Identification of Marker Trait Associations in a Barley Four Way Cross

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Abstract
Until recently, genetic mapping in cereals has been exclusively applied to populations derived from simple crosses. Genetic studies are now targeting broader population structures to take advantage of association mapping and whole genome analysis techniques. This paper presents a study of a Doubled Haploid (DH) population derived from the complex cross, Chieftan/Barque//Manley/VB9104, which is a significant departure from conventional mapping population structures.

The population has been extensively phenotyped for malt quality and adaptation characteristics, through evaluation as a mainstream breeding population within the SA Barley Improvement Program (SABIP). Of the 841 (DH) lines, 837 lines were evaluated in double row trials, with 350 individuals promoted to stage one, 70 individuals to stage two and 10 individuals to stage three. One line (WI3408) has subsequently progressed to pilot scale malting and brewing trials, with potential for commercial release. Using high throughput technology the stage one DH lines (350) were extensively screened with fluorescently labelled simple sequence repeats (SSRs) based on their association with the traits of interest for this population. There was a 60% rate of polymorphism between one or more parents. In total, 200 SSRs were assayed and analysed using both Gene Flow and Gene Mapper data bases.

Key words: Barley, Whole genome analysis, Simple sequence repeats (SSRs)

Introduction
Conventional barley breeding is based upon crossing two elite lines with the assumption that some of the progeny would out perform both parental lines. However, as knowledge of the structure of the genome increased, breeders have begun to use the backcrossing technique to target specific traits of interest in varieties that were well adapted to their environment.

Significant developments in marker technology has allowed for marker saturation of key regions in the genome, whole genomic studies can now be carried out on current breeding populations. This development has been made possible by the availability of markers which are closely linked to traits of interest within the breeding program.

This population will assess the ability of feed varieties to improve the agronomic performance of elite, malting barley varieties for Australian growing conditions, and the possibilities of breeding for a molecular ideotype.

Materials and Methods
Germplasm
Initial crosses were made between Chieftan and Barque and similarly between Manley and VB9104, resulting in 'single cross' F_1 plants. These F_1 plants were then inter-crossed, and 841 DH lines were derived from nine different inter-cross F_1 plants. The number of these DH lines generated per plant ranged from four to 332 with 206 lines and 332 lines being the two largest sets.

Phenotyping
Of the 841 DH lines, 837 were evaluated in double row trials in 1999 and re-evaluated again in 2004. All lines from 1999 trials were harvested, and data collected on screenings and quality traits, were

assessed by near infra-red spectroscopy (NIR). There were 350 selected individuals promoted to stage one yield trials in 1999, grown as one replicate at three sites, with seven cultivars as grid checks. Agronomic observations were recorded, yield measured and IOB wet-chemistry quality data obtained. 70 individuals were advanced to stage two yield trials in 2000 and evaluated in unreplicated trials at eight sites. Data was collected as in stage one but with more detailed quality analyses. Ten individuals were advanced to stage three yield trials in 2001, which were evaluated in replicated trials at eight sites.

Genotyping

The minimum density of good quality polymorphic SSR markers for linkage map construction were selected based on their map positions around known quantitative trait loci (QTL) and specific traits of interest (Table 1).

Table 1. Characteristics of the four parental lines, Chieftan, Barque, Manley and VB9104. The key genetic locations presumed to influence the traits are also shown.

	Chieftan	Barque	Manley	VB9104	Chromosome
Scald	R	S	S	MR	3H
SFNB	S	R	S	MR	7H
Mildew	R	MR	S	S	2H,4H
Height	SD	T	T	T	3H
Maturity	L	E	L	E	2H
Leaf rust	R	S	S	S	5H,7H
Lodging	R	MS		MS	2H
Extract	H	L	H	M	1H,2H,5H
DP	M	L	VH	M	1H,4H,5H
Viscosity	L	H	L	MH	1H
Fermentability	M	L	VH	M	4H,6H
CCN(Ha4)	S	R	S	S	5H
Yield	M	H	L	H	2H,3H,4H,7H
Grain size	SM	ML	M	VL	2H
ppd		sens		sens	2H
eps		E		E	2H

SFNB – spot form net bloch; DP - diastatic power; CCN – cereal cyst nematode; *ppd* – photo period response; *eps* – earliness *per se*; R – resistant; S – susceptible; MR – moderately resistant; MS – moderately susceptible SD – semi-dwarf; T – tall; L – late; E – early; H – high; L – low; M – moderate; VH – very high; MH – moderately high; SM – small to medium; ML – medium to large; M – medium; VL – very large; sens – sensitivity.

Genomic DNA extractions were performed as described in Rogowsky *et al*. (1991) with some minor adjustments to facilitate the high throughput grinding of leaf material. For the genotyping of this population fluorescently labelled and unlabelled primers was chosen, with the majority of the analysis being accomplished with labelled primers. Labelled and unlabelled primers were amplified using a touchdown polymerase chain reaction (PCR) with an annealing temperature of either 50^{o} C or 55 ^{o} C. Each 12.5µL reaction contained 2µL DNA, reaction buffer (Qiagen), Q solution (Qiagen), 1.50mM $MgCl_{2,}$ 10µM of each forward and reverse primer, 0.24mM dNTPs, 0.25 unit of Qiagen Taq polymerase. Unlabelled SSRs were analysed on 8% acrylamide gels, stained with ethidium bromide and visualised using a gel documentation system. To facilitate multi-pooling for ABI 3100/3700 analysis PCR assays were performed as described above using forward primers labelled at the 5-prime end with FAM, HEX and NED in combination with GenScan 400HD [ROX] size standard. The raw data was analysed using Genotyper software. In the later stage of the project SSRs were assayed using the Multiplex Ready method as described by Hayden *et al*., (2005). This technique consisted of multi-pooling four fluorescent labels FAM, VIC, NED and PET. The PCR reaction was reduced from

12.5µL to 7µL and contained 3µL DNA, and a 4µL reaction mix containing reaction buffer (5x Mpx-Rdy), 10µM of dye-labelled tagF primer, 10 µM tagR primer, 4 µM locus-specific primer, 0.15 unit of Immolase. These samples were separated on the ABI 3730 platform using GeneScan 500 LIZ size standard and raw data analysed with GeneMapper software. This facilitated a three fold increase in throughput and a significant reduction in cost.

Due to the size of the population a 384 well plate format was used in conjunction with the Corbett CAS-3800 pipetting robot.

Results

From the total number of parental screens (520), 290 SSRs were polymorphic. Two hundred of these made the final selection based on quality, chromosome location and association with selected traits, with some markers distinguishing between each parental barley genotype i.e. producing four alleles (Figure 1). Although not apparent from the parental screens some SSR markers were monomorphic.

Initial work involved multipooling eight labelled SSR markers or 3,072 data points per 384 well plate. Since the availability of the Multiplex Ready system it was possible to successfully multi pool 24 SSR markers or 9,216 data points per 384 well plate. Due to the optimisation of primers before PCR both noise and artefacts had been greatly reduced and in most instances removed, thereby, increasing the quality and success of data.

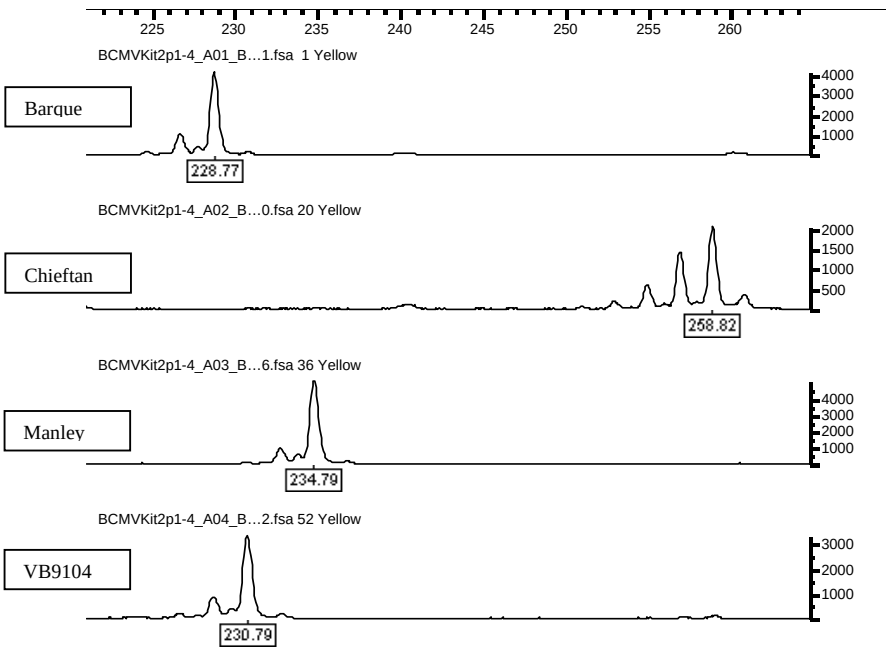

Figure 1. Results of parental screen using a fluorescent labelled SSR marker which distinguishes between the four barley genotypes.

Discussion

The full set of DH lines of the Barque/Chieftan//Manley/VB9104 cross is similar to a very large conventional mapping population. However, the population is derived from inter-crossed F_1 plants and from four parents that are not all necessarily homogeneous and homozygous. Some markers appeared to have more than four alleles present, suggesting that one or more of these parents could be heterogeneous and/or heterozygous. Preliminary analysis suggested that the additional alleles were derived from Barque. However, further investigation also supported that VB9104 is not a fixed line and is heterozygous for some loci. From this completed data a high density map is in the process of

being generated on the 350 stage one lines. This will facilitate measurement of frequency changes to alleles in response to pragmatic selection, and the identification of conserved linkage blocks associated with superior agronomic or quality phenotypes.

This analysis will determine the genetic basis of the elite line WI3408, which is a member of the largest family of 332 lines. WI3408 is currently being considered for commercial release, targeting medium-low rainfall production environments and export markets requiring high diastase and highly fermentable malt.

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