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Development of microsatellite markers for *Miscanthus sinensis* (Poaceae) and cross-amplification in other related species[1]

Hai-Fei Zhou[2], Shan-Shan Li[2,3], and Song Ge[2,3,4]

[2]State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China; and [3]The Graduate University of the Chinese Academy of Sciences, Beijing 100039, China

- *Premise of the study*: We developed microsatellite loci for the biomass crop *Miscanthus sinensis* to investigate genetic diversity and population structure of *M. sinensis* and its closely related species.
- *Methods and Results*: Fourteen microsatellite loci were identified from an enriched genomic library of *M. sinensis* and tested in one *M. sinensis* population. The number of alleles per locus ranged from 1 to 15, with a mean of 7.0. The observed and expected heterozygosities varied from 0.318 to 0.864 and from 0.424 to 0.901, respectively. Of them, 12 primers could be applied to three other species in *Miscanthus* (*M. sacchariflorus, M. floridulus,* and *M. lutarioriparius*).
- *Conclusions*: These markers will be important for further analyzing population genetics and evolutionary history, as well as facilitating molecular breeding of *Miscanthus sinensis* and its related species.

Key words: cross-species amplification; microsatellites; *Miscanthus sinensis*; SSR.

Bioenergy contributes a relatively small proportion of the total energy currently used in the world, but the proportion is expected to grow in the near future (Sims et al., 2006). In recent decades, concerns about global warming have stimulated interests in using plant biomass for energy. *Miscanthus* ×*giganteus* Greef & Deuter is the most cultivated species in Europe with respect to biomass production. However, it is propagated vegetatively from an artificial hybrid between *M. sinensis* Anderss. and *M. sacchariflorus* (Maxim.) Hack and thus has extremely limited variability, which hampers its breeding and potential as a bioenergy crop (Hernández et al., 2001). As the wild parents of *M.* ×*giganteus*, *M. sinensis* and *M. sacchariflorus* are widely distributed in different habitats in Asia, and thus are the most important resources for broadening the genetic base of *M.* ×*giganteus* for breeding (Clifton-Brown et al., 2008). Cross-taxa amplification of maize microsatellite primers was attempted in *Miscanthus* species (Hernández et al., 2001), and nine microsatellite markers specific to *Miscanthus sinensis* have been developed (Hung et al., 2009). However, the number of markers for *Miscanthus* species is still limited, and some of them did not perform efficiently in our initial population screening. Here we report the development of 14 microsatellite markers for *M. sinensis* and apply them to other related species, including *M. sacchariflorus, M. floridulus* (Labill.) Warb., and *M. lutarioriparius* L. Liu ex Renvoize & S. L. Chen for molecular breeding and population genetic investigations. These loci provide useful markers for further studying population genetics and managing these species as potential biomass resources.

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[4]Author for correspondence: gesong@ibcas.ac.cn

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METHODS AND RESULTS

Leaf samples were collected from one population (20 to 22 individuals) each of four *Miscanthus* species and dried in silica gel immediately upon collection. Information on the populations sampled is provided in Appendix 1, and specimen vouchers were deposited at the Institute of Botany, Chinese Academy of Sciences. Total genomic DNA was extracted individually from dried leaves using a CTAB method (Doyle and Doyle, 1987). Small insert libraries enriched for microsatellite repeats were constructed from one individual of *M. sinensis* sampled from Gongji, Huadian City, Jilin Province, China (Appendix 1) following the protocol of Glenn and Schable (2005). The genomic DNA was digested (into ~500 bp small fragments) with a 4-base cutting restriction enzyme *Rsa*I (New England Biolabs, Ipswich, Massachusetts USA) and subsequently ligated to the SuperSNX linkers (SuperSNX24 Forward 5′-GTTTAAGGCCTAGCTAGCAGCAGAATC; SuperSNX24+4P Reverse 5′-GATTCTGCTAGCTAGGCCTTAAACAA). The digestion-ligation mixture was independently hybridized with 3′biotinylated oligo probes TG12/AG12/AAG8 and captured by magnetic streptavidin Dynabeads (Dynal Biotech, Oslo, Norway) for enrichment of microsatellite-containing 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 Corp., Madison, Wisconsin, USA) and transformed into competent cells of *E. coli.* Four hundred positive colonies were randomly selected and sequenced on a 3130XL DNA analyzer (Applied Biosystems, Foster City, California, USA), and 280 contained repeats. Of them, 98 sequences were selected for primer design with Primer Premier 5.0 (Premier Biosoft International, Silicon Valley, California, USA) and evaluated for PCR amplification using 22 individuals from the *M. sinensis* population (Appendix 1). Finally, 14 pairs of primers (Table 1) were chosen for genotyping because they showed single and clear bands.

PCR amplifications were performed in 15-μl volumes on GeneAmp PCR System 9700 thermocyclers (Applied Biosystems). Final concentrations for optimizing reactions were 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl2, 50 mM KCl, 0.1% BSA, 200 mM of each dNTP, 5% DMSO, 0.5 U ex*Taq* polymerase (TaKaRa Bio, Otsu, Shiga, Japan), 10 ng genomic DNA, 0.5 μM marker-specific reverse primer, 0.033 μM marker-specific M13-tailed forward

TABLE 1. Characteristics of 14 microsatellite primer pairs in *M. sinensis*.

Locus	Primer sequence (5′–3′)	Fragment length (bp)	Repeat Motif	Number of Alleles	H_O	H_E	Genbank Accession No.
MSSR4	F: TTCTGTGAGATTCTGGTATGCC R: CAACTTGCTTGGGACTGA	215	(AC)16(AAC)11	11	0.318	0.872	HQ283196
MSSR10	F: CCTGGGATTCTTTGATTTGAG R: GGATTTGGCTTTCGCTGTC	159	(AG)19	7	0.500	0.798	HQ283197
MSSR11	F: TTGAAGAGGGTAGCGGTTG R: TAGTTAGGGGCTGTTTGGA	305	(AG)11	7	0.636	0.599	HQ283195
MSSR14	F:ACTAAAGGCGAAAGCTAGGAGG R:CAGATGCTGGCTGTTGGTGATGT	155	(AC)18A9	5	0.5	0.424	HQ283198
MSSR17	F: CTATGATGATGGCAACG R: TCCAAAACAGTGAGGGT	200	(AT)7 (AC)19	1	/	/	HQ283199
MSSR18	F: TTTTCTGCCCACTACTGCTA R: TGTGATCTTCTATGCTTCCA	270	(AG)17	9	0.727	0.770	HQ283200
MSSR21	F: TATGGGTGAATGTTGGTTT R: GCCCGTTTGTGCGAGTGC	187	(AC)8A8	4	0.682	0.709	HQ283201
MSSR25	F: TGACAGGCACAGAAAGC R: CCAACCATCAAGCAGGAG	256	(AC)5(AG)28	15	0.773	0.901	HQ317215
MSSR30	F: GACCTTTCAGCCACCCTC R: AACGACTCCTGCTCCTATCA	221	(AGG)8	4	0.773	0.678	HQ283204
MSSR36	F: TAAGCCCAAACAAAGGAAAT R: CAAATGGCAATAGTGAGCAA	228	(AG)13	3	0.591	0.534	HQ283205
MSSR37	F: CAGATGCCATTACTGTAGCGA R: ACCACAACGAAACCAAAAC	243	(AC)5(AG)2(AC)11	6	0.864	0.669	HQ283206
MSSR38	F: GAGTGAGCAGTGGCAACG R: ATCTGGCTGGACAACCTTTT	248	(GAT)7	1	/	/	HQ283207
MSSR42	F:TGCCACGCCTTCTTCACCTATC R:GCATCCAGCCATCCACCCTC	180	(TG)8 (AG)14	13	0.773	0.844	HQ283202
MSSR70	F: GCACGCATGAGCCAAACTG R: TCGGTCGGTGCTTGTCTCG	280	(TC)4(TG)14(AG)19	12	0.636	0.754	HQ283202
Mean				7.0	0.648	0.713	

TABLE 2. Results of cross-species amplification in *Miscanthus*.

	Number of Alleles						
Locus	*M. sacchariflorus* (22)	*M. lutarioriparius* (20)	*M. floridulus* (20)	*M. sinensis* (22)	Total	Size range (bp)	PIC
MSSR4	9	18	13	11	32	208–276	0.942
MSSR10	8	6	13	7	24	110–271	0.870
MSSR11	7	15	14	7	21	314–358	0.886
MSSR14	3	3	10	5	12	119–160	0.693
MSSR17	4	3	4	1	5	170–186	0.370
MSSR18	8	13	6	9	19	262–307	0.882
MSSR21	4	5	11	4	15	173–212	0.854
MSSR25	2	2	14	15	25	204–280	0.824
MSSR30	3	2	6	4	7	209–225	0.606
MSSR36	3	4	12	3	13	235–264	0.724
MSSR37	4	3	na	6	10	217–269	0.733
MSSR38	1	1	1	1	3	261–268	0.554
MSSR42	3	Na	na	13	14	152–200	0.784
MSSR70	5	11	12	12	26	278–428	0.880
Mean	4.6	6.6	9.7	7.0	16.1		0.757

PIC, Polymorphic information content; na, amplification failure. Information on population samples were provided in Appendix 1. Figure in parentheses following species names indicates the number of individuals sampled in the species.

primer and 0.5 μm HEX-, TAMRA-, or FAM-labeled M13 primer (adapted from Schuelke, 2000). A touch-down cycling profile was as follows: initial denaturation of 95°C for 3 min, followed by 30 cycles of 95°C for 20 s; 65°C (–0.3 descending each cycle) for 20 s; and 72°C for 30 s, and a final extension step at 72°C for 10 min. PCR products were analyzed on a 3730XL sequencer and genotypes were scored using the GeneMapper v4.0 software (Applied Biosystems). Sequences of microsatellite loci were deposited in GenBank (accession nos. HQ283195-HQ283207,HQ317215). The number of alleles per locus, fragment length, and the observed and expected heterozygosity (H_O and H_E) were calculated with Arlequin 3.1 (Excoffier et al., 2005). Polymorphic information content (PIC) for each locus was estimated with Cervus 3.0 (Marshall et al., 1998). Cross-amplification was conducted on one population of each three *Miscanthus* species (Table 2) following the same protocol described above.

A total of 14 microsatellite loci were isolated from *M. sinensis* (Table 1). Of them, MSSR17 and MSSR38 were monomorphic in *M. sinensis* but polymorphic when the other three species were taken into account. MSSR25 showed highest polymorphism with 15 alleles. The observed heterozygosity ranged from 0.318 (MSSR4) to 0.864 (MSSR37) (mean = 0.648), while the expected heterozygosity varied from 0.424 (MSSR14) to 0.901(MSSR25) (mean = 0.712). The results of cross-species amplification were shown in Table 2. Twelve out of 14 loci were amplified successfully in *M. sacchariflorus*, *M. floridulus*, and *M. lutarioriparius*. MSSR42 failed to amplify in *M. floridulus* and *M. lutarioriparius*,

while MSSR37 failed in *M. floridulus.* The mean number of alleles per species was 4.6, 6.6, 9.7, and 7.0 for *M. sacchariflorus*, *M. lutarioriparius*, *M. floridulus*, and *M. sinensis* populations, respectively (Table 2). The total number of alleles ranged from 3 (MSSR38) to 32 (MSSR4) (average 16.1). PIC value ranged from 0.370 (MSSR17) to 0.942 (MSSR4) with an average of 0.757. These microsatellite markers were highly polymorphic in all four species.

CONCLUSIONS

The microsatellite primer pairs described here are useful for further molecular breeding and genetic studies of *M. sinensis*. Interspecific amplification of these markers also reveals the potential for their suitability in the closely related species. The application of these microsatellite loci may provide a tool for understanding the population demography, population structure, gene flow, and mating system of *Miscanthus* species.

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APPENDIX 1. Information on the populations of four *Miscanthus* species sampled in this study.

Species	*Locality*	GPS coordinate	Accession No.
M. sinensis	Gongji, Huadian City, Jilin Province	42°59′41″N, 126°54′42″E	M03
M. sacchariflorus	Dangcha, Hengshan County, Shaanxi Province	37°59′58″N, 109°47′19″E	D22
M. floridulus	Huaping Natural Reserve, Guangxi Province	25°32′21″N, 109°52′34″E	M11
M. lutarioriparius	Shahu, Xiantao City, Hubei Province	30°09′6″N, 113°42′25″E.	N08