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RESEARCH ARTICLE

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Association mapping for morphological traits relevant to registration of barley varieties

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Abstract

Elucidating marker-trait associations would have fruitful implications in distinctness, uniformity, and stability (DUS) tests of new varieties required for both variety registration and granting plant breeders' rights. As the number of new varieties with narrow genetic bases increases, the necessity for deployment of molecular markers to complement morphological DUS traits gets particular attention. We used simple sequence repeats (SSRs) and sequence related amplification polymorphisms (SRAPs) markers in association mapping of morphological traits in a collection of 143 barley landraces and advanced breeding lines. This panel represented a diverse and uniform sample in terms of both quantitative and categorical traits whilst it was structurally partitioned by number of ear rows (six- and two-rowed) and seasonal growth habit (winter and spring types) characteristics. SSRs were more powerful compared with SRAPs in separating six- and two-rowed genotypes based on both model-based Bayesian and neighbor joining clustering methods. A number of associated SSR and SRAP markers were found for 15 out of 36 DUS traits after considering Bonferroni correction through linear models (GLM and MLM) and chi-square-based tests (SA and AAT). This is also the first report of association of awn roughness and grain color with molecular markers in barley. Moreover, SSR marker BMAC0113 appeared associated with time of ear emergence (TEE), confirming previous findings. These markers could be beneficial to complement and speed up DUS testing of new varieties, as well as for improving management of barley reference collections.

Additional keywords: LD mapping; *Hordeum vulgare* L.; DUS traits; SSRs & SRAPs markers.

Abbreviations used: AAT (allelic association test); AFLP (amplified fragment length polymorphism); AR (awn roughness); DUS (distinctness, uniformity, stability); ED (ear density); GLM (general linear model); GSLN (spiculation of inner lateral nerves of lemma); KCAL (color of grain aleurone layer); MLM (mixed linear model); NER (number of ear rows); SA (stratified analysis); SGH (seasonal growth habit); SRAP (sequence-related amplified polymorphism); SSR (simple sequence repeats); TEE (time of ear emergence).

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Introduction

Conducting distinctness, uniformity and stability (DUS) tests of new varieties are prerequisites for both variety registration (enlisting) and granting plant breeder's rights (PBRs) to variety owners. Moreover, varieties of crop plants should have sufficient merits or value for cultivation and use (VCU) before earning eligibility for commercialization (Cooke & Reeves, 2003). DUS tests are generally relied on recording morphological characteristics during two independent growing periods, within the plant variety protection (PVP) framework of the International Union for the Protection of New Varieties of Plants (UPOV, 1991). Due to plentiful advantages of molecular markers over morphological traits (Lombard *et al.*, 2000), the Working Group on Biochemical and Molecular Techniques and DNA-Profiling in Particular (BMT) of

UPOV has considered their use in DUS tests through positively assessed models. Molecular markers tightly linked to genes controlling morphological traits, which could be considered as their predictors are preferred over markers with neutral functionality (UPOV, 2013), since the use of the latter for DUS testing would eventually minimize the distinctness level of new varieties to merely one base pair of DNA, thus endangering Plant Breeders' Rights (ISF, 2012).

Various molecular markers with unknown function or without association with morphological characteristics were used in DUS testing of crops (Giancola *et al.*, 2002; Gunjaca *et al.*, 2008; Noli *et al.*, 2008), vegetables (Bernet *et al.*, 2003; Kwon *et al.*, 2005), and fruit trees (Rotondi *et al.*, 2003; Ibañez *et al.*, 2009). Lombard *et al.* (2000), using amplified fragment length polymorphisms (AFLPs), could distinguish rapeseed cultivars according to growth type (winter *vs*. spring), country of origin, and breeding company of varieties. Bonow *et al.* (2009) based on simple sequence repeats (SSRs) derived from upstream region of MADS-box genes and other expressed sequence-tags could discriminate closely related rice varieties according to their subspecies, *i.e., japonica* and *indica*. In barley, robust diagnostic PCR-based markers was used for identification of spring and winter types (Cockram *et al.*, 2009) and, in tomato, some disease resistance genes were used for DUS testing (Arens *et al.*, 2010).

Association mapping (AM) has been suggested as a suitable approach for investigating the genetic basis of marker-trait correlations. AM, also known as linkage disequilibrium (LD) mapping, relies on existing natural populations or designed populations of plants to overcome the constraints inherent to linkage mapping. LD mapping exploits ancestral recombination events that occurred in the population and takes into account all major alleles present in the population to identify significant marker-trait associations (George & Cavanagh, 2015). Moreover, this method has alleviated the time-consuming necessity of creating segregation populations, in addition to its finer mapping resolution of genetic loci (Cockram *et al.*, 2010). However, AM suffers from complexity of existing population stratifications that should be corrected before analysis. Many factors have led to this population structure such as geographical origin, breeding methods, and selection during domestication (Balding, 2006).

Barley (*Hordeum vulgare* L.) was domesticated in the Fertile Crescent about 10,000 years ago (Morrell & Clegg, 2007), and is currently the fourth most important cereal crop after wheat, rice and maize. Germplasm of cultivated barley as a diploid and self-pollinated species, is highly partitioned in terms of number of ear rows (NER) (six- *vs*. two-rowed samples) and seasonal growth habit (SGH) (spring *vs*. winter-type samples) (Hamblin *et al.*, 2010; Pasam *et al.*, 2012; Wang *et al.*, 2012). Such population structure in addition to differential relatedness among individuals should be corrected before association analysis as they result in higher rates of type I error and consequently spurious associations (Waugh *et al.*, 2009). The most widely used methods for adjustment of population structure are model-based clustering method based on Bayesian statistics (Pritchard *et al.*, 2000) and data reduction technique by principal components analysis (PCA) (Price *et al.*, 2006). Incorporation of marker-based estimates of cluster membership (Q matrix) along with familiar relatedness (K matrix) into mixed linear model as covariates has better consequences in controlling the rate of false positives without reduction in statistical power (Yu *et al.*, 2006; Astle & Balding, 2009). However, some studies indicated the sufficiency of either of matrices in association analysis as simultaneous use of them resulted in overcorrection for population structure, hence a reduction in statistical power (Würschum *et al.*, 2011; Wang *et al.*, 2012).

Unlike the characterization of molecular bases of ear row number (Komatsuda *et al.*, 2007) and vernalization requirement (von Zitzewitz *et al.*, 2005; Karsai *et al.*, 2005; Cockram *et al.*, 2015) in barley, these partitioning loci have also been investigated through genome wide association study (GWAS) (Cockram *et al.*, 2008). Wang *et al.* (2012) used single nucleotide polymorphisms (SNPs) for association analysis of 32 morphological DUS traits and 10 agronomic characters in a sample of registered barley cultivars in UK and reported significant associations for half of the assessed DUS traits and nine agronomic characters. In addition, the genetic locus conferring anthocyanin pigmentation in auricles, awn tips and lemma nerves was fine-mapped on chromosome 2H (Cockram *et al.*, 2010). SSR and AFLP markers and genome-wide SNP marker loci were used in separate association mapping studies for mapping gene controlling rachilla hair length of grain on chromosome 5H (Kraakman *et al.*, 2006; Waugh *et al.*, 2010). Pasam *et al.* (2012) in reported associated SNP markers to agronomic traits of which heading date and plant height are conventionally used in DUS testing of barley varieties. Therefore, development of diagnostic markers for DUS traits that inherit in a Mendelian manner would be more promising than the direct use of quantitative DUS characters (like plant height) with complex heritability (Jones *et al.*, 2013). As recently, the prediction of five DUS traits in barley has been enabled through a KASP genotyping platform (Cockram *et al.*, 2012; Jones *et al.*, 2013).

We used different statistical models to find SSR and sequence related amplification polymorphisms (SRAP)

markers associated with 36 DUS traits in a panel of 143 barley landraces and cultivars. These samples represented a diverse and uniform plant material in terms of morphological characteristics after being purified through ear-to-row selection cycles. For this purpose, linear models and chi-square-based tests were employed for association mapping of quantitative and categorical DUS traits respectively.

Material and methods

Plant material

A panel of 143 barley landraces, cultivars and advanced breeding lines including 63 six-rowed and 80 two-rowed accessions from eight countries (Iran, Egypt, China, US, England, India, Pakistan, and Algeria) was used for an association study. The accessions were provided by the Dryland Agricultural Research Institute, Iran. The heterogeneous landraces underwent three generations of pure line selection through ear-to-row method in research fields of DARI during 2008-09. Based on growth habit type, the panel was divided into 62 spring, 68 winter, 12 facultative types and one with unknown growth type. The vast majority of six-rowed barley samples were winter type (88.8%) and nearly two-third (70%) of two-rowed samples were spring type (Table S1 [suppl.]).

Field trials and phenotyping

The 143 accessions were sown in an experimental field of the Seed & Plant Certification & Registration Institute, following a lattice square (12×12) design with two replications, in autumn of 2013 and 2014, and harvested in spring of the following years. In order to alleviate the vernalization requirements of winter and facultative types, the seeds were soaked in water for 24 hours and then stored at 2°C for 40 days before cultivation.

A total number of 36 morphological traits were measured in the accessions studied, of which 9 were quantitative (continuous) and 27 were qualitative (categorical). The traits were chosen from UPOV's DUS test guideline in barley (UPOV, 1994), DUS test protocol of Community Plant Variety Office (CPVO, 2012), India's DUS test guideline (PPV & FRA, 2011), and characteristics used by Wang *et al.* (2012). Among categorical variables, nine were binary (presence or absence), 13 were ordinal (a visual scale of the expression intensity of a characteristic), and five were nominal (like color or shape of an organ). Moreover, four ordinal variables *i.e.* color of grain aleurone layer (KCAL), awn roughness (AR), anthocyanin color of nerves of lemma (GACN), and spiculation of inner lateral nerves of lemma (GSLN) were also regarded as binary variables. The ear density (ED) of samples was measured as a continuous variable, as well as an ordinal characteristic (Table S2 [suppl.]).

SSR and SRAP genotyping

Genomic DNA was extracted using the CTAB method (Saghai Maroof *et al.*, 1984) from a bulk of 15 plants of each accession. A set of 149 SSR (Ramsay *et al.*, 2000; Wenzl *et al.*, 2006; Varshney *et al.*, 2007) and EST-SSR (derived from expressed sequence tags) (Varshney *et al.*, 2006) loci with even distribution over seven barley chromosomes were used for amplification (Table S3 and Fig. S1 [suppl.]). Moreover, a set of 30 primer combinations (5×6 me-em primers) was used to amplify SRAP markers (Li & Quiros, 2001). Markers with minor allele frequency (MAF) below 0.05 and markers with more than 15 % missing data were removed from analyses.

Inference of population structure and family-based relatedness

The population structure of 143 barley accessions was inferred by the Bayesian clustering method using STRUCTURE v.2.3.4 (Pritchard *et al.*, 2000). We modeled a burn-in period of 50,000 cycles followed by 100,000 Markov Chain Monte Carlo (MCMC) iterations. The number of clusters or sub-population (K) was set from 1 to10 and repeated 20 times for each K for the stability of each model set for no-admixture and correlated allele frequencies. The most probable number of sub-populations (clusters) was determined by plotting the quantity of ΔK as a function of K (Evanno *et al.*, 2005) using the online software STRUCTURE HARVESTER (Earl & vonHoldt, 2012) to generate membership coefficient (Q) matrix. In order to assign each individual to a particular group a cut-off limit of 60% membership (Q-matrix) was considered (Jakob *et al.*, 2014). Accessions with values below this threshold were considered as admixed individuals.

Analysis of molecular variance (AMOVA) was performed using GenAlex v.6.41 (Peakall & Smouse, 2006) using 999 permutations to estimate population differentiation among pre-defined sub-populations *i.e.* NER (two-rowed *vs*. six-rowed), and SGH (winter type *vs*. spring type). Principal coordinate analysis (PCoA) was run on Nei's genetic distance matrix (Nei, 1973). Furthermore, unweighted neighbor joining (NJ) dendrogram was constructed by DARwin V.5.0.158 software (Perrier & Jacquemoud-Collet, 2006), based

on Jaccard's similarity matrix calculated in NTSYS v.2.02 software (Rohlf, 1998).

Statistical analysis

Shapiro–Wilk normality test was used to test the distributions of the nine quantitative DUS traits at 0.05 significance level. The association of DUS traits with NER groups was tested by using Fisher's exact chi-square test for binary and nominal traits, Mann-Whitney U test for ordinal traits and Student t test for quantitative traits. Best linear unbiased predictions (BLUPs) of genotypic means were calculated from the fixed genotypic effects of quantitative traits and were used for association analysis. Statistical analyses were carried out using the SAS software (SAS Inst., 2002).

Association analysis of DUS traits

A general linear model (GLM) and a mixed linear model (MLM) (Yu *et al.*, 2006) were used for testing associations between markers and DUS traits using TASSEL v.3.0 software (Bradbury *et al.*, 2007). The Q was harvested from STRUCTURE analysis where ΔK was highest, and incorporated into the GLM approach as a set of covariates to correct for the effect of population structure. For MLM analysis, Jaccard's dissimilarity coefficients between individuals were added to the model ($Q+K_J$) to account for relatedness between individuals. Moreover, kinship values (scaled between 0 and 2) computed by TASSEL was also incorporated into the MLM analysis ($Q+K_T$).

Furthermore, allelic association tests (AAT) were used for categorical (nominal and binary) traits based on allele frequencies between states using PLINK v.1.07 software (Purcell *et al.*, 2007). Moreover, Cochran-Mantel-Haenszel test for 2×2×K stratified tables were used in stratified analysis (SA) using PLINK, in which NER groups was defined as K to consider the population structure in the analysis.

The *p* values of associated markers were tested against Bonferroni adjusted significance thresholds at 0.05 and 0.01 significance levels. Therefore, two conservative (0.05/684) and stringent (0.01/684) cut-offs were employed in all association analyses.

Results

Phenotypic variation and heritability

Nine quantitative traits were measured on 143 barley samples planted in two consecutive years (Table S4 [suppl.]). Based on Shapiro–Wilk normality test, only time of ear emergence (p =0.125) and plant length (p =0.367) followed normal distributions, whereas the other seven traits did not fit a normal distribution (p <0.01). Estimates of heritability (H^2_b) based on two-year data of five quantitative traits were 0.73 for plant height, 0.88 for time of ear emergence, and 0.95 for ear length, ear density and 1000-seed weight. These results are, in general, congruent or even above heritability estimates reported for the same traits in other studies in barley (Pasam *et al.*, 2012; Maurer *et al.*, 2016).

The accessions studied showed similar state in expression of four out of 27 categorical morphological characteristics. All 80 two-rowed accessions had sterile lateral spikelets with full development. The grains of all samples were husked, hairless in ventral furrow, and bearing clasping lodicules. The states of the remaining categorical (ordinal, binary, and nominal) characteristics were polymorphic between samples (Table S5 [suppl.]).

Genetic structure of barley panel

Since seasonal growth habit and ear row type are considered as the major sources of barley population structure, we compared the differences of the traits and marker allele frequency between two and six-rowed samples (NER group) as well as winter and spring accessions (SGH group). Four out of nine quantitative DUS traits, namely ear length, ear density, 1000-seed weight, and coleoptile length were significantly ($p \leq 0.01$) different between NER groups as revealed by t-test (Fig. S2 [suppl.]). In addition to these traits, first leaf length was also significantly ($p \leq 0.01$) different between SGH groups. Mann-Whitney U test revealed significant differences between NER groups ($P \leq 0.05$) for all the ordinal characteristics except three traits *i.e.* glaucosity of flag leaf sheet, anthocyanin color of lemma nerves, and awn length. However, the number of non-significant ordinal traits increased to seven when the U-value was calculated between SGH groups. In that respect, growth habit, awn roughness, length of first segment of rachis, and color of grain aleurone layer were also non-significant between SGH groups. A similar pattern was observed for binary and nominal DUS traits, in terms of reduction of significant traits between SGH groups compared to NER groups. All traits, except ear shape and spiculation of inner lateral nerves of lemma, presented differential distribution ($p \leq 0.05$) between NER groups as tested by Fisher's exact test. On the other hand, the values of six binary and nominal DUS traits (*i.e.* color of grain aleurone layer, hairiness of lower leaves sheaths, awn roughness, anthocyanin color of nerves of lemma, rachilla hair type of grain, and spiculation of inner lateral nerves of

lemma) were not significantly different between SGH groups (data not shown).

Comparison of allele frequency between two- and six-rowed accessions showed that the 56% of markers had allele frequencies with more than two-fold differences between two groups. This indicates that performing association tests in such panel without accounting for population structure will result in an increased rate of false positives (Balding, 2006). The result revealed that four and nine percent of alleles were specific to two and six-rowed barley, respectively. Similarly, around half of the markers (52%) had allele frequencies differing more than twofold between winter and spring barley samples and three and nine percent of the markers were specific to spring and winter growth habit, respectively.

The population structure in the panel of 143 barley accessions was analyzed using 149 SSR and 140 SRAP markers and a model-based approach in STRUCTURE. Based on both marker data, the [LnP(D)] appeared to be an increasing function of *K* for all the values observed. But the highest value for Δ*K* was detected at *K*=2, where the most significant increase of [LnP(D)] was observed from 1 to 2 (Fig. 1). At *K*=2, the panel was clearly categorized into two-rowed and six-rowed barleys (Fig. 2). Although the accessions were assigned into two groups based on two marker types, grouping by SSR data was more distinct compared with that of SRAP markers. Matthies *et al.* (2012), in a GWAS study of malting and kernel quality in barley, reported that grouping using SSRs was more accurate than using DArTs, a type of bi-allelic markers. The allocation of each individual to either groups was carried out based on the 60% membership threshold which was mostly corresponded to accessions row number, leaving two two-rowed (122 and 142) and four six-rowed (40, 41, 125, and 143) samples with admixed structure. In the resulting Q-matrix, all 63 six-rowed samples fell in Q1 sub-population except one individual (32). Interestingly, this individual resided in two-rowed subgroup derived from NJ dendrogram. Moreover, 13 individuals of 80 two-rowed samples remained in Q1 (six-rowed) group, of which 10 individuals stayed in a separate subgroup according to the dendrogram. The dominant stratification of the population according to NER was also confirmed by PCoA. The first two principal coordinates explained 20.5 and 8.0% of total marker variation, respectively. The first coordinate separated the accessions based on row number (Fig. 2). Overall, the clustering of accessions was consistent among various methods, and the genetic diversity within these groups was further explored. The extent of genetic differentiation among predefined sub-populations revealed by various methods was assessed by AMOVA. The percentage of molecular variance among sub-populations (Rst) was 0.24 when considering the NER as sub-populations. This value declined to 0.12 when the SGH of samples were regarded as sub-populations.

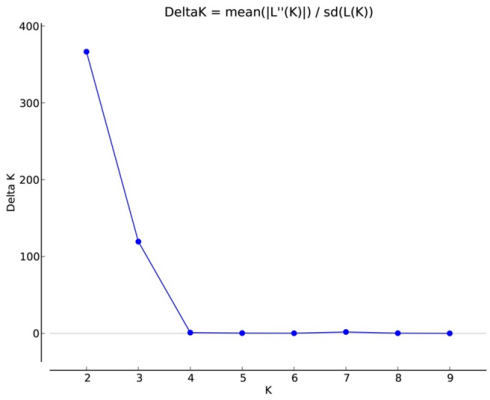

Figure 1. Delta K values as a function of K, according to Evanno et al. (2005). The higher value (K=2) was used to build Q-matrix, in which two clusters represented the number of ear rows of 143 barley samples

Comparison of models

Several models, linear (GLM and MLM) and allele frequency-based (AAT and SA), were used to detect associations between markers and 36 DUS traits. Using the GLM model, a total of 66 marker-trait associations was found considering -$\log_{10}$ Bonferroni threshold (0.05/684=4.13). The associations were reduced to 48 when a more stringent threshold (0.01/684=4.83) was considered. Moreover 61 and 72 associations were found after Bonferroni correction (0.05/684=4.13) in MLM models (Q+K_J and Q+K_T) respectively, though some of them were different from those of GLM results. As their predictive value failed in pairwise marker-trait evaluations, they were considered as spurious associations.

For both binary and nominal (treated as binary) traits, the results of SA and AAT analyses based on allele frequencies between morphological states were mostly congruent with the results of GLM model, though the number of associations exceeding the Bonferroni threshold were much higher than that of the linear approach. Comparison of expected and observed P values in Q-Q plots showed that for all the categorical DUS traits, the observed *p* values from SA method greatly deviated from the expected *p* values in case of no genetic associations. For all six traits, the observed *p* values from SA had inflated probabilities compared to AAT and GLM tests, though the two latter models showed different behavior in Q-Q plots. The observed *p* values from GLM for four traits (*i.e.* hairiness of lower leaves sheaths, awn roughness,

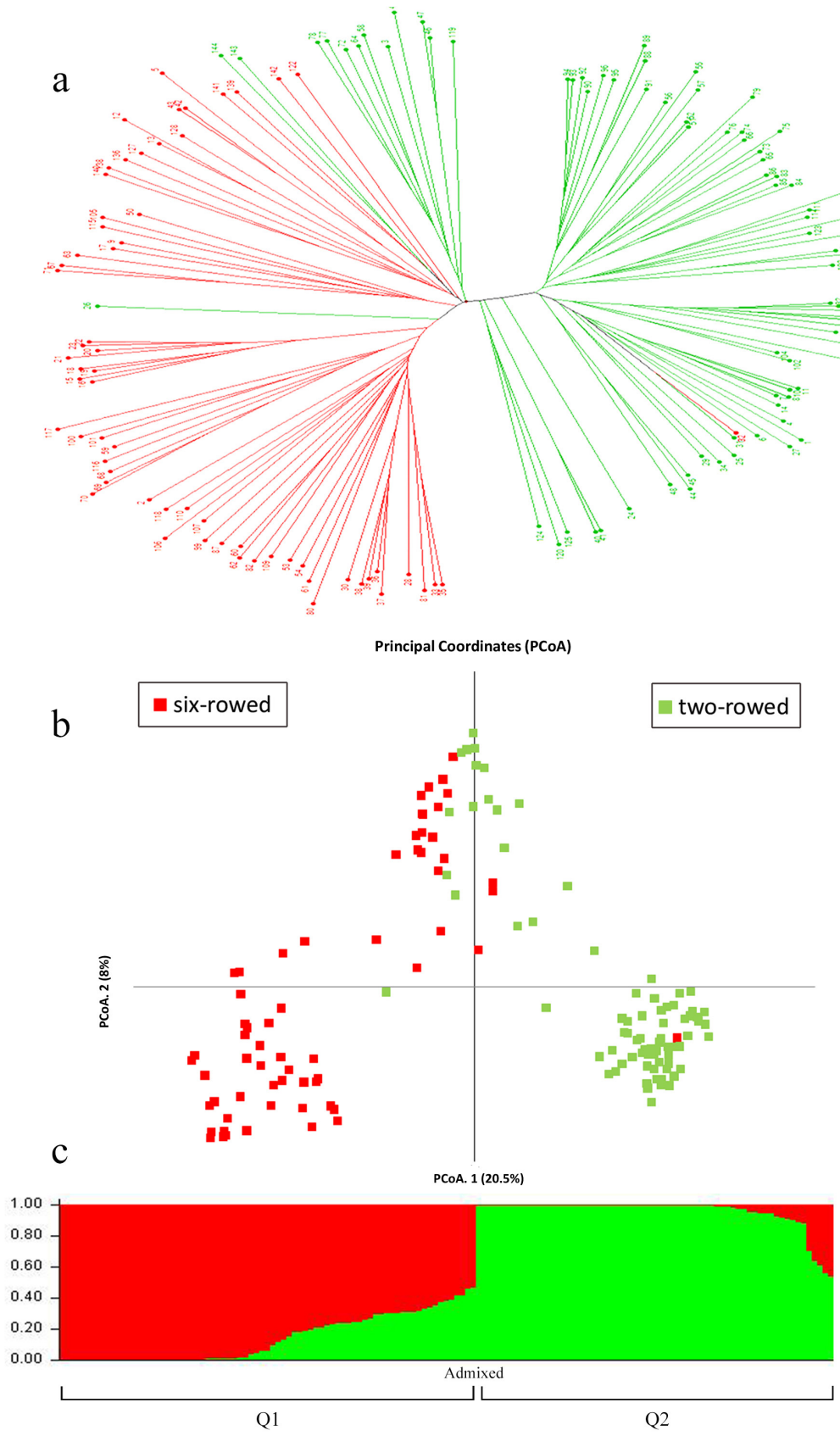

Figure 2. Differentiating six- and two-rowed barley samples with unweighted neighbor joining tree based on Jaccard's dissimilarity (a), principal coordinate analysis on two first axes (b), and model-based clustering method based on Bayesian statistics into Q1 and Q2 clusters (c). Accessions belonging to the six- and two-rowed groups are in red (left side) and green (right side) respectively.

Table 1. Significant markers exceeding the stringent Bonferroni threshold (4.83) detected in general linear model (GLM), stratified analysis (SA), and allelic association test (AAT)

DUS trait	Associated marker	Map	Allele-stateassociation		R^2	-log10 adjusted *p*		
			Allele	State of expression		GLM	SA	AAT
Anthocyanin color of awn tips (AACT)	BMAG0518	2H	5	absent	0.45	24.26	17.36	18.43
	SCSSR02306	5H	1	absent	0.31	13.52	13.78	14.19
	BMAC0213	1H	8	absent	0.26	9.87	10.38	11.95
	GBM1366	2H	1,2	present, absent	0.22	7.77	6.58	9.88
	GBMS0183	3H	4	absent	0.23	7.52	5.44	8.42
	EBMAC0854	2H	3	present	0.19	6.61	13.02	9.88
	EBMATC0039	2H	1,2	present, absent	0.19	5.92	6.01	9.89
	me4-em5	–	4	absent	0.17	5.75	6.13	8.24
	GBMS0160	2H	8	absent	0.17	5.64	5.06	7.27
Awn roughness (AR)	GBM1309	2H	1,2	rough, smooth	0.30	10.36	15.08	8.74
	BMAG0345	1H	3	smooth	0.23	7.01	9.99	7.62
	GBM1110	3H	1	rough	0.22	6.62	10.4	5.47
Grain color (GC)	EBMAC0624	6H	3	black	0.18	6.07	13.19	8.73
	BMAG0345	1H	3	yellow	0.16	5.15	8.84	6.50
Rachilla hair type of grain (GRHT)	HVI3	–	1	short	0.34	9.86	8.42	8.15
	GBM1408	2H	1,2	short, long	0.25	6.90	8.17	6.01
	BMAG0140	2H	1	short	0.25	6.58	8.89	5.90
	HV13GEIII	3H	1	short	0.25	5.97	8.82	5.16
Spiculation of lateral nerves of lemma (GSLN)	BMAC0310	4H	2	absent	0.34	10.12	18.24	7.38
Color of grain aleurone layer (KCAL)	BMAG0345	1H	3	colored	0.33	10.56	17.73	9.23
Attitude of sterile spikelets (SSA)	EBMAC0788	4H	8	Parallel to weakly divergent	0.32	8.68	13.65	7.03
Tip shape of sterile spikelets (SSTS)	BMAG0504	1H	1,3	pointed, rounded	0.28	9.53	9.58	9.81
	GBM1525	4H	1	rounded	0.20	5.89	4.07	6.61
	BMAG0807	6H	1	rounded	0.20	5.39	6.63	6.63
	EBMAC0624	6H	3	pointed	0.17	7.08	14.5	10.00
Hairiness of lower leaves sheaths (LLHL)	EBMAC679	4H	2	present	0.21	4.96	8.89	4.56

rachilla hair type of grain, and anthocyanin color of awn tips), were closer to expected *p* values showing less inflation (Fig. S3 [suppl.]). Overall, for binary and nominal DUS traits, we declared as associated markers those which surpassed the stringent threshold (4.83) in all three analyses *i.e.* the GLM (using TASSEL software) and AAT and SA (using PLINK software).

Marker-trait associations

A total of 684 alleles (in form of binary data) consisting 544 SSR alleles and 140 SRAP bands remained for association analysis, after removing markers with MAF<0.05 and those with more than 15% missing data.

Overall, there were several SSR markers that showed association with DUS traits compared to just one SRAP marker. However, employing the stringent threshold resulted in deletion of majority of putative associated SRAP markers as they probably represented spurious associations. Among these SSRs, EBMAC0039, GBM1366, and GBM1408 had only two alleles (like bi-allelic markers) that associated with binary traits. The first two associated with presence *vs*. absence of anthocyanin color in awn tips (AACT) and the latter was also correlated with short *vs*. long type of rachilla hair in grain (GRHT).

Table 2. Significant associated markers detected in general linear model (GLM) and two mixed linear models (MLM) in which Jaccard's (K_J) and kinship coefficients calculated by TASSEL (K_T) were incorporated to calculate a Kinship matrix. Markers exceeding the stringent Bonferroni threshold (4.83) by GLM method are denoted in bold font

DUS trait	Associated marker	Map	Allele-state association		GLM		MLM ($Q+K_J$)		MLM ($Q+K_T$)	
			Allele	State of expression	-log10 adjusted *p*	R^2	-log10 adjusted *p*	R^2	-log10 adjusted *p*	R^2
Intensity of anthocyanin color of awn tips (AIAC)	**BMAG0518**	2H	5	absent or very weak	6.55	0.22	–	–	–	–
	EBMAC0541	3H	2	strong	5.40	0.19	–	–	–	–
	BMAC0213	1H	8	absent or very weak	4.57	0.18	–	–	–	–
Awn roughness (AR)	**GBM1400**	6H	3	smooth	13.10	0.40	3.64	0.21	1.89	0.14
	GBM1309	2H	1	rough	7.73	0.27	0.23	0.08	–	–
	GBM1463	5H	1	smooth	6.11	0.23	0.27	0.09	–	–
	GBM1221	4H	1	smooth	5.53	0.21	–	–	–	–
	GBM1438	5H	3	smooth	4.61	0.20	–	–	–	–
	BMAG0345	1H	3	intermediate, rough	4.64	0.19	–	–	–	–
Ear density (ED)	**GBM1400**	6H	3	very lax	5.58	0.20	–	–	–	–
	GBM1221	4H	1	very lax	4.18	0.16	–	–	–	–
Color of grain aleurone layer (KCAL)	**BMAG0345**	1H	3	weakly/strongly colored	10.37	0.31	–	–	–	–
	EBMAC0624	6H	3	strongly colored	6.53	0.23	–	–	–	–
Plant height (PH)	GBM1464	7H	6	–	4.72	0.19	–	–	–	–
1000-seed weight (SW)	GBM1293	5H	2	–	4.58	0.09	–	–	–	–
Time of ear emergence (TEE)	**EBMAC560**	1H	1,2	–	9.60	0.31	–	–	–	–
	BMAG0518	2H	5	–	8.56	0.29	–	–	–	–
	BMAC0113	5H	6	–	6.24	0.24	–	–	–	–
	GBM1309	2H	1	–	4.37	0.19	–	–	–	–
Anthocyanin color of nerves of lemma (GACN)	BMAG0740	4H	9	–	4.34	0.20	2.27	0.16	0.63	0.10

Namely, eight SSRs and one SRAP marker were significantly associated with AACT, where most of them were on chromosome 2H. The highest percentage of total phenotypic variation (R^2) explained by the associated markers (0.45) was observed for BMAG0518 (Table 1). This marker also showed association with intensity of expression of anthocyanin (AIAC), explaining 22% total variation of the trait. SSR marker EBMAC0541 on 3H, explained 19% of AIAC total variation (Table 2). Four SSR markers *i.e.* GBM1400, GBM1309, GBM1463, and GBM1221 on respective chromosomes 6H, 2H, 5H, and 4H were associated with AR (awn roughness) when the awns phenotypes were scored as smooth, intermediate, and rough. The percentage of variation explained by GBM1400 was 40%, and was confirmed by both MLM models. The other three markers contributed in 21 to 27% of trait phenotypic variation (Table 2). When AR was scored as binary (smooth *vs*. rough), in addition to GBM1309, two other SSR markers *i.e.* BMAG0345 and GBM1110 were also identified as associated markers using GLM analysis (Table 1). Based on GLM analysis, two SSR markers BMAG0345, and EBMAC0624 were significantly associated with KCAL (color of grain aleurone layer) when its phenotype was scored as ordinal (whitish, weakly colored, and strongly colored). These markers explained 31 and 21% of color variation respectively (Table 2). However, based on binary scoring (whitish *vs*. colored) of KCAL, only SSR marker BMAG0345 on chromosome 1H showed significant association with the trait categories (Table 1). Association analysis revealed significant effect of BMAC0310 (4H) marker on GSLN (spiculation of inner lateral nerves of lemma) with explaining 34% of phenotypic variance when GSLN was considered as binary DUS trait (absent *vs*. present) (Table 1). No association was identified using the GLM method when GSLN was scored as ordinal trait (absent or very weak, weak, medium, strong, and very strong). SSR marker BMAG0740 on 4H was associated with GACN (anthocyanin color of nerves of lemma), explaining 20% of phenotypic variation. Four SSR markers HVI3, GBM1408, BMAG0140, and HV13GEIII were significantly associated with GRHT trait variation, as revealed by three analyses *i.e.*, GLM, AAT, and SA. These markers explained 25 to 34% of hair type variation (short *vs*. long) (Table 1). Two

SSR markers EBMAC0624 (6H) and BMAG0345 (1H) were associated with grain color variation, explaining 18 and 16% of grain color variation respectively (Table 1).

In two-rowed barleys, GLM analysis revealed significant association between SSR markers EBMAC0788 (4H) and attitude of sterile spikelets. Four SSR markers EBMAC0624, BMAG0504, GBM1525, and BMAG0807 showed significant association with tip shape of sterile spikelets variation. One SSR marker (EBMAC679) on 4H was associated with hairiness of lower leaves sheaths variation, explaining 21% of hairiness variation (Table 1). In association analysis, DUS traits *i.e.* KCAL, AR, GACN and GSLN were scored as ordinal (a visual scale of the expression intensity), as well as binary (presence or absence) (Table S2 [suppl.]) to increase the chance of finding marker-trait correlations. Moreover, for ED (ear density) both ordinal and measurement scales were incorporated into GLM analysis. This approach led to detection of similar associated marker in either of states deployed (Table 2).

The GLM analysis showed significant association SSR marker GBM1400 (6H) with ED variation. This marker showed association when phenotypic states of ED were scored as quantitative (number of grains divided by ear length) as well as ordinal (very lax, lax, medium, dense, and very dense). Additionally, GBM1221 on 4H was also associated with ED variation when trait was scored as ordinal variable. Three SSR markers EBMAC560, BMAG0518 and BMAC0113, on respective chromosomes 1H, 2H, and 5H showed significant association with time of ear emergence (TEE), explaining 31, 29, and 24% of total phenotypic variation, respectively. The SSR marker GBM1309 (2H) was also associated with TEE, when conservative cut-off was considered. The GLM analysis revealed significant association between EST-SSR marker GBM1464 (7H) and plant height explaining 19% of trait total variation. SSR markers GBM1293 on chromosome 5H was significantly associated with thousand-seed weight, which explained 9% of trait variation (Table 2).

Discussion

Employing two MLM models in present study resulted in more associations (which could be considered spurious) as compared to GLM analysis with incorporated Q-matrix. These potentially false positives were detected in evaluating their predictive value through pairwise marker-trait alignments. Thus we inferred that adding other covariates (K_J and K_T) into two MLM models resulted in over-correction, as the Q-matrix was solely enough in the model by delineating population structure of 143 barley genotypes according to their NER. Similar result was found by Wang *et al.* (2012) when seven statistical approaches were used in genome-wide association mapping of agronomic and DUS traits. Two mixed linear models outperformed other four approaches in controlling false positive rates. Among these, the MLM (K) with incorporation of just a matrix of pairwise identical-by-state allele-sharing showed better statistical power compared with other MLM (P+K) with additional factor accounted for a matrix derived from *k*-means clustering of top three principal component axes. These results suggested that using K matrix alone was sufficient to correct the population structure caused by SGH.

In the present study, some SSR markers showed association with the same traits. For example, BMAC0213 (1H) and MBAG0518 (2H) were associated with AACT and AIAC. However, the latter marker was also associated with TEE. Similarly, BMAG0345 (1H) and EBMAC0624 (6H) were associated with grain color (GC) and KCAL. In addition, these markers were also associated with AR and tip shape of sterile spikelets (SSTS), respectively. GBM1400 (6H) and GBM1221 (4H) showed association with AR and ED based on separate GLM models and GBM1309 (2H) was associated with AR and TEE. The results suggest that these could be due to pleiotropic effects of QTLs or linkage of independent QTLs or loci.

The 143 barley landraces and advanced breeding lines used in the present study represented a diverse and uniform panel in terms of morphological DUS characteristics. The SSR and SRAP markers could group the accessions according to their NER based on both hierarchical and model-based clustering methods. The Q-matrix resulted from population structure and predefined NER groups were taken into account in linear models (GLM and MLM) and in chi-square-based test (SA) respectively. By considering a Bonferroni-adjusted threshold, 33 associated markers (32 SSRs and 1 SRAP) with DUS traits were found, in which 13 SSR markers (markers with GBM or GBMS prefix) were originally derived from expressed sequence tags of barley (Varshney *et al.*, 2006). Some associated SSR markers were found to be correlated with traits in previous QTL bi-parental mapping and association analyses. Among them, SSR marker BMAC0113 linked to QTLs for days to heading in barley (Pillen *et al.*, 2003, 2004) was validated again through GLM analysis as it showed association with TEE. Moreover,

EST-SSR marker GBM1464 that showed association with plant height in the present study was previously reported that had a functional association (Varshney *et al.*, 2008).

"Character-specific molecular markers" are positively considered by UPOV's BMT to be used in DUS testing, provided that they can reliably estimate traditional characteristics (UPOV, 2013). Until now, the function of molecular markers evaluated for variety registration was unknown (Giancola *et al.*, 2002; Rotondi *et al.*, 2003; Bernet *et al.*, 2003; Kwon *et al.*, 2005; Gunjaca *et al.*, 2008; Noli *et al.*, 2008; Ibañez *et al.*, 2009). Moreover, these markers were irrelevant to morphological traits that are currently used in DUS testing of new plant varieties. However, in a few cases, microsatellite markers in and around the genes were used for discrimination of closely related rice varieties (Bonow *et al.*, 2009). Also, AFLPs could distinguish rapeseed cultivars according to growth habit (winter *vs*. spring), country of origin, and breeding company of varieties (Lombard *et al.*, 2000). Besides to introduction of PCR-based markers for prediction of SGH in barley (Cockram *et al.*, 2009), a suite of 25 SNP markers based on KASP™ genotyping platform were recently assessed for predicting 15 DUS traits in UK barley varieties (Cockram *et al.*, 2012; Jones *et al.*, 2013). Our study is the first report of introducing associated SSR and SRAP markers with 15 morphological traits used in DUS testing of barley, of which seven traits are in common with 15 DUS characters diagnosed by SNP markers (Cockram *et al.*, 2012). Moreover, SSR markers associated with anthocyanin color of awn tips, intensity of anthocyanin color of awn tips, and hairiness of lower leaves sheaths were in similar chromosome positions to the markers reported by previous GWAS studies of barley DUS traits (Cockram *et al.*, 2010; Wang *et al.*, 2012). Furthermore, this is the first report of association of awn roughness and grain color with SSR markers.

With increasing number of candidate varieties for both registration and PBRs, the necessity for deployment of molecular markers in DUS testing will grow. Considering the drawbacks of conventional traits from their limitations in number, influenceability from environmental factors, to difficulties in scoring of some traits (Lombard *et al.*, 2000; Jones *et al.*, 2013), the markers associated with DUS traits introduced in the present study could be used as complement of conventional DUS testing in barley. They also could be an alternative to the morphological traits that are used at present when they represent the functional polymorphisms at the genes controlling the DUS traits. Furthermore, these associated markers (SSR and SRAP) are especially cost-effective for test authorities with less access to high-throughput SNP genotyping platforms.

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