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Isolation and characterization of polymorphic microsatellite markers in the endangered species *Bretschneidera sinensis* Hemsl.

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ABSTRACT. *Bretschneidera sinensis* is an endangered species that is mainly distributed in South China. As a tertiary relict and the single species in the Bretschneideraceae family, it has a high conservation value. To investigate the influence of human disturbance on its mating system, 63 new microsatellites were developed using restriction-site-associated DNA sequencing and their polymorphisms were tested on 30 samples from one population. Among the 63 microsatellites, the number of alleles per locus ranged from 2 to 16. The observed and expected heterozygosities ranged from 0.133 to 0.967 and from 0.127 to 0.912, respectively. These microsatellites may be used for studying the mating system of *B. sinensis* as well as the within-population hereditary structure.

Key words: Bretschneideraceae; Conservation genetics; Genetic diversity; Genetic markers; Mating system; RAD-seq

INTRODUCTION

Bretschneidera sinensis is a tree species mainly distributed in South-east China, extending to northern Thailand and northern Vietnam. It grows up to 10-20 m tall in low to middle elevation forest. As a tertiary relict and the single species in the family Bretschneideraceae, it is of high research value. Due to habitat loss and logging, *B. sinensis* is becoming increasingly rare. According to recent studies, most populations generally contain less than 30 individuals (Xu et al., 2013). In the field, this species also suffers from reproductive failure due to low seed productivity and seed germination rates (Qiao et al., 2011; Liang et al., 2013), which further increasing its extinction risk. At present, it is included in the China national key protected wild plants and the China plant red data book (Fu and Jin, 1992), and is listed as endangered by the international union for conservation of nature and natural resources (Sun, 1998).

B. sinensis has hermaphroditic flowers that form long racemes and are mainly pollinated by hymenopteran insects (Qiao et al., 2012). A pollination ecology study showed that *B. sinensis* has a mating system consisting of a mixture of outcrossing and selfing (Qiao et al., 2012). The mating system plays a key role in maintaining genetic diversity within populations. However, it is currently unknown how such mating systems are influenced by habitat fragmentation and the reduced population size in *B. sinensis*. In the present study, we report 63 newly developed polymorphic microsatellite loci for *B. sinensis* that may be used in future mating system studies.

MATERIAL AND METHODS

A total of 31 leaf tissues of *B. sinensis* were sampled from two populations: 30 from Nan-Kun Mountain Nature Reserve (NKMNR, 113°48'41"-113°56'32"E, 23°35'14"-23°43'05"N) in Guangdong Province and one from Hua-Ping National Nature Reserve (HPNNR, 109°48'54"-109°58'20" E, 25°31'10"-25°39'36"N) in Guangxi Province. For microsatellite locus identification, the HPNNR sample and one sample randomly chosen from NKMNR were used to construct restriction-site-associated DNA sequencing (RAD-seq) library. Total genomic DNA was exacted from these two tissues. The extraction was performed using a modified CTAB method (Doyle, 1991). The two DNA samples were then subjected to RAD-seq using a HiSeq2500 sequencer (Illumina Inc., San Diego, CA, USA). After trimming the adapters and removing low-quality sequences, we obtained 21,533,218 and 23,378,532 clean sequences from the two DNA samples, respectively. The sequences were then assembled using STACK v. 1.24 (Catchen et al., 2011, 2013). From the assembled sequences, a total of 417 microsatellites (di- and trinucleotides; containing at least seven repeats) were identified using MSATCOMMANDER v. 0.8.2 (Faircloth, 2008) of which 211 could be used for further primer design.

Polymerase chain reaction (PCR) amplifications were conducted for the 211 sequences for the NKMNR individual in a 20-μL volume containing 2 μL 10X PCR buffer (Mg^{2+} plus), 0.5 mM dNTPs, 0.4 μM each primer, 50 ng DNA template, and 1 U *Taq* polymerase (TaKaRa, Biotechnology Co., Ltd., Daliang, China). The PCR amplification conditions were as follows: initial template denaturation at 95°C for 5 min; then 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 45 s; followed by a final extension at 72°C for 10 min. The amplified products were electrophoresed on a 2% agarose gel. A total of 170 sequences successfully amplified the target regions.

Six NKMNR individuals were preliminary used to examine the polymorphism of the 170 microsatellite loci. The amplified PCR products were electrophoresed on an ABI 3730 sequencer (Applied Biosystems, Carlsbad, CA, USA), and their sizes were measured using the ABI GeneMapper software v. 4.1. The loci that showed clear and stable polymorphisms were further used for allelic variation assessments on 30 individuals from NKMNR (Table 1).

Table 1. Characteristics and genetic diversity of 63 microsatellite primers developed in *Bretschneidera sinensis*.

Locus	Repeat motif	Primer sequences (5'-3')	Size range (bp)	5'-fluorescence label	N_A	H_O	H_E	F	EMBL accession No.
BLS-3	$(TA)_9$	F:AGGAAGAAATCAAACAGGATTTGG	158 - 170	HEX	5	0.633	0.672	0.0589	LN886552
		R:TGTTAGCACCTACTACCAAGGG							
BLS-5	$(CT)_{12}$	F:TGACTACTTTCTGCTTGCCAC	220 - 256	FAM	11	0.900	0.887	-0.0149	LN886553
		R:ACTCGTTGCCTAATCTCTGAAAC							
BLS-6	$(CT)_{12}$	F:TACCCGATAAGCCCTGTGG	144 - 166	FAM	7	0.870	0.736	-0.1860	LN886554
		R:TGGAAATCGCAGAGGAATTAGG							
BLS-9	$(GA)_9$	F:CCATCCATTCCCTTTGAATTTCTG	167 - 169	HEX	2	0.276	0.242	-0.1429	LN886555
		R:GAGCCGAGATGGGCAGATG							
BLS-10	$(TA)_9$	F:TCTGTAGTGGTTCAATATTAAGTGG	179 - 183	FAM	3	0.429	0.512	0.1660	LN886556
		R:TCTTGGGATATAGACCAGAAATACG							
BLS-12	$(TC)_9$	F:CTCGCACATTGGCCATTCC	222 - 224	FAM	2	0.448	0.506	0.1165	LN886557
		R:ACATGTGATGTGAGTACAACATGG							
BLS-13	$(AC)_{11}$	F:TGTCGATGTGAGTTTGCCTTA	206 - 214	FAM	3	0.600	0.518	-0.1613	LN886558
		R:CATCTCAATACAATTCAAGCATCA							
BLS-15	$(TC)_{12}$	F:ATAATGCAGAATCCACACCACTT	237 - 245	FAM	5	0.733	0.727	-0.0087	LN886559
		R:GAGGGGTTCAAAATGAGTAACAG							
BLS-52	$(AT)_9$	F:CTCGAGCTTTGCTTGAAAGG	225 - 243	HEX	7	0.704	0.825	0.1497	LN886560
		R:GATGCCGAACTTGGCTCTG							
BLS-53	$(AT)_8$	F:GTATCCTAGTTTGGCTGGCG	230 - 256	HEX	12	0.821	0.753	-0.0933	LN886561
		R:GCTTCCCTGTTCTTGTAAGATTCG							
BLS-57	$(CT)_{13}$	F:GCATGCATATTGGCTCTTTACG	258 - 268	HEX	5	0.667	0.663	-0.0061	LN886562
		R:TGTCACATGCACAAGAATGG							
BLS-61	$(CT)_8$	F:TCGATTTGCTTCTAATGCACCC	168 - 172	FAM	3	0.636	0.532	-0.2025	LN886563
		R:CCCTTCCCACCAGAGATTCC							
BLS-63	$(CT)_{11}$	F:ACACTTAACACCTCTACTTGGTTG	208 - 220	HEX	4	0.724	0.745	0.0289	LN886564
		R:TGGCATAACTCAAGTGTACAGG							
BLS-66	$(CT)_8$	F:TCTCTTGCCACGCGATTTC	211 - 219	HEX	4	0.633	0.606	-0.0465	LN886565
		R:GGACTGGCCAAGGAAGGG							
BLS-67	$(CT)_8$	F:GACTTGTACATGCCGAAGGTG	258 - 266	HEX	3	0.700	0.656	-0.0684	LN886566
		R:CAGTGGAAATAGCCAACATTGC							
BLS-71	$(AG)_9$	F:TCTTAAGTTGCAGGCCGAC	211 - 237	HEX	12	0.967	0.869	-0.1139	LN886567
		R:AGCAACATCTTCCTAGTGAGTTC							
BLS-73	$(CT)_{10}$	F:CCGCTGGGTCCTCCATAG	188 - 196	FAM	3	0.276	0.246	-0.1228	LN886568
		R:TCAAGAATTTGAATCAGTAGCAACC							
BLS-74	$(AG)_{10}$	F:GAAGCAAGAGTTCTCTGCCG	190 - 192	FAM	2	0.567	0.508	-0.1179	LN886569
		R:GCTCTTAGTACCGAATCATGGC							
BLS-75	$(CT)_8$	F:TCAAACTGCTGCAAAGAGGC	169 - 173	FAM	3	0.552	0.482	-0.1487	LN886570
		R:TTAAAGCACCCGAAAGCCC							
BLS-76	$(AT)_{12}$	F:TGACCGGTATTTGCATAAAGG	259 - 276	HEX	9	0.500	0.793	0.3738*	LN886571
		R:CTCAACACACCCTTCCATTGAG							
BLS-85	$(CT)_8$	F:TCACAGGCACAATACCCTAATTTC	177 - 183	FAM	4	0.667	0.572	-0.1682	LN886572
		R:GGCGGCTCCATTGACTTAC							
BLS-94	$(AC)_9$	F:ACAAGCAACAGGTTGTCAGG	192 - 202	HEX	5	0.552	0.738	0.2558	LN886573
		R:CAGGTTCAATTCCAGTCGGC							
BLS-98	$(AG)_9$	F:GAGAGAGGGAGTCTTGATCTTTGC	264 - 308	HEX	4	0.481	0.587	0.1826	LN886574
		R:GTCATGGAAGCAGGCGTTG							
BLS-100	$(AC)_9$	F:GCTACAAATGCCATCCGC	261 - 279	HEX	7	0.786	0.749	-0.0504	LN886575
		R:TGGCAGCTTGACAGAATGG							
BLS-112	$(CT)_8$	F:GGTCCACTCACAAGATGAGC	255 - 257	HEX	2	0.367	0.305	-0.2083	LN886576
		R:TGTAGTTGTCGCTCCTTCC							
BLS-118	$(AC)_8$	F:CTTCTTCATTCTTCAGTTCCCAAG	225 - 227	HEX	2	0.133	0.127	-0.0545	LN886577
		R:GCTCAAACATCTGTAGCCCAAG							
BLS-121	$(AC)_{12}$	F:ACCTACTATAAACCCGTTCAGTC	168 - 228	FAM	16	0.690	0.912	0.2473	LN886578
		R:GTGTTCGAAATCAAATAGGATGGC							
BLS-122	$(AT)_7$	F:CTGAATGGGTGGTAGGTCATC	168 - 172	FAM	3	0.200	0.244	0.1812	LN886579
		R:GCAACCTGTCCTGTGAACC							
BLS-123	$(AT)_8$	F:ACCATGCTTGATTATGCACCC	178 - 180	FAM	2	0.467	0.499	0.0667	LN886580
		R:GTTTGCACACCATGCCTTC							
BLS-128	$(ATC)_9$	F:CGACATTTCGCTCTGTAAAGCAC	129 - 156	FAM	5	0.759	0.696	-0.0922	LN886581
		R:CAGACCAGGATCTTGATTTCGG							
BLS-130	$(AG)_{13}$	F:TTATCTACTATAGGACTTCCAGCC	211 - 265	HEX	9	0.933	0.857	-0.0907	LN886582
		R:TGTTCCGCTTATTGCTGCC							
BLS-133	$(AG)_8$	F:GCTTGTTTGGTGACCTGGG	210 - 222	HEX	3	0.200	0.188	-0.0675	LN886583
		R:GGATGCACATCTTCCAAACTATTC							
BLS-134	$(CT)_9$	F:GGTCAAATCGACAAATAACGACTG	218 - 226	HEX	5	0.250	0.736	0.6643*	LN886584
		R:ATCCTAAAGCTCTAATACCATGAAG							
BLS-145	$(AAG)_6$	F:TTTCACCGTCCGATTTGTTC	180 - 213	FAM	3	0.633	0.538	-0.1799	LN886585
		R:AGTTTGGGTGCACGGATTG							
BLS-149	$(GAT)_7$	F:AGTGTAATTCATCCTTCGACTTG	161 - 176	FAM	3	0.333	0.412	0.1933	LN886586
		R:CTCCCATCTAATTTCCCACCTTC							
BLS-152	$(CT)_{10}$	F:AGGGAGGTTCAATCAATTCCTG	196 - 198	FAM	2	0.517	0.509	-0.0169	LN886587
		R:AGCAGCAGACTTATGCATTCC							
BLS-153	$(CT)_9$	F:GAAACCACCTTGGCCCTTG	167 - 171	FAM	3	0.467	0.429	-0.0899	LN886588
		R:CTCTCTCAAATCGCGGGTATC							
BLS-154	$(AT)_8$	F:TCCCATCAGTTCATGTTGTACG	258 - 260	HEX	2	0.286	0.444	0.3609	LN886589
		R:ACTTAAGCCCGATTTATGCAAG							
BLS-156	$(AT)_{10}$	F:GCGGCTGAAACCTATGCAG	301 - 337	HEX	6	0.759	0.742	-0.0224	LN886590
		R:AGAGATTTGTTTGGACCGCC							
BLS-170	$(AG)_{12}$	F:TGAAGCATTAATTTGATACGCCC	235 - 253	HEX	9	0.733	0.819	0.1058	LN886591
		R:GACCAGTGCCATCAATCCC							
BLS-172	$(AG)_{12}$	F:AGCTACAAGATGGTTGGGC	227 - 233	HEX	3	0.267	0.377	0.2970	LN886592
		R:CACTCGTAACAACTGCACCC							
BLS-174	$(CT)_{11}$	F:ATCTTGGTCACCTCCTCAAG	166 - 170	FAM	3	0.483	0.623	0.2276	LN886593
		R:CAACTCATCGGCTCGTTGC							
BLS-175	$(ATC)_6$	F:TCCCTGATGAACGGTCACG	177 - 186	FAM	2	0.333	0.364	0.0852	LN886594
		R:CCTTTCGCCGGCAATCTAC							
BLS-181	$(CT)_7$	F:TTACTGTGCAGCCGATCCC	180 - 186	FAM	3	0.300	0.422	0.2927	LN886595
		R:GCCAGAGCCAATATGGTTGC							
BLS-183	$(AAG)_8$	F:CACCAGTTTCCAAGACCGC	256 - 271	HEX	6	0.793	0.755	-0.0514	LN886596

Continued on next page

Table 1. Continued.

Locus	Repeat motif	Primer sequences (5'-3')	Size range (bp)	5'-fluorescence label	N_A	H_O	H_E	F	EMBL accession No.
		R:AGGCGGGTCTTCAGTTGAG							
BLS-186	$(CT)_{13}$	F:CTCAGCTCAAGCCTTTGCC	141 - 157	FAM	8	0.889	0.829	-0.0740	LN886597
		R:CACAACAAGTGTTACTGCTTTGG							
BLS-193	$(AC)_9$	F:ACCAAACCGCAATGGCTAC	240 - 296	HEX	8	0.593	0.787	0.2505*	LN886598
		R:TCAAGTGGGTTCGAGTACTTTG							
BLS-195	$(AT)_{13}$	F:AACAGAGGGCAGTTGTCAG	203 - 257	HEX	14	0.933	0.884	-0.0573	LN886599
		R:AAACAGGCCTCCCACAAAG							
BLS-202	$(AG)_{14}$	F:GGGTTTGGTTAACTGGTGGC	215 - 229	HEX	5	0.733	0.716	-0.0249	LN886600
		R:GTCTGTGATACTGAGGTTCAAGTG							
BLS-205	$(CT)_9$	F:ACTTCTCTTATTGCAAACCAGC	156 - 180	FAM	7	0.500	0.451	-0.1111	LN886601
		R:AGTTCCATGACAAGGCCAAC							
BLS-208	$(CT)_{10}$	F:GATGCTGACCACATCCCAG	269 - 275	HEX	4	0.360	0.522	0.3143	LN886602
		R:TGGACTTGATTTAGAGGTTTGCAG							
BLS-211	$(AG)_{10}$	F:CTTGCCTGCTTGTGTGGAC	165 - 171	FAM	4	0.400	0.397	-0.0087	LN886603
		R:AGCTGTCGATGGTTATGTTTC							
BLS-212	$(AC)_{10}$	F:AGTGGACGTCAGTTGAGGG	170 - 174	FAM	3	0.731	0.639	-0.1473	LN886604
		R:ACAGGAATGCAATTAGACGC							
BLS-213	$(CT)_9$	F:GTGATTCAACGACAGTTACATTTCC	200 - 206	HEX	4	0.577	0.544	-0.0608	LN886605
		R:TGAAAGGTTTGCTTCAGGTCG							
BLS-218	$(AT)_8$	F:AATGCGAACCAAGACGGTG	160 - 166	FAM	4	0.233	0.271	0.1398	LN886606
		R:ATTGGCAACTAAACGGAGATG							
BLS-221	$(AG)_{10}$	F:TGAGTTCCATGAAGAACGATGC	185 - 191	FAM	4	0.679	0.607	-0.1201	LN886607
		R:AGATCTGGCCATACAAAGCG							
BLS-227	$(AG)_{11}$	F:GGGCCACATGAGGGATTTG	196 - 218	FAM	9	0.857	0.833	-0.0294	LN886608
		R:GCCCATCTTTCCTGGATGC							
BLS-236	$(CT)_{11}$	F:TCCCTAGCTCTACCGCAAC	249 - 251	HEX	2	0.567	0.494	-0.1492	LN886609
		R:TGTACAATGCCAGCGCAAC							
BLS-238	$(AG)_{10}$	F:TTTACCCACGCGCCTTAGC	155 - 159	FAM	3	0.310	0.376	0.1765	LN886610
		R:CGATGCCCGAACCCATTTG							
BLS-242	$(AG)_{10}$	F:ACATGAAGCAAAGCAAATTACCC	183 - 211	FAM	10	0.655	0.804	0.1878	LN886611
		R:CCTTCTCTCCCTTTGAGCAAG							
BLS-243	$(AG)_9$	F:CAGTTGGGTGTTGCAGGC	149 - 163	FAM	7	0.767	0.763	-0.0053	LN886612
		R:GACGACTCAAACCTCAGTTAGC							
BLS-245	$(AT)_8$	F:AGGTGATACTAACCATTGAGCG	269 - 299	HEX	9	0.852	0.811	-0.0519	LN886613
		R:ACCATAATGTAGAACATCAATTGCC							
BLS-246	$(AG)_{10}$	F:TGCACTCCCATTAAGCTGG	171 - 205	FAM	7	0.759	0.691	-0.0990	LN886614
		R:CGGCTTCCTGCAACGAATG							

All loci use the same annealing temperature of 55°C. N_A: number of alleles; H_O: observed heterozygosity; H_E: unbiased expected heterozygosity; F: fixation index. *Indicates a deviation at $P < 0.05$ from Hardy-Weinberg equilibrium after Bonferroni correction.

Parameters of genetic diversity for 63 loci were calculated using GENEPOP v. 4.3 (Rousset, 2008). The same software was also used to detect deviations from Hardy-Weinberg equilibrium (HWE) and genotypic linkage disequilibrium (LD) among all pairs of loci. The significance levels of HWE and LD tests were adjusted using Holm's sequential Bonferroni correction (Holm, 1979).

RESULTS AND DISCUSSION

In total, 63 microsatellites showed polymorphisms. The number of alleles per locus varied from 2 to 16 (Table 1). The H_O and H_E ranged from 0.133 to 0.967 and from 0.127 to 0.912, respectively. Three loci (BLS-76, BLS-134, and BLS-193) showed significant deviations from HWE, which was caused by heterozygote deficiency. After Bonferroni correction, significant LD was only detected in three locus pairs, loci BLS-53 and BLS-57, BLS-128 and BLS-243, and BLS-13 and BLS-212.

The identified polymorphic loci are useful to investigate genetic diversity and mating system of this species. These genetic markers may also be used to investigate the fine-scale spatial genetic structure and gene flow in NKMNR and HPNNR. All loci will provide valuable information for sound conservation of this species. In this study, the 63 microsatellites were tested individually. To reduce costs and save time, multiplex PCRs will be tested for these microsatellites for large samples in the future.

Conflicts of interest

The authors declare no conflict of interest.

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