Assessment of the Genetic Diversity and Population Structure of Lotus Cultivars Grown in China by Amplified Fragment Length Polymorphism

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ADDITIONAL INDEX WORDS. *Nelumbo*, genetic variation, molecular marker

ABSTRACT. To assess the genetic diversity among lotus (*Nelumbo*) accessions and evaluate the correlation between genetic variation and morphological classification, we sampled 138 accessions: two of *N. lutea*, 112 of *N. nucifera*, 17 of hybrids between *N. nucifera* and *N. lutea*, and seven Japanese cultivars. The 11 selected combinations of amplified fragment length polymorphism (AFLP) primers produced 138 polymorphic loci, and the percentage of polymorphism was 28.7%. The unweighted pair group method with arithmetic mean (UPGMA) dendrogram clustered all the accessions into two groups: Group I comprised *N. lutea* and its hybrids with *N. nucifera*; Group II included *N. nucifera* and its hybrids with *N. lutea* and Japanese cultivars. Population structure analysis identified four main clusters: *N. lutea* clustered mainly in C1, whereas *N. nucifera* clustered in C2, C3, and C4, which was consistent with the UPGMA and principal coordinate analysis results. The Japanese cultivars were related more closely to *N. nucifera* (genetic similarity coefficient = 0.74) than to *N. lutea* (0.46); hence, the Japanese cultivars can be classified as *N. nucifera*. Moreover, rhizome lotuses formed a separate subclade, whereas seed lotuses were interspersed among flower lotuses, which demonstrated that rhizome lotuses were distinct from flower and seed lotuses. Plant size, flower color, and other morphological criteria used commonly to classify lotuses were correlated with genetic variation to a certain extent but not sufficiently for accurate classification. It appears that it is necessary to use both DNA markers and morphological characteristics to classify lotus species and cultivars.

Lotus is in the family Nelumbonaceae, which consists of only one genus with two species, *Nelumbo nucifera* and *Nelumbo lutea* (Kanazawa et al., 1998; Savolainen and Chase, 2003). *Nelumbo nucifera* is found throughout Asia and Australia.

In China, *N. nucifera* is distributed on both sides of the major freshwater lakes in the valleys of the Yangtze River, Yellow River, and Pearl River. *Nelumbo lutea* is distributed across North America and northern South America but grows mainly in the eastern and central United States (Borsch and Wilhelm, 1994). Although these two species differ in external morphology such as plant size, leaf shape, petal shape, and color, as a result of their geographical isolation, they share the same number of chromosomes ($2n = 16$) and the similar karyotype and can be hybridized easily. It has been proposed that *N. lutea* should be considered a subspecies of *N. nucifera* (Huang et al., 1992). However, other scholars consider the two taxa to be

Received for publication 14 Apr. 2011. Accepted for publication 5 July 2011. This work was supported by the National Natural Science Foundation of China (30671474).

We thank Liming Xu and Jiarong Zhao for their kind help in the experimental sampling and the analysis of morphological traits.

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separate species in the genus *Nelumbo* (Chen et al., 2008; Han et al., 2007a, 2007b, 2009; Kubo et al., 2009; Li et al., 2010; Tian et al., 2008b).

Nelumbo nucifera is an important ornamental aquatic plant with beautiful flowers and unusual leaves (Ni and Zhao, 1987; Wang and Zhang, 2005). It is also a source of herbal medicine with strong antipyretic, cooling, astringent, and demulcent properties, which can be attributed to alkaloids and flavonoids in the leaves, leaf stems, and rhizomes (Mukherjee et al., 1997; Qian, 2002; Sinha et al., 2000). *Nelumbo lutea* is a species as magnificent as *N. nucifera*, but it is cultivated less commonly as an ornamental plant. It is considered to be an invasive, difficult-to-eradicate weed in its original habitat (Borsch and Wilhelm, 1994; Wiersema, 1997) and probably originally confined to flood plains of major rivers and their tributaries in the east-central United States and carried northward and eastward by aborigines who used the seeds and tubers for food. The rhizome and seeds of *N. lutea* are commonly eaten by Native Americans, and those of *N. nucifera* are still a part of the Oriental diet. In Japan, the lotus has been cultivated as an ornamental and food plant since it was introduced from China 1000 years ago (Masuda et al., 2006). Approximately 350 cultivars of lotus are known in Japan, including more than 100 landraces (Kubo et al., 2009).

In China, the lotus has been cultivated for more than 3000 years, and rich germplasm resources have been generated by artificial hybridization and selection (Ni and Zhao, 1987; Wang and Zhang, 2005). More than 600 lotus cultivars have been recorded in China and have been classified into three main types on the basis of their morphological characteristics and agricultural properties: 1) flower lotus; 2) seed lotus; and 3) rhizome lotus (Wang and Zhang, 2005; Zou et al., 1997). The flower lotus cultivars have been subdivided further on the basis of petal characteristics into several types, which include: 1) few-petaled lotus, with less than 24 petals per flower; 2) double-petaled lotus, in which most of the stamens have been transformed into petals; 3) duplicated-petaled lotus, in which pistils have been transformed into petals or bubbled; 4) versicolor lotus, with red strips or blocks dotted on the white petal; and 5) thousand-petaled lotus, in which the stamens, pistils, and receptacles have all been transformed into petals (Wang and Zhang, 2005). However, such characteristics might not be useful for classification because of their continuous variation and high degree of plasticity in response to changes in environmental conditions. In light of these limitations, further analysis of the genetic differences among lotus cultivars by molecular methods is necessary.

In recent years, the rapid development of molecular marker technologies and DNA fingerprinting analysis has provided new techniques to assess the genetic diversity of plants and animals such as random amplified polymorphic DNA (RAPD), AFLPs, intersimple sequence repeats (ISSRs), and simple sequence repeats (SSRs) (Ge et al., 2005; Oraguzie et al., 2001; Torres-Díaz et al., 2007; Wang et al., 2011). These approaches have also been used to analyze the genetic diversity of the lotus (Chen et al., 2008; Guo et al., 2007; Han et al., 2007a, 2007b, 2009; Kubo et al., 2009; Li et al., 2010; Pan et al., 2007, 2010; Tian et al., 2008a, 2008b) and have revealed rich genetic variation in lotus germplasm. Although AFLP markers are dominant in most cases, they can detect more variation at the whole genome level than ISSR or RAPD markers and are more efficient, because large numbers of loci can be screened readily (Na et al., 2009; Portis et al., 2005). At present, only one

study that used AFLP markers to evaluate the genetic diversity of rhizome lotuses has been published (Peng et al., 2004). However, the limited number of AFLP markers (two pairs) and lotus accessions (12 rhizome lotuses) that were used was not sufficient to assess the genetic variation of the lotus.

In the study reported here, 11 AFLP primer pairs were selected to evaluate the genetic diversity and relationships among 138 lotus accessions. The accessions represented a large collection of genotypes of the two species (*N. lutea* and *N. nucifera*): flower lotuses, seed and rhizome cultivars, ancient lotuses, Japanese cultivars, and tropical lotuses. The objectives were to: 1) evaluate the genetic relationship between *N. nucifera* and *N. lutea*; 2) assess the genetic diversity among flower, seed, and rhizome lotuses; and 3) confirm which morphological characteristics can be used in the classification of lotuses.

Materials and Methods

PLANT MATERIALS. In total, 138 lotus accessions were used in this study (Table 1): two of *N. lutea*, 112 of *N. nucifera* (76 flower lotuses, nine seed lotuses, 26 rhizome lotuses, and one ancient lotus), 17 of hybrids between *N. nucifera* and *N. lutea*, and seven Japanese cultivars. All accessions have been collected and lodged at Wuhan Botanical Garden, Chinese Academy of Sciences in Wuhan, Hubei Province, China.

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM ANALYSIS. Approximately 2 g of fresh young leaves were harvested from plants and used for the extraction of total genomic DNA in accordance with the cetyltrimethylammonium bromide procedure described by Doyle and Doyle (1990). The quality and quantity of the DNA were checked by agarose gel electrophoresis and ultraviolet spectrophotometry. The DNA solution was diluted to 50 ng·μL⁻¹ with 1 mol·L⁻¹ Tris-EDTA buffer and used as a template in the polymerase chain reaction (PCR) amplification for the AFLP analysis.

The AFLP analysis was performed in accordance with the protocol developed by Vos et al. (1995). Genomic DNA was digested with the restriction enzymes *EcoRI* and *MseI* amplified by PCR. The PCR products were separated by electrophoresis on polyacrylamide gels and stained by silver nitrate solution. To improve the efficiency, all 224 possible AFLP primer combinations were used first to amplify 10 representative samples. From the preliminary screening, 11 primer combinations that gave repeatable, clear, and abundant polymorphic bands were selected for the analysis of all 138 accessions. The sequences of the 11 primer combinations selected are listed subsequently:

E01/M04, 5'-GACTGCGTACCAATTCATC-3'/5'-GATGAGTCCT GAGTAACTA-3';
E02/M18, 5'-GACTGCGTACCAATTCAGA-3'/5'-GATGAGTCCT GAGTAACAC-3';
E03/M03, 5'-GACTGCGTACCAATTCACA-3'/5'-GATGAGTCCT GAGTAACTG-3';
E03/M13, 5'-GACTGCGTACCAATTCACA-3'/5'-GATGAGTCCT GAGTAACGC-3';
E03/M18, 5'-GACTGCGTACCAATTCACA-3'/5'-GATGAGTCCT GAGTAACAC-3';
E04/M05, 5'-GACTGCGTACCAATTCAC-3'/5'-GATGAGTCCT GAGTAACAA-3';
E04/M18, 5'-GACTGCGTACCAATTCAC-3'/5'-GATGAGTCCT GAGTAACAC-3';

Table 1. Name, collection number in Wuhan Botanic Garden (WBG), origin, morphological characteristics, and amplified fragment length polymorphism (AFLP) group for 138 lotus accessions analyzed by AFLP.

No.	Accession name	Collection no.	Species or origin	Sample type	Plant size ^z	Petal form ^y	Flower color ^x	AFLP group	
								Group	Subgroup
1	Oriole	WBG307	Hybrid	Flower	Ms	sD	Y	I	Ia1
2	Pond Oriole	WBG308	Hybrid	Flower	Ms	sD	Y	I	Ia1
3	Jasmine Lotus	WBG205	Hybrid	Flower	Ms	F	Y	I	Ia1
4	Golden Lotus	WBG242	<i>Nelumbo lutea</i>	Flower	L	F	Y	I	Ia1
5	Yellow Garden	WBG938	Hybrid	Flower	Ms	sD	Y	I	Ia1
6	Fragrant Seeping	WBG213	Hybrid	Flower	Ms	sD	Y	I	Ia1
7	Yellow Dancer	WBG117	Hybrid	Flower	L	F	Y	I	Ia1
8	Golden Bird	WBG210	Hybrid	Flower	Ms	F	Y	I	Ia1
9	America Lotus	WBG1042	<i>N. lutea</i>	Flower	Ms	F	Y	I	Ia2
10	Learning Dance	WBG1143	Hybrid	Flower	Ms	sD	Y	I	Ia2
11	Cool Charming	WBG836	Hybrid	Flower	Ms	D	Y	I	Ib
12	Resplendence	WBG807	Hybrid	Flower	Ms	D	Y	I	Ib
13	Full of Gold and Jade	WBG907	Hybrid	Flower	Ms	D	Y	I	Ib
14	Little Birthday	WBG315	<i>Nelumbo nucifera</i>	Flower	Ms	D	R	II	Ila1
15	September Purple	WBG842	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila1
16	Zhuahongcha Bowl	WBG1219	Japanese cultivar	Flower	Ms	F	R	II	Ila1
17	Yiliang Thousands Petals	WBG320	<i>N. nucifera</i>	Flower	L	T	R	II	Ila2
18	Thousands Petals	WBG1321	<i>N. nucifera</i>	Flower	L	T	R	II	Ila2
19	Pink Double Palace	WBG1130	<i>N. nucifera</i>	Flower	L	Du	R	II	Ila2
20	Puzhehei Red Lotus	WBG324	<i>N. nucifera</i>	Flower	L	D	R	II	Ila2
21	Red Thousand Petals	WBG1133	<i>N. nucifera</i>	Flower	L	D	R	II	Ila2
22	Good Luck Lotus	WBG224	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila2
23	Embroidery Jade	WBG1135	Japanese cultivar	Flower	Ms	D	R	II	Ila2
24	Red Peony	WBG1137	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila2
25	Camellia Red	WBG1249	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila2
26	Wan'er Hong	WBG1123	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila2
27	Red River	WBG109	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila2
28	Red Bowl	WBG1129	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila2
29	Frost Morning Moon	WBG416	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila2
30	Pink Exquisite	WBG532	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila2
31	Tender Face	WBG403	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila2
32	Lushan Red	WBG1125	Japanese cultivar	Flower	Ms	D	R	II	Ila2
33	Duplicate-petalled Green	WBG1026	<i>N. nucifera</i>	Flower	Ms	Du	W	II	Ila3
34	Gardenia Bowl Lotus	WBG418	<i>N. nucifera</i>	Flower	Ms	Du	W	II	Ila3
35	Brilliant Moon	WBG622	<i>N. nucifera</i>	Flower	Ms	D	W	II	Ila3
36	Green-White Lotus	WBG732	<i>N. nucifera</i>	Flower	Ms	Du	W	II	Ila3
37	A Double Handful Snow	WBG817	<i>N. nucifera</i>	Flower	Ms	sD	W	II	Ila3
38	Golden Pearls on Jade Plate	WBG1347	<i>N. nucifera</i>	Flower	Ms	F	W	II	Ila3
39	Luminescent Pearl	WBG630	<i>N. nucifera</i>	Flower	Ms	F	W	II	Ila3
40	Bright Moon	WBG1337	<i>N. nucifera</i>	Flower	Ms	sD	W	II	Ila3
41	Green on Jade Plate	WBG721	<i>N. nucifera</i>	Flower	Ms	Du	W	II	Ila3
42	Jifei Lian	WBG1213	Japanese cultivar	Flower	Ms	F	R	II	Ila4
43	Sparkle	WBG621	<i>N. nucifera</i>	Flower	Ms	F	R	II	Ila4
44	Cherry Lotus	WBG1209	Japanese cultivar	Flower	Ms	F	R	II	Ila4
45	Color of Girl's Face and Peach Flower	WBG710	<i>N. nucifera</i>	Flower	Ms	D	V	II	Ila5
46	Versicolor	WBG910	<i>N. nucifera</i>	Flower	Ms	D	V	II	Ila5
47	Red Lady	WBG935	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila6
48	Rosy Dawn Light	WBG1250	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila6
49	Red Skirt with Gold Embroidery	WBG838	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila6
50	Early Peach-red	WBG524	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila6
51	Table Lotus	WBG1127	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila6
52	Red Stay when Green Faded	WBG824	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila6
53	Autumn Beauty	WBG618	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila6
54	Yangcheng Bowl	WBG1016	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila6

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Table 1. Continued.

No.	Accession name	Collection no.	Species or origin	Sample type	Plant size ^z	Petal form ^y	Flower color ^x	AFLP group	
								Group	Subgroup
55	Hundred Petals	WBG1109	<i>N. nucifera</i>	Seed	Ms	sD	R	II	Ila7
56	Fujian Red Flower	WBG1114	<i>N. nucifera</i>	Seed	Ms	F	R	II	Ila7
57	Hunan White	WBG1112	<i>N. nucifera</i>	Seed	L	F	V	II	Ila7
58	Hunan White Stamen	WBG1116	<i>N. nucifera</i>	Seed	L	F	R	II	Ila7
59	Hunan	WBG1118	<i>N. nucifera</i>	Seed	L	F	R	II	Ila7
60	Red Screen	WBG639	<i>N. nucifera</i>	Flower	L	F	R	II	Ila7
61	Rouge	WBG114	<i>N. nucifera</i>	Flower	Ms	F	R	II	Ila7
62	White Small Gentleman	WBG1223	Japanese cultivar	Flower	Ms	F	W	II	Ila7
63	Red in Snow	WBG406	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila7
64	Thai #1	WBG408	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila7
65	Winter's Red	WBG407	<i>N. nucifera</i>	Flower	Ms	F	R	II	Ila7
66	Zhuzhou Red Lotus	WBG212	<i>N. nucifera</i>	Flower	L	F	R	II	Ila8
67	Black Lotus	WBG739	<i>N. nucifera</i>	Flower	L	F	R	II	Ila8
68	Qinglianzi	WBG1218	<i>N. nucifera</i>	Flower	L	F	R	II	Ila8
69	Big Red Coat	WBG1217	<i>N. nucifera</i>	Flower	L	F	R	II	Ila8
70	East Mountain Red Coat	WBG1126	<i>N. nucifera</i>	Seed	L	F	R	II	Ila8
71	Baiyangdian Red	WBG1106	<i>N. nucifera</i>	Flower	L	F	R	II	Ila9
72	Chinese Antique Lotus	WBG1202	<i>N. nucifera</i>	Ancient	L	F	R	II	Ila9
73	Ohga Lotus	WBG1203	Japanese cultivar	Ancient	L	F	R	II	Ila9
74	Fujian White	WBG1110	<i>N. nucifera</i>	Seed	L	F	W	II	Ila9
75	Red Lotus	WBG1122	<i>N. nucifera</i>	Seed	L	F	R	II	Ila9
76	Red Coat	WBG1325	<i>N. nucifera</i>	Seed	L	F	R	II	Ila9
77	Hefei Lotus	WBG537	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
78	Zhou Ou	WBG1424	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
79	Hangzhou White Flower	WBG1422	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
80	Daqingkai	WBG1522	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
81	Yacheng	WBG1404	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
82	Beijing White Flower	WBG1421	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
83	Slow Lotus	WBG1418	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
84	Xiaomaojie	WBG1423	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
85	Big Square	WBG1426	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
86	Bamboo Joint	WBG1427	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
87	Flower Lotus	WBG1532	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
88	Jia Yu	WBG1533	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
89	June Early	WBG1434	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
90	Beijing Pink Flower	WBG1412	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
91	Two White Flowers	WBG1531	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
92	Lutouzhong	WBG1519	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
93	Falling Flowers	WBG1520	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
94	Big Lying Dragon	WBG1415	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
95	Long June Early	WBG1429	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
96	Snow Lake	WBG1431	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
97	Winter Lotus	WBG1408	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
98	Pig Tail	WBG1432	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
99	Fresh Flowers	WBG1521	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
100	Hainan	WBG1417	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
101	Early Lotus	WBG1518	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
102	Paozi	WBG1411	<i>N. nucifera</i>	Rhizome	L	F	W	II	Ila10
103	Little Buddha's Seat	WBG1227	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila11
104	Pink Evening Glow	WBG1131	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila11
105	Pearls on Red-plate	WBG113	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila11
106	Apricot Blossom in Spring Rain	WBG1333	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila11
107	Love Poem	WBG640	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila11
108	Dripping Dew	WBG1009	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila11
109	Drunken Cloud	WBG641	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila11

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Table 1. Continued.

No.	Accession name	Collection no.	Species or origin	Sample type	Plant size ^z	Petal form ^y	Flower color ^x	AFLP group	
								Group	Subgroup
110	Little Drunken Fairy	WBG733	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila11
111	Chrysanthemum Pot	WBG534	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila11
112	Jingzhou Peony-Pink	WBG940	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila12
113	Drunken Beauty	WBG116	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila12
114	Pingshan Lotus	WBG734	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila12
115	Spring Red	WBG134	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila13
116	Rouge Dew	WBG1015	<i>N. nucifera</i>	Flower	Ms	Du	R	II	Ila13
117	The Sound of Autumn	WBG830	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila13
118	Red Rolling Silk Ball	WBG1003	<i>N. nucifera</i>	Flower	Ms	Du	R	II	Ila13
119	Fair Dawn	WBG609	<i>N. nucifera</i>	Flower	Ms	sD	R	II	Ila13
120	Full Happiness	WBG610	<i>N. nucifera</i>	Flower	Ms	sD	R	II	Ila13
121	Little Beauty	WBG631	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila13
122	Poem of Autumn	WBG724	<i>N. nucifera</i>	Flower	Ms	Du	R	II	Ila13
123	Peach with Raindrops	WBG1012	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila13
124	Red Bowl	WBG1002	<i>N. nucifera</i>	Flower	Ms	D	R	II	Ila13
125	Little Duplicate Red	WBG1105	<i>N. nucifera</i>	Flower	Ms	Du	R	II	Ila13
126	Pearls in Green House	WBG708	<i>N. nucifera</i>	Flower	Ms	Du	R	II	Ila13
127	Red Exquisite	WBG302	<i>N. nucifera</i>	Flower	Ms	Du	R	II	Ila13
128	Rose Red Duplicate	WBG1619	<i>N. nucifera</i>	Flower	Ms	Du	R	II	Ila13
129	Spring Hope	WBG812	<i>N. nucifera</i>	Flower	Ms	sD	P	II	Ila14
130	Pink Tower	WBG102	<i>N. nucifera</i>	Flower	Ms	D	P	II	Ila14
131	Pure Heart	WBG901	<i>N. nucifera</i>	Flower	Ms	sD	W	II	Ila15
132	Little Antelope	WBG912	<i>N. nucifera</i>	Flower	Ms	sD	W	II	Ila15
133	Little Oriole	WBG918	Hybrid	Flower	Ms	sD	Y	II	Ilb
134	Golden Sun	WBG917	Hybrid	Flower	Ms	D	Y	II	Ilb
135	Colorful Glow	WBG937	Hybrid	Flower	Ms	D	V	II	Ilb
136	Orange of Loquat	WBG342	Hybrid	Flower	Ms	D	V	II	Ilb
137	Charming Lips	WBG332	Hybrid	Flower	Ms	Du	V	II	Ilb
138	Red Sun	WBG338	Hybrid	Flower	Ms	D	V	II	Ilb

^zMs = medium to small plant; L = large plant.

^yF = few petals; sD = semidouble petals; D = double petals with most of the stamens transformed into petals; Du = duplicated petals with stamens and pistils transformed into petals or bubbled; T = thousand petals with stamens, pistils, and receptacles all transformed into petals.

^xY = yellow flower; W = white flower; R = red flower; P = pink flower; V = versicolor flower.

E05/M02, 5'-GACTGCGTACCAATTCAAG-3'/5'-GATGAGTCCT GAGTAACCA-3';
 E05/M16, 5'-GACTGCGTACCAATTCAAG-3'/5'-GATGAGTCCT GAGTAACCC-3';
 E06/M05, 5'-GACTGCGTACCAATTCAGT-3'/5'-GATGAGTCCT GAGTAACAA-3';
 E07/M04, 5'-GACTGCGTACCAATTCAAC-3'/5'-GATGAGTCCT GAGTAAC-3'.

STATISTICAL DATA ANALYSIS. The AFLP bands at a given locus were scored as 1 (present) or 0 (absent) to create a binary matrix set (bands of the same size were considered to belong to the same locus).

Genetic similarity among all accessions was calculated by using NTSYS-pc version 2.1 software (Rohlf, 2000) on the basis of the simple matching coefficient and then a dendrogram was constructed by UPGMA. The binary data were also subjected to principal coordinate analysis (PCA) using EIGEN modules.

The population structure of the lotus accessions was estimated on the basis of the AFLP data set using the software STRUCTURE Version 2.2 (Pritchard et al., 2000). The assumed number of populations (*K*) was varied between two and 10. Five runs for each *K* value were conducted using an admixture model and correlated allele frequencies. A burn-in

period of 10,000 followed by 50,000 iterations was used for clustering of populations.

Results

GENETIC DIVERSITY OF LOTUS ACCESSIONS. Eleven AFLP primer combinations were selected from all the combinations of AFLP primers and yielded abundant polymorphic markers among the 138 lotus accessions. The scored DNA fragments ranged in size from 75 to 300 bp. In total, 481 bands were obtained from PCR amplification using the 11 AFLP primer combinations, of which 138 (28.7%) were polymorphic across all samples. The number of polymorphic loci per primer combination varied from five to 21 with a mean of 13.

Genetic similarity among the lotus accessions was estimated using NTSYS-pc 2.1. The genetic similarity coefficient among the accessions varied from 0.21 to 0.99 with an average of 0.68 (data not shown). To further assess the genetic similarity of different kinds of lotus accessions used in this study, the material was divided into seven groups on the basis of origin and use: *N. nucifera*, *N. lutea*, hybrid between *N. nucifera* and *N. lutea*, Japanese cultivar, flower lotus, seed lotus, and rhizome lotus (Table 2). The similarity coefficient between *N. nucifera* and *N. lutea* (0.42) was much lower than those within these two

Table 2. Genetic similarity coefficients of lotus accessions on the basis of amplified fragment length polymorphism analysis.^z

	<i>Nelumbo nucifera</i>	<i>Nelumbo lutea</i>	Hybrid	Japanese cultivar	Flower lotus	Seed lotus	Rhizome lotus
<i>N. nucifera</i>	0.74						
<i>N. lutea</i>	0.42	0.68					
Hybrid	0.51	0.61	0.59				
Japanese cultivar	0.74	0.46	0.52	0.74			
Flower lotus	0.69	0.55	0.53	0.69	0.66		
Seed lotus	0.75	0.41	0.52	0.74	0.69	0.76	
Rhizome lotus	0.79	0.37	0.50	0.71	0.69	0.80	0.90

^zNumbers on the diagonal are the average coefficients of the lotus accessions within each group; the numbers below the diagonal are the average coefficients between the groups.

groups (0.74 and 0.68, respectively). The coefficient within the hybrid accessions (0.59) was the lowest for all intragroup similarity. Moreover, the coefficient between the hybrid accessions and those of *N. nucifera* (0.51) was lower than the coefficient between the hybrid accessions and those of *N. lutea* (0.61).

The mean similarity coefficient within the Japanese cultivars (0.74) was the same as that between the Japanese cultivars and the accessions of *N. nucifera*. This value was much higher than the coefficient between the Japanese cultivars and *N. lutea* (0.46) and represented that the Japanese cultivars were related more closely to *N. nucifera* than to *N. lutea*. The coefficient within the groups of rhizome lotus cultivars was 0.90, which was greater than those within the groups of flower lotuses (0.66) or seed lotuses (0.76) and indicated that the genetic relationships among the cultivars of rhizome lotuses were closer than those among the flower and seed lotuses.

CLUSTER ANALYSIS. From the UPGMA analysis of the AFLP data, a dendrogram was constructed for the 138 lotus accessions (Fig. 1). The accessions were all divided into two groups (Groups I and II) with a similarity coefficient of 0.45. The 13 accessions in Group I corresponded to *N. lutea* and hybrids of *N. lutea* with *N. nucifera* and were all flower lotuses with yellow petals. Group II was composed of accessions of *N. nucifera*, hybrids of *N. nucifera* with *N. lutea*, and Japanese cultivars. The cultivars in this group were a mixture of flower, seed, and rhizome lotuses. Flower color was diverse, from white to red, and flower type varied in regard to the number of petals.

Group I was divided further into two subgroups (Ia and Ib) with a similarity coefficient of ≈ 0.58 . Group Ia consisted of 10 accessions: the two *N. lutea* accessions ('Golden Lotus' and 'America Lotus') and their hybrids with *N. nucifera*. Group Ib (No. 11-13) included the cultivar Cool Charming and its hybrid progenies with *N. nucifera*. The origin of 'Cool Charming' is unknown, but it was found here to have a close genetic relationship with hybrids between *N. lutea* and *N. nucifera*; thus, it might itself be a hybrid. 'Golden Lotus' (No. 4) of *N. lutea* and its hybrids (No. 1 to -3 and 5 to 8) were clustered in Group Ia1, whereas 'America Lotus' (No. 9) and its hybrid (No. 10) formed Group Ia2.

Group II could also be subdivided into two subgroups (IIa and IIb) with a similarity coefficient of 0.62. One hundred nineteen accessions (No. 14 to 132), which included all the *N. nucifera* and Japanese cultivars, were clustered in Group IIa. Flower color in this subgroup was mainly white, pink, or red. The six accessions in Group IIb were hybrids between *N. lutea* and *N. nucifera* and had yellow or versicolor flowers. Two of the hybrids, 'Little Oriole' (No. 133) and 'Golden Sun' (No. 134), which both had yellow petals, were the progeny of 'Golden Lotus', but the parents of the other four hybrids were

unknown. Group IIa comprised 15 subclusters (IIa1 to IIa15), and most of the accessions gathering in each group had a consistent overall plant size and flower color (Table 1). The accessions in Groups IIa8 and IIa9 (flower and seed lotuses) and IIa10 (rhizome lotus) shared a common feature of large-sized plant. The flower and seed lotuses in these groups mainly had red petals, whereas the rhizome lotuses mainly had white petals. Medium to small-sized accessions were clustered in other subgroups, and the clustering was consistent with petal color.

The PCA revealed a similar grouping of the accessions as the dendrogram constructed by UPGMA (Fig. 2). For instance, in the dendrogram, accessions 1 to 13 and 133 to 138, which corresponded to *N. lutea* and hybrids between *N. lutea* and *N. nucifera*, were located in Groups I and IIb, respectively, and were genetically very different from *N. nucifera* (Group IIa). These *N. lutea* and hybrid accessions were also separated from *N. nucifera* by PCA.

POPULATION STRUCTURE OF LOTUS ACCESSIONS. The results are shown in Figure 3. The number of clusters, *K* (which corresponds to the number of populations), was inferred to be four on the basis of likelihood values. Lotus accessions 1 to 13 belonged to cluster C1, which was consistent with their clustering into Group I in the dendrogram constructed by UPGMA and the results of the PCA. Accessions 17 to 65 were clustered mainly in C2. Cluster C3 contained mainly accessions 66 to 102. Cluster C4 included accessions 115 to 127. The other accessions (No. 128 to 138) belonged to multiple clusters, which indicated their complex genetic composition. These estimates are quite similar to the results obtained with the UPGMA cluster analysis.

The two accessions of *N. lutea*, 'Golden Lotus' (No. 4) and 'America Lotus' (No. 9), were consistently in cluster C1. The ancient lotuses 'Chinese Antique Lotus' (No. 72) and 'Ohga Lotus' (No. 73) were consistently in cluster C3. The hybrids and other Japanese cultivars were distributed through multiple clusters. The seed lotuses were found in cluster C2 or C3, and the rhizome lotuses were distributed in C3, which indicated that they were genetically uniform. In contrast, the flower lotuses showed up in multiple clusters.

Discussion

THE GENETIC RELATIONSHIP BETWEEN *N. NUCIFERA* AND *N. LUTEA*. Lotuses are basal eudicots and have a longer evolutionary history than most flowering plants. They have accumulated considerable genetic diversity, particularly as a result of the geographical isolation of the two species (Ni and Zhao, 1987; Wang and Zhang, 2005). The 138 lotus accessions that were analyzed in the study were derived from *N. nucifera* and *N. lutea* directly or indirectly through interspecific or intraspecific

Fig. 1. Dendrogram of 138 lotus accessions obtained by unweighted pair group method with arithmetic mean cluster analysis of data from amplified fragment length polymorphism markers. Simple similarity values are given at the bottom of the dendrogram. Numbers on the right of the dendrogram indicate the 138 lotus accessions and correspond with Table 1. Roman numerals I and II indicate the two groups that were discriminated by a similarity coefficient of 0.45. Groups Ia and Ib, and IIa and IIb are the subgroups of Groups I and II, respectively. Ia1 to Ia2 and IIa1 to IIa15 are the subclusters within subgroups Ia and IIa, respectively.

Fig. 2. Principal coordinates analysis of 138 lotus accessions on the basis of amplified fragment length polymorphism markers. The numbers at each dot indicate the 138 lotus accessions and correspond with Table 1. The accessions within the four groups formed are identical to those in Groups Ia, Ib, IIa, and IIb in the dendrogram constructed by unweighted pair group method with arithmetic mean.

Fig. 3. Population structure of 138 lotus accessions on the basis of amplified fragment length polymorphism markers. Each individual accession is depicted by a thin rectangle that is broken into four colored segments that represent the estimated membership fractions of the four clusters (C1, C2, C3, and C4). Numbers under each thin rectangle indicate the 138 lotus accessions and correspond with Table 1.

hybridization. The accessions were divided into two groups [I and II (Fig. 1)]. The two accessions of *N. lutea* were assigned to Group I and thus were separated from the accessions of *N. nucifera*, which were all in Group II. The genetic similarity coefficient between the two species was low [0.42 (Table 2)]. The STRUCTURE analysis demonstrated that *N. lutea* was consistently in cluster C1 (Fig. 3), whereas the accessions of *N. nucifera* were found in clusters C2, C3, and C4. These results confirmed that *N. lutea* was genetically distinct from *N. nucifera* and supported the conclusion that *N. nucifera* and *N. lutea* are two separate species in the genus *Nelumbo* as has been reported previously with different types of molecular marker

(Chen et al., 2008; Han et al., 2007a, 2007b, 2009; Kubo et al., 2009; Li et al., 2010; Tian et al., 2008b). It is likely that the genetic differentiation of *N. lutea* and *N. nucifera* is the result of long periods of geographical isolation and a lack of gene flow.

Despite their geographical isolation, there are no hybridization barriers between the two separate species and they can be crossed easily (Huang et al., 1992). In recent years, in an attempt to obtain cultivars with yellow flowers, hybridization between *N. lutea* and *N. nucifera* has been practiced extensively. In the current study, 17 hybrids derived from the two species were sampled, and there was a greater number of hybrids with yellow flowers than with versicolor flowers, which

might be the result of the preference of breeders for cultivars with yellow petals. On the basis of morphological characteristics, Wang and Zhang (2005) proposed to classify hybrids of *N. lutea* and *N. nucifera* as a single class. However, the 17 hybrids analyzed here were found to be dispersed between both groups of the genetic dendrogram (Table 1; Fig. 1). The differences between the hybrids in the two groups might be caused by different genetic compositions. The major genetic contribution to hybrids in Group I would appear to be derived from *N. lutea*, whereas the major genetic contribution to hybrids in Group II appeared to be derived from *N. nucifera*; this difference was confirmed by the STRUCTURE results. The hybrids in Group I were consistently in cluster C1, whereas those in Group II had a more complex genetic composition and were distributed among clusters C1, C2, C3, and C4 (Fig. 3). This result indicates that the selection and backcrosses were made to either *N. lutea* to give the hybrids found in Group I or *N. nucifera* to give the hybrids found in Group II.

THE GENETIC RELATIONSHIP BETWEEN LOTUS CULTIVARS FROM CHINA AND JAPAN. In this study, seven Japanese cultivars (which included the ancient lotus ‘Ohga Lotus’) were analyzed to evaluate their genetic relationship with *N. nucifera* and *N. lutea*. These cultivars were all clustered in Group IIa and were scattered among the *N. nucifera* accessions from China (Figs. 1 and 2). The coefficient of genetic similarity between the Japanese cultivars and *N. nucifera* was much higher than that between the Japanese cultivars and *N. lutea* [0.74 versus 0.46 (Table 2)], which suggested that the Japanese cultivars probably belong to *N. nucifera*. This result was supported by previous studies that used internal transcribed spacer, ISSR, and SSR markers (Han et al., 2007a; Kubo et al., 2009) and is consistent with the geographical distribution of *N. nucifera*.

The ancient Chinese lotus ‘Chinese Antique Lotus’ (No. 72) and the Japanese cultivar Ohga Lotus (No. 73) both originated from the seeds of ancient lotuses that had been buried underground for more than 1000 years and were discovered at Pulandian, Liaoning Province, China, and Chiba-ken, Japan, in 1951, respectively (Kanazawa et al., 1998; Wang and Zhang, 2005). Despite their different geographical locations, the two ancient lotuses had similar morphological features such as large plant size, few petals and red flowers, and similar genetic compositions. In two previous studies that used RFLP and SSR markers, these ancient lotuses were found to be located close to each other in the phylogenetic trees that were generated with high bootstrap values, 78% and 96.6%, respectively (Kanazawa et al., 1998; Kubo et al., 2009). In the present study, the genetic similarity coefficient between them was 0.96 (data not shown), and both were in cluster C3 (Fig. 3). These results are supported by available historical evidence that lotuses were imported from China to Japan in ancient times (Masuda et al., 2006) and confirms that Japanese cultivars should be classified as *N. nucifera* (Han et al., 2007a; Kanazawa et al., 1998; Kubo et al., 2009).

GENETIC RELATIONSHIPS AMONG FLOWER, SEED, AND RHIZOME LOTUSES. Through traditional artificial selection and agricultural use, three categories of *N. nucifera* have been established: flower, seed, and rhizome lotuses (Wang and Zhang, 2005; Zou et al., 1997). This classification is based mainly on morphological traits. In the current study, we analyzed the genetic relationships within and among the three groups. The coefficient of genetic similarity was highest within the rhizome lotuses (0.90) as compared with the flower and seed lotuses [0.66 and 0.76, respectively (Table 2)]. Rhizome lotus cultivars

that were analyzed (No. 77 to 102) clustered together and formed a separate subclade, Group IIa10 (Table 1; Fig. 1), which indicated the existence of a close genetic relationship among rhizome lotuses and a distinct genetic background from flower and seed lotuses. Moreover, the genetic backgrounds of the rhizome lotuses were relatively homogeneous and clustered in C3 (Fig. 3), which might be explained by the fact that rhizome lotus cultivars are mostly bred and propagated by asexual reproduction, through underground rhizomes, to fix valuable traits. They have the common characteristics of thick and edible underground rhizome. Consequently, the presence of thick and edible rhizomes is most likely to be used as a morphological characteristic for classification.

There was no obvious distinction between the flower and seed lotus cultivars. Seed lotus cultivars were interspersed among the cultivars of flower lotuses, which indicated that seed lotuses were phylogenetically close to flower lotuses. This finding was confirmed by the PCA and STRUCTURE results (Figs. 2 and 3). Flower and seed lotuses might share a common origin and might have been selected from similar, if not the same, gene pools. Many flower and seed lotus cultivars have been hybridized with each other. Any lotus accession whose flowers are of ornamental value can be classified as a flower lotus. Seed lotuses have beautiful flowers with well-developed pistils and stamens and can also produce abundant seeds. Seed lotuses can be classified separately from flower lotuses provided that they give high yield with fine-quality seed. So seed lotus can be considered as a special type of flower lotus. This might explain why flower and seed lotuses were not distinguished genetically from each other.

Zhang and Wang (2006) found that lotuses could be divided into two ecotypes, namely temperate and tropical cultivars. The former typically have an annual growth cycle, an enlarged rhizome that is dormant during the winter, and include flower, seed, and rhizome lotuses that are cultivated in inland China. The latter keep growing throughout the year, have a thin rhizome, and include lotuses that are cultivated in southern China and southeast Asia. We analyzed three tropical lotuses (No. 63 to 65). These accessions bloom quite freely during the cold months of the year, but their underground rhizome does not become enlarged even in Wuhan (south-central region). The tropical accessions were related more closely to *N. nucifera* than to *N. lutea* and were interspersed with the flower and seed lotuses that are cultivated in inland China [in Group IIa7 (Figs. 1 and 3)]. The results suggest that the three tropical lotuses belong to *N. nucifera*. On the basis of morphological characteristics and genetic analysis, our results supported the two ecotypes proposed by Zhang and Wang (2006).

THE CORRELATION BETWEEN GENETIC CLASSIFICATION AND MORPHOLOGICAL CHARACTERISTICS. In China, lotus cultivars have been classified mainly on the basis of morphological characteristics such as plant size, flower type and color, leaf shape, and color (Wang and Zhang, 2005; Zou et al., 1997), especially flower lotuses. Plant size has been considered to be an important feature in lotus classification. In the present study, the accessions were classified in general into small to medium and large plants (Table 1). For lotuses in Group I, namely *N. lutea* and its hybrids with *N. nucifera*, plant size was small to medium in general. *Nelumbo lutea* grows poorly in Wuhan, and most of the hybrids that were bred from this species were not vigorous. In Group II, plant size was correlated with the subgroups in the phylogenetic tree to some extent. Most of

the large-sized cultivars were in Groups IIa8, IIa9, and IIa10, whereas small to medium-sized cultivars were located in other subgroups. However, given that plant size is controlled by multiple genes and is also affected by environmental conditions, it might not be appropriate to use plant size as a basis for cultivar classification.

In most cases, morphological classification is based on flower characteristics (e.g., color and number of petals and size and shape of flowers). We detected a correlation between petal color and genetic relationships. The cultivars with yellow petals were clustered mainly in Group I (*N. lutea* and its hybrids with *N. nucifera*). In Group II, the partitioning of the subgroups was consistent with flower color. However, petal number did not correlate strongly with genetic variation. For example, double-petaled lotuses were distributed throughout the dendrogram.

In summary, morphological traits such as plant size and petal color were correlated to some degree with genetic background but not sufficiently to allow lotuses to be classified accurately on the basis of these traits. DNA markers were more informative and accurate in assessing genetic diversity and relationships among lotus accessions. Therefore, it is necessary to use both DNA markers and morphological characteristics to classify lotuses.

Conclusions

The results confirmed the significant genetic differences between *N. lutea* and *N. nucifera*; this is most likely the result of geographical isolation. The Japanese lotus cultivars had a very close genetic relationship with *N. nucifera* cultivated in China and thus could be classified as *N. nucifera*. Rhizome lotus cultivars were separated genetically from flower and seed lotuses, perhaps as a result of their vegetative propagation by rhizome and their selection on the basis of rhizome characteristics. Although plant size and petal color correlated to some degree with genetic variation, as assessed by DNA markers, morphological traits alone are not adequate for the classification of lotuses.

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