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*Full Length Research Paper*

# Genetic variations between two ecotypes of Egyptian clover by inter-simple sequence repeat (ISSR) techniques

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**The inter-simple sequence repeat (ISSR) markers have been used in order to determine genetic variation and relationship between two clover ecotypes. Ten (10) primers for ISSR were used in this study but only six were successful in generating reproducible and reliable amplicons for different types of the Egyptian clover. The results reveal the polymorphism level by ISSR primers. HB10 ISSR-primer was higher than the rest of the ISSR primers in polymorphic 100%. The Fahl monocut ecotype had 29 present bands, 3 absent bands in total of 32 bands; among those there were two unique bands. The multicut ecotype were given different pattern of bands, Gemmiza1 (21 present and 11 absent), Giza6 (21 present and 11 absent) and Serw1; (23 present and 9 absent). There were three unique bands appearance in the two ecotypes. Fahl was given two with HB11 and HB13; the Serw1 multicut cultivar had one unique bands with HB08. Similarity indices among the four Egyptian clover cultivars based on ISSR analysis was estimated and the highest value appeared between Fahl and Gemmiza1 as well as Giza6 and Serw1 followed by Fahl and Serw1. The lowest similarity value appeared between Gemmiza1 and Serw1 followed by Gemmiza1 and Giza6.**

**Key words:** Egyptian clover, molecular marker, Fahl, Gemmiza1, Giza6, Serw1.

## INTRODUCTION

Among fodder crops, Egyptian clover (Berseem) has high commercial importance and potential value. 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 five to six times after cutting, including numerous varieties in the Egyptian clover. The cycle of berseem capital is estimated by ten billion US dollars. Berseem is the main forage crops for livestock to produce milk and /or meat in Egypt. Moreover, berseem is the guard on Egyptian soil fertility (Zayed, 2013). Egypt has poor rangeland, although vast areas of more than 10

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million ha exist. The highest total dry matter yield of 15.861 ton $ha^{-1}$ was recorded for Gemmiza1 cultivar. Egypt depends mainly on Egyptian clover as the key forage crop. The cultivated area of berseem in Egypt can reach more than 1.2 million 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 (FAO, 2010). 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).

Four Egyptian clover (*Trifolium alexandrinum* L) cultivars representing two ecotypes were used in the present study. Fahl cultivar is prevalent in whole Egypt and is good for single cut as it has poor regeneration ability, whereas Serw1, Giza6 and Gemmiza1 give 5-6 cuts of good fodder. Techniques based on molecular marker analysis (that is, RFLP, RAPD, ISSR-PCR) may provide 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 (Beckmann and Soller, 1990; Lagercrantz et al., 1993; Tautz and Renz, 1984). 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 genetic study in the past (Ulloa et al., 2003). The advantage of ISSR over RAPD is its being more reproducible (Fernandez et al., 2002; Greene et al., 2004).

In previous studies, polyacrylamide gel electrophoresis (PAGE) was performed for native and SDS protein and isozyme variations. RAPD was conducted using eight 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 Sakha4 and Gemmiza1, while the lowest similarity (0.53) was observed between Giza6 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 (Soliman et al., 2010; Zayed et al., 2010). In the present study, ISSR markers have been used in order to determine genetic variation and relationship between two ecotypes.

**Table 1.** List of ISSR primers and their nucleotide sequences.

| Primer name | Sequence |
|---|---|
| H8 | $(GA)_6GG$ |
| H9 | $(GT)_6GG$ |
| H10 | $(GA)_6CC$ |
| H11 | $(GT)_6CC$ |
| H12 | $(CAC)_3GC$ |
| H13 | $(GAG)_3GC$ |

## MATERIALS AND METHODS

### Plant material

Four Egyptian clover (*Trifolium alexandrinum* L) cultivars representing two ecotypes were used in the present study: one cultivar monocot ecotype (FAh1) and three cultivars multicut ecotype (Serw1, Giza6 and Gemmiza1). Fahl cultivar has 16 chromosome and prevalent in whole Egypt as single cut because it has poor regeneration ability. Serw1cultivar has 16 chromosome, multicut, and cultivated in salinity soil and north of Egypt. Giza6 cultivar has 16 chromosome, multicut, and cultivated in Upper Egypt. Gemmiza1cultivar has 16 chromosome according to Soliman et al. (2010), multicut, and cultivated in all Egypt. Serw1, Giza6 and Gemmiza1 cultivars are distributed in Egypt and can give 5-6 cuts of good fodder. They have higher green fodder yield and has good regeneration ability after cutting.

### Genomic DNA extraction and purification

Extraction of total DNA was performed using methods for medicinal and aromatic plants according to Anna et al. (2001). 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. (µg/ml) = $OD_{260}$ × 50 × dilution factor according to Barbas et al. (2001).

### Inter simple sequence repeats (ISSRs)

Ten (10) 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 volume containing 1x PCR buffer, 4 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmole primers, 2 units Taq DNA polymerase and 25 ng template DNA. PCR amplification was performed in a Perkin Elmer 2400 thermocycler (Germany). PCR 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 10 min at 72°C in the final cycle.

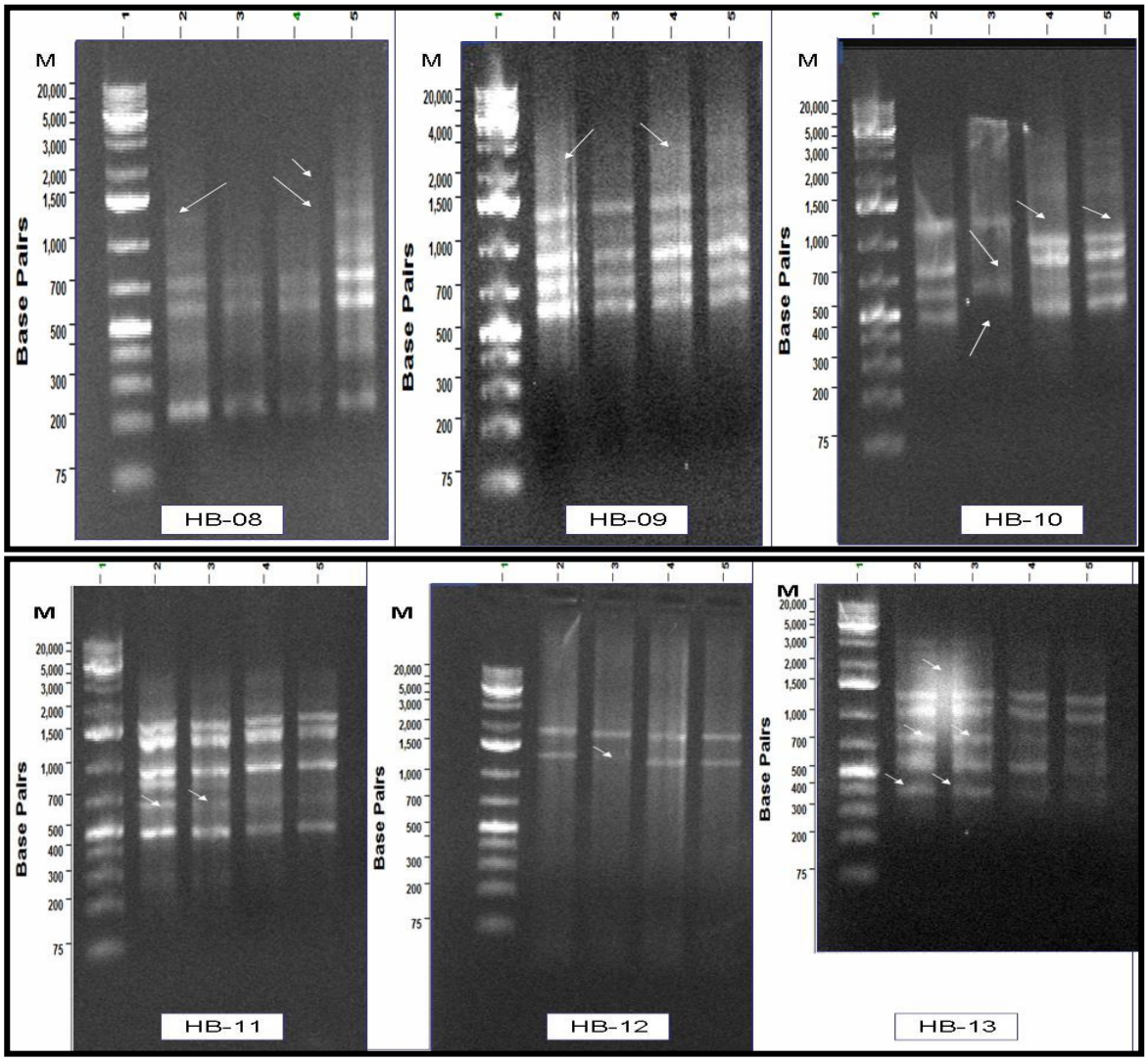


**Figure 1.** Illustration of ISSR–PCR reaction 6HB primers, HB08, HB09, HB10, HB11, HB12 and HB13 with two ecotypes (Mono and Multicut). 1= 1 kp DNA marker; 2 = Fahl (Monocut); 3=Gemmiza1 (Multicut); 4=Giza6 (Multicut); 5 = Serw1 (Multicut).

### Detection of PCR Products

The products of ISSR-based PCR analyses were detected using agarose gel electrophoresis (1.2% in 1X TBE buffer), then stained with Ethidium bromide (0.3 µg/ml) and then visually examined with UV trans-illuminator 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 shows the DNA banding patterns obtained with ISSR-PCR techniques of the four cultivars Fahl, Gemmiza1, Giza6 and Serw1 using six different ISSR primers. The identified bands that resulted from the ISSR- primer HB8, HB9, HB10, HB11, HB12 and HB13 profile are presented in Figure 1 and Table 2. The polymorphism level shown by ISSR primers confirmed that HB ISSR-primer HB10 was higher than the rest of the ISSR primers (Table 3). The primer HB08 give 7 bands with two ecotypes, one monocut Fahl cultivar and

**Table 2.** Present and absent bands ISSR-PCR products by HB primers in Four Egyptian clover cultivars.

| Primer | MW (bp) | Ecotype 1 | Ecotype 2 | | |
|---|---|---|---|---|---|
| | | Monocut | Multicut | | |
| | | Fahl | Gemmiza1 | Giza6 | Serw1 |
| **HB08** | | | | | |
| 1 | 1652 | 0 | 0 | 0 | 1 |
| 2 | 1257 | 1 | 0 | 0 | 1 |
| 3 | 909 | 1 | 0 | 0 | 1 |
| 4 | 712 | 1 | 1 | 1 | 1 |
| 5 | 582 | 1 | 1 | 1 | 1 |
| 6 | 400 | 1 | 0 | 0 | 1 |
| 7 | 219 | 1 | 1 | 1 | 1 |
| **HB09** | | | | | |
| 1 | 1277 | 1 | 1 | 1 | 1 |
| 2 | 1055 | 1 | 0 | 1 | 0 |
| 3 | 882 | 1 | 1 | 1 | 1 |
| 4 | 731 | 1 | 1 | 1 | 1 |
| 5 | 615 | 1 | 1 | 1 | 1 |
| **HB10** | | | | | |
| 1 | 5000 | 0 | 1 | 0 | 0 |
| 2 | 1217 | 1 | 1 | 0 | 0 |
| 3 | 919 | 0 | 0 | 1 | 1 |
| 4 | 747 | 1 | 0 | 1 | 1 |
| 5 | 595 | 1 | 1 | 0 | 1 |
| 6 | 462 | 1 | 0 | 1 | 1 |
| **HB11** | | | | | |
| 1 | 1593 | 1 | 1 | 1 | 1 |
| 2 | 1354 | 1 | 1 | 1 | 1 |
| 3 | 978 | 1 | 1 | 1 | 1 |
| 4 | 830 | 1 | 0 | 0 | 0 |
| 5 | 657 | 1 | 1 | 1 | 0 |
| 6 | 500 | 1 | 1 | 1 | 1 |
| **HB12** | | | | | |
| 1 | 1959 | 1 | 1 | 1 | 1 |
| 2 | 1304 | 1 | 0 | 1 | 1 |
| **HB13** | | | | | |
| 1 | 1278 | 1 | 1 | 1 | 1 |
| 2 | 997 | 1 | 1 | 1 | 1 |
| 3 | 756 | 1 | 1 | 0 | 0 |
| 4 | 605 | 1 | 0 | 0 | 0 |
| 5 | 557 | 1 | 1 | 1 | 0 |
| 6 | 421 | 1 | 1 | 0 | 0 |
| Total | 32 | 29 | 21 | 21 | 23 |

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

three cultivar, multicut, Gemmiza1, Giza6, and Serw1. As well, the primer HB08 was observed to produce unique bands with the ecotype multicut for Serw1 cultivar at 1652 bp. Moreover, the primer HB08 was given 3 bands monomorphic, 4 bands polymorphic with 57.1% polymorphism (Table 3). Primer HB09 showed 5 bands with the two ecotypes. The bands were distributed in 4 monomorphic and 1 polymorphic with 20% polymorphism ratio (Table 3 and Figure 1). Primer HB10 was more variable than the other primers, which give 6 bands as a total polymorphic, 100% polymorphism, and one unique band with multicut ecotypes Egyptian clover Gemmiza1

**Table 3.** Primer name, total band, monomorphic, polymorphic, polymorphism ratio, unique bands and cultivar name.

| Primer Name | Total band | Monomorphic Band | Polymorphic band | Polymorphism % | Unique band number | Cultivar name | MW (Bp) | Ecotype |
|---|---|---|---|---|---|---|---|---|
| HB08 | 7 | 3 | 4 | 57.1 | 1 | Serw1 | 1652 | Multicut |
| HB09 | 5 | 4 | 1 | 20.0 | | | | |
| HB10 | 6 | 0 | 6 | 100 | 1 | Gemmiza1 | 5000 | Multicut |
| HB11 | 6 | 4 | 2 | 33.3 | 4 | Fahl | 830 | Monocut |
| HB12 | 2 | 1 | 1 | 50.0 | | | | |
| HB13 | 6 | 2 | 4 | 66.7 | 4 | Fahl | 605 | Monocut |
| Total | 32 | 14 | 18 | 56.3 | 10 | | | |

**Table 4.** Similarity indices among four Egyptian clover cultivars based on ISSR-PCR analysis.

| Cultivars | Fahl | Gemmiza1 | Giza6 |
|---|---|---|---|
| Gemmiza1 | 0.82 | | |
| Giza6 | 0.80 | 0.78 | |
| Serw1 | 0.81 | 0.70 | 0.82 |

at 5000 bp (Tables 2, 3 and Figure 1).

The cultivar Fahl (monocut ecotype) had four bands with ISSR primer HB10 which have molecular weight 1217, 747, 595 and 462 bp according to data in (Table 2 and Figure 1). On the other hand, the multicut ecotype cultivars which include three Egyptian clover cultivars Gemmiza1, Giza6 and Serw1 have different distribution regards to those four bands. The multicut ecotype cultivar Gemmiza1 were involved with monocut ecotype Fahl in 1217 and 595 bp as well as they disagrees in three bands (Table 2). The Fahl and multicut ecotype Giza6 were involved in two bands 747 and 462 bp. Furthermore, Fahl and Serw1 were involved in three bands 747, 595 and 462 bp but not 1217 bp (Table 2 and Figure 1).

Six bands appeared with primer HB11 out of which four bands were monomorphic, two bands polymorphic with 33.3% polymorphism ratio. Unique band at 830 bp was observed in Fahl cultivar only, and it was absent in other cultivars (Tables 2, 3 and Figure 1).

The primer HB12 produced two bands in both ecotypes at 1959 and 1304 pb except in Gemmiza1 cultivar band 1304 was absent. As well as, one band monomorphic, 1 band polymorphic with 50% polymorphism ratio and non-unique bands (Tables 2, 3 and Figure 1).

The primer HB13 amplified six bands with the two ecotypes with 2 bands monomorphic and 4 bands polymorphic with 66% polymorphism ratio. One unique band was found in Fahl monocut ecotype at 605 bp and was absent in multicut ecotype cultivars (Tables 2, 3 and Figure 1). All ecotypes were involved in bands number 1 and 2 that founded at 1278 and 997 bp (Tables 2, 3 and Figure 1). It was noted that the two ecotypes were evolved in band at 557 bp except serw1 which was absent in it (Tables 2, 3 and Figure 1).

It is worth mentioning that the Fahl had 29 present bands, 3 absent bands and two unique bands across the six primer. The multicut ecotypes were given different band pattern, Gemmiza1 (21 present, 11 absent), Giza6 (21 present and 11 absent) and Serw1 (23 present and 9 absent) (Tables 2, 3 and Figure 1). These differences stem from the location of the class environment where the temperature and humidity, product features carry the harsh conditions, as is the case in Serw1 and the genetic structure of both ecotypes (Table 2). Both ecotypes were also found to be varied from each other as indicated by various molecular markers (Zayed, 2013; Zayed et al., 2010). In addition, Soliman et al. (2010) found the monocut ecotype Fahl which was primitive than multicut ecotype.

The present results are in line with the results obtained by Tarrad and Zayed (2009) who studied the multicut ecotype cultivar and observed disagreement 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 analysis showed that the highest value appeared between Fahl and Gemmiza1 as well as Giza6 and Serw1 (82%) followed by Fahl and Serw1(81%). The lowest similarity value appeared between Gemmiza1 and Serw1 followed by Gemmiza1 and Giza6 (Table 4). These results are in agreement with that of Tarrad and Zayed (2009) who reported that the highest similarity indices was 0.85 between the two cultivars Sakha4 and Gemmiza1, while a lowest similarity index (70%) was observed between the Giza6 and Helaly.

## Cluster analysis

The dendrogram shown in Figure 2 shows that the cultivars were divided into two main clusters; cluster 1 had Fahl and Gemmiza1 cultivars and cluster 2 had

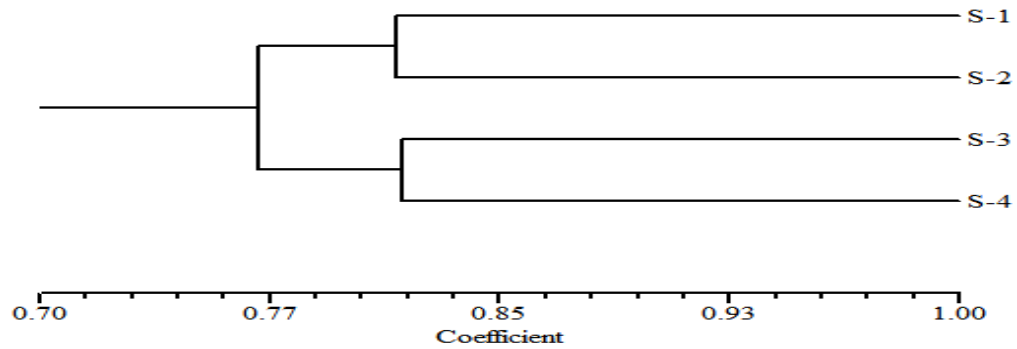


**Figure 2.** UPGMA clustering of Egyptian clover cultivars using the ISSR primers. S-1 = Fahl; S-2 = Gemmiza1; S-3 = Giza6; S-4 = Serw1.

Giza6 and Serw1 cultivars. These results are not in agreement with that of Tarrad and Zayed (2009) who reported the Gemmiza1 and Fahl had so far genetic distance and similarity indices equal to 50%.

The multicuts cultivars differed in the origins as follow: Gemmeiza1 developed through poly-crossing selections within collected landrace from Dusuok 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, and gives 4-6 cuts/ season (Middle Delta and Middle Egypt). The cultivar Serw1 developed through poly-crossing among 14 selected landraces characterized with high forage productivity under salt-affected soil at Serw1 Research Station. It is salt tolerant and gives 4-6 cuts/season (North Delta and salt affected soil). Moreover, the cultivar Giza6 developed through selection among farmer's seed lots. It has late flowering, heat tolerant good yielder and gives 4-6 cuts/season (Middle and Upper Egypt). On the other hand, Abd El-Naby et al. (2012) studied ISSR primers with Fahl and Sakha4 and its hybrids; analyses of ISSR gave a total number of 60 bands from five primers. Also, they found the number of polymorphic bands to be 44; while polymorphism percentage was 73.4%. Furthermore, Soliman et al. (2010) found the results may be important to distinguish the difference between monocut and multicut. They reported that Fahl is more primitive Miskawi. The selection of vigorous plants may be used to improve new cultivars with economic value and can increase forage production per unit area (Abd El-Naby et al., 2012; Abd El-Naby et al., 2009; Abo-Feteih et al., 2010). 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 conditions are met: no mutations, no gene flow (no immigration /emigration), large population size (no genetic drift), no selective forces and no non-random mating. The allele frequency had different values in both ecotypes (Table 5). The dominant allele was frequented in cultivars Fahl, Gemmiza1, Giza6 and Serw1 with values 0.8, 0.64, 0.68 and 0.74, respectively. These data mean the cultivar had differed in allele frequency.

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

| Allele | Fahl | Gemmiza1 | Giza6 | Serw1 |
|---|---|---|---|---|
| Dominant(p) present | 0.80 | 0.64 | 0.68 | 0.74 |
| Recessive (q)absent | 0.20 | 0.36 | 0.32 | 0.26 |

An average polymorphic information content (PIC) value of 0.218 across all scored ISSR bands, as well as an average (Marker index) of 3.709 across all primers obtained with both ecotypes berseem clover were different than that of AFLP-based genetic diversity studies in various crops (Muminovic et al., 2004; Powell et al., 1996). 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 groclimatic zones (Table 6).

## Conclusion

The ISSR markers have been used in order to determine genetic variation and relationship between two Egyptian clover ecotypes. Six primers out of ten for ISSR-PCR technique succeeded and gave reliable amplicons for different types of Egyptian clover ecotypes. The results reveal polymorphisms level by ISSR primers. HB10 ISSR-primer was better than the rest of the ISSR primers in polymorphic 100%. The Fahl monocut ecotype had 29 present bands, 3 absent bands in 32 total bands; also

**Table 6.** Comparative analysis of banding patterns generated by ISSR for four berseem clover.

| Primer Name | Polymorphism (%) | Range of fragment size (pb) | PIC* | MI** |
|---|---|---|---|---|
| HB08 | 57.1 | 1652.4-219 | 0.268 | 1.072 |
| HB09 | 20.0 | 1276.7- 614.6 | 0.110 | 0.11 |
| HB10 | 100.0 | 1216.9- 462.2 | 0.319 | 1.595 |
| HB11 | 33.3 | 1593.2- 499.8 | 0.153 | 0.306 |
| HB12 | 50.0 | 1959.3 -1303.7 | 0.183 | 0.183 |
| HB13 | 66.7 | 1278 - 421.3 | 0.276 | 1.104 |
| Mean | 54.8 | | 0.218 | 3.709 |

*PIC = Polymorphic information content; **MI = marker index.

Fahl had two unique bands. The multicut ecotype Gemmiza1, Giza6 and Serw1 were given different pattern of bands 21 present, 11 absent; 21 present and 11 absent and 23 present and 9 absent, respectively. The three unique bands appeared in two ecotypes. Fahl was given two bands HB11 and HB13; the Serw1 multicut cultivar had one unique bands with HB08. Similarity indices among the four Egyptian clover cultivars based on ISSR analysis was estimated and the highest value appeared between Fahl and Gemmiza1 as well as Giza6 and Serw1 followed by Fahl and Serw1, while the lowest similarity value was between Gemmiza1 and Serw1 followed by Gemmiza1 and Giza6.

## Conflict of interests

The authors did not declare any conflict of interest.

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