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Molecular analysis of some endemic and near-endemic medicinal plants located at Saint Katherine, Egypt

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Abstract: *The present study focused on genetically studies of endemic and near endemic medicinal plants from Saint Katherine Protectorate, Egypt, using ISSR and RAPD markers. The three plants Nepeta septemcrenat "near-endemic", thymus decussates "near-endemic" and Phlomis aurea "endemic" were collected from Wadi Elfaraa, Saint Katherine, Egypt. For RAPD-PCR analysis five primers has been evaluated. A total of 35 DNA bands were detected, 17 bands were polymorphic, 18 were monomorphic. The percentage of polymorphic bands ranged from 25% to 72.72% with an average of 44.18%. The amplified DNA bands ranges in size between 89 to 1457 bp. For ISSR analysis, DNAs of the three selected plants were subjected to PCR against 7 ISSR Primers . A total of 46 DNA bands were detected, 23 bands were polymorphic, 23 were monomorphic. The percentage of polymorphic bands ranged from 16.667% to 77.778% with an average of 47.89%. The amplified DNA bands ranges in size between 112 to 7403 bp Genetic distance between populations for RAPD-PCR ranged from 0.66 to 0.71 while ISSR- PCR ranged from 0.61 to 0.78. The efficiency of the discriminatory power " Dj" is an extension of the PIC and provides an estimate of the probability that two randomly chosen individuals show different banding patterns for the same primer and, thus, are distinguishable from one another. Discriminatory power is a direct indication of primer efficiency. The Dj values for RAPD-PCR markers tested ranged from 0.73 to 0.87 and for ISSR-PCR markers tested ranged from 0.78 to 0.86.*

Keywords*: discriminatory power, endemic and near-endemic plants, medicinal plants, molecular markers, Saint Katherine.*

I. Introduction

With its high mountains and deep wadies in the southern Sinai Peninsula and its relatively unexplored desert ecosystem of wild plants, Saint Katherine Protectorate (SKP) is as one of region's most amazing areas not only for its natural landscapes, but also for its medicinal plant diversity that has national and global interest.
The SKP contains a wide range of habitats and landscapes that are a consequence of varying climatic conditions, a wide range of altitudes, and variable topography [1]. SKP is one of the most floristically diverse spots in the Middle East and with 44% of Egypt's endemic plant species. 1261 species were recorded in Sinai [2]. 472 plant species have been recorded as surviving and still occurring in south Sinai [3] of these 19 species are endemic [2] and about 170 are with known by medicinal properties used in traditional therapy and remedies. It is currently recognized as one of the central regions for flora diversity in the Middle East by the World Conservation Union, WWF & IUCN 1994 [4].

In the past few years, the Sinai peninsula has attracted a great deal of scientific attention both as a new axis of development of Egypt and as an important phytogeographic province. The recent socioeconomic and the tourist industry development of Sinai are based on previous evaluation of its natural resources soil, water, animal and plant wild-life. Proper knowledge of these resources will help plan for the future development and conservation of its natural treasures. Studies dealing with the evaluation of these natural resources and monitoring of the changes taking place are needed, especially ecological studies [5]. The preservation of genetic diversity is important, because it provides long-term evolutionary potential for changing environmental conditions [6].

Medicinal plants have a very important role from the medical and economical point of view all over the world, where the folk medicine made a wide base used for searching new drug. Family Lamiaceae comprises many species of special economic and medicinal importance due to their different constituents [7].It is represented in Egypt by 21 genera [8] among them *Thymus, Nepeta and Phlomis*. Some species of *Nepeta* genus are important medicinal plants and their extracts have been used for medicinal purposes [9], *Nepeta septemcrenata* known to be used by the native Bedouins in folk medicine as antipyretic, sedative, cardiotonic, eye wash and as a gargle in sore throat[10]. *Phlomis* genus has been instrumental in the discovery of natural medicinal products [11]. *Phlomis* species are used to treat various conditions such as diabetes, gastric ulcer, hemorrhoids, inflammation, and wounds, *Phlomis aurea* is recognized for its antidiabetic properties due to its ability to protect liver and pancreas integrity by reducing the oxidative stress in diabetes or by stimulating the production of enzymes implicated in glucose metabolism [12]. *Thymus* species are well known as medicinal

plants because of their biological and pharmacological properties. In traditionalmedicine, leaves and flowering parts of *Thymus* species are widely used as tonic and herbal tea, antiseptic, antitussive, and carminative as well as treating colds [13, 14].In recent years, molecular markers derived from DNA using electrophortic techniques have provided powerful markers for the study of several aspects in all biological fields including systematic and genetic relationships of plant species and sub-specific ranks.Currently techniques of choice is the RAPD-PCR and ISSR-PCR. RAPD-PCR is based on using the PCR as proposed by [15] to amplify DNA sequences with single short 9-10bp primers of arbitrary nucleotide sequence. It requires small
amounts of DNA, easy to perform and reveals dominant molecular markers of ultimate potentialities in several fields of plant science including systematic and evolution [16]. Many authors have utilized the RAPD-PCR approach to study genetic diversity and species relationships in some plant genera. The applications of RAPD in plant biodiversity also included investigation of genetic diversity some species of lamiacea were used; *Nepeta septemcrenata species* [17] , *Cunila Incisa Benth* [18], *Salvia* [19], *Thymus* [20] and *Thymes* [21].

ISSR marker is involving PCR amplification of DNA by a single 16-18 bp long primer composed of a repeated sequence anchored at the 3' or 5' end of 2-4 arbitrary nucleotides [22]. The technique is rapid, simple, inexpensive and more reproducible than RAPD [23]. ISSR used to study the genetic diversity of plants for examples; *Nepeta* [24], *thyme* [25] , *Mentha aquatica* L. [26], *satureja* [27], *Salvia* [28], *Dracocephalum thymiflorum L.*[29] and *Thymus* [30]. RAPD and ISSR markers have been extensively used for genetic diversity for example: the investigation for some species of lamiaceae by RAPD and ISSR; *Salvia* [19] , *Phlomis kurdica* and *Phlomis oppositiflora* [31], *Ocimum* [32] and *Morus* [33]. The goal of this research was to study the molecular fingerprint for the three endemic and near-endemic plants by using RAPD and ISSR techniques.

II. Materials And Methods

2.1. Plant materials:

Fresh leaves of Nepeta septemcrenata , thymus decussates and Phlomis aurea were collected from Wadi Elfaraa in Saint Katherine Protectorate during the Spring season May, 2013.Scientific names, Families and Geographic location " Trimble model" shown in Table 1.

2.2. ***Genomic DNA isolation:***

Young fresh leaves of each studied plant were collected and then samples of one gram was treated with liquid nitrogen and transferred to the laboratory of biotechnology in Desert Research Center (DRC), Cairo, Egypt for DNA extraction according to the method of Welsh and McCleland [34].

2.3. ***Random amplified polymorphism DNA (RAPD)technique:***

Asset of 5-base oligonucleotide primers A1, A4, A9, B8, and B9 according to [15] as shown in Table 2.a. Agarose gel of 1.5 % containing ethidium bromide 0.5µg/ml in 1X TBE buffer at 95 volts was used for resolving the PCR products. Ladder Promaga lnc. of DNA was used as a DNA standard size marker "in bp" of the resulted 13 bands 10000, 8000, 6000, 5000, 4000, 3500, 2500, 2000, 1500, 1000, 750, 500 and 250 bp. PCR products were visualized on UV light and photographed using a gel documentation system "Bio-Rad® Gel Doc-2000". Gels were analyzed by gel documentation system.
Amplified products were visually examined and the presence or absence of each size class was scored as 1 or 0; respectively as shown in table 3.a.

2.4. Inter Simple Sequence Repeat (ISSR-PCR) technique:

Inter-Simple Sequence Repeats "ISSRs" has only recently been developed as an anonymous, RAPDs-like approach that accesses variation in the numerous microsatellite regions dispersed throughout the various genomes "particularly the nuclear genome" and circumvents the challenge of characterizing individual loci that other molecular approaches require. Microsatellites are very short usually 10-20 base-pair stretches of DNA that are "hyper variable", expressed as different variants within populations and among different species [35]. DNA isolation of plant samples for each studied species was conducted according to the method outlined by [36]. ISSR - PCR reactions were conducted using 7 selective primers A11. A12, A15, 17898A, 17898B, 17899A and 17899B table 2.b.

2.5. Statistical analysis:

RAPD-PCR and ISSR-PCR amplified fragments were scored as present 1 or absent 0. Only clear and major bands were scored according to [37] Table 3.a,b. RAPD and ISSR analyses were performed by the Nei genetic similarity index [38] on the basis of the equation: SI = 2Nij / (Ni + Nj), where Nij is the number of common bands shared between samples i and j, Ni and Nj are the total number of DNA bands for genotypes i and j, respectively. A dendrogram was constructed Figure 3.a,b,c. Dendrogram using the neighbor joining method of cluster analysis.

The discriminatory power (Dj= 1-Cj) and confusion probability (Cj) of the j^{th}assay [39] were calculated according to the following equation: $Cj= \sum Ii= 1\ pi\ (Npi - 1)/N-1$ Where, pi represents the frequency of the i^{th} pattern, N the sample size, and I is the total number of pattern.

III. Results

3.1. RAPD analysis

Five RAPD primers were used to differentiate between the three plants. The size of the amplified fragments ranged from 89 to 1457 bp generated 35 DNA bands, 17 bands were polymorphic, and 18 were monomorphic. The number of bands varied from 4 (primer OPA- 09 & OPB-08) to 11 (primer OPA-01) with an average of 17.5 markers per primer Table 4.a. and figure 1. The polymorphism level ranged from 25% to 72.72% with an average of 44.18%.Minimum and maximum number of polymorphic bands were obtained with the primer B8 1 and A1 8, respectively Table 4.a. Cluster analysis based on the similarity index of [40] revealed two main clusters Fig. 3.a One of them separate S.1 *Nepeta septemcrenata* as a genetically dissimilar genotype with all other one's. While the second cluster was separated into two sub-clusters comprised the first involved S.2 *Phlomis aurae* and the second sub-cluster S.3 *Thymus decussatus*; this indicates that *Thymus decussates* and *Phlomis aurea* are genetically close to each other than *Nepeta septemcrenata*. as illustrated in table (5.a) similarity indices ranged from 0.74 between Nepeta septemcrenata and Phlomis aurae, to 0.81 between Phlomis aurae and Thymus decussates.

The discriminatory power "Dj" Table 6 which indicate primer efficiency for RAPD ranged from0.734 to 0.865.

3.2. ISSR analysis

Seven ISSR primers were used.Fig 2 and table 4.b. The size of the amplified fragmentss ranged from 112- 7403 bp. 7 ISSR primers generated 46 bands, which 23 bands polymorphicand 23 were monomorphic. The number of bands varied from 5 "primer 17898B" to 9 "primer 17898A" with an average of 6.57 markers per primer. The polymorphism level, calculated as the number of polymorphic bands per primer ranged from 16.667% to 77.778% Minimum and maximum number of polymorphic bands were obtained with the primer HB12 "1" and 17898A "7", respectively Table 3. Cluster analysis revealed two main clusters one of them separate S.1 *Nepeta septemcrenata* as a genetically dissimilar genotype with all other one's. While the second cluster was separated into two sub-clusters comprised the first involved S.2 *Phlomis aurae* and the second sub-cluster S.3 *Thymus decussatus*; this indicates that *Thymus decussates* and *Phlomis aurea* are genetically close to each other than *Nepeta septemcrenata* Fig. 3.b. Similarity indices ranged from 0.73 between Nepeta septemcrenata and Thymus decussates to 0.86 between Phlomis aurae and Thymus decussates. Table 5.b.

The discriminatory power "Dj" for ISSR ranged from 0.777 To 0.859.

3.3. Combined RAPD and ISSR analysis

The RAPD and ISSR data were combined for similarity and cluster analyses. Fig. Cluster analysis performed on combining data of both markers revealed two main clusters.one of them separate S.1 *Nepeta septemcrenata* as a genetically dissimilar genotype with all other one's. While the second cluster was separated into two sub-clusters comprised the first involved S.2 *Phlomis aurae* and the second sub-cluster S.3 *Thymus decussatus*; this indicates that *Thymus decussates* and *Phlomis aurea* are genetically close to each other than *Nepeta septemcrenata*. Fig 3.c. Similarity indices ranged between 0.74 to 0.83. Similarity indices show that the highest similarity value 0.83 was recorded between *Phlomis aurea* and *Thymus decussates*, indicating that these two plants were closely related to each other than *Nepeta septemcrenata* which show the lowest similarity value with *Phlomis aurae* 0.74 and *Thymus decussates* 0.76 .Table 5.c. The discriminatory power "Dj" for RAPD-PCR markers tested ranged from 0.734 to 0.865, with average 0.806. and for ISSR-PCR markers tested ranged from an estimate of the probability that two randomly chosen individuals 0.777 To 0.859, with average 0.811. table 6.

IV. Discussion

In the present study, RAPD and ISSR markers were applied to assess the genetic diversity between three plants (*Nepeta septemcrenata, Phlomis aurea, Thymus decussates*). The ISSR primers generated more polymorphic bands 23 than RAPD primers 17, so ISSRs were found to be more efficient over RAPDs in estimating genetic diversity. Similar results have been obtained for several other plants including Salvia [19] , Phlomis [31]. Morus alba [33], Ocimum [32]. Dendrogram constructed using the neighbor joining method of cluster analysis revealed two main clusters for RAPD and ISSR. Cluster analysis was also carried out on two sets of marker profiling data based on RAPD and ISSR combination which grouped the three plants into two main clusters. First cluster included *Nepeta septemcrenata* in all of three studied marker systems.and the second cluster was separated into two sub-clusters, one of them involved *Phlomis aurea* and the other involved Thymus decussates. we expected high genetic distance between *Nepeta septemcrenata* and the two other plants *Phlomis*

aurea and Thymus decussates which more closely related. Variations in DNA sequences lead to polymorphism and greater polymorphism are indicative of greater genetic diversity. According to similarity indices The high similarity between *Phlomis aurea* and *Thymus decussatus* was obtained in RAPD 0.81,ISSR 0.83 and RAPD+ISSR Combined 0.83 .These results indicate that these two species are closely related. This result in harmony with [19] and [31] Primer efficiency is an important parameter, especially in cases where random primers are used for genetic diversity analysis.discriminatory power "Dj", introduced by [39] is the most effective method to know the primer ability in distinguishing the cropplant genotypes using random markers. The *Dj* values for both RAPD and ISSR markers ranged from0.734 to 0.865 RAPD with average 0.80 and 0.777 To 0.859 ISSR with average 0.81 respectively. The efficiency of the *Dj* is an extension of the PIC and provides an estimate of the probability that two randomly chosen individuals show different banding patterns for the same primer and, thus, are distinguishable from one another. Discriminatory power, a direct indication of primer efficiency, prevents the use of those primers which show less efficiency in distinguishing the genotypes. In the current study Dj values Dj values lower for RAPDs than for ISSRs. [41] revealed similar results.

V. Conclusion

The three plants under study *Nepeta septemcrenata*, *Phlomis aurea* and *Thymus decussates* are very rare, endangered and severely threatened by both natural and human factors.Also characterized by high medical value so study genetic fingerprint help in plants preservation and benefits. RAPD and ISSR markers were applied to assess the genetic diversity between three plants. The two sets of marker profiling data based on RAPD and ISSR combination which grouped the three plants into two main clusters. First cluster included *Nepeta septemcrenata* in all of three studied marker systems. and the second cluster was separated into two sub-clusters, one of them involved *Phlomis aurea* and the other involved Thymus decussates. we expected high genetic distance between *Nepeta septemcrenata* and the two other plants *Phlomis aurea and Thymus decussates* which more closely related.

VI. Figures And Tables

Table 1: scientific names, family, location and vernacular names of the studied plant

Scientific names/Family	Location/GPS	Vernacular names
Nepeta septemcrenatat (Lamiaceae)	Wadi Elfaraa: North:28.539368 East: 33.967015 Altitude: 1873	(زيتيه) Zayteya
Phlomis aurea Lamiaceae	Wadi Elfaraa: North: 28.539368 East: 33.967015 Altitude: 1873	Awarwar (عوروز)
Thymus decussates (Lamiaceae)	Wadi Elfaraa: North:28.539368 East: 33.967015 Altitude: 1873	(زعيتران)Zyteran

Table 2.a: List of RAPD Operon primers and their nucleotide sequences

Primer Code	Primer Sequences	Primer Code	Primer Sequences
A1	**5CAGGTTTTTC3**	B8	**5TGCTCTCTGCCC3**
A4	**5AAGTTTGCTC3**	B9	**5GGTGACGCAG3**
A9	**5GGGTAACGCC3**		

Table 2.b: List of ISSR Operon Primers and their nucleotide sequences

Primer Code	Primer Sequences	Primer Code	Primer Sequences
HB11	**(GT)6CC**	17898B	**(CA)6 GT**
HB12	**(CAC)3GC**	17899A	**(CA)6 AG**
HB15	**(GTC)3GC**	17899B	**(CA)6 GG**
17898A	**(CA)6 AC**		

Table 3.a: RAPD polymorphism in three plants genotypes tested using RAPD-PCR with five primers

Primer	Amplicon	BP	1	2	3	M
A1	AF1	1457	1	1	1	
	AF2	719	1	0	0	M^+
	AF3	679	0	1	1	M^-
	AF4	588	1	0	0	M^+
	AF5	521	0	1	0	M^+
	AF6	418	0	0	1	M^+
	AF7	294	1	1	1	
	AF8	206	0	1	0	M^+

	AF9	198	0	0	1	M^+
	AF10	156	0	1	1	
	AF11	89	1	1	1	
A4	AF12	1230	0	0	1	M^+
	AF13	1020	1	0	1	M^-
	AF14	865	1	0	0	M^+
	AF15	780	0	1	1	M^-
	AF16	534	1	1	1	
	AF17	426	1	1	1	
	AF18	275	1	1	1	
	AF19	233	1	1	1	
	AF20	148	1	1	1	
A9	AF21	746	0	1	1	M^-
	AF22	525	1	1	1	
	AF23	321	1	1	1	
	AF24	278	1	1	0	M^-
B8	AF25	1428	1	0	1	M^-
	AF26	721	1	1	1	
	AF27	330	1	1	1	
	AF28	221	1	1	1	
B9	AF29	1274	1	1	1	
	AF30	1149	1	1	1	
	AF31	853	1	1	1	
	AF32	658	0	1	0	M^+
	AF33	562	1	0	1	M^-
	AF34	344	1	1	1	
	AF35	251	1	1	1	

Table 3.b: ISSR polymorphism in the three plants tested using ISSR-PCR with seven primers

primer	Amplicon	BP	1	2	3	**M**
HB11	AF01	692	1	1	1	
	AF02	557	0	1	1	M^-
	AF03	491	1	0	0	M^+
	AF04	223	1	0	0	M^+
	AF05	147	1	0	1	M^-
	AF06	112	1	1	1	
HB12	AF07	6035	0	1	1	M^-
	AF08	1030	1	1	1	
	AF09	604	1	1	1	
	AF10	442	1	1	1	
	AF11	329	1	1	1	
	AF12	230	1	1	1	
HB15	AF13	7403	0	1	1	M^-
	AF14	2182	1	1	1	
	AF15	1356	0	1	1	
	AF16	775	1	1	1	
	AF17	569	1	1	1	
	AF18	368	1	1	1	
	AF19	263	1	1	1	
	AF20	141	1	0	0	
17898A	AF21	4742	0	1	0	M^+
	AF22	4626	1	0	0	M^+
	AF23	2240	0	1	0	M^+
	AF24	2041	1	0	1	
	AF25	1298	1	1	1	
	AF26	841	1	1	0	
	AF27	652	1	1	1	
	AF28	344	1	1	0	
	AF29	287	0	0	1	
17898B	AF30	1261	1	1	1	
	AF31	1003	0	0	1	M^+
	AF32	827	1	1	1	
	AF33	646	1	1	1	
	AF34	466	1	1	1	
17899A	AF35	1728	0	1	0	M^+
	AF36	1333	1	1	1	
	AF37	749	0	1	1	M^-
	AF38	622	1	1	0	M^-
	AF39	445	1	1	1	
	AF40	260	0	1	1	M^-

17899B	AF41	851	0	1	1	M^-
	AF42	689	1	1	1	
	AF43	400	1	1	1	
	AF44	511	1	0	0	M^+
	AF45	288	1	1	1	
	AF46	191	0	1	1	M^-

Table 4.a: Amplification results of the five RAPD primers for the three plants tested

Primers	**TAF**	**PB**	**P%**	**Genotypes**						**TSM**
				G1		G2		G3		
				AF	SM	AF	SM	AF	SM	
A1	11	8	72.72%	5	3	7	4	7	1	
A4	9	4	44.444%	7	2	6	1	8	1	
A9	4	2	50%	3	1	4	0	3	1	
B8	4	1	25%	4	0	3	1	4	0	
B9	7	2	28.71%	6	0	6	2	6	0	

TAF= total number of amplified fragments, PB = polymorphic bands, P% = polymorphism percentage, AF = amplified fragment, SM = genotype specific marker including either the presence or absence of agiven band, TSM + total number of specific markers.

Table 4.b: Amplification results of the seven ISSR primers for the three plants tested

primers	**TAF**	**PB**	**P%**	**Genotypes**						**TSM**
				G1		G2		G3		
				AF	SM	AF	SM	AF	SM	
HB11	6	4	66.667%	5	3	3	1	4	0	4
HB12	6	1	16.667%	5	1	6	0	6	0	1
HB15	8	3	37.5%	6	3	7	0	7	0	3
17898A	9	7	77.778%	6	1	7	2	7	5	8
17898B	5	1	20%	4	0	4	0	5	1	1
17899A	6	4	66.667%	3	2	5	1	4	2	5
17899B	6	3	50%	4	3	5	0	5	0	3

TAF= total number of amplified fragments, PB = polymorphic bands, P% = polymorphism percentage, AF = amplified fragment, SM = genotype specific marker including either the presence or absence of agiven band, TSM + total number of specific markers.

Table 5. a- Similarity indices obtained for RAPD

	Nepeta septemcrenata	*Phlomis aurea*	*Thymus decussatus*
Nepeta septemcrenata	1.00		
Phlomis aurea	0.74	1.00	
Thymus decussatus	0.79	0.81	1.00

Table 5.b- Similarity indices obtained for ISSR

	Nepeta septemcrenata	*Phlomis aurea*	*Thymus decussatus*
Nepeta septemcrenata	1.00		
Phlomis aurea	0.74	1.00	
Thymus decussatus	0.73	0.86	1.00

Table 5.C- Similarity indices obtained for RAPD and ISSR

	Nepeta septemcrenata	*Phlomis aurea*	*Thymus decussatus*
Nepeta septemcrenata	1.00		
Phlomis aurea	0.74	1.00	
Thymus decussatus	0.83	0.76	1.00

Table 6. Levels of genetic information (Discriminating power, Dj**)** generated by 5 RAPD-PCR primers and 7 ISSR-PCR Primers on the three endemic and near endemic plants tested.

RAPD **Primer**	Dj	**ISSR Primer**	Dj
A1	**0.855**	**HB11**	***0.777***
A4	**0.865**	**HB12**	***0.829***
A9	**0.734**	**HB15**	***0.859***
B8	**0.741**	**17898A**	***0.84***
B9	**0.839**	**17898B**	***0.77***
		17899A	***0.803***
		17899B	***0.804***
average Dj	**0.8068**		***0.8117***

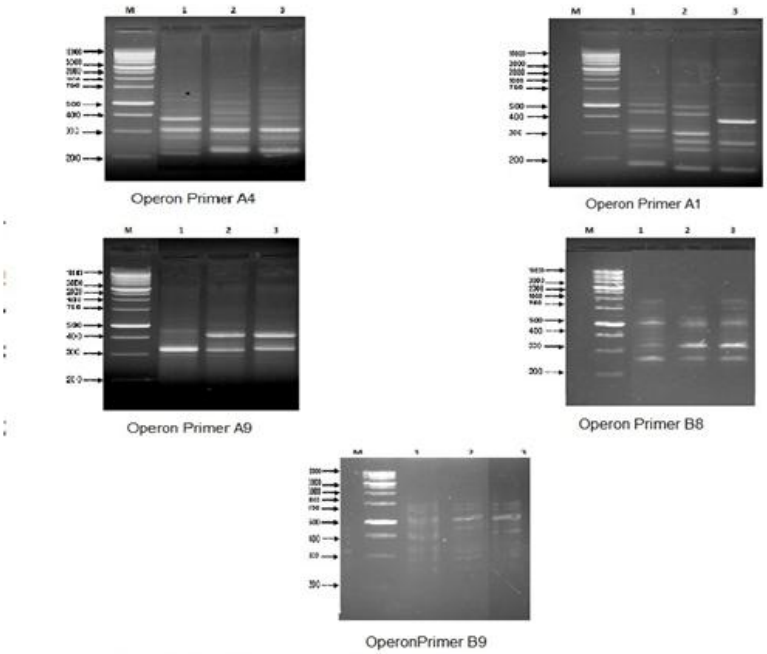

Figure 1. Polymorphic bands generated by different RAPD Primers

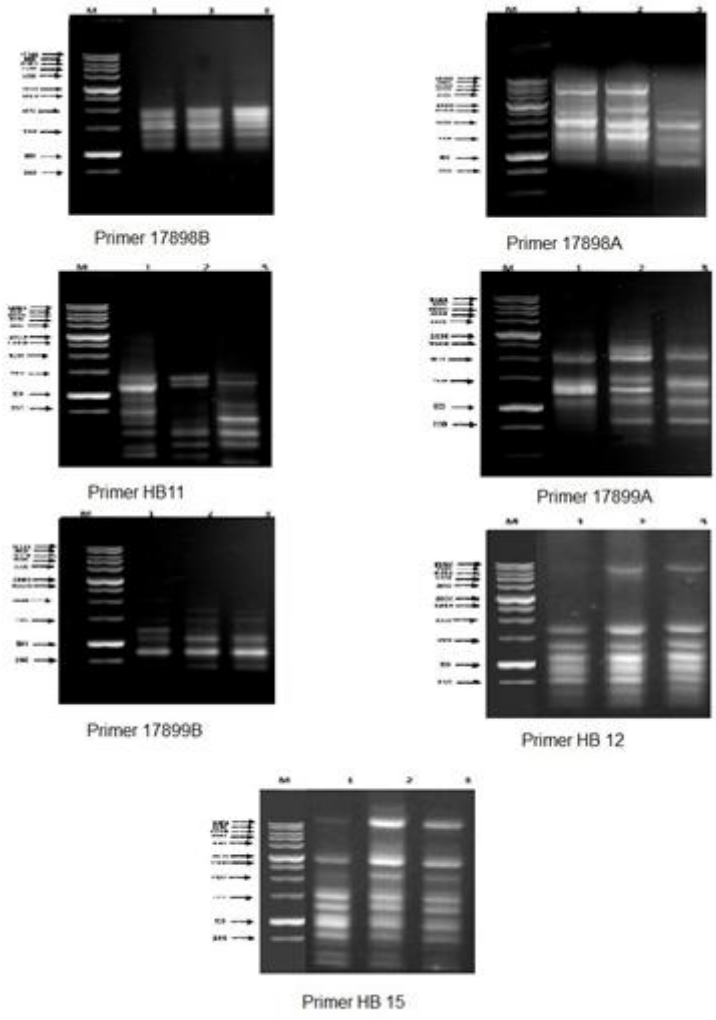

Figure 2. Polymorphic bands generated by different ISSR primers.

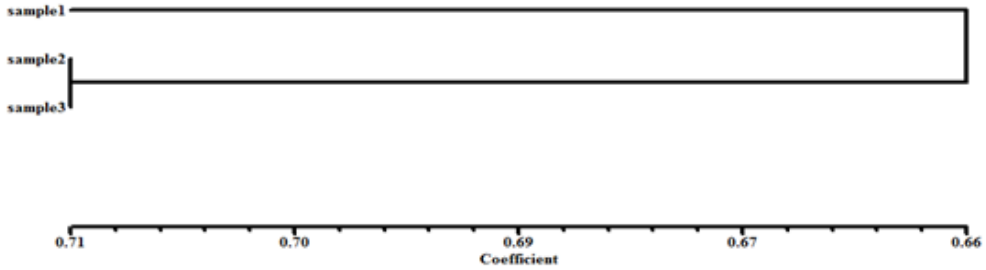

Fig. 3.a: Dendrogram constructed according to UPGMA cluster analysis, based on the similarity index of Nei (1978), showing the genetic relationships between three plants based on RAPD Primers analysis.

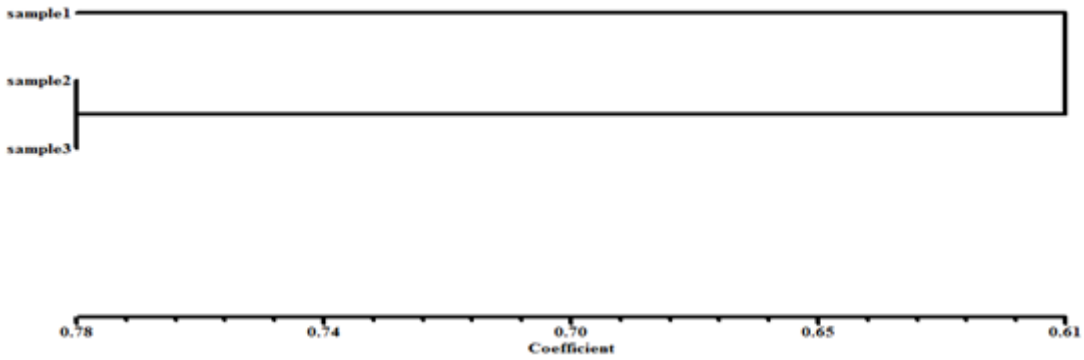

Fig. 3.b: Dendrogram constructed according to UPGMA cluster analysis, based on the similarity index of Nei (1978), showing the genetic relationships between three plants based on ISSR Primers analysis.

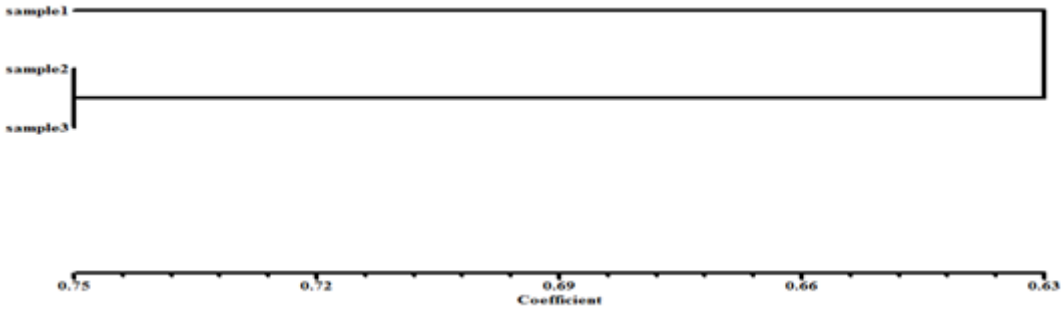

Fig.3.c: Dendrogram constructed according to UPGMA cluster analysis, based on the similarity index of Nei (1978), showing the genetic relationships between three plants based on RAPD and ISSR Primers analysis.

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