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Isolation and characterization of novel microsatellite markers for *Avena sativa* (Poaceae) (oat)[1]

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- *Premise of the study:* A new set of microsatellite primers was developed for *Avena sativa* and characterized to assess the level of genetic diversity among cultivars and wild genotypes.
- *Methods and Results:* Using an enrichment genomic library, 14 simple sequence repeat markers were identified. The loci of these markers were characterized and found to be polymorphic in size among 48 genotypes of oat from diverse geographical locations. The number of alleles per locus ranged from two to eight, while the observed heterozygosity ranged from 0.031 to 0.75.
- *Conclusions:* These newly identified microsatellite markers will facilitate genetic diversity studies, fingerprinting, and genetic mapping of oat. Moreover, these new primers for *A. sativa* will aid future studies of polyploidy and hybridization in other species in this genus.

Key words: *Avena sativa*; enriched library; microsatellite; oat; Poaceae.

Oat (*Avena sativa* L.) is a cereal crop of global importance used for food, feed, and forage (Tinker et al., 2009). Differing from other cereal grains such as wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.), oat is rich in the antioxidants α-tocotrienol, α-tocopherol, and avenanthramides, as well as total dietary fiber including the soluble fiber β-glucan (Oliver et al., 2010). The development of specific molecular markers such as microsatellite markers can be very useful for cultivar fingerprinting, assessing genetic diversity of germplasm, and aiding in molecular breeding to improve crop characteristics. Although oat is an important grain and forage crop in many parts of the world, few modern genetic studies using microsatellite markers have been reported, especially when compared with other crops. This is partly due to the low availability of reliable and easy-to-use molecular markers (Wight et al., 2010). In this study, we report the isolation and characterization of 14 novel polymorphic microsatellite loci generated from the genome of *A. sativa*. These new markers were evaluated using accessions obtained from different cultivation areas.

METHODS AND RESULTS

Genomic DNA of one individual of *A. sativa* cv. Xiayoumai was extracted from fresh leaves following the procedure of Doyle and Doyle (1990), and this was used to construct a microsatellite-enriched library according to the method of Wu et al. (2012). Total genomic DNA was digested with *Msp*I and *Eco*RI and ligated to the corresponding adapters. Combining a simple sequence repeat (SSR) motif-containing anchored primer and an adapter primer, the SSR-containing sequences were selectively amplified and the amplicons were then recombined by redigestion and ligation for the next round of amplification. Then, taking only the SSR motif-containing sequence as the primer, recombinants that contained SSRs were selectively amplified. Microsatellite-enriched DNA fragments were ligated into the plasmid vector pCR2.1 (Invitrogen, Carlsbad, California, USA) and introduced into *Escherichia coli* TOP 10 (Tiangen, Beijing, China) cells. These bacteria were cultured on Luria broth (LB) agar plates containing kanamycin (Tiangen). Colonies were picked and transferred to the wells of 96-well plates containing LB supplemented with kanamycin, and then grown overnight. Positive colonies were screened by PCR to confirm the presence of inserts. A total of 186 clones were sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) and electrophoresed on a 3730xl DNA sequencer (Applied Biosystems).

Using the criterion of at least seven repeat units in the target sequence, primers were designed from 38 of these sequences using Primer3 (Rozen and Skaletsky, 2000). The criteria for primer design were: a GC content between 40% and 60% and approximately 20 bp in length and an annealing temperature of approximately 60°C. Each primer pair was designed to amplify a fragment ranging between 100 and 300 bp in length.

The abilities of the 38 selected primer pairs to generate reliable and polymorphic PCR products were tested using the genomic DNA of 48 *Avena* genotypes available in the germplasm collection in the National Genebank of China (Appendix 1); relevant feature information for the microsatellite sequences are provided in Appendix S1. For microsatellite assays, genomic DNA of each genotype was extracted using the cetyltrimethylammonium bromide (CTAB) method mentioned above. PCR amplifications were performed using approximately 50 ng template DNA in 20 μL reaction mixtures containing 1× PCR buffer (Tiangen), 1.5 mM $MgCl_2$, 0.75 μM of each primer, 0.2 mM of each dNTP, and 0.5 U of *Taq* DNA polymerase (Tiangen). The following PCR program was used: 94°C for 5 min; then 35 cycles at 94°C for 30 s, annealing at optimal temperature (see Table 1) for 30 s, and 72°C for 60 s; and a final elongation step at 72°C for 7 min. The PCR products were separated on 6% denaturing polyacrylamide gels and visualized by silver nitrate staining. The observed heterozygosity (H_o) was calculated using PowerMarker version 3.25 software (Liu and Muse, 2005) (Table 2). A BLAST search was performed against previously published *A. sativa* nucleotide sequences, but these newly identified loci showed no homology with any other available sequences.

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Table 1. Characteristics of the 14 microsatellite primers developed for *Avena sativa*. For each primer pair the following data are given: forward and reverse sequence, repeat type, size of the original fragment, optimal annealing temperature when run individually, and the GenBank accession number. All values were obtained using 48 samples of Chinese oats located in Inner Mongolia, Hebei, Shanxi, and Qinghai provinces.

Primer name	Primer sequence (5′–3′)	Repeat motif	Size (bp)	T_a (°C)	GenBank accession no.
AM01	F: TCTGGAAACCCTAGTCCCACTT R: CCCTAAACCCATAGCCACTGAT	$(AG)_{10}$	282	57	JN558763
AM02	F: AGTAGGACTAGATGGCAGAGCG R: TTGTGCTGGCCCAACCCTGATT	$(AG)_{28}$	281	58	JN558764
AM03	F: GTACGTGTATCCCCGTGCTCTA R: GGTTGCATCCTGAACCTATGGC	$(AG)_{39}$	255	55	JN558765
AM04	F: GGATAGGAGGCCATCGGTTTCT R: GGCGATAACTTCCTACCCACTC	$(AG)_{37}$	189	57	JN558766
AM05	F: TCCAATACATCAGCACCAACCT R: TCGATTGCGGTGTCTGCTGAAC	$(AG)_{28}$	168	57	JN558767
AM06	F: TTGCTTGACTTCAATGTCTTGT R: AATTACTATGCCACTGGCGTCT	$(AG)_{19}$	253	58	JN558768
AM07	F: GAAGAGCCCCACGAGAAGACTG R: GGAGGCAGAGCACCGCAGGAGG	$(AG)_{14}$	132	55	JN558769
AM08	F: AGAAGGCTGTCGCTGCTTGACT R: ATCTAGGGAAAGGGCATGGTAT	$(AG)_{14}$	195	55	JN558770
AM09	F: GCGGTAACAAACAGCGACGAGA R: CGTTTACTAATAATAGGCCGTG	$(GA)_{18}$	127	58	JN558771
AM10	F: ACTGCTCCCTCCAACCTCTTCG R: GCGAGTTCGTTGGGGTTGGTCT	$(AG)_{17}$	285	60	JN558772
AM11	F: CTCGACTGGAGTACGAGATGGA R: TCCAGGCTAACTAAAACCAACG	$(AG)_{11}$	188	56	JN558773
AM12	F: GTTCCCAGCCGTCGATTCACAA R: GCGAACTCTTGAAGCAAGCAGC	$(AG)_{13}$	214	58	JN558774
AM13	F: CAAGCAGCAAACAGTCCCCTAT R: ACAGCTCCAACCATTTGGACAC	$(AG)_{18}$	254	58	JN558775
AM14	F: ATTGATTAACAGAATGGCGTTG R: CTACATCTCGATCTCGATCTGC	$(AG)_{8}$	108	57	JN558776

Note: T_a = annealing temperature.

CONCLUSIONS

Although there are reports of oat SSR markers from as early as 2000 (Li et al., 2000), they are difficult to develop and fewer markers are available than for other cereal crops. To date, only 348 oat SSR markers have been published, far fewer than for other graminaceous cereal crops such as wheat and barley, and many of these have turned out to be biallelic and are not universally polymorphic (Wight et al., 2010). Moreover, there are no reports of SSR markers developed specifically for *A. nuda*. In this study, 14 out of 38 novel microsatellite marker loci for *A. sativa* were identified and found to be polymorphic in naked oat. Thus, these primers are a new set of microsatellite markers for the genus *Avena* that will be useful for studies of genetic diversity and the conservation of genetic resources, but could also find application in breeding assistance and cultivar identification.

Table 2. Results of initial primer screening in populations of *Avena sativa* and *A. nuda*.

	A. sativa (N = 32)[a]			*A. nuda* (N = 16)[a]		
Locus	A	H_e	H_o	A	H_e	H_o
AM01	3	0.23	0.13	3	0.34	0.13
AM02	4	0.28	0.16	3	0.28	0.06
AM03	8	0.79	0.09	8	0.78	0.19
AM04	7	0.80	0.13	5	0.68	0.06
AM05	5	0.80	0.13	5	0.78	0.06
AM06	3	0.66	0.03	3	0.41	0.00
AM07	2	0.09	0.03	2	0.06	0.06
AM08	2	0.15	0.03	2	0.06	0.06
AM09	8	0.62	0.19	7	0.76	0.38
AM10	5	0.57	0.75	4	0.38	0.44
AM11	3	0.62	0.13	4	0.60	0.13
AM12	6	0.72	0.03	6	0.75	0.13
AM13	3	0.57	0.06	5	0.58	0.13
AM14	7	0.81	0.22	8	0.85	0.25

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity.
[a]The sample size for each population is given in parentheses.

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Appendix 1. Taxa used in this study were obtained from the National Genebank of China, Institute of Crop Sciences (ICS), Chinese Academy of Agricultural Sciences, Beijing, China.

Avena sativa: ZY000732, ZY000740, ZY000745, ZY000752, ZY000760, ZY000764, ZY000766, ZY000770, ZY000783, ZY000788, ZY000797, ZY000798, ZY000809, ZY000810, ZY000834, ZY000849, ZY000876, ZY000893, ZY000909, ZY000911, ZY000913, ZY001328, ZY001340, ZY001358, ZY001463, ZY002507, ZY002508, ZY002516, ZY002551, ZY002552, ZY002561, ZY002593.

Avena nuda: ZY000001, ZY000012, ZY000064, ZY000078, ZY000212, ZY000252, ZY000254, ZY000289, ZY000492, ZY000519, ZY000572, ZY000654, ZY001505, ZY002131, ZY002149, ZY003265.