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# Karyotyping and identifying all of the chromosomes of allopolyploid *Brassica juncea* using multicolor FISH

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ABSTRACT

Chromosome identification and karyotype using fluorescence *in situ* hybridization (FISH) provides a technical platform for genome and cytogenetic studies. *Brassica juncea* (brown mustard, $2n = 4\times = 36$; genome AABB) is an allopolyploid species that originated from a spontaneous hybridization of *Brassica rapa* and *Brassica nigra* and contains many valuable traits. In this study, a multicolor FISH procedure allowing the identification of all 18 chromosomal pairs was developed by two-step hybridizations with probes on the same metaphase chromosomes. The distribution patterns and chromosomal localizations of six repeat sequences (satellite repeat pBrSTR, 5S rDNA, 45S rDNA, B genome-specific repeat pBNBH35, and centromeric satellite repeats CentBr1 and CentBr2) on *B. juncea* chromosomes were characterized. Comparative karyotype analyses showed that the genome is relatively stable in comparison with its diploid progenitor species and revealed intraspecific karyotypic diversity among three accessions of *B. juncea*. This study provides valuable information about the genome evolution of *B. juncea* and a toolkit that will be helpful for chromosome identification.



## 1. Introduction

The genus *Brassica* contains many agriculturally important species, including vegetable, condiment, and oilseed crops. The genetic relationships between six diploid and allopolyploid cultivated *Brassica* species were described by the triangle of U [1]. The three monogenomic diploids are *B. rapa* (AA, $2n = 20$), *B. nigra* (BB, $2n = 16$), and *B. oleracea* (CC, $2n = 18$). The three allopolyploids are *B. juncea* (AABB, $2n = 36$), *B. napus* (AACC, $2n = 38$), and *B. carinata* (BBCC, $2n = 34$), which have evolved as a result of hybridization between different monogenomic diploids. *B. juncea* (brown mustard, $2n = 4\times = 36$; genome AABB) is an allopolyploid species derived from a spontaneous hybridization of *B. rapa* and *B. nigra*. It is well adapted to cultivation in dryland areas and can grow as a major oilseed crop in the Indian subcontinent during winter [2]. *B. juncea* possesses valuable traits, such as nonshattering siliques and disease resistance, that are not found in the A or C genomes of *B. napus* [3,4]. It is thus a potential gene source for improving disease resistance, yield, and environment adaptation of *B. napus* [2].

Karyotyping, a useful tool for cytogenetic studies, reveals the number and characteristics of chromosomes and can be used to elucidate the origin, ploidy, and phylogenetic relationships among plants [5–12]. Several karyotypes had been

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published for *Brassica* species, mostly related to *B. rapa*, *B. oleracea*, and *B. napus*, which are of greatest agronomic interest [10,13,14]. In previous studies, karyotyping was based on mitotic and meiotic chromosomes and used several staining methods including Giemsa staining, C banding, CMA3/DAPI fluorescence staining, silver staining, and fluorescence *in situ* hybridization (FISH) with repetitive DNA sequences [13,15–21], as well as BAC-FISH [22–24]. Combining BAC-FISH and FISH with repetitive sequences, Xiong and Pires developed a complete *B. napus* molecular cytogenetic karyotype integrating genetic with cytogenetic maps and unambiguously identified each chromosome of both the A and C genomes [10]. Recently, He et al. [25] performed fine karyotyping of radish (*Raphanus sativus* L.), allowing the ready identification of each individual somatic metaphase chromosome. However, despite the economic importance of *B. juncea*, few cytogenetic studies in this species have been reported. Dual-color FISH with 5S and 25S rDNA for karyotyping allowed the identification of 10 of 18 chromosome pairs in *B. juncea* [26]. Dual-color FISH with rDNA probes, together with genomic *in situ* hybridization (GISH), discriminated the 28 chromosomes of *B. juncea* [27].

To eludicate the evolutionary relationships, distribution, and organization of repetitive elements in the genus *Brassica* and closely related species, several researchers have used FISH to map repetitive DNA sequences on *Brassica* chromosomes [17]. Using a 45S rDNA repetitive sequence as a probe, Fukui et al. [13] developed a first FISH pattern karyotype of *B. rapa*. Subsequently, Snowdon [14] used the FISH technique for chromosomal localization of 25S rDNA and 5S rDNA repetitive DNA sequences and proposed a karyotype of *B. rapa*. The subtelomeric tandem repeats pBrSTR, pBnSTR, and pBoSTR have also been localized on chromosomes of *B. rapa*, *B. nigra* and *B. oleracea*, respectively [28]. Based on sequence data and FISH analysis, two classes of 176 bp CentBr tandem arrays were characterized in the centromeres of different chromosomes of *B. rapa* [29,30], *B. olearacea*, and *B. napus* [10]. *B. nigra* lacks CentBr repeats [30], in contrast to their localization in pericentromeres with another tandem repetitive sequence, pBNBH35 [31].

In the present study, to better determine the chromosomal localizations of repetitive sequences and the evolutionary genomic structures of *B. juncea*, we developed a two-step hybridization allowing routine identification of all the chromosomes of this allopolyploid. In the first step, repetitive DNA sequences including 45S rDNA, 5S rDNA, pBrSTR, and a BAC clone, KBrB092N24 were used as probes. In the second step, a second FISH was performed with two pericentromeric satellite repeat sequences (CentBr1 and CentBr2) and a B genome-specific pericentromeric repeat sequence to distinguish the A and B genomes of *Brassica*. Accessions of *B. juncea* with different origins were compared to elucidate the genome evolution of *B. juncea*.

## 2. Materials and methods

### 2.1. Plant materials

Three *B. juncea* accessions, PI 537018, CGN12009, and CGN07154, were used. Seeds were germinated on moist filter paper in Petri dishes at 20–22 °C. Seedlings with roots were then grown in a greenhouse.

### 2.2. Chromosome preparation

Immature flower buds (about 2 mm long) were harvested from plants grown in the greenhouse for mitotic chromosome spreads. Flower buds were fixed in ice-cold 90% acetic acid for 10 min and stored in 70% ethanol at −20 °C until used. Fixed buds were washed in distilled water and digested enzymatically in a mixture of 3% (w/v) cellulase and 1.5% (w/v) pectinase for 2 h at 37 °C. Slides were prepared as described previously [32].

### 2.3. DNA probes and FISH

The BAC clone KBrB092N24 and six repetitive DNA sequences were used for karyotyping *B. juncea*. Repetitive DNA sequences included 45S rDNA, 5S rDNA, CentBr1, CentBr2, pBrSTR, and a B genome-specific pericentromeric repeat sequence pBNBH35. 45S and 5S rDNA were PCR-amplified using M13 forward and reverse primers as described previously [32]. CentBr1 and CentBr2 had been previously characterized [10] and the PCR cloning of these repeats was performed [33]. pBrSTR [29] was PCR-amplified from *B. rapa* genome DNA using the forward primer 5′-TTATAGTGGTTAGTCCACCAATTTAGG-3′ and the reverse primer 5′-GTTGTCTCCATTTCC CTAGAAAA-3′. The B genome-specific pericentromeric repeat pBNBH35 sequence was PCR-cloned as described by Schelfhout et al. [31].

Repetitive sequences were labeled with fluorescein-12-dUTP, Cy3-dCTP, and Cy5-dUTP or simultaneously with fluorescein-12-dUTP and Cy3-dCTP, using nick translation as previously described [10]. FISH was performed using the method of Kato et al. [32] with minor modifications [34]. The chromosome preparations were reused for the second FISH detection with CentBr1 and CentBr2 centromeric tandem repeat probes and B genome-specific pericentromeric repeat [29]. The chromosomes were stained in Vectashield mounting medium containing DAPI (H-1200; Vector Laboratories). Visualization was performed with a ZEISS Imager M2 fluorescent microscope with a 63× plan oil immersion lens, and digital images were captured using the AxioCamMRm software. Images were cropped, size-adjusted, and contrast-optimized using only functions affecting the whole image with Adobe Photoshop.

## 3. Results

A standardized karyotype of allopolyploid *B. juncea* for each accession was constructed by multicolor FISH using BAC clone KBrH092N24 and previously reported repetitive DNA sequences: 45S rDNA, 5S rDNA, and pBrSTR in the first-step hybridization, and CentBr1, CentBr2 (two pericentromeric 176 bp satellite repeats), and pBNBH35 in the second-step hybridization. All somatic chromosomes showed distinctive karyotype patterns according to FISH signals (Figs. 1–3).

KBrH092N24 produced robust and consistent cytological signals on the long arms of chromosomes A2 and A7 from the A genome (Fig. 1-A, C, red signals), but produced no strong signals on B-genome chromosomes of *B. juncea*. Using the repetitive sequence pBrSTR as a probe, different strengths of signals were observed at the subtelomeric regions on several chromosomes from both A and B genomes of *B. juncea* (Figs. 1–3). However, the signals of pBrSTR were stronger on chromosomes of A-genomic

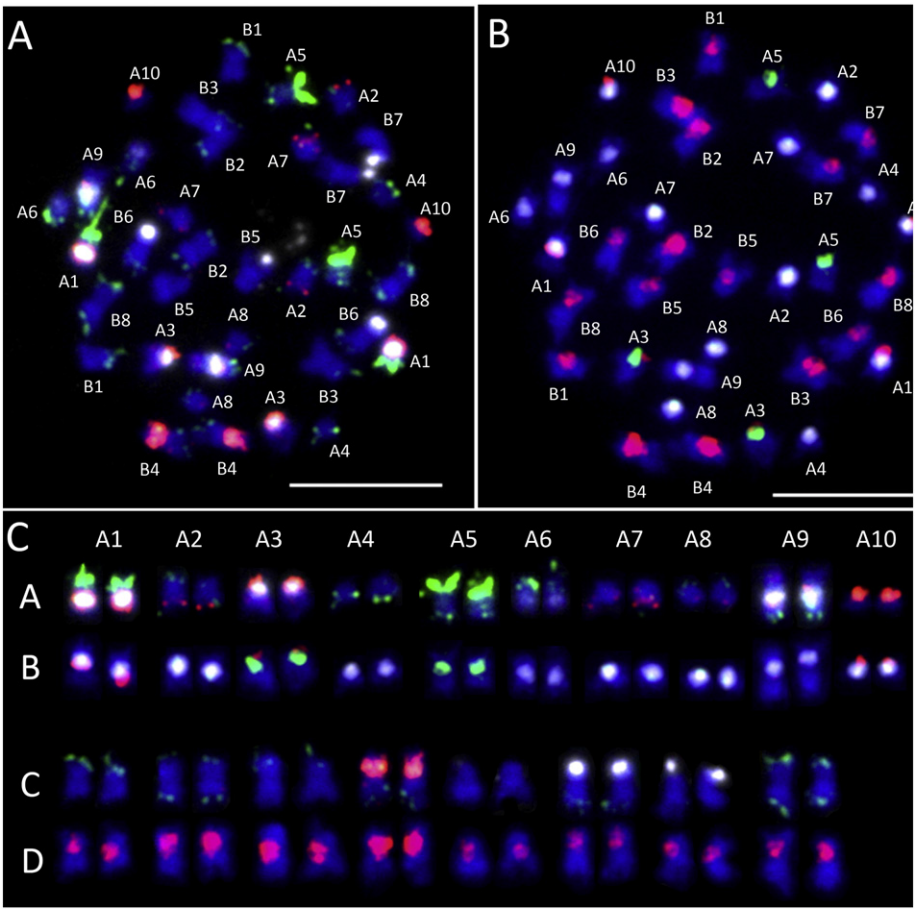



**Fig. 1 – Karyotyping of *B. juncea* accession PI 537018. (A) Multicolor FISH using repetitive sequences 45S (white), 5S (orange), BAC KBrB 092N24 (red), and pBrSTR (green) in the first-step hybridization. (B) The same cell as shown in A, reprobed with CentBr1 (white), CentBr2 (green), and the B genome-specific repeat pBNBH35 (red) in the second-step hybridization. (C) Combined results from A and B, with each chromosome of *B. juncea* identified according to its signal pattern. In lane a, all chromosomes from the A genome were cut out from panel A and lined up following the order of genetic nomenclature. In lane b, all chromosomes from the A genome were cut out from panel B and the position and organization of the centromere are shown for each chromosome. In lane c, chromosomes from B genome were cut out from panel A and lined up to show the distribution and organization of repeat sequences. In lane d, B-genome chromosomes were cut out from panel B to show the signals of the pBNBH35 repeat located in the pericentromeric region. Scale bars represent 10 μm.**

than on those of B-genomic origin. On the B genome of *B. juncea*, pBrSTR signals were located on 12 of the 16 chromosomes of accession PI 537018 (Fig. 1).

According to the karyotypes of our studies, all three accessions of *B. juncea* had 16 45S rDNA loci, with 10 and 6 loci on A- and B-genome chromosomes, respectively. Three pairs of intense fluorescence signals on chromosomes A1, A3, and A9 and two pairs of weak signals on chromosomes A5 and A6 were observed on the A genome of *B. juncea* (Figs. 1–3). Among the ten loci on A-genome chromosomes, eight were localized in interstitial regions and one pair was localized at the distal end of the short arm of chromosome A3. In addition, two pairs of medium signals on chromosomes B6 and B7 and one pair of small signals on chromosome B5 were detected on the B genome of *B. juncea* accession PI 537018 (Fig. 1-A, C). All three loci were mapped at the distal ends of the short arms of the chromosomes of B-genome chromosomes.

Fluorescence- and Cy3-labeled 5S rDNA probes were hybridized to mitotic metaphase chromosomes and produced yellow signals by FISH (Fig. 1-A, C). 5S rDNA probes hybridized to ten sites on chromosomes of *B. juncea*, with strong signals on chromosomes A1 and B4. 5S rDNA colocalized with 45S rDNA on chromosomes A1 and A3 and also in the pericentromeric region of chromosome A9 (Fig. 1-A).

In the second FISH, the chromosomal localizations of the centromeric satellite repeats, CentBr1 and CentBr2 from A genome and pBNBH35 from B genome clearly distinguished the A-genome from the B-genome chromosomes of *B. juncea*. The karyotype of the allopolyploid *B. juncea* consisted of 18 pairs of chromosomes with ten pairs containing Cent1 or Cent2 signals and eight pairs containing pBNBH35 red signals, and this number is equal to the sum of the chromosome numbers of its diploid ancestors, *B. rapa* and *B. nigra*.

Among the ten pairs of chromosomes from A genome in *B. juncea*, eight pairs showed white CentBr1 signals and two pairs showed green CentBr2 signals (Fig. 1-B). The pBNBH35 repeat localized specifically in the centromeric regions of eight pairs of the B-genome chromosomes, and different signal

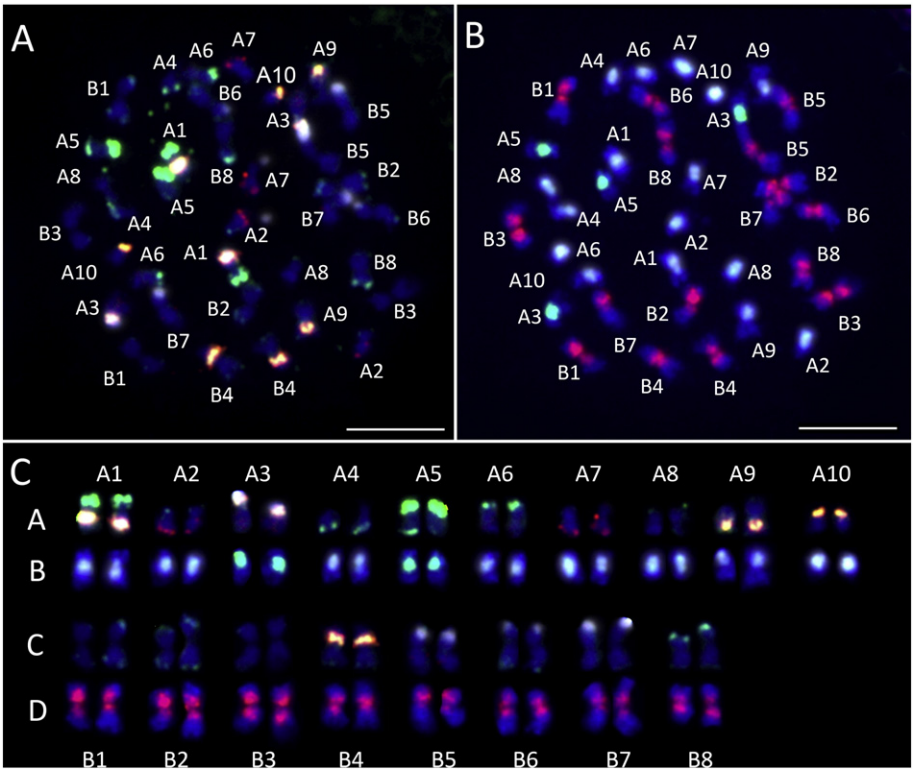



**Fig. 2 – Karyotyping of *B. juncea* accession CGN12009. (A) Multicolor FISH using repetitive sequences 45S (white), 5S (orange), BAC KBrB 092 N24 (red), and pBrSTR (green) in the first-step hybridization. (B) The same cell as shown in A reprobed with CentBr1 (white), CentBr2 (green) and B-genome specific repeat pBNBH35 (red) in the second-step hybridization. (C) Combined results from A and B, with each chromosome of *B. juncea* identified according to its signal pattern. In lane a, all chromosomes from A genome were cut out from panel A and lined up following the order of genetic nomenclature. In lane b, all chromosomes from A genome were cut out from panel B and the position and organization of the centromere for each chromosome are shown. In lane c, chromosomes from B genome were cut out from panel A and lined up to show the distribution and organization of repeat sequences. In lane d, B-genome chromosomes were cut out from panel B to show the signals of the pBNBH35 repeat located in the pericentromeric region. Scale bars represent 10 μm.**

intensities, representing copy number variation of the repeat, were detected in accession PI 537018 (Fig. 1-B). Multicolor FISH with three centromeric repeats as probes at the second step could be used not only to distinguish A- or B-genome chromosomes, but also to identify different chromosomes in the B genome of *B. juncea*.

FISH on metaphase complements, using the above six repeats and one BAC clone as probes, together with two-step multicolor FISH, produced diagnostic markers for reliably identifying all chromosomes of *B. juncea*.

Chromosome A1 is easily distinguished by the largest 45S rDNA and strong 5S signals on its long arm. Strong signals from pBrSTR are located at the end of the short arm.

Chromosome A2 shows faint pBrSTR signals at the tip of the short arm and KBrB092N24 BAC clone signals on its long arm.

Chromosome A3 has the largest nucleolar organizing region (NOR) signals and 5S signals on the short arms. It is distinguished from other chromosomes by strong CentBr2 pericentromere repeat signals.

Chromosome A4 is a small chromosome with weak pBrSTR signals at the end of the long arm.

Chromosome A5 is easily identified by the strongest pBrSTR signals at the end of the short arm and by the green CentBr2 signals at the centromeric position. The weakest 45S signals are located within the pericentromeric region of the long arm.

Chromosome A6 presents medium pBrSTR signals at the end of the short arm. Medium-strength 45S signals are present in the pericentromeric region of the long arm.

Chromosome A7 shows KBrB092N24 signals on the long arm. It is distinguished from chromosome A2 by an absence of pBrSTR signals.

Chromosome A8 is a small chromosome with a faint pBrSTR signal at the end of the short arm.

Chromosome A9 is the longest chromosome in the A genome of *B. juncea*. It contains a 45S rDNA locus in the pericentromeric region and a medium-strength pBrSTR signal at the end of the long arm.

Chromosome A10 is the smallest chromosome and contains a 5S rDNA locus on the short arm.

Chromosome B1 shows a medium pBrSTR signal at the end of the short arm.

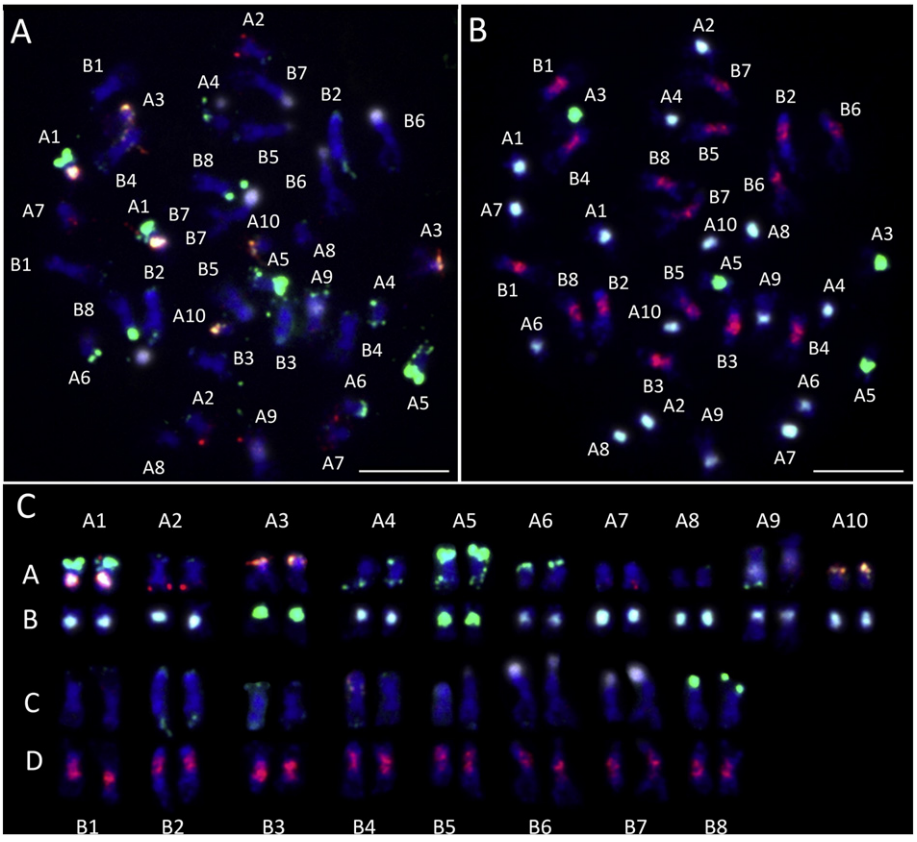



**Fig. 3 – Karyotyping of *B. juncea* accession CGN07154. (A) Multicolor FISH using repetitive sequences 45S (white), 5S (orange), BAC KBrB 092 N24 (red), and pBrSTR (green) in the first-step hybridization. (B) The same cell as shown in A reprobed with CentBr1 (white), CentBr2 (green) and B genome-specific repeat pBNBH35 (red) in the second-step hybridization. (C) Combined results from A and B, with each chromosome of *B. juncea* identified according to its signal pattern. In lane a, all chromosomes from A genome were cut out from panel A and lined up following the order of genetic nomenclature. In lane b, all chromosomes from A genome were cut out from panel B and the position and organization of the centromere are shown for each chromosome. In lane c, chromosomes from B genome were cut out from panel A and lined up to show the distribution and organization of repeat sequences. In lane d, B-genome chromosomes were cut out from panel B to show the signals of the pBNBH35 repeat located in the pericentromeric region. Scale bars represent 10 μm.**

Chromosome B2 shows pBrSTR signals at both ends of chromosome arms and strong pBNBH35 signals in the centromeric region.

Chromosome B3 shows faint pBrSTR signals at the end of the short arm and centromeric pBNBH35 signals.

Chromosome B4 is easily distinguished by the largest 5S rDNA locus on the short arm.

Chromosome B5 contains a small 45S rDNA locus at the end of the short arm and shows a small pBrSTR signal at the end of the long arm.

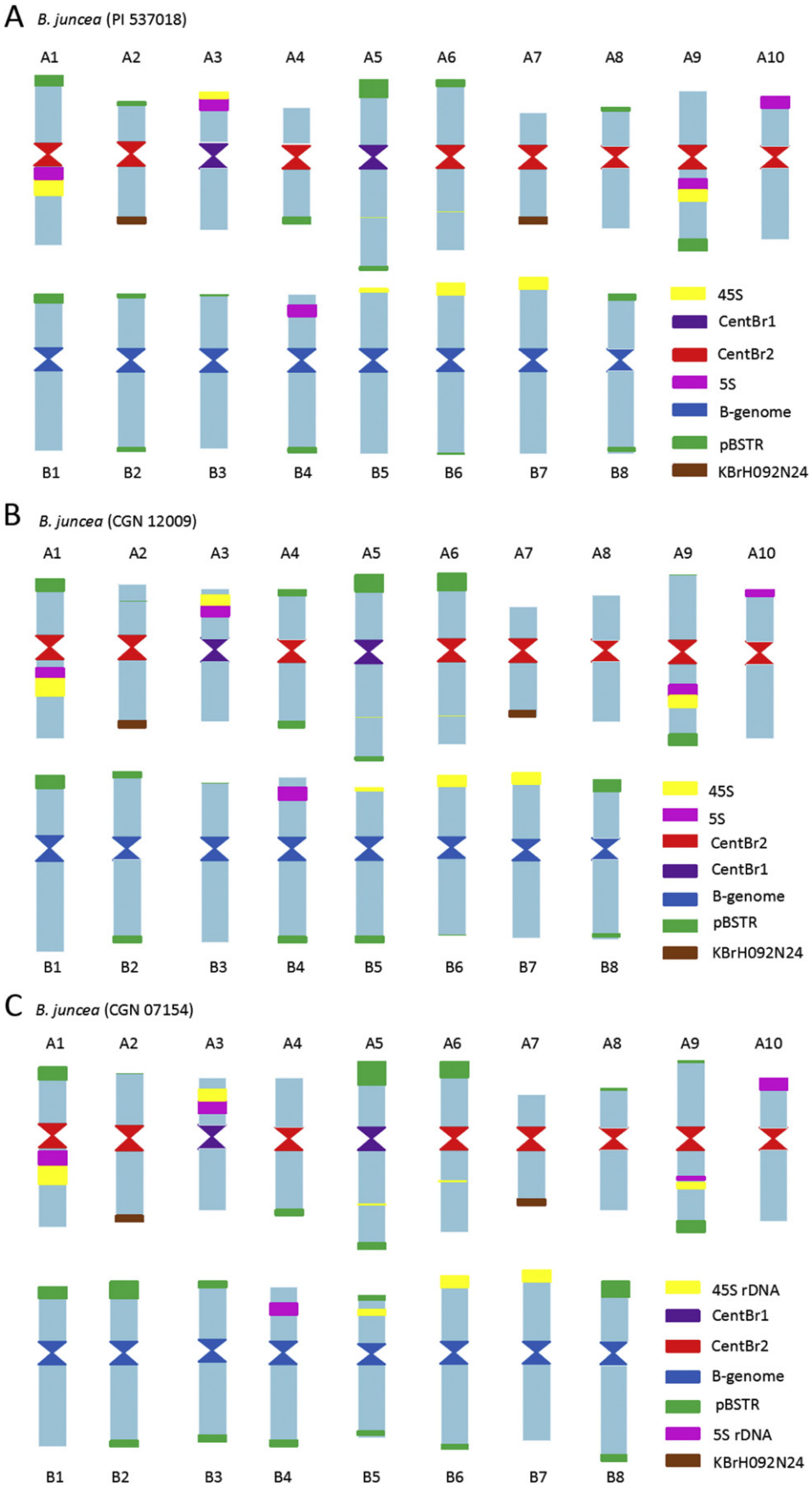
Chromosome B6 has a medium 45S rDNA locus at the end of the short arm and shows a small pBrSTR signal at the end of the long arm.

Chromosome B7 contains a medium 45S rDNA locus at the end of the short arm.

Chromosome B8 shows medium and small pBrSTR signals at the ends of the short and long arms, respectively.

The karyotypes of the three *B. juncea* accessions, PI 537018, CGN12009, and CGN07154, were analyzed by multicolor FISH and the genome diversities were comparatively studied. No significant genome diversity was detected among the three accessions except for a few minor signal variations. For example, accession CGN07154 showed very faint 5S rDNA signals on chromosome B4 in comparison with the other two accessions and new pBrSTR loci were observed on the ends of some chromosomes. In comparisons of the karyotypes with previously published results [10,28–30], the genome of *B. juncea* appeared relatively stable compared with its diploid progenitor species, but the number of 5S rDNA loci and the signal strength of 45S rDNA on several chromosomes were

**Fig. 4 -Idiograms of the three accessions, PI 537018, CGN 12009, and CGN 17054 of *B. juncea*. The yellow, pink, purple, red, blue, green, and brown colors indicate the positions of 45S rDNA, 5S rDNA, CentBr1, CentBr2, pBNBH35, pBrSTR, and BAC KBrH092N24, respectively. The remaining light blue zones are assumed to be mostly euchromatic. (A) Accession PI 537018. (B) Accession CGN 12009. (C) Accession CGN 17054.**


A
B. juncea (PI 537018)
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
B1
B2
B3
B4
B5
B6
B7
B8
45S
CentBr1
CentBr2
5S
B-genome
pBSTR
KBrH092N24
B
B. juncea (CGN 12009)
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
B1
B2
B3
B4
B5
B6
B7
B8
45S
5S
CentBr2
CentBr1
B-genome
pBSTR
KBrH092N24
C
B. juncea (CGN 07154)
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
B1
B2
B3
B4
B5
B6
B7
B8
45S rDNA
CentBr1
CentBr2
B-genome
pBSTR
5S rDNA
KBrH092N24

variable. Idiograms of the three accessions of *B. juncea* are shown in Fig. 4.

## 4. Discussion

Repetitive DNA sequences including tandem repeats are responsible for heterogeneity in genome size and composition and are the major contributors to plant chromosome structure [30]. FISH localization using tandem repeats generates chromosomal markers useful for characterizing chromosomal structure and genome organization and for performing evolutionary, genetic, and taxonomic studies among species [29]. In *Brassica*, tandem repeats, including 45S rDNA, 5S rDNA, CentBr1, CentBr2, pBnSTR, and pBNBH35, have previously been physically localized on chromosomes [13,14,28–31]. In this study, we located all of these repetitive DNA sequences and constructed a robust karyotype of *B. juncea* that provides detailed information about the chromosomal structure and genome organization of each chromosome of this species.

The BAC clone KBrH092N24, which contains repeat sequences, was used as a chromosomal marker to identify chromosomes A2 and A7 in the A genome as well as chromosomes C2 and C6 in the B genome of *B. napus* [10]. In agreement with previous results, strong cytological signals from KBrH092N24 were detected on the long arms of chromosomes A2 and A7, but no obvious signals were produced on the B-genome chromosomes of *B. juncea*. Thus, the repeat sequences contained in KBrH092N24 may represent a recent repeat that appeared before the differentiation of the A- and C genomes but after the divergence of these two genomes from the B genome of *Brassica*.

The chromosomal locations of BAC clone KBrB072L17, which contains repetitive sequence pBrSTR, had been reported to lie on chromosomes 1S, 2S, 4L, 5S, 5L, 6S, 6L, 8S, and 9L in *B. rapa* and in the A genome of *B. napus* [10]. Chromosomal localization of pBrSTR, pBnSTR, and pBoSTR on metaphase chromosomes of *B. rapa*, *B. nigra*, and *B. oleracea*, respectively, showed that those repeat sequences were conserved across the *Brassica* A, B, and C genomes [30]. However, no hybridization was detected on chromosomes of *B. nigra* when pBrSTR was used as a probe [30]. According to our results, the signals of pBrSTR were stronger on *B. juncea* chromosomes of A-genomic than on those of B-genomic origin. The strengths of FISH signals of repeated sequence usually represent copy number variation among chromosomes in the same species. In this case, the divergence of the pBrSTR and pBnSTR repeats between A- and B genomes could be another reason for the change in signal strength. In addition, the distribution pattern of pBrSTR on A genome of *B. juncea* was similar to that on *B. rapa* [30], suggesting that the pBrSTR was relatively conserved after the polyploidization event in *B. juncea*.

The differences in numbers of 45S rDNAs that have been reported in *Brassica* may reflect differentiation of *Brassica* chromosomes within a species [13]. Variation of 45S rDNA loci, with 12–14 signals [6], and with 16 signals [26,27] has been described for *B. juncea* by different groups using different accessions as materials. In the present study, all three accessions of *B. juncea* had 16 45S rDNA loci. 45S rDNA proved to be an excellent marker for chromosome identification and the FISH signal distributions of 45S rDNA on the A-genome chromosomes of *B. juncea* were very similar to those on the A genomes of *B. napus* and *B. rapa* [6], except for chromosome A5, which showed very faint 45S rDNA signals in this study.

Centromere-specific retrotransposons (CRB) of *Brassica* have been reported [33] to be core centromeric sequences of *Brassica* species, and two main CRB named CentBr1 and CentBr2 were found on both the A and C genome-derived chromosomes. CRB were not detected in *B. nigra* or on any of the B-genome chromosomes in *B. juncea* and *B. carinata* [28,30,35], but rather were localized in pericentromeres with another tandem repetitive sequence pBNBH35 [31]. Thus, multicolor FISH using CentBr1, CentBr2, and pBNBH35 simultaneously as probes provided a reliable method of identifying A- and B-genome chromosomes of *B. juncea*.

Recent studies of natural allopolyploids [36] and synthetic polyploids [37] have demonstrated the high prevalence of chromosomal instability and karyotype variability immediately after genome duplication [38]. It is interesting to note that the genome was relatively stable compared with those of its diploid progenitor species and that intraspecific karyotypic diversity was detected among 3 accessions of *B. juncea*. Comparative mapping of *B. juncea* and *Arabidopsis thaliana* using intron polymorphism markers also indicated that the B genome of *B. juncea* maintained a genomic architecture similar to that of its diploid progenitor in *B. nigra*, suggesting the absence of large-scale perturbations during the formation of the allopolyploid *Brassicas* [39]. One explanation of the conflicts is that the survival of the natural allopolyploid *Brassicas* and wheat (*Triticum aestivum* L.) was based on suppression of pairing between homeologous chromosomes [39]. Another is natural selection, which limits the chromosomal instability and karyotype variability of allopolyploids [38]. Chromosomal instability was negatively correlated with pollen viability in synthetic wheat polyploids [37] and with fertility in synthetic *B. napus* [11].

## 5. Conclusions

Repetitive DNA sequences, such as tandem repeats, are responsible for heterogeneity in genome size and composition and are the major contributors to plant chromosome structure. In this study, we constructed a robust karyotype of *B. juncea* and located 6 tandem repeats including 45S rDNA, 5S rDNA, CentBr1, CentBr2, pBnSTR, and pBNBH35, providing detailed information about the chromosomal structure and genome organization of all of the chromosomes of this species. Comparative karyotype analyses indicated that the genome is relatively stable in comparison with its diploid progenitor species. Intraspecific karyotypic diversity was detected among 3 accessions of *B. juncea*. This study has provided a useful toolkit for chromosome identification and valuable information about the genome evolution of *B. juncea*.

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