GENETIC VARIATION BETWEEN TWO ECOTYPES OF EGYPTIAN CLOVER BY ISSR TECHNIQUE

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Berseem is the added value in Egyptian economic world. The cycle of berseem capital equal tens B.US$ Berseem is the main forage crops for livestock and have produce milk and/or meat production in Egypt. So too berseem is the guard on Egyptian soil fertility (Zayed, 2013). Egypt has poor rangeland, although vast areas of more than 10 million ha exist. Egypt depends mainly on Egyptian clover (berseem) as the key forage crop. The cultivated area of berseem ranges from 1,050,000 ha to 1,260,000 ha in Delta and the Nile Valley annually. There is big competition between berseem and wheat, especially on old land where the productivity is the highest for both crops. Although there is a wide gap between the available and the required feed, there is a very rapid development in the animal wealth to meet the high demand for animal products (FAO, 2012). Egypt depends largely on Egyptian clover (berseem) as the main forage crop and on crop residues and by-products. The cultivated area of berseem ranges from 1,050,000 to 1,260,000 hectares annually in the Delta and Nile Valley. There is a competition between berseem and wheat, especially on old land, where the productivity is the highest for both crops (FAO, 2003 & 2010).

Techniques based on molecular marker analysis (*i.e.* RFLP, RAPD and ISSR-PCR) may provide a more efficient and accurate screening method. Simple sequence repeats comprise short oligonucleotide sequences, two to six bases long, repeated in tandem array, which occur very frequently in eukaryotic genomes (Tautz and Renz, 1984; Beckmann and Soller, 1990; Lagercrantz *et al*., 1993). They are widely distributed within genomic DNA and are present in both the introns of genes and in non-coding regions. The ISSR-PCR technique uses primers that are complementary to a single SSR and was anchored at the 5′ or 3′ end with a one- to three-base degenerate oligonucleotide (anchor) (Zietkiewicz *et al*., 1994). This anchor ensures that the primer binds only to one end of a complementary SSR locus. The great number of amplicons generated consists of the region between neighboring and inverted SSRs. As a result, the highly complex banding pattern obtained will often differ greatly between cultivars of the same species. Inter-simple sequence repeats (ISSR) have also been widely utilized for genetically study in past (Ulloa *et al.,* 2003). The advantage of ISSR over RAPD is its being more reproducible (Fernandez *et al.,* 2002; Greene *et al.,* 2004).

RAPD was conducted using 8 arbitrary 10-mer primers. Combined analysis based on four isozymes, PAGE protein electrophoresis and RAPD analyses revealed high similarity of 0.85 between the cultivars Sakha 4 and Gemmiza 1, while the lowest similarity (0.53) was observed between Giza 6 and Helaly (Tarrad and Zayed, 2009; Zayed *et al.*, 2010).

The Miskawy, Saidi and Fahl ecotypes differ in their morphological yield, regeneration ability after cutting and stage of maximum growth. The Miskawy and Fahl have high inter-varietal variability in terms of green yield, plant height, number of branches and tillers per plant. Helaly is a derivative of Miskawi ecotype (Zayed *et al.*, 2010; Soliman *et al.*, 2010). In the present work, protein ISSR markers have been used in order to determine genetic variation and relationship between two ecotypes of Egyptian clover (4 cultivars). Egyptian clover is divided into two ecotypes of recovery status after cutting: first ecotype a single cut mower which cannot renew itself after the cutting while the second ecotype renews itself from 5-7 times after cutting, including numerous varieties in the Egyptian clover.

MATERIALS AND METHODS

Plant Material

Four Egyptian clover (*Trifolium alexandrinum* L) cultivars representing two ecotypes [Fahl cultivar = monocut ecotype; Gemmiza 1, Serw 1 and Giza 6 cultivar = multicut ecotype] were used in the present study cultivar Fahl is prevalent in whole of Egypt and is good for single cut as it has poor regeneration ability, whereas Serw1, Giza 6 and Gemmiza 1 give 5-6 cuts of good fodder. Four Egyptian clover (*Trifolium alexandrinum* L) cultivars representing two ecotypes were used in the present study. The first cultivar is Fahl (no of chromosome is 16), it is prevalent in whole of Egypt and is good for single cut as it has poor regeneration ability. The second cultivar is Serw 1(no of chromosome is 16), it is multicut and one ecotype and cultivated in salinity soil and north Egypt). The third cultivar Giza 6 (no of chromosome is 16, multicut ecotype and cultivated in upper Egypt). The fourth cultivar Gemmiza 1 (no of chromosome is 16; according Soliman *et al.* (2010), multicut and two ecotype and cultivated in all Egypt) are distributed in Egypt and can give 5-6 cuts of good fodder. It has higher green fodder yield and has good regeneration ability after cutting.

Genomic DNA extraction and purification: extraction of total DNA was performed using according to Anna *et al.* (2001) method. To remove RNA contamination, RNase A (10 mg/ml, Sigma, USA) was added to the DNA solution and incubated at 37°C for 30 min. Estimation of the DNA concentration in different samples was done by measuring optical density at 260 nm according to the following equation: Conc. (ug/ml) = OD_{260} X 50 X dilution factor according to Carlos *et al.* (2001).

Inter simple sequence repeats (ISSRs)

Six primers for ISSR were used in the present study but only 6 were successful in generating reproducible and reliable amplicons for different types of Egyptian clover. Names and sequences of the selected primers are shown in Table (1). The amplification reaction was carried out in 25 µl reaction volumes containing 1 x PCR buffer, 4 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmole primers, 2 units Taq DNA polymerase and 25 ng templates DNA. PCR amplification was performed in a Perkin Elmer 2400 thermocycler (Germany), programmed to conditions and amplification was programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 40°C for 2 min, and an extension step at 72°C for 2 min, following by extension cycle for 7 min at 72°C in the final cycle.

Detection of PCR Products

The products of ISSR-based PCR analyses were detected using agarose gel electrophoresis (1.2% in 1 X TBE buffer), then stained with Ethidium bromide (0.3 ug/ml) and then visually examined with UV transilluminator and photographed using a CCD camera (UVP, UK).

Data analysis

Clear, unambiguous and reproducible bands recovered through different techniques were considered for scoring. Each band was considered a single locus. Data were scored as (1) for the presence and (0) for the absence of a given DNA band. Band size was estimated by comparing with 1-kb ladder (Invitrogen, USA) using Totallab, TL120 1D v2009 (nonlinear Dynamics Ltd, USA). The binary data matrices were entered into the NTSYSpc (Ver. 2.1) and analyzed using qualitative routine to generate similarity coefficient and used to construct a dendrogram using unweighted pair group method with arithmetic average (UPGMA) and sequential hierarchical and nested clustering (SHAN) routine.

RESULTS AND DISCUSSION

Figure (1) depicts the DNA banding patterns obtained with ISSR-PCR techniques of the four cultivars; Fahl, Gemmiza 1, Giza 6 and Serw 1. The polymorphism levels appeared by ISSR primers confirmed that, HB-10 ISSR-primer was higher than the rest of ISSR primers (Table 4 and Fig. 1). The results of ISSR-primers HB-8, -9, -10, -11, -12 and HB-13 profile (Fig. 1) are present in Table (2). Primer HB-08 gave 7 bands with the two ecotypes, *i.e.*, four cultivars; one monocut Fahl cultivar and three multicut cultivar; Gemmiza 1, Giza 6, and Serw 1. As well as, primer HB-08 showed unique band with the multicut ecotype for Serw 1 cultivar at 1652.4 bp. Cultivar Serw 1 was released so, the unique bands may be expressed for this advantage. Moreover, primer HB-08 was gave 3 bands monomorphic, 4 bands polymorphic and 57.1% polymorphism (Table 3). Primer HB-09 showed 5 bands with the two ecotypes.

The bands were distributed in 4 monomorphic, one polymorphic with 20% polymorphism (Table 4 and Fig. 1). Primer HB-10 was more variable than other primers, which gave 7 bands as a total polymorphic (100%) polymorphism and one unique bands with multicut ecotype Egyptian clover, Gemmiza 1 at 5000 bp (Tables 2, 3 and Fig. 1).

The cultivar Fahl (monocut ecotype) had four present bands with primer HB-10 which have molecular weight 1216.9 bp, 747.4 bp, 594.9 bp and 462.2 bp according to data in (Table 2 and Fig. 1). On the other hand, the cultivars are multicut ecotype which has three Egyptian clover Gemmiza 1, Giza 6 and Serw 1. The multicut ecotype cultivar Gemmiza 1 involved with monocut ecotype Fahl in 1216.9 bp and 594.9 bp as well as they disagrees in three bands (Table 2). The Fahl and multicut ecotype Giza 6 were involved in two bands 747.4 bp and 462.2 bp, respectively. Furthermore, Fahl and Serw 1 were involved in three bands 747.4 bp, 594.9 bp and 462.2 bp (Table 3 and Fig. 1).

Six bands appeared with primer HB-11, 4 bands were monomorphic, 2 bands polymorphic with 33.3% polymorphism. Unique band (no. 4 with 830.3 bp) was observed in Fahl cultivar only, and it was absent in the other cultivars (Tables 2, 3 and Fig. 1). Primer HB-12 produced 2 bands in both ecotypes; bands number 2 of 1303.7 bp was absent in Gemmiza 1. While, one band was monomorphic, one band was polymorphic with 50% polymorphism and non unique bands (Tables 2, 3 and Fig. 1).

Primer HB-13 depicted 6 bands with the two ecotypes which distributed in 2 bands monomorphic and 4 bands polymorphic with 66% polymorphism. Unique band in Fahl monocut ecotype at MS of 4,605.2 bp was present which was absent in multicut ecotype cultivars (Tables 2, 3 and Fig. 1). It was noted that the two ecotypes had band number 5 at 557.1 bp except serw1 cultivar (Tables 2, 3 and Fig. 1).

It is worth mentioning that Fahl cultivar had 29 present bands, 3 absent bands and two unique bands across the six primers. The multicut ecotype; Gemmiza 1, Giza 6 and Serw1 showed 21; present, 10 absent bands; 21 present and 11 absent bands and 23 present and 9 absent bands respectively (Tables 2, 3 and Fig. 1).

These differences stems from many factors, the location of the class environment where the temperature and humidity, product features carry the harsh conditions, as is the case in Serw 1 and the genetic structure of both ecotypes (Table 2). Both ecotypes were also found to be varied from each to other as indicated by various molecular markers (Zayed *et al.*, 2010; Zayed 2013). In addition to, Soliman *et al.* (2010) found that monocut ecotype Fahl was primitive than multicut ecotype.

The present results agreed with the results obtained by work of Tarrad and Zayed (2009) who studied the multicut

ecotype cultivars and observed disagrees in the field performance, isozymes and RAPD-PCR reaction based on genetic material and performance of genetic materials within cuts.

Similarity and Dissimilarity

Similarity indices among the four Egyptian clover cultivars based on ISSR-PCR analysis showed that the highest value appeared between Fahl and Gemmiza1 as well as Giza 6 and Serw 1 (82%) followed by Fahl and Serw 1 (81%). The lowest similarity value appeared between Gemmiza 1 and Serw 1 (70%) followed by Gemmiza 1 and Giza 6 (78%) (Table 4). These results agreed with Tarrad and Zayed (2009).

Cluster analysis

The dendrogram shown in Fig. (2) cleared that the four cultivars were divided into two main clusters according to ISSR-PCR results, the cluster 1 included Fahl and Gemmiza 1. The Cluster 2 contained the two cultivars, Giza 6 and Serw 1. These results weren't agreed with Tarrad and Zayed (2009) who reported the Gemmiza 1 and Fahl had so far genetic distance and similarity indices equal 50%.

The multicut cultivars were different in their origins as follows: Gemmiza 1 developed through poly-crossing selections within collected landraces from Dosuok District, Kafr El-Sheikh Governorate, Egypt for high forage yield potential and prolonged re-growth period at Sakha Research Station. It has a vigorous agronomical traits, early flowering, higher tillering ability, gives 4-6 cuts/season (Middle Delta and Middle Egypt). The cultivar Serw 1 developed through poly-crossing among 14 selected landraces characterized with high forage productivity under salt-affected soil at Serw Res. Station. It is salt tolerant, gives 4-6 cuts/season (North Delta and salt affected soil). Moreover, the cultivar Giza 6 developed through selection among farmer's seed lots. It has late flowering, heat tolerant good yielder, gives 4-6 cuts/season (Middle and Upper Egypt). On the other hand, Abd El-Naby *et al.* (2012) used ISSR primers with Fahl and Sakha 4 and their hybrids; analyses of ISSR-PCR gave a total number of 60 bands with five primers. Also, they found that the number of polymorphic bands was 44; while polymorphism percentage was 73.4%.

Furthermore, Soliman *et al.* (2010) found results that may be important to distinguish the difference between monocut and multicut. They reported that Fahl is more primitive than Miskawi. The selection of vigorous plants may be used to improve for new cultivars with economic value and can to increase forage production per unit area (Abd El-Naby *et al*., 2009; Abo-Feteih *et al*., 2010; Abd El-Naby *et al*., 2012). Moreover, the relationship study between two cultivars Fahl and Miskawi can be better performed using Cubic, Quadratic model (Zayed *et al.*, 2010).

Allele Frequency

A population is said to be in Hardy- Weinberg equilibrium when 5 condi-

tions are present: no mutations, no gene flow (no immigration /emigration), large population size (no genetic drift), no selective forces and random mating. The allele frequency had different values in both ecotypes (Table 5). The dominant allele was frequented in cultivars Fahl, Gemmiza 1, Giza 6 and Serw 1 with values of 0.8, 0.64, 0.68 and 0.74, respectively. These data mean the cultivar that differed in allele frequency.

An average PIC value of 0.218 across all scored ISSR bands, as well as an average MI of 3.709 across all primers obtained with both ecotypes berseem clover was different than that of AFLP-based genetic diversity studies in various crops (Powell *et al*., 1996; Muminovic *et al.*, 2004). Though both AFLP and RAPD are dominant markers, the easiness associated with RAPD analysis as well as high PIC and MI obtained with berseem clover justifies its use for fingerprinting and identification of cultivars for different a groclimatic zones (Table 6).

SUMMARY

The ISSR markers have been used in order to determine genetic variation and relationship between two clover ecotypes. Six primers for ISSR-PCR technique and succeeded reliable amplicons for different types of Egyptian clover ecotypes. The results revealed polymorphisms level revealed by ISSR primers. HB-10 ISSR-primer was better than of the rest ISSR primers in polymorphic 100%. The Fahl monocut ecotype had 29 present bands, 2 absent bands in 31 total bands; also Fahl have two unique bands. The multicut ecotype Gemmiza 1, Giza 6 and Serw1 were given 20 present, 11 absent; 21 present and 10 absent and 23 present and 11 absent respectively. The three unique bands were appeared in two ecotypes. Fahl was given two with HB-11 and HB-13; the Serw multicut cultivar had one unique bands with HB-08. Similarity indices among the four Egyptian clover cultivars based on ISSR analysis was estimated the highest value was appeared between Fahl and Gemmiza 1 as well as Giza 6 and Serw 1 followed by Fahl and Serw 1. The lowest similarity value was appeared between Gemmiza 1 and Serw 1 followed by Gemmiza 1 and Giza 6.

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Table (1): List of six ISSR primers and their nucleotide sequences.

No.	Primer	Sequence	No.	Primer	Sequence
1	H8	$(GA)_6GG$	4	H11	$(GT)_6CC$
2	H9	$(GT)_6GG$	5	H12	$(CAC)_3GC$
3	H10	$(GA)_6CC$	6	H13	$(GAG)_3GC$

Table (2): Presence and absence of bands of six ISSR-PCR products in the Four Egyptian clover cultivars.

Primer	MW(bp)	Ecotype 1	Ecotype 2		
		Monocut	Multicut		
HB08		Fahl	Gemmiza 1	Giza 6	Serw 1
1	1652.4	0	0	0	1
2	1256.8	1	0	0	1
3	909.0	1	0	0	1
4	711.9	1	1	1	1
5	581.8	1	1	1	1
6	400.0	1	0	0	1
7	219.0	1	1	1	1
HB09		Fahl	Gemmiza 1	Giza 6	Serw 1
1	1276.7	1	1	1	1
2	1055.0	1	0	1	0
3	882.2	1	1	1	1
4	730.6	1	1	1	1
5	614.6	1	1	1	1
HB10		Fahl	Gemmiza 1	Giza 6	Serw 1
1	5000.0	0	1	0	0
2	1216.9	1	1	0	0
3	919.4	0	0	1	1
4	747.4	1	0	1	1
5	594.9	1	1	0	1
6	462.2	1	0	1	1
HB11		Fahl	Gemmiza 1	Giza 6	Serw 1
1	1593.2	1	1	1	1
2	1354.2	1	1	1	1
3	978.0	1	1	1	1
4	830.3	1	0	0	0
5	656.9	1	1	1	0
6	499.8	1	1	1	1
HB12		Fahl	Gemmiza 1	Giza 6	Serw 1
1	1959.3	1	1	1	1
2	1303.7	1	0	1	1
HB13		Fahl	Gemmiza 1	Giza 6	Serw 1
1	1278	1	1	1	1
2	996.9	1	1	1	1
3	755.9	1	1	0	0
4	605.2	1	0	0	0
5	557.1	1	1	1	0
6	421.3	1	1	0	0
Total		29	21	21	23

*bp= base pairs, (1) = present and (0) =absent

Table (3): Primer name, total number, monomorphic, polymorphic band, polymorphism ratio, unique bands and cultivar name.

No.	Primer code	Total band	Monomorphic Band	polymorphic band	polymorphism %	unique band No.	Cultivar name	MW bp	Ecotype
1	HB08	7	3	4	57.1	1	Serw 1	1652.4	Multicut
2	HB09	5	4	1	20.0				
3	HB10	6	0	6	100.0	1	Gemmiza 1	5000.0	Multicut
4	HB11	6	4	2	33.3	4	Fahl	830.3	Monocut
5	HB12	2	1	1	50.0				
6	HB13	6	2	4	66.7	4	Fahl	605.2	Monocut
Total		32	14	18	56.3	10			

Table (4): Similarity indices among the four Egyptian clover cultivars based on ISSR-PCR analysis.

Cultivars		Ecotype 1	Ecotype 2 multicut	
		Fahl	Gemmiza 1	Giza 6
Ecotype 2 multicut	Gemmiza 1	0.82	1.0	0.78
	Giza 6	0.80	0.78	1.00
	Serw 1	0.81	0.70	0.82

Table (5): Allele frequency (p and q) in two cut ecotypes of Egyptian clover based on ISSR-PCR analysis.

Allele	Fahl	Gemmiza 1	Giza 6	Serw 1
Dominant (p) present	0.8	0.64	0.68	0.74
Recessive (q) absent	0.2	0.36	0.32	0.26

Table (6): Comparative analysis of banding patterns generated by ISSR for four berseem clover.

No.	Primer code	Polymorphism (%)	Range of fragment size (bp)	PIC	MI
1	HB08	57.1	1652.4 - 219.0	0.268	1.072
2	HB09	20.0	1276.7 - 614.6	0.110	0.110
3	HB10	100.0	1216.9 - 462.2	0.319	1.595
4	HB11	33.3	1593.2 - 499.8	0.153	0.306
5	HB12	50.0	1959.3 -1303.7	0.183	0.183
6	HB13	66.7	1278.0 - 421.3	0.276	1.104
Mean		54.8		0.218	3.709

PIC = Polymorphic information content, MI = Marker index

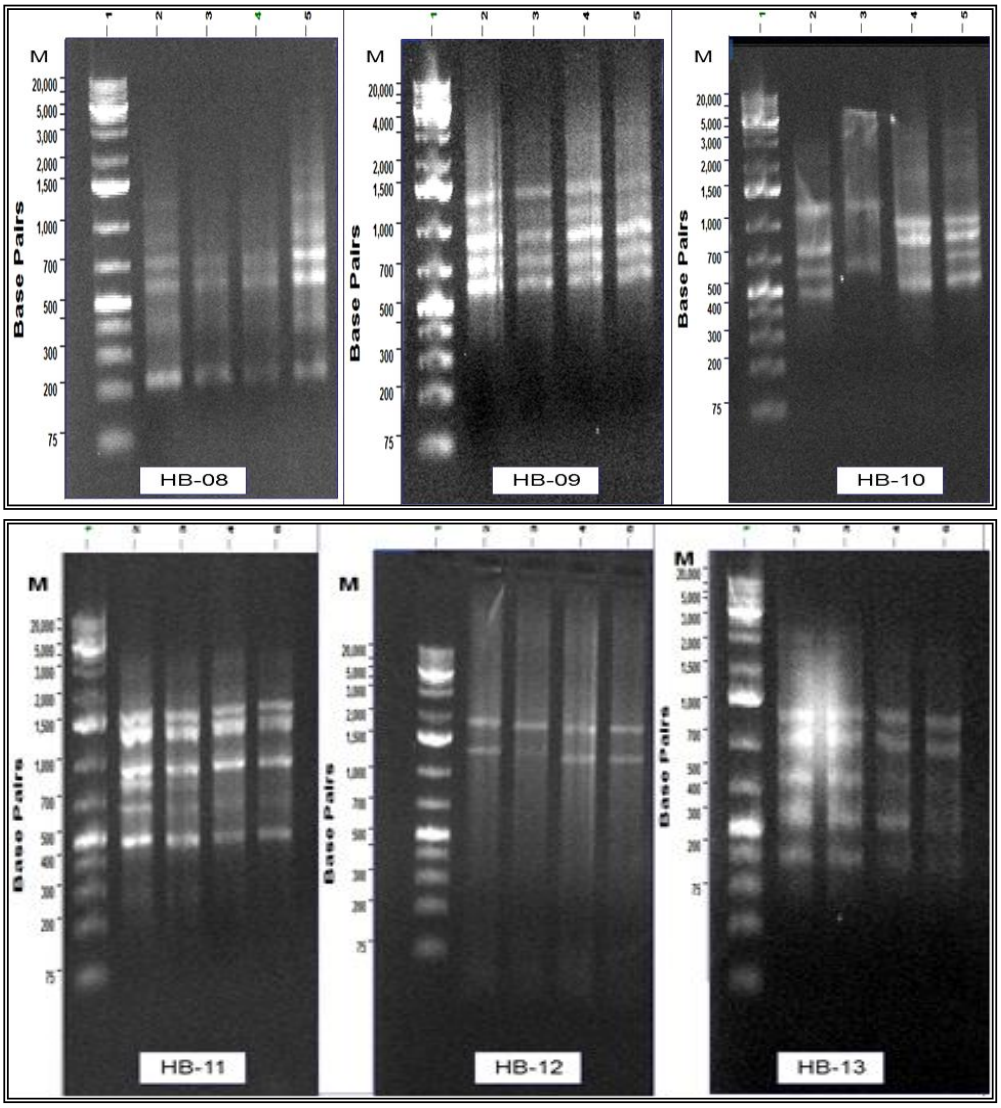

Fig. (1): Illustration ISSR-PCR reaction 6HB-primers, HB-08, 09,10,11,12 and HB-13 with two ecotypes (Mono and Multicut), 1 = Fahl (Monocut), 2 = Gemmiza 1 (Multicut), 3 = Giza 6 (Multicut) and 4 = Serw 1 (Multicut).

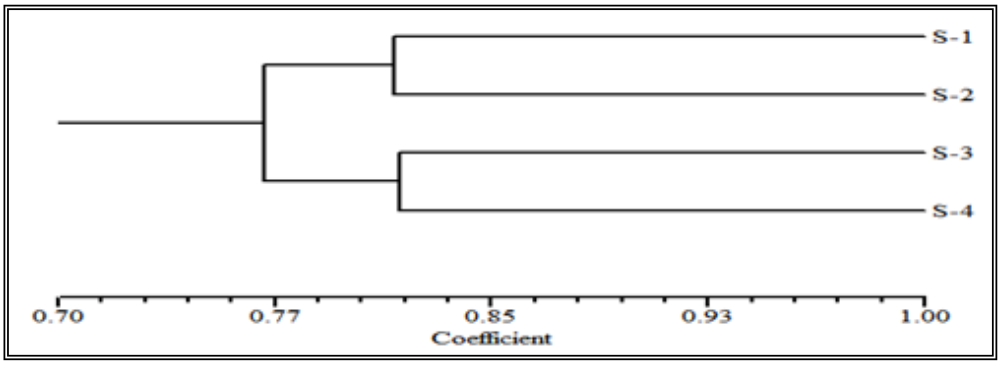

Fig. (2): UPGMA clustering of Egyptian clover cultivars using the six ISSR primers, S-1 = Fahl, S-2 = Gemmiza 1, S-3 = Giza 6 and S-4 = Serw 1.