

# Detection of QTLs controlling alpha-amylase activity in a diversity panel of 343 barley accessions

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**Abstract** The  $\alpha$ -amylase activity of cultivated barley is critically important to the brewing industry. Here, we surveyed variation in malt  $\alpha$ -amylase activity in 343 cultivated barley accessions from around the world. Population structure analysis based on genotype data at 1536 SNPs clustered these accessions into two groups, one comprising South-East Asian and Ethiopian accessions and one group containing the other accessions. A genome-wide association study identified significant quantitative trait loci (QTLs) for  $\alpha$ -amylase activity on all seven chromosomes of barley. Accessions showing high and low  $\alpha$ -amylase activity were crossed with the high-quality Japanese malting barley cv. Harun Nijo to develop F<sub>2</sub> mapping populations. We identified two QTLs on chromosome 6H in a cross between Harun Nijo (high activity) × Weal (highest activity). Single QTLs were identified each on 3H, 4H, and 5H from a cross between Harun Nijo (high activity) × VLB-1 (low activity), indicating that the high  $\alpha$ -amylase activity in Harun Nijo might be derived from loci on these chromosomes. The addition of the high  $\alpha$ -

amylase activity QTL alleles from chromosome 6H in cv. Weal further increased the  $\alpha$ -amylase activity conferred by alleles of Harun Nijo. These results demonstrate that a target haplotype can be successfully improved using a strategy comprising diversity analysis of ex situ collections followed by introducing effective new alleles.

**Keywords**  $\alpha$ -amylase · Genome wide association study · *Hordeum vulgare* · QTL · SNP

## Introduction

The malting process involves germination of barley grains and activation of enzymes that convert starch to sugar, which brewing yeast ferments into ethanol. Germinating barley grain contains  $\alpha$ -amylases,  $\beta$ -amylases, limit dextrinases, and  $\alpha$ -glucosidases, which combine to degrade amylose and amylopectin into glucose (Schwarz and Li 2011). The  $\alpha$ -amylases catalyze the hydrolysis of (1,4)- $\alpha$ -linkages in amylose and amylopectin in an endo-manner to yield linear and branched (1,6:1,4- $\alpha$ -linked) dextrans (Schwarz and Li 2011). Since  $\alpha$ -amylases are the only sufficiently thermostable amylolytic enzymes, they are largely responsible for the starch degradation throughout the full duration of mashing (Hayes et al. 2003). The  $\alpha$ -amylases in grain are therefore of great importance to brewers.

The genetic and protein factors responsible for  $\alpha$ -amylase activities have attracted the interest of geneticists and breeders. In cereals, there are two classes of  $\alpha$ -

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amylases, AmyA and AmyB. Barley and wheat contain AmyA  $\alpha$ -amylases, which are further classified by isoelectric point (IEP) into Amy1 (high IEP) and Amy2 (low IEP) (Huang et al. 1992). Muthukrishnan et al. (1984) found that barley chromosome 7H contains three or more genes closely related to one of the  $\alpha$ -amylase cDNA clones, and chromosome 6H carries six or more genes closely related to a different  $\alpha$ -amylase cDNA clone. Khursheed and Rogers (1988) further showed that the genes encoding the low PI (or type A) isozymes are on chromosome 7H, while the genes encoding the high PI (or type B) isozymes are on chromosome 6H. The genome of barley cultivar Morex contains 12  $\alpha$ -amylase (amy) family sequences, which can be classified into four subfamilies, with gene duplication events having occurred in the subfamilies amy1 (four genes) and amy2 (three genes), located on chromosomes 6H and 7H, respectively (Mascher et al. 2017). Amy2 has a known crystal and molecular structure (Kadziola et al. 1994) and documented allelic variation (Ko et al. 1996). SNP markers in *Amy1* are significantly associated with important malting parameters in 117 European spring and winter barley cultivars (Matthies et al. 2009). Szűcs et al. (2009) localized 20 previously reported QTLs for  $\alpha$ -amylase activity on a consensus genetic map of the Oregon Wolfe Barley population. In addition, Marquez-Cedillo et al. (2000) detected 8 of these 20 QTLs in one doubled haploid Harrington/Morex cross population, including *Amy1* (chromosome 6H) and *Amy2* (7H) loci. Harrington (two-row) and Morex (six-row) are both elite malting barley cultivars from North America, but they have different pedigrees and exhibit different allelic combination in the *Amy1* and *Amy2* regions. Though genetic variation in  $\alpha$ -amylases has been well documented, it has not yet been reported to improve the malting quality of cultivars.

The aim of this study was to identify QTLs controlling enzyme activity of  $\alpha$ -amylases and use that information to improve malting quality in barley. The International Barley Core Collection is a set of materials representing world barley diversity (Knüpffer and Hintum 2003), with samples from Europe, West Asia and North Africa (WANA), the Americas, and South-East Asia. These materials have been used in genome-wide association studies linking SNPs with grain cadmium concentration (Wu et al. 2015) and plant salt tolerance (Hanan et al. 2014). Here, we used these accessions to identify new QTL alleles linked to differences in  $\alpha$ -amylase activity. We then tested a strategy

for the improvement of barley germplasm. We carried out a QTL analysis using two representative accessions from the Barley Core sub-collection selected according to their  $\alpha$ -amylase activities. We crossed those accessions with the Japanese elite malting cultivar Haruna Nijo to increase the  $\alpha$ -amylase activity even further.

## Materials and methods

### Plant materials

A Barley Core sub-collection (BCS) consisting of 318 cultivated accessions selected from the International Barley Core Collection and 25 accessions of Ethiopian landraces from the Okayama University collection were used for malt  $\alpha$ -amylase analysis and genome-wide marker genotyping. Twenty plants of each accession were planted in the experimental field at Okayama University, Kurashiki, Japan (34° 35' N and 133° 46' E), and their seed samples were harvested in 2008 for  $\alpha$ -amylase analysis. A single plant from each accession was used to extract DNA for marker genotyping. For the QTL analysis, two mapping populations were derived from the cross Haruna Nijo (BCS052)  $\times$  Weal (BCS143) (92 F<sub>2</sub> plants) and Haruna Nijo  $\times$  VLB-1 (BCS033) (93 F<sub>2</sub> plants). These populations and their parents (20 individuals each) were planted and harvested in 2015. Seed samples were used for  $\alpha$ -amylase analysis, and F<sub>2</sub> leaf segments were used to extract DNA for marker genotyping.

### Malt $\alpha$ -amylase assay

Malt samples of the 343 BCS accessions, 92 and 93 F<sub>2</sub> individuals from the mapping populations and three samples each of their parents were prepared by the malting facilities at the Bioresources Research and Development Department of Sapporo Breweries Ltd. in Ota, Japan. Samples were processed using an automatic micro-malting system (Phoenix Systems, Adelaide, Australia). Briefly, steeping time of each sample was 52 h prior to germination. Steeping included a 7-h air rest after every 5-h steep in 15 °C water. Germination lasted 2 days at 15 °C and the kilning scheme was 14 h at 55 °C, 8 h at 65 °C, 3.5 h at 75 °C, and 4 h at 83.5 °C. After malting, each sample was powdered with a desktop sample mill IFM-180G (Iwatani, Japan) and then used for enzyme extraction. The activity of malt  $\alpha$ -

amylase in each sample was measured using an  $\alpha$ -amylase assay kit (Ceralpha Method, catalog # K-CERA, Megazyme u.c., Ireland) following the manufacturer's protocol. After the enzyme reaction, absorbance at 405 nm was measured for each sample with the absorptiometer POWERSCAN HT (DS Pharma Biomedical Co., Ltd., Japan). Reactions were done in triplicate for each sample, and the average was used as  $\alpha$ -amylase activity. For parental samples of QTL analysis, three plants for each parent were analyzed. Statistical difference of averages are tested by analysis of variance ( $n > 2$ ) or Student's *t*-test ( $n = 2$ ).

#### DNA extraction and SNP genotyping

To genotype the 343 BCS accessions, genomic DNA was extracted with the DNeasy Plant mini kit (QIAGEN, Germany) according to the manufacturer's protocol. DNA sample quality was checked by gel electrophoresis. Genotyping was done using the Illumina GoldenGate® assay with the 1536 SNP platform of barley oligonucleotide pool assay 1 (BOPA1, Close et al. 2009). To genotype the  $F_2$  plants, genomic DNA was extracted with GENE PREP STAR PI-480 (KURABO, Japan) according to the manufacturer's protocol (PLANT ver. 1). After a quality check, DNA samples were genotyped with an Illumina GoldenGate® assay using a 384 SNP platform developed from BOPA1 based on the genetic map position of Close et al. (2009) to avoid duplicate markers. All genotyping data were analyzed with the software GenomeStudio (Illumina, USA).

#### Genome-wide association of $\alpha$ -amylase activity in the Barley Core sub-collection accessions

Population structure was estimated from the BCS genotyping data with STRUCTURE ver. 2.3.4 (Pritchard et al. 2000) using an Admixture Model and MCMC chain length of 10,000 following a burn-in period of 10,000. Heterozygous alleles identified in the Illumina GoldenGate® assay were eliminated from the analysis. Markers with less than 10% minor allele frequency were also eliminated from the analysis. The appropriate number of ancestral population groups (*Q*) was estimated by calculating  $\Delta K$  (Evanno et al. 2005) and then used for association mapping. Marker-phenotype associations were detected using TASSEL ver. 5 (Bradbury et al. 2007) with a general linear model.

#### QTL mapping by $F_2$ linkage analysis

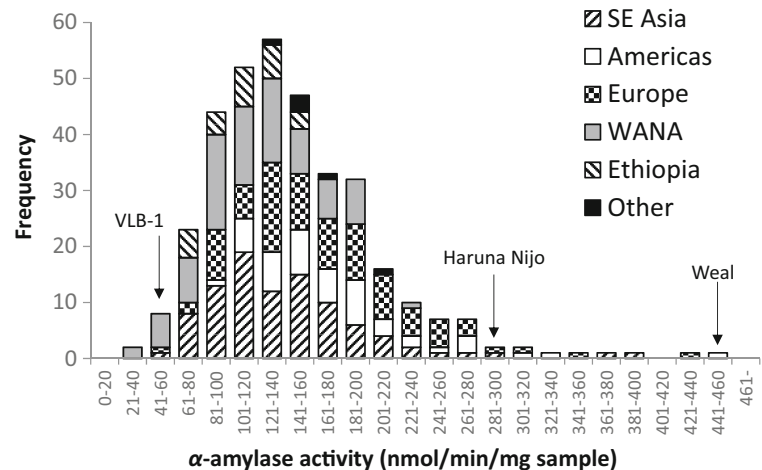
Two sets of genetic maps were constructed based on genotyping data from Haruna Nijo  $\times$  Weal ( $H \times W$ ) and Haruna Nijo  $\times$  VLB-1 ( $H \times V$ ) populations using Antmap ver. 1.2 (Iwata and Ninomiya 2006). Linkage groups were calculated with threshold values ranging from 0.3 (default) to 1.0, from 0.3 to 0.5 for  $H \times W$  and  $H \times V$ , respectively. A QTL analysis of malt  $\alpha$ -amylase activity was conducted using the composite interval mapping procedure in Windows QTL Cartographer ver. 2.5 (Wang et al. 2012) with a 1000 permutation test at  $p = 0.05$ .

## Results

#### Variation in malt $\alpha$ -amylase activity in the Barley Core sub-collection

The Barley Core sub-collection (BCS) accessions used in this study were selected to reflect the subgroup accessions in the original Core Collection. We tested these barley accessions to quantify the variation of  $\alpha$ -amylase activity in barley worldwide. Among the 343 BCS accessions we tested,  $\alpha$ -amylase activity varied widely, with a unimodal peak toward lower activity (Fig. 1). The average activity value was 146.4 nmol/min/mg, with a range from 32.9 to 460.0 (Table S1). In addition, there were significant differences in  $\alpha$ -amylase activity between subsets of the accessions ( $F = 10.93$ , Fig. 1). The average  $\alpha$ -amylase activity was higher in accessions from the Americas (181.2,  $n = 47$ ) and Europe (170.1,  $n = 86$ ), but lower in those from WANA (117.8,  $n = 83$ ) and Ethiopia (110.1,  $n = 25$ ). Accessions showing more than 300.0 nmol/min/mg were Weal (BCS143, USA; 460.0 nmol/min/mg), Strickhoff (BCS167, Switzerland; 429.8 nmol/min/mg), Kawaring 2 (BCS043, India; 382.1 nmol/min/mg), Revelatum Type 13 (BCS024, China; 366.0 nmol/min/mg), K16478 (BCS215, Russia; 359.3 nmol/min/mg), Acora (BCS096, Peru; 322.1 nmol/min/mg), Conquest (BCS113, Canada; 312.9 nmol/min/mg), and Colonia (BCS154, Germany; 308.6 nmol/min/mg). High  $\alpha$ -amylase genotypes were distributed in different regions of the world, but many of the low  $\alpha$ -amylase accession (below 60.0 nmol/min/mg) were collected from WANA countries.

**Fig. 1** Frequency distribution of  $\alpha$ -amylase activity in Barely Core sub-collection (BCS) accessions. Accessions were grouped by the collection subsets (WANA: West Asia and North Africa, SE Asia: South-East Asia), and Ethiopian accessions were from Okayama University



### Population structure analysis and genome-wide association study on Barley Core sub-collection

We genotyped barley accessions to estimate the linkage between the  $\alpha$ -amylase activity and SNP markers. Of the 1536 BOPA1 SNP markers, 1336 markers (87%) were polymorphic among the BCS accessions. Haplotypes of BCS accessions are shown in order of their subsets in Fig. S1, and some clear regional differences were revealed by the haplotypes - Ethiopian accessions, for example, showed almost identical haplotypes. We eliminated the heterozygous markers and carried out further analysis to estimate the population structure of the BCS accessions. In the STRUCTURE analysis,  $\Delta K$  (Evanno et al. 2005) was calculated from  $\ln(K)$  (natural logarithm for optimum number of population structure) because the  $\ln$  probability did not result a smaller  $K$  value (Fig. S2A). The largest  $\Delta K$  value was obtained at  $K = 2$ , which was assumed to be an appropriate number of ancestral populations (Fig. S2B). The  $Q$  (inferred ancestry) value of each accession was calculated ( $Q1$  and  $Q2$  for ancestral populations 1 and 2, Table S1). For each accession, the dominating ratio of  $Q1$  or  $Q2$  was used to categorize it into a group (Table S1). Group 1 mainly included accessions from South-East Asia and Ethiopia, and group 2 mainly included accessions from the Americas, Europe, Australia, and WANA. Group 1 had an average  $\alpha$ -amylase activity of 133.65 nmol/min/mg, whereas group 2 activity was 154.97 nmol/min/mg.

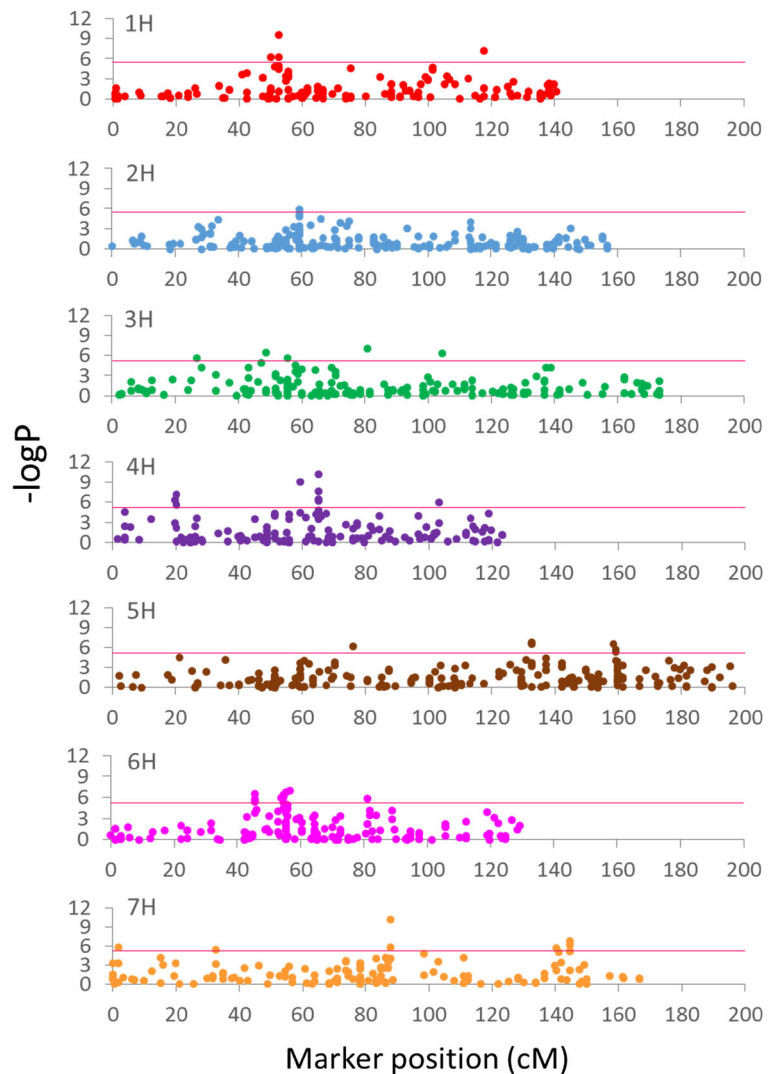
For a genome-wide association study, markers showing less than 10% frequency among all accessions were omitted, and  $\alpha$ -amylase activity was associated with a total of 1258 markers in the 343 BCS accessions. As

shown in Fig. 2, associations below the significance threshold of  $P < 0.01$  with Bonferroni correction ( $7.95e-06$ ) were detected for 22 regions on seven chromosomes (chromosome 1H: 2 markers, 2H: 1 marker, 3H: 4 markers, 4H: 4 markers, 5H: 3 markers, 6H: 3 markers, and 7H: 4 markers) (Table S2). Of these, four markers had  $R^2$  values greater than 10% (Table 1): markers 5297-796 (10.7%: 1H, 52.5 cM), 2421-520 (10.0%: 4H, 59.4 cM), ABC09431-1-1160 (11.4%: 4H, 65.1 cM), and 1696-557 (11.4%: 7H, 88.0 cM).

### QTL analysis of $\alpha$ -amylase activity in $F_2$ populations

Based on the  $\alpha$ -amylase activity data for the 343 BCS accessions, we selected two accessions, Weal and VLB-1, with high and low  $\alpha$ -amylase activity, respectively, to cross with the Japanese elite malting cultivar Haruna Nijo for QTL analysis. There were large differences in the average  $\alpha$ -amylase activities between the parental alleles of Haruna Nijo (177.1 nmol/min/mg, s.d. = 13.9)  $\times$  Weal (400.5 nmol/min/mg, s.d. = 10.1) ( $H \times W$ ) population and the Haruna Nijo  $\times$  VLB-1 (141.4 nmol/min/mg, s.d. = 8.9) ( $H \times V$ ) population. The  $\alpha$ -amylase activity in both  $F_2$  populations showed a continuous distribution, with a range of 107.8–379.4 nmol/min/mg in  $H \times W$  and 87.7–307.1 nmol/min/mg in  $H \times V$  (Fig. 3). Among the 384 genotyped SNPs, 179 markers in  $H \times W$  and 189 markers in  $H \times V$  showed polymorphisms between the parents of  $H \times W$  and  $H \times V$ . Linkage between these markers was calculated using Antmap (Iwata and Ninomiya 2006), resulting a 163-marker genetic map for  $H \times W$  and a 169-marker map for  $H \times V$  (Fig. S3).

**Fig. 2** Manhattan plot of  $\log P$  values for the linkage between markers and  $\alpha$ -amylase activity. The red line represents the significance threshold ( $-\log P = 7.95e-06$ )



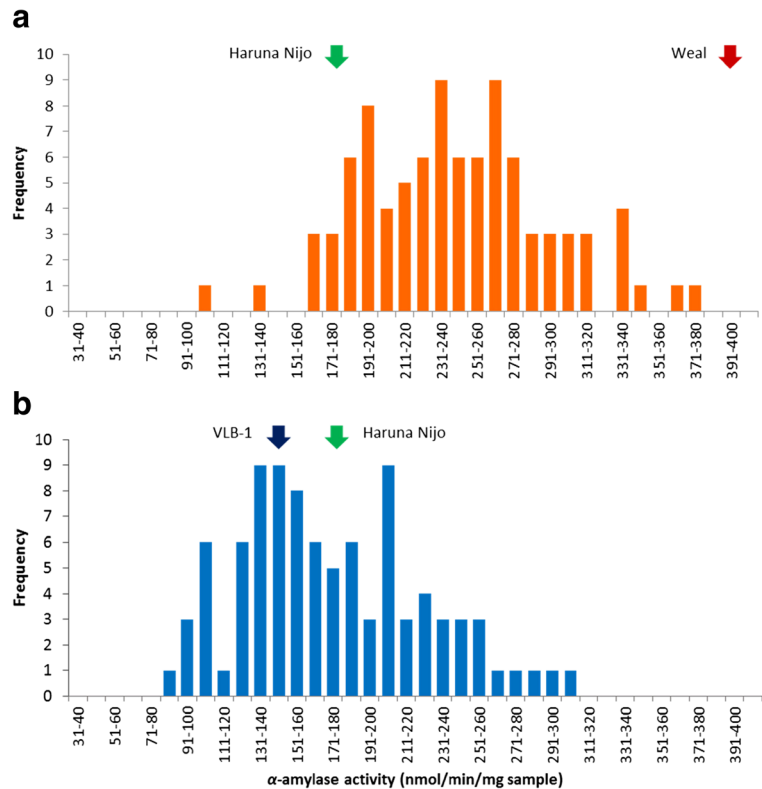
In the  $H \times W$  cross, the composite mapping procedure detected two QTLs on 6H of 51.0 cM (5771-91,  $LOD = 6.16$ ,  $R^2 = 0.19$ ) and 65.2 cM (4235-1617,  $LOD = 5.82$ ,  $R^2 = 0.18$ ), both with positive  $\alpha$ -amylase activity alleles from Weal (Fig. 4a, b, Table 2). The  $\alpha$ -

amylase activity difference between alleles at the marker 5771-91 was also significant ( $P < 0.00$ ), with average of 205.2 nmol/min/mg for the Haruna Nijo allele ( $n = 19$ ) and 273.0 nmol/min/mg for the Weal allele ( $n = 22$ ). The difference between alleles at 4235-1617 was also

**Table 1** The markers associated with malt  $\alpha$ -amylase activity in BCS

Marker #	Marker name	Chromosome	Position (cM)	$P$ value	$R^2$ (%)
44	5297-796	1H	52.5	2.95E-10	10.7
630	2421-520	4H	59.4	1.04E-09	10.0
645	ABC09432-1-1-160	4H	65.1	5.62E-11	11.4
1211	1676-557	7H	88.0	7.89E-11	11.4

**Fig. 3** Distribution of  $\alpha$ -amylase activity in  $F_2$  plants of the crosses **a** Haruna Nijo  $\times$  Weal and **b** Haruna Nijo  $\times$  VLB-1. Parental scores ( $n = 3$ ) are indicated by arrows



significant ( $P < 0.00$ ), with an average of 210.0 nmol/min/mg for the Haruna Nijo allele ( $n = 24$ ) and 272.3 nmol/min/mg for the Weal allele ( $n = 26$ ).

In the  $H \times V$  cross, the composite interval mapping procedure detected three QTLs associated with  $\alpha$ -amylase activity: one on 3H of 94.1 cM (10114-1946, LOD = 4.63,  $R^2 = 0.20$ ), one on 4H of 50.5 cM (3549-743, LOD = 3.99,  $R^2 = 0.13$ ), and one on 5H of 49.5 cM (8561-968, LOD = 4.03,  $R^2 = 0.04$ ) (Fig. 4 d, e, Table 2). The positive alleles at all three QTLs are from Haruna Nijo. The  $\alpha$ -amylase activity difference between alleles at the marker 10114-1946 was significant ( $P < 0.00$ ), with a mean of 202.4 nmol/min/mg for the Haruna Nijo allele ( $n = 25$ ) and 149.9 nmol/min/mg for the VLB-1 allele ( $n = 20$ ). The difference between alleles at marker 3549-7436 was also significant ( $P < 0.00$ ), with a mean activity of 206.2 nmol/min/mg for the Haruna Nijo allele ( $n = 17$ ) and 152.2 nmol/min/mg for the VLB-1 allele ( $n = 28$ ). The difference between alleles at the 8561-968 marker was also significant ( $P = 0.01$ ), with a mean activity of 189.9 nmol/min/mg for the Haruna Nijo allele ( $n = 23$ ) and 153.6 nmol/min/mg for the VLB-1 allele ( $n = 22$ ).

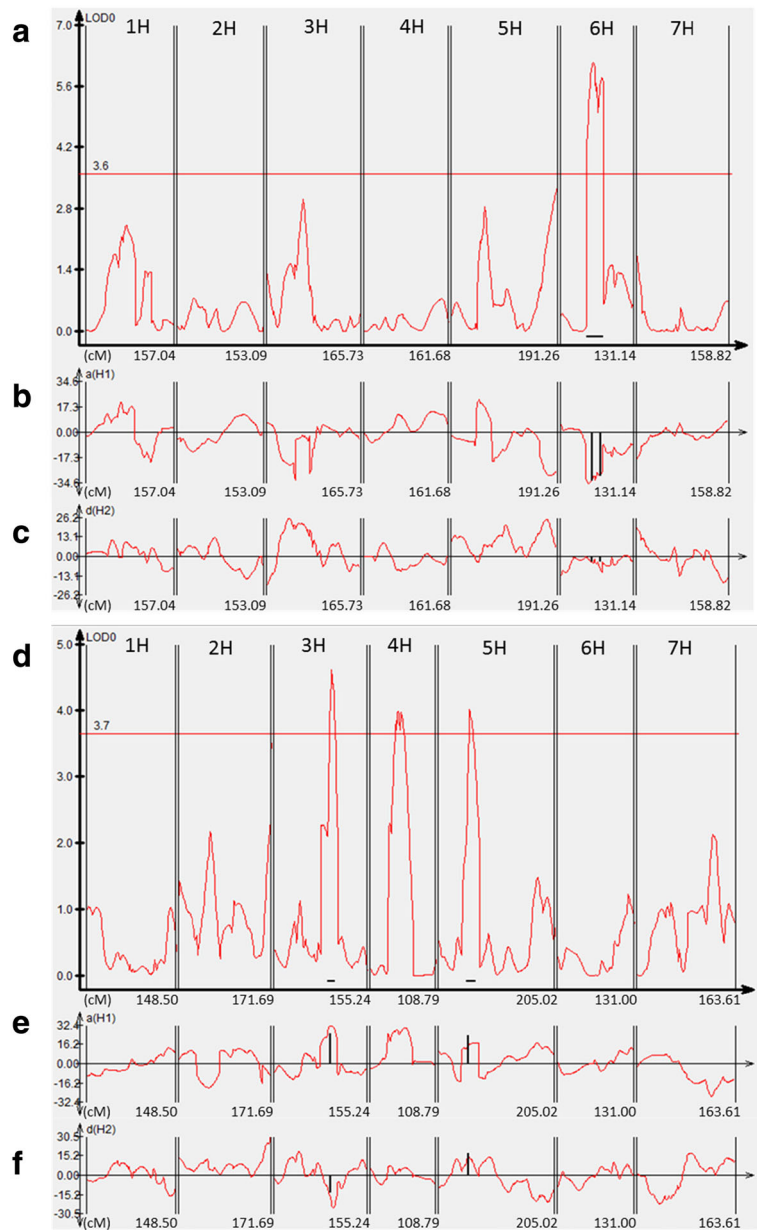
## Discussion

### Diversity and population structure in Barley Core sub-collection accessions

According to Knüpfper and Hintum (2003), the International Barley Core Collection is composed of sets of samples from WANA, the Americas, Ethiopia (which are currently not available from the local organization so we used tentative accessions from Okayama University in this study), Europe, South-East Asia, and others. Most of the accessions from WANA, Ethiopia, and South-East Asia are landraces, but the majority of accessions from Europe and the Americas are cultivars, including malting barleys. Since these malting barley cultivars have high  $\alpha$ -amylase activity values, they contribute to increase the range of  $\alpha$ -amylase activity linked to genetic diversity in this analysis.

Based on our population structure analysis using 1336 markers, the barley accessions were divided into two groups. Using a selection of 100 accessions from the International Barley Core Collection, Wu et al. (2015) reported seven groups after genotyping with 1536 SNPs. Hanen et al. (2014) used 380 South-East

**Fig. 4** QTL scans for LOD score (a Haruna Nijo × Weal and d Haruna Nijo × VLB-1), additive effect (b Haruna Nijo × Weal and e Haruna Nijo × VLB-1), and dominance effect (c Haruna Nijo × Weal and f Haruna Nijo × VLB-1) in the mapping crosses



Asian accessions and found two groups after genotyping with 384 SNPs. The number of populations predicted therefore likely depends on the number of accessions and markers used in each study. In this work, we used the same 1536 SNPs as Wu et al. (2015); however, the larger number of accessions in our study might have resulted in the rather simple population structure estimate we obtained ( $n = 2$ ).

Pourkheirandish et al. (2015) proposed that the two ancestral forms of cultivated barley correspond to the

rachis brittleness types *Btr1btr2* and *btr1Btr2*. Since original East Asian barleys trace their ancestry to *Btr1btr2* and other cultivated barleys trace theirs to *btr1Btr2*, it is assumed that the BCS populations largely reflect the ancestral types of cultivated barley. By contrast, Ethiopian barleys are categorized as Occidental (non-Asian) barleys but are assigned to the same population group with East Asian barleys in this analysis. A possible cause of this discrepancy, and the small number of estimated populations, is that most of the SNPs

**Table 2** The positions of significant QTLs for  $\alpha$ -amylase activity in barley malt of  $F_2$  populations and their associated markers

Population <sup>a</sup>	Chr.	QTL position (cM)	Neighborhood markers	Marker position (cM)	LOD score	Additive effect <sup>b</sup>	Dominant effect <sup>b</sup>	$R^2$
H × W	6H	51.01	5771-91 3378-619	47.63 61.75	6.16	-33.93	-4.22	0.187
	6H	65.21	ABC02895-1-4-231 4235-1617	63.42 66.85	5.82	-30.24	-4.24	0.176
H × V	3H	94.11	10114-1946 7241-553	94.07 97.46	4.63	24.71	-14.28	0.198
	4H	50.71	3549-743 1523-1136	50.53 70.65	3.99 <sup>c</sup>	26.19	-1.25	0.132
	5H	49.51	8561-968 65-778	49.48 50.57	4.03	23.01	16.84	0.041

<sup>a</sup>“H × W” or “H × V” show the  $F_2$  populations derived from the crosses between “Haruna Nijo” and “Weal” or between “Haruna Nijo” and “VLB-1”, respectively

<sup>b</sup>Additive or dominant effects for “Haruna Nijo” allele

<sup>c</sup>This QTL was not detected by QTL Cartographer but its LOD score was significant

detected by BOPA1 are from donors of Occidental origin (Sato et al. 2011). Genotyping with these SNPs may be prone to ascertainment bias, which could reduce their applicability for detecting polymorphism between Ethiopian and South-East Asian barleys. Alternative marker systems that are polymorphic between Ethiopian and South-East Asian barleys may help elucidating the structure independent populations and raise the mapping resolution by reducing the detection of false positive markers polymorphic between Ethiopian and South-East Asian barleys.

#### QTL detection by genome-wide association study and composite interval mapping of $F_2$ populations

The *Amy1* and *Amy2* loci encode two  $\alpha$ -amylase isozymes that differ in their biochemical and biophysical characteristics (Hayes et al. 2003). Szűcs et al. (2009) localized QTLs controlling  $\alpha$ -amylase activity on every barley chromosome, including these two loci. Our genome-wide association study also identified QTLs in the vicinity of *Amy1* (45 and 56 cM on 6H) and *Amy2* (88 cM on 7H). The accessions showing distinct values for  $\alpha$ -amylase activity with different alleles at these positions can be used to analyze *Amy1* and *Amy2* by bi-parental mapping.

The  $F_2$  mapping population of Haruna Nijo × Weal (H × W) allowed us to detect QTLs at 51.0 cM (5771-91, LOD = 6.16,  $R^2$  = 0.19) and 65.2 cM (4235-1617, LOD = 5.82,  $R^2$  = 0.18) on

6H. Some plants showed recombination between these two QTL positions, suggesting that these QTLs correspond to different genetic factors. One of these QTLs is likely to be the *Amy1* locus based on its mapping position according to Szűcs et al. (2009). The other  $F_2$  mapping population, Haruna Nijo × VLB-1 (H × V), revealed three QTLs: 94.1 cM (10114-1946, LOD = 4.63,  $R^2$  = 0.20) on 3H, 50.5 cM (3549-743, LOD = 3.99,  $R^2$  = 0.13) on 4H, and 49.5 cM (8561-968, LOD = 4.03,  $R^2$  = 0.04) on 5H, with all positive alleles from Haruna Nijo. Our genome-wide association study also mapped QTLs around these positions. Highly significant QTLs were also identified on 1H and 4H by the genome-wide association study. These QTLs are good candidates to improve  $\alpha$ -amylase activity within the accessions in this study. Since the malt  $\alpha$ -amylase activity is influenced by the protein content, further genome-wide association study on the protein content with larger amount of malt samples may reveal that some of the  $\alpha$ -amylase QTLs identified in this study correspond to QTLs of malt protein content.

Mascher et al. (2017) showed 12  $\alpha$ -amylase (*amy*) family sequences in cv. Morex, including subfamilies *amy1* and *amy2*, located on chromosomes 6H and 7H, respectively (Table S2). The comparison of physical positions between these  $\alpha$ -amylase family sequences and closest marker sequences to QTLs strongly support that some of these  $\alpha$ -amylase family sequences are *Amy1*, *Amy2*, and QTLs found in this study. The mapping resolution of this study is not high enough to estimate physical positions of QTLs but may provide useful information



and materials to phenotype  $\alpha$ -amylase (amy) family sequences.

### Practical application of QTLs in malting barley breeding

Haruna Nijo is a Japanese malting barley cultivar that has an excellent malting quality for brewing beer. It has been used extensively as the foundational genotype for current Japanese barley breeding (Sato et al. 2016). Our BCS diversity analysis indicated that Haruna Nijo is among the higher  $\alpha$ -amylase activity barleys (287.6 nmol/min/mg within the BCS range from 32.9 to 460.0 nmol/min/mg). In the QTL analysis of Haruna Nijo  $\times$  VLB-1, higher  $\alpha$ -amylase activity was derived from the Haruna Nijo alleles at the QTLs on 3H, 4H, and 5H, which are different from the genomic positions of *Amy1* (6H) and *Amy2* (7H). The QTL analysis of Haruna Nijo  $\times$  Weal indicated that even higher  $\alpha$ -amylase activity can be achieved on the genetic background of Haruna Nijo by introducing alleles from Weal, which had the highest  $\alpha$ -amylase activity value among the BCS accessions at 460.0 nmol/min/mg. Notably,  $\alpha$ -amylase activity in Haruna Nijo was improved by introducing new alleles at QTLs on 6H, one of which is likely an allele of *Amy1*. These results demonstrate that the quality of the foundational genotype in Japanese malting barley can be improved using an exotic germplasm.

### Conclusion

The BCS represents the genetic diversity found in the world's barley, and our analysis of the  $\alpha$ -amylase activity in those accessions provides a general spectrum of the genetic variation in this activity. The wide range of natural  $\alpha$ -amylase activities can serve as the basis for improvement in barley. Indeed, we showed that the QTL alleles of the Japanese foundation malting cultivar Haruna Nijo could be improved upon by introducing novel alleles from an accession with high  $\alpha$ -amylase activity, and even from an accession with low activity. These results thus demonstrate the successful improvement of a target haplotype using a combination of ex situ collections and genome-wide DNA markers.

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**Author contribution statement** KS initiated the project and designed the experiments. KS developed the materials. KM,

TSZ, and SM conducted the experiments. SM, HH, and KS analyzed the data. HH and KS wrote the manuscript.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical standards** The experiments conducted in this study comply with domestic and international laws.

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