Chromosome characterization and physical mapping of 18S rDNA in *Lilium longiflorum* originated interspecific hybrids using combined genomic and fluorescent *in situ* hybridization

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Abstract

This study was aimed at differentiating parental genomes, examining intergenomic composition, and mapping mitotic metaphase chromosomes by localizing parental and 18S rDNA probes in seven interspecific hybrid progenies that originated from *Lilium longiflorum*. Since *in situ* hybridization has not been previously used in lily breeding, flow cytometry was used in conjunction with genomic and fluorescent *in situ* hybridization to determine the genomic contribution of each parent to the interspecific progenies. A significant variation was observed in the DNA content, chromosome length, and 18S loci in F_1 as compared to the female and male parents. *L. longiflorum* showed nearly two times higher DNA content than the male parents and *L. longiflorum* × Asiatic progenies, but eight times higher than *L. longiflorum* × *L. hansonii*. Genomic *in situ* hybridization results revealed that both female and male parents contributed an equal number of chromosomes to their interspecific F_1 offspring. Fluorescent *in situ* hybridization mapping revealed that 18S rDNA had 8, 6 and 7 loci in *L. longiflorum* parents, i.e., White heaven, Bright tower, and White tower, respectively, whereas each Asiatic cultivar and *L. hansonii* used as male showed 8 and 12 loci respectively. Interspecific progenies showed 8 and 7 loci in LA, and 10–11 in LM hybrids. These cytogenetic results implied equal genetic and chromosomal contribution from both parents to their intergenomic progenies. Therefore, this combined (Schwarzacher et al., 1992)cytogenetic method has the potential to be an affordable and time-saving approach in lily breeding that could determine the status of hybrids and their genomic origin while achieving physical mapping and detecting genes in different genomes.

Introduction

Lily, one of the most economically important cut flowers, belongs to the genus *Lilium*, comprising over 100 species with more than 8000 registered cultivars (Zhou et al., 2008a; Zhou et al., 2008b; van Tuyl and Arens, 2010; Naing et al., 2014). *Lilium longiflorum* (L-genome), Asiatic (A-genome), and Oriental (O-genome) hybrids are considered to be the three primary hybrid groups, belonging to the sections Leucolirion, Sinomartagon, and Archelirion, respectively (Barba-Gonzalez et al., 2005; Mazharul et al., 2019). *L. longiflorum* Thunb. (2n = 2x = 24) is an important species involved in modern breeding programs, which has white, funnel-shaped flowers with a distinctive fragrance, low temperature tolerance, and a non-tunicate bulb but susceptible to the virus (Karlov et al., 1999; Lim et al., 2008). Asiatic lilies are widely used in breeding owing to their upright-facing, early to late blooming, and vigorous and long-living flowers with a wide range of colors and their resistance against *Fusarium oxysporum* and viruses (Merhaut and Newman, 2005; Lim et al., 2008). *L. hansonii*, belonging to the Martagon section, is a dwarf native species of the Korean Peninsula, growing under shady deciduous trees. It has 3–4 layers of whorled leaves and 8–10 pendulous flowers with yellow petals and numerous spots that have a long vase life (Lim, 2014; Ahn et al., 2017; Kumari et al., 2018). However, weak growth and virus susceptibility of *L. hansonii* is causing a decline in the number of cultivated plants every year. At present, there is no reported an interspecific lily hybrid originating from *L. hansonii*. LA (*L. longiflorum* × Asiatic) hybrids play an important role in commercial breeding as they introduce various flower colors, sizes, shapes, and

distinct fragrances (Lim et al., 2008; Lucidos et al., 2013; Tang et al., 2020), and in some cases, resistance to *Fusarium* and other viruses (Liu et al., 2008). Therefore, interspecific hybridization among *L. longiflorum*, Asiatic, and *L. hansonii* may contribute to the creation of modern cultivars with desirable horticultural traits such as dwarfness and 6–8 medium-sized flowers with colorful petals, which could meet market demand.

In a majority of the crops, interspecific hybridization is one of the most essential methods for creating novel hybrids and improving desired features. In a taxonomic section, it is quite simple method to induce novel hybrids through normal pollination. Due to pre- and post-fertilization barriers, it is difficult to achieve interspecific hybrids. Moreover, poor pollen tube growth caused by stigmatic incompatibility is the first barriers, where incompatible pollen grains are unable to digest specific compounds in the stigma, depleting the stigma's own stores and resulting in poor growth. The second obstacle is seeds lacking endosperm cause the embryos to abort in the early stages of the development. Various techniques have been developed to overcome pre-fertilization barriers, such as cut-style pollination, grafted style method, intra-styllar pollination, mentor pollen, and *in vitro* pollination (Van Tuyl et al., 1997). Interspecific crossing between *L. longiflorum* and Asiatic hybrids most often result in sterile F_1 progenies due to failure of chromosome pairing during meiosis (Van Tuyl et al., 1997; Karlov et al., 1999). For efficient breeding, 2n gametes can be used, which play an important role in transferring desired parental genes to the F_1 and chromosome recombination during meiosis (Schwarzacher et al., 1992; Karlov et al., 1999).

Fluorescent *in situ* hybridization (FISH) is an advanced technique used to detect DNA sequences and allele copy numbers in different positions on chromosomes (Ramzan et al., 2017; Jo et al., 2019; Islam et al., 2020). Analysis of nuclear organizing regions can be achievable using FISH technique (Fujiwara et al., 2007; Hwang et al., 2011). However, genomic *in situ* hybridization (GISH) is the most useful technique to differentiate whole parental genomes (Schwarzacher et al., 1992; Karlov et al., 1999; Chester et al., 2010; Van Laere et al., 2010; Silva and Souza, 2013; Ramzan et al., 2017), alien chromosome segments, genome association in allopolyploids, and intergenomic recombination (Karlov et al., 1999; Nakazawa et al., 2011; Silva and Souza, 2013; Ramzan et al., 2016). Both FISH and GISH are usually employed separately in *Lilium* breeding, which represents an expensive and time-consuming approach. Moreover, whole parental genomes and particular nucleic acid sequences cannot be both identified by using either FISH or GISH alone, and no combined GISH–FISH protocol has been published yet on lilies. Therefore, the purpose of this study was to introduce novel interspecific lily hybrids having parental attributes and to investigate status of hybrids, origin of genomes, chromosomal abnormalities, and chromosomal recombination in interspecific *L. longiflorum* × Asiatic, and *L. longiflorum* × L. *hansonii* hybrid progenies through combined GISH and FISH.

Materials And Methods

Plant material

Three *L. longiflorum* cultivars (L-genome), three Asiatic (A), and two *L. hansonii* (M) were used for interspecific hybridization experiments to produce F_1 progenies. *L. longiflorum* cultivars “White heaven”, “Bright tower”, and “White tower” and Asiatic cultivars “Conception”, “Gironde”, and “Sky shim 2” were collected from a local commercial company. Whereas, highly genetically diversified *L. hansonii* was collected from various locations of Ulleung-do Island, Republic of Korea and cultivated in the greenhouse condition. All the bulbs were diploid (2n = 2x = 24) and were grown in a greenhouse at Kyungpook National University, Republic of Korea. The greenhouse temperature was maintained at 14–18°C during the night and 20–25°C during the daytime. A detailed breeding scheme is shown in Table 1. As *L. longiflorum*, Asiatic, and Martagon lilies belong to different taxonomical sections, interspecific hybridization was performed through the CSM (Lim et al., 2005) to avoid any fertilization barrier as well as to protect the hybridized ovary from any undesired pollen entry. A brief diagram of CSM breeding in lily has been shown in Fig. 1.

Table 1
The interspecific cross combinations between *L. longiflorum* × Asiatic hybrid and *L. longiflorum* × *L. hansonii* used in this study.

Crossed code	Cross combined	Female		Male		Crossing method	No. of Crossed flower
		Cultivar name	Genome	Cultivar name	Genome		
113521	LL×AA	White Heaven	LL	Conception	AA	CSM	32
132025	LL×AA	Bright Tower	LL	Gironde	AA	CSM	41
143024	LL×AA	White Tower	LL	Sky Shim 2	AA	CSM	23
128115-7	LL×MM	White Tower	LL	*L. hansonii*	MM	CSM	31
128130-5	LL×MM	White Tower	LL	*L. hansonii*	MM	CSM	52
128133-8	LL×MM	White Tower	LL	*L. hansonii*	MM	CSM	18

Flow Cytometry Analysis

Genome sizes and ploidy levels were determined by flow cytometry. For the analysis, 0.5 cm^2 fresh leaf tissue was placed in a petri dish (SPL 10060) and chopped with 500 µL Nuclei Extraction Buffer (CyStain UV Precise P, Sysmex) using a sharp blade. The extraction buffer, containing exposed nuclei, was filtered through a 30 µm-nylon mesh filter into a 3 ml-tube and stained in a staining buffer (CyStain UV Precise P, Sysmex). After a short incubation of 30 s, nuclear suspensions were analyzed using a flow cytometer

(Partec PA, Ploidy Analyzer, Germany). Mean DAPI fluorescence of the target samples was compared with an internal standard, Oriental-Trumpet “Yelloween” (Ramzan et al., 2016; Kwon et al., 2017). Genome size (2C) was calculated based on the ratio of relative DAPI fluorescence of a sample to that of the internal standard. The calculation procedure was as follows: DNA content of the standard × (mean of relative DAPI fluorescence of a sample/mean of relative DAPI fluorescence of the standard) (Hembree et al., 2020).

Chromosome Observation

Young, actively growing root tips were collected from interspecific F_1 hybrids. To ensure the presence of metaphase chromosomes, root tips were pretreated with α-bromonapthalene for 4 h at 20°C, followed by fixation in 3:1 (v/v) solution of 100% ethanol: glacial acetic acid for 24 h at room temperature (25°C). The fixed root tips were stored in 70% ethanol at − 20°C until use (Takahashi et al., 1997; Kwon et al., 2017). For slide preparation, root tips were rinsed 2–3 times in distilled water, incubated in a pectolytic enzyme mixture containing 0.2% (w/v) cellulase RS, 0.2% (w/v) pectolyase Y23, and 0.2% (w/v) cytohelicase in 10 mM citrate buffer (pH 4.3) at 37°C for 70 min. Dissection and squashing of macerated root tips were made in a drop of 70% acetic acid that was subsequently air-dried. Finally, well-spread chromosomes were identified using a fluorescent microscope (BX61, Japan).

Gish–fish Analysis

DNA isolation and preparation

For GISH, non-shared parental genomic DNA was isolated from *L. longiflorum* “Woori Tower” using the CTAB method with some modifications. DNA concentration was measured by NanoDrop 2000 (Thermo Fisher Scientific) using 1 µL per sample, and three measurements were performed for each sample. The isolated parental genomic DNA was used as a probe and was labeled with digoxigenin-11-dUTP using a standard nick translation kit according to the manufacturer’s instructions (Roche Diagnostics GmbH, Germany). Labeled genomic DNA was used in a volume of 20 µL, including 1300 ng genomic DNA, 4 µL DIG-Nick translate, and extra distilled water. For FISH, a 18S rDNA probe was directly labeled with biotin-16-dUTP by nick translation in accordance with the manufacturer’s protocol (Roche, Germany). The blocking DNA was obtained from herring sperm (Invitrogen, USA) that was fragmented into 100–300 bp fragments and subsequently autoclaved.

Combined genomic-fluorescence in situ **hybridization**

The combined GISH–FISH hybridization procedure can be divided into three parts: chromosome pretreatment, combined hybridization, and probe detection (Fig. 1). The selected slides were treated with 100 µg/ml RNase for one hour at 37°C, washed three times with 2× SSC for 5 min each, and fixed for 10 min with 4% paraformaldehyde. Again, the slides were washed thrice with 2× SSC for 5 min each time,

dehydrated with a 70%, 90%, and 99.9% ethanol series for 3 min each, and air-dried. Hybridization was performed in a mixture containing 50% (v/v) deionized formamide, 20× SSC buffer, 10% (w/v) sodium dextran sulfate (SDS), 50% dextran sulfate (DS), 5 ng/µl *L. longiflorum* "Woori tower" genomic DNA, 20 µg/ml each of the 18S rDNA probes, and 30–45 ng/µl blocking DNA. DNA was denatured by heating of the hybridization mixture at 74°C for 10 min, followed by incubation in an ice jar for 15 min. For each slide, 40 µl of hybridization solution was used, and the slides were denatured at 82°C for 5 min, followed by an overnight incubation in a humidified chamber at 37°C. After hybridization, the slides were washed with 2× SSC buffer for 5 min at room temperature and subsequently washed with 0.1× SSC buffer in a shaking jar at 42°C for 30 min. Digoxigenin-labeled probes were detected with antidigoxigenin fluorescein (Roche Diagnostics GmbH, Germany) and amplified with fluorescein anti-sheep and fluorescein anti-rabbit (Vector Laboratories). Similarly, biotin-labeled probes were detected using streptavidin-labeled Cy3 and amplified with biotinylated goat anti-streptavidin (Amersham Biosciences, UK). Finally, the treated slides, containing chromosomes, were counterstained with 3 mg/ml 4, 6-diamidino-2-phenylindole (DAPI) in Vectashield mounting medium (Vector Laboratory Inc., USA). Different methods, such as nick translation, random-primed labeling, and polymerase chain reaction, were used to label the probe during marker labeling. Various methods, such as autoclaving, shearing the DNA with a tiny needle in a syringe, or sonication, are used to prepare blocking DNA. Chromosome slide preparation included selection of well-spread chromosomes prepared from a young root tip using an enzyme mixture at 37°C. Slide pretreatment was comprised of enzymatic digestion of chromosomes in order to unmask DNA prior to hybridization. Hybridization involved attachment of both blocking and probe/genomic markers with chromosomes to identify the specific loci/origin of the genome of the respective chromosome. During detection, attachment of the designed antibody against the target marker along with blocking buffer was performed to detect the specific fluorochrome.

Results

Ploidy and DNA content analysis

Ploidy levels and genome size of female *L. longiflorum* cultivars, male Asiatic cultivars, *L. hansonii* and their F_1 progenies were assessed by flow cytometry and estimated by the comparison to the control "Yelloween" (Table 2). Genome size of F_1 progenies varied from 51.6 to 59.9 pg, which was similar to the male parents, i.e., 48.9–55.4 pg, but lower than that of the female plants, i.e., 68.9–70.1 pg.

Table 2
Ploidy and putative 2C genome size in female plants and F_1 progenies.

Plant type	Plant material/code	Genome	Mean	2C genome size (pg)	Estimated ploidy (x)
Female	Control (Yelloween)	OT	50.4 ± 0.3	60.2 ± 0.2	2n = 2x = 24
	White heaven	LL	55.9 ± 0.6	69.4 ± 0.6	2n = 2x = 24
	Bright tower	LL	53.9 ± 1.0	70.1 ± 0.4	2n = 2x = 24
	White tower	LL	51.4 ± 1.1	68.9 ± 1.0	2n = 2x = 24
	Conception	AA	51.1 ± 0.4	53.7 ± 0.9	2n = 2x = 24
	Gironde	AA	49.6 ± 0.2	55.4 ± 1.0	2n = 2x = 24
Male	Sky Shim 2	AA	50.1 ± 0.0	52.6 ± 0.9	2n = 2x = 24
	L. hansinii (111114)	MM	50.3 ± 0.8	49.2 ± 1.6	2n = 2x = 24
	L. hansinii (121038-1)	MM	50.0 ± 0.5	48.9 ± 0.8	2n = 2x = 24
F_1	113521	LA	51.0 ± 0.3	57.1 ± 0.4	2n = 2x = 24
	132025	LA	52.1 ± 0.2	59.9 ± 0.7	2n = 2x = 24
	143024	LA	55.8 ± 0.1	56.1 ± 1.0	2n = 2x = 24
	128115-7	LM	54.6 ± 0.0	52.2 ± 0.1	2n = 2x = 24
	128130-5	LM	51.3 ± 0.1	51.9 ± 0.0	2n = 2x = 24
	128133-8	LM	48.4 ± 0.2	51.6 ± 0.3	2n = 2x = 24

[z] means ± standard error. 113521: *L. longiflorum* 'White Heaven' × Asiatic lily 'Conception', 132025: *L. longiflorum* 'Bright Tower' × Asiatic lily 'Gironde', 143024: *L. longiflorum* 'White Tower' × Asiatic lily 'Sky Shim 2', 128115-7: *L. longiflorum* 'White Tower' × *L. hansonii* (111114), 128130-5: *L. longiflorum* 'White Tower' × *L. hansonii* (111114), 128133-8: *L. longiflorum* 'White Tower' × *L. hansonii* (121038-1).

Fish Analysis Of Interspecific Progenies With 18s Rdna Probes

FISH results of three female lilies, i.e., “White heaven,” and “Bright tower”, “White tower”, five male lilies, i.e., “Conception”, “Gironde”, “Sky Shim 2”, *L. hansonii* (111114, 121038-1), and six interspecific hybrid progenies with 18S rDNA as a probe are presented in Fig. 3. Biotin-labeled 18S rDNA loci (red fluorescence) are marked by yellow arrows (Fig. 3), and karyotype ideograms and FISH summary are shown in Fig. 5, 7 and Table 3. Each of the seven interspecific hybrids and their respective female parents were found to be diploid (2n = 2x = 24). The number of 18S rDNA loci varied among parents and *L. longiflorum*-originated progenies. The female parents i.e., “White heaven,” and “Bright tower”, and “White tower,” showed eight, six, and seven 18S rDNA loci, respectively. Whereas, male Asiatic lilies and *L. hansonii* showed eight and twelve 18S loci each, respectively. Out of eight 18S rDNA loci in *L. longiflorum* “White heaven,” four were equally found on the long arm and the centromeric region. Similarly, “Bright tower” (two loci on the long arm and four on the centromeric region) and “White tower” (three loci on the long arm and four on the centromeric region) showed variation in terms of 18S rDNA loci distribution. In male parents, four loci on both long arm and centromeric region (Conception, Sky Shim 2), five loci on long arm and three on centromere (Gironde), eight loci on long arm and four on centromere (111114), and nine loci on long arm and three on centromere (121038-1) were observed as shown in Table 3 and Fig. 3.

Table 3
Distribution of 18S rDNA signals on the chromosome of *L. longiflorum* used as female parents.

Cultivar	No. of 18S rDNAs	Location of 18S rDNA		Chromosome pair containing 18S rDNA
		Long arm	Short arm	
White Heaven	8	4	4	Ch# 3, 4, 7, 9, 11
Bright Tower	6	2	4	Ch# 3, 4, 6, 7
White Tower	7	3	4	Ch# 3, 4, 7, 11
Conception	8	4	4	Ch# 1, 3, 4, 7, 11
Gironde	8	5	3	Ch# 3, 4, 6, 9, 11
Sky Shim 2	8	4	4	Ch# 1, 3, 7, 11
L. hansinii (111114)	12	8	4	Ch# 1, 2, 3, 4, 7, 8,11, 12
L. hansinii (121038-1)	12	9	3	Ch# 1, 2, 3, 4, 5, 6, 7, 10, 11

Genomic Composition Of Interspecific Progenies

A minimum of 18 individual progenies of each cross combination were examined using GISH, as shown in Table 4, and representative images are illustrated in Fig. 4. According to the GISH results, all the seven interspecific progenies showed true hybridity and possessed three different genomes, namely, Longiflorum (L), Asiatic (A), and Hansonii (M), which have been clearly distinguished on the respective chromosomes. An equal number of 24 chromosomes in each individual, representing seven interspecific hybrids, was observed without any break points and recombination (Table 4). Since *L. longiflorum* was used as a female genomic DNA probe, 12 chromosomes of Longiflorum (113521, 132025, 143024, 128115-7, 128130-5, and 128133-8) and 12 chromosomes of Asiatic (113521, 132025, and 143024), and Hansonii (128115-7, 128130-5, and 128133-8) were characterized (Fig. 4 and Table 4).

Table 4
Genomic composition in F_1 hybrids lilies.

Crossed code	Expected genome	No. individual examined	Ploidy level	Chromosome constitution		No. of recombinant sites	Hybridity status
				Female	Male		
113521	LA	22	2x	12	12	0	True
132025	LA	23	2x	12	12	0	True
143024	LA	19	2x	12	12	0	True
128115-7	LM	32	2x	12	12	0	True
128130-5	LM	18	2x	12	12	0	True
128133-8	LM	19	2x	12	12	0	True

However, due to the higher genetic diversity of *L. hansonii*, the number of 18S rDNA loci increased in F_1 hybrid progenies. In LL × AA-crossed progenies (113521, 132025), among the detected 18 rDNA loci, four were detected on the long arm (one locus from a female and three loci from a male) and four loci on the short arm (two loci from a female and two from a male). The remaining LL × AA hybrid (143024) showed seven 18S loci, including four on the long arm (one locus from a female and three loci from a male) and three loci on the short arm (two loci from a male and one locus from a male).

The number of 18S rDNA loci was higher in LL × MM progenies (128115-7, 128130-5, and 128133-8). Out of the 11 detected loci (128115-7), 7 were located on the long arm (1 locus from a female and 6 loci from a male) and 4 on the short arm (2 loci equally from a female and male). 128130-5 and 128133-8 progenies showed 10 and 11 18S rDNA loci, including 6 and 7 found on the long arm and 4 loci each on the short arm (2 loci each from female and male), respectively. Among the six interspecific progenies, all 18S rDNA loci were detected on the chromosome pairs 3, 4, and 7, except for 132025 (LL × AA) hybrids.

Karyotype analysis of the female, male parents and interspecific progenies

Diploid *L. longiflorum*, “White heaven”, “Bright tower”, and “White tower” as female parents, “Conception”, “Gironde”, “Sky Shim 2”, *L. hansonii* (111114, 121038-1) used as male parents showed 24 chromosomes each. The longest and shortest chromosome of female parental lilies ranged from 17.19–18.83 µm and 9.07–11.18 µm (Table 5), while it was 10.71–17.23 µm and 8.71–10.53 µm in male parents, respectively. Total length of all chromosomes in female and male parents were 259.90-281.94 µm and 211.54-245.89 µm, respectively. However, among the six interspecific hybrid progenies, number of chromosomes and their karyotype varied. Moreover, number of metacentric, sub-metacentric, and telocentric chromosomes also varied among them. The highest number of submetacentric chromosomes was scored among the female, male parents and their F_1 progenies. Among F_1 progenies, the longest and the shortest arm ranged from 15.27–19.33 µm and 7.59–11.10 µm, respectively, and total length of all chromosomes was 229.20-271.58 µm.

Table 5
Distribution of 18S rDNA signals on the chromosomes *L. longiflorum* originated F_1 interspecific hybrids.

Crossed code	18S rDNA			Locations of 18S rDNA		Chromosome containing18S rDNA
	Number	From female	From male	Long arm (□/□)	Short arm (□/□)	
113521	8	Ch# 3,4,7	Ch# 1,3,4,7,11	1/3	2/2	Ch# 1,3,4,7,11
132025	8	Ch# 3,4,6	Ch# 3,4,6,9,11	1/3	2/2	Ch# 3,4,6,9,11
143024	7	Ch# 3,4,7	Ch# 1,3,7,11	1/3	2/1	Ch# 1,3,4,7,11
128115-7	11	Ch# 3,4,7	Ch# 1,2,3,4,7,8,11, 12	1/6	2/2	Ch# 1,2,3,4,7,8, 11, 12
128130-5	10	Ch# 3,4,7,	Ch# 1,2,3,4,5,8,11	1/5	2/2	Ch# 1,2,3,4,5,6,7,8,11
128133-8	11	Ch# 3,4,7	Ch# 1,2,3,4,5,6,10,11	1/6	2/2	Ch# 1,2,3,4,5,6,7,10,11

Table 6
Chromosome characteristics in female, male parents and their F_1 interspecific hybrids.

Crossed code	No. of chromosome	Longest chromosome (μm)	Shortest chromosome(μm)	Total length (μm)	Karyotype formula
White heaven	24	18.28 ± 0.8	11.18 ± 0.2	278.26 ± 0.5	2n = 2x = 2m + 20sm + 2t
Bright tower	24	18.83 ± 0.3	11.10 ± 0.6	281.94 ± 0.1	2n = 2x = 2m + 16sm + 6t
White tower	24	17.19 ± 0.4	9.07 ± 0.8	259.90 ± 0.7	2n = 2x = 4m + 20sm
Conception	24	15.44 ± 0.1	10.21 ± 0.2	245.89 ± 0.7	2n = 2x = 4m + 18sm + 2t
Gironde	24	16.61 ± 0.7	10.53 ± 0.5	211.54 ± 0.3	2n = 2x = 4m + 16sm + 4t
Sky Shim 2	24	17.23 ± 0.2	8.71 ± 0.1	235.32 ± 0.8	2n = 2x = 4m + 18sm + 2t
L. hansinii (111114)	24	12.67 ± 0.3	9.54 ± 0.6	229.94 ± 0.7	2n = 2x = 4m + 6sm + 14t
L. hansinii (121038-1)	24	10.71 ± 0.4	9.19 ± 0.3	223.76 ± 0.1	2n = 2x = 4m + 6sm + 14t
113521	24	17.51 ± 0.2	10.10 ± 0.2	252.74 ± 0.7	2n = 2x = 4m + 18sm + 2t
132025	24	18.84 ± 0.3	9.78 ± 0.7	271.58 ± 0.5	2n = 2x = 2m + 18sm + 4t
143024	24	19.33 ± 0.5	11.10 ± 0.1	242.94 ± 0.3	2n = 2x = 2m + 16sm + 6t
128115-7	24	15.83 ± 0.4	9.65 ± 0.3	235.89 ± 0.9	2n = 2x = 14sm + 10t
128130-5	24	15.74 ± 0.8	9.38 ± 0.5	229.20 ± 0.8	2n = 2x = 2m + 14sm + 8t
128133-8	24	15.27 ± 0.3	7.59 ± 0.3	231.92 ± 0.1	2n = 2x = 4m + 12sm + 8t

Discussion

The genus *Lilium* Tourn. ex L. is one of the largest genera in the plant kingdom. *L. longiflorum* Thumb. was reported to have the 2C value of about 72 pg (Karlov et al., 1999), and it was found to genetically varied among the lily cultivars used in this study as female and male parents. As the female and male parents used in this study were both diploid (2n = 2x = 24), the obtained interspecific progenies should

have to be diploid, and this was confirmed by both flow cytometry and chromosomal observations. Similarly, triploid lily was obtained by crossing between tetraploid and diploid lilies (Gao et al., 2014). Interspecific hybridization is a challenging task in the genus *Lilium* breeding due to both pre- and post-fertilization barriers, which can be overcome by employing CSM and GSM crossing (Van Tuyl et al., 1997; Barba-Gonzalez et al., 2005). However, using the CSM method, a relatively low success rate of interspecific hybridization was achieved in this study, yet it was higher in comparison to the OT hybrids (Cao et al., 2019).

Moreover, F_1 sterility is another challenge in the creation of desired hybrids. According to Zhou et al. (2008), only a few individuals out of a large number of interspecific hybrid progenies, including LA, can produce functional n gametes. Therefore, using 2n functional gametes is a good option for overcoming F_1 sterility and accomplishing intergenomic recombination as well as for increasing genetic variations (Ramanna, 1992; Lim et al., 2001; Barba-Gonzalez et al., 2005; Zhou et al., 2015). Genetic recombination and homologous crossing-over are important features of interspecific breeding, especially for LA and OA progenies (Lim et al., 2001; Van Tuyl et al., 2002; Sun et al., 2014). In this study, both female and male parents provided equal portions of genetic material to their interspecific progenies without any notable homologous recombination. *L. longiflorum* × Asiatic hybrid progenies usually show quantitative differences between genotypes that result in chromosome pairing failure; however, in this study, almost normal pairing was observed. Chromosome recombination frequently occurs in BC1 progenies, whereas little intergenomic recombination has been reported in *L. longiflorum* × *L. hansonii* progenies in our previous studies which is under further investigation to find out the recombination origin through meiotic stages analysis (Lim et al., 2003).

FISH mapping of 18S rDNA in *L. longiflorum* showed 6–8 loci usually on the third, fourth, and eleventh pair of chromosomes (Table 4), similarly to (Lim et al., 2001), whereas different FISH mapping of 45S was observed in *L. lancifolium* (Zhou et al., 2008b). Moreover, in Asiatic lilies, 45S rDNA loci are stable on the first, fourth, and seventh pair of chromosomes (Cao et al., 2019). However, *L. hansonii* was found to be a highly genetically diversified species in Ulleung-do Island, Korea (Robledo et al., 2009), and no *L. hansonii*-originated interspecific hybrids have been reported yet. As a result, LM progenies inherited more 18S rDNA loci from the male *L. hansonii* parent, resulting in a rise in their number.

Both GISH and FISH are the most advanced cytogenetic techniques used to assess hybridity status, chromosomal abnormalities, homologous crossing-over, and FDR mechanisms as well as identification of the origin of F_1 progeny (Robledo et al., 2009; Younis et al., 2015; Kwon et al., 2017; Ramzan et al., 2017). All of these characteristics can be assessed by combining genomic and FISH. Therefore, the development of the combined GISH–FISH analysis would be a new addition to cytogenetic studies on lily breeding. The use of parental DNA and 18S rDNA for tagging the desired chromosomal loci helped in analyzing chromosome attributes and determining the karyomorphology of the interspecific F_1 progenies and their female parents.

Conclusions

The obtained interspecific hybrid progenies had equal genetic characters inherited from the female and male parents, according to the results of chromosome characterization and FISH mapping. In addition, this study illustrates the potential of combined GISH–FISH analysis for providing information about hybridity status, chromosomal abnormalities, and genomic origin of *L. longiflorum*-derived interspecific hybrid progenies and can be useful as a time-saving and low-cost cytogenetic method in breeding programs.

Declarations

Author Contributions: Mazharul Islam, Lee Hyemin, Deen Mohammad Deepo designed the manuscript and Reshma Yesmin, Fahad Ramzan and Hong-Yul Kim worked on the acquisition of data, analysis, and interpretation. Prof. Ki-Byung Lim gave his valuable time for critical revisions of the manuscript.

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Figures

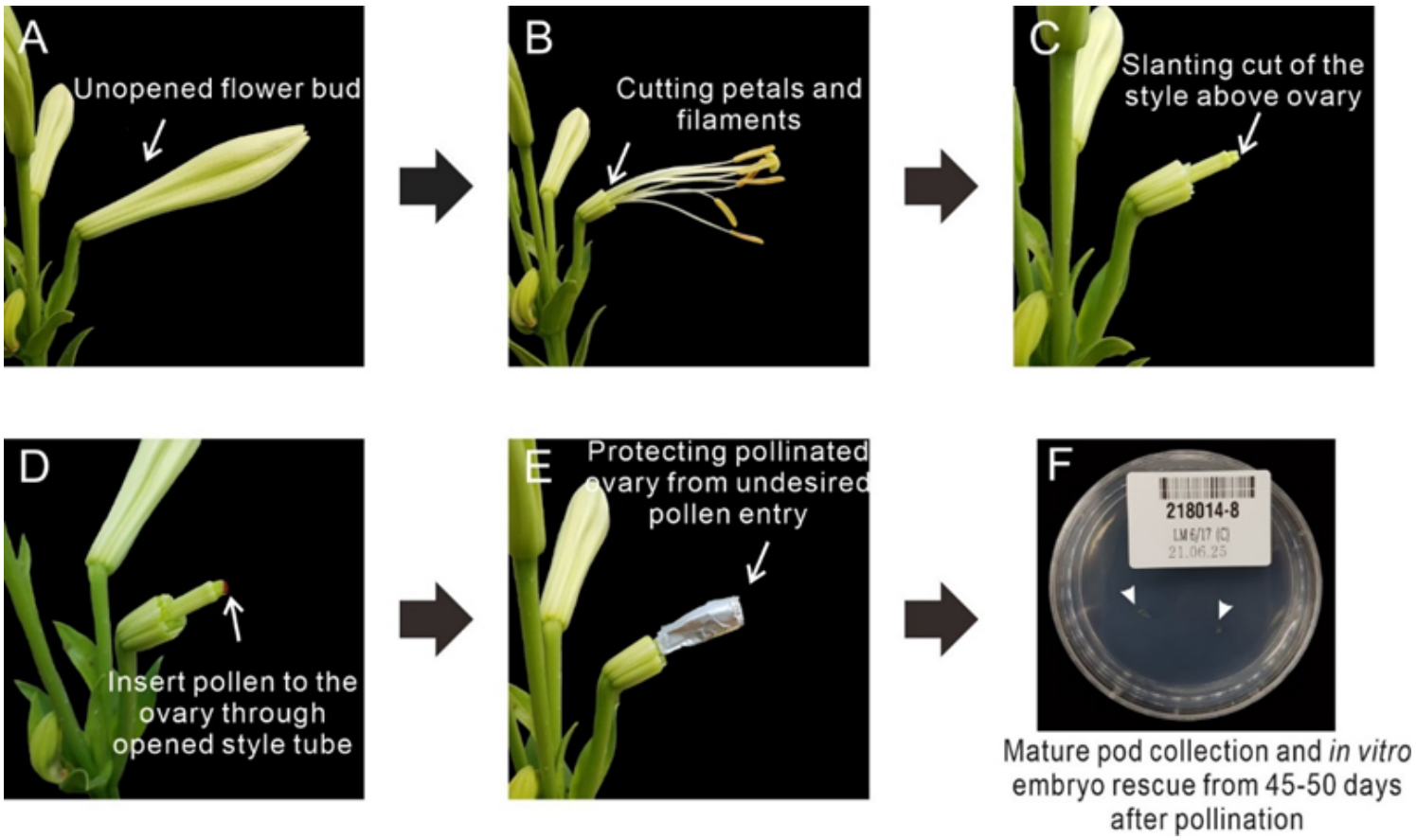

Figure 1

Interspecific hybridization using Cut Style Method in lily breeding. A: Pre-bloomed mature virgin flower bud, B: Removal of petals and the filaments below ovary-style junction, C: Style cutting with 45o angel above 0.5cm from the ovary, D: Pollination with desired pollen, E: Covered with aluminum foil to prevent contamination of other pollen, and F: Embryos rescue from mature pods are placed on the medium at 45-50 DAP, respectively.

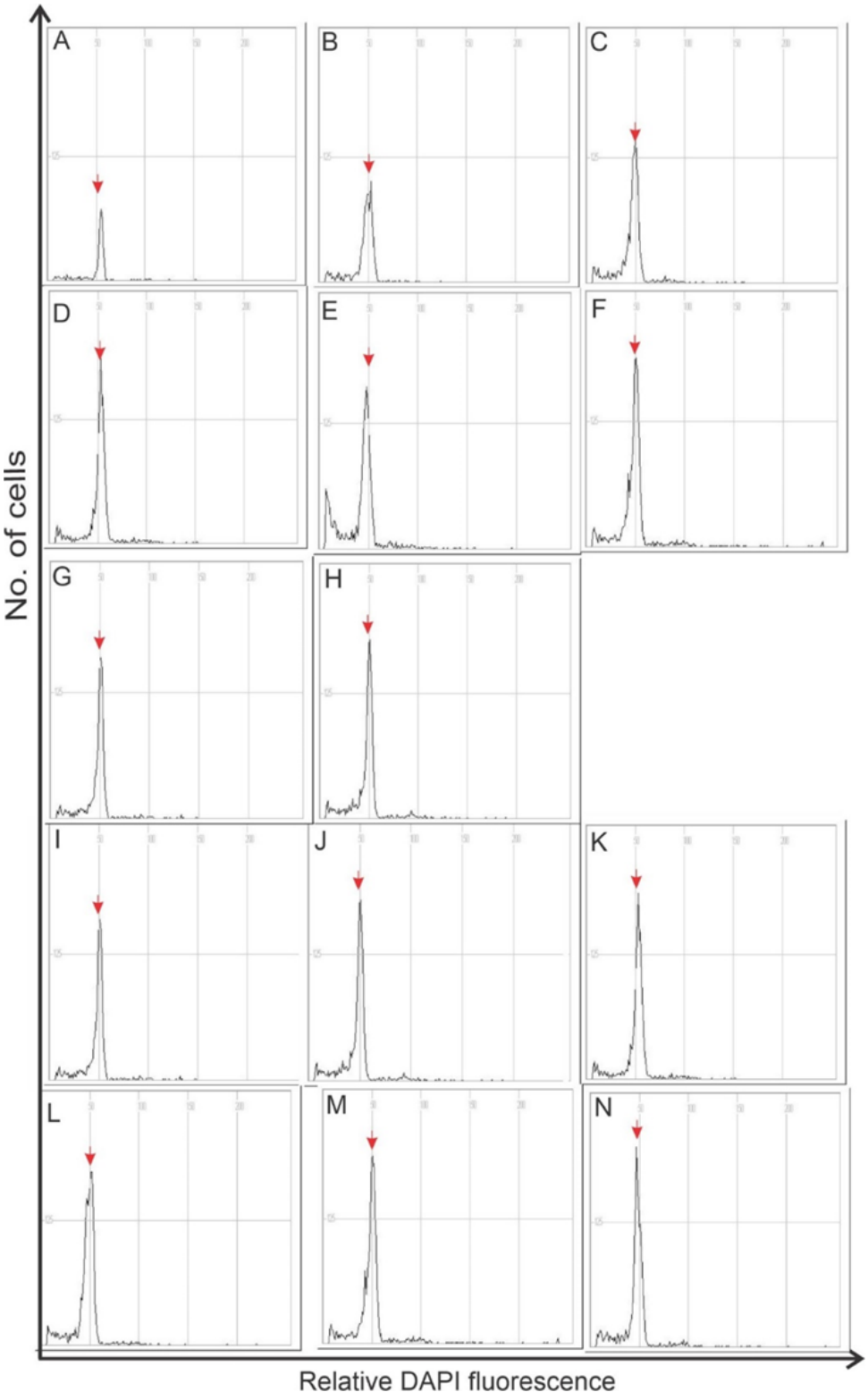

Figure 2

The flowcytometry histogram of female L. longiflorum, male Asiatic, L. hansonii, and their interspecific progenies. A: 'White Heaven', B: 'Bright Tower', C: 'White Tower', D: 'Conception', E: 'Gironde', F: 'Sky Shim 2', G: L. hansonii (111114), H: L. hansonii (121038-1), and their interspecific progenies I: 113521, J: 132025, K: 143024, L: 128115-7, M: 128130-5, N: 128133-8. Red arrows indicate the '50' peak position meaning diploid lily.

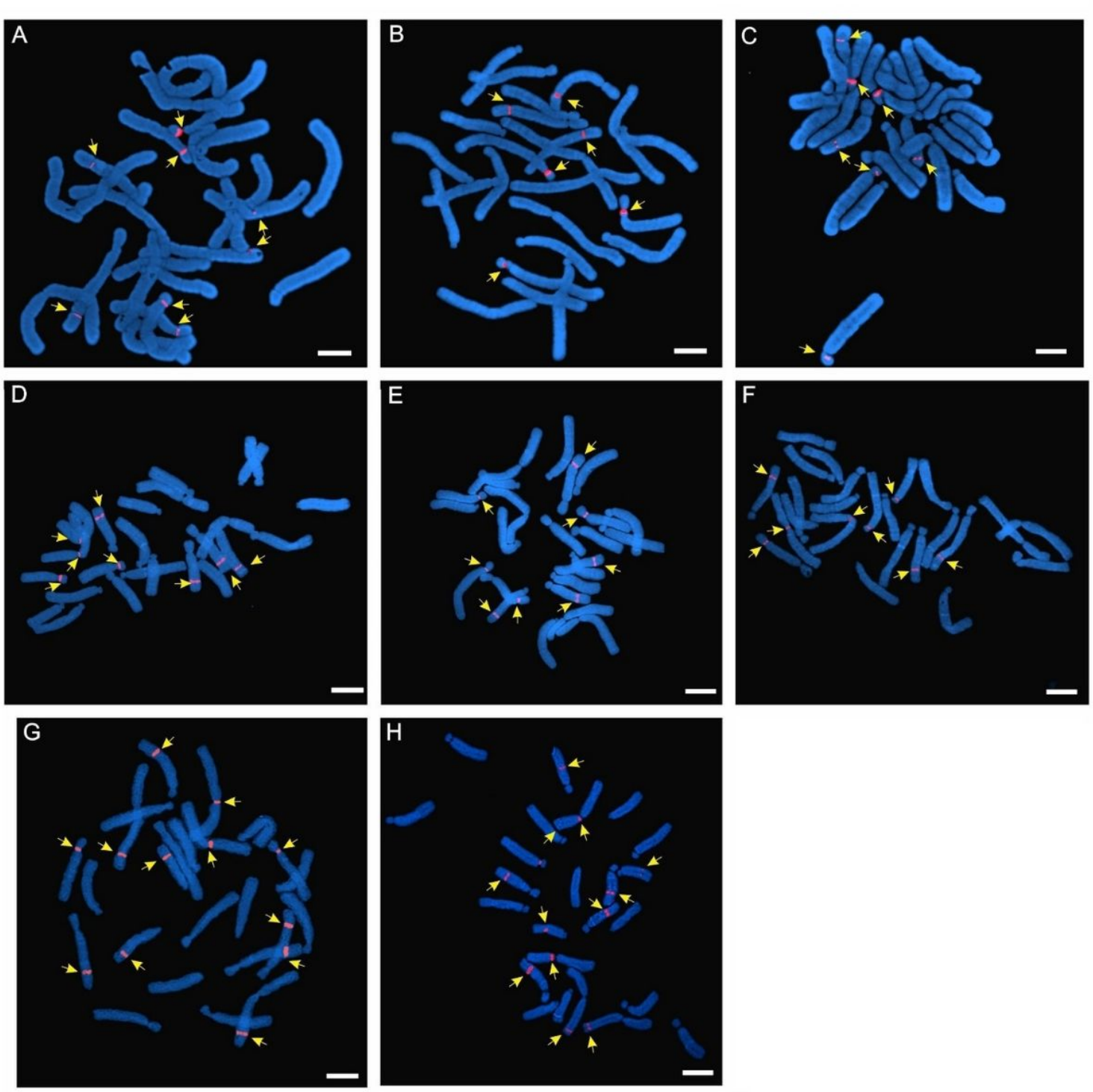

Figure 3

Fluorescence in situ hybridization analysis using 18S rDNA probes at metaphase stage of female, male parents. A: ‘White Heaven’, B: ‘Bright Tower’, C: ‘White Tower’, D: ‘Conception’, E: ‘Gironde’, F: ‘Sky Shim 2’, G: L. hansonii (111114), H: L. hansonii (121038-1). Yellow arrows indicate the 18S loci. Bar = 5μm.

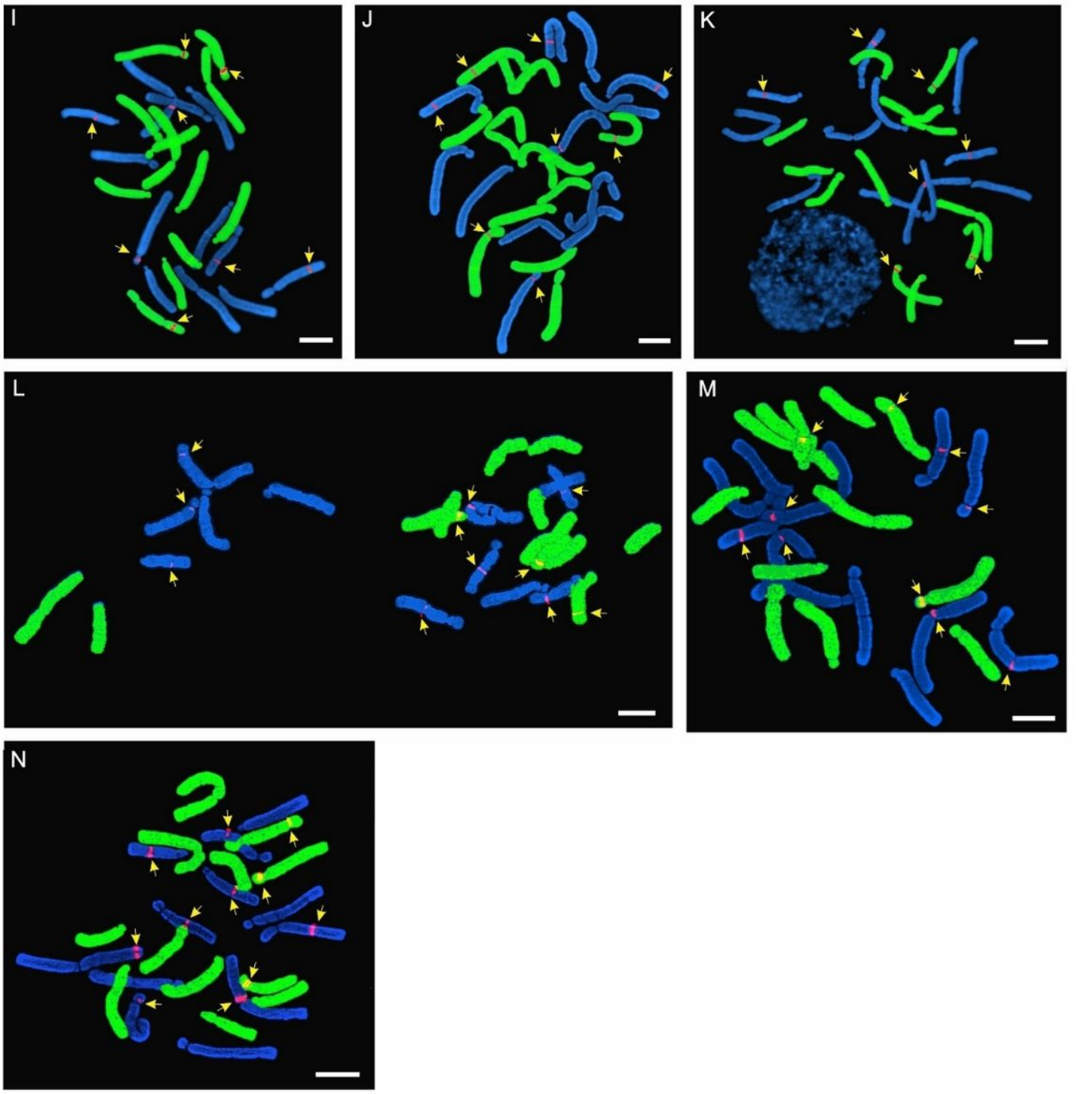

Figure 4

Genomic-fluorescence in situ hybridization analysis using L. longiflorum genome and 18S rDNA probes at metaphase stage of F1 hybrids lilies. Here, I: 113521, J: 132025, K: 143024, L: 128115-7, M: 128130-5, N: 128133-8. L. longiflorum chromosomes represented as green and Asiatic, and L. hansonii chromosomes represented as blue (DAPI). Yellow arrows indicate the 18S loci. Bar = 5 μm.

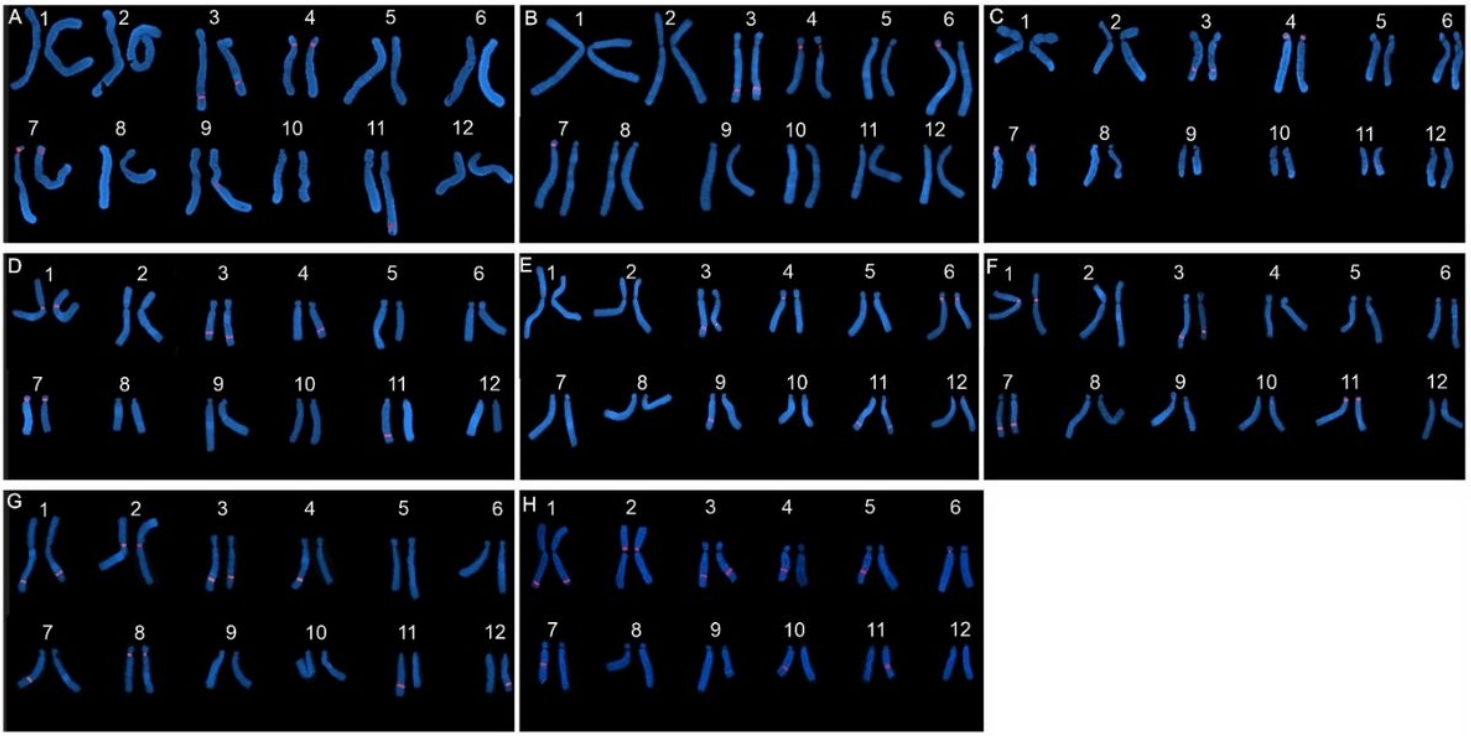

Figure 5

Karyotype analysis in female and male plants. Female parents, A: ‘White Heaven’, B: ‘Bright Tower’, C: ‘White Tower’, and male parents D: ‘Conception’, E: ‘Gironde’, F: ‘Sky Shim 2’, G: L. hansonii (111114), H: L. hansonii (121038-1).

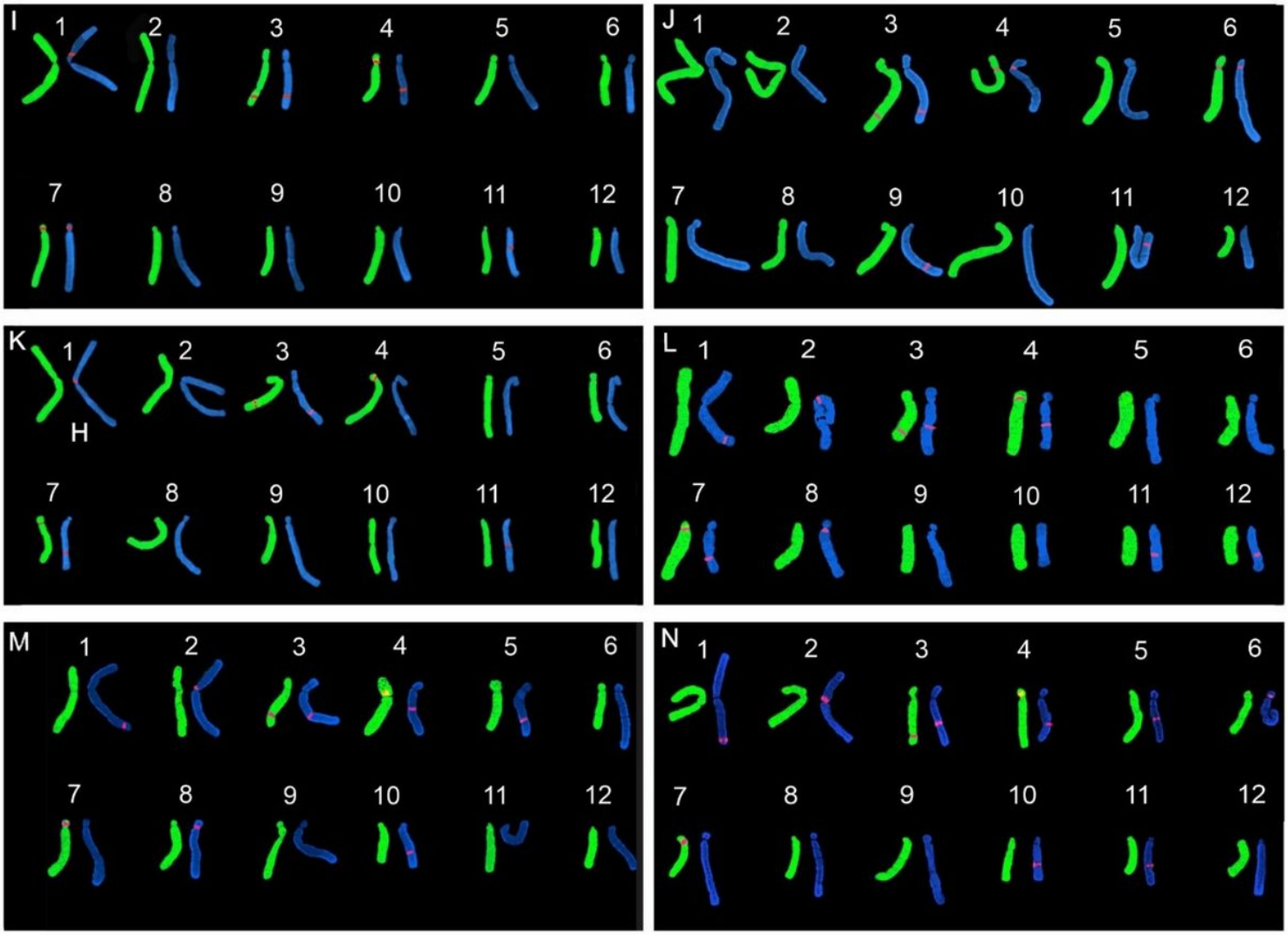

Figure 6

Karyotype analysis in F1 hybrid lilies. I: 113521, J: 132025, K: 143024, L: 128115-7, M: 128130-5, N: 128133-8. L. longiflorum chromosomes were represented as green and Asiatic, L. hansonii chromosomes were represented as blue. 18S rDNA loci were represented as red.

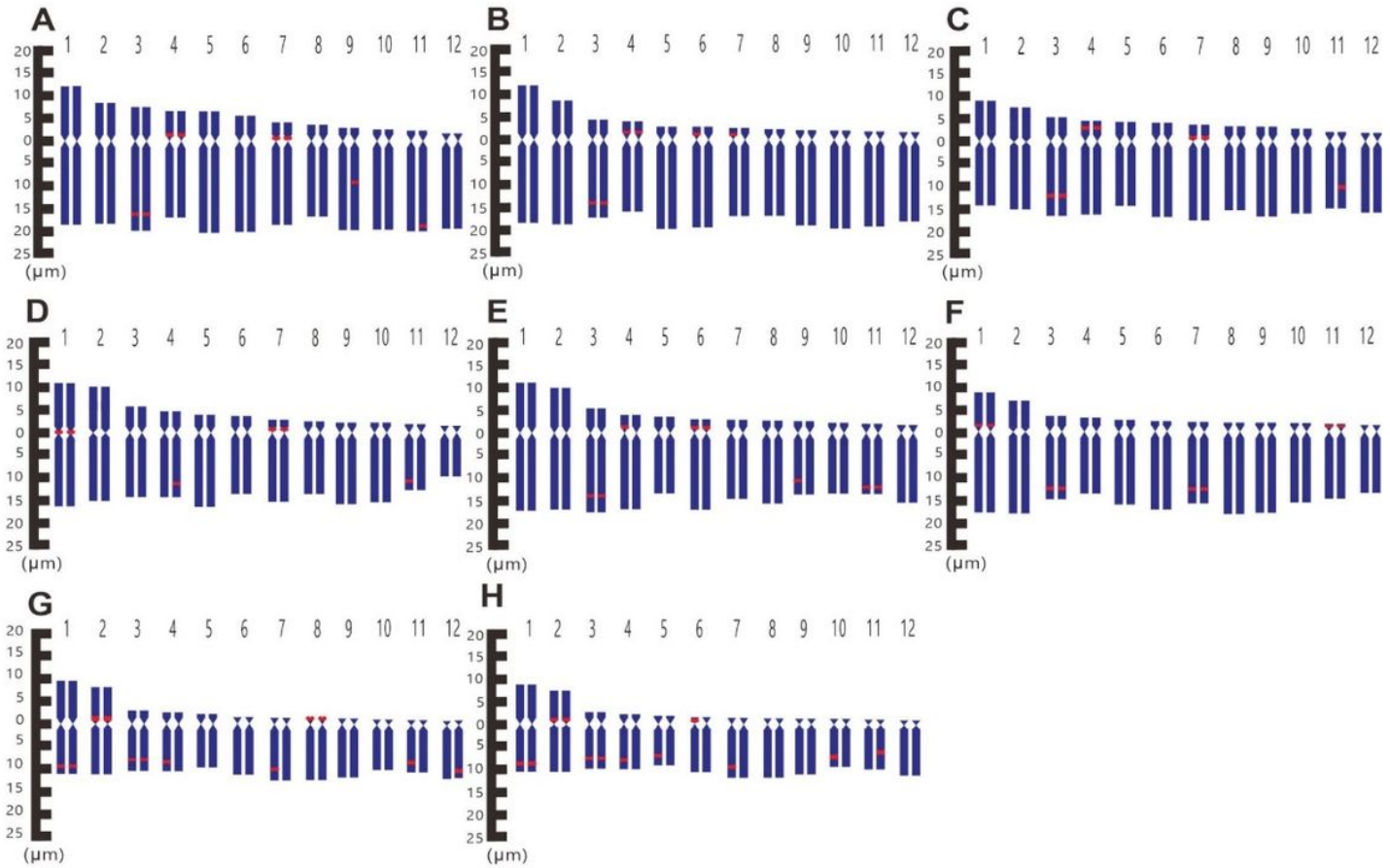

Figure 7

Ideograms of chromosomes of female and male parents. Female parents, A: 'White Heaven', B: 'Bright Tower', C: 'White Tower', and Male parents D: 'Conception', E: 'Gironde', F: 'Sky Shim 2', G: L. hansonii (111114), H: L. hansonii (121038-1). 18S rDNA loci were represented as red.

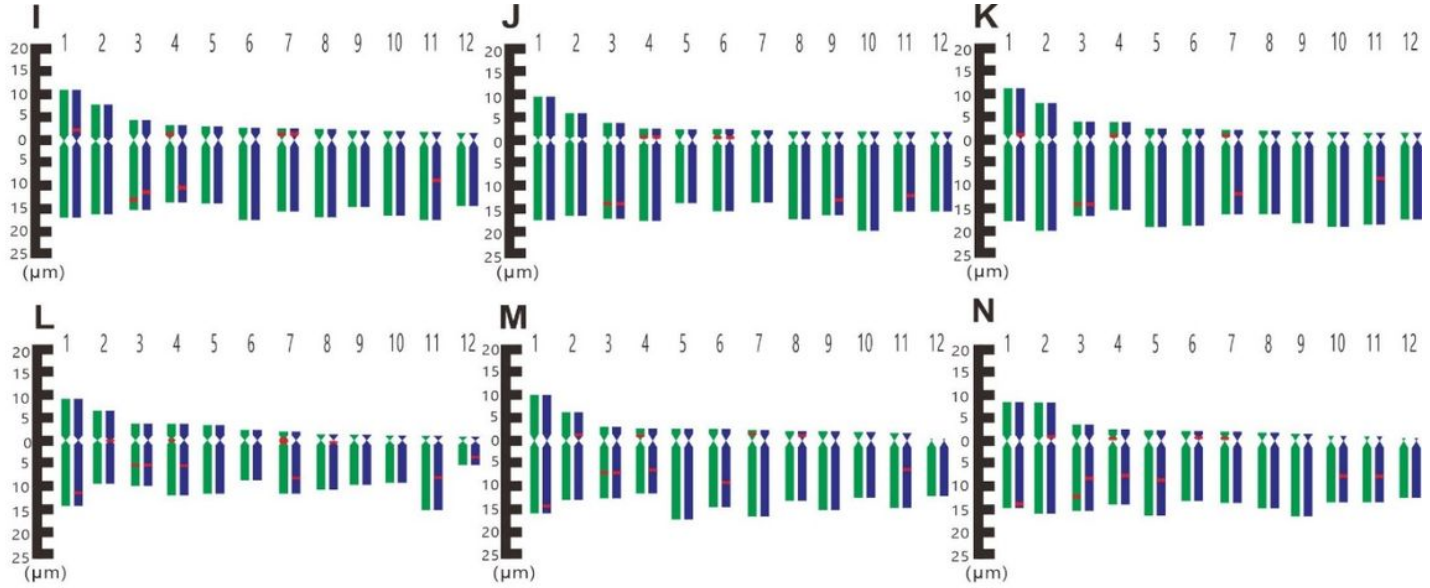

Figure 8

Ideograms of chromosomes of interspecific F1 hybrids progenies. I: 113521, J: 132025, K: 143024, L: 128115-7, M: 128130-5, N: 128133-8. L. longiflorum chromosomes were represented with green and Asiatic, L. hansonii chromosomes were represented with blue. 18S rDNA loci represented as red.