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Molecular Phylogeny of Some Iraqi Species of *Alcea* L. Based on ITS Sequences

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Abstract. Phylogenetic analysis was performed using ITS gene sequences. Sequences were obtained from 11 species of *Alcea* (*Alcea apterpcarpa* (Fenzl.)Boiss., *A. arbelensis* Boiss. et Hausskn. , *A. biennis* Winter. , *A. dissecta* (Bakerf.) Zohary , *A. fasciculiflora* Zohary , *A. guesti* Zohary , *A. mosulensis* I.Riedl. , *A. pisidica* Hub.-Mor., *A. rematoflora* (Boiss and Heldr.)Alef. , *A. setosa* (Boiss.)Alef. , and *A. sulphurea* Boiss. et Hoh. of the Malvaceae family. Taxonomy and evolutionary relationships among these taxa were employed to evaluate the genetic diversity of the species utilizing one nuclear internal transcribed spacer (ITS). The quality of each extracted DNA sample was assessed spectrophotometrically using a NanoDrop device and agarose gel electrophoresis to determine the DNA yield and purity range for all sample extracts utilizing the extraction procedures. The 260/280 ratio in this study was in the range of 1.08–1.28. The concentration of the extracted DNA samples , varied from 2.2–11.5 ng/µl. The results showed that elevated bootstrap values substantiated the eight main clades within *Alcea*. The sequences of *A. apterpcarpa* and *A. guestii* exhibited approximately 74% similarity, indicating a close relationship with *A. pisida* and *A. biennis*, which demonstrated 85% similarity. *A*dditionally, the close relationship between *A. faciculiflora* and *A. dissecta* were found to be 56%, whereas the close relationship with *A. setosa* was determined to be 65% .In addition, analysis of this dataset revealed six primary clades: *A. pisida*, two containing *A. biennis*, the third consisting of *A. sulphura*, the fourth represented by *A. remotoflora*, the fifth including *A. areblences*, and the sixth constituted by *A. mosuleusis*. Three principal clades were identified within *the Alcea* species: the *A. areblences* clade, which includes *A. remotoflora*, exhibiting 94% bootstrap support; the *A. mosuleusis* clade, encompassing other species, with 49% bootstrap support; and the *A. mosuleusis* clade, encompassing other species, with 49% bootstrap support.

Keywords. *Alcea* , ITS, PCR Amplification, Phylogenetic analysis.

1. Introduction

Malvaceae Juss. is a large family containing 244 genera and 4225 species , and is a worldwide family composed of herbs, shrubs , and small trees[1,2]. In Iraq, the Malvaceae family is represented by 5 genera and 29 species [3]. *Alcea* L. is taxonomically attributed to the Malveae tribe and the Malvoideae subfamily of Malvaceae. It comprises approximately 75 taxa widely distributed throughout Europe, Central Asia, Southern Russia, North America, North Africa, and Afghanistan [2,4, 5, 6].The name of this genus is derived either from alce (remedy) in reference to its therapeutic

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value or from alke (strength) because of its vigorous growth[7]. *Alcea* is represented in Iraq by5 species [3].

Molecular markers are an effective means of elucidating the evolutionary relationships among species and populations. Because DNA markers are less affected by their surroundings and permit straightforward detection at the DNA level, they have several benefits over morphological features [8-12].

Previous research has elucidated the phylogeny of Malvaceae through cpDNA analysis uing restriction site data [13], revealing adequate variation in ITS to clarify phylogenetic relationships both within and among genera in Malvoideae[14, 15]. Molecular investigations of Malvales and Malvoideae (within Malvaceae) have yielded initial evidence regarding phylogenetic relationships both within the subfamily and the tribe Malveae. The tribe Gossypieae was determined to be sister to Malveae based on rbcL, atpB[16],and ndhF[17]sequence data.All five tribes were included in a recent phylogenetic analysis of tribe Hibisceae, utilizing chloroplast ndhF and rpl16 intron sequences [18]. Molecular evidence from chloroplast and nuclear markers of Lavatera (Malvaceae) was examined in [19].Moreover, numerous studies have indicated that certain genera are not monophyletic as now defined. This group includes Abutilon and Sida[19]. Established based on the analysis of RAPD markers, [20]assessed the genetic profiles of 18 *Alcea* species using RAPD markers and discovered significant variation (0.13 to 0.69) among them. *Alcea* is a sister genus to *Althaea*[21]; nonetheless, the inclusion of *Alcea* in molecular systematic research is typically restricted to widely cultivated *A. rosea*, leaving the monophyly of *Alcea* in relation to *Althaea* unresolved.

Badrkhani*etal.*[22]employed a sequence-related amplified polymorphism (SRAP) marker to assess the genetic diversity and genetic relationships among 14 *Alcea* species collected from northwest Iran. The genetic similarity between *A. sophiae* and *A. flavovirens* was minimal (0.17); however, the highest similarity was observed between *A. digitata* and *A. longipedicellata*(0.68). Examination of DNA sequence data from ITS regions revealed significant genetic heterogeneity (polymorphism) among species [23-27].

Cheng *et al.*[28] conducted a study on molecular identification and genetic relationships among *Alcea* species (Malvaceae) uing ISSR markers. Cheng *et al.*[28] investigated 14 species from 16 randomly selected areas. Of 128 bands generated from 10 primers in the amplification of genomic DNA, 119 polymorphic bands (94.33%) were identified. Yin and Khodayari [29] investigated 34 *Alcea* species in Iran with (Random Amplified Polymorphic DNA (RAPD).

No data exists regarding its population genetic structure, genetic diversity, and morphological variability in Iraq. Consequently, owing to the significance of these plant species, we synthesized molecular data for these species. For this investigation, we used 11 flora specimens.

This study sought to assess the evolutionary relationships and genetic diversity among certain *Alcea* species by using nuclear ITS genetic sequence data from Iraq.

2. Materials and Methods

In the present study, we collected 11 species of Alcea (Alcea apterpcarpa, A. arbelensis, A. biennis, A. dissecta, A. fasciculiflora, A. guestti, A. *mosulensis*, A, pisidica, A. rematoflora, A. setosa and A. sulphurea) from different locations in Iraq. The samples were enclosed in perforated aluminum foil, securely sealed, and placed in polyethylene bags, accompanied by an adequate amount of silica gel to absorb moisture from the leaves.

2.1. Extraction of DNA and Polymerase Chain Reaction Amplification

DNA was extracted from young leaves using the Plant Genomic DNA Mini Kit in accordance with the manufacturer's guidelines with certain modifications. DNA samples 50µl were preserved in a freezer at -20°C. Polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. The ITS region of nuclear ribosomal DNA consisted of ITS 1 and 4 and was amplified using the primers listed in Table 1.

Table 1. ITS gene sequencing.

Primers	Sequences (5`- 3`)
ITS/1	TCCGTAGGTGAACCTGCGG
ITS/4	TCCTCCGCTTATTGATATGC

An aliquot (12µL) from the GoTaq Green Master Mix was placed in a PCR tube. Aliquots: 1.5µL from (10 µM) of upstream primer and 1.5µL of (10 µM) downstream primer were added. Five microliters of DNA template were taken, and 5 µL of nuclease-free water was added. The mixture was vortexed for 30 seconds and then placed in a PCR machine.

2.2. Programmer of PCR

The various phases of cycle sequencing were executed in a Gene Amp PCR System 9700 Thermocycler, utilizing the protocol outlined in Table (2).

Table 2. Stages of ThermocyclerCycle Sequencing of PCR.

Stage	Temperature	Time (min)
Initial denaturation	95	4
No. of cycles = 32 cycle		
Denaturation	95	1
Anneling	57	1
Extention	72	2
Final extension	72	10

3. Results and Discussion

The purity of each extracted DNA sample was confirmed spectrophotometrically using a NanoDrop spectrophotometer and agarose gel electrophoresis. Table (3) highlights the DNA yield and purity ranges obtained for all sample extracts using these extraction methods. The 260/280 ratio in this study was in the range of 1.08–1.28. The concentration of the extracted DNA samples, which varied from 2.2 to 11.5 ng/µl, was measured using a Nanodrop Spectrophotometer.

Table 3. The DNA purity range obtained from *Alcea* sample extracts using NanoDrop spectrophotometer.

No.	Species	DNA purity(260/280)	DNA concentration (ng/µl)
1	*Alceaapterpcarpa*	1.24	2.5
2	*Alceaarbelensis*	1.13	11.5
3	*Alceabiennis*	1.39	2.2
4	*Alceadissecta*	1.29	5.1
5	*Alceafasciculiflora*	1.82	3.1
6	*Alceaguestti*	1.46	2.5
7	*Alceamosulensis*	1.4	5.8
8	*Alceapisidica*	1.08	5.2
9	*Alcearematoflora*	1.54	10.5
10	*Alceasetosa*	1.19	3.8
11	*Alceasulphurea*	1.5	7.1

The purity of the extracted DNA was assessed by 1% agarose gel electrophoresis, as shown in Figure 1. The results showed no fragmentation of the extracted DNA. The isolated DNA was directly utilized for PCR amplification of the ITS gene (900 bp), as illustrated in Figure 1. Phylogenetic analysis of the selected *Alcea* species was conducted using ITS multi-alignments (Figure 1 and 2). PCR analysis revealed a distinct band in 11 samples with a size of 700-750bp. The nucleotide sequences of the discovered species exhibited 98-100% identity with those in the Genbank database.

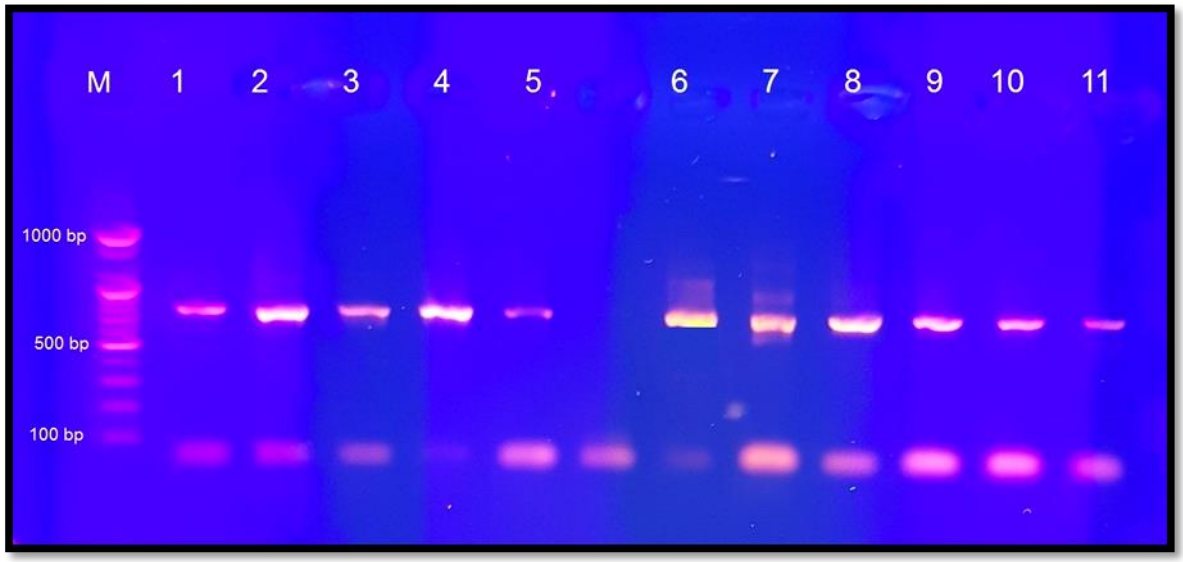


Figure 1. DNA profile of ITS region, M: DNA ladder (100bp) ; 1: Alcea apterpcarpa, 2:A. arbelensis, 3:A. biennis, 4:A. dissecta,5:A. fasciculiflora, 6:A. guestti, 7:A**.** *mosulensis*, 8:A, pisidica,9: A. rematoflora, 10:A. setosa and 11: A. sulphurea

Analysis of the DNA sequence data from the ITS regions indicated a high level of genetic variability (polymorphism) among the samples tested. The results revealed that eight major clades within *Alcea* were supported by high bootstrap values. The sequences of *A.apterpcarpa* and *A. guestii* were almost 74% identical, and they were closely associated (85 %) with *A. pisida* and *A. biennis*, which were 85%. A close relationship was observed between *A. faciculiflora* and *A. dissecta* at 56%, and a closer association with *A. setosa* at 65% (Figure 2).

In addition, the analysis of this dataset identified six main clades, one containing *A. pisida*, two with *A. biennis*, the third clade was composed of *A. sulphura*, fourth *A. remotoflora*, the fifth clade was *A. areblences* and *A. mosuleusis* formed the sixth clade (Figure 2).Most of the species were distinct and existed in separate clades. Three principal clades were identified within the *Alcea* species: the *A. areblences* clade, which includes *A. remotoflora*, exhibiting 94% bootstrap support, and the *A. mosuleusis* clade, encompassing other species, with 49% bootstrap support (Figure 2). The recently documented species in various regions of Iraq and the results of this study suggest that numerous additional species may evolve owing to species diversification in the studied area. This indicates that additional genetic and morphological taxonomic investigations are required, as regions harboring *Alcea* species represent a hotspot that necessitates greater focus[30,31].

A few studies have examined genetic variability in *Alcea*,[35] and found a 93 percent polymorphism level in *A. rosea* folks in Iran, with a wide range of genetic similarities. Yin and Khodayari[29]reported that *Alcea* is a relatively complex taxonomic group, and that several morphological characteristics make it difficult to identify and classify *Alcea* species [21].Owing to this complexity, it is essential to investigate other methodologies that may enhance the conventional taxonomical approach [33]. Because of the minimal number of distinguishing morphological features, such as , leaf ordering and carpel structure, *Alcea* includes a difficult taxonomy[33]. Some morphological characteristics ,such as hairs and , the number of corolla and corolla colors, are different between similar species.

The phylogenetic gap between closely related species is caused by additions or deletions in certain base pairs. The long-term effects of abiotic stresses in the region, such as low precipitation rates and local geology, which lead to species adaptation and diversification, may be the source of this distance. The phylogenetic gap among coexisting species in the region indicates the presence of phylogenetic diversity within taxa of the same genus *Alcea*[31].

Molecular-phylogenetic evidence also supports Alcea'smonophyly, according to [21]; however, it is not very useful for establishing links between species and their boundaries.

All *Alcea* species exhibit close-knit communities, whereby geographically proximate communities are far more effectively linked through gene flow than populations separated by substantial distance. Kazemi *et al.*[34], through RAPD identification research, showed that *A. rosea* species in Iran had a 93 percent polymorphism ratio, with significant genetic similarities ranging from 0.31 - 0.75. In contrast, using RAPD markers [20]examined the genomic profiles of 18 *Alcea* species and discovered significant variation (0.13 -0.69 them).

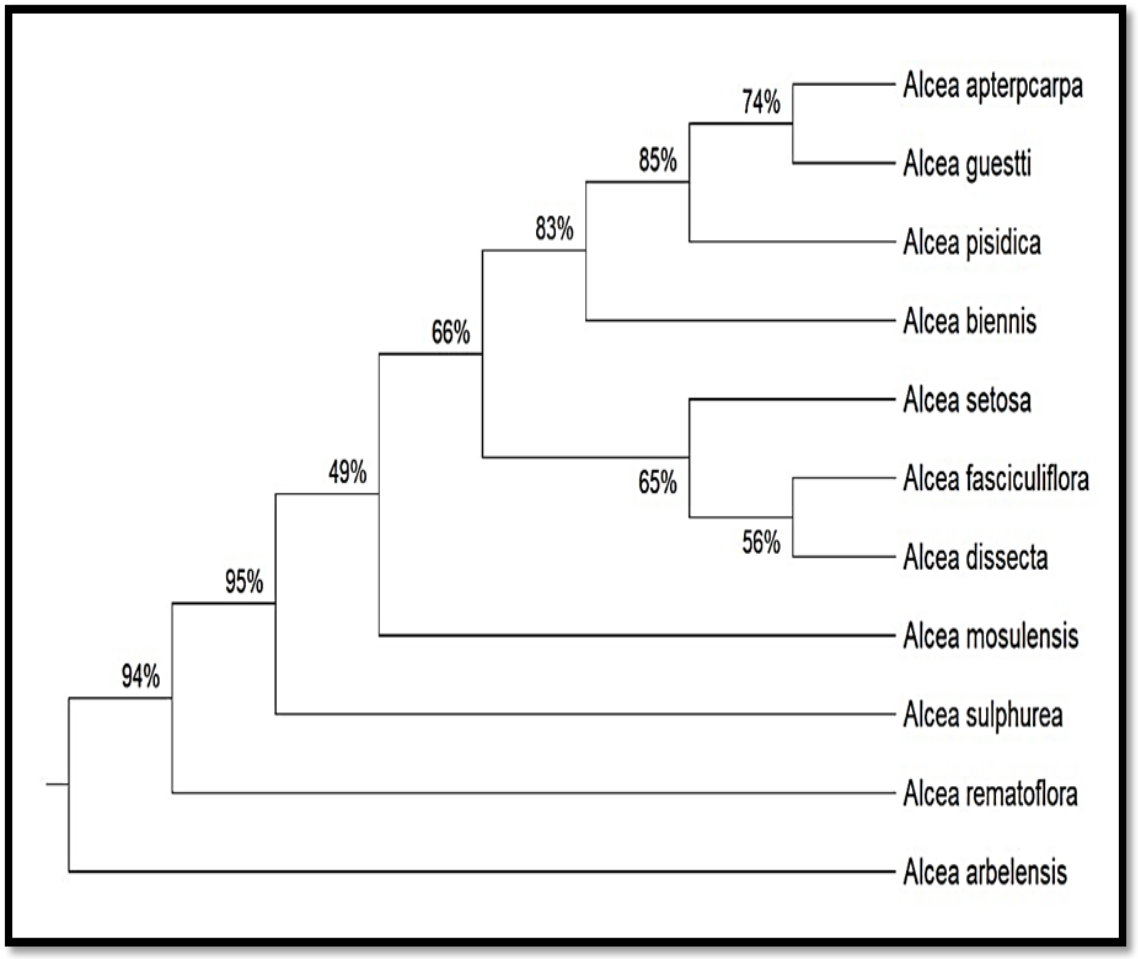


Figure 2. Phylogenetic relationships among *Alcea* species serving as the outgroup.

Conclusion

The classification of Iraqi *Alcea* species has proven challenging owing to their limited morphological characteristics, resulting in a complex taxonomy for the genus. The present work reveals that PCR-based fingerprinting techniques are informative for determining the breadth of genetic diversity as well as for determining the pattern of genetic relationships among species and categorizing them . This work represents a crucial initial step in comprehending the population genetics of *Alcea* species; its true significance is seen when considered as a foundational basis. The amalgamation of morphological data with complementary molecular techniques, including cpDNA for historical context, SSRs for detailed analysis, and SNP-based genotyping, will be crucial for elucidating the comprehensive evolutionary narrative and devising the most effective, evidence-based conservation strategies for this species. Furthermore, we advocate for the concurrent use of various molecular markers to provide future data.

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