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The statistical analysis of quality traits in plant improvement programs with application to the mapping of milling yield in wheat

A. B. Smith[AF], *B. R. Cullis*[A], *R. Appels*[B], *A. W. Campbell*[C], *G. B. Cornish*[D], *D. Martin*[E], *and H. M. Allen*[A]

[A] Wagga Wagga Agricultural Institute, Private Mail Bag, Wagga Wagga NSW 2650, Australia.
[B] CSIRO Plant Industry, PO Box 1600, Canberra, ACT 2601, Australia.
[C] Centre for Rural and Environmental Biotechnology, Faculty of Sciences, University of Southern Queensland, Toowoomba, Qld 4350, Australia.
[D] SARDI, Grain Quality Laboratory, Glen Osmond, SA, 5064, Australia.
[E] The Leslie Research Centre, PO Box 2282, Toowoomba, Qld 4350, Australia.
[F] Corresponding author; email: alison.smith@agric.nsw.gov.au

Abstract. It is well known that the response to selection for grain yield is improved with the use of appropriate experimental designs and statistical analyses. The issues are more complex for quality traits since the data are obtained from a 2-phase process in which samples are collected from the field then processed in the laboratory. This paper presents a method of analysis for quality trait data that allows for variation arising from both the field and laboratory phases. Initially, an analysis suitable for standard varietal selection is presented. This is extended to include molecular genetic marker information for the purpose of detecting quantitative trait loci. The technique is illustrated using two doubled haploid wheat (*Triticum aestivum* L.) populations in which the trait of interest is milling yield.

Additional keywords: mixed model, statistical analyses, milling QTL, molecular genetic map, wheat, 2-phase experiment.

Introduction

Despite the importance of selection for quality traits in crop improvement programs, little attention has been given to statistical aspects of analysing quality trait data. The data are usually obtained from a 2-phase process in which lines are grown in designed field experiments (phase I) then grain samples from the field plots are processed in the laboratory (phase II). It is well known that the response to selection for trait data that are measured in the field, in particular grain yield, can be improved with the use of sound field design and statistical analysis techniques (see Cullis and Gleeson 1991; Cullis *et al.* 1998). Little research has been conducted into the more complex design and analysis issues of quality trait data but the use of appropriate methodology is likely to enhance selection. This is important both for varietal selection and for the identification of quantitative trait loci (QTLs). In this paper we focus on the genetic mapping of milling yield in wheat (*Triticum aestivum* L.) but the issues and concepts apply to many other quality traits and crops.

Milling yield data may be affected by non-genetic variation from both the field and laboratory phases. The field experiment phase is characterised by spatial variation, so called because it is linked to the spatial location of plots in the field. This may reflect natural variation (due to fluctuations in soil fertility, for example) and extraneous variation (often linked to management practices). Experimental design and subsequent analysis should be aimed at controlling this variation and include row–column and neighbour balanced designs. In terms of analysis, many authors have recognised the need to model spatial variation in the field (Wilkinson *et al.* 1983; Besag and Kempton 1986; Gleeson and Cullis 1987; Cullis and Gleeson 1991; Gilmour *et al.* 1997). Gilmour *et al.* (1997) presented a general approach that is widely used in Australian plant breeding programs for the analysis of grain yield. It has been shown to improve both the accuracy and the precision of varietal yield estimates.

Like grain yield data, milling yield data may be affected by field variation but additionally by variation from the laboratory phase. In terms of the latter, potential sources include variation associated with the order in which samples are milled within a day, and variation from day to day. The experimental laboratory design, namely the order of milling

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samples, should be aimed at controlling this variation. However, there are additional design issues, arising from the 2-phase nature of the design. The literature on the design of 2-phase experiments is limited. McIntyre (1955) provided some broad guidelines but the types of experiments considered were relatively simple compared with milling yield experiments. When varietal selection is the aim, key issues are the number of field replicates to be milled, the number of laboratory checks, or duplicates of field samples, and the allocation of varieties to days and positions within a day. Current practice often involves the milling of a single (or composite) field replicate with little regard as to the order of processing. As pointed out by McIntyre (1955), the use of several field replicates is essential to obtain a valid estimate of error for varietal comparisons. Laboratory checks (subsamples from a uniform source of grain) or duplicates of field samples (single grain samples split into two and milled separately) may be included for a number of reasons. They allow the partitioning of the total error in milling yield data into field and laboratory components. Without them we may be unable to identify spatial variation in the field, thereby sacrificing the associated gains in response to selection. Laboratory checks are an inefficient means of achieving this since they use resources without increasing information on the material of interest. Therefore, unless there is insufficient grain, the use of duplicate samples is preferred. Duplicates are standard practice in other types of 2-phase experiments (McIntyre 1955; Brien 1983; Wood *et al.* 1988). With respect to the order of processing, we should aim to accommodate laboratory variation, but should also be mindful of the link between the field and laboratory design. For example, it may be advantageous to break any confounding between field and laboratory trend by ensuring that varieties that are neighbours in the field are milled far apart.

As is the case with experimental design, there is little literature on the analysis of quality trait data. Brien (1983) provided a useful framework for determining the analysis of variance (ANOVA) table appropriate for orthogonal 2-phase designs. Wood *et al.* (1988) considered designs with non-orthogonal block structure. Quality trait experiments are generally more complex than those considered by Brien (1983) and Wood *et al.* (1988). In this paper we present a mixed model analysis for quality trait data that is consistent with the approaches of Brien (1983) and Wood *et al.* (1988), but allows greater flexibility for modelling spatial and laboratory variation.

As discussed by Moreau *et al.* (1999), statistical design and analysis concepts are often neglected in genetic mapping experiments for detecting QTLs. They stressed the need for accounting for spatial field variation when mapping grain yield and made the comment that 'computer programs for QTL detection based on interval mapping do not take [this variation] into account'. They stated that the programs could be used on spatially adjusted means, rather than raw data, but that '... an integrated one-step approach seems better than proceeding in two steps'. The comments of Moreau *et al.* (1999) for grain yield apply equally to the detection of QTLs in quality trait data. In this paper we consider the mapping of milling yield in 3 doubled haploid (DH) populations. We use a 1-step mixed model approach in which QTLs are detected via regressions on pairs of marker covariates (Whittaker *et al.* 1996), this being done simultaneously with the modelling of field and laboratory trend. A detailed account of the mixed model approach to interval mapping is presented in Eckermann *et al.* (2001, this issue). The examples demonstrate the implications of ignoring field and laboratory variation when mapping milling yield in wheat.

Description of data

Phenotypic data

The plant material comprises doubled haploid (DH) lines from two mapping populations, derived from crossing the variety Cranbrook with Halberd (Cran × Hal) and CD87 with Katepwa (CD × Kat) (see Kammholz *et al.* 2001, this issue). The lines were grown in replicated field trials, each trial being laid out as a rectangular array of rows and columns (Table 1). All trials were designed as randomised complete blocks with two replicates for DH lines and often extra replicates of parental or check varieties. For some DH lines there was only sufficient seed to sow a single replicate. The CD × Kat population was split into quick (Q) and mid (M) maturing lines and grown in separate trials (Table 1).

Grain samples from the field trials were milled in laboratories using various regimes. Usually only a subset of the field plots was sampled, the exception being the trial for the quick maturing CD × Kat lines (Table 2). The samples for Cran × Hal were milled using a Quadramat Junior Mill, whereas all others were milled using a Buhler Mill. This is reflected in the number of samples processed per day. The samples for Cran × Hal were not milled according to any experimental design protocols. The two field replicates for a

Table 1. Field trials for two wheat mapping populations
Q, quick; M, mid maturing lines

Population	Location	Year	DH lines	Other lines	Rows	Columns	DH lines with 1 rep.
Cranbrook × Halberd	Roseworthy	1996	170	3	29	12	–
CD87× Katepwa	Roma (Q)	1998	60	4	10	14	–
CD87 × Katepwa	Roma (M)	1998	154	4	24	14	4

Table 2. Milling process for grain samples from field trials
Q, quick; M, mid maturing lines

Population	DH lines	DH lines with 1 rep	Field checks	Days	Samples per day	Field samples	Samples of lab. check
Cranbrook × Halberd	167	2	48	16	16–22	290	17
CD87 × Katepwa (Q)	60	4	–	18	9	140	22
CD87 × Katepwa (M)	118	4	1	34	9	264	42

given DH line were milled consecutively. A single laboratory check was milled each day, always in the first half of the day. The number of samples varied from day to day. The samples from all other trials were milled according to designs based on some simple statistical principles. In the absence of information about efficient 2-phase designs for milling data, the following designs were adopted. The design for CD × Kat (mid maturing trial) was based on a resolvable incomplete block design for the lines sampled from the field. Days on which samples were milled were regarded as blocks with complete replicates comprising 17 blocks. Field replicates were confounded with laboratory replicates. Laboratory checks were added to the design with a systematic grid of one check per day, cycling through the positions each day. Extra checks were added to maintain a complete 2-way layout of days by samples per day. The design for CD × Kat (quick maturing) was similar except that a neighbour balanced design was used instead of the incomplete block design.

Maps

The genetic maps were produced using protein (seed storage protein) and DNA markers. The DNA markers included RFLPs, AFLPs, and microsatellites (Chalmers *et al.* 2001, this issue). The map for Cran × Hal comprised 813 markers on 21 pairs of chromosomes, while for CD × Kat there were 414 markers on 18 pairs of chromosomes. For the purposes of interval mapping we chose markers approximately equally spaced at a distance of 10 cM (centimorgans). In terms of the choice of distance there were two key factors, namely the resolution of the map and the statistical correlation between marker covariate data. At small separations marker locations may be unreliable and the correlation between the associated covariate data so strong that the pair of markers is uninformative in terms of QTL detection (Hackett *et al.* 2001). The choice of 10 cM separation resulted in a map of 358 markers for Cran × Hal and 215 markers for CD × Kat.

Statistical models

Extended mixed model for the analysis of quality trait data

In this section we extend the mixed model approach of Gilmour *et al.* (1997) for the analysis of field data to the analysis of 2-phase quality trait data such as milling yield in wheat.

Let $\boldsymbol{y}^{(n \times 1)}$ be the vector of milling yield data for n samples. The number of samples is given by $n = n_p + n_d$ where n_p is the number of field plots from which samples are taken and n_d is the number of duplicated field samples or laboratory checks. The base-line model for the milling yield for the ith sample is given by

$$y_i = \boldsymbol{d}_i'\boldsymbol{\mu} + \boldsymbol{a}_i'\boldsymbol{g} + \boldsymbol{b}_i'\boldsymbol{f} + l_i \quad (1)$$

where $\boldsymbol{g}^{(n_g \times 1)}$ is the vector of random effects for the DH lines and $\boldsymbol{f}^{(n_p \times 1)}$ is the vector of random field plot effects. For simplicity we assume that all n_p plots in the field are sampled for milling. Elements in the vector $\boldsymbol{\mu}^{(n_t \times 1)}$ represent means for n_t different types of sample. For example, laboratory and field checks have their own means that are distinct from the mean for the DH lines. The vectors $\boldsymbol{a}_i^{(n_g \times 1)}$, $\boldsymbol{b}_i^{(n_p \times 1)}$, and $\boldsymbol{d}_i^{(n_t \times 1)}$ pick out the DH line, field plot, and sample type associated with the ith sample. Then, for example, if the ith sample is a laboratory check, there are no DH line or plot error effects so that all elements in $\boldsymbol{a}_i$ and $\boldsymbol{b}_i$ are zero. The term l_i represents the error for the sample arising from the laboratory process.

The model in Eqn 1 can be used when selection is the primary aim of the milling yield experiment. Fitting the model will provide estimates of genetic merit together with some measure of confidence. The latter is vital otherwise response to selection may be hindered. In order to calculate valid confidence measures we must fit terms in the model to properly define the error structure. In an analysis of variance (ANOVA) setting we would speak of the definition of 'strata'. In milling yield data there are two error strata: associated with the field and the laboratory. Following McIntyre (1955) and Brien (1983) the appropriate error term on which to base confidence measures for the estimated genetic effects is the field plot error term. Thus in an

Table 3. ANOVA table showing sources of variation for milling yield data
Effects are classified as fixed or random

Term	Model effects	Fixed or random	Variance structure
	Field		
Genotype	$\boldsymbol{g}$	Random	$\sigma_g^2 \boldsymbol{I}_{n_g}$
Error	$\boldsymbol{f}$	Random	Ω
	Laboratory		
Error	$\boldsymbol{l}$	Random	$\boldsymbol{R}$

ANOVA sense the genetic effects are nested within the field plot stratum. This structure is shown in the ANOVA decomposition in Table 3. The decomposition corresponds to the model in Eqn 1, the key to defining the error structure being the inclusion of the terms for field and laboratory error. This facilitates the calculation of valid confidence measures for the genetic effects.

We note that if a single (or composite) field replicate only is milled then genetic and plot error effects are confounded. Since it is not possible to estimate a plot error variance, no valid confidence measures can be calculated. It may not be possible to separate field and laboratory error. This may occur when there are insufficient duplicate field samples or laboratory checks or when one of the error components dominates. We may also choose to confound field and laboratory errors, constructing a laboratory design to achieve this. In either case, if it is not possible to separate the two sources of error the term $\boldsymbol{f}$ is dropped from the model and l_i then represents the pooled effect of field and laboratory error for the ith sample.

The model in Eqn 1 is known as a mixed model since it involves a mixture of fixed effects ($\boldsymbol{\mu}$) and random effects ($\boldsymbol{g}$, $\boldsymbol{f}$ and $\boldsymbol{l}$). Each vector of random effects is assumed to have a Gaussian distribution with zero mean. The genetic effects g_j are assumed to be independently and identically distributed (IID) with variance σ_g^2 (see Table 3). The joint distribution of the field and laboratory error effects has a variance matrix of the form:

$$\begin{bmatrix} \boldsymbol{\Omega} & \boldsymbol{0} \\ \boldsymbol{0} & \boldsymbol{R} \end{bmatrix}$$

where $\boldsymbol{\Omega}^{(n_p \times n_p)}$ and $\boldsymbol{R}^{(n \times n)}$ are symmetric positive definite matrices. Initially, we assume the effects in each set of errors to be IID with $\boldsymbol{\Omega} = \sigma_p^2 \, \boldsymbol{I}_{n_p}$ and $\boldsymbol{R} = \sigma_l^2 \, \boldsymbol{I}_n$. Graphical diagnostics based on estimates of plot error effects and of laboratory error effects from the IID model are used to identify departures from the IID assumptions. For example, smooth spatial field trends may be identified. This can be accommodated in the analysis by extending the base-line model in Eqn 1 to include a correlation model for $\boldsymbol{\Omega}$ (see Gilmour *et al.* 1997, for details on possible model choices). The diagnostics may also reveal the need to add (fixed and/or random) terms to the base-line model in order to accommodate field and/or laboratory trends. For example, we may add a term for random day effects to accommodate non-systematic day-to-day variation in the milling process. The modelling is a sequential process involving the use of diagnostics and formal tests of significance (see Gilmour *et al.* 1997).

Incorporation of molecular marker information

Estimation of the size and location of an isolated QTL

If the aim of the milling experiment is to conduct interval mapping for the DH lines, we can extend the model in Eqn 1 by proposing a sub-model for the genetic effects. The model for the jth DH line is given by:

$$g_j = \beta_L \, x_{Lj} + \beta_R \, x_{Rj} + u_j \tag{2}$$

where x_{Lj} and x_{Rj} are covariate values for the jth DH line for a pair of flanking (left and right) markers and u_j is the residual genetic effect, that is, due to genetic influences other than the two markers. The covariates indicate the marker types so take only two possible values, namely '+1' or '–1'. The size and location of a potential QTL between flanking markers are determined by the regression coefficients β_L and β_R. This regression approach to interval mapping was developed by Whittaker *et al.* (1996) (also see Eckermann *et al.* 2001, this issue).

Substituting Eqn 2 into Eqn 1 and writing in matrix notation gives:

$$\boldsymbol{y} = \boldsymbol{D}\boldsymbol{\mu} + \beta_L \boldsymbol{A}\boldsymbol{x}_L + \beta_R \boldsymbol{A}\boldsymbol{x}_R + \boldsymbol{A}\boldsymbol{u} + \boldsymbol{B}\boldsymbol{f} + \boldsymbol{l} \tag{3}$$

where $\boldsymbol{A}^{(n \times n_g)}$, $\boldsymbol{B}^{(n \times n_p)}$, and $\boldsymbol{D}^{(n \times n_t)}$ are matrices with rows given by $\boldsymbol{a}_i'$, $\boldsymbol{b}_i'$, and $\boldsymbol{d}_i'$ (i = 1... n). The vectors $\boldsymbol{x}_L$, $\boldsymbol{x}_R$, and $\boldsymbol{u}$ have elements x_{Lj},x_{Rj} and u_j (j = 1... n_g) and the vector $\boldsymbol{l}$ has elements l_i. When regressions for mapping are included in the model the distributional assumptions relate to the residual genetic effects u_j rather than the genetic effects as a whole. We denote their variance by σ_u^2. The ANOVA decomposition for this model is shown in Table 4.

Standard interval mapping programs carry out an identical regression to that in Eqn 3 but make no allowance for field or laboratory trends and use a simple error term, which is a composite of residual genetic effects, and field and laboratory error. Often the data comprise DH line means. If we let $\boldsymbol{y}_+^{(n_g \times 1)}$ be the vector of mean milling yields for the DH lines the regression model used in standard interval mapping programs is given by:

$$\boldsymbol{y}_+ = \mu + \beta_L \boldsymbol{A}_+ \boldsymbol{x}_L + \beta_R \boldsymbol{A}_+ \boldsymbol{x}_R + \boldsymbol{e}_+ \tag{4}$$

where μ is the constant term, $\boldsymbol{A}_+^{(n_g \times n_g)}$ is an indicator matrix analogous to $\boldsymbol{A}$ in Eqn 3, and $\boldsymbol{e}_+$ represents the (composite) error term for the regression.

Table 4. ANOVA table showing sources of variation for mapping of milling yield data
Effects are classified as fixed or random

Term	Decomposition	Model effects	Fixed or random	Variance structure
		Field		
Genotype	Left marker	$\boldsymbol{x_L}$	Fixed	
	Right marker	$\boldsymbol{x_R}$	Fixed	
	Residual genetic	$\boldsymbol{u}$	Random	$\sigma_u^2 \boldsymbol{I}_{n_g}$
Error		$\boldsymbol{f}$	Random	$\boldsymbol{\Omega}$
		Laboratory		
Error		$\boldsymbol{l}$	Random	$\boldsymbol{R}$

In general, estimates of the regression coefficients and associated significance tests from analyses based on Eqn 3 and Eqn 4 will differ. Agreement will occur only in the simplest situation, for example, when all DH lines are equally replicated and there are no non-orthogonal sources of variation (such as spatial variation in the field or day effects in the laboratory).

Detection of multiple QTLs

If the existence of multiple QTLs is proposed, the model in Eqn 3 can be extended to a multiple regression with the inclusion of a number of marker pairs. The selection of markers to be included is a difficult problem (see e.g. Moreau *et al.* 1999). In the data sets under study here the number of DH lines was insufficient to enable the fitting of all markers simultaneously (as done in Whittaker *et al.* 1996). We chose a selection procedure commencing with the fitting of a sequence of pair wise regression models using successive pairs of markers. Marker pairs with estimated regression coefficients of opposite sign were discarded immediately since these are deemed inadmissible. Of the remainder, pairs were selected if the test of the hypothesis that both regression coefficients are zero was rejected (see Whittaker *et al.* 1996). The threshold for significance was set at $P < 0.01$. The chosen set of marker pairs was then included in a multiple regression and re-assessed for admissibility. Regression coefficients from the final model comprising all admissible marker pairs were used to estimate the size and location of the QTLs (see Whittaker *et al.* 1996).

Mixed model estimation

Estimates of the fixed and random effects in a mixed model are obtained as solutions to the mixed model equations (Henderson 1950). This provides best linear unbiased estimates (BLUEs) of the fixed effects and best linear unbiased predictors (BLUPs) of the random effects. Variance parameters can be estimated using the method of residual maximum likelihood (REML) (Patterson and Thompson 1971).

We used the samm (Butler *et al.* 2000) functions in S-Plus (Mathsoft 1999) to estimate all models. These functions call the core routines of ASREML (Gilmour *et al.* 1999), which is an efficient computer program for the estimation of linear mixed models.

Examples

Cranbrook × Halberd cross

We use the mixed model technique described in the statistics section to map the milling yield data from the trial conducted at Roseworthy in 1996 (see Table 1 and Kammholz *et al.* 2001, this issue). We stress that the results must be interpreted with caution since there was no experimental design in the laboratory phase. Unlike a designed experiment we have no recourse to randomisation theory and we have very limited information with which to partition field and laboratory error. This example, however, highlights some important experimental design issues and the impact of accounting for sources of variation.

Initially, we considered the analysis in the absence of mapping, that is, based on the model in Eqn 1. This is of interest in its own right when selection is the primary aim. It is also useful as the first step when mapping is the aim since it simplifies identification of appropriate trend models for field and laboratory variation. The trend models thus identified are then maintained (but the parameters re-estimated) as pairs of marker covariates are added to the model.

We began with a simple variance component model, that is, with the assumption of IID effects for the genetic effects, field plot errors, and laboratory errors. Field and laboratory variation contributed similar amounts to the total error (Table 5). Graphical diagnostics based on estimates of plot error effects and laboratory error effects from the IID model led to the identification of smooth spatial field trends and random effects associated with day-to-day variation in the laboratory. The former was accommodated in the model using a separable correlation structure for Ω with an identity (independence) model in the column direction and an autoregressive process of order 1 in the row direction (denoted ID × AR1). Both the field trend model and the random day effects were significant. To illustrate the spatial field trend, Fig. 1 contains the estimated plot error effects graphed against field row number for each field column. Clearly, the milling yield of a sample is affected by the spatial location of the field plot from which it originates. For example, samples from low numbered rows in columns 8–12 have much lower (approximately 2% lower) milling yields than those in high numbered rows in the same columns. The

Table 5. REML estimates of variance components from IID model for mixed model analysis of milling yield data

Q, quick; M, mid maturing lines

Population	Location	Year	Genetic variance	Error variance	
				Field	Laboratory
Cranbrook × Halberd	Roseworthy	1996	6.20	1.27	1.12
CD87 × Katepwa	Roma (Q)	1998	0.86	0.61	0.49
CD87 × Katepwa	Roma (M)	1998	4.15	1.32	0.29

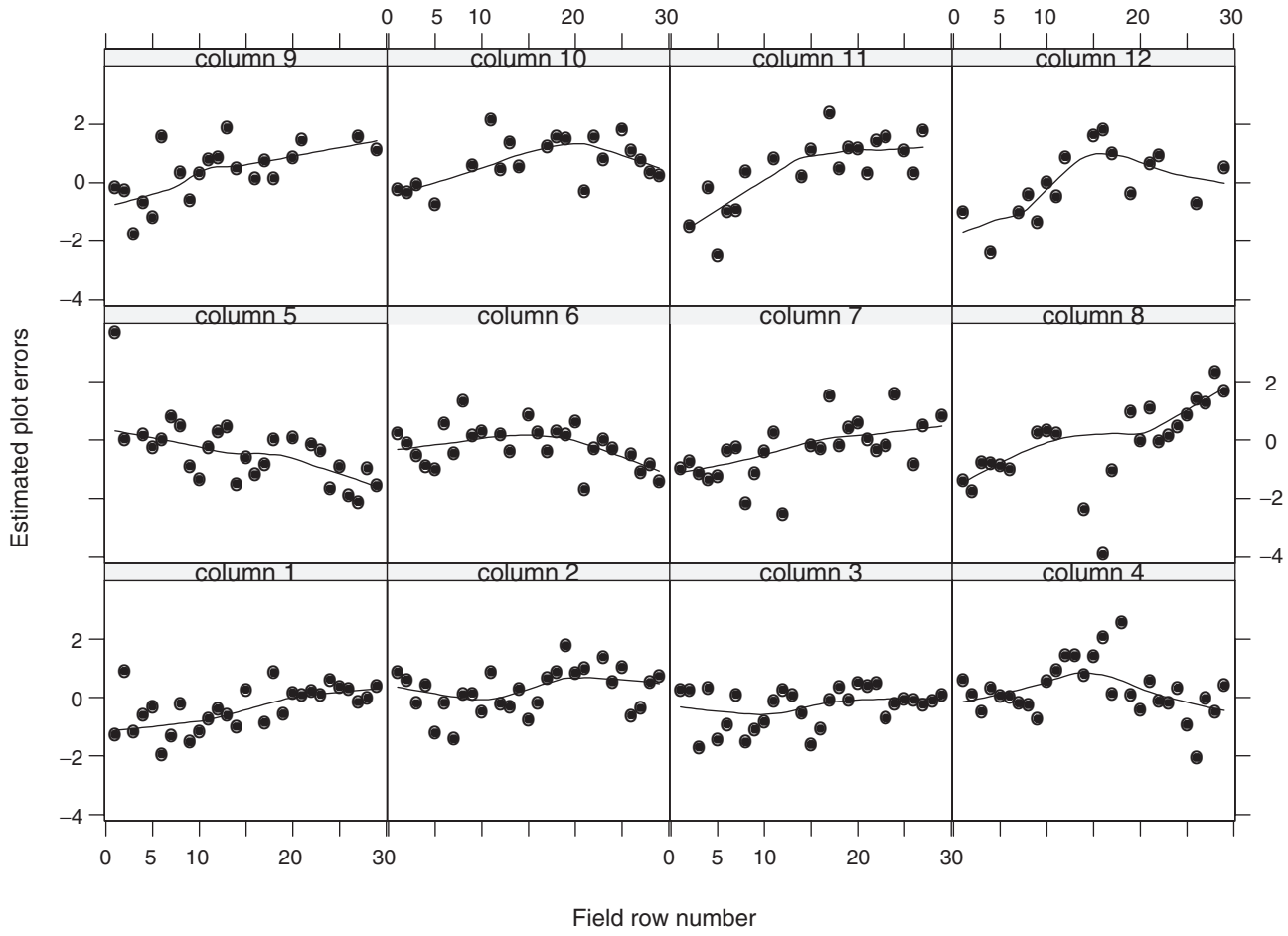

Fig. 1. Field plot error effects for Cranbrook × Halberd milling yield data graphed against row number for each column.

day on which samples were milled also had an impact in the Cran × Hal data, with a difference of 2.5% in the average milling yield between the lowest/highest yielding days. The cause of variation between days was unknown. Experience in analysing milling yield data has shown that unexplained day-to-day variation is common.

There was substantial disagreement between the estimates of the DH means from the mixed model and the raw means (Fig. 2). This is mainly due to the fact that the two field replicates of an individual line were milled on the same day so that variation between lines milled on different days was confounded with the day-to-day variation associated with the laboratory process. Since the latter was large this has resulted in large adjustments for some DH lines. In selecting the top 20 lines only 13 of the lines from the analysis would also be chosen using the raw means.

We then considered mapping the milling yield data and fitted a sequence of models, maintaining the trend models just described, but adding successive pairs of marker covariates. The covariates were defined such that a value of '+1' indicated a Cranbrook marker type and '–1' a Halberd marker type. The pair-wise regressions led to the selection of 9 pairs of markers for the multiple regression (Table 6).

The same regression approach was applied to the raw means of the DH lines. That is, using the model in Eqn 4 rather than Eqn 3. This resulted in the selection of 11 pairs of markers (Table 6). The raw means were also analysed using Map Manager (Manly *et al.* 2000). The LRS (likelihood ratio statistic) for the marker pairs selected in both the modelling and standard approaches is given in Table 6. The statistical model underlying the standard approach and Map Manager (Manly *et al.* 2000) is very similar so general agreement is expected. Discrepancies may arise for several reasons, including the use of a different type of test statistic and variations in interval sizes. Table 6 shows reasonable agreement between the results for the standard approach and Map Manager (Manly *et al.* 2000). There are, however, substantial differences between these and the results from the mixed modelling approach.

The 9 marker pairs identified in the mixed modelling approach were then included together in the regression model and re-assessed. This resulted in a final regression

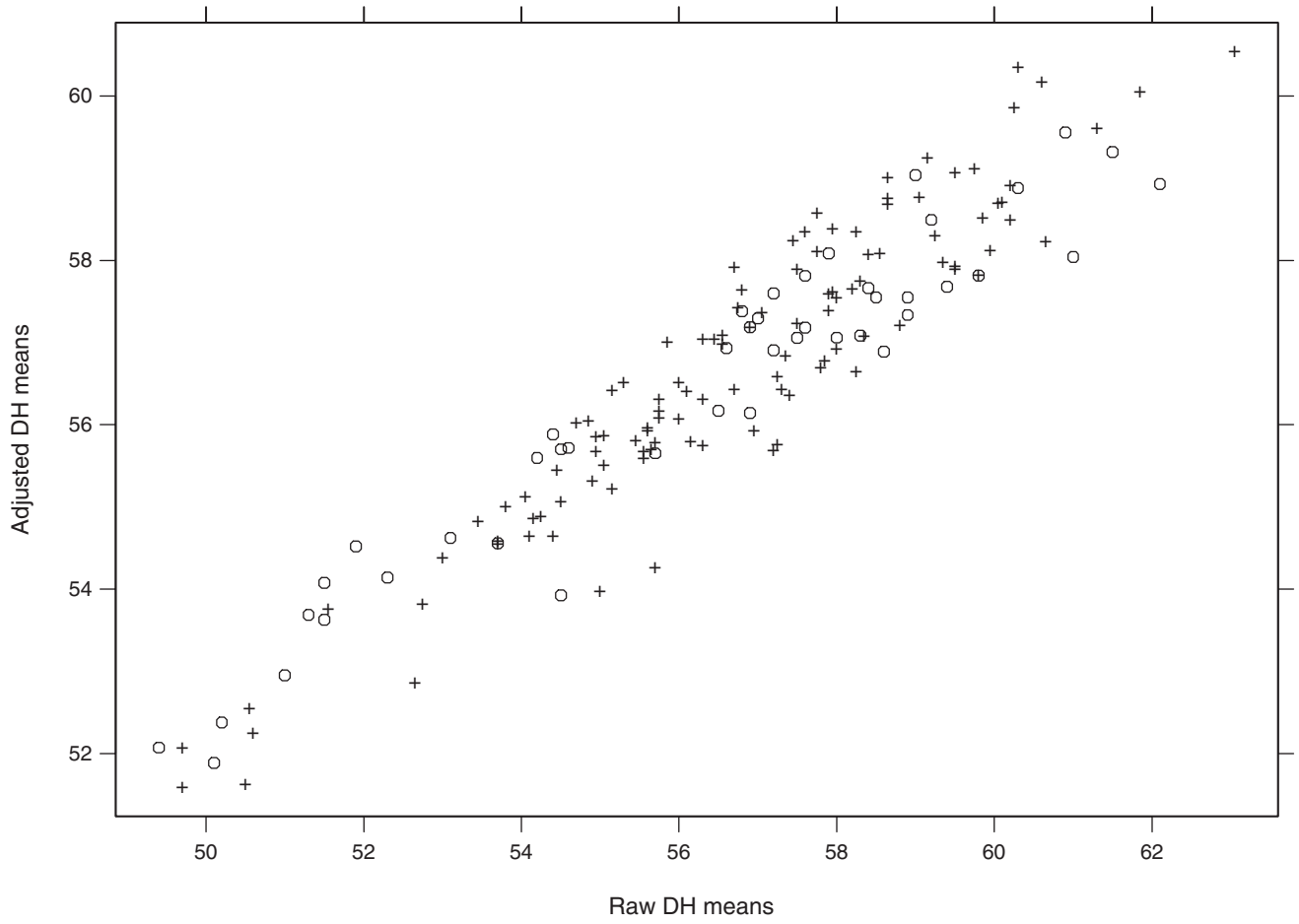

Fig. 2. DH means from Cranbrook × Halberd milling yield data. Means based on two replicates are indicated '+'; means from one replicate are indicated '○'.

Table 6. Marker pairs selected from pair-wise regressions for Cranbrook × Halberd using mixed modelling and standard regression approaches: regression test statistics and LRS statistic from Map Manager

Critical value ($P = 0.01$) for all test statistics is 9.2 (significant values in bold type)

Chromo-some	Flanking markers Left	Right	Modelling	Standard	Map Manager
2B	PAGC.MCGA2	wmc154	7.7	**10.4**	7.4
2B	wmc154	PACA.MCCC1	8.4	**10.4**	**9.5**
2D	PAGA.MCAG121	BCD410(C)	8.5	**10.0**	**9.2**
3B	PAGC.MCCT7	Iha.B1.2	**11.4**	**9.4**	7.4
3B	PAAT.MCAC5	PAAA.MCAT3	**10.2**	**10.2**	8.0[A]
4A	pAAT.mCGT2	CDO506	**9.9**	7.4	6.6
5A	PAGG.MCTG2	ABG397	**12.3**	**12.9**	**12.7**
5A	ABG397	BCD21	**14.9**	**14.3**	**18.3[A]**
5B	PACT.MCCA1	PAGG.MCAA5	**12.4**	**16.4**	**9.9[A]**
6B	PAGG.MCGT260	gwm626	8.1	**10.4**	**14.1**
7B	PACA.MCAA1	PACA.MCTG1	**10.0**	–[B]	5.6
7D	PAAC.MCTT1	CD01400	**14.0**	**14.3**	**13.2[A]**
7D	CD01400	wmc405	**13.2**	**12.2**	**10.5**

[A] Map Manager included more than one pair in this interval; largest LRS given.
[B] Inadmissible pair (regression coefficients with opposite sign).

Table 7. QTLs identified in mixed modelling multiple regression approach to mapping Cranbrook × Halberd milling yields

Chromo-some	Flanking markers Left/right	Regression coefficient	Distance[A]	QTLs Size of effect	*P*-value for effect
3B	PAAT.MCAC5	–0.49	5.8	–0.576	0.037
	PAAA.MCAT3	–0.06			
5B	PACT.MCCA1	0.19	37.5	0.745	0.007
	PAGG.MCAA5	0.48			
7D	PAAC.MCTT1	–0.37	4.1	–0.691	0.0004
	CDO1400	–0.32			

[A] Distance is measured in cM from the left marker.

Table 8. QTLs identified in standard multiple regression approach to mapping Cranbrook × Halberd mean milling yields

Chromo-some	Flanking markers Left/right	Regression coefficient	Distance[A]	QTLs Size of effect	*P*-value for effect
2B	wmc154	–0.50	3.3	–0.588	0.012
	PACA.MCCC1	–0.08			
2D	PAGA.MCAG121	0.60	4.7	0.847	0.0001
	BCD410(C)	0.24			
5B	PACT.MCCA1	0.20	39.5	0.922	0.002
	PAGG.MCAA5	0.62			
7D	PAAC.MCTT1	–0.51	3.3	–0.823	0.0001
	CDO1400	–0.31			

[A] Distance is measured in cM from the left marker.

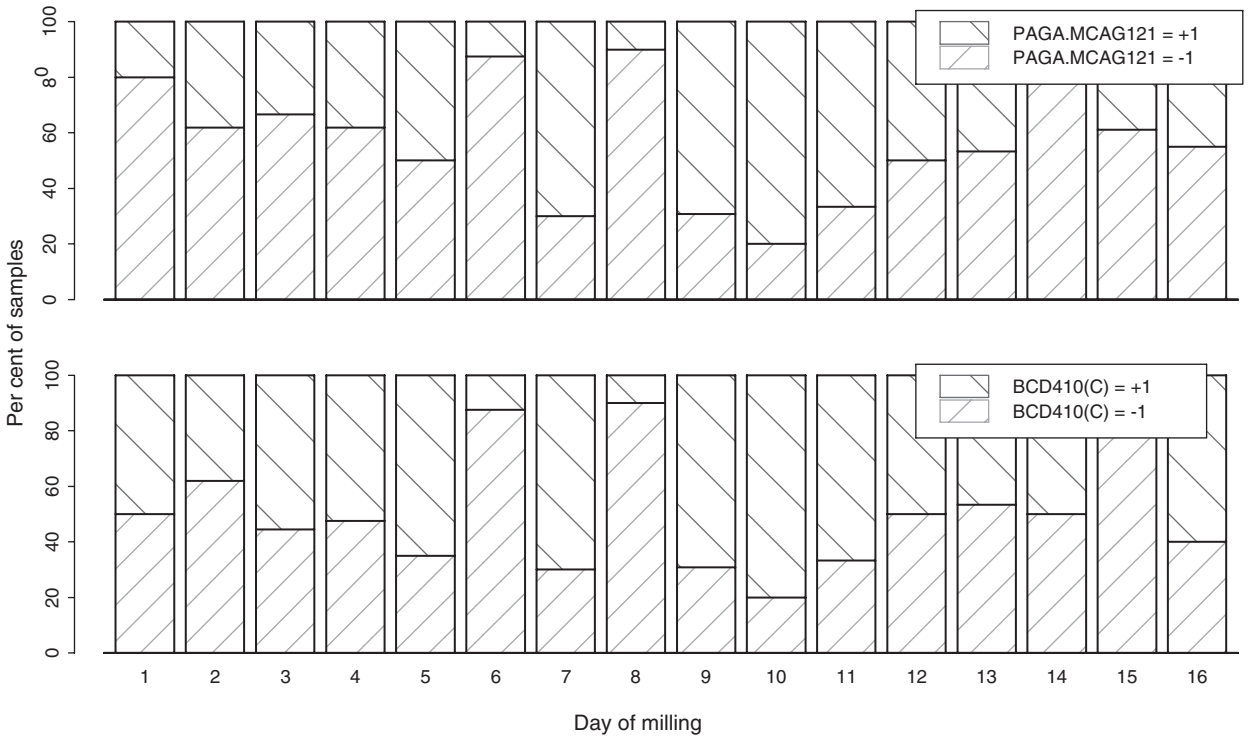

Fig. 3. Distribution of covariate values for each day of milling for flanking markers of QTL identified on chromosome 2D for Cranbrook × Halberd using a standard mapping approach.

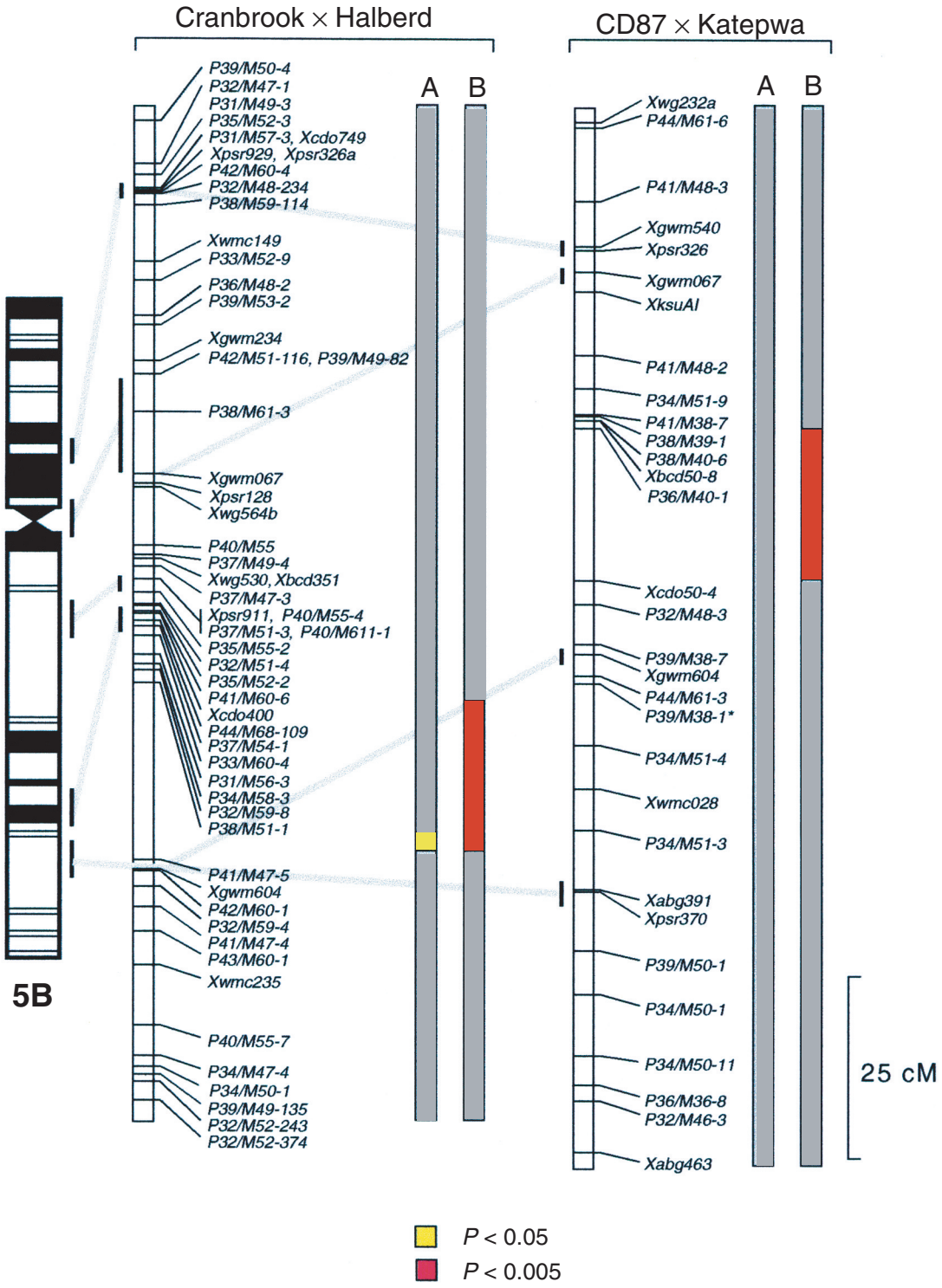

Fig. 4. Comparison of QTLs obtained for milling yield from (A) Map Manager and (B) the mixed modelling approach, for two crosses, Cranbrook × Halberd and CD87 × Katepwa. The schematic chromosome on the left is the standard C-banded karyotype for chromosome 5B. For the genetic maps a scale of 50 cM (centiMorgans) is provided and the grey lines indicate the alignment of the maps using shared markers. The alignment of the genetic maps with the physical maps utilised the locations of markers published previously, as described in Chalmers *et al.* (2001, this issue).

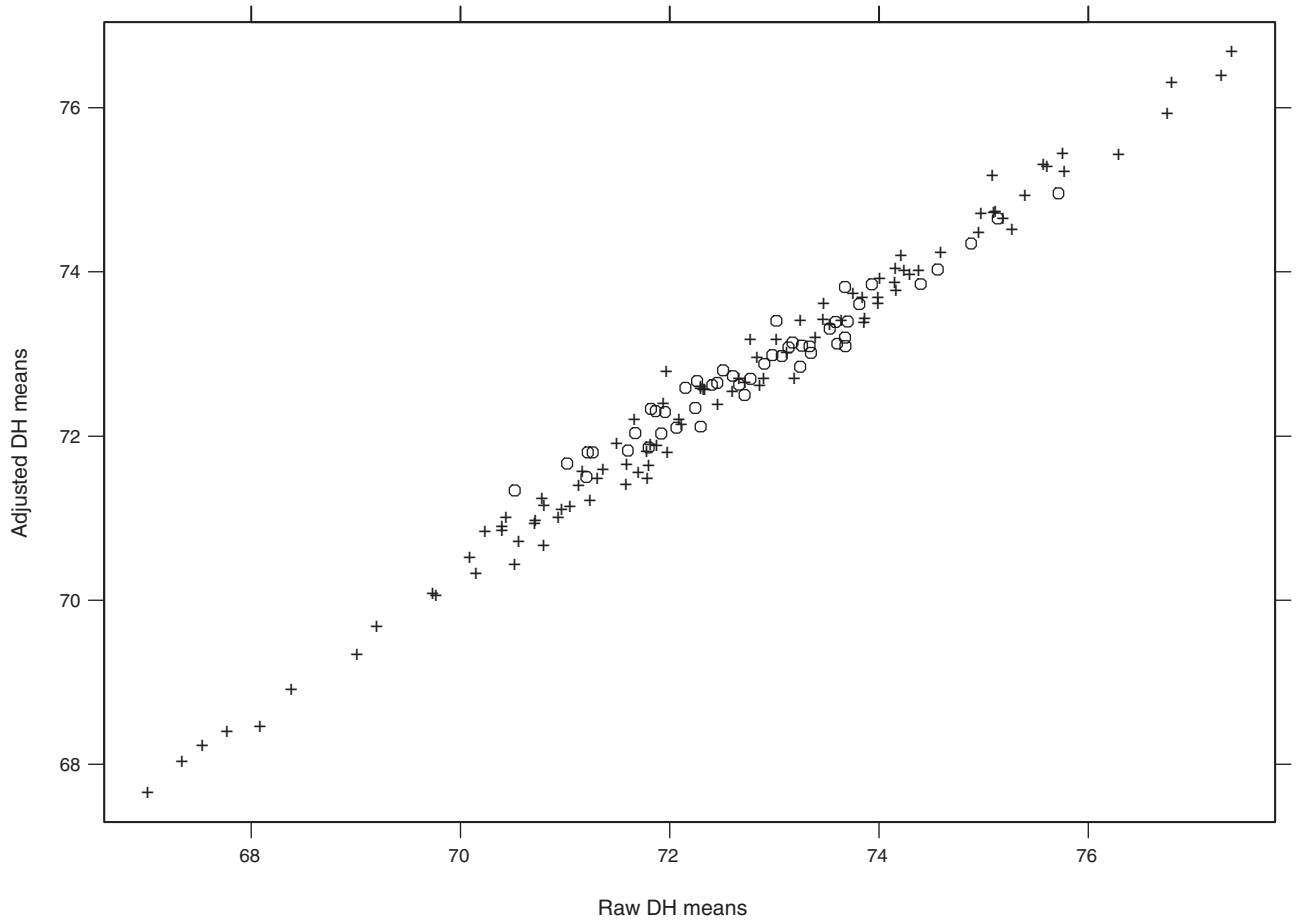

Fig. 5. DH means from CD87 × Katepwa milling yield data. The maturity of DH lines is indicated as '+' (mid) or '○' (quick).

model with 3 marker pairs. The regression coefficients, size of the effect, and location (distance from left flanking marker) of the associated QTLs are given in Table 7. Also given is the *P*-value for the significance of the QTL effect. In terms of the standard regression approach the 11 significant marker pairs from the pair-wise regressions were included together, leading to a final model with 4 marker pairs (Table 8).

Both the mixed modelling and standard mapping approaches have identified QTLs between the same pairs of markers on chromosomes 5B and 7D. The size and significance of the effect was over-estimated using the standard approach. Of greater concern, however, is that the two approaches have identified other QTLs in different locations. The standard approach has not detected the QTL on chromosome 3B, but has detected QTLs on 2B and 2D. Differences between the two approaches may have been anticipated on the basis of the difference between the adjusted and raw DH means and the unequal replication of DH lines (see Fig. 2). However, the apparently highly significant QTL on 2D requires further investigation. It appears to reflect effects associated with days of milling, a very significant source of variation in the Cran × Hal data, rather than a true QTL. The distribution of covariate values for the two markers across days of milling was very uneven (see Fig. 3). The ideal situation would be characterised by half the samples on any given day having a marker covariate value of '+1' and the other half '–1'. If this were the case then even in the presence of large day effects there would be no adjustment to the regression coefficients in the model. But with the uneven distribution (Fig. 3) the fitting of day effects has resulted in the disappearance of the QTL between these markers.

Figure 4 summarises the locations of QTL outputs for chromosome 5B from the mapping program Map Manager (Manly *et al.* 2000) and the mixed model analysis. This chromosome was chosen because it also shows a significant QTL in a second cross that was analysed (CD87 × Katepwa, see below). The QTL location data from Map Manager and the pair-wise regression step of the mixed modelling approach are shown with the respective significance of the QTLs along the chromosome.

CD87 × Katepwa cross

Due to practical considerations the quick (Q) and mid (M) maturing lines were grown in separate field trials. They were then milled separately in the laboratory, that is, on a

different set of days. We note that the splitting of the lines in both phases was not optimal for the purposes of mapping the combined set. In the analysis we accommodated the separation by fitting separate means, genetic variances, and field and laboratory error variances for each trial. This is analogous to the spatial analysis of a series of variety trials (Cullis *et al.* 1998). The estimated variance components from the IID model are shown in Table 5. The genetic variance for the M lines was far greater than for the Q lines. Field and laboratory variation contributed similar amounts to the total error in the Q line trial but in the M line trial, field error dominated. Note that data for 2 laboratory checks and one field sample in the M data set and one field sample in the Q data set were found to be erroneous so were omitted. In the Q line trial there were temporal trends in milling yield within a day and this was modelled using a separable correlation structure for $\boldsymbol{R}$, namely an ID×AR1 model (for days and samples within days). It was not possible to identify any field trend. In the M line trial there was field trend in the row direction that was modelled as for the Cran × Hal data set. There were also significant random effects associated with days of milling. The estimated means from the mixed model and the raw means show reasonable agreement (Fig. 5). The greater strength of agreement compared with the Cran × Hal data is due in part to the use of the laboratory designs.

We then considered mapping the milling yield data. The covariates were defined such that a value of '+1' indicated a CD87 marker type and '–1' a Katepwa marker type. The pair-wise regressions using the mixed modelling approach led to the selection of 3 pairs of markers for the multiple regression (Table 9). Standard pair-wise regressions applied to the raw means of the DH lines also led to the selection of 3 pairs of markers, but a different set than that obtained from the spatial modelling (Table 9). There was general agreement between the test statistics for the standard pair-wise regressions and the LRS from Map Manager (Manly *et al.* 2000). The results from the mixed modelling approach were quite different.

The regression coefficients, size of the effect, and location (distance from left flanking marker) of the associated QTLs from the multiple regressions for the mixed modelling and standard approaches are given in Tables 10 and 11. Also given is the *P*-value for the QTL significance. Since the QTL on chromosome 2D identified using the standard approach was not isolated, no size or position could be calculated.

Discussion

Quality trait data such as milling yield in wheat routinely exhibit variation associated with both the field and the laboratory. This variation must be accounted for at the design

Table 9. Marker pairs selected from pair-wise regressions for CD87 × Katepwa using mixed modelling and standard regression approaches: regression test statistics and LRS statistic from Map Manager

Critical value ($P = 0.01$) for all test statistics is 9.2 (significant values in bold type)

Chromo-some	Flanking markers		Modelling	Standard	Map Manager
	Left	Right			
1D	wmc432	P35.M395	**9.9**	2.0	1.5[A]
2D	BCD175	wmc025.1	5.1	**14.2**	**9.7**[A]
2D	wmc025.1	ABC.451	7.7	**17.7**	9.1[A]
5B	P41.M482	P34.M519	**9.2**	5.2	4.2
6B	P34.M503	P42.M501	**9.9**	**9.4**	8.7

[A] Map Manager included more than one pair in this interval: largest LRS given.

Table 10. QTLs identified in mixed modelling multiple regression approach to mapping CD87 × Katepwa milling yields

Chromo-some	Flanking markers		QTLs		
	Left/right	Regression coefficient	Distance[A]	Size of effect	*P*-value for effect
1D	wmc432	0.25	20.3	0.448	0.014
	P35.M395	0.15			
5B	P41.M482	–0.21	4.2	–0.347	0.010
	P34.M519	–0.14			
6B	P34.M503	0.08	9.3	0.368	0.005
	P42.M501	0.28			

[A] Distance is measured in cM from the left marker.

Table 11. QTLs identified in standard multiple regression approach to mapping CD87 × Katepwa mean milling yields

Chromo-some	Flanking markers Left/right	Regression coefficient	Distance[A]	QTLs Size of effect	*P*-value for effect
2D	BCD175	0.36			
	wmc025.1	0.30			
2D	wmc025.1	0.30			
	ABC.451	0.35			
6B	P34.M503	0.37	4.3	0.570	0.0004
	P42.M501	0.20			

[A] Distance is measured in cM from the left marker.

stage and in the subsequent statistical analysis. In this paper we have presented a mixed model analysis that accommodates potential sources of variation. The analysis was originally developed for the purpose of standard selection of lines with superior milling yield. We have extended the approach for the identification of isolated QTLs for the trait. A further, natural application is marker-assisted selection.

A difficulty with mapping and marker-assisted selection is the choice of markers to include in the regression model. Standard mapping programs avoid this problem since they only perform a sequence of pair-wise marker regressions. Many authors (e.g. Whittaker *et al.* 1996; Hackett *et al.* 2001) conduct QTL mapping using a multiple regression, that is, the simultaneous regression on a number of marker pairs. Usually the number of markers is far in excess of the number of lines so only a subset of marker covariates can be included at any one time. In this paper a sequence of pair-wise marker regressions was used as the first step for marker selection. Many other selection methods have been considered (e.g. Moreau *et al.* 1999) but the relative merits of the methods are unclear. Whittaker *et al.* (1996) pointed out that for QTL detection the selection of the 'best' subset of variables to include is a more complex problem than in the standard regression context. For example, in order to estimate QTL locations and effects it is not individual markers that are candidates for inclusion or exclusion from the model, but rather *pairs* of flanking markers.

In terms of experimental design there are a number of issues to be resolved. If standard selection is the aim, some progress should be possible since the focus of the design is the lines themselves. Research is required to provide efficient arrangements of the lines in the field and in the laboratory. Adequate replication is required in both phases. With better designs, that is, with the spatial field location and milling ordering of lines in some way balanced for field and laboratory trend, there should be much smaller adjustments to line mean yields than observed in the Cranbrook × Halberd data set, for example. If mapping or marker-assisted selection is the aim, the experimental design issues are more complex since the marker covariates would then need to be allocated in some optimal way.

The finding of a QTL for milling yield on chromosome 5B in both the Cranbrook × Halberd and CD87 × Katepwa crosses is of particular interest. The alignment of the two genetic maps with the physical map of chromosome 5B (Fig. 4) suggests that in both crosses the QTL is located in the distal region of the long arm. It is therefore possible that the same QTL is being assayed in the two crosses. The markers that flank this 5B-QTL are currently under further investigation, in order to validate the possibility that they define a region of the chromosome carrying a significant QTL for milling yield.

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