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The structure of the Xy_p sex chromosome complex in male meiosis of two beetles: *Tenebrio molitor* (Tenebrionidae) and *Chrysolina graminis* (Chrysomelidae)

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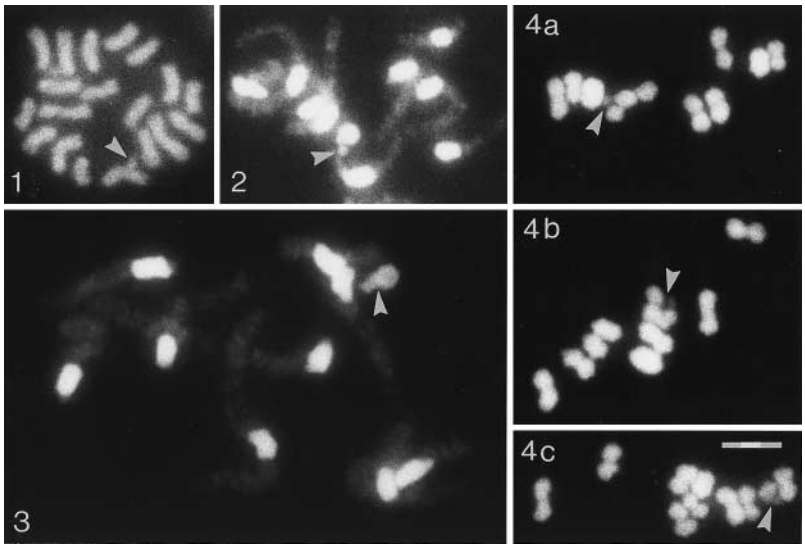
Abstract. Sex chromosome association was studied in male meiosis of *Tenebrio molitor*, a darkling beetle, using chromosome spreads prepared from testes. Staining with a DNA-specific fluorescent dye showed that the sex chromosomes formed a cone-shaped element at pachytene. The two chromosomes were closely associated. Concomitantly with chromatin condensation at late prophase I, a slender unstained gap developed between the large X and the tiny y chromosome, indicating that the sex chromosome pair is achiasmatic. The gap persisted in metaphase I spermatocytes. The electron microscopic analysis of ultrathin serial sections through metaphase I spermatocytes also revealed one asymmetric bivalent per metaphase plate. This bivalent contained a block of electron-dense material located between a large and a small mass of chromatin. It is plausible to assume that the asymmetric body represented the XY pair. The electron-dense mass corresponded in location to the chromosomal portion not stained by the DNA-specific dye and is believed to consist of non-chromatin material. In order to determine whether electron-dense material is routinely found in the achiasmatric sex chromosome pairs in male meiosis of beetles, primary spermatocytes of a leaf beetle, *Chrosolina graminis*, were studied as well. As in *T. molitor*, a homogeneously textured mass was detected in one bivalent, the sex chromosome pair, but in contrast to this species transparent vacuoles were scattered throughout the material in *C. graminis*.

Key words. Bivalent; chiasma substitute; darkling beetle; leaf beetle; pachytene; metaphase spermatocyte.

In conventional meiosis, homologous chromosomes pair in the early stages of prophase I and crossovers develop. Three-layered elements, the synaptonemal complexes, play a central role in this process. In late prophase I, i.e. diplotene and diakinesis, chiasmata become visible, which are responsible for the association of homologues throughout metaphase I until segregation takes place in anaphase of the first meiotic division. The physical adhesion of homologues with one another until the onset of anaphase I is required for proper meiotic segregration. When pairing fails or when homologues dissociate prematurely, univalents arise. These usually segregate independently from one another in anaphase I, and aneuploid gametes form at a high rate.

Yet there are examples of achiasmatic meiosis affecting either individual chromosome pairs or the entire chromosome complement of one sex. Recently, a total of 6 modes has been described by which meiocytes deal with achiasmatric chromosomes in meiosis I [1]. According to this classification, achiasmatic bivalents can be formed in four different ways. These are through modified synaptonemal complexes that persist beyond pachytene, adhesion of non-sister chromatids comparable to somatic pairing, 'stickiness' of heterochromatin, and through distinct 'segregation bodies' consisting of material structurally different from chromatin. In specific cases, the spindle appears to be able reliably to segregate univalents, which are not directly physically linked with one another. Finally, amphitelic orientation (for terminology, see ref. 2) of univalents at metaphase I and pairing of the chromatids in meiosis II appear to ensure correct segregation as well. For further details of achiasmatic meiosis see ref. 1.

To the Coleoptera, an insect order with more than 350,000 described species, sex chromosomes in males often show a particular configuration at different meiotic stages. Cytogenetic studies have revealed a large X chromosome adjacent to or associated with the tiny Y chromosome at metaphase I. In between the X and Y chromosomes, an area of Feulgen-negative material was observed [3]. A specific terminology has been developed for this heteromorphic XY association. The small size of the Y chromosome is commonly indicated by using a small letter (y). The entire complex is reminiscent of a parachute and, therefore, the term Xy_p (p = parachute) is customary. The fine structure of the sex chromosomes in pachytene cells has been described in some Coleoptera species [4], but as far as I am aware, information on the ultrastructure of the Xy_p chromosome aggregate of beetles in metaphase I spermatocytes is not available. This information is necessary to decide whether the achiasmatic sex chromosome association in beetles follows one of the above mentioned mechanisms.



Figures 1 to 4 represent light micrographs of chromosome spreads prepared from testicular material of *Tenebrio molitor*. The preparations were stained with DAPI. All the micrographs are printed at the same magnification. Bar represents 5 μm.
Figure 1. Metaphase spermatogonium. A total of 20 chromosomes is visible. The tiny y chromosome is marked (arrowhead). Primary contrictions are not detectable.
Figure 2. Late prophase I spermatocyte. The autosomal bivalents consist of brightly stained heterochromatin and weakly labelled euchromatin. The sex chromosome pair is entirely heterochromatic and marked (arrowhead).
Figure 3. Pachytene spermatocyte. The autosomes possess large heterochromatic portions flanked by weaker stained euchromatic segments. The sex chromosome pair forms a cone-shaped body (arrowhead).
Figure 4a–c. Metaphase I spermatocytes. The autosomes uniformly appear as dumbbell-shaped bodies, while the sex chromosome pair forms an asymmetric element (arrowheads) with – owing to the advanced condensation – the tiny y chromosome barely detectable.

In order to fill this gap, I have selected two species of two different families for an analysis of the sex chromosome pair at metaphase I. The species were the mealworm, *Tenebrio molitor* (Tenebrionidae), and the leaf beetle, *Chrysolina graminis* (Chrysomelidae). The karyotypes of both species are known. *T. molitor* possesses nine autosomal chromosome pairs and an Xy_p complex in the male sex [e.g. 5, 6]. All chromosomes have large blocks of pericentromeric heterochromatin [5, 7, 8]. Males of *C. graminis* have 11 autosomal bivalents and there is also an Xy_p sex chromosome complex [9].

Materials and methods

Animals. Animals of a wild-type laboratory strain of *T. molitor* were reared on rolled oats [cf. 10, 11]. Adults of *C. graminis* were collected in the field about 1 km west of Groß Wesenberg (Schleswig Holstein, Germany) in May of 1994, transferred to the laboratory, and prepared for electron microscopy the next day (see below).
Chromosome preparations. Testes were dissected from last instar larvae and young pupae of *T. molitor* in a saline solution [12] and fixed (10 min) in a mixture of ethanol, chloroform and acetic acid (6:3:1). The testes were then minced on a microscopic slide in a droplet of 70% acetic acid. The cell suspension was placed on a hot plate for about 1 min, allowing part of the droplet to evaporate. Remaining acetic acid was discarded and the coverslips were air-dried [13]. Chromatin was stained with 5 μg/ml DAPI (4′,6-diamidino-2-phenylindole · 2 HCl) (Serva) dissolved in McIlvain buffer (100 mM citric acid, 200 mM Na_2HPO_4, pH 7). The preparations were analysed and photographed using a Laborlux 12 photomicroscope equipped with epifluorescence illumination and a Fluotar ×100 objective (Leitz, Wetzlar).
Electron microscopy. Testes of young pupae (*T. molitor*) or adults (*C. graminis*) were prepared for electron microscopy according to ref. [12]. In brief, the dissected gonads were transferred to 1 ml Ringer solution containing 2.5% glutaraldehyde. After 5 min, 3 ml of 8% tannic acid (Merck) in phosphate buffer (0.067 M, pH 6.8) were added. The gonads were postfixed in phosphate-buffered OsO_4 (1%, 1 h), dehydrated in ethanol and embedded in Epon 812. Series of ultrathin sections, about 70 nm thick, were analysed with a Philips EM 400 transmission electron microscope operated at 80 kV.

Results

Light microscopy. The analysis of spermatogonial chromosome complements of *T. molitor* confirmed previous observations [5–7]. A total of 20 chromosomes was counted. One element was much smaller than the other chromosomes and this represented the y chromosome (fig. 1). Neither previous cytogenetic work involving the mealworm (see above) nor this study revealed distinct

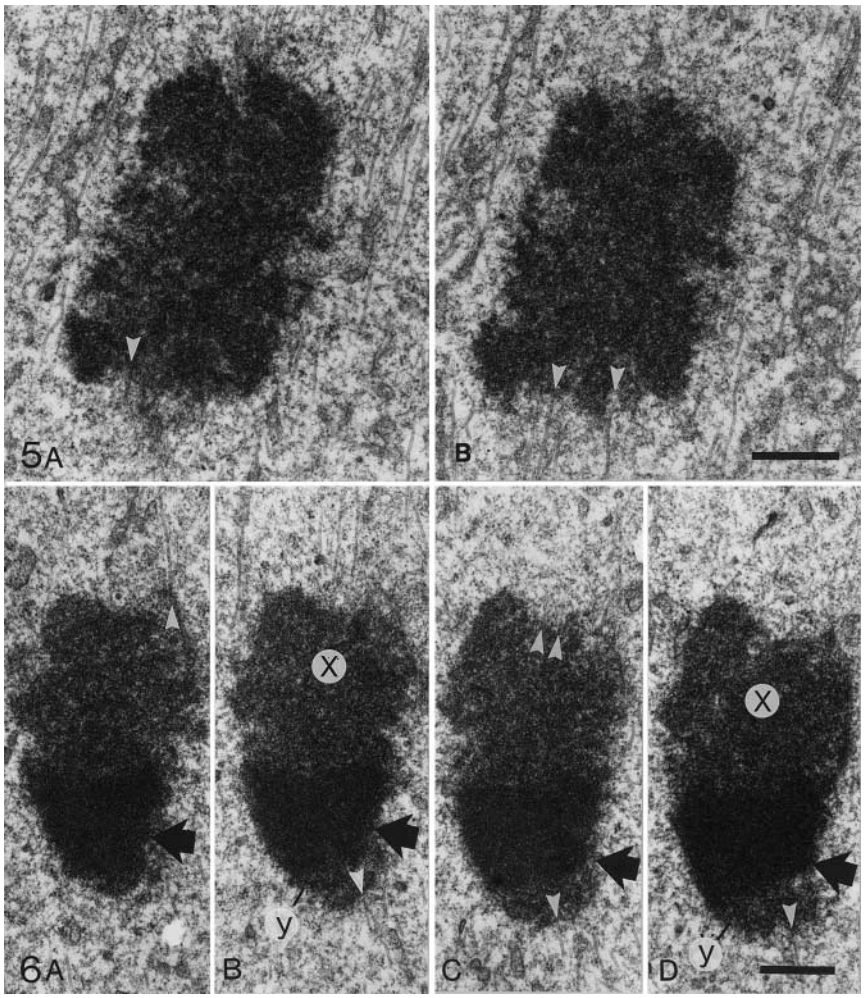


Figure 5. Electron micrographs of two consecutive serial sections through an autosomal bivalent in metaphase I of *Tenebrio molitor*. The bivalent is symmetrical and spindle microtubules are connected with both chromosomal surfaces (arrowheads). Bar represents 0.5 μm.

Figure 6. Electron micrographs of four consecutive serial sections through the sex chromosome pair of *Tenebrio molitor*. The X chromosome (X) is connected with the y chromosome (y) by a homogeneously staining block of electron-dense material (arrows). Spindle microtubules are visible at the poleward chromsomal surfaces (arrowheads). Bar represents 0.5 μm.

primary constrictions as we expect to see in monokinetic chromosomes (fig. 1). All chromosomes appeared as straight or slightly curved rods. The autosomes of pachytene spermatocytes consisted of brightly stained segments, the heterochromatin, flanked by weakly stained portions, the euchromatin (fig. 3). The sex chromosome pair was cone-shaped and appeared entirely heterochromatic in this type of preparation (fig. 3). The sex chromosomes were closely attached to one another. At the time of the late prophase I chromatin condensation, the sex chromosome pair developed an unstained gap located in an asymmetric position (fig. 2). This gap is referred to as the 'DAPI-negative region' in the present text. Condensation of the autosomal bivalents was advanced, but euchromatin and heterochromatin could still be distinguished in late prophase I cells (fig. 2). This did not apply to metaphase I chromosome complements. The 9 autosomal bivalents formed symmetrical dumbbell-shaped bodies, which were brightly stained and had an equatorial weakly-stained gap (fig. 4a–c). This gap signalled the adhesion site between the homologous chromosomes. It clearly did not represent a primary constriction, because the bivalents were oriented with their long axes parallel to the pole-to-pole axes of the metaphase I spindles (see below). The sex chromosome pair formed an asymmetric body. The tiny y chromosome was separated by a DAPI-negative region from the large X chromosome (fig. 4a–c).

Electron microscopy. The present study was concerned with the structure of the sex chromosome pair in *T. molitor*. For information on spindle structure and assembly and the processes of meiosis and spermiogenesis in this beetle the reader is referred to previous articles of the author. It should be added that the first meiotic spindles show a conventional bipolar design [10, 11]. The analysis of a serially sectioned metaphase I chromosome plate revealed that the autosomal bivalents were cylinders oriented with their long axes parallel to

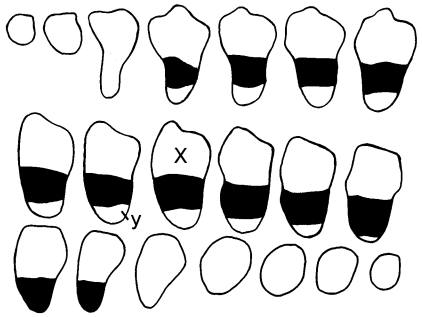


Figure 7. Twenty profiles of the sex chromosome complex in a metaphase I spermatocyte of *Tenebrio molitor*. The electron-dense block located between X and y chromosomes is depicted in black. The third profile (top row) represents a grazing section through the slender portion of the sex chromosome pair. Owing to this technical factor, the presence of electron-dense material was not clearly detectable in this particular section.

the spindle axis (fig. 5A, B). In this case the metaphase plate was fully recorded. Although the chromosomal surface was slightly sculptured in a region halfway between the poleward surfaces, the site of adhesion between the homologues could not be detected in the chromosome of some metaphase plates. It was, however, clearly visible in others [cf. ref. 14]. The presence of early or late metaphase I spermatocytes probably accounts for this variation. In contrast to the autosomal bivalents, which were symmetrical bodies, the sex chromosome pair of metaphase I spermatocytes was blunt at one end and rounded at the opposite end. This was clearly detectable in individual sections (fig. 6A–D) and confirmed in a full series throughout the sex chromosomes complex (fig. 7). Additionally, a homogeneous electron-dense mass was found in an asymmetric position within the sex chromosome pair. The location of this material was interpreted as corresponding to the DAPI-negative region in meiotic chromosome spreads (see above). Thus, it represented non-chromatin material. Furthermore, the large chromatin body at one side of the electron-dense mass constituted the X chromosome, while the chromatin element at the opposite face represented the tiny y chromosome (fig. 6A–D). The analysis of a complete series of ultrathin sections through the sex chromosome pair showed that the electron-dense mass was present throughout the entire chromosomal width and apparently linked X and y chromosomes (fig. 7). The architecture of the sex chromosome aggregate was confirmed in two other metaphase I spermatocytes, but in these cells only the sex chromosomes were recorded in micrographs.

In order to determine whether electron-dense material is of wider occurrence in sex chromosome pairs of the Xy_p type in beetles, metaphase I spermatocytes of another species, *C. graminis*, were also studied using transmission electron microscopy. The gross morphology of the sex chromosome pair of two primary spermatocytes of this leaf beetle was as in *T. molitor*. This similarity included the size difference between X and y chromosomes. The two species differed, however, in that the electron-dense mass of *C. graminis* showed vacuoles of varying size. Some were prominent and had a diameter of about 400 nm (fig. 8A–D).

Discussion

The behaviour of the sex chromosome pair was followed in male meiosis of *T. molitor* using fluorescence microscopy. The analysis of chromosome spreads stained with a DNA-specific fluorescent dye (DAPI) confirmed that the mealworm possesses an Xy_p sex chromosome complex typical of many beetles [3] and that all chromosomes have large blocks of heterochromatin [5, 7, 8]. The sex chromosomes appeared entirely heterochromatic in male meiosis. In pachytene, the sex chromosomes were closely associated and formed a cone-shaped element. Heterochromatin is known to participate in a variety of heterologous associations in vertebrates and inverte-

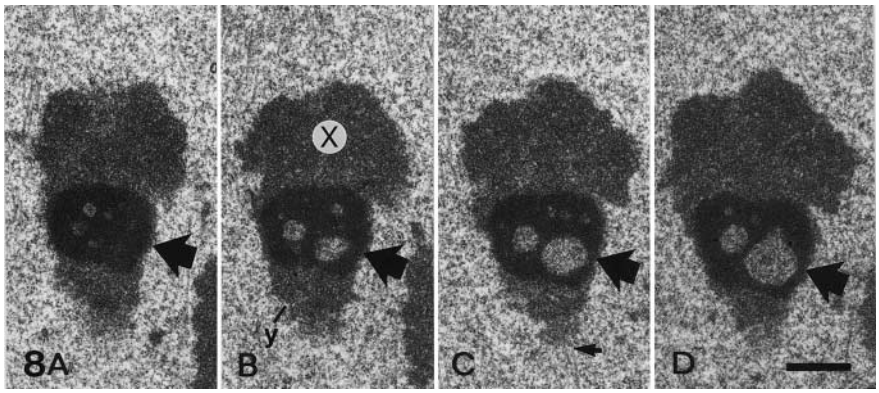


Figure 8. Electron micrographs of four consecutive serial sections through the sex chromosome pair of *Chrosolina graminis*. The X chromosome (X) is connected to the tiny y chromosome (y) by a block of electron-dense material (large arrows). Vacuoles with a content slightly more electron-dense then the spindle matrix are scattered throughout the electron-dense block. A small arrow marks material of lesser electron density at the poleward chromosomal surface of the y chromosome, where spindle microtubules are inserted. Bar represents 0.5 μm.

brates [e.g. refs. 15–18]. It is a strong possibility that heterochromatin located in both chromosomes is responsible for the initial close association between X and y chromosomes in male meiosis of *T. molitor*.

It is only during diplotene and diakinesis of *T. molitor* that a DAPI-negative region becomes detectable between X and y in chromosome preparations. Thus, the close association between these chromosomes does not persist. Furthermore, the failure to detect DAPI-stained material in the region between the sex chromosomes strongly suggests that there is no chiasmatic association between them. The width of the DNA-free region between X and y chromosomes in male meiosis of beetles appears to vary depending on the species. The gap is small in *T. molitor* (this study) and relatively wide in *Tribolium castaneum*, another darkling beetle examined recently [19]. The consistent proximity of the sex chromosomes in late prophase I through metaphase I of *T. molitor* indicates that they are still physically linked. The ultrastructural study of metaphase I spermatocytes showed that the space between them contains an electron-dense mass. This does not stain with DAPI and therefore represents non-chromatin material. Thus, the Xy_p sex chromosome pair of *T. molitor* possesses a 'segregation body' according to a recent classification of achiasmatic chromosome adhesions [1], and this serves as a chiasma substitute to ensure proper disjunction at meiosis I.

The idea that heterochromatin is involved in the association of the sex chromosomes in male prophase I of beetles has been advanced by previous workers in the field [20]. The satellite DNA of *T. molitor* constitutes up to 60% of its genome and contains a 142 base pair repeat which is present in all chromosomes. Individual repeats do not show much variation, indicating a mechanism acting to maintain the homogeneity of the satellite DNA [21–23]. A function in sex chromosome association during the early stages of meiosis in males could well be a selecting factor that keeps satellite DNA conserved in the sex chromosome pair. Repetitive DNA certainly plays a role in the organization of the centremeres of autosomes, and this could explain the homogeneity of the satellite DNA in autosomes.

The precise nature of the non-chromatin material found between X and y chromosomes in metaphase I spermatocytes of *T. molitor* is not known. Its appearance in ultrathin sections of conventionally embedded testes does not provide much information. It is more electron-dense than the chromatin and this suggests that it is highly osmiophilic and/or has a high affinity to heavy metals used to stain the ultrathin sections. Both sex chromosomes of *T. molitor* possess nucleolus-organizing regions (NORs) in subtelomeric positions of the X and y chromosomes [24] and the possibility exists that the non-chromatin material is of nucleolar origin, as has previously been assumed [20]. The composition of the DNA-free region of the Xy_p complex has been assessed in a series of weevils. The authors [25] determined that it contains argyrophilous proteins. The presence of RNA within the achiasmatic sex bivalents was rendered unlikely because treatments known to extract RNA, namely ethanolic barium hydroxide or perchloric acid, did not affect the argyrophily of the Xy_p complex. Unfortunately, staining techniques involving silver nitrate are not particularly specific and the use of refined cytochemical methods in these experiments is overdue. Extraction with an RNAse and/or proteinases appears to be a promising approach in the future. In metaphase I, the non-chromatin material forms a thick plate between the sex chromosomes. This indicates that direct interaction of DNA elements, as suggested for the XY pairing in the achiasmatic male meiosis of *Drosphila melanogaster* [26], is not necessary to ensure proper segregation.

The fine structure of the achiastmatic sex bivalent in metaphase I spermatocytes of *C. graminis* was essentially similar to that in *T. molitor*. The tiny y chromosome was separated by electron-dense material from the voluminous X chromosome. Thus, the present fine structure study confirmed a previous cytogenetic analysis showing an Xy_p complex in this beetle [9] and this type of sex chromosome association is probably the rule in the genus *Chrysolina* [27, 28]. The presence of vacuoles in the material linking the two sex chromosomes of *C. graminis* and their absence in *T. molitor* should not be overlooked. It may indicate that the chemical composition of the 'segregation body' varies in a species-specific manner. It cannot be excluded, however, that variation in fine structures simply reflects differences in the response to preparation between both species.

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