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PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF MICROSATELLITE LOCI FOR A BIOENERGY GRASS, *MISCANTHUS SACCHARIFLORUS* (POACEAE)[1]

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- *Premise of the study:* Microsatellite loci were developed for the biomass C_4 grass, *Miscanthus sacchariflorus*, and proved to be suitable markers for population genetic studies and germplasm management of this species.
- *Methods and Results:* Twenty-three polymorphic microsatellite loci were identified from an enriched genomic library of *M. sacchariflorus*. The polymorphism was assessed in 50 individuals from two populations in China. The number of alleles per locus varied from two to 18, with a mean of 8.13. The observed and expected heterozygosities ranged from 0.2 to 1.0 and from 0.198 to 0.898, respectively.
- *Conclusions:* These new markers will be useful for further investigation of genetic diversity and population genetic structure as well as molecular breeding of *Miscanthus* species.

Key words: genetic diversity; germplasm resources; microsatellites; *Miscanthus sacchariflorus*; Poaceae; simple sequence repeat markers.

Miscanthus Andersson (Poaceae) is a genus of C_4 photosynthetic grass species. Two of these species, *M. sacchariflorus* (Maxim.) Hack. and *M. sinensis* Andersson, have received considerable attention as potential bioenergy crops because of their ability to produce very high yields of biomass. *Miscanthus* ×*giganteus* J. M. Greef & Deuter, the most cultivated species in Europe with respect to biomass production, is an artificially interspecific hybrid between *M. sinensis* and *M. sacchariflorus*. As an indigenous species to a wide geographic range in Asia, *M. sacchariflorus* provides suitable "climate- and soil-matched" genotypes for most agricultural climates, and thus became a target species for breeding and selection of bioenergy crops (Clifton-Brown et al., 2008). Microsatellites have been proven to be useful markers for population genetic studies and assessment of genetic resources due to their advantages as molecular markers, such as codominance and hypervariability. Although a few dozen microsatellites have been isolated in *M. sinensis*, a close relative of *M. sacchariflorus* (Hung et al., 2009; Zhou et al., 2011; Ho et al., 2011), some of these loci could not amplify efficiently in *M. sacchariflorus* and the number of markers available for *M. sacchariflorus* is still limited. Many microsatellites transferred from distinct species such as maize or *Brachypodium distachyon* (L.) P. Beauv. by cross-taxa amplification have also failed in *Miscanthus* species (Hernández et al., 2001; Zhao et al., 2011). Here we describe the isolation and evaluation of 23 novel microsatellite loci in *M. sacchariflorus*, which will be used in further assessment of the genetic diversity and germplasm characterization to facilitate molecular marker–assisted selection and breeding of this species and its relatives.

METHODS AND RESULTS

The microsatellite-enriched library was constructed from a single individual of *M. sacchariflorus* (Fuxin County, Liaoning Province, China, 42°00′03.4″N, 121°46′27.2″E) following the protocol of Glenn and Schable (2005). Specimen vouchers were deposited at the Institute of Botany, Chinese Academy of Sciences (PE). Leaf samples were collected and dried in silica gel immediately upon collection. Genomic DNA was extracted from dried leaves using a cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). The genomic DNA was digested (into fragments smaller than 500 bp) with the enzyme *Rsa*I (New England Biolabs, Ipswich, Massachusetts, USA) and ligated into the SuperSNX linkers (SuperSNX24 Forward: 5′-GTTTAAGGCCTAGCTAGCAGCAGAATC; SuperSNX24 + 4P Reverse: 5′-GATTCTGCTAGCTAGGCCTTAAACAA). The digestion-ligation mixture was independently hybridized with 3′ biotinylated oligo probes $(TG)_{12}$, $(AG)_{12}$, and $(AAG)_8$ and captured by magnetic streptavidin Dynabeads (Dynal Biotech, Oslo, Norway) for enrichment of repeat fragments. Captured and enriched DNA was recovered by PCR amplification using the SuperSNX24 forward primer. The recovered DNA was directly ligated into a pGEM-T Easy Vector (Promega Corporation, Madison, Wisconsin, USA) and transformed into competent cells of *Escherichia coli*.

A total of 350 positive colonies were sequenced, and 160 contained repeats. Ninety-eight sequences showed clear microsatellite motifs and contained sufficient flanking regions for primer design. Fifty-six primers were designed with Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA). PCR amplifications were performed in a 15 μL volume on GeneAmp PCR System 9700 thermocyclers (Applied Biosystems, Foster City, California, USA). Final concentrations for optimizing reactions were 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 0.1% bovine serum albumin (BSA), 200 μM of each dNTP, 5% dimethyl sulfoxide (DMSO), 0.5 U ex*Taq* polymerase (TaKaRa Biotechnology Co., Dalian, Liaoning, China), 10 ng genomic DNA,

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0.5 μM marker-specific primer pairs, 0.033 μM marker-specific M13-tailed (TGTAAAACGACGGCCAGT) forward primer, and 0.5 μm HEX-, TAMRA-, or FAM-labeled M13 primer (adapted from Schuelke, 2000). A PCR cycling profile was as follows: initial denaturation of 95°C for 3 min, followed by 28 cycles of 95°C for 30 s, 58–54°C for 40 s, and 72°C for 40 s, and a final extension step at 72°C for 10 min. PCR products with different fluorescences were analyzed on an ABI3730XL sequencer with GS500 LIZ size standard (Applied Biosystems), and genotypes were scored using GeneMapper version 4.0 software (Applied Biosystems). All primer pairs were assayed in individual PCRs using samples from two *M. sacchariflorus* populations in China: pop-D40 containing 22 individuals (Heilongkou, Shangluo City, Shaanxi Province, 34°01′19.1″N, 108°58′02.0″E) and pop-D49 consisting of 28 individuals (Shijiazhuang City, Hebei Province, 38°03′17.2″N, 114°21′51.2″E). Specimen vouchers were deposited at PE (accession no.: D40 and D49, respectively). Finally, a total of 23 novel polymorphic microsatellite loci were isolated from *M. sacchariflorus*; characteristic information is provided in Table 1. None of these loci were the same as those reported previously using BLASTN similarity analysis in GenBank. All microsatellite loci except DSSR24 showed a dinucleotide repeat motif (Table 1). The number of alleles per locus, fragment length, and the observed and expected heterozygosities (H_o and H_e) were calculated with Arlequin 3.1 (Excoffier et al., 2005). Tests of deviation from Hardy–Weinberg equilibrium for each locus in each population were conducted using GenePop version 4.0 software (Rousset, 2008).

In pop-D40, the number of alleles per locus varied from two to 10, with an average of 5.65, and the H_o and H_e per locus ranged from 0.182 to 1.0 and from 0.169 to 0.844, respectively (Table 2). In pop-D49, the number of alleles per locus varied from two to 13, with an average of 5.83, and the H_o and H_e per locus ranged from 0.179 to 1.0 and from 0.223 to 0.853, respectively (Table 2). With all samples considered together, DSSR11 and DSSR24 were dimorphic in two *M. sacchariflorus* populations. DSSR25 showed the highest polymorphism with 18 alleles, followed by DSSR2 with 13 alleles. The mean number of alleles per locus was 8.13. The H_o and H_e per locus ranged from 0.200 (DSSR12) to 1.0 (DSSR13) and from 0.349 (DSSR12) to 0.898 (DSSR17) excluding two dimorphic loci, respectively (Table 2). Six loci (DSSR13, DSSR20, DSSR24, DSSR40, DSSR32, DSSR35) in pop-D40 showed significant deviation from expectation under HWE while eight markers (DSSR2, DSSR13, DSSR20, DSSR23, DSSR24, DSSR34, DSSR40, DSSR39) in pop-D49 showed significant deviation ($P < 0.01$) (Table 2).

Table 1. Characteristics of 23 microsatellite loci in *Miscanthus sacchariflorus*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Size range (bp)	GenBank accession no.
DSSR2	F: TGAACACGATGGCGAGAACG	$(AC)_8(GA)_{14}$	55	196–236	JQ730686
	R: GCACGGGCAACCGAGGAA				
DSSR9	F: CGCACCACTCCCTGACAAT	$(AC)_{17}$	55	284–322	JQ730687
	R: GCCAGTAACATCCCCAACG				
DSSR11	F: CCCAAAGGAACAAGAACAA	$(AC)_{13}$	54	291–293	JQ730688
	R: TCCAAGGTGATTTTAGTGATAGT				
DSSR12	F: ACGCAACCTTGAAAGCGAGTA	$(TG)_6(AG)_{13}$	54	140–152	JQ730689
	R: GCATCATCATTCCGCCTGTC				
DSSR13	F: AAGGCAGAAAGGTAAGGAGC	$(AC)_{11}$	54	222–234	JQ730690
	R: GCGAGTCGGATTGTTAGTAGC				
DSSR15	F: GCAGGCTGGTAACAAGACAATG	$(AG)_{21}$	55	206–264	JQ730691
	R: TCCACTCCAACTGAATCAAATG				
DSSR17	F: TCAAAGCCACCTCTTCTCACC	$(AG)_{24}$	58	134–190	JQ730692
	R: CCCGTAACCATCCATTATCAG				
DSSR18	F: CCACCGCTCATCAGTGAAATC	$(TG)_{15}(AG)_{13}$	58	204–222	JQ738127
	R: ACACGGGGAGGACAAGCATAG				
DSSR20	F: AGAGGTCCTGGGTTGTTTT	$(AC)_{11}$	56	105–109	JQ730693
	R: GTCTCGGTAGGGTGTCGTG				
DSSR21	F: TCGCTCTGGCGGATGGAAAT	$(AC)_{28}$	56	178–196	JQ730694
	R: CGGAGACGGTGAAAGTGAAAGG				
DSSR22	F: GAAACAGGCACTAAAAGGCAT	$(AG)_{36}$	58	187–217	JQ730695
	R: CTCCACTATCTATTCTACCCAAAG				
DSSR23	F: AATCAAACCCGAACAAAATG	$(AG)_{14}$	58	116–154	JQ730696
	R: ACTGCTGCTTCACGCAAATC				
DSSR24	F: AAGGGCGAGGCAAAGCAAG	$(ATG)_7$	56	90–94	JQ730697
	R: AGATTTCGTGACAAAGGGAGC				
DSSR32	F: GATTAGACTGTTTGGTAGGGATTC	$(AG)_{23}$	54	286–322	JQ730698
	R: CTGGTTTTGCGAGATTTCAT				
DSSR34	F: ACCCTCCTCTTGGGCATCTT	$(AC)_{13}$	56	185–223	JQ730699
	R: ATCTTACTTCAACGGTGTCC				
DSSR35	F: CGTCGTTGCTGTCTGTTAT	$(AG)_{16}$	56	274–298	JQ730700
	R: GTTTTCCTCCCTTGTTTCT				
DSSR37	F: CGGAACACTAACTGAATGACCA	$(AG)_{10}$	56	244–260	JQ730701
	R: GAGGGCAGACGAAAACCAC				
DSSR39	F: AGAAAGACGAAACGAAATGA	$(AG)_{13}$	57	198–240	JQ730702
	R: GATGAGACCTGCCAAAACTA				
DSSR40	F: GGTGTTGTGTGACTTCTTGTG	$(AC)_{15}$	54	147–163	JQ730703
	R: TGTGTCTCTTGTCTTTGATTTG				
DSSR45	F: GAATCAAGAGAAGTTCAATCAGA	$(AC)_{17}$	55	182–198	JQ730704
	R: GTTTTCAGAAGAGCACAGTTT				
DSSR47	F: GACTGTCAGGTAGCCGAGA	$(AC)_{16}(AG)_{15}$	57	156–180	JQ730705
	R: TTACGACGACTAAACCATCA				
DSSR49	F: ACATAGAGAGGCTTACCCATA	$(AC)_{15}$	57	148–178	JQ730706
	R: AACTTCACCACCGACAGAT				
DSSR56	F: TCTCCCCGCATAAATACGC	$(AC)_{11}$	55	249–277	JQ730707
	R: GAAGCATCTCCTCGGTGACG				

Note: T_a = annealing temperature.

TABLE 2. Results of initial primer screening in two populations of *Miscanthus sacchariflorus*.

	pop-D40 (N = 22)			pop-D49 (N = 28)			Total (N = 50)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
DSSR2	10	0.955	0.823	8	0.893	0.756	13	0.920	0.866
DSSR9	8	0.682	0.818	8	0.893	0.796	12	0.800	0.892
DSSR11	2	0.182	0.169	2	0.250	0.223	2	0.220	0.198
DSSR12	2	0.227	0.384	3	0.179	0.314	3	0.200	0.349
DSSR13	4	1.000	0.594	4	1.000	0.605	4	1.000	0.594
DSSR15	6	0.636	0.807	6	0.857	0.625	10	0.760	0.807
DSSR17	8	0.864	0.783	13	0.964	0.853	17	0.920	0.898
DSSR18	7	0.864	0.765	5	0.464	0.531	8	0.640	0.739
DSSR20	2	1.000	0.512	2	0.893	0.503	2	0.940	0.503
DSSR21	5	0.591	0.626	5	0.536	0.495	6	0.560	0.562
DSSR22	6	0.818	0.781	6	0.250	0.513	9	0.500	0.788
DSSR23	10	0.636	0.854	12	1.000	0.826	18	0.840	0.883
DSSR24	2	1.000	0.512	2	1.000	0.509	2	1.000	0.505
DSSR32	8	0.545	0.626	6	0.821	0.650	11	0.700	0.797
DSSR34	6	0.500	0.719	7	0.321	0.641	8	0.400	0.817
DSSR35	10	0.364	0.844	6	0.857	0.752	12	0.640	0.825
DSSR37	3	0.409	0.443	4	0.607	0.576	5	0.520	0.557
DSSR39	5	0.773	0.772	6	0.714	0.567	8	0.740	0.764
DSSR40	4	0.955	0.621	5	0.964	0.621	7	0.960	0.629
DSSR45	3	0.409	0.519	5	0.964	0.712	5	0.720	0.657
DSSR47	9	0.636	0.816	8	0.964	0.712	10	0.820	0.859
DSSR49	2	0.636	0.444	6	0.179	0.265	6	0.380	0.371
DSSR56	8	0.591	0.547	5	0.714	0.545	9	0.660	0.567
Mean	5.65	0.664	0.643	5.83	0.708	0.591	8.13	0.689	0.671

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

CONCLUSIONS

The novel microsatellite loci developed here showed polymorphism in *M. sacchariflorus* populations. These markers will be used to gain a better understanding of various evolutionary questions including population genetic diversity and differentiation, population demography, and gene flow of *Miscanthus* species. They are also valuable tools in further molecular breeding and population genetic studies of *M. sacchariflorus* and its relatives.

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