Multiple marker-traits associations for maize agronomic traits

Sanja Mikic[1*], Ankica Kondic-Spika[1], Ljiljana Brbaklic[2], Dusan Stanisavljevic[1], Dragana Trkulja[1], Marina Tomicic[1], Aleksandra Nastasic[1], Borislav Kobiljski[2], Slaven Prodanovic[3], and Gordana Surlan Momirovic[3]

ABSTRACT

Association analysis is a relatively novel approach in quantitative traits studies that allows high resolution mapping and time efficient and direct application on breeding material. Since the markers, which are close to the quantitative trait loci stable across environments and genetic backgrounds, may be valuable for marker assisted selection, we chose microsatellite markers previously linked to traits of interest in various mapping studies. A set of 36 microsatellite markers positioned near important maize (*Zea mays* L.) agronomic loci was used to evaluate genetic diversity and determine population structure. To verify the associations between the markers and traits, a panel of diverse maize inbred lines was genotyped with microsatellites and phenotyped for flowering time, yield and yield components. A relatively high level of polymorphism detected in number of alleles per locus (8.2), average polymorphic information content value (0.64), and average gene diversity (0.684) lines showed the analyzed panel of maize inbred contained significant genetic diversity and was suitable for association mapping. The population structure estimated by model-based clustering method grouped maize inbred lines into three clusters. The association analysis using the general linear and mixed linear models determined significant correlations between several agronomic traits and three microsatellites on chromosomes 3, 5, and 8, namely *umc1025*, *bnlg1237*, and *bnlg162* consistent across the environments, explaining from 4.7% to 18.2% of total phenotypic variations. The results suggest that the chromosome regions containing quantitative trait loci (QTLs) associated with multiple yield-related traits consistently across environments are potentially important targets for selection.

Key words: Association analysis, inbred lines, linkage, pleiotropy, SSR markers, *Zea mays*.

[1]Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia.
[*]Corresponding author (sanja.mikic@ifvcns.ns.ac.rs).
[2]Biogranum, Toplice Milana 20, 21000 Novi Sad, Serbia.
[3]University in Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Belgrade-Zemun, Serbia.

Received: 6 October 2015.
Accepted: 25 April 2016.
doi:10.4067/S0718-58392016000300006

INTRODUCTION

A large number of maize (*Zea mays* L. subsp. *mays*) traits important from an agronomic perspective have a complex nature, are governed by numerous genes and influenced by environmental factors and interaction between genes and environment. Many findings support the hypothesis that complex traits are controlled by few quantitative trait loci (QTLs) with large effects and many QTLs with small effects (Salvi and Tuberosa, 2005; Buckler et al., 2009; Poland et al., 2011).

For a long time, QTL mapping has been the method of choice to explore the number, locations and effects of genes that affect a particular trait. The major shortcomings of QTL mapping are the ability of the method to identify maximum two alleles in genetically narrow-based biparental populations, low resolution power and the inconsistency in detected QTLs in different genetic backgrounds and environments. This makes the information on QTLs found in different mapping studies non-interchangeable and thus hinders its application in breeding. Holland (2007) explains that the instability of QTL for complex traits occurs because a biparental mapping population is a very small subset of an available diverse gene pool and each population in QTL mapping studies encompasses a different gene set controlling the trait.

Association analysis, an approach that tests correlations between genotypic and phenotypic variations in a diverse set of unrelated individuals, addresses these problems. It takes advantage of historical recombination in generations of meiosis during genotype development and is based on linkage disequilibrium between a marker and a QTL. Association analysis provides a higher resolution mapping, identification of more alleles, simultaneous analysis of multiple traits and direct application on breeding material.

Estimation of population structure, which can arise from adaptation to local conditions or selection, is essential to prevent declaration of false-positive associations. If population structure and relatedness among individuals are accounted for, association analysis can be a powerful tool to identify QTL with small effects, as well as to avoid declaring false positive associations (Yu et al., 2006).

The phenomenon of a stark disproportion between the number of QTL published and practical application of the markers co-localizing with detected QTL was highlighted by Xu and Crouch (2008). However, mapping studies can be consolidated to narrow down the DNA region where QTLs concur or overlap. A marker residing with quantitative trait loci mapped in various mapping studies across different environments and genetic backgrounds

could indicate the presence of one or more genetic factors, such as genes, transcription factor coding region or promotor, in the QTL vicinity underlying biological processes and eventually leading to the several phenotypic manifestations. Gene expression analysis postulate the existence of hotspots, a single polymorphism which can influence the expression of several genes mapped to the same locus (Breitling et al., 2008).

The aim of our study was to assess the genetic diversity and population structure of maize inbred lines, to validate the QTLs for traits of interest previously mapped in different studies and determine if consistent QTLs for multiple yield-related and flowering traits on the local breeding material and in agroecological conditions of southern Pannonian Basin may be valuable for marker assisted selection.

MATERIALS AND METHODS

A panel of 96 diverse maize inbred lines developed at the Institute of Field and Vegetable Crops, Novi Sad, Serbia, was selected for the analysis. It contained some historical relevant inbreds and a majority of elite commercial lines from Iowa Stiff Stalk Synthetic (BSSS), Lancaster and Iodent heterotic groups, including lines developed from local Serbian maize varieties with mixed origin and from exotic germplasm with limited information about their full pedigrees.

The trial was conducted during 2011 and 2012 in a randomized complete block design with three replicates in three locations: Rimski Sancevi (45°20' N, 19°51' E, 84 m a.s.l.), Srbobran (19°09' E, 45°46' N, 88 m a.s.l.) and Sombor (45°33' N, 19°48' E, 79 m a.s.l.) The plot size for each genotype was 6 m^2 and consisted of two rows, each 4 m long. The distance between rows was 0.75 m and 0.22 m within rows, with a density of 60 600 plants ha^{-1}. The planting was performed by machine, whereas harvest was done manually and standard production technology practices were applied.

The phenotypic evaluation of inbred lines was performed for the following traits: days to pollination, days to silk emergence, anthesis silk interval (d), plant height (cm), ear height (cm), ear diameter (cm), ear length (cm), row number (per ear), kernel number per row, total leaf number (per plant), number of leaves above the ear (per plant), 1000 kernel weight (g) and grain yield per plant (g). Days to pollination, days to silk emergence, and anthesis silk interval were evaluated at Rimski Sancevi and Srbobran during both years, whereas the other traits were analyzed in all five environments.

Genomic DNA was extracted from the seedlings using CTAB protocol according to the modified method of Doyle and Doyle (1990). Out of 50 markers initially chosen for their associations with QTLs for traits of interest in various mapping studies, 14 were excluded from the analyses due to their monomorphic manifestation or difficulties in PCR optimization. Remaining 36 fluorescently labelled SSR markers, evenly distributed throughout the genome and which primer sequences are available at the Maize Genetics and Genomics Database (MaizeGDB, http://www.maizegdb.org), were used for molecular characterization. Total PCR mix contained 2.5 µL 25 ng genomic DNA, 1 µL 0.2 mM dNTP, 1 µL 1×Taq buffer with KCl, 0.8 µL 2 mM $MgCl_2$, 0.2 µL 1 U Taq polymerase, 0.5 µL 0.5 pmol of each primer and 3.5 µL nuclease-free water. PCR began with DNA denaturation at 94 °C for 5 min, followed by 38 cycles at 94 °C for 30 s, 53-60 °C for 45 s, 72 °C for 45 s and the final extension for 7 min at 72 °C. The 10 µL reaction volume for fragment analysis contained: 2 µL mixture of differently labelled PCR products, 0.2 µL GeneScan500 LIZ size standard and 7.8 µL Hi-Di formamide. The PCR products were separated by capillary electrophoresis on ABI Prism 3130 and their sizes were determined with Gene Mapper Software Version 4.0 (Applied Biosystems).

The descriptive statistics for the phenotypic data were obtained in STATISTICA 12.6 (Statsoft, Tulsa, Oklahoma, USA). The broad-sense heredity of the traits was calculated using the following equation:

$$H^2 = \sigma^2_G/(\sigma^2_G + \sigma^2_e)$$

where σ^2_G is the genotypic variance component, and σ^2_e is the residual variance component. Genotypic data analysis determined the number of allele, observed heterozygosity, gene diversity and polymorphism information content (PIC) using Powermarker version 3.25 (Liu and Muse, 2005). Population structure of the diverse population was calculated using model-based clustering method based on parametric model of frequency distribution with unknown number of subpopulations integrated into STRUCTURE software (Pritchard et al., 2000). The hypothetical number of subpopulations (K) ranged from 1 to 10 with 5 independent runs per K. The burn-in period and run length of Markov Chain Monte Carlo algorithm was set to 100 000 × 100 000. The admixture model for the ancestry of individuals was chosen allowing the possibility that inbred lines may have mixed ancestry. The assumption that the allele frequencies in each population are independent draws from a distribution was set as a default. No prior information was used to define subpopulations. Optimal number of subpopulations K was chosen based on method proposed by Evanno et al. (2005). The method determined the most probable number of genetic clusters by calculating the *ad hoc* statistic delta K (ΔK), which identifies the highest rate of change in the log-likelihood between successive K values by the equation:

$$\Delta K = m(|L(K + 1) - 2\,L(K) + L(K - 1)|)/s[L(K)]$$

where *m* is the mean of the absolute value, *L(K)* is the mean likelihood over all runs for each *K* in STRUCTURE and *s[L(K)]* is the standard deviation of *L(K)* (Evanno et al., 2005). Maize genotypes with a membership coefficient less than 0.75 were considered as mixed origin.

Associations between the markers and the traits were tested using general linear model (GLM) and mixed linear model (MLM) in TASSEL 2.1 (Bradbury et al., 2007). Trait data were normalized and minor alleles with frequencies less than 5% were removed from marker data using algorithms implemented in Tassel. Estimation of population structure (Q matrix) based on the average value of five iterations

of log probability of data obtained by the STRUCTURE were incorporated in GLM as a fixed covariate as well as 1000 permutations for the correction of multiple testing. The marker-environment interaction was included in the model and was tested along the marker-trait associations using F-test with multiple degrees of freedom. Bonferroni correction for multiple testing (α/n) was applied to further reduce the possibility of declaring false positives. Coefficient of determination R^2 was computed to determine the percentage of phenotypic variation explained by a marker. Additionally, the kinship matrix (K) defining the level of genetic covariance between pairs of genotypes as a random effect was used along with Q matrix for population structure and family relatedness correction (Q + K) in MLM analyses.

RESULTS AND DISCUSSION

In total, 296 alleles were detected in 36 SSR loci with mean number of 8.2 alleles per locus (Table 1). One third of SSR markers had 10 or more alleles. The largest number of alleles (21) was observed at *umc1035* locus, while the smallest number of alleles (4) was identified at seven loci. Heterozygosity was observed at locus *umc1122* indicating residual heterozigosity or a possible mutation. The average number of alleles per locus was relatively large regarding the number of marker and genotypes used. Liu et al. (2003) found much larger average number of alleles per locus (21.7) with 96 SSR markers in a 260 diverse set of inbred lines. In the study of Li et al. (2006) the average numbers of alleles obtained with 64 microsatellites in 46 maize inbred lines, were much smaller, 6.3. The average PIC value was 0.64, similar to those found in other studies (Li et al., 2006; Reid et al., 2011), showing a significant level of genetic polymorphism. Relatively high level of polymorphism detected in the analyzed panel of maize inbred lines showed it contain significant genetic diversity and was suitable for of association mapping.

Table 1. Parameters of genetic diversity in maize inbred lines obtained with microsatellites.

SSR locus	Bin	Allele number	Observed heterozigosity	Gene diversity	PIC	Allele size (bp)
dupssr26	1.04	6	0.000	0.562	0.51	112-142
umc1035	1.06	21	0.000	0.866	0.85	110-212
umc1122	1.06	9	0.021	0.832	0.81	141-167; null
bnlg1556	1.07	11	0.000	0.806	0.78	150-184
bnlg125	2.04	11	0.000	0.669	0.63	164-196
phi083	2.04	4	0.000	0.614	0.56	121-129
bnlg1520	2.09	7	0.000	0.651	0.59	165-195
bnlg1523	3.03	9	0.000	0.480	0.46	177-236
umc1025	3.04	8	0.000	0.781	0.75	101-117
phi053	3.05	6	0.000	0.680	0.62	120-192
dupssr23	3.06	10	0.000	0.725	0.68	64-119
umc1022	4.01	6	0.010	0.472	0.40	65-97
umc2176	4.03	7	0.000	0.630	0.58	130-154
bnlg2291	4.06	10	0.000	0.751	0.72	153-196
phi093	4.08	4	0.000	0.664	0.60	280-286; null
umc1109	4.10	4	0.000	0.594	0.53	103-115
dupssr10	5.04	18	0.000	0.834	0.83	156-198
umc1221	5.04	8	0.000	0.757	0.73	69-95
bnlg1237	5.05	7	0.000	0.565	0.47	151-184
umc1792	5.08	5	0.000	0.603	0.54	113-125
bnlg238	6.00	12	0.000	0.832	0.81	135-179
umc1083	6.02	10	0.000	0.741	0.71	90-129; null
umc1014	6.04	8	0.000	0.792	0.76	113-140
bnlg1792	7.02	8	0.000	0.684	0.63	108-139
phi034	7.02	5	0.010	0.570	0.48	118-139
umc1944	7.04	6	0.000	0.705	0.65	117-139
umc1075	8.01	4	0.010	0.663	0.59	136-146
umc1360	8.02	4	0.000	0.639	0.58	139-149
bnlg162	8.05	11	0.000	0.813	0.79	214-260
bnlg666	8.05	15	0.000	0.778	0.76	111-158
phi027	9.03	4	0.000	0.557	0.46	141-156; null
bnlg430	9.03	5	0.000	0.569	0.53	99-111
bnlg1209	9.04	10	0.000	0.709	0.68	164-196
bnlg1525	9.07	13	0.000	0.811	0.79	157-200
phi059	10.02	4	0.000	0.549	0.45	139-156; null
umc2003	10.04	6	0.000	0.673	0.61	71-89; null
Average	-	8.2	0.001	0.684	0.64	-

PIC: Polymorphism information content.

The model based method grouped genotypes into three populations using an ad hoc statistic ΔK based on the rate of change in the log probability of data between successive K values (Figure 1). The ΔK value reached its peak for K = 3 (Figure 1), indicating that this set of inbreds could be divided until into three groups. The first group contained 28 genotypes, the second 16 and the third 36. The remaining 16 inbred lines having the membership coefficient Q less than 0.75 were grouped in the mixed group (Figure 2). The inbred lines clustered in general according to their pedigrees. The largest group contained inbred lines that belonged to BSSS heterotic group and originated mostly from B73, B14, B37, B84, and A681 lines, their crosses and different cycles of reselection. The second largest group encompassed 28 inbreds from Lancaster Sure Crop (LSC) heterotic group, lines that derived various crosses and from C103, Mo17, Oh43 and other local germplasm. The third groups consisted of 16 inbred lines which have mostly Iodent heterotic pattern and few adapted inbred lines from exotic South American gene pool and improved inbreds from local Serbian germplasm. In this study, three clusters identified using a

Figure 1. Estimation of the true number of clusters using ΔK method according to Evanno et al. (2005) from 10 iterations obtained with Bayesian clustering analyses implemented in Structure. Optimization of hypothetical number of clusters (K), varying from K = 1 to 10, was performed using second order dimensionless statistics (ΔK). The modal value (the peak) indicates the most probable number of clusters.

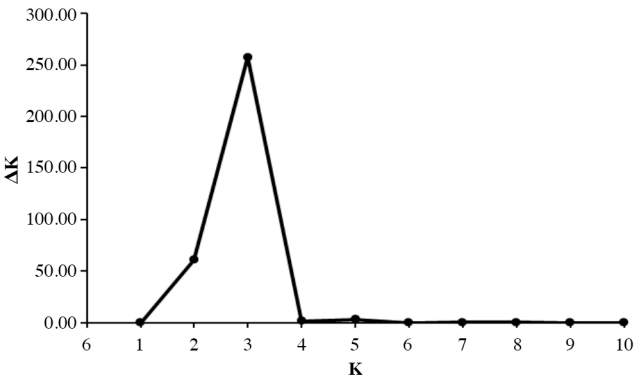
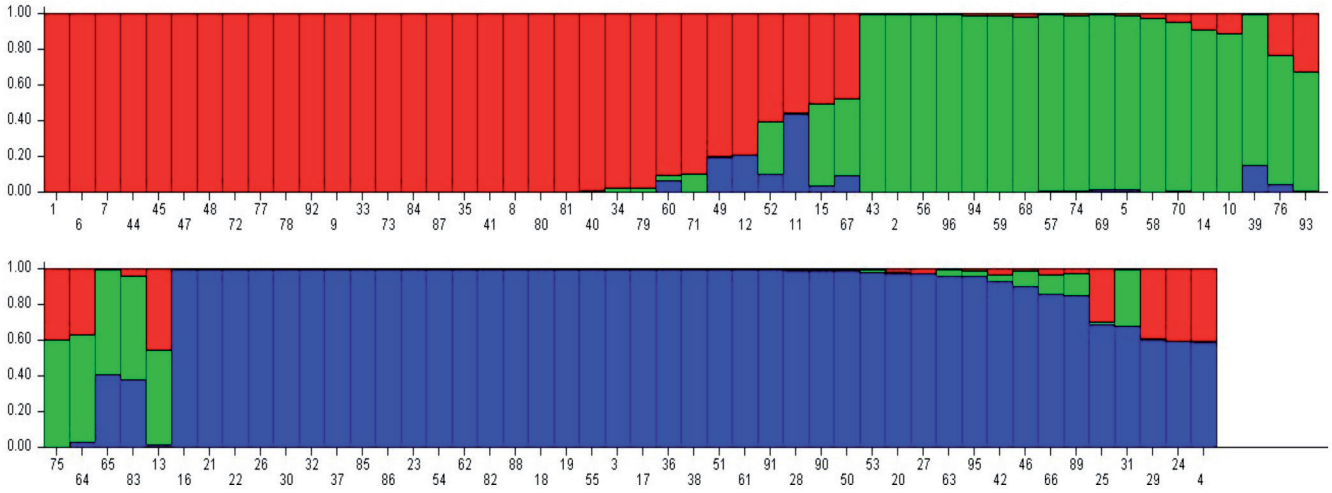


Figure 2. Population structure of maize inbred lines estimated with Bayesian assignment probabilities using microsatellite data. Each genotype is presented as a vertical bar showing its coefficient membership values in three clusters: BSSS (blue), LSC (red) and Iodent (green). The inbred lines with coefficient membership less than 0.75 in all three clusters were placed in the mixed group.



BSSS: Iowa Stiff Stalk Synthetic, LSC: Lancaster Sure Crop.

STRUCTURE analysis grouped inbred lines in three major heterotic groups, which is in congruence with their origins giving the biologically justification for the choice of the cluster number, as it was suggested by Pritchard et al. (2000). The correct estimates of population structure and accounts for its effects in linear models are crucial to avoid discovery of false-positive associations. Furthermore, the method that controls both population structure and relatedness proved to be powerful for association analysis (Yu et al., 2006).

For all investigated traits, the 96 inbred lines comprised a range of phenotypic variation in the tested environments (Table 2). Coefficient of variation ranged from 4.7% for days to silk emergence to 36.8% for anthesis silk interval revealing a large amount of genetic variation among the inbred lines. Among the 13 traits, number of rows per ear had the highest (88%), while yield per plant had the lowest (30%) broad sense heritability.

Association analysis indicated significant correlations between several agronomic traits and microsatellites tested in different environments (Table 3). Only the loci that showed consistent associations with three or more traits were presented. In total, 87 and 85 marker-trait associations were determined using GLM and MLM model, respectively. No significant Marker × Environment interactions were observed for all traits using GLM.

The associations for flowering time seemed to be consistent in most the environments, whereas the number of associations for yield and yield related traits varied across the markers for both models. Seven markers were correlated with grain yield. The most stable associations with this trait were identified with *bnlg162*, in three out of five environments using both models.

Based on the largest number of associations between the marker and the analyzed traits consistent in more environments and identified by both GLM and MLM models, three markers, namely *umc1025*, *bnlg1237* and *bnlg162*, were selected (Table 4). Slightly more significant associations between analyzed traits and markers *umc1025* and *bnlg1237* were identified by MLM in comparison to GLM, whereas more associations was found using GLM for *bnlg162* marker. Although MLM took into account information on both population structure and kinship and was, thus, more rigorous in claiming associations than GLM, it had greater statistical power and here detected more true associations than GLM.

Marker *umc1025* on chromosome 3 was associated with seven traits in two to four environments (Table 4). The phenotypic variation explained by the marker ranged from 5.6% for ear diameter to 14% for kernel number per row. Two traits, days to pollen shedding and ear diameter, had most consistent associations with the marker across environments. Grain yield was associated with *umc1025* in two and three environments, depending on the model. This marker accounted for 6.4%-8.1% of yield phenotypic

Table 2. Descriptive statistics and broad sense heritability (H^2) of maize inbred lines.

Trait	Mean ± SD	Minimum	Maximum	CV (%)	H^2
Days to pollination	81.1 ± 4.0	65.8	88.4	5.0	0.42
Days to silking	77.2 ± 3.6	63.5	83.6	4.7	0.53
Anthesis silk interval	4.0 ± 1.5	0.9	7.3	36.8	0.38
Plant height	178.2 ± 20.0	130.8	223.5	11.2	0.54
Ear height	71.6 ± 13.6	42.3	101.5	19.0	0.84
Leaves above the ear	6.0 ± 0.7	3.9	7.4	11.3	0.59
Total leaf number	12.3 ± 1.1	8.9	15.3	9.1	0.81
Ear length	14.7 ± 1.8	11.2	19.9	12.2	0.69
Ear diameter	3.9 ± 0.3	3.3	4.6	7.4	0.65
Row number	14.3 ± 1.7	10.8	18.9	11.6	0.88
Kernel number	24.4 ± 3.8	16.6	35.2	15.5	0.56
1000 kernel weight	254.4 ± 34.2	166.0	311.2	13.4	0.66
Yield per plant	100.9 ± 0.6	51.7	140.3	17.3	0.30

SD: Standard deviation; CV: coefficient of variation.

Table 3. Number of environments with significant associations between a marker and several maize traits using general (GLM) and mixed linear model (MLM).

Marker	Trait	Total nr env.	Number of environments with significant associations				
			GLM	MLM	Unique for GLM	Unique for MLM	Common for GLM and MLM
bnlg162	Days to pollination	4	4	3	1	0	3
	Days to silking	4	4	4	0	0	4
	Total leaf number	5	1	1	0	0	1
	Leaves above the ear	5	1	1	0	0	1
	Ear length	5	3	3	0	0	3
	Kernel number	5	2	1	1	0	1
	1000 kernel weight	5	1	2	1	2	1
	Yield per plant	5	3	3	1	1	2
umc1025	Anthesis silk interval	4	2	1	1	0	1
	Days to pollination	4	4	4	0	0	4
	Days to silking	4	3	3	0	0	3
	Total leaf number	5	2	2	0	0	2
	Ear diameter	5	3	4	0	1	3
	Kernel number	5	1	3	0	2	1
	Yield per plant	5	2	3	0	1	2
bnlg1237	Days to pollination	4	4	4	0	0	4
	Days to silking	4	4	4	0	0	4
	Ear diameter	5	1	1	1	1	1
	Ear length	5	4	5	0	4	1
	Yield per plant	5	0	1	0	1	0
umc1221	Ear height	5	5	1	4	0	1
	Total leaf number	5	1	0	1	0	0
	Ear diameter	5	1	2	0	1	1
	Yield per plant	5	1	1	1	1	0
dupssr23	Days to pollination	4	4	2	2	0	2
	Days to silking	4	2	1	1	0	1
	Total leaf number	5	3	2	1	0	2
umc1022	Plant height	5	3	3	0	0	3
	Ear height	5	5	5	0	0	5
	Leaves above the ear	5	1	0	1	0	0
bnlg238	Anthesis silk interval	4	4	3	1	0	3
	Ear height	5	1	1	1	1	0
	Yield per plant	5	1	1	0	0	1
phi027	Ear length	5	1	1	0	0	1
	Kernel number	5	1	2	0	1	1
	Yield per plant	5	1	1	0	0	1
phi034	Ear length	5	1	3	0	2	1
	Kernel number	5	1	2	0	1	1
	Yield per plant	5	1	1	0	0	1

nr env.: Number of environments.

variation. A QTL with positive additive effect on grain yield near *umc1025* was mapped in three environments in the study of Lima et al. (2006). Li et al. (2010) also found a QTL flanked with *umc1025*, which explained 10.4% of the phenotypic variation of yield.

Association analysis revealed significant correlations between marker *bnlg1237* on chromosome 5 and five traits in one to five environments. Marker associations with days to pollination, days to silking and ear length were significant in all environments for both models, except the latter which were constant in four environments using GLM. In previous studies, *bnlg1237* was associated with QTL for 1000 kernel weight (Lu et al., 2006), QTL for ear length (Ma et al., 2007), QTL for plant height (Tang et al., 2007) and QTLs for grain quality (Zhang et al., 2008).

Marker *bnlg162* on chromosome 8 was linked with eight traits in a range of environments. Phenotypic variation explained by the marker varied from 6% up to 18.2% depending on the trait concerned and to less extent to the environment. The most stable associations with the markers were identified for number of days to pollination and silking in all environments, whereas ear length and yield were consistent in three out of five environments. The marker *bnlg162* was correlated with QTL for plant height and flowering time (Frascaroli et al., 2007; 2009), QTL for root length, dry root weight and number of roots (Feng et al., 2013) and QTL for protein, starch and oil content (Wassom et al., 2008; Li et al., 2009).

Multiple associations between various traits and one marker can indicate existence of a gene or QTL with large single pleiotropic effect on different traits in proximity to the marker. It may be possible that these genes or QTLs participate in a complex molecular system that control biochemical, metabolic or physiological pathways and indirect pleiotropic effects on fitness yield and yield related traits (Hao et al., 2010). Multiple associations between various traits could also be due to closely located QTLs in linkage disequilibrium created by selection, genetic drift or admixture of populations with different gametic frequencies (Mueller, 2004). Yang et al. (2012) found consistent and

Table 4. Significant associations between markers *umc1025*, *bnlg1237*, *bnlg162* and maize traits.

		umc1025			*bnlg1237*			*bnlg162*		
Trait	E	p-GLM	p-MLM	R^2 (%)	p-GLM	p-MLM	R^2 (%)	p-GLM	p-MLM	R^2 (%)
ASI	E2	0.001*	1.2E-03*	9.3	ns	ns	-	ns	ns	-
	E3	0.001*	0.080	8.8	ns	ns	-	ns	ns	-
POLL	E1	0.001*	1.3E-03*	10.3	2.4E-04*	1.2E-03*	10.0	8.9E-05*	1.0E-04*	15.1
	E2	1.1E-03*	2.0E-04*	9.4	3.9E-04*	2.2E-04*	9.0	5.5E-05*	1.1E-03*	12.3
	E3	3.5E-04*	0.001*	10.9	8.5E-04*	1.1E-03*	9.2	3.5E-04*	0.001*	13.2
	E4	0.001*	1.9E-04*	13.9	0.001*	1.2E-03*	8.2	1.1E-04*	0.026	13.0
SILK	E1	1.8E-4*	1.0E-04*	8.5	0.001*	1.2E-03*	9.9	3.4E-04*	0.001*	16.3
	E2	0.001*	6.6E-04*	6.3	3.1E-04*	0.001*	8.2	1.4E-04*	1.2E-03*	17.2
	E3	1.3E-04*	4.3E-04*	5.8	1.4E-04*	1.9E-04*	7.8	1.4E-04*	0.001*	14.4
	E4	ns	ns	-	1.2E-03*	1.4E-04*	8.5	5.5E-04*	4.2E-04*	11.8
TLN	E2	1.3E-03*	0.001*	9.0	ns	ns	-	ns	ns	-
	E3	ns	ns	-	ns	ns	-	0.001*	0.001*	9.4
	E4	1.7E-04*	0.001*	6.3	ns	ns	-	ns	ns	-
LAE	E4	ns	ns	-	ns	ns	-	0.001*	0.001*	10.9
ED	E1	0.047	0.001*	5.6	ns	ns	-	ns	ns	-
	E2	0.001*	0.001*	5.8	ns	ns	-	ns	ns	-
	E4	1.9E-04*	1.6E-04*	7.1	6.4E-04*	1.34E-03*	6.1	ns	ns	-
	E5	4.9E-05*	1.7E-04*	7.5	ns	ns	-	ns	ns	-
EL	E1	ns	ns	-	0.001*	4.5E-05*	8.0	0.001*	1.8E-04*	9.4
	E2	ns	ns	-	0.036	0.001*	8.4	7.7E-05*	4.4E-05*	7.5
	E3	ns	ns	-	0.001*	3.1E-04*	7.5	ns	ns	-
	E4	ns	ns	-	3.4E-04*	1.1E-03*	8.0	ns	ns	-
	E5	ns	ns	-	7.4E-04*	4.2E-04*	6.8	5.8E-05*	2.2E-05*	8.6
KN	E2	ns	ns	-	ns	ns	-	3.8E-04*	0.015	-
	E3	0.008	0.001*	12.5	ns	ns	-	2.1E-04*	9.8E-04*	6.0
	E4	0.031	1.9E-04*	14.0	ns	ns	-	ns	ns	-
	E5	0.001*	3.7E-04*	10.2	ns	ns	-	ns	ns	-
TKW	E1	ns	ns	-	ns	ns	-	0.001*	0.044	15.1
	E3	ns	ns	-	ns	ns	-	0.012	1.1E-04*	12.4
	E5	ns	ns	-	ns	ns	-	0.031	2.8E-04*	18.2
YPP	E1	7.1E-04*	1.2E-03*	6.5	ns	ns	-	3.4E-04*	5.4E-04*	9.9
	E3	ns	ns	-	ns	ns	-	0.044	0.001*	8.0
	E4	0.021	1.4E-04*	6.4	ns	ns	-	7.2E-04*	1.2E-03*	10.9
	E5	6.5E-04*	1.1E-04*	8.1	0.002	3.4E-04*	4.7	0.001*	0.015	9.3

ASI: Anthesis silk interval; POLL: days to pollination, SILK: days to silking; TLN: total leaf number; LAE: leaf number above the ear; ED: ear diameter; EL: ear length; KN: kernel number per row; TKW: thousand kernel weight; YPP: yield per plant; R^2: percentage of phenotypic variation explained by the marker. E: environment; E1: Rimski Šančevi 2011; E2: Srbobran 2011; E3: Rimski Šančevi 2012; E4: Srbobran 2012; E5: Sombor 2011; ns: nonsignificant for neither model; *Bonferroni correction threshold (α/number of markers) $0.05/36 = 1.38 \times 10^{-3}$.

dense QTL clusters for yield and related traits in four chromosome bins 3.04-3.05, 7.02, 8.04-8.05 and 9.04-9.05. Two of them, 3.04 and 8.05, are congruent with the region of associations between a marker and multiple traits identified in this study. Since the bin size of maize chromosome is relatively large and corresponds to a typical QTL interval size of 10 cM (Holland, 2007), the location of QTLs in the same bin cannot provide precise conclusion about the nature of multiple traits correlations. Nonetheless, these loci may indicate the chromosome regions that harbor genetic factors important for controlling yield-associated traits and represent hotspots that deserve a more careful approach and closer examination.

CONCLUSIONS

The panel of selected maize inbred lines demonstrated significant genetic diversity suitable for association analysis. Its population structure analysis classified inbred lines concordantly to their origins and heterotic group indicating that Bayesian method could give more insight into often limited pedigree information. Significant associations found between the markers and the traits seemed to be more stable and consistent for flowering time than yield and yield-related traits, thus only former can be useful in marker-assisted breeding for the validated environments. The chromosome regions containing QTLs associated with multiple traits could be important targets for selection during breeding.

ACKNOWLEDGEMENTS

This research was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia (TR31073).

REFERENCES

Bradbury, P.J., Z. Zhang, D.E. Kroon, T.M. Casstevens, Y. Ramdoss, and E.S. Buckler. 2007. TASSEL: software for association mapping of complex traits in diverse samples. Bioinformatics 23:2633-2635.

Breitling, R., Y. Li, B.M. Tesson, J. Fu, C. Wu, T. Wiltshire, et al. 2008. Genetical genomics: spotlight on QTL hotspots. PLoS Genetics 4:e1000232.

Buckler, E.S., J.B. Holland, P.J. Bradbury, C.B. Acharya, P.J. Brown, C. Browne, et al. 2009. The genetic architecture of maize flowering time. Science 325:714-718.

Doyle, J.J., and J.L. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. Molecular Ecology 14:2611-2620.

Feng, X., W. Li, Y. Feng, W. Hu, L. Duan, J. Zhang, et al. 2013. Identification of significant loci for drought resistance and root traits at seedling stage with a set of maize introgression lines. Maydica 58:231-237.

Frascaroli, E., M.A. Cane, P. Landi, G. Pea, L. Gianfranceschi, M. Villa, et al. 2007. Classical genetic and quantitative trait loci analyses of heterosis in a maize hybrid between two elite inbred lines. Genetics 176:625-644.

Frascaroli, E., M.A. Cane, M.E. Pe, G. Pea, M. Morgante, and P. Landi. 2009. QTL detection in maize testcross progenies as affected by related and unrelated testers. Theoretical and Applied Genetics 118:993-1006.

Hao, Z., X. Li, X. Liu, C. Xie, M. Li, D. Zhang, et al. 2010. Meta-analysis of constitutive and adaptive QTL for drought tolerance in maize. Euphytica 174:165-177.

Holland, J.B. 2007. Genetic architecture of complex traits in plants. Current Opinion in Plant Biology 10:156-161.

Li, M., X. Guo, M. Zhang, X. Wang, G. Zhang, Y. Tian, et al. 2010. Mapping QTLs for grain yield and yield components under high and low phosphorus treatments in maize. Plant Science 178:454-462.

Li, M.S., X.H. Li, S. Salvi, R. Tuberosa, L.X. Yuan, F. Rotondo, et al. 2006. Genetic relationships among CIMMYT subtropical QPM and Chinese maize inbred lines based on SSRs. Maydica 51:543-549.

Li, Y., Y. Wang, M. Wei, X. Li, and J. Fu. 2009. QTL identification of grain protein concentration and its genetic correlation with starch concentration and grain weight using two populations in maize (*Zea mays* L.) Journal of Genetics 88:61-67.

Lima, M.L.A., C.L. Souza, D.A.V. Bento, A.P. Souza, and L.A. Carlini-Garcia. 2006. Mapping QTL for grain yield and plant traits in a tropical maize population. Molecular Breeding 17:227-239.

Liu, K., M. Goodman, S. Muse, J.S. Smith, E. Buckler, and J. Doebley. 2003. Genetic structure and diversity among maize inbred line as inferred from DNA microsatellites. Genetics 165:2117-2128.

Liu, K., and S.V. Muse. 2005. PowerMarker: integrated analysis environment for genetic marker data. Bioinformatics 21:2128-2129.

Lu, G.H., J.H. Tang, J.B. Yan, X.Q. Ma, J.S. Li, S.J. Chen, et al. 2006. Quantitative trait loci mapping of maize yield and its components under different water treatments at flowering time. Journal of Integrative Plant Biology 48:1233-1243.

Ma, X.Q., J.H. Tang, W.T. Teng, J.B. Yan, Y.J. Meng, and J.S. Li. 2007. Epistatic interaction is an important genetic basis of grain yield and its components in maize. Molecular Breeding 20:41-51.

Mueller, J.C. 2004. Linkage disequilibrium for different scales and applications. Briefings in Bioinformatics 5:355-364.

Poland, J.A., P.J. Bradbury, E.S. Buckler, and R.J. Nelson. 2011. Genome-wide nested association mapping of quantitative resistance to northern leaf blight in maize. Proceedings of the National Academy of Sciences 108:6893-6898.

Pritchard, J.K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Reid, L.M., K. Xiang, X. Zhu, B.R. Baum, and S.J. Molnar. 2011. Genetic diversity analysis of 119 Canadian maize inbred lines based on pedigree and simple sequence repeat markers. Canadian Journal of Plant Science 91:651-661.

Salvi, S., and R. Tuberosa. 2005. To clone or not to clone plant QTLs: present and future challenges. Trends in Plant Science 10:297-304.

Tang, J., W. Teng, J. Yan, X. Ma, Y. Meng, J. Dai, et al. 2007. Genetic dissection of plant height by molecular markers using a population of recombinant inbred lines in maize. Euphytica 155:117-124.

Wassom, J.J., J.C. Wong, E. Martinez, J.J. King, J. Debaene, J.R. Hotchkiss, et al. 2008. QTL associated with maize kernel oil, protein, and starch concentrations, kernel mass, and grain yield in Illinois high oil × B73 backcross-derived lines. Crop Science 48:243-252.

Xu, Y., and J.H. Crouch. 2008. Marker-assisted selection in plant breeding: from publications to practice. Crop Science 48:391-407.

Yang, G., Y. Li, Q. Wang, Y. Zhou, Q. Zhou, B. Shen, et al. 2012. Detection and integration of quantitative trait loci for grain yield components and oil content in two connected recombinant inbred line populations of high-oil maize. Molecular Breeding 29:313-333.

Yu, J., G. Pressoir, W. Briggs, I. Bi, M. Yamasaki, J. Doebley, et al. 2006. A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nature Genetics 38:203-208.

Zhang, J., X.Q. Lu, X.F. Song, J.B. Yan, T.M. Song, J.R. Dai, et al. 2008. Mapping quantitative trait loci for oil, starch, and protein concentrations in grain with high-oil maize by SSR markers. Euphytica 162:335-344.