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Development of wild barley-derived DArT markers and their integration into a barley consensus map

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Abstract Wild barley-specific genomic libraries were developed for the purpose of creating a 'comprehensive' genomic representation of the primary *Hordeum* genepool capable of more robust genotyping of barley. In order to enrich for wild barley-specific sequences in the DArT libraries, suppression subtraction hybridization (SSH) was performed using cultivated barley as the subtraction driver and wild barley as the tester. Four doubled-haploid populations were genotyped with the comprehensive barley DArT array, including two from wild × cultivated crosses (Damon/Harrington and Shechem/Harrington) and two from cultivated × cultivated crosses (Albacete/Barbarrouse and TX9425/Naso Nijo). Analysis of genotyping data revealed that the SSH process was somewhat ineffective at enriching for unique sequences in this application of DArT marker development. However, the addition of markers derived from wild barley proved to be an effective means for increasing the number of polymorphic markers obtainable from a single DArT assay. Genetic maps of the four component populations were developed and 607 newly developed DArT markers were integrated with a barley consensus map to create a new synthetic map of the barley genome containing 3542 markers. This significantly increased the resolution of the consensus map and improved the power of the map to provide a reference for profiling genetic diversity within the primary *Hordeum* genepool. The improvement in the genotyping capability of the comprehensive DArT genomic representation and the higher resolution of the synthetic map facilitates an even greater flexibility of DArT markers to be utilized as a fast, high-throughput platform for molecular marker-based barley breeding.

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Introduction

Modern cultivated barley (*Hordeum vulgare* subsp. *vulgare*) is one of the world's 12 major food crops, and one of the five major cereal grains, which together account for over half of all calories consumed by humans. The majority of barley grown for human consumption is used for malting and ultimately in the brewing process for beer manufacturing. Therefore, many of the selections made by breeders have been to increase malting quality and yield. Barley is a self-pollinating crop and as a result of numerous inbred pedigrees has come to have a very narrow genetic base (Rasmusson and Phillips 1997). This lack of genetic diversity creates limitations in breeding novel traits into cultivated germplasm.

Much research has begun to emerge in the use of wild barley as a novel source of genetic diversity for improvement of cultivated germplasm (Pillen et al. 2003, 2004; von Korff et al. 2004, 2005, 2006). In order to increase the diversity for disease resistance in cultivated barley, we developed two doubled-haploid (DH) mapping populations from intra-specific wild × cultivated barley crosses. Two wild barley accessions, Damon 11–11 and Shechem 12–32 (hereafter referred to as Damon and Shechem), were previously identified as having resistance to six major diseases of barley (Fetch et al. 2003). Each of these accessions was crossed to the North American two-rowed malting quality standard cultivar Harrington, and two F_1-derived DH populations were developed using the *Hordeum bulbosum* method (Pickering and Fautrier 1993). These populations were used for the linkage mapping reported in this study and also as a tool in quantitative trait loci (QTL) analyses published in a separate work.

Linkage mapping is the first essential step in the identification of loci affecting quantitative traits such as yield, quality, and, in some cases, disease resistance. The barley research community has worked assiduously to produce numerous linkage maps of the barley genome. Over the past two decades, different maps have been generated with markers based on restriction fragment length polymorphism (Graner et al. 1991; Heun et al. 1991; Kleinhofs et al. 1993), amplified fragment length polymorphism (Qi et al. 1998), and simple sequence repeats (Ramsay et al. 2000). Many researchers also have integrated populations and/or marker types to produce consensus maps (Karakousis et al. 2003; Langridge et al. 1995; Marcel et al. 2007; Qi et al. 1996; Stein et al. 2007; Varshney et al. 2007; Wenzl et al. 2006). The recent trend, however, has been to switch to more high-throughput and cost-efficient hybridization-based markers such as single nucleotide polymorphism (SNP) and Diversity Arrays Technology (DArT) (Rostoks et al. 2005; Wenzl et al. 2004, 2006, Hearnden et al. 2007).

In order to tailor these high-throughput marker systems toward wild barley genotyping applications, wild barley-specific genomic libraries were developed in conjunction with Diversity Arrays Technology Pty. Ltd. to produce wild barley-derived DArT markers. These markers were combined with the previously described cultivated-barley-derived DArT markers (Wenzl et al. 2004) for the purpose of creating a 'comprehensive' genomic representation of the primary *Hordeum* genepool capable of more robust genotyping of barley. In order to enrich for wild barley-specific sequences in the DArT libraries, suppression subtraction hybridization (SSH) was performed using cultivated barley as the subtraction driver and wild barley as the tester prior to cloning. The efficacy of utilizing the subtraction technique to enhance the diversity of clones on the array and the use of wild- and cultivated-barley-specific DArT libraries to genotype wild and cultivated barley is discussed. We report the development of genetic maps of four DH populations genotyped with the comprehensive barley DArT array, including two from wild × cultivated crosses (Damon/Harrington and Shechem/Harrington) and two from cultivated × cultivated crosses (Albacete/Barbarrouse and TX9425/Naso Nijo). We also report the development of a synthetic map of barley that incorporates 607 additional DArT markers from these four populations into the barley DArT consensus map reported by Wenzl et al. (2006). This synthetic map differs from traditional consensus maps as it does not integrate the datasets of multiple mapping populations derived from independent crosses but rather utilizes information on locus positions of individual component maps to create one combined map.

Materials and methods

Plant materials

Two DH populations were developed from crosses between each of the *H. vulgare* subsp. *spontaneum* accessions Damon and Shechem and the barley cultivar Harrington. F_1 progeny of these two-rowed × two-rowed crosses were then hybridized with *Hordeum bulbosum* to produce the DH populations via haploid embryo rescue and colchicine-induced chromosome doubling (Pickering and Fautrier 1993). These DH populations were provided by R. Pickering (New Zealand Institute for Crop & Food Research Ltd, Christchurch, New Zealand). The wild barley accessions were both collected in Israel: Damon near the Haifa Peninsula and Shechem near the modern-day city of Nablus in the West Bank. Both carry resistance to six major diseases of barley (Fetch et al. 2003). The Damon/Harrington (D/H) and Shechem/Harrington (S/H) populations have 114 and 160 progeny, respectively.

For the purpose of incorporating wild barley-derived DArT markers into the barley DArT consensus map, we added mapping data for two additional populations that were genotyped with the comprehensive barley array. The Albacete/Barbarrouse (A/B) and TX9425/Naso Nijo (T/N) populations are both from crosses between *H. vulgare* subsp. *vulgare* cultivars. Albacete and Barbarrouse are both six-rowed winter feed barleys of European origin, whereas TX9425 and Naso Nijo are Chinese feed and Japanese malting cultivars, respectively. A/B and T/N are both DH populations produced by anther culture (Davies 2003) and have 224 and 188 progeny, respectively.

Suppression subtraction hybridization (SSH)

SSH was performed as previously described (James et al. 2008), using all the same restriction enzymes as well as adaptor and primer sequences. Two sets of suppression subtraction hybridizations were performed, both of which utilized cv. Harrington as the subtraction driver. Harrington was chosen as the driver in order to enrich for sequences that would be polymorphic in the D/H and S/H mapping populations. Of the two sets of tester DNA samples utilized, tester 1 contained a mixture of the two wild barley parents previously discussed (Damon and Shechem), and tester 2 contained a mixture of 31 *H. vulgare* subsp. *spontaneum* accessions from the Wild Barley Diversity Collection (Steffenson et al. 2007; Table 1). These 31 accessions were selected for maximum genetic diversity based on ten microsatellite markers (Steffenson et al. 2007) and included four accessions (Damon, Shechem, 41–1, and OUH602) used in various wild × cultivated barley mapping populations (Baum et al. 2003; Yun et al. 2005). For each set of hybridizations, subtraction was carried out in either one or two stages. Amplification of subtraction products and subsequent cloning led to the production of four 384-well plates of wild barley-derived DArT clones (markers).

Comprehensive barley DArT genomic representation

The full comprehensive barley *PstI/BstNI* genomic representation (array) consists of 16,384-well plates of DArT markers. In addition to the four wild barley-derived plates produced by SSH described above, there were five additional plates of wild barley-derived markers produced by Triticarte Pty. Ltd. with standard DArT protocols for *PstI/BstNI* digestion-based array development (Jaccoud et al. 2001; Wenzl et al. 2004). Of these five plates, two and three of them were produced from mixtures of accessions 28–31 and 1–31, respectively (Table 1). The remaining seven plates were widely utilized *PstI/BstNI*-derived markers ('bPb' markers) that originated from cultivated barley, six of which were enriched for polymorphic markers and the other of non-polymorphic control clones (Wenzl et al. 2004, 2006).

DArT marker analysis

Genomic DNA was isolated from the progeny of each population for subsequent genotyping with DArT molecular markers. The FASTprep kit (Q-BIOgene, Irvine, CA) was used to extract DNA from young tillers of 5-week-old plants of the D/H and S/H populations. The DNeasy 96 Plant Kit (Qiagen, Valencia, CA) was used to extract DNA from young leaves of the A/B progeny, and a modified CTAB method was used to extract DNA from 3-week-old seedlings of the T/N population (Stein et al. 2001). All four populations and their respective parents were

Table 1 Accessions of the Wild Barley Diversity Collection (WBDC) utilized in the production of wild barley-derived DArT markers

#	Identifier		Origin		
	Name	Other designator	Country	City/Province	Source[a]
1	WBDC008	38627	Jordan	Irbid	ICARDA
2	WBDC025	38693	Pakistan	Balochistan	ICARDA
3	WBDC052	39850	Jordan	Mafraq	ICARDA
4	WBDC060	39891	Egypt	Marsa Matruh	ICARDA
5	WBDC064	39914	Syria	Idlib	ICARDA
6	WBDC071	39933	Libya	Al Marj	ICARDA
7	WBDC077	39941	Russia	Daghestan	ICARDA
8	WBDC101	40063	Jordan	Tafila	ICARDA
9	WBDC102	40064	Jordan	Ma'an	ICARDA
10	WBDC109	40090	Syria	Sweida	ICARDA
11	WBDC121	40138	Iran	Fars	USDA
12	WBDC123	40142	Iran	Khorasan	USDA
13	WBDC140	40186	Lebanon	Baalbek	ICARDA
14	WBDC145	40194	Lebanon	Zahle	ICARDA
15	WBDC156	107425	Iraq	Mosul	ICARDA
16	WBDC172	112673	Iran	Hamadan	IPK
17	WBDC180	112847	Libya	Tubruq	IPK
18	WBDC189	116105	Turkey	Gaziantep	ICARDA
19	WBDC196	116125	Turkey	Gaziantep	ICARDA
20	WBDC198	119402	Syria	Homs	ICARDA
21	WBDC207	123949	Uzbekistan	Fergana	ICARDA
22	WBDC217	126933	Armenia	Yerevan	ICARDA
23	WBDC218	131375	Kazakhstan	Dzhambul	ICARDA
24	WBDC227	132552	Azerbaijan	Lankaran	ICARDA
25	WBDC233	38668	Afghanistan	Baghlan	USDA
26	WBDC335	135624	Turkmenistan	Kazanjik	ICARDA
27	WBDC347	40152	Uzbekistan	Surkhandar'ya	USOU
28	WBDC348	Damon 11–11[b]	Israel	Damon	U. of Haifa
29	WBDC349	Shechem 12–32[b]	Israel	Shechem	U. of Haifa
30	WBDC350	41–1[c]	Israel	Unknown	ICARDA
31	WBDC355	OUH602[c]	Trans caucasus region	Unknown	Okayama U.

[a] *Source* ICARDA (International Center for Agricultural Research in the Dry Areas). USDA (United States Department of Agriculture), IPK (Institute of Plant Genetics and Crop Plant Research), USOU (School of Botanical Sciences, University of Southampton, UK)

[b] More precise locations referenced in previous literature (Fetch et al. 2003)

[c] Utilized in previous mapping studies (Baum et al. 2003; Górny 2001; Yun et al. 2005)

genotyped with the comprehensive barley genomic representation. DArT assays, including sample preparation, hybridization, scanning, and marker scoring, were conducted by Triticarte Pty. Ltd. and performed as previously described (Wenzl et al. 2006).

Linkage map development

Linkage map construction for all populations was performed, and resultant maps provided, by Triticarte Pty. Ltd. utilizing purpose-built software for

semi-automatic map construction (EasyMap; Diversity Arrays Technology P/L, Canberra, Australia). Briefly, this software combines pre-map quality filtering of markers/lines with several iterations of map construction followed by post-map quality filtering of markers, lines, and individual genotype calls. In each of these iterations, the whole set of markers is ordered using the RECORD algorithm (Van Os et al. 2005) and split into linkage groups based on a combination of criteria (recombination frequency, linkage group length, cM tension). The order of markers within each linkage group is then re-optimized, all possible pairs of linkage-group ends are tested for compatibility, compatible ends are joined, and the marker order in the resultant larger linkage group(s) is re-optimized. Potential genotyping errors ($LOD_{error} > 4$; Lincoln and Lander 1992) are then identified and substituted with missing data. Finally, the marker order of affected linkage groups is re-optimized again, and Kosambi cM distances are reported.

A total of seven individuals and 117 markers were removed from the analysis of the D/H population due to data quality issues, which may have arisen from poor DNA quality and/or excessive (or highly variable) DNA concentrations of some samples (P. Wenzl, unpublished data). Quality filtering of the S/H population removed 39 markers, but did not result in the removal of any individuals. In the A/B and T/N populations, four markers but no individuals were removed due to poor quality. The maps generated by EasyMap were compared with a barley consensus map (Wenzl et al. 2006) to identify and rectify cases of pseudo-linkage and to orient linkage groups. The D/H and S/H populations resulted in seven linkage groups, whereas the A/B and T/N populations resulted in 11 and 10 linkage groups, respectively. There was more than one linkage group for some chromosomes in the A/B (1H, 2H, and 3H) and T/N (3H, 5H, and 6H) populations. Albacete carries a reciprocal translocation with breakpoints at or near the centromeres of chromosomes 1H and 3H (Lacasa-Benito et al. 2005; Farre 2008). As the A/B population segregated for chromosome arrangement, standard and translocated, no attempt was made to generate single 1H and 3H linkage groups from the A/B mapping data. Rather, the linkage groups were independently determined at the individual arm level. With the exception of very few rearrangements of closely linked markers, common markers in all linkage groups from the four populations were ordered identically to those previously reported for the known seven chromosomes of barley (Fig. 1). All component and synthetic map figures included in this manuscript were produced utilizing MapChart software (Voorrips 2002).

Synthetic map development

The locus positions of the four maps genotyped with the comprehensive barley array, as well as those from the cultivated barley consensus map (Wenzl et al. 2006), were merged with PhenoMap software to create a synthetic map (GeneFlow Inc., Centreville, VA.). PhenoMap utilizes a multi-phase heuristic procedure to merge component maps and estimate the order and relative distance between markers. The integration is based upon markers found in common between linkage groups, which form the framework of the synthetic chromosome. The process begins with the selection of a base linkage group, often the one with the most common markers, which establishes the order of a subset of common markers. Following this step, two additional but separate phases are required to complete the process. The first establishes the order of all common markers, and the second integrates markers unique to each linkage group. To add the rest of the common markers (phase 1), the remaining linkage groups are processed in order such that those containing the highest number of remaining unordered common markers are processed first. Unordered common markers are placed (ordered) in the growing synthetic map in relation to the nearest common flanking markers. The distance between each adjacent pair of common markers is calculated as an average interval size from all maps containing those markers. Once all common markers have been added, unique markers are placed on the synthetic framework (phase 2). Distances of unique markers from the component maps are calculated based on their relative distance to the nearest flanking common markers and scaled to the equivalent distance on the synthetic map.

In the cases where there were multiple linkage groups per chromosome (A/B and T/N populations), the position for the first marker of the distal linkage groups was arbitrarily chosen, as each chromosome must be loaded into PhenoMap as one group. Since the relative distance between markers affects the outcome of the synthetic map, for these instances, the position of the first marker in each distal linkage

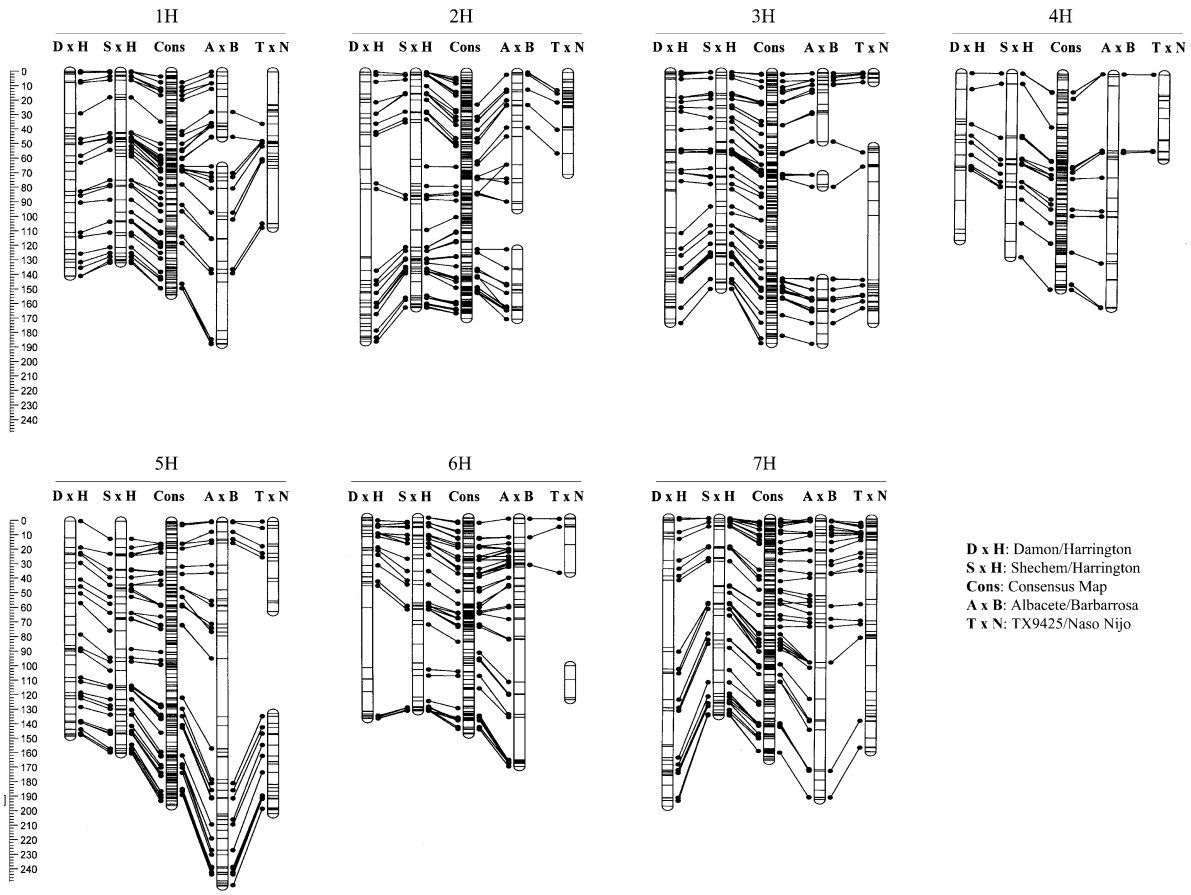

Fig. 1 Colinearity of locus order in component maps. Loci in component maps are displayed schematically by *horizontal lines* across the *bars* representing chromosomes. Loci that are common between adjacent pairs of populations are depicted by *dots* and connected by *lines*

group was established based on previously reported map positions (Wenzl et al. 2006). The position of the first marker in those secondary linkage groups that was in common with the consensus map (in four out of five cases this was the first marker in the group, in the other case it was the second) was taken from the consensus map and used as the position in the component map. The positions of the remaining markers in those groups were then adjusted accordingly based on their calculated intervals.

Results

Component DArT marker linkage maps

From a total of 6144 DArT markers on the comprehensive array surveyed for polymorphism, 573.3 ± 31.6 (9.3 ± 0.5%) were polymorphic and mapped in the four component populations (Table 2). The map lengths of these populations ranged from 709.5 cM (T/N) to 1177.1 cM (A/B) with an average length of 988.2 ± 201.1 cM. There were a number of redundant markers, i.e. those mapping to the identical location in each population, within each map. When the redundant markers were collapsed into bins, the number of unique bins per population ranged from 182 (T/N) to 240 (S/H) with an average of 219.8 ± 26.9. The distance between unique bins ranged from 3.90 cM (T/N) to 4.95 cM (A/B) and on average was 4.47 ± 0.54 cM. Across all four populations, the average number of unique bins was 28.5 ± 4.7, 33.8 ± 7.7, 36.0 ± 5.2, 16.3 ± 3.3, 39.3 ± 6.4, 27.5 ± 12.2, and 38.5 ± 5.9 for chromosomes 1H to 7H, respectively.

Of the markers mapped in the four component populations genotyped with the comprehensive

Table 2 Map features of the four component linkage maps created from populations genotyped with the comprehensive barley DArT genomic representation and used in synthetic map development

Population[a]	D × H	S × H	A × B	T × N	Average ± SD
Length (cM)	1077.5	988.8	1177.1	709.5	988.2 ± 201.1
Number of markers	559	620	563	551	573.3 ± 31.6
Number of unique bins	219	240	238	182	219.8 ± 26.9
Inter-bin distance (cM)	4.92	4.12	4.95	3.90	4.47 ± 0.54

[a] Population used to create linkage map: Damon/Harrington (D × H), Shechem/Harrington (S × H), Albacete/Barbarrouse (A × B), TX9425/Naso Nijo (T × N), and the average ± SD

Table 3 Pair-wise comparison of the number of common markers between and unique markers (shown in bold) in the four component maps and the barley DArT consensus map used in synthetic map development

	Component map[a]				
	Consensus	D × H	S × H	A × B	T × N
Consensus	**2005**	419	453	329	302
D × H	–	**56**	286	142	125
S × H	–	–	**81**	126	119
A × B	–	–	–	**146**	154
T × N	–	–	–	–	**159**

[a] Population used to create component linkage map: Damon × Harrington (D × H), Shechem × Harrington (S × H), Albacete × Barbarrouse (A × B) and TX9425 × Naso Nijo (T × N)

genomic representation, at least 300 of them in any given population were also mapped in the barley DArT consensus map (Table 3). In pair-wise comparisons of the four component maps, the number of markers in common ranged from 119 (S/H and T/N populations) to 286 (D/H and S/H). With the exception of very few closely linked minor rearrangements, common markers in all linkage groups from all four populations and the barley consensus map were ordered identically (Fig. 1). The number of markers unique to each map was 56, 81, 146, and 159 for the D/H, S/H, A/B, and T/N populations, respectively (Table 3).

On average, 77.0 ± 5.7% and 23.0 ± 5.7% of the markers mapped in the four component populations were derived from cultivated and wild germplasm, respectively (Table 4). Of those markers derived from wild barley, 57.5 ± 11.9% and 42.5 ± 11.9% were produced via standard DArT and SSH methods, respectively. Of those markers produced via standard DArT methods, 37.7 ± 7.1% and 62.3 ± 7.1% were derived from wild barley accessions 28–31 and 1–31, respectively. Similarly, for those produced via SSH, 66.8 ± 8.1% and 33.2 ± 8.1% were derived from accessions 28–29 and 1–31, respectively.

Of the cultivated barley-derived markers mapped in the D/H and S/H populations, 71.3 ± 2.4% were present (positive or greater hybridization on the array as compared to the other parent; scored as '1') in the cultivated parent, cv. Harrington, and 28.7 ± 2.4% were present in the wild parents (Table 5). Of the markers derived from wild barley, 33.2 ± 2.8% and 66.8 ± 2.8% were present in cv. Harrington and the wild parents, respectively.

A synthetic map of barley

Map synthesis was conducted five times for each chromosome, once utilizing each component map as the base or reference map, to determine the most optimal map to use as the reference for map synthesis. The most optimal map to use as the reference map for synthesis was determined based upon which synthetic map resulted in the least number of incorrectly ordered loci in the four remaining component maps after synthesis, and, to a lesser extent, the degree of map expansion in the resultant synthetic map as compared to the consensus map. Genome-wide, utilizing the consensus map as the base map resulted in the fewest number of incorrectly ordered markers and a moderate expansion of map length as compared to utilizing the other component maps as the base map (Table 6). Since the consensus map resulted in the fewest number of incorrectly ordered loci and also contained the greatest number of common markers between component maps, it was chosen as the base map for synthesis for all chromosomes.

Table 4 Comparison of germplasm sources and methods of marker development of mapped DArT markers

	Population[a]					Total array[e]
	D × H	S × H	A × B	T × N	Average ± SD	
Wild[b]	20.0	20.2	20.2	31.6	23.0 ± 5.7	56.3
Via DArT[c]	65.2	68.8	53.5	42.5	57.5 ± 11.9	55.6
From 28–31[d]	43.8	37.2	27.9	41.9	37.7 ± 7.1	40.0
From 1–31[d]	56.2	62.8	72.1	58.1	62.3 ± 7.1	60.0
Via SSH[c]	34.8	31.2	46.5	57.5	42.5 ± 11.9	44.4
From 28–29[d]	71.8	59.0	75.5	61.0	66.8 ± 8.1	50.0
From 1–31[d]	28.2	41.0	24.5	39.0	33.2 ± 8.1	50.0
Cultivated[b]	80.0	79.8	79.8	68.4	77.0 ± 5.7	43.8
Total	100.0	100.0	100.0	100.0	100.0	100.0

[a] Population used to create linkage map: Damon/Harrington (D × H), Shechem/Harrington (S × H), Albacete/Barbarrouse (A × B), TX9425/Naso Nijo (T × N), and the average ± SD

[b] Percentage of mapped markers derived from wild or cultivated barley germplasm

[c] Percentage of mapped markers derived from wild barley developed via standard DArT procedures or with the additional step of suppression subtraction hybridization (SSH)

[d] Percentage of mapped markers within each method of marker development that were derived from different mixtures of wild barley accessions (28–29, 28–31, or 1–31; Table 1)

[e] Breakdown of percentages of DArT markers present on the comprehensive genomic representation (array) used to genotype these populations by source of germplasm and method of marker development

Table 5 Comparison of polymorphic DArT marker features in two wild × cultivated barley populations

Population[a]	D × H	S × H	Average ± SD
Total	100.0	100.0	100.0
From cultivated[b]	80.0	79.8	79.9 ± 0.1
Present in wild	30.4	27.1	28.7 ± 2.4
Present in cultivated	69.6	72.9	71.3 ± 2.4
From wild[b]	20.0	20.2	20.1 ± 0.1
Present in wild	68.8	64.8	66.8 ± 2.8
Present in cultivated	31.3	35.2	33.2 ± 2.8

[a] Wild × cultivated population genotyped with the comprehensive barley DArT array: Damon/Harrington (D × H) and Shechem/Harrington (S × H) and average ± SD

[b] Percentage of polymorphic DArT markers derived from cultivated or wild barley, and, of those, the percentage that were present (positive or greater hybridization on the array as compared to the other parent; scored as '1') in the respective cultivated (Harrington) or wild (Damon or Shechem) barley parent

Table 6 Comparison of resultant genome-wide incorrectly ordered loci and degree of map expansion during synthetic map development

Reference map[a]	Incorrectly ordered loci[b]	Map length[c] (cM)
D × H	33.50	70.7
S × H	30.75	36.0
A × B	31.50	155.5
T × N	31.75	68.8
Consensus	20.75	77.4

[a] Reference map used for synthetic map construction: Damon/Harrington (D × H), Shechem/Harrington (S × H), Albacete/Barbarrouse (A × B), TX9425/Naso Nijo (T × N), and consensus maps

[b] Average number of resultant incorrectly ordered loci in the remaining four non-reference maps

[c] Difference in map length between the synthetic and consensus maps

Mapping of the four component populations genotyped with the comprehensive barley array and their subsequent synthesis with the barley DArT consensus map led to the integration of 607 previously unincorporated DArT markers. The number of newly integrated markers ranged from 44 (4H) to 129 (7H) and on average was 86.7 ± 26.8 per chromosome (Table 7). The number of unique bins in the synthetic map increased as well, as compared to the consensus map, by 257. By chromosome, the increase in bins ranged from 23 (4H) to 53 (7H) and on average increased by 36.7 ± 11.1 bins. The overall

Table 7 Number of loci, unique bins, average/median inter-bin distance, and map length in the consensus and synthetic maps for chromosomes 1H through 7H

	1H	2H	3H	4H	5H	6H	7H	Genome
Number of loci								
Consensus	423	522	470	278	379	362	501	2,935
Synthetic	489	620	552	322	474	455	630	3,542
Difference[a]	66	98	82	44	95	93	129	607
Number of bins								
Consensus	158	210	165	124	180	135	181	1,153
Synthetic	184	241	204	147	228	172	234	1,410
Difference[a]	26	31	39	23	48	37	53	257
Average/median inter-bin distance (cM)								
Consensus	1.0/0.8	0.8/0.5	1.1/0.8	1.2/0.7	1.1/0.8	1.1/0.7	0.9/0.7	1.0/0.7
Synthetic	0.8/0.5	0.8/0.5	0.9/0.6	1.0/0.6	0.9/0.6	1.0/0.6	0.9/0.6	0.9/0.6
Difference[a]	−0.2/−0.3	0/0	−0.2/−0.2	−0.2/−0.1	−0.2/−0.2	−0.1/−0.1	0/−0.1	−0.1/−0.1
Map length (cM)								
Consensus	151.9	166.7	184.6	148.6	194.2	147.6	165.4	1,159.0
Synthetic	142.7	200.1	184.8	147.1	198.0	162.8	200.9	1,236.4
Difference[a]	−9.20	33.40	0.20	−1.50	3.80	15.20	35.50	77.40

[a] Values for the consensus map subtracted from the synthetic map

length of the map increased by 77.4 cM. By chromosome, differences ranged from a decrease of 9.2 cM for chromosome 1H to an increase of 35.5 cM for chromosome 7H, and on average increased by 11.1 ± 17.6 cM per chromosome. The change in average inter-bin distance (IBD) ranged from a decrease of 0.2 cM (1H, 3H, 4H, and 5H) to no change (2H and 7H), and the change in median IBD ranged from a decrease of 0.3 cM (1H) to no change (2H). Genome-wide, the synthetic map contains 3542 loci and is 1236.4 cM in length. There are 1410 unique bins with an average/median IBD of 0.9/0.6 cM. There are 489, 620, 552, 322, 474, 455, and 630 loci mapped on chromosomes 1H–7H, respectively.

Discussion

We developed several new DArT marker libraries that were derived from a broader representation of the primary *Hordeum* genepool, thus increasing the diversity of markers on the array and providing a platform for more robust genotyping of barley. The full comprehensive barley array consists of seven 384-well plates of clones derived from cultivated barley (Wenzl et al. 2004), as well as nine 384-well plates of clones derived from wild barley. The wild barley-derived DArT markers were developed using both standard DArT and SSH methods. Using four DH barley populations genotyped with the comprehensive DArT array, we incorporated novel wild barley-derived markers and produced maps of highly comparable order to the DArT consensus map. The integration of these maps with the consensus map led to an improved synthetic map with 20.7% more markers (3542 in total).

Suppression subtractive hybridization (SSH) for DArT marker development

Of the 6144 DArT markers present on the comprehensive DArT array, 56.3% were newly developed and derived from wild barley (Table 4). Despite that majority, 77.0% of the markers mapped in the four component populations were from the minority (43.8%) derived from cultivated barley. This is not surprising, however, as the cultivated-barley-derived markers have been enriched for polymorphism over time (Wenzl et al. 2004). The number of wild barley-derived markers mapped in the four component populations and the methods from which they were

developed are indicative of the effectiveness of those methods of marker development.

It has been reported that as few as 40% of the alleles present in wild barley are also present in cultivated germplasm (Ellis et al. 2000). The SSH technique is designed to enrich for those sequences (markers) that are unique to wild barley and therefore theoretically should result in a higher percentage of mapped wild barley-derived markers than those produced via standard DArT methods. This should especially be the case for the D/H and S/H populations as the wild and cultivated parents of these two populations were utilized as tester and driver lines in the SSH process, respectively. Out of the 3456 wild barley-derived markers on the array, 55.6 and 44.4% were produced via standard DArT and SSH methods, respectively (Table 4). Therefore, it would be expected that the number of wild barley-derived markers developed via SSH that were mapped in the four component populations, and especially the D/H and S/H populations, would be greater than 44.4% of the total of mapped wild barley-derived markers. Chi-squared analysis revealed that there was an interaction between method of marker development and number of polymorphic markers mapped per population ($P = 0.00001$); however, there was a less than expected number of markers produced via SSH mapped in the four component populations (42.5%), and even fewer in the D $\times$ H and S $\times$ H populations alone (33.0%; Table 4). This indicates that the SSH process was not effective at enriching for DArT markers specific to our targeted input samples, which is contradictory to previously published results (James et al. 2008). However, there was a positive effect on the number of polymorphic markers obtained with the SSH process when a smaller number of accessions were utilized in the process. Of the 1536 markers on the comprehensive genomic representation developed via SSH, 50% were derived from only two accessions (Damon and Shechem), with the remaining 50% derived from a mixture of 31 diverse wild barleys. Yet in all populations assayed for polymorphism, there was always a greater proportion of polymorphic markers (average 66.8 $\pm$ 8.1%) derived from accessions Damon and Shechem as compared to those produced from the full set of 31 wild barley accessions (Table 4). This indicates that the SSH process for DArT marker development may be more effective at enriching for polymorphic markers when a limited number of lines is used. One possible reason for this is that unique markers may get "diluted" when many genotypes are mixed in the tester for SSH. Only a small number of markers developed uniquely from the wild barley accessions used would segregate in the populations tested in this work. It is quite likely that the benefits of using subtraction from a larger pool of wild barleys would manifest themselves clearly in a broad diversity/association analysis of wild barley accessions.

Efficacy of genotyping diverse barley germplasm with the comprehensive representation

Utilization of the comprehensive DArT array to genotype two DH wild $\times$ cultivated barley populations (D/H and S/H) enabled us to determine the efficacy of utilizing wild- and cultivated-barley-derived DArT markers for genotyping wild and cultivated barley. Examining the number of markers derived from wild and cultivated barley that are polymorphic in wild $\times$ cultivated DH populations does not reveal as much information regarding the effectiveness of surveying wild and/or cultivated germplasm with either wild- or cultivated-derived markers as does examining, for instance, whether any given marker derived from wild or cultivated barley was actually present (positive or greater hybridization on the array as compared to the other parent; scored as '1') in wild or cultivated germplasm. To explain further, there are four possible outcomes for any given polymorphic marker: (1) the marker is derived from cultivated barley and present in the cultivated parent; (2) the marker is derived from cultivated barley and present in the wild parent; (3) the marker is derived from wild barley and present in the wild parent; or (4) the marker is derived from wild barley and present in the cultivated parent. Cases 1 and 3 represent what may be regarded as expected; that is, markers cloned from cultivated or wild germplasm are more likely to be present in the respective cultivated or wild genotype. Case 2 represents instances where a marker cloned initially from cultivated germplasm was actually present in the wild parent and is indicative of the effectiveness of using the cultivated DArT libraries to genotype wild barley. Similarly, case 4 type markers are indicative of the effectiveness of surveying cultivated germplasm with wild-derived DArT markers.

Of all polymorphic markers mapped in the two wild × cultivated barley populations (D/H and S/H), 79.9 ± 0.1% and 20.1 ± 0.1% were derived from cultivated and wild barley germplasm, respectively (Table 5). Of the mapped cultivated-derived markers, 28.7 ± 2.4% were present in Damon and Shechem. Given the large number of cultivated-derived DArT markers available for screening, this indicates that a moderate number of polymorphic markers could be obtained by genotyping wild barley with the cultivated-barley-derived markers. Although there are several reports of wild relatives being genotyped with DArT markers (Xia et al. 2005; Yang et al. 2006; Olivera 2008), in most cases both wild and cultivated germplasm were utilized during array development, and in the case where wild germplasm was not included there was an over-representation of '0' scores in the wild accessions (Wenzl et al. 2004). Therefore, broadening the diversity of materials used for marker/array development via the addition of wild barley-derived markers serves to reduce ascertainment bias when genotyping wild and cultivated material. The presence of ascertainment bias in genotyping wild species should be considered in other genome profiling technologies that utilize standard SNP technology, as almost invariably the materials used for SNP marker discovery are cultivated and often represent very narrow sampling of diversity. Discovery of the majority of SNP markers has come from the extensive expressed sequence tag (EST) resources that are available. However, of the over 500,000 *H. vulgare* ESTs in current databases, less than 5% are derived from the subspecies *spontaneum*. (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). Of the mapped wild barley-derived markers, 33.2 ± 2.8% were present in Harrington in the D/H and S/H populations. This is also indicative of the potential for increasing the number of polymorphic markers obtainable in a single array (regardless of whether it is cultivated or wild barley being genotyped) by assaying the wild barley-derived markers. Despite a large percentage of wild barley-derived markers present in Harrington, due to the limited overall number of polymorphic wild barley-derived markers, this did not equate to a large number of wild-derived markers present in Harrington (39.5 ± 6.4). However, since DArT marker development is an iterative process, whereby polymorphic markers are enriched on the array over time (Wenzl et al. 2004, 2006), efforts aimed at enrichment for polymorphism in the wild barley-derived markers would significantly increase the power of the array to produce polymorphic markers within any cultivated or wild barley germplasm.

Individual component and synthetic maps

Of the 573.3 ± 31.6 markers mapped in the four component populations (Table 2), the number of markers in common between any two populations ranged from 119 (S/H and T/N) to 286 (D/H and S/H) and on average was only 158.7 ± 63.7 (Table 3). This indicates that there is a considerable amount of genetic diversity within the parental germplasm used in these populations. Of particular interest is that out of over 550 polymorphic markers in the D/H and S/H populations (which share a common parent in cv. Harrington) only 286 of them were common to both. Damon and Shechem, the two *H. vulgare* subsp. *spontaneum* parental accessions used in those populations, were collected about 40 km apart in Israel. Nevo et al. (1998) reported high levels of diversity in wild barley from Israel, Iran, and Turkey, and determined that ecological factors such as temperature and water availability, rather than geographical origin, play a greater role in determining the extent of genetic diversity between different populations. Although Damon and Shechem were both collected in mesic environments where diversity was shown to be lower than in high-stress environments, there was still a high level of polymorphism between them. This indicates that the diversity present in wild barley, even from ecologically similar locations in close proximity, is sufficient to mine for novel loci conferring traits of economic importance.

The addition of wild barley-derived markers to the comprehensive array significantly improves the power of discovery of polymorphic markers. The four populations genotyped with the comprehensive array were from both cultivated × cultivated and wild × cultivated crosses between diverse sets of parents. These parents included wild barley accessions (Damon and Shechem), a landrace (Albacete), and old (Barbarrouse) and modern (Harrington, TX9425, Naso Nijo) cultivars. They also were from divergent geographic regions (North America, Europe, and Asia) and differed in end use (feed/malting), row type (two-rowed/six-rowed), and habit (spring/

winter). The increase in diversity on the array enabled us to obtain over 550 polymorphic markers in each population, regardless of the genetic distance of the parents in the cross. However, there is still a tendency for a few regions of the genome to contain gaps in the component maps for each population. This was most apparent in the short T/N map (709.5 cM), as there were three chromosomes represented by two linkage groups (3H, 5H, and 6H), and two more (2H and 4H) that were markedly shorter and only covered a portion of the chromosome (Fig. 1). The map of the A/B population also has regions that are under-represented by markers. It has two chromosomes represented by two linkage groups (1H and 2H) and one chromosome represented by three linkage groups (3H). However, two of these breaks are due to the whole-arm reciprocal translocation present on chromosomes 1H and 3H in Albacete. Because of the translocation, chromosomes 1H and 3H in the A/B population were mapped as independent arms. However, it is also the longest of the four component maps, which is due, in part, to it having the highest number of gaps (31) over 10 cM. In contrast, the D/H and S/H component maps contain no chromosomes with multiple linkage groups and have adequate genome-wide coverage. Therefore, the lack of coverage in the T/N and A/B maps in certain regions of the genome is more likely due to the genetic similarity of the parents in those populations, which are all cultivated and of Asian and European origin, respectively, than the power of a single genotyping assay to provide adequate coverage of the genome. However, it does highlight the potential advantage of tailoring DArT marker development towards specific genotyping experiments.

Regardless of the lack of coverage in certain regions of the genome in some populations, all of the component maps were ordered, with the exception of a few minor closely linked rearrangements, identically to the previously reported DArT consensus map (Wenzl et al. 2006). The ordering of closely linked markers within component maps is inherently difficult due to the limited resolution provided by the population sizes used for map construction. Therefore, it is unavoidable that there will be a small degree of incorrect ordering between very closely linked markers in some populations. This effect is reduced, however, upon synthetic map construction, as the order of the majority of markers is predetermined in PhenoMap software upon selection of the consensus map as the reference map, which is already a merged map of ten populations and contains more markers assayed across a larger number of lines. For this reason, as well as to retain consistency within the synthetic map-building process, the consensus map was chosen as the reference map for all chromosomes. Genome-wide, this resulted in the least number of incorrectly ordered loci in the four non-reference maps as compared to the synthetic map. On average, there were 20.75 incorrectly ordered loci genome-wide (three per chromosome) in each component map (Table 6). Graphical genotypes for each population, ordered as determined by map synthesis, are presented in Supplemental 1.

Although choosing different component maps as the base for synthesis could have reduced the degree of map expansion somewhat, map expansion was deemed to be a lesser qualifier for determining the optimal base map than marker order. The algorithm used to compute the Kosambi cM distances in the component and merged maps of the consensus map versus the four component maps of the synthetic map was slightly different. Marker distances in the consensus map and its component maps were calculated with the regression algorithm utilized in Joinmap 3.0 (Van Ooijen and Voorrips 2001; Wenzl et al. 2006). However, map distances in the four populations used in this study were calculated by simply adding the cM distances between adjacent markers, an algorithm that tends to result in slightly larger distance estimates. Therefore, it is expected that chromosome lengths may increase upon integration of the component maps into the consensus map.

There were a number of markers, both previously mapped and unique to the four component populations, which mapped to multiple loci. Within the four component maps, there were seven markers that mapped to two loci within the same chromosome (1H, 2H, 3H, 6H, and 7H). There also were 32 markers that mapped to multiple loci on different chromosomes, although one of these had already been identified as a multi-locus marker (at the same two loci). Including the 22 previously identified multi-locus markers, the total number of multi-locus markers mapped within the 'bPb' DArT markers was 60 (2.8%); however, 36 of the multi-locus markers are redundant to at least one other multi-locus marker and may represent identical clones. Considering the

overall redundancy within the 'bPb' DArT markers, this may still be a good estimate of the frequency of multi-locus DArT markers, which is considerably lower than the levels associated with the non-DArT markers included in the consensus/synthetic maps (8.8%).

The synthetic map contains 3542 markers, of which 2935 (2085 DArT and 850 other loci) were from the barley consensus map and 607 from the newly incorporated DArT markers derived from the four component maps in this study (65% wild barley-derived). A chart of the complete synthetic map, including marker names and a visual of the randomly distributed 607 integrated markers, is included in Supplemental 2. Detailed information on all markers present in the map, including chromosome and position, is included in Supplemental 3. The alignment of markers in common between the barley consensus map and the synthetic map is identical; only the distances between markers and resolution of the map varied by chromosome (Fig. 2).

Wenzl et al. (2006) reported 1629 unique bins (loci) in the barley consensus map; however, we used a slightly different method of collapsing loci into

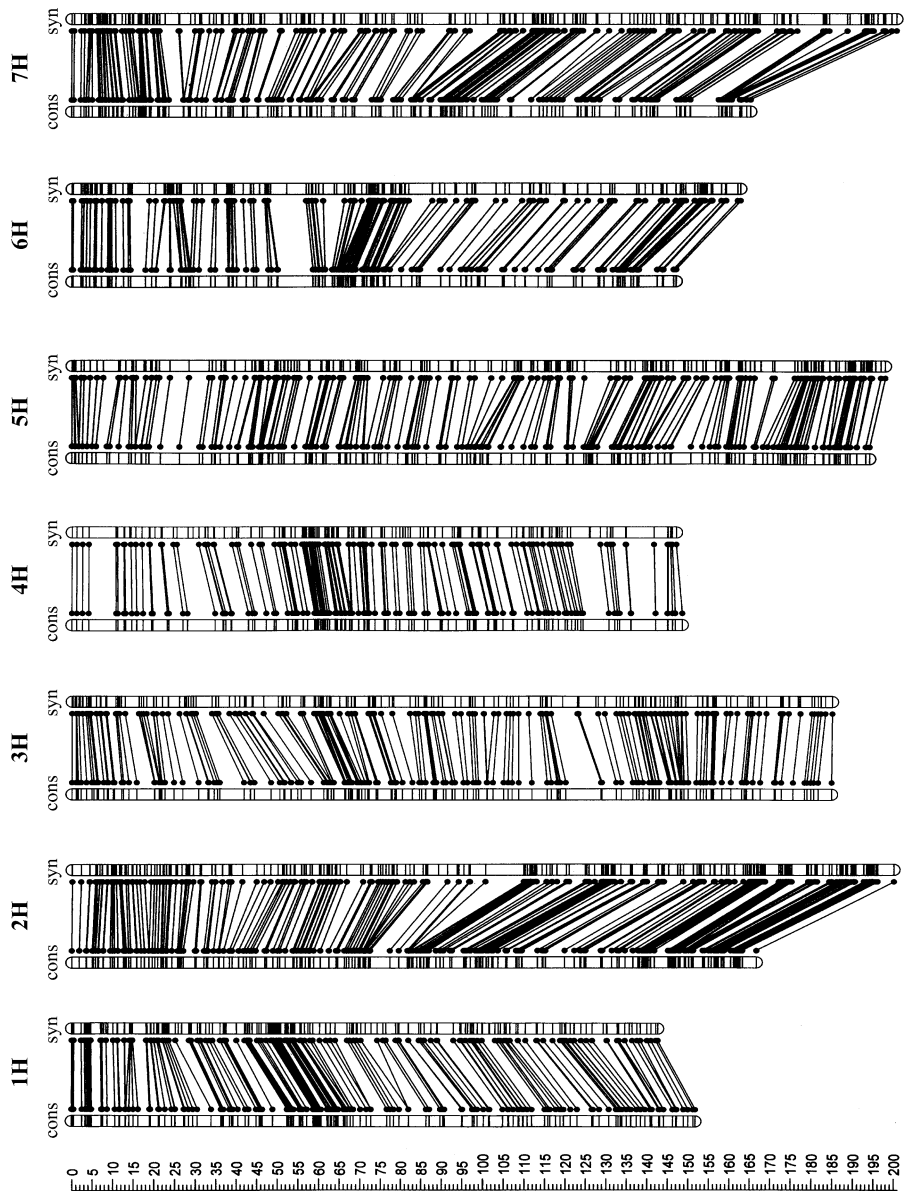

Fig. 2 Alignment of the synthetic and consensus maps. Comparison of positions of common loci between the consensus map (cons) and the synthetic map built with PhenoMap software using the consensus map as the reference map (syn). The positions of homologous loci in the two maps are highlighted by a pair of *dots* and connected by *lines*

bins. Our method was based upon distances between markers, rather than segregation signatures, as was utilized in the construction of the consensus map (Wenzl et al. 2006). When calculating orders of closely segregating collapsed bins for consensus map construction, Wenzl et al. (2006) arbitrarily assigned a distance of 0.001 cM between such bins in order to maintain a fixed bin order. In order to be more conservative in our estimates of redundancy as well as to be consistent across the consensus and synthetic maps for the sake of comparison, we calculated the number of bins based on the rule that any markers less than 0.005 cM apart would be considered cosegregating and therefore collapsed into bins. The number of bins in the consensus map calculated with these methods was 1153 and the average/median IBD was 1.0/0.7 cM (Table 7). Therefore, synthesis of the consensus map with the four component maps resulted in a higher resolution map with 257 more bins and a decrease in average/median IBD of 0.1/0.1 cM. The degree to which map resolution changed varied by chromosome; however, for each chromosome, the average/median IBD never decreased, but rather at least stayed the same or improved. The greatest improvement in resolution was for chromosome 1H, for which the average/median IBD decreased by 0.2/0.3 cM. On the other hand, for chromosome 2H, neither the average nor median IBD changed.

Another useful determinant of map resolution is the number/size of gaps between markers (or bins) present in the map. There were no IBDs in either the consensus or synthetic maps greater than 10 cM, and the number of 5–10 cM gaps present in each map did not change overall (9 each). However, some 5–10 cM gaps that were present in the consensus map were filled, while some were newly introduced upon introgression of the four component maps during synthesis. This again varied by chromosome, with the overall result being that two 5–10 cM gaps were filled on each of chromosomes 3H and 4H, and one introduced into each of four different chromosomes (2H, 5H, 6H, and 7H). Overall, in terms of saturation of markers, the coverage between the two maps did not vary significantly.

A majority of the newly incorporated markers (57.7%) mapped to loci redundant to either previously mapped markers or other newly incorporated markers. Comparing the number of newly incorporated markers with the increase in bin number is useful for estimating the level of marker redundancy present within the 'bPb' DArT markers. Wenzl et al. (2006) reported an estimate of DArT marker redundancy in barley at 38%. A recent sequence analysis of all cultivated-barley markers combined with part of the wild barley markers produced a similar estimate (40%) (A. Kilian and P. Wenzl, unpublished data). Estimates of DArT marker redundancy in other species are about the same or lower (Akbari et al. 2006; Wittenberg et al. 2005). These figures are much lower than the 57.7% redundancy found within the sample of newly incorporated markers. This is not surprising, however, for two reasons. One is that marker redundancy as measured by genetic mapping will always tend to be higher than redundancy measured by sequencing as a result of closely linked but physically different markers. Secondly, there will almost always be a higher frequency of marker redundancy as more DArT markers are developed. Despite the use of more diverse wild barley as the source, there is still a high degree of homology between *H. vulgare* subsp. *vulgare* and subsp. *spontaneum*, and therefore there is still the chance of randomly cloning sequences identical to those previously cloned. One way to keep redundancy stable during additional DArT marker development in barley would be to utilize additional combinations of restriction enzymes. Despite high levels of marker redundancy and no change in the number of 5–10 cM gaps, the synthetic map has an improved overall resolution, as there was an increase in the number of bins, and a decrease in the average/median IBD.

The higher resolution of the synthetic map and the development and incorporation of wild barley-derived DArT markers significantly improved the power of the map to provide a reference for profiling genetic diversity within barley germplasm. Over the last few years, there have been several reports on the development of high-resolution consensus maps in barley (Marcel et al. 2007; Stein et al. 2007; Varshney et al. 2007; Wenzl et al. 2006). Breeding efforts in barley, from marker-assisted selection to potential association mapping (Steffenson et al. 2007), will benefit from these high-resolution maps with many precisely ordered loci. The incorporation of wild barley-derived markers and the ability to readily screen them in a high-throughput manner will facilitate the molecular improvement of cultivated barley via more precise introgression and manipulation of genomic fragments from *H. vulgare* subsp. *spontaneum*.

Continual enhancement of the comprehensive barley DArT array–through polymorphism enrichment and/or removal of redundant sequences, development of new markers utilizing novel diverse germplasm, and/or the use of additional restriction enzyme combinations in marker development—could greatly improve the already powerful hybridization-based genotyping platform to the point of reasonably obtaining 1,000–2,000 polymorphic markers in a single assay.

In summary, the purpose of this study was to enhance the barley DArT genotyping platform by developing novel markers derived from wild barley, thus increasing the power of the barley DArT array to detect polymorphism in more diverse germplasm without bias in marker scoring. We utilized this 'comprehensive' array to genotype four DH barley populations. Utilizing PhenoMap software, which is an effective alternative approach to the commonly used Joinmap 4.0 software (Van Ooijen 2006) for merging genetic mapping data (Wenzl et al. 2006), we merged the individual component maps with the barley DArT consensus map to create a new synthetic map of the barley genome, which incorporated 607 novel markers from the four component populations. The new synthetic map has 3542 DArT, SSR, RFLP, and STS markers and agricultural trait loci. The improvement in the genotyping capability of the comprehensive DArT genomic representation and the higher resolution of the synthetic map enables an even greater flexibility of DArT markers to be utilized as a fast, high-throughput platform for molecular barley breeding.

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