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C. J. Schelfhout · R. Snowdon · W. A. Cowling · J. M. Wroth

A PCR based B-genome-specific marker in *Brassica* species

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Abstract Previous hybridisation studies showed that the repetitive DNA sequence pBNBH35 from *Brassica nigra* (genome BB, 2*n*=16) bound specifically to the B-genome and not to the A- or C-genomes of *Brassica* species. We amplified a sub-fragment of pBNBH35 from *B. nigra* by PCR, cloned and sequenced this sub-fragment, and confirmed that it was a 329-bp sub-fragment of pBNBH35. PCR and hybridisation techniques were used to confirm that the pBNBH35 sub-fragment was *Brassica* B-genome-specific. Fluorescence in situ hybridisation (FISH) in *B. nigra*, *B. juncea* (AABB, 2*n*=36) and *B. napus* (AACC, 2*n*=38) showed that the pBNBH35 sub-fragment was present on all eight *Brassica* B-genome chromosomes and absent from the A- and C-genome chromosomes. The pBNBH35 repeat was localised to the centromeric region of each B-genome chromosome. FISH clearly distinguished the B-genome chromosomes from the A-genome chromosomes in the amphidiploid species *B. juncea*. This is the first known report of a B-genome repetitive marker that is present on all B-genome chromosomes. It will be a useful tool for the detection of B chromosomes in interspecific hybrids and may prove useful for phylogenetic studies in *Brassica* species.

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C. J. Schelfhout (✉) · W. A. Cowling · J. M. Wroth
School of Plant Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia,
35 Stirling Hwy,
Crawley, WA, 6009, Australia
e-mail: cschelfh@agric.uwa.edu.au
Tel.: +61-8-64882560
Fax: +61-8-64881108

R. Snowdon
Institut für Pflanzenbau und Pflanzenzüchtung I, Justus Liebig Universitat,
Heinrich-Buff-Ring 26-32,
35392 Giessen, Germany

W. A. Cowling
Canola Breeders Western Australia Proprietary Limited,
15/219 Canning Highway,
South Perth, WA, 6151, Australia

Introduction

Large regions of eukaryote genomes are characterised by repetitive DNA sequences (Flavell 1980; Jelinek and Schmid 1982) which generally appear in one of several different forms—tandemly repeated sequences, retro-elements and other unique classes such as telomeric sequences or rDNA units (Heslop-Harrison 2000). These repetitive DNA sequences have been useful as markers in phylogenetic studies (Halldén et al. 1987) as repetitive sequence motifs tend to be highly conserved within species but vary across species (Heslop-Harrison 2000). Species-specific sequences have been identified in *Brassica* species, and homology often exists between A- and C-genome repetitive sequences while there are fewer similarities between B-genome and A- or C-genome sequences (Hosaka et al. 1990; Chèvre et al. 1991). This is consistent with the results of genomic studies of *Brassica* species that have identified close relationships between the A- and C-genomes and more distant associations with the B-genome (Song et al. 1988; Quiros et al. 1991; Truco et al. 1996).

Prior to this communication no *Brassica* B-genome specific sequences had been shown to be present on all eight B-genome chromosomes. Gupta et al. (1992) cloned two *B. nigra* genome-specific sequences, one of which—pBNBH35—proved to be highly B-genome specific. Southern hybridisation analysis showed that this 496-bp sequence hybridised to all species carrying the *Brassica* B-genome (Gupta et al. 1992). Southern hybridisation analysis suggested that pBNBH35 is a highly dispersed sequence. Kapila et al. (1996) showed that pBNBH35 was present on five monosomic addition lines of *B. nigra* in a *B. napus* background (Chèvre et al. 1991), however they were unable to identify this sequence on all eight *B. nigra* chromosomes, and there have been no further studies reported.

The objective of the investigation reported here was to convert pBNBH35 into a PCR-based marker and confirm its specificity to all eight chromosomes of the *Brassica* B-genome using PCR, DNA hybridisation and in situ

hybridisation techniques. The potential use of this sequence as a B-genome-specific marker is discussed.

Materials and methods

Plant material

The plant material used in this study is shown in Table 1. Seeds were sown in pots containing a standard potting mix and slow-release fertiliser and were propagated in an air-conditioned glasshouse at 20–25°C. At the six-leaf stage, the youngest two leaves from each plant were collected, snap frozen in liquid nitrogen and stored at −80°C.

Total genomic DNA extraction and quantification

Sufficient leaf tissue to half-fill a 1.5-ml tube was removed from −80°C storage and macerated in 600 µl of DNA extraction buffer (10 g l^{-1} *N*-lauroyl-sarcosine, 3.2 g l^{-1} EDTA, 12.1 g l^{-1} Trizma base, 12.6 g l^{-1} sodium sulfite, 5.8 g l^{-1} sodium chloride, pH 8.5). The tubes were kept on ice for 10 min, after which 600 µl of phenol: chloroform:iso-amyl alcohol (25:24:1) was added. The contents were mixed for 1 min and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a fresh tube along with 40 µl of 3 *M* sodium acetate and 250 µl isopropanol, and the tubes were inverted to encourage precipitation. After 10 min at room temperature the tubes were centrifuged at 13,000 rpm for 10 min. The supernatant was discarded and the DNA pellet washed with 500 µl 70% ethanol, then centrifuged at 13,000 rpm for 5 min. Excess ethanol was decanted and the DNA pellet gently vacuum-dried for 10 min. DNA was resuspended in 100 µl of R40 (2 µl RNase A in 1 ml TE buffer). Total genomic DNA concentration was estimated against a DNA mass ladder (Gibco, Gaithersburg, Md.) by electrophoresis on a 1.5% agarose gel followed by ethidium bromide staining and UV light visualisation.

PCR assay

Primers were designed to amplify a sub-fragment of the pBNBH35 sequence published by Gupta et al. (1992) (Fig. 1). A PCR protocol was optimised to amplify this sequence in species containing the Brassica B-genome. Template DNA (1 µl of 100 ng/µl) from each of the respective *Brassica* species (Table 1) was added to 24 µl of master mix [2.5 µl 10× reaction buffer (Sigma, St. Louis, Mo.), 4.0 µl 1.25 m*M* dNTPs (Promega, Madison, Wis.), 1 µl 10 m*M* Primer 1 (Geneworks), 1 µl 10 m*M* Primer 2 (Geneworks), 1 µl REDTaq polymerase (Sigma) and 15.3 µl MilliQ water]. The PCR analyses were run on the following program in a Hybaid thermocycler: one cycle of 4 min at 94°C; 30 cycles of 1 min at 94°C, 2 min at 60°C, 2 min at 72°C; a final hold at 4°C.

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                                                          >
  1  GATCCTGCAC CATACCTATT GAGCTCACTG TTTTGAAGAT GGCATCTGAA
 51  GAGAGAGTCC CTTTGATCCT TGGCACTCCT TTCCTCACAA CAGTTGGAGC
101  TTGCATTGAC TTTCCCAACA AGAAGGTCAC CCTCATGCAT GCCAACAAAG
151  CTGTCTCCTA TCCTTTCCAA CCTCCAATGG AATCAGACTA TTGTGGGACA
201  ATCACTCGTG ATTCACAAGT TGTTGAGAAG CCCCAAGATG AAGAGGTTGT
251  TGGTAAGAAA GAAGGTCTTG ATGGAGAGTC CTCTAAATAG AAGTGTGGTG
301  AGAACTTGGA AATGCTAAAA AGGAGGGGAT GAGTGGAGTC TCAAAGGCCA
                                  <
351  CTCATGGCAA GAAGAAGATG GTGAAAGAGC CTCATCCTCC ACCTCTTGAT
401  TTGCTCCCAC ACACTCTCAC TCTCCATCCA AAGAATCTCA AGGATGGTCA
451  TTGAGTACAA GATCAAGCAC AAGGTCAGAT CCACACCATT CTCAAG
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Fig. 1 pBNBH35, a 496-bp B-genome-specific sequence published by Gupta et al. (1992) with primers designed (*bold*) to amplify a 329-bp sub-fragment

Sequence analysis

The size of the PCR products was determined by electrophoresis on a 1.5% agarose gel, ethidium bromide staining and visualisation under UV light. The PCR products were isolated from the gel and cleaned on PCR cleanup columns (Roche, Indianapolis, Ind.). Cleaned samples were again checked for size, then ligated into a pGEM T-Easy (Promega) plasmid vector and transformed into *Escherichia coli*-competent cells (JB 109, Promega) before overnight cultivation in LB media at 32°C. A sample of the clone containing the pBNBH35 sub-fragment was stored at −80°C in glycerol stocks. Sequence analysis was conducted on two independently cloned PCR products isolated from *B. nigra*. Sequencing was performed using the BigDye terminator protocol (Applied Biosystems, Foster City, Calif.) and sequence comparisons made using Vector NTI software. Homology of the amplified sub-fragment of pBNBH35 to published DNA sequences was examined using a BLASTN database query (Web Angis-BioManager website: http://biomanager.angis.org.au/) on the Genebank main and EST databases.

Specificity of PCR product

To check the B-genome specificity of pBNBH35, DNA was extracted from each *Brassica* species in U's triangle (U 1935) (Table 1) and used as template DNA in the PCR-based assay described above. The cleaned PCR products (10 µl) were screened on a 1.5% agarose gel as described above. Fragment size was estimated against a 100-bp ladder (Promega).

Slot blot hybridisation assay

Genomic DNA (20 ng) from *B. juncea* and *B. napus* was denatured in an equal volume of 0.4 *N* sodium hydroxide and blotted onto Hybond N+ nylon membrane that had been pre-soaked in 2× SSC

Table 1 *Brassica* species used in this study

Brassica species	Variety name	Genome description
B. rapa	Pak Choy (Chinese cabbage)	2*n*=20 (AA)
B. nigra	Black mustard (ATC 90745)	2*n*=16 (BB)
B. oleracea	Cabbage	2*n*=18 (CC)
B. napus	Canola cv. Mystic	2*n*=38 (AACC)
B. juncea	Indian mustard (line JN29)	2*n*=36 (AABB)
B. carinata	Ethiopian mustard	2*n*=34 (BBCC)

(0.3 *M* NaCl, 0.03 *M* Na Citrate) using a Bio-Rad (Hercules, Calif.) slot blot apparatus. Following blotting, the membrane was rinsed in 2× SSC, blotted again and dried at 60°C for 30 min. Membranes were pre-hybridised for 3–4 h at 65°C in the pre-hybridisation solution [per 30-ml tube: 0.3 g dextran sulphate, 17.55 ml sterile water, 6 ml of 20× SETS (3 *M* NaCl, 20 m*M* EDTA, 0.6 *M* Tris-HCl, 11 m*M* tetra-sodium pyrophosphate, pH 8), 6 ml 50× Denhardt's solution and 3 ml of 10% sodium dodecyl sulphate (SDS)]. The pBNBH35 sub-fragment (100 ng) was used as a probe in the hybridisation. It was prepared by denaturation for 10 min at 100°C with 1.2 µl random primers (50 µg ml^{-1}) and immediate quenching on ice. This solution was then combined with 3.2 µl sterile water, 2.0 µl oligo buffer (Promega), 0.6 µl Klenow (Promega) at 5 U μl^{-1} and 3 µl [^{32} P]-dCTP (20 MBq) and incubated at 37°C for 2 h. Following the incubation period 30 µl 0.8 *M* EDTA was added to stop the labelling reaction, and the contents of the tube were briefly centrifuged. The probe solution was then centrifuged for 10 min at 10,000 rpm in a sephadex spin column to remove unincorporated nucleotides. The pre-hybridisation solution was replaced with the hybridisation solution (per tube: 0.5 g dextran sulphate, 2.7 ml sterile water, 1 ml SETS, 1 ml 50× Denhardt's and 50 µl 10% SDS). The probe solution was added and the membrane hybridised overnight at 65°C. The following day the hybridisation solution was discarded, and the membrane was given two 15-min washes in 2× SSC. Membranes were exposed to film (Fuji medical X-ray) for approximately 4 days at −80°C and developed in an auto-developer (All-pro imaging). The slot blot matrix was crossed-checked for the presence of bands.

Fluorescence in situ hybridisation (FISH) assay

Binding of the amplified sub-fragment (pBNBH35-sub) to chromosomes within the *Brassica* complex was examined by FISH in order to confirm its B-genome specificity and distribution within B-genome chromosomes of *B. juncea*.

Four-day-old root tips were obtained from *Brassica* species (Table 1) for observation of mitotic chromosomes. Seeds were germinated on canola germination media [MS salts + B5 vitamins, 3% (w/v) sucrose, 0.8% purified agar and myo-inositol (100 mg l^{-1}] at 25°C for 3 days and moved to 4°C for 24 h prior to the excision of 1-cm-long root tips. To accumulate metaphases we placed the excised root tips in 2 m*M* 8-hydroxyquinoline for 2 h at room temperature on a shaker and then transferred them to 4°C for 2 h without shaking. The root tips were blotted dry on filter paper and transferred to Farmer's fixative (3:1, ethanol:acetic acid). After 24 h, the root tips were transferred to fresh Farmer's fixative and stored at −20°C. Root tips were transferred to 100% ethanol for long-term storage at ambient temperatures.

To make chromosome preparations we removed the root tips from the ethanol and washed them briefly in sterile distilled water. Fifteen root tips were selected from each *Brassica* species (Table 1). One-millimeter segments of the root tip were excised and transferred to 50 µl enzyme solution consisting of 2% (w/v) cellulase (Calbiochem) and 20% (v/v) pectinase (Sigma) dissolved in enzyme buffer (40 m*M* citric acid, 60 m*M* tri-sodium citrate, pH 4.8; filter-sterilised through a 0.2-µm filter). The material was digested for 2 h at 37°C. The enzyme solution was then replaced with 50 µl hypotonic solution (75 m*M* KCl), renewed once and left at room temperature for 40 min. The hypotonic solution was replaced with 50 µl 60% acetic acid, renewed once and left for 25 min at room temperature, with gentle mixing every few minutes. The second acetic acid solution was replaced with 50 µl Farmer's fixative and renewed once. The root tips were gently sheared with a pipette to separate cells, and 15–20 µl of the cell suspension was loaded onto pre-cleaned cold microscope slides. We immediately added 30 µl of Farmer's fixative, and the slides were allowed to air dry slowly. The slides were viewed by phase-contrast microscopy to identify suitable metaphase stage cells for hybridisation.

For preparation of the FISH probe the pBNBH35 sub-sequence was directly labelled with the fluorochrome Cy3-11-dUTP (Amersham Life Science, UK) via PCR amplification. Unincorporated fluorochrome was removed using a PCR cleanup column (Qiagen, Valencia, Calif.), and the labelled probe was eluted at a concentration of approximately 10 ng μl^{-1} in a hybridisation solution containing 50% formamide, 2× SSC and 10% dextran sulphate.

Approximately 15 µl of probe solution was applied to the area of the slide to be hybridised, covered with a 24×24-mm cover slip and sealed with rubber cement. Denaturation was achieved by incubation at 80°C for 4 min on a heated metal plate. The slides were immediately transferred to 37°C for overnight hybridisation. The following day the rubber seal and cover slip were carefully removed and the slides washed at 42°C for 5 min in 2× SSC followed by two washes for 5 min in 0.2× SSC and a final wash of 5 min in 2× SSC. The slides were stored in 4× SSC containing 0.5% Tween prior to staining.

DAPI-Antifade (20 µl) (Appligene-Oncor) was added to each slide, covered with a cover slip, left for 2 min, then squashed under a paper towel to remove excess stain. Slides were stored in the dark until viewing. They were viewed under a Leica DM-R fluorescence microscope with a single-bypass and photographed with a Cohu 4912 uncooled CCD camera. Individual images were merged using the Leica Q-FISH software. No image manipulation was necessary.

Results

Sequence analysis of the two independently cloned sub-fragments of pBNBH35, amplified by PCR from *B. nigra*, confirmed their homology with the sequence published by Gupta et al. (1992) (Fig. 1). When aligned using VECTOR NTI software both of the analysed sequences showed 92% homology with pBNBH35. The BLASTN programme indicated no homology greater than 10% with any other sequence in the Genebank main and EST databases. The PCR product showed B-genome specificity

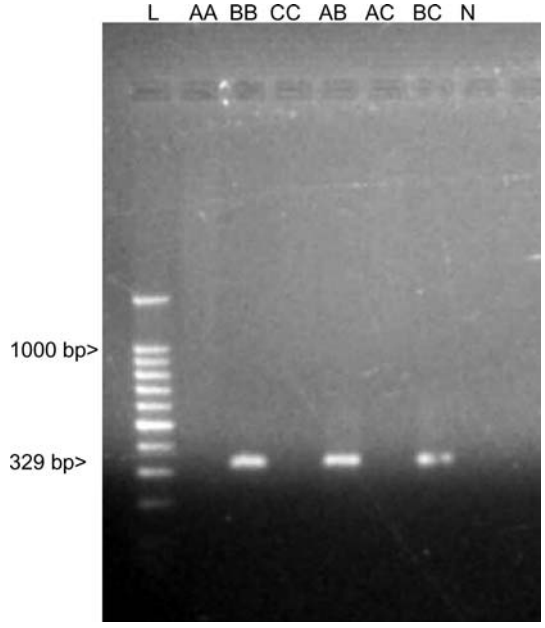

Fig. 2 PCR based assay of six *Brassica* species with different genome compositions. L 100-bp ladder, AA *Brassica rapa*, BB *B. nigra*, CC *B. oleracea*, AB *B. juncea*, AC *B. napus*, BC *B. carinata*, N negative control

when amplified from several *Brassica* species from U's triangle (U 1935) (Fig. 2).

The pBNBH35 sub-fragment was amplified only from those samples where the template DNA contained the *Brassica* B-genome (*B. nigra*, *B. juncea* and *B. carinata*) (Fig. 2). A single band, equivalent to the expected size of 329 bp from pBNBH35 (Fig. 1), was generated in each *Brassica* species with the B-genome (Fig. 2).

Slot blot hybridisation of the pBNBH35 sub-fragment gave positive signals with *B. juncea* (AABB) but not with *B. napus* (AACC) (data not shown).

Results from in situ hybridisation with the pBNBH35-sub-fragment are shown in Figs. 3 and 4. The molecular cytogenetic results supported the results of the PCR assay and the Southern hybridisations. Strong FISH signals from the pBNBH35 sub-fragment were observed on *B. nigra* chromosomes, whereas no signals were seen in *B. napus*. Strong hybridisation signals were also observed on 16 chromosomes in *B. juncea* (2*n*=36), presumably corresponding to the 16 B-genome chromosomes. No pBNBH35 sub-fragment signals were observed in the remaining 20 chromosomes of *B. juncea*.

The pBNBH35 repeat was localised in large blocks surrounding the centromeres of all B-genome chromosomes, with a gap often visible at the centromere (Fig. 3). Signals did not extend to the telomeric regions.

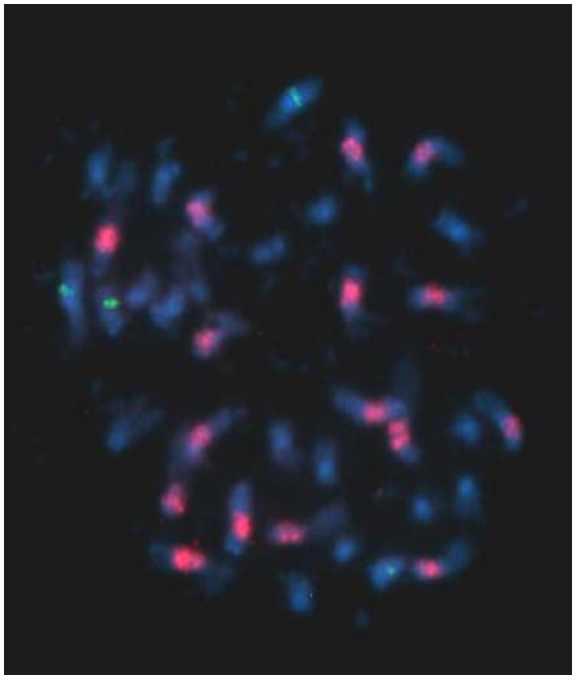

Fig. 3 Fluorescence in situ hybridisation to *B. juncea* mitotic metaphase chromosomes with Cy3-labelled B-genome-specific sequence pBNBH35 (*red*). *Green* signals show a FITC-labelled A-genome-specific repeat sequence (Snowdon, unpublished results) that hybridises to four A-genome chromosomes in *B. juncea*. Chromosomes are counterstained with the *blue* fluorescent dye DAPI

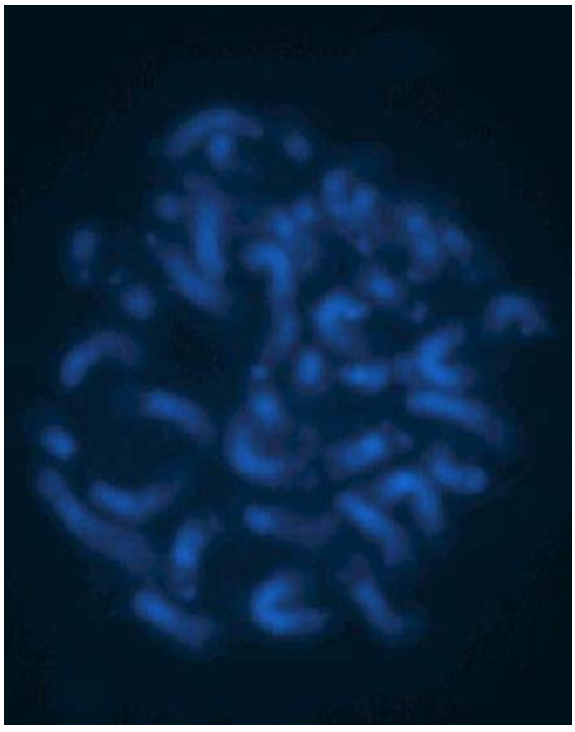

Fig. 4 *B. napus* probed with Cy3-labelled, B-genome-specific sequence. pBNBH35: no *red* labels

Discussion

FISH has become the method of choice for analysis of the chromosomal distribution of repetitive DNA sequence elements in plant genomes (Heslop-Harrison 2000). In the investigation described here, FISH was used to investigate the previously unknown distribution of a *Brassica* B-genome repeat sequence on B-genome chromosomes. We confirmed that pBNBH35 is a repetitive sequence with B-genome specificity, as indicated by Gupta et al. (1992), and we converted the sequence into a PCR product that is amplified only in species containing the *Brassica* B-genome. The FISH assay showed that this sequence is localised in high-copy number on either side of the centromeres of all eight *Brassica* B-genome chromosomes and appears with low frequency or is absent in the interstitial and telomeric regions. This is the first report confirming a B-genome-specific marker that is distributed across all B-genome chromosomes.

The PCR sub-fragment from pBNBH35 resolved as a unique single band on agarose gel at the expected size of 329 bp, and two independently cloned products from this band produced identical sequences with the predicted size of 329 bp. The pBNBH35 sub-fragment has the properties of a highly conserved dispersed tandem repeat sequence, and there was no evidence for sequence homology with any known retro-element. Strong signals surrounding the centromeres of the chromosomes indicate high-copy numbers in pericentromeric heterochromatin, however there are virtually no signals towards the telomeric regions. Such a distribution, frequently observed in genomic in situ hybridisation (GISH) with *Brassica*

hybrids (Fahleson et al. 1997; Skarzhinskaya et al. 1998; Snowdon et al. 2000), reflects the generally low number of dispersed repeat sequences in the interstitial and telomeric chromosome regions of *Brassica* and related genera (Heslop-Harrison and Schwarzacher 1996).

A variety of applications can be envisaged for this PCR-based, B-genome-specific marker. The marker could be used for detecting the B-genome in the progeny of wide interspecific and intergeneric crosses where this has not been possible for studies in the past (Roy 1984; Prakash and Chopra 1988; Chèvre et al. 1991; Rao et al. 1993) where it may be present in whole chromosomes (addition lines) or introgressions. This marker would complement existing randomly amplified polymorphic DNA markers where linkage to B-genome alleles have been useful in aiding the selection of beneficial traits in *Brassica* wide crosses (Chèvre et al. 1997). Previous work has demonstrated the utility of GISH to distinguish *Brassica* B-genome chromosomes from those of the A- and C-genomes (Snowdon et al. 1997). However GISH gives only cytogenetic information on anonymous genome-specific repeat sequences. In contrast, the availability of a PCR-based tandemly repeated marker enables a combination of exact cytological characterisation by FISH with molecular genetic analyses. The latter can give considerably more insight into the presence or absence of a given sequence or its near homologues and enables a much more accurate estimation of repeat copy numbers. Furthermore, tandem repeats tend to remain phylogenetically well conserved after they are amplified to high-copy numbers in genomes. Hence this B-genome-specific marker will also be useful in comparative mapping and phylogenetic studies among the Brassicaceae. For example, there has been speculation from cytological studies that *B. nigra* might be more closely related to *Sinapis* species than it is to the *Brassica* A- and C-genome diploids. The availability of PCR-based repeat sequence markers will allow detailed investigation of the sequences and distributions of genome-specific tandem repeats throughout different crucifer genera and give new molecular phylogenetic information on Brassica genome evolution.

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