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Microsatellite marker development in tung trees (*Vernicia montana* and *V. fordii*, Euphorbiaceae)[1]

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- *Premise of the study:* The tung tree is valuable for tung oil, which is derived from the oilseeds of the tree. Microsatellite markers were developed in tung trees (*Vernicia montana* and *V. fordii*) for use in population genetic studies and, particularly, analyses of genetic relationships among germplasms.
- *Methods and Results:* A total of 20 microsatellite markers were developed and characterized in *V. montana* and tested for cross-amplification in *V. fordii*, using the Fast Isolation by AFLP of Sequences COntaining Repeats protocol. These markers showed rich polymorphism when assessed in 120 samples of *V. montana* and six samples of *V. fordii*.
- *Conclusions:* The developed microsatellite markers will be of great use for investigating genetic diversity of germplasms, analyzing population structure, and facilitating molecular breeding in tung trees.

Key words: genetic diversity; microsatellite; tung tree; *Vernicia fordii*; *Vernicia montana.*

The tung tree, also called Tung Oil Tree or China Wood-Oil Tree, a small Asian tree of the spurge family (Euphorbiaceae), is valuable for tung oil, which is derived from the oilseeds of the tree. Among all the vegetable oils, tung oil is distinctive because of its high level of eleostearic acid consisting of cis-9, trans-11, and trans-13 double bonds and is commonly used in formulations of inks, dyes, coatings, and resins (Sonntag, 1979). Recently, studies have proven tung oil to be a raw material for biodiesel production after blending with other biodiesel (Chen et al., 2010). Exploiting tung oil as a feedstock for biodiesel and launching the breeding and improvement of tung tree varieties are drawing great attention in China and South Asia. There are two species of tung trees: *Vernicia montana* Lour. and *V. fordii* (Hemsl.) Airy Shaw, both native to southern China and South Asia. Taxonomically, *V. montana* is easily distinguished from *V. fordii* by its inflorescence and fruit characters. Although *V. fordii* has been planted and tung oils have been used for hundreds of years in China, the main germplasm of tung trees (in particular *V. montana*) is scattered throughout southern China, Myanmar, and Vietnam into Thailand. To facilitate breeding and improvement of varieties, there is an urgent need for investigating genetic diversity at the population level and identifying genetic relationships among germplasms in tung trees. Microsatellite markers are a powerful tool for investigating genetic diversity and identifying genetic relationships among germplasms. Here, we report 20 microsatellite markers (Table 1) for tung trees that will be helpful for investigating genetic diversity at the population level and identifying the genetic relationship among germplasms.

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METHODS AND RESULTS

The FIASCO (Fast Isolation by AFLP of Sequences COntaining Repeats) protocol developed by Zane et al. (2002) was used to construct a microsatellite-enriched library for *V. montana*. Genomic DNA was isolated and purified from fresh leaves of one individual *V. montana* planted at XTBG (Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences) using the Plant Genomic DNA Kit (TianGen, Beijing, China). About 500 ng genomic DNA was digested with 5 U of *Mse*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA) in a 50 μL volume, and then the digested DNA was electophoresised on 1.5% agarose gel in TAE buffer, and the 250–800 bp *Mse*I-digested size fraction was isolated using QIAquick Gel Extraction Kit (QIAGEN, Valencia, California, USA). The 250–800 bp DNA fragment was ligated to an *Mse*I adaptor pair (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′), using 2 U of T4 DNA ligase (Fermentas, Burlington, Ontario, Canada) in a volume of 30 μL. Tenfold diluted digestion-ligation mixture was directly amplified with 1 μL of the *Mse*I-N primer (5′-GATGAGTCCTGAGTAAN-3′) (25 μM), 1 U of *Taq* polymerase (Takara, Dalian, Liaoning, China), 2 μL of 10× PCR buffer, 0.4 μL of dNTP (2.5 mM each) in a total volume of 20 μL, following the program: 95°C for 3 min, 25 cycles of 94°C for 30 s, 53°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 5 min.

For enriching the fragments containing the microsatellite loci, the PCR products were denatured at 95°C for 5 min, and then hybridized with two kinds of 5′-biotinylated probes $(AC)_{15}$ and $(AG)_{15}$ in a 250 μL hybridization solution (SSC 4.2× and SDS 0.07%) at 50°C for 2 h. Following the procedure of Liu et al. (2010), streptavidin-coated magnetic beads (Promega, Madison, Wisconsin, USA) were used to separate and capture the DNA fragments hybridized to the probe. Recovered DNA fragments were amplified with the *Mse*I-N primer as described above. The PCR products were purified and then ligated into a pGM-T vector (Tiangen, Beijing, China) and transformed into competent Trans5α (Transgen, Beijing, China). Transformants were plated and insert-containing clones were selected by blue/white screening. One hundred thirty-one positive clones of 260 total were sequenced. Of these, 104 clones possessed microsatellite motifs, and 73 sequences with relatively long flanking regions were suitable for primer design using PRIMER 3 software (Rozen and Skaletsky, 2000). These sequence records were deposited at GenBank (JF713027–JF713046).

To test the polymorphisms of the 73 microsatellite primer pairs designed in tung tree, we screened 120 samples of *V. montana* collected from the main distribution areas of tung tree in China and planted at XTBG, covering 30 germplasms (accession No: vm001-030, see the Appendix for the

TABLE 1. Characteristics of 20 microsatellite makers developed in *V. montana*. Shown for each marker are the forward and reverse sequence, repeat type, allele size (in bp), fragment size range (in bp) in 120 individuals from 30 germplasms (four samples each), optimal annealing temperature (T_a), and GenBank accession number.

Marker	Primer sequences (5′–3′)	Repeat	Size (bp)	Size Range (bp)	T_a (°C)	GenBank Accession No.
TT05	F: AACTTGTCTGTGCATGTGCC	$(CA)_7$	102	100–108	55	JF713027
	R: TGCGTAACGTTCGAGTTGTC					
TT06	F: TTCTATGGCTTGAAGGGGTG	$(CA)_{17}$	190	188–198	55	JF713028
	R: TGCACTTGAATGTTTGTGCC					
TT07	F: CGCCTCGGTAATGCTCTAAC	$(AC)_9$	163	150–170	55	JF713029
	R: TCCCATTTCCTGAAAGCAAG					
TT11	F: CCAGTGGAAATGCAACAGAA	$(AC)_9$	132	132–140	55	JF713030
	R: AAAACAAAACCCAATGCAGC					
TT14	F: CGTATTGTCATCGTCTCCCA	$(TG)_{13}$	104	88–110	55	JF713031
	R: TCAGTCCTTCTCCTTATTTGAACA					
TT16	F: TGCTTGCCCAGTTTAGGTCT	$(CA)_{10}$	127	120–136	55	JF713032
	R: GCACACTTACCAAAACACACAA					
TT17	F: ATGAAGGCACTGTGAAGGGA	$(GT)_6$	270	265–320	55	JF713033
	R: CCATCCCAAATCCTCTAGCA					
TT28	F: GCTCTAGTTGGCCCTTCAAA	$(TG)_{13}$	185	170–190	55	JF713034
	R: AAGGGATGTTGCAGCTATGG					
TT29	F: ATGAGAGCATTGCACCACAC	$(TC)_{10}$	117	106–124	55	JF713035
	R: ATTTGCCATTCAAGTCTCGC					
TT31	F: TGGCAGCAAAGAAACTGAGA	$(AG)_{16}$	169	160–170	55	JF713036
	R: GAGTCCTGAGTAAGCCGTCG					
TT32	F: AATCAGTGGCAAGAAGTGGG	$(AG)_{22}$	269	240–274	55	JF713037
	R: GTCCTGAGTAAGGGCATCCA					
TT34	F: TTCCACACGAATTTTCTCCTG	$(AT)_8(GT)_{11}$	137	137–145	55	JF713038
	R: TGCAGATATTCTGCTGCCAC					
TT39	F: TCAATGCACTCAAGTCTGGC	$(GA)_{15}$	107	96–110	55	JF713039
	R: TCAGGTTCTTGTTTTTGCCC					
TT42	F: GTTCGATATCCAAGCCCTGT	$(TG)_{17}$	158	140–170	55	JF713040
	R: CCGCCCCTGCATACATATAG					
TT50	F: CGGGTCAAACCCACAAGATA	$(AG)_{14}$	180	170–200	55	JF713041
	R: AACTGACATTGTAAGGCAGCTC					
TT53	F: TGGTGACAGCTTTGCGTAAC	$(TG)_{11}(GA)_{11}$	149	139–159	55	JF713042
	R: TGATTAGCATTGCAGCAAAAA					
TT55	F: TCCTGAGTAAACAATCAACATCTCA	$(AT)_6(AG)_8$	119	119–129	55	JF713043
	R: TGGGACTAGCCTTGCCTCTA					
TT57	F: CAATAACAATGCGACAATGC	$(AG)_9$	159	157–163	55	JF713044
	R: AGACCACCCTGTTTTTGCTG					
TT69	F: GCACTATCCCCTTACGCAAC	$(GA)_{21}$	143	134–146	55	JF713045
	R: GTGCTTGTTCTGCTCCCTTC					
TT71	F: CACTCCTAGGTGAAATGCCC	$(CT)_{18}$	238	218–240	55	JF713046
	R: TGCTCCAAAAATAGGAGTGGA					

sample information). The genomic DNA of each sample was isolated as described above. PCR amplifications were performed in 25 μL reaction mixtures containing 1.2 U of *Taq* polymerase (Transgen), 2.5 μL of 10× PCR buffer, 0.5 μL of dNTP (2.5 mM each), 1 μL of each primer (10 μM), and ~10 ng genomic DNA. The thermal profile used was initial denaturation for 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 56°C, 40 s at 72°C, and final extension for 7 min at 72°C. PCR products were resolved on an 8% polyacrylamide denaturing gel and visualized by silver staining. Product sizes were determined by comparison to a 20 bp DNA ladder (Transgen).

Of 73 primer pairs tested, 42 primer pairs successfully amplified PCR products, and 20 primer pairs produced unambiguous and polymorphic amplification with the anticipated size in 30 samples of *V. montana*. The 20 polymorphic markers amplified two to eight alleles with an average of four alleles per locus.

TABLE 2. Results of the initial microsatellite screening in 120 individuals of *V. montana* from 30 germplasms (four samples each). Number of alleles (N_a), observed (H_o) and expected (H_e) heterozygosities, and polymorphism information content (PIC) of each marker are shown.

Marker	N_a	*Ho*	*He*	*PIC*	Marker	N_a	H_o	H_e	PIC
TT05	4	0.608	0.6777	0.6181	TT32	3	0.392	0.5664	0.5045
TT06	3	0.550	0.6032	0.5337	TT34	5	1.000	0.7010	0.6482
TT07	6	1.000	0.7296	0.6868	TT39	6	1.000	0.7375	0.6968
TT11	3	0.508	0.6462	0.5730	TT42	8	0.597	0.7864	0.7543
TT14	4	0.483	0.6250	0.5781	TT50	6	1.000	0.8256	0.8010
TT16	4	1.000	0.7466	0.6993	TT53	4	0.700	0.7070	0.6532
TT17	2	0.042	0.4708	0.3600	TT55	3	0.387	0.5191	0.4640
TT28	4	1.000	0.7364	0.6877	TT57	3	0.387	0.5662	0.5042
TT29	3	0.383	0.5462	0.4877	TT69	4	1.000	0.6857	0.6280
TT31	3	1.000	0.6182	0.5453	TT71	3	0.742	0.5308	0.4707

Expected heterozygosity (H_e) or gene diversity ranged from 0.47 to 0.83 with an average of 0.65, and the polymorphism information content (PIC) ranged from 0.36 to 0.80 (Table 2) with an average of 0.59, calculated by GENEPOP 4.0 software (Raymond and Rousset, 1995). Cross-amplification tests were performed in six samples of *V. fordii* from three germplasms (two samples each, accession No: vf001-003, see the Appendix). All 20 microsatellite markers developed successfully amplified PCR products in *V. fordii* and exhibited similar polymorphisms (allele number ranged from two to six with an average of four per locus; H_e ranged from 0.52 to 0.74 with an average of 0.62) to that in *V. montana*. These results indicated that the 20 microsatellite markers harbored rich polymorphisms in the tung tree.

CONCLUSIONS

We developed and characterized 20 microsatellite markers from *V. montana* and tested their polymorphisms in *V. fordii* by cross-amplification. These markers will be helpful for investigating the genetic diversity at the population level or identifying the genetic relationships among germplasms, facilitating breeding and variety improvement of the tung tree.

LITERATURE CITED

CHEN, Y.-H., J.-H. CHEN, C.-Y. CHANG, AND C.-C. CHANG. 2010. Biodiesel production from tung (*Vernicia montana*) oil and its blending properties in different fatty acid compositions. *Bioresource Technology* 101: 9521–9526.

LIU, Y., X.-J. GE, Q.-B. SUN, AND T. CAO. 2010. Development of microsatellite markers for the moss *Ptychomitrium gardneri* (Ptychomitriaceae). *American Journal of Botany* 97: e14–e16.

RAYMOND, M., AND F. ROUSSET. 1995. Genepop (version 1.2): population genetics software for exact tests and ecumenicism. *The Journal of Heredity* 86: 248–249.

ROZEN, S., AND H. J. SKALETSKY. 2000. Primer 3 on the WWW for general users and for biologist programmers. *In* S. Krawetz and S. Misener [eds.], Bioinformatics methods and protocols: Methods in molecular biology, 365–386. Humana Press, Totowa, New Jersey, USA.

SONNTAG, N. O. V. 1979. Composition and characteristics of individual fats and oils. *In* D. Swern [ed.], Bailey's industrial oil and fat products, 289–477. John Wiley & Sons, New York, USA.

ZANE, L., L. BARGELLONI, AND T. PATARNELLO. 2002. Strategies for microsatellite isolation: A review. *Molecular Ecology* 11: 1–16.

APPENDIX 1: Information on the original source of tung trees in the germplasm collection. All tung tree germplasms were planted at XTBG and their voucher specimens were deposited at XTBG Herbarium.

***V. montana* germplasm**: Vm001, collected from Qidong, Hunan (26°47'N, 111°38'E), voucher: QY2006vm01; Vm002, collected from Qidong, Hunan (26°49'N, 111°39'E), voucher: QY2006vm02; Vm003, collected from Menghai, Yunnan (21°57'N, 100°18'E), voucher: QY2005vm01; Vm004, collected from Menghai, Yunnan (21°58'N, 100°25'E), voucher: QY2005vm02; Vm005, collected from Mojiang, Yunnan (23°58'N, 100°25'E), voucher: QY2005vm03; Vm006, collected from Jingdong, Yunnan (24°27'N, 100°50'E), voucher: QY2005vm04; Vm007, collected from Fuyang, Zhejiang (30°3'N, 119°57'E), voucher: QY2007vm01; Vm008, collected from Fuyang, Zhejiang (30°5'N, 119°59'E), voucher: QY2007vm02; Vm009, collected from Zhenfeng, Guizhou (25°22'N, 105°42'E), voucher: QY2006vm03; Vm010, collected from Fangcheng, Guangxi (21°48'N, 108°21'E), voucher: QY2006vm04; Vm011, collected from Huanjiang, Guangxi (24°49'N, 108°15'E), voucher: QY2006vm05; Vm012, collected from Louang phrabang, Northern Laos (19°51'N, 102°30'E), voucher: QY2008vm01; Vm013, collected from Tiane, Guangxi (25°6'N, 107°10'E), QY2006vm06; Vm014, collected from Shanghang, Fujian (25°26'N, 116°24'E), voucher: QY2008vm02; Vm015, collected from Jinggu, Yunnan (23°30'N, 100°42'E), voucher: QY2005vm05; Vm016, collected from Mojiang, Yunnan (23°25'N, 101°41'E), voucher: QY2008vm03; Vm017, collected from Jinggu, Yunnan (23°29'N, 100°42'E), voucher: QY2008vm04; Vm018, collected from Simao, Yunnan (23°29'N, 100°42'E), voucher: QY2008vm05; Vm019, collected from Changsha, Hunan (28°13'N, 113°0'E), voucher: QY2007vm03; Vm020, collected from Changsha, Hunan (28°18'N, 113°6'E), voucher: QY2007vm04; Vm021, collected from Changsha, Hunan (28°23'N, 113°1'E), voucher: QY2007vm05; Vm022, collected from Rongshui, Guangxi (25°4'N, 109°15'E), voucher: QY2007vm06; Vm023, collected from Jingchuan, Guangxi (25°18'N, 110°17'E), voucher: QY2007vm07; Vm024, collected from Lingui, Guangxi (25°13'N, 110°13'E), voucher: QY2007vm08; Vm025, collected from Biaozhou, Jiangxi (28°41'N, 115°54'E), voucher: QY2008vm06; Vm026, collected from Fenyi, Jiangxi (27°48'N, 114°39'E), voucher: QY2008vm07; Vm027, collected from Nanchang, Jiangxi (28°39'N, 115°53'E), voucher: QY2008vm08; Vm028, collected from Nanchang, Jiangxi (28°29'N, 115°58'E), voucher: QY2008vm09; Vm029, collected from Yaan, Sichuan (30°40'N, 114°4'E), voucher: QY2008vm10; Vm030, collected from Menghai, Yunnan (21°59'N, 100°27'E), voucher: QY2006vm07.

***V. fordii* germplasm**: Vf001, collected from Menghai, Yunnan (21°53'N, 100°20'E), voucher: QY2008vf01; Vf002, collected from Qidong, Hunan (26°37'N, 111°42'E), voucher: QY2008vf02; Vf003, collected from Huanjiang, Guangxi (24°46'N, 108°13'E), voucher: QY2008vf03.