Development of Microsatellite Markers in the Oil-Producing Species Vernicia fordii (Euphorbiaceae), A Potential Biodiesel Feedstock

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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE MARKERS IN THE OIL-PRODUCING SPECIES *VERNICIA FORDII* (EUPHORBIACEAE), A POTENTIAL BIODIESEL FEEDSTOCK[1]

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- *Premise of the study:* Tung tree, *Vernicia fordii*, is native to China. Little has been done on genetics and breeding at the molecular level in this species, let alone utilizing microsatellite (simple sequence repeat [SSR]) markers. Therefore, a set of SSR molecular markers was developed for studies on molecular genetics and breeding in tung tree.
- *Methods and Results:* We designed 78 SSR markers using a protocol based on the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol. Assessed in 81 *V. fordii* accessions, 40 of these markers were polymorphic and 12 of them showed monomorphism. When tested using six *V. montana* accessions, 52 of the markers were capable of PCR amplification and 25 were polymorphic.
- *Conclusions:* The newly developed SSR markers are effective and helpful in the evaluation of genetic germplasm and molecular breeding in tung tree.

Key words: diversity evaluation; Euphorbiaceae; FIASCO protocol; microsatellite marker; *Vernicia fordii.*

Tung tree, *Vernicia fordii* (Hemsl.) Airy Shaw (Euphorbiaceae), is a native economic tree species in China. It is the most important species used to produce industrial oil (tung oil) and has been cultivated for over a thousand years in China. Today, the remnant plantation areas of *V. fordii* include Sichuan, Hunan, Hubei, Guizhou, and Chongqing provinces, as well as adjacent regions (Zhang and Peng, 2011). In addition to its irreplaceable role in industry for the manufacture of paints and coatings, tung oil has been reported to be a promising feedstock in biodiesel production (Shang et al., 2010). *Vernicia fordii* is adaptive to drought and barren mountainous areas. Thus, its development will both meet the energy demands without endangering the food supply chain and provide employment in poor mountainous regions. Molecular markers are efficient in revealing genetic diversity (Peng et al., 2000) and in assisting tree breeding (Li et al., 2008; Zhao et al., 2011). In this study, we developed a set of microsatellite (simple sequence repeat [SSR]) markers based on the specific genomic sequences of *V. fordii*, and evaluated their efficiency in amplifying the DNA of the related species *V. montana* Lour.

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METHODS AND RESULTS

Leaf tissues collected from adult tung trees were immediately preserved in silica gel for fast drying and then stored in a −70°C freezer. The dried leaf tissues were ground into fine powder in liquid nitrogen just before DNA extraction. Genomic DNA was isolated using a modified cetyltrimethylammonium bromide (CTAB)–based plant DNA extraction method (Doyle and Doyle, 1987; Zhang et al., 2013). The SSR-containing fragments were isolated using a protocol based on the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol (Zane et al., 2002). Total genomic DNA (~500 ng) was digested by the *Mse*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA) at 37°C for 3.5 h and then ligated to an *Mse*I adapter pair (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) with T4 DNA ligase (Fermentas International, Burlington, Ontario, Canada) in a 25-μL reaction mixture. The ligation product was diluted (1 : 10) and amplified by PCR with the adapter-specific primers *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′) (25 μM). The amplified DNA fragments were enriched for SSR repeats by magnetic bead selection with 5′-biotinylated $(AC)_{13}$ and $(AG)_{13}$ probes, respectively. PCR products were purified using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China). The purified DNA fragments were ligated into the pMD18-T vector and transformed into DH5α cells (TaKaRa Biotechnology Co., Dalian, Liaoning, China). Positive clones were detected by PCR using the M13-tailed PCR method. All PCR reactions were performed using the following procedure: an initial denaturation of 5 min at 95°C; followed by 30 cycles of 40 s at 94°C, 30 s at 55°C, and 45 s at 72°C; and a final extension at 72°C for 8 min (Pan et al., 2009).

Among 400 colonies, a total of 196 (49%) fragments were found to contain SSR repeats, and the sequences were deposited in GenBank. For the SSR sequences containing adequate flanking regions, PCR primers were designed with Primer3 software (Rozen and Skaletsky, 2000). Eighty-one individuals of *V. fordii* from a local population (Huangpi, 31°06′15.66″N, 114°11′53.46″E) were used to test the polymorphism of the microsatellite markers. Vouchers were deposited at Wuhan Botanical Garden (Appendix 1). PCR products were separated by 6% denaturing polyacrylamide gels. Allele sizes were estimated visually

TABLE 1. Characterization of 40 polymorphic SSR markers developed in *Vernicia fordii.*

Locus	Primer sequences (5′–3′)	Repeat motif[a]	Size range (bp)	T_a (°C)	GenBank accession no.
vfSSR04	F: ATCGGGACACAAAGAGAACG	$(AG)_{19}$	160–186	60	JQ323357
	R: TTCCTCCGTTGGTGTTTCTC				
vfSSR05	F: CCAGCATCTTCTTGTTCTTCC	$(TC)_{18}$	200–224	60	JQ323359
	R: GAATTCAAAAGTGGTACAGC				
vfSSR06	F: TGCCATTGCTAAGGAAGAAGA	$(CA)_{11}$	220–228	60	JQ323360
	R: CACGTGGAGCATCTTCAAAA				
vfSSR07	F: AGAAAACGAGCAGGAGACCA	$(GA)_{10}$	200–208	60	JQ323361
	R: CGGATGCGAAAGAAAAGAGA				
vfSSR08	F: GGTATATCGGGCCCTTTGAG	$(TG)_{16}$	190–300	60	JQ323362
	R: TCAATTCAAGCATCCCAAGT				
vfSSR09	F: GATCGAGTGCTTCATGTGCT	$(TG)_{16}$	134–146	60	JQ323363
	R: TGACTAGGAAATCTCACTTTAG				
vfSSR10	F: TGAAAGTAGGGGCACAGCTT	$(AG)_{17}$	130–150	60	JQ323364
	R: TTCACACTCATGGCACTGCT				
vfSSR12	F: CATCCCATGTCCTTTTCTGG	$(CA)_9$	180–205	60	JQ323366
	R: CTTCATAGGCATGGCCACAT				
vfSSR15	F: TGGGTATACAAGAGGCTAGGTT	$(CT)_{21}$	130–280	60	JQ323369
	R: CTTGACCTCTTGCTCTGTGCT				
vfSSR16	F: GAAGATCACCCTTCCGACAA	$(AT)_5(GT)_{11}$	200–240	60	JQ323370
	R: CTTCTATCAAGGTTTTCATGCT				
vfSSR17	F: AGAAGGGCGTTCAGCATATC	$(GA)_{35}$	345–430	60	JQ323371
	R: CCCAGATCCTTCTTCTTCTCC				
vfSSR18	F: CGAGTGGTTGACAAGGAAGTT	$(CT)_{35}$	100–105	60	JQ323372
	R: TGCTCCTCACTCTCCCATGT				
vfSSR20	F: CCATCATCTTTTCTCATTTCAC	$(CA)_8(TTC)_4$	148–170	60	JQ323375
	R: CCATATTGGCCAAACATCAA				
vfSSR21	F: TGGCCCCAAAAGAAACATAG	$(CA)_8,(AG)_{23}$	120–124	60	JQ323377
	R: TCAACAAATATCTCTTCACGCTTC				
vfSSR22	F: TTCCTAGAAAAGGGGCGTCT	$(CA)_{12}(GA)_{14}$	220–240	60	JQ323378
	R: GCATCATTTGGAGGTCTGGT				
vfSSR25	F: GCCTACAGTCTACAGTTCCAAAAA	$(AG)_{21}$	106–180	60	JQ323381
	R: CAAAAATTGAGACAACACATGACA				
vfSSR26	F: AATGAAAGAGCACTGCATGG	$(TC)_{10},(AC)_7$	220–240	60	JQ323382
	R: TCCAAACACCAAAGCCCTAC				
vfSSR27	F: TGTATAGACTGAGGAATGCAAGC	$(TG)_{10}$	200–240	60	JQ323383
	R: TTCCCTTGCTCTACATACCATTC				
vfSSR28	F: GAATTCCCTAAGAGGCAATAAGC	$(TC)_{13}(AC)_{16}$	152–162	60	JQ323384
	R: TGAATTTGAAGATAAAGAGAGC				
vfSSR33	F: TGTAATTTTACATGCTGGTG	$(TG)_8,(AG)_8$	200–225	60	JQ323390
	R: AGAATGCATGTGCTGTTGC				
vfSSR35	F: AATGTATGATTGCATGAGAA	$(AC)_9,(AT)_6$	185–215	60	JQ323392
	R: CTGGCCATCCATTGATATT				
vfSSR36	F: GACCCACTAACACAAATTGC	$(TG)_7$	172–174	60	JQ323393
	R: TGGATCTAGCATGTGCTCACT				
vfSSR37	F: AGGTTGCTTCTGGCTCTCC	$(TC)_5,(AC)_8$	220–240	60	JQ323394
	R: TCCCAAAAGTGGGATGTGA				
vfSSR40	F: CGGAGTTAGTGGCATGT	$(TC)_9,(TG)_{13}$	126–136	60	JQ323397
	R: CCTTCAAAAACAAAACAGAAGC				
vfSSR41	F: AAGACCGGCGAAAGCTAAC	$(ATT)_9,(CA)_9$	104–108	58	JQ323398
	R: CAAGCCCAACATTTCTACC				
vfSSR44	F: GGGGAGCTCAAAGAAAAGA	$(CA)_{11}$	250–275	60	JQ323401
	R: CTTTATATGCACAATCATTGAC				
vfSSR45	F: GTTGGAAACGGAGGTAGAA	$(TG)_8$	114–158	58	JQ323402
	R: AAGCAGAAAAGGAGAGACAAAA				
vfSSR49	F: ATTACATGAATGTTCGGGATCT	$(GA)_{63}$	172–176	60	JQ323407
	R: AAGCTGTAGGCGTCGGATA				
vfSSR50	F: TGAACCAGAGAAACAAACG	$(AG)_{34},(AAG)_{12}$	140–190	60	JQ323409
	R: AACCAGAACTCTTCTTCTTTTT				
vfSSR53	F: GAGAAGGATGAGGGTGGTC	$(AG)_{10},(TG)_{10}$	145–154	60	JQ323413
	R: TCTCTCACACAGCCACCAA				
vfSSR56	F: CAAACTGTAATACCCTAAGGA	$(TG)_{17},(TA)_7$	160–195	60	JQ323417
	R: CAGTGGCAGCATCTCTTTT				
vfSSR57	F: GTAATTTTACATGCTGGTG	$(TG)_9(AG)_8$	200–210	60	JQ323418
	R: AGAATGCATGTGCTGTTGC				
vfSSR58	F: AAAATAACCGTATAAGACA	$(TG)_{22}$	138–144	60	JQ323419
	R: TCCCAAGTTTCTTTGGACATT				
vfSSR59	F: TCTTGACAAAAAGGGGAAGA	$(AG)_{70}$	154–170	60	JQ323420
	R: TTGCATCATCAAAATCACA				

TABLE 1. Continued.

Locus	Primer sequences (5′–3′)	Repeat motif[a]	Size range (bp)	T_a (°C)	GenBank accession no.
vfSSR61	F: GGTGAATACTTCGTTGGTCTT R: CTCAACACTATGCACATAACCA	$(AC)_{14}$	230–256	60	JQ323422
vfSSR63	F: TGTTTGTTTCTATCTTCCCTCTTTT R: GCGTAACGTTTCACTCTCC	$(TTTG)_6$	138–160	60	JQ323424
vfSSR65	F: TTGGGAGATAGCCAAAGCA R: AGAGAGGTGGGTACTGAAGTG	$(GA)_4(CAA)_2$	185–200	60	JQ323426
vfSSR67	F: GTGAAGAGGGGTGAGTCAA R: TTTGGTTCTGTCTATGTGG	$(AT)_4(GT)_9$	148–156	60	JQ323428
vfSSR73	F: ACAACAAAACTAGAGAAAC R: CTTCGGAGCGTCACTTCTT	$(CT)_{39},(GT)_{12}$	218–224	58	JQ323434
vfSSR76	F: TGCGGAACAGAGAACTAAGAGA R: CCCCTAATATGGTTGCCTACTTT	$(AC)_8$	118–122	60	JQ323437

Note: T_a = annealing temperature.
[a]Commas signify a gap (i.e., no SSR) between the two motifs in the complex SSR.

TABLE 2. Population genetic parameters for the polymorphic SSR markers developed in *Vernicia fordii* and *V. montana*.

	V. fordii				*V. montana*			
Locus	*A*	*I*	H_o	H_e	*A*	*I*	H_o	H_e
vfSSR04	2	0.6523	0.7160	0.4625	2	0.6365	0.6667	0.4848
vfSSR05	2	0.6870	0.8642	0.4969	2	0.6931	0.6667	0.5455
vfSSR06	3	1.0719	0.8642	0.6521	2	0.6931	0.6667	0.5455
vfSSR07	4	1.0849	0.4198	0.6202	2	0.4506	0.3333	0.3030
vfSSR08	2	0.6855	0.8025	0.4954	2	0.6792	0.8333	0.5303
vfSSR09	2	0.6931	1.0000	0.5031	2	0.6792	0.5000	0.5303
vfSSR10	5	1.1861	0.9136	0.6461	2	0.2868	0.1667	0.1667
vfSSR12	2	0.2641	0.0988	0.1380	2	0.6931	1.0000	0.5455
vfSSR15	6	1.6130	0.8025	0.7930	0	0.0000	0.0000	0.0000
vfSSR16	2	0.6710	0.6173	0.4809	2	0.6792	0.5000	0.5303
vfSSR17	2	0.0665	0.0000	0.0245	2	0.4506	0.3333	0.3030
vfSSR18	2	0.1158	0.0000	0.0485	2	0.6931	1.0000	0.5455
vfSSR20	4	1.1440	0.7901	0.6438	2	0.6931	1.0000	0.5455
vfSSR21	3	0.9060	0.2469	0.5398	0	0.0000	0.0000	0.0000
vfSSR22	4	1.0514	0.9383	0.6056	0	0.0000	0.0000	0.0000
vfSSR25	3	0.2440	0.0864	0.1067	2	0.2868	0.1667	0.1667
vfSSR26	2	0.6411	0.4596	0.3773	0	0.0000	0.0000	0.0000
vfSSR27	3	0.7197	0.6049	0.4580	2	0.5623	0.5000	0.4091
vfSSR28	6	0.7571	0.1852	0.3614	0	0.0000	0.0000	0.0000
vfSSR33	5	0.6464	0.2222	0.3057	0	0.0000	0.0000	0.0000
vfSSR35	6	1.3909	1.0000	0.7017	3	0.8676	0.3333	0.5455
vfSSR36	3	0.7359	0.8148	0.5003	2	0.4506	0.3333	0.3030
vfSSR37	3	0.9113	0.3580	0.5442	0	0.0000	0.0000	0.0000
vfSSR40	2	0.6411	0.4596	0.3773	0	0.0000	0.0000	0.0000
vfSSR41	3	1.0067	0.7778	0.6163	2	0.6931	1.0000	0.5455
vfSSR44	3	0.7510	0.9259	0.5152	2	0.4506	0.3333	0.3030
vfSSR45	3	0.3532	0.0000	0.1626	3	1.0114	0.3333	0.6667
vfSSR49	3	0.5519	0.3210	0.3116	0	0.0000	0.0000	0.0000
vfSSR50	3	0.7917	0.9383	0.5270	3	0.8676	0.3333	0.5455
vfSSR53	3	0.7508	0.9630	0.5150	0	0.0000	0.0000	0.0000
vfSSR56	3	0.4039	0.1358	0.1941	0	0.0000	0.0000	0.0000
vfSSR57	2	0.4970	0.2469	0.3190	3	0.9184	1.0000	0.6212
vfSSR58	3	0.9134	1.0000	0.5709	0	0.0000	0.0000	0.0000
vfSSR59	2	0.3085	0.1852	0.1691	0	0.0000	0.0000	0.0000
vfSSR61	3	0.6380	0.1852	0.3574	3	0.5661	0.3333	0.3182
vfSSR63	2	0.0665	0.0000	0.0245	0	0.0000	0.0000	0.0000
vfSSR65	4	0.6992	0.1481	0.3432	2	0.6931	1.0000	0.5455
vfSSR67	2	0.0665	0.0000	0.0245	2	0.6365	0.3333	0.4848
vfSSR73	2	0.1158	0.0000	0.0485	2	0.6931	1.0000	0.5455
VfSSR76	2	0.4301	0.2099	0.2626	0	0.0000	0.0000	0.0000
Mean	3	0.6411	0.4596	0.3773	2	0.5140	0.4417	0.3621

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *I* = Shannon information index.

using a 10-bp DNA ladder as a size reference. Of the 78 designed primer pairs, only 26 (33.33%) failed to generate PCR amplification products, and 52 (66.67%) could successfully yield PCR products. Forty of the 52 efficient SSR markers were polymorphic and the polymorphism rate reached 76.92% (40/52) (Table 1). Population-level Hardy–Weinberg equilibrium tests were conducted using POPGENE version 1.32 (Yeh et al., 1999). *P* values indicated there was no significant departure from Hardy–Weinberg equilibrium ($P < 0.01$). The number of alleles per locus ranged from two to eight with an average of 2.9750, and the average observed heterozygosity, expected heterozygosity, and Shannon information index were 0.4596, 0.3773, and 0.6411, respectively (Table 2). The same set of 78 SSR markers was used to test six accessions of the related species, *V. montana*. Of these tested SSR markers, 52 (66.67%) also amplified in *V. montana*, and 25 (48.08%) of the 52 markers that amplified showed polymorphism. The number of alleles per locus ranged from two to three, and the average Shannon information index was 0.5140 (Table 2).

CONCLUSIONS

This set of highly polymorphic SSR markers could be applied to further studies on genetic diversity in *V. fordii*. Because the SSR markers were tested in the local tung tree population, we expect that a higher level of genetic diversity will be detected in the equal-size tung tree population consisting of nationwide collections. The SSR-revealed genetic diversity in this species is important for conservation and proper utilization of tung tree germplasm. The newly developed SSR markers are also helpful for marker-assisted breeding in this important biodiesel plant species.

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Appendix 1. Voucher information for the accessions used in this study. Vouchers are deposited at Wuhan Botanical Garden, Chinese Academy of Sciences.

Species	Voucher specimen accession no.[a]	Collection locality[b]	Geographic coordinates	*N*
V. fordii	PT18	Huangpi	31°06′15.66″N, 114°11′53.46″E	81
V. montana	PT258	WBG	30°32′45.16″N, 114°24′52.38″E	6

Note: *N* = number of individuals; WBG = Wuhan Botanical Garden.

[a] The abbreviation "PT" is based on the first letter of the collector's surname (Peng Junhua) and "Tung tree."

[b] City in Wuhan, Hubei Province, China.