# Cytogenetic markers of *Brassica napus* L. chromosomes

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**Abstract.** *Brassica napus* has relatively small and poorly differentiated chromosomes. The total length, arm ratio and localisation of 18S-5.8S-26S rRNA genes formed the basis for the preparation of the ideogram of metaphase chromosomes. The morphometric features of the *B. napus* chromosomes allow for their classification into three morphological groups, but it is difficult to distinguish particular chromosome pairs within the groups. rRNA genes are present in 12 chromosomes of the diploid complement and are located in three chromosomal positions: secondary constrictions, terminal and pericentromeric regions. All rDNA loci at the secondary constriction are active. The signals of *in situ* hybridisation with rDNA co-localise with CMA positive bands in most of the loci. It was found that rRNA genes are good markers for some *B. napus* chromosomes, but still more cytogenetic markers are needed for the identification of all chromosome pairs.

**Keywords:** Ag-NOR, *Brassica napus*, fluorescence *in situ* hybridisation, karyotyping, molecular cytogenetics, rDNA, silver staining.

## Introduction

*Brassica napus* spp. *oleifera* (oilseed rape) is one of the most productive oilseed crops. It is an amphidiploid species formed from interspecific hybridisation between two diploid progenitors: *B. campestris* (2n = 2x = 20; genome AA) and *B. oleracea* (2n = 2x = 18; genome CC). The genome of *B. napus* contains a full chromosome set of both ancestral species (2n = 4x = 38; genome AACC). The origin of *B. napus* was proposed by U (1935) on the basis of cytological observations. This hypothesis was supported by evidence from isozyme analysis (CHEN et al. 1989) and organelle and nuclear RFLP analysis (SONG et al. 1988, DELSNEY et al. 1990, SONG, OSBORN 1992, PARKIN et al. 1995). Chromosome pairing

(ATTIA, RÖBBELEN 1986) and comparative analysis revealed colinearity between A and C genomes of *B. napus* (SCHEFFLER et al. 1997).

The agronomic and economic importance of oilseed rape stimulates the development of genetic and molecular investigations leading to the construction of several genetic and molecular maps (PARKIN et al. 1995, FOISSET et al. 1996), while cytogenetic investigations are very limited and lag behind molecular study. Chromosomes of *B. napus* are relatively small and exhibit little morphological variation with only a few bands and a simple banding pattern (OLIN-FATIH, HENEEN 1992, OLIN-FATIH 1994, 1996). This makes it difficult to identify them in a complement. On the basis of morphological features of metaphase or prometaphase chromosomes, several karyotypes of *B. napus* have been published, but it was only possible to classify chromosomes into groups according to centromere position (OLIN-FATIH, HENEEN 1992, OLIN-FATIH 1994, SKARZHINSKAYA 1998).

Genetic and plant breeding investigations for crop improvement and introduction of the desirable genes are involving modern biotechnology techniques, such as a somatic hybridisation, transformation (De BLOCK, DEBROUWER 1991) and cell and protoplast in vitro culture (OLIN-FATIH 1996), which can result in chromosome variation. Such investigations require cytogenetic markers for chromosome identification and also for integration of the genetic and cytogenetic maps. Molecular cytogenetics, and especially in situ hybridisation by localisation of specific DNA sequences on chromosomes, give such possibilities. One of the most often applied groups of chromosome markers are rRNA genes. In some plants, for example *Hordeum vulgare* (LEITCH, HESLOP-HARRISON 1993) and *Arabidopsis thaliana* (MURATA et al. 1997), the localisation of 5S and 45S rDNA by in situ hybridisation allows to distinguish all chromosomes of these species.

In this paper we present the ideogram of *B. napus* based on morphometric features of the metaphase chromosomes after differential staining and localisation of rDNA and active rRNA genes.

## Material and methods

### Chromosome preparation

Seeds of *B. napus* cv. Leo obtained from a Polish plant breeding station were germinated on moist filter paper in petri dishes at 20-22°C. To obtain desirable frequency of metaphases, suitable for karyotyping, seedlings with 2-3 cm long roots were treated with 2 mM 8-hydroxyquinoline for 2 h at room temperature and for 2 h at 4°C. Then the material was fixed in a mixture of ethanol and glacial acetic acid (3:1) for 3 h and stored at −20°C until use. Fixed root tips were washed in citric buffer (0.01 M, pH 4.8) and digested with an enzymatic solution of 2% pectinase (Sigma), 0.9% cellulase (Calbiochem) and 0.1% cellulase "Onozuka R-10" (Serva) in citric buffer at 37°C for 60-90 min. Squashing was done in 45%

acetic acid. Cover-slips were removed after freezing on dry ice and the slides were air-dried.

## Staining methods

Double fluorescent staining with CMA (chromomycin A3) and DAPI (4', 6 diamidino-2-phenylindole) was done according to SCHWEIZER method (1976). Preparations were stained with CMA solution (0.5 mg/ml, Serva) for 1 h in darkness, briefly rinsed in distilled water and air-dried. Then the slides were stained with DAPI solution (2 μg/ml, Serva) for 30 min in darkness, briefly rinsed in distilled water and mounted in antifade buffer (Citifluor, Ted Pella, Inc.).

Silver staining was done using the modified method of HIZUME et al. (1980). The chromosome preparations were treated with a borate buffer (Merck, pH 9.2) for 20 min and air-dried. Several drops of freshly prepared 50% silver nitrate in deionised water were added to each preparation. Slides were covered with a polyamide cloth and incubated in a moist chamber at 45°C for 1-1.5 hours, washed in distilled water, air-dried and mounted in DPX.

Fluorescence in situ hybridisation was carried out according to the method described by MALUSZYNSKA and HESLOP-HARRISON (1993). The rDNA fragment, containing the whole 45S rDNA unit isolated from *Arabidopsis thaliana*, was directly labelled with Cy3 using nick translation. Preparations were pretreated with RNase (100 mg/ml, 37°C, 1 h), washed in 2 × SSC (0.3 M sodium chloride + 0.03 M trisodium citrate, pH 7), dehydrated in an ethanol series (70%, 90%, and 100%) and air-dried. Slides were denatured together with a predenatured DNA probe (70°C for 5 min) in a humid chamber at 85°C for 7-9 min. Hybridisation was performed overnight at 37°C. Stringent washing in 20% formamide in 0.1 × SSC at 42°C was followed by several washes in 2 × SSC. Preparations were counterstained with 2 μg/ml DAPI and mounted in Citifluor.

Slides were examined under an Olympus AX-70 Provis epifluorescence microscope. Photographs were taken using a Kodak Gold 100 or 400 ASA film, scanned, then computer-processed using AnalySIS 3.0 and Picture Publisher 7.0 software and finally printed using a high resolution colour ink jet printer in the CMYK technology.

## Karyotyping

The lengths of chromosomes and their arms were measured on several well-spread metaphase plates stained with DAPI, and the relative length as well as arm ratio were calculated. The chromosome nomenclature followed is that of LEVAN et al. (1964). Considering to centromere position the chromosomes were classified into three morphological groups. The length of the satellite was not included when NOR-bearing chromosomes were measured.

The morphometric features and staining patterns of individual chromosomes are presented in the ideogram (Figure 2). The chromosome pair numbers corre-

spond to those on the DAPI stained metaphase plate (Figure 1a). The following four banding types were assigned to the particular chromosomes in the ideogram: DAPI⁻ (negative) bands, CMA⁺ (positive) bands, position of rDNA loci after in situ hybridisation and Ag⁺ (positive) bands (Ag-NORs).

# Results

## Chromosome number and morphology

The diploid karyotype of *B. napus* consists of 19 pairs of chromosomes and this number is equal to the sum of chromosome numbers in its diploid ancestors: *B. campestris* and *B. oleracea*. Chromosomes of *B. napus* are small and similar in shape and size. The lengths of chromosomes range from 1.52 to 3.30 µm. The low morphological diversity enables their classification into three groups: median/metacentric (*m*), submedian/submetacentric (*sm*) and subterminal/subtelocentric (*st*). However, unquestionable determination of individual chromosomes is only possible for a few chromosomes in the karyotype, while the determination of chromosome origin, from A or C genome is practically impossible on the basis of morphometric analysis alone.

The *m* group is composed of ten chromosome pairs (1-10) that are similar in size, while the *sm* group includes five pairs (11-15). The last four pairs of the chromosomes (16-19) belong to the *st* group. Despite the slight differences in chromosome length in this group, pairs 18-19 are easily distinguishable by the presence of the satellites.

## Cytogenetic markers for identification of *B. napus* chromosomes

Figures 1a-b show the same metaphase plate after chromomycin A3 (CMA) and DAPI double staining. The fluorescent banding pattern of *B. napus* chromosomes stained by CMA is generally the reverse of the DAPI pattern. Pericentromeric or satellite regions of some chromosomes are brighter after CMA staining (CMA⁺) and duller after DAPI staining (DAPI⁻). This suggests the presence of G : C rich blocks of chromatin in these parts of the chromosomes. CMA⁻/DAPI⁺ bands were not observed in the *B. napus* karyotype.

Chromosomes in the *m* group display a different pattern of bands after CMA/DAPI staining. Chromosomes 1, 4 and 7 have the prominent CMA⁺/DAPI⁻ band in the pericentromeric region. Chromosome 9 has a fine CMA⁺ band near the centromere. Other pairs of chromosomes do not show any bands. No chromosomes in the *sm* group display any banding pattern. In the *st* group two pairs of the NOR-bearing chromosomes (18, 19) usually have a prominent CMA⁺/DAPI⁻ band that includes the distal part of the short arm and frequently also the satellite region. A very weak CMA⁺ band was sometimes observed in the distal part of the short arm of chromosome 17.
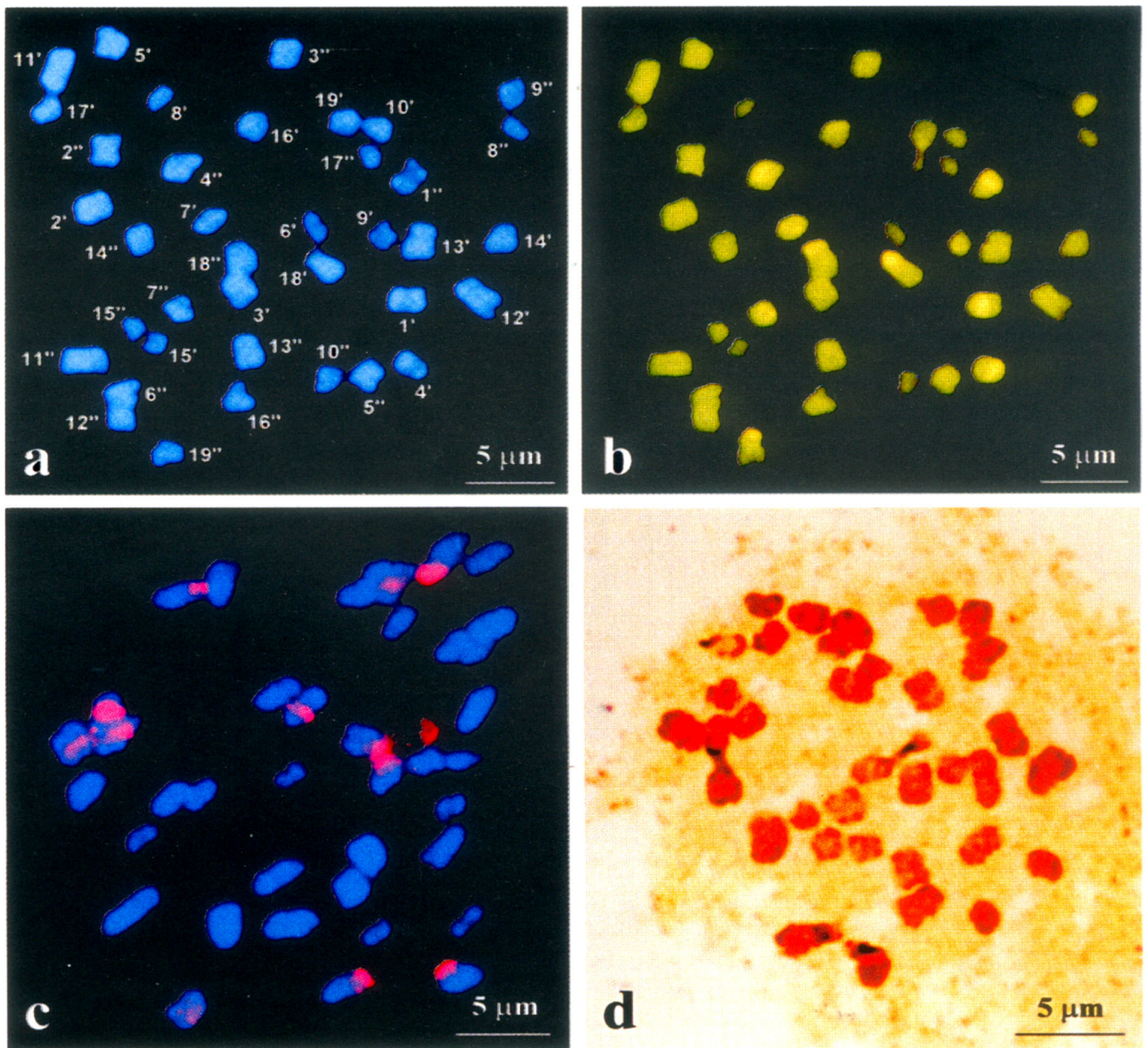
Figure 1. Mitotic chromosomes of *Brassica napus*
(a) DAPI staining – negative bands; (b) CMA staining – positive bands; (c) FISH with 45S rDNA as a probe; rDNA loci – red fluorescence; counterstaining with DAPI – blue fluorescence; (d) silver staining – deep brown stained Ag$^+$ bands (AgNORs) at secondary constrictions
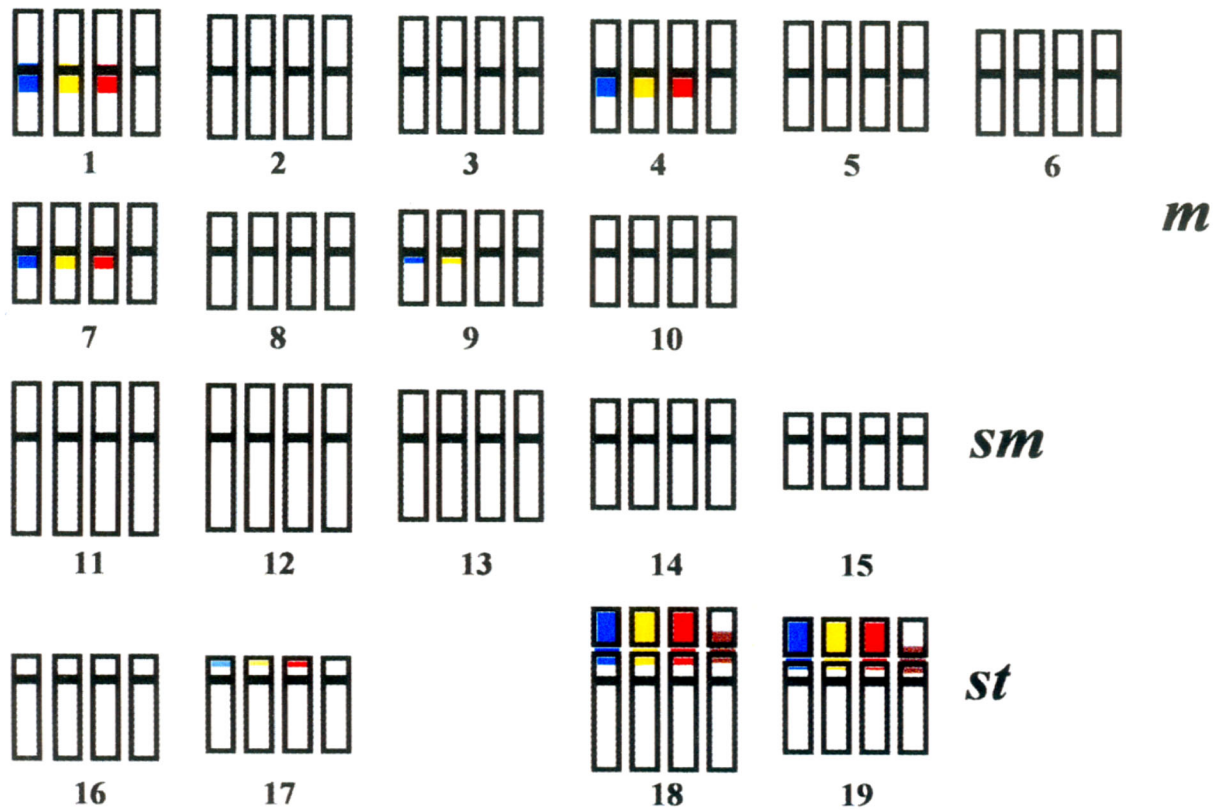
Figure 2. Ideogram of chromosomes of *Brassica napus*

*m* – medial (metacentric), *sm* – submedial (bubmetacentric), *st* – subterminal (subtelocentric) chromosomes. Colours on ideogram represent markers obtained in individual stainings: blue – DAPI band; yellow – CMA$^+$ band; red – 45S rDNA hybridisation signal; brown – Ag$^+$ band

The number and localisation of 18S-5.8S-26S rRNA gene sites was determined by in situ hybridisation with a 45S rDNA probe (Figure 1c). *B. napus* chromosomes carry six pairs of the loci which occupy three different chromosomal positions. The most typical localisation of rRNA genes is in the secondary constriction and satellite region (chromosome pairs 18, 19). One pair of loci is situated at the terminal part of the short arm of chromosome 17. The remaining three pairs are distributed in pericentromeric regions of chromosomes 1, 4 and 7, which all belong to group *m*. Some rDNA sites differ in size and degree of chromatin condensation. The largest hybridisation sites are observed in the secondary constriction and satellite region. The distribution of hybridisation signals is generally the same as the localisation of $CMA^+$/DAPI$^-$ bands. The only exception is chromosome 9, where a signal of hybridisation is not present in the $CMA^+$ band. In the short arm of chromosome 17, at an rDNA site, the $CMA^+$/DAPI$^-$ band is very weak or completely invisible. After silver staining only the loci on the secondary constrictions have $Ag^+$ bands (Figure 1d). Both pericentromeric and terminal rDNA are transcriptionally inactive or below detection level.

Figure 2 shows the ideogram of *B. napus*, which takes into consideration the morphology and size of the chromosomes as well as the localisation of individual bands on the chromosomes. Seven of the nineteen pairs of chromosomes have at least one marker.

## Discussion

Morphometric analysis is a fundamental method of chromosome identification. In some plant species, for instance in *Allium cepa* or *Crepis capillaris*, the determination of chromosome length and centromere position is sufficient for qualification of all chromosome pairs in the karyotype. Chromosomes of *Brassica* species are small and both intra- and intergenomically poorly differentiated. This makes their karyotyping difficult, especially in allotetraploids, where chromosomes can be assigned only to larger groups.

In current studies *B. napus* chromosomes were classified into three groups. These results are similar to those reported recently by SKARZHINSKAYA et al. (1998). However, in earlier studies of late prophase chromosomes OLIN-FATIH and HENEEN (1992) assigned 7, 6, 5 and 1 pair of chromosomes to groups of medial, submedial, subterminal and terminal chromosomes, respectively. These disparities may be due to different staining methods and dissimilar patterns of chromatin condensation between metaphase and late prophase chromosomes investigated by the above authors.

In karyotypes of *B. napus* based on C-banding (OLIN-FATIH, HENEEN 1992, OLIN-FATIH 1994, 1996) and Giemsa staining (SKARZHINSKAYA et al. 1998) only one pair of satellite chromosomes was described. In our study, after in situ hybridisation with rDNA as well as after NOR-specific silver staining, two pairs

of chromosomes with distinct secondary constrictions and satellites were observed in metaphase plates. Similarly, SNOWDON et al. (1997a) found one pair of chromosomes with typical satellites and one pair with NOR-like structures that were somewhat shorter than typical satellites. In some metaphase plates we also observed a lower number of satellites. This may be caused by loss of the satellite regions during chromosome preparation and staining procedure.

In *B. napus* cytogenetic investigations designation of homologous chromosomes is an important part of determination of their genomic origin. In some allotetraploid species genomic in situ hybridisation (GISH) was an efficient tool for discrimination of ancestral genomes and chromosome rearrangements during evolution (KENTON et al. 1993). In the family Brassicaceae application of GISH enabled chromosome identification of R genome in hybrids *Raphanus sativus* × *B. napus* and ancestral genomes in two allotetraploid species: *B. carinata* and *B. juncea*. However, due to a considerable homoeology between A and C genomes, their recognition by GISH in *B. napus* was not successful (SNOWDON 1997b).

Two repetitive, centromeric sequences used for in situ hybridisation with *Brassica* chromosomes also did not permit to distinguish chromosomes of A and C genomes in the *B. napus* complement (HARRISON, HESLOP-HARRISON 1995). These DNA probes do not hybridise with chromosomes of genome B but cross-hybridise with pericentromeric regions of several chromosomes that belong to genomes A and C. Thus these sequences are not useful for determination of the genomic origin of *B. napus* chromosomes.

Another kind of repetitive DNA sequences is ribosomal DNA (18S-5.8S-26S rRNA genes). In somatic cells of *B. napus* these genes are present in twelve loci. It makes them a good marker for six of the 19 pairs of chromosomes in this species. Results obtained in this study are consistent with those recently reported by SNOWDON et al. (1997a) and confirm an earlier observation (MALUSZYNSKA, HESLOP-HARRISON 1993) that the number of rDNA sites in allotetraploid *Brassica* species is one less than the sum of loci in their ancestral species.

Analysis of the distribution of rRNA genes on a chromosome makes it possible to determine the genomic origin of the chromosome. For example, pericentromeric localisation is characteristic for genome A only. Such loci are present in *B. campestris* and its amphidiploids. The chromosomes of *B. napus*, which have terminally distributed rRNA genes originated from genome C; there are no such rDNA sites in genome A. The chromosomes with rRNA genes on the secondary constriction are present both in A and C genomes and are indistinguishable in the *B. napus* chromosome complement.

Fluorescent staining with chromomycin A3 and DAPI used in this study is a very good tool for *Brassica* chromosome investigations. This technique is simple, reliable and enables the analysis of small and uniform chromosomes. Additionally, chromomycin A3 is a fluorochrome, which binds preferentially with G-C rich chromatin. In most plant species ribosomal DNA is rather G-C rich

(SCHWEIZER 1980), so the staining with chromomycin A3 may be used for quick, preliminary detection of rDNA loci. This study shows that the main signals of in situ hybridisation with rDNA co-localise with $CMA^+$ bands, providing additional markers for *B. napus* chromosomes.

In this study we found that *B. napus* has two pairs of chromosomes with active rRNA genes. All of them are located on the secondary constriction. There are few reports about the nucleolar activity in *Brassica* species. So far only diploid species have been analysed using silver staining. In *B. campestris* (CHENG, HENEEN 1995) and *B. oleracea* (CHENG et al. 1995) one pair of rDNA loci showed expression. They are situated in the secondary constriction region. In this study it was found that both *B. campestris* and *B. oleracea* NORs are active in *B. napus* and nucleolar dominance was not observed in this allotetraploid species (HASTEROK and MALUSZYNSKA, unpublished).

It is necessary to introduce new cytogenetic markers, which enable accurate determination of individual chromosome pairs. So far, despite attempts to use RFLP probes for in situ hybridisation (SNOWDON 1997), chromosome-specific markers have not been available for *B. napus* chromosomes.

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