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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE MARKERS IN *ILEX KAUSHUE* (AQUIFOLIACEAE), A MEDICINAL PLANT SPECIES[1]

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- *Premise of the study:* Microsatellite markers were developed for *Ilex kaushue* (Aquifoliaceae), a medicinal plant with extremely small wild populations that exists in fragmented habitats, to assess and protect its genetic diversity.
- *Methods and Results:* Using 454 GS FLX Titanium sequencing, 16 microsatellite primer sets were isolated and characterized. Fifteen of these markers were polymorphic. The number of alleles per locus ranged from one to nine across 22 individuals from both cultivated and wild populations. The observed and expected heterozygosity in these two populations ranged from 0.000 to 1.000 and from 0.000 to 0.785, respectively.
- *Conclusions:* These markers will be useful in studies on genetic diversity of *I. kaushue*.

Key words: Aquifoliaceae; genetic diversity; *Ilex kaushue*; microsatellite marker.

Ilex kaushue S. Y. Hu (Aquifoliaceae) is an evergreen tree growing in dense forests at elevations between 400 and 1000 m in southern China (Chen et al., 2008). The species is one of the primary sources of ku-ding-cha (Hao et al., 2013), a tea used in traditional medicine that has been consumed for thousands of years in China. Modern medicinal research has demonstrated that ku-ding-cha has significant pharmacological effects, including as an antidiabetes and antiobesity drug, as well as an antioxidant (Hao et al., 2013). *Ilex kaushue* lives in a fragmented habitat with extremely small population sizes, and was included in a conservation program carried out in 2011 by the State Forestry Administration of China (Chen et al., 2014). Furthermore, *I. kaushue* is an economically important crop due to its widespread use for tea, and there has been rapid development in its cultivation in southern China (Guo et al., 2005).

Evidence supports that small natural populations and modern plant breeding can lead to a reduction in overall genetic diversity (Tanksley and McCouch, 1997; Leimu et al., 2006). Low levels of genetic diversity put wild populations at risk and jeopardize the continued ability to improve crops (Reif et al., 2005). Therefore, assessment and preservation of genetic diversity of *I. kaushue* are important concerns. Although Zhang et al. (2003) developed molecular markers (RAPD) for use in the study of *I. kaushue*, further genetic diversity research is necessary at the population and species levels to assess and protect its germplasm resources. Assessment and conservation of genetic diversity of a species requires development of efficient codominant microsatellite markers. In this study, 16 microsatellite loci for *I. kaushue* were isolated and characterized, which will be useful for assessment and conservation of genetic diversity of *I. kaushue.*

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METHODS AND RESULTS

We sampled 12 *I. kaushue* trees in a natural population (Baisha County/Hainan Province, China [QS]: 19°08′50.38″N, 109°16′14.9″E) and 10 trees in a cultivated population (Dapu County/Guangdong Province, China [DM]: 24°16′40.31″N, 116°28′02.83″E). Voucher specimens of each population were deposited in the Guangxi Institute of Traditional Medical and Pharmaceutical Sciences herbarium (GXMI; accession numbers Ik-012-ZQW and Ik-008-HYF, respectively; Appendix 1). Genomic DNA (gDNA) was extracted from silica gel–dried leaves using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). We mixed gDNA of all wild-collected individuals to be shotgun sequenced by Sangon Biotech (Shanghai, China) using 454 GS FLX Titanium (Roche Applied Science, Branford, Connecticut, USA). The 454 sequencing technique is described in detail in Margulies et al. (2005).

We obtained 29,247 reads ranging from 32 to 691 bp with an average read length of 401 bp, for a total of 11,736,223 bases. All reads were further screened for microsatellite motifs implemented in the program SSRHunter 1.3 with the default parameters (Li and Wan, 2005). A total of 1109 sequences containing 1104 dinucleotide, 259 trinucleotide, and nine tetranucleotide repeats were obtained. Of these sequences, those containing at least six dinucleotide or trinucleotide repeats and sufficient lengths at either end of the repeat motif were chosen for primer design using Primer Premier 5.0 (Clarke and Gorley, 2001); a total of 631 sequences, containing 691 dinucleotide and 82 trinucleotide repeats, were subjected to primer design. The settings for Primer Premier were as follows: (i) each search range of sense primer and antisense primer was at each end of the repeat motif; (ii) the primer length was between 17 and 25 bp; (iii) the PCR product size was between 100 and 400 bp long; (iv) the annealing temperature of primers was between 50°C and 64°C, and the difference in annealing temperature between the forward and reverse primers was <4°C; (v) the GC content was between 40% and 60%; (vi) there was not obvious hairpin structure within the primer; and (vii) other parameters followed the default settings of "High" stringency in the search criteria. A total of 78 primer pairs were successfully designed for a total of 99 repeats including 65 dinucleotide,

TABLE 1. Characteristics of 16 microsatellite markers in *Ilex kaushue*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Allele size range (bp)	GenBank accession no.
KDC1	F: CTTACTCCCTTTGGTGCTC	$(AG)_{13}$	60	181–191	KP943496
	R: CTCTTTTAGTCATTTTGCCC				
KDC10	F: GGCCCTCCTGTAATTTTTC	$(TA)_7$	58	133–139	KP943497
	R: GGTCGGTCCCATTCTTGT				
KDC11	F: TCTCAGGGTGCCTAAATA	$(GA)_7$	56	122–138	KP868632
	R: AACTAAGGTGTTTAAGGTCC				
KDC12	F: GTAGACGACAATAATGGCGG	$(TGG)_6$	60	329–335	KP868633
	R: CTCCACCGATTGCTACTATTG				
KDC16	F: CGAGCGGAAAGCAGAAATC	$(GTG)_6$	60	238	KP943498
	R: AGCCGAGGCAGAGGTAAAGA				
KDC27	F: GACAACCAAACACAGAAAAG	$(AG)_8$	57	186–192	KP868635
	R: CAAAAGGACCAGTAACCC				
KDC29	F: GAGTGGTTTGTATGGTCTTGT	$(TG)_7(GT)_5$	60	203–207	KP868636
	R: CAGTGGTTAGCCTTTGATTC				
KDC32	F: AGGTGATAAAGGAGAGGTCG	$(AT)_5(AG)_7$	60	127–135	KP868637
	R: CTCCCTCTCGTATACCACCT				
KDC41	F: CACTAGTTGCATTGGTGCT	$(TTC)_{10}$	58	282–306	KP868638
	R: TGTTTAATGAACCCACCTC				
KDC49	F: CAACTAACCCTATGTGTC	$(AG)_{14}$	53	120–142	KP868639
	R: TTGTTAGAAAATCCTCG				
KDC50	F: GCATGGTCTTTTGAAAACGA	$(GA)_{14}$	58	272–286	KP943499
	R: GGGACGGCATAGAACTGTAAT				
KDC58	F: AGAGGACAACGAAGATTAGG	$(CT)_9$	60	346–350	KP868641
	R: GAGAGGGTGGACTGAGAGAT				
KDC61	F: CATTCCACTGACACAACCG	$(GA)_8$	60	238–244	KP868642
	R: GAGCCTCCTCCTTCATTGT				
KDC62	F: GTGTTGTTGATGGTGGGTT	$(GA)_5(GA)_7$	59	166–176	KP868643
	R: ACGTTAGACCCACTCTCATC				
KDC63	F: CGACATTTACAGTCTAGC	$(GT)_8$	56	170–174	KP868644
	R: CTCAACCTTTAACTCTCTC				
KDC66	F: CCAACAAATCAATAGGGAC	$(GA)_9$	56	142–156	KP868645
	R: AACTTTTAAGAGCAGTGCC				

Note: T_a = annealing temperature when run individually.

18 trinucleotide, and 16 compound repeats. These primers were tested for polymorphism in 22 individuals from the two populations.

PCR reactions were performed in a 20-μL reaction volume containing 50–100 ng of gDNA, 0.5 μM of each primer, and 10 μL of 2× *Taq* PCR MasterMix (0.1 U/μL *Taq* polymerase, 0.5 mM dNTP each, 20 mM Tris-HCl [pH 8.3], 100 mM KCl, and 3 mM $MgCl_2$ [Tiangen Biotech, Beijing, China]). PCR amplifications were conducted under the following conditions: 95°C for 5 min; followed by 35 cycles at 94°C for 45 s, at the annealing temperature for each specific primer (optimized for each locus, Table 1) for 45 s, 72°C for 45 s; and a final extension step at 72°C for 5 min. PCR products were resolved on 6% polyacrylamide denaturing gel using a 10-bp or 25-bp DNA ladder (Invitrogen, Carlsbad, California, USA) as a reference and were visualized by silver staining.

Sixteen primer pairs were successfully amplified; these products exhibited the expected sizes and showed clearly defined banding patterns with a maximum of two alleles in each locus per individual. The number of alleles per locus (*A*) and the observed and expected heterozygosity (H_o and H_e) of the two populations were estimated by GenAlEx version 6 (Peakall and Smouse, 2006). Linkage

TABLE 2. Results of initial primer screening in two populations of *Ilex kaushue*.

	QS population (natural, $N = 12$)				DM population (cultivated, $N = 10$)				
Locus	*A*	H_o	H_e	*P* value	*A*	H_o	H_e	*P* value	A_T
KDC1	3	0.667	0.653	1.000	2	0.300	0.375	0.480	4
KDC10	3	0.417	0.569	0.193	2	0.600	0.500	0.976	4
KDC11	4	0.667	0.559	0.607	3	0.300	0.585	0.057	6
KDC12	3	0.417	0.569	0.504	2	0.600	0.480	1.000	3
KDC16	1	—	—	—	1	—	—	—	1
KDC27	4	0.750	0.726	1.000	1	—	—	—	4
KDC29	1	—	—	—	3	0.800	0.660	1.000	3
KDC32	4	0.833	0.712	0.286	2	0.500	0.495	1.000	5
KDC41	5	0.833	0.708	0.533	3	0.800	0.655	1.000	6
KDC49	6	0.917	0.764	0.846	5	0.900	0.675	0.953	9
KDC50	6	1.000	0.785	0.791	4	0.700	0.655	0.849	7
KDC58	3	0.583	0.569	1.000	1	—	—	—	3
KDC61	3	0.583	0.531	1.000	2	0.500	0.495	1.000	4
KDC62	5	0.667	0.726	0.809	2	0.600	0.500	1.000	5
KDC63	2	0.333	0.500	0.282	3	0.600	0.645	1.000	3
KDC66	4	0.917	0.691	0.547	3	0.800	0.665	0.024	6

Note: *A* = number of alleles; A_T = total number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *N* = sample size for each population; *P* value = test for deviation from Hardy–Weinberg expectations.

disequilibrium (LD) and deviation from Hardy–Weinberg equilibrium (HWE) were calculated by GENEPOP version 4.2 (Raymond and Rousset, 1995).

Across the cultivated and wild populations, *A* varied from one to nine, and a total of 73 alleles were scored in 22 individuals (Table 2). H_o and H_e in the natural and cultivated populations ranged from 0.000 to 1.000 and from 0.000 to 0.785, respectively. No pairs of loci showed significant LD. The *P* value of tests for HWE ranged from 0.024 to 1.000 (Table 2). Only locus KDC66 in population DM significantly deviated from HWE ($P < 0.05$), which may due to overdominant selection or admixture from different resources given the high level of heterozygosity for this locus.

CONCLUSIONS

A total of 16 nuclear microsatellite markers were developed for *I. kaushue*. Fifteen of these markers showed varying levels of polymorphism and one marker exhibited monomorphism. These loci will be useful for assessment and conservation of genetic diversity of *I. kaushue*.

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Appendix 1. Voucher information for *Ilex kaushue* used in this study.

Population	Voucher specimen accession no.[a]	Collection locality[b]	Geographic coordinates	*N*
QS	Ik-012-ZQW	Qingsong Township, Baisha County, Hainan Province	19°08′50.38″N, 109°16′14.91″E	12
DM	Ik-008-HYF	Dama Town, Dapu County, Guangdong Province	24°16′40.31″N, 116°28′02.83″E	10

Note: *N* = number of individuals.

[a]Vouchers deposited in the Guangxi Institute of Traditional Medical and Pharmaceutical Sciences herbarium. ZQW = Qi-Wei Zhang, collector; HYF = Yun-Feng Huang, collector.

[b]Locality and Chinese province.