ASSESSMENT OF GENETIC COMPONENTS AND GENETIC DIVERSITY OF SIX EGYPTIAN CLOVER *(Trifolium alexandrinum* L.) GENOTYPES USING ISSR AND URP MARKERS

A. N. EL-BANNA[1] AND MONA M. F. GHAZY[2]

1. *Genetics Dept., Faculty of Agriculture, Kafrelsheikh University, 33516, Egypt*
2. *Forage Research Dept., Crops Research Institute, Agriculture Research Center, Egypt*

The genus *Trifolium* belongs to the family leguminosae and includes hundreds species, of which red clover (*Trifolium pretense* L.), white clover (*T. repens* L.), alsike clover (*T. hybridum*) and Egyptian clover (*Trifolium alexandrinum* L.) are among the most important species. They widely used as high quality forage (Zayed *et al.*, 2012). Berseem or Egyptian clover is the main source of annual winter forage crop in Egypt, Turkey, India and Pakistan and most of Mediterranean region. Berseem forage represents about 60% of forage animal feed requirements in Egypt and is superior to grasses in protein and mineral contents so it has a high nutritional value for animal feed (Laghari *et al.*, 2000). In addition, it contributes to soil fertility and improves its physical characteristics (Bakheit, 2013). The concept of biotechnology applications has risen early eighties of last century and several articles of biotechnology on Egyptian clover were published. Molecular markers have been proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. They are recognized as a good method for the identification of plant species (Wadl *et al*., 2009; Abdel-Tawab *et al*., 2004) and genetic diversity (Bered *et al.*, 2005; Abdel-Tawab *et al.*, 2009) due to the high resolution and reliability in the identification of cultivars. It has been shown that different markers might reveal different classes of variation (Powell *et al*., 1996; Russell *et al*., 1997). It is correlated with the genome fraction surveyed by each kind of marker, their distribution throughout the genome and the extent of the DNA target which is analyzed by each specific assay (Dávila *et al*., 1999). These markers could be helpful in the breeding programs aimed to develop new cultivar through early detection and screening of plants (Dean *et al.*, 2011). Also, to improve plants taxonomy or to correct cultivars number in collections (Gismondi *et al.*, 2013 and 2014). Genetic diversity of plants was detected using various methods, such as restriction fragment length polymorphism (RFLP), analysis of chloroplast DNA (cpDNA) (Arzate-Fernández *et al*., 2005) randomly amplified polymorphic DNA (RAPD), simple sequence

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repeats (SSRs) and inter simple sequence repeats (ISSRs) have enabled researchers to investigate genetic diversity among various plant species across natural populations (Archak *et al.*, 2003; Wolfe *et al*., 1998). The efficiency of these methods has already been demonstrated in phylogenetic and population genetic studies (Arzate-Fernández *et al*., 2005). Local populations of traditional cultivars provide a valuable resource for plant breeding as well as for the preservation of genetic diversity (Nevo, 1998). Randomly amplified polymorphic DNA (RAPD) method has often been used for estimating genetic variation within and among some cultivars of Egyptian clover (Azab *et al*., 2011; Zayed *et al.*, 2010). In addition, the usefulness of inter simple sequence repeat (ISSR) markers that amplify the genomic sequence between two simple sequence repeats (SSR or microsatellites) using anchored simple sequence repeat (ASSR) primers has also been reported for the identification of molecular differences among *Trifolium* species (Dabkevičien *et al*., 2011). Repeat sequences from Korean weedy rice, originally referred to as universal rice primer (URP), have been used for the fingerprinting of diverse genomes of plants, animals and microbes (Kang *et al*., 2002; Banerjee *et al*., 2014). The principle of URP-PCR method is similar to randomly amplified polymorphic DNA (RAPD) (Williams *et al*., 1990), except the use of longer primer (20 nucleotides) and relatively high annealing temperature for PCR reaction. The URP-PCR condition allows producing reproducible PCR fingerprints compared to RAPD. Recent report showed that URP can be used in identification of 13 varieties of three *Pleurotus* species, oyster mushrooms that were bred in Korea (Kang *et al*., 2001). URP marker is used for the first time to fingerprint Egyptian clover cultivars.

The purpose of this study was to evaluate the agronomic performance, genetic diversity and real genetic variation of six Egyptian clover cultivars available to Berseem growers in Egypt, and to develop a molecular profile using ISSR and URP markers.

MATERIALS AND METHODS

Six clover genotypes *T. alexandrinum* i.e. Helaly, Sakha 4, Gemmiza 1, Serw 1, Giza 6 and a promising accession, Sakha composite were provided by Sakha Forage Research Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt. A two-year field experiment was carried out at Sakha Agricultural Research Station farm, during 2014/2015 and 2015/2016 successive winter seasons to study the performance of these genotypes. Molecular analysis was carried out at the Department of Genetics, Faculty of Agriculture, Kafrelsheikh University, Egypt.

Field experiment

Egyptian clover genotypes were sown in randomized complete block design (RCBD) with plot size 6 m^2 (1.5 m x 4 m), with three replications, sowing dates were at the 7^{th} and 20^{th} of October in two

successive seasons, respectively. Seeds were sown broadcast with seeding rate of 20 kg fed^{-1}. The crop was raised using recommended cultural practices. Four cutting treatments spread over the growing season were practiced. For each cut, the fresh forage yield was determined in kg $plot^{-1}$ and dry forage yield was calculated by multiplying fresh forage yield by dry matter percentage. Total fresh forage yield and total dry forage yield were measured.

Statistical analysis

The collected data were subjected to proper statistical analysis according to the procedures outlined by Snedecor and Cochran (1989) using MSTAT-C program (1986). Homogeneity test of variance was computed by Bartlett's test (Bartlett, 1937). Means were compared at 0.05 level of significance by the least significant difference (LSD) according to Gomez and Gomez (1984). Phenotypic (σ^2p) and genotypic (σ^2g) variances were calculated using the formulae from the combined analysis over years according to Al-Jibouri *et al.* (1958). Phenotypic coefficient of variability (PCV) and genotypic coefficient of variability (GCV) were estimated using the formula developed by Burton (1952). Heritability (h^2) in the broad sense was calculated using the following formula: $h^2 = \sigma^2g/\sigma^2p$ X 100. The parameters were calculated according to Comstock and Robinson (1952) and Johanson *et al.* (1955).

Genomic DNA extraction and PCR condition

Total genomic DNA was isolated from two weeks old seedlings using the Walbot and Warren method (Walbot and Warren, 1988). PCR was performed in a final reaction volume of 20 µl. PCR reaction mixture contained 20 ng DNA templates, 10 pmol of each primer, 2 mM dNTP, 1.5 µl 10x PCR Dream Taq green buffer and 0.1 µl of 5 U/µl Dream Taq DNA polymerase. The conditions for PCR were as follows: an initial denaturing step was performed at 94°C for 3 min followed by 35 cycles at 94°C for 30 s, a 30 s annealing at different temperature (primer specific), an extension at 72°C for 1 min and a final extension at 72°C for 5 min. All the reactions were performed on an eppendorf mastercycler ep384 (Eppendorf, Germany). The PCR products were run on a 1.5% agarose gel, visualized by ethidium bromide and documented with a Gen Genius Bio Imaging System (Syngene, UK). Reproducibility of the DNA profiles was tested by repeating the PCR amplifications two to three times with each of the respective primers. The bands were considered reproducible and scorable only after observing and comparing them in separate amplifications for each primer. Clear and intense bands were scored, while faint bands against background smear were excluded. ISSR and URP profiles were analyzed using MyImage Analysis v2.0 software, Thermo Scientific.

Screening of primers

A set of six ISSR and five URP primers were used for analysis of the genetic variation among clover cultivars (Table 1).

Gel scoring and data analysis

Marker index for ISSR and URP primers was calculated in order to characterize the capacity of each primer to detect polymorphic loci among the genotypes. It is the sum total of the polymorphism information content (PIC) values of all the bands produced by a particular primer. PIC value was calculated using the formula: PIC = $1\text{-}\Sigma pi^2$, where *pi* is the frequency of the i^{th} allele (Smith *et al*., 1997). Band informativeness (Ib) was calculated as given by Prevost and Wilkinson (1999), Band informativeness of a given band: $Ib = 1 - (2 \times |0.5 - p|)$, where *p* is the proportion of the total genotypes containing the band. The bands obtained by scoring the ISSR and URP profiles were treated as binary characters and coded accordingly (presence = 1, absence = 0). Jaccard similarity index (Jaccard, 1908) was determined among the cultivars studied to be used in clustering. The genotypes showing similarity in their ISSR and URP characteristics were grouped by using UPGMA (Unweighted Paired Group with Arithmetic Average). SPSS-10 package was used for statistical analysis.

RESULTS AND DISCUSSION

Agronomic traits

The mean squares of fresh and dry forage yield for different cuts of clover and seasonal fresh forage yield over the two seasons 2013/2014 and 2014/2015 are presented in Table (2). Data showed that years were highly significant for all cuts and seasonal fresh forage yield. The mean squares of genotypes were highly significant for four cuts and seasonal fresh yield, while genotypes x years interaction was not significant. In contrast, years were not significant for all cuts and seasonal dry forage yield. The mean squares of genotypes were highly significant for four cuts and seasonal dry forage yield, while the interaction between genotypes x years were not significant except in the first cut which was highly significant. Mean performance for the four cuts and seasonal fresh and dry forage yield of the six cultivars for combined data over two years were presented in Table (3). For fresh forage yield, the seasonal fresh forage yield ranged from 81.77 kg $plot^{-1}$ (Sakha comp.) to 87.99 kg $plot^{-1}$ (Helaly). Helaly was the best cultivar for the four fresh cuts and seasonal yield with the mean values of 19.19, 21.12, 23.59, 23.87 and 87.99 kg $plot^{-1}$, respectively. While Gemmeiza1 has the lowest mean (17.97 kg $plot^{-1}$) in the first cut, Giza 6 (19.61 and 21.44 kg $plot^{-1}$) in the second and third cuts, respectively, and Sakha composite. (22.18 and 81.77 kg $plot^{-1}$) in the fourth cut and seasonal yield, respectively. For dry forage yield, Sakha 4 produced the highest mean value for the first cut

(2.03 kg plot^{-1}). While Gemmeiza1 has the highest value in the second cut (2.70 kg plot^{-1}). Serw 1 recorded the highest values in the third, fourth and seasonal dry yield with the mean values of 3.33, 3.10 and 10.92 kg plot^{-1}, respectively. Sakha composite has the lowest mean values of dry forage yield for all cuts and seasonal yield except for the first cut. The results indicated that Helaly genotype relatively dominate other genotypes for seasonal fresh and dry yield. Helaly was bred for relatively high ability to rapid regrowth which may be associated with alternative conditions. Therefore, Helaly proved to be characterized with high potential for seasonal fresh and dry forage yield kg plot^{-1} in comparison with the other genotypes under study. Similar results were obtained by El-Nahrawy (2007).

Grand mean (Ẍ), genotypic variance (σ^2g), phenotypic variance (σ^2p), heritability (h^2), phenotypic coefficient of variability PCV% and genotypic coefficient of variability GCV % for cuts and seasonal fresh and dry forage yield traits for combined data over two years were presented in Table (4). The grand mean of fresh forage yield increased starting from the 1st cut (18.57 kg plot^{-1}) up to the 4th cut (22.9) with seasonal yield of (84.28 kg plot^{-1}). The phenotypic coefficient of variation (PCV) was higher than the genotypic coefficient of variation (GCV) and considerable consistency of values was observed between PCV and GCV percentage for all cuts and seasonal yield. The values suggested some effects of environment for these traits. Also, the results indicated that high estimates of heritability (broad sense) were found for all cuts and seasonal yield with the exception of the 1st cut which showed moderate heritability values (45.1%). For dry forage yield, the results showed that the grand mean (Ẍ) also increased from 1.78 kg plot^{-1} at the 1st cut to 3.08 kg plot^{-1}at the 3rd cut and seasonal yield 10.00 kg plot^{-1}, as well as the PCV was higher than he GCV for all cuts and seasonal yield. Also, the results indicated that the heritability in the broad sense were high as estimated for all cuts and seasonal yield. The results of Ẍ, phenotypic and genotypic coefficient of variation for fresh and dry forage yield, the Ẍ increased from cut to other and PCV was higher than GCV for all cuts and seasonal yield for fresh and dry forage yield and these results showed considerable consistency of values observed between PCV and GCV percentage for all cuts and seasonal yield suggesting some effects of environment on these traits, similar results were obtained by Radwan *et al.* (1983) and El-Nahrawy (2007).

Molecular analysis

Out of nine ISSR primers used for initial screening with the six clover cultivars, only six primers amplified polymorphic patterns. These six ISSR primers revealed high polymorphism percentages with the amplified DNA (Fig. 1 and Table 1). They produced a total of 49 scorable bands from which 38 were polymorphic (77.6%) among the genotypes. The number of scorable markers produced per

primer ranged from 3 to 14. The highest number of bands (14) was obtained with primer P1, while the lowest number (3) was obtained with primer M822. Different primers showed the same variation in their ability to detect polymorphism, the highest percentage of polymorphism was recorded by primers P2 and UBC813 (100%), while the lowest value was recorded by primer UBC812 (20%). The six polymorphic primers exhibited variation with regard to band informativeness (Ib), primer UBC812 showed the lowest Ib (0.07), while the highest Ib (0.52) and value were exhibited by primer P1. The PIC values, a reflection of allele diversity and frequency among the cultivars, were not uniformly higher for all the ISSR loci tested. The PIC value ranged from 0.35 (P1) to 0.06 (UBC812). URP primers produced 40 scorable bands among studied genotypes, out of which 36 bands (90%) were polymorphic (Table 1). The size of amplified products ranged from 400 bp to 1300 bp. The number of scorable markers produced per primer ranged from 5 to 12. Primer URP6R produced the highest number of bands (12), while primer URP4R produced the lowest number of bands (5). Twelve out of 40 URP bands were found to be cultivar specific. These markers were scored for the presence of unique bands for a given cultivar. The fragments of 480 and 1300 bp are specific for the cultivars Sakha4 and Sakha composite, respectively (Fig. 1). These primers showed variation in the polymorphism information content (PIC) and band informativeness (Ib). The PIC values were higher for all the URP loci tested. The PIC value ranged from 0.26 (URP2F) to 0.39 (URP4R). The primer URP2F showed the highest values of Ib (0.48).

Genetic diversity and clustering based on ISSR and URP polymorphism data

The similarity matrices resulting from ISSR and URP primers data were performed to generate correct relationships based on large and different genome regions. Similarity coefficients index for the clover genotypes based on 6 ISSR primers ranged between 0.37 and 0.72 (Table 5), similarity index was the highest between Sakha 4 and Sakha composite (72%), while was the lowest between Helaly and Giza 6 (37%). The cluster analysis of ISSR markers separated the clover genotypes into two distinct clusters (Fig. 2A). The first cluster included only Helaly, while the second cluster included the remaining genotypes and was further divided into two subclusters. The first subcluster had Giza 6 genotype and second subcluster contained Serw, Sakha 4, Sakha composite and Gemmiza1 genotypes.

According to the URP data, a dendrogram was developed for the six genotypes and indicated two main clusters; the first cluster included Helaly cultivar with the lowest genetic similarity of 23 to 41% with other genotypes (Fig. 2B and Table 5). The second cluster contained two subclusters, one consisted of Giza 6 and Serw 1 and the second group

contained Sakha 4, Sakha composite and Gemmeiza 1. The highest similarity index was recorded for Sakha and Sakha composite (62%).

The dendrogram obtained from the combined data on amplified products with all the 11 primers of the two techniques showed different levels of genetic similarity among clover cultivars. There were two major clusters formed among all cultivars separating Helaly uniquely in the first cluster and the second consisted of the rest of cultivars (Fig. 2C and Table 5). The second cluster was further subdivided into two subclusters, in the first one, Sakha 4, Sakha composite and Gemmeiza 1, while the second subcluster consisted of Sakha 6. The lowest genetic similarity (29-43%) was recorded between Helaly and the other genotypes under study.

The results indicated that the percentage of URP polymorphic bands (90%) was higher than that of ISSR (77.6%). Moreover, PIC and Ib values (Table 1) detected by the five URP primers was much higher than that of the six ISSR primers which suggested that the URP markers were superior to ISSR markers in the capacity of revealing more informative bands. The ISSRs have proven to be a reliable, rapid, simple, cost effective, easy to generate, and versatile set of markers that do not require previous knowledge of the genome sequence to generate DNA markers (Bornet and Branchard, 2001; Bornet *et al.*, 2002). Furthermore, previous investigators have demonstrated that ISSR analysis usually detects a higher level of polymorphism than that detected with Restriction Fragment Length Polymorphism (RFLP) or Randomly Amplified Polymorphic DNA (RAPD) analyses (Kantety *et al*., 1995; Nagaoka and Ogihara, 1997). Thus, ISSR markers are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology and are widely applied in plant genetic analyses (McClean *et al*., 2002; Gonzalez *et al*., 2005; Manimekali and Nagarajan, 2006). Tarrad and Zayed (2009) and Zayed *et al.* (2010) analyzed some Egyptian clover cultivars based on RAPD, ISSR and AFLP. The generated DNA profiles proved highest level of molecular variance (24.5%). Also they found that the biochemical genetic markers were differed between Egyptian clover cultivars. The present investigation shows that URP-PCR fingerprinting is a valuable tool for rapid identification and differentiation of Egyptian clover cultivars. Repeat sequences from Korean weedy rice, originally referred to as universal rice primer (URP), have been used for the fingerprinting of diverse genomes of plants, animals and microbes (Kang *et al.*, 2002), but to our knowledge this is the first report of using URP primers for clover fingerprinting studies. Combination of ISSR and URP markers has been used to determine the genetic affinities between six Egyptian clover cultivars. The results emphasized the advantages of using a combination of several molecular systems over the use of one system in order to obtain higher resolution to discriminate among the Egyptian clover cultivars.

This study gives an insight into the rich variability present in the Egyptian clover genotypes at agronomical and molecular levels, which can prove to be a good source of genes for further improvement programs. Moreover, most of the studied species had specific fragments that could serve as markers for species identification.

SUMMARY

Six Egyptian clover genotypes, i.e., Helaly, Sakha 4, Gemmeiza 1, Serw1, Giza6 and Sakha composite were used in this study. Data was calculated on four cuts and seasonal yield for fresh and dry forage yield. The results indicated that the mean square values were highly significant for genotypes for all studied traits. Helaly cultivar gave the highest values in four cuts and seasonal yield for fresh forage yield. High heritability values were recorded for all studied traits except 1st fresh yield which recorded moderate value. Phenotypic coefficient of variability (PCV) was higher than genotypic coefficient of variability (GCV) in all cuts and seasonal yields. Genetic variations among the six Egyptian clover cultivars were evaluated using universal rice primers (URP) and inter simple sequence repeat (ISSR) markers. URP fingerprinting detected more polymorphic loci (90%) than the ISSR fingerprinting (77.55%). Mean PIC (polymorphism information content) and band informativness (Ib) for each of these marker systems were (0.21, 0.26 for ISSR and 0.32, 0.46 for URP) respectively, suggested that URP marker systems were effective than ISSR in determining polymorphisms. Pairwise similarity index values ranged from 0.37 to 0.72 for ISSR, 0.23 to 0.62 for URP and 0.29 to 0.65 for combined ISSRs and URP indicated the genetic distinctness among the studied genotypes.

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Table (1): Presence of different bands using six ISSR and five URP primers with the Egyptian clover.

Primer code	Primer sequence (5' → 3')	NB	PB	P%	UB	PIC	Ib
P1(ISSR1)	CACACACACACAGT	14	12	85.70	4	0.35	0.52
P2(ISSR2)	CACACACACACA	13	13	100.00	6	0.33	0.38
Q2(ISSR3)	TCTCTCTCTCTCTCTCC	4	1	25.00	1	0.07	0.08
M822(ISSR4)	TCTCTCTCTCTCTCTCA	3	1	33.30	0	0.09	0.11
UBC812(ISSR5)	GAGAGAGAGAGAGAGAA	5	1	20.00	1	0.06	0.07
UBC813(ISSR6)	CTCTCTCTCTCTCTCTT	10	10	100.00	5	0.34	0.47
Total and average		49	38	60.66	17	0.32	0.27
URP-1F(URB1)	ATCCAAGGTCCGAGACAACC	8	7	87.50	2	0.31	0.46
URP-6R(URB2)	GGCAAGCTGGTGGGAGGTAC	12	11	91.70	4	0.32	0.44
URP-2F (URB3)	GTGTGCGATCAGTTGCTGGG	7	6	85.70	3	0.26	0.48
URP-4R(URB4)	AGGACTCGATAACAGGCTCC	5	5	100.00	1	0.39	0.46
URP2R(URB5)	CCCAGCAACTGATCGCACAC	8	7	87.50	2	0.32	0.44
Total and average		40	36	90.48	12	0.38	0.46

NB: Number of amplified bands, PB: Polymorphic bands, P%: Polymorphism percentage,
UB: Unique bands, PIC: Polymorphism information content, Ib: band informativness.

Table (2): Mean squares for four cuts and seasonal fresh and dry forage yield of the six clover cultivars for combined data over the two seasons.

S.O.V	df	1st Cut		2nd Cut		3rd Cut		4th Cut		Seasonal yield	
		Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Years (Y)	1	19.30**	0.123	13.40**	0.220	16.10**	0.010	39.500**	0.200	372.20**	1.82
Rep./Year	4	0.65	0.990	0.21	0.068	0.53	0.014	2.700	0.010	1.62	0.85
Genotypes (G)	5	0.86**	0.370**	4.28**	0.230**	3.80**	0.400**	3.030**	0.320**	33.10**	2.05**
G x Y	5	0.12	0.030**	0.03	0.004	0.01	0.010	0.053	0.003	0.96	0.09
Error	20	0.17	0.010	0.31	0.030	0.31	0.030	0.350	0.015	2.31	0.06

**Significant at 0.01 level of probability

Table (3): Mean performance for four cuts and seasonal fresh and dry forage yield kg plot^{-1} of the six clover cultivars for combined data over the two seasons.

Cultivars	1st Cut		2nd Cut		3rd Cut		4th Cut		Seasonal yield	
	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Helaly	19.19	1.68	21.12	2.58	23.59	3.28	23.87	2.58	87.99	10.13
Sakha4	18.47	2.03	19.99	2.47	21.90	2.88	22.29	2.58	83.11	9.97
Gemmeiza1	17.97	1.37	21.75	2.70	22.50	3.28	23.21	2.62	85.73	9.97
Serw 1	18.76	1.83	20.52	2.65	21.79	3.33	23.64	3.10	84.68	10.92
Giza 6	18.64	2.02	19.61	2.25	21.44	3.00	22.59	2.65	82.30	9.92
Sakha comp.	18.40	1.72	19.75	2.25	21.58	2.70	22.18	2.42	81.77	9.08
LSD 0.05	0.494	0.081	0.668	0.217	0.674	0.200	0.713	0.146	1.829	0.298
LSD 0.01	0.674	0.110	0.910	0.296	0.919	0.273	0.973	0.199	2.495	0.407

Table (4): Grand mean (x^-), genotypic variance (σ^2g), phenotypic variance (σ^2p), heritability (h^2), genotypic coefficient of variability GCV% and phenotypic coefficient of variability PCV% for the 4 cuts and seasonal fresh and dry forage yield traits for combined data over the two seasons.

Cultivars	1st Cut		2nd Cut		3rd Cut		4th Cut		Seasonal yield	
	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Ẍ	18.57	1.78	20.50	2.48	22.13	3.08	22.9	2.70	84.3	10.00
σ^2g	0.12	0.14	0.71	0.04	0.64	0.07	0.50	0.05	5.40	0.30
σ^2p	0.27	0.07	0.92	0.06	0.85	0.09	0.75	0.06	7.20	0.40
h^2	45.10	82.60	76.50	62.30	75.10	75.90	66.40	83.20	74.20	82.1
GCV%	1.90	13.50	4.10	7.90	3.60	8.30	3.10	8.70	2.75	5.70
PC%	2.80	14.80	4.70	9.90	4.17	9.60	3.80	9.50	3.20	6.30

Table (5): Similarity indices among the Egyptian clover cultivars based on ISSR, URP and combined ISSR and URP bands.

ISSR data	Helaly	Gemmeiza 1	Sakha 4	Sakha composite	Serw1
Gemmeiza 1	0.56				
Sakha 4	0.40	0.59			
Sakha composite	0.48	0.69	0.72		
Serw1	0.40	0.53	0.63	0.56	
Giza 6	0.37	0.44	0.49	0.51	0.41
URP data	Helaly	Gemmeiza 1	Sakha 4	Sakha composite	Serw1
Gemmeiza 1	0.23				
Sakha 4	0.28	0.59			
Sakha composite	0.23	0.60	0.62		
Serw1	0.24	0.39	0.46	0.48	
Giza 6	0.41	0.44	0.46	0.48	0.55
Combined data	Helaly	Gemmeiza 1	Sakha 4	Sakha composite	Serw1
Gemmeiza 1	0.39				
Sakha 4	0.43	0.54			
Sakha composite	0.36	0.62	0.65		
Serw1	0.32	0.46	0.54	0.50	
Giza 6	0.29	0.39	0.50	048	0.44

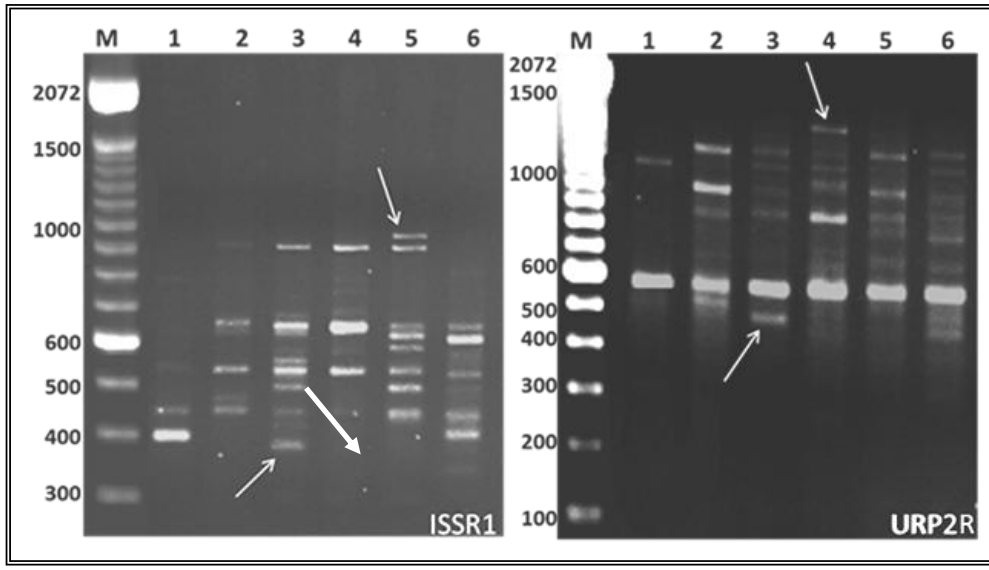
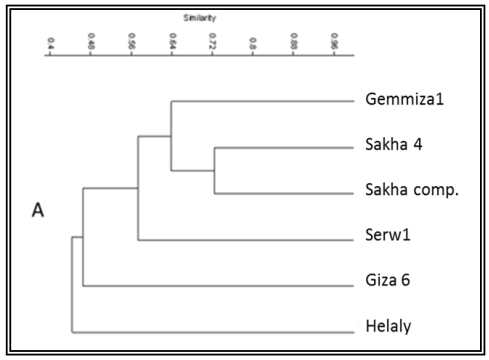
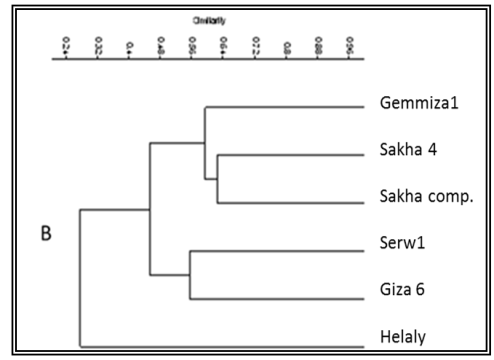


Fig. (1): DNA fingerprint profile of the six clover cultivars obtained with primer ISSR1 and URP2R. as an example. M is the 100 kb DNA ladder (Invitrogen), Lanes 1-6, Clover cultivars, 1 - Helaly,2- Gemmeiza1, 3- Sakha 4, 4- Sakha composite, 5- Serw1, 6-Giza 6. Arrows indicated cultivar specific markers.





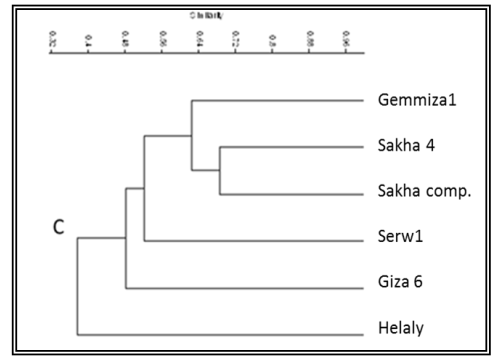


Fig. (2): Dendrogram generated using UPGMA analysis, showing relationships between clover cultivars using ISSR, URP and combining ISSR and URP data (A) ISSR. (B) URP (C) ISSR and URP. Similarity matrices computed according to Jaccard's coefficient.