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Karyotype Analysis of Korean *Lilium maximowiczii* Regal Populations

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Detailed karyotypes analyses were carried out in 30 populations of Korean L. maximowiczii Regel. The populations were constituted with 43 diploids (*2n=2x*=24) and 38 triploids (*2n=3x*=36). The lengths of mitotic metaphase chromosomes ranged from 13.22 μm to 25.18 μm, with a total length of 203.78 μm in diploid plants. In triploid plants, the chromosome lengths ranged from 14.28 μm to 27.75 μm, with a total length of 214.42 μm. There was only one cytotype identified by karyotype analysis in both diploid and triploid populations. The basic set of somatic chromosome complement consists of two metacentrics (chromosomes **a** and **b**), two subtelocentrics (chromosomes **c** and **d**), and eight telocentrics (chromosomes **e**, **f**, **g**, **h**, **i**, **j**, **k**, and **l**) in both diploid and triploid populations. Four loci of 45S rRNA genes were observed by FISH, and three of the four loci correspond to the secondary constrictions. One additional 45S rRNA locus was observed by FISH at the near telomere of the long arm of a set of telocentric chromosomes. There were two 5S rRNA gene loci, which were closely placed on the long arm of a set of subtelocentric chromosomes that were not the 45S rRNA loci–carrying chromosomes. In conclusion, the karyotypes in both diploid and triploid populations were identical, implying that the triploid organisms are autotriploids.

Key words: autotriploid, chromosomal landmarks, FISH, Maximowicz's lily

INTRODUCTION

Twelve lily species, mainly belonging to the sections *Sinomartagon*, *Martagon*, and *Leucolirion*, are native to Korea (Kim, 1996). *Lilium* species found in Korea include all three types of the leaf arrangement (with single whorls, multiple whorls, and scattered leaves) within the genus (Lighty, 1968). *L. maximowiczii*, which is believed to be the most closely related to the origin of triploid *L. lancifolium* based on morphological and cytological characteristics (Noda, 1966 and 1974), is widely distributed in Eastern Asia, including Japan, Korea, Manchuria, northern China, and Ussuri in Russia (Asano, 1986; Baranova, 1969).

Wilson (1925) reported a wide distribution of *L. maximowiczii* around the mountain plateau of Hamkyungnam–do Province in North Korea. Lighty (1968) explored *Lilium* populations in South Korea and reported a limited geographical distribution of *L. maximowiczii* only in Gangwon–do Province. *L. maximowiczii* and *L. leichtlinii* var. *leichtlinii* are the most common lilies in Japan and are widely distributed throughout the Japanese archipelago from the seashore to the mountains over 2,000 meters in elevation (Shimizu, 1969). Jeong (1991) and other researchers collected specimens sporadically from Keumo–do Island, the southernmost part of Korea, to Gangwon–do Province, the northernmost part of South Korea; however, some specimens are suspected to have been misidentified, and their identities are uncertain (KBIS, 2013; Kim *et al*., 2015). We extensively examined the reported sites of the specimens in the southern part of Korea, but were unable to find *L. maximowiczii* plants in many places now. *L. maximowiczii* and *L. leichtlinii* var. *leichtlinii* have been thought to comprise only diploid (*2n=2x*=24) plants in Japan (Morinaga and Fukushima, 1931; Kumazawa and Kimura, 1947; Kurita, 1948; Stewart, 1947; Noda, 1956, 1966, 1974, and 1977; Ogihara, 1960; Song and Seo, 1988) and Korea (Son, 1982; Song, 1987; Jeong, 1991; Sultana *et al*., 1010). However, detailed ploidy analyses have not been attempted on these specimens.

Recently, we found that *L. maximowiczii* plants grow in very scattered populations and are distributed in steep mountain ranges in Chungcheongbuk–do, Gyeonggi–do, and Gangwon–do Provinces, Korea. Their habitats do not overlap with those of diploid *L. lancifolium* in South Korea (Kim *et al*., 2015). Furthermore, we found that triploid plants are far more common than diploid plants in our analysis of Korean *L. maximowiczii* populations, and that triploid and diploid populations have only one cytotype from the conventional chromosome analysis (Kim *et al*., 2015). These results made it clear that Korean *L. maximowiczii* includes both diploid and triploid forms in the *Lilium* genus.

On the other hand, Noda (1977) reported A and B cytotypes of karyotypes in the Japanese *L. maximowiczii* collections. While the arm index and general chromosome morphology were similar between two cytotypes, chromosomes in the A cytotypes were larger than those of the B cytotypes. He also reported the presence

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of different numbers of B–chromosomes in the Japanese accessions. It is interesting that while Japanese accessions have numerous B–chromosomes, none of the Korean accessions have B–chromosomes, which requires further analysis of detailed karyotype variations in Korean *L. maximowiczii*. Conventional staining methods have some limitations in unequivocal identification of individual chromosomes of similar size and morphology in *Lilium* species (Song, 1987). Fluorescence in situ hybridization (FISH), using ribosomal DNAs as probes, provides information of nucleolar organizing regions (NORs) (Heslop–Harison *et al*., 1991) and has been used to analyze chromosomes in several *Lilium* species (Lim *et al*., 2001; Marasek *et al*., 2004; Hwang *et al*., 2011).

The purpose of this study is to understand detailed karyotype variations in diploid and triploid populations of Korean *L. maximowiczii*, using chromosomal landmarks such as chromosome length, arm ratio, secondary constrictions, DAPI bands, and FISH signals using 45S rRNA and 5S rRNA genes.

MATERIAL AND METHODS

Materials

Forty–three diploid bulbs were collected from fourteen populations, and thirty–eight triploid bulbs were collected from sixteen populations (Table 1). These accessions were planted in pots containing a mixture of perlite and peat moss (1:3) and cultivated at the university farm of Kangwon National University, South Korea. The active root tips were harvested in the early morning,

Table 1. Locality and number of plants analyzed from each native Korean *L. maximowiczii* population

Population number	Locality	Ploidy	No. of plants analyzed[z]	Accession number[y]
1	Daegi–ri, Wangsan–myeon, Gangneung–si, GW	2x	10	GWL1458
2	Yongsan–ri,Daegwanryeong–myeon, Pyeongchang–gun, GW	2x	5	GWL1451
3	Songgye–ri, Sillim–myeon, Wonju, GW	2x	2	GWL1484
4	Hoenggye–ri,Daegwanryeong–myeon,Pyeongchang–gun,GW	2x	4	GWL1302
5	Mahyeon–ri, Sangseo–myeon, Hwacheon–gun, GW	2x	2	GWL1473
6	Woechon–ri, Cheolwon–ub, cheolwon–gun, GW	2x	2	GWL1485
7	Samhwa–ri, Hanam–myun, Hwacheon–gun, GW	2x	2	GWL1459
8	Mahyeon–ri, Keunnam–myun, cheolwon–gun, GW	2x	2	GWL1481
9	Sanyang–ri, Sangseo, Hwacheon–gun, GW	2x	2	GWL1486
10	Sanyang–ri, Sangseo–myeon, Hwacheon–gun, GW	2x	2	GWL1487
11	Sanyang–ri, Sangseo–myeon, Hwacheon–gun, GW	2x	3	GWL1488
12	Sanyang–ri, Sangseo–myeon, Hwacheon–gun, GW	2x	2	GWL1489
13	Eron–ri, SeoSeok–myun, Hongcheon–gun, GW	3x	2	GWL1457
14	Mandae–ri, Haean–myeon, Yanggu–gun, GW	3x	2	GWL1300
15	Bangdong–ri, Kirin–myun,Inje–gun, GW	3x	2	GWL1469
16	Bangdong–ri, Kirin–myun, Inje–gun, GW	3x	2	GWL1490
17	Mahyeon–ri, Keunnam–myun, cheolwon–gun, GW	3x	2	GWL1491
18	Mahyeon–ri, Sangseo–myeon, Hwacheon–gun, GW	3x	2	GWL1477
19	Eoron–ri, Nam–myeon, Inje–gun, GW	3x	2	GWL1466
20	Tosung–ri, Galmal–eup, Cheolwon–gun, GW	3x	2	GWL1492
21	Sagok–ri, Geunnam–myeon, Cheolwon–gun, GW	3x	3	GWL1493
22	Yookdan–ri, Keunnam–myun, Cheolwon–gun, GW	3x	2	GWL1494
23	Buchon–ri, Sangseo–myeon, Hwacheon–gun, GW	3x	2	GWL0115
24	Samhwa–ri, Hanam–myeon, Hwacheon–gun, GW	3x	5	GWL1460
25	Suib–Ri, Seojong–myeon, Yangpyeong–gun, GG	3x	2	GWL1253
26	Song–am–ri, Sabuk–myeon, Chuncheon–si, GW	3x	2	GWL1425
27	Saeun–ri, chilsung–myun, Goesan–gun, CB	3x	3	GWL1118
28	Cheondong–ri,Danyangub, Danyang–gun, CB	2x	6	GWL1495
29	Cheondong–ri,Danyangub, Danyang–gun, CB	3x	3	GWL1496
30	Cheondong–ri,Danyangub, Danyang–gun, CB	2x	7	GWL1497

[z] *2x=43 plants in 13 sites, 3x=38 plants in 15 sites*

[y] GWL serial number is the accession number of registration to RDA Gene bank Korea

Abbreviation: GWL, Gangwon–do lily germplasm; GW, Gangwon–do; GG, Gyeonggi–do; CB, Chungchungbuk–do

and pretreated in 0.2% colchicine solution at 4°C for 24 h. The materials were rinsed three times with tap water, then fixed in acetic acid : ethanol (1:3) for 24 h at room temperature. The root tips were then rinsed three times with tap water and stored in 70% ethanol at –20°C.

Karyotype analysis

The stored root tips were rinsed thoroughly with water before softening in 1 N HCl at 60°C for 5 min. Chromosomes of root tip cells at metaphase were stained in 1% (w/v) aceto–carmine and prepared using a squash technique described by Fernandez *et al*. (1998) with some minor modifications. Five well–spread sets of metaphase chromosomes from each accession were photographed. The karyotype analysis was carried out according to the method developed by Noda (1977). The total chromosome length, the length of long arm and short arm, the arm ratio index (ratio of long arm length to short arm length), and the centromeric index (percentage of short arm length to the total length of chromosome) were measured using Micro Measure 3.3 software (Reeves and Tear, 2000). Nomenclature for the centromeric position on chromosome was classified according to the arm index (1.0–1.7: metacentric; 1.7–3.0: submetacentric; 3.0–7.0: subtelocentric; ≥7.0: telocentric) (Levan *et al*., 1964).

Slide preparation for fluorescence in situ hybridization (FISH)

The stored root tips were rinsed several times with water, then cut to about 1 mm in length and put into 1.5 ml tubes with enzyme solution (pH 4.5) containing 0.3% (w/v) cellulase RS, 0.2% (w/v) pectolyase Y23, 0.2% (w/v) macerozyme R10, 0.07 M KCl, and 1 mM methylene diamine tetra–acetic acid (EDTA), then incubated at 37°C for 10 min. The root tips were then gently rinsed in distilled water. One softened root tip was placed on each slide, immediately mixed with 10 μl 1% aceto–carmine using a needle, covered with a cover glass (20 × 20 mm), and squashed with thumb. The positions of the well–spread chromosomes were marked under the microscope. The slide was placed in liquid nitrogen, and after the cover glass was removed using a razor blade, it was quickly transferred to absolute ethanol for 5 min, air–dried, and stored again at –20°C until use.

Probes for FISH

The 5S rDNA and 45S rDNA probes were directly labeled with fluorescein–12–dUTP and tetramethylrhodamine–5–dUTP, respectively (Duchefa, Enzo Life science, USA). Nick translation was performed according to the manufacturer's instructions (Roche, Germany). To remove unbound nucleotide and enhance probe concentration, the protocol described by Karafiátová *et al*. (2013) was followed with some modifications. The labeled probe product was transferred into a 1.5 ml tube; precipitated by adding 3 M sodium acetate (NaAc; pH 5.2), cool absolute ethanol, and 100 μg salmon sperm DNA (Sigma–Aldrich, Germany); then incubated at –20°C overnight. The probes were centrifuged at 4°C at 14,000 × g for 15 min. The supernatant was discarded, and 200 μl 70% ethanol was added before centrifuging the probes again. The supernatant was again discarded, and the probes were air–dried in the dark. The probes were dissolved in hybridization buffer containing 50% deionized formamide (v/v), 10% dextran sulfate (w/v), 0.5% sodium dodecyl sulfate, and 2X saline–sodium citrate buffer (SSC). The probe quality was confirmed on a 1% agarose gel, and the probes were stored at –20°C.

Fluorescence in situ hybridization and microscopy

The *in situ* hybridization procedure (Zhang *et al*., 2014) consisted of four steps. 1) Pretreatment of chromosomes: the slide was treated with 100 μg mL^{-1} RNase in 2X SSC for one hour at 37°C then stabilized in 4% paraformaldehyde for 10 min at room temperature. After each treatment, the slide was washed with 2X SSC three times for 5 min each. The slide was then dehydrated in a series of 70%, 95%, and 100% ethanol for 5 min each and air–dried at room remperature. 2) Denaturation of chromosomes: the slide was treated with 70% formamide in 2X SSC at 70°C for 2 min, quickly dehydrated in a series of 70%, 95%, and 100% ethanol at –15°C for 5 min each, and air–dried at room temperature. 3) Hybridization: hybridization buffer containing the probe and block DNA was denatured at 75°C for 10 min, quickly put on ice for 10 min, and briefly spun down. This mix was added (30 μl) to each slide, and the slide was covered with a cover glass (20 × 40 mm). The DNA was denatured at 80°C for 5 min then incubated at 37°C overnight in a humid box. 4) Post–hybridization and detection: The cover glass was removed, and the slide was washed with 2X SSC two times at 42°C for 5 min each, with 50% formamide for 10 min at 42°C, and again with 2X SSC two times at 42°C and one time at room temperature for 5 min. The slide was then dehydrated in a series of 70%, 95%, and 100% ethanol at room temperature for 5 min each and air–dried at room temperature in the dark. The chromosomes were counterstained with 30 μl 4',6'–diamidino–2–phenylindole (DAPI) in Vectashield (Vector Laboratories, Inc., USA), examined using a fluorescent microscope (Nikon Eclipse 80i, Japan), and captured using a Nikon digital camera (D90, Japan). Images were edited in Adobe Photoshop CS4 to facilitate the analysis of individual chromosomes.

RESULTS

Somatic chromosomes of *L. maximowiczii*

The diploid plants have the somatic chromosome numbers of $2n=2x=24$ (Fig. 1). We did not detect aneuploidy or B–chromosomes. Chromosome length at metaphase ranged from 13.22 μm to 25.18 μm, and the total chromosome length of the haploid complement was 203.78 μm. The 12 somatic chromosomes included two metacentric (chromosome **a** and **b**), two subtelocentric (chromosome **c** and **d**), and eight telocentric chromosomes (chromosome **e**, **f**, **g**, **h**, **i**, **j**, **k**, and **l**) (Table 2). The chromosomes were designated according to the system established by Noda (1977).

The triploid plants have the somatic chromosome

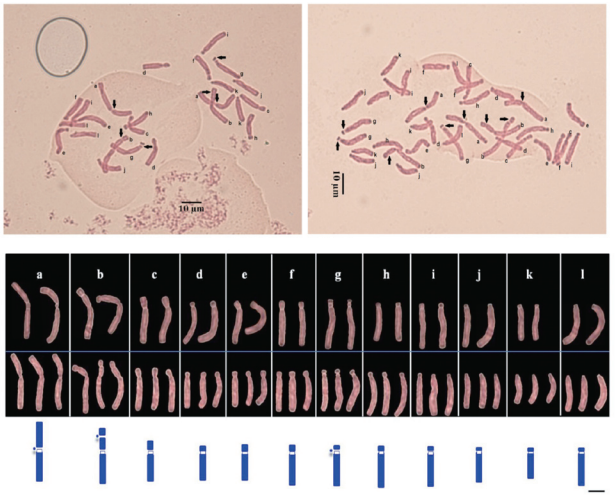

Fig. 1. Mitotic metaphase chromosomes and karyotypes of diploid and triploid *L. maximowiczii* using aceto-carmine stain. Idiogram shows the secondary constructions on the chromosomes of *L. maximowiczii*. Arrows indicate secondary constrictions. The bars represent a length of 10 μm.

numbers of $2n=3x=36$ (Fig. 1). We did not detect aneuploidy or B–chromosomes in the triploid accessions. Chromosomes of the triploids were measured slightly longer than those of diploid with a range from 14.28 μm to 27.75 μm. There were three sets of two metacentric chromosomes (**a** and **b**), three sets of two subtelocentric chromosomes (**c** and **d**), and three sets of eight telocentric chromosomes (**e**, **f**, **g**, **h**, **i**, **j**, **k**, and **l**) (Table 3). The total chromosome length of the monoploid complement was 214.42 μm.

Chromosome identification

Individual chromosomes in both diploid and triploid plants were distinguished by chromosome length, chromosome index (arm ratio), secondary constrictions, DAPI bands, and rRNA loci by conventional aceto–carmine, DAPI staining and FISH (Fig. 2 and 3, Tables 2 and 3). Three sets of chromosomes exhibited constant secondary constrictions proximal to the centromere in the short arm (chromosome **a** and **g**) and near the telomere in the short arm (chromosome **b**). There were five sets of chromosomes showing DAPI bands, which are believed to be constitutive heterochromatic regions. The DAPI bands were at the interstitial regions in short arms of metacentric chromosomes and the middle of long arms of one subtelocentric chromosome and two telocentric chromosomes. Four loci for 45S rRNA genes were observed. Three of the 45S rRNA loci detected by FISH corresponded to the secondary constrictions. One additional 45S rRNA locus was observed by FISH at the proximal telomere of the long arm of a set of telocentric chromosomes on chromosome **f** (Table 2 and 3, Fig. 2 and 3). Two 5S rRNA gene loci were observed on the long arm of a set of subtelocentric chromosomes (chromosome **c**) that do not possess 45S rRNA loci.

The chromosomes were identified based on the following chromosome landmarks, indices, and lengths. Chromosome **a** is metacentric and the longest chromosome. It also contains a 45S rRNA locus on the minor secondary constriction near the centromere of the short arm and a DAPI band in the interstitial site of the short arm. Chromosome **b** is metacentric and the second–longest chromosome. It possesses a 45S rRNA locus at a large secondary constriction near the telomere of the short arm and a DAPI band at the interstitial site in short arm. Chromosome **c** is a subtelocentric chromosome containing two 5S rRNA loci near the telomere of the long arm. Chromosome **d** is a subtelocentric chromosome that includes a DAPI band proximal to the centromere in the long arm. Chromosome **e** is the longest chromosome among telocentric chromosome that includes a DAPI band proximal to the centromere in the long arm. Chromosome

Table 2. Karyotype summary of natural diploids of Korean *L. maximowiczii* populations

Chromosome number	Length each (μm)	Long arm length (μm)	Short arm length (μm)	Arm Ratio (L/S)	Chromosome type	Location of rDNA gene		DAPI band
						5S	45S	
a	25.18±0.45	14.63±0.30	10.55±0.25	1.39±0.03	metacentric	–	S	S
b	22.54±0.34	13.74±0.20	8.81±0.17	1.56±0.02	metacentric	–	S	S
c	14.85±0.27	11.58±0.23	3.28±0.08	3.53±0.13	subtelocentric	L	–	–
d	14.79±0.35	12.77±0.35	2.02±0.05	6.32±0.28	subtelocentric	–	–	L
e	17.74±0.36	15.76±0.38	1.98±0.08	7.91±0.37	telocentric	–	–	L
f	16.06±0.33	14.25±0.34	1.80±0.09	7.96±0.68	telocentric	–	L	–
g	17.51±0.30	15.95±0.29	1.56±0.05	10.22±0.54	telocentric	–	S	–
h	16.81±0.35	15.37±0.34	1.44±0.05	10.66±0.79	telocentric	–	–	–
i	16.38±0.29	15.12±0.27	1.26±0.03	12.01±0.48	telocentric	–	–	L
j	14.61±0.25	13.63±0.24	0.98±0.06	13.95±0.17	telocentric	–	–	–
k	13.22±0.30	12.37±0.30	0.85±0.02	14.55±0.64	telocentric	–	–	–
l	14.09±0.40	13.47±0.40	0.62±0.02	21.70±1.26	telocentric	–	–	–
Total	203.78	168.63	35.15					

[z]Mean ± standard error, n = 100; S: short arm; L: long arm

Table 3. Karyotype summary of natural triploids of Korean *L. maximowiczii* populations

Chromosome number	Length each (μm)	Long arm length (μm)	Short arm length (μm)	Arm Ratio (L/S)	Chromosome type	Location of rDNA gene		DAPI band
						5S	45S	
a	27.75±0.23	16.09±0.16	11.65±0.11	1.38±0.02	metacentric	-	S	S
b	22.89±0.22	15.35±0.19	7.54±0.11	2.03±0.04	metacentric	-	S	S
c	18.03±0.21	15.76±0.21	2.27±0.07	6.95±0.17	subtelocentric	L	-	-
d	17.63±0.23	15.90±0.23	1.73±0.04	9.18±0.17	subtelocentric	-	-	L
e	19.07±0.21	17.60±0.22	1.47±0.04	11.97±0.23	telocentric	-	-	L
f	15.78±0.24	14.63±0.24	1.15±0.04	12.74±0.25	telocentric	-	L	-
g	15.47±0.23	14.42±0.23	1.05±0.04	13.68±0.31	telocentric	-	S	-
h	15.59±0.20	14.60±0.19	0.99±0.03	14.69±0.38	telocentric	-	-	-
i	15.23±0.22	14.31±0.22	0.92±0.03	15.49±0.40	telocentric	-	-	L
j	15.32±0.19	14.53±0.19	0.79±0.03	18.44±0.42	telocentric	-	-	-
k	14.97±0.16	14.28±0.17	0.69±0.03	20.70±0.43	telocentric	-	-	-
l	16.69±0.26	16.14±0.26	0.55±0.02	29.35±0.80	telocentric	-	-	-
Total	214.42	183.60	30.81					

[z]Mean ± standard error, n=150; S: short arm; L: long arm

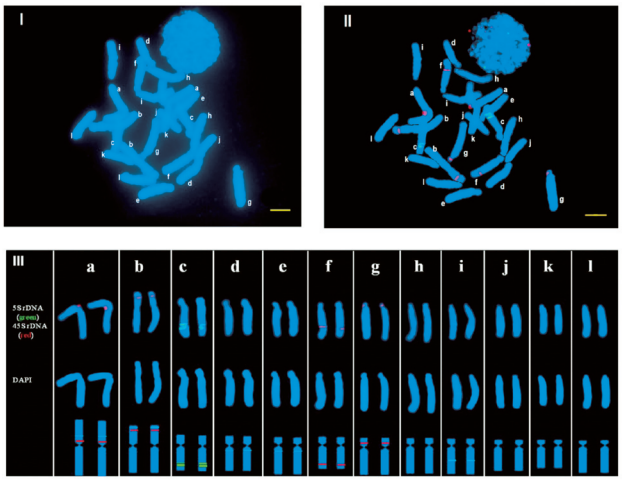

Fig. 2. Fluorescence *in situ* hybridization of mitotic metaphase chromosomes in diploid *L. maximowiczii*. (Ⅰ): DAPI counter staining, (Ⅱ): Green fluorescence indicates 5S rDNA loci and red fluorescence indicates 45S rDNA loci, (Ⅲ): Karyotype. The bar represents a length of 10 μm.

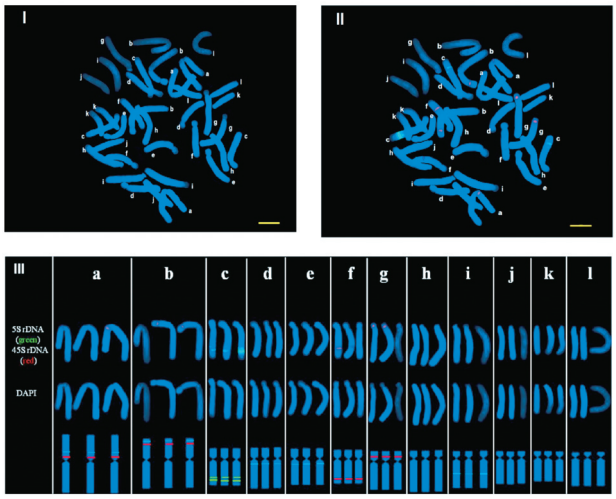

Fig. 3. Fluorescence *in situ* hybridization of mitotic metaphase chromosomes in triploid *L. maximowiczii*. (Ⅰ) DAPI counter staining, (Ⅱ); Green fluorescence indicates 5S rDNA loci and red fluorescence indicates 45S rDNA loci, (Ⅲ): Karyotype. The bar represents a length of 10 μm.

g is a telocentric chromosome that contains a 45S rRNA locus at a major secondary constriction in the short arm.

Chromosome **f** is a telocentric chromosome that possesses a 45S rRNA locus near the telomere of the long arm, where the secondary constriction is scarcely visible by conventional chromosome staining. Chromosome **i** is a telocentric chromosome and includes a strong DAPI band in the middle of the long arm. Chromosome **h** is a telocentric chromosome with a similar chromosome shape to chromosome **j** but is distinguished by its smaller arm ratio. Chromosome **k** is telocentric and the shortest chromosome. Chromosome **l** is a telocentric chromosome but nearly resembles an acrocentric chromosome due to the very short length of the short arm.

DISCUSSION

Species in the genus *Lilium* are distributed across temperate regions of Eurasia and North America (Asano 1986). The basic chromosome number is 12 in the *Lilium* species, and most of the *Lilium* species are diploid ($2n = 2x = 24$) except for the triploids of *L. lancifolium* (Noda, 1978; Kim *et al*., 2006) and *L. maximowiczii* (Kim *et al*., 2015). *L. maximowiczii* is an endemic lily in Korea. It is found in inland of the Korean Peninsula. Until the recent report of triploids by our group (Kim *et al*., 2015), *L. maximowiczii* has been known as diploids in nature (Morinaga & Fukushima, 1931; Kumazawa and Kimura, 1947; Kurita, 1948; Stewart, 1947; Noda, 1956,

1966, and 1974; Ogihara, 1960; Son, 1982; Song and Seo, 1988; Sultana *et al*., 1010). The triploid plants were found in the alpine regions of Gangwon–do and Chungcheongbuk–do Provinces, which are located in the middle of the main Korean mountain range, so–called "Baekdudaegan", that stretches across most of the length of the Korean Peninsula (Kim *et al*., 2015). The triploid plants were robust and not inferior to the diploids in any of the environments we observed (Kim *et al*., 2015). Noda (1974) also observed the same phenomenon in artificial triploids. Willson (1925) described two distinguishable types of plants, a weak plant about 2 feet tall and a robust plant above 6 feet tall, in Hamkyungnam–do, North Korea. This distinction may be due to the difference in ploidy levels.

Although the plant morphology of *L. maximowiczii*, including its flowers, is highly similar to that of *L. lancifolium*, they show different characteristics from each other: stoloniferous underground stem in *L. maximowiczii* and bulbil attachment in *L. lancifolium*. Recently, Lee *et al*. (2015) confirmed that *L. tigrinum* is phylogenetically very distant from other Sinomartagon species including *L. maximowiczii*. The diploid populations of *L. lancifolium*, which are distributed narrowly in the western and southern coastal areas, as well as islands of the Korean peninsula (Kim *et al*., 2005 and 2006), never overlap with *L. maximowiczii* populations. Thus, it presently appears unlikely that triploid *L. maximowiczii* arise from the combination of diploid *L. maximowiczii* and diploid *L. lancifolium*.

The current study shows that there is only one cytotype in both diploid and triploid *L. maximowiczii* plants, indicating that the triploids are autotriploids. Production of polyploids from unreduced gametes has been found in other lily varieties by interspecific hybridization (Barba–Gonzalez, 2004; Barba–Gonzalez, 2005; Chung *et al*., 2013; Fernandez *et al*., 1998). However, the triploid form of *L. maximowiczii* appeared to have originated by fertilization between a functional unreduced gamete (2n) and a normal reduced gamete (n) within a diploid population of *L. maximowiczii*. Autotriploid plants have often been observed in diploid populations of *Lilium* in nature (Samejima, 1958; Noda and Schmitzer, 1990).

Diploid *L. maximowiczii* populations are widely distributed throughout the main islands of the Japanese archipelago (Noda, 1956; Song and Seo, 1988; Asano, 1986), but *L. maximowiczii* populations in Korea are mainly triploid and show very limited distribution from the central inland area to the northern area where elevations are from 200 m to 1,400 m. According to the distribution map of *L. maximowiczii* (Asano, 1986), *L. maximowiczii* populations in Korea are located at marginal areas of the distribution map. The big populations (population No. 28, 29 and 30) at the Mt. Sobaek are composed of both diploids and triploids, and cover more than 1 Km in diameter around the mountain slopes at mid to high elevations (700 m~1,400 m). These populations in Mt. Sobaek are sympatric with *L. cernum*, *L. distichum*, and *L. tsingtauense* within a 4 Km diameter region. These habitations of many sympatric species make it hard to deny allotriploid production in these species, since flowering times of these species overlap partially. Authors have never witnessed these habitats sympatric with many species in China and Japan except for *L. tigrinum* that is mainly dispersed via human activity. We found only autotriploids of *L. maximowiczii* populations in Korea and we did not find any *L. tigrinum* at Mt. Sobaek. The main Korean mountain range, so–called "Baekdudaegan", is recognized as a major refugia area for *L. cernum*, *L. tsingtauense*, *L. amabile* and *L. distichum* (Chung *et al*., 2014a; Chung *et al*., 2014b; Chung and Chung, 2014) during the last glacial period. Korean habitats of *L. maximowiczii* might have emerged from the same area as the refugia during the last glacial period. These high land area habitats of Korea show severe temperature fluctuation during the flower bud development (June: Max. 35°C – Min. 5°C, during the last 50 years) (KMA, 2015). Considering 2n gamete production can be stimulated by both low and high temperatures in many species (Ramsey and Schemske, 1990; Pecrix *et al*., 2011; Yamada *et al*., 2005) and in *Lilium* (van Tuyl and Stekelenburg, 1989), this extremely low or high temperature of Mt. Sobaek might have affected 2*n* gamete production in *L. maximowiczii*, consequently producing polyploids. This hypothesis requires a systematic investigation of triploid formation in *L. maximowiczii* before conclusion can be drawn. Unfortunately, we are unable to discuss Japanese *L. maximowiczii*, because highland populations in Japan are unexplored as yet (Asano, 1986; Kurita, 1948; Noda, 1956; Noda, 1977).

Although it is well known about general trends for B–chromosome detection in many plants, their mode of origin remains unclear (Jones and Houben, 2003; Jones *et al*., 2008). *Lilium* species have been known to carry large genomes (Leitch *et al*., 2007). Besides establishing the occurrence of B–chromosomes in several species (Kayano, 1957), preferential transmission and maintenance in embryo–sac mother cells and Mendelian transmission in pollens have been investigated in *L. callosum* (Kayano, 1957; Kimura and Kayano, 1961). An origin of new aberrant small chromosomes was analyzed using hybrid lilies recently (Xie *et al*., 2014). Noda (2003) also reported about B–chromosome in Tiger lily.

Noda (1977) reported the karyotypes of the A and B cytotypes of the Japanese *L. maximowiczii* collections. While the arm index and general chromosome morphology were similar between the two cytotypes, chromosomes in the A cytotypes were larger than those of the B cytotypes. Noda (1977) and Song and Seo (1988) also reported the presence of different numbers of B–chromosomes in the Japanese accessions. Sultana *et al*. (2010) also noted two cytotypes in Korean *L. maximowiczii* by FISH analysis of 45S rRNA loci. However, the origin of the *L. maximowiczii* in their study is uncertain. We analyzed 2 to 10 plants from each of 30 populations of diploids and triploids that were collected from diverse sites in Gangwon–do and Chungcheongbuk–do Provinces of Korea. However, we could not assign A or B cytotypes unequivocally in our samples, so we posit a single cytotype in Korean accessions of *L. maximowic-*

zii. Moreover, none of the Korean collections carried B–chromosomes. B–chromosomes are dispensable elements; their inheritance is non–Mendelian and irregular (Jones and Houben, 2003). In maize, sequences of B–chromosomes were derived from different A–chromosomes (Cheng and Lin 2003). The B–chromosomes in the Japanese *L. maximowiczii* accessions must have derived from A–chromosomes. B–chromosome has never been found in Korea not only in a diploid form (Kim *et al*., 2005; Kim *et al*., 2006) but also in triploid forms (unpublished data) of Tiger lily. It is interesting that Japanese accessions of *L. maximowiczii* and *L. lancifolium* have numerous B–chromosomes, but Korean accessions do not. This apparent disparity may require further analysis.

With the combination of chromosome landmarks and FISH using rRNA loci, 9 of the 12 chromosomes of *L. maximowiczii* were easily identifiable. It was difficult to distinguish between chromosomes **h**, **j**, and **l** unequivocally. The three chromosomes **a**, **b**, and **g**, showing secondary constrictions, were also observed previously by conventional staining (Noda, 1977). These two major secondary constriction sites could be the major nucleolus organizing regions (NORs), where the 45S RNA genes reside in *L. maximowiczii*. In addition to these two loci, we detected one more 45S rRNA locus on chromosome **f**, where the secondary constriction was not detected. The FISH signal on chromosome **f** was weaker than that of the three major NOR loci on chromosomes **a**, **b**, and **g**. Thus, the NOR loci on chromosomes **a**, **b**, and **g** would be the major nucleolar loci, and the locus on chromosome **f** would be the transcriptionally silent minor locus (Pikaard, 1999). Sultana *et al*. (2010) reported the karyotypes of 12 accessions in 9 *Lilium* species by FISH analysis of 45S rRNA and 5S rRNA loci. They arranged chromosomes according to their sizes from largest to smallest and reported that five chromosomes (chromosomes 1, 2, 6, 7, and 11) carry 45S rRNA genes in *L. maximowiczii*. By chromosome shape, we could assign the identities **a**, **b**, **g**, **f**, and **k** to these five chromosomes (chromosomes 1, 2, 6, 7, and 11, respectively). The NOR loci on chromosomes **a**, **b**, and **g** in our study were also observed in their study. The 45S rRNA loci on chromosomes **a** and **b** were highly conserved across *Lilium* species (Sultana *et al*., 2010). However, chromosome 6 showed only one hybridization signal in their study, whereas both hybridization signals were observed on chromosome **f** in our analysis. We also failed to detect additional 45S rRNA hybridization signals in our study. These discrepancies may be explained by the different sources of plant materials. The *L. maximowiczii* accessions were the collections from the alpine regions in Gangwon–do and Chungcheongbook–do Provinces, Korea, whereas the accessions of Sultana *et al*. (2010) were obtained from the Lily Experimental Station, Taean, Chungcheongnam–do Province, Korea. No further geographical information on the origin of this accession was available. The number of chromosomes carrying 45S rRNA genes range from two in *L. henryi* to six in *L. duchartrei* (Marasek *et al*., 2004; Wang *et al*., 2012), and the loci in chromosomes **a** and **b** are highly conserved in most of the *Lilium* species studied (Sultana *et al*., 2010). The 5S rRNA locus on chromosome **c** is conserved in all of the *Lilium* species that were studied by Sultana *et al*. (2010). However, duplicated loci were only observed in the *Sinomartagon* section, such as *L. maximowiczii*, *L. tigrinum*, and *L. amabil*e, but not in other *Lilium* species. Thus, the 5S rRNA duplication must have occurred only after the divergence of *Sinomartagon* section lilies.

In conclusion, we are reporting the karyotypes of diploid and triploid forms of *L. maximowiczii* using chromosomal landmarks such as chromosome length, arm ratio, secondary constrictions, DAPI bands, and FISH signals using 45S rRNA and 5S rRNA genes. Only one cytotype was observed in the karyotype analysis of both diploid and triploid populations, and the karyotypes of the diploid and triploid forms were identical, indicating that the triploid form emerged by autotriploidy.

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