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Painting analysis of meiotic metaphase I configurations of the germ line-limited chromosomes in *Acricotopus*

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Abstract

Meiotic metaphase I configurations and pairing behavior of the germ line-limited chromosomes (=Ks) in the chironomid *Acricotopus lucidus* were analysed by chromosome painting using specific probes of the three soma chromosomes (=Ss) and of their individual arms. The Ks are derived from the Ss and possess large S-homologous sections. Beside regular K and S bivalents we also observed frequently K multivalents, e.g. trivalents, mainly quadrivalents, but also penta- and hexavalents, composed of the same K type in metaphases I. Chiasmata predominately occur within the S-homologous sections, probably ensuring a correct segregation and the transmission of a set of Ks to the next generation. Because K bivalents are almost exclusively autobivalents in *A. lucidus* formed by earlier sister chromatids, this multivalent formation with crossover also between homologous but nonidentical Ks leads to genetic recombination within a K type. Rarely, quadrivalents composed of nonhomologous Ks but derived from the same S were found. Therefore, these multivalents most probably resulted from crossover between homologous sections of morphologically different K types. This may result in new K types and might be important for the evolution of K type diversity in *A. lucidus*. In some cases, pairing-like associations between SIII and K4, which is derived from SIII, were observed in metaphases I, indicating the possibility of crossover events and recombination between these chromosomes and so between the somatic and the germ line restricted chromosome complements. Possible functions of additional copies of S sequences carried in the germ line are discussed.

Key words: FISH, germ line-limited chromosomes, meiosis

Introduction

Additional chromosomes limited to the germ line and eliminated from the future somatic nuclei during early embryonic divisions were established in the dipteran families Cecidomyiidae, Chironomidae and Sciaridae (White 1973, Tobler 1986, Gerbi 1986, Goday & Esteban 2001).

In *Acricotopus lucidus* a member of the Orthocladiinae, a subfamily of the Chironomidae, these germ line-limited chromosomes (=Ks) together with the soma chromosomes (=Ss) pass through a complex chromosome cycle exhibiting different special mitoses with partial and complete elimination of the Ks, and with monopolar movement of the K complement (Bauer & Beermann 1952, Beermann 1956, Redi *et al.* 2001). In the cecidomyiids *Wachtliella* and *Miastor* it was demonstrated that the Ks are essential for normal development of the germ cells (Geyer-Duszynska 1966, Bantock 1970) .

Recently, in *A. lucidus* fluorescence in-situ hybridization (FISH) with painting probes specific for the Ss onto spermatogonial mitoses demonstrated that the Ks contain large S-homologous sections and that with one exception, each K-type is derived from a specific S (Staiber & Schiffkowski 2000). These results produce strong evidence for the hypothesis that the Ks are developed from the Ss by endopolyploidization, rearrangements and the accumulation of germ line-specific sequences mainly in the centromeric regions (Staiber *et al.* 1997).

Pairing behavior of Ks in meiosis and autobivalent formation of Ks in *A. lucidus* were investigated in earlier studies by G-banding (Staiber 1989, 1991a). But there were some questions as to meiotic pairing of Ks, e.g. multivalent formation and pairing-like K-S-assoziations, that cannot be answered by chromosome banding. So, in the present study the pairing configurations of Ks and Ss in meiotic metaphase I stages were analysed by FISH using chromosome and chromosome arm-specific painting probes of the Ss.

Materials and methods

Chromosome preparation

Squash preparations were made by the dry ice method from testes of young prepupae of *Acricotopus lucidus* (Diptera, Chironomidae) previously treated with a hypotonic solution of 0.5% sodium citrate for 20 min and fixed in 3:1 ethanol acetic acid (Staiber *et al.* 1997).

Labeling of probes

DNA probes specific for the three Ss and for each of their chromosome armes were generated by microdissection of polytene salivary gland Ss and DOP-PCR (degenerate oligonucleotide primed-polymerase chain reaction; Telenius *et al.* 1992) as described in Staiber & Schiffkowski (2000). The painting probes were produced by a second 50 µl PCR with 2.5-5 µl of the stock DOP-PCR DNA probe and adding 40 µmol digoxigenin-11-dUTP or 66 µmol fluorescein-12-dUTP (Roche) to the premixed reagents of a DOP-PCR kit (Roche), or by nick translation with biotin-16-dUTP.

Fluorescence in situ hybridization

Painting probes were coprecipitated with ethanol, redissolved in a mixture of 50% deionized formamide, 10% dextrane sulfate, 2x SSC, 1x Denhardt solution and 0.1% SDS in 40 mmol/L phosphate buffer pH 7.0 and hybridized to the chromosome preparations in a humidified chamber overnight at 37°C. Post-hybridizatiom washes, probe detection and signal amplification were carried out as described in Staiber & Schiffkowski (2000). In three-color FISH using chromosome-specific paints, the biotinyltated SI probe was visualized by Cy5-conjugated streptavidin (blue; Dako), the digoxigenated SII probe by rhodamine-conjugated anti digoxigenin Fab fragments (red; Roche) and the fluorescein-labeled SIII probe by layers of a rabbit anti fluorescein antibody (Dako) and fluorescein-conjugated goat antirabbit antibody (green; Vector Laboratories). In two-color FISH using S chromosome arm-specific paints the biotinylated short arm probes were detected by Cy5-conjugated streptavidin and the digoxigenin-labeled long arm probes by anti-digoxigenin-fluorescein Fab fragments (Roche). Preparations were stained with 0.2 µg/ml 4',6-diamidino-2-phenylindole (DAPI) in phosphate buffered saline (three-color FISH) or mounted in Vectashield fluorescence antifade solution (Vector Laboratories) containing 2.5 µg/ml propidium iodide (two-color FISH). Digital images were captured with a confocal laser scanning microscope (Biorad) and with an epifluorescence microscope (Zeiss) equipped with a digital camera (Pixera; DAPI-stained preparations), and processed with a Corel Draw software package.

Results

The Ks of *A. lucidus* possess large S-homologous sections beside heterochromatic segments containing K-specific DNA sequences. The use of painting probes of the three Ss and their individual arms produced by microdissection of the polytene salivary gland chromosomes and DOP-PCR allows the display of the S-homologous sections and the analysis of the pairing behavior and meiotic configurations of the Ks. Results of three-colour FISH using a combination of all S probes to metaphases I are presented in Figures 1a-c. In addition, Figures 1a'-c' show the DAPI counterstaining to visualize chromosomes and pairing configurations and to support the identification of the K and S bivalents. Nine different K types (K1-K9) were found in a G-banding analysis of gonial mitoses of *A. lucidus* (Staiber 1988). In the metaphases I in Figure 1, the Ks were identified by the DAPI staining pattern of K-specific heterochromatin (Staiber 1991b) and by their size. The identification is possible for the Ss and those Ks that are painted by the SI (blue) and SIII (green) probes but is difficult for the similar sized and banded SII-painted Ks (red), which are K3, K6 and K7. Because the latter could not be distinguished from each other with certainty in meiotic stages they were not labeled in the figures. In metaphases I mainly open K bivalents with one chiasma but also ring K bivalents with two chiasmata occurred. Predominately, the chiasmata are located in the painted S-homologous sections of the Ks. The S bivalents are frequently found to be spatially arranged in a group as seen in the top and side

views of the metaphases I in Figures 1a & 1b. All K types are painted from one of the S probes, except K2. Figure 1c clearly demonstrates that both arms of K2 are labeled by different S probes, SI and SII. The painting patterns of K2 bivalents resulting from two-colour FISH with combinations of chromosome arm specific probes of SI and SII (Figures 2a & 2b) indicate more precisely that the long arm of the K2 is derived from the long arm of SI (green) and the short arm from the short arm of SII (blue).

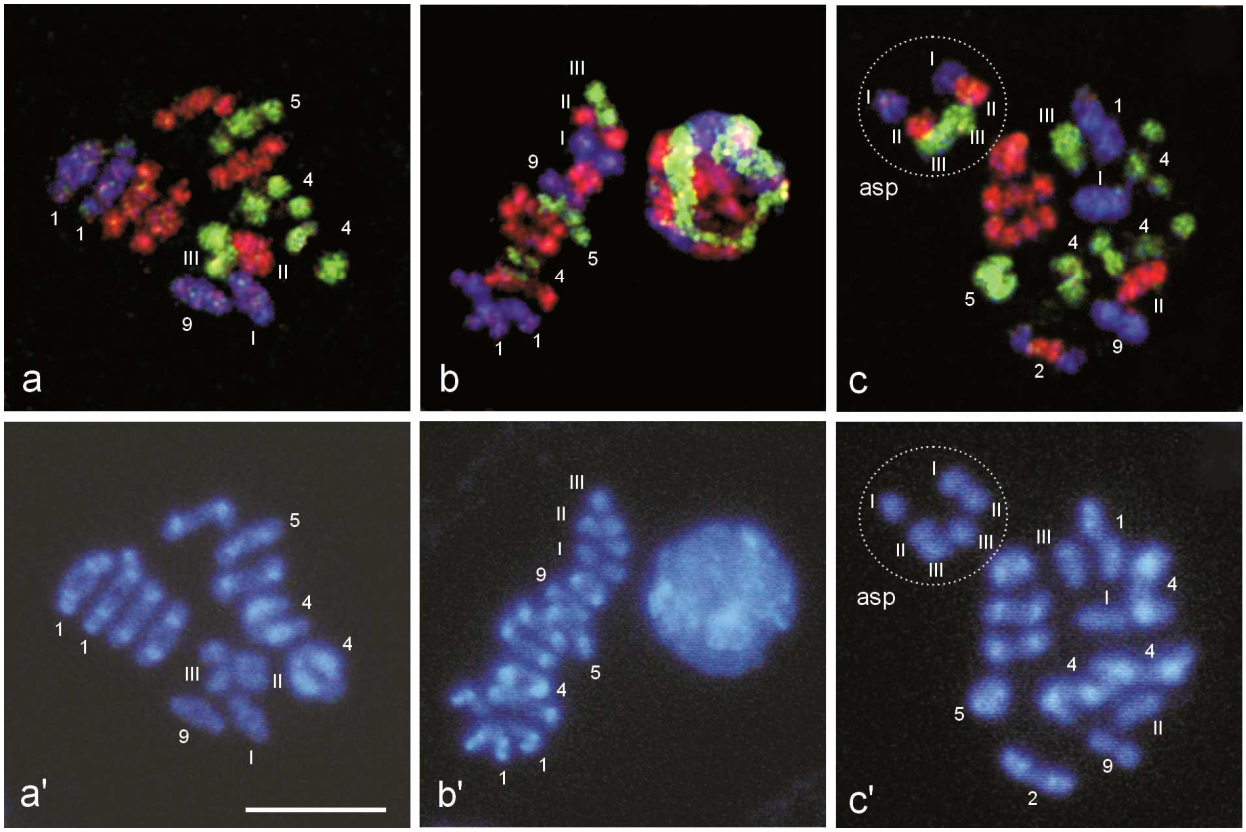

Figure 1. (**a-c**) Three-colour FISH using painting probes of the three Ss of *A. lucidu*s onto spermatocyte metaphases I. The probes of SI (blue), SII (red) and SIII (green) visualize the Ss and the S-homologous sections in the K bivalents. (**a'-c'**) DAPI counterstaining. (**a, b**) The S bivalents (I-III) are arranged in a group. (**b**) Side view of a metaphase I with an interphase nucleus. (**c**) Metaphase I with S and K bivalents and a mitotic metaphase of the related aberrant spermatocyte (=*asp*, white cycle) containing only unpaired Ss. Those Ks that are painted by the SII probe (red; K3, K6 and K7) are not labeled in the three metaphases I because they cannot distinguished from each other with certainty. I, II, III = SI, SII, SIII. 1-9 = K1-K9. Bar represents 10 µm.

The three metaphases in Figures 1a-c also exemplify the regular occurring variation in the number of Ks (18 Ks, nine K bivalents, in Figure 1b', and 20 Ks, ten K bivalents, in Figures 1a', c') and in the composition of K types (4x K1, 4x K4 in Figure 1a; 4x K1, 2x K4 in Figure 1b; 2x K1, 6x K4 in Figure 1c) in the K complements of *A. lucidus*.

Simultaneously with the first meiotic division of the regular spermatocyte the corresponding aberrant spermatocyte (=*asp* in Figures 1c & 2b) passes through a mitotic division. Both types of spermatocytes are products of the last inequal gonial mitosis (=differential mitosis) resulting in that the regular spermatocyte received Ss and all Ks, and the aberrant only Ss. In contrast to the preceding gonial mitoses in which the homologous Ss are always closely paired in metaphases

(not shown), the Ss exhibit no pairing in the mitotic metaphases of aberrant spermatocytes (Figures 1c & 2b).

More detailed information about the pairing behavior and associations of the S-homologous K sections resulted from two-colour FISH with combinations of chromosome arm-specific painting probes of SI, SII and SIII (Figures 2a-c) to metaphases I.

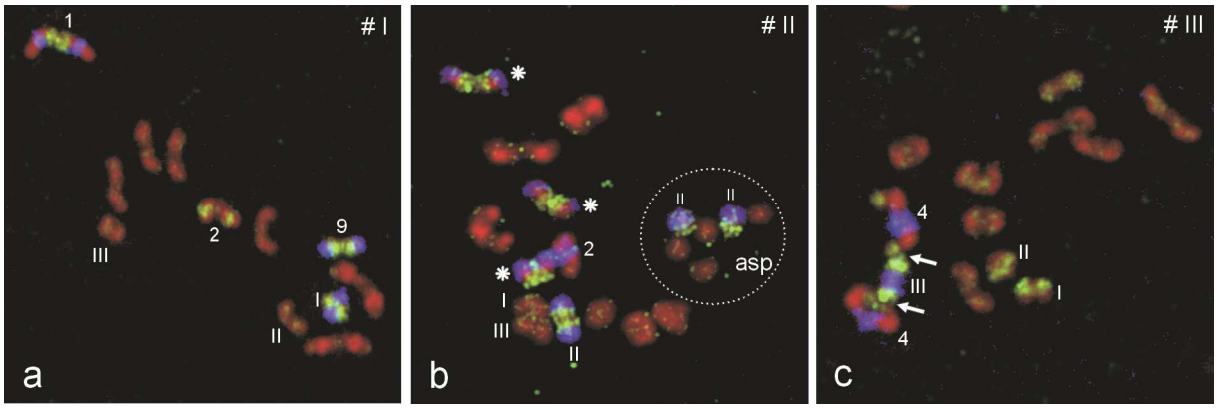

Figure 2. Painting of metaphases I with chromosome arm-specific probes of (**a**) SI (= #I), (**b**) SII (= #II) and (**c**) SIII (= #III). Short arm (Cy5, blue) and long arm (fluorescein, green) probes selectively hybridize to the related S-homologous K sections. Chromosomes are counterstained with propidium iodide. (**b**) The differentiation between the K3, K6 and K7 bivalents (*) painted by the SII arm probes was not possible. Mitotic metaphase of the related aberrant spermatocyte (=asp, white cycle). (**c**) Close pairing-like association between the long arms of the SIIIs and the homologous sections of K4s of two different K4 bivalents (arrows). I, II, III = SI, SII, SIII. 1-9 = K1-K9.

Hybridization of the short arm probes was visualized with Cy5 (blue) and of the long arm probes with fluorescein (green). The chromosomes were counterstained with propidium iodide. Except for K1, the short arms of each of the other K types were painted by the short arm probe of the related S, the long arms by the long arm probe. The long arm probe of SIII (Figure 2c, green) contains a small portion of sequences of the repetitive AlSo family (Staiber *et al.* 1997) which is located specifically in the centromeric regions of all Ss. In *A. lucidus* the nucleolus expression disrupts the SIII centromere heterochromatin into a lot of small fragments; some of these were microdissected together with the long chromosome arm of the polytene SIII during probe generation. Therefore the centromeres of the SIs and SIIs were also labeled in the metaphase of Figure 2c.

Associations between homologous chromosome sections can be recognized very well in metaphases painted with arm-specific probes. An example for this is the closely pairing-like assoziation between the SIII bivalent and two K4 bivalents in Figure 2c. In this case there seems to be a selective pairing of the long arm (green) of each of the SIII homologs with the long arms of two K4s of two different K4 bivalents. Pairing-like associations like this were found in several metaphases I, not only between SIII and K4 bivalents (Figures 3a,b) but also between a SIII bivalent and a K4 trivalent (Figure 3c). But in none of these cases can one say with certainty that a chiasma is formed between SIII and K4. Pairing-like associations between other K types and Ss were not observed.

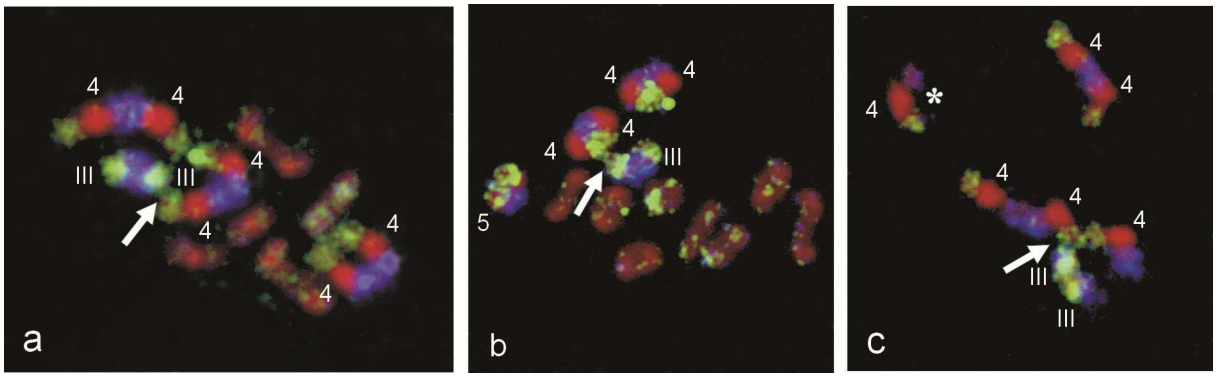

Figure 3. (**a-c**) Close pairing-like associations between SIIIs and K4s (arrows). Sections of metaphases I. FISH with short (blue) and long (green) chromosome arm probes of SIII. Propidium iodide counterstaining. (**c**) Pairing of SIII and a K4 of a chain trivalent. Also present is a K4 univalent (*) and a K4 bivalent.

When a spermatocyte is entering into meiosis and chromosomes of a distinct K type are present more than two times, as e.g. K1 and K4 in Figure 1a with four, or K4 in Figure 1c with six exemplares, then these Ks can form not only regular bivalents but also multivalents. Such multivalents, mainly quadrivalents, but also higher multivalent configurations were found in metaphases I. Most frequently they were composed of K4s (Figures 4a-d, a'-d'). Different types of quadrivalents, mainly chain and ring quadrivalents (Figures 4a, b), but also "cross" (not shown) and "frying pan" quadrivalents (Figure 4c) of K4s occurred (Sybenga 1975).

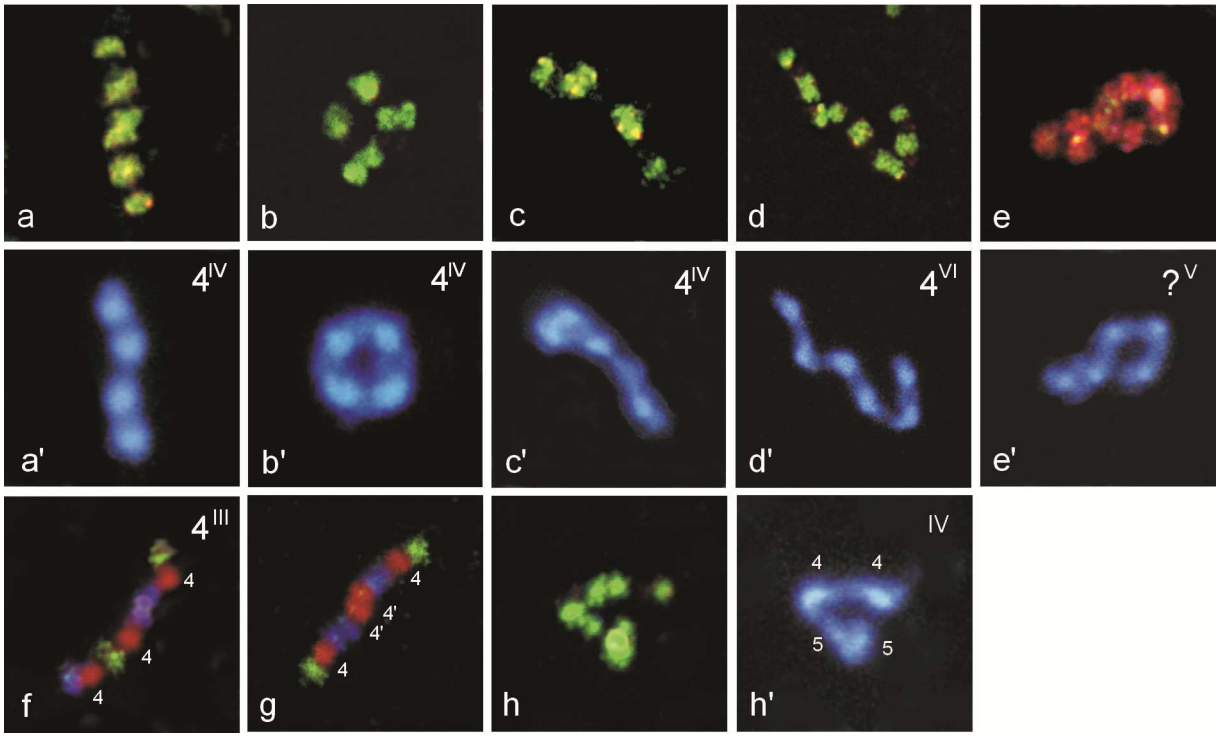

Figure 4. (**a-h**) K multivalent configurations from three- and two-color FISH metaphases I of *A. lucidus*. (**a-d, h**) Multivalents painted by the SIII (green) and (**e**) by the SII probe (red). (**f, g**) FISH using a combination of short (blue) and long (green) arm-specific painting probes of SIII. (**a'-e', h'**) DAPI and (**f, g**) propidium iodide staining. (**a**) Chain, (**b**) ring and (**c**) 'frying-pan' quadrivalent of K4s (4^{IV}). (**d**) Chain hexavalent of K4s (4^{VI}). (**e**) Pentavalent of Ks painted by the SII probe ($?^{V}$). (**f**) Chain trivalent of K4s (4^{III}). (g) Chain quadrivalent formed by two aberrant (4') and two regular (4) K4s. (**h**) Quadrivalent (IV) composed of two different K types, K4 and K5, both containing SIII-homologous sections.

K4 can easily be identifed by its characteristic morphology founded in the exceptional size and bright fluorescence of its central heterochromatic segment, that was not be painted by the SIII probe (Figure 1) and so containing great amounts of germ line-specific DNA sequences. Two examples for higher multivalents are the chain hexavalent composed of K4s in Figures 4d, d' and the chain pentavalent composed of a K type painted by the SII probe (red) in Figures 4e, e'. That multivalent formation in the same K type is founded in crossover events and chiasma formation between homologous chromosome arms is clearly seen in the painting pattern of the K4 trivalents in Figures 3c and 4f resulting from use of arm-specific probes of SIII. But, in rare cases, also multivalents composed of different K types were found. An example is the K4/K5-quadrivalent in Figures 4h, h'. Both K types are painted by the SIII probe and so are derived from SIII. A chiasma is formed most probably between the short arms of K4 and K5.

Aberrant Ks, that sometimes occur spontaneously can be easily recognized by the arm-specific painting. Figure 4g shows a chain quadrivalent composed of two regular K4s located on both ends and of two aberrant K4s in the middle. The latter are smaller than regular K4s possessing only the short arm (blue) and a great part of the central heterochromatic segment. The chiasma between the aberrant K4s is formed within the heterochromatic segments. The chiasmata between aberrant and normal K4s are formed within the short arms.

Discussion

The painting of spermatocyte metaphases I using probes of the three Ss, as shown in Figures 1a-c, clearly demonstrates that the Ks of *A. lucidus* contain large S-homologous sections each derived from a distinct S. This strongly supports the hypothesis that the Ks are descendents of the Ss. In metaphases I not only regular K bivalents, but also multivalents composed of the same K type occur. The overwhelming majority of the K bivalents in *A. lucidus* are so-called autobivalents (more than 99%), i.e. bivalents of identical chromosomes, as demonstrated by X-ray-induced marker Ks (Staiber 1991a). The identical partners are former sister chromatids that had carried out a monopolar migration without separation during the preceding last gonial mitosis (differential mitosis). From equal crossovers between identicals in autobivalents, no genetic recombination within a K type is to be expected. So, multivalent formation that resulted from crossovers between non-identical homologous Ks, as shown in Figures 4a-f, is important for recombination and exchange within a K type.

In rare quadrivalents found to be composed of two different K types, both types were painted by the same S probe and are derived from the same S, e.g. K4 and K5 in Figure 4h from SIII. This indicates that crossover can occur between homologous sections of otherwise nonhomologous Ks. Such multivalents are thought to provide recombination between different Ks. Crossover events between nonhomologous Ks may result in new K types and might be important for the evolution of K type diversity in *A. lucidus*.

A variation in number and composition of Ks was found earlier in gonial K complements of larvae of *A. lucidus* (Staiber 1988; see also Figure 1a-c in this study). K4 univalent and K4 trivalent formation as seen in Figure 3c may lead, via irregularities in anaphase I segregation (Sybenga 1975), to gametes with different numbers and compositions of their K complements. This, in addition to irregularities resulting from spontaneous non-disjunction of Ks in the first mitosis of the arising pole cells in early embryos (Staiber 2000) and from differences in the elimination of about half of the Ks in the primordial germ cells of newly hatched larvae (germ line elimination; Bauer & Beermann 1952), could explain the variations in the K complements.

The paracentromeric heterochromatin bands, common to all Ks except K9, are not painted by the S probes. This is clearly to be seen in Figure 1 & 2. The heterochromatin bands contain the highly repetitive, germ line-specific AlKe DNA sequence family (Staiber *et al.* 1997), that is supposed to be involved in the differentiation of the Ks from the Ss and in the recognition of the Ks during their soma and germ line elimination.

One possible function of the S-homologous K sections in meiosis is that they may be necessary for the regular occurrence of crossover events between the K homologs. The resulting chiasma formation in these sections ensures their correct anaphase I segregation (Staiber & Schiffkowski 2000). This is necessary for transmission of a full set of Ks to the next generation.

Experiments of Geyer-Duszynska (1966) and Bantock (1970) have indicated that the Ks contain not only heterochromatic 'junk' DNA, but may have important functions for gamete differentiation and possibly also for germ line determination. Tobler (1986) introduced some further possible functions of the Ks, among them that the Ks 'might represent a genetic reservoir carried in the germ line for evolutionary purposes'. If the latter is true, then there should exist mechanisms to transfer sequences of the S-homologous K sections from the 'K genome' back to the 'S genome'. This might occur via recombination events between homologous sections of Ks and Ss.

The close pairing-like associations between homologous sections of SIII and K4 (Figures 2c & 3a-c) indicate that meiotic pairing of Ss and Ks and crossover might be possible. But, one cannot exclude, that the observed physical associations between SIII and K4 may reflect only 'somatic' pairing between closely related DNA sequences rather than crossovers. Recombination events between Ss and Ks would act against genetic isolation of the S-homologous sequences in the Ks and would lead to a conservation of these sequences. Two facts strongly support the supposition that the S-homologous sequences in the Ks might be conserved. First, the presence of S-homologous banding patterns and especially the expression of puffs and a Balbiani ring found in X-ray-translocated and polytenized K segments in the larval salivary gland chromosomes of *A. lucidus* (Staiber & Thudium 1986). Second, the clear hybridization of the S-derived painting probes in the S sections of the Ks indicates a high homology of sequences of Ss and Ks.

One can speculate that *A. lucidus* uses its Ks (among other things) as a 'backup' of S sequences, e.g. to replace mutated gene sequences of the Ss. But, as mentioned before, it could also be advantagous for *A. lucidus* to carry the S sections in the germ cells for evolutionary purposes,

e.g. to get changed genes or new sequence arrangements from the sequence pool of the Ks for 'testing' them in the 'S genome'.

One way to examine the above supposition of S-K recombination and conservation of the K sequences is to compare homologous gene sequences from Ss and Ks. Appropriate for such a comparison are highly conserved genes as e.g. rRNA genes, that are also used for molecular phylogenetic studies (Pelandakis & Solignac 1993, Hwang *et al.* 1998). That loci containing rDNA sequences are present in the K complement of *A. lucidus* was recently demonstrated by FISH for K4 (Staiber & Schiffkowski 2000) using the rDNA cluster of *Chironomus tentans* as a probe.

The karyotypes of the aberrant and the regular spermatocyte in Figure 1c are quite different: 2S-sets versus 2S- and 2K-sets. Both spermatocytes originate from the same germ line cell and are the result of the differential gonial mitosis. After replication, the aberrant spermatocyte enters into mitosis, while the regular spermatocyte is able to start meiosis. Possibly only the Ks carry factors controlling and regulating the entry into meiotic divisions (Maines & Wasserman 1998).

The extent of painted sections of the Ks in the metaphases I in Figures 1a-c clearly demonstrates that for each S, a multiple dose of homologous sections is present in the K complement. But it does not seem to give a dose problem of the additional copies of the S sections and sequences in the germ line cells of *A. lucidus.* Perhaps it might be favourable to have additional copies of specific genes, e.g. genes for fertility factors, in the germ line.

Aberrant Ks, as e.g. the changed K4s in the multivalent in Figure 4g, may arise spontaneously. But since no new K type has established in the last fifteen years in the laboratory stock of *A. lucidus* used for this investigation, there seems to exist a mechanism in *A. lucidus* that stabilizes and favours the existing nine different K types.

The present painting analysis of configurations of Ks in spermatocyte metaphases I produces new insights into pairing behavior, recombination and structural evolution of the Ks in *A. lucidus,* but raises new questions as to their molecular evolution.

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