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Genetic diversity and relationships of lotus (*Nelumbo*) cultivars based on allozyme and ISSR markers

Hong-Li Tian [a,d], Jian-Hua Xue [b], Jun Wen [a,c], Grant Mitchell [e], Shi-Liang Zhou [a,*]

[a] *State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Xiangshan, Beijing 100093, China*
[b] *Department of Life Sciences, Daqing Normal College, Daqing 163712, China*
[c] *Department of Botany, National Museum of Natural History, Smithsonian Institution, Washington, DC 20013-7012, USA*
[d] *Graduate School of the Chinese Academy of Sciences, Beijing 100039, China*
[e] *Liang Lian Lotus & Waterlily Farm, R.M.B. 824 Bunga 2550, NSW, Australia*

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Abstract

Genetic diversity and genetic relationships of lotus (*Nelumbo* Adanson) cultivars were evaluated using allozyme and ISSR markers. The samples used covered 11 accessions of possible hybrids between *Nelumbo nucifera* and *Nelumbo lutea* and 92 accessions of *N. nucifera* including 69 flower lotus, 13 rhizome lotus, 5 seed lotus and 5 wild lotus. For allozyme studies, a total of 31 alleles at 23 loci of 18 enzyme systems were detected of which 5 (21.7%) loci *Aat*, *Idh*, *Mdh*-2, *Pgd*, *Sod* were polymorphic. The loci of *Aat* and *Idh* included two alleles, *Mdh*-2, *Pgd* and *Sod* included three alleles. Eighteen genotypes were detected with the 13 alleles of the 5 polymorphic loci. The parameters of average allele number, observed heterozygosity, expected heterozygosity and Shannon information index of 92 *N. nucifera* samples were 1.35 ± 0.71, 0.06 ± 0.21, 0.05 ± 0.14, 0.10 ± 023, respectively. Thirteen ISSR primers generated 93 loci, of which 37.63% were polymorphic across all samples. The percentage of polymorphic loci, average allele number, expected heterozygosity and Shannon information index of 92 *N. nucifera* samples were 26.67%, 1.30 ± 0.46, 0.10 ± 0.18 and 0.15 ± 0.25, respectively for the ISSR data. The 'Bottleneck effect' and rapid propagation of clones after the ice ages may explain the low genetic diversity of lotus. The dendrograms based on ISSR and allozymes were not congruent. Based on the ISSR data, the 103 samples were divided into the *N. nucifera* group (Group I), and the group containing inter-specific hybrids between *N. nucifera* and *N. lutea* (Group II). The flower lotus, rhizome lotus, and seed lotus each has multiple sources of origin. Plant size, a criterion commonly used in the classification of cultivars of lotus, is not correlated with genetic variation. Flower color is correlated with the cultivar classification to some degree, but its variation is complex in the hybrids.

Keywords: *Nelumbo nucifera* Gaerten.; Lotus; Genetic diversity; Genetic relationships; Allozyme; ISSR

1. Introduction

Nelumbo is well known as an ancient taxon, belong to the family Nelumbonaceae and it consists of two eastern Asian and North American disjunct species, *N. nucifera* Gaerten and *N. lutea* Willd. The sacred lotus, *N. nucifera* Gaerten. is one of the important ornamental and economic plants in many parts of Asia. Lotus is one of the top ten traditional garden flowers in China. It is extensively cultivated in water gardens for its beautiful flowers and pleasant fragrance, and for cultural and religious reasons. The cultivars selected from natural variations have been cultivated in home gardens since ancient times in China, and some traditional cultivars have even been handed down to present day. Since the mid-20th century especially since the 1980s, there has been a rapid increase in interest in the collecting and breeding of lotus cultivars. At present, over 600 cultivars are now under cultivation (Wang and Zhang, 2005).

Identification of the numerous available cultivars is a major challenge for horticulturists. It is therefore necessary to clarify the origin of cultivars and test the classification systems with molecular genetic data. The traditional classifications are primarily based on plant size, flower diameter, flower color and uses. Since the 1980s, the origin of the cultivars was considered as an important criterion and the classification then tended to be more natural. In an evolutionary context, horticulturalists have been using the following evolutionary criteria/trends—(1)

* Corresponding author. Tel.: +86 10 62836503.
E-mail address: slzhou@ibcas.ac.cn (S.-L. Zhou).

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flower form evolving from few-petaled (single), to semidouble-petaled, to double-petaled, and finally to completely multi-layer-petaled; (2) flower color changing in the following evolutionary sequence: red → pink → white → green → varied color → versicolor; (3) plant size evolving from large to small such as the ''bowl lotus'' type as an example of the latter. Flower color is an important character for non-hybrid cultivar classification for many ornamental plants, say, *Osmanthus fragrans* (Hu et al., 2004). The color of lotus is mostly white or pink. Since the introduction of *N. lutea* into China, the color of cultivated lotus has become more diverse. In general, rhizome lotus is mostly white; seed lotus is primarily pink, while flower lotus is very rich in color variation. However, complexity in morphological variations and interbreeding between cultivars make it difficult to discern the direction of morphological changes and these rules seldom hold true. Cultivars with similar morphology do not necessarily have the closest evolutionary relationship.

Like many plants in cultivation, selection of mutants from the wild or cultivated forms and hybridization are the two major ways to develop new lotus cultivars. Many existing lotus cultivars have a complex origin and the parents of many cultivars have not been recorded. It is difficult to identify cultivars by morphology alone because the lotus cultivars were selected from genetic mutation, and are either mutants of gene expression or hybrids among *N. nucifera* cultivars. Homonyms and synonyms may also commonly exist among the names of cultivars. Several recent studies have documented the genetic diversity and constructed genetic relationships among cultivars using molecular markers such as AFLP (Peng et al., 2004), ISSR (Xue et al., 2006), RAPD (Kim et al., 1998), and mtDNA RFLP (Kanazawa et al., 1998). These studies have focused on samples from local regions or a small number of cultivars. Furthermore, the dominant nature or uniparental inheritance of these markers makes it difficult to discern the origin of certain cultivars bred via hybridization with unknown parents. When the sample size was small, a few loci may help to identify the different cultivars. However, with the increase of sample size, inconsistency among loci was high and the efficiency of molecular markers became a question. Furthermore, most cultivars contain only the genome of *N. nucifera*. Due to the low genetic diversity of *N. nucifera*, the difference among cultivars may be those of a few specific genes and not the whole genome.

In this study, we chose dominant and co-dominant markers (ISSR and allozyme) to analyze a large number of cultivars and a few wild lotus. The objectives of this study were to assess genetic diversity of lotus, determine genetic relationships of cultivars, test the established rules of evolution of morphological variation used in cultivar classification, and compare the efficacy of different molecular markers.

2. Materials and methods

2.1. Plant material

Fresh leaves from 103 lotus samples were collected and used in this study. The 103 collections included 80 flower lotus, 13 rhizome lotus, 5 seed lotus and 5 wild lotus. Among the flower lotus, there were 11 inter-specific hybrids between *Nelumbo nucifera* and *Nelumbo lutea* (Table 1).

2.2. Allozyme analysis

Small pieces of fresh leaves (about 0.2 g) from each of the 103 cultivars were ground on ice with 0.2-mL extracting buffer. The protocol followed Zhou et al. (1998a, 1998b). Three buffer systems were used to analyze 18 enzyme systems (Table 2).

2.3. ISSR analysis

The DNA extraction followed the CTAB procedure (Doyle and Doyle, 1987). Thirteen primers selected from 48 primers were used for ISSR reactions (Table 3). The PCR reaction mixture was 25 μL containing genomic DNA (about 25 ng), 1 unit Taq polymerase enzyme, 3 mM $MgCl_2$, 0.2 mM dNTP, 0.3 μM primer and 1× PCR buffer. Amplification was carried out in a thermocycler (PTC-100, Bio-RAD Corporation). The PCR program was 94 °C for 1.5 min followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 44 °C for 45 s, and DNA elongated at 72 °C for 1.5 min. The program ended with one more cycle of 45 s at 94 °C, 45 s at 44 °C, and 5 min at 72 °C. Amplified products were separated by electrophoresis on 1.5% agarose gels (Promega corporation), stained in ethidium-bromide (0.5%). The 100 bp DNA ladder (New England BioLabs Inc.) was used as a standard molecular weight. Photographs were taken using the automatic imaging system GEL-DOCUMENT 2000 (BIO-RAD Corporation).

2.4. Data analysis

Allozyme bands were identified and recorded according to Zhou et al. (1998a), and a binary matrix set of the genotype was obtained. Electrophoresis photographs of ISSR were analyzed with the software of Quantity-one version 4.3.1 (BIO-RAD corporation) combining manual verification. ISSR bands were scored as 1 (present) or 0 (absent) on a locus (bands of the same size were considered to belong to the same locus) to create a binary matrix set. Parameters—the percentage of polymorphic loci (*P*), observed average number of alleles (*A*), effective average number of alleles (*Ae*), observed heterozygosity (*Ho*), Nei (1973) expected heterozygosity (*He*), Shannon's information index (*I*) and Nei's genetic distance (GD) were all calculated with POPGENE version 1.31 (Yeh et al., 1999), according to the two set data. The UPGMA dendrogram was obtained using the Nei's genetic distance with the software MEGA version 3.0 (Kumar et al., 2004). We also used software STRUCTURE version 2.2 (Pritchard et al., 2000) to estimate the data structure. Seven runs ($K = 2$–8) were performed using admixture model and all runs utilized 10,000 iterations after a burn-in 5,000 iterations based on ISSR dataset.