BULLETIN OF THE IRAQ NATURAL HISTORY MUSEUM
Iraq Natural History Research Center & Museum, University of Baghdad
https://jnhm.uobaghdad.edu.iq/index.php/BINHM/Home
Copyright © Bulletin of the Iraq Natural History Museum Online ISSN: 2311-9799, Print ISSN: 1017-8678

Bull. Iraq nat. Hist. Mus.
(2026) 19 (1): 263-274. https://doi.org/10.26842/binhm.7.2026.19.1.0263

ORIGINAL ARTICLE

PHYLOGENETIC RELATIONSHIPS AMONG CERTAIN SPECIES OF *ASTRAGALUS* L., 1753 (FABALES, FABACEAE) IN SOUTHEASTERN OF IRAQ

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***Received:** 7 Oct. 2025, **Revised:** 3 March 2026, **Accepted:** 9 March 2026, **Published:**20 June 2026*

ABSTRACT

In the current study, eight species of *Astragalus* L.,1753 (Fabales, Fabaceae): *A.annularis* Forssk,1775, *A. asterias* Steven,1832, *A. bruguieri* Boiss.,1849, *A. dactylocarpus* Boiss., 1843, *A. fasciculifolius* Boiss.,1843, *A. hamosus* L.,1753, *A. spinosus* (Forssk.) Muschi., 1907 and *A. tribuloides* Delile,1813 in southeastern Iraq were investigated for molecular phylogenetic analysis. For this purpose, the Internal Transcribed Spacer (ITS) was sequenced to examine the evolutionary relationships among the species. The species in two clades were separated based on proximity in the phylogenetic tree. The first clade consisted of three subclades: subclade I comprises three species, including *A. asterias*, *A. tribuloides*, and *A. dactylocarpus*; the relationship between them was 94% and 85%, respectively. Subclade II comprised only *A. bruguieri*, the resemblance of these subclades to subclade one was 78%. Two species were positioned within subclade III (*A. faciculifolius* and *A. spinosus*) with 98% similarity. The second clade included only two species, *A.annularis* and *A. hamosus*; with a similarity of 90%. The data obtained were reliable for their usability in solving taxonomic problems of the studied species.

Keywords: Angiosperm, Fabaceae, ITS, PCR Amplification, Phylogenetic relationship.

INTRODUCTION

Fabaceae is one of the largest angiosperm families, comprising 19,500 species belonging to 730 genera (Azani *et al*., 2017). Fabaceae is widely distributed and encompasses numerous economically significant species, and it has been categorized into three subfamilies, based on their floral morphology (Lewis *et al*., 2005). Recent investigations have utilized nuclear and chloroplast sequences to endorse a novel categorization scheme that comprises six subfamilies (Cercidoideae, Caesalpinioideae, Detarioideae, Dialioideae, Duparquetioideae, and Papilionoideae) with morphological corroboration (Azani *et al*., 2017; Koenen *et al*., 2020; Zhang *et al*., 2020).

The genus *Astragalus* L., 1753, belongs to Fabaceae family, Papilionoideae subfamily, Astragalinae subtribe, and Galegeae tribe (Lymberakis and Poulakakis, 2010; Li *et al*., 2014; Khal *et al*., 2020). *Astragalus* is one of the largest plant genera globally, comprising over 2,400 species. In Iraq, Rechinger (1964) mentioned that there were 31 species of the genus. Additionally, Townsend and Guest (1974) identified 116 species in the Flora of Iraq.

Astragalus is a prominent medicinal herb in traditional medicine, utilized for treating many diseases, stress, anorexia, tremors, exposure to traumas, viral infections, unhealed puppies, fever, diaphoresis, uterine prolapse, edema (nephritis), odynophagia, anti-inflammatory conditions, diabetes, uterine carcinoma, colorectal carcinoma, and ovarian carcinoma. This genus may interact with immunosuppressive medications and influence blood glucose levels and blood pressure (Foster, 1998; Li *et al*., 2014).

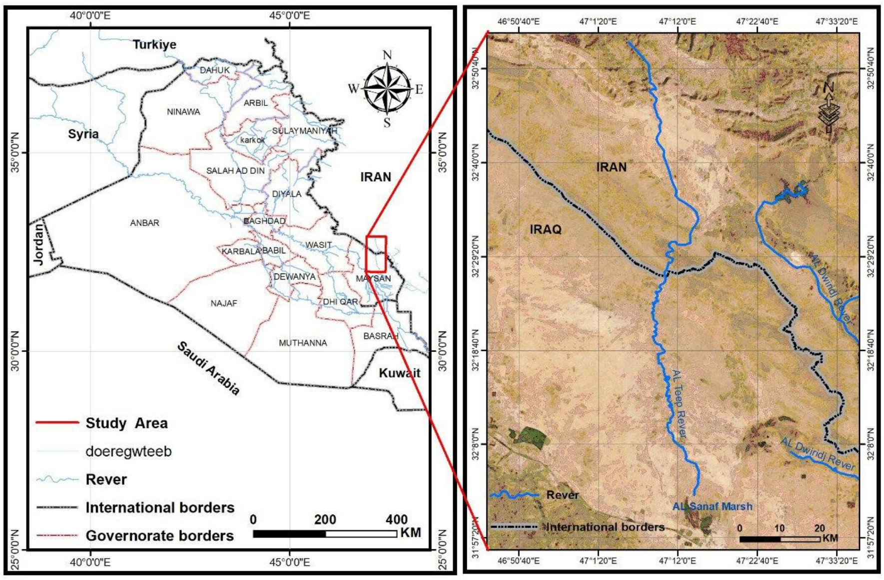
Various researchers have adopted molecular markers over morphological or chemical description methods, as they serve as excellent tools for delineating genetic variation and establishing interspecies relationships (Mandel *et al*., 2019). Currently, DNA barcoding is one of the most prevalent techniques for species differentiation and phylogenetic analysis of related taxa (Mosa *et al*. 2019). Comprehending the aspects of Fabaceae variety and evolution, together with the analysis of the genes that underpin these characteristics, necessitates a robust and well-defined phylogeny (Koenen *et al*., 2020). A dependable species tree is essential for the development of nature reserves, the protection of wild species, and the conservation of biodiversity (Zhao *et al*., 2021). Gene-based analyses have effectively elucidated relationships among lineages of angiosperm families (Zeng *et al*., 2017; Yang *et al.,* 2018; Mandel *et al*., 2019; Zhang *et al*., 2020), and ferns (Shen *et al*. 2017; Qi *et al*. 2018). Genomic databases have been produced for 20 legume species (Cannon *et al*., 2015), 30 legume species (Vatanparast *et al*., 2018), and 42 species (Koenen *et al*., 2020) to examine evolutionary relationships among subfamilies and tribes.

Based on utilization of genetic markers such as cpDNA and nrDNA, many researchers have examined the evolutionary relationships within the genus (Scherson *et al*., 2008; Su *et al*., 2021). Numerous publications have indicated that the ITS region is frequently sought after in molecular studies (Yao *et al*., 2010). The richness in this region makes it suitable for phylogenetic analysis at the species, genus, and family levels (Monsefi *et al*., 2015; Khal e*t al*., 2020). Numerous molecular markers, including CDDP (Ahmed *et al*., 2023), ISSR (Essadki *et al*., 2006; Aliabadi *et al*., 2023), and CDDP/SSR/ITS (Khal *et al*., 2023) have recently been employed to investigate phylogenetic connections among Astragalus species. Significant efforts have been made in Turkey (Gedik *et al*., 2019; Karaman *et al*., 2022), Iraq (Khal *et al*., 2023), and Iran (Roofigar and Jalili, 2021; Aliabadi *et al*., 2023) concerning the morphological diversity of *Astragalus* species.

There is a lack of understanding of the genetic diversity of *Astragalus* species. In addition, the decline of these species in Iraq has intensified due to climate change. Therefore, the present work aims to utilize ITS markers to ascertain the relationship, among eight species of this genus. A molecular taxonomy of these plants also unnecessary significantly enhances the comprehension of their evolutionary history.

MATERIALS AND METHODS

Collection and identification of the specimens: In the present study, the specimens were collected from different places in the Wadi Al-Tib Region (N 32º27 E 47º16) in southeastern Iraq (Map 1), during the period 2023-2024. The species were identified at the herbarium of the College of Science at the University of Maysan, using the taxonomic keys from the Flora of Lowland Iraq (Rechinger, 1964), Flora of Iraq (Townsend and Guest, 1974), and Flora of Turkey and the East Aegean Islands (Davis, 1988). The specimens were initially placed into polyethylene bags and securely sealed with perforated aluminum foil to allow the leaves to absorb moisture.They were then sealed with a small amount of silica gel. After that, liquid nitrogen was used to grind the leaves.



Map (1): The plants sampling location in Wadi Al-Tib Region southeastern in Iraq (Designed by ArcMap v10.8 software)

DNA extraction: The Plant Genomic DNA Mini Kit was used to extract DNA from leaves in accordance with the manufacturer's instructions. 50 µl of extracted DNA samples were kept at -20°C.

DNA quantification using NanoDrop: DNA quantification was estimated using the at 260/280 absorbance ratio by Spectrophotometer NanoDrop. Two µL of the DNA sample were loaded, and the concentration was determined and documented.

Polymerase Chain Reaction (PCR) Amplification: An aliquot of 13µL from the 2xGoTaq Green Master Mix was transferred to a PCR tube. 1µL of upstream primer and 1µL of downstream primer were added. Five µL of DNA template were taken, and 5µL of nuclease-free water were added. The mixture was vortexed for “30 s” and transferred to the Polymerase chain reaction (PCR) technique. PCR involves amplification of the ITS region of nuclear ribosomal DNA. The primers used were $^{5'}$TCCGTAGGTGAACCTGCGG$^{3'}$ and $^{5'}$TCCTCCGCTTATTGATATGC$^{3'}$, according to El-Badan *et al*., (2024). The amplification procedure included an initial denaturation phase lasting 4 minutes at 95ºC, followed by 32 cycles. Each cycle comprised a denaturation phase at 95ºC for 1 minute, an annealing phase at 57ºC for 1 minute, and an elongation phase at 72ºC for 2 minutes. The primer extension phase was extended to 10 minutes at 72ºC during the final cycle in a Gene Amp PCR System 9700 Thermocycler.

The PCR results were analyzed using 1.5% agarose gel electrophoresis at 70 volts for 60 minutes. The PCR products were transferred to Macrogen, Korea for sequencing using the Sanger method.

Phylogenetic Tree: To construct the phylogenetic tree, the method of Hall (1999) was followed for assembling and aligning sequences using BioEdit software, and the method of Saitou and Nei (1987) to clarify the relationship among species by estimating genetic distance using *MEGA12* software.

RESULTS AND DISCUSSION

DNA quality and quantity assessment: A NanoDrop device and agarose gel electrophoresis were used to confirm the purity of each extracted DNA sample spectrophotometrically. A 260/280 nm ratio of 2.21 suggested that the isolated DNA exhibited high purity. Table (1) delineates the DNA yield and purity range acquired from the sample extracts using the extraction procedures. The 260/280 ratio in this study ranged from 1.01 to 2.21. The quantity of extracted DNA samples was determined using a NanoDrop spectrophotometer, and the concentration ranged from 3.1 to 35.7 ng/µl.

Table (1): The range of DNA purity measured and concentration using a NanoDrop spectrophotometer from sample extracts.

No.	Species	DNA purity (260/280)	DNA concentration (ng/µl)
1	*A. annularis* Forssk.	1.76	3.7
2	*A. asterias* Steven	2.21	35.7
3	*A. bruguieri* Boiss.	1.92	6.2
4	*A. dactylocarpus* Boiss.	1.77	22.9
5	*A. fasciculifolius* Boiss.	1.81	12.1
6	*A. hamosus* L.	1.77	3.1
7	*A. spinosus* (Forssk.)Muschi	1.01	16.8
8	*A. tribuloides* Delile	2.12	31.3

DNA extraction and PCR amplification: As shown in Plate (1), the quality of the extracted DNA was assessed by gel electrophoresis. The extracted DNA was directly used for PCR amplification of the ITS gene, as found in Plate (1). The phylogenetic analysis of species was estimated based on ITS (Pl. 1, Diag. 1), the PCR analysis showed clear band in 8 samples, with a band of 700-750 bp corresponding to the amplification of the forward and reverse ITS sequences.

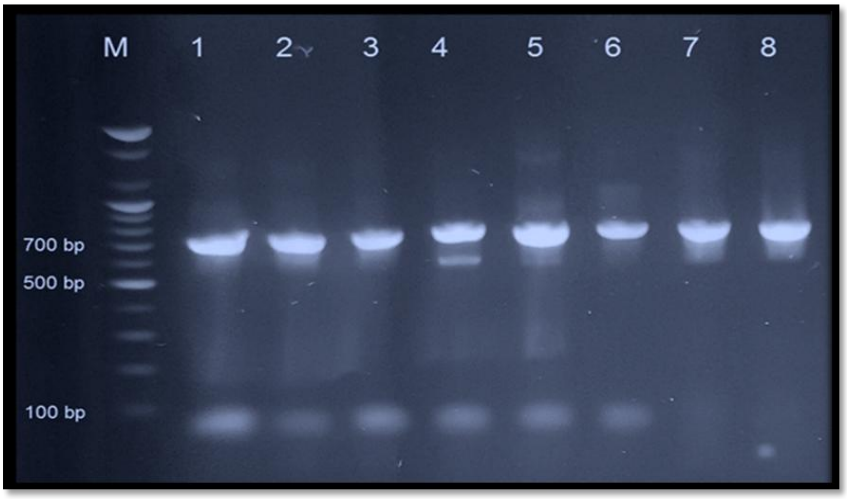


Plate (1): Agarose gel electrophoresis of PCR products from using primers ITS.M: DNA ladder(100bp), Samples; (1) *A. tribuloides*, (2) *A. bruguieri*, (3) *A. dactylocarpus*, (4) *A. hamosus*, (5) *A. fasciculifolius*, (6) *A. spinosus*, (7) *A. asterias*, (8**)** *A. annularis*.

Phylogenetic Analysis: The ITS region provides resolution and clade support for relationships among species and traditionally related genera (Al-Taie and Al-Saadi, 2025**)**. The presence of a phylogenetic gap between closely related species is caused by additions or deletions in certain base pairs. The persistent effects of abiotic stress in the region, including low precipitation levels and local geology, which result in species adaptability and diversification, thereby accounting for this distance. Phylogenetic diversity exists among taxa, as evidenced by the phylogenetic gap between cohabiting species in the area (Khalil, 2022).

Recent advancements in molecular biology and DNA sequencing technology enable the characterization of organismal genomes, and numerous contemporary genomic studies across various species are providing significant insights into their biology and applications. Numerous molecular investigations of the Fabaceae have been undertaken, focusing on various regions of both nuclear and chloroplast DNA. For instance, phylogenetic relationships among *Vicia* L., (1753) and its related species have been derived from trnL sequences (Fennel *et al.*, 1998); phylogenetic analysis of the genus *Astragalus* L. utilized nrDNA ITS and matK sequences (Wojciechowski, 2005) and TrnL sequences were employed (Kaplan *et al.*, 2021).

Eight species of *Astragalus*, belonging to five sections: *Oxyglottis*, *Chronopus*, *Poterion*, *Buceras*, and *Haematodes*, were recognized. The most prevalent species was *A. spinosus* (section: *Poterion*), whereas the least prevalent species were *A. dactylocarpus* (section: *Chronopus*) and *A. bruguieri* (section: *Poterion*) in the Wadi Al-Tib Region in southeastern Iraq.

The studied species of *Astragalus* were used to generate a neighbor-joining tree through a series of nucleotide sequence alignments. The phylogenetic tree categorized eight species into two main clades (Diag. 1). The first clade consisted of three subclades. Subclade I consists of three species: *A. asterias*, *A. tribuloides*, and *A. dactyocarpus.* The relationship between them was 94% and 85%, respectively. While subclade II consisted of only *A. bruguieri*, the similarity between these subclades was 78%. Two species were aligned in subclade III: *A. faciculifolius* and *A. spinosus,* with a similarity of 98%. The second clade included only two species: *A. annularis* and *A. hamosus*; the similarity was 90%.

Moreover, the bootstrap values in this study were lowest for *A. asterias*, *A. tribuloides*, *A. dactylocarpus*, and *A. bruguieri* at 78%, while the highest value was observed between *A. faciculifolius* and *A. spinosus* at 98%. A separate study conducted in northern Iraq (Khal *et al*., 2023) and Turkey (Dizkirici *et al*., 2014) indicated that *Astragalus* species achieved a robust 99% bootstrap support, and USA *neo-Astragalus* exhibited 88% bootstrap supports (Sanderson and Wojciechowski, 2000).

Osalu *et al*. (2005) examined 200 species using ITS sequences to elucidate their evolutionary relationships, while Dizkirici *et al.* (2014) studied 56 species in Turkey, Mehr *et al*. (2012) investigated 24 species in Iran, and Khal *et al.* (2023) analyzed 33 species in northern Iraq. This study selected the ITS region to examine the evolutionary relationships among eight species of this genus located in southeastern Iraq.

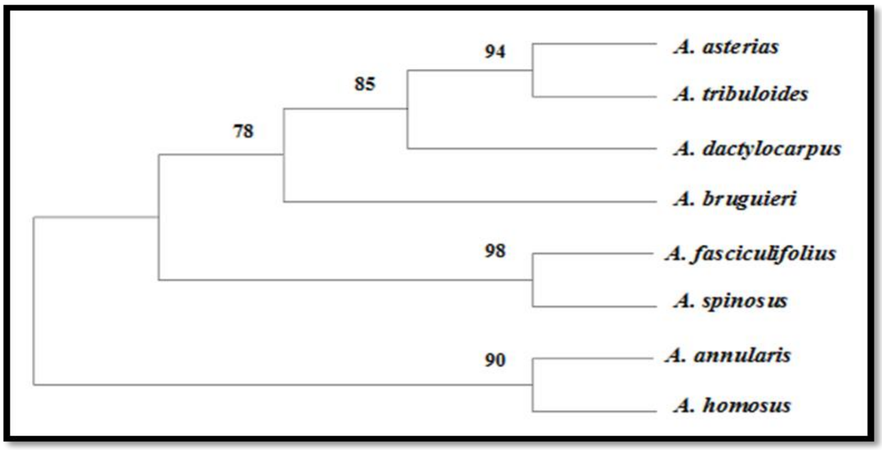


Diagram (1): Dendrogram shows relationships among *Astragalus* species based on molecular data.

CONCLUSIONS

The current work included eight species of *Astragalus* and was conducted to examine the use of ITS for molecular assessment and to further understand of the main evolutionary relationships among the studied species. This study demonstrates how crucial DNA barcoding is for identifying and categorizing taxa. Our results, based on ITS sequences, reveal genetic distances ranging from 78% to 98% among the studied species. Molecular techniques elucidate the comprehensive evolutionary narrative and identify the most effective species conservation strategies. Furthermore, we advocate the simultaneous use of various molecular markers, including cpDNA, RAPD, ISSR, and SNPs to provide future data.

CONFLICT OF INTEREST STATEMENT

The author declares no conflict of interest with any other parties.

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العلاقات التطورية بين بعض أنواع جنس *Astragalus* L.,1753 (Fabaceae،Fabales) في جنوب شرق العراق

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الاستلام: 2025/10/7، المراجعة: 2026/3/3، القبول: 2026/3/9، النشر: 2026/6/20

الخلاصة

خـــلال التحريات الحالية، فحصت ثمانية أنواع من جنس *Astragalus* L., 1753 (Fabaceae،Fabales) و التي ضمت: *A.annularis* Forssk.,1775 و*A. asterias* Steven, 1832 و*A. bruguieri* Boiss., 1849 و *A. dactylocarpus* Boiss., 1843 و *A. fasciculifolius* Boiss., 1843 و*A. homosus* L., 1753 ، و*A. spinosus* (Forssk.) Muschi., 1907 و *A. tribuloides* Delile, 1813 في جنوب شرق العراق ، وذلك لدراسة تطورها الجزيئي. لهذا الغرض، تم فحص تسلسل Internal Transcribed Spacer (ITS) لدراسة العلاقات التطورية بين الأنواع. اذ فصلت الأنواع في مجموعتين رئيسيتين بناءً على قربها في الشجرة التطورية. تتكون المجموعة الأولى من ثلاث مجموعات فرعية: تضم المجموعة الفرعية الأولى ثلاثة أنواع: *A. asterias* و*A. tribuloides* و*A. dactyocarpus*، وكانت نسبة التشابه بينها 94% و 85%على التوالي. أما المجموعة الفرعية الثانية، فضمت *A. bruguieri* فقط ، وبلغت نسبة التشابه بينها وبين المجموعة الفرعية الأولى 78%. وتم تصنيف نوعين ضمن المجموعة الفرعية الثالثة *A. faciculifolius* و*A. spinosus* بنسبة تشابه بلغت 98%. وتضم المجموعة الثانية نوعين فقط *A. annularis* و*A. hamosus*، وبلغت نسبة التشابه بينهما 90%. كانت البيانات التي تم الحصول عليها موثوقة من حيث قابليتها للاستخدام في حل المشكلات التصنيفية للأنواع المدروسة.