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CHROMOSOMA

C-banding and non-homologous associations

II. The "parachute" Xy_p sex bivalent and the behavior of heterochromatic segments in *Epilachna paenulata*

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Abstract. A chromosome complement formed by 16 autosomes and an Xy_p sex chromosome system was found in *Epilachna paenulata* Germar (Coleoptera: Coccinellidae). All autosomes were metacentric except pair 1 which was submetacentric. The X and the Y chromosomes were also submetacentric but the Y was minute. The whole chromosome set carried large paracentric heterochromatic C-segments representing about 15% of the haploid complement length. Heterochromatic segments associated progressively during early meiotic stages forming a large single chromocenter. After C-banding, chromocenters revealed an inner networklike filamentous structure. Starlike chromosome configurations resulted from the attachment of bivalents to the chromocenters. These associations were followed until early diakinesis. Thin remnant filaments were also observed connecting metaphase I chromosomes. Evidence is presented that, in this species, the Xy_p bivalent resulted from an end-to-end association of the long arms of the sex chromosomes. The "parachute" Xy_p bivalent appeared to be composed of three distinct segments: two intensely heterochromatic C-banded corpuscles formed the "canopy" and a V-shaped euchromatic filament connecting them represented the "parachutist" component. The triple constitution of the sex bivalent was interpreted as follows: each heterochromatic corpuscle corresponded to the paracentric C-segment of the X and Y chromosomes; the euchromatic filament represented mainly the long arm of the X chromosome terminally associated with the long arm of the Y chromosome. The complete sequence of the formation of the Xy_p bivalent starting from nonassociated sex chromosomes in early meiotic stages, and progressing through pairing of heterochromatic segments, coiling of the euchromatic filament, and movement of the heterochromatic corpuscles to opposite poles is described. These findings suggest that in *E. paenulata* the Xy_p sex bivalent formation is different than in other coleopteran species and that constitutive heterochromatic segments play an important role not only in chromosome associations but also in the Xy_p formation.

Introduction

Nonhomologous chromosome association involving several or all chromosomes in a diploid cell seems to be a rather frequent process in insect meiosis. These associations are generally produced through heterochromatic segments located at the telomere region or at the paracentric area. An extreme case of a generalized end-to-end random association involving all bivalents was observed in *Gryllus argentinus* (Drets and Stoll 1974). In this cricket, manifold chromosome connections occurred through heterochromatic terminal segments which persisted until mid diakinesis. Condensed heterochromatic segments were found connecting all bivalents, a fact related to the presence of telomeric C-segments in mitotic chromosomes.

In beetles heterochromatic segments usually undergo different degrees of ectopic pairing and chromocenter formation (Virkki 1951). Similar nonhomologous pairing and chromocenter fusion were first reported in plants by Heitz (1933) who noticed the tendency of heterochromatic segments from different chromosomes to associate into large chromocenters.

In the present paper, observations on the karyotype, size and distribution of constitutive C-segments, their behavior during chromocenter formation and the organization of the Xy_p "parachute" sex bivalent in *Epilachna paenulata* Germar are described.

Material and methods

Specimens of *E. paenulata* were collected during summertime in suburban areas of Montevideo, Uruguay or in plantations of *Cucurbita pepo* and *C. maxima* usually parasitized by the insect. The animal was also successfully reared in the laboratory which allowed the study of specimens of known age the whole year round. The method used to rear *E. paenulata* consisted of introducing two or three females and one male into a flask containing fresh leaves of *C. pepo* or small pieces of the fruit (about 0.5 cm long) and a slightly humidified, sterile filter paper stacked at the bottom. Flasks were maintained at 20°–22° C and checked daily for eggs. Eggs were transferred to another flask as soon as they were laid to prevent the parents from eating them. Eclosion occurred after approximately 2 weeks, and larvae were fed with fresh leaves of *C. pepo* grown in a greenhouse. The complete larval cycle was about 5–6 weeks. Two-day-old adult male specimens were selected to obtain early meiotic stages. A high number of cells per slide was obtained by dissecting and pooling testes of 5–7 animals. Cell dispersion technique and saline solution have been reported previously (Drets and Stoll 1974), except that the trypsin step was skipped to avoid an excessive digestion and chromosome loss. Cells were fixed in methanol acetic acid (3:1) and

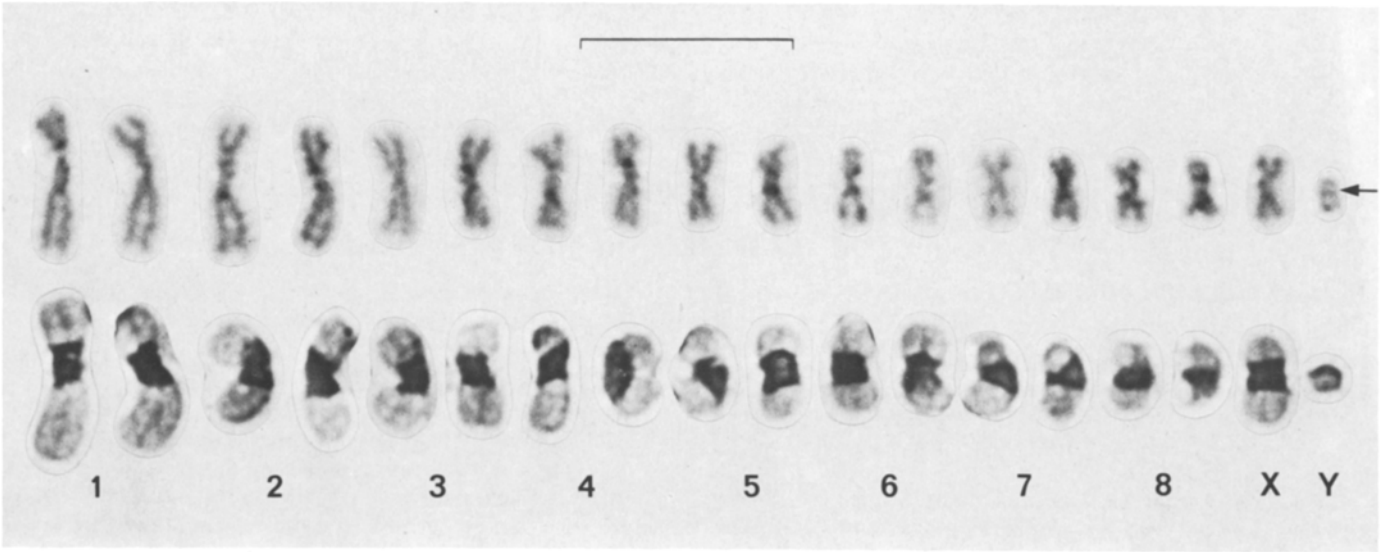


Fig. 1. Male karyotype of *Epilachna paenulata*. Upper row: Prometaphase, Giemsa staining. Practically all autosomes are metacentric. *Arrow* points to the paracentric heterochromatic segment of the Y. Lower row: Male C-banded karyotype. Most of the chromosomes show large heterochromatic segments. Bar represents 10 μm

air or flame dried. C-banding was induced according to Arrighi and Hsu (1971). Goodpasture and Bloom's technique (1975) was used to detect nucleolar organizer regions. Slides were rehydrated in ethanol (100%, 70%, 50%, 20%) for 2 min each and McIlvaine's buffer pH 4.5 for 5 min and stained either with quinacrine HCl (5 mg/ml McIlvaine's buffer pH 4.5) at 35° C for 17 min or with Hoechst 33258 fluorochrome (0.05 μg/ml PBS buffer, pH 7.0) for 10 min. Quinacrine-stained slides were washed (3 × 5 min) and mounted in McIlvaine's buffer. Hoechst-stained slides were mounted in PBS buffer.

Chromosome length measurements were made from photomicrograph enlargements (× 2000) using a curvimeter with a precision of 0.1 mm. Bright-field, phase-contrast observations and photomicrography were carried out with a Zeiss Photomicroscope II (Oberkochen) provided with Planapochromat and Neofluar phase immersion × 100 lenses. Fluorescence observations were made with BG3, BG12 excitation filters and 53/47 barrier filters. Photomicrographs of Giemsa-stained slides were taken using High Contrast Copy film (Kodak, Rochester) developed in Microdol developer (Kodak) for 9 min at 20° C. Fluorescence photomicrographs were taken on Plus X film (Kodak) and developed in D76 for 7 min at 20° C.

Results

The karyotype of *E. paenulata* was found to be 2n = 16 A + XX for females and 2n = 16 A + Xy_p for males. Seven autosome pairs were metacentric, and pair 1 was a large submetacentric. Sex chromosomes were also submetacentric, but the Y was minute, representing only 2.2% of the length of the haploid set (Fig. 1). The lower row shows the karyotype after C-banding. Large paracentric segments of constitutive heterochromatin were detected in all chromosomes. The arrow (upper row) points to the centromere region of the Y chromosome which shows a small segment of heterochromatic material stained with Giemsa. Centric hetero-

Table 1. Quantitative characteristics of the *E. paenulata* mitotic chromosomes

Pair no.	Centromere index ±0.02	Relative length (%) ±0.05 (haploid set)	Relative length (%) of C-segment (haploid set)
1	0.37	17.4	2.0
2	0.47	15.9	2.0
3	0.48	11.9	1.6
4	0.45	11.3	1.6
5	0.45	9.9	1.7
6	0.48	9.2	1.6
7	0.44	8.3	1.6
8	0.49	7.7	1.5
X	0.38	8.4	1.3
Y	0.36	2.2	0.3
		Total	15.2

Number of chromosomes measured = 450

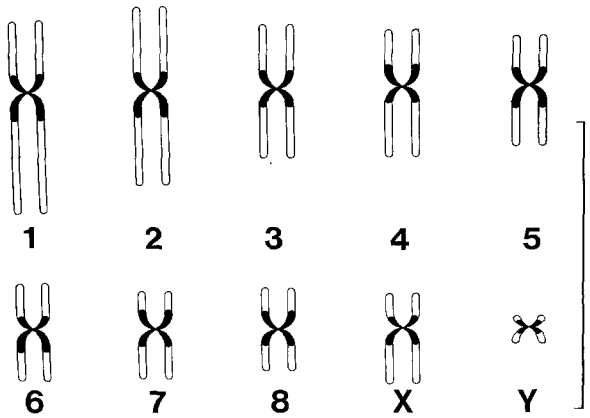
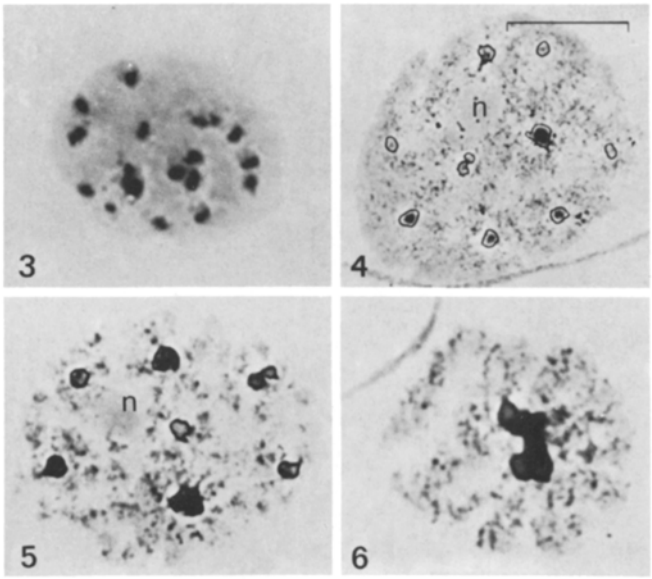
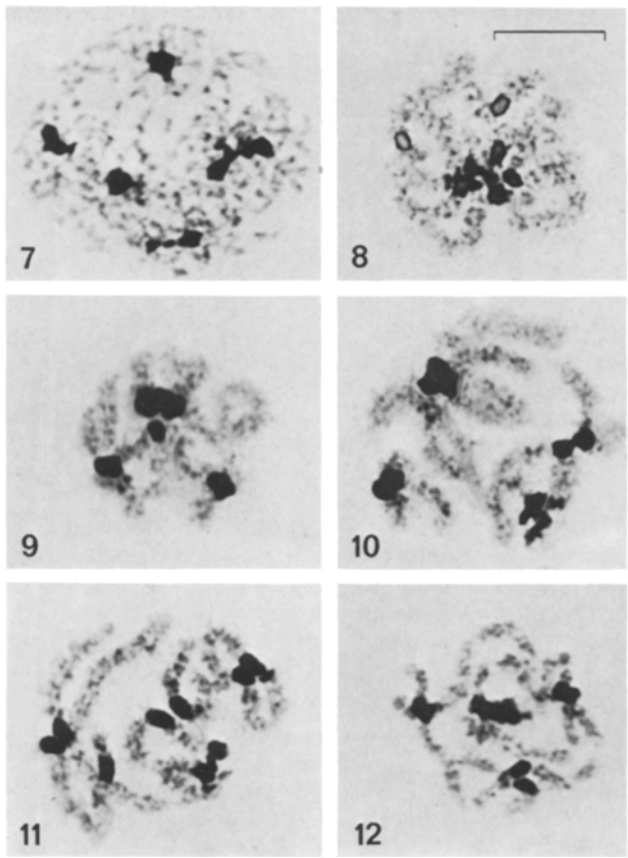


Fig. 2. Diagrammatic representation of the haploid male chromosome set illustrating size and distribution of constitutive C-segments. The drawing was based on averaged measurements from 25 well-spread metaphase nuclei. Total length of the Y chromosome is approximately 1 μm. Bar represents 10 μm



Figs. 3–6. Change in number of detectable chromocenters as meiosis progresses in gonial cells of *E. paenulata*. **Fig. 3.** Eighteen small chromocenters are visible in a gonial nucleus. **Fig. 4.** Late leptotene. Chromocenters have already fused and a haploid number of heterochromatic corpuscles is observed. This photomicrograph of an unstained fixed cell was taken with phase contrast. The high chromocenter density enhanced the contrast of the phase image particularly at its periphery, forming an unusually dark halo. **Fig. 5.** Detectable number of chromocenters decreases as meiosis progresses. **Fig. 6.** A single intensely heteropycnotic mass is found in spermatocytes after chromocenter association is completed. **Figs. 3**, **5** and **6** Giemsa staining. *n* nucleolus. Bar represents 10 μm



Figs. 7–12. Nonhomologous bivalent associations into chromocenters and their progressive detachment. **Fig. 7.** Leptotene. Of the five still visible chromocenters, one shows a double and one a triple composition with the components connected by heterochromatic bridges. **Fig. 8.** Leptotene. Chromocenters are centrally grouped but not all of them are clearly fused. **Fig. 9.** Early pachytene. Several chromocenters are fused forming single large heterochromatic masses. Bivalents are seen radiating from chromocenters. **Fig. 10.** Pachytene cell showing seven chromosome arms radiating from one of the chromocenters. **Fig. 11.** Midpachytene. Chromocenters have started to separate from former associations but are still aligned side-by-side. The bivalent chromomeric structure is apparent. **Fig. 12.** Late pachytene. Chromosomes have shortened, but detachment from chromocenters is not yet complete. Giemsa staining. Bar represents 10 μm

chromatin was more difficult to detect in the C-banded Ys because of their smallness and the swelling produced by the banding procedure (Fig. 1, lower row). Measurements made on these segments allowed us to estimate that they represented about 15% of the total length of the haploid karyotype. Centromere indices, relative chromosome length (%), and relative length of heterochromatic C-segments (%) are presented in Table 1.

Figure 2 summarizes diagrammatically all these observations. Due to the gradient in chromosome size and the absence of chromosome markers, the numbers given to chromosome pairs are arbitrary.

There was a direct correlation between the number of chromocenters observed in gonial cells and the heterochromatic segments found in mitotic metaphase chromosomes. Moreover, a variable number of chromocenters ranging from eighteen to one was observed in spermatocytes according to the meiotic stage. Figure 3 illustrates a gonial cell with eighteen chromocenters which corresponded to the expected diploid number of chromosomes. In late leptotene cells, the number of chromocenters was reduced to nine, suggesting that meiotic homologous chromosome pairing had already occurred (Fig. 4). About 15% of the 750 cells examined had nine chromocenters. However, because our cell dispersion method mixes all cell types on the same slide, the percentage of cells showing a haploid number of chromocenters was probably higher. Figures 5–8 show the progression of chromocenter association. In Figure 6, a large and irregularly shaped intensely heteropycnotic single chromocenter is seen in early pachytene.

Due to the fusion of heterochromatic segments all bivalents became associated into chromocenters. During late pachytene, fused heterochromatic material started to detach, and chromosome arms were observed radiating from remaining large chromocenters (Figs. 9–12). Large heteropycnotic C-segments were also detected during late diplotene and early diakinesis (Fig. 13).

Sex chromosomes were separate and close to the nuclear membrane during late leptotene. In particular, it was possible to recognize both arms and the heterochromatic paracentric segment of the X chromosome (Fig. 14). During early pachytene, an end-to-end association between the X and the Y was found (Fig. 15). This association is also illustrated in Figure 16, where the short arms (p), long arms (q), and both heterochromatic segments are visible. Moreover, two marker secondary constrictions were observed in these association filaments from early pachytene to early diplotene. Figure 16 shows that the filament was bent. A

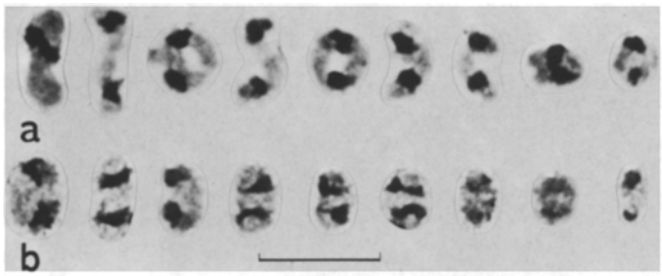
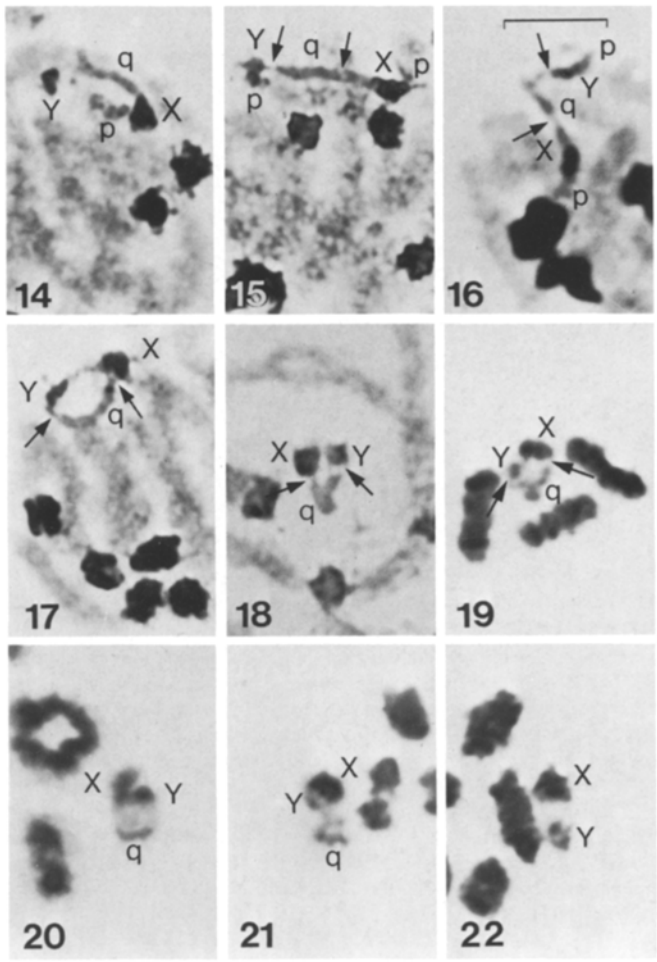


Fig. 13. C-banded bivalents showing large heterochromatic segments in late diplotene (**a**) and early diakinesis (**b**). Despite the C-treatment, the chromosome structure was conserved allowing the visualization of chiasma terminalization. One and two chiasmatic bivalent configurations are seen in **a**. Bar represents 10 μm

pseudo-ring sex bivalent was formed later when an extra association occurred between both heterochromatic segments (Fig. 17). During midpachytene the sex bivalent presented two intensely stained heterochromatic corpuscles of different size and a folded long euchromatic filament with two secondary constrictions (arrows). These constrictions corresponded to the ones detected during former stages (Fig. 18). The general appearance of this chromosome configuration suggested the image of the classical "parachute", but, in our material, the chromosome segment that should have corresponded to the Y chromosome was interpreted as the result of a long arm association and further folding. During the formation of the "parachute" heterochromatic segments of the sex chromosomes converged and became associated through extra "euchromatic" filaments (Figs. 17–19). These connections greatly resembled those observed during chromocenter associations (Fig. 27). The triple composition of the Xy_p sex bivalent and the morphological changes undergone during pachytene allowed us to subdivide this meiotic stage into three substages (I, II, and III).

The double nature of the heterochromatic "pole" of the Xy_p could be followed until early diakinesis (Figs. 19–21). In these figures, it is also noticeable that the heterochromatic corpuscles have a different size than in earlier stages. Unfolding of the euchromatic filament occurred during diplotene accompanying the repulsion at this stage. This is illustrated in Figure 20 which shows that segment q extended again and that it was connected by thin filaments to the heterochromatic corpuscles. Spiralization of the q segment was noticed during early diakinesis (Fig. 21) as well as a rotation of about 45° of the small heterochromatic corpuscle with respect to the large one. In late diakinesis the smaller pole of the sex bivalent was found to consist of two small corpuscles suggesting that the q segment shortened still more and that the Y heterochromatic corpuscle had moved to this pole (Fig. 22).

Final stages of the Xy_p association and later changes of the heterochromatic corpuscles appear illustrated in Figure 23 which is composed of six chromosome cut-outs ordered from early diakinesis to late metaphase I. First, similar configurations to the ones appearing in Figures 21 and 22 are shown (Fig. 23a, b). Then, the detachment of the euchromatic segment (arrow) from the small "pole" (Fig. 23c) and its progressive incorporation into the large pole (Fig. 23d, arrow and e) is illustrated. The result was



Figs. 14–22. Formation and behavior of the sex bivalent Xy_p. **Fig. 14.** Late leptotene. The X and the Y chromosomes are close to the nuclear membrane but they are still not associated. **Fig. 15.** Early pachytene I. End-to-end association has occurred between the long arms of the X and the Y chromosomes. **Fig. 16.** Early pachytene II. Bending of associated sex chromosomes. Heterochromatic and euchromatic chromosome segments allow precise identification of the long arms (*q*) and short arms (*p*) in both sex chromosomes. *Arrows* point to secondary constrictions detected also at later stages. **Fig. 17.** Early pachytene III. A thin filament connecting both heterochromatic segments resulted in a "ring" chromosome configuration. **Fig. 18.** Midpachytene. Attraction between the two heterochromatic segments is more advanced. The euchromatic filament (*q*) is folded and the two secondary constrictions observed in Figure 16 (*arrows*) are more elongated. The whole chromosome configuration presents now the classical aspect of a "parachute" bivalent but formed by three distinct segments. **Fig. 19.** Early diplotene. The X and the Y heterochromatic segments are still in close contact but the folded chromosome arm (*q*) has started to spread apart. **Fig. 20.** Late diplotene. Unfolding of chromosome segment *q* is completed. A euchromatic thick arc-shaped filament resulted from the repulsion process. Thin filaments connect heterochromatic and euchromatic segments and correspond to extremely stretched secondary constrictions observed in earlier stages. **Fig. 21.** Early diakinesis. Spiralization of the euchromatic segment (*q*) is observed. Both heterochromatic components have rotated a little, and a more elongated shape of the sex bivalent is noticeable. **Fig. 22.** Late diakinesis. The smaller heterochromatic pole of the Xy_p presents two corpuscles at this stage. **Figs. 14–21** Giemsa staining; **Fig. 18** C-banding. Bar represents 5 μm

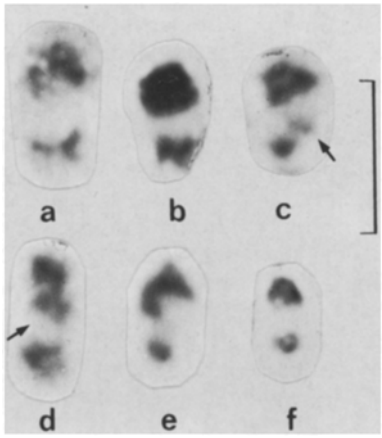


Fig. 23a–f. Six chromosome cut-outs illustrating aspects of final presegregational stages of the "parachute" sex bivalent. **a and b** Early and late diakinesis. **c** Early metaphase. **d, e** Midmetaphase. **f** Late metaphase. The sequence suggests that euchromatic material (*arrows*) was moving from the double-corpuscled pole (**b**) to the large heterochromatic pole as metaphase progressed (**e**). Both members of the sex bivalent Xy_p shown in the last cut-out (**f**) are highly contracted and ready for meiotic segregation. Giemsa staining. Bar represents 5 μm

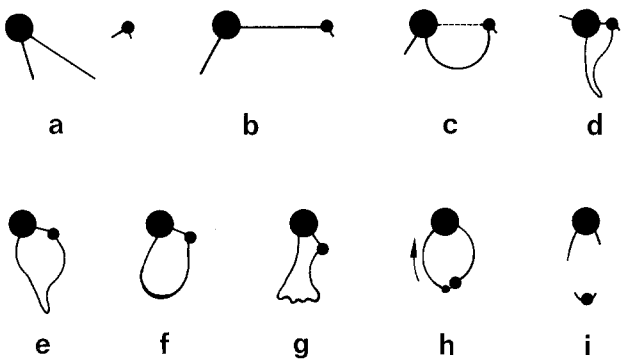
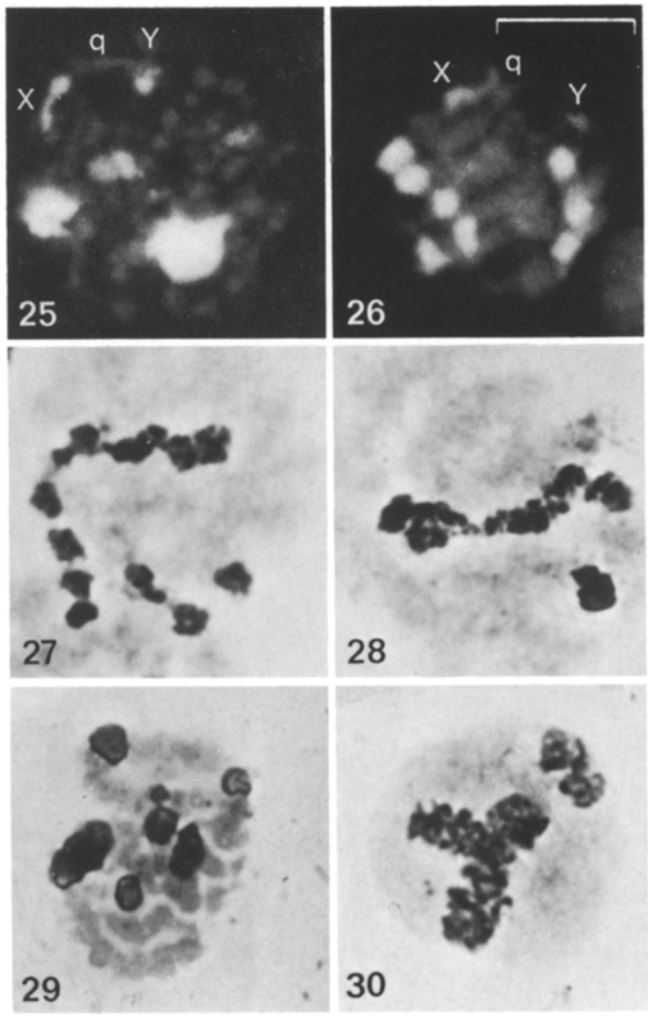


Fig. 24a–i. The different cytological images found during sex chromosome association and formation of the "parachute" Xy_p bivalent. The heterochromatic segment of the X chromosome is represented by a large dot and that of the Y, by a small dot. *Straight lines* in (**a**) and (**b**) represent chromosome arms. *Dashed line* in (**c**) denotes the "attraction" between heterochromatic segments. Thickening of the connecting long filament (**f**) indicates the initiation of chromosome arm coiling which is depicted in (**g**). Also, rotation and movement of the smaller corpuscle from the upper pole to the lower one is suggested (**f–h**). *Arrow* in (**h**) represents the displacement of the euchromatic material illustrated in Figure 23c–e. Finally, the sex chromosomes appear separated in anaphase I (**i**)



Figs. 25–30. Chromocenters and heterochromatic associations. **Fig. 25.** Early pachytene. Quinacrine staining. Two large and two smaller brightly fluorescent chromocenters are seen. A chromosome association similar to the one in Figure 16 is observed close to the nuclear membrane. The X and the Y heterochromatic segments fluoresce brighter than the connecting filament *q*. **Fig. 26.** Early pachytene. Hoechst 33258 staining. Chromocenters and heterochromatin of sex chromosomes show intense fluorescence. Sex chromosomes are not yet associated. **Figs. 27, 28.** Giemsa staining. Chromocenter associations and fusions in *E. paenulata*. Alignment and connecting filaments are observed before chromocenters proceed to fuse into a single heterochromatic mass. **Fig. 29.** C-stained pachytene cell. Fused chromocenters are intensely C-stained particularly at their periphery. **Fig. 30.** Large Y-shaped chromocenter after C-banding procedure showing coiled and intermingled filaments. Bar represents 10 μm

a classical Xy_p configuration both in appearance and in structure, this bivalent being ready for normal meiotic chromosome segregation (Fig. 23f).

All the cytological observations made on the Xy_p sequence (Figs. 14–23) are summarized in Figure 24. The sequence starts with the two unassociated sex chromosomes (a). End-to-end association takes place at the long arms (b). Bending of associated long arms completes the "parachute" (d) and repulsion of euchromatic filament and further coiling appears in (e)–(g). The three-corpuscle stage is shown in (h); the condensed long arm of the X chromosome representing the smallest corpuscle. Segregation of the sex chromosomes is shown in (i).

Observations on fluorescent slides stained with quinacrine HCl or with Hoechst 33258 confirmed that sex chromosomes were located close to the nuclear membrane and that they were associated through their long arms (Figs. 25 and 26). Moreover, these figures show that chromocenters fluoresced intensely with both fluorochromes.

Associations usually took place through an orderly side-by-side chromocenter alignment and resulted in the formation of long chromocenter chains connected by euchromatic

filaments which could be observed during early stages (Figs. 27 and 28). Thin chromatin filaments were also detected connecting bivalents during metaphase I.

Fused chromocenters reacted intensely to C-banding, showing a crustlike aspect with their peripheries darkly delineated (Fig. 29). An interesting finding was that with C-banding large chromocenters showed a coiled and intermingled filamentous structure (Fig. 30). Apparently, the treatment revealed an underlying structure showing that fused chromocenters were not amorphous masses of chromosome material as usually observed with Giemsa staining but an intricate network of filaments.

Discussion

The chromosome formula found in *E. paenulata* corresponded to the diploid number found in most species of the subfamily Epilachninae (Agarwal 1961; Smith and Virkki 1978; Vidal et al. 1977). The number of paracentric segments detected in mitotic chromosomes was similar to the number of chromocenters observed in gonial cells. The fact that approximately 15% of the leptotene cells presented nine chromocenters, which is the haploid number in this species, suggested that chromosome pairing had occurred. A similar reduction in the number of prochromosomes has been described in *Plantago ovata* (Stack and Brown 1969) and in *Impatiens balsamina* and *Salvia nemorosa* (Chauhan and Abel 1968).

Heterochromatic associations and chromocenter fusions in *E. paenulata* strongly suggested that these were significant steps in meiotic chromosome pairing. The observable number of chromocenters was reduced from diploid to haploid, and chromocenter fusions later formed a single intensely heteropycnotic mass with bivalent arms radiating from it. Similar images have been found in other animals such as *Epicanta* and *Pyrota* (Virkki 1962).

Filament chromosome interconnections observed in metaphase I have also been found in a variety of cell types involving centromeric heterochromatin and homologous parts of chromosomes. They have not been considered as artifacts but as the result of chromatin fusions occurring in living cells and representing association remnants (Godin and Stack 1975).

Beetles present different types of sex chromosome mechanisms, the Xy_p sex bivalent being one of the most frequent systems found in polyphagan families of Coleoptera (Smith and Virkki 1978). The type of Xy_p bivalent sex system found in *E. paenulata* has been classically attributed to the association of the X chromosome with the Y, which usually is minute.

Stevens (1906) noticed a resemblance between the "heterochromosome group" and a "parachute" with the X chromosome representing the canopy of the parachute and the Y, the load or parachutist. Smith (1950) introduced the symbol Xy_p to designate this peculiar sex bivalent. Two interpretations have been proposed for this chromosome configuration:

(1) Terminal pairing and chiasmata have occurred between chromosome segments in long or short arms of each sex chromosome (Kacker 1970; Saha 1973; Sanderson 1967; Smith 1951). However, White (1973) pointed out that no clear evidence of chiasmatic associations has been reported in the Xy_p probably because sex bivalents in beetles are very small and compact during metaphase. On the other hand, the early investigators using the classical techniques were probably not able to observe minor structural details.

(2) Stevens reported in an early paper (1906) that pairing of the Xy_p was due to associated nucleolar material. Suomalainen (1947) also claimed that nucleolar material connected the X to the Y chromosome. Furthermore, John and Lewis (1960) found in two coccinellids and in *Tenebrio molitor* that a nucleolar moiety appeared between the X and the Y, thus, ruling out the presence of chiasmata in this material. Virkki (1967) described in *Trox* and in *Pleocoma* prenucleolar associations of Xp and Yp which usually were bent in a horseshoe shape.

No nucleolar material associated to the Xy_p bivalent was detected with Goodpasture and Bloom's silver technique suggesting that in *E. paenulata,* the nucleolar material is autosomally organized.

The behavior of the parachute heterochromatic segments agreed with the general autosomal heterochromatin attraction and adhesion occurring in this species. It was, thus, difficult to exclude the heterochromatin association as an explanation of the origin of the double composition of the heterochromatic "pole" of the "parachute", typically represented by the configuration illustrated in Figure 18. According to our view, the parachute canopy results from a nonchiasmatic association of the heterochromatic segments of the X and Y chromosomes, and the parachutist portion of the Xy_p represents only the terminally associated long arms of the sex chromosomes. This differs from the usual assumption that the parachute canopy is the X and the parachutist, the Y.

Figures 20–23 illustrate that the number of heterochromatic corpuscles changed in both "poles" of the Xy_p according to the meiotic stage, a fact not previously reported in the literature.

Despite the small size of the chromosomal structures (0.5 μm–1.5 μm), changes in number and position of the corpuscles were clearly detected, suggesting that at least one of the corpuscles originated from the coiling of the euchromatic filament q (Fig. 21) and that the other one resulted from the displacement of heterochromatic material (Figs. 22 and 23). This kind of movement of heterochromatic material is apparently a mechanism to ensure the normal segregation of the X and the Y during anaphase I.

Interestingly, John and Shaw (1967) observed in *Dermestes lardarius, D. frischii, D. ater, D. haemorroidalis,* and *D. maculatus* a fingerlike allocyclic segment of Xp and extra threads joining it to y_p. They believe this segment to be responsible for organizing the sex nucleolus. A similar structure has been reported in *Pleocoma* (Virkki 1967). These images resembled, to a great extent, the ones observed in our material (Fig. 23e). Since a detailed description of chromosome configurations in the preceding meiotic stages was not given, a comparison with our findings is not possible.

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