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Multicolor FISH mapping of the dioecious model plant, *Silene latifolia*

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Abstract *Silene latifolia* is a key plant model in the study of sex determination and sex chromosome evolution. Current studies have been based on genetic mapping of the sequences linked to sex chromosomes with analysis of their characters and relative positions on the X and Y chromosomes. Until recently, very few DNA sequences have been physically mapped to the sex chromosomes of *S. latifolia*. We have carried out multicolor fluorescent in situ hybridization (FISH) analysis of *S. latifolia* chromosomes based on the presence and intensity of FISH signals on individual chromosomes. We have generated new markers by constructing and screening a sample bacterial artificial chromosome (BAC) library for appropriate FISH probes. Five newly isolated BAC clones yielded discrete signals on the chromosomes: two were specific for one autosome pair and three hybridized preferentially to the sex chromosomes. We present the FISH hybridization patterns of these five BAC inserts together with previously described repetitive sequences (X-43.1, 25S rDNA and 5S rDNA) and use them to analyze the *S. latifolia* karyotype. The autosomes of *S. latifolia* are difficult to distinguish based on their relative arm lengths. Using one BAC insert and the three repetitive sequences, we have constructed a standard FISH karyotype that can be used to distinguish all autosome pairs. We also analyze the hybridization patterns of these sequences on the sex chromosomes and discuss the utility of the karyotype mapping strategy presented to study sex chromosome evolution and Y chromosome degeneration.

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Introduction

There are only a very few plant species possessing heteromorphic sex chromosomes. Of these, *Silene latifolia* Poiret (syn. *Melandrium album* Garcke or white campion) is perhaps the most popular plant model used to study sex determination and sex chromosome evolution in plants. The sex chromosomes in this species were identified independently by Blackburn (1923) and Winge (1923). The standard diploid number of chromosomes in this species is $2n$=22A + XX in females and $2n$= 22A + XY in males. It is generally accepted that sex chromosomes evolved from a pair of autosomes. From this point of view the *Silene* genus provides a unique opportunity to study the origin of sex chromosomes and genome evolution. Despite different reproductive strategies (many of the species are gynodioecious or hermaphroditic (Desfeux et al. 1996) and large differences in genome size (Siroky et al. 2001), all *Silene* species have the same chromosome number $2n$=24. This species has also become an object of molecular studies (recently reviewed by Negrutiu et al. 2001).

One of the tools that can be used to analyze sex chromosomes is isolation of specific DNA sequences and their physical (FISH, fluorescent in situ hybridization) mapping in related species. FISH mapping of unique sequences in plants usually requires relatively long DNA probes (>10 kb). Due to this fact libraries with long genomic sequences [phage vectors, bacterial artificial chromosomes (BAC)] are used as a source of such probes. FISH karyotypes of some plant species have been constructed recently using BAC clones: for example, *Lotus japonicus* (Pedrosa et al. 2002), *Pinus* sp. (Hizume et al. 2002) or *Picea abies* (Vischi et al. 2003). However,

their use might lead to ambiguous results, especially for plants with large genomes in which many repetitive sequences are widespread and probes that share repeated sequences can be attracted unspecifically to multiple sites (for review see Heslop-Harrison 2000).

To date, only two sequences have been isolated that give discrete FISH signals on sex chromosomes of *S. latifolia*. The repetitive sequence X-43.1 (Buzek et al. 1997) hybridizes to the subtelomeric region of both arms of the X chromosome, but on the Y chromosome the signal is localized asymmetrically, only on the q arm. Moore et al. (2003) identified a gene, *DD44*, linked to the sex chromosomes. A genomic clone containing this gene was physically mapped to the distal part of the q arm of the X chromosome and to the p arm of the Y chromosome. Both these sequences have recently been used to orient the sex chromosomes with respect to the pseudoautosomal region (Lengerova et al. 2003).

We present here a multicolor FISH karyotype of *S. latifolia* constructed according to the presence and intensity of the FISH signals on individual chromosomes. We used combinations of several repetitive sequences (X-43.1, 25S rDNA, 5S rDNA) previously mapped on the chromosomes of *S. latifolia* (Buzek et al. 1997; Siroky et al. 2001) and five new markers isolated after screening a sample BAC library to construct a FISH karyotype of this model species. Each of the chromosomes possesses a unique combination of signals, which enables their precise identification. Special attention was paid to BAC clones that hybridized preferentially to the sex chromosomes.

Materials and methods

Plant material

Root tips of germinated seeds from the collection of the Institute of Biophysics were used as a source of *Silene latifolia* metaphase chromosomes. The seeds were sterilized and grown in water at 4°C for 3 days to synchronize germination followed by another 3 days of growth at room temperature. Aphidicolin, a DNA polymerase inhibitor (Sigma, Indianapolis, Ind.), was then added (30 μM) to synchronize the cell cycle. After 16 h in the presence of this inhibitor, the seedlings were extensively washed with water (5 h) and then exposed to 15 μM oryzalin (Sigma) (for 5 h) to accumulate the dividing cells in metaphase. During the last hour of oryzalin treatment, root tips of the seedlings were cut off, and metaphase protoplasts were isolated as described in Hladilova et al. (1998). Slides were prepared by dropping fixed protoplast suspensions onto slides.

Construction and screening of a sample BAC library

Young leaves of *S. latifolia* male plants were used as a source of interphase nuclei to isolate high-molecular-weight DNA as described in Zhang et al. (1995). Interphase nuclei were imbedded in agarose blocks and digested with *Hind*III restriction enzyme. Cleaved DNA was separated on a 1% agarose gel by PFGE, and fragments in the size ranges of 100-500 kb were isolated for ligation with the *Hind*III-digested and dephosphorylated pBeloBAC11 vector (Promega, Madison, Wis.). Competent *Escherichia coli* strain DH10B (GibcoBRL, Gaithersburg, Md.) cells were transformed by electroporation, and white colonies were individually picked out on microtiter plates. A sample consisting of 960 clones was analyzed. High-density grids were prepared by transferring the BAC clones from 384-well plates onto nylon membranes using a GeneTAC G3 robot, and bacteria were grown for about 5 h at 37°C. The membranes were then placed on Whatman filter papers soaked with the following solutions respectively—0.5 *M* NaOH, 1.5 *M* NaCl; 0.5 *M* Tris-Cl (pH 7.5), 1.5 *M* NaCl; 2× SSC (2× SSC is 0.3 *M* NaCl, 0.03 *M* sodium citrate)—remaining 5 min on each different treatment. Remnants of the bacterial colonies were removed by rolling a glass pipette over the membrane placed between two filter papers. The membranes were air-dried for 30 min and then treated with 1 mg/ml Pronase E solution in 1× SSC for 15 min (upside down with gentle shaking). They were then transferred into chloroform and treated three times for 1 min, followed by last washes—twice for 1 min and once for 10 min in 0.3 *M* NaCl. Air-dried membranes were baked for 2 h at 80°C and later used for hybridization with the Alkphos Direct hybridization kit (Amersham Pharmacia Biotech, Piscataway, N.J.). *S. latifolia* female genomic DNA and pBluescript DNA (Stratagene, La Jolla, Calif.) were used as probes for screening. A 100-ng aliquot of DNA was labeled according to the manufacturers' protocol, and hybridization was performed at 55°C for 16 h. The washing steps consisted of two washes of 10 min each in primary wash buffer at 55°C and two washes of 5 min each in secondary wash buffer at room temperature. Detection was performed using CDP-Star detection reagent (Amersham Pharmacia Biotech). BAC DNA of selected clones was isolated using the QIAGEN Plasmid Midi kit (QIAGEN, Valencia, Calif.), and the approximate length of the inserts was established by restriction analysis. Aliquots of 3 μg BAC DNA were digested with four different restriction enzymes (*Hind*III, *Ear*I, *Hha*I and *Ssp*I) and separated on a 0.8% agarose gel. The length of the fragments was analyzed using DNAFRAG computer software (freeware).

Fluorescent in situ hybridization

As a probe for FISH we used X-43.1 (208-bp repeat unit isolated by Buzek et al. 1997) cloned in pGEM-T Easy (Promega) and 25S rDNA (2.5-kb long *Eco*RI fragment of 25S rDNA isolated by Kiss et al. (1989) from tomato) cloned in pBluescript SK+ (Stratagene). X-43.1 and 25S rDNA were amplified by PCR using the SP6 and T7 or T3 and T7 primers, respectively. A part of the 5S rDNA gene cloned in pBluescript SK- (Stratagene) was amplified with specific primers (Fulnecek et al. 1998). All PCR products were purified using the QIAGEN Nucleotide Removal kit (QIAGEN). High-purity BAC DNA was isolated using the QIAGEN Plasmid Midi kit with a modification for low-copy plasmids. For green labeling we used SpectrumGreen direct-labeled dUTP and the Nick Translation kit (both Vysis) or for red labeling, Fluorolink Cy3-dUTP (Amersham Pharmacia Biotech) in combination with the Nick Translation Mix (Roche).

Preparations were heated at 60°C for 30 min, treated with 100 μg/ml RNase A (QIAGEN) in 2× SSC for 1 h at 37°C, washed three times for 5 min each time in 2× SSC, treated with 20 mg/ml pepsin in 0.01 *N* HCl for 12 min, washed in 2× SSC as before, post-fixed in 3.7% formaldehyde (Merck) for 10 min, washed once again, dehydrated in an increasing ethanol series and air-dried. Chromosome and probe denaturation, hybridization and post-hybridization washes were performed as described by Heslop-Harrison et al. (1991) with minor modifications. A stringent wash was done in 0.1× SSC at 42°C twice for 5 min each time. The hybridization mixture contained 50% (v/v) formamide (Sigma), 10% (w/v) dextran sulfate (Sigma), 2× SSC and 5–100 ng of probe per slide. Typically 20–30 μl of hybridization mixture was applied on each slide. The slide was then heated for 5 min at 75°C on a thermal cycler for denaturation, followed by a controlled renaturation protocol: 65°C for 2 min, 55°C for 2 min, 45°C for 2 min, 37°C for 16 h. After the first round of hybridization, the slides were treated as described in Heslop-Harrison et al. (1992) to get rid of

the bound probes and then later reprobed. We generally hybridized up to four probes on the same slide.

Slides were analyzed on an Olympus Provis microscope, and image analysis was performed using ISIS software (Metasystems). Five well-spread metaphases were analyzed for construction of the idiogram. For each autosome, the average arm ratio (r=q/p) and relative length (l=[(p+q)×100]/11A) were established. Ten representative figures with clear FISH signals were taken and used for analysis of the presence and intensity of the signals on individual chromosomes.

Results

We used multicolor FISH to identify and characterize individual chromosomes of *S. latifolia*. Since not many cytogenetic markers are available, we constructed a sample collection of large male genomic clones in a BAC vector and tested their suitability for FISH studies in order to develop new markers. As a result, we isolated three new clones hybridizing preferentially on the sex chromosomes and two clones hybridizing to unique autosomal loci. We used a robotics system to array 960 bacterial colonies with cloned inserts on nylon membrane and screened these by DNA-DNA hybridization. The main goal was to select clones yielding discrete signals on chromosomes, especially on the sex chromosomes. To select clones with a lower amount of repetitive sequences, we hybridized the membranes with female genomic DNA; clones that hybridized weakly were considered to have this character. Female genomic DNA was applied so as not to exclude clones that could contain Y chromosome-specific repetitive sequences. The same membrane was later hybridized with pBluescript (which displays partial homology to cloning vector pBeloBAC11) to correct for the influence of the different sizes of the bacterial colonies. Following this screening we obtained 75 clones and tested their suitability for FISH mapping. Of these BAC clones, 25 did not yield any clear signal, 25 clones gave a dispersed signal on all chromosomes, 15 hybridized to subtelomeric regions of all chromosomes, five clones hybridized to 25S rDNA clusters, two gave discrete signals on autosomes and, finally, three clones hybridized on autosomes together with a strong signal on the sex chromosomes. These latter five clones were subsequently used for karyotyping. We digested BAC DNA with four different restriction enzymes (*Hha*I, *Hind*III, *Ear*I, *Ssp*I) and estimated their approximate length using the computer program DNAFRAG (freeware). The digested BAC DNA fragments were blotted onto a nylon membrane and hybridized to total male genomic DNA. Some clones yielded strong hybridization signals, indicating the presence of abundant repeats (for example, 6B3 and 9B7), while a single autosome pair-specific clone, 3E3, hybridized significantly weaker (Fig. 1). The

Table 1 List of *Silene latifolia* DNA sequences used as FISH probes

Probes	Type	Length
25S rDNA[a]	Repetitive sequence	2.5 kbp
5S rDNA[b]	Repetitive sequence	500 bp
X-43.1[c]	Repetitive sequence	208 bp
9B7	BAC clone	44 kbp
3E3	BAC clone	30 kbp
2G7	BAC clone	40 kbp
7H5	BAC clone	34 kbp
6B3	BAC clone	28 kb

[a] Kiss et al. 1989
[b] Fulnecek et al. 1998
[c] Buzek et al. 1997

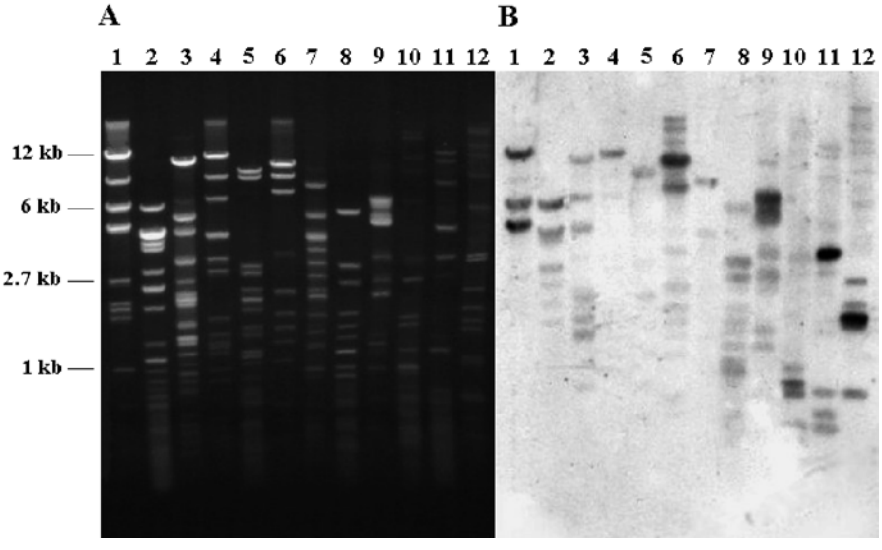

Fig. 1 A,B Restriction analysis and Southern hybridization of selected BAC clones. BAC clones were digested with restriction enzymes, separated on a 1% agarose gel and stained with ethidium bromide (**A**). DNA fragments were then transferred onto nylon membrane and hybridized with total male genomic DNA (**B**). *Lanes*: *1* BAC 2G7/ *Hind*III, *2* BAC 2G7/ *Hha*I, *3* BAC 2G7/*Ear*I, *4* BAC 3E3/*Hind*III, *5* BAC 3E3/*Hha*I, *6* BAC 6B7/*Hind*III, *7* BAC 3E3/*Ear*I, *8* BAC 6B3/*Hha*I, *9* BAC 6B3/*Ear*I, *10* BAC 9B7/*Alu*I, *11* BAC 9B7/*Hha*I, *12* BAC 9B7/*Ssp*I

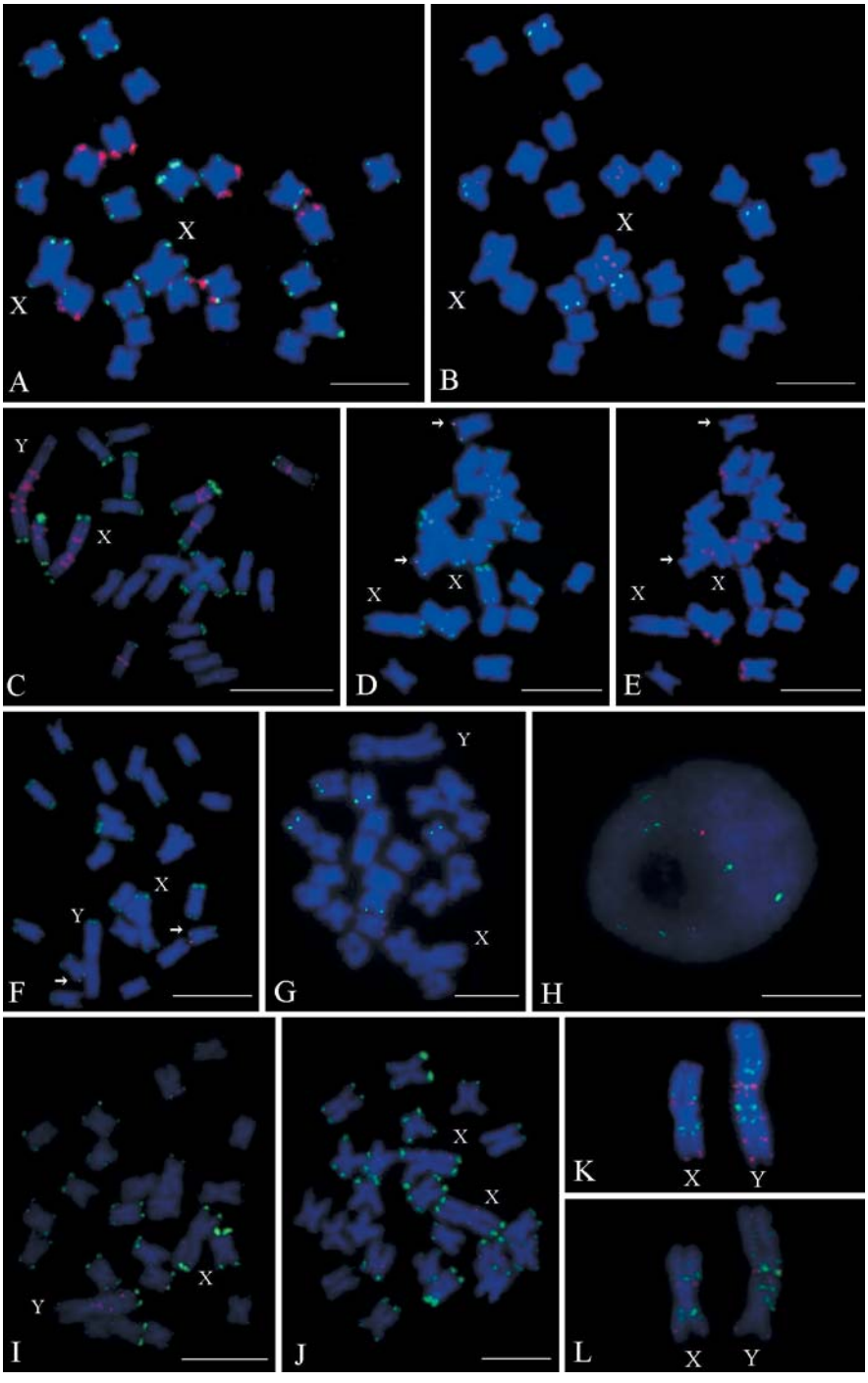

Fig. 2A–L Multicolor FISH on *Silene latifolia* metaphase chromosomes and interphase nuclei. *Bars*: 10 μm. **A**, **B** Slides were sequentially hybridized with four different probes labeled directly by nick translation using Cy3- or SpectrumGreen-conjugated nucleotides. Chromosomes were counterstained with DAPI. **A** Slides with metaphase chromosomes hybridized with the X-43.1 repetitive sequence (*green*) and 25S rDNA (*red*). **B** After analysis, the old probe was removed and the slides reprobed with 5S rDNA (*green*) and BAC clone 9B7 (*red*). **C** Hybridization of BAC clone 9B7 (*red*) and repetitive sequence X-43.1. Note strong hybridization signals on the sex chromosomes. **D** BAC clone 2G7 (*red*) is specific for one autosome pair with the X-43.1 signal (*green*) on both arms. It is the same chromosome that carries 25S rDNA (*red*) on its q arm (**E**). **F** BAC clone 3E3 (*red*) also gives a discrete signal on only one autosome pair (X-43.1 is *green*). **G** 3E3 (*red*) was also hybridized with 5S rDNA (*green*) on both chromosomes and interphase nuclei (**H**) and is a good candidate for studies of nuclear organization. The other two BAC clones, 7H5 and 6B3 (both *red*), were also mapped with respect to X-43.1 (*green*) (**I** and **J**, respectively). Both these clones hybridize preferentially to the sex chromosomes. **K** Co-localization of BAC 9B7 (*green*) and BAC 7H5 (*red*) on the X and Y chromosome. **L** Co-localization of BAC 9B7 (*green*) and BAC 6B3 (*red*)

sizes of the inserts of the selected BAC clones were unusually short: the average insert size of the library was 125 kb (data not shown). Results of the length analysis and a list of the probes used for FISH mapping are summarized in Table 1.

FISH experiments were performed without any suppressor DNA (genomic DNA or its Cot1 fraction). One of the most effective techniques to map more DNA sequences is the sequential hybridization of several probes on one slide. We were able to establish a basic FISH karyotype using the sequences previously characterized (X-43.1, 25S rDNA, and 5S rDNA) and a new marker, BAC clone 9B7. First, we hybridized slides with X-43.1 (green) and 25S rDNA (red) (Fig. 2A). Figure 2 clearly shows that X-43.1 hybridizes to subtelomeric regions of all the autosomes, but that the patterns on the chromosomes differ in the intensity and presence of the signal on the p and q arms. Both the p and q arms of the X chromosome carry the X-43.1 signal, while it is asymmetrically localized only on the q arm of the Y chromosome (Fig. 2C). 25S rDNA gives signals on five autosome pairs, four times on the p arm and once on

Table 2 Numerical karyotype of *S. latifolia* chromosomes. Autosomes are arranged according to their relative length (*SD* standard deviation)

Chromosome number	r[a] ± SD	l[b] ± SD
1	1.27±0.16	10.6±0.31
2	1.57±0.10	9.8±0.28
3	1.67±0.22	9.8±0.37
4	1.45±0.13	9.4±0.49
5	1.42±0.23	9.4±0.55
6	1.63±0.26	9.2±0.22
7	1.41±0.25	9.0±0.28
8	1.16±0.21	8.8±0.41
9	1.49±0.25	8.8±0.43
10	1.45±0.33	8.0±0.27
11	1.41±0.21	7.8±0.37
X	1.28±0.12	15.0±0.22
Y	1.11±0.03	20.3±0.18

[a] Arm ratio r=q/p
[b] Relative length l=[(p+q)×100]/11A. In the case of the sex chromosomes their l values represent the relative length calculated from the male diploid genome

the q arm. The same slide was later stripped of old probe and reprobed with BAC clone 9B7 (red) and 5S rDNA (green) (Fig. 2B). BAC 9B7 obviously contains a repetitive motif that is present in the centromeres of most of the autosomes (seven pairs) and has accumulated on the sex chromosomes X and Y (Fig. 2C). 5S rDNA hybridizes to three pairs of autosomes; twice it is localized on the same autosome as 25S rDNA. Five representative metaphases were analyzed by measuring the lengths of the p and q arms of each chromosome (Table 2). On the basis of the FISH patterns and the results of measurement of the arm ratios, centromeric indexes and relative lengths of the chromosomes we constructed an idiogram of *S. latifolia* chromosomes (Fig. 3). Since it is quite hard to distinguish individual autosome pairs according to arm ratio or centromeric index due to variability in the centromere position (Grabowska-Joachimiak and Joachimiak 2002), we decided to arrange the autosomes only according their relative length. Seven pairs of autosomes can now easily be identified with the combination of four probes used.

The other BAC clones were then mapped to this basic karyotype. Two BACs were chromosome-specific: BAC 2G7 hybridized to the same chromosome that carries the 25S rDNA signal on its q arm (Fig. 2D, E) and BAC 3E3 hybridized to the q arm of one autosome pair (Fig. 2F). Such clones could be also used to study interphase nuclear topology. An example of bicolor hybridization of BAC 3E3 and 5S rDNA on metaphase chromosomes and interphase nuclei is presented in Fig. 2G and H, respectively. The last two BACs (7H5 and 6B3) hybridized preferentially to the Y chromosome, but signals were also present on the X chromosome and autosomes, although their intensity on the autosomes was usually significantly weaker (Fig. 2I, J). We used bicolor FISH for fine mapping of the sex chromosomes and mapped clones 7H5 and 6B3 with respect to clone 9B7

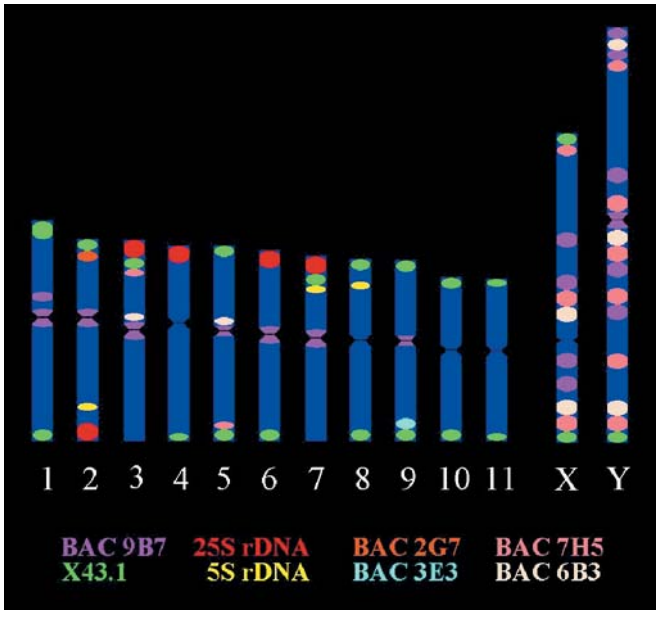

Fig. 3 Idiogram of *S. latifolia* chromosomes with location of FISH signals. Autosome pairs are ordered according their relative length. All autosome pairs possess specific FISH patterns and can, therefore, be easily distinguished

(Fig. 2K, L). All data obtained are summarized in the schematic FISH karyotype presented in Fig. 3.

Discussion

A detailed study of the *S. latifolia* karyotype was carried out by Ciupercescu et al. (1990), who classified the chromosomes into three groups: group A, comprising six pairs of metacentric chromosomes; group B, comprising five pairs of submetancentric chromosomes; the X and Y sex chromosomes. The idiogram proposed in their paper was based on a classical cytogenetic classification according to arm ratio, centromeric index and relative length of autosomes. A different approach was used by Grabowska-Joachimiak and Joachimiak (2002), who constructed karyotypes of *S. latifolia* and *S. dioica* based on Giemsa staining and the C-banding technique. In their study the classification into three chromosome groups as proposed by Ciupercescu et al. (1990) was not confirmed, and a new scheme was presented. Nine metacentric and two submetacentric autosome pairs were identified, and the authors constructed an idiogram with fixed and polymorphic heterochromatin bands. Nevertheless, it is still hard to distinguish individual pairs of *S. latifolia* autosomes based only on cytological measurements.

Here we present a FISH karyotype analysis based on the localization and intensity of the FISH signals. In this karyotype, chromosomes are ordered only according to their relative length. We used previously characterized cytogenetic markers like rDNA clusters or subtelomeric repetitive sequences in combination with newly isolated markers from the sample BAC library. BAC libraries are

now successfully used in *Arabidopsis*, where they serve as excellent cytogenetic markers for individual chromosomes or chromosomal arms (Lysak et al. 2001; Siroky et al. 2003). Despite the advantages of BAC clones, their utility for mapping in plants possessing large (like *S. latifolia*, 2C=5.4 pg) genomes might be questionable. These genomes contain a huge portion of repetitive sequences (tandem repeats, transposable elements) that are spread throughout the genome (Heslop-Harrison 2000). The use of BAC library clones in plants with large genomes (like barley) was tested by Lapitan et al. (1997). In their studies an excess of suppressor DNA or Cot1 fraction was applied to avoid non-specific hybridization. Most of the BAC clones tested in our study hybridized as dispersed signals on all chromosomes and it was not possible to obtain a discrete signal even when a large excess of suppressor DNA was added. However, when using the five selected clones, we obtained discrete signals without any suppression. The BAC clones yielding discrete signals were only about 40 kb long and therefore represented the smallest BAC clones in our sample library. As shown by Southern hybridization (Fig. 1), these clones contained mainly moderately low or low-copy sequences, except for BAC 9B7; the latter contained correspondingly more abundant genomic repeats, which probably accounted for its FISH signal. All of the BAC clones selected will be used for chromosome mapping on related *Silene* species.

The preferential hybridization of three BAC clones to the sex chromosomes of *S. latifolia*, with the strongest hybridization to the Y chromosome, reflects the accumulation of the repetitive sequences on the Y chromosome. The Y chromosome is the largest chromosome in the *S. latifolia* genome, and it has been suggested that represents an early stage of sex chromosome evolution (Negrutiu et al. 2001). According to mathematical models, non-recombining parts of the Y chromosome should accumulate repetitive DNA (Charlesworth 1991), leading to its bigger size. In plants, Y chromosome-specific repetitive sequences have been found in the sorrel *Rumex acetosa*, which possesses the old highly degenerated Y chromosomes (Shibata et al. 1999), and in the liverwort *Marchantia polymorpha* (Okada et al. 2001). In *S. latifolia*, a similar density of long-terminal-repeat retrotransposons on the sex chromosomes and autosomes has been demonstrated (Matsunaga et al. 2002). Our results demonstrate for the first time the accumulation of repetitive sequences on the Y chromosome in *S. latifolia*. These sequences are now isolated and characterized in detail.

BAC 9B7 hybridized to the centromeres of most autosomes but not to the centromeric regions of the X chromosome. Plant centromeres are predominantly composed of repetitive DNA sequences, including tandem repeats as well as retroelements (Cheng et al. 2002; Nagaki et al. 2003). Some of the repeat families localized in the centromeres are also present in other chromosome regions (Nagaki et al. 2003), which can also be the case of the repeats present in clone 9B7. The absence of repeats originated from BAC 9B7 in centromeres of the X chromosomes of *S. latifolia*, in contrast to their amplification in other parts of the sex chromosomes, could be a result of an amplification of other sequences in centromeres during their evolutionary competition for chromosomal niches.

The FISH karyotyping presented here provides a valuable tool to study the genome and chromosome structure of the *Silene* genus. The specific hybridization patterns of three BAC clones (9B7, 7H5, and 6B3) on the X and Y chromosomes will enable the structural divergence of the X and Y chromosomes to be compared. This approach can also be used to answer various questions concerning sex chromosome structure and evolution in this model dioecious species.

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