Assessment of Genetic Variations in Some *Vigna* Species by RAPD and ISSR Analysis

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Abstract: Genetic variations of seven *Vigna* species were evaluated using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers. Amplification of genomic DNA of the seven genotypes by RAPD analysis, five primers generated 64 fragments, of which 31 were polymorphic with an average of 12.8 bands/ primer. The amplified products varied in size from 2556 to 255 bp. Eleven selected ISSR primers produced 128 bands across seven genotypes of which 89 were polymorphic with an average of 11.64 / primer. The size of amplified bands ranged from 2838 to 264 bp. Similarity index values ranged from 0.913 to 0.120 (RAPD) 0.822 to 0.118 (ISSR) and 0.899 to 0.115 (RAPD and ISSR). The results indicated that both of the marker systems RAPD and ISSR, individually or combined can be effectively used in determination of genetic relationship among *Vigna* species. It could be concluded that, the information of genetic similarities and diversity among *Vigna* genotypes are necessary for breeding programs.
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1. Introduction

The genus *Vigna* belonging to family Fabaceae and divided into seven subgenera, it includes over 150 species. Many *Vigna* species are cultivated for food. The morphological markers were not quite enough to expose the genetic diversity and do not reflect real genetic relationships. Therefore, molecular markers have several advantages over the traditional phenotypic markers. They are unaffected by environment and detectable in all stages of development. The molecular genetic techniques have been adopted for the management and manipulation of plant genomes. DNA markers are the most powerful and widely used because they can portray genome sequence composition (Karp *et al*., 1997).

Over the years, the methods for detecting and assessing genetic diversity have extended from analysis of discrete morphological traits to molecular traits. Several DNA marker systems are now common use in diversity studies of plants, the most commonly used marker systems are restriction fragment length polymorphism (RFLP) (Soller and Beckmann, 1983), random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990), amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995), inter-simple sequence repeats (ISSRs) (Zietkiewicz *et al.*, 1994) and microsatellites or simple sequence repeats (SSRs) (Becker and Heun, 1994).

Among them to characterize DNA variation patterns within species and among closely related taxa in *Vigna* species have been RAPD (Dikshit *et al.*, 2007), AFLP (Yoon *et al*., 2007), RFLP (Kaga *et al.*, 2000), ISSR (Ajibade *et al.*, 2000) and SSRs (Dikshit *et al.*, 2007) .

The utility of PCR-based RAPD or ISSR variations as phylogenetic markers for investigating evolutionary relationships among plants has been clearly established (Maugham *et al.*, 1996). Random amplified polymorphic DNA (RAPD) is a valuable tool for identifying genetic variation because it is inexpensive, quick, and simple (Williams *et al*., 1990).It permits the identification of DNA polymorphisms and can be used to amplify particular fragments of genomic DNA (Bielawski *et al.*, 1996).Inter-simple sequence repeats (ISSRs) is a type of molecular marker, proposed by (Zeitkiewicz *et al.*, 1994) for fingerprinting. ISSRs fingerprinting has been commonly used to study population genetics, taxonomy and phylogeny of many plant species (Wolf and Randle, 2001). ISSR primers can confirm specific amplified DNA polymorphic fragments within the variety (Leian *et al.*, 2005).

Since knowledge of genetic diversity is essential for evolving systematic breeding and conservation strategies, the present molecular

diversity analysis using RAPD and ISSR techniques were carried out on seven *Vigna* species.

2. Material and Methods

Seeds of seven *Vigna* species were used *V. mango* L., (Mang.), *V. angularis* L. (Angul.), *V. umbellata* (Thumb) (Umb), *V. unguiculata* L. (Ung-Ger.) and *V. radiate* L. (Radia.) were kindly obtained from the germplasm collection of the Institute for Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. While another two species, *V. sinesis* (Endl.) (Sin-Egy) and Egyptian *V. unguiculata* L. (Ung-Egy.) from Horticulture Research Institute, Agricultural Research Center, Giza, Egypt.

DNA isolation

Total genomic DNA was extracted from the young leaves of the seven *Vigna* species by using Bio Basic Kits. Its integrity was checked on agarose gel electrophoresis according to Sambrook *et al*. (1989).

DNA amplification

RAPD fingerprinting:

RAPD amplification was carried out with five primers (Metabion International AG.) in a 25µL volume, containing (5 µL of 5x buffer, 3.0 µL of dNTPs (2.5mM) 3 µL of $Mgcl_2$ (25 mM), 3.0 µL primer (2.5 µ L), 0.3 µ L of Taq polymerase (5U/ µL) and 2.0 µL of genomic DNA (50 ng/ µL) . Amplification was performed in PTC-100 PCR version 9.0 from M J Research-USA. programmed for an initial denaturation at 94^{o}C 5 min, 40 cycles of 1 min denaturation at 94^{o}C, 1 min annealing at 40^{o}C and 2 min extension at 72^{o}C followed by final extension for 5 min at 72^{o}C according to (Williams *et al.*, 1990). Each PCR reaction was repeated twice in order to ensure that RAPD banding patterns were consistent and reproducible and only stable products were scored.

ISSR fingerprinting:

The isolated DNA was performed according to (Jonathan and Wendel, 1990) ISSR-PCR reaction for the seven genotypes of *Vigna* species was conducted using 12 primers. Amplification was carried out in 25 µl reaction mixture contained 2 µl of genomic DNA, 1 µl of the primer, 2.5 µl of 10X Taq DNA polymerase reaction buffer, 1.5 unit of Taq DNA polymerase and 200 mM of each dNTP. Amplifications were performed in DNA amplification Thermocycler (PTC-100 PCR version 9.0 from M J Research-USA). The apparatus is programmed to execute the following conditions, denaturation step of 5 min at 94°C, followed by 35 cycles composed of 30 s at 94°C, 90 s at the annealing temperature, and 90 s at 72°C. A final extension of 72°C for 5 min. Amplifications were performed at least twice and only reproducible products were taken into account for further data analysis.

Gel electrophoresis: Amplification products of RAPD and ISSR were separated on 1.5% agarose gels in 1X TAE buffer with DNA ladder (1Kb) and detected by staining with ethidium bromide according to Sambrook *et al.*, (1989). Then, the PCR products were visualized by UV-transilluminator and photographed by gel documentation system, Biometra - Bio Doc. Analyze.

Data analysis

Pairwise comparison of genotypes based on the presence (1) or absence (0) of reproducible polymorphic DNA bands was used to generate the similarity coefficients by SPSS program version-10 (Norman *et al.,* 1975). the similarity coefficients was used to construct a dendrogram by the unweighted pair group method with arithmetical average (UPGMA).

3. Results and Discussion

RAPD analysis:

The RAPD profiles of the amplification products of the five random primers are shown in Figure (1), the sequences of the primers and the number of bands generated by each primer is given in Table (1). Five primers were used to screen seven genotypes of *Vigna* species produce a total number of 64 amplified DNA products were generated across the studied genotypes with an average of 12.8 bands / primer. Out of the total bands, 31 were polymorphic and 23 were unique ones. The total number of the amplified RAPDs produced by each primer varied from a minimum number of 2 amplified products by primer OPX-17 to a maximum of 23 amplified products by primer OPA-03.

The size of amplified bands also varied with different primers. The largest 2556 bp band was amplified by primer OPD-13, while the smallest size was amplified by primer OPW-04 and detected about 264 bp.

The genetic similarity coefficients based on RAPD markers of the seven studied *Vigna* species Table (2) illustrated that the highest similarity value was 0.913 which recorded between *V. unguiculata* L. Ger. and *V. unguiculata* L. Egy while the lowest similarity value was 0.120 between *V. sinesis* Egy. and *V. Radiata*. Similarity coefficient matrices were

used to generate a dendrogram of *Vigna* species based on UPGMA analysis Fig. (2), which grouped the seven genotypes into three main clusters. The first cluster included, *V. unguiculata* L. Ger. and *V. unguiculata* L. Egy. with highly related value (0.913) then come *V. sinesis* Egy. which is moderately related with *V. unguiculata* L. Ger. (0.556) and highly related (0.889) with *V. unguiculata* L. Egy., while the second cluster contained two genotypes, *V. Radiata* and *V. umbellata* with similarity index value of (0.785). Then, came the third cluster comprised *V. angularis* L. and *V. mango* L. which are moderately related to each other (0.756).

These results demonstrated that, RAPD analysis through UPGMA dendrogram revealed substantial polymorphism and gave precise phylogenetic relationship among investigated *Vigna* species. These results were in agreement with the results obtained by (Saini *et al.,* 2010) they concluded that the information of genetic relationship among *V. radiata* L. genotypes by RAPD analysis may be useful for selecting the diverse parents and monitoring the genetic diversity in the breeder's working collection of mung bean (*V. radiate* L.).

Several studies have reported using RAPD markers for the identification of the genetic relationships among cultivars of some *Vigna* species including, *V. unguiculata* (Ba *et al*., 2004), *V. angularis* (Yee *et al*., 1999) and *V. radiata* (Lakhanpaul *et al*., 2000 and Lavanya *et al.*, 2008).

ISSR Analysis:

Twelve selected ISSR primers were used and obtained from Pioneer (Lab. Technology Co.). Table (3). These primers were used to analyze the seven investigated *Vigna* genotypes. ISSR marker profiles produced by the eleven primers are shown in Fig. (3). Out of 12 primers, eleven gave rise to reproducible amplification products, while one primer (UBC 886-11) did not give any amplification. Eleven primers produced a total number of bands of 128 across seven genotypes with average of 11.64/ primer, of which 89 were polymorphic bands and 24 were unique. The total number of the amplified bands produced by each primer varied from a minimum number of 4 amplified products by primer UBC 829-11 to a maximum of 17 amplified products by primer UBC 834-11. The size of amplified bands also varied with different primers. The largest 2838 bp band was amplified by primer UBC 829-11, while the smallest size was amplified by primers UBC 807-11, UBC840-11 and UBC846-11 and detected about 255 bp.

Similarity coefficient values for the seven *Vigna* genotypes based on ISSR markers ranged from (0.822) between *V. sinesis* Egy. and *V. unguiculata* L. Egy. to (0.118) between *V. angularis* L. and *V. unguiculata* L. Ger. Table (4).

The ISSR data were used to generate UPGMA dendrogram (Fig.4) the obtained dendrogram showed that the seven *vigna* species are grouped into three groups. The first one includes *V. sinesis*. Egy., *V. unguiculata* L. Egy. and *V. unguiculata* L. Ger. Where, *V. sinesis*. Egy. was highly related with *V. unguiculata* L. Egy. (0.822) and closely related with *V. unguiculata* L. Ger. (0.745). The second group comprised *V. umbellata* and *V. angularis* L. they are moderately related to each other (0.673), then come *V. radiate* L. and *V. mango* L. represented the third group at a peripheral position and closer to *V. radiate* L. with similarity value of (0.615).

The results indicated that ISSR markers have been successfully utilized for assessing the genetic diversity and revealed a remarkable molecular discrimination between the seven *Vigna* species under study. Similar results were reported by (Ajibade *et al.,* 2000) where they found that the ability of the ISSR technique to effectively distinguish species in the genus *Vigna.* Moreover, (Galvan *et al.,* 2001) concluded that ISSR would be a better tool than RAPD for phylogenetic studies. Nagaoka and Ogihara (1977) have also reported that the ISSR primers produced several times more information than RAPD markers in wheat. The number of potential ISSR markers depends on the frequency of microsatellites, which changes with species (Depeiger *et al*., 1995), So that the Potential for integrating ISSR–PCR into plant improvement programme is enormous and their applications in different crop species are sufficiently reviewed (Reddy *et al*. 2002).

Table (1): Code and sequence of five DNA random primers used for identifying the *Vigna* species and the number of the amplified DNA bands.

Primer Code	Sequence 5'- 3'	polymorphic bands	total bands
OPA-03	5'-AGTCAGCCAC-3'	12	23
OPC-19	5'-GTTGCCAGCC-3'	3	9
OPD-13	5'-GGGGTGACGA-3'	4	10

OPW-04	5'-CAGAAGCGGA-3'	11	20
OPX-17	5'-GACACGGACC-3'	1	2
Total	-	31	64

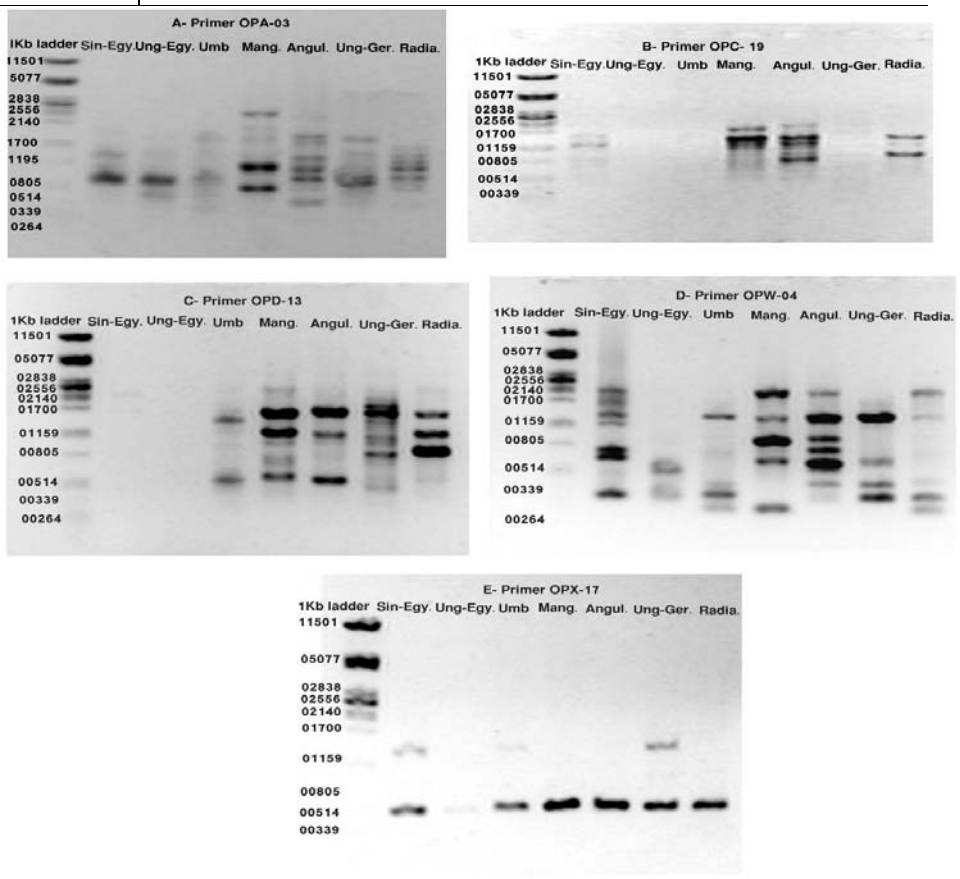


Fig. (1): RAPD fingerprints of the seven *Vigna* species generated by the seven primers: a) OPA-03 b) OPC-19 c) OPD-13 d) OPW-04 e) OPX-17.

Table (2): Similarity coefficients of the seven *Vigna* species based on RAPD markers.

Case	Matrix File Input						
Sin-Egy.	1.000						
Ung-Egy.	0.889	1.000					
UMB	0.296	0.593	1.000				
Mang.	0.185	0.556	0.519	1.000			
Angul.	0.174	0.370	0.311	0.756	1.000		
Ung-Ger.	0.556	0.913	0.333	0.259	0.593	1.000	
Radia.	0.120	0.370	0.785	0.685	0.444	0.467	1.000
	Sin-Egy.	Ung-Egy	UMB	Mang.	Angul.	Ung-Ger.	Radia.

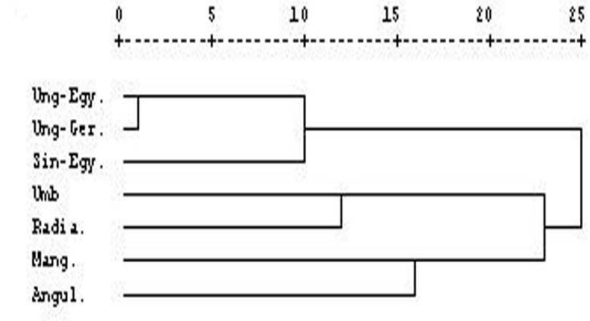
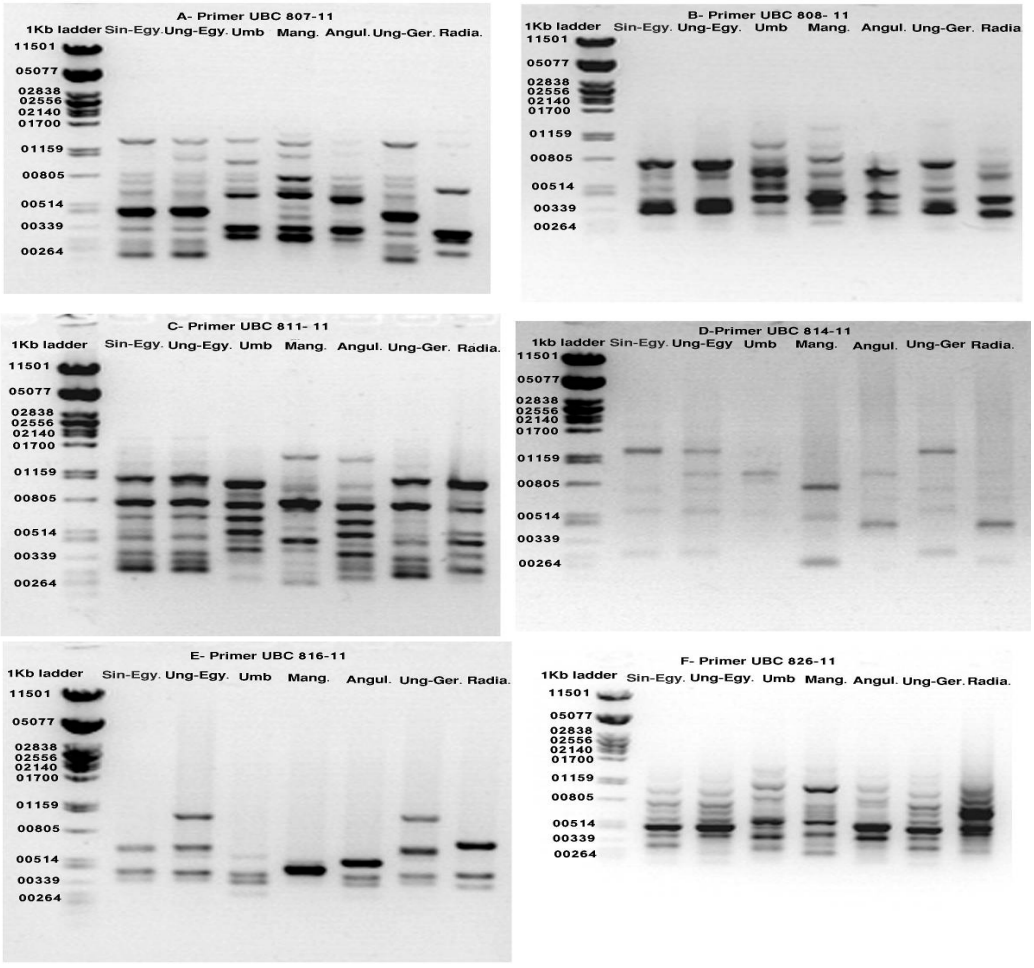


Fig (2): UPGMA dendrogram indicating the genetic relationships among *Vigna* species. based on RAPD markers.

Table (3): Code and sequence of the twelve ISSR primers used for identifying the *Vigna* species and numbers of the amplified DNA bands.

Primer Code	Sequence	CG %	No. of Polymorphic Bands	No. of total bands
UBC 807-11	AGAGAGAGAGAGAGAGT	47.1	8	13
UBC 808-11	AGAGAGAGAGAGAGAGC	52.9	4	10
UBC 811-11	GAGAGAGAGAGAGAGAC	52.9	10	15
UBC 814-11	CTCTCTCTCTCTCTCAT	47.1	7	7
UBC 816-11	CACACACACACACACAT	47.1	5	8
UBC 826-11	ACACACACACACACACC	52.9	9	12
UBC 827-11	ACACACACACACACACG	52.9	10	13
UBC 829-11	TGTGTGTGTGTGTGTGT	52.9	4	4
UBC 834-11	AGAGAGAGAGAGAGAGTT	44.4	10	17
UBC 840-11	GAGAGAGAGAGAGAGATT	44.4	10	15
UBC 846-11	CACACACACACACACAAT	44.4	12	14
UBC 886-11	AAACTCTCTCTCTCTCT	41.2	0	0
total	-	-	89	128



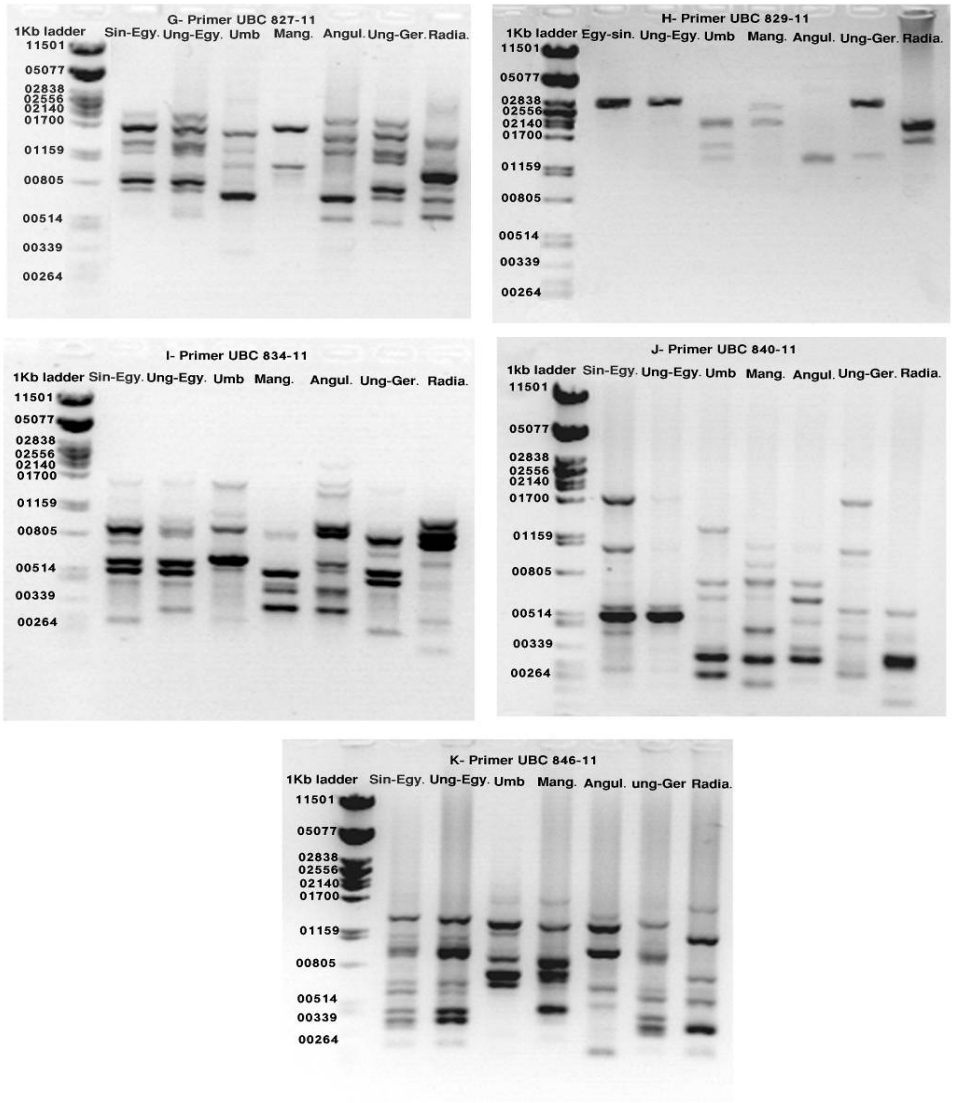


Fig. (3): ISSR fingerprints of the seven species of *Vigna* generated by the twelve primers:

a) UBC 807-11 b) UBC 808-11 c) UBC 8011-11 d) UBC 814-11 e) UBC 816-11 f) UBC 826-11

g) UBC827-11 h) UBC 829-11 i) UBC 834-11 j) UBC 840-11 k) UBC 846-11 l)UBC 886-11

Table (4): **Similarity coefficients of the *Vigna* species based on ISSR markers.**

Case	Matrix File Input						
Sin-Egy.	1.000						
Ung- Egy.	0.822	1.000					
UMB	0.137	0.457	1.000				
Mang.	0.059	0.339	0.275	1.000			
Angul.	0.314	0.490	0.673	0.337	1.000		
Ung- Ger.	0.745	0.810	0.437	0.120	0.118	1.000	
Radia.	0.294	0.235	0.396	0.615	0.455	0.237	1.000
	Sin-Egy.	Ung-Egy	UMB	Mang.	Angul.	Ung- Ger.	Radia.

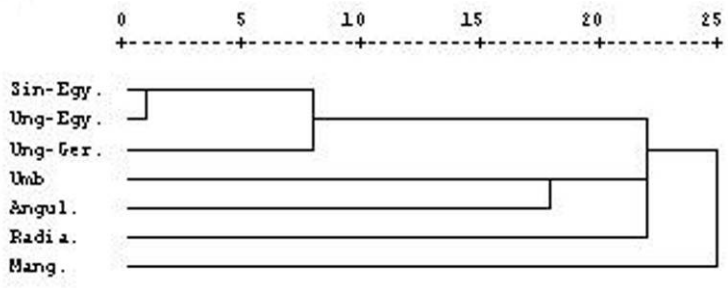


Fig (4): UPGMA dendrogram indicating the genetic relationships among *Vigna* species based on ISSR markers.

RAPD and ISSR analysis:

The similarity coefficients of the seven *Vigna* species based on RAPD and ISSR markers ranged from 0.899 to 0.115 among the seven genotypes. *V. sinesis* Egy. and *V. unguiculata* L. Egy. showed the highest similarity index (0.899), while the lowest value was (0.115) between *V. sinesis* Egy. and *V. mango* L. genotypes. Table (5) Cluster analysis performed from combining data of both markers generated a dendrogram separated the genotypes into three clusters. The first cluster included *V. sinesis* Egy., *V. unguiculata* L. Egy. and *V. unguiculata* L. Gre., where *V. sinesis* Egy. was highly related to *V. unguiculata* L. Egy. (0.899) and closely related to *V. unguiculata* L. Gre. (0.633). The second cluster comprised *V. angularis* L. and *V. radiate* L. (0.794), then come *V. umbellata* and *V. mango* L. (0.709) in the third cluster. Fig (5).

the present work used a combination of RAPD and ISSR markers to determine the further genetic affinities between *Vigna* species at the DNA level, the results indicated that, close correspondence between the similarity matrices of both RAPD and ISSR individually or combined, hence both the marker systems can be effectively used in determination of genetic relationship among *Vigna* species. Similar studies have been widely applied in a variety of plant genera, such as *Cicer* (Iruela *et al.* 2002), Vigna (Ajibade *et al.*, 2000), and *Vicia* (Potokina *et al.*, 1999).

Table (5): Similarity coefficients of the *Vigna* species based on RAPD and ISSR markers.

Case	Matrix File Input						
Sin-Egy.	1.000						
Ung- Egy.	0.899	1.000					
UMB	0.137	0.412	1.000				
Mang.	0.115	0.347	0.709	1.000			
Angul.	0.162	0.412	0.221	0.221	1.000		
Ung- Ger.	0.633	0.676	0.262	0.144	0.350	1.000	
Radia.	0.147	0.250	0.447	0.415	0.794	0.265	1.000
	Sin-Egy.	Ung- Egy	UMB	Mang.	Angul.	Ung-Ger.	Radia.

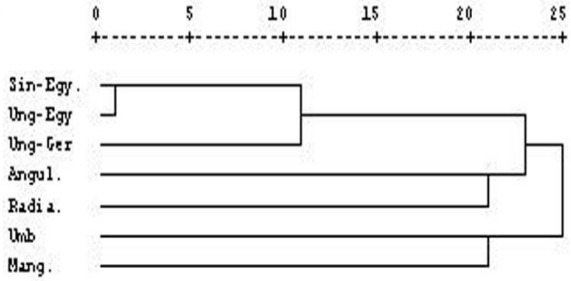


Fig (5): UPGMA dendrogram indicating the genetic relationships among *Vigna* sp. based on: Combination RAPD and ISSR.

The results indicated that RAPD, ISSR and combined RAPD and ISSR analysis provided the possibility of identifying the investigated *Vigna* genotypes. High degree of genetic similarity was observed among Egyptian species (*V. sinesis* (Endl.) and *V. unguiculata* L.) .Also a close relationship was found between the two Egyptian *Vigna* genotype and one of the German genotype (*V. unguiculata* L) as shown by high values of similarity index between them.

Another four German *Vigna* species, *V. mango* L., *V. angularis* L., *V. umbellata* (Thumb), and *V. radiate* L. recorded closely or moderately relationships between each other, on the other hand, they were less related with the two Egyptian species

as recorded by low values of the genetic similarity index.

In addition, the three dendrograms showed minor differences in the cluster pattern of the different *Vigna* species as revealed by RAPD, ISSR and combined RAPD and ISSR, as expected, the two Egyptian genotypes tended to cluster together with high degree of genetic similarity regardless the type of molecular marker. Similarly, German genotypes tended to cluster together into two separated groups. A high degree of biodiversity between Egyptian and German species was recorded except German *V. ungulate* L. genotype was clustered with the two Egyptian genotypes, *Vigna sinesis* (Endl) and *Vigna unguiculata* L. as mentioned above. This close relationship between species from different geographic region was supported by (Liavanya *et al*. 2008 and Pathak *et al*., 2010) they reported lack of correlation between geographic region and genetic diversity in other legumes or these genotypes may have been derived from the same pedigree and have common ancestor, at least for one of the parent (Saker *et al*., 2005 and Saraladevi *et al*., 2008). It could be concluded that, the information of genetic similarities and diversity among *Vigna* genotypes are necessary for breeding programs.

4. Conclusion:

The assessment of genetic diversity and identification of seven *Vigna* species were evaluated by RAPD and ISSR analysis. The obtained results indicated that both of the marker systems RAPD and ISSR, individually or combined can be effectively used in determination of genetic relationship among *Vigna* species. The two Egyptian genotypes *Vigna sinesis* (Endl) and *Vigna unguiculata* L. tended to cluster together with high degree of genetic similarity regardless the type of molecular marker. Similarly, German genotypes *V. mango* L., *V. angularis* L., *V. umbellata* (Thumb) and *V. radiate* L. tended to cluster together into two separated groups. A high degree of biodiversity between Egyptian and German species was recorded except German *V. ungulate* L. genotype was clustered with the two Egyptian genotypes. It could be concluded that The *Vigna* species have sufficient amount of genetic diversity and a wide range in genetic base of the studied genotypes which can be used for crop improvement.

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