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Source: Applications in Plant Sciences, 3(7)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1500034

Applications in Plant Sciences 2015 3(7): 1500034

Primer Note

Development and characterization of microsatellite loci for *Ficus hirta* (Moraceae)[1]

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- *Premise of the study:* Microsatellite primers were developed to investigate population genetic structure in *Ficus hirta* (Moraceae).
- *Methods and Results:* Sixteen microsatellite primers were developed and optimized for *F. hirta* using Illumina paired-end sequencing of pre-receptive and receptive developmental-phase female flowers. Out of 16 primers, nine were found to be polymorphic in four populations of *F. hirta*. Alleles per locus ranged from two to 15 across the 94 *F. hirta* individuals, while within-population observed and expected heterozygosity per locus ranged from 0.000 to 0.955 and from 0.000 to 0.882, respectively. In addition, the 16 primers were tested in 29 additional *Ficus* species, with all found to amplify in at least 11 of these species and with most amplifying in a majority of the species.
- *Conclusions:* This set of microsatellite primers is the first specifically developed for *F. hirta* and will facilitate studies of genetic diversity within and genetic differentiation among populations of *Ficus* species.

Key words: *Ficus hirta*; microsatellites; Moraceae; polymorphism; transcriptome sequencing.

Figs (*Ficus* L., Moraceae) and their pollinating wasps (Hymenoptera: Agaonidae) are a textbook example of pollination mutualism and one of the best cases of coevolution known (Jousselin et al., 2003). With more than 750 species, figs are a common component of tropical and subtropical habitats and are completely dependent on their pollinator wasps for pollen dispersal and the production of viable seeds. In turn, the pollinating wasps gall a subset of the developing fig seeds and so are completely dependent on the fig for the completion of their life cycle. *Ficus hirta* Vahl is a dioecious shrub or small tree with a broad distribution at low elevations in Southeast Asia, where it plays an important role in forest succession and renewal in human-disturbed mountainous landscapes. Consequently, understanding the genetic diversity of this species is relevant to forest restoration and conservation, as well as coevolutionary studies.

Microsatellite loci have become a powerful tool for analyzing population genetic structure, gene flow, and levels of inbreeding. Many microsatellite primers have been developed for genetic studies of figs (Zavodna et al., 2005; Crozier et al., 2006; Ikegami et al., 2009; Zhang et al., 2011). Moreover, microsatellite primers for *F. hirta*'s pollinator have been developed (Tian et al., 2011). Six of the microsatellite primers developed for other fig species are polymorphic in *F. hirta*; however, two of these loci exhibit significant excesses of homozygotes within populations, suggestive of null alleles (Yu et al., 2010). Additional marker loci are needed for population genetic studies of *F. hirta*; therefore, we have used a next-generation transcriptome sequencing approach (RNA-Seq) to develop microsatellites specifically for this species.

METHODS AND RESULTS

***Plant material and RNA/DNA extraction*—**Fresh, female flowers from pre-receptive and from receptive male syconia of *F. hirta* growing at the South China Botanical Garden (IBSC; Guangdong Province) were excised with tweezers and then immediately frozen in liquid nitrogen for RNA extraction. A voucher specimen (*Huiyu 368*) has been preserved in the IBSC herbarium. Total mRNA was extracted from each of the two samples using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). For each sample, an RNA-Seq library was constructed using the Illumina TruSeq RNA Sample Preparation Kit (Illumina, San Diego, California, USA) following the manufacturer's recommendations, and then sequenced on an Illumina HiSeq 2000 platform. Both cDNA library construction and Illumina sequencing were carried out by Novogene (Beijing, China). In total, 56,047,406 (pre-receptive) and 60,136,746 (receptive) raw paired-end reads at least 100 bp in length were obtained. For testing new microsatellite loci, we sampled 94 individuals of *F. hirta* from four distinct, natural populations: three in China (DA: 19°33′02″N, 110°21′84″E; MH: 18°47′42″N, 110°23′27″E; WN: 21°58′46″N; 100°27′01″E) and one in Vietnam (YC: 20°15′182″N, 105°42′725″E). All of these individuals had morphological features typical of *F. hirta* var. *hirta*. Genomic DNA of each individual was extracted from silica gel–dried leaves using the modified CTAB method (Doyle and Doyle, 1987), and one voucher specimen per population was preserved in the IBSC herbarium (*Huiyu-DA1*, *Huiyu-MH1*, *Huiyu-WN1*, and *Huiyu-YC1*).

***Microsatellite marker development*—**The program MISA (Thiel et al., 2003) was used to identify and localize putative microsatellite motifs in the

[1]Manuscript received 27 March 2015; revision accepted 24 April 2015.

This study was supported by the National Natural Science Foundation of China (31370409) and the Natural Science Foundation of Guangdong Province (S2013020012814).

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doi:10.3732/apps.1500034

F. hirta transcriptome sequence library. To be identified as a potential microsatellite locus, mononucleotide repeats were required to extend at least 10 repeats, dinucleotide repeats were required to extend at least six repeats, and repeats of all other motif lengths were required to extend at least five repeats. MISA identified 35,558 putative microsatellite loci. With this detailed information, the program Primer3 (Rozen and Skaletsky, 1999) was then used to design 11,468 primer pairs containing di-, tri-, tetra-, penta-, and hexanucleotide repeat motifs, with a melting temperature (T_m) ranging from 57°C to 63°C and fragment size from 100 to 300 bp.

***Microsatellite screening*—**Primer pairs were synthesized (Invitrogen Trading Shanghai Co. Ltd., Shanghai, China) for 56 loci. The 56 loci were randomly selected with the constraint of having expected product sizes between 150 and 300 bp. Dinucleotide repeat loci had at least nine repeats and trinucleotide repeat loci had at least six repeats. These primer pairs were subsequently tested for proper PCR amplification in *F. hirta* using genomic DNA. PCRs were performed in a 20-μL volume containing 3 μL of 10× reaction buffer (containing Mg^{2+}), 2 μL dNTP mix (10 mM; 2.5 mM each), 0.4 μM primers, 1 μL of 50 ng DNA, and 1 unit of *Taq* polymerase (TaKaRa Biotechnology Co., Dalian, China). PCR cycling was performed in the following conditions: one cycle of denaturation at 95°C for 3 min; 35 cycles for 45 s at 94°C, 45 s annealing at 60°C, and 45 s extension at 72°C; followed by a final 10-min extension at 72°C. The PCR products were checked on 1.5% agarose gels. Sixteen of the 56 primer pairs tested were found to amplify successfully in *F. hirta* (Table 1). To assess polymorphism of the 16 microsatellite primers, genomic DNA templates from the 94 *F. hirta* individuals from the four natural populations were used. PCR amplification was performed using forward primers labeled with fluorescent dyes (TAMRA, HEX, and FAM) with the same PCR reaction and cycling conditions as above. The PCR products were detected on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA) using Genotyper 4.0 and GeneScan 500 LIZ (Applied Biosystems) as an internal lane standard. Fragment lengths were calculated using GeneMapper 4.0 (Applied Biosystems) software. Nine of the 16 microsatellites were found to be polymorphic in *F. hirta* (Table 1).

***Microsatellite marker data analysis*—**Genotypes were scored by analyzing the electropherograms from the fragment analysis using GeneMarker version 1.95 (SoftGenetics, State College, Pennsylvania, USA). Descriptive statistics, including the number of alleles and the observed and expected heterozygosities for each *F. hirta* population, were estimated using GenAlEx version 6.1 (Peakall and Smouse, 2006) (Table 2). For each population, the number of alleles per locus at the nine loci ranged one to 11 with a total of 85 alleles summed across loci. Observed heterozygosity values ranged from 0.000 to 0.955, and expected heterozygosity ranged from 0.000 to 0.882. FSTAT 2.9.3 (Goudet, 2001) was used to test for deviations from Hardy–Weinberg equilibrium for each locus in each population and linkage disequilibrium between loci. Using a sequential Bonferroni correction for multiple comparisons (Rice, 1989), these tests revealed that two loci (FH1, FH21) were not in Hardy–Weinberg equilibrium (as expected for dioecious *F. hirta*), and that levels of linkage disequilibrium between loci were not significant.

***Cross-species amplifications*—**The 16 primer pairs that amplified successfully in *F. hirta* were tested for amplification in one individual of each of 29 additional *Ficus* species, using the same procedures as above. All 16 were found to amplify in at least 11 of these species, with most amplifying in a majority of the species (Appendix 1).

CONCLUSIONS

The microsatellite loci described in this study provide an important resource for studying the factors affecting the distribution of genetic diversity in *F. hirta* and other *Ficus*

TABLE 1. Characteristics of 16 microsatellite loci developed for *Ficus hirta*.

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	GenBank accession no.
FH1	F: GCAGCAGTTGTGAGGGAAGA	$(ATA)_6(AT)_8$	266	60	KP903721
	R: TAGTGAAGAAGCGCATCCGG				
FH3	F: CTCCCACCCACAAATCCCTC	$(ACC)_7$	231	60	KP903722
	R: GGTCCTCCAAACTCTTCGCA				
FH5	F: TCGTCGAAGGCTCAGATTCG	$(AG)_9$	184	60	KP903723
	R: GCATTGTGTGGGGGAAGAGA				
FH7	F: AATCTTACTGGCGCGGGAAA	$(GA)_9$	150	60	KP903724
	R: GTGCTGCGGATTTCGATTCC				
FH10	F: TGCTGGGGATAGGTCTTGGA	$(TC)_9TAGCTTCTT(TC)_6$	141	60	KP903725
	R: AATATCCAGAGCCGAAGCCG				
FH14	F: AGACACACCCCTTTGGAAGC	$(AAG)_6$	155	60	KP903726
	R: TTCCCAAAAGAGCTGAGCCG				
FH21	F: AAGATCGTGGTGGTGAGCAG	$(AGT)_6$	252	59	KP903727
	R: CGTGGTGCTCACAAACCTTG				
FH23	F: GCCGAGAGTGGAGAGCATTT	$(GAC)_5$	232	60	KP903728
	R: TTCGAGAAAGATGCAGCCGT				
FH47	F: TTTCCGATCGATCTCAGCCG	$(AGA)_6$	251	60	KP903729
	R: AAAATGGAGCCTCTCTGCCC				
FH15	F: TCAATTCTGTGGGTGTGCGA	$(TTTA)_5$	192	60	KP903730
	R: TCCCTTTCTAATGTCGAGTTGAA				
FH18	F: ATCGAATCCGCCATGGCTAG	$(TCG)_7$	280	60	KP903731
	R: TGTCGTCCTCATCAAAGGGC				
FH32	F: GGGTAGGTACAACCAGGCAC	$(TTA)_6$	268	60	KP903732
	R: ACCCAACCAACCAACCAAGA				
FH35	F: AGAGCAAAGGGAAGACACCG	$(TTC)_6$	262	60	KP903733
	R: CAAACCTACCTCGGTGACCC				
FH37	F: GGCACTTCAAGCAGCAATCC	$(GGT)_6$	234	60	KP903734
	R: CCAAGATCGACCTCACCACC				
FH42	F: GATCCGGAACACTTGCATGC	$(CAT)_6$	207	60	KP903735
	R: TGGTTTCTGGTTTCGCCACT				
FH43	F: CGCCCTTTTCGTTCTGTTGG	$(AATA)_5$	275	60	KP903736
	R: CTGATTCTTCGCGATGCAGC				

Note: T_a = annealing temperature.

[a]The first nine primer pairs (bold locus names) were determined to be polymorphic in *F. hirta*.

TABLE 2. Nine microsatellite locus–specific measures of genetic diversity across four populations of *Ficus hirta*.[a]

	DA (n = 24)			MH (n = 24)			WN (n = 24)			YC (n = 22)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
FH1	6	0.350	0.751	7	0.389	0.713	5	0.208	0.663	8	0.333	0.685
FH3	7	0.708	0.635	6	0.792	0.734	8	0.625	0.649	5	0.682	0.657
FH5	6	0.458	0.533	9	0.588	0.657	6	0.708	0.661	7	0.727	0.702
FH7	10	0.667	0.807	9	0.955	0.847	11	0.750	0.874	11	0.818	0.882
FH10	9	0.875	0.720	6	0.789	0.720	4	0.333	0.572	10	0.909	0.823
FH14	2	0.348	0.386	5	0.542	0.577	2	0.083	0.080	5	0.227	0.211
FH21	1	0.000	0.000	2	0.000	0.278	2	0.000	0.330	2	0.000	0.496
FH23	3	0.125	0.190	3	0.087	0.235	1	0.000	0.000	2	0.091	0.087
FH47	4	0.917	0.699	6	0.875	0.760	4	0.875	0.708	6	0.955	0.728

Note: A = number of alleles sampled; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.

[a]Geographic coordinates for populations: DA = 19°33′02″N, 110°21′84″E; MH = 18°47′42″N, 110°23′27″E; WN = 21°58′46″N, 100°27′01″E; YC = 20°15′182″N, 105°42′725″E.

species. We can use these loci to further investigate large-scale and fine-scale population genetic structure in *F. hirta*, building on our past work in this species investigating relative rates of pollen and seed migration (Yu et al., 2010) and the constraints to postglacial range expansion arising from restricted pollen or seed dispersal (Yu and Nason, 2013). Our results also demonstrate the application of RNA sequencing as a valuable tool for developing microsatellite loci in nonmodel species.

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Appendix 1. Amplification of 16 microsatellite primer pairs developed for *Ficus hirta* in 30 *Ficus* taxa.

Ficus taxa (subgenus)	FH1	FH3	FH5	FH7	FH10	FH14	FH15	FH18	FH21	FH23	FH32	FH35	FH37	FH42	FH43	FH47
F. deltoidea Jack (*Ficus*)	—	—	—	—	+	—	—	—	+	—	—	+	+	+	+	+
F. esquiroliana H. Lév. (*Ficus*)	+	+	+	+	+	+	+	+	+	—	+	+	+	+	+	+
F. formosana Maxim. (*Ficus*)	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	—
F. gasparriniana Miq. var. *viridescens* (H. Lév. & Vaniot) Corner (*Ficus*)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. hirta Vahl (*Ficus*)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. ischnopoda Miq. (*Ficus*)	—	—	—	—	+	+	—	—	—	+	—	+	—	+	+	—
F. stenophylla Hemsl. (*Ficus*)	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+
F. vasculosa Wall. ex Miq. (*Pharmacosycea*)	+	+	+	+	+	—	+	+	+	+	+	+	+	+	+	+
F. cyrtophylla (Wall. ex Miq.) Miq. (*Sycidium*)	+	+	+	+	+	—	+	+	+	+	+	+	+	+	+	—
F. heterophylla L. f. (*Sycidium*)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. subincisa Buch.-Ham. ex Sm. (*Sycidium*)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. tinctoria G. Forst. subsp. *gibbosa* (Blume) Corner (*Sycidium*)	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. auriculata Lour. (*Sycomorus*)	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+
F. hainanensis Merr. & Chun (*Sycomorus*)	+	—	+	—	+	+	+	+	+	—	+	+	+	+	+	+
F. hispida L. f. *(Sycomorus)*	—	+	—	+	+	—	—	+	+	—	+	+	+	+	+	+
F. sarmentosa Buch.-Ham. ex Sm. var. *henryi* (King ex Oliv.) Corner (*Synoecia*)	+	—	—	—	+	—	+	+	+	+	+	+	+	+	+	—
F. altissima Blume (*Urostigma*)	—	+	—	+	+	+	—	+	+	—	+	+	+	+	+	+
F. annulata Blume (*Urostigma*)	+	—	+	+	+	+	—	+	+	—	+	+	+	+	+	+
F. benjamina L. (*Urostigma*)	+	+	—	+	+	+	—	+	+	—	+	+	+	+	+	—
F. concinna (Miq.) Miq. (*Urostigma*)	+	+	—	+	+	+	—	+	+	—	+	+	+	+	+	+
F. curtipes Corner (*Urostigma*)	—	+	—	—	—	+	—	+	+	—	+	+	+	+	+	—
F. cyathistipula Warb. (*Urostigma*)	+	+	—	—	+	+	—	—	+	+	—	+	+	+	+	—
F. drupacea Thunb. (*Urostigma*)	—	+	—	+	+	—	—	+	+	—	+	+	+	+	+	+
F. drupacea var. *pubescens* (Roemer & Schultes) Roth & Corner (*Urostigma*)	+	—	+	+	+	—	+	+	+	+	+	+	+	+	+	+
F. elastica Roxb. (*Urostigma*)	+	—	—	+	+	+	—	+	+	—	+	+	+	+	+	+
F. glaberrima Blume (*Urostigma*)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. lyrata Warb. (*Urostigma*)	+	+	+	+	+	+	—	+	+	+	+	+	+	+	+	+
F. maclellandii King (*Urostigma*)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. microcarpa L. f. (*Urostigma*)	+	+	+	+	+	+	—	+	+	—	+	+	+	+	+	+
F. stricta (Miq.) Miq. (*Urostigma*)	—	+	—	+	+	+	—	+	+	+	+	+	+	+	+	+

Note: + = primers amplified successfully in the species; — = primers did not amplify in the species.