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Molecular diversity in maize inbred lines for turcicum leaf blight resistance

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Abstract

Maize (*Zea mays* L.), third most important cereal crop in the world, its productivity can be limited partly by an important foliar disease called turcicum leaf blight caused by *Exserohilum turcicum*. In order to design efficient breeding programmes for resistance to leaf blight, the germplasm must be thoroughly characterized. This study evaluated the diversity of maize inbred lines using 26 simple sequence repeat (SSR) primers. The polymorphism information content (PIC) value of the SSR loci ranged from 0.61 to 0.71, with an overall mean of 0.65. It was highest for the primer bnlg1335 (0.71) and lowest for the primer bnlg1666 (0.61). The markers produced one to four alleles, with an average of 2.5 alleles per marker. Using the DARwin 6.0 programme, the inbred lines were grouped into different clusters. The cluster A was solitary, with the inbred line VL171488-2 having a resistant reaction against leaf blight. This line can be used in crossing programmes with divergent parents to develop leaf blight resistant hybrids. The results of this study can be used to design efficient breeding programmes for resistance to leaf blight.

Keywords

Dendrogram, Jaccard's similarity coefficient, maize, SSRs, TLB

Introduction

Maize is an important cereal crop in the world for feed, food, fodder and raw materials of industries (1). Maize, coupled with rice and wheat, supplies at least 30% of the calories consumed by approximately 4.5 billion people in 94 developing countries (2). In India, it is cultivated over an area of 9.89 m ha with a production and productivity of 31.60 million tonnes and 3199 kg ha^{-1} respectively. In Andhra Pradesh, maize is cultivated in an area of 0.3 m ha, with a production and productivity of 1.78 million tonnes and 5917 kg ha^{-1} respectively (3).

The turcicum leaf blight, commonly known as northern leaf blight, is a foliar disease of maize caused by the ascomycetes fungus, *Setosphaeria turcica* and its conidial stage *Exserohilum turcicum* (Pass.) Leonard and Suggs. (syn. *Helminthosporium turcicum* Pass) affects the photosynthesis and reduces kernel yield to an extent of 28 to 91% (4, 5). The disease is characteried by long elliptical, greyish green or brown leaf lesions which emerge first on the lower leaves and gradually extend throughout the foliage. If the disease starts at an early stage, it causes premature death of blighted leaves. As a result, the crop loses their nutritive value as fodder, have reduced germination capacity, vigor, grain yield, total sugar content,

restricted starch formation, chaffy kernels and infected plants are liable to infection with stalk rots. Several disease management options have been recommended to reduce the impact of maize foliar diseases. Among these practices, planting of resistant cultivars can effectively reduce the rate of disease development and is widely recommended. Breeding for resistance is a practical, cost-effective means to manage the disease (6). The development of resistance against turcicum leaf blight will have large effect on the maize crop improvement programmes.

Table 1. List of parental lines used in the present study

S. No.	Entry	Source of origin	S. No.	Entry	Source of origin
Lines			**Testers**		
1	VL171488-2	ARS, Peddapuram	12	BML6	MRC, Rajendranagar
2	VL18828	ARS, Peddapuram	13	BML7	MRC, Rajendranagar
3	VL19978-6	ARS, Peddapuram	14	LM13	PAU, Ludhiana
4	VL19705-8	ARS, Peddapuram	15	LM14	PAU, Ludhiana
5	VL19255	ARS, Peddapuram	**Checks**		
6	VL18142	ARS, Peddapuram			
7	CAL1733-13	ARS, Peddapuram	16	P3396	Pioneer
8	VL175869-14	ARS, Peddapuram	17	DKC8171	Monsanto Bayer
9	SNL19564-20	ARS, Peddapuram	18	P3546	Pioneer
10	SNL19582-22	ARS, Peddapuram	19	DKC9120	Monsanto Bayer
11	SNL19588-23	ARS, Peddapuram	20	PAC751	Adventa

Genetic diversity using molecular markers provides the potential to examine changes in crop diversity over time and place. Among the several PCR-based markers, microsatellite markers based on Simple Sequence Repeats (SSRs) are widely exploited due to their ease of use, reproducibility, rapid analysis, low cost, simple scoring patterns and greater allelic diversity (1). “In the present era, DNA-based markers (molecular markers) are frequently used for genetic diversity and grouping of the populations. Among the various high throughput DNA-based marker techniques available, single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs) are the markers of choice because they are co-dominant in nature, locus-specific, reproducible, highly informative and easy to use” (1). Moreover, SSR markers are more informative than biallelic SNP markers because they can detect multiple alleles per locus. It was reported that SSRs are 7 to 11 times more accurate than SNPs (7). “The SSR markers will allow detection of polymorphisms at the DNA level which will facilitate the separation of inbreds at DNA level by the estimates of genetic distance” (8).

Materials and Methods

Field Evaluation

A total of 15 parental lines along with 5 checks were screened against TLB under artificial epiphytotics using sorghum grain inoculation technique at Agricultural college farm, Bapatla, Andhra Pradesh, situated at $15^0$55' North latitude and 80^0 30' East longitude and an altitude of 5.49 m above mean sea level. Each entry is planted in one row of 1.6 meters length, adopting a spacing of 60 x 20 cm for screening against *Exserohilum turcicum*. The block was flanked by 3 border rows of susceptible cultivar, P3396. List of entries used in the present investigation are presented in Table 1.

Turcicum leaf blight severity was recorded on five plants in each entry at the time of tasseling, twenty days after tasseling and at maturity using 1-9 disease rating scale (9, 10) and is presented in Table 2.

Molecular Evaluation

DNA Isolation

DNA was extracted from the leaf tissue collected at 15 days after sowing from all the genotypes using the method described by (11, 12) with certain modifications.

Principle

The prerequisite for DNA isolation is to extract DNA from the cells. Addition of the extraction buffer containing CTAB assists breakage of nuclear membrane and cell lysis. This results in the dispersion of all the cell components in to the buffer. Tris HCl assures that the pH of the solution is maintained around 8.0. EDTA forms complexes (chelates) with the several metal ions such as magnesium and calcium which are required as cofactors by the majority of DNases. NaCl being a salt decreases the solubility of DNA in the buffer solution and also increases the osmotonicity of the buffer to facilitate the process of cell lysis. Phenol: chloroform: Isoamyl alcohol (25:24:1) removes proteins by denaturing them and aggregate in the intermittent phase along with cell debris. For DNA precipitation isopropanol is used. RNA is removed by the RNase treatment. After precipitation the pellet is washed with 70% ethanol for removing any salts retained after precipitation. The DNA is preferably dissolved in a base solution like Tris EDTA rather than in sterile distilled water since Tris solution provides buffering for the DNA and EDTA in the buffer ensures that the DNA is stable by inactivating DNases.

Simple Sequence Repeats (SSRs)

A total of 26 primers was used to access the genetic diversity in 15 parental lines along with 5 checks. The primer information for the selected SSR markers was retrieved from the maize GDB database and is available in public domain (13) and summary of the SSR primers used in the present study are given in Table 3.

Table 2. Disease scoring scale (1-9) for turcicum leaf blight

Rating scale	Degree of infection (per cent DLA*)	PDI**	Disease reaction
1.0	Nil to very slight infection (≤10%).	≤11.11	Resistant (R) Score: ≤ 3.0 DLA : ≤ 30% PDI: ≤ 33.33
2.0	Slight infection, a few lesions scattered on two lower leaves (10.1-20%).	22.22	
3.0	Light infection, moderate number of lesions scattered on four lower leaves (20.1-30%).	33.33	
4.0	Light infection, moderate number of lesions scattered on lower leaves, a few lesions scattered on middle leaves below the ear (30.1-40%).	44.44	Moderately resistant (MR) Score: 3.1–5.0 DLA : 30 –50% PDI: 33.34-55.55
5.0	Moderate infection, abundant number of lesions scattered on lower leaves, moderate number of lesions scattered on middle leaves below the ear (40.1-50%).	55.55	
6.0	Heavy infection, abundant number of lesions scattered on lower leaves, moderate infection on middle leaves and a few lesions on two leaves above the ear (50.1-60%)	66.66	Moderately susceptible (MS) Score: 5.1-7.0 DLA : 50.1 – 70% PDI: 55.56-77.77
7.0	Heavy infection, abundant number of lesions scattered on lower and middle leaves and moderate number of lesions on two to four leaves above the ear (60.1-70%).	77.77	
8.0	Very heavy infection, lesions abundant scattered on lower and middle leaves and spreading up to the flag leaf (70.1-80%).	88.88	Susceptible (S) Score: >7.0 DLA : >70% PDI: >77.77
9.0	Very heavy infection, lesions abundant scattered on almost all the leaves, plant prematurely dried and killed (>80%).	99.99	

Table 3. List of SSR primers used for PCR amplification in the present investigation

The below SSR primers are selected based on the earlier reports of (1) (25)

S. No	Primer	F/R	Sequence	T^m
1	bnlg198	F	GTTTGGTCTTGCTGAAAAATAAAA	50.9
		R	GCTGGAGGCCTACATTATTATCTC	54.8
2	bnlg1335	F	GAAGGTTGCTCTTCCACTGG	55.8
		R	TGGTTTGTGCAAGTGTCACC	56.1
3	umc2210	F	GATGCTACCATTTCAGTGAGCGAT	57.2
		R	AGCGGGTCGATCTTTCTCTTAGTT	57.7
4	umc1665	F	CAATCAGGAGCCAGGGAGATG	57.5
		R	CTTAAACTTGTCGAGACGGTCCTG	57.3
5	umc1029	F	AACACCTGCTGGATATGGATCACT	58.0
		R	GGAAGAAAAATGTCGACCTGCTC	56.2
6	bnlg1666	F	GCTGGTAGCTTTCAGATGGC	56.2
		R	TGTCCCTCCTCCAGTTTCAC	56.5
7	bnlg240	F	AAGAACAGAAGGCATTGATACATAA	52.6
		R	TGCAGGTGTATGGGCAGCTA	58.8
8	umc1728	F	AGTACTTTCAGGCAGGGACCTTCT	59.6
		R	AACGCACTTCTTGTAGCTGTAGGG	58.9
9	phi056	F	ACTTGCTTGCCTGCCGTTAC	58.5
		R	CGCACACCACTTCCCAGAA	57.9
10	umc1293	F	GTATCCGTTTCTCATGCAACACAC	56.4
		R	GATCTCGATCTGCTTCATCATCTG	55.1
11	umc1811	F	AGATAGCCGCCGAGACCAAG	55.1
		R	ACTCACTCGACGGACTTCTCGAC	53.5
12	umc1144	F	ATGGCCCACTCATCATATCTCTGT	56.0
		R	TGTGTTGATTAGCAGCGGATAAAA	57.8
13	umc1551	F	CACCGGAACACCTTCTTACAGTTT	63.4
		R	CGAAACCTTCTCGTGATGAGC	65.1
14	umc2077	F	AAACTCACTGAACATGATCCTGGC	54.8
		R	CTGGTTCGGATGCAAGTAGTCAG	56.6

15	umc1086	F	CATGAAAGTTTTCCTGTGCAGATT	65.0
		R	GGGCAACTTTAGAGGTCGATTTATT	67.7
16	umc1559	F	CTTGCTAGAGTCGGTGAACAACAA	56.1
		R	AACCAAGCTCCTTAATGAGGTCAC	56.9
17	umc1644	F	CCATAAACTGTTCCTTTGGCACAC	58.3
		R	CTTTCACGTGTTAAGGGAGACACC	56.2
18	umc2169	F	ACTACTCCTCGGATAGCCACG	53.7
		R	GACGAGTAGAGGCTCTGGGAC	56.0
19	bnlg2086	F	CGGAACCTGCTGCAGTTAAT	55.9
		R	GAGATGCAGGAATGGGAAAA	53.7
20	phi330507	F	GTAAAGTACGATGCGCCTCCC	58.0
		R	CGGGGTAGAGGAGAGTTGTG	56.9
21	umc1568	F	AAGTCCAGCCAAGTTCATCAAAGA	56.8
		R	ACTGTAACTAAACTGGGTGTGCCC	59.0
22	nc003	F	ACCCTTGCCTTTACTGAAACACAACAGG	61.0
		R	GCACACCGTGTGGCTGGTTC	61.8
23	umc2214	F	ACCCCCTGATTCTCTCTTACGTTT	57.6
		R	CTGGATGAGGAGGAAGAATACGAG	56.3
24	phi085	F	AGCAGAACGGCAAGGGCTACT	61.3
		R	TTTGGCACACCACGACGA	57.5
25	bnlg238	F	CTTATTGCTTTCGTCATACACACACATTCAT	57.7
		R	GAGCATGAGCTTGCATATTTCTTGTGG	58.5
26	phi054	F	AGAAAAGAGAGTGTGCAATTGTGATAGAG	60.8
		R	AATGGGTGCCTCGCACCAAG	56.9

F – forward primer , **R**- Reverse primer, **T^m** – Annealing temperature

Genomic DNA amplification in PCR using SSR markers

The polymerase chain reaction (PCR) was carried out using a programmable thermocycler (prima 96 ™, HIMEDIA). Amplifications were performed in 10 µl reaction mixture having the following components 3.00 µl of DNA template (50 ng/µl); 1.50 µl of Taq buffer (10X); 0.35 µl of DNTPs (2.5 mM); 0.75 µl of forward and reverse primer each; 0.25 µl of Taq polymerase (5 U/µl); 7.40 µl of Autoclaved double distilled H_2O. Amplification was performed in a Himedia Thermal Cycler programmed as mentioned below

Agarose gel electrophoresis for resolution of SSR markers

Amplified products thus obtained were separated on 3% agarose gel using horizontal gel electrophoresis assembly at 80-100 volt for 2 h in 1X TAE buffer. The DNA fragments were then visualized under UV-transilluminator anddocumented using gel documentation system (SYNGENE Gene flash, U.K.)

Analysis of SSR data

Scoring of data

Data were scored for computer analysis on the basis of the presence or absence of the PCR products. If a product was present in a genotype, it was designated as '1' and if absent it was designated as '0'. The data were entered into MS-Excel data sheet. The data were maintained in the data sheet format for further analysis. The Microsoft Excel 2013 (Microsoft, USA) sheet was used for further statistical analysis.

Cluster analysis

The genetic associations between the genotypes were calculated by using the Jaccard's similarity coefficients for pair wise comparisons based on the proportions of shared bands produced by the primers (14). Similarity matrix was generated using DARwin software 6.0 version (15). The similarity coefficients were used for cluster analysis and dendrogram was constructed by Unweighted Pair-Group Method with Arithmetic Average (UPGMA).

Determination of Polymorphism Information Content (PIC)

The Polymorphism Information Content (PIC) value for each locus was calculated on the basis of allele frequency by the given formula (16).

$$PIC=1-\sum_{f=1}^{n}(P_{ij})^2$$

Where, P_{ij} is the frequency of the j^{th} band for marker i, and summation extends over n^{th} band.

Results and discussion

Field Screening

Among the inbreds, six lines *viz*., VL171488-2, VL18828, VL19705-8, VL18142, VL175869-14, SNL19582-22 and one tester, LM13 were categorized as resistant with disease score ≤ 3.0. Three lines *viz*., VL19978-6, VL19255, SNL19588-23 and one tester, LM14 were categorized as moderately resistant lines. One line, CAL1733-13 and two male testers, BML6 and BML7 were categorized as moderately susceptible lines. Only one line, SNL19564-20 with disease score 7.5 was categorized as susceptible to disease TLB. Disease reaction of entries were presented in Table 4.

Table 4. Disease reaction of 15 parental lines and 5 checks

S. No.	Entries	Disease score	PDI	Disease Reaction	S. No.	Entries	Disease score	PDI	Disease Reaction
	Lines					**Testers**			
1	VL171488-2	3	6.7	R	12	BML6	5	11.1	MR
2	VL18828	3	6.7	R	13	BML7	4	8.9	MR
3	VL19978-6	4	8.9	MR	14	LM13	3	6.7	R
4	VL19705-8	3	6.7	R	15	LM14	4	8.9	MR
5	VL19255	3.5	7.8	MR					
6	VL18142	2	4.4	R		**Checks**			
7	CAL1733-13	6	13.3	MS	16	P3396	6	13.3	MS
8	VL175869-14	2	4.4	R	17	DKC8171	6	13.3	MR
9	SNL19564-20	5	11.1	MR	18	P3546	3	6.7	R
10	SNL19582-22	2.5	5.6	R	19	DKC9120	4	8.9	MR
11	SNL19588-23	4	8.9	MR	20	PAC751	4	8.9	MR

SSR Polymorphism

To know the extent of genetic diversity among 20 genotypes, DNA profiling of these genotypes was generated by using SSR markers which were reported to be associated with TLB resistance in maize. Out of 26 SSR primers, only seven primer pairs showed clear polymorphism with PIC value of 0.60 indicating their usefulness. Amplification pattern of these primers are presented in Fig 1. The resuts obtained by using these seven polymorphic primers are presented in Table 4. The markers produced one to four alleles and the average of alleles per marker was 2.5. The polymorphism information content (PIC) value of the SSR loci ranged from 0.61 to 0.71. PIC was highest for the SSR primer bnlg1335 (0.71) followed by umc1029 (0.68) and was lowest for the primer bnlg1666 (0.61). The overall mean of PIC was 0.65 (Table 4). The higher the PIC value, the more informative is the SSR marker. Hence, primers with PIC value more than 0.6 were found to be highly informative. The similar results were obtained earlier (17) having PIC range from 0.58 to 0.81 with mean of 0.71; (18) with mean PIC of 0.54; (19) having PIC range from 0.32 to 0.85 with mean of 0.68 and (1) having PIC range 0.04 to 0.86.

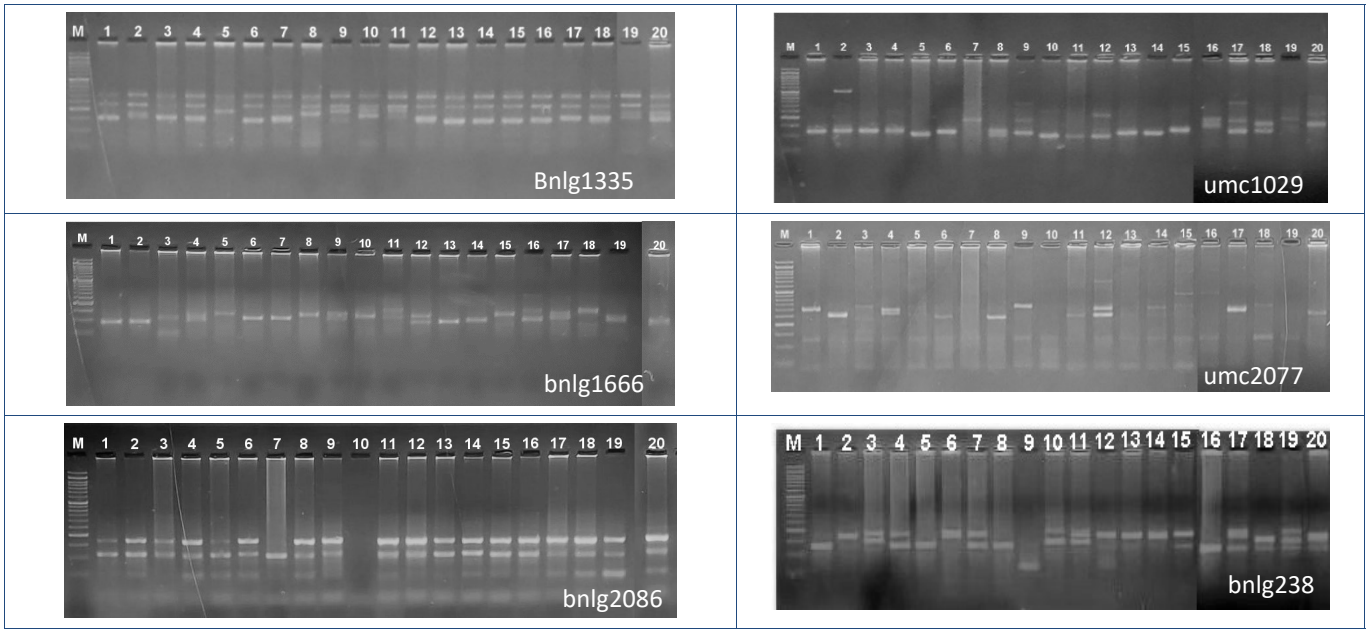

Fig. 1. SSR marker profiles of 15 parental inbreds along with 5 checks generated by various primers.

Similarity Index

The binary data from the polymorphic primers were used for computing Jaccard's similarity indices. The similarity index values obtained for each pair wise comparison among the 20 genotypes. The overall range of similarity among the 20 genotypes was found to be 0.17 to 0.78. Among the 20 genotypes the highest similarity index (0.78) was observed between BML7 and LM14 followed by (0.77) between LM13 and BML7 indicating that they are genetically less divergent (or) more similar as compared to other genotypes. The lowest similarity index (0.17) was observed between SNL19582-22 and VL171488-2 indicating that they are more divergent (or) less similar.

Similar results were obtained earlier (20) found genetic similarities ranged from 0.27 to 0.99 and (21) found similarity matrix value ranges from 0.17 to 0.84.

Cluster Analysis

The similarity coefficients during the present investigation were used as input data for UPGMA based clustering method. Twenty inbreds were grouped into 2 major clusters (A and B). Cluster A (VL171488-2) was solitary and cluster B was subdivided into ten subclusters at 0.52 Jaccards similarity coefficient (Fig. 2). Cluster A consists of single inbred.

These results were in accordance with earlier work categorization of genotypes into 3 distinct clusters (22); noted 3 distinct clusters (23); categorization of the genotypes into 4 clusters (16) and categorization of genotypes into 3 clusters based on UPGMA dendrogram (24).

TLB Resistance and SSR markers

The field screening of these genotypes identified very good variability for resistance indicating the importance of these genotypes in the breeding programmes. The inbred lines, VL171488-2, VL18828, VL19705-8, VL18142, VL175869-14, SNL19582-22 and LM13 recorded resistance reaction to the pathogen. Most of the reports indicated the involvement of more number of genes and the polygenic nature of inheritance of this trait. When these genotypes were tested for polymorphism using SSR markers, they showed very good variation in the banding pattern indicating their usefulness in differentiating the genotypes.

All the resistant genotypes produced a band of 120bp for the marker, bnlg 1335 and 160bp for the marker, bnlg 2086 except in the genotype, VL174488-2 indicating these sequences may not be involved in the resistance to TLB in maize. The primer pair, umc1029, produced a specific band of 80bp in the genotype, VL18828 and a 200bp

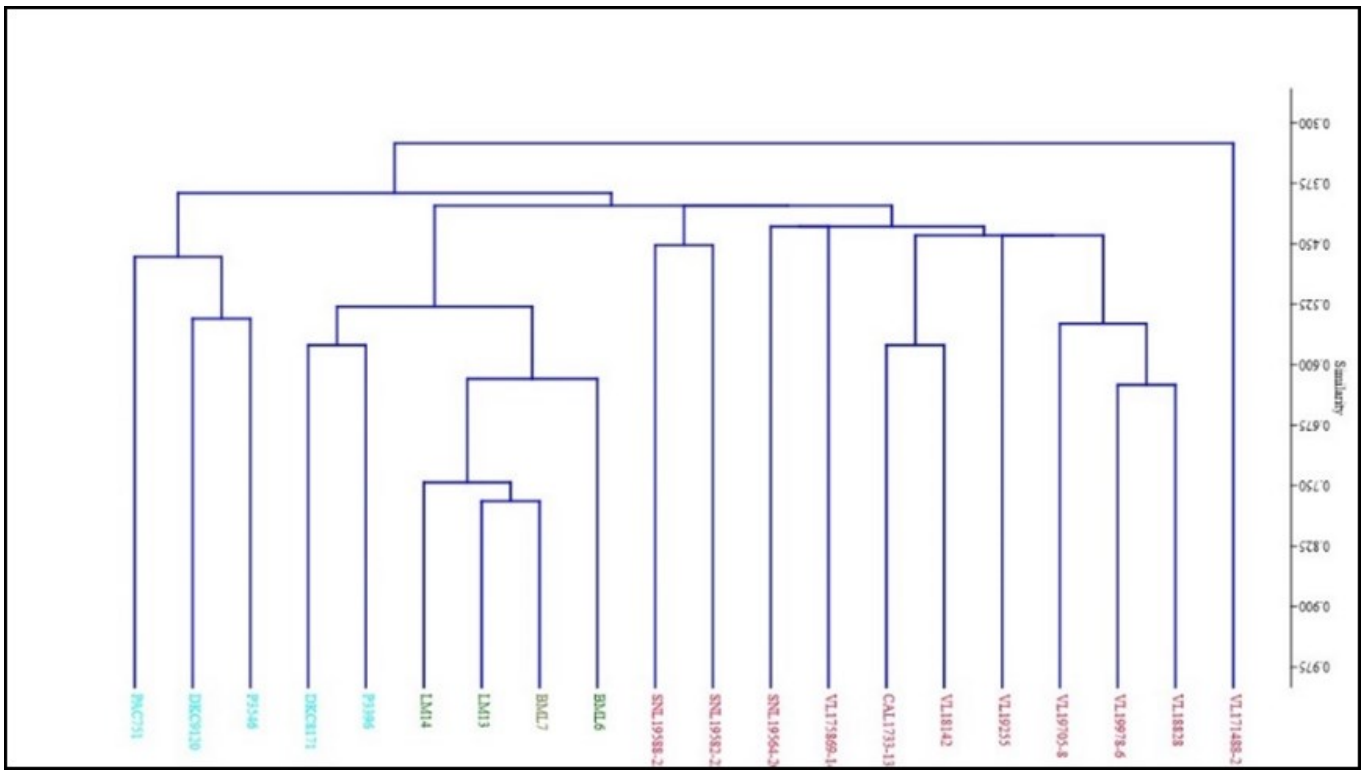

Fig. 2. UPGMA dendrogram generated based on Jaccards similarity index using SSR markers showing variability among 15 parental inbreds in maize (*Zea mays* L.).

The subcluster I of cluster B consists of 3 inbreds *viz*., VL18828, VL19978-6, VL19705-8; Subcluster II consists of single genotype VL19255; Subcluster III consists of 2 inbreds *viz*., VL18142, CAL1733-13; Subcluster IV, V, VI and VII were solitary clusters. VIII subcluster was the largest having 6 genotypes of 4 testers BML6, BML7, LM13, LM14 and 2 checks P3396 and DKC8171; IX subcluster had 2 genotypes P3546 and DKC9120 and the subcluster X had the single genotype *i.e* PAC751.

band in the genotype, P3546. The markers, bnlg238 and bnlg1666, produced 170bp and 60bp bands only in the inbreds, VL18142 and SNL19582-22 and VL19705-8 & VL175869 respectively, indicating these markers are useful for the identification of these genotypes from others and the new sequences present in these genotypes may be associated with the TLB resistance.

Thus, the present study identified lot of variability in the expression of the reported markers associated with

TLB resistance in resistant genotypes indicating the quantitative inheritance and involvement of a greater number of alleles. Further, there is a need to use a greater number of TLB associated markers to get the clear picture of resistance in maize.

Conclusion

In the present study 26 SSR markers were used for assessment of molecular diversity in 15 parental inbred lines along with 5 checks. Out of these primers seven primers *viz*., bnlg1335, bnlg1666, bnlg2086, bnlg238, Phi054, umc1029 and umc2077 recorded PIC value more than 0.6; These markers also noted lot of variation in the resistant genotypes indicating their usefulness. Further these markers also produced varied expression of bands in the resistant genotypes indicating the polygenic nature of the trait. Further, these resistant sources can be exploited in developing a novel maize genotypes with desirable yield and TLB resistance.

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Authors contributions

DK carried out the molecular genetic studies and drafted the manuscript. TH supervised the study and participated in design and coordination. ISK participated in gathering of primers for molecular analysis and sufficient guidance throughout the research work. DR performed the statistical analysis and necessary suggestions. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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