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ORIGINAL RESEARCH

Physical Mapping of rRNA Gene Loci and Inter-specific Relationships in Wild *Lilium* Distributed in Korea

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Abstract Molecular cytogenetic analyses using fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) were carried out to elucidate inter-specific relationships among wild *Lilium* species distributed in Korea. FISH revealed four to eight 45S rRNA gene loci, which are located on chromosomes 1–7, 10, and 11 among the different species. In contrast, the 5S rRNA gene locus was conserved on the long arm of chromosome 3, occasionally with two adjacent sites on the same chromosome arm in a few species. The 5S rDNA site was located adjacent to the 45S rDNA site in only three species, *Lilium distichum*, *Lilium hansonii*, and *Lilium tsingtauense*. GISH analysis using genomic DNA probes detected strong hybridization of genomes between diploid and triploid *Lilium lancifolium* species, demonstrating that triploid plants were derived from diploid *L. lancifolium* and not from *Lilium maximowiczii*. Phylogenetic analysis of the ITS and NTS sequences supported the cytogenetic data as well as Comber's classification of the genus *Lilium*.

Keywords FISH/GISH · ITS/NTS · *Lilium* species · rRNA gene

Introduction

The genus *Lilium*, which belongs to the family Liliaceae, is comprised of approximately 100 species and is widely distributed across temperate regions of Asia, Europe, and North America (Woodcock and Stearn 1950; Synge 1980). The relatively mild climate of East Asia is highly favorable for lily plants where approximately 23 species are distributed and 11 of these species are native to Korea (Kim 1996).

All the species of the genus *Lilium* are diploid ($2n=2x=24$) (Stewart 1947) except for *Lilium lancifolium* for which triploids ($2n=3x=36$) occur as well (Noda 1986). Chromosome morphology for a majority of *Lilium* species has been determined (Stewart 1947). In addition, cytological investigation by C-banding has been employed in many different *Lilium* species (Holm 1976; Kongsuwan and Smyth 1977; Song 1987; Smyth et al. 1989). Karyotype analysis of Korean wild *Lilium* has also been done by C-banding (Son and Song 1978; Son 1982; Song 1987). C-banding has been widely used for chromosome analysis since the 1970s (Gill and Kimber 1974; Vosa 1976). However, C-banding identifies only constitutive heterochromatin which provides insufficient cytogenetic data for complete species identification. Also, in *Lilium* C-band patterns change rapidly, hence proving to be inadequate in identifying closely related species (Smyth et al. 1989). Moreover, *Lilium* chromosomes are relatively large (Stewart 1947) and it is difficult to represent genomes of this size with C-banded DNA (Smyth et al. 1989). Recently, fluorescence *in situ* hybridization (FISH) has identified both 45S and 5S rRNA genes on the chromosomes for detecting and analyzing genome organization (Jiang and Gill 2006). The rDNAs (45S and 5S), which are repeated sequences in the genome (Lapitan 1992; Sastri et al. 1992), are commonly used for the physical mapping of plant chromosomes due to their universal occurrence and redundancy (Hasterok et al. 2001). The application of FISH to plant chromosomes using rDNAs began in the late 1980s and has become a

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powerful tool for localizing specific DNA sequences on plant chromosomes (Schwarzacher et al. 1989; Mukai et al. 1993). Simultaneous FISH of 45S and 5S rDNAs has been useful in various plants including *Hordeum* (Taketa et al. 1999), *Brassica* (Snowdon et al. 2000; Hasterok et al. 2001), *Cucumis* (Koo et al. 2002), *Nicotiana* (Lim et al. 2005), and *Tulipa* (Mizuochi et al. 2007). Genomic *in situ* hybridization (GISH), which uses genomic DNA as a probe, is an additional chromosome mapping technique for detecting parental genomes in allopolyploids and hybrids (Lim et al. 2003a; Zhou et al. 2008; Choi et al. 2008, 2009). It was proposed that triploid *L. lancifolium* may have originated from diploid *L. lancifolium* or *Lilium maximowiczii*, as these three species are very similar morphologically (Noda 1986). To date, only a few molecular cytogenetic studies have been reported in *Lilium* interspecific hybrids (Karlov et al. 1999; Lim et al. 2000, 2001; Marasek et al. 2004) and in European mountain *Lilium* (Yakovlev et al. 2003). However, to our knowledge, no molecular cytogenetic studies have been performed in wild *Lilium* plants distributed in Korea.

The 45S rRNA genes are arranged in tandemly repeated clusters within the nucleolar organizer regions (NORs), including the coding regions for the three rRNAs (18S, 5.8S, and 26S) and two internal transcribed spacers (ITS) that flank the 5.8S rRNA gene (Gründler et al. 1991). The 5S rRNA genes, which are located outside the NORs, are also arranged in highly repeated tandem arrays that consist of a 120 bp coding region flanked by non-transcribed spacers (NTS) (Sastri et al. 1992; Danna et al. 1996). The spacer sequences of both rRNA genes show very high sequence heterogeneity among different species and these can be used potentially for estimating the phylogenetic relationships of species. ITS sequence variability was found to be useful for phylogenic reconstruction in *Lilium* (Dubouzet and Shinoda 1999a, 1999b; Nishikawa et al. 1999, 2001).

In the present study we have conducted molecular cytogenetic analyses using FISH and GISH to detect variability in the rRNA gene loci and to analyze the interspecific relationships among the species. Additionally, we attempted to elucidate the phylogenetic relationships between *Lilium* species distributed in Korea based on their ITS and NTS sequences.

Materials and Methods

Plant Materials

Plants of *Lilium* species, *L. lancifolium* Thunberg diploid (2*x*), *L. lancifolium* Thunberg triploid (3*x*), *L. maximowiczii* Regel, *Lilium amabile* Palibin, *Lilium callosum* Siebold et Zuccarini, *Lilium cernuum* Komarov, *Lilium concolor* Salisbury var. *partheneion* Baker, *L. concolor* var. *buschianum* Baker, *Lilium dauricum* Ker-Gawl, *Lilium distichum* Nakai ex Kamibayashi, *Lilium hansonii* Leichtlinm, and *Lilium tsingtauense* Gilg, were obtained from Lily Experimental Station (Taean, Chungnam province), Hantaek Botanical Garden (Kyunggi province), Breeding Institute of Korean Native Plants (Wonju, Kangwon province), Yeongwol (Kangwon province) and Ockcheon (Chungbuk province) of Korea and transplanted to the plant growth facility in Chungnam National University. For *L. concolor*, two taxa, *L. concolor* var. *partheneion* and *L. concolor* var. *buschianum*, were used in this study.

Chromosome Preparation

Root tips from pot-grown plants were pretreated with 0.1% colchicine solution for 5 h at room temperature and fixed in ethanol : glacial acetic acid (3:1, *v*/*v*) for 24 h. Root tips were thoroughly washed and then macerated using an enzyme cocktail (2% cellulase, 1.5% macerozyme, and 1% pectolyase in 1 mM EDTA; pH 4.2) at 37°C for 1 h, followed by rinsing in distilled water. Root tips were tapped with a fine forceps in a few drops of fresh fixative and air-dried.

Probe Preparation

The 45S rDNA probe for FISH was directly labeled with biotin-16-dUTP using the specific 17S rDNA primer set (5′-TACCTGGTTGATCCTGCCAG-3′, 5′-TTGTCAC TACCTCCCCGTGT-3′) by PCR of diploid *L. lancifolium* genomic DNA (Takaiwa et al. 1984). The PCR reaction mixture (25 μl) was composed of 5 pM of each primer set, 10 ng of template DNA, 2.5 units of Ex *Taq* polymerase (Takara, Japan), 0.2 mM of dNTPs, and 0.2 mM of biotin-16-dUTP (Roche, Germany). The 5S rDNA probe was labeled via nick translation of the diploid *L. lancifolium* plasmid DNA (PCR-cloned with the specific 5S rDNA primer set, 5′-GGATCCCATCAGAACTCCGAAG-3′ and 5′-GGTGCTTTAGTGCTGGTATG-3′) with digoxigenin-11-dUTP (Fukui et al. 1994). Total genomic DNA of diploid *L. lancifolium* and *L. maximowiczii* was used as probe DNA in GISH experiments to triploid *L. lancifolium*. The GISH probe was labeled by Biotin Nick Translation Mix (Roche, Germany) according to the manufacturer's instructions.

In Situ Hybridization

FISH and GISH were performed with a modification of the technique described by Choi et al. (2009). Chromosomal DNA was denatured on slides in 70% formamide at 65°C for 90 sec and then dehydrated using an ethanol gradient (70%, 95%, and 100%) at −20°C for 3 min each. The probe

mixture containing 50% formamide (*v*/*v*), 10% dextran sulfate (*w*/*v*), 5 ng/μl salmon sperm DNA and 500 ng/ml of each probe was denatured at 99°C for 15 min and immediately chilled on ice for 5 min; 25 μl of the probe mixture was applied to the denatured chromosomal DNA and covered with a glass cover slip. Slides were then placed in a humid chamber at 37°C for 18 h. Each probe was detected with avidin-FITC (Vector Lab, USA) and anti-digoxigenin rhodamine (Roche, Germany). The preparations were counterstained with Vectashield (Vector Lab, USA) containing 1.5 μg/μl 4′, 6-diamidino-2-phenylindole (DAPI). All images were acquired using a Leica Epi-fluorescence microscope equipped with a FITC-DAPI two-way or FITC-rhodamine-DAPI three-way filter set (Leica, German) and processed with a cooled CCD camera (CoolSNAP, Photometrics, USA) and the Meta Imaging Series™ 4.6 software.

Cloning and Sequencing of ITS and NTS

Genomic DNA was extracted from young leaves using the QIAGEN DNeasy Plant Mini Kit (Qiagen, USA) from all species. The ITS1-5.8S-ITS2 region of 45S rDNA was amplified by PCR using the primer set 5′-CACACCGCCCGTCGCTCCTACCGA-3′ and 5′-ACTCGCCGTTACTAGGGGAA-3′ (Ngan et al. 1999). The NTS and coding region of the 5S rDNA was also amplified by PCR using the same primer set and PCR components as described above. The 45S and 5S rDNA fragments of each species were isolated using a gel extraction kit (Cosmo Genetech, Korea), ligated into pGEM-T-Easy vector (Promega, USA), and transformed into *Escherichia coli* DH5α cells (Sambrook et al. 1989). Sequence data was analyzed using BLAST (NCBI, USA).

Phylogenetic Analysis

Phylogenetic analysis was performed based on ITS and NTS sequences according to the maximum parsimony tree method using MEGA software ver. 4.1 (http://www.megasoftware.net).

Results

FISH

FISH was used to analyze the variability of rRNA gene loci on the somatic metaphase chromosomes of *Lilium* species (Figs. 1 and 2; Table 1). FISH results are presented in Fig. 1, where each row represents chromosomes from individual metaphases. Diploid *L. lancifolium* had five 45S rRNA gene loci, where each locus was detected on the short arm of chromosomes 1, 2, and 7 and on the long arm of chromosomes 6 and 11 (Figs. 1a and 2). Triploid *L. lancifolium* had also five 45S rRNA gene loci on the same positions as in the chromosomes of diploid *L. lancifolium* (Fig. 1b and 2). *L. maximowiczii* exhibited the same FISH pattern of 45S rRNA gene loci as observed in the *L. lancifolium*, but one of the 45S rRNA gene locus was eliminated in the homologous pair of chromosome 6 (Figs. 1c and 2). In *L. amabile* FISH revealed 45S rDNA sites on the short arm of chromosomes 1 and 7 and on the long arm of chromosome 6 (Figs. 1d and 2). In *L. callosum, L. concolor* var. *partheneion* and *L. concolor* var. *buschianum* the 45S rRNA gene loci were detected on the same chromosomes as in the *L. lancifolium* (Figs. 1e, g, h and 2). *L. cernuum* had the same 45S rRNA gene loci on chromosomes 1, 2, 7, and 11 as observed in the three species mentioned above, but a different 45S rRNA gene locus on chromosome 6, where it was observed at the pericentromeric region of the short arm (Figs. 1f and 2). The 45S rDNA sites of *L. dauricum* were detected on the same loci of chromosomes 1, 2, 6, and 7 as seen in *L. callosum* (Figs. 1i and 2). Three species, *L. distichum, L. hansonii* and *L. tsingtauense* exhibited co-localization of both 45S rRNA and 5S rRNA genes on chromosome 3, where the 45S rDNA hybridized to the long arm proximal from the 5S rDNA site (Figs. 1j–l and 2). The 45S rRNA gene loci of the three species were detected on the long arm of chromosomes 3, 4, 5, 10, and/or 11 at the intercalary region. In addition *L. hansonii* had 45S rDNA sites on the long arm of chromosome 1 and on the short arm of chromosomes 2 and 7 at the pericentromeric region (Figs. 1k and 2). Only a single locus was identified on the homologue of chromosome 7 in this species. *L. tsingtauense* had a 45S rRNA gene locus on chromosome 10 instead of chromosome 11 as observed in the other two species (Figs. 1l and 2).

The FISH results revealed that the chromosomal location of the 5S RNA gene was conserved on chromosome 3 for the species examined. One major 5S rRNA gene locus with intense signal was most commonly detected at the interstitial region on the long arm of chromosome 3 (Figs. 1a–l and 2). Two closely located 5S rRNA gene loci were observed on the long arm of chromosome 3 in three species, *L. lancifolium* (2*x*, 3*x*), *L. maximowiczii* and *L. amabile*. Two adjacent sites of 5S rDNA were consistent in *L. lancifolium* and *L. maximowiczii* (Figs. 1a–c and 2), but in the other species these sites were found to be variable on homologue pair of chromosme 3.

The number of 45S rRNA gene loci differed significantly between the species evaluated (Table 1). The lowest number of 45S rRNA gene loci observed in *L. amabile* was three (Fig. 1d; Table 1), while the highest number of loci, eight, were found in *L. hansonii* (Fig. 1k; Table 1). However, the most commonly observed number of 45S rRNA gene loci was four (*L. dauricum, L. distichum* and *L. tsingtauense*) (Fig. 1i, j, l; Table 1) and five (diploid and

triploid *L. lancifolium, L. maximowiczii, L. callosum*, *L. cernuum, L. concolor* var. *partheneion,* and *L. concolor* var. *buschianum*) (Fig. 1a–c and e–h; Table 1).

GISH

GISH was performed to elucidate cytogenetic relationships among the species diploid *L. lancifolium*, triploid *L. lancifolium* and *L. maximowiczii*. The total genomic DNA probe of diploid *L. lancifolium* labeled with biotin-16-dUTP strongly hybridized with the chromosomes of triploid *L. lancifolium* (Fig. 3a, b). On the other hand, when the genomic DNA probe of *L. maximowiczii* labeled with biotin-16-dUTP was applied to the chromosomes of triploid *L. lancifolium*, no distinct hybridization was observed on the chromosomes (Fig. 3c, d).

Phylogenetic Analysis

ITS1 and ITS2 with intervening 5.8S coding regions of the 45S rRNA gene, and NTS with coding regions of the 5S rRNA gene were sequenced from the *Lilium* species

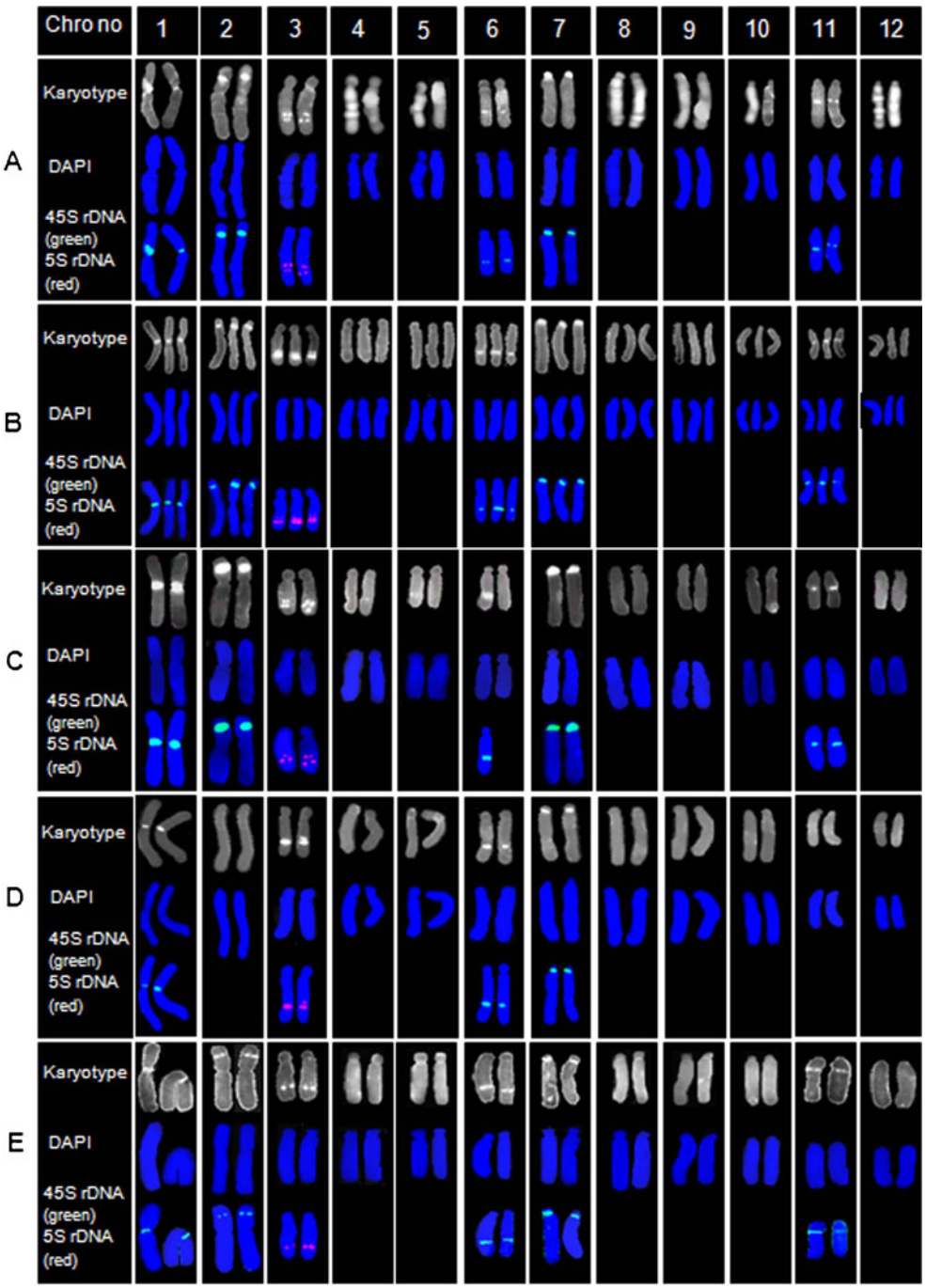
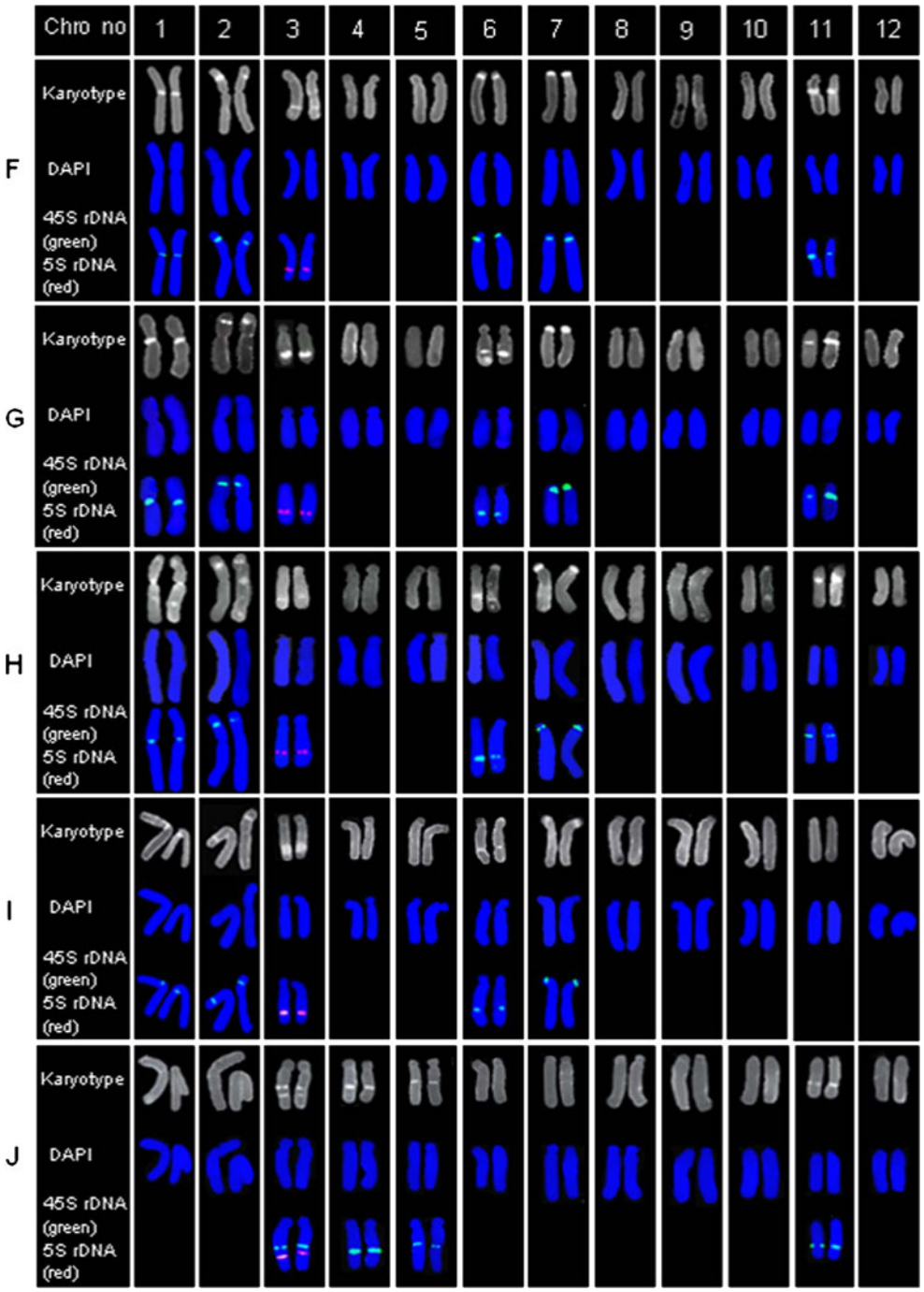


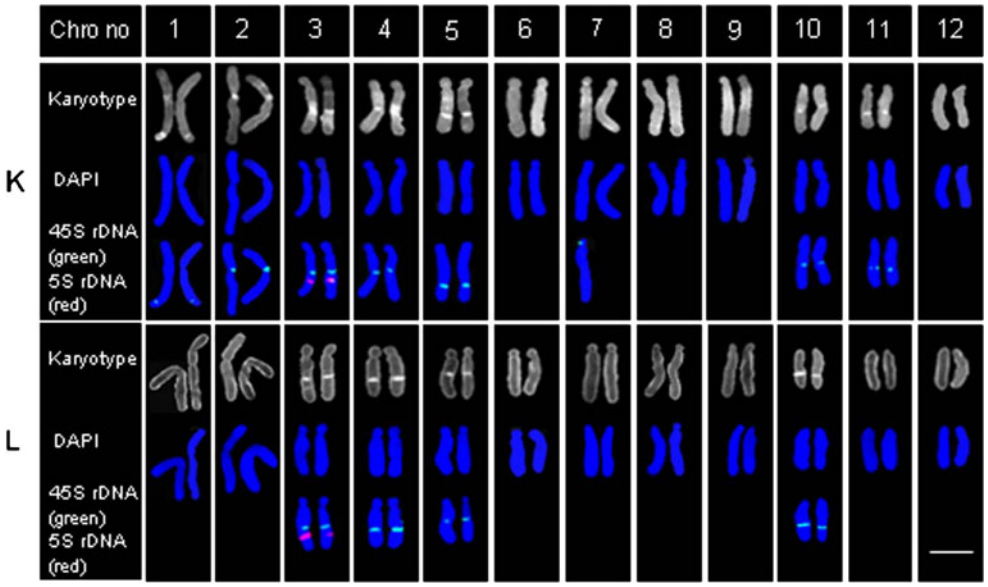
Fig. 1 FISH karyotype of *Lilium* species using 45S and 5S rDNA probes: *Lilium lancifolium* 2*x* (**a**), *L. lancifolium* 3*x* (**b**), *Lilium maximowiczii* (**c**), *Lilium amabile* (**d**), *Lilium callosum* (**e**), *Lilium cernuum* (**f**), *Lilium concolor* var. *partheneion* (**g**), *L. concolor* var. *buschianum* (**h**), *Lilium dauricum* (**i**), *Lilium distichum* (**j**), *Lilium hansonii* (**k**), and *Lilium tsingtauense* (**l**). For the species, each row represents metaphase chromosomes (*black* and *white*), DAPI-stained chromosomes (*blue*) and rDNA FISH (45S, *green* and 5S, *red*), respectively. *Bar*, 5 μm

Fig. 1 (continued)



studied (data not shown). Nucleotide sequences of the ITS region were divergent among species: 2–13 bp in ITS1 and 4–22 bp in ITS2. The NTS regions were also variable in length, ranging from 349 bp to 519 bp except for each 120 bp coding region (data not shown). A maximum parsimony tree was constructed based on ITS and NTS sequences of all species studied (Fig. 4). For the phylogenetic analysis, the values of consistency index and retention index were 0.841 and 0.851, respectively, indicating low homoplasy and thereby high phylogenetic utility of ITS and NTS sequences. The phylogenetic tree showed that the species were divided in two major groups (clade I and clade II), where clade I included a majority of the species and clade II included only three species, *L. distichum, L. hansonii* and *L. tsingtauense*. These two groups were supported by bootstrap values of 84% and 58%, respectively. In clade I, the closest relationship was observed in *L. amabile*, *L. callosum* and *L. concolor* var. *partheneion* with a 100% bootstrap value. It was found that diploid and triploid *L. lancifolium* were closely

Fig. 1 (continued)



related with 82% bootstrap value and *L. maximowiczii* was distantly related with *L. lancifolium*, although these three species belong to the same clade. In clade II, *L. distichum* showed the closest similarity with *L. tsingtauense* supported by a 70% bootstrap value, than with the other species *L. hansonii*.

Discussion

FISH using 45S and 5S rDNA probes has been performed for the first time in our knowledge, to reveal cytogenetic relationships in Korean native *Lilium* species. FISH analysis showed variation in the number and position of rRNA genes between the species that have been evaluated (Figs. 1 and 2; Table 1). The number of 45S rRNA gene loci was variable, ranging from three (*L. amabile*) to eight (*L. hansonii*), whereas the 5S rRNA gene was located on a single locus. A polymorphic 5S rRNA gene locus that had two adjacent sites on the long arm of chromosome 3 was consistent in *L. lancifolium* (2*x*, 3*x*) and *L. maximowiczii* (Figs. 1a–c and 2), and found variable on single of homologous pair of the same chromosome in *L. amabile*. Moreover, many variable 45S rDNA sites were shown (pericentromeric, interstitial and subtelomeric regions of both short and long arms) in each species (Fig. 2; Table 1).

For diploid *L. lancifolium*, five 45S rRNA gene loci were detected on chromosomes 1, 2, 6, 7, and 11. FISH of 45S rDNA in this species did not differ from previous C-banding results (Son 1982; Song 1992), where the NORs of secondary constrictions (SC) were reported on the same locations of chromosomes 1, 2, 7, and 11. Moreover, diploid *L. lancifolium* had an additional 45S rDNA site on the long arm of chromosome 6. Triploid *L. lancifolium* had the same 45S rDNA loci on chromosomes 1, 2, 6, 7, and 11, as in diploid *L. lancifolium*. Previous cytological studies (Son 1982; Noda 1986; Song 1997) showed four NOR bearing chromosomes with a variable number of nucleolar constrictions on each set of four chromosomes from different plants and localities in this species. Noda (1986) reported 18 nucleolar types, distinguished by differentiation in the maximal number of nucleolar constrictions on four chromosomes, of which the 3–2–2–1 nucleolar type was most common in triploid *L. lancifolium* distributed in Japan, Korea and North-East China. Song (1997) also reported two (3–2–3–0, 2–2–3–0) and seven nucleolar types (1–2–2–1 was most common) in triploid and diploid *L. lancifolium*, respectively, from the Jejudo population in Korea.

In *L. maximowiczii,* FISH revealed 45S rRNA gene loci on chromosomes 1, 2, 7, 11, and a homologue of chromosome 6. However, only two loci on chromosomes 2 and 7 were described in this species by Son (1982). The deletion of NOR from the homologous pair of chromosome 6 was also observed in *L. maximowiczii* distributed in Japan (Song and Seo 1988). The number and position of 45S rRNA gene loci in *L. amabile, L. callosum* and *L. concolor* var. *partheneion* was consistent with previous C-banding patterns (Son 1981; Song 1991). The 45S rRNA gene loci of *L. cernuum* were different from its C-banding pattern obtained by Son and Song (1978). They reported two SCs on the short arm of chromosomes 2 and 5, whereas FISH revealed 45S rRNA gene loci on the short arm of chromosomes 1, 2, 6 and 7 and on the long arm of chromosome 11.

The localization of 45S rDNA sites in *L. dauricum* corresponded to the SCs described by Stewart (1947). *L. distichum* had four 45S rRNA gene loci on chromosomes

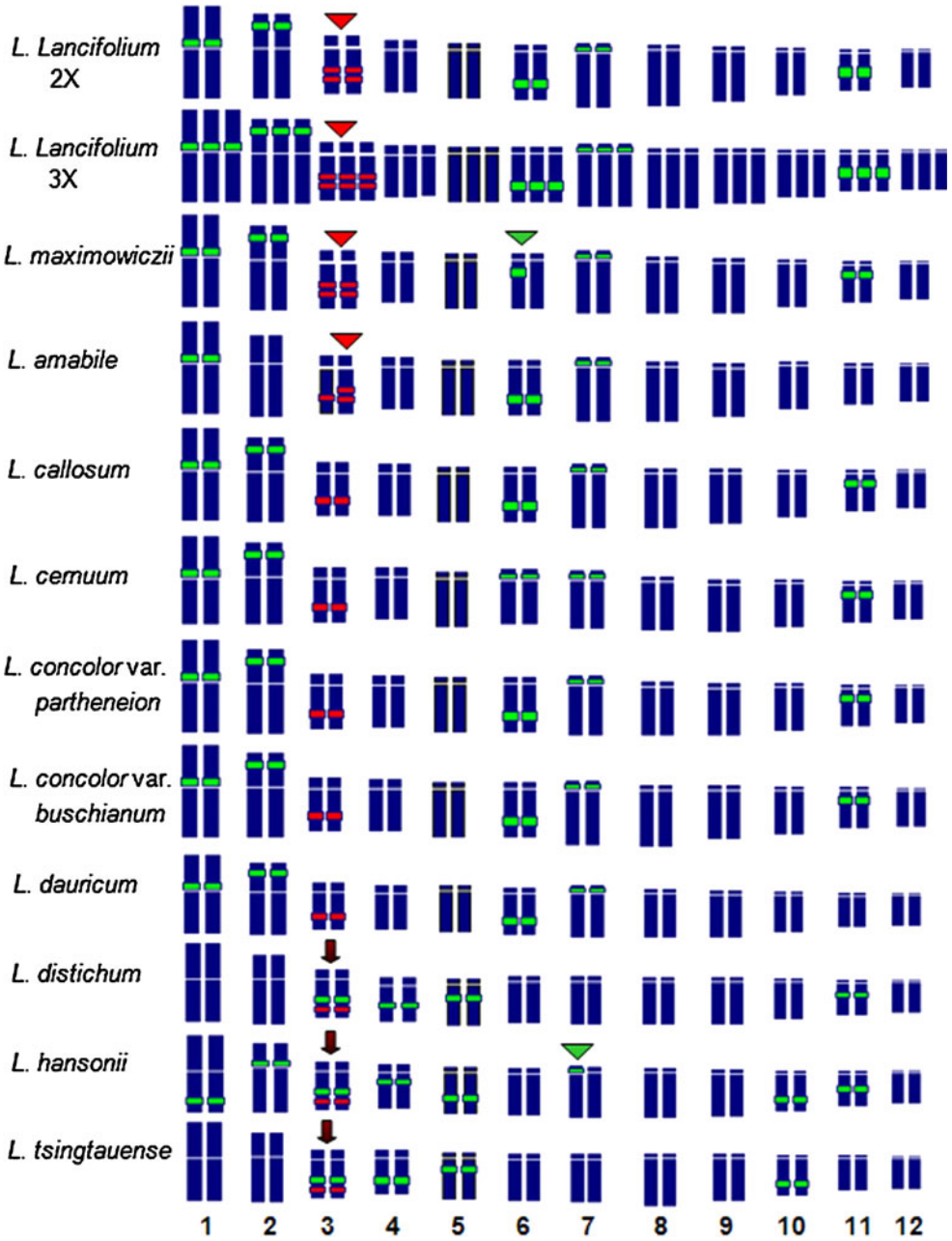


Fig. 2 Ideograms showing the distribution of rDNAs (45S, *green* and 5S, *red*) on the chromosomes in *Lilium* species. The green triangle indicates deletion of a 45S rRNA gene locus in one of the homologous pair. The red triangle indicates the position of two adjacent 5S rDNA. The arrowheads indicate the co-localization of 45S and 5S rDNA sites on chromosome 3 in *Lilium distichum, Lilium hansonii*, and *Lilium tsingtauense*

3, 4, 5, and 11. Son (1982) detected the NORs at the same positions on chromosomes 3, 4, 5, 7, and 11. In *L. hansonii*, the 45S rRNA gene loci on chromosomes 2, 3, 4, 5, 7, 10, and 11 were not different from the results detected by Smyth et al. (1989) and Noda (1973). In addition, the 45S rDNA site was identified on the long arm of chromosome 1 in this species, and only a single signal was found on the homologue of chromosome 7. For *L. tsingtauense*, the 45S rRNA gene loci were consistent with its C-band pattern on chromosomes 3, 4, 5, and 10 (Son 1971).

In *Lilium* species almost all chromosomes, 1–7, 10, and 11 carried the 45S rRNA gene loci except chromosomes 8, 9, and 12, while chromosome 3 was the only pair with the 5S rRNA gene locus. This observation was in agreement with previous studies in European mountain *Lilium* (Yakovlev et al. 2003) and *Lilium henryi* (Marasek et al. 2004). In most of the species, 45S and 5S rDNA sites were physically separated and mapped independently on the chromosomes, except for the three species of *L. distichum*, *L. hansonii*, and *L. tsingtauense*, where both sites were co-localized on chromosome 3. The close proximity of 45S–5S rDNA sites showed that these three species are more closely related to each other than to the remaining species. The molecular phylogeny of *Lilium* species based on ITS and NTS sequences supported that *L. distichum*, *L.*

Table 1 The number and position of 45S and 5S rRNA gene loci on specific chromosomes in *Lilium* species

Species	Section[a]	45S rDNA site		5S rDNA site	
		No of locus	Positions on specific chromosome	No of locus	Positions on specific chromosome
Lilium lancifolium 2*x*	*Sinomartagon*	5	Chr 1 (p/PC), Chr 2 (p/ST), Chr 6 (p/PC), Chr 7 (p/PC), Chr 11 (q/INT)	1	Chr 3[b] (q/INT)
L. lancifolium 3*x*	*Sinomartagon*	5	Chr 1 (p/PC), Chr 2 (p/ST), Chr 6 (q/INT), Chr 7 (p/PC), Chr 11 (q/INT)	1	Chr 3[b] (q/INT)
Lilium maximowiczii	*Sinomartagon*	5	Chr 1 (p/PC), Chr 2 (p/ST), Chr 6[c] (q/INT), Chr 7 (p/PC), Chr 11 (q/INT)	2	Chr 3[b] (q/INT)
Lilium amabile	*Sinomartagon*	3	Chr 1 (p/PC), Chr 6 (q/INT), Chr 7 (p/PC)	1	Chr 3[b] (q/INT)
Lilium callosum	*Sinomartagon*	5	Chr 1 (p/PC), Chr 2 (p/ST), Chr 6 (q/INT), Chr 7 (p/PC), Chr 11 (q/P)	1	Chr 3 (q/INT)
Lilium cernuum	*Sinomartagon*	5	Chr 1 (p/PC), Chr 2 (p/ST), Chr 6 (p/PC), Chr 7 (p/PC), Chr 11 (q/P)	1	Chr 3 (q/INT))
Lilium concolor var. *partheneion*	*Sinomartagon*	5	Chr 1 (p/PC), Chr 2 (p/ST), Chr 6 (q/INT), Chr 7 (p/PC), Chr 11 (q/P)	1	Chr 3 (q/INT)
L. concolor var. *buschianum*	*Sinomartagon*	5	Chr 1 (p/PC), Chr 2 (p/ST), Chr 6 (q/INT), Chr 7 (p/PC), Chr 11 (q/P)	1	Chr 3 (q/INT)
Lilium dauricum	*Sinomartagon*	4	Chr 1 (p/PC), Chr 2 (p/ST), Chr 6 (q/INT), Chr 7 (p/PC)	1	Chr 3(q/INT)
Lilium distichum	*Martagon*	4	Chr 3 (q/INT), Chr 4 (q/INT), Chr 5 (q/INT), Chr 11 (q/P)	1	Chr 3[d] (q/INT)
Lilium hansonii	*Martagon*	8	Chr 1 (q/ST), Chr 2 (p/PC), Chr 3 (q/INT), Chr 4 (q/P), Chr 5 (q/INT), Chr 7[c] (p/PC) Chr 10 (q/INT), Chr 11 (q/INT)	1	Chr 3[d] (q/INT)
Lilium tsingtauense	*Martagon*	4	Chr 3 (q/INT), Chr 4 (q/INT), Chr 5 (q/P), Chr 10 (q/INT)	1	Chr 3[d] (q/INT)

Abbreviations: *p* short arm, *q* long arm, *PC* pericentromeric, *P* proximal, *INT* interstitial, *ST* subtelomeric

[a] The sections follow Comber's classification (Comber 1949) of the genus *Lilium*

[b] Additional 5S rRNA gene locus

[c] Deletion of a 45S rRNA gene locus in one of the homologous pair

[d] Co-localization of 45S and 5S rDNAs

hansonii, and *L. tsingtauense* are in closer proximity to each other, than other species which had 45S and 5S rDNA sites on different chromosome pairs.

FISH is generally used to localize rRNA gene loci and specific DNA sequences in the genome, while GISH can provide more information regarding the origin and divergence of genomes at the whole genome level. GISH analysis using genomic DNA from probable diploid progenitor species as probes provided important information of the genome evolution and divergence of allopolyploid species (Mukai et al. 1993; Jiang and Gill 1994, 2006). Noda (1986) proposed that the triploid *L. lancifolium* may either be the allotriploid produced by natural hybridization between a diploid form and another closely related *L. maximowiczii* which has occurred in the remote past, or the autotriploid directly originated from the diploid form through the production of unreduced gamets. Based on geographical distribution and habitat differentiation of diploid and triploid *L. lancifolium* in Korea, Kim et al. (2006) proposed that the possibility for allotriploids originated from the inter-specific hybridization between these diploid species may be hardly accepted yet, because the distribution pattern of *L. maximowiczii* has never been found sympatrically and parapatrically with the "diploid" *L. lancifolium.* In this study, GISH results revealed that the genomic DNA probe of diploid *L. lancifolium* showed strong hybridization signals with the triploid *L. lancifolium* chromosomes (Fig. 3a, b), whereas no GISH signals were observed between *L. maximowiczii* GISH probe and triploid *L. lancifolium* chromosomes (Fig. 3c, d). This data showed that the triploid *L. lancifolium* genome appeared to be closely related with the diploid *L. lancifolium* genome but not with the other genomes examined. The GISH result strongly agreed with the Kim's hypothesis, who described that, since no tetraploid *Lilium* species has been found under natural conditions so far, only diploid that can produced functional unreduced gametes could be the resource of triploid.

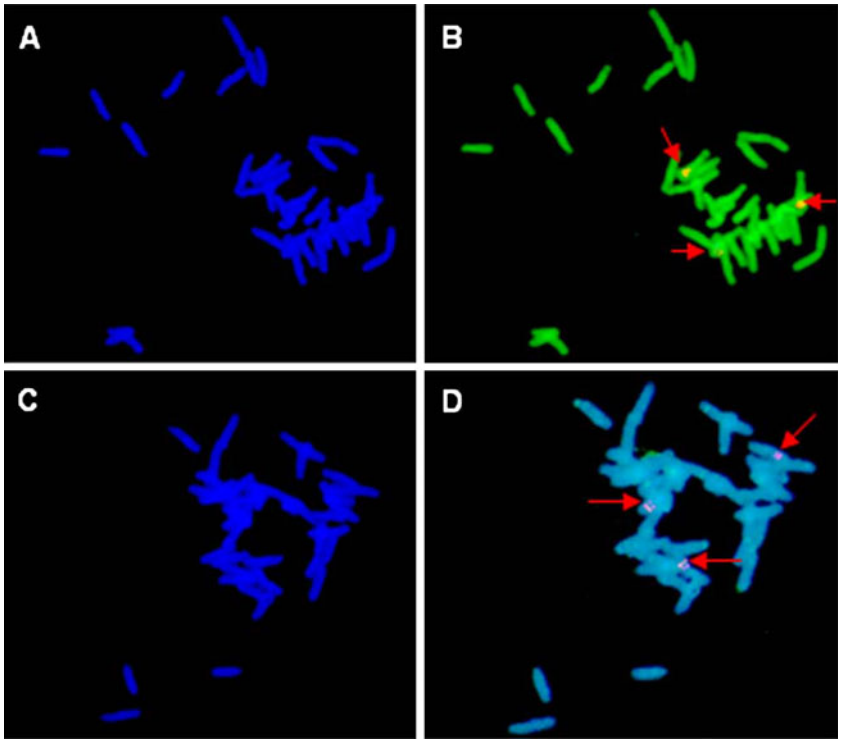


Fig. 3 GISH analyses of *Lilium lancifolium*. GISH of *L. lancifolium* 3*x* with total genomic DNA probe of *L. lancifolium* 2*x* (**a**–**b**): DAPI counter staining (*blue*; **a**), biotin-labeled genomic DNA probe of *L. lancifolium* 2*x* (*green*, strong hybridization signal was detected) and digoxigenin-labeled 5S rDNA probe (*red*; **b**). GISH of *L. lancifolium* 3*x* with total genomic DNA probe of *Lilium maximowiczii* (**c**–**d**): DAPI counter staining (*blue;* **c**), biotin-labeled genomic DNA probe of *L. maximowiczii* (*green*, no distinct hybridization signal was detected) and digoxigenin-labeled 5S rDNA probe (*red*; **d**). Bar, 5 μm

Previously GISH was successfully used in *Lilium* to identify the genome composition of triploid lily cultivars (Zhou et al. 2008), to determine hybrid status (Lim et al. 2000; Marasek et al. 2004) and to trace homologous recombination in backcrossed progenies (Karlov et al. 1999; Lim et al. 2000, 2003a, 2003b).

The phylogenetic approach by ITS and NTS sequences was consistent with the classification of the genus *Lilium* (Comber 1949). Our study showed that the rDNA sequences of *Lilium* species could be useful to unravel the genetic relationships between species. Clade I included most of the species which belonged to the Section *Sinomartagon*, and clade II included the species belonging to the Section *Martagon* (Fig. 4). There was no misplacement of any species between the Sections. The phylogenetic analysis also revealed that *L. lancifolium* (2*x*, 3*x*) grouped together, but not with *L. maximowiczii*. The phylogenetic relationships of these three species supported the current GISH results. In clade II, *L. distichum* showed closer relationships with *L. tsingtauense* than *L. hansonii*.

In conclusion, FISH and GISH have been effectively used for demonstrating the genomic organization of rRNA gene loci and analyzing cytogenetic relationships among *Lilium* species distributed in Korea. The phylogenetic relationships determined by ITS and NTS sequences were in agreement with FISH and GISH data in this study, as well as with previous classifications of the genus *Lilium*.

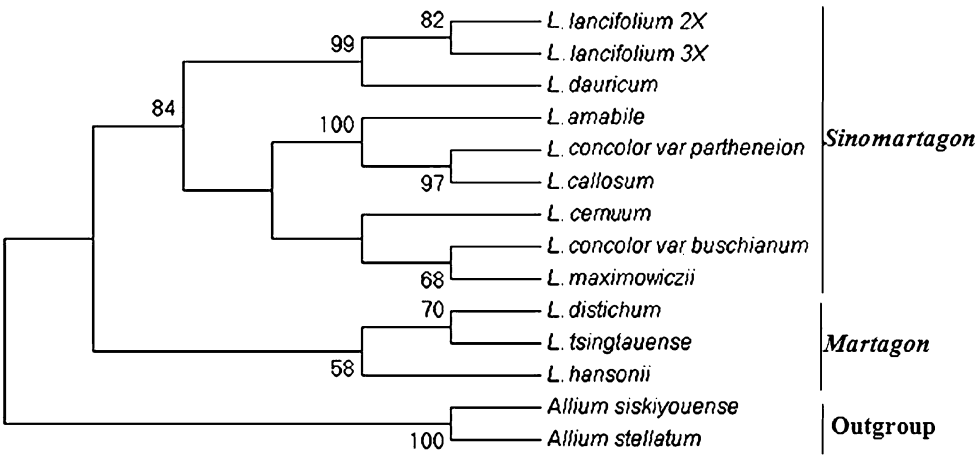


Fig. 4 Phylogenetic tree constructed from ITS and NTS sequences using maximum parsimony method. *Allium siskiyouense* and *Allium stellatum* used as the outgroup species. The numbers at the nodes represent bootstrap values (%) for a 1,000 replicate analysis

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