Development of 15 Polymorphic Microsatellite Markers for Ficus virens (Moraceae)

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PRIMER NOTE

DEVELOPMENT OF 15 POLYMORPHIC MICROSATELLITE MARKERS FOR *FICUS VIRENS* (MORACEAE)[1]

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- *Premise of the study: Ficus virens* (Moraceae) is distributed widely in South and Southeast Asia, Melanesia, and northern Australia, and it is also cultivated outside its original northern range limit in southwestern China. Therefore, the species is well suited to explore the mechanism of range limits of *Ficus* species. However, little is known about its genetic background.
- *Methods and Results:* Fifteen polymorphic microsatellite markers were developed using the biotin-streptavidin capture method. Polymorphism was tested in 85 *F. virens* individuals sampled from three populations. The number of alleles ranged from three to 17. The observed and expected heterozygosity of each population varied from 0.0667 to 0.9286 and 0.0650 to 0.8890, respectively. Cross-species amplification was also carried out in eight other *Ficus* species.
- *Conclusions:* These 15 markers will be valuable for studying the genetic variation and population structure of *F. virens* and related *Ficus* species.

Key words: cross-amplification; *Ficus virens*; genetic diversity; microsatellite; Moraceae; southwest China.

As keystone species, fig trees (*Ficus* L., Moraceae) play a significant role in both tropical and subtropical ecosystems by providing food for many vertebrates (Shanahan et al., 2001). Some *Ficus* species are widely cultivated outside their natural distribution range, providing a valuable opportunity to explore the mechanism of range limits in the genus (Lin et al., 2007). *Ficus virens* Aiton is a monoecious fig tree species in the subgenus *Urostigma* (Endl.) Miq. (Bartholomaeus et al., 2009). Its natural distribution is in South and Southeast Asia, Melanesia, and northern Australia (McPherson, 2005). It is also planted widely in southwestern China outside its original distribution regions, extending to the most northern regions for *Ficus* species in China (Zhang et al., 2014). Therefore, the fine-scale population genetic structure and gene flow among the northern populations of *F. virens* can offer valuable information for interpreting the mechanism of range limits of *Ficus* species. In this study, we have developed high-resolution microsatellite loci to provide crucial information for fine-scale genetic studies of this species (Rout and Aparajita, 2009).

METHODS AND RESULTS

***Plant material and DNA extraction*—**Young and healthy leaves of *F. virens* were collected in Chengdu (30.68271°N, 104.10363°E), Mianyang (31.47661°N, 104.78943°E), and Chongqing (29.8217°N, 106.42913°E) in southwestern China (Appendix 1). The sampled leaves were kept in allochroic silica gel for drying. Genomic DNA was extracted from 0.03 g dry leaves using a Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China).

***Development of microsatellite primers*—**Microsatellite primers were developed using the biotin-streptavidin capture method following the procedure of Kijas et al. (1994) and Zhang et al. (2016). *Mse*I (New England Biolabs, Beverly, Massachusetts, USA) was applied to digest the genomic DNA in a 25-μL reaction volume overnight at 37°C. Then the fragments were ligated to *Mse*I adapters (F: 5′-TACTCAGGACTCAT-3′, R: 5′-GACGATGAGTCCTGAG-3′) and amplified with *Mse*I-N primer (5′-GATGAGTCCTGAGTAAN-3′) with the following conditions: 3 min denaturation at 95°C; followed by 20 cycles of 30 s denaturation at 94°C, 1 min annealing at 53°C, and 1 min extension at 72°C; with a final extension of 72°C for 8 min. The PCR products were mixed with 5′-biotinylated probe $(AC)_{15}$ followed by 5 min denaturation at 95°C, and 2 h hybridization at 48°C. Hybridization products were captured with streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA). The enriched product was amplified with *Mse*I-N as primers for an additional 30 cycles according to the conditions described above. Purification was then performed with a multifunctional DNA Extraction Kit (Sangon Biotech, Shanghai, China), the products were ligated into pGM-T vector (Tiangen Biotech), and then transformed into *Escherichia coli* strain DH5α (Tiangen Biotech). In total, 108 positive clones were selected and sequenced. These were tested by PCR using $(AG)_{10}$ and $M13^+/M13^-$ as primers.

The selected positive clones were sequenced with primer $M13^+/M13^-$ on an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, California, USA) at Sangon Biotech. Eighty-two clones contained simple sequence repeats, 47 of which were discarded because their sequences were too short for primer design. The remaining clones were used to design primers with Primer Premier version 5.0 (PREMIER Biosoft International, Palo Alto, California, USA). The criteria for primer design were as follows: (1) product size from 100 to 300 bp; (2) primer size from 16 to 23 bp with an optimum size of 20 bp; (3) primer melting temperature from 45°C to 65°C with an optimum temperature of 55°C; and (4) GC content of primers from 40% to 60%.

Thirty-five pairs of microsatellite primers were designed and successfully amplified. The polymorphism of each designed primer was tested using 23 randomly selected individuals. PCRs were performed in a 15-μL volume containing 1.5 μL of 10× PCR buffer (with Mg^{2+}), 0.3 μL dNTPs (2 mM each), 0.1 μL each primer

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TABLE 1. Characteristics of 15 microsatellite loci developed in *Ficus virens*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	*A*	GenBank accession no.
V33	F: TGGTGAAGCAAAGACCGAAAC R: CCAAAGAAGACACGCAAAGAT	$(CT)_7$	112	58	3	KU975125
V42	F: ATGGTAATGACCTGTGCTA R: AGTTGCTCTGTTATTGGTC	$(AG)_{10}(AG)_{17}$	154	58	4	KU975126
V70	F: CATTCTCCGACGAAAGTGT R: TCAAGCAAAAGACGAACTG	$(AC)_{11}$	190	58	12	KU975127
V98	F: GTATGGAAAGAGTCGAAGGGTG R: GGGTTATGTTTGGCAAGAGG	$(CT)_9$	156	58	9	KU975128
V99	F: CGTAGGCTTAGTTTCAGGACC R: TAGGCCACCAAGCATCGTTAG	$(AG)_8$	111	58	9	KU975129
V131	F: TCAAACCAAACATCCCCATAAC R: CTTGAACAAGGAATAGGGCTCT	$(AG)_9$	149	58	10	KU975130
V149	F: ATGTAGGACTTTTGGAACC R: AGCAGCACTGAAGGACATG	$(TC)_{10}$	202	58	8	KU975131
V159	F: AATTCGAGAAGTTCGTGGGT R: ACAACTCTAGGGATTGGTGCA	$(TC)_{14}$	164	58	15	KU975132
V171	F: GAGATGGCGAAAACAACA R: CAAAACCCAACGGTATCA	$(AG)_{12}$	131	58	6	KU975133
V183	F: CCCATTGTGGTTCCCTG R: GCTTGATGCTTGGGTTG	$(GA)_{17}$	234	58	17	KU975134
V188	F: ATAGAGGGGCAAGGCAGTA R: CCTTGGGCCTTAGATGACA	$(GA)_{10}(GA)_{15}(AG)_6$	263	58	15	KU975135
V212	F: CGCCCGTGAAACGATACATA R: GGTTGCTCATTGTATTGTCC	$(TC)_6(CT)_5(AC)_{10}(AT)_5$	132	58	5	KU975136
V217	F: CCCACCAAAGGAGATTAG R: ATACCAAGAAAGGACGCT	$(AG)_{14}$	175	58	6	KU975137
V230	F: CCGCAGGGTTGCATTG R: GGCCGAGGACCATCTT	$(GA)_{18}(AG)_6$	119	56	10	KU975138
V254	F: ACTCATATCATCAAAACACGTT R: AATTCAGCTGCAGTGTAGTAGT	$(TC)_9$	190	58	11	KU975139

Note: *A* = number of alleles sampled; T_a = annealing temperature.

(10 μM), 2 μL of 50 ng/μL DNA, 0.2 μL Blend Taq (2.5 units; Toyobo Co. Ltd., Osaka, Japan), and 12.6 μL of DNase-free water. PCR cycling was performed using the program described by Zhang et al. (2016). The products were first checked on 1% agarose gels, then settled on 8% denaturing polyacrylamide gels, and visualized by silver staining with pUC19 DNA/*Map*I (*Hpa*II) (Fermentas International, Burlington, Ontario, Canada) as the ladder. Of the 35 microsatellite primers, 20 were monomorphic and 15 were polymorphic.

Genetic variation of three populations (Chengdu, Mianyang, and Chongqing) was measured using selected polymorphic primers to further test the amplification stability of each primer. The forward primers of each polymorphic locus were labeled with fluorescent dyes (5′TAMRA, 5′ROX, 5′6-FAM [FITC]; Sangon Biotech) for scoring fragment length on an ABI 3130 automated sequencer (Applied Biosystems), using GeneScan 500 LIZ (Applied Biosystems) as an internal lane standard. The fragment lengths were calculated by GeneMapper 4.0 (Applied Biosystems) software.

***Data analysis*—**Linkage disequilibrium and Hardy–Weinberg equilibrium (HWE) were calculated using GENEPOP 4.0 (Rousset, 2008), and occurrence

TABLE 2. Genetic diversity of the 15 polymorphic microsatellite in three *Ficus virens* populations.[a]

	Chengdu (*n* = 27)				Mianyang (*n* = 30)				Chongqing (*n* = 28)			
Locus	*A*	H_o	H_e	HWE	*A*	H_o	H_e	HWE	*A*	H_o	H_e	HWE
V33	3	0.1111	0.1063	1.0000	3	0.0667	0.0650	1.0000	2	0.0714	0.1352	0.1081
V42	4	0.5556	0.6173	0.6996	3	0.6333	0.6428	1.0000	4	0.1071	0.6716	0.0000*
V70	7	0.2593	0.7936	0.0001*	9	0.6333	0.8450	0.1580	8	0.6071	0.7532	1.0000
V98	7	0.5926	0.7524	0.1233	7	0.4333	0.6939	0.0017*	6	0.4643	0.5874	0.1137
V99	6	0.5926	0.5720	0.7068	6	0.4667	0.6344	0.4789	5	0.4643	0.6626	0.1137
V131	10	0.5926	0.7874	0.2444	9	0.5667	0.7539	0.7033	9	0.5000	0.8272	0.3023
V149	7	0.7037	0.7051	0.6971	7	0.6667	0.6322	1.0000	6	0.7500	0.6754	0.2510
V159	7	0.4444	0.6790	0.0000*	9	0.6333	0.8244	0.0000*	12	0.9286	0.8253	0.6714
V171	5	0.7778	0.6934	0.2405	5	0.5667	0.6889	1.0000	6	0.6786	0.6818	0.4655
V183	13	0.6296	0.8683	0.0004*	12	0.7000	0.8633	0.1580	13	0.6429	0.8890	0.0729
V188	13	0.8148	0.8532	0.6456	11	0.6000	0.7683	0.1320	12	0.7143	0.8138	0.6714
V212	4	0.1852	0.6440	0.0014*	4	0.2667	0.7483	0.0124*	5	0.0714	0.7679	0.0000*
V217	5	0.2963	0.3539	0.2698	5	0.3000	0.4500	0.1786	4	0.3214	0.4841	0.2034
V230	6	0.7407	0.7298	0.7068	9	0.6667	0.6606	1.0000	7	0.6071	0.7876	0.1995
V254	10	0.2963	0.8176	0.0000*	8	0.0667	0.8367	0.0000*	7	0.2857	0.7506	0.0003*

Note: *A* = number of alleles sampled; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; *n* = number of individuals sampled.

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

*Significant deviation from HWE ($P < 0.05$).

Table 3. Amplification of 15 microsatellite loci developed for *Ficus virens* in eight other *Ficus* species.

Locus	*Ficus altissima* (*n* = 5)	*Ficus benjamina* (*n* = 5)	*Ficus deltoidea* (*n* = 5)	*Ficus hispida* (*n* = 5)	*Ficus microcarpa* (*n* = 5)	*Ficus montana* (*n* = 5)	*Ficus rumphii* (*n* = 5)	*Ficus sarmentosa* (*n* = 5)
V33	—	+	—	—	+	—	—	—
V42	+	—	+	—	+	—	+	—
V70	—	+	+	+	+	+	+	—
V98	+	+	+	+	+	+	+	—
V99	+	+	—	—	+	+	+	+
V131	+	+	+	+	+	+	+	—
V149	+	+	—	+	+	+	+	+
V159	—	+	—	+	+	—	—	—
V171	+	+	—	—	+	+	—	+
V183	+	+	+	—	+	+	+	+
V188	+	+	+	+	+	+	+	+
V212	+	—	—	—	—	—	—	—
V217	+	+	—	+	+	+	+	+
V230	+	+	—	+	—	+	+	+
V254	+	+	+	+	+	+	+	+

Note: + = primers could be successfully amplified; — = primers could not be amplified.

of null allele frequencies was tested with MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004). The genetic variations were estimated for two sampled populations by FSTAT 2.9.3.2 (Goudet, 2001), using the parameters of the number of alleles at each locus, observed heterozygosity, and expected heterozygosity.

Linkage disequilibrium was not detected in any loci. The number of alleles per locus ranged from three to 17, with an average of 9.333 (Table 1). The observed heterozygosity and expected heterozygosity of each population ranged from 0.0667 to 0.9286 and 0.0650 to 0.8890, respectively (Table 2). Five loci (V70, V159, V183, V212, V254) in Chengdu, four loci (V98, V159, V212, V254) in Mianyang, and three loci (V42, V212, V254) in Chongqing showed significant departure from HWE. Two loci (V212, V254) showed consistent deviation from HWE. This could be caused by the presence of null alleles (present in six loci: V70, V98, V159, V183, V212, and V254) or by homozygote excess.

***Cross-amplification in additional species*—**Cross-amplification of polymorphic microsatellite primers was performed on eight related species (*n* = 5 for each species), including *F. altissima* Blume, *F. benjamina* L., *F. deltoidea* Jack, *F. hispida* L. f., *F. microcarpa* L. f., *F. montana* Burm. f., *F. rumphii* Blume, and *F. sarmentosa* Buch.-Ham. ex Sm.

All 15 polymorphic primers amplified successfully (amplified a distinct band when the PCR products were checked on 1% agarose gel) in at least one of the eight additional *Ficus* species tested (Table 3). Two of the markers (V188, V254) were successfully amplified in all eight species, while one locus (V212) was only successfully amplified in *F. altissima*. Successful amplification across species did not appear to be correlated with the closeness of the genetic relationship of the species tested with *F. virens*. However, the cross-species sample size was limited and thus it is still likely that these markers will be most useful for closely related species.

CONCLUSIONS

In this study, 15 microsatellite markers were developed specifically for *F. virens* and showed considerable polymorphism in all three studied populations. These markers can be used in further studies to explore the mating system, population genetic structure, and gene flow of *F. virens*.

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Appendix 1. Voucher and locality information for *Ficus virens* specimens used in this study.[a]

Voucher accession no.[b]	Collection locality	Geographic coordinates
HWNU-FV20160805001-XqY, XyD	Chengdu, Sichuan	30.68271°N, 104.10363°E
HWNU-FV20160801002-LT, RhF	Mianyang, Sichuan	31.47661°N, 104.78943°E
HWNU-FV20160818003-LT, RhF	Chongqing	29.8217°N, 106.42913°E

[a] Vouchers are deposited in the Herbarium of China West Normal University, Nanchong, Sichuan, China.
[b] Collectors: XqY = Xiqian Yang; XyD = Xiangyue Ding; LT = Lu Tan; RhF = Ronghua Fu.