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ORIGINAL PAPER

Development and assessment of DArT markers in triticale

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Abstract Triticale (*X Triticosecale* Wittm.) is a hybrid derived by crossing wheat (*Triticum* sp.) and rye (*Secale* sp.). Till date, only a limited number of simple sequence repeat (SSRs) markers have been used in triticale molecular analyses and there is a need to identify dedicated high-throughput molecular markers to better exploit this crop. The objective of this study was to develop and evaluate diversity arrays technology (DArT) markers in triticale. DArT marker technology offers a high level of multiplexing. Development of new markers from triticale accessions was combined with mining the large collection of previously developed markers in rye and wheat. Three genotyping arrays were used to analyze a collection of 144 triticale accessions. The polymorphism level ranged from 8.6 to 23.8% for wheat and rye DArT markers, respectively. Among the polymorphic markers, rye markers were the most abundant (3,109) followed by wheat (2,214) and triticale (719). The mean polymorphism information content values were 0.34 for rye DArT markers and 0.37 for those from triticale and wheat. High correlation was observed between similarity matrices derived from rye, triticale, wheat and combined marker sets, as well as for the cophenetic values matrices. Cluster analysis revealed genetic relationships among the accessions consistent with the agronomic and pedigree information available. The newly developed triticale DArT markers as well as those originated from rye and wheat provide high quality markers that can be used for diversity analyses and might be exploited in a range of molecular breeding and genomics applications in triticale.

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Introduction

Triticale (*X Triticosecale* Wittm.) is an intergeneric hybrid between wheat (*Triticum* sp.) and rye (*Secale* sp.) and has been synthesised at a range of ploidy levels (i.e. tetraploid, hexaploid, octoploid or decaploid) and genome constitutions. Of these different types, hexaploid triticales (AABBRR) have been subjected to extensive breeding and currently are the most commonly grown worldwide (Ammar et al. 2004). Like both its parents, triticale can be classified as spring or winter with respect to growth habit.

The interest in triticale emerges from the fact that it generally combines the high yield potential and high grain protein content of wheat with the disease resistance and environmental tolerance of rye (Barnett et al. 2006; Oettler 2005). Triticale is mainly used either for feed or forage, but

holds some promise for human nutrition (Nascimento et al. 2004; Oettler 2005). More recently, triticale has received attention as a potential industrial crop (e.g. biomass source for fuel production, biocomposite materials or chemicals) (Hills et al. 2007).

A variety of DNA-based molecular markers useful for plant improvements are available (Semagn et al. 2006a). These markers are powerful tools for identification of genotypes, tagging important traits and studying population structure and genetic diversity. Simple sequence repeats (SSRs) are commonly used because of their abundance and high level of polymorphism; their main disadvantage is high cost and research effort required for their development. To mitigate these problems, SSR markers developed for one species can be used in closely related species (Brown et al. 1996). Kuleung et al. (2004) found 57 and 39% transferability for 148 wheat and 28 rye SSR markers, respectively, with many producing additional DNA fragments in triticale. However, this transferability rate implies screening of a much higher number of wheat or rye SSR markers to identify those that can serve as reliable markers in triticale. In addition, factors such as ploidy levels and mutations may complicate the transferability (Dirlewanger et al. 2002). In a newly formed polyploid hybrid, rapid genomic modifications such as sequence modification and/or elimination usually occur in order to harmonize coexistence of different genomes in the same nucleus (Ma and Gustafson 2008; Ozkan et al. 2001; Soltis and Soltis 1999). These changes occur at a high rate immediately after hybridization and continue at a low rate during the first five generations and beyond (up to 35 generations) (Ma and Gustafson 2008).

Diversity arrays technology (DArT) assay is based on microarray hybridization that detects the presence versus absence of individual DNA fragments in "genomic representations" generated from genomic DNA samples through a process of complexity reduction (Jaccoud et al. 2001; Wenzl et al. 2004). The technology offers high-throughput DArT markers that can be rapidly developed for practically any genome, as both marker discovery and routine analysis are performed using the same hybridization-based assay. This type of markers was developed for a variety of species from Arabidopsis (Wittenberg et al. 2005) to cassava (Xia et al. 2005). In the context of triticale, DArT markers already developed for wheat (Akbari et al. 2006; Mantovani et al. 2008; Peleg et al. 2008) and rye (Bolibok-Brągoszewska et al. 2009) could provide a valuable resource.

The present study reports on the development and assessment of DArT markers in triticale. The objectives of this study were: (1) to develop a triticale diversity array for DArT genotyping, (2) to evaluate transferability of rye and wheat DArT markers in triticale, (3) to assess utility of DArT technology in diversity analyses on a set of 144 accessions representing a broad range of historic and current triticale germplasm.

Materials and methods

Plant material

This study is based on a collection of 174 (95 used for triticale array development and 144 for diversity study) spring and winter hexaploid triticale accessions (Table 1 and Supplemental Table S1). The accessions were kindly provided by many winter and spring triticale researchers around the world including Dr. E. Bauer, University of Hohenheim, Germany; Dr. G. Ittu, RICIC-Fundulea, Romania; Dr. R. Metzger, Corvallis Oregon, USA; Mr. P. Martinek, Kromirez, Czech Republic; Dr. S. Nalepa, Gilroy, CA, USA; Dr. A. Lukaszewski, Riverside, CA, USA and Dr. D. Salmon, Lacombe, AB, Canada.

Source of DNA

Genomic DNA of accessions originating from Canada and CIMMYT was extracted from leaves of 3-week old triticale seedlings using the DNeasy Plant Mini Kit following manufacturer's instructions (Qiagen Inc., Mississauga, Canada). DNA extractions of triticale from SW Seed Company were carried out using the protocol developed by Cheung et al. (1993). An additional cleaning step with 400 µl CHISAM (chloroform/isoamyl alcohol) was included. DNA from four individuals per genotype was bulked. DNA of all remaining accessions was extracted from the leaves of young plants using a standard CTAB protocol.

DNA concentration in each sample was adjusted to ca. 100 ng/µl. The DNA samples were shipped to Diversity Arrays Technology Pty. Ltd., Yarralumla, Australia for array development and genotyping.

Development of DArT markers for triticale

A set of 95 triticale accessions (Table 1 and Supplemental Table S1) was used to create a library of DArT clones using methods described previously (Akbari et al. 2006, Jaccoud et al. 2001). In this study, The *Pst*I/*Taq*I method of complexity reduction was selected as it was also used for development of DArT markers for wheat (Akbari et al. 2006) and rye (Bolibok-Brągoszewska et al. 2009). DNA of all samples used for array development was digested with *Pst*I and *Taq*I restriction enzymes and adaptors were simultaneously ligated to *PstI* overhangs (Akbari et al. 2006). After amplification with adaptor-specific primers (Wenzl et al. 2004) and verification of these resulting

Table 1 Country of origin, growth habit and pedigree for triticale accessions used for genetic diversity analyses

No.	Accession	Country[a]	Habit[b]	Pedigree
1	00L002010	CAN	S	88L012114/99P163
2	00L003004[c]	CAN	S	88L012114/99P143
3	00L003005	CAN	S	88L012114/99P143
4	00L033001	CAN	S	T153/97L008
5	02L006002	CAN	S	92L012010/94L043021
6	02L008002	CAN	S	94L028002/01P050
7	02L015004	CAN	S	94L043021/92L012010
8	02L017002[c]	CAN	S	94L043023/92L012010
9	03P110	MEX	S	POLLMER_4/5/TAPIRI//2*MUSK_7/4/FERAS_1/6/NIIR_1/HARE_265//ERIZO_9/5/7631-ED4B/Rl4137/7431A68E4/3/0TTER"S"/4/WAPITI/PANDA"R"203
10	03P124	MEX	S	PASSI_8/ERIZO_8//POLLMER_4/5/PIKA 5/YOGUI_1/4/7631-ED4B/Rl4137/7431A68E4/3/0TTER"S"
11	05P033[c]	MEX	S	POLLMER_2.2.1*2//FARAS/CMH84.4414
12	05P128	MEX	S	
13	05P148	MEX	S	MASSA/NIMIR_3/3/YOGUI_1/TARASCA 87_3/HARE_212/4/ARDI_1/TOPO 1419//ERIZO_9/3/PORSAS_2
14	05P179	MEX	S	
15	05P184	MEX	S	DAHBI_6/3/ARDI_1/TOPO 1419//ERIZO_9/4/2*ZEBRA 79/LYNX*2//FAHAD_1
16	05TF124[c]	USA	W	
17	05TG139[c]	USA	W	
18	06P005	MEX	S	
19	06P008	MEX	S	ERIZO_10/BULL_1-1//MANATI_1/4/SIKA 26/TESMO_3//LYNX/3/FEHAD_2
20	06P016	MEX	S	SUSI_2/5/TAPIR/YOGUI_1//2*MUSX/3/ERIZO_7/4/FARAS_1/6/VARSA_2/7/754.3/IBEX//BUF_2
21	06P022	MEX	S	DAHBI_6/3/ARDI_1/TOPO 1419//ERIZO_9/4/SONNI_3
22	06P024	MEX	S	
23	06P032	MEX	S	DAHBI_6/3/ARDI_1/TOPO 1419//ERIZO_9/4/ERIZO_11*2/MILAN
24	06P033	MEX	S	DAHBI_6/3/ARDI_1/TOPO 1419//ERIZO_9/4/RONDO/2*ERIZO_11
25	06P036	MEX	S	GAUR_2/HARE_3//JLO 97/CIVET/5/DIS B5/3/SPHD/PVN/YOGUI_6/4/KER_3/6/150.83//2*TESMO_1/MUSK 603
26	06P039	MEX	S	ARDI_1/TOPO 1419//ERIZO_9/3/PORSAS_2/4/POLLMER_3.5.1/5/KISSA_7-3//SIKA 26/HARE_337
27	06P045	MEX	S	POLLMER_2.2.1*2//FARAS/CMH84.4414
28	06P049	MEX	S	BULL_10/MANATI_1/3/2*POLLMER_1.2//ANOAS_5/STIER_13
29	89TT108[c]	MEX	S	
30	89TT126[c]	MEX	S	
31	90TT42	MEX	S	
32	94L028005	CAN	S	
33	94L038025	CAN	S	
34	94L039008	CAN	S	
35	94L040011	CAN	S	FAHAD 8-2/5/7631-ED4B/RL4137//7431A-68E4/3/OTTER-S/4/WAPITI/M2A(2)-BEAGLE X PANDA'R'203
36	94L040023	CAN	S	

Table 1 continued

No.	Accession	Country[a]	Habit[b]	Pedigree
37	94L040034	CAN	S	
38	94L041024[c]	CAN	S	
39	94L043019	CAN	S	
40	94L043035	CAN	S	
41	94L043037	CAN	S	
42	94L043060	CAN	S	
43	94L043063	CAN	S	
44	94L043067	CAN	S	
45	94L043069	CAN	S	
46	94L043089	CAN	S	
47	94L043092	CAN	S	
48	94L043107	CAN	S	
49	94L043112	CAN	S	
50	94L043135	CAN	S	
51	94L044009	CAN	S	
52	96L014004	CAN	S	PRONGHORN/88L012075
53	96L014013	CAN	S	PRONGHORN/88L012076
54	97L007014	CAN	S	
55	97L007019	CAN	S	
56	97L010037	CAN	S	
57	97L013021[c]	CAN	S	
58	97L013022	CAN	S	
59	97L014001	CAN	S	
60	97L014006	CAN	S	
61	97L020010	CAN	S	
62	99L003001	CAN	S	98LI03/PRONGHORN
63	99L003024	CAN	S	98LI03/PRONGHORN
64	99L004010	CAN	S	98LI02/PRONGHORN
65	99L005002	CAN	S	98LI04/PRONGHORN
66	99L005003	CAN	S	98LI04/PRONGHORN
67	99L005011	CAN	S	98LI04/PRONGHORN
68	99L005016	CAN	S	98LI04/PRONGHORN
69	99L005018	CAN	S	98LI04/PRONGHORN
70	99L006001[c]	CAN	S	98LI06/PRONGHORN
71	99L006012	CAN	S	98LI06/PRONGHORN
72	99L006018	CAN	S	98LI06/PRONGHORN
73	AC Ultima[c]	CAN	S	DAGRO/IBEX//CIVET#2
74	AD206[c]	Former USSR	W	AD1//BEZ1/SARATOV Krz
75	Agrano	DEU	W	
76	Algalo[c]	SWE	W	
77	Avangard[c]	RUS	W	
78	B163[c]	USA	W	
79	B164[c]	USA	W	
80	Benetto	POL	W	
81	Bienvenu	FRA	F	
82	Bobcat[c]	CAN	W	7631-ED4B/RL4137//7431A-68E4/3/Panther 'S'/4/ 87ED01
83	BOH17-11[c]	POL	W	
84	BOH 504[c]	POL	W	

Table 1 continued

No.	Accession	Country[a]	Habit[b]	Pedigree
85	Bunker[c]	CAN	S	PIKA-5/YOGUI-1//85L012006
86	CHD1190[c]	POL	W	
87	Clercal[c]	FRA	W	
88	Cultivo	NLD	W	
89	CW140881	MEX	S	
90	DC3[c]	POL	W	
91	Dinaro	POL	W	
92	Ego[c]	SWE	W	
93	FE4[c]	CAN	S	AC ALTA*5/PURENDO-38
94	Grenado	POL	W	
95	Inpetto	NLD	W	
96	IP21YTD4-11[c]	USA	W	
97	Kolor[c]	CZE	W	
98	Kortego	NLD	W	
99	Krakowiak[c]	POL	W	
100	KS88T/165[c]	USA	W	
101	Lasko[c]	POL	W	
102	Magistral[c]	FRA	W	
103	MAH201089[c]	POL	W	
104	Matinal	FRA	W	TORPEDO/MAGISTRAL//COLOSSAL
105	Meridal[c]	CHE	W	
106	Moderato	POL	W	
107	Moreno[c]	POL	W	
108	Mungis[c]	DEU	W	
109	NE03T407[c]	USA	W	
110	NE03T449[c]	USA	W	
111	NE422T[c]	USA	W	TRICAL/UB-UW26
112	No190[c]	BLR	W	
113	NT01435[c]	USA	W	85LT111/6A365//NE69150/3/76H75
114	NT01451[c]	USA	W	OMI-4MI-3MI/NE91T410
115	Piano[c]	DEU	W	
116	Pika[c]	CAN	W	81DEO1
117	Prego[c]	POL	W	
118	Presto[c]	POL	W	
119	Pronghorn[c]	CAN	S	WAPITI/79Q133001007
120	Puerto	NLD	W	
121	Ruslan[c]	RUS	W	
122	Saka3006[c]	DEU	W	
123	Salvo[c]	POL	W	M2A//274/320///7680
124	Sirius[c]	CHE	W	
125	Stan 1[c]	USA	W	
126	Stil[c]	ROM	W	5735TW/6741TW
127	SW Falmoro	NLD	W	
128	SW Fargo	NLD	W	
129	SW Talentro	NLD	W	
130	SZD61[c]	POL	W	
131	SZD1403(Gniewko)[c]	POL	W	
132	SZD1504(Gniewko)[c]	POL	W	

Table 1 continued

No.	Accession	Country[a]	Habit[b]	Pedigree
133	T05MXI_C38ITSN-28	MEX	S	ALPACA_1/3/ZEBRA31/CIVET//URON_5/7/CIN/PI//PATO/3/BGL/4/DRIRA/5/DLF99/3/M2A/SNP//BGL/4/TESMO_1/6/FAHAD_1/8/GAUR_3/ANOAS_2//BANT_1
134	T06M_F7HR-34[c]	MEX	S	LIRON_2/5/DIS B5/3/SPHD/PVN//YOGUI_6/4/KER_3/6/BULL_10/MANATI_1/7/DAHBI_6/3/ARDI_1/TOPO 1419//ERIZO_9
135	T06M_F7HR-40	MEX	S	HX87-244/HX87-255/5/PRESTO//2*TESMO_1/MUSX 603/4/ARDI_1/TOPO 1419//ERIZO_9/3/SUSI_2
136	T14317-P1[c]	MEX	S	
137	T14317-P3[c]	MEX	S	
138	TF2[c]	ROM	W	WT/TCL3
139	Titan[c]	ROM	W	PLAI//12TK/121TJ
140	Trilstar[c]	ROM	W	340TO/DROPIA//188TR
141	Trimaran[c]	DEU	W	
142	Tyndal[c]	CAN	S	NIMIR-1/HARE-265//ERIZO-9/3/88L012
143	Valentin[c]	RUS	W	
144	Vinoko[c]	SWE	W	

[a] Country of origin. *BLR* Belarus, *CAN* Canada, *CHE* Switzerland, *CZE* Czech Republic, *DEU* Germany, *FRA* France, *MEX* Mexico, *NLD* Netherlands, *POL* Poland, *ROM* Romania, *RUS* Russia, *SWE* Sweden, *USA* United States of America, *USSR* Soviet Union

[b] Growth habit. *F* facultative type, *S* spring type, *W* winter type

[c] Accessions also used for the development of the dedicated triticale DArT array

amplicons on 1.2% agarose gels equal volumes of amplicons from each accession were mixed and cloned into the pCR 2.1 vector.

Array composition and DArT genotyping methods

Three arrays, consisting of previously developed rye and wheat probes and newly developed triticale probes, were used to genotype all 144 accessions listed in Table 1. The first array contained 12,280 wheat probes, 1,536 rye probes and 1,536 triticale probes. The second array contained 13,440 wheat probes and 1,536 triticale probes. The third array contained 11,520 rye probes. The arrays were printed with 48 metal pins using a MicroGridII arrayer (Biorobotics, Cambridge, UK) onto poly-L-lysine-coated slides (Erie Scientific, Portsmouth, USA). Arrays were hybridized with fluorescently labeled targets from all genotypes used for the array development. All targets were prepared using *PstI/TaqI* complexity reduction method as described above with two replicated targets analyzed for each genotype so that the technical reproducibility of markers discovered could be easily evaluated. Only markers with the maximum of a single discordant score among technical replicates were included in the results.

After overnight hybridization at 62°C, the slides were washed and scanned using a Tecan LS300 (Grödig, Salzburg, Austria) confocal laser scanner. Hybridization and washing conditions led to identification of hybridizing sequences with 95% or higher identity to the immobilized probes.

Image analysis and polymorphism scoring

Microarray image analysis was performed using DArTsoft program, version 7.4.3, integrated into and interfaced with DArTdb Laboratory Information Management System, so that images were extracted from database for processing and data stored directly in the database. Spots were localized in a fully automatic manner using a "seeded region growing" approach to image segmentation. Polymorphism and scoring was performed using DArTsoft v.7.4.3 using fuzzy c-means clustering. Scoring of markers was done in a dominant manner (0/1) for the array features with bimodal distribution of relative signal intensities. DArTsoft also extracted the percentage of DNA samples with defined '0' or '1' allele calls (call rate), the polymorphism information content (PIC) values and several additional technical quality parameters. The markers used in this study were selected with a call rate >79%, and PIC >0.01.

Diversity analysis

A collection of 144 accessions were screened on the genotyping array as previously described (Akbari et al.

2006). Two replicates sets: the Polish cultivar Gniewko (no. 131 and 132) and the CIMMYT advanced breeding line T14317 (no. 136 and 137), were part of the collection. Some of the Canadian advanced breeding lines were closely related and were also included in collection to evaluate the discrimination power of DArT markers. The country of origin, growth habit, and pedigree (where available) of the accessions tested are presented in Table 1, with those that were part of the set of accessions used for triticale array development annotated.

DArT polymorphism was used to construct a binary matrix which was subsequently transformed to a genetic similarity matrix using Dice coefficient of similarity (Dice 1945). The genetic similarity matrix was analyzed using the unweighted pair group method with arithmetic average (UPGMA) algorithm and a dendrogram was produced (Rohlf 1997). To determine the discrimination power of markers from different sources, data acquired from rye, triticale and wheat were separately employed to generate the matrices of genetic similarity and cophenetic values. The cophenetic matrix encodes the similarity relationships between a set of objects as defined by the output of a tree (Rohlf 1997). The correlations between individual matrices of genetic similarity as well as individual and combined matrices were calculated and statistically tested by the Mantel matrix-correspondence test with 9,999 random permutations (Mantel 1967). The matrices are plotted one against the other, element by element and the product-moment correlation, r, is given as a measure of the degree of relationship between them. The degree of fit can be interpreted as follows: $r \geq 0.9$, very good fit; $0.8 \leq r < 0.9$, good fit; $0.7 \leq r < 0.8$, poor fit; $r < 0.7$, very poor fit (Rohlf 1997). Similar analyses were computed for individual and combined cophenetic value matrices. Computations were performed with appropriate procedures of the software NTSYSpc version 2.02e (Exeter Software, Setauket, NY). The goodness-of-fit (r_{coph}) and robustness (1,000 replicates) of the dendrogram were tested using the freeware PAST version 1.83 (http://folk.uio.no/ohammer/past). Additionally, the resulting scoring from the 144 accessions was used to determine the present frequency of each DArT marker in the collection.

Results

DArT array development and transferability of markers from other species

The diversity study involved 144 accessions of spring and winter triticale from 14 countries (Table 1) tested with large collections of wheat and rye DArT probes (25,720 and 13,056, respectively) as well as 3,072 triticale (Table 2). DArTsoft identified 6,042 DArT markers as polymorphic (Tables 2, 3 and Supplemental Table S2). The overall marker quality was good, with approximately 72% of markers with call rates above 90% and a mean call rate of 93% (Fig. 1a). The quality of markers in the final genotyping array is expected to be even higher, as the probes printed in the marker discovery arrays do not go through the same quality control process as probes for the routine genotyping arrays. The accession call rates ranged from 80 to 97% (Fig. 1b), with only 20% of them having call rates below 90% which was probably due to low DNA quality and/or heterozygosity/heterogeneity of the populations used for DNA extraction.

Among polymorphic markers those for rye were the most abundant (3,109), followed by wheat (2,214) and triticale (719) (Table 3). Of the total number of polymorphic markers, the chromosome location was known for 455 wheat DArT markers, including 177 markers for the A-genome, 258 for the B-genome, and 20 for the D-genome (Table 3). Based on their present (allele "1") frequency, the 6,042 markers can be classified as low, moderate, and highly present (1,407, 2,001 and 2,634, respectively) in triticale accessions (Table 3). The mean PIC values ranged from 0.34 for rye DArT markers to 0.41 for those from wheat D-genome while a value of 0.36 for all the 6,042 DArT markers was observed among the germplasm collection (Table 3).

From a total of 6,042 DArT markers evaluated, 4,964 and 5,746 markers were polymorphic when triticale accessions were separated on their spring or winter growth habit, respectively. The mean PIC values were very similar between the spring (0.37) and winter (0.36) triticale accessions (Supplemental Table S2).

A comparison of markers from different sources

Unequal numbers of polymorphic markers were identified for each donor source (Tables 2, 3) and were used to generate individual and combined genetic similarity matrices and the appropriate dendrograms. To estimate differences between dendrograms for each marker set, a

Table 2 Polymorphism level of DArT probes with different origin among 144 triticale accessions

Donor species	Total number of DArT probes	No. of polymorphic markers in triticale	Polymorphism level (%)[b]
Rye	13,056	3,109	23.8
Triticale	3,072	719	23.4
Wheat[a]	25,720	2,214	8.6

[a] Wheat probes were developed from bread and durum wheat

[b] Percentage of probes polymorphic among 144 triticale accessions

Table 3 Distribution of polymorphic rye, triticale and wheat DArT markers in germplasm collection based on presence frequency and their polymorphism information content range

	Presence frequency (%) of polymorphic markers in triticale germplasm[a]			Total number of markers/Mean PIC ± SD
	0.7–33	33–66	66–99.3	
Rye				
Number of markers	727	1,012	1,370	3,109
PIC range	0.01–0.46	0.42–0.50	0.01–0.47	0.34 ± 0.14
Triticale				
Number of markers	127	224	368	719
PIC range	0.06–0.46	0.43–0.50	0.01–0.46	0.37 ± 0.11
Wheat A-genome[b]				
Number of markers	51	74	52	177
PIC range	0.06–0.43	0.43–0.50	0.05–0.45	0.36 ± 0.13
Wheat B-genome[b]				
Number of markers	76	74	108	258
PIC range	0.03–0.45	0.43–0.50	0.02–0.46	0.35 ± 0.12
Wheat D-genome[b]				
Number of markers	3	11	6	20
PIC range	0.29–0.37	0.44–0.50	0.23–0.43	0.41 ± 0.11
Wheat unlocated				
Number of markers	423	606	730	1,759
PIC range	0.02–0.46	0.42–0.50	0.01–0.47	0.37 ± 0.09
All combined				
Total number of markers	1,407	2,001	2,634	6,042
PIC range	0.01–0.46	0.42–0.50	0.01–0.47	0.36 ± 0.13

PIC polymorphism information content, *SD* indicates standard deviation

[a] Presence frequency was calculated based on total number of successfully scored accessions for a given marker, accessions with missing data were excluded

[b] Polymorphic markers known to be located on A-,B-, and D-genomes were identified for all seven chromosome pairs except for chromosomes 6 on D-genome

new set of matrices based on the cophenetic values were constructed and compared. The product-moment correlation calculated between the genetic similarity matrices of individual sets (rye, triticale and wheat) was always higher than 0.80 (Table 4) suggesting that these markers from different donor sources give similar estimates of genetic relationship among the accessions tested. Similarly, the correlation between matrices of cophenetic values from the dendrograms based on individual markers sets was higher than 0.80 (Table 4) indicating good correspondence between these dendrograms. High correlations were also observed when matrices based on individual sets (rye, triticale and wheat) and the entire data set (all combined) were compared (Table 4). As a result of the good overall fit between dendrograms based on individual and combined data sets, only the dendrogram generated from the combined set is presented and further discussed.

Genetic relationship among triticale accessions

Genetic similarity among 144 accessions based on all 6,042 DArT markers identified ranged from 0.47 to 0.99. The most divergent were Avangard (no. 77), a winter triticale from Russia, and 06P049 (no. 28) a spring triticale line from Mexico, with a similarity coefficient of 0.47. A very good fit of the genetic similarity matrix data to the tree topology ($r_{coph} = 0.89$) was observed (data not shown). The estimated reliability of the branches of the tree was also supported by bootstrap values which are shown, if higher than 50%, in the dendrogram (Fig. 2).

Based on genetic distances, all accessions tested can be divided into three major clusters, with cluster I containing only two accessions, Valentin (no. 143) and AD206 (no. 74). Cluster II comprised winter accessions while cluster III includes spring accessions with a single exception. The exception is winter line Matinal placed in cluster III.

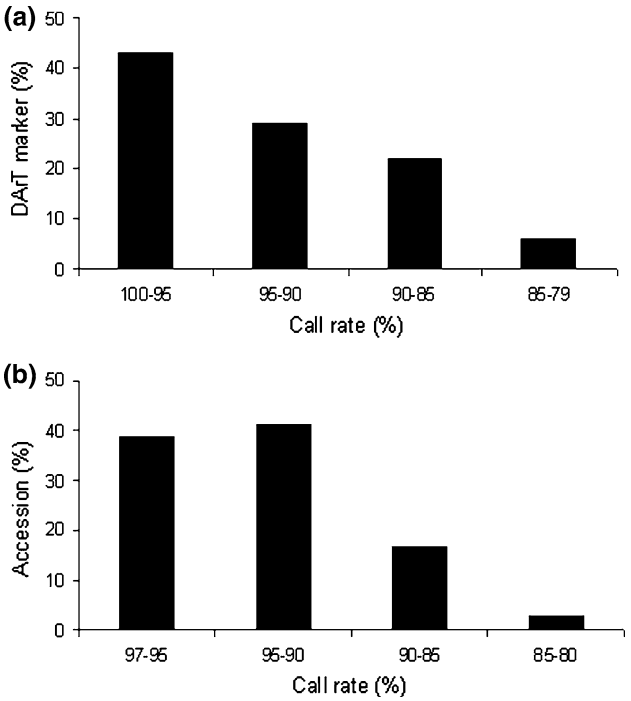

Fig. 1 Distribution of call rate among the 6,042 DArT markers (**a**) and the 144 triticale accessions (**b**)

Table 4 Correlation of cophenetic value (above the diagonal) and similarity coefficient (below the diagonal) derived from rye, triticale, wheat and combined sets of markers

	Rye	Triticale	Wheat	All combined
Rye	–	0.81*	0.83*	0.98*
Triticale	0.89*	–	0.95*	0.84*
Wheat	0.88*	0.96*	–	0.88*
All combined	0.97*	0.96*	0.97*	–

* Statistically significant ($P = 0.0002$)

Clusters II and III were further subdivided into three subgroups, each generally matching its geographic origin: Europe and North America (II-a), Poland and Netherlands (II-b), North America (II-c), France (III-d), Canada and Mexico (III-e), and Mexico (III-f).

Discussion

The total number of DArT markers identified, 6,042, is a function of the overall level of DNA polymorphism in the analyzed germplasm collection and the complexity reduction method used. Of the polymorphic markers, 33 and 44% were in the moderate and high frequency classes and only 23% in the low frequency class, suggesting a modest frequency of loci with rare alleles in the analyzed sample of accessions. However, the fuzzy c-means clustering implemented in DArTsoft does not efficiently detect markers with the frequency below 1–2%. This software treats rare presence ("1" allele) or absence ("0"allele) as outliers and not as small clusters. As high PIC markers have higher utility in genetic mapping and marker-trait association analysis, the bias against markers with very rare alleles does not limit the applications of arrays in breeding and genetics of triticale. Relative to the conventional linkage mapping based on allele segregation among progeny of a specific cross, in the association mapping approaches (i.e. linkage disequilibrium-based mapping strategies), low frequency markers do not remain unnoticed. However, the power of testing marker-trait associations seems to be reduced when this type of markers are included in the test (Crossa et al. 2007; Malosetti et al. 2007).

A larger number of polymorphic markers (3,019) detected here that originated from rye could indicate greater degree of polymorphism of R-genome. This appears reasonable given the allogamous nature of rye. The transferability of rye DArT markers to wheat and vice versa has not yet been tested hence, the degree of homeology between wheat and rye DArT markers is not very clear even though high hybridization stringency was applied (>95% sequence identity). Further studies are needed to clarify the specificity levels of those markers and to identify their chromosome locations. For selected sets of rye or wheat genome-specific markers, single-marker assay format could be developed upon request, given that DArT markers are sequence-ready clones of genomic DNA. These markers would enable analyses of chromosome composition, as well as detection of recombination and translocation events in triticale, replacing labor intensive methods such as chromosome banding and in situ hybridization. A recent analysis of DArT clones in oat has shown that over one-third of them contain DNA sequence with strong homology to functionally annotated genes (Tinker et al. 2009).

In this study, 56% of the wheat markers with previously known chromosomal location originated from B-genome. This may be a direct consequence of different numbers of DArT markers initially mapped to different wheat genomes. Higher numbers of DArT markers mapped to the B-genome was reported in hexaploid (Semagn et al. 2006b) and tetraploid wheat (Mantovani et al. 2008); the same tendency was also observed for other types of markers (Lotti et al. 2000; Röder et al. 1998).

Only 20 markers originating from the D-genome were identified here. This reflects the general constitution of hexaploid triticale, AABBRR. Variable number of rye chromosome pairs was previously reported to be replaced by D chromosomes of wheat in hexaploid spring triticale lines (Gustafson and Bennett 1976; Pilch 1989) as well as

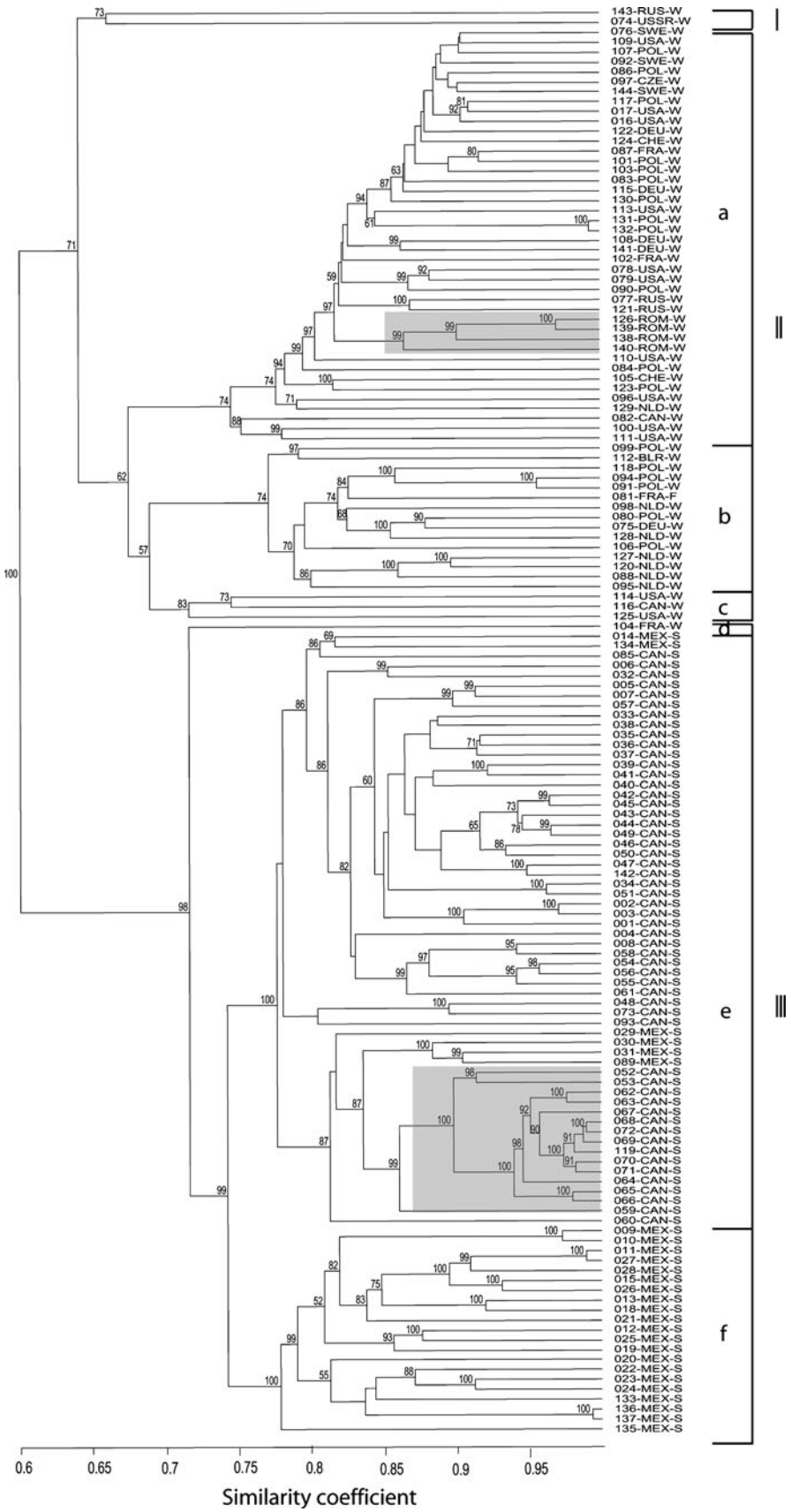

Fig. 2 Dendrogram of 144 triticale accessions originating from 14 countries estimated by Dice similarity coefficient based on 6,042 DArT markers with different origins. Bootstrap support values (1,000 replicates) are shown if greater than 50%. Shadowed areas indicate the Romanian group and the Canadian Pronghorn-derived lines group. The designation is the accession number followed by country of origin and growth habit (refer to Table 1)

replacement of various A- and B-genome chromosome pairs (Gustafson et al. 1989). These substitutions were less common in winter triticale lines (Lukaszewski 1988). Recently, amplification of the D-genome-specific SSR markers in both spring and winter triticale have been reported (Kuleung et al. 2006; Leonova et al. 2005; Tams et al. 2004). Alternatively, the D-genome markers reported here might identify loci in the A-, B-, or R-genomes. Their specificity and actual chromosome locations require further study. Such D-genome-specific markers could be valuable molecular tools in breeding programs aiming introgression of D-genome chromatin into hexaploid triticale. It is believed that some characteristics of hexaploid triticale could be improved by introducing D-genome chromosomes or their segments (Budak et al. 2004; Lukaszewski and Curtis 1994).

The mean PIC value (0.37) observed for the new DArT markers in triticale is fairly high for randomly selected bi-allelic loci, and comparable to those previously observed for barley (0.38) (Wenzl et al. 2006). Comparable mean PIC values were observed in the present study for rye (0.34) and for all wheat markers (0.37), and could indicate that overall these sets of markers explained similar genetic variability of the rye and wheat genomes of triticale. Tams et al. (2004) observed a greater variation in the wheat genomes (0.60) than in rye genome (0.45) in triticale, while Kuleung et al. (2006) reported similar mean values for rye and wheat markers (0.53 and 0.55, respectively) based on SSR markers. However, the mean PIC values of the polymorphic DArT markers in triticale that originated from rye and wheat are lower than those observed either by Tams et al. (2004) or Kuleung et al. (2006) for the SSR markers. The lower PIC values of DArT markers seem to be advantageous in cluster analyses because they lead to a more defined genetic structure of accessions from distant geographic regions or genetic origin (Mantovani et al. 2008; Stodart et al. 2007).

A high correlation of similarity matrices as well as cophenetic value matrices was observed for rye, triticale and wheat DArT markers. These individual DArT marker sets seemed to be equally suitable for triticale genome analysis. Similar findings were reported by Kuleung et al. (2006) when wheat and rye SSR markers were compared in triticale.

The cluster analysis on combined marker sets separated the lines basically into two large clades based on the growth habit. This can be interpreted in two ways: either DArT markers were linked to the growth habit trait, or there is a substantial separation of the winter and spring triticale genepools. Given the large number (6,042) of polymorphic markers analyzed, linkage effect is unlikely. On the other hand, the reluctance of winter triticale breeders to make crosses between spring and winter accessions is well-known. Most likely a majority of these markers are linked to various traits of agronomic relevance as previously observed for barley (Wenzl et al. 2006) and wheat (Crossa et al. 2007). The clear separation of the two genepools is probably a reflection of the different breeding efforts made in spring and winter triticales and little gene flow between them. Many of spring triticales tested here were generated and developed at CIMMYT, Mexico, and distributed around the world, while winter accessions were developed primarily in Europe, with some separation of breeding efforts. Similar distinct clusters of spring and winter types were reported by Amiour et al. (2002) based on allelic frequencies of glutenin and secalin loci, and by Royo et al. (1995) based on 14 different agronomic and morphological characteristics. However, Kuleung et al. (2006) using 57 wheat and rye SSR markers did not observe a very distinct separation of spring and winter types. In the present study, the French winter accession Matinal (no. 104), subcluster III-d, originating from a cross between winter cultivar Torpedo and winter cultivar Magistral followed by a cross to the spring cultivar Colossal (Table 1) did not group with its parent Magistral (no. 102) in cluster II. The inclusion of Matinal in cluster III could be attributed to common ancestry with spring CIMMYT lines since one of its parents, Colossal, was selected from an early generation of CIMMYT material (Bouguennec, personal communication). On the other hand, the French facultative cultivar Bienvenu (no. 81) grouped with winter accessions in subcluster II-b.

The historic winter triticale line AD206 (no. 74) and the Russian winter triticale line Valentin (no. 143) formed a separate cluster (cluster I) suggesting their genetic distinction from other Russian or European winter lines. Kuleung et al. (2006) using SSR markers also reported as a singleton a Russian winter triticale line (Spontannyj Kanova 1185), although the other winter or spring Russian lines tested were grouped together.

Subcluster II-a included winter triticale accessions from Europe and North America. No distinct subgrouping of accessions from the same breeding source was observed, with the exception of the Romanian lines. Tams et al. (2004, 2005), who used wheat and rye SSR markers to study the genetic diversity of European winter triticale, reported no clear grouping within the tested germplasm pool. Only the genotypes developed by Nordsaat (Germany) and RICIC (Romania) constantly formed distinct subclusters in their studies. They suggested that the absence of clear clustering is because European triticales are bred for adaptability to a wide range of environments and for only one end-use (i.e. feed grain). It could also be indicative of active germplasm exchange programs among different European breeding centers. The strong collaboration between two European triticale breeding programs, Poland and Netherlands, is

reflected by subcluster II-b (Vrolijk, personal communication). Two American winter lines (no. 114 and no. 125) and the Canadian winter line Pika (no. 116) formed subcluster II-c, while all remaining winter North American lines grouped with the European lines in subcluster II-a. This may again reflect considerable germplasm exchange among breeding programs. Such exchange is probably the basis for grouping in Subcluster III-e with Canadian and a few CIMMYT spring lines. Canadian and CIMMYT breeding programs collaborated closely in the initial efforts to develop spring triticale (Ammar et al. 2004). However, the majority of CIMMYT lines were grouped together in a distinct subcluster (III-f).

The replicate samples of cv. Gniewko (no. 131 and 132) clustered with a very high similarity coefficient of 0.9932 (Fig. 2 subcluster II-a) and those of T14317 line (no. 136 and 137) with a very high similarity coefficient of 0.9934 (Fig. 2 subclaster III-f). These replicates represent DNA samples extracted from different plants within each accession, and show variation in only 39 (T14317) or 44 (Gniewko) of the 6,042 DArT markers tested. This level of difference (0.6 and 0.7%, respectively) can be interpreted as intravarietal heterogeneity. Similar levels of heterogeneity were observed in cultivars of barley (0.5–1.3%) (Wenzl et al. 2004) and wheat (0.2–1.4%) (Akbari et al. 2006). Such a high resolution level of DArT markers suggests the fact that they could be used in seed purity and genetic identity tests in triticale.

Accessions sharing a parent or a similar pedigree tended to cluster together. This serves as an internal control, providing confidence that the DArT marker assay is reliable. For example, the Romanian winter accessions sharing TF2 (no. 138) line in their pedigrees were tightly grouped (Fig. 2 shadowed area in subcluster II-a). Titan (no. 139) has the cv. Plai as a parent (Table 1) which is also found in Stil' (no. 126) and Trilstar's (no. 140) pedigrees. A parent of cv. Stil, 5735TW, originates from the Plai/143TW cross and a parent of cv. Trilstar, 188TR, is derived from the Plai//235TJ/58TJ cross while one of Plai parents is TF2 (Ittu, personal communication). Among spring lines, all Canadian lines (no. 52, 53 and 62–72) sharing Pronghorn (no. 119) as a parent cluster together (Table 1; Fig. 2 shadowed area in subcluster III-e); Canadian sister lines (no. 002 and 003) as well as CIMMYT sister lines (no. 011 and 027) were also tightly grouped (Table 1; Fig. 2 subcluster III-e and -f, respectively). Most pedigrees of triticale accessions are complicated, unknown, or not entirely reliable. Therefore, it is impossible to estimate the relationship among various accessions, based on co-ancestry coefficient (*f*). In addition, the assumption in calculating *f* is that all ancestors are homozygous and homogeneous which may not be the case in triticale. Tams et al. (2004, 2005) reported in triticale that genetic relationships based on molecular markers did not fully agree with the pedigree information.

DArT marker profiling effectively depicted genetic relationships among the triticale accessions with clear separation of spring and winter types. This appears to support the distinction of the two main gene pools of hexaploid triticale. Moreover, the accessions grouped in subclusters that generally matched their geographic origin and/or active germplasm exchange between various breeding programs. DArT-derived clusters also reflected accessions pedigree backgrounds, differentiating genetically closely related lines. Therefore, the set of 6,042 DArT markers tested here, that includes markers newly developed for triticale as well as markers transferred from rye and wheat, represent a very efficient tool for investigating genetic diversity in triticale. These markers will effectively complement existing technologies in triticale breeding and genomics. It is known from previous development and applications of DArT markers in other plant species that they can be used very effectively for fine mapping, facilitating quantitative trait locus identification, genome background screening, whole-genome marker-assisted selection or accelerated introgression of selected genomic regions. With this completed work, it will be now possible to establish DArT-based molecular linkage maps in spring and winter triticale, in order to facilitate genome analysis and marker-assisted selection for breeding purposes.

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