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Molecular mapping of the Oregon Wolfe Barleys: a phenotypically polymorphic doubled-haploid population

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Abstract A phenotypically polymorphic barley (*Hordeum vulgare* L.) mapping population was developed using morphological marker stocks as parents. Ninety-four doubled-haploid lines were derived for genetic mapping from an F_1 using the *Hordeum bulbosum* system. A linkage map was constructed using 12 morphological markers, 87 restriction fragment length polymorphism (RFLP), five random amplified polymorphic DNA (RAPD), one sequence-tagged site (STS), one intron fragment length polymorphism (IFLP), 33 simple sequence repeat (SSR), and 586 amplified fragment length polymorphism (AFLP) markers. The genetic map spanned 1,387 cM with an average density of one marker every 1.9 cM. AFLP markers tended to cluster on centromeric regions and were more abundant on chromosome 1 (7H). RAPD markers showed a high level of segregation distortion, 54% compared with the 26% observed for AFLP markers, 27% for SSR markers, and 18% for RFLP markers. Three major regions of segregation distortion, based on RFLP and morphological markers, were located on chromosomes 2 (2H), 3 (3H), and 7 (5H). Segregation distortion may indicate that preferential gametic selection occurred during the development of the doubled-haploid lines. This may be due to the extreme phenotypes determined by alleles at morphological trait loci of the dominant and recessive parental stocks. Several molecular markers were found to be closely linked to morphological loci. The linkage map reported herein will be useful in integrating data on quantitative traits with morphological variants and should aid in map-based cloning of genes controlling morphological traits.

Keywords Barley · Genetic map · Molecular markers · Segregation distortion · Morphological markers

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Introduction

Barley (*Hordeum vulgare* L.) is an excellent system for genome mapping and map-based analyses. The three main advantages of barley for genetic studies are that: (1) barley is a diploid (2n=14) with seven cytologically distinct chromosomes containing approximately 5.3×10^9 bp DNA (Bennett and Smith 1976); (2) barley chromosomes are homoeologous to those of common wheat (Moore et al. 1995), which allows barley to serve as a model system for the more complex polyploid cereals; and (3) barley doubled-haploid (DH) reference populations can be produced with relative ease, allowing repeated phenotyping and genotyping to generate genetic maps of qualitative and quantitative trait loci (Chen and Hayes 1989).

Genetic linkage maps are useful for understanding genome organization, for establishing syntenic relations, as a platform for map-based cloning, and for quantitative trait locus (QTL) detection. The North American Barley Genome Mapping Project (NABGMP) has built maps in elite germplasm in order to facilitate the direct application of these maps to plant breeding via QTL analysis (reviewed by Hayes et al. 1996). Over 1,000 loci have been placed on these maps, providing a comprehensive catalog of markers and complete genome coverage (Kleinhofs et al. 1993; http://barleygenomics.wsu.edu/).

Several types of molecular markers, including restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), sequence-tagged sites (STSs), simple sequence repeats (SSRs), and amplified fragment length polymorphisms (AFLPs), have been used in barley mapping, and each has advantages and disadvantages. RFLPs are co-dominant markers, are repeatable, and are well distributed on the barley genome (Graner et al. 1991; Kleinhofs et al. 1993). Their main disadvantages are that they require large amounts of relatively pure DNA and are time-consuming and technically challenging. Markers based on the polymerase chain reaction (PCR), such as RAPDs, STSs, SSRs, and AFLPs, require significantly less DNA (in some cases 100 times less). RAPDs are relatively quick and simple but suffer from a lack of reproducibility (Kleinhofs et al. 1993; Dahleen et al. 1997). STSs combine the high speed and low cost of RAPDs with the high specificity of RFLPs, but they are limited in number and generally show reduced polymorphism (Tragoonrung et al. 1992; Mano et al. 1999). SSRs are usually co-dominant markers (Saghai Maroof et al. 1994) that are numerous in barley. Additionally, multiplexing of SSRs has increased the number that can be scored per experiment (Narvel et al. 2000). AFLPs (Becker et al. 1995; Qi and Lindhout 1997; Waugh et al. 1997) tend to be more repeatable than RAPDs and can generate larger number of scorable polymorphisms per primer combination (Becker et al. 1995; Qi et al. 1998). Sequencing of AFLP loci has shown, however, that these are not always specific to a single genomic location (Shan et al. 1999). RFLPs and SSRs, on the other hand, have the advantage of certainty of locus identity because these map to the same place in multiple mapping populations, thereby facilitating map integration and comparative mapping (Qi et al. 1996).

An aspect that has lagged behind the characterization of DNA-level variation is the integration of molecular marker information with morphological variants in barley. Alleles at morphological trait loci may determine extreme phenotypes important for agricultural production (Robertson 1985) and may provide starting points for the comparative analysis of plant genomes. Several morphological traits of agronomic importance have been mapped using molecular markers such as the *denso* dwarfing gene (Laurie et al. 1993), the *liguless* gene (Pratchett and Laurie 1994), and the *vrs1* locus (Komatsuda et al. 1997). Most mapping populations, however, are segregating for only a limited number of morphological traits. A detailed molecular marker map in a population segregating for several morphological markers would be of great use because it would serve as a base for map integration and map-based cloning of morphological trait loci. The Oregon Wolfe Barley (OWB) population is a set of spring barley DH lines, developed from the F_1 of a cross between dominant and recessive morphological marker stocks (Wolfe 1972), that show an exceptional degree of phenotypic variation in a single reference population. Here, we report the results of mapping 713 molecular and 12 morphological markers in this population.

Materials and methods

Plant materials and morphological traits

Recessive and dominant morphological marker spring barley stocks (Wolfe and Francowiak 1991; Wolfe 1972), were crossed, and a population of 94 DH lines was derived from the F_1 using the *Hordeum bulbosum* method (Chen and Hayes 1989). Twelve morphological traits were scored: (1) *nud*=naked caryopsis, (2) *Lks2*=short awn 2, (3) *vrs1*=six-rowed spike, (4) *Zeo*=Zeocriton dwarf 1, (5) *wst*=white streak 7 (Ribbon grass), (6) *alm*=albino lemma, (7) *Pub*=pubescent leaf blade, (8) *Hsh*=hairy leaf sheath, (9) *Rh*=hairy rachis, (10) *Blp*=black lemma and pericarp, (11) *rob*=orange lemma, (12) *srh*=short rachilla hair. The morphological markers were designated following the nomenclature described by Francowiak (1997).

Molecular marker analyses

Genomic DNA was isolated from fresh leaves using a proteinase K extraction method and was phenol-chloroform-purified, ethanol-precipitated, and resuspended in sterile water. DNA was diluted to approximately 500 ng/ml for later use. Twenty-six RAPD primers from the A, B, C, D, and E sets from Operon Technologies (Alameda, Calif.) were used. These were designated as OP plus a letter indicating the primer and a number indicating the size of the amplified band. The STS marker MSU21 was amplified as described by Trangoonrung et al. (1992). An *hvknox3* intron fragment length polymorphism (IFLP) was characterized as described by Müller et al. (1995). Amplification products from RAPD and STS reactions were separated on 1.4% agarose gels by electrophoresis (80 V, 3 h) and scored visually. RFLP protocols and RFLP locus designations followed those described in Kleinhofs et al. (1993). RFLP markers of known location (Kleinhofs et al. 1993) on barley genetic maps were used as anchor probes for each barley chromosome. The primer sequences for the 33 microsatellites used in this study were obtained from GrainGenes (http://wheat.pw.usda.gov/) and from the Scottish Crop Research Institute (Invergowrie, Dundee, Scotland). The PCR reactions were performed in a 96-well microtiter plates using a Perkin Elmer 9600 Thermal cycler (PE Applied Biosystems, Foster City, Calif.) and a PTC-100 MJ Research (MJ Research, Watertown, Mass.). The 10-µl PCR reaction mix consisted of 0.1 µ*M* of each primer [in the case of using fluorescently labeled primers, the forward primer was unlabeled and the reverse primer was labeled at its 5′ end with a fluorochrome: 6-FAM (6-carboxyfluorescein), TET (tetrachloro-6-carboxyfluorescein), or HEX (hexachloro-6-carboxyfluorescein]; 0.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.), 0.2 m*M* of each dNTP (except in the case of radioactive detection of products when we used: 0.2 m*M* of each dCTP, dGTP, and dTTP, and 50 uM dATP; 1 µCi (37 kBq) of α-[^{33}P]dATP), 1× *Taq* buffer, 2 m*M* $MgCl_2$, 25 ng of template DNA, and distilled H_2O. The PCR amplification consisted of an

initial denaturation step of 3 min at 94°C, followed by 30 cycles of three steps of 30–60 s each: denaturation at 94°C, annealing (55°–65°C depending on the primer), and elongation at 72°C. A final elongation step at 72°C for 5 min was performed. For [^{33}P]-labeled products, the product was denatured after PCR at 94°C for 8 min, and 10 µl of stop solution (95% formamide, 20 m*M* EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added. A 6% polyacrylamide denaturing gel containing 8 *M* urea was pre-run for 30 min. Samples (7 µl) were loaded and separated at 75 W constant power using a DNA sequencing unit (FB-SEQ-3545). After electrophoresis the gels were immediately covered with plastic wrap, dried, and exposed to X-ray film. For fluorescently labeled primers, each marker was amplified in a separate PCR reaction. One to three PCR products were loaded per lane in a polyacrylamide gel, together with an internal lane standard, GS500, labeled with TAMRA (*N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine) according to manufacturer's instructions (PE Biosystems, Foster City, Calif.) in order to achieve high-throughput. Amplified microsatellites were analyzed using an ABI PRISM 377 automated DNA sequencer and the appropriate computer software (Genescan and Genotype, PE Biosystems, Foster City, Calif.). AFLP analysis followed protocols supplied with GIBCO BRL (Gaithersburg, Md.) AFLP Core Reagent Kit and AFLP Starter Primer Kit for Analysis System I. The following 55 *Eco*RI and *Mse*I primer combinations were used: E32M47, E32M48, E32M49, E32M50, E32M59, E32M60, E32M61, E32M62, E33M47, E33M48, E33M59, E33M60, E33M61, E33M62, E35M47, E35M48, E35M49, E35M50, E35M59, E35M60, E35M61, E35M62, E36M47, E36M48, E36M49, E36M50, E36M59, E36M60, E36M61, E36M62, E37M47, E37M48, E37M49, E37M50, E37M59, E37M60, E37M61, E37M62, E38M47, E38M48, E38M49, E38M50, E38M59, E38M60, E38M61, E38M62, E40M47, E40M48, E40M49, E40M50, E41M47, E41M48, E41M50, E41M61, E41M62, following the nomenclature proposed by Qi and Lindhout (1997). *Eco*RI primers were labeled using γ-[^{32}P]-ATP and T4 kinase. Samples (2.5 µl were electrophoresed on a 5% polyacrylamide denaturing gel consisting of 7.5 *M* urea, 20:1 acrylamide:bisacrylamide gel solution, and 0.5× TBE (45 m*M* Tris, 45 m*M* boric acid, 1.25 m*M* disodium EDTA, pH 8.3). The gels were subsequently dried and exposed to X-ray film at room temperature for 1–2 days. Polymorphisms were scored visually.

Linkage map construction

Linkage analysis of the molecular and morphological trait data was performed using Gmendel 3.0 (Hollaway and Knapp 1994). Linkage groups were first calculated using a maximum recombination percentage of 25% and a LOD score of 7. Markers showing severe segregation distortion were dropped, and markers with the most complete data were retained. Linkage groups were then recalculated using a LOD of 3.8 and a recombination percentage of 35%. Marker orders were checked by Monte Carlo and Bootstrap simulations using annealing temperatures of 300 "inner" and 200 "outer." The assignment of linkage groups to chromosomes was based on markers in common with previously published maps (Kleinhofs et al. 1993; Qi et al. 1996). The software package JoinMap Version 2.0 (Stam and Van Oijen 1995) was then used to contruct a genetic map that included AFLP, STS, and RAPD markers with the parameters set for DH-derived progeny. Linkage groups were assigned to the corresponding barley chromosome based on the anchor RFLP markers previously assigned by Gmendel. Segregation of the markers was tested for segregation distortion against the expected 1:1 ratio by calculating chi-square values. For final map construction all the data were used from morphological markers, RFLPs, and SSRs, while only AFLPs and RAPDs that had fewer than five data points missing and that were not distorted at ($P \leq 0.10$) were used. Grouping of markers was carried out with LOD values of 4.00 through 6.50 with a step increase of 0.25. Groups found to contain specific markers mapping to the same chromosome were combined if linked by more than three markers with a LOD score of at least 3.0. Recombination fractions were converted to centiMorgans with the Kosambi mapping function (Kosambi 1944). The values used for the JoinMap response files were LOD=0.01, rec=0.499, JUMP=3 to 5, and one to three ripples (Stam and van Ooijen 1995). The anchor markers were used as fixed orders in the final map.

Results and Discussion

Map construction

Eight hundred and thirty markers were split into 41 linkage groups at a LOD threshold grouping of 6.0. RFLP and morphological markers described in the core map were used as anchor markers (shown in bold in Fig. 1) to assign linkage groups to the seven barley chromosomes. *Pub* was present in a group of ten markers that included MWG884, ABG004, MWG883, Bmac0144d, HVM62, ABC805, and ABC172. These markers had been mapped previously to the long arm of chromosome 3 (3H) and therefore were grouped with the rest of the chromosome 3H markers. This forced merging, however, required relaxing some of the parameters in the map construction module of JoinMap. Chromosome 1 (7H) had the highest number of markers (163), while chromosome 5 (1H) had the lowest number (80). The full linkage map that included 725 markers comprised a total linkage distance of

Table 1 Genetic lengths (in centiMorgans, cM) and distribution of markers on the seven chromosomes of the Oregon Wolfe Barley population

Chromosome	Markers						
	cM	Morphological	SSR	RFLP	AFLP	Other[a]	Total
1 (7H)	191	2	4	13	143	1	163
2 (2H)	186	3	8	13	75	2	101
3(3H)	218	2	3	13	85	1	104
4 (4H)	162	1	3	13	65	1	83
5 (1H)	159	2	4	12	62	0	80
6 (6H)	203	1	3	10	78	1	93
7 (5H)	268	1	8	13	78	1	101
Total	1387	12	33	88	586	7	725

[a] Other: includes one STS, one IFLP and five RAPD markers

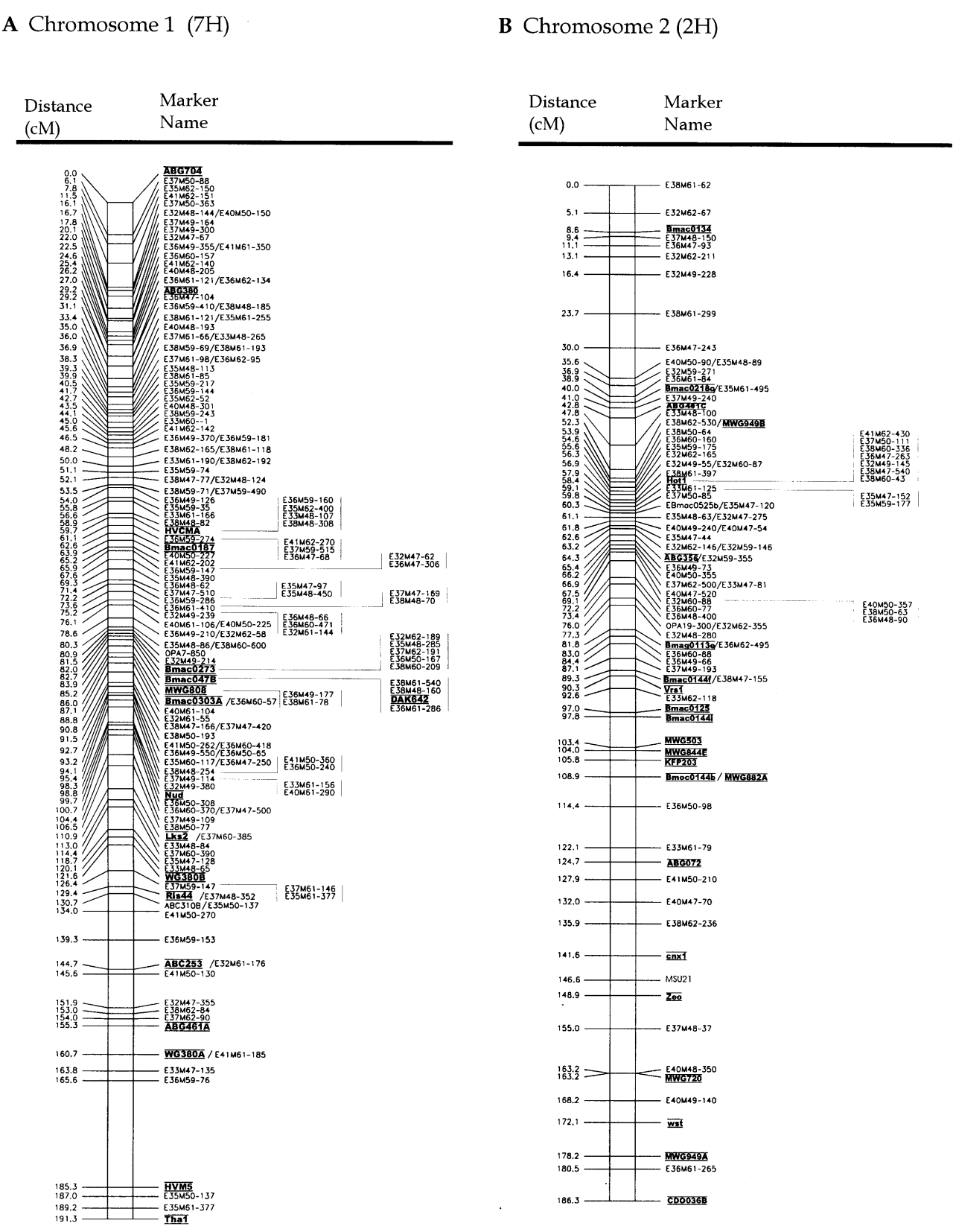

Fig. 1A–G The OWB barley marker map. **A** through **G** correspond to barley chromosomes 1 to 7, with the short arm at the *top*. Anchor markers are *underlined* and overlined in *bold type*

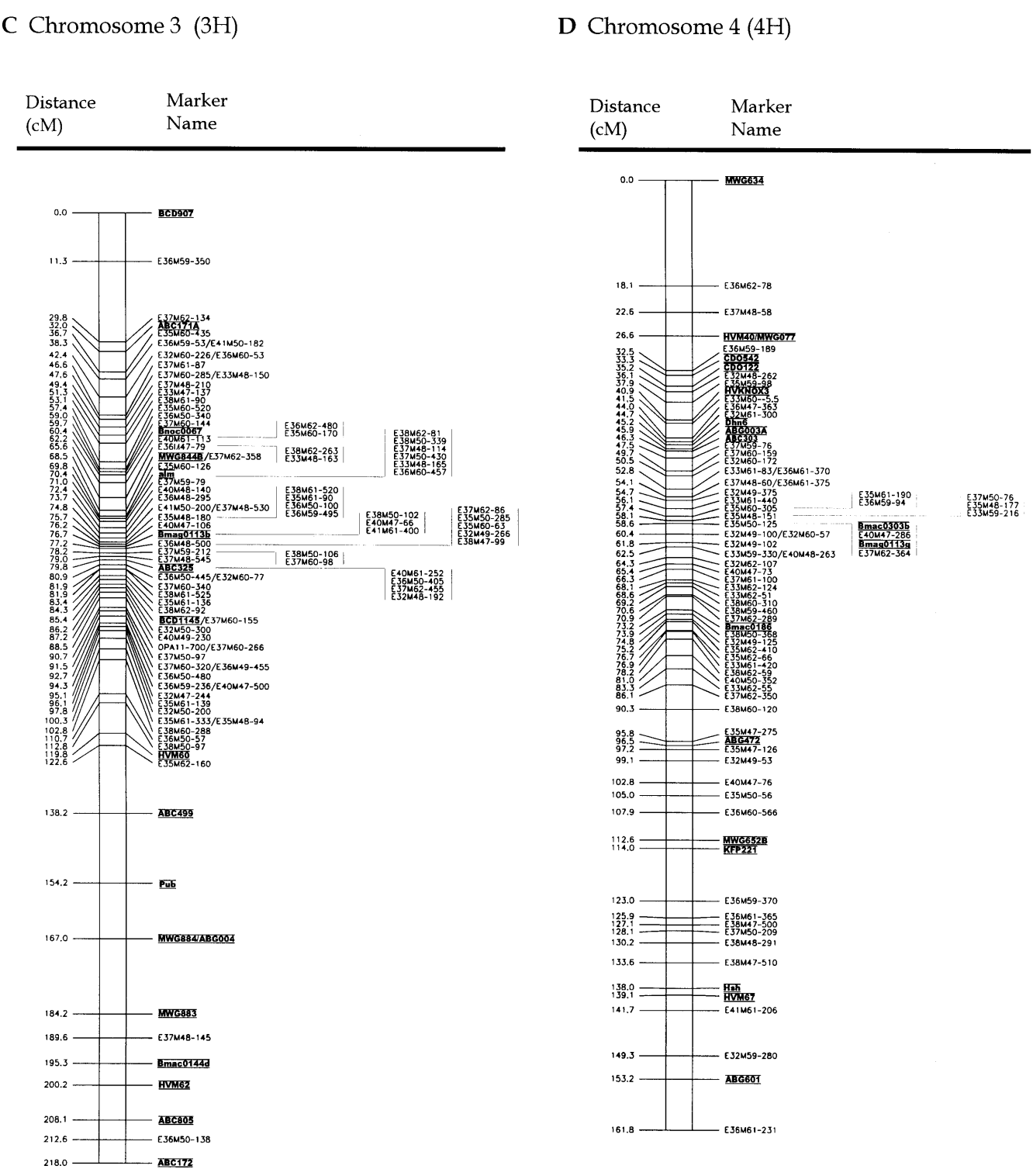

Fig. 1C–D

1,387 cM (Table 1). The average two-locus interval in the full map was 1.9 cM. The order of the markers and observed genetic distances were generally consistent with previously published maps (Kleinhofs et al. 1993; Qi et al. 1996; Hayes et al. 1997).

Polymorphism and utility of molecular markers

By using 55 primer combinations, we could observe 1,111 polymorphic AFLP marker loci. The number of polymorphic markers per primer combination ranged from 6 for E32M61 to 36 for E36M59, with an average of 20.2. Two hundred and eighty-four (26%) of the

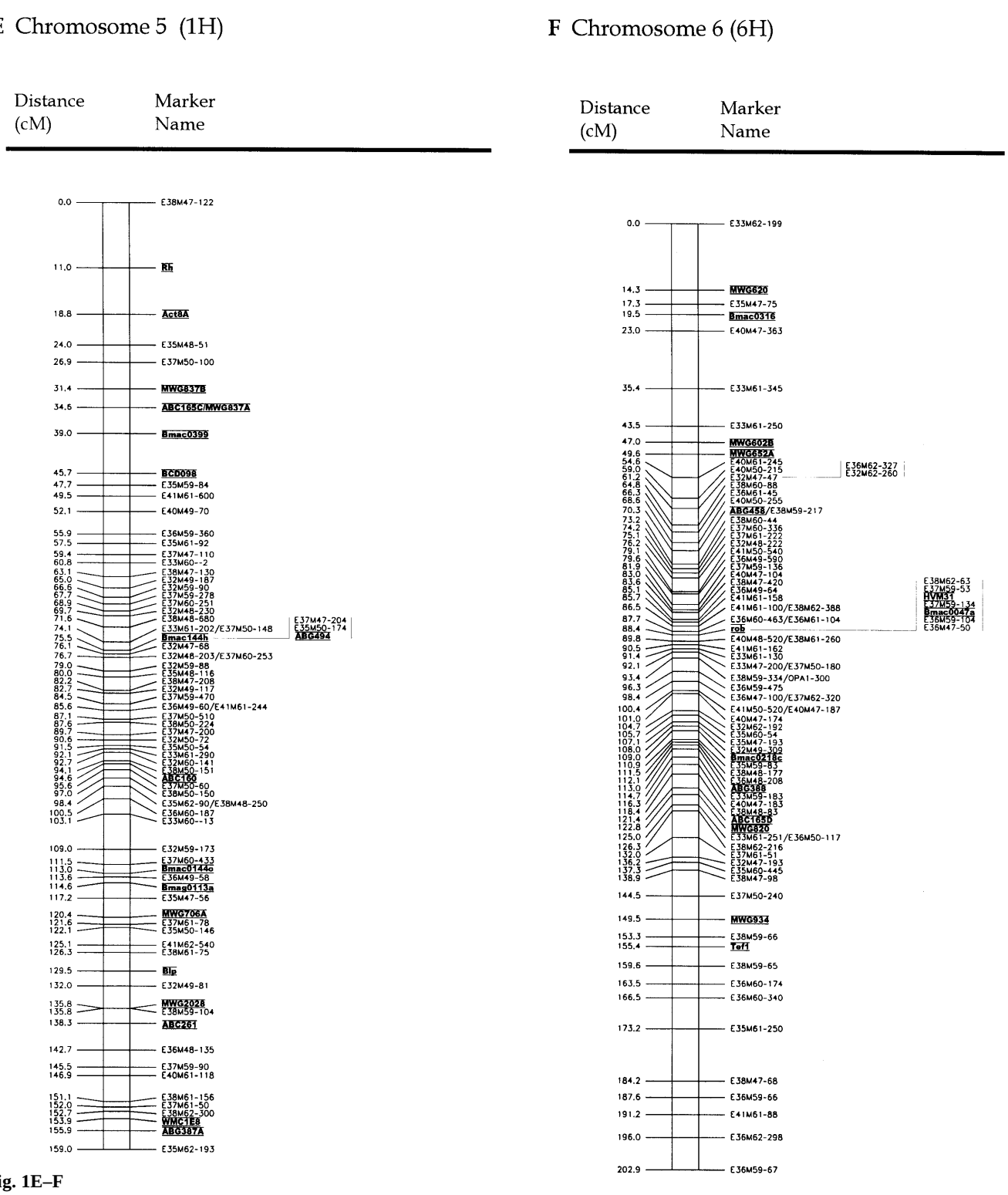

Fig. 1E–F

AFLPs deviated from the expected 1:1 segregation ratio for the two parental alleles ($P \leq 0.05$). In contrast with these results, Qi et al. (1998) detected a low level of segregation distortion (8%) for AFLPs in an F_9 recombinant inbred barley population, while Becker et al. (1995) found that 6% of AFLPs were distorted in a DH barley population. Using 26 random primers, we cored 54 RAPD marker loci, of which 29 (54%) deviated from the expected 1:1 segregation ratio for the two parental alleles ($P \leq 0.05$). The average number of polymorphisms per primer was 2.1. Manninen (2000) observed a similar degree of segregation distortion (47%) for RAPDs in a DH barley population.

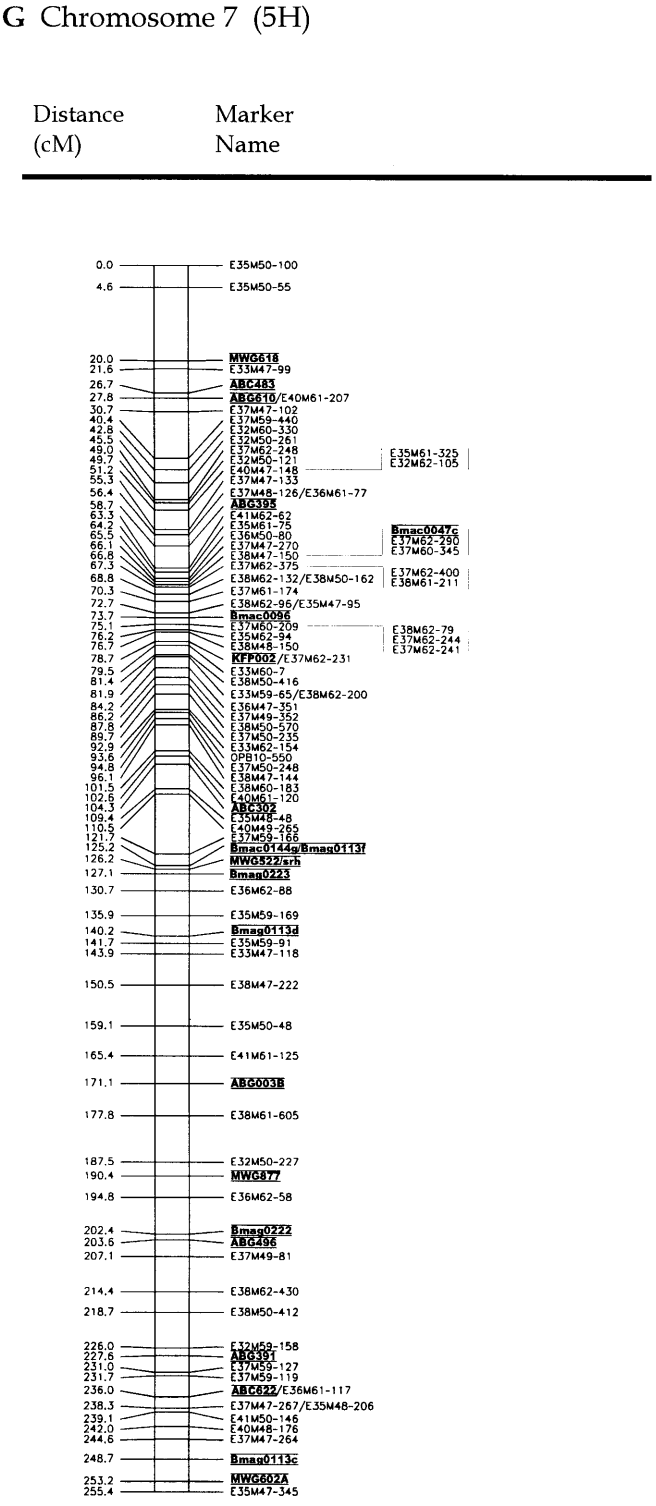

Fig. 1G

RAPDs were of limited utility due to segregation distortion, low information content per primer, and low data quality. Only 5 (20%) of 25 RAPDs were associated with linkage groups. Compared to other published barley genetic maps (Kleinhofs et al. 1993; Hayes et al. 1997), the OWB map presented here provides nearly complete genome coverage. Therefore, the unlinked RAPDs were not located in regions that are unmapped in this OWB population. AFLPs had several advantages over RAPDs: (1) they had much less segregation distortion than RAPDs; (2) they generated a higher number of polymorphisms per primer combination; and (3) most AFLPs (71%) mapped to existing linkage groups. The only STS marker scored (MSU21) showed segregation distortion at $P \leq 0.05$. Out of 87 RFLP markers 16 (18%) were distorted at $P \leq 0.05$; while 9 (27%) out of 33 SSR markers were distorted at $P \leq 0.05$. The RFLP markers used in this study had been previously mapped and were purposefully chosen to give complete coverage of all barley linkage groups. SSRs appeared to be superior to AFLPs because although they had a similar rate of segregation distortion they all mapped into existing linkage groups. Furthermore, SSRs can be multiplexed to generate several data points per experiment, thus increasing their utility as molecular markers (Narvel et al. 2000).

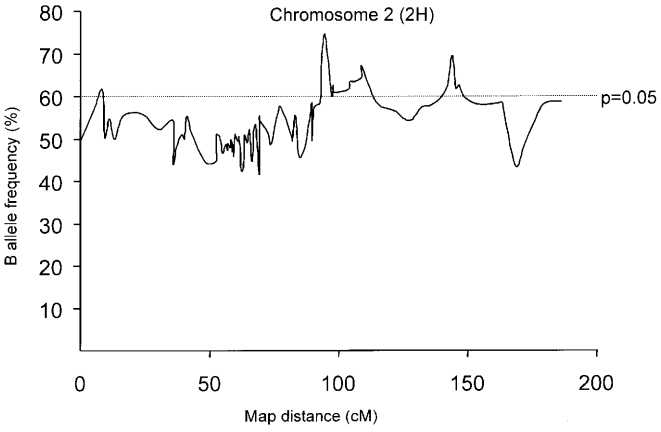

Fig. 2 Frequency distribution of B alleles (recessive stock) on chromosome 2 (2H) of the OWB population

Regions of segregation distortion

Three major regions of segregation distortion, located on chromosomes 2 (2H), 3 (3H), and 7 (5H), were observed (Figs. 2–4). The regions on 2H and 3H were skewed towards the recessive allele, while the region on 5H was distorted towards the dominant allele. Segregation distortion has rarely been observed in DH populations derived by the *H. bulbosum* method (Hayes et al. 1997; Zhu et al. 1999). It is commonly observed, however, in barley mapping populations derived from anther culture (Graner et al. 1991; Zivy et al. 1992; Devaux et al. 1995; Manninen 2000). The possible genetic causes of segregation distortion are the abortion of male or female gametes, the selective fertilization of particular gametic combinations, and gametophytic selection in the doubled-haploid process (Xu et al. 1997; Manninen 2000). Regions of segregation distortion have been previously reported on chromosomes 2H, 3H, and 4H of barley (Manninen 2000) and also on 6H (Qi et al. 1998). In the OWB germplasm, linkage drag associated with alleles at

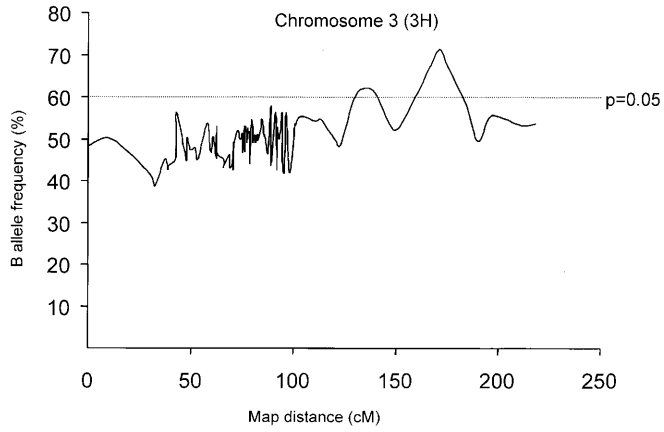

Fig. 3 Frequency distribution of B alleles (recessive stock) on chromosome 3 (3H) of the OWB population

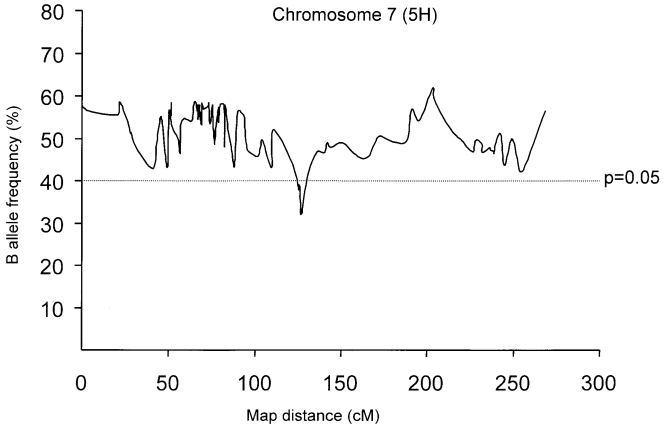

Fig. 4 Frequency distribution of B alleles (recessive stock) on chromosome 7 (5H) of the OWB population

some morphological trait loci may have given certain gametes, or certain combinations of gametes, a selective advantage during the generation of the DH population causing segregation distortion. For example, the region showing segregation distortion on chromosome 2H was near the *Zeo* locus, where alleles determine normal plant height or dwarfness. The recessive allele (normal plant height) may have conferred a selective advantage to the gametes, or combinations of gametes, or plants with recessive alleles. Manninen (2000) reported that chromosomal regions showing segregation distortion on 2H and 3H were associated with plant regeneration rate. It was interesting to note, however, that the chromosomal regions where distorted segregation was observed in his study were not always the same as the regions linked to anther-culture response.

In a review of segregation distortion across a wide range of species and mapping populations, it was noted that recombinant inbred populations had the higher frequencies of distorted markers (40%) compared with the 20% to 30% observed for F_2, DH, or backcross populations (Xu et al. 1997). These relatively high rates of segregation distortion may be due to the fact that parents of most mapping populations are purposefully selected to be genetically diverse in order to provide a high level of detectable polymorphism (Xu et al. 1997). Similarly, the recessive and dominant stocks used to develop the OWB population represent a cross of two morphologically diverse parental genotypes in which a large degree of distortion would be expected.

Table 2 Number of AFLP markers observed and expected in the OWB population based on the observed length (in centiMorgans) of each chromosome of barley

Chromosome	Observed	Expected	chi-square
1 (7H)	143	82	45.37***
2 (2H)	75	76	0.01
3 (3H)	85	93	0.69
4 (4H)	65	70	0.36
5(1H)	62	64	0.06
6 (6H)	78	85	0.58
7 (5H)	78	111	9.81***

*** Significantly different at $P \leq 0.001$

Clustering and distribution of AFLP markers

To assess the distribution of AFLP markers over the barley genome, we compared the number of AFLP markers mapped on each chromosome to the expected number based on a random distribution over the entire genome, as described by Castiglioni et al. (1999). Chromosomes 1 (7H) and 7 (5H) had significant deviations from the expected number. Chromosome 1 (7H) had a much higher number of AFLP markers than those expected by chance, while chromosome 7 (5H) had fewer than expected (Table 2). Becker et al. (1995), in contrast, observed a strong correlation (r=0.91) between the length of the barley chromosomes as measured in cntiMorgans and the number of AFLPs per chromosome.

Clustering of AFLP markers were observed on all chromosomes. These clustered AFLPs appeared to be mostly on centromeric regions as reported by Qi et al. (1998) and Becker et al. (1995) in barley and Castiglioni et al. (1999) in maize and may be located in regions of suppressed recombination. In a recent study of the relationship of physical and genetic distances in barley, recombination rates were observed to be generally highly heterogenous along barley chromosomes and tended to be higher in a few small areas next to large segments where recombination is severely suppressed (Künzel et al. 2000). They observed that the regions of higher recombination (≤1Mb/cM) that corresponded to approximately 5% of the barley genome contained 47% of the RFLP markers used in their study.

Utility of the genetic map

We have generated a genetic map which combines different types of molecular marker data with morphological

trait data. Several molecular markers were found to be closely linked to morphological loci. For example, the AFLP marker E37M60-385 cosegregated with the *Lks2* locus, the AFLP marker E38M62-81 cosegregated with the *alm* locus, the RFLP MWG522 and the AFLP marker E40M48-640 cosegregated with the *srh* locus, while seven markers (four AFLPs, one RFLP and one SSR) co-segregated with the *rob* locus (Fig. 1). Therefore, the complete linkage maps reported herein may aid in map-based cloning of genes controlling morphological traits. For the isolation of agronomically important genes from plants, Briggs (1991) indicated that a powerful method may be based on merging QTL maps with detailed genetic maps of populations derived from mutant lines. For example, the location of QTLs for height in maize may coincide with that of dwarfing genes (Veldboom et al. 1994). A detailed genetic map of a population segregating for extreme alleles at morphological trait loci could also be an excellent vehicle for testing if a QTL in agronomically relevant populations is due to less extreme alleles at morphological trait loci where other alleles confer more extreme phenotypes, as proposed by Robertson (1985). The Oregon Wolfe Barley population could be used to test such hypotheses.

The OWB mapping population size was designed to be easy and efficient (e.g., fits a 96-well format, which is ideal for PCR). Although this population size is not large, it is comparable to other populations being used for linkage mapping of the triticeae. For example, the population used by the International Triticeae Mapping Initiative has a core mapping subset of 88 inbreds (Van Deynze et al. 1995). Furthermore, we plan to expand the size of the OWB population in the future.

The full power of this population as an interactive, collaborative teaching, and research tool will come as participants generate additional genotype and phenotype data. Consider, for example, a University lab generating abundant DNA-level polymorphism while a high school science class measures plant height and heading date. If each group operates in isolation, the marker data generates just another map, and the plant growth data are just another quantitative data set. However, through this collaborative network, the two can be integrated, and through QTL analysis, the determinants of the maturity and plant height can be assigned to chromosome positions. Chromosome location information, in turn, provides tools for physiology, developmental genetics, and finer structure genetic analysis. We hope that this networking will also lead to long-term partnerships. More information on the "Oregon Wolfe Barleys" can be found on the World Wide Web at http://www.css.orst.edu/Barley/WOLFEBAR/Wolfnew.htm. This site serves as a resource for obtaining seed, data retrieval, and data reporting. These freely available resources are part of an effort to improve the teaching of genetics using an agriculturally important crop.

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