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CHROMOSOMA

Characterization of Heterochromatin in Different Species of the *Scilla siberica* Group (Liliaceae) by in situ Hybridization of Satellite DNAs and Fluorochrome Banding

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Abstract. Satellite DNAs have been isolated from the monocotyledonous plants *Scilla siberica, S. amoena, S. ingridae* (all are highly GC-rich), and *S. mischtschenkoana* by using the $Ag^+ - Cs_2SO_4$ density centrifugation technique. Hybridization in situ has been performed with ^{3}H-cRNA to these satellite DNAs in all four species. In each species, the endogenous satellite DNA is located mainly in intercalary and major heterochromatin bands associated with terminal regions and nucleolar organizer regions (NORs) but not in centromeric regions. Patterns observed after cross-species hybridization show a high degree of satellite DNA homology between *S. siberica, S. amoena,* and *S. ingridae*. By contrast, satellite DNA of *S. mischtschenkoana* consists largely of different, non homologous DNA sequences, with two exceptions: (*i*) the NORs of all four species contain similar satellite sequences, and (*ii*) a strong homology exists between the satellite DNA of *S. mischtschenkoana* and centromeric DNA of *S. siberica* but not with those of *S. amoena* and *S. ingridae*. – Heterochromatin has also been characterized by the AT-specific fluorochromes quinacrine (Q) and DAPI and the GC-specific agent chromomycin A_3 (CMA_3), in combination with two counterstaining techniques. While CMA_3-fluorescence is largely in agreement with data on base composition and location of the specific satellite DNAs, the results with Q and DAPI are conflicting. Prolonged fixation has been found to change the fluorescence character in certain instances, indicating that other factors than the base sequence of the DNA also play a role in fluorochrome staining of chromosomes. The results are discussed in relation to the taxonomy and phylogeny of the four species.

Introduction

Constitutive heterochromatin of plant and animal cells frequently contains highly repetitive DNA sequences which can be isolated as a separate fraction, either as satellite DNA by ultracentrifugation techniques or by gel electrophoresis

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after digestion with restriction enzymes. Little, however, is known of the function of these genome components and the principles governing their evolution (John and Miklos, 1979). It is known that heterochromatic chromosome segments containing highly repetitive DNA can, in certain cases, influence the amount and location of meiotic recombination (Miklos and Nankivell, 1976; Yamamoto and Miklos, 1978). Recently, transcription of satellite DNA sequences has been reported in certain stages of amphibian oogenesis (Varley et al., 1980). Among certain groups of higher plants striking interspecific differences in the amount and distribution of constitutive heterochromatin have been found, and such groups are promising systems for studies of plant taxonomy at a DNA level and the evolutionary role of heterochromatin and satellite DNA in plants (e.g., Singh and Röbbelen, 1975; Greilhuber and Speta, 1976, 1977, 1978; Greilhuber, 1977, 1979; Schweizer and Ehrendorfer, 1976; Kenton, 1978; Linde-Laursen et al., 1980). In the liliacean species *Scilla siberica* much is known about its major satellite DNA components (Timmis et al., 1975; Deumling et al., 1976; Deumling, 1978) and the cytochemistry of its heterochromatin (Caspersson et al., 1969; Baumann, 1971; Greilhuber, 1973; Schweizer, 1973, 1976a, b, 1977). Therefore, we have studied the heterochromatin of this species in comparison with that of the related species *S. amoena, S. ingridae*, and *S. mischtschenkoana.* Three cytochemical techniques, namely staining of the chromosomes of these *Scilla* species with fluorescent dyes (quinacrine, DAPI, chromomycin A_3), Giemsa-C-banding, and in situ-hybridization of ^{3}H-satellite cRNAs with these chromosomes, have been used to examine both the distribution of homologous satellite DNA sequences in heterochromatic regions of these species and the relationship of fluorchrome staining of chromosomes to the DNA base composition in certain chromosomal segments.

Materials and Methods

Plants. Plants were cultivated as singles in pots in the Botanical Garden of the University of Vienna (HBV). *Scilla amoena* L. was taken from an old established garden population, *S. siberica* Haw. in Andr. cv. 'alba', the triploid *S. siberica* cv. 'Spring Beauty', *S. ingridae* Speta and *S. mischtschenkoana* Grossh. were obtained from Tubergen Ltd., Haarlem, The Netherlands. For taxonomic annotations see Greilhuber and Speta (1978).

Pretreatment and Fixation. Root tips were treated with colchicine (0.1% colchicine in aqua dest.) for 48 hours at 4° C, and fixed in methanol-acetic acid (3:1), at least overnight and occasionally up to several weeks, at 4° C.

Slide Preparations for Fluorescence Microscopy and in situ Hybridization. Meristem-containing root tip regions were dissected in the fixative, rinsed for a few seconds in 45% acetic acid, and squashed directly on glass slides. The coverslips were removed after freezing the slide over a cold plate, and after air drying the slides were stored in the refrigerator for 1 to several days. For fluorochromy in some cases the enzymatic maceration technique was employed (see below).

DNA Extraction and Fractionation. DNA was extracted from leaves using the preparation procedure and purification on hydroxyapatite described previously (Deumling, 1978). The purified DNA was fractionated in Ag^+-Cs_2SO_4 density gradients into main band and satellite fractions. The Ag^+/DNA molar ratio was 0.27 for *S. amoena, S. ingridae*, and *S. siberica*, and ratios of 0.2, 0.27, and 0.3 were used for *S. mischtschenkoana.* In this latter species, an Ag^+/DNA ratio of 0.2 resulted in the best separation of satellite DNA which, however, was still not complete.

In situ Hybridization. Squashed root tips, fixed and pretreated as described above, were used. Part of the slides had been stained with fluorochromes (see below) prior to in situ hybridization. In situ hybridization was carried out with ^{3}H-satellite cRNAs using the formamide method described previously in detail (Deumling, 1978).

Sequential Fluorochrome Staining. Quinacrine was used as the first fluorescent stain. Slides were rinsed in McIlvaine's citric acid/Na_2HPO_4 buffer, pH 7.0, stained in a 0.005% solution of quinacrine dihydrochloride (Atebrin, Gurr) in the same buffer for 15 min, rinsed briefly and mounted in McIlvaine's buffer (pH 4.2). The slides were stored in a moist chamber in the dark at 4° C, usually overnight, before examination. A Leitz epi-illumination system was used (Ploemopak II). The filter block H_2 or E_3 was used combined with BG 38 and BG12 (3 mm) as additional excitor filters and K510 as barrier filter. Metaphase plates were selected and photographed on Kodak Tri X Pan film. Then the coverslip was removed by rinsing with buffer, and the slides were destained for two days in two changes of 96% ethanol. Remaining fluorescence was negligible.

DAPI (4′-6-diamidino-2-phenylindole, Serva, Heidelberg, Germany) was used as the second dye (Schweizer and Nagl, 1976; Schweizer, 1976a, b). The substance was dissolved in McIlvaine's buffer (pH 7.0) and used at a concentration of 0.2 µg/ml. After preincubation in buffer of pH 7.0 the slides were flooded with DAPI solution and stained for 10 min in the dark. After staining the slides were rinsed and mounted in the same buffer. For observation, the filter block A was used in combination with excitor filter UG 1 and barrier filter K 490. The same metaphase plates were then photographed again.

Chromomycin A_3 ("reinst", from Serva, Heidelberg, Germany) was used as the subsequent stain (Schweizer, 1976a, 1980). The substance, dissolved in McIlvaine's buffer, pH 7.0, containing 10 mM $MgCl_2$, was used at a concentration of 0.2 mg/ml. The coverslips were removed from the DAPI-stained slides, which were rinsed in the $MgCl_2$ containing buffer and flooded with the CMA_3 staining solution for 15 to 40 min. After staining and rinsing, the slides were mounted in glycerol/McIlvaine's buffer, pH 7.0 (1:1), or were counterstained with the nonfluorescent compounds distamycin A or methylgreen (see Schweizer, 1981). A freshly prepared solution of 0.2 mg/ml distamycin A-HCl (serva) in McIlvaine's buffer of pH 7.0 was applied for 15 min, and after rinsing the slides were mounted as described. Alternatively, chromomycin A_3-stained slides were poststained for 5 min with a chloroform-extracted 0.1% solution of methyl green GA (Chroma, Stuttgart, Germany) in McIlvaine's buffer (pH 4.9), rinsed and mounted as described. Prior to investigation the slides were often "aged" for about one week in the refrigerator. The filter block E_3 was used with BG 38 and BG 12 (3 mm) as additional excitor filters and K 510 as barrier filter. Relocated metaphases were documented again. The filter combination used completely abolished remaining DAPI fluorescence so that a pure chromomycin fluorescence could be observed.

Sequential Giemsa-C-Banding. Fluorochromed slides were subjected to acetic-acid-, $Ba(OH)_2$-, and $2\times$SSC-treatments as described by Schwarzacher et al. (1980).

Giemsa C-Banding. Schwarzacher et al. (1980) have described an improved method which combines maceration of meristems and enzymatic digestion of cell walls by pectinase-cellulase treatment with the various acid-, base-, and buffer treatments that seem to be indispensable for successful C-banding in plants. The technique allows to band successfully long-stored fixed material, resulting in better contrast of staining and also revealing small bands, especially centromeric heterochromatin. With this procedure, some tiny additional bands were detected in *Scilla siberica* and *S. amoena* which were not revealed previously. These were mapped according to the principles outlined by Greilhuber and Speta (1978).

Karyograms. In *Scilla siberica* cv. "Spring Beauty" and *S. amoena* the karyograms were completed on the basis of the previously published data, which were also used for *S. mischtschenkoana* (Greilhuber and Speta, 1978). New karyograms were worked out for *S. siberica* cv. "alba" and *S. ingridae* based on 20 and 7, respectively, well spread metaphase plates. All karyograms refer to single individuals which were used in the present study, and are presented in a C-value proportional size. On the basis of apparent interspecies homology, chromosome numbering was modified in *S. mischtschenkoana.*

Genome Size Determinations. DNA contents were determined by the two-wavelength Feulgen cytophotometry using a Leitz MPV 2 cytophotometer and *Allium cepa* (1 C = 16.75 pg) as an internal standard. Hydrolysis was performed in 5 N HCl at 20° C for 60 min (for details see Greilhuber, 1977).

Results

Heterochromatin Distribution

All species studied have a common chromosome number of x = 6 and are also similar in relative sizes and centromere positions of their chromosomes. Each chromosome type in one species probably has its homologous correlate in the other species. However, for chromosomes 3 and 4 the respective interspecific homologies are disputable. In *S. siberica, S. amoena,* and *S. ingridae* the NORs are loacted near the centromere in the short arm of chromosome no. 6, whereas *S. mischtschenkoana* has two nucleolar chromosomes, no. s 5 and 6, with terminal NORs in the short arms. In the latter species, a variable terminal heterochromatic segment may be located near the NOR of chromosome no. 5 (Fig. 1).

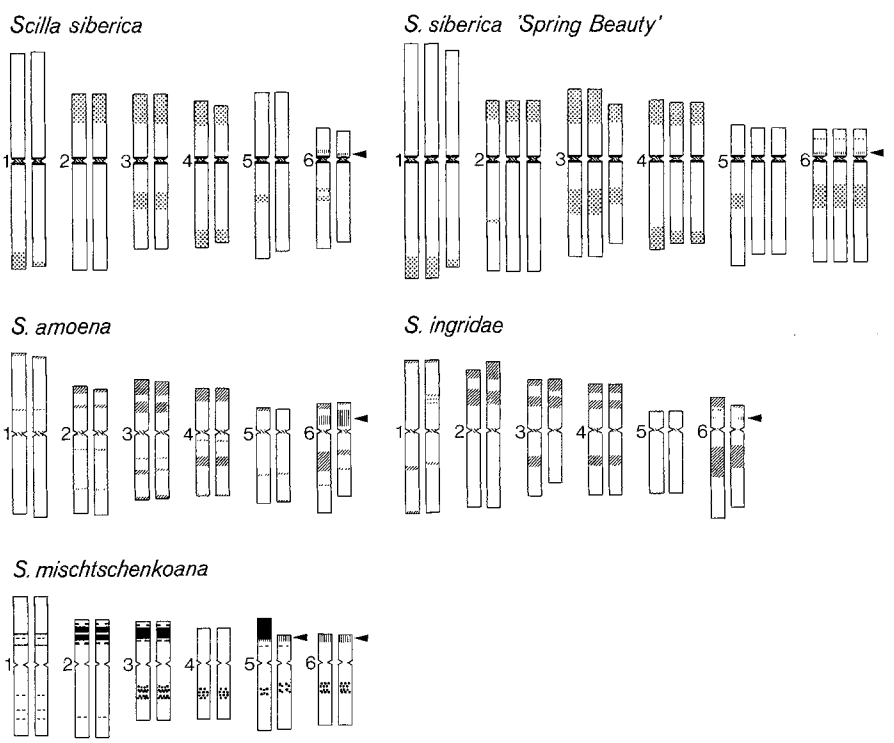


Fig. 1. Synopsis of fluorochrome banded karyotypes (presented in correct proportion with the respective average DNA content of the corresponding species; the various differentially fluorescent heterochromatin types are graphically distinguished, compare Table 1; arrows mark NORs). Figure reference: *Scilla siberica* Figure 3b, c; *S. siberica* "Spring Beauty" Figures 2a, 3a, 7a–d; *S. amoena* Figure 2b; *S. mischtschenkoana* Figure 5a–d

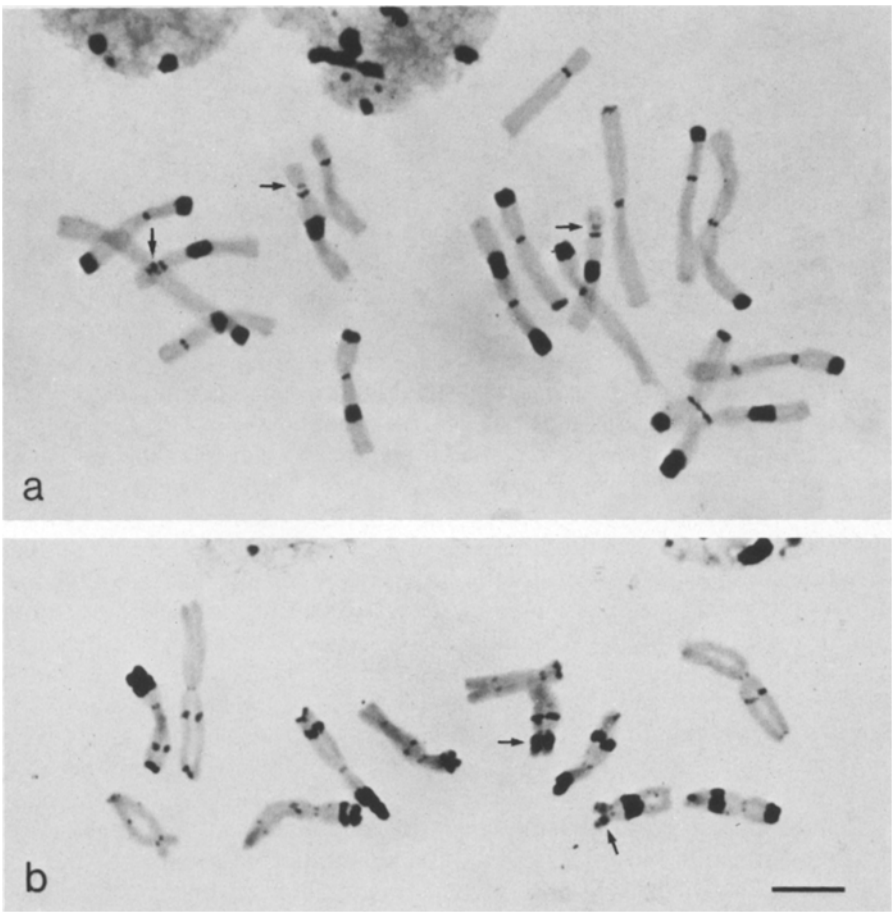


Fig. 2a and b.[1] Giemsa C-banding in **a** *Scilla siberica* "Spring Beauty", **b** *S. amoena*. *Arrows* mark NORs

All four species have appreciable amounts of constitutive heterochromatin as identified by Giemsa C-banding (Figs. 1, 2, 5d). NORs and centromeric regions are generally positively stained after C-banding, but the bulk of the constiutive heterochromatin is found at intercalary and terminal positions, which, for convenience, we classify as "major bands" or "major heterochromatin fraction" as opposed to "NOR-adjacent regions" and "centromeric regions". It has been estimated that 16–20% of the DNA is located within C-bands in *S. siberica*, ca. 16% in *S. amoena*, 18–27% in *S. ingridae*, and about 11% in *S. mischtschenkoana* (Table 1). Apart from the differences in heterochromatin arrangement found in different *Scilla* species, there are also heterochromatin variations (polymorphisms) observed in different individuals of the same species, and even in homologous chromosomes of the same individual, especially in *S. ingridae* and *S. siberica* (Vosa, 1973; Greilhuber and Speta, 1978). Neverthe-

[1] Bars in Figures 2–10 represent 10 μm

less, for each species a characteristic general mode of C-band arrangement can be discerned. The patterns are relatively simple in *S. siberica*, but more complex in *S. amoena* and *S. ingridae*. In *S. mischtschenkoana*, the material identified by C-banding is distributed in small clusters rather than in solid blocks (Figs. 2a, 5b).

Quantified karyograms of individual plants used for the present study are presented in Fig. 1 and in part extend an earlier investigation on the nature of C-bands, which categorized these regions as an additive component of the genome rather than a substitution for euchromatin (Greilhuber and Speta, 1978). Lengths of heteromorphic homologous chromosomes should vary depending on the size of the C-bands, if the hypothesis of heterochromatin as an additive component of the genome is correct (Greilhuber and Speta, 1978). Conversely, no such regular size differences should be found if heterochromatin was incorporated into the genome as a substitute for euchromatin (Vosa, 1973, 1979) or, more specifically, by transformation of euchromatin into heterochromatin (King, 1980). Our chromosome measurements in *S. ingridae* clearly showed that heterochromatin is an additive genome component. In *S. siberica* cv. "alba" evaluations of chromosomal length differences were more difficult because of the relatively inconspicuous C-band heteromorphisms found. In this case, 20 complete and well spread metaphase plates were measured and statistically evaluated (t-test for paired observations). Significant C-band dependent length differences were found in the long arms of chromosome 5 (0.05% level) and 6 (0.5% level), and the short arm of chromosome 4 (2.5% level), whereas no significant difference was observed in the whole long arms of chromosomes 1 and 4. The euchromatin quantity appears to be different (0.5% level) only in the long arm of chromosome 1. From our measurements we conclude that, at least in *Scilla*, the heterochromatin neither replaces euchromatin nor represents transformed euchromatin, but represents a variable additive component of the genome. Additionally, the results show that euchromatin regions can also vary in these karyotypes, independent from heterochromatin variation, probably as a consequence of orthodox rearrangements such as duplications and deletions. Apparent examples of euchromatin variation are the markedly different short arms of chromosome 5 in *S. siberica* cv. "alba" and cv. "Spring Beauty", and the long arms in chromosome 1 of *S. siberica* cv. "alba".

The contrasting conclusion of Vosa (1973; for critique see Greilhuber and Speta, 1978) that C-bands mostly substitute for euchromatin in *Scilla siberica* was recently extended to the vegetatively propagating triploid clone *S. siberica* cv. "Spring Beauty" (Vosa, 1979). The reasoning is based on a karyogram which markedly deviates from that published by Greilhuber and Speta (1978). In order to avoid future confusion we point to an obvious misidentification of one homologue each of chromosomes 2 and 3 in the karyogram shown by Vosa, apparently caused by the omission of a C-band which clearly shows up in his photograph, and to obvious discrepancies of his karyogram in that chromosome arms which are actually of different length are shown to be of the same size. From the photographs published, the identity of Vosa's material with that used in this study and that of Greilhuber and Speta (1978) is evident.

The basic karyotype similarity of *S. siberica, S. amoena, S. ingridae,* and *S. mischtschenkoana* would suggest that there has been little evolutionary chromosomal diversification, except for the variation in constitutive heterochromatin. However, this is not the case, as considerable differences in nuclear DNA

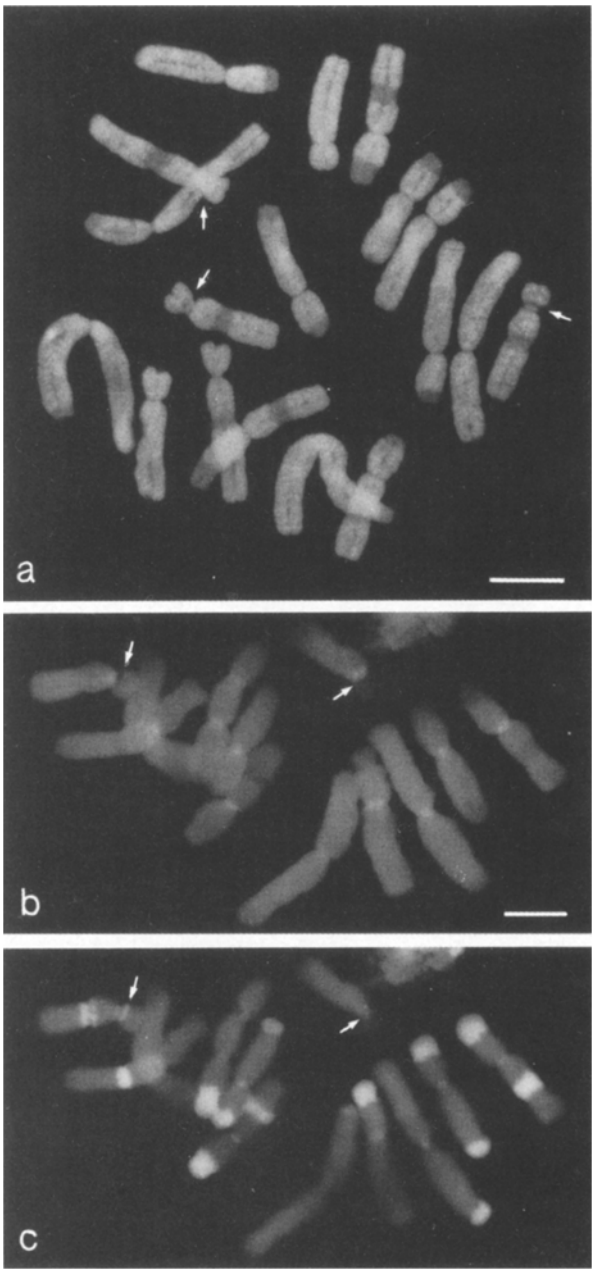


Fig. 3a–c. Fluorochrome banding in *Scilla siberica*. **a** Q-banding in *S. siberica* "Spring Beauty", **b and c** sequential banding of *S. siberica* (2×) with **b** DAPI and **c** chromomycin-distamycin. *Arrows* mark NORs

content have been found among different species. *S. siberica* has the largest genome (1 C=32 pg, on the average), while *S. amoena* (1 C=23.5 pg), *S. ingridae* (1 C=24.4 pg) and *S. mischtschenkoana* (1 C=21.6 pg) all reveal lower DNA contents. This relation is shifted only slightly, when the euchromatin amounts are estimated for these species.

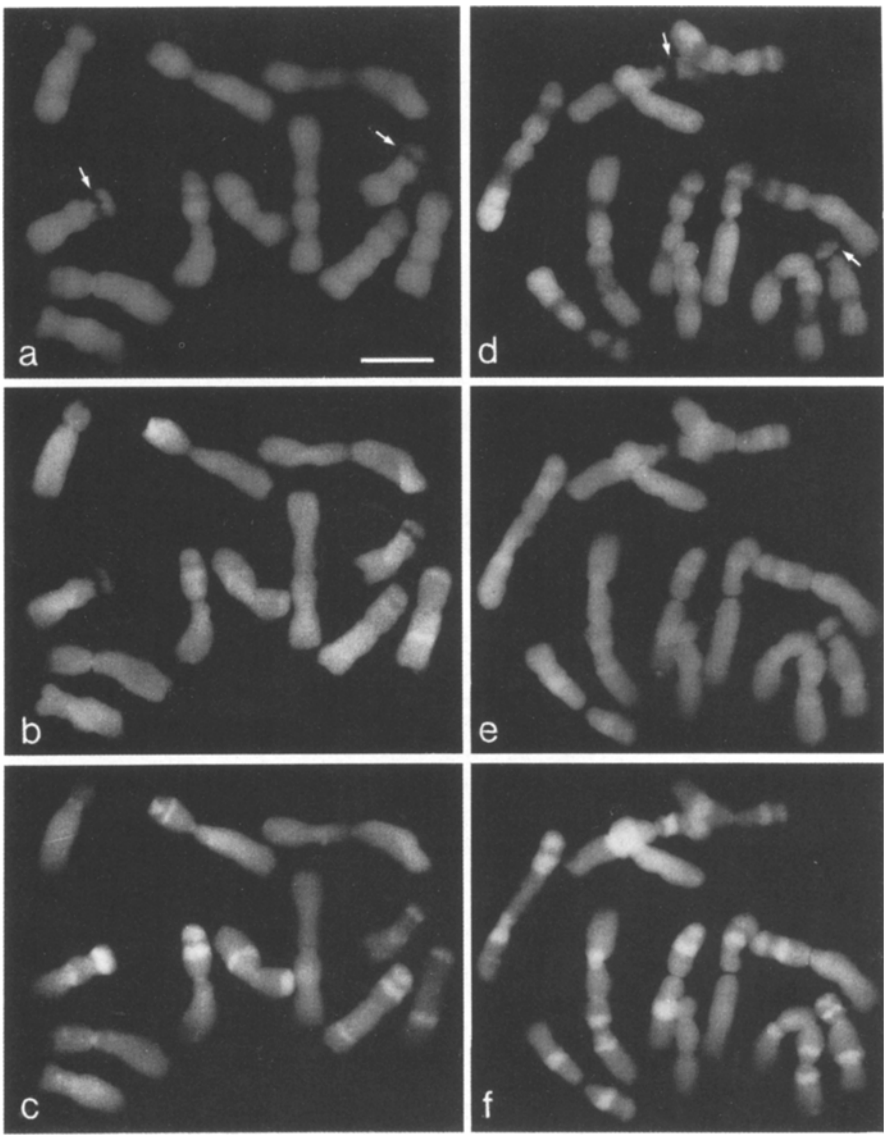


Fig. 4a–f. Sequential fluorochrome banding in **a–c** *Scilla amoena* and **d–f** *S. ingridae*. **a** and **d** Quinacrine, **b** and **e** DAPI, **c** and **f** chromomycin-distamycin. *Arrows* mark NORs in **a** and **d**

Heterochromatin Characterization with Fluorochromes

In certain cases a dependence of the differential heterochromatin fluorescence from the duration of fixation (shorter or longer than approximately 2 days) was noted. This led to the conclusion that chromatin constituents other than DNA, probably proteins, also play a role in the cytological fluorochrome staining. After staining with quinacrine, a dye reported to be specific for chromatin containing AT-rich DNA sequences (Weisblum and DeHaseth, 1972; Comings,

Table 1. Survey of nuclear and DNA characters, and fluorescence properties of metaphase chromosomes of *Scilla* species

	siberica	*amoena*	*ingridae*	*mischtschenkoana*
DNA content (1C) in pg	32.3	23.7	23.9	21.6
Heterochromatin (% of the karyotype)	16–20	16	19–27	11
Late melting component (% of DNA)	12	6	10	max. 3
Late melting component (%GC)	80	80	80	–
Satellite DNA (% of total DNA)	19.0	6.5	10.5	ca. 6.0
Mainband DNA (% GC)	50.0	50.0	50.0	50.0
Fluorescence properties				
Major heterochromatic regions	Q--,[b] DAPI--, CMA_3+	Q-(v), DAPI o+(v), CMA_3+	Q-(v), DAPI o+(v), CMA_3+	Q o (v), DAPI+, CMA_3 o(v)
NORs	Q--, DAPI--, CMA_3+	Q--, DAPI--, CMA_3+	Q--, DAPI--, CMA_3+	Q--, DAPI--, CMA_3+
Centromeric regions	h; Q+(v) DAPI+ CMA_3+	h; Q+(v) CMA_3+	h; Q+(v) CMA_3+	Q o DAPI o CMA_3 o

[a] --strongly diminished; −moderately diminished; o non-differentially fluorescent; +moderately enhanced; + +strongly enhanced; h heterogeneous; v variable fluorescence

[b] Q quinacrine, DAPI 4′-6-diamidino-2-phenylindole, CMA_3 chromomycin A_3

1978; Latt, 1976), the fluorscence is significantly diminished[1] in the major heterochromatin bands and in NOR-associated regions of *S. amoena, S. ingridae,* and *S. siberica*, if the material was fixed for short times (Figs. 3a, 4a, d), while centromeric or paracentromeric regions show slightly enhanced fluorescence (see Table 1). This pattern is altered after prolonged fixation: only the NOR-associated heterochromatin of all three species as well as the major heterochromatin bands of *S. siberica* remain differentially stained whereas almost no differential fluorescence can be noted in the other regions differentiated after short periods of fixation.

The quinacrine pattern observed in chromosomes of *S. mischtschenkoana* (Fig. 5) differ qualitatively from the pattern in the three species described above. In the major bands, fluorescence was only very slightly enhanced after short fixation times. The heterochromatic terminal block of chromosome no. 5 showed the weakest differential fluorescence. NORs and centromeric regions were stained similarly as in the three other *Scilla* species. Prolonged times of fixation result in a complete absence of differential fluorescence in major bands and

[1] The terms "diminished", "reduced", "indifferent", "enhanced", and "positive" are to be understood as relative to the fluorescence intensity of euchromatin

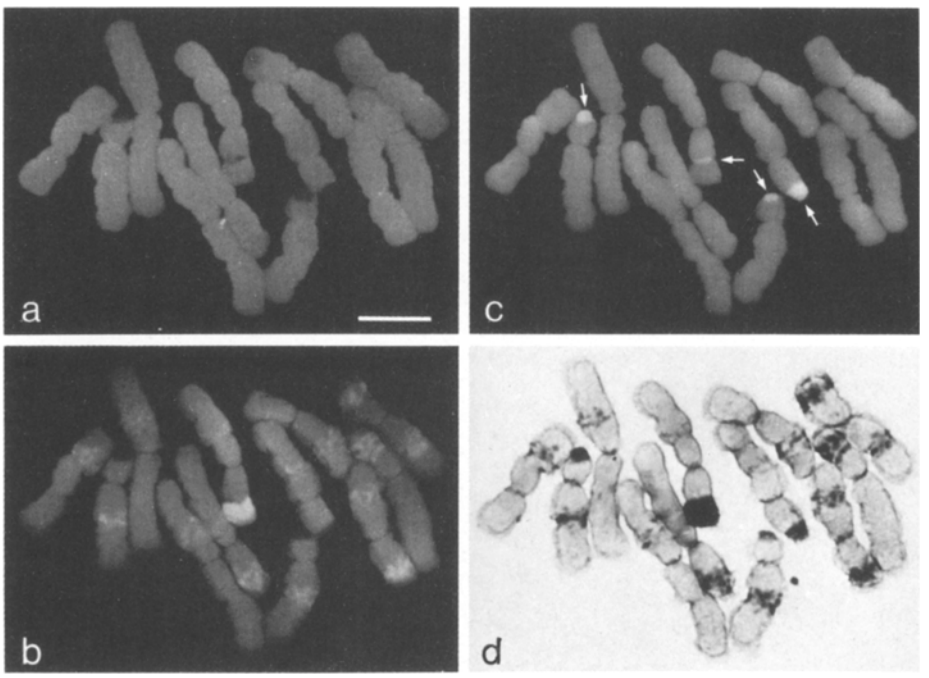


Fig. 5a–d. *Scilla mischtschenkoana*, sequential banding with **a** quinacrine, **b** DAPI, **c** chromomycin (DAPI prestaining), **d** Giemsa C-technique. *Arrows* in **c** mark NORs

centromeric regions but not in NORs. With all four species, treatment of root tips with cellulase and pectinase, applied after prolonged fixation, changed chromosome fluorescence characteristics in the same way as obtained in freshly fixed material without enzymatic maceration.

Staining with DAPI, a dye with known specificity for AT-rich DNA in solution (Chandra and Mildner, 1979), has shown results different from those obtained with quinacrine. In freshly fixed material, major bands show moderately diminished fluorescence in *S. siberica* (Fig. 3b) but slightly enhanced fluorescence in *S. amoena* and *S. ingridae* (Fig. 4b, e). In *S. mischtschenkoana* (Fig. 5b), such bands appear distinctly positive. Fluorescence is strongly diminished in the NORs of all four species. The prominent paracentromeric regions in *S. siberica* showed moderately enhanced fluorescence, but practically no differential centromeric fluorescence was observed in the remaining species, except that gaps were noted at the positions of the variable centromeric Q-bands described above (Figs. 4b, e, 5b). Long-term fixation (3 days or longer) yielded almost complete loss of differential fluorescence in the major bands of *S. amoena* and *S. ingridae* (enzymatic maceration of fixed tissue, however, resulted in higher contrast of enhanced fluorescence compared to staining of freshly fixed material). However, the DAPI patterns were stable in *S. siberica* and *S. mischtschenkoana*, irrespective of fixation times or enzymatic treatment.

After counterstain-enhanced fluorochromy with chromomycin A_3, the major heterochromatin bands of *S. amoena, S. ingridae*, and *S. siberica* (Figs. 3c,

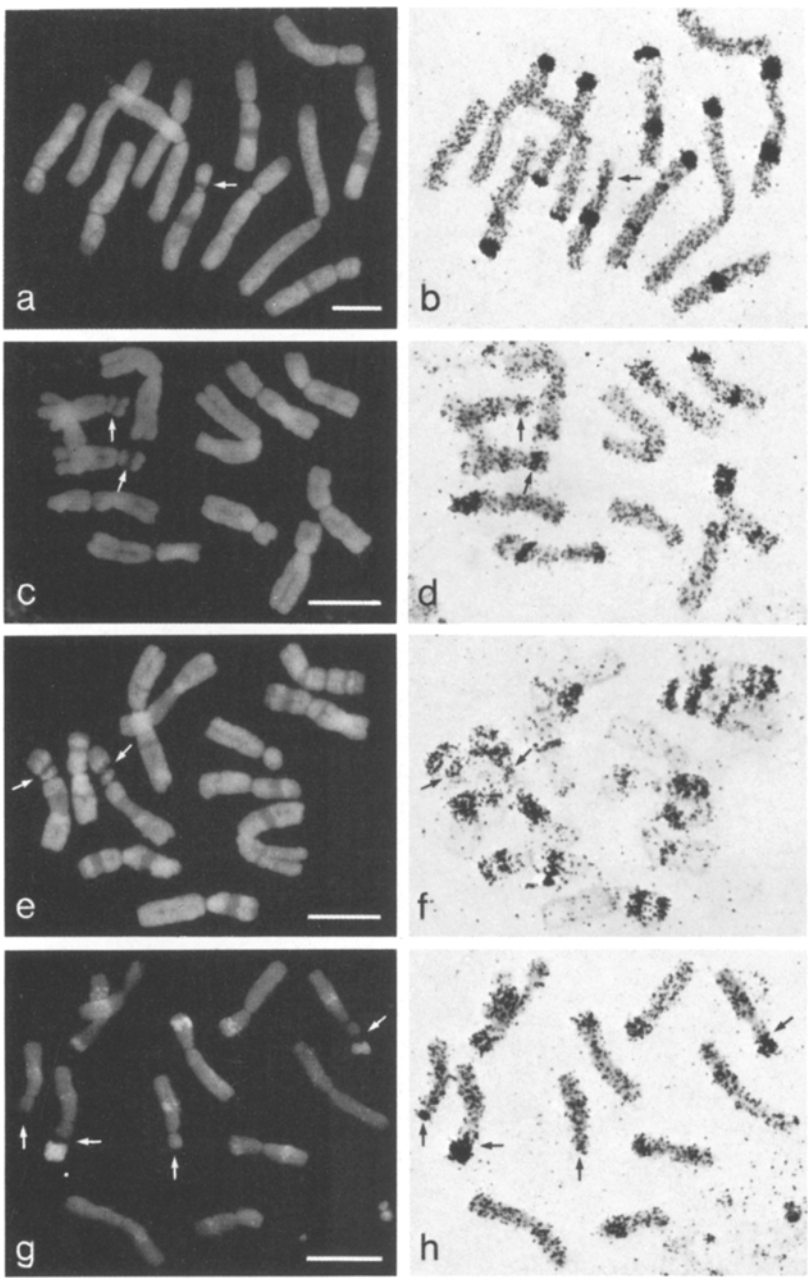


Fig. 6a–h. Sequential in situ hybridization of satellite-DNA-cRNA onto fluorochrome stained chromosomes. **a** and **b** *Scilla siberica* "Spring Beauty", quinacrine, autologous hybridization, **c** and **d** *S. amoena*, quinacrine, autologous hybridization, **e** and **f** *S. ingridae*, heterologous hybridization with *S. amoena* satellite-DNA-cRNA, **g** and **h** *S. mischtschenkoana*, DAPI, autologous hybridization. *Arrows* mark NORs

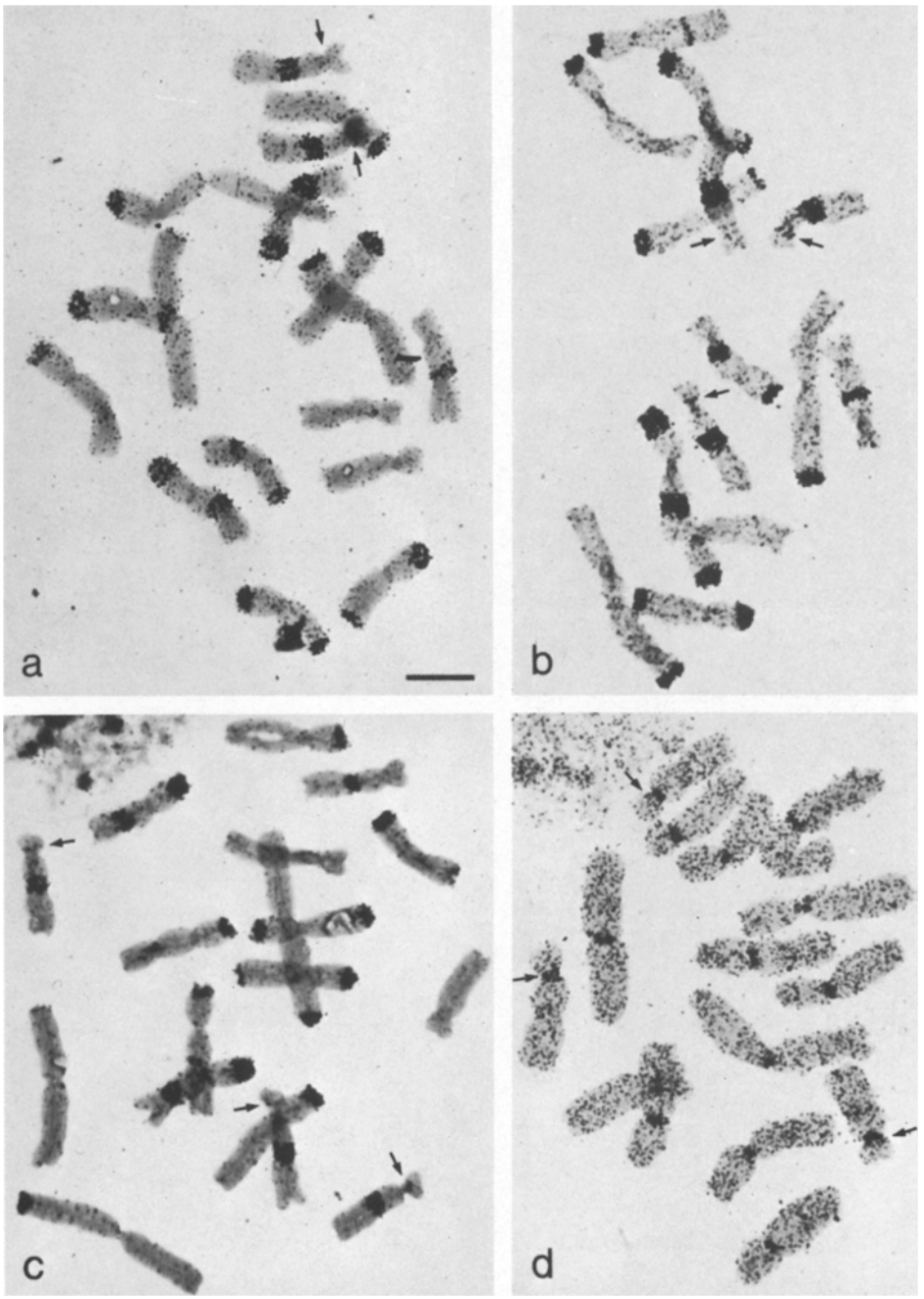


Fig. 7a–d. *Scilla siberica* "Spring Beauty" chromosomes. In situ hybridization with satellite-DNA-cRNA of **a** *S. siberica*, **b** *S. amoena*, **c** *S. ingridae*, **d** *S. mischtschenkoana*. Note heavy labelling of intercalary and terminal constitutively heterochromatic regions and NORs in **a–c**, but heavy labelling of centromeric regions in **d**. *Arrows* mark NORs

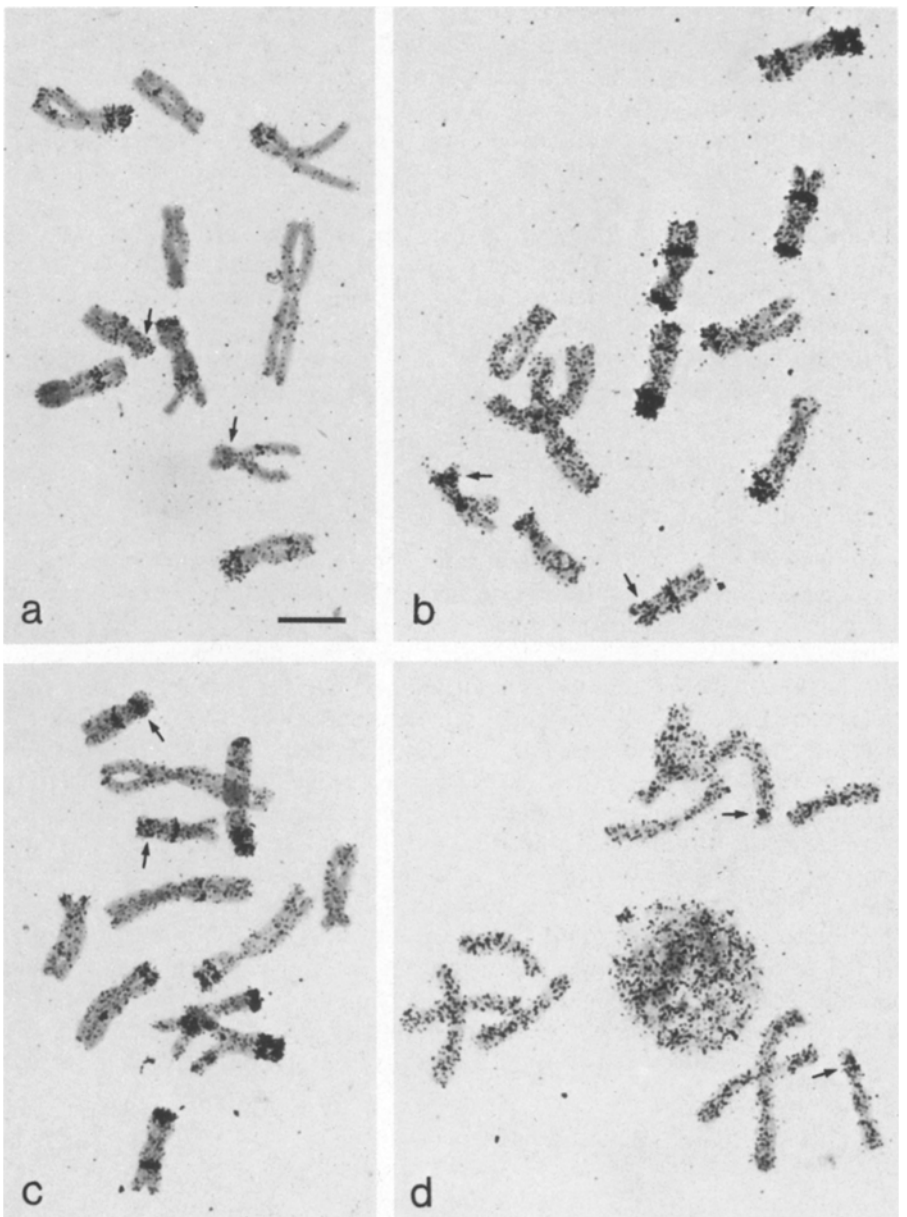


Fig. 8a–d. *Scilla amoena* chromosomes. In situ hybridization with satellite-DNA-cRNA of **a** *S. siberica*, **b** *S. amoena*, **c** *S. ingridae*, **d** *S. mischtschenkoana*. Note heavy labelling of intercalary and terminal constitutively heterochromatic regions and NORs in **a–c**, but absence of specific heterochromatin labelling with the exception of NORs in **d**. *Arrows* mark NORs

4c, f) showed bright fluorescence as did also the NORs and the central part of the centromeric regions. In *S. mischtschenkoana* (Fig. 5c), NOR-associated heterochromatin showed also distinctly enhanced fluorescence whereas, in the other heterochromatic regions, only the (DAPI-positive) terminal block of the long arm of chromosome 5 exhibited a slightly diminished fluorescence, especially in freshly fixed material (the rest of the heterochromatin was not differentiated).

Sequential C-banding (Fig. 5d) revealed perfect congruence of C-staining and fluorescence in major bands and NORs. In centromeric regions, however, C-banding reveals generally more positively stained chromosomal material than either of the fluorchromes alone, but seems to cover the total of differentially fluorescing material, with the exception of *S. mischtschenkoana,* where the C-banding positive centromeres do not show consistently differential fluorescence.

In situ Hybridization of Satellite DNAs

Homologous Hybridization

In situ hybridization of cRNA to satellite DNA on chromosomes of the same species has been studied after heterochromatin localization by fluorochromy with quinacrine (in *S. siberica, S. amoena*, and *S. ingridae*) or DAPI (in *S. mischtschenkoana*), Fig. 6a–d, g, h. For control, non-fluorochromed preparations have also been used for hybridization (Figs. 7a, 8b, 9c, 10d). In all species there is strong hybridization of satellite-DNA-cRNA to all major heterochromatin regions and to the NOR-associated segments. The hybridization to the latter is especially remarkable in the case of *S. mischtschenkoana*, considering the different fluorescence characteristics of major bands and NOR-associated heterochromatin. In all cases, the label over the latter is too strong to result from a mere contamination (Table 1). Low but significant label over centromeric regions is only found in *S. siberica* (Figs. 6b, 7a), indicating that these chromosome regions contain little DNA homologous to satellite DNA. A low amount of label is also seen over euchromatic chromosome regions which may result from the presence of some contaminating main band DNA in the satellite fraction. However, at present it cannot be excluded that some satellite DNA sequences are also located outside the large heterochromatin blocks (Moar et al., 1975).

Heterologous Hybridizations

All combinations of cross hybridization among *S. siberica, S. amoena,* and *S. ingridae* have given results that are indistinguishable from the corresponding homologous hybridizations (Figs. 6b, 7b, c, 8a, c, 9a, b). This suggests that these species are highly similar with regard to their satellite DNA sequences. Minor quantitative differences in the hybridization would not be distinguished under the conditions employed.

However, differences have been obtained after cross-hybridization of the above three species and *S. mischtschenkoana.* When cRNA to satellite DNA of *S. mischtschenkoana* is allowed to hybridize to the chromosomes of the other species, the only common feature observed has been a slight labelling

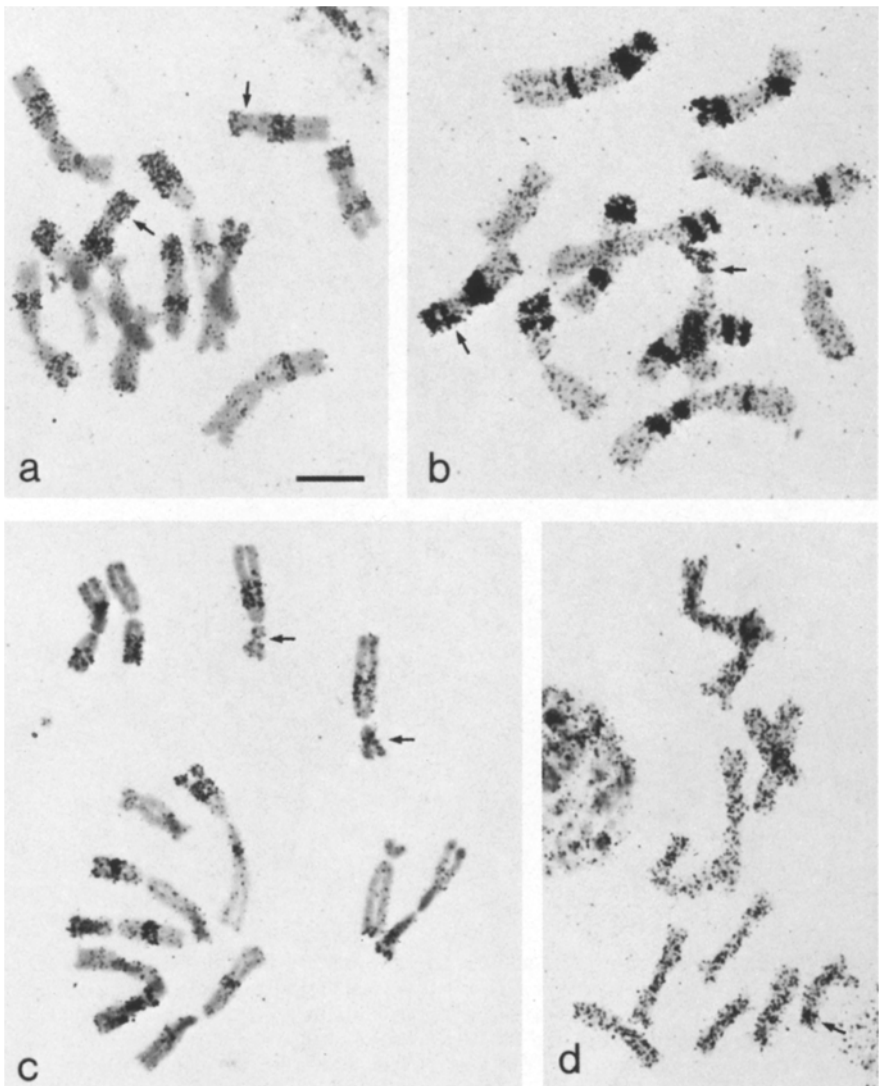


Fig. 9a–d *Scilla ingridae* chromosomes. In situ hybridization with satellite-DNA-cRNA of **a** *S. siberica*, **b** *S. amoena*, **c** *S. ingridae*, **d** *S. mischtschenkoana*. Note heavy label over intercalary and terminal constitutively heterochromatic regions and NORs in **a–c**, but almost total absence of specific heterochromatin labelling in **d**, where only one NOR appears significantly labelled. *Arrows* mark NORs

of the NORs (Figs. 6d, 7d, 8d). No significant label has been found in the major heterochromatic regions and around centromeres of *S. amoena* and *S. ingridae*. By contrast, *S. siberica* chromosomes (Fig. 6d) not only show some label over the major heterochromatin bands but also remarkably strong label over the centromeric (or paracentromeric) regions, indicating a high degree of homology between centromeric DNA sequences from *S. siberica* and certain satellite DNA sequences from *S. mischtschenkoana*. Cross hybridization of satel-

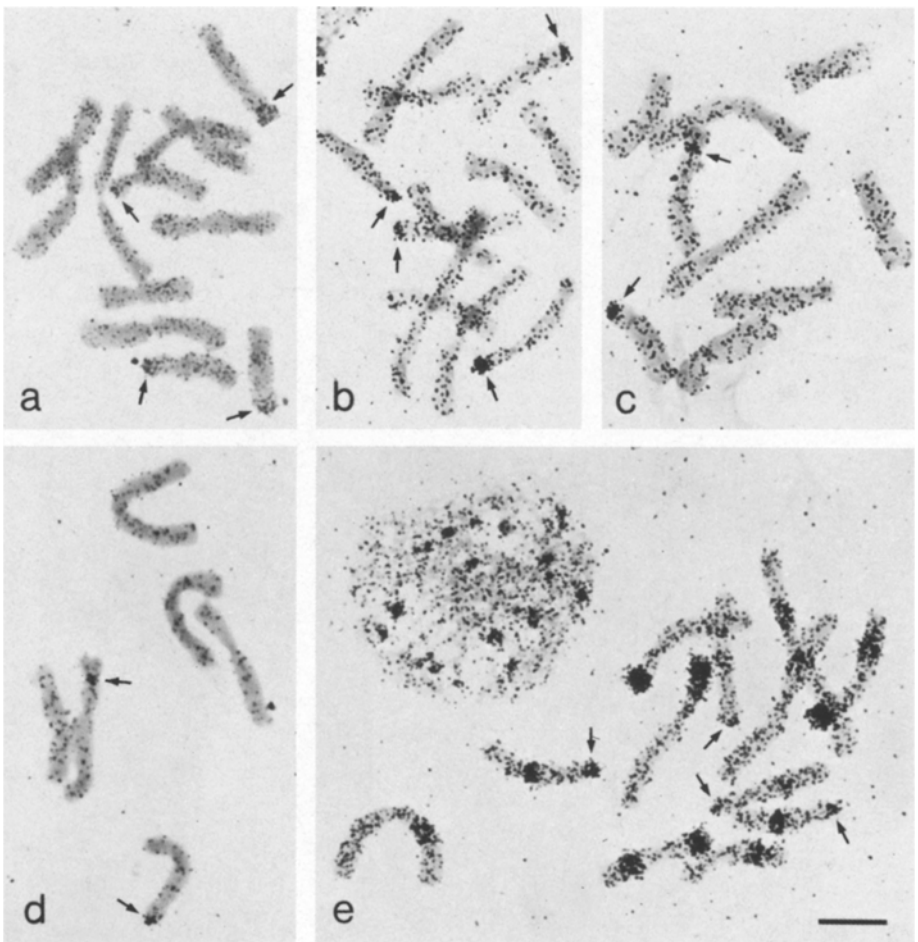


Fig. 10a–e. *Scilla mischtschenkoana* chromosomes. In situ hybridization with satellite-DNA-cRNA of **a** *S. siberica*, **b** and **c** *S. amoena*, **d** *S. ingridae*, **e** *S. mischtschenkoana*. With heterologous hybridization (**a–d**) only NORs show heavy labelling; hybridization with *S. siberica* satellite-DNA-cRNA (**a**) also yields moderate label over the DAPI-positive satellite and a weak positive labelling over the intercalary and subterminal heterochromatin. Autologous hybridization (**e**) yields a strong labelling of all major heterochromatic regions and NOR-associated segments. *Arrows* mark NORs

lite cRNA from the two species *S. amoena* and *S. ingridae* with chromosomes of *S. mischtschenkoana* resulted only in some labelling of the NOR-associated regions (Fig. 10b–d). After hybridization using cRNA to satellite DNA from *S. siberica*, however, some label is also seen over the major heterochromatin regions, especially in the (DAPI-positive) heterochromatic segment of chromosome no. 5 (Fig. 10a). This additional labelling is probably due to the fact that in the total satellite DNA the highly repetitive fraction is present in a higher proportion in the satellite DNA of *S. siberica* than in *S. amoena* and *S. ingridae* (Table 1). This is consistent with the identity of the Hae III restriction fragments of satellite DNA in *S. amoena*, *S. ingridae*, and *S. siberica* (Deumling, 1981).

Discussion

This study presents, in four closely related species of the monocotyledonous plant genus *Scilla*, the direct comparison of localization by in situ hybridization of heterochromatin DNA (satellite DNA with the same defined nucleotide sequence, present in *Scilla amoena, S. ingridae,* and *S. siberica*, Deumling, 1981, as well as one satellite DNA with different nucleotide sequence, in *S. mischtschenkoana*) with the patterns obtained after various selective staining procedures, especially fluorochrome-based ones.

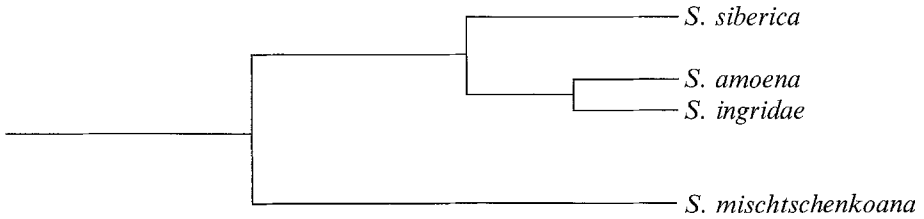
On the whole, regions of preferential satellite DNA hybridization are always coincident with certain C-banding positive chromosome segments. The chromosome staining characteristics seen after treatment with quinacrine, DAPI, or chromomycin A_3, however, shows, in several instances, marked differences when compared to the results of the in situ hybridization. While distinct enhanced fluorescence was regularly observed with CMA_3 in all chromosome segments containing GC-rich DNA sequences, even when made and stained under different conditions, the fluorescence of Q and DAPI can be influenced by the experimental conditions, i.e. duration of storage of the material in the methanol/acetic acid fixative, or by treatment of the tissue with pectinase and cellulase. It is obvious that these inconsistencies are not due to the DNA base sequence.

In three species, *S. amoena, S. ingridae,* and *S. siberica*, major C-bands were all CMA_3-bright as expected for chromatin containing GC-rich DNA sequences. Quinacrine and DAPI, however, which consequently should result in correspondingly reduced fluorescence in these C-bands, did not show a clear correlation in all cases: with quinacrine, the fluorescence was strongly diminished in the major heterochromatin bands of *S. siberica*, as expected. However, especially with material fixed for longer time, a change to almost indifferent fluorescence was noticed in the species *S. amoena* and *S. ingridae*. With DAPI, major bands showed also diminished fluorescence in *S. siberica,* while these bands were non-differentiated or even slightly positive in *S. amoena* and *S. ingridae*. Similar discrepancies in DAPI and quinacrine fluorescence of the major heterochromatin bands were also observed in *S. mischtschenkoana* (see below).

Digestion of satellite DNAs from the three species, *S. amoena, S. ingridae,* and *S. siberica*, however, using the restriction enzyme Hae III, resulted in an identical pattern of DNA restriction fragments as revealed by gel electrophoresis. Moreover, the sequence of the dominant fragment was determined to be identical in all three species (Deumling, 1981). Since this unusually thermostable and highly repetitive satellite DNA component represents a substantial part of the DNA in the major C bands of all three species, this highly GC-rich portion of the DNA should cause a clear and consistent reduction of quinacrine and DAPI fluorescence. Thus one has to conclude that characterization of plant heterochromatin by fluorochromes is not always a reliable marker related to the specific DNA sequences present. The incongruity of the heterochromatin staining with DAPI and quinacrine remains unexplained.

In *S. mischtschenkoana*, the satellite DNA shows slightly different buoyant density in Ag^+-Cs_2SO_4 gradients and does not show, in thermal denaturation kinetics, the major thermostable component characteristic for the other three species. Moreover, digestion with Hae III reveals a number of fragments different

from those obtained in the other species, though there are several restriction fragments which contain a very small amount of the protosequence of the *S. siberica* satellite DNA as revealed by Southern blotting and hybridization with ^{32}P-cRNA of the respective satellite DNA fractions (Deumling, unpublished). Satellite DNA from *S. mischtschenkoana* did hybridize, with chromosomes of the same species, to the major heterochromatin and NOR-associated regions, and also exhibiting a somewhat more disperse reaction over the whole chromosomes. Extensive crosshybridization with the other species was not obtained but, nevertheless, the few sequence homologies maintained were sufficient to allow some in situ cross hybridization with the satellite DNA present in heterochromatic regions of the other three species (mainly NOR-adjacent chromatin). When using satellite DNA from *S. mischtschenkoana*, it hybridized, on chromosomes of *S. siberica*, not only with NOR-adjacent heterochromatin but also with centromeric heterochromatin, i.e. a chromosomal heterochromatin region containing only very little of the endogeneous satellite DNA isolated. It also hybridized, to a very small extent, with the major heterochromatin blocks. These results as well as those of the other cross hybridization experiments (see Results) show that *S. mischtschenkoana* is taxonomically distant from the other *Scilla* species. They show, too, that *S. amoena* and *S. ingridae* are more closely related to each other than to *S. siberica*. Additional support for these conclusions is given by quantitative karyotype analysis and genome size determinations. The phylogenetic argumentation can be summarized as follows: Unique traits of *S. mischtschenkoana* are: two terminal NOR-pairs, clusters of small C-bands rather than solid blocks, particular, unorthodox fluorescence characteristics, and content of a non-GC-rich satellite DNA in the major heterochromatin fraction (see Table 1). Common traits of *S. siberica, S. amoena,* and *S. ingridae* are: one near-centromeric NOR-pair, the major C-bands arranged as terminal and intercalary blocks, which contain the same GC-rich satellite DNA as judged from cross in situ hybridization and sequencing (Deumling, 1981). Unique traits of *S. siberica* mainly are: simple C-band arrangement, relatively much centromeric heterochromatin, fluorescence characteristics of the major bands as typical for GC-rich DNA, roughly a 1:1 proportion of relative heterochromatin to satellite DNA content, a large genome size. Common traits for *S. amoena* and *S. ingridae* are: complex C-band arrangement, little centromeric heterochromatin, fluorescence characteristics of the major bands somewhat atypical for GC-rich DNA, a roughly 2:1 proportion of the relative contents of heterochromatin and extracted satellite DNA, a smaller genome size. On this basis, the four species investigated in the present paper can be clustered as follows:



The intrakaryotypic distribution of the diverse heterochromatin types in the *Scilla* species investigated follows certain fixed patterns rather than being

random. For instance, in *S. siberica* the DAPI-positive fraction is found only in centromeric position, the quinacrine-dull major fraction only in terminal and certain intercalary regions. A similar regularity is also recognized in *S. amoena, S. ingridae,* and *S. mischtschenkoana.*

The DNA sequence itself does not seem to be correlated necessarily with the position on the chromosome, since, as an example, strongly homologous sequences are located centromerically in *S. siberica* but intercalary-subterminal in *S. mischtschenkoana.* The interspecific homology of the satellite DNA and its location in similar chromosomal loci in *S. siberica, S. amoena,* and *S. ingridae* may be explained by a common origin of the major heterochromatin blocks. The location of homologous highly repetitive sequences, however, in the intercalary major bands of *S. mischtschenkoana,* on the one hand, and in the centromeric heterochromatin of *S. siberica,* on the other hand, is hardly explained by a monophyletic origin: (i) concerted displacement by inversions is unlikely for reasons of the karyotype structure, and (ii) selective elimination of centromeric or intercalary heterochromatin in a hypothetical ancestor species originally containing homologous DNA sequences in both regions, is not supported by the present evidence in this group of the genus *Scilla.* (iii) As a further possibility, an independent amplification of the same sequence in different chromosomal loci in different species could be considered as suggested by Fry and Salser (1977) who assume a limited set of potential satellite sequences in the eukaryotic genome ("library") from which certain sequences can be selected and amplified, even in long-term separated species (Fry and Salser, 1977; Brutlag, 1980). Selection of specific satellite sequences by evolutionary pressure seems a necessary postulate of this hypothesis, although there is no evidence available to explain the choice of such a specific sequence.

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