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Determination Of The Genetic Relationships Among Five Species From Families Boraginaceae,Papaveraceae, Plantaginaceae And Polygonaceaeby Rapd And Issr Analysis In Iraq

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ABSTRACT

Isolation of the genomic DNA from leaves of the species under study belonging to different families with concentrations ranging between (70-163ng/ml) and purity of (1.75-2) which was estimated by a Nanodropdevice at the wavelengths of 260 - 280 nm, Random Amplified Polymorphic DNA (RAPD) and The Inter-simple sequence repeat (ISSR).The discrepancies between the replicated pieces of each species (their numbers and molecular sizes) were detected after the replication products of the samples were migrated onto the agarose gel and stained. With the stain of Red safe staining solution,(20) selected primers showing varied outcomes of multiplication among the species examined, as those primers showed with (RAPD) Marker (189) polymorphic bands out of (197) total main bands, While with (ISSR) Marker (52) polymorphic bands out of (56) total main bands.

For RAPD-PCR analysisit gave the primers OP-E20, OP-L05, OP-L20 and OP-M05 and OP-V19 unique fingerprints for each genotype, but this did not apply to other primers.While, ISSR- PCR analysisthe primers UBC842 and A35, UBC480, and A34 are uniquely fingerprinted for each genotype, but this did not apply to other primers.

These methods were good in diagnosing the species understudy

Keywords: Iraq, genotype, RAPD, ISSR, primer.

INTRODUCTION:

Among molecular techniques that have been applied in the field of molecular genetics are the Random Amplification Polymorphic DNA (RAPD) and The Inter-simple sequence repeat (ISSR)[1]. Scientists interested in studying molecular diagnostics have also developed these techniques according to the case of study. Genetic variations at the species level are led to the emergence of other techniques that are more advanced and more accurate, such as the technique of sequencing DNA, which is today one of the most important, best and most

accurate methods of molecular diagnosis as it depends on determining the sequence of nitrogenous bases of the piece.

When compared to other approaches, RAPD and ISSR procedures are fast and straightforward, because the marking sequences are not necessary and may yield plentiful polymorphism plots. Until today, genetic variation has been studied using molecular analyses with little effort. [2].

The Boraginaceaefamily of sedge or borage family belongs to the order Lamiales, which includes herbs, shrubs and trees, the inflorescence is a scorpiod(Helicoid), Researchers have differed in the number of its genera and species. [3] and [4] estimated the number of its genera as 100 genera and about 2000 species, and it is represented in Iraq by 26 genera and about 93 species**.**

However, studies that dealt with the family from a molecular point of view, which focus on the evolutionary aspect, are still few. This may be due to the high cost of materials needed by these studies, depending on the molecular data, [5] divided the family into three clads: Echiochileae, Eritrichieae and Trichodesmeae,This study is considered one of the first studies in Iraq.

One of the most important species of this family,the species Arnebiadecumbens (Vent.) Coss. & Kralik: It is known locally as Al-Kahil[6], which is an annual herb with a usually hairy surface covering. The inflorescence is scorpion-shaped, and it is a very common species in Iraq that spreads in desert and sandy areas, and it has red roots with a bright red dye [3].This plant is used in alternative medicine as mentioned by [7] that it is biologically and pharmacologically useful because it contains antiviral, antibacterial, anti-inflammatory and antitumor properties, and these activities strengthen the immune system, in addition to the presence of chemical compounds extracted from the leaves of the plant, the most important of which are flavonoids, which are important secondary metabolic compounds in addition to acids. phenolic, Anthocyanidin, Carotenoids, Procyanidins, and Sterols [8].The species did not have molecular studies in Iraq.

The species HeliotropiumbacciferumForssk.: Known locally as the scorpion's tail, it is found in Iraq and Kuwait [3]. It is a perennial herb native to Algeria and medically important [9], its powder is used medically in the treatment of skin diseases, wounds, stomach ulcers, varicose and warts, and its leaves are used locally to treat skin diseases such as Abscess and boils, while the aerial parts are used as decoctions). To treat laryngitis, the juice is also used to treat skin burns [10].

The Papaveraceae(Papaver family) belongs to the order Ranunculales. [11] mentioned that it is a perennial, annual or biennial herb, rarely in the form of shrubs. It includes 26 genera, five of which are found in Iraq. that includes 26-42 genera and 690-800 species, widely distributed in temperate and subtropical regions of the world, [12].

Analyzes of chloroplast DNA sequences of the family Papaver alpinum L. show that it is of monophyletic origin, and the establishment of sister-group relationships is more complex as analyzes involving only diploid P. alpinum species revealed lineages that do not match the DNA regions different nuclear weapons [13].

The speciesGlauciumflavum(L.)Curt.: The 'yellow horned or 'marine' Papavera is a herbal plant growing on sandy and rocky beaches that have unique leaves of silver, yellow blooms and horny fruits, and its popular name. It is mostly prevalent on the Mediterranean and Western European coastlines [14].It is a medicinal plant whose therapeutic effects are due to the presence of Glaucine as the main alkaloid.

The Plantaginaceae the plantain family of the order Plantaginales is found in the form of herbs and sometimes small shrubs, including one genus and about 270 species [15]. According to the study of [16], it is a global family that includes three genera and about 275 species spread in diverse habitats all over the world. In Iraq, [3] mentioned that it is a small family with three genera and 285 species spread in tropical and temperate regions.During [17] study of 92 genera belonging to the family using the ITS region is one of the most widely sequenced DNA regions in molecular systems and has been used to evaluate intragenetic and intrafamilial relationships in the genera Veroniceae, Plantago and Cheloneae. The matK-trnK gene is useful for assessing relationships within families and between races and has also been shown to be useful at higher taxonomic levels.

The species Plantago ovataForsk.: Known locally as Civet [18], it is a perennial herb with a smooth hairy surface covering, devoid of stems, distributed mostly in temperate regions and a few in tropical regions such as Asia, especially the Mediterranean region [19]. The species P.ovata has gained worldwide importance due to its wide diversity in terms of medical importance, It is used as a treatment for chronic bacillary dysentery, as a laxative and for chronic amoebic diseases [18]. it uses black or brown seeds that are tasteless and odorless to treat hemorrhagic colon disease, as it works to detoxify the body by removing food residues from the human body and lowering cholesterol [20].

The Polygonaceae family belong to the order Caryophyllales, a family of flowering plants found all over the world [21]. [3] indicated that it is annual or perennial herbs or shrubs and includes 559 genera and more than 1150 species in Iraq. [22] identified 48 genera and 1200 species of herbs, shrubs and small trees, while [23] stated that the family generally consists of 30 to 49 genera with about 750 species worldwide and about 33 species in Iraq. Schuster et al (2015)[24] demonstrated the evolutionary history of a family member by building on an existing molecular data set (nrITS, matK and trnL-trnF) analyzed with maximum probability and Bayesian methods.The results of his study indicate that Oxygonum is a possible sister strain isolated to all other members of the family.

The species Rumexvesicarius L.: It is called sorrel locally or the tooth of the old man. It is an annual herb that grows in sandy soils and fields in Iraq, Syria and North Africa. It is an edible wild plant and is collected in the spring. The leaves have a sour taste and are eaten fresh or cooked [3]. It has many important medicinal uses such as treating liver diseases, indigestion, diuretic, laxative, tonic, analgesic, and antibacterial, and it can be used to reduce biliary disorders and control cholesterol and blood sugar levels[25].

Due to the importance of these families from a medcinal point of view, we suggested studying it from molecular aspects as an attempt to cover more accurate details, so the study aimed to investigate the following aspects:

a) Determine the evolutionary relations between them by adopting the usual PCR technique (RADP and ISSR analysis).
b) Finding the molecular DNA characteristic of the studied species to diagnose it.
c) Determine the genotype of most types of genera under study.

MATERIALS AND METHODS :

Collection of Plant Samples

The current study relied on the fresh samples collected during field trips to the Bahr Al-Najaf depression. Five species belonging to different families were selected in Table (1).as work was done in the central laboratory for post graduate studies in the Biology department / faculty of Education for Girls / KufaUniversity for the period from 5/11/2020 to 31/4/2021.

Table (1):Names of the species and families studied

No	species	Families
1.	Arnebiadecumbens(Vent.) Coss. & Kralik	Boraginaceae
2.	HeliotropiumbacciferumForssk.	Boraginaceae
3.	GlauciumflavumCrantz	Papaveraceae
4.	Plantago3149vateForsk	Plantaginaceae
5.	RumexvesicariusL.	Polygonaceae

The DNA Isolation

DNA was extracted from samples selected by the name ofthe Kit ZR Plant/Seed DNA MiniPrep provides a simple and fast method for obtaining pure DNA including genomic DNA, mitochondrial DNA and chloroplasts from plant tissues, and as it was measurement of DNA concentration and purity determination, For this purpose, a spectrophotometer (Nanodrop) was used by placing a small drop of 0.7 µl of DNA extract on the sensitive lens of the device after calibrating it with a similar drop of Elution buffer to clear it, Then the values of DNA concentration for each plant extractand its purity were recorded on the wavelengths 260 and 280.

The primers used in the polymerization interactionas it was 20 types of primers were used in the reaction prepared by Bioneer in a lyophilized form, the primers were diluted by adding distilled water ddHO to get a concentration of 100 pkamol according to the attached leaflet from the supplying company, then the required concentration was prepared by taking 10 µl of the original solution and completing the volume to 100 µladd distilled water to make it ready for use Table (2).

Table (2): Primer name and sequence of nitrogenous bases for RAPD and ISSR Markers

	NO.	Primer	Sequence
RAPD	1	OP-E20	AACGGTGACC
	2	OP-L05	ACGCAGGCAC
	3	OP-L20	TGGTGGACCA
	4	OP-M05	GGGAACGTGT
	5	OP-M06	CTGGGCAACT
	6	OP-M14	AGGGTCGTTC
	7	OP-M20	AGGTCTTGGG
	8	Op-P04	GTGTCTCAGG
	9	OP-V19	GGGTGTGCAG
	10	OP-V14	AGATCCCGCC
ISSR	1	UBC842	GAGAGAGAGAGAGAGAYG
	2	UBC858	TGTGTGTGTGTGTGTGRT
	3	UBC807	AGAGAGAGAGAGAGAGT
	4	UBC862	AGCAGCAGCAGCAGCAGC
	5	A35	AGAGAGAGAGAGAGAGCT
	6	UBC480	GAGAGAGAGAGAGAGAYT
	7	UBC815	CTCTCTCTCTCTCTCTG
	8	A34	GCGCGTGTGTGTGTGT
	9	N35	GAGACC
	10	813	CTCTCTCTCTCTCTCTT

Polymerase Chain Reaction (PCR)

The two molecular indicators adopted in this study, RAPD and ISSR, were applied in PCR technique is taking 5 µL of template DNA and 2 µL of primer were added into the prepared master reaction tube and the final volume of the reaction was completed by adding distilled deionized water to a volume of 20 µL solution. Thermocycler has been placed in a special program for each group of primers, as shown in Tables (3) and (4):

Table (3):Thermal cycle stages of the polymerase chain reaction technology RAPD

Step	Temperature	Time	No.of Cycles
Initial Denaturation	94C˚	5 min	1 Cycles
Denaturation	94C˚	30 Sec	40 Cycles
Annealing	36C˚	45 Sec	
Extension	72C˚	45 Sec	
Final Extension	72C˚	7 min	1 Cycles

Table (4): Thermal cycle stages of the polymerase chain reaction technology ISSR

Step	Temperature	Time	No.of Cycles
Initial Denaturation	94C°	3 min	1 Cycles
Denaturation	94C°	1 min	40 Cycles
Annealing	35C°	1 min	
Extension	72C°	1 min	
Final Extension	72C°	10 min	1 Cycles

After the end of the reaction time, the tubes were removed from the thermal polymerase device and 10 µl was withdrawn from the tubes and loaded on the pits of the previously prepared agarose gel at a concentration of 1.5%, with the DNA ladder loaded on one side. At 70 volts, the PCR products were exposed to UV rays on agarose gel by the gel documentation system for imaging purposes[26], The gene characterization data may be transformed into tables indicating whether or not for each sample of the samples investigated the beam is present or not, 1 for the presence of the band and 0 for the absence of the band, after identification of the number and molecular weight of the bands.These samples were used to study the genetic similarity using the Dice Co-efficient equation [27].By using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) method PAST version 1.62 [28] was used and based on the above program, the genetic dimension and the drawing of a genetic tree were done.

RESULTS AND DISCUSSION

It was noted that the species included in the study varied from the first sight in their purity and concentration. For example, the highest concentration of DNA was recorded in speciesP. ovata, which amounted to 163 ng/m, and the lowest in speciesA.decumbens at a rate of 70 ng/m, and the rest of the species ranged between these two rates, and the highest purity of the species was P. ovata at a rate of 2 Table (5).

Table (5):Measurement of DNA concentration and purity determination

No	The species	Nucleic acid Conc. (ng/ml)	Purity 260/280
1.	Arnebiadecumbens	70	1.7
2.	Rumexvesicarius	77	1.8
3.	Plantago ovata	163	2
4.	Heliotropiumbacciferum	110	1.75
5.	Glauciumflavum	144	1.9

Results of DNA replication based on RAPD markers

This study included the use of 10 primers for RAPD-PCR analysis for all the tested genomic DNA, and Current results utilizing the RAPD markers reveal a variation in

polymorphism and monomorphic band presence between the genotypes investigated; as it gave the primers OP-E20, OP-L05, OP-L20 and OP -M05 and OP-V19 unique fingerprint for each genotype, but this did not apply to other primers, these primers gave amplified products that can be detected and thus were useful, these primers are listed and sequenced, and the results of fingerprinting plant species are summarized in the table (6). The primer OP-E20gave 18 main bands distributed between only one single band and 13polymorphic, and their molecular sizes ranged between (200-2000) base pairs, and the number of duplicated bands was 137 bands. The highest number of duplicating bandswas 10 in A.decumbens and G.flavum, while the lowest number was 5 in R.vesicarius and H.bacciferum, gave this primer a discriminating ability that reached 9.52381 and an efficiency of 9.473684, while the formal variation of this primer was 100% Fig (1) table (6).

The primer OP-L05 gave 16 main bands, 15 of which were divergent, only two were monomorphic and 11 polymorphic, and their molecular sizes ranged between (125-3000) base pairs, and the number of duplicated bandswas 118, the number of multiplexed bandswere 11 in species G.flavum. This primer gave a discriminatory ability of 7.936508 and an efficiency of 8.421053, while the formal variation of this primer reached 93.75% Fig (2)table (6). The primer OP-M14 gave 19 main bands, the number of polymorphic was 27, and their molecular sizes ranged between (250-2250) base pairs, and the number of duplicated bandswas 150, the highest number of duplicated bands9 in two species H.bacciferum and G.flavum , while the lowest number was 5 in species A.decumbensandPlantagoovate, This primer gave a discriminating ability that reached 10,05291 and an efficiency of 10, while the formal variation of this primer was 100% Fig (3) table (6).

In general, the average number of bands for all the primers used in this study reached 197 bands for each primer at a large rate if it is known that it falls within the maximum ranges of RAPD indicators. The primers nucleotides used as well as the number of primer binding sites to the DNA template depends on the type of genome and the extent to which the enzyme identifies those sites [29].

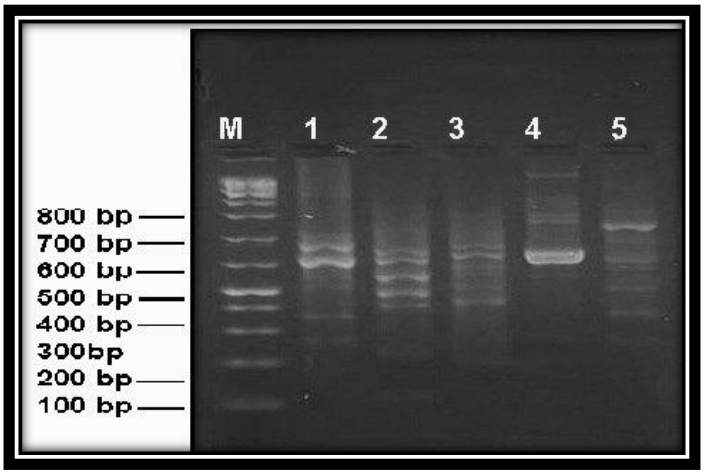


Fig (2): Products of polymerization doubling using OP-L05,1.5 % agarose gel electrophoresis at 70 volts for 45 minutes (M Ladder represents 100 base pairs, numbers from 1-18 represent plant samples and according to the sequence below).

1. *A.decumbens* 2.*R. vesicarius*.3. *P.ovata* 4. *H. bacciferum* 5. *G. flavum*

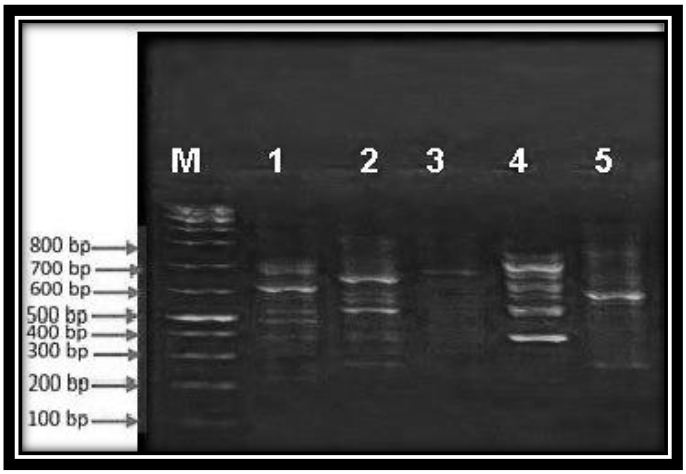
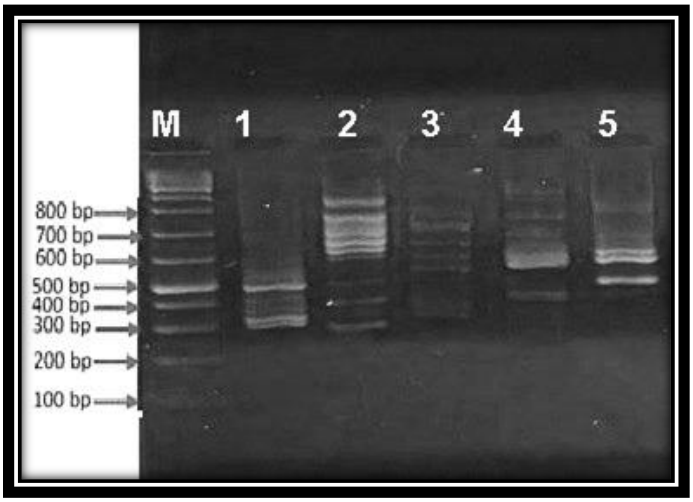


Fig (1): Products of polymerization doubling using OP-E20,1.5 % agarose gel electrophoresis at 70 volts for 45 minutes (M 70 ladder represents 100 base pairs, numbers from 1-18 represent plant samples and according to the sequence below).

1. *A. decumbens* 2 . *R. vesicarius*.3. *P.ovata* 4. *H.bacciferum* 5. *G.flavum*

Fig (3): Products of polymerization doubling using OP-M14,1.5 % agarose gel electrophoresis at 70 volts for 45 minutes (M Ladder represents 100 base pairs, numbers from 1-18represent plant samples and according to the sequence below).

1. *A. decumbens* 4. *R. vesicarius*.3.*P.ovata* 4. *H. bacciferum*5.



The results of using primers in RAPD reactions showed a difference in the number of the resulting bands and their molecular weights according to the different primers used and resulting from the difference in the number of complementary sites in the genome of each species, which confirmed the separation between the studied ranks, especially the genetically close ones, in addition, the unique band that were identified can be adopted. As genetic markers for the species to which it belongs,these results are in agreement with what was indicated by a study[30].

The study showed that the molecular weight of the studied species ranged between (125-4000) base pairs, as the primer OP-M05 recorded the highest molecular weight, which reached 4000 base pairs, and the lowest molecular weight, which was recorded by the primer OP-L05, which amounted to (4000) base pairs, and that there is a relationship between the primer sequence associated with the DNA template and the size of the duplicated bands [31].

The pattern shown by the primer OP-V19 was distinct and most suitable for the species because it showed three unique bands with different molecular weights, while the primers OP-E20, OP-L20 and OP-M05 showed one unique band. This heterogeneity may be due to several factors, such as the structure of primers, the quantity of the template, or the low number of linkage sites in the genome [32]; [33].

The primers efficiency group showed the ability of the primer to give a greater proportion of polymorphic bands according to a number of amplification ranges. Therefore, the efficiency of the primer does not mean the primer that gave the largest number of multiplexed bands, but rather its ability to show the differences between the studied species [34]. The percentage of polymorphism was 100% among the studied species except for the primer OP-L05, in which the percentage of polymorphism reached 93.75% Table (6).

Table (6): shows the outputs of the RAPD prefixes of total amplified bands, polymorphic bands, and monomorphic with their efficiency ratios and Primer discriminatory power for the studied species

No	The primer	No. of total amplified bands	No. of polymorphic bands	No. of Monomorphic bands	Primer discriminatory power%	Polymorphism %
1.	**OP-E20**	19	18	1	9.473684	100
2.	**OP-L05**	17	15	2	8.421053	93.75
3.	**OP-L20**	19	18	1	9.473684	100
4.	**OP-M05**	22	21	1	11.05263	100
5.	**OP-M06**	19	19	0	10	100
6.	**OP-M14**	19	19	0	10	100
7.	**OP-M20**	24	24	0	12.63158	100
8.	**OP-P04**	17	17	0	8.947368	100
9.	**OP-V19**	24	21	3	11.05263	100
10.	**OP-V14**	17	17	0	8.947368	100
		197	189			

Genetic dimension values based on ISSR Markers

This study included the use of 10 primers for ISSR-PCR analysis for all genomic DNA tested in Table (7), and the results of the current study showed the discrepancy between the studied genotypes through the presence of heterogeneous, duplicated and monomorphicin addition to the presence of identical bands, and gave the primers UBC842 and A35, UBC480, and A34 are uniquely fingerprinted for each genotype, but this did not apply to other primers, and this agrees with [35]. The primer UBC842 gave 7 main bands distributed among 4 different bands, one of the single bands, and 3 identical bands, and their molecular sizes ranged between (250-1200) base pairs, and the number of duplicated bands was 99, the highest number of duplicated bands was 7 in the species A. decumbens, while the lowest number was 4 in the species P. ovata, and this primer gave a discriminating ability of 7.692308 and an efficiency of 9.589041, while the formal variation of this primer was 57.14286% Fig (4) Table (7).This primer UBC862 gave 8 main bands distributed between 6 different bands and 2 identical bands, and their molecular sizes ranged between (200-1100) base pairs, and the number of duplicated bands was 74 bands, the highest number of duplicated bands was 6 in species A. decumbens, and the lowest number was 2 in the two species,H. bacciferum and G. flavum and, this primer gave a discriminating ability that reached 11.53846 and an efficiency of 10.9589, while the morphological heterogeneity of this primer was 75% Fig (5) Table (7).

The primer A35 gave 10 main bands distributed among 5 different bands, only one single, 3 identical and 2 polymorphic, and their molecular sizes ranged between (225-1500) base pairs,

and the number of duplicated bands reached 115, the highest number of multiplexed bands was 9 in the species H. bacciferum, while the lowest number 4 in the species P. ovate, and this primer gave a discriminating ability of 9.615385 and an efficiency of 13.69863, while the formal variation of this primer was 50% Fig (6) Table (7).

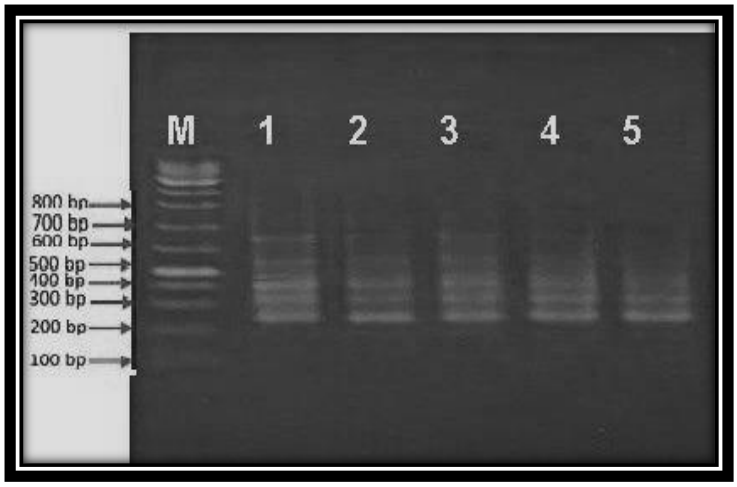


Fig (4): Products of polymerization doubling usingUBC842,1.5 % agarose gel electrophoresis at 70 volts for 45 minutes (M Ladder represents 100 base pairs, numbers from 1-18 represent plant samples and according to the sequence below).

1. *A. decumbens* 2.*R.vesicarius*.3.*P. ovata* 4.*H. bacciferum*5 . *G. flavum*

Fig (6): Products of polymerization doubling using A35,1.5 % agarose gel electrophoresis at70 volts for 45 minutes (M Ladder represents 100 base pairs, numbers from 1-18 represent plant samples and according to the sequence below).

1.*A.decumbens* .2 *R. vesicarius.3. P.ovata* 4. *H. bacciferum* 5. *G. flavum*

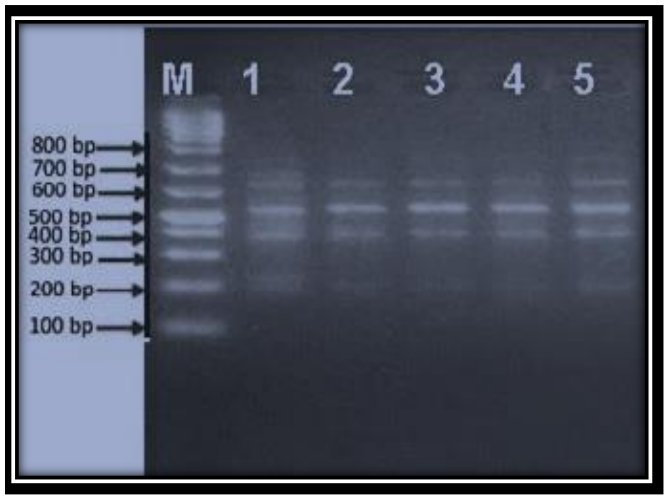


Fig (5): Products of polymerization doubling using UBC862,1.5 % agarose gel electrophoresis at70 volts for 45 minutes (M Ladder represents 100 base pairs, numbers from 1-18 represent plant samples and according to the sequence below).

1.*A. decumbens* 2 .*R.vesicarius.3. P. ovata* 4 . *H. bacciferum*5. *G. flavum*

Primers showed 52 variant bands out of 56 main bands. Of the multiplexed bands 65 with the primer 813, the highest number of the main and dissimilar bands was 12 by the prefixes UBC480 and A34, and the lowest number of the main bands was 4 with the prefixes UBC858, UBC807

and 813, while the lowest number of the divergent bands was 1 with the primer UBC858, The difference in the number of main and differentiated bands is mainly due to the structure of the primer, and that some prefixes recognize a large number of link sites, which is more useful than the primers that recognize a smaller number of link sites. The results of the ISSR indices study showed that the molecular size of the resulting bands was varied for all studied species, ranging between (150-1500) base pairs, as the primer UBC480 recorded the lowest molecular size of 150 base pairs, and the highest molecular size was recorded by the primer A35, which amounted to 1500 base pairs. Basal and the size of the duplicating pieces are usually associated with the primer sequence associated with the DNA template [36].

The primer A34 gave the highest number of monomorphic2 while the lowest number was 1 among the prefixes UBC842, A35 and UBC480, while the rest of the prefixes did not give any monomorphic(Table 7). the presence of such bands indicates that the primer knew the linking site of the bands This is an opportunity to increase the production of a distinct structure signature [37].

It is useful to test many primers to determine the structures, as the highest discriminatory ability was obtained with UBC480 and A34 primers, which amounted to 23.07692, while UBC858 gave the lowest discriminatory ability, which amounted to 1.923077[38] Table (7).

Table (7): shows the outputs of the ISSR prefixes of total amplified bands, polymorphic bands, and monomorphic with their efficiency ratios and Primer discriminatory power for the studied species

No	The primer	No. of total amplified bands	No. of polymorphic bands	No. of Monomorphic bands	Primer discriminatory power%	Polymorphism %
1.	**UBC842**	5	4	1	7.692308	57.14286
2.	**UBC858**	1	1	0	1.923077	25
3.	**UBC807**	2	2	0	3.846154	50
4.	**UBC862**	6	6	0	11.53846	75
5.	**A35**	6	5	1	9.615385	50
6.	**UBC480**	13	12	1	23.07692	100
7.	**UBC815**	4	4	0	7.692308	66.66667
8.	**A34**	14	12	2	23.07692	100
9.	**N35**	4	4	0	7.692308	66.66667
10.	**813**	2	2	0	3.846154	50
		56	52	3	L56	

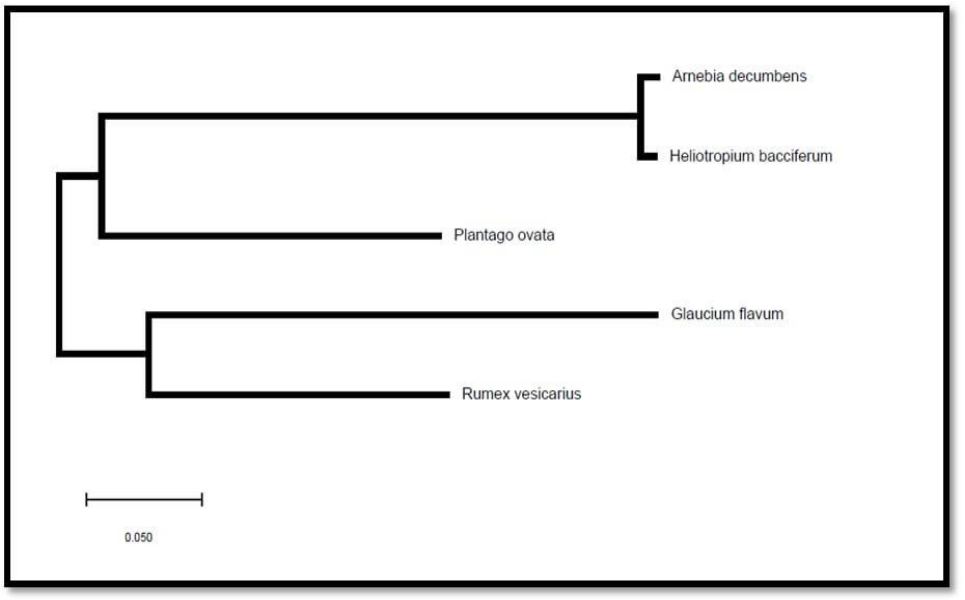


Figure 7: UPGMA Dendrogram showing genetic linkages of RAPD and ISSR markers among species

Conclusions

A molecular study has proven its power in elucidating the evolutionary relationships between species and its importance in dividing species in the form of aggregates and cladede.

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