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New Record for the Flora of Iraq: *Myosotis incrassata* Guss. (Boraginaceae)

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ABSTRACT

The genus *Myosotis incrassata* Guss. is one of the largest genera of tribe Myosotideae, subfamily Cynoglossoideae, and family Boraginaceae. The aim of this study is to validate the *M. incrassata* is new record in Iraq, so that an extensive sampling characterization of macro- and micro-morphology, some characteristics of pollen grains, such as shape, color, size, sculpture, and number have been studied. To further validate the taxonomic position of *M. incrassata* within the Boraginaceae family, phylogenetic analyzes have been performed. The collected plant specimens differ from the other species that are already present in Iraq, where *M. incrassata* differs from others in that the calyces in fruit are rarely appressed, less than 3 mm, and not crowded. Pollens was yellow in color, single, 3-colporate, circular in equatorial view, spheroid in polar view, small-sized. The molecular studies have confirmed the genetic relationship and placement of this newly recorded species within the broader context of the family, solidifying its classification and evolutionary lineage.

1.Introduction

Myosotis L. (Boraginaceae, subfamily Cynoglossoideae), commonly known as forget-me-not, comprises approximately 100 species, primarily distributed across temperate regions of both the Northern and Southern Hemispheres, with a few taxa found in alpine areas of the tropics (Winkworth et al., 2002). Western Eurasia represents one of the two main centers of diversity for *Myosotis*, harboring around 60 taxa (Al-Shehbaz, 1991). The second principal center of diversity is New Zealand, which is home to more than 35 taxa (Winkworth et al., 2002). In Iraq, there are 7 species of *Myosotis* mentioned by Al-Rawi (1964). But, unfortunately, the precise number of *Myosotis* species present in Iraq remains undetermined due to the seventh volume of the Flora of Iraq not being published. In Turkey, Mill (1978) mentioned 23 species of the same genus including *M. incrassata*. However, previously Riedl (1967) had introduced 18 species of *Myosotis* in Iran.

According to previous literature, none of the researchers who conducted a comprehensive survey of Iraqi districts, including the Kurdistan Region, mentioned the presence of *M. incrassata* in their findings (Khalaf, 1980, Ridda and Daood, 1982, Faris, 1983, Abbas, 1991, Fatah, 2003, Ahmad, 2013, Hameed et al., 2016, Darwesh, 2017, Abbas and Saeed, 2021, Mrad and Saeed, 2023). The family Boraginaceae is considered a monophyletic group based on its shared morphological, molecular, and phytochemical characteristics. However, other phylogenetic analyzes indicate that the traditional circumscription of Boraginaceae renders it paraphyletic with respect to the families Hoplestigmataceae, Hydrophyllaceae, and Lennoaceae (Nazaire and Hufford, 2012).

The present study aimed to confirm the presence of *M. incrassata* in Iraq and to study the morphological characters of the species as a new record for the Iraqi Flora. In addition, to further verification, pollen grain and phylogenetic study were investigated.

2. Materials and Methods

2.1 Taxon Sampling

Extensive field excursions were conducted across various regions of Iraqi Kurdistan, encompassing the districts of Amadiya (MAM), Rowanduz (MRO), Sulaimaniyah (MSU), Kirkuk (FKI), and Arbil (FAR), during the spring and summer seasons of 2024. The primary objective was to obtain plant specimens for comprehensive analysis. The identification process involved the utilization of specialized taxonomic keys, notably the Flora of Turkey. Subsequently, the collected specimens underwent herbarium treatment to ensure their preservation as formal herbarium specimens. Concurrent with the specimen collection, pertinent ecological observations were accurately documented, and a detailed map (Figure 1) was prepared to illustrate the geographical context of the study area. During various field trips to Kurdistan Region districts, the plant species was collected within MSU districts, and the new record species would key out as belonging to genus *Myosotis* following the key in 'Flora of Turkey'. However, the flower morphology of the new record is more similar to that of Myosotis. In order to confirm the generic position of the new record, we conducted a simple molecular systematic study

2.2 Pollen grain study

For the pollen grain study, anthers were fixed in FAA (Formalin glacial acetic acid-ethyl alcohol 95%, 1:3:6), then a single anther was removed and placed in a drop of water or 50% glycerol (to prevent the material from drying out) and dissected with a scalpel to extrude the pollen grains; the anther wall material was removed and a cover slip applied. Then, the pollens stained with safranin 0.5% (Sardar, 2017). A Sony camera has been used for photographing the pollen grains and different plant parts, and the scientific terms that were used in the study have been followed in Erdetman (1952) study (Table 1).

Table 1: Showing morphological features of Pollen grains of Myosotis incrassata based on 10 qualitative and quantitative characters.

Character	Measurement
Polar axis µm (P)	(11.5–13.5) 12.5
Equatorial axis µm (E)	(6.5–8.5) 7.5
P/E ratio	1.66
Pollen shape	Prolate
Polar view	Spheroidal
Equatorial view	Circular

Aperture (shape & number)	3-colporate
Color	Yellow
Releasing type	Monad
Pollen outline	Elliptic

2.3 DNA extraction

Genomic DNA was extracted from younger leaf tissue sample using a cetyltrimethylammonium bromide (CTAB) protocol, modified from Doyle and Doyle (1990). The CTAB method including 2% PVP 40 (polyvinylpyrolidone), was used. No more than 10 mg of plant tissue was included per extraction to avoid decrease of DNA quality and yield.

2.4 PCR and DNA sequencing

Noncoding regions of nrDNA ITS was amplified by using the primer shown in (Table 2). The primer was ordered from Macrogen Company, Seoul, Korea. The total volume of amplification reactions was 25 µL and master mix (Amplicon, Odense Denmark) made up of 12.5 µL (the Master Mix consisting of 3 mM MgCl2, 0.2% Tween® 20, 20 mM Tris-HCl pH 8.5, (NH4)2S04, 0.2 units/µl Ampliqon Taq DNA polymerase, 0.4 µM of each primer, and 0.4 mM of each dNTP.), 3 µL genomic DNA extract (density of 10 ng/µl); 2 µL of each primer; 5.5 µL free nuclease water. Polymerase chain reaction (PCR) for amplification of DNA fragments was carried out by using the following amplification program: 94°C for 5 minutes; 35 cycles of (94°C for 1 minute, annealing at the temperature at 58 °C for 1 minute, and 72°C for 2 min), with a final extension at 72°C for 5 minutes. The resultant PCR products were checked on 1.5% agarose gel run in TAE buffer. The gel was stained with Safe red dye and photographed under UV transilluminator. Cleaned amplification products were sent for sequencing to the National Science and Technology Development Agency (NSTDA) in Thailand. Forward and reverse sequences from each template were manually edited and combined into a single consensus sequence with Geneious v5.4 (Biomatters, available from www.geneious.com). The sequence was submitted to National Center for Biotechnology Information GenBank and obtained accession number (PQ640283).

Table 2: list of primers and their sequences that have been used in the study.

Primer for regions	Product size	Sequence 5'---- 3'		References
		Foreword	Reverse	
ITS	400-600 bp	ATGCGATACTTGGTGTGAAT	TCCTCCGCTTATTGATATGC	(White et al., 1990)

2.5 Sequence Alignment and Phylogenetic Analysis

All the DNA sequences were edited and aligned with ClastalW option available in BioEdit, Version 7.0.4.1 (Hall, 2001) and manual adjustment, there were 23 accessions for ITS region (Table 3), including the out group species. Bayesian inference (BI) and Maximum parsimony (MP) analyses were conducted for each dataset separately that included 23 terminal taxa with all sequences available. For MP, PAUP_ 4.0a164 (Swofford, 2000) was also used. Using heuristic search with 100 replicates of random taxon additions, Tree-Bisection-Reconnection (TBR) branch swapping, MulTrees on, and steepest decent off was performed. The maximum numbers of saved trees were 100 for each replicate. The consistency index (CI), retention index (RI), rescaled consistency (RC), and homoplasy index (HI) were assessed, and the bootstrap values were computed from 100 repetitions (Felsenstein, 1985). Prior to performing BI, MrModeltest2 version 2.3's Akaike information criterion (AIC) was used to evaluate the best substitution models (Nylander et al., 2004). The estimated best-fit model for the ITS region was the general time reversible model of nucleotide substitution with gamma-shaped rate variation (GTR+I+G). MrBayes v.3.2 was utilized for BI analysis (Ronquist and Huelsenbeck, 2003). The software generated automated estimates of the priors for state frequencies, rates, and variance between sites. Two independent analyses were performed using two million generations for the ITS dataset, using four Markov chains (one cold and three heated) for each generation and a temperature parameter of 0.1. Every 100 generations, samples of trees

were taken. A tree with a maximum of 50% (majority rule consensus tree) was then displayed after burn-in phase samples had deleted 25% of the initial tree analyzed. The value of posterior probability (PP) was calculated and the final tree was visualized by using FigTree software version 1.4.3 (Rambaut, 2016).

Table 3: Samples included in nrDNA ITS phylogenetic analyses obtained from NCBI genebank.

Taxa	DNA source (location and Voucher)	Accession no
Myosotis arvensis	Mu,M. et al. 2024, Germany	PP763535
M. discolor	Meudt,H.M. et al. 2012, New Zealand	JX128891
M. incrassata	Hasan,H. and Sardar,A. 2024, Iraq	PQ640283
M. laxa	Sherafati,M.et al. 2014, Iran	AB989077
M. olympica	Sherafati,M.et al. 2014, Iran	AB989057
M. propinqua	Sherafati,M.et al. 2014, Iran	AB989074
M. ramosissima	Mu,M. et al. 2024, Germany	PP763562
M. refracta	Sherafati,M.et al. 2014, Iran	AB989060
M. sparsiflora	Mu,M. et al. 2024, Germany	PP763549
M. stricta	Sherafati,M.et al. 2014, Iran	AB989059
M. sylvatica	Stojilkovic,V. 2020, Slovenia	MT636737
M. wumengensis	Wei,L. and Liu,Q. 2016, China	KY201317
Paracaryum cristatum	Sherafati,M. et al. 2021, Tehran, Iran	LC625910
P. rugulosum	Pourghorban,Z. et al. 2019, Iran	MN720430
P. sintenisii	Pourghorban,Z. et al. 2019, Iran	MN720431
P. strictum	Pourghorban,Z. et al. 2019, Iran	MN720432
Rochelia bungei	Khoshsokhan,M. et al. 2010, Iran	AB564695
R. cardiosepala	Khoshsokhan,M. et al. 2010, Iran	AB564701
R. disperma	Khoshsokhan,M. et al. 2010, Iran	AB564708
R. persica	Khoshsokhan,M. et al. 2010, Iran	AB564697
Mertensia davurica	Chacon,J. et al. 2016, Germany	KU927736
M. sibirica	Chacon,J. et al. 2016, UK	KU927739
Brachybotrys paridiformis	Chacon,J. et al. 2016, Russia	KU927662

3. Results

3.1Morphological Study

M. incrassata Guss., Fl. Sic. Syn. 1:214 (1842). Syn: M. idaea Boiss. & Heldr. in Boiss., Diagn. ser. 1(11): 121 (1849); M. cretica Boiss. & Heldr., loc. cit. (1849). Ic: Grau, op. cit. 7: t. 5 (1968).

Annual herb, height (8–11) cm. Stems ascending-erect, green or green-yellow, (4–6×0.07–0.12) cm, much branched at base, patent-hairy at base and hairs antrorse above. Leaves simple, alternate-spiral; basal leaves ovate or lanceolate, margin entire, apex obtuse-acute, base attenuate, green-yellow, (10–11×2.7–4.0) mm, sessile; lower cauline leaves lanceolate to cultrate, margin entire, apex acute, base acute or attenuate, green, (10–12×2.0–3.0) mm; upper cauline leaves narrowly lanceolate to cultrate, margin entire, apex acute, base truncate, subamplexicaul, green, (7.0–8.5×2.0–2.3) mm. Inflorescence terminal and sub-terminal cyme, ebracteate, peduncle teret, green, (2.5–3.0×0.3–0.5) mm. Flowers actinomorphic, pentamerous, (2.0–4.3×2.5–3.0) mm, pedicel terete, green, (1.0–1.5×0.4–0.55) mm. Calyx gamosepalous, 5-lobed ± to base, narrowly lanceolate, margin entire, apex acuminate, base obtuse, green, (1.0–1.5×0.8–1.8) mm, persistent calyx, accrecent in fruit, bigger than calyx, (1.8–2.3×1.5–2.0), apex acute, base ½ open, margin entire. Corolla rotate blue-violet, glabrous, with tube and limb, tube (1.0–1.5×0.5–1.0) mm; limb 5-lobed, (0.5–0.75×1.5–2.0) mm, lobes saucer-shaped, margin undulate, apex obtuse, lobes width (1.0–1.5) mm; throat of 5, violet, semicircular, antipetalous scales (appendages), (0.1–0.2×0.2–0.3) mm (broader than long, where base of anther situated below scale base), apex round. Stamens 5, epipetalous, altering with corolla lobes, inserted on the upper half of corolla tube; filaments filiform, yellow, (0.2–0.35×0.05–0.07) mm, anthers oval-ellipsoid,

basifixed attachment with the filaments, dark yellow, (0.2–0.3×0.2–0.25) mm. Pistil one, ovary superior, 4-locular, pyramidal, dark yellow, (0.5–0.7×0.5–0.65) mm; style gynobasic, included, filiform, yellow, (0.2–0.35×0.1–0.2) mm; stigma entire, globoid, yellow, (0.05–0.075×0.05-0.1) mm. Fruit Schizocarpic with 4 nutlets without rim, nutlets, (1.0–1.15×0.65–0.85) mm, ovate-ellipsoid, brown, attachment scar with two marginal groove, rounded small at base (Figure 3 and Plate 1).

Syntypes: [Sicily] monte di Cammerata: Etna a monte Rosso, *Oranger,Philippi*; Busambra, *Tineo.*

MSU: ESUH/ Piramagrun Mountain (northeast of Sulaymaniyah), 1600-1800 m, 26.5.2024, H. Hasan and A. Sardar, 9012, flowering from May to June and Fruiting time started from June to July.

3.2 Molecular study

Detailed information about alignment characteristics and statistics of MP analyses are summarized in (Table 4). Tree topologies resulting from BI and MP are shown in (Figure 2). The BI and MP analyses of the ITS region produced congruent trees with well-supported conflict. According to the phylogenetic hypothesis based on the ITS data set, five clades (A-E) containing the 23 specimens, including the outgroup, were constructed. The matrix consisted of 663 nuclear ribosomal DNA (nrDNA) characters of the ITS region. The model of molecular evolution selected by AIC was applied to both analyses. The systematic analysis revealed that all genera with more than one species formed highly supported monophyletic clades. The genus *Myosotis*, including *M. incrassate*, formed two clades that were sister to the clade comprising *Paracaryium* and *Mertensia*, while *Rochelia* occupied a basal position relative to all other genera (Figure 2). Within *Myosotis*, two major clades emerged. Clade A contained most species, including *M. incrassata*, and Clade B encompassed other species. Both clades exhibited high support values.

Table 4: A summary of alignment and tree statistics of nrDNA ITS region analyses.

Parameters/Regions	ITS
Aligned length	663
Number of parsimony informative characters	214
Number of variable parsimony uninformative characters	335
Number of constant characters	114
Tree length (steps)	1033
CI (Consistency Index)	0.797
RI (Retention Index)	0.740
RC (Rescaled Index)	0.590
HI (Homoplasy index)	0.203
Model	GTR+G

3.3 Palynology

Pollens small yellow in color, single, 3-colporate, circular in equatorial view, spheroid in polar view, small-sized, equatorial axis (6.5–8.5) µm, polar axis (11.5–13.0) µm, numerous, pollen shape prolate. The mature pollen grains possess diminutive dimensions. When observed from a lateral perspective, they exhibit an elliptical shape with two shallow indentations on either side. This configuration is also apparent when viewed from above. In the latter orientation, the pollen grains bear a striking resemblance to three small, spherical entities adhering together in a tetrad arrangement (Plate 2). Based on a literature review and scrupulous specimen comparisons, as well as observation of the palynology of other *Myosotis* species, the species is proposed as a new record for the Flora of Iraq.

3.4 Geographical distribution

The species is distributed as individual plants within the studied area , on the dry sandy habitat, rocky soils (figure 1).

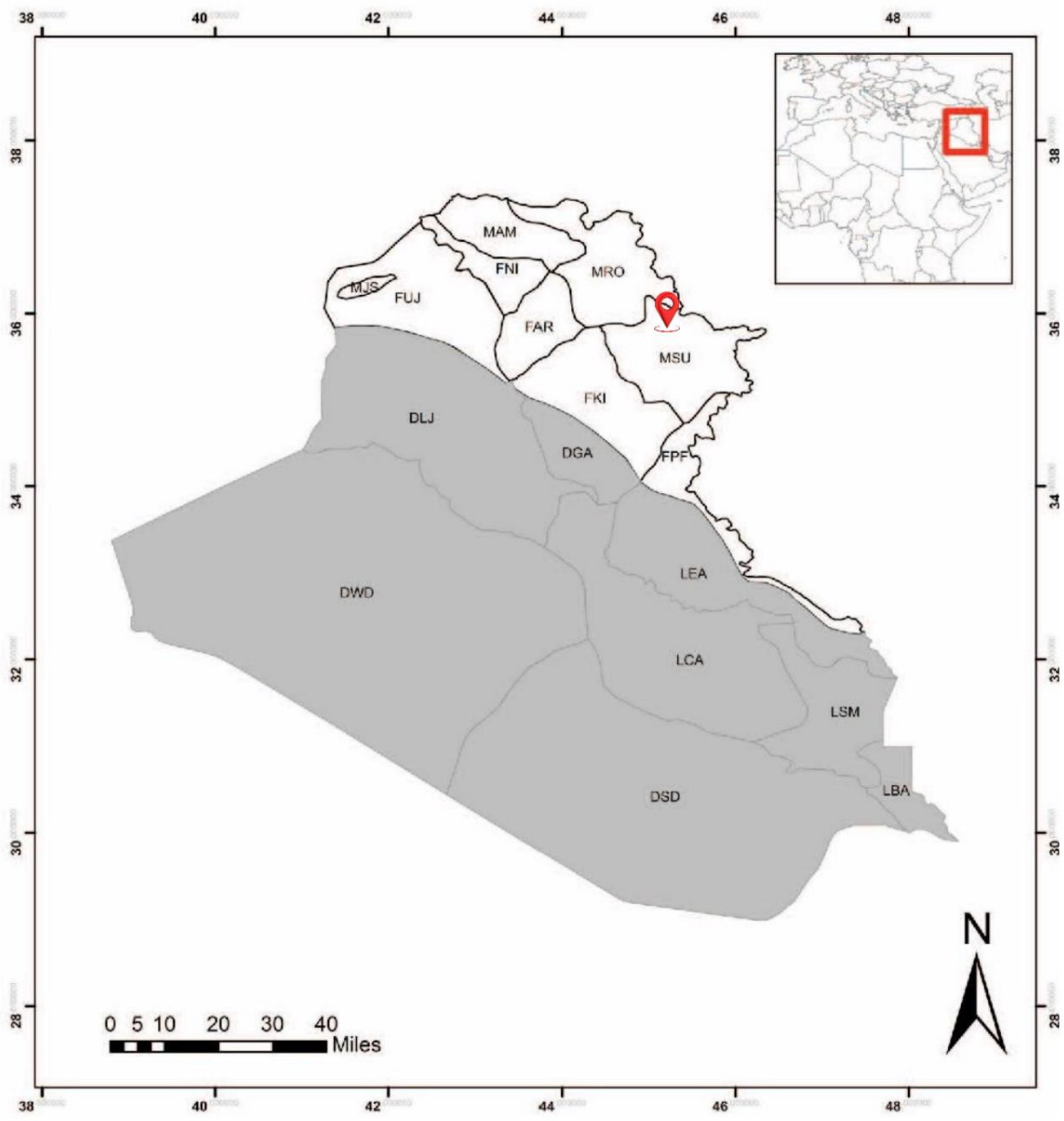


Figure 1: Physiographic districts of Iraq modified from Guest (1966), the area in white shows the districts of the Kurdistan Region.

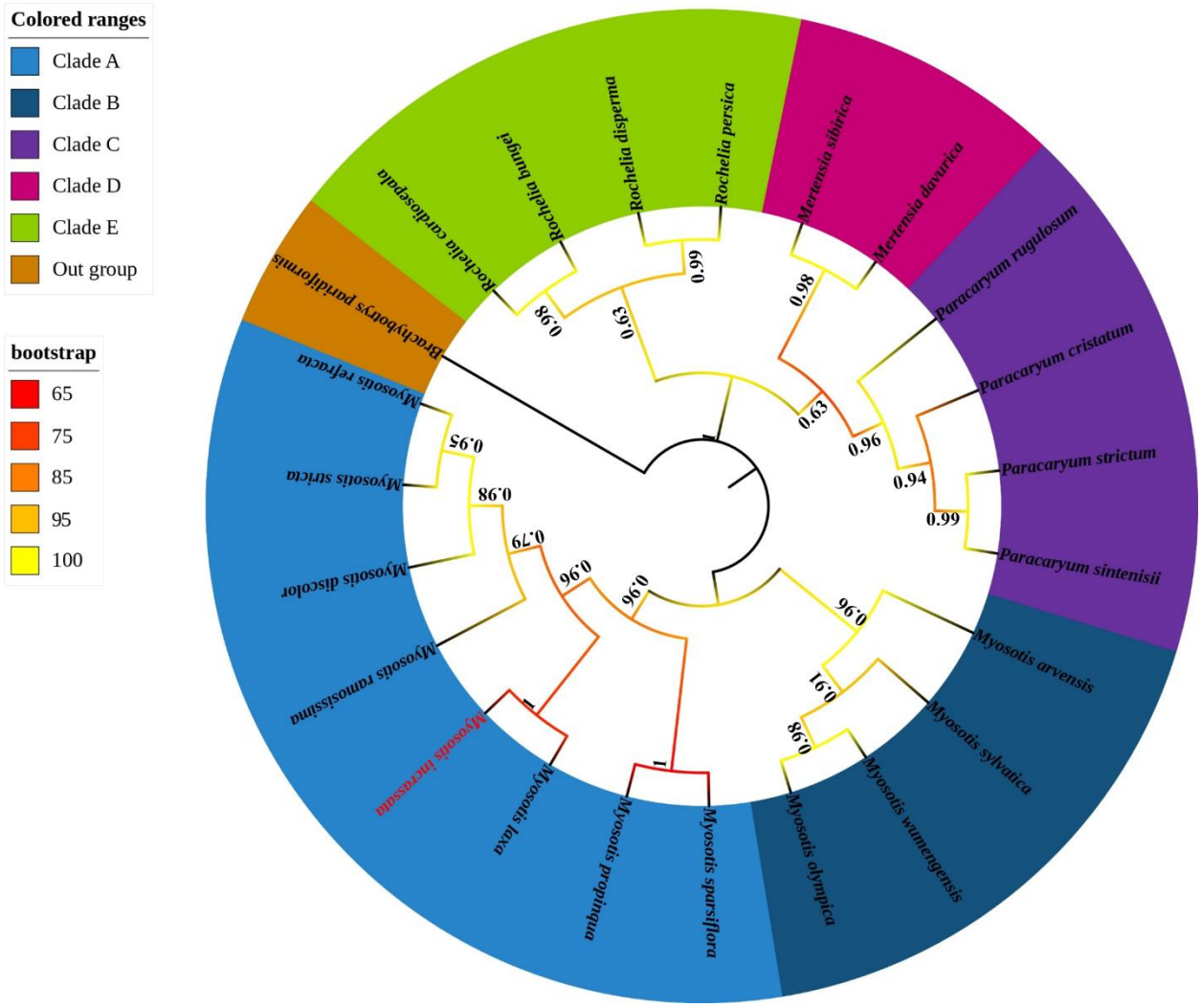


Figure 2: Strict consensus phylogenetic tree of selected *M. incrassata* based on maximum parsimony (MP) and Bayesian inference (BI) analysis of ITS region. Bootstrap proportions are represented by color of the MP analysis and numbers above the branches indicate posterior probability of the BI analysis and clades are denoted by color.

4. Discussion

The present study delves into the exploration of *Myosotis incrassata*, a plant species that represents a novel new record to the Boraginaceae family in Iraq. This research endeavor encompasses a multifaceted approach, encompassing morphological characterization, palynological investigations, phylogenetic analysis, and an examination of the environmental conditions conducive to the species' presence. Through an extensive review of literature pertaining to the genus *Myosotis*, with particular emphasis on previously published studies, and a meticulous examination of specimens housed within the National Herbarium of Iraq (BAG), the Herbarium of the College of Science at the University of Salahaddin-Erbil, Iraq (ARB), and the Herbarium of the College of Education at the University of Salahaddin-Erbil, Iraq (ESUH), the researchers did not uncover any specimens attributable to *M. incrassata*. Consequently, this finding represents a novel record for the Flora of Iraq, with the species having been discovered in the Piramagrun mountain region.

According to molecular study, our molecular analyses identified five distinct clades within *Myosotis*; molecular characterization of additional species may reveal others. Although these major groups are genetically well differentiated, the relationships between them are strongly resolved. This topology aligns with previous studies on *Myosotis* (Winkworth et al., 2002, Meudt et al., 2015, Hao et al., 2017). The newly recorded species was nested within the clade A (Figure 3). The primary objective of our endeavor was to ascertain that the genus we encountered aligned with the taxonomic classification of *Myosotis*, and our findings have successfully corroborated this hypothesis.

Myosotis incrassata, a recently documented species in Iraq, exhibits distinct morphological characteristics that differentiate it from other species found within the region. The most notable feature lies in the appearance of its calyces during the fruiting stage. Unlike other species, the calyces of *M. incrassata* are rarely appressed, meaning they do not lie flat or close against the stem. Additionally, these calyces measure less than 3 millimeters in length and are not densely clustered or crowded together. These key morphological traits, particularly the non-appressed and sparsely distributed calyces of relatively small size, serve as distinguishing characteristics that firmly establish *M. incrassata* as a unique and newly recorded species within the Iraqi Flora. This finding is congruent with Mill's (1978) work, in which he initially documented the presence of *M. incrassata* in Turkey.

Identifying species from fresh material is typically straightforward in most cases. However, many morphological characteristics employed in identification keys (Fischer et al., 2008, Rottensteiner, 2014), such as calyx openness and pedicel growth direction during fruiting, are often unreliable on herbarium specimens due to deformation of plant material during the preservation process. While, there are many morphological features share between *M. arvensis* and *M. incrassata*, but they are do not locate at the same clade based on phylogenetic study (Notov and Zhukova, 2022). In contrast to *M. laxa*, *M. incrassata* exhibits a distinct difference in the attachment scar of the nutlet. The attachment scar in M. incrassata is not spongy, whereas in M. laxa, it is spongy. However, these two species share numerous morphological similarities, resulting in their placement within the same clade (Pihu et al., 2009). Moreover, certain reliable identification features are found in nutlets, which are frequently absent from herbarium specimens. Therefore, pollen grain size was already used for delimitation between species. *M. incrassata* has a small size, is monad, tri-colporate, prolate, has a surface psilate, and has heteroaperturate pollen grain. This feature was congruent with Meudt (2016).

The significance of this study lies in its contribution to the ever-expanding body of knowledge surrounding the botanical diversity of Iraq. By documenting the presence of *M. incrassata*, researchers gain valuable insights into the distribution patterns and ecological niches occupied by this species within the country's borders. Furthermore, the comprehensive examination of morphological,

palynological, and phylogenetic characteristics not only aids in the accurate identification and classification of the species but also lays the foundation for future investigations into its evolutionary history and potential applications.

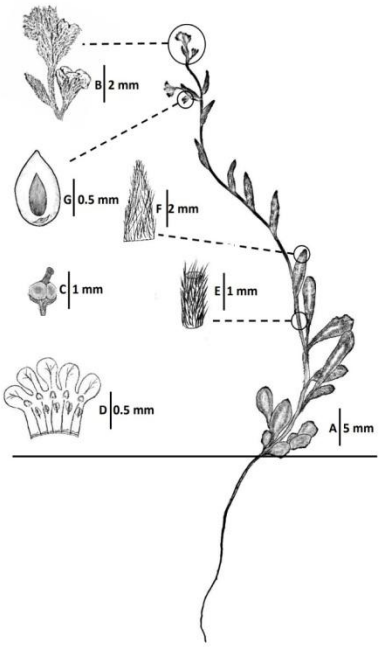


Figure 3: *Myosotis incrassata* (A) habit, (B) flower, (C) pistil, (D) opened corolla showing anther and throat appendage, (E) stem, showing hairs, (F) blade upper surface, (G) Fruit.

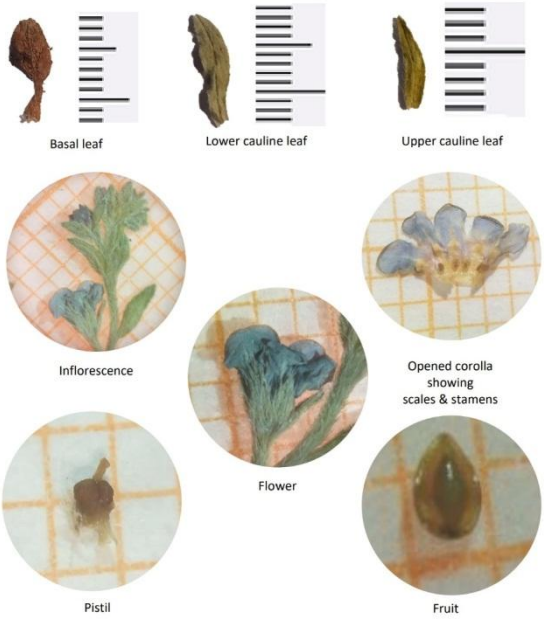
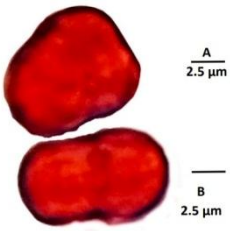


Plate 1: Plant parts of *M. incrassata*.



5. Conclusion

The present study has made a significant contribution to the field of botany in Iraq by confirming the presence of the plant species *Myosotis incrassata*, which represents a new record for the country's flora. This discovery adds to the existing knowledge of plant diversity in Iraq and underscores the importance of continued research and exploration in the region. Based on a comprehensive literature review conducted as part of the study, the number of species belonging to the genus *Myosotis* found in the mountainous regions of Iraq now stands at eight. These species are *M. alpestris*, *M. discolor*, *M. olympica*, *M. ramosissima*, *M. laxa*, *M. refracta*, *M. silvatica*, and the newly recorded *M. incrassata*.

The identification of *M. incrassata* in Iraq not only expands the known distribution range of this plant species but also highlights the potential for further discoveries within the country's diverse ecosystems. This finding reinforces the need for ongoing efforts in documenting and conserving Iraq's rich biodiversity, as well as promoting sustainable management practices to ensure the preservation of these natural resources for future generations.

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