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C.D. Li · B.G. Rossnagel · G.J. Scoles

The development of oat microsatellite markers and their use in identifying relationships among *Avena* species and oat cultivars

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Abstract Microsatellites have many desirable marker properties. There has been no report of the development and utilization of microsatellite markers in oat. The objectives of the present study were to construct oat microsatellite-enriched libraries, to isolate microsatellite sequences and evaluate their level of polymorphism in *Avena* species and oat cultivars. One hundred clones were isolated and sequenced from three oat microsatellite-libraries enriched for either $(AC/TG)_n$, $(AG/TC)_n$ or $(AAG/TTC)_n$ repeats. Seventy eight clones contained microsatellites. A database search showed that 42% of the microsatellite flanking sequences shared significant homology with various repetitive elements. Alu and retrotransposon sequences were the two largest groups associated with the microsatellites. Forty four primer sets were used to amplify the DNA from 12 *Avena* species and 20 *Avena sativa* cultivars. Sixty two percent of the primers revealed polymorphism among the *Avena* species, but only 36% among the cultivars. In the cultivars, the microsatellites associated with repetitive elements were less polymorphic than those not associated with repetitive elements. Only 25% of the microsatellites associated with repetitive elements were polymorphic, while 46% of the microsatellites not associated with repetitive elements showed polymorphism in the cultivars. An average of four alleles with a polymorphism information content (PIC) of 0.57 per primer set was detected among the *Avena* species, and 3.8 alleles with a PIC of 0.55 among the cultivars. In addition, 54 barley microsatellite primers were tested in *Avena* species and 26% of the primers amplified microsatellites from oat. Using microsatellite polymorphisms, dendrograms were constructed showing phylogenetic relationships among *Avena* species and genetic relationships among oat cultivars.

Keywords Enriched-library · SSR · Repetitive elements · Allelic diversity · Evolution

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C.D. Li · B.G. Rossnagel · G.J. Scoles (✉)
Department of Plant Sciences and Crop Development Centre,
University of Saskatchewan, 51 Campus Drive,
Saskatoon, SK S7N 5A8, Canada
e-mail: graham.scoles@usask.ca
Tel.: +1-306-966 5857, Fax: +1-306-966 5015

Introduction

Microsatellites or simple sequence repeats occur ubiquitously and abundantly in eukaryotic genomes. As molecular markers, they combine many desirable marker properties including high levels of polymorphism and information content, an unambiguous designation of alleles, even dispersal, selective neutrality, high reproducibility, co-dominance, and rapid and simple genotyping assays. Microsatellites have become the molecular markers of choice for a wide range of applications in genetic mapping and genome analysis (Liu et al. 1996; Chen et al. 1997; Ramsay et al. 1999), genotype identification and variety protection (Smith and Helentjaris 1996), seed-purity evaluation and germplasm conservation (Brown et al. 1996; Hahn and Grifo 1996), diversity studies (Xiao et al. 1996), paternity determination and pedigree analysis (van de Ven and McNicol 1996; Ayres et al. 1997; Bowers et al. 1999), gene and quantitative trait locus analysis (Koh et al. 1996; Blair and McCouch 1997), and marker-assisted breeding (Ayres et al. 1997; Weising et al. 1998). Considerable efforts have been made to develop microsatellite markers for important crops including rice (Chen et al. 1997), wheat (Devos et al. 1995; Roder et al. 1995), barley (Liu et al. 1996), maize (Senior et al. 1998), sorghum (Brown et al. 1996), soybean (Akkaya et al. 1992), beans (Yu et al. 1999), *Brassica* species (Szewc-McFadden et al. 1996), alfalfa (Diwan et al. 1997), sunflower (Brunel 1994), and tomato (Smulders et al. 1997).

The development of microsatellite markers in plant species has included the utilization of the sequences in public databases, and the construction and screening of un-enriched or enriched libraries. Wang et al. (1994) searched for all possible microsatellites, ranging from

mononucleotide to tetranucleotide repeats, from 54 plant species in EMBL and GenBank databases. A total of 130 microsatellites were found, but only a few were from *Avena* DNA sequences. Obviously there are not enough sequences available in current databases to develop microsatellite markers for oat.

Oat belongs to the genus *Avena*, and includes diploid (2n=14), tetraploid (2n=28) and hexaploid (2n=42) species. Most cultivated oats belong to the hexaploid species *Avena sativa*. The development and application of molecular markers in oat has been relatively slow compared with other crops. Restriction fragment length polymorphism (RFLP) and random amplified polymophic DNA (RAPD) are still the major markers for oat genomic mapping and marker assisted-breeding (O'Donoughue et al. 1995; Ronald et al. 1997; Kianian et al. 1999). Recently, amplified fragment length polymorphism (AFLP) was used to identify markers linked to BYDV tolerance in oats (Jin et al. 1999). There is still no report on the use of microsatellite markers in oat.

The present study included: (1) the construction and characterization of microsatellite-enriched libraries with $(GAA/CTT)_n$, $(AG/CT)_n$ and $(AC/TG)_n$ repeats; (2) the characterization of relationships among *Avena* species and cultivars using microsatellite markers; and (3) the testing of barley microsatellite primers for amplification from the *Avena* genome.

Materials and methods

Plant materials

Seed from 12 wild oat species was provided by Dr. H. Bockelman, USDA-ARS, National Small Grains Collection, Aberdeen, Idaho, USA, and by Dr. J. Chong, Agriculture & Agri-Food Canada (AAFC), Cereal Research Centre, Winnipeg, Manitoba, Canada (Table 1). Seed for the 20 oat cultivars were obtained from the Crop Development Centre (CDC), University of Saskatchewan (Table 1). For constructing the microsatellite-enriched library, DNA was extracted from *Avena sativa* cv Ogle using the CTAB method (Saghai Maroof et al. 1984). For microsatellite assays, DNA from each cultivar or species was extracted from young leaves using a modified micro-CTAB method (Procunier et al. 1990).

Microsatellite-enriched library construction

Libraries were enriched for microsatellites using 3′-biotinylated oligos bound to streptavidin-coated paramagnetic beads. The protocol was modified based on Jarret et al. (1997) and Fischer and Bachmann (1998). Oat genomic DNA (*A. sativa* cv Ogle) was digested with various blunt-end restriction enzymes. The enzyme combination of *Dra*I+*Alu*I+*Rsa*I was finally chosen to digest the DNA for construction of microsatellite-enriched libraries, since this enzyme combination digested most of the oat genomic DNA to 200–900 bp fragments. Five micrograms of genomic DNA was digested with the restriction combination. DNA fragments from 300 to 900 bp were excised and purified from an agarose gel using a QIAquick gel extraction kit (QIAGEN). The resulting DNA was ligated to a synthesized blunt-end linker (35 base pairs) (provided

Table 1 List of accessions from the genus *Avena* and oat cultivars included in study

Sample	Species or cultivar name	Accession	Genome	Sources
1	Brawn	–	AACCDD	Wisconsin
2	Belle	–	AACCDD	Wisconsin
3	Triple Crown	–	AACCDD	Sweden
4	Calibre	–	AACCDD	Saskatoon
5	AC Preakness	–	AACCDD	Winnipeg
6	CDC Pacer	–	AACCDD	Saskatoon
7	Jim	–	AACCDD	Minnesota
8	Gem	–	AACCDD	Wisconsin
9	AC Juniper	–	AACCDD	Lacombe
10	AC Assiniboia	–	AACCDD	Winnipeg
11	AC Stewart	–	AACCDD	Ottawa
12	86Ab4582	–	AACCDD	Idaho
13	AC Mustang	–	AACCDD	Lacombe
14	Novosadski 4126	–	AACCDD	Russia
15	P8640A-1–31–5-4	–	AACCDD	Iowa
16	GA921019	–	AACCDD	Guelph
17	Ripon	–	AACCDD	Czech Republic
18	Jerry	–	AACCDD	North Dakota
19	CDC Boyer	–	AACCDD	Saskatoon
20	Q287178	–	AACCDD	Australia
21	*A. longiglumis*	CIav 9071	A1A1	USDA-ARS
22	*A. canariensis*	IB 105	AcAc	AAFC
23	*A. strigosa*	CIav 2921	AsAs	USDA-ARS
24	*A. clauda*	CAV 5566	CpCp	AAFC
25	*A. abyssinica*	PI 58728	AABB	USDA-ARS
26	*A. barbata*	PI268214	AABB	USDA-ARS
27	*A. maroccana*	CIav 8331	AACC	USDA-ARS
28	*A. murphyi*	CAV2832	AACC	AAFC
29	*A. sterilis*	CIav 8336	AACCDD	USDA-ARS
30	*A. fatua*	UM33	AACCDD	CDC
31	*A. byzantina*	Kanota	AACCDD	CDC
32	*A. sativa*	Ogle	AACCDD	CDC

by Dr. R. Jarret, USDA-ARS, Albany, Calif.). The ligation mixture was denatured at 98°C for 5 min and then hybridized with 20 pmol of 3′-biotinylated microsatellite oligos [$(AC)_{12}$, $(AG)_{12}$ or $(GAA)_8$] in 6× SSC for 20 min at 60°C (for a GAA repeat) or 70°C (for AC and AG repeats). Hybridizing molecules were captured with 30 μg of Dynabeads M-289 (DYNAL). Non-specific-bound DNA was washed off using 2× SSC, 0.1% SDS for 10 min at room temperature (twice) and 1× SSC for 10 min at 60°C or 70°C. The enriched DNA fragments were eluted from the Dynabeads with 0.1 N NaOH and amplified using PCR for 30 cycles of 94°C for 3 min, 60°C for 30 s and 72°C for 3 min with 10 s added to the extension time in each cycle. The PCR products were cloned into the vector pCR2.1 (Invitrogen).

Library screening

The enriched library was screened by in situ colony hybridization (Sambrook et al. 1989). The synthetic oligos $(AC)_{12}$, $(AG)_{12}$ or $(GAA)_8$ were 5′end-labelled with (α-^{32}P)ATP and used as probes to screen the library. The filters were hybridized in 6× SSPE, 1% SDS at 55°C for 4 h; washed twice in 2× SSPE, 0.1% SDS at room temperature; washed once in 1× SSPE, 0.1% SDS at 55°C; and then exposed to film for autoradiography.

Pre-screening of microsatellite-containing clones

To avoid sequencing clones of limited value, a pre-screening step was introduced to eliminate clones containing a microsatellite repeat too close to one of the cloning sites. The procedure was based on three primer combinations: (1) an M13 forward primer and a microsatellite internal primer; (2) an M13 reverse primer and a microsatellite internal primer; and (3) M13 forward and M13 reverse primers. The bacterial clones which hybridized with the microsatellite oligos were picked up using toothpicks and rinsed in a 50-μl PCR reaction containing 1× PCR buffer (GIBCO), 1.5 mM $MgCl_2$, 200 μM of DNTP, 10 pmol of each primer and 1 unit of *Taq* polymerase. The PCR was performed at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. The PCR products were separated on 1.5% agarose gel. A clone was not investigated further if the PCR product from any of these three reactions was less than 150 bp.

Sequencing

Based on the pre-screening results, plasmids were purified using a Miniprep kit (QIAGEN) and sequenced using an Applied Biosystems 3700 by the DNA sequencing laboratory at the Plant Biotechnology Institute, Saskatoon.

Barley microsatellite primers

Fifty four barley microsatellite primers were provided by Dr. P. Langridge, The University of Adelaide, Australia. The primer names, sequences and repeat types were published by Liu et al. (1996).

Primer design and evaluation

PCR primers for each microsatellite were designed using the “PRIMERSELECT” software from Lasergene. All default parameters were used to select primer pairs which flanked the microsatellite and would amplify a fragment of 100 to 300 bp. The Tm of the primers was between 40°C and 55°C [Tm was calculated as: Tm=59.9+41(%GC) – (675/Primer length)] in a 0.05 M salt solution. PCR reactions were carried out in a Thermolyne Amplitron II thermocycler. Each 25-μl reaction contained 50 ng of genomic DNA, 1× reaction buffer (GIBCO), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 10 pmol of each primer and 1 unit of *Taq* polymerase. Depending on the Tm of the primers used, amplification was performed employing one of the following three “Touchdown” PCR profiles (Don et al. 1991): (1) a PCR profile consisting of 18 cycles of 94°C for 1-min denaturing, and 72°C for 1-min extension. Annealing temperatures (30 s) were progressively decreased by 0.5 degree every cycle from 64°C to 55°C. The PCR reaction continued for 30 additional cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The reaction ended with a 10-min extension at 72°C; (2) similar PCR conditions to those in (1) except that the annealing temperature was decreased from 67°C to 58°C over 18 cycles, the reaction was then continued for 20 additional cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min; (3) similar PCR conditions to those in (1) except that the annealing temperature was decreased from 62°C to 53°C over 18 cycles and this was continued for 20 additional cycles. The third program was mainly used to run the barley microsatellite primers. PCR products were separated on a sequencing gel containing 6% polyacrylamide, 7 M urea and 1× TBE at 85 W constant power for 3 h (BioRad sequencing system). The gel was fixed, stained and dried using a DNA silver-staining kit (Promega).

Data analysis

The polymorphism information content (PIC) of each microsatellite was determined as described by Weir (1996). $PIC=1-\Sigma P_i^2$, where P_i is the frequency of the ith allele in the examined cultivars or species. Genetic similarities were estimated using Jaccard’s coefficient (Sokal and Sneath 1963). The 12 *Avena* species or the 20 oat cultivars were clustered based on the matrix of genetic similarities using the Unweighted Pair Group Method Using Arithmetic Average (UPGMA) clustering algorithm (NTSYSpc, Version 2.0). Flanking sequences of microsatellites were aligned with the DNA sequences in EMBL using the Basic Local Alignment Search Tool (Altschul et al. 1990). The default parameter settings were used and only the sequence showing the highest score was recorded.

Results

Isolation of microsatellite clones from the enriched libraries

Using either 5′end-labelled $(AC)_{12}$, $(AG)_{12}$ or $(GAA)_8$ as probes, microsatellite-enriched (AC/TG)*n*, (AG/TC)*n* and (GAA/CTT)*n* libraries were screened by colony hybridization. Seventy six percent (AC/TG repeat), 83% (AG/TC repeat) and 92% (GAA/TCC repeat) of clones showed positive hybridization with their respective probe (data not shown). After pre-screening by PCR with a microsatellite oligonucleotide as a primer paired with an M13 forward or reverse primer, plasmids from positive clones were isolated for sequencing (Table 2).

Table 2 Number of sequenced clones, microsatellite-containing sequences and primers designed from each microsatellite-enriched library

Library	Clones sequenced	Microsatellite clones	Duplicated clones	Primers designed
(AC/TG)*n*	40	29	5	23
(AG/TC)*n*	30	22	2	18
(AAG/TTC)*n*	30	27	2	20
Total	100	78	9	61

Table 3 Oat microsatellite clones with homology to repetitive DNA sequences in accessions in the EMBL database

Clone	Accession	Known repeat	Identity (%, bp)
AM4	ASAC397	3′-Alu	70, 51
AM5	AC005545	5′-Alu	72, 47
AM6	HSAD1502	5′-Alu	76, 43
AM7	HS147M19	5′-Alu	75, 62
AM15	AC004726	3′-Alu	75, 45
AM18	HSA147M19	5′-Alu	69, 52
AM27	HSAC2316	5′-Alu	65, 60
AM37	AC005279	3′-Alu	93, 18
AM47	AC002454	3′-Alu	75, 48
AM51	HS714137	3′-Alu	68, 58
AM56	AC004891	5′-Alu	75, 52
AM62	HSAC2382	5′-Alu	77, 44
AM23	HVE1321IR	Retrotransposon	77, 45
AM29	AF090446	Retrotransposon	69, 62
AM40	PVA005726	Retrotransposon	73, 61
AM44	AF090446	Retrotransposon	76, 43
AM45	AF078801	Retrotransposon	66, 87
AM57	AF031569	Retrotransposon	93, 32
AM63	HSDS12	Retrotransposon	89, 46
AM64	TAWIS21AI	Retrotransposon	70, 79
AM14	AF102233	Mini-Tn5	71, 56
AM24	AC004986	L2 repeat	69, 59
AM28	AC003976	L1 repeat	73, 49
AM25	HSDPRA26	Satellite DNA	61, 88
AM48	HS326L13	L1 repeat	72, 55
AM54	HSAC404	5′-MIR	67, 71
AM58	AC005831	5′-MIR	75, 56
AM59	ASA005500	Repetitive DNA	87, 31
AM65	AC004804	L1 repeat	72, 54

Seventy eight microsatellite-containing clones were identified. Comparison of sequences showed that nine clones were duplicated, and eight clones were to found to have no primer sequence at one or both ends. Microsatellite-containing clones accounted for 72%, 73% and 90% of the sequenced AC/TG, AG/TC and AAG/TTC clones, respectively. By combining Southern hybridization and sequencing results, the enriched libraries were estimated to contain 55%, 60% and 83% microsatellite clones for the AC/TG, AG/TC and GAA/CTT repeats, respectively. In addition, four minisatellite sequences and one satellite sequence were identified from the 22 non-microsatellite clones (data not shown).

Sequence analysis of microsatellite-containing clones

The flanking sequences of microsatellites were used to search the EMBL database. Fourteen sequences (20%) were unique and did not share homology with any known sequences in the database. Twenty nine (42%) of the microsatellite flanking sequences showed significant homology with various repetitive elements, at least at one end. These clones are listed in Table 3. Alu-like sequences were the most common repetitive element associated with microsatellites (12 clones). Retrotransposon-like sequences were the second largest group (eight clones).

Another group of microsatellite clones were not associated with any repetitive elements, but had in common a 22-bp highly conserved sequence (more than 90% homology) at one end of the microsatellite. Ten clones were found to possess this sequence. This included AC/TG, AG/TC and GAA/TCC repeats. This microsatellite group has also been found in the microsatellite-containing clones of cowpea and yam (Li and Scoles, unpublished). The significance of the conserved sequence to the evolution of microsatellites is discussed elsewhere (Li et al., in preparation).

Designing PCR primers to amplify microsatellites from *Avena*

Using "PRIMERSELECT" software and its default parameter settings, 61 primer sets were designed from the 78 microsatellite clones. The primer sequence, repeat types, length of the amplified fragment and melting temperatures are listed in Table 4.

Microsatellite polymorphism in *Avena* species and cultivars

The first 44 primer sets in Table 4 were chosen to amplify DNA from *A. sativa cv* Ogle (the cultivar used to construct microsatellite-enriched libraries) using three Touchdown PCR profiles: (1) Touchdown 64°C to 55°C; (2) Touchdown 67°C to 58°C; and (3) Touchdown 62°C to 53°C. Under the most-stringent PCR conditions, 31 primer sets amplified a single band with a size similar to that predicted from the cloned sequences and 11 primer sets produced multiple bands, while two primer sets failed to amplify DNA. These primer sets were further used to amplify DNA from 12 *Avena* species and 20 hexaploid cultivars (Table 1) under the most-stringent PCR conditions (Tables 5 and 6).

Twenty six (62%) primers showed clear polymorphism among the 12 *Avena* species. Four other primer sets could not be scored because of their complicated "stutter" band pattern on polyacrylamide gels, although they were polymorphic. The remainder were not polymorphic. Most primer sets amplified a weak but readable band from some of the diploid and tetraploid species. A few primer sets failed to amplify DNA from one or more of the diploid and tetraploid species. These cases were treated as missing data rather than as new alleles. The allele number and polymorphism information content of each primer set are listed in Table 5. Two to eight different alleles were observed among the 12 *Avena* species for different primers with an average of four alleles per primer set. The PIC varied from 0.28 to 0.79 with an average of 0.57.

Microsatellite polymorphism was relatively low among the *A. sativa* cultivars, with only 16 primer sets (36%) showing polymorphism. Two of the primer sets (AM1 and AM30) identified two bands that each showed

Table 4 Microsatellite primer sequences, repeat types, amplified fragment length and average melting temperatures

Primer name	Primer sequence	Repeat type	Size bp	Tm °C
AM1	5′GGA TCC TCC ACG CTG TTG A 5′CTC ATC CGT ATG GGC TTT A	$(AG)_{21}(CAGAG)_6$	204	46
AM2	5′TGA ATT CGT GGC ATA GTC ACA AGA 5′AAG GAG GGC ATA GGG AGG TAT TT	$(AG)_{24}$	144	49
AM3	5′CTG GTC ATC CTC GCC GTT CA 5′CAT TTA GCC AGG TTG CCA GGT C	$(AG)_{35}$	280	51
AM4	5′GGT AAG GTT TCG AAG AGC AAA G 5′GGG CTA TAT CCA TCC CTC AC	$(AG)_{34}$	166	48
AM5	5′TTG TCA GCG AAA TAA GCA GAG A 5′GAA TTC GTG ACC AGC AAC AG	$(AG)_{27}$	172	46
AM6	5′AAT GAA GAA ACG GGT GAG GAA GTG 5′CCA GCC CAG TAG TTA GCC CAT CT	$(AG)_{20}$	209	52
AM7	5′GTG AGC GCC GAA TAC ATA 5′TTG GCT AGC TGC TTG AAA CT	$(AG)_{21}$	156	48
AM8	5′CAA GGC ATG GAA AGA AGT AAG AT 5′TCG AAG CAA CAA ATG GTC ACA C	$(AG)_{15}$	254	47
AM9	5′CAA AGC ATT GGG CCC TTG T 5′GGC TTT GGG ACC TCC TTT CC	$(AG)_{19}$	217	48
AM10	5′AAA ATC GGG GAA GGA AAC C 5′GAA GGC AAA ATA CAT GGA GTC AC	$(AG)_{20}$	186	46
AM11	5′TCG TGG CAG AGA ATC AAA GAC AC 5′TGG GTG GAG GCA AAA ACA AAA C	$(AG)_{12}(AAAG)_3$	225	49
AM12	5′TGC TGA AGT GAA CAA TCG C 5′CCT TCT CCA ACA ACT CTA C	$(AG)_{20}$	310	44
AM13	5′CGG CGT GAT TTG GGG AAG AAG 5′CTA GTA ACG GCC GCC AGT GTG CTG	$(AG)_{15}$	201	54
AM14	5′GTG GTG GGC ACG GTA TCA 5′TGG GTG GCG AAG CGA ATC	$(AC)_{21}$	133	48
AM15	5′GTG ACC GTA AAC GAT AAC AAC 5′AAG CAA GAC GCG AGA GTA GG	$(AC)_{14}$	229	47
AM16	5′CGG GTT GGC ATC GAC TAT 5′TGA CCA GGC TCT AAC ACA	$(AG)_4..(AC)_{16}$	114	44
AM17	5′CGA GAT TTC GGT GTA GAC 5′CCG GGA ATT AAC GGA GTC	$(AC)_{13}$	250	44
AM18	5′CAA TGT CGT CGG TGT GAG TTT 5′TAC GAG TGT GGC ACG AGC	$(AC)_{14}$	270	47
AM19	5′ATA GAA CGG CAT GAT AAC GAA ATA 5′GCG CGA CAA CAG GAC CTT C	$(AC)_3..(AC)_6..(AC)_5..(AC)_7$	251	48
AM20	5′TGT CGA TTT CTT TAG GGC AGC ACT 5′TCG CGA GAA AGA TGG AAA GGA GA	$(TG)_{10}(CG)_5$	258	50
AM21	5′ACG TTG GTC TCG GGT TGG 5′AAA TCC TTG ACT TCG CTC TGA	$(AT)_5..(AC)_5..(AC)_5$	210	46
AM22	5′ATT GTA TTT GTA GCC CCA GTT C 5′AAG AGC GAC CCA GTT GTA TG	$(AC)_{22}$	138	46
AM23	5′TCT TTA AGG ATT TGG GTG GAG 5′AAT CTT CGA GGG TGA GTT TCT	$(AC)_{19}$	247	45
AM24	5′GTT ATT GAT TTC CTG ATG TAG AGA 5′AGA GCC AAG AAA GCA ACT G	$(AAG)_5..(TCA)_5$	170	45
AM25	5′AGC CTG GAC ATG TAA TCT GGT 5′AGC CCT GGT CTT CTT CAA CA	$(AC)_8..(AC)_4(CT)_4$	229	47
AM26	5′ATA AAG GGG GCA TTG GAT T 5′AAC ATA TTG GGC ATT CAC AT	$(AAG)_{14}$	224	41
AM27	5′CAA AGG CCA AAT GGT GAG 5′CCG CAA AGT CAT ATG GAG CAT	$(AAG)_{10}$	161	45
AM28	5′GAC CTC TTG AGT AAG CAA CG 5′TGG TCT TCC TAT CCA CAA TG	$(GAA)_8$	135	46
AM29	5′TCC CGC AAA ATC ATC ACG A 5′AAG GGA GCA TTG GTT TTG TT	$(GAA)_9$	143	43
AM30	5′TGA AGA TAG CCA TGA GGA AC 5′GTG CAA ATT GAG TTT CAC G	$(GAA)_{14}$	203	43
AM31	5′GCA AAG GCC ATA TGG TGA GAA 5′CAT AGG TTT GCC ATT CGT GGT	$(GAA)_{23}$	186	47
AM32	5′AGT GAA GGC GAT GGC GAA 5′GGA TAA TGC ACC CGA GTT GC	$(GAA)_{19}$	295	47
AM33	5′GCA AAG GTT AAA TGG TGA GA 5′GCC AAC ATA TTG TGC ATA CA	$(GAA)_{15}$	246	43
AM34	5′GAG TAA GCA AAG GTC AAA TG 5′GTT AGC ACT TCC CAC AAA ATC A	$(GAA)_{10}$	181	44

Table 4 (continued)

Primer name	Primer sequence	Repeat type	Size bp	Tm °C
AM35	5′CGT GAC CTT TAT ATC ACC ACT 5′GTG GCT CGT GAT ATT GGC AC	$(GAA)_{14}$	216	47
AM36	5′CTT CCC GCA AAG TTA TCA T 5′AGG GGC ATT GGC TTT GTC	$(GAA)_9$	142	43
AM37	5′CTT CCA CAA GGC AAC GAG TC 5′GGT TAG CAC TTC CCG CAA A	$(GAA)_9$	213	47
AM38	5′TGA TGA CCT CTT GAG TAA GCA 5′TGC CTT TCG TGG ACT TAC TA	$(GAA)_9$	178	45
AM39	5′TTG GGC ATG CCC TTG TT 5′GCC TTG GAG AGT AAA TTC TC	$(GAA)_8$	238	43
AM40	5′CTC TGG GGG TGG TAG TTC CT 5′GAA AGA CAG GCC TCC ACA AAT	$(GAA)_7$	249	49
AM41	5′CCA AAG GAA ACA AGT CAA TAG 5′TTC CCG CAA AGT CAT CAT	$(GAA)_{10}$	205	42
AM42	5′GCT TCC CGC AAA TCA TCA T 5′GAG TAA GCA AAG GCC AAA AAG T	$(GAA)_{16}$	193	45
AM43	5′AGC CCC TAC AAA GCC ATC A 5′CAA GCA AAG GAC GAA CAA TAG	$(GAA)_{17}$	162	46
AM44	5′CGT TGG CCC CTT TTT TCA GTG 5′AGG GGC ATT GGC TTT GTC C	$(GAA)_{11}$	174	49
AM45	5′AGG GAA AAA CAA AAC GTG AGA GTA 5′ATG CAA CAG ATA GAC AAG GGA TTA	$(AC)_9$	191	47
AM46	5′TTG GCA AGG CGA GGT CT 5′CCA AAA GGC TAC AAC ATC ACA C	$(AC)_9$	105	46
AM47	5′GCA CCG GTT AAA AAG GAG TCA G 5′TTT CTT CTT ACC CAC CCA CCA C	$(AC)_{14}$	274	50
AM48	5′ATT CGT GGC TCC TGT GC 5′GTG TAC GTT AAC TCC CCT CTA T	$(AC)_{25}$	191	46
AM49	5′TAG AAA AAG GAA AGT TAG GGT TAG 5′TGA AAT TGA CTG TTA TGT GGT TAC	$(T)_5$..$(TC)_3(AC)_3$	220	45
AM50	5′CTT GAG CGC TAG ATG GTT CC 5′CTC TGT TAC TCA AGT GTT TCA ATA	$(AT)_6$..$(AC)_5$	273	47
AM51	5′AAT CGG ACC TAT GCA AGT TTC AG 5′AGT CGG ACG CTC TAT CAT TCT CA	$(AC)_3$..$(AT)_5$	229	49
AM52	5′ATA GTG CCA TGA CCA ATC TT 5′GTC TAG CCC ACT CAA ACA CCA	$(AC)_4$..$(AC)_4$	229	46
AM53	5′TCG CCA TTA ATA AGA GGG AAG G 5′GCT GCT GTT GGG TGG TTA GTG	$(AC)_{10}$	261	50
AM54	5′AAA CCC GTG CAA GAA ACC AA 5′TGC GGG AGG AGG GAA GAC	$(AC)_9$	186	47
AM55	5′TAT TTG CGG GCT GAA GTT TGT A 5′GGT CAT TTG ATA TCT CCA TTC TA	$(T)_7$..$(AC)_4$	163	45
AM56	5′CGC GAC GGC TTT GTG TT 5′CCG CGC CTC TTT GGT ATT	$(AG)_8$	156	45
AM57	5′TCA CGC GAT GGG CAG AA 5′CGC GCT TCC AAG GTA TTA TC	$(CCT)_3(AAG)_3$	182	46
AM58	5′GTT TAG ATG GGG GTG GCT TAG 5′TTT CTT GTT CTT TGG ATT TTA TTT	$(CT)_5$..$(AC)_3$	215	45
AM59	5′TCT GGG CTA CGA AAA TGT T 5′CCG GCG GAA ATG GAG TG	$(G)_{12}$..$(GA)_5$	275	44
AM60	5′GAA GCG CGT CAG TTG ATG 5′AGC CCA CGT TCA CTC CAT	(NNT)8..(AT)5	242	45
AM61	5′TCG GAG CCG GTA TGG AAG C 5′GGT GGC AAG GGG TGT ATG AG	$(TTTC)_4$..$(CCT)_6$	206	51

polymorphism. Two to eight different alleles were found in the 20 cultivars with a PIC ranging from 0.1 to 0.85 (Table 6).

Polymorphism of barley microsatellites in *Avena*

Fifty four sets of barley microsatellite primers were used to amplify genomic oat DNA. Fourteen sets of the primers (26%) amplified distinguishable microsatellite bands from the *Avena* species; six primer sets showed no detectable product. The other 34 primer sets produced multiple products from the *Avena* DNA. The 14 barley microsatellite primers identified 2–5 alleles in the 12 *Avena* species with a PIC ranging from 0.31 to 0.77 (Table 5). The average allele number and PIC were 3.8 and 0.55, respectively, which is similar to results with oat microsatellite primers (Table 5).

Table 5 Alleles and polymorphic information content (PIC) of oat (A prefix) and barley (H prefix) polymorphic microsatellites in *Avena* species

Primers	No. alleles	PIC	PCR[a]
AM1	5	0.79	2
AM2	2	0.28	2
AM3	5	0.74	2
AM4	6	0.76	2
AM5	2	0.28	2
AM6	6	0.74	2
AM11	4	0.72	1
AM14	4	0.65	2
AM15	5	0.61	2
AM17	4	0.49	2
AM19	2	0.28	2
AM21	6	0.78	2
AM22	8	0.88	2
AM23	4	0.56	2
AM24	3	0.41	2
AM25	3	0.61	2
AM26	2	0.28	1
AM27	3	0.49	2
AM28	2	0.37	2
AM30	6	0.81	1
AM31	6	0.81	2
AM35	2	0.38	2
AM38	4	0.66	1
AM40	3	0.4	2
AM41	3	0.49	2
AM42	3	0.55	2
Average	4	0.57	
HVM3	3	0.44	1
HVM4	3	0.42	1
HVM11	2	0.38	3
HVM20	5	0.77	1
HVM22	4	0.58	3
HVM34	4	0.74	3
HVM44	4	0.45	3
HVM51	4	0.42	3
HVM54	4	0.31	3
HVM60	3	0.46	3
HVM65	5	0.72	3
HVM68	2	0.44	3
HVBAREI	2	0.46	3
HVWAX	4	0.65	1
Average	3.8	0.55	

[a] PCR used for the specific primer set (1: Touchdown 64°C to 55°C; 2: Touchdown 67°C to 58°C; 3: Touchdown 62°C to 53°C)

Table 6 Alleles and polymorphic information content (PIC) of oat (A prefix) and barley (H prefix) polymorphic microsatellites in oat cultivars. The primers AM1 and AM30 detected two polymophic loci

Primer	No. alleles	PIC	PCR[a]
AM1a	8	0.85	2
AM1b	2	0.18	2
AM2	3	0.49	2
AM3	5	0.73	2
AM4	5	0.68	2
AM11	5	0.68	1
AM14	6	0.69	2
AM17	2	0.48	2
AM21	3	0.59	2
AM22	2	0.47	2
AM23	2	0.18	2
AM25	3	0.64	2
AM30a	3	0.65	1
AM30b	2	0.46	1
Am31	3	0.51	2
AM38	3	0.56	1
AM41	2	0.1	2
AM42	3	0.59	2
HVM3	2	0.15	1
HVM20	5	0.50	1
HVWAX	3	0.51	1
Average	3.4	0.51	

[a] PCR used for the specific primer set (1: Touchdown 64°C to 55°C; 2: Touchdown 67°C to 58°C)

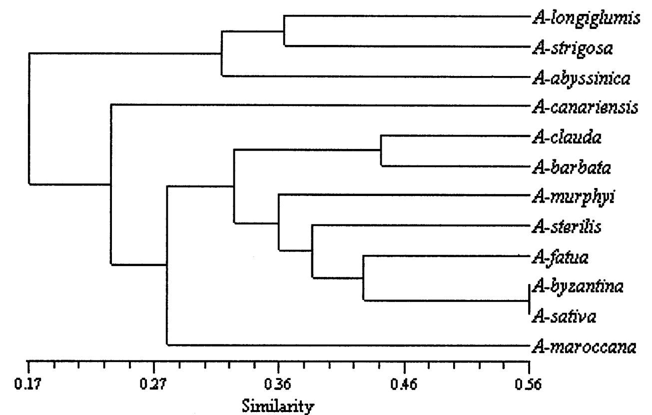

Fig. 1 Phylogenetic relationship of the 12 *Avena* species constructed using 40 microsatellite polymorphisms

Phylogeny of the *Avena* species

Using microsatellite polymorphisms, a dendrogram of the 12 *Avena* species was constructed with an Unweighted Paired Group Method Using Arithmetic Averages (UPGMA)-clustering algorithm (Fig. 1). Two major groups were identified from the dendrogram. The first included two A-genome diploid species (*Avena longiglumis* and *Avena strigosa*) and one AABB tetraploid species (*Avena abyssinica*). The second contained A- and C-genome diploid species, AABB and AACC tetraploid species, and AACCDD hexaploids. Within this group the A genome diploid species (*Avena canariesis*) and the AACC tetraploid species (*Avena maroccana*) were relatively distant from the other species. The tetraploid species *Avena murphyi* showed the closest relationship with the hexaploid species. The four hexaploid species clustered into a closely related group and *Avena fatua* clustered closer to the cultivated species (*A. sativa* and *Avena byzantina*) than did *Avena sterilis*.

Relationship of the cultivars

Although microsatellite polymorphism was relatively low in the cultivars, the 20 cultivars could be grouped using the information of the 19 polymorphic primers (Table 6). As the pedigree of most cultivars was not

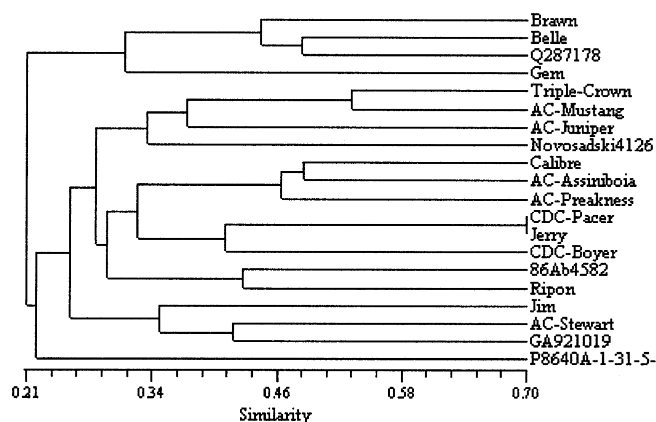

Fig. 2 Dendrogram of the 20 *A. sativa* cultivars constructed using microsatellite polymorphisms

available, it is hard to judge if the dendrogram (Fig. 2) reflects the genetic relationship of the 20 cultivars. However, several groups in the dendrogram were consistent with their origin (Table 1). For example, the three cultivars originating from Wisconsin were grouped into a single group (Table 1; Fig. 2)

Discussion

Polymorphism of microsatellites in oats

Microsatellites have been developed in several important crops. The percentage of microsatellites with polymorphism detected, the number of alleles per locus and the polymorphic information content varies with the crop and the materials (species or cultivars) evaluated (Akkaya et al. 1992; Saghai Maroof et al. 1994; Roder et al. 1995; Szewc-McFadden et al. 1996; Smulders et al. 1997; Senior et al. 1998; Sun et al. 1998; Yu et al. 1999). The percentage of microsatellites detecting polymorphism ranged from 22% in cultivars to 66% in wild species. The number of alleles per locus was as high as 15.5.

In the present study, 62% of the microsatellites were polymorphic with an average of four alleles per locus in the *Avena* species, but only 36% of the microsatellites showed polymorphism in the oat cultivars with an average of 3.4 alleles per locus. The polymorphism level is low for the cultivars but this is comparable with results from bread wheat cultivars (also hexaploid), where 36% of the microsatellites were polymorphic with an average of 3.2 alleles per locus (Roder et al. 1995). The low polymorphism of other DNA markers in cultivated oat has been suggested to be related to the complex genome of oat, which contains a large fraction of repetitive DNA and three related genomes (O'Donoughue et al. 1995).

Microsatellites associated with repetitive elements

Plant genomes contain a large amount of repetitive DNA. A recent study demonstrated that 41% of the microsatellites isolated from enriched libraries of barley were associated with known repetitive elements (Ramsay et al. 1999). In the current study, 42% of the microsatellites isolated from oat showed an association with known repetitive elements (Table 3). Considering the fact that the flanking regions of many microsatellites had no corresponding database entry, together with the lack of well-characterized repetitive elements in barley and oat, this figure is probably an underestimate. Thus, the association of microsatellites with repetitive elements may be common in grass genomes. When microsatellite polymorphism was tested in 12 *Avena* species, 69% of the microsatellites associated with known repetitive elements were polymorphic and 58% of the microsatellites not associated with known repetitive elements showed polymorphism (Tables 3 and 5). However in the 20 oat cultivars, only 25% of the microsatellites associated with known repetitive elements were polymorphic, while 46% of the microsatellites not associated with known repetitive elements showed polymorphism (Tables 3 and 6). It is to be expected that polymorphism for any marker will be lower in cultivars than in wild species, as cultivars usually have a narrower genetic base than wild species. However, the microsatellites associated with repetitive elements showed an extremely low level of polymorphism in the oat cultivars compared to the microsatellites not associated with repetitive elements. This phenomenon was also observed with direct amplified minisatellite polymorphisms in rice (Zhou et al. 1997) and oat (unpublished data). The possible explanation is that repetitive elements (minisatellites, satellites, retrotransposons and transposons) have played an important role in species differentiation during the early stages of plant evolution and have been relatively stable after the completion of species differentiation. The evidence to support this is that the different genomes of the same species and of different species have unique repetitive elements. These unique repetitive elements can be used to distinguish different species and genomes (Somers et al. 1996; Zhou et al. 1997). Therefore, being a part of the repetitive element, microsatellites associated with the repetitive elements are relatively stable and show low polymorphism in cultivars. If this is true, microsatellites isolated from expression libraries would be expected to show a higher ratio of polymorphic microsatellites than those from genomic libraries. Roder et al. (1995) also suggested that the isolation of microsatellites enriched in single-copy and low-copy number sequences may improve the success rate in wheat.

The phylogeny of *Avena* species

The genus *Avena* includes diploid, tetraploid and hexaploid species. Although there is considerable morphologi-

cal, biochemical, geographical and cytotaxonomical evidence available, much is still unknown about the evolution of the genus (Leggett 1992; Leggett and Thomas 1995). Numerous translocations and unknown levels of homoeology among the various diploid genomes (Leggett and Thomas 1995) complicate the phylogeny. There is now good evidence from molecular techniques that the A and D genomes are very closely related (Leggett and Markhand 1995; Linares et al. 1996, 1998). It is possible that a D-genome species never existed (Leggett 1996) and that the D genome is a derived A genome. There is also good evidence that the B genome of some AABB tetraploids is also closely related to the A genome (Leggett 1996).

The microsatellite polymorphisms investigated in this study appear to shed very little light on the phylogeny of *Avena* species at the DNA level. As shown in Fig. 1, the first branch of the dendrogram includes two A-genome diploids (*A. longiglumis* and *A. strigosa*) and one tetraploid species (*A. abyssinica*) with AABB genomes, but does not include the third A-genome diploid species (*A. canariensis*) or the other AABB tetraploid (*A. barbata*). It has been proposed that *A. abyssinica* evolved from two A-genome diploids like *Avena barbata* (AABB) (Leggett 1992) and this would explain the grouping. However, this fails to explain the lack of clustering of either the other A-genome diploid (*A. canariensis*) or the tetraploid *A. barbata*, which is thought to be of near-autoploid origin (Leggett and Thomas 1995), with this group.

It is satisfying that the four hexaploid species did cluster together and that the two cultivated hexaploid species clustered away from the two wild hexaploid species. However, the two AACC tetraploids did not cluster together although Leggett and Thomas (1995) place them in the same section within the genus *Avena.* Although these results do not help to elaborate on the evolution of the genus, this phylogenetic tree is supported by other work with minisatellites and satellite DNA (Li et al. 2000).

Using microsatellites from other crops to amplify DNA from the oat genome

Microsatellites are an ideal marker system; however, the development of microsatellite markers is expensive and time consuming. Roder et al. (1995) showed that only one pair of wheat microsatellite primers (6%) amplified microsatellites from barley and rye. In the present study, 26% of the 54 barley microsatellite primers amplified microsatellites from oat. This number may well be an underestimate as the number of useful microsatellites depends on the PCR conditions. For example, only 5% of the barley microsatellite primers amplified the oat microsatellites using a Touchdown profile from 64°C to 55°C while the number increased to 26% using a Touchdown profile from 62°C to 53°C. This is reasonable considering the sequence divergence in different crops. Thus in some minor crops it may well be worthwhile attempting to use microsatellites developed from a related crop species before embarking on microsatellite isolation.

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