Karyological analysis of an interspecific hybrid between the dioecious *Silene latifolia* and the hermaphroditic *Silene viscosa*

Michaela Markova, Martina Lengerova, Jitka Zluvova, Bohuslav Janousek, and Boris Vyskot

Abstract: The genus *Silene* is a good model for studying evolution of the sex chromosomes, since it includes species that are hermaphroditic and dioecious, while maintain a basic chromosome number of $2n = 24$. For some combinations of *Silene* species it is possible to construct interspecific hybrids. Here, we present a detailed karyological analysis of a hybrid between the dioecious *Silene latifolia* as the maternal plant and a related species, hermaphroditic *Silene viscosa*, used as a pollen partner. Using genomic probes (the genomic in situ hybridization (GISH) technique), we were able to clearly discriminate parental genomes and to show that they are largely separated in distinct nuclear domains. Molecular GISH and fluorescence in situ hybridization (FISH) markers document that the hybrid genome of somatic cells was strictly additive and stable, and that it had 12 chromosomes originating from each parent, including the only X chromosome of *S. latifolia*. Meiotic analysis revealed that, although related, respective parental chromosomes did not pair or paired only partially, which resulted in frequent chromosome abnormalities such as bridges and irregular non-disjunctions. GISH and FISH markers clearly document that the larger genome of *S. latifolia* and its largest chromosome component, the X chromosome, were mostly employed in chromosome lagging and misdivision.

Key words: sex chromosome, *Silene*, interspecific hybrid, meiotic pairing, misdivision.

Résumé : Le genre *Silene* est un bon modèle pour étudier l'évolution des chromosomes sexuels puisqu'il comprend à la fois des espèces hermaphrodites et dioïques qui ont toutes le même nombre de chromosomes ($2n = 24$). Dans certains cas, il est possible de produire des hybrides interspécifiques. Les auteurs présentent ici une analyse caryologique détaillée d'un hybride entre l'espèce dioïque *Silene latifolia* (parent maternel) et l'espèce apparentée hermaphrodite *Silene viscosa* (parent paternel). Au moyen de sondes génomiques (technique GISH), les auteurs ont réussi à distinguer nettement les génomes parentaux et à montrer qu'ils logent dans des domaines nucléaires distincts. Des analyses moléculaires avec des marqueurs GISH et FISH ont révélé que le génome somatique hybride est strictement additif et stable, incluant 12 chromosomes de chacun des deux parents dont l'unique chromosome X du *S. latifolia*. Des analyses méiotiques ont montré que, bien qu'apparentés, les chromosomes parentaux ne s'appariaient pas ou seulement en partie ce qui produisait de nombreuses anomalies telles que des ponts et des non-disjonctions irrégulières. Les marqueurs GISH et FISH ont clairement documenté que le génome plus large du S. latifolia et son plus grand chromosome, le chromosome X, étaient plus souvent impliqués dans les anomalies sous forme de chromosomes retardataires ou incorrectement divisés.

Mots clés : chromosome sexuel, *Silene*, hybride interspécifique, appariement méiotique, division anormale.

[Traduit par la Rédaction]

Introduction

Silene latifolia (syn. *Melandrium album*; white campion) is a dioecious plant, which possesses a pair of well-distinguishable sex chromosomes in the diploid genome, XX in females and XY in males (Vyskot and Hobza 2004). Recently, this species became an important model object for studies of the early stages of the sex chromosome evolution (Negrutiu et al. 2001). The main advantages of this model in comparison with mammalian model systems (including human), for example, are that the sex chromosomes in this species arose relatively recently (Nicolas et al. 2005) and that there are related *Silene* species that are not dioecious (Desfeux et al. 1996). Existence of hermaphrodite relatives of dioecious species gained much attention from researchers studying sex determination during the early years of the discipline. Correns (1928) made crosses between hermaphroditic and dioecious species to discriminate which sex in *S. latifolia* is heterogametic. He succeeded only in crosses where an *S. latifolia* female was pollinated by a hermaphro-

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ditic *Silene viscosa* (the reciprocal cross was not successful in spite of all efforts). All of the plants originating from the cross showed reduced stamen development, therefore Correns described them as females. As the female progeny were uniform, the result confirmed Correns's opinion that the heterogametic sex in *S. latifolia* is the male sex. Zluvova et al. (2005) performed detailed histological study of *S. latifolia* × *S. viscosa* hybrids (which were also obtained by standard cross and the reciprocal cross yielded no viable seeds) to study evolutionary changes in the genes during sex chromosome evolution. Conclusions of this study suggest that some Y-linked genes evolved very rapidly, so that their function cannot be substituted by the action of their homologues from the related hermaphroditic species.

The presence of 2 kinds of chromosomal sets (a set coming from the dioecious organism and a set coming from the hermaphrodite) in one cell also offers further possibilities for the study of changes that occur during sex chromosome evolution. Here, we study the interactions of the dioecious and hermaphroditic chromosome sets on the cytological level. Special attention is given to the behaviour of the X chromosome in this system. This approach enables one to answer the question as to whether there is any difference between the X chromosome and autosomes in their abilities to retain the competence of meiotic pairing or even recombination with their putative homologues from the *S. viscosa* chromosome set. It cannot be highly expected that large differences between the X chromosome of *S. latifolia* and its relative of *S. viscosa*, which could affect meiotic pairing, are present; in mammals, the X chromosome structure is relatively uniform even when distant taxa are compared (Kohn et al. 2004). If there are changes on the level of genetic control of meiotic pairing, genetic control of meiotic recombination, chromatin structure, or chromosome structure, than interactions between some of the chromosomes or between both sets will be prevented. If the competence of meiotic pairing between the X and its homologue were retained, it would also be possible to find a homologue of the X chromosome in *S. viscosa*.

Materials and methods

Plant material

Wild type *S. latifolia* and *S. viscosa* plants came from the seed collection of the Institute of Biophysics in Brno, Czech Republic. Flower buds of *S. latifolia* females were covered with paper bags before the flowers opened to prevent cross pollination. After anthesis, flowers were hand pollinated with pollen from *S. viscosa*.

Slide preparation

Root tips of germinating seeds of *S. latifolia, S. viscosa*, and their hybrids were used for preparation of metaphase chromosomes. The seeds were sterilized and grown in water at 4 °C for 3 d to synchronize germination, followed by another 3 d of growth at room temperature. The cell cycle was synchronized with 30 µmol/L aphidicolin (Sigma, St. Louis, Mo.). After 16 h, the roots were washed with water and exposed to 15 µmol/L oryzalin (Sigma) for 5 h to accumulate the dividing cells in metaphase. Metaphase protoplasts were isolated as described by Hladilova et al. (1998). Protoplast suspensions were fixed in ethanol – acetic acid (3:1) and dropped onto slides. Young floral buds of hybrid plants and their parents were collected and fixed, and squash preparations were made to analyse male meiosis.

Fluorescence in situ hybridization

Four different FISH probes were used as chromosome markers: an 18 kb genomic clone containing the *DD44X* allele (Moore et al. 2003), which was cytogenetically mapped on the q arm of the X chromosome of *S. latifolia* (Lengerova et al. 2003); X-43.1 is a subtelomeric repetitive sequence specific for *S. latifolia* chromosomes (Buzek et al. 1997); 25S rDNA (2.5 kb long *Eco*RI fragment of tomato 25S rDNA) was isolated by Kiss et al. (1989); and part of the 5S rDNA isolated by Fulnecek et al. (1998). Total genomic DNA isolated from both parents as described in Zhang et al. (1995) was used for genomic in situ hybridization. For green labelling, SpectrumGreen direct-labelled dUTP and the nick translation kit (both from Vysis, Chicago, Ill.) was used; for red labeling, Fluorolink Cy3-dUTP (Amersham, Piscataway, N.J.) in combination with nick translation mix (Roche, Basel, Switzerland) was used. The FISH procedure was performed as described by Lengerova et al. (2004). DAPI C-banding was performed according to Buzek et al. (1997). After analysis, slides were washed as described in Heslop-Harrison et al. (1992) to remove the bound probes and were then reprobed. Slides were checked on an Olympus Provis AX70 fluorescent microscope with separate filter sets for DAPI, Cy3, and SpectrumGreen dyes, photographed with a monochrome AxioCam MR CCD camera (Zeiss, Jena, Germany), and visualized using ISIS software (Metasystems, Altlussheim, Germany).

Genome size estimation

To assess relative DNA content in *S. latifolia* and *S. viscosa* genomes, a modified method according to Matsunaga et al. (1994) was used. After GISH, mitotic spreads of hybrids were quantitatively counterstained with DAPI (0.2 mg/mL in 2× SSC) for 30 min. After extensive washing in 2× SSC slides were mounted in Vectashield (Vector Labs, Burlingame, Calif.) and analyzed under a fluorescence microscope. Based on hybridization signals, the chromosomes were sorted according to their parental origin. The fluorescence of DAPI counterstain was registered for chromosomes from each parent (10 metaphases each) and using an analytical tool in the ISIS software package (MetaSystems), the integrated fluorescence (number of fluorescent pixels multiplied by pixel intensities) was recorded. Paired comparison of genome sizes was performed using a signed-rank Wilcoxon test.

Phylogenetic analysis

Sequences of intergenic spacers (ITS1 and ITS2) were grouped and analysed as a single sequence. Alignment was performed using Clustal X software (BCM search launcher, http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). A phylogenetic tree was created using the program TreeTop (http://www.genebee.msu.su/services/phtree_reduced.html) using both the topological and clustering algorithms with 100 repetitions.

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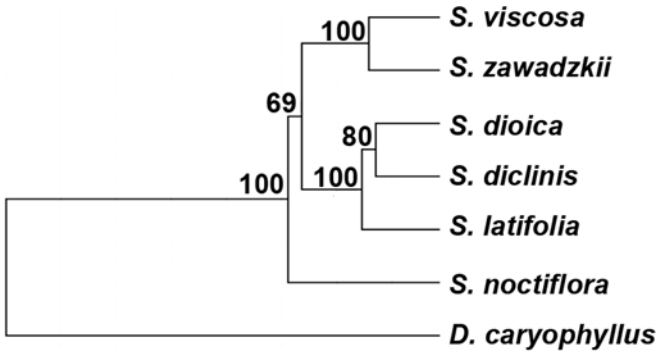
Fig. 1. Relationship of the dioecious species of the section *Elisanthe* (*Silene dioica, Silene diclinis*, and *Silene latifolia*) and other members of the previous genus *Melandrium* (*Silene noctiflora, Silene zawadzkii*, and *Silene viscosa*) as revealed by construction of a phylogenetic tree based on analysis of cumulated ITS1 and ITS2 regions of 45S rDNA. *Dianthus caryophyllus* is included as an outgroup member. Numbers indicate bootstrap values. Note a close relationship of *Silene viscosa* with *S. zawadzkii*, both of them showing good crossability to the dioecious *Silene* species of the section *Elisanthe* (Prentice 1978; Zluvova et al. 2005). *Silene noctiflora* appears to be more distant. This fact is in accordance with the data obtained in previous crossing experiments (Prentice 1978).



Results

As seen from the phylogenetic tree, *S. latifolia* and *S. viscosa* are closely related (Fig. 1). Using GISH on metaphase chromosomes with both parental genomes, we were able to clearly distinguish the parental genomes (Fig. 2). During mitotic metaphase in root meristems, 12 chromosomes of *S. latifolia* (including the only X chromosome, the largest in the female genome) and 12 *S. viscosa* chromosomes were regularly observed (Fig. 2*c*). To compare the size of *S. viscosa* and *S. latifolia* genomes, the relative contents of DNA of each chromosome during metaphases of the interspecific hybrid were measured. Subsequent GISH labelling enabled us to distinguish the chromosomes according to their parental origin.

Using non-parametric statistics (signed-rank Wilcoxon test), the *S. latifolia* genome was found to be significantly larger than the *S. viscosa* genome ($P = 0.006$); comparing relative DNA contents, we found out that the *S. latifolia* genome is approximately 1.2 times larger, which was confirmed by comparison of the data. The X chromosome of *S. latifolia* is about 2.0 times larger than an average autosome of *S. viscosa* and 1.8 times larger than an average autosome of *S. latifolia*. This fact obviously reflects a higher frequency of repetitive DNA sequencies in *S. latifolia*. These repeats are largely accumulated at the subtelomeres of the vast majority of *S. latifolia* chromosomes (Fig. 3*e*). During evolution, at least some of them diverged to such extent that they are species specific (e.g., X-43.1, Fig. 3*f*).

Since the *S. latifolia* genome is significantly larger than the *S. viscosa* genome and this size difference may lead to chromosome lagging during anaphase and consequently to misdivision and aneuploidy, we have tested the interspecific hybrid for possible mitotic aberrations. No irregularities were found during individual phases of mitosis (Fig. 2*a* for interphase, 2*b* for prophase, 2*c* for metaphase, 2*d* for anaphase, and 2*f* for telophase). To better characterize the somatic stability of the hybrid nucleus, FISH probes were used. Regular division in anaphase was verified using the 5S rDNA probe. Figures 2*e* and 2*g* show that 4 chromosomes possessing the 5S rDNA signals divide in anaphase into 4 chromatids and create 2 symmetrical daughter cells.

Using 25S and 5S rDNA probes, the structure and number of chromosomes were verified. We counted the signals of these repetitive sequences in both parents and in the hybrid (Fig. 3). *Silene latifolia* had 5 pairs of 25S rDNA signals and 3 pairs of 5S rDNA signals (Fig. 3*a*), whereas *S. viscosa* had 7 pairs of 25S rDNA and 1 pair of 5S rDNA (Fig. 3*b*). The *S. latifolia* × *S. viscosa* hybrids had 5 signals of 25S rDNA from *S. latifolia* and 7 signals of *S. viscosa* (12 altogether) and 3 plus 1 signals of 5S rDNA, respectively (4 altogether, Fig. 3*c*). Thus we conclude that the hybrid has an exactly additive character. The last FISH probe used in this study was employed as an X-chromosome marker, DD44, which hybridizes specifically to the pair of sex chromosomes in *S. latifolia*. In *S. viscosa*, DD44 yields a clear signal on a single pair of chromosomes. This fact was confirmed in the hybrid (Fig. 3*d*).

To address the question of the chromosome pairing and homologue of the X chromosome, we analysed the meiotic cycle of this hybrid, particularly the pairing of chromosomes. The DD44 probe served as an X-chromosome marker. In pachytene, the chromosomes were close together along some small regions, but they did not recombine in diplotene. Some indications of pairing occurred in pachytene and diplotene, but we mostly observed only 2–3 pairs of a possible 12 (Figs. 4*a*, 4*b*). However, in metaphase I, no bivalents were formed (Fig. 4*c*). For comparison, we present the regular chromosome pairing in the *S. latifolia* male (Fig. 4*d*). In anaphase I, the X chromosome, which comes from *S. latifolia*, tended to lag (Figs. 4*e*-*g*). Provided the X chromosome pairs with its *S. viscosa* homologue, in anaphase I we could expect 2 signals of DD44 on the X chromosome in one half of the anaphase figure and 2 signals on the second half on the *S. viscosa* chromosome. However, we observed 2 pieces of the X chromosome from *S. latifolia*, both labelled with 1 signal of DD44 (Fig. 4*e*). This phenomenon can be interpreted as a premature misdivision of the X chromatids in anaphase I. Similarly, autosomes did not divide symetrically either (Figs. 4*f*, 4*g*). We observed frequent bridges in anaphase II, probably caused by asymmetric separation of chromatids (Figs. 4*h*, *i*). This situation was found in more than 80% of anaphases scored. Four tetrads were finally created (Fig. 4*j*).

Discussion

In the presented work, we have studied an interspecific hybrid in which genomes of 2 related species are combined to form a hybrid containing only 1 X chromosome (from the dioecious *S. latifolia).* This approach enabled us to answer the question as to whether there is any difference between the X chromosome and the autosomes in their abilities to retain the competence of meiotic pairing or even recombina-

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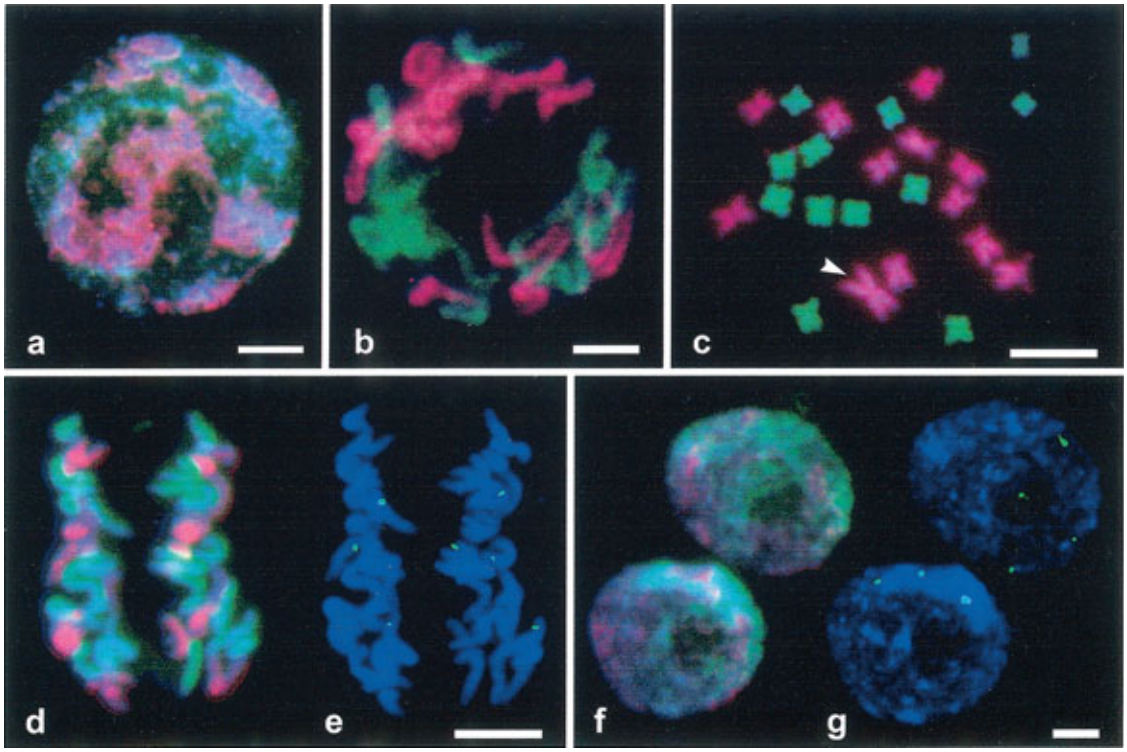
Fig. 2. Root tip meristematic nuclei of the hybrid plant *Silene latifolia* × *Silene viscosa* analysed with GISH and FISH probes: *S. latifolia* female genomic DNA (red), *S. viscosa* genomic DNA (green), and DAPI counterstaining (blue). (*a*) Interphase. (*b*) Prophase. (*c*) Metaphase (the X chromosome is indicated). (*d*) Anaphase. (*f*) Telophase. Slides *d* and *f* were later rehybridized with 5S rDNA (green signals), resulting in figures *e* and *g*, respectively. Bars indicate 10 μm.



tion with their putative homologues from the *S. viscosa* chromosome set.

Because the *S. latifolia* genome is significantly larger than the *S. viscosa* genome and this size difference may lead to chromosome lagging during anaphase and consequently to misdivision and aneuploidy, we have tested the interspecific hybrid for possible mitotic aberrations. We conclude that this phenomenon obviously does not occur in the somatic *S. latifolia* × *S. viscosa* hybrid nuclei. No irregularities throughout the mitotic cycle were observed. Parental genomes occupy different domains of hybrid nucleus, which is a phenomenon widely observed in plants (Leitch et al. 1991; Schwarzacher et al. 1992).

The hybrid plants have a nearly normal male developmental program (Zluvova et al. 2005), so anthers are formed and meiosis is completed. During meiosis I, we can, in principle, expect 1 of the 3 main patterns of chromosome behaviour: chromosome homologues could either (*i*) pair, recombine, and be equally distributed as two-chromatid bodies to daughter nuclei without larger problems (Nicolas et al. 2005); (*ii*) just pair without recombination (Nasrallah et al. 2000); or (*iii*) not pair or pair to a highly restricted extent (Singh 2003). We have observed only a very limited chromosome pairing not enabling the recognition of the homologue of the X chromosome and no obvious chromosome recombination occurred. Because the loss of homologous pairing is genome wide, it can be concluded that the cause of non-pairing is not represented by chromosome rearrangements but rather by a divergence of elements controlling meiotic pairing.

On the molecular level, a number of sites or regions have been identified that appear to facilitate chromosome pairing. The one commonality of these regions is that they all map near to or comprise repetitive sequences (Hawley 1980; McKim et al. 1988; McKee 1996; Sanford and Perry 2001). The most well-characterized pairing site is a 240 bp repeat sequence in the intergenic spacer found between ribosomal RNA genes clustered on the *Drosophila* X and Y chromosomes. When present in multiple copies, this sequence facilitates the pairing and subsequent segregation of the X and Y chromosomes during meiosis in *Drosophila* males (McKee 1996). Because repetitive sequences between *S. latifolia* and *S. viscosa* largely diverged, this could also concern the regions facilitating chromosome pairing.

Owing to the lack of chromosome pairing, chromosomes behaved like univalents. In anaphase I, they moved randomly to different poles. We observed frequent chromatin bridges in anaphase I and telophase I. As shown by GISH, these irregular misdivisions were stained with the *S. latifolia* probe and the bridges very often included the X chromosome marker. This process may lead to telocentrics and isochromosomes (Singh 2003). In principle, a similar process is repeated in meiosis II, where chromatids are split randomly into daughter nuclei.

In anaphase I, we also observed frequent bridge formation largely coming from the *S. latifolia* genome and especially from its X chromosome. We conclude that owing to loss of chromosome pairing and recombination between the *S. latifolia* and *S. viscosa* genomes, their chromosomes are not regularly separated, which leads to numerous chromosome

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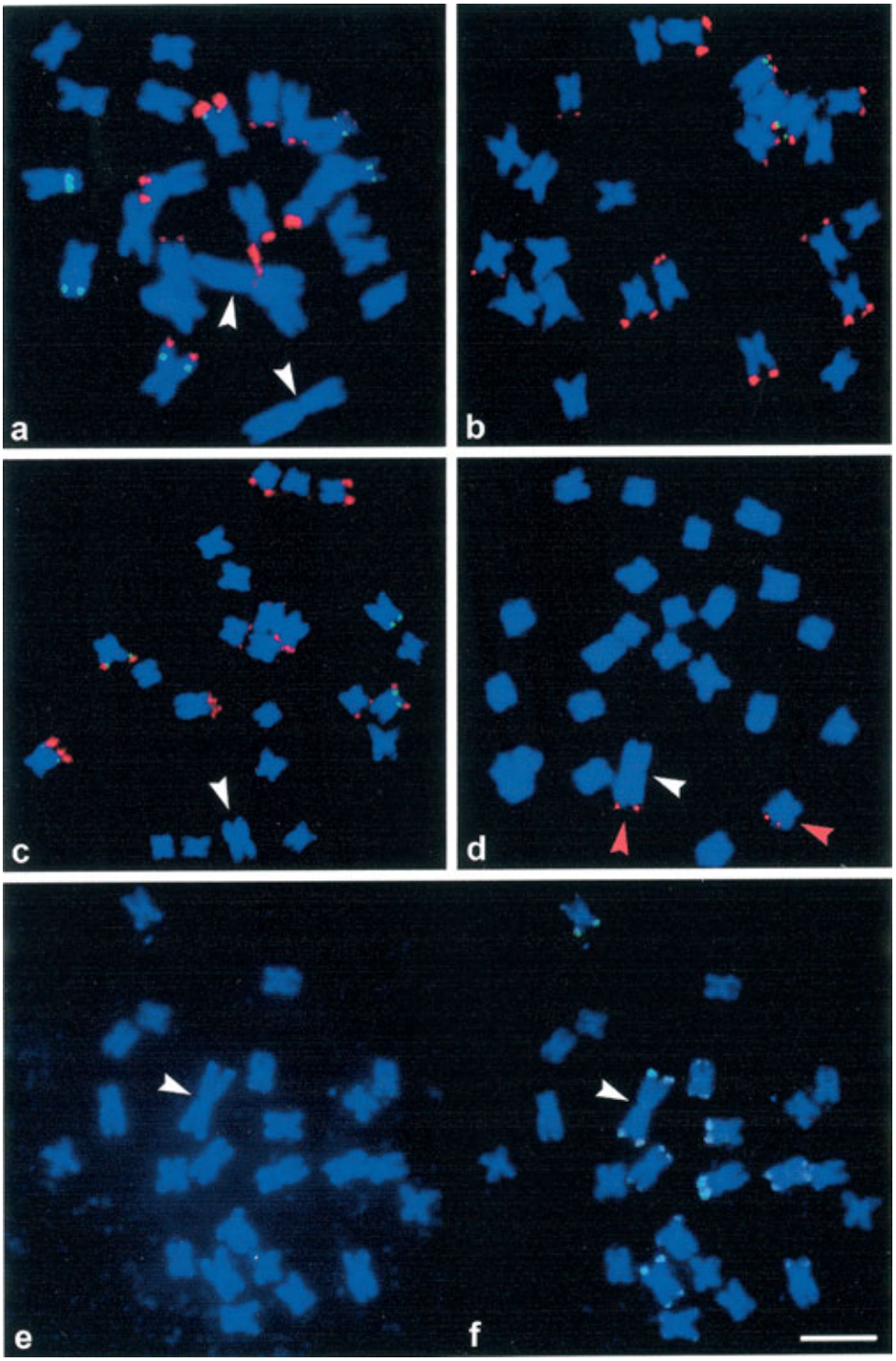
Fig. 3. Karyotypes of the hybrid and its parents. Localization of 25S rDNA (red) and 5S rDNA (green) signals on mitotic metaphase chromosomes of *Silene latifolia* female (*a*), *Silene viscosa* (*b*), and their hybrid (*c*). DD44 marker as visualised on the chromosomes of the hybrid, the signals indicated by arrows (*d*). DAPI-C banding on the hybrid showing dense subtelomeric bands in *S. latifolia* chromosomes (*e*), which are identified with the X43.1 *S. latifolia* specific marker (*f*). Chromosomes are counterstained with DAPI (blue) and the X chromosomes are indicated. Bar represents 10 μm.



abnormalities often seen in sterile, unbalanced hybrids. We also assume that the hybrid genome, which tolerates the parental size differences during mitosis, is vulnerable to this size problem both in meiosis I and meiosis II. The size of the genome is obviously not critical, but it does reflect a difference in the duration of meiosis (Bennett 1971). A similar explanation can be applied for the lagging X chromosome. In this case, the X chromosome serves as a cytogenetic marker for its extreme size rather than for its function in sexual development. Surprisingly, meiosis is completed in

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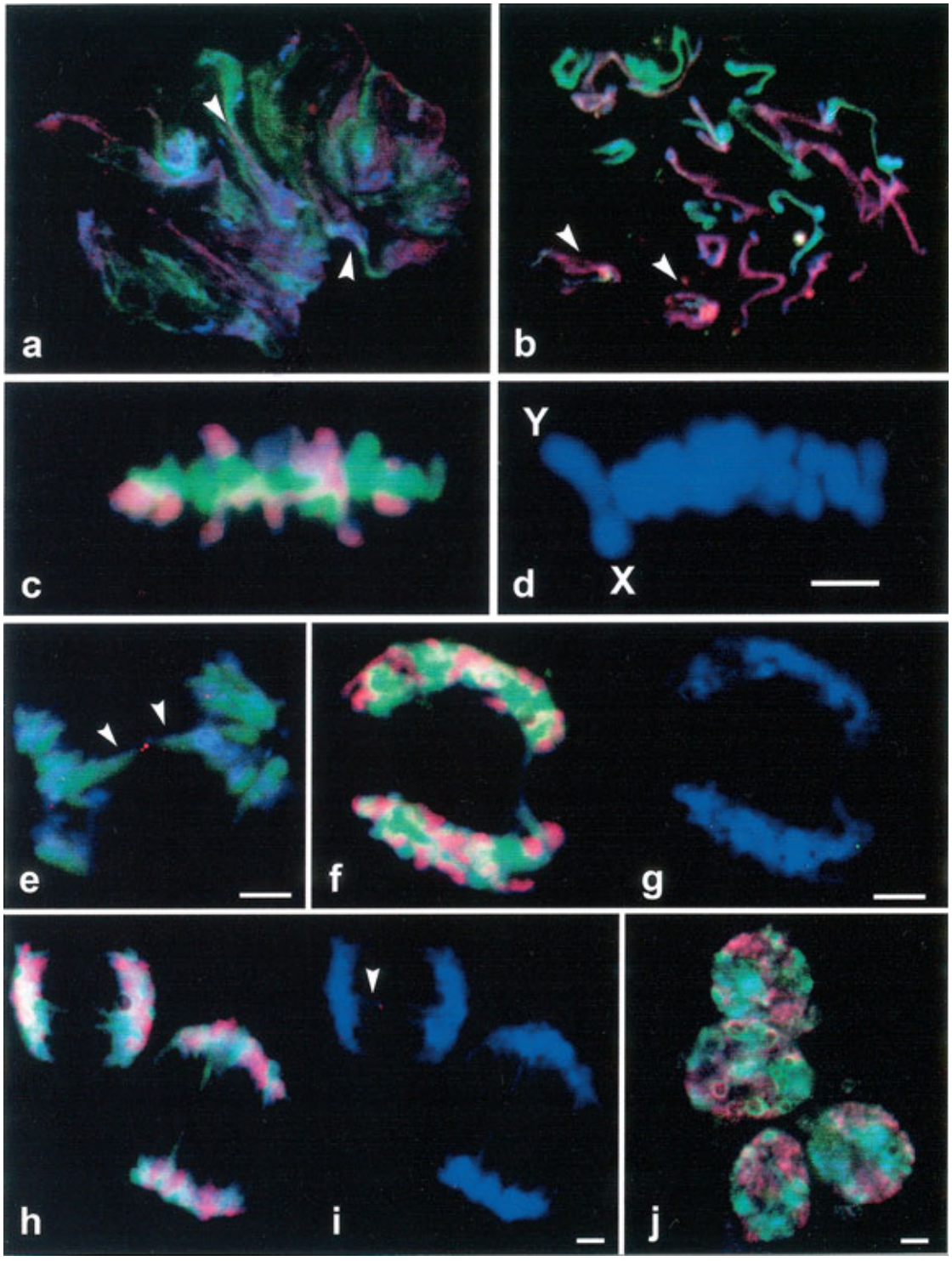
Fig. 4. Analysis of meiosis in PMCs of the hybrid *Silene latifolia* × *Silene viscosa* (*a–c*, *e–j*) and *S. latifolia* as a control (*d*). (*a–c*) PMCs illuminated by GISH with *S. latifolia* female genomic DNA (red) and *S. viscosa* genomic DNA (green), and counterstained with DAPI (blue). (*a*) Pachytene stage. (*b*) Diakinesis (arrows show coupled chromosomes). (*c*) Metaphase I (no bivalents are formed in the hybrid). (*d*) For comparison, bivalents in metaphase I of *S. latifolia* are shown and X and Y chromosomes are indicated. (*e*) Anaphase I of the hybrid hybridized with *S. latifolia* female genomic DNA (green) and DD44 (red) to demonstrate a misdivision of the X chromosome. (*f*) Anaphase I hybridized with *S. latifolia* female genomic DNA (green) and *S. viscosa* genomic DNA (red). (*g*) The same figure as *f*, but hybridized with 5S rDNA (green) to show irregularities in the division of autosomes. (*h*) Anaphase II hybridized with *S. latifolia* female genomic DNA (green) and *S. viscosa* genomic DNA (red). (*i*) The same figure as in *h* hybridized with DD44 (red) and 5S rDNA (green). (*j*) Pollen tetrads hybridized with *S. latifolia* female genomic DNA (green) and *S. viscosa* genomic DNA (red). Slides were counterstained with DAPI (blue). The positions of the X chromosome are identified according to the DD44 marker and *S. latifolia* genomic probe. Bars represent 10 μm.



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tetrads; however, owing to their variable genome components, they are not able to form equivalent spores.

When hybrids are viable, their degree of chromosome pairing in meiosis I indicates homology of DNA sequences. There is a great variety of extent of pairing between parental chromosomes in plant interspecific hybrids, usually presented by fractions of bivalents and univalents (Singh 2003). In the hybrid *S. latifolia* × *S. viscosa* presented here, we can speak about minimal pairing, since we observed only some indications of synopsis or association rather than bivalents. In conclusion, our data show that *S. latifolia* and *S. viscosa* chromosomes are no longer able to pair in meiosis owing to evolutionary changes occurring after the divergence of their common ancestor. Because the loss of homologous pairing is genome-wide, it can be concluded that the cause of nonpairing is not represented by chromosome rearrangements but rather by a divergence of elements controlling meiotic pairing.

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