Molecular Genetic Identification of Some Egyptian Hibiscus Samples

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Abstract: Seven local samples of Roselle (*Hibiscus sabdariffa* L.) collected from different locations from Egypt (Siwa, Cairo and Aswan). Molecular genetic analysis elucidated the genetic distances among them. Polymerase Chain Reactions based on Randomly Amplified Polymorphic DNA (RAPD-PCR) and Inter Simple Sequence Repeats (ISSR-PCR) analysis performed to establish molecular markers as a fingerprint for these seven samples. Eleven 10-mer arbitrary RAPD primers out of total twenty seven and eight ISSR primers had successfully generated reproducible polymorphic products. RAPD and ISSR profiles were pooled together to elucidate the genetic relationships among the examined seven *Hibiscus* samples. The constructed dendrogram tree divided the studied samples into two main genetic clusters. First cluster includes the sample Light Red LR (Siwa). Second cluster comprises six samples [Dark Red DR1 (Siwa), DR2 (Siwa), DR (Cairo), DR (Cairo), White W (Cairo) and DR (Aswan)].
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Key words: Hibiscus, *Hibiscus sabdariffa* L., Molecular markers, RAPD-PCR, ISSR-PCR.

1-Introduction

Hibiscus sabdariffa L. (Roselle) belongs to the family Malvaceae. It is an annual herb cultivated for its leaves, stem, seeds and calyces (Babalola *et al*., 2001 and Fasoyiro *et al*., 2005a). It is best grown in tropical and subtropical regions (El-Meleigy, 1989). *Hibiscus* was cultivated in Egypt for multipurpose uses. It is well known in Egypt with the name of 'Karkadeh'. Its purplish sepals (calyx and epicalys) are the most important economic parts of the plant which is used in food as jam and jelly and cosmetic industries as a source of natural coloring agent (El-Meleigy, 1989 and Fasoyiro *et al*., 2005b). The pigments (anthocyanins), which responsible primarily for red color, were delphinidine-3-glucoside and cyaniding-3-glucoside (Kalt *et al*., 1992). *Hibiscus sabdariffa* L. cultivars are rich in riboflavin, niacin, calcium and iron (Babalol *et al*., 2001 and Qi *et al*., 2005). It is also contains antioxidants including flavonoids, gossypetine, hibiscetine and sadderetine (Fasoyiro *et al*., 2005b). Moreover, the extract of sepals also proved experimentally to have highly antibacterial properties (Omobuwajo *et al*., 2000).

In Egypt, 'Karkadeh' is considered a very popular beverage and valuable medicinal plant due to its effect on lowering and/or adjusting the blood pressure without producing any side effect (Faraji and Tarkhani, 1999). Also, it has favorable effect on the functions of stomach. It possesses a high intestinal antiseptic action and can be used to resist various infections of intestinal diseases (Owolabi *et al*., 1995).

Random Amplified Polymorphic DNA analysis (RAPD-PCR) can be used quickly and efficiently for cultivar identification. RAPD analysis has been used to study genetic relationships in a number of fruit trees including almond (Bartolozzi *et al*., 1998), plum varieties (Ortiz *et al*., 1997), peach varieties (Chaparro *et al*., 1994; Warburton and Bliss, 1996), peach rootstocks (Lu *et al*., 1996). RAPD markers have been used in peach genetics and breeding programs (Chaparro *et al*., 1994 and Dirlewanger and Bodo 1994).

The past limitation associated with pedigree data and morphological, physiological and cytological markers for assessing genetic diversity in cultivated and wild plant species have largely been circumvented by the development of DNA markers such as Random Amplified Polymorphic DNAs (RAPD) This method has proven useful for germplasm identification, elucidation of genetic relationships of numerous plant cultivars and species (Williams *et al*., 1990; Halward *et al*., 1992; Levi and Rowland, 1997; Barik *et al.,* 2006; Suvakanta *et al*,2006; Refaei *et al*, 2010 and Shilpa *et al*.,2012). Also, such technique is simple to use and do not require the use of radioactive materials as well as it able to detect a significant degree of polymorphism (Williams *et al*., 1990). The generated DNA polymorphism reflects both the distance between two annealing sites and the pattern of their distribution throughout the genome of a particular cultivar or species (Casas *et al*., 1999).

Inter Simple Sequence Repeats (ISSR-PCR) analysis involves the Polymerase Chain Reaction

(PCR) amplification of regions between adjacent, inversely oriented microsatellites, using a single Simple Sequence Repeats (SSR) motifs (di-, tri-, tetra, or penta- nucleotides) containing primers anchored at the 3` or 5` end by two to four arbitrary, often degenerate nucleotides (Zietkiewicz *et al*., 1994). The potential supply of ISSR marker depends on the variety and frequency of microsatellites, which changes with species and the SSR motifs that are targeted (Depeiges *et al*., 1995). RAPD and ISSR markers have been used both for DNA fingerprinting (Martín and Sánchez-Yélamo, 2000; Moreno *et al*., 1998; Blair *et al*., 1999). A large number of polymorphic markers are required to measure genetic relationships and genetic diversity in a reliable manner. Molecular markers like RAPD and ISSR have been widely used in many plant species for identification, varieties analysis, population studies and genetic linkage mapping (Williams *et al*., 1990; Rout *et al*., 2003; Mohapatra and Rout 2005 Hammad, 2009 and Gyana *et al*,2010).

The purpose of the present study was to evaluate neutral DNA RAPD (Random Amplified Polymorphic DNA) and DNA ISSR (Inter Simple Sequence Repeats) markers as a tool to distinguish between collected *Hibiscus* samples from different locations in Egypt.

2-Materials and methods:

Plant materials:

Seven samples (*Hibiscus sabdariffa* L.) were collected from different locations from Egypt, their names and geographical location shown in Table 1.

Table 1: Names and geographical location of *sabdariffa* L.)

No.	Cultivar	Location
1	Red Light (PR)	Siwa
2	Red Dark (DR1)	Siwa
3	Red Dark (DR2)	Siwa
4	Red Light (PR)	Ain Shams
5	Red Dark (DR)	Ain Shams
6	White (W)	Ain Shams
7	Red Dark (DR)	Aswan

DNA extraction

Yong freshly excised leaves were collected for each *Hibiscus* sample. DNA was extracted as described by Dellaporta *et al*., (1983). About 0.1g of plant tissues was ground in liquid N_2 with 1ml extraction buffer (100mM Tris-HCl pH8.0, 50mM EDTA and 0.5M NaCl) + 0.2ml 20% SDS and incubated at 65ºC for 20 minutes. One ml of phenol, chloroform and isoamyl alcohol (25: 24:1) was added and centrifuged at 10,000 rpm for 10 minutes. Supernatants were transferred to new tubes, and 1ml of chloroform and isoamyl (24:1) was added and then centrifuged at 10,000 rpm for 10 minutes. Supernatants were transferred to a new tube and then 1ml of isopropanol and overnight in -18ºC. Centrifugation was performed at 10,000 rpm for 10 minutes. The pellets containing DNA were re-suspended in 1ml ethanol and centrifuged at 10,000 rpm for 2 minutes. DNA pellets were resuspended in 200 1x TE buffer (10mM Tris-HCl pH8.0 and 1mM EDTA). DNA was quantities by quantitatively determined and gel electrophoresis.

Polymerase Chain Reaction (PCR):

RAPD-PCR amplification

PCR conditions were optimized by varying concentrations of template DNA, *Taq* DNA polymerase and Mg^{2+} ion. A total of eleven random DNA oligonucleotide primers were independently used according to Williams *et al*., (1990). Primers that gave reproducible and scorable amplifications were used in the analysis of all the seven genotypes, listed in Table (2) with their sequences.

The PCR amplification was performed in a 25µl reaction volume containing the following: 2.5µl of dNTPs (2.5 mM), 1.5µl of $MgCl_2$ (25 mM), 2.5µl of 10x buffers, 2.0µl of primer (2.5 µM) and 2.0µl of template DNA (50ng/µl), 0.3µl of *Taq* polymerase (5U/µl) and 14.7µl of sterile dd H_2O. The reaction mixtures were overlaid with a drop of light mineral oil per sample. Amplification was carried out in Techni TC-512 PCR system. The reaction was subjected to one cycle at 95ºC for 5 minutes, followed by 35 cycles at 96ºC for 30 seconds, 55ºC for 30 seconds, and 72ºC for 30 seconds, then a final cycle of 72ºC for 5 minutes. PCR products were run at 100V for one hour on 1.4% agarose gels to detect polymorphism between *Hibiscus* samples under study. After electrophoresis, the RAPD patterns were visualized with UV transilluminator. RAPD markers were scored from the gels as DNA fragments present or absent in all lanes. Gels were photographed using a Polaroid camera.

ISSR-PCR amplification

ISSR-PCR reactions were conducted using eight primers according to Williams *et al*., (1990). Amplification was conducted in 25µl reaction volume containing the following reagents: 2.5µl of dNTPs (2.5 mM), 2.5µl $MgCl_2$ (2.5 mM), and 2.5µl of 10 x buffer, 3.0µl of primer (10pmol), 3.0µl of template DNA (25ng/ µl), 1µl of *Taq* polymerase (1U/µl) and 12.5µl of sterile dd H_2O. The PCR was programmed for one cycle at 94ºC for 4 minutes followed by 45 cycles of 1 minutes at 94ºC, 1 minutes at 57ºC, and 2 minutes at 72ºC. The reaction was finally stored at 72ºC for 10 minutes. The PCR products were separated on a 1.5% agarose gels and fragments sizes were estimated with the 100bp ladder marker (Sizyme com).

Statistical analysis:

The DNA bands generated by each primer were counted and their molecular sizes were compared with those of the DNA markers. The bands scored from DNA profiles generated by each primer were pooled together. Then the presence or absence of each DNA band was treated as a binary character in a data matrix (coded 1 and 0, respectively) to calculate genetic similarity and to construct dendrogram tree among the studied seven *Hibiscus* samples. Calculation was achieved using Dice similarity coefficients (Dice, 1945) as implemented in the computer program.

3-Results and discussions

RAPD markers:

Screening of the seven Egyptian *Hibiscus* samples by RAPD-PCR analysis showed that every one of the 11 primers (eleven primers out of total twenty-seven primers) generated electrophoretic DNA patterns (clear bands on the RAPD amplifications) for the samples studied (Figure 1 and 2).

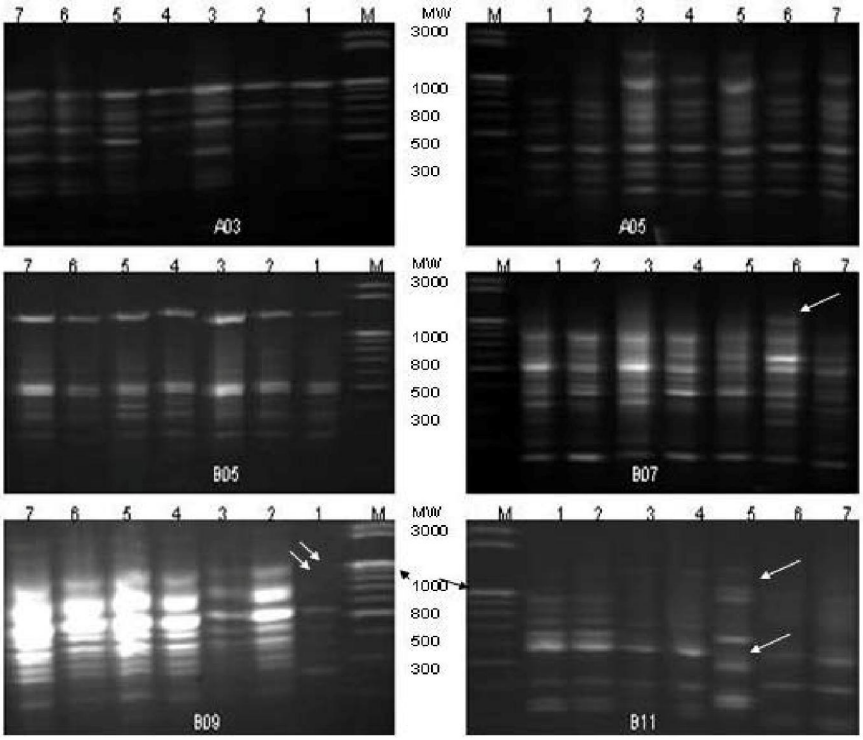

Figure 1: RAPD fingerprints of seven Hibiscus samples in Egypt using A03, A05, B05, B07, B09 and B11 random primers. Arrows point to the unique fragments.

The degree of polymorphism for each primer was expressed as polymorphic/ amplified bands ratio. The primers, sequences and data of amplification are listed in Table (2). Total number of amplified bands was 140 bands with 45.21% polymorphism. These results were similar to other previous studies for phylogenetic relationships of *Jatropha* genotypes which elucidated a high average of polymorphism (Gupta *et al*., 2008).

Table 2: Primers Sequences, Names, Polymorphic, Monomorphic bands and Polymorphism percent detected by RAPD analysis in Hibiscus samples discrimination.

Primer No.	Sequences	Monomorphic band	Polymorphic band	Unique band	Polymorphism %
OP-A03	5` TCGGCCATAG 3`	9	0	0	0.00
OP-A05	5` CCTTGACGCA 3`	7	4	0	36.36
OP-B05	5` GTGACCCCTC 3`	4	6	0	60.0
OP-B07	5` GGCGGTCTTT 3`	9	6	0	40.0
OP-B09	5` CTCACCGTCC 3`	5	7	2	58.0
OP-B11	5` TGTCTCGGTG 3`	5	9	2	64.0
OP-D01	5` TGTCTCGGTG 3`	9	6	1	40.0
OP-D02	5` GGACCCAACC 3`	7	9	2	56.0
OP-D07	5` GGACCCAACC 3`	8	3	1	27
OP-F05	5` GGGCGGTACT 3`	7	8	1	53.33
OP-L20	5` AGGTTGCAGG 3`	5	7	1	61.53
Total		75	65	10	45.21

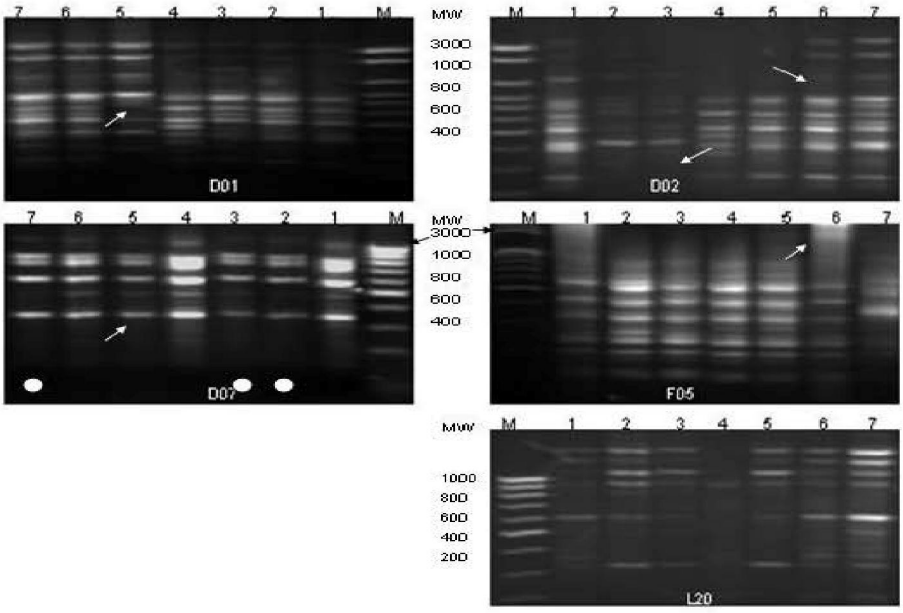

Figure 2: RAPD fingerprints of seven Hibiscus samples in Egypt using D01, D02, D07, F05 and L20 random primers. Arrows point to the unique fragments and circles point to similar patterns.

In our study, total number of clear bands obtained from each primer ranged from 9 for primer OP-A03 to 16 for primer OP-D01 with an average of 14.4 bands per primer with a wide range (3000 bp to 200 bp) of variability. With combination of suitable primers, all accessions could be identified by having accession-specific band, or by lacking a band that was present in all other accessions.

The primer D07 generated similar amplified pattern characteristic for the Dark Red samples of *Hibiscus* collected from Siwa and Aswan (No. 2, 3 and 7). It was possible to score some unique bands for specific samples. Primer B09 (No. 1) showed two negative bands for Light Red *Hibiscus* of Siwa. Dark Red *Hibiscus* of Cairo (No. 5) could be distinguished. Two positive and one negative band were regard to primers B11 and D01, respectively. D07 and L20 generated one positive and one negative band, respectively for Light Red Cairo sample (No. 4). Primers F05, D02 and B07 had good markers for White *Hibiscus* of Cairo as a positive unique band (No. 6). This positive unique band as a DNA fragment can be notified as an loci for Proantho Canidin in this sample (White color), which contain the highest level of Proantho Canidin (2.3) and lowest level of total phenol content, Delphindin-3-glucoside, Cyanidin-3-glucoside, Carotene and Quercitin (Table 1). It was not possible to identify a specific marker to differentiate between locations or specific marker to differentiate between colors of *Hibiscus*.

ISSR markers:

Using ISSR-PCR amplification, eight primers were representative of most types of repeated sequence as ISSR markers to assess the level of polymorphism in the seven *Hibiscus* samples. Total number of amplified bands was 117 with 47.0% polymorphism (Table 3).

This was not unexpected, since the ISSR technique amplifies microsatellite regions that are potentially polymorphic (Morgante and Olivieri, 1993). The ISSRs have a high capacity to reveal polymorphism and offer great potential to determine intra- and intergenomic diversity as compared to other arbitrary primers like RAPDs (Zietkiewicz *et al*., 1994). In this study, electrophoretic patterns obtained with ISSR markers are illustrated in Figure 3. All primers amplified several DNA regions giving a complex amplification pattern that made us able to analyses simultaneously many loci and to detect more polymorphic fragments with a unique amplification reaction. Primer 48B led to the greatest number of polymorphic bands thus having a high polymorphic/amplified bands ratio, equal to 66.66%. But, primer HB11 led to the greatest number of monomorphic bands thus having a high monomorphic/amplified bands ratio, equal to 25.00 %.

Amplification of primer HB14 gave distinguishable pattern for the two Light Red *Hibiscus* samples either from Siwa or Cairo (No. 1 and 4), which has loci of color. Two markers, negative bands, could be successfully distinguished for only one sample No. 1

(Light Red of Siwa) by primers 44A and 49A. White *Hibiscus* from Cairo could be distinguished by primers HB09 and HB10 showed four negative bands, respectively (No. 6). This negative bands may be corresponds to allele for high level of Proantho Canidin and low level of total phenol content, Delphindin-3-glucoside, Cyanidin-3-glucoside, Carotene and Quercitin (Table 1). This bands as DNA fragments corresponds for high level of total phenol content, Delphindin-3-glucoside, Cyanidin-3-glucoside, Carotene, Quercitin and then Dark Red *Hibiscus* from Aswan could be distinguished by primers 48B, HB10 and HB14 that shown one positive, two negative and three negative bands, respectively (No. 7). Kalt *et al*., (1992) reported that the pigments (anthocyanins) which are responsible primarily for Red color were delphindin-3-glucoside and cyaniding-3-glucoside. In general, the detection of high levels of polymorphism makes ISSR analysis a powerful tool for assessing genetic diversity according to (Leil *et al*., 2006) who revealed that, none of the individual studied plants were genetically identical according to ISSR analysis. The generated profiles based on RAPD and ISSR analysis revealed high levels of polymorphism among the studied samples. The observed data of these primers recorded a sum of 257 bands. These bands were identified as 120 polymorphic bands and 137 monomorphic bands in all genotypes under study. The polymorphic bands were scored as 25 unique and 112 non-unique ones. These unique bands were used to discriminate between the seven studied *Hibiscus* samples. In addition, the other recorded non-unique polymorphic bands provided a considerable number of combined class patterns that can be used for further discrimination between the studied samples.

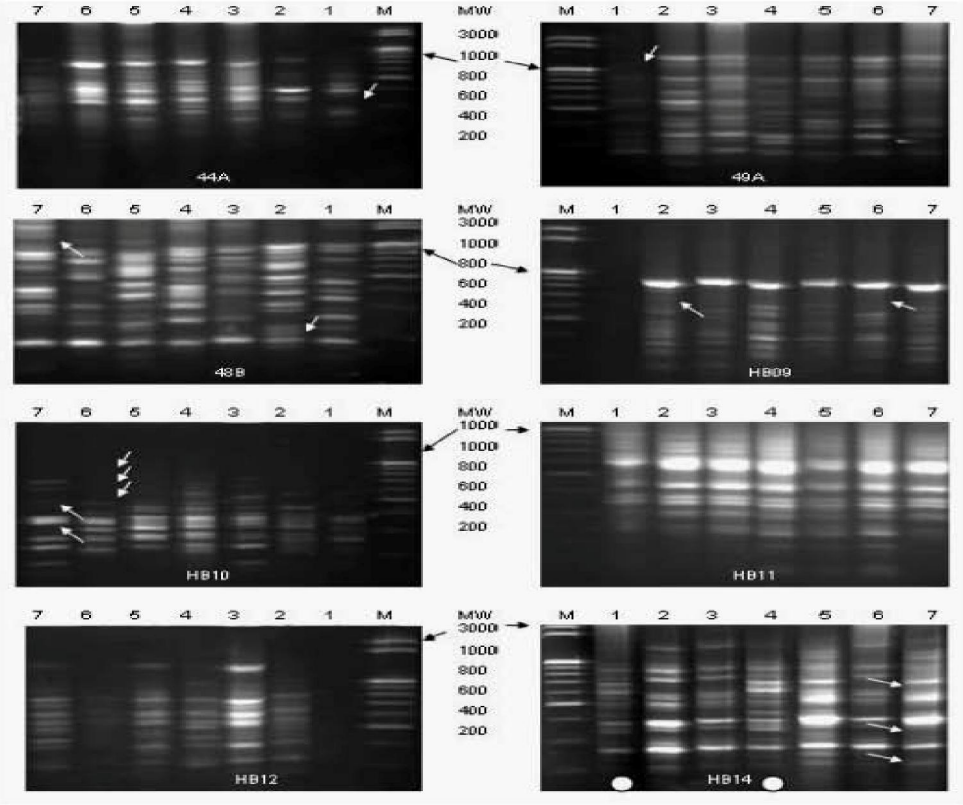

Figure 3: ISSR fingerprints of seven Hibiscus samples in Egypt using 8 specific primers. Arrows point to unique fragments. White circles reveal to similar pattern.

The highest similarity value was recorded between the two samples LR (Siwa) and DR (Siwa) as well as between the two samples DR (Siwa) and DR (Cairo) and also between the two samples LR (Cairo) and DR (Cairo), respectively. The lowest similarity was recorded between the two samples DR1 (Siwa) and DR2 (Siwa).

Dendrogram trees:

Dendrogram tree based on the similarity index of RAPD analysis (Figure 4) showed that, the three genotypes of Dark Red *Hibiscus* from Siwa and Cairo were grouped in one cluster. White red W Cairo and Dark red DR Aswan were separately in one cluster and Light red LR Siwa grouped alone in one

cluster. From dendrogram tree based on ISSR DR (Figure 5) *Hibiscus* either from Siwa or Cairo grouped in one cluster. The differences found among the dendrograms generated by RAPDs and ISSRs could be partially explained by the different number of PCR products according to Loarce *et al*., 1996 and Karp *et al.*, 1997.

Table 3: Primers names, Polymorphic, Monomorphic bands and Polymorphism percent detected by ISSR analysis in Hibiscus samples discrimination.

Primer No.	Sequences	Monomorphic band	Polymorphic band	Unique band	Polymorphism %
44 A	$(CA)_8GT$	6	7	1	53.84
49 A	$(CA)_8AG$	8	5	1	38.46
48B	$TC(CTC)_4C$	5	10	2	66.66
HB-09	$(GT)_6GG$	7	7	3	50.00
HB-10	$(GA)_8CC$	6	10	5	62.50
HB-11	$(GT)_8CC$	12	4	0	25.00
HB-12	$(CAC)_5GC$	8	5	0	38.46
HB-14	$(CTC)_5GC$	10	7	3	41.17
Total		62	55	15	47.00

Dendrogram tree based on combination between RAPD, ISSR (Figure 6) divided the studied samples to two main clusters. One sample (LR Siwa) was separated alone wide from other studied samples. Second main cluster divided to three sub cluster, both DR Siwa were grouped together in one sub-cluster, cultivars from Cairo grouped together in another sub-cluster but DR Aswan separated alone in sub-cluster. Cairo was separately in one cluster based on RAPD, ISSR and combination between RAPD and ISSR analysis. Dendrogram tree can not separates or identify the deferent samples (dependent on the location). Gyana (2010) describe the relationships among twelve species of *Phyllanthus* collected in India by help of molecular markers(RAPD and ISSR). In total, 259 marker loci were assessed; out of which249 were polymorphic revealing 96.13% polymorphism. Nei's similarity index varied from 0.35 to 0.76 for RAPD (Random Amplified Polymorphic DNA) and from 0.31 to 0.76 for ISSR marker systems.

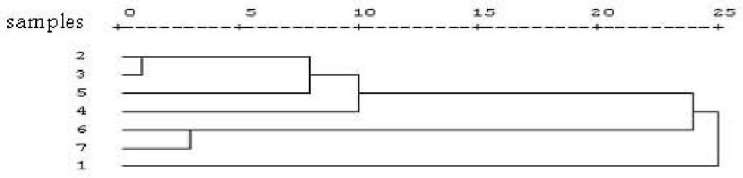

Figure 4: Dendrogram analysis of Hibiscus samples using RAPD analysis.

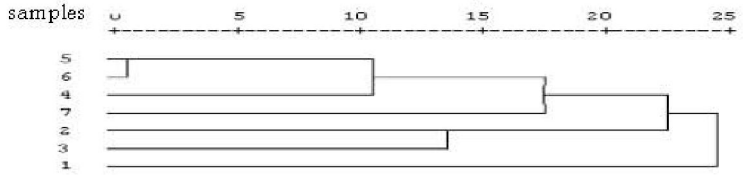

Figure 5: Dendrogram analysis of Hibiscus samples using ISSR analysis.

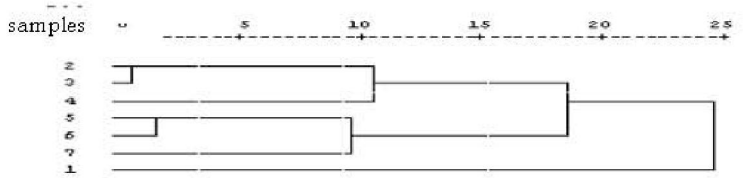

Figure 6: Dendrogram analysis of Hibiscus samples using RAPD and ISSR analysis.

Conclusion

RAPD and ISSR-PCR analysis could be good techniques to distinguish and identify between the variable samples and suggested relation between some unique bands. Studied samples of *Hibiscus* collected from different location have different pattern of amplified DNA fragments in both techniques not dependent on the location. These results suggested that *Hibiscus* genotypes from different locations were mixed.

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