Kor. J. Hort. Sci. Technol. 30(6):751-756, 2012 DOI http://dx.doi.org/10.7235/hort.2012.12168

Centromere Repeat DNA Originated from *Brassica rapa* is Detected in the Centromere Region of *Raphanus sativus* Chromosomes

Yoon-Jung Hwang[1†], Hee-Ju Yu[2†], Jeong-Hwan Mun[3], Kwang Bok Ryu[1], Beom-Seok Park[3], and Ki-Byung Lim[1*]

[1]*Department of Horticultural Science, Kyungpook National University, Daegu 702-701, Korea*
[2]*Department of Life Sciences, The Catholic University of Korea, Bucheon 420-743, Korea*
[3]*Department of Agricultural Biotechnology, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, Korea*

Abstract. Fluorescence in situ hybridization (FISH) is a powerful tool for the detection of DNA sequences in the specific region of the chromosomes. As well as for the integrated physical mapping, FISH karyotype analysis has to be preceded. Karyotype of *Raphanus sativus* 'Wonkyo 10039' was analyzed by a dual-color FISH technique; using various repetitive DNA probes, including 5S rDNA, 45S rDNA, and centromere retrotransposon. The length of the somatic metaphase chromosome ranged from 1.35 to 2.06 μm with a total length of 15.29 μm. The chromosome complements comprised of eight pairs of metacentrics and one pair of submetacentric. Bleached DAPI Band analysis revealed a heterochromatin region, covering 28.6% to 50.4% each chromosomes. 5S and 45S rDNA sequences were located on two and three pairs of chromosomes, respectively. The centromere retrotransposon of *Brassica* (CRB) is a major component in *Brassica* related species that has been maintained as a common centromere component. CRB signals were detected on the centromere and pericentromeric region of *R. sativus* 'Wonkyo 10039' and three basic *Brassica* species (*B. rapa*, *B. nigra*, and *B. oleracea*). These results will provide a valuable background for physical mapping and elucidation of the evolutionary relationship among the *Brassica* related species.

***Additional key words*:** bleached DAPI band, Brassicaceae, genome, heterochromatin, radish

Introduction

Radish, *Raphanus sativus* L., belongs to the genus *Raphanus* in the family Brassicaceae, which is classified into two sections of *Raphanus* DC. and *Hesperidopsis* Boiss (Kitamura, 1958). The phylogenic relationship between *Raphanus* and *Brassica* species suggests that *Raphanus* originated from hybridization between *Brassica rapa/oleracea* and *Brassica/nigra* lineages (Yang et al., 2002). Radish is an important edible vegetable in daily life and widely cultivated root crop in East Asia (Kaneko et al., 2007).

Molecular cytogenetic studies such as karyotype analysis and fluorescence in situ hybridization (FISH) provide comprehensive information pertaining to the phylogenetic relationship between the related species and study of plant evolution (Cao, 2003; Lou et al., 2010). To date, cytogenetic studies of radish have been conducted using conventional techniques such as Heidenhain's iron hematoxylin staining and the Feulgen squash method (Kaneko et al., 2007; Mukherjee, 1979; Richharia, 1937), as well as karyotype analysis using the molecular cytogenetic method (Hasterok et al., 2006; Schrader et al., 2000). However, none of these previous studies have clearly characterized the individual chromosome of *R. sativus*. In *Brassica* related species, it is difficult to identify the centromeric region without any centromere specific markers because of the small size and highly condensed condition of the centromere regions. Ribosomal DNAs have been classified into two different types, 45S (18S-5.8S-26S) and 5S rDNA, which are arranged in tandem repeats and located in one or more specific locations. Ribosomal DNA has been shown to be a useful marker for analysis of karyotypes with similar size and shape, particularly, in various *Brassica* species (Fukui et al., 1998;

***Corresponding author:** kblim@knu.ac.kr
[†]These authors are contributed equally to this work.
※ Received 29 August 2012; Revised 18 September 2012; Accepted 19 September 2012. We thank Dr. Soo-Hyung Park of the Vegetable Research Division, NIHHS, RDA for providing WK39. This work was supported by grants from the Next-Generation Biogreen21 program (PJ008019), Rural Development Administration, Korea.

Hasterok et al., 2006; Hwang et al., 2009; Lim et al., 2005; Snowdon et al., 1997). The bleached DAPI band (BDB) method is also advantageous in identification of the centromeric region in *Brassica* and its related species without the use of any probes (Hwang et al., 2009; Lim et al., 2005).

In this study, the dual color FISH technique was used to provide detailed characterization of each chromosome and the chromosomal distribution of 5S and 45S rDNA loci, as well as the centromeric retrotransposon of *Brassica* (CRB) in *R. sativus* cv. Wonkyo 10039 (WK39), which is a reference species of ongoing radish genome sequencing project in Korea.

Materials and Methods

Chromosome Preparation

The seeds of *R. sativus* 'WK39' (National Institute of Horticultural & Herbal Science, RDA, Korea, 2n = 2x = 18, RR) were sown on moist filter paper in a Petri dish and incubated at 25°C for 36 h for germination. The young root tips (1.0-2.0 cm long) were treated with 2 mM 8-hydroxyquinoline at 22°C for 3 h. The materials were subsequently fixed in Carnoy's solution (glacial acetic acid:ethanol = 1:3) and stored at -20°C. The fixed roots were then washed in 0.01 M citric acid-sodium citrate buffer prior to enzymatic treatment, after which the meristematic tips were digested in 0.3% of pectolyase, cellulase, and cytohelicase at 37°C for 60 min. Finally, the root tips were washed in distilled water and then squashed in a drop of 60% acetic acid.

Probe Preparation

A 9 kb fragment of 45S rDNA (18S-5.8S-25S rDNA) (Gerlach and Bedbrook, 1979) was labeled with biotin-16-dUTP by the Nick translation method. The 5S rDNA was obtained after PCR amplification according to the method described by Hwang et al. (2009) and labeled with digoxigenin-11-dUTP by Nick translation method.

Fluorescence in Situ Hybridization

The FISH procedure described by Lim et al. (2005) was used. Briefly, the hybridization mixture consisted of 50% deionized formamide, 10% dextransulfate, 2X SSC, and sheared salmon sperm DNA. The probes (5S rDNA, 45S rDNA, CRB) were mixed to a final concentration of about 20 ng·mL^{-1} and then denatured at 70°C for 10 min. The probe mixture was added to each slide and was covered using a cover slip. The hybridization was carried out in a humid chamber at 37°C for 24 h. The slides were subsequently washed in 2X SSC at room temperature for 5 min and 0.1X SSC at 42°C for 30 min, after which the fluorescence signal was detected using FITC conjugated anti-digoxygenin antibodies (Roche, Germany) and streptavidin Cy3 (Zymed Lab., USA). The slides were then mounted and counterstained in Vectashield containing 2 μL·mL^{-1} DAPI (4', 6-diamidino-2-phenylindole).

Microscopic Observation of Karyotype Analysis

The images were observed using an Olympus BX 61 fluorescent microscope equipped with a CCD camera and then analyzed with the Genus image analysis workstation software (Genus version 3.8, Applied Imaging Corporation, UK). More than five well-spread metaphase chromosomes were used for karyotype analysis. The paired chromosomes were confirmed based on their morphological characteristics, FISH signals, BDB pattern, and length of short and long arm. The chromosome morphological classification was conducted according to Levan et al. (1964).

Flow Cytometry Analysis

For genome size measurement, 1 cm^2 of young leaves was mixed with 0.2 mL of nuclear extraction buffer solution (solution A of the plant high-resolution DNA kit, Partec GmbH, Munster, Germany) and incubated at room temperature for 15 min. The extracted nuclear solution was then stained with staining solution (10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v) PVP K-30, 0.1% (v/v) Triton X-100, Propidium Iodide) and analyzed by flow cytometry (Partec CA-II, Munster, Germany). These whole processes were carried out at the Iribov breeding support laboratory in The Netherlands. Tetraploid *Brassica oleracea* was used as a reference for which the DNA contents calculated as 2.8 pg. The formulas for converting the number of nucleotide pairs (or base pairs) to picograms of DNA and vice-versa are based on Dolezel et al. (2003).

Results

Measurement of Relative Nuclear DNA Content

We confirmed that the number of chromosomes was 2n = 2x = 18 by conventional chromosome counting under the microscope (Fig. 1A). The relative nuclear DNA content in *R. sativus* 'WK39' was determined to be 552.6 Mbp by flow cytometry, which was equivalent to 1.2 pg per 2C level (Table 1).

Characteristics of Chromosome Morphology

The mitotic chromosome number of *R. sativus* 'WK39' was 2n = 2x = 18 and nine pairs of homologous chromosomes were obtained (Fig. 1). The somatic metaphase chromosome lengths ranged from 1.35 ± 0.06 μm (chromosome i, shortest)

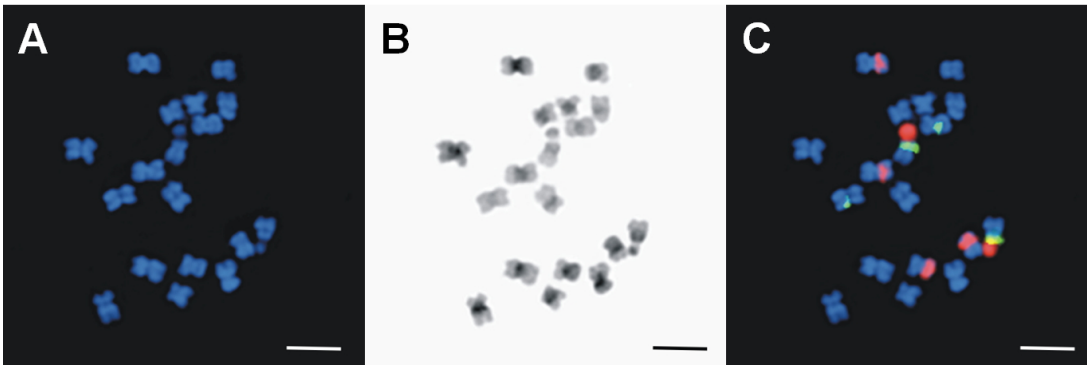

Fig. 1. FISH analysis of two types of rDNA (5S and 45S) sequences on somatic metaphase chromosome complements of *R. sativus*. A. DAPI staining of the metaphase complements. B. Centromeric region clearly revealed after BDB treatment. C. Merged signals of 5S (green fluorescence) and 45S (red fluorescence) on mitotic metaphase complement. Bars = 5 μm.

Table 1. Relative nuclear DNA content of *R. sativus* 'WK39' by flow cytometry.

Species	Ploidy level	Flow cytometry	
		Relative DNA content (2C)	
'WK39'	2n = 2x = 18	1.2 ± 0.3[z] pg	1105.2 Mbp[y]

[z]Values indicate mean ± standard deviation.
[y]Genome size (bp) = (0.978 × 10^9) × DNA content (pg).

Table 2. FISH karyotype analysis using chromosome lengths, number and positions of various repetitive DNAs, and BDB technique.

Chr. no.	Chromosome length (μm)			Centromere index (%)[z]	Chromosome type	FISH			BDB[v] (%)
	Short arm (S)	Long arm (L)	Total length (S + L)			5S rDNA	45S rDNA	CRB[w]	
a	0.96 ± 0.04	1.11 ± 0.01	2.06 ± 0.04	46.6	m[y]	-	L	+	34.0
b	0.51 ± 0.01	0.93 ± 0.02	1.44 ± 0.02	35.4	sm[x]	S	S	+	48.6
c	0.87 ± 0.03	1.05 ± 0.05	1.92 ± 0.02	45.3	m	L	-	+	31.8
d	0.85 ± 0.03	1.05 ± 0.06	1.90 ± 0.05	44.7	m	-	-	+	32.1
e	0.79 ± 0.05	1.10 ± 0.08	1.89 ± 0.03	41.8	m	-	-	+	28.6
f	0.76 ± 0.05	0.93 ± 0.03	1.69 ± 0.08	45.0	m	-	-	+	29.0
g	0.61 ± 0.11	1.00 ± 0.10	1.61 ± 0.01	37.9	m	-	S	+	32.9
h	0.63 ± 0.07	0.79 ± 0.02	1.42 ± 0.09	44.4	m	-	-	+	50.4
i	0.53 ± 0.02	0.82 ± 0.05	1.35 ± 0.06	39.3	m	-	-	+	39.6
	6.51	8.78	15.29						

[z]Short arm/total length × 100.
[y]Metacentric.
[x]Submetacentric.
[w]Centromeric retrotransposon of *Brassica*.
[v]Bleached DAPI band.

to 2.06 ± 0.04 μm (chromosome a, longest) with a total length of 15.29 μm (Table 2). According to Levan et al. (1964), the karyotype formula was 8 m + 1 sm, indicating that the homologous chromosome was composed of eight pairs of metacentrics (chromosome a, c, d, e, f, g, h, and i) and one pair of submetacentric (chromosome b). The centromere index varied from 35.4% (chromosome b) to 46.6% (chromosome a), which coincides with a chromosome composed metacentrics or submetacentrics. The BDB covered 28.6% to 50.4% of each chromosome, which revealed that the heterochromatin regions were positioned on centromere region in whole chromosomes and the nucleolar organizing region (NOR) in chromosome b. The heterochromatin domain was located asymmetrically in the short arm and NOR of chromosome b. The BDB block intensity was the greatest on chromosome e, with the intensity decreasing on the following order: chromosome a, d, g, i, f, h, and c.

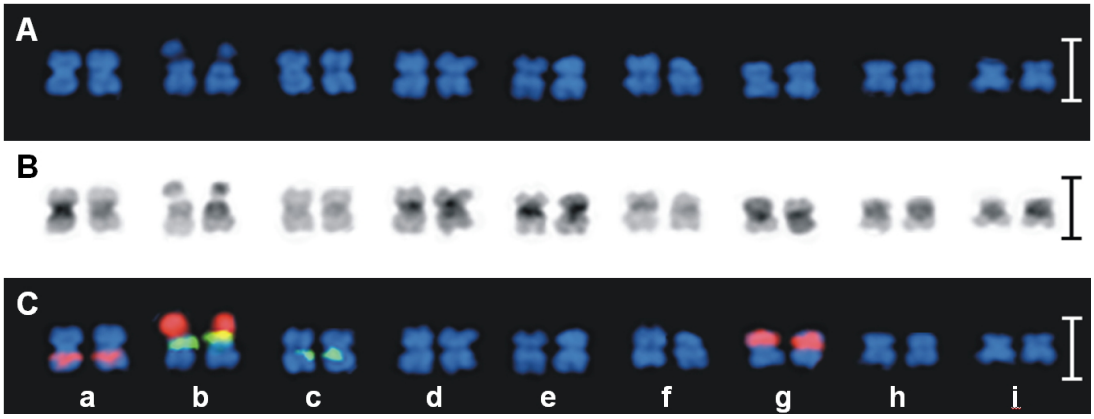

Fig. 2. FISH mapping of 5S and 45S rDNAs on metaphase chromosomes. A. Somatic metaphase chromosomes stained with DAPI. B. Karyotype analysis by BDB technique. C. Merged FISH signals of 5S (green) and 45S (red) rDNA. Bars = 3 μm.

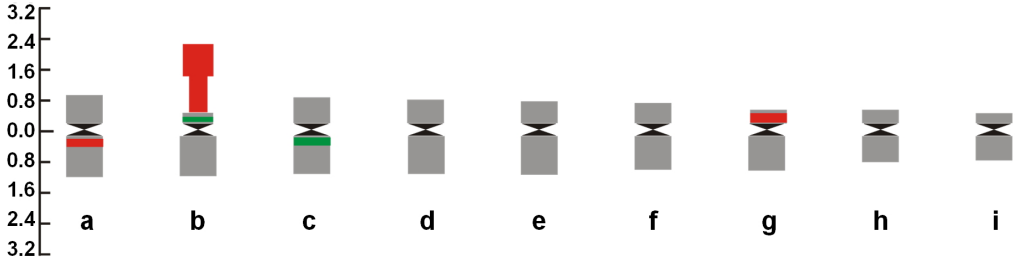

Fig. 3. FISH idiogram of *R. sativus* 'WK39' showing the location of 5S (green) and 45S (red) rDNA signals.

Ribosomal DNA loci in *R. sativus* 'WK39'

There were two pairs of 5S (green fluorescence) and three pairs of 45S rDNAs (red fluorescence) detected in *R. sativus* 'WK39' (Figs. 2 and 3). The 45S rDNA signals were observed on the long arm of chromosome a, short arm of chromosome g, and NOR region of chromosome b. Chromosome b showed the largest signals, which covered the entire NOR region, while chromosomes g and a were arranged in decreasing order based on their intensity. In addition, the 5S rDNA signals of chromosomes b and c were located in the interstitial region of the short and long arm, respectively, which were adjacent to the centromere. 5S and 45S rDNAs were co-localized on chromosome b.

Centromeric Retrotransposon of *Brassica* loci in *R. sativus* 'WK39'

The FISH karyotype was used on somatic metaphase chromosomes by centromere retrotransposon of *B. rapa* (CRB) as a probe for identification of the centromere and pericentromere in *R. sativus* 'WK39' and three basic *Brassica* species (*B. rapa*, *B. nigra*, and *B. oleracea*) (Fig. 4). The CRB probes hybridized at the centromere and pericentromeric region of all chromosomes in *R. sativus* 'WK39' and basic three *Brassica* species (*B. rapa*, *B. nigra*, and *B. oleracea*).

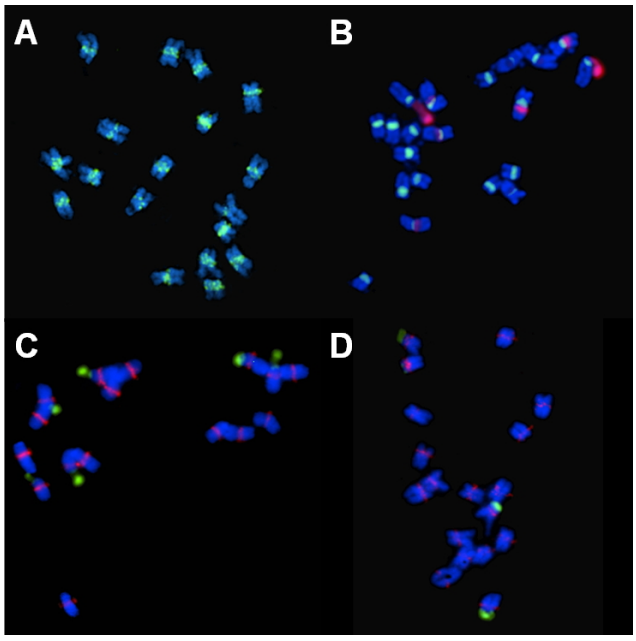

Fig. 4. Fluorescence in situ hybridization with centromeric retrotransposon of *Brassica* on A, *R. sativus* 'WK39' (green fluorescence, RR genome); B, *B. rapa* (green fluorescence, AA genome); C, *B. nigra* (red fluorescence, BB genome); and D, *B. oleracea* (red fluorescence, CC genome).

Discussion

The genome size is one of the key factors to understanding

evolutionary events in plants. The family Brassicaceae includes various species that were evaluated as important materials in evolution studies because of their relatively small genome size and complex polyploidy histories. Previous studies reported that the genome size of three basic diploid *Brassica* species ranged from 529 Mbp (*B. rapa*) to 696 Mbp (*B. oleracea*) and *R. sativus* was estimated to be 573 Mbp (Johnston et al., 2005). Based on the result of this study, the genome size of *R. sativus* 'WK39' was calculated as 552.6 Mbp. Additionally, BDB results, which enable constitutive heterochromatic domains to be distinguished, indicating that 36.3% of the total genome was composed of the heterochromatic domain in *R. sativus* 'WK39'.

Because of the morphological similarities and unambiguous centromeric regions in small sized chromosomes (1-3 μm), especially in *Brassica* species, it is difficult to identify their homologous chromosomes by conventional staining methods and in fluorochromes (Geber and Schweizer, 1988; Hwang et al., 2009). Until now, none of the previous studies reported the precise and accurate karyotype in *R. sativus*, but only reveals the location of the ribosomal signals (Hasterok et al., 2006; Schrader et al., 2000). Previous studies reported that 5S and 25S rDNA signals, 25S rDNA determined the 45S rDNA (18S-5.8S-25S) site, were slightly differ from our results. There were four loci of 5S and 25S rDNAs detected in *R. sativus* 'Peglette', four and six loci in *R. sativus* '186', four and five loci in *R. sativus* 'Opolanka' (Hasterok et al., 2006; Schrader et al., 2000). We observed two pairs (four loci) of 5S rDNA and three pairs (six loci) of 45S rDNA in *R. sativus* 'WK39'. This discrepancy between our results and those of other studies might be due to the intravarietal polymorphism within individual varieties (Hasterok et al., 2006). In general, the rDNA hybridization patterns, indicated that chromosome b of *R. sativus* 'WK39' is highly similar to chromosome A3 of *B. rapa*, and both have a distinctive NOR structure (Mun et al., 2010). Moreover, chromosome a, c, and g correspond with chromosome A9 (chromosome 1), A1 (chromosome 3), and A7 (chromosome 7), respectively (Lim et al., 2005).

CRB is a complete retrotransposon located in three positions in the centromeric repetitive of *Brassica* (CentBr) array. CRB and CentBr are assumed to be the major components of the centromeric region in *Brassica* species (Lim et al., 2007). Lim et al. (2007) reported that CRB was maintained as a common centromere component in *Brassica* species. In our results, we also observed the CRB signals were located in centromeric region in three basic *Brassica* species and *R. sativus*. These results assumed that CRB originated from common ancestor of *Brassica* species and it inserted before divergence of *Brassica* species and *R. sativus*.

In this study, we constructed a detailed FISH karyotype in *R. sativus* 'WK39' using rDNA and centromeric retrotransposon. These results will provide a useful reference for elucidation of the phylogenetic relationships among species in the family Brassicaceae as well as a guide line to integrate genetic and sequence data generated from the ongoing genome sequencing project of radish.

Literature Cited

Cao, W. 2003. Cytogenetic and molecular genetic evidence on evolution of genus *Triticum*, p. 223-247. In: A.K. Sharma and A. Sharma (eds.). Plant genome: Biodiversity and evolution. Phanerogam-Angiosperm, Science Publisher, Enfield (NH).

Dolezel, J., J. Bartos, H. Voglmayr, and J. Greilhuber. 2003. Letter to the editor: Nuclear DNA content and genome size of trout and human. Cytometry 51A:127-128.

Fukui, K., S. Nakayama, N. Ohmido, H. Yoshiaki, and M. Yamabe. 1998. Quantitative karyotyping of three diploid *Brassica* species by imaging methods and localization of 45S rDNA loci on the identified chromosomes. Theor. Appl. Genet. 96:325-330.

Gerber, G. and D. Schweizer. 1988. Cytochemical heterochromatin differentiation in *Sinapis alba* (Crucuferae) using a simple air-drying technique for producing chromosome spreads. Plant Syst. Evol. 158:97-106.

Gerlach, W.L. and J.R. Bedbrook. 1979. Cloning and characterization of ribosomal rDNA genes from wheat and barley. Nucleic Acids Res. 7:1869-1885.

Hasterok, R., E. Wolny, M. Hosiawa, M. Kowalczyk, S. Kulak-Ksiazczyk, T. Ksiazczyk, W.K. Heneen, and J. Maluszynska. 2006. Comparative analysis of rDNA distribution in chromosome of various species of Brassicaceae. Ann. Bot. 97:205-216.

Hwang, Y.J., H.H. Kim, S.J. Kwon, T.J. Yang, H.C. Ko, B.S. Park, J.D. Chung., and K.B. Lim. 2009. Karyotype analysis of three *Brassica* species using five different repetitive DNA markers by fluorescence in situ hybridization. Kor. J. Hort. Sci. Technol. 27:456-463.

Johnston, J.S., A.E. Pepper, A.E. Hall, Z.J. Chen, G. Hodnett, J. Drabek, R. Lopez, and J. Price. 2005. Evolution of genome size in Brassicaceae. Ann. of Bot. 95:229-235.

Kaneko, Y., C. Kimizuka-Takagi, S.W. Bang, and Y. Matsuzawa. 2007. Radish, p. 141-160. In: C. Kole (ed.). Genome mapping and molecular breeding in plants, Vol. 5. Vegetables Springer-Verlag, Berlin Heidelberg, Germany.

Kitamura, S. 1958. Varieties and transitions of radish, p. 1-19. In: I. Nishiyama (ed.). Japanese radish. Japan Science Society Press, Tokyo, Japan.

Levan, A., K. Fredga, and A.A. Sandberg. 1964. Nomenclature for centromeric position on chromosomes. Hereditas 52:517-523.

Lim, K.B., J.H. De Jong, T.J. Yang, J.Y. Park, S.J. Kwon, J.S. Kim, M.H. Lim, J.A. Kim, M.A. Jin, S.H. Kim. Y.P. Lim, J.W. Bang, H.I. Kim, and B.S. Park. 2005. Characterization of rDNA and tandem repeats in the heterochromatin of *Brassica rapa*. Mol.Cell.19:436-444.

Lim, K.B., T.J. Yang, Y.J. Hwang, J.S. Kim, J.Y. Park, S.J.

Kwon, J.A. Kim, B.S. Choi, M.H. Lim, M. Jin, H. de Jong, I. Bancroft, Y.P. Lim, and B.S. Park. 2007. Characterization of the centromere and peri-centromere retrotransposons in *Brassica rapa* and their distribution in related *Brassica* species. Plant J. 49:173-183.

Lou, Q., M. Iovene, D.M. Spooner, C. Robin Buell., and J.M. Jiang. 2010. Evolution of chromosome 6 of *Solanum* species revealed by comparative fluorescence in situ hybridization mapping. Chromosoma 119:435-442.

Mukherjee, P. 1979. Karyotypic variation in ten strains of Indian radish (*Raphanus sativus* L.). Cytologia 44:347-352.

Mun, J.H., S.J. Kwon, Y.J. Seol, J.A. Kim, M. Jin, J.S. Kim, M.H. Lim, S.I. Lee, J.K. Hong, T.H. Park, S.C. Lee, B.J. Kim, M.S. Seo, S. Baek, M.J. Lee, J.Y. Hahn, Y.J. Hwang, K.B. Lim, J.Y. Park, J. Lee, T.J. Yang, H.Y. Yu, I.K. Choi, B.S. Choi, S.R. Choi, N. Ramchiary, Y.P. Lim, F. Fraser, N. Drou, E. Soumpourou, M. Trick, I. Bancroft, A.G. Sharpe, I.A.P. Parkin, J. Batley, D. Edwards, and B.S. Park. 2010. Sequence and structure of *Brassica rapa* chromosome A3. Genome Biol. 11:R94.

Richharia, R.H. 1937. Cytological investigation of *Raphanus sativus*, *Brassica oleracea*, and their F_1 and F_2 hybrids. J. Genet. 34:19-44.

Schrader, O., H. Budahn, and R. Ahne. 2000. Detection of 5S and 25S rRNA genes in *Sinapis alba*, *Raphanus sativus* and *Brassica napus* by double fluorescence in situ hybridization. Theor. Appl. Genet. 100:665-669.

Snowdon, R.J., W. Köhler, and A. Köhler. 1997. Chromosomal localization of rDNA loci in the *Brassica* A and C genomes. Genome 40:582-587.

Yang, Y.W., P.Y. Tai, Y. Chen, and W.H. Li. 2002. A study of the phylogeny of *Brassica rapa*, *B. nigra*, *Raphanus sativus*, and their related genera using noncoding regions of chloroplast DNA. Mol. Phylogenet. Evol. 23:268-275.