Relationship between hybrid performance and genetic diversity based on ISSR-PCR markers in Pepper (*Capsicum annuum. L.*)

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ABSTRACT

Capsicum annuum is widely cultivated around the world. The enormous genetic diversity available for pepper breeding has facilitated the development of new varieties and hybrids. Several pepper breeders coincide in that the level of heterosis exhibited by pepper hybrids is directly related with the genetic distance between their parental lines. Therefore, it is important to develop reliable techniques for the estimation of genetic distance. The search for superior hybrid parents in pepper breeding programs is commonly based on the estimation of the General Combining Ability (GCA) and Specific Combining Ability (SCA) of inbred lines. However, the application of this procedure is expensive and time consuming. The development of DNA based molecular markers represents an alternative procedure for the identification of promising parental lines for high performance of hybrid production. The Inter Sequence Repeat Polymerase Chain Reaction (ISSR-PCR) markers have been widely used for the estimation of genetic distance among closely related individuals. Thus molecular markers, such as ISSR-PCR, could be used for germplasm classification and clustering. This could be a valuable information for heterosis prediction. The aim of this research was to study the relationship between the genetic distances, measured using ISSR markers among parental lines, and the heterosis observed as yield, of the F_1 hybrids as estimations of GCA, SCA. Heterosis were performed using seven elite lines and their F_1 hybrids. The 28 genotypes (7 lines and 21 hybrids) were distributed in the field following a complete block design with three replicates. The genotypes tested were statistically different for fruit yield/plant. Among all the hybrids and parental lines, the F_1 (P_4 x P_6) produced the highest yield. Also, GCA and SCA were statistically significant, with P_6 showing the highest GCA effect, and the F_1 (P_6 x P_7) the highest SCA. The F_1 (P_4 x P_6) showed the highest heterosis (108.95%). Genetic distances calculated by ISSR markers produced a dendogram with seven nodes for the parental lines. However, the correlation between the matrix of genetic distances among parental lines and the matrix of heterosis gave positive significant correlation (r = 0.574).

Key Words: *Capsicum annuum* — ISSR_PCR — cluster analysis — genetic diversity — heterotic group.

INTRODUCTION

Pepper *(Capsicum annuum)* is widely cultivated around the world as well as in Egypt. The main producers are Hungary, India, Mexico, China, and Korea. The more probable ancestor of *Capsicum annuum* is the "Piquín" pepper (*C. annuum* var. *aviculare*). Piquín is extensively distributed from South America up to the south of The United States. However, probably the main area of domestication includes Central America and México **(De Witt and Boslland, 1996).**

The enormous genetic diversity available in Egypt has facilitated the development of new varieties and hybrids. The methods used for pepper breeding in Egypt include pedigree, single seed descend, and a combination of both (**Hassan, 1993).**

Plant breeders working with several species have reported a direct relationship between the level of heterosis exhibited by the F_1, and the divergence between their parents **(Lee** *et al***., 1989**; **Sekhon and Gupta, 1995).** Therefore, estimation of genetic distance can be useful for prediction of high performance crossings **(Smith** *et al***., 1990).**

Furthermore, diallelic designs can be used to estimate the genetic components of phenotypic variation. However, the application of these techniques is expensive and time consuming. The use of molecular markers has been proposed as an alternative procedure **(Toby** *et al*., **1999).** Molecular markers based on polymorphisms of the DNA are specially useful for this enterprise, because they are not affected by the environment **(Tatineni** *et al*., **1996)**.

The Random Amplified Polymorphic DNA (RAPD-PCR) and Inter Sequence Repeat Polymerase Chain Reaction (ISSR-PCR) technique has been widely used to quantify the genetic variation due to its simplicity and power to detect differences, even among closely related individuals, in species of *Brassica* **(Jain** *et al.*, **1994**), , *Pisum* **(Hoey** *et al*., **1996),***Wheat* **(Liu** *et al.,***1999***)* and *Pepper* (**Geleta** *et al.* **2004** and **Kumar** *et al.*, **2001).** Additionally, this type of markers is extremely useful to estimate genetic distances, mainly due to their productivity in terms of number of markers per essay, sensibility, quickness, and possibility of automation **(Laucou** *et al*., **1998).**

The main aim of this research was to determine the relationship between genetic distance, based on ISSR-PCR markers, and the heterosis for fruit yield in pepper. In addition, *GCA* and *SCA* were quantified to study several parental lines and the heterosis shown by their F_1 hybrids.

MATERIALS AND METHODS

This research was conducted at the Agricultural Experimental Station of the Genetics Department, Faculty of Agriculture at Moshtohor, Benha University, during summer seasons of 2004 to 2006.

Plant Material:

Seven pepper parental germplasm **(***Capsicum annuum. L***.)** i.e. P_1: (PI:15925601_U.S.A.),P_2: (PI: 5927201_U.S.A.), P_3: (PI:16736101_ *Turkey*), P_4: (PI: 13582401_ *Afghanistan*), and P_5: (PI: 13587301_ *Pakistan*) were kindly provided by University of Georgia, Plant Genetic Resources Conservation Unit, U.S.A., while seeds of the lines P_6:(YellowWax_U.S.A.) and P_7: (Aswany_Local) were obtained from the germplasm preservation laboratory of the Horticulture Department, Faculty of Agriculture, Moshtohor, Benha University.

Field Experiments:

Crosses were made among the parental germplasm and a half diallel set (21 hybrids) was obtained. The 28 genotypes (Seven parental lines and 21 hybrids) were distributed in the field, following a complete block design with three replicates. Agricultural practices were applied as recommended for pepper production. Plant height (PH), fruit length(FL), fruit diameter(FD), fruit weight (FW),fruit number per plant (FN) and fruit yield per plant (FY) were used for the morphological characterization of the all genotypes.

DNA extraction:

Young leaves were collected in 1.5 ml eppendorf tube, quickly frozen in liquid nitrogen and ground with konte pestles into fine powder. DNA was extracted according to **Doyle and Doyle, (1990)** mini preparation protocol. The purity of extracted DNA was tested on 1% agarose gel using 0.5x TE (Tris EDTA) buffer and stained with 10 mg/ml ethidium bromide. The gel was exposed to UV-light and photographed. Optimizations of the working dilutions were made using various dilution

ratios. Finally, the dilution produced amplification with the ISSR primer and three samples for screening was in ratio of 1:1000 after determining the concentration with a TD-700Fluorometer.

ISSR-PCR:

ISSR-PCR was carried out according to **(Williams** *et al.,* **1990).** The primers used were 11 to18 mer olignucleotide; nine primers were selected as potentially useful. The codes and sequences of the used primers are shown in Table(1).

PCR reactions were optimized and mixtures (25µl total volume) were composed of dNTPs (200µM), $MgCl_2$ (1.5mM),1x buffer, primer(0.2µM), DNA (50ng),Taq DNA polymerase (2units).Amplification was carried out in a thermo Cycler programmed for 94°C for 3min(one cycle); followed by 94°C for 30sec,40°C for 45 sec and 72°C for 1 min (35 cycle), 72°C for 10 min (one cycle) then 4°C (infinitive).

Amplification products (25µl) were mixed with 3µl loading buffer and separated on 1.3%agarose gel and stained with 0.5 µg/ml ethidium bromide, and visualized under ultraviolet light and photographed. DNA fragment sizes were determined by comparisons with the 1kb plus DNA ladder marker.

Table(1): Name and sequences of the used primers with ISSR molecular markers.

ISSR Primer	Nucleotide sequence 5' to 3'
814_A	$(CT)^8TG$
844_A	$(CT)^8AC$
844_B	$(CT)^8GC$
17898_A	$(CA)^6AC$
17899_A	$(CA)^6AG$
HB_9	$(GT)^6GG$
HB_{11}	$(GT)^6CC$
HB_{12}	$(CAC)^3GC$
HB_{13}	$(GAG)^3GC$

Data analysis:

The statistical design applied was the Method-2 / Model-1 of **Griffing (1956).** Heterosis was estimated as the deviation of the F_1 from the average of its two parents **(Bhatt, 1971).**

The obtained data of ISSR analysis was entered in a computer file as binary matrices where "0" stands for the absence of a band and in each individual sample. Similarity coefficients were calculated according to dice matrix **(Nei and Li, 1979; Rohlf, 1993).** Parents were grouped by cluster analysis with the similarity matrix and unweighted pair group method based on arithmetic mean (UPGMA).

RESULTS AND DISCUSSION

1.Performance of parental lines and hybrids:

Highly significant differences among genotypes were detected for plant height ,fruit length, fruit diameter, fruit weight, fruit number and fruit yield per plant (Table-2), indicating abundant genetic variability for fruit yield per plant. The highest yielding parent was P_1, even it was statistically similar to P_6; Parental line P_7 presented the lowest fruit yield per plant. Among all the hybrids and parental lines, the F_1 (P_4 x P_6) produced the highest yield (Table -3).

As can be seen in Table-2, the mean squares of *GCA* and *SCA* were highly significant; in accordance with **Sprague and Tatum (1942).** These results suggest the presence of additive and dominance effects in the genetic control of fruit yield.

Table (2): Analysis of variance (mean squares) for six characters and estimated combining ability for fruit yield / plant character in pepper**.**

S.O.V	d.f	PY	FN	FW	FD	FL	PH
Replication	2	140.75	201.04	71.49	0.001	0.061	2.118
Genotypes	27	269269.67**	67488.31**	86832.42**	1.713**	16.477**	792.041**
GCA	6	186.38**					
SCA	21	25460.90**					
Error	54	88929.50**					

The lines P_4 and P_6 showed the highest general combining ability; the highest specific combining ability corresponded to P_6x P_7; however, P_4 x P_6, P_1 x P_5, P_2 x P_5, and P_2 x P_3, produced hybrids of similar yield to P_6 x P_7.

As can be seen in Table-3, the F_1 (P_4 x P_6) showed the highest heterosis with 108.29.5%, and a relatively high *SCA* effects . The heterosis values found in this study are well over others reported in pepper (**Milerue and Nikornpun, 2000; Owen, 1992).**

As mentioned before, P_4 and P_6 exhibited the highest *GCA* effect for fruit yield. However, these lines just participated in three out of six of the best hybrids. On the other hand, P_3 with an intermediate *GCA* effect, produced two out of six of the best hybrids in terms of heterosis and in one out of five of the highest values of *SCA*. These results suggest that the *SCA* effects were important in controlling these traits.

In the present investigation, the mean values of the hybrids were significantly larger for FY, FN, FW, FD,FL and PH, when compared with the mean of parental lines (Mid-parent) indicating that heterosis was present for these traits.

Comparatively, FY showed a greater level of heterosis in most of F_1 hybrids. However, the parental performance (additive gene action) contributed less to F_1 performance. The hybrid mean was significantly lower than the mean of the parental lines for FW, revealing the lack of dominance and positive heterosis for this character.

Some characters, such as FY, FD and PH, demonstrated average in the desired direction indicating the presence of true heterosis. The reason for the F_1 hybrids showing PH for these characters might be explained by desirable genetic complementation between the inbred genotypes (**Dubreuil** *et al.* **1996**).

In this study, although it was not consistent for all hybrids, generally hybrids obtained from very closely or distantly related parents showed low heterosis but crosses produced from parents of intermediate divergent classes tended to show higher heterosis for FY, FL and FW; this suggests that intermediate divergence of parental lines could give higher heterosis for these traits. **Liu** *et al.* **(1999)** reported similar results in wheat.

2.Genetic relationship using Inter Sequence Repeat Polymerase Chain Reaction analysis(ISSR-PCR):

a. ISSR-PCR analysis.

In the present study, the genetic variability among different genotypes of pepper based on ISSR-PCR analysis has been studied.

Screening nine random primers with seven genotypes of pepper resulted in nine primers that produce different polymorphic bands and amplified DNA fragments as shown in Tables (4) and Fig.(1).

The nine primers used in the study generated a total of 104 amplification products, among which 100 were found to be polymorphic; this resulted in 96.15% polymorphism. All the primers produced polymorphic amplification products, however, the extent of percent polymorphism varied with each primer (81.82 to 100%).

The PCR products of primer 844_A ranged from two bands in P_6 and P_7 to eight bands in P_1 (Tables 4 and Fig.1A); this primer produced two monomorphic bands in all genotypes; the other bands were polymorphic as they were present in some genotypes and absent in the other. Some genotypes had some specific bands and could be used to distinguish among them; for instance P_4 has one positive specific marker at *(M.W.)* of 473.33*bp*.

The PCR products of primer 844_B and analysis of there products are illustrated in Fig.1B and Tables(4); this primer produced 3-8 bands for the studied genotypes; and one positive specific marker was found in P_1 with *M.W.* of 400.0*bp*.

The result of ISSR analysis using primer 814_A were illustrated in Fig.1C and Tables(4);the total number of bands varied with a lowest number of one band in P_2,P_5 and P_7 to a highest number of seven bands in P_3; there was one monomorphic band in all genotypes and four positive specific markers were found in P_1 and P_3, with *M.W.* of 672.55,490.28,453.97 and 400*bp*, respectively.

The PCR products of primer 17898_A ranged from one band in P_4 to six bands in P_1 (Tables 4 and Fig.1D); this primer produced one

monomorphic band in all genotypes; some genotypes had some specific bands and could be used to distinguish among then; for instance P_6 has one positive specific marker at *M.W.* of 658.236*bp*.

The PCR products of primer 17899_A and analysis of there products are illustrated in Fig.1E and Table(4); this primer produced 3-8 bands for the studied genotypes; this primer don't produce any monomorphic bands in all genotypes. Three positive specific markers were found in P_2 and P_7 with *M.W.* of 475.07, 429.60 and 311.37*bp*), respectively.

Table (4): Number of amplified fragments and specific markers of seven pepper genotypes based on ISSR-PCR analysis with nine primers.

Genotypes		ISSR Primers								
		844A	**844B**	**814A**	**17898A**	**17899A**	**HB9**	**HB11**	**HB12**	**HB13**
	TAF	11.0	9.0	8.0	9.0	14.0	9.0	12.0	21.0	11.0
	MB	2.0	0.0	1.0	1.0	0.0	0.0	0.0	0.0	0.0
	PB	9.0	9.0	7.0	8.0	14.0	9.0	12.0	21.0	11.0
	PB%	81.82	100.0	87.50	88.89	100.0	100.0	100.0	100.0	100.1
P_1	*AF*	8.0	7.0	4.0	6.0	8.0	4.0	5.0	8.0	7.0
	SM	0.0	1.0	2.0	0.0	0.0	0.0	1.0	5.0	0.0
P_2	*AF*	6.0	3.0	1.0	3.0	8.0	5.0	3.0	4.0	8.0
	SM	0.0	0.0	0.0	0.0	2.0	1.0	0.0	0.0	1.0
P_3	*AF*	5.0	4.0	5.0	2.0	8.0	3.0	5.0	7.0	3.0
	SM	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0	1.0
P_4	*AF*	2.0	7.0	0.0	1.0	0.0	4.0	3.0	6.0	3.0
	SM	1.0	0.0	0.0	0.0	0.0	1.0	1.0	0.0	0.0
P_5	*AF*	4.0	6.0	1.0	3.0	3.0	5.0	6.0	11.0	3.0
	SM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0
P_6	*AF*	2.0	6.0	0.0	5.0	4.0	5.0	3.0	8.0	2.0
	SM	0.0	0.0	0.0	1.0	0.0	0.0	0.0	1.0	0.0
P_7	*AF*	2.0	7.0	1.0	3.0	7.0	5.0	3.0	11.0	2.0
	SM	0.0	0.0	0.0	0.0	1.0	1.0	1.0	0.0	0.0
Total	*AF*	29.0	40.0	12.0	23.0	38.0	31.0	28.0	55.0	28.0
	SM	1.0	1.0	4.0	1.0	3.0	3.0	3.0	6.0	3.0

TAF=total amplified fragment., MP= monomorphic bands., PB=polymorphic bands.
AF=amplified fragment, SM=specific marker.

The result of ISSR analysis using primer HB_9 were illustrated in Fig.1F and Tables(4). The total number of bands varied with a lowest number of three bands in P_3 and a highest number of five bands in three genotypes; there were three positive specific markers were found in P_2,P_4 and P_7 with *M.W.*459.09, 481.82 and 693.25*bp*.

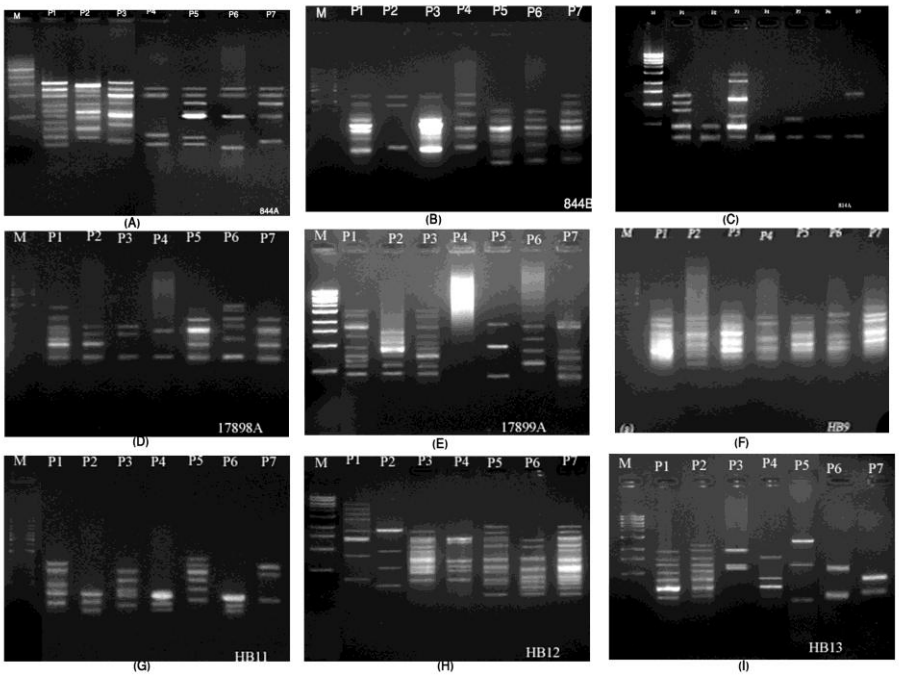

Fig.(1):DNA polymorphism of the seven pepper genotypes using ISSR_PCR with nine primers.

The result of ISSR analysis using primer HB_{11} were illustrated in Fig.1G and Tables(4);the total number of bands varied with a lowest number of three bands in P_2,P_4,P_6 and P_7, a highest number of six bands in P_5. Three positive specific markers were found in P_1,$P_4$and P_7 with *M.W.* 400.0, 368.56 and 553.66*bp*, respectively.

The PCR products of primer HB_{12} ranged from four bands in P_2 and found 11 bands in P_5 and P_7 (Tables 4 and Fig.1H); this primer don't produce any monomorphic bands in all genotypes; some genotypes had some specific bands and could be used to distinguish among then; for instance P_1 and P_6 had six positive specific markers at *M.W.* of (918.79, 814.45, 771.44,700.0,400.0 and 270.59*bp*),respectively.

The result of ISSR analysis using primer HB_{13} were illustrated in Fig.1I and Tables(4);the total number of bands varied with a lowest number of two bands in two genotypes P_6 and P_7 to a highest number of eight bands in P_2; this primer does not produce any monomorphic band in

all genotypes. However, three positive specific markers were found in P_2,P_3 and P_5 with *M.W.* 824.96, 791.92 and 612.8273*bp*,respectively.

Table (5):Trait specific ISSR markers in the seven pepper genotypes.

Molecular Marker	Band Number	MW*(bp)*	Morphological Trait	Parents
844A	3	473.33	PH	P_4
844B	1	400.0	FW,FN,FY	P_1
814A	1	672.55	PH,FL,FD	P_1, P_3
	2	453.97		
17898A	2	658.23	FL,FD,FY	P_6
17899A	2	475.07	FD	P_2
	3	429.60		
	6	311.37	PH,FW,FN	P_7
HB9	3	459.09	FD	P_2
	4	481.82	PH	P_4
	1	693.25	PH,FW,FN	P_7
HB11	3	400.0	FW,FN,FY	P_1
	1	368.56	PH	P_4
	2	553.66	PH,FW,FN	P_7
HB12	1	918.79	FW,FN,FY	P_1
	2	814.45		
	3	771.44		
	4	700.0		
	8	400.0		
	7	270.59	PH,FW,FN	P_6
HB13	1	824.96	FD	P_2
	1	791.92	PH,FL,FD	P_3
	1	612.82	PH	P_5

ISSR-PCR analysis was performed using primers to obtain markers to assist selection for some of the aforementioned traits in pepper. Primers 844_A,844_B, 814_A, 17898A, 17899_A, HB_9,HB_{11}, HB_{12} and HB_{13} produced ISSR fragments with different lengths could be used as markers to assist selection for some of the yield related traits in pepper as shown in Tables (5).

Some of these ISSR products could be used in markers assisted selection programs. Primer 844_A produced an amplified fragment with length of 473.33 *bp* related to plant height and could be used as marker to assist selection for this trait. On other hand, primers 844_B and 17898_A produced fragment with length of 400.0 and 658.23 *bp*, respectively,

which could be used as negative marker in selection for FW,FN,FL,FD and FY traits, in accordance with (**Geleta** *et al.* **2004** and **Kumar** *et al.*, **2001).**

b. Nei's similarity coefficient.

Similarity indices and consensus tree were developed on the basis of the scorable banding patterns of the seven pepper genotypes using the nine ISSR primers as shown in Table (6); the two most closely related genotypes P_1 and P_2 with highest genetic distances ranged from (0.475). On the other hand, the two most closely related genotypes P_4 and P_7 with low genetic distances ranged from (0.176); the average of similarity among genotypes was (0.336).

Table (6): Genetic distances among the seven pepper parents as estimated using ISSR data.

Genotype	P_1	P_2	P_3	P_4	P_5	P_6	P_7
P_1	1.0						
P_2	0.475	1.0					
P_3	0.403	0.358	1.0				
P_4	0.210	0.200	0.176	1.0			
P_5	0.299	0.220	0.333	0.333	1.0		
P_6	0.333	0.278	0.327	0.239	0.327	1.0	
P_7	0.333	0.328	0.259	0.196	0.431	0.373	1.0

c. Cluster analysis.

Nei's genetic distance (Fig.2) showed that the genetic distances for each genotype combination ranged from (0.24) to (0.48) and the studied genotypes formed two main clusters. The first main cluster separated at genetic similarity of (0.32) and created four sub clusters, the first subcluster included an individual genotype P_3 at genetic similarity of (0.40),the second subcluster included P_1 and P_2 at genetic similarity of (0.48), the third subcluster included P_5 and P_7 at genetic similarity about (0.44) and the forth subcluster included an individual cultivar P_6 at genetic similarity of (0.36). In reaction to second main cluster separated at genetic similarity of (0.24) and created one subcluster, included an individual cultivar P_4 at genetic similarity of (0.24).

The aforementioned results confirmed that ISSR profiling is a powerful method for identification and molecular classification which agreed with(**Geleta** *et al.* **2004** and **Kumar** *et al.*, **2001).**

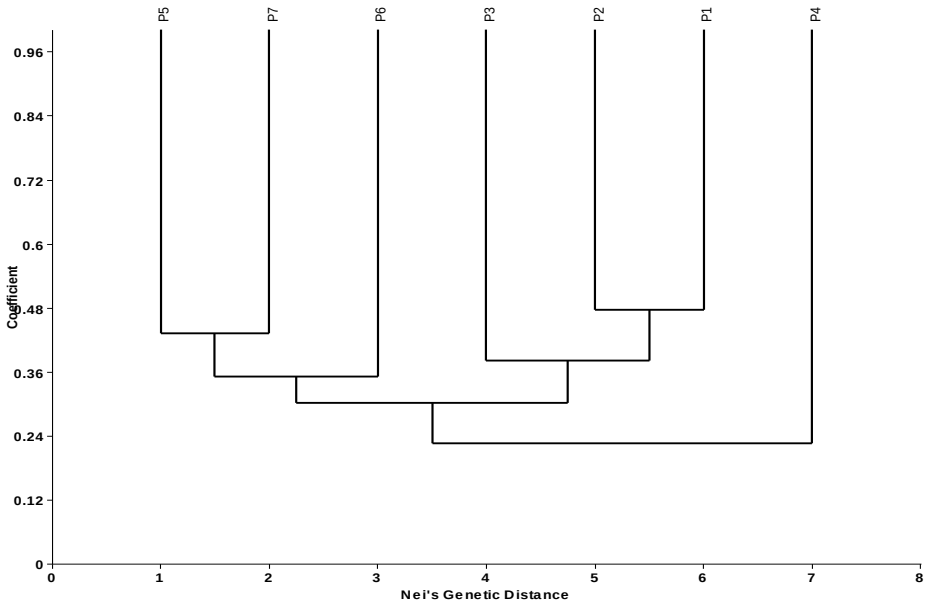

Fig.(2):Dendogram obtained from UPGMA cluster based on ISSR data from the seven pepper genotypes.

3.Genetic relationship between genetics distance and heterosis :

The correlation coefficients between the Nei's genetic distances matrix and the heterosis matrix was medium positive significant correlation (r = 0.574).

The correlation coefficients of ISSR distance with SCA effects of FY was low negative significant correlation(r = -0.022). It is assumed that the SCA effect expressed by a hybrid is related to the genetic distance between its parental lines (**Lee** *et al.* **1989**).

The genetic distances among parental lines are related with the heterosis of their hybrids (Table-7). However, P_4 and P_6 were the more divergent parents and produced the highest fruit yield and the second highest heterosis values. This shows an isolated tendency, since it was observed that parental lines with smaller divergence had low values of heterosis **(Sekhon and Gupta, 1995).**

Similar results have been obtained in maize **Dudley** *et al.* **(1991)**. Also, **Barbosa** *et al.* **(1996) and Burkhamer** *et al.* **(1998)** reported low

correlations between genetic distances and heterosis in wheat. **Charcosset** *et al*. **(1991)** indicate that the detection of molecular markers which are not close to the genes responsible for the assayed trait may decrease the correlation between genetic distances and heterosis. **Bernardo (1992),** mention that it is essential to identify a specific marker related to the segments of the genome which determine the expression of the traits of interest to find a high correlation between genetic distances and heterosis.

Table (7): Genetic distances and heterosis values of seven pepper genotypes and their F_1 hybrids.

Genotypes	Genetic Distances						
	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**
P1	1.0	0.475	0.403	0.210	0.299	0.333	0.333
P2	0.134	1.0	0.358	0.200	0.220	0.278	0.328
P3	0.028	0.772	1.0	0.176	0.333	0.327	0.259
P4	0.105	0.922	0.804	1.0	0.333	0.239	0.196
P5	0.115	0.771	0.429	0.589	1.0	0.327	0.431
P6	-0.019	0.333	0.645	1.083	0.255	1.0	0.373
P7	-0.166	0.343	0.211	-0.024	-0.058	0.775	1.0
Heterosis Values							

It may be expected that genetic distances, calculated using molecular markers, will become a useful way to predict heterosis until genes controlling important traits are placed on highly saturated genetic linkage maps and the adequate markers, those strongly linked, can be chosen to calculate the genetic distance.

The results of this study showed that genetic distances, in general, correlated medium with heterosis and hybrid performance. Previous studies in various crop species such as maize **Benchimol** *et al***. (2000**), rice (**Kwon** *et al.* **2002**), wheat **(Burkhamer** *et al*. **1998),** alfalfa **(Riday** *et al.* **2003**) and chickpea (**Sant** *et al.* **1999**) also showed low or medium correlations of genetic distance with heterosis.

Although higher levels of heterosis for the majority of characters studied show the possibility of hybrid breeding in pepper, extreme divergence for characters such as fruit-related traits is not desirable.

However, if the objective of a breeding programme is, for example, to develop a hybrid of medium sized-fruit, smaller-and larger-fruited parents can be used in the crossing programmes.

In this study, both morphological and ISSR distances differentiated pepper lines into heterotic groups from which superior hybrids can be derived. However, the method expressed little or no promise for predicting F_1 performance for most characters studied.

The parental genotypes used in this study had diverse morphological backgrounds and were from different market types. As each type of pepper must conform to its own unique set of characteristics in order to be commercially acceptable, pepper hybrid breeding should deal with parental lines of similar varietal groups (market types) unless the breeding programme is to develop hybrids or pure lines of different forms.

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