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RESEARCH ARTICLE

# Development of expressed sequence tag derived-simple sequence repeats in the genus *Lilium*

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**Abstract**
Although lily is the second largest flower crop in cutting flower commodity, only six simple sequence repeats SSRs have been reported. Thus, we developed expressed sequence tag derived-SSRs (EST-SSRs) for the *Lilium* genus. Among 2,235 unique ESTs, 754 ESTs contained SSR motifs, among which 165 ESTs were amenable to primer design. Among these 165 EST-SSRs, 131 EST-SSRs showed amplification in at least one *Lilium* species, and 76 EST-SSRs showed amplification in at least nine species. Of the 76 EST-SSRs, 47 showed amplification in all *Lilium* species analyzed. Using 10 breeding lines, we selected 19 EST-SSRs that had the highest number of alleles and polymorphism information content. The polymorphism information content values of these selected EST-SSRs ranged from 0.49 to 0.94 with an average of 0.76, which are higher than other plant species. The phylogenetic dendrogram derived from the amplification profiles of the 19 high polymorphic EST-SSRs was congruent with the genetic background of the 84 selected lily accessions and hybrids, which are available in commerce. Thus, the developed EST-SSRs will be very useful in germplasm management, genetic diversity analysis, cultivar finger printing, and molecular breeding in the lily.

**Keywords** lily; EST-SSR; Polymorphism content information; Cross-species SSR amplification

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## Introduction

Lily is one of the major flowering crops and is second to the rose in the cut flower market worldwide. Lilies are herbaceous perennials that form underground scaly bulbs that are used for asexual propagation in commerce. Breeders have produced hybrid varieties from crosses between a few *Lilium* species via conventional breeding and selections based on morphological characteristics such as flower colors and shapes.

The genus *Lilium* contains approximately 90 species that are widely distributed in temperate Asia, Europe, and North America (Synge, 1980; Asano, 1989). Early botanists classified lilies into six sections based on their morphological traits (Comber, 1949), which generally agreed with their chromosome morphology and heterochromatin distributions (Smyth et al., 1989). The morphological and cytological classifications were subsequently refined by molecular studies, such as randomly amplified polymorphic DNA (Yamagushi, 1995; Ikinci and Oberprieler, 2010), internal transcribed spacer regions of

rRNA genes (Nishikawa et al., 1999, 2001), and inter-simple sequence repeat (Yamagushi et al., 2002). However, molecular marker studies in lily are far behind other plant species, which might be due to the large genome sizes of lily species. For example, only three genetic linkage-mapping studies of lily have been reported to date. Abe et al. (2002) reported the genetic analysis of floral anthocyanin pigmentation traits in Asiatic hybrid lilies using randomly amplified polymorphic DNA and inter-simple sequence repeat. Van Heusden et al. (2002) used amplified fragment length polymorphism markers to map four quantitative trait loci (QTLs) for *Fusarium oxysporum* and a single locus of *LMoV* in an Asiatic backcross population. Shahin et al. (2011) analyzed QTLs for several ornamental traits and disease resistance with amplified fragment length polymorphism, DArT, and fine-map NBS profiling. However, these reported linkage analyses and QTLs for important agronomical traits lead to questions about the anchor markers for landing the loci on the particular chromosome.

The major advantage of molecular systems over classical systems in systematic and genetics is avoiding the incorrect phenotyping of homoplasious characters and environmental effects (Thomas, 1960; Bretting and Widrlechner, 1995; van Tienderen et al., 2002; Selkoe and Toonen, 2006). Among the many different molecular marker systems, the simple sequence repeat (SSR) system has many advantages over other systems, such as high polymorphism, abundance, high reproducibility, and high transferability (reviewed in Park et al., 2009). The repeating motifs are one to six nucleotides long and very abundant in eukaryotic genomes. SSR analysis uses polymerase chain reaction (PCR) for amplification of the repeat motif, so that the sequence information of the region flanking the repeat motif is required for primer design. Although DNA sequencing techniques have rapidly evolved to accommodate all organisms with large genome sequences, it is still necessary to sequence species with very large genomes, such as lilies, which have variable C-values ranging from 22 pg to 104 pg DNA with an average of 56.3 pg (1 pg ~ 965 million bp) (Peruzzi et al., 2009). Alternatively, mining SSR motifs in the genome database deposited in GenBank is amenable to many species. Among the different datasets in the genome database, sequences of mRNA or expressed sequence tags (ESTs) are being used to design EST-SSR primers. Numerous reports are available on the use of EST-SSR for genome studies in plant species (Park et al., 2009; references therein).

Molecular markers are highly required to distinguish varieties to protect breeders during the propagation of vegetative species like lily. Although SSR is the most reliable molecular marker system, only one report is available for SSRs in the genus *Lilium* (Horning et al., 2003), in which only six SSR loci were surveyed in a population of *Lilium philadelphicum*. Here, we report the sequences, characterization, and distribution of SSRs derived from an EST database in GenBank. In addition, cultivar identification is presented as an example of the practical application of mined EST-SSRs.

## Materials and Methods

### SSR mining

ESTs from four *Lilium* species and one hybrid lily were retrieved from National Center for Biotechnology Information (NCBI). Redundant sequences among the mined ESTs were removed per the protocol of Huang et al. (2010) at a cutoff of 90% identity. SSRs longer than 12 bp and a motif length 2~21 bp were isolated using the Pearl computer program (Temnyk et al., 2001). Primers were designed to produce amplicons ranging in length between 150~400 bp at melting temperatures between 53~65°C using the ARGOS software program (Kim, 2004).

### Plant materials and DNA extraction

*Lilium* species plant materials and accessions were provided by Dr. Dae-Kee Hong at the Kangwon Branch of Rural Development and Administration, Korea and from the *Lilium* gene bank at Kangwon National University. The *Lilium* species are listed in Supplemental Table 1. Genomic DNA was isolated from young leaves using the DNeasy Plant Maxi kit (Qiagen, USA) using the supplier's instructions. DNA quantity was adjusted to 50ng/μℓ by 0.8% agarose gel electrophoresis.

### PCR and electrophoresis

PCR was conducted in a 25-μℓ reaction mix containing 2.5

**Table 1.** EST sequence sources and SSRs (≥12 bp).

| *Lilium* species | Source ESTs from NCBI | | Non-redundant ESTs | |
|---|---|---|---|---|
| | ESTs (n) | Total length (bp) | ESTs (n) | Total length (bp) |
| *Lilium davidii* | 45 | 18,641 | 38 | 16,340 |
| *Lilium formosanum* | 1,324 | 385,734 | 743 | 223,432 |
| *Lilium longiflorum* | 991 | 591,281 | 842 | 510,039 |
| *Lilium regale* | 210 | 109,246 | 184 | 96,276 |
| *Lilium* hybrid division VII | 565 | 199,932 | 428 | 155,082 |
| Total | 3,135 | 1,304,834 | 2,235 | 1,001,169 |
| ESTs with SSRs | N/A | N/A | 754 | 13,835 |

**Table 2.** Number of repeat motifs in di-, tri-, and tetranucleotide and compound motifs.

| Motif length | Repeat number | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | 4 | 5 | 6 | 7 | 8 | 9 | 10 | >10 | |
| Di | - | 1 | 3 | 9 | 8 | 2 | - | 3 | 26 |
| Tri | 66 | 45 | 7 | 4 | 5 | 6 | 3 | 1 | 137 |
| Tetra | 1 | - | - | - | - | - | - | - | 1 |
| Compound | 1 | - | - | - | - | - | - | - | 1 |
| Total | 68 | 46 | 10 | 13 | 13 | 8 | 3 | 4 | 165 |

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 was as follows: 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and a final extension of 5 minutes at 72°C. The amplified products were electrophoresed either in a 6% denaturing polyacrylamide gel in a conventional PAGE system for 2 hrs at 1,800 V or in a LiCor 4300 automatic electrophoresis system for 4 hrs. The separated DNA fragments by conventional PAGE were visualized by silver staining (Promega, USA).

**Data analysis**

Alleles of each genotype were coded numerically from 1 to n. If the alleles of different genotypes were common, the codes were the same; otherwise, they were different. The polymorphism information content (PIC) was calculated per the equation, $PIC = 1- \Sigma Pi^2$, where p is the frequency of the $i^{th}$ allele (Shete et al., 2000). Phenetic relationships between genotypes were revealed by the use of dendrograms based on the unweighted pair-group method with arithmetic mean using the Numerical Taxonomy and Multivariate Analysis computer program (Roholf, 1989).

## Results and Discussion

**Sequence sources and SSR DNA mining**

On June 15, 2010, the number of ESTs of *Lilium* species registered at the NCBI was 3,135, which were reduced to 2,235 after removal of redundant clones (Table 1). Among the 2,235 ESTs, ESTs containing SSRs comprised 754 clones in which only 165 ESTs were amenable to primer design for SSR amplification ranging from 150 to 400 bp. Of the 165 SSRs, the species distribution was 23 from *Lilium formosanum*, 92 from *L. longiflorum*, 17 from *L. regale*, and 33 from *Lilium* hybrid division VII.

Trinucleotide (137, 80.3%) and dinucleotide (26, 15.8%) motifs comprised the greatest motif categories. The most frequent numbers of repeats were 7 and 8 in dinucleotide repeats and 4 and 5 in trinucleotide repeats, respectively (Table 2, Supplemental Table 2). Of the 26 dinucleotide motif SSRs, the CT/GA motif was the most frequent, followed by the AT/TA motif. Of the 137 trinucleotide repeats, four motifs, GGC/CCG (21/137, 15.3%), GGT/CCA (14/137, 10.2%), GCG/CGC (12/137, 8.7%), and GAG/CTC (10/137, 7.3%), appeared more than 10 times, suggesting that GC-rich repeats are more frequent than AT-rich SSR motifs (Supplemental Table 2).

Our results are comparable to those in other plants. Poncet et al. (2006) isolated 434 unique SSRs from 5,534 coffee ESTs, among which trinucleotides were most abundant (34.34%), followed by di- (25.75%) and hexanucleotides (22.04%) (Pocent et al., 2006). Liang et al. (2009) reported 881 EST-SSRs from 24,238 peanut (*Arachis hypogea* L.) ESTs, and 290 SSR primer pairs were successfully designed in which the frequency of trinucleotide repeats was 63.9%, followed by di- (32.7%) and tetranucleotides (1.7%); the four dominant motifs were AG/TC (27.7%), AAG/TTC (17.4%), AAT/TTA (11.9%), and ACT/TGA (7.26%). More recently, Zheng et al. (2010) isolated 2,810 EST-SSRs from 22,295 cDNA fragment sequences in *Epimedium sagittatum*, in which di- and trinucleotide repeat motifs comprised 55.2% and 30.4% of the identified motifs, respectively, and the dominant motif was AAG/TTC (23.6%), followed by AG/TC (19.3%), ACC/TGG (11.1%), AT/TA (7.5%), and AAC/TTG (5.9%). The frequency of SSRs containing ESTs in *Lilium* species in our analysis was 33.7% (754/2235), which was comparably higher than in coffee (7.8%, 434/5534), peanut (7.8%, 434/5534), and *E. sagittatum* (12.6%, 2810/22295). Additionally, the trinucleotide motif frequency in our analysis was higher than in these studies. In a classical study on SSRs, Wang et al. (1994) surveyed short DNA motifs in the European Molecular Biology Laboratory and GenBank databases and found that the mono-, di-, tri-, and tetranucleotide repeats were all present in non-coding regions, but 57% of the trinucleotide motifs were present in the coding regions. The high frequency of trinucleotide motifs in the coding region was attributed to the fact that other types of repeats were eliminated from the coding region because of frameshift mutations. Recently, Victoria et al. (2011) demonstrated by *in silico* comparative SSR analyses that dinucleotide motifs are most common in Chlorophyta, Bryophyta, and Lycophyta, but trinucleotide motifs are the most common in Magnoliophyta flowering plants; the most frequent repeat motifs also differed in each plant group. In our analysis, GC-rich trinucleotide motifs in *Lilium* were predominantly present, suggestive of high GC content in the genome of *Lilium* species, which was also reported for grass species (Morgante et al., 2002).

**SSR amplification and species relationships among *Lilium* species**

Cross-species amplification of EST-SSRs was assessed in 11

**Table 3.** Frequencies of self and non-self-amplification of EST-SSRs.

| | No. of Markers | Self amplification | Non-self amplification | Amplification failed |
|---|---|---|---|---|
| Longiflorum | 92 | 62 (67.39%) | 10 (10.87%) | 20 |
| Hybrid | 33 | 17 (51.51%) | 7 (21.21%) | 9 |
| Regale | 17 | 14 (82.35%) | 2 (11.76%) | 1 |
| Formosanum | 23 | 11 (47.83%) | 8 (34.78%) | 4 |
| Total | 165 | 104 (63.03%) | 27 (16.36%) | 34 |

different *Lilium* species and three cultivars of each Asiatic and Oriental hybrid. Of the 165 EST-SSRs, 131 EST-SSRs showed amplification in at least one species, and 76 EST-SSRs showed amplification in at least nine species of the 12 analyzed lilies. Of the four species in which EST-SSRs were mined, *L. formosanum* showed the highest non-self-amplification frequency of 34.8%, while *L. regale* showed the lowest non-self-amplification frequency of 11.76% (Table 3). Among the *Lilium* species, *L. amabile*, *L. callosum*, *L. lancifolium*, *L. leichtlini*, *L. formosanum*, and three Asiatic hybrid cultivars showed amplification in all 26 universal EST-SSRs. Of these universally applicable 26 EST-SSRs, 13 were amplifiable in all *Lilium* plant specimens analyzed (Supplemental Figure 1). The primer sequences of all EST-SSRs are shown in Supplemental Table 3.

Although the species used in this analysis under-represent the genus *Lilium*, our data are still valuable to obtain insights for cross-species amplification of the EST-SSRs in the genus *Lilium*. Information from cross-species SSR amplification is useful for analyzing under-used minor species in the genus since development of SSR markers requires high resource input. Usually, EST-SSRs are more transferable cross-species than are genomic SSRs. For example, 43 of the 78 EST-SSRs were transferable from *Triticum* to *Hordeum* species, indicating that the flanking sequences of the EST-SSRs were conserved not only within the genus but also between related genera in the Poaceae family (Gupta et al., 2003). However, only two of the 11 genomic markers from *Swietenia humilis* showed cross-species amplification in the Meliaceae family (White and Powell, 1997). In the genus *Lilium*, there is only a single report on SSRs (Horning et al., 2003), in which six SSRs were isolated from the *Lilium philadelphicum* genomic library, but cross-species transferability was not attempted in this study. In the current study, cross-species amplification was conducted on species in the *Lilium* genus. Thus, analysis of EST-SSR amplification in the current report will be useful in genetic studies of other species in the Liliaceae family, since the SSRs were not reported previously.

### Analysis of the genetic diversity and relationships among lily cultivars

SSR amplification was assessed in 10 breeding lines maintaining in the Lily Breeding Laboratory at Kangwon National University, Korea. The breeding lines are listed in Supplemental Table 1. Of the 165 SSRs, 131 SSR primer sets produced PCR amplicons, and 100 primer sets produced monomorphic amplification. Of the remaining 31 SSR primers, we selected 19 SSR primer sets, producing a high number of reproducible allelic bands to analyze the genetic diversity and

**Table 4.** Number of alleles observed and PIC values of the selected highly polymorphic 19 EST-SSRs.

| Locus | No. of alleles observed | Forward primer | Reverse primer | Motif unit | Polymorphic Information Content(PIC) |
|---|---|---|---|---|---|
| L5 | 21 | AACTCCACAATAAGAGGGAAG | TGTTGTACTTGGCTGTTACATT | $(GA)_9$ | 0.93 |
| L9 | 5 | CAATCCTCTGTGTCAATAACTG | GTAACAACCGGATCTTTAACTC | $(CAG)_6$ | 0.55 |
| L20 | 13 | CCAACAATTTTGATTACATGG | ATTCAAGCAATATCTCATCCTC | $(GGA)_9$ | 0.90 |
| L27 | 9 | CCTACATGTGCATCTCAAATAC | TAACAGATCCAGCAAAGATATG | $(AAC)_4$ | 0.83 |
| L59 | 16 | ACTGGGGAGAATATCAAGAAC | AAAAACCAACTACAACACATCA | $(ACC)_9$ | 0.88 |
| L60 | 11 | CCACAATAAACGATGATGTCT | TAAGCATCATATCAAGCATAGC | $(TCC)_6$ | 0.82 |
| L61 | 9 | GATTGCACTCTATCAGTCACAG | TAATCCCTTTATGAAGATGGTC | $(GCC)_5$ | 0.76 |
| L66 | 7 | CTATTTCCCCTCCTTTGACC | AGATGGTGTCTGTTGAAGTTTT | $(CT)_7$ | 0.80 |
| L67 | 13 | CAGAGATACAAAGCAAAAACAA | AAGAGTGGAGGATCTGAAGAG | $(CT)_8$ | 0.88 |
| eL16 | 21 | TTTCTCGGTTGGCCCCTATG | AGATGAGACATTGCCGGCTG | $(CTC)_4$ | 0.94 |
| eL17 | 11 | CTGATCTGGTAGACGAGCACGA | AGATGCTCACAAACACCGTCAA | $(AAT)_4$ | 0.78 |
| eL28 | 5 | TGCGCTCTGTAGTGTGTTCCAT | CAGACATGCCATGAAAACGAAG | $(TAA)_6$ | 0.49 |
| eL42 | 8 | AAGCATGCTGAGCTGTTGTCAG | CTGCTTGAGTTGGTGTTGTTCG | $(GCA)_4$ | 0.84 |
| eL47 | 4 | TCCCAATGAAGAACACCCTCTC | GACCTGGAAGAAGTCGGTGATG | $(CCT)_4$ | 0.63 |
| eL64 | 8 | AGTCAGATGCAGGAGAGGATGG | GTCCTCCGCTTCCACAAGTTC | $(GAG)_4$ | 0.81 |
| eL65 | 5 | CGAAATTAGGGTTAGGGTTCCG | GTCGGAGAAATTGCTCGAATTG | $(GCG)_4$ | 0.76 |
| eL70 | 8 | CAGGAGCTTAGGTGCTGCTGTT | TAGTGCTGCTCAGTTGTGTGGG | $(GCG)_4$ | 0.78 |
| eL75 | 9 | TACATCTGCTGGGTCCATCCTT | TGACAGCATTGTGAATGGAAGC | $(TGC)_4$ | 0.76 |
| eL81 | 5 | CCCTTTGATGAAGCAGAAGTGC | TTGCACAGAAAATCACGATGCT | $(GCC)_4$ | 0.70 |
| Mean | 9.95 | | | | 0.76 |

relationships among 84 lily cultivars consisting of *L. formology* (FL), *L. formosanum* (FS), *L. brownii* (BR), *L. longiflorum* (LL), Asiatic hybrids (AA), OO (Oriental hybrids), LA (*L. longiflorum*/Asiatic hybrids), OA (Oriental/Asiatic hybrids), and OT (Oriental/Trumphet hybrids). PIC values of the 19 SSR makers ranged from 0.49 to 0.94 with an average of 0.76 (Table 4), which were higher than groundnut (Cuc et al., 2008), maize (Enoki et al., 2002), cassava (Moyib et al., 2007), and soybean (Hwang et al., 2009). The PIC values in our analysis appeared unrelated to repeat length and motif number as shown in eL16 and eL28 (Table 4). Similar results were reported for groundnut (Cuc et al., 2008).

We then selected 19 high polymorphic EST-SSRs for an analysis of the genetic relationship of the 84 lily accessions and hybrids (Fig. 1). The phylogenetic dendrograms derived from the SSR profiles are congruent with the genetic backgrounds of the hybrids that were derived from complex crossings (Asano, 1989). The clustering pattern showed that the accessions or hybrids formed distinct groups in the phylogenetic dendrogram with few exceptions. The odd lines may be due to complex crossings between interspecies or interhybrids during line development. The analyzed lilies were divided into two groups: Oriental and non-Oriental. The lilies belonging to the latter group were further divided into two subgroups: Asiatic and Longiflorum. While the Asiatic subgroup was composed mainly of AA lines, the Longiflorum subgroup contained lines from FL, FS, BR, LL, and LA. AA lines were derived from progenies of multiple crosses [(*L. hollandium*/*L. lancifolium*)/*L maculatum*/*L. amabile*]; thus, their clusters were separated from other Asiatic groups. LA lines were hybrids of LL and FL (Lim et al., 2008), which is supported by the clustering pattern. The four LA lines (Brindisi, Royal Trinity, Ceb Dazzle, and Courier) in the AA cluster must be re-evaluated, since they tied together in the deepest sub-branches. The six FS lines formed a cluster that was separated from the clusters of FL and BR, indicating that the markers employed in this analysis could be used for line selection in interspecific hybrids between these three species (Lim et al., 2008;

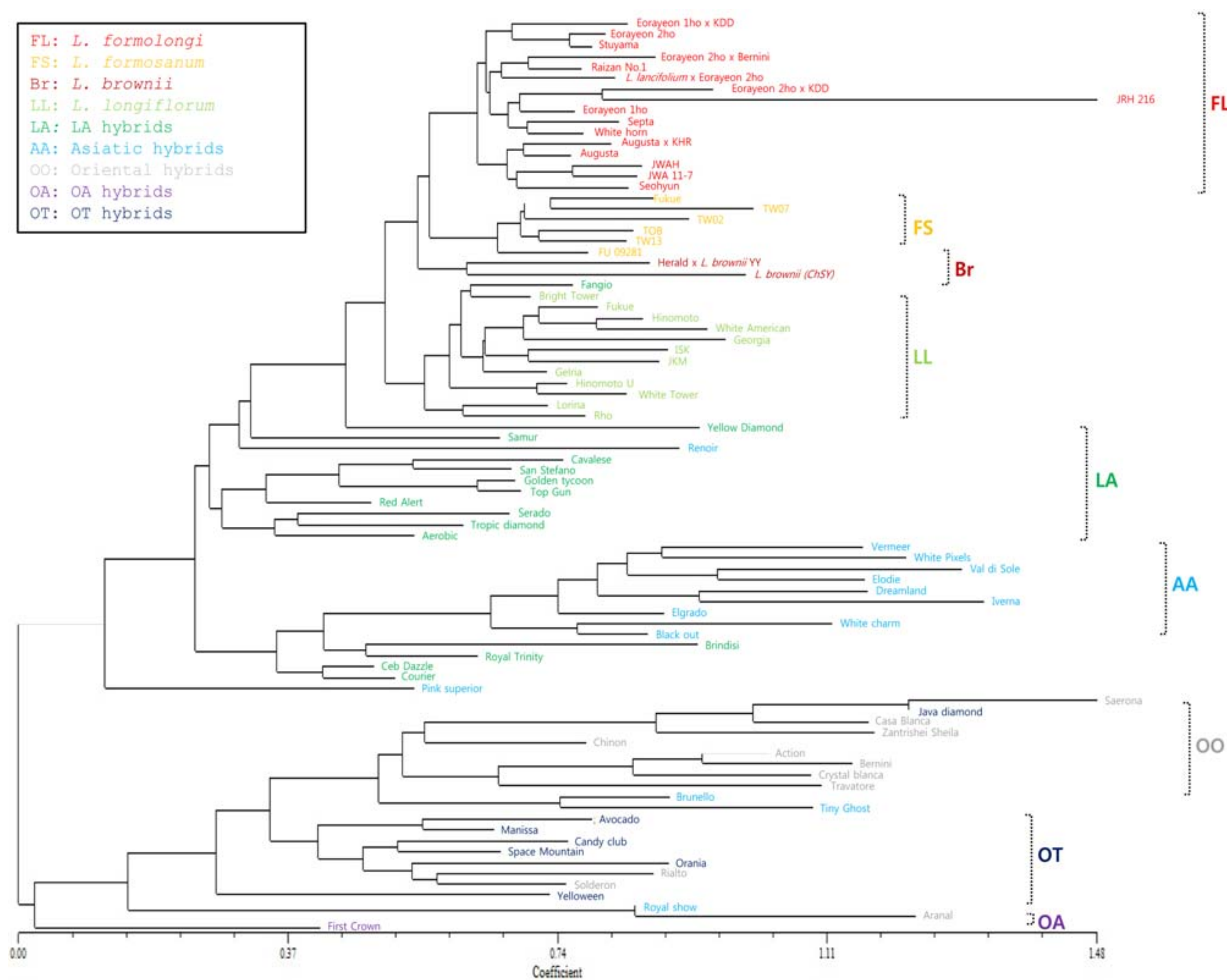

**Figure 1.** A phylogenetic dendrogram of 84 lily cultivars and accessions on the basis of 19 SSR profiles.

Babara-Gonzalez et al., 2008). The Oriental line groups were distinct from the Asiatic groups. The three Asiatic lines, Brunello, Tiny Ghost, and Royal Show, might have an Oriental genetic background due to introgression by crosses during breeding line development. It is difficult to trace an accurate pedigree in each line, as the crosses between the lines were often too complex. OO lines are Oriental hybrids that were derived from progenies (*L. specisum*/*L. auratum*)/*L. parkmanni*/(*L. japonicum*/*L. rubellum*). OT lines are Oriental trumpet hybrids that were derived from progenies of Oriental hybrids and Chinese aurelianese, which are hybrids between (*L. sargentia*/*L. regale*)/*L. sulphureum* (Asano, 1989). Out-group placement of the OA line, First Crown, may be because OA lines are hybrids between Oriental and Asiatic lines.

## Conclusion

SSRs are robust molecular markers used for various purposes. Although lily is a major flowering crop, SSRs have not been developed except for a report of six SSRs in *L. philadelphiicum* (Horning et al., 2003). Because underground scaly bulbs are used for commercial propagation, development of an efficient system to distinguish varieties is required to protect a breeder's right in the molecular breeding of lily. We have developed 165 EST-SSRs: 23 from *L. formosanum*, 92 from *L. longiflorum*, 17 from *L. regale*, and 33 from *Lilium* hybrids. Of the 165 EST-SSRs, we identified 26 EST-SSRs that can be used for the amplification of many *Lilium* species, so that these markers may be valuable in marker-assisted selection in interspecific hybrid breeding programs for lily cultivar development. We also identified 19 EST-SSRs that generated the highest polymorphisms among the Asiatic and Oriental cultivars. These latter 19 markers can be applied for cultivar identification by different marker combinations. Thus, these EST-SSRs will provide valuable molecular resources for germplasm management, genetic diversity analysis, cultivar fingerprinting, and molecular breeding in lily.

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