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# Phylogenetic relationships of genus *Lotus* L. (fabaceae) in Iraq

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## ABSTRACT

The research includes a wide general molecular analysis of 9 species all belonging to the genus *Lotus* L. growing freely in Iraq. The banding pattern has a lot of meaning when it comes to recognizing species relationships. There was also an attempt to use DNA markers in the Polymerase Chain Reaction (PCR) technique to identify the nine *Lotus* taxa and find genetic variability among them. A commercial kit was used to extract total genomic DNA from dried and fresh leaves of the species investigated. The Random Amplified Polymorphic DNA (RAPD-PCR) approach was used to do the molecular analysis, which included ten random markers.

**Key words**: *Lotus* L. (fabaceae), Genetic Variation, RAPD.
**Article type:** Research Article.

## INTRODUCTION

The Papilionaceae family contains 35 wild genera and over 300 species in Iraq, as well as a number of cultivated species (Al-Mosawi 1987), but the Iraqi Flora lists approximately 46 genera (Townsend 1974). *Lotus aegeus*, *L. corniculatus, L. gebelia*, *L. gebelia* var. *hirsutissimus*, *L. halophiluss, L. lanuginosus, L. corniculatus* var. *tenuifolius*, *L. corniculatus* var. *rosacous*, and *L. gracini* grow in the Lowland of Iraq, according to Richenger (1964). The DNA marker protocols based on PCR usage have been commonly used in plant genomic analysis, the genetic standard, through which nucleic acids can be analysed, the apparent criterion in determining a particular trait or selection of good plants or mixing strains between species (Hailu & Asfere 2020). Mendel has been used in many studies, including genetic diversity, which is characterized by inaccurate results in the selection and genetic matching of plant species. Changes in morphology are known to be notably due to environmental conditions (Ovesna *et al.* 2002). Molecular markers can be defined as small segments that can track a gene or genes useful for the genetic structures examined, and that enables us to use small fragments of DNA as an indication or a marker where can easier-to-build traits adopted in determining many morphological traits and proteins. However, DNA markers were highly efficient in identifying a lot of genetic traits, since DNA was easily extracted from plant species (Alkubaisi 2019). For these reasons, the researchers interested in taxonomy have turned to these modern methods by which can identify the genetic similarity and distance of plant species through the use of biotechnologies that have allowed taxonomists to realize and distinguish between species. Molecular markers are almost one of the most important techniques through which DNA genetic variations can be detected (Aljubouri 2015). These techniques use DNA, which has allowed scientists through this modern technology to solve many of the problems and difficulties they encountered in previous methods that were heavily dependent on morphological markers. These markers have many important features, since DNA is stable, not affected by environmental conditions and is at every stage in all parts of the plant (Aldaraji 2014). Another point that characterizes DNA where it is abundant allows the genetic content of all chromosomes to be covered, as well as the original representation of the structure of the gene at the DNA level with the potential for long-term conservation of DNA (Alkubaisi 2019). The use of this technique can significantly reduce the problems of inserting many desirable traits into a single genetic structure, and can also be significantly exploited in solving

diversity problems and calculating or estimating genetic symmetry (Ramsay *et al.* 2000). Despite the high cost of this technique, it greatly helps in many elections, because it shortens a lot of time compared to other traditional techniques, and also allows for the early extraction of DNA from plant development at any stage of its growth period and facilitates the detection of a particular gene site that is responsible for a particular trait (Sayed 2001). Molecular markers provide access to any location of the required genes at the earliest possible genetic distance. This depends on the similarity of molecular markers to the gene, since it gives a high correlation probability, for instance, if the trait is quality and is controlled by a single gene or pair of genes, the connection between the marker and the gene is fulfilled. In another context, if the trait is quantitative and controlled by many genes, then we have to study the multiple molecular markers of these genes in order to allow them to be used in many hybridizations and improvement processes (Poux & Douzery 2014).

## MATERIALS AND METHODS

### DNA extraction using commercial kit

Total genomic DNA was extracted and purified from 100 mg dry leaves by squashing with liquid nitrogen and mechanical destruction, following the ZR Quick-DNA Plant/Seed Miniprep Kit protocol (ZR Technologies, Germany). Then 10 mg $\mu L^{-1}$ RNase treatment at 37 °C for 30 min was used to remove RNA. Electrophoresis was done in 1.5% agarose gel at 100V for at least 60 min in 1X TBE buffer and the isolated DNA was visualized by staining with Red safe and using ultraviolet transilluminator, and then documented by gel documentation system. The suitable concentrations were adjusted to 50 mg $\mu L^{-1}$ and stored at -20 °C to -80 °C.

### RAPD amplification

PCR reactions were carried out using 10 random decamere-nucleotide primers, OP-P04, OP-I02, OP-V02, OP-V19, OP-R06, OP-V09, OP-M05, OP-H01, OP-V14, and OP-D01 (Operon Technologies Inc., USA). Each polymerase chain reaction was carried out in a 25 µL volume containing quantities mentioned in Table 1.
Amplification was performed in thermal cycler (Corbett Research, Australia) using the conditions mentioned in Table 2.

**Table 1.** Mixture of the specific interaction for diagnosis gene.

| Components | Concentration |
|---|---|
| Taq PCR PreMix | 5 µL |
| primer | 10 picomols $\mu L^{-1}$ (2 µL) |
| DNA | 1.5 µL |
| Nuclease-Free Water | 16.5 µL |
| Final volume | 25 µL |

**Table 2.** The optimum condition of detection gene.

| No. | Phase | Tm (°C) | Time | No. of cycle |
|---|---|---|---|---|
| 1- | Initial Denaturation | 94 °C | 5 min. | 1 cycle |
| 2- | Denaturation -2 | 94 °C | 30 sec | |
| 3- | Annealing | 36 °C | 45 sec | 40 cycle |
| 4- | Extension-1 | 72 °C | 45 sec | |
| 5- | Final Extension | 72 °C | 7 min. | 1 cycle |

## RESULTS AND DISCUSSION

### A: Analysis of genetic distance

The genetic distance among 9 *Lotus* taxa was calculated using genetic program (Numerical Taxonomy and Multivariate Analysis System Version 1.80 package) depending on shared bands between taxa when increasing bands number that lead to decreasing genetic distance and vice versa.

### The first group (I)

This group included 8 taxa and included subgroups IA and IB. The first subgroup (IA) also includes second subgroup, $IA_1$ and $IA_2$. The group $IA_1$ contains the taxa *Lotus aegeus, Lotus corniculatus,* while the group $IA_2$

contains the taxa *Lotus gebelia*, *Lotus gebelia* var. *hirsutissimus*, while the group IB also contains second subgroup $IB_1$ and $IB_2$. The group $IB_1$ contains the taxa *Lotus halophilus* and *Lotus corniculatus* var. *rosacous,* while the group $IB_2$ contain the taxa *Lotus corniculatus* var. *tenuifolius* and *Lotus lanuginosus.*

**The second group (II)**

This group included only one taxa, *Lotus gracini*. The dendrogram (Fig. 1) showing the genetic relationships among taxa of *Lotus* as between *L. gebelia* and *L. gebelia* var. *hirsutissimus* and they seem very closely related together. This similarity may be due to their common geographical localities and their flowering period as well as the same morphological, taxonomical, palynological and anatomy characters they have. So that, the stems are erect and cylindrical, the base blade are attenuate, the calyx teeth shape are subulate and seed colour are brownish. In addition, the shape of pollen grain are prolate. On the other hand, the trichomes shape are straight-curved, trichomes apex are acute and base immersion of trichomes are superficiality. Furthermore, the papillae on corolla surface are absence, while the anatomy characters mentioned in the ordinary epidermal cell of stem in anticlinal wall are straight. On the other hand, the *L. aegeus* is similar to *L. corniculatus* in the morphological and anatomical characters like the blade margin and trichomes shape, as well as presence of papillae on corolla. In addition, the type of stomata in abaxial epidermis of leaves are anomocytic, while the shape of stem stomata are widely ellipsoid. The similarity between *L. halophilus* and *L. corniculatus* var. *rosacous* included their indumentum which is densely villose and papillae on corolla. The *L. corniculatus* var. *tenuifolius* and *L. lanuginosus* are similar in morphological characters. So that, fruit shape are curved and absences of papillae on corolla. In addition, in anatomical characters such as stem epidermis are paracytic type. The second group included only one species, *L. gracini*, which was quite distant from the rest of the species, reaching its genetic distance (0.741). This variation is due to adaptation of living conditions as well as mutations and genetic mutations between taxa to suit the environment in which they live especially, since this taxa was recently added to Iraqi flora by (Mousa, 2019). The *L. garcinii* has succeeded in migration to the new environment and this is evident through the collection of two consecutive years 2017 and 2018. According to the results of genetic distance and relationships illustrated, the ability to resolve genetic variation among different *Lotus* taxa may be related to the number of polymorphic bands detected with marker technique employed in this study. All of the 10 RAPD primers were effectively used to amplify the genomic DNA of the 9 *Lotus* taxa by using PCR. These primers have produced multiple band profiles with a number of amplified DNA fragments varying from 8 to 15. Eleven representative agarose gels and amplified bands were scored for each RAPD primer based on as either present (1) or absent (0). The primer OP-P04 exhibited the minimum number of fragments (8), whiles the highest number (15) of fragments were amplified with primers OP-H01. The lowest number of polymorphic bands (88.88%) was 1 with OP-V02, which produced 7 polymorphic bands, followed by OP-H01 which have 93.33%, polymorphic bands. Its produced 9 polymorphic bands and the other primer displayed the highest number of bands and all were polymorphic (100%). From a total of 116 band that were detected, 114 were polymorphic (98.221%; Table 4). The primers (OP-P04, OP-I02, OP-V02, OP-V19, OP-R06, OP-V09, OP-M05, OP-H01, and OP-V14) were used for RAPD-PCR investigations since they offered the best results in terms of amplification and polymorphism for the *Lotus* taxa. the first set of primers produced a total of 105 major bands across the eight species. Two bands of the 105 PCR products produced were monomorphic across the examined species. The remaining 103 bands were polymorphic across the taxa tested, indicating that genotypes of *Lotus* taxa differ significantly.

**Table 3.** Similarity coefficient matrix of pairwise of 9 taxa.

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | | | | | | | | |
| 2 | 0.43478 | 0 | | | | | | | |
| 3 | 0.47917 | 0.63265 | 0 | | | | | | |
| 4 | 0.6 | 0.61039 | 0.7037 | 0 | | | | | |
| 5 | 0.50538 | 0.49474 | 0.47475 | 0.69231 | 0 | | | | |
| 6 | 0.48 | 0.58824 | 0.41509 | 0.71765 | 0.47573 | 0 | | | |
| 7 | 0.54348 | 0.44681 | 0.53061 | 0.71429 | 0.47368 | 0.4902 | 0 | | |
| 8 | 0.52577 | 0.43434 | 0.61165 | 0.65854 | 0.46 | 0.47664 | 0.21212 | 0 | |
| 9 | 0.55556 | 0.6 | 0.42105 | 0.74194 | 0.44144 | 0.47458 | 0.52727 | 0.47826 | 0 |

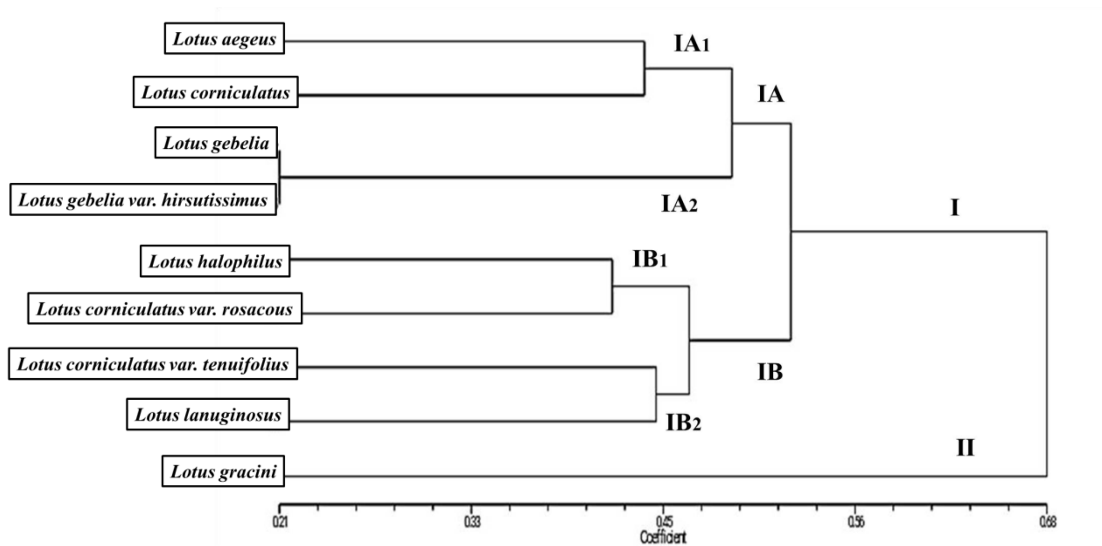


**Fig. 1.** Dendrogram showing the genetic relationships generated from RAPD markers for the following species: 1-*Lotus aegeus* 2- *Lotus corniculatus* 3- *Lotus halophilus* 4- *Lotus gracini* 5-*Lotus corniculatus* var. *tenuifolius* 6-*Lotus corniculatus* var. *rosacous* 7-*Lotus gebelia* 8- *Lotus gebelia* var. *hirsutissimus* 9- *Lotus lanuginosus*.

**Table 4.** The codes and sequences of 101 RAPD primers used for PCR amplification of genomic DNA from 9 *Lotus* genotypes. Total number and size range of amplified bands as well as the number of polymorphic and monomorphic bands were obtained for each primer.

| Primers | Total bands | Polymorphic bands | Rate of the polymorphism of the Primers (%) | Rate of Primers efficiency (%) | Discrimination power |
|---|---|---|---|---|---|
| OP-P04 | 8 | 8 | 100 | 6.89 | 7.01 |
| OP-I02 | 12 | 12 | 100 | 10.34 | 10.52 |
| OP-V02 | 9 | 8 | 88.88 | 7.758 | 7.01 |
| OP-V19 | 13 | 13 | 100 | 11.20 | 11.40 |
| OP-R06 | 11 | 11 | 100 | 9.48 | 9.64 |
| OP-V09 | 10 | 10 | 100 | 8.62 | 8.77 |
| OP-M05 | 14 | 14 | 100 | 12.06 | 12.28 |
| OP-H01 | 15 | 14 | 93.33 | 12.93 | 12.28 |
| OP-V14 | 13 | 13 | 100 | 11.20 | 11.40 |
| OP-D01 | 11 | 11 | 100 | 9.48 | 9.64 |
| Total | 116 | 114 | 98.221 | | |

Note: NT = Number of total bands; NP = Number of Polymorphic bands; Polymorphic = NP/NT × 100.

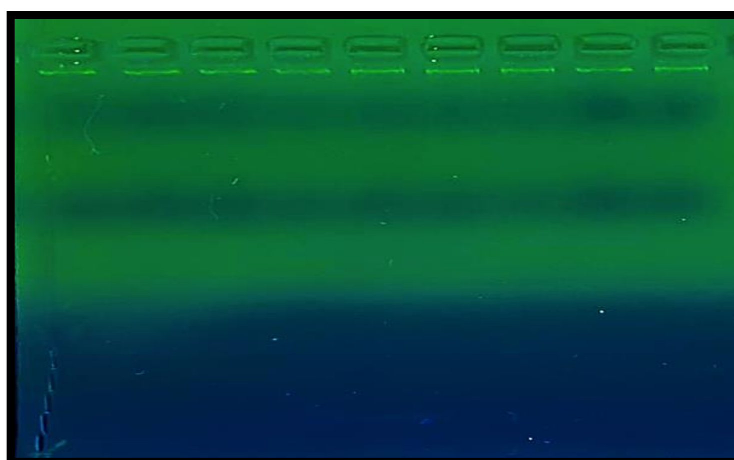

**Fig. 2.** Gel electrophoresis of genomic DNA extraction from 9 *Lotus* species, 1% agarose gel at 5 vol /cm for 1:15 hour.

**Table 5.** The *lotus* taxa and the primers that appeared the unique bands, the number, and molecular weight of these bands.

| No. | Taxon | Primer | Molecular weight(bp) of unique bands | Unique band number |
|---|---|---|---|---|
| 1 | *L. halophiluss* | OP-P04 | 550 | 4 th |
| | | OP-V09 | 400 | 7th |
| 2 | *L. gracini* | OP-I02 | 1000 | 3rd |
| 3 | *L. lanuginosus* | OP-V19 | 1250 | 1st |
| 4 | | | 300 | 10th |
| | *L. corniculatus var. rosacous* | OP-V19 | 275 | 11th |
| | | | 175 | 13th |
| 5 | *L. corniculatus* | OP-V09 | 300 | 9th |

A total of 103 polymorphic bands were found, ranging in size from 7 to 14. The primers OPM05 and OPH01 produced the greatest polymorphic bands (14), while the OPP04 primer produced the fewest polymorphic bands (7). The number of bands created by each primer differed; OPV14 amplified the highest bands (71) while OPD01 the least (25). Variable factors like primer structure, template quantity, and the genome's lack of annealing sites influence the number of bands amplified by different primers (Kernodle *et al.* 1993).

**Table 6.** Distinct characteristic of random primers included in the study: primer's name, total number of bands, number of polymorphic bands and rate (%) of polymorphism in taxa of *Lotus*.

| No. | Primer | Total number of main bands | Number of polymorphic bands |
|---|---|---|---|
| | OP-P04 | 8 | 7 |
| 2 | OP-I02 | 12 | 11 |
| 3 | OP-V02 | 9 | 8 |
| 4 | OP-V19 | 13 | 9 |
| 5 | OP-R06 | 11 | 11 |
| 6 | OP-V09 | 10 | 8 |
| 7 | OP-M05 | 14 | 14 |
| 8 | OP-H01 | 15 | 14 |
| 9 | OP-V14 | 13 | 13 |
| | Total | 105 | 95 |

In this work, polymorphisms were generated among the nine *Lotus* species, and some primers produced unique band that may be utilized as a DNA marker to identify between the local *Lotus* species. In some cases, single base alterations in genomic DNA may be the cause of DNA polymorphism between samples. Furthermore, single nucleotide alterations in a primer sequence were observed to result in a complete change in the pattern of amplified DNA segments.

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