Analysis of Genetic Diversity in Maize (*Zea mays* L.) Variety by Using SSR Markers

ABSTRACT

Thepresent study was carried out to analyze the genetic diversity of five maize varieties at K. K. Waghcollage of agricultural Biotechnology, Nashik during May to October 2022 Genomic DNA was isolated from maize varieties by using CTAB method. The PCR amplification obtains with set of five SSR primers.Thedendrogram was constructed based UPGMA method from the jaccard's similarity coefficient and the varieties subjected to cluster analysis. Among the five SSR primer used only three found polymorphic with total 13 alleles. The PIC value 0.358 to 0.868of Phi-085, ssr-08 and ssr-06 shows polymorphism 50%, 80% and 83.34% respectively. The cluster analysis indicates the varieties were divided into 3 clades and one Simplicifolicus. Interpreting the results P. madhu is divers among the all varieties. The cluster analysis indicates the P. madhu variety shows the diversity and useful for different programs

Key Words: *Maize, DNA, RNase, SSR Marker, Genetic diversity, UPGMA.*

1. INTRODUCTION

Maize (*Zea mays* L.) is an annual crop of the grass family that includes crops such as wheat, rye, barley, rice, sorghum and sugarcane. Corn (*Zea mays* L.) is her third most important grain in the world after wheat and rice and is known as the "Queen of Cereals". It is of global importance as food for humans, animal feed, and numerous other industrial products such as glucose, starch and oil. It is an important food crop and the primary source of dietary energy and protein for most area in the world. Maize was domesticated in Mexico and from there it was introduced to other parts of the world with different agroclimatic conditions. Corn is mainly used as feed (60%), followed by food (24%), industrial products (starch) (14%), beverages and seeds (1% each). Corn is considered the third most important food crop in India[12]. first place is rice, second place is wheat. Total corn production is about 31.51 tons. It accounts for 9% of the national production. Corn can be regarded as a basic raw material and raw material for industrial products such as proteins, alcoholic beverages, food sweeteners, pharmaceuticals, cosmetics, films, fibers, rubber, packaging, paper, starch, and oils. It is also used as an important fodder for animals. Corn can be grown all year round in almost all states of India. Cultivation purposes may differ from use as grain, fodder, vegetables, sweet corn, baby corn and popcorn. The largest corn production in India is from Karnataka 16.45%, Madhya Pradesh 11.37%, Maharashtra 10.91%. Tamil nadu 8.63%, West Bengal 7.76%, Rajsthan 7.20%, Bihar 7.06%, Andhra Pradesh 6.19%, Uttar Pradesh 5.72%, Telengana5.55% and other state 13.15% contribute [15].

Maize is a large, pronounced C4 annual crop that varies in height from 4 meters and produces large, narrow, opposite leaves that alternate along the length of a stiff stem. Maize (*Zea mays* L.) is a tall, deep-rooted, warm weather annual grass. A single long stalk will develop from seed. Long smooth leaves are attached at the stem nodes. Seed producing shoots originate from the base of the main stem.Corn can grow well in a variety of soils, from loamy sand to clay loam. However, soils with high organic matter content, high water holding capacity and neutral pH are considered suitable for high productivity. As a plant sensitive to water stress, especially excessive soil moisture and salinity stress; poorly drained lowlands and high salinity fields should be avoided. Therefore, it is necessary to choose a well-drained field for cultivating corn[4].

Comment [D1]: Provide source

Comment [D2]: Provide source.

Comment [D3]: Check the numbering of the references well

Comment [D4]: Kindly correct all the references in the text.

Genetic diversity analyze the SSR is PCR-based and co-dominant in genetic and polyallelic marker systems. Several studies have extensively used molecular markers to assess the genetic relatedness of maize varieties. Many mapped SSR markers are available in maize that can be used for molecular characterization.Microsatellites are generally identified by (1) screening of small insert or microsatellite-enriched genomic libraries by hybridization with oligonucleotide primers followed by sequencing, and/or (2) searching DNA sequence databases[2]
In this study, we performed DNA fingerprinting of maize varieties using SSR markers to identify these cultivars and to authenticate the genetic purity of different seed lots for developing specific varieties. We characterized a DNA fingerprinting database for further use. Objective: Determination of genetic diversity among five different maize cultivars (*Zea mays* L.) by SSR markers.

2. Material and Methods

2.1 Plant material collected:

Table No. 1: List of Maize varieties

Sr. No.	Maize varieties	Characteristics
1	PhuleRajshri	Suitable for Kharif& Rabi season and high starch content (72.25 %)
2	PhuleMadhu	Medium maturity with sweetness (Brix %): 14.89%
3	PhuleMaharshi	Suitable for Kharif& Rabi season with Medium maturity.
4	PhuleManjri	Suitable for grain as well as fodder purpose.
5	Rasi-3499	Medium late maturity, Uniform cobs and excellent tip filling

The plant material used in this study was collected from M. P.K.V., Rahuri.

2.2 DNA Isolation

The extraction of plant DNA was carried out by CTAB (Cetyl-Tri Methyl Ammonium Bromide) method as described by Doyle and Doyle (1987) with minor modifications from sample of leaves from 5 varieties. . Fresh leaf samples (1g) ground to fine powder in liquid nitrogen, were transferred to 2 ml centrifuge tubes containing 2 ml pre-warmed CTAB extraction buffer (1M Tris-HCl, 0.5M EDTA, 2% CTAB, PVP 1%, 200µl β-mercaptoethanol, pH 8.0). Sample tubes were incubated in water bath at 65 °C for 1 hr with occasional swirling followed by centrifugation at 10,000 rpm for 10 min at 20 °C. Aqueous phase was pipetted out and Equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added to the tubes and mixed thoroughly again centrifugation at 10,000 rpm for 10 min at 20 °C. Aqueous phase was pipetted out and pre-chilled iso-propanol 0.6 volume of the content in tube was added followed by gentle inversion until fibrous mass was visible. Tubes were shifted at 4˚C for overnight. Tubes were then centrifuged at 5000 rpm for 10 minutes at 4 °C to obtain a precipitate. The supernatant was drained by gently inverting the tubes. The tubes were left inverted with lids open on blotting paper to drain the residual iso-propanol. After a while, the DNA pellet was washed twice with 70% ethanol, and kept overnight at room temperature for drying the pellet. 30 µl of TE buffer (pH 8.0) was added to dissolve the pellet. After 6-8 hrRNase (1 mg/ml) was added to the DNA of 2 µl/10 ul of crude DNA. Mixture was incubated in a water-bath for 40 min at 37˚C with intermittent mixing. DNA concentration is calculated by biospectrometer with the following formula:

$$\text{dsDNA concentration} = 50\mu g/ml \times OD_{260} \times \text{dilution factor}$$

Comment [D5]: Be consistent with the references.

2.2 Primers and PCR amplification

A set of five SSR primers used in the study (Table 2.) After the initial screening, five SSR markers amplified in experiment.

Table No. 2: SSR primer selected for study

Sr. no.	SSR primers	Nucleotide Sequences	Annealing Temperature (˚C)
1	umc-1061	F-AGCAGGAGTACCCATGAAAGTCC R-TATCACAGCACGAAGCGATAGATG	58
2	umc-1063	F-AGGCCACTGAGCAGGTGAAG R-GTGATGGTAGAGGAGTCCTTGGTG	57.3
3	phi-085	F-AGCAGAACGGCAAGGGCTACT R-TTTGGCACACCACGACGA	58.7
4	ssr-06	F-GATATGTCGAGCTTCGCTGGAG R-CGCACACTAAAGCATCCTTAACCT	55
5	ssr-08	F-CAGCCACAGTGAGGCACATC R-CAGAGACTCTCCATTATCCCTCCA	54.6

2.3 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed in a thermal cycler (Eppendorf, Hamburg, Germany) with thermal PCR reaction mix contained 50ng of DNA, 10X PCR Hi Buffer (100 mMTris-HCl, pH 9.1, 500 mM KCl,17.5 mM $MgCl_2$), 10 mMdNTP solution set (MolBio HIMEDIA), 5 pmol each of forward and reverse primer and 1.5 U/ µl of Taq DNA polymerase (MolBio HIMEDIA) in a reaction volume of 20 µl. Protocol for the PCR cycles initial denaturation, denaturation, annealing, extension, final extension and hold by repeatative cycles of 35(Table No.3).

Table No. 3 :PCR program and conditions

Steps	Reaction	Temperature	Time duration	Cycle
I	Initial denaturation	94 °C	4 min	1
II	Denaturation	94 °C	1 min	35
	Annealing	55-59 °C	1:30 min	
	Extension	72 °C	2 min	
III	Final extension	72 °C	7 min	1
	Hold	4 °C	∞	1

2.4 SSR data analysis

Scoring of the SSR alleles was manually performed sequentially from smallest to largest band with respect to band position relative to the ladder. Varieties showing two allelic bands of equal intensity were considered heterozygous for the locus. Polymorphism information content (PIC) values for each primer were estimated using the formula given by Goodman and Bird[13]. H. PIC = 1-, where Pij is the frequency of her jth allele of the ith primer and the sum is for the "n" pattern. Power Marker version 3.25 was also used to calculate mean number of alleles, genetic diversity, and polymorphic information content (PIC) values.[14] Based on the electrophoretic banding patterns of the 5 SSR markers, we estimated pairwise genetic distances between varieties and constructed a dendrogram using UPGMA clustering. Multivariate using classical methods was based performed using the computer software program in Paleontological Statistics Software Package for Education and Data Analysis PAST 4.0.

The molecular analysis depicts an array of characters which were converted into system (Sneath and Sokal 1973) based on presence of amplification of band for each allele. The presence of clear and distinct band was scored as giving '1' and '0' to absence of band corresponding among all 5 varieties. Similarity matrix was generated using the SIMQUAL function of PAST version 4.0. software. The Jaccard's similarity coefficients were used for cluster analysis and Phylogenetic tree or Dendrogram was constructed using the Unweighted Pair-Group method (UPGMA) by function of PAST software. The numberof alleles refers to the number of scored bands. The frequency of an allele was obtained by dividing the number of accessions where it was found by the total number of accessions. Finally, the data obtained from the SSR morphological characterization of maize varieties was pooled together to generate a combined dendrogram to get the overall picture of variation in the Maize varieties. [18]

3. Results and Discussion

In the present study, five maize varieties were screened with SSR markers.Among theFiveset of SSR marker 3 were polymorphic and 2 monomorphic. Primer Phi-085, umc-1061 and umc-1063 shows the amplification of 100 bp to 200bp, ssr-06 and ssr-08 shows the amplification of 200bp to 1000bp. The ssr-06 primer shows unique bands with amplicon size 200bp and 1000bp in P. Rajashri respectively. The total 21 bands were obtained in between 100 to 1000bp, among them 15 polymorphic and 6 monomorphic.(Plate No. 1) According to previous studies umc-1061 primer shows the amplification of 105 bp in size[25]. The selected ssr primer shows high level of polymorphism represent by their corresponding PIC value ranged from 0.358 (phi085) to 0.868 (ssr06) with an average PIC of 0.662 (Table No. 4).PIC value ranged from 0.421 and 0.88 was obtain with an average 0.58 is observed in some studies [25].

Table no. 4: %of polymorphism, heterozygosity and PIC value obtain using SSR locus

Primer name	Polymorphic band	Monomorphic band	% of polymorphism	Heterozygosity	PIC value
phi-085	1	1	50.00	0.47	0.36
ssr -06	10	2	83.34	0.88	0.87
umc-1061	0	1	00.00	0.00	0.00
ssr-08	4	1	80.00	0.79	0.76
umc-1063	0	1	00.00	0.00	0.00

Based on the dendogram tertiary data quoted from the gel picture of SSR marker with the five primer, we can observe the following things, the whole dendogram can be divided into 3 clades that are clade I (with two), clade II (with two), clade III (connecting with clade I and clade II) and one simplicifolicus. Interpreting the results clade I denotes the similarity between Rasi-3499 and P. Manjri shows similarity (0.75) within each other, clade II shows higher similarity (0.818) between P. Maharshi and P. Rajshri and clade III include the clade I and clade II show similarity clustering. (Table No.5)The more less similar in between P. Madhu and P. Rajshri shows 0.5.Simplicifolicus with variety PhuleMadhu having great variation among all variety. PhuleMadhu show higher genetic diversity among all 5 varieties.(Fig. No. 1)

Knowing the genetic diversity of maize is important for understanding the genetic makeup that helps breeders select the parents they need for their breeding programs. SSR markers are reported to be powerful tools for detecting genetic diversity in maize populations [19]. These markers can be applied for phylogenetic characterization at the molecular level. It

also helps maize breeders efficiently assign lines to heterogeneous groups and provide appropriate parent selection for the development of new hybrid varieties [20]. Genetic diversity testing is important for selecting individual varieties from closely related groups to initiate new breeding experiments. The PIC value average current study was 0.433.The P. madhu is more diverse than other varieties. Genotyping is one of the most reliable methods for establishing such phylogenetic relationships between sets of hybrids[21]. This parameter indicates the power of the SSR locus and its ability to detect differences between varieties based on genetic relatedness [22]. Primers ssr-06 and ssr-08 were found to be the most suitable for testing genetic diversity. Genetic distance measures the degree of relatedness between individuals within a particular population [23-24].

According to Bocianowski's paper, SSR markers that are important for quantitative traits are likely to be associated with QTLs that determined those traits. The microsatellite markers detected in period study should help advance our understanding of genetic diversity and other quantitative traits [1].The large extent genetic diversity is present in most of the species due tothe environmental conditions. Variety of molecular techniques using standard molecular markers is used to estimate this genetic diversity. Among these molecular markers, SSR molecular marker technique is most acceptable, codominant in nature, reproducible, highly polymorphic [6], which have specific primers, and is species specific [7]. Some studies suggested that these SSRs can be used for the estimation of genetic diversity in the improvement of maize [8-10]. Due to these properties SSR markers extensively used in various applications in crop improvement [11].

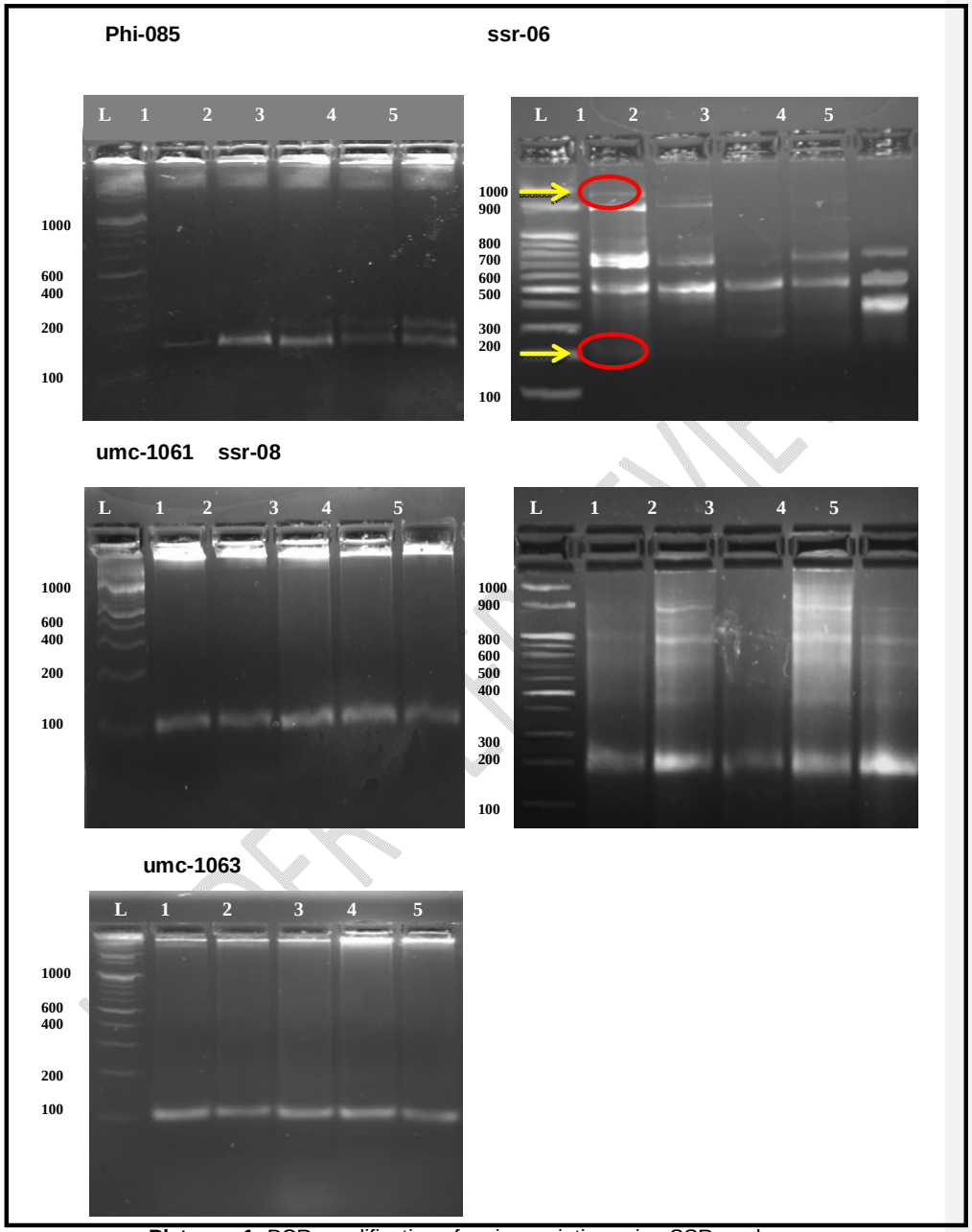

Plate no. 1 :PCR amplification of maize varieties using SSR markers

(L-100 bp ladder, 1-P.Rajashri, 2-P.Madhu, 3-P.Maharshi, 4-P.Manjri, 5- Rasi-3499)

Table No. 5: Similarity indice by jaccard'scoeficient

	P. Rajshri	P.Maharshi	P.Madhu	P.Manjri	Rasi 3499
P. Rajshri	1				
P.Maharshi	**0.818**	1			
P.Madhu	**0.5**	0.5	1		
P.Manjri	0.615	0.75	0.583	1	
Rasi 3499	**0.538**	0.666	0.636	0.75	1

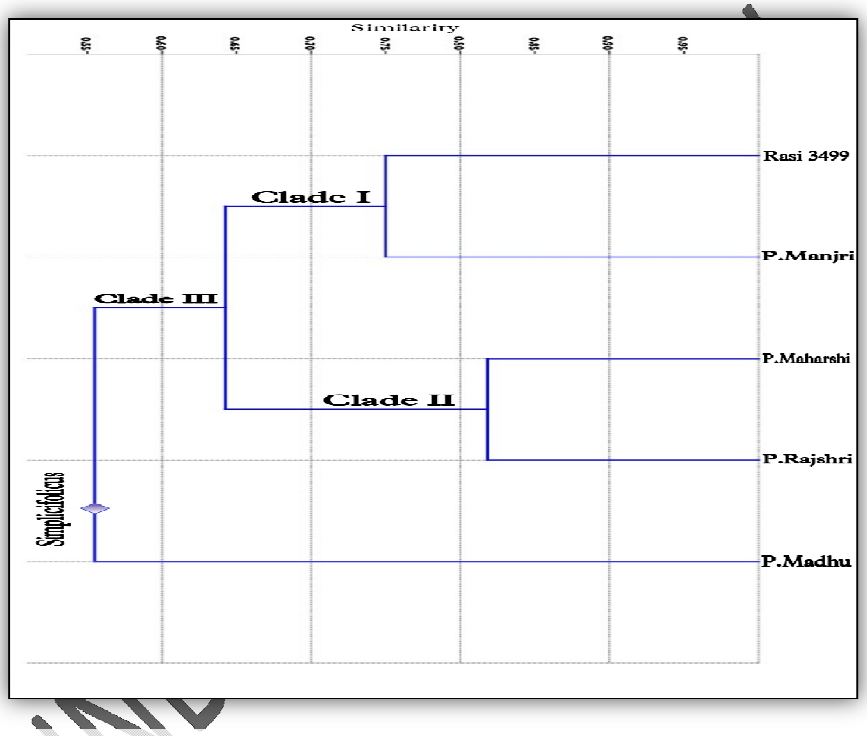

Fig.No. 1: Dendrogram generated using UPGMA analysis showing relationship among Maize varieties using SSR marker

4. Conclusions

This study determined the existence of molecular diversity and interrelationships in five maize genotypes. Polymorphic information content (PIC) values clearly indicated that ssr06 and ssr08 were the best SSR markers for hybrid identification. Hybrid P. Madhu was placed separately from other hybrids variety in the cluster analysis by SSR markers. SSR markers can be used to accurately characterize lines at the molecular level, to guide maize breeders in efficiently assigning lines to heterogeneous groups, and in selecting parents for developing new hybrids. At the same time, unique allelic patterns of individual genotypes can be distinguish by SSR marker.This application is becoming an important in plant variety protection [26].

Comment [D6]: This is not conclusion.

References

1. Bocianowski J, Nowosad K, Wróbel B, Szulc P. Identification of associations between SSR markers and quantitative traits of maize (Zea mays L.). Agronomy. 2021 Jan 19;11(1):182.
2. Carvalho, Bantte, K. and Prasanna B. M. (2002). Simple sequence repeat polymorphism in Quality Protein Maize (QPM) lines. Euphytica. 129(3):337-344.
3. Doyle, J. J. and Doyle J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19: 11-15.
4. Kumar B., Karjagi C.G., Jat S.L., Parihar C.M., YathishK.R.,Singh V., Hooda K.,Dass A., Mukri G., Sekhar J.C., Kumar R. and Kumar R., Maize biology : an introduction, Directorate of maize research-ICAR, Delhi, april,2012.
5. Gazal A, Dar ZA, Lone AA, Shikari AB, HabibM (2016) Genetic Diversity of Maize (*Zea mays* L.) Varieties Assessed by SSR Markers under Temperate Conditions.I.J. of plant research,Vegetos 29:3
6. De loose M. and Gheysen G., Identification methods based on molecular techniques, UPOV, (International Union for the Protection of New Varieties of Plants), Working group on biochemical and molecular techniques and DNA-Profiling in Particular, Wageningen, The Netherland (1995)
7. Pestova E., Ganal M. W. and Rodder M. S., Isolation and mapping of microsatellite markers specific for the D genome of bread wheat, Genome, 43(4), 689-697 (2000)
8. Liu K., Goodman M. J., Muse S., Smith J. S., Buckler E. and Deabley J., Genetic structure and diversity among inbred lines as inferred from DNA micro satellite, Genetica, 165(4), 2117-2128 (2003)
9. Legesse B. W., Myburg A. A., Pixley K. V. and Botha A. M., Genetic diversity of African maize inbred lines revealed by SSR markers, Hereditas, 144(1), 10-7 (2007)
10. Xia X. C., Reif J. C., Hoisington D. A., Melchinger A. E., Frisch M. and Warburton M. L., Genetic diversity among CIMMYT maize inbred lines investigated with SSR markers; I. Lowland Tropical Maize, Crop Sci., 44, 2230-2237 (2004)
11. Stachel M., Lelly T., Grausgruber H. and Vollman J., Application of microstallites in wheat (Triticumaestivum L.) for studying genetic differentiation caused by selection for adaptation and use, Theor. Appl. Genet100, 242-248 (2000)
12. Murray, M. G., and WF324241 Thompson. "Rapid isolation of high molecular weight plant DNA."Nucleic Acids Res. Spec. Publ 8.19 (1980): 4321-4326.
13. Singh T. P. and Prasad S. K. (1990). Genetic diversity under different environments in maize (*Zea mays* L.). Indian J. Gene. Plant Breeding. 50(4):407-411.
14. Bantte K, Prasanna BM (2003) Simple sequence repeat polymorphism in quality protein maize (QPM) line. Euphytica 129: 337-344
15. Liu, Kejun, and Spencer V. Muse. "PowerMarker: an integrated analysis environment for genetic marker analysis."J. Bioinform. 21.9 (2005): 2128-2129.
16. Valiyeva, L. S. Rahimova, G.K. Nabiyeva N.A. (2018). Application of SSR markers for study of genetic polymorphism of dark grain maize varieties Cur.Chall. Plant Genet.,Geno. Bioinfo. &Biotech.: 39–41.
17. Lalsanglur, Sharma A., Yadev R., Meitei S., Yadev M., Arora N., Dawar S. and Gaur D.K.,8th Agriculture statics at a glance.,2021.
18. Hammer, Øyvind, D. AT Harper, and Paul D. Ryan. "PAST: Paleontological statistics software package for education and data analysis." Palaeontol. Electron.4.1 (2001): 9.
19. Hoxha, S., M. R. Shariflou, and P. Sharp. "Evaluation of genetic diversity in Albanian maize using SSR markers." *Maydica* 49.2 (2004): 97-103..

20. Singh, S.B.; Gupta, B.B.; Singh, S.P. Genetic divergence in exotic inbreds of maize (*Zea mays* L.). Prog. Agric. 2007, 7, 1–4.
21. Govindaraj, M.; Vetriventhan, M.; Srinivasan, M. Importance of genetic diversity assessment in crop plants and its recent advances: An overview of its analytical perspectives. Genet. Res. Int. 2015, 431487.
22. Reyes-Valdés, M.H. Informativeness of Microsatellite Markers. In Microsatellites. Methods in Molecular Biology (Methods and Protocols); Kantartzi, S., Ed.; Humana Press: Totowa, NJ, USA, 2013; Volume 1006, pp. 259–270.
23. Garcia, A.A.F.; Benchimol, L.L.; Barbosa, A.M.M.; Geraldi, I.O.; Souza, C.L., Jr.; de Souza, A.P. Comparison of RAPD, RFLP, AFLP and SSR markers for diversity studies in tropical maize inbred lines. Genet. Mol. Biol. 2004, 27, 579–588.
24. Liersch, A.; Bocianowski, J.; Popławska, W.; Szała, L.; Sosnowska, K.; Cegielska-Taras, T.; Nowosad, K.; Matuszczak, M.; Bartkowiak-Broda, I. Creation of gene pools with amplified fragment length polymorphis markers for development of winter oilseed rape (*Brassica napus* L.) hybrid cultivars. Euphytica 2019, 215, 22
25. I. Afaf, Shehata,A. Haila, Al-Ghethar,A. Ali, Al-Homaidan, Application of simple sequence repeat (SSR) markers for molecular diversity and heterozygosity analysis in maize inbred lines, Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451(2009)
26. A. Kumari, S. Sinha, R. Kumari, S.S. Mandal and S. Sahay, Genetic diversity analysis in maize (*Zea mays* L.) using SSR markers, J. pharmacogn. phytochem.2018; SP1: 1116-1120