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Occurrence of Spontaneous Polyembryony in *Lilium lancifolium* Thunb.

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We examined spontaneous polyembryony in *Lilium lancifolium* Thunb. by determining the occurrence of polyembryonic seeds in intraspecific crosses and open pollination, and by analyzing the genetic origins of polyembryos with simple–sequence repeat (SSR) markers. Forty–one polyembryonic seeds (0.2% of total) were detected from 17,583 seeds. Of the polyembryonic seeds, 36 were duplets and five were triplets. Eighteen seedling sets (three triplets and 15 duplets) were cultured and grown to maturity. Flow cytometry revealed that all were diploids, and results of analyses with SSR markers indicated that all the multiple embryos, except for two triplet seeds, originated from a zygotic embryo through cleavage embryony. The SSR profiles confirmed that the seedlings from polyembryos resulted from cross–fertilizations and did not from unfertilized somatic embryos. Six seedlings rescued from the two triplets occurring in 2x × 3x combinations were heterozygous with respect to SSR, and thus they may have their origin in multiple embryo sacs and/or polyspermy. The occurrence of polyembryonic seeds was remarkably greater in one accession collected from Wan–do (Wan island) than the others. This suggested that the frequency of polyembryony depends on the genotypes of *L. lancifolium*.

Key words: polyspermy, SSR marker, Tiger lily, triplet embryos, twin embryos

INTRODUCTION

Polyembryony results when two or more embryos develop from a single fertilized egg and/or somatic cells within a single seed. The phenomenon occurs regularly in many plants and animals. The goal of this work was to describe and characterize polyembryony in *Lilium lancifolium* Thunb., and to understand its variability among the natural accessions.

Species of *Lilium* have been used as experimental model plants for more than a century. Indeed, double fertilization was first discovered in this genus (Navashin, 1898). With regard to polyembryony in lily, Overton (1891) described the development of a synergidous embryo in *L. martagon*, and considered to be the result of fertilization of one of the synergids (two cells of the embryo sac that flank the egg) by a sperm cell delivered by a second pollen tube. Seeds with two embryos, one haploid and one diploid, were observed in several species of lily by Cooper (1943), presumably due to development of one of the haploid embryo sac cells into an embryo, without fertilization. Also, X–ray–irradiated lily pollen resulted in increase of the frequency of seeds with twin embryos; i.e., the frequency increased in plants pollinated with this pollen than those pollinated with untreated pollen (Morgan and Rappleye 1951).

Khvedynich and Kravets (1991) proposed fertilization of synergids in interspecific crosses. Kravets and Khvedynich (2008) mentioned the possibility of antipodal polyspermy in *L. regale* and *L. henryi* and suggested that several pollen tubes may enter the embryo sac from the antipodal end and fertilize one or more antipodal cells. Recently, authors found that polyembryonic seeds occurred spontaneously in *L. lancifolium* in the case of crossing between the strains.

Based on this background, the occurrence of polyembryony and polyspermy may be common in some *Lilium* species. The observations described above were made during the early pro–embryonic stages and lacked following genetic studies. Confirmation of the genetic origin of polyembryos usually relied on isozyme analysis (Iglesias *et al*., 1974; Torres *et al*., 1978; Ashari *et al*., 1988; Mestre *et al*., 1997); however, the analysis is limited because of the lack of detectable isozyme variation and the available isozyme loci.

Simple–sequence repeats (SSR) gains credibility as tools for the determination of the origin of plant embryos (Tautz, 1989; Morgante & Olivieri, 1993; Russell *et al*., 1997; Ruiz *et al*., 2000; Horning *et al*., 2003; Yildiz *et al*., 2013; Trapero *et al*., 2014). This method relies on the existence of repeated regions in DNA that are susceptible to mutation, but are flanked by conserved regions, making them useful as markers. We have employed SSR markers in this study of polyembryony in *L. lancifolium* and its variability.

MATERIALS AND METHODS

Plant materials and hybridization

Diploid *L. lancifolium* plants are narrowly distributed in west and south coastal regions of Korea (Kim *et al*., 2006). We selected six diploid accessions from islands of the West Sea area and six from islands of the South Sea area of the Korean peninsula (Table 1). Their accession numbers were registered in the Agricultural Gene Bank of Rural Development and Administration,

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Table 1. *Lilium lancifolium* accessions used in this experiment

Accession No.* (Abbreviation of island where the plant was collected)	Location of island where the plant was collected	Ploidy
282192 (AC)	Acha–do, Seodomyeon, Incheon city	2x
282465 (BO)	Boleumdo, Seodomyeon, Incheon city	2x
282968 (JU)	Jumoon–do, Seodomyeon, Incheon city	2x
282266 (DU)	Duckjeokdo, Duckjeok–myeon, Incheon city	2x
282443 (MO)	Mo–do, Duckjeok–myeon, Incheon city	2x
282346 (AH)	Ahnmyeon–do, Ahnmyeongun, Chungcheongnamdo	2x
282378 (JI)	Jin–do, Jindogun, Jeollanamdo	2x
282907 (WA)	Wan–do, Wando–up, Jeollanamdo	2x
GWL1374 (OE)	Oenaro–do, Wando–up, Jeollanamdo	2x
282383 (GO)	Goheung, Goheunggun, Jeollanamdo	2x
282756 (KE)	Keum–O–do, Nammyeon, Jeollanamdo	2x
GWL1127 (YO)	Yokji–do, Yokjimyeon, Jeollanamdo	2x
Total	12 populations	

*Accession numbers are registered numbers of the Gene Bank of Rural Development and Administration, Korea.

Korea. The region and accessions were Acha–do (282192), Boleum–do (282465), Jumoon–do (282968), Duckjeok–do (282266), Mo–do (282443), Ahnmyeon–do (282346), Jin–do (282378), Wan–do (282907), Oenaro–do (GWL1374), Goheung (282383), Keum–O–do (282756), and Yokji–do (GWL1127).

Crosses were conducted using standard procedures. Anthers were collected from selected plants, stigmas were capped with aluminum foil to prevent unwanted pollination, selected pollinations were performed, and then pollinated stigmas were capped. Crosses were performed mainly between populations from either the West or South Sea areas. Non–crossed flowers were open pollinated in the Gene–Bank field of Kangwon National University, Chuncheon, Korea. This field includes both diploid and triploid groups of *L. lancifolium*. Cross– and open–pollinated fruits were collected in each combination before the capsules were open (Table 2).

Morphological observations

Morphological observations of mature seeds were conducted for three years, from 2013 to 2016, after collecting the capsules at the end of September in the open field. Dried papery seeds were observed to put them on a light box, which allows us to determine if the thin and semi–transparent *Lilium* seeds are mono– or polyembryonic, without dissection (Morgan and Rappleye, 1951; Nguyen *et al*., 2015). Occasionally when it was ambiguous whether a seed was polyembryonic due to a small additional embryo, it was necessary to confirm their status under a dissecting microscope.

Polyembryonic seed culture

In previous breeding experiments (Nguyen *et al*., 2015), we discovered that underdeveloped embryos of *L. lancifolium* were difficult to germinate in soil. Consequently, all polyembryonic seeds that had small or underdeveloped embryos were cultured on the modified Murashige–Skoog (MS) charcoal medium (3% sucrose, 3 g charcoal L^{-1}, pH 6.8). The seeds were surface sterilized in 70% ethanol and subsequently sterilized in 0.5% sodium hypochlorite solution for 15 minutes, rinsed three times in distilled water, placed on MS complete medium, and germinated under 12 h light followed by 12 h dark at 23°C.

Ploidy analysis

The ploidy levels of the parent and hybrid plants were analyzed with a flow cytometer (Partec PA–1, Gorlits, Germany) according to the previously published method of Kim *et al*. (2016). DNA content was determined as C–values using standard *Allium cepa* cells (2C, approximately 33.5 pg; Lim *et al*., 2001).

DNA extraction

Genomic DNA was isolated from young leaves using the DNeasy Plant Maxi kit (Qiagen, USA) according to the manufacturer's instructions. DNA quantity was adjusted to 50ng/μl and then subjected to 0.8% agarose gel electrophoresis. We used 35 expressed sequence tag–simple sequence repeat (EST–SSR) markers for analysis. These 35 EST–SSRs were selected from 76 EST–SSRs that were developed previously in Lilium (Lee *et al*., 2011; Song *et al*., 2012).

PCR and electrophoresis

PCR analyses were conducted in a 25–μL reaction mix containing 20 ng of template DNA, 2.5 µL of 10 × reaction buffer (50 mM KCl, 20 mM Tris–HCl, pH 8.0 and 2.0 mM MgCl2), 2.5 mM of each deoxyribonucleotide triphosphate, 0.1 μM primers, 20 ng template DNA, and 0.5 U Taq DNA polymerase (Intron Bio, Korea). The

Table 2. Crosses between accessions of each *L. lancifoliu*m population and the number of fruits harvested

Cross combination	No. of fruits harvested
AC × WA	5
AC × OE	7
AC × open pollination	8
BO × WA	5
BO × MO	6
BO × open pollination	7
JU × WA	5
JU × OE	9
JU × open pollination	7
DU × WA	5
DU × OE	7
DU × open pollination	6
MO × WA	11
MO × OE	5
MO × open pollination	12
AH × WA	4
AH × AC	3
AH × open pollination	5
JI × AC	7
JI × WA	9
JI × open pollination	12
WA × AC	12
WA × MO	9
WA × open pollination	9
OE × AC	9
OE × MO	12
OE × open pollination	7
GO × AC	5
GO × MO	4
GO × open pollination	5
KE × AC	7
KE × MO	6
KE × open pollination	5
YO × AC	5
YO × MO	4
YO × open pollination	5
Total of cross–pollinated fruits	161
Total of open–pollinated fruits	88

reaction conditions were as follows: 94°C for 2 minutes; and then 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute; with a final extension at 72°C for 5 minutes. The amplified products were analyzed by electrophoresis using a 6% denaturing polyacrylamide gel and a conventional PAGE apparatus for 2 hours at 1,800 V, or a LiCor 4300 automatic electrophoresis system for 4 hours. The DNA fragments separated by conventional PAGE were visualized by silver staining (Promega, USA).

Difference in the frequency of polyembryony

The differences in the frequency of polyembryony events between the 12 evaluated accessions were analyzed by Pearson's Chis–squared nonparametric test at $p = 0.05$, reflecting the observed and expected frequencies of polyembryonic seeds in each accession. Statistical analysis of the data was carried out using the Excel.

RESULTS

Occurrence of polyembryonic seeds

Polyembryony has been observed in the germination of large numbers of hybrid progenies in an edible lily breeding program in *L. lancifolium* (Nguyen *et al*., 2015). We detected polyembryonic seeds in seven of the 12 accessions screened. The rate of polyembryonic seeds ranged from 0% in most accessions to 1.2% in the accession 282907(WA) (Table 3). Accession 282907(WA) produced significantly more polyembryonic seeds than the other accessions, according to the Chi–square test at $p = 0.05$ (Table 3).

Morphology of polyembryonic seeds

Various types of duplet embryos were observed among the polyembryonic seeds. They could be grouped into four types based on the morphology of the embryos. These groups were as follows. Group I: embryos were unattached and approximately equal in size (Fig. 1a), Group Ⅱ: embryos were fused or conjoined and one of the members was much smaller than the others (Fig. 1b), Group Ⅲ: embryos were conjoined and abnormally curved (Fig. 1c), and Group IV: embryos were attached and underdeveloped (Fig. 1d). The most frequent embryo type belonged to Group Ⅱ. Seeds with duplet embryos of equal size (Group I) germinated normally in soil (Fig. 1f). Seeds belonging to Group Ⅱ and containing a small embryo attached to the original zygotic embryo did not germinate easily in soil (Fig. 1g). Four duplet seeds were separated into three embryos (Fig. 1h) at an early germination stage. These seeds were referred to as "triplets" (Table 2). Many underdeveloped embryos of duplets (Fig. 1g) and triplets (Fig. 1h) died early in germination, leading us to "rescue" 15 duplet and three triplet seeds (Fig. 1i) from 41 polyembryonic seeds through *in vitro* culture (Table 4).

Ploidy and confirmation of polyembryony

Eighteen seedling sets (15 duplets, three triplets) rescued were analyzed by flow cytometry to determine their ploidy. All of these seedlings of duplets and triplets were diploids (Table 4). The survived 39 seedlings from these embryos and their parents were analyzed by expressed–sequence tags (EST)–SSR to trace their genetic origin. Of 35 SSR primers (Lee *et al*., 2011; Song *et al*., 2012), 22 produced maternal–parent–specific amplicons and 25 produced paternal–parent–specific amplicons. Of the 25 paternal–specific primer sets, we selected 14 (L01, L04, L05, L09, L50, L59, L60, L61,

Table 3. Maternal effect on the frequency of polyembryony in natural accessions

Accession	No. of seeds tested	No. of polyembryonic seeds	Type of polyembryony	Rate of polyembryonic seeds (% of total)	Chi square value
282192 (AC)	3,834	5	duplet	0.1	1.6
282465 (BO)	1,479	0	–	0	3.4
282968 (JU)	567	0	–	0	1.3
282266 (DU)	756	0	–	0	1.7
282443 (MO)	1,371	1	duplet	0.07	1.5
282346 (AH)	456	3	duplet	0.07	3.7
282378 (JI)	2,733	3	duplet	0.1	1.7
282907 (WA)	1,981	23	duplet + 4 triplets	1.2	75.5**
GWL1374 (OE)	2,240	5	duplet	0.2	0.003
282383 (GO)	574	1	triplet	0.2	0.074
282756 (KE)	945	0	–	0	2.2
GWL1127 (YO)	1,019	0	–	0	2.3
Total of 12 accessions	17,955	41			

Overall Chi square value = 94.9 (p = 0.05); **, Chi square$_{11}$ = 19.68 (p = 0.05)

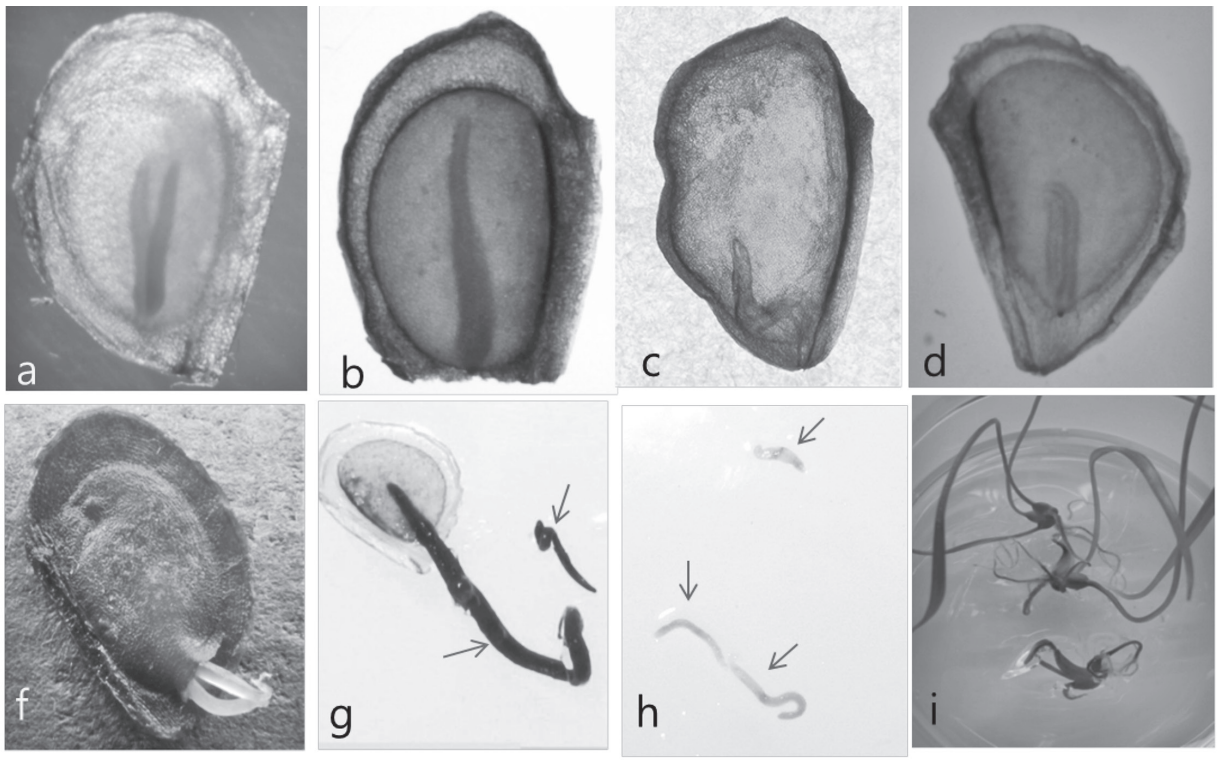

Fig. 1. Photographs of polyembryonic seeds and seedlings. **a**: equal–sized duplets; **b**: unequal duplets; **c**: abnormal duplets; **d**: underdeveloped duplets; **f**: germination of duplet embryos of equal size in soil; **g**: germination of unequal duplets indicated by arrows; **h**: germination of triplets indicated by arrows; **i**: seedlings of triplets.

eL16, eL34, eL61, eL65, eL75, eL81) that produced reproducible allelic bands in polyembryonic seedlings. Consistent results were obtained from genotyping the 39 polyembryonic seedlings using the maternal–specific 14 SSR markers. The SSR profiles permit us to conclude that both duplet embryos developed from zygotic embryos, based on the presence of both maternal and paternal bands, and the SSR profiles were identical among them (Fig. 2. L05, eL65). We concluded that these duplets are equivalent to the so–called 'zygotic polyembryony' found in other species (Tisserat *et al.*, 1979; Schnell and Knight 1992; Martínez–Gómez and Gradziel

Table 4. Confirmation of ploidy and genetic nature of surviving seedling sets

Accessions	No. of polyembryonic seeds in indicated type (Given seed number)		Rescued seed in vitro in indicated type		Ploidy[z] and SSR confirmation of seedling sets	
	Duplet	Triplet	Duplet	Triplet	Duplet	Triplet
282192 (AC)	5 (1, 2, 3, 4, 5)	0	1, 3	–	2x, zygotic twin	–
282443 (MO)	1 (6)	0	6	–	2x, zygotic twin	–
282346 (AH)	3 (7, 8, 9,)	0	9	–	2x, zygotic twin	–
282378 (JI)	3 (10, 11, 12)	0	11	–	2x, zygotic twin	–
282907 (WA)	19 (13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31)	4 (32, 33, 34, 35)	13, 14, 16, 19, 22, 25, 29, 31	33 [y], 34 [x]	2x, zygotic twin	33: 2x, all identical 34: 2x, all different
GWL1374 (OE)	5 (36,37, 38, 39, 40, 41)	0	37, 40	–	2x, zygotic twin	–
282383 (GO)	0	1(43)	–	43 [w]	–	2x, all different
Total	38 seeds	5 seeds	15 seedss	3seeds	all 2x	all 2x

[z]Confirmed by flow cytometry.

[y]Obtained from 2x × 2x cross between 282907 and 282192 accessions.

[x]Obtained from open pollination of 282907(2x) × 282360(3x) combination.

[w]Obtained from open pollination of 282382(2x) × 282396(3x) combination.

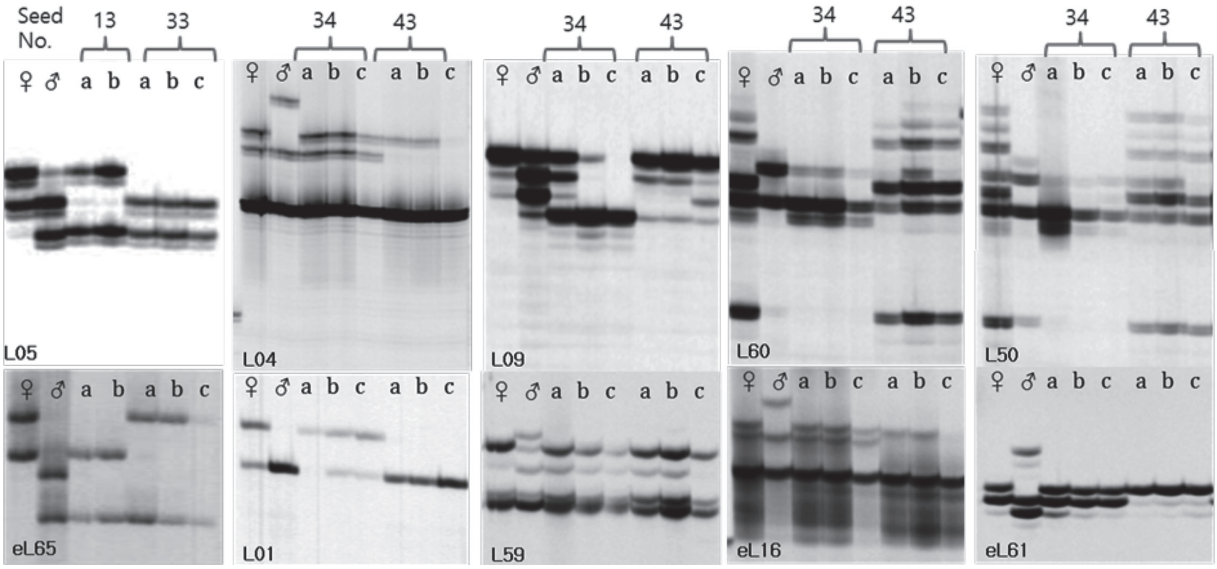

Fig. 2. Expressed–sequence tag simple–sequence repeat (EST–SSR) profiles in polyembryonic seedlings of duplet (No. 13) and triplets (No. 33, 34, 43), and their parents. Seedlings of seed No. 13 (duplet) and 33 (triplet) appeared to be genetically identical in SSR markers L05 and eL65. Seedlings of seed No. 34 and 43 appear to be heterozygous in eight SSR markers (L01, L04, L09, L59, L60, L50, eL16, and eL61), indicating three independent fertilizations.

2003; Aleza *et al*., 2010; Zenkteler *et al*., 2012; Trapero *et al*., 2014), and result from monozygotic cleavage and/or suspensor embryony after normal fertilization. Genetically identical triplet seedlings were also found in No. 33 triplet seed (Table 4, Fig. 2, L05, eL65). The seedlings from the other two triplet seeds derived from open pollinations were genetically different, with paternal SSR bands. Figure 2 shows the SSR profiles of L01, L04, L09, L50, L59, L60, eL16, and eL61, with bands that reveal heterozygosity between the parents in seeds No. 34 and 43. The SSR profiles of these seeds indicate that they were derived from independent fertilization: one plant may have resulted from fertilization of the egg, while the other plants may have arisen from fertilization of synergids. Interestingly, these two polyspermic seeds were obtained from open pollination of 2x × 3x; their accessions of paternal parents were confirmed by SSR profiles (Fig. 2) to 28290729(2x) × 282360(3x) and 282383(2x) × 282396(3x) combinations (Table 3).

DISCUSSION

In this study, we analyzed accessions of *L. lancifolium* for polyembryony. Its occurrence was invariably

low in this study, with a mean of 0.2% of the seeds that produced polyembryos. There was one notable exception: accession 282907(WA), collected from a remote coastal area locating at south of the Korean peninsula, showed remarkably more frequent (1.2%) polyembryony than the other accessions. Polyembryony has also been noted to be genetically determined in other horticultural species (Aron *et al*., 1988; Batygina and Vinogradova, 2007; Kishore *et al*., 2012; Trapero *et al*., 2014). Another peculiarity of this accession has been noted: it produced 2n gametes in intraspecific crosses (Nguyen *et al*., 2015).

Although we confirmed that all polyembryonic seedlings were diploid, we cannot rule out the possibility of haploid embryos, as they have been shown to be present at early proembryo stages in other *Lilium* species (Cooper, 1943). If it was, we failed to rescue small embryos in 21 dried duplet seeds that might contain haploids (Table 4). Additionally, small seedlings (Fig. 1g) of duplets did not survive in spite of early germination, suggesting the possibility that these were also haploid. These postulations remain unanswered as further studies.

In this study, the parent gametophyte mating combinations and the genotypes of polyembryonic plants of each cross combination were validated through SSR analysis (Fig. 2 and Table 4). Seedlings of the two triplet seeds (no. 34, 43) were shown to be heterozygous in various EST–SSR profiles (Fig. 2). These polyspermic seedlings were obtained from open pollination of 2x × 3x combinations. We tried many times to produce polyspermic seeds using 282907(2x) × 282360(3x) and 282382(2x) × 282396(3x) combinations (see Table 4), but failed to generate them again. It is difficult at the moment to hypothesize the exact mechanisms of polyspermy in *Lilium* species. *Lilium* species have embryo sacs of the Fritilaria type, which is composed of a haploid egg apparatus, tetraploid secondary polar nuclei, and triploid antipodals (Cooper, 1934, 1935; Maheshwari, 1948; Zhou *et al*., 2011). Possible synergidous polyspermic events have been studied and described in *L. martagon* (Overton, 1891) and in *L. henryi* (Khvedynich and Kravets, 1991), and possible antipodal polyspermic events in *L. henryi* (Kravets and Khvedynich, 2008). Genetic confirmation by SSR profiles of triplets in this study is the first that shows a polyspermic origin of polyembryonic seedlings in *Lilium*. More detailed studies, with adequate reproducibility, are necessary to generalize about of polyspermic events in *L. lancifolium*. As cited earlier, polyspermy development in *L. martagon* from fertilization of a synergid by the sperm of an additional pollen tube, and antipodal polyspermy in *L. regale* and *L. henryi* have been noted (Overton 1891; Kravets and Khvedynich 2008). Thus, polyspermic origin of polyembryony in *L. lancifolium* remains a possibility.

AUTHOR CONTRIBUTIONS

Conceptualization: YSS JHK. Data curation: YSS. Funding acquisition: JHK. Investigation: YSS JHK. Methodology: JHK. Resources: JHK. Writing – original draft: JHK. Writing – review & editing: AW.

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