Determination of Genetic Relationship Using RAPD in Some Species of TRIGONELLA L. from the Fabaceae Family in Central and Northern Iraq

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Abstract

The current research included the study of six wild plant species belonging to the genus Trigonella L. of the legume family in central and northern Iraq, and they have medical and economic importance, and because these species are closely related phenotypically and even from an anatomical point of view, the aim of the study is to use the genetic aspect to distinguish between species taxonomically, it was shown through the study The current genetic distance between species using the RAPD marker, as ten primers were used. It varied in showing the number of bands, their location and size, as it was observed that the bundles were absent in some species and their appearance in other types using the same apparent, which indicated the existence of genetic variation between species, as the total number of bands reached 381 bands, and the number of polymorphic bands was 243, and the study recorded the emergence of 138 monomorphic bands While the number of unique bands reached 32 bands distributed over species and different molecular sizes, the study also showed the percentage of polymorphism as it reached 100%, while the discriminating ability reached 16.460 % , It was found through the dendritic diagram that the shortest genetic distance was between the two species T.astroites and T.elliptica, which amounted to 0.51856, while the longest genetic distance was recorded between the two species T.monspliaca and T.stellata and was 0.68599, and this discrepancy is attributed to the occurrence of adaptation to living in the environments in addition to Due to the occurrence of genetic mutations and modifications between species, therefore, the use of the RAPD marker distinguishes between species genetically and the genetic dimension between them.

Keywords: *RAPD, Trigonella L., Fabaceae.*

INTRODUCTION

The genus Trigonella L. belongs to the Fabaceae family , which is the third largest botanical family of seedless plants after the family of Asteraceae and the family of Orchidaceae, and is the second family in terms of economic importance after the Poaceae family. The Fabaceae family includes 700 species and more than 20 thousand species in the world (Lpwc ,2013a; FAO ,2015) In Iraq, the family consists of 35-58 genera and 300-339 species spread in mountainous, dry wild and sometimes agricultural areas (Ghzanfar, 2015). The plants of the genus Trigonella are medicinal plants (Sun et al, 2020). These plants are located in dry areas throughout the Mediterranean, Western Asia, Europe, North and South Africa, North America and South

Australia. This genus has 62 species (Chaudhary et al,2018).

Genetic studies have played an important role in the field of plant classification, by studying the convergence and genetic distance between genera, species, and plant varieties, and identifying the distinctive varieties of cultivated species (Al-Sakmani et al, 2018). Recently, researchers have resorted to using various techniques, including the Polymerase chain reaction technique (PCR), And the Random Amplified Polymorphic DNA marker, known as the RAPD marker and the ISSR Inter –simple sequence repeat technique (Yassin, 2011) in the study of genetic variation in plants as well as bacteria and animals and identifying the genetic locations of the traits that are located on or close to genes and the possibility of transmission from parents to The Sons (Hassan and Al-Sangari, 2018). In the current study, six wild species of the genus Trigonella belonging to the Fabaceae family were selected for their economic and medical importance. No previous detailed study on these species was observed in terms of genetics and the use of genetic markers such as RAPD marker in them. Therefore, the current research aims to study the genetic relationships based on the genetic dimension between species. of the genus Trigonella L. using RAPD random multiplication marker and distinguishing between the species under study.

Materials and methods:

1. Specimens Collection plant and Identification:

In the current study, it was based on dry samples of leaves of species belonging to the genus Trigonella, which are Trigonella astroites, Trigonella coerulscens, Trigonella elliptica, and Trigonella elliptica. Trigonella monspliaca, Trigonella stellata, and Trigonella uncinata Plant samples were collected through several field tours of the study area during the period starting from April 2022 until September 2022, as the plant samples of species belonging to the genus Trigonella were collected from different locations in central and northern Iraq, and these geographical locations are (Governorate Baghdad, Erbil Governorate, Al-Ishaqi District, Dujail District, Balad, Baiji, Tikrit and Al-Alam), The samples were kept in two groups, the first group was compressed, dried and preserved for the purpose of diagnosis after writing down the place and date of collection and the name of the collector with the sample number, while the second group took the leaves of all the species under study then washed with distilled water and dried at room temperature away from sunlight then grinded and placed in Vials Writing information on it for the purpose of using it in the molecular study to isolate genetic material and use as marker RAPD, the samples were diagnosed through many international botanical encyclopedias, including Turkish, Iranian and Saudi, and through the assistance of professors specialized in Iraqi herbariums, including the National Herbarium in Abu Ghraib and the Herbarium of the College of Agriculture at the University of Baghdad.

2. DNA isolation:

Isolation of DNA from dry leaves of the species under study of the genus Trigonella L. obtained directly from field trips using CTAB (Cetyltrimethyl ammonium bromide) mentioned by (Weigand et al, 1993) which are summarized in the following steps:

1. The weight of 1 gm of dry leaves of all species under study of the genus Trigonella, and after washing them with distilled water, they were cut and then 0.2 gm of PVP (Polyvinyl Pyrolidine) was added to them, then ground by adding liquid nitrogen in a ceramic dish until it became as powdery as possible.

2. Putting the powder in glass tubes and adding 5 ml of the extraction solution kept in a water bath at 65 0 C. The tubes were incubated in the shaking water bath at the same temperature for a period of 60-90 minutes.

3. The tubes were left to reach room temperature, then 4 ml of chloroform solution: Isoamyl alcohol (1:24) ml were added to each tube with continuous stirring for 15 minutes.

4. The tubes were transferred to a centrifuge and the mixture was discarded at a speed of 4000 rpm for 15 minutes.

5. The upper aqueous layer was removed by means of a micropipette to another tube, and the same previous volume of chloroform solution was added and discarded at the same speed again.

6. The upper aqueous layer was removed using a fine pipette and placed in new sterile tubes. 5 ml of cooled isopropanol alcohol was added and mixed with gentle stirring until white strands representing DNA appeared.

7. The DNA strands were pulled by a glass rod with a curved end and placed in another tube containing 2 ml of washing solution and left for 20 minutes.

8. The strands were raised to be added to sterile tubes containing 200-300 microliters of the dissolving solution, by stirring from time to time until the DNA was completely dissolved, and then the DNA samples were kept at a temperature of -20 °C for use in subsequent experiments.

3. Measurement of DNA concentration and purity:

The DNA concentration was estimated by measuring the absorbance of the ultraviolet spectrum using a spectrophotometer and at a wavelength of (260) nm. The samples to be estimated were diluted to 100 times using (TE) solution by taking 20 ml of the sample and adding it to 1980 ml. Soluble solution (according to the cell of the device used cuvette) and by applying the following equation:

DNA concentration µg/µL = absorbance reading per 1 mL of sample x inverse of the dilution (100) x 50/1000

As for the purity, it is estimated by dividing the absorbance reading at wavelength (260) by the absorbance reading at wavelength (280) nm. (Maniatis et al, 2001).

4- Determination of DNA molecular size

The molecular sizes of DNA were estimated by electrophoresis on an agarose gel, using the DNA lambda size index of known molecular weight.

Table 1: Random primers used in the study of RAPD marker with their sequences

Relay initiator	Primer number	Sequence
CAGGCCCTTC	OPA-01	1
GATGACCGCC	OPC-05	2
AACGGTGACC	OPE-20	3
GGAGTACTGG	OPF-16	4
CTCTCCGCCA	OPG-13	5
GGTCGGAGAA	OPH-01	6
CATTCGAGCC	OPK-01	7
GTTGGTGGCT	OPM-01	8
GGGACGATGG	OPQ-01	9
GTCCGTATGG	OPT-19	10

5.RAPD interactions:

RAPD reactions were performed based on Williams and his group, 1990 on DNA samples of six species of the genus Trigonella under study according to the following steps:

1- The concentration of DNA in all samples under study was controlled through dilution

with TE solution to obtain the concentration required to conduct RAPD reactions, and it is approximately 25-50 ng / microliter for each sample. The master reaction mixture was prepared by mixing the reaction components in an Eppendroffe tube with a capacity of 0.5 sterile ml as per Table 2:

Table 2: Solutions used in RAPD reactions for processing and insertion of a thermal cycle

Components	Final concentration	Size for one sample
Distilled sterile water	-	14.8microliters
The PCR structuredolution Buffer is strong X10 with Mg Cl2	1X	2.5 microliters
DNTPs	200 micromolers	2.5 microliters
Primer	10Becomol	2.0 microliters
Taq polymerase DNA	1unit/reaction	2.0 microliters
DNA template	25-50 ng/µl	3 microliters

2- Prepare the master reaction mixture by mixing the reaction components in a sterile 0.5 ml Eppendroffe tube, as shown in Table 4.

3- The mixture was expelled in the Microfuge device for a period of 3-5 seconds to complete the mixing of the reaction components, and it must be taken into account that the work inside the Hood is sterile, gloves are worn, and the tubes are placed in ice, then the tubes are placed in the thermo polymer device, then the program is applied as in Table 5 (Maniatis and his group, (2001.

4- After the end of the reaction time, the tubes were removed from the thermo polymer device and 5-7 microliters were withdrawn from the tubes and mixed with 2-3 microliters of loading buffer.

5- Loading the mixture into the pre-prepared agarose gel at a concentration of 1.5% with lambda DNA size guide cut with the two cutting enzymes EcoR I and Hind III.

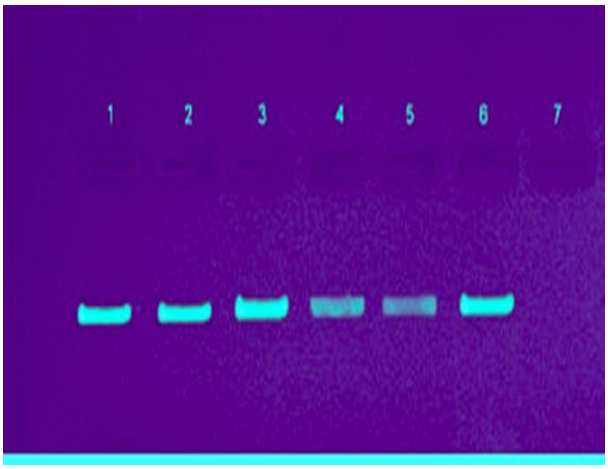
6- After that, the samples were removed by operating the electrophoresis device for a period of 90-180 minutes.

7- Then dye the gel by immersing it in a safe red dye for 45-90 minutes with stirring, then exposing it to a source of ultraviolet radiation on a UV-transilluminator and photographing the gel using a high-resolution digital camera.

Results and discussion

The DNA bundles were detected by electrophoresis on agarose gel of the six studied species and plant leaves by mixing 3 microliters of DNA sample with 5 microliters of loading buffer as in Figure 1.

Figure 2: DNA bands of plant species of the genus Trigonella stage on 1% agarose gel



1- T.astroites 2- T.coerulescens

3- T.elliptica 4- T.monspliaca

5- T.stellata 6- T.uncinata

7- control

The concentration and purity of DNA extracted from Trigonella species studied to obtain DNA were estimated at concentrations

ranging between 70-200 ng/microliter and purity ranged between 1.5-2 ng/microliter from dry plant samples using a kit prepared by the Korean company FAVORGEN.BIOTECH CORP. It was carried out in the DNA Lab, a scientific research laboratory, and it is a method that gave good results, which indicates the efficiency of the extraction method used.

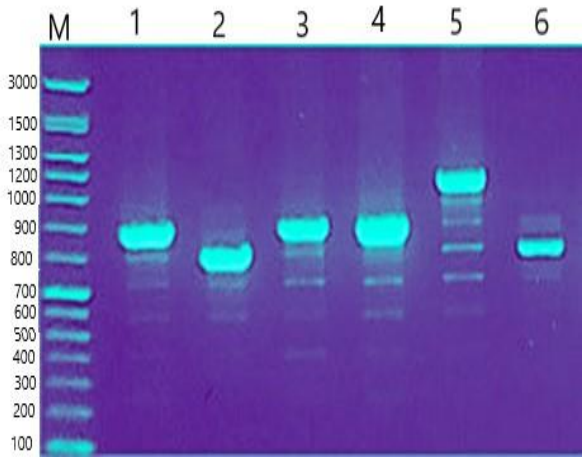
Polymerase reaction for RAPD-PCR technology

In the current study, ten primers were used, and the primers gave positive results, and the copying process took place in all species, and each primer succeeded in distinguishing between the types studied, as polymorphic bands, monomorphic bands, and unique bands appeared, and some species did not appear as bundles, as detailed below:

1. OPA-01 primer

This primer showed 7 sites for produced bands, including one site for monomorphic band with a molecular size of 700 base pairs, while the rest of the sites was polymorphic, their molecular sizes ranged between 400-1200 base pairs, and the total number of bands reached 23 bands, and the highest number of bands was 6 bands in T. stellata either. The lowest number of bands was 2 in T.uncinata, as shown in Figure 2.

Figure 2: Results of amplification of DNA extracted from Trigonella species using the OPA-01 primer after electrophoresis on an agarose gel. (M) represents the molecular size (3000bp).



1-T.astroites 2- T.coerulescens

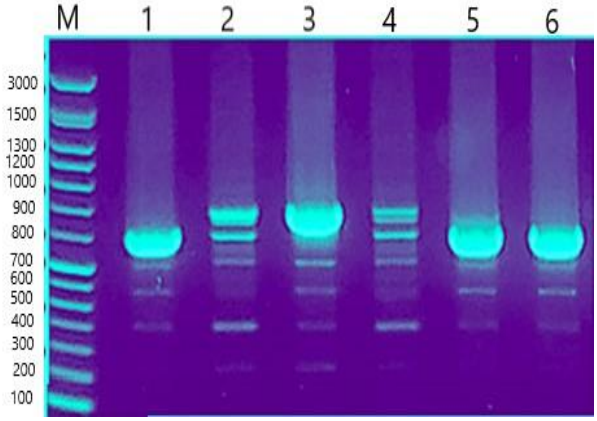
3- T.elliptica 4- T.monspliaca

5- T.stellata 6- T.uncinata

2. OPC-05 primer

This primer produced 5 sites of bands distributed between 4 different sites and one site of similar molecular sizes with a molecular size of 700 base pairs, their molecular sizes ranged between 400-900 base pairs, and the total number of bands produced was 20 bands, and the highest number of bands produced was 4 bands in T.coerulescens and. T.monspliaca, while the lowest number of bands produced was 3 bands in all remaining species, as shown in Figure 3.

Figure 5: Results of amplification of DNA extracted from Trigonella species using the OPC-05 primer after electrophoresis on an agarose gel. The (m) represents molecular size (3000bp)



1-T.astroites 2-T.coerulescens

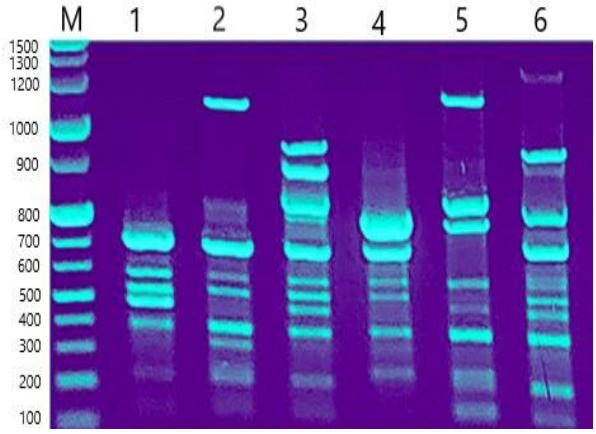
3-T.elliptica 4- T.monspliaca

5- T.stellata 6- T.uncinata

3. OPE-20 primer

This primer recorded 14 sites for the produced bands, including 11 different sites and 3 sites with similar molecular sizes for all studied species. Its molecular sizes ranged between 100-1200 base pairs, and the total number of bands was 54 bands. The highest number of bands was 12 bands in T. uncinata, and the lowest was Number of bands 5 bands in T.astroites as shown in Figure 4.

Figure 4: Results of amplification of DNA extracted from Trigonella species using the OPE-20 primer after electrophoresis on an agarose gel. The (m) represents molecular size (1500bp)



1-T.astroites 2-T.coerulescens

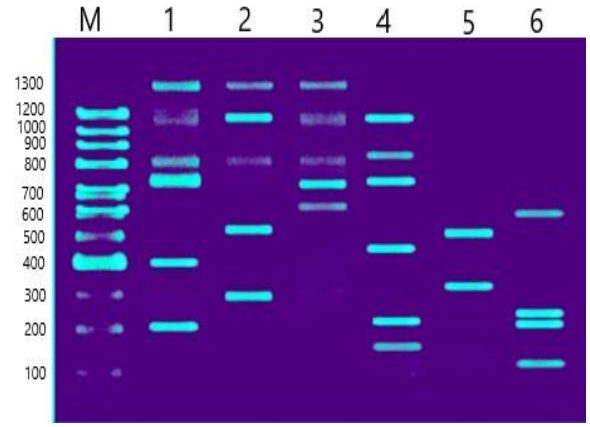
3-T.elliptica 4- T.monspliaca

5- T.stellata 6- T.uncinata

4. OPF-16 primer

This primer gave 13 sites of varying molecular size, their molecular sizes ranged between 100-1300 base pairs, and the total number of produced bands reached 28 bands. The highest number of bands produced was 6 bands in T. astroites and T. monspliaca, while the lowest number of bands produced was 2 bands in T. stellata, as shown in Figure 5.

Figure 5: Results of amplification of DNA extracted from Trigonella species using the OPF-16 primer after electrophoresis on an agarose gel. (M) is the molecular size index (1300bp).



1- T.astroites 2- T.coerulescens

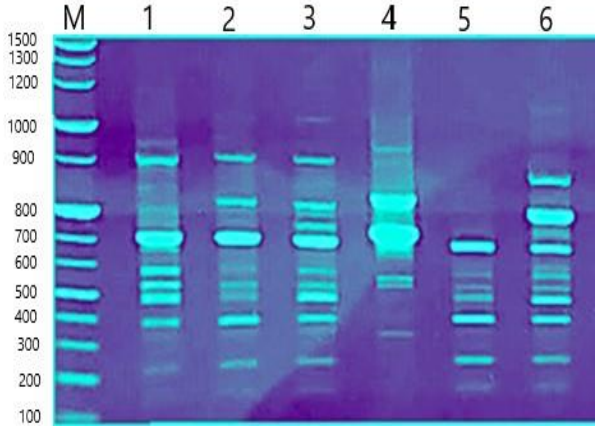
3- T.elliptica 4- T.monspliaca

5- T.stellata 6- T.uncinata

5. OPG-13 primer

This primer produced 13 sites of bands distributed between 10 different sites and 3 sites of similar molecular sizes for all studied species, and their molecular sizes ranged between 200-1000 base pairs, the total number of bands was 50 bands, the highest number of bands was 11 bands in T. elliptica, while the lowest was recorded. The number of bands in T.monspliaca was 6, as shown in Figure 6.

Figure 6: Test results of DNA extracted from Trigonella species using the OPG-13 primer after electrophoresis on an agarose gel. (M) is the molecular size index(1500bp).



1- T.astroites 2- T.coerulescens

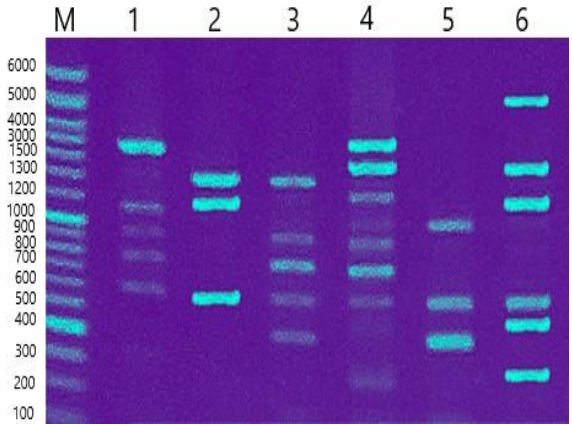
3- T.elliptica 4- T.monspliaca

5- T.stellata 6- T.uncinata

6. OPH-01 primer

This primer showed 13 sites for the produced bands, among them one site for monomorphic bands with a molecular size of 600 base pairs, while the rest of the sites were polymorphic and their molecular sizes ranged between 200-5000 base pairs. The total number of bands produced was 30 bands, and the highest number of bands produced was 8 bands in the species T.monspliaca, while the lowest number of bands produced was 2 bands in T.coerulescens, as shown in Figure 7.

Figure 7: Test results of DNA extracted from Trigonella species using the OPH-01 primer after electrophoresis on an agarose gel. (M) is the molecular size index (6000 bp).



1- T.astroites 2- T.coerulescens

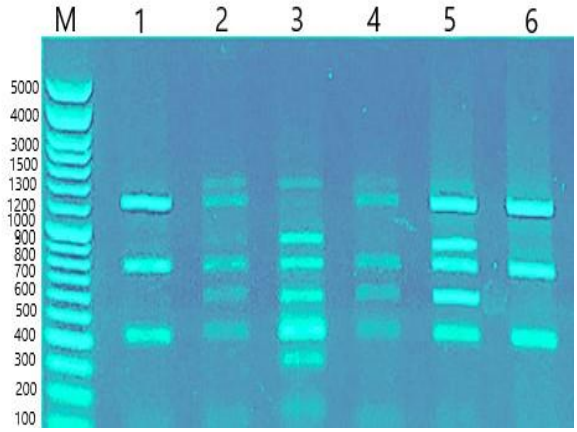
3- T.elliptica 4- T.monspliaca

5- T.stellata 6- T.uncinata

7. OPK-01 primer

This primer produced 9 sites of bands distributed among 7 different sites and 2 sites of similar molecular sizes for all studied species, their molecular sizes ranged between 100-1300 base pairs and the total number of bands was 29 bands, and the highest number of bands was recorded as 7 bands in T. elliptica, while it was The lowest number of bands is 3 bands in the two species T.astroites and T.uncinata, as shown in Figure 8.

Figure 8: Test results of DNA extracted from Trigonella species using the OPK-01 primer after electrophoresis on an agarose gel. (M) is the molecular size index (5000).



1- T.astroites 2- T.coerulescens

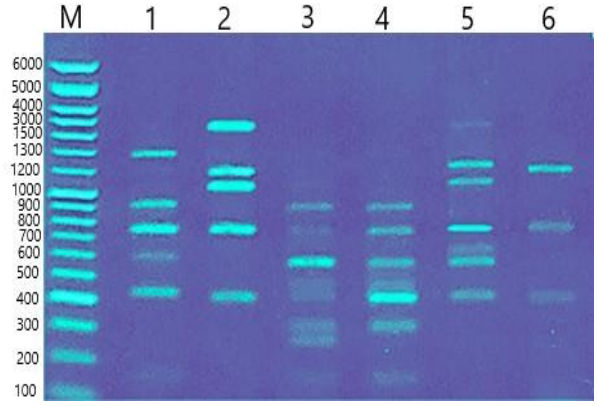
3- T.elliptica 4- T.monspliaca

5- T.stellata 6- T.uncinata

8. OPM-01 primer

This primer recorded 12 sites for the produced bands, among which 10 sites were polymorphic and 2 sites of similar molecular sizes for all the studied species, and their molecular sizes ranged between 100-3000 base pairs. The total number of bands was 32, and the highest number of bands was 7 in T. stellata, while the lowest number of bands was 3 in T.uncinata, as shown in Figure 9.

Figure 9: Test results of DNA extracted from Trigonella species using the OPM-01 primer after electrophoresis on an agarose gel. (M) is the molecular size index (6000 bp).



1- T.astroites 2- T.coerulescens

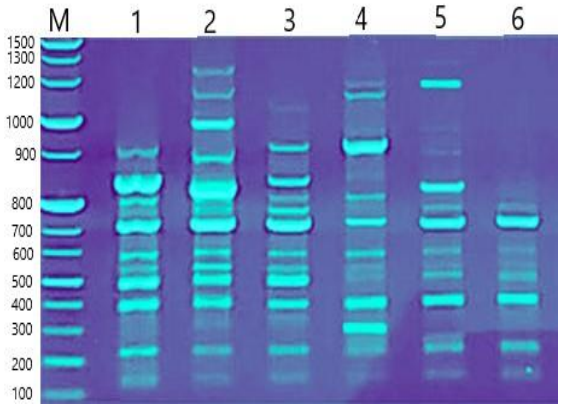
3- T.elliptica 4- T.monspliaca

5- T.stellata 6- T.uncinata

9- OPQ-01 primer

This primer showed 16 sites of produced bands distributed between 8 different sites and 8 sites of similar molecular size in all studied species, their molecular sizes ranged between 100-1300 base pairs and the total number of produced bands was 63 bands, and the highest number of produced bands was 13 bundles in type T .coerulescens, while the lowest number of bundles produced was 8 bands in T.uncinata, as shown in Figure 10.

Figure 10: Test results of DNA extracted from Trigonella species using the OPQ-01 primer after electrophoresis on an agarose gel. (M) is the molecular size index (1500bp).



1- T.astroites 2- T.coerulescens

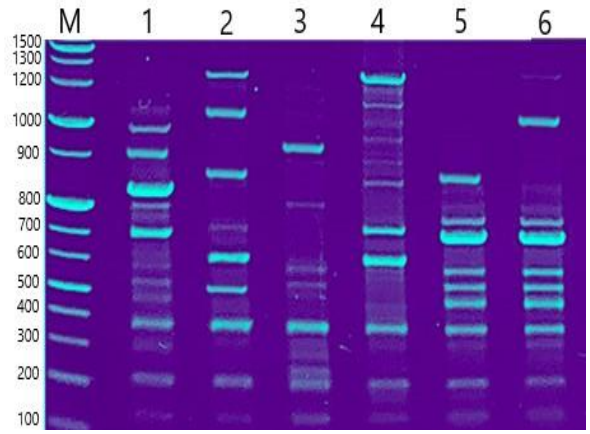
3- T.elliptica 4- T.monspliaca

5- T.stellata 6- T.uncinata

10. OPT-19 primer

This primer gave 17 sites for bands distributed among 15 different sites and 2 sites of similar molecular sizes for all studied species, and their molecular sizes ranged between 200-1250 base pairs, and the total number of bands was 63 bands, and the highest number of bundles was 11 bands in T. astroites, while The lowest number of bands was 7 in T. elliptica, as shown in Figure 11.

Figure 12: Test results of DNA extracted from Trigonella species using the OPT-19 primer after electrophoresis on an agarose gel. (M) is the molecular size index (1500bp).



1- T.astroites 2- T.coerulescens

3- T.elliptica 4- T.monspliaca

5- T.stellata 6- T.uncinata

The current study included the use of 10 primers, all of which proved their ability to give polymorphic bands among the studied species. The total number of bands, depending on all the primers used, was 381, and the polymorphic bands for all the primers were 243, and the current study of the studied species recorded the emergence of 138 monomorphic bands distributed over 9 primers random and with different particle sizes , While the number of unique bands reached 32 bands distributed among the studied species and with different molecular sizes, the primers' characteristics (polymorphism, efficiency, and discriminatory ability for all primers) were calculated, as the primer OPF-16 recorded the highest polymorphism rate of 100%, while the primer OPQ-01 showed the lowest. Its percentage of polymorphism reached 23.809%, The current study showed a clear variation in the efficiency of the primers used that were tested in this study, as the highest efficiency rate was recorded for the primers OPQ-01 amounted to16.535 % and the lowest efficiency rate for the primer OPC-05 amounted to 5.249%, while the highest discriminatory ability was recorded by the primer OPT -19, which amounted to 16.460%, while the primer OPC-05 recorded the lowest discriminatory ability of 5.761%, as in Table 3.

Table 3: Total and differentiated beams with their efficiency ratios and discriminating ability for the studied species for the RAPD marker

The discriminating ability of the primer%	primer efficiency %	Polymorphism of the primer %	The number of polymorphic bands	The total number of bands	primer symbol
6.995	6.036	73.913	17	23	OPA-01
5.761	5.249	70	14	20	OPC-05
14.814	14.173	66.666	36	54	OPE-20
11.522	7.349	100	28	28	OPF-16
13.168	13.123	64	32	50	OPG-13
9.876	7.874	80	24	30	OPH-01
6.995	7.611	58.620	17	29	OPK-01

8.230	8.398	62.5	20	32	OPM-01
6.172	16.535	23.809	15	63	OPQ-01
16.460	13.648	76.923	40	52	OPT-19
			243	381	The total

It was found through the current study using RAPD-PCR indicators to separate the six species belonging to the genus Trigonella and using 10 random primers. The study showed variation between species depending on the DNA genetic material and the appearance and absence of packages. And the variation in the genetic aspect and the extent of the genetic distance between the species under study, as in Table 4, and showing the dendritic diagram as in Figure 13, where the genetic dimensions were shown between the species under study, and the species were divided into two groups:

1. First Group A:

This group included three species divided into two secondary groups A1 and A2, and group A1 contained the two types T.astroites and T.elliptica, while A2 included one species, T.monspliaca, and the shortest genetic distance in this group was 0.51856 between the two types T.astroites and T. elliptica.

2. The second group B:

This group also included three species and was divided into two secondary groups, B1 and B2. Group B1 contained the type T.coerulescens, while group B2 contained the two types T.stellata and T.uncinata. The shortest genetic distance was recorded in this group between the two types T.stellata and T.uncinata, as it was 0.54232.

It is inferred from the cluster diagram that the two closest species to each other are T.astroites and T.elliptica, followed by the two species T.stellata and T.uncinata. As for the type T.coerulescens, it was isolated from the two species T.stellata and T.uncinata with genetic characteristics, while the species was isolated from T.monspliaca rest of the studied species, and this discrepancy is due to the occurrence of adaptation to living in the environments in addition to the occurrence of genetic mutations and modifications between species to suit the environment in which these species live.

Table 4: The genetic distance between the studied species of the genus Trigonella.

Types	1	2	3	4	5	6
1	0					
2	557600.	0				
3	518560.	0.57247	0			
4	557600.	0.57977	0.57247	0		
5	601110.	0.55001	0.64168	0.68599	0	
6	579770.	0.55760	0.63510	0.66736	0.54232	0

1- T. astroites

2- T.coerulescens

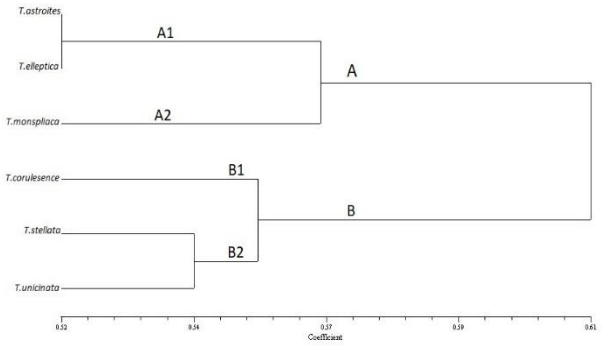
3- T.elliptica

4- T.monspliaca

5- T.stellata

6- T.uncinata

Figure 13: Cluster chart of RAPD index in species of the genus Trigonella



Discussion:

Through the current study, it was found that the highest polymorphism of the RAPD marker was recorded by the primer OPF-16, reaching 100%, while the lowest percentage of polymorphism was shown by the primer OPQ-01, amounting to 23.809%. This came in contrast to what was shown by the study of Dangi et al (2004) on the plant T. foenum-gracium, as he studied 22 primers for the RAPD marker on cultivated varieties of this type, and it was found that the polymorphism amounted to 70.12%, noting that the number of primers used in our current study is 10 primers From the genus Trigonella, as for the study of Al-Yasiri et al (2021) on the RAPD index in the genus Trigonella, it showed the presence of 62 bands of DNA using 5 primers, and the number of polymorphic bands was 26 bands, and the polymorphism rate reached 94.21%, and this came in contrast to our current study, as the polymorphism The formality is higher, using 10 primers, which gave 100% to the OPF-16 primer, and the total number of bands was 381, and the total number of polymorphic bands was 243 bands.

As for the study of Hora et al (2016) on the RAPD and ISSR markers, they used 400 primers for the RAPD marker for eight cultivars of T. foenum-gracium, showed a polymorphism of 42.91% for the RAPD marker, and this came in contrast to our current study, as the polymorphism was 100% for the RAPD marker, Their study gave the highest genetic similarity among the cultivars, and it was 0.85, while the genetic similarity in our current study of the RAPD marker was between 0.51-0.68.

Our current study of the RAPD marker showed that the shortest genetic distance was between the two species T.astroites and T.elliptica is 0.51856, and this is due to the similarity in phenotypic, anatomical and chemical characteristics between the two species that were expressed in the genetic aspect, while the farthest genetic distance was between the two species T.monspliaca and T. stellata is 0.68599, and the reason for this may be due to the variation in morphological, anatomical and chemical characteristics between the two species.

Reference

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