

# GENETIC VARIATION AND PHYLOGENETIC RELATIONSHIPS AMONG MAIZE TYPES AND TEOSINTE AS REVEALED BY ISOZYMES AND RAPD MARKERS

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**M**aize or corn (*Zea mays* L.,  $2n = 20$ ) is one of the most diverse grain crops found in nature. Selection pressure by both humans and nature has resulted in many different types of corn that vary in physical and chemical characteristics. The most common types of corn include dent, flint, flour, sweet, pop, and waxy corn (Knott *et al.*, 1995). The physical appearance of each kernel type is determined by its pattern of endosperm composition as well as quantity and quality of endosperm (Zilic *et al.*, 2011).

Teosinte is the wild relative of maize. Both teosinte and maize belong to the genus *Zea*, which has four species: *Z. luxurians*, *Z. diploperennis*, *Z. perennis* and *Z. mays*. *Zea mays* is in turn divided into four subspecies; *mexicana huehuetenangensis*, *parviglumis* and *mays*. *Zea mays* ssp. *mays* is the only cultivated species (maize), the other species and subspecies are wild grasses, referred to as teosintes (Doebley, 1990).

Analysis of genetic divergence in maize can provide some interesting information about differentiation, adaptability and interrelationships of genotypes and giving graphical assessment of genetic

variability. 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. Isozymes are good biochemical markers used as a powerful tool both in characterization of cultivar and in genetic and phylogenetic studies for many plant species (Orasmo *et al.*, 2007; Frigo *et al.*, 2009). Isozymes were developed as first co-dominant markers (Ivy *et al.*, 2010). Although, nowadays new DNA-based molecular techniques are used, isozymes still represent a powerful tool for evaluation of genetic variability within and between populations of plants (Zeidler, 2000). Several recent studies have confirmed that the use of isozymes analysis as a biochemical marker can be useful for genomic assessment in *Zea mays* L. breeding programs (Pereira *et al.*, 2008; Dowd *et al.*, 2010).

Applications of molecular markers, particularly in the last two decades, have led to new insights into domestication events in maize (Matsuoka *et al.*, 2002b), assessing the genetic diversity in the

maize gene pool, understanding phylogenetic relationships and gene flow between maize landraces and the wild relative, teosinte (Van-Heerwaarden *et al.*, 2011; Warburton *et al.*, 2011), helping in tracking the migration routes of maize from the centers of origin (Vigouroux *et al.*, 2008) and understanding the fate of genetic diversity during maize domestication. Molecular markers are stable and detectable in all plant tissues regardless of the growth, differentiation, development or status of cells. They have also many advantages such as abundance in polymorphism, no pleiotropic effect; less affected by environment and subjected to rapid detection (Agarwal *et al.*, 2008). Among molecular markers, random amplified polymorphic DNAs (RAPDs) which have been extensively used in genetic research owing to their speed and simplicity (Williams *et al.*, 1990). This technique has potential to differentiate and detect differences among genotypes (Schulman, 2007; Bernardo, 2008).

The present work was undertaken to highlight the genetic variation and phylogenetic relationships among maize types (dent, flint, sweet and pop corn) and their wild relative (teosinte) using peroxidase and esterase isozymes in addition to RAPD markers.

## MATERIALS AND METHODS

### *Plant materials*

A local ecotype of teosinte (*Zea mexicana*) and four different types of yellow maize (*Zea mays*) including types of

the dent, flint, sweet and pop corn were chosen for this study (Table 1). The grains of these genotypes were provided by germplasm bank of the Department of Maize Research, Agricultural Research Center, Giza, Egypt.

### *Germination and harvest*

Twenty grains of each genotype were planted in pots at the Department of Genetics, Faculty of Agriculture, Kafr El-Sheikh University, Kafr El-Sheikh, Egypt. Four weeks after planting, the second and the third leaf were harvested and stored at -20°C until performing the isozymes and RAPD assays.

### *Electrophoretic analysis of isozymes*

Two isozymes systems (peroxidase and esterase) were applied in non-denaturing polyacrylamide gel electrophoresis for detecting isozymes variation among the five studied genotypes. Leaf samples (0.250 gm) were frozen with liquid nitrogen and ground in a mortar and pestle with 1 ml of 20% sucrose to extract peroxidase and esterase isozymes. Peroxidase isozymes were localized on the gel using the staining solution composed of 250 mg of benzidine dihydrochloride (moistened with 4 drops of glacial acetic acid) in 100 ml H<sub>2</sub>O and 10 drops of 1% H<sub>2</sub>O<sub>2</sub> was added immediately before being used according to Scandalios (1964). However, esterase isozymes patterns were detected on the gel as described by Vallejos (1983) using  $\alpha$  and  $\beta$ -naphthyl acetate (40 mg of each) and 250 mg Fast blue RR dissolved in 98 ml of 0.1 M

phosphate buffer (pH 6.5).

### ***DNA extraction***

For isolating genomic DNA, equal quantities of leaf tissue harvested from 20 seedlings per genotype at 2-3 leaf stage were bulked and used in DNA isolation using CTAB-chloroform based method according to Saghai-Marroof *et al.* (1984).

### ***Random amplified polymorphic DNA (RAPD) analysis***

RAPD analysis was carried out using seven decamer random primers (OP-A9, OP-B5, OP-B6, OP-B7, OP-B8, OP-B10 and OP-B14) which were purchased from Bio Basic Inc, Canada. The list of primers and their sequences are presented in Table (2). The optimization of PCR conditions for each primer was performed in a 25 µL reaction volume including 2 µL of 40 ng of genomic DNA. Final concentrations were 1 x buffer (Mg<sub>2</sub><sup>+</sup> free), 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs mix, 0.8 µM primer, 1 U Taq DNA Polymerase (ROVALAB, Germany). Amplifications were carried out in a thermal cycler according to manufacture instructions as follow: the initial amplification program started with denaturation at 94°C for 5 min, followed by 35 cycles consisting of 30 sec. of denaturation at 94°C, 45 sec. of annealing at 30°C and 1.5 min of elongation at 72°C. The program ended with a final elongation step at 72°C for 2 min. Amplification products were separated on 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet

light. A known DNA Ladder (50 bp DNA Ladder ready-to-use, Cat-no: 300003, GeneON) was run against the PCR products.

### ***Data analysis***

The data generated from isozymes (native gel) banding patterns in addition to the banding patterns of the seven RAPD primers were introduced to SPSS package program according to binary values of (1) and (0) for the presence and absence of bands, respectively. The genetic distances among the genotypes were assessed based on Jaccard's similarity coefficient (Jaccard, 1901) using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) analysis (Nei, 1973).

## **RESULTS AND DISCUSSION**

### ***Genetic polymorphism among the studied genotypes***

#### ***a. Isozymes polymorphism***

Genetic variations at the biochemical level were studied using native-polyacrylamide gel electrophoresis (Native-PAGE). Considerable variations in both isozymes systems; peroxidase and esterase, were found among all the studied genotypes (Fig. 1). A total of 51 bands with 80.39% polymorphism were detected using the two isozymes systems, ten bands were monomorphic and the other 41 bands were polymorphic (Table 3). This polymorphism level was higher than the polymorphism data recorded by Stuber and Goodman (1983) from 23 isozymes loci

which provide unique patterns for 73% of the 406 maize inbred lines they surveyed. In the current study, the PAGE migration pattern of peroxidase gave a total of 24 bands for the studied maize types and their ancestor teosinte, 16 bands were polymorphic (66.67% polymorphism) while eight bands were found to be common in all genotypes. Native-PAGE analysis for esterase isozymes exhibited a total of 27 bands, 25 polymorphic bands (92.59% polymorphism) and two monomorphic bands. This result indicated that esterase isozymes exhibited higher polymorphic patterns than peroxidase among the studied genotypes. Esterases in plants have been intensively explored as biochemical markers due its high allele polymorphism (Carvalho *et al.*, 2003; Resende *et al.*, 2004) and its high number of loci by using the polyacrilamide gel electrophoresis system (Carvalho and Machado, 2004). On the other hand, the electrophoretic bands; for both peroxidase and esterase isozymes, showed wide variation in their intensities ranging from faint to dark, reflecting different activities in the seedling tissues of these genotypes. These results might reflect a case of variations in isozymes banding patterns among tested genotypes. The observed isozymes patterns with both peroxidase and esterase in selected genotypes can be explained in terms of allelic differences. Isozymes are all functionally similar forms of enzymes including all polymers of subunits produced by different gene loci or by different alleles at the same locus. Their electrophoretic mobilities are the result of different size and shapes of enzyme mole-

cules and their variation is a good indicator of genetic diversity (Shannon, 1968). So, electrophoresis separation of isozymes has been widely used both in taxonomic and genetic studies in maize. In this regard, Pereira *et al.* (2008) utilized isozymes markers for assess the changes in the genetic variability and distance in a Brazilian composite population of popcorn following four cycles of recurrent selection for yield. They found that the variability detected in this population was less than the breeds of the Northern Flint or Southern Dent (Labate *et al.*, 2003). Many of the Mexican and South American landraces of maize have already been characterized using isozymes (Sanchez *et al.*, 2000 & 2007). Furthermore, Gimenes and Lopes (2000) used isozymes analysis in studying 15 maize populations derived from three indigenous maize races.

#### ***b. RAPD Polymorphism***

Using RAPD analysis with the five genotypes, a total of 116 bands were obtained from seven decamer primers (Fig. 2). The number of bands ranged from 6 bands for the primer OP-B10, to 24 bands for the primers OP-B6 and OP-B7 (Table 3). The primer OP-B6 produced the highest number of polymorphic bands (24 bands). On the other hand, the primer OP-B10 generated only four polymorphic bands. The percentage of polymorphic loci was 87.07% which indicates a high level of polymorphism. This level of polymorphism was higher than the values observed in some maize studies, such as Bruel *et al.* (2006) who obtained 84.44%

of polymorphism in case of studying genetic divergence between maize inbred lines using RAPD markers. In addition, Carvalho *et al.* (2002) had reported 75.8% polymorphism with ISSR markers in maize. The obtained level of polymorphism depends on the degree of divergence between the genotypes under study. Molecular characterization of maize landraces of India (Prasanna *et al.*, 2010; Sharma *et al.*, 2010) and more recently of America and Europe (Warburton *et al.*, 2011), led to significant insights with regard to the genetic diversity and population structure. Teosinte became one of the best-characterized systems in plant molecular population genetics, including studies based on DNA samples recovered from archeological specimens (Jaenicke-Despres *et al.*, 2003).

### ***Genetic relationships among the studied genotypes***

#### ***a. Genetic relationships based on isozymes markers***

Similarity coefficient between each pair of genotypes for peroxidases as well as esterases is presented in Table (4). The maximum similarity coefficient of 0.842 and 0.792 for peroxidases and esterases, respectively, was found between dent and flint types indicating a high degree of genetic similarity between them. In addition, the study showed that there is an association between the dendrograms obtained by peroxidases and esterases markers (Fig. 3), which represent the phylogenetic relationships of teosinte with maize types. The

dendrograms of both isozymes systems revealed two main clusters. The first cluster has most distant which include teosinte, whereas the second cluster included the four maize types, which were divided into two sub-clusters. The first sub-cluster included dent and flint types since they associated with the highest genetic similarity coefficient (0.842 and 0.792 for peroxidases and esterases, respectively) and the second sub-cluster included the types of sweet and pop corn with similarity coefficient of 0.722 and 0.667 for peroxidases and esterases, respectively. This result is in agreement with Reddy *et al.* (2012), who reported that today's dent corns originally came from crosses between late-flowering Southern dent corns (Gourdseed) and early-flowering Northern flints. The dendrogram of peroxidases (Fig. 3a) revealed that the two main clusters were less distant than the dendrogram of esterases (Fig. 3b). These results showed high diversity between teosinte and each type of maize.

This result is in consistent with archaeological data and previous studies on maize domestication (Koo and Jiang, 2008; Fu *et al.*, 2010). The theory that maize originated from teosinte has also been considered in detail by Kato (1984). His study showed a very similar chromosome knob configuration in teosinte and maize, and supported the idea that gene flow from teosinte to maize occurred since the beginning of maize cultivation. This gene flow is considered to be responsible for the large genetic and phenotypic vari-

ability in maize today. According to Matsuoaka *et al.* (2002a), the diversification of maize races was a result of continuous hybridizations between diverse populations of maize with teosinte in many including novel environments. This resulted in the introgression of a large diversity into maize and might have established the maize races known today. This allowed the introgression of new traits into the different types of maize and selection within them, creating many different morphological and physiological phenotypes in maize. This confirms earlier evidence of allozyme alleles that occurred in *ssp. mexicana* and in sympatric maize populations, suggesting that these alleles had probably introgressed into maize from *mexicana* *ssp.* (Doebley, 1990). These results are in accordance with the findings of Abdel-Tawab *et al.* (1982), who reported a close relationship between maize and teosinte (*Zea mexicana*) based on protein electrophoretic patterns.

#### ***b. Genetic relationships based on RAPD markers***

The RAPD data were used to estimate the genetic similarities and the phylogenetic relationship among the five genotypes. A similarity coefficient matrix among all genotypes is presented in Table (5). The similarity values among the five genotypes ranged from 0.322 to 0.588. The highest similarity index (0.588) was observed between the flint and sweet types, followed by the similarity index between sweet and pop corn (0.532), whereas the lowest one (0.322) was rec-

orded between dent and teosinte. Moeller and Schaal (1999), using RAPD markers in Native American maize collections of Great Plains, showed a similarity index that varied from 0.44 to 0.80.

The UPGMA dendrogram based on the similarity index separated the five genotypes into two main clusters (Fig. 4). Teosinte was the most divergent in the first cluster and the second cluster has been less distance, which consisted of all maize types. These results were in agreement with the results of isozymes which revealed high diversity among maize types and teosinte. There were close relationship among the types of flint and sweet corn, which clustered with a similarity coefficient of 0.588. This association is consistent with their common origin since both of them contain the flint gene pool. Historical records indicated that sweet corn germplasm is largely derived from the Northern Flint race of corn (Gerdes and Tracy, 1994). On the other hand, the highest estimated distance was observed between the ancestor germplasm teosinte and dent corn type. Many studies assessed the degree of genetic relatedness between maize and teosinte. Doebley and Iltis (1980) reported that the genus *Zea* was divided into sections *Luxuriantes* (teosinte) and *Zea*. These subgeneric boundaries of *Zea* are defined on UPGMA-based dendrogram derived from RAPD analysis. Gene flow among maize and teosinte populations has occurred readily since maize's domestication 9,000 years ago (Ellstrand *et al.*, 2007; Dyer *et al.*, 2009). Van-Heerwaarden *et al.* (2011)

explained the genetic evidence for an apparent highland origin of modern maize by gene flow from *Z. mays* ssp. *mexicana* (teosinte).

On the other hand, many studies explained the genetic diversity in maize types. Studies comprising 28 open pollinated varieties of maize (Parentoni *et al.*, 2001) showed that flint and semi-flint genotypes as well as the dent and semi-dent germplasm were placed in different groups by RAPD markers. Recently studies of Munhoz *et al.* (2009) and Leal *et al.* (2010) reported that investigation the genetic diversity of popcorn is extremely important especially in comparison with wild forms of popcorn, common maize, sweet maize and ancestral species. Hence, in popcorn, the genetic basis is narrow as it is originated only from the dent type of common maize (Kantety *et al.*, 1995).

In conclusion, these results demonstrate the efficiency of isozymes and RAPD markers in separation and identification the variability of maize types evaluated in present work with their ancestor teosinte. By characterizing these genotypes, it is possible to identify the most genetically distinct genotypes and to use them in breeding programs to maximize the use of genetic resources.

### SUMMARY

Genetic variation and phylogenetic relationships among four types of yellow maize (*Zea mays* L.); dent, flint, sweet and pop corn, and their wild relative; teosinte

(*Zea mexicana*), were assessed using isozymes and random amplified polymorphic DNA (RAPD) markers. The results indicated that the percentage of polymorphic loci; for peroxidase and esterase isozymes, were 66.67% and 92.59%, respectively. By applying RAPD analysis, 116 bands were obtained from seven primers with 87.07% polymorphism. The UPGMA dendrogram based on genetic distance segregated the five genotypes into two main clusters. Both isozymes and RAPD markers separated teosinte into the first cluster, whereas the four maize types were grouped together in the second cluster. Dent and flint types were much close to each other with high similarity indices; 0.842 and 0.792 based on peroxidase and esterase isozymes, respectively. Furthermore, the flint type closely related to sweet type in RAPD cluster (similarity index of 0.588). This high variability detected among maize types and teosinte can be used in breeding programs to maximize the use of genetic resources.

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Table (1): Pedigree and description of the five studied genotypes; teosinte and types of dent, flint, sweet and pop corn.

Genotype	Grain type	Scientific name	Pedigree	Description
Teosinte	-	<i>Zea mays</i> spp. <i>Mexicana</i>	Damietta District	Maize ancestor
SC173	Dent corn	<i>Zea mays</i> indentata	Gz647 x Gz666	Hard endosperm is present on the sides and base of the kernel. The remainder of the kernel is filled with soft starch; when the grain starts drying the soft starch at the top of the kernel contracts, producing the depression for which it is named.
SCSk142	Flint corn	<i>Zea mays</i> indurate	Gz653 x Sk6057/7-2	Kernels are characterized by their high percentage of hard endosperm around a small soft centre.
SCSk-sw1	Sweet corn	<i>Zea mays</i> rugosa	ZPL620/121 x ZPKŠ 8/1-131	The developing grain of sweet maize is higher in sugar content due to one or more recessive mutations blocking conversion of sugar to starch.
SCSk-pop1	Pop corn	<i>Zea mays</i> everta	HP6208 x HP6252	Kernels are characterized by a high proportion of hard endosperm, which is much higher than in any other maize kernel.

Table (2): List of random amplified polymorphic DNA (RAPD) primers and their nucleotide sequence.

Primer code	Sequence (5'→3')
OP-A9	GGGTAACGCC
OP-B5	TGCGCCCTTC
OP-B6	TGCTCTGCCC
OP-B7	GGTGACGCAG
OP-B8	GTCCACACGG
OP-B10	CTGCTGGGAC
OP-B14	TCCGCTCTGG

Table (3): Level of polymorphism among teosinte and maize types (dent, flint, sweet and pop corn) based on isozymes and RAPD markers.

Markers	Genotypes					TB	PB	MB	P%
	Teosinte	Dent	Flint	Sweet	Pop				
Isozyme markers									
Peroxidase	12	17	18	18	13	24	16	8	66.67
Esterase	8	21	22	14	16	27	25	2	92.59
Total	20	38	40	32	29	51	41	10	80.39
RAPD markers									
OP-A9	4	7	5	4	4	8	7	1	87.50
OP-B5	6	14	9	10	7	18	16	2	88.89
OP-B6	15	0	13	14	12	24	24	0	100.00
OP-B7	13	12	15	17	17	24	17	7	70.83
OP-B8	7	6	9	8	3	14	13	1	92.86
OP-B10	4	4	4	5	4	6	4	2	66.67
OP-B14	19	8	12	10	3	22	20	2	90.91
Total	68	51	67	68	50	116	101	15	87.07

TB, Total Band; PB, polymorphic bands; MB, monomorphic bands; P%, polymorphism %.

Table (4): Similarity matrix among teosinta and the four maize types based on peroxidases (above the diagonal) and esterases (below the diagonal) markers.

	Teosinte	Dent	Flint	Sweet	Pop
Teosinte	-	0.526	0.500	0.500	0.562
Dent	0.318	-	0.842	0.667	0.500
Flint	0.200	0.792	-	0.636	0.476
Sweet	0.158	0.458	0.440	-	0.722
Pop	0.263	0.542	0.462	0.667	-

Table (5): Similarity matrix among teosinta and the four maize types based on RAPD markers.

	Teosinte	Dent	Flint	Sweet
Dent	0.322			
Flint	0.378	0.457		
Sweet	0.417	0.434	0.588	
Pop	0.405	0.384	0.462	0.532

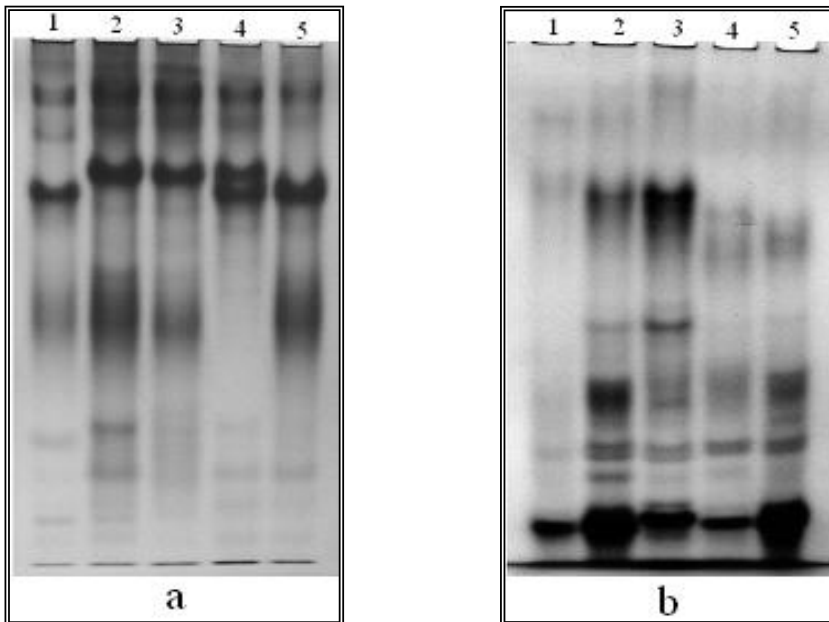


Fig. (1): Electrophoretic patterns of peroxidases (a) and esterases (b) for teosinte and the four maize types in non-denaturing gel electrophoresis. Lane 1: teosinte, lane 2: dent, lane 3: flint, lane 4: sweet and lane 5: pop corn.

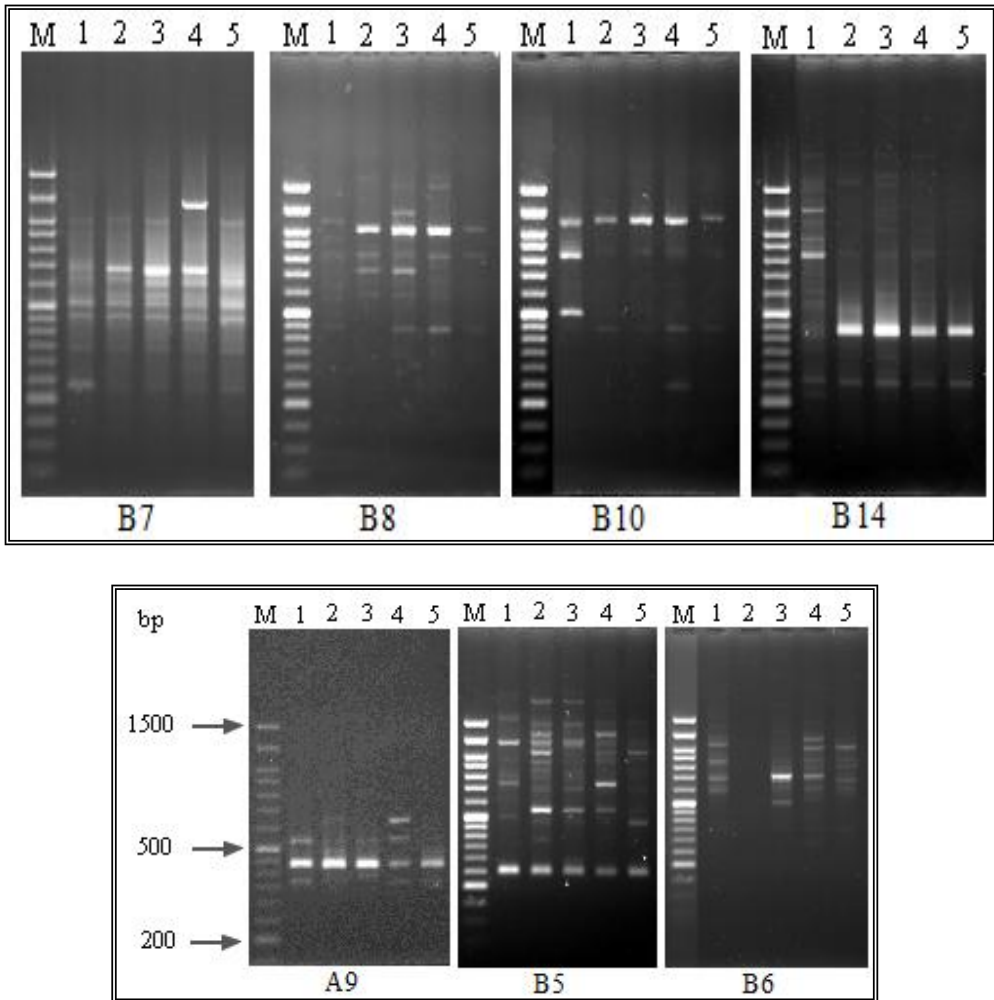


Fig. (2): RAPD profiles of teosinte and the four maize types. M: 50 bp DNA leader; 1: Teosinte; 2: dent; 3: flint; 4: sweet and 5: pop corn.

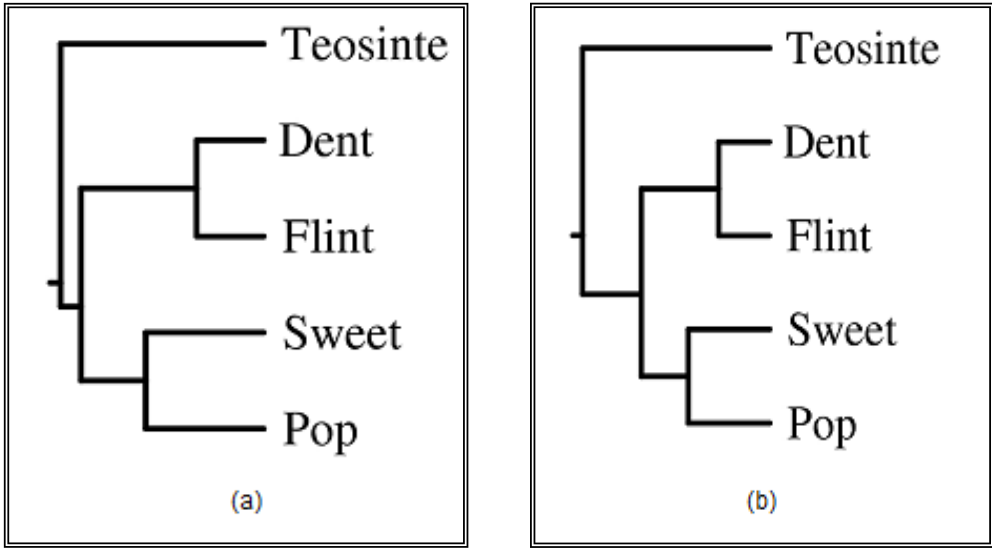


Fig. (3): The dendrogram of teosinte and maize types (dent, flint, sweet and pop corn) based on (a) peroxidase and (b) esterase isozymes data using UPGMA method.

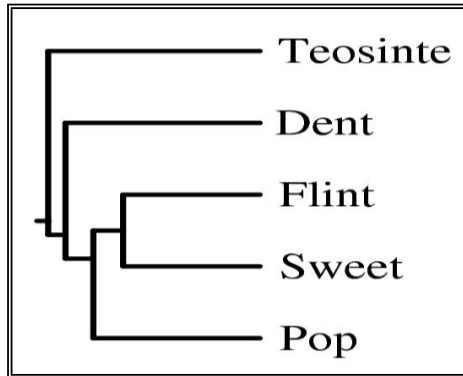


Fig. (4): The dendrogram of teosinte and maize types (dent, flint, sweet and pop corn) based on RAPD data using UPGMA method.