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Research Paper

Association analysis of resistance to cereal cyst nematodes (*Heterodera avenae*) and root lesion nematodes (*Pratylenchus neglectus* and *P. thornei*) in CIMMYT advanced spring wheat lines for semi-arid conditions

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To identify loci linked to nematode resistance genes, a total of 126 of CIMMYT advanced spring wheat lines adapted to semi-arid conditions were screened for resistance to *Heterodera avenae*, *Pratylenchus neglectus*, and *P. thornei*, of which 107 lines were genotyped with 1,310 DArT. Association of DArT markers with nematode response was analyzed using the general linear model. Results showed that 11 markers were associated with resistance to *H. avenae* (pathotype Ha21), 25 markers with resistance to *P. neglectus*, and 9 significant markers were identified to be linked with resistance to *P. thornei*. In this work we confirmed that chromosome 4A (~90–105 cM) can be a source of resistance to *P. thornei* as has been recently reported. Other significant markers were also identified on chromosomal regions where no resistant genes have been reported for both nematodes species. These novel QTL were mapped to chromosomes 5A, 6A, and 7A for *H. avenae*; on chromosomes 1A, 1B, 3A, 3B, 6B, 7AS, and 7D for *P. neglectus*; and on chromosomes 1D, 2A, and 5B for *P. thornei* and represent potentially new loci linked to resistance that may be useful for selecting parents and deploying resistance into elite germplasm adapted to regions where nematodes are causing problem.

Key Words: association mapping, bread wheat, DArT, cereal cyst nematode, root lesion nematode, QTL.

Introduction

Wheat is one of the three most important food crops and has been cultivated for thousands of years in Europe, West Asia, and North Africa. It is grown on 20% of the global cultivated land area and is the main food resource for 40% of the world's population (Braun *et al.* 2010). Increases in cereal production are limited by many biotic and abiotic factors, including—in rainfed regions—plant parasitic nematodes (Dixon *et al.* 2009). The cereal cyst nematode (CCN) *Heterodera avenae* and root lesion nematodes (RLNs), including *Pratylenchus neglectus* and *Pratylenchus thornei*, are considered to cause the most damage to temperate cereals and are the most economically important species globally (Nicol and Rivoal 2008, Rivoal and Nicol 2009, Vanstone *et al.* 2008). In wheat, cereal nematodes are managed through strategies such as crop rotation, in addition fallow and tillage practices are used. Crop rotation, using resistant varieties and lines with different tillage techniques, is recommended for controlling CCNs, while management of RLNs is more dependent on resistant varieties as RLNs have a broader host range (Andersen and Andersen 1982,

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Dababat *et al.* 2015, Williams *et al.* 2002).

The use of resistant and tolerant wheat varieties offers the most effective, economic, and environmentally friendly option of controlling nematodes. Sources of resistance to cereal nematodes include cultivated wheat varieties, synthetic wheat and its wild relatives such as *Aegilops tauschii* (Coss.) and *Triticum turgidum* (Van Slageren 1994). Genetic resistance to the cereal nematodes complex is still being sought; the effectiveness of resistance depends on the strength and durability of the resistance source, and on the correct identification of the nematode species and/or pathotypes (Nicol and Rivoal 2008). To date, 11 CCN resistance genes conferring predominately dominant or partial resistance have been catalogued. Previously, CCN resistance genes *Cre1* and *Cre8* have been reported in *T. aestivum* (Slootmaker *et al.* 1974, Williams *et al.* 2002); *Cre2*, *Cre5*, and *Cre6* in *Ae. ventricosa* (Delibes *et al.* 1993, Jahier *et al.* 1996, Ogbonnaya *et al.* 2001); *Cre3* and *Cre4* in *Ae. tauschii* (Eastwood *et al.* 1994, Eastwood 1995); *Cre7* in *Ae. truincialis* (Romero *et al.* 1998); *CreX* and *CreY* in *Ae. variabilis* (Barloy *et al.* 2007); and *CreR* in *Secale cereale* (Asiedu *et al.* 1990). *Cre1* is inherited as a dominant allele and confers resistance to Australian and several European cyst nematodes, while *Cre2* is resistance to the *H. avenae* populations Ha71 (Spanish), Ha11 (British), and Ha12 and Ha41 (French), but is ineffective against HgI–HgIII (Swedish) and Ha13 (Australian) (Delibes *et al.* 1993, Ogbonnaya *et al.* 2001). *Cre3* and *Cre6* are also inherited as dominant alleles but exhibit stronger resistance than *Cre1* against the Ha13 pathotype, though they are susceptible to the European pathotypes Ha11 and Ha12 (Ogbonnaya *et al.* 2001). *Cre2*, *Cre4*, *Cre5*, and *Cre8* are partially resistant to Ha13.

Similarly, sources of resistance to RLNs have been previously reported in wheat and its wild relatives including *Ae. tauschii*, *T. urartu*, *T. monococcum*, and *T. turgidum* (Sheedy 2004, Sheedy *et al.* 2012, Thompson and Haak 1997). Some bread wheat varieties show complete or partial resistance to *Pratylenchus spp.* (Thompson 2008, Thompson *et al.* 2008). Resistance to *P. neglectus* is partial and quantitative; only one gene "*Rlnn1*" originating from the wheat cv. Excalibur to *P. neglectus* mapped on chromosome 7AL in a double haploid population of wheat has been identified and validated thus far (Thompson *et al.* 2009, Williams *et al.* 2002), and some other major quantitative trait loci (QTL) for *P. thornei* were identified on chromosomes 2BS, 6DS, and 6DL. Similarly, Zwart *et al.* (2010) identified two QTL for *P. neglectus* resistance on 2BS and 6DS in a synthetic backcross-derived population. Two major QTL for *P. neglectus* resistance (2BS, 6DS) and three for *P. thornei* resistance (2BS, 6DS, 6DL) were detected in the wheat landrace accession "AUS2845" from Iran (Smiley and Nicol 2009). In another study, QTL for resistance to *P. thornei* have been identified on the 1B, 2B, 3B and 4D, 6D chromosomes in the investigated sources (Toktay *et al.* 2006). In a recent study, seven new QTL were identified on chromosomes 1D, 4D, 5B, 5D, and 7D for resistance to CCNs, and 4A, 5B, and 7B for resistance to *P. neglectus* in synthetic hexaploid lines. (Mulki *et al.* 2013). To date limited number of QTL for nematode resistance have been validated by traditional QTL mapping approach.

Association mapping or linkage disequilibrium (LD) mapping is an alternative approach to biparental phenotype–genotype association that does not require development of parental crosses and higher mapping resolution may be achieved with many more meiotic recombination events. In view of the advantages and applications of association mapping, it can be applied to develop molecular markers for nematode resistance in wheat. In this study, we analyzed the association of Diversity Arrays Technology (DArT) markers with resistance to CCNs and RLNs in a spring wheat nursery of the International Maize and Wheat Improvement Center (CIMMYT). The resistant lines are warranted, but also the related markers for these traits can be effectively used, to lower large phenotyping cost and increase accuracy of combining genes. Therefore, the major objective of this study was to find novel sources of resistance to *H. avenae* and *Pratylenchus sp.* in a core set of spring bread wheat panels and to map the genomic regions associated with their resistance for further pyramiding into elite varieties.

Materials and Methods

Genetic resources

A total of 126 CIMMYT advanced spring wheat lines from the 29th Semi-Arid Wheat Screening Nursery (29th SAWSN) were phenotypically evaluated for resistance to *H. avenae*, *P. thornei*, and *P. neglectus* under controlled growth room conditions at the Biological Control Research Station in Adana, Turkey. A subset of 107 lines was genotyped and used for the association study. These lines (normally F_6) derived from a set of 53 advanced lines and represent typical high-yielding germplasm developed at CIMMYT-Mexico with a certain degree of tolerance to moisture stress. The detailed pedigree for this set is presented in **Supplemental Table 1**.

Phenotyping

For *H. avenae* inoculum preparation, soil samples were collected from a field in Adana, Turkey (latitude 39°24′13″; longitude 32°37′14″). This population belongs to *H. avenae* Ha21 pathotype of the Ha1 group. *H. avenae* cysts were extracted according to Cobb's decanting and sieving method (Cobb 1918) and by using the Fenwick-Can technique (Fenwick 1940). Cysts were collected and surface sterilized with 0.5% NaOCl for 10 min and rinsed several times in distilled water. The cysts were kept in a refrigerator at 4°C for 4 months before being transferred to room temperature (10–15°C) to induce hatching. The freshly hatched second stage juveniles (J2) were then used as inoculum in screening tests.

Single wheat seeds were planted in standard small tubes (16 cm in height × 2.5 cm in diam.) filled with a sterilized

Table 1. List of checks used in this study to screen *Heterodera avenae*, *Pratylenchus neglectus*, and *Pratylenchus thornei*, and their resistance reactions

Cross Name	CIMMYT-TK Acc #	Type	OC	*H. avenae*	*P. thornei*	*P. neglectus*	References
SERI	951027	SW	MX	S	S	S	Toktay *et al.* 2012
MILAN	990659	SW	MX	S	ND	ND	
SILVERSTAR	031017	SW	AUS	R	ND	ND	
CROC_1/AE.SQUARROSA (224)//OPATA	020615	SW	MX	R	ND	ND	de Majnik *et al.* 2003
GATCHER	20611	SW	AUS	ND	S	S	Toktay *et al.* 2012
CROC_1/AE.SQUARROSA (224)//OPATA	20616	SW	MX	ND	R	R	Thompson *et al.* 2009
CROC_1/AE.SQUARROSA (224)//OPATA	20617	SW	MX	ND	R	ND	Thompson *et al.* 2009
GS50A	20596	SW	AUS	ND	ND	R	Thompson *et al.* 2009

TK ACC # = Turkish accession number; OC = origin country; SW = spring wheat; MX = Mexico; AUS = Australia; S = Susceptible; R = Resistant; ND = Not determined.

mixture of sand, field soil, and organic matter (70:29:1 v/v). The field soil and sand were sieved and sterilized at 110°C for two h on two successive days, whereas the organic matter was sterilized at 70°C for five h. After plant emergence, 7 tubes (replicates) were selected per genotype and were inoculated with 400 freshly hatched second stage juveniles (J2) in three holes made around the stem base. In the week after nematode inoculation, plants were gently watered to increase the efficiency of nematode penetration. Plants were left to grow in a growth chamber with 16 h of artificial photoperiod at a temperature of 22 ± 3°C, and with 70% relative humidity. The experiments were conducted in Randomized Complete Block with 7 replications and repeated once for data validation. Based on the period of time needed for cyst formation, plants were harvested 9 weeks after J2 inoculation. Soil from each tube was collected in a 2-litre pot filled with water for cyst extraction; this step was repeated three times to ensure that all cysts were successfully extracted, while roots were washed on nested sieves with 850 µm and 250 µm mesh sizes to free cysts from the root system. Cysts from both root and soil extractions were collected on the 250 µm sieve and counted under a stereomicroscope.

The nematode reproduction values obtained were used to classify plant resistance relative to the control varieties presented in **Table 1**. Wheat genotypes were recorded with mean cyst number and classified into 5 groups based on mean number of females and cysts recorded per plant (Dababat *et al.* 2014), and according to the check lines used where resistant lines normally have less than 5 cysts per root system. The groups were: Resistant (R) = equal or fewer cysts than in a known resistant check; Moderately Resistant (MR) = slightly more cysts than in a resistant check; Moderately Susceptible (MS) = significantly more cysts than in a resistant check, but not as many as in the susceptible check; Susceptible (S) = as many cysts as in the susceptible check and number of cysts per root system considered damaging; and Highly Susceptible (HS) = more cysts than in the susceptible check.

For the RLNs, each seed was inoculated with 400 nematodes of *P. neglectus* or *P. thornei* originated from nematodes reared on carrot discs as described by Moody *et al.* (1973). Nine weeks after nematode inoculation, the plants were harvested, shoots were removed, and *P. thornei* and *P. neglectus* individuals were extracted from the roots and soil using the modified Baermann funnel (Southey 1986). The total number of *P. thornei* and *P. neglectus* nematodes per plant was calculated based on the number of nematodes counted under a microscope. Genotypes were divided into five groups based on the number of nematodes per plant, taking into account the reaction of check varieties with known resistance to nematodes presented in **Table 1**. The nematode numbers were showed normal distribution so data transformation did not used in both RLN and CCN data.

Data were analyzed with standard analysis of variance procedures in GenStat 14 program for windows (VSN international, http://www.vsni.co.uk/software/genstat). Differences between treatments were investigated using an LSD test, with statistical differences considered significant at $P \leq 0.05$.

DNA extraction and genotyping

Genomic DNA was extracted from bulked leaves of 10 two-week-old seedlings using a cetyltrimethylammonium bromide procedure (Saghai-Maroof *et al.* 1984) modified according to CIMMYT protocols (CIMMYT 2005). Lines were genotyped using the DArT platform (Diversity Arrays Technology Pty Ltd., Wenzl *et al.* 2004), which produced 1,726 markers for this population where 1,310 polymorphic DArT markers with known position were scored and designated by the prefix "*wPt*", followed by a unique numerical identifier.

Gene diversity, marker allele frequency, and genetic map construction

Gene diversity and marker allele frequency were calculated using Tassel 3.0 (Bradbury *et al.* 2007). Markers with minor allele frequency (less than 5%) were culled from the data set to reduce false positives, leaving a final number of 1,205 markers for the marker-trait association analysis. Chromosomal positions of the remaining DArT markers were determined based on the Triticarte wheat map alignment version 1.2 (http://www.triticarte.com.au). Map

positions of significant markers ($P < 0.05$) were visualized with WinQTLCart 2.5 software (Wang *et al.* 2006).

Population structure

Population structure was determined by principal component analysis (PCA) using all 1,310 polymorphic DArT markers with known positions in TASSEL 3.0. PCA has been shown to have the equivalent effect as population structure estimated through the maximum likelihood approach (Zhao *et al.* 2007). The advantages of using PCA for population structure are that it: (1) is fast; (2) is easily implemented; (3) allows accurate testing of significance of a natural null model; and (4) provides a strong indication of how many axes of variation are meaningful (Patterson *et al.* 2006).

Linkage disequilibrium

Linkage disequilibrium (LD) was estimated between genome anchored DArT over the 126 accessions considered. Linkage disequilibrium (LD) between markers was estimated as squared allele frequency correlation estimates (r^2) between markers using the LD function in TASSEL 3.0. The significance of pairwise LD was computed using 1,000 permutations. The r^2 parameter was estimated for unlinked loci (inter-chromosomal pairs) and for loci in the same chromosome (intra-chromosomal pairs). In the case of intra-chromosomal pairs, LD statistics were calculated per chromosome and subsequently aggregated over all chromosomes and plotted against genetic distance measured in centi Morgans (cM). A critical value for r^2, as evidence of linkage, was derived using the 95% percentile of unlinked loci, beyond which LD is likely caused by real physical linkage (Breseghello and Sorrels 2006). The trend of LD decay was plotted using a Loess regression to smooth the data, which revealed an overall correlation between genetic distance of markers in the same chromosome and LD.

Association analysis

The general linear model (GLM) implemented in TASSEL software, version 3.0.115, was used to calculate the associations between the markers and each trait in turn. GLM was preferred over the mixed linear model (MLM) because the Q-Matrix and the K-Matrix used by the MLM approach leads to a loss of degree of freedom, therefore reducing the power of detecting associations in small populations (Stich *et al.* 2008). For the GLM, genotypic data, phenotypic data and the Q matrix were integrated as covariates to correct for the effects of population structure. To correct for multiple testing, a false discovery rate (FDR) was used to calculate marker-specific FDR critical values at the significance level (q) of 0.05 (Benjamini and Hochberg 1995, Thissen *et al.* 2002). Analyses were done separately for each nematode species, then marker alleles with FDR at $q < 0.05$ were declared to be significantly associated with *H. avenae*, *P. thornei*, and *P. neglectus* resistance, respectively.

Results

Phenotypic variation

The results of phenotypic data for the 126 lines from the 29th SAWSN population evaluated against *H. avenae*, *P. neglectus*, and *P. thornei* are shown in (**Table 2**, **Fig. 1**). The percentage of lines grouped as MR varied from 14% for *H. avenae*, 28% for *P. neglectus*, and 34% for *P. thornei*. Heritability values (h^2) for *H. avenae*, *P. neglectus*, and

Table 2. Analysis of variance (ANOVA), Range, mean, standard deviation (SD), least significant difference (LSD), and heritability (h^2) values for number of cyst of *Heterodera avenae*, *Pratylenchus neglectus*, and *Pratylenchus thornei* evaluated in 126 CIMMYT advanced spring wheat lines from the 29th SAWSN nursery and 4 check varieties

Heterodera avenae (HA)					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	129	25735.5	199.5	22.1	<0.001
Residual	774	6978.0	9.0		
Range	3–32				
Mean	14.4				
St. deviation	6.01				
LSD (0.05)	3.15				
h^2	0.95				
Pratylenchus neglectus (PN)					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	129	12544903.0	97247.0	35.7	<0.001
Residual	774	2111430.0	2728.0		
Range	21–612				
Mean	319.9				
St. deviation	127.1				
LSD (0.05)	54.8				
h^2	0.97				
Pratylenchus thornei (PT)					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	129	18652971.0	144597.0	41.3	<0.001
Residual	774	2712180.0	3504.0		
Range	27–629				
Mean	348.5				
St. deviation	153.3				
LSD (0.05)	62.1				
h^2	0.97				

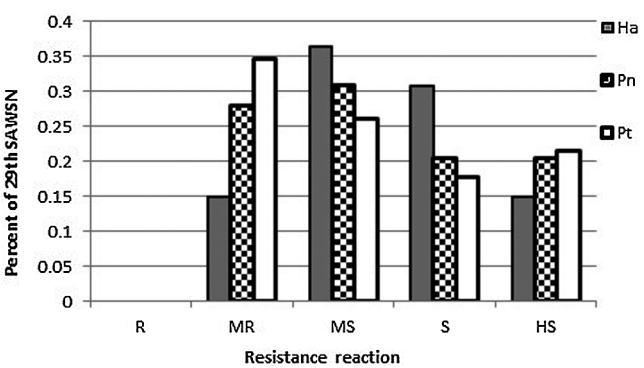

Fig. 1. Resistance reactions of 126 wheat lines from the 29th SAWSN nursery to cereal cyst nematodes *Heterodera avenae* (Ha), *Pratylenchus neglectus* (Pn), and *Pratylenchus thornei* (Pt). R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible, and HS = highly susceptible.

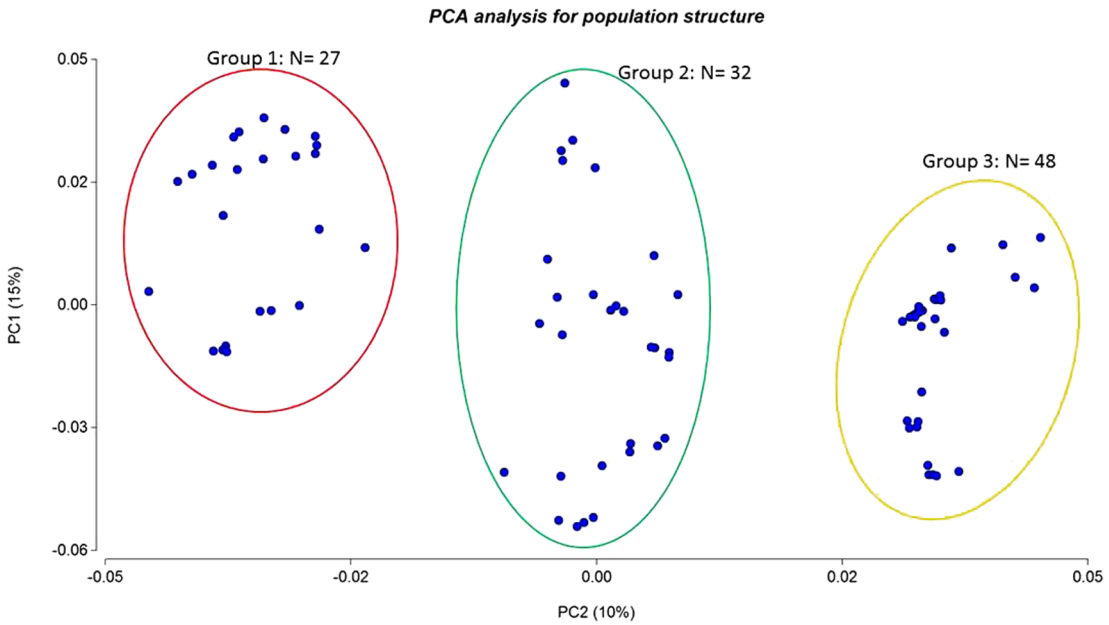

Fig. 2. PCA analysis showing the population structure for a set of 107 lines from CIMMYT's 29th SAWSN spring wheat nursery used in the association analysis. Three distinctive groups can be identified.

P. thornei were very high (0.95, 0.97, and 0.97, respectively) and genotypic differences for resistance to *H. avenae*, *P. neglectus*, and *P. thornei* were highly significant ($P \leq 0.001$), as revealed by ANOVA (**Table 2**).

Population structure

Analysis of population structure revealed three distinct groups among the 107 subset of lines that were genotyped (**Fig. 2**). This separation of the groups indicated a pattern based on the similarity of the pedigree of the crosses (e.g. crosses sharing a common grandparent). Then, group 1 consisted of 27 lines, many of which included the line SOKOLL in their background. Group 2 was formed by 32 lines, where many of them had SOKOLL, SERI, ROLF07, and ACHTAR in their pedigrees. Group 3 consisted of 48 lines, with many lines derived from the lines BABAX/LR42//BABAX, CNO79, ONIX, and PASTOR among others. Sister lines from the same cross (full-siblings) tended to cluster within the same group, though this was not always the case.

Linkage disequilibrium

Linkage disequilibrium (LD) was estimated by r^2 at $P \leq 0.01$ from all 44,486 pairs of markers and decayed rapidly with genetic distance as presented in **Supplemental Fig. 1**. On a genome-wide level, 48% of all pair loci were in significant LD, with an average r^2 of 0.14.

SNP markers assigned to their map position were further used to estimate intra-chromosomal LD. About 18% of intra-chromosomal pair of loci were in significant LD with $r^2 = 0.62$; $P \leq 0.01$, while 1,045 SNP pairs were in perfect LD ($r^2 = 1$) (**Table 3**). The extent and distribution of LD were graphically displayed by plotting intra-chromosomal r^2 values for loci in significant LD at $P \leq 0.01$ against the genetic distance in centi Morgans and a second degree LOESS curve was fitted as presented in **Supplemental Fig. 1**. The critical value for significance of r^2 was estimated at 0.21, and thus all values of $r^2 > 0.21$ were estimated to be due to genetic linkage. The average LD decay distance was approximately 22 cM for locus pairs with $r^2 > 0.05$, at the whole genome level.

Marker-trait associations

Marker-trait associations (MTA) were calculated separately for each of the resistance measures (to *H. avenae*, *P. neglectus*, and *P. thornei*, respectively). Manhattan plots showing genome-wide *P* values of association (e.g. $-\log_{10} P$ values; threshold at 1.5) before FDR are shown in (**Fig. 3**). For all three nematodes, a total of 60 significant MTAs at $P \leq 0.05$ level were initially detected. After applying the FDR ($q \leq 0.05$), 45 true MTAs were retained (**Table 4**): 11 for resistance to *H. avenae*, 25 for resistance to *P. neglectus*, and 9 for resistance to *P. thornei*. The percentage of phenotypic variation (R^2) explained by the associated markers (for all 3 nematodes) ranged from 3.8 to 10.8%. The frequency of the positively associated, significant marker alleles for *H. avenae*, *P. neglectus*, and *P. thornei* ranged from 0.39 to 0.95. Chromosome group 3 (e.g., chr. 3A, 3B and 3D) had the lowest number of MTAs across all 3 traits (3 markers, one on chromosome 3A and two on chromosome 3B), whereas chromosome group 4 had the highest number of MTAs (14, all on chromosome 4A). For other chromosome groups, the number of significant MTAs ranged from 4 (chromosome groups 2 and 6) to 8 MTAs (chromosome group 1) (**Table 4**).

Table 3. Mean allele frequency correlations (r^2) for all pairs, number (No.), and percentage (%) of pairs in significant in linkage disequilibrium (LD; $P \leq 0.01$); No. and % of physically linked pairs (r^2 > critical r^2, $P \leq 0.01$); and No. and % of pairs in complete LD ($r^2 = 1$)

Chromo-some	Pairs Total	Mean r^2 of all pairs	No. significant pairs	% significant pairs	No. Physically linked pairs	% Physically linked pairs	Mean r^2 for physically linked pairs	No. of pairs in complete LD	% of pairs in complete LD
1A	5,886	0.12	3,158	53.65	726	12.33	0.64	84	1.43
1B	3,225	0.27	2,138	66.29	1,331	41.27	0.57	42	1.30
1D	406	0.07	126	31.03	30	7.39	0.61	8	1.97
2A	1,575	0.21	782	49.65	361	22.92	0.80	47	2.98
2B	2,425	0.09	1,295	53.40	218	8.99	0.53	17	0.70
2D	780	0.14	325	41.67	119	15.26	0.73	28	3.59
3A	1,725	0.13	844	48.93	229	13.28	0.70	54	3.13
3B	4,625	0.09	2,312	49.99	460	9.95	0.58	53	1.15
3D	3,325	0.20	1,148	34.53	877	26.38	0.66	192	5.77
4A	1,525	0.13	880	57.70	268	17.57	0.46	12	0.79
4B	210	0.07	47	22.38	20	9.52	0.63	6	2.86
4D	1	0.00	0	0.00	0	0.00	–	0	0.00
5A	231	0.10	97	41.99	24	10.39	0.57	5	2.16
5B	2,475	0.07	1,086	43.88	208	8.40	0.50	22	0.89
5D	6	0.20	5	83.33	1	16.67	0.85	0	0.00
6A	4,025	0.09	1,512	37.57	418	10.39	0.59	38	0.94
6B	3,525	0.09	1,495	42.41	406	11.52	0.50	29	0.82
6D	91	0.21	39	42.86	28	30.77	0.60	6	6.59
7A	2,325	0.08	870	37.42	186	8.00	0.56	18	0.77
7B	2,025	0.13	1,210	59.75	317	15.65	0.55	12	0.59
7D	4,075	0.38	1,986	48.74	1,809	44.39	0.80	373	9.15
ALL	44,486	0.14	21,355	48.00	8,036	18.06	0.62	1,046	2.35

Markers mapped in response to H. avenae

The 11 significant markers associated with resistance to *H. avenae* were identified on chromosomes 1A, 1D, 5A, 5B, 5D, 6A, 6B, and 7A (**Table 4**, **Supplemental Fig. 2**) and explained between 3.8% and 8.7% of the phenotypic variation. Markers *wPt-743099* on chromosome 6B (position 65.8 cM, $P < 0.002$) and *wPt-4131* on chromosome 5A (position 37.9 cM, $P < 0.002$) were strongest against *H. avenae* and responsible for 8.5% and 8.7% of the resistance, respectively.

Markers mapped in response to P. neglectus

Twenty-five significant markers associated with resistance to *P. neglectus* were located on chromosomes 1A, 1B, 2B, 3A, 3B, 4A, 6B, 7A and 7D (**Table 4**, **Supplemental Fig. 2**) and explained between 4.0% and 10.7% of the phenotypic variation. Chromosome 4A contained 14 significant markers, all located between 77.6 and 98.4 cM. Marker *wPt-9183* on chromosome 4A (position 95.7 cM, $P < 0.0008$) had the strongest effect against *P. neglectus*, explaining 10.7% of the resistance. Another three markers on chromosome 4A (*wPt-6502*, *wPt-8167*, *and wPt-8271*; positioned between 89.9 and 94.3 cM) and one on chromosome 3B (*wPt-8886*, position 64.7 cM) explained individually 10.2% of the phenotypic variation and were highly significant ($P < 0.001$).

Markers mapped in response to P. thornei

Nine significant markers with resistance to *P. thornei* were mapped to chromosomes 1D, 2A, 3B, 5B and 7A and explained between 4.1 and 10.7% of the resistance (**Table 4**, **Supplemental Fig. 2**). Marker *wPt-665480* on chromosome 1D (position 17.9 cM, $P < 0.0009$) had the strongest phenotypic effect, explaining 10.8% of the phenotypic variation. Marker *wPt-7151* on chromosome 7AS (position 10.5 cM, $P < 0.043$) appeared to provide resistance (~4% of the resistance) to both *P. neglectus* and *P. thornei*.

Discussion

Several studies have shown that genome wide association analysis is an effective strategy for identifying markers linked to agronomical important traits in wheat (Crossa *et al.* 2007, Kollers *et al.* 2013, Mulki *et al.* 2013, Tadesse *et al.* 2014, Yu *et al.* 2011, Zhang *et al.* 2013). Screening for cereal nematodes by classical techniques is time consuming and labor demanding. Therefore, finding new effective QTL's would be of great value to tackle those unseen diseases by the use of marker assisted selection.

Linkage disequilibrium

In this study, we used 1,205 DArT markers distributed among the genomes and with known chromosome positions to calculate LD (r^2) between DArT markers. Based on the marker average interval (2.3 cM) and the LD decay at the genome, sub-genome, and chromosome levels in the advanced lines (4 to 34 cM), we suggest that genome wide association analysis described in this study is likely an efficient way of detecting genome-wide MTA. The genome-wide LD decay distance in our panel (average 22 cM) is larger than the normal 5 to 10 cM estimates of LD decay for different panels of elite wheat varieties (e.g. Breseghello

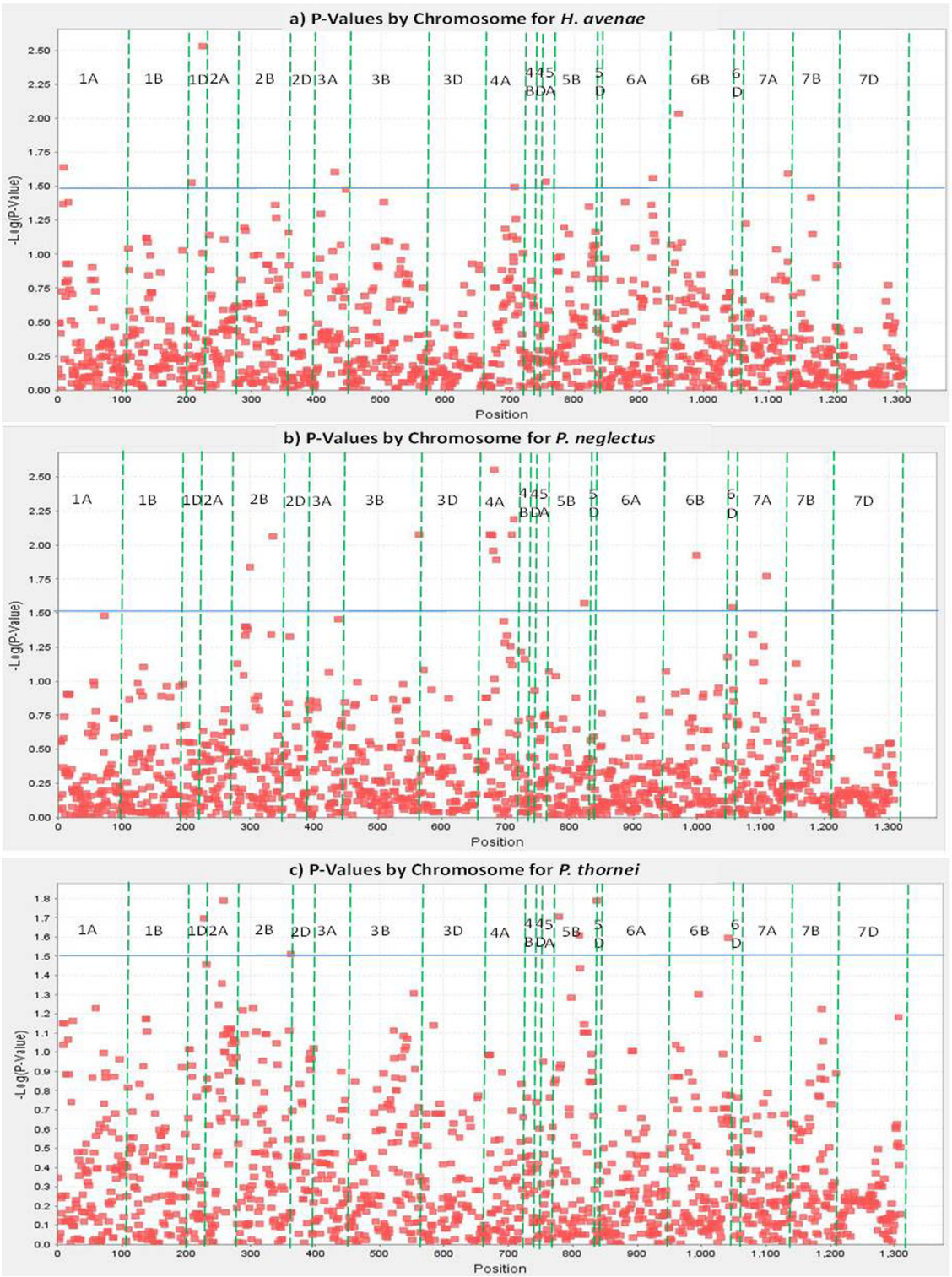

Fig. 3. Manhattan plots of *P* values (before FDR) indicating genomic regions associated with three nematode resistant traits a) *Heterodera avenae*, b) *Pratylenchus neglectus*, c) *Pratylenchus thornei*. X-axis shows DArT markers along each wheat chromosome; *y*-axis is the –log10 (*P*-value).

and Sorrels 2006, Hao *et al.* 2011, Tadesse *et al.* 2014), but similar to that reported by Zhang *et al.* (2013) and Dreisigacker *et al.* (2012) in a panel of 94 varieties with narrow diversity in terms of geographic origin and a similar CIMMYT screening nursery. In the current study, the 107 advanced lines developed from a long term breeding program, in which linkage blocks most likely have been built up. Despite the fact that CIMMYT's wheat breeding program

Table 4. Chromosome position, *P* value, R^2, FDR (*q*), allele number, and frequency of the favorable allele associated with resistance to *Heterodera avenae*, *Pratylenchus neglectus*, and *Pratylenchus thornei*

Trait	DArT	Chr	pos	*P*	*q*	R^2	Allele	Frequency
H. avenae	wPt-731843	1A	16.7	0.0169	0.0188	0.054	0	0.81
H. avenae	wPt-664586	1A	17.5	0.0304	0.0313	0.045	0	0.83
H. avenae	wPt-730408	1A	74.5	0.0087	0.0125	0.071	0	0.93
H. avenae	wPt-671653	1D	67.2	0.0086	0.0094	0.066	0	0.46
H. avenae	wPt-4131	5A	37.9	**0.0027**	0.0063	0.087	0	0.54
H. avenae	wPt-5120	5B	29.4	0.0110	0.0156	0.062	0	0.78
H. avenae	wPt-2373	5B	155.4	0.0315	0.0344	0.045	0	0.68
H. avenae	wPt-1210	5D	4.4	0.0469	0.0469	0.038	1	0.81
H. avenae	wPt-3247	6A	107.8	0.0336	0.0375	0.045	1	0.69
H. avenae	wPt-743099	6B	65.8	**0.0027**	0.0031	0.086	0	0.93
H. avenae	wPt-672075	7A	10.4	0.0250	0.0255	0.053	0	0.74
P. neglectus	wPt-8016	1A	125.4	0.0175	0.0250	0.056	1	0.62
P. neglectus	wPt-9639	1B	14.6	0.0376	0.0423	0.044	0	0.67
P. neglectus	wPt-2600	2B	58.6	0.0142	0.0212	0.059	1	0.79
P. neglectus	wPt-5672	2B	63.2	0.0276	0.0346	0.050	0	0.74
P. neglectus	wPt-1036	3A	190.2	0.0458	0.0462	0.040	0	0.56
P. neglectus	wPt-8886	3B	64.7	**0.0010**	0.0038	0.102	0	0.84
P. neglectus	wPt-4596	4A	77.6	0.0068	0.0135	0.073	0	0.81
P. neglectus	wPt-3349	4A	84.8	0.0065	0.0115	0.071	1	0.81
P. neglectus	wPt-2982	4A	84.8	0.0181	0.0269	0.056	1	0.84
P. neglectus	wPt-9196	4A	84.8	0.0346	0.0385	0.044	1	0.60
P. neglectus	wPt-7807	4A	87.9	0.0084	0.0192	0.070	0	0.39
P. neglectus	wPt-6502	4A	89.9	**0.0010**	0.0096	0.102	1	0.84
P. neglectus	wPt-730913	4A	93.1	0.0070	0.0173	0.071	1	0.81
P. neglectus	wPt-5578	4A	93.2	0.0406	0.0442	0.041	1	0.70
P. neglectus	wPt-8167	4A	94.3	**0.0010**	0.0058	0.102	0	0.84
P. neglectus	wPt-8271	4A	94.3	0.0010	0.0077	0.102	0	0.84
P. neglectus	wPt-9645	4A	94.6	0.0362	0.0404	0.043	1	0.75
P. neglectus	wPt-9183	4A	95.7	**0.0008**	0.0019	0.107	0	0.84
P. neglectus	wPt-1155	4A	98.4	0.0069	0.0154	0.072	0	0.66
P. neglectus	wPt-4424	4A	98.4	0.0150	0.0231	0.060	0	0.67
P. neglectus	wPt-9642	6B	55	0.0198	0.0288	0.053	1	0.95
P. neglectus	wPt-6208	6B	66	0.0270	0.0327	0.052	0	0.62
P. neglectus	wPt-7151	7A	10.5	0.0479	0.0481	0.042	0	0.79
P. neglectus	wPt-3992	7A	85.9	0.0218	0.0308	0.052	1	0.75
P. neglectus	wPt-664400	7D	116.9	0.0321	0.0365	0.046	1	0.98
P. thornei	wPt-665480	1D	17.9	**0.0009**	0.0026	0.108	1	0.93
P. thornei	wPt-3855	1D	21.4	0.0149	0.0184	0.057	1	0.93
P. thornei	wPt-8490	2A	28.9	0.0430	0.0447	0.046	0	0.83
P. thornei	wPt-5029	2A	46	0.0216	0.0237	0.054	1	0.82
P. thornei	wPt-742715	3B	14.2	0.0368	0.0381	0.042	1	0.79
P. thornei	wPt-744750	5B	44.5	0.0132	0.0158	0.059	1	0.81
P. thornei	wPt-1304	5B	111.7	0.0238	0.0263	0.049	0	0.94
P. thornei	wPt-7151	7A	10.5	0.0434	0.0474	0.044	0	0.79
P. thornei	wPt-6668	7A	12.4	0.0166	0.0211	0.056	1	0.63

P values in **bold** correspond to markers that passed the Bonferroni test.

possesses good genetic diversity (Reif *et al.* 2005), several sister lines were retained in the 29th SAWSN nursery, so for the same reasons as Zhang *et al.* (2013), the genetic diversity of the mapping population used in this study may be lower, resulting in higher LD levels.

Significant markers linked to previously identified QTL

The advanced CIMMYT lines analyzed in this study was of quantitative character of resistance to *H. avenae*, *P. neglectus*, and *P. thornei*, suggesting that resistance consists of minor genes only. Resistant genes *Cre1* and *Cre3* (to a lesser extent) have been introgressed into CIMMYT spring wheat using marker-assisted selection in the 17th and 18th Semi-Arid Wheat Yield Trials (SAWYT), as well as in the 30th Semi-Arid Wheat Screening Nursery (30th SAWSN), which is evidenced in the pedigree and selection history of the lines (data not shown). Within the nursery used in the present study (29th SAWSN), there is no evidence of the presence of *Cre1* or *Cre2* genes, though one identified marker (*wPt-743099*) was located on chromosome 6B. Chromosome 6B also harbored one known gene, *Cre8*. This identified new DArT markers is either located in the genomic region of *Cre8* or putatively new QTL that need to be confirmed in the further experiments. Similarly, markers *wPt-664586*, *wPt-731843*, *wPt-671653*, *wPt-2373*, *wPt-5120*, and *wPt-1210*, found on chromosomes 1A, 1A, 1D, 5B, 5B, and 5B, respectively, concur with QTL previously reported for the CCN resistance found in the Trident/

Molineux double haploid population (Williams *et al.* 2006) and in synthetic hexaploid wheats (Mulki *et al.* 2013).

This study mapped 17 *P. neglectus* resistance markers in regions of known QTL. Of these, 14 mapped to chromosome 4A, two mapped to chromosome 2B, and one mapped to chromosome 7AL. Four tightly-linked DArT markers located at ~89.9–95.7 cM on chromosome 4A (*wPt-8167*, *wPt-8271*, *wPt-6502*, and *wPt-9183*) each explained more than 10% of the phenotypic variation to *P. neglectus*. These markers were located close to DArT marker *wPt-9675* (104.6 cM), which was previously identified by Mulki *et al.* (2013) as explaining 4% of *P. neglectus* resistance. The region of 85–105 cM on chromosome 4A could therefore be a source of new QTL that confer resistance to *P. neglectus*. Two other markers were identified in concurrence with genomic regions previously reported; namely *wPt-5672/wPt-2600* and *wPt-3992*, which mapped to chromosomes 2B and the long arm of 7A, respectively.

Resistant QTL to *P. thornei* have previously been reported on chromosomes 1B, 2B, 3B, 4D, 6A, 6D, and 7A (Smiley and Nicol 2009, Toktay *et al.* 2006, Zwart *et al.* 2010). The present study mapped 3 markers (*wPt-742715*, *wPt-7151*, and *wPt-6668*) to chromosomes known to host resistant QTL to *P. thornei* (3BS, 7A, and 7A, respectively). Interestingly, a previously reported resistant QTL to *P. thornei* was identified in a primary synthetic line from CIMMYT (CROC_1/AE.SQUARROSA (224)//OPATA) on chromosome 3B (Toktay *et al.* 2006). It is not surprising that this study identified one resistant marker on chromosome 3B (wPt-742715) that was responsible for 4% of the resistance, since some of the lines used also have this synthetic line in their pedigree. According to our results, the genomic region on chromosome 7AS from 10.5 cM to 12.4 cM seems to be responsible for the resistance (to some extent) to all 3 nematode diseases (*H. avenae*, *P. neglectus*, and *P. thornei*). To our knowledge, this is the first study that identifies a QTL effective for more than one nematode genera and species and should be further exploited.

Significant markers linked to novel cereal nematode resistance QTL

In addition to the resistant markers that were in agreement with genomic regions reported to harbor resistant QTL for CCNs and RLNs in the literature, the present study also identified novel markers conferring resistance to CCNs and RLNs in genomic regions that—to our knowledge—have not yet been reported. Four novel alleles conferring resistance to *H. avenae* identified in the current study were linked to markers *wPt-4131*, *wPt-3247* and *wPt-672075*. Among them, marker *wPt-4131* had the strongest effect against *H. avenae*, explaining 8.7% of the phenotypic variation. In the case of *P. neglectus*, 7 novel alleles conferring resistance were located on chromosomes 1A, 1B, 3A, 6B, 7AS, and 7D, linked to markers *wPt-8016*, *wPt-9639*, *wPt-1036*, *wPt-8886*, *wPt-6208*, *wPt-7151* and *wPt-664400*. Among these, *wPt-8886* had the strongest effect against *P. neglectus*, explaining 10.2% of the phenotypic variation. For *P. thornei*, 6 novel resistant alleles were identified on chromosomes 1D, 2A, and 5B, linked to markers *wPt-665480*, *wPt-3855*, *wPt-5029*, *wPt-8490*, *wPt-744750* and *wPt-1304*. Among these, marker *wPt-665480* was the one with the strongest effect against *P. thornei*, explaining 10.7% of the phenotypic variation.

Conclusions

This study demonstrated the value of genome-wide association mapping for identifying QTL linked to *H. avenae*, *P. neglectus*, and *P. thornei* resistance using CIMMYT advanced spring wheat lines. The results regarding potentially new loci conferring resistance to *H. avenae*, *P. neglectus*, and *P. thornei* will need to be confirmed by creating biparental mapping populations and other type of genetic stocks (i.e. NILs) for fine mapping and validation of potential QTL. Nevertheless, resistant genotypes from the present study can be used as parents to enrich the allelic frequency of resistance for *H. avenae*, *P. neglectus*, and *P. thornei* in breeding programs. If QTL are confirmed and markers are developed, marker-assisted selection could be used for quick deployment of resistance by combining and pyramiding/stacking these potential loci into elite wheat breeding lines. Moreover DArT markers have been substituted by DArTseq markers based on genotyping by sequencing technology. So DArT markers linked to any of the nematode resistance could be directly implemented in breeding programs after validation. Due to the minor effect of the novel QTL from the present study, breeding efforts aiming the incorporation of effective resistance to nematodes will be most likely a combination of known major genes (e.g., *Cre1*, *Cre3* and *Cre6*) with 2 or 3 genes with minor effect. Stacking single genes for resistances to *H. avenae*, *P. neglectus*, and *P. thornei* is advised; however the use of inter species resistant QTL such as the 7AS QTL will create a base line to build up resistance to nematodes present in a wide range of environments especially where pathotypes exist in case of *H. avenae*.

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