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Heterogeneity of rDNA distribution and genome size in *Silene* spp.

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Abstract

Genus *Silene* L. (*Caryophyllaceae*) contains about 700 species divided into 44 sections. According to recent taxonomic classification this genus also includes taxa previously classified in genera *Lychnis* and *Melandrium*. In this work, four *Silene* species belonging to different sections were studied: *S. latifolia* (syn. *Melandrium album*, Section Elisanthe), *S. vulgaris* (Inflatae), *S. pendula* (Erectorefractae), and *S. chalcedonica* (syn. *Lychnis chalcedonica*, Lychnidiformes). Flow cytometric analysis revealed a genome size of 2.25 and 2.35 pg/2C for *S. vulgaris* and *S. pendula* and of 5.73 and 6.59 pg/2C for *S. latifolia* and *S. chalcedonica*. All four species have the same chromosome number including the pair of sex chromosomes of the dioecious *S. latifolia* ($2n = 2x = 24$). Double target fluorescence *in-situ* hybridization revealed the chromosomal locations of 25S rDNA and 5S rDNA. A marked variation in number and localization of rDNA loci but no correlation between the numbers of rDNA clusters and genome size was found. FISH and genome size data indicate that nuclear genomes of *Silene* species are highly diversified as a result of numerous DNA amplifications and translocations.

Introduction

Species belonging to the *Silene* genus are known to have diverse reproductive systems – hermaphroditic, gynodioecious, and dioecious (Desfeux *et al.* 1996). For taxonomic classification, see Oxelmann & Lindén (1995) and citations herein. Previously separated genera *Lychnis* and *Melandrium* are now grouped in the single genus *Silene* (Chowdhuri 1957, Greuter *et al.* 1984). *Silene* has recently become a model to study the evolution of plant sex chromosomes (for a review, see Charlesworth & Guttman 1999).

The genomes of eukaryotes contain many families of repeated DNA sequences, including ribosomal DNA**.** The rRNA genes represent multigene families which are believed to have arisen by gene duplication. 45S rDNA, consisting of 18S, 5.8S and 25S rDNA and separated by two internal transcribed spacers (ITS), and 5S rDNA are highly conserved, (Erdmann & Wolters 1986) present in high copy numbers and organized in long tandem arrays mostly at telomeric and subtelomeric regions (Leitch 2000). Positions of rDNA sequences provide useful markers for chromosome identification (Castilho & Heslop- Harrison 1995).

The sequence diversity of ITS was also used for assessing intrageneric and intergeneric relationships in the tribe *Silenae* (Oxelman & Lindén 1995, Desfeux & Lejeune 1996), showing that the genus *Silene* is monophyletic.

We have studied the number and location of 25S rDNA and 5S rDNA sequences by double target FISH and the genome size of four *Silene* species. These studies provide new information on the karyotypes and genome organization of the genus *Silene*, and on the evolution of both classes of rDNA sites.

Materials and methods

Chromosomal preparation

Seeds of *Silene latifolia* Poiret, *S. pendula* L., *S. vulgaris* (Moench) Garcke, and *S. chalcedonica* L. from the collection of the Institute of Biophysics, Brno were used. Hairy root cultures were derived from leaf fragments of *S. latifolia, S. vulgaris,* and *S. pendula* by infection with *Agrobacterium rhizogenes,* strain A4RS. Long-term cultures were kept on B5 medium without hormones. Germinating seeds (36 h at 26°C in dim light) of *S. chalcedonica* were used to obtain root tips. For chromosome preparation, see Hladilova *et al.* (1998). In order to synchronize both hairy root cultures and germinating seeds, the DNA polymerase inhibitor aphidicolin (30 μmol/L, Sigma) was added for 12 h. After extensive washing with the medium (hairy root cultures) or water (seeds) root tips were released from the aphidicolin block. Metaphases were accumulated with 15 μmol/L oryzalin (4 h, Elanco). Using cellulolytic enzymes, protoplasts were obtained, fixed in 3:1 (v/v) ethanol : acetic acid, dropped onto microscope slides and stored desiccated at −20°C. After hydrolysis in 5 N HCl for 10 min, washing and air-drying, chromosomes were stained with 5% Giemsa (Merck). Active nucleoli in interphase nuclei from root tip meristems were identified using a colloid developer mixed with 50% silver nitrate according to Howell & Black (1980).

Fluorescent in-situ *hybridization*

As a FISH probe for 45S rDNA an internal 2.5-kbp *Eco*RI fragment of 25S rRNA gene cloned in pBluescript II SK+ (Stratagene) was used (Kiss *et al.* 1989). Purified insert was labelled with digoxigenin-11-dUTP (Roche) by nick translation. A biotinylated 5S rDNA probe was prepared from a 116-bp-long insert (*Eco*RI–*Xba*I) of a part of the 5S rDNA gene cloned in pBluescript II SK− by PCR using specific primers (Fulneček *et al.* 1998) and biotin-14-dATP (Gibco). The FISH hybridization mixture (20 μl per slide), consisting of 200 ng of each labelled probe, 6 μg autoclaved salmon sperm DNA (Serva), 4 μl of a 50% solution of dextran sulphate (Sigma), 10 μl formamide (Sigma), and 2 μl of 20 × SSC, was denatured at 76°C for 15 min and immediately cooled on ice. Slides were treated with RNase (50 μg/ml, 2 h at 37°C), washed in 2 × SSC, dehydrated in an ethanol series and air-dried. To remove remnants of cytoplasm, slides were subjected to pepsin treatment (10 μg/ml in 10 mN HCl, 10 min at 20°C), washed and dehydrated as before. After post-fixation in ethanol : acetic acid for 30 min slides were denatured in 7 : 3 (v/v) formamide : 2 × SSC for 2 min at 72°C, immediately dehydrated through 50%, 70% and 100% ethanol (−20°C) and air-dried. Then the probe was added and hybridized for 18 h at 37°C. Stringent washing consisted of 10 min in 1:1 (v/v) formamide : 2 × SSC, at 42°C. Detection of digoxigenin-labelled targets was done by FITC in a three-step procedure using a fluorescent antibody enhancer set (Roche) simultaneously with Cy3 detection of biotin by Cy3-avidin (Amersham), biotinylated anti-avidin (Vector), and a second incubation in Cy3-avidin. Chromosomes were counterstained with DAPI. FISH signals were observed using an Olympus AX 70 fluorescent microscope equipped with filter sets for DAPI, FITC, and Cy3. Images were captured using a digital camera and ISIS software (Meta Systems). For classification of chromosomes bearing FISH signals, digital images were processed using ImagePro (Media Cybernetics) software. Counterstain colour was extracted

from the image files and negative images were printed. The position of centromere and the length of chromosomes was determined and images were manually aligned to FISH signals.

Flow cytometry

For flow cytometric analysis of nuclear genome size, plants were cultivated in a greenhouse. Five plants of each species were analysed and each sample was measured four times. Small pieces of young leaf tissue of both analysed and standard species were chopped together in a Petri dish containing 1 ml of LB01 buffer supplemented by propidium iodide and RNase (50 μg/ml each) as described by Vagera *et al.* (1994). The suspension of nuclei was filtered through a 50-μm nylon mesh and the relative fluorescence intensity was analysed using a Partec PAS flow cytometer (Partec GmbH, Münster), equipped with a high-pressure mercury arc lamp. Two reference standards were used. Tomato (*Lycopersicon esculentum* cv. Stupické; 1.96 pg/2C, Doležel *et al.* 1992) served as an internal standard for DNA content estimation in *S. latifolia* and *S. chalcedonica* and maize (*Zea mays* cv. C-777; 5.43 pg/2C, Lysák & Doležel 1998) for *S. vulgaris* and *S. pendula*. The genome size of analysed plants was estimated using the ratio between the fluorescence peaks of a sample and a reference standard as follows:

$$\text{2C DNA content} = \frac{\text{sample } G_1 \text{ peak mean}}{\text{standard } G_1 \text{ peak mean}} \times \text{2C DNA content of reference standard [pg/2C]}$$

Results and discussion

Except for some tetraploids, the majority of *Silene* species contain $2n = 2x = 24$ chromosomes; only two diploid species possess $2n = 20$ chromosomes (Degraeve 1980). Using the arm ratio (r = q/p), we classified the chromosomes in all the species

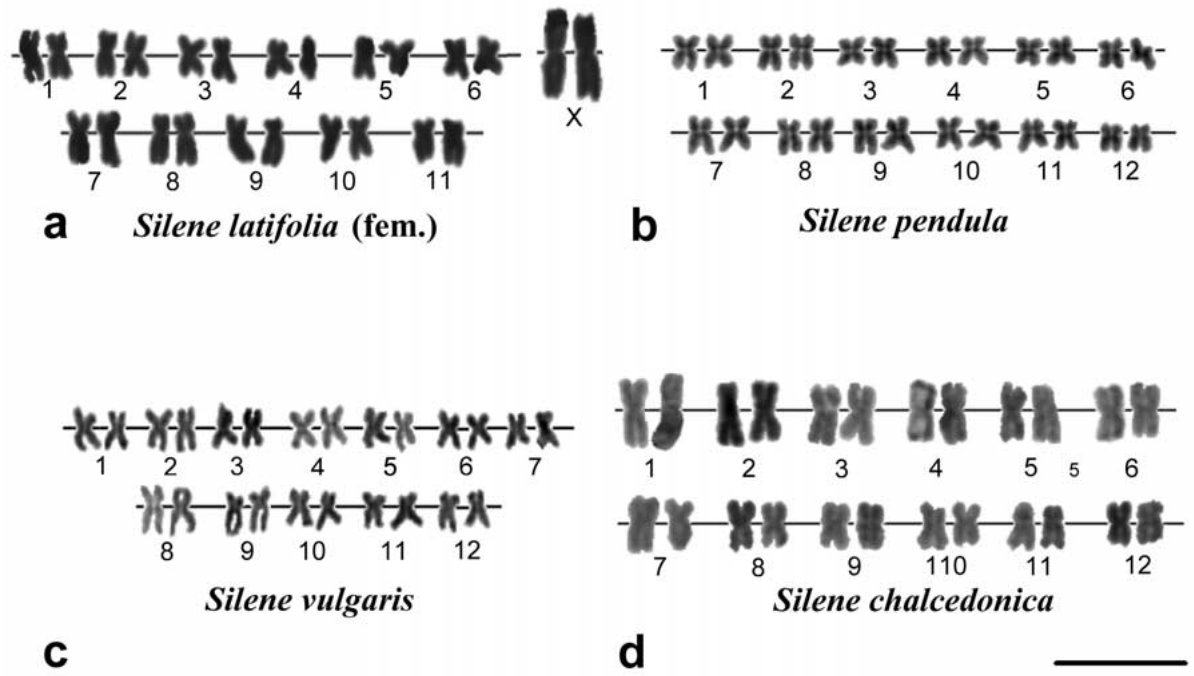

Figure 1. Karyotypes of *Silene* species. Giemsa-stained chromosomes are arranged in metacentric (upper rows) and submetacentric (lower rows) groups. (**a**) *S. latifolia* female karyotype; X denotes the pair of X chromosomes. (**b**) *S. pendula*, (**c**) *S. vulgaris* and (**d**) *S. chalcedonica*. Bar = 10 μm.

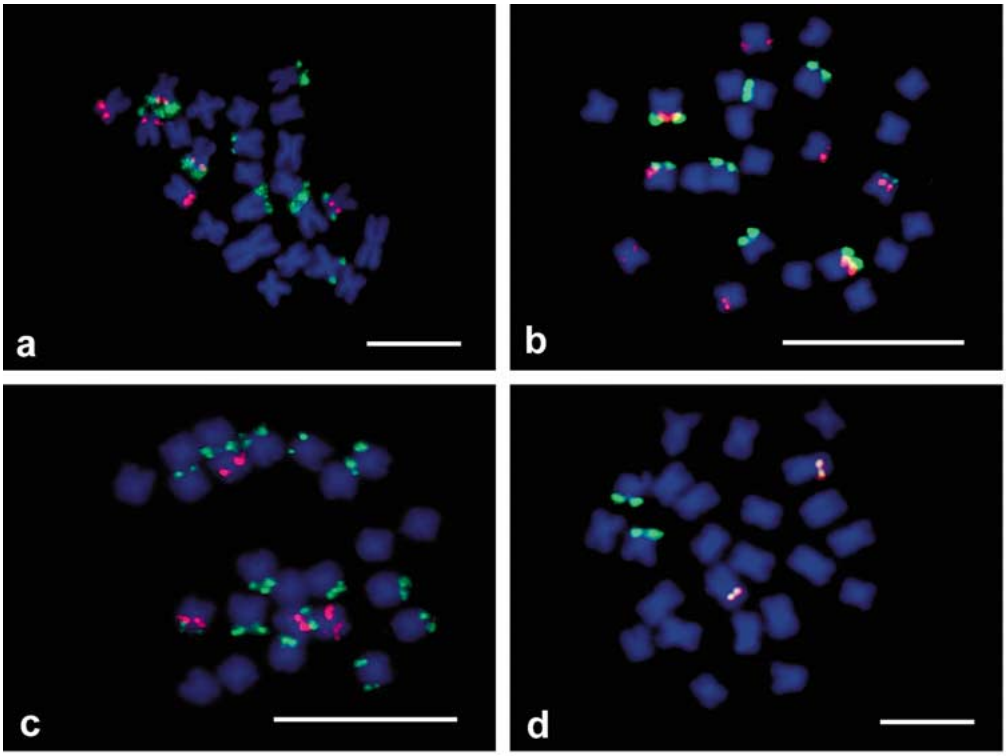

Figure 2. Bicolour FISH with 25S and 5S rDNA probes on *Silene* chromosomes. Metaphase cells of (**a**) *S. latifolia*, female, (**b**) *S. pendula*, (**c**) *S. vulgaris* and (**d**) *S. chalcedonica* with 25S rDNA loci (green signals) and 5S rDNA loci (red signals). Chromosomes were counterstained with DAPI. Bars = 10 μm.

tested as metacentric and submetacentric. Female cells of *S. latifolia* (Figure 1a) contain six pairs of metacentric and five pairs of submetacentric in addition to a pair of larger metacentric X chromosomes. The metacentric Y chromosome of male cells was even larger than the X (not shown). Similarly, the karyotype of *S. vulgaris* (Figure 1c) consisted of seven pairs of metacentric and five pairs of submetacentric chromosomes. Despite marked differences in the length of chromosomes between the two other species, *S. pendula* (Figure 1b) and *S. chalcedonica* (Figure 1d) had six pairs of both metacentrics and submetacentrics.

After FISH, each species revealed four groups of chromosomes according to their labelling patterns: chromosomes with either 25S or 5S rDNA loci, chromosomes with both types of signals, and chromosomes without signals. In *S. latifolia* and *S. pendula* (Figure 2a & b), there were two pairs of chromosomes having both 25S and 5S rDNA signals. The results with 25S rDNA probe for *S. latifolia* are in concordance with those of Ciupercescu *et al.* (1990). Due to the enhanced sensitivity of the FISH technique, an additional 25S rDNA site on the *p* arm of chromosome 8 was unveiled. This locus was adjacent to a 5S rDNA signal, as on the *q* arm of chromosome 7 (Figure 3a). An additional 5S rDNA site was found on chromosome 6. Similarly, in *S. pendula*, there are two chromosome pairs with adjacent, subtelomerically located 25S and 5S rDNA signals. In *S. vulgaris* and *S. chalcedonica* (Figures 2c, d & 3c, d), both the 5S and 25S rDNA signals together were observed on only one pair of chromosomes. The highest number (seven pairs) of 25S rDNA loci was observed in *S. vulgaris* possessing the smallest genome size, while the smallest number (only two pairs) was found in *S. chalcedonica* with the largest genome. The 25S rDNA signals were localized at terminal chromosome positions. The only exception was

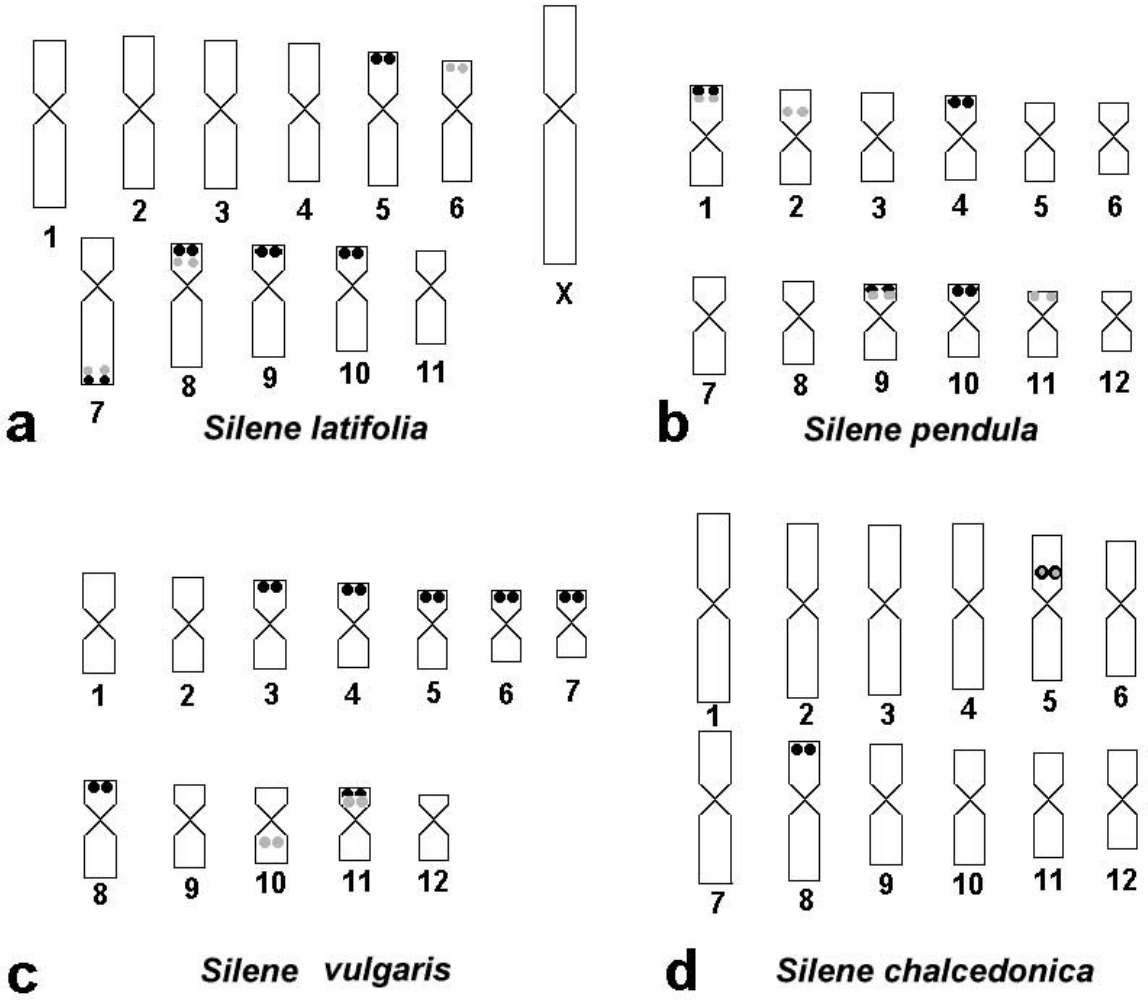

Figure 3. Idiograms of *Silene* chromosomes with hybridization sites of 25S (black circles), and 5S (grey circles) rDNA probes (overlapping signals = concentric circles).

the one pair in *S. chalcedonica*. The highest number of 5S-rDNA-positive sites was detected in a small genome of *S. pendula* (four pairs), while *S. chalcedonica* had only one pair (Figures 2 & 3). In *S. chalcedonica*, the 25S and 5S rDNA probes collocalized on the same position of the shorter arm of chromosome 5. The 5S rDNA loci are occasionally found closely adjacent to 45S rDNA in plant species (Roose *et al.* 1998, Taketa *et al.* 1999). Sone *et al.* (1999) even found a collocalization of these genes in *Marchantia polymorpha* by FISH and molecular mapping. Also in our study, 25S and 5S rDNA signals were detected in close proximity (chromosomes 7 and 8 in *S. latifolia*, 1 and 9 in *S. pendula*, and 11 in *S. vulgaris*; Figures 2 & 3) but the signals did not overlap. The FISH data for 25S and 5S rDNA in *S. chalcedonica* were similar to those described for two endemic species from Portugal, *Silene cintrana* and *S. rothmaleri* (Pontes *et al.* 2000). The majority of *S. latifolia*, *S. pendula,* and *S. chalcedonica* nuclei had only one nucleolus. A larger number of nucleoli was detected in these species in only about 15% of nuclei. A greater heterogeneity in nucleolar number was observed in *S. vulgaris*: mononucleolar nuclei represented only 54% and the rest of nuclei had two to six nucleoli (Figure 4).

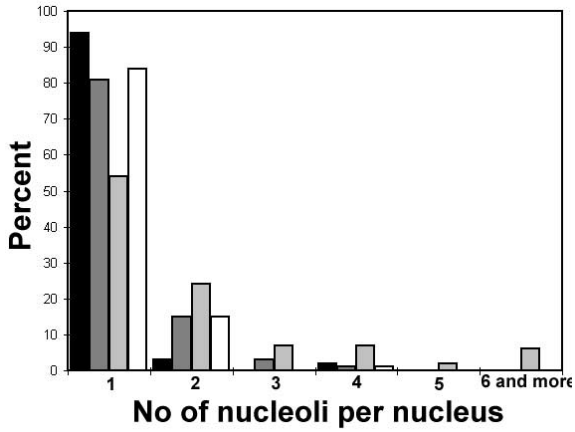

Figure 4. Numbers of nucleoli per nucleus (%) for *S. latifolia*, (black columns); *S. pendula*, (grey columns); *S. vulgaris*, (light grey columns); and *S. chalcedonica* (white columns). For each species, at least 150 nuclei were scored.

The nuclear DNA content was estimated using flow cytometry. Due to large differences in genome size of the analysed species, two different reference standards were used. All analyses resulted in histograms with clearly defined fluorescence peaks corresponding to G_1 nuclei of the sample and the standard. Although all species possess the same chromosome number, they differed greatly in their genome size ranging from 2.25 to 6.59 pg/2C (Table 1). Two groups of *Silene* species could be distinguished according to their DNA content; the first group (*S. pendula* and *S. vulgaris*) has a low DNA content and the second group (*S. latifolia* and *S. chalcedonica*) has a more than two-fold larger one (Table 1). This grouping holds true also for other *Silene* species (Bennett *et al.* 1998). While *S. coeli-rosa* has 2.0 pg/2C, three other species have a more than two-fold larger genome size (*S. dioica* 5.4, *S. latifolia* 5.8, *S. nutans* 6.4 pg/2C). This reflects the phylogenetic distances obtained by Desfeux & Lejeune (1996) after the Neighbour-Joining method of tree construction: (i) *S. latifolia* and *S. dioica* with a very similar DNA content belong to the same section, Elisanthe; (ii) *S. pendula* and *S. vulgaris*, although classified in different sections (Erectorefractae, Inflatae), are closely related having a similar DNA content; (iii) *S. nutans* (section Siphonomorpha) and *S. coeli-rosa* (Eudianthe) differ markedly in 2C values from the above mentioned species and are distant relatives. In our work, no correlation between the numbers of rDNA clusters and the genome size of the *Silene* species tested was found. This indicates that differences in DNA content are not due to global genome amplification.

Table 1. Mean nuclear DNA content of four *Silene* species.

Species	Nuclear DNA content (pb/2C)	
	Mean	SD
S. latifolia (male)*	5.85	0.01
S. latifolia (female)*	5.73	0.01
S. pendula	2.35	0.06
S. vulgaris	2.25	0.05
S. chalcedonica	6.59	0.14

*According to Vagera *et al.* (1994).

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