Original article

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

Fahad Ramzan a, Hyoung Tae Kim a, Adnan Younis b, Yasir Ramzan c, Ki-Byung Lim a,⇑

a Department of Horticulture, Kyungpook National University, Daegu 41566, South Korea
b Institute of Horticultural Sciences, University of Agriculture, Faisalabad 38040, Pakistan
c Wheat Research Institute, AARI, Faisalabad, Pakistan

1. Introduction

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 (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). Hermaphroditic 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).

https://doi.org/10.1016/j.sjbs.2020.12.019

1319-562X/© 2020 The Authors. Published by Elsevier B.V. on behalf of King Saud University.

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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 bulbs, and is comparatively resistant to abiotic stressors such as cold, heat, drought, salinity, nutrient deficiencies, and infections with viruses and Fusarium (Askar, 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 (Steel 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 Nararvena, 2006; Winter and Kahl, 1995). Inter simple sequence repeat (ISSR) 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 (Gilbert 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, cytogenetic 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 digoxigenin-11-DUTP, respectively, by nick translation (Roche, Germany). The hybridization mixture, containing 50% denized 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 digoxigenin and biotin were detected using FITC conjugated anti-digoxigenin 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).
2.4. DNA analyses using ISSR

DNA extraction was performed using a modified hexade-
cyltrimethylammonium 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/L) 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 PCR Master Mix (0.625 U
µL⁻¹ Tag DNA polymerase; 2 mM MgCl₂; 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 denatura-
tion at 94 °C for 5 min, 45 cycles of 94 °C for 30 s, primers anneal-
ing 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 stan-
dard molecular marker of 100 bp + 3 K DNA Ladder (Smobio, Tai-
wan) 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 + | Dark orange | Yellow + | Red+ | Yellow + | Yellow + | Yellow + | White | Yellow + | Yellow + | Yellow + |
| Color type             | Uni  | Double | Uni  | Double | Yes  | No    | Yes  | No    | Yes  | Yes  | Yes  | Yes  |
| Flower shape (Petal recurved) | Yes | No | Yes | No | Yes | No | Yes | No | Yes | Yes | Yes | Yes |
| Flower position        | Down | Up | Down | Up | Up | Side | Side | Side | Up | Up | Up | Up |
| Spot Distribution      | Many | Less | Many | Less | Medium | Medium | Medium | Many | Less | No | Medium | Medium |
| Spot size              | Big | Small | Big | Small | Medium | Medium | Medium | Small | No | Medium | Medium |
| Bulbil production ability | Yes | No | Yes | No | No | No | No | Yes | No | No | Yes | Yes |
and F2-9 progeny showed less spot distribution on petals while F2-4 showed the highest spot distribution among F2 progeny. In addition, spot size was intermediate in F2 progeny than their parents 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 chromosomes 1, 2, 6, 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, F2-4, F2-5, F2-7, and F2-8) contained a novel signal on chromosome 8 which was not found in any parent or F1 hybrid. It has been seen that 45S signals distribution 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 ratios 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 F2-2, F2-3, F2-7 were higher (9.3 ± 1.4, 8.5 ± 1.1, and 8.0 ± 1.1, respectively).

---

**Table 2**

| Plant type | Number of 45S rDNAs | Location of 45S rDNA | Chromosome number containing 45S rDNA | Number of 5S rDNAs | Location of 5S rDNA |
|------------|---------------------|----------------------|---------------------------------------|-------------------|-------------------|
| P1         | 10                  | Short Arm 6 Long arm 4 | Ch#1,2,6,7,11                         | 2 s + 1w          | 0                 |
| P2         | 9                   | Short Arm 6 Long arm 3 | Ch#12.6,7 + 11 sgd                    | 2 s + 1w          | 0                 |
| F1         | 10                  | Short Arm 6 Long arm 4 | Ch#12,6,7,11                          | 2 s                | 0                 |
| F2-1       | 10                  | Short Arm 6 Long arm 4 | Ch#12,6,7,11                          | 2 s                | 0                 |
| F2-2       | 10                  | Short Arm 7 Long arm 3 | Ch#12,7.11 + 6,8 Sg                   | 2 s + 1w          | 0                 |
| F2-3       | 10                  | Short Arm 6 Long arm 4 | Ch#12,6,7,11                          | 2 s + 1w          | 0                 |
| F2-4       | 9                   | Short Arm 6 Long arm 3 | Ch#12,7.11 + 6 Sg                     | 2 s + 1w          | 0                 |
| F2-5       | 10                  | Short Arm 7 Long arm 3 | Ch#12,7.11 + 6,8 Sg                   | 2 s + 1w          | 0                 |
| F2-6       | 11                  | Short Arm 7 Long arm 4 | Ch#12,6,7,11 + 8 Sg                   | 3 s                | 0                 |
| F2-7       | 10                  | Short Arm 7 Long arm 3 | Ch#12,7.11 + 6,8 Sg                   | 1 s + 2w          | 0                 |
| F2-8       | 10                  | Short Arm 7 Long arm 3 | Ch#12,7.11 + 6,8 Sg                   | 1 s + 2w          | 0                 |
| F2-9       | 10                  | Short Arm 6 Long arm 4 | Ch#12,6,7,11                          | 1 s + 2w          | 0                 |

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

---

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.
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 0.6352 and 0.7547 was observed in F2-4 and F2-9, respectively, whereas the minimum value of 0.5299 was observed in F2-4 hybrids. The highest similarity value between F2 hybrids (0.7463) was observed between F2-2, F2-3, F2-4, and F2-8 and F2-9, and the lowest value (0.5000) was found as the similarity of F2-4 and F2-9.

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   | 32.4 ± 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      |
significantly different between F2 individuals. The maximum genetic
distance occurred between F2-4 and F2-5 (0.6783), and the mini-
mum 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 pro-
geny were closer to the male parent than to the female parent.
The F2-4 hybrid was distant from other F2 progeny based on phy-
genetic 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 rela-
tionship 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 num-
ber and location of 45S and 5S signals are useful to find variation
and hybridity status in progeny. Our results indicated genetic vari-
ation between parent and progeny as well as among progeny,
based on 45S and 5S signal distribution. Wang et al. (2017) con-
ducted 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

---

**Table 4**

Characteristics of the selected primers used for generating ISSR amplification and number of bands per primer for ‘L. lancifolium × Asiatic Lilium ‘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 | (TG)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 | Ne | He | I |
|-------------|----|----|----|---|
| 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 | 1.9503 ± 0.21 | 1.6429 ± 0.31 | 0.3665 ± 0.14 | 0.5396 ± 0.17 |

---

**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 |
| F3(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 | 0.3980 | 0.4552 | 0.4205 | 0.3231 |
| F2-9       | 0.5671 | 0.5411 | 0.5284 | 0.3335 | 0.3335 | 0.3980 | 0.6931 | 0.4552 | 0.4205 | 0.3231 | 0.2927 | **** |

---

Fig. 4. Dendrogram of progenies produced from selfing. P1 = L. lancifolium, P2 = Asiatic Lilium hybrid ‘Dreamland’, P3(F1) = L. lancifolium × Asiatic Lilium hybrid ‘Dreamland’, F2-1 to F2-9 = F1-1 (selfing of F1 hybrid).
mechanisms (Schubert and Wobus, 1985). Visualization of active mobility may be due to transposition or unequal recombination. 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 becoming an important tool for discriminating closely related cultivars. Additional morphological characters are also useful for comparing plant genomes (Scoltis and Soltis, 2003). 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. 

Mauro 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.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) and was funded by the Ministry of Education (Project No. NRF-2016R1D1A1B04932913) and the Regional Subgenebank Support Program of Rural Development Administration, Republic of Korea (No.PJ012891).

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

References

Anderson, N.O., Younis, A., Optiz, E., 2009. Development of colored, non-vernalization requiring seed propagated ilies. Acta. Hort. 836, 193–198.

Askar, H.M., 2015. Influences of flower of tiger lily in vivo and buffaloes culture in vitro. Afr. J. Biotechnol. 14, 2616–2621.

Barrett, S.C., 2002. The evolution of plant sexual diversity. Nature Reviews Genetics 3, 274–284.

Barrett, S.C., 2010. Understanding plant reproductive diversity. Philos. Trans. Royal Soc. B. Biol. Sci. 365, 105–109.

Biswas, M.K., Nath, U.K., Howlader, J., Bagchi, M., Natarajan, S., Kayum, M.A., Kim, H., Park, J.L., Kang, J., Nou, I., 2018. Exploration and exploitation of Novel SSR Markers for Candidate Transcription Factor Genes in Lilium Species. Genes 9 (97), 1–14.

Bueren, E., Backer, G., Vriend, H., Osterhard, G., 2010. The role of molecular markers and marker assisted selection in breeding for organic agriculture. Euphytica. 175 (1), 51–64.

Chakravarthi, B.K., Naravane, R., 2006. SSR marker-based DNA fingerprinting and diversity study in rice (Oryza sativa L.) African. J. Biotechn. 5 (9), 684–688.

Charlesworth, D., Wright, S.I., 2001. Breeding systems and genome evolution.Curr. Opin. Genet. Develop. 11, 163–168.

Cole, C.T., 2003. Genetic variation in rare and common plants. Annu. Rev. Ecol. Evol. S. 34, 213–237.

Darwin, C., 1876. Effects of cross and self-fertilization in the vegetable kingdom. John Murray, London.

Dwivedi, S.L., Crouch, J.H., Mackill, D.J., Xu, Y., Blair, M.W., Ragot, M., Upadhyaya, H. D., Ortiz, R., 2007. The molecularization of public sector crop breeding: progress, problems and prospects. Adv. Agron. 95, 163–318.

Eckert, C.G., Samis, K.E., Dart, S., 2006. Reproductive assurance and the evolution of uniparental reproduction in flowering plants. In: Harder, L.D., Barrett, S.C.H. (Eds.), The Ecology and Evolution of Flowers. Oxford University Press, Oxford, pp. 183–203.

Elmeer, K., Alghanem, M., Al-Latif, L., Alhembary, H., 2017. Efficiency of RAPD and ISSR markers for the detection of polymorphisms and genetic relationships in date palm. Biotechnol. 16, 19–26.

Fisher, R.A., 1941. Average excess and average effect of a gene substitution. Ann. Eugenics 11, 51–63.

Glimm, S., Galtier, N., 2012. Genome evolution in outcrossing versus selfing versus asexual species. Springer, Evolutionery Genomics, pp. 311–335.

Gilbert, J.E., Lewis, R.V., Wilkinson, M.J., Galagari, P.D.S., 1999. Developing an appropriate strategy to assess genetic variability in plant germplasm collections. Theo. Appl. Genet. 98, 1125–1131.

Goodwillie, C., Kalisz, S., Eckert, C., 2005. The evolutionary enigma of mixed mating systems in plants: Occurrence, theoretical explanations, and empirical evidence. Annu. Rev. Ecol. Evol. Syst. 36, 47–79.

Guilien, A.K., Hirai, Y., Tanoue, T., Hirai, H., 2004. Transcriptional repression mechanisms of nuclear organizer regions (NORs) in humans and Drosophila melanogaster. Chromosoma 122, 237–267.

Hamrick, J.L., Godt, M.J.W., 1996. Effects of life history traits on genetic diversity in plant species. Philos. T. Roy. Soc. B. 351, 1291–1298.

Hamrick, J.L., Godt, M.J.W. 1990. Allzyme diversity in plant species. In: Brown, A.H. D., Clegg, M.T., Kahler, A.L., Weir, B.S. (Eds.), Plant Pop. Genet. Breed. Genet. Reso. Sunderland: Sinauer Associates, pp. 43–63.

Hernandez-Verdun, D., 1986. Structural organization of the nucleus in mammalian cells. Methods Achiev. Exp. Pathol. 12, 26–62.

Howell, W.M., 1977. Visualization of ribosomal gene activity: silver stains proteins associated with rRNA transcribed from oocyte chromosomes. Chromosa 62, 361–367.

Huang, J., Ma, L., Yang, F., Fei, S.Z., Li, L., 2008. 45S rDNA regions are chromosome fragile sites expressed as gaps in vitro on metaphase chromosomes of root-tip meristem cells in Lolium spp. PLoS ONE 3 (5), 1–7.

Hwang, Y.J., Kim, H.H., Kim, J.B., Lim, K.B., 2011. Karyotype analysis of Lilium tigrinum by FISH. Hortic. Environ. Biotechnol. 52, 292–297.

Ingvason, P.K., 2002. A metapopulation perspective on genetic diversity and differentiation in partially self-fertilizing plants. Evolution. 56, 2368–2373.

Jain, S.K., 1976. The evolution of inbreeding in plants. Ann. Rev. Ecol., Evol., Systemat. 7, 469–495.

Jarne, P., Auld, J.R., 2006. Animals mix it up too: the distribution of self-fertilization among hermaphroditic animals. Evolution 60, 1816–1824.

Jarne, P., Charlesworth, D., 1993. The evolution of the selling rate in functionally hermaphroditic plants. Annu. Rev. Ecol. Evol. Syst. 24, 441–469.

Jiang, J., Gill, B.S., 2006. Current status and the future of fluorescence in situ hybridization (FISH) in plant genome research. Genome. 49, 1057–1068.

Joshi, S.P., Prabhakar, K., Ranjekar, P.K., Gupta, V.S., 2011. Molecular markers in plant genome analysis. Curr. Sci. 77 (2), 230–240.

Khajudparn, P., Prajongjai, T., Poolsawat, O., Tantasawat, P.A., 2012. Application of AFLP analysis of Lilium longiflorum and Lilium rubellum by chromosome banding and fluorescence in situ hybridization. PLoS ONE. 3 (5), 1–7.

Lim, K.B., Wennekes, J., De Jong, J.H., Jacobsen, E., Van Tuyl, J.M., 2001b. Karyotype characterization of the centromere and peri-centromere retro transposons in Lilium longiflorum and Lilium rubellum. Chromosoma 110, 323–334.

Lim, M.H., Jin, M., Kim, H.I., De Jong, J.H., Bancroft, I., Lim, Y.P., Park, B.S., 2007. Characterization of the centromere and peri-centromere retro transposons in Brassica campestris and their distribution in related Brassica species. Plant J. 49, 173–183.
Makinen, A., Zijlstra, C., de Haan, N.A., Mellink, C.H., Bosma, A.A., 1997. Localization of 18S, 28S and 5S ribosomal RNA genes in the dog by fluorescence in situ hybridization. Cytogenet. Cell Genet. 78, 231–235.

Marasek, A., Hasterok, R., Wiejacha, K., Orlikowska, T., 2004. Determination by GISH and FISH of hybrid status in Lilium. Hereditas 140, 1–7.

Mckae, E.A., 1998. Lilies: A Guide for Growers and Collectors. Timber Press, Portland, Oregon, pp. 211–238.

Muakrong, N., Phetcharat, C., Tanya, P., Srinives, P., 2014. Breeding field crops for ornamental purpose: a case in Jatropha spp. Agrivita. 36 (3), 229–234.

Nei, M., Li, W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76, 5269–5273.

Nei, M., 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. USA 70, 3321–3323.

Noda, S., 1991. Chromosomal variation and evolution in the genus Lilium. In: Tsuchiya, T., Gupta, P.K. (Eds.), Chromosome engineering in plants: genetics, breeding, evolution, part B. Elsevier, pp. 507–524.

Nybbom, H., 2004. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Mol. Ecol. 13, 1143–1155.

Omondi, E.O., Debener, T., Linde, M., Abukutsa-Onyango, M., Dinssa, F.F., Winkelmann, T., 2016. Molecular markers for genetic diversity studies in African leafy vegetables. Adv. Biosci. Biotechnol. 7, 188–197.

Ornduff, R., 1969. Reproductive biology in relation to systematics. Taxon 18, 121–133.

Ramzan, F., Younis, A and Lim, k.b. 2017. Application of Genomic In Situ Hybridization in Horticultural Science. International Journal of Genomics. 2017, 1-12.

Richards, A.J., 1986. Plant breeding systems. George Allen & Unwin. United Kingdom, London.

Salmi, M., Rahimnalek, M., Eltemam, M.H., 2017. Genetic Variability of outcross and selfed fennel based on morphological and ISSR markers. J. Agr. Sci. Tech. 19, 157–172.

Schubert, I., 2007. Chromosome evolution.Curr. Opin. Plant Biol. 10, 109–115.

Schubert, I., 2007. Chromosome painting in plants. In: Sharma, A.K., Sharma, A. Methods in Cell Sci., 23, 57–69.

Scoltis, D.E., Solits, P., 2003. The role of phylogenetic in comparative genetics. Plant Physiol. 132, 1790–1800.