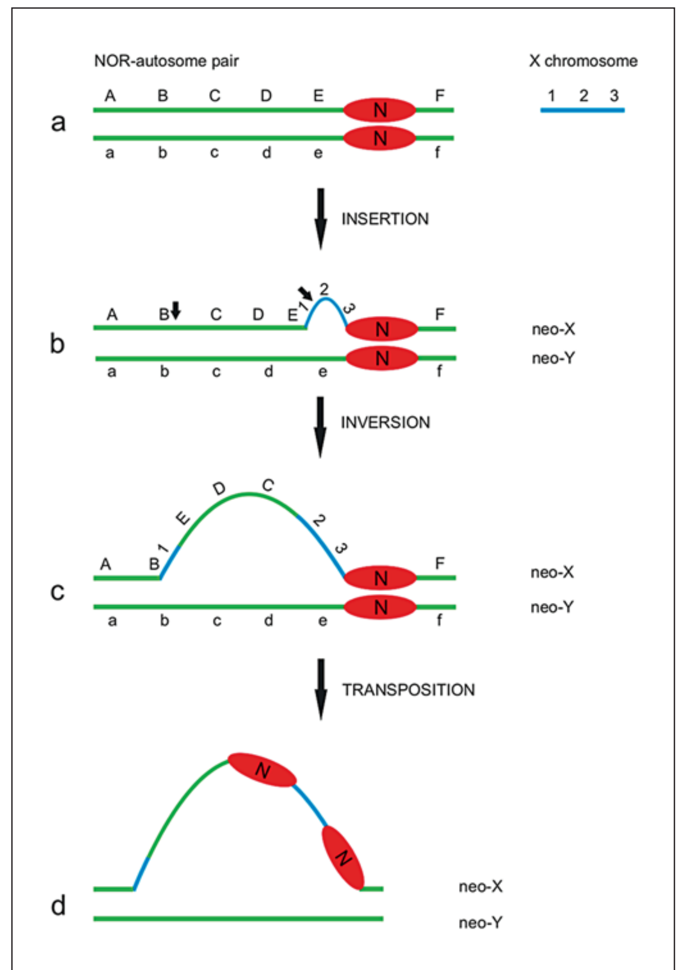



**Fig. 10.** Schematic interpretation of chromosomal rearrangements in the evolution of the neo-Xneo-Y sex-chromosome system in *Dysdercus albofasciatus* males. **a** The ancestral NOR-autosome pair (green) with a subterminal rDNA cluster (red) and the single ancestral X chromosome (blue) such as found in *D. chaquensis* and *D. ruficollis*. **b** A neo-X chromosome arose by insertion of the ancestral X chromosome into the NOR-autosome, close to the rDNA cluster; the other NOR-autosome became a neo-Y chromosome. **c** A large inversion in the neo-X chromosome, which involved a small segment of the ancestral X chromosome; this resulted in the lack of homology between the neo-Y chromosome and a large part of the neo-X chromosome from the NOR to the smaller segment of the ancestral X chromosome. **d** The rDNA cluster of the neo-Y chromosome was transposed, possibly by unequal recombination, to the neo-X chromosome next to the larger ancestral X-chromosome segment; this neo-Xneo-Y sex chromosome constitution including 2 rDNA clusters in the neo-X chromosome, each located at 1 end of the larger ancestral X chromosome segment, is found in *D. albofasciatus*. Letters and numbers above chromosomes indicate chromosome segments; arrows in **b**, chromosome breaks.

## Acknowledgements

This work was made possible through a Research co-operation project between CONICET (Buenos Aires, Argentina) and Academy of Sciences of the Czech Republic (ASCR, Prague, Czech Republic). Experiments were funded by grants X164 of the University of Buenos Aires, PICT 2007-00635 of ANPCyT from Argentina and 206/06/1860 of the Grant Agency of the Czech Republic (Prague) and from the Entomology Institute project Z50070508. F.M. and M.V. acknowledge support from grant IAA600960925 of the Grant Agency of the ASCR (Prague). S.K. was supported by project MZE 0002716202 of the Grant Agency of the Ministry of Agriculture of the Czech Republic, Prague.

## References

Arnheim N, Krystal M, Schmickel R, Wilson G, Ryder O, et al: Molecular evidence for genetic exchange among ribosomal genes on nonhomologous chromosomes in man and apes. Proc Nat Acad Sci USA 77:7323–7327 (1980).

Ayling LJ, Griffin DK: The evolution of sex chromosomes. Cytogenet Genome Res 99:125–140 (2002).

Bachtrog D: A dynamic view of sex chromosome evolution. Curr Opin Genet Dev 16:578–585 (2006).

Bedo DG: Karyotypic and chromosome banding studies of the potato tuber moth, *Phthorimaea operculella* (Zeller) (Lepidoptera, Gelechiidae). Can J Genet Cytol 26:141–145 (1984).

Bergero R, Charlesworth D: The evolution of restricted recombination in sex chromosomes. Trends Ecol Evol 24:95–102 (2009).

Bressa MJ, Papeschi AG, Mola LM, Larramendy ML: Meiotic studies in *Dysdercus* Guérin Méneville 1831 (Heteroptera: Pyrrhocoridae). I. Neo-XY in *Dysdercus albofasciatus* Berg 1878, a new sex chromosome determining system in Heteroptera. Chromosome Res 7:503–508 (1999).

Bressa MJ, Fumagalli E, Ituarte S, Frassa MV, Larramendy ML: Meiotic studies in *Dysdercus* Guérin Méneville 1831 (Heteroptera: Pyrrhocoridae). II. Evidence on variations of the diffuse stage between wild and laboratory-inbred populations of *Dysdercus chaquensis* Freiberg, 1948. Hereditas 137:125–131 (2002).

Bressa MJ, Papeschi AG, Fumagalli E, van Doesburg PH, Larramendy M: Cytogenetic and nucleolar meiotic cycle analyses in *Dysdercus imitator* Blote, 1931 (Heteroptera, Pyrrhocoridae) from Argentina. Folia Biol 51:135–141 (2003).

Bressa MJ, Larramendy M, Papeschi AG: Heterochromatin characterization in five species of Heteroptera. Genetica 124:307–317 (2005).

Cattani MV, Papeschi AG: Nucleolus organizing regions and semi-persistent nucleolus during meiosis in *Spartocera fusca* (Thurnberg) (Coreidae, Heteroptera). Hereditas 140:105–111 (2004).

Cattani MV, Greizerstein EJ, Papeschi AG: Male meiotic behaviour and nucleolus organizing regions in *Camptischium clavipes* (Fabr.) (Coreidae, Heteroptera) analyzed by fluorescent banding and in situ hybridization. Caryologia 57:267–273 (2004).

Charlesworth D, Charlesworth BW, Marais G: Steps in the evolution of heteromorphic sex chromosomes. Heredity 95:118–128 (2005).

Chickering AM: An unusual complex in *Lethocerus*. Anat Rec 37:156 (1927).

Chickering AM, Bacorn B: Spermatogenesis in the Belostomatidae. IV. Multiple chromosomes in *Lethocerus*. Pap Mich Acad Sci 17:529–533 (1933).

Criniti A, Simonazzi G, Cassanelli S, Ferrari M, Bizzaro D, et al: Distribution of heterochromatin and rDNA on the holocentric chromosomes of the aphids *Dysaphis plantaginea* and *Melanaphis pyraria* (Hemiptera: Aphididae). Eur J Entomol 106:153–157 (2009).

Flores SV, Evans AL, McAllister BF: Independent origins of new sex-linked chromosomes in the *melanica* and *robusta* species groups of *Drosophila*. BMC Evol Biol 8:33 (2008).

Franco M, Bressa MJ, Papeschi AG: Karyotype and male meiosis in *Spartocera batatas* (Fabricius) and meiotic behaviour of multiple sex chromosomes in Coreidae, Heteroptera. Eur J Entomol 103:9–16 (2006).

Fuková I, Nguyen P, Marec F: Codling moth cytogenetics: karyotype, chromosomal location of rDNA, and molecular differentiation of sex chromosomes. Genome 48:1083–1092 (2005).

Fuková I, Traut W, Vítková M, Nguyen P, Kubíčková S, et al: Probing the W chromosome of the codling moth, *Cydia pomonella*, with sequences from microdissected sex chromatin. Chromosoma 116:135–145 (2007).

Gokhman VE, Kuznetsova V: Comparative insect karyology: current state and applications. Entomol Rev 86:352–368 (2006).

Haaf T, Schmid M: An early stage of ZW/ZZ sex chromosome differentiation in *Poecilia sphenops* var. *melanistica* (Poeciliidae, Cyprinodontiformes). Chromosoma 89:37–41 (1984).

Henking H: Untersuchungen über die ersten Entwicklungsvorgänge in den Eiern der Insekten. II. Über Spermatogenese und deren Beziehung zur Eientwicklung bei *Pyrrhocoris apterus* L. Z wiss Zool 51:685–736 (1891).

Iturra P, Veloso A: Further evidence for early sex chromosome differentiation of Anuran species. Genetica 78:25–31 (1989).

Jacobs DH: The evolution of a neo-$XY_1Y_2$ sex chromosome system by autosome-sex chromosome fusion in *Dundocoris nodulicarinus* Jacobs (Heteroptera: Aradidae: Carventinae). Chromosome Res 12:175–191 (2004).

Jande SS: An analysis of the chromosomes in the four species of the family Belostomatidae (Heteroptera, Cryptocerata). Res Bull (N.S.) Panjab Univ 10:25–34 (1959).

Jones KW, Singh L: Snakes and the evolution of sex chromosomes. Trends Genet 1:55–61 (1985).

Kejnovsky E, Hobza R, Cermak T, Kubat Z, Vyskot B: The role of repetitive DNA in structure and evolution of sex chromosomes in plants. Heredity 102:533–541 (2009).

Kubickova S, Cernohorska H, Musilova P, Rubes J: The use of laser microdissection for the preparation of chromosome-specific painting probes in farm animals. Chromosome Res 10:571–577 (2002).

Lockwood APM: 'Ringer' solutions and some notes on the physiological basis of their ionic composition. Comp Biochem Physiol 2:241–289 (1961).

Manna GK: Chromosomes in evolution in Heteroptera; in Sharma AK, Sharma A (eds): Chromosomes in Evolution of Eukaryotic Groups, Vol II, pp 189–225 (CRC Press, Boca Raton 1984).

Marec F: Synaptonemal complex in insects. Int J Insect Morphol Embryol 25:205–233 (1996).

Messthaler H, Traut W: Phases of sex chromosomes inactivation in *Oncopeltus fasciatus* and *Pyrrhocoris apterus* (Insecta, Heteroptera). Caryologia 28:501–510 (1975).

Mola LM, Papeschi AG: Citogenética de *Dysdercus chaquensis* (Heteroptera: Pyrrhocoridae). Rev Soc Entomol Argent 56:20 (1997).

Moses MJ, Poorman PA, Roderick TH, Davisson MT: Synaptonemal complex analysis of mouse chromosomal rearrangements IV. Synapsis and synaptic adjustment in two paracentric inversions. Chromosoma 84:457–474 (1982).

Nanda I, Schartl M, Feichtinger J, Epplen JT, Schmid M: Early stages of sex chromosome differentiation in fish as analysed by simple repetitive DNA sequences. Chromosoma 101:301–310 (1992).

Nokkala S, Nokkala C: Chromosomes in two bug species of *Hebrus* (Hebridae, Heteroptera). The occurrence of neo-XY sex chromosome system in Heteroptera. Caryologia 52:27–30 (1999).
Panzera F, Perez R, Panzera Y, Alvarez F, Scvortzoff E, et al: Karyotype evolution in holocentric chromosomes of three related species of triatomines (Hemiptera-Reduviidae). Chromosome Res 3:143–150 (1995).
Papeschi AG: C-banding and DNA content in three species of *Belostoma* (Heteroptera) with large differences in chromosome size and number. Genetica 76:43–51 (1988).
Papeschi AG: DNA content and heterochromatin variation in species of *Belostoma* (Heteroptera, Belostomatidae). Hereditas 115:109–114 (1991).
Papeschi AG, Bressa MJ: Evolutionary cytogenetics in Heteroptera. J Biol Res 5:3–21 (2006).
Papeschi AG, Mola LM, Bressa MJ, Greizerstein EJ, Lia V, et al: Behaviour of ring bivalents in holokinetic systems: alternative sites of spindle attachment in *Pachylis argentinus* and *Nezara viridula* (Heteroptera). Chromosome Res 11:725–733 (2003).
Pigozzi MI, Solari AJ: Differential immunolocalization of a putative Rec8p in meiotic autosomes and sex chromosomes of triatomine bugs. Chromosoma 112:38–47 (2003).
Piza SdT: Cromossômios de *Dysdercus* (Hemiptera-Pyrrhocoridae). An Esc Sup Agric 'Luiz de Queiroz' 4:209–216 (1947a).
Piza SdT: Comportamento dos cromossômios sexuais de *Dysdercus mendesi* na segunda divisão dos espermatócitos. Bragantia 7:269–271 (1947b).
Piza SdT: Interpretacão do typo sexual de *'Dysdercus mendesi'* Bloete (Hemiptera Pyrrhocoridae). Genet Iber 3:107–112 (1951).
Ray-Chaudhuri SP, Manna GK: A new type of segregation of the sex chromosomes in the meiotic divisions of the cotton stainer *Dysdercus koenigii* (Fabr.). J Genet 51:191–197 (1952).
Rebagliati P, Papeschi AG, Mola LM: Meiosis and fluorescent banding in *Edessa meditabunda* and *E. rufomarginata* (Heteroptera: Pentatomidae: Edessinae). Eur J Entomol 100:11–18 (2003).
Roy V, Monti-Dedieu L, Chaminade N, Siljak-Yakovlev S, Aulard S, et al: Evolution of the chromosomal location of rDNA genes in two *Drosophila* species subgroups: *ananassae* and *melanogaster*. Heredity 94:388–395 (2005).
Ruthmann A, Dahlberg R: Pairing and segregation of the sex chromosomes in $X_1X_2$-males of *Dysdercus intermedius* with a note on the kinetic organization of heteropteran chromosomes. Chromosoma 54:89–97 (1976).
Sahara K, Marec F, Traut W: TTAGG telomeric repeats in chromosomes of some insects and other arthropods. Chromosome Res 7:449–460 (1999).
Schempp W, Schmid M: Chromosome banding in Amphibia. VI. BrdU-replication patterns in Anura and demonstration of XX/XY sex chromosomes in *Rana esculenta*. Chromosoma 83:697–710 (1981).
Schrader F: The formation of tetrads and the meiotic mitoses in the male of *Rhytidolomia senilis* Say (Hemiptera, Heteroptera). J Morph 67:123–141 (1940).
Schubert I: Chromosome evolution. Curr Opin Plant Biol 10:109–115 (2007).
Schubert I, Wobus U: In situ hybridization confirms jumping nucleolus organizing regions in *Allium*. Chromosoma 92:143–148 (1985).
Solari AJ: Autosomal synaptonemal complexes and sex chromosomes without axes in *Triatoma infestans* (Reduviidae; Hemiptera). Chromosoma 72:225–240 (1979).
Steinemann M, Steinemann S: Degenerating Y chromosome of *Drosophila miranda*: a trap for retrotransposons. Proc Natl Acad Sci USA 89:7591–7595 (1992).
Steinemann M, Steinemann S: Enigma of Y chromosome degeneration: Neo-Y and Neo-X chromosomes of *Drosophila miranda* a model for sex chromosome evolution. Genetica 102/103:409–420 (1998).
Steinemann S, Steinemann M: Retroelements: tools for sex chromosome evolution. Cytogenet Genome Res 110:134–143 (2005).
Suja JA, del Cerro AL, Page J, Rufas JS, Santos JL: Meiotic sister chromatid cohesion in holocentric sex chromosomes of three heteropteran species is maintained in absence of axial elements. Chromosoma 109:35–43 (2000).
Toscani MA, Pigozzi MI, Bressa MJ, Papeschi AG: Synapsis with and without recombination in the male meiosis of the leaf-footed bug *Holhymenia rubiginosa* (Coreidae, Heteroptera). Genetica 132:173–178 (2008).
Traut W: Pachytene mapping in the female silkworm *Bombyx mori* L. (Lepidoptera). Chromosoma 58:275–284 (1976).
Traut W: Sex determination in the fly *Megaselia scalaris*, a model system for primary steps of sex chromosome evolution. Genetics 136: 1097–1104 (1994).
Traut W: The evolution of sex chromosomes in insects: differentiation of sex chromosomes in flies and moths. Eur J Entomol 96:227–235 (1999).
Traut W, Sahara K, Otto TD, Marec F: Molecular differentiation of sex chromosomes probed by comparative genomic hybridization. Chromosoma 108:173–180 (1999).
Traut W, Sahara K, Marec F: Sex chromosomes and sex determination in Lepidoptera. Sex Dev 1:332–346 (2007).
Ueshima N: Hemiptera II: Heteroptera; in John B (ed): Animal Cytogenetics, Vol 3: Insecta 6, pp V+117 (Gebrüder Borntraeger, Berlin-Stuttgart 1979).
Vítková M, Fuková I, Kubíčková S, Marec F: Molecular divergence of the W chromosomes in pyralid moths (Lepidoptera). Chromosome Res 15:917–930 (2007).
Volff JN, Nanda I, Schmid M, Schartl M: Governing sex determination in fish: Regulatory putsches and ephemeral dictators. Sex Dev 1: 85–99 (2007).
Vyskot B, Hobza R: Gender in plants: sex chromosomes are emerging from the fog. Trends Genet 20:432–438 (2004).
Whiting MF: Phylogeny of the holometabolous insect orders: molecular evidence. Zoologica Scripta 31:3–15 (2002).
Yoshido A, Marec F, Sahara K: Resolution of sex chromosome constitution by GISH and telomere-FISH in some species of Lepidoptera. Chromosoma 114:193–202 (2005).

**© Free Author Copy – for personal use only**

ANY DISTRIBUTION OF THIS ARTICLE WITHOUT WRITTEN CONSENT FROM S. KARGER AG, BASEL IS A VIOLATION OF THE COPYRIGHT.

Written permission to distribute the PDF will be granted against payment of a permission fee, which is based on the number of accesses required. Please contact permission@karger.ch