Euphytica (2021) 217:77
https://doi.org/10.1007/s10681-021-02811-1

Genotyping by Multiplexed Sequencing (GMS) protocol in Barley

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Received: 21 October 2020 / Accepted: 19 March 2021 / Published online: 1 April 2021

Abstract Genotyping by sequencing (GBS) and single nucleotide polymorphism (SNP) chip technologies are the primary SNP genotyping technologies used today. However, these genotyping technologies have some drawbacks that limit their usefulness in analysis. We have developed a robust protocol called genotyping by multiplexed sequencing (GMS) using SNP markers, providing informative genotypic data with greater flexibility. The genotypes derived from direct sequence reads reduce ambiguity in genetic analysis. The advantages of this protocol include: (1) This PCR-based direct sequencing protocol generates information from markers of interest and provides a more streamlined and accurate analysis process, by multiplexing hundreds of informative markers into a single sequencing run. (2) The marker sets are easily customized to the species of interest and can readily be changed. In this study we have taken the GMS protocol developed in wheat and adapted it to barley. We have identified 577 SNP markers that work well using this protocol providing adequate genome coverage for genomic selection and tag 267 QTL's for genes of interest. Good markers have an adequate read

Supplementary information The online version contains supplementary material available at (https://doi.org/10.1007/s10681-021-02811-1)

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depth of at least 5 amplicons and are reliably present across the population.

Keywords Barley · Multiplex · Sequencing · SNP · Single Nucleotide Polymorphism · MAS · Marker Assisted Selection · Genomic Selection · QTL Analysis · Genome Wide Association Study

Introduction

Barley ranks a distant 4th in grain production worldwide with a total of 140.6 metric tons annually. The United States produced approximately 169.6 million bushels of barley at an estimated price of 761.2 million dollars in 2019 (USDA NASS). Many barley traits contribute to malting quality, as well as food and feed quality. Here we describe a more efficient and adaptable protocol for tracking important barley traits for marker-assisted selection (MAS). Marker-assisted selection provides opportunities for enhancing the response from selection because molecular markers can be applied in early generations, with high precision and reductions in cost (Miedaner and Korzun 2012). Marker assisted selection (MAS) has been an effective tool for ensuring the transfer of quantitative trait loci (QTL) with high effects into elite barley lines. Genotyping by Multiplexed Sequencing (GMS) is a highly adaptable and effective genotyping tool for tracking markers of interest with high accuracy, which can translate to a more efficient and affordable MAS process in future breeding projects. Multiplexing on a small scale has been demonstrated by (Tamang et al. 2019; Poudel et al. 2018; Richards et al. 2016). The primer pools described therein have low marker complexity with approximately 12 markers per pool. The protocol presented here provides an avenue to multiplex more than 300 markers in a single pool.

Many marker systems have been developed over the years in order to detect genetic differences among DNA samples and SNPs have become the most useful in high throughput next generation sequencing (NGS). SNPs are the most commonly used marker in genetic studies because they represent the greatest source of genetic variability in eukaryotic organisms (Agarwal, Shrivastava and Padh 2008; Brookes 1999; Bybee et al. 2011; Campbell et al. 2015; Liu 2015; Onda et al. 2018; Voss-Fels and Snowdon 2014; Ruff et al. 2020). SNPs occur throughout the genome in both genic and non-genic regions, occurring more often in non-genic regions with no phenotypic correlation (Agarwal et al. 2008). SNPs found in genic regions are invaluable to breeders and researchers alike, because they may directly impact an organism's phenotype (Allen, et al. 2011; Chan 2009) (Ruff et al. 2020). SNPs are generally bi-allelic and have a low mutation rate making them ideal for mapping, genomic studies, and MAS (Syvanen 2001; Batley and Edwards 2007; He et al. 2009; Liu et al. 2014; Poland et al. 2012; Zhao et al. 2011; Bernardo et al. 2015). This protocol includes 267 SNP markers with known QTL associations (Table 2).

GBS and SNP chip technologies are currently the most popular protocols for SNP genotyping to date. However, both technologies have their problems. GBS results can be arduous to analyze if the whole genome is not known, because it produces random DNA sequences within the genome. The random selection and amplification of DNA sequences through the GBS process results in missing data between samples. Therefore, imputation is required, reducing the accuracy of GBS. Currently the 50 k Illumina Infinium iSelect chip is the primary chip used for genotyping in barley; however, a 9 k chip preceded the 50 k chip and was incorporated into the 50 k chip during its development (Bayer et al. 2017). The majority of SNP's on the 50 k iSelect chip have been derived from variants called in exome capture data of a wide range of European barley germplasm (Bayer et al. 2017). SNP chip technology is based on a hybridization technique which results in a dot plot that may misrepresent true allelic states, particularly at heterozygous loci. Also, once the SNP chip is developed researchers cannot easily add new markers, making adaptation and customization impossible. This is not the case for the GMS protocol which makes adding and customizing the marker pool less difficult. In barley, the GMS protocol produces greater genetic accuracy in the event of a false heterozygote call at a locus which has a paralogue in the genome. The GMS protocol is designed to be used in genomic selection and sequencing may be sequenced on either the Illumina or Ion Torrent NGS platforms (Ruff et al. 2020).

Materials and methods

Barley markers for this project were selected due to informative nature and chromosome coverage for use in genomic selection. They were derived from markers developed with the 50 k barley SNP chip (Bayer et al. 2017). To ensure performance when multiplexing, five hundred markers were selected for each of three pools for a total of 1500 barley markers. Primers were then designed from sequence data using Sequenom MassArray Design 4.0 (Sequenom, San Diego, CA.), which reduces the chance of primer dimers and standardizes the annealing temperature. These three marker pools were then multiplexed on 25 lines of the Oregon Wolfe Barley (OWB) bi-parental population. The OWB population was developed by Dr. Bob Wolfe by systematically crossing recessive alleles into one parent and dominant alleles into the other. The population consists of 175 barley doubled haploid progeny (Costa et al. 2001). Figure 1 provides an overview of the PCR based, direct sequence read GMS protocol. Due to duplicate markers with different but synonymous names the total resulting markers in all pools were reduced to 830 unique markers. To ensure data quality duplicate samples were genotyped to confirm output calls. The majority of cleaning is completed in the analysis pipeline which includes throwing out calls with inadequate read depth below 5 amplicons per sample. The protocol used here has been adapted to barley from the GMS protocol used in wheat and published earlier in 2020 (Ruff et al. 2020).

PCR 1

The first PCR produces the target amplicons using a 10 µl reaction. Locus-specific forward primers have an M13 tail attached to the 5′ end. Reagents include 2.35 µl H_2O, 1 µl 10 × MCLAB Taq PCR Buffer with 20 mM $MgCl_2$ (MCLAB, San Francisco, CA.), 0.45 µl $MgCl_2$ at 1.125 mM, 1 µl dNTP's at 100 mM, 1 µl primer pool at 125 nM, 4 µl sample DNA at 6.6 ng/µl, and 0.2 µl HOTaq DNA Polymerase (MCLAB, San Francisco, CA.) at 5 unit/µl. Thermocycler conditions were: 10 min at 94 °C for initial denaturation, then cycle 35 times at 94 °C denaturation for 20 sec, 56 °C annealing for 2 min, and 68 °C extension for 30 sec; followed by final 3 min elongation at 72 °C.

PCR 2

PCR 1 amplicons are diluted 1:1 and used as template DNA in the second PCR. PCR 2 adds a unique barcode and standard Ion A Adapter to the M13 tail of the forward primer, which then becomes the forward primer in successive amplicons. Barcodes are used for sample identification and the standard Ion A Adapter is used for attachment to the Ion Proton chip for sequencing. Reagents of PCR 2 include 0.875 µl H_2O, 0.5 µl 10 × MCLAB Taq PCR Buffer with 20 mM $MgCl_2$ (MCLAB, San Francisco, CA.), 0.2 µl MgCl2 at 25 nM, 0.025 µl dNTP's at 100 mM, 0.2 µl P1B Primers at 10 µM, 2 µl unique barcoded adapters at 2 µM, 2 µl diluted PCR #1 product, and 0.2 µl HOTaq DNA Polymerase (MCLAB, San Francisco, CA.) at 5 unit/ µl. Thermocycler conditions: 94 °C 10 min for initial denaturation, then cycle 15 times at 94 °C denaturation for 20 sec, 60 °C annealing for 30 s, and 72 °C extension for 1 min, followed by final 3 min elongation at 72 °C.

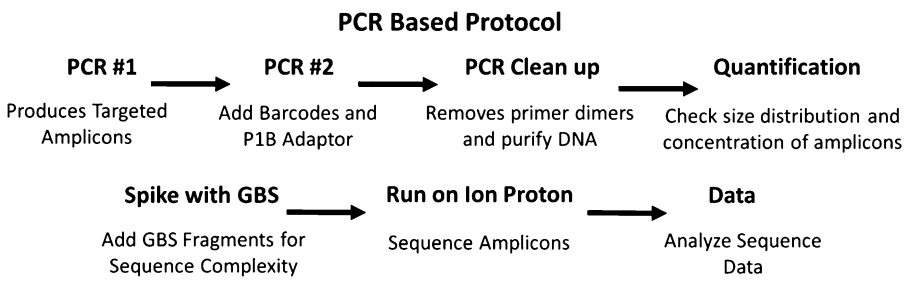
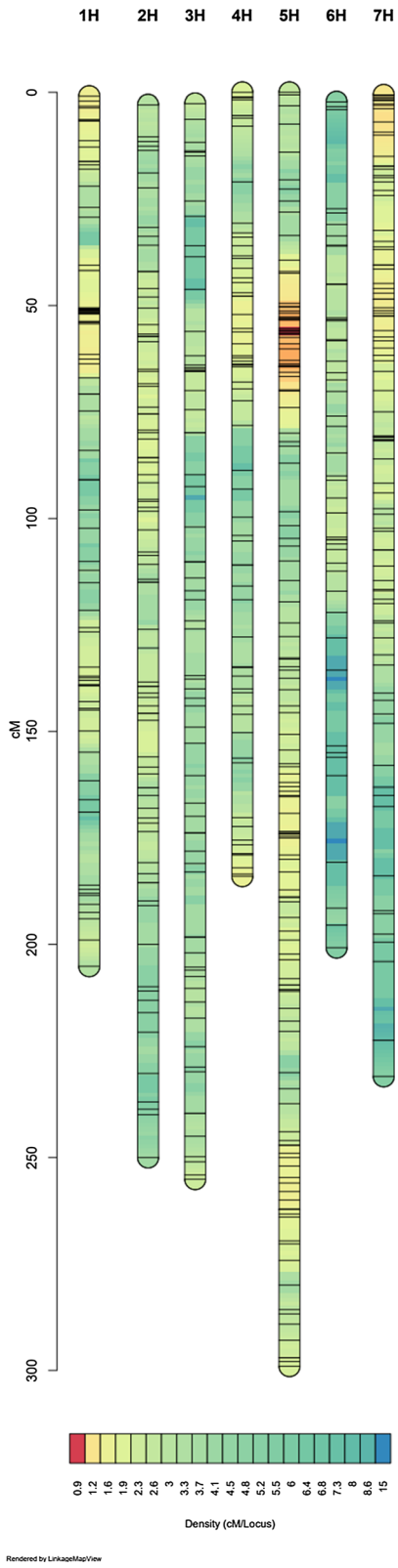


Fig. 1 Summary of the GMS process



◀**Fig. 2** Heat map of marker coverage across all 7 barley chromosomes

Table 1 Average cM distance between markers, largest cM gap, and total markers across all 7 barley chromosomes

	Avg. cM	Max cM	> 10 cM	Tags
1H	2.69	17.22	2	77
2H	2.98	10.97	2	85
3H	3.83	15.35	3	67
4H	2.83	13.09	3	66
5H	2.12	11.42	1	143
6H	4.23	23.33	4	48
7H	2.56	18.54	2	91
Avg	3.03	15.70	2.43	82.43

PCR clean up and amplicon quantification

After PCR 2 approximately 6 µl are taken from each well of the plate and combined into a single 1.5 mL Eppendorf tube. The final combined PCR product is purified using the QIAquick PCR Purification Kit (Qiagen Hilden Germany). The PCR product is further purified using Agencourt AMPure XP beads (BeckmanCoulter, Indianapolis, IN) using 0.8 µl of beads per 1 µl of PCR product. Then size selection is done with 4% E-Gel SizeSelect Gel (Life Technologies, Carlsbad, CA), selecting for products between 144 and 233 bp. further gel purification is performed with a QIAquick Gel Extraction Kit (Qiagen) resulting in an elution at 30 µl EB. The final size selection is accomplished on a 2% E-Gel Size Select Gel (Life Technologies, Carlsbad, CA) targeting products between 140 and 250 bp and followed by a final purification with the QIAquick PCR Purification Kit. The purified and size-selected product was quantified using Qubit dsDNA HS assay kit (Life Technologies, Carlsbad, CA), which provided an initial DNA concentration. The final quantification is performed with the Agilent DNA 7500 Kit (Agilent, Santa Clara, CA), which allows for the final dilution to a minimum concentration of 80 pmol/L and have amplicon sizes between 185 and 260 bp. The GMS product is spiked with a compatible and diverse NGS library for sequence complexity. The GMS library must then be

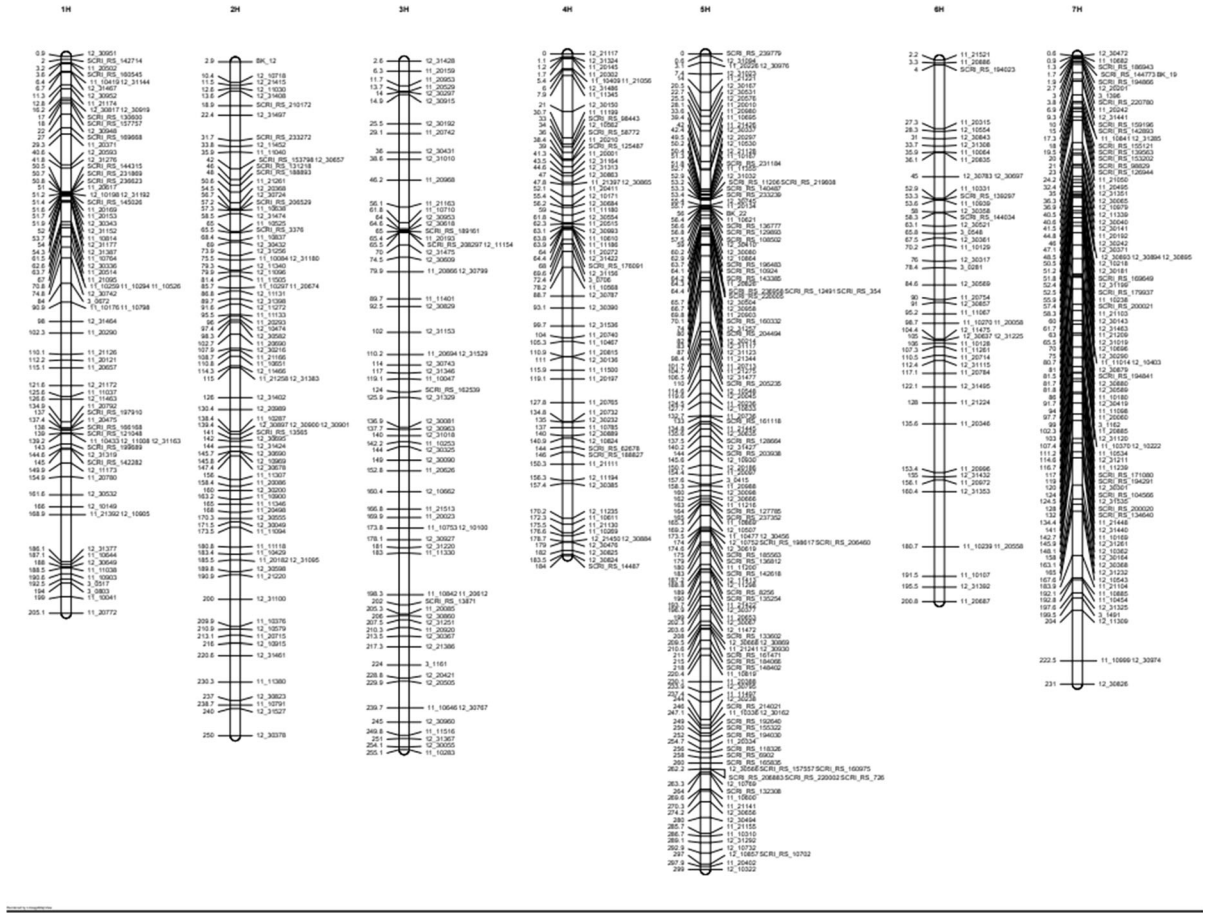


Fig. 3 Genetic map of barley markers. Barley marker location was determined from the BOPA consensus map

loaded onto the Ion Proton chip for sequencing. GBS libraries are loaded onto the Ion Proton chip using the Ion Chef (Life Technologies, Carlsbad, CA) with the Ion PI^{TM} Hi-Q Sequencing 200 and the Ion PI^{TM} Chip Kit v3. The loaded chips are then sequenced on an Ion Torrent Proton NGS platform (Life Technologies, Carlsbad, CA).

Sequence analysis pipeline

Individual OWB lines were de-multiplexed from raw sequence data using the unique barcode attached in PCR 2. Then raw sequence data from each OWB line are analyzed with a custom Python script. The first step uses cd-hit-est which collapses the raw sequence files into approximately similar sequence reads (Li and Godzik 2006). Each one of these clusters of unique sequence reads are considered a bin. The bins are then aligned to target sequence keyfiles with Muscle (Edgar 2004) at 100% homology excluding the SNP of interest. This output is organized in a FASTA file which notes the number of reads per sample and percent match to the marker key-file using a custom Python script. These results can then be looked into individually for further analysis within each line and marker for genotyping clean up if genotyping ambiguity exists.

Results and discussion

Duplicate samples of OWB DNA were analyzed for each of the 25 OWB samples to provide redundancy on ambiguous calls and provide confidence in analysis. During the development of this protocol it was

Table 2 Markers with known associated QTL/gene, 267 markers total

Marker name	TRAIT	QTL/gene
12_30817	Barley stripe rust	BSR-1H
SCRI_RS_142714	Diastatic power (DP)	QDp.FW6-1H
11_21174	Barley stripe rust	BSR-1H
12_30919	Barley stripe rust	BSR-1H
12_30817	Disease resistance	RPsh-1H
12_30951	Barley stripe rust	BSR-1H
12_30952	Barley stripe rust	BSR-1H
12_30948	Barley protein (BP)	QMe.StMo-1H.2; QKp.HaMo-1H.1
SCRI_RS_130600	Barley stripe rust	BSR-1H
SCRI_RS_157757	Barley stripe rust	BSR-1H
SCRI_RS_169668	Barley stripe rust	BSR-1H
12_30958	α-amylase (AA), diastatic power, β–glucanase activity, malt β–glucan, S/T ratio	QAa.HaTR-5H, QBgnm.HaTR-5H.2, QDp.HaTR-5H, QFcd.HaTR-5H, QMe.HaTR-5H.2, QGN.BlKy-5H, QS/T.HaTR-5H
12_31019	Fine-coarse difference	QFcd.DiMo-5H
12_31023	Malt extract	QFge.HaTR-5H.1
12_30948	Barley stripe rust	BSR-1H
12_31276	Plant height	QHt1H.27
12_31276	Low temperature tolerance	Fr-H3
12_30336	Kernel plumpness (PL)	QMe.StMo-1H.3
12_31177	Low temperature tolerance	Fr-H3
12_30336	Low temperature tolerance	Fr-H3
11_10764	Low temperature tolerance	Fr-H3
SCRI_RS_160545	Low temperature tolerance	Fr-H3
11_21095	Low temperature tolerance	Fr-H3
11_10259	Low temperature tolerance	Fr-H3
12_31467	Plant height	QHt1H.47
11_10176	β-glucan (BG)	NA
SCRI_RS_144315	Photoperiod sensitivity	PPD-H2
SCRI_RS_166168	Photoperiod sensitivity	PPD-H2
SCRI_RS_231869	Photoperiod sensitivity	PPD-H2
SCRI_RS_236623	Photoperiod sensitivity	PPD-H2
SCRI_RS_121048	Photoperiod sensitivity	PPD-H2
SCRI_RS_197910	Photoperiod sensitivity	PPD-H2
11_10433	Photoperiod sensitivity	PPD-H2
12_31163	Photoperiod sensitivity	PPD-H2
12_31192	Photoperiod sensitivity	PPD-H2
12_31319	Photoperiod sensitivity	PPD-H2
SCRI_RS_199689	Photoperiod sensitivity	PPD-H2
SCRI_RS_142282	Photoperiod sensitivity	PPD-H2
SCRI_RS_145026	Photoperiod sensitivity	PPD-H2
11_20754	Photoperiod sensitivity	PPD-H2
11_20169	Photoperiod sensitivity	PPD-H2
11_20153	Heading Date QTL	PPD_H2

Table 2 continued

Marker name	TRAIT	QTL/gene
12_11173	Grain test weight	QTw1H.101
12_31152	Malt extract (ME)	× QMe.StMo-1H.4; × QMe.HaMo-1H.2; QMe.Gutiérrez et al. (2011)
12_10905	Malt extract (ME)	× QMe.HaMo-1H.2
11_20772	Heading date	QHd1H.140
11_10041	α-amylase (AA)	
12_21415		AK375690
SCRI_RS_153798	Photoperiod sensitivity	PPD-H1
SCRI_RS_210172	Photoperiod sensitivity	PPD-H1
SCRI_RS_233272	Photoperiod sensitivity	PPD-H1
BK_12	Photoperiod sensitivity	PPD-H1
BK_14	Photoperiod sensitivity	PPD-H1
SCRI_RS_131218	Photoperiod sensitivity	PPD-H1
SCRI_RS_188893	Photoperiod sensitivity	PPD-H1
BK_12	Heading date	PPD-H1
11_21261	Grain protein content	QGpc.HaMo-2H.1, QMe.DiMo-2H, QMe.GaHN-2H
12_20593	Grain protein content, malt extract	QGpc.HaMo-2H.1, QMe.DiMo-2H, QMe.GaHN-2H
11_10084	NADH-ubiquinone reductase complex 1 MLRQ subunit	MLOC_39185.1
12_30582	Maturity	Eam6
12_10474		
12_30724	Maturity	Eam6
SCRI_RS_206529	Maturity	Eam6
11_21166	Maturity	Eam6
11_10651	*Glycoside hydrolase family 9*	QBgn.W-2H2
11_10287	Spike morphology	VRS1
12_30897	Spike morphology	VRS1
12_30900	Spike morphology	VRS1
12_30901	Spike morphology	VRS1
SCRI_RS_13565	Spike morphology	VRS1
SCRI_RS_3376	Spike morphology	VRS1
11_11307	Grain protein content	MLOC_44190.1
11_11094	Grain protein content, diastatic power, test weight, kernel plumpness, S/T ratio	
12_31095	Plump	QKp2H.113–116
12_10579	Grain yield	QYld2H.132
12_30823	Grain protein content	QGpc.StMo-2H.3
12_31180	β-glucan	QBgn.W-2H3
12_31408	*CslF4, CsFl8, CsFl10, CsFl12, CslH*	QBgn.SW-2H1
11_10837	Kernel weight (KW)	Qme.DiMo-2H
12_10915	Kernel weight (KW)	NA
11_20953		
12_31010	Grain yield	QYld3H.52
11_20866		AK365832

Table 2 continued

Marker name	TRAIT	QTL/gene
11_21163		MLOC_55793.1
SCRI_RS_162539	Heading Date QTL	QHd.FW6-3H
11_10842		
SCRI_RS_13871	Dwarfing	Denso
SCRI_RS_189161	Dwarfing	Denso
11_20085	Dwarfing	Denso
12_11154	Dwarfing	Denso
SCRI_RS_208297	Dwarfing	Denso
12_31251	Dwarfing	Denso
11_10283	Malt extract (ME)	NA
11_20145	β-glucan (BG)	× gQBgnm.StMo-4H(malt)
SCRI_RS_125487	Spike morphology	Int-C
SCRI_RS_58772	Spike morphology	Int-C
SCRI_RS_98443	Spike morphology	Int-C
11_20411	Spike morphology	Int-C
11_20302	β-glucan (BG)	NA
12_31156		AK368758
SCRI_RS_176091	α-amylase (AA)	QAa.FW6-4H.1
SCRI_RS_14487	Barley stripe rust	BSR-4H
SCRI_RS_62678	Barley stripe rust	BSR-4H
SCRI_RS_188827	Barley stripe rust	BSR-4H
11_20515	Barley stripe rust	BSR-4H
11_10610	Low temperature tolerance	VRN-H2
11_20272	Low temperature tolerance	VRN-H2
12_21450	Low temperature tolerance	VRN-H2
12_30476	Low temperature tolerance	VRN-H2
12_30884	Low temperature tolerance	VRN-H2
12_30889	Low temperature tolerance	VRN-H2
12_31422	Low temperature tolerance	VRN-H2
11_11186	Low temperature tolerance	VRN-H2
12_30824	β-amylase	Bmy1
12_30825	β-amylase	Bmy1
11_21130	Diastatic power (DP)	× QDp.DiMo-4H
12_30865	Grain Test Weight	QTw4H.33
12_30554	Heading Date	QHd4H.97
11_21221	Malt extract	QFge.HaTR-5H.1
12_31094	Malt extract	QFge.HaTR-5H.1
12_20576	α-amylase (AA), diastatic power, fine-coarse difference, grain protein content	QAa.DiMo-5H, QBgnw.DiMo-5H, QDp.DiMo-5H, QFcd.DiMo-5H, QGpc.DiMo-5H.1
11_20980	α-amylase (AA), diastatic power, fine-coarse difference, grain protein content	QAa.DiMo-5H, QBgnw.DiMo-5H, QDp.DiMo-5H, QFcd.DiMo-5H, QGpc.DiMo-5H.1
12_10864	Diastatic power, grain protein content	QDp.StMo-5H, QGpc.StMo-5H
SCRI_RS_205235	Wort β-glucan; α-amylase (AA)	QBg.FW6-5H; QAa.FW6-5H.1
12_20297		MLOC_3683.5

Table 2 continued

Marker name	TRAIT	QTL/gene
12_10633	Malt extract, β-glucanase activity (malt and kilned), grain protein content, test weight	QMe.DiMo-5H.2, QBgsg.StMo-5H, QBgsk.StMo-5H, QGpc.HaMo-5H, QTw.HaMo-5H
12_21128	Malt extract, β-glucanase activity (malt and kilned), grain protein content, test weight	QMe.DiMo-5H.2, QBgsg.StMo-5H, QBgsk.StMo-5H, QGpc.HaMo-5H, QTw.HaMo-5H
11_10167	Malt extract, β-glucanase activity (malt and kilned), grain protein content, test weight	QMe.DiMo-5H.2, QBgsg.StMo-5H, QBgsk.StMo-5H, QGpc.HaMo-5H, QTw.HaMo-5H
SCRI_RS_160332	Barley stripe rust	BSR-5H
11_11355	Barley stripe rust	BSR-5H
12_31427	Barley stripe rust	BSR-5H
SCRI_RS_128664	Barley stripe rust	BSR-5H
SCRI_RS_161118	Barley stripe rust	BSR-5H
SCRI_RS_231184	Barley stripe rust	BSR-5H
SCRI_RS_239779	Barley stripe rust	BSR-5H
SCRI_RS_11206	Barley stripe rust	BSR-5H
SCRI_RS_219608	Barley stripe rust	BSR-5H
SCRI_RS_140487	Barley stripe rust	BSR-5H
SCRI_RS_203938	Barley stripe rust	BSR-5H
SCRI_RS_233239	Barley stripe rust	BSR-5H
12_30098	α-amylase, S/T ratio	QAa.ChHa-5H, QAa.St.Mo-5H.1, QAa.HaMo-5H, QAa.HaTR-5H, QS/T.DiMo-5H.2
11_20134	α-amylase, S/T ratio	QAa.ChHa-5H, QAa.St.Mo-5H.1, QAa.HaMo-5H, QAa.HaTR-5H, QS/T.DiMo-5H.2
SCRI_RS_127785	Low temperature tolerence	FR-H2
BK_22	Low temperature tolerence	FR-H2
12_10752	Low temperature tolerence	FR-H2
SCRI_RS_108502	Low temperature tolerence	FR-H2
SCRI_RS_129893	Low temperature tolerence	FR-H2
SCRI_RS_135254	Low temperature tolerence	FR-H2
SCRI_RS_136777	Low temperature tolerence	FR-H2
SCRI_RS_136812	Low temperature tolerence	FR-H2
SCRI_RS_142618	Low temperature tolerence	FR-H2
SCRI_RS_185563	Low temperature tolerence	FR-H2
SCRI_RS_198617	Low temperature tolerence	FR-H2
SCRI_RS_206460	Low temperature tolerence	FR-H2
SCRI_RS_237352	Llow temperature tolerence	FR-H2
SCRI_RS_8256	Low temperature tolerence	FR-H2
12_30456	Malt extract (ME); α-amylase (AA)	NA
11_11200	Kernel plumpness (PL)	NA
11_21422	Kernel weight and plumpness, test weight	QGwe.HaTR-5H.2, QKp.HaMo-5H.2, QTw.HaMo-4H.2
12_30619	Plant height	QHt5H.114
12_20045	Kernel weight and plumpness, test weight	QGwe.HaTR-5H.2, QKp.HaMo-5H.2, QTw.HaMo-4H.2
12_30377	Cellulose synthase	QBgn.S-5H1

Table 2 continued

Marker name	TRAIT	QTL/gene
12_11472	Malt β-glucan, extract viscosity	QBgnm.HaTR-5H.1, QEv.HaTR-5H
SCRI_RS_133602	Low temperature tolerence	FR-H1/VRN-H1
SCRI_RS_148402	Low temperature tolerence	FR-H1/VRN-H1
12_30668	Low temperature tolerence	FR-H1/VRN-H1
12_30869	Low temperature tolerence	FR-H1/VRN-H1
12_30930	Low temperature tolerence	FR-H1/VRN-H1
SCRI_RS_161471	Low temperature tolerence	FR-H1/VRN-H1
11_21241	Low temperature tolerence	FR-H1/VRN-H1
SCRI_RS_184066	Low temperature tolerence	FR-H1/VRN-H1
11_11497		morex_contig_392559
SCRI_RS_192640	TTKSK stem rust	RPG4/RPG5
11_10869	TTKSK stem rust	RPG4/RPG5
12_30162	TTKSK stem rust	RPG4/RPG5
SCRI_RS_155322	TTKSK stem rust	RPG4/RPG5
SCRI_RS_194030	TTKSK stem rust	RPG4/RPG5
11_20988	TTKSK stem rust	RPG4/RPG5
SCRI_RS_118326	TTKSK stem rust	RPG4/RPG5
SCRI_RS_6902	TTKSK stem rust	RPG4/RPG5
SCRI_RS_10924	TTKSK stem rust	RPG4/RPG5
SCRI_RS_165835	TTKSK stem rust	RPG4/RPG5
11_20826	TTKSK stem rust	RPG4/RPG5
12_30666	TTKSK stem rust	RPG4/RPG5
SCRI_RS_143385	TTKSK stem rust	RPG4/RPG5
SCRI_RS_157557	TTKSK stem rust	RPG4/RPG5
11_20309	TTKSK stem rust	RPG4/RPG5
SCRI_RS_12491	TTKSK stem rust	RPG4/RPG5
SCRI_RS_132308	TTKSK stem rust	RPG4/RPG5
SCRI_RS_160975	TTKSK stem rust	RPG4/RPG5
SCRI_RS_196483	TTKSK stem rust	RPG4/RPG5
SCRI_RS_206883	TTKSK stem rust	RPG4/RPG5
SCRI_RS_214021	TTKSK stem rust	RPG4/RPG5
SCRI_RS_220002	TTKSK stem rust	RPG4/RPG5
SCRI_RS_220005		RPG4/RPG5
SCRI_RS_236958	TTKSK stem rust	RPG4/RPG5
SCRI_RS_354	TTKSK stem rust	RPG4/RPG5
SCRI_RS_726	TTKSK stem rust	RPG4/RPG5
11_11216	Malt extract	QMe.DiMo-5H.3
12_30656	Soluble:total protein (ST)	× QAa.StMo-5H.2
11_20826		
12_30504	Wort protein (WP)	QAa.StMo-5H.2
12_10732	α-amylase (AA), diastatic power, fine-coarse difference, grain protein content	QAa.HaTR-5H, QBgnm.HaTR-5H.2, QDp.HaTR-5H, QFcd.HaTR-5H, QMe.HaTR-5H.2, QGN.BlKy-5H, QS/T.HaTR-5H
12_10769	Malt extract	QMe.DiMo-5H.3

Table 2 continued

Marker name	TRAIT	QTL/gene
12_11413	α-amylase (AA), diastatic power, fine-coarse difference, grain protein content, S/T ratio	QAa.HaTR-5H, QBgnm.HaTR-5H.2, QDp.HaTR-5H, QFcd.HaTR-5H, QMe.HaTR-5H.2, QGN.BlKy-5H, QS/T.HaTR-5H
12_10322	α-amylase (AA), malt β-glucan, diastatic power, fine-coarse difference, malt extract, grain nitrogen, S/T ratio	QAa.HaTR-5H, QBgnm.HaTR-5H.2, QDp.HaTR-5H, QFcd.HaTR-5H, QMe.HaTR-5H.2, QGN.BlKy-5H, QS/T.HaTR-5H
11_10310	Kernel plumpness (PL)	QAa.StMo-5H.2; QAa.HaMo-5H;QS/T.HaMo-5H
12_31292	Wort protein (WP); soluble:total protein (ST); α-amylase (AA); soluble/total ratio (ST); α-amylase (AA)	QAa.StMo-5H.2; QAa.HaMo-5H;QS/T.HaMo-5H
11_20402	α-amylase (AA), malt β-glucan, diastatic power, fine-coarse difference, malt extract, grain nitrogen, S/T ratio	QAa.HaTR-5H, QBgnm.HaTR-5H.2, QDp.HaTR-5H, QFcd.HaTR-5H, QMe.HaTR-5H.2, QGN.BlKy-5H, QS/T.HaTR-5H
SCRI_RS_10702	Soluble:total protein(ST)	QSt.FW6-5H.2
12_31123	α-amylase (AA), diastatic power, β–glucanase activity, malt β-glucan, S/T ratio	QAa.HaTR-5H, QBgnm.HaTR-5H.2, QDp.HaTR-5H, QFcd.HaTR-5H, QMe.HaTR-5H.2, QGN.BlKy-5H, QS/T.HaTR-5H
11_20402	Malt extract (ME); wort protein (WP); soluble:total protein (ST); α-amylase (AA); malt extract (ME); β-glucan (BG)	QAa.StMo-5H.2; QAa.HaMo-5H;QS/T.HaMo-5H
12_10857	α-amylase (AA); wort protein (WP); soluble/total ratio (ST)	× QAa.StMo-5H.2; × QAa.HaMo-5H;QS/T.HaMo-5H
12_10322	Malt extract (ME); wort protein (WP); soluble:total protein (ST); α-amylase (AA); malt extract (ME); β-glucan (BG)	QAa.StMo-5H.2; QAa.HaMo-5H;QS/T.HaMo-5H
12_31123	Malt extract (ME); wort protein (WP); soluble:total protein (ST); α-amylase (AA); malt extract (ME);	QAa.StMo-5H.2; QAa.HaMo-5H;QS/T.HaMo-5H
SCRI_RS_10702	Dormancy	*Qsd2* (dormancy)//co-located with QAa.FW6-5 h.2(alpha-amylase)
SCRI_RS_205235	Dormancy	*Qsd1* (dormancy)//co-located with *QAa.FW6-5 h.1*(alpha-amylase)//co-located with *QBg.FW6-5H*(wort beta-glucan)
SCRI_RS_194023	Plump grain	QPl.FW6-6H.1
12_30697	RNA recognition and motif containing protein	MLOC_16674.1
12_31308	Pentatricopeptide	MLOC_16674.1
11_20835	Diastatic power (DP)	QGpc.DiMo-6H
SCRI_RS_139297	α-amylase (AA)	QAa.FW6-6H
11_10331	α-amylase (AA); malt extract (ME)	NA
SCRI_RS_144034	Plump grain	QPl.FW6-6H.2
12_20201		MLOC_75066.1
SCRI_RS_186943	Starch type	GBSSI waxy
11_10841	Starch type	GBSSI waxy
SCRI_RS_155121	Starch type	GBSSI waxy

Table 2 continued

Marker name	TRAIT	QTL/gene
SCRI_RS_159196	Starch type	GBSSI waxy
SCRI_RS_139563	Starch type	GBSSI waxy
SCRI_RS_153202	Starch type	GBSSI waxy
12_31285	Starch type	GBSSI waxy
BK_19	Starch type	GBSSI waxy
SCRI_RS_142893	Starch type	GBSSI waxy
SCRI_RS_144773	Starch type	GBSSI waxy
SCRI_RS_126944	Starch type	GBSSI waxy
SCRI_RS_194866	Starch type	GBSSI waxy
SCRI_RS_98829	Starch type	GBSSI waxy
SCRI_RS_220780	Vernalization	VRN-H3
12_30894	Vernalization	VRN-H3
12_30893	Vernalization	VRN-H3
12_30895	Vernalization	VRN-H3
SCRI_RS_179937	Vernalization	VRN-H3
12_30893	Heading Date	QHd7H.36–38
12_10218	Plant Height	QHt7H.39
12_30879	Malt extract (ME)	× QMe.StMo-7H; QBgnm.StMo-7H.1, QKp.HaMo-7H;QDp.HaMo-7H;QGpc.HaMo-7H
12_30290	Alpha-amylase, diastatic power, beta-glucanase activity, malt beta-glucan, S/T ratio	QAa.StMo-7H.3, QDp.StMo-7H.3, QBgnm.StMo-7H.1, QBgsk.StMo-7H.1, QS/T.HaTR-7H
12_30181	Alpha-amylase, diastatic power, malt beta-glucan, beta-glucanase activity (kilned), S/T ratio	QAa.StMo-7H.3, QDp.StMo-7H.3, QBgnm.StMo-7H.1, QBgsk.StMo-7H.1, QS/T.HaTR-7H
12_30880	Alpha-amylase, diastatic power, beta-glucanase activity, malt beta-glucan, S/T ratio	QAa.StMo-7H.3, QDp.StMo-7H.3, QBgnm.StMo-7H.1, QBgsk.StMo-7H.1, QS/T.HaTR-7H
12_10403	Alpha-amylase, diastatic power, malt beta-glucan, beta-glucanase activity (kilned), S/T ratio	QAa.StMo-7H.3, QDp.StMo-7H.3, QBgnm.StMo-7H.1, QBgsk.StMo-7H.1, QS/T.HaTR-7H
12_10718	Malt beta-glucan, malt extract	QBgn.StMo-2H.1, QMe.StMo-2H.1
12_31441	Grain test weight	QTw7H.70
SCRI_RS_169649	HULL adherence	NUD
SCRI_RS_171080	Hull adherence	NUD
SCRI_RS_194291	Hull adherence	NUD
11_21103	Hull adherence	NUD
12_31211		MLOC_54855.1
SCRI_RS_104566	Hull adherence	NUD
SCRI_RS_194841		NUD
11_10534	β-glucan (BG)	QMe.StMo-7H.3; QDp.StMo-7H.12; QAa.StMo-7H.1;QKp.HaMo-7H; QDp.HaMo-7H; QGpc.HaMo-7H
SCRI_RS_134640		NUD
SCRI_RS_200020	Hull adherence	NUD
SCRI_RS_200021	Hull adherence	NUD

Table 2 continued

Marker name	TRAIT	QTL/gene
12_31199	Protein	QGpc6H.86
11_21209	Protein	QGpc7H.130
12_31325	Alpha-amylase, grain protein content, test weight, kernel plumpness	QAa.HaTR-7H, QGpc.HaTR-7H, QKp.HaTR-7H, QTw.HaTR-7H.1
12_31387	Malt beta-glucan, malt extract	QBgnm.StMo-1H.2, QFge.HaTR-1H.2

discovered that adding sequence diversity resulted in higher quality sequence reads. To test the accuracy of this new genotyping protocol in barley, our results were compared to the published 50 k Illumina iSelect genotyping array results of the OWB population. The results matched 99.998% at each locus across all 25 OWB lines sequenced with the GMS protocol. Out of the 4,824 data points only one was called differently than the published data, excluding missing calls. A sample of 201 barley markers were selected for comparison which had the greatest polymorphic information content (PIC) and least missing data. The average missing data for each marker across all 25 OWB lines was 5%, with the largest being 12%. Of the 577 barley markers; 77 map to chromosome 1H, 85 to 2H, 67 to 3H, 66 to 4H, 143 to 5H, 48 to 6H, and 91 to 7H (Fig. 1). Marker chromosome and map location was derived from the BOPA consensus map (Close et al. 2009). Marker distribution across the genome has an average cM distance less than 3.03 per chromosome with an average of 82 markers per chromosome (Fig. 2)(Table 1). Polymorphic marker content was 47% on our panel of 25 OWB lines. The markers used in this study are not all informative, as many markers were selected to maximize genome coverage for use in genomic selection.

The first two pools of 500 markers were selected as markers of interest and markers with a known function. The third pool was selected to optimize chromosome coverage and fill in chromosomal gaps. This resulted in a total of three barley marker pools for genotyping. Chromosomal coverage is fair with few gaps larger than 10 cM (Fig. 2). The average map distance across all chromosomes is 3.03 cM, so any QTL would theoretically be in linkage disequilibrium with at least one marker. Average linkage disequilibrium was observed to decay below a critical level (r^2-value 0.2) within a map distance of 5–10 cM (Pasam et al. 2012). There is at least one example of cM distance greater than 10 on each chromosome, chromosome 6H has 4 chromosomal gaps larger than 10 cM.

Genetic maps for all 7 barley chromosomes were generated from the chromosomal position identified in the 50 k iSelect SNP Chip consensus map (Fig. 3). Of the 468 markers with a known function or associated QTL present in the three pools, 267 produced genotypic data (Table 2). Of the remaining 213 markers with known function that did not work only 32 amplified the loci, but did not produce results because they did not match the sequence keyfile used for genotyping. This failure was largely due to primer dimers. When comparing the most robust genetic variability from this protocol matches the OWB consensus genotype at 99.998% which provides strong evidence for the efficacy of this protocol. This protocol is robust and capable of being customized to any genome or marker of interest, which may be very useful in marker-assisted selection and association mapping. Due to these markers being selected from known markers in the 50 k iSelect chip, which were derived from a wide range of European barley germplasm, there could be ascertainment bias as winter and facultative barley are under-represented in the 50 k chip.

Acknowledgements We would like to thank the USDA-ARS for their invaluable assistance and funding for this research project, as well as our colleagues Dr. Jamie Sherman and Dr. Pat Hayes for their assistance in selecting markers for the pools and refinement of the manuscript. Finally, I would like to thank Washington State University, the Department of Plant Pathology, and the ARCS fellowship program for their financial assistance, education, and professional development.

Author contribution statement Jonathan Eagle was the lead researcher overseeing the advancement of the research specifically pertaining to barley protocol development. Travis Ruff, USDA Biologial Science Technician overseeing a similar project in wheat and offering assistance and advice for protocol optimization and manuscript revision. Marcus Hooker assisted in the development and optimization of the analysis pipeline and manuscript revision. Sajal Sthapit, fellow graduate student who assisted in research and manuscript revision. Elliott Marston, fellow graduate student who assisted in research and manuscript revision. Karol Marlowe, USDA Biological Science Technician who assisted in sequencing, protocol optimization, and manuscript revision. Dolores Covarrubias, former WSU lab technician who assisted in sequencing and protocol optimization. Daniel Skinner assisted in early development of the analysis pipeline. Patrick Hayes, collaborator assisting in marker selection for traits of interest and manuscript revision. Jamie Sherman, fellow collaborator in marker selection for traits of interest and manuscript revision. Deven See, USDA ARS WRSGGL lab director providing funding, mentorship, and specialized knowledge for the development of the GMS protocol.

Declarations

Conflict of interest There are no relevant financial or non-financial competing interests to report.

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