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Classification and diversity of sacred and American *Nelumbo* species: the genetic relationships of flowering lotus cultivars in Japan using SSR markers

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Abstract

The water lotus, genus *Nelumbo*, contains two species, the sacred (*Nelumbo nucifera*) and American lotuses (*Nelumbo lutea*). Hundreds of flowering lotus cultivars are currently known. However, their classification is unclear. For the classification of *Nelumbo* cultivars, in addition to 35 simple sequence repeat (SSR) markers recently developed, we have developed 17 and 16 of new *Nelumbo* SSR markers from SSR-enriched genomic libraries and expressed sequence tag (EST) data, respectively. Out of these 68 SSRs, along with SSRs recently published by others, 52 showed clear polymorphisms in 98 *Nelumbo* samples. A total of 300 alleles were observed, ranging from 2 to 11 alleles per locus, with an average of 5.77. Alleles specific for the American lotus-derived cultivars and a cluster of the American lotus-derived cultivars on a neighbour-joining tree confirmed genetic differences between *N. lutea* and *N. nucifera*. In addition, a possible differentiation between Chinese and Japanese cultivars was also suggested. Parentage analysis using the SSR markers confirmed four known parentages and predicted currently-unknown parentages of six cultivars. The present data have demonstrated that site-specific, co-dominant SSR markers enable more accurate classification, identification and comparison of *Nelumbo* species.

Keywords: lotus; neighbour-joining tree; *Nelumbo*; simple sequence repeat (SSR)

Introduction

The water lotus (genus *Nelumbo*) has recently been classified into the family Nelumbonaceae [reviewed in Savolainen and Chase, (2003)]. This genus contains two species, *Nelumbo nucifera* (Indian or sacred lotus) and *Nelumbo lutea* (American lotus). The former is distributed in Asia and north Australia, and the latter is found in north and south America (Borsch and Barthlott, 1994). These two species have similar characters except for differences in petal colour and leaf size, and they can be crossed easily. *Nelumbo* represents one of the most famous water flowers in the world. In particular, ornamental flower cultivars are called 'flowering lotuses'. More than 900 flowering lotus cultivars have been released mostly from China (Wang and Zhang, 2005). In Japan, about 350 cultivars are currently known, including 100 or more Japanese landraces (Kaneko, 2002).

Nelumbo cultivars have been classified based on morphological characters of the flower, leaf, plant size, rhizome, etc. [reviewed in Watanabe, (1990) and

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Wang and Zhang, (2005)]. In most cases, flower characters (e.g. colour and number of petals, sharpness of petal vein, and size and shape of flower) are used for the standards of the classification [see Watanabe, (1990) and Supplementary Table S1, available online only at http://journals.cambridge.org for instances]. The evolutionary and breeding histories of lotuses have recently been taken into account in the classification system. However, morphological characters are often hard to distinguish because of continuous variations and environmental effects. Several DNA analyses have been conducted with different molecular markers for the classification of *Nelumbo* samples (e.g. Kanazawa *et al.*, 1998; Zou *et al.*, 1998; Katori *et al.*, 2003; Huang *et al.*, 2003, 2004; Peng *et al.*, 2004; Xue *et al.*, 2006; Guo *et al.*, 2007; Han *et al.*, 2007a, b, 2009; Chen *et al.*, 2008; Tian *et al.*, 2008b). These previous DNA analyses have the disadvantage that they basically detect haplotype or dominant alleles. *Nelumbo* has protogynous flowers, which are usually outcrossed via insects, although self-pollination is also possible. The resultant heterozygosity can be maintained as long as *Nelumbo* undergoes vegetative propagation with rhizomes. In this context, dominant or organellar markers may only clarify part of the genetic information in *Nelumbo*, which may retain relatively high heterozygosity. That is, genetic information from the parent that provides null alleles with a dominant marker may be unclear or no information of the male lineage could be obtained with an organellar marker. Therefore, co-dominant markers are ideal for studying this species. Although allozymes have recently been used in *Nelumbo* as co-dominant markers (Tian *et al.*, 2008b), the number of available allozyme markers is very limited.

Recently, we developed simple sequence repeat (SSR) markers in *Nelumbo* (Kubo *et al.*, 2009). SSR is a DNA repeat consisting of 1–6 nucleotide repeat units. Because of their high reproductivity, relatively high polymorphism content, co-dominant inheritance and abundance in eukaryotic genomes, SSRs have frequently been used for genetic analysis such as taxonomic classification, genetic diversity studies and making genetic maps [reviewed in Varshney *et al.*, (2005)]. Although a few studies on SSR markers in *Nelumbo* have recently been conducted (Pan *et al.*, 2007; Tian *et al.*, 2008a; Kubo *et al.*, 2009), these markers have not yet been used for the classification.

In this study, we conducted the classification of our *Nelumbo* collections based on SSR markers. Additional *Nelumbo* SSR markers from SSR-enriched libraries and registered EST data were developed. In total, 98 *Nelumbo* cultivars were tested. We included 21 cultivars, most of which had been collected from the 'Ogura-ike Reclaimed Land'. The Ogura-ike Reclaimed Land was the biggest pond in Kyoto, Japan, and was famous for its lotus flowers. Such cultivars as many as 70 (namely the 'Ogura-ike group' cultivars) with various morphological characters are known (Kaneko, 2002; Uchida, 2006). However, their genetic relationships are unknown. One of our aims in this study was to investigate the relationships of the various cultivars in this group. A neighbour-joining (NJ) tree based on the SSRs showed that the *Nelumbo* cultivars were separated into two main groups, one of which included American lotus-derived cultivars. This confirmed the genetic difference between American and sacred lotuses. The other group comprised sacred lotuses, and it could be further divided into four subgroups. A possible genetic differentiation between Chinese and Japanese cultivars might have been suggested. We also conducted parentage analysis using the SSR alleles, which resulted in the confirmation and prediction of parents in several *Nelumbo* cultivars. The utility of SSR markers for the classification of *Nelumbo* species and the relationships of the cultivars are discussed.

Materials and methods

Plant materials and DNA isolation

The 98 *Nelumbo* cultivars used in this study are listed in Table 1. Most of them have been obtained from the Uchida Flowering Lotus Farm (Kumiyama, Kyoto, Japan) and the Kyoto Flower Center (Seika, Kyoto, Japan). Detailed information on their origins and pedigrees is described in Supplementary Table S1, available online only at http://journals.cambridge.org. These cultivars are being maintained at Kannondo, Joyo, Kyoto, Japan. An American lotus cultivar, 'America-kibasu #1', was kindly provided by the Experimental Station for Landscape Plants, Graduate School of Agricultural and Life Sciences, the University of Tokyo (Hanamigawa-ku, Chiba, Japan). This cultivar was introduced from USA to Japan and is thought not to have been hybridized with any sacred lotus. Total DNA was extracted from each cultivar as described previously (Kubo *et al.*, 2009).

Development of SSR markers

The SSR-enriched DNA libraries were constructed from total DNA of the 'Oguramadara-seedling' as described in Nunome *et al.* (2006). A 5′-biotin-conjugated $(GA)_{15}$ or $(GT)_{15}$ oligonucleotide (Invitrogen, Carlsbad, CA, USA) was hybridized for enrichment of SSR-containing DNA fragments. The SSR-enriched fragments have been sequenced and their nucleotide sequences have been deposited in the DDBJ/EMBL/GenBank databases under accession nos. AB481132–AB481148. Registered *Nelumbo* EST data (Cai *et al.*, unpublished) were retrieved from the

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Table 1. List of *Nelumbo* samples used in this study

Nelumbo nucifera cultivar	
1	Balitobasu
2	Benigani
3	Beniwanren
4	Byodoin
5	Chuugokukodaibasu[b]
6	Chugokushokumibasu
7	Chuunichiyuugiren[b]
8	Daikijakuto
9	Daikoho
10	Daimyohren[b]
11	Dainichirenge-3 gou
12	Enyoten
13	Fukudanojo-shiro[a]
14	Fuminohana
15	Futamatajo
16	Genshibasu[b]
17	Goshozakura
18	Gunma-akabasu
19	Gyokushuuren[b]
20	Gyozankohren[b]
21	Hakumanman[b]
22	Hakumanyo
23	Hekidairen[b]
24	Higashikanze-beni[a]
25	Hikonejobasu
26	Hokkeren[b]
27	Indobasu-shiro
28	Isuien
29	Ittenshikai[b]
30	Johdairen[b]
31	Kanren[b]
32	Kashohren[b]
33	Kinki
34	Kinzuiren[b]
35	Kokyowaren
36	Korakuen
37	Kurobohigashi[a]
38	Kyotogosho
39	Maiyohren[b]
40	Makishima-shiro[a]
41	Makotobasu[b]
42	Mekawa-nishi[a]
43	Minamikanze[a]
44	Misuaka[a]
45	Myorenji[a]
46	Nakayuden-beni[a]
47	Nanbei-Brazil
48	Nehru-basu[b]
49	Ninomaruike[a]
50	Oguradaikoku[a]
51	Oguramadara[a]
52	Oguramadara-seedling
53	Oguranishi[a]
54	Oguranoakebono[a]
55	Oguranohonoo[a]
56	Ohgahasu[b]
57	Ooshimasaki[a]
58	Ootorii
59	Pinkhitoe
60	Rosea Plena
61	Saienjishohren[b]
62	Saifukuji-shiro[a]
63	Sakosotoyashiki[a]
64	Sasayamajobasu
65	Sayakaze
66	Seigetsuren[b]
67	Seiryokoren
68	Shinnyoren[b]
69	Shirokunshishohren[a]
70	Shiroshakuyaku
71	Shisendohsaikoren[b]
72	Shokkohren[b]
73	Shusuichoten
74	Suihiren[b]
75	Taiseikin
76	Tatsutabasu
77	Tenjikumadarabasu[b]
78	Tenponotsutsumi
79	Ukesho[a]
80	Ukeshohonbeni[a]
81	Wakasamatagorobasu
82	Yodohime[a]
83	Yozanko
84	Zuikohren[b]
Nelumbo lutea and its hybrid cultivar (including possible ones)	
85	Aamujo
86	America-byakuren[b]
87	America-kibasu #1
88	Bichuko
89	Kenmai
90	Kitaine
91	Kurodanibyakuren
92	Maihiren[b]
93	Ohio-ren (Kannondo)
94	Ohjibasu (Kannondo)
95	Ooji-Kawakatsu
96	Ougyokuhai
97	Rinnohren[b]
98	Yazuma (Kohai 2)

[a] 'Ogura-ike group' cultivar (see text).
[b] Cultivar names from Watanabe (1990).

See Supplementary Table S1, available online only at http://journals.cambridge.org for detail.

GenBank database (accession nos. EE985570–EE987522, EH613358–EH613607 and EL740203–EL740207) and used for the development of the *Nelumbo* SSR markers. Primer pairs flanked by SSRs of six or more dinucleotide repeats were designed with manual inspection from the genomic (NS186–NS295) and EST data (NSe01–NSe18).

PCR amplification and detection of polymorphisms

SSR fragments were amplified by PCR using each SSR primer pair, either of which was fluorescence-labelled (Sigma-Aldrich, St Louis, MO, USA). Recently published *Nelumbo* SSRs (Pan *et al.*, 2007; Tian *et al.*, 2008a; Kubo *et al.*, 2009) were also tested. The PCR reaction, fragment analysis of amplified products and allele scoring were performed as described previously (Kubo *et al.*, 2009). SSR markers with missing data were omitted from further analyses.

Data processing, phylogenetic analysis and assessment of parentages

Expected (H_e) and observed heterozygosities (H_o) of each SSR locus, and other gene diversity indices were calculated using the Cervus 3.03 program (Kalinowski *et al.*, 2007) and Popgene 1.32 software (Yeh *et al.*, 1997). Based on the allele data, pairwise genetic distances were calculated using the Microsat 1.5 program (Minch *et al.*, 1998). We used the $1 - P$ distance measurement, where P is the proportion of shared alleles for the SSR markers (Bowcock *et al.*, 1994). This genetic distance measure is suitable for closely-related samples and is frequently used for SSR loci. An NJ tree was constructed using the Phylip 3.67 package (Felsenstein, 2007). Bootstrap (BS) analysis was performed from 1000 replicates using the Microsat 1.5.

Parentage analysis was performed based on the allele data using Cervus 3.03. No mismatch was permitted at any paired locus for the analysis. In predicting cultivars of unknown parentage, only pairs showing relatively greater genetic distance (pairwise genetic distance ≥ 0.30) were considered as candidate parentages so as not to simply choose a cultivar pair with similar genotypes. We did not examine the maternity of five cultivars ('Aamujo', 'Bichuko', 'Maihiren', 'Ougyokuhai' and 'Rinnohren'), which are predicted to be offsprings of an American lotus cultivar 'Ohjibasu', because the original 'Ohjibasu' cultivar was unavailable.

Results

Development of Nelumbo *SSR markers from genomic libraries and EST data*

In addition to 35 SSR markers developed previously (Kubo *et al.*, 2009), in this study we further isolated SSR fragments from SSR-enriched genomic libraries. Of the 53 clones sequenced, 17 SSR markers were newly

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Table 2. *Nelumbo* simple sequence repeat markers developed in this study

Marker	Sequence (5′–3′)[a]	Motif	Size[b]	Accession nos.
SSRs developed from Nelumbo genomic libraries				
NS186	F:CTGCTACTCTTTCCAAACTGAG R:GTTGAAGAGACAATGGCCTAC	$(GA)_{21}$	142	AB481132
NS207	F:ACATATAGGGTAGTAGAGGTTCA R:ATGAAAATATCCTGGTTTCTACCC	$(GT)_{20}$	147	AB481133
NS217	F:CAGGTGCAACGACGATTCAAAAA R:GTTGACCGATAAAAAAATTGGCTC	$(GT)_{14}$	118	AB481134
NS219	F:CAACGACGATTCAAAAACATTGTATA R:CTTGACCGATAAAAAAATTGGCTC	$(GT)_{14}$	105	AB481135
NS224	F:GTCTGACAGTGCTGAAGCAATT R:CAAGAAATGTCCATGAAGAGGAC	$(GA)_{16}$	143	AB481136
NS227	F:AGTGACGCAGGAAAAAGTTAAAAC R:CCTTGTTTATCAGATAAACATCGCT	$(GA)_{16}$	135	AB481137
NS236	F:TGAGACAAGATCTCAGATCTGAGA R:AACAGTTCCAAACATTTGTCCCTTAG	$(GA)_{23}$	254	AB481138
NS248	F:ACATGGATCCATAGACTTGAAAAC R:TGTAGTTGATTTTGTTGGGCTTC	$(TG)_{11}(AG)_{11}$	137	AB481139
NS260	F:AGGCATGTTTAATGTAGCATCAAAT R:TTATTGTGGCAAGATCATTCCTC	$(GA)_{13}$	128	AB481140
NS262	F:AGCCCCTGTTTTTTGTCTCATA R:AAACTATAACAGGGGCTTCAAC	$(AG)_{16}$	103	AB481141
NS264	F:GCCTAGATATGGTTAAGACTTAAGA R:GTATCTACCTAGATACTATGAAATGG	$(GT)_{14}$	128	AB481142
NS268	F:GGGAAAAATTATATCACGATACAA R:GCATCTGGCCATTTGTAATATTGT	$(TG)_{10}(AG)_{8}$	108	AB481143
NS281	F:CCTCTATTATAGTTCAGTTGTCTTTA R:GTGACTGCTTCTCTCTTTTAATGA	$(GT)_{12}$	132	AB481144
NS290	F:GGCAGGAAACAACACTAAATACTGA R:ACTCACTT(G/T)TCGAT(G/T)TCCCTCC	$(AG)_{21}$	112	AB481145
NS292	F:gtACTATTAAAGGAATTGTGCAATG R:AACTCTATAGCCAAAGGAATAGAAA	$(TG)_{11}(AG)_{9}$	127	AB481146
NS294	F:CCACAACTACAATTTCAAAGAATATAC R:TCAATTTTTTGCTTTCGACCACCA	$(GA)_{14}$	118	AB481147
NS295	F:CTGATTTCTGAGAACCTTGTTG R:AAGTAGGTGGTAAGACTGTTC	$(GA)_{16}$	124	AB481148
SSRs developed from Nelumbo EST data				
NSe01	F:TAATGCTAAGTACCATGTATGCG R:CTACTGCTACTGGTAAATTTCC	$(AG)_{11}$	100	EE985585
NSe02	F:GGTACACAAGCGTTACAGACT R:GTGAAAGTGTGCTATGTCCTC	$(GA)_{10}$	160	EE985688
NSe03	F:TGAGACCCAGAGTGGAGAACA R:TACCAACAGCGATCCGGTTGAT	$(AG)_{13}$	140	EE985848
NSe04	F:CAAGTCACTATTGCTGTTTGCT R:CTCTCATAAACGCCATTAAACC	$(GA)_{18}$	102	EE986142
NSe07	F:TCTTTGTATGGGTCATGGTCAA R:GACTCCTCATTGAACTTAGATC	$(AG)_{8}$	127	EE986306
NSe08	F:TAATTGATGTATGAACTATGAAGGCA R:CAATGGGATTAGAGCAAACAAGT	$(GA)_{10}$	110	EE986577
NSe09	F:GAAATCAATATTTTGGCGTGTCAC R:CTATGTAAAAGCACCCACATTCA	$(GTT)_{4}$, $(AT)_{8}$	174	EE986603
NSe10	F:GTATCTTTCAGCTATCCACATTC R:CCATATGGACTCTCTATGATTTCA	$(GA)_{13}$	141	EE986609
NSe11	F:AGTACTCAAGAAGAAGTTCGGAA R:GATTACCACTCGTTATAGCAGA	$(AG)_{10}$	159	EE986755
NSe12	F:GGAACTTGAGCTAAAATGGAATAG R:GACAACCAAACCGGGTTATTTT	$(GA)_{10}$	112	EE986865
NSe13	F:GCAATTCCTCACCAATGTCAAATAT R:GGTATACATTAACCTTATCCTGAC	$(GA)_{9}$	124	EE986966
NSe14	F:TCTGAGTTTGTTGAATTTCTCCTT R:TCTCATTTAATAAGACAATGGTCC	$(AG)_{10}$	117	EE987185

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Table 2. *Continued*

Marker	Sequence (5′–3′)[a]	Motif	Size[b]	Accession nos.
NSe15	F:ATGTAAGACTTGGAAGGCATAC R:AAGGAACAATCCATGCTCGA	$(GA)_{10}$	135	EE987264
NSe16	F:CAATTGTTACATCTGTTCCAGAC R:AAAACATGCAGCAAGCTAAGC	$(GA)_9$	130	EH613554
NSe17	F:GTCTTATTGCAAACAAGCTAG R:ATCAGCAACCTCAGCATCAGAT	$(TA)_7$, $(GA)_{11}$	208	EH613505
NSe18	F:TCAAATCTGTATTACACTCGCAC R:GTTTGGCATGTGTACCTACATT	$(GA)_9$	122	EH613595

[a] Small cases are nucleotide addition assumed from the restriction sites.
[b] Estimation of PCR products according to the sequenced fragment used for primer design.

designed (Table 2, NS186–NS295). Three markers had compound SSRs that had different types of repeat motifs. Thus, we developed 52 SSR markers from *Nelumbo* genomic DNA in both studies. Another 16 SSR markers (Table 2, NSe01–NSe18) were developed from 2207 *Nelumbo* ESTs in the database (Cai *et al.*, unpublished). In the 68 *Nelumbo* SSR fragments, the lengths of GA/AG and GT/TG dinucleotide repeats ranged from 6 to 26, with an average of 14.5.

Polymorphisms of SSRs in Nelumbo *cultivars*

Polymorphisms of the SSR markers were examined in 98 *Nelumbo* samples (Table 1). Of the 94 SSRs used [68 developed by Kubo *et al.* (2009) and this study; 26 developed by Pan *et al.* (2007) and Tian *et al.* (2008a)], 52 primer pairs that clearly amplified polymorphic bands were chosen. One or two alleles were constantly detected at each locus in all the *Nelumbo* samples examined. A total of 300 alleles were detected with the 52 SSR markers (Table 3). These markers revealed 2–11 alleles with an average of 5.77. H_o ranged from 0.071 to 0.582 per locus, with an average of 0.4312. The 52 primers were applicable to amplification in *N. lutea* (American lotus) and its hybrid cultivars. Alleles of different sizes (e.g. Fig. 1, arrows) were observed with 37 SSR markers (Table 3, daggers). These alleles are likely to be derived from American lotuses because most have been identified as homozygous loci in the *N. lutea* cultivar ‘America-kibasu #1’. In contrast, all of these loci were heterozygous in eight possible American lotus-derived cultivars (Table 1, ‘Aamujo’, ‘Bichuko’, ‘Kenmai’, ‘Kurodanibyakuren’, ‘Maihiren’, ‘Ohio-ren (Kannondo)’, ‘Ohjibasu (Kannondo)’ and ‘Ougyokuhai’). This indicated that these eight cultivars are inter-specific hybrids between *N. lutea* and *N. nucifera*. Unexpectedly, the remaining five possible American lotus-derived cultivars [‘America-byakuren’, ‘Kitaine’, ‘Ooji-Kawakatsu’, ‘Rinnohren’ and ‘Yazuma (Kohai 2)’] had no American lotus-specific allele at any of the loci examined.

Relationships of Nelumbo *cultivars based on an NJ tree*

We calculated the genetic distance of each cultivar (Supplementary Table S2, available online only at http://journals.cambridge.org) from the allele data, and we constructed an NJ tree using the cultivar ‘America-kibasu #1’ as an outgroup. The ingroup 97 *Nelumbo* samples were separated into two main groups (I and II; Fig. 2). Group I was composed of the eight American lotus-derived cultivars described above (Fig. 2, yellow). Group II included the rest of the cultivars and could be subdivided into four subgroups (IIa–d). The subgroup IIa consisted of 17 cultivars, 12 of which were ‘Oguraike group’ cultivars (Fig. 2, blue). The subgroups IIb and IIc contained 7 and 31 cultivars, respectively, most of which were Japanese cultivars. The subgroup IId included two ‘antique lotuses’ (‘Chuugokukodaibasu’ and ‘Ohgahasu’) and their progenies (‘Chuunichiyuugiren’ and ‘Shusuichoten’; Fig. 2, green). ‘Chuugokukodaibasu’ and ‘Ohgahasu’ were closely positioned on the NJ tree with a high BS value (96.6%). This result confirmed their close genetic relationship, as previously reported (Kanazawa *et al.*, 1998; Han *et al.*, 2007a). Four ‘Oguraike group’ cultivars (‘Minamikanze’, ‘Misuaka’, ‘Myorenji’ and ‘Ooshimasaki’) were also included in this subgroup. The subgroup IId showed a higher proportion of Chinese cultivars (11 out of 36) than the other subgroups.

Among these groups and subgroups, only one division of the main groups I and II was supported by a BS value of more than 50% (59.6%). However, some distal clades were relatively well supported with BS values of 51.4–100%.

Assessment of genetic identity with SSR markers

To date, several synonyms and parentages have been known in *Nelumbo* cultivars. In this study, we examined such relationships based on the SSR alleles. Our samples included two pairs of synonyms, ‘Daimyohren’–‘Ittenshikai’

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Table 3. Characterization of the 52 simple sequence repeat loci examined in this study

Locus[a]	Allellic range[b]	Allele nos. observed	H_o[c]	H_e[d]	Reference
NSh02†	139–149	6	0.439	0.642	Kubo *et al.* (2009)
NS001R	166–183	4	0.439	0.472	Kubo *et al.* (2009)
NS002†	121–141	7	0.439	0.528	Kubo *et al.* (2009)
NS005†	124–145	7	0.500	0.539	Kubo *et al.* (2009)
NS010	72–121	6	0.408	0.652	Kubo *et al.* (2009)
NS012†	112–130	7	0.500	0.704	Kubo *et al.* (2009)
NS020†	129–149	6	0.418	0.571	Kubo *et al.* (2009)
NS034	98–124	10	0.520	0.709	Kubo *et al.* (2009)
NS037b†	120, 125	2	0.082	0.097	Kubo *et al.* (2009)
NS049†	261–287	6	0.561	0.666	Kubo *et al.* (2009)
NS077†	142–156	5	0.510	0.644	Kubo *et al.* (2009)
NS080†	195–218	7	0.582	0.607	Kubo *et al.* (2009)
NS088†	122–141	4	0.490	0.552	Kubo *et al.* (2009)
NS092†	83–110	6	0.551	0.623	Kubo *et al.* (2009)
NS094†	131, 141	2	0.071	0.088	Kubo *et al.* (2009)
NS116†	106–114	4	0.510	0.534	Kubo *et al.* (2009)
NS124	116–143	9	0.429	0.685	Kubo *et al.* (2009)
NS139	135–165	7	0.541	0.631	Kubo *et al.* (2009)
NS149†	125–155	9	0.510	0.638	Kubo *et al.* (2009)
NS154†	150, 152, 154	3	0.082	0.098	Kubo *et al.* (2009)
NS160†	106–126	5	0.500	0.550	Kubo *et al.* (2009)
NS169†	87–125	10	0.214	0.372	Kubo *et al.* (2009)
NS207	140–174	11	0.500	0.796	This study
NS217†	91–155	9	0.571	0.659	This study
NS219†	89–152	8	0.582	0.643	This study
NS224†	130–146	5	0.571	0.572	This study
NS227†	129–159	8	0.551	0.641	This study
NS248	104–217	6	0.378	0.561	This study
NS260†	111–129	5	0.184	0.391	This study
NS262†	91–116	7	0.286	0.375	This study
NS290†	91–108	5	0.480	0.570	This study
NS292†	121–182	7	0.388	0.529	This study
NS294†	93–115	6	0.510	0.727	This study
NSe01†	98–128	7	0.490	0.537	This study
NSe03†	135–150	7	0.561	0.698	This study
NSe07†	119, 125	2	0.071	0.088	This study
NSe08†	105–112	4	0.500	0.551	This study
NSe10†	132–144	4	0.531	0.664	This study
NSe11†	163–169	4	0.510	0.544	This study
NSe12	105–111	4	0.561	0.561	This study
NSe13	122–131	4	0.204	0.322	This study
NSe17†	210–230	5	0.531	0.549	This study
NSe18	119, 121	2	0.480	0.502	This study
PR01	193–211	9	0.429	0.671	Pan *et al.* (2007)
PR06	146–233	4	0.245	0.347	Pan *et al.* (2007)
PR07†	247–250	4	0.480	0.538	Pan *et al.* (2007)
PR08†	200, 202, 204	3	0.520	0.519	Pan *et al.* (2007)
PR09	151–178	6	0.439	0.628	Pan *et al.* (2007)
PR11	162–226	7	0.500	0.611	Pan *et al.* (2007)
Nelumbo-06†	263–288	8	0.541	0.628	Tian *et al.* (2008a)
Nelumbo-14	147–182	4	0.316	0.595	Tian *et al.* (2008a)
Nelumbo-15†	98, 122, 124	3	0.214	0.271	Tian *et al.* (2008a)
Mean		5.77	0.4312	0.5363	

[a] Daggers (†) indicate markers, with which American lotus-specific alleles were detected.
[b] Estimation of nucleotide size by fragment analysis.
[c] Observed heterozygosity.
[d] Expected heterozygosity.

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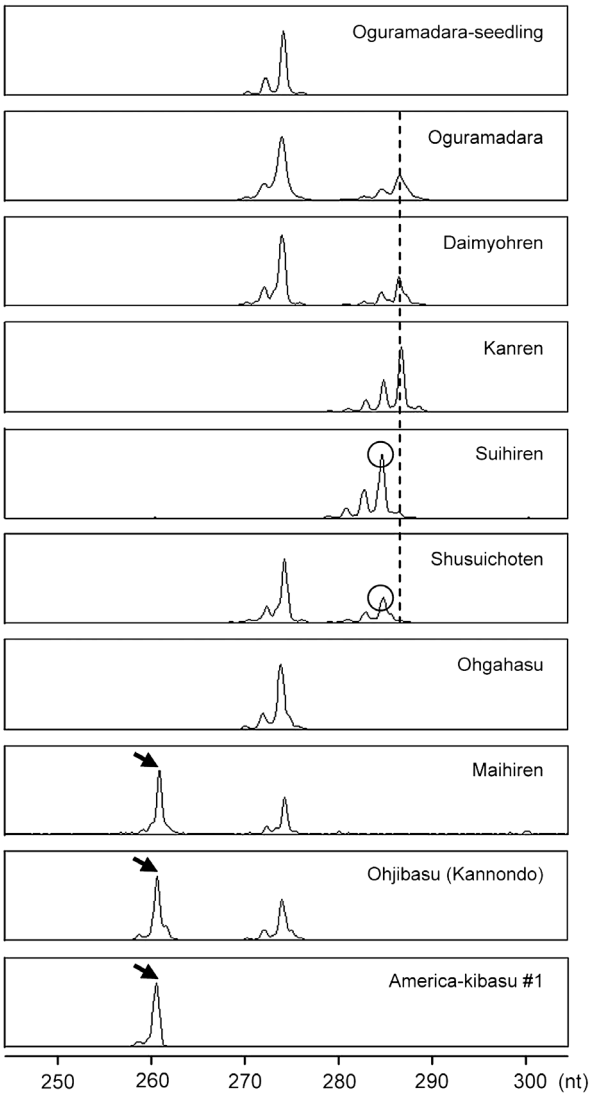

Fig. 1. The SSR polymorphisms in *Nelumbo* cultivars. Fragment analysis of the SSR marker NS049 in ten cultivars is shown as an example. The American lotus-specific allele is indicated with an arrow. A small nucleotide difference observed in 'Suihiren' and 'Shusuichoten' is enclosed with a circle, and the corresponding allele in 'Oguramadara', 'Daimyohren' and 'Kanren' is marked with a dashed line. A molecular weight standard is shown at the bottom.

and 'Gyokushuuren'–'Hokkeren' (Watanabe, 1990). In the former case, all the detected alleles were identical. Furthermore, two additional cultivars ('Oguramadara' and 'Tenjikumadarabasu') had the same allele sets as 'Daimyohren' and 'Ittenshikai' (Fig. 2, purple). The latter case ('Gyokushuuren'–'Hokkeren') was rejected in this study because of several mismatches between their alleles (data not shown). However, 'Gyokushuuren' and 'Hokkeren' appeared to be genetically similar, because they were in the same cluster on the NJ tree (BS = 72.2%; Fig. 2, subgroup IId). In addition to the known synonyms, each of the following three groups had identical alleles: 'Dainichirenge 3-gou'–'Johdairen'; 'Kitaine'–'Yazuma (Kohai 2)' and 'Oguranishi'–'Saifukuji-shiro'–'Shinnyoren'. There was a single-allele difference in each of the following four cultivar groups: 'Gyozankoren'–'Dainichirenge 3-gou' or 'Johdairen'; 'Higashikanze-beni'–'Nakayuden-beni'; 'Kurobohigashi'–'Shokkohren'; and 'Saienjishohren'–'Hakumanman' or 'Hekidairen'.

Confirmation and prediction of parentages with SSR markers

Parentages of *Nelumbo* cultivars were examined based on the SSR alleles. Although the parentages of some *Nelumbo* cultivars based on dominant inter-SSR markers have been examined previously (Huang *et al.*, 2004), co-dominant SSR markers have the advantage of enabling simultaneous comparison of the alleles derived from both parents in a given sample. We investigated nine cultivars, in which either or both parents were included in the samples (except for the offspring of 'Ohjibasu', see 'Materials and methods'). These were 'Chuunichiyuugiren', 'Daikoho', 'Kenmai', 'Maihiren', 'Oguramadara-seedling', 'Ootorii', 'Sayakaze', 'Shusuichoten' and 'Tenponotsutsumi' (Supplementary Table S1, available online only at http://journals.cambridge.org). Among these cultivars, the following relationships were confirmed: 'Ohgahasu' as paternity of 'Maihiren', 'Oguramadara' as maternity of 'Oguramadara-seedling' and 'Ootorii', and 'Tenjikumadarabasu' as maternity of 'Tenponotsutsumi'. For example, 'Maihiren' shared at least one allele with 'Ohgahasu' at each locus (Supplementary Table S3, available online only at http://journals.cambridge.org, light-blue). There was also no incongruence between the 'Ohgahasu'-derived alleles and the American lotus-specific alleles at 33 loci in 'Maihiren' (Supplementary Table S3, available online only at http://journals.cambridge.org, yellow). This observation did not contradict the pedigree of 'Maihiren' as an inter-specific hybrid between 'Ohjibasu' and 'Ohgahasu', although we did not directly examine its original maternal parent. In contrast, the remaining five possible parentages were negative in this study because of incongruence at several loci (data not shown). However, each of the following cultivar groups, 'Daikoho'–'Taiseikin', 'Kenmai'–'Maihiren' and 'Shusuichoten'–'Chuunichiyuugiren'–'Chuugokukodaibasu', appeared to share similar genetic backgrounds because they each fell into the same group or subgroup on the NJ tree (Fig. 2).

In addition to already-known parentages, potential paternal parents were predicted for three cultivars. (1) 'Aamujo' was derived from the natural pollination of

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Fig. 2. An NJ tree constructed by 97 *Nelumbo* samples based on 52 SSRs. The American lotus cultivar, 'America-kibasu #1', was used for an outgroup. Numbers on the node are BS values (>50%) from 1000 replicates. A cluster composed of American lotus-derived cultivars (group I) and four subgroups (IIa–d) is indicated on the right. Colours represent the following cultivars: American lotus-derived cultivars confirmed (yellow) and questionable (brown); 'Ogura-ike group' cultivars (blue); antique lotuses and their progenies (green); possible synonyms of 'Daimyohren' (purple); and other cultivars (black).

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'Ohjibasu' (Kaneko, 2002; Uchida, 2006), but its paternal parent is unknown. In this case, the 'Ogura-ike group' cultivar 'Sakosotoyashiki' was predicted to be a paternal parent because either allele was congruent with 'Aamujo' at each locus examined (Supplementary Table S3, available online only at http://journals.cambridge.org, light-blue). 'Genshibasu', 'Higashikanze-beni' and 'Nakayuden-beni' were also congruent as having paternity of 'Aamujo'. Like the case of 'Maihiren', no incongruence was observed between the alleles of these predicted paternal parents and the American lotus-specific alleles. (2) The paternal parent of 'Ootorii' was predicted to be 'Kurobohigashi' or 'Shokkohren' without any incongruence at paired alleles. This prediction was strongly supported by an allele of 108 nucleotides at locus NS290 (Supplementary Table S3, available online only at http://journals.cambridge.org, asterisks), which was specific in the three cultivars. (3) The paternal parent of 'Tenponotsutsumi' was predicted to be 'Kanren' or 'Shisendohsaikoren'. These three cultivars formed a single cluster on the NJ tree (Fig. 2, subgroup IIc) and shared similar morphological characteristics such as colour and number of petals.

Finally, possible cases of inbreeding were predicted in four cultivars. 'Byodoin', 'Hikonejobasu' and 'Oguramadara-seedling' were predicted to have been derived from 'Oguramadara' and its possible synonyms ('Daimyohren', 'Ittenshikai' and 'Tenjikumadarabasu'). At each locus, alleles in 'Byodoin', 'Hikonejobasu' and 'Oguramadara-seedling' were always the same with either or both of the alleles in the predicted parents (Supplementary Table S3, available online only at http://journals.cambridge.org, purple). This evidence raised the hypothesis that the three cultivars arose by self-pollination or by a cross between very closely-related cultivars. A significant decrease of heterozygous loci in 'Byodoin', 'Hikonejobasu' and 'Oguramadara-seedling' compared with the parents (8, 10 and 11 vs. 42, respectively) may also support this hypothesis (Supplementary Table S3, available online only at http://journals.cambridge.org, underlines). A typical case was found in an 'Ogura-ike group' cultivar. The parentage of 'Minamikanze' was predicted to be 'Dainichirenge-3 gou'. When their genotypes were compared, alleles in 'Minamikanze' were homozygous at all loci with regard to those in 'Dainichirenge-3 gou'. This may represent a fixation of alleles probably because of successive selfings or inbreedings.

Discussion

In this study, we conducted the classification of flowering lotus (*Nelumbo*) cultivars based on the SSR markers. To accomplish this, 33 additional SSR markers were developed from genomic and EST sequences. Together with the 35 markers that we previously developed (Kubo *et al.*, 2009) and 26 markers developed by other researchers (Pan *et al.*, 2007; Tian *et al.*, 2008a), we tested a total of 94 SSRs. Fifty-two of these markers were capable of genotyping 98 *Nelumbo* cultivars. Some alleles showed small nucleotide differences among cultivars (e.g. Fig. 1, circles). Therefore, SSR fragment analysis with a DNA sequencer or equivalent would be effective for accurate genotyping. Some of our SSRs also amplified products of similar size in a tree, *Platanus* (data not shown), which is a sister taxon to *Nelumbo* [reviewed in Savolainen and Chase, (2003)]. Therefore, our SSR markers may be useful for the analysis of species closely-related to *Nelumbo*.

Previously, we reported the presence of American lotus-specific alleles in SSR markers (Kubo *et al.*, 2009). In the present study, this assumption was confirmed in the American lotus-derived cultivars ('Aamujo', 'Bichuko', 'Kenmai', 'Kurodanibyakuren', 'Maihiren', 'Ohio-ren (Kannondo)', 'Ohjibasu (Kannondo)' and 'Ougyokuhai') plus 'America-kibasu #1'. In contrast, another five cultivars predicted to be derived from the American lotus ('America-byakuren', 'Kitaine', 'Ooji-Kawakatsu', 'Rinnoh-ren' and 'Yazuma (Kohai 2)'; Fig. 2, brown) did not show any such alleles. A similar incongruence between genetic data and pedigree has also been reported by Huang (2004). These observations raise the possibility that the identities or pedigrees of the five cultivars in question were incorrect. Alternatively, these cultivars might contain an American lotus-specific allele at loci that we have not yet analysed.

The SSR-based phylogenetic tree showed that the eight American lotus-derived cultivars were separated from the sacred lotus group (Fig. 2, yellow). This confirmed that American lotuses (*N. lutea*) are genetically distinct from sacred lotuses (*N. nucifera*), as reported previously with various types of molecular markers (Kanazawa *et al.*, 1998; Katori *et al.*, 2003; Peng *et al.*, 2004; Han *et al.*, 2007a; Chen *et al.*, 2008; Tian *et al.*, 2008b). The sacred lotus cultivars in our samples formed four subgroups (IIa–d). It is noteworthy that most Chinese cultivars examined in this study were in the subgroup IId (Fig. 2), unlike previous studies (Huang *et al.*, 2003; Katori *et al.*, 2003). Some of these Japanese cultivars may have been imported from China in ancient times, but the detailed relationships of Chinese and Japanese cultivars have yet been studied sufficiently. The present data suggest a possible genetic differentiation of some Japanese cultivars from Chinese lotuses. Because major studies of flowering lotuses have mainly examined Chinese cultivars with few Japanese samples, the present results may be helpful in assuming the differentiation of Chinese and Japanese lotuses. To infer a correlation

between the genetic classifications and morphological characteristics, we plotted petal characters on the NJ tree (Supplementary Fig. S1, available online only at http://journals.cambridge.org). There was no strong overall correlation between petal characters and genetic relationships, except for the American lotus group and small local clusters that formed on the NJ tree. For example, cultivars with white petals were distributed throughout the tree, without forming an apparently large cluster. This was also the case with the characters of nail-red petal and petal number. These results reflect the complex history of petal characters during the differentiation of *Nelumbo* cultivars. We are currently unable to trace the origins of these petal characters because each of the subgroups was poorly supported with BS values. The low BS values could be because of the relatively small genetic diversity of *Nelumbo* species (Xue *et al*., 2006). However, this assumption contradicts the fact that the gene diversity indices from the present data were not low (Supplementary Table S4, available online only at http://journals.cambridge.org). It is more likely that resolution of the NJ tree was disturbed by intra-specific hybrids between distantly-related cultivars, as seen in our parentage analysis. On the other hand, because relatively higher BS values were obtained in distal clades (Fig. 2), the present SSRs seem to be more effective for distinguishing the closely-related cultivars.

One of our aims in this study was to examine the relationships among cultivars of the 'Ogura-ike group', which constitutes one of the unique Japanese lotus cultivar groups. Twelve of these cultivars ('Higashikanze-beni', 'Makishima-shiro', 'Mekawa-nishi', 'Nakayuden-beni', 'Ninomaruike', 'Oguradaikoku', 'Oguranishi', 'Oguranoakebono', 'Oguranohonoo', 'Saifukuji-shiro', 'Sakosotoyashiki' and 'Yodohime') were grouped into a cluster with five other cultivars ('Genshibasu', 'Gunma-akabasu', 'Nehru-basu', 'Shinnyoren' and 'Zuikohren'; Fig. 2, subgroup IIa). This was in good agreement with the observation that 'Genshibasu' and 'Nehru-basu' have similar morphological characteristics to 'Sakosotoyashiki' (Uchida, 2006). Another cluster was found with four 'Ogura-ike group' cultivars ('Minamikanze', 'Misuaka', 'Myorenji' and 'Ooshimasaki'; Fig. 2, subgroup IId). These results suggest that the 'Ogura-ike group' cultivars in each cluster are genetically close and perhaps originated from a few closely-related cultivars.

Besides the genetic relationships among cultivars, comparison of SSR alleles allowed us to examine the identity and pedigree of each cultivar. We confirmed an already known synonym ('Daimyohren'–'Ittenshikai'), in which two additional cultivars ('Oguramadara' and 'Tenjikumadarabasu') may be included. Three cultivar groups with identical genotypes and four cultivar groups with almost identical genotypes were also identified. Each member of a cultivar group may represent a novel synonym or a close relative. However, slight morphological variations have also been observed in some cases. For example, 'Daimyohren' has a bigger plant size and more spots than 'Ittenshikai' (Kaneko, 2002). 'Tenjikumadarabasu' has many more spots than the other three possible synonyms. 'Oguranishi' and 'Shinnyoren' have single white flowers but they show morphological differences in petal angle and receptacle (data not shown). It is unclear which factor(s) cause such slight variations. Although it is unlikely that genotypes would be identical at all 52 loci by chance, the possible synonyms might contain small DNA differences that were undetectable in this study. There might be a small chance of somaclonal mutation, such as bud sport, because *Nelumbo* mutants in flowering lotuses (full double flower mutants 'Myohren' and 'Qianban lian') have been known (Watanabe, 1990; Wang and Zhang, 2005).

The present study also demonstrated the usefulness of SSR markers for parentage analysis. Relatively few parentages were confirmed and suggested here: four known parentages of 'Maihiren', 'Oguramadara-seedling', 'Ootorii' and 'Tenponotsutsumi'; and six currently-unknown parentages of 'Aamujo', 'Byodoin', 'Hikonejobasu', 'Minamikanze', 'Ootorii' and 'Tenponotsutsumi'. However, the number of predicted parentages is likely to have been underestimated because we applied stringent conditions for the parentage analysis to avoid false positives. In contrast, incongruences between the genetic data and the reported pedigrees were found. In this study, we verified morphological characteristics for most of the examined cultivars and omitted questionable ones to the best of our knowledge. The identities or pedigrees of these cultivars in question need to be clarified.

In conclusion, the utility of SSR markers for classification and identification has been demonstrated in *Nelumbo*. Fragment analysis of the SSRs enables more accurate genotyping of *Nelumbo* samples, especially for closely-related cultivars. Further studies using broader-based samples and data exchange among different research groups will uncover the classification and the pedigree of *Nelumbo* cultivars in future. The sequence-specific, co-dominant SSR markers would provide one of the most ideal tools for this purpose.

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