

The use of microsatellite polymorphisms to characterise and compare genetic variability in *Avena strigosa* and *A. barbata*

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Abstract Microsatellite (SSR) polymorphism was assessed across 90 diploid *Avena strigosa* Schreb. and tetraploid *Avena barbata* Pott ex Link accessions obtained from the USDA-ARS National Small Grains Collection using 105 genomic SSRs. Eleven polymorphic SSRs that detected 69 different alleles were identified and used to genotype the 90 accessions, which were chosen from a larger set of 385 accessions based on geographical source-diversity and variable reaction responses to five Australian pathotypes of the crown rust pathogen *Puccinia coronata* Corda f. sp. *avenae* Eriks. Eight diploid and eight tetraploid clades were identified among the 90 accessions. Diploid accessions displayed the lowest genetic diversity, with all accessions being at least 86 % similar, and included accessions from countries in the Americas such as Canada, USA, Argentina, Uruguay and Brazil,

and European accessions from France, Romania and Poland. Although both species formed distinct clusters in the dendrogram, a few instances of diploids showing high similarity with tetraploids and vice versa were observed. An AMOVA analysis revealed 86 % of the total genetic variation to be distributed within the two oat species, while between-species differences accounted for only 14 %. Heterozygosity (H) index values of 0.32 and 0.40 were obtained for diploids and tetraploids respectively. Our study effectively differentiated *A. strigosa* and *A. barbata*, and identified 11 SSRs suitable for future characterisation of accessions of the two species.

Keywords *Avena strigosa* · *A. barbata* · Analysis of molecular variance · Genetic similarity · SSR Simple sequence repeats

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Introduction

In addition to being the progenitors of present day oat cultivars, diploid, tetraploid and hexaploid oat species are also known to possess large amounts of genetic variability, including new genes for resistance to the crown rust fungus *Puccinia coronata* f. sp. *avenae* (*Pca*) (Leonard et al. 2004). Many of these resistance genes have been introgressed into the cultivated hexaploid species *Avena sativa* L. from diploid (Sadana and Simons 1960) and hexaploid (Kiehn et al. 1976) *Avena* species.

Characterising genetic variability using molecular markers can assist in understanding phylogenetic and evolutionary patterns. In recent times, microsatellite or SSR markers have become the preferred DNA-based marker system, being used extensively in studies of variability and genotype characterisation. These markers are PCR-based (Weber and May 1989), co-dominant (Litt and Luty 1989) and consist of short tandem repeats of between 1 and 6 base pairs (Queller et al. 1993). As compared to RFLP and AFLP analysis, assays involving SSRs are rapid, cost effective (Jannink and Gardner 2005), work well with minute or even degraded samples of DNA, and display consistency in scoring of alleles with clear comparisons across various gels (Queller et al. 1993).

Studies aimed at identifying relationships between diploid, tetraploid and hexaploid species of oat and cultivars of *A. sativa* using oat and barley SSR primer sets were carried out by Li et al. (2000), who reported that 62 % of the SSRs tested were polymorphic among *Avena* species as against 36 % among the oat cultivars. Fu et al. (2007) characterised SSR variation in 369 accessions of *A. sterilis* L. collected from 26 countries, and two groups possessing *Pc* and *Pg* genes, using 26 SSR primer pairs derived from oat and other related genera. The authors found that 90 % of SSR variation resided within the accessions of a country, and further suggested that grouping based on disease response might be more informative than those based on the geographical origin of accessions. Ideally, a combination of rust disease phenotyping and SSR screening of accessions might provide a more detailed and meaningful grouping. As such, in the process of characterisation, it would also be interesting to determine or establish a correlation between rust disease response patterns and genetic similarity levels (via clustering patterns) among accessions of a species. The present study examined genetic diversity in a set of accessions of the diploid oat species *Avena strigosa* and tetraploid species *Avena barbata* chosen to represent diverse geographic origins and phenotypic responses to five Australian *Pca* pathotypes (Cabral 2009). The objectives were to utilise SSR loci (Li et al. 2000; Pal et al. 2002) to characterise genetic variability among the *A. strigosa* and *A. barbata* accessions, in addition to assessing genetic variability within and between regions from which the accessions originated, and to identify polymorphic SSRs most suitable for

this characterisation, which could potentially be developed into species or genome-specific SSR markers.

Materials and methods

Plant material

A total of 90 oat accessions from the USDA-ARS National Small Grains Collection were chosen to characterise with the polymorphic SSR markers, based on their geographic origins and phenotypic responses to crown rust. The accessions were originally collected from 28 different countries (Table 1), and displayed 16 different phenotypic responses to five *Pca* pathotypes of diverse pathogenicity (Cabral 2009). These tests were conducted at the Plant Breeding Institute Cobbity Australia. Six accessions (four of which were diploid (CIav8087, CIav9065, PI78821, PI83721) and two tetraploid (PI367283, PI367338)) were resistant to all five pathotypes. The remaining 84 accessions produced compatible responses (infection types “33+” or “3+”) to at least one of the five pathotypes, with 38 accessions (20 diploid and 18 tetraploid) being susceptible to all five pathotypes (Cabral 2009).

All accessions were subjected to cytological tests involving chromosome counts of root tips to confirm their ploidy status (results not shown). About 6–8 seeds from each of the 90 accessions were germinated separately on moist filter paper in glass Petri plates. Five to six days later 1–2 cm of newly emerged root was excised, pre-treated in ice water and placed in a refrigerator for 24 h. The root tips were later fixed in a solution of three parts absolute alcohol and one part acetic acid (3:1) contained in glass vials. Fixed root tips were blotted off and stained with 45 % aceto carmine for 30 min, after which vials were heated to near-boiling over a spirit lamp. The stained root tips were blotted off on filter paper, placed on a glass slide and fine cross sections of root tips were carefully excised with a surgical blade. A drop of 45 % acetic acid was added (to de-colourise the cytoplasm) to the sample over which a cover slip was placed, prior to crushing the specimen. The slide was briefly run over a spirit lamp and observed under a compound microscope, first with 10×/0.25 magnification and later with 40×/0.65 magnification to enable chromosome counts.

Table 1 Description of the 90 diploid *A. strigosa* and tetraploid *A. barbata* oat accessions used in the experiment, along with their accession numbers, working-designations (WD), country of collection and Native or Non Native (N/NN) origin with respect to the Mediterranean region

No	Accession	Species	WD	Country	N/NN	No.	Accession	Species	WD	Country	N/NN
1	Clav1782	<i>A. strigosa</i>	Rus25	Russian Fed.	NN	46	PI337809	<i>A. barbata</i>	Tur131	Turkey	N
2	Clav2520	<i>A. strigosa</i>	Fr26	France	N	47	PI337821	<i>A. barbata</i>	Gre133	Greece	N
3	Clav2521	<i>A. strigosa</i>	Fr27	France	N	48	PI337827	<i>A. barbata</i>	Gre136	Greece	N
4	Clav2525	<i>A. strigosa</i>	UK30	UK	NN	49	PI337863	<i>A. barbata</i>	Ity140	Italy	N
5	PI78821	<i>A. strigosa</i>	Aus31	Australia	NN	50	PI337893	<i>A. barbata</i>	Ity149	Italy	N
6	PI83721	<i>A. strigosa</i>	Aus33	Australia	NN	51	PI337896	<i>A. barbata</i>	Ity151	Italy	N
7	PI83723	<i>A. strigosa</i>	Aus35	Australia	NN	52	PI337905	<i>A. barbata</i>	Ity158	Italy	N
8	Clav2920	<i>A. strigosa</i>	UK37	UK	NN	53	PI337910	<i>A. barbata</i>	Lib161	Libya	N
9	Clav3214	<i>A. strigosa</i>	US39	USA	NN	54	PI337912	<i>A. barbata</i>	Lib163	Libya	N
10	PI111261	<i>A. strigosa</i>	Rom40	Romania	NN	55	PI337945	<i>A. barbata</i>	Tun185	Tunisia	N
11	PI131640	<i>A. strigosa</i>	Pol43	Poland	NN	56	PI337953	<i>A. barbata</i>	Ity186	Italy	N
12	PI131641	<i>A. strigosa</i>	Pol44	Poland	NN	57	PI337963	<i>A. barbata</i>	Fra191	France	N
13	PI131642	<i>A. strigosa</i>	Pol45	Poland	NN	58	PI337963	<i>A. barbata</i>	Fra192	France	N
14	Clav4639	<i>A. strigosa</i>	Brz46	Brazil	NN	59	PI337872	<i>A. barbata</i>	Alg194	Algeria	N
15	PI158245	<i>A. strigosa</i>	Sp49	Spain	N	60	PI337975	<i>A. barbata</i>	Alg195	Algeria	N
16	PI158247	<i>A. strigosa</i>	Por50	Portugal	NN	61	PI282715	<i>A. barbata</i>	Isr223	Israel	N
17	Clav5057	<i>A. strigosa</i>	Rus51	Russian Fed.	NN	62	PI282721	<i>A. barbata</i>	Isr227	Israel	N
18	Clav5082	<i>A. strigosa</i>	Uru52	Uruguay	NN	63	PI282774	<i>A. barbata</i>	Isr237	Israel	N
19	Clav6858	<i>A. strigosa</i>	Uru53	Uruguay	NN	64	PI287191	<i>A. barbata</i>	Isr242	Israel	N
20	Clav6956	<i>A. strigosa</i>	Can54	Canada	NN	65	PI287259	<i>A. barbata</i>	Ity257	Italy	N
21	Clav7010	<i>A. strigosa</i>	Brz55	Brazil	NN	66	PI293342	<i>A. barbata</i>	Cy259	Cyprus	N
22	Clav7280	<i>A. strigosa</i>	US58	USA	NN	67	PI293343	<i>A. barbata</i>	Cy260	Cyprus	N
23	Clav8087	<i>A. strigosa</i>	Sp59	Spain	N	68	PI295885	<i>A. barbata</i>	Isr264	Israel	N
24	Clav9011	<i>A. strigosa</i>	Den62	Denmark	NN	69	PI317945	<i>A. barbata</i>	Isr296	Israel	N
25	Clav9012	<i>A. strigosa</i>	Bul63	Bulgaria	NN	70	PI317953	<i>A. barbata</i>	Isr298	Israel	N
26	Clav9014	<i>A. strigosa</i>	Can64	Canada	NN	71	PI320584	<i>A. barbata</i>	Isr308	Israel	N
27	Clav9020	<i>A. strigosa</i>	Arg67	Argentina	NN	72	PI320588	<i>A. barbata</i>	Isr311	Israel	N
28	Clav9021	<i>A. strigosa</i>	Can68	Canada	NN	73	PI320689	<i>A. barbata</i>	Isr360	Israel	N
29	Clav9022	<i>A. strigosa</i>	Net69	Netherlands	NN	74	PI337731	<i>A. barbata</i>	Gre379	Greece	N
30	Clav9043	<i>A. strigosa</i>	Arg75	Argentina	NN	75	PI337741	<i>A. barbata</i>	Ity383	Italy	N
31	Clav9065	<i>A. strigosa</i>	Can77	Canada	NN	76	PI337750	<i>A. barbata</i>	Lib390	Libya	N
32	Clav9112	<i>A. strigosa</i>	Can80	Canada	NN	77	PI337763	<i>A. barbata</i>	Fra396	France	N
33	PI274609	<i>A. strigosa</i>	Pol83	Poland	NN	78	PI337795	<i>A. barbata</i>	Mo401	Morocco	N
34	PI274610	<i>A. strigosa</i>	Pol84	Poland	NN	79	PI367283	<i>A. barbata</i>	Spn403	Spain	N
35	PI287315	<i>A. strigosa</i>	Ger85	Germany	NN	80	PI367293	<i>A. barbata</i>	Spn404	Spain	N
36	PI291990	<i>A. strigosa</i>	Isr86	Israel	N	81	PI367318	<i>A. barbata</i>	Por412	Portugal	NN
37	PI292226	<i>A. strigosa</i>	Isr88	Israel	N	82	PI367322	<i>A. barbata</i>	Por414	Portugal	NN
38	PI361911	<i>A. strigosa</i>	Rom92	Romania	NN	83	PI367338	<i>A. barbata</i>	Por418	Portugal	NN
39	PI436103	<i>A. strigosa</i>	Chi96	Chile	NN	84	PI367375	<i>A. barbata</i>	Spn427	Spain	N
40	PI436106	<i>A. strigosa</i>	Chi99	Chile	NN	85	PI391417	<i>A. barbata</i>	UK428	UK	NN
41	PI436107	<i>A. strigosa</i>	Chi100	Chile	NN	86	PI411366	<i>A. barbata</i>	Isr430	Israel	N
42	PI436108	<i>A. strigosa</i>	Chi101	Chile	NN	87	PI411369	<i>A. barbata</i>	Tur431	Turkey	N
43	PI165960	<i>A. barbata</i>	Ind126	India	NN	88	'GLABROTA'	<i>A. strigosa</i>	AG	Australia	NN
44	PI296214	<i>A. barbata</i>	Isr127	Israel	N	89	CI3815	<i>A. strigosa</i>	CI3815	Australia	NN
45	PI320603	<i>A. barbata</i>	Isr128	Israel	N	90	'SAIA'	<i>A. strigosa</i>	'Saia'	Australia	NN

SSR Primers

One hundred and five oat genomic SSR markers of the AM series (AM1–AM60 and AM80–AM124) were selected from the GrainGenes2.0 database (<http://wheat.pw.usda.gov/cgi-bin/graingenes>) (Table 2). For amplification via PCR, working DNA concentrations of

10 ng/μl were prepared for each of the 105 forward and reverse primer sequences from the stock solutions. The SSRs were screened initially on a sub-set of 24 accessions, to identify those that were polymorphic (data not shown). The lines within the sub-set were selected based on observed differences in individual reaction responses to the *Pca* pathotypes and their origin.

Table 2 List of the 105 genomic SSR markers along with their repeat sequences, obtained from the online GrainGenes2.0 *Avena* database

No.	Locus	Repeat	No.	Locus	Repeat	No.	Locus	Repeat
1	AM1	(AG) ₂₁ (CAGAG) ₆	36	AM36	(GAA) ₉	71	AM90	(AC) ₁₅
2	AM2	(AG) ₂₄	37	AM37	(GAA) ₉	72	AM91	(AC) ₂₀
3	AM3	(AG) ₃₅	38	AM38	(GAA) ₉	73	AM92	(AC) ₁₃
4	AM4	(AG) ₃₄	39	AM39	(GAA) ₈	74	AM93	(AC) ₁₂
5	AM5	(AG) ₂₇	40	AM40	(GAA) ₇	75	AM94	(AC) ₁₁
6	AM6	(AG) ₂₀	41	AM41	(GAA) ₁₀	76	AM95	(AC) ₁₃
7	AM7	(AG) ₂₁	42	AM42	(GAA) ₁₆	77	AM96	(AC) ₁₀
8	AM8	(AG) ₁₅	43	AM43	(GAA) ₁₇	78	AM97	(AC) ₃₅
9	AM9	(AG) ₁₉	44	AM44	(GAA) ₁₁	79	AM98	(AC) ₁₀
10	AM10	(AG) ₂₀	45	AM45	(AC) ₉	80	AM99	(AC) ₁₂
11	AM11	(AG) ₁₂ (AAG) ₃	46	AM46	(AC) ₉	81	AM100	(AC) ₁₅
12	AM12	(AG) ₂₀	47	AM47	(AC) ₁₄	82	AM101	(AC) ₃
13	AM13	(AG) ₁₅	48	AM48	(AC) ₂₅	83	AM102	(AC) ₉
14	AM14	(AC) ₂₁	49	AM49	(T) ₅ (TC) ₃	84	AM103	(AC) ₁₆
15	AM15	(AC) ₁₄	50	AM50	(AT) ₆ (AC) ₅	85	AM104	(AG) ₃₆
16	AM16	(AG) ₄ (AC) ₁₆	51	AM51	(AC) ₃ (AT) ₅	86	AM105	(AC) ₂ (AC) ₅
17	AM17	(AC) ₁₃	52	AM52	(AC) ₄ (AC) ₄	87	AM106	(AC) ₄ (AC) ₅
18	AM18	(AC) ₁₄	53	AM53	(AC) ₁₀	88	AM107	(AC) ₃ (AC) ₄
19	AM19	(AC) ₃ (AC) ₆	54	AM54	(AC) ₉	89	AM108	(AG) ₅ (AG) ₂
20	AM20	(TG) ₁₀ (CG) ₅	55	AM55	(T) ₇ (AC) ₄	90	AM109	(AC) ₈ (AG) ₅
21	AM21	(AT) ₅ (AC) ₅	56	AM56	(AG) ₈	91	AM110	(AC) ₃ (AG) ₄
22	AM22	(AC) ₂₂	57	AM57	(CCT) ₃ (AAG) ₃	92	AM111	(AC) ₁₁ (AT) ₆
23	AM23	(AC) ₁₉	58	AM58	(CT) ₅ (AC) ₃	93	AM112	(AG) ₃ (AC) ₉
24	AM24	(AAG) ₅ (TCA) ₅	59	AM59	(G) ₁₂ (GA) ₅	94	AM113	(AG) ₁₅ (AC) ₉
25	AM25	(AC) ₈ (AC) ₄	60	AM60	(NNT) ₈ (AT) ₅	95	AM114	(AG) ₂₄ (AC) ₁₄
26	AM26	(AAG) ₁₄	61	AM61	(TTTC) ₄ (CCT) ₆	96	AM115	(AC) ₉
27	AM27	(AAG) ₁₀	62	AM81	(AC) ₉	97	AM116	(CAA) ₅₈
28	AM28	(GAA) ₈	63	AM82	(AC) ₉	98	AM117	(GAA) ₁₄
29	AM29	(GAA) ₉	64	AM83	(AC) ₁₁	99	AM118	(GAA) ₈
30	AM30	(GAA) ₁₄	65	AM84	(AC) ₉	100	AM119	(GAA) ₁₁
31	AM31	(GAA) ₂₃	66	AM85	(AC) ₁₀	101	AM120	(GAA) ₁₂
32	AM32	(GAA) ₁₉	67	AM86	(AC) ₉	102	AM121	(GAA) ₁₆
33	AM33	(GAA) ₁₅	68	AM87	(AC) ₁₃	103	AM122	(GAA) ₆
34	AM34	(GAA) ₁₀	69	AM88	(AC) ₈	104	AM123	(GAA) ₆
35	AM35	(GAA) ₁₄	70	AM89	(AC) ₁₆	105	AM124	(GAA) ₁₉

Genomic DNA extraction

DNA was extracted by the SDS method (Goldenberger et al. 1995) with minor modifications, using leaf samples taken from 11 day-old seedlings. Leaves from a single seedling per accession, were used for DNA extraction. Samples collected in 2 µl Eppendorf tubes containing two steel ball bearings were ground in a Retch MM300 Shaker. Warm extraction buffer was added to the ground sample and tubes were incubated in a water bath at 65 °C for 15 min. Next, 5 M potassium acetate was added and samples were kept at –20 °C for 30 min, after which they were centrifuged at 12,000 rpm for 15 min and the resulting supernatant was transferred to fresh tubes. An equal volume of chilled isopropanol (–20 °C) was added to the tubes, which were centrifuged at 12,000 rpm for 15 min and the supernatant discarded. Pellets were dissolved in distilled water, and the tubes were kept at 4 °C for 30 min, and later centrifuged. The supernatant was transferred to a new tube, to which 3 M sodium acetate was added. Samples were rinsed twice, first with 100 % ethanol and later with 70 % ethanol, and centrifuged, discarding the supernatant after each treatment. Pellets were air-dried and dissolved in 120 µl distilled water, to make up the DNA stock solutions. Stocks were treated with RNase, incubated at 37 °C for 3 h, and gDNA concentrations were measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Working DNA concentrations of 25 ng/µl of each sample were prepared for amplification via PCR.

PCR amplification and electrophoresis

Reaction volumes of 15 µl were prepared in 96-well PCR plates and run on a Mastercycler[®] ep (Eppendorf, Germany). Each reaction contained 2 µl of 50 ng of plant gDNA, 1.5 µl of 10× PCR buffer (Applied Biosystems, Australia) 1.5 µl of 200 µM dNTPs (Roche Diagnostics, Australia), 0.7 µl of 2 mM MgCl₂, 0.75 U of Taq DNA polymerase (Applied Biosystems, Australia) and 1.5 µl each of 10 ng/µl forward and reverse primers. A normal PCR of 20 cycles, which consisted of initial and subsequent denaturation cycles of 94 °C for 3 min and 45 s, respectively, and an extension at 72 °C for 1 min, was followed by a ‘Touchdown’ PCR (Don et al. 1991) of 20 cycles. The ‘Touchdown’ PCR involved initial primer annealing temperature-

decrements of 0.5 °C until the actual primer melting temperature (T_m) was attained, followed by an extension at 72 °C for 1 min. A final extension at 72 °C for 7 min preceded the completion of the reaction. PCR products were visualised on 3 % agarose gels or on 6 % polyacrylamide gels (PAGE). The PAGE gels were run in a 1 % TBE buffer at 60 W for 90 min, on a 45 cm vertical electrophoresis unit (Edwards Scientific). To each well, 2.5 µl of the PCR product was loaded alongside a 25 bp ladder. Gels were fixed in 10 % acetic acid for 45 min, rinsed twice in dH₂O, and stained with 0.2 % silver nitrate for 40 min. Excess stain was rinsed off with dH₂O, and gels were developed for 10–15 min before being immersed in a stopper solution (5 % acetic acid + 2 % glycerol) for 5 min. Finally, gels were rinsed twice with dH₂O, air dried and bands were scored the following day.

Data analysis

Bands were scored as either 1 (present) or 0 (absent). However to obtain unbiased comparisons (Chapuis and Estoup 2007), null alleles were omitted from the calculations. Polymorphism information content (PIC) (Weir 1996) values were calculated for each marker using the formula $PIC = 1 - \sum P_i^2$, where P_i is the frequency of the i th allele among the selected genotypes. The heterozygosity index (H) (Nei 1978) was calculated by averaging PIC scores of all 11 polymorphic markers. This was done because PIC and (H) are reported to be essentially the same (Anderson et al. 1993). While the PIC value was calculated taking into consideration all 90 genotypes, (H) values were calculated separately for diploid and tetraploid species. Genetic similarity among accessions was evaluated using UPGMA, and a dendrogram was constructed using the Simple Matching matrix of the NTSYS 2.02 software. A bootstrap (Efron 1979) analysis using the Dice coefficient was carried out with the aid of the Winboot software (Yap and Nelson 1996) in order to test the statistical significance and reliability of the similarity patterns obtained from the dendrogram. While a principal coordinate analysis (PCA) was used to depict the distribution of diploid and tetraploid accessions in space, an AMOVA was performed using Genalex 6.1 (Peakall and Smouse 2006), computing F_{ST} (Wright 1951) to partition total variance in the two species. Additionally, based on the assumption of a Mediterranean centre of origin, all

accessions were grouped as Native (N) or Non Native (NN) in order to identify possible patterns in variability that could be due to origin/place of collection.

Results

Of the 105 SSR markers tested on the sub-set of 24 oat genotypes, 30 produced distinct amplicons and were therefore used to genotype all 90 accessions on 6 % PAGE gels. A total of 11 polymorphic SSR loci, detecting 63 different alleles across the 90 genotypes, were identified (Table 3). An example of allele variability of marker AM53 on agarose gel is shown in Fig. 1. Marker AM1 amplified 13 different alleles, followed by AM53 with 12 alleles, AM102 with 11 alleles, AM89 with nine alleles, AM25 with seven alleles, AM94 with five alleles, AM21 and AM61 with three alleles, and finally two alleles each for AM33, AM110 and AM118 (Table 3). While all 11 markers

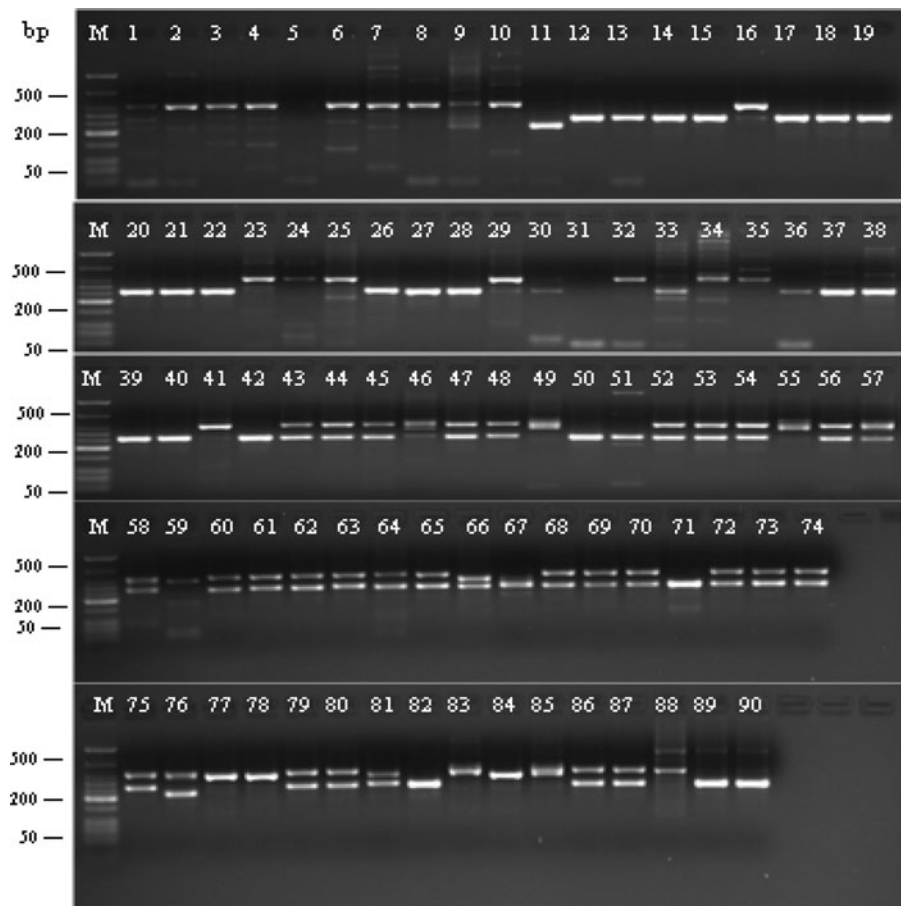
produced clear amplicons, AM102 failed to amplify in 95 % of the diploid accessions. Both diploid and tetraploid accessions produced bands between 160 and 360 base pairs in length, and bands greater than 360 bases were observed in only a few tetraploid accessions. AM25 produced amplicons of approximately 210 base pairs that were unique to the three diploid genotypes 'Saia', CI 3815 and 'Glabrota', also included in the study.

A dendrogram (Fig. 2) of similarity depicted genetic relationships among the 90 accessions, with clusters resolved arbitrarily (Mather and Doornkamp 1970) at 95 % similarity for effective visualisation of groups. Diploid and tetraploid accessions each formed eight clusters and shared an overall genetic similarity of 87 %. Further, seven groups were identified each comprising accessions that were genetically identical with the markers used. Of these, five contained accessions that all produced identical reaction infection types to five *Pca* pathotypes, while two groups

Table 3 Characteristics of the 11 polymorphic SSR markers with their repeat motifs, PCR size, forward (F) and reverse (R) primer sequences, melting temperatures (T_m), allele number and polymorphism information content (PIC) values

Locus	Repeat motif	Size (bp)	Primer sequence	T_m (°C)	No. of alleles	PIC
AM1	(AG) ₂₁ (CAGAG) ₆	204	F-5'GGATCCTCCACGCTGTTGA R-5'CTCATCCG ATGGGCTTTA	46	13	0.64
AM21	(AT) ₅ ...(AC) ₅ (AC) ₅	210	F-5'ACGTTGGTCTCGGGTTGG R-5'AAATCCTTGACTTCGCTCTGA	46	3	0.17
AM25	(AC) ₈ (AC) ₄ ...(CT) ₄	229	F-5'AGCCTGACATGTAATCTGGT R-5'AGCCCTGGTCTTCTTCAACA	47	7	0.48
AM33	(GAA) ₁₅	246	F-5'GCAAAGGTTAAATGGTGAGA R-5'GCCAACATATTGTGCATACA	43	2	0.09
AM53	(AC) ₁₀	261	F-5'TCGCCATTAATAAGAGGGAAGG R-5'GCTGCTGTTGGGTGGTTAGTG	50	12	0.83
AM61	(TTTC) ₄ (CCT) ₆	206	F-5'TCGGAGCCGGTATGGAAGC R-5'GGTGGCAAGGGGTGTATGAG	51	3	0.04
AM89	(AC) ₁₆	193	F-5'GGCGGTTGGAGAGTGTCTT R-5'AGGTGAAGGCGAGTGAAG	58	9	0.72
AM94	(AC) ₁₁	187	F-5'TCTGGAAGTGGTAGTGGGTGT R-5'AACCCATCCACCATAGGC	58	5	0.49
AM102	(AC) ₉	213	F-5'TGGTCAGCAAGCATCACAAT R-5'TGTGCATGCATCTGTGCTTA	51	11	0.88
AM110	(AC) ₃ (AG) ₄	193	F-5'TTCATCGCTGTGACCATGTG R-5'TCCGTGAGTTCCCTCAGTTC	58	2	0.40
AM118	(GAA) ₈	190	F-5'TCCATTGCATGACCTCTTGA R-5'ACTCCCCGAAAATCATCAC	58	2	0.09

Fig. 1 PCR amplification of the marker AM53 showing allelic variability among 90 *Avena* accessions, on 3 % agarose gels. *M* is the 25 base pair DNA ladder (New England Bio-labs)



contained accessions that differed in response to the five *Pca* pathotypes (Cabral 2009).

While all tetraploid accessions with the exception of PI293342 (*A.bCyp259*) from Cyprus clustered together, diploid accessions formed two distinct groups of five (C1–C5) and three (C6–C8) clusters respectively. Among the genetically less similar (84 %) clusters of diploid accessions were Clav1782 (*A.sRus25*) from Russia, PI78821 (*A.sAus31*) from Australia, Clav9011 (*A.sDen62*) from Denmark, PI287315 (*A.sGer85*) from Germany, Clav8087 (*A.sSp59*) from Spain, Clav9014 (*A.sCan64*) from Canada, and the three genotypes ‘Saia’, CI3815 and ‘Glabrota’.

Diploid NN accessions from Canada, USA, Argentina, Uruguay and Brazil contained identical alleles, while a similar situation was recorded for European accessions from France, Romania and Poland. Diploid accessions from non-proximal regions (countries distant from each other, on separate continents/landmass) with identical

alleles included genotypes from the UK and USA on the one hand and Poland and Uruguay on the other.

Results of a bootstrap analysis, while failing to validate the robustness of the diploid and tetraploid clusters, identified two groups that were supported significantly, appearing in greater than 95 % of the sampled bootstrap trees (Felsenstein 1985). The first group comprised diploid accessions PI295885 and PI317945 from Israel, appearing in 99.7 % of sampled trees, and the second group comprised Spanish accessions PI367283 and PI367293, which appeared in 96.8 % of bootstrap samples (Fig. 2). Although the bootstrap analysis provided poor support for the clusters identified, a principal coordinate analysis (PCA) depicted two distinct groups of diploid and tetraploid accessions (Fig. 3).

Heterozygosity (*H*) values were 0.32 and 0.40 for diploid accessions and tetraploid accessions, respectively (data not shown). The average PIC values for all 11 markers across 90 genotypes was 0.44, ranging

Fig. 2 UPGMA similarity dendrogram generated from data of 11 polymorphic SSRs screened on 90 diploid and tetraploid accessions, each forming eight clusters (C1–C8) with an overall genetic similarity of 87 %. Bootstrap values for two groups of accessions appearing in greater than 95 % of sampled bootstrap trees are listed. *Ellipses* represent the seven groups each with genetically identical accessions

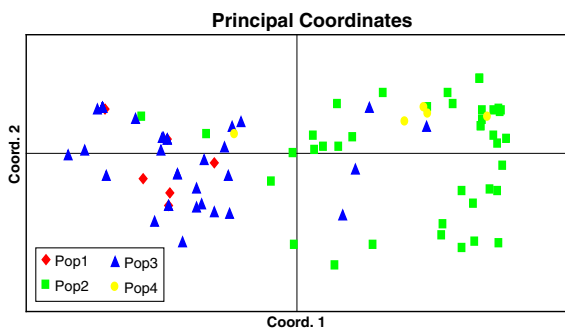
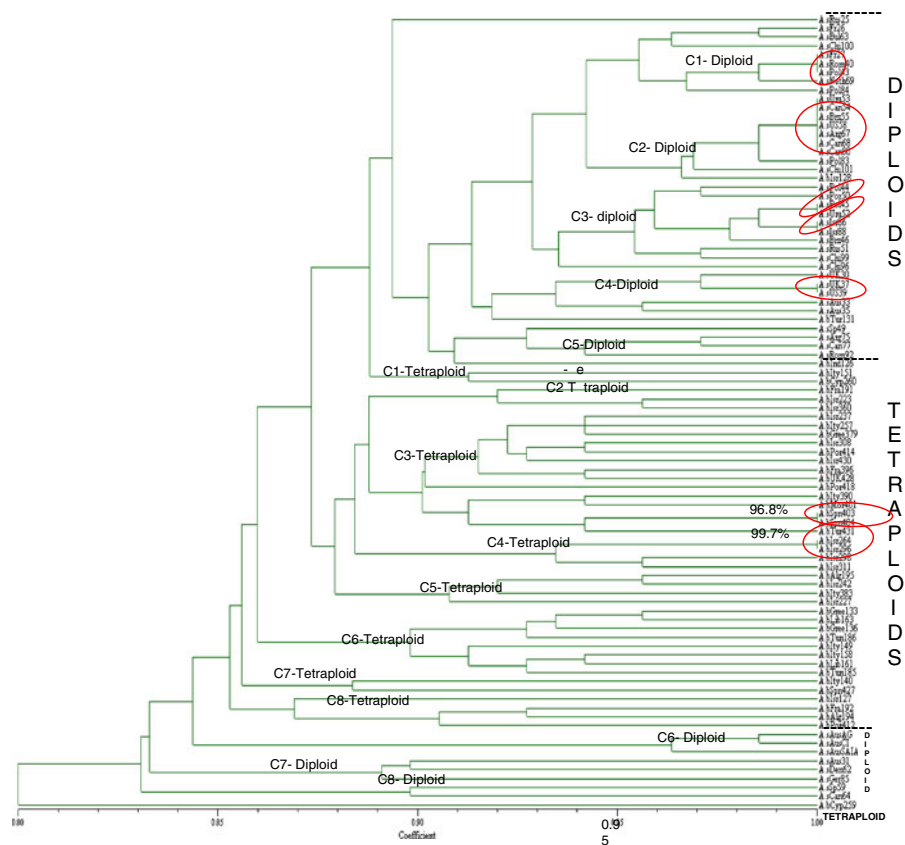


Fig. 3 Principal coordinates for the 90 accessions, with diploid accessions [Pop 1-Native (N) and Pop 3-Non Native (NN)] and tetraploid accessions [Pop 2-Native (N) and Pop 4-Non Native (NN)] forming distinct groups. Accessions were designated as either Native (N) or Non-native (NN) based on the assumption of a Mediterranean centre of origin

between 0.04 and 0.83. In an AMOVA (Table 4), the majority of the total genetic variation (86 %) was distributed among accessions of the two oat species. Only 14 % of the total variation could be explained by differences between the two species ($P = 0.01$). The PhiPt value, indicative of the comparative variability

Table 4 Analysis of molecular variance (AMOVA) carried out for diploid and tetraploid accessions ($\Phi_{HiPT} = 0.136$ at $P = 0.01$)

Source	df	SS	MS	Est. variance	%
Between species	1	37.9	37.9	0.74	14
Among accessions within species	88	412.4	4.68	4.69	86
Total	89	450.3		5.42	100

among accessions of a species, was significant in a pair-wise comparison between *A. strigosa* and *A. barbata* ($\Phi_{HiPt} = 0.136$ $P = 0.010$). However, no significant genetic variation could be detected between respective N and NN groups of both species.

Discussion

This study found that the diploid *A. strigosa* accessions examined were less diverse than the tetraploid *A.*

barbata accessions examined. About 14 % of the total genetic variation was due to differences between the two species, while no significant genetic variation could be detected between native and non-native accessions of both species. The 11 polymorphic SSR markers identified effectively differentiated *A. strigosa* and *A. barbata*, and could therefore be used in future studies involving the above two species, without the need to re-screen the same series of AM markers to identify polymorphic SSRs. A single marker, AM102, while producing amplicons in tetraploid *A. barbata* species, failed to amplify in 95 % of diploid *A. strigosa* accessions and might thus be a potential source for the development of a species specific marker(s).

The UPGMA dendrogram depicted two distinct groups, diploid *A. strigosa* and tetraploid *A. barbata* accessions, with an overall genetic similarity of 87 %. The two species clusters each comprised eight sub-clusters. Although the bootstrap analysis provided poor support for the clusters identified, these clusters were also seen in a PCA as two distinct groups of diploid and tetraploid accessions, and hence are most likely not artifactual. While diploid accessions formed two groups of five (C1–C5) and three (C6–C8) clusters respectively, all tetraploid accessions clustered together, with the exception of PI293342 from Cyprus. Among the genetically less similar (84 %) were clusters of the diploid accessions C6 ('Saia', CI3815, 'Glabrota'), C7 (PI78821 from Australia, CIav9011 from Denmark, PI287315 from Germany), C8 (CIav8087 from Spain, CIav9014 from Canada) and CIav1782 (Russia). All of these accessions produced compatible ITs to the five pathotypes, with the exception of PI78821 (Australia) and CIav8087 (Spain), which both produced resistant ITs to the five pathotypes. Further, seven groups were identified each comprising accessions that were genetically identical. Of these, five groups of accessions of *A. strigosa* produced identical reaction ITs to the five *Pca* pathotypes. The remaining two groups, each containing accessions of *A. barbata*, produced slightly variable ITs in response to the five pathotypes. These observations might suggest a correlation between accessions of a cluster that are genetically identical, and their corresponding identical/similar disease infection types in response to a given number of pathotypes of *Pca*. However, further investigations are needed before any such conclusions can be made. In

contrast, Fu et al. (2007) found that accessions of *A. sterilis* carrying a known number of *Pc* and *Pg* resistance genes had similar levels of SSR variation, but clustered separately.

The markers detected greater genetic similarity among diploid accessions than among tetraploid accessions. Also, the (H) values for diploid accessions (0.32) as against tetraploid accessions (0.4) suggested lower genetic diversity in the former. This could be due to the smaller genomes of diploid species, with fewer repeated or altered sequences due to a less rapid rate of genome restructuring (Gale and Devos 1998) compared to tetraploid species, as a consequence of evolution. Similar observations of greater genetic diversity among tetraploid forms than among diploid forms of *Rorippa amphibian* (L.) Besser, tested with eight microsatellite loci were reported by Luttkhuizen et al. (2007). In contrast, diploid accessions of *A. strigosa* have been reported to possess relatively greater levels of genetic variability with respect to genes for rust resistance (Dyck 1966). Hence, an alternative explanation could be that a majority of the genetically identical diploid accessions from the Americas might have descended from the same genotype(s) of a particular region, and were subsequently introduced to the given countries over a period of time. In studies of SSR diversity in accessions of *Triticum aestivum* L., Huang et al. (2002) observed that not all accessions originating from a geographic region clustered in the same group. The authors concluded that either similar genetic variation occurred independently among accessions in the different geographical regions or that the artificial transfer of accessions between regions resulted in the incorrect/false determination of geographical origin. Additionally, the greater genetic similarity among diploid accessions observed in this study might have resulted from the order of cataloguing and/or selection of accessions, wherein a majority of diploid accessions with phenotypic and/or morphological similarities and possible common descent/parentage were combined in this collection.

A lack of amplification in 95 % of the diploid accessions tested with marker AM102, which has the dinucleotide repeat sequence (AC)₉, could be due to the reportedly lower frequency of AC/TG repeats in plants than in humans (Morgante and Oliviere 1993; Powell et al. 1996). However, if this is true, a similar lack of amplification should have also been observed

for tetraploid accessions. Additionally, this observation cannot be deemed conclusive because marker AM53 with a repeat of (AC)₁₀ produced distinct amplicons in diploid accessions. Therefore, a possible explanation might be the less frequent occurrences of the corresponding primer binding sites containing the specific number of repeats, i.e. (AC)₉, in diploid genotypes. Based on this observation, SSRs with similar attributes could be exploited for genome/species differentiation, because repetitive sequences associated with SSRs are relatively stable and genome-specific (Li et al. 2000). The corresponding amplicons generated by marker AM102 in the tetraploid genotypes examined could be due to the larger genomes undergoing translocations, mutations and/or slippage to generate DNA polymorphisms over time (Phillips and Vasil 1994). Thus, these genomes would now contain altered nucleotide sequences that are complimentary to the given primer sequence, thus generating amplicons. Further investigations are needed to examine this selective amplification of marker AM102, including sequencing of gel-excised bands or PCR products of the marker to design species or genome-specific primers.

Results of an AMOVA revealed that only 14 % of the total genetic variation observed with SSRs was due to differences between *A. strigosa* and *A. barbata*. The remaining 86 % of total genetic variation was distributed among accessions within the two oats species. A PhiPt value of 0.136 (at $P = 0.01$) indicated a significant genetic differentiation among accessions of both species. While both species carry the A genome, *A. barbata* has an additional B genome. Given that only 14 % of the total genetic variation is attributable to differences between the two species, it is likely that most of the SSRs characterised in this study are associated with the shared A genome. Additionally, because AABB genome tetraploid species like *A. barbata* are reported to have evolved from the diploid species *A. strigosa* (Ladizinsky and Zohary 1968), allelic similarities between both species are to be expected.

The AABB tetraploids are reported to have originated or resulted from duplication of AsAs diploids (Ladizinski and Zohary 1968; Katsiotis and Forsberg 1995). The A and As genomes of AABB tetraploids and AsAs diploids, respectively, are morphologically identical, while the B genome has evolved from the diploid As genome (Rajhathy and

Thomas 1974). The above arguments might support our findings of A and B genomes not being very different. On the contrary, a lower/lack of similarity between diploid As and tetraploid B genomes detected via karyotype studies (Rajhathy and Morrison 1959) and molecular *in situ* hybridisation and Southern blots (Fominaya et al. 1988; Irigoyen et al. 2001) contradict the AsAs diploid duplication theory, suggesting instead that the AABB tetraploid *A. barbata* might have resulted from a hybridisation event between two A genome diploid species, rather than from a single AsAs diploid. Based on this assumption, genetic diversity in the tetraploid *A. barbata* should far exceed the diversity of the diploid species. Hence, the mild difference in genetic diversity between *A. strigosa* and *A. barbata* detected in this study might be attributed to a modest number of 90 accessions that were screened with 11 polymorphic SSRs. As such, it is likely that larger data sets/collections might satisfy the above assumption of diversity in the tetraploid *A. barbata* far exceeding diversity of the diploid species.

The use of SSR markers for the characterisation of genetic variability among accessions of wild and cultivated oat species is a first step in the process of identifying genetically diverse accessions that might be potential carriers of new and useful traits like rust resistance. Furthermore, the use of phenotypic/disease data (Fu et al. 2007) might help refine the classification process. Such phenotypic classifications could greatly enhance the association of genetic markers and specific traits via studies of the non-random association of alleles at loci/linkage disequilibrium (Bresgello and Sorrells 2006) in closely related accessions of a species, when chromosomal locations are known. Because detailed SSR linkage maps of diploid and tetraploid species of *Avena* are yet to be created, the exact genomic/chromosomal location of these SSRs and disease resistance genes in diploid and tetraploid genomes are not known. These factors suggest that although oat SSR markers might be able to clearly detect allelic differences among genotypes and distinguish between accessions of different ploidy levels, they might not provide a detailed explanation of diversity patterns in oat species. Therefore, the use of SSR markers in combination with other related genome sequencing technologies might be a better approach to studying and quantifying genetic diversity.

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