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MORPHOLOGICAL AND MOLECULAR DIVERSITY AMONG TEOSINTE ACCESSIONS USING SIMPLE SEQUENCE REPEAT MARKERS

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Teosinte, (*Zea mays* ssp*. parviglumis*), which has large-spikelets, is the wild progenitor of maize and serves as an important resource for studying maize evolution and genetics. Teosinte exhibits significant genetic polymorphism, which has been exploited only to a limited extent for the genetic diversification and improvement of maize (Adhikari *et al*., 2021; Joshi *et al.*, 2021). Despite striking morphological differences between teosinte and maize, particularly in plant architecture, inflorescence, and seed structure, both species are remarkably similar at the DNA level, sharing the same number of chromosomes and a highly conserved gene arrangement. The genetic variation within teosinte represents a valuable resource that must be harnessed in breeding programs to develop more diverse maize lines and introduce novel genetic variations for crop improvement (Adhikari *et al.*, 2021). Teosinte carries potent gene combinations associated with traits such as tolerance to biotic and abiotic stresses, as well as desirable quality traits (Garg *et al.*, 2019; Kumar *et al.*, 2020; Sahoo *et al.*, 2021). These beneficial genes can be introgressed into maize to enhance allelic diversity, adaptability, and the overall genetic base of maize germplasm (Sahoo *et al.*, 2021).

A fundamental and crucial factor in designing any breeding programme is the genetic diversity present within or between the breeding materials. This variation can be harnessed either by developing new varieties or by selecting suitable parents for targeted breeding efforts (Adhikari *et al.*, 2021). Numerous studies have analyzed different maize germplasm collections to assess their genetic diversity (Ertiro *et al.*, 2017). A significant number of lines exhibiting prolific behaviour were identified in backcross-derived lines from maize × teosinte crosses (Adhikari *et al.*, 2019). This highlights the potential of teosinte as a valuable donor for enhancing maize prolificacy. Understanding the extent of genetic diversity within germplasm is essential for the effective utilization of this variation in crop improvement. Such understanding can facilitate the development of inbred lines with broad genetic variability through the selection of diverse parental combinations (Ertiro *et al.*, 2017), ultimately aiding in the establishment of heterotic groups and the generation of valuable source materials for breeding programmes.

For the diversity analysis of wild and modern maize, several marker technologies can be employed, including phenological, morphological, biochemical, and molecular markers. Considering the potential genetic variation in wild sources, the present study aims to assess diversity among thirty-nine teosinte accessions based on both genetic evaluation and morphological traits.

The teosinte accessions were procured from CIMMYT SeedBank through NBPGR. The seeds were sown in experimental area of the School of Agricultural Biotechnology, Punjab Agricultural University (PAU), Ludhiana during the Spring 2021 (Fig 1). Observations were recorded on Days to anthesis (DA), Days to silking (DS), Anthesis-silking interval (ASI), Tassel angle (TA), Tassel: Anthocyanin colouration of anthers (ANCA), Plant height (PH) and Tassel: Altitude of lateral branches (ATB). The data of accessions were converted to binary numbers as per the DUS testing guideline of the Protection of Plant Varieties and Farmers' Rights Authority, Government of India (2007). The set of data collected was subjected to various analyses i.e., Principal component analysis, Correlation analysis and Cluster analysis using different packages of R software 4.2.0.

Genotyping for diversity analysis was carried out using already available Simple Sequence Repeat

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Fig. 1. (A) Seeds of selected genotypes procured from CIMMYT seed bank
(B) Field experiment of 39 accessions at the School of Agricultural Biotechnology, PAU, Ludhiana
(C) Variation of different qualitative characters in Teosinte accessions

(SSR) markers. The data were then subjected to PIC calculations. Furthermore, a dendrogram was generated using an unweighted pair group with arithmetic mean (UPGMA) by the SHAN clustering function of NTSYS-pc version 2.02 software based on similarity coefficients.

Morphological diversity

The present study involved the analysis of seven different morphological traits in thirty-nine teosinte accessions, with data collected from five plants per accession. This study aims to evaluate the variability among these accessions. Regarding plant height, 17 (43.64%) of the accessions had taller plants, 15 (38.86%) had medium-height plants, and the remaining 7 (17.50%) accessions showed shorter plant height. Plant height plays an imperative role in plant lodging. Consequently, maize breeders give special attention to this trait in maize breeding. Plants with small heights, and internodes will be smaller, thus low plant density may hinder better penetration of solar radiation to the canopy. The tall plants may have long internodes thus reducing intra-plant shading and can accumulate more photosynthates. Therefore, teosinte accessions with less shading could be utilized for the high-density planting of maize (Adhikari *et al*., 2021). Remarkably, different accessions showed variations in the tassel-related traits (Fig. 1). In our study, 23 accessions exhibited narrow angles while 16 accessions showed wide angles. Kumar *et al*. (2019) studied the characteristics of teosinte introgressed lines derived from the cross of teosinte with wide flag leaf angle with inbred line DI-103 that have narrow flag leaf angle. The introgressed backcross lines AM-2, AM-4, AM-5 and AM-12 showed a wider angle and the AM-7 line showed narrow angle.The smaller tassel allows more penetration of light to the leaf canopy, resulting in increased grain yield (Lambert *et al*., 2014).

For the anthocyanin colouration of anthers, 11 (28.20%) out of the 39 accessions possessed no anthocyanin, while strong anthocyanin colouration was observed in 28 (71.80%) of the accessions. The anthocyanin colouration of anthers was present in teosinte and AM-7 line developed from the cross of teosinte with maize inbred line. (Kumar *et al*., 2019). Most of the tassels between the 39 accessions were straight (51.28%), while 41.02% displayed a curved shape, and a minority were strongly curved (7.7%). Increasing field planting density is one method of boosting maize output. Therefore, one important factor for yield improvement is breeding for plant types that support high plant densities.

The duration of the anthesis period displayed variation, ranging from a minimum of 90 days to a maximum of 98 days, with an average of 92.43 days. Similarly, during the silking phase, the time spanned from 98 days to 107 days, with an average of 101.85 days (Table 1). The anthesis-silking interval of teosinte accessions ranged from 7 to 14 days with a mean of 9.36 days. Teosinte has protogynous behaviour whereas in maize it is a crucial characteristic even under stress conditions (Magoja and Pischedda, 1994). Protogynous lines were found in teosinte-derived maize populations in several studies (Adhikari *et al.,* 2019). As a result, these lines have a lot of potential in stress conditions and could be used in drought improvement programmes.

Morphological characterization was conducted to analyse the variations both within and between the teosinte accessions. In our analysis, correlation

analysis on seven different traits showed strong correlations between them (Fig. 1). Days to 50% silking is an important trait used to determine the earliness to maturity of the maize crop. The correlation analysis showed that days to silking had a highly significant positive correlation with plant height (Halidu *et al*., 2015). These results are similar to our study, as we have observed a significant and positive correlation of Plant height with Days to Silking (0.168) (Fig. 2).

Table 1. Mean, Range and Standard Deviation of teosinte accessions

Trait	Mean	Range	Standard Deviation
Days to anthesis	92.43	90-98	2.245
Days to silking	101.85	98-107	2.538
Anthesis-silking Interval	9.363	7-14	3.201

The principal component analysis (PCA) was performed for morphological traits under investigation. Four principal components (PCs) were identified with eigenvalues more than 1. PC_1, PC_2, PC_3 and PC_4 accounted for 27.3%, 24.8%, 20% and 14% of the total variation, respectively (Table 2). In the PCA analysis, ASI, ATB and DA contribute more towards diversity within the accessions and in this context, these traits can be focused more while performing selection during wide hybridisation as PC_1 component showed more variability and traits i.e., DA, DS, ASI, TA, ATB, and PH are major contributors towards diversity and can be incorporated in maize breeding programmes. Thus, it means that the enhancement of one trait can bring out the improvement of other traits (Saleh *et al*., 2022). The PCA analysis resulted in 5 vectors accounting for 88.40 % and 90.30 % of the total variability produced by various traits from the diallel cross of maize inbred lines under study in Bauchi and Misau, respectively (Saleh *et al*., 2022).

A hierarchical cluster analysis was performed with the 39 accessions and these were grouped into 3 clusters using the Scree plot and Elbow method based on 7 traits (Fig 2). Cluster 1 consisted of 14 accessions, cluster 2 of 7 and cluster 3 of 18 accessions (Supplementary Table 1). Cluster 1 had a maximum intra-cluster distance value of 68.759 followed by Cluster 2 (60.212) and Cluster 3 (41.681). The positioning of accessions apart from each other suggests that these teosintes have a somewhat unique morphology. The demarcation of teosinte accessions into phenetic groups suggests that accessions are from different localities. This is an indication of the range of variation in terms of morphological characters among accessions (Smith *et al*., 1981). Our results showed that 39 accessions grouped into three clusters because of morphological and genotypic variations. This might be due to a greater number of environmental factors affecting plant competition and survival. This study produced three different clusters with few number of teosinte accessions in each cluster which signifies that the collection is very diverse and these teosinte accessions in the clusters contain distinct characters from each other.

Molecular diversity

Among the 25 polymorphic markers, 23 produced two alleles each, while five generated a maximum of three alleles each. The Polymorphism Information Content (PIC) values ranged from 0.062 to 0.888, with an average PIC value of 0.4237 (Supplementary Table 2). The highest PIC value was associated with the primer umc1017 (0.888), whereas the lowest PIC values were attributed to the primer umc1077 (0.062).

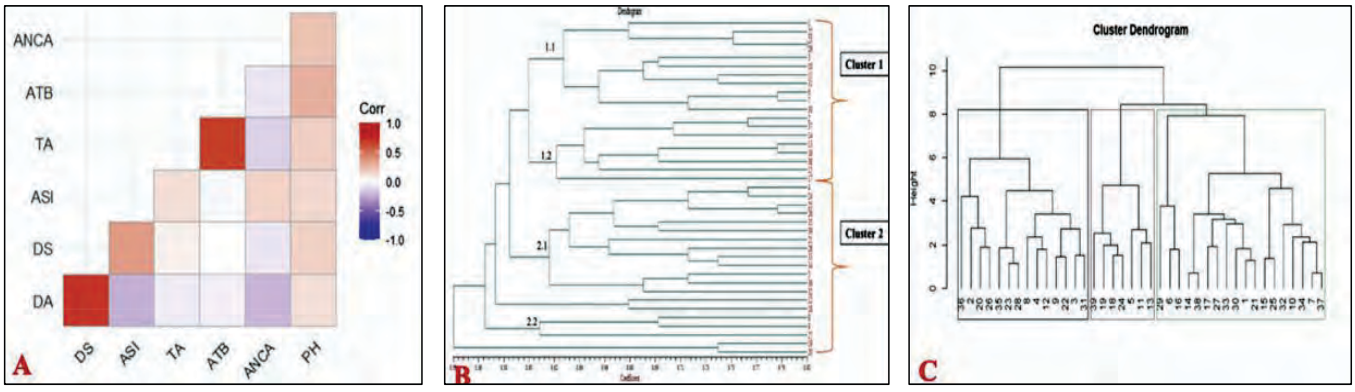

Fig. 2. (A) Correlation between different morphological traits viz; DA, DS, TA, ANCA, PH and ATB, (B) Dendrogram showing the relationship of 39 teosinte accessions in Three clusters based on EuclideanD² distance, (C) Dendrogram showing the relationship of 39 teosinte accessions based on SSR markers

Supplementary Table 1. Grouping of teosinte accessions in different hierarchical clusters

Cluster	Number of accessions	Name of accessions
Cluster 1	14	MGB-CI 16, K 67-4, W.S. 92-9, MGB-CI 5, MGB-CI 15, MGB-CI 2, MGB-CI 7, K 68-3 ,K 68-2, K 69-12, K 69-7, MGB- CI 1, K 68-1, MGB- CI 11
Cluster 2	7	MGB-CI 19, W.S.T. 92-6, W.S.T. 92-3, MGB-CI 3, K-69-3, TEOSIN 69-15, TEOSIN 62-87
Cluster 3	18	MGB-CI 8, K 69-4, E86-2, TEOSIN 62-?, MGB-CI 18, W.S.T. 92-1, MGB- CI 6, MGB- CI 13, MGB- CI 9, K 67-1, T.K.R. 93-3, E86-1. MGB- CI 4, MGB- CI 12, K 69-8, MGB- CI 14, K65-2, MGB- CI 17

Supplementary Table 2. PIC values for SSRs markers

Primer	PIC value
bnlg176	0.150
umc1641	0.418
umc1077	0.062
umc1034	0.594
umc1231	0.239
umc1017	0.888
umc2136	0.194
umc1953	0.404
bnlg1720	0.737
bnlg2323	0.683
umc1196a	0.408
phi087	0.710
umc1031	0.645
bnlg1231	0.326
umc1805	0.332
bnlg118	0.0999
bnlg1371	0.206
bnlg1759a	0.207
phi126	0.520
umc1034	0.373
umc2007	0.557
umc2177	0.397
umc2320	0.484
umc2126	0.194
umc1577	0.752

Supplementary Table 3. Grouping of teosinte accessions in two clusters

Cluster	Number	Name
Cluster 1	19	K 67-1, MGB-CI 2, MGB-CI 1, MGB-CI 11, K 68-1, K 69-7, K 69-9, MGB-CI 8, K 65-2, K 69-4, MGB-CI 9, K 67-4, MGB CI 17, E 86-2, TEOSIN 69-15, TEOSIN 62-?, MGB-CI 18, E86-1, TEOSIN 62-87
Cluster 2	20	K 68-2, T.K.R. 93-2, MGB-CI 3, MGB-CI 4, MGB CI 15, MGB-CI 19, T.K.R 93-3, MGB-CI 6, MGB-CI 12, MGB-CI 14, K 68-3, MGB-CI 16, MGB-CI 7, K 69-12, W.S.T. 92-1, W.S.T. 92-3, K 69-3, MGB-CI 5, W.S.T.92-6, W.S. 92-9

The 25 SSR markers used in the study generated multiple alleles in our study of which marker *(bnlg 176, umc 1231, umc 1953)* generated 2 alleles and marker *(umc1077, umc 1017, umc 1031, umc 1805, bnlg 1371)* generated 3 alleles each. These results are in concurrent with observations of Islam *et al*. (2023) in which 18 polymorphic SSR loci from 10 chromosomes in diversity studies between maize genotypes. Similarly, Kumari *et al.* (2018) studied diversity in eight different genotypes of maize using nine pairs of polymorphic markers generated 18 polymorphic alleles. The number of alleles detected per locus varied between from 2 *(bnlg 1484)* to 7 alleles *(bnlg1194, bnlg1526* and *Phi119).* Moreover, in a diversity study between six teosinte accessions by Loaisiga *et al.* (2012), the total no. of alleles that were amplified for all the primers varied from 2-3.

The PIC value of markers signifies the strength of the marker to differentiate between genotypes. The difference is based on allele number and frequency of alleles per locus. The PIC content in our investigation ranged from 0.062 - 0.888 which is in parity with the findings of Sserumaga *et al.* (2014) PIC value = 0.16 - 0.91 and Patel *et al*. (2017) (PIC value = 0.46 - 0.86). The PIC less < 0.5 is possibly due to the presence of a conserved region in the group of genotypes studied. Moreover, some problems associated with microsatellites as markers also impact PIC values. The primer umc1017 possessed the highest PIC (0.888) as per our study making it the most suitable marker for genetic diversity study.

The clustering of the accessions was done by UPGMA method. All teosinte accessions were grouped into two major clusters (Fig. 2). The first cluster contained 19 accessions whereas, the second

Table 2. Eigenvalue, the contribution of variability and eigenvectors for the principal component axis of teosinte accessions

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Eigen values	**1.909**	**1.738**	**1.401**	**0.993**	0.593	0.284	0.082
Proportion	0.273	0.248	0.200	0.142	0.085	0.041	0.012
Cumulative proportion	0.273	0.521	0.721	0.863	0.948	0.988	1.000
DA	0.305	-0.606	0.225	0.252	-0.166	-0.054	0.628
DS	0.437	-0.541	-0.171	-0.173	-0.184	0.088	-0.645
ASI	0.203	0.011	-0.607	-0.613	0.152	0.106	0.424
TA	0.510	0.380	0.190	-0.188	-0.244	-0.682	0.004
ATB	0.499	0.413	0.209	0.084	-0.179	0.703	0.060
ANCA	-0.089	0.136	-0.610	0.440	-0.636	-0.054	0.044
PH	0.397	0.079	-0.317	0.543	0.648	-0.129	-0.061

cluster comprised 20 accessions (Supplementary Table 3). The first cluster was further grouped into two sub-clusters (1.1 and 1.2) and the second cluster was sub-grouped into two sub-clusters (2.1 and 2.2) (Fig. 2). The accessions TEOSIN 69-15, TEOSIN 62-, K 65-2, K 69-7, MGB-CI 3 and MGB-CI 4 show high similarity coefficients (0.78) with the largest similarity coefficient values. On the other hand, the accessions named W.S.T 92-3 and MGB-CI 5 showed the lowest similarity coefficient (0.39).

The two major clusters were generated based on a difference in similarity coefficient, which can be attributed to variation in terms of the genic composition of these accessions. The 45 maize genotypes were grouped into two major clusters which were further subdivided into several sub-clusters. Kanagarasu *et al.* (2013) used 10 SSR molecular markers for diversity analysis of 27 maize inbred lines and cluster analysis revealed five major clusters at 0.62 similarity coefficient. Patel *et al.* (2017) grouped 8 genotypes into three major clusters and Kumar *et al.* (2016) clustered 13 genotypes into five clusters in a dendrogram using similarity coefficient values. These studies are in line with the present investigation.

From this investigation, it can be concluded that there is more heterogeneity among the teosintes accessions. These diverse wild accessions can be utilised for various breeding programmes. Assessment of genetic diversity between different genotypes serves as an essential tool to exploit the genetic variability which is the foremost requirement of any successful breeding programme. The PCA result suggests that morphological evolution during domestication occurs and this could contribute to the variability, and thus can be employed for breeding programmes. The marker *umc 1017* emerged as the most efficient for genotype identification, as specified by the high PIC value of 0.888. Based on SSR primers, the similarity coefficient showed that W.S.T 92-3 and MGB-CI 5 were the most diverse accessions. The genetic variability detected between the teosinte accessions can be exploited for heterosis and developing genetically diverse populations in maize breeding improvement programmes. This work will be useful in determining the parameters that contribute to the variability and in choosing suitable accessions for breeding and utilization in the maize improvement attributes. The identification of accessions with superior traits may facilitate future breeding strategies and the selection of accessions that can be used in hybridisation. Furthermore, these accessions can be screened against *Chilo partellus*, Fall armyworm and Banded leaf and sheath blight resistance, as till now there are imited sources of resistance available. According to the results of our study, selecting accessions for hybridization can be done from clusters that exhibit substantial inter-cluster distance.

Authors' contribution

Conceptualization of research (PS, YV); Designing of the experiments (PS, YV, RK[3]); Contribution of experimental materials (YV, PS, RK[3]); Execution of field/lab experiments and data collection (RK[1] , DB); Analysis of data and interpretation (RK[1], MJ); Preparation of the manuscript (RK[1], PS, DB).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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