Marker-trait association analysis for drought tolerance in smooth bromegrass

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Abstract

Background: Little information is available on the application of marker-trait association (MTA) analysis for traits related to drought tolerance in smooth bromegrass. The objectives of this study were to identify marker loci associated with important agronomic traits and drought tolerance indices as well as fining stable associations in a diverse panel of polycross derived genotypes of smooth bromegrass. Phenotypic evaluations were performed at two irrigation regimes (normal and deficit irrigation) during two years; and association analysis was done with 626 SRAP markers.

Results: The results of population structure analysis identified three main subpopulations possessing significant genetic differences. Under normal irrigation, 68 and 57 marker-trait associations were identified using general linear model (GLM) and mixed linear mode1 (MLM), respectively. While under deficit irrigation, 61 and 54 markers were associated with the genes controlling the studied traits, based on these two models, respectively. Some of the markers were associated with more than one trait. It was revealed that markers Me1/Em5-11, Me1/Em3-15, and Me5/Em4-7 were consistently linked with drought-tolerance indices.

Conclusion: Following marker validation, the MTAs reported in this panel could be useful tools to initiate marker-assisted selection (MAS) and targeted trait introgression of smooth bromegrass under normal and deficit irrigation regimes, and possibly fine mapping and cloning of the underlying genes and QTLs.

Background

Smooth bromegrass (*Bromus inermis* Leyss.), the most commonly cultivated perennial bromegrass species, is a long-lived, sod-forming and cool-season grass species [1, 2]. It is adapted to dry regions and grown mainly for hay, pasture and soil conservation [1].

Drought is the major abiotic constraint affecting plant growth and production worldwide [3, 4]. Development of suitable cultivars with more drought tolerance is crucial for enhancing sustainable production of plants and provides a strategy to combat the effects of climate change [5]. However, drought tolerance is a complex and quantitative trait, involving multiple metabolic pathways. A promising strategy to facilitate selection and breeding for drought tolerance is marker-assisted selection (MAS) to identify genetic markers linked to the traits related to drought tolerance. The basic prerequisite for MAS is the availability of markers that are tightly linked to genes or quantitative trait loci (QTLs) which can be used to select for traits that are difficult to measure or dependent on the developmental stage [6, 7].

The application of molecular markers allows locating the genes of interest in the genome, thus avoiding the time and the space needed in breeding programs [8]. Sequence-related amplified polymorphism (SRAP) is a PCR-based molecular marker technique which can be adapted for a variety of purposes in different crops, including genetic diversity analysis, map construction, gene tagging, genomic and cDNA fingerprinting, and map based cloning [9, 10]. Moreover, it is an advanced molecular marker for genetic research in grass and forage species which detects nucleotide sequence polymorphisms by using a pair

of primers of an arbitrary nucleotide sequence [11]. Analysis of SRAP data has frequently been employed for the construction of linkage maps [12, 13] and identification of QTLs [14, 15]. However, the utilization of SRAP for grass and forage researches such as association analysis in smooth bromegrass is rare.

The first step towards MAS, as an important tool for accelerating varietal improvement and rate of genetic gain, is to dissect marker-trait associations (MTAs) [16]. Association mapping and linkage analysis are two commonly used and complementary approaches to dissect complex traits [17, 18]. However, QTL mapping has a low resolution and requires a lot of time and resources [19, 20]. Genome-wide association studies (GWAS) or association mapping, also known as linkage disequilibrium (LD) mapping, have recently become a popular alternative to bi-parental QTL mapping for identifying MTAs in plant populations [21, 22]. Compared to linkage mapping in traditional bi-parental populations, GWAS overcomes several of the drawbacks of QTL mapping (classical linkage analysis). It offers higher mapping resolution, is less time consuming and requires fewer resources, and evaluates a wide range of alleles rapidly [23, 24].

In order to avoid identifying spurious associations between markers and traits in association analysis, it is necessary to evaluate the population structure [25]. In addition, utilizing a mixed-model approach involving multiple levels of relatedness simultaneously, has an important role in avoidance of both types of error (types I and II) [6, 26]. Association analysis can be performed by using general linear model (GLM) and mixed linear model (MLM). In MLM, both the kinship (relatedness) matrix (K) and population structure (Q) are incorporated, whereas in the GLM, only population structure information is used as a covariate [6].

In recent years, the application of association analysis in forage grasses is discussed in several reports. In perennial ryegrass (*Lolium perenne*) the application of association mapping for some traits such as flowering time, leaf length, carbohydrate content, submergence tolerance, salinity and drought tolerance has been evaluated [27-31]. In tall fescue (*Festuca arundinacea*), SSR loci related to agronomic traits [32] and heat-tolerance-related traits [33] have been detected. Kempf et al. (2017) applied SRAP and SSR markers in marker–trait association analysis for agronomic and compositional traits of sainfoin (*Onobrychis viciifolia*) [7]. In orchardgrass, studies have been carried out for detection of the loci related to drought tolerance, seed yield, forage yield [34], rust resistance [35], and heading date [36]. Such studies have demonstrated that GWAS is an efficient method for identifying genomic regions associated with complex quantitative traits. However, in smooth bromegrass the application of association mapping in identifying links between genes or markers with complex quantitative traits such as drought tolerance is still in its infancy. This study was conducted to: i) identify genetic loci associated with the key agronomic traits and drought tolerance, under normal and water deficit conditions using SRAP markers; and ii) discover stable marker loci linked to the agronomic and drought tolerance related traits.

Results

Phenotyping

There were significant differences between irrigation regimes for most of the measured traits. Except for flag leaf length (FLL), flag leaf width (FLW), panicle length (PL), and degree of winter growth (DWG), the magnitude of mean performance was significantly decreased for all of the evaluated traits, under water-deficit condition. Dry matter yield (DMY) was decreased by deficit irrigation 42% on average (Table 1).

The range of phenotypic coefficient of variation (PCV) was from 4.89% for plant height (PH) to 23.77% for number of stems per plant (NS) under normal irrigation and from 6.89% for days to anthesis (DA) to 27.42% for NS under deficit irrigation (Table 1). Genetic coefficient of variation (GCV) ranged from 6.08% for PH to 27.32% for NS under normal irrigation and from 7.07% for DA to 30.87% for NS under deficit irrigation. Except for crown diameter (CD), the values of genetic variation under deficit irrigation were higher than the ones for normal irrigation. Based on PCV and GCV, higher range of genetic variation was observed for PH and relatively lower range of genetic variance was detected for DA and DWG (Table 1).

The estimates of broad-sense heritability were calculated for each irrigation regime, separately and are given in Table 1. Under both irrigation regimes, moderate to high values of heritability were estimated for all of the evaluated traits. According to the results, heritability estimates ranged from 61.33 % for DWG to 94.05 % for DA under normal irrigation and from 66.01 % for DMY to 95.13 % for days to panicle emergence (DPE) under deficit irrigation regime. For all of the measured traits, estimates of heritability were higher under normal irrigation than deficit irrigation (Table 1).

Genotyping

In total, 959 bands were generated from 30 SRAP primer combinations, of which 626 bands were polymorphic (Table 2). The total number of bands scored per primer combination ranged from 13 (Me3/Em3) to 28 (Me4/Em2), with an average of 21 bands per primer combination. The percentage of polymorphic bands ranged from 55.17% (Me4/Em3) to 85.19% (Me3/Em4) with an average of 69.85%. The relative informativeness of each marker can be evaluated on the basis of its PIC value. In the present study, PIC value ranged from 0.35 (Me1/Em5) to 0.50 (Me2/Em4, Me3/Em4, and Me5/Em3), with the average of 0.45. The highest and lowest MI values were 12.60 (Me4/Em2) and 5.85 (Me3/Em3), respectively. Markers Me4/Em2 and Me3/Em1 showed the highest and lowest RP values, respectively (Table 2).

Population structure and association analysis

The optimum number of sub-populations (K) was determined using the largest value of Delta K in the STRUCTURE 2.3.4 software. The maximum likelihood and D*K* were used to calculate the number of subpopulations (*K*). The maximum value of D*K* obtained at *K*= 3, suggested that there were three subpopulations in the smooth bromegrass panel (Table 3; Figures 1 and 2). Each of these three subpopulations had its own characteristics. Subpopulation 1 contained seven genotypes of G30, G31,

G32, G33, G34, G35, and G36 (Figure 2). All of these genotypes were belonged to Hungary, and had higher persistence than other genotypes. Subpopulation 2 included genotypes G11, G15, G21, G26, and G28, which all of them were belonged to Hungary except for G15 (Iranian genotype). Genotypes of this subpopulation were early flowering and had lower productivity than other genotypes. The remaining genotypes were located in the third subpopulation (Figure 2). This subpopulation showed late flowering and had higher productivity than other ones. All of the genotypes of this subpopulation were Iranian except for G12, G13, and G25 (Hungarian genotypes). As observed, structure analysis was able to separate genotypes based on geographical or ecological data.

Association analysis between SRAP markers and the phenotypic mean of traits (over years for normal and water-deficit irrigations) was separately conducted based on both GLM and MLM models. Under normal irrigation, based on the GLM model (*P* values <0.01 and a cut-off value of 0.05 for the FDR) 68 SRAP markers showed significant associations with means of the studied traits, at 0.01 probability level (Table S1). The percentage of phenotypic variation (coefficient of determination, R^2) of an individual trait explained ranged from 11.75% to 31.32% (Table S1). Under deficit irrigation, 61 markers had significant associations with the studied traits, at 0.01 probability level (Table S1). The percentage of phenotypic variation (R^2) of a trait explained varied from 9.89% to 26.28% (Table S1). However, in the MLM model, kinship or relatedness matrix was considered as a factor, and the number of significant markers decreased as compared to GLM model. So that, under normal irrigation 57 markers and under deficit irrigation 54 markers indicated significant associations at 0.01 probability level (Table 4). In this model, the percentage of phenotypic variation, under normal irrigation ranged from 7.71% to 20.89% and under deficit irrigation varied from 6.76% to 17.61% (Table 4). Moreover, association analysis was also done for drought tolerance and susceptibility indices. Results revealed that 19 and 20 markers showed significant associations with calculated indices based on GLM and MLM model, respectively (Tables 5 and S2).

Analysis based on both GLM and MLM models showed markers which were associated with more than one trait at the same time. For example, under both normal and deficit irrigation marker Me1/Em6-7 showed simultaneously significant associations with DA, PH, FLL, and FLW based on both GLM and MLM models. Marker Me2/Em5-21 had concurrently significant associations with DA, PH, and FLL, under both irrigation regimes and in both GLM and MLM models (Tables 4 and S1). In addition, under normal irrigation, marker Me2/Em1-14 showed significant associations with DPE, DA, PH, FLL, and FLW, based on GLM model; and showed significant associations with the traits of DPE, DA, PH, and FLL based on MLM model. However, under deficit irrigation this marker had significant associations with DA and PH based on both models (Tables 4 and S1). Based on MLM model, marker Me1/Em5-11 showed associations with DMY and DWG under both normal and deficit irrigation regimes (Table 4). Moreover, markers Me1/Em5-11 and Me1/Em3-15 showed significant associations with MP, GMP, and STI, based on both GLM and MLM models (Tables 5 and S2).

Association analysis was conducted in each irrigation regime separately to assess stable associations. In total, 30 and 21 trait associated markers showed sufficiently stable expression across irrigation regimes, based on GLM and MLM models, respectively. For instance, in GLM model, markers Me1/Em6-7,

Me2/Em1-14, Me2/Em2-13, Me4/Em6-22, Me2/Em5-21, Me2/Em3-19, Me2/Em1-12, and Me1/Em6-16 showed significant and stable associations in both irrigation regimes with DA. Similarly, markers Me1/Em5-11, Me1/Em5-24, Me2/Em6-16, Me5/Em2-20, and Me1/Em2-19 displayed significant and constant associations in both irrigation regimes with DWG (Table S1). In MLM model, markers Me1/Em6-7, Me2/Em1-14, Me2/Em2-13, Me4/Em6-22, and Me2/Em5-21 had significant and stable associations in both irrigation regimes with DA. Also, markers Me4/Em4-14, Me4/Em6-2, Me5/Em2-1, Me1/Em2-21, and Me1/Em1-13 were constantly associated with FLW in both moisture conditions. In the same way, marker Me1/Em5-11 was associated with DMY (Table 4).

Discussion

Significant genetic variations among genotypes in terms of all of the evaluated traits demonstrate the difference in genes controlling yield, its components, and drought-tolerance. Moreover, the non-static performance of genotypes in two irrigation regimes emphasizes the importance of marker-trait association analysis in the two moisture environments, separately.

Most of the evaluated traits were significantly affected by water deficit due to decreased water potential of the soil and decline in net assimilation and photosynthesis of leaves [37, 38]. Similar results were reported in other researches [39, 40]. Wide genetic variation observed for all of the evaluated traits is promising genetic progress for these genotypes. Moreover, higher estimates for PCV and GCV under the deficit irrigation regime compared with normal irrigation indicate that water deficit have increased genetic variation for most of the studied traits and therefore, selection under deficit irrigation would be more effective. The findings in this case are contraditionary. For example, some researchers reported that genetic gain through selection is usually higher under normal irrigation than deficit irrigation [39, 41]. However, other researchers have also reported that the genetic advance through selection was higher under deficit irrigation [42, 43]. Moderate to high heritability estimates observed for all of the evaluated traits indicates that the improvement of these traits would be possible through selection and also emphasizes that detecting of marker–trait associations is possible for these traits [44].

The high percentage of polymorphism indicated that SRAP marker combinations used in the present study could be used as powerful tools for discriminating of smooth bromegrass genotypes. Results of this study also revealed that the primer combination Me4/Em2 with the highest polymorphism percentage and high values of PIC, MI, and RP indices is informative and powerful enough for identification and discrimination of smooth bromegrass genotypes.

Based on the population structure analysis, genotypes of smooth bromegrass were separated into three groups with different genetic structures. As expected, structure analysis was able to separate genotypes based on their origin. Furthermore, association analysis of important agronomic traits under normal and water deficit conditions showed that the number of significant marker-trait associations (MTAs) were lower in the MLM than GLM model. SRAP markers identified based on the MLM model can be considered as the most interesting candidates for future studies using MAS. Achleitner et al. [45] stated that the

combination of Q and K matrices provides the highest reduction in the coefficient of determination and presumably is the best correction for population structure.

Marker-trait associations were mostly different in normal and deficit irrigation regimes. The results showed that a greater number of genes were probably involved in controlling traits at deficit irrigation regime than normal one. The percentage of variation which is explained by identified associations was low (7.71–20.89% under normal irrigation and 6.76–17.61% under water-deficit irrigation). This low R^2 value for each trait may be attributed to the role of many minor genes controlling the trait, outcrossing nature of smooth bromegrass, markers exhibiting minor quantitative effect, rare alleles, and complex allelic interactions [46, 47]. These results are in agreement with the findings of Lou et al. [32] and Sun et al. [33] in tall fescue.

Based on the results of GLM and MLM models, some markers had simultaneously significant associations with more than one trait. These markers may be effectively used for the improvement of several traits, concurrently [33, 34]. Multi-association among different traits could be attributed to the co-expression mediated by expression of quantitative trait loci or e-QTLs [48]. For instance, marker Me1/Em6-7 showed simultaneously significant associations with DA, PH, FLL, and FLW under both irrigation regimes and based on both GLM and MLM models. Similarly, marker Me2/Em5-21 concurrently showed significant associations with DA, PH, and FLL. In addition, under normal irrigation, marker Me2/Em1-14 showed significant associations with DPE, DA, PH, FLL, and FLW, based on GLM model and also showed significant associations with DPE, DA, PH, and FLL based on MLM model. At deficit irrigation this marker had significant associations with DA and PH based on both models. These concurrent associations of markers with several traits may be attributed to pleiotropic effects or to several tightly linked genes affecting several traits [33, 49].

Determination of the genetic basis of drought tolerance involves correlating the occurrence of molecular markers with phenotypic scores for prediction of DNA genomic regions involving factors influencing the plant response [50]. Marker–trait association analysis identified 19 and 20 loci related with drought tolerance and susceptibility indices based on GLM and MLM models, respectively. Among these SRAP markers Me1/Em5-11 and Me1/Em3-15 showed significant associations with MP, GMP, and STI, based on both GLM and MLM models. Moreover, in both models, markers Me5/Em5-9 and Me5/Em3-10 showed a significant association with DSI. If the effectiveness of these regions in the genetic control of drought tolerance is confirmed, these markers could be potentially used for the improvement of drought tolerance in smooth bromegrass.

Most of the MTAs were different under the two irrigation regimes, indicating the environmental effects in these associations [51]. These results showed that different genes may contribute to the same trait in different environments [52] or there might be a change in the expression level of the same gene between the two environments [48]. In the present study, 21 markers showed a stable association with different traits under both irrigation regimes. Diapari et al. [53] stated that associated markers which were detected in two or more different environments are more reliable than those present in only one environment.

In conclusion, the utility of association analysis approach as a powerful tool for identifying and detecting genes and markers linked to complex traits of agricultural and economic importance was demonstrated. Satisfactory levels of polymorphism and genetic diversity were observed for the studied traits in the polycrossed population. Three subpopulations were identified in smooth bromegrass genotypes and 90 significant MTAs using GLM and MLM models were detected under contrasting water conditions. Among these, three MTAs were identified for drought tolerance. Moreover, it was demonstrated that SRAP markers can be used in the future breeding programs to enhance drought tolerance of smooth bromegrass. Some SRAP markers were associated with the key agronomic traits of this species. Environmental specificity of MTAs demonstrates that genotype × environment interactions affect association analysis; nevertheless, 30 and 21 MTAs showed significantly stable expression across normal and deficit irrigations based on GLM and MLM models, respectively. The markers identified in the present study are useful genomic resources for MAS in the future breeding programs of smooth bromegrass.

Methods

Plant materials and field experiment

Genotype panel used for the present study consisted of 216 clones randomly selected from a large nursery comprised of 1800 single spaced-plant polycrossed progenies resulting from 36 parental ecotypes of smooth bromegrass (*Bromus inermis*). These 36 genotypes were randomly sampled from polycross progenies of a set of 25 parental genotypes (Table 6). These 25 parental plants were randomly taken from a diverse and large nursery including natural ecotypes collected from wide geographical areas of Iran. Foreign ecotypes were provided by the Hungarian Institute for Agrobotany (HIFA), Tapioszele, Hungary. Parental genotypes were polycrossed based on a half-sib mating design with eight replications in 2009, and bulked seeds from all replications were harvested separately and were grown in a greenhouse during the winter of 2009. Established seedlings were space-planted in the field on March 1st 2010 and grown during 2011 and 2012. For the present study, 216 clones were propagated in a greenhouse during the winter of 2012 and then were space-planted (50-cm grid) in the field according to a randomized complete block design with six replications. Genotypes were evaluated under two levels of irrigation including a normal and a water deficit condition for 2014-2015.

Under normal and water deficit conditions, plants were irrigated when 50 and 90% of the total available soil water was exhausted from the root zone, respectively; following accepted methods of determination of evapotranspiration [54]. In each year of the experiment, water stress was differentially imposed during the growing season from the first of May to the first of October. The irrigation intervals during the growing season and between the two irrigation treatments were variable depending on the weather conditions. Soil samples were taken daily prior to each irrigation at three depths, namely 0–20, 20–40 and 40–60 cm, using a hand auger to determine the gravimetric soil water content and detect the irrigation times [55]. A basin system was used for irrigation. In this system, water was delivered to the field via a pump

station and polyethylene pipes. The applied water volume for each irrigation treatment was measured using a volumetric counter.

This experiment was carried out in the field at Research Farm of Isfahan University of Technology, situated in Lavark, Najaf-Abad, Isfahan, Iran (32° 30’ N, 51° 20’ E; 1630 m amsl) during two years. The soil was calcareous, non-saline and non-sodic. The region has a mean annual temperature of 14.5 ˚C and mean annual precipitation of 140 mm, generally without rain during the summer (from late May to mid-October), making supplemental irrigation necessary for growing crops during this period.

Phenotyping

During the year that plants were established (2013) no data was recorded. The number of days from March 1st until appearance of three panicles and onset of pollen shedding in each plant were documented as days to panicle emergence (DPE) and days to anthesis (DA), respectively. The distance from the plant base to the top of the three highest panicles at full anthesis was considered as the plant height (PH). Flag leaf length (FLL), flag leaf width (FLW), panicle length (PL), and number of stems per plant (NS) were recorded at the pollination stage. In each year of the experiment, three harvests of aboveground biomass were undertaken. The first harvest was in late spring after pollination, the second in late summer, and the third in late autumn. At each harvest, the aboveground biomass of each plant was cut manually at 5 cm above the ground, dried at 75˚C for 48 h and then weighed to obtain dry matter yield (DMY). The average forage weight (g per plant) from the three cuts was used for analysis. Crown diameter (CD) was recorded as the plant basal cover width remaining after the first harvest. Degree of winter growth (DWG) was visually scored from one (weakest) to nine (most robust) according to the plant viability, vitality, canopy size, and appearance at the end of the cool season.

Six selection indices including tolerance index (TOL) [56], mean productivity (MP) [56], geometric mean productivity (GMP) [57], drought susceptibility index (DSI) [58], and stress tolerance index (STI) [57] were calculated based on the dry matter yield under normal and water deficit irrigations, according to the following formulations:

$TOL = Y_{pi}-Y_{si}$

$MP = (Y_{pi}+Y_{si})/2$

$GMP = (Y_{pi} \times Y_{si})^{0.5}$

$DSI= [1- (Y_{si} / Y_{pi})] / [1- (Y_{ms} / Y_{mp})]$

$STI = [(Y_{pi} \times Y_{si}) / (Y_{mp})^{2}]$

where Y_{si} designates the yield of the ith genotype grown under deficit irrigation, Y_{pi} designates that of the ith genotype grown under normal irrigation, Y_{ms} is the yield mean over all genotypes grown under deficit irrigation, and Y_{mp} is the yield mean over all genotypes grown under normal irrigation.

Genotyping

Genomic DNA was extracted from young leaf tissues using the modified method described by Murray and Thompson [59]. DNA quality and concentration were determined by electrophoresis in 1% agarose gel. Genotyping using SRAP markers was performed following the method of Li and Quiros [9]. Among the SRAP markers available, 30 primer combinations were screened by polymerase chain reaction (PCR). PCR reactions were conducted in volumes of 10 μL, using a BIO-RAD thermocycler. Each PCR reaction was consisted of 1.5 μL of DNA, 1 μL of forward primer, 1 μL of reverse primer, 5 μL of master mix (Amplicon), and 1.5 μL of distilled water. For SRAP analysis, samples were subjected to the following thermal profile: the first five cycles were run at 94˚C for 1 min, 35˚C for 1 min, and 72˚C for 1 min, for denaturing, annealing, and extension, respectively. Then the annealing temperature was raised to 50˚C for another 35 cycles, followed by another extension step of 10 min at 72˚C. Amplification products were separated by electrophoresis on 12% non-denatured polyacrylamide gels and stained by AgNO3 solution [60].

Statistical analyses

Phenotypic data analysis

Data were tested for normality by Kolmogorov– Smirnov test and homogeneity of variance was tested by Bartlett test. Analysis of variance was performed for the normal and water-deficit irrigations separately based on the split-plot in time (year) model using Proc MIXED of SAS release 9.4 [61]. Components of variance were estimated for individual irrigation regimes according to the method of Steel and Torrie [62]. Broad-sense heritability (h^2_b) for normal and deficit irrigation regimes was estimated on a phenotypic mean basis averaged over replications and years as described by Nguyen and Sleper [63]:

$$h^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_{gy}/y + \sigma^2_{gr}/r + \sigma^2_e /ry)$$

In which σ^2_g is the genotype, σ^2_{gy} is the genotype × year, σ^2_{rg} is the genotype × rep, and σ^2_e is the residual variance, y is the number of years, and r is the number of replicates. To estimate the level of genetic variation, the phenotypic coefficient of variation (PCV) and genetic coefficient of variation (GCV) were calculated as:

$$PCV = (\sigma_p / \mu)\ 100$$

$GCV = (\sigma_g / \mu)\ 100$

where σ_p is the standard deviation of the phenotypic variance, σ_g is the standard deviation of the genotypic variance, and μ is the phenotypic mean [64].

Molecular data analysis

Polymorphic SRAP markers were scored as binary data with presence (1) or absence (0). For each of the SRAP markers, the following indices were computed.

Polymorphism information content (PIC) was calculated according to the formula of Roldán-Ruiz et al. [65]:

$PIC_i = (2f_i \times [1\text{-}f\ ]$

where i is the ith primer, f_i is the frequency of the amplified allele, and $(1\text{-}f_i)$ is the frequency of the null allele. Resolving power (RP) was estimated as:

$RP = \sum 1\text{-}\ [2\times\ (0.5 - f_i)]$

Marker index (MI) was determined according to Powell et al. [66]:

$MI_i = PIC_i \times N_i \times b_i$

where N_i is the total bands for the ith primer, and b_i is the percentage polymorphic bands of the ith primer.

Population structure and association analysis

Structure analysis and stratification of the studied population into subpopulations with different genetic structures was done based on SRAP marker data in STRUCTURE software version 2.3.4 [25]. This analysis was performed applying an admixture model, a burn-in of 10,000 iterations followed by 100,000 Monte Carlo Markov Chain (MCMC) replicates. The membership of each genotype was run for the range of genetic clusters (K) from K= 2 to K= 10 with five repetitions for each K. Delta k approach by Evanno et al. [67] was used to determine the optimum number of sub-populations, using STRUCTURE HARVESTER [68].

Association analysis was run by both GLM and MLM [26] to calculate *P*-values for marker–trait associations, using TASSEL version 4.2.1 [69]. The phenotypic mean of traits (P-matrix) over two years was applied to identify significant associations under normal and water deficit irrigations, separately. To correct for population structure in GLM and MLM models, a Q-matrix that was derived from structure

analysis (at maximum DK), was used as a covariate. Moreover, a kinship matrix (K-matrix) was calculated based on the results of marker genotype data using TASSEL version 4.2.1 [69] and was used in MLM. A correction for multiple testing was performed with the FDR (false discovery rate) method, using the QVALUE R package [70].

Abbreviations

CD: Crown diameter; DA: Days to anthesis; DMY: Dry matter yield; DPE: Days to panicle emergence; DSI: Drought susceptibility index; DWG: Degree of winter growth; FLL: Flag leaf length; FLW: Flag leaf width; GCV: Genetic coefficient of variation; GLM: General linear model; GMP: Geometric mean productivity; GWAS: Genome wide association study; MAS: Marker assisted selection; MLM: Mixed linear model; MP: Mean productivity; MTA: Marker-trait association; NS: Number of stems per plant; PCV: Phenotypic coefficient of variation; PH: Plant height; PL: Panicle length; QTL: Quantitative trait loci; SRAP: Sequence related amplified polymorphism; STI: Stress tolerance index; TOL: Tolerance index.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

FS, MMM and AM conceived and designed the experiments; FS performed the experiments, analyzed the data and wrote the manuscript with the supervision of MMM and AM; all authors discussed the results

and reviewed the manuscript.

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Tables

Table 1 Mean performance, phenotypic coefficient of variation (PCV), genotypic coefficient of variation (GCV), and broad- sense heritability (h^2_b) of traits recorded under normal and water deficit conditions in smooth bromegrass genotypes

Traits	Mean ± SD		Change (%)	PCV (%)		GCV (%)		h^2_b (%)		
	Normal	Stress		Normal	Stress	Normal	Stress	Combined	Normal	Stress
DPE (day)	58.89 ± 7.62	56.61 ± 6.84	3.87*	9.37	10.49	8.95	10.24	95.32	91.27	95.13
DA (day)	80.36 ± 6.83	77.83 ± 6.17	3.15*	6.55	7.07	6.35	6.89	95.60	94.05	95.06
PH (cm)	96.57 ± 16.22	75.55 ± 13.69	21.77**	6.08	12.41	4.89	11.68	73.05	64.72	88.59
FLL (mm)	141.62 ± 28.40	152.54 ± 29.96	-7.71*	10.36	11.50	8.88	10.26	84.73	73.46	79.62
FLW (mm)	6.99 ± 1.33	7.18 ± 1.73	-2.72n.s	12.25	14.39	11.76	13.46	92.73	92.17	87.51
PL (cm)	16.03 ± 2.13	16.00 ± 2.34	0.19n.s	9.14	10.21	8.42	9.42	89.08	84.92	85.17
NS (No. plant^{-1})	187.58 ± 78.75	149.57 ± 68.14	20.26**	27.32	30.87	23.77	27.42	86.40	75.75	78.89
DMY (g/plant)	99.25 ± 46.61	57.12 ± 28.75	42.45**	23.13	27.45	19.09	22.30	77.99	68.13	66.01
CD (cm)	25.76 ± 5.25	23.28 ± 4.19	9.63**	14.54	12.97	13.74	12.02	89.66	89.34	85.89
DWG	2.47 ± 0.97	3.58 ± 1.35	-44.94**	20.66	19.88	16.18	16.84	75.19	61.33	71.80

*, **, and *** significant at the 0.05, 0.01, and 0.001 probability level respectively; ns: not significant

GCV, Genotypic coefficient of variation; PCV, Phenotypic coefficient of variation; SD, Standard deviation

CD, Crown diameter; DA, Days to anthesis; DMY, Dry matter yield; DPE, Days to panicle emergence; DWG, Degree of winter growth; FLL, Flag leaf length; FLW, Flag leaf width; NS, Number of stems per plant; PH, Plant height; PL, Panicle length

Table 2 Information and diversity statistics for sequence related amplified polymorphism (SRAP) markers used for association analysis in smooth bromegrass

No.	Oligo name	Oligo sequence 5′ → 3′	NPB/NB	PPB	PIC	MI	RP
1	Me1/Em1	TGAGTCCAAACCGGTTG GACTGCGTACGAATTTGC	24/33	72.73	0.47	11.28	23.94
2	Me1/Em2	TGAGTCCAAACCGGTTG GACTGCGTACGAATTACG	26/36	72.22	0.43	11.18	24.61
3	Me1/Em3	TGAGTCCAAACCGGTTG GACTGCGTACGAATTTAG	23/32	71.88	0.49	11.27	20.06
4	Me1/Em4	TGAGTCCAAACCGGTTG GACTGCGTACGAATTCAG	19/27	70.37	0.44	8.36	19.78
5	Me1/Em5	TGAGTCCAAACCGGTTG GACTGCGTACGAATTCGA	24/37	64.86	0.35	8.40	25.83
6	Me1/Em6	TGAGTCCAAACCGGTTG GACTGCGTACGAATTTGA	25/37	67.57	0.37	9.25	26.72
7	Me2/Em1	TGAGTCCAAACCGGTGT GACTGCGTACGAATTTGC	20/31	64.52	0.49	9.80	23.00
8	Me2/Em2	TGAGTCCAAACCGGTGT GACTGCGTACGAATTACG	19/27	70.37	0.49	9.31	22.22
9	Me2/Em3	TGAGTCCAAACCGGTGT GACTGCGTACGAATTTAG	22/31	70.97	0.38	8.36	21.50
10	Me2/Em4	TGAGTCCAAACCGGTGT GACTGCGTACGAATTCAG	26/34	76.47	0.46	11.96	27.50
11	Me2/Em5	TGAGTCCAAACCGGTGT GACTGCGTACGAATTCGA	24/37	64.86	0.48	11.52	29.17
12	Me2/Em6	TGAGTCCAAACCGGTGT GACTGCGTACGAATTTGA	18/25	72.00	0.50	9.00	17.00
13	Me3/Em1	TGAGTCCAAACCGGATA GACTGCGTACGAATTTGC	14/19	73.68	0.46	6.44	12.50
14	Me3/Em2	TGAGTCCAAACCGGATA GACTGCGTACGAATTACG	18/22	81.82	0.49	8.82	22.80
15	Me3/Em3	TGAGTCCAAACCGGATA GACTGCGTACGAATTTAG	13/16	81.25	0.45	5.85	16.70
16	Me3/Em4	TGAGTCCAAACCGGATA GACTGCGTACGAATTCAG	23/27	85.19	0.50	11.50	26.90
17	Me3/Em5	TGAGTCCAAACCGGATA GACTGCGTACGAATTCGA	16/21	76.19	0.46	7.36	19.70
18	Me3/Em6	TGAGTCCAAACCGGATA GACTGCGTACGAATTTGA	15/19	78.95	0.41	6.15	18.60
19	Me4/Em1	TGAGTCCAAACCGGAGC GACTGCGTACGAATTTGC	19/30	63.33	0.44	8.36	20.00
20	Me4/Em2	TGAGTCCAAACCGGAGC GACTGCGTACGAATTACG	28/45	62.22	0.45	12.60	31.61
21	Me4/Em3	TGAGTCCAAACCGGAGC GACTGCGTACGAATTTAG	16/29	55.17	0.45	7.20	18.17
22	Me4/Em4	TGAGTCCAAACCGGAGC GACTGCGTACGAATTCAG	23/34	67.65	0.43	9.89	26.00
23	Me4/Em5	TGAGTCCAAACCGGAGC GACTGCGTACGAATTCGA	20/29	68.97	0.39	7.80	20.94
24	Me4/Em6	TGAGTCCAAACCGGAGC GACTGCGTACGAATTTGA	23/39	58.97	0.47	10.81	28.22
25	Me5/Em1	TGAGTCCAAACCGGTGC	25/37	67.57	0.46	11.50	26.44

		GACTGCGTACGAATTTGC					
26	Me5/Em2	TGAGTCCAAACCGGTGC GACTGCGTACGAATTACG	21/30	70.00	0.47	9.87	15.78
27	Me5/Em3	TGAGTCCAAACCGGTGC GACTGCGTACGAATTTAG	23/33	69.70	0.50	11.50	22.33
28	Me5/Em4	TGAGTCCAAACCGGTGC GACTGCGTACGAATTCAG	24/33	72.73	0.40	9.60	23.50
29	Me5/Em5	TGAGTCCAAACCGGTGC GACTGCGTACGAATTCGA	19/32	59.38	0.49	9.31	21.83
30	Me5/Em6	TGAGTCCAAACCGGTGC GACTGCGTACGAATTTGA	16/25	64.00	0.48	7.68	13.06

MI, Marker index; NB, No. of bands; NPB, No. of polymorphic bands; PIC, Polymorphic information content; PPB, Percentage of polymorphic bands; RP, Resolving power

Table 3 Calculated statistics to detect optimum number of subpopulations (*K*) in structure analysis of smooth bromegrass genotypes (D*K* method; Evanno et al. 2005), using the STRUCTURE program

K	Reps	Mean LnP (K)	Stdev LnP (K)	Ln′ (K)	Ln′′ (K)	ΔK
2	5	-15785.10	8.81	-	-	-
3	5	-15648.70	18.45	136.40	4616.68	250.19*
4	5	-20128.98	5968.36	-4480.28	5961.88	1.00
5	5	-18647.38	4062.75	1481.60	1102.94	0.27
6	5	-18268.72	2143.97	378.66	5471.46	2.55
7	5	-23361.52	5990.88	-5092.80	3066.18	0.51
8	5	-25388.14	5288.39	-2026.62	8905.46	1.68
9	5	-36320.22	8671.94	-10932.08	15386.38	1.77
10	5	-31865.92	4503.99	4454.30	-	-

Mean LnP (*K*), mean of LnP(D) of repetitions for each *K*; Stdev LnP (*K*), standard deviation of repetitions; Ln′ (*K*), Ln (*K*)*n* – Ln (*K*)*n* – 1; Ln′′ (*K*), Ln′ (*K*)*n* – Ln′ (*K*)*n* – 1; D*K*, |Ln′′ (*K*)|/stdev LnP (*K*). *, *K*-value with largest D*K*

Table 4 Association of SRAP markers with phenological, morphological, and agronomic traits of smooth bromegrass genotypes under normal and water deficit conditions based on mixed linear model (MLM)

Traits	Normal irrigation			Deficit irrigation		
	Marker	P value	R^2 (%)	Marker	P value	R^2 (%)
DPE	Me1/Em4-13*	0.0054	10.08	Me4/Em1-5*	0.0047	9.52
	Me3/Em1-13	0.0058	9.94	Me4/Em2-11	0.0049	9.46
	Me2/Em1-14	0.0059	9.88	Me2/Em4-7	0.0062	9.01
	Me2/Em2-13	0.0067	9.61	Me1/Em4-13*	0.0080	8.52
	Me4/Em1-5*	0.0070	9.54			
	Me1/Em3-10	0.0098	8.82			
DA	Me1/Em6-7*	0.0012	11.20	Me1/Em6-7*	0.0001	12.79
	Me2/Em1-14*	0.0013	10.99	Me2/Em1-14*	0.0004	11.33
	Me1/Em4-13	0.0017	10.56	Me2/Em1-12	0.0011	9.87
	Me2/Em2-13*	0.0032	9.53	Me4/Em6-22*	0.0017	9.23
	Me4/Em6-22*	0.0051	8.70	Me2/Em5-21*	0.0043	7.82
	Me3/Em1-13	0.0069	8.15	Me5/Em3-3	0.0085	6.78
	Me2/Em5-21*	0.0088	7.71	Me2/Em2-13*	0.0086	6.76
PH	Me1/Em6-7*	0.0008	20.89	Me1/Em6-7*	0.0016	18.83
	Me2/Em1-14	0.0013	19.40	Me2/Em5-21*	0.0024	17.61
	Me2/Em5-21*	0.0014	19.20	Me5/Em5-15	0.0046	15.68
	Me5/Em3-10	0.0046	15.59	Me1/Em2-2	0.0067	14.51
	Me5/Em6-1	0.0060	14.79	Me4/Em2-11	0.0070	14.35
	Me4/Em3-14	0.0062	14.67			
	Me2/Em4-2	0.0072	14.21			
	Me2/Em5-22	0.0084	13.75			
FLL	Me2/Em5-21	0.0011	16.46	Me1/Em6-7*	0.0027	14.97
	Me1/Em6-7*	0.0015	15.62	Me2/Em5-8	0.0029	14.86
	Me5/Em6-11	0.0025	14.46	Me3/Em1-6	0.0047	13.53
	Me5/Em3-3	0.0045	12.96	Me2/Em1-20	0.0061	12.83
	Me2/Em1-14	0.0054	12.47	Me2/Em2-4	0.0063	12.74
	Me1/Em6-16	0.0075	11.62	Me2/Em4-23	0.0077	12.19
				Me5/Em1-21	0.0078	12.17
				Me4/Em3-13	0.0087	11.85
FLW	Me4/Em4-14*	0.0011	14.76	Me1/Em2-21*	0.0011	14.70
	Me4/Em6-2*	0.0012	14.51	Me4/Em6-2*	0.0023	13.12
	Me5/Em2-1*	0.0014	14.09	Me4/Em4-14*	0.0029	12.59
	Me1/Em2-21*	0.0042	11.73	Me5/Em2-1*	0.0047	11.52
	Me1/Em6-7	0.0042	11.71	Me4/Em1-14	0.0068	10.65
	Me5/Em5-7	0.0072	10.48	Me1/Em1-13*	0.0093	9.93
	Me1/Em1-13*	0.0079	10.27			

Table 4 (continued)

Traits	Normal irrigation			Deficit irrigation		
	Marker	P value	R^2 (%)	Marker	P value	R^2 (%)
PL	Me2/Em3-5	0.0049	13.64	Me5/Em2-13	0.0015	15.49
	Me4/Em1-9	0.0084	12.13	Me2/Em2-18	0.0043	12.93
				Me2/Em2-12	0.0075	11.53
				Me1/Em1-6	0.0088	11.12
NS	Me2/Em2-16	0.0033	16.43	Me5/Em4-10	0.0027	17.56
	Me2/Em6-18	0.0039	15.98	Me4/Em2-24	0.0030	17.26
	Me5/Em4-7*	0.0041	15.83	Me5/Em4-7*	0.0034	16.92
	Me5/Em4-11	0.0049	15.23	Me5/Em2-20	0.0048	15.79
	Me2/Em6-10	0.0084	13.57	Me4/Em6-20	0.0096	13.59
	Me4/Em3-11	0.0094	13.25			
DMY	Me1/Em3-15	0.0030	14.42	Me1/Em5-11*	0.0028	14.14
	Me4/Em2-12	0.0061	12.61	Me1/Em5-23	0.0033	13.75
	Me4/Em1-17	0.0081	11.84	Me2/Em4-16	0.0098	10.93
	Me1/Em5-11*	0.0094	11.43			
CD	Me4/Em2-12	0.0004	20.20	Me5/Em4-22*	0.0040	13.52
	Me5/Em4-11	0.0049	13.87	Me1/Em5-11	0.0050	12.95
	Me5/Em4-22*	0.0095	12.00	Me2/Em4-16	0.0095	11.24
DWG	Me1/Em5-11*	0.0011	19.30	Me2/Em4-7	0.0024	17.21
	Me5/Em4-21	0.0015	18.42	Me1/Em5-11*	0.0032	16.35
	Me5/Em3-5	0.0028	16.69	Me5/Em5-18	0.0040	15.70
	Me5/Em2-20*	0.0036	15.98	Me2/Em6-16*	0.0046	15.32
	Me1/Em5-24	0.0045	15.31	Me5/Em2-20*	0.0046	15.30
	Me5/Em1-4	0.0047	15.14	Me1/Em5-3	0.0048	15.16
	Me2/Em6-16*	0.0094	13.04	Me1/Em2-19	0.0058	14.59
	Me2/Em2-1	0.0099	12.86	Me2/Em5-5	0.0074	13.84
				Me1/Em3-15	0.0089	13.27

* Stable markers under normal and water deficit conditions

CD, Crown diameter; DA, Days to anthesis; DMY, Dry matter yield; DPE, Days to panicle emergence; DWG, Degree of winter growth; FLL, Flag leaf length; FLW, Flag leaf width; NS, Number of stems per plant; PH, Plant height; PL, Panicle length

Table 5 Association of SRAP markers with drought tolerance and susceptibility indices of smooth bromegrass genotypes based on mixed linear model (MLM)

Indices	Marker	P value	R^2 (%)
TOL	Me5/Em5-16	0.0009	19.96
	Me1/Em2-1	0.0013	19.00
	Me2/Em6-8	0.0013	18.94
	Me2/Em4-16	0.0032	16.37
	Me4/Em2-12	0.0049	15.10
	Me1/Em2-6	0.0062	14.38
	Me5/Em6-3	0.0064	14.27
	Me2/Em6-9	0.0098	12.98
MP	Me1/Em5-11	0.0024	14.67
	Me1/Em3-15	0.0044	13.17
GMP	Me1/Em5-11	0.0022	15.04
	Me1/Em3-15	0.0063	12.28
	Me5/Em4-7	0.0088	11.41
DSI	Me5/Em5-16	0.0016	18.11
	Me5/Em5-9	0.0062	14.16
	Me1/Em2-6	0.0064	14.05
	Me5/Em3-10	0.0064	14.03
STI	Me1/Em5-11	0.0015	17.10
	Me5/Em4-7	0.0055	13.61
	Me1/Em3-15	0.0097	11.98

DSI, Drought susceptibility index; GMP, Geometric mean productivity; MP, Mean productivity; STI, Stress tolerance index; TOL, Tolerance index

Table 6 Information on the parental plants of genetic materials used in this study

Parental plant	Population code	Origin
1	2000/25	Iran, Hamedan
2	2000/18	Iran, Isfahan-Fozve
3	2000/50	Iran, Isfahan-Fozve
4	2000/40	Iran, Isfahan-Semirom
5	2000/43	Iran, Isfahan-Fozve
6	2000/4	Iran, Isfahan-Fozve
7	2000/18-2	Iran, Isfahan-Fozve
8	2000/T-9	Iran, Hamedan
9	2000/60	Iran, Semnan
10	2000/10	Iran, Kordestan
11	2000/24	Iran, Isfahan-Fozve
12	RCAT040601	Hungary
13	RCAT041016	Hungary
14	RCAT041861	Hungary
15	RCAT042133	Hungary
16	RCAT042134	Hungary
17	RCAT064831	Hungary
18	RCAT064835	Hungary
19	RCAT064837	Hungary
20	RCAT064839	Hungary
21	2000/36	Iran, Isfahan-Fozve
22	2000/14	Iran, Isfahan-Fozve
23	2000/48	Iran, Isfahan-Fozve
24	2000/20	Iran, Isfahan-Fozve
25	2000/30	Iran, Isfahan-Fozve

Figures

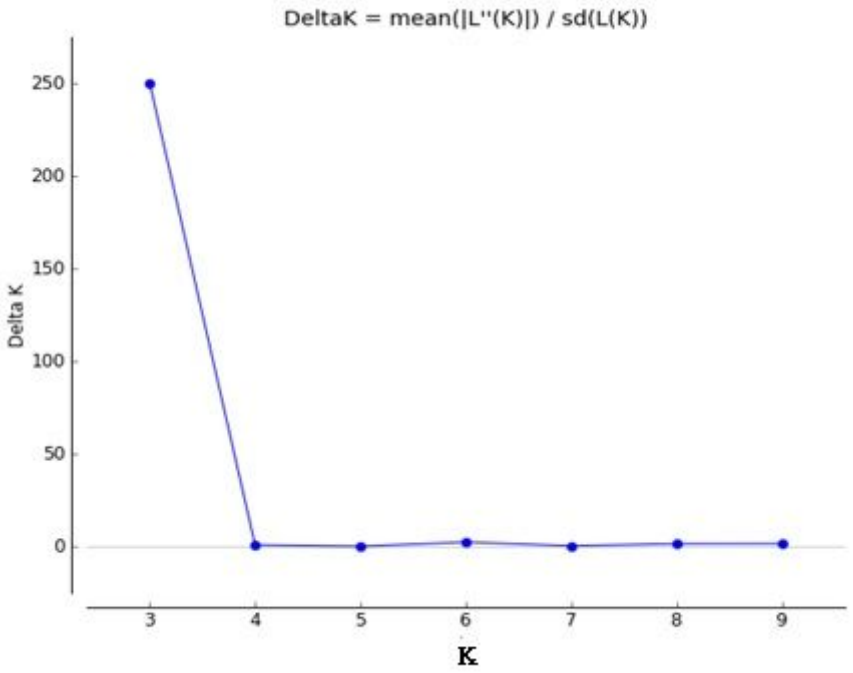

Figure 1

Population structure analysis in a diversity panel of smooth bromegrass genotypes (Δk was used to determine the optimum k value for population structure using the Bayesian clustering method)

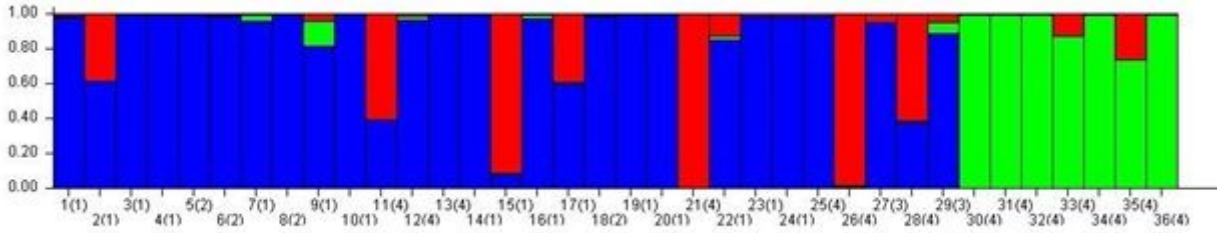

Figure 2

Genetic relatedness of smooth bromegrass genotypes analyzed by STRUCTURE program. Numbers on the y-axis indicate the membership coefficient. The color of the bar indicates the three subpopulations identified through the STRUCTURE program. Genotypes with the same color belong to the same subpopulation

Supplementary Files

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- Supplementary.doc