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CYTOGENETIC ANALYSIS OF DIPLOID *BRASSICA* SPECIES

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The chromosomes of *Brassica* species are small and poorly differentiated, and their identification is extremely difficult using conventional cytogenetic methods. Progress in molecular analysis of *Brassica* species requires cytogenetic maps of their chromosomes. Chromosome-specific markers are needed to distinguish particular pairs of homologous chromosomes and for karyotyping. In this study, three morphological groups of chromosomes in *B. campestris* (genome A) and *B. oleracea* (genome C) and two in *B. nigra* (genome B) were distinguished by the morphometric features of the chromosomes, based upon arm ratio and absolute length. Using fluorescence in situ hybridization and differential stainings it was possible to establish further markers for five pairs of chromosomes in genome A, three in genome B and two in genome C, and to present idiograms of the chromosomes for three diploid *Brassica* species. However, for clear identification of all chromosome pairs more cytogenetic markers are needed.

Key words: Ag-NOR, *Brassica*, fluorescence in situ hybridization, karyotyping, molecular cytogenetics, rDNA, silver staining.

INTRODUCTION

Many *Brassica* species have great economic significance because of their value as oil seeds or vegetables. Some species encompass many varieties and morphologically diverse types of plants. The importance of these crops makes their genetic analysis and chromosome mapping necessary. One of the most important goals of these investigations is to integrate the genetic maps obtained by analysis of molecular markers with the physical maps constructed by localization of DNA sequences on chromosomes. Unfortunately, chromosomes of *Brassica* species are relatively small and very poorly differentiated in their morphology, so they are difficult to analyze cytogenetically.

The main diploid *Brassica* species are *B. campestris* (2n = 2x = 20), *B. nigra*, (2n = 2x = 16) and *B. oleracea* (2n = 2x = 18), which represent the A, B and C genomes, respectively. Their chromosome numbers were determined many years ago (Karpechenko, 1922) but it is still difficult to identify particular chromosomes in the complement. Several karyotypes based on investigations of mitotic (Olin-Fatih and Heneen, 1992; Cheng et al., 1995a; Fukui et al., 1998) or meiotic (Cheng et al., 1994; Mackowiak and Hennen, 1999) chromosomes have been reported. Analyses included metaphase and prometaphase chromosomes, different chromosome staining, and a chromosome image analysis system (Fukui et al., 1998). In the karyotyping of some *Brassica* species the position of rRNA genes has been used as a chromosome marker (Snowdon et al., 1997; Fukui et al., 1998).

In the present paper we show chromosomes and idiograms of three diploid *Brassica* species after complex cytogenetic studies which identified chromosomes with markers: 5 chromosomes for genome A, 3 chromosomes for genome B, and 2 chromosomes for genome C.

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MATERIALS AND METHODS

CHROMOSOME PREPARATION

The seeds of diploid species *B. nigra* (var. *occidentalis*; 2n = 2x = 16; BB) obtained from the seed collection of the Polish Academy of Sciences Botanical Garden, Powsin, Warsaw, *B. oleracea* (var. *capitata*, cv. Kamienna Glowa; 2n = 2x = 18; CC) and *B. campestris* (var. *rapifera*, cv. Goldball; 2n = 2x = 20; AA), both obtained from Polish plant breeding station, Katowice, were germinated on filter paper moistened with tap water in Petri dishes. Seedlings 2–3 days old were immersed in 2 mM aqueous solution of 8-hydroxyquinoline for 1.5–2 h at room temperature, then fixed in acetic alcohol (1:3) and stored at -20°C until used. Standard protocols were used to obtain chromosome preparations. Briefly, fixed seedlings were washed for 15 min in 0.01 M citric acid-sodium citrate buffer at pH 4.8 and digested in 2% (w/v) cellulase (Calbiochem) and 20% (v/v) pectinase (Sigma) for 40–60 min at 37°C. Then the root tips were placed in the center of a slide in a drop of 45% acetic acid and squashed. After freezing the coverslips were removed, and the preparations were air-dried and stored at -20°C until required.

STAINING METHODS

For general DNA staining and fluorescent pattern analysis, the chromosomes were stained with both chromomycin A3 (CMA) and 4',6-diamidino-2-phenylindole (DAPI) (Schweizer, 1976). Preparations were stained with 0.5 mg/ml CMA solution (Serva) for 1–1.5 h in the dark, briefly rinsed in distilled water and air-dried. Then the slides were stained with 2 mg/ml DAPI solution (Serva) for 30 min in the dark, again briefly rinsed in distilled water and finally mounted in an antifade buffer (AF1 Citifluor). After staining, the slides were aged in the dark for 3–5 days prior to examination.

The silver staining was performed according to Hizume et al. (1980). The slides were immersed in a borate buffer (pH 9.2) for 15–30 min and air-dried. Then a few drops of freshly prepared 50% aqueous silver nitrate were applied to each preparation. The slides were covered with nylon mesh and incubated in a humid chamber at 45°C for 1–2 h, then washed in distilled water and air-dried. The photographs were taken using 100 ASA film.

Fluorescence in situ hybridization was performed according to Maluszynska and Heslop-Harrison (1993). The ribosomal DNA clone containing 18S-5.8S-25S rRNA genes isolated from *Arabidopsis thaliana* was directly labelled with Cy3 by nick translation. The slides were pretreated with RNase (100 μg/ml, 37°C, 1 h) and denatured together with a pre-denatured DNA probe (70°C for 5 min) in a humid chamber at 85°C for 7 min. Hybridization was performed overnight at 37°C. Stringent washing was performed in 20% formamide in 0.1 × SSC at 42°C followed by several washes in 2 × SSC. Finally the preparations were counterstained with 2 mg/ml DAPI and mounted in antifade buffer (AF1, Citifluor). The slides were examined using an Olympus AX-70 Provis epifluorescent microscope equipped with filter sets for DAPI, CMA3 and Cy3. Photographs were taken using 400 ASA film, scanned, and computer-processed using the AnalySIS 3.0 software system and finally printed with a high-resolution color printer.

KARYOTYPE CONSTRUCTION

The photographs of the chromosomes were scanned at 300 dpi resolution, magnified and measured using the AnalySIS 3.0 software. The total and relative lengths of each chromosome as well as arm ratio were determined. Chromosome nomenclature was according to Levan et al. (1964) with some modifications. Based on the arm ratio value (q/p) all chromosomes were classified into three different groups – median (q/p = 1.0–1.7), submedian (q/p = 1.71–3.0) and subterminal (q/p > 3.0). When NOR-bearing chromosomes were measured the length of the satellite was not included.

The morphometric features and banding patterns of individual chromosomes are presented on the idiograms (Fig. 3). The chromosome pair numbering corresponds to those on the $DAPI^-$ stained metaphase plates (Fig. 1), and the following banding types were assigned to the particular chromosomes on the idiogram: $DAPI^-$ (negative) band, CMA^+ (positive) band, position of rDNA loci after in situ hybridization, and Ag positive band (Ag-NOR).

RESULTS

BRASSICA CAMPESTRIS (2n = 2x = 20, genome AA)

The chromosomes of genome A are fairly distinctive in their morphology and size (Fig. 1a). The absolute lengths of the chromosomes are in the range of 1.77–3.73 μm. On the basis of centromere position it is possible to classify the chromosomes into three

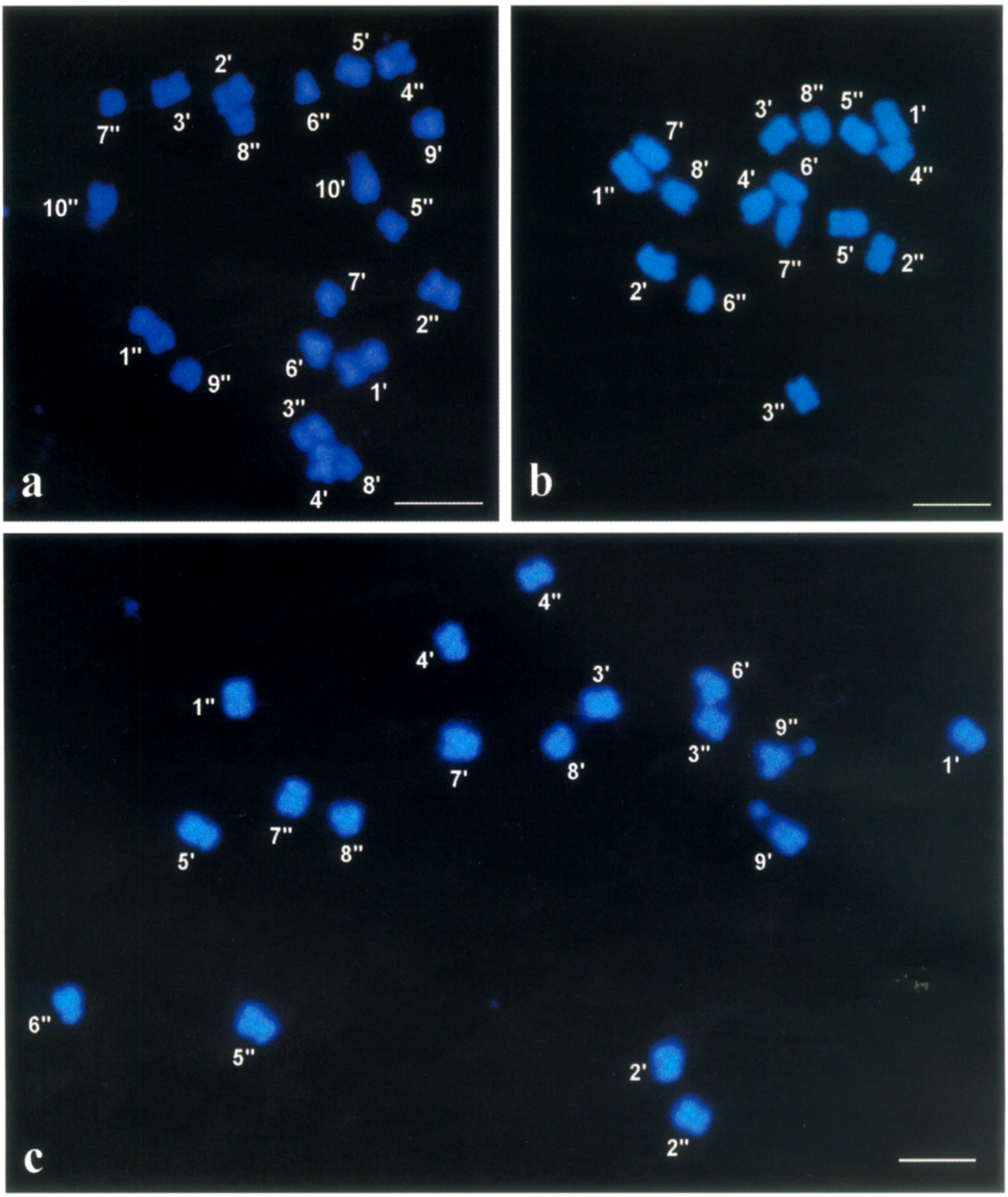

Fig. 1a–c. Mitotic chromosomes of diploid *Brassica* species stained with DAPI. (**a**) *B. campestris* (genome A), (**b**) *B. nigra* (genome B), (**c**) *B. oleracea* (genome C). Negative bands are visible on some chromosomes in genomes A and C. Bars = 5 µm

groups only: median (*m*), submedian (*sm*) and subterminal (*st*). The homologous chromosomes can be accurately determined for only very few pairs, for example satellite chromosomes. The *m* group includes five pairs of chromosomes (1–5) and the *sm* group contains three pairs (6–8). The last two pairs of chromosomes (9–10) belong to the group *st*. Chromosome 10 has a secondary constriction and a prominent satellite on the short arm.

Double fluorescent staining with chromomycin A3 (CMA) and DAPI showed the presence of bright (CMA^+) and dull ($DAPI^-$) stained regions on five pairs of chromosomes. The fluorescent banding pattern of *B. campestris* chromosomes stained with

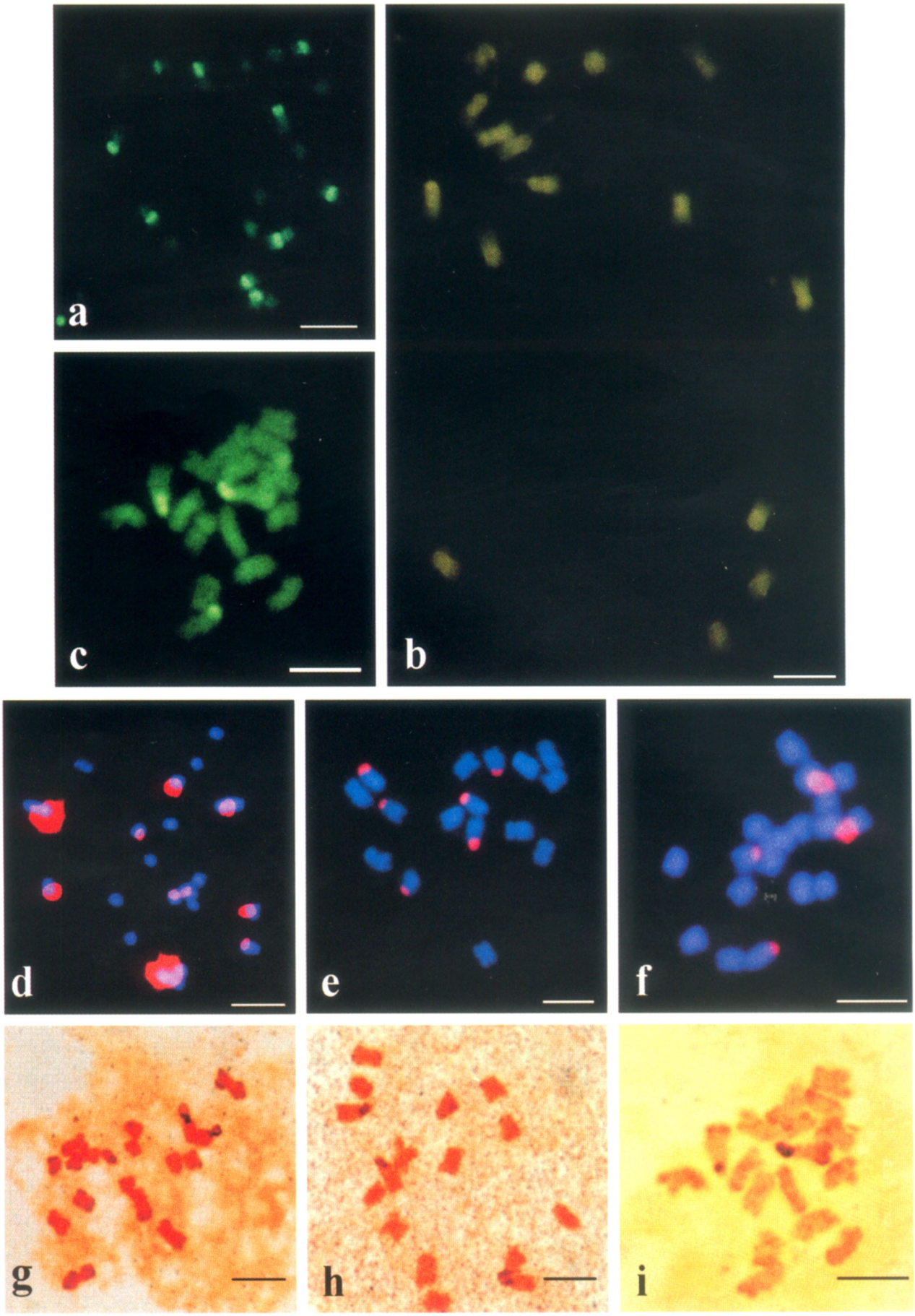

Fig. 2a–i. Mitotic chromosomes of diploid *Brassica* species. (**a**) *B. campestris* – 10 CMA^+ bands, (**b**) *B. nigra* – no CMA^+, (**c**) *B. oleracea* – 4 CMA^+ bands, (**d**) *B. campestris* – 10 sites, (**e**) *B. nigra* – 6 sites, (**f**) *B. oleracea* – 4 sites, (**g**) *B. campestris* – 2 Ag-NORs, (**h**) *B. nigra* – 4 Ag-NORs, (**i**) *B. oleracea* – 2 Ag-NORs. Figs. a–c – CMA staining; Figs. d–f– FISH with 45S rDNA; Figs. g–i – silver staining. Bars = 5 μm.

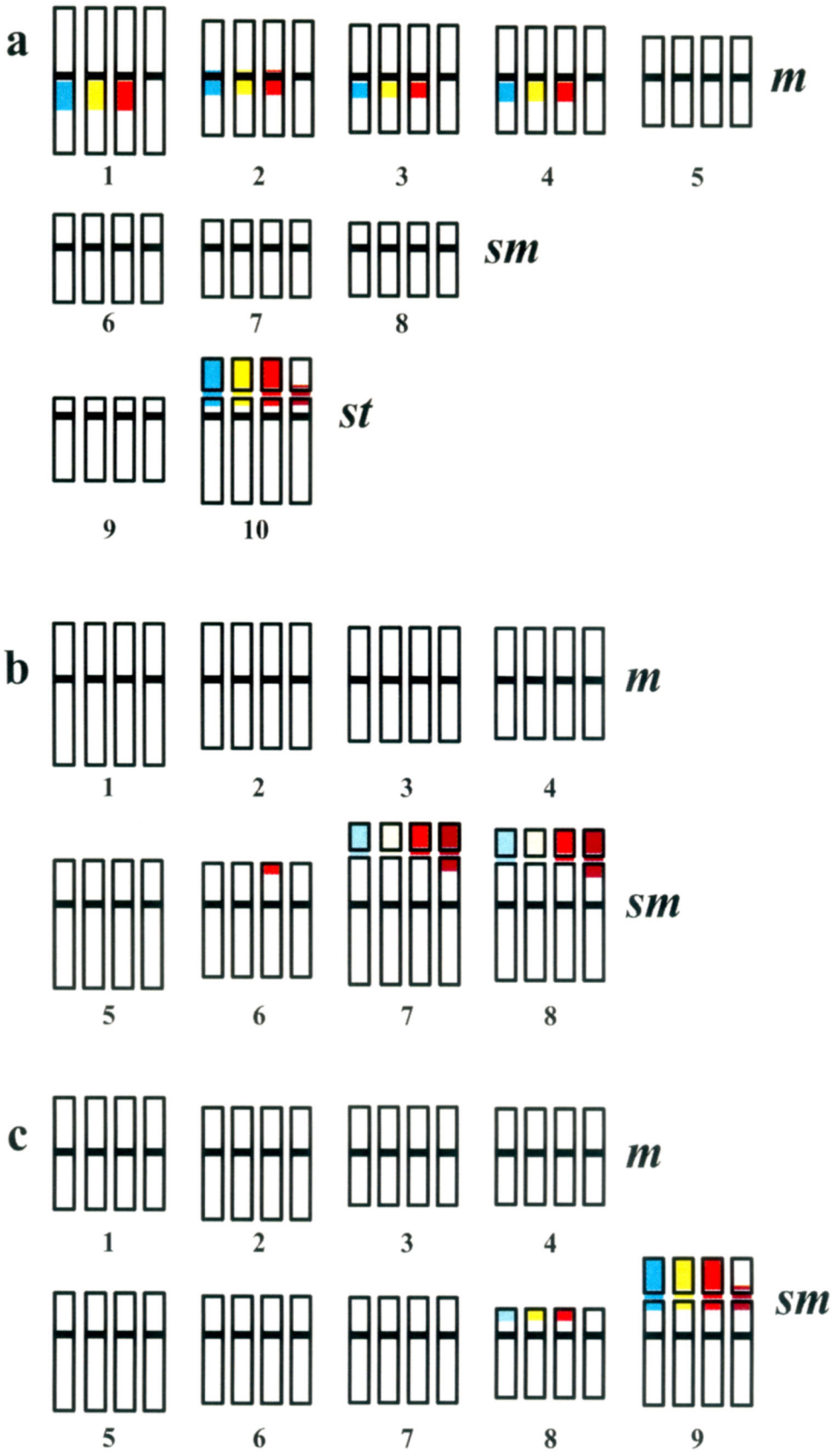

Fig. 3a–c. Idiogram of the chromosomes of diploid *Brassica* species. (**a**) *B. campestris*, (**b**) *B. nigra*, (**c**) *B. oleracea*. The groups of chromosomes on the idiograms: *m* – medial (metacentric), *sm* – submedial (submetacentric), *st* – subterminal (subtelocentric). Colors on the idiogram represent bands obtained in individual stainings: blue – $DAPI^-$; yellow – CMA^+; red – 45S rDNA hybridization signal; brown – Ag-NOR.

CMA is exactly the reverse of the DAPI pattern (Figs. 1a, 2a). The bands are similar in size. They occupy two different chromosomal locations: either the pericentromeric region on the long arm (chromosomes 1, 2, 3, 4) or the distal part of the short arm and the secondary constriction (chromosome 10).

Fluorescence in situ hybridization with rDNA as the probe revealed that five pairs of *B. campestris* chromosomes carry 18S-5.8-25S rRNA genes (Fig. 2d). The hybridization sites differ in size; the biggest one is located on the secondary constrictions of chromosome 10. Generally the distribution of hybridization signals corresponds to the localization of the $CMA^+/DAPI^-$ fluorescent bands. Figure 2g shows the results of silver staining, which is an indicator of the transcriptional activity of rRNA genes. Only two out of ten rDNA loci are active. The active loci are distributed on the secondary constriction of chromosome 10. All pericentromeric loci are inactive or their activity is below detection level.

BRASSICA NIGRA (2n = 2x = 16, genome BB)

The chromosomes of this species are similar in size (absolute length 2.47–3.57 μm) and morphologically very uniform (Fig. 1b). Chromosomes 1–4 have median positioning of the centromere, while chromosomes 5–8 are of the submedian type. Subterminal chromosomes are not present in the *B. nigra* genome. Two pairs of chromosomes (7 and 8) have the secondary constriction and satellite on the short arm.

Double fluorescent staining with chromomycin A3 and DAPI revealed the lack of distinct CMA^+ and $DAPI^-$ bands on the chromosomes of the B genome (Figs. 1b, 2b). Fluorescence in situ hybridization showed the presence of three pairs of rDNA loci, which are similar in size (Fig. 2e). One pair of loci occupies the terminal part of the short arm of chromosome 6. Two sites are located at the secondary constriction and satellite of chromosome pairs 7 and 8. As revealed by silver staining, only these two pairs of loci are transcriptionally active (Fig. 2h).

BRASSICA OLERACEA (2n = 2x = 18, genome CC)

The chromosomes of *B. oleracea* are rather poorly differentiated in morphology and size (Fig. 1c). The absolute lengths of the chromosomes are in the range of 2.17–2.97 μm. The median group encompasses chromosome pairs 1–4, while chromosomes 5–8 belong to the submedian group. In less condensed plates (prometaphases), however, chromosomes 8 and 9 showed subterminal localization of the centromere. The chromosome complement of *B. oleracea* possesses only one pair of chromosomes (pair no. 9), with the secondary constriction and satellite on the short arm.

Two pairs of chromosomes in the complement have distinctive banding patterns after CMA staining (Fig. 2c). The smaller CMA^+ band is located in the terminal part of the short arm of chromosome 8. The larger of the two occupies the secondary constriction/satellite region of chromosome 9. Additionally, chromosomes 9 have a prominent $DAPI^-$ band in a corresponding region of the secondary constriction (Fig. 1c). An insignificant $DAPI^-$ band at the telomere of the short arm of chromosome 8 is also visible in some metaphase plates. The number and localization of rDNA loci after in situ hybridization (Fig. 2f) correspond with the results of CMA staining. After silver staining only the rDNA locus on chromosome 9 is positively stained; the minor pair of loci did not show activity (Fig. 2i).

Chromosome morphology and size as well as chromosome marker localization are compared on the idiograms of individual genomes (Figs. 3a,b,c).

DISCUSSION

KARYOTYPES OF *BRASSICA* SPECIES

Because of the economic importance of the *Brassica* species, study of their cytogenetic makeup began very early (Takamine, 1916; Karpechenko, 1922). So far several karyotypes for *Brassica* species have been presented. In principle they were based on morphometric analysis of mitotic (Olin-Fatih and Heneen, 1992; Cheng et al., 1995a; Fukui et al., 1998) or meiotic (Cheng et al., 1994) chromosomes after Giemsa staining or C-banding. Our results are generally consistent with previous reports. Among the analyzed diploid *Brassica* species, chromosomes of genome A are morphologically the most diverse. In this genome, three distinct groups of chromosomes (medial, submedial and subterminal) can be distinguished easily, and prominent differences in morphology allow some chromosomes in the complement to be qualified as homologous pairs. In contrast, the chromosomes of genome B are much more uniform, and their karyotyping is the most difficult.

In the studies mentioned above, prometaphase chromosomes were used for karyotype construction. As they are less condensed, they show more morphological details and offer better resolution of banding

patterns after differential staining. Our study analyzed mainly metaphase chromosomes, since they are more suitable for measurement and centromere position determination. However, comparisons of *B. oleracea* karyotypes performed using prometaphase and metaphase chromosomes yielded different numbers of chromosomes in each group. For example, chromosomes 8 and 9 are typically subterminal in prometaphase (Olin Fatih and Heneen, 1992; Cheng et al., 1995a), but in metaphase they show submedial positioning of the centromere. These differences are probably due to different degrees of condensation of the hetero- and euchromatin in the chromosome arms. The long arms may contract earlier than the short arms (Olin-Fatih, 1994). Chromosomes of *Brassica* species are very small and poorly differentiated. In such a situation, "pure" karyotyping based only on morphometric analysis is inaccurate and may lead to many misclassifications.

Analysis of small, uniform chromosomes such as those in the genus *Brassica* requires good chromosome resolution, and the presence of additional markers is very important for identification. In this study we used fluorescent staining, which offers better resolution and contrast for chromosome analysis than any traditional methods such as Giemsa or Feulgen staining. Fluorescent staining with chromomycin A3 and DAPI is simple and reliable, delivering markers for identification of some chromosomes in the complement. CMA binds preferentially to G-C-rich regions and DAPI to A-T-rich regions (Schweizer, 1979). In most plant species, ribosomal DNA is rather G-C rich (Hemleben et al., 1988), thus staining with chromomycin A3 may be used for quick, preliminary detection of rDNA loci. This was confirmed for *B. campestris* and *B. oleracea* in the present study. The hybridization sites of rDNA colocalize with CMA^+ bands in the A and C genomes.

DISTRIBUTION AND ACTIVITY OF rRNA GENES

In situ hybridization allows detection of genes and other DNA sequences both on chromosomes and in interphase nuclei. Using this technique the number of loci and localization of 18S-5.8S-25S rRNA genes have been established for many plant species (Maluszynska et al. 1998). The number of rDNA loci in *Brassica* species estimated per haploid genome by in situ hybridization vary slightly in published results. Only the data for *B. campestris* are in full agreement – five sites of hybridization are always observed in mitotic chromosomes. *B. nigra* has either two (Maluszynska and Heslop-Harrison, 1993) or three (Snowdon et al., 1997; Fukui et al., 1998; this study) pairs of rDNA loci. Similarly, in *B. oleracea* either two or three pairs of loci were found (Maluszynska and Heslop-Harrison, 1993; Cheng et al., 1995b; Snowdon et al., 1997; Armstrong et al., 1998; this study). Various numbers of rDNA loci in *B. oleracea* have been determined by restriction fragment length polymorphism (RFLP) analyses (Delsney et al., 1990; McGrath et al., 1990; Kianian and Quiros, 1992). These discrepancies may be connected with the huge intraspecific polymorphism of *B. nigra* and *B. oleracea*. Many forms and crop types significantly differ in their morphology. These phenotypic differences may reflect significant differences in genotypes and in rDNA localization and activity. On the other hand, differences in the number of hybridization sites may also be due to experimental conditions such as type of DNA probe, stringency, etc.

In situ hybridization revealed that all *Brassica* genomes have more than one rDNA locus per haploid genome. Until now there have been few reports about the activity of rRNA genes in *Brassica* species (Cheng and Heneen, 1995; Cheng et al., 1995b). In all species only rRNA genes located at the secondary constriction regions are transcriptionally active. Our observations are generally consistent with the results of Cheng and Heneen (1995) and Cheng et al. (1995b). Two out of ten loci and two out of four loci are active in diploid cells of *B. campestris* and *B. oleracea*, respectively. However, we found that four out of six loci on metaphase chromosomes of *B. nigra* show expression. This observation conflicts with the results of Cheng and Heneen (1995), who failed to detect Ag-NORs on metaphase chromosomes but detected six Ag-stained sites in telophase. It is likely that these differences, like those described above, may be due to intraspecific polymorphism in the *Brassica* species and the different cultivars used in the experiments.

PHYLOGENY OF DIPLOID *BRASSICA* SPECIES

Little is known about the evolution of the diploid species of *Brassica* and the origin of the different genomic chromosome numbers. Catcheside (1937) observed secondary pairing of the chromosomes in meiosis of *B. oleracea*. This homeology may be due to duplication of the basic number of chromosomes. On the basis of morphometric features of pachytene chromosomes, Röbbelen (1960) found six basic types of chromosomes in the *Brassica* genus. This suggests that *Brassica* species might be secondary polyploids

with a basic chromosome number of x = 6. Chromosome pairing in haploids of *B. campestris* indicated homology between chromosomes as well (Armstrong and Keller, 1982). This finding has been confirmed by the results of molecular analyses. RFLP marker mapping revealed that *Brassica* genomes are highly duplicated (Song et al., 1988; Quiros, 1995). The results of other cytogenetic and molecular analyses (Hussein and Abobakr, 1976; Chen and Heneen, 1991) have indicated that the basic number of chromosomes could be three. Comparative mapping between *Arabidopsis thaliana* and *Brassica* species revealed that diploid *Brassica* genomes contain three copies of a basic genome similar in size to that of *Arabidopsis*. This indicated that a high rate of chromosomal rearrangement was involved in the evolution of *Brassica* species (Lagercrantz, 1998). Comparison of the linkage maps of three diploid *Brassica* species for colinearity indicated inter- and intragenomic homology, and pointed to a phylogenetic relationship among the chromosomes of the A, B and C genomes. Truco et al. (1996) hypothesized that the *Brassica* chromosomes originated from an ancestral genome of x = 6.

RFLP analyses of both nuclear (Song et al., 1988, 1991) and chloroplast genomes (Warwick and Black, 1991, 1993) suggest a closer relationship between genomes A and C. This is consistent with cytogenetic observations such as chromosome behavior in meiosis (Cheng et al., 1994) or the morphometric features of the chromosomes (Röbbelen, 1960; Hasterok and Maluszynska, 1997). On the other hand, RFLP analysis of rDNA in diploid *Brassica* species implies a closer affinity between the B and C genomes. The number and localization of rDNA loci in genomes B and C resemble each other more than they resemble those in genome A. To gain a better understanding of the phylogeny of the genus *Brassica*, more genome- and chromosome-specific markers are needed for cytogenetic studies.

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