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Molecular Characterization for some species of *Galium* L. by Random Amplified Polymorphic DNA (RAPD) Markers Polymorphism

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Abstract

The five distinct species of Galium L. (Rubiaceae) were identified in this work utilizing the PCR methodology. In Iraq, species genotyping with the RAPD-PCR approach will be mainly focused on. Five primers (OPB18, OPC2, OPB11, OPC14, and OPC8) in Galium species yielded a total of 50 polymorphic amplified products from 120 bands, and the Jaccard's coefficient varied from 0.1081 to 0.3714. The dendrogram divided the investigated species according to the bivariate (1-0) data and genetic similarities utilizing the UPGMA cluster algorithm. Thus, it is possible to conclude that the RAPD methodology is useful for analyzing molecular Characterization and determining the links between different plant populations. Molecular genetic marker-based biodiversity analyses are valuable tools for reintroducing and conserving rare and endangered species. These species were classified utilizing phenotypic characteristics, and their accurate taxonomic names have been identified by building a phylogeny tree utilizing RAPD-PCR to assess for kinship. Members of Galium have tight relationships, as seen by the UPGMA-based dendrogram, which is a polymorphic association that suggests that every species has a distinct evolutionary tendency and identity.

Keywords: Molecular markers, RAPD-PCR, *Galium*, Polymorphism

Key findings: Utilizing RAPD molecular markers, the study revealed a highly polymorphic relationship among the family (Rubiaceae) species with a particular identity and unique evolutionary trend.

INTRODUCTION

With over 42 tribes, 11,500 species, and 660 genera, the family Rubiaceae is the fourth-largest family of angiosperms (Soza & Olmstead, 2010a). Among the Rubiaceae, Galium is one of the larger genera (Willis, 1985; Mabberley, 1987). One of the most significant developments in evolution over the last ten years is molecular taxonomy, which uses RNA or DNA data to diagnose or infer relationships between living things, therefore aiding in the resolution of most taxonomic issues.

Accurate identification of plant accession is necessary for their protection and sustainable usage. Using species identification methods has evolved due to the development of DNA-based markers (Botstein et al., 1980). Various novel methods for rapid and precise plant species identification have been made available to those concerned about protecting plants' genetic assets by significant advancements in molecular genetics in recent years. Numerous methods have proven effective in addressing common taxonomic and evolutionary queries and examining the amount and distribution of diversity within species gene pools (Karp et al., 1996; Karp et al., 1997). Highly polymorphic nature, codominant inheritance (determining heterozygous and homozygous states of diploid organisms), frequently occurring in the genome, selective neutral behavior (any organism's DNA sequences have been neutral to environmental situations or management practices), easy access (accessibility), quick and simple assay, high reproducibility, and ease of data exchange between labs are all desirable characteristics for ideal DNA markers (Joshi et al., 1999).

While frequency data from markers like random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellites give the means for categorizing persons into nominal genotypic groups and are mainly appropriate for intra-species genotypic difference investigation, sequencing-based molecular methods offer better resolution at the intra-genus and above level (Robinson et al., 1999). Understanding this difference is crucial for population research, mainly when choices on the conservation of plant resources are based on the diversity data. For example, an up-to-date investigation on Napier grass (Pennisetum purpureum) revealed that AFLP is incompatible with morphological and RAPD data; re-registration of all Napier grass accessions depending to DNA barcoding is recommended as a way to address the remaining issues with accessions' identities (Struwig et al., 2009). This review's primary goal is to give readers a basic grasp of the molecular tools produced lately and how they may be used to conserve plant resources.

Since molecular data represent variations at the level of genes and aren't immediately impacted by changes in the environment like those with phenotypic qualities, molecular taxonomists feel that molecular data are far more probable than phenotypic data to reveal the actual ethnic origin of organisms (Judd et al., 1999). Since species from various sections of Galium have similar habits, many species are polymorphic and widely dispersed, and species groupings sometimes lack sufficient geographic and morphological differentiation, Galium presents taxonomic challenges (Schischkin, 2000).

In his 1753 description of this genus, Linnaeus confirmed the presence of 26 species. Depending on the kind of fruit (glabrous and hispid), they have been separated into two categories. Boissier (1881) examined ninety species, dividing them into eleven subsections and three divisions (Cruciata et al.). Ehrendorfer et al. (1976) identified 145 species for the European flora, dividing them into ten divisions. Ehrendorfer and Schonbeck-Temesy (1982) categorized Turkey's flora into ten divisions, with 101 species reported. While Boulos (1995) and Boulos (2000) identified only 10 species of Galium in Egypt, Tackholm (1974) listed twelve. Research by Abdel Khalik et al. (2007; 2008 a, b, c)

Molecular technologies can also obtain vital data on the genetic distances between the different species. Utilizing random primers and genomic DNA to create numerous copies of DNA chains, PCR machines create RAPD, a PCR-based marker (Simner et al., 2015). Primer binding to both the partially and fully matched specimen DNA strands of the templates is permitted under less restrictive circumstances, producing strain-specific heterogeneous DNA products.

A single set of primers and a small quantity of DNA may amp up numerous loci utilizing RAPD, which is quick, easy, and affordable (Sharaf-Eldin et al., 2018). Nevertheless, RAPD findings might not be repeatable in other labs and are limited to identifying salient traits (Ramos et al., 2008). The literature research also showed that there has yet to be any documentation of the phylogenetic (RAPD, molecular markers) analysis of the Galium species up to this point.

Utilizing RAPD technology to analyze each species' DNA, the current study aims to investigate genetic diversity, infer the genetic relationships between species depending on their degree of genetic similarity, and provide a thorough overview of the phylogenetic relationships among the different species of the genus Galium L. as suggested by molecular

phylogenetic studies involving RAPD-PCR methodologies.

METHODS AND MATERIALS

Plant specimens gathering and extraction of DNA

The genus Galium L. comprises five plant species obtained from various herbarium samples located in the Baghdad area of Iraq. According to phenotypic characteristics and the use of relevant scientific references, a Plant Taxonomist at the Biology Department, College for Pure Science, Kerbala University, Iraq, categorized these species. The trial ran from February 2023 until December of the same year. The Geneaid company's process was followed to extract the genomic DNA.

RAPD-PCR assay

Amplification of the RAPD-PCR was performed with the help of five primers (Table 1). So as to carry out the RAPD-PCR amplification procedure, a reaction mixture of 25 µL was utilized. This combination included 5 µL of Taq PCR PreMix, 1 µL of each primer (10 pmol), and 2 µL of genomic DNA (20 ng/ µL). When adjusting the final amounts to 25 µL, sterile distilled water was utilized as the adjustment agent. The RAPD-PCR-based amplification was carried out in the following manner with the assistance of the Multi Gene OptiMax Gradient Thermal Cycler, which was manufactured in Germany. Initial denaturation was performed at 95 degrees Celsius for five minutes. Forty cycles of 95 degrees Celsius for thirty seconds, one minute at the annealing temp of 45 degrees Celsius, and finally, extension was performed at 72 degrees Celsius for one minute. The last extensions were performed at 72 degrees Celsius for five minutes.

Gel electrophoresis and data analysis

Utilizing gel electrophoresis, which required passing PCR product through a gel consisting of 2% agarose in TBE solution, the results of the RAPD were separated, which was done so as to separate the findings. One method for determining the size of the DNA bands on the gel was to use a 100-base pair DNA ladder in conjunction with the staining procedure for ethidium bromide staining. After that, a UV illuminator (of the CBS brand Scientific Electrophoreses USA) was used to examine the agarose gel. The frequency of polymorphisms and the bands' number produced by each primer have been identified

independently (Al-Tameme, 2018).

By the presence or lack of the bands (allele), the amplification bands were assigned scores of '1' and '0', respectively. Calculating were made to identify the RAPD fragment frequencies for every unique species. Estimating the size of amplification bands was accomplished utilizing the Photo Capt Molecular Weight method.

The next equation was used so as to identify the proportion of polymorphism that the primer had (Grundmann et al., 1995):

$$\text{Percentage (\%)Polymorphism per primer} = \frac{\text{Number of variant bundles in the primer}}{\text{total number of primer bands}} \times 100$$

Calculating were made utilizing the next equation so as to identify the proportion of discriminating power possessed by each primer:

$$\text{Discrimination power per primer (\%)} = \frac{\text{Number of differential bands of the primer}}{\text{number of differential bands of all primers}} \times 100$$

Calculating were made utilizing the next equation to identify the percentage of each primer's effectiveness in terms of efficacy:

$$\text{The efficiency of each primer (\%)} = \frac{\text{Total number of primer bands}}{\text{total number of all primer bands}} \times 100$$

RESULTS AND DISCUSSION

Utilizing the Geneaid DNA kit, the results of the DNA extraction process from five different plant species are shown in Figure 1 and Table 2, respectively. The majority of the species that were found to be of interest had a high level of DNA purity, which ranged from 1.8 to 2. According to Ramos et al. (2014), there is a possibility that the variation in the amount of DNA is connected to the chemical composition that makes up each biological species.

In addition, the current findings demonstrated that five primers were found to have successfully matched the fingerprints of the five species of plants. Furthermore, the results demonstrated the polymorphic bands dependent on the genetic variants among these species when genotyping was performed (Table 2). As seen in Figure 3, the bands' number on each plate exhibited a greater degree of genetic diversity. Utilizing seven different primers, the

genotyping findings revealed various band patterns for each investigated species. There were a total of 120 polymorphic bands in the ensemble. The most exciting aspect of the scientific work presented here was that all the bandwidths and locations exhibited the most significant polymorphism. On the other hand, the polymorphic bands had a rate of one hundred percent (Table 4).

Some primers were considered excellent for genotyping all plant species, and they created extremely unique and sharp bands by primers (Figure 2). The productivity of the five essential primers exhibited a varying level of production. On the other hand, other primers displayed weak bands and mixed up with one another simultaneously.

Table 2 demonstrates that the OPC14 primer represented one of the best primers since it displayed the highest bands' number, efficacy, and capacity to discriminate and distinguish between bands. Additionally, the primer in question was differentiated by seventeen heteromorphic bands. These bands were mirrored in the effectiveness of the primer, and its capacity to differentiate between different types of DNA amounted to 26.15%. Following it was the primer OPB18, which displayed 15 heteromorphic bands with an efficacy and discriminatory capacity of 23.17%, which was subsequently followed by the primers OPC2 and OPB11. On the other hand, the primer OPC8 demonstrated the lowest bands' number, which amounted to seven bands, and it had an efficacy and discriminatory capacity of 10.76% (Table 2).

It was determined that the coefficient distance between the five plant species was the basis for the phylogenetic tree of the five species. The creation of the tree for the different plant species revealed, based on RAPD-PCR, that three species were grouped in clade-1, and two species were clustered in clade-2, which was done to identify the affinity connection between the species. The RAPD-PCR approach, which depends on the distance coefficient between species, was shown via the evolutionary tree constructed among the five species. Table 3 and Figure 3

The investigation of DNA changeability was dependent on RAPD markers, which could explain the high degree of variation observed. Genotyping of different species resulted in lower similarity between the studied species. RAPD-PCR with an arbiter primer was generally used to distinguish between related closed biotypes or some strains of the same species. However, the RAPD-PCR genotyping findings vary from other genotyping patterns

found in several previous researches (Anis and Al-Dulaimi, 2020; Al-Tamimi, 2019; Abdul et al., 2014). Every plant species of interest was a reliant on taxonomic unit and had a unique evolutionary line, based on the genotyping pattern (Greenberg and Donoghue, 2011).

CONCLUSIONS

The RAPD technique was found effective in determining the genetic distance between plants from various locations, genera and species of the Rubiaceae family. Necessary conclusions can be drawn by comparing RAPD data with botanical data. Computer processing of RAPD data created a dendrogram that showed the phylogenetic relationship between some cactus populations and the genetic diversity of the family as a whole.

Table 1. RAPD primers were used in genotyping the plants under study.

S.No.	Primer	Sequence 5' to 3'
1	OPB18	**CCACAGCAGT**
2	OPC2	**GTGAGGCGTC**
3	OPB11	**GTAGACCCGT**
4	OPC14	**TGCGTGCTTG**
5	OPC8	**TGGACCGGTG**

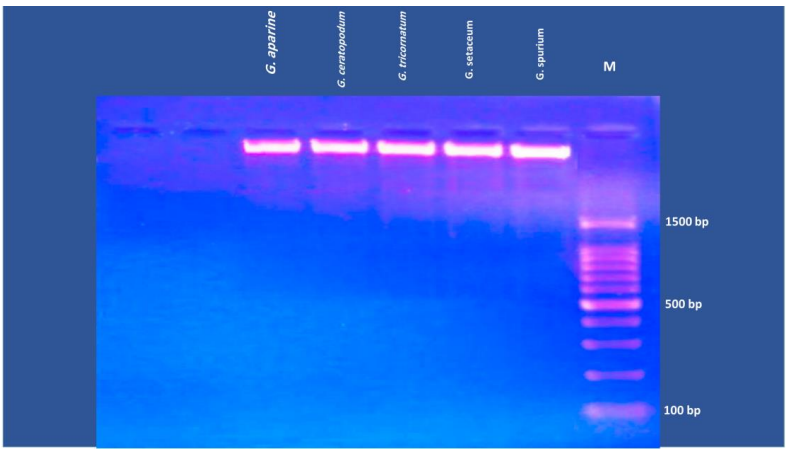

Figure 1. Genomic DNA from five plant species was electrophoresed on a 1% agarose gel at 70 volts for 30 minutes.

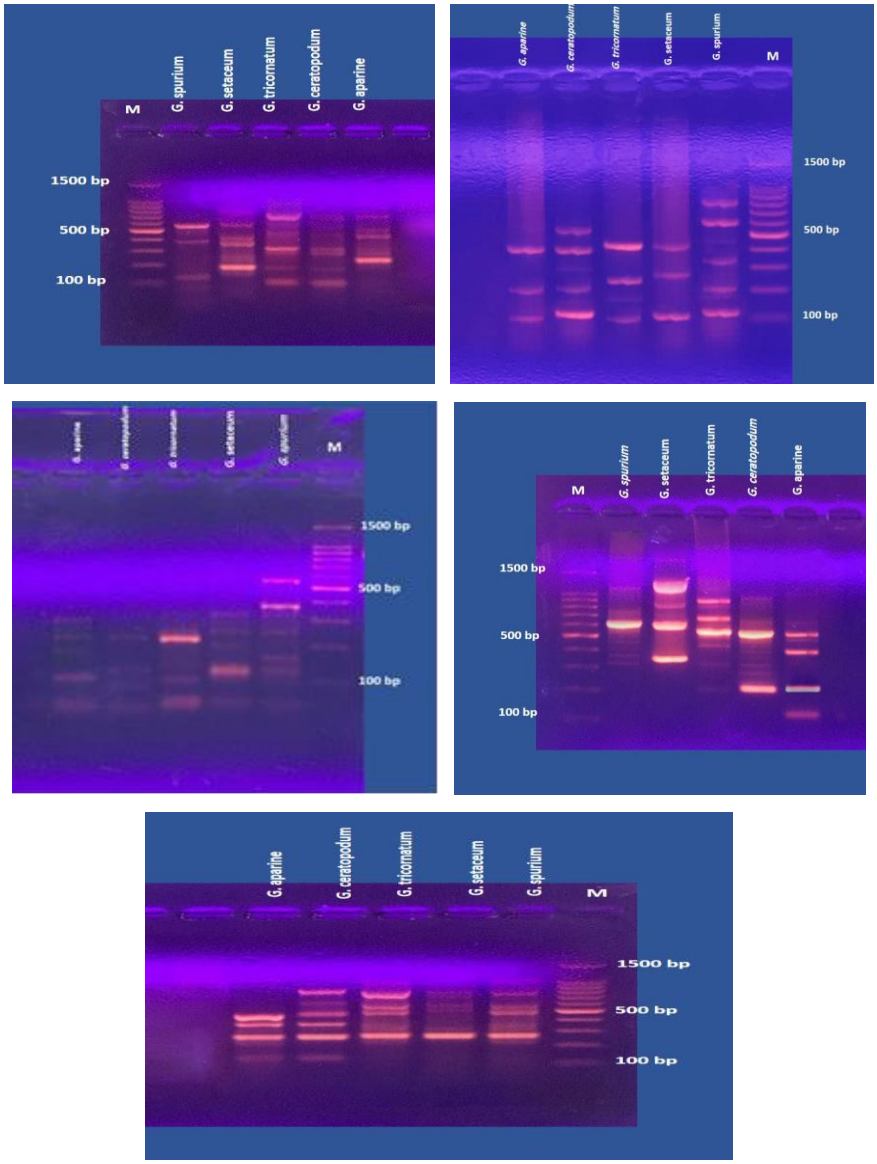

Figure 2. RAPD-PCR product of primers: OPB18, OPC2, OPB11, OPC14 and OPC8, , respectively.

Table 2. *Galium* species genetic polymorphism is based on a technique of RAPD-PCR by five primers, total bands productivity, heterogenetic, primer efficiency %, and discrimination.

Priners	Molecular size in bp	Main bands	Amplified bands	Monomorphic band	Polymorphic band	Polymorphism (%)	Efficiency	Discriminatory Value (%)

OPB18	800-125	15	23	0	15	100	0.652	23.07
OPC2	800-130	13	26	0	13	100	0.5	20
OPB11	580-100	13	23	0	13	100	0.565	20
OPC14	1200-100	17	28	0	17	100	0.607	26.15
OPC8	800-150	8	20	1	7	87.5	0.35	10.76
Total					65			

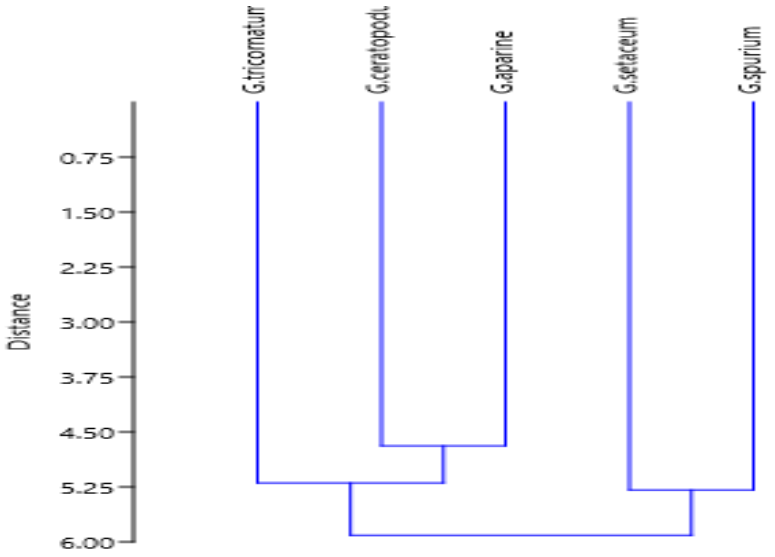

Figure 3. Phylogenetic tree dendrogram (homologous coefficient %) among various Galium species based on the RAPD–PCR primers.

Table 3. The similarity matrix was computed with the Jaccard coefficient among various species.

Similarity and distance indices

	G.spurium	G.setaceum	G.tricornati	G.ceratopo	G.aparine
G.spurium	1	0.24324324	0.18604651	0.15217391	0.125
G.setaceum	0.24324324	1	0.20512821	0.13953488	0.10810811
G.tricornati	0.18604651	0.20512821	1	0.31707317	0.27777778
G.ceratopo	0.15217391	0.13953488	0.31707317	1	0.37142857
G.aparine	0.125	0.10810811	0.27777778	0.37142857	1

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