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Occurrence and Survival of Autotriploids in Natural Diploid Populations of *Lilium lancifolium* Thunb.

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To determine the occurrence and survival of triploid plants in natural diploid populations of tiger lily (*Lilium lancifolium*), ploidy analysis was conducted on natural open–pollinated seeds produced from plants grown on isolated islands, and on hybrid seeds produced by artificial crossing between plant populations originating on different Korean islands. Normal seeds were classified into five grades depending on the ratio of embryo/endosperm lengths, including 5/5, 4/5, 3/5, 2/5, and 1/5. Triploids were not observed among seedlings produced from natural open pollinations on isolated islands. Triploids were detected only in seedlings of underdeveloped seed grades (3/5 and 2/5) from artificial crosses between populations from different isolated islands. The triploid occurrence frequency was calculated as 0% for natural open–pollinated seedlings and 0.058% for artificial crosses (six triploids from 10,303 seedlings). Triploids were produced from crosses between isolated populations located at least 70 km apart; no triploids were detected in inter–population crosses of plants originating on the same islands. Triploid seedlings had very low viability in soil. We analyzed factors affecting triploid occurrence and survival in natural diploid populations of *L. lancifolium*. The results suggest that triploids originate from fertilization between plants that are genetically isolated due to geographical isolation and/or genotypic differences.

Key words: genetic distance, genetic isolation, inter–population cross, *Lilium lancifolium*, triploid

INTRODUCTION

The genus *Lilium* contains many horticulturally important species. Although the native *Lilium* species of Orientals, Asiatics, Trumpets, and Longiflorum are distributed in narrow regions in northeast Asia including the Korean Peninsula (Asano, 1986, 1989; Lightly, 1968; Rong *et al*., 2011; Synge, 1980; Willson, 1925), their distribution is not fully characterized. Many field expeditions confirmed their narrow distribution in Korea and Japan; these observations were consistent with reported geographical collection sites for GenBank accessions (Asano, 1986; Hiramatsu *et al*., 2011; Kim *et al*., 2006b). Tiger lily (*L. lancifolium*) is the most broadly distributed species in the genus, although its distribution is relatively limited. *L. lancifolium* is the only species that forms polyploid complexes of diploids and triploids in the genus *Lilium* (Noda, 1986, Kim *et al*., 2006b). *L. lancifolium* triploids are distributed only in inland areas of Korea and northeast China; however, they are widely distributed in coastal regions, oceanic islands, and inland areas of Japan (Noda, 1986, Kim *et al*., 2005, 2006b). Triploids undergo vigorous asexual propagation via bulbils, and the level of genetic variation is as high as that of sexual reproduction (Kim *et al*., 2006a; Shim *et al*., 2007). This suggests that *L. lancifolium* may propagate sexually in nature (Ellstrand and Roose, 1987; Noda *et al*., 1978, 1986; Shim *et al*, 2007). Noda (1986) proposed that *L. lancifolium* triploids originate in two ways: (1) autotriploids are generated from crosses between diploid plants with an unreduced gamete (2n) and a normal reduced gamete (n); (2) allotriploids are generated from natural interspecific hybridization between diploid *L. lancifolium* and closely related diploid species such as *L. leichtlinii* var. *maximowiczii*. Neither of these hypotheses has been experimentally verified.

Triploid *L. lancifolium* have a wide geographic distribution. By contrast, diploid *L. lancifolium* have limited distributions in the islands and coastal regions of the South and West Korean Peninsula and northern Tsushima Island, Japan (Fig. 1). The islands surrounding the Korean Peninsula were formed 12,500 to 7,000 years ago when the last quaternary glacial period ended (Kim *et al*., 1999). Thus, *L. lancifolium* diploids on Korean islands must have been reproductively isolated from each other for at least 7,000 years. The majority of *L. lancifolium* diploid populations (94.6%) are observed in narrow zones of coastal cliffs, approximately 2–3 meters above sea level, and in marginal forest lands, but they are rarely found in the forest (Kim *et al*., 2004). These observations indicate that they are well maintained in natural environments without human interference.

L. lancifolium diploids and triploids are not sympatrically distributed with other lily species or ploidy lines in West Sea and South Sea Islands of the Korean Peninsula. This observation raises the following questions. Why have autotriploid *L. lancifolium* never been

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observed in those isolated islands? What mechanism excludes the occurrence of autotriploids, if they arise? What are the genetic differences among *L. lancifolium* populations in these isolated islands?

Variations in the functional development of unreduced gametes are observed in non–hybrid crop species (Bretagnolle and Thompson, 1995) and in interspecific *Lilium* hybrids (Lim *et al*., 2001). The frequency of autotriploid occurrence may be different among different diploid accessions of *L. lancifolium*. Further systematic investigations are required to understand the emergence of natural autotriploids, to determine if autotriploids and diploids can grow under sympatric conditions, to identify the genetic relationships between allopatric and sympatric populations, and to determine if the frequency of functional unreduced gametes is genotype–dependent.

As a first step to understand the occurrence of *L. lancifolium* autotriploids, this study investigated autotriploid genesis from natural populations and from artificial crosses between populations, estimated the frequency of unreduced gametes, and monitored the survival of autotriploids under field conditions.

MATERIALS AND METHODS

Plant materials

Diploid *L. lancifolium* bulbils were collected from 31 populations on six islands in the West Sea and 30 populations on seven islands in the South Sea of the Korean Peninsula (Table 1, Fig. 1). The bulbils were germinated in 3–inch pots (diameter) containing a soil mix of peat : peat : pearlite : sand (4:1:1). The young seedlings were transferred to the field at Kangwon National University Farm at Chuncheon, Korea, in April, and grown until maturity and flowering.

Hybridization

Crosses were conducted using standard procedures. Anthers were removed from selected plants, stigmas were capped using aluminum foil, selected pollinations were performed, and pollinated stigmas were re–capped. Crosses were performed between populations from the same islands, between populations from different islands of either the West or South Sea, and between populations from the West Sea Islands and populations from the South Sea Islands.

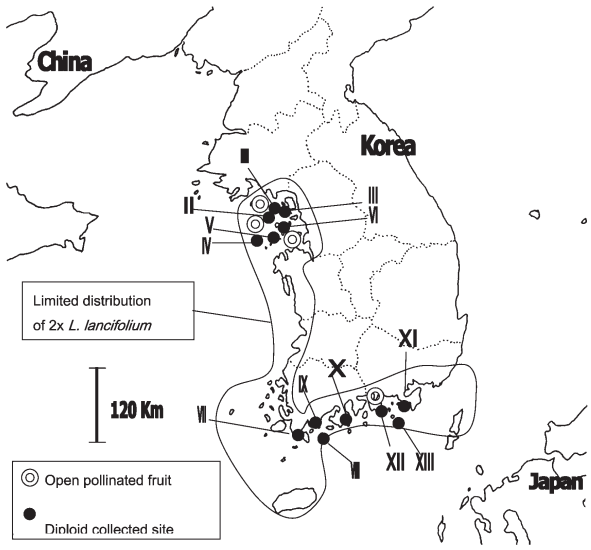


Fig. 1. Geographic distribution of diploid populations and sites of fruit collection from native populations.

Table 1. *L. lancifolium* populations used for the present experiments and genetic crosses

Island	Location	Ploidy	No. of Plants collected	No. of fruits from nature	Cross combinations (Numbers in parentheses are the numbers of fruits harvested)
Acha–do (I)	Seodo–myeon, IC	2x	5	20	I × I (12), I × IV (5), I × VII (5)
Boleum–do (II)	Seodo–myeon, IC	2x	6		II × I (2), II × III (3), II × VIII (5), II × XI (1), II × XII (5), III × XI (3)
Joomoon–do (III)	Seodo–myeon, IC	2x	5		III × I (2), III × XI (3), III × XIII (4)
Baika–do (IV)	Deokjeok–myeon, IC	2x	3		IV × VII (2), IV × IX (2)
Duckjuck–do (V)	Deokjeok–myeon, IC	2x	8	13	V × I (6), V × II (3), V × VIII (3), V × X (3), V × VII (6), V × XIII (18)
Mo–do (VI)	Bukdo–myeon, IC	2x	4	5	VI × VI (2), VI × VIII (1)
Geumgapri (VII)	Uisinmyeon, JN	2x	3		VII × VII (1), VII × I (15), VII × IV (4), VII × X (3)
Sinji–do (VIII)	Sinjimyeon, JN	2x	4		VIII × VIII (5)
Jungdori (IX)	Wandoeup, JN	2x	3		IX × I (10), IX × IV (4), IX × VII (4), IX × VIII (1), IV × IV (4), IX × X (4)
Deokheungri (X)	Dongilmyeon, JN	2x	2		X × I (1), X × X (1)
Mijori (XI)	Mijomyeon, JN	2x	2	16	
KeumO–do (XII)	Nammyeon, JN	2x	6	14	XII × I (5), XII × II (2), XII × VIII (1)
Yokji–do (XIII)	Yokjimyeon, KN	2x	6		XIII × I (3), XIII × II (1), VIII × III (2), XIII × VIII (4), XIII × XII (4)

Note: Islands I–VI are in the West Sea, and islands VII–XIII are in the South Sea of the Korean Peninsula (See Fig. 1). IC: Incheon City; JN: Jeonlanam–do; KN: Kyungsangnam–do.

Fruit harvesting for natural open–pollinated seeds

Open–pollinated fruits and seeds were collected from Acha–do (I), Duckjuck–do (V), Mo–do (VI), Mijori (XI), and Yokji–do (XIII) at the end of October (Table 1) before the capsules were open. The geographic locations are shown in Fig. 1.

Seed classification

The seeds obtained from the crosses were visually inspected using a light box and classified into the following four categories: normal seeds containing an embryo, embryoless seeds containing only endosperm, exalbuminous seeds containing only a shriveled embryo, and empty seeds. Normal seeds were further classified into the following five grades depending on the ratio of embryo/endosperm lengths: 5/5, 4/5, 3/5, 2/5, and 1/5. Seeds with grades 5/5 and 4/5 were considered as fully matured, whereas seeds with grades 3/5, 2/5, and 1/5 were considered as underdeveloped seeds with underdeveloped embryos.

Germination test

Seeds representing each grade were soaked in water for 24 hours and transferred to 5°C for two weeks. Then, these cold–stratified seeds were germinated in a soil mix of peat :peat: pearlite : sand (4:1:1) on April 10 under field conditions in Chuncheon, Korea. Seeds with grades 3/5, 2/5, and 1/5 had very poor germination and very low survival under field conditions (Table 3); therefore, these underdeveloped seeds were germinated *in vitro*. The seeds were sterilized in 70% ethanol and 0.5% sodium hypochloride solution for 15 minutes and rinsed in distilled water three times. Then, they were placed on MS complete medium (0.3% agar, pH 5.8, 3% sugar) and germinated under the condition of 12 hours day/night at 23°C.

Ploidy analysis

Ploidy of the parental and hybrid plants was analyzed by flow cytometry (Partec PA–1, Germany) according to the previously published method of Kim *et al.* (2005). C value was determined using standard *Allium cepa* (2C, approximately 33.5pg) cells (Lim *et al.*, 2001). The flow cytometry results for plants with triploid DNA were verified by performing root–tip cell chromosome counting according to the previously published protocol of Fernandez *et al.* (1998). At least five metaphase cells with good chromosome spread were analyzed for verification of chromosome numbers.

Plant materials and DNA extraction

Lilium lancifolium accessions were obtained from the *Lilium* gene bank at Kangwon National University. 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 50 ng/μl and then subjected to 0.8% agarose gel electrophoresis. We used 42 expressed sequence tag–simple sequence repeat (EST–SSR) markers for analysis. These 42 EST–SSRs were selected from 76 EST–SSRs that were developed previously in *Lilium* (Lee *et al.*, 2011).

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 $MgCl_2$), 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 reaction conditions were as follows: 94°C for two minutes; and then 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1minute; 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 two hours at 1,800 V, or a LiCor 4300 automatic electrophoresis system for four hours. The DNA fragments separated by conventional PAGE were visualized by silver staining (Promega, USA).

RESULTS

Seed production

Seed set from open pollinations in natural populations was 51%, whereas that of artificial crosses was 82% (Table 2). The occurrence of abnormal seeds (e.g.,

Table 2. Normal seed production in natural open pollinations and artificial crosses of diploid *L. lancifolium*

Pollination type (2× × 2×)	No. of flowers pollinate	No. of fruits (% fruit set)	Total no. of normal seeds (Total no. of ovules)	Number of seeds per capsule ± SD					Proportion of fruit set (A) and normal seed production (B)
				Total no. of seeds	Normal seeds	Embryoless seeds	Exalbumi–nous seeds	Empty seeds	
Open pollination in Inatural habitat[Z]	–	68 (–)	9,346 (18,142)	266.8 ± 69.3	137.4 ± 57.3	1.9 ± 1.7	0.1 ± 0.01	127.4 ± 44.1	(B) 0.515
Inter–population cross[Y]	212	174 (82.1)	16,612 (49,607)	285.1 ± 7.9	92.9 ± 6.9	25.3 ± 3.3	0.1 ± 0.03	166.8 ± 9.7	(A) 0.821

[Z] Open pollination under natural conditions on isolated islands without other Lilium species and no other ploidy.
[Y] Cross includes crosses between endogenous populations isolated on different islands and inter–population crosses located on the same islands.

embryoless seeds) was higher in artificial crosses than in open pollinations of natural populations.

Seed germination

Seeds with grades 5/5 and 4/5 had greater than 81% survival from germination to mature growth stages under field conditions. Germination and growth were progressively reduced according to the degree of seed underdevelopment as follows: 3/5 > 2/5> 1/5 (Table 3). Germination and growth of underdeveloped seeds were promoted by *in vitro* culture. For seeds with grade 3/5, only 3 of 60 seeds germinated in soil (5%), whereas 33 out of 60 seeds germinated *in vitro* (45%). No seeds with grades 2/5 and 1/5 germinated in soil; these seeds had to be germinated *in vitro* for ploidy analysis.

For all seeds examined, the percentage of underdeveloped seeds was 2.4% and the percentage of fully matured seeds was 97.6% (Table 4). Because the number of underdeveloped seeds was low, it was not possible to perform replicate experiments for statistical analysis (Table 4). The percentage survival of seeds germinated in soil was 77.5% for fully matured seeds, but 45.5% for underdeveloped seeds. The total number of underdeveloped seeds harvested with grade 1/5 was 16, including three seeds from natural open pollinations and 13 seeds from artificial crosses (Table 4). Seeds with grade 1/5 completely failed to germinate under *in vitro* conditions.

Occurrence of triploids

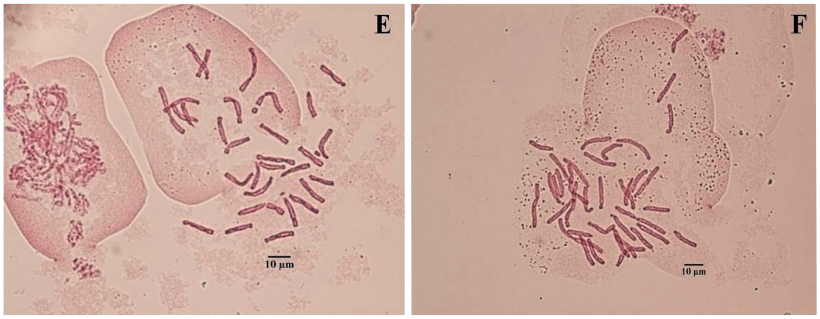
Triploids were not observed among seedlings pro-

Table 3. Germination of diploid seeds in soil and *in vitro*

Seed grade	Germination condition	No. of seeds sown	No. of seeds germinated	% Germination (±SD)	No. of seedlings survived (%)
5/5	Soil	400	344	86.0 ± 1.68	324(81)
4/5	Soil	400	372	93.3 ± 1.91	344(86)
3/5	Soil	60	45	75.7 ± 1.9	3(5)
	In vitro	60	57	95.0 ± 0.7	27(45)
2/5	Soil	20	8	40.0	0(0)
	In vitro	20	17	85.0	6(30)
1/5	Soil	10	0	0	0(0)
	In vitro	10	6	60.0	0(0)

Table 4. Seed germination and seedling survival for each seed grade from different hybridizations of diploid *L. lancifolium* populations

Type of pollination 2× × 2×	Germination ratio and surviving ratio	Germination ratio (%), survival ratio (%) and proportion of seeds and survived seedlings in indicated method of germination and type of seed grade				
		In soil		*In vitro*		
		5/5 (fully developed)	4/5 (fully developed)	3/5 (underdeveloped)	2/5 (underdeveloped)	1/5 (underdeveloped)
Open pollination in natural island populations	No. of germinated seeds / no. of seeds sown (%)	7,430 / 8,541 (86.9)	511 / 600 (85.2)	176 / 197 (89.3)	3 / 5 (60.0)	0/3 (0)
(9,346 seeds were used)	No. of survived seeds / no. of germinated seeds (%)	6,090 / 7,430 (81.9)	465 / 511 (77.5)	92 / 176 (52.3)	0 (0)	0 (0)
Inter–population crosses (12,258 seeds were used)	No. of germinated seeds / no. of seeds sown (%)	8,269 / 8,826 (93.7)	2,910 / 3,119 (93.3)	180 / 193 (93.3)	85 / 107 (79.4)	0/13 (0)
	No. of survived seeds / no. of germinated seeds (%)	7,573 / 8,269 (91.6)	2,620 / 2,910 (90.0)	99 / 180 (55.0)	11 / 85 (12.9)	0 (0)
Subtotal (21,604 seeds)		19,120 / 21,086 (88.5)		444 / 518 (85.7)		
Proportion of fully matured and underdeveloped seeds		21,086 / 21,604 = 0.976		518 / 21,604 = 0.0239		
Proportion of seedlings survival		16,748 / 21,604 = 0.775		202 / 444 = 0.455		



2n=2x=24 2n=3x=36

Fig. 2. Somatic chromosomes of diploid (E) and triploid (F) *L. lancifolium*.

duced by natural open pollinations. Triploids were detected only in seedlings of underdeveloped seeds generated by artificial crosses (Table 5). The percentage of triploid occurrence was 27.3% and 3.0% for seeds with grade 2/5 and 3/5, respectively. These results indicate that the triploid occurrence was 0.0% in natural open pollinations and 0.058% in artificial crosses (six triploids out of 10,303 seedlings).

We performed SSR analysis to determine whether the triploids were generated from self–fertilization or cross–fertilization (Fig. 3). We obtained four triploid plants from six cross combinations (Table 7). All triploid plants were verified to have both maternal and paternal SSR alleles; therefore, triploids were derived from cross–fertilization.

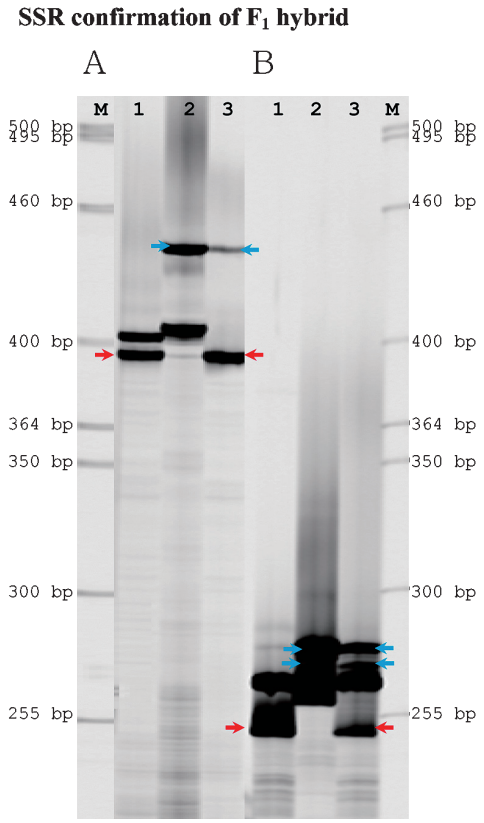


Fig. 3. Simple sequence repeat analysis of F_1 autotriploid plants from a cross of Jungdori (IX) × Deokheungri (X) (see Table 7). (A) SSR L05. (B) SSR eL64. Lane 1, Jungdori (IX); lane 2, Deokheungri (X); lane 3, F_1 triploid. Red and blue arrows indicate maternal and paternal alleles in the F_1 hybrid, respectively.

DISCUSSION

Analysis of 2n gamete formation

Six triploid seedlings were identified among 10,303 seedlings generated by artificial crosses between populations of diploid *L. lancifolium*, which results in a triploid occurrence frequency of 0.058% (Table 5). Triploids are produced by unreduced gametes that arise during microgametogenesis or megagametogenesis. In our study, triploids were identified among seedlings of underdeveloped seeds with grades of 3/5 and 2/5 that were cultured *in vitro*. These results indicate that triploid seeds are subjected to early abortion, which is consistent with an early report (Brink and Cooper, 1947). We deduce two possible explanations: (1) embryo development in triploid seed is not normal, and (2) germination and seedling survival for triploid seeds is very low. Therefore, the frequency of triploid occurrence might be higher than the calculated frequency due to the fact that underdeveloped seeds with a high probability of triploidy fail to germinate (Table 4), especially among seeds with grades 2/5 and 1/5. The embryoless seeds (early embryo abortion) in Table 2 may be generated as a result of 2n gamete production. If seeds with grade 1/5 have the same frequency of triploid occurrence as seeds with grade 2/5 (27.3%), then the occurrence of 2n gametes in the total number of sown seeds is calculated as 0.31% (Table 6), which is lower than the 0.56% rate of occurrence calculated for other species (Ramsey and Schemske, 1998). The frequency of 2n gamete seeds can be calculated as 38.5, which is derived by multiplying the rate of polyploidy occurrence by the total number of sown seeds (Table 6). The occurrence of 2n gametes (0.31%) is calculated by dividing the sum of the number of seeds with each grade by 12,258 (the total number of seeds sown in this experiment; Table 4).

Table 5. Occurrence of diploids and triploids from open pollinations and artificial crosses of diploid *L. lancifolium*

Pollination type	No. of seedlings analyzed (A)	Ploidy level	No. of 2x and 3x surviving seedlings in each seed developmental grade (B)					Ploidy type	Proportion for occurrence of ploidy (B/A)
			Fully developed seeds		Underdeveloped seeds				
			5/5	4/5	3/5	2/5	1/5		
Open pollination in natural diploid populations	6,647	2x	6,090	465	92	0	0		1
		3x	0	0	0	0	0	Autotriploid	0
Crosses between natural diploid populations	10,303	2x	7,573	2,620	96	8	0		0.999418
		3x	0	0	3	3	0	Autotriploid	0.000582
% Autotriploids in germinated seeds			0	0	3.0	27.3	0		

Table 6. Estimation of 2n gamete occurrence in the crosses with diploid populations of *L. lancifolium*

Research item	Rate in each underdeveloped seed grade		
	3/5	2/5	1/5
Germinated seeds / sown seeds (%)	180 / 193 (93.2)	85 / 107 (79.4)	0 / 13(0)
Surviving seedlings (% of sown seed)	99 (51.4)	11 (10.2)	0 (0)
% Polyploids in surviving seedlings (see Table 5)	3 / 99 = 3.0	3 / 11 = 27.3	–
Estimated rate of polyploids in sown seeds if all survived	193 × 0.03 = 5.8	107 × 0.273 = 29.2	13 × 0.273 = 3.5
Total estimation of 2n gamete occurrence in total sown seeds (see Table 4)	(5.8+29.2+3.5) / 12,258 = 0.0031 (0.31%)		

Effect of genetic distance and genetic differentiation

Most natural autopolyploids likely formed via some degree of hybridization (Soltis and Soltis, 1989; Stebbins 1985; Tayale and Parisod, 2013), but very little information is available regarding the genetic consequences of autopolyploidy in natural populations. Furthermore, it is impossible to find the genetic consequences of autotriploidy in natural populations.

Genetic distance contributes to the occurrence of triploids in many crops (Joyab and Bruneauac, 2004; Ramsey and Schemske. 1998; Soltis *et al*., 2009). In the current study, triploids were not identified among seedlings generated from open pollination of natural populations from isolated islands (Table 7). The tiger lily (*L. lancifolium*) pollinator in the Korean Peninsula is the tiger swallowtail, which visits neighboring flowers and can travel to neighboring populations located on nearby islands. *L. lancifolium* populations on the same island and on nearby islands can share genetic material and have similar gene structures. However, the feasibility of pollen transport by the tiger swallowtail from West Sea island populations to South Sea island populations (and vice versa) is extremely low due to the distance of 300 km (Fig. 1). In our study, triploids were produced from artificial crosses between populations locating on different islands more than 70 km apart (Table 7 and Fig. 1), and no triploids were detected in inter–population crosses of plants from the same island (see combinations in Table 1). This result suggests that triploids were pro-

Table 7. Limited cross combinations generated autotriploids

Crosses producing autotriploids (Site no.)	No. of seedlings analyzed	Geographic distance (km)*	No. of autotriploids obtained in each underdeveloped seed grade			Population locations**
			3/5	2/5	1/5	
Jungdori (IX) × Deokheungri (X)	360	70	1	0	0	S × S
Deokheungri (IX) × Baika–do (IV)	372	330	1	1	0	S × W
Geumgapri (VII) × Acha–do (I)	1,485	380	0	2	0	S × W
Yokji–do (XIII) × Baika–do (IV)	73	420	1	0	0	S × W

*Geographical distance between populations from West Sea and South Sea Islands is >300 km (see Fig. 1).
**W: West Sea Islands; S: South Sea Islands.

Table 8. Summary of probable factors affecting autotriploid occurrence in *L. lancifolium*

Factors	Proportion	Reference data and results
A: Fruit set	0.82	Artificial 2× × 2× cross (Table 2)
B: Seed set	0.515	Table 2 (in nature)
C: Underdeveloped seed	0.0239	Table 4 (*in vitro* + in nature)
D: Survival rate of underdeveloped seed	0.455	Table 4 (*in vitro* + in nature)
E: Proportion of triploid	0.000582 (0.0582%)	Table 5 (*in vitro*)
	0	Table 5 (in nature)
Chance of autotriploid occurrence *in vitro*	0.0000026	A × B × C × D × E
Chance of autotriploid occurrence in nature	0	

duced during fertilization between plants with low genetic similarity due to geographical isolation and/or distinct genotypes. Triploid seedlings were only produced during artificial crosses between ovule plants (♀) from South Sea populations and pollen plants (♂) from West Sea populations. Our previous SSR study showed that *L. lancifolium* populations from West Sea Islands are genetically differentiated from those of South Sea Islands (Song *et al.*, 2012). However, further genetic analysis using other markers is required to completely elucidate the extent of genetic differentiation.

Calculation of triploid occurrence in nature

The probability of triploid occurrence may be affected by many factors including fruiting and seedling survival (Table 8). The average fruit set in artificial crosses was 0.82, the average seed development in fruit was 0.515 in natural open–pollination conditions (Table 2), the proportion of underdeveloped seeds was 0.0239 (Table 4), and the survival rate of seedlings after germination of underdeveloped seeds was 0.455 (Table 4). The frequency of triploid occurrence among germinated and surviving seedlings was 0.000582. Each parameter for triploid formation presented in Table 8 is an independent event, so the total frequency must be included in the analysis. Therefore, the probability of triploid occurrence in the final calculation would be 0.0000026 under *in vitro* conditions and 0 under natural conditions. This allows us to rule out the occurrence of triploids under natural conditions; we calculate that 1 out of 400,000 zygotic embryos can be triploid under *in vitro* conditions. In our study, triploid plants were generated during artificial crosses between diploid plants that have been geographically and genetically isolated for many millennia.

Persistence of insular populations

Triploids must be produced in natural diploid populations (Bretagnolle and Thompson, 1995), and triploids have been observed in diploid populations (Einset, 1952; Rick, 1945; Tyagi, 1988). Triploids are generated by the fusion of a reduced (n) gamete and an unreduced (2n) gamete that were produced in plants with large genetic distances (Ramsey and Schemske. 1998). However, triploids are rare in nature because they cannot compete with diploid plants and have greater infertility.

L. lancifolium can reproduce both sexually and asexually. They can easily grow and propagate via bulbils. We surveyed more than 30 Korean islands since 1997, but we have not identified any triploids on those islands (Kim *et al.*, 2005, 2006b). By contrast, *L. lancifolium* triploids are frequently found in inland areas of the Korean Peninsula (Kim *et al.*, 2004). In the isolated islands, no *Lilium* species other than *L. lancifolium* have been identified. Korea is believed to be a center of origin of the *L. lancifolium* species within the *Lilium* genus. Because *L. lancifolium* triploids are frequently found in inland areas of Korea, a question emerged regarding why *L. lancifolium* triploids did not evolve in the insular populations of Korean islands, which have been isolated for approximately 12,500 years since the late quaternary geological period. There must be mechanisms or factors that exclude triploids in those isolated populations. Considering the results of this research, we propose the following four possible mechanisms that can explain the absence of triploid *L. lancifolium* on isolated Korean islands: (1) triploid occurrence in natural populations is low because the occurrence and germination of underdeveloped seeds (which produced the observed triploid seedlings) is very low; (2) triploid seedlings have very low viability in soil; (3) crosses between genetically isolated populations were not successful; and (4) isolated islands have no other endogenous *Lilium* species other than diploid *L. lancifolium* during flowering period, so the probability of allotriploid production is zero. The most important factor excluding autotriploid genesis is the gene–flow block due to ecological and geographical isolation of the islands. A systematic investigation of triploid *L. lancifolium* in inland areas will be performed in future studies to further elucidate the mechanisms involved in triploid formation in tiger lily.

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