Full Length Article

Association Mapping of Grain Weight, Length and Width in Barley (*Hordeum vulgare*) Breeding Germplasm

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Abstract

Grain weight, grain length and grain width are important target traits in the malting barley breeding, due to their attributes for determining the market value of barley grain. In this study, a diversity collection of 112 accessions was used to identify quantitative trait loci (QTLs) for them by association mapping approach with simple sequence repeat (SSR) markers and detect elite alleles related to the improvement of grain size. The heritability calculated for grain weight over multi-environment experiments was 83.6%, while the values of grain length and width were 70.0% and 69.9%, respectively. Population structure analysis showed that all the accessions were divided into two subgroups that originated from East Asia and North America. A total of five QTLs for grain weight were identified on chromosomes 1H, 2H, 5H and 6H, respectively, and the QTLs on 5HS and 6HS were detected in more than one environment. The *A4* allele of *GBM1215* on 6HS increased grain weight significantly in all three environments. Four and two QTLs were detected for grain length and width, respectively. QTL for grain width on 2HS was detected in two environments and the *A1* allele produced wider grain. These findings might be valuable for marker-assisted selection of breeding lines to improve grain weight and width. © 2017 Friends Science Publishers

Keywords: Barley (*Hordeum vulgare* L.); Association mapping; Grain weight; Grain length; Grain width

Introduction

Barley (*Hordeum vulgare* L.) is the fourth most important cereal crop in the world and its grain is mainly used for animal feed, malting and brewing (Virender and Zhang, 2003; Walker *et al.*, 2013). As one of the most adaptable cereals, the growing zone of barley ranges from subarctic to subtropical areas (Wenzel *et al.*, 2015). Tibetan Plateau and its vicinity are considered as one of the centers of genetic diversity and domestication of cultivated barley (Feng *et al.*, 2006a; Feng *et al.*, 2006b; Dai *et al.*, 2012). A larger number of wild barleys have been found in Tibet, Qinghai and Sichuan province of China, with high genetic diversity (Feng *et al.*, 2006b; Dai *et al.*, 2012). It was reported that Chinese cultivated barley originated from the two-rowed wild barley from Tibet was significantly different from those found by other centers of genetic diversity (Dai *et al.*, 2012).

Grain size including grain weight, grain length and grain width are important attributes for determining the market value of barley grain. Heavy and plump grains are always associated with superior malting quality, owing to

their highsu malt extract, and better feed quality with more starch per grain (Hadjichristodoulou, 1990). Grain weight is also one component of yield (Hadjichristodoulou, 1990; Passarella *et al.*, 2005), which is mainly determined by grain length and grain width (Sun *et al.*, 2013; Rasheed *et al.*, 2014). Thus, enhancing grain size is an efficient approach to improve both end-use quality and yield potential of barley.

Grain weight, length and width are complex quantitative traits, which are controlled by polygenes or QTLs (Sun *et al.*, 2013) and influenced by environment (Xing and Zhang, 2010; Walker *et al.*, 2013), i.e. temperature during pre-anthesis and grain filling (Wallwork *et al*., 1998; Passarella *et al.*, 2005), drought stress (Royo *et al.*, 2000), etc. In the past few decades, with the help of molecular markers and marker-based genetic maps, identifications of QTLs for grain weight in barley have been reported in different barley germplasms. According to those reports, QTLs for grain weight are almost distributed on all the seven chromosomes of barley (Kjaer and Jensen, 1996; Tinker *et al.*, 1996; Bezant *et al.*, 1997; Marquezcedillo *et al.*, 2001; Li *et al.*, 2005; Szücs *et al.*, 2009; Worch *et al.*,

2011; Walker *et al.*, 2013). Matthies *et al.* (2012) detected several QTLs for grain weight on chromosomes 1H, 3H and 7H by association mapping using simple sequence repeat (SSR) and Diversity Arrays Technology (DArT) markers, most of which were consistent with QTLs detected in reported bi-parental populations. On chromosome 2H, the intervals of several reported QTLs for grain weight were overlapped in different germplasms (Bezant *et al.*, 1997; Marquezcedillo *et al.*, 2001; Szücs *et al.*, 2009; Mikolajczak *et al.*, 2016; Wang *et al.*, 2016). Moreover, major QTLs for grain weight on chromosome 4H (Pillen *et al.*, 2003; Korff *et al.*, 2006; Maurer *et al.*, 2016) were also identified around the vernalization gene '*Vrn-H2*' (Yan *et al.*, 2004). QTLs for grain length and grain width were detected throughout most chromosomes (Backes *et al.*, 1995; Ayoub *et al.*, 2002; Walker *et al.*, 2013). The putative candidate gene of one major grain length QTL on chromosome 4H that explained 22.3% of the phenotypic variance was homologous to *An-1* in rice, which encodes a bHLH protein regulating cell division, grain length, and awn elongation (Zhou *et al.*, 2016).

Linkage mapping based on bi-parental cross population and association mapping (AM) are considered as two main approaches in QTLs detection. AM method that had been firstly applied in human genetics for complex diseases was based on linkage disequilibrium (LD) in diversity collection of germplasms. In plant, the population for AM covers larger number of alleles used in breeding, and takes most historical recombination events into account (Flint-Garcia *et al.*, 2003; Gupta *et al.*, 2005; Breseghello and Sorrells, 2006a), with advantages of increased involved genetic variations and resolution for QTL detection. Currently, AM has been widely applied in identifying phenotype-markers associated in barley (Long *et al.*, 2013; Zhou and Steffenson, 2013; Ziems *et al.*, 2014).

Although lots of QTLs for grain weight and size related traits have been reported in recent years, comparison of effects between different QTL alleles from different germplasms were reported rarely, which made it difficult for breeders to select suitable parental lines. Moreover, prevailing artificial plant breeding in China adopts only a small number of main lines that had unfortunately narrowed the genetic base of modern cultivated barley and made a great bottleneck on genetic enhancement for yield. It seems that introducing extra germplasms and further dissecting elite alleles become an effective remedy.

In this study, a diversity association mapping panel of 112 barley accessions from China, Mexico and USA was evaluated over three environments using simple sequence repeat (SSR) markers with a mixed linear model approaches (MLM), and the effects for enhancing grain weight, length and width were further compared between different QTL alleles at detected sites by association mapping. The goals of this study were to identify loci associated with grain weight, length and width, and detect the elite allelic variations enhancing grain size.

Materials and Methods

Plant Materials

The diversity barley panel contained 112 accessions, including elite parental lines, advanced breeding materials, current cultivars and landraces with the origins of Gansu (China), Qinghai (China), Sichuan (China), Tibet (China), Mexico and USA, which were provided by the Barley Research Centre of Sichuan Agriculture University (Table 1). The whole genotypes consisted of 104 six-rowed and 8 two-rowed accessions. Among the collection, 77 are hull-less barleys and 35 are hulled barleys. Of the 104 six rowed barely lines, 73 are naked barleys and the others are hulled barleys.

Trait Evaluation

Field trials were conducted in the northwest of the Chengdu basin, Sichuan Province, China (103˚88' E, 30˚82' N; 548 m above sea level) in the years of 2014 (CD2014) and 2015 (CD2015) and the hilly area of Nanchong, Sichuan Province, China (105˚97' E, 30˚78' N; 680 m above sea level) in 2015 (NC2015). Both Chengdu and Nanchong are under a humid subtropical monsoon climate with the average annual rainfall from 887.3 mm to 927.6 mm. Each field experiment was performed in a randomized complete block design with two replicates. Each plot of the field experiment had five rows with 1.5 m long and spaced 0.35 m apart. After harvest, the grain weight was measured as the mean weight of 1000 individual grains (TGW), while grain length (GL) and grain width (GW) was defined as the longest and the widest distance through an average of 100 grains, respectively. All of three traits were conducted by the SC-G plant grain analysis system (Wseen company, China).

Genotyping

A total of 319 SSR markers, which were selected according to the consensus genetic map by Varshney *et al.* (2007) were used to genotyping. Each chromosome contained about 45 SSR markers for a balanceable coverage of barley chromosome. Genomic DNA was isolated from bulked young leaf tissue of 10 seedlings per accession using CTAB procedure (Irfan *et al.*, 2013). PCR reactions were performed in a total volume of 15 μL containing 1x Buffer, 2 mmol L^{-1} MgCl$_2$, 0.25 mmol L^{-1} dNTPs, 0.25 μmol L^{-1} of each primer, 1 U *rTaq*-polymerase and 20 ng genomic DNA as template. The PCR profile was as follows: one cycle of 94℃ for 5 min; 35 cycles of 94℃ for 45 sec, 55–60℃ (primer depended) for 45 s and 72℃ for 1 min; and a final extension at 72℃ for 10 min. The PCR products were separated in 6% (w/v) denaturing polyacrylamide gels with 1 x TBE Buffer and then were visualized by silver staining.

Table 1: The code, name, origin and row type of 112 barley accessions

Code	Name	Origin	Row type
1	GQ2	Gansu (China)	six
2	GQ3	Gansu	six
3	GQ4	Gansu	six
4	9516	Gansu	six
5	DLH	Gansu	six
6	HZBQK	Gansu	six
7	KJZBQK	Gansu	six
8	MQK	Gansu	six
9	HQK	Gansu	six
10	CQK	Gansu	six
11	HQK	Gansu	six
12	SYQK	Gansu	six
13	CQK	Gansu	six
14	DMQK	Gansu	six
15	LLGQK	Gansu	six
16	LLLQK	Gansu	six
17	XLQK	Gansu	six
18	QLZQK	Gansu	six
19	BQ1	Qinghai (China)	six
20	BQ2	Qinghai	six
21	BQ3	Qinghai	six
22	BQ5	Qinghai	six
23	BQ6	Qinghai	six
24	BQ7	Qinghai	six
25	KL2	Qinghai	six
26	KL10	Qinghai	two
27	KL12	Qinghai	six
28	HYTQK	Qinghai	six
29	LDQK	Qinghai	six
30	HZSCR	Qinghai	six
31	DMDGLLQK	Qinghai	six
32	HZBLLQK	Qinghai	six
33	HZLLQK	Qinghai	six
34	HYLLL	Qinghai	six
35	DTBLS	Qinghai	six
36	CMBQK	Qinghai	six
37	LDBQK	Qinghai	six
38	M112BQK	Qinghai	six
39	YSBQK	Qinghai	six
40	HUBLLQK	Qinghai	six
41	DTBLL	Qinghai	six
42	DTHJN	Qinghai	six
43	HZHCM	Qinghai	six
44	HZCM	Qinghai	six
45	HZLCM	Qinghai	six
46	YHCMQK	Qinghai	six
47	XHXQK	Qinghai	six
48	XHHQK	Qinghai	six
49	HLWLQK	Qinghai	six
50	MYQLQK	Qinghai	six
51	ZYDM	Qinghai	six
52	HYHQK	Qinghai	six
53	REGBXHK	Qinghai	six
54	GHHQK	Qinghai	six
55	LDLQK	Qinghai	six
56	HZZPQK	Qinghai	six
57	MZQK	Qinghai	six
58	FB0310	Qinghai	six
59	FBO226	Sichuan (China)	six
60	SPZQK	Sichuan	six
61	QNQK	Sichuan	six
62	BYQK	Sichuan	six
63	AQ4	Sichuan	six
64	AQ5	Sichuan	six
65	BDCQK	Tibet (China)	six

Table 1: Continued

Code	Name	Origin	Row type
66	BDQK	Tibet	six
67	FB0641	Tibet	six
68	FB0642	Tibet	six
69	FB0647	Tibet	six
70	FB0648	Tibet	six
71	FB0650	Tibet	six
72	FB0651	Tibet	six
73	Golas Bley	Mexico	two
74	B3034	Mexico	two
75	Tibetannia	Mexico	two
76	K5	Mexico	two
77	ARUMIR	USA	six
78	WAS3	USA	two
79	APM-HB1905	USA	six
80	H.SAT.V.HAX.F.FURB	USA	six
81	SAH	USA	six
82	AHOR443170	USA	six
83	AHOR2194170	USA	six
84	BANG-IU	USA	six
85	BRABHVTIB	USA	six
86	H.VULJ.L.TRIF	USA	six
87	M66.85-BI12168	USA	six
88	ORE"S"BBB-177	USA	six
89	IBNBF8-582SEL.6AP	USA	six
90	IBNBF8-588SEL.1AP	USA	six
91	IBNBF8-594SEL.2AP	USA	six
92	FB0598	USA	six
93	FB0604	USA	six
94	FB0605	USA	six
95	FB0606	USA	six
96	FB0607	USA	six
97	FB0611	USA	six
98	FB0612	USA	six
99	FB0613	USA	six
100	FB0614	USA	six
101	FB0615	USA	six
102	FB0616	USA	six
103	FB0619	USA	six
104	JNABBB-204	USA	six
105	JNABBB-205	USA	six
106	FB0844	USA	six
107	FB0609	USA	two
108	XQ0758	USA	six
109	31721	USA	two
110	GY135	USA	six
111	GY137	USA	six
112	GY138	USA	six

Among the 319 SSR markers, 147 markers with clear and sharp PCR bands were selected for further analysis of genetic diversity and association mapping in this study (Table 2).

Statistical Analysis

Analysis of variance (ANOVA) for the phenotypic data was carried out using the general linear model function (GLM) of the SPSS statistical package (SPSS Inc., Chicago, IL). The genotypic variance V^2_g, genotype-environment interaction V^2_{ge}, and environmental variances V^2_e were estimated for each trait to measure their heritability (h^2_m) by the following formula: $h^2_m = V^2_g/(V^2_g+V^2_{ge}/e+V^2_e/re)$, where e is number of trials and r is number of replications (Long *et al.*, 2013).

Table 2: Number of alleles and PIC values caculated based on the panel of 112 barley genotypes

Code	Locus	Chromosome	Position[a]	Annealing Temperature (°C)	Na[b]	Ne[c]	PIC[d]
1	*EBmac0560*	1HS	58.76	55	2	1.02	0.0177
2	*GBM1412*	1HS	59.11	55	2	1.03	0.0351
3	*Bmag0350*	1HS	59.83	55	10	6.21	0.8388
4	*Bmag0211*	1HS	60.42	60	6	4.41	0.7730
5	*GBM1451*	1HS	60.52	55	5	2.61	0.6157
6	*GBM1234*	1HS	61.06	55	2	1.55	0.3565
7	*EBmac0659*	1HS	61.81	55	2	1.02	0.0177
8	*GBM1336*	1HL	63.01	55	2	1.06	0.0605
9	*Bmac0090*	1HL	63.66	55	10	6.15	0.8374
10	*EBmac0695*	1HL	65.31	55	3	2.13	0.5311
11	*HVM20*	1HL	66.25	55	5	3.27	0.6942
12	*scssr10477*	1HL	79.09	55	5	1.32	0.2430
13	*GBM1092*	1HL	91.79	55	1	1.00	0.0000
14	*GBM5162*	1HL	93.12	55	2	1.02	0.0177
15	*Bmag0579*	1HL	132.84	55	8	2.63	0.6202
16	*GBM1461*	1HL	135.94	55	6	4.09	0.7554
17	*GBM1434*	1HL	136.38	55	2	1.87	0.4641
18	*scssr08238*	1HL	139.81	55	5	1.98	0.4949
19	*GBM1204*	1HL	139.81	55	3	2.67	0.5594
20	*GBM1187*	2HS	19.51	55	2	1.72	0.4193
21	*scssr10226*	2HS	46.63	55	3	1.81	0.4456
22	*scssr07759*	2HS	48.58	55	4	1.74	0.4245
23	*GBM1446*	2HS	58.13	55	3	1.68	0.4033
24	*GBM1251*	2HS	58.8	55	5	2.79	0.6427
25	*HvXan*	2HS	62.69	55	2	1.29	0.2254
26	*scssr03381*	2HS	63.61	55	5	4.51	0.7779
27	*GBM1459*	2HS	64.35	55	5	4.29	0.7671
28	*GBM5230*	2HS	65.16	55	2	1.09	0.0853
29	*GMS003*	2HS	66.05	60	7	1.64	0.3887
30	*EBmac0640*	2HS	68.78	55	4	1.17	0.1429
31	*GBM1203*	2HS	69.58	55	2	1.11	0.1014
32	*EBmac0525*	2HS	70.98	55	1	1.00	0.0000
33	*Bmag0518*	2HL	72.01	60	11	5.04	0.8015
34	*Bmag0829*	2HL	74.56	55	6	2.65	0.6229
35	*Bmag0711*	2HL	79.52	55	8	3.33	0.6993
36	*GBM1468*	2HL	84.06	55	4	1.83	0.4538
37	*GBM1408*	2HL	89.44	55	2	1.13	0.1172
38	*EBmatc0039*	2HL	93.26	60	3	1.39	0.2801
39	*GBM1440*	2HL	96.34	55	4	2.67	0.5594
40	*GBM1208*	2HL	102.85	55	5	2.31	0.5669
41	*GBM1149*	2HL	107.89	55	4	2.61	0.6172
42	*EBmac0415*	2HL	117.86	55	4	1.93	0.4831
43	*GBM1200*	2HL	124.8	55	1	1.00	0.0000
44	*GBM1498*	2HL	125.71	55	3	2.32	0.5662
45	*Bmag0749*	2HL	147.93	55	5	2.69	0.6284
46	*GBM1475*	2HL	149.47	55	3	1.59	0.3692
47	*scssr08447*	2HL	156.49	55	5	2.55	0.6079
48	*GBM1280*	3HS	3.83	55	2	1.43	0.2991
49	*GBM1450*	3HS	13.94	55	2	1.39	0.2817
50	*GBM1382*	3HS	16.32	55	4	3.82	0.7384
51	*EBmac0705*	3HS	20.49	55	5	3.01	0.6676
52	*scssr10559*	3HS	23.26	55	6	1.94	0.4846
53	*GBM1284*	3HS	31.95	55	2	1.05	0.0436
54	*Bmag0023*	3HS	46.28	55	1	1.00	0.0000
55	*GBM1163*	3HS	60.27	55	2	1.09	0.0853
56	*GBM1110*	3HS	60.27	55	3	2.19	0.5429
57	*GBM1495*	3HL	62.57	55	2	1.11	0.1014
58	*GBM1405*	3HL	86.33	55	4	1.51	0.3367
59	*GBM1233*	3HL	89.66	55	3	1.61	0.3774
60	*Bmag0013*	3HL	113.7	60	12	6.35	0.8426
61	*GBM1420*	3HL	152.53	55	5	3.28	0.6955
62	*GBM1501*	4HS	0	55	2	1.52	0.3418
63	*HVM40*	4HS	22.4	60	8	3.22	0.6894
64	*GBM1323*	4HS	28.96	55	3	1.88	0.4686
65	*scssr20569*	4HS	44.87	55	4	1.84	0.4579

Table 2: Continued

Table 2: Continued

66	*Bmag0808*	4HL	53.04	55	7	3.43	0.7082
67	*EBmac0906*	4HL	54.98	55	5	1.87	0.4650
68	*Bmac0181*	4HL	58.51	55	5	3.45	0.7098
69	*Bmac0030*	4HL	58.6	55	9	3.21	0.6876
70	*Bmag0490*	4HL	62.19	55	11	3.13	0.6809
71	*GBM1299*	4HL	72.04	60	3	1.37	0.2686
72	*EBmac0658*	4HL	75.59	55	4	1.81	0.4466
73	*EBmac0635*	4HL	93.06	55	11	2.26	0.5582
74	*EBmac0679*	4HL	94.5	55	10	2.48	0.5974
75	*EBmac0701*	4HL	96.15	55	15	2.61	0.6159
76	*GBM1220*	4HL	99.86	55	4	1.33	0.2496
77	*HVMLOH1A*	4HL	102.27	55	4	2.06	0.5148
78	*HVM67*	4HL	120.5	60	4	1.98	0.4950
79	*GBM1388*	4HL	122.35	55	2	1.93	0.4807
80	*Bmag0138*	4HL	124	55	6	2.69	0.6276
81	*GBM1453*	4HL	132.27	55	3	1.88	0.4668
82	*scssr02306*	5HS	6.13	55	3	2.51	0.6022
83	*GBM1176*	5HS	18.59	55	2	1.44	0.3047
84	*scssr07106*	5HS	20.44	55	4	1.86	0.4635
85	*GBM5028*	5HS	27.41	55	3	1.61	0.3793
86	*EBmac0970*	5HS	40.77	55	2	1.96	0.4885
87	*Hvm30*	5HS	41.93	55	4	1.49	0.3327
88	*Bmag0751*	5HS	42.87	55	10	3.77	0.7346
89	*Bmag0337*	5HS	44.99	55	8	3.68	0.7285
90	*Bmag0323*	5HS	50.85	55	14	11.06	0.9096
91	*Bmac0096*	5HS	53.12	60	6	3.64	0.7251
92	*GBM1399*	5HL	69.86	55	3	1.77	0.4342
93	*EBmac0684*	5HL	73.79	55	6	2.27	0.5585
94	*GBM1506*	5HL	75.45	55	6	2.13	0.5312
95	*scssr15334*	5HL	77.79	55	6	2.19	0.5444
96	*GBM1483*	5HL	80.64	55	2	1.11	0.1014
97	*Bmag0812*	5HL	90.32	55	10	4.62	0.7835
98	*scssr05939*	5HL	90.64	55	3	1.67	0.4022
99	*GBM1227*	5HL	91.82	55	2	1.19	0.1626
100	*GBM1231*	5HL	102.27	55	3	1.15	0.1270
101	*GBM1438*	5HL	104.74	55	3	1.35	0.2616
102	*GBM1295*	5HL	107.11	55	3	2.47	0.5945
103	*GBM5008*	5HL	118.98	55	4	1.53	0.3479
104	*GBM1363*	5HL	120.68	55	2	1.36	0.2637
105	*GMS061*	5HL	122.09	60	8	3.76	0.7339
106	*EBmatc0003*	5HL	127.69	60	4	2.19	0.5452
107	*GBM1470*	5HL	130.55	55	1	1.00	0.0000
108	*GBM1166*	5HL	133.5	55	2	1.32	0.2449
109	*GBM1490*	5HL	144.14	55	3	1.97	0.493
110	*GBM1164*	5HL	156.88	55	2	1.09	0.0853
111	*84c21j33*	6HS	0	55	4	2.93	0.6585
112	*Bmac0316*	6HS	7.16	55	6	1.42	0.2970
113	*GBM1270*	6HS	36.52	55	2	1.23	0.2052
114	*GBM1215*	6HS	39.54	55	6	2.69	0.6283
115	*GBM1212*	6HS	55.1	55	3	1.41	0.2929
116	*Bmac0040*	6HS	61.07	55	7	2.14	0.5330
117	*Bmag0344*	6HS	67.8	55	1	1.00	0.0000
118	*scssr05599*	6HL	96.34	55	4	3.12	0.6797
119	*GBM1140*	6HL	97.31	55	2	1.99	0.4998
120	*GBM1274*	6HL	123.45	55	1	1.00	0.0000
121	*GBM1276*	6HL	124.29	55	2	1.93	0.4824
122	*GBM1275*	6HL	124.29	55	3	1.98	0.4959
123	*GBM1087*	6HL	127.7	55	3	2.97	0.6632
124	*GBM1404*	6HL	129.76	55	1	1.00	0.0000
125	*GBM1126*	7HS	8.8	55	4	2.67	0.6258
126	*Bmag0206*	7HS	15.25	55	12	5.61	0.8216
127	*GBM5060a*	7HS	unknown	55	1	1.00	0.0000
128	*GBM1326a*	7HS	unknown	55	1	1.00	0.0000
129	*GBM5060b*	7HS	31.24	55	7	3.42	0.7079
130	*GBM1326b*	7HS	31.24	55	7	3.58	0.7208
131	*EBmac0603*	7HS	35.39	60	5	1.74	0.4267

Table 2: Continued

Table 2: Continued

132	*Bmag0914*	7HS	46.85	55	7	2.32	0.5686
133	*GBM1464*	7HS	53.43	60	6	1.93	0.4824
134	*Bmac0282*	7HS	59.31	55	1	1.00	0.0000
135	*Bmac0187*	7HS	72.57	60	8	3.43	0.7080
136	*GBM1432*	7HS	72.81	55	1	1.00	0.0000
137	*Bmag0321*	7HL	79.24	60	9	4.09	0.7559
138	*EBmac0827*	7HL	80.57	55	8	2.93	0.6584
139	*GBM1115a*	7HL	unknown	55	1	1.00	0.0000
140	*GBM1115b*	7HL	81.85	55	5	4.48	0.7767
141	*Bmag0369*	7HL	83.31	60	5	1.91	0.4749
142	*GBM1303*	7HL	86.43	55	9	3.78	0.7358
143	*GBM1297*	7HL	88.56	55	2	1.46	0.3157
144	*GBM1174*	7HL	93.86	55	2	1.25	0.1983
145	*GBM5225*	7HL	101.73	55	3	2.61	0.6171
146	*GBM1456*	7HL	136.79	55	2	1.65	0.3922
147	*scssr04056*	7HL	148.22	55	11	4.18	0.7608

[a]Genetic position (in cM) of marker on chromosome based on reported by (Varshney *et al.*, 2007)
[b]Observed number of alleles
[c]Effective number of alleles
[d]Polymorphism information content

Pearson correlation coefficients between grain weight, grain length and grain width in different field trials were calculated using SPSS.

For each marker, its genetic diversity parameters, observed number of alleles and effective number of alleles per locus were calculated using POPGENE software version 1.32 (Yeh and Boyle, 1999). Additionally, Polymorphism information content (PIC) values were computed by the formula: PIC=1-$\sum P_i^2$, where P_i is the frequency of the ith SSR allele, with the software PowerMarker version 3.25 (Liu and Muse, 2005).

Population Structure Estimate

The population structure of the diversity panel was investigated based on a set of 28 unlinked SSR markers, which were located on different chromosome arms. The genetic distance between two chosen markers on the same chromosome arm was more than 20 cM to avoid genetic linkage (Kulwal *et al.*, 2012). Population structure was estimated using principal component analysis (PCA) based on simple matching of alleles performed by software TASSEL 2.0 (Yu *et al.*, 2006) and by the Bayesian clustering analysis with STRUCTURE software version 2.3.3 (Pritchard *et al.*, 2000) running 10 times independently, with *K* ranging from 1 to 11 in each run using default setting of admixture model for the ancestry of individual and correlated allele frequencies. To confirm the true number of subgroups (*K*), Δ*K* calculated as described by Evanno *et al.* (2005) was plotted against *K* for the second approach. The best number of population subgroup was determined by Δ*K* with their peaking value (Evanno *et al.*, 2005).

Association Analysis

Association analysis was carried out using mixed linear model (MLM) approach as described in the software package TASSEL 2.0 (Bradbury *et al.*, 2007). SSR markers with minor allele frequency less than 5% were removed from the data set to reduce false associations. Furthermore, to control both Type I and Type II errors, *Q* values obtained by the software STRUCTURE and kinship matrix generated using the program TASSEL 2.0 were implemented as covariates in MLM analysis (Bradbury *et al.*, 2007).

A threshold of P=0.01 [-Log$_{10}$(P)=2] was set for declaring the significant marker-trait associations. Significant markers representing the same QTL were defined as having a linkage distance less than 12 cM and LD (measured as r^2 from pair-wise analysis) greater than 0.2 (Locatelli *et al.*, 2013).

Allele Effects Evaluate

Phenotypic effects of major alleles were evaluated in comparison to the 'null allele' (plus missing and rare alleles) for each locus (Breseghello and Sorrells, 2006b), and T-test between major allele and 'null allele' was conducted using the SPSS statistical package (SPSS Inc., Chicago, IL).

Results

Phenotypic Analysis

Large phenotypic variations were observed for grain weight, grain length and grain width in different trials, and the phenotype values of each trait followed normal distribution in the population (Table 3), indicating a multi-gene genetic programming model. The heritability (h^2_m) for grain weight, grain lenth and grain width across the three environments were 83.6%, 70.0% and 69.9%, respectively (Table 3), suggesting the grain length and grain width were more sensitive to environment than grain weight.

ANOVA also showed that the mean square (MS) values of environment (E) for grain length and grain width were much greater than their MSs of genotype (G) (Table 4). Moreover, G×E interaction (GEI) variances were significant for grain weight, length and width (P<0.01) reflecting that different genotypes had different sensitivity to environmental alternatives. However, their values of MS were often less than G and E (Table 4). Positive correlations were detected among these traits (Table 5). The average of correlation coefficient between grain weight and grain width was greater than that between grain weight and grain length across multi-environments (Table 4). It seemed that grain width had stronger influence on grain weight.

Genetic Diversity

In this study a total of 147 SSR markers distributed on all seven chromosomes were screened to evaluate the panel diversity, with 19 markers on the chromosome 1H, 28 on 2H, 14 on 3H, 20 on 4H, 29 on 5H, 14 on 6H and 23 on 7H. Most of the loci detected for the SSR markers were distributed on short and long chromosome arm uniformly, except that 16 out of 20 loci were clustered on the long arm of chromosome 4H. Diversity statistics calculated for polymorphic locus were summarized in Supplementary Table 2. The total number of alleles ranged from 1 to 15, with an average of 4.5 alleles per locus. The effective number of alleles varied from 1.0 to 11.1, with an average of 2.3. The monomorphic loci, such as *GBM1092*, *GBM1200*, *EBmac0525*, *Bmag0023*, *GBM1470*, *GBM1274*, *GBM1404*, *Bmag0344*, *GBM1115a*, *GBM1432*, *GBM5060a*, *GBM1326a* and *Bmac0282*, were excluded and the rest ones were selected for further AM analysis. For the left 134 loci, PIC value was estimated from 0.0177 to 0.9086 (*Bmag0323*). The average PIC values of the seven chromosomes ranged from 0.3883 (6H) to 0.5265 (4H) (Fig. 5).

Population Structure

The model-based Bayesian clustering showed that the average Ln[P(D)] (log-probability of data) value increased continuously with *K* increasing from 1 to 11, and the most apparent inflection was obtained when *K* was 2 (Fig. 6). The result of subgroup numbers (*K*) was further inferred using Δ*K* estimation, and the maximum peak value of Δ*K* was also obtained at *K*=2 (Fig. 1). Thus, the panel of 112 barley accessions was divided into two distinct subgroups using model-based Bayesian clustering. Interestingly, principal component analysis strong supported the similar result that the first subgroup accessions mostly originated from East Asia (Fig. 2), in which all of them are hull-less barley, while the second subgroup accessions were mostly from North America (Fig. 2), in which most of them are hulled barley except for the accessions WAS3, LDLQK, MZQK, B3034

Table 3: Phenotypic distribution and heritability of grain weight, grain length and grain width in the AM panel

Trait	Trial	Mean ± S.E.	Range Max	Min	Asymp. Sig.[a]	h^2_m (%)
TGW (g)	CD2014	27.9±0.5	41.6	13.5	0.887	83.6
	CD2015	29.1±0.5	45.4	17.9	0.949	
	NC2015	28.7±0.6	43.6	14.5	0.593	
GL (mm)	CD2014	6.42±0.07	8.03	4.82	0.998	70.7
	CD2015	6.45±0.07	8.06	4.56	0.852	
	NC2015	6.88±0.07	8.76	4.99	0.817	
GW (mm)	CD2014	2.59±0.03	3.21	1.57	0.636	69.9
	CD2015	2.84±0.03	3.44	2.02	0.983	
	NC2015	2.82±0.03	3.51	1.94	0.933	

TGW, grain weight; GL grain length; GW, grain width
[a]Two-tail and Kolmogorov-Smirnov test for normal distribution

Table 4: ANOVA analyses of grain weight, grain length and grain width in multi-environment trials

Trait	Source of variation	DF	MS	MS %[a]
TGW	Genotype (G)	111	146.44**	53.92
	Environment (E)	2	87.22**	32.12
	GEI	222	37.90**	13.96
GL	Genotype (G)	111	1.55**	8.89
	Environment (E)	2	15.00**	86.53
	GEI	222	0.85**	4.86
GW	Genotype (G)	111	0.29**	5.94
	Environment (E)	2	4.36**	90.92
	GEI	222	0.15**	3.15

TGW, grain weight; GL grain length; GW, grain width
**indicate significant difference at P<0.01
[a]MS%=100% x $MS_{(G, E\ or\ GEI)}/(MS_G + MS_E + MS_{GEI})$

Table 5: Correlation coefficients among grain weight, grain length and grain width in different trials

Traits	Grain length	Grain width
Grain weight	0.599**	0.588**
	0.378**	0.523**
	0.264**	0.687**
Grain lenth		0.673**
		0.579**
		0.581**

[¶]For each trait, the correlation coefficients from CD2014, CD2015 and NC2015 are represented in the first, second and third rows, respectively
**indicate significance at P<0.01

and Tibetannia. All of genotypes in the first subgroup are six-row barley except accession "K5", while most of two-row barleys are clustered into the second subgroup of North America.

Association Mapping

Marker-trait associations were identified using the Q+K model. A significant QTL was declared when the P value for marker-trait association was less than 0.01 [-Log$_{10}$ (P) =2] in the QTL regions. A total of 11 QTLs were detected for three traits investigated in this study across three environments (Table 6).
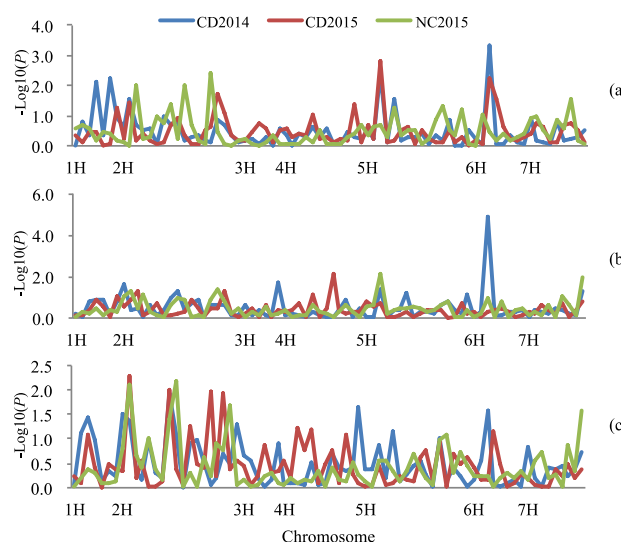
Table 6: Markers showing significant association with grain weight, grain length and grain width in mixed linear model (Q+K)

Trait	Locus	Chr.	Pos. (cM)	CD2014		CD2015		NC2015	
				-Log$_{10}$(P)	R^2 (%)	-Log$_{10}$(P)	R^2(%)	-Log$_{10}$(P)	R^2(%)
Grain weight	*Bmag0579*	1HL	132.84	2.1	2.71	-	-	-	-
	scssr08238	1HL	139.81	2.2	2.75	-	-	-	-
	scssr10226	2HS	46.63	-	-	-	-	2.0	2.73
	GBM1468	2HL	84.06	-	-	-	-	2.5	4.16
	GBM1408	2HL	89.44	-	-	-	-	2.1	2.76
	GBM5028	5HS	27.41	2.4	2.60	2.8	2.08	-	-
	GBM1215	6HS	39.54	3.3	4.80	2.3	1.86	-	-
Grain length	*HVMLOH1A*	4HL	102.27	-	-	2.2	4.17	-	-
	GBM5028	5HS	27.41	-	-	-	-	2.1	2.25
	GBM1215	6HS	39.54	4.9	4.49	-	-	-	-
	GBM1456	7HL	136.79	-	-	-	-	2.0	2.05
Grain width	*scssr10226*	2HS	46.63	-	-	2.3	5.82	2.1	3.26
	GBM1468	2HL	84.06	-	-	2.0	5.67	-	-
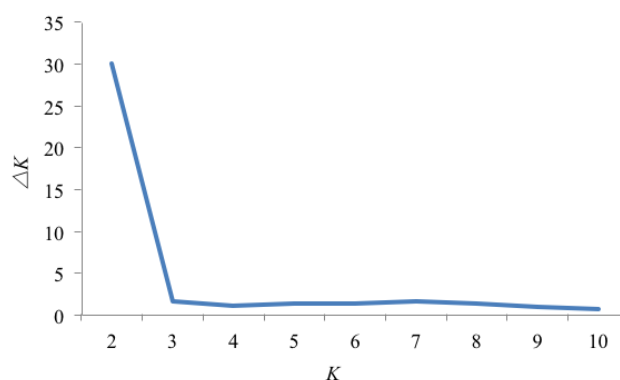
Fig. 1: The best possible subgroup number for 112 spring barley accessions using Δ*K* approaches
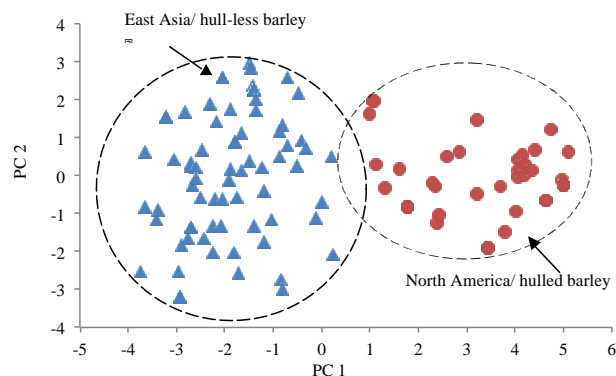
Fig. 2: Scatter plot of principal component 1 (PC1) drawn against principal component 2 (PC2). Each triangle or dot sign indicates an individual genotype in different subgroups of the AM panel. East Asia and North America accessions are represented by triangles and dots, respectively

Fig. 3: Association mapping of 112 barley genotypes using the MLM for (a) grain weight, (b) grain length and (c) grain width. The P values were converted into [-Log$_{10}$(P)]

Grain Weight

A total of five QTLs associated with seven markers were detected on chromosome arms 1HL, 2HS, 2HL, 5HS and 6HS in this study (Table 6; Fig. 3a). On 1HL, two SSR loci

Bmag0579 and *scssr08238* were significantly associated with grain weight in the CD2014 environment, and the genetic distance between these two loci was 6.97cM with the value of the linkage disequilibrium (D′) between them was 0.85, indicating that they were located on the same QTL region with the most significant locus *scssr08238* (Table 6; Fig. 3a). On 2HL, two loci were detected to be significantly associated with grain weight in the trail of NC2015 within 5.38 cM region (Table 6). The most significant locus was *GBM1468*, and thus the QTL was designated as *QTgw-GBM1468*. The QTL on chromosome 2HS was prominent in NC2015 environment, whereas QTLs on chromosomes 5HS linked with locus *GBM5028* and 6HS linked with locus *GBM1215* were stable detected in both CD2014 and CD2015 environments. None of QTLs was significant in all three trials and all associated markers

explained just a small portion of the phenotypic variation, ranging from 1.86% to 5.82%, as expected for complex polygenic trait.

Grain Length

Four QTLs were found to be significantly associated with grain length (Table 6; Fig. 3b). The QTL linked with *GBM1215* locus located on chromosome 6HS had the strongest associations and explained 4.49% of the phenotypic variation. Moreover, this QTL had the highest [-Log$_{10}$ (P)] in our study (Table 6; Fig. 3b). Four QTLs related to grain length were detected only in a single environment and also explained just a small portion of the phenotypic variation (Table 6; Fig. 3b).

Grain Width

Two QTLs on chromosome 2H were identified for grain width (Table 6; Fig. 3c). One QTL associated with locus *scssr10226* explained 3.26% and 5.82% of the phenotypic variation in NC2015 and CD2015 environment, respectively. Another one linked with locus *GBM1468* explained 5.67% of the phenotypic variation and was detected only in CD2015 environment (Table 6; Fig. 3c).

Phenotypic Effects of Major Alleles

Among these QTLs for investigated traits, three QTLs for grain weight and width stably expressed in two environments and one QTL for grain length with the highest [-Log$_{10}$ (P)] (Table 6) were selected to evaluate phenotypic effects of their major alleles. At the locus of *GBM5028* associated with grain weight, a total of two major alleles *A1* and *A2* were observed and the representative accessions for them were DLH (accession code: 5) and 9516 (accession code: 4) (Table 1), respectively. Accessions carrying *A2* allele produced significantly lower grain weight in CD2014 environment, which decreased about 2.5 g of TGW (Fig. 4a). Among the three major alleles at the locus of *GBM1215* associated with grain weight, the allele of *A4* increased the average of 3.5 g of TGW significantly, while *A3* decreased the TGW significantly (Fig. 4a). *GBM1215* was associated with grain length significantly. Among the three detected alleles, *A3* decreased grain length significantly in both the trials of CD2014 and NC2015, *A4* increased grain length significantly in the trials of CD2014, while the allele *A5* was associated with opposite effects in CD2014 and NC2015 (Fig. 4b). At the locus of *scssr10226* associated with grain width, the allele *A3* produced significantly narrower grains, while *A1* was significantly associated with increased grain width (Fig. 4c).

Discussion

In our study, a total of five QTLs were significantly associated with grain weight. The QTL linked to *scssr10226*
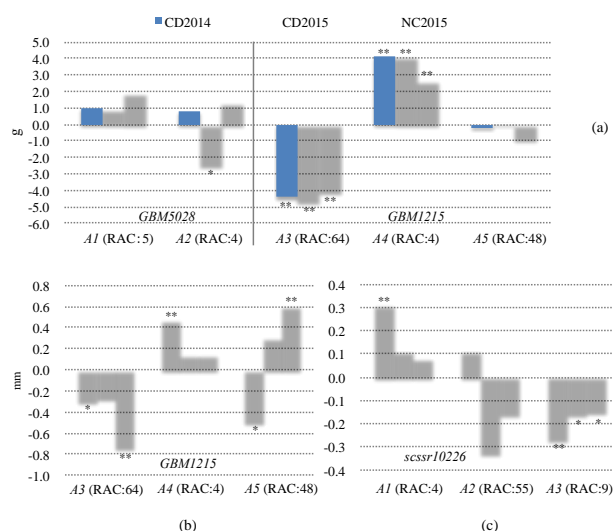

Fig. 4: Phenotypic effect of the major alleles at SSR loci associated with (a) grain weight, (b) grain length and (c) grain width in the trials of CD2014, CD2015 and NC2015. Representative accession code (RAC). *, ** mean significance of phenotypic effect of the major allele at the 0.05 and 0.01 level when comparing with 'null alleles', respectively
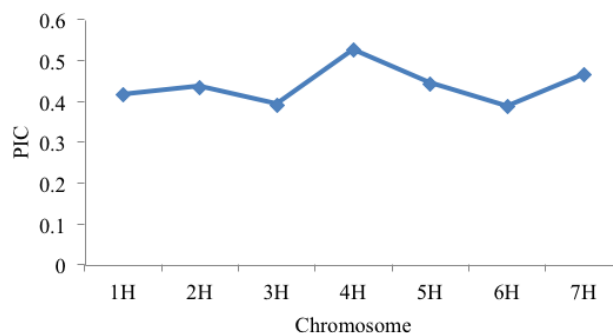

Fig. 5: Average PIC value of each chromosome in the panel of 112 barley genotypes

on the chromosome 2HS in this study is consistent with several reported QTLs, such as QTLs in the interval of *HVM36-GMS3* (Pillen *et al.*, 2003; Korff *et al.*, 2006) and *GBM1214* related to grain weight with [-Log$_{10}$ (P)] score more than 10 (Mikolajczak *et al.*, 2016), while *scssr10226* is tightly linked to *GBM1214* and *GMS3* based on the comparisons of genetic maps reported by Korff *et al.* (2004) and Varshney *et al.* (2007). The significant QTL *QTgw-GBM1468* identified in this study on 2HL corresponded to the QTL identified by Li *et al.* (2005) and Bauer *et al.* (2009), and the QTL on chromosome 5HS in our study is in the similar genetic position to that reported by Saal *et al.* (2011). In addition, few QTLs are reported around the interval of *QTgw-scssr08238* on chromosome 1H.
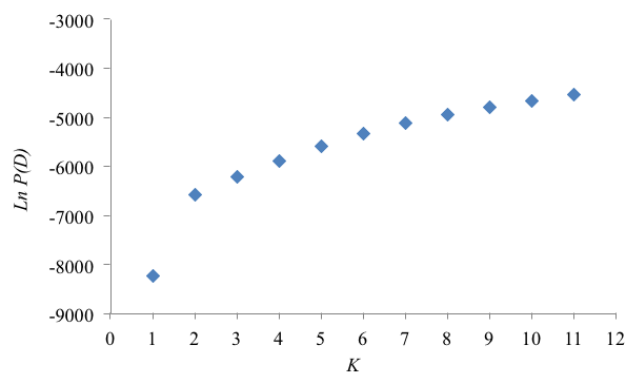
Fig. 6: Scatter plot of LnP(D) (Log probability of data) , averaged over ten replications drawn against *K*. apparent inflection found at *K*=2
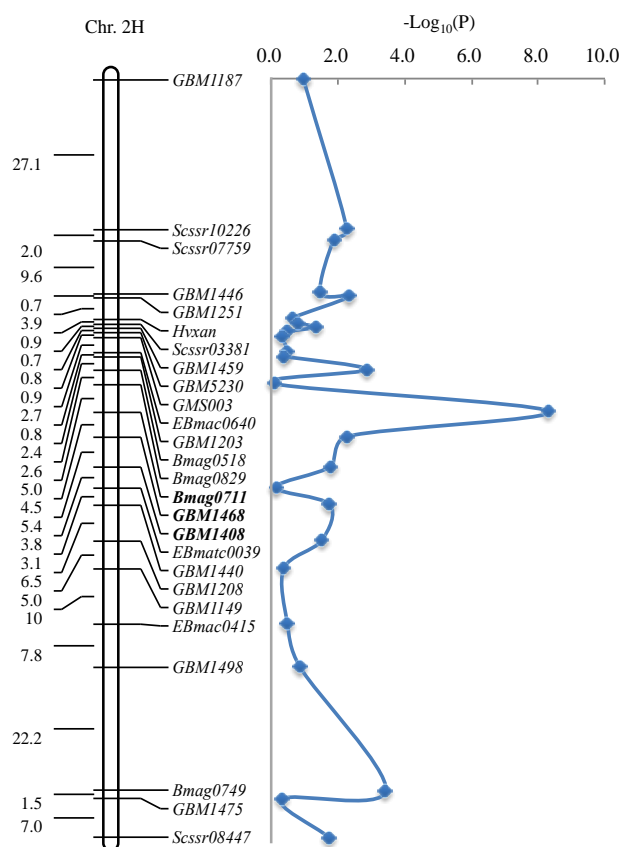

Fig. 7: Association mapping for row type on chromosome 2H of barley. Markers in bold indicates the interval most significantly related to row type

Among the four QTLs for grain length, the QTL on the long arm of chromosome 7H is similar in position to that report by Walker *et al.* (2013). Nevertheless, the others detected in this study seem to be different from previous reports (Backes *et al.*, 1995; Ayoub *et al.*, 2002; Walker *et al.*, 2013; Zhou *et al.*, 2016). For the two QTLs related to

grain width on chromosome 2H, one linked to the locus *GBM1468* that is also significantly associated with row type (Fig. 7) is nearby locus *Vrs1* (Varshney *et al.*, 2007; Huang and Wu, 2011), which determines barley row type that is associated with kernel size, as QTLs associated with grain shape and weight have been detected in the interval of HVBKASI-Vrs1 (Marquezcedillo *et al.*, 2001; Ayoub *et al.*, 2002).

In our study, grain weight was highly positively correlated with grain length and grain width. Correlations between them partially explained the phenomenon of QTL clustering in some chromosome regions, as half of QTLs for gain length and width were co-localized with QTLs for grain weight (Table 6; Fig. 3), suggesting that grain weight was jointly influenced by both grain length and width. Walker *et al.* (2013) also reported that grain weight was often positively related to grain width and QTL cluster was found for both of them. However, for the grain length, grain weight was not always highly or positively correlated with it (Backes *et al.*, 1995; Schnaithmann and Pillen, 2013). In this study, grain width was significantly positively correlated with grain length, but no QTL cluster was detected for them, indicating that grain width and grain length may be independent traits in genetic basis and the correlation between them is mostly caused by artificial selection, and this also happened in barley reported by Schnaithmann and Pillen (2013) and in wheat reported by Gegas *et al.* (2010).

For the SSR locus *GBMS5028* associated with grain weight and grain length, *YABBY* gene family containing *FAS* gene (Cong *et al.*, 2008) and *YAB1, CRC, DL* and *INO* genes (Han *et al.*, 2015) which played important roles in the development of leaf, flower, and fruit in tomato was found to be tightly linked to it (http://webblast.ipk-gatersleben.de/barley/; http://www.ncib.nlm.nih.gov/). *GBM1408* associated with grain weight was linked to gene that was homologous to 1-aminocyclopropane-1-carboxylate (*ACC*) synthase gene, which regulates a number of plant processes, ranging from seed germination to organ senescence (Bleecker and Kende, 2000). For *GBM1456* locus related to grain width, its linked gene *GDSL* esterase/lipase belonged to the α/β hydrolase fold superfamily of proteins, regulating coleoptile elongation in rice as *GDSL-containing enzyme rice 1* gene (*GER1*) (Riemann *et al.*, 2007). Actually, the SSR primers for *GBM1456* were developed from the sequence of *GDSL* esterases/lipases gene (http://wheat.pw.usda.gov/).

For complex quantitative traits, QTLs identified by AM often explains smaller percentage of the phenotypic variation than using bi-parental mapping (Massman *et al.*, 2011; Zhou and Steffenson, 2013; Zhou *et al.*, 2014). In this study, the detected QTLs for grain size related traits explained no more than 6% of the phenotypic variation, which are probably caused by (1) artificial selection in cultivated germplasm pool used in our study which fixing that larger-effect QTL/gene more rapidly under the process

of domestication or breeding, and (2) insufficient marker density in this study (Massman *et al.*, 2011; Zhou and Steffenson, 2013).

Comparison of phenotypic effects between different QTL alleles from different germplasms is useful for breeders to choose suitable parental lines. For instance, at the site of *GBM1215* associated with grain weight, if using parental lines only containing *A3* and *A5* alleles to construct bi-parental QTL mapping population, QTL allele of *A5* would be favor QTL allele increasing grain weight. Actually, *A4* was the most interesting QTL allele for enhancing grain weight.

In conclusion, we identified eleven QTLs including five for grain weight, four for grain length and two for grain width and elite alleles improving these traits at these QTLs loci. Several QTLs identified had similar genetic regions to previous reported indicating that these QTLs might be useful for enhancing grain weight, length and width in barley breeding program.

Acknowledgments

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