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RFLP-PCR Fingerprinting ITS region of Fabaceae species in Hillah city in Iraq

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Abstract

The aerial parts (fresh leaves) of thirteen species belong to family of Fabaceae were collected from various fields in Hillah city to investigate polymorphism in the ITS region of nrDNA by using polymerase chain reaction processed with restriction fragment length polymorphism (RFLP-PCR). Based on the data generated, it was possible to identify all of the species that were placed in three groups that are compatible with the three subfamilies within Fabaceae. So, we can be considered that the RFLP-PCR method is very reliable to identify different species belonging to the same family
Key words: *Fabaceae, ITS region, RFLP-PCR, Mimosoideae, Caesalpinoideae, Papilionoideae*

- **Introduction:**

Fabaceae (Leguminosae) is "the third largest family in flowering plant after Orchidaceae and Compositae with approximately 730 genera with 19300 species [1]". Legumes are second only to Poaceae (the grasses) in agricultural and economic importance. The family includes horticultural varieties and many species harvested as crops and for oils, fiber, fuel, timber, medicines, and chemicals. Traditionally, it is divided into three subfamilies—the Mimosoideae, Caesalpinoideae, and Papilionoideae, the latter being the largest and most diverse of the three subfamilies.

Although this family has been studied morphologically and phytochemically in some detail [2], there are many open questions concerning the classification of particular genera within tribes and subfamilies. Molecular techniques are being used increasing in plant systematics [3]. Nuclear ribosomal DNA internal transcribed spacer ITS nrDNA sequences have been widely used in plant molecular phylogenetics of genera, subfamiliar taxa and families, e.g. in Agavaceae [4] and Polemoniaceae [5].

In the past several years, considerable progress has been made in constructing objective phylogenetic hypotheses in the family, many of which are summarized in the most recent volume of the Advances in Legume Systematics series [6] and are reflected in the latest comprehensive taxonomic treatment of the family [7]. Several of these corroborate the earlier intuitive studies. Most of these phylogenetic studies have been confined to genera in a single tribe, or in some cases to a few closely related tribes, and many have utilized molecular characters, such as chloroplast DNA (cpDNA) restriction mapping [8], nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) sequences [9], or genetic variation among and within a different populations in a taxa by using RAPD and FAMEs analysis [10].

The ITS region of rDNA (ribosomal DNA) is "constituted by highly variable regions, which can be used for studies of taxonomical groups with recent diversification or even among the populations. On the other hand, the ribosomal DNA possesses highly conserved regions, which can be applied for organisms comparing with ancient diversification [11]". The same conserved regions can be very useful for designing so called “universal primers” [12]. However, studies so far have been limited by the nature of the markers utilized. [13] explained the use of PCR amplification with specific or universal primers, followed by restriction digestion and electrophoretic separation of the fragments, this approach was introduced recently in plant phylogenetic studies. Restriction fragment length polymorphism (RFLP) markers have the advantages of potentially unlimited numbers and therefore better genome coverage, and high polymorphism. Depending on the technology of restriction enzyme, in several studies the products of the polymerization using restriction enzymes for the purpose of finding similarities and differences between

the genotypes of the target areas of DNA . RFLP is based on the creation or deletion of recognition site of a restriction endonuclease by nucleotide variations in the polymorphic site; consequently digestion of the PCR product containing the polymorphism with an appropriate restriction endonuclease results in disparate electrophoretic patterns by polymorphism genotype.

In Iraq, There is a few studies concern with using molecular techniques to the characterization of family under study , therefore, the aim of this work was to indicate the efficiency of using of RFLP-PCR on nrDNA technique to investigate phylogenetic relationships among thirteen species belonging to Fabaceae family

- **Materials and Methods**

1. **Plant collection.** Thirteen species were collected randomly from various regions of Hillah city listed in Table 1, including one taxa from the subfamily Mimosoideae, four taxa from the subfamily Caesalpinioideae, eight taxa from the subfamily Papilionoideae, these samples were identified according to traditional methods, mainly by using flora of Iraq [14].

Table (1) Species under study with subfamilies in Fabaceae family.

No.	Species	Subfamillies
1	*Albizia julibrissin* Durazz.	Mimosoideae
2	*Alhagi camelorum* Fisch.	Papilionoideae
3	*Bauhinia alba* L..	Caesalpinioideae
4	*Caesalpinia gilliesii* L.	Caesalpinioideae
5	*Cassia didymobotrya* Fres.	Caesalpinioideae
6	*Cassia senna L.*	Caesalpinioideae
7	*Dolichos lablab* L.	Papilionoideae
8	*Glycyrrhiza glabra* L.	Papilionoideae
9	*Medicago sativa* L.	Papilionoideae
10	*Melilotus officinalis* (L.) Pall.	Papilionoideae
11	*Trifolium purpureum* L.	Papilionoideae
12	*Trigonella foenum-graecum* L.	Papilionoideae
13	*Vicia faba* L.	Papilionoideae

2. DNA Extraction. Total genomic DNA was extracted from fresh leaves using a Wizard®Genomic DNA Purification Kit (Promega, USA) was employed for the extraction, following the protocol recommended by the manufacturers.

3. PCR Assay

Two primer pairs namely ITS3, ITS4, ITS5, and P2 were used as previously reported by [12] and [15] (Bioneer, Korea) . These primers were used for PCR amplification and direct double-stranded DNA sequencing. The sequence of these primers is as follows:
ITS3" (5- GCATCGATGAAGAACGCAGC-3)
ITS4" (5- TCCTCCGCTTATTGATATGC-3)
ITS5"(5-GGAAGTAAAAGTCGTAACAAGG-3)
P2"(5- CTCGATGGAACACGGGATTCTGC-3)

4. ITS amplification

The PCR mixture (25 µl) consisted of 5 µl of 20x Master Mix (Promega, USA), 2 µl (10 pmole) of each primer and 1 µl template DNA, made up to 25 µl with molecular-grade water. The PCR mixture was amplified by thermal cycler PCR System (Verity, Applied Biosystem) using the following conditions: first denaturized temperature of 95 for 5 min followed by 35 cycles including initial denaturation temperature of 94 for 1 min; annealing temperature 55°C for 1 min; extensions temperature, 72°C for 2 min, and final extension temperature, 72°C for 10 min. The PCR products were run on 1% agarose gel (Bio Basic Canada Inc.) and electrophoreses were performed at 100 V in TBE buffer. The gel was pre-stained with 0.05% ethidium bromide. The DNA bands were detected by Desktop Gel imager scope 21 ultraviolet transilluminator.

5. RFLP-PCR Assay

ITS-1 and ITS-2 region were digested with two restriction enzymes (*Cfo*I and *Hha*I). The digest reactions for *Cfo*I enzyme were performed in 20 µL, containing 0.75 µL restriction enzyme, 0.3 µL Stock BSA (Bovin Serium Albumin) , 2 µL Multicore buffer and 5.95 µL D.D.W. Another mixture for *Hha*I restriction enzyme were composed of 1µL enzyme, 5-8µL PCR products and 11-14 µL 1×buffer. These mixture incubation at 37Cº in 3 hours for *Cfo*I enzyme and 15 min for *Hha*I enzyme.

- **Results**

The results revealed of deportation efficiency of the extraction method by using Wizard®Genomic DNA Purification Kit with the exception of the two species *Alhagi camelorum* and *Bauhinia alba* which did not demonstrate the efficiency of the extraction through the emergence of a faint band, which

led to disappearance during electrophoresis, in spite of the attempt to be extracted several times to amplified ITS-1 region, In addition the specie *Cassia senna* when amplified ITS-2 region **(Figure 1)**

The results of the amplified DNA extracted from leaves by using a pair primers P2/ITS-5 to amplify the sequence nitrogenous bases to the target area of ITS-1 spacer, which lies between (16S-18S) the small secondary units and 5.8S rRNA in regions of chromosomes Cistronic regions, the results recorded amplification output polymerization length ranges between 350-500 base pairs, and referred the species *Albizia julibrissin* and *Caesalpinia gilliesii* have two genotype reached as the result of polymerization of about 350 and 420 base pairs respectively, while the species *Cassia didymobotrya, Cassia senna, Dolichos lablab, Glycyrrhiza glabra, Medicago sativa* and *Melilotus officinalis* have a same pattern of genetic approximately 500 base pairs, whilst the polymerization which reached approximately 400 base pairs appeared in the species *Trifolium purpureum*, *Trigonella foenum-graecum*, and *Vicia faba* **(Figure 1-A).**

On another hand the polymerization products that have been used where the pair primers ITS-3 / ITS-4, which targeted the amplified ITS-2 region, which lies between the 5.8S and large secondary unit (23S- 28S) in regions of chromosomes, that the length of the output amplifying polymerization of between 400-450 base pair. The first genotype size of 400 base pairs in all species under study expect *Trifolium purpureum* and *Melilotus officinalis* which have the second genotype size is estimated at about 450 base pairs **(Figure 1-B).**

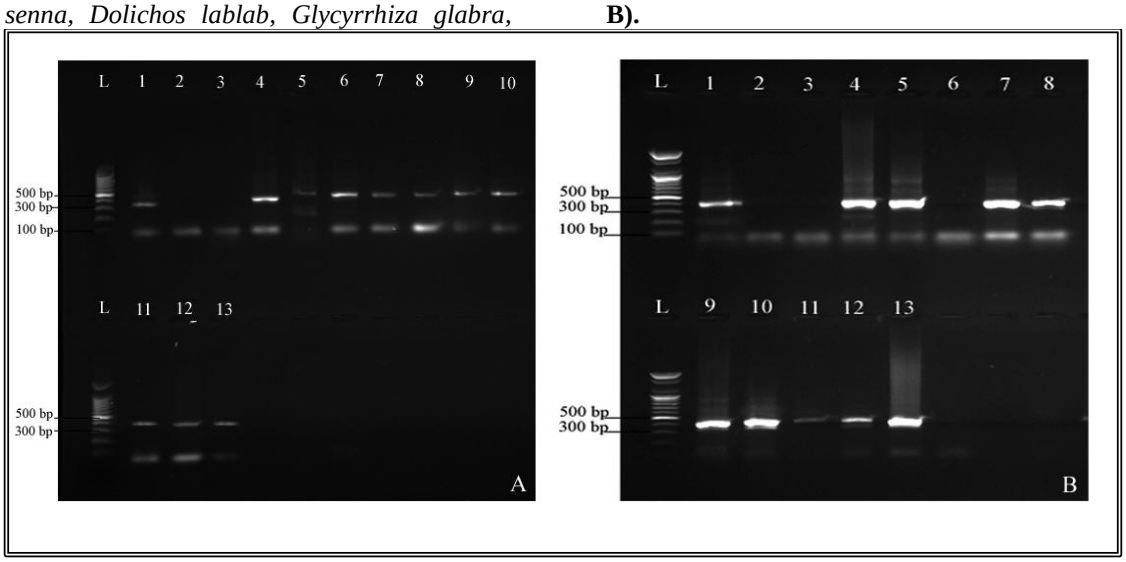

Figure 1. Gel electrophoresis of PCR products from 13 samples of DNA amplified using primers. (A) represented primer ITS5/P2 for ITS-1 region and (B) represented primer ITS3/ITS4 for ITS-2 region. Lane L molecular size marker one step 100 bp ladder (Promega). Lane 1-13 species under study (1- *Albizia julibrissin 2- Alhagi camelorum* 3- *Bauhinia alba* 4- *Caesalpinia gilliesii* 5- *Cassia didymobotrya* 6- *Cassia senna* 7- *Dolichos lablab* 8- *Glycyrrhiza glabra* 9- *Medicago sativa* 10- *Melilotus officinalis* 11- *Trifolium purpureum* 12- *Trigonella foenum-graecum* 13- *Vicia faba*

Digestions with restriction enzymes *Cfo*I and *HhaI* gave different pattern, in spite of these enzyme have the same ability to distinguish sequence (GCG/C), which presented at 16 fragment and 33 fragment from PCR product treated with *Cfo*I restriction enzyme ITS-1 and ITS-2 regions respectively, but in *HhaI* enzyme appear 22 fragment in polymerization ITS-1 region and 25 fragment in polymerization ITS-2 region; for this reason, the result indicated a higher level of genomic polymorphism obtained with two enzymes during digestion PCR product for ITS-2 region.

RFLP-PCR by *Cfo*I restriction enzyme conducted in this study to template DNAs representing 13 species revealed that the primer pairs ITS5/P2 used generated single-band of the appropriate size as a monomorphic pattern in *Albizia julibrissin, Caesalpinia gilliesii, Cassia senna, Glycyrrhiza glabra* **,** *Medicago sativa* , *Trifolium purpureum* and *Trigonella foenum-graecum*, but *Dolichos lablab, Melilotus officinalis* and *Vicia faba* have a dimorphic pattern, while the polymorphic represented in *Cassia didymobotrya*. And the length of fragment that approximately 500 bp which found in

numerous species such as *Caesalpinia gilliesii*, *Dolichos lablab* , *Glycyrrhiza glabra* , *Medicago sativa*, *Melilotus officinalis* and *Vicia faba*. Whilst the primer pairs ITS3/ITS4 revealed all the species have a polymorphic pattern expect *Albizia julibrissin* and *Dolichos lablab* have a dimorphic pattern and *Trifolium purpureum* has a monomorphic pattern of genotype. In addition our result appeared the large number of species have the segment genetic length approximately 300 bp as shown in **(Figure 2)**

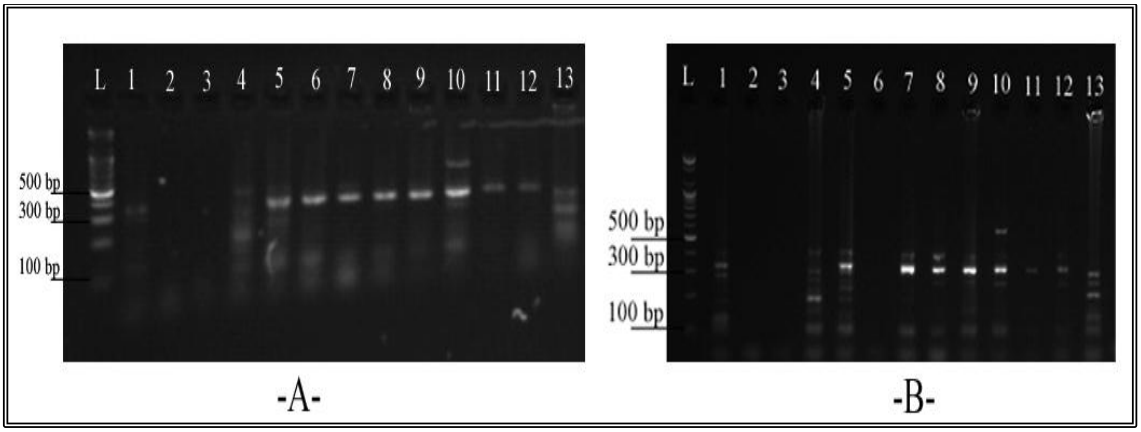

Figure 2. Restriction digestion patterns obtained after electrophoresis on a 2% agarose gel of 13 samples of DNA amplified using primers and digested with *Cfo*I. (A) represented primer ITS5/P2 for ITS-1 region and (B) represented primer ITS3/ITS4 for ITS-2 region. Lane L molecular size marker one step 100 bp ladder (Promega), Lane 1-13 species under study (1- *Albizia julibrissin 2- Alhagi camelorum* 3- *Bauhinia alba* 4- *Caesalpinia gilliesii* 5- *Cassia didymobotrya* 6- *Cassia senna* 7- *Dolichos lablab* 8- *Glycyrrhiza glabra* 9- *Medicago sativa* 10- *Melilotus officinalis* 11- *Trifolium purpureum* 12- *Trigonella foenum-graecum* 13- *Vicia faba*

Digestions with *HhaI* enzyme appeared in the primer pairs ITS5/P2 as polymorphic in all species under study and length of fragment that approximately 400 bp which found in *Albizia julibrissin, Caesalpinia gilliesii, Cassia didymobotrya , Cassia senna, Dolichos lablab, Glycyrrhiza glabra, Medicago sativa, Melilotus officinalis, Trifolium purpureum, Trigonella foenum-graecum* and *Vicia faba*. On the other hand the primer pairs ITS3/ITS4 indicated the species which highly similarity are *Medicago sativa, Melilotus officinalis, Trifolium purpureum, Trigonella foenum-graecum* and *Vicia faba* because all of them had a fragment length approximately 420 bp. in addition we can classified the species according the number of band pattern into three groups, a monomorphic as in *Glycyrrhiza glabra* , and dimorphic pattern in *Dolichos lablab* , *Medicago sativa, Trifolium purpureum* and *Trigonella foenum-graecum* , whereas a polymorphic which is a the third pattern found on the other species as shown in **(Figure 3)**

Thus, banding patterns which presented the same restriction patterns obtained with two enzymes confirmed the genetic similarity between *Caesalpinia gilliesii* and *Cassia didymobotrya* as one group, and another group included *Dolichos lablab, Glycyrrhiza glabra, Medicago sativa, Melilotus officinalis, Trifolium purpureum, Trigonella foenum-graecum* and *Vicia faba*, while it was possible to isolate the specie *Albizia julibrissin* as a third group.

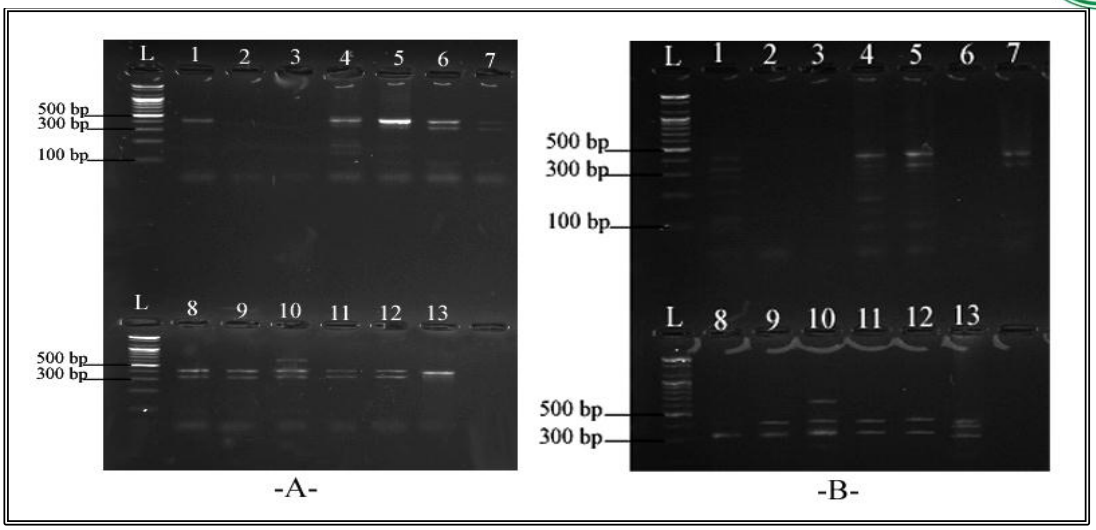

Figure 3. Restriction digestion patterns obtained after electrophoresis on a 2% agarose gel of 13 samples of DNA amplified using primers and digested with *Hha*I. (A) represented primer ITS5/P2 for ITS-1 region and (B) represented primer ITS3/ITS4 for ITS-2 region. Lane L molecular size marker one step 100 bp ladder (Promega). Lane 1-13 species under study (1- *Albizia julibrissin* 2- *Alhagi camelorum* 3- *Bauhinia alba* 4- *Caesalpinia gilliesii* 5- *Cassia didymobotrya* 6- *Cassia senna* 7- *Dolichos lablab* 8- *Glycyrrhiza glabra* 9- *Medicago sativa* 10- *Melilotus officinalis* 11- *Trifolium purpureum* 12- *Trigonella foenum-graecum* 13- *Vicia faba*

- **Discussion**

Although it is generally agreed that the Fabaceae family consists of three independent groups and understandings of our phylogeny within the family has changed with the passage of time. Phylogenetic studies consistently show that the sub-families Mimosoideae and Papilionoideae are monophyletic, but the sub-family Caesalpinioideae is paraphyletic [16]and [17] . Substantial changes at tribal level and generic level in the three sub-families have occurred.

The results of the study have agreed with [18] when he pointed the ITS regions vary greatly in size and sequence. In addition the small size of ITS region (< 700 bp in angiosperms) and the presence of highly conserved sequences flanking each of two spacers this region easy to amplify, even from herbarium material using universal eukaryotic primers designed by [12].

Unfortunately there is no previous data base on these species in Iraq and in Neighbouring countries such as Iran and Saudi Arabia for comparison, but the early studied emphasis the ITS regions vary greatly in size and sequence like [9] when they studied four genera and 31 species in Fabaceae and pointed the length of ITS-1 nearly the same size approximately 221-231bp and 207-217bp in ITS-2, while [19] nearly 235bp in ITS-1 and 208bp in ITS-2. Also 205bp in ITS-1, 220bp in ITS-2 as in [20].

Also, Genomic differences estimated by the number of polymorphic fragments out of the total detected fragments in RFLP. The difference may be due to the frequency in noncoding regions, which may have a higher level of genetic polymorphism [21]., However, the RFLP methodology is limited by several factors. It requires a relatively large amount of good quality because The mechanisms of RFLP are based on the variation of nucleotides at restriction sites of homologous sequences between two genomes, and it is not amazing to detect a similar estimation of genomic diversity using this method.

Our results emphasis close relationship between *Caesalpinia gilliesii* and *Cassia didymobotrya* which it is compatible with the results of the morphological study by a return to the same subfamily Caesalpinioideae, whereas the species *Dolichos lablab*, *Glycyrrhiza glabra*, *Medicago sativa*, *Melilotus officinalis*, *Trifolium purpureum*, *Trigonella foenum-graecum* and *Vicia faba* which have same genetic pattern belong to subfamily Papilionoideae ,while it was possible to isolate the specie *Albizia julibrissin* according to similarity between morphological and genetically characteristics because it belong to subfamily Mimosoideae, This result

was further supported by the evidence from pollen morphology [2]and [22].

On the other hand, A mount of variation within the individual, however, was not indicative of the low number of potential problems that may cause phylogenetically misleading signal when using ITS amplification or Sexual reproduction is a factor that reduces the genetic variation of a population since some species have predominant out-crossing systems. Such systems should help protect the variation of populations. Differences were observed between these populations is much more than those of self-crossing species [23] Thus, The reason for the compatibility and incongruity between morphological and molecular evolution needs to be further studied. To solve this problem, more additional genes including nuclear genes are needed.

- **Conclusion**

We have evaluated RFLP-PCR as power full marker for assessing genetic variation and determining the relationships among species of Fabaceae family. And polymorphisms obtained with RFLP marker have different underlying causes at the molecular level and thus may differ for their informativeness in the analysis of genetic relationships.

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