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TECHNICAL NOTE

Development and characterization of microsatellite loci for lotus (*Nelumbo nucifera*)

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Abstract This paper reports the development of microsatellite primers for *Nelumbo nucifera* Gaerten. By screening genomic libraries enriched with 10 kinds of probes, Seventeen polymorphic loci were isolated and primers were designed. Polymorphism of these 17 loci was assessed in 24 individuals. All the 17 loci are polymorphic and the number of alleles ranged from two to seven. Observed heterozygosity and expected heterozygosity ranged from 0.0000 to 0.9176 and from 0.2837 to 0.7917 respectively. These microsatellite loci should be useful for studying the genetic diversity of *N. nucifera*.

Keywords *Nelumbo nucifera* · Genetic diversity · Microsatellite primers · SSR

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Nelumbo is a small genus of aquatic angiosperm, comprising only two species, *Nelumbo nucifera* Gaerten. and *Nelumbo lutea* Willd. *Nelumbo nucifera* is found throughout Asia and north part of Australia and *N. lutea* is mainly distributed in the eastern North America.

Sacred lotus, *Nelumbo nucifera* Gaerten., is one of the most well known ornamental and economic plants in the world. It is extensively cultivated for the beauty of its flowers and unusual leaves in water gardens. Its rhizomes are an everyday vegetable consumed throughout China as well as other Asian countries and its seeds are used as a Chinese herbal medicine or a tonic. Lotus is also deeply embedded in Eastern culture and religion. In China lotus, *N. nucifera* has been cultivated for more than 3,000 years (Ni and Zhao 1987). Selection from wild forms and hybridization between cultivars are the two major ways for new cultivar breeding. Therefore, the wild populations are the important resource. However, distribution of wild lotus is extremely limited and endangered in China. Especially in recent years, with the development of aquaculture, the wild lotus is in the hard times of habitat fragmentation. *Nelumbo nucifera* has been listed in endangered species in China (Dong and Zheng 2005). *Nelumbo lutea* (American lotus), unlike its Asia relative *N. nucifera*, is less cultivated for ornament. However, wild *N. lutea* is in endangered status. For some lakes, specimens of *N. lutea* have ever collected, but there are not any now. For example, in Wisconsin, it is difficult to find this plant. So, it is stringent to protect wild American lotus. The success of any genetic resource conservation is dependent on understanding the genetic diversity of this species. However, it is scarce about the reports of genetic diversity of *N. lutea.*

Since very limited genetic diversity is harboured in wild populations, (for example, Xue et al. 2006) and it is difficult to give precise evaluations on the genetic variation,

Table 1 Characterization of the 17 microsatellite loci for *Nelumbo nucifera*, based on a sample of 24 individuals

Name	Motif sequence	Primer sequence (5′–3′)	Size (bp)	*Ta* (°C)	*A*	H_E	H_O	HW	GenBank accession no.
Nelumbo-04	$(GA)_{18}$	F: ATTATCTGCCCAAGCCTTCT	242	55	2	0.4539	0.0000	0.0000	EU368651
		R: TGTTCCATTTGATCTGCGTC							
Nelumbo-06	$(GA)_7GC(GA)_9$	F: GCATTCCCTTTATCCCACTC	265	56	2	0.3369	0.0000	0.0000	EU368652
		R: GGAGCCCATGTCACTCTACG							
Nelumbo-13	$(GA)_{22}$	F: CGGCTAGAAACCCTAGATTTCTATA	163	58	3	0.6782	0.6250	0.0000	EU368653
		R: ACACTCTTCAGTTCAGTCTTCACCT							
Nelumbo-14	$(AG)_{14}$	F: ATTTCATTTTGTATCTTTGATTTCT	150	54	3	0.4885	0.2917	0.2243	EU368654
		R: GACTGGATTGTACTTCTGAGTTCTA							
Nelumbo-15	$(AGT)_5$	F: TCCTTGGGGGTTGAGACTTAGA	123	58	3	0.2970	0.0417	0.0002	EU368655
		R: CAAGCAATGAACACGAGGAAAC							
Nelumbo-16	$(GGT)_6$	F: CGGTCACTTGCTAATTCAA	201	50	4	0.4495	0.3333	0.0027	EU368656
		R: AAGACTACCTTCACCTCCC							
Nelumbo-17	$(TG)_8(AG)_6$	F: GTGGCAATCCTTAAAGCTA	218	50	5	0.7101	0.9167	0.0017	EU368657
		R: TCTGTTTAGAAGCAATGTG							
Nelumbo-18	$(GA)_{10}$ GG $(GA)_6$	F: TTGGGGATTTCCTAACTGGT	286	55	7	0.5851	0.4583	0.1283	EU368658
		R: TCATTGTCTCAACAACTGGC							
Nelumbo-21	$(TG)_7$	F: TTTATTTTGGGAAGAGGAATA	193	52	3	0.3511	0.0000	0.0000	EU368659
		R: CTGACTTTTGTGAAATTCTGC							
Nelumbo-22	$(CT)_{13}$	F: AGCTTAGGGCTTTTATCTGCAC	170	58	6	0.6995	0.3750	0.0000	EU368660
		R: ATGGCAATGATAGAAAAGGGAG							
Nelumbo-23	$(TGG)_6$	F: CAGCTTCACCTTCTGCACGAA	287	58	3	0.5505	0.8750	0.0006	EU368661
		R: GCCCAAGAACAATGGAGGAAC							
Nelumbo-24	$(CCT)_7$	F: ACTTCTTCAGCTCCACTGTCC	203	55	3	0.5133	0.3333	0.2721	EU368662
		R: GTAGTGGTAGCAAGAGGACGC							
Nelumbo-25	$(TG)_6$	F: TGAGTTCTCCTCGCAATA	195	50	2	0.2837	0.0000	0.0003	EU368663
		R: AAAGAAGCCTTGATGGAA							
Nelumbo-27	$(CT)_{25}G(TC)_6$	F: TAAGCTAAGATAGGAATCCAACTAG	178	55	4	0.6746	0.7917	0.0000	EU368664
		R: AAAAGGATAGGAGATTAGTAGGTGA							
Nelumbo-32	$(TC)_{19}$	F: ATAATGGATTTTGGAGGTCTTG	210	55	5	0.7491	0.1667	0.0000	EU368665
		R: CTCTTCTTCATTCCTTTGGTTT							
Nelumbo-33	$(TC)_{11}$	F: ACTACTGGAATCTGCTGCAAGC	252	58	5	0.7057	0.8333	0.0100	EU368666
		R: CTGAAAGTGAACAGGCATCGTG							
Nelumbo-34	$(TC)_{21}CCTCC(CT)_7$	F: TGGTTGGCACTGTAATCTTC	151	52	6	0.7917	0.5833	0.0025	EU368667
		R: CTGTTTCGACTCTAGGCTTC							
Mean over all loci					3.88	0.5481	0.3897		

Size, expected fragment size from sequencing data; *Ta*, Annealing temperature; *A*, Number of alleles; H_E, Expected heterozyosity; H_O, Observed heterozygosity; HW, Significant deviation from Hardy-Weinberg level at $P = 0.05$ and the GenBank accession numbers of the fragment sequences on which primers were designed

population structure and gene flow. The microsatellite or the simple sequence repeat (SSR) markers seem to be a suitable alternative technique for the characterization of genetic diversity in both wild and cultivated lotus due to it's reliable, informative, co-dominant nature and ease of exchange of data among different studies. In this paper we report the procedures of SSR development from enriched libraries and the primers designed for lotus.

Fresh leaves were collected from Honghu Lake, Hubei Province, China. Genomic DNA (about 200ng/μl) was extracted using the CTAB method (Doyle and Doyle 1987) and then purified with the Wizard DNA Clean-Up System (Promega). A micro-satellite enriched library was built following Glenn and Schable (2005) with some modifications. The total DNA was digested with *Rsa* I (New England Biolabs) and ligated to double-strand Super SNX-24 linker (forward 5′-GTT TAA GGC CTA GCT AGC AGA ATC-3′, reverse 5′-pGAT TCT GCT AGC TAG GCC TTA AAC AAA-3′). The ligated DNA was denatured and hybridized to a mixture of the following single-strand bio-tinylated microsatellite probes (1 μM each): $(AG)_{12}$, $(AT)_{12}$, $(CG)_{12}$, $(GT)_{12}$, (for $ACG)_{12}$, $(ACT)_{12}$, $(CCA)_8$, $(AACT)_8$, $(AAGT)_8$ and $(AGAT)_8$ for enrichment. Hybridized DNA was captured with streptavidin coated paramagnetic beads (Dynal Biotech Dynabeads M-280 Streptavidin) and collected with magnetic particle collecting unit (MPC, Dynal Biotech Dynal MPC-S). The enriched DNA was amplified by PCR and enriched again. The PCR was carried out in a 25 μl volume containing 1 × HiFi T*aq* PCR SuperMix (TransGen Biotech, Beijing, China), 0.4 μM superSNX-24 linker-forward as a primer and 2 μl of enriched DNA fragments. The PCR program started with an initial step at 95 °C for 2 min, followed by 30 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 1.5 min, and ended with a final extension at 72°C for 30 min. The products from the second enrichment were PCR amplified, purified and ligated into pGEM-T easy vector (Promega), and transformed into Top 10 competent cells of *E. coli* (TransGen Biotech, Beijing, China). Positive clones were checked through PCR amplification of inserts using SP6 and T7 primers and the fragments of different size were sequenced on ABI 3730xl DNA analyzer (Applied Biosystems, USA). Sequences from both strands were assembled and edited using Sequencher 4.6 (Gene Codes Corporation, MI, USA) and microsatellite loci were sought with SSRHunter 1.3.0 (Qiang Li, Nanjing Agricultural University, Nanjing, China). Primers were designed with Primer premier 5.0 (Premier Biosoft International).

Primer pairs were checked using 24 *Nelumbo* samples (23 *N. nucifera* cultivars and one *N. lutea*). PCR amplification reactions were carried out using 1 × HiFi T*aq* PCR SuperMix (TransGen Biotech, Beijing, China), 0.25 μM of each primer and 25 ng DNA. PCR profile started with an activation step at 95°C for 3 min, followed by 30 cycles of 30 s at 94°C, 20 s at the annealing temperatures given in Table 1, and 1 min at 72°C, and ended with a final extension at 72°C for 10 min. The products were checked with 2.5% agarose gels. The amplified products showing the band of expected size were separated on 6% denaturing polyacrylamide sequencing gels using silver staining.

Five hundred and fifty-three clones were found positive. Two hundred and twenty (40%) positive clones with inserts of different sizes of the fragments were sequenced for the targeted fragments. Eighty-eight (40%) contained SSRs. Of the 88 sequences, 32 (36%) were unique, and 2 sequences were not suitable for primer design. So, 30 pairs of SSR primers were designed for amplifying 30 microsatellite loci and 17 pair primers showed the expected band.

We calculated alleles, Observed and expected heterozygosities and detected significant deviations from Hardy-Weinberg (HW) proportions (Table 1) and linkage disequilibrium (LD) between primer pairs using the software GENEPOP version 3.4 based on 24 *N. nucifera* samples (Raymond and Rousset 1995). All tested 17 primers showed polymorphism and all primer stets amplified one or two bands in *N. lutea*. The number of alleles ranged from two to seven, Observed heterozygosity and expected heterozygosity ranged from 0.0000 to 0.9176 and from 0.2837 to 0.7917 respectively (Table 1). The polymorphism is higher than other marker RAPD and ISSR (Xue et al. 2006). All loci except Nelumbo-14, Nelumbo-18, Nelumbo-24 and Nelumbo-33 loci showed significant deviation from Hardy-Weinberg equilibrium ($P < 0.01$). The test samples (almost were cultivars) should be the most plausible explanation for deviation from Hardy-Weinberg equilibrium. In this study, there was significant linkage disequilibrium ($P < 0.01$) between the loci pairs of Nelumbo-14 and Nelumbo-13; Nelumbo-15 and Nelumbo-17; Nelumbo-16 and Nelumbo-6 or Nelumbo-32; Nelumbo-24 and Nelumbo-14, Nelumbo-17, Nelumbo-25 or Nelumbo-33. Except loci Nelumbo-24, no significant LD was found after correction for multiple tests. This test indicates that primers here we designed could be useful in the studies of genetic diversity of *Nelumbo* and will provide useful implications for resource conservation.

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