



Agronomic and molecular evaluation of maize inbred lines for drought tolerance

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Abstract

Drought is a severe threat to maize yield stability in Serbia and other temperate Southeast European countries occurring occasionally but with significant yield losses. The development of resilient genotypes that perform well under drought is one of the main focuses of maize breeding programmes. To test the tolerance of newly developed elite maize inbred lines to drought stress, field trials for grain yield performance and anthesis silk interval (ASI) were set in drought stressed environments in 2011 and 2012. Inbred lines performing well under drought, clustered into a group with short ASI and a smaller group with long ASI, were considered as a potential source for tolerance. The former contained inbreds from different heterotic groups and with a proportion of local germplasm. The latter consisted of genotypes with mixed exotic and Lancaster germplasm, which performed better in more drought-affected environments. Three inbreds were selected for their potential drought tolerance, showing an above-average yield and small ASI in all environments. Association analysis indicated significant correlations between ASI and grain yield and three microsatellites (*bnlg1525*, *bnlg238* and *umc1025*). Eight alleles were selected for their favourable concurrent effect on yield increase and ASI decrease. The proportion of phenotypic variation explained by the markers varied across environments from 5.7% to 22.4% and from 4.6% to 8.1% for ASI and yield, respectively. The alleles with strongest effect on performance of particular genotypes and their interactions in specific environments were identified by the mean of partial least square interactions analysis indicating potential suitability of the markers for tolerant genotype selection.

Additional key words: anthesis silk interval; inbreds; microsatellites; yield; *Zea mays*.

Abbreviations used: ANOVA (analysis of variance); ASI (anthesis silk interval); CTAB (cetyltrimethylammonium bromide); GEI (genotype × environment interaction); GLM (general linear model); LOESS (locally weighted scatter plot smoothing); PCR (polymerase chain reaction); PLS (partial least square); QTL (quantitative trait locus); RS (Rimski Šančevi); SR (Srbobran); SSR (simple sequence repeat).

Authors' contributions: Conceived and designed the experiments: AKS and BK. Performed the laboratory experiments: SM and LB. Conducted field experiments: DS and BM. Statistical analysis and data interpretation: SM and MZ. Contributed new reagents and provided field resources: AN. Supervised and coordinated the work: GŠM. Wrote the paper: SM.

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Introduction

Drought is a complex abiotic stress that causes significant yield losses in rainfed grown field crops worldwide (Mir *et al.*, 2012). In Serbia and other temperate Southeast European countries, drought is a severe threat to maize (*Zea mays* L.) yield stability occurring occasionally but with significant yield reduction of more

than 60% during a dry year (Stricevic *et al.*, 2011). Due to limited irrigation capacities, maize production in Serbia, similarly to many developing countries, is traditionally and mostly rainfed. In the last couple of decades, a trend of higher air temperatures and decreased precipitation during maize growing season has been observed (Kresovic *et al.*, 2014), threatening Serbia to become drought-affected more often and more

intensely. The need for prompt and efficient solutions propelled maize breeding programmes to prioritise development of drought tolerant genotypes. The elusive nature and partially understood molecular mechanisms of plant tolerance to drought could be a reason why breeding for drought tolerant maize is often very challenging (Tuberosa & Salvi, 2006). Moreover, it is often difficult to make accurate predictions of drought events and anticipate crop responses once they occur, which further impede breeding efforts.

Grain yield in water stress free conditions is a chief selection criterion for improving adaptation to abiotic and biotic stress in most maize breeding programmes in Serbia. Inbred lines development and evaluation of their hybrid performance under rainfed conditions during the favourable weather conditions carry a risk of hybrid underperformance when a drought occurs. The choice of maize inbred lines for drought tolerance, based on their performance in well-watered conditions, can be hampered by low correlation between predictions of performance in such contrasting environments and low selection efficiency due to low yield heritability and genetic variance caused by drought (Blum, 2005; Cairns *et al.*, 2013). Inbred lines with good yield potential *per se* and good combining abilities, tolerant to abiotic stresses, could be a valuable breeding material for developing high-yielding and drought-tolerant hybrids.

To address this goal, Bruce *et al.* (2002) and Monneveux *et al.* (2006) proposed the indirect selection for secondary traits correlated to drought tolerance such as anthesis silk interval (ASI), stay-green, early flowering, leaf growth, leaf rolling, tassel size, grain size, plant height and number of primary tassel branches. The results of Monneveux *et al.* (2008) suggested that the relationship between grain yield and secondary traits was prone to change during selection as some traits became more while others became less important for yield selection. Bruce *et al.* (2002) highlighted reduced bareness, ASI and stay-green as the key secondary traits for drought tolerance selection. Drought prolongs ASI, delaying silk growth and emergence, and accelerates pollen and stigma senescence, resulting in poor fertilisation and incomplete kernel set. Since genotypes with shorter ASI tackle drought more efficiently, maize breeders often use ASI to estimate a cultivar response to stress and predict its grain yield under drought stress (Edmeades *et al.*, 2000).

Drought also increases genotype \times environment interaction (GEI) further impeding breeding process (Cattivelli *et al.*, 2008). Moreover, both conventional and molecular breeding strategies rely on understanding GEI, which can be further dissected on QTL \times environment interaction. Determination of genetic factors

and environmental variables that contribute most to GEI variation could assist selection of superior and stable genotypes (Cossa *et al.*, 1999). Multi-environment trials are being increasingly used for analysing GEI, assessing the stability of quantitative traits and finding associations among molecular markers and quantitative trait variation based on association analysis (Federer & Cossa, 2012). Besides, association analysis has become a method of choice in elucidating genetic factors that control important agronomic traits, as it enables the evaluation of genotypes in a range of environments and distinction of environments in which specific QTL or combinations of QTLs were expressed (Reynolds *et al.*, 2009; Maccaferri *et al.*, 2011). One of many statistical models developed for studying GEI is the partial least square (PLS) regression model (Aastveit & Martens, 1986), which has proved to be a useful tool to find environmental variables or genetic marker data influencing GEI of a particular trait and to explain a considerable proportion of GEI variability (Vargas *et al.*, 1999).

The success of maize breeders in selecting inbred lines for hybrid development under water-limited conditions depends greatly on effective screening and evaluation of the source of drought tolerance with desirable quantitative traits, identification of molecular markers linked to QTLs controlling the target traits and GEI under drought. Therefore, the aim of this study was (i) to identify maize inbred lines with short ASI and high grain yield in drought conditions, (ii) to select markers associated with ASI and yield, specific to tested environments and (iii) to assess environmental variables responsible for GEI and marker allele-genotype interactions with four drought affected environments.

Material and methods

Ninety six diverse maize inbred lines from the Institute of Field and Vegetable Crops, Novi Sad, Serbia, containing elite material from Iowa Stiff Stalk Synthetic (BSSS), Lancaster and Iodent heterotic groups, inbreds developed from local Serbian maize varieties of mixed origin and from exotic germplasm were selected for the analysis.

The experiment was set in a completely randomised design with three replications at Rimski Šančevi (19° 51' E, 45° 20' N, altitude 84 m) and Srbobran (19° 09' E, 45° 46' N, altitude 88 m), during drought-affected years of 2011 and 2012 and on chernozem soil. The genotypes were evaluated in two-row plots, 4 m long, with 0.75 m between rows and 0.22 m within rows and a plant density of 60,600 plants/ha. The field trials were

managed following the standard agricultural practices. Anthesis silk interval was recorded as the number of days between the dates when 50% of the plants shed pollen and when on 50% of the plants 2 cm long silk emerged. At physiological maturity, all plants were hand-harvested and grain yield (g/plant) was measured.

The maize genomic DNA was extracted from the 10-day-old seedlings using modified cetyltrimethylammonium bromide (CTAB) protocol. Thirty six fluorescently labelled simple sequence repeat (SSR) markers with primer sequences obtained from the Maize Genetics and Genomics Database (<http://www.maizegdb.org>) were used. Total polymerase chain reaction (PCR) mix contained 25 ng of genomic DNA, 0.2 mM dNTP, 1 × Taq buffer with KCl, 2 mM MgCl₂, 1U Taq polymerase and 0.5 pmol of each primer. PCR began with DNA denaturation at 94°C for 5 min, followed by 38 cycles at 94°C for 30 s, 53°C-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 visualized and analysed with Gene Mapper Software 4.0 (Applied Biosystems).

Descriptive statistics for ASI and grain yield in all four environments (location × year combination), across years, locations and as overall means were performed and graphically presented with boxplots in the R software (R Core Team, 2015; <http://www.R-project.org/>). One way analysis of variance (ANOVA) and Tukey multiple comparisons of means for 95% and 99% confidence levels were applied to test differences between mean values among four environments for two traits.

The broad-sense heritability of the traits in was calculated for each and across environments as a proportion of genetic variance component in total phenotypic variance. For a single environment, heritability was calculated as:

$$H^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_e^2),$$

whereas heritability across environments were calculated using the formula:

$$H^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_{ge}^2 / e + \sigma_e^2 / re),$$

where σ_g^2 , σ_{ge}^2 and σ_e^2 are genetic, genotype × environment and residual variance, respectively, e denotes the number of environments and r presents the number of field replications.

Cluster analyses with different distances (Euclidean and Manhattan) for calculating dissimilarities between

observations and different clustering methods (un-weighted and weighted pair-group average methods, single linkage, complete linkage and Ward's method) were tested to find the most appropriate method for grouping the inbred lines according to their yield and ASI performance. The R package Pvcust was used to calculate probability values for each cluster using approximately unbiased method based on multiscale bootstrap resampling (Suzuki & Shimodaira, 2006). Different cluster means were tested for significance with ANOVA and compared with Tukey honest significant difference test in the R software.

Correlations between ASI and grain yield in each environment were calculated with Spearman's rank correlation coefficient, whereas the averaged correlations for years, locations and environments were calculated using Fisher's z -transformation. A smooth curve between ASI and grain yield was fitted with locally weighted scatter plot smoothing (LOESS) in the R software.

General linear model (GLM) was applied to test the significance of associations between the markers and the traits in the software Tassel 2.1 (Bradbury *et al.*, 2007). Estimation of population structure (Q) obtained by the software Structure (Pritchard *et al.*, 2000) was implemented in GLM as a fixed covariate. The optimal number of groups obtained from the Structure was chosen according to log probabilities of data $\Pr [X|K]$ (Pritchard *et al.*, 2000) and the method of Evanno *et al.* (2005). The Bonferroni correction for multiple testing (α/n) at 0.05 was applied to reduce the possibility of declaring false positives. The percentage of phenotypic variation explained by a marker was determined by coefficient of determination (R^2).

The partial least squares (PLS) regression was used to identify the most important environmental variables that explained GEI for ASI and yield (model 1) and the most important marker alleles effecting GEI for ASI (model 2). In the first model, explanatory variables were represented with a set of 12 standardized environmental variables measured during growing seasons and were contained in an independent data (Z) matrix. The environmental variables were: mean temperature (tm, °C), maximum temperature (mx, °C), precipitation (pr, mm), relative humidity (rh, %) and temperature variation (tv, °C), measured during June (abbreviated as 6), July (7) and August (8). For the second model, explanatory variables that were included in the independent data (Z) matrix were effects of marker alleles that were found to show significant associations with both traits across the environments. The dependent variable Y matrices for ASI and/or grain yield were presented with four rows corresponding to environments and 96 columns corresponding to inbred lines.

The independent data (Z) matrices were related to the double-centred genotype by location dependent (Y) matrix representing grain yield or ASI data across four environments. The data matrices were expressed as:

$$Y = TQ' + F \text{ and } Z = TP' + E,$$

where matrix T contained Z scores, matrix P contained Z loadings, matrix Q consisted of Y loadings and F and E were the residuals of variation. The relationship among the Y and Z matrices was presented using the latent variable T . The number of T variables that explained as much as possible of the variance between Y and Z was determined by cross-validation procedure (Stone, 1974). The data visualizations of PLS analysis were performed in the R software.

Results

The prominent water deficit and high daily temperatures were observed during June, July and August in 2011 and 2012 not only in Serbia, but in many parts of Southeast Europe (Fig. S1 [suppl.]). This period coincided with flowering, fertilisation and grain filling, the stages that most determine maize yield. During these three months, precipitation sums were lower than

the long-term average (1981-2010), by 53.1% to 67.4% in 2011 and by 57.1% to 63.1% in 2012, depending on the locality (Table S1 [Suppl.]). The average daily temperatures were higher than the long-term average by 2.2°C in Srbobran to 3.1°C in Rimski Šančevi in 2012 and similar to the long-term average in 2011. There were, on average for two locations, 40% and 110% more days with maximum temperatures above 30°C during June, July and August in 2011 and 2012, respectively, comparing to the long-term average.

Considerable phenotypic variation of ASI and grain yield with coefficients of variation 38.6% and 16%, respectively, was observed among inbred lines across four environments. The highest yield of 117.85 g/plant was observed in SR11 environment (Fig. 1). In 2012, environments RS12 and SR12 had the lowest mean values for yield 81.72 g/plant and 82.26 g/plant, respectively, not significantly differing from each other ($p = 0.9428$). The variations in yield were more pronounced in RS than in SR, as well as in 2011 in comparison to 2012. Anthesis silk interval was the shortest in 2011 with no significant differences ($p = 0.3684$) between 2.75 and 2.34 days in locations RS and SR, respectively. In 2012, both RS and SR locations had significantly ($p = 0.0000$) higher values of ASI, 5.19 and 4.74 days, respectively, comparing to those in the previous year, but not between each other ($p = 0.2551$). Unlike

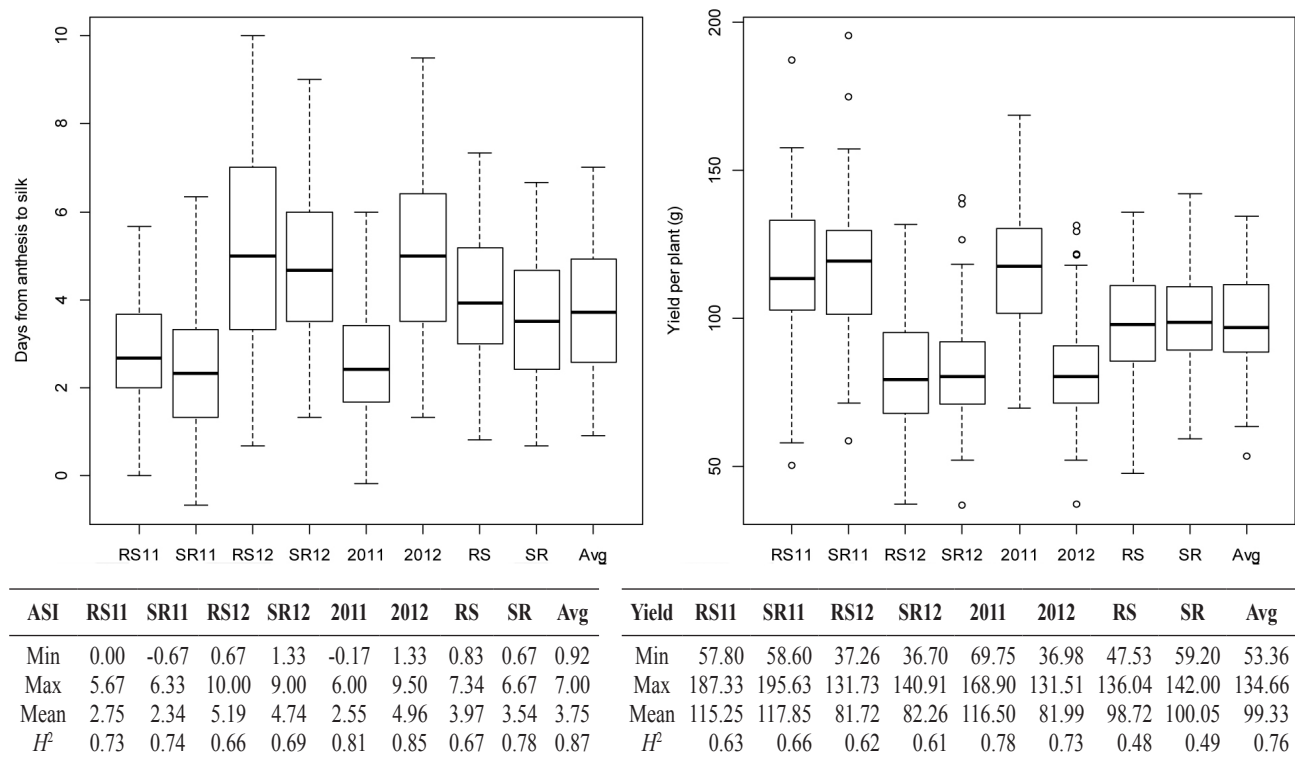


Figure 1. Boxplots with median, extremes, quartiles (above) and minimum, maximum, mean values and heritability (H^2) for maize (*Zea mays* L.) anthesis silk interval (ASI) (left) and grain yield (right) representing four environments (RS11, SR11, RS12 and SR12), their averages across years (2011 and 2012), locations Rimski Šančevi (RS) and Srbobran (SR) and the total average (Avg).

grain yield, ASI variations were greater in 2012 than in 2011. The broad sense heritability of ASI and yield was high in each environment, ranging from 0.66 to 0.74 for ASI and from 0.61 to 0.66 for yield (Fig. 1). The heritability values were slightly lower in environments under severe drought than in environments under moderate drought. Notably, heritability for yield observed across locations was considerably lower than heritability estimated across years.

Ranking the genotypes by their two-year yield average and plotting its values against the genotypes' corresponding ASI revealed a trend showing that better performing genotypes had shorter interval between male and female flowering time (Fig. S2 [suppl.]). The inbred lines with ASI of six and seven days mostly grouped among the worst ranked genotypes with a grain yield up to 85 g/plant. The inbred lines with four to five days between pollen shedding and silk appearance had an intermediate yield of 85 to 110 g/plant, whereas the best yielding inbreds with more than 110 g/plant had on average one to three days of ASI. Most of the good performing inbred lines had high yield in 2011 and intermediate or low yield in severely drought affected 2012 showing their capacity to cope with mild to intermediate drought stress. Only nine inbred lines, namely G11, G13, G24, G36, G39, G42, G43, G69 and G94, had an above total average yield in all four environments. Three of them (G36, G39 and G42) scored a yield above 115 g/plant and ASI less than two days in all environments.

The phenotypic correlations between ASI and yield per plant were significant and moderate in each environment, in averaged years and averaged locations as well as in overall mean (Fig. S3 [suppl.]). The Spearman's coefficient ranged from -0.38 ($p < 0.000$) in SR12 to -0.64 ($p < 0.000$) in RS12. An average correlation between days from pollen shedding to silk emergence and grain yield of maize inbred lines was $\rho = -0.55$ ($p < 0.000$).

The weighted pair-group method with arithmetic mean clustering method with Manhattan distance measure showed to be the best choice for grouping the inbred lines according to their yield and ASI performance among the tested clustering procedures and different similarity measures. Multiscale bootstrap resampling p values for clusters was high ($p \geq 95\%$), indicating that the clusters were well supported by data (Fig. 2a). The analysis clustered the genotypes into four groups. The first group comprised 32 lines with intermediate ASI and low yield: the average ASI was 3.26 days while the mean yield was 94.02 g/plant. The second group contained six lines with high ASI values and high yield, with an average ASI of 4.57 days and a mean yield of 108.60 g/plant. The third group of 25 inbreds

had low ASI values (2.19 days on average) and high yield (average yield of 119.81 g/plant). The last fourth group encompassed 33 lines with high ASI and low yield values, 5.29 days and 87.12 g/plant, respectively. All four groups differed significantly in their mean ASI and yield values ($p = 0.0000$), except the group two and the group four ($p = 0.1329$) for ASI, and the group one and the group four ($p = 0.0121$) and the groups two and three ($p = 0.0326$) for yield.

For both ASI and grain yield, the analysis of variance showed that the GEI was significant ($p < 0.01$). The main effect of environments explained 29.3% and 34.4%, whereas differences between genotype means contributed 40.2% and 28.6%, and GEI did with 15.8% and 20.9% of the total sum of squares for ASI and grain yield, respectively (data not shown).

The cross-validation procedure for the number of significant PLS dimensions suggested that two dimensions (latent vectors) were of relevance for prediction. Partial least square analysis of GEI, showed that the variance of explanatory variables explained by the first PLS dimension, namely mean maximum temperatures in July (mx7) and August (mx8) and precipitation in June (pr6) was large ($\geq 75\%$) for both ASI and yield, while the variance of relative humidity in July (rh7) and August (rh8) was large ($\geq 75\%$) for ASI and moderate ($> 60\%$) for grain yield (Fig. 3). Therefore, of all environmental variables, maximum daily temperatures and relative air humidity during flowering and grain setting and precipitation at pre-flowering and flowering stages most strongly affected GEI for ASI and grain yield.

The population structure of the genotypes was obtained in Structure software and the inbred lines were grouped into three clusters, as determined according to the proposed methods (Fig. 2b). The clusters were consistent with three major maize heterotic groups: BSSS, Lancaster and Iodent. The consistency of the clusters with inbred lines' genetic background (Fig. 2c) justified the choice of the cluster number. Maize inbred lines developed from crosses between different heterotic groups and exotic non-tropical germplasm and local Serbian germplasm had the membership coefficient (Q) less than 0.75 and were considered as mixed origin.

Association analysis revealed several marker-trait associations for ASI and grain yield and the markers significantly associated with those traits in at least two environments were retained for further consideration (Table 1). Five and four markers showed stable associations with ASI and yield, respectively. More significant associations were found for ASI than for grain yield. Three markers on the chromosomes 3, 6 and 9, namely *umc1025*, *bnlg238* and *bnlg1525*, were associ-

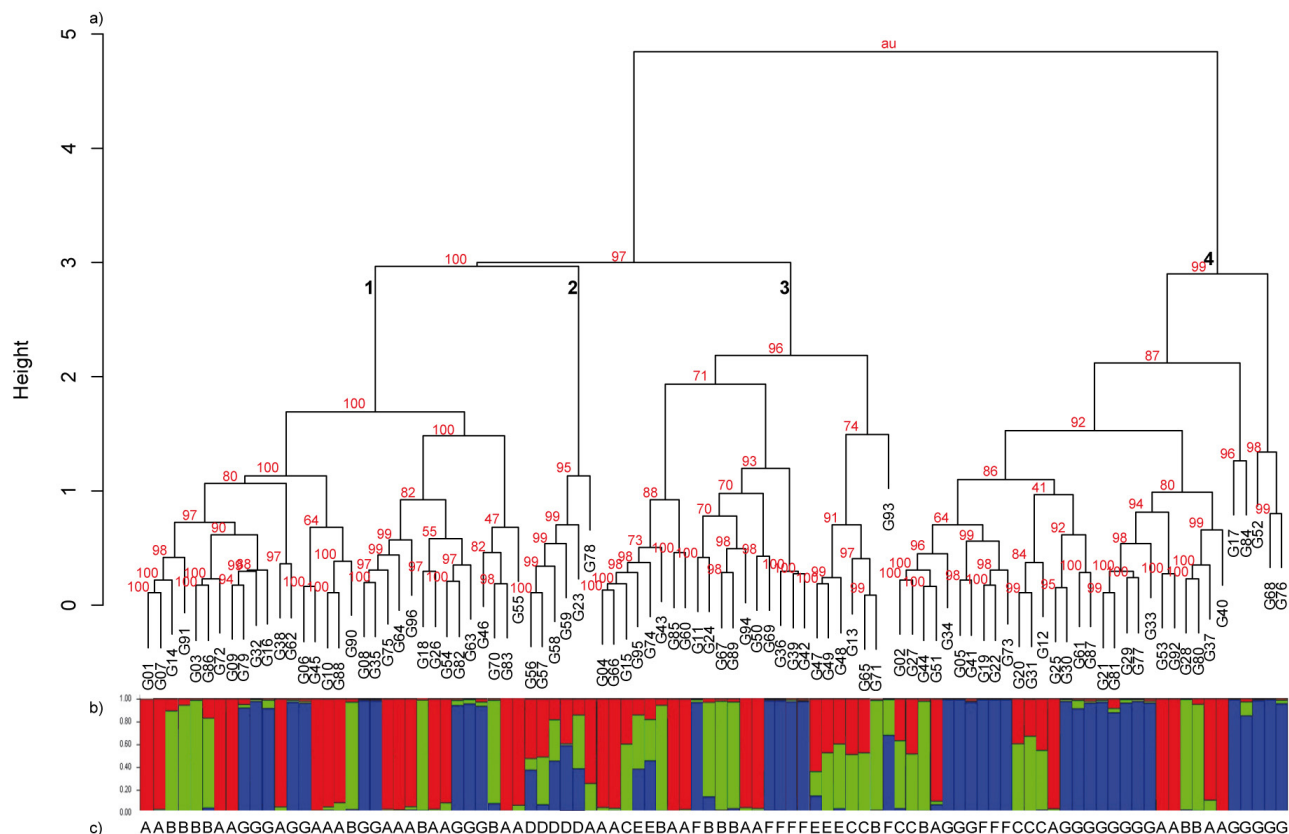


Figure 2. a) Dendrogram of maize inbred lines obtained by weighted clustering method and Manhattan distance showing four groups of inbred discriminated by their anthesis silk interval (ASI) and yield performances. The first group (1) contained inbreds with intermediate ASI and low yield; the second group (2) consisted of inbreds with long ASI and high yield; the third group (3) clustered inbreds with short ASI and high yield and the fourth group (4) encompassed inbreds with long ASI and low yield. Approximately unbiased (au) p -values indicate how strong the cluster is supported by data (high au values ≥ 95 are strongly supported by data). b) Population structure of maize inbred lines estimated with SSR markers using software STRUCTURE. The vertical bars show the coefficient membership values (Bayesian probabilities) assigning the inbred lines in groups: Lancaster (red), BSSS (blue) and Iodent (green). The inbred lines with coefficient membership less than 0.75 in all three clusters were placed in the mixed group. c) The germplasm group: A, Lancaster; B, Iodent; C, mixed Lancaster and Iodent origin; D, mixed exotic non-tropical germplasm from Argentina with Lancaster; E, mixed Serbian local germplasm with Lancaster or Iodent; F, non-B73 BSSS; and G, reselected B73 inbreds.

ated with both traits. They were significant in all environments for ASI and in two to three environments for grain yield. The proportion of phenotypic variation explained by the markers varied across environments from 5.7% up to 22.4% and from 4.6% up to 8.1% for ASI and yield, respectively.

The effects of 31 alleles from these three markers on ASI and yield were estimated (Fig. 4). In total, nine alleles affected ASI reduction and 11 alleles affected yield increase. Eight alleles were selected for their favourable concurrent effect on yield increase and ASI decrease. Those were alleles 163 bp and 174 bp of marker *bnlg1525*, alleles 135 bp, 161 bp, 163 bp and 167 bp of marker *bnlg238* and alleles 107 bp and 111 bp of *umc1025*. One *umc1025* allele had a favourable effect of ASI reduction, but also a negative one on yield in three out of four environments. The

allele 115 bp of marker *umc1025* and alleles 184 bp and 186 bp of marker *bnlg1525* had positive effects on yield and inconsistent influence on ASI across the environments.

To identify the most important marker alleles, as explanatory variables, affecting genotype performance in each environment and their interactions, the multivariate dimension reduction PLS regression approach was employed (Fig. 5). According to the cross-validation procedure, the first two latent dimensions were found to be highly significant ($p < 0.01$) for prediction and explained 52.3% and 22.9% of the total interaction variance for ASI. Marker alleles that formed acute angles with the x-axis (first dimension) and were further away from the origin better explained the interaction variation, regardless of their positive or negative effect on the trait. The PLS revealed that the first di-

Table 1. Marker-trait associations for maize ASI and grain yield traits significant in at least two environments

Marker	Environment	Bin	df ₁ , df ₂	F	p	R ² (%)	
ASI							
<i>bnlg1525</i>	RS11	9.07	11, 81	3.4958	0.0005*	10.1	
	SR11			3.1704	0.0013*	9.4	
	RS12			3.1977	0.0012*	9.3	
<i>bnlg238</i>	SR12	6.00	10, 82	3.2955	0.0009*	8.3	
	RS11			3.9572	0.0002*	13.9	
	SR11			3.3748	0.0010*	16.1	
<i>umc1022</i>	RS12	4.01	5, 87	3.5589	0.0006*	22.4	
	SR12			4.8186	0.0000*	18.9	
	RS11			4.3915	0.0013*	7.9	
<i>umc1025</i>	SR11	3.04	7, 86	4.6018	0.0009*	6.7	
	RS12			4.8346	0.0006*	6.9	
	SR12			4.4871	0.0011*	5.7	
<i>umc1360</i>	RS11	8.02	3, 90	3.8469	0.0011*	10.7	
	SR11			3.8900	0.0010*	9.3	
	RS12			3.8854	0.0010*	8.8	
	SR12			3.7717	0.0013*	10.4	
	RS11			5.6878	0.0013*	9.4	
	SR11			5.8945	0.0011*	7.6	
	RS12			5.8277	0.0012*	10.4	
	SR12			5.9078	0.0010*	8.0	
Yield							
<i>bnlg1525</i>	RS11	9.07	11, 81	3.2150	0.0011*	7.9	
	SR11			2.9620	0.0024	7.0	
	RS12			3.4824	0.0005*	7.8	
<i>bnlg238</i>	SR12	6.00	10, 82	3.2597	0.0010*	6.9	
	RS11			3.3062	0.0012*	7.1	
	SR11			3.3404	0.0011*	6.3	
<i>umc1025</i>	RS12	3.04	7, 86	3.9572	0.0002*	5.9	
	SR12			2.1748	0.0274	7.2	
	RS11			4.0445	0.0007*	6.5	
<i>umc1083</i>	SR11	6.02	9, 78	2.8324	0.0105	8.5	
	RS12			2.8114	0.0110	6.8	
	SR12			4.0845	0.0006*	8.1	
	RS11			3.4704	0.0012*	4.7	
	SR11			3.1265	0.0029	5.3	
	RS12			3.8134	0.0005*	4.6	
	SR12			3.2341	0.0022	5.2	

df: degrees of freedom; * $p < \text{Bonferroni correction threshold } (\alpha/\text{number of markers}) 0.05/36 = 1.38 \times 10^{-3}$; R²: percentage of phenotypic variation explained by the marker; RS11: Rimski Šančevi 2011; SR11: Srbobran 2011; RS12: Rimski Šančevi 2012; SR12: Srbobran 2012.

mension was dominated by differences between environments with shorter ASI (RS11 and SR11) vs. environments with longer ASI (RS12 and SR12), as the majority of inbred lines with high ASI and low yield grouped around more drought affected environments RS12 and SR12 and vice versa. In general, marker alleles grouped around the environments and among the genotypes on which had the strongest effect, either negative or positive. Seven of eight alleles with favourable effect on both ASI and yield grouped with maize inbred lines with shorter ASI and higher yield around two less drought affected environments (RS11 and SR11). The remaining favourable allele 174 bp of

bnlg1525 grouped among poorer performing genotypes in more drought affected environment RS12. A few good performing genotypes in terms of their average ASI and grain yield values (e.g. genotypes 4, 11 and 93) were placed among low-yielding and long-ASI inbreds in RS12, the environment that was severely affected by drought. Despite their good overall performance, these three inbred lines had the lowest yield and the largest ASI in this environment and were nearest to the marker allele *umc1025-109* with the strongest effect on ASI increase (2.37 days) and yield decrease (-21.12 g/plant) in RS12 comparing to the other three environments (Fig. 5).

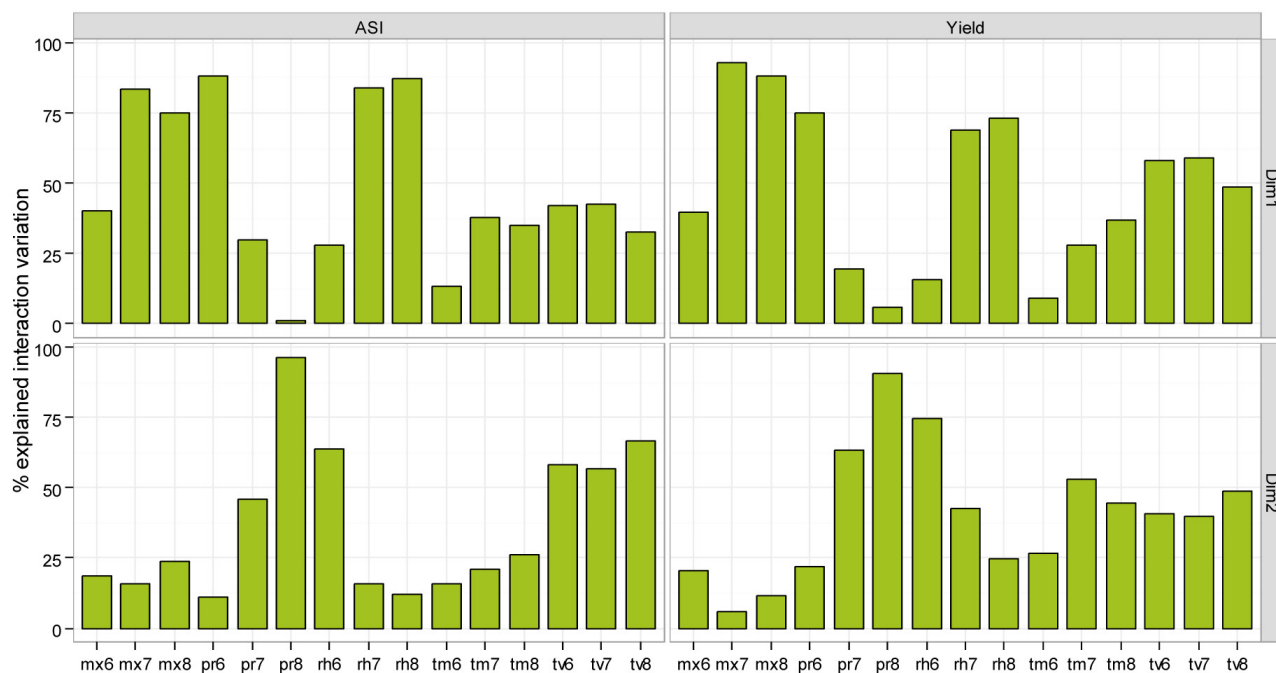


Figure 3. Variation of environmental variables by PLS dimensions for ASI and grain yield of maize inbred lines: mx, maximum temperature; pr, precipitation; rh, relative humidity; tm, average temperature; tv, temperature variation; 6, June; 7, July; 8, August.

Discussion

As demonstrated, drought is a complex phenomenon that refers not only to severe water deficit due to insufficient precipitation during the growing season, but also to a synergy of unfavourable weather conditions, such as extremely high temperatures, low relative humidity, strong insolation, as well as soil water scarcity leading to plant dehydration and heat stress and ultimately jeopardising crop production. Simulations of drought conditions in artificial growing facilities, *i.e.* experimental pot or hydroponics, do not necessarily imply straightforward yield prediction on the field (Hervé & Serraj, 2009). This problem was partially overcome by rainout shelters that control water supply shielding the crops from the rain; however, they are prone to experimental error due to the edge effect and uncontrolled microclimate modifications under the shelters and are associated with certain costs (Yahdjian & Sala, 2002). Considering all these facts and in order to estimate maize inbred lines performance under real growing conditions, we opted for setting the experiment in rain-fed conditions confining it to two drought-affected years.

Different genotypic responses to moderate and severe drought conditions in 2011 and 2012, respectively, were reflected in considerable variation of grain yield and ASI among maize inbred lines. The diverse set of inbred lines selected for the analysis seemed to cover a lot of the genetic variation of maize and could

be a valuable source of favourable alleles for the investigated traits. Our results were in accordance with Almeida *et al.* (2014) who observed that genetic variance was reduced for grain yield and increased for ASI under drought stress. Significant negative correlations between ASI and yield in this study were congruent with other findings (Bolanos & Edmeads, 1996; Zheng *et al.*, 2009; Almeida *et al.*, 2014).

The broad sense heritability for ASI and yield was high in all single environments, indicating large genetic variance present in diverse evaluated maize material. The heritability for both traits was lower in more drought affected environments, similarly to previous findings (Bolanos & Edmeads, 1996; Cairns *et al.*, 2013), due to reduced genetic variance and higher error variance. When environments were combined across years and locations, reduced heritability was observed in locations RS and SR (Fig. 1). This was due to a larger proportion of GEI variation that attributed to the phenotypic variation when considered environments under severe and moderate drought in a single location in comparison to the environments under similar drought intensity in a single year. Since the yield stability can be hindered by considerable GEI manifested in low heritability, especially in the environments with contrasting stress intensities, higher estimates of yield heritability and, consequently, its selection efficiency could be achieved by increasing number of testing environments (Frey *et al.*, 2016), complementing phenotypic selection with highly heritable secondary traits

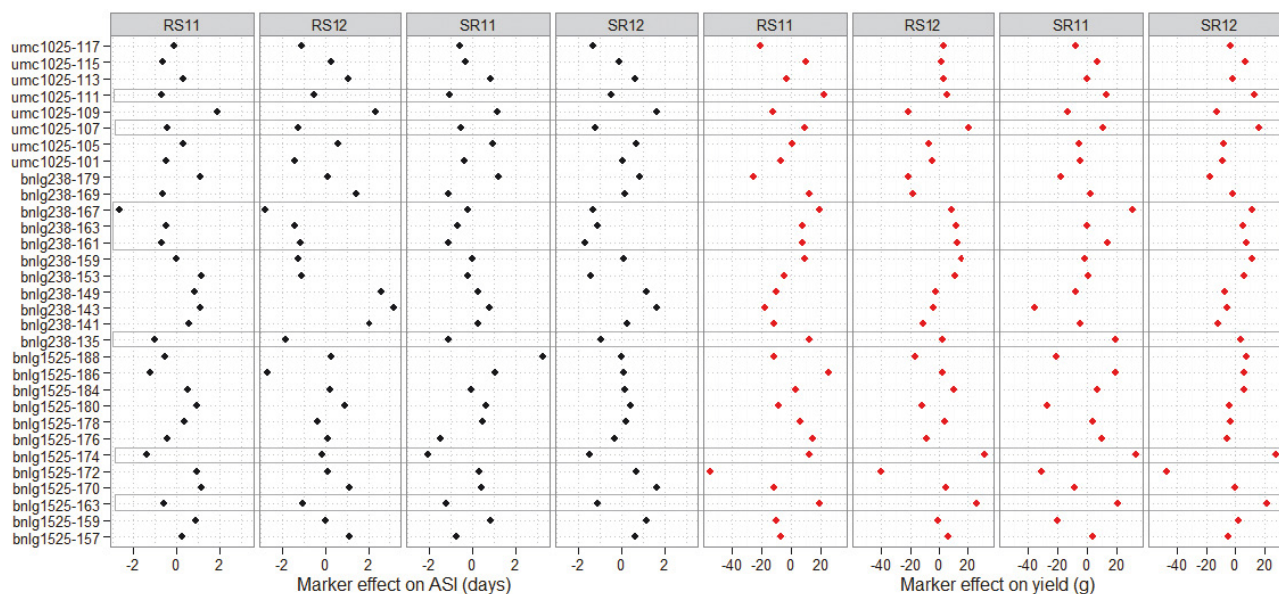


Figure 4. Effects of *bnlg1525*, *bnlg238* and *umc1025* marker alleles on ASI and grain yield of maize inbred lines in four environments: RS11, Rimski Šančevi 2011; SR11, Srbobran 2011; RS12, Rimski Šančevi 2012; SR12, Srbobran 2012. The highlighted alleles had simultaneous effects on ASI reduction and yield increase. The effects of marker alleles on ASI are in black and red for yield.

genetically correlated with yield (Araus *et al.*, 2012), and using environment specific markers in assessing drought tolerance that can shed more light on GEI (Messmer *et al.*, 2009).

Cluster analysis proved to be effective in discriminating maize genotypes with different ASI and yield performance under drought (Babic *et al.*, 2012; Khodarahmpour, 2012). Besides two expected clusters of inbred lines with short ASI and high yields and the inbred lines with large ASI and low yield, two other groups of maize lines were distinguished in our study, one with intermediate ASI and low yield and the other one with large ASI and high yield. The latter consisted mostly of inbred lines developed by crossing exotic non-tropical germplasm from Argentina with germplasm from Lancaster heterotic group (G23, G56, G57, G58 and G59). These inbreds performed better in more drought-affected environments in 2012 than in less drought-affected environments in 2011. The allele *bnlg238-159* was unique for these genotypes and had positive effect on yield in 2012 only, possibly contributing to their good performance under severe drought. The other cluster interesting from the breeder point of view contained inbred lines with short ASI and high yield mostly from Lancaster, Iodent heterotic groups, mixed Lancaster-Iodent origin, Serbian local germplasm mixed with Lancaster or Iodent inbred lines (Stojaković *et al.*, 2000) and a few non-B73 inbreds from BSSS heterotic group. Majority of the inbreds had stable performances in all environments and contained the alleles identified to be favourable across the

environments. Noteworthy, marker allele *bnlg238-167* that affected yield increase and ASI reduction was unique for five inbred lines (G47, G48, G49, G74 and G95) containing a portion of germplasm from a local Serbian landrace. The PLS analysis grouped these lines with *bnlg238-167*, which could contribute to the adaptation to local conditions and drought tolerance. Besides, three maize inbred lines (G36, G39 and G42) from the same cluster were highlighted for their high yields and small ASI in all environments (Fig. S2 [suppl.]). These BSSS inbreds contained a fraction of B14 and B37 germplasm and were developed in several cycles of reselection. They were parental lines for commercial hybrids that also proved to have good performance in extensive yield trials under drought stress (Ivanović *et al.*, 2008; Mitrović *et al.*, 2011). However, testing general and special combining abilities of the evaluated inbred lines were out of the scope of this paper.

The PLS regression revealed that the precipitation in June, maximum daily temperatures and relative humidity in July and August contributed most to GEI. Interestingly, precipitations in July and August seemed not to affect the interaction much. However, low relative humidity in July and August appeared to be an important factor for contrasting inbred lines reaction to air drought in different environments, since high air temperature and low relative humidity during the critical flowering stage can disturb the pollen shed and timely exertion of receptive silks causing pollen deterioration and silk senescence further impeding fertilisa-

same marker flanked a QTL responsible for physiological traits associated with water stress tolerance at seedling stage (Liu *et al.*, 2011). Marković *et al.* (2008) identified a chromosome region involved in ASI expression close to *bnlg1525*. The marker *umc1025* was used to identify a QTL with an additive effect on grain yield explaining 3% of its total phenotypic variation in the study of Lima *et al.* (2006) and a QTL with a dominant gene action explaining 10.4% of grain yield variation as reported by Li *et al.* (2010). Liu *et al.* (2008) mapped a QTL near the marker *bnlg238* with a partial dominant effect and 10% of the explained phenotypic variation of grain yield under low nitrogen stress. The proportion of phenotypic variation of the significant marker-trait associations were quite low for grain yield ($\leq 8.1\%$) as expected for a quantitative trait, whereas R^2 ranged from 5.7% to 22.4% for ASI depending on the marker and environment. The average R^2 values of all the markers that were significantly associated with ASI, except *umc1022*, indicated the presence of major QTLs, as defined by Flint-Garcia *et al.* (2005). The presence of these major QTLs implies potential application of the markers with favourable effects on ASI for marker-assisted introgression during maize inbred lines development for improved drought tolerance.

Due to a considerable GEI, the effects of marker alleles on investigated traits were not the same across environments. Marković *et al.* (2008) observed that some of maize families had short ASI in one location, while in the other ASI was considerable longer. Similarly, the expression of the QTL mapped for grain yield, plant and ear height and number of ears per plant changed across environments, resulting the differential performance of the maize families (Lima *et al.*, 2006). Furthermore, several studies suggested that different loci and combinations of their alleles were expressed under different intensity of drought (Messmer *et al.*, 2009; 2011). Undoubtedly, the large number of QTLs that control complex traits, such as yield, under drought conditions, the high proportion of QTLs that interact with environments and their epistatic effects could make the routine use of molecular markers in identifying and developing drought tolerant genotypes a rather challenging task. The high environmental variability could be addressed by identifying sets of environments with ideally low or non-crossover GEI and QTLs that are specific for and stable in those environments (Lima *et al.*, 2006). This ideal scenario is not always achievable as the lack of repeatability of the GEI patterns over years and unpredictable crossover interaction are common in maize growing regions (Stojaković *et al.*, 2015). Nevertheless, evaluation of maize breeding material and mining exotic and locally

adapted germplasm as a source for drought tolerance, identification of marker alleles responsible for good performance of specific genotypes and their interactions in specific environments are essential for selecting inbred lines used to develop hybrids with high yielding potential, wide adaptability and stability in the light of the unpredictable nature of drought events.

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