

Testing water-soluble carbohydrate QTL effects in perennial ryegrass (*Lolium perenne* L.) by marker selection

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Abstract Water-soluble carbohydrates (WSC) are an important factor determining the nutritional value of grass forage and development of genetic markers for selection of WSC traits in perennial ryegrass would benefit future breeding programmes. Quantitative trait loci (QTLs) for WSC have been published for an F₂ ryegrass mapping family. Markers showing significant associations with these QTLs were used to design narrow-based populations with homozygosity for target QTLs. Founders were selected from within the mapping family. The divergent populations produced were analysed for WSC content in the glasshouse and the field. There was evidence of complex interactions between WSC content and other factors and traits, including the scale of assessment, time/degree of sward establishment and other forage quality parameters. Differences between the divergent pairs of the various populations were small. However, differences observed between the founder selection groups were maintained and the roles of the QTL regions in regulating forage WSC content were confirmed. In general, the individual divergent populations exploited only a limited extent of the large phenotypic variation available within the mapping family. However, this study sets the scene for exploring the opportunities for marker-assisted breeding strategies for complex traits in obligate

out-breeding species, and the challenges of doing this are discussed.

Abbreviations

VIVO	Apparent digestibility of organic matter in vivo
ASH	Ash content
CWD	Cell-wall digestibility
DM	Dry matter
G × E	Genetic by environment
HPLC	High performance liquid chromatography
MAS	Marker-assisted selection
NIRS	Near infrared spectroscopy
NDF	Neutral detergent fibre
N	Nitrogen
QTL	Quantitative trait locus
OMD	Organic matter digestibility
WSC	Water-soluble carbohydrate

Introduction

Temperate grasslands support most of the world's cattle and sheep livestock systems. In the UK around 75% of feed requirements for these animals are presently obtained from grass and grass forage (Wilkins and Humphreys 2003). Perennial ryegrass (*Lolium perenne* L.) is the most widely used species (Burgon et al. 1997) and, as feed costs can make up as much as 35% of the total production cost (Nix 2009), the nutritional value of this fodder has a major impact on the efficiency and profitability of animal production systems. It is important that ruminant feeds provide the animal with an easily fermentable source of energy to the rumen (Miller et al. 2001) and carbohydrates generally provide this. However, the energy content of grass forages can sometimes be rather low for efficient fermentation in the

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rumen and Miller et al. (2001) showed experimentally that feeding grass cultivars containing a high water-soluble carbohydrate (WSC) content gave rise to increased milk and meat production. Furthermore, their study demonstrated that the high sugar content of these cultivars also reduced nitrogen partitioning into urine and thus can help to lower ammonia emissions to the environment.

Considerable genetic variation for WSC content exists within perennial ryegrass (Humphreys 1989a; Turner et al. 2001, 2002). Recently this variation has been characterised by quantitative trait locus (QTL) mapping in an F₂ mapping family which was created to segregate for WSC. Several regions of the genome were identified which are expected to contain genes or gene clusters controlling carbohydrate metabolism (Turner et al. 2006). Over recent years many authors have advocated the use of markers in plant breeding, predicting their use will improve both efficiency and precision (Bernardo and Yu 2007; Collard and Mackill 2008; Liu et al. 2003; Van Berloo and Stam 2001; Xu and Crouch 2008; Yonezawa and Ishii 2005). Although few results have been published, widespread use of markers has been employed in breeding programmes for major world crops, particularly by many commercial companies (de Oliveira et al. 2005; Eathington et al. 2007; Ribaut and Ragot 2007; Sugiura et al. 2004), either alone or in conjunction with phenotypic selection (Ender et al. 2008; Liang et al. 2004; Liu et al. 2006; Tar'an et al. 2003). WSC content, like many traits of agronomic importance such as yield, yield stability and adaptation to environment (Araus et al. 2008) is a complex and polygenic trait and this poses challenges for marker selection as many genes with small individual effects have to be tackled simultaneously. Nevertheless, some successes with markers associated with polygenes have been demonstrated (Colton et al. 2006; Knoll and Ejeta 2008; Rebetzke et al. 2008; Shen et al. 2001; Steele et al. 2006; Zhang et al. 2007). However, there appears to be little published evidence showing the transfer of QTLs for polygenic traits by marker selection through to subsequent generations in obligate out-breeding crops such as the temperate forage grasses (Humphreys et al. 2006). Marker-based crosses for a range of nutritional traits including WSC have been carried out in Italian ryegrass (VandeWalle et al. 2007), but the amount of variation explained by the WSC QTLs was rather low and no significant effects were observed in the selection populations. The significance of individual WSC QTL for ryegrass breeding therefore remains unknown. No candidate genes have currently been isolated from these WSC QTL regions, but markers associated with them can be identified and used for testing both QTL effects and marker selection.

The primary objective of the work reported here was to carry out a test-crossing programme to examine the effects of the individual perennial ryegrass WSC QTLs published

by Humphreys et al. (2003) and subsequently confirmed in Turner et al. (2006). Accordingly, markers were used to design individual narrow-based populations with contrasting homozygosity for each of four target QTL regions. Plant phenotype was not considered and the founders were selected on the basis of marker genotype alone. The narrow-based populations produced by the crosses were assessed in the glasshouse and in the field. No crosses were designed on the basis of combined QTL scores in this study but, following confirmation in 2006 of all the major QTL with stability across years (Turner et al. 2006), further work employing a QTL index is currently underway. A further objective was to assess the results from these experiments in the context of the development of marker-assisted breeding strategies for complex traits in obligate out-breeding species.

Materials and methods

Plant material

The founders used to create narrow-based populations with homozygosity for target QTLs were chosen from the WSC F₂ mapping family on the basis of genotypes for markers located in the vicinity of each of four genomic regions identified by QTL analysis on preliminary WSC data (Humphreys et al. 2003). All these regions were later confirmed by subsequent analysis of a full data set for three replicate years, although fructan and/or total WSC QTLs were not always detected (Turner et al. 2006). Only one further major QTL region (on chromosome 6) was subsequently detected by Turner et al. (2006). The QTL effects reported in the 2006 publication are summarised in Table 1. Each of the allele-specific QTL founder selections was based on markers linked to a single QTL. The markers used mostly defined broad genomic regions to ensure good coverage of the QTL region (Fig. 1) and the QTL effects within these regions are also shown in Table 1. The number of F₂ plants chosen differed because the strategy was to have as many founders per selection as possible. For clarity to the reader, the founder selections and narrow-based populations produced from them are named in the form QTL1-high and QTL1-low (for the example of the divergent selections on the chromosome 1 QTL region) as not all the 'high' alleles identified in the F₂ came from the same mapping family parent, i.e., 'a' was not always the high allele and 'b' was not always low. These high and low attributions were based on allele additive effects for total WSC whether or not a QTL for this trait had been declared in the QTL study (see Table 1). The number of founders plus the F₂ mapping family linkage phase and the mean carbohydrate content of the different founder groups (from measurements

Table 1 QTL-effect data for the WSC F₂ mapping family

Chromosome	Trait	LOD	Flanking markers	Additive effect	Variation explained (%)
Multiple QTL mapping results for LOD score, QTL flanking markers (2-LOD interval), additive effect and percentage variation explained (modified from Turner et al. 2006)					
1	Tiller base spring polymeric fructan	9.9	rv0659–rv0033	+28.81	21.0
	Tiller base autumn polymeric fructan	3.5	rv0913–rv1391	−4.53	8.2
	Tiller base autumn polymeric fructan	3.7	E42M3308–02ga1	+31.22	8.9
	Tiller base spring total WSC	7.0	rv0659–rv0033	+27.30	16.2
2	Leaf autumn glucose	8.6	R3349–Fp12.2	+15.67	16.1
	Leaf autumn sucrose	12.4	E39M4906–rv1154	+6.27	24.4
5	Tiller base spring glucose	6.5	GSY60.2–rv0814	−3.71	14.9
6	Leaf autumn glucose	6.1	B6104–rv0307	−2.65	14.7
	Leaf autumn fructose	5.1	B6104–rv0307	−2.90	12.4
	Leaf autumn oligofructan DP > 3	24.4	rv1423–E39M4908	−17.29	59.0
	Leaf spring polymeric fructan	7.21	RZ87–Alkinv.1/4	−17.88	16.3
	Leaf autumn polymeric fructan	22.5	CDO395–E39M4908	+42.75	43.2
	Leaf spring total WSC	5.81	RZ87–Alkinv.1/4	−24.74	15.0
	Leaf autumn total WSC	15.9	CDO395–E39M4908	+34.66	38.7
Interval mapping results for total WSC for the marker-selection regions whether or not a significant QTL was declared previously. The highest LOD score within the region, QTL flanking markers (1-LOD-fall interval if appropriate: the outer selection marker is in parentheses if the 1-LOD-fall interval extends beyond it), additive effect and percentage variation are explained					
1	Tiller base spring total WSC	2.58	(PGI)–(CDO580)	+17.13	6.7
	Tiller base autumn total WSC	0.54	Not appropriate	+6.46	1.3
2	Leaf spring total WSC	0.81	Not appropriate	+8.05	2.0
	Leaf autumn total WSC	1.75	E38M4704–(M15-185)	−12.12	4.6
5	Tiller base spring total WSC	1.93	(GSY60.2)–(CDO127)	−17.87	4.8
	Tiller base autumn total WSC	0.12	Not appropriate	−5.78	0.3
6	Leaf spring total WSC	3.07	RZ87–Alkinv.2/3	−18.46	8.0
	Leaf autumn total WSC	1.49	(RZ87)–M65-1	+10.71	3.9

Positive additive effect values indicate that the Aurora allele, the high-sugar parent, conferred the positive effect

made during the characterisation of the mapping family) are shown in Table 2.

Polycrosses of the plants in each of the allele-specific QTL selection groups were carried out in pollen-proof isolation houses during summer 2001 to generate seed of narrow-based populations with homozygosity for target QTLs for further evaluation. The crosses of the allele-specific selections for QTLs on chromosomes 1 and 2 generated sufficient seed for field testing within the framework of an EU-project. The NIMGRASS (EC FAIR CT98-4063) project investigated the development of molecular marker-aided selection for nitrogen-use efficiency and feeding value of forage grasses with the ultimate aim of developing systems of grassland management which decrease the nitrogen footprint of livestock production. Multiplication of selections for QTLs on chromosomes 5 and 6 were on a smaller scale and failed to produce sufficient seed for field plots. Therefore, all the polycrosses were repeated with the same founder groups in 2002 to enable assessment in small

field plots. Seed was harvested in July in both years and cleaned prior to sowing.

Seed from 2001 was germinated in trays in August 2001 and 20 individuals from each selection were grown on in Humax John Innes No3 with wetting agent. These plants were maintained in a frost-free, unlit glasshouse throughout the year in 15-cm diameter pots. The plants were renewed each year from a small group of tillers. Tiller base and leaf material were sampled from February 2002 to October 2004 for analysis of the individual components of WSC as in Turner et al. (2006). All material was immediately frozen in liquid nitrogen, stored at -80°C , freeze-dried and then finely chopped prior to extraction for WSC. Leaf material for DNA extraction was also frozen in liquid nitrogen and, if necessary, stored at -80°C until extraction.

Small 1-m² field plots were sown at a seed rate of 3 g/m² in a randomised block design with three replicates in August 2001 for the chromosomes 1 and 2 crosses and 2-m² field plots for all crosses were sown at the same seed rate in

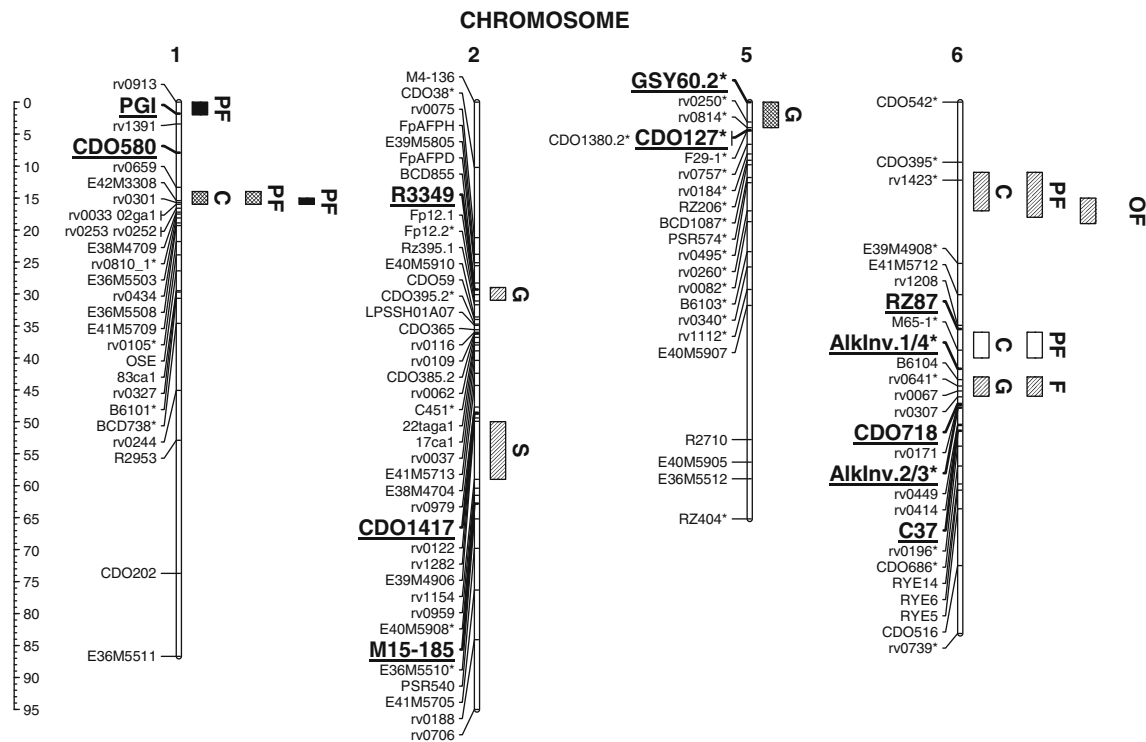


Fig. 1 Regions of the ryegrass genetic linkage map used to select founders for creating narrow-based populations with homozygosity for target QTLs, and their relationships to WSC QTLs. Markers used for selection are indicated in a *larger, bold and underlined font*. Chromosome designations conform to the Triticeae numbering. Asterisk indicates markers with a distorted segregation ratio ($P < 0.05$). Fruc-

tan and WSC QTL data are from Turner et al. (2006); QTL bars represent the 1 LOD-fall interval from MQM mapping. *Open bars* are leaf QTLs in the spring, *hatched bars* are leaf QTLs in the autumn, *cross-hatched bars* are tiller base QTLs in the spring and *filled bars* are tiller base QTLs in the autumn. *C* total WSC, *PF* polymeric fructan, *OF* oligomeric fructan DP > 3, *S* sucrose, *G* glucose, *F* fructose

August 2002. Plots were cut back and broad-leaved weeds controlled with a single spray at the end of September. Before the winter a further cut was carried out in October. The plots received 100 kg nitrogen (N) per ha in the sowing year. In all subsequent years they received 500 kg N per ha divided into eight applications; two in early spring and one after each cut. Cuts for analysis were taken from the plots at 5- to 6-week intervals from April to October for the following 3 years with a Haldrup plot harvester set at a cutting height of approximately 6 cm. The fresh weight of the full sample was measured in the field. A sub-sample of between 300 and 400 g fresh matter was dried overnight in an oven set at 80°C and dry matter (DM) yield calculated from %DM of the sub-sample (data not shown). Around half the dry sample was milled to pass through a 2-mm screen in a hammer mill and this was further sub-sampled for WSC analysis.

The selections concerning QTLs on chromosomes 1 and 2 were also assessed in larger plots in the multi-location NIMGRASS field trials. Four different populations were evaluated in these trials carried out by Zelder BV at Ottersum (Netherlands) and Aston Le Walls (UK), respectively, and by NPZ at Isle Poel in Northern Germany, as part of a

comparative study of 36 entries differing in nitrogen-use efficiency and forage quality. At each site the trials had a randomised block design with two N-levels, 400 kg/ha (high N) and 240 kg/ha (low N), respectively, three replicates per N-level and a plot area of 6 m². The plots were sown at a seed rate of approximately 3 g/m² in October 2001. In 2002, the trials were cut four times, except in Germany where there were three cuts. After cutting, fresh weight and DM content of the crop obtained from each plot were determined, DM yield calculated and sub-samples taken for measurement of forage quality characteristics.

Measurement of carbohydrate content and forage quality

Carbohydrates from plants in the glasshouse were extracted and analysed following the procedures of Turner et al. (2006). Individual sugars were separated and quantified by isocratic high performance liquid chromatography (HPLC). Total WSC was calculated by summing the content of individual sugars from HPLC analysis.

Total WSC content of the field samples from the small plots was measured by near infrared spectroscopy (NIRS). Samples of the dried and ground material were scanned at

Table 2 (I) F_2 mapping family linkage phase, number of plants, carbohydrate content and estimates of allele frequency for the allele-specific selection founder groups; (II) allele frequencies in the narrow-based populations produced by crossing the respective founder groups

I. Founder group	QTL1 high	QTL1 low	QTL2 high	QTL2 low	QTL5 high	QTL5 low	QTL6 high	QTL6 low
F_2 mapping family linkage phase	aa	bb	bb	aa	bb	aa	bb	aa
Number of plants	14	12	12	15	5	6	6	12
Polymeric fructan content								
Leaf spring	65.8 ± 8.1	54.7 ± 8.1	60.4 ± 8.9	63.3 ± 7.4	45.7 ± 6.0	59.2 ± 10.9	58.5 ± 16.8	25.7 ± 4.7
Leaf autumn	156.3 ± 11.2	172.0 ± 13.3	162.1 ± 14.7	144.1 ± 9.2	178.2 ± 32.3	131.0 ± 15.2	132.9 ± 17.5	161.4 ± 10.2
Tiller base spring	155.4 ± 7.1	125.3 ± 13.6	148.1 ± 6.6	116.4 ± 8.9	111.1 ± 9.1	128.8 ± 18.9	133.7 ± 15.6	120.4 ± 11.0
Tiller base autumn	293.6 ± 11.5	277.2 ± 10.8	280.4 ± 9.2	278.2 ± 13.7	289.0 ± 11.1	280.5 ± 25.0	272.7 ± 10.6	263.1 ± 7.4
Total WSC content								
Leaf spring	186.3 ± 14.6	186.2 ± 14.2	189.6 ± 14.8	183.6 ± 11.9	187.4 ± 16.0	174.3 ± 8.6	178.5 ± 19.8	150.8 ± 9.4
Leaf autumn	280.0 ± 9.7	276.7 ± 7.5	282.7 ± 11.3	270.1 ± 7.1	278.6 ± 20.8	258.7 ± 16.5	254.4 ± 14.8	276.6 ± 9.6
Tiller base spring	280.5 ± 10.1	262.5 ± 19.0	276.7 ± 10.7	236.1 ± 10.3	259.6 ± 13.3	231.0 ± 17.3	247.4 ± 13.5	243.5 ± 11.4
Tiller base autumn	336.4 ± 11.8	332.5 ± 11.7	330.1 ± 9.0	325.6 ± 14.3	342.4 ± 6.2	327.4 ± 24.1	325.5 ± 13.3	310.0 ± 6.4
Frequency of high allele (%)								
QTL1 cdo580	100	4	58	60	33	75	64	56
QTL2 M15-185	63	50	100	0	63	30	75	44
QTL5 rv0054	54	83	71	60	100	0	82	65
QTL6 rv0414	46	25	50	43	30	33	100	0
chr 6 rv1423	57	63	58	60	63	67	20	68
Mean frequency of high allele at non-selected QTL regions (%)	55	55	59	56	47	51	60	58
II. Population	QTL1 high	QTL1 low	QTL2 high	QTL2 low	QTL5 high	QTL5 low	QTL6 high	QTL6 low
Number of plants	20	20	20	20	20	20	20	20
Frequency high allele (%)								
QTL1 cdo580	90	15	39	74	45	86	58	55
QTL2 M15-185	95	15	100	0	47	19	61	34
QTL5 rv0054	45	71	75	32	94	0	68	71
QTL6 rv0414	68	30	25	64	37	31	90	0
chr 6 rv1423	45	53	89	68	61	83	26	58
Mean frequency of high allele at non-selected QTL regions (%)	64	42	57	60	48	55	53	55

The WSC data for leaves and tiller bases (mg g^{-1} dry matter) are means with standard errors and were calculated from the 3-year entry means of the data collected in the study of Turner et al. (2006). The frequency of the high QTL-allele was based on one QTL-specific PCR-based marker per QTL

2-nm intervals over the wavelength range from 1,100 to 2,498 nm in reflectance mode, using a NIRSystems 6500 spectrophotometer (FOSS UK, Warrington, UK). Data were collected using WINISI software (Infrasoft International, Port Matilda, USA) and spectra were stored as $\log 1/R$ where R is the diffuse reflectance. The calibration model used for the prediction of WSC was developed by modified partial least squares regression as described by Lister and Dhanoa (1998).

Several forage quality characteristics were measured on samples from the NIMGRASS field plots at DFE [Departement voor Fytotechnie en Ecofysiologie (CLO); now under 'services' at the Instituut voor Landbouw—en Visserijonderzoek (ILVO), Melle, Belgium]. These samples were obtained by pooling per N-level equal amounts of forage samples from each of the three replicates available per entry to reduce the amount of analytical work to be done. The quality traits were ash content (ASH), N content,

content of neutral detergent fibre (NDF), and WSC content all expressed as percentage of dry matter. Further the organic matter digestibility (OMD), apparent digestibility of organic matter in vivo (VIVO), and cell-wall digestibility (CWD) were determined. All quality traits were analysed by NIRS using calibration equations from DFE. In addition, some wet chemical analyses of a balanced subset of 144 out of about 2,500 samples were carried out at DFE and used to improve the NIRS predictions of the three digestibility traits.

DNA extraction and molecular marker analysis

DNA was extracted from the plant material using the QIAGEN DNEasy Plant Mini Kit (QIAGEN, Crawley, UK). Marker analysis methods were previously described by Armstead et al. (2002, 2004) and Turner et al. (2006).

Statistical analysis

All statistical analyses were carried out with the standard menu-driven procedures included in GenStat[®] for Windows[®], Version 11.1 (Payne et al. 2008). One-way analysis of variance (ANOVA) treating year as a random effect and entry as a fixed effect was used to partition genetic and environmental variation in the populations. Broad sense heritability [$H = V_g / (V_g + V_e/n)$, where V_g is the genetic variance, V_e is the error variance and n is the number of replications for each entry] was calculated from the ANOVA output. Leaf and tiller base tissues and the different QTL selections were analysed separately for the glasshouse experiments. Two-way ANOVA, treating population and season as fixed effects and year as a random effect, was used, and predicted means, F test probabilities (FPROB) and least significant differences (LSD) for interaction level means at the 5% level are presented. Years were analysed independently by one-way ANOVA (population as fixed effect and replicate as random effect) for the small-plot field data to enable comparison with the large-plots. Fertiliser application rates were analysed independently for the large-plot field data. Population was the fixed effect and location was used as the random effect in a one-way ANOVA.

Results

Production of the narrow-based populations and characterisation of genetic effects

The founders were chosen using RFLP scores collected early during the linkage mapping of the F_2 family. The founders as well as the QTL populations were checked with one high-throughput PCR-based marker for each target

QTL region (Table 2). Homozygosity for these markers was confirmed for all the founder groups except QTL1-low, where one plant must initially have been mis-genotyped. Allele frequency data for the SSR rv1423, a marker associated with the additional QTL on chromosome 6 not included when the founder groups were determined, is also shown. This QTL is in repulsion with the selected QTL6 genomic region: this is clearly evident from the allele frequencies of the QTL6 founder groups and it had consequences on the carbohydrate content of the founder groups. WSC content was elevated in all tissues of the QTL6-low group except for leaves in the spring, which was the trait producing the target QTL on chromosome 6 (Table 2).

The allele frequency data for the narrow-based QTL populations show some changes from the founder groups (Table 2). Homozygosity of the selected QTL in the high populations was only maintained in QTL2-high, but the frequency of the high allele nevertheless remained high in the other high populations. Shifts in the frequencies of alleles at non-selected QTL also occurred in some of the populations, notably for the QTL2 marker M15-185 in the QTL1 populations. However, the mean allele frequency for the non-selected QTL markers was fairly neutral for all the pairs of divergent founder groups and for all the pairs of QTL populations except for QTL1.

Genetic and environmental (year) variances were partitioned by ANOVA for two traits, total WSC and fructan content, using the data from the experimental material grown in the glasshouse (Table 3). Data for the mapping family are included for reference. Partitioning of genetic by environment ($G \times E$) effects was not possible, but these must be smaller than the residual mean squares which also include sampling and analysis errors and random plant to plant variation, so their order of magnitude can be inferred. In general, variation within the QTL populations as a whole was similar to that available from the mapping family and the data for the two sets of material follow the same pattern. Variation due to year effects was much larger than for genetic effects, but $G \times E$ interaction effects were relatively small. Consequently the estimates of the broad sense heritability of these traits were mostly moderate to high.

Populations grown under glasshouse conditions

Leaf WSC content results for the different divergent populations are shown in Table 4. Year was used in the replicate term during ANOVA to remove this variation from the population means [the effect of year was highly significant ($P < 0.001$) in all cases] but the range of the year means is indicated. Spring and autumn carbohydrate content were always significantly different ($P < 0.01$ or greater) except for oligofructan (DP > 3) in the QTL1 populations and glucose in the QTL2 populations. There were considerably

Table 3 Partitioning of genetic (plant) and environmental (year) variation by mean squares from analysis of variance (ANOVA) together with broad sense heritabilities for polymeric fructan- and total WSC content traits in the WSC F₂ mapping family (copied from Turner et al. 2006) and in the narrow-based populations from the glasshouse experiment

	Leaves				Tiller bases			
	Spring		Autumn		Spring		Autumn	
	WSC F ₂	QTL populations	WSC F ₂	QTL populations	WSC F ₂	QTL populations	WSC F ₂	QTL populations
Total WSC								
Year	626,340	526,739	1,514,758	156,870	131,565	162,053	255,284	759,928
Plant	6,639	3,429	4,475	3,605	7,058	11,453	5,672	9,151
Residual	4,064	2,432	2,412	1,514	3,653	5,554	5,063	4,665
Broad sense heritability	0.388	0.291	0.461	0.580	0.571	0.515	0.425	0.490
Polymeric fructan								
Year	516,465	11,973	1,412,383	228,911	100,324	44,664	175,660	267,975
Plant	2,695	2,844	6,908	4,653	5,465	8,305	6,103	8,707
Residual	1,496	1,894	2,088	1,581	2,942	3,565	4,250	3,294
Broad sense heritability	0.445	0.334	0.698	0.660	0.462	0.571	0.304	0.622

Data are for all plants in each case: 188 mapping family plants and 3 years; 160 plants from the populations combined and 3 years

fewer effects of population, but some interactions with season. Leaves of QTL1-high plants had significantly ($P < 0.001$) elevated polymeric fructan, higher sucrose and total WSC and lower oligofructan ($DP > 3$). In contrast, there were only small differences between the carbohydrate contents of the QTL2-high and low populations for all the measured WSC fractions. None were significant and there were no interactions with season. Although there were also few differences for the high and low populations for QTL5, there were some interactions with season. Leaf polymeric fructan and total WSC were higher in QTL6-high than in QTL6-low in the spring, but not in the autumn.

Tiller base oligofructan (DP3) and total WSC content were not significantly different in spring and autumn (Table 5), but the effect of season was highly significant ($P < 0.001$) for all the other carbohydrates measured in this study. There were only significant effects of population ($P < 0.05$ or greater) for the QTL5 and QTL6 divergent populations, and little evidence of interaction between population and season effects. The QTL5-high and QTL5-low populations showed good divergence for monosaccharide, disaccharide and total WSC contents of tiller bases in both spring and autumn. In some cases this was accompanied by changes in the opposite direction for the content of some oligo- or poly-fructans. The QTL6-high population also had lower tiller base fructan and WSC in both spring and autumn.

Populations grown in small field plots

The plot experiments integrated effects over larger numbers of plants per population and over the growing season as the

mini-swards contained many individuals of each population and samples were taken on six dates over the year. The chemical composition was measured on cut forage which will have been predominantly leaf blade but with some sheath (tiller base) material also present. These experiments showed many of the same trends in WSC content as the data for individual plants from the populations sampled at two times of the year (spring and autumn) in a glasshouse. The differences in WSC content between the divergent populations were all in the expected directions for the crosses carried out in 2001 (Table 6). In the first year these differences were small and were not significant. However, with further sward establishment the differences were greater in years 2 and 3, and were significant at $P < 0.01$. The QTL1 crosses carried out in 2002 did not repeat these effects although the differences in WSC content were in the expected direction. There was also little divergence in the early years between the QTL2 populations from 2002, but by year 3 a significant difference ($P < 0.05$) was observed. There were consistently larger effects shown by the QTL5 populations; the WSC content of the high populations was significantly higher ($P < 0.001$) over all the 3 years of the experiment. The QTL6 populations showed little divergence and no significant effects.

Populations grown in large field plots

The performance of the QTL populations in larger field plots is summarised in Table 7. The trials grew well and sward height was sufficient to enable reliable recovery of harvested material by machinery for 3–4 cuts in the first year after establishment. These yielded about 9.6 tonnes

Table 4 Leaf WSC content (mg g⁻¹ dry matter) in spring and autumn for the QTL populations grown under glasshouse conditions

QTL tested	WSC fraction	Population				Probability			LSD (0.05)
		Spring		Autumn		Population	Season	Interaction	
		QTL high	QTL low	QTL high	QTL low				
QTL1	Oligofructan DP3	22.1 (7–40)	20.8 (6–32)	9.8 (6–19)	5.8 (5–8)	NS	$P < 0.001$	NS	4.9
	Oligofructan DP > 3	9.9 (6–14)	22.3 (17–31)	11.8 (3–22)	20.1 (11–38)	$P < 0.001$	NS	NS	6.7
	Polymeric fructan	64.4 (60–79)	42.0 (28–50)	96.0 (74–130)	80.0 (59–117)	$P < 0.001$	$P < 0.001$	NS	16.5
	Sucrose	102.3 (67–118)	81.9 (62–94)	39.0 (25–67)	36.4 (23–63)	$P < 0.001$	$P < 0.001$	$P < 0.01$	9.1
	Glucose	16.9 (2–30)	16.7 (9–30)	13.7 (8–22)	13.8 (10–21)	NS	$P < 0.01$	NS	3.2
	Fructose	25.7 (18–34)	22.3 (14–33)	17.9 (12–28)	18.4 (11–27)	NS	$P < 0.001$	NS	3.4
	Total WSC	241.6 (169–273)	206.4 (150–253)	188.3 (164–222)	174.5 (146–210)	$P < 0.001$	$P < 0.001$	NS	17.6
QTL2	Oligofructan DP3	31.8 (7–36)	22.5 (8–31)	7.5 (5–12)	8.6 (6–13)	NS	$P < 0.001$	NS	5.0
	Oligofructan DP > 3	16.4 (7–22)	15.3 (10–20)	13.8 (5–27)	6.2 (4–10)	NS	$P < 0.01$	NS	6.2
	Polymeric fructan	71.0 (65–77)	68.6 (53–81)	93.2 (71–133)	101.4 (61–163)	NS	$P < 0.001$	NS	18.4
	Sucrose	106.6 (73–117)	104.2 (77–118)	42.4 (22–78)	42.9 (26–73)	NS	$P < 0.001$	NS	10.1
	Glucose	17.3 (3–28)	17.2 (5–27)	15.7 (8–28)	14.3 (7–25)	NS	NS	NS	
	Fructose	23.7 (16–31)	24.4 (14–32)	20.3 (13–34)	18.8 (13–29)	NS	$P < 0.001$	NS	3.5
	Total WSC	258.5 (187–295)	252.4 (187–293)	192.9 (169–228)	192.4 (165–241)	NS	$P < 0.001$	NS	18.4
QTL5	Oligofructan DP3	21.2 (7–32)	16.4 (7–22)	7.6 (6–10)	9.0 (7–12)	NS	$P < 0.001$	NS	4.7
	Oligofructan DP > 3	15.5 (14–20)	20.1 (4–34)	10.8 (8–17)	4.5 (0–13)	NS	$P < 0.001$	$P < 0.01$	6.2
	Polymeric fructan	52.6 (40–62)	63.0 (50–89)	111.1 (75–154)	123.5 (104–164)	NS	$P < 0.001$	NS	18.1
	Sucrose	98.2 (64–115)	99.2 (73–120)	38.3 (22–78)	48.3 (31–89)	NS	$P < 0.001$	NS	9.6
	Glucose	19.9 (6–32)	14.5 (9–21)	12.5 (8–20)	15.7 (10–27)	NS	$P < 0.01$	$P < 0.001$	3.2
	Fructose	23.6 (15–33)	19.5 (14–25)	16.5 (11–25)	19.2 (10–31)	NS	$P < 0.001$	$P < 0.05$	3.3
	Total WSC	231.2 (150–269)	232.9 (180–270)	196.9 (173–230)	220.1 (199–241)	NS	$P < 0.001$	NS	19.2
QTL6	Oligofructan DP3	17.4 (6–24)	13.8 (5–22)	7.6 (5–13)	7.9 (5–13)	NS	$P < 0.001$	NS	4.8
	Oligofructan DP > 3	19.5 (15–25)	28.5 (13–43)	16.5 (10–26)	11.2 (4–24)	$P < 0.05$	$P < 0.001$	$P < 0.05$	7.5
	Polymeric fructan	54.0 (38–84)	43.1 (23–73)	79.7 (47–125)	97.0 (70–139)	$P < 0.001$	$P < 0.001$	$P < 0.05$	17.4
	Sucrose	99.9 (70–114)	93.9 (62–107)	34.0 (22–57)	38.2 (25–65)	NS	$P < 0.001$	NS	8.9
	Glucose	20.6 (8–33)	19.8 (8–37)	13.3 (8–20)	12.6 (8–20)	NS	$P < 0.001$	NS	4.4
	Fructose	25.7 (16–35)	22.8 (14–33)	16.1 (11–26)	18.3 (13–27)	NS	$P < 0.001$	NS	3.3
	Total WSC	237.3 (163–274)	222.1 (143–285)	167.4 (144–216)	185.1 (164–225)	$P < 0.01$	$P < 0.001$	$P < 0.05$	17.3

Data are 3-year means for 20 plants from two-way ANOVA for population and season (spring and autumn) fixed effects. The ranges of the individual year means, rounded to whole integers, are given in parentheses. Probabilities of significant effects (Genstat FPROB values) below the 5% level are entered as non-significant (NS)

LSD least significant difference at the 5% level for comparing the population × season interaction level means presented

of dry matter per ha at 400 kg/N and 6.5 tonnes at 240 kg/N. These yield data include nearly all the forage produced in 2002; there was a little regrowth after the last cut but sward height was below the threshold for accurate mechanical harvesting. Site to site differences in dry matter yield were small and location was used in the replicate term for ANOVA. At both N-levels the trial showed a significant difference ($P < 0.05$) in DM yield between the QTL1-high and QTL1-low populations whereas QTL2-high and QTL2-low did not differ significantly. There were no significant differences in dry matter content and the fresh matter yield of the QTL1-high population was also mostly higher than for the QTL1-low population.

The two high populations (QTL1-high and QTL2-high) significantly ($P < 0.05$) outperformed their respective low populations (QTL1-low and QTL2-low) for nutritive value at both N levels, indicating that the divergent selections on both QTL regions under study had been successful (Table 7). Forage WSC content was significantly higher ($P < 0.05$) in both QTL1-high and QTL2-high. These populations generally had a lower N content and a higher true and apparent digestibility of organic matter and cell-wall digestibility. The NDF content, which represents the main component of structural carbohydrates, tended to be lower for the high WSC selections, although the differences between the contrasting populations were not statistically significant. There were no significant differences in ash content.

Table 5 Tiller base WSC content (mg g⁻¹ dry matter) in spring and autumn for the QTL populations grown under glasshouse conditions

QTL tested	WSC fraction	Population				Probability			LSD (0.05)
		Spring		Autumn		Population	Season	Interaction	
		QTL high	QTL low	QTL high	QTL low				
QTL1	Oligofructan DP3	7.9 (0–19)	6.3 (3–11)	5.7 (0–14)	6.2 (1–14)	NS	NS	NS	
	Oligofructan DP > 3	25.7 (3–45)	31.8 (4–53)	0.0 (0–0)	3.6 (0–9)	NS	<i>P</i> < 0.001	NS	8.9
	Polymeric fructan	180.2 (177–185)	168.4 (150–191)	271.0 (230–323)	256.0 (231–295)	NS	<i>P</i> < 0.001	NS	27.7
	Sucrose	52.3 (43–66)	55.3 (35–87)	26.6 (17–45)	22.1 (16–29)	NS	<i>P</i> < 0.001	NS	6.7
	Glucose	34.9 (16–61)	31.2 (18–53)	6.6 (6–9)	11.2 (9–16)	NS	<i>P</i> < 0.001	<i>P</i> < 0.05	5.2
	Fructose	32.9 (25–46)	28.9 (23–35)	11.0 (10–13)	14.5 (9–20)	NS	<i>P</i> < 0.001	<i>P</i> < 0.01	3.4
	Total WSC	331.2 (322–348)	322.0 (285–382)	321.7 (267–405)	315.0 (278–383)	NS	NS	NS	
QTL2	Oligofructan DP3	6.3 (2–12)	6.3 (0–11)	7.3 (1–20)	8.0 (2–20)	NS	NS	NS	
	Oligofructan DP > 3	27.2 (14–36)	27.6 (11–37)	0.0 (0–0)	0.10 (0–0)	NS	<i>P</i> < 0.001	NS	8.0
	Polymeric fructan	163.9 (145–189)	172.3 (152–183)	266.8 (225–322)	275.0 (251–317)	NS	<i>P</i> < 0.001	NS	23.9
	Sucrose	48.6 (38–55)	56.2 (55–60)	28.2 (17–46)	29.3 (19–46)	NS	<i>P</i> < 0.001	NS	6.8
	Glucose	28.2 (13–51)	31.8 (14–57)	10.6 (7–18)	12.5 (7–22)	NS	<i>P</i> < 0.001	NS	6.0
	Fructose	29.9 (22–42)	33.0 (23–47)	10.6 (10–11)	11.6 (10–15)	NS	<i>P</i> < 0.001	NS	3.6
	Total WSC	304.3 (262–361)	327.3 (289–364)	323.8 (266–417)	336.6 (296–417)	NS	NS	NS	
QTL5	Oligofructan DP3	5.2 (0–10)	9.6 (7–16)	6.1 (1–16)	5.3 (0–13)	NS	NS	NS	
	Oligofructan DP > 3	32.9 (7–47)	22.4 (5–35)	0.2 (0–1)	0.7 (0–1)	NS	<i>P</i> < 0.001	NS	8.0
	Polymeric fructan	165.6 (136–189)	178.8 (163–205)	290.8 (261–350)	277.6 (246–313)	NS	<i>P</i> < 0.001	NS	23.7
	Sucrose	56.8 (51–67)	45.4 (36–59)	29.3 (17–50)	21.0 (15–29)	<i>P</i> < 0.001	<i>P</i> < 0.001	NS	7.0
	Glucose	37.4 (19–65)	25.1 (12–48)	13.9 (8–25)	7.6 (6–10)	<i>P</i> < 0.001	<i>P</i> < 0.001	NS	5.0
	Fructose	33.5 (26–44)	22.8 (25–42)	11.4 (10–13)	12.9 (9–15)	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001	3.0
	Total WSC	331.5 (294–364)	304.4 (288–335)	351.8 (298–455)	325.1 (289–382)	<i>P</i> < 0.05	NS	NS	27.3
QTL6	Oligofructan DP3	7.7 (6–10)	9.8 (0–20)	9.2 (1–23)	6.6 (1–15)	NS	NS	NS	
	Oligofructan DP > 3	26.0 (11–53)	26.2 (2–47)	0.0 (0–0)	0.9 (0–2)	NS	<i>P</i> < 0.001	NS	8.3
	Polymeric fructan	147.6 (126–167)	173.7 (155–193)	259.2 (243–287)	289.1 (256–333)	<i>P</i> < 0.001	<i>P</i> < 0.001	NS	25.8
	Sucrose	57.5 (48–76)	53.4 (44–71)	23.6 (16–32)	22.6 (16–32)	NS	<i>P</i> < 0.001	NS	6.5
	Glucose	35.9 (17–67)	35.6 (19–65)	11.5 (6–21)	11.0 (10–14)	NS	<i>P</i> < 0.001	NS	5.8
	Fructose	32.8 (26–44)	31.2 (25–42)	11.3 (10–13)	11.6 (10–13)	NS	<i>P</i> < 0.001	NS	3.0
	Total WSC	307.9 (280–330)	330.3 (295–357)	315.4 (284–376)	342.0 (301–410)	<i>P</i> < 0.01	NS	NS	30.1

Data are 3-year means for 20 plants from two-way ANOVA for population and season (spring and autumn) fixed effects. The ranges of the individual year means, rounded to whole integers, are given in parentheses. Probabilities of significant effects (Genstat FPROB values) below the 5% level are entered as non-significant (NS)

LSD least significant difference at the 5% level for comparing the population × season interaction level means presented

Discussion

In general, differences between the pairs of divergent populations were small and not always significant although some QTL effects were more evident than others. However, in many cases the divergent populations did show overall phenotypic differences of the same order of magnitude as those found between their corresponding founder groups. The apparently contradictory results for the QTL6 populations can be explained by the second QTL region in repulsion phase on the chromosome outside the selection interval, identified after the founders for the study were chosen (Turner et al. 2006). The recombination frequency between these two QTL regions was of the order of just

2–4% in this study. Some other effects were not those that were expected. This is most likely to have resulted from uncontrolled segregation at the non-selected and other currently unknown QTL regions although genotype data suggested there were no major shifts, on average, in allele frequency for example markers at non-selected regions of the genome. The exception was for the QTL2 region of the genome in both the QTL1 populations. It is interesting that the *S* and *Z* loci of the ryegrass self-incompatibility system are located on chromosomes 1 and 2, respectively (Thorogood et al. 2002). However, although some allele combinations have been found to be favoured under some circumstances, the *S* and *Z* loci are generally thought to segregate independently and it is difficult to postulate a

Table 6 WSC content (% dry matter) of bulk forage from small plots

Population	%WSC		
	Year 1	Year 2	Year 3
2001 crosses			
QTL1 high	21.52	26.15	22.06
QTL1 low	19.94	23.62	19.66
QTL2 high	21.09	25.45	21.04
QTL2 low	20.12	22.91	18.30
Probability	NS	<0.001	<0.001
LSD ($P = 0.05$)		1.55	1.34
2002 crosses			
QTL1 high	24.10	21.86	20.85
QTL1 low	23.37	21.10	20.83
QTL2 high	23.17	20.81	22.33
QTL2 low	23.82	21.44	21.01
QTL5 high	25.49	21.51	21.84
QTL5 low	22.41	17.66	18.81
QTL6 high	23.00	20.36	20.39
QTL6 low	23.03	19.81	20.99
Probability	<0.001	<0.001	<0.001
LSD ($P = 0.05$)	1.32	1.39	1.26

Data are means for the six cuts between April and October, analysed by one-way ANOVA ($n = 3$). Probabilities of significant effects (Genstat FPROB values) below the 5% level are entered as non-significant (NS) LSD least significant difference for comparing population means at the 5% level

mechanism by which the incompatibility system might have produced the results seen in this study. The current results for the QTL2 marker M15-185 could be explained by over-representation of some QTL1 founder plants in the resultant populations, but inspection of genotype data for these founders shows that this would also have caused shifts in allelic frequency on chromosomes 5 and 6 which were not evident. The specific interaction in these crosses thus remains unclear without further work, but it is not expected that the distortion to the average high/low allelic balance across all the non-selected markers analysed would have resulted in a significant phenotypic effect compared to that for the QTL1 region under test.

This study has shown considerable evidence of relationships between WSC content and environmental factors and other traits. However, although high variance was associated with some environmental effects, like ‘year’ in the glasshouse experiments, $G \times E$ effects were mostly much smaller. In consequence means calculated across environments detected significant genetic effects and heritability values for the populations were moderate to high. In general, the genetic effects were easier to distinguish as the scale on which they were assessed was increased. The field plots integrated data over larger number of individual

plants than was possible in the glasshouse and this reduced the variance of the data and increased the detection of significant effects. The fact that the nature of the genetic effects was similar at all scales is of value from a plant breeding perspective. Much pasture plant breeding is done at the scale of individual plants but the ultimate assessment of plant performance is in a sward setting with the additional stress of competition. The closeness of the relationship between performance of individual plants in the glasshouse and swards in the small plots field plots can be examined by correlation analysis of top level total WSC means for the eight QTL populations. In fact there was no significant correlation with all eight populations ($r = 0.409$, $P = 0.157$), but inspection of the data indicated that this was due to one outlier as a result of the WSC value for the QTL5-low population in the glasshouse. Omitting this population resulted in a significant correlation ($r = 0.939$, $n = 7$, $P = 0.002$). It is not unusual for WSC content to be increased at lower N fertiliser rates and vice versa (Hoekstra et al. 2007). However, in the large plot field experiment, low N application also increased the magnitude of the divergence observed between the pairs of populations. The pots in the glasshouse and the small field plots all received high N, so conditions for detecting QTL effects in these experiments might have been less discriminating. In the field plots the differences also generally became more distinct over time as the swards established. The complexity of the relationship between forage WSC content and digestibility was also clearly illustrated. Although WSC is not the only determinant of digestibility it has been widely accepted that increased WSC leads to increased digestibility (Humphreys 1989b; Wilkins and Humphreys 2003). This was demonstrated by the QTL2 populations in this study, but not by those for QTL1. This work has again highlighted a number of aspects of the complexity of the regulation of WSC content and its relationships to other traits.

The QTL populations in this study maintained the divergence displayed by their founders, confirming the importance of the tested QTL regions in regulating WSC metabolism. This is in agreement with results for Italian ryegrass in which small effects were recovered from crosses based on small-effect QTLs (VandeWalle et al. 2007). The realisation of a greater proportion of the available variation within a diverse population would probably require controlled segregation at more than one of the main regulatory loci. Perennial ryegrass test crosses based on a marker summation index for five QTLs were more successful in recovering QTL effects for nitrogen-use efficiency (Dolstra et al. 2007). However, in order to favour its use, marker selection must have advantages over conventional methods. Studies where the rates of progress by different selection methods have been compared have frequently shown phenotypic selection to be extremely effective (Davies et al. 2006;

Table 7 Mean performance of four QTL populations at high and low N-levels in a multi-site trial held in 2002 in the UK, the Netherlands and Germany

Population	Trait									
	WSC (%)	N (%)	NDF (%)	ASH (%)	OMD (%)	VIVO (%)	CWD (%)	DMY (kg/m ²)	FMY (kg/m ²)	DM (%)
High N										
QTL1 high	15.30	2.21	53.54	9.50	82.79	74.12	70.95	1.095	5.397	21.2
QTL1 low	13.15	2.54	53.98	9.35	82.65	73.98	70.32	0.917	4.144	23.7
QTL2 high	14.70	2.43	53.28	9.31	83.29	74.64	71.05	0.865	3.987	23.5
QTL2 low	13.08	2.42	54.44	9.77	81.22	72.48	68.28	0.951	4.383	23.1
Probability	<0.001	<0.001	0.066	NS	0.002	0.002	0.002	0.020	0.028	NS
LSD ($P = 0.05$)	1.52	0.10	1.59		1.34	1.40	1.90	0.179	1.053	
Low N										
QTL1 high	19.13	1.97	51.90	8.72	83.48	74.83	70.92	0.772	3.282	24.7
QTL1 low	15.98	2.17	53.25	9.16	82.28	73.59	70.03	0.553	2.361	24.4
QTL2 high	18.15	2.08	51.97	8.73	83.34	74.69	70.70	0.632	2.622	25.3
QTL2 low	15.78	2.12	53.41	9.26	81.20	72.47	67.77	0.643	2.768	24.6
Probability	<0.001	<0.001	0.066	NS	0.002	0.002	0.002	0.020	0.028	NS
LSD ($P = 0.05$)	1.52	0.10	1.59		1.34	1.40	1.90	0.179	1.053	

The data were analysed by ANOVA and are means (per site/ N-level) over cuts for the seven forage quality traits; ash content (*ASH*), nitrogen content (*N*), neutral detergent fibre (*NDF*), water-soluble carbohydrate content (*WSC*), organic matter digestibility (*OMD*), apparent digestibility of organic matter in vivo (*VIVO*), cell-wall digestibility (*CWD*) and dry matter content (*DM%*). The yield characteristics, fresh matter yield (*FMY*) and dry matter yield (*DMY*), are means of summations over cuts. LSD is the least significant difference for comparing population means at the 5% level

Wilde et al. 2007). Currently, traditional phenotypic screening may well return the highest gains for complex traits like WSC content, yield and stress tolerance. In this context it is pertinent to consider the rates of gain which may be achievable by the two methods. Over recent years phenotypic selection methods have produced a 2% gain per year in the WSC content of perennial ryegrass varieties bred by IGER (Richard Hayes, personal communication; 5-year mean WSC content for new variety releases increased by 5.4% DM from 18.3% DM in 1983 to 23.7% DM in 1998, a relative gain of 29.5% over 15 years). Data from the current study that were generated under conditions most like those used in the IGER breeding programme were those for the high-N large plots in the NIMGRASS field trial. These data show an end-point mean gain/loss of 6–7% from the median WSC content of 14.12% DM. This is equivalent to three years of progress in the breeding programme, but is the maximum gain possible on these markers. No further gain is possible on an individual QTL location once complete homozygosity has been achieved. Continued progress then depends on the identification of new alleles of greater additive effect at existing QTL locations (much diversity is available within perennial ryegrass) or new QTL locations, although genomic selection (Heffner et al. 2009) might provide an effective alternative route. However, fixation of specific QTL by marker selection seems unlikely to be the best strategy for overall improve-

ment of WSC content, although it might be interesting to compare the relative rates of gain from marker selection using a QTL index or genomic selection with phenotypic selection from the same or similar populations.

Improvement of polygenic traits by marker selection remains a major challenge. Many genes with small individual effects have to be selected together. The literature contains various estimates of the number of QTL regions that it is feasible to select concurrently (Araus et al. 2008; Bernardo 2008). What is not in doubt is that the population size required for marker selection increases rapidly as more QTLs are added. This is complicated further by epistatic effects and interactions with environment (Babu et al. 2004). The problems only increase when outbreeding crop species are considered. Several authors have expressed the opinion that routine use of marker selection may not be feasible, especially if the trait is only one of several being concurrently selected for, because it is extremely difficult to control segregation/recombination across the genome (Bernardo 2008; Collins et al. 2008). This severely restricts the scope to concurrently synthesise the ideal genotype at many QTL locations. Additionally, it should not be forgotten that it may be necessary to maintain appropriate levels of heterozygosity in order to avoid inbreeding depression and to maximise heterotic effects. The use of an intense index-selection for gene fixation may produce rapid inbreeding. Strategies for the most effective use of markers

for population improvement in outbreeding crops have still to be developed. Further population genetic modelling is required to develop population improvement strategies both to create and to maintain optimum gene frequencies in gene pools whilst imposing optimum selection pressures. In the immediate future markers are unlikely to provide a means of reducing the time-scale for development of new varieties in these crops.

In conclusion, this study has confirmed the role of the QTL regions identified by Turner et al. (2006) in the regulation of forage WSC content in perennial ryegrass, and demonstrated the truly polygenic nature of the trait. It has also indicated some of the challenges and opportunities ahead for the use of marker selection in breeding programmes handling complex traits in out-breeding crops. The rate of uptake of markers for selection purposes in out-breeding crops will depend, on a case by case basis, on the trait of interest and the ease and cost of phenotypic screening. In many cases further detailed, long-term efforts to examine the nature of complex traits at both physiological and molecular levels are needed in order to develop markers that are effective and to exploit their full potential. However, in our opinion, there are a number of cogent reasons to continue such work on developing marker strategies for some applications in these crops. These include their use for improving the efficiency of selection for traits which are particularly difficult or expensive to measure, where the positive allele is recessive, or which show strong genotype \times environment or genotype \times developmental-stage effects.

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References

- Araus JL, Slafer GA, Royo C, Serret MD (2008) Breeding for yield potential and stress adaptation in cereals. *Crit Rev Plant Sci* 27:377–412
- Armstead IP, Turner LB, King IP, Cairns AJ, Humphreys MO (2002) Comparison and integration of genetic maps generated from F2 and BC1- type mapping populations in perennial ryegrass. *Plant Breed* 121:501–507
- Armstead IP, Turner LB, Farrell M, Skøt L, Gomez P, Montoya T, Donnison IS, King IP, Humphreys MO (2004) Synteny between a major heading-date QTL in perennial ryegrass (*Lolium perenne* L.) and the Hd-3 heading date locus in rice. *Theor Appl Genet* 108:822–828
- Babu R, Nair SK, Prasanna BM, Gupta HS (2004) Integrating marker-assisted selection in crop breeding—prospects and challenges. *Curr Sci India* 87:607–619
- Bernardo R (2008) Molecular markers and selection for complex traits in plants: learning from the last 20 years. *Crop Sci* 48:1649–1664
- Bernardo R, Yu J (2007) Prospects for genome-wide selection for quantitative traits in maize. *Crop Sci* 47:1082–1090
- Burgen A, Bondesen OB, Verburgt WH, Hall AG, Bark NS, Robinson M, Timm G (1997) The forage seed trade. In: Fairey DT, Hampton JG (eds) Forage seed production. 1. Temperate species. CAB International, Wallingford, pp 271–286
- Collard BCY, Mackill DJ (2008) Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philos Trans R Soc B* 363:557–572
- Collins NC, Tardieu F, Tuberosa R (2008) Quantitative trait loci and crop performance under abiotic stress: where do we stand? *Plant Physiol* 147:469–486
- Colton LM, Groza HI, Wielgus SM, Jiang J (2006) Marker-assisted selection for the broad-spectrum potato late blight resistance conferred by gene RB derived from a wild potato species. *Crop Sci* 46:589–594
- Davies J, Berzonsky WA, Leach GD (2006) A comparison of marker-assisted and phenotypic selection for high grain protein content in spring wheat. *Euphytica* 152:117–134
- de Oliveira EJ, Alzate-Marin AL, Borem A, de Azeredo Fagundes S, de Barros EG, Moreira MA (2005) Molecular marker-assisted selection for development of common bean lines resistant to angular leaf spot. *Plant Breed* 124:572–575
- Dolstra O, Denneboom C, de Vos ALF, van Loo EN (2007) Marker-assisted selection for improving quantitative traits of forage crops. In: Guimarães EP, Ruane J, Scherf BD, Sonnino A, Dargie JD (eds) Marker-assisted selection: current status, future perspectives in crops, livestock, forestry, fish. FAO, Rome, pp 59–65
- Eathington SR, Crosbie TM, Edwards MD, Reiter RS, Bull JK (2007) Molecular markers in a commercial breeding program. *Crop Sci* 47(S3):S154–S163
- Ender M, Terpestra K, Kelly JD (2008) Marker-assisted selection for white mold resistance in common bean. *Mol Breed* 21:149–157
- Heffner EL, Sorrells ME, Jannink J-L (2009) Genomic selection for crop improvement. *Crop Sci* 49:1–12
- Hoekstra NJ, Schulte RPO, Struik PC, Lantinga EA (2007) Pathways to improving the N efficiency of grazing bovines. *Eur J Agron* 26:363–374
- Humphreys MO (1989a) Water-soluble carbohydrates in perennial ryegrass breeding. I. Genetic differences among cultivars and hybrid progeny grown as spaced plants. *Grass Forage Sci* 44:231–236
- Humphreys MO (1989b) Water-soluble carbohydrates in perennial ryegrass breeding. III. Relationships with herbage production, digestibility and crude protein content. *Grass Forage Sci* 44:423–430
- Humphreys M, Turner L, Skøt L, Humphreys M, King I, Armstead I, Wilkins P (2003) The use of genetic markers in grass breeding. *Czech J Genet Plant Breeding* 39 (Special issue). Biodiversity and Genetic Resources as the Bases for Future Breeding. In: Proceedings 25th EUCARPIA Fodder Crops and Amenity Grasses Section Meeting, Brno, Czech Republic, 1–4 September 2003, pp 112–119
- Humphreys MW, Yadav RS, Cairns AJ, Turner LB, Humphreys J, Skøt L (2006) A changing climate for grassland research. *New Phytol* 169:9–26
- Knoll J, Ejeta G (2008) Marker-assisted selection for early-season cold tolerance in sorghum: QTL validation across populations and environments. *Theor Appl Genet* 116:541–553
- Liang F, Deng Q, Wang Y, Xiong Y, Jin D, Li J, Wang B (2004) Molecular marker-assisted selection for yield-enhancing genes in the progeny of “9311 \times *O. rufipogon*” using SSR. *Euphytica* 139:159–165
- Lister SJ, Dhanoa MS (1998) Comparison of calibration models for the prediction of forage quality traits using near infrared spectroscopy. *J Agric Sci* 131:241–242

- Liu P, Zhu J, Lou X, Lu Y (2003) A method for marker-assisted selection based on QTLs with epistatic effects. *Genetica* 119:75–86
- Liu Q-Q, Li Q-F, Cai X-L, Wang H-M, Tang S-Z, Yu H-X, Wang Z-Y, Gu M-H (2006) Molecular marker-assisted selection for improved cooking and eating quality of two elite parents of hybrid rice. *Crop Sci* 46:2354–2360
- Miller LA, Moorby JM, Davies DR, Humphreys MO, Scollan ND, Macrae JC, Theodorou MK (2001) Increased concentration of water-soluble carbohydrate in perennial ryegrass (*Lolium perenne* L.). Milk production from late-lactation dairy cows. *Grass Forage Sci* 56:383–394
- Nix J (2009) Farm management pocketbook, 40th edn (for 2010). Imperial College London, Wye Campus. ISBN: 9780954120184
- Payne RW, Murray DM, Harding SA, Baird DB, Soutar DM (2008) GenStat® for Windows®, introduction, 11th edn. VSN International, Hemel Hempstead
- Rebetzke GJ, van Herwaarden AF, Jenkins C, Weiss M, Lewis D, Ruuska S, Tabe L, Fettell NA, Richards RA (2008) Quantitative trait loci for water-soluble carbohydrates and associations with agronomic traits in wheat. *Austr J Agric Res* 59:891–905
- Ribaut J-M, Ragot M (2007) Marker-assisted selection to improve drought adaptation in maize: the backcross approach, perspectives limitations, and alternatives. *J Exp Bot* 58:351–360
- Shen L, Courtois B, McNally KL, Robin S, Li Z (2001) Evaluation of near-isogenic lines of rice introgressed with QTLs for root depth through marker-aided selection. *Theor Appl Genet* 103:75–83
- Steele KA, Price AH, Shashidhar HE, Witcombe JR (2006) Marker-assisted selection to introgress rice QTLs controlling root traits into an Indian upland rice variety. *Theor Appl Genet* 112:208–221
- Sugiura N, Tsuji T, Fujii K, Kato T, Saka N, Touyama T, Hayano-Saito Y, Izawa T (2004) Molecular marker-assisted selection in a recurrent backcross breeding for the incorporation of resistance to rice stripe virus and panicle blast in rice (*Oryza sativa* L.). *Breed Res* 6:143–148
- Tar'an B, Michaels TE, Pauls KP (2003) Marker-assisted selection for complex trait in common bean (*Phaseolus vulgaris* L.) using QTL-based index. *Euphytica* 130:423–432
- Thorogood D, Kaiser WJ, Jones JG, Armstead I (2002) Self-incompatibility in ryegrass 12. Genotyping and mapping of the S and Z loci of *Lolium perenne* L. *Heredity* 88:385–390
- Turner LB, Humphreys MO, Cairns AJ, Pollock CJ (2001) Comparison of growth and carbohydrate accumulation in seedlings of two varieties of *Lolium perenne*. *J Plant Physiol* 158:891–897
- Turner LB, Humphreys MO, Cairns AJ, Pollock CJ (2002) Carbon assimilation and partitioning into non-structural carbohydrate in contrasting varieties of *Lolium perenne*. *J Plant Physiol* 159:257–263
- Turner LB, Cairns AJ, Armstead IP, Ashton J, Skøt K, Whittaker D, Humphreys MO (2006) Dissecting the regulation of fructan metabolism in perennial ryegrass (*Lolium perenne*) with quantitative trait locus mapping. *New Phytol* 169:45–58
- Van Berloo R, Stam P (2001) Simultaneous marker-assisted selection for multiple traits in autogamous crops. *Theor Appl Genet* 102:1107–1112
- VandeWalle M, Calsyn E, Baert J, de Riek J, van Bockstaele E (2007) Application of marker assisted selection for yield and nutritional traits in Italian ryegrass. Breeding and seed production for conventional and organic agriculture. In: Proceedings of the XXVI meeting of the EUCARPIA fodder crops and amenity grasses section, XVI meeting of the EUCARPIA *Medicago* spp. group, Perugia, Italy, pp 312–315
- Wilde F, Korzun V, Ebmeyer E, Geiger HH, Miedaner T (2007) Comparison of phenotypic and marker-based selection for *Fusarium* head blight resistance and DON content in spring wheat. *Mol Breed* 19:357–370
- Wilkins PW, Humphreys MO (2003) Progress in breeding forage grasses for temperate agriculture. *J Agric Sci* 140:129–150
- Xu Y, Crouch JH (2008) Marker-assisted selection in plant breeding: from publication to practice. *Crop Sci* 48:391–407
- Yonezawa K, Ishii T (2005) Marker-based selection as a tool for enhancing the efficiency of two conventional breeding methods of self-fertilizing crop plants, the generation-accelerated bulk breeding and doubled haploid breeding. *Breed Sci* 55:397–407
- Zhang L-H, Mojtahedi H, Kuang H, Baker B, Brown CR (2007) Marker-assisted selection of Columbia root-knot nematode resistance introgressed from *Solanum bulbocastanum*. *Crop Sci* 47:2021–2026