Using of RAPD-PCR markers to detect the genetic relationship of number of *Bellevalia Lapeyr* and *Ornithogalum L.* species developing in central and northern of Iraq

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**Abstract.** This study evaluates eleven wild plant species belong to two genera of Asparagaceae family, naming *Bellevalia lapeyr* and *Ornithogalum L.* and discuss the similarities and differences among all species by using eighteen random primers for the Random amplification polymorphic DNA (RAPD) markers were used to study the genetic relationship and genetic distance among these species. The study aimed to determination genetic relationships among all the eleven species by using RAPD markers and the method of extraction DNA from plants was CTAB with liquid nitrogen. The result show 16 primers success in giving 293 bands, of which 263 were polymorphic bands, 25 unique bands and 5 were absent. *B.chrisii* type also succeeded in showing two unique bands at OPG-08 primer without registering any other bands. The lowest genetic distance was 0.306 between *B.longipes* and *B.parva*, while the highest genetic distance was 0.943 between *B.chrisii* and *O.pyrenaicum*. It is concluded from our study based on the results of the RAPD test that the studied species were divided into two main groups, each group was divided into subgroups, the first group included three groups and the second main group included two main groups. The study found that the *O.pyrenaicum* type is distinguished by being far from the rest of the species.

**Keywords.** Asparagaceae, Genetic relationship, *Bellevalia, Ornithogalum, RAPD.*

1. Introduction

Asparagaceae family of plants belong to monocots plants, and it is a widely spread family in the temperate zones, tropical and subtropical zones. It includes around 114-143 genera and 3632 [1] species, and they were mentioned, for the first time, by the scientist Jussieu [2] and considered part of the lily family. Asparagaceae family is characterized by a perineal herbal nature or seasonal. The species of this family are characterized by their economic importance as they are used for ornamental purposes of as fodder or livestock [3]. According to the scientific researches, the two genera *Bellevalia lapeyr* and *Ornithogalum L.* belong to the Asparagus family [4], but they were considered for a long time that they belong to Liliaceae family. In the Turkish flora, [5, 6, 7] mentioned that *Bellevalia*...
belongs to the Asparagus family. [8] classified it within the same family in the Iranian Flora and [9] mentioned *Bellevalia* in Asparagus family in his study to the Italian Flora. Moreover, the study of [10] categorized both of the genera within the liliaceous family in the Iraqi Flora. The molecular markers are regarded as markers of the stable genetic material, which have specific locations on the genome, through which we can identify the specific genetic differences and the type of mechanisms responsible for a certain characteristic and also the inhibitory genes. Amongst the molecular markers is the DAN Markers, through which we can detect the genetic variations in any piece of the genetic material whether it was DNA or RNA or cytoplasmic DNA or cpDNA (mtDNA), whose sizes range from one pair of nitrogen base to a couple of thousands [11]. Researchers and authors endeavored to use techniques that depend on the plant genetic material to identify the kinship relations amongst the plant species and genetic distance between them [12]. One of these techniques is the Polymerase Chain Reaction (PCR), and one of the markers that depend on the PCR technique is the Random Amplified Polymorphic DNA. The number of the amplified pieces is dependent upon the primer length, the genome size, and its sequence sample, rapid and needs small quantities of DNA (20-25) nanograms [13]. The purpose behind using these techniques, which rely on the genetic material is to solve several problems related to the classification of plants, identifying the genetic print and variation as well as using the results of these techniques in asserting the deduction of new dynasties and the selection of high quality and specifications species that contribute to enhancing the agricultural production [14]. Therefore, The current study aims to identifying the genetic relation among eleven wild species, which belong to *Bellevalia lapeyr* and *Ornithogalum* L.

2. Materials and Methods

2.1. Samples collection and identification

A total Plant samples were collected during one season from March to May 2019 from many areas in the middle and north of Iraq, which included four governorates: Salahaldeen (Sherqat, Haweejah, AlAlam, AlAbbasi, Balad and Amerli), Nineveh (Rabee'a, Zummar and Qayyarah), AlTa’meem governorate (Kirkuk center, Haweejah and Lower Zab, Erbil governorate (Koysanjaq) and the information related to each species were registered. The information about specimens was recorded; and they identified by the second author. Also assured by comparing them with the specimens available at the National herbarium of Iraq.

2.2. Genomic DNA extraction

The DNA was isolated from the fresh leaves of the plants and 2 grams were taken from the leaves, using the (CTAP) in accordance with the method mentioned by [15, 16] and which is used by [17]. The DNA was extracted from the fresh leaves, purified and then its concentration and pureness were measured by the Nanodrop.

2.3. RAPD – PCR reactions [18]

According to the method mentioned by [19], the reactions of RAPD for the DNA samples of the plant species *Bellevalia* and *Ornithogalum* L. The number of primers used were provided by Promega company they were 18 random primers and their sequence is shown in table (1). The reaction solution was prepared.
Table 1. Shows the random primers used in the study of RAPD – PCR.

| No. | Primer code | Nucleotide sequence 5' to 3' |
|-----|-------------|-------------------------------|
| 1   | OPA-01      | CAGGCCCTTC                   |
| 2   | OPA-06      | GTGCCCTGAC                   |
| 3   | OPB-14      | TCCGCTCTGG                   |
| 4   | OPB-20      | GGACCCTTAC                   |
| 5   | OPC-08      | TGGACCGGTTG                  |
| 6   | OPC-16      | CACACTCCAG                   |
| 7   | OPD-03      | GTGGCCGCTCA                  |
| 8   | OPD-18      | GAGAGCCAAC                   |
| 9   | OPE-03      | CCAGATGCAC                   |
| 10  | OPE-11      | GAGTCTCAGG                   |
| 11  | OPF-05      | CGAAATTCCC                   |
| 12  | OPF-20      | GTCTAGAGG                    |
| 13  | OPG-08      | TACGTCAC                     |
| 14  | OPG-14      | GATGAGACC                    |
| 15  | OPH-08      | GAACACCC                     |
| 16  | OPH-16      | TCTAGCTGG                    |
| 17  | OPI-02      | GAGAGAGAG                    |
| 18  | OPI-19      | AATGGGGAG                    |

Tubes with capacity of 0.2 ml contained the reaction mixture were prepared as shown in table (2), put in the ice to complete the required additions and then the mixture was cast away for 2-5 seconds by the Microfuge to make reaction components mix well. After that the tubes were put in the Thermo cycler according to the program shown in table (3).

Table 2. Materials used in the main reaction mixture of PCR.

| No. | Components                     | Final concentration | Final volume of each sample/µL |
|-----|--------------------------------|---------------------|-------------------------------|
| 1   | Premix                         |                     | 2                             |
| 2   | Nuclease free water            | -                   | 15                            |
| 3   | Primer                         | 10 pmol             | 1                             |
| 4   | DNA template                   | 50 ng/µg            | 2                             |

Table 3. The steps of the PCR reaction program.

| Stage          | Temperature | Time   | No. of cycles |
|----------------|-------------|--------|---------------|
| initiation     | 94          | 7 min. | 1             |
| denaturation   | 93          | 45 sec.|               |
| annealing      | 36          | 45 sec.| 40            |
| extension      | 72          | 90 sec.|               |
| Final extension| 72          | 7 min. | 1             |

When time of reaction was ended, the mixture was kept in the fridge. An amount of 5 microliter was withdrawn from each tube to be borne in the Holes of Agarose gel which was prepared previously in a concentration of 1.5% and the marker was put. Samples electrophoresis gel was conducted for 60 minutes in 70 volt, after that the gel was subjected to ultraviolet ray in UV-Transilluminator and photographed with a high resolution camera.

2.4. Estimation of the genetic distance using RAPD markers
In depending on the RAPD reactions results, the genetic distance for the plants species in question was estimated by means of the Genetic statistical package (2.02i NTSYS-PC.Version), which relies on the bands shared between the genetic structures according to the equation mentioned by [20]. A tree diagram that elucidates the genetic relation between the species was drawn by using SAS program.

3. Results and Discussion

Results of the current study revealed that there are two types of primers; the productive primers and the non-productive primers, which are two OPB-14 and OPD-03, that showed no bands on the Agarose gel. All the productive primers, which are 16 random primer, produced Polymorphic loci. The sizes of the molecular bands ranged between (100-1500 bp). Using of many number of primers is regarded as an marker of the increase of the genetic distance accuracy [14], and eventually results in more accurate results which can identify the genetic relation amongst the species as 293 bands appeared, 263 of them are polymorphic band, 25 are Unique bands and 5 are Absent bands. The unique bands appeared in several primers such as OPA-01, OPE-11, OPG-14 and OPA-06 and their appearance varied on the molecular volumes 400, 800, 1000 and 1250 bp. Both the species B.flexuosa and B.parva were distinguished in a number of the productive unique bands when using the primers OPC-08, OPD-18, OPF-20 and OPH-16. The species O.brachystachys recorded three unique bands at the primers which are: OPA-06, OPE-03 and OPI-19, while the species B.saviczi produced two unique bands at the two primers OPA-01 and OPA-06 in two different locations and the same was for B.kurdistanica which recorded two unique bands at the primers OPD-18 and OPF-05 at two different locations and we notice the superiority of the primer OPC-08 in terms of the number of the bands, followed by OPF-20. So, we concluded that all the primers shared the feature of showing unique bands for different species except for the primers OPB-20 and OPC-16 which did not produce unique bands. This emergence demonstrates Genomic DNA sequence of that species per se and it can be considered as a diagnostic feature to discriminate between the species. The second type of the distinguished bands was represented by the absent bands, which emerged at the primers OPA-16, OPE-03, OPF-05, OPG-14 and OPI-02. The appearance of the absent bands is regarded as a type of mutations that took place at the location of the primer identification of one genetic structure that led to abolishing the identification location and eventually that band did not emerge [14]. In table (4), we observed the primers varied in their proficiency values, which result from their capability to show that variance among the genetic structures of plants species in question. The primer OPG-08 was less proficient and with less distinguishing capability (0.6) and (0.7) respectively, while the primer OPI-19 was characterized with the highest proficiency and distinguishing capacity amongst the primers (8.8) and (8.9) respectively in terms of producing 26 different bands. From the other hand, the proficiency and the distinguishing capability of the two primers OPE-11 and OPF-05 were similar. Therefore, it is concluded that more bands are more proficiency the primer has. At the same time, the increase of the primers used in RAPD markers results in increasing the accuracy of identifying the genetic distance and the increasing the possibility of detecting larger area for the genome [21]

Table 4. Represents the results of the random primers used in RAPD.

| No | Primer-name | No. of loci | Polymorphic bands | Total bands | unique bands | absent bands | primer's efficiency | Discrimination power |
|----|-------------|-------------|-------------------|-------------|--------------|--------------|---------------------|---------------------|
| 1  | OPA-01      | 6           | 6                 | 20          | 1            | -            | 6.8                 | 7.6                 |
| 2  | OPA-06      | 4           | 4                 | 13          | 2            | -            | 4.4                 | 4.9                 |
| 3  | OPB-20      | 5           | 5                 | 22          | -            | -            | 7.5                 | 8.3                 |
| 4  | OPC-08      | 9           | 9                 | 22          | 4            | -            | 7.5                 | 8.3                 |
| 5  | OPC-16      | 3           | 3                 | 16          | -            | 1            | 5.4                 | 6.0                 |
| 6  | OPD-18      | 8           | 8                 | 24          | 2            | -            | 8.1                 | 9.1                 |
| 7  | OPE-03      | 6           | 6                 | 25          | 2            | 1            | 8.5                 | 9.5                 |
|    | Primer | M  | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | Total |
|----|--------|----|------|------|------|------|------|------|------|------|------|------|------|-------|
|  8 | OPE-11 | 6  | 6    | 6    | 18   | 1    | -    | -    | -    | -    | -    | -    | -    | -     |
|  9 | OPF-05 | 6  | 6    | 6    | 18   | 2    | 1    | -    | -    | -    | -    | 6.1  | 6.8  | 1.2   |
| 10 | OPF-20 | 6  | 6    | 6    | 17   | 3    | -    | -    | -    | -    | -    | 5.8  | 6.4  | 1.2   |
| 11 | OPG-08 | 2  | 2    | 2    | 2    | 2    | -    | -    | -    | -    | -    | 0.6  | 0.7  | 0.7   |
| 12 | OPG-14 | 5  | 5    | 5    | 23   | 1    | 1    | -    | -    | -    | -    | 7.8  | 8.7  | 1.5   |
| 13 | OPH-08 | 8  | 8    | 8    | 21   | 1    | -    | -    | -    | -    | -    | 7.1  | 7.9  | 1.2   |
| 14 | OPH-16 | 4  | 4    | 4    | 12   | 1    | -    | -    | -    | -    | -    | 4.0  | 4.5  | 0.5   |
| 15 | OPI-02 | 3  | 3    | 3    | 14   | 1    | 1    | -    | -    | -    | -    | 4.7  | 5.3  | 0.4   |
| 16 | OPI-19 | 8  | 8    | 8    | 26   | 2    | -    | -    | -    | -    | -    | 8.8  | 9.8  | 1.6   |
|    | Total  | 89 | 89   | 293  | 25   | 5    |      |      |      |      |      |      |      | 5     |

Figure 1. Reaction results for primer OPB-20 for plants under study in agarose gel electrophoresis with concentration 1.5 and Marker.

Figure 2. Reaction results for primer OPA-06 for plants under study in agarose gel electrophoresis with concentration 1.5 and Marker.

Figure 3. Reaction results for primer OPE-03 for plants under study in agarose gel electrophoresis with concentration 1.5 and Marker.

Figure 4. Reaction results for primer OPC-16 for plants under study in agarose gel electrophoresis with concentration 1.5 and Marker.

Figure 5. Reaction results for primer OPI-02.

Figure 6. Reaction results for primer OPG-14.
for plants under study in agarose gel electrophoresis with concentration 1.5 and Marker

From table (5), we find that the values of the genetic distance ranged between (0.306 - 0.943). the least genetic distance was 0.306 between the species B.longipes and B. parva, and thus they are the most similar among the species because they belong to the same genus and in spite of the difference between them in some morphological characteristics, but the a proximity in the genetic distance value demonstrates the similarity in the genetic material. From the other hand, the highest genetic distance was 0.943 between B.chrisii and O.pyrenaicum they were least similar to those which belong to two different genera; B.chrisii belongs to the genus Bellevalia and O.pyrenaicum belong to the genus Ornithogalum and this lead to why they are genetically distant.

Table 5. The genetic distance amongst the species in this study.

|  | S1  | S2  | S3  | S4  | S5  | S6  | S7  | S8  | S9  | S10 | S11 |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 0.000 | 0.446 | 0.000 | 0.446 | 0.433 | 0.000 |
| 0.720 | 0.315 | 0.433 | 0.378 | 0.000 |
| 0.718 | 0.543 | 0.606 | 0.306 | 0.403 | 0.000 |
| 0.460 | 0.489 | 0.446 | 0.352 | 0.446 | 0.461 | 0.000 |
| 0.642 | 0.734 | 0.692 | 0.520 | 0.533 | 0.461 | 0.460 | 0.000 |
| 0.777 | 0.850 | 0.734 | 0.629 | 0.707 | 0.703 | 0.720 | 0.720 | 0.693 | 0.509 | 0.000 |

S1: B.chrisii, S2: B.flexuosa, S3:B.kudistanica, S4: B.longipes, S5: B.macrobotrys, S6: B.parva S7: B.pycnantha S8:B.saviczii, S9:O.brachystachys, S10:O.neurostegium S11:O.pyrenaicum.

In depending on the values of the genetic distance shown in table (5), the tree diagram was drawn, according to which the plants species were divided into two main groups, as follows:

3.1. The first main group:

It included seven species, included : B.chrisii, B.flexuosa, B.kudistanica, B.longipes, B.macrobotrys, B.parva and B.pycnantha.

3.1.1. The first secondary group:

It involves the species B.chrisii, this species is alone in a genetic group indicated in the dissimilarity of its genetic material with other species and it has a genetic distance of 0.446.

3.1.2. The second secondary group: It contains three species:

The genetic distance of B.flexuosa and B.macrobotrys was 0.315, while it was 0.433 between B.macrobotrys and B.kudistanica and the same genetic distance is between B.flexuosa and B.kurdistanica. This indicates that the three species belong to one common origin and approved by the morphological classification results as they belong to the same genus.
3.1.3. The third secondary group:

It involves *B.longipes*, *B.parva* and *B.pycnantha* with genetic distance of 0.306 between the two species *B.longipes* and *B.parva*; and 0.461 between *B.parva* and *B.pycnantha*. From the other hand, the genetic distance between *B.longipes* and *B.pycnantha* was 0.352 and the genetic convergence of these species is in accordance with their similarity in several morphological features.

3.2. The second main group:

It includes *B.saviczii*, *O.brachystachys*, *O.neurostegium* and *O.pyrenaicum*. They are divided into two secondary groups involve:

3.2.1. The first secondary group:

It included three species: *B.saviczii*, *O.brachystachys* and *O.neurostegium*. The genetic distance between *B.saviczii* and *O.brachystachys* was 0.432 and between the species *O.brachystachys* and *O.neurostegium* was 0.389. The presence of these species in one group indicates that they share the same origin, despite the fact that the species *B.saviczii* belongs to the *Bellevalia* genus and the other two species belong to the *Ornithogalum* genus according to the morphological classification.

3.2.2. The second secondary group:

It included one species, which is *O.pyrenaicum* and its presence in an independent genetic group confirms that its genetic material is different from the rest of the species according to the results of RAPD – PCR, this species showed no band at the primers OPC – 08, OPF – 20 and OPI – 19. From the other hand, the species *O.pyrenaicum* was characterized with four unique bands at the primers OPA – 01, OPD – 18, OPH – 08 and OPI – 02 at the molecular size (300, 1500, 1500 and 1250) respectively, with three absent bands when using the primers OPC – 16, OPF – 05 and OPI – 02.

![Figure 7. The genetic relationship between the plants species according to the Genetic distance values of RAPD markers.](image)

The use of plenty molecular markers indicates its efficiency and accuracy in giving the results besides being inexpensive. Regarding the molecular studies of plants species which they are not available except for the study of [22] for three species belong to the genus *Ornithogalum*, they are: *O.brevipedicellatum*, *O.oligophyllum*, and *O.pamphylicum*. Study used the PCR technology using two
specific genera, trnL and rbcL. The study of [23] was a cellular study of the number, size and shape of the chromosomes of three species that belong to Bellavalia genus, whereas [24] studying the shape and number of chromosomes for the species O.alpigenum, which belong to Ornithogalum genus. The results show the primer OPC-08 was unique to the appearance of 4 unique bands, 3 unique bands recorded by type B.parva, two with molecular size ranging from (1250 to 1500), the third band of the same species with a molecular size (300bp), and the fourth unique bands of the primer OPC-08 recorded by B.flexuosa at molecular size (250bp) thus being the most unique prime in the number of unique bands. Whereas, the primers OPB-20 and OPC-16 did not produce any unique bands. The two species B.parva and B. flexuosa similar to each other in having 4- unique bands, this meaning they are similar in many morphological characters like color of flowers are violet and the long leaves so they are related to each other, while the species O.pyrenaicum have shown no bands in many primers, this mean that the species is genetically divergent from the rest of the species.

4. Conclusion

The Results showed that there was a high genetic variance amongst the plant species, they were characterized with the emergence of different bands and distinguished bands (unique and absent). Also, the results showed the efficiency of using RAPD-PCR markers in defining the genetic relation between these species and the genetic print.

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