

RESEARCH ARTICLE

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Development and application of molecular markers linked to single and double flowers in northern Chinese lotus (*Nelumbo nucifera*)

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Abstract

Lotus (*Nelumbo* Adans.) is a versatile plant with ornamental, edible, medicinal, water purification, and cultural significance. The wide variation in petal numbers, particularly the double-petal cultivars, contributes to its ornamental value. However, traditional breeding methods, which require growing seedlings to maturity to identify single or double flowers, are time-consuming (ranging from one to three years) and costly. This study mapped the quantitative trait locus (QTL) for single/double-petal traits to the 0.90–4.40 Mb region on chromosome 5 using the F₁ generation of the traditional cultivar ‘Dan Sajin’. Within this region, 78 single nucleotide polymorphism (SNP) and insertion-deletion (InDel) loci were identified, showing significant genotypic differences between single- and double-petal traits. A C/T SNP locus at Chr.5: 1,400,097 bp (marker H8) was selected from 42 single-petal and 35 double-petal lotus samples, demonstrating the highest resolution for distinguishing between the two flower types. In three hybrid combinations of Asian lotus (excluding single-petal lotus from south of the Yangtze River), seedlings with CC or CT genotypes consistently produced single-petal flowers (100% at maturity), while those with the TT genotype yielded double-petal flowers with an 85.7% probability. The H8 marker promises to significantly reduce production costs in lotus breeding, offering a simple, rapid, low-cost, and highly efficient method.

Keywords Asian lotus, Double flower, Molecular marker, *Nelumbo*, QTL mapping, Seedling stage identification

Introduction

The lotus (*Nelumbo* Adans.) is one of China’s top ten traditional ornamental flowers, also valued for its culinary (Guo et al. 2009) and medicinal uses (Chen et al. 2019; Sharma et al. 2017). Furthermore, it plays a vital role in the purification and restoration of aquatic ecosystems (Liu et al. 2016a; Zhang 2019). The *Nelumbo* genus includes only two species: the Asian lotus (*N. nucifera*

Gaertn.) and the American lotus (*N. lutea* Willd.). Asian lotus cultivars are highly diverse, with flower colors ranging from pink and red to variegated and white, and flower forms spanning single-, semi-double, double-, and thousand-petal types. In contrast, the American lotus remains predominantly wild, with yellow flowers and fewer cultivars (Liu et al. 2019). To date, 2,080 lotus cultivars have been officially recorded globally. Among these, 40.3% are single-petal cultivars, typically with 20–30 petals per flower, while 56.1% are double-petal cultivars, each containing more than 30 petals (Liu et al. 2019). Despite differing breeding objectives—whether enhancing ornamental traits through double-petal improvement, increasing seed yield with single- or semi-double-petal cultivars (Li et al. 2013; Liu et al. 2021a), or developing varieties for both ornamental and edible purposes (Lu 2022; Yang et al. 2021)—flower phenotype identification

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still depends on traditional methods, which require growing seedlings to maturity. However, extensive cultivation and breeding practices (Liu et al. 2021a, b) reveal that approximately two-thirds of seedlings from hybridized or self-pollinated lotus seeds take one to three years to bloom, significantly increasing the time and cost associated with developing new lotus cultivars. Therefore, the development of molecular markers for early identification of single- or double-petal traits in seedlings could streamline decisions on whether to select single-petal seedlings for seed production or retain double-petal plants for ornamental lotus breeding.

Research on the single/double flower trait has been conducted across various plant species, including petunia, carnation, lily, camellia, cyclamen, hollyhock, hydrangea, rose, domestic apple, gentian, peach, crabapple, peony, dianthus, plum, and lotus. These studies have investigated aspects such as morphological anatomy (Dadpour et al. 2011; MacIntyre and Lacroix 1996; Naghiloo et al. 2014; Zhang et al. 2019), genetic patterns (Guan et al. 2024; Sink 1973; Suyama et al. 2015), gene mapping (Meng et al. 2019; Roman et al. 2015; Wang et al. 2020; Yu et al. 2021), genome/transcriptome analysis (Huo et al. 2023; Wang et al. 2022; Zhang et al. 2024), and gene function validation (Akita et al. 2011; Gattolin et al. 2020; Liu et al. 2024; Ma et al. 2015; Noor et al. 2014; Sharifi et al. 2015). Notably, molecular markers linked to the double-flower trait have been developed, including one restriction fragment length polymorphism (RFLP) and two simple sequence repeat (SSR) markers for carnation (*Dianthus caryophyllus*) (Scovel et al. 1998; Yagi et al. 2014), one sequence-related amplified polymorphism (SRAP) and one SSR marker for petunia (*Petunia hybrida*) (Liu et al. 2016b), a retrotransposable element (*Tgs1*) insertion into *GsAG1* in Japanese gentian (*Gentiana scabra*) (Tasaki et al. 2017), one SSR marker for hydrangea (*Hydrangea macrophylla*) (Waki et al. 2018), and one SSR and five insertion-deletion (InDel) markers for Mei (*Prunus mume*) (Shi et al. 2023). These findings indicate that the single/double-petal trait is likely controlled by a few major-effect genes, with potential contributions from minor-effect genes, and can be roughly mapped to chromosomes. Consequently, with an appropriate mapping population, the use of high-density genome-wide single nucleotide polymorphism (SNP) and InDel markers, rather than low-density SSRs and SRAPs, offers promise for identifying markers tightly linked to petal number in lotus. In lotus, studies on the double-flower trait or flower shape formation (Sun et al. 2025) have focused on anatomy (Huang et al. 2014; Jiang et al. 2009), gene expression and function (Kashiwamura et al. 2020; Liu et al. 2012), stamen and carpel petaloidy (Lin et al. 2018, 2021), methylation (Lin et al.

2019), and genome-wide association studies (GWAS) (Gao et al. 2022). However, no molecular markers have been developed for identifying single/double-petal traits at the seedling stage, which limits breeding efficiency for single- and double-flower cultivars in the lotus industry. Over the past decade, bulked segregant analysis (BSA) combined with next-generation sequencing (BSA-seq) has greatly enhanced the efficiency and accuracy of quantitative trait locus (QTL) mapping while reducing costs. BSA-seq also extends its applicability and facilitates multi-omics integration, making it particularly effective for the rapid identification of major-effect QTLs and the development of molecular markers (Li and Xu 2022; Qu et al. 2023). In this study, BSA and whole-genome resequencing were first used to conduct preliminary QTL mapping of the petal number trait in lotus. Within the identified chromosomal regions, SNP and InDel sites closely associated with the single- and double-petal traits were detected and screened using whole-genome sequencing (WGS) technology. After verification with typical single- and double-petal lotus as well as segregating populations from three hybrid combinations, a molecular marker was identified. This SNP/InDel marker enables the prediction of whether a lotus seedling will produce single or double flowers at maturity, based on SNP/InDel genotype detection at the seedling stage. This approach facilitates the cost-effective and efficient breeding of superior lotus cultivars.

Material and methods

Plant materials

A total of 180 lotus accessions were tested, including 42 single-petal cultivars (species), 35 double-petal cultivars, 89 single- and double-petal segregating progeny from three artificial hybrid combinations, and 14 progenies derived from the self-pollination of two single-petal F_2 individuals (Fig. 1; Table S1). The 42 and 35 samples consisted of wild types, classic cultivars, and self-produced individuals, representing antique lotus, traditional cultivars, and modern hybrids. These samples included both temperate and tropical lotus from various geographic regions, ensuring their broad representation. In this study, lotus individuals with 20–30 petals per flower were classified as single-petal samples, while those with more than 35 petals, showing significant petalization of the stamens (and pistils), were classified as double-petal samples (Fig. 1).

All plant materials were cultivated at the 'International Nelumbo Collection' (certified by the International Waterlily and Water Gardening Society in 2016) at Chen-shan Botanical Garden in Shanghai. Species authenticity was confirmed by Dr. Fengluan Liu and Senior Engineer Naifeng Fu, with single- and double-petal traits recorded



Fig. 1 Morphology of single- and double-petal flowers in lotus. Upper panels: single-petal flowers, with no petalization occurring in the stamens; lower panels: double-petal flowers, characterized by the significant petalization of the stamens and pistils

for at least five flowers per sample during the blooming period. For various analytical purposes, the lotus accessions were categorized into five groups: (a) Initial QTL mapping for single- and double-petal traits using BSA, (b) SNP/InDel detection within the mapped chromosomal regions *via* WGS, (c) Screening for closely linked SNP/InDel markers by PCR, (d) Validation of candidate SNP/InDel markers in three hybrid combinations, and (e) Flowering phenotype observation of self-fertilized offspring with the CC genotype in the F_2 population.

Initial QTL mapping for single- and double-petal traits in lotus by BSA

The BSA method was employed for QTL mapping of petal number in lotus. The traditional single-flower cultivar 'Dan Sajin' (No. 18) was used as the parent (Table S1). This cultivar exhibits a single-petal phenotype with 23–27 petals, yet carries a heterozygous genotype for the single/double-flower trait, and has likely existed for over 430 years (Liu et al. 2020a). Extensive self-pollination of 'Dan Sajin' generated F_1 progeny, from which 30 single-petal and 21 double-petal individuals were selected to prepare separate DNA bulks, followed by WGS at 30× depth. Using the MutMap+ strategy (Fekih et al. 2013), heterozygous SNPs from all eight chromosomes of 'Dan Sajin' served as a reference. The Δ (SNP-index) was calculated between the two DNA bulks, with a sliding window of 1000 kb and a step size of 100 kb used to fit the curve. The initial QTL region on the lotus chromosomes associated with the petal number trait was identified, based on 95% and 99% confidence intervals of the curve. Additionally, the Euclidean distance (ED) algorithm (Hill et al. 2013) and the G-statistic method (Magwene et al. 2011) were also applied for QTL localization.

Initial detection *via* WGS and primer design for SNP/In Del markers linked to petal number

In plant QTL mapping studies, initial mapping regions for specific traits typically span 3–5 Mb and contain numerous candidate molecular markers, necessitating further refinement. In this study, WGS was conducted to identify significantly differentiated and homozygous SNP/InDel loci between representative single- and double-petal lotus within the chromosomal intervals of the initial QTL mapping for petal number. These SNP/InDel loci were subsequently screened to identify molecular markers closely linked to the single- and double-petal traits in two lotus groups. The single-petal group consisted of 10 wild types, six cultivars, and three DNA bulks, totaling 21 samples (including three samples of American lotus). The double-petal group included six representative cultivars and five DNA bulks, totaling 15 samples (Tables S1, S2). Clonal replicates of certain species (e.g. No.02, 10) were included in both single- and double-petal groups, with the single-petal group also containing seven samples with heterozygous genotypes for petal-number-regulating genes (Tables S1, S2). Linkage disequilibrium (LD) decay within the QTL region was calculated using PopLDdecay v3.31, with MaxDist set to 1,000, based on the correlation coefficient (r^2) (Zhang et al. 2019).

Sequencing depth was 10× for individuals and 20× for DNA bulks. Data filtering and SNP/InDel calling followed previously established protocols (Liu et al. 2021a). The molecular marker screening process consisted of two steps. First, within the chromosomal interval where the QTL for petal number was mapped, loci where the proportion of shared genotypes in 21 single-petal and 15 double-petal samples was greater than or equal to 80% were excluded. Second, from the remaining loci, those meeting the following criteria were manually selected as

target molecular markers linked to petal number: >90% genotype consistency within either the wild single-petal or double-petal groups and distinct genotypes between the two groups, preferably homozygous for both. Forward and reverse primers for each target SNP/InDel locus were designed using online tools from the National Center for Biotechnology Information (NCBI) website (<https://www.ncbi.nlm.nih.gov/>).

Screening of SNP/InDel markers linked to single/double-petal traits via PCR

The manually selected SNP/InDel markers were validated and filtered through PCR amplification in 42 single-petal (No.01–42) and 35 double-petal (No.43–77) cultivars. The PCR products were sequenced bidirectionally using forward and reverse primers by Sangon Biotech (Shanghai) Co., Ltd., and the resulting DNA sequences were aligned using BioEdit software. The molecular markers were evaluated based on their genotyping accuracy in distinguishing between single- and double-petal individuals. The SNP/InDel marker exhibiting the highest consistency between the genotypes derived from bidirectional sequencing and the single- or double-petal phenotypes across the tested samples was considered the most closely linked marker for petal number in lotus.

Validation of candidate SNP/InDel markers linked to single and double flowers

To validate the predictive value of the candidate SNP/InDel markers for petal number, 89 flowering individuals (No.78–166) from F₁ and F₂ generations of three hybrid combinations were selected as test subjects (Table S1). DNA was extracted from leaf tissue, and the candidate molecular markers were amplified by PCR, followed by sequencing and sequence alignment. The accuracy of predicting single- or double-petal traits was calculated to evaluate the applicability of these SNP/InDel markers for predicting flowering petal number during the seedling stage in lotus hybrid breeding.

DNA extraction, PCR amplification, and SNP/InDel detection

DNA extraction: Genomic DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle JJ and Doyle JL. 1987), with the sugar removal step repeated three times. The DNA was then diluted to 50 ng·μL⁻¹ and stored at 4°C. For DNA bulks, genomic DNA was extracted from individual samples and pooled in equal volumes to a concentration of 50 ng·μL⁻¹.

SNP/InDel calling: Library construction, WGS, and data filtration followed the methods outlined in a previous study (Liu et al. 2021a). Clean reads were mapped

to the eight chromosomes of ‘China Antique’ (<https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=4430>, Assembly number: GCA_014319735.1) using Burrows-Wheeler Aligner-maximum exact matches (BWA-mem, version 0.7.17) (Li 2013) and sequence alignment/map (SAM, version 1.10) tools (Li et al. 2009). PCR duplicates were marked using Picard (<https://github.com/metabrainz/picard>, version 2.23.4), and SNPs and InDels were detected with the Genome Analysis Toolkit (GATK, version 4.1.8.1) (Poplin et al. 2017).

PCR amplification: The total PCR reaction volume was 10 μL, containing 5 μL 2×ES Taq mix (Kangwei Biotech, Beijing, China), 1.5 μL DNA template (50 ng/μL), 0.5 μL of each forward and reverse primer (10 μM), and 2.5 μL ddH₂O. The PCR program was as follows: pre-denaturation at 94°C for 3 min, followed by 28 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30–40 s, with a final extension at 72°C for 7–10 min.

Results

Chromosomal intervals of QTLs for single- and double-petal traits in lotus

Self-pollination was performed on the ‘Dan Sajin’ lotus, which exhibits a single-petal phenotype but carries heterozygous loci for the single/double-petal traits. After sowing and cultivating 210 offspring, 95 plants flowered in the first year: 71 with single petals, 21 with double petals, and three with ambiguous traits, displaying 25–35 petals per flower. The ratio of single-petal to double-petal individuals was approximately 3:1, indicating that the single/double-petal trait in ‘Dan Sajin’ is governed by a major gene allele, with single-petal being dominant and double-petal recessive. BSA results from the Δ(SNP-index) and ED algorithms identified a significant QTL region on chromosome 5, spanning 900,000–4,400,000 bp, within the 99% confidence level (Fig. 2a, b). This region contains 204 genes (Nn5g26733–Nn5g26940). In contrast, the G-statistic algorithm did not identify a QTL interval at the 95% confidence level (Fig. 2c). These findings align with those of Gao et al. (2022), who identified a similar region using a GWAS approach, thus confirming the reliability of the results. Furthermore, LD analysis of 1,072 SNPs across the QTL interval revealed consistently high r^2 values (0.6–0.7) with minimal fluctuations (Fig. S1), indicating strong linkage between genetic variations across the entire region.

SNP/InDel markers closely linked to single- and double-petal traits

Within the 0.90–4.40 Mb region of chromosome 5, 57 SNP and 21 InDel loci were identified that met the

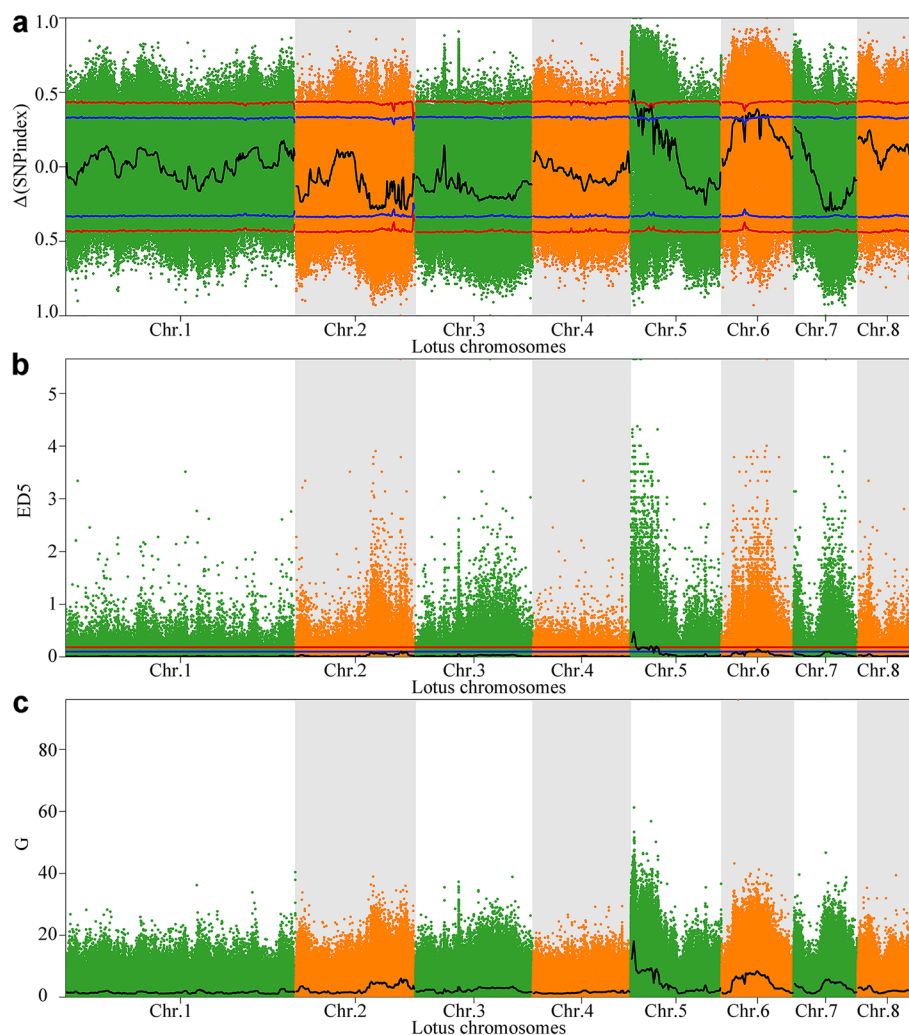


Fig. 2 Quantitative trait locus (QTL) mapping petal numbers in lotus. Mapping was performed using the $\Delta(\text{SNP-index})$ (a), Euclidean distance (ED) (b), and G-statistic algorithm (c). The scatter plot represents raw values, and the black curve depicts fitted values. The blue and red lines indicate the 95% and 99% confidence interval boundaries, respectively. No significant QTLs were detected using the G-statistic method. Chr, chromosome

primary screening criteria based on WGS data from 21 single-petal and 15 double-petal samples (Table S2). After further manual selection, six SNP, four InDel, and three SNP + InDel markers were chosen for validation with expanded samples (Table 1). Each marker contained one to six loci, displaying two distinct genotypes between the majority of single-petal and double-petal samples (Table S2). For instance, the H8 marker, located at Chr.5: 1,400,097 bp and 1,400,289 bp, showed a CC genotype in 88.9% of nine single-petal samples from seven wild Asian lotus, and a TT genotype in 96.7% of 15 double-petal samples. This suggests that seedlings with a CC genotype at these two SNPs are likely to produce single-petal flowers, while those with a TT

genotype are more likely to bloom with double petals. Notably, three samples of American lotus (*N. lutea*), although phenotypically single-petal, predominantly exhibited genotypes more consistent with double-petal samples (Table S2), indicating a different genetic mechanism regulating petal number compared to ‘Dan Sajin’.

Primers were designed for the 13 SNP/InDel markers (Table 1), and PCR amplification followed by genotyping was performed on a representative global collection of 42 single-petal and 35 double-petal lotus. Molecular markers with poor genotype–phenotype consistency were eliminated, leaving only those with higher detection accuracy. DNA sequence alignment revealed that the SNP locus at Chr.5: 1,400,097 bp in marker H8

Table 1 Main characteristics and primers of 13 SNP/InDel markers linked to lotus petal numbers

No	Marker code	Genomic position of marker	Reference base	Alternate base	Forward/Reverse primer (5'–3')	Marker start/end position	Product size
1	H8	1,400,097	C	T	F: GGCCAATGAATGAACAGA ATCCAT	1,400,028	363
		1,400,289	C	T	R: GATCCAGTCCCAGTTACA CACT	1,400,390	
2	J10	1,439,767	G	C	F: CACTCCCTTCCTCACCAA AGAT	1,439,546	783
		1,440,107	G	C	R: ATCCGTCCATAAATGCTG ACAAAC	1,440,328	
3	Z11	1,444,687	T	C	F: AGAAACGCCATTCAACCT AGCAT	1,444,596	810
		1,445,009	T	C	R: G ^a CACGTATTGTCAGTTAGC TCTTT	1,445,405	
		1,445,345	A	G			
4	M13	1,532,453	CAATCCAACACCTAGTGC GGTACCATAATCAAGTCTAGG GGTTATGTGTAACTTTCCAGT TTTTGCCATT	C	F: GCCTCAACATACCCAAGT TTGAC R: TGCCCAACCTATCTCCCA TTTC	1,532,391	791
		1,532,467	G	A,*			
		1,532,476	C	T,*		1,533,181	
5	D4	1,604,287	A	T	F: TGCTCCACACAAAGCACA CTAT R: CGAATCCTGCTAACTGCA AGTGAT	1,604,029	590
						1,604,618	
6	Q17	2,577,017	AT	ATT,A	F: TG ACTCCCACCACCAGA ACTAAT R: TGGTTAGCATCTCAATAC GCTTGT	2,576,757	673
						2,577,429	
7	Z33	3,359,140	C	CA,CAAAAA	F: TATCGTGGCTGGAAGTTA AAGGAT	3,358,876	569
		3,359,374	T	C	R: GGATAAAGCTATGGAACC CAGTGTAT	3,359,444	
8	Z37	3,700,603	A	G,*	F: CG TATACCGAGCCATG TCAG	3,700,348	629
		3,700,610	T	TG,*	R: CG ATTCTGAGCCAAGCAAG CTAT	3,700,976	
9	P16	3,704,917	A	T	F: CTACGTCTACCTTGTTCTTGT GATTT	3,704,782	740
		3,705,073	C	T	R: CACCCTAACCAACTGCCA ATGT	3,705,521	
		3,705,091	T	A			
		3,705,173	G	A			
		3,705,271	C	T			
10	V22	3,940,693	G	GGAAGAA	F: AGCATTGGTCCCTGAAC TTTGT	3,940,465	774
		3,940,858	A	G	R: CCAGATTGCGAGTTTGA GTAGT	3,941,238	
11	Z66	4,064,693	A	G	F: CG GTTGGCTGAGACTG AGATAG	4,064,451	689
					R: AGTCGGTCGGGTTTTCAT CTGT	4,065,139	
12	X24	4,130,861	T	TC	F: G GTACCTACTACACCCCTC AAC	4,130,263	755
					R: CG TCGATGGGAAGCTAAAA GGT	4,131,017	

Table 1 (continued)

No	Marker code	Genomic position of marker	Reference base	Alternate base	Forward/Reverse primer (5'–3')	Marker start/end position	Product size
13 ^{b)}	R18 a	4,374,883	G	A	F: C GAATGGGAGGAAGGG CATT	4,374,639	No DNA bands
		4,374,980	TGA	T	R: A GGCTACTGGCTTTCGTGT AAT	4,375,227	
	R18 b	4,374,883	G	A	F: GCGGACCAGCGTAGTTA AT	4,374,468	
		4,374,980	TGA	T	R: CCTGTAATTGAGGATTCG GGTCTT	4,375,213	

a) The replaced nucleotide is highlighted in font, with factors such as primer hairpin formation or primer dimer minimized or eliminated. b) No DNA bands were amplified from the R18 marker, despite the development of two primer pairs for it. SNP/InDel refers to single nucleotide polymorphism/insertion-deletion. * indicates that the base cannot be determined

exhibited the highest concordance between genotype and flowering phenotype, distinguishing single- and double-petal traits (Table S1).

In 42 single-petal lotus samples, the H8 marker's 1,400,097-SNP detected 24 CC, 3 CT, and 15 TT genotypes (Fig. 3), with a prediction accuracy of 64.3% (27/42) for CC/CT genotypes correlating with the single-petal phenotype (Table S1; Fas. S1). Excluding 11 samples from south of the Yangtze River (No. 30–40) and two American lotus (No. 41–42), the prediction accuracy increased to 93.1% for the 13 northern Asian lotus (No. 01–13) and

16 cultivars of unknown origin (No. 14–29), with only two samples (No. 28–29) from the latter showing the TT genotype. In 35 representative double-petal lotus, only one sample (No. 74) displayed the CC genotype, while the remaining samples were TT (Table S1; Fas. S2; Fig. 3), resulting in a prediction accuracy of 97.1% (34/35) for the TT genotype correlating with the double-petal phenotype. The chi-square test indicated a strong association between the CC/CT and TT genotypes and the single/double-petal phenotypes ($\chi^2=52.5$, $p<0.001$, Cramer's $V=0.906$).

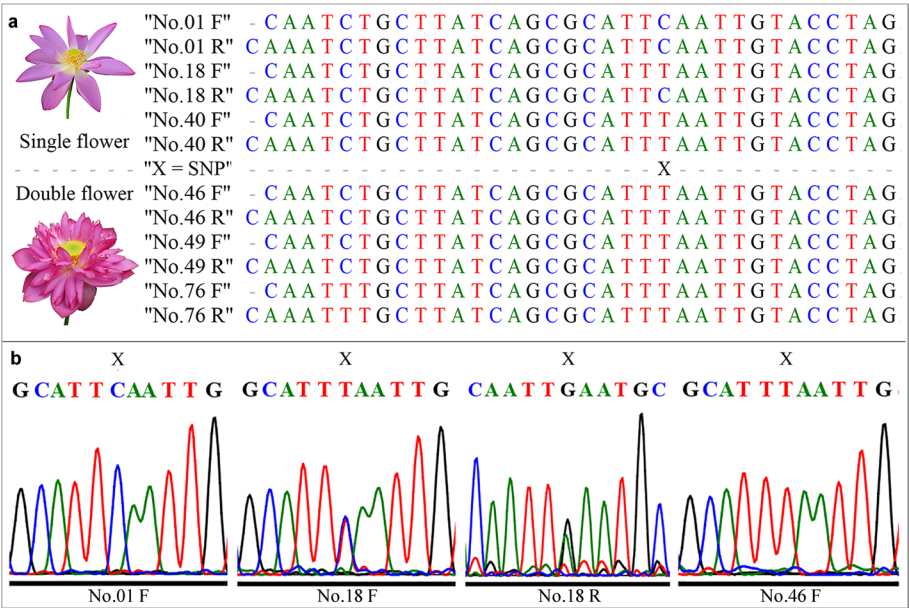


Fig. 3 Genotyping of the H8 marker in single- and double-petal lotus. Bidirectional DNA sequence alignments (a) and electropherograms (b) for representative individuals. Sample No. 01 has the CC genotype, No. 18 displays a heterozygous TC genotype, and No. 40, 46, 49, and 76 show the TT genotype. X indicates the single nucleotide polymorphism (SNP) locus at Chr5: 1,400,097 bp in the H8 marker. DNA sequence alignments and electropherograms for all samples are presented in Fas. S1, S2, and Zip. S1

The remaining 12 molecular markers, such as J10 and X24, were ineffective in distinguishing between single- and double-petal phenotypes when tested with a small number of lotus samples and were subsequently discarded. Therefore, the H8 SNP site is identified as the molecular marker most tightly linked to the single/double-petal traits in northern Chinese lotus.

Application of SNP marker H8 to northern Chinese lotus

Using molecular markers to predict single- or double-petal flowering at the seedling stage can significantly reduce labor and resource costs in lotus breeding by enabling the selective retention of the desired type. Among 89 flowering offspring from three hybrid combinations (crosses I, II, and III), the 1,400,097 bp SNP of the H8 marker demonstrated 100% accuracy in identifying single-petal individuals and an 85.7% accuracy rate for double-petal individuals (Table 2; Table S1; Fas. S3–S5; Zip. S1). Specifically, offspring seedlings from crosses of northern Chinese lotus, when tested for the H8-1,400,097-SNP genotype as CC or CT, will inevitably develop single-petal flowers at maturity, while those with the TT genotype will likely bloom with double petals with an 85.7% probability.

Discussion

Genetic pattern of petal number trait in lotus

Double-petal flowers in most plants arise from single-petal flowers through the petalization of pistils and stamens. The genetic regulation of this trait involves various models, such as major genes, minor genes, polygenes, or ploidy variations. Guozhen Huang, a renowned lotus researcher, proposed that double petals in lotus are a recessive trait. In a cross between the single-petal ‘Baihua Jian’ lotus and the double-petal ‘Hong Qianye’, the F₁ generation all displayed single-petal traits, with the F₂ generation segregating into both single- and double-petal types (Huang et al. 1983). Jiang et al. (2009b) obtained double-petal individuals in the F₁ generation by using single-petal parents without American lotus genes. After more than a decade of breeding over 700 cultivars (species)

collected from the ‘International Nelumbo Collection’, our team observed that most hybrid combinations follow a genetic pattern where the single-petal trait is dominant and the double-petal trait is recessive (unpublished data).

However, some exceptions were noted in our breeding and cultivation, such as certain tropical double-petal lotus producing single-petal progeny when crossed. Additionally, double-petal individuals sometimes appear in F₁ offspring from crosses between Asian lotus and purely single-petaled American lotus (unpublished data). In this study, three single-petal lotus frequently exhibited the TT genotype associated with double petals, rather than the expected CC genotype for single petals, at 78 SNP/InDel loci within the Chr.5: 0.90–4.40 Mb region. This unexpected pattern was consistently observed in subsequent validation of the H8 marker using samples No. 01–77 (Tables S1, S2). These samples included single-petal germplasms from south of the Yangtze River in China (No. 30–35), single-petal tropical lotus from Thailand, Vietnam, and India (No. 36–40), and the American lotus (No. 41–42), which only possesses the single-petal trait. Thus, the molecular markers within the Chr.5: 0.90–4.40 Mb interval for petal number in ‘Dan Sajin’ cannot effectively distinguish between single-petal and double-petal samples from southern China or American lotus. For lotus from regions south of the Yangtze River, particularly tropical varieties, this phenomenon may be attributed to their complex genetic backgrounds (Liu et al. 2020b, 2023). Liu et al. (2020b) revealed through WGS of 296 lotus accessions that wild lotus from central (e.g. the Yangtze River Basin) and northern China share close genetic relationships and a uniform genetic background, which is distinct from lotus found in southern China (Yunnan) and Southeast Asia. This genetic diversity could contribute to the observed genotypic patterns. As for American lotus, due to limited germplasm availability with well-characterized genetic backgrounds over a short period, extensive future work will be required to validate unique genotypes within the QTL interval. Therefore, through long-term artificial selection and natural evolution, particularly in Asia, lotus in different regions may have evolved distinct mechanisms for regulating the single- and double-petal traits.

Our previous breeding work (Liu et al. 2021b) indicated that the single-petaled ‘Dan Sajin’, used as the self-pollinating parent for BSA analysis, is a classic horticultural cultivar with a heterozygous allele controlling the single/double-petal traits. Due to the non-heritable nature of variegated flower color, which only propagates vegetatively through rhizomes, it is speculated that ‘Dan Sajin’ has existed for approximately 430 years (Liu et al. 2020a). This suggests that ‘Dan Sajin’ may not have participated in the hybridization process or gene flow of

Table 2 Accuracy analysis of H8 marker in identifying single/double-flowering during the seedling stage

Genotype and phenotype	Cross I	Cross II	Cross III	Identification accuracy ^{a)}
CC+CT	21	20	13	54/54
Single-flower plant	21	20	13	100.0%
TT	13	15	7	30/35
Double-flower plant	11	13	6	85.7%

a) DNA sequence alignment and single nucleotide polymorphism (SNP) genotype data for the three crosses are provided in Table S1 and Fas. S3–S5

modern lotus, potentially preserving a relatively pure genetic background. Such a background is advantageous for the precise mapping and isolation of genes controlling the single/double-petal traits. In this study, the 3:1 segregation ratio of single- to double-petal individuals in the self-cross progeny of 'Dan Sajin' provided the initial Chr.5: 0.90–4.40 Mb interval, which overlaps with the chromosomal region for the petal number trait identified by Gao et al. (2022) using GWAS analysis of 88 lotus samples. Additionally, within the 3.5 Mb interval of the petal-number QTL, LD r^2 values remained stable between 0.6 and 0.7, approximately 0.1 higher than those observed in rhizome lotus and wild lotus from tropical, central, and northern Asia (Liu et al. 2020b). This suggests that this QTL region may represent a low-recombination zone or be under strong selection pressure, implying that the QTL effect may stem from one or a few major-effect variations. These results indicate that the single/double-petal traits in most lotus resources from China, particularly those from north of the Yangtze River, may be regulated by a major gene in a dominant-recessive manner.

Regarding the failure of the G-statistic method to detect any significant QTL interval, this study attributes this to the inherent calculation principles and sensitivity of the method (Magwene et al. 2011). The G-statistic approach requires a large sample size, sufficient sequencing depth, and accurate genotyping. Thus, increasing the BSA analysis with 10 additional double-petaled individuals from the self-pollination of 'Dan Sajin' (totaling 30 plants) may improve the likelihood of identifying significant QTL regions.

Sample selection for screening SNP/InDel marker associated with petal numbers

In this study, when selecting lotus samples for screening SNP/InDel markers within the Chr.5: 0.90–4.40 Mb interval, both horizontal geographical distribution and vertical chronological differences were considered. Additionally, two clonal individuals of the same species were included as replicate samples to minimize errors in WGS and reduce individual developmental variations induced by environmental factors. Separate DNA bulks from single-petal and double-petal F_2 individuals were also prepared as test samples, expanding the genotypic diversity of many SNP/InDel loci within the initial QTL mapping interval. This approach facilitated the exclusion of SNP/InDel loci inconsistent with the single/double-petal phenotype, thereby narrowing down the number of target molecular markers. Furthermore, four heterozygous samples (No. 14–16, No. 18), derived from selfing or crossing and verified by test crosses, were selected (Tables

S1, S2). Their SNP (InDel) genotypes, matching those of the three single-petal DNA bulks (No. 17, 119–123, and 147–151), were predominantly 0|1 in the WGS data, confirming their heterozygosity for the single/double-petal gene. This provided an important reference for filtering molecular markers closely linked to the lotus petal number trait.

Strategy for screening molecular markers linked to petal number trait in lotus

SNPs and InDels, when combined with high-throughput sequencing technologies such as WGS, exhibit extremely high density and allow for precise chromosomal localization. These advantages make them indispensable for identifying molecular markers closely linked to target traits and for further mining of functional genes. In this study, a total of 57 SNPs and 21 InDels within the Chr.5: 0.90–4.40 Mb interval were identified, capable of distinguishing between certain single-petal and double-petal samples. A strategy involving PCR amplification, Sanger sequencing, and sequence alignment validation was then employed to screen these markers. However, this strategy has certain limitations, particularly with heterozygous InDel markers. While Sanger sequencing can typically detect heterozygous SNP sites, characterized by clear double peaks at the corresponding base on the electropherogram, the detection of heterozygous InDels—especially large insertions or deletions (> 10 bp) or complex variations—presents a greater challenge. In these cases, Sanger sequencing often results in noisy or unreadable electropherograms following the InDel sites, necessitating additional confirmation methods such as determining molecular weight *via* the fluorescently labeled primer method (Schuelke 2000; Liu et al. 2025) or polyacrylamide gel electrophoresis (PAGE). However, these methods are technically demanding, costly, and procedurally complex, which conflicts with the practical requirements of breeding for "ease of operation," "low cost," and "rapid processing." Consequently, the current study ultimately adopted a strategy based on PCR amplification, Sanger sequencing, and sequence alignment to screen and validate the 13 SNP/InDel markers.

Potential application of the H8 marker for marker-assisted breeding in lotus

In this study, DNA sequences and electropherograms for the 166 lotus samples (Zip. S1) revealed that the H8-1,400,097-SNP locus exhibited high stability and accuracy in genotyping when sequenced bidirectionally (Fig. 3). This approach eliminates the need for labor-intensive methods such as kompetitive allele-specific

PCR (KASP), cleaved amplified polymorphic sequences (CAPS), or dCAPS markers, and is simpler than detecting InDels using complex methods like PAGE or fluorescently labeled primers. Furthermore, the electropherograms allow for the detection of heterozygous samples for the single/double-petal genes, greatly facilitating research that requires these individuals as intermediates or for other specific applications.

The H8 marker offers three key advantages for molecular-assisted breeding of lotus: (a) Simplicity: Plant DNA extraction and PCR amplification are routine molecular biology techniques that can be performed in most laboratories. (b) Rapidity: The entire process, from DNA extraction to PCR amplification, sequencing, and sequence alignment, can be completed within 1.5 days in China. (c) Cost Reduction: By using the H8 marker to determine whether to select for single- or double-petal traits at the seedling stage, production costs per plant can be reduced by approximately 50%.

The H8 marker was identified within a relatively large QTL region (approximately 3.5 Mb) on chromosome 5 of lotus, which may explain its tight linkage with the petal number trait. However, it was not sufficient to distinguish between single- and double-petal individuals in tropical lotus. Fine mapping using backcross populations or recombinant inbred lines (RILs) would be beneficial for narrowing down the QTL interval, thus improving the precision of the H8 marker or identifying more closely linked molecular markers. However, such efforts require considerable time and extensive experimental validation.

Additionally, the petal number trait—whether single or double—is a key ornamental characteristic and a crucial breeding target in many horticultural species, including rose, peony, carnation, and chrysanthemum. Breeding efforts for this trait in these species have traditionally relied on phenotype-based selection at flowering, which is inefficient, time-consuming, and costly. Although the genes controlling petal number may vary among species, the successful identification of the H8 marker in this study provides a promising methodological reference for the development of molecular markers for petal number in other ornamental plants.

Conclusions

Predicting flower phenotype—single or double petal—during the seedling stage of lotus enables breeders to select the desired flower form, significantly reducing breeding costs. In this study, the QTL associated with single- and double-petal traits in Asian lotus was mapped to the 0.90–4.40 Mb region on chromosome 5. Within this region, an SNP at Chr.5: 1,400,097 bp (marker H8) was identified, which is tightly linked to petal number traits. In hybrid combinations using

Asian lotus, excluding single-petal types from south of the Yangtze River, seedlings with CC or CT genotypes at the H8 marker exhibited a 100% probability of producing single-petal flowers at maturity, while those with the TT genotype showed an 85.7% probability of blooming with double petals. This molecular marker, distinguished by its simplicity, rapidity, low cost, and high efficiency, holds significant promise for application in the lotus breeding industry.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s44281-025-00086-3>.

Supplementary Material 1. Table S1. Basic information of test lotus, accessions used for each molecular marker, and genotypes for marker H8

Supplementary Material 2. Table S2. Single nucleotide polymorphism (SNP) and insertion-deletion (InDel) loci with significant genotypic differences between single- and double-flowered lotus

Supplementary Material 3. Figure S1. Linkage disequilibrium (LD) decay curve of single nucleotide polymorphisms (SNPs) within the quantitative trait locus (QTL) interval

Supplementary Material 4. Fas. S1. Sequence alignment of 42 single-flower accessions for marker H8 (viewable using the software provided in Supplementary Material 10; applies to Supplementary Materials 4–9)

Supplementary Material 5. Fas. S2. Sequence alignment of 35 double-flower accessions for marker H8

Supplementary Material 6. Fas. S3. Sequence alignment of Cross I for marker H8

Supplementary Material 7. Fas. S4. Sequence alignment of Cross II for marker H8

Supplementary Material 8. Fas. S5. Sequence alignment of Cross III for marker H8

Supplementary Material 9. Supplementary Zip. S1. Sequencing signal maps of 166 lotus accessions for marker H8

Supplementary Material 10. Supplementary Software S1. BioEdit software used for viewing

Acknowledgements

Not applicable.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by JXH, YNM, GHH, SY, and ZNG. The first draft of the manuscript was written by JXH, YNM, and GHH. The final draft of the manuscript was reviewed and edited by TMAS and FLL. FLL conceived and supervised the project. All authors read and approved the final manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (Grant Number 32071821).

Data availability

All data generated or analyzed in the study are available within the paper and its supplementary files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Authors declare that they have no competing interests.

Received: 1 May 2025 Revised: 25 August 2025 Accepted: 28 August 2025
Published online: 10 December 2025

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