배추에서 Ribosomal DNAs 분포의 비교분자세포유전학적 분석

Franklin H. Mancia¹ · 김정선² · 황윤정^{3*}

¹삼육대학교 대학원 환경원예학과, ²농촌진흥청 국립농업과학원, ³삼육대학교 화학생명과학과

Comparative Molecular Cytogenetic Analysis of Ribosomal DNAs Distribution in *Brassica* Species

Franklin H. Mancia¹, Jung Sun Kim², and Yoon-Jung Hwang^{3*}

¹Department of Environmental Horticulture, Sahmyook University, Seoul 01795, Republic of Korea

²Department of Agricultural Biotechnology, National Institute of Agricultural Science, Rural Development Administration, Jeonju 54874, Republic of Korea

³Department of Chemistry Life Science, Sahmyook University, Seoul 01795, Republic of Korea

Abstract Three *Brassica* species, namely, *Brassica rapa*, *B. nigra*, and *B. oleracea* are considered economically important as they are grown for human consumption and biogas production. Like other crops facing agricultural constraints, selective crossing or hybridization in cruciferous vegetables has helped farmers to improve them. This study conducted a comparative evaluation across and within the species through cytogenetic analysis to provide fresh insights into their chromosome structures and evolutionary relationships. A new karyomorphological parameter confirmed symmetric karyotypes in all the accessions, thereby allowing for the depiction of the ancestral chromosome karyotype. Several lines of *B. rapa*, *B. nigra*, and *B. oleracea* were subjected to physical mapping using a fluorescence *in situ* hybridization technique to elucidate the chromosomal distribution of the two types of rDNAs. The signal number and distribution of 18S rDNA across the metaphase chromosomes of *B. rapa* accessions did not vary as compared to 5S rDNA, which was also observed in several lines of *B. nigra*. In contrast, the number and distribution of 5S rDNA loci across the chromosomes in several lines of *B. oleracea* were found to be more conserved than those corresponding to the 18S rDNA. Overall, this study revealed the evolutionary dynamics of rDNA, which may play an important role in shaping the chromosome karyotypes of *Brassica* species.

Keywords FISH, interspecies, karyotype evolution, polymorphism

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* Corresponding Author (E-mail: hyj@syu.ac.kr, Tel: +82-3399-1718)

Introduction

The genus *Brassica* in the mustard family comprises 38 species, which include economically important crops (Kim et al. 2014). Of these crops, many are valuable sources of vegetables (Masarirambi et al. 2020), oils (Sun 2017), feed and forage (Westwood and Mulcock 2012), and medicines (Saeidnia and Gohari 2012). Owing to their high market demand, several genera of Brassicaceae have been subjected to plant breeding, and considerable research, including the identification of agronomic traits, diversity analyses, and genetic mapping (Nishio 2014, Sharma et al. 2014, Velasco et al. 2017). Moreover, three diploid species, that is, *Brassica rapa* ($2n = 2 \times = 20$),

Brassica nigra ($2n = 2 \times = 16$), and *Brassica oleracea* ($2n = 2 \times = 18$) are denoted as A, B and C genomes, respectively, in the triangle of U (U 1935). Based on their close relationships, interspecific hybridization has been effectively conducted among the *Brassica* diploids for the production of new species and the introgression of useful adaptive traits for targeted crop improvement (Dhaliwal et al. 2017, Elvis et al. 2020, Navabi et al. 2010). Furthermore, the progress in understanding the genome structures of *Brassica* species has been greatly underpinned by molecular cytogenetic studies (Chèvre et al. 2018, Hong et al. 2008).

Fluorescence *in situ* hybridization (FISH) is a widely used technique in plant molecular cytogenetic research that has been

proved promising in relating DNA sequence information to chromosome biology, thereby allowing a comprehensive view of the evolution of plant species (Bačovský et al. 2018, Jiang & Gill 2006, Zheng et al. 2016). A unique FISH pattern of repetitive DNA sequences on individual chromosomes can help discriminate plant genomes and analyze the somatic pairing of homologous chromosomes (Hasterok et al. 2005b, Hwang et al. 2009, Iourov et al. 2005). Moreover, this technique aids in the identification of species using taxonomic and phylogenetic studies, validation of karyotypic and chromosomal evolution, analysis of chromatin features associated with gene expression, and provides a valuable complementary approach to genome sequencing and map-based cloning research strategies (Braz et al. 2018, Deng et al. 2019, Jiang & Gill 2006).

Important applications of molecular cytogenetic analysis have been reported for *Brassica* crops, which include the following: information about genome organization in diploid and polyploid species leading to the establishment of a common ancestral genome and description of the three differentially fractionated *Brassica* ancestral sub-genomes, and an enhanced understanding of intergenic relationships and structural rearrangements in interspecific *Brassica* hybrids (Chèvre et al. 2018). Previously, FISH-mapping has been conducted in several *Brassica* crops to evaluate their karyotypic structures, genome compositions, and chromosomal distribution of molecular cytogenetic markers such as ribosomal DNAs (Kamiński et al. 2020, Koo et al. 2004, Hwang et al. 2009, Perumal et al. 2017, Waminal et al. 2016, Wang et al. 2007, Xiong & Pires 2011, Zheng et al. 2014). Likewise, the distribution and number of ribosomal DNA (rDNA) sites have been evaluated in several genotypes of *B. rapa*, *B. nigra*, and *B. oleracea*, which have revealed polymorphism in 5S rDNA and 35S rDNA (Hasterok et al. 2006, Zheng et al. 2015).

Despite the abundance of molecular cytogenetic maps generated for rDNA in the three *Brassica* diploids, FISH-based mitotic karyotypes need to be studied for these genotypes. Such analyses are important to make the genus *Brassica* a model for conducting extensive studies on complex chromosomal evolution in plants. Therefore, we conducted the present study to evaluate the cytogenetic diversity of *Brassica* genotypes

that might prompt biologists to delve comprehensively into research regarding species diversification. The data from this study would enhance our current understanding of the genetic diversity that could facilitate breeding in *Brassica* species.

Materials and Methods

Chromosome slide preparation

Seeds used in this research were provided by National Agrobiodiversity Center (<http://genebank.rda.go.kr>), Korea. The seeds were stored inside a properly sealed container and placed at 4°C. A petri dish with moist filter paper was used to germinate the seeds at 24°C in dark conditions. The root tips were harvested after they attained a length of 1-2 cm. Then, these were soaked in 8-hydroxyquinoline at 18°C for 5 hrs. A mixture of ethanol and acetic acid in a ratio of 3:1 (v/v) was used to fix the pre-treated roots overnight at room temperature. The fixative substance was decanted and replaced by 70% ethanol and stored at 4°C. The pre-treated young roots were enzymatically digested to prepare a chromosome slide. It was carried out by following the protocol of Zheng et al. (2014) with some minor modifications. Root tips were treated with 2% cellulase R-10 (Sigma, USA), cytohelicase (Sigma, USA), and pectolyase γ -23 (Duchefa, Germany) at 37°C for 80 min to digest the cellular components excluding DNA materials. Carefully, the enzyme solution was discarded and replaced with distilled water. Next, water was eliminated without disturbing the delicate roots and homogenized with Carnoy's solution. The steam-drop method by Kirov et al. (2014) was followed to prepare well-distributed and morphologically intact chromosomes on a slide.

Polymerase chain reaction and DNA probes

The total genomic DNA from *B. rapa* L. (50 ng $\cdot$ μ l⁻¹) was extracted using the CTAB method of Devi et al. (2013). A pair of primers (forward, 5'-GGA TGG GTG ACC TCC CGG GAA GTCC-3'; reverse, 5'-CGC TTA ACT GCG GAG TTC TGA TGGG-3') were used to amplify the 5S rDNA (Yang et al. 1998). PCR conditions involved an initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 1

min, annealing at 55°C for 45 sec, extension at 72°C for 1 min, and a final extension at 72°C for 3 min. The primers used to amplify the 18S rDNA are as follows: forward, 5' -AAC CTG GTT GAT CCT GCC AGT-3' ; reverse, 5' -TGA TCC TTC TGC AGG TTC ACC TAC-3' (Matsuda et al. 2011). The amplification was conducted with an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C at 10 min. The amplified DNA products were labeled with dig 11-dUTP or biotin 16-dUTP through nick translation (Roche, Germany). The reaction mixture constituted 2% TaKaRa Ex Taq, 10% 10× exTaq buffer, 10% dNTP mix, 1% template DNA (< 500 ng), 20% primer mix (2 μm), and 54% DNase/RNase-Free Water (Takara Bio, Japan).

In situ hybridization and imaging

The FISH protocol by Lim et al. (2001) was performed with some modifications. The DNA probe (50-100 ng · μl⁻¹) was added to the hybridization mixture (50% dextran sulfate, 10% SDS, 20× SSC, 100% formamide, 5 ng · μL⁻¹ salmon sperm DNA). Next, the mixture was denatured at 90°C for 10 min and transferred on ice for 5 min before adding onto the slide. The somatic metaphase chromosomes on a slide were denatured at 90°C for 5 min. The slide was kept inside a moistened container at 37°C for 16-18 hrs. Stringent washing was performed by immersing the slide in a copulin jar containing 20% deionized formamide in 0.1× SSC at 42°C for 10 min (Hasterok et al. 2005a). The probes labeled with digoxigenin-11-dUTP and biotin 16-dUTP were detected with anti-digoxigenin-rhodamine and streptavidin-cy3 (Sigma-Aldrich, USA). Finally, the preparations were mounted in 100 ug · μl⁻¹ DAPI (Roche, USA) in Vectashield (Vector Laboratories, USA). Visualization was conducted using a model BX63 microscope (Olympus, Japan) equipped with a CoolSNAP cf camera.

Chromosome evaluation

The metaphase chromosomes from several accessions of the three *Brassica* species were analyzed using the computer application developed by Altinordu et al. (2016). The karyotype

formula, chromosome size variation, and total haploid chromosome lengths were used to characterize the chromosome complements. Karyotype asymmetry was assessed using indices for interchromosomal level, which illustrate the heterogeneity of chromosome sizes within a complement. Likewise, indices were used for the intrachromosomal asymmetry, marking the predominance of certain chromosomes with terminal/subterminal or median centromeres. Interchromosomal indices include: 1) A₂ (0 indicates 'no variation' and values > 0 indicate 'increasing variation'), and 2) CV_{CL} (0 indicates 'no variation' and 100 indicates 'very heterogeneous'); intrachromosomal indices include: 1) S/A_I (1 indicates 'full symmetric' and 4 indicates 'full asymmetric'), 2) TF% (increasing values indicate 'symmetric' and decreasing values indicate 'asymmetric'), 3) A (0 indicates 'symmetric' and values ≥ 1 indicate 'asymmetric'), and 4) M_{CA} (0 indicates 'symmetric' and 100 indicates 'asymmetric').

Results

FISH-based karyotype analysis

Conventional chromosome analysis coupled with FISH mapping using two types of rDNAs provided information on diverse cytological characters such as chromosome number, ploidy level, chromosome size variation, karyotype formula, and haploid chromosome length (Table 1). Further, insights into chromosomal and genome evolution were provided based on the generated cytogenetic maps in several *Brassica* genotypes (Figs. 1-3). Moreover, karyotype asymmetry was quantified in the different accessions of *Brassica* diploids with intrachromosomal and interchromosomal indices.

B. rapa chromosome evaluation

The number and distribution of 5S and 18S rDNA loci across the somatic metaphase chromosomes were determined, and the latter being highly conserved in all eight genotypes of *B. rapa* (Fig. 1). Six rDNA-bearing chromosomes were primarily painted with FISH signals on the pericentromeric regions, while the rest were on the terminal regions. Co-localization of 5S and 18S rDNA loci on two pairs of chromosomes (Chrs. #2 and #3) was observed in all the genotypes. Moreover, an extra

Table 1. Karyotypic characteristics and karyotype asymmetry assessment for different accessions of *B. rapa*, *B. nigra*, and *B. oleracea*.

Taxa	IT No. ^z	Place of origin	2n	KF ^y	CSV ^x (μm)	hcl ^w (μm)	CS ^v	Inter- and Intrachromosomal indices					
								A ₂ ^u	CV _{CL} ^t	S/A _I ^s	TF% ^r	A ^q	M _{CA} ^p
<i>B. rapa</i> subsp. <i>pekinensis</i>	032747	Korea	20	12m+8sm+2st	5.4-11.2	81	2	0.24	23.6	1.5	36.9	0.28	28.5
<i>B. campestris</i> var. <i>rapifera</i>	108867	Korea	20	12m+6sm+2st	4.3-8.9	60.8	2	0.20	20.29	1.5	38.2	0.25	28.9
<i>B. rapa</i> var. <i>perviridis</i>	112272	Japan	20	12m+6sm+2st	4.4-9.4	65.7	3	0.16	16.23	1.5	35.8	0.29	29.24
<i>B. rapa</i> subsp. <i>rapa</i>	175952	Korea	20	12m+6sm+2st	4.7-10	70.2	2	0.15	14.7	1.5	36.7	0.28	27.7
<i>B. rapa</i> var. <i>chinensis</i>	185735	Korea	20	12m+6sm+2st	4.6-11.2	64.3	3	0.21	20.9	1.5	37.2	0.26	27.4
<i>B. rapa</i> subsp. <i>narinosa</i>	221794	Japan	20	12m+6sm+2st	5.3-11.1	64.9	4	0.18	17.9	1.5	37	0.27	27.2
<i>B. rapa</i> subsp. <i>oleifera</i>	223924	Italy	20	12m+6sm+2st	6-11.8	81.4	4	0.17	17.3	1.5	36	0.30	29.8
<i>B. rapa</i> var. <i>narinosa</i>	293391	China	20	12m+6sm+2st	5.1-11.8	78	3	0.21	21.1	1.5	35.6	0.31	31
Mean								0.19	19	1.5	36.7	0.28	28.7
<i>B. nigra</i>	305656	Germany	16	10m+6sm	3.7-5.8	36.5	0	0.08	7.88	1.4	37.6	0.23	22.8
<i>B. nigra</i>	305086	Germany	16	10m+6sm	3.3-4.7	32.2	2	0.10	10.14	1.4	39	0.22	22.4
<i>B. nigra</i>	279264	UK	16	10m+6sm	4.2-5.7	39	0	0.06	6.34	1.4	38.9	0.22	22.4
<i>B. nigra</i>	279261	Italy	16	10m+6sm	3-4.8	30	0	0.10	10.2	1.4	38.3	0.23	23.1
<i>B. nigra</i>	279259	Pakistan	16	10m+6sm	4.2-5.9	40.3	0	0.07	7.21	1.4	38.2	0.23	23.4
<i>B. nigra</i>	279258	India	16	10m+6sm	3.8-5.2	40	1	0.09	9.2	1.4	38.2	0.23	23.5
<i>B. nigra</i>	279253	Ethiopia	16	10m+6sm	2.8-4.5	28	2	0.11	10.5	1.4	39.4	0.21	21
<i>B. nigra</i>	279251	Pakistan	16	10m+6sm	2.8-4.1	26	2	0.09	8.51	1.4	38	0.24	24
Mean								0.09	8.75	1.4	38.5	0.22	22.8
<i>B. oleracea</i> var. <i>capitata</i>	136508	India	18	10m+8sm	4.2-7.8	48.3	0	0.11	10.9	1.4	40	0.21	21
<i>B. oleracea</i> var. <i>capitata</i>	180722	Egypt	18	10m+8sm	3-4	32	0	0.07	7.28	1.4	39	0.23	23
<i>B. oleracea</i> var. <i>capitata</i>	188172	Tanzania	18	10m+8sm	3-4.6	32	0	0.09	9.08	1.4	38.9	0.22	22
<i>B. oleracea</i> var. <i>capitata</i>	206707	Uzbekistan	18	10m+8sm	3.3-4.5	34	0	0.27	7.37	1.4	37	0.27	27
<i>B. oleracea</i> var. <i>gemmifera</i>	267606	UK	18	10m+8sm	4.2-6.6	46.1	0	0.09	8.70	1.4	37	0.26	26
<i>B. oleracea</i> var. <i>gongylodes</i>	257512	Japan	18	10m+8sm	3.6-5.3	39	1	0.09	8.8	1.4	37.5	0.25	25
<i>B. oleracea</i> var. <i>alboglabra</i>	210060	Thailand	18	10m+8sm	3-4.9	32	1	0.11	10.6	1.4	37.1	0.25	25
<i>B. oleracea</i> var. <i>alboglabra</i>	278477	Netherlands	18	10m+8sm	3-4.1	31	0	0.08	7.8	1.4	39	0.23	23
<i>B. oleracea</i> var. <i>alboglabra</i>	278479	Netherlands	18	10m+8sm	3-5.4	36.1	0	0.12	12.3	1.4	37	0.25	28
Mean								0.11	9.2	1.4	38	0.24	24.4

^zThe seeds and IT no. used in this study were obtained from the National Agrobiodiversity Center, Korea., ^yKF: Karyotype Formula, ^xCSV: Chromosome Size Variation, ^whcl: total sum of haploid chromosome length, ^vCS: Colocalized rDNA Site pair, ^uA₂ index (Zarco 1986), ^tCV_{CL} (Lavana & Srivastava 1992, Paszko 2006, Watanabe et al. 1999), ^sS/A_I (Eroğlu 2015), ^rTF% (Huziwara 1962), ^qA (Watanabe et al. 1999), ^pM_{CA} (Peruzzi & Eroğlu 2013).

locus and hemizygous site of 5S rDNA were observed in *B. rapa* var. *perviridis* (Figs. 1A-c and B-c, Chr. #1), *B. rapa* var. *chinensis* (Figs. 1A-e and B-e, Chr. #1), *B. rapa* subsp. *narinosa* (Figs. 1A-f and B-f, Chr. #1 and #5), *B. rapa* subsp. *oleifera* (Figs. 1A-g and B-g, Chrs. #1 and #5), and *B. rapa* var. *narinosa* (Figs. 1A-h and B-h, Chr. #5).

The average values for A₂ and CV_{CL} (Coefficient of Variation of Chromosome Length) indices across eight *B. rapa* genotypes are 0.19 and 19, respectively. Additionally, four indices were

measured to determine the intrachromosomal asymmetry of the karyotypes. The average values corresponding to each index were observed as follows: S/A_I (1.5), TF % (36.7), A (0.28), and M_{CA} (Mean Centromeric Asymmetry) (28.7) (Table 1).

B. nigra chromosome evaluation

Two types of rDNAs were detected along the somatic metaphase chromosomes corresponding to the eight *B. nigra* genotypes (Fig. 2A). Three pairs of 18S rDNA were continuously

Fig. 1. FISH-based mapping of 18S (red) and 5S rDNAs (green) in eight *B. rapa* accessions. A) Karyotypes of *B. rapa* subsp. *pekinensis* (a), *B. campestris* var. *rapifera* (b), *B. rapa* var. *perviridis* (c), *B. rapa* subsp. *rapa* (d), *B. rapa* var. *chinensis* (e), *B. rapa* subsp. *narinosa* (f), *B. rapa* subsp. *oleifera* (g), *B. rapa* var. *narinosa* (h). B) Haploid ideograms of 8 genotypes showing the conserved distribution and locus number of 18S rDNA. *polymorphic sites in chromosome-bearing 5S rDNA, +hemizygous sites in chromosome-bearing 5S rDNA (Scale bar = 10 μ m).

Fig. 2. Chromosomal localization of 18S (red) and 5S rDNAs (green) in eight *B. nigra* accessions. A) Karyotypes of *B. nigra* (IT no. 305656) (a), *B. nigra* (IT no. 305086) (b), *B. nigra* (IT no. 279264) (c), *B. nigra* (IT no. 279261) (d), *B. nigra* (IT no. 279259) (e), *B. nigra* (IT no. 279258) (f), *B. nigra* (IT no. 279253) (g), *B. nigra* (IT no. 279251) (h). B.) Haploid ideograms of eight genotypes depicting the conserved distribution and locus number of 18S rDNA. *polymorphic sites in chromosome-bearing 5S rDNA (Scale bar = 10 μ m).

Fig. 3. Detection of rDNAs in metaphase chromosomes of *B. oleracea* accessions. A) Karyotypes of *B. oleracea* var. *capitata* from India (a), *B. oleracea* var. *capitata* from Egypt (b), *B. oleracea* var. *capitata* from Tanzania (c), *B. oleracea* var. *capitata* from Uzbekistan (d), *B. oleracea* var. *gemmifera* from the UK (e), *B. oleracea* var. *gongylodes* from Japan (f), *B. oleracea* var. *alboglabra* from Thailand (g), *B. oleracea* var. *alboglabra* from the Netherlands (h), *B. oleracea* var. *alboglabra* from the Netherlands (i). B) Haploid ideograms of 10 genotypes showing the conserved distribution and locus number of 5S rDNA (green). +hemizygous sites in chromosome-bearing 18S rDNA (red) (Scale bar = 10 μ m).

observed across all genotypes (Chrs. #4, #6, and #7). Similar to our observations for the *B. rapa* genotypes, 5S rDNA exhibited three different loci patterns in *B. nigra* (Fig. 2B). In four genotypes of *B. nigra* (IT no. 305656, 279264, 279261, and 279259), only one major 5S rDNA was found in the short arm of Chr. #3 (Fig. 2B-a, c-e). In the other genotype (IT no. 279258), an extra locus of 5S rDNA was observed in the telomeric region of Chr. #4 (Fig. 2B-f). Interestingly, three other genotypes (IT no. 305086, 279253, and 279251) carried additional site of 5S rDNA in Chr. #6 (Fig. 2B-b, g, and h).

Interchromosomal asymmetry was measured for all eight genotypes (Table 1). The average values of A_2 and CV_{CL} indices were observed as 0.09 and 8.75, respectively. Similarly, intrachromosomal asymmetry was measured, and the average values of S/A_1 , TF %, A, and M_{CA} were found as 1.4, 38.5, 0.22, and 22.8, respectively.

B. oleracea chromosome evaluation

In contrast to *B. rapa* and *B. nigra*, the number and distribution

of 5S rDNA loci across nine *B. oleracea* genotypes were found to be conserved (Fig. 3A). Seven genotypes possessed two loci of 18S rDNA in Chr. #3 and #7 (Fig. 3B). However, two genotypes namely, *B. oleracea* var. *gongylodes* (Fig. 3B-g) and *B. oleracea* var. *alboglabra* (Fig. 3B-f), contained a hemizygous site of 18S rDNA juxtaposed to 5S rDNA in Chr. #2. The homogeneity of chromosome sizes and predominance of chromosomes with terminal/sub terminal centromeres were evaluated in each chromosome complements. The average values of A_2 and CV_{CL} indices were observed as 0.11 and 9.2, respectively (Table 1). Additionally, the average values for other indices such as S/A_1 , TF%, A, and M_{CA} were 1.4, 38, 0.24, and 24.4, respectively.

Discussion

Homogeneous karyotypes were observed for the morphotypes of the three *Brassica* diploids (Table 1). Based on our results, the three *Brassica* diploids species exhibited high intra-

chromosomal symmetry with more chromosomes having centromeres positioned in the median and submedian regions. However, marginally higher interchromosomal asymmetry was observed across the accessions of *B. rapa* than those of *B. nigra* and *B. oleracea* (Table 1). Further, the results obtained upon assessment of karyotype asymmetry have suggested that the *Brassica* species (being examined in this study) did not undergo consequential and extreme genomic makeover resulting from genome evolution (i.e., genome triplication and diploidization events) (Tang and Lyons 2012). The genomes remain to an ancestral type with more metacentric and submetacentric chromosomes (Baltisberger and Hörandl 2016). However, Peruzzi and Eroğlu (2013) have highlighted that a symmetric karyotype does not necessarily imply 'primitivity'. Overall the three species did not vary drastically in the karyotypic symmetry and chromosomal size homogeneity.

Interspecific variation of rDNA locus has been observed among several lines of *B. rapa*, *B. nigra*, and *B. oleracea*. Although, Hasterok et al. (2005a) have reported similar findings, the inclusion of new accessions for this specific analysis would ascertain the earlier outcome. Moreover, the interspecific differences of 5S rDNA chromosomal locations observed in several accessions of *B. rapa* and *B. nigra* (Figs. 1B and 2B), have also been observed in *Arabidopsis thaliana* and *Cucumis* spp. (Tutois et al. 2002, Zhang et al. 2016). Further, variation of rDNA loci has been documented to occur in a population of the same plant species (Breda et al. 2012). Cytogenetic changes and inferred dynamism have been reported to be triggered by genomic rearrangement, which results from contemporary hybridization and rDNA region instability due to deleterious recombination of repeats (Rosato et al. 2017, Kobayashi 2014). The interspecific variation of 5S rDNA locus has demonstrated how a specific gene locus can reorganize through interchromosomal translocation, which can further substantially impact genome function (Simon et al. 2018). However, the conjecture about the propensity of 5S rDNA gene to integrate into different genomic locations in *Brassica* is still unclear.

Nevertheless, the insertion of a transposable element in the 5S rDNA unit of *Triticum aestivum* showed differences in

the distribution and intensity of signals on the chromosomes (Sergeeva et al. 2017), suggesting a similar phenomenon to occur in *Brassica* species. In addition, the RNA interference (RNAi) pathway has been determined to play a role in the dispersal of rDNA repeats from their wild-type loci to other chromosomal regions (Begnis et al. 2018). This circumstance might explain the unequal number of rDNA locus and its differential distribution within the species. The heterogeneity of 5S rDNA concerning to its copy number and sequence has been determined to exist across and within species (Cloix et al. 2002). Heterochromatic repeats in the centromere and rDNA regions are predisposed to rapid divergence leading to genetic variations (Koo et al. 2011, Mehrotra and Goyal 2014). Moreover, the expression of 5S rDNA is moderated by two silencing pathways, methylation-dependent (RNAi) and methylation-independent (MOM pathway), resulting in only a fraction of genes being expressed in *A. thaliana* (Douet and Tourmente 2007). These variations might indicate differential usage of 5S rDNA loci in distinct ecotypes (Simon et al. 2018). On the contrary, a more homogenous distribution of 5S rDNA among the accessions of *B. oleracea* was observed (Fig. 3B), consistent with the results reported by Hasterok et al. (2006).

Conserved locus number and chromosomal distribution of 18S rDNA can be observed among *B. rapa* and *B. nigra* accessions (Figs. 1B and 2B). Similarly, a conserved number of rDNA sites were reported in varieties and subspecies of *Cucumis* spp. (Zhang et al. 2016). Ribosomal DNA homogeneity has been observed across the major clades of early land plants, which suggests an evolutionary rDNA stasis during land colonization and diversification (Rosato et al. 2016). On the contrary, *B. oleracea* accessions exhibited polymorphism in 18S rDNA for the site number and location (Fig. 3B). One accession of *B. oleracea* was reported to contain three loci of 45S rDNA (Armstrong et al. 1998), as opposed to two loci found in 80% of the genotypes studied in this paper. Several genotypes of *Lolium multiflorum* Lam, *Brachypodium pinnatum*, and *Brachypodium sylvaticum* have also shown variability in 45S rDNA loci that might be associated with their fragile sites (Bustamante et al. 2014, Breda et al. 2012). Differential characteristics such as the variability of rDNA site distribution

or location and signal intensities would indicate structural changes in the nuclear genome at the chromosome level involved in speciation and diversification (Bustamante et al. 2014, Storme and Mason 2014).

The polymorphisms in site number and distribution of 5S and 18S rDNA observed within each species strongly suggest an intraspecific genome size variation. A recent study has shown that more rDNA and Gypsy elements are present in *B. rapa* 'Z1' compared to *B. rapa* 'Chiifu', which greatly impacted genome size intraspecific variability (Boutte et al. 2020). The three diploid *Brassica* species have multiple 5S and 18S rDNA repeat clusters across all accessions, but *B. oleracea* has only one locus of 5S rDNA. In addition, the studied species manifest evident interspecific genome size variation. Recent reports indicate the smallest genome size was found in *B. rapa* with 442.9 Mb, followed by the other two diploids (*B. nigra* and *B. oleracea*) with 570-608 Mb and 630 Mb, respectively (Zhang et al. 2018, Perumal et al. 2020, Liu et al. 2014). A positive relationship between rDNA copy number and genome size for 162 species of plants and animals was reported (Prokopowich et al. 2003). This relationship is unquestionably important in facilitating evolutionary processes and maintaining genome stability (Kobayashi 2011). Genome sizes of two subspecies of *Japonica* were observed to vary due to differences in the copy number of the repetitive sequences (Haiyan et al. 2012, Rubio-Piña et al. 2016). Nuclear DNA content variation among plant genomes, mediated by the loss and gain of repeated DNA sequences, is connected to their adaptation to different environments (Cavallini & Natali 1991).

Overall, the obtained molecular cytogenetic data from mostly unreported *Brassica* genotypes have exhibited complex evolutionary dynamics of rDNA loci within and across the *Brassica* species, which have mounted evidence about the generative roles of repetitive sequences in shaping the chromosome karyotypes in plants (Li et al. 2017, Volkov et al. 2017).

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