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MORPHO-MOLECULAR CHARACTERIZATION OF MAIZE INBRED LINES ACCELERATING PARENTAL SELECTION FOR HYBRIDIZATION

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ABSTRACT

Characterization of genetic diversity is the foundation step for crop improvement, which provides a basis for analyzing combining ability and heterosis of inbred genotypes during a hybridization program. An investigation was carried out at the field laboratory of the Genetics and Plant Breeding department in Bangabandhu Sheikh Mujibur Rahman Agricultural University, to elucidate the genetic architecture by evaluating 12 morphological and 4 molecular (SSR) markers within 52 diverse S1 genotypes, and to assess the relationship of molecular and morphological GD. An almost equal amount of PCV and GCV coupled with high heritability and genetic advance for the traits cob weight (gm), NKPC, and NKPR lead to the selection of promising genotypes based on these characters. Correlation coefficient and scatter plot matrix established a positive and strong relationship of KL (mm), KW (mm), and KT (mm) with 100 kernel weight (gm) suggesting the importance of kernel morphology. Mahalanobis D2 statistics revealed the highest inter-cluster distance between I and II. The percentages of molecular variance within the population and among the population were 76% and 14%, respectively. The optimum K-value was 5. Heatmap relying on molecular GD exposed MMIL-28, MMIL-54, and MMIL-96 as the most diverse lines. SHE analysis hypothesized the increase of richness and diversity over time. Less correlation between the divergence generated from morphological traits and molecular markers suggested that the morphological variation may be determined by environmental factors and also by genetic factors. A strategy for the effective selection of predicting parental lines for a future hybridization program was developed.

Keywords: Maize, Inbred lines, Genetic diversity, SSR markers, Population Structure

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INTRODUCTION

Hybrid maize has proven to be helpful to reduce food insecurity all over the world although, the generation of hybrid maize requires a series of fieldwork as maize is a cross-pollinated plant. Hybrids are produced by crossing between two or more unrelated inbred parents. Thus, generating genetically superior varieties which are better than their conventional parents. Hybrid maize generates high yields, better adaptability, increased value, and reduced production costs. In 1908, Shull reported that inbred lines of maize showed general deterioration in yield and vigor, but that hybrids between two inbreds completely recovered. In many cases, their yield exceeded that of the varieties from which the inbred were derived.

Generating typically distant inbred lines is the first prerequisite of hybrid maize development which takes about 75% of the effort in a corn breeding program conventionally. An inbred is genetically uniform for all traits and will always breed true to form. It has been a rich resource for fundamental and applied investigations in maize for studies in genetics and hybrid breeding. Moreover, if we could select the plants at the inbred level, i.e. if the inbreds on their own could predict the performance of the hybrid testcross, we could considerably reduce expenses



(Bittman and Kowalenko, 2004). Information on the genetic diversity and population structure of the base germplasm is crucial for better progress of selection tactics in any crop improvement program (Adu et al., 2019). Maize has enormous genetic diversity that offers incredible opportunities for genetic enhancement despite the environmental challenges as there is no lack of favorable alleles in the global maize germplasm that contribute to higher yield, abiotic stress tolerance, disease resistance, or nutritional quality improvement. However, these desirable alleles are often scattered over a wide array of landraces or populations (Prasanna, 2012). Thus, such huge maize genetic diversity needed to be characterized by morphological traits, pedigree analysis, heterosis data, biochemical and DNA molecular markers (Smith and Smith, 1989; Ranatunga et al., 2009). Analysis of genetic diversity and relationships among the elite breeding materials can significantly aid in crop improvement. Advances in genomic mapping with molecular markers have provided the means to characterize maize inbred at the DNA level with unprecedented power and resolution (Lee, 1990). A molecular marker is a piece of DNA that is closely associated with a gene (or genes) responsible for a certain trait. Molecular markers help to identify traits like maturity or height along the DNA trail. Using molecular markers, researchers can better predict which plants have desired beneficial traits. This saves time and resources because the first selection is made in the lab even before field trials begin. As a result, breeders begin field trials with an improved pool of candidate genotypes/hybrids that are more likely to succeed in customers' fields. According to a review of Maniruzzaman et al. (2018), in maize, microsatellites have proved to be a valuable tool for genome mapping (Taramino and Tingey, 1996). Microsatellites or simple sequence repeats (SSRs) are DNA markers with short stretches of tandem repeated di-, tri- or tetra-nucleotide motifs (Weber, 1990). They are codominant, highly reproducible, and polymorphic, multi-allelic, and have become the marker of choice for genetic analysis in crops (Gupta and Varshney, 2000). Global maize production is increasingly being challenged by diverse environmental stresses (Deinlein et al., 2014; Zuo et al., 2015) like heat, chilling injury, oxidative stress, drought, heavy metal accumulation, salinity, etc. Thus, we aim to explore some local and exotic lines of tropical maize to develop S1 inbred to elucidate the genetic architecture of those genotypes through morpho-molecular characterization and finally screen out the most diverse accession that can be further used in hybridization program to develop new hybrid genotypes.

LITERATURE REVIEW

Inbred lines have been a rich resource for fundamental and applied investigations in maize. They constitute a sampling of the genetic diversity in Zea mays L. that has been captured and partitioned into an array of uniform, reproducible genotypes (Lee, 1994). These are genotypes that are developed to be used as parents in the production of hybrid cultivars in the breeding of cross-pollinated species. The success of a crop breeding program relies on the choice of the best parents possessing complementary and desired traits. Thus, breeders continuously select potential parent populations from diverse sources including landraces, modern cultivars, obsolete or primitive cultivars, wild or semi-wild species (Robsa Shuro, 2017). Advances in genomic mapping with molecular markers have provided the means to characterize maize inbred at the DNA level with unprecedented power and resolution. Such information has become increasingly valuable for planning and conducting research (Lee, 1994).

The major objective of most of the maize breeding programs is to develop better yielding hybrids than the existing cultivars, so hybrid breeding remains the choice of the method considering its success over the years (Chancel and Guleria, 2019; Sreckov et al., 2010). Inbred lines are the prerequisite for hybrid variety development in crop plants. Genetic resources are the building blocks and also fundamental not only to a crop improvement program but also for the very survival of the species in time and space. Characterization of genetic diversity of maize germplasm is of great importance in hybrid maize breeding (Xia et al., 2005). Several methods

have been reported to decipher the pattern and magnitude of variabilities such as descriptive analysis comprising genetic variability, heritability and genetic advance, principal component analysis, biplot analysis, correlation analysis, D2, cluster analysis etc.

Genetic diversity is the total number of genetic characteristics in the genetic makeup of a species, it ranges widely from the number of species to differences within species and can be attributed to the span of survival for a species. In case of exploiting the potential of hybrid breeding in maize, maize inbred are developed from a limited number of elite lines and elite line synthetics, a practice that heightens the risk of decreased genetic diversity in commercial maize production fields (Hallauer et al., 1987). A better understanding of the genetic diversity ensures the breeder in planning crosses for hybrid and line development, in assigning lines to heterotic groups, and in plant variety protection (Pejic et al., 1998). Meanwhile, there have been frequent warnings about the genetic vulnerability of maize (Goodman, 1990). This made the maize breeders realize the need for both maintaining genetic diversity and improving the management of genetic resources (Goodman, 1994). The developments during the past few decades in DNA marker technology are enormous and an array of DNA markers is made available as a tool to assess the genetic diversity in plants and animals.

The genetic diversity between the genotypes is important as the genetically diverged parents can produce high heterotic effects (Ghaderi et al., 1979). Several studies on maize have shown that inbred lines from diverse stocks tend to be more productive than crosses of inbred lines from the same variety (Vasal, 1998). The manifestation of heterosis usually depends on the genetic divergence of the two parental varieties (Chandel and Guleria, 2019). Knowledge of germplasm diversity and the relationship among elite breeding materials has a significant impact on the improvement of crop plants. In maize, this information is useful in planning crosses for hybrid and line development, in assigning lines to heterotic groups, and in plant variety protection. Evaluation of genetic diversity is important to know the source of genes for a particular trait within the available germplasm (Tomooka et al., 2011). The importance of genetically diverse genotypes as a source of obtaining transgressive segregants with desirable combinations has been reported by several breeders (Peter and Rai, 1978). Genetic diversity can be assessed by common morphological traits or molecular markers. Molecular markers have become the study of choice for plant genetic diversity. The utility of SSRs for assigning lines to heterotic groups and relating the SSR-based genetic distance with hybrid yield or heterotic performance in maize has been explored by a few research groups in Asia (e.g., Xu et al., 2005; Mohammadi et al., 2002; Prasanna and Hoisington, 2003; Xie et al., 2008).

In conventional breeding programs, the genetic relationship and gene diversity among inbred lines or accessions are evaluated based on the pedigree of the inbred lines, morphophysiological characters, and the extent of heterosis expressed in the hybrid (Lasley et al., 1994; Vathana et al., 2019). An understanding of the genetic diversity and population structure among inbred lines would make a significant contribution to the development and release of new varieties because it is useful for allocating lines for heterotic groups, arranging crosses for inbred lines and hybrids (Zheng et al., 2013). Molecular markers make differences in the DNA sequence visible, which can be related to different phenotypes. So, in the Inbreeding programs, molecular markers are used to select traits at the DNA level. On the one hand, they facilitate the choice for the elite parental lines to be used in crossbreeding and, on the other hand, the decision on which offspring to continue breeding with or to choose for multiplication (i.e. Seed production) (Robsa Shuro, 2017).

RESEARCH METHODOLOGY

The research was conducted from July 2018 and ended in March 2020. The location of the research was in the field and molecular laboratory of Genetics and Plant Breeding, Bangabandhu Sheikh Mujibur Rahman Agricultural University.

To get desired promising maize inbred lines, controlled self-pollination was carried out and S1 seeds were developed by artificial inbreeding of the base materials (Table S1). During the 2nd generation, through selection and elimination based on the physiological development of the plants, 52 S1 lines were chosen for further investigation (Table S2). Data collection was set considering the parameters plant height (PH), No. of cobs (NC), cob length (cl), cob width (CW), cob weight (Cwt.), No. of rows per cob (NPRC), the number of kernel per row (NKPR), no. of kernel per cob (NKPC), kernel length (KL), kernel width (KW), kernel thickness (KT), and 100 kernel weight (HKW). The estimation of variance components, phenotypic and genotypic coefficients analysis, heritability and genetic advance estimation (Carena et al. 2010), correlation analysis (Saboor et al. 2018), path coefficient analysis (Wright, 1934), and (Dewey and Lu, 1959) was conducted using R 4.1.0 software. Scatter Plot Matrix of Kernel Related Traits with mini histograms was developed using statistical data analysis software in excel. Assessment of genotypic divergence along with cluster differentiation and a dendrogram was generated using Mahalanobis D2 statistic between populations (Mahalanobis,1936). DNA Extraction and SSR marker

DNA was extracted following the modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (Ferrari et al., 2007). After several successive centrifugations at projected rpm DNA pellets formed at the bottom of the tube were separated from the supernatants. The pellets were then rinsed with 70% ethanol and centrifuged for 5 min. at 12000 rpm. Finally, the pellets were suspended in 150µl 1X TE buffer and stored at -20 C. A spectrophotometer was used to quantify the extracted DNA, while the DNAs were run on 1% agarose gel to determine their quality. Four SSR markers were used for the molecular evaluation of 52 maize genotypes (Table S3). PCR was performed in 10µl reactions containing 3 µl DNA, 4 µl PCR Master Mix (TakaRa, Dalian, China) and 0.5 µl each of 10 µM forward and reverse primers. Initial denaturation at 94°C for 4 min for 1 cycle, followed by 35 cycles of template denaturation at 94°C for 1 min, primer annealing at 65°C for 50 sec. according to the used primer, primer extension at 72°C for 2 min. and the final extension at 72°C for 5 min. The PCR product was preserved for a short time at 4°C temperature in the thermal cycler in case of necessity (Table S4). Agarose gel was prepared by dissolving the 3.6g agarose powder in 240ml TAE buffer and 9µl Ethidium Bromide added for visualization. 2µl of 10X loading dye was added to each well containing 10µl PCR product and 3µl of the mixer was loaded in the wells of gel with the help of a pipette. DNA size marker like 1.5 kb DNA ladder was loaded for size determination. Image lab software was used to document the PCR mix separation. The gel was exposed to UV light and the gel image was saved as a TIFF file.

Analysis of molecular variance (AMOVA) was used to partition the variation among and within-group (population) components (Excoffier et al., 1992). Population structure was estimated by a Bayesian Marko Chain Monte Carlo model (MCMC) that was implemented in STRUCTURE v2.3.4 (Pritchard et al., 2000). Ten runs were performed for each K in which the value of K fell between 1 and 10. By using the Monte Carlo chain replicates of 100,000, the burn-in period for every run was 100,000 steps. The most probable K-value was determined by Structure Harvester (Earl and Vonholdt, 2012) by plotting the number of clusters (K) against delta K (Δ K). SHE analysis by Shannon-Wiener Diversity Index was employed to evaluate the diversity index to its formed components by using the formula H=Ln (S) + Ln (E), (Buzas and Hayek, 2005) with PAST software. Matrices of Roger's genetic distance (Rogers, 1972) were calculated between each pair of lines in the study using DARwin software 6.0 (Perrier and Jacquemoud-Collet, 2016). Heatmap Relying on pair-wise genetic distance was generated exploiting the software Heatmapper, according to the method described by Babicki et al., (2016). A dendrogram was constructed from the genetic distance matrix using the neighborjoining algorithm with DARwin software 6.0.



RESULTS AND DISCUSSION

Mean Performances of 52 Maize Inbred Lines Relying on Phenotypic Traits

The top five best-performing maize lines relying on twelve phenotypic traits are presented in Table S5. The frequency distribution graphs explained the number of genotypes that intended to fall in the same group (Figure 1). The histograms of mean values resulted in significant differences among the inbred lines for growth, yield, and yield-related traits due to the segregating behavior of genes during inbreeding. The height of most of the plants ranged from 185 cm to 225 cm, counted 30 genotypes. While considering cob-related traits, it was estimated that the number of cobs was 3 in almost 22 genotypes following 2 in 21 plants. Length of each cob (cm) was found highest in between 14 cm to 16 cm, counted 17 genotypes. About 11 genotypes were found with cob width (mm) ranging from 46 mm to 48 mm. Finally, cob weight (gm) averaged 125g to 145g for most of the genotypes specifying 10 genotypes in the population. 20 genotypes were found having kernel rows from 21 to 25, which was the highest in the population. 18 genotypes had 13 to 14 kernels per row and 12 cobs had the number of kernels per cob ranging 191 to 240 followed by 11 ears ranging 291 to 340 kernels. The average range of Kernel length (mm) was found 10 mm to 12 mm covering 38 genotypes, kernel width (mm) was 9 mm to 10 mm in 25 genotypes and kernel thickness was observed averaging 4.5 mm to 5 mm in 20 genotypes following 17 genotypes ranging 5 mm to 5.5 mm. Finally, hundred kernel weight, which was the strongest yield contributing factor in this study, was found averaging 30 gm to 35 gm in most of the genotypes, counted 22 genotypes. This report was relevant to the study of Jilo et al. (2018) who suggested a wide range of variability of traits such as cob length (cm), number of rows per cob, number of kernels per row, and 1000 seed weight.





Figure 1: Frequency distribution histograms of 52 maize inbred lines correspond to 12 yield interacting traits.

Estimation of Variability Parameter

Range of values obtained from 52 maize inbred lines for 12 traits, mean of the traits with standard error, genotypic (GV) and phenotypic (PV) variance, genotypic (GCV), and phenotypic coefficient of variation (PCV), genetic advance (GA), genetic advance as percent (GAM) of mean and broad-sense heritability (h2b) has been furnished in Table S6.

genotypic (GCV= coefficient of variance, PCV= Phenotypic coefficient of variance, GAM=Genetic advance over mean, h2b= heritability, PH= Plant Height (cm), NC= Cob number/plant, CL= Cob length (cm), CW= Cob width (mm), Cwt.= Cob weight (gm), NRPC= Number of rows per cob, NKPR= No. of Kernel per row, NKPC= Number of kernels per cob, HKW= 100 kernel weight (gm), TW=

Test Weight (gm/50ml), KL= Kernel Length(mm), KW= Kernel width (mm), KT= Kernel Thickness (mm))



Figure 2: Comparison of genetic parameters for 12 traits in 52 maize inbred lines.

Genotypic and phenotypic coefficients of variation

High GCV was observed for the number of cobs (33.94), cob weight (36.85), and the number of kernels per cob (39.60), and the number of rows per cob (25.28). On the contrary, moderate values were observed for 100 kernel weight (gm) (15.61), plant height (cm) (18.31), cob length

(cm) (15.29), number of kernels per row (19.11), and kernel width (12.05). PCV was observed more or less similar to GCV except for, number of cobs per plant (42.51), kernel length (11.57), kernel width (14.37), and kernel thickness (15.56) where it was higher than GCV. The percent genotypic and phenotypic coefficient of variation (GCV and PCV) for plant height (cm), cob length (mm), cob width (mm), cob weight (gm), number of rows per cob, number of kernels per cob, and 100 kernel weight (gm) was very close to each other (Figure 1) providing evidence that these parameters were under the control of additive gene effects, and characters were less influenced by the environment. PCV was found higher from the GCV of the characters' number of cobs (42.51>33.94), kernel length (mm) (11.57> 9.60), kernel width (mm) (14.67>12.05), and kernel thickness (mm) (15.57>7.18) (Table S5, Figure 2), showing the higher influence of environment and low genetic contribution. In a study on elite yellow maize inbred lines Ogunniyan and Olakojo, (2014) found high GCV and PCV for the number of cobs per plant and cob weight (gm), moderate for plant height (cm) which was supportive to our study. GCV and PCV were high in an experiment conducted by Ayodeji and Comfort, (2019). Therefore, selection based on phenotype alone can be effective for the improvement of the traits. These results were also agreed with Chandel and Guleria (2019). Phenotypically superior plants with the higher influence of environment and low genetical contribution may yield poor recombinant in segregation, thus selection may not be effective, as reported by Mustafa et al., (2015) and Jilo et al., (2018).

Heritability and genetic advance

Traits such as plant height (cm) (h2b= 99.95%), cob length (h2b=91.16%), cob width (mm) (h2b=97.16%), cob weight (gm) (h2b=99.98%), number of rows per cob (h2b= 98.82%), number of kernels per row (h2b= 93.55%), number of kernels per cob (h2b= 100%) and 100 kernel weight (gm) (h2b= 93.51%), exhibited high heritability accompanied with high to moderate, closely related genotypic and phenotypic coefficient of variation (Table S6). The estimates of high heritability (>70%) coupled with high genetic advance (>20%) were recorded (Figure 2) for plant height (cm) (h2b= 99.95%, GAM=37.72%), cob length (mm) (h2b= 91.43%, GAM= 30.11%), cob weight (gm) (h2b= 99.98%, GAM= 75.89%) number of rows per cob (h2b= 98.82%, GAM=51.76%), number of kernels per row (h2b= 93.55%, GAM= 38.08%), number of kernels per cob (h2b= 100, GAM=81.58%) and 100 kernel weight (gm) (h2b= 98.33%, GAM=31.88%) in, which exhibited good scope for improving these traits through phenotypic selection due to the additive gene action. However, cob width (mm) with high heritability but low genetic advance, number of cobs, kernel length (mm), kernel width, and kernel thickness (mm) with moderate heritability and low genetic advance have nonadditive gene action, thus, simple selection may not be rewarding. The maximum heritability was recorded for the number of kernels per cob (100%) and the minimum for the kernel thickness (21.25%) (Figure 2). Heritability estimates are of tremendous significance to the breeder, as their magnitude indicates the accuracy with which a genotype can be recognized by its phenotypic expression. High heritability accompanied with a closely related genotypic and phenotypic coefficient of variation found in this study refers that most likely the heritability is due to additive gene effects and for these traits, selection may be effective in early generations. The high heritability of those traits indicated that the influence of the environment on these characters is negligible or low. Therefore, selection can be effective based on phenotypic expression of these traits in the individual plant by implementing simple selection methods. High heritability was observed for plant height (cm), ear height, and 1000 seed weight by Ferdoush et al. (2017) and also for plant height (cm), cob height (cm), cob width (mm), number of kernels per cob and 1000 seed weight by Chandel and Guleria (2019). Besides, moderate heritability for ear length, kernel length (mm), kernel width (mm), and kernel thickness (mm) were found by Haydar et al., (2015). High heritability does not always indicate a high genetic gain; heritability should be used together with the genetic advance in predicting the ultimate

effect for selecting superior varieties. (Muchie and Fentie, 2016). Hybridization followed by selection is desirable and transgressive segregates would be the better option for improving these traits. Heritability for all the characters was high, indicating the low influence of the environment on the studied characters. Heritability estimates along with genetic advances are more helpful in predicting the gain under selection (Johnson et al., 1955). In the present study, high heritability coupled with high genetic advance was recorded for plant height (cm), cob length (cm), cob weight (gm), number of rows per cob, number of kernels per row, number of kernels per row cob and 100 kernel weight (gm), indicating that the heritability was due to additive gene effects and selection may be effective for these four traits. Comparing all the genetic parameters through the twelve consecutive traits, the number of kernels per cob, followed by cob weight (gm) was found to have the highest potentiality to be selected based on the phenotypic gene actions (Figure 2).

Genotypic and Phenotypic Correlation Co-Efficient Studies of Interrelated Traits in Maize Figure 2 showed the genotypic (above diagonal) and phenotypic (below diagonal) correlation co-efficient results of the study (Table S7). The current observation exhibited that number of kernels per cob significantly and positively correlated with cob weight (gm) (Figure 3) both genotypically (0.9109) and phenotypically (0.9109). This type of result was also found by (Al-Amin et al., 2019). Cob weight was also highly linked with cob width, the number of rows per cob, and kernel length in both cases, which was also reported by Ali et al.(2017) and Jatto (2015). A significantly negative association (rg, -0.8338; rp, -0.5381) (Table S6) was observed between the number of kernels per row with kernel width.

(PH= Plant Height (cm), NC= Cob number/plant, CL= Cob length (cm), CW= Cob width (mm), Cwt.= Cob weight (gm), NRPC= Number of rows per cob, NKPR= No. of Kernel per row, NKPC= Number of kernels per cob, HKW= 100 kernel weight (gm), KL= Kernel Length(mm), KW= Kernel width (mm), KT= Kernel Thickness (mm))

In both phenotypic and genotypic coefficient correlation evidence the

major yield contributing trait, hundred kernels weight was found to have a significant positive correlation with cob width, kernel length, kernel width, and kernel thickness, and high negative association with the number of rows per



Figure 3: Estimation of genotypic correlation coefficients (rg) (above diagonal) and phenotypic correlation coefficients (rp) (below diagonal) for yieldrelated traits of maize S1 Inbred.

cob, number of kernels per row and number of kernels per cob. Non-significant relation was found in plant height, the number of cobs, cob length, and cob weight with hundred kernel weight. Though kernel thickness showed mostly negative genotypic correlations but found to have a significantly positive phenotypic correlation with the number of cobs per plant (Figure 3). The correlation value denotes the nature and extent of association existing between pairs of characters. The correlation between various traits is because of the presence of linked genes. It plays a pivotal role in the selection of the right traits for breeding purposes as it acts as a measure that indicates traits to be considered to increase yield. In this study, both in phenotypic and genotypic coefficient correlation signify the major yield contributing trait, hundred kernels weight having a significant positive correlation with cob width, kernel length, kernel width,

and kernel thickness. So, these agronomic traits can be considered during the selection of genotypes for improving yield potential.

Scatter Plot Matrix of Kernel Related Traits

A scatter plot matrix is a grid (or matrix) of scatter plots used to visualize bivariate relationships between combinations of variables. However, with the present set of the population considering the kernel morphology attributes, scatter plot matrix established a relationship between kernel length (mm) and the rest of the considered kernel-related traits where a positive and strong relationship was revealed between kernel length (mm) and 100 kernel weight (gm) (Figure 4).

(HKW= 100 kernel weight (gm), KL= Kernel Length(mm), KW= Kernel width (mm), KT= Kernel Thickness (mm))

The matrix revealed kernel length (mm), kernel width (mm), and kernel thickness (mm) had a high strong and positive association with 100 kernel weight (gm). kernel length (mm) exhibited a strong but negative association with kernel thickness (mm). However, kernel width (mm) showed a positive association with kernel length (mm) and kernel thickness (mm).

Therefore, it is imperative for the breeders that kernel morphology traits must be emphasized while designing a breeding program underlying inbred development to improve farmers' most desire traits i.e. 100 kernel weight (gm). Nonetheless, considering the data plan it was also evident (Figure 4) that the traits kernel



Figure 4: Scatter plot matrix considering only the kernel-related traits in target maize inbred population.

width (mm) and 100 kernel weight (gm) exhibited normal distribution while for the rest of the case unexpected bimodal distribution patterns were revealed signifying that individuals for those traits are likely to fall into two different groups where one group is under-expressed and other groups may have the property of overexpression.

Mahalanobis D2 Statistical Analysis

52 maize inbred lines were grouped into 6 different clusters by using hierarchical clustering techniques by Mahalanobis D2 statistical analysis based on 12 agronomic traits. The cluster distribution pattern is presented in Table 1. The maximum number of genotypes (14) was in cluster III, followed by cluster I (13). The cluster V contained only 4 lines and occupied the least position. The D2 analysis carried out involving 52 inbred lines for 12 characters revealed that altogether 6 clusters have been formed (Table 1). The clustering pattern of the lines revealed that the lines developed from the base materials exhibited different characteristics. Inter and intra-cluster distance (D = $\sqrt{D2}$) values were worked out from divergence analysis and are presented in Table S8. From the table, it was revealed that the inter-cluster distance was larger than the intra-cluster distance indicating wide diversity among the inbred lines of different groups. The maximum intra-cluster distance (D = 3.65) was observed in cluster I followed by cluster II (D =3.41) and VI (D =3.4). The highest inter-cluster distance (D =7.35) was observed between clusters I and V followed by clusters I and II (D = 6.18) and, clusters III and V (D=6.09).

	']	Table 1: D2 hierarchical clustering Based on Phenotypic traits
Cluster	Size	Member
Ι	13	MMIL-2, MMIL-7, MMIL-17, MMIL-30, MMIL-32, MMIL-42, MMIL-47, MMIL-58, MMIL-61, MMIL-71, MMIL-74, MMIL-78, MMIL-82
II	6	MMIL-4, MMIL-26, MMIL-27, MMIL-72, MMIL-96, MMIL-97
III	14	MMIL-10, MMIL-39, MMIL-45, MMIL-46, MMIL-49, MMIL-54, MMIL-55, MMIL-65, MMIL-66, MMIL-67, MMIL-80, MMIL-81, MMIL-94, MMIL-98
IV	8	MMIL-12, MMIL-28, MMIL-37, MMIL-57, MMIL-62, MMIL-68, MMIL-92, MMIL-93
V	4	MMIL-13, MMIL-64, MMIL-90, MMIL-91
VI	7	MMIL-14, MMIL-16, MMIL-18, MMIL-19, MMIL-20, MMIL-21, MMIL-22

These findings were supported by Haydar et al. (2015). The inbred lines belonging to the clusters separated by high statistical distance could be used in a hybridization program for obtaining a wide spectrum of variation among the segregates. In this context, inbred lines from clusters I and V should be selected as parents in the hybridization program for yield improvement in maize inbred lines. The distance between clusters I and III was minimum (4.07) indicating that the inbred lines belonging to these clusters were comparatively less diverse. Haydar et al. (2015), Marker and Krupakar (2009), Zaman and Alam (2013) reported that the clustering revealed instability due to relatively lesser divergence, where the widely divergent cluster remains distinct in a different environment. It is expected that the crosses between the lines of clusters I and II would exhibit high heterosis and are also likely to produce new recombinants with desired characters. However, parental selection only based on phenotypic data for hybridization may not be feasible during early generations of segregation. Genetic variability and population structure In this study, AMOVA was used to determine the proportion of genetic variation partitioned among and within the 52 maize genotypes exploiting four SSR markers in Figure 5.

It revealed the percentages of molecular variance within the population as well as among the population as 76% and 24 %, respectively. This significant difference was due to the cross-pollinating nature of maize. There was a good correspondence between the AMOVA and molecular genetic distance in differentiating the maize genotypes into different clusters.

The STRUCTURE v2.3.4 (Pritchard et al., 2000) was used to assess the population structure and genetic relations among the



Figure 5: Scatter plot matrix considering only the kernel-related traits in target maize inbred population.

genotypes employing four SSR markers. To find the optimal K-value, the number of clusters (K) was plotted against Δ K by using the Structure Harvester website (Earl and Vonholdt, 2012), which provided K vs. Δ K graph that showed a sharp peak at K = 5 (Figure 7). The population structure is represented in the bar plot showing five subsets (five clusters of genotypes) with five different colors (Red, Green, Blue, Yellow, Purple) (Figure 8). Genotypes with membership proportions (Q-value) >80% were considered as pure and part of their corresponding cluster while genotypes with membership proportions (Q-value) lesser than 80% were adjudged as admixtures.

According to the analysis number of members per each population are follows: as Pop1= 14, Pop2= 11, Pop3= 6, Pop4= 10. Pop5= 8 and Admixture= 3 genotypes respectively (Table S9). STRUCTURE 2.3.4 software identified subsets of all genotypes by detecting allele frequency differences within the data and assigns individuals to a different subset of populations based on analysis of likelihoods.



Figure 6: K vs Del K showing peak value at K=5



Figure 7: Bar plot showing population structure sorted by the value of Q with genotypes in X-axis and inferred value in Y-axis indicating five different subtypes of the population

The optimal K-value indicated that there have five subsets of populations among the studied genotypes with similar genetic constitutes, excluding the environmental effects. Principal Coordinate Analysis (PCoA)



Figure 8: Plot of Coordinate 1 and Coordinate 2 from principal coordinate analysis (PCoA) based on genetic distance matrix calculated from 4 SSR data. Figure 9: Plot of Coordinate 1 and Coordinate 3 from principal coordinate analysis (PCoA) based on genetic distance matrix calculated from 4 SSR Data.



In this study, the association between the lines was also examined using the principal coordinates analysis in combination with SSR data, and the genetic distances were projected onto a bi-dimensional plane (Figure 8 and 9). The two-dimensional graphical view of principal coordinates revealed the spatial distribution where 52 inbred genotypes were scattered distributed throughout the ordinates. PCo1 exploited against PCo2 revealed that four inbred lines e.g. MMIL-22, MMIL-66, MMIL-93, and MMIL-90 were far away from the centroid signifying their potentiality as parents of the hybrid breeding program. Nonetheless, the PCo1 Vs PCo3 plot showed six inbred lines e.g. MMIL-22, MMIL-46, MMIL-47, MMIL-49, MMIL-55, and MMIL-58 were far away from the centroid. The results of the PCoA in our experiment revealed that large genetic diversity existed among the 52-maize inbred. Nyaligwa et al., (2015) found the percentages of variance for the first 2 principal coordinates were 52.7% and 14.3% with a total variance of 67.0% and thus, classified 79 inbred lines into two major groups (Group A and B). in this study, The genotypes were placed far away from the centroid were more genetically diverged compared to the genotypes placed near the centroid were likely to be genetically more similar (Moniruzzaman et al., 2018). However, the centroid may be defined as the vector representing the middle point of the cluster, which contained at least one number for each variable.

SHE analysis by Shannon-Wiener diversity index

In this study with S1 population of maize inbred lines was counted as the sample size. The SHE analysis showed that the two diversity indices including the richness (S) and Shannon index (H) in the S1 population had the same increasingly trend lines gradient while the evenness index (E) line was reversely downward line gradient (Figure 10).

A diversity index is a quantitative measure that reflects how many different types there are in a dataset (a community) and that can simultaneously take into account the phylogenetic relations among the individuals distributed among iversity those types, such as richness, divergence, or evenness. (Tucker et al., 2017). This figure precisely showed that with the increase of the sample number, the richness diversity will raise and to maximum rate (about to 4) and if it happened, the evenness of species will descend into less, as well. Reaching maximum diversity will swiftly happen in the following self-



Figure 10. SHE analysis plot showing a decrease of evenness (E) and increase of species richness (S) along with Shannon-Wiener diversity index (H) through the sample size (N)

pollinated generations because of line head which started from 36 and finished to 132 Ln (N). **Heatmap relying on pairwise genetic distance**

The concept of dissimilarity may be used in a more general way, to determine the pairwise difference between genotypes. As an example, this was used by Silveira and Hanashiro (2009) to study the impact of similarity and dissimilarity between superior and subordinate in the quality of their relationship. The dissimilarity matrix combined with heatmap is an effective method for visualizing genetic distance (GD) among genotypes. The pairwise genetic distance values were graphically represented by a heatmap in Figure 11, which were indicated by gradient colors from red (low values) to lemon (high values), ranging from 0 to 4.3267. Genotype 21 (MMIL-54) was the most genetically dissimilar compared to other genotypes in



the dissimilarity matrix followed by 42 (MMIL-96) (Figure 12). Furthermore, a considerable dissimilarity was also shown by (MMIL-22), (MMIL-30), (MMIL-28), (MMIL-81), (MMIL-82), (MMIL-97), (MMIL-27), (MMIL-45), (MMIL-14) and (MMIL-21) to the other members of the exploited inbred poll. On the contrary, the genotypes revealing most similarity matrix within the entire population were MMIL-18, MMIL-19, MMIL-26, MMIL-32, MMIL-37, MMIL-39, MMIL-42, MMIL-71, MMIL-74, MMIL-78, MMIL-80, MMIL-91, MMIL-92, MMIL-90, MMIL-90 (Figure 12).



(The distance between genotypes is indicated by the gradient of color; the lemon color denotes the highest dissimilarity, and the red color means the lowest genetic distance. Also, the red color represents the diagonal.)

Figure 11: Heatmap generated exploiting the dissimilarity matrix revealing the genetic distances among the genotypes.

CONCLUSION

Comparing the results, it was determined that the parental selection from current population of study is best while selecting through kernel related traits. The highest association of kernel width and length with hundred kernel weight shows that yield will be better if selected based on kernel architecture. Rather, cluster based on phenotypic characters differed from SSR derived population suggesting association of environmental effects. This strategy was useful predicting the most diverse population as well as specific genotypes both based on phenotypic traits and molecular markers for the future hybridization program.

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Conflict of interest

The Authors declare no conflict of interest.

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Annexures: (Supplementary Tables)

Serial no.	Base Materials	Source of Collection
	Inbred and Outcrossed Lines	
1	MIL-BK-01	China
2	MIL-Whi-01	China
3	IML-BG-01	China
4	MIL-Mym-01	Local Market, Bangladesh
5	MIL-Mym-02	Local Market, Bangladesh
6	ML-Syl-01	Local Market, Bangladesh
7	IML-Arron-01	Local Market, Bangladesh
8	MIL-AG-01	Local Market, Bangladesh
9	MIL-AG-02	Farmers field, Bangladesh
10	MIL-BKC	Farmers field, Bangladesh
11	IML-2TY-02	Farmers field, Bangladesh
12	IML-2TY-01	Farmers Field, Bangladesh
13	MIL-POP-01	Farmers Field, Bangladesh
	Hybrid Lines	
14	Fortune	
15	Titan	
16	D.7001	
17	981	Collected from different local and foreign
18	Everest	seed companies
19	Elite	
20	D.949	
21	Hiramon-917	

Table S1. List of the local and exotic base materials used

Table S2: Selected Promising S1 genotypes from the base materials with DNA Sample number

Genotypes	DNA	Genotypes	DNA	Genotypes	DNA	Genotypes	DNA
ID	Sample	ID	Sample	ID	Sample	ID	Sample
	number		number		number		number
MMIL-2	1	MMIL-37	14	MMIL-63	27	MMIL-93	40
MMIL-4	2	MMIL-39	15	MMIL-65	28	MMIL-94	41
MMIL-7	3	MMIL-28	16	MMIL-66	29	MMIL-96	42
MMIL-10	4	MMIL-42	17	MMIL-68	30	MMIL-97	43
MMIL-12	5	MMIL-46	18	MMIL-71	31	MMIL-27	44
MMIL-13	6	MMIL-47	19	MMIL-72	32	MMIL-67	45
MMIL-16	7	MMIL-49	20	MMIL-74	33	MMIL-90	46
MMIL-18	8	MMIL-54	21	MMIL-78	34	MMIL-98	47
MMIL-19	9	MMIL-55	22	MMIL-80	35	MMIL-20	48
MMIL-22	10	MMIL-57	23	MMIL-81	36	MMIL-17	49
MMIL-26	11	MMIL-58	24	MMIL-82	37	MMIL-45	50
MMIL-30	12	MMIL-61	25	MMIL-91	38	MMIL-14	51
MMIL-32	13	MMIL-62	26	MMIL-92	39	MMIL-21	52

C/N	Montron	Chromosome		Security	AT	
5 /1N	Marker	no.	no. Sequence			
1	umc1122	1	F	3'CACAACTCCATCAGAGGACAGAGA5'	65°C	
			R	3'CTGCTACGACATACGCCA GGC5'		
2	umc1152	10	F	3'CCGAAGATAACCAAACAATAATAGTAGG 5'	65°C	
			R	3'ACTGTACGCCTCCCCTTCTC5'		
3	Phi015	8	F	3'GCAACGTACCGTACCTTTCCGA5'	65°C	
			R	3'ACGCTGCATTCAATTACCGGGAAG5'		
4	Phi022	9	F	3'TGCGCACCAGCGACTGACC 5'	65°C	
			R	3'GCGGGCGACGCTTCCAAAC5'		

Table S3. List of SSR primers used in the molecular evaluation of maize varieties

Table 64 DCD (Creals must and r	and for ampliful	ng the tenget DN	for 10.1 DCD product
Table 54. PUK (Uvcie drolocoi l	sed for ambinivi	ng the target DNA	
	-,			

Steps	Temperature (°C)	Duration	Cycles	Activity
1	94	4 min	1	Initial Denaturation
2	94	1 min	35	Denaturation
3	65	50 s		Annealing
4	72	2 min	1	Extension
5	72	5 min	1	Final extension
6	4	∞		Storage

Traits	PH	NC	CL	CW	Cwt.	NRPC	NKPR	NKPC	HKW	KL	KW	KT
Rank	(cm)	(no.)	(cm)	(mm)	(g)	(No.)	(No.)	(No.)	(gm)	(mm)	(mm)	(mm)
1	MMIL-21	MMIL-74	MMIL-72	MMIL-91	MMIL-91	MMIL-4	MMIL-16	MMIL-91	MMIL-53	MMIL-64	MMIL-58	MMIL-7
	108	6	20.1	54.79	237.4	42	20	575	50.57	13.566	11.24	6.862
2	MMIL-42	MMIL-26	MMIL-90	MMIL-64	MMIL-90	MMIL-97	MMIL-20	MMIL-64	MMIL-52	MMIL-91	MMIL-74	MMIL-74
	111	5	19.5	54.03	233	42	20	547	48.67	13.316	11.076	6.164
3	MMIL-78	MMIL-96	MMIL-82	MMIL-90	MMIL-64	MMIL-72	MMIL-21	MMIL-90	MMIL-74	MMIL-93	MMIL-37	MMIL-17
	113	5	18.8	52.1	226.2	38	20	541	46.82	13.234	10.612	6.122
4	MMIL-47	MMIL-4	MMIL-26	MMIL-57	MMIL-26	MMIL-26	MMIL-91	MMIL-26	MMIL-57	MMIL-90	MMIL-27	MMIL-58
	136	4	18.3	50.43	221.4	37	20	535	46.7	12.886	10.496	5.988
5	MMIL-7	MMIL-27	MMIL-93	MMIL-16	MMIL-27	MMIL-27	MMIL-13	MMIL-4	MMIL-58	MMIL-12	MMIL-67	MMIL-2
	142	4	18	50.36	201.6	37	18	529	45.99	12.358	10.156	5.776

Table S5: Top ten promising S1 inbred lines based on their mean performance as observed during robi season 2018-19

Table S6. Descriptive statistics of seventeen yield contributing traits in selected advance inbred lines (S1) of maize

Characters	Range	Mean±SE	GV (z ² g)	PV(zp)	GCV (%)	PCV (%)	GA	GAM (%)	h2b
Plant Height (cm)	108-233	181.2981±0.49	1102.7526	1103.2524	18.3166	18.3208	68.3924	37.7237	99.95
No. of cobs	1-7	2.7885 ± 0.51	0.8956	1.4054	33.9385	42.5143	1.5563	55.8121	63.73
Cob length (cm)	8.6-21.1	15.2558±0.51	5.4394	5.9492	15.2877	15.988	4.594	30.1132	91.43
Cob Width (mm)	36.56-55.79	45.0696±0.51	17.4549	17.9647	9.2699	9.4043	8.4835	18.8231	97.16
Cob weight (gm)	48.97-238.4	126.7013±0.51	2179.6334	2180.1432	36.8477	36.852	96.163	75.8974	99.98
No. of Rows per cob	15-43	25.8654 ± 0.51	42.7658	43.2756	25.283	25.4333	13.3919	51.7754	98.82
No. of Kernel per row	7-21	14.2308±0.51	7.3967	7.9065	19.1113	19.7589	5.4189	38.0788	93.55
No. of Kernel per cob	87-576	304.2308±0.51	14515.3575	14515.8673	39.6014	39.6021	248.1838	81.5775	100
Kernel length (mm)	8.048-14.566	11.0569±0.51	1.1264	1.6362	9.5987	11.5687	1.814	16.406	68.84
Kernel Width (mm)	5.36-12.24	9.1249±0.51	1.2095	1.7193	12.0524	14.3697	1.9002	20.8243	70.35
Kernel Thickness (mm)	3.438-7.164	5.1692 ± 0.51	0.1376	0.6474	7.176	15.5654	0.3523	6.8153	21.25
100 kernel weight (gm)	23.76-47.82	35.0683±0.51	29.9545	30.4643	15.6069	15.7391	11.1798	31.8801	98.33



Table S7: Estimation of genotypic correlation coefficients (rg) (above diagonal) and phenotypic correlation coefficients (rp) (below diagonal) for yield-related traits of maize hybrids

Traits	РН	NC	CL	CW	CWt.	NRPC	NKPR	NKPC	KL	KW	КТ	HKW
РН	1	0.0554 NS	0.3119 *	0.1565 NS	0.3594 **	0.2337 NS	0.1997 NS	0.325 *	0.1444 NS	-0.1759 NS	-0.3385 *	0.0483 NS
NC	0.0569 NS	1	0.053 NS	-0.241 NS	-0.004 NS	0.2646 NS	-0.3121 *	-0.016 NS	-0.0713 NS	0.2022 NS	-0.1135 NS	0.1177 NS
CL	0.3043 **	0.2168 *	1	0.3402 *	0.6179 **	0.5652 **	0.3542 **	0.617 **	0.2891 *	-0.1893 NS	0.0647 NS	-0.0296 NS
CW	0.1578 NS	-0.0882 NS	0.3699 **	1	0.8006 **	0.3319 *	0.5241 **	0.6349 **	0.6871 **	-0.0711 NS	-0.2956 *	0.3037 *
CWt.	0.3596 **	0.0061 NS	0.5953 **	0.7916 **	1	0.729 **	0.5163 **	0.9109 **	0.6752 **	-0.0959 NS	-0.3849 **	0.1527 NS
NRPC	0.2345 *	0.2754 **	0.569 **	0.3435 **	0.7262 **	1	0.139 NS	0.817 **	0.2443 NS	0.0114 NS	-0.4671 **	-0.2018 NS
NKPR	0.1985 *	-0.088 NS	0.4019 **	0.5425 **	0.5032 **	0.1612 NS	1	0.6159 **	0.3921 **	-0.8338 **	-0.5993 **	-0.3384 *
NKPC	0.325 **	-0.0092 NS	0.5917 **	0.6268 **	0.9109 **	0.8128 **	0.5972 **	1	0.5041 **	-0.3478 *	-0.617 **	-0.2389 NS
KL	0.1316 NS	0.289 **	0.3928 **	0.656 **	0.5687 **	0.2621 **	0.4564 **	0.4215 **	1	-0.0033 NS	-0.4172 **	0.4329 **
KW	-0.136 NS	0.4633 **	0.0076 NS	0.0329 NS	-0.0721 NS	0.0686 NS	-0.5381 **	-0.2885**	0.3017 **	1	0.3527 *	0.7022 **
КТ	-0.1373 NS	0.4927 **	0.2883 **	0.0151 NS	-0.1639 NS	-0.1177 NS	-0.0419 NS	-0.2792 **	0.3358 **	0.6196 **	1	0.6798 **
HKW	0.0506 NS	0.1711 NS	0.0098 NS	0.3186 **	0.1534 NS	-0.1849 NS	-0.2917 **	-0.2362 *	0.4284 **	0.6545 **	0.4255 **	1

Column1	c1	c2	c3	c4	c5	c6
c1	3.65					
c2	6.18	3.41				
c3	4.07	5.07	2.98			
c4	4.47	4.86	4.16	2.93		
c5	7.35	4.7	6.09	4.79	2.69	
сб	5.66	5.42	4.51	4.96	5.24	3.4

Table S8: Average intra- (bold) and inter-cluster distance (D2) for 52 maize S1 inbred lines obtained based on 12 morphological characters.

Table S9: Population Structure Based on SSR data

Number of Population	Size	Members
Population 1	14	MMIL-2, MMIL-4, MMIL-7, MMIL-10, MMIL-12, MMIL-13, MMIL- 16, MMIL-22, MMIL-30, MMIL-46, MMIL-47, MMIL-49, MMIL-55, MMIL-98
Population 2	11	MMIL-27, MMIL-58, MMIL-61, MMIL-62, MMIL-64, MMIL-65, MMIL-66, MMIL-67, MMIL-68. MMIL-93, MMIL-94
Population 3	6	MMIL-18, MMIL-28, MMIL-37, MMIL-54, MMIL-57, MMIL-72
Population 4	10	MMIL-20, MMIL-32, MMIL-42, MMIL-71, MMIL-74, MMIL-78, MMIL-80, MMIL-90, MMIL-91, MMIL-92
Population 5	8	MMIL-14, MMIL-17, MMIL-21, MMIL-39, MMIL-45, MMIL-81, MMIL-82, MMIL-97
Admixture (<80%)	3	MMIL-19, MMIL-26, MMIL-96