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Genetic variability and diversity of the main resources of lily assessed via phenotypic characters, pollen morphology, and ISSR markers

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ABSTRACT. Lily (*Lilium* spp), which belongs to *Lilium*, is one kind of monocotyledon. As a perennial ornamental plant with extremely high esthetic, edible, and medicinal value, lily has gained much favor due to its mostly showy flowers of various colors and elegant shape. In this research, we studied experimental materials in a sample of 49 individuals including 40 cultivars, nine species of wild lily, and their variants. The collection of 40 cultivars covered all six hybrids in the genus, i.e., Asiatic hybrids, Oriental hybrids, Longiflorum hybrids, LA hybrids, LO hybrids, and OT hybrids. Genetic diversity and inter-relationships were assessed through analysis of phenotypic characteristics, pollen morphology, and ISSR molecular markers. Quantitative characters were selected to analyze phenotypic variation, with results indicating greater variability in petiole length as compared to other characters. Pollen morphological observations suggested that the largest variation coefficient between all hybrids and wild species was the lumina. ISSR makers demonstrated that both cultivars

and wild species possess a high level of genetic diversity. Specifically, the genetic diversity of wild lily was higher than cultivars.

Key words: Lily; Genetic diversity; Phenotypic characters; ISSR markers; Pollen morphology

INTRODUCTION

The genus *Lilium* belongs to the family Liliaceae, and comprises approximately 110-115 species that are distributed in the temperate and cold regions of the Northern Hemisphere (Mcrae, 1998; Liang and Tamura, 2000), particularly in East Asia, Europe, and North America (Woodcock and Stearn, 1950; Synge, 1980; Wang et al., 2015). Approximately 55 species occur naturally in the southwest and north of China, which is considered to be the global center of wild lily diversity (De Jong, 1974). Molecular analysis indicates that lilies arose in Eurasia, nearly 68 million years ago (Leitch et al., 2007). Lilies have been bred for more than 200 years (Peng, 2002; Cui et al., 2014), and more than 10,000 cultivars have been developed to date (Younis et al., 2014). Despite genetic similarities, species of the genus *Lilium* have evolved with notable phenotypic diversity, which is of considerable evolutionary significance (Patterson and Givnish, 2002). The first botanist to classify this genus as a whole was Endlicher (1836), who divided it into five sections based on morphological characteristics: Amblirion, Martagon, Pseudolirium, Eulirion, and Cardiocrinum. Comber (1949) proposed the most authoritative classification of the genus, classifying naturally growing lilies into seven sections and nine subsections based on 13 morphological characteristics and two germination types. The seven sections were Martagon Rchb., Pseudolirium Endl., Liriotypus Asch. and Graeb., Archelirion Baker, Sinomartagon Comber, Leucolirion Wilson, and Daurolirion Comber, respectively (Comber, 1949). These have remained the most widely accepted taxonomical classifications.

Despite this taxonomic system, it is difficult to classify several species of *Lilium* based on morphological traits alone, given that some traits are shared by distantly related species (Nishikawa et al., 1999; Du et al., 2014a). Taxonomists and botanists have gradually recognized the importance of pollen morphology in clarifying the classification of *Lilium.* Pollen grains should be regarded as a functional unit, with the exine ornamentation as a compromise between the following four major functions: protective, harmomegathic, reservoir, and clustering (Muller, 1979; Du et al., 2014b). Baranova (1985) divided the morphological types of pollen into three categories: Martagon, Callose, and Concolor, depending on the pollen number, shape and arrangement of columellae. Based on previous and present studies, Du (2014b) suggested that pollen from *L. formosanum* should be classified as a new type, Formosanum, and proposed that the trends of pollen types were from Martagon to Callose, to Concolor, to Formosanum, as demonstrated by the evolution of an exine ornamentation. In addition, the pollen morphology of some Chinese species has been described using scanning electron microscopy (SEM) in several relevant studies (Li and Qin, 1993; Zhang et al., 2006; Wu et al., 2007). These implicated that pollen had not only the commonness of genus but also the specificity of single species. Significant differences in pollen size and pollen ornamentation were observed, which provided a reference for the delimitation of species or subsection, and highly reflected levels of genetic diversity.

To date, various methods have been applied to evaluate the degree of genetic diversity including morphological characteristics, pollen morphology, chromosome traits, isozymes, DNA markers, and others (Gupta and Varshney, 2000). DNA markers have proven to be valuable tools

in the characterization and evaluation of genetic diversity within and between species and cultivars (Guasmi et al., 2012). Commonly used DNA marker systems include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and more recently, simple sequence repeats (SSRs). These methods have several limitations such as the low reproducibility of RAPD, high-cost of AFLP, and the need to know the flanking sequences to develop species-specific primers for SSR polymorphism. Inter-simple sequence repeats (ISSR) are among the most useful DNA markers that have overcome most of these limitations, and have been efficiently and widely used in current studies addressing the genetic variability and diversity of plants and crops (Taheri et al., 2012; Karimi et al., 2014). At present, only a few studies have examined the diversity of *Lilium* species using molecular markers, including RAPD (Huang et al., 2009), ITS (Sultana et al., 2011; Du et al., 2014a) and ISSR (Guo et al., 2011; Xi et al., 2012; Zhao et al., 2014; Cui et al., 2014).

This research was designed to evaluate the genetic diversity and relationships among lily cultivars. This was achieved through assessment of morphological characters, pollen morphology, ISSR molecular markers, and the use of advanced statistical analyses. The results gained using three independent lines of evaluation provide an objective and accurate assessment of genetic diversity and phylogenetic relationships. This study further provides a theoretical foundation and important reference for lily germplasm research, development, utilization, and cultivation of new varieties with independent intellectual property rights. Additionally, our findings have significance in studies of crossbreeding-assisted molecular markers (Peruzzi et al., 2009).

MATERIAL AND METHODS

Plant materials

The study was carried out with 49 accessions, which consisted of 16 Asian hybrids, seven Oriental hybrids, two Longiflorum hybrids, four LA hybrids, one LO hybrid, nine OT hybrids, and nine species of wild lily. A list of accessions used along with species name, cultivar names and classification is presented in **Tables S1**, **S2**, and **S3**.

Our collection of 40 cultivation species were planted in the experimental greenhouse of Beijing Forestry University (116.3°E, 40.0°N), and maintained at 22°-25°C /17°-20°C temperatures, under natural irradiance conditions. The lily bulbs were cultivated in standard plastic boxes (60 cm x 40 cm x 25 cm) using a matrix formulation for Peat:Perlite:vermiculite grown = 3:1:1. Approximately 10-15 bulbs were planted in each box, with the final number of bulbs dependent on bulb size. Prior to planting the bulbs into boxes, 30 min disinfection with a 1:500 dilution of carbendazim was performed.

Tissue culture techniques were used to preserve nine species of wild lily used in this experiment. The culture medium was basic MS medium with 0.5 mg/L NAA, 1.0 mg/L BA, 6 g Agar and 30 g sugar. The pH value of culture medium was in the range of 6.2-6.4.

Measurement of morphological traits

A previous study conducted by Zhang et al. (2000) and Xiang et al. (2005) demonstrated that 18 neutral like traits, including ten quantitative traits and eight qualitative traits, can be used to distinguish lily species. The ten quantitative characteristics selected for analysis of phenotypic variation were plant height, ground diameter, inner petal width, ovary length, style length, pedicel length, flower diameter, anther length, petiole length, and flower number. Eight quantitative characteristics were also analyzed, including leaf shape, flower type, flower gesture, flower fragrance, anthers inserted mode, anthotaxy, flower color, and tepal spotting.

Pollen morphology observation

Morphological observations were conducted using SEM. Pollen for SEM was taken from flower buds just prior to the opening of the anthers. In order to collect pollen, isolated anthers were placed on Petri dishes and kept at room temperature for 24 h, allowing drying of the pollen. Dry pollen grains were then mounted onto the surface of polished aluminum stubs using double-sided adhesive tape. Each stub was sputter coated with a gold layer and taped to the object stage. Observation and image acquisition were made using a Hitachi S-3400 SEM following Avetissian (1950). All microscopy procedures were performed at the Biotechnology Centre, Beijing Forestry University. Biometric measurements were made using Image-Pro plus 6.0 (Media Cybernetics, USA). For each sample, measurements were made on 30 mature pollen grains, which were correctly formed and chosen randomly. Parameters determined included the polar axis (P), equatorial diameter (E), P/E ratio, lumina, and muri width. These five parameters, as well as exine ornamentation, were selected for Q-cluster analysis.

Data analysis

SPSS 18.0 was used for all statistical analyses. Relationships between the 18 morphological traits assessed were examined using Pearson's correlation coefficients and principal component analysis (PCA). Differences between cultivars were examined based on the maximum, minimum, average, standard deviation, range, and variation ratio of pollen characteristics of 30 individuals.

Cluster analysis was utilized to test for differences between the 39 cultivars under study (**Table S1**). Key traits obtained from the result of PCA were considered, i.e. petiole length, pedicel length, leaf shape, flower gesture, flower type, ground diameter, style length, tepal spotting, flower number, plant height, flower diameter, anther length, and flower anthers inserted mode. Another cluster analysis was performed that included data from 22 cultivars and 2 species (**Table S2**). Pollen morphological characters including P, E, P/E ratio, lumina, muri width, and exine ornamentation were selected for Q-cluster analysis. The Euclidean coefficient distance factor was adopted as the genetic distance of clustering units.

DNA extraction and ISSR-PCR

The 40 cultivars, nine wild lilies and their variants were investigated using ISSR markers (**Table S3**). Plant genomic DNA was extracted from young leaves using a genome DNA kit Tiangen DP305-03 (Tiangen Biotech, Beijing, China) following the manufacturer protocol. For wild lily species, robust leaves used for DNA extraction were obtained from tissue culture seedlings. DNA quality was estimated on an agarose gel (1.2%) stained with ethidium bromide. Purified genomic DNA was quantified using a Nano Drop 2000. Extracted DNA was kept at -20°C prior to molecular marker testing.

Of the 15 primers tested for ISSR amplification, 11 produced reliable banding patterns with high reproducibility and clear band resolution, and thus, were used during the present study. The PCR program was set as follows: an initial denaturation step at 94°C for 5 min followed by 45 cycles of 50 s at 94°C, 45 s for annealing at the primer-specific melting temperature, and 75 s at 72°C (elongation step). A final extension for 8 min at 72°C followed this cycle.

RESULTS AND DISCUSSION

Correlation analysis between the traits of lily cultivars

The results of correlation analysis between morphological characteristics of the 39 lily cultivars are demonstrated in Table 1. The main nutritional traits for lily included plant height, ground diameter, pedicel length, petiole length, and leaf shape. Positive correlations were apparent between pedicel length and both plant height (r = 0.40), and petiole length (r = 0.63). In contrast, leaf shape demonstrated significant negative correlations with petiole length (r = -0.82) and pedicel length (r = -0.57).

The main reproductive traits for lily included flower diameter, flower number, flower color, and flower fragrance. Inner petal width demonstrated significant positive correlations with ovary length (r = 0.73) and style length (r = 0.74). However, the inner petal width also showed significant negative correlations with flower number (r = -0.52) and flower fragrance (r = -0.65). There was a significant positive correlation between ovary length and style length (r = 0.82). Conversely, ovary length demonstrated significant negative correlations with flower number (r = -0.49), flower fragrance (r = -0.49), and flower gesture (r = -0.46). Style length was positively correlated with anther length (r = 0.42), flower type (r = 0.45) and tepal spotting (r = 0.41), whereas it was negatively correlated with flower number (r = -0.44), flower gesture (r = -0.50) and flower fragrance (r = -0.73). Anther length displayed a significant positive correlation with anthers insert mode (r = 0.49) as well as style length (r = 0.42). Flower number was negatively correlated with tepal spotting (r = -0.48), inner petal width (r = -0.52), ovary length (r = -0.49) and style length (r = -0.44). Flower fragrance and anther length were significantly negatively correlated (r = -0.46).

Correlations were also apparent between reproductive traits and nutritional traits. Plant height displayed a significant positive correlation with inner petal width (r = 0.45) and ovary length (r = 0.43). Inner petal width was positively correlated with pedicel length (r = 0.58) and petiole length (r = 0.48). Ovary length had a significant positive correlation to pedicel length (r = 0.47). Overall, data indicated that the selection of nutritional traits benefited the development of lily cultivars with larger flowers.

PCA of lily cultivar traits

PCA was performed on the correlation matrix of the evaluated variables. Six principal components were needed to account for 76.69% of the total variation in the data, whereby 23.66% was represented by the first component, corresponding mainly to three characteristics: petiole length, pedicel length, and leaf shape. The second principal component represented 15.58% of cumulative variance and was positively correlated with the following variables: flower gesture, flower type, ground diameter, and style length. The third principal component accounted for 10.97% of the total variation and was associated with two characteristics: tepal spotting and flower number. The fourth principal component represented 9.67% of the total variation and was associated with two characteristics: plant height and flower diameter. The fifth principal component accounted for 8.62% of the total variation, and was associated with two characteristics: anther length and flower anthers inserted mode. Finally, the sixth principal component represented 8.20% of the total variation and was associated with flower color (Tables 2 and 3).

Therefore, 13 key traits, i.e., petiole length, pedicel length, leaf shape, flower gesture, flower type, ground diameter, style length, tepal spotting, flower number, plant height, flower diameter, anther length, and flower anthers inserted mode, were selected from 18 neutral like traits.

Table 1. Correlation coefficient matrix of lily morphological characteristics, showing the significance of correlations.

	Plant height	Ground diameter	Inner petal width	Ovary length	Style length	Pedicel length	Flower diameter	Anther length	Petiole length	Flower number	Leaf shape	Flower type	Flower gesture	Flower fragrance	Anthers inserted mode	Anthotaxy	Flower color	Tepal spotting
Plant height	1																	
Ground diameter	0.218	1																
Inner petal width	0.452**	0.132	1															
Ovary length	0.432**	0.246	0.733**	1														
Style length	0.403*	0.288	0.743**	0.819**	1													
Pedicel length	0.402*	0.159	0.575**	0.468**	0.516**	1												
Flower diameter	0.304	-0.006	0.390*	0.182	0.150	0.211	1											
Anther length	0.205	-0.039	0.332*	0.289	0.418**	0.311	0.181	1										
Petiole length	0.123	-0.081	0.483**	0.306	0.402*	0.633**	0.159	0.254	1									
Flower number	-0.178	0.069	-0.519**	-0.494**	-0.440**	-0.213	-0.158	-0.116	-0.056	1								
Leaf shape	-0.311	0.149	-0.544**	-0.449**	-0.509**	-0.570**	-0.153	-0.350*	-0.820**	0.211	1							
Flower type	0.244	0.201	0.136	0.364*	0.447**	-0.007	-0.076	0.216	-0.171	-0.209	0.002	1						
Flower gesture	-0.052	-0.410**	-0.256	-0.457**	-0.498**	0.030	0.050	-0.019	0.049	0.283	0.061	-0.386*	1					
Flower fragrance	-0.403*	-0.166	-0.651**	-0.489**	-0.734**	-0.790**	-0.129	-0.455**	-0.575**	0.218	0.586**	-0.244	0.176	1				
Anthers inserted mode	0.279	-0.053	0.312	0.201	0.272	0.375*	0.075	0.493**	0.469**	0.218	-0.462**	0.044	0.004	-0.424**	1			
Anthotaxy	-0.352*	-0.245	-0.028	-0.035	-0.096	-0.247	-0.203	-0.262	-0.129	-0.269	0.103	-0.001	0.076	0.223	-0.261	1		
Flower color	0.234	0.096	-0.108	-0.012	-0.040	-0.083	-0.116	-0.227	-0.193	0.256	0.118	0.188	-0.226	0.072	0.241	-0.230	1	
Tepal spotting	0.063	-0.146	0.384*	0.344*	0.409**	-0.109	-0.030	0.218	0.047	-0.480**	-0.181	0.098	-0.316*	-0.166	-0.029	-0.084	-0.066	1

** and * indicate the significance of $P \leq 0.01$ and $P \leq 0.05$, respectively.

Table 2. Results of principal components analysis showing the total variance explained by each principal component (PC).

PCs	Total variance explained								
	Initial eigenvalues			Extracting square and loading			Rotation square and loading		
	Total	Variance (%)	Cumulative variance (%)	Total	Variance (%)	Cumulative variance (%)	Total	Variance (%)	Cumulative variance (%)
1	5.818	32.323	32.323	5.818	32.323	32.323	4.258	23.657	23.657
2	2.459	13.659	45.982	2.459	13.659	45.982	2.804	15.580	39.237
3	2.027	11.263	57.246	2.027	11.263	57.246	1.974	10.969	50.206
4	1.284	7.135	64.380	1.284	7.135	64.380	1.740	9.666	59.872
5	1.199	6.659	71.039	1.199	6.659	71.039	1.552	8.624	68.496
6	1.018	5.653	76.692	1.018	5.653	76.692	1.475	8.196	76.692
7	0.944	5.242	81.934						
8	0.707	3.928	85.863						
9	0.532	2.956	88.819						
10	0.461	2.560	91.379						
11	0.371	2.062	93.441						
12	0.314	1.744	95.185						
13	0.253	1.405	96.590						
14	0.221	1.226	97.817						
15	0.136	0.755	98.571						
16	0.106	0.589	99.160						
17	0.097	0.540	99.700						
18	0.054	0.300	100.000						

Table 3. Principal component (PC) rotation scores (loadings), % variance explained, and total cumulative variance explained.

Variables	Rotation component matrix					
	PC1	PC2	PC3	PC4	PC5	PC6
Plant height	0.278	0.247	0.034	0.652	0.118	0.307
Ground diameter	-0.015	0.690	-0.514	0.197	-0.070	-0.034
Inner petal width	0.644	0.307	0.379	0.409	-0.034	-0.100
Ovary length	0.496	0.586	0.347	0.253	-0.051	-0.028
Style length	0.575	0.651	0.305	0.153	0.132	-0.074
Pedicel length	0.814	0.126	-0.203	0.287	0.055	-0.102
Flower diameter	0.089	-0.149	0.079	0.815	0.050	-0.149
Anther length	0.276	0.122	0.187	0.091	0.823	-0.260
Petiole length	0.892	-0.179	-0.001	-0.020	0.067	-0.038
Flower number	-0.166	-0.296	-0.648	-0.212	0.285	0.345
Leaf shape	-0.853	0.071	-0.225	-0.029	-0.111	-0.050
Flower type	-0.075	0.694	0.138	-0.075	0.239	0.068
Flower gesture	-0.018	-0.749	-0.224	0.080	0.091	-0.189
Flower fragrance	-0.762	-0.338	-0.003	-0.138	-0.259	0.081
Anthers inserted mode	0.542	-0.074	-0.037	-0.031	0.543	0.388
Anthotaxy	-0.031	-0.071	0.225	-0.433	-0.530	-.329
Flower color	-0.104	0.159	-0.062	0.024	-0.055	0.913
Tepal spotting	0.014	0.189	0.820	0.014	0.144	0.016
Eigenvalue	5.818	2.459	2.027	1.284	1.199	1.018
Proportion (%)	32.323	13.659	11.263	7.135	6.659	5.653
Cumulative proportion (%)	32.323	45.982	57.246	64.38	71.039	76.692

Analysis of pollen morphology and genetic diversity of lily cultivars

A description of the pollen grain morphology of the lilies studied is provided below and illustrated with SEM photographs (Figures 1 and 2). Pollen grains occur as monads. The pollen morphology of lily was characterized as long-ellipsoidal in the polar view, and different cultivars showed different exine ornamentation. The exine patterns appeared to be reticulate with muri formed by different kinds of columellae.

Given that only one specimen of LO hybrids and Longiflorum hybrids was included in materials, the study of variation within cultivars and species was analyzed in the rest of series of hybrids, i.e., Asiatic hybrids, LA hybrids, OT hybrids, Oriental hybrids, and wild lily. Results highlighted that the lumina demonstrated the largest variation coefficient across all hybrids, as well as wild species. The second largest variation coefficient was different between cultivars and wild species. For the cultivars, the second largest variation coefficient was the muri width, and for wild species was the P/E value. In general, for the same pollen morphological index, the coefficient of variation between different series was greater than the variation within the series. On the whole, regardless of cultivars or wild species, the five pollen characteristics assessed during the present study highly reflected the levels of genetic diversity apparent (Table 4) .

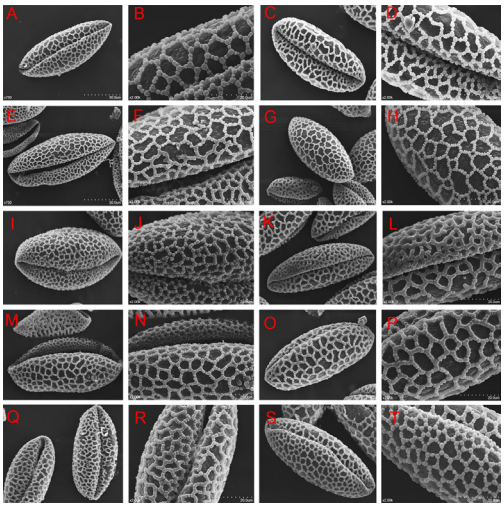

Figure 1. A. and **B.** 'Prato'; **C.** and **D.** 'Tiny ghost'; **E.** and **F.** 'Tiny dino'; **G.** and **H.** 'Tiny bee'; **I.** and **J.** 'Cancun'; **K.** and **L.** 'Pollyanna'; **M.** and **N.** 'Navona'; **O.** and **P.** 'Detroit'; **Q.** and **R.** 'Blackout'; **S.** and **T.** 'Loreta'.

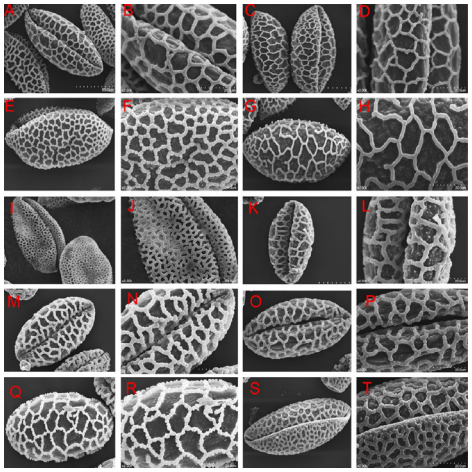

Figure 2. A. and **B.** 'Yaoyan'; **C.** and **D.** 'Red alert'; **E.** and **F.** 'Honesty'; **G.** and **H.** 'Triumphator'; **I.** and **J.** 'Miyabi'; **K.** and **L.** 'Huang jinjia'; **M.** and **N.** 'Concador'; **O.** and **P.** 'Red Dutch'; **Q.** and **R.** 'Tiber'; **S.** and **T.** 'Elite'.

Table 4. Variation in pollen characteristics of 30 different lily cultivars.

Different cultivation	Different pollen measure index	Max. value	Min. value	Average value	Standards	Extreme value	Variation ratio (%)
Asiatic hybrids	P	167.00	87.60	118.18	18.90	79.40	15.99
	E	61.50	30.20	44.68	7.44	31.30	16.64
	P/E	3.47	1.93	2.67	0.33	1.54	12.35
	Lumina	13.81	4.19	7.10	2.09	9.62	29.49
	Muri width	2.39	1.32	1.74	0.29	1.07	16.63
LA hybrids	P	128.00	71.40	115.17	7.52	25.00	6.53
	E	69.20	34.10	54.08	8.21	25.70	15.18
	P/E	2.91	1.69	2.18	0.38	1.26	17.50
	Lumina	17.02	3.07	10.13	3.29	14.84	32.43
	Muri width	2.88	1.67	2.01	0.48	1.55	23.76
OT hybrids	P	123.00	72.40	97.07	17.93	50.60	18.47
	E	56.10	34.10	45.46	6.34	22.00	13.94
	P/E	2.57	1.69	2.14	0.28	0.87	13.25
	Lumina	16.41	2.07	9.90	2.91	14.34	29.41
	Muri width	4.30	1.67	2.76	0.61	2.62	22.05
Oriental hybrids	P	125.00	96.00	107.94	10.65	29.00	9.86
	E	52.90	37.60	45.93	5.82	15.30	12.67
	P/E	2.87	2.03	2.38	0.35	0.84	14.52
	Lumina	14.69	4.20	8.91	3.13	10.49	35.17
	Muri width	2.39	1.02	1.81	0.37	1.37	20.29
Wild lily	P	99.60	76.80	84.94	8.34	22.80	9.82
	E	44.50	26.80	33.30	6.60	17.70	19.83
	P/E	3.48	1.80	2.63	0.54	1.69	20.35
	Lumina	9.21	3.40	6.06	1.68	5.81	27.71
	Muri width	1.73	0.97	1.26	0.21	0.75	16.88
Different spices of lily	P	167.00	72.40	110.06	20.48	94.60	18.61
	E	76.30	26.80	46.10	9.81	49.50	21.27
	P/E	3.48	1.65	2.44	0.43	1.83	17.52
	Lumina	18.91	2.07	8.50	3.16	16.84	37.15
	Muri width	4.30	0.97	1.96	0.58	3.33	29.76

Analysis of Q-cluster and genetic diversity of lily cultivars

As shown in Figure 3, the results of Q-cluster analysis indicated that the 39 cultivars could be classified into nine groups. The first group consisted of No. 6, No. 8, No. 9, No. 10, No. 11, No. 12, No. 13, No. 15, No. 16, and No. 17 cultivars of lily, belonging to Asiatic hybrids, in addition to No. 18 ‘Red alert’, which represented LA hybrids. The second group was composed of No. 1, No. 2, No. 3, No. 4, and No. 5 cultivars of lily, pertaining to Asiatic hybrids, and No. 19 ‘Suncrest’, pertaining to LA hybrids. The third group included three cultivars, No. 26, No. 27, and No. 28, belonging to OT hybrids. The fourth group was composed of No. 20, No. 22, No. 23, No. 24, No. 25, No. 31, and No. 32 cultivars of lily, involving four series: LO, LA, OT hybrids, and Longiflorum hybrids. Members of this group appeared to be more complex, representative of LO hybrids was No. 22 ‘Triumphator’, and of LA hybrids was No. 20 ‘Honesty’. No. 25 ‘Yelloween’, No. 31 ‘S’ and No. 32 ‘V’ belonged to OT hybrids, No. 24 ‘Miyabi’, and No. 23 ‘White heaven’ pertained to Longiflorum hybrids. The fifth group only consisted of No. 36 ‘Marlon’, belonging to Oriental hybrids. The sixth

group included three cultivars: No. 38 and No. 39 were Oriental hybrids, while No. 7 ‘After eight’ was Asiatic hybrids. The seventh group was composed of No. 29, No. 30, No. 33, No. 34, No. 35, and No. 37 lily cultivars, including Oriental hybrids and OT hybrids two series; among them No. 39 ‘Robina’, No. 30 ‘Golden’ and No. 33 ‘Concador’ were OT hybrids; No. 34 ‘Legend’ and No. 35 ‘Siberia’ belonged to Oriental hybrids. In the eighth group, No. 21 ‘Dazzling’ was LA hybrids. In the ninth group, No. 14 ‘Elite’ was Oriental hybrids.

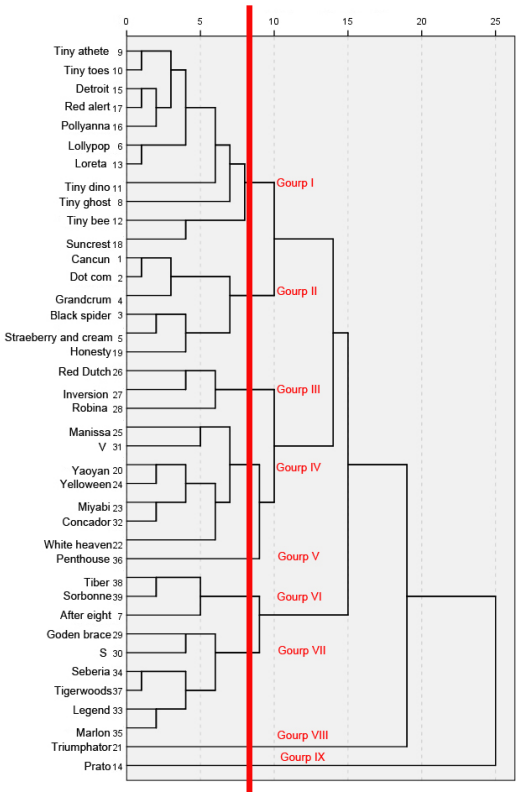

Figure 3. Q-cluster analysis of lily varieties via phenotypic traits.

A further cluster analysis was performed using six pollen morphological traits (Figure 4). Twenty-two cultivars and 2 species were clustered into eight groups: The first group included No. 3, No. 5, No. 7, No. 16, No. 19, and No. 20 lily cultivars. It contained representatives of three series, the Asiatic hybrids, Longiflorum hybrids, and OT hybrids. The second group was wild lily No. 24. The third group only consisted of No. 4 lily cultivars, belonging to the Asiatic hybrids. The fourth group was composed of three cultivars No. 12, No. 13 and No. 15, involving LA hybrids and LO hybrids. The fifth group included three cultivars: No. 2, No. 9 and No. 21, pertaining to the Asiatic hybrids and Oriental hybrids. The sixth group was comprised of No. 6, No. 8, No. 10, No. 14, No. 17, No. 18, and No. 22, belonging to the Asiatic hybrids, Oriental hybrids, LA hybrids, and OT hybrids, as well as wild lily No. 23. The seventh group and eighth group were No. 1 and No. 11, respectively, both Asiatic hybrids.

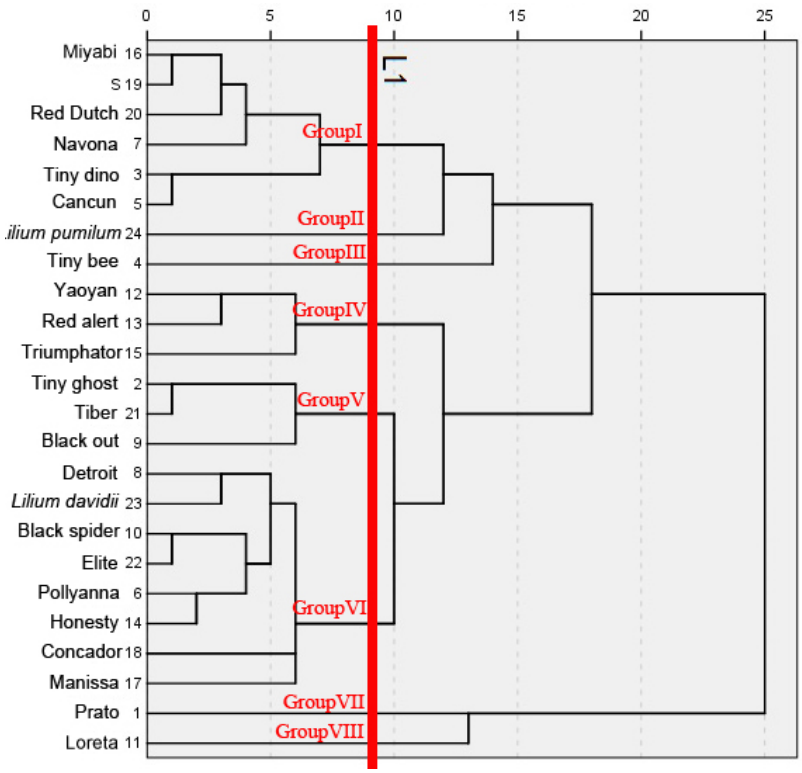

Figure 4. Q-cluster analysis of lily varieties via pollen morphological traits.

The results showed that cultivars of lily were highly heterozygous. And only single level of clustering method could not obtain the entirely consistent result with the current classification criteria presented by Royal Horticultural. In order to get an accurate clustering result, several methods should be combined scientifically and reasonably at different levels. To reveal the genetic relationships among all samples at different levels, cluster analysis was carried out using both phenotypic characteristics and pollen morphology. Cluster analysis allows for objective examination of the classification results using existing data and provides new ideas for modifying or improving the existing classification systems.

Analysis of ISSR markers and genetic diversity of lily cultivars

Of the 15 primers, 11 primers produced reliable banding patterns with high reproducibility and clear band resolution, and were tested for ISSR amplification (Table 5). Of 180 fragments produced, 179 polymorphic bands (99.44%) were used to evaluate genetic relationships within 40 cultivars. The percentage of polymorphic bands (PPB), observed number of alleles (N_A), effective number of alleles (N_E), Nei's gene diversity (h), and Shannon's information index (I) was 99.44%, 1.9944, 1.2461, 0.1692, and 0.2870, respectively. There were 76 amplified bands and 74 polymorphic loci for nine wild lilies and their variants, and the major indexes were as follows: PPB = 97.37%, N_A = 0.9737, N_E =1.6207, h = 0.1639, I = 0.5389.

The percentage of polymorphic loci in Asiatic hybrids, Oriental hybrids, OT hybrids, LA hybrids, Longiflorum hybrids, and LO hybrids was 72.22, 56.67, 51.67, 36.67, 12.78, and 0%, respectively (Table 6). The genetic diversity among different groups of *Lilium* cultivars is shown in Table 7. Nei's gene diversity (h) ranged from 0.0000 to 0.1526, with a total of 0.1692, and Shannon's diversity index (I) ranged from 0.0000 to 0.2505.

Table 5. List of primers, sequences, and annealing temperatures employed for the ISSR analysis.

No.	Primer	Sequence (5'-3')	Tm (°C)
1	3A59	$(CT)_7GTG$	50
2	3A20	$(CT)_7AGT$	50
3	3A8	$(TC)_7GGA$	52
4	3A37	$(CA)_7TGA$	52
5	3A61	$(CT)_7TGT$	48
6	UBC815	$(CT)_8G$	52
7	UBC844	$(CT)_8RC$	52
8	UBC845	$(CT)_8RG$	52
9	UBC841	$(GA)_8YC$	52
10	3A30	$(CT)_7GAA$	50
11	3A2	$(CT)_7ATC$	48

Tm: melting temperature.

Table 6. Percentage of polymorphic loci in different groups of *Lilium* cultivars.

Different cultivation	Samples	Total of bands	Polymorphic bands	PPB (%)
Asiatic hybrids	16	180	130	72.22
LA hybrids	4	180	66	36.67
LO hybrids	1	180	0	0
Longiflorum hybrids	2	180	23	12.78
OT hybrids	9	180	93	51.67
Oriental hybrids	8	180	102	56.67
Total	40	180	179	99.44

PPB: percentage of polymorphic bands.

Table 7. Genetic diversity among different groups of *Lilium* cultivars.

Different cultivation	Samples	N_A	N_E	h	I
Asiatic hybrids	16	1.7222	1.2326	0.1526	0.2505
LA hybrids	4	1.3667	1.1943	0.1191	0.1829
LO hybrids	1	1.0000	1.0000	0.0000	0.0000
Longiflorum hybrids	2	1.1278	1.0904	0.0529	0.0773
OT hybrids	9	1.5167	1.2202	0.1376	0.2173
Oriental hybrids	8	1.5667	1.2028	0.1366	0.2233
Total	40	1.9944	1.2461	0.1692	0.2870

N_A: Observed number of alleles; N_E: effective number of alleles; h: Nei's gene diversity; I: Shannon's information index.

The results of the present study indicated that both cultivars and wild species demonstrated a high level of genetic diversity. Specifically, the genetic diversity of wild lilies was higher than cultivars.

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Supplementary material

Table S1. Samples assessed for phenotypic traits.
Table S2. Samples assessed for pollen morphology.
Table S3. Samples assessed for ISSR markers.
http://www.geneticsmr.com/year2016/vol15-1/pdf/gmr7638_supplementary.pdf