Indian J. Hort. 70(2), June 2013: 266-273

Analyses of genetic relationships in *Nelumbo nucifera* using *atpB-rbc*L chloroplast spacer and AFLP markers

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ABSTRACT

Despite the economic importance of *Nelumbo nucifera*, there have been meagre studies on genetic diversity with few accessions in the species. In the present study a total of 120 accessions in wild lotus and three cultivated types (flower lotus, seed lotus and rhizome lotus) were analyzed with *atpB-rbc*L chloroplast spacer and AFLP marker to determine their genetic relationships. The analysis of the *atpB-rbc*L chloroplast spacer revealed no significant genetic differentiation between wild lotus and cultivars and among the three cultivated types. The results of the AFLP analysis further proved the high genetic similarities among them. AFLP cluster indicated that the wild lotus did not distribute in a single independent group but interspersed in the different flower lotus groups. It meant that the flower lotus probably originated from different groups of the wild lotus. Nevertheless, seed lotus and rhizome lotus only clustered in one group with a quite high genetic similarity indicating that they had close genetic relationships. The quite high similarity among the accessions of seed and rhizome lotus indicated that the two types might have arisen from a single domestication event that led to a genetic bottleneck that limited diversity within the two types.

Key words: AFLP, *atpB-rbc*L chloroplast spacer, genetic relationships, *Nelumbo lutea*, *Nelumbo nucifera.*

INTRODUCTION

The lotus is an important aquatic economic plant, not only as a dainty and ornamental flower but also as a source of herbal medicine (Qian, 12). Nelumbonaceae also known as the lotus family is a small family of perennial, aquatic angiosperms which traditionally consists of the two species *Nelumbo nucifera* Gaertn. and *Nelumbo lutea* (Willd.) Pers. based on morphological characters (Borsch and Barthlott, 2). More recently, evidence has suggested that *N. lutea* should be considered to be a sub-species of *N. nucifera* (Li *et al*., 10; Diao *et al*., 4). Through traditional artificial selection and natural evolution, three big types-flower lotus, seed lotus and rhizome lotus have been formed in *N. nucifera*. Although the genetic relationships among the three types of lotus were investigated in earlier studies (Han *et al*. 6; An *et al*., 1), there is still need for more elucidation especially because of the few number of accessions used in the earlier studies.

The present study was conducted to determine the genetic variability in *Nelumbo nucifera* using *atpB-rbc*L chloroplast spacer and AFLP marker. The aims of the study were to provide a better understanding of genetic relationships between wild and cultivated *N. nucifera* and among the three cultivated-groups in *N. nucifera* for breeding programmes.

MATERIALS AND METHODS

A total of 119 accessions of *Nelumbo nucifera* which included wild lotus and cultivars including the three types flower lotus seed lotus and rhizome lotus were used in the genetic analyses. *N. lutea* was used as the out-group in the study. Accessions codes, types and sources are shown in Table 1. About 5 g of young leaves from each accession were collected for analysis. Nine accessions were analyzed with *atpB-rbcL* chloroplast spacer. These included four flower lotus accessions, two seed lotus accessions, two rhizome lotus accessions and a wild lotus. *N. lutea* and *Magnolia denudata* were used as out-group. All sequences were submitted to GenBank and located GenBank accession numbers EF377288 to EF377297. Genomic DNA of every sample was isolated according to the CTAB protocol of Doyle and Doyle (Doyle and Doyle, 5). The *atpB-rbc*L chloroplast spacer was amplified by PCR using the universal primer pair *atpB*-1 (5'-ACATCKARTACKGGACCAATAA-3') and *rbc*L-1 (5'-AACACCAGCTTTRAATCCAA-3') (Chiang *et al*., 3). Each 25 µl PCR mixture contained: 50 ng of total genomic DNA, 400 mM each dNTP, 1U *Taq*

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Table 1. One hundred and twenty lotus accessions used in the present study.

Code	Accession name	Type	Source
1	*Nelumbo nucifera* cv. 'Zhuzhou Pink'	Wild lotus	CLRC
2	*Nelumbo nucifera* cv. 'Sunyatesn Lotus'	Flower lotus	CLRC
3	*Nelumbo nucifera* cv. 'Sino-Japanese Friendship Lotus'	Flower lotus	CLRC
4	*Nelumbo nucifera* cv. 'Shaoxing Pink'	Wild lotus	CLRC
5	*Nelumbo nucifera* cv. 'Sichuan Pink'	Wild lotus	CLRC
6	*Nelumbo nucifera* cv. 'West-lake Pink'	Wild lotus	CLRC
7	*Nelumbo nucifera* cv. 'Xuanwu Pink'	Wild lotus	CLRC
8	*Nelumbo nucifera* cv. 'Hong Lake Pink'	Wild lotus	LCWU
9	*Nelumbo nucifera* cv. 'Chang Lake Pink'	Wild lotus	LCWU
10	*Nelumbo nucifera* cv. 'Liangzi Lake Pink'	Wild lotus	LCWU
11	*Nelumbo nucifera* cv. 'Longgan Lake Pink'	Wild lotus	LCWU
12	*Nelumbo nucifera* cv. 'Heilongjiang Pink'	Wild lotus	CLRC
13	*Nelumbo nucifera* cv. 'Weishan Pink'	Wild lotus	CLRC
14	*Nelumbo nucifera* cv. 'Echeng Pink'	Wild lotus	CLRC
15	*Nelumbo nucifera* cv. 'East-lake Pink'	Wild lotus	CLRC
16	*Nelumbo nucifera* cv. 'Antique Lotus of Zhongnanhai'	Wild lotus	CLRC
17	*Nelumbo nucifera* cv. 'Chinese Antique Lotus'	Wild lotus	CLRC
18	*Nelumbo nucifera* cv. 'White River Platform'	Flower lotus	CLRC
19	*Nelumbo nucifera* cv. 'Brave Man'	Flower lotus	CLRC
20	*Nelumbo nucifera* cv. 'Jade Girl of Fujian'	Flower lotus	CLRC
21	*Nelumbo nucifera* cv. 'Big Leaf White'	Flower lotus	CLRC
22	*Nelumbo nucifera* cv. 'Big White'	Flower lotus	CLRC
23	*Nelumbo nucifera* cv. 'Taibai Lotus'	Flower lotus	CLRC
24	*Nelumbo nucifera* cv. 'Shijiazhuang White'	Flower lotus	WIB
25	*Nelumbo nucifera* cv. 'Lushan White'	Flower lotus	WIB
26	*Nelumbo nucifera* cv. 'Qianling White'	Flower lotus	WIB
27	*Nelumbo nucifera* cv. 'Pink Lotus'	Flower lotus	CLRC
28	*Nelumbo nucifera* cv. 'Pink River Platform'	Flower lotus	CLRC
29	*Nelumbo nucifera* cv. 'Nehru Lotus'	Flower lotus	CLRC
30	*Nelumbo nucifera* cv. 'Tangzhaotisi Lotus'	Flower lotus	CLRC
31	*Nelumbo nucifera* cv. 'Big White Layer'	Flower lotus	CLRC
32	*Nelumbo nucifera* cv. 'Bright Green'	Wild lotus	CLRC
33	*Nelumbo nucifera* cv. 'Pink Thousands Petals'	Wild lotus	CLRC
34	*Nelumbo nucifera* cv. 'White Peony'	Flower lotus	CLRC
35	*Nelumbo nucifera* cv. 'Little Versicolor'	Wild lotus	CLRC
36	*Nelumbo nucifera* cv. 'Big Versicolor'	Wild lotus	CLRC
37	*Nelumbo nucifera* cv. 'Duplicate Pink'	Flower lotus	CLRC
38	*Nelumbo nucifera* cv. 'Thousands Petals'	Flower lotus	CLRC
39	*Nelumbo nucifera* cv. 'Red Spot'	Flower lotus	CLRC
40	*Nelumbo nucifera* cv. 'Bright Sky'	Flower lotus	CLRC

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Code	Accession name	Type	Source
41	*Nelumbo nucifera* cv. 'East-lake Spring'	Flower lotus	CLRC
42	*Nelumbo nucifera* cv. 'Warrior of White Uniform'	Flower lotus	CLRC
43	*Nelumbo nucifera* cv. 'Beautiful Bowl'	Flower lotus	CLRC
44	*Nelumbo nucifera* cv. 'Dancing Phoenix'	Flower lotus	CLRC
45	*Nelumbo nucifera* cv. 'Red-edged Jade-plate'	Flower lotus	CLRC
46	*Nelumbo nucifera* cv. 'Spring at Desk'	Flower lotus	CLRC
47	*Nelumbo nucifera* cv. 'Full Happiness'	Flower lotus	CLRC
48	*Nelumbo nucifera* cv. 'Small Cape Jasmine'	Flower lotus	CLRC
49	*Nelumbo nucifera* cv. 'Candle Light'	Flower lotus	CLRC
50	*Nelumbo nucifera* cv. 'Butterfly's Love'	Flower lotus	CLRC
51	*Nelumbo nucifera* cv. 'Peach with Raindrops'	Flower lotus	CLRC
52	*Nelumbo nucifera* cv. 'Red Tree Peony'	Flower lotus	CLRC
53	*Nelumbo nucifera* cv. 'Jingzhou Peony-red'	Flower lotus	CLRC
54	*Nelumbo nucifera* cv. 'Guangzhou Bowl Lotus'	Flower lotus	CLRC
55	*Nelumbo nucifera* cv. 'Brocade Beauty'	Flower lotus	CLRC
56	*Nelumbo nucifera* cv. 'Rouge Dew'	Flower lotus	CLRC
57	*Nelumbo nucifera* cv. 'Lucky Snow'	Flower lotus	CLRC
58	*Nelumbo nucifera* cv. 'White Pigeon'	Flower lotus	CLRC
59	*Nelumbo nucifera* cv. 'Jade Bowl'	Flower lotus	CLRC
60	*Nelumbo nucifera* cv. 'Sleeping Beauty'	Flower lotus	CLRC
61	*Nelumbo nucifera* cv. 'Sunset Clouds'	Flower lotus	CLRC
62	*Nelumbo lutea*	Flower lotus	LCWU
63	*Nelumbo nucifera* cv. 'Dancing Concubine Lotus'	Flower lotus	LCWU
64	*Nelumbo nucifera* cv. 'Galaxy Peony'	Seed lotus	GRSWL
65	*Nelumbo nucifera* cv. 'Outer space's Charming'	Seed lotus	GRSWL
66	*Nelumbo nucifera* cv. 'Wind Rolled up Red-flag'	Flower lotus	GRSWL
67	*Nelumbo nucifera* cv. 'Spread out Sleeve'	Seed lotus	GRSWL
68	*Nelumbo nucifera* cv. 'March in Snow'	Flower lotus	GRSWL
69	*Nelumbo nucifera* cv. 'Hunan Lotus'	Seed lotus	GRSWL
70	*Nelumbo nucifera* cv. 'Jiangsu Seed Lotus'	Seed lotus	GRSWL
71	*Nelumbo nucifera* cv. 'Zhejiang Lotus'	Seed lotus	GRSWL
72	*Nelumbo nucifera* cv. 'Jingxi White Lotus'	Seed lotus	GRSWL
73	*Nelumbo nucifera* cv. 'Guangchang Hundred Leaves'	Seed lotus	GRSWL
74	*Nelumbo nucifera* cv. 'Guangchang Lotus'	Seed lotus	GRSWL
75	*Nelumbo nucifera* cv. 'Hongjian Lotus'	Seed lotus	GRSWL
76	*Nelumbo nucifera* cv. 'Fujian White'	Seed lotus	GRSWL
77	*Nelumbo nucifera* cv. 'Fujian No.17'	Seed lotus	GRSWL
78	*Nelumbo nucifera* cv. 'Eouza No.3'	Seed lotus	LCWU
79	*Nelumbo nucifera* cv. 'Wax Gourd'	Seed lotus	GRSWL
80	*Nelumbo nucifera* cv. 'East Mountain Red Coat'	Seed lotus	GRSWL

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Code	Accession name	Type	Source
81	*Nelumbo nucifera* cv. 'Outer Space Lotus No.1'	Seed lotus	GRSWL
82	*Nelumbo nucifera* cv. 'Outer Space Lotus No.2'	Seed lotus	GRSWL
83	*Nelumbo nucifera* cv. 'Outer Space Lotus No.3'	Seed lotus	GRSWL
84	*Nelumbo nucifera* cv. 'Outer Space Lotus No.4'	Seed lotus	GRSWL
85	*Nelumbo nucifera* cv. 'Jingguang No.1'	Seed lotus	GRSWL
86	*Nelumbo nucifera* cv. 'Jingguang No.2'	Seed lotus	GRSWL
87	*Nelumbo nucifera* cv. 'Ion No.7'	Seed lotus	GRSWL
88	*Nelumbo nucifera* cv. 'Ion No.8'	Seed lotus	GRSWL
89	*Nelumbo nucifera* cv. 'Ion No.9'	Seed lotus	GRSWL
90	*Nelumbo nucifera* cv. 'Ion No.10'	Seed lotus	GRSWL
91	*Nelumbo nucifera* cv. 'Hubei No.1'	Rhizome lotus	LCWU
92	*Nelumbo nucifera* cv. 'Hubei No.2'	Rhizome lotus	LCWU
93	*Nelumbo nucifera* cv. 'Hubei No.3'	Rhizome lotus	LCWU
94	*Nelumbo nucifera* cv. 'Hubei No.4'	Rhizome lotus	LCWU
95	*Nelumbo nucifera* cv. 'Hubei No.5'	Rhizome lotus	LCWU
96	*Nelumbo nucifera* cv. 'New No.1'	Rhizome lotus	LCWU
97	*Nelumbo nucifera* cv. '9217'	Rhizome lotus	LCWU
98	*Nelumbo nucifera* cv. 'Red Beauty'	Rhizome lotus	LCWU
99	*Nelumbo nucifera* cv. 'Big Mauve'	Rhizome lotus	LCWU
100	*Nelumbo nucifera* cv. 'Eouza No.1'	Rhizome lotus	LCWU
101	*Nelumbo nucifera* cv. 'Eouza No.2'	Rhizome lotus	LCWU
102	*Nelumbo nucifera* cv. 'Eouza No.4'	Rhizome lotus	LCWU
103	*Nelumbo nucifera* cv. 'Maojie'	Rhizome lotus	WIB
104	*Nelumbo nucifera* cv. 'Yacheng'	Rhizome lotus	WIB
105	*Nelumbo nucifera* cv. 'Sesame Lake'	Rhizome lotus	WIB
106	*Nelumbo nucifera* cv. 'Qingmaojie'	Rhizome lotus	WIB
107	*Nelumbo nucifera* cv. 'Damaojie'	Rhizome lotus	WIB
108	*Nelumbo nucifera* cv. 'Big square'	Rhizome lotus	WIB
109	*Nelumbo nucifera* cv. 'Bamboo Joint'	Rhizome lotus	WIB
110	*Nelumbo nucifera* cv. 'Pig Tail'	Rhizome lotus	WIB
111	*Nelumbo nucifera* cv. 'Hunanpao'	Rhizome lotus	WIB
112	*Nelumbo nucifera* cv. 'June Early'	Rhizome lotus	WIB
113	*Nelumbo nucifera* cv. 'Xiangtanhuaye'	Rhizome lotus	WIB
114	*Nelumbo nucifera* cv. 'Wuxi White'	Rhizome lotus	WIB
115	*Nelumbo nucifera* cv. 'Daqingkai'	Rhizome lotus	WIB
116	*Nelumbo nucifera* cv. 'Xingcheng'	Rhizome lotus	WIB
117	*Nelumbo nucifera* cv. 'Big Snow Magpie'	Rhizome lotus	WIB
118	*Nelumbo nucifera* cv. 'Big Magpie'	Rhizome lotus	WIB
119	*Nelumbo nucifera* cv. 'Shanxi white'	Rhizome lotus	WIB
120	*Nelumbo nucifera* cv. 'Wuzishi C1'	Rhizome lotus	WIB

CLRC: China Lotus Research Center, WIB: Wuhan Institution of Botany, GRSWL: Guangchang Research School of White Lotus, LCWU: Lotus Center of Wuhan University

polymerase (Promega, Madison, USA), 1× Triton-X PCR buffer, 2.0 mM $MgCl_2$, and 0.4 mM of each primer. PCR amplification conditions were 30 cycles of 94^{0}C denaturing for 45 s, 49^{0}C annealing for 1 min. 15 s, and 72^{0}C extension for 1 min. 15 s, followed by 72^{0}C extension for 10 min. and 4^{0}C for storing. The interesting bands were excised from a 2% agarose gel in 0.5×TBE buffer and purified using a gel extraction kit (TakaRa Biotechnology Co. Lid, Dalian, China). PCR products were cloned using a pGEMT-easy vector system (Promega, Madison, USA). A total of 500 ng of purified plasmid DNA was then sequenced in both directions on an ABI3730XL sequencer (Sunbiotech Co. Ltd, Beijing, China). Sequences were aligned using CLUSTALW (Thormann *et al*., 15). Phylogenetic analyses of the complete data set of sequences were conducted using the test version of PAUP4.0b10 (Swofford, 4), and heuristic searches were done with COLLAPSE, MULPARS and TBR branch-swapping options to save all of the equally most parsimonious trees. Bootstrap analyses of 1000 replications were performed to show relative support for individual clades.

The protocol of AFLP was based on Milla *et al*. (11). Separation of amplification fragments was accomplished on 6% polyacrylamide gels at 68 W for 3 h. Analysis was carried out by silver-straining of the gel and overnight drying before being photographed. The protocol of silver-straining was based on Han *et al*. (7). The amplified fragments were scored for band presence (1) or absence (0) and two binary qualitative data matrices were constructed. Data analyses were performed by using the NTSYS-pc version 2.1 computer program package (Rohlf, 13). Pair-wise comparisons were calculated using the Jaccard'S similarity coefficient (Kosman and Leonard, 9). The similarity values were used to generate a dendrogram via the un-weighted pair group method with arithmetic average (UPGMA).

RESULTS AND DISCUSSION

The *atpB-rbc*L chloroplast spacer sequences with an aligned length of 887 characters were applied for the analysis of ten accessions. Of these 887 characters, 801 (90.3%) were constant characters; 84 (9.5%) were parsimony-uninformative variable characters; and 2 (0.2%) were parsimony-informative characters across all the ten accessions. Parsimony analyses of the data yielded the strict consensus of the most parsimonious trees (Fig. 1). The accessions *Nelumbo nucifera* cv. 'Hong Lake Pink' and *Nelumbo nucifera* cv. 'Nehru Lotus' clustered together with a bootstrap of 62%. There are no distinct genetic differentiations between wild lotus and cultivars and among the three types of lotus.

A total of 215 distinct bands in size from 100 to 750 bp were scored with seven primer combinations, of which 214 (99.53%) were polymorphic. The number of scored loci amplified by each primer combination varied from 9 to 43 with an average of 30.57 per reaction (Table 2).

In the dendrogram (Fig. 2), 120 accessions were also distinctly separated into two major groups at the Jaccard's similarity coefficient level of 0.71 in *Nelumbo nucifera*. The Jaccard similarity coefficient ranged from 0.56 to 0.98. The group I included all flower lotus and wild lotus accessions with the exception of three seed lotus and two rhizome lotus accessions (70, 71, 73, 105, 108). The group II consisted of groups II A and II B. Group II A included most of the big-flower type accessions while group II B includes most of the medium-small flower type accessions. In the group II

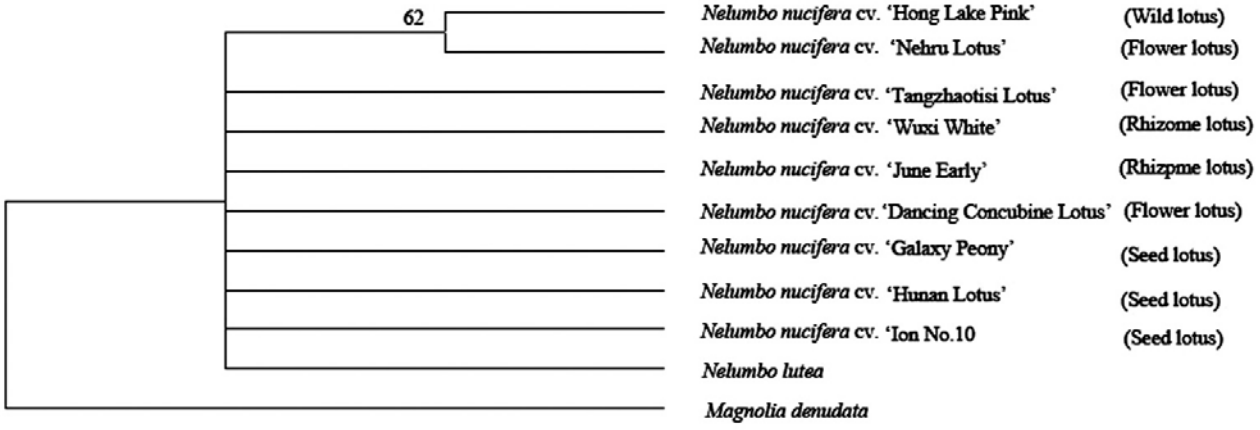

Fig. 1. The strict consensus of the only one parsimonious tree based on *atpB-rbcL* chloroplast spacer sequence. Number of bootstrap replicates = 1000. Bootstrap values (only > 50% is showed) are above branches.

Table 2. Numbers of total bands and polymorphic bands and percentage polymorphic bands for each of seven primer combinations (e is the preamplification primer sequence for *Eco*RI site (5-GACTGCGTACCAATTC) without any selective nucleotides and m is the preamplification primer sequence for MseI site (5-GATGAGTCCTGAGTAA).

Selective amplification primer combinations	Total bands	Polymorphic bands	% polymorphic bands
eACC/mCGA	33	33	100
eACA/mCAT	9	8	88.89
eAGT/mCTC	30	30	100
eAGT/mCGA	25	25	100
eAAG/mCAA	35	35	100
eATG/mCAT	40	40	100
eATG/mCGA	43	43	100
Total	215	214	99.53

A the same color accessions clustered together. The group III included almost all the rhizome and seed lotus accessions and four flower lotus accessions (24, 25, 26, 68). The analysis of the *atpB-rbc*L chloroplast spacer revealed no significant genetic differentiation between wild lotus and cultivars and among the three cultivated types. On the contrary, AFLP analysis distinctly separated all the 120 accessions indicating that AFLP was a better molecular marker for analysis of genetic diversity in *Nelumbo nucifera* than *atpB-rbc*L chloroplast spacer.

In the analysis of AFLP, the wild lotus accessions clustered with flower lotus indicating that flower lotus had preserved more primal characters than seed lotus and rhizome lotus. The wild lotus interspersed within the flower lotus accessions. It suggested that the flower lotus might have originated from different wild lotus. Among wild lotus, distinct genetic differentiation among its accessions was detected. This was in agreement with the results of our earlier study (Han *et al*., 8), which indicated obvious regional differences in wild lotus.

The seed lotus and rhizome lotus clustered in a single group with a quite high genetic similarity indicating close relationships between the two types. In flower lotus, big-flower type accessions and medium-small type accessions had obvious genetic variation, indicating that height of the lotus was an important criterion in the classification system of flower lotus. In the big-flower type accessions, the accessions with the same flower color clustered together, suggesting that color of the lotus was also an important criterion in the classification system of big-flower type lotus.

Wild lotus is an important genetic resource for the breeding programme of flower lotus, because it showed rich diversities in features of flower lotus. The rich genetic diversity in flower lotus suggested that the type might arise from different domestication events. The quite high similarity among the accessions of seed and rhizome lotus indicated that the two types might have arisen from a single domestication event that led to a genetic bottleneck that limited diversity within the two types.

ACKNOWLEDGEMENT

This work was supported by the National Science and Technology Supporting Program (No. 2012BAD27B01).

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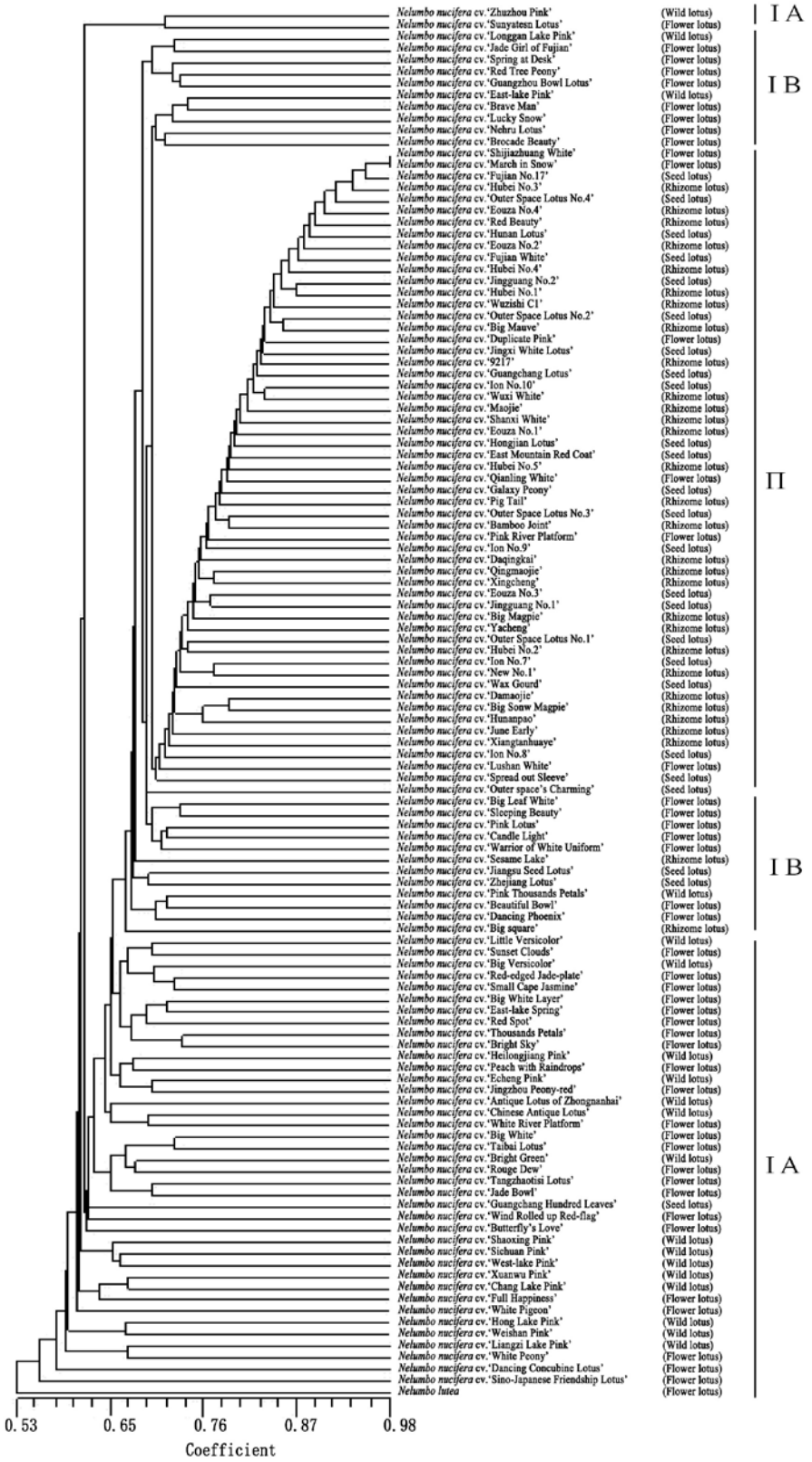

Fig. 2. Dendrogram illustrating genetic relationships among 120 accessions generated by UPGMA cluster analysis calculated from AFLP data.

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Received: April, 2012; Revised: October, 2012;
Accepted: December, 2012