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Morphological characterization under different ecological habitats and physical mapping of 5S and 45S rDNA in *Lilium distichum* with fluorescence *in situ* hybridization

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Abstract

Background: This study was performed to investigate the phenotypic, karyomorphological, and habitat environment characteristics of *Lilium distichum* that grows naturally in South Korea. Currently, this species follows limited distribution areas and its natural populations are at the brink of extinction mainly due to fragmentation or destruction its natural habitat.

Results: This species was distributed between approximately 1,000 and 1,500 m above sea level with an average temperature of 22°C. The soil characteristics surrounding the natural habitats included loamy and silt loam soils having organic matter content (10.82%), pH (5.22), electrical conductivity (EC) (0.37 dS/m), total nitrogen (0.45%), and cation exchange capacity (34.3 $cmol^{+}/kg$). The peak period of blossoming was between 27 July and 1 August. The maximum number of flowers was observed in Mount Deogyu (2.8), whereas the minimum number of flowers was observed in Mount Jiri (1.3) and Mount Seorak (1.2). Results regarding the number of verticillate leaf, bract counts, and verticillate leaf length and width were highest in Mount Odae, while lowest in the Mount Seorak region. The chromosome complement of *L. distichum* is $2n = 2x = 24$; the length of somatic metaphase chromosomes ranges from 17.01 ± 0.32 μm (chromosome 10) to 32.06 ± 0.35 μm (chromosome 1) with a total length/genome of 261.92 μm. In *L. distichum*, the presence of 1 pair (two loci) of 5S ribosomal DNA (rDNA) and 8 pairs (16 loci) of 45S rDNA was revealed on metaphase chromosomes. One pair of 5S rDNA signal was observed in interstitial region of long arm of chromosome 3 which co-occurred with 45S rDNA. Among the eight pairs of 45SrDNA, three pairs of 45S rDNA signals were observed in short arm of chromosome (chromosome 2, 6, and 7) which were located close to centromere. The other five pairs of 45S rDNA signals were positioned in the interstitial region of long arm (chromosome 3, 4, 5, 10, and 11).

Conclusions: This study provides baseline information regarding the effective exploitation and use of *L. distichum* resources for breeding research to be used as cut flower and potted plants.

Keywords: Chromosome; Karyotype; Habitat; Korea; 5S rDNA; 45S rDNA

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Background

The genus *Lilium* contains 110 species and out of these, approximately 60 species are distributed in South Korea, China, Japan, and other regions of East Asia, whereas 25 species thrive in North America and 14 species in Europe (Beattie and White 1993; Kong et al. 2013). Molecular analysis reveals that lilies arose in Eurasia, nearly 68 million years ago, and then distributed throughout the world (Leitch et al. 2007), and until now more than 10,000 cultivars have been developed (Younis et al. 2014a, b). The distribution of the lily is mainly based on environmental conditions like altitude, temperature, humidity, light intensity, etc. Plants in this genus have evolved with a fair amount of phenotypic diversity despite the genetic similarities, and this diversity of characteristics is of considerable evolutionary significance (Patterson and Givnish 2002). Comber (1949) exploited 15 morphological characteristics (e.g., seed germination form, leaf order, leaf shape, species size, bulb form, flower nectary, flower form, bulb color, stem property, stigma size, appearance of stem root, etc.) to classify naturally grown lilies into seven sections.

Korea offers diverse ecological characteristics which exhibit richness in *Lilium* germplasm, and 15 species that grow naturally have been documented until now (Kim 2008; Lee et al. 2011). Most of the lilies thriving in South Korea are colored, and those presenting vivid flower colors and shapes receive the greatest ornamental and commercial value. The high ornamental value of the *Lilium distichum* of the Martagon section is mainly based on its yellowish orange flowers with purple spots slightly 'flat faced' in appearance with uneven distribution of floral petals to make it like a fan shape. Liang and Tamura (2000) reported *L. distichum* as a boreal species usually found on Mount Seorak and the Jeju mountains of South Korea whereas, Rong et al. (2011) documented that this species is also well distributed in Amur of Siberia, Russian Far East, and Manchuria region of China. Recently, Chung et al. (2014) found this species in grasslands or temperate deciduous forests in the Baekdudaegan and Nakdongjeongmaek in Korea at altitudes of 900-1600 m.

Fluorescence *in situ* hybridization (FISH) is an amenable molecular cytogenetic approach in which chromosomal markers are used to detect the position of specific genes, like ribosomal DNAs for physical mapping. FISH signals provide a unique insight into the genome structure to categorize individual chromosomes that may lead to provide adequate information for evolutionary studies. Two types of ribosomal RNA genes (45S and 5S ribosomal DNA (rDNA)) are important chromosomal markers for chromosome identification and characterization as they are structured in hundreds to thousands of tandem repeats and vary in intra- or interspecies in number, hybridization signal intensity, and location (Lim et al. 2005). Most of the *Lilium* species were known as diploid ($2n = 2x = 24$) with basic chromosome number of $x = 12$. The chromosome nomenclature of lilies is based on the descending order of the short arm, but the detailed cytogenetic studies are limited in *L. distichum* (Sultana et al. 2010). Also the molecular mapping is hindered due to large genome size (76 pg/2C) in *Lilium* (Anderson et al. 2010). Previous studies on *L. distichum* have focused on cross-fertilization (Kim 2008; Khan et al. 2012) and *in vitro* breeding (Kim 1996; Chen et al. 2007; Eum et al. 2008), whereas ecological, morphological, and cytogenetic investigations are limited. Furthermore, most previous studies did not look into the finer details of each lily species, and thus, the current classification of the Jeju-do Kochang lily has sparked confusion among taxonomists and academicians (Lighty 1970; Kim 1996; Fox 2006). The historical report of Makino (1901) stated that the Jeju-do Kochang lily is morphologically distinct from those grown in the Korean Peninsula. On the basis of this observation, he suggested a new name for the species *Lilium miquelianum*. Consequently, Fox (2006) combined the existing literature and suggested the occurrence of an interspecific hybrid, in which the Kochang lily of Jeju-do represents an intermediate form of the Korean wheel lily (*L. distichum*) and the Kochang lily. The occurrence of *L. distichum* at mid to high altitudes suggests that the history of this species would be close to a state of survival within large refugia that may lead to the high or moderate level of genetic variability within population (Chung et al. 2014). Based on these developments, the present study was conducted to examine the morphological characteristics, the natural habitat surroundings, the distribution of *L. distichum*, and chromosome analysis to facilitate its taxonomic and cytogenetic classification. Present study findings can be exploited in breeding programs, cultivar development, and gene resource preservation.

Methods

Distribution and natural habitat

This study conducted an on-site growth investigation for 4 years, from 2008 to 2011, in the natural habitat of the *L. distichum* of South Korea, from Mount Jiri, which is the southern region, to the northern Mount Seorak (Figure 1). Prior to the field survey, the location of the natural habitat of the *L. distichum* and habitat-related information were collected through various channels. Based on collected materials, information on the habitat location of the *L. distichum* was organized, which resulted in the creation of a primary location distribution map of this species. Blooming time was predicted using the information gathered from the preliminary survey; after which, the investigation was carried out. Errors in the existing location were adjusted based on the data of the *L. distichum* habitat (Mount Jiri, Mount Deogyu, Mount Odae, Mount Hambaek, and Mount Seorak) distribution area. Growth conditions were visually confirmed, and new habitats discovered during the

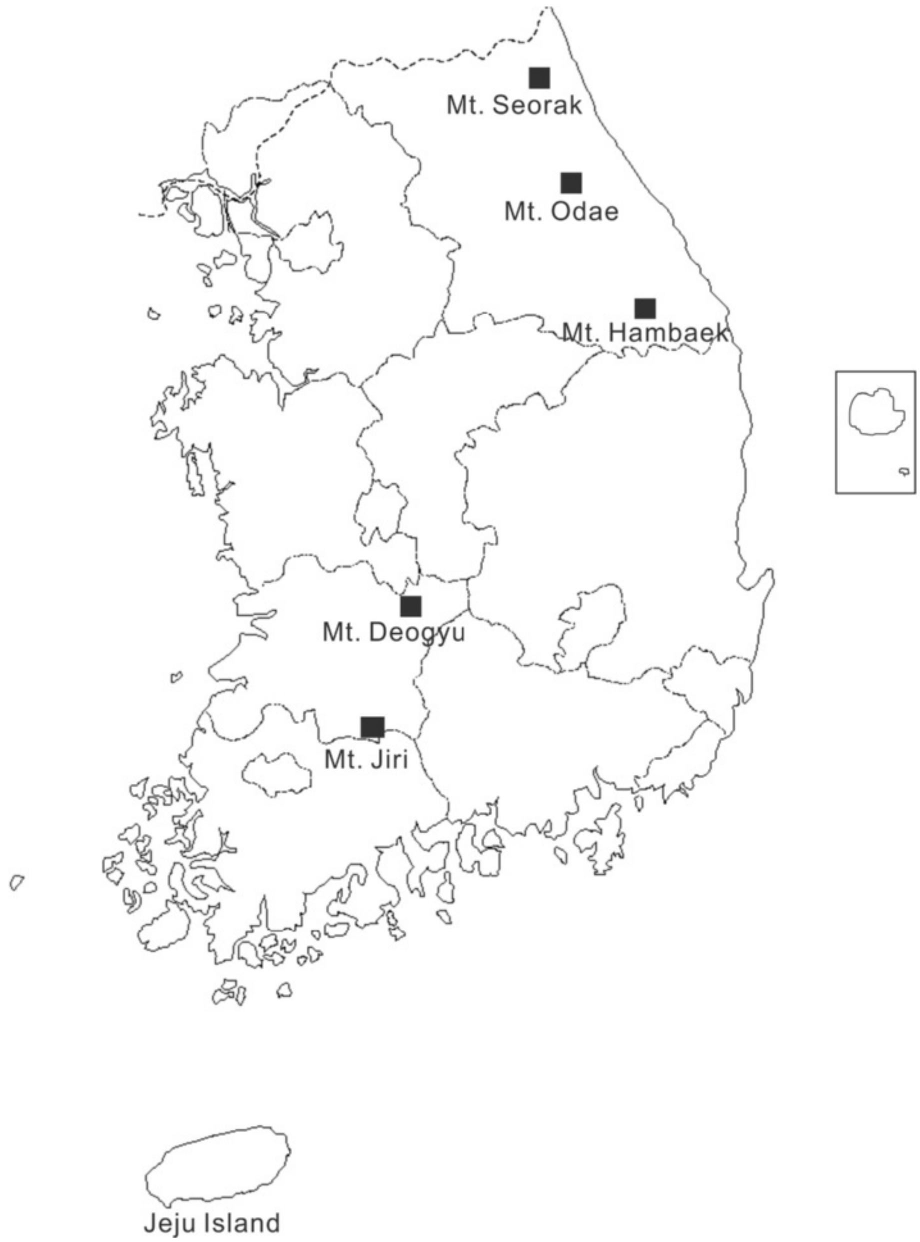

Figure 1 **Map of surveyed habitats of *L. distichum* in Korea.**

exploration were integrated into the map using a portable GPS (Garmin Colorado 300, Garmin, Kansas, TX, USA).

Physicochemical analysis of soil

To investigate pedological characteristics, leaf mold of the upper layer was removed, and the three soil samples within a depth of 10 to 15 cm were collected from five survey sites surrounding the natural habitat of the Kochang lily. The soil samples were combined, air-dried, pulverized, and filtered through a 1-mm strainer prior to analysis. Soil pH and electrical conductivity (EC) were measured using a soil suspension created using distilled water (1:5). Organic matter was analyzed using the Tyurin method (Nikitin 1999), all nitrogen content was determined using the Kjeldahl method (Jackson 1962), and cation exchange capacity (CEC) was measured using the method adopted by Hendershot and Duquette (1986). For the physical characteristics of the soil, a hydrometer was used to microscopically inspect the texture of each soil sample.

Natural habitat environment and vegetation

To examine the environment of the natural habitat, temperature, humidity, types of vegetation, and light intensity (data logger and photometer, LX1330B Lux meter (Chemie GmbH, Unna, Germany)) were measured. For the vegetation survey, a minimal central unit of the natural habitat (three entities) consisting of an arbitrary quadrat measuring 5 m in width and length was installed to study the herbs and tree plants within the zone (Table 1).

Morphological analysis

A survey of plant morphological characteristics was conducted using 40 specimens (8 specimens from each of the 5 habitats) of the flowers, leaves, stems, and bulbs of the *L. distichum*, following the Lily Characteristics Investigation Protocol of the National Seed Management Office.

Chromosome preparation

The young root tips of 3 cm in length were collected and placed for 4 h in α-bromonaphthalene solution under room temperature (RT). Then, the treated root tips were washed with running tap water prior to keep at −20°C in 70% ethanol solution. After fixing, the root tips were washed with distilled water and then treated with enzyme mix [pectolyase (0.3%), cytohelicase (0.3%), cellulase (0.3%)] in 150 mM citrate buffer for 1 h at 37°C. The treated root tips were squeezed in 60% acetic acid droplet followed by air drying overnight and finally stored at −20°C prior to FISH experiment.

Probe DNA preparation (Ribosomal DNA)

45S rDNA, having 9-kb fragment of 18S-5.8S-25S rDNA genes, was isolated from *Triticum aesitivum* and labeled with biotin-16-dUTP (Roche, GmbH, Mannheim, Germany). 5S rDNA was obtained after amplification of genomic DNA extracted by using PCR synthesis kit (Roche, GmbH, Mannheim, Germany) from *Lilium* spp.

Fluorescence *in situ* hybridization

FISH was carried out as adopted by Lim et al. (2007). The slides were pretreated with RNase A in 2× saline-sodium citrate (SSC; 100 $\mu L \cdot mL^{-1}$, DNase free; Cayman Chemical, Ann Arbor, MI, USA) for 1 h at 37°C. The slides were washed in 2× SSC three times and then post-fixed in paraformaldehyde solution (4%) for 10 min. 5S and 45S rDNA were tagged with digoxygenin-11-dUTP and biotin-16-dUTP by nick translation mix (Roche, Mannheim, Germany), respectively. The hybridization mixture containing deionized formamide (50%), dextran sulfate (10%), 2× SSC, and 20 $\mu g \cdot mL^{-1}$ of probe DNA (all from Merck & Co., Inc. Whitehouse Station, NJ, USA) was denatured for 10 min at 70°C. The hybridization mixture was then transferred to slides and covered with cover slips. Each slide was denatured for 5 min at 80°C and incubated in a humid chamber for 1 h at 37°C. After hybridization step, each slide was washed with 0.1× SSC for 30 min at 42°C, followed by detection of digoxygenin and biotin by using fluorescein isothiocyanate FITC-conjugated anti-digoxygenin antibody (Roche, Mannheim, Germany) and streptavidin Cy3 (Zymed Laboratories Inc., San Francisco, CA, USA). The chromosomes were counterstained with 2 $\mu L \cdot mL^{-1}$ of 4′,6-diamidino-2-phenylindole (DAPI) in vecta-shield (Vector Laboratories Inc., Burlingame, CA, USA) and observed under the fluorescent microscope (Nikon BX 61, Nikon, Huntington, New York, USA). Images were taken through a charge coupled device (CCD) and then images processing were done through the Genus FISH imaging system (Applied Imaging Corporation, Genus version 3.8 program, San Jose, CA, USA). Confirmation of putative homologous chromosomes was done on the basis of their morphological characteristics and FISH results.

Karyotype analysis

A minimum ten cells with metaphase spreads chromosomes were used for karyotype analysis. The length of individual chromosome was measured through computer software, and chromosome numbers were determined on the basis of short arm length order as adopted by Stewart (1947). Three plant samples were used from each survey site and average was computed. Chromosome types were then observed based on arm ratio value (Levan et al. 1964).

Table 1 Geographical description and the environmental conditions of *L. distichum* habitats at anthesis in Korea

Habitat (Mt)	Latitude (°)	Altitude (m)	Flowering date	Temp (°C)	RH (%)	Light intensity (klx)	
						Habitat	Field
Jiri	35.21	1,178~1,340	27 July	20.4	34	6~27	116~140
Deogyu	35.51	1,300~1,549	29 July	21.9	38	5~26	110~120
Hambaek	37.08	1,100~1,300	30 July	23.0	37	5~27	110~130
Odae	37.46	1,089~1,300	30 July	20.0	40	3~30	90~130
Seorak	38.06	1,100~1,200	1 August	23.6	35	3~30	90~130
Range	-	1,089~1,549	27 July to 1 August	20.0~23.6	34~40	3~30	90~140

Results

Distribution and the natural growth environment

Among the surveyed sites, the lowest mountain in which the *L. distichum* has been sighted was Mount Odae (1,089 m); the highest mountain was Mount Deogyu (1,549 m). Besides the five surveyed mountains, the *L. distichum* has been observed in Mount Bangtae, Mount Hwangbyun, Mount Bakji, and Mount Baekjuk of Gangwon-do, Mount Gaji of Gyeongsangnam-do, Mount Juwang of Gyeongsangbuk-do, and other habitats.

According to the present investigation, the peak period of blossoming of the *L. distichum* in South Korea was between 27 July and 1 August, 2011 (Table 1). In Mount Jiri area habitat, early blossoming was observed, i.e., July 27, whereas in the Mount Seorak habitat, flowering occurred on 1 August. Blossoming tends to be delayed at higher latitudes. At the peak period of blossoming, the average temperature ranged from 20.0°C to 23.6°C and humidity ranged from 34% to 40%. Luminosity at the periphery of the natural habitat ranged from 3 to 30 klx, which was 22% to 33% of the external luminosity (range: 90 to 140 klx). The natural habitat of *L. distichum* had soil that contained leaf mold, which generally occurs at the lower regions of the deciduous forest.

Physicochemical analysis of soil

The physicochemical characteristics of the soil collected from the five natural habitats of the *L. distichum* are presented in Table 2. The surveyed sites consisted of loam and silt loam soils. Mount Seorak and Mount Jiri possessed loam soil, whereas Mount Deogyu, Mount Hambaek, and Mount Odae had silt loam soil. The average composition ratio of sand, silt, and clay in the natural habitats was 33.3%, 55.9%, and 10.8%, respectively. For the chemical analysis of the soil, the average ratio of organic matter in all the surveyed sites was 10.82%. The average ratio of organic matter of Mount Jiri and Mount Hambaek was 15.8% and 12.8%, respectively, which was relatively higher than the other regions. The average soil pH was 5.22, indicating that it was slightly acidic. In the Mount Hambaek area, the soil pH was highest at 5.8 and lowest in Mount Jiri at 4.7. The pH of Mount Hambaek was relatively higher than the overall forest average of 5.48.

Natural habitat environment and vegetation analysis

The herbaceous plants commonly found in the five surveyed sites included *Athyrium yokoscense*, *Lychnis cognata*, *Astilbe chinensis*, and *Veratrum patulum*. Xylophytes included *Tripterygium regelii*, *Sasa borealis*, *Quercus mongolica*, *Magnolia sieboldii*, *Fraxinus rhynchophylla*, *Acer komarovii*, and *Betula ermanii* (Figure 2). The characteristics of the herbaceous plants were that its environment for optimal growth and development was a forest with penumbra or soil with good drainage and a humid environment.

Morphological characteristics

Flower

The flower characteristics of the *L. distichum* are presented in Table 3. The average flower per stem count was 2.1 in the five natural habitats of the *L. distichum*. The highest counts were observed in Mount Deogyu (2.8) and Mount Odae (2.6), whereas the lowest counts were observed in Mount Jiri (1.3) and Mount Seorak (1.2). The diameter of the flowers was within the range of 7.2 to 8.0 cm; the average was 7.5 cm (Table 3). In Mount Deogyu habitat, the smallest flower size (7.2 cm) was observed, whereas the largest flower size (8.2 cm) was recorded in Mount Odae. Flower color was determined as bright orange (Royal Horticultural Society (RHS) 28B, RHS color chart) in all habitats (Table 3). Some flowers in Mount Deogyu showed orange color (RHS 28A, RHS color chart); however, no major differences were observed for flower color in other habitat under study. Fragrance was observed in the flowers growing in all habitats, but some flowers had strong fragrance while others had weak fragrance.

The inflorescence was of umbel form when the flower count per stem were two; however, when the flower count was more than two, both umbel and raceme forms were observed. Petal length ranged from 3.9 to 4.4 cm

Table 2 Physio-chemical properties of soil texture of *L. distichum* habitats in Korea

Habitat	Chemical characteristics					Physical characteristics			
	Organic matter (%)	pH	EC (dS/m)	T-N (%)	CEC ($cmol^+$/kg)	Sand (%)	Silt (%)	Clay (%)	Soil texture
Jiri	15.8	4.7	0.34	0.48	34.3	51.6	39.4	9.0	Loam
Deogyu	9.7	5.2	0.39	0.59	38.6	14.8	75.2	10.0	Silt loam
Hambaek	12.8	5.8	0.55	0.51	38.0	29.3	60.3	10.4	Silt loam
Odae	8.6	5.4	0.31	0.42	38.7	25.1	60.5	14.4	Silt loam
Seorak	7.2	5.0	0.27	0.24	21.9	45.9	43.9	10.2	Loam
Average	10.82	5.22	0.37	0.45	34.3	33.3 ± 15.2	55.9 ± 14.4	10.8 ± 2.1	-

Figure 2 Photographs of wild vegetation around *L. distichum* habitats in Korea. (A) *Lychnis cognata Maxim.* **(B)** *Astilbe rubra Hook.f. & Thomson var. rubra.* **(C)** *Athyrium yokoscense* ((*FR. et SAV.*) *H. CHRIST*). **(D)** *Erythronium japonicum* (*Balrer*) *Decne.* **(E)** *Acer komarovii Pojark.* **(F)** *Fraxinus rhynchophylla Hance.* **(G)** *Betula ermanii Cham.* **(H)** *Tripterygium regelii K. Koch.* **(I)** *Acer pseudosieboldianum* (*Pax*) *Kom.* **(J)** *Quercus mongolica Fisch. ex Ledeb.*

and the average was measured at 4.2 cm (Table 3). The average petal length of the lilies thriving in Mount Seorak was 3.9 cm, whereas petal length in Mount Hambaek was relatively small (4.0 cm). The average petal length of the lilies thriving on Mount Deogyu and Mount Odae was 4.4 cm, which was the longest. Petal width ranged from 1.4 to 1.7 cm and the average was 1.5 cm. Calyx length ranged from 3.8 to 4.4 cm, with the average measuring at 4.1 cm. In the Mount Seorak region, the average calyx length was 3.8 cm, whereas those in Mount Jiri were 4.3 cm and those in Mount Odae was 4.4 cm. Calyx width ranged from 1.1 to 1.3 cm and the average was 1.2 cm. The average calyx width of those in Mount Deogyu and Mount Seorak was 1.1 cm, whereas those in Mount Hambaek and Mount Odae measured 1.3 mm. The average number of dots distributed on the petals was 87 for all regions surveyed (Table 3), and the average was highest in habitat of Mount Deogyu (108) while the average was lowest in Mount Jiri (69). However, the number of dots in the surveyed entities ranged from 42 to 174, indicating a wide range regardless of the region or size. Dot color was red-violet and the sizes ranged from 0.5 to 1.9 mm.

The flower shape of *L. distichum* was categorized into three types (Figure 3) as follows: one petal was separated from the other five petals and inserted independently (Figure 3B,C,D), Turk's cap-like form (Figure 3E) and bilateral star-like form (Figure 3F). The three flower shapes were observed in all regions, with the petal insertion type as the predominant form.

Stem

The morphological characteristics of the stem revealed that the average total length of the stem was 59.6 cm. The longest stem was observed in Mount Odae (79.5 cm), and the shortest was in Mount Seorak (46.2 cm). Overall height up to the verticillate leaves was 33.7 cm with the tallest observed in Mount Odae (41.7 cm) and the shortest in Mount Seorak (25.4 cm). Based on the total average height, the height to the verticillate leaves ranged from 53% to 63% in terms of ratio to total length (Table 4).

Table 3 Characteristics of *L. distichum* flowers under various habitats in Korea

Habitat	Number of florets	Diameter (cm)	Color[a]	Inflorescence	Petal		Sepal		Number of spot
					Length (cm)	Width (cm)	Length (cm)	Width (cm)	
Jiri	1.3 ± 0.5	7.6 ± 0.8	28B	U	4.2 ± 0.3	1.4 ± 0.3	4.3 ± 0.2	1.2 ± 0.1	69 ± 17.3
Deogyu	2.8 ± 1.3	7.2 ± 0.6	28A/B	U/R	4.4 ± 0.1	1.5 ± 0.3	4.0 ± 0.4	1.1 ± 0.2	108 ± 58.0
Hambaek	1.8 ± 0.5	7.8 ± 0.5	28B	U	4.0 ± 0.4	1.7 ± 0.6	4.1 ± 0.3	1.3 ± 0.1	96 ± 48.2
Odae	3.6 ± 0.9	8.0 ± 0.8	28B	U/R	4.4 ± 0.3	1.6 ± 0.2	4.4 ± 0.4	1.3 ± 0.1	83 ± 41.9
Seorak	1.2 ± 0.4	6.7 ± 0.9	28B	U	3.9 ± 0.3	1.3 ± 0.1	3.8 ± 0.3	1.1 ± 0.2	76 ± 37.6
Average	2.1 ± 1.0	7.5 ± 0.5	28A/B	U/R	4.2 ± 0.2	1.5 ± 0.2	4.1 ± 0.2	1.2 ± 0.1	86.4 ± 15.6

[a]RHS mini color chart. U, umbel; R, raceme.

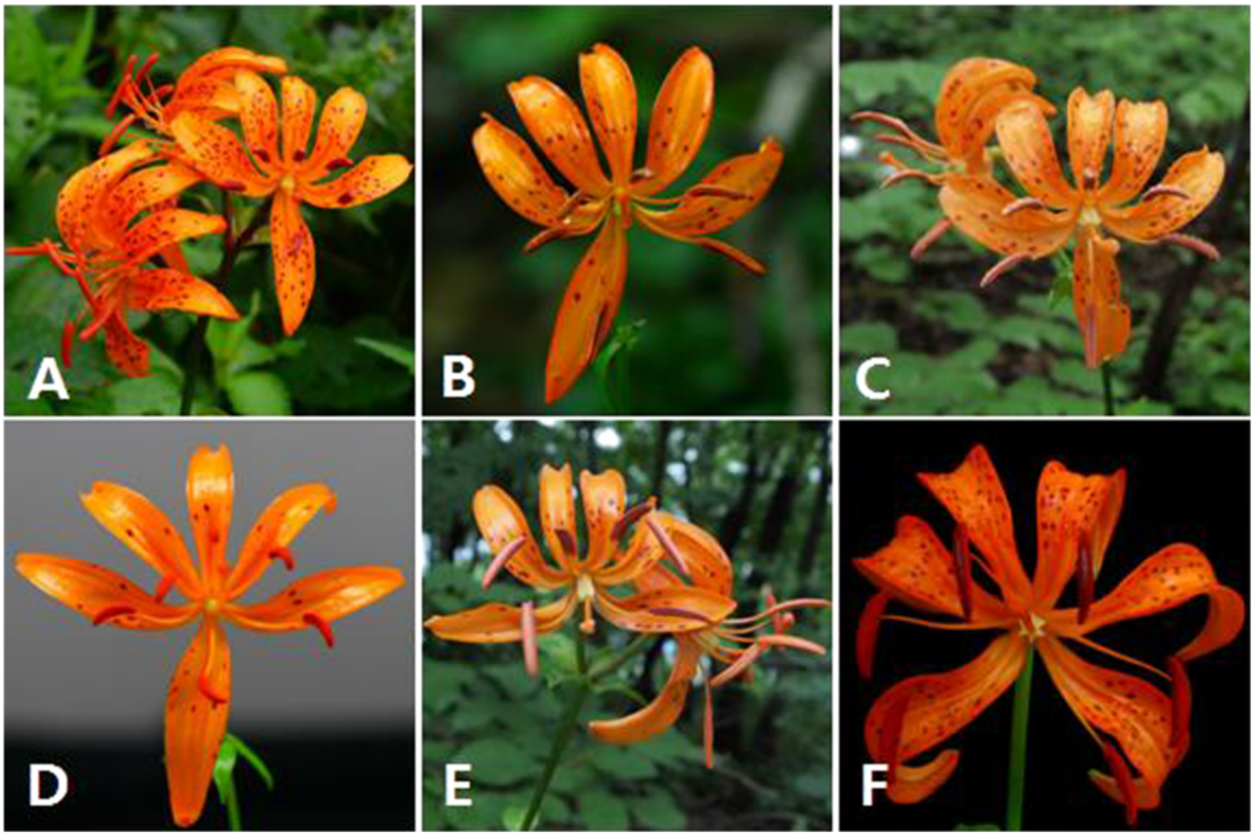

Figure 3 Flower of *L. distichum* showing various corolla shapes in different native habitats. (A) Mt. Deogyu. **(B)** One petal was separated from the other five petals and inserted independently in Mt. Seorak. **(C)** One petal was separated from the other five petals and inserted independently in Mt. Odae. **(D)** One petal was separated from the other five petals and inserted independently in Mt. Jiri. **(E)** Turk's cap-like form in Mt. Hambaek. **(F)** Bilateral star-like form in Mt. Jiri.

The diameter of the stem was measured at a point 2 cm above and below the verticillate leaves. The average diameter above the verticillate leaves was 2.9 cm and below was 4.3 cm. In Mount Odae, the upper surface measured 3.7 cm (shortest) and the lower surface was 5.2 cm (longest); in Mount Seorak, the upper surface measured 2.1 cm and the lower surface was 2.1 cm. Based on the part below the verticillate leaves, the upper diameter size ranged from 62% to 71% in terms of its ratio with the lower portion. In terms of the tactility of the stem surface, all regions showed a mixed sensation of roughness and sleekness regardless of the parts above and below the verticillate leaves, except for those from Mount Deogyu, which was sleek in both parts of the stem.

Leaf

The leaf characteristics of the *L. distichum* are presented in (Table 5) which showed that the total average number

Table 4 Characteristics of *L. distichum* stem under various habitats in Korea

Habitat	Length (cm)		Location of verticillate on stem (%)	Diameter (cm)		Surface[e]
	Total length[a]	Length[b]		Under[c]	Above[d]	
Jiri	55.8[a] ± 14.5	34.8[b] ± 9.1	62.4	3.5[c] ± 0.9	2.2[d] ± 0.6	Mixed[e]
Deogyu	64.2 ± 17.8	37.3 ± 8.2	58.1	4.8 ± 0.8	3.0 ± 0.5	Smooth
Hambaek	52.2 ± 12.6	29.5 ± 9.8	56.5	4.3 ± 0.7	2.3 ± 0.6	Mixed
Odae	79.5 ± 8.6	41.7 ± 5.2	52.5	5.5 ± 0.5	3.5 ± 0.5	Mixed
Seorak	46.2 ± 10.3	25.4 ± 3.6	55.0	3.3 ± 0.6	2.0 ± 0.4	Mixed
Average	59.6 ± 12.9	33.7 ± 6.4	52.5~62.4	4.3 ± 0.9	2.9 ± 0.9	

[a]Total length from bottom to under bract, [b]length from bottom to verticillate leaves, [c]under verticillate leaves, [d]above verticillate leaves, [e]smooth and rough both type of surface.

Table 5 Characteristics of *L. distichum* leaves under various habitats in Korea

Habitat	Number of VL	Number of above VL	Number of under VL	Number of bracts	Length of VL	Width of VL	LSI
Jiri	8.3 ± 2.5	3.5 ± 0.6	1.8 ± 0.5	3.8 ± 1.5	11.3 ± 1.9	2.9 ± 0.9	3.9
Deogyu	10.2 ± 1.9	6.6 ± 2.2	2.0 ± 1.3	5.7 ± 2.1	11.8 ± 2.2	3.4 ± 0.9	3.5
Hambaek	10.4 ± 2.1	5.4 ± 1.3	1.2 ± 0.8	5.4 ± 3.7	11.3 ± 1.8	3.0 ± 0.7	3.8
Odae	10.2 ± 1.8	8.0 ± 1.4	1.2 ± 1.0	7.0 ± 2.0	14.4 ± 0.9	3.8 ± 0.3	3.8
Seorak	7.2 ± 1.8	3.8 ± 1.3	1.6 ± 0.5	2.8 ± 1.1	10.1 ± 1.2	3.2 ± 0.7	3.1
Average	9.2 ± 1.4	5.5 ± 1.9	1.6 ± 0.4	4.9 ± 1.7	11.8 ± 1.6	3.3 ± 0.3	3.6

VL, verticillate leaf; LSI, leaf shape index (length/width).

of verticillate leaves was 9.2. The average number of verticillate leaves per plant in each surveyed site was recorded the highest in Mount Hambaek (10.4) whereas, in Mount Deogyu and Mount Odae, 10.2 leaves were observed. The average number of leaves below the verticillate leaves was 1.6 in all surveyed sites. The average leaf shape index as measured using the vertical length and the horizontal length of the verticillate leaves was 3.6. Based on these results, the verticillate leaf count, number above and below, bract counts, and verticillate leaf length and width were relatively high in Mount Odae, and all aspects were lower in the Mount Seorak region.

Bulb

The bulb color was white (RH 155D, RHS color chart) in all regions surveyed. The height and width measured when assessing the bulb size ranged, respectively, from 2.1 to 2.6 cm and from 1.9 to 2.9 cm. In Mount Deogyu and Mount Hambaek, habitats showed the maximum size (2.6 cm) of bulbs and maximum width (2.9 cm) was found in Mount Hambaek. The average bulb size was 2.4 cm and average width was 2.5 cm. The bulb shape ratio was approximately 1:1, indicating a symmetrical shape (Figure 4). The average bulb circumference was 8 cm; the biggest (9.3 cm) was observed in Mount Hambaek habitats and the smallest (6.4 cm) in the Mount Jiri area (Table 6).

The highest average total number of disassembled scales was in Mount Deogyu (92.4), whereas the lowest was in Mount Jiri (34.8). A single node was observed as the predominant number, whereas those without nodes or two nodes varied among regions (Figure 5).

FISH karyotype analysis

We measured the individual chromosome length to determine the chromosome number on the basis of the decreasing short arm length. All plant materials collected from different habitats had chromosome complements of 2n = 2x = 24. Karyotype analysis revealed that the length of somatic metaphase chromosomes ranges from 17.01 ± 0.32 μm (chromosome 10) to 32.06 ± 0.35 μm (chromosome 1) with a total length of 261.92 μm. The chromosomes are comprised of two pairs of metacentrics (chromosomes 1 and 2), five pairs of subtelocentrics (chromosomes 3, 4, 5, 6, and 7), and five pairs of telocentrics (chromosomes 8, 9, 10, 11, and 12) (Table 7). FISH

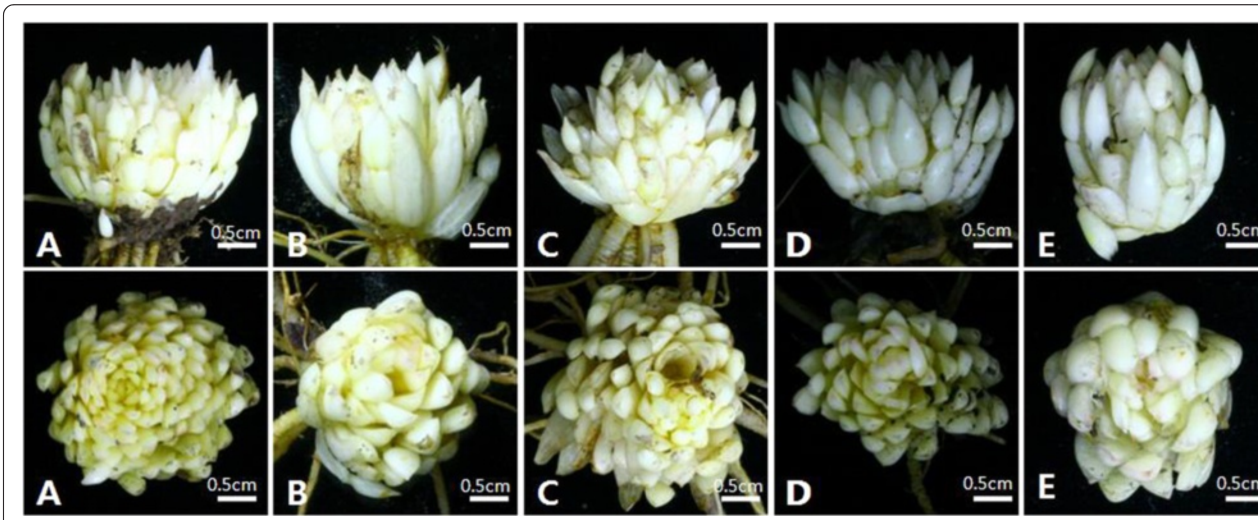

Figure 4 Variations in bulbs shape of *L. distichum* in different natural habitats. **(A)** Mt. Deogyu, **(B)** Mt. Seorak, **(C)** Mt. Odae, **(D)** Mt. Hambaek, **(E)** Mt. Jiri.

Table 6 Characteristics of *L. distichum* bulb in different habitats in Korea

Habitat	Height (cm)	Width (cm)	A/B[z]	Circumference	Number of scale			
					Total	No joint	1 joint	2 joints
Jiri	2.1 ± 0.3	1.9 ± 0.4	0.9	6.4 ± 1.0	34.8 ± 4.7	11.8 ± 1.7	20.8 ± 3.8	9.0 ± 0
Deogyu	2.6 ± 0.4	2.7 ± 0.5	1.0	8.7 ± 1.5	92.4 ± 27.0	18.2 ± 7.6	44.0 ± 18.1	30.2 ± 20.6
Hambaek	2.6 ± 0.4	2.9 ± 0.7	1.1	9.3 ± 1.3	56.3 ± 13.8	12.3 ± 5.0	32.3 ± 10.2	11.8 ± 4.3
Odae	2.4 ± 0.2	2.6 ± 0.6	1.1	8.2 ± 1.5	71.0 ± 32.0	18.3 ± 9.6	37.0 ± 19.1	24 ± 5.6
Seorak	2.4 ± 0.2	2.5 ± 0.4	1.0	7.6 ± 0.5	43.0 ± 6.6	10.7 ± 4.9	27.0 ± 3.6	5.3 ± 3.0
Average	2.4 ± 0.2	2.5 ± 0.4	1.0 ± 0.1	8.0 ± 1.1	59.5 ± 23.0	14.3 ± 3.7	32.2 ± 9.0	17.8 ± 11.3

[z]Height/width.

assay with 5S and 45S rDNA signals detected 2 loci and 16 loci, respectively. The positions of 5S and 45S rDNA sites were summarized in Table 7 and Figure 6. Chromosomes 1 and 2 had very similar morphology, the long arm of chromosome 1 was slightly longer than that of chromosome 2. In addition, one 45S rDNA site localized at the pericentromeric region of the short arm of chromosome 2. From chromosome 3, the length of short arm dramatically decreased compared to that of chromosomes 1 and 2. The 5S and 45S rDNA signals localized on the interstitial region of the long arm, with the 45S rDNA being more proximal to the centromere. The chromosomes 4, 5, 6, and 7 were observed as subtelocentrics which have also almost similar short arm length. These chromosomes can be distinguished according to the increasing long arm length and location of 45S rDNA sites. The 45S rDNA sites are positioned in the interstitial region of the long arms of chromosomes of 4 and 5, with site in chromosome 4 being middle part of the long arm and one-third away from the telomere in chromosome 5. On the other hand, the 45S sites are localized in the pericentromeric regions of the short arms of chromosomes 6 and 7. The chromosomes 8 and 9 were observed as telocentrics and arranged according to decreasing long arm length. The chromosomes 10, 11, and 12 were observed as telocentrics which can be discriminated by the order of their increasing long arm length. 45S rDNA sites localized on the chromosome 10 and 11, with site chromosome 10 being one-third way from the telomere and middle part of the long arm in chromosome 11.

Discussion

Genomic and evolutionary studies in *Lilium* have attained progress with pheno-morphological attributes, geographical distribution, and molecular and cytogenetic investigations. It was found that environmental conditions of the habitats greatly influence the diversity of the species as well as morphological characteristic of a particular species (Rodnikova 2012). Physiological and morphological characteristics in plants are interrelated with adaptive effects to environmental conditions (Dyer et al. 2006), and plants from more productive habitats having more conducive environmental conditions often have faster growth rates and it results into vigorous plant health with quality flowers (Grime et al. 1997). But many morphological features in plants are gene regulated and sometime environment has no indicative effect on them (Barkoulas et al. 2007). Development of plants from 2*n* gametes also contributes to variations in plants (Younis et al. 2014a, b). These significant variations in different

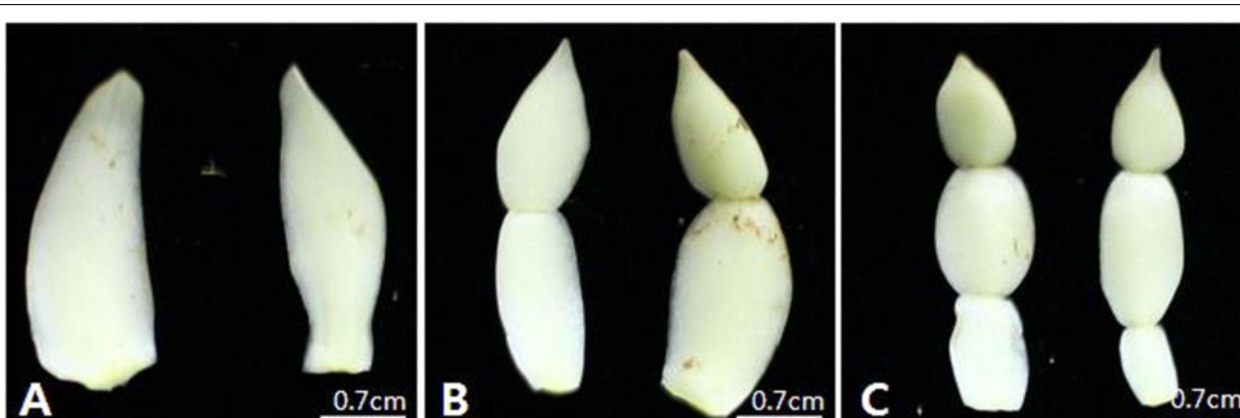

Figure 5 Different shapes of scale in *L. distichum* under different habitats. **(A)** Mt. Deogyu, **(B)** Mt. Seorak, **(C)** Mt. Odae.

Table 7 Cytogenetic characteristics of *L. distichum*

Chromosome number	Chromosome length (μm)			rDNA FISH		Chromosome type
	Short arm (S)	Long arm (L)	Total (S + L)	5S	45S	
1	12.88[a] ± 0.85	19.18 ± 0.23	32.06 ± 0.35	-	-	m[d]
2	11.84 ± 0.17	16.83 ± 0.13	28.67 ± 0.04	-	S (2)[b]	m
3	5.40 ± 0.01	19.93 ± 0.16	25.33 ± 0.16	L(2)	L(2)[c]	st[e]
4	4.40 ± 0.11	13.44 ± 0.75	17.84 ± 0.15	-	L(2)	st
5	3.97 ± 0.05	15.82 ± 0.36	19.79 ± 0.32	-	L(2)	st
6	3.84 ± 0.12	16.44 ± 1.22	20.28 ± 0.35	-	S(2)	st
7	3.52 ± 0.13	19.44 ± 0.39	22.96 ± 0.26	-	S(2)	st
8	2.40 ± 0.18	18.81 ± 0.83	21.12 ± 0.65	-	-	t[f]
9	2.31 ± 0.01	18.39 ± 0.41	20.79 ± 0.41	-	-	t
10	2.02 ± 0.03	14.99 ± 0.32	17.01 ± 0.32	-	L(2)	t
11	1.94 ± 0.11	15.75 ± 0.26	17.69 ± 0.15	-	L(2)	t
12	1.44 ± 0.06	16.94 ± 0.68	18.38 ± 0.62	-	-	t
Total	55.96	205.96	261.92			2 m + 5st + 5 t

[a]Mean ± standard deviation, [b]short arm, [c]long arm, [d]metacentric, [e]subtelocentric, [f]telocentric.

morphological characteristics among different populations might be due to differences in habitats, and this phenotypic plasticity facilitates any plant to alter its growing pattern as it comes under different stresses (Guo et al. 2007; Jugran et al. 2013).

As expected, *L. distichum* exhibited considerable levels of phenotypic and cytogenetic variations. It is important to note that *L. distichum* occurred in temperate forests at mid to high altitudes on Korean Peninsula, and it suggests that during the glacial period, this species survived in mid elevations, where it may have found some proper sites for its survival (Chung et al. 2014). The pattern of diversity for this species proposes the survival of multiple refugia in natural habitats, which would have supported plant species to sustain its population and a high rate of recurrent gene flow (Chung and Chung 2014). In a previous study, it was found that habitat had a significant interaction with morphological variations in *Lilium* that suggests that ecological diversification contribute to phenotypic variability (Lim et al. 2014). Geographical variation in morphological expressions revealed various survival strategies that had long been recognized as enabling the plants to acclimatize in changing habitats, and it also specifies the evolutionary pattern of plant population (Park et al. 2014; Du et al. 2014).

This report previously mentioned that naturally grown lilies of South Korea were superior breeding materials based on its height, flower shape, flower color, and size. However, the biggest problem involved fragrance, with most of the naturally grown lilies emitting an unpleasant smell when its lipids undergo decomposition (Jeong et al. 1991). The fragrance of the *L. distichum* has been associated with various strengths and weaknesses. In this study, the flowers of *L. distichum* contained sweet and pleasant smell. This was suspected to be a mutant that may have emerged after its continuous propagation, which suggests that the unpleasant fragrance of naturally growing lilies could be eradicated by selective breeding. The Kochang lily generally produces an umbel inflorescence (Jeong and Kim 1991). However, a higher flower count increases its tendency to produce racemose inflorescence.

Previous studies have shown that the predominant blossoming direction was side facing, but in this study, the blossoming direction of the *L. distichum* was not only to the side but also to side-upward direction. Flower shape and blossoming direction are often used as major criteria in classifying lilies. Irregular distribution pattern of petal around the face of flowers results into different forms and shape of *L. distichum* flowers, and three flower shapes were observed in all habitats under study, with the petal insertion type as the predominant form.

Among the naturally growing lilies in the Martagon section, only *Lilium medeoloides*, the Korean wheel lily, and the *L. distichum* have been reported to have nodes in its scales (Lighty 1968). However, the *L. distichum* are the only ones with two nodes in its scales. All scales have very weak cohesion; scales were easily separated during the investigation process, which has also been previously described by Jeong et al. (1991). The weak bulb adhesion of the *L. distichum* was probably adapted through evolution.

It has been previously mentioned that the *L. distichum* thrives in sites 800 m above sea level and in the forest near the summit of the mountain, 1,000 m above sea level (Jeong et al. 1991). It has been previously shown that the natural habitat of the *L. distichum* in Mount Seorak is between 900 and 1,700 m above sea level (Lighty 1969). Previous reports have shown that the

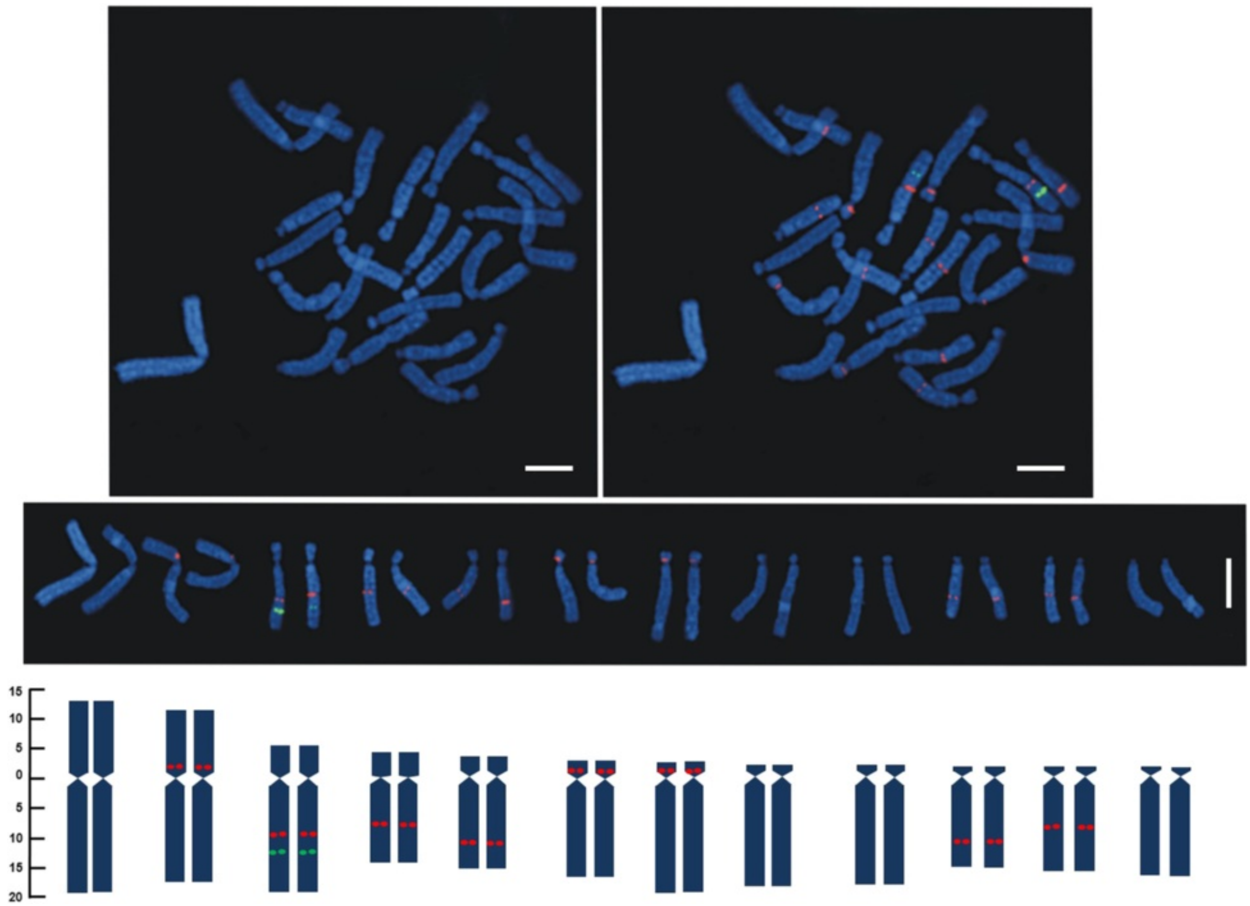

Figure 6 Fluorescence *in situ* hybridization of 5S and 45S rDNA on mitotic metaphase chromosomes of *L. distichum*. Green and red colors indicate the position of the 5S rDNA and 45SrDNA, respectively. Size bars = 10 μm.

natural habitat of the *L. distichum* included the river valley, the side of a hill, slope of a mountain, and shady areas of the forest, where the soil is usually covered with humus (Baranova 1969). Lily growth and development is generally favorable in damp and shady silt loam (Wu et al. 2006). The environment of the surveyed sites and the environmental characteristics mentioned in previous reports were similar to our findings. The average forest soil of South Korea consisted of 37.3% sand, 44.8% silt, and 17.9% clay (Jeong et al. 2002). Sand and clay were below South Korea's average, but the silt composition was approximately 10% higher. When the soil samples of Mount Jiri and Mount Seorak were compared with the other three regions, the sand ratio was relatively higher. This is assumed to have a correlation with earlier morphological characteristics since plants found in Mount Jiri and Mount Seorak showed relatively lower growth and development.

In terms of the xylophytes, most were distributed in the alpine regions halfway above the mountains. *Magnolia*, *Q. mongolica*, *B. ermanii*, and *S. borealis* occurred >1,000 m above sea level and at the foot of a mountain, shady areas inside the mountain forest, or in an area with high humidity. These results suggest that the natural habitat of *L. distichum* are grasslands and gaps of *Q. mongolica* dominated deciduous forests having *Acer*, *Tripterygium*, *Lychnis*, etc. taxa, which can thus be used as indicator plants of the *L. distichum* (Chung et al. 2014).

FISH analysis in *L. distichum* revealed the positions of rDNA sequences on *Lilium* chromosomes. This technique assists in the identification of individual chromosome and depicting molecular understanding of *Lilium* genome (Lee et al. 2014). The chromosome nomenclature of the genus *Lilium* has been settled by Stewart (1947) who classified 12 chromosomes in decreasing order of short arm length. The identification of certain chromosomes based on morphological characteristics is important. Besides the short arm length, long arm length is also an important part for the discrimination of chromosome number without any identification marker. The ribosomal RNA genes (45S rDNA and 5S loci) consist of tandem repeats, which proved to be efficient markers for *in situ* hybridization because they have the ability to conserve the DNA sequences present in high copy numbers, permitting the application of cloned heterologous probes (Lim et al. 2005; Weiss-Schneeweiss et al. 2007; Hwang et al. 2015). Present FISH results showed considerable variations in the size, number, and position of 45S rDNA and 5S loci in *L. distichum* that cater effective molecular markers to identify karyotype structure variations associated with plant evolution and speciation (Hwang et al. 2011).

Conclusions

This research provides comprehensive morphological, ecological, and cytogenetic information regarding rare species *L. distichum* which can be helpful for its successful management, conservation, and preservation. The significant phenotypic differences and substantial genetic variations among populations in different habitats suggest preserving the maximum genetic diversity of *L. distichum* through *in situ* protection of plant population and their natural habitats, by providing natural resources. Present results also provide insights about organization and physical location of 5S rDNA and 45S rDNA gene loci and their multiple copies which can be used for constructing chromosome physical maps and, in turn, provide a valuable method for genome identification and open new breeding avenues for *Lilium* cultivar improvement.

Abbreviations

CEC: cation exchange capacity; EC: electrical conductivity; FISH: fluorescence *in situ* hybridization; LSI: leaf shape index; rDNA: ribosomal DNA; RHS: Royal Horticultural Society; VL: verticillate leaf.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LKB and KCK designed the experiment together. YA wrote and revised the manuscript, SCM and HYJ conducted the FISH analysis, YA and LKB analyzed and interpreted the data, and KYI and LKB made the survey of the habitats of *Lilium*. All authors read and approved the manuscript.

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