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Cytogenetic analysis of partial elimination of germ line limited chromosomes from primary germ cells in *Acricotopus lucidus* (Diptera, Chironomidae)

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Summary

Unusual mitotic behavior of additional chromosomes present only in the germ line was reported for the dipteran families Cecidomyiidae, Chironomidae and Sciaridae, which are known to have complex chromosome cycles. In the chironomid *Acricotopus lucidus* all germ line limited chromosomes (=Ks) are eliminated from the future somatic nuclei during germ line-soma differentiation in early, still syncytial, embryonic divisions by remaining in the equatorial plane. But also in the germ line about half of the Ks are eliminated in the first mitotic division of the primary germ cells in the young gonads of first instar larvae. Until now not much is known about the cytogenetic processes in the course of germ line elimination of the Ks. In this study, the cytogenetic changes in the primary germ cells before, during and after the elimination mitosis were analysed in detail with DAPI stained gonad preparations.

Key words Elimination mitosis, Germ line limited chromosomes, *Acricotopus lucidus,* DAPI fluorescence

Introduction

Eliminations of X chromosomes and/or of full or partial complements of germ line limited chromosomes (named E = eliminated, K = "Keimbahn" or L = limited chromosomes) are known from the complex chromosome cycles found in the dipteran families Cecidomyiidae, Chironomidae and Sciaridae (Bauer and Beermann 1952, White 1973, Gerbi 1986).

In the Orthocladiinae, a subfamily of the Chironomidae, Bauer and Beermann (1952) reported from the elimination of Ks as well as from the soma as also from the germ line. The elimination of all Ks from the future somatic nuclei (=soma elimination) occurs in the germ line-soma differentiation during early syncytial embryonic mitoses in that the Ks remain in the equatorial zone, while the soma chromosomes (=Ss) segregate regularly. In the germ line about half of the Ks are eliminated in the first mitosis of the primary germ cells in the young gonads of first instar larvae (= germ line elimination). This reduction in the number of Ks in the germ line stem cells is then compensated in the last gonial mitosis prior to meiosis by the migration of all Ks as unseparated sister chromatids to only one cell pole (=differential mitosis). The Ss segregate regularly. The daughter cell, which receives all the Ks together with the Ss pass through a normal meiosis, while the daughter cell with only a diploid set of Ss develops to a nurse cell in the female and to an aberrant spermatocyte in the male (Bauer and Beermann 1952, Redi *et al*. 2001).

Experiments on cecidomyiid embryos resulting in the elimination of all Ks from the future germ line nuclei produced only sterile imagines in both sexes. Therefore, the Ks probably carry fertility factors (Geyer-Duszynska 1966, Bantock 1970).

The origin and the structural evolution of the Ks of the orthocladiid *Acricotopus lucidus* was investigated by chromosome painting of mitotic and meiotic metaphases using specific probes of the Ss (Staiber and Schiffkowski 2000, Staiber and Wahl 2002). The painting results clearly demonstrated, that the Ks are descendants of the Ss and possess large S-homologous sections in addition to heterochromatic segments containing germ line specific repetitive sequences.

Less is known of the cytogenetic behavior of the Ks during elimination from the primary germ cells in the Orthocladiinae. Up to now only one figure with drawings of meta- and anaphases of elimination mitoses of *Psectrocladius obvius* and *Acricotopus lucidus* has been published (Figure 2 in Bauer and Beermann 1952). Nothing is known about the mechanism discriminating between the half of the Ks that are eliminated, and the half that segregate regularly. In *Acricotopus*, a G-banding study of K complements of gonial cell showed after elimination mitosis that the K complements vary in number and composition of the nine different K types within one and between different larvae (Staiber 1988).

Therefore, the aim of the present study was to study and to document in detail the cytogenetic changes in the primary germ cells in *Acricotopus* before, during and after the process of elimination of about the half of the Ks.

Materials and methods

The animals used in this study were taken from a laboratory stock of *Acricotopus lucidus* (Diptera, Chironomidae). The rearing conditions are described in Staiber and Behnke (1985).

In order to analyse elimination mitosis in primordial germ cells (=germ line elimination of Ks) whole newly hatched larvae (0 h) and first instar larvae up to 48 h, kept at room temperature, were fixed in ethanol-glacial acetic acid (3:1) and then stored at -20°C. Squash preparations of first instar larvae were made in 45% acetic acid, frozen on dry ice, dehydrated in an ethanol series (70%, 80%, 96%) and air dried. Preparations were stained with 0.1 µg/ml 4',6'-diamidino-2-phenylindole (DAPI) in phosphate-buffered saline (PBS), washed with PBS and mounted in Vectashield fluorescence antifade solution (Vector Laboratories).

Labeling of the painting probes of the three somatic chromosomes (SI-SIII) and fluorescence *in situ* hybridization (FISH) are described in Staiber and Schiffkowski (2000).

Digital images of the DAPI fluorescence were captured with a Canon A80 camera using a Zeiss epifluorescence microscope (filter combination BP 365/FT 385/LP 397) equipped with a Neofluar 100/1.3 oil objective. They were first converted into 16 bit grey scale images and then inverted using a CorelDraw software package.

Results and discussion

An elimination of about half of the Ks from the germ line occurs in the chironomid *Acricotopus lucidus* in the primary germ cells of young gonads of first instar larvae (Fig. 1a-n) (Bauer and Beermann 1952, Bauer 1970). This reduction in the number of Ks is compensated in the last so-called differential gonial mitosis by a migration of the unseparated Ks to only one cell pole (Staiber 1994) as shown in Figure 2a,b. The fluorescence in situ hybridization (FISH) of the differential mitosis in Figure 2a using painting probes of the three Ss (SI, blue; SII, red; SIII, green) also clearly demonstrates that the Ks are descendants of the Ss and are composed of large (painted) S-homologous sections and of heterochromatic segments (Fig. 2b) containing germ line specific repetitive sequences (Staiber and Schiffkowski 2000, Staiber 2002).

In whole mounted carmine-stained newly hatched first instar larvae, as shown in Figure 1a, the gonads were found to be located in the region of the abdominal segments 6 to 7. The insert in Figure 1a presents a pair of gonads each containing only one primary germ cell. This is characteristic for *Acricotopus* and corroborates the observations of Bauer and Beermann (1952). It was very difficult to dissect gonads from newly hatched larvae (length 0.6 - 0.8 mm) for cytogenetic analysis. Therefore, whole body squash preparations were made after the dry ice method and were subsequently stained with DAPI.

Combining fluorescence and phase contrast microscopy, the gonads can be easily identified by the oval shape (Fig. 1f). The envelope around the primary germ cell is formed by some sheath cells. Most of the prometa-, meta-, ana- and telophase stages of elimination mitoses were found

in gonads of first instar larvae from 27 h to 31 h after hatching. Male and female larvae cannot be distinguished at this stage of development.

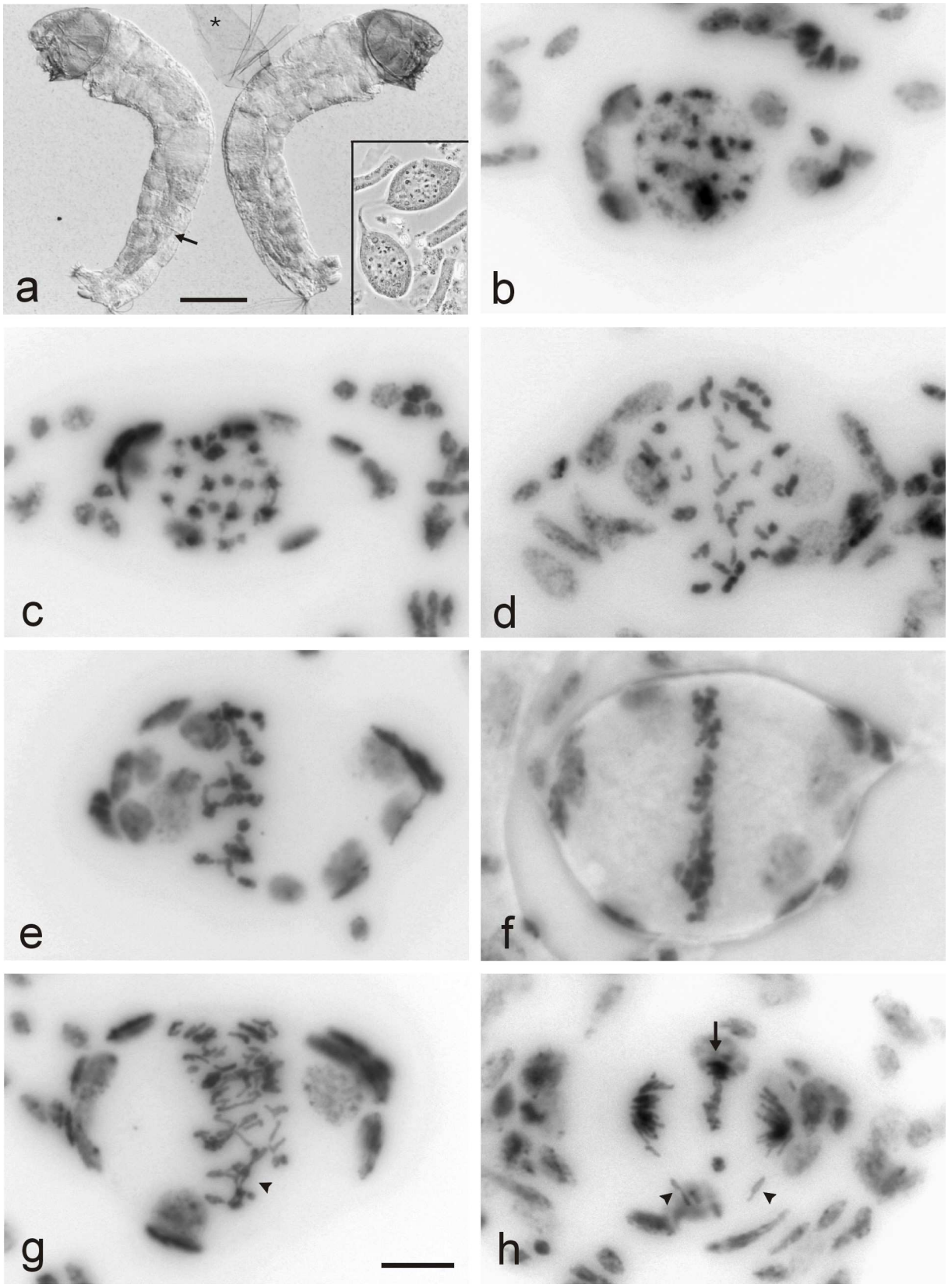

Fig. 1a-h. (**a**) Two carmine-stained newly hatched first instar larvae of *Acricotopus lucidus*. The arrow indicates the position of one gonad. The asterisk marks a part of an egg shell. Differential interference contrast. Bar represents 100 µm. Insert: Pair of gonads each containing only one primary germ cell.

(**b**-**n**) Young gonads with primary germ cells in different stages of elimination mitosis. Inverse DAPI fluorescence. For detailed description of stages see text. (**b**) Primary germ cell in 'resting stage' with semi-condensed chromosomes. (**c**) Prophase. (**d**) Early prometaphase with 6 Ss and about 30 Ks. (**e**) Late prometaphase. (**f**) Metaphase. Fluorescence/phase contrast. (**g**) Early anaphase. Arrowhead indicates the Ks that probably will be eliminated. Bar represents 10 µm. (**h**) Late anaphase. About half of the Ks remain in the equatorial plane (arrow). Delayed pole movement of two sister chromatids (arrowheads).

In the primary germ cell nuclei of newly hatched larvae the individual chromosomes are found to be present as separate entities arrested in a specific semi-condensed state (Fig. 1b). The chromosomes are composed of more condensed central domains surrounded by fuzzy chromatin and are spherically arranged towards the periphery of the nucleus. This characteristic chromosome arrangement can be observed in *Acricotopus* already in the nuclei of the pole cells shortly after their formation and in the primordial germ cells in the course of their dorsal migration towards the future gonads during early gastrulation (data not shown). Such a 'resting stage' of germ line cells ranging from pole cell formation to hatching of the larvae was also reported for *Sciara.* De Saint Phalle and Sullivan (1996) described this stage in *Sciara coprophila*, a species having also germ line limited chromosomes, as a 'prophase with the chromosomes condensed on the nuclear membrane, like fingers grasping a ball', and Perondini and Ribeiro (1997) in *Sciara ocellaris*, as an 'atypical interphase exhibiting semi-condensed chromosomes'.

In transition from the 'resting stage' to prophase of the elimination mitosis in *Acricotopus*, the fuzzy chromatin around the pycnotic center of the chromosome domains condenses (Fig. 1c), but the chromosomes remain arranged at the periphery of the nucleus. With the breakdown of the nuclear membrane at the beginning of the prometaphase, the typical spherical arrangement of the chromosomes disappears. In the prometaphase cell in Figure 1d, about 30 Ks are present in addition to the 6 Ss. At the end of prometaphase the chromosomes move towards the equatorial zone (Fig. 1e).

In the fluorescence/phase contrast image of the metaphase in Figure 1f all chromosomes are arranged exactly in the equatorial plane. But, here as well as in the prometaphase it is not possibile to differentiate between Ss and Ks, or Ks that will be eliminated from Ks that will segregate normally by cytogenetic characteristics, as i.e. a differential condensation state.

In the early anaphase, in Figure 1g, in the upper part of the mid zone the chromosomes, which have separated regularly, are moving to the opposite poles, whereas in the middle part some sister chromatids and in the lower part nearly all chromatids (arrowhead) seem to stick together. In the late anaphase, in Figure 1h, about half of the Ks move together with the Ss towards each pole, while the other half of the Ks remain at the equator. In the lower part of the spindle two K chromatids have separated late and migrate behind the other Ks to the poles (arrowheads). As shown in Figure 1i (late anaphase) one chromatid, probably a K, has moved widely to the right pole, while its long arm is still connected with its sister chromatid in the equatorial plane. The reason why the sister chromatids behave differently is unclear. This unequal segregation would lead to daughter cells with different sets of Ks. This was observed earlier in a G-banding study analysing the number and composition of gonial K complements of

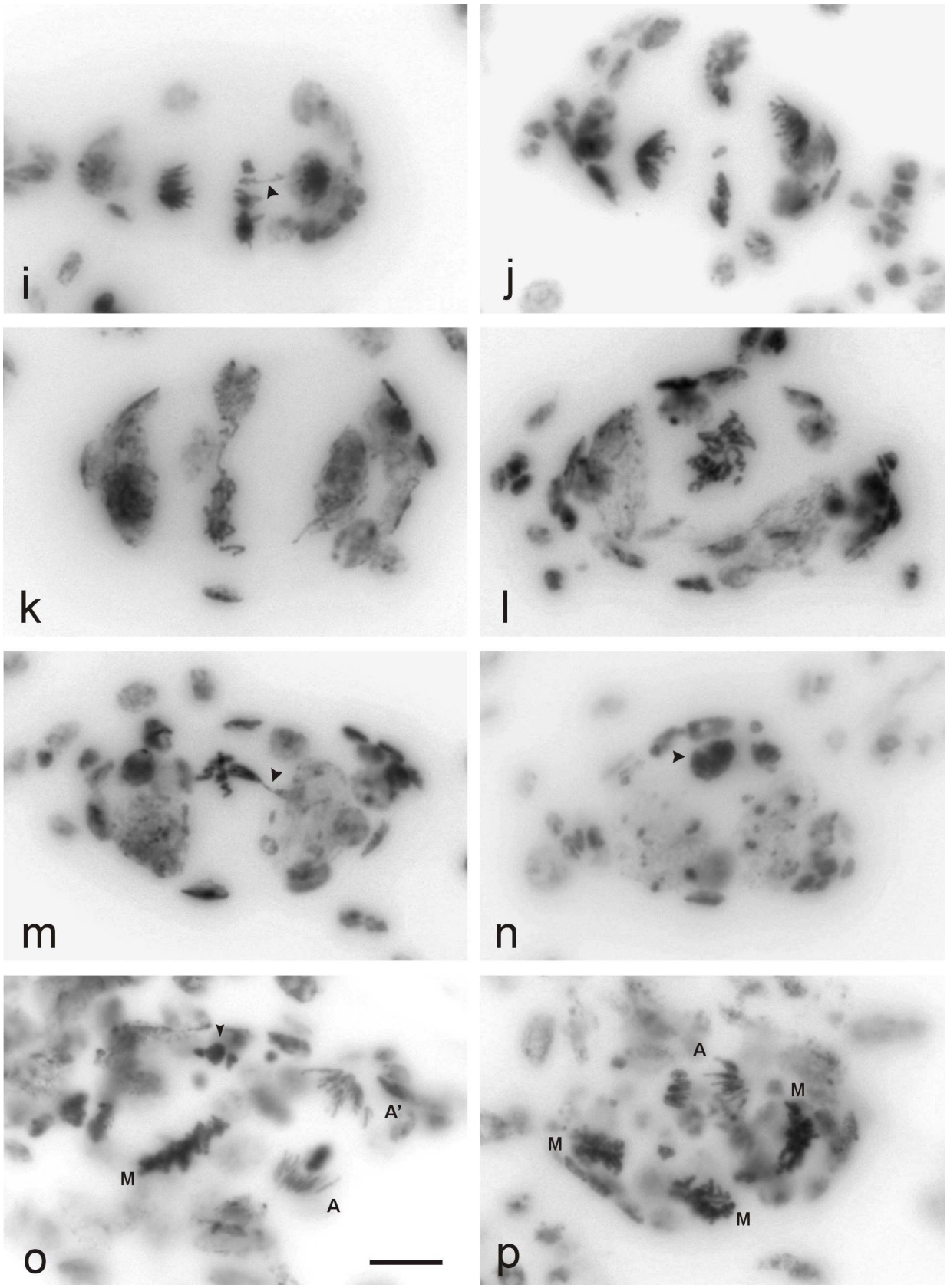

Fig. 1i-p. (**i**) Late anaphase. One pole moving chromatid is still connected with its sister chromatid in the equator (arrowhead). (**j**) Late anaphase. The eliminated Ks are still accurately arranged in the equator. (**k**) Telophase. (**l**) Interphase nuclei with slightly spread eliminated Ks. (**m**) Chromatin bridge (arrowhead) ranging from the eliminated Ks to the right interphase nucleus. (**n**) Interphase nuclei with a large lump of pycnotic chromatin (arrowhead) derived from the eliminated Ks. (**o**) Second gonial mitosis. The cells divide asynchronously. One cell is in metaphase [M], the other in late anaphase [A, A']. Arrowhead

indicates heteropycnotic K chromatin droplets from the first mitosis. Bar represents 10 µm. (**p**) Third gonial mitosis. Three cells are in metaphase [M] and one in mid anaphase [A].

young fourth instar larvae (Staiber 1988). Within one gonad the number of Ks in different cells can vary between 0-2 Ks. Surprisingly, the K complements of cells from sister gonads differ only in 0-2 K types. If the elimination of Ks in both gonads would be completely random one would expect a higher variation.

While in the late anaphase, in Figure 1j, the eliminated Ks are still strongly condensed and are arranged exactly in the equatoral plane, then in telophase (Fig. 1k) during the decondensation of the Ks and Ss in the daughter nuclei, the eliminated Ks decondense to some extent and spread in the mid zone. This spreading of the eliminated Ks can be seen more clearly at the beginning of the interphase (Fig. 1l). In some cases chromatin bridges were observed extending from the eliminated Ks to one of the interphase nuclei (arrowhead in Fig. 1m). Later on in the interphase the eliminated Ks fuse together (Fig. 1n), forming 1 to 3 different sized lumps of strongly condensed chromatin (Fig. 1n) which shows bright DAPI-fluorescence. Whether the eliminated Ks are in or excluded from the cytoplasma of the daugther cells cannot be determined, because the cell membranes are not visible within the gonads, neither in phase contrast nor in differential interference contrast.

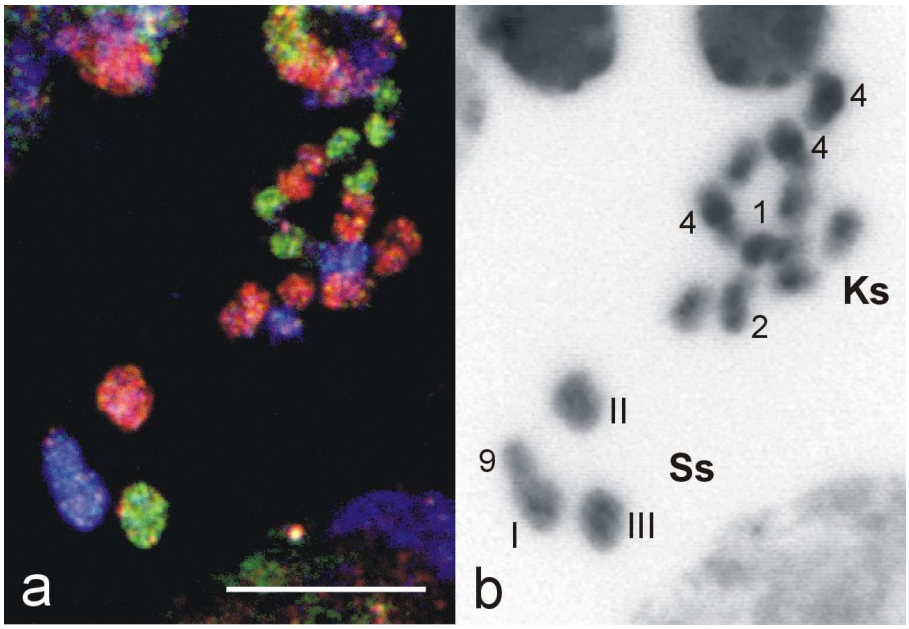

Fig. 2. (**a**) Three colour FISH of a differential mitosis of a spermatogonial cell of *Acricotopus lucidus* with probes of the three somatic chromosomes, SI (blue), SII (red) and SIII (green). The Ss and the S-homologous sections of the Ks (n=11) are painted in the appropriate colours. In this last gonial mitosis the Ks move as unseparated pairs of chromatids to only one pole (upper one), while the paired Ss (2n=6) still remain in the equatorial plane. The special K9 is still partially paired with one of the SI and will follow the other Ks. When all the Ks have reached the cell pole the Ss will begin to segregate regularly. This unequal mitosis doubles the number of Ks in the upper daughter cell, the future functional spermatocyte, which then pass through regular meiosis. The other daughter cell without the Ks develops to an aberrant spermatocyte. (**b**) Inverse image of the DAPI-stained chromosomes. The paracentromeric hetero-chromatic regions of the Ks, that are not painted in (**a**), are clearly seen.

Elimination mitoses does not proceed synchronously in sister gonads. Some larvae were found in which in the one gonad the primary germ cell was still in prophase, while in the other gonad the elimination mitosis was already completed, resulting in two daughter cells and lumps of eliminated chromatin (not shown). This indicates that the primary germ cells initiate the elimination mitosis autonomously. Within a gonad the second and the third mitosis do not proceed synchronously. In the second mitosis in Figure 1o, one cell is still in metaphase, while the other is already in late anaphase, and in the third mitosis in Figure 1p three cells are in metaphase and one in anaphase. Sometimes pycnotic chromatin droplets derived from the elimitated Ks can be observed still in the second mitosis (arrow in Fig. 1o).

The movements of all chromosomes during prometaphase and the arrangement into the equatoral plane of the Ks that will be eliminated and as well as the escaping Ks and of the Ss is caused by opposite directed microtubule forces acting on the centromeres of the sister chromatids. The reason why the sister chromatids of the eliminated Ks do not segregate is unclear. In *Sciara coprophila* incomplete sister chromatid separation is responsible for the programmed elimination of X chromosomes (De Saint Phalle and Sullivan 1996). Possibly such a failure of sister chromatid separation is also the mechanism of the elimination of Ks in the first mitosis of the primary germ cells in *Acricotopus*.

The study of the programmed elimination of all Ks from the future somatic nuclei during germ line-soma differentiation in early embryogenesis of *Acricotopus* may give further information on the mechanism effecting the non-segregation of germ line limited chromosomes in elimination mitoses.

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