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Original article

Genetic assessment of the effects of self-fertilization in a *Lilium* L. hybrids using molecular cytogenetic methods (FISH and ISSR)

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ABSTRACT

Self-fertilization (also termed selfing) is a mode of reproduction that occurs in hermaphrodites and has evolved several times in various plant and animal species. A transition from outbreeding to selfing in hermaphroditic flowers is typically associated with changes in flower morphology and functionality. This study aimed to identify genetic effects of selfing in the F2 progeny of F1 hybrid developed by crossing *Lilium lancifolium* with the Asiatic *Lilium* hybrid 'Dreamland.' Fluorescence *in situ* hybridization (FISH) and inter-simple sequence repeats (ISSR) techniques were used to detect genetic variations in plants produced by selfing. The FISH results showed that F1 hybrid were similar to the female parent (*L. lancifolium*) regarding the 45S loci, but F2 individuals showed variation in the number and location of the respective loci. In F2 progeny, F2-2, F2-3, F2-4, F2-5, and F2-8 hybrids expressed two strong and one weak 5S signal on chromosome 3, whereas F2-7 and F2-9 individuals expressed one strong and two weak signals. Only two strong 5S signals were detected in an F2-1 plant. The ISSR results showed a maximum similarity value of 0.6269 between the female parent and the F2-2 hybrid. Regarding similarity to the male parent, a maximum value of 0.6119 was found in the F2-1 and F2-2 hybrids. The highest genetic distance from *L. lancifolium* and the Asiatic *Lilium* hybrid 'Dreamland' was observed in the F2-4 progeny (0.6352 and 0.7547, respectively). Phylogenetic relationships showed that the F2 progeny were closer to the male parent than to the female parent. Self-fertilization showed effects on variation among the F2 progeny, and effects on the genome were confirmed using FISH and ISSR analyses.

1. Introduction

Self-fertilization (also termed selfing) is a mode of reproduction type which occurs in hermaphrodites and evolved several times in various plant and animal species (Goodwillie et al., 2005; Jarne and Auld, 2006; Jarne and Charlesworth, 1993). One advantage of selfing is the high proportion of successful gene transmission, thereby selecting for genes that are responsible for selfing. The rate of selfing thus increases when these genes appear in originally outcrossing populations. Moreover, selfing has a high rate of reproductive success when few mating partners are available (Darwin, 1876; Fisher, 1941; Jain, 1976). At a population level, evolution driven by selfing may be effective for maintaining genetic variation (Glémin and Galtier, 2012). Self-fertilization is an important breeding technique and is facilitates expression of parental genes in the progeny (Eckert et al., 2006).

A transition from outbreeding to selfing is a recurrent and important evolutionary event in angiosperms (Stebbins, 1950, 1957, 1974; Barrett, 2002). This transition is of scientific interest to ecologists, taxonomists, and evolutionary biologists owing to its effect on individual and population genetics (Charlesworth and Wright, 2001; Barrett, 2010). A transition from outbreeding to selfing in hermaphroditic flower is typically associated with changes in flower morphology and functionality (Darwin, 1876; Ornduff, 1969; Richards, 1986). Hermaphrodite plants can fertilize own egg cells and those of other plants, which enables them to transfer more genes to subsequent generations when compared with the exclusively outbreeding plants (Fisher, 1941).

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The ability to resort to selfing can sustain recolonization periods, which is useful to conserve germplasm; consequently, selfing can help to improve the genetic structure of populations (Ingvarsson, 2002). Compared to typically outcrossing species, plant species that predominantly rely on selfing or mixed modes of mating show more differences in their genetic structures (Hamrick and Godt, 1996; Nybom, 2004). However, outcrossing can have distinct negative effects on endangered plant species due to decreasing genetic diversity (Cole, 2003).

The genus *Lilium* is highly heterozygous in nature because of frequent interspecies crossing; however, exact measurement of heterozygosity within this genus is difficult because of a limitation in molecular markers that can be used to characterize heterozygosity (Biswas et al., 2018). In *Lilium*, breeding of old cultivars with germplasm of wild species is a common approach to develop new cultivars (Anderson et al., 2009). *Lilium* has more than 200 years of breeding history. Asiatic germplasm has been in use for about 50 years in cultivar breeding programs and was a part of a major breakthrough in lily breeding. Therefore, Asiatic hybrids, which belong to Division 1, are currently being used predominantly, compared with the other divisions (Lim et al., 2008; McRae, 1998; Shimizu, 1987). *Lilium lancifolium* Thunb., an Asiatic species, has a strong stem, is typically vigorous, produces small black aerial bulbils, and is comparably resistant to abiotic stressors such as cold, heat, drought, salinity, nutrient deficiencies, and infections with viruses and *Fusarium* (Asker, 2015; Li and Gao, 2013; Lim and Van Tuyl, 2006; Wang et al., 2014). This species shows considerable genetic diversity in the progeny of new populations (Hamrick and Godt, 1990), and it has been used as a primary parent to produce various Asiatic hybrids (Suzuki and Yamagishi, 2015).

Cytogenetic techniques such as *in situ* hybridization are useful for evaluating chromosomes, genome evolution, genomic function and structure, introgression of alien genes, and to distinguish chromosomes regarding their origin of different genomes of horticultural crops (Ramzan et al., 2017). Fluorescence *in situ* hybridization (FISH) is a molecular cytogenetic method to identify the distribution of specific DNA sequences and to observe variations in chromosomal structures. Chromosomal characterization of various plant taxa has been accomplished using FISH analysis (Jiang and Gill, 2006; Younis et al., 2015), and this technique was used in lilies for chromosome mapping (Zhang et al., 2005), karyotype analysis (Hwang et al. 2011, Lim et al., 2001a, 2001b), and identification of hybrids (Marasek et al. 2004). Chromosome identification and karyotyping is a primary aim in cytogenetic research, which is typically followed using microscopic examination of chromosome morphology. Chromatin sites in individual chromosomes can be identified using DNA sequencing and FISH analysis (Schubert et al., 2001).

In plant breeding analysis, DNA markers are widely applied to analyze genetic diversity, marker-assisted selection, genetic homogeneity, and parental genomic contribution to the progeny (Steele et al., 2004; VanToal et al., 1997). The diversity of morphological traits is mainly affected by environmental factors and trait limitations. Plant maturity is a factor to identify phenotypic variation and diversity of traits. Cluster analyses of loci throughout the plant genome is becoming increasingly simpler owing to technical advances. Molecular markers are the most important tools for assessing genetic relationships within and among species and are used to examine genetic variation (Chakravarthi and Naravaneni, 2006; Winter and Kahl, 1995). Inter simple sequence repeats (ISSRs) are typically used as a combination of SSR and RAPD techniques to produce higher resolution of polymorphisms than RAPD, and ISSRs are typically more stable and sensitive than other markers. Furthermore, this approach is highly reproducible (Gllbert et al., 1999; Omondi et al., 2016). It has been shown that ISSR markers are a fast, simple, reliable, and cost-effective method to investigate genetic diversity of closely related cultivars, identify varieties and cultivars, and to characterize progeny (Kumar et al., 2008).

The aim of this study was to identify effects of self-pollination on the genomic structure of the progeny using FISH and ISSR techniques. In addition, cytogentic techniques were used to find the genetic diversity and genetic relationship among hybrids.

2. Materials and methods

2.1. Plant material and growing conditions

An F1 hybrid was obtained by crossing *L. lancifolium* with the Asiatic *Lilium* hybrid 'Dreamland.' The F1 hybrid was self-pollinated to produce an F2 generation (Fig. 1). Bulbs of parent plants and progeny were planted and grown in a greenhouse at the Department of Horticultural Sciences, Kyungpook National University, Republic of Korea.

2.2. Chromosome preparation

Actively growing root tips were pre-treated using α-bromonaphtalene at 20 °C for 3 h, and then fixed in acetic acid–ethanol solution (1:3, v/v) at room temperature for 24 h. The samples were stored in 70% ethanol at −20 °C until further analysis. For chromosome preparation, root tips were rinsed thoroughly and treated with an enzyme mixture (0.3% pectolyase, 0.3% cellulase, and 0.3% cytohelicase in 150 mM citrate buffer) at 37 °C for 1 h. The root tips were squashed in a drop of 60% acetic acid and then air-dried (Hwang et al., 2011).

2.3. Fish

FISH was performed according to Lim et al. (2007). Briefly, the slides were pre-treated using RNase A in 2 × SSC (DNase-free, 100 μL mL^{-1}) for 1 h at 37 °C, washed in 2 × SSC three times and were then post-fixed in a 4% *para*-formaldehyde solution for 10 min. 45S and 5S rDNA were directly labeled using biotin-16-dUTP and digoxygenin-11-dUTP, respectively, by nick translation (Roche, Germany). The hybridization mixture, containing 50% deionized formamide, 10% dextran sulfate, 2 × SSC and 20 μL mL^{-1} of probe DNA, was subsequently denatured at 70 °C for 10 min and applied to the slides which were then sealed using a cover slip. The slides were heated to 80 °C for 5 min, followed by incubation at 37 °C in a humid chamber overnight. After hybridization, the slides were washed using 0.1 × SSC at 42 °C for 30 min, after which digoxygenin and biotin were detected using FITC conjugated anti-digoxygenin antibodies (Roche, Germany) and streptavidin Cy3 (Zymed Lab., USA), respectively. The chromosomes were then counterstained with 2 μL mL^{-1} of 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield (Vecta Laboratories Inc., USA) and examined using a Nikon BX 61 fluorescence microscope (Nikon, Japan). Images were captured using CCD and then processed using the Genus image analysis workstation software (Genus version 3.8, Applied Imaging Corporation, USA). Potentially homologous chromosomes were confirmed based on their morphological characteristics, FISH, and DAPI bands. At least five cells showing well-spread metaphase chromosomes were used for karyotype analyses. Individual chromosome length was measured using the software and the chromosome number was determined based on short arm length order according to Lim et al. (2001a, 2001b).

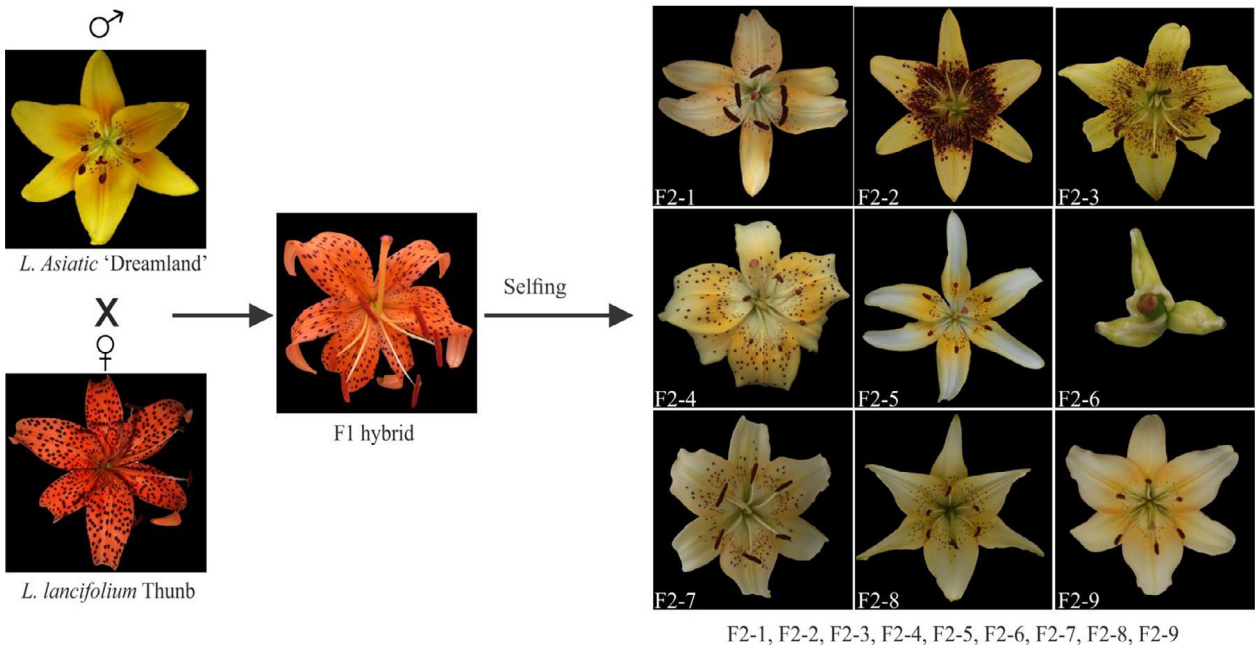

Fig. 1. Crossing Scheme of progeny developed by selfing.

2.4. DNA analyses using ISSR

DNA extraction was performed using a modified hexadecyltrimethylammonium bromide method as described by Zhou et al. (1999). The purity of DNA extracts was measured using an absorbance ratio at 260 nm and 280 nm (A260/A280). Only DNA extracts with an A260/A280 ratio of 1.8–2.1 were used (at a concentration of 10 ng μL^{-1}) as template for polymerase chain reaction (PCR) amplification.

Twenty-one primers were used for ISSR analysis (Bioneer, Republic of Korea). DNA amplification was performed in 25 μL reaction volume containing 12.5 μL 2 × PCR Master Mix (0.625 U μL^{-1} Tag DNA polymerase; 2 mM $MgCl_2$; 0.2 mM each dNTP) (Biofact, Republic of Korea), 2 μL primers (10 pmol μL^{-1}), 6.5 μL twice-distilled water, and 4 μL DNA (25 ng/μL). PCR reactions were performed using a thermo cycler (Master cycler, Eppendorf, Germany) and the following cycling conditions: initial denaturation at 94 °C for 5 min, 45 cycles of 94 °C for 30 s, primers annealing at 45 °C–50 °C (depending on the respective primers) for 30 s, and extension at 72 °C for 60 s, followed by a final extension step at 72 °C for 10 min (Tables 1 and 2).

Polymorphisms were scored as presence (1) or absence (0) after visualization on 2% agarose gels prepared in 1 × TBE buffer. A standard molecular marker of 100 bp + 3 K DNA Ladder (Smobio, Taiwan) was used to determine molecular size of the amplified bands. Gels containing ethidium bromide were examined using UV light. Gel image for primer number FBL- ISSR-13 is presented in Fig. 5.

2.5. Data analyses

Seven ISSR marker DNA bands were analyzed using PopGen-1.31 software. The observed number of alleles, Nei's gene diversity ([H]; Nei, 1973), Shannon's information index (I), and Nei's genetic distance were calculated with using POPGENE V 1.31 software. Relationships between different lily species were evaluated using a dendrogram based on genetic distances according to Nei and Li (1979) using an unweighted pair group method and cluster analyses.

3. Results

3.1. Phenotypic description of parents and progeny

Phenotypic characteristics of parents and progeny is presented in Table 1. Results indicated that most of the F2 progenies were closer to male parent (P2) with respect to flower color and flower position. Similar results were obtained for flower position where most of F2 progeny showed upside flower position. F2-1, F2-5

Table 1
Qualitative phenotypic characteristics of P1 (*L. lancifolium*), P2 (*L. Asiatic* 'Dreamland'), F1 (*L. lancifolium* × *L. Asiatic* 'Dreamland'), F2 (selfing of F1) progeny.

Character	P1	P2	F1	F2-1	F2-2	F2-3	F2-4	F2-5	F2-6	F2-7	F2-8	F2-9
Flower color	Dark orange	Yellow + Orange	Dark orange	Yellow + White	Red+ White	Yellow + White	Yellow + White	Yellow + White	White	Yellow + White	Yellow + White	Yellow + White
Color type	Uni	Double	Uni	Double	Double	Double	Double	Double	Uni	Double	Double	Double
Flower shape (Petal recurved)	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes
Flower position	Down	Up	Down	Up	Up	Up	Side	Side	Up	Side	Side	Up
Spot Distribution	Many	Less	Many	Less	Medium	Medium	Many	Less	No	Medium	Medium	Less
Spot size	Big	Small	Big	Small	Medium	Medium	Medium	Small	No	Medium	Medium	Less
Bulbil production ability	Yes	No	Yes	Yes	No	No	No	Yes	Yes	No	No	Yes

Table 2
FISH probe information of 45S and 5S rDNA signals on the chromosomes of P1 (*L. lancifolium*), P2 (Asiatic *Lilium* hybrid 'Dreamland'), F1 (*L. lancifolium* × Asiatic *Lilium* hybrid 'Dreamland'), F2-1 to F2-9 (selfing of F1 hybrid) respectively.

Plant type	Number of 45S rDNAs	Location of 45S rDNA		Chromosome number containing 45S rDNA	Number of 5S rDNAs	Location of 5S rDNA	
		Short Arm	Long arm			Short arm	Long arm
P1	10	6	4	Ch#1,2,6,7,11	2 s + 1w	0	2 s + 1w
P2	9	6	3	Ch#1,2,6,7 + 11 sg	2 s + 1w	0	2 s + 1w
F1	10	6	4	Ch#1,2,6,7,11	2 s	0	2 s
F2-1	10	6	4	Ch#1,2,6,7,11	2 s	0	2 s
F2-2	10	7	3	Ch#1,2,7,11 + 6,8 Sg	2 s + 1w	0	2 s + 1w
F2-3	10	6	4	Ch#1,2,6,7,11	2 s + 1w	0	2 s + 1w
F2-4	9	6	3	Ch#1,2,7,11 + 6 Sg	2 s + 1w	0	2 s + 1w
F2-5	10	7	3	Ch#1,2,7,11 + 6,8 Sg	2 s + 1w	0	2 s + 1w
F2-6	11	7	4	Ch#1,2,6,7,11 + 8 Sg	3 s	0	3 s
F2-7	10	7	3	Ch#1,2,7,11 + 6,8 Sg	1 s + 2w	0	1 s + 2w
F2-8	10	7	3	Ch#1,2,7,11 + 6,8 Sg	2 s + 1w	0	2 s + 1w
F2-9	10	6	4	Ch#1,2,6,7,11	1 s + 2w	0	1 s + 2w

S: strong 5S rDNA Signals.
W: weak 5S rDNA Signals.

and F2-9 progeny showed less spo on petals while F2-4 showed highest spot distribution among F2 progeny. In addition, spot size was intermediate in F2 progeny than their t P1, P2 and F1 parents.

3.2. FISH analysis in progeny developed by selfing

Parents and progeny were diploid (2n = 24). Ten 45S rDNA signals were observed in the female parent (*L. lancifolium*) on chromosomes 1, 2, 6, 7, and 11. The karyotype of the male parent showed four pairs of 45S signals expressed on chromosomes 1, 2, 6, and 7, whereas a single 45S signal was observed on chromosome 11 (Table 2). The 45S signals of the F1 hybrid were identical to that of *L. lancifolium*. All F2 individuals had eight 45S signals on chromosome 1, 2, 7, and 11; however, a paired signal on chromosome 6 which was found in two parents and the F1 hybrid was transformed to a single signal in five F2 individuals (F2-2, F2-4, F2-5, F2-7, and F2-8) as shown in Fig. 2. Interestingly, five individuals (F2-2, F2-5, F2-6, F2-7, and F2-8) contained a novel signal on chromosome 8 which was not found any parent or F1 hybrid. It has been seen that 45S signals idstrubtion based on long arm and short arm also showed variation (Fig. 3). Highest number of 45S signals in short arm was observed in F2-2, F2-5, F2-6, F2-7, F2-8 and highest number of 45S signals in long arm was observed in F2-1, F2-3, F2-6 and F2-9 respectively.

Regarding 5S rDNA distribution based on intensity and frequency, two strong and one weak signals on chromosome number 3 were expressed in the female (*L. lancifolium*) and in the male (Asiatic *Lilium* hybrid 'Dreamland') parent, whereas only two strong signals were observed in the F1 hybrid. The F2 individuals F2-2, F2-3, F2-4, F2-5, and F2-8 hybrids expressed two strong and one weak signal on chromosome number 3, whereas F2-7 and F2-9 hybrids expressed one strong and two weak signals. Two strong 5S signals were detected in the individual F2-1. Comparable to the 45S signal pattern, F2-6 expressed three strong 5S signals which significantly discriminated this individual from others.

3.3. Chromosomal karyotype of progeny developed by selfing

Short arm, long arm, and complete chromosome lengths were 8.0 ± 1.4 μm, 32.5 ± 0.7 μm, and 40.5 ± 1.5 μm, respectively, in the female parent, and 9.2 ± 1.8 μm, 39.7 ± 1.1 μm, 49.0 ± 2.3 μm, respectively, in the male parent (Table 3). Short arm length was longer in the F1 hybrid (9.3 ± 1.8 μm) than in the parent plants. Long arm (36.5 ± 1.0 μm) and complete chromosome (45.9 ± 2.2 μm) lengths of the F1 hybrid were intermediate between male and female parent. In the F2 generation, the highest values of short arm, long arm, and complete chromosome length were observed in F2-2 (8.0 ± 1.7 μm, 35.8 ± 1.0 μm, and 43.9 ± 2.0 μm, respectively). The lowest values of short arm, long arm, and complete chromosome length were observed in F2-9 (5.6 ± 0.9 μm, 24.0 ± 0.6 μm, and 29.6 ± 1.1 μm, respectively). The range of arm ratio was 6.2 ± 0.6 to 9.3 ± 1.4. Arm rations in the female and male parent were 6.4 ± 0.7 and 6.9 ± 0.7, respectively. F1 arm ratio was the same as that of the female parent, whereas F2 arm ratio showed substantial variation. The arm ratios of F2-3, F2-2, F2-7 were higher (9.3 ± 1.4, 8.5 ± 1.1, and 8.0 ± 1.1, respectively).

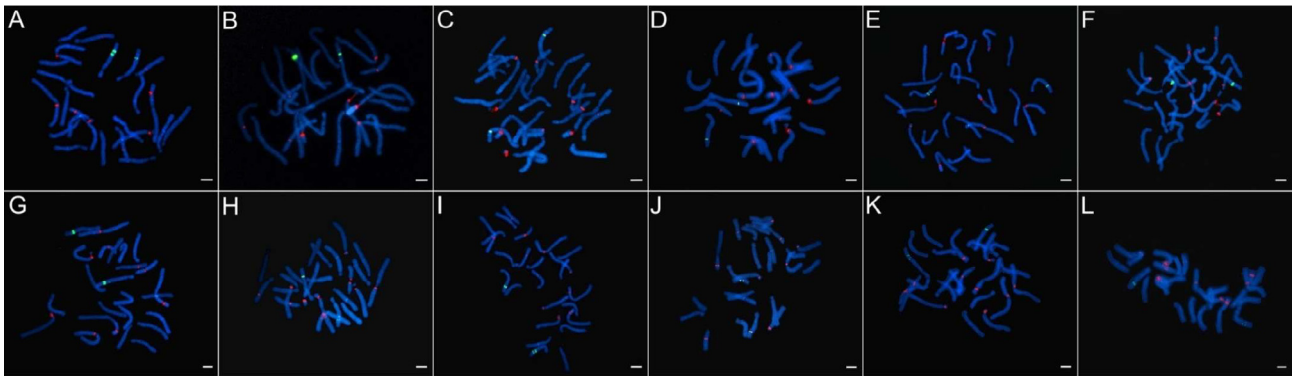

Fig. 2. Fluorescence *in situ* hybridization of 5S and 45S rDNA on mitotic metaphase chromosomes of A. P1 (*L. lancifolium*), B. P2 (Asiatic *Lilium* hybrid 'Dreamland'), C. F1 (*L. lancifolium* × Asiatic *Lilium* hybrid 'Dreamland'), D to L. F2-1 to F2-9 (selfing of F1 hybrid) respectively. Green and red signals indicate the positions of 5S and 45S rDNAs, respectively. Size bars = 10 μm.

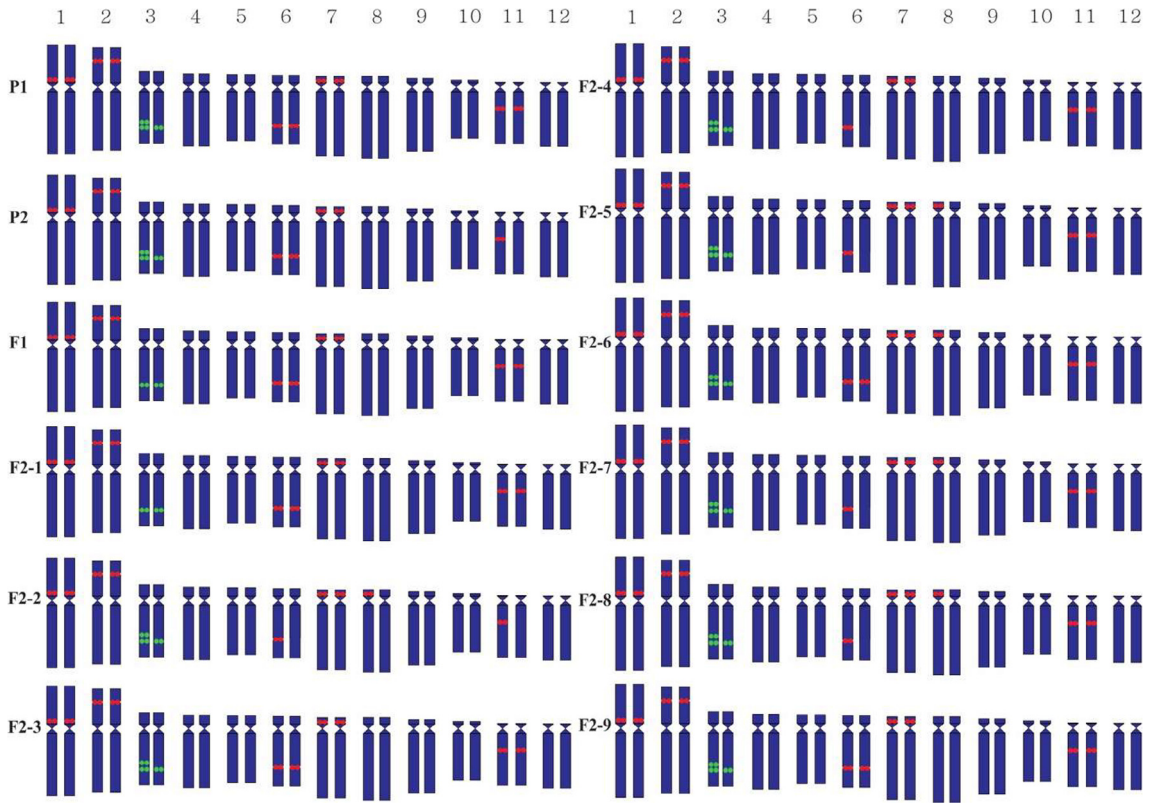

Fig. 3. FISH karyotype detail of 45S rDNA and 5S rDNA, signals on the chromosomes of P1 (*L. lancifolium*), P2 (Asiatic *Lilium* hybrid 'Dreamland'), F1 (*L. lancifolium* × Asiatic *Lilium* hybrid 'Dreamland'), F2-1 to F2-9 (selfing of F1 hybrid), respectively. Green and red marks indicate the positions of 5S and 45S rDNAs, respectively.

Table 3
Karyotype results of chromosomes of P1 (*L. lancifolium*), P2 (Asiatic *Lilium* hybrid 'Dreamland'), F1 (*L. lancifolium* × Asiatic *Lilium* hybrid 'Dreamland'), F2-1 to F2-9 (selfing of F1 hybrid) respectively.

Plant type	Short arm (μm)	Long arm (μm)	Total (μm)	Arm ratio (μm)
P1	8.0 ± 1.4	32.5 ± 0.7	40.5 ± 1.5	6.4 ± 0.7
P2	9.2 ± 1.8	39.7 ± 1.1	49.0 ± 2.3	6.9 ± 0.7
F1	9.3 ± 1.8	36.5 ± 1.0	45.9 ± 2.2	6.4 ± 0.7
F2-1	8.0 ± 1.6	33.0 ± 0.7	41.0 ± 1.8	7.6 ± 0.9
F2-2	8.0 ± 1.7	35.8 ± 1.0	43.9 ± 2.0	8.5 ± 1.1
F2-3	7.3 ± 1.6	33.1 ± 0.8	40.5 ± 1.7	9.3 ± 1.4
F2-4	7.7 ± 1.6	31.9 ± 0.7	39.7 ± 1.9	7.9 ± 1.0
F2-5	7.0 ± 1.2	31.2 ± 0.7	38.2 ± 1.5	7.2 ± 0.9
F2-6	7.4 ± 1.5	32.9 ± 0.9	40.4 ± 1.8	7.7 ± 0.9
F2-7	5.9 ± 1.2	26.5 ± 0.8	32.5 ± 1.4	8.0 ± 1.1
F2-8	7.6 ± 1.5	34.2 ± 0.8	41.9 ± 1.7	7.6 ± 0.9
F2-9	5.6 ± 0.9	24.0 ± 0.6	29.6 ± 1.1	6.2 ± 0.6

3.4. ISSR analyses

Twenty-one primers were used for initial screening, and seven primers produced high percentages of polymorphism. A total of 126 highly reproducible ISSR bands ranging in size from 180 to 2,250 bp were obtained of which 96.83% (122 out of 126) were polymorphic, demonstrating a high level of genetic diversity among the progeny (Table 4).

The observed number of alleles ranged from 1.9048 to 2.0000 as shown in table 5. The effective maximum number of alleles (Ne) was produced by the primer FBLISSR-11 (1.6606), whereas the minimum value was produced by FBLISSR-13 (1.4725). Nei's genetic diversity index (He) ranged from 0.3003 to 0.3827. The highest Shannon's information index value was produced by the primer FBLISSR-11 (0.5655), and the lowest value by the primer FBLISSR-13 (0.4693).

Similarity coefficients ranged from 0.4701 to 0.7463 (Table 6). The F1 hybrid was closer to the female parent (0.5597) than to the male parent (0.6045). In F2 progeny, a maximum similarity value of 0.6269 to the female parent was observed in F2-2, and a minimum value of 0.5299 was observed in F2-4. Regarding similarity values to the male parent, a maximum value of 0.6119 was found in F2-1 and F2-2, and a minimum value of 0.4701 was observed in F2-4 hybrids. The highest similarity value between F2 hybrids (0.7463) was observed between F2-2, F2-3, F2-3 and F2-6, and F2-8 and F2-9, and the lowest value (0.5000) was found as the similarity of F2-4 and F2-9.

The highest genetic distance from *L. lancifolium* and the Asiatic *Lilium* hybrid 'Dreamland' was observed in F2-4 (0.6352 and 0.7547) progeny. Genetic distances ranged from 0.2927 to 0.6931. A maximum genetic distance from the F1 parent was observed in F2-5 (0.5411) progeny. Genetic distances were signif-

Table 4
Characteristics of the selected primers used for generating ISSR amplification and number of bands per primer for '*L. lancifolium* × Asiatic *Lilium* hybrid 'Dreamland" progeny.

Code	Base sequence	Annealing Temperature (°C)	Total number of bands	Polymorphic bands	Percentage of Polymorphisms (%)	Band Range (bp)
FBL-ISSR 2	(CT)8 G	47	17	17	100	210–2250
FBL-ISSR 4	(TC)7 GGA	45	17	17	100	200–2250
FBL-ISSR 11	(CT)7 GCA	42	27	27	100	200–1200
FBL-ISSR 12	(CT)7 ATG	48	13	12	92.31	350–2250
FBL-ISSR 13	(CT)7 TGA	48	16	16	100	210–1400
FBL-ISSR 18	(GACA)4C	47	18	17	94.44	250–1450
FBL-ISSR 19	(CT)7 TGT	45	21	19	90.47	180–1450

Table 5
Genetic diversity of progeny from selfing.

Primer Code	Na[a]	Ne[b]	He[c]	I[d]
FBL-ISSR 2	2.0000 ± 0.00	1.6599 ± 0.28	0.3783 ± 0.12	0.5581 ± 0.14
FBL-ISSR 4	2.0000 ± 0.00	1.6272 ± 0.31	0.3619 ± 0.13	0.5390 ± 0.15
FBL-ISSR 11	2.0000 ± 0.00	1.6606 ± 0.26	0.3827 ± 0.10	0.5655 ± 0.11
FBL-ISSR 12	1.9231 ± 0.28	1.5789 ± 0.36	0.3323 ± 0.17	0.4942 ± 0.21
FBL-ISSR 13	2.0000 ± 0.00	1.4725 ± 0.27	0.3003 ± 0.12	0.4693 ± 0.14
FBL-ISSR 18	1.9565 ± 0.21	1.6429 ± 0.31	0.3665 ± 0.14	0.5396 ± 0.17
FBL-ISSR 19	1.9048 ± 0.30	1.5330 ± 0.32	0.3175 ± 0.16	0.4777 ± 0.20

[a] Na: observed number of alleles.
[b] Ne: effective number of alleles.
[c] H: Nei's gene diversity.
[d] I: Shannon's information index.

Table 6
Nei's original measures of genetic identity (top) and genetic distance (bottom) of progeny from selfing.

Plant type	P1	P2	P3(F1)	F2-1	F2-2	F2-3	F2-4	F2-5	F2-6	F2-7	F2-8	F2-9
P1	****	0.5672	0.5597	0.5672	0.6269	0.5672	0.5299	0.6045	0.5373	0.5896	0.5373	0.5672
P2	0.5671	****	0.6045	0.6119	0.6119	0.5672	0.4701	0.5448	0.5970	0.5896	0.5224	0.5821
P3(F1)	0.5804	0.5034	****	0.6791	0.6045	0.6194	0.6119	0.5821	0.6493	0.6418	0.6194	0.5896
F2-1	0.5671	0.4911	0.3870	****	0.6119	0.6119	0.5746	0.6194	0.6269	0.7239	0.7164	0.7164
F2-2	0.4670	0.4911	0.5034	0.4911	****	0.7463	0.5448	0.7090	0.7015	0.6493	0.6269	0.7164
F2-3	0.5671	0.5671	0.4790	0.4911	0.2927	****	0.6493	0.5896	0.7463	0.6642	0.5970	0.6716
F2-4	0.6352	0.7547	0.4911	0.5540	0.6074	0.4319	****	0.5075	0.6493	0.5821	0.5896	0.5000
F2-5	0.5034	0.6074	0.5411	0.4790	0.3440	0.5284	0.6783	****	0.5896	0.5821	0.5896	0.6343
F2-6	0.6212	0.5158	0.4319	0.4670	0.3545	0.2927	0.4319	0.5284	****	0.6791	0.5970	0.6567
F2-7	0.5284	0.5284	0.4435	0.3231	0.4319	0.4092	0.5411	0.5411	0.3870	****	0.6791	0.7239
F2-8	0.6212	0.6493	0.4790	0.3335	0.4670	0.5158	0.5284	0.5284	0.5158	0.3870	****	0.7463
F2-9	0.5671	0.5411	0.5284	0.3335	0.3335	0.3980	0.6931	0.4552	0.4205	0.3231	0.2927	****

icantly different between F2 individuals. The maximum genetic distance occurred between F2-4 and F2-5 (0.6783), and the minimum genetic distance between F2-3 and F2-6, F2-8 and F2-9, and between F2-2 and F2-3 (0.2927).

The dendrogram results showed significant variation among F2 progeny (Fig. 4). Phylogenetic relationships showed that F2 progeny were closer to the male parent than to the female parent. The F2-4 hybrid was distant from other F2 progeny based on phylogenetic relationships. Significant phylogenetic relationships were observed between F2-1 and F2-7, F2-8 and F2-9, and between F2-2 and F2-3. Among F2 progeny, the F2-5 hybrid showed a close relationship to the male and female parent.

4. Discussion

In self-pollination, genetic variation plays an important role for discriminating progeny. Therefore, FISH results regarding the number and location of 45S and 5S signals are useful to find variation and hybridity status in progeny. Our results indicated genetic variation between parent and progeny as well as among progeny, based on 45S and 5S signal distribution. Wang et al. (2017) conducted an experiment to identify locations and frequency of 45S rDNA in parents and progeny of *Lilium*. The male parent expressed eight pairs of 45S signals, whereas the female parent (*Lilium*

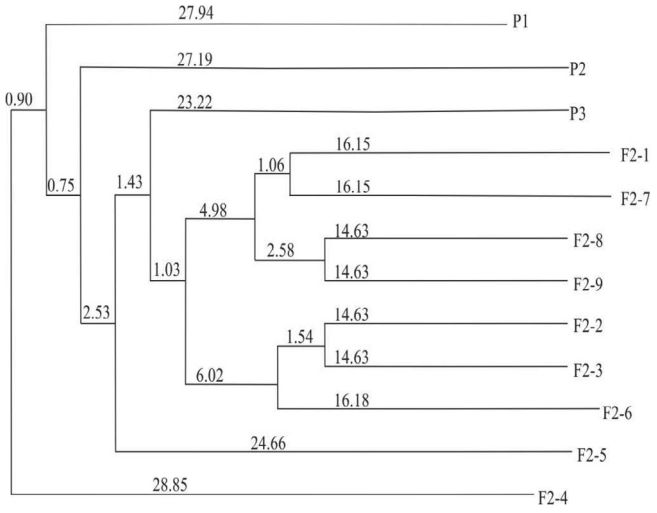

Fig. 4. Dendrogram of progenies produced from selfing. P1 = *L. lancifolium*, P2 = Asiatic *Lilium* hybrid 'Dreamland', F1-1 = *L. lancifolium* × Asiatic *Lilium* hybrid 'Dreamland', F2-1 to F2-9 = F1-1 (selfing of F1 hybrid).

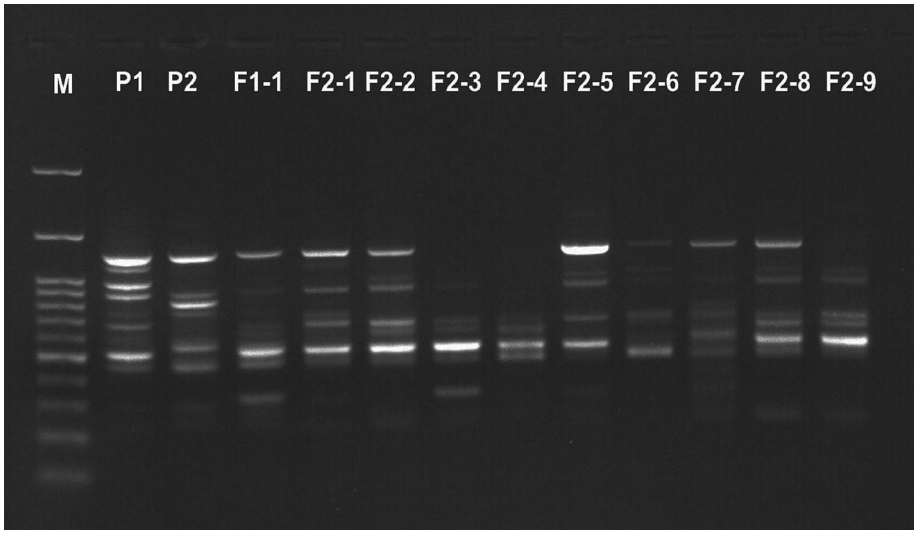

Fig. 5. ISSR profiles of progeny developed by selfing amplified by FBL- ISSR-13 primer. Lane M = DNA marker Ladder, Lane P1 = *L. lancifolium*, P2 = *L. Asiatic* 'Dreamland', Lane F1-1 = *L. lancifolium* × *L. Asiatic* 'Dreamland', Lane F2-1 to F2-9 = (Selfing of F1 hybrid).

'Renoir') exhibited six pairs and one individual 45S signal. Progeny individuals (B-1, B-2 and B-4) exhibited eight 45S rDNA signals, whereas only B-3 progeny expressed nine 45S rDNA signals. FISH results confirmed the hybridity status in progeny through variation in genetic structure of chromosomes (Wang et al., 2017).

FISH details of a self-fertilization scheme help to understand the genetic structure of progeny. In the present study, the F1 hybrid was closer to the female parent (*L. lancifolium*) regarding 45S loci, but F2 hybrids showed genetic variation in the number and location of 45S signals. These signals thus characterized the individuals and showed genetic improvement in the chromosomal structure because of the appearance of 45S signal on chromosome 8 which was not present in either parent. Variation in the number of 5S signals were in lined with the results of 45S signals. Addition and deletion of rDNA was also associated with nucleolus organizer regions. For the breeding of new cultivars FISH analysis is important to identify hybrids. Genetic difference and relationships are confirmed by FISH signals in previous studies. A previous study examined *Lilium* hybrids 'Royal Lace' (triploid), Brunello (tetraploid), and their crossing progeny using FISH and found that 'Royal Lace' parent expressed 11 and Brunello expressed 16 45S signals. The progeny was aneuploid and showed 19 45S signals. A typical 45S signal on chromosome 2 was inherited from the Brunello parent, and it was confirmed that all 45S signals were inherited from both parents. The distribution of 45S occurred mostly near the centromere or the long arm while only signals were observed near the secondary constriction regions (Xin et al., 2017).

It is important to note that some F2 hybrids exhibited 45S signals on short arm of chromosome 8, which was not observed in the parental karyotypes. Therefore, the total number of 45S signals in the F2-6 hybrid was higher (11) than that in the parents and other progeny. This genetic modification in progeny chromosomes may be due to relocation of the nucleolus organizer regions (NORs). These regions are chromosomal locations which have multicopy groups of ribosomal RNA genes (5.8S, 18S, and 28S). These genes are also considered rDNA (Hernandez-Verdun, 1986). NORs typically show high intragenomic mobility and polymorphism. This mobility may be due to transposition or unequal recombination mechanisms (Schubert and Wobus, 1985). Visualization of active and inactive NORs can be achieved using FISH as this method is based on rDNA probes (Howell, 1977; Makinen et al., 1997). The signal intensity and size of NORs is typically associated with the ribosome production level and the number of rDNAs (Guillen et al., 2004).

In F2 progeny, 45S signals were comparatively reduced and differed from the parental karyotypes. In hybrids, the reduction of 45S rDNA is due to chromosome breakages near the centromere or telomeres (Schubert, 2007). In *Allium*, the numbers and positions of NORs which contain the rDNA gene differed in clones of one genotype, therefore it can be concluded that NORs of several chromosomes can in fact move from one locus to another (Huang et al., 2008). Evolution of the *Lilium* karyotype brought about numerous variations in the overall chromosome structure. These variations produced various chromosome characteristics such as sites and number of intercalary satellites or secondary constrictions (Noda, 1991).

Plant selection based on the analysis of molecular markers is an important means of improving selection methods (Dwivedi et al. 2007; Xu and Crouch 2008). In the past two decades, commercial plant breeding companies used molecular markers to improve breeding selection, to enhance reproductive efficiency, and to reduce the duration of variety development (Bueren et al, 2010; Joshi et al, 2011). In the present study, ISSR marker provided genetic evidence of differences and relationships between parent and progeny. Moreover, the obtained results on genetic diversity were in line with those of Zhao et al. (2014) who observed high genetic diversity in 20 *Lilium* species using ISSR markers.

High genetic diversity obtained from seven primers used on progeny developed from selfing showed substantial genetic differences and diversity among the progeny. Genetic relationships and distances to the parent showed differences among F2 hybrids. F2-2 exhibited the highest genetic similarity to the male parent, whereas F2-4 showed the highest genetic distance to the male parent. Khajudparn et al. (2012) used ISSR analyses to discriminate outcrossed F1 hybrids from the self-pollinated progeny. F1 hybrids were morphologically similar to self-pollinated progeny and female parents. Outcross hybrids were significantly different as ISSR bands of the male parent were observed. Salami et al. (2017) used ISSR markers to assess the impact of selfing and outcrossing on phenotypic characteristics and genetic diversity in the progeny of fennel. Genetic diversity was reduced due to selfing and an Iranian population was affected more by selfing than a European population.

Genes that are responsible for the expression of certain plant characteristics are restricted to certain sections of a chromosome. The genome is a group of these genes contained in a single gamete (King and Stansfield, 1990). Marker-based DNA fingerprinting has become an important tool for discriminating closely related cultivars (Elmeer et al., 2017). Furthermore, phylogenetic analyses are useful for comparing plant genomes (Scoltis and Soltis, 2003). Phylogenetic relationships can be used to assess gene frequencies and respective differences among progeny and parents. A phylogenetic tree produced from ISSR results can illustrate the output of crossing programs. In a selfing scheme, closer genetic relationship of the F2 individuals with the male than with the female parent demonstrated the stronger genetic contribution of the male parent to F2 hybrids than to the F1 hybrid. Phylogenetic results also showed a close relationship between F2 hybrids such as between F2-1 and F2-7, between F2-8 and F2-9, and between F2-2 and F2-3. Muakrong et al. (2014) developed F1 hybrids by crossing *Jatropha curcas* (green flower) with *J. integerrima* (red flower) to produce an F2 generation by self-fertilization. In F1 individuals, white and pink colored flowers occurred, whereas in the F2 generation nine different color variations occurred. This showed that selfing caused variation in genetics and expression of flower color. Our results provide molecular and cytogenetic information to identify genetic variations between hybrids and to assess parental contribution after self-fertilization. In addition, selfing had considerable effect on the genetic structure of the progeny.

5. Conclusions

This study confirmed the impact of selfing in the resulting progeny. Reproduction by self-fertilization cause substantial genetic variation in the F2 progeny. Genetic effects were confirmed by FISH and ISSR analyses. Genetic difference among the F2 hybrid in FISH findings and genetic relationship of F2 progeny with the male parent in ISSR analysis gave a key genetic information for such valuable breeding material and it can be a valuable source for further breeding programs.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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