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Synapsis with and without recombination in the male meiosis of the leaf-footed bug *Holhymenia rubiginosa* (Coreidae, Heteroptera)

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Abstract In organisms with chiasmatic meiosis two different relationships have been described between crossing over and synapsis: in one group of organisms synapsis depends on the initiation of meiotic recombination while in the other group it is independent of this initiation. These patterns have been observed mainly in organisms where all meiotic bivalents in the set have similar behaviors. In some heteropteran insects a pair of chromosomes named m chromosomes is known to behave differently from autosomes regarding synapsis and recombination. Here we used immunodetection of a synaptonemal complex component and acid-fixed squashes to investigate the conduct of the small m chromosome pair during the male meiosis in the coreid bug *Holhymenia rubiginosa*. We found that the m chromosomes form a synaptonemal complex during pachytene, but they are not attached by a chiasma in diakinesis. On the other hand, the autosomal bivalents synapse and recombine regularly. The co-existence of these variant chromosome behaviors during meiosis I add further evidence to the absence of unique patterns regarding the interdependence of synapsis and recombination.

Keywords Heteroptera · Holokinetic chromosomes · Meiosis · Recombination · Synapsis

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Introduction

During meiosis homologous chromosomes align in a process that involves two phases: a pairing stage where homologs recognize and reach a rough alignment and a synapsis stage where paired homologs are aligned precisely and held together by a specialized structure, the synaptonemal complex (SC). Pairing involves a long-distance recognition independent of the formation of SCs or other obvious physical connections. On the other hand, synapsis is defined by the formation of an SC in most organisms (reviewed in Zickler and Kleckner 1999; Kleckner 2006). In most meiotic systems organisms that form synaptonemal complexes also have chiasmatic meiosis. However, classic studies in lepidopteran females and other organisms demonstrated that the formation of the SC can occur in the absence of crossing over (Rasmussen 1977; Traut 1977; reviewed in von Wettstein et al. 1984). More recently, it has been shown that SC formation in budding yeast and mammals depends on the function of Spo11 that introduces the double-strand breaks (DSBs) that initiate recombinational interactions between the homologous chromosomes (Keeney et al. 1997; Romanienko and Camerini-Otero 2000). On the other hand, synapsis occurs normally in the absence of DSBs in *Drosophila* females and in *Caenorhabditis elegans* (Dernburg et al. 1998; McKim et al. 1998). Altogether, these evidences from naturally occurring meiotic variants and from experimental systems point to the existence of different requirements for SC formation in different organisms.

Heteropteran insects constitute suitable models to obtain further insight in the processes and relationships between synapsis and recombination. Bugs possess holokinetic chromosomes and classic cytogenetic studies have described different chromosome types with different behavior

in male meiosis (Ueshima 1979). Autosomes are generally chiasmatic and segregate reductionally during anaphase I, while sex chromosomes are achiasmatic, behave as univalents and generally divide equationally during the first meiotic division. In correlation with this behavior of autosomes and sex chromosomes it has been shown in species from different families that the autosomes form SCs while the sex chromosomes—XY and X_1X_2Y systems—do not have typical meiotic axes. Furthermore, the meiotic cohesin REC8 is present on autosomal SCs but not on the sex chromosomes (Pigozzi and Solari 2003) that also lack the main SC component SCP3 (Suja et al. 2000). In addition to the differential behavior of autosomes and sex chromosomes in spermatocytes, another feature that makes unique the meiotic system of Heteroptera is the presence of one chromosome pair called ''m chromosomes''. The m chromosomes were originally described by Wilson (1909) in species of Coreidae referring to the smallest autosomal pair which behaves differently from both autosomes and sex chromosomes during male meiosis. At present they have been reported in 14 out of 45 families of Heteroptera. The m chromosomes have been described as asynaptic and achiasmatic during male meiosis after conventional staining of squashed spermatocytes. During diakinesis they approach each other and at metaphase I they are invariably associated end-to-end (touch-and-go pairing) forming a pseudo-bivalent that segregates reductionally at anaphase I. Although early reports described the m chromosomes as the smallest pair of the complement, it is their special meiotic behavior and not its size that defines them. The only exception to the lack of synapsis and chiasmata in the m chromosomes has been described in the coreid bug *Coreus marginatus* where some male meiotic cells showed a small SC corresponding to the m chromosome pair, that later appears as a chiasmatic bivalent in diplotene (Nokkala 1986; Suja et al. 2000).

Confirmation of the synaptic behavior of the m chromosomes during male meiosis is scarce because in most species they are very small in size, isopycnotic during early prophase I and therefore undistinguishable from leptotene to the beginning of diplotene. To overcome this difficulty we used an antibody against the cohesin subunit SMC3 to observe the meiotic axes during the meiotic prophase I in spermatocytes of *Holhymenia rubiginosa*, a coreid species carrying one pair of small m chromosomes. In addition, we analyze the behavior of autosomes, m chromosomes and the sex chromosome during post-diplotene stages using acid-fixed squashes. We found that the m chromosomes of this species synapse regularly during pachytene and are achiasmatic in diakinesis, while autosomes and the sex chromosome X have the typical features described for other Heteroptera regarding axial element formation and recombination.

Materials and methods

Materials

Nymphs (23 specimens) and adults (25 specimens) of *Holhymenia rubiginosa* Breddin were collected in natural populations in Buenos Aires province.

Haematoxylin staining

Testes were dissected out and fixed in 6:3:1 (ethanol: chloroform: glacial acetic acid). Slides were performed by the squash technique in a drop of acetic haematoxylin.

Immunostaining and SC spreads

Testes were dissected and processed according to a surface-spreading procedure described by Suja et al. (2000) with minor modifications. Preparations were dried briefly at room temperature, rinsed in 0.4% Photoflo and then air-dried. Slides were used immediately for immunostaining or kept at –70°C for later use. Immunostaining was performed as previously described (Pigozzi and Solari 2003) using a polyclonal antibody against SMC3 raised in rabbit (Chemicon) to label the SCs followed by detection with FITC-labeled goat-anti-rabbit. Slides were mounted in Vectashield with DAPI as counterstain and examined in a Leica fluorescence microscope equipped with a CCD camera. Some spreads were stained using a colloidal silver method for the observation of meiotic axes at the light microscope.

Results

Meiotic chromosomes in haematoxylin-stained squashes

The diploid chromosome number is 2n = 24 + 2m + X0 (Fig. 1a). During early prophase I the X chromosome is detected as a positively heteropycnotic body close to the nuclear periphery (Fig. 1b–d). After pachytene the chromatin decondenses during a short diffuse stage (Fig. 1d); as chromosomes recondense the 12 bivalents and the two m chromosomes can be recognized (Fig. 1e–g). The m chromosomes can be identified from late diplotene/diakinesis, when they are always observed separated (Fig. 1f, g). As meiosis proceeds the m chromosomes approach and associate in a pseudo-bivalent. At metaphase I autosomal bivalents arrange in a ring, the m pseudo-bivalent lies at its center and the X univalent is observed outside it (Fig. 1h). At anaphase I autosomes and the m chromosomes segregate reductionally and the X chromatids divide

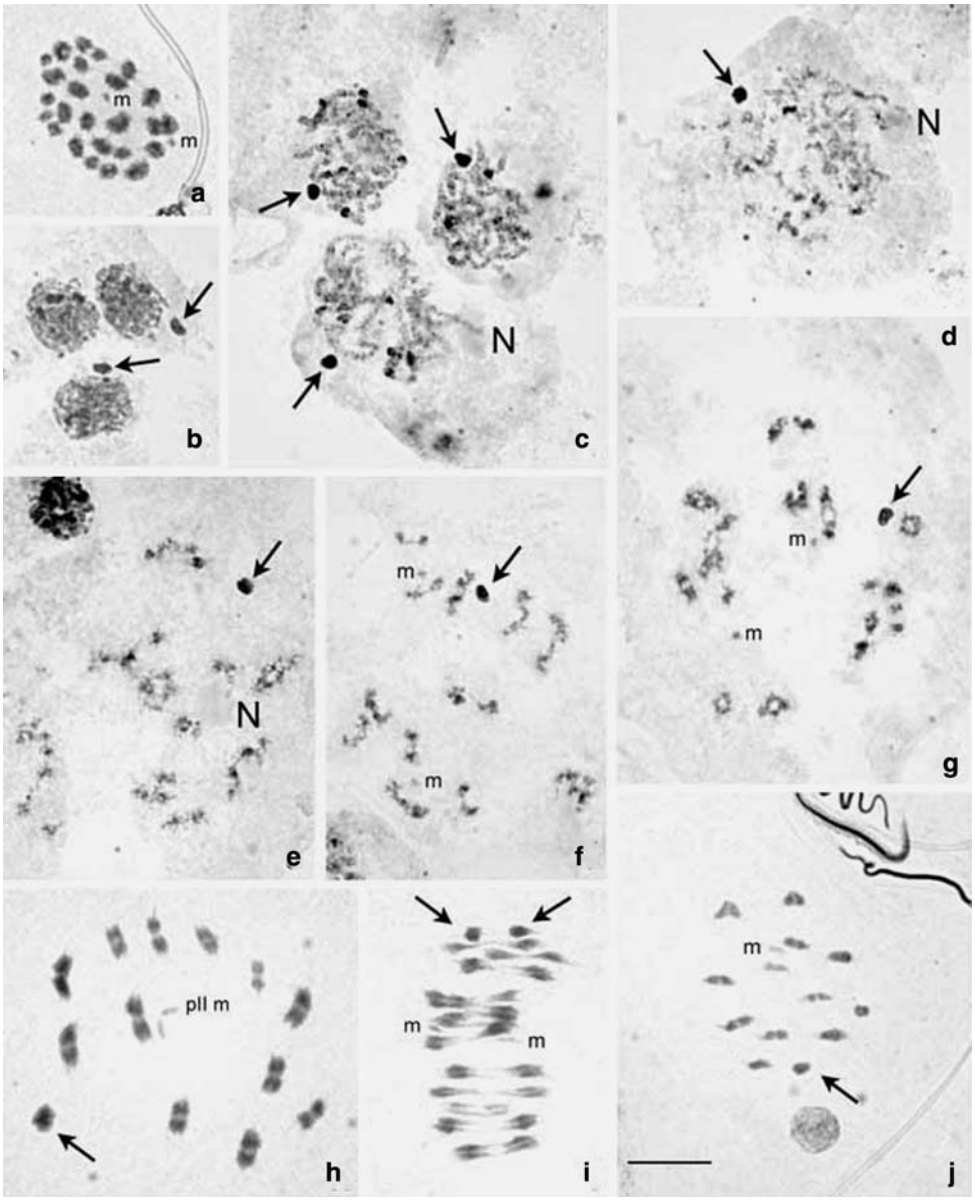

Fig. 1 Haematoxylin stained male meiotic cells. (**a**) Spermatogonial metaphase. (**b**) Leptotene–Zygotene. (**c**) Pachytene. (**d**) Diffuse stage. In **b–d** the positively heteropycnotic X univalent lies at the nuclear periphery. (**e**) Early Diplotene. (**f**) Late Diplotene. The m chromosomes are separated and negatively heteropycnotic. (**g**) Early Diakinesis. The m chromosomes are still separated. (**h**) Metaphase I. The m chromosomes are associated in a pseudobivalent (pII m). (**i**) Anaphase I. (**j**) Metaphase II. Arrows point at the sex chromosome; m = m chromosomes; N = nucleolus. Bar 10 μm

equationally (Fig. 1i). Second meiotic division follows without an interkinetic stage, and a similar metaphase arrangement is observed: the autosomes form a ring, the m chromosomes lie at its center and the X lies outside it (Fig. 1j).

Immunolocalization of SMC3 in spermatocytes

SMC3 localizes to the axial elements since early prophase allowing the recognition of stages from leptotene to diplotene. During leptotene SMC3 labels the axial elements with a discontinuous pattern (Fig. 2a). From zygotene on, the formation of a central element cannot be directly ascertained in this species because the evolutionary divergence of structural SC components prevents their recognition in insects by antibodies to the homologous mammalian proteins (reviewed in Page and Hawley 2004). However, the presence of thin and thick threads of SMC3 can be correlated to unsynapsed and synapsed axes, as shown in immunostained spermatocytes of grasshoppers (Viera et al. 2004; Calvente et al. 2005). In *H. rubiginosa* typical zygotenes, that is, thin SMC3-labeled axes diverging from thick synaptic segments, were not observed. Instead, zygotene nuclei show discontinuos axial labeling, with the thicker stretches of SMC3 representing partially synapsed bivalents (Fig. 2b). By early pachytene, most bivalents completed the formation of their synaptonemal complexes with few gaps lacking SMC3 labeling (Fig. 2c). When SC formation is complete, autosomal SCs are shorter, fully labeled and the m chromosomes can be

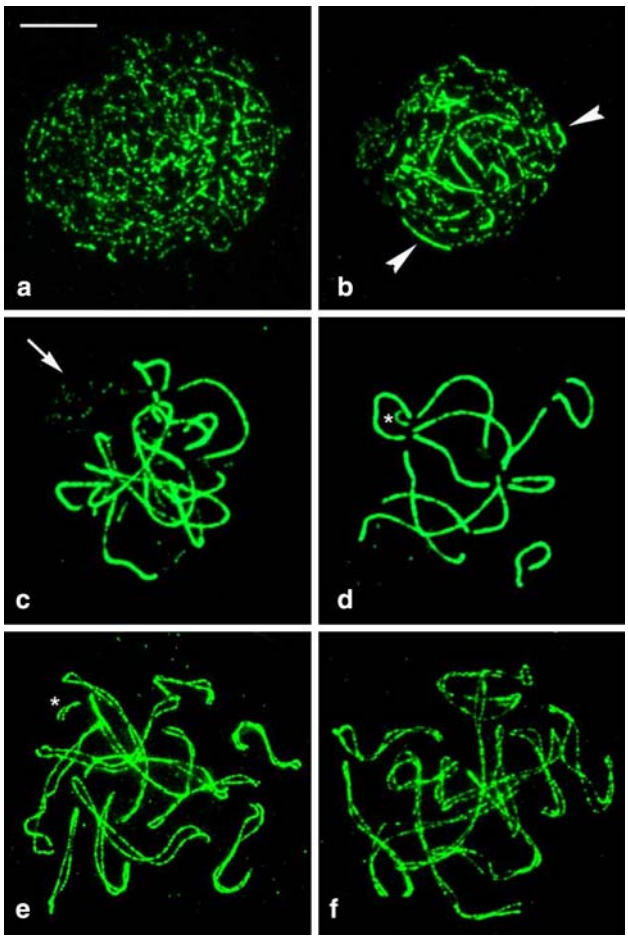

Fig. 2 Immunodetection during meiotic prophase. (**a**) Leptotene. Short stretches of meiotic axes are labeled with SMC3. (**b**) Zygotene. Thick labeled filaments represent the synapsed lateral elements (arrowheads). Notice the discontinuous labeling of the asynapsed axial elements. (**c**) Early pachytene. Most bivalents have formed their synaptonemal complexes but there are unlabeled gaps along some of them. Some faint SMC3 dots are on the chromatin of the single X chromosome (arrow). (**d**) Late pachytene. Twelve fully labeled SCs are recognizable that correspond to autosomal bivalents. The shorter SC (asterisk) corresponds to the m chromosomes that are synapsed in this nucleus. (**e**) Diplotene. The autosomal axial elements begin to separate. The m chromosomes are still together (asterisk). (**f**) Diplotene. Individual bivalents cannot be recognized with certainty and the axial elements are discontinuous. Bar10 μm

recognized as the smallest SC (Fig. 2d) or as separated axes (see below). Nuclei going through the brief diffuse stage that occurs in this species after pachytene could not be related to a particular SMC3 pattern. Some nuclei were observed in the spreads showing a cloudy FITC staining which may represent the diffuse stage. These nuclei showed a solid staining pattern with DAPI in the PFA-fixed spreads used for immunostaining, preventing a direct comparison to the appearance of the chromatin observed during this stage in acid-fixed spermatocytes. At the beginning of diplotene, the autosomal bivalents show again continuous axial elements that appear in slight repulsion, and the m chromosomes appear as a bivalent that starts to separate (Fig. 2e). In a large number of diplotene nuclei the labeling of the axial elements was fainter and discontinuous preventing the recognition of individual autosomal bivalents and the m chromosomes (Fig. 2f). It was not possible to determine if these nuclei represent an early diplotene substage, when the axial elements are reforming after the diffuse stage or if they show the final disassembling of the meiotic axes before diakinesis. No SMC3 labeling was detected in diakinesis/metaphase I. Besides a few faint spots, no SMC3 labeling was observed associated to the X chromosome (Fig. 2c). Silver stained pachytenes showed the autosomal and the m chromosome axes but no axial core could be identified associated to the X chromatin mass (not shown).

The synaptic or asynaptic state of the m axes during pachytene was scored in a total of 60 nuclei where the m chromosome axes could be distinctively recognized (Fig. 3a, b). In 30% of these nuclei the m axes were not synapsed, and in the rest they were forming a small SC. In diplotene nuclei with identifiable m chromosomes the axes were always together (Fig. 2e). Thus, it is likely that pachytenes showing separated m chromosomes represent an early substage and that the m chromosomes complete their synapsis regularly but later, asynchronously respect to the autosomal bivalents.

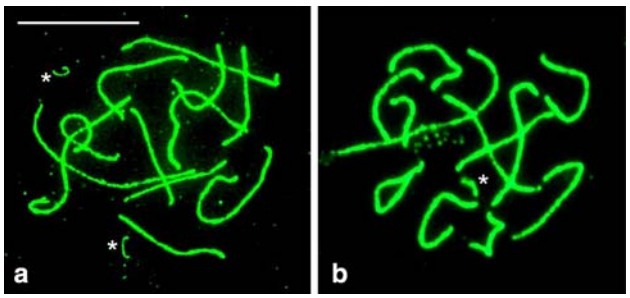

Fig. 3 The m chromosomes during pachytene. (**a**) Asynaptic m chromosomes (asterisks) in a pachytene nucleus. Notice the thinner SMC3-labeled axes of the asynapsed m chromosomes compared to the autosomal SCs. (**b**) Pachytene showing synaptic m chromosomes. The SMC3-labeled axes of autosomes and m chromosomes have comparable thickness. Bar 10 μm

Discussion

In this paper we described the behavior of the meiotic axes from leptotene to diplotene in spermatocytes of *Holhymenia rubiginosa* using immunodetection of the cohesin component SMC3, with special attention to the behavior of the m chromosomes. This procedure gives excellent details about axial element formation before pachytene and also

gives clear labeling of the axes in diplotene, which have not been observed previously in SC spreads of heteropterans with immunodetection of other SC components or with silver staining.

We have shown that in *Holhymenia rubiginosa* within the same meiotic nucleus regular synapsis and segregation of homologous chromosomes occur with crossing over and in its absence. While autosomes align and form bivalents conjoined by synaptonemal complexes, the smallest chromosomes in the set or m chromosomes, synapse regularly in pachytene but they are invariably separated during diakinesis, when autosomes are forming bivalents and show chiasmata. The X chromosome, which divides equationally at anaphase I, lacks a cohesin axis in agreement with observations in other heteropterans with the typical inverted sex-chromosome behavior (Solari 1979; Suja et al. 2000; Pigozzi and Solari 2003). These findings indicate that heteropterans with synaptic m chromosomes have developed mechanisms that act during male meiosis that are able to control simultaneously: (1) SC formation between recombining chromosomes (regular autosomes); (2) SC formation and disjunction without crossing over (m chromosomes) and, (3) disjunction in the absence of SCs and crossing over (sex chromosomes).

Most reports on the behavior of the m chromosomes during male meiosis in Heteroptera described them as asynaptic and achiasmatic. An exception was observed in a detailed analysis of the m chromosomes of *Coreus marginatus* (Nokkala 1986), where a small bivalent, corresponding to the synapsed m chromosomes, is present at the base of the pachytene bouquet in male meiosis. The m chromosomes desynapse later in some spermatocytes leading to the occurrence of m chromosome univalents (achiasmatic m-chromosomes), but they remain together in some nuclei during diplotene-diakinesis (chiasmatic m-chromosomes). The synaptic behavior of the m chromosomes in some spermatocytes of *C. marginatus* was confirmed by electron microscopy observations of SC spreads, along with the occurrence of univalents and bivalent m chromosomes at diplotene/diakinesis (Suja et al. 2000). Finally, the m chromosomes of *C. marginatus* are always associated during metaphase I and segregate reductionally during anaphase I like the other autosomal bivalents. Differently from these observations in *C. marginatus*, we did not find different spermatocytes populations with chiasmatic and achiasmatic m bivalents in *H. rubiginosa*. In this species the m chromosomes form regularly a synaptonemal complex during pachytene and they remain associated in early diplotene nuclei. The observation that they are always separated at diakinesis supports the idea that synapsis occur in the absence of crossing over in the m chromosome pair of *H. rubiginosa*. This achiasmatic behavior is followed by the regular formation of a pseudobivalent during metaphase I that ensures the regular segregation of the m chromosomes during the first division. These results show that, in the same meiotic nucleus, different chromosome pairs display regular synapsis and segregation with crossing over and in its absence. A similar phenomenon is observed in *D. melanogaster* females where the tiny fourth chromosome lacks crossing over but it still forms an SC like the recombining autosomal pairs (Carpenter 1975). In fact, synapsis can be accomplished in *Drosophila* oocytes in complete absence of meiotic exchanges, as shown by the formation of normal SCs in meiotic mutants which eliminate crossing over (McKim et al. 1998). So far there are no molecular or genetic evidences regarding the requirements for pairing and synapsis in heteropterans, but the formation of an SC between the non-recombining m chromosomes of *H. rubiginosa* suggests that crossing over might not be a pre-requisite for SC formation in some heteropteran species. If this is a phenomenon restricted to some m chromosome pairs or it is a more extended feature among Heteroptera, could be clarified with SC analysis in bugs with achiasmatic male meiosis (Ituarte and Papeschi 2004; reviewed in Papeschi and Bressa 2006).

The observation of regular synapsis of the m chromosomes in *H. rubiginosa* suggests that the original definition of m chromosomes as the smallest chromosome pair of the complement, asynaptic and achiasmatic should be reconsidered. Species with m chromosomes of relatively large size have already been described (Bressa et al. 2005) and synapsis could be a common feature that remains unnoticed because of the difficulties to identify the m chromosome pair during early meiosis in conventional chromosome preparations. Further clarification is also needed concerning the existence or not of recombining m chromosomes in different species since at least in *Coreus marginatus* they are chiasmatic, at least in some meiotic cells.

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