

Research article

Transcript-specific, single-nucleotide polymorphism discovery and linkage analysis in hexaploid bread wheat (*Triticum aestivum* L.)

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Summary

Food security is a global concern and substantial yield increases in cereal crops are required to feed the growing world population. Wheat is one of the three most important crops for human and livestock feed. However, the complexity of the genome coupled with a decline in genetic diversity within modern elite cultivars has hindered the application of marker-assisted selection (MAS) in breeding programmes. A crucial step in the successful application of MAS in breeding programmes is the development of cheap and easy to use molecular markers, such as single-nucleotide polymorphisms. To mine selected elite wheat germplasm for intervarietal single-nucleotide polymorphisms, we have used expressed sequence tags derived from public sequencing programmes and next-generation sequencing of normalized wheat complementary DNA libraries, in combination with a novel sequence alignment and assembly approach. Here, we describe the development and validation of a panel of 1114 single-nucleotide polymorphisms in hexaploid bread wheat using competitive allele-specific polymerase chain reaction genotyping technology. We report the genotyping results of these markers on 23 wheat varieties, selected to represent a broad cross-section of wheat germplasm including a number of elite UK varieties. Finally, we show that, using relatively simple technology, it is possible to rapidly generate a linkage map containing several hundred single-nucleotide polymorphism markers in the doubled haploid mapping population of Avalon × Cadenza.

Keywords: wheat, next-generation sequencing, KASPar genotyping, single-nucleotide polymorphism.

Introduction

With the world's population forecasted to reach nine billion by 2050, food security has become a critical global challenge for the 21st century. It has been estimated that cereal production needs to increase by 50% by 2030 (Foresight: The Future of Food and Farming, 2011). Wheat is the dominant cereal crop grown in temperate countries, and globally, one of the three most important crops for human and livestock feed (Shewry, 2009). Consequently, increasing wheat yields is now one of the top priorities for agricultural research (Reaping the benefits: Science and the sustainable intensification of global agriculture, 2009). When compared with other crops, the increase in wheat yields has slowed since the 'green revolution' of the 20th century (Alston *et al.*, 2010). While diploid crops such as rice and barley have benefited from extensive genetic analysis and molecular breeding programmes, the complexity of the wheat genome has hindered these types of studies.

The allohexaploid genome of modern bread wheat (AABBDD) is derived from the hybridization of the diploid DD genome of

Aegilops tauschii with the AABB tetraploid genome of *Triticum turgidum* (Dubcovsky and Dvorak, 2007). The genetic bottleneck caused by this polyploid speciation event approximately 10 000 years ago has been intensified by the domestication of wheat, resulting in a decline of genetic diversity within modern inbred, elite cultivars (Haudry *et al.*, 2007). To increase the genetic diversity of bread wheat, it has been suggested that greater use could be made of the various wild relatives of wheat and that by introducing genes from these relatives it might be possible to tap novel sources of stress, pest and disease resistance (Reynolds *et al.*, 2011). However, such strategies, sometimes referred to as pre-breeding, can be resource intensive (Valkoun, 2001). A crucial step in making wheat pre-breeding more efficient is the development of molecular markers capable of tracking the introduced genomic regions in large numbers of lines. There are several challenges to developing molecular markers in a polyploid species such as wheat, for instance, the molecular markers must be capable of distinguishing between the relatively large numbers of polymorphisms seen in homoeologous and paralogous genes compared with

the relatively infrequent varietal polymorphisms (Barker and Edwards, 2009). Homoeologous/paralogous and varietal single-nucleotide polymorphisms (SNPs) have previously been studied and used in polyploid crops (Akhunov *et al.*, 2009; Bernardo *et al.*, 2009; Bundock *et al.*, 2009; Edwards *et al.*, 2009; Imelfort *et al.*, 2009); however, these studies have also shown that distinguishing intervarietal markers from intergenomic polymorphisms is complicated and prone to error. For example, a previous *in silico* study by our group showed that of the ~71 000 putative SNPs identified in the wheat EST database, only ~3500 appear to be intervarietal (~5%) with the majority being SNPs in homoeologous and paralogous genes (Barker and Edwards, 2009). These results, together with the large size of the wheat genome (~17 300 Mb), mean that despite the global importance of wheat, there are still relatively few validated varietal SNP markers in regular use. While this situation remains, it is likely that, when compared with other crops such as maize and rice, wheat will continue to lag behind in terms of marker-assisted selection in breeding programmes (Mochida and Shinozaki, 2010).

The development of SNP-based genotyping platforms has led to an increase in the number of protocols available for analysing the genetic variation in numerous species (Perkel, 2008). However, as with the development of SNPs, large-scale genotyping in polyploid species is still a significant challenge because of the presence of the homoeologous and paralogous genes. Despite these challenges, a number of platforms have recently been developed to perform high-density genotyping (large numbers of SNPs, with small numbers of individual plants), and these have been successfully employed to genotype wheat (Akbari *et al.*, 2006; Akhunov *et al.*, 2010; Bérard *et al.*, 2009; Chao *et al.*, 2010). However, these technologies can be difficult to optimize and as such they have yet to be generally adopted by the wheat community leaving few options for wheat breeders and geneticists who wish to carry out medium- to low-density genotyping on large or very large numbers of individual plants.

In the present study, we undertook to mine the UK wheat germplasm for varietal SNPs. To do this, we used both the available wheat expressed sequence tags (ESTs) present in the public database and next-generation sequencing (NGS), together with a novel sequence alignment and assembly approach to identify varietal SNPs in lines of interest to European wheat breeders. We then investigated whether these SNPs could be validated by a relatively new, high-throughput genotyping procedure, the KBioscience Competitive Allele-Specific polymerase chain reaction (KASPar) assay (Orrù *et al.*, 2009). Finally, we made use of the existing Avalon × Cadenza mapping population to examine whether the validated SNPs could be efficiently placed onto the existing linkage groups identified within this doubled haploid population.

Results

SNP discovery

Two complimentary approaches were taken to identify putative varietal SNPs. First, screening of the publicly available wheat EST database (generated from numerous cDNA libraries, from a random collection of varieties relating to different stages of grain development and various stress treatments) yielded ~3500 putative varietal SNPs in 8668 sequences (Table S1). These data were supplemented by sequencing normalized

whole-seedling cDNA from five wheat varieties (Avalon, Cadenza, Rialto, Savannah, Recital) on the Illumina GAllx platform (Table 1). For each line, we generated between 24 and 45 million, 75-base pair (bp) paired-end reads. SNP discovery, carried out as described in experimental procedures, resulted in the identification of 14 078 putative SNPs in 6255 distinct reference sequences covering 2.7 megabases in total. On average, this equates to five varietal SNPs per kilobase in the reference sequences containing one or more SNP (Table S2).

SNP validation and characterization

A subset of 1659 putative SNPs were selected for validation with genomic DNA. Of these, 213 (from 199 contigs covering 25 773 bases) were derived from sequences obtained from the NCBI database and 1446 (1237 contigs covering 5 59 059 bases) from the NGS carried out as part of this study. For the SNPs mined from the NGS data, these were selected on the basis of their predicted polymorphism level in the sequenced varieties. In most cases, loci were limited to one SNP per cDNA contig, to maximize the genome coverage. SNPs were validated using the KASPar genotyping platform on 21 hexaploid wheat varieties, a diploid and a tetraploid wheat (Table 2). The wheat varieties were selected following a survey of UK academics and wheat breeders to ensure that material of use to the whole community was represented, e.g. the inclusion of parents of other published mapping populations. Of the 1659 putative varietal SNPs, 1114 (67%) were polymorphic between the different varieties, 70 (4%) were monomorphic in the hexaploid varieties and 475 (29%) failed to generate a useful amplification signal (Table S3). Where a SNP probe failed to generate a useful signal, no attempt was made to redesign the primer. To compare the different SNP generation techniques, these statistics were assessed separately. Of the 213 SNPs generated from the EST database, 174 (82%) were polymorphic between the 23 varieties, four were monomorphic (2%) and 35 failed to generate a useful signal (16%; Table S1), while of the 1446 SNPs from NGS data, 940 (65%) were polymorphic, 66 were monomorphic (4.5%) and 440 failed to generate a useful signal (30%; Table S2). Based upon the SNP genotyping data of the 23 varieties screened, the polymorphism information content (PIC) values of the validated SNPs varied from 0.080 to 0.375 with an average value of 0.300 (Figure 1). The PIC values of the SNPs generated from the EST database (average 0.306) were not significantly different from those generated from NGS data (average 0.298).

To test whether the non-polymorphic SNPs were homoeologous rather than homologous SNPs, we screened 28 on the Chinese Spring nullisomic lines (Sears, 1954; Devos *et al.*,

Table 1 Summary of wheat lines used and next-generation sequences obtained

Variety	No. of sequences	No. sequences uniquely assigned to reference	No. of SNPs (compared to Avalon)
Avalon	33 199 506	17 419 590	N/A
Cadenza	34 296 300	16 938 920	3251
Rialto	25 522 334	13 241 931	2944
Savannah	24 349 192	12 271 061	3492
Recital	43 961 514	22 151 006	3792

Table 2 Details of wheat varieties used in this study

Wheat Line	Breeder/Source	Ploidy	Planting season	Pedigree
Avalon	PBIC	Hexaploid	Winter	Maris-Ploughman × Bilbo
Alchemy	Limagrain UK Ltd	Hexaploid	Winter	Claire × (Consort × Woodstock)
Alcedo	NIAB	Hexaploid	Winter	(Record, AUT × Poros) × Carstens-VIII
Bacanora	CIMMYT	Hexaploid	Spring	(Jupateco-73 × BlueJay) × Ures-81
Brompton	Elsoms Seeds Ltd	Hexaploid	Winter	CWW 92.1 × Caxton
Cadenza	PBIC	Hexaploid	Spring	Axona × Tonic
Chinese Spring (L 42)	JIC	Hexaploid	Spring	Chinese Land Race
Claire	Limagrain UK Ltd	Hexaploid	Winter	Wasp × Flame
Hereward	RAGT seeds Ltd	Hexaploid	Winter	Norman 'sib' × Disponent
Opata	CIMMYT	Hexaploid	Spring	Bluejay × Jupateco-73
Recital	C.C. Benoist	Hexaploid	Winter?	Mexique-267 (R-267) × 9369
Renan	INRA	Hexaploid	Winter	(Mironovskaya-808 × Maris-Huntsman) × Courtot
Rialto	PBIC	Hexaploid	Winter	Haven × Fresco
Robigus	KWS UK Ltd	Hexaploid	Winter	Z836 × 1366 (PUTCH)
Savannah	Zeneca Seeds UK Ltd.	Hexaploid	Winter	Riband × Brigadier
Shamrock	Verneuil Recherche	Hexaploid	Winter	COMPL TIG 323-1-3m × CWW 4899/25
Soissons	Maison Florimond Desprez, Franc	Hexaploid	Winter	Iena (Jena) × HN35
Synthetic	JIC	Hexaploid	Spring	Unknown
Weebil	CIMMYT	Hexaploid	Spring	Unknown
Xi19	Limagrain UK Ltd	Hexaploid	Winter	(Cadenza × Rialto) × Cadenza
TA1618	WGGRC	Diploid	Spring	<i>Aegilops Tauschii</i> (D genome)
Croc	WGGRC	Tetraploid	Spring	<i>Triticum turgidum</i> subsp. <i>Dicoccoides</i> (AB genome)
Synthetic 39	WGGRC	Hexaploid	Spring	TA1618 × Croc

PBIC, Plant Breeding International Cambridge, Ltd. (UK); NIAB, National Institute of Agricultural Botany; CIMMYT, International Maize and Wheat Improvement Centre; JIC, John Innes Centre; INRA, French National Institute for Agricultural Research; WGGRC, Wheat Genetic and Genomic Resources Center.

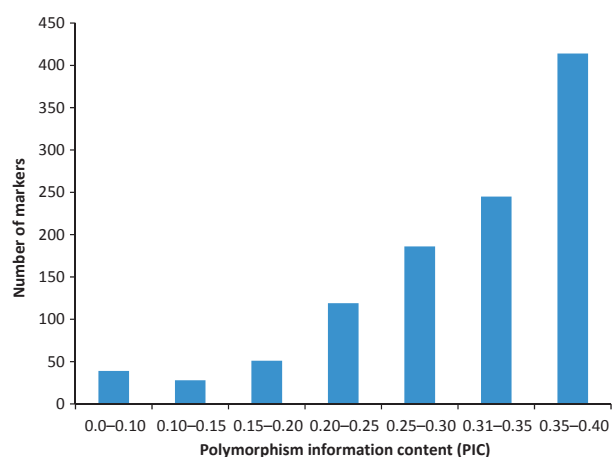


Figure 1 Distribution of polymorphism information content (PIC) of SNPs among the 23 wheat varieties. The PIC was calculated for each marker according to Botstein *et al.* (1980), using Excel Microsatellite Toolkit add-in software (Park, 2001).

1999); of these, we were able to confirm that 26 (93%) were in fact homoeologous SNPs and were therefore specific for either the A, B or D genome, but not a specific variety.

To investigate the genetic relationship between the 23 lines, we carried out a hierarchical cluster analysis using all 1114 validated SNPs between the varieties used in this study (Figure 2). Several varieties clustered tightly together including the pairs of Cadenza and Xi19; Claire and Alchemy; Opata and Weebil and

Hereward and Shamrock. The synthetic varieties formed a clear outlying group, with the other varieties falling into two main groupings. The first included subgroups of Renan, Robigus and Avalon and Hereward, Shamrock, Brompton, Rialto and Alchemy. The second group contained more diverse material including the French lines Recital and Soissons, and the German variety Alcedo.

Genetic map construction

An Avalon × Cadenza doubled haploid population comprising 190 individuals was scored for 548 loci (53 from the EST data set and 495 from the NGS data set) using KASPar genotyping. Of these, 38 loci showed significant segregation distortion and were removed from the data set before constructing the linkage groups. Using the information generated, in conjunction with the 574 non-SNP markers previously available for this population, we were able to map 480 SNP markers (50 from the EST data set and 430 from the NGS data set) to 21 linkage groups representing chromosomes. A further 20 loci mapped to unassigned linkage groups and 10 loci were unlinked (Table 3, Figure 3, Table S4). The linkage groups ranged from 53.0 to 240.5 centiMorgans (cM) in size, with 9–108 markers. The total map length was 2999 cM, with an average spacing of 2.8 cM between loci. The total map lengths of each of the three genomes were approximately similar: 1131 cM for the A genome, 1075 cM for the B genome and 792 cM for the D genome (Table 3). However, there was a significant difference in the distribution of SNP marker loci between the three genomes with the A genome having 354 markers (163 SNPs), the B genome having 526 markers (267 SNPs) and the D genome having 174

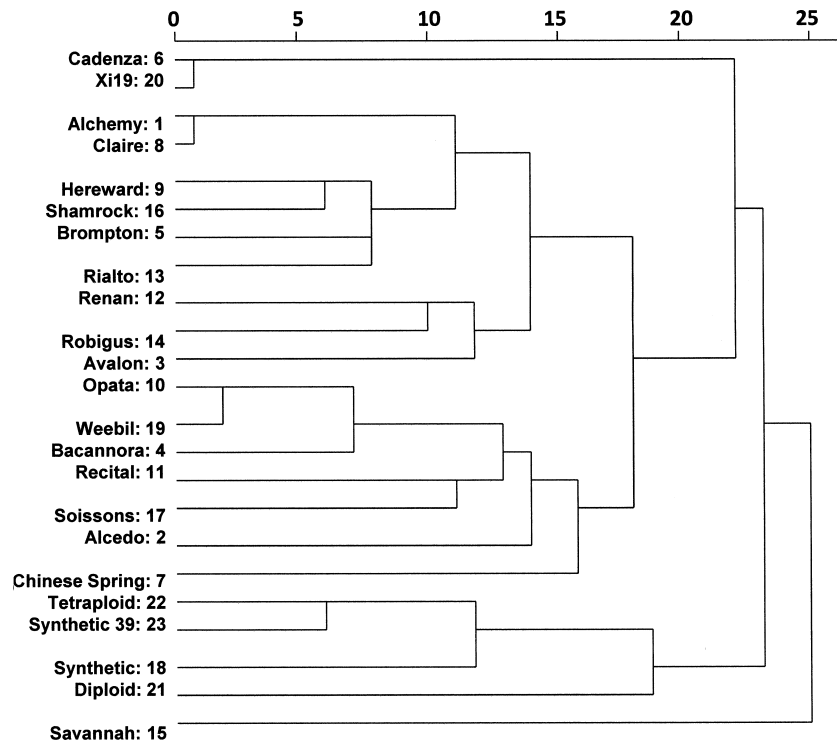


Figure 2 Indicator of genetic relationships between the of 23 wheat lines screened with 1114 KASPar probes. Hierarchical cluster analysis was performed in PASW (SPSS Inc.) using the Squared Euclidean Distance method.

markers (50 SNPs). The homoeologous chromosome groups also showed variation in size and marker density; the combined group 5 chromosome linkage maps measured the largest at 613 cM, while the group 4 linkage groups measured a total of 267 cM. Marker density was highest in the group 1 linkage maps (average 1.7 cM between loci) and lowest in the group 7 linkage maps (average 3.8 cM between loci).

Our mapped SNP data set contained 48 loci that we had provisionally mapped to specific chromosome regions using the Kansas deletion lines (Endo and Gill, 1996; Qi *et al.*, 2003). Comparison of the map locations determined using both the Kansas deletion lines and the genetic mapping confirmed the map location for 29 of the 48 SNP markers (60%). A further 10 SNP loci mapped to the same homoeologous group, which gives a total of 81% of the loci in common mapping to the same homoeologous group. To extend this analysis, we also BLASTN searched the 500 mapped SNP marker reference sequences against the deletion-mapped ESTs produced by Qi *et al.* (2004). Of the 155 SNP markers contained within these two data sets, 107 (69%) mapped to the same homoeologous group.

The same 500 mapped sequences were also BLASTN searched against the *Brachypodium distachyon* genome sequence (International Brachypodium Initiative., 2010) resulting in 266 matches. A syntenic match was assumed where two or more consecutive wheat mapped SNPs matched the same *B. distachyon* chromosome. These criteria resulted in 205 of the 266 mapped wheat SNPs being placed on the *B. distachyon* genome. SNP coverage was generally too low to explore synteny in detail, but where sufficient markers existed, as for wheat linkage group 2 versus *B. distachyon* chromosome 5, the syntenic relationship was consistent with known chromosome relationships (Figure 5).

The putative functions of 500 sequences containing validated and mapped SNP markers were determined, via BLASTX searches, against the National Centre for Biotechnology Information (NCBI) non-redundant protein sequence database using an e-value cut-off of 10^{-5} (Table S5). This search indicated that 213 sequences (43%) had detectable similarity to sequences in the database, while 287 sequences (57%) failed to detect any sequences with a similarity at the level at or above the cut of value. Of the 213 sequences with detectable sequence similarities, we were able to identify 184 unique hits, as determined by the SNP having either a unique hit in the database or having a similar hit, but with a different map location. The remaining 29 SNPs were distributed between 13 contigs; one contig having four SNPs, one contig having three SNPs and eleven contigs having two SNPs each.

Optimization of primer design for homoeologous-specific KASPar markers

Although no attempt was made to design homoeologous-specific probes, 111 of the 1114 validated KASPar probes (~10%) appeared to be specific for a single homoeologous gene. In practical terms, these probes can be used to routinely screen for heterozygotes in segregating populations, as well as establishing the purity of inbred lines. Because such probes might be of considerable interest to wheat breeders, we examined whether it was possible to convert generic KASPar probes to homoeologous-specific assays. To do this, we used the publicly available 5 × sequence coverage of the Chinese Spring wheat genome (http://www.cerealsdb.uk.net/search_reads.htm) to design homoeologous-specific KASPar reverse primers near the existing varietal SNP 'BS00000329'. In its original form, KASPar probe BS00000329 detects either an 'A' or an 'AG'

Table 3 Summary of linkage groups and mapped loci

Linkage Group	Chromosome	Size (cM)	Total Number of Loci	Number of Bristol SNP loci	Average spacing between loci (cM)
1	1A	104.9	52	28	2.0
2	1B	148.9	108	58	1.4
3	1D	108.9	50	20	2.2
4	2A	180.5	53	22	3.4
5	2B	181.1	85	43	2.1
6	2D	96.9	35	11	2.8
7	3A	155.5	37	18	4.2
8	3B	224.6	74	33	3.0
9	3D	53.0	15	1	3.5
10	4A	143.1	40	18	3.6
11	4B	53.7	26	16	2.1
12	4D	70.2	9	1	7.8
13	5A	179.5	51	23	3.5
14	5B	240.5	104	53	2.3
15	5D	193.1	20	8	9.7
16	6A	155.7	67	35	2.3
17	6B	132.0	82	37	1.6
18	6D	57.9	10	2	5.8
19	7A	212.3	54	19	3.9
20	7B	94.2	47	27	2.0
21	7D	212.3	35	7	6.1
Total		2998.8	1054	480	2.8
	A genome	1131.4	354	163	3.2
	B genome	1075.0	526	267	2.0
	D genome	792.3	174	50	4.6
	Group 1	362.8	210	106	1.7
	Group 2	458.4	173	76	2.6
	Group 3	433.1	126	52	3.4
	Group 4	267.0	75	35	3.6
	Group 5	613.1	175	84	3.5
	Group 6	345.6	159	74	2.2
	Group 7	518.7	136	53	3.8

genotype. Using the available 5 × Chinese Spring genomic sequences, we were able to design a new KASPar reverse primer that converted the original generic probe into a homoeologous-specific KASPar probe detecting either an 'A' or a 'G' genotype (Figure 5b,c).

Discussion

SNP discovery

This study represents the first large scale assembly of genotyping and genetic map information for elite UK wheat varieties based on individual SNP markers. The SNPs described in this study were mined from two sources; the NCBI wheat EST database and next-generation cDNA sequencing data. Both sources of data specifically targeted the identification of varietal SNPs in coding regions, to generate molecular markers potentially linked to genes of interest and include both synonymous and non-synonymous point mutations. The EST data yielded ~3500 putative varietal SNPs from 8668 sequences, with an average of 4.3 varietal SNPs per kilobase (Barker and Edwards, 2009). The existing EST data were supplemented by mining NGS data for varietal

SNPs, a technique that yielded 13239 putative varietal SNPs in 6255 sequences, with an average of five varietal SNPs per kilobase across the five varieties sequenced. Both SNP discovery techniques identified a similar number of varietal SNPs per kilobase and are in agreement with previous estimates for varietal SNPs in wheat (Ravel *et al.*, 2007; Barker and Edwards, 2009).

SNP validation and characterization

A subset of putative varietal SNPs (1659) were selected for validation using the KASPar genotyping platform. The KASPar system gave a conversion rate of ~67% (1114 SNPs) using a standard set of PCR conditions, which is extremely important for routine MAS applications. However, this figure of 67% could probably be increased with primer design and PCR optimization if absolutely necessary. This conversion rate is lower than previous studies on diploid species such as mouse (77%; Petkov *et al.*, 2004), but relatively high for a complex polyploid such as wheat (Edwards *et al.*, 2009). Of the putative varietal SNPs, 4% were monomorphic in the hexaploid varieties, but polymorphic between the hexaploid varieties and the diploid and/or the tetraploid. This pattern suggests that these markers represent intravarietal homoeologous SNPs. To test this theory, we screened 28 markers from this category against the Chinese Spring nullisomic lines and confirmed that 26 (93%) were in fact homoeologous SNPs. These markers are likely to have been misidentified as varietal SNPs in the discovery pipeline. Nevertheless, 4% is low when considering that intravarietal homoeologous SNPs account for ~74% of all SNPs in wheat (Barker and Edwards, 2009) and clearly demonstrates that our data sets have been significantly enriched for varietal SNPs. The failure of the remaining SNP markers to validate (29% failed to produce a defined or useful cluster) is likely to be because of the presence of paralogous sequences, primer design issues (such as primers spanning intron/exon boundaries or incorporating different homoeologous SNPs) and/or the need to optimize PCR conditions. We made no attempts to optimize any failed assays, and therefore, it is possible that these SNPs could be validated with alternative primers.

The PIC values of the validated markers ranged from a minimum of 0.08–0.375, with an average value of 0.300 (Figure 1), a figure comparable to other estimates in wheat (Chao *et al.*, 2009; Edwards *et al.*, 2009). No significant difference was observed between the PIC values of markers from different genomes or different homoeologous groups. A hierarchical cluster analysis performed on the genotyping data at the SNP loci used in this study indicates possible genetic relationships between the 23 lines (Figure 2). Although the SNP markers were selected based upon their polymorphism between just a few lines (Avalon, Cadenza, Rialto, Savannah and Recital), the expected clustering of varieties was observed, suggesting that the SNP markers were representative of the genome. In particular, the close relationship between the varieties Cadenza and Xi19 (derived from a Cadenza cross) and Claire and Alchemy (derived from a Claire cross) is illustrated in the dendrogram. The different varieties are split into two main clades with the synthetics as an outlying group. The first clade includes subgroups of Renan, Robigus and Avalon and Hereward, Shamrock, Brompton, Rialto and Alchemy; all winter wheat varieties. The second clade contains more divergent material including the French lines Recital and Soissons, and the German variety Alcedo. A close relationship in this grouping is indicated between Opatá, Weebil and Bacanora, all spring wheat lines

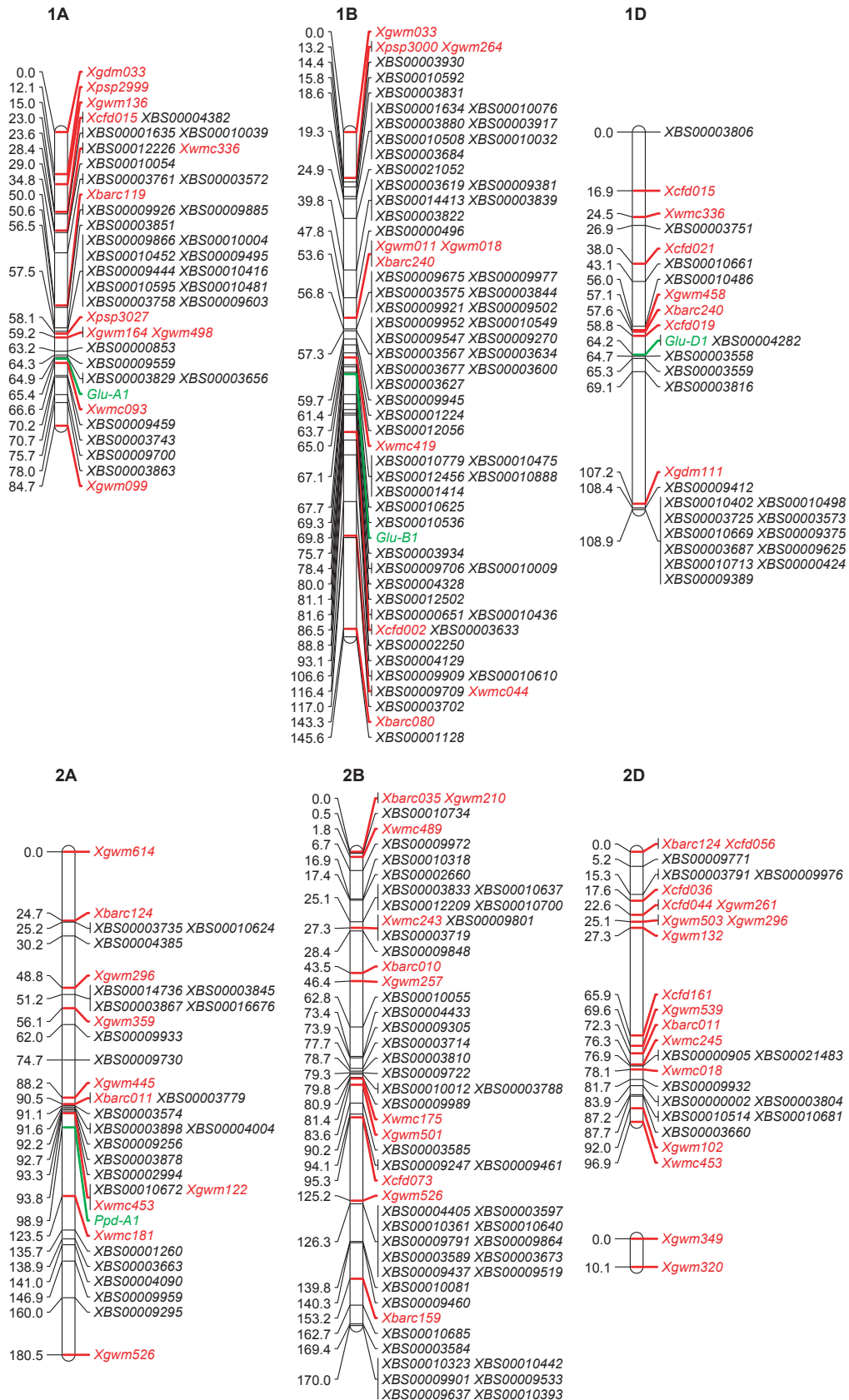


Figure 3 Genetic linkage maps of wheat derived from 190 Avalon × Cadenza doubled haploid lines. Each linkage group was assigned to a chromosome indicated above the linkage group and chromosomes are arranged with the short arm above the long arm. Simple sequence repeat (SSR) markers are shown in red, known genes are shown in green and SNP loci mapped in this study are shown in black (designated XBS). Map distances, calculated using the Kosambi mapping function (Kosambi, 1944), are shown in centiMorgans (cM).

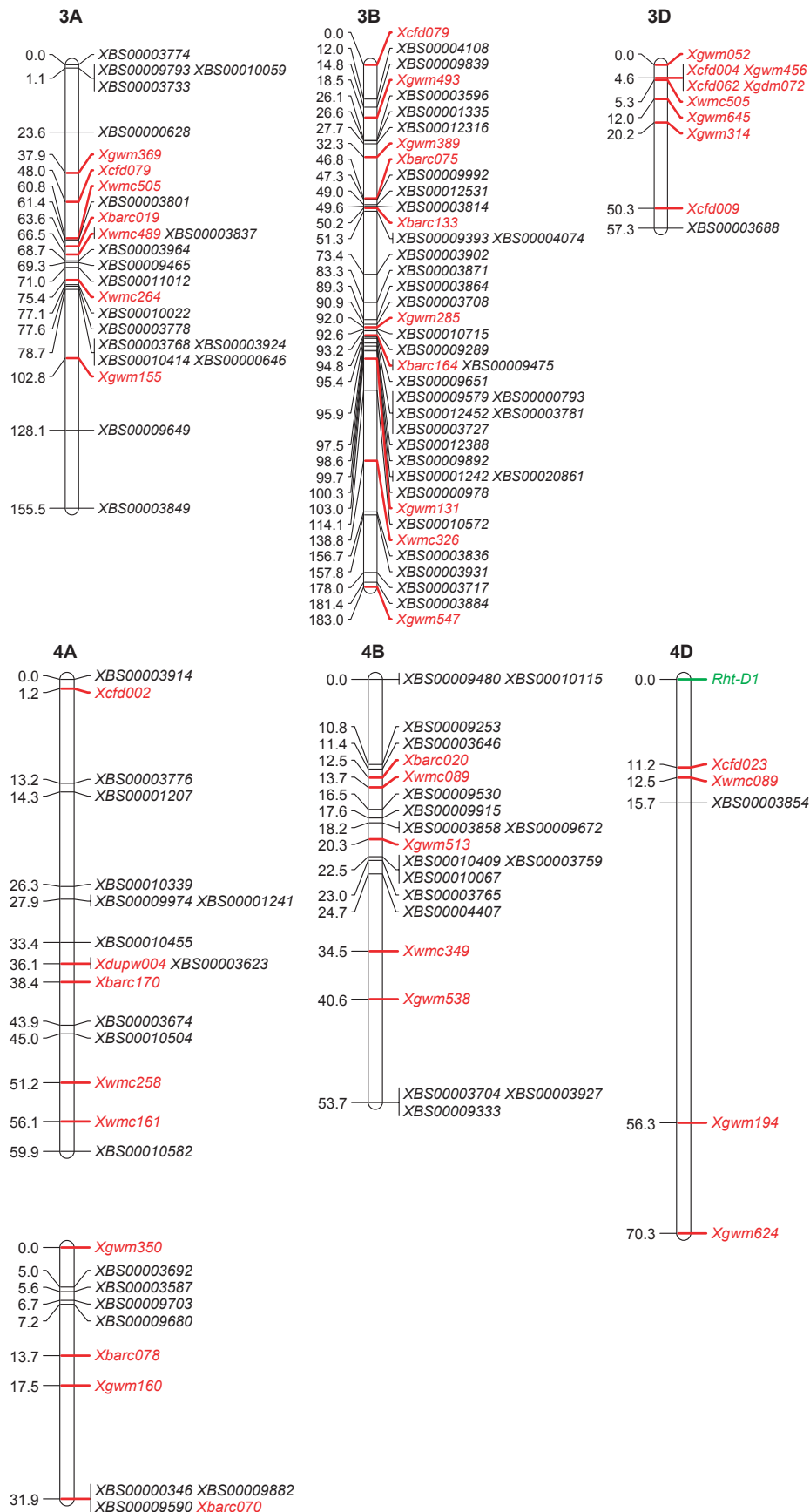


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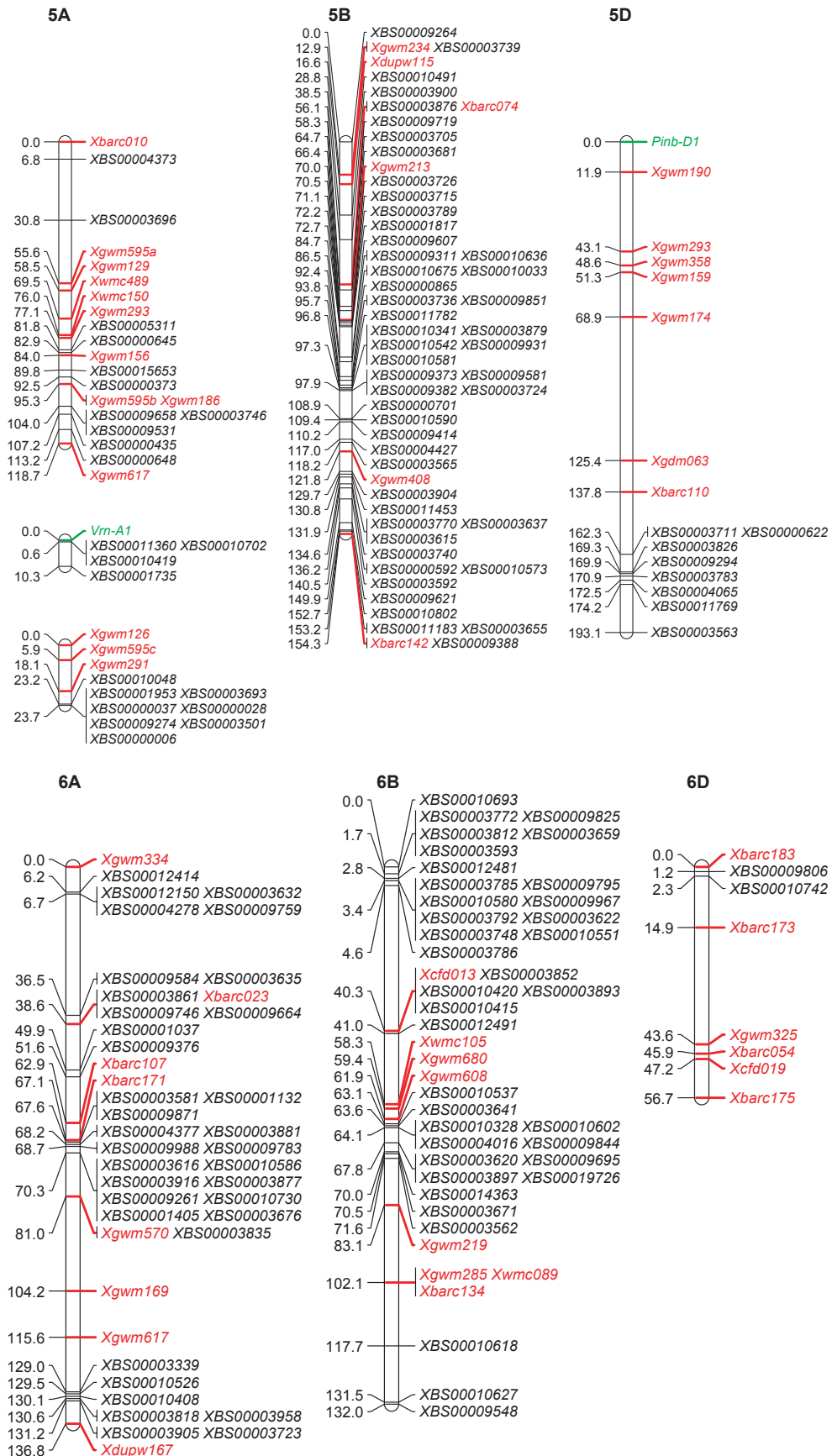


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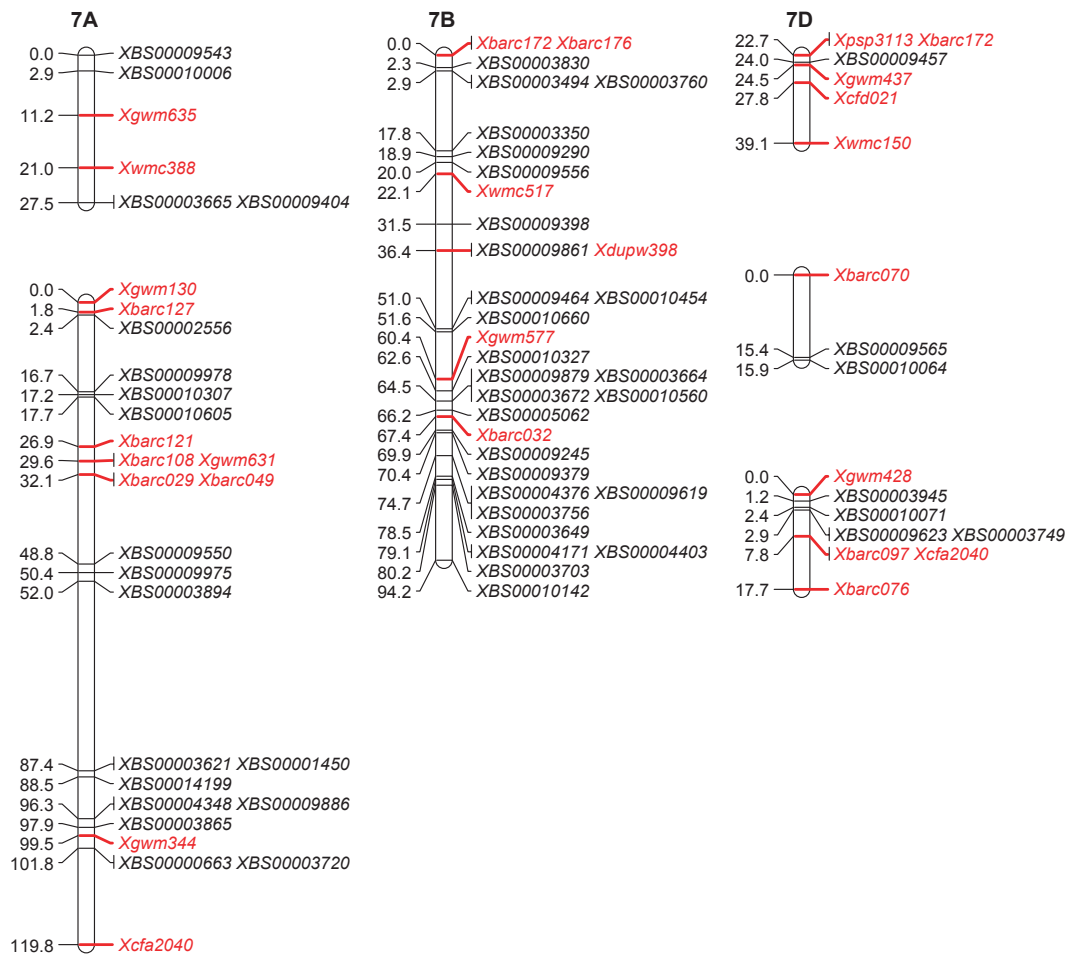


Figure 3 Continued

developed by CIMMYT. Savannah does not appear to cluster with the other hexaploid varieties, reflecting the exotic genetic background to this line. The hierarchical cluster analysis illustrates the genetic relationship between the varieties used in this study, but also indicates the reduction in germplasm variation within elite lines and the need for introducing genetic diversity into breeding programmes (Haudry *et al.*, 2007).

Genetic map

A linkage map is often the first step towards understanding genome assembly and evolution and provides an essential framework for mapping agronomic traits of interest (Xia *et al.*, 2010). We present here the first SNP-based linkage map for wheat, constructed using 500 transcript-linked varietal SNPs generated in this study in combination with 574 previously developed markers (Figure 3). The total map length was 2999 cM, similar in length to previous framework linkage maps produced in wheat using microsatellite markers (2569 cM; Somers *et al.*, 2004), DArT markers (2937 cM; Akbari *et al.*, 2006) and a combination of markers (3522 cM; Quarrie *et al.*, 2005). While each of the three genomes had similar map lengths (1131 cM for the A genome; 1075 cM for the B genome and 792 cM for the D genome), there was a significant difference between the distribution of markers (i.e. SSR, SNP, AFLP and DArT loci) between the three genomes (A genome 354 mark-

ers; B genome 526 markers; D genome 174 markers), a ratio of 33 : 50 : 17, and this difference was more apparent when calculated for the 480 SNP markers alone (34 : 56 : 10). The ratio of total markers on the three genomes is similar to that observed by Quarrie *et al.* (2005) who found the distribution of markers between the three genomes at a ratio of 40 : 40 : 20 for the A, B and D genomes, respectively. Other comparable ratios from wheat linkage maps for the A/B/D genomes were 33 : 41 : 25 (Röder *et al.*, 1998), 30 : 39 : 31 (Somers *et al.*, 2004) and 28 : 60 : 12 (Bernardo *et al.*, 2009).

As might be expected, the distributions of markers on each of the several homoeologous chromosome groups were not uniform with large gaps of more than 30 cM on D genome chromosomes and concentrations of markers around the centromeres that were particularly noticeable on A and B chromosomes. Our SNP marker set contained a considerably lower number of D genome markers than previously developed marker sets; this is a likely consequence of SNP markers targeting genic regions, while other marker types are distributed more evenly throughout the genome. The lack of D genome markers compared with the A and B genomes has been attributed to the loss of polymorphism in coding regions during the genetic bottleneck that accompanied the development of modern elite cultivars and indicates that future SNP discovery efforts will be required to specifically target the D genome to offset the

effects of lower levels of genetic diversity in this genome (Caldwell *et al.*, 2004; Chao *et al.*, 2009). Marker distribution between the homoeologous linkage groups ranged from 7 to 20% of the total markers, with group 4 containing the lowest number of markers, a pattern also seen in the linkage map of Somers *et al.* (2004). In a genome-wide study of nucleotide diversity by Akhunov *et al.* (2010) the group 4 chromosomes were found to have low levels of diversity, with chromosomes 4A and 4B having similar diversity to the D genome. It has been hypothesized that homoeologous group 4 chromosomes have a lower number of genes than the remaining six homoeologous groups (Qi *et al.*, 2004) and hence a lower level of recombination and resulting genetic diversity (Akhunov *et al.*, 2010).

The genetic map locations of the 500 SNP loci generated in this study were compared with their predicted map location from physical mapping experiments. Of 48 genetically mapped loci, 81% mapped to the same homoeologous group predicted by the Kansas deletion lines. A similar comparison of 155 SNP loci with deletion-mapped ESTs revealed that 69% mapped to the same homoeologous group (Qi *et al.*, 2004). While this is a relatively high level of correlation, discrepancies in the data are not unexpected because of the numerous secondary and tertiary deletions seen in the deletion lines (Qi *et al.*, 2004). Genetic map locations of SNP loci that can also be placed on physical maps are a potentially useful resource for identifying and characterizing these additional deletions.

The same 500 mapped sequences were also BLASTN searched against the *B. distachyon* genome sequence resulting in 205 wheat SNPs being placed on the *B. distachyon* genome. Although SNP coverage was generally low when mapped markers were divided between wheat chromosomes, where sufficient markers existed, as for wheat linkage group 2 versus *B. distachyon* chromosome 5, the relationship was consistent with known chromosomal relationships (International Brachypodium Initiative., 2010; Figure 4). We found that the synteny between wheat group 2 and *B. distachyon* chromosome 5 was generally in line with expectations; however, we did find that two SNPs (BS0010442 and BS00009533) that mapped to the same point in linkage group 2 were placed at opposite ends of *B. distachyon* chromosome 5. To investigate this further, we BLAST searched both the rice and *Sorghum bicolor* genomes with the sequences flanking BS0010442 and BS00009533. We found no match against the rice genome, but both SNPs mapped to within 135 kilobases of each other on *S. bicolor* chromosome 6. From these results, we tentatively conclude that the ancestral state of these markers is to lie in close proximity and that a chromosomal rearrangement, such as a reciprocal telomeric translocation, has altered their relative positions in the lineage leading to *B. distachyon*.

Screening the 500 sequences against the NCBI non-redundant protein sequence database generated 213 hits, of which 184 were unique. Owing to the nature of the normalized cDNA libraries used, the unique sequences identified did not show a significant bias to sequences associated with either leaf or root gene expression (the tissues used to generate the cDNA libraries); instead, they reveal an interesting mix of sequences, such as various disease resistance-like genes, sequences involved in DNA repair and various genes involved in numerous metabolic pathways. While this analysis indicated that a number of sequences (13) had multiple SNPs, it also revealed that 17 SNPs showed similarity to the same database entries, but mapped to different regions of the genome. For instance, three SNPs

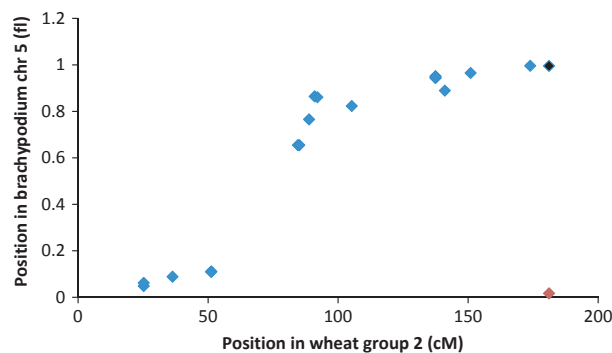


Figure 4 Scatterplot showing the syntenic relationship between the wheat SNP map positions generated in this study for linkage group 2 and the corresponding location in *Brachypodium distachyon*, shown as fraction of length (fl) along chromosome 5. The wheat SNP flanking sequences were matched to the *B. distachyon* genome using BLASTN with an e-value cut-off of $1e^{-10}$. Wheat linkage group 2 is shown here as it has the highest number of markers mapped onto the *B. distachyon* genome. The point marked in red is SNP BS0010442, and that in black is SNP BS00009533, both of which map to 181 cM in wheat linkage group 2.

showed sequence similarity to rice sequence H0322F07.7; one of these SNPs, BS00009929, mapped to an unassigned linkage group, while the second SNP, BS00010640, mapped to 2B and the third SNP, BS00014363, mapped to 6B. The presence of homoeologous and paralogous sequences throughout the wheat genome has been documented (Gupta *et al.*, 2008). However, the specific example shown here and the remaining 16 examples, in which similar sequences could be mapped to individual locations, suggest that KASPar-based genotyping is sufficiently sensitive and robust to map individual paralogous genes in the wheat genome.

Homoeologous versus non-homoeologous-specific KASPar probes

In wheat, the majority (~90%) of the KASPar probes detect both the polymorphic and non-polymorphic homoeologous loci. While such probes are suitable for screening inbred wheat varieties, screening heterozygous material is more problematical. To overcome this, we investigated the possibility of converting a standard KASPar probe (BS0000329; Figure 5b) to a homoeologous-specific KASPar probe. Using the 5 × Chinese Spring wheat genome sequence, we were able to design three homoeologous-specific reverse primers, which when used in conjunction with the original varietal SNP-specific KASPar primers generated one of two possible results; a non-polymorphic probe (when the homoeologous copy being amplified did not contain a varietal SNP; Figure 5c) or a polymorphic probe (when the homoeologous copy being amplified did contain a varietal SNP; Figure 5d). Use of the redesigned homoeologous-specific primers to screen an F2 population confirmed that they were capable of discriminating between homozygotes and heterozygotes (Figure 5e). Examination of the genomic sequence around several other KASPar loci suggested that it should be possible to design homoeologous-specific primers for the majority of the SNPs generated in this investigation. However, in the absence of both suitable primer design software and a more complete genome sequence, this process is time-consuming taking approximately 30 min per probe and requires further experi-

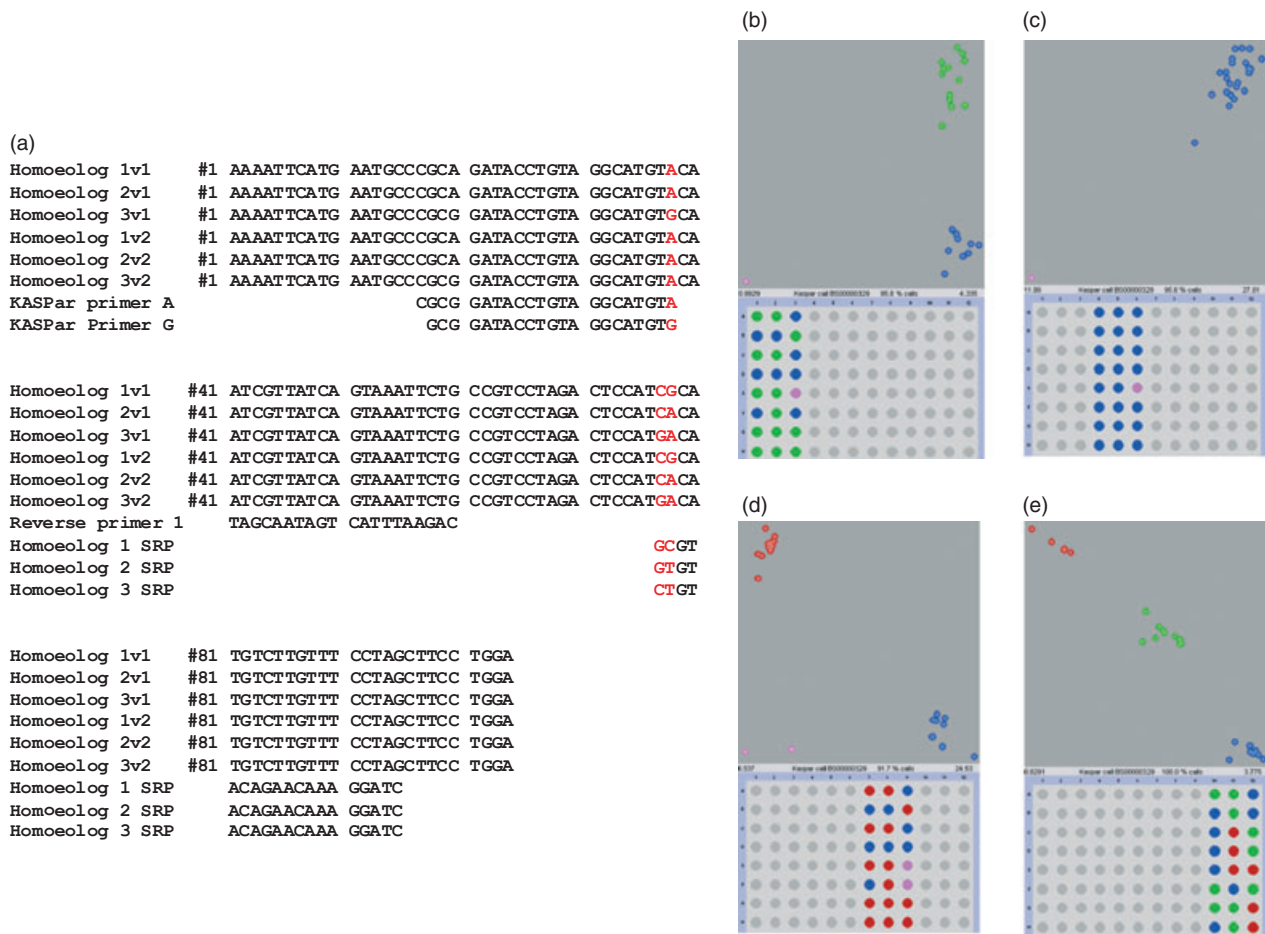


Figure 5 Effect of primer design of specificity of the KASPar reaction. (a) Sequence of region around SNP BS00000329 in the three wheat homoeologous genes and across two varieties (v1 and v2) together with the six probes designed for this region. The varietal SNP (A/G) and the homoeologous SNPs are highlighted. The reverse primer 1 and the three homoeologous-specific primers (SRP) are shown as designed in the reverse complement to the BS00000329 sequence. (b–d) KASPar plots following amplification with the various primer combinations. For b–c, the standard 20 hexaploid varieties were used together with a negative control (E3, E6 and E9) and a diploid, tetraploid and resulting synthetic line. In all cases, the KASPar primers A and G were used. In (b), the generic reverse primer was used; in (c), Homoeolog 1 SRP was used while in (d) Homoeolog 3 SRP was used. No results are presented for Homoeolog 2 SRP as this gave the same result as Homoeolog 1 SRP. (e) KASPar plot of F₂-derived material following amplification with the KASPar primers A and G together with the Homoeolog 3 SRP primer. In (e), both homozygotes (Blue and red spots) and heterozygotes (green spots) are shown as discrete clusters.

mental work to validate the appropriate primer combination. Therefore, it is our belief that currently it is more productive to design nonspecific KASPar probes and convert these to homoeologous-specific probes only when they are found to be useful for a specific purpose, for instance, when they are found to be tightly linked to a locus of interest.

Conclusions

To the best of our knowledge, this is the first report of a public linkage map for hexaploid wheat containing several hundred individual SNP markers. Bernardo *et al.* (2009) have reported a linkage map of wheat consisting of 923 single feature polymorphisms (SFPs) obtained by using Affymetrix arrays on 71 recombination inbred lines from the cross Ning 7840 × Clark. However, as reported, attempts to convert the array-based SFP markers to useful SNP markers that can be used in single locus assays were time-consuming and only partially successful with 33 working SNaPshot markers being generated from 58 SFPs

tested. In addition, we believe this report is also the first demonstration of KASPar-based technology to both genotype wheat varieties and generate a linkage map. To generate the SNP-based linkage map described, we carried out 102 220 individual KASPar reactions (538 probes × 190 plants). These reactions were carried out within a 24-h period, using simple microplate technology and a relatively inexpensive and widely available fluorescence resonance energy transfer plate reader. Using this and similar technology, we believe that it will be possible for wheat breeders to achieve one of their most important goals, to rapidly and cheaply genotype thousands of plants with a large and flexible number of markers.

Although we believe that the work described here meets the requirements of both wheat breeders and academics, it is likely that other technologies will be developed, which will increase the rate of SNP-based genotyping/mapping and decrease the cost of the individual data points. However, our studies have also shown that there is need for further SNPs, especially for the D genome and the homoeologous group 4 chromosomes,

but with continued SNP development genome-wide association studies will soon become possible in wheat. The extensive genomics resources developed by whole-genome shotgun sequencing of Chinese Spring, coupled to re-sequencing multiple breeding lines, promises to dramatically increase the number of informative SNPs, permitting unprecedented levels of precision genetic analysis in wheat breeding.

Our work has also highlighted the need to capture all of the information associated with the SNP markers, for instance, the sequence surrounding the SNP being assayed. This information will allow the SNP to be adapted and used in a variety of genotyping platforms as and when they are developed. It is for this reason that via this publication, we have made all the data associated with the SNP, including the available flanking sequences (usually 120 base pairs or greater) available via the associated supplementary data (Tables S1 and S2). We hope that by making this information available, it will encourage other wheat geneticists to ensure that such markers are made public and free to use. Only by following this principle will we help to ensure that wheat breeders across the world have the tools required to breed new varieties.

Experimental procedure

Plant material

The wheat lines included in this study were selected to give the best representation of the UK wheat germplasm. Twenty-three different wheat varieties were grown for nucleic acid extraction (for details see Table 2). The Avalon × Cadenza population (supplied by the John Innes Centre) of doubled haploid (DH) individuals, derived from F1 progeny of a cross between cvs Avalon and Cadenza, was developed by Clare Ellerbrook, Liz Sayers and the late Tony Worland (John Innes Centre), as part of a Defra funded project led by ADAS. The parents were originally chosen (to contrast for canopy architecture traits) by Steve Parker (CSL), Tony Worland and Darren Lovell (Rothamsted Research, West Common, Harpenden, Hertfordshire, AL5 2JQ, UK).

All plants were grown in pots in a peat-based soil and maintained in a glasshouse at 15–25 °C under a light regime of 16 h light, 8 h dark. Root and leaf tissues were harvested from 6-week-old plants. All harvested tissues were immediately frozen on liquid nitrogen and stored at –80 °C until nucleic acid extraction.

Preparation of normalized cDNA libraries

Total RNA was extracted from root and leaf tissue of five wheat varieties (Avalon, Cadenza, Rialto, Savannah, Recital) using the TRIzol RNA extraction protocol (Invitrogen Ltd., Paisley, UK) according to the manufacturer's instructions and purified using the RNeasy MinElute Kit (QIAGEN Ltd., Crawley, UK). Complementary DNA (cDNA) was synthesized from total RNA using the MINT kit (Evrogen, Moscow, Russia) according to the manufacturer's instructions. Double-stranded cDNA was purified using the QiaQuick PCR purification kit (QIAGEN Ltd.). The purified cDNA samples were then normalized using the TRIMMER kit (Evrogen) according to the manufacturer's instructions and purified using the QiaQuick PCR purification kit (QIAGEN Ltd.).

Next-generation sequencing

For each variety, 5 µg of normalized cDNA was processed for sequencing by the University of Bristol Transcriptomics Facility.

Independent sequence libraries were generated by following the manufacturer's protocol for sequencing of genomic DNA (Illumina Inc., San Diego, CA), with a modification to allow multiplexing of samples. Five sets of custom adapters were designed based upon the Illumina PCR primers plus an extra 4-base barcoding tag. Sequencing was carried out on a paired-end flowcell, using Illumina's v.4 Cluster Generation and multiple v.4 36-Cycle Sequencing Kits. The Illumina Genome Analyzer Ix, was run for 2 × 76 bases of data acquisition. Image analysis and base calling was performed with SCS 2.3 software (Illumina Inc, San Diego, CA).

SNP discovery

Putative varietal SNPs were mined from two sources. First, public wheat Expressed Sequence Tag (EST) data held at NCBI was used as described by Barker and Edwards (2009). In this data set, 3500 SNPs were deemed to have a high probability of being varietal (as opposed to homoeologous), and from these, we selected 213 for validation. In the second approach, SNPs were mined from NGS data as follows. A reference sequence data set comprising 91 368 sequences was produced by combining the data from NCBI wheat unigene build 38 with the NGS data used in this study. The NCBI sequences were sampled as pseudoreads of 75 bases, combined with the NGS data and *de-novo* assembled using ABYSS (Simpson *et al.*, 2009) on the Bristol Bluecrystal HPC cluster. NGS from the five UK varieties were mapped to our custom reference using ELAND (Illumina Inc.) with a seed length of 32 bases and the resulting sorted export files used for downstream analysis. Uniquely mapped reads were analysed using a series of custom PERL scripts designed to identify only differences between varieties as opposed to those between each variety, and the reference sequence. In this way, homoeologous SNPs (which are not useful markers) were excluded from our SNP discovery pipeline. SNPs were called where at least two alternative bases were found at a reference position, each represented by 2 or more independent reads or 5% of all reads examined (whichever was the greater). Only bases at the centre of a three base window of PHRED quality ≥20 were included in the analysis. Sequences were discarded if they showed more than 5% sequence variation from the reference over their length or if they mapped equally well to more than one locus as in either case they were deemed to have uncertain mapping. Finally, where multiple reads started at the same position in the reference, all but one were ignored to guard against clonal reads being sampled more than once. The NGS data will be made available at: <http://www.cerealsdb.uk.net/NGSdata/AllenSupplement>.

SNP validation

Genomic DNA was prepared from leaf tissue using a phenol-chloroform extraction method (Sambrook *et al.*, 1989). Genomic DNA samples were treated with RNase-A (New England Biolabs UK Ltd. Hitchin, UK) according to the manufacturer's instructions and purified using the QiaQuick PCR purification kit (QIAGEN Ltd.).

For each putative varietal SNP, two allele-specific forward primers and one common reverse primer (Table S6) were designed (KBioscience, Hoddesdon, UK). Genotyping reactions were performed in a Gene Pro Thermal cycler (Bioer Technology, Hangzhou, China) in a final volume of 5 µL containing 1X KASP Reaction Mix (KBioscience), 0.07 µL Assay mix (containing 12 µM each allele-specific forward primer and 30 µM reverse pri-

mer) and 10–20 ng genomic DNA. The following cycling conditions were used: 15 min at 94 °C; 10 touchdown cycles of 20 s at 94 °C, 60s at 65–57 °C (dropping 0.8 °C per cycle); and 26–35 cycles of 20 s at 94 °C, 60 s at 57 °C. Fluorescence detection of the reactions was performed using an Omega Fluorostar scanner (BMG LABTECH GmbH, Offenburg, Germany), and the data were analysed using the KlusterCaller 1.1 software (KBioscience). The polymorphic information content (PIC) was calculated for each marker according to Botstein *et al.* (1980), using Excel Microsatellite Toolkit add-in software (Park, 2001). Hierarchical cluster analysis was performed in PASW (SPSS Inc. IBM Corporation, Somers, NY).

Genetic map construction

The software program MapDisto v. 1.7 (Lorieux, 2007) was used to place the SNP markers in the previously established genetic map for Avalon × Cadenza (<http://www.wgin.org.uk/resources/MappingPopulation/TAMapping.php>). A chi-square test was performed on all loci to test for segregation distortion from the expected 1 : 1 ratio of each allele in a DH population, and any loci showing significant distortion were removed from the data set before constructing the linkage groups. Loci were assembled into linkage groups using likelihood odds (LOD) ratios with a LOD threshold of 6.0 and a maximum recombination frequency threshold of 0.40. The linkage groups were ordered using the likelihoods of different locus-order possibilities and the iterative error removal function in MapDisto and drawn in MapChart (Voorrips, 2002). The Kosambi mapping function (Kosambi, 1944) was used to calculate map distances (cM) from recombination frequency.

Comparative mapping of SNPs to ESTs and the *Brachypodium distachyon* and *Sorghum bicolor* genomes

The consensus sequence surrounding mapped SNPs were matched to published, mapped wheat EST sequences (<http://wheat.pw.usda.gov/cgi-bin/westsq/locus.cgi>) and to the *B. distachyon* genome sequence (<http://www.brachypodium.org/>) using NCBI BLASTN (Altschul *et al.*, 1990) with an e-value cut-off of $1e^{-10}$. Only the top BLASTN hit was used for comparative mapping.

Selected wheat SNP flanking sequences were also BLASTN searched to the *S. bicolor* genome at <http://www.phytozome.net> using Phytozome version 6.

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× Cadenza population public. For further details of the Avalon × Cadenza mapping population, please refer to the Wheat Genetic Improvement Network web site at: <http://www.wgin.org.uk/resources/MappingPopulation/TAMapping.php>.

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Supporting information

Additional Supporting information may be found in the online version of this article:

- Table S1** EST-derived SNPs.
- Table S2** NGS-derived SNPs.
- Table S3** Genotyping calls.
- Table S4** Mapping data.
- Table S5** BLAST results.
- Table S6** KASPar assays and primer sequences.

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