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Full Length Article

Genetic Diversity between and within *Lilium* Species and Hybrids Revealed by ISSR Markers

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Abstract

Genetic variation and relationships among 22 *Lilium* accessions including 14 wild species and 8 hybrids were examined by using inter simple sequence repeat (ISSR) markers. Among 100 primers used for PCR amplification of genomic DNA, 10 primers (10%) generated polymorphic DNA fragments. A total of 148 ISSR fragments were generated from 10 primers, 97.97% of which were polymorphic among the 22 *Lilium* accessions. The genetic variation revealed by ISSR markers indicated that 22 *Lilium* accessions showed high polymorphism, abundant genetic variation, and the genetic diversity within wild species was higher than that within hybrids. Cluster analysis based on ISSR data by Unweighted Pair Group Method with Arithmetic Means (UPGMA) showed that 22 *Lilium* accessions could be placed in two groups with a similarity ranging from 0.4459 to 0.9392. This revealed that wild species were genetically distant from hybrids, and was basically consistent with the results of traditional classification based on morphological characters in *Lilium*. The results of this study revealed that molecular techniques might be helpful or even necessary to the precise classification of *Lilium* and the necessity of further study on the genetic relationships between wild *Lilium* species and hybrids. At the same time, the results also revealed that *L. wenshanense* might be of great value to new variety breeding of *Lilium*. This study will provide the basis for the systematic study and the rational utilization of *Lilium* germplasm resources. © 2019 Friends Science Publishers

Keywords: *Lilium*; Germplasm resources; Genetic diversity; Relationship; ISSR; Molecular marker

Introduction

The genus *Lilium* contains around 110 accepted species native to cold and temperate regions of the Northern Hemisphere between 10 and 60º latitudes (Bakhshaie *et al.*, 2016). Lilies are herbaceous perennials that form underground scaly bulbs (Lee *et al.*, 2011b), and have unsurpassed beauty and great commercial significance (Van Tuyl *et al.*, 2011). Lilies have been cultivated as food and medicine in China for at least 2,000 years (Haw, 1986). Many hybrids have been developed through conventional breeding by utilizing several *Lilium* species, and hybrids showing hetero genetic characteristics in flower color, shape of flowers, and various morphological characters (Lee *et al.*, 1996). At present, *Lilium* hybrids are one of the most important cut flowers and potted plants, wild *Lilium* species and hybrids are also used as large, showy flower garden plants in the world (Van Tuyl *et al.*, 2011).

Discrimination of *Lilium* germplasm has been done by using morphological markers, isozyme markers and molecular markers (Yamagishi, 1995; Bakhshaie *et al.*, 2016; He *et al.*, 2018; Kumari *et al.*, 2018). Among them, morphological markers are unstable under the influence of environment, and the use of isozyme markers is limited by the number of informative markers (Yamagishi, 1995). However, molecular markers may be useful tools for these purposes because of circumventing the above–mentioned disadvantages. Researches on genetic diversity and relationships in *Lilium* by using molecular markers are of great theoretical significance and practical value in molecular identification, genetic improvement, germplasm conservation, construction of core germplasm, gene location of important agronomic traits, molecular marker assisted breeding of *Lilium* germplasm resources, etc.

As a microsatellite–based technique, ISSR is a widely used PCR–based molecular marker system with the advantages of easy scoring, high cost–effectiveness, high sensitivity and good reproducibility (Tang *et al.*, 2014). It combines the universality of random amplified polymorphic DNA (RAPD) with most of the advantages of simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) etc. (Bornet and Branchard, 2001) It is now broadly used by researchers in various fields of plant biology (Godwin *et al.*, 1997; Abe *et al.*, 2002; Reddy *et al.*, 2002; Arzate–Fernández *et al.*, 2005; Wu *et al.*, 2015; Chaudhary *et al.*, 2018).

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In the present study, we presented the use of ISSR as genetic markers for 22 *Lilium* accessions including 14 wild species and 8 hybrids. This study aimed to analyze the genetic variation and relationships of *Lilium*, thus providing the basis for the systematic study and the rational utilization of *Lilium* germplasm resources.

Materials and Methods

Plant Materials

Fourteen *Lilium* species (*L. lancifolium*, *L. pumilum*, *L. davidii*, *L. davidii* var. *unicolor*, *L. regale*, *L. lophophorum*, *L. dauricum*, *L. sargentiae*, *L. henryi*, *L. wenshanense*, *L. taliense*, *L. leucanthum*, *L. distichum* and *L. auratum*) and eight hybrids (Tigerwoods, Siberia, Starfighter, Sorbonne, Robina, Royal Trinity, Conca D'or and Yelloween) were used as experimental materials in the present study (Table 1). They were collected from Yunnan, Sichuan, Gansu, Hubei, Heilongjiang, Jilin province. All samples were authenticated based on the morphological characters (Wang and Tang, 1980; Long *et al.*, 1999), and stored in the molecular biology laboratory, School of Ethnic Medicine, Yunnan Minzu University, China.

DNA Isolation and PCR Amplification

DNA samples were isolated from silica–gel dried leaf tissue using a modified CTAB procedure (Yamagishi *et al.*, 2002). PCR was carried out in a 25 μL reaction mixture containing 40 ng of template DNA, 2.0 m*M* $MgCl_2$, 1.5 units of *Taq* DNA polymerase, 200 μ*M* each of dCTP, dGTP, dATP and dTTP, 800n*M* of primer (with reference to the ISSR primer sequences published on www.UBC.ca.com) and to Yamagishi's 3A ISSR primer sequences (Yamagishi *et al.*, 2002), primers synthesised by Sangon Biotech (Shanghai) Co., Ltd., 1 × reaction buffer. DNA amplification was performed by a programmed temperature control system (Eppendorf AG22331 Hamburg, Germany). The thermal cycle program consisted of three steps: 94ºC for 5 min; 35 cycles of 94ºC for 1 min, 51.8ºC for 1 min and 72ºC for 2 min; 72ºC for 8 min, followed by maintaining the mixture at 4ºC. Amplification products were analyzed by electrophoresis on 1.5% agarose gels using 0.5 × TBE buffer at a constant 50V and then visualized by staining with ethidium bromide. Total genomic DNA extraction and PCR reactions were carried out three times, and only reproducible and well–defined bands were scored (Pérez de la Torre *et al.*, 2012).

Statistical Analysis

For ISSR analysis, the banding patterns were recorded by a gel imaging system (Syngene, GeneGenius, Cambridge, UK). Bands with the same mobility and molecular weight were treated as identical fragments. In the data matrices, presence or absence of each ISSR fragment was coded as "1" or "0" for each of the primer pairs (Huang and Sun, 2000). With the amplification data of all informational primers, the similarity was estimated according to the number of shared amplification products (Nei, 1978) by using POPGEN software (Version1.32) (Yeh *et al.*, 2002), and a dendrogram based on similarity coefficients was generated by using NTSYSpc software (Version2.1) (Rohlf, 2000) according to UPGMA.

Results

Primer Screening and PCR Amplification

One hundred primers were screened using the template DNA from *L. lancifolium*, *L. davidii*, Siberia and Sorbonne to identify primers that generated informative arrays of PCR products, and 10 informative primers were finally selected for further analysis in this study. The selected 10 primers generated from 11 to 23 bands with an average of 14.8 bands per primer, while the proportion of polymorphic bands varied from 85.71 to 100% (Table 2). Two examples of PCR amplification of DNA samples from the 22 *Lilium* accessions with primers UBC835 and 3A25 were showed respectively (Fig. 1–2).

Genetic Diversity Analysis

ISSR analysis using the 10 informative primers generated a total of 148 DNA bands in 22 *Lilium* accessions, and the percentage of polymorphic loci (*PPL*) was 97.97%. On the species level, the mean observed allele (*Ao*) was 1.9797, the mean effective allele (*Ae*) was 1.6688, the Nei's gene diversity (*H*) was 0.3786, the shannon's information index (*I*) was 0.5558, indicating that all 22 *Lilium* accessions showed high polymorphism, abundant genetic variation. At the same time, the genetic variation within 14 wild species was compared with that within 8 hybrids, and all index values of genetic diversity indicated that the genetic diversity within wild species was higher than that within hybrids (Table 3).

Genetic Similarity Analysis

The similarity matrix obtained by multivariant analysis using Nei's coefficient is shown in Table 4. From the similarity matrix, the similarity coefficients ranged from 0.4459 to 0.9392 among the samples. The results indicated that *L. lophophorum* was the most closely related to *L. dauricum* due to both belonging to Sect. *Lophophorum*. Among wild species, *L. lophophorum* was the most closely related to *L. dauricum*. Among hybrids, Tigerwoods was the most closely related to Siberia, similarity coefficient between them was 0.8581.

Table 1: The experimental materials used for the ISSR analysis

No.	Name	Lines	No.	Name	Lines
1	*L. lancifolium*	W	12	*L. leucanthum*	W
2	*L. pumilum*	W	13	*L. distichum*	W
3	*L. davidii*	W	14	*L. auratum*	W
4	*L. davidii* var. *unicolor*	W	15	Tigerwoods	O
5	*L. regale*	W	16	Siberia	O
6	*L. lophophorum*	W	17	Starfighter	O
7	*L. dauricum*	W	18	Sorbonne	O
8	*L. sargentiae*	W	19	Robina	O/T
9	*L. henryi*	W	20	Royal Trinity	L/A
10	*L. wenshanense*	W	21	Conca D'or	O/T
11	*L. taliense*	W	22	Yelloween	O/T

Note: W: Wild species; O: Oriental hybrids; L/A: *Longiflorum*×Asiatic hybrids; O/T: Oriental × Trumpet hybrids

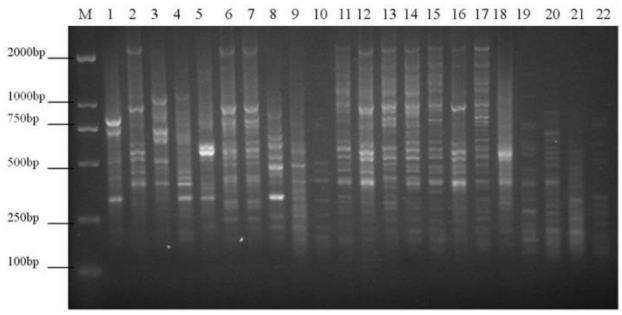

Fig. 1: Electrophoresis of ISSR-PCR with primer UBC835
M: Marker DL2000; 1-22: 22 *Lilium* accessions

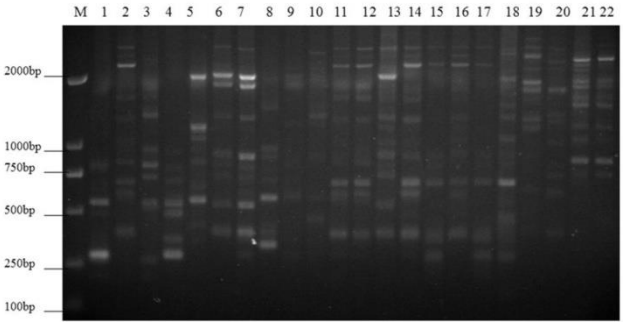

Fig. 2: Electrophoresis of ISSR-PCR with primer 3A25
M: Marker DL2000; 1-22: 22 *Lilium* accessions

Clustering Analysis

The similarity matrix above was used to generate a dendrogram (Fig. 3) by UPGMA analysis in order to determine the grouping of different accessions. The result illustrated that the 22 *Lilium* accessions could be placed in two groups (A and B) at a threshold value of 0.57. The first group (A) contained all wild species and could be further separated into three subgroups at a threshold value of 0.59. The first subgroup (A_1) contained five species in which *L. lancifolium*, *L. davidii*, *L. davidii* var. *unicolor* and *L. henryi* belonging to Sect. *Sinomartagon*. The second subgroup (A_2) contained two species (*L. regale* and *L. sargentiae*) both belonging to Sect. *Lilium*. The third subgroup (A_3) contained seven species in which *L. pumilum* and *L. taliense* belonging to Sect. *Sinomartagon*, *L. lophophorum* and *L. dauricum* belonging to Sect. *Lophophorum*, *L. leucanthum* belonging to Sect. *Lilium* and *L. distichum* belonging to Sect. *Martagon*, respectively. The second group (B) contained all hybrids and could be further separated into two subgroups at a threshold value of 0.627. The first subgroup (B_1) contained all the Oriental hybrids (Tigerwoods, Siberia, Starfighter and Sorbonne). The second subgroup (B_2) contained all the Oriental×Trumpet hybrids (Robina, Conca D'or and Yelloween) and Royal Trinity belonging to *Longiflorum*×Asiatic hybrids. In 22 *Lilium* accessions, all wild species and all hybrids were placed in two different groups, indicating that wild species were genetically distant from hybrids.

Discussion

Breeders have realized that a large amount of genetic variation within crop plants exists unexploited in wild germplasm as a result of domestication (Acosta–Gallegos *et al.,* 2007), these wild relatives can play important roles on the *Lilium* breeding. In China, *Lilium* hybrids are mainly introduced from abroad. As a result of the artificial hybridization that has been going on for nearly 100 years, genetic relationships within hybrids are getting closer and closer, and genetic distances between hybrids and their original wild ancestors are getting larger and larger. Therefore, the genetic background of hybrids is very complicated. In the present study, the genetic variation revealed by ISSR markers indicated that the genetic diversity within wild species was higher than that within hybrids, and cluster analysis based on ISSR data indicated that wild species were genetically distant from hybrids. The results of this study revealed the necessity of further study on the genetic relationships between wild *Lilium* species and hybrids.

For a long time, there have been so many arguments in classification, origin, evolution and other issues of subordinate taxa in *Lilium.* Nowadays, classification of *Lilium* according to Comber (1949) is universally acceptable that the species of *Lilium* are classified into seven sections, ie, *Lilium*, *Martagon*, *Pseudolirium*, *Archelirion*, *Sinomartagon*, *Leucolirion* and *Oxypetala* on basis of their 15 morphological characteristics. Since then, numerous researchers have explored and evaluated the classification methods of *Lilium* (Haruki *et al.,* 1997; Dubouzet and Shinoda, 1999; Hayashi and Kawano, 2000; Nishikawa *et al.,* 1999, 2001; Lee *et al.,* 2011a). On the basis of morphological characteristics such as flower shape, leaf arrangement, the existence form and curvature of stamens, the species of *Lilium* in China could be classified into four sections, i.e., Sect. *Lilium*, Sect. *Lophophorum*, Sect. *Sinomartagon* and Sect. *Martagon* (Wang and Tang, 1980). In the wild species that *L. lancifolium*, *L. davidii*, *L. davidii* var. *unicolor* and *L. henryi*

Table 2: The names and sequences of primers and amplified results

Primer	Primer sequence(5′→3′)	Total band number	Polymorphic band number	Percentage of polymorphic band (%)
UBC811	GAGAGAGAGAGAGAGAC	15	15	100
UBC825	ACACACACACACACACT	12	12	100
UBC835	AGAGAGAGAGAGAGAGYC	15	15	100
UBC842	GAGAGAGAGAGAGAGAYG	12	11	91.67
UBC895	AGAGTTGGTAGCTCTTGATC	14	12	85.71
UBC899	CATGGTGTTGGTCATTGTTCCA	11	11	100
3A13	CACACACACACACAGTC	17	17	100
3A25	CTCTCTCTCTCTCTGAC	15	15	100
3A37	CACACACACACACATGA	23	23	100
3A39	AGAGAGAGAGAGAGCTT	14	14	100
Total		148	145	

Table 3: Genetic variation of 22 *Lilium* accessions detected by ISSR (standard deviation in parentheses)

Taxon	*NP*	*PPL*(%)	A_o	A_e	*H*	*I*
Wild species	137	96.48	1.9648(0.1850)	1.6475(0.2948)	0.3699(0.1327)	0.5445(0.1677)
Hybrids	104	86.67	1.8667(0.3414)	1.5898(0.3513)	0.3349(0.1680)	0.4928(0.2269)
At species level	145	97.97	1.9797(0.1414)	1.6688(0.2936)	0.3786(0.1286)	0.5558(0.1584)

NP: The number of polymorphic loci; A_o: Mean observed number of alleles; A_e: Mean effective number of alleles; *H*: Nei's genetic diversity; *I*: Shannon's information index; *PPL*: The percentage of polymorphic loci

Table 4: Similarity matrix for Nei's coefficient of 22 *Lilium* accessions obtained from ISSR marker analysis

No.	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	0.6014	0.6757	0.6554	0.5946	0.5541	0.5608	0.5676	0.6014	0.6284	0.5676	0.5405	0.5878	0.5608	0.5878	0.5811	0.6216	0.5878	0.5811	0.5743	0.5203	0.5811
2		0.6554	0.5946	0.5068	0.7095	0.6486	0.5473	0.6216	0.5811	0.6824	0.7500	0.6757	0.6757	0.6081	0.6554	0.5743	0.5946	0.5473	0.5811	0.5405	0.5338
3			0.6419	0.5946	0.5946	0.5878	0.5541	0.6014	0.5743	0.5405	0.5676	0.5743	0.5338	0.4932	0.5541	0.5270	0.5203	0.4865	0.4932	0.5068	0.5270
4				0.6149	0.6284	0.6351	0.5878	0.6081	0.5811	0.5473	0.6284	0.6216	0.6216	0.5676	0.5878	0.6014	0.5811	0.5068	0.4730	0.4865	0.5473
5					0.5946	0.5473	0.7027	0.6284	0.5743	0.5676	0.6351	0.6284	0.6014	0.5473	0.6216	0.5946	0.6149	0.5541	0.6014	0.5338	0.5676
6						0.9392	0.5405	0.6419	0.5473	0.6622	0.7162	0.6689	0.6554	0.5338	0.5946	0.5541	0.6014	0.5000	0.5338	0.4662	0.5270
7							0.5068	0.5811	0.5135	0.6014	0.6689	0.6216	0.6216	0.5270	0.5743	0.5608	0.5946	0.4662	0.4865	0.4459	0.4797
8								0.6149	0.5473	0.5541	0.6081	0.5203	0.5608	0.5608	0.5811	0.5405	0.6014	0.5541	0.5203	0.5068	0.5946
9									0.6757	0.6284	0.6149	0.6486	0.5541	0.5270	0.5743	0.5743	0.5811	0.5743	0.5676	0.5405	0.5743
10										0.6014	0.5473	0.5946	0.5676	0.5946	0.5743	0.6014	0.6351	0.5743	0.5676	0.5541	0.6014
11											0.8108	0.7365	0.8176	0.7230	0.7162	0.6081	0.6554	0.5811	0.6419	0.5338	0.5270
12												0.7095	0.8041	0.6419	0.7568	0.5946	0.6689	0.5541	0.6014	0.5338	0.4865
13													0.7568	0.5676	0.6149	0.5608	0.5946	0.5608	0.5405	0.5405	0.5068
14														0.7297	0.7365	0.6284	0.6757	0.6014	0.5946	0.5541	0.5203
15															0.8581	0.8311	0.7703	0.6419	0.6622	0.5946	0.6419
16																0.7432	0.8041	0.6622	0.6689	0.6149	0.5946
17																	0.7365	0.5811	0.6554	0.5743	0.6351
18																		0.6284	0.6216	0.5811	0.6419
19																			0.8446	0.7500	0.7838
20																				0.7703	0.7095
21																					0.7095

belonging to Sect. *Sinomartagon* were clustered into the first subgroup, *L. regale* and *L. sargentiae* belonging to Sect. *Lilium* were clustered into the second subgroup, *L. lophophorum* and *L. dauricum* belonging to Sect. *Lophophorum* were closely related to each other and their similarity coefficient was 0.939 indicated that the result of cluster analysis was basically consistent with that of morphological classification in this study. Although some species are distantly related to each other, but its morphological traits especially flower type are very similar, so it is difficult to classify *Lilium* only based on morphological characteristics (Nishikawa *et al.*, 2001). To some extent, this view was confirmed that the cluster forms of *L. pumilum*, *L. taliense*, *L. auratum*, *L. leucanthum* and *L. distichum* were different from traditional morphological classification results. That *L. pumilum* and *L. taliense* were distantly related to *L. lancifolium*, *L. davidii*, *L. davidii* var. *unicolor* and *L. henryi*, and were clustered into different subgroups indicated that Sect. *Sinomartagon* was very complicated with strong differentiation within section, and might be diverse origins which were consistent with the previous reports (Nishikawa *et al.*, 1999, 2001; Lee *et al.*, 2011a). The results that the similarity coefficient between *L. sargentiae* and *L. regale* was 0.7027, that between *L. regale* and *L. leucanthum* was 0.6351 and that between *L. sargentiae* and *L. leucanthum* was 0.6081 were basically consistent with the view that *L. sargentiae*, *L. regale* and *L. leucanthum* belonging to Sect. *Lilium* had close relationships and the former two were more closely related to each other (Nishikawa *et al.*, 1999).

At present, there are few reports about *L. wenshanense* which is native to the grassy slope at

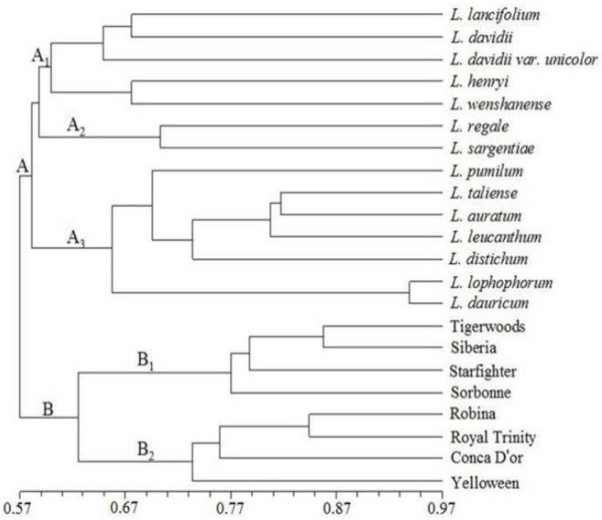

Fig. 3: Dendrogram of 22 *Lilium* accessions generated from ISSR data using UPGMA analysis. The scale is based on Nei's coefficients of similarity

altitudes ranging from 1,000 to 2,200 m in Wenshan of Yunnan, China. The results that *L. wenshanense* were distantly related to the tested hybrids indicated that *L. wenshanense* might be of great value to new variety breeding of *Lilium* in the future according to its excellent characters such as flower shape and flower color.

Conclusion

All 22 *Lilium* accessions showed high polymorphism, abundant genetic variation, and the results of cluster analysis were basically consistent with those of traditional classification based on morphological characters in *Lilium*. However, the relationships among several species belonging to Sect. *Sinomartagon* were far away, and their clustering in different subgroups indicated that the origin of this Sect. was very complicated. In cluster analysis, seven species belonging to different sections were finally got together in a subgroup, indicating that the precise classification of *Lilium* should be combined with morphological and molecular techniques. At the same time, the genetic variation revealed by ISSR markers indicated that the genetic diversity within wild species was higher than that within hybrids, and cluster analysis based on ISSR data indicated that wild species were genetically distant from hybrids. Results further revealed the necessity of study on the genetic relationships between wild *Lilium* species and hybrids. In addition, *L. wenshanense*, a rarely reported elite wild germplasm, was distantly related to the tested hybrids revealed that it might be of great value to new variety breeding of *Lilium*. This study will provide the basis for the systematic study and the rational utilization of *Lilium* germplasm resources.

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