Analysis of genetic relationships and identification of lily cultivars based on inter-simple sequence repeat markers

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ABSTRACT. Inter-simple sequence repeat (ISSR) markers were used to discriminate 62 lily cultivars of 5 hybrid series. Eight ISSR primers generated 104 bands in total, which all showed 100% polymorphism, and an average of 13 bands were amplified by each primer. Two software packages, POPGENE 1.32 and NTSYSpc 2.1, were used to analyze the data matrix. Our results showed that the observed number of alleles (N_A), effective number of alleles (N_E), Nei's genetic diversity (H), and Shannon's information index (I) were 1.9630, 1.4179, 0.2606, and 0.4080, respectively. The highest genetic similarity (0.9601) was observed between the Oriental x Trumpet and Oriental lilies, which indicated that the two hybrids had a close genetic relationship. An unweighted pair-group method with arithmetic means dendrogram showed that the 62 lily cultivars clustered into two discrete groups. The first group included the Oriental and OT cultivars, while the Asiatic, LA, and Longiflorum lilies

were placed in the second cluster. The distribution of individuals in the principal component analysis was consistent with the clustering of the dendrogram. Fingerprints of all lily cultivars built from 8 primers could be separated completely. This study confirmed the effect and efficiency of ISSR identification in lily cultivars.

Key words: Lily; Inter-simple sequence repeat; Cultivar identification; Genetic relationship

INTRODUCTION

Lily is a very important flower in international flower market. Since the introduction of China's wild lily into Europe during the 18th century, lilies became common ornamental plants whose varieties are largely bred for cut flowers. The breeding history of lilies can be traced back more than 200 years (Peng, 2002). As of 2008, more than 9465 lily varieties have been registered (Lim et al., 2008), which are classified into nine types by the UK Royal Horticultural Society based on the species that were used as parents for hybridization (Sato and Miyoshi 2007). Lily varieties update rapidly. With the breeding and popularization of a large number of new varieties, it is increasingly important to safeguard the interests of producers and breeders by identifying cultivars to ensure their authenticity and purity. In addition, retaining the genetic diversity between varieties is the fundamental guarantee for sustainable breeding and pest resistance. Traditionally, morphological traits are often used to identify cultivars and evaluate genetic diversity, but they have some disadvantages including long life cycle, less useful marks, and low reliability. Molecular marker technology provides a new way to identify cultivars and evaluate genetic diversity. DNA-based markers allow direct comparisons of different cultivars at the molecular level. Compared with other molecular markers such as randomly amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) markers (Zietkiewicz et al., 1994) have showed more polymorphism and reproducibility (Qian et al., 2001). In addition, the ISSR technique is easy and economical, and it has been used successfully in genetic diversity studies of many plants, including *Jatropha curcas* (Grativol et al., 2011), spring orchid (Wang et al., 2009a), *Auricularia auricula* (Tang et al., 2010), clematis (Gardner and Hokanson, 2005), and loquats (Wang et al., 2010). ISSR markers can also be used to identify cultivars of *Curcuma* (Taheri et al., 2012) and strawberry (Arnau et al., 2002); detect the authenticity of hybrid offspring in *Coffea* (Ruas et al., 2003), mungbean (Khajudparn et al., 2012), and *Phyllostachys* (Lin et al., 2010) including lily (Wang et al., 2009b; Wu et al., 2009); and test asexual reproduction strain stability in lily (Liu and Yang, 2012; Xi et al., 2012). To date, there have been very few reports using ISSRs to differentiate varieties of lily hybrids.

In this paper, ISSR molecular markers were used to analyze the genetic diversity and build DNA fingerprints of lily cultivars to provide molecular evidence to protect and identify varieties.

MATERIAL AND METHODS

Plant materials

The plant samples included 62 lily varieties, which were planted in a greenhouse. The

details of the hybrid categories that were used in the study are listed in Table 1. All samples were stored in the Flowers Institute, Yunnan Academy of Agricultural Sciences, Yunnan, China.

Table 1. Code, hybrids type, and color of 62 lily cultivars used in the ISSR analysis.

Code	Cultivar name	Hybrid type	Petals color	Code	Cultivar name	Hybrid type	Petals color
D9	Casa Blanca	O	White	OT4	Manissa	OT	Yellow
D11	Cobra	O	Deep pink	OT5	May wood	OT	Red
D14	Constanta	O	White	OT8	Robinna	OT	Deep pink
D24	Marco Polo	O	Light pink	OT17	Birmingham	OT	Yellow
D32	Siberia	O	White	A3	Avelino	A	Yellow
D34	Simplon	O	White	A4	Brunello	A	Orange
D36	Sorbonne	O	Pink	A7	Clair	A	Deep red
D37	Star Gazer	O	Deep pink	A9	Elite	A	Orange
D38	Tiber	O	Pink	A13	Italia	A	Pink
D60	Lido	O	Pink	A17	Lemon Tree	A	Yellow
D61	Chili	O	Pink	A18	Lyon	A	Orange
D62	Mont de marsan	O	White	A25	Pisa	A	Yellow
D63	Santander	O	White	A26	Poll Yanna	A	Yellow
D64	Rialto	O	White	A32	Solden Horn	A	Yellow
D65	Montezuma	O	White	A39	Umbria	A	White
D66	Cherbourg	O	White	LA4	Royal Song	LA	Red
D67	Burlesca	O	Light pink	LA5	Turandot	LA	Pink
D68	Parasol	O	Pink	LA6	Ceb Dazzle	LA	Yellow
D69	Oberto	O	Pink	LA8	Royal Discovery	LA	Orange
D70	Albisola	O	Pink	LA10	Royal Ballade	LA	Pink
D72	Bracciano	O	Pink	LA11	Ceb Glow	LA	Yellow
D73	Aberlour	O	Pink	LA12	Birgi	LA	Red
D78	Kordesa	O	Pink	LA13	Freya	LA	Yellow
D82	Caldeira	O	White	LA17	Royal Sunset	LA	Orange
D83	Key west	O	Pink	L1	Gelria	L	White
D84	Caruso	O	Pink	L2	Snow Queen	L	White
D42	E7	OT	Yellow	L3	White Fox	L	White
OT14	Kraton	OT	Yellow	L4	White heaven	L	White
OT15	Travatore	OT	Yellow	N1	New Longiflorum I	L	White
OT18	Biaritz	OT	Yellow	N2	New Longiflorum II	L	White
OT1	Conca Dor	OT	Yellow	N5	New Longiflorum III	L	White

White O, OT, A, LA and L respectively represent Oriental lily hybrids, Oriental-Trumpet lily hybrids, Asiatic lily hybrids, Longiflorum-Asiatic lily hybrids, and Longiflorum lily hybrids.

DNA extraction

For each type of lily, two fresh leaves were collected from 10 different plants. Total genomic DNA was extracted from the pooled samples from the 10 individuals using the cetyltrimethylammonium bromide method of Jin et al. (2003) and Tian et al. (2010), with minor modifications. The DNA concentration was estimated using the absorbance at 260 nm (A260). The purity of the DNA extracts was measured using the ratio of the absorbance at 260 and 280 nm (A260/A280). Only DNA with an A260/A280 ratio of 1.8 to 2.1 was used (at a dilution of 10 ng/μL) as a template for polymerase chain reaction (PCR) amplification.

Primer screening and PCR amplification

A total of 36 ISSR primers were synthesized by the Shanghai Sangon Biological Company. After the initial screening, 8 of the 36 primers produced clear banding patterns and were used for further analysis (Table 2).

Table 2. Eight primers were selected for ISSR fingerprinting of 62 lily cultivars.

Primer code	Sequence	Size in bp	Annealing temperature (°C)
R-808	$(AG)_8C$	17	52
R-815	$(CT)_8G$	17	52
R-818	$(CA)_8G$	17	52
R-801	$(TC)_7GGA$	17	52
R-868	$(GAA)_6$	18	48
R-857	$(AC)_8AG$	18	54
R-809	$(CT)_8RA$	20	53
R-895	$(AG)_2TTGGTAG(CT)_2TGATC$	20	58

Different concentrations of template DNA and Taq DNA polymerase were tested for the optimal amplification of products. The optimized amplification reaction mixture (25 µL) contained 20 ng DNA template with the PCR buffer [50 mM KCl; 10 mM Tris-HCl, pH 8.3; 1.5 mM MgCl2; and 0.001% (w/v) gelatin], 0.5 µM each primer, 200 µM dNTPs, 1.0 U Taq DNA polymerase, and PCR-grade dH_2O.

Amplification was performed using a thermocycler (Eppendorf Mastercycler Gradient, Germany). The thermal cycling parameters were an initial denaturation at 94°C for 5 min; 42 cycles of a denaturation step at 94°C for 40 s, an annealing step at 52°C for 45 s (with variable temperatures for different primers), and an extension step at 72°C for 90 s; and a final elongation step at 72°C for 8 min.

Electrophoresis and ISSR data analysis

The PCR products were electrophoresed on 2% horizontal agarose gels (Promega) in 1X Tris, borate, ethylenediaminetetraacetic acid buffer at 5 V/cm. The agarose gels were stained with 2.5 µg/mL ethidium bromide and photographed on an ultraviolet transilluminator. The experiment was performed twice, and only the reproducible bands were recorded. ISSR fragments were treated as a unit characteristic that were scored as present (1) or absent (0) for each of the markers. The POPGENE (version 1.32) (Yeh and Boyle, 1997; Zhao et al., 2010) and NTSYS-pc (version 2.10) (Lewontin, 1972) software were used to analyze the binary data. The percentage of polymorphic loci (PPB), observed number of alleles (N_A), effective number of alleles (N_E), Shannon's information index (*I*) (Lewontin, 1972), and Nei's genetic diversity (*H*) (Nei, 1973) were calculated using POPGENE 1.32. The genetic distances obtained were used to create a dendrogram using unweighted pair-group method with arithmetic means (UPGMA) cluster analysis (Senthil Kumar et al., 2009; Zhao et al., 2010). NTSYS-pc 2.10 was used to perform principal component analysis (PCA) (Senthil Kumar et al., 2009; Tian et al., 2010).

RESULTS

Products of ISSR amplification

A total of 104 clear and reproducible bands were amplified using the eight selected primers, which all showed 100% polymorphism. The eight primers each produced a different number of bands, which ranged from 10 (primer-808) to 18 (primer-857), with an average of 13 bands that were amplified by each primer. The size of the bands varied between 150 and 1200

bp. All samples could be distinguished based on the differences in their ISSR banding patterns.

Genetic relationships

The genetic diversity parameters of all samples and the five types of lilies are summarized in Table 3. N_E and H are two of the most commonly used indicators of genetic variation (Miao et al., 2008; Zeng et al., 2010). For all 62 lily samples, N_A, N_E, H, and I values were 1.9630, 1.4179, 0.2606, and 0.4080, respectively. Among the five types of lilies, the LA lilies exhibited the highest genetic diversity ($H = 0.2127$; $I = 0.3219$). The lowest genetic diversity was observed among the Asiatic lilies ($H = 0.1683$; $I = 0.2656$). The two genetic diversity parameters (H and I) followed a similar trend among the five types of lilies, namely, LA hybrids > Oriental hybrids > Longiflorum hybrids > OT hybrids > Asiatic hybrids.

Table 3. Genetic parameters for five hybrid types of lily from ISSR analysis.

Lily type	Sample size	N_A	N_E	H	I
Oriental	28	1.7315	1.3252	0.1969	0.3050
Oriental x Trumpet	8	1.5648	1.2844	0.1725	0.2657
Asiatic	11	1.6204	1.2653	0.1683	0.2656
Longiflorum x Asiatic	9	1.3593	1.3593	0.2127	0.3219
Longiflorum	7	1.4907	1.3313	0.1891	0.2784
Total	63	1.9630	1.4179	0.2606	0.4080

N_A = observed number of alleles; N_E = effective number of alleles; H = Nei's gene diversity; I = Shannon's Information index.

The similarity coefficients were used to construct the genetic similarity matrix of 62 lily genetic materials based on the bands that were amplified by PCR with the eight ISSR primers. The similarity coefficients between pairs of the various types of lilies ranged from 0.8419 to 0.9561 (Table 4). The highest genetic similarity (0.9561) was observed between the OT and Oriental lilies, which was followed by that between the LA lilies and the Asiatic and Longiflorum lilies. These results indicated that the derived hybrids and their parents were genetically more closely related at the molecular level. The morphology of LA hybrids is more like Asian lilies; their similarity coefficient (0.9345) was higher than that of LA hybrids with Longiflorum lilies (0.8797). The pairwise analysis of the genetic distance between individuals revealed the considerable genetic diversity of the different lily hybrid series.

Table 4. Similarity coefficients of five lily hybrid series.

pop ID*	O	OT	A	LA
OT	0.9561			
A	0.8752	0.8793		
LA	0.8459	0.8540	0.9345	
L	0.8419	0.8459	0.8630	0.8797

Each letter represents a lily hybrids that same with Table 1.

Cluster analysis

The dendrogram (Figure 1) that was generated by the UPGMA cluster analysis of the Jaccard's similarity coefficients showed that the 62 lily cultivars clustered into two discrete

groups. The first group included all Oriental and OT lilies. Asiatic, LA, and Longiflorum lilies were combined in the second cluster. The clustering results were consistent with the classification of the different varieties within each lily hybrid series. Two cultivars of the same hybrid series with a close genetic relationship may not necessarily have the same flower color. For example, the first cluster included 27 Oriental lily varieties, and the cultivar "Siberia" (D32) was closer to "Cobra" (D11) than to the white flower cultivar "Casablanca" (D9). Five OT lilies (OT14, OT1, OT4, OT15, and OT18) clustered at Jaccard's similarity coefficient 0.81 in advance, and then they were grouped together with four other OT lilies and Oriental hybrids. Oriental lilies were one parent of the OT hybrids, which caused a close genetic relationship between these two lily varieties. The second cluster included the Asian, LA, and Longiflorum lilies, and the Longiflorum hybrids clustered into a separate group. The LA lily is a relatively new hybrid series that was bred from Asian and Longiflorum hybrids, which have a flower shape and color that is more similar to Asian hybrids than Longiflorum hybrids.
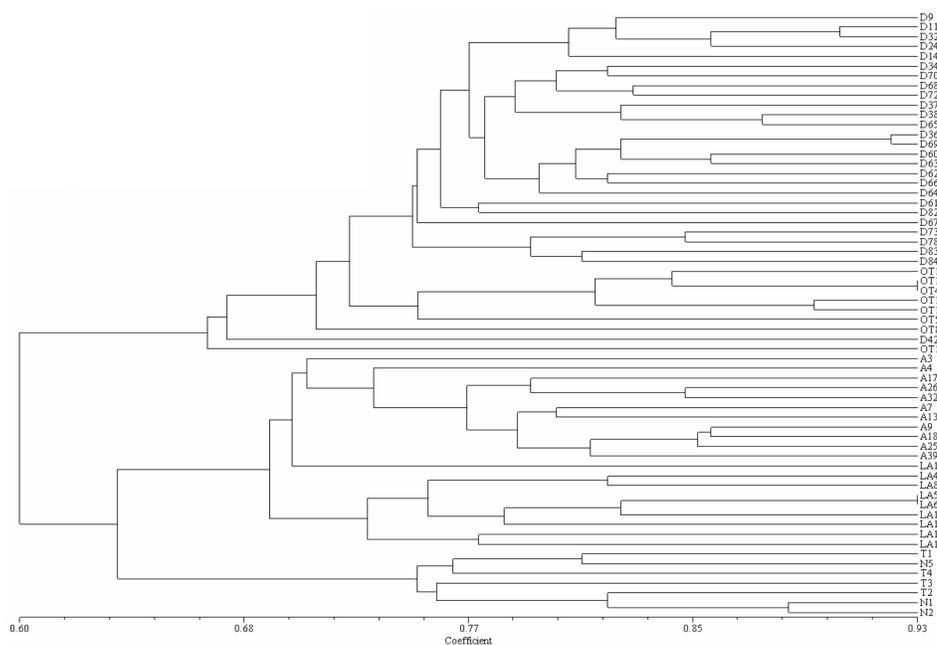

Figure 1. Unweighted pair-group method with arithmetic means dendrogram estimating the genetic relationships among 62 lily cultivars based on Jaccard's coefficient. Cultivar names are listed in Table 1.

PCA analysis

The genetic relationships among the 62 lily samples were further analyzed by PCA (Figure 2). The first three principal components explained 19.86, 7.79, and 5.69% of the total variation. The overall distribution of individuals in the PCA pattern was consistent with the clustering pattern of the UPGMA dendrogram. The Oriental, Asiatic, and Longiflorum lilies could be distinguished by the first principal component, whereas the OT and LA lilies can be separated by the second and third principal components.

Figure 2. Principal component analysis of 62 lily cultivars. Cultivar names are listed in Table 1.

DISCUSSION

This study confirmed the effect and efficiency of lily cultivar identification by ISSR. ISSR markers can reveal more genomic polymorphisms than techniques such as restriction fragment length polymorphisms, simple sequence repeats, and RAPDs. Rao et al. (2007) used ISSR to analyze chickpea cultivars and indicated that six polymorphic ISSR primers could be used to evaluate the genetic diversity of all varieties, while nearly 30 primers were required for RAPD. A similar result was also obtained from the lily genetic relationship analysis with RAPD and ISSR. In RAPD fingerprinting, 22 primers generated 224 bands in total, and only 163 bands showed polymorphism (Zuo et al., 2005). In this study, the 8 primers amplified 104 polymorphic loci, and the polymorphism rate was 100%. Furthermore, ISSR markers have another advantage in lily: there are no strict requirements for the quality of extracted DNA, which is particularly important for lilies because of their high polysaccharide content in all nutritive organs as compared with other plants. Therefore, ISSR genotyping is a suitable method for studying the genetic diversity of lilies and for the first time were used for the molecular identification of lily cultivars.

The result of the ISSR analysis of 62 lily cultivars showed that the genetic similarity coefficients had minor changes (0.8419-0.9561) between different hybrid series, and the Nei genetic diversity index had significant differences (0.1683-0.2127). The differences in genetic parameters indicated that lily cultivars had rich genetic diversity. Digital fingerprinting of all lily cultivars built from 8 primers could be separated completely. This observed number of al-

leles may have been influenced by the sampling strategy, as lily cultivars belonged to different hybrid series. For Oriental lilies, the number of alleles observed (N_A = 1.7315) is larger than the effective number of alleles (N_E = 1.3252), which might be because of the large number of Oriental lily samples. From the perspective of morphological differences, if different cultivars within the same hybrid series have similar phenotypes, their N_A and N_E will be closer.

Lily cultivars have more ploidy types, and their genomes are highly heterozygous (Li et al., 2011). Distinct differences exist between hybrid series because of the species, the number of original parents, and the number of generations of hybridization. At present, various hybridization techniques produced the richness in the types of lily hybrids. The complex lily hybrid series were bred by integrating the advantages of different pre-existing hybrid series. The LA (Longiflorum x Asiatic) and OT (Oriental x Trumpet) lily hybrids are considered major breakthroughs in the lily breeding industry (Zhou et al., 2008). For the composite hybrid series, the cultivars usually cluster with the parental series in the dendrogram. For example, the LA lily "Royal Ballade" first clustered with the Asian lilies group instead of other varieties of LA hybrids, which indicated a close genetic relationship between the two lilies. This study revealed that two varieties with similar petal shapes among Oriental lilies grouped together. Longiflorum hybrids have only two original parents and are easy to cross. The seedlings from germination to flowering only need 1.5 years, and the period of bulb incubation is much shorter than that of Asian lilies, Oriental lilies, and other hybrids. Therefore, the Longiflorum lilies experienced higher hybrid genomic recombination and obtained abundant genetic diversity (H = 0.1891; I = 0.2784). Five OT lilies of the same color comprised a cluster, and then they joined with the four OT cultivars that had a different flower color than that of the former five varieties. OT5 and OT8 are the red flower varieties, while D42 was a potted variety with short plants, which was a trait that made it different from the other eight cut flower varieties. The main color of OT17 is yellow, but a red halo and red glands were distributed in the petals, which may be because of its red parent.

In this study, ISSR markers were used to evaluate the genetic relationships of different lily hybrid series. The cluster and PCA based on ISSR polymorphism indicates that ISSR markers can be used to quickly and accurately identify lily hybrids and determine the exact type of an unknown lily variety, even though lily cultivars have a complex genetic background.

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