Hereditas 136: 144–150 (2002)

Karyotyping of *Brassica* amphidiploids using 5S and 25S rDNA as chromosome markers

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Kulak, S., Hasterok, R. and Maluszynska, J. 2002. Karyotyping of *Brassica* amphidiploids using 5S and 25S rDNA as chromosome markers. — *Hereditas 136*: 144–150. Lund, Sweden. ISSN 0018-0661. Received November 9, 2001. Accepted March 3, 2002

Species of *Brassica* have small, morphologically similar chromosomes, which makes karyotyping difficult using conventional cytogenetic methods. Molecular cytogenetic methods, like fluorescence in situ hybridisation (FISH) have the potential to improve karyotyping through the use of chromosome- or genome-specific markers. Simultaneous application of more than one DNA probe can greatly enrich the results obtained compared with separate single target FISH experiments. This paper demonstrates the use of multicolour fluorescence in situ hybridisation with 5S and 25S rDNA for karyotyping three amphidiploid species: *B. napus*, *B. juncea* and *B. carinata*. Using this method, it was possible to identify eight out of nineteen pairs of chromosomes in *B. napus*, ten out of eighteen pairs in *B. juncea* and six out of sixteen pairs in *B. carinata*. Additionally, rDNA sites allow the determination of the genomic origin of all marked chromosomes in *B. napus* and *B. juncea*.

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Brassica juncea (brown mustard, $2n = 4x = 36$; genome AABB) and *B. carinata* (Abyssinian mustard, $2n = 4x = 34$; genome BBCC) are less well studied than other species of the genus *Brassica* which are of great agronomical importance as vegetables, oilseed or fodder plants. *B. napus* ($2n = 4x = 38$; genome AACC) has been intensively investigated but identification of particular chromosomes and karyotyping is still very limited. These allotetraploids arose by spontaneous interspecific hybridisation from putative diploid progenitors: *B. campestris* (turnip, $2n = 2x = 20$; genome AA), *B. nigra* (black mustard, $2n = 2x = 16$; genome BB) and *B. oleracea* (cabbage, $2n = 2x = 18$; genome CC) and constitute the classic ''U-triangle'' (U 1935). Recently, *Brassica* species have been the subject of extensive molecular and cytogenetic analyses (SKARZHINSKAYA et al. 1998; OCHS et al. 1999; JACKSON et al. 2000). In cytogenetic studies, one of the most important goals is chromosome identification and karyotype construction. However, the chromosomes of *Brassica* are small, morphologically similar and quite numerous in allotetraploids. Hence their analysis based upon morphometric features only, is extremely difficult and requires additional chromosome- and genome-specific markers.

To date several karyotypes have been published for *Brassica* species. Most of them relate to *B. campestris*, *B. oleracea* and *B. napus*, the species of the greatest agronomical interest. The karyotypes were based on the analyses of mitotic (OLIN-FATIH and HENEEN 1992; CHENG et al. 1995; SNOWDON et al. 1997a; FUKUI et al. 1998; HASTEROK and MALUSZYNSKA 2000a,b) and rarely meiotic (CHENG et al. 1994; MAĆKOWIAK and HENEEN 1999) chromosomes after different staining methods, like Giemsa staining, C-banding, CMA3/DAPI fluorescent staining, silver-staining and fluorescence in situ hybridisation (FISH) with 45S rRNA. Tandem organisation and high copy number of rRNA genes make them suitable markers for chromosome identification and karyotyping in many different plant genera such as *Aegilops* (CASTILHO and HESLOP-HARRISON 1995; BADAEVA et al. 1996), *Arabidopsis* (FRANSZ et al. 1998), *Hordeum* (TAKETA et al. 1999) and *Trifolium* (ANSARI et al. 1999). Recently, simultaneous FISH of 5S and 25S rDNA has been applied for the study of the chromosomes of closely related genera of the Brassicace family *Sinapis*, *Raphanus* and for *Brassica napus* (SCHRADER et al. 2000). The use of the same set of DNA probes in a comparative study of the six ''U-triangle'' species of *Brassica* provided unexpectedly high numbers of chromosome- and genome-specific landmarks showing significant differences in the number and localisation of rDNA loci between three basic *Brassica* genomes (HASTEROK et al. 2001). These differences reflect to some extent the phylogenetic relationships among the diploid *Brassica* species.

The results presented here are a combination of morphometric study and multicolour FISH with 5S and 25S rDNA probes to *B. juncea*, *B. carinata* and

B. napus chromosomes, which allowed karyotype construction for three allopolyploid *Brassica* species.

MATERIALS AND METHODS

Chromosome preparation

Seeds of *B. napus* cv. Leo, *B. juncea* cv. Malopolska (obtained from the plant breeding station Ulhowek, Poland) and *B. carinata* cv. s-67 (Holetta R. Center, Ethiopia) were germinated on moist filter paper in Petri dishes at 20–22°C. Further treatment is according to HASTEROK et al. (2001). Briefly, seedlings with roots, 1–2 cm long, were treated with 2 mM 8-hydroxyquinoline for 1.5–2 h at room temperature (RT), fixed in methanol – glacial acetic acid (3:1) and stored at −20°C until use. Fixed roots were washed in citrate buffer (0.01 M citric acid + 0.01 M sodium citrate, pH 4.8) and digested enzymatically in a mixture of 1 % (w/v) cellulase (Calbiochem), 1 % (w/v) cellulase "Onozuka R-10" (Serva) and 20 % (v/v) pectinase (Sigma) for 1.5–2 h at 37°C. The material was squashed in a drop of 45 % acetic acid. After freezing, the coverslips were removed and the preparations were immediately post-fixed in prechilled ethanol – glacial acetic (3:1), dehydrated in absolute ethanol and air-dried.

DNA probes and fluorescence in situ hybridisation

Labelling of the rDNA probes and fluorescence in situ hybridisation were carried out according to the methods described in HASTEROK et al. (2001). Briefly, a 2.3 kb subclone of the 25S rDNA coding region of *Arabidopsis thaliana* (UNFRIED and GRUENDLER 1990) was labelled with digoxigenin-11-dUTP (Roche) by nick translation following the protocol provided by the kit's manufacturer (Roche). The 5S rDNA from the wheat, clone pTa794 (GERLACH and DYER 1980) was amplified and labelled with rhodamine-4-dUTP using PCR with universal M13 'forward' (5′-CAG GGT TTT CCC AGT CAC GA-3′) and 'reverse' (5′-CGG ATA ACA ATT TCA CAC AGG A-3′) sequencing primers. Conditions for PCR labelling were as follows: 94°C × 1 min, 35 cycles of 94°C × 40 s, 55°C × 40 s, 72°C × 1 min, 1 cycle of 72°C × 5 min.

Chromosome preparations were pretreated with RNase in 2 × SSC (DNase-free, 100 µg/ml, 1 h at 37°C) followed by several washes in 2 × SSC and post-fixation in 4 % aqueous formaldehyde solution. The hybridisation mixture consisted of 50 % deionised formamide, 10 % dextran sulphate, 2 × SSC and 0.5 % SDS, probe DNA was mixed to a concentration of 100–130 ng per slide. The hybridisation mixture was denatured at 75°C for 10 min, the slides were denatured together at 70°C for 5 min and the probes hybridised overnight in a humid chamber at 37°C. The slides were washed stringently in 20 % deionised formamide in 0.1 × SSC, and the immunodetection of digoxigenated DNA probe was carried out by FITC-conjugated anti-digoxigenin antibodies (Roche). Finally, preparations were mounted in 2 µg/ml DAPI (Serva) in Vectashield (Vector Laboratories). Images were acquired with a Hamamatsu CCD camera attached to an Olympus Provis epifluorescence microscope then processed uniformly and superimposed using Micrografx Picture Publisher software.

Karyotyping

The lengths of chromosomes and arm length ratios were measured on several metaphase plates after fluorescence in situ hybridisation with 5S and 25S rDNA and DAPI counterstaining. The chromosome nomenclature of LEVAN et al. (1964) was adopted with slight modifications. According to the arm length ratio (q/p) all chromosomes were identified as either median (q/p = 1.0–1.7) or submedian (q/p = 1.71–3.0). Nucleolar organising chromosomes were separated from the rest and presented, regardless their length, at the end of the karyotype. For the purpose of chromosome measurement, the length of satellites was excluded. The karyotypes presented are based on the chromosomes extracted from the original images presented in the respective figures.

RESULTS AND DISCUSSION

Both 5S and 25S rDNA probes hybridised to numerous chromosomes of the complements of the allotetraploid species of *Brassica*, allowing genome- and in some cases also chromosome-specific markers especially for *B. juncea* and *B. napus* chromosomes to be identified. These markers are based on eight different rDNA-bearing chromosomal types, which are described in detail by HASTEROK et al. (2001).

Brassica juncea karyotype (AABB)

The mitotic complement comprises 36 chromosomes (Fig. 1a) which is equal to the sum of the chromosome numbers in its putative ancestors. The lengths of chromosomes range from 1.38 to 3.25 µm. As in the previous study carried on diploid *Brassica* species (HASTEROK and MALUSZYNSKA 2000b) and *B. napus* (HASTEROK and MALUSZYNSKA 2000a), morphometric analysis based on the comparison of the length of chromosomes and the position of the centromere allows only rough classification of chromosomes into median (Nr 1–6) and submedian (Nr 7–15) groups (Fig. 2a). The only chromosomes of the complement

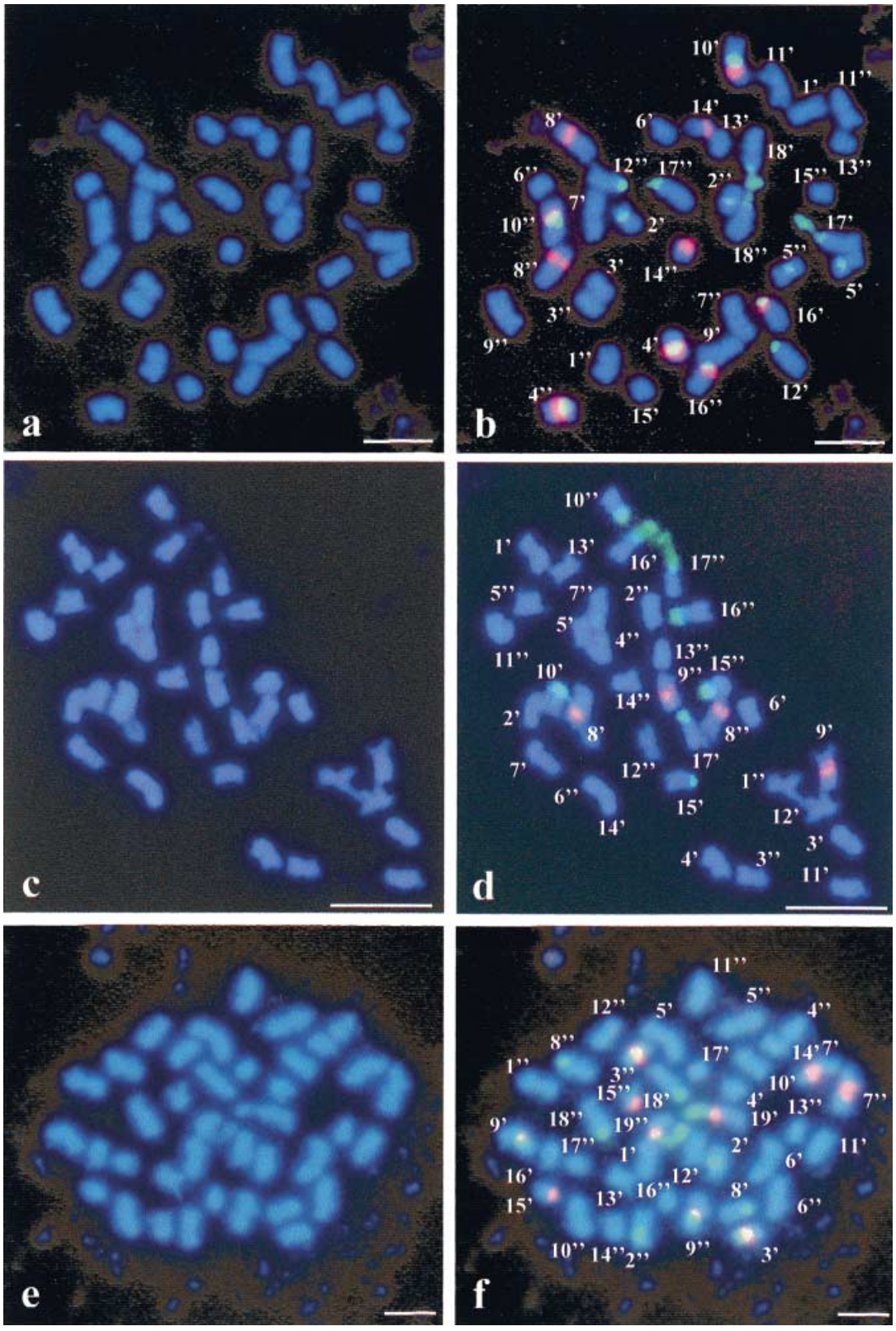

Fig. 1. Somatic metaphase chromosomes of *B. juncea* (**a–b**), *B. carinata* (**c–d**) and *B. napus* (**e–f**). (**a, c, e**) DAPI staining, (**b, d, f**) double-target fluorescence in situ hybridisation of 5S rDNA (red) and 25S rDNA (green) probes. Bars represent 5 μm.

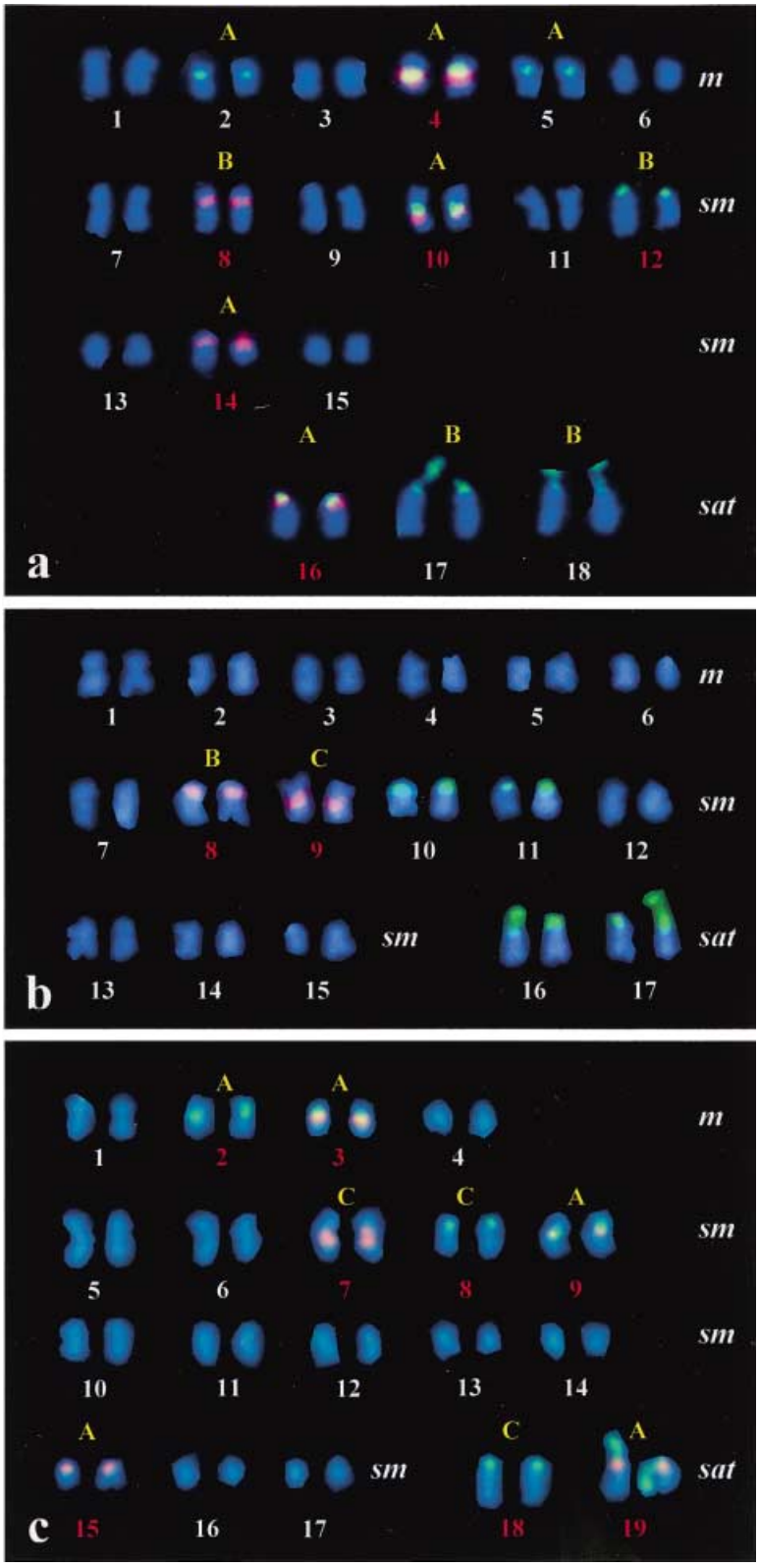

Fig. 2. Ideograms of *B. juncea* (**a**), *B. carinata* (**b**) and *B. napus* (**c**) somatic chromosomes based on the metaphase plates shown in Fig. 1. Yellow upper case letters in the ideograms signify the genomic origin of tagged chromosomes. White numbers show the general numbering of chromosomes, which are arranged in descending order according to total length. Red numbers indicate those chromosomes, which possess unique features within the whole karyotype and therefore can be unambiguously identified. Symbols: *m* (median), *sm* (submedian) and *sat* (satellite) indicate morphological groups of the chromosomes.

that can be easily recognised, but not confirmed as strictly homologous, are the NOR-bearing chromosomes with usually prominent secondary constrictions in the short arm (Nr 17–18). Relatively large variation in chromosome size and morphology observed in this species is due to the chromosomes derived from genome A, which is the most asymmetric among the three genomes investigated.

Figure 1b shows the results of double-target FISH of 5S and 25S rDNA probes to the same mitotic chromosomes that are shown in Fig. 1a. Ten sites of 5S rDNA (red fluorescence) and sixteen sites of 25S rDNA (green fluorescence) tag 20 out of 36 chromosomes. Morphometric characteristics and the rDNA markers are sufficient to identify some chromosomes and are therefore chromosome-specific (Nr 4, 8, 10, 12, 14, 16).

Chromosomes 4, 10 and 16 have co-localised 5S and 25S rRNA genes, which among the "U-triangle" *Brassica* species is an exclusive feature of the A genome (HASTEROK et al. 2001). Similar juxtaposition of rRNA genes has also been found in some related species, e.g. *Raphanus sativus* and *Sinapis alba* (SCHRADER et al. 2000). The position of rDNA sites in all tagged chromosomes is genome-specific allowing unequivocal determination of the genomic origin of these chromosomes (Fig. 2a).

Chromosome Nr 16 is particularly interesting. It is the NOR-bearing chromosome (type I) but unlike its counterpart in *B. napus* (Nr 19; Fig. 2c) it consistently fails to show a distinct secondary constriction/satellite region. In other cultivars of *B. juncea* we have observed this chromosome with a normal secondary constriction, while the corresponding regions of chromosomes 17 and 18 were significantly contracted (unpubl.). The striking consistency of this phenomenon suggests that these differences cannot be caused simply by the fact that secondary constrictions are naturally "fragile" and that satellites might sometimes be lost during preparation. Rather, they may reflect differences in the activity of rRNA genes between individual loci (FLAVELL 1986).

Brassica carinata karyotype (BBCC)

The chromosomes are inordinately uniform both in size (length 1.56–2.40 μm) and morphology (Fig. 1c). These features preclude chromosome identification on the basis of morphometric analysis alone. According to centromere position only two groups of chromosomes can be discriminated: chromosomes Nr 1–6 are median, while chromosomes 7–15 are submedian (Fig. 2b). Two pairs of satellite chromosomes (Nr 16–17) with distinct secondary constrictions are observed in this karyotype.

Fluorescence in situ hybridisation with 5S and 25S rDNA to mitotic chromosomes (Fig. 1d) revealed the presence of four 5S rDNA sites (red fluorescence). Usually, only eight sites of 25S rDNA (green fluorescence) were observed, which is two sites less than expected on the basis of the number of 25S rDNA sites in the respective ancestral species. None of chromosomes in the complement of *B. carinata* carries both 5S and 25S rRNA genes, closely linked or colocalised on the same chromosome. In contrast to *B. juncea*, rDNA landmarks in *B. carinata* chromosome pairs (10–11 and 16–17) are indistinguishable from each other (Fig. 2b), which is the consequence of great similarity between the B and C genomes and rather similar chromosomal distribution of rDNA landmarks. The only exceptions are chromosome pairs 8 and 9 from genomes B and C, respectively, which usually can be discriminated according to slight differences in size and localisation of their 5S rDNA sites.

In some *B. carinata* plants, nine 25S rDNA sites were observed, although plants carrying eight sites constituted the vast majority in the population. SNOWDON (1997) reported the presence of ten sites for this species. This proves some degree of polymorphism in the number of 45S rDNA loci not only between different cultivars but also among individuals of the same cultivar. Variation in the number of rDNA loci among individuals of the same cultivar was also observed in other plant species, for example in rye, where 5S rDNA sites were always present in chromosome 1R and 5R and only occasionally in chromosome 3R (CUADRADO and JOUVE 1997).

Brassica napus karyotype (AACC)

Karyotypes for this species have already been published on the basis of different chromosome staining methods, e.g. C-banding (OLIN-FATIH and HENEEN 1992), FISH with 45S rDNA (HASTEROK and MALUSZYNSKA 2000a) and computer image analysis (FUKUI et al. 1998). The length of chromosomes ranges between 1.5–3.3 μm (Fig. 1e). Morphometric analysis identifies three groups of chromosomes (Fig. 2c). Chromosomes Nr 1–4 are median, 5–17 submedian and the last two (Nr 18 and 19) are satellited.

Double in situ hybridisation with 25S and 5S rDNA provides additional landmarks (Fig. 1f). Eight pairs of chromosomes carry rRNA gene clusters. Three pairs of chromosomes possess both types of rDNA (Nr 3, 9, 19) and belong to genome A. Three other pairs of chromosomes (Nr 7, 8, 18) carrying a single locus of either 5S or 25S rDNA belong to the C genome, while chromosome pair 2 with a pericentromeric site of 25S rDNA and chromosome pair 15 with a small terminal site of 5S rDNA belong to the

A genome (Fig. 2c). As in *B. juncea*, the NOR-bearing chromosome Nr 18 from genome C does not frequently exhibit a secondary constriction whilst another NOR-bearing chromosome (Nr 19; genome A) has always a distended secondary constriction.

Interestingly, in this cultivar of *B. napus* both kinds of rDNA sites were found in only eight pairs of chromosomes, which is one pair less than was observed in another cultivar (HASTEROK et al. 2001) and two pairs less than was reported by SNOWDON et al. (2000). Intraspecific variation in the number and chromosomal localisation of rDNA is also apparent in some diploid species of *Brassica*. For instance, MALUSZYNSKA and HESLOP-HARRISON (1993) observed rDNA sites in two pairs of chromosomes in *B. nigra* and three pairs in *B. oleracea*, while subsequent studies revealed the presence of three and two pairs of these loci respectively (FUKUI et al. 1998; HASTEROK and MALUSZYNSKA 2000c; HASTEROK et al. 2001). Intraspecific variation in the number and chromosomal location of ribosomal genes has been observed in many other plant species, for example in cotton (HANSON et al. 1996) and rice (SHISHIDO et al. 2000). This might be the result of different chromosomal rearrangements, especially in species with numerous rDNA loci.

The analysis of rDNA localization on chromosomes of diploid *Brassica* species discriminates 8 types of chromosomes, which are confined to particular genomes A, B or C (HASTEROK et al. 2001). This allows the identification of at least some chromosomes belonging to the two different genomes in amphidiploid species. Usually, the more efficient and accurate technique for distinguishing genome components of allopolyploids or interspecific/intergeneric hybrids is genomic in situ hybridisation (GISH) (PAROKONNY et al. 1992; MUKAI et al. 1993a; MOLNÁR-LÁNG et al. 2000). This technique has also been applied successfully to assay the presence of alien chromatin in interspecific or intergeneric hybrids mainly in Triticeae (MUKAI et al. 1993b; MORAIS-CECÍLIO et al. 1997). There are some problems with the application of GISH to *Brassica* species (SNOWDON et al. 1997b). Using total genomic DNA it was possible to distinguish all chromosomes of the A and B genomes in *B. juncea* and the B and C genomes in *B. carinata* but not the A and C genomes in *B. napus*. The close similarity between the A and C genomes has also been shown using molecular markers (PARKIN et al. 1995) and in situ hybridisation with repetitive centromeric sequences (HARRISON and HESLOP-HARRISON 1995). Therefore it is desirable to seek additional genome- as well as chromosome-specific DNA sequences, which might allow the identification of more or indeed all the chromosomes of these genomes.

ACKNOWLEDGEMENTS

The authors thank Dr. Glyn Jenkins (University of Wales Aberystwyth, UK) for his valuable comments on the manuscript.

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Erratum

Kulak, S., Hasterok, R. and Maluszynska, J. 2002. Karyotyping of *Brassica* amphidiploids using 5S and 25S rDNA as chromosme markers.—*Hereditas 136*: 144–150. Lund, Sweden. ISSN 0018-0661. Received November 9, 2001. Accepted March 3, 2002.

In the article, Figure 1 on page 146 is incorrect and should be replaced with the Figure 1 on the following page.

Ed.

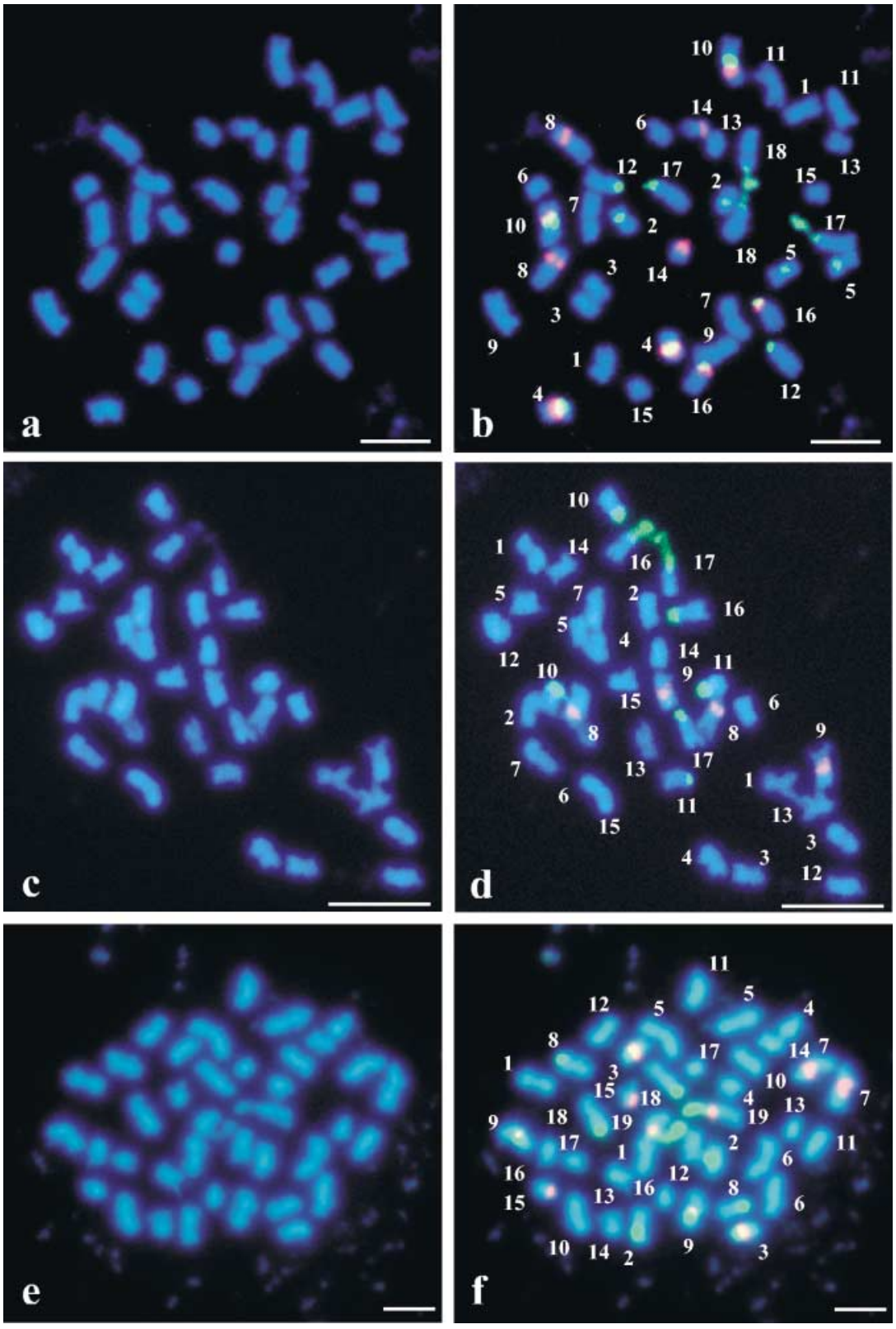

Fig. 1. Somatic metaphase chromosomes of *B. juncea* (**a–b**), *B. carinata* (**c–d**), and *B. napus* (**e–f**). (**a, c, e**) DAPI staining, (**b, d, f**) double-target fluorescence in situ hybridisation of 5S rDNA (red) and 25S rDNA (green) probes. Bars represent 5 μm.