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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE MARKERS FROM THE TRANSCRIPTOME OF *FIRMIANA DANXIAENSIS* (MALVACEAE S.L.)[1]

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- *Premise of the study: Firmiana* consists of 12–16 species, many of which are narrow endemics. Expressed sequence tag (EST)–simple sequence repeat (SSR) markers were developed and characterized for size polymorphism in four *Firmiana* species.
- *Methods and Results:* A total of 102 EST-SSR primer pairs were designed based on the transcriptome sequences of *F. danxiaensis*; these were then characterized in four *Firmiana* species—*F. danxiaensis*, *F. kwangsiensis*, *F. hainanensis*, and *F. simplex*. In these four species, 17 primer pairs were successfully amplified, and 14 were polymorphic in at least one species. The number of alleles ranged from one to 13, and the observed and expected heterozygosities ranged from 0 to 1 and 0 to 0.925, respectively. The lowest level of polymorphism was observed in *F. danxiaensis*.
- *Conclusions:* These polymorphic EST-SSR markers are valuable for conservation genetics studies in the endangered *Firmiana* species.

Key words: *Firmiana danxiaensis*; Malvaceae; microsatellites; transcriptome; transferability.

Firmiana Marsili (Malvaceae sensu lato [s.l.]) is distributed in the Asiatic continent, Malaysia, and the Pacific Islands (Kostermans, 1957). This genus consists of 12–16 species, many of which are narrow endemics (Mabberley, 1997; Tang et al., 2007). Some species of *Firmiana* are cultivated as ornamental plants for their graceful shape and beautiful flowers. The wood of *F. simplex* W. Wight has been used by the Chinese to make high-quality traditional Asian musical instruments for thousands of years. This genus could be divided into two groups, i.e., *Firmiana* and *Erythropsis*, based on morphological and molecular data (Fan et al., unpublished data). There are five *Firmiana* species endemic to China. Among them, *F. danxiaensis* H. H. Hsue & H. S. Kiu is distributed only in Mount Danxia, Guangdong Province; *F. kwangsiensis* H. H. Hsue occurs only in Jingxi County, Guangxi Province; *F. hainanensis* Kosterm. and *F. pulcherrima* H. H. Hsue are confined in the central-south mountains of Hainan Province; and *F. major* (W. W. Sm.) Hand.-Mazz. is restricted to Yunnan Province and southwestern Sichuan Province (Tang et al., 2007). All of these species are listed as threatened in the *China Species Red List* (Wang and Xie, 2004).

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Because of the lack of efficient molecular markers, there have been no studies on genetic diversity analyses and conservation genetics of *Firmiana* species. The transcriptome of *F. danxiaensis* was recently sequenced using the Illumina platform, and sequences for tens of thousands of transcripts were obtained by de novo assembly (Chen et al., unpublished data). Based on these sequences, 17 novel polymorphic expressed sequence tag (EST)–simple sequence repeat (SSR) markers were developed and characterized for size polymorphism among four *Firmiana* species: *F. danxiaensis*, *F. kwangsiensis*, *F. hainanensis*, and *F. simplex*. Of these species, *F. kwangsiensis* belongs to the *Erythropsis* group, while the other species belong to the *Firmiana* group of the genus *Firmiana*.

METHODS AND RESULTS

One seedling of *F. danxiaensis* was collected from Danxia Mountain, Guangdong Province, China, and planted in the greenhouse of Sun Yat-sen University. One year later, fresh leaves of the seedling were collected for RNA extraction, and the subsequent protocols for the transcriptome sequencing followed Chen et al. (2011). A total of 26.95 million 90-nucleotide paired-end reads were obtained and de novo assembled into 33,522 contigs using Trinity (Grabherr et al., 2011) with a minimal length of 300 bp and an average length of 834 bp. Functional annotation for these contigs was performed using the automatic annotation tool Blast2GO (Conesa and Gotz, 2008). Using MISA (http://pgrc.ipk-gatersleben.de/misa), we detected 2069 SSRs in 1897 contigs. Among them, trinucleotide repeats (58.1%) were the most common, followed by dinucleotide (36.1%), tetranucleotide (3.3%), hexanucleotide (1.6%), and pentanucleotide repeats (0.9%). Using Primer3 (Rozen and Skaletsky, 2000; http://frodo.wi.mit.edu/primer3), 102 pairs of primers were designed from contigs containing at least nine repeats for dinucleotide and trinucleotide motifs, or seven repeats for tetranucleotide motifs. Contigs containing these 102 SSR loci were deposited in GenBank (Appendix S1, Table 1).

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TABLE 1. Characteristics of 17 EST-SSR markers developed in *Firmiana danxiaensis*.

Locus	Primer sequences (5′–3′)	Repeat motif[a]	T_a (°C)	Expected allele size (bp)	*A* (Allele size range [bp])[b]	GenBank accession no.	Putative function[c]
Fir_SSR2[d]	F: TCCAATGTCATGCTCTCCT R: GTGGCGGTTCTGGTTATG	$(CCA)_5…(CAC)_5$	50	248	9 (216–246)	KF048040	no hit
Fir_SSR5[e]	F: TGTGATGCTGACGAGGAG R: ATGAACCGCTATAACGACAA	$(CCT)_5…(TTG)_5$	50	166	1 (166)	KF048041	flowering time control protein FCA-like
Fir_SSR9[d]	F: AACAAGTGCGTTGAGAGG R: GAGCGAAGTGAAAGAGAAAG	$(GGA)_6…(TC)_7$	50	169	8 (164–184)	KF048042	no hit
Fir_SSR12[d]	F: TCATCACCATCAACAACAAC R: GCACTGAATCAATCACCATT	$(CAG)_5…(CAT)_6$	50	197	7 (189–217)	KF048043	B3 domain-containing transcription factor NGA1-like
Fir_SSR16[e]	F: GTTCCAGTTACAGCCTCAG R: CAGCAGGGTGATGATGAATA	$(GCA)_8…(ACA)_5…(ACA)_8$	57	164	1 (164)	KF048044	bZIP transcription factor bZIP28
Fir_SSR25[d]	F: CCGTTGACATCTGTAATATCTC R: GCTGGACATGGTGGTTAG	$(TTC)_6…(TC)_6$	57	156	8 (145–177)	KF048045	nudix hydrolase chloroplastic-like
Fir_SSR28[d]	F: TGGCATCTTCAAGGCATTA R: GCTGTGACTAGGAGGAGA	$(ACC)_6…(CAG)_6$	55	149	7 (133–166)	KF048046	probable calcium-binding protein CML48-like
Fir_SSR32[e]	F: CGTGGAGAGTCATCTTGG R: GGTAGTTCTACTTGATCGGATA	$(TC)_7…(CA)_6$	50	204	5 (202–210)	KF048047	exostosin-like protein
Fir_SSR59[d]	F: CAATTACTCTACTCAGCATC R: GTGTGGAGGTTATGACTAA	$(CT)_{10}$	50	181	11 (172–202)	KF048048	ubiquitin-conjugating enzyme E2-18
Fir_SSR63[e]	F: GAAACTCGTGAAACAGGAAA R: TGGAAGAACAGTGAAGAAGA	$(AG)_{10}$	50	214	9 (199–223)	KF048049	scarecrow-like protein 14-like
Fir_SSR80[e]	F: CTTGGCTGCTTCAGAGTG R: TTGGAGTAGTATATGTGGTGAG	$(AG)_9$	50	214	8 (212–226)	KF048050	orange protein
Fir_SSR81[d]	F: CTGTCAATCAAATCCCTTCA R: ATTCCTTCCACTGTGTCTT	$(AG)_9$	50	167	5 (159–173)	KF048051	SWIB/MDM2 domain-containing protein
Fir_SSR83[e]	F: TACACGATTAGCAGCAGAA R: AGCAGAGAAGGAGAGGAG	$(AG)_9$	50	216	10 (211–239)	KF048052	probable lactoylglutathione lyase, chloroplast-like
Fir_SSR85[e]	F: CTACTGTTCGGCTACTCAA R: AGATAGAGAAGAAGGCTGAC	$(TC)_9$	50	189	1 (189)	KF048053	disease resistance protein RPS2
Fir_SSR93[e]	F: GCAGTCAACATGAACTTACT R: CTTGCCATCTCCATACCAT	$(TG)_9$	50	115	13 (107–159)	KF313464	no hit
Fir_SSR98[d]	F: TCGGAATTGGTTGTTCAGA R: GAGGTGCAAGCTCAGATT	$(CA)_9$	50	250	3 (238–256)	KF048055	virion binding
Fir_SSR101[e]	F: CGGTTACAGGTGTTGAGTT R: TTACGGCGAGGCAGATAA	$(CT)_9$	57	181	6 (179–195)	KF313465	mitochondrial carrier

Note: F = forward primer; R = reverse primer; T_a = annealing temperature.

[a] Ellipses in repeat motifs signify intervening non-repeat sequence (≤100 bp) between two microsatellite repeats.

[b] Values from *F. danxiaensis*, *F. kwangsiensis*, *F. hainanensis*, and *F. simplex*.

[c] Annotation of putative homology acquired by searching the National Center for Biotechnology Information (NCBI) nonredundant database with BLASTX with the expected value $<10^{-10}$.

[d] Primers labeled with the fluorescent tag 6-FAM.

[e] Primers labeled with the fluorescent tag HEX.

Table 2. Results of initial primer screening in four *Firmiana* species.

	F. danxiaensis (*N* = 8)			*F. kwangsiensis* (*N* = 8)			*F. hainanensis* (*N* = 8)			*F. simplex* (*N* = 8)		
Locus	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e
Fir_SSR2	2	0.250	0.233	3	1.000	0.633	2	0.250	0.233	5	0.500	0.800
Fir_SSR5	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
Fir_SSR9	2	0.125	0.125	7	0.875	0.833	4	0.875	0.692	3	0.375	0.592
Fir_SSR12	1	0.000	0.000	1	0.000	0.000	4	0.750	0.650	5	0.625	0.808
Fir_SSR16	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
Fir_SSR25	2	0.125	0.125	2	1.000	0.533	5	0.875	0.825	5	0.375	0.608
Fir_SSR28	3	0.250	0.342	1	0.000	0.000	3	0.250	0.425	4	0.375	0.692
Fir_SSR32	1	0.000	0.000	1	0.000	0.000	3	0.250	0.433	4	0.125	0.725
Fir_SSR59	3	0.500	0.433	4	0.375	0.642	4	0.250	0.517	5	0.250	0.758
Fir_SSR63	3	0.250	0.567	5	0.875	0.758	2	0.375	0.525	6	0.250	0.783
Fir_SSR80	2	0.125	0.125	1	0.000	0.000	5	0.500	0.708	5	0.125	0.758
Fir_SSR81	2	0.125	0.125	3	0.000	0.700	3	0.125	0.242	4	0.375	0.675
Fir_SSR83	2	0.125	0.125	3	0.375	0.342	4	0.750	0.742	9	0.750	0.925
Fir_SSR85	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
Fir_SSR93	3	0.250	0.242	4	0.500	0.675	8	0.750	0.883	4	0.375	0.642
Fir_SSR98	3	1.000	0.592	2	0.625	0.525	2	0.625	0.458	1	0.000	0.000
Fir_SSR101	2	0.125	0.125	3	1.000	0.633	2	0.375	0.458	6	0.625	0.733

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity.

Polymorphisms of these primer sets were assessed in four *Firmania* species. Among them, *F. danxiaensis* was collected from Mt. Danxia, Guangdong Province (25°01′N, 113°44′E); *F. hainanensis* was collected from Jianfengling and Bawangling, Hainan Province (18°45′N, 108°53′E; 19°07′N, 109°07′E); *F. kwangsiensis* was collected from Jingxi County, Guangxi Province (23°00′N, 106°40′E); and *F. simplex* was collected from Taroko National Park, Taiwan (24°10′N, 121°31′E), Mt. Malan, Shenzhen (22°37′N, 114°19′E), and Daye, Hubei Province (30°04′N, 114°56′E). Voucher specimens for the four species were deposited at the Herbarium of Sun Yat-sen University (Appendix 1). We extracted the genomic DNA for all individuals from silica-dried leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). For the 102 primer pairs, we first selected three individuals of each species to screen universal primers for all four *Firmiana* species. PCR amplifications were performed in 20-μL reaction volumes, containing 25 ng of genomic DNA, 2 μL 10× buffer (with Mg^{2+}), 0.25 mM of dNTPs, 0.2 μM of each primer, and 1 U of Easy-*Taq* DNA polymerase (TransGen Biotech Co. Ltd., Beijing, China). The PCR reactions were conducted with the following conditions: initial denaturing at 94°C for 2 min; followed by 35 cycles of 94°C for 30 s, appropriate annealing temperature (Table 1) for 30 s, and 72°C for 40 s; and a final extension at 72°C for 5 min. The PCR products were first electrophoresed on a 10% polyacrylamide denaturing gel and visualized by silver nitrate staining. The band size was estimated by comparison with a 25-bp DNA ladder (Fermentas, Vilnius, Lithuania). Of the 102 primer sets, 17 were successfully amplified in the four *Firmiana* species (Table 1).

The above 17 primer sets were then labeled with fluorescent dye, and PCR amplifications were carried out for the 32 individuals of the four *Firmiana* species (eight for each species). Their PCR products were analyzed on an Applied Biosystems 3730xL DNA Analyzer (Applied Biosystems, Foster City, California, USA), and the allele size was estimated using Peak Scanner Software version 1.0 (Applied Biosystems). The genetic diversity indices, including the number of alleles (*A*), observed heterozygosity (H_o), and expected heterozygosity (H_e), were calculated using the software POPGENE (Yeh and Boyle, 1997). The results showed 14 of these 17 primer sets were polymorphic in at least one *Firmiana* species. *A* ranged from one to three in *F. danxiaensis*, from one to seven in *F. kwangsiensis*, from one to eight in *F. hainanensis*, and from one to nine in *F. simplex*. H_o and H_e ranged from 0 to 0.500 and 0 to 0.592 in *F. danxiaensis*, from 0 to 1 and 0 to 0.833 in *F. kwangsiensis*, from 0 to 0.875 and 0 to 0.883 in *F. hainanensis*, and from 0 to 0.750 and 0 to 0.925 in *F. simplex*, respectively (Table 2).

CONCLUSIONS

In our study, 17 EST-SSRs were identified from the transcriptome of *F. danxiaensis* and were amplified in three other *Firmiana* species. Because the four species of *Firmiana* belong to different clades, these 17 SSR markers show good transferability in the genus. These polymorphic markers are valuable for conservation genetic studies in these endangered *Firmiana* species.

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APPENDIX 1. Voucher specimen information for taxa used in this study. Specimens are deposited at the Herbarium of Sun Yat-sen University (SYSU), China.

Species	Voucher specimen collection no.	Collection locale
F. danxiaensis	*Q. Fan 9610*	Guangdong
F. kwangsiensis	*Q. Fan 11295*	Guangxi
F. hainanensis	*Q. Fan 11310*	Hainan
	Q. Fan 11325	Hainan
F. simplex	*Fan and Chen 1209*	Guangdong
	Fan and Sun 1210	Guangdong
	Fan and Shi 0502	Taiwan

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