PHYLOGENETIC RELATIONSHIPS AMONG TEOSINTE, MAIZE AND ITS HYBRIDS

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Annual teosinte *(Zea mays* ssp. *mexicana*) was introduced to Egypt in the last century but never gained wide importance as a summer fodder because of difficult in seed production and relatively slow early growth (Radwan *et al.,* 2000). Teosinte has special merits over fodder maize including multiple cutting, high nutritive value and ease of production. Teosinte differs from corn by abundant tillering which results in tufted plants, and the ability to recover and produce new growth from the crown buds after cutting (Kellogy and Birchler 1993 and Rammah 1995). Hand-crossing studies demonstrated that *Z. mays* ssp. *mexicana* and maize exhibit genetically based cross-incompatibility (Baltazar *et al.* 2005). Unusually, the flow of genes has occurred in both directions (reciprocal introgression) (Wilkes 1977) although a number of factors tend to favor gene flow from teosinte to maize rather than from maize to teosinte (Baltazar *et al.* 2005). There is also evidence of a restriction to cross ability in some populations of *Z. mays* teosintes when teosinte is the female and maize the male parent and this has been linked to a teosinte gene or gene cluster known as teosinte crossing barrier1 (Tcb1) (Evans and Kermicle, 2001). The incompatibility is asymmetric, being very strong when maize is the pollen parent, but weaker when teosinte is the pollen parent (Baltazar *et al.* 2005; Kermicle and Evans2005). Maize-Teosinte hybrids have been of considerable interest to both maize and teosinte breeders. The close genetic relationship between the two subspecies has stimulated interest in enriching the gene pool of maize with useful genes from maize. Likewise, maize-teosinte or teosinte-maize hybrids have also received attention for enhancing the fodder production potential of teosinte by taking advantage of hybrid vigor shown by the hybrids.

Hybrids of ssp. *mays* x ssp. *mexicana* crosses have statistically significant heterosis compared to the wild teosinte but not when compared to the cultivated parent (Guadagnuolo *et al.* 2006). Genetic distance GD among the germplasm lines has been quantified by means of morphological, biochemical and molecular analyses and by means of heterosis (Menkir *et al.* 2004 and Laborda *et al.* 2005). The

degree of heterotic effect of F_1 populations correlated with GD of the parental lines, as parents are more divergent, the heterosis is higher and vice-versa (Prasad and Singh 1986).

Cultivated maize derived from teosinte and their morphological differences resulted from human selection in the process of domestication (Matsuoka *et al*. 2002 and Doebley 2004). Despite being one of the cultivated species with greater genetic diversity, molecular analysis of the maize genome suggests that a single domestication event reduced diversity when compared with teosinte (Vigouroux *et al*. 2002 and Warburton *et al*. 2008). Most maize commercial varieties in the world has limited genetic diversity, whereas today the germplasm base in maize breeding programs is relatively narrow (Tarter *et al*. 2004).

With the development of molecular marker techniques, DNA polymorphisms have been used as markers to measure genetic diversity in many plant species. Some scientists have been trying to predict yield heterosis on the molecular level. The relationship between molecular marker distance and heterosis remains unclear. Some of the reports state significant association (Lanza *et al*. 1997; Amorim *et al*. 2006 and Srdic *et al*. 2007) whereas, the others state non-significant or no association between markers based GD and heterosis (Shieh and Thseng 2002; Legesse *et al*. 2008 and Devi and Singh 2011).

Molecular markers allow a direct comparison of the similarity of genotypes at the DNA level. Restriction fragment length polymorphisms (RFLPs; Botstein *et al*. 1980) have been used quite extensively for this purpose. However, RFLP assays are labor intensive and time consuming and, therefore, increasingly substituted by other marker techniques such as randomly amplified polymorphic DNA (RAPDs; Williams *et al*. 1990), Amplified fragment length polymorphism, (AFLPs; Zabeau and Vos, 1993), and simple sequence repeats (SSRs; Tautz, 1989). RAPDs marker has been used to investigate GD across the diverse species including segregating lines of maize (Ajmone-Marsan *et al*. 1993), to predict the best crosses among lines for hybrid development (Lanza *et al.,* 1997) and to assess genetic diversity among maize collections (Moeller and Schaal 1999).

Study of genetic diversity is the process by which variation among individuals or group of individuals or populations is analyzed by a specific method or a combination of methods. Maize breeders frequently use genetic diversity evaluation as an alternative method for germplasm selection. The objectives of this study were to estimate the variation of teosinte, maize parents and its hybrids for mean performance and degree of divergence, to assess the correlation of morphological genetic distance (GD_{mor}) with mean performance and to predict the best crosses among most distant hybrids selected from morphological clusters by RAPD molecular marker.

Materials and Methods

A local ecotype of teosinte (*Zea mays* spp. *mexicana*) and eight different maize genotypes (*Zea mays* L.) including three inbred lines, two single crosses, one three- way cross and two composite populations were used in this investigation (Table 1). The maize genotypes were kindly furnished by the Department of Maize Research, Agricultural Research Center, Giza, Egypt.

This investigation was carried out at Giza Agricultural Research Station, ARC during 2006, 2007 and 2008 summer seasons. Crosses of a local teosinte with eight different maize genotypes used to produce eight hybrids and their eight reciprocal crosses during 2006 season. The parents and their hybrids were sown in the field during 2007 and 2008 seasons using randomized complete block design with three replications. Each parent, hybrids and its reciprocal hybrids were grown in a plot represented by three ridges. Each ridge was 4 m long and 60 cm wide with single-plant hills spaced 20 cm apart (20 plants ridge^{-1}). Hills were over seeded then thinned to one plant/hill after complete emergence.

The morphological traits of the parents and their hybrids were measured on ten randomly selected plants in the field such as plant height (cm), number of basal tillers plant^{-1}, stem diameter (cm) at the third internodes above soil, length and width of the fourth basal leaf (cm), fourth leaf area (cm^2) estimated according to Stickler *et al*. (1961), leafiness % = (leaf weight)/ (leaf + stem weights)*100 on dry basis estimated from a random sub- sample of stem, dry weight plant^{-1} (g) and crude protein (%) according to A.O.A.C. (1980). Data of the two seasons combined after homogeneity of variance estimation using Bartlett test according to Gomez and Gomez (1984).

Assessment of genetic distance

The mean performance of growth characters, forage yield and quality traits of single plants at the first cut (60-days) over two seasons were considered in the analysis. Genetic distance was calculated to measure genetic diversity among nine parents, nine parents with 8 hybrids and 8 reciprocal hybrids using NTSYSpc software, version 2.0 (Rohlf, 1997). The cluster analysis was based on Nei's values (Nei, 1972) using the unweighted pair-group method with arithmetical average (UPGMA) and the relationships among them were visualized using a dendrogram.

The genomic DNA was isolated from leaf tissues of 4-week old seedlings from each teosinte, maize genotyps SC 10 and TWC 310, its 2 hybrids and 2 reciprocal hybrids using Dellaporta protocol (Dellaporta *et al.* 1983).

RAPD amplification

Ten primers, OPA 11-20 (Operon Technologies Inc.) and six primers, Ready-To-Go RAPD Primers (Amersham Biosciences) used for PCR amplification (Table 2) according to Williams *et al.* (1993). Polymerase chain reaction (PCR) was performed in a volume of 25 μL containing 100 mM of Tris-Hcl pH 8.8, 50 mM KCl, 0.01% Triton X-100, 1.14 mM MgCl$_2$, 0.175 mM of each dNTP, 0.5 μM primer, 25 ng of genomic DNA and 1 unit of Taq DNA polymerase. DNA amplification was performed in a DNA Thermal cycler UNO II (Biometra) programmed for an initial denaturation step of 5 min at 95°C, then 40 cycles at 95°C (1 min), 36°C (1 min), 72°C (2 min) for denaturation, primer annealing and primer extension, respectively, and a final primer extension at 72°C for 7 min. Amplified products were analyzed by electrophoresis in 1.5% agarose gels with 5 ng/ml ethidium bromide and photographed on a UV transillumenator.

RAPD analysis

RAPD fragments for each primer were scored as 0 for absent or 1 for presence in each parent, hybrids and reciprocal hybrids. The data was obtained only from seven polymorphic primers that produced reproducible and informative marker patterns. This data was transformed into a binary matrix. Genetic distance, cluster analysis and dendrogram of 3 parents with 2 hybrids and 2 reciprocal hybrids were constructed using NTSYSpc software as a measure of genetic distance from each primer independently.

Results and Discussion

Mean performance of teosinte, maize and their hybrids

The mean performance and ranges of teosinte, maize parents, hybrids (maize x Teosinte) and reciprocal hybrids (teosinte x maize) are given in Table (3). Teosinte showed the lowest values for all traits except tillers plant^{-1}, leafiness and crude protein percentage, which gave the highest values (10.68, 92.43 and 19.48, respectively). On the other hand, maize parent followed the opposite trend which gave the lowest values for tillers plant^{-1}, leafiness and crude protein percentage (1.0, 47.19 and 14.63,

respectively). Their hybrids exhibited substantial improvement over teosinte ranging from 8.86% in leaf length to 115.68% in dry weight except, tillers plant^{-1}, leafiness and crude protein percentage, which gave the lowest values from teosinte. The reciprocal hybrids showed greater increases over teosinte than the hybrids in all traits except crude protein percentage. These results are in agreement with Radwan *et al.* (2000).

Correlation coefficients between GD_{mor} of teosinte with maize parents and mean performance of morphological traits were calculated in maize parents, hybrids and their reciprocal (Table 4). The results showed that maize parents had highly significant positive correlation between GD_{mor} with all traits except tillers plant$^{-1,}$ leafiness and crude protein percentage, but hybrids had highly significant positive correlations between GD_{mor} with dry weight only. Reciprocal hybrids showed highly significant positive correlation in plant height, stem diameter and highly significant negative correlation with leafiness. GD_{mor} had highly significant positive correlation with plant height of maize parents and reciprocal hybrids with values 0.968 and 0.908, respectively. These results corroborate with previous studies (Lee *et al* 2007; Devi and Singh 2011). The ability to predict heterosis levels using genetic distance between the parents varied for the different traits. For some traits, it was possible to explain a significant proportion of the heterosis variation while other traits were difficult to predict (Flint-Garcia *et al*. 2009). Knowledge about germplasm diversity and genetic relationships among breeding materials could be valuable aid in crop improvement strategies. A number of methods are currently available for analysis of genetic diversity in germplasm accessions, breeding lines and populations. These methods have relied on pedigree data, morphological data, agronomic performance data, biochemical and molecular (DNA-based) data.

Morphological performance cluster analysis

The dendrogram, which represent the phylogenetic relationships of parents, parents pooled with each hybrid and reciprocal hybrid are given in Fig. (1). The dendrogram separated parents into two main clusters (Fig.1a). Teosinte was most distant in the first cluster and the second cluster has been less distance, which consisted of all maize parents. These results revealed the highly diversity between maize and teosinte. The dendrogram of pooled parents with hybrids revealed two main clusters (Fig. 1b). The first one was most distant, which include teosinte and all hybrids and the second included all maize parents. The first cluster had two sub clusters, teosinte in the first sub cluster and all hybrids in the second. These results showed high diversity between teosinte and each of maize parents and hybrids

although the hybrids closely related with teosinte in monophyletic cluster. The dendrogram of pooled parents with reciprocal hybrids revealed that the two main clusters were less distant Fig. (1c). The first cluster consists of teosinte, all maize parents and one hybrid (teosinte x SC 10). The second cluster included seven hybrids, which were divided into two sub clusters. The first sub cluster included six hybrids and the second sub cluster included one hybrid (teosinte x TWC 310). High diversity between reciprocal hybrids and all parents (teosinte and maize) except one hybrid (teosinte x SC 10) that is closely related with teosinte in the same monophyletic cluster with maize parents was observed.

The hybrids (maize x teosinte) closely related with teosinte whereas, the reciprocal hybrids (teosinte x maize) deviated from teosinte and closely related with maize. These results agree with Wang *et al*. (2008), who generated F_1 hybrids by using *Z. mays* ssp. *mexicana* as the female parent and cultivated maize inbred line Ye515 as the male parent to create new maize germplasm. In this study, when teosinte was used as male, the diversity was clear whereas, teosinte was used as female the hybrid (teosinte x SC 10) deviated from teosinte x maize hybrids and closely related with teosinte. Although, the SC 10 was ancestor of TWC 310 the diversity between their hybrids with teosinte differed from reciprocal hybrids. Therefore, we used RAPD-PCR to assess the diversity among teosinte, SC 10, TWC 310 and its hybrids at the molecular level.

RAPD analysis

Seven random primers out of the sixteen primers initially screened gave reproducible RAPD patterns and therefore were used to quantify the GD and perform dendrogram among teosinte, TWC 310, SC 10, hybrids and reciprocal hybrids. A total of 133 RAPD loci with minimum of 12 per primer AB-2 (Amersham Bioscience) to maximum of 28 loci per primer OPA-14 (Operon Technologies Inc.) were amplified (Fig. 2). Of these, 132 loci were polymorphic whereas one was monomorphic.

The level of polymorphism (99.25%) obtained was higher than in some maize studies, such as Lanza *et al*. (1997) and Bruel *et al*. (2006) who obtained 80.6% and 84.44%, respectively of polymorphism studying genetic divergence between inbred lines using RAPD markers. The level of polymorphism obtained depends on the degree of divergence between the genotypes under study.

The RAPD markers successfully grouped parents and its hybrids into two main clusters based on the dendrogram (Fig. 3), the first cluster was the most distant and the second less distant. The first cluster consisted of two maize parents (TWC 310 and SC 10) and two hybrids (teosinte x TWC 310 and TWC 310 x teosinte). The second cluster had teosinte parent and two hybrids (teosinte x SC 10 and SC

10 x teosinte). The maize parent TWC 310 closely related with hybrid (teosinte x TWC 310) whereas, the hybrid (teosinte x SC 10) closely related with the hybrid (SC 10 x teosinte). The phylogenetic relationship between teosinte and two maize parents revealed the high diversity between them based on RAPD molecular marker. These results from morphological performance and RAPD marker are in agreement with isozyme and chloroplast DNA analysis (Doebley 1990b), which confirmed by microsatellite genotyping (Matsuoka *et al.* 2002) and nucleotide diversity (Goloubinoff *et al.*, 1993; Hilton and Gaut, 1998). They concluded that all maize closed in a single monophyletic lineage and teosinte is extremely diverse. Although the relationship between molecular marker distance and heterosis remains unclear, RAPD molecular marker could be used as a tool for determining the extent of genetic diversity among maize genotypes (Liu *et al.* 1997; Lanza (1997) and its relatives and progenitors (Asif *et al*, 2006).

This study indicated possibility of using mean performance to estimate the diversity of teosinte, maize and its hybrids. In addition, the teosinte improvement may be generated by using *Z. mays* ssp. *mexicana* as the female parent and maize genotype TWC 310 as the male parent.

SUMMARY

A local ecotype of teosinte (Zea mays ssp. mexicana), eight different maize genotypes (Zea mays L.), their hybrids and reciprocal were used to estimate the variation of mean performance among them, assess the correlation of morphological genetic distance with mean performance and predict the best crosses from the most distant hybrids for teosinte improvement. The obtained data revealed that parental mean performance differed from hybrids performance. Correlation coefficients between morphological genetic distant (GDmor) of teosinte with maize parents and mean performance of the morphological traits showed that maize parents had highly significant positive correlation between GDmor with all traits except tillers plant-1, leafiness and crude protein percentage, but maize x teosinte hybrids had highly significant positive correlations between GDmor with dry weight only. Teosinte x maize hybrids (reciprocal) showed highly significant positive correlation in plant height, stem diameter and highly significant negative correlation with leafiness.

Cluster analysis based on mean performance of morphological traits displayed a clear separation of the teosinte, maize parents, their hybrids and reciprocal. When teosinte was used as male parent, the hybrids were closely related with teosinte while, teosinte x SC 10 hybrid and maize parents were closely related with teosinte when teosinte was used as female parent. Although the SC 10 maize parent is

ancestor of TWC 310 maize parent, high diversity between teosinte x SC 10 and teosinte x TWC 310 was established. The cluster analysis of RAPD marker showed that teosinte was most distant with either teosinte x TWC 310 hybrid or TWC 310 x teosinte hybrid. Therefore, the maize genotype TWC 310 could be used as a promising genotype for teosinte improvement.

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Table 1. Teosinte and maize parental genotypes, its pedigree and origin

Genotype	Sub-species	Pedigree	Origin
Local teosinte	Mexicana	Damietta District	Egypt
Inbred line 6	Mays	Rg-15 g.s. (Syn. Laposta x Ci 64) (S.C.14)	Egypt
Inbred line170	Mays	C.M.103	India
Inbred line171	Mays	C.M.104	India
SC 10	Mays	(Sd 7 x Sd 63)	Egypt
SC 129	Mays	(Gz 612 x Gz 628)	Egypt
TWC 310	Mays	(SC 10 x Sd 34)	Egypt
Giza 2	Mays	A composite population	Egypt
Laposta	Mays	A composite population	CIMMYT

Table 2. Primers used for RAPD analysis

Operon Technologies Inc.		Amersham Biosciences	
Primer	Sequence	Primer	Sequence
OPA-11	5'-CAATCGCCGT-3'	AB-1	5'-GGTGCGGGAA-3'
OPA-12	5'-TCGGCGATAG-3'	AB-2	5'-GTTTCGCTCC-3'
OPA-13	5'-CAGCACCCAC-3'	AB-3	5'-GTAGACCCGT-3'
OPA-14	5'-TCTGTGCTGG-3'	AB-4	5'-AAGAGCCCGT-3'
OPA-15	5'-TTCCGAACCC-3'	AB-5	5'-AACGCGCAAC-3'
OPA-16	5'-AGCCAGCGAA-3'	AB-6	5'-CCCGTCAGCA-3'
OPA-17	5'-GACCGCTTGT-3'		
OPA-18	5'-AGGTGACCGT-3'		
OPA-19	5'-CAAACGTCGG-3'		
OPA-20	5'-GTTGCGATCC-3'		

Table3. The range and mean performance ± standard error of teosinte, maize parents and its hybrids for the studied traits over two seasons.
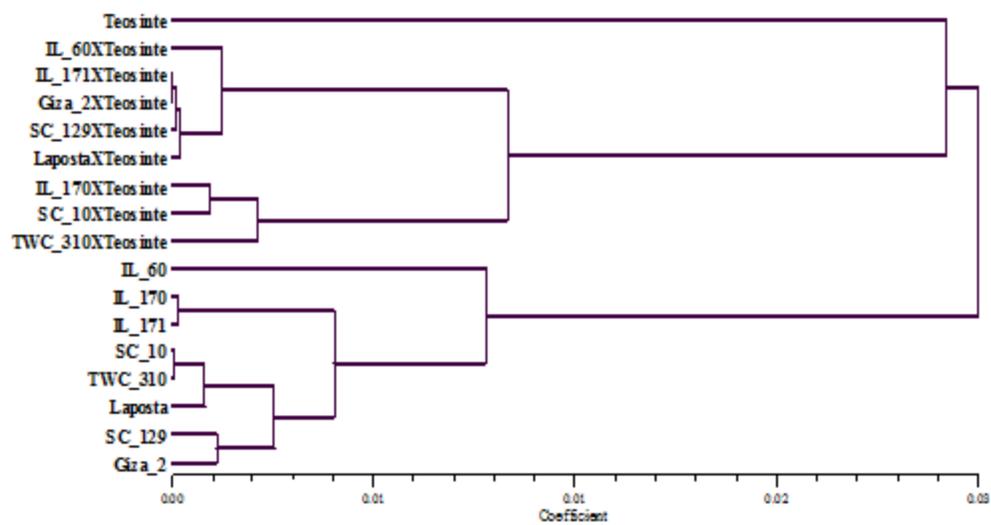
Trait	Teosinte	Maize Parents			Maize x Teosinte (hybrids)			Teosinte x Maize (Reciprocal hybrids)		
		Mean ± standard error	Range Min.	Max.	Mean ± standard error	Range Min.	Max.	Mean ± standard error	Range Min.	Max.
Plant height (cm)	66.28	132.03 ± 3.42	89.80	179.92	108.44± 3.66	83.33	131.80	110.86± 3.75	85.00	124.20
Tillers plant-1	10.68	1.00± 0.00	1.00	1.00	4.11±0.29	3.33	4.80	8.24± 0.44	5.80	10.10
Stem diameter(cm)	1.90	2.37± 0.06	1.90	2.85	2.89± 0.12	2.42	3.27	3.17± 0.11	2.30	3.70
Leaf length	81.47	85.12±1.50	73.00	96.05	88.69± 2.75	74.33	107.13	90.91± 2.28	82.50	101.10
Leaf width	4.38	6.13± 0.14	4.40	7.58	6.06± 0.18	4.80	6.85	6.46±0.15	6.00	7.60
Leaf area (cm^2)	266.18	396.27± 14.52	239.95	537.02	403.94± 21.28	265.70	548.32	439.05± 19.47	381.00	530.00
leafiness	92.43	47.19±0.75	42.30	52.85	61.13± 0.57	57.03	65.80	70.10± 1.01	64.90	75.72
Dry weight (g)	90.07	98.94± 3.82	57.25	138.50	194.26±11.33	160.07	234.75	335.31±15.69	210.6	515.10
Crude protein (%)	19.48	13.55± 0.13	12.15	14.63	18.24± 0.11	17.00	19.70	16.57± 0.18	14.52	18.52

Table 4. Phenotypic correlation between genetic distance of teosinte with maize and mean performance of maize parents, hybrids and reciprocal hybrids
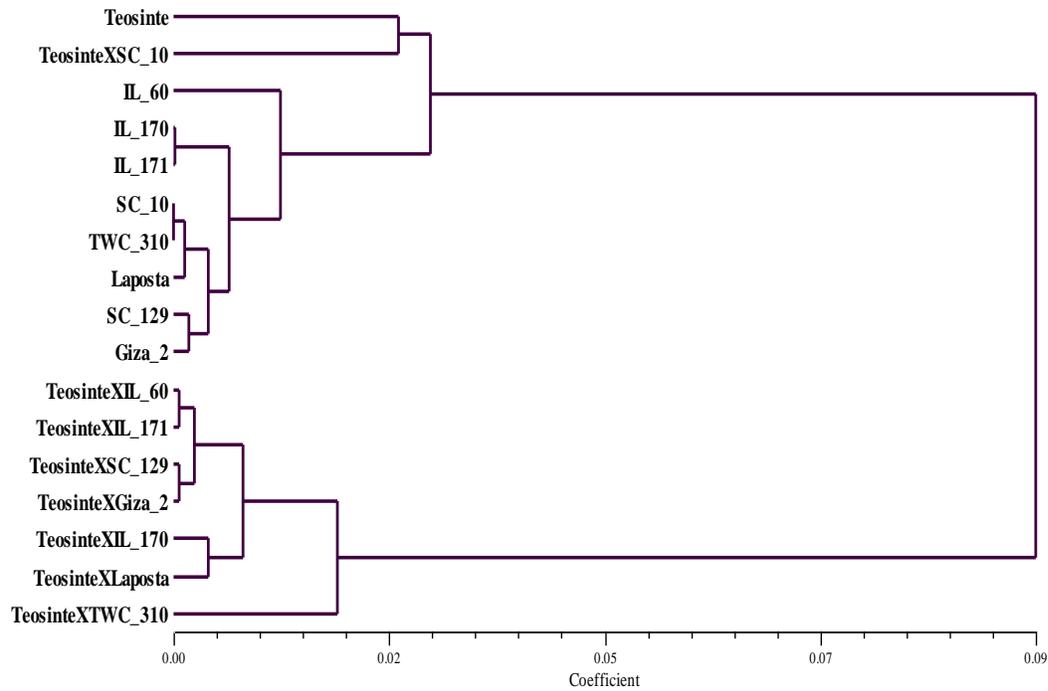
| Trait	Maize Parents	Maize x Teosinte (hybrids)	Teosinte x Maize (Reciprocal hybrids)
Plant height (cm)	0.968**	0.205	0.908**
Tillers plant^{-1}	0.000	-0.034	-0.262
Stem diameter (cm)	0.503**	0.291	0.700**
Leaf length	0.823**	0.358	0.007
Leaf width	0.666**	0.058	-0.160
Leaf area (cm^2)	0.788**	0.284	-0.164
Leafiness	0.392	-0.138	-0.717**
Dry weight (g)	0.828**	0.583**	0.132
Crude protein (%)	-0.306	0.173	-0.337
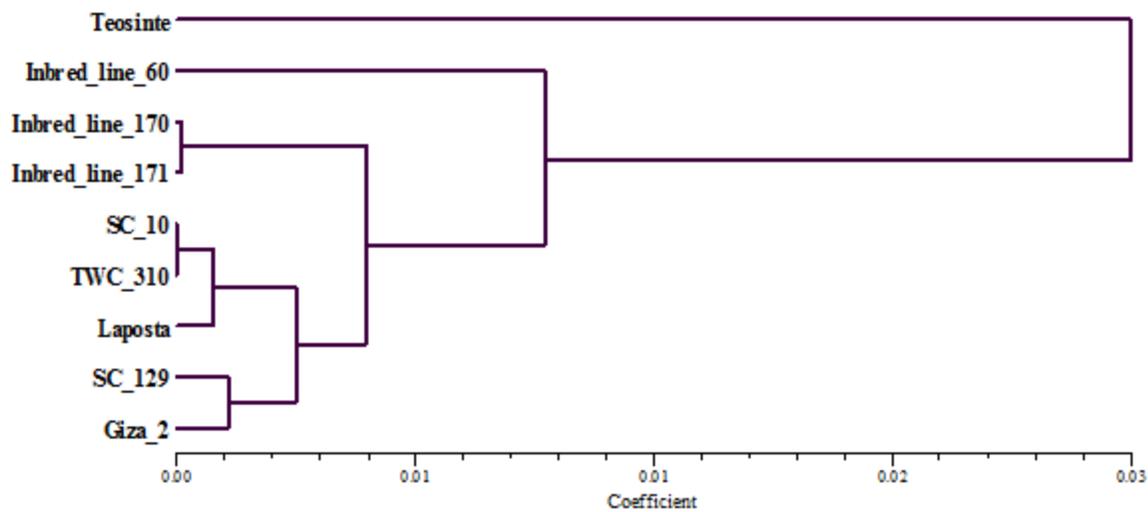
(A)

(B)

(C)

Fig.1. The dendrogram of teosinte, maize parents and its hybrids in morphological data using Nei's distance based on UPGMA method: (A) The dendrogram of teosinte and maize parents; (B) The dendrogram of parent's genotypes and their hybrids; (C) The dendrogram of parent's genotypes and their reciprocal hybrids.
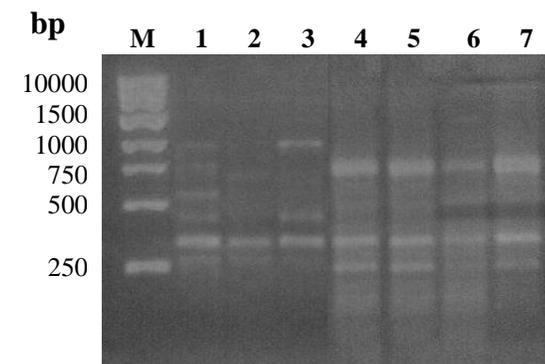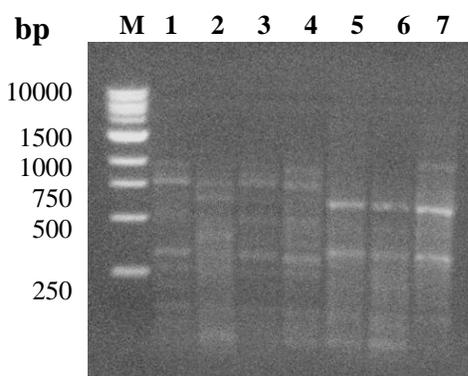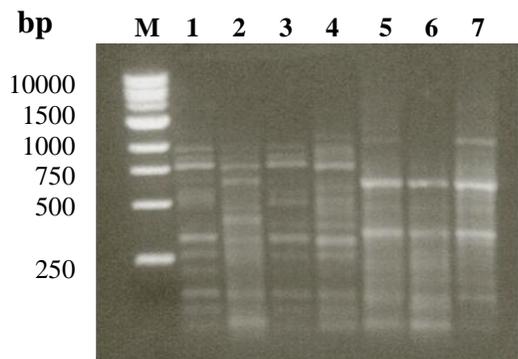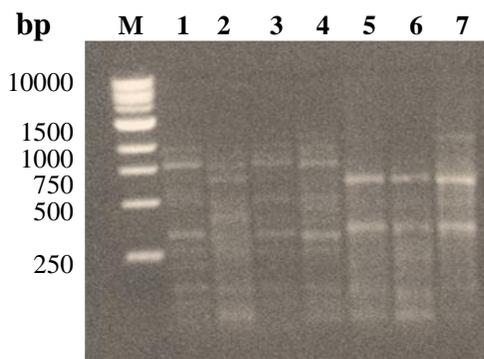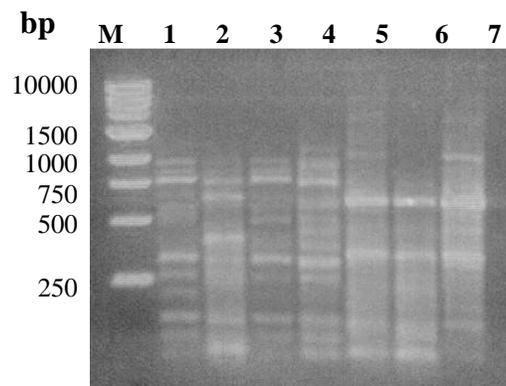
AB-1
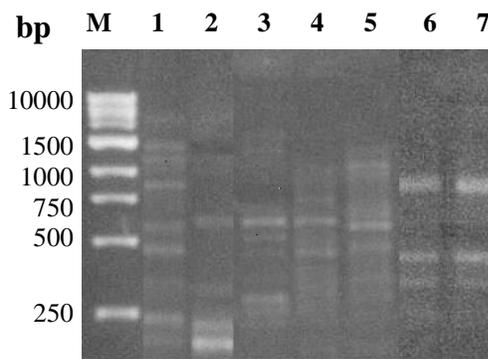
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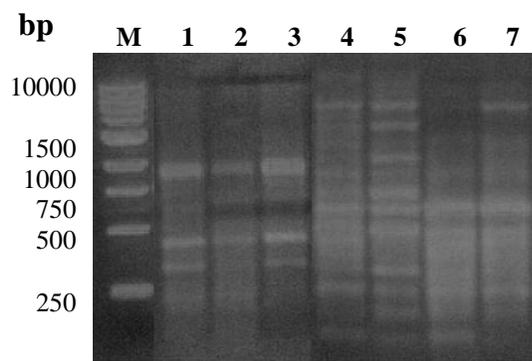
AB-3

OPA-12

OPA-14

OPA-16

OPA-18

Fig. 2. RAPD profiles of teosinte, maize (TWC 310 and SC 10), hybrids and reciprocal hybrids. M: 1kb DNA leader; 1: TWC 310; 2: SC 10; 3: Teosinte x TWC 310; 4: TWC 310 x Teosinte; 5: Teosinte x SC 10; 6: SC 10 x Teosinte; 7: Teosinte .
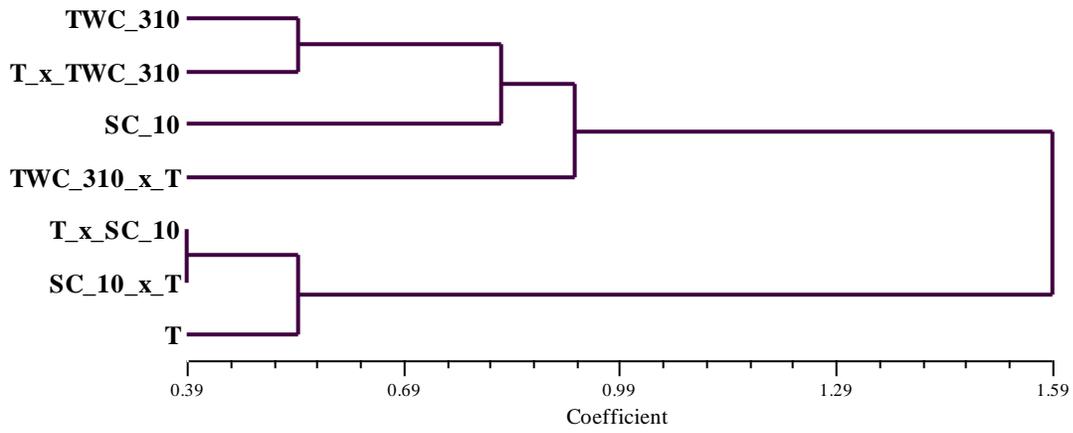
16

Fig. 3: The dendrogram of teosinte (T), maize (TWC 310 and SC 10), hybrids and reciprocal hybrids in RAPD data using Nei's distance based on UPGMA method.