Association study for drought tolerance of flint maize inbred lines using SSR markers

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Research Article

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Abstract

This study assessed the genetic and phenotypic variation of 12 flint maize inbred lines and performed association analysis of 11 drought-related traits using 360 simple sequence repeats (SSRs), detecting 1,604 alleles, with an average of 4.4 alleles per locus. The average values of gene diversity (GD) and polymorphism information content (PIC) were 0.648 and 0.598, respectively. In principal component analysis (PCA), shoot fresh weight (SFW), shoot dry weight (SDW), stem weight (SW), leaf weight (LW), root fresh weight (RFW), root dry weight (RDW), and leaf area (LA) traits contributed greatly to the PIC. Association analysis was performed using a general linear model with a Q-matrix (Q GLM) and a mixed linear model with Q and K-matrices (Q + K MLM). Twelve SSR markers for drought tolerance trait were detected by Q GLM, and all maize inbred lines were clearly divided into two groups in accordance with their drought tolerance. Duplicated significant marker-trait associations (SMTAs) between Q GLM and Q + K MLM identified eight marker-trait associations involving four SSR markers that were associated with the traits of SW, SFW, RFW, and RDW with a significant level of $P < 0.05$. The umc1175 and umc2092 were associated with SW and SFW; umc1503 was associated with RFW, SFW, and SW; and umc2341 was associated with RDW. The detection of loci associated with drought-related traits in this study may provide better opportunities to improve maize drought tolerance by marker-assisted selection (MAS). These results will be useful for breeders in producing tolerant varieties as well as markers for using MAS in maize breeding programs.

Introduction

Maize is one of the most important agricultural and economic crops, and it is the main source of food for humans and forage for livestock. Among cereal crops, global production is highest for maize, followed by wheat and rice, while maize ranked second after wheat in terms of harvested area (FAOSTAT 2020). With the global expansion of maize harvested areas, world maize production and yields have been increasing. World production, area harvested, and yield for maize recorded 1162.4 million tons, 202.0 million ha, and 5.8 t/ha, respectively, in 2020 (FAOSTAT 2020, http://www.fao.org/faostat/en/#compare). Maize can be divided into several types based on the starch composition of the kernel's endosperm, such as normal (including dent and flint), waxy, pop, and sweet. Especially, normal maize is widely cultivated and used in food and feed worldwide. As the world population is increasing, the scientific community must use all available ways to help farmers meet the ever-increasing demand for food, forage, and other resources. Drought is a primary abiotic stress affecting crop production and harvested areas worldwide because of water limitations. Moreover, maize is more sensitive to drought stress than other crops, such as winter wheat (Webber et al. 2018). Drought stress in maize, especially during the vegetative growth stage, can lead to a decreased growth rate, extension of the vegetative growth stage, and redirection of the roots (Ao et al. 2020). The seedling stage after the emergence stage until the 5-leaf stage of maize is especially sensitive to environmental stress, such as drought; although this stage requires less water than the later vegetative and reproductive stages, drought stress will have a greater effect on development at the early growth stages compared with the later development stages, such as flowering and anthesis-silking interval (Bell 2017; Maiti et al. 1996; Cao and Wj 2004). It was estimated that there was a 15–20% decrease of maize production yearly because of drought stress and that these losses were expected to increase further (Chen et al. 2012). In consideration of continuous climate change and more frequent occurrence of drought, genetic improvements for maize have focused on enhancing drought tolerance (Campos et al. 2004; Lopes et al. 2011). Therefore, the use of drought-tolerant maize inbred lines and cultivars is one of the best strategies for reducing water deficiency in the crop (Tani et al. 2019). However, the degree of tolerance for drought stress varies for growth stages and condition, variety or accession, and agro-ecological region (Toscano et al. 2019).

Drought tolerance is derived from complex quantitative traits that are associated with different shoot and root morphological characters (Yadav and Sharma 2016). The traditional breeding method depends on phenotypic

selection in the field, which is time-consuming and laborious for accurate evaluation and development of a new maize cultivar (Duvick et al. 2004). Such a phenotypic evaluation and selection for breeding programs may be also inaccurate because of environmental factors. However, polymerase chain reaction (PCR)-based molecular markers are not influenced by environmental factors and can be used to detect more accurately genetic diversity and population structure among breeding materials and to predict hybrid performance and heterosis (Kashiani et al. 2012; Solomon et al. 2012). Marker-assisted selection (MAS) using molecular markers allows breeders to select target phenotypes based on genotypes for genetic improvement and selection of crops (Zhang et al. 2011). To utilize MAS, it is necessary to identity the molecular markers and genetic regions associated with target traits (Yu et al. 2005). Association analysis especially enables identification of significant marker-trait associations (SMTAs) for MAS that have various advantages such as reduction of experimental time and cost compared with quantitative trait loci (QTL) mapping (Flint-Garcia et al. 2005; Yu and Buckler 2006). Molecular markers have been widely utilized for QTL mapping and association studies and for MAS for crop breeding and genetic research (Mohan et al. 1997). Among various molecular marker systems such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs), and single nucleotide polymorphism (SNP), microsatellites or SSRs are short tandem parts with simple repeated fragments of one to six nucleotide motifs and exist in both coding and non-coding regions (Miah et al. 2013). SSR markers provide valuable information about genetic diversity, genetic relationships, and population structure in crop germplasms because of being highly polymorphic, generally codominant, reproducible, and broadly distributed throughout the plant genome (Powell et al. 1996; Park et al. 2009). These SSR markers has been successfully applied for crop characterization and studies of genetic diversity and desired gene association analysis (Kalivas et al. 2011).

Molecular markers associated with drought tolerance in maize will provide insights for selecting inbred lines and cultivars, which will help in maize breeding programs for enhancing yield and productivity as well as drought tolerance. Thus, this study performed association analysis of 360 SSR markers and 11 traits associated with drought tolerance among 12 drought tolerant and susceptible flint inbred lines, which were selected from our previous study by using morphological characters (Adhikari et al. 2019). The objective of our study is not only to research the genetic diversity and population structure of flint maize inbred lines by SSR molecular markers, but it is also to confirm molecular markers related to drought tolerant traits using association analysis. The results of this study are expected to provide useful information for future maize breeding programs for drought tolerant lines.

Materials And Methods

Plant materials and morphological analysis

The 12 flint maize inbred lines used in this study were divided into two groups, drought-tolerant and susceptible groups, which were selected from our previous study using ten morphological traits (Adhikari et al. 2019) (Table 1). Among these inbred lines, six inbred lines (FLD1, FLD13, FLD16, FLD18, FLD29, FLD31) were drought-tolerance inbred lines, and the other six inbred lines (FLD12, FLD23, FLD24, FLD33, FLD35, FLD37) were susceptible to drought condition. For drought-tolerance trait, these 12 inbred lines were scored as tolerant (1) or susceptible (2) based on our previous study. For association analysis, ten morphological traits from Adhikari et al. (2019) were used to generate the difference between values of control and drought condition; these were plant height (PH), leaf area (LA), leaf and stem weight (LW and SW), shoot and root fresh weight (SFW and RFW), root length (RL), total chlorophyll content (TCC), and shoot and root dry weight (SDW and RDW) (Adhikari et al. 2019).

Table 1
List of maize inbred lines used for the study

Entry No.	Drought Tolerance*	Accession Name	Source
FLD01	S	00hf1	Eongdan14
FLD13	S	hc2	NK487
FLD16	S	hc6	Unknown
FLD18	S	HF1	Unknown
FLD29	S	07S8004	IP144
FLD31	S	07S8011	1P161
FLD12	T	hc5	Ho-5
FLD23	T	KS118	Unknown
FLD24	T	SIM6	Maysin collection
FLD33	T	06S8001	ISU pop T-C 8644-27/ISU POP 5
FLD35	T	06S8013	ISU INB. 1368/(B87/B73-12)B#
FLD37	T	06S8030	EV43-SR/9B-5
* T: Drought Tolerant; S: Drought Susceptible lines			

DNA extraction and SSR amplification

Genomic DNA in young leaves was obtained using the Dellaporta et al. (1983) method with minor modifications. A total of 360 SSR markers, distributed across the ten maize chromosomes (average 36 loci per each chromosome), were used for analysis for genetic variation, population structure, and association between markers and traits in the 12 flint maize inbred lines (Table 2). Information of SSR markers, such as chromosome location and sequences of forward and reverse primer, were derived from MaizeGDB (http://www.maizegdb.org/).

Table 2
Means and standard deviations of eleven traits for drought-tolerant and susceptible groups.

	Tolerance**	PH (cm)	LA (cm^2)	LW (g)	SW (g)	SFW (g)	RFW (g)	RL (cm)	TCC	SDW (g)	RDW (g)
FLD1	S	-8.3	-34.0	-1.2	-2.6	-3.8	-2.1	-2.6	-2.6	-0.7	-0.3
FLD13	S	-5.8	-16.8	-0.4	-1.6	-2.0	-0.6	-16.9	-6.5	-0.5	-0.2
FLD16	S	-9.5	-13.8	-0.9	-1.2	-2.2	-1.1	-1.4	-6.1	-0.6	-0.1
FLD18	S	-11.4	-32.4	-1.5	-3.5	-5.1	-1.6	-3.8	-4.0	-0.8	-0.1
FLD29	S	-15.7	-30.9	-0.8	-0.5	-1.3	-0.2	-7.4	-7.7	-0.2	-0.1
FLD31	S	-15.8	-10.1	-1.0	-0.5	-1.5	-0.5	-8.1	-4.4	-0.4	-0.1
	Mean*	-11.1 ± 4.1	-23.0 ± 10.6	-1.0 ± 0.4	-1.7 ± 1.2	-2.6 ± 1.5	-1.0 ± 0.7	-6.7 ± 5.7	-5.2 ± 1.9	-0.5 ± 0.2	-0.2 ± 0.1
FLD12	T	-6.4	-1.8	-0.2	-0.7	-0.9	-0.7	-5.9	-7	-0.2	0.0
FLD23	T	-3.8	-0.3	-0.4	-0.2	-0.6	-0.2	-1.6	-8.3	-0.2	0.0
FLD24	T	-3.6	-16.7	-0.5	-0.6	-1.1	-0.5	-1.4	-8.6	-0.2	0.0
FLD33	T	-7.2	-17.0	-0.2	-0.1	-0.3	-0.4	-5.8	-3.7	0.0	-0.1
FLD35	T	-8.9	-3.5	-0.5	-0.9	-1.4	-0.3	-0.8	-5.1	-0.1	-0.1
FLD37	T	-1.5	-6.6	-0.1	-0.1	-0.2	-0.4	-0.9	-3.5	-0.1	0.0
	Mean*	-5.2 ± 2.7	-7.6 ± 7.4	-0.3 ± 0.2	-0.4 ± 0.4	-0.7 ± 0.5	-0.4 ± 0.2	-2.7 ± 2.4	-6.0 ± 2.2	-0.1 ± 0.1	0.0 ± 0.0
** S: Susceptible; T: Tolerant.											
*Average values for each group are expressed as mean ± standard deviation.											

An SSRs amplification test was carried out using EX *Taq* PCR kit (Takara, Ohtsu, Japan). For PCR of the SSRs loci, a total volume of 20 µL of product conducted 20 ng of genomic DNA, 1 × EX *Taq* buffer, 0.5 µM of forward and reverse primers, 0.2 mM dNTP mixture, and 1 unit of EX *Taq* Polymerase. The PCR protocol proceeded as follows: first step was initial denaturation at 94°C for 5 min; and second step was denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 2 min. After the second step, temperature for the annealing stage was decreased by increments of 1°C following every annealing stage until a final annealing temperature of 55°C. The second step was then repeated 36 times. After completing the two steps, a final third step was carried out for 5 min at 72°C for extension.

Electrophoresis and fragment detection

For the PCR products, DNA electrophoresis analysis was performed with a mini vertical electrophoresis system (MGV-202-33, CBS Scientific Company, San Diego, USA). Three µl of the PCR product was mixed with 3 µl of formamide loading dye (98% formamide, 0.02% BPH, 0.02% xylene C, and 5 mM NaOH). Two µl of the sample was loaded onto a 6% acrylamide-bisacrylamide gel (19:1) in 0.5X TBE buffer and electrophoresed at 250 V for 40 ~ 60 min. The separated DNA fragments were then visualized using ethidium bromide (EtBr).

Data and Statistical analyses

The number of alleles, gene diversity (GD), polymorphic information content (PIC), and major allele frequency (MAF) for drought-tolerant and susceptible inbred lines were identified using PowerMarker software (Liu and Muse 2005). Genetic similarities (GS) between each pair of lines were calculated with the Dice similarity index (Dice 1945). The similarity matrix was then used to construct a dendrogram based on an unweighted pair group method with arithmetic mean (UPGMA), with the help of SAHN-Clustering from NTSYS-pc (Rohlf 1998). Moreover, a principal component analysis (PCA) was performed to estimate relationships for phenotypic variance among maize inbred lines using the NTSYSpc software package (Rohlf 1998).

Population structure among the 12 drought-tolerant and susceptible inbred lines was confirmed by model-based program STRUCTURE software (Pritchard and Wen 2003). This software was executed five times for each simulation subgroups (*K* value) from 1 to 10 with a burn-in of 100,000 and a run length of 100,000 in an admixture model. The delta *K* value based on degree of change for log probability by Evanno et al. (2005) was calculated with STRUCTURE HARVESTER (http://taylor0.biology.ucla.edu/structHarvester/). The subgroup was assigned by using the run result with maximum likelihood among five runs of estimated numbers, with lines with membership probabilities of ≥ 0.80 assigned to subgroups, while lines with less than 0.80 were assigned to an admixed group (Stich et al. 2005). Association analysis was performed using TASSEL 3.0 (Bradbury et al. 2007), which was used to confirm marker-trait associations using a mixed linear model (Q + K MLM). The Q + K MLM method was performed by combining the population structure (Q) matrix derived from the STRUCTURE and the kinship (K) matrix derived from the TASSEL at $P < 0.05$ (Pritchard and Wen 2003; Bradbury et al. 2007). Furthermore, basic statistical analysis was performed using applications in Microsoft Office Excel 2016. Student's t-test at $P < 0.05$ and 0.01 was used for estimation of the statistical difference between the six tolerant and six susceptible lines. Moreover, correlation for 11 phenotypic traits was calculated. Both analyses used IBM SPSS Statistics version 21.

Results

Phenotypic analysis and statistical test

Phenotypic variation of ten agronomic traits between control (well-watered) and drought condition in tolerant and susceptible maize inbred groups are summarized in Table 2. The average PH decrease of susceptible lines in drought condition was − 11.1 ± 4.1 cm, ranging from − 5.8 (FLD13) to -15.8 (FLD31) cm. On the other hand, the average PH decrease of tolerant lines was − 5.2 ± 2.7 cm, ranging from − 1.5 (FLD37) to -8.9 (FLD35) cm. The average LA decrease of the susceptible group in drought condition compared with well-watered condition was − 23.0 ± 10.6 cm^2, ranging from − 10.1 (FLD31) to -34.0 (FLD1) cm^2, while the average value for the tolerant group ranged from − 0.3 (FLD23) to -17.0 (FLD33) cm^2, with an average of -7.6 ± 7.4 cm^2. In the case of LW, the average value of susceptible lines was − 1.0 ± 0.4, with a range from − 0.4 (FLD13) to -1.5 (FLD18) g. However, drought-tolerant lines had an average value of -0.3 ± 0.2, with a range from − 0.1 (FLD37) to -0.5 (FLD24, 35) g. The average SW decrease of susceptible lines in drought condition was − 1.7 ± 1.2 g, with a range of -0.5 (FLD29, 31) ~ -3.5 (FLD18) g. The average value for SW in the tolerant group ranged from − 0.1 (FLD33, 37) to -0.9 (FLD35) with an average of -0.4 ± 0.4. For the SFW trait, the average value of the susceptible group was − 2.6 ± 1.5, with a range of -1.3 (FLD29) ~ -5.1 (FLD18) g, while the tolerant group showed an average value of -0.7 ± 0.5, with a range from − 0.2 (FLD37) to -1.4 (FLD35) g. The average RFW decrease of the tolerant group in drought condition was − 0.4 ± 0.2 g, ranging from − 0.2 (FLD23) to -0.7 (FLD12) g. Meanwhile, the average RFW decrease of the susceptible group was − 1.0 ± 0.7 g, ranging from − 0.2 (FLD29) to -2.1 (FLD1) g. The average value for RL in the susceptible and tolerant groups showed − 6.7 ± 5.7 and − 2.7 ± 2.4 cm, respectively. Moreover, the RL trait of the susceptible lines ranged from − 1.4 (FLD16) to -16.9 (FLD13) cm, but that of the tolerant lines ranged from − 0.8 (FLD35) to -5.9 (FLD12) cm. The average TCC decrease of the susceptible lines in

drought condition was − 5.2 ± 1.9, with a range of -2.6 (FLD1) ~ -7.7 (FLD29). The average value for TCC in the tolerant group ranged from − 3.5 (FLD37) to -8.6 (FLD24) with an average of -6.0 ± 2.2. The average SDW decrease of the susceptible group was − 0.5 ± 0.2 g, ranging from − 0.2 (FLD29) to -0.8 (FLD18) g, while the average value for the tolerant group ranged from 0.0 (FLD33) to -2.0 (FLD12, 23, 24) g, with an average of -0.1 ± 0.1 g. The average RDW decrease of the susceptible lines in drought condition was − 0.2 ± 0.1 g, with a range of -0.1 (FLD16, 18, 29, 31) ~ -0.3 (FLD1) g. The RDW in all tolerant lines except FLD33 and FLD 35 (-0.1) showed no change in drought condition with an average of 0.0 ± 0.0 (Table 2, Fig. 1).

Significant differences in phenotypic variation between the tolerant and susceptible maize inbred groups were evaluated by t-test (Fig. 1). The results showed a statistically significant difference in PH, LA, LW, SFW, SDW, and RDW between the tolerant and susceptible maize inbred groups at $P < 0.05$ and 0.01. Correlation analysis was performed to confirm genetic relationships among 11 agronomic traits in the 12 flint inbred lines (Table 3). Among all 55 combinations, 16 combinations showed comparatively higher positive or negative coefficients, namely SW and SFW (0.982**), SFW and SDW (0.934**), SW and SDW (0.908**), LW and SFW (0.891**), SW and RFW (0.868**), SFW and RFW (0.865**), LW and SDW (0.857**), RFW and SDW (0.841**), Tolerance and SDW (-0.812**), LW and SW (0.790**), Tolerance and LW (-0.766**), SDW and RDW (0.759**), Tolerance and RDW (-0.730**), LW and RFW (0.724**), RFW and RDW (0.711**), and LA and LW (0.710**), at $P < 0.01$ (Table 3).

Table 3
Correlation analysis among 11 drought-related traits of 12 flint maize inbred lines.

Traits	PH	LA	LW	SW	SFW	RFW	RL	TCC	SDW	RDW
Tolerance	-0.679*	-0.677*	-0.766**	-0.602*	-0.681*	-0.520	-0.449	0.214	-0.812**	-0.730**
PH		0.444	0.632*	0.233	0.367	0.096	0.254	-0.128	0.347	0.206
LA			0.710**	0.660*	0.706*	0.611*	0.167	-0.296	0.627*	0.613*
LW				0.790**	0.891**	0.724**	-0.060	-0.298	0.857**	0.528
SW					0.982**	0.868**	0.075	-0.380	0.908**	0.666*
SFW						0.865**	0.037	-0.372	0.934**	0.654*
RFW							-0.120	-0.517	0.841**	0.711**
RL								0.092	0.189	0.406
TCC									-0.303	-0.478
SDW										0.759**

* and ** show the significant differences at the 0.05 and 0.01 probability levels, respectively.

Moreover, the morphological data was used to perform PCA analysis. The results showed that the first and second principal components accounted for 59.6% and 13.7% of the total variance, respectively (Table 4). The SFW, SDW, SW, LW, RFW, RDW, and LA traits contributed in a positive direction on PC1, and RL contributed in a positive direction on PC2. Based on PC1, all maize inbred lines except FLD29 were clearly separated into two maize inbred groups by their drought tolerance (Fig. 2).

Table 4
Eigen vector and cumulative variance of the first and second principal components.

Traits	Eigen vector	
	PC1	PC2
Shoot fresh weight (SFW)	0.964	-0.086
Shoot dry weight (SDW)	0.943	0.033
Stem weight (SW)	0.932	-0.133
Leaf weight (LW)	0.894	0.040
Root fresh weight (RFW)	0.888	-0.364
Root dry weight (RDW)	0.792	0.145
Leaf area (LA)	0.788	0.185
Plant height (PH)	0.435	0.593
Root length (RL)	0.129	0.829
Total chlorophyll content (TCC)	-0.480	0.339
Cumulative variance (%)	59.6	13.7

Genetic diversity among 12 flint inbred lines related to drought tolerance

A total of 360 SSR loci were used to evaluate a genetic diversity index, including GD, PIC, and MAF, among the 12 flint inbred lines (Table 5). The 360 SSR loci appeared in a total of 1,604 alleles in the 12 flint inbred lines. The number of alleles per locus ranged from 2 to 11, and the average number of alleles per locus was 4.4 (Table 5, Supplementary Table 1). The average GD was 0.648, with a range of 0.153–0.903. The average PIC value was 0.598, with a range of 0.141–0.895. The average MAF was 0.466, with a range of 0.167–0.917 (Table 5). To clearly understand genetic diversity and variation in the 12 drought-related inbred lines, this study verified the allele numbers, GD, PIC, and MAF in the six drought-tolerant and six drought-susceptible inbred lines. Those values for the 360 SSR loci in the tolerant and susceptible maize inbred groups are shown in Table 6. The total number of alleles was 1,241 and 1,174 with an average of 3.4 and 3.3 in each group of the six flint inbred lines, respectively. Furthermore, the averages of the GD, PIC, and MAF values were 0.609, 0.551, and 0.494, respectively, in the six drought-tolerant inbred lines. Meanwhile, these values for the six drought-susceptible inbred lines were 0.581, 0.521, and 0.521, respectively (Table 6).

Table 5
Total number of alleles and genetic diversity index for 360 SSR loci in the twelve-flint maize inbred lines.

Chromosome	No. of MK	Total alleles	Mean of alleles	GD	PIC	MAF
Chr.1	32	142	4.4	0.651	0.602	0.466
Chr.2	37	170	4.6	0.660	0.608	0.453
Chr.3	35	149	4.3	0.639	0.590	0.474
Chr.4	49	236	4.8	0.662	0.617	0.454
Chr.5	30	128	4.3	0.652	0.596	0.453
Chr.6	30	134	4.5	0.645	0.594	0.467
Chr.7	48	213	4.4	0.642	0.592	0.469
Chr.8	40	177	4.4	0.640	0.591	0.473
Chr.9	31	142	4.6	0.663	0.616	0.457
Chr.10	28	113	4.0	0.630	0.573	0.491
Total	360	1,604	-	-	-	-
Mean	36.0	4.4	-	0.648	0.598	0.466
Min	-	2	-	0.153	0.141	0.167
Max	-	11	-	0.903	0.895	0.917

Table 6
Comparison of total number of alleles and genetic diversity index between tolerant and susceptible groups.

Parameter	Tolerant inbred lines (n = 6)	Susceptible inbred lines (n = 6)
No. of alleles	1,241	1,174
Mean	3.4	3.3
Gene Diversity	0.609	0.581
Min	0.000	0.000
Max	0.833	0.833
PIC	0.551	0.521
Min	0.000	0.000
Max	0.810	0.810
MAF	0.494	0.521
Min	0.167	0.167
Max	1.000	1.000

Population structure analysis in flint maize inbred lines

To confirm the genetic structure and relationships among the 12 flint inbred lines related to drought tolerance, this study used a model-based STRUCTURE program to subdivide into appropriate subgroups. Because it was difficult to separate subgroups using five replicate sets ranging from 1 to 10 from the LnP(D) of the data, this study applied the ad hoc measure ΔK (Evanno et al. 2005). Although the highest ΔK value was revealed for $K = 2$ in all 12 flint inbred lines using the 360 SSR loci, all inbred lines were not clearly separated on the basis of drought tolerance (Fig. 3). Moreover, a distance-based dendrogram from the UPGMA analysis was constructed using the 360 SSR loci (Fig. 4). All flint inbred lines were classified into two maize inbred groups at a genetic similarity of 0.281. Group I consisted of five inbred lines, composed of two drought-tolerant lines (FLD12, 23) and three drought-susceptible lines (FLD1, 16, 18); while Group II consisted of seven inbred lines, composed of four drought-tolerant lines (FLD 24, 33, 35, 37) and three drought-susceptible lines (FLD13, 29, 31) (Fig. 4).

Association analysis using Q GLM and Q+K MLM

Association analysis between a total of 360 SSR markers and 11 phenotypic traits in the 12 flint maize inbred lines was performed by Q GLM and Q + K MLM. This study detected 205 marker-trait associations involving 120 SSR markers associated with the 11 agronomic traits using Q GLM at $P < 0.05$ (Supplementary Table 2). When we used Q + K MLM, four SSR markers, umc1175, umc1503, umc2092, and umc2503, were associated with SW, SFW, RFW, and RDW traits at a significance level of $P < 0.05$ (Table 7). Among these SMTAs, umc1175 was associated with two traits, SFW and SW, on chromosome 4. Meanwhile, umc1503 was associated with three traits, RFW, SFW, and SW, on chromosome 4. Moreover, umc2092 were associated with two traits, SFW and SW, on chromosome 7. SSR marker umc2503 was associated with only one trait, RDW, on chromosome 8 (Table 7).

Table 7
Information on overlapping SMTA markers between Q GLM and Q + K MLM

SSR Marker	Chr.	Phenotypic Traits	Q GLM	Q + K MLM
umc1175	4	SFW	0.006	0.040
		SW	0.006	0.042
umc1503	4	RFW	0.000	0.048
		SFW	0.000	0.048
		SW	0.000	0.048
umc2092	7	SFW	0.006	0.040
		SW	0.006	0.042
umc2503	8	RDW	0.002	0.030

Discussion

Drought is a major limiting factor for maize plant growth, development, and productivity (Djemel et al. 2018). In our previous study, we selected six drought-tolerant and six susceptible maize inbred lines by using drought tolerance indices, namely PH, LA, LW, SW, SFW, RFW, RL, TCC, SDW, and RDW (Table 1, Adhikari et al. 2019). Drought stress influences diverse morpho-physiological characteristics including plant biomass, root length, and shoot length (Jaleel et al. 2008). In this study, we compared the average value for ten traits between drought-tolerant and susceptible

groups (Table 2). The results showed that there was a statistically significant difference in PH, LA, LW, SFW, SDW, and RDW between the tolerant and susceptible groups by t-test at $P < 0.05$ and 0.01, although there was no statistical significance between the groups for some traits, SW, RFW, and RL (Fig. 1). This result is supported by correlation analysis, which showed a high correlation coefficient between drought tolerance with LW, SDW, and RDW at $P < 0.01$ and with PH, LA, and SFW at $P < 0.05$ (Table 3).

Correlation analysis helps to confirm the interrelationship between traits related to plant growth and enables recognition of traits that can be used for selecting drought tolerant maize inbred lines at the early growth stage (Akinwale et al. 2018). The ratio of root to shoot has been reported to increase under drought conditions because roots are less sensitive to water deficiency compared with shoots (Wu and Cosgrove 2000). This study also obtained similar results with the ratio of root to shoot for the drought susceptible group being 0.328 in normal condition and 0.377 in drought condition and that of the tolerant group being 0.384 in well-watered condition and 0.400 in water deficient condition (data not shown). Furthermore, the ratio of root to shoot of the susceptible group was more variable than that of the tolerant group, which suggests that the tolerant inbred lines are less sensitive than the other group.

Root dry weight has the potential to be an important trait for selection against water stress (Mehdi et al. 2001). This study also confirmed the association in Tolerance and RDW (Table 3). In this study, PCA was performed to evaluate differentiation among the drought tolerant and susceptible maize inbred lines and to select informative traits for drought tolerance (Table 4, Fig. 2). The results showed that all maize inbred lines, except FLD29, were clearly divided into two groups based on PC1. The SFW, SDW, SW, LW, RFW, RDW, LA, and RL traits greatly contributed in the positive direction on PC1 and PC2. Thus, these agronomic traits may be considered useful for selection and discrimination among maize inbred lines for drought tolerance in breeding programs.

Information about genetic diversity and relationships and the population structure of breeding materials is useful for the development of new varieties or elite inbred lines in plant breeding programs. In this study, 360 SSR loci (SSR loci per chromosome ranged from 28 for Ch.10 to 49 for Ch. 4) covering the whole maize genome were used to detect genetic variation in 12 flint maize inbred lines related to drought tolerance (Table 5, Supplement Table 1). A total of 1,604 alleles were detected with an average number of 4.4 alleles per locus, and the average GD, PIC, and MAF was 0.648, 0.598, and 0.466, respectively (Table 5). In addition, this study compared the values of a genetic diversity index between the six drought tolerant and six susceptible maize inbred lines. The average GD, PIC, and MAF values for the tolerant group were 0.609, 0.551, and 0.494, respectively, and 0.581, 0.521, and 0.521, respectively, for the susceptible group (Table 6). Consequently, the tolerant group showed relatively higher genetic variation than the susceptible group.

The population structure using the 360 SSR markers in this study was investigated using a model-based clustering method (STRUCTURE) and distance-based phylogenetic methods (NTSYS). In a model-based clustering pattern based on a probability threshold > 0.8, all inbred lines could be divided into two distinct Groups I and II and an Admixed group. Most of the maize inbred lines (FLD23, 24, 33, 35, 37 of drought tolerant lines and FLD13, 18, 29, 31 of drought susceptible lines) were designated by Group I. One drought tolerant inbred line, FLD16, is the only member of Group II. The remaining two inbred lines, FLD12 of tolerant and FLD1 of susceptible, belong to the Admixed group (Fig. 3). A UPGMA dendrogram based on genetic distance was divided into two main groups, and 2 ~ 3 subgroups was observed in each main group (Fig. 3). Although two different methods based on model and distance were used, there was no clear separation pattern based on drought tolerance using the 360 SSR markers, and cluster analysis based on genetic distance yielded more information on the genetic diversity of all inbred lines than the model-based method. Moreover, three inbred lines, FLD1, 12, and 16, which were contained in Group II and the Admixed group, were clustered into Group I-1 in the distance-based dendrogram (Fig. 4). Although there is pedigree data of nine inbred lines, three inbred lines, FLD16, 18, and 23, are unknown (Table 1). The population structure information will enhance understanding of

the structural organization of the unknown lines for pedigree and source information. Furthermore, this genetic diversity and population structure information of the 12 flint maize inbred lines is expected to help in optimizing the selection of cross combinations in the development of new maize cultivars.

Recently, association analysis has been used as an alternative to QTL mapping because it is effective in detecting molecular markers related to targeted morphological traits, such as drought tolerance (Liu and Qin 2021). In our study, 360 SSR loci (average 36 SSRs per chromosome) were used and distributed across the ten maize chromosomes. However, false positives (Type-I error) are a major problem in association analysis and lead to invalid associations because of population structure (Q) and unequal relatedness (K) (Zhang et al. 2010). To prevent false positives, we used two different methods for association analysis, a general linear model based on a Q-matrix (Q GLM) and a mixed linear model based on a Q and K matrix (Q + K MLM) (Tables 7, Supplementary Table 2). Population structure analysis using the Q GLM model identified 193 marker-trait associations, but only eight associations were found using the Q + K MLM model, based on population structure and kinship. In general, the Q + K MLM method detects relatively fewer SMTAs (Yu et al. 2006; Kwon et al. 2012). Moreover, this result indicated that the Q + K MLM method is better for decreasing the false positive rate in association analysis. Among marker-trait associations by Q GLM, 12 SSR markers (umc2400, umc2378, umc1872, bnlg2046, umc1969, bnlg1126, umc2334, phi022, umc1088, umc1707, bnlg1117, and umc1716) were detected for the drought tolerance trait. We performed distance-based UPGMA analysis again with the selected 12 SSR markers for verification. The result showed that all maize inbred lines clearly divided into two maize inbred groups in accordance with their drought tolerance at a genetic similarity of 0.123, although there was no clear pattern using the 360 SSR markers (Fig. 5). This result indicates that this set of SSR markers can be useful for selecting drought tolerance in future maize breeding programs. The eight overlapping SMTAs between Q GLM and Q + K MLM were associated with only shoot and root-related traits, excluding PH, TCC, and leaf-related traits (Table 7). In particular, umc1175, umc1503, and umc2092 on chromosomes 4 and 7 were simultaneously associated with the SFW and SW traits. Moreover, two SSR markers, umc1503 and umc2503 on chromosomes 4 and 8, were associated with root-related traits RFW and RDW. These results were supported by higher correlation coefficients being detected between SFW and SW (0.982**), SW and RFW (0.868**), and SFW and RFW (0.865**) than the other combinations.

Some SSR markers in this study have been detected by other association analysis or QTL mapping studies, although the same SSR markers were not exactly consistent with the same traits in this study. For example, a previous report of QTL mapping by Benke et al. (2014) found that umc2092 was associated with shoot water content, but it was also associated with shoot and stem-related traits SFW and SW in this study. A higher shoot fresh weight indicates a higher uptake of water during well-watered conditions (Yaqoob et al. 2012). The umc1175 and umc1503 were tightly linked to the *akh1* (*aspartate kinase-homoserine dehydrogenase1*, bin 4.05) and *ubi2* (*ubiquitin2*, 4.09) genes, respectively, on chromosome 4 (http://www.maizeGDB.org). Finally, umc2503 was tightly linked to the *rgp2* (*ras-related protein*) gene on chromosome 8 (http://www.maizeGDB.org).

The results of this drought tolerance study for maize provide useful information for understanding the change of leaf, shoot, and root-related traits of 12 tolerant and susceptible flint maize inbred lines in drought condition, and the SSR markers related to these traits will provide useful information for MAS in maize breeding programs. Also, the identification of the loci associated with drought tolerance in this study may provide better opportunities for maize breeders to enhance maize drought tolerance by MAS.

Declarations

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Authors' contributions

JKL and KJS wrote the manuscript and designed the experiments. KJS, HP and SJJ performed the experiment and analyzed the data, and ZF helped to draft the manuscript. All authors commented on previous versions of the manuscript and approved the final manuscript.

Date availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Conflict of interest The authors declare that they have no conflicting interests.

Ethical approval This article does not contain any studies with human subjects or animals performed by any of the above authors.

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Figures

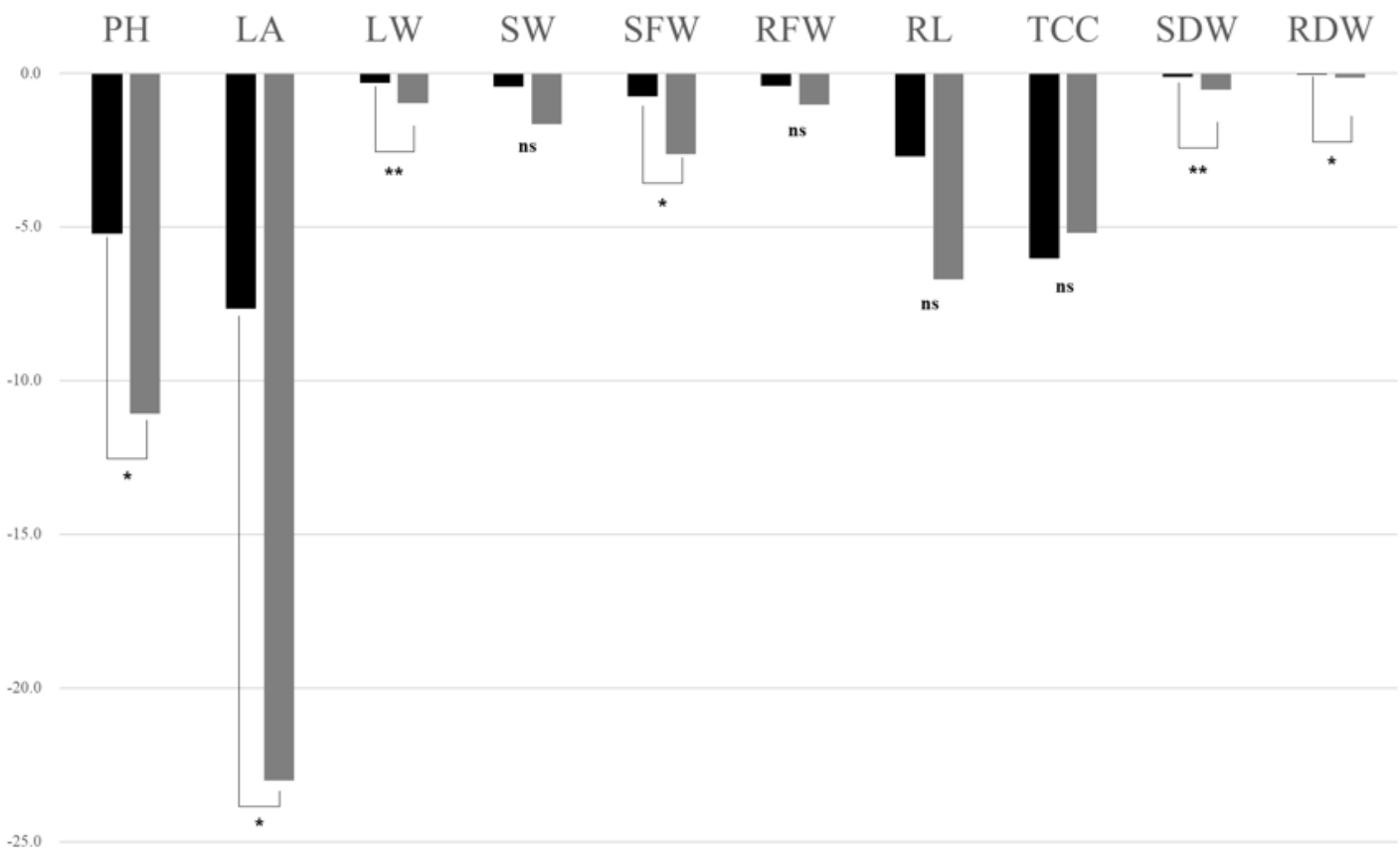

Figure 1

Bar graph of ten drought-related traits between tolerant (black) and susceptible (gray) groups. * and ** show the significant differences by t-test at the 0.05 and 0.01 probability level, respectively.

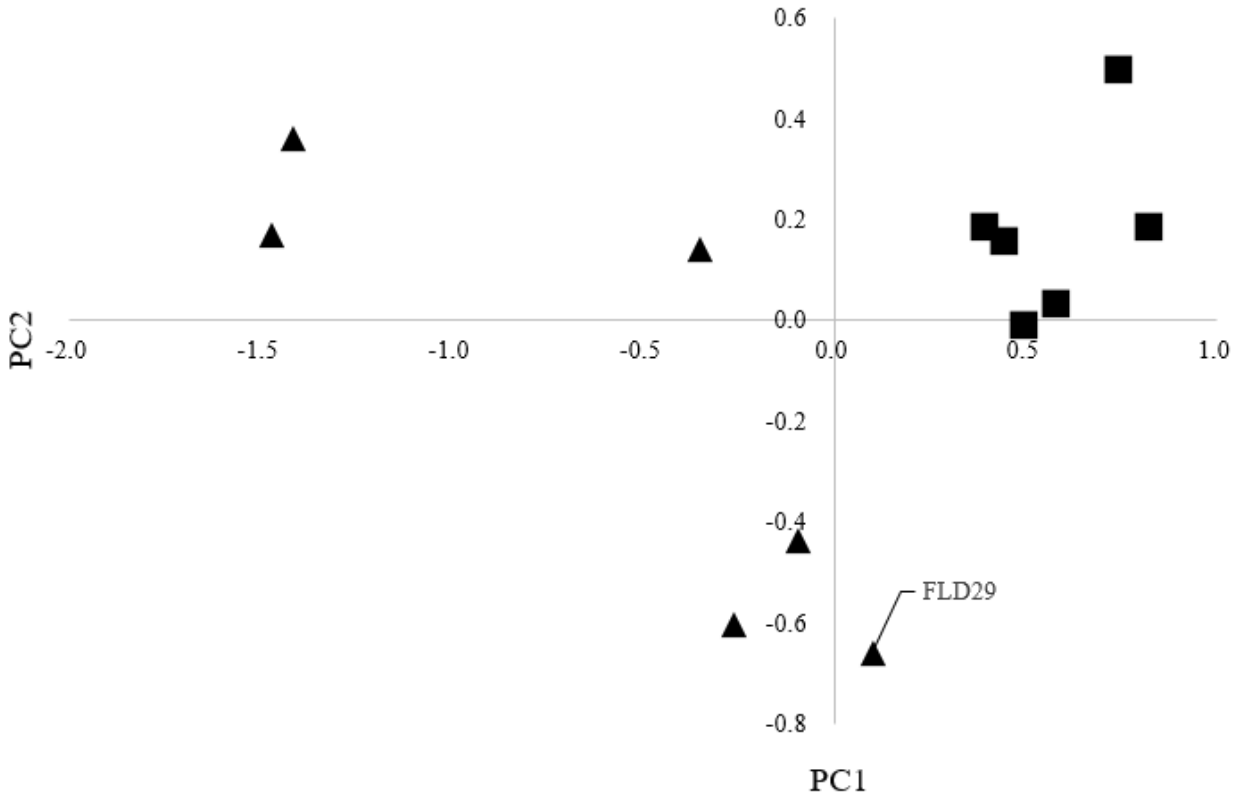

Figure 2

Projection of the 12 flint maize inbred lines in the first and second principal components.

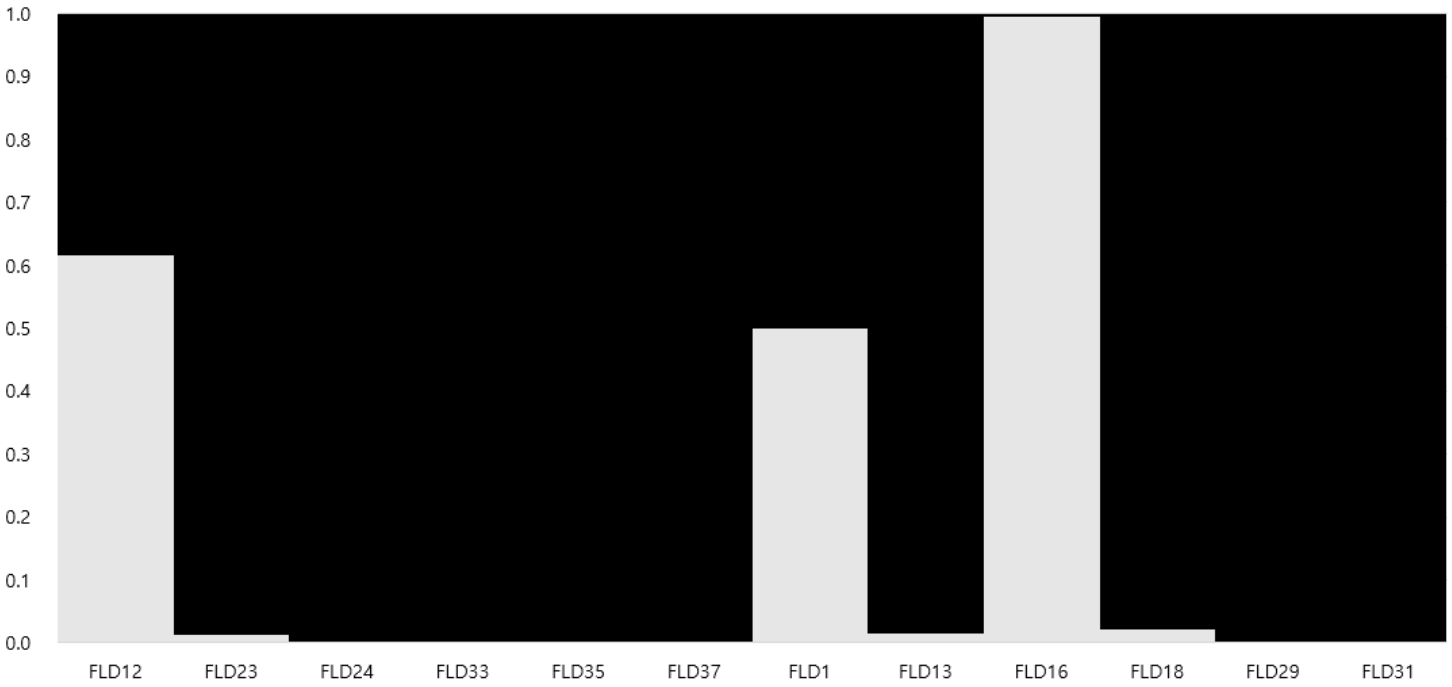

Figure 3

Population structure pattern in 12 maize inbred lines based on 360 SSR markers.

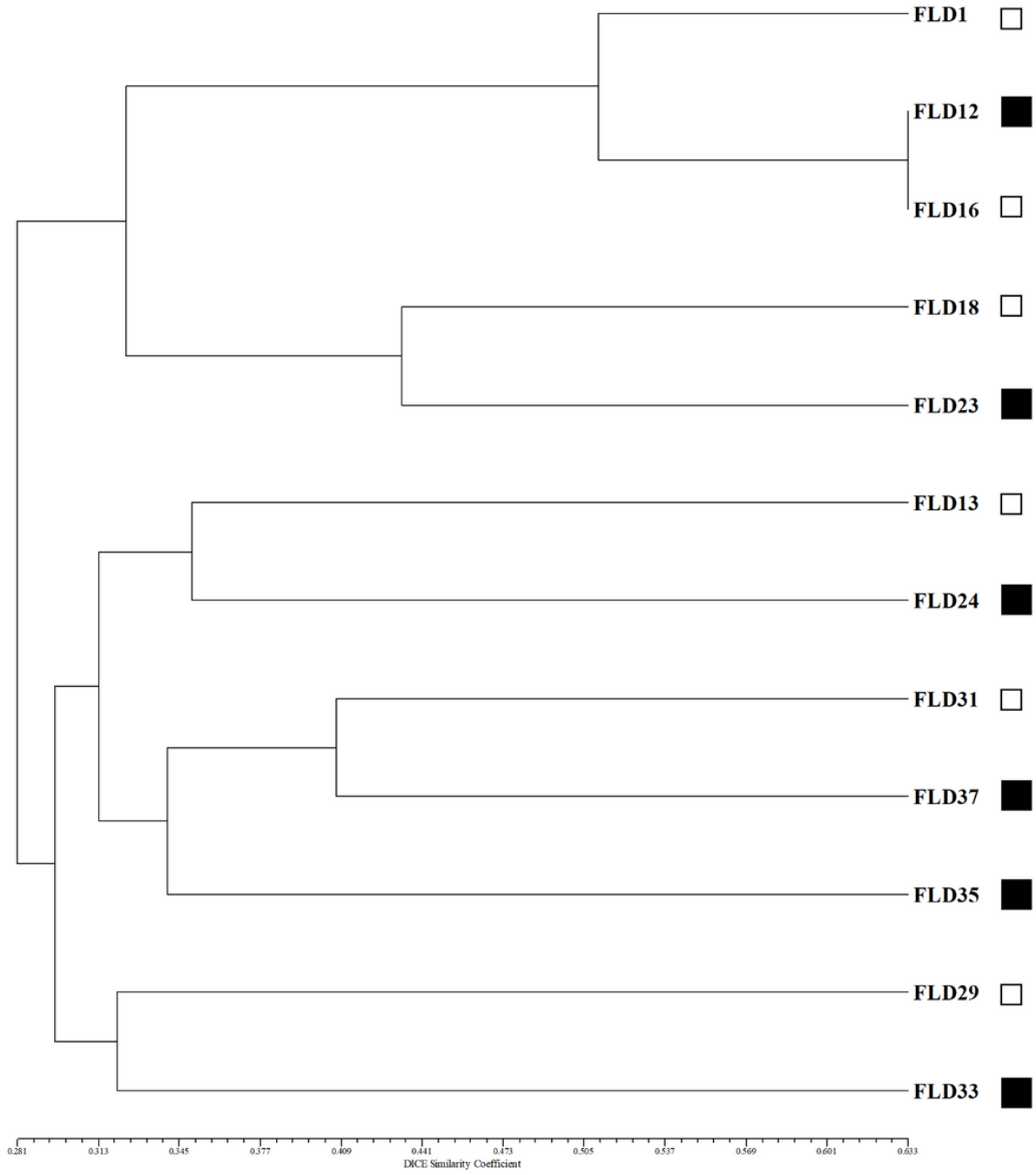

Figure 4

UPGMA dendrogram of the 12 flint inbred lines based on 360 SSR markers.

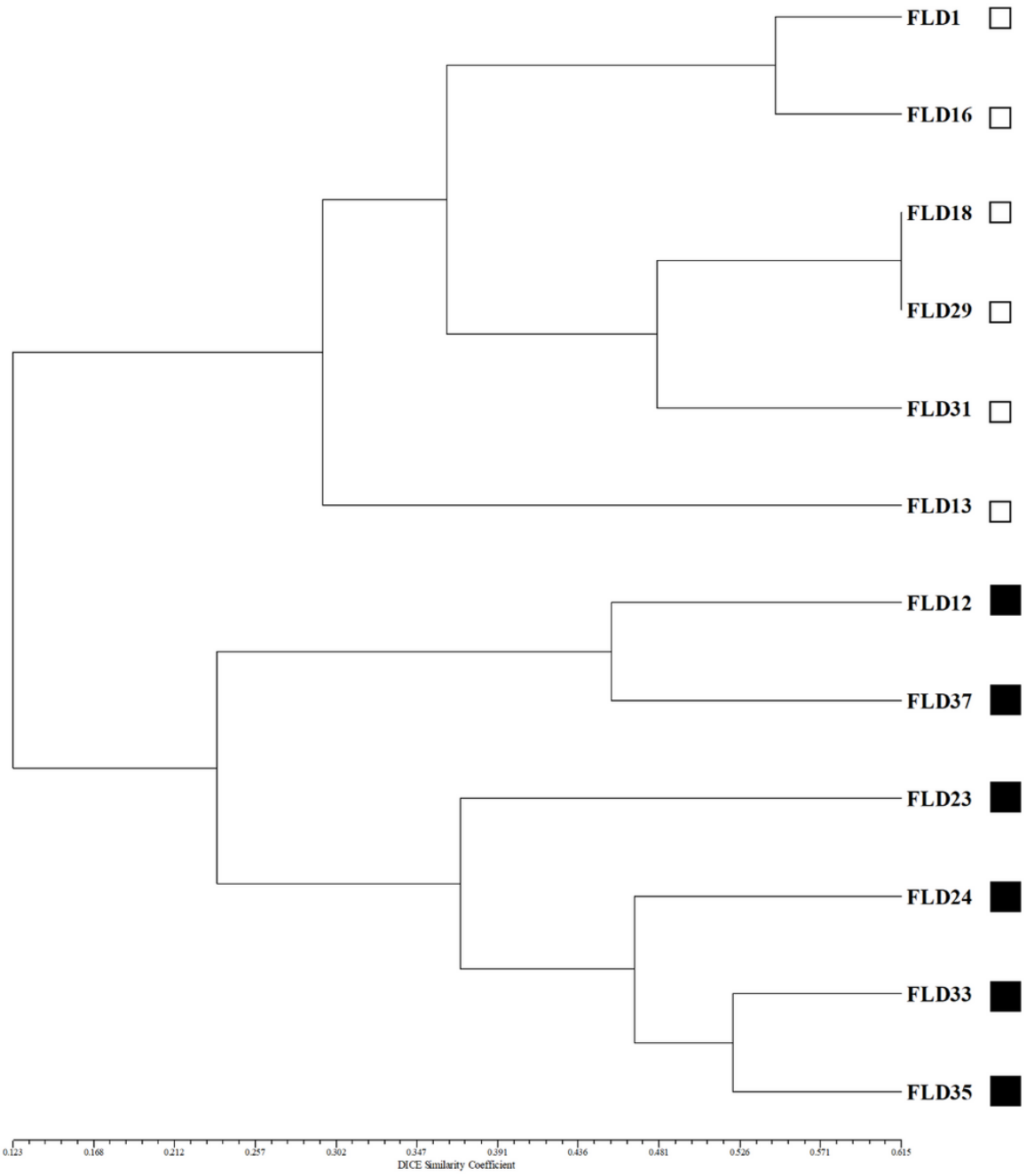

Figure 5

UPGMA dendrogram of the 12 flint inbred lines based on 12 SSR markers by selecting Q GLM.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.xlsx
- SupplementatyTable2.xlsx