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Primer Note

Isolation and identification of EST-SSR markers in *Chunia bucklandioides* (Hamamelidaceae)[1]

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- *Premise of the study: Chunia bucklandioides* (Hamamelidaceae), endemic to Hainan, China, is listed as threatened in the IUCN Red List and is now only found on Mt. Diaoluo and Mt. Jianfeng. Thus, microsatellite markers were developed for future conservation genetic studies of this species.
- *Methods and Results:* A total of 115 primers were designed on the basis of the transcriptome data of *C. bucklandioides*. Of them, 59 successfully amplified in *C. bucklandioides* and polymorphisms were detected in 11; the number of alleles per locus varied from two to five, the observed heterozygosity ranged from 0.000 to 0.941, and the expected heterozygosity ranged from 0.000 to 0.699. A total of 13 primers amplified in *Mytilaria laosensis*, and five primers amplified in *Exbucklandia tonkinensis* and *E. populnea*.
- *Conclusions:* The markers screened here provide a basis to assess genetic structure and further establish conservation strategies for *C. bucklandioides*.

Key words: *Chunia bucklandioides*; Hamamelidaceae; microsatellite markers; transcriptome.

Hamamelidaceae, a family of woody plants ranging from tall trees to small shrubs, is an ancient family of approximately 26 genera and 100 species (Endress, 1993). The genera in this family are small: 14 are monotypic, six contain only two to three species, and others are composed of five to 14 species. Furthermore, most species in this family are narrow endemics or are very restricted in their distribution mostly due to past climatic changes (Endress, 1993). Fourteen species of Hamamelidaceae are currently listed as threatened in the IUCN Red List of Threatened Species (IUCN, 2015). To date, only sporadic studies have emphasized the genetic study and conservation of these species (Yu et al., 2014; Hatmaker et al., 2015).

Chunia bucklandioides H. T. Chang (Hamamelidaceae), the only species in *Chunia* H. T. Chang, was listed as threatened in the IUCN Red List in 1997. It is a tall tree endemic to Hainan, China, and the wood can be applied in agricultural implements, furniture, and construction. However, it is now found only on Mt. Diaoluo and Mt. Jianfeng (IUCN, 2015). Here, we developed and characterized 11 polymorphic expressed sequence tag–simple sequence repeat (EST-SSR) markers and tested their cross-transferability in three related species—*Mytilaria laosensis* Lecomte, *Exbucklandia tonkinensis* (Lecomte) H. T. Chang, and *E. populnea* (R. Br. ex Griff.) R. W. Brown—on the basis of the phylogenetic tree of Hamamelidaceae (Shi et al., 1999). We expect that these markers will be useful for future conservation genetic studies of the species.

METHODS AND RESULTS

The total RNAs were extracted from the fresh leaves of one individual of *C. bucklandioides* (Mt. Diaoluo; Appendix 1) using the optimized cetyltrimethylammonium bromide (CTAB) method (Gambino et al., 2008). A normalized cDNA library was constructed and sequenced using the HiSeq 2000 system (Illumina, San Diego, California, USA). A total of 55.34 million 100-bp paired-end reads were produced and de novo assembled into 88,011 contigs (N50: 1056 bp) using Trinity (Grabherr et al., 2011). With the MISA tool (Thiel et al., 2003; http://pgrc.ipk-gatersleben.de/misa), 11,100 SSRs were detected in 9456 contigs. Of them, dinucleotide repeat motifs (72.73%) were the most common, followed by tri- (24.69%), tetra- (2.23%), penta- (0.19%), and hexanucleotide (0.16%) repeats. Using Primer3 (Rozen and Skaletsky, 1999), 115 paired primers were designed on the basis of randomly selected contigs containing SSR loci, which were deposited in GenBank (Appendix S1).

A total of 48 individuals of *C. bucklandioides* representing two populations were used to evaluate the polymorphisms of the target SSR loci, and 28 individuals of *M. laosensis*, *E. tonkinensis*, and *E. populnea* were used to test their transferability (Appendix 1). Total genomic DNA was extracted from silica-dried leaves of these individuals using the modified CTAB method (Doyle, 1987). Voucher specimens of these species were deposited at the Herbarium of Sun Yat-sen University, Guangzhou, Guangdong Province, China.

The PCR amplification trials were performed on two individuals from each of the two *C. bucklandioides* populations according to Fan et al. (2013), with appropriate annealing temperature (52–55°C; Table 1). For the 59 primer pairs that showed clear peaks with expected allele size, six individuals from each population were selected to tentatively assess their size polymorphism. The products were inspected with the Fragment Analyzer Automated CE System (Advanced Analytical Technologies [AATI], Ames, Iowa, USA) using the Quant-iT PicoGreen dsDNA Reagent Kit (35–500 bp; Invitrogen, Carlsbad, California, USA). The raw data were further processed to obtain allele size and

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TABLE 1. Characteristics of 19 microsatellite loci isolated from *Chunia bucklandioides* that showed polymorphism in *C. bucklandioides* or that could be amplified in closely related taxa.

Locus	Primer sequences (5′–3′)	Repeat motif	Expected allele size (bp)	T_a (°C)	GenBank accession no.	Putative function [organism][a]
N31	F: ATTAGTCCATAACGGCTAGT R: CCAAGAGAAGACAATGAACC	$(CTA)_5$	161	52	KX254740	—
N34	F: GCTTCCTCGTCCTTCTCT R: CGGCATCATTCTAATCATCTC	$(GAC)_6$	311	52	KX254743	PREDICTED: RING-H2 finger protein ATL67-like [*Nelumbo nucifera*]
N50	F: TGAGCATCTGATTACGAAGA R: CCAATCTCCGATACGACTT	$(TTGT)_6$	204	52	KX254759	Conserved hypothetical protein [*Ricinus communis*]
N54	F: CGGGAGATGATAAAGGATACA R: GGATCGGAGAAGCATTCG	$(AT)_8$	237	52	KX254763	Hypothetical protein ZeamMp042 [*Zea mays* subsp. *mays*]
N91	F: GCTACCTGACCTCTTCTTC R: GATTACTCGGACGGTGAC	$(CT)_6$	361	55	KX254800	Uncharacterized protein LOC100854009 [*Vitis vinifera*]
N1	F: ATCGCCATTCTTGCTCTC R: GCTCCAATACACGCCATA	$(AG)_6$	300	52	KX254710	—
N6	F: GCCTCCGTTAATTGTGTAC R: AGCCTTCGATGTAGTGATG	$(AT)_6$	317	52	KX254715	Hypothetical protein, partial (mitochondrion) [*Nicotiana tabacum*]
N49	F: GGAACAACCACGAAGAAGA R: GTCTACTCTGCCACAACTATA	$(AAG)_7$	219	52	KX254758	PREDICTED: nucleolar protein 56-like [*Vitis vinifera*]
N65	F: TCACTTCTACCTCGCAATG R: ACAGTCTTCTCTTCAATGGA	$(CTT)_5$	187	52	KX254774	Uncharacterized protein LOC100262883 [*Vitis vinifera*]
N89	F: CCGCAACAATATCGTCATT R: GGAAGAAGGTGGAGAACAT	$(TCA)_5$	257	52	KX254798	Uncharacterized protein LOC100262883 [*Vitis vinifera*]
N90	F: ATAGATAGACACTGGTGGATAG R: AACAGGCTCACATTACATCA	$(GGT)_5$	163	52	KX254799	—
N97	F: CGTAAGGTGTGCGATTCT R: AGAGTTGCCAACAGAGATG	$(AAC)_5$	305	52	KX254806	Uncharacterized protein LOC105794361 [*Gossypium raimondii*]
N98	F: GCAGCAGTGAGTCAAGTG R: CCTATCCTCCATCTCATCCA	$(GAG)_5$	242	52	KX254807	Uncharacterized protein LOC105111436 [*Populus euphratica*]
N23	F: TTGGAGTGATGGTTGAGG R: GTTCGGAGAAGAGGAAAGTA	$(AC)_6$	194	55	KX254732	—
N43	F: ATTCAACGGAGTTAGGACAT R: GATTGACGAGAACACATCAT	$(TA)_7$	147	52	KX254752	—
N45	F: CCTGATTACAATGAAGTCTTGG R: AGTAGTTCTGCCTTGAAGTT	$(GA)_7$	189	52	KX254754	Tau class glutathione transferase GSTU43 [*Theobroma cacao*]
N64	F: TGACGGTGGTAAGAAGGTA R: GAACGCAACAGGCATCTA	$(AT)_9$	199	52	KX254773	—
N84	F: CCTTGTCTCCTCATTGTCTT R: GCTCTGCTGTTGCTTACT	$(AT)_7$	270	52	KX254793	PREDICTED: serine/arginine repetitive matrix protein 1 [*Gossypium raimondii*]
N114	F: ACCAGACGACCACTACAG R: CGAAGCATAAGGAGATTGGA	$(AGATG)_5$	149	52	KX254823	—

Note: T_a = annealing temperature.
[a] *E*-value < 10^{-6}.

Table 2. Amplification and polymorphism of 19 microsatellite loci in populations of the four species.[a]

	Chunia bucklandioides											
	Mt. Diaoluo (N = 24)			Jianfengling (N = 24)				*Mytilaria laosensis* (N = 16)				*Exbucklandia tonkinensis* (N = 9) and *E. populnea* (N = 3)
Locus	A	H_o	H_e[b]	A	H_o	H_e[b]	Allele size (bp)	A	H_o	H_e[b]	Allele size (bp)	Allele size (bp)
N31	1	0.000	0.000	1	0.000	0.000	161	1	0.000	0.000	161	161
N34	1	0.000	0.000	1	0.000	0.000	309	1	0.000	0.000	315	315
N50	2	0.435	0.340	3	0.000	0.169***	187–204	1	0.000	0.000	195	195
N54	1	0.000	0.000	1	0.000	0.000	234	1	0.000	0.000	238	218
N91	1	0.000	0.000	1	0.000	0.000	359	1	0.000	0.000	364	364
N1	1	0.000	0.000	1	0.000	0.000	300	1	0.000	0.000	296	—
N6	1	0.000	0.000	1	0.000	0.000	317	1	0.000	0.000	317	—
N49	1	0.000	0.000	2	0.083	0.080	211–226	3	0.200	0.380*	284–311	—
N65	1	0.000	0.000	5	0.091	0.504***	167–187	3	0.375	0.537	155–173	—
N89	2	0.773	0.474	2	0.941	0.524	257–266	1	0.000	0.000	257	—
N90	2	0.217	0.258**	2	0.174	0.159	145–164	2	0.125	0.375*	161–164	—
N97	1	0.000	0.000	1	0.000	0.000	305	1	0.000	0.000	308	—
N98	1	0.000	0.000	1	0.000	0.000	242	1	0.000	0.000	203	—
N23	4	0.565	0.699	4	0.304	0.521*	187–199	—	—	—	—	—
N43	2	0.500	0.486**	2	0.200	0.420	133–147	—	—	—	—	—
N45	2	0.000	0.423***	2	0.217	0.496**	181–189	—	—	—	—	—
N64	2	0.130	0.122	3	0.095	0.459***	184–199	—	—	—	—	—
N84	2	0.227	0.416**	2	0.059	0.327***	266–272	—	—	—	—	—
N114	2	0.087	0.083	1	0.000	0.000	140–150	—	—	—	—	—

Note: — = no amplification; A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sampled individuals from each population.

[a] Population and locality information are provided in Appendix 1.

[b] Significant deviations from Hardy–Weinberg equilibrium after sequential Bonferroni corrections: *** represents significance at the 0.1% nominal level; ** represents significance at the 1% nominal level; * represents significance at the 5% nominal level.

number using PROSize version 2.0 software (AATI). The results showed that 11 loci were polymorphic in *C. bucklandioides*, and 48 loci were monomorphic. Further PCR amplification was performed on 48 individuals of *C. bucklandioides* with these 11 polymorphic primer pairs. The statistical parameters, including the number of alleles per locus (A), observed heterozygosity (H_o), and expected heterozygosity (H_e), were calculated with GenAlEx version 6.5 (Peakall and Smouse, 2012). GENEPOP 4.3 was used to measure the departure from Hardy–Weinberg equilibrium (HWE) (Rousset, 2008). The results showed that A varied from two to five, and H_o and H_e ranged from 0.000 to 0.941 and from 0.000 to 0.699, respectively. Four and six loci showed significant deviation from HWE in the Mt. Diaoluo and Mt. Jianfeng populations, respectively (see Table 2).

Finally, the cross-amplification of the 59 primers that successfully amplified in *C. bucklandioides* was also tested in *M. laosensis*, *E. tonkinensis*, and *E. populnea*. Of them, 13 amplified in *M. laosensis*, and five amplified in *E. tonkinensis* and *E. populnea* (Table 2).

CONCLUSIONS

Here, we isolated and characterized a set of 11 polymorphic EST-SSR markers, which may be useful for future conservation genetic studies of *C. bucklandioides.* The cross-genus amplification and polymorphism were also tested in three related species.

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Appendix 1. Voucher specimen information for populations used in this study. Specimens are deposited at the Herbarium of Sun Yat-sen University, Guangzhou, Guangdong Province, China.

Species	Voucher no.	Collection locality[a]	Geographic coordinates	*N*
Chunia bucklandioides H. T. Chang	*Fan and Li 13194*	Jianfengling, Hainan	18°44′58.90″N, 108°55′07.20″E	24
	Fan and Li 13040	Mt. Diaoluo, Hainan	18°41′40.22″N, 109°50′39.28″E	24
Mytilaria laosensis Lecomte	*Fan, Li and Liu 13481*	Heishiding, Guangdong	23°27′13.81″N, 111°52′19.63″E	4
	Fan, Li and Liu 13497	Tongledashan, Guangxi	23°12′28.00″N, 111°24′13.00″E	4
	Fan, Li and Liu 13502	Xinyi, Guangdong	22°24′12.62″N, 111°30′38.26″E	4
	Fan, Li and Liu 13528	Yangchun, Guangdong	21°54′23.95″N, 111°30′32.71″E	4
Exbucklandia tonkinensis (Lecomte) H. T. Chang	*Liu Lxp-09-6584*	Taoyuandong, Hunan	26°34′06.32″N, 114°04′46.71″E	3
	Fan, Li and Liu 13484	Heishiding, Guangdong	23°25′52.00″N, 111°52′43.89″E	3
	Fan, Li and Liu 13540	Yangchun, Guangdong	21°51′31.73″N, 111°25′18.75″E	3
Exbucklandia populnea (R. Br. ex Griff.) R. W. Brown	*Fan 13585*	Malipo, Yunnan	23°11′20.52″N, 104°49′17.23″E	3

Note: *N* = number of individuals sampled.
[a]City and province in China.