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Molecular taxonomy of *Vicia* L. species (Fabaceae) from the mountainous regions of Iraqi Kurdistan based on ITS barcoding

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Abstract

Accurate species identification within *Vicia* remains challenging due to high morphological variability, phenotypic plasticity, and frequent hybridization. This study presents the first molecular assessment of nine wild *Vicia* species from the mountainous area of Iraqi Kurdistan using the ITS region. A dataset of 32 ITS sequences (nine Iraqi accessions and 23 GenBank references) was analyzed for genetic divergence and species discrimination. Intraspecific K2P distances ranged from 0.002 to 0.018, while interspecific distances varied from 0.005 to 0.111, generally exceeding intraspecific divergence. Barcode gap analysis revealed a clear species-level separation in *Vicia ervilia* L., *Vicia faba* L., *Vicia. narbonensis* L., and *Vicia sericocarpa* Fenzl, whereas only partial overlap was observed in *Vicia tenuifolia* Roth., *Vicia villosa* Roth., *Vicia palaestina* Boiss., *Vicia michauxii* Spreng., and *Vicia sativa* L. The species identification tests (TaxonDNA) offered approximately 90% correct assignments, but the All-Species Barcode provided only 56% of correct assignments, reflecting the loss of resolution of closely related taxa. Maximum Likelihood phylogenetic inference confirmed that most Iraqi accessions clustered with conspecific references from neighboring countries. However, the weak support for some lineages raised questions about the resolving power of ITS for assessing recently diverged or hybridizing taxa.

Generally, ITS barcodes were suitable for confirming the species identity and phylogenetic reconstruction of Iraqi *Vicia* but were insufficient to completely resolve the closely related lineages. This study provides the first molecular dataset for *Vicia* in Iraq and underscores the need for multilocus or genomic-scale approaches to solidify species delimitation and support regional plant genetic resource conservation.

Key words: DNA barcoding, genetic divergence, species delimitation, phylogenetics, plant conservation

Introduction

Fabaceae (syn.: Leguminosae), is the third-most extensive family of flowering plants after Orchidaceae and Asteraceae; it has almost 19,400 species under about 730 genera (Welbaum 2024). Many legumes serve as food crops because of their protein content and nutritional value (Rajput *et al.* 2024). Another advantage of this family in agriculture is soil enrichment via symbiotic nitrogen fixation, thereby forming an integral part of sustainable agriculture (Chen and Zhou 2024).

Within Fabaceae, the genus *Vicia* L. consists of almost 150 to 210 species distributed worldwide, with centers of diversity in the Mediterranean and western Asia, as evidenced by many checklist studies (i.e. Perrino *et al.* 2013, Calabrese *et al.* 2015, Cueto *et al.* 2018, Ben Mahmoud *et al.* 2024, El Zein *et al.* 2025). About 40 species are economically important, *V. faba* L. being among these, as it is widely cultivated for food and forage (Van de Wouw *et al.* 2001). The exact number of species is a question due to outdated monographs and inconsistent taxonomic

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treatments (Al-Ghamdi 2013; Vasconcelos *et al.* 2020; Ibáñez *et al.* 2020; Salehi *et al.* 2021). In Iraq, more than 20 species of *V.* are recorded, with the majority occurring in the mountainous regions of Kurdistan, one of the biodiversity hotspot in the Middle East (Townsend & Guest 1974). Despite extensive morphological diversity and cytological variation, molecular studies of Iraqi *V.* are lacking, leaving many taxonomic questions unresolved.

Since the nineteenth century, significant taxonomic work has been performed on the genus *V* in Iraq through herbarium collections, floristic surveys, and morphological analyses. The first records of *V.* in Kurdistan came through early expeditions, beginning with Kotschy in 1841 and continuing through the publication of Boissier's Flora Orientalis in 1867. Handel-Mazzetti (1914) and Zohary (1946) enriched Iraq and neighboring countries' species inventories.

In the Flora of Iraq (Townsend & Guest, 1974), there were five proposed sections of the genus, and there were keys for 23 species, most of which were in Kurdistan. Additional records from Rechinger (1964), Al-Rawi (1988), and Fatah (2003) showed that the number of taxa increased to nearly 24. More recent studies have utilized integrative methodologies: Mohamad (2010) integrated morphological, cytological, and phytochemical features for 23 species and 4 varieties, whereas Al-Joboury (2017) studied interspecific relationships of 5 species using morphology, pollen features, and numerical taxonomy.

Although these studies offer morphological and ecological insights into Iraqi *V.*, molecular data are still lacking, particularly with overlapping morphological characters, putative cryptic species, and extensive hybridization. Therefore, molecular markers, such as the ITS region, are necessary to verify species delimitations and identifications and to provide a molecular, independent line of evidence for taxonomic credibility.

Previously, the classification of the *V.* genus was mainly based on leaf structure and pod features (Nam *et al.* 2012). However, phenotypic plasticity and a high rate of hybridization obscure taxonomic boundaries and cause vague identifications (Coyne *et al.* 2020). Such constraints make the morphological approach a very difficult means of solving species relationships and give extra emphasis to molecular methodology as an alternative and more reliable approach in genus species resolution.

Molecular taxonomy is a valuable tool when compared to traditional morphology because it enables the inference of phylogenetic relationships and species identification through DNA sequences (Hugenholtz *et al.* 2021). DNA barcoding is now one of the most efficient species identification methods because it can be used in areas with limited taxonomic knowledge or underdeveloped infrastructure (Hollingsworth 2011). The plastid loci (matK, rbcL, trnH-psbA) and the nuclear internal transcribed spacer (ITS) region are now regarded as the standard barcodes for plants (Loera-Sánchez *et al.* 2020).

Among these, ITS is preferred due to its high sequence variability, broad amplification success with universal primers, and proven effectiveness in resolving taxonomic ambiguities in *V.* (Besse 2014; Shiran *et al.* 2014; Raveendar *et al.* 2017). Studies in Palestine have validated the discriminatory capabilities of ITS with universal 18S and 28S primers in *V.*, *Lens, Pisum*, and *Lathyrus* (Omar *et al.* 2019). The marked advantages have been repeatedly demonstrated by comparative studies; ITS coupled with plastid markers yield superior phylogenetic resolution, as validated by Wu *et al.* (2020, 2021) and Bosmali *et al.* (2022). Han *et al.* (2021) and Kaplan *et al.* (2021) highlighted the use of ITS in identifying cryptic species and demonstrated that ITS can effectively inform conservation through the clear demarcation of lineages for distinct genetic entities.

Although multi-locus approaches offer greater accuracy, the ITS region remains a cost-effective and practical marker for species-level identification and phylogenetic reconstruction in *V.*, making it particularly suitable for the present study.

Iraqi Kurdistan is a unique biodiversity hotspot in the Middle East, shaped by complex topography and climate variations. Its high-altitude habitats, from mountain slopes to alpine meadows, support an extraordinary diversity of *V.* species. Taxonomically, the genus is infamously complicated, exhibiting great variation in morphology and cytological differences in chromosome numbers, thus preventing a uniform classification (Tabor *et al.* 2002).

Although substantial documentation exists on the basis of herbarium records, morphology, cytology, phytochemistry, and ecological data (e.g., Mohamad 2010; Al-Joboury 2017), molecular studies of Iraqi *V.* remain scarce. Many specimens in regional herbaria have been morphologically identified, but problems, including overlapping traits, suspected cryptic species, and hybridization, remain unresolved. These issues have left the phylogenetic placement of Iraqi taxa poorly understood and their biodiversity under-documented.

Consequently, independent molecular evidence is urgently required to confirm species boundaries, detect misidentifications, and provide a framework for conserving plant genetic resources. The ITS region, with its proven utility in *V.*, offers a suitable marker to address these gaps.

To address these gaps, the present study assesses the effectiveness of the nuclear ITS region in distinguishing nine wild *V.* species from the mountainous regions of northeastern Iraqi Kurdistan and reconstructing their phylogenetic

relationships. The newly generated sequences are deposited in GenBank, providing the first publicly available molecular dataset for Iraqi *V.* and establishing a foundation for future taxonomic, phylogenetic, and conservation studies.

Materials and methods

Sampling and Collection of Plant Material

From April 2022 to July 2023, five field expeditions were organized to complete the list of *Vicia* L. wild species existing in the mountainous terrain of the northeastern region of Iraqi Kurdistan. These expeditions led to the collection of samples from nine *V.* species (Fabaceae) listed in Table 1. Plants were sampled during the flowering stage to confirm the presence of different species. Although the species' sampling locations differed, a flora was constructed for each site, and molecular analyses were conducted to verify the initial morphological identifications, which were made through the flora of Iraq (Townsend & Guest 1974) and flora of Iran (Rechinger 1979). Following the deposition of the voucher specimens in the Baghdad University Herbarium, College of Education for Pure Sciences/Ibn Al-Haitham (BUE), they were assigned voucher numbers, as shown in Table 1. The voucher specimens were prepared and stored according to standard herbarium procedures (Figure 1).

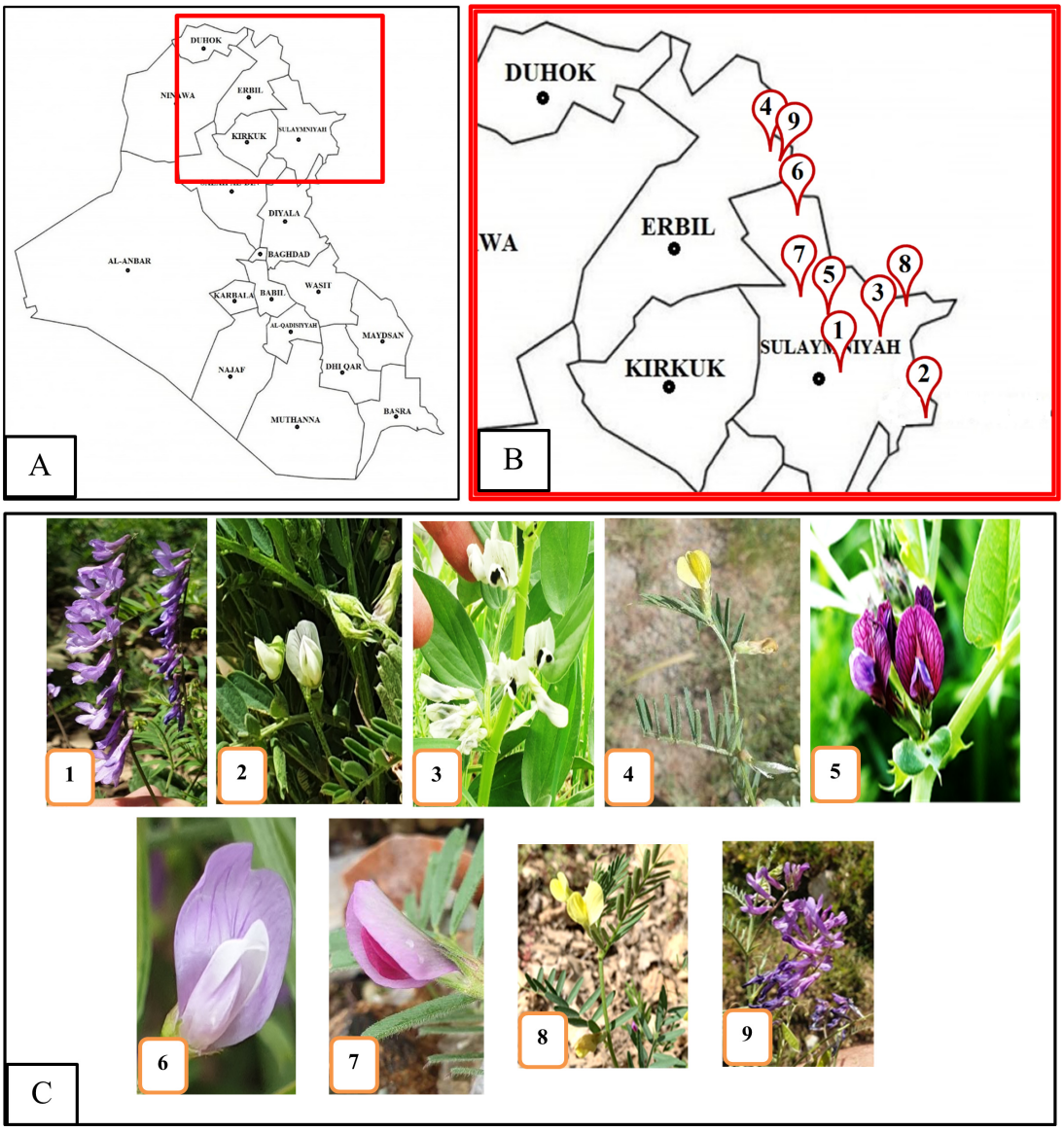

FIGURE 1. (A) Map of Iraq; **(B)** Enlarged map showing Collection sites of nine *Vicia* species; **(C)** Representative photographs of the nine *Vicia* species studied: (1) *V. tenuifolia*; (2) *V. ervilia*; (3) *V. faba*; (4) *V. michauxii*; (5) *V. narbonensis*; (6) *V. palaestina*; (7) *V. sativa*; (8) *V. sericocarpa*; (9) *V. villosa.*

DNA Extraction

Genomic DNA was extracted from refrigerated leaf samples at 25°C using the CTAB method of Aboelmaaty and Oraby (2019), which is a modification of the method of Doyle and Doyle (1987). The extraction began with the sample pulverization with liquid nitrogen. DNA quality and quantity were assessed using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA), and the absorbance ratios were used to determine the purity. The DNA samples were kept at -20°C until further analysis.

TABLE 1. Sample information of Iraqi *Vicia* species: voucher number, locality, coordinates, and collection date.

No.	Taxon	Collection Site	Coordinates (WGS84)	Date	Voucher number
1	*Vicia ervilia* L.	Byara–Tawela Road, Balkha, Sulaymaniyah, Iraqi Kurdistan	35°11′57.8″N 46°09′12.2″E	May 2022	1046
2	*Vicia faba* L.	Penjwen Road, Sulaymaniyah, Iraqi Kurdistan	35°26′36.6″N 45°52′36.1″E	April 2022	1047
3	*Vicia michauxii* Spreng.	Choma–Haji Omaran Road, Erbil, Iraqi Kurdistan	36°40′55.9″N 45°00′07.6″E	May 2022	1048
4	*Vicia narbonensis* L.	Kani Panka, Halabja–Sulaymaniyah Road, Iraqi Kurdistan	35°22′51.9″N 45°43′17.6″E	April 2022	1049
5	*Vicia palaestina* Boiss.	Ahmad Awa (The Way of Zalm), Halabja, Iraqi Kurdistan	35°18′36.7″N 46°03′57.9″E	March 2023	1050
6	*Vicia sativa* L.	Kani Panka, Sulaymaniyah Road, Iraqi Kurdistan	35°22′51.9″N 45°43′17.6″E	April 2022	1051
7	*Vicia sericocarpa* Fenzl	Blkian Road, Penjwen, Sulaymaniyah, Iraqi Kurdistan	35°36′19.2″N 45°57′44.8″E	April 2022	1052
8	*Vicia tenuifolia* Roth.	Gulan Mountain, Sulaymaniyah, Iraqi Kurdistan	3515′23.4″N 45°21′27.5″E	April 2022	1045
9	*Vicia villosa* Roth.	Przha Road, Choman, Erbil, Iraqi Kurdistan	36°34′52.0″N 44°59′28.2″E	July 2023	1053

PCR Amplification and Sequencing

The nuclear ITS region was amplified using the primers ITSp3F (5′-YGACTCTCGGCAACGGATA-3′) and ITSu4R (5′-RGTTTCTTTTCCTCCGCTTA-3′) following Cheng *et al.* (2016). The PCR reactions were undertaken under the following conditions: an initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s; and a final extension at 72 °C for 5 min. Successful amplification was confirmed by electrophoresis on 2% agarose gels stained with ethidium bromide.

The PCR products were purified and sequenced from both directions by Macrogen Inc. (Seoul, South Korea) using the Sanger method according to the company's standard protocols. High-quality sequences were submitted to the NCBI GenBank database, with accession numbers appearing in Table 2.

ITS Sequence Alignment, Genetic Distance Analysis, and Phylogenetic Inference

Raw ITS sequences were edited in BioEdit v7.2.5 (Hall 1999) to remove ambiguous bases, and multiple alignments were generated with ClustalW in MEGA v11 (Kumar *et al.* 2018) under default parameters. Alignments were visually inspected and adjusted manually where necessary.

To improve phylogenetic robustness, additional sequences (n = 24, including the outgroup) were retrieved from GenBank, representing the same or closely related *Vicia* species from diverse regions. Sequences were selected based on ≥97% identity and ≥95% query coverage with the studied specimens (Raveendar *et al.* 2017). Accession numbers and details are provided in Table 2.

For the nine Iraqi *Vicia* species, sequence length and GC content were calculated in MEGA v11. Pairwise genetic distances were estimated under the Kimura 2-parameter (K2P) model, chosen by Bayesian Information Criterion. Barcode gap analysis was performed following Rather *et al.* (2023) and visualized in histograms. Species-level identification success was further assessed using TaxonDNA v1.8 (Meier *et al.* 2006) with Best Match (BM) and Best Close Match (BCM) criteria at a 1% threshold, and with the All-Species Barcodes test at a 3% threshold.

Phylogenetic relationships were inferred using the Maximum Likelihood (ML) method in MEGA v11 under the K2P model, with 1,000 bootstrap replicates. *Lathyrus cassius* was used as the outgroup taxon because it belongs to the same family (Fabaceae) as *Vicia* but represents a distinct genus that has been consistently employed as an outgroup in previous phylogenetic studies of the group. Bootstrap values ≥70% were considered to indicate reliable clades.

TABLE 2. List of Iraqi and reference Vicia sequences used in this study with GenBank accession numbers (including the outgroup).

No.	Taxon	GenBank ITS Accession	Origin (Country)
1	***Vicia ervilia* L.**	**PP218517**	**Iraq**
2	*V. ervilia*	KJ787181	Iran
3	***Vicia faba* L.**	**PP218518**	**Iraq**
4	*V. faba*	KJ787180	Iran
5	*V. faba*	FJ212318	China
6	*V. faba*	HM470590	Iran
7	***Vicia michauxii* Spreng.**	**PP218520**	**Iraq**
8	*V. michauxii*	KJ787163	Iran
9	*V. michauxii*	HM470630	Iran
10	***Vicia narbonensis* L.**	**PP218522**	**Iraq**
11	*V. narbonensis*	KJ787157	Iran
12	*V. narbonensis*	HM470591	Iran
13	*V. narbonensis var. narbonensis*	HM470592	Iran
14	***Vicia palaestina* Boiss.**	**PP218523**	**Iraq**
15	*V. palaestina*	MN736419	Turkey
16	*V. palaestina*	KJ864938	Palestine
17	*V. palaestina*	JX506267	Turkey
18	***Vicia sativa* L.**	**PP218525**	**Iraq**
19	*V. sativa*	OP467026	India
20	*V. sativa subsp. nigra*	HM470608	Iran
21	*V. sativa var. sativa*	KJ787134	Iran
22	***Vicia sericocarpa* Fenzl**	**PP218526**	**Iraq**
23	*V. sericocarpa*	KJ787133	Iran
24	*V. sericocarpa*	HM470629	Iran
25	***Vicia tenuifolia* Roth.**	**PP218514**	**Iraq**
26	*V. tenuifolia*	KJ787150	Iran
27	*V. tenuifolia*	HM470619	Iran
28	*V. tenuifolia subsp. dalmatica* (A.Kern.) Greuter	HM470636	Iran
29	***Vicia villosa* Roth.**	**PP218527**	**Iraq**
30	*V. villosa*	DQ312199	Iran
31	*V. villosa*	PV138118	China
32	*V. villosa*	HM470611	Iran
33	*Lathyrus cassius* |Outgroup	LC311098	Iran

Results

Sequence Characteristics

The ITS dataset comprised 33 sequences, including nine newly generated Iraqi accessions and 24 retrieved from GenBank, with *Lathyrus cassius* designated as the outgroup. The final alignment was 752 bp in length, of which 314 sites were constant, 41 variable, 41 parsimony-informative, and 36 segregating sites. The GC content among the Iraqi *Vicia* species showed moderate variation, ranging from 48.6% in *V. tenuifolia* and *V. ervilia* to 50.0% in *V. faba* and *V. narbonensis*. Intermediate values were observed in *V. palaestina* (49.2%), *V. michauxii* (49.7%), *V. sativa* (48.7%), *V. sericocarpa* (49.0%), and *V. villosa* (48.8%) (Table 3).

TABLE 3. Nucleotide composition and GC content of ITS sequences in the studied *Vicia* species.

No.	Studied Species	T(U)	C	A	G	Total	GC%
1	*V tenuifolia*	27.8	21.9	23.6	26.7	424	48.6
2	*V ervilia*	28.0	21.4	23.4	27.3	440	48.6
3	*V faba*	27.1	22.9	22.9	27.1	436	50.0
4	*V michauxii*	27.5	22.7	22.9	27.0	437	49.7
5	*V narbonensis*	28.3	22.7	21.7	27.3	428	50.0
6	*V palaestina*	29.4	22.5	21.4	26.7	378	49.2
7	*V sativa*	27.9	22.0	23.3	26.8	437	48.7
8	*V.sericocarpa*	27.9	22.2	23.1	26.8	437	49.0
9	*V villosa*	27.6	22.2	23.6	26.7	424	48.8

Intra- and Interspecific Genetic Divergence and Barcode Gap Analysis.

Intraspecific K2P distances varied across species. The lowest intraspecific divergence was observed in *V. sericocarpa* (mean = 0.002), whereas the highest occurred in *V. sativa* (mean = 0.018, max = 0.049). Several species showed negligible or zero intraspecific variation, including *V. ervilia* (represented by a single accession).

Interspecific divergences were substantially higher, ranging from **0.005 to 0.111**, with the lowest value between *V. tenuifolia* and *V. villosa*, and the highest between *V. ervilia* and *V. sativa*. Mean interspecific distances across species varied between 0.033 and 0.059, consistently exceeding average intraspecific values.

Barcode gap assessment revealed variable discriminatory efficiency of the ITS marker among the studied species (Table 4; Figure 2). Clear barcode gaps (✓) were detected in *V. ervilia*, *V. faba*, *V. narbonensis*, and *V. sericocarpa*, where the minimum interspecific distance exceeded the maximum intraspecific divergence. Conversely, species such as *V. tenuifolia*, *V. michauxii*, *V. palaestina*, *V. sativa*, and *V. villosa* exhibited no clear separation (✗), reflecting partial overlap between intra- and interspecific distances. Notably, *V. sativa* displayed the largest intraspecific divergence, further complicating species delimitation. (Table 5).

TABLE 4. Intra- and interspecific K2P distances, nearest-neighbor comparisons, and barcode gap outcomes among the studied *Vicia* species.

No.	Species	Min Intra	Max Intra	Mean Intra	Min Inter	Max Inter	Mean Inter	Nearest species	Barcode gap (✓/✗)
1	*V_tenuifolia*	0.000	0.025	0.011	0.005	0.089	0.033	*V_villosa*	✗
2	*V_ervilia*	NA	NA	NA	0.033	0.111	0.059	*V_michauxii*	✓
3	*V_faba*	0.000	0.009	0.003	0.012	0.074	0.035	*V_michauxii*	✓
4	*V_michauxii*	0.000	0.024	0.013	0.012	0.088	0.037	*V_faba*	✗
5	*V_narbonensis*	0.02	0.012	0.006	0.017	0.081	0.041	*V_faba*	✓
6	*V_palaestina*	0.000	0.019	0.007	0.017	0.098	0.045	*V_villosa, V_tenuifolia*	✗
7	*V_sativa*	0.002	0.049	0.018	0.014	0.111	0.046	*V_faba*	✗
8	*V.sericocarpa*	0.000	0.006	0.002	0.019	0.087	0.042	*V_faba*	✓
9	*V_villosa*	0.000	0.015	0.009	0.005	0.091	0.035	*V_tenuifolia*	✗

To further evaluate species-level resolution, identification success was assessed using TaxonDNA (Best Match/Best Close Match). At the 1% threshold, the ITS dataset (n = 32) achieved high performance: 29 sequences (90.62%) were correctly identified, 1 (3.12%) was ambiguous, and 1–2 (3.12–6.25%) were misidentified depending on the criterion. Misassignments involved V. tenuifolia and *V. villosa*, which showed reciprocal matches at low divergence (0.46–0.99%), and one *V. michauxii* sequence that matched *V. sativa* at 1.62%. An additional ambiguous case was observed in *V. villosa*, reflecting its close relationship with *V. tenuifolia*. These results corroborate the barcode gap analysis, demonstrating moderate-to-high discriminatory power of ITS, but reduced resolution in closely related lineages.

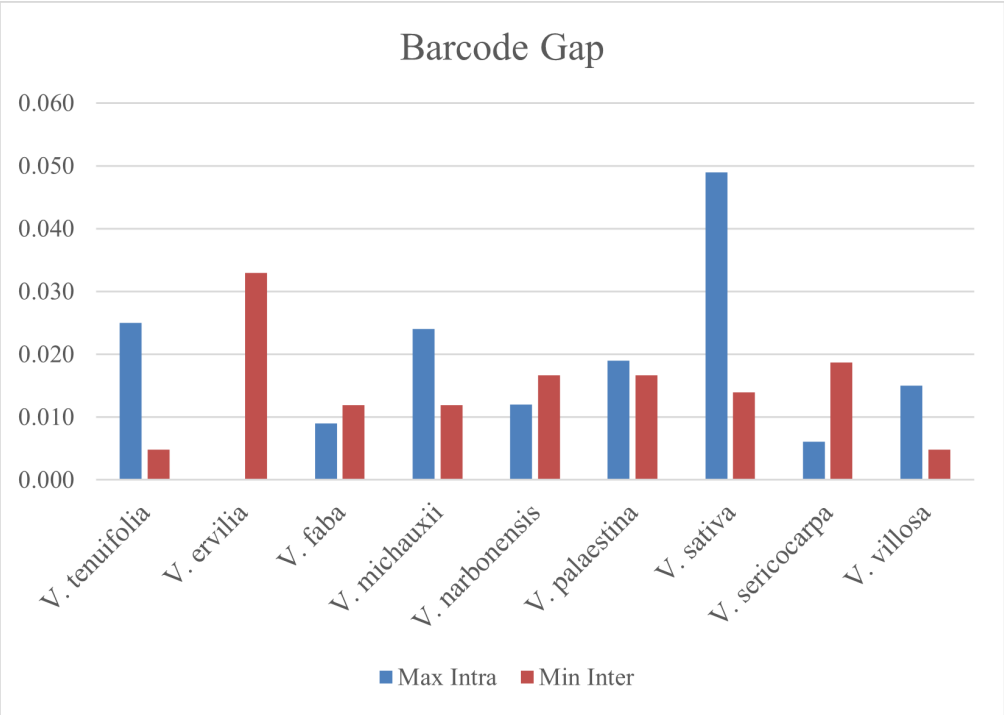

FIGURE 2. Barcode gap analysis showing maximum intraspecific (blue) and minimum interspecific (red) K2P distances for each *Vicia* species.

The All-Species Barcodes test implemented further evaluated the discriminatory efficiency of ITS across the dataset. The analysis showed that 18 sequences (56.25%) were correctly identified at the species level, whereas 13 sequences (40.62%) yielded ambiguous identifications due to overlap among closely related taxa. Only a single case (3.12%) represented an incorrect assignment. These results highlight the limited resolution of ITS in certain lineages, particularly where intra- and interspecific divergences overlapped, consistent with the outcomes of the barcode gap and BM/BCM analyses. Figure 3.

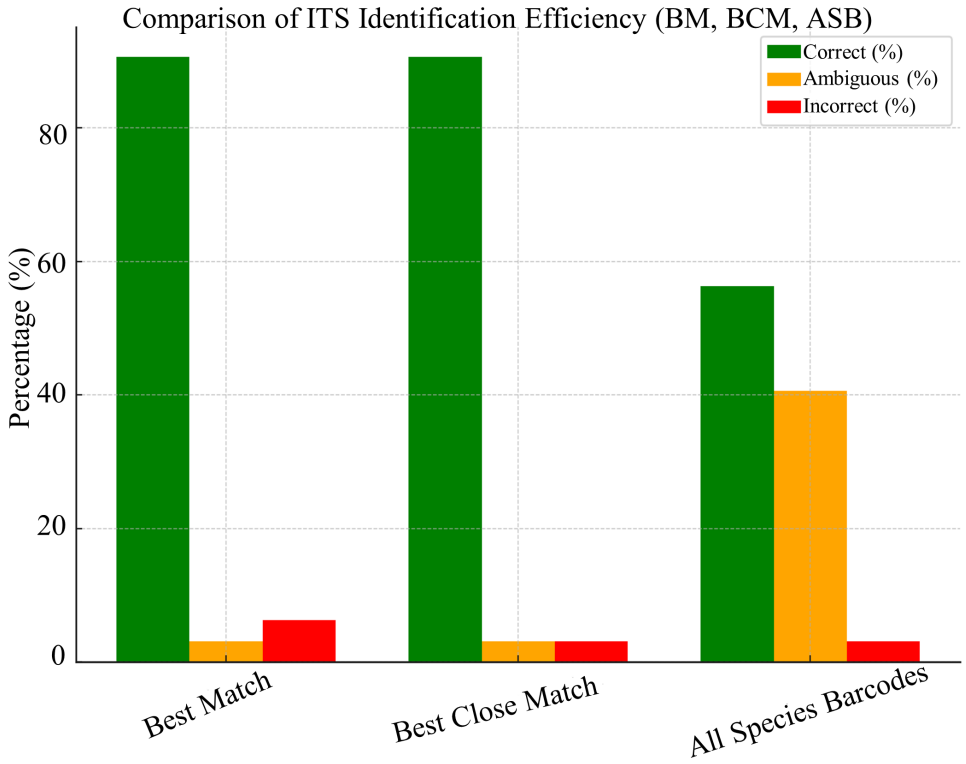

FIGURE 3. Results of the All-Species Barcode (ASB) test showing proportions of correct, ambiguous, and incorrect identifications.

Phylogenetic Inference

The Maximum Likelihood (ML) tree reconstructed from the ITS dataset under the Kimura 2-Parameter (K2P) model with 1,000 bootstrap replicates provided insights into the phylogenetic placement of the Iraqi *Vicia* accessions (Figure 4). Overall, the topology largely supported their molecular identification and revealed affinities with conspecific sequences from neighboring regions.

Vicia tenuifolia clustered with Iranian accessions, forming a moderately supported clade (BS = 32). *V. palaestina* grouped with Turkish and Palestinian sequences, albeit with weak to moderate support (BS = 24–38). *V. villosa* formed a distinct cluster with multiple GenBank accessions (BS = 62–81), showing a close relationship with *V. tenuifolia*. *V. narbonensis formed a monophyletic cluster with moderate to high support (BS = 33–95), clearly separating the Iraqi accession from related GenBank sequences.*

V. ervilia formed a strongly supported clade with Iranian references (BS = 100), confirming species identity. Both *V. faba* and *V. michauxii* clustered with their respective GenBank sequences with high support (BS = 92–94). *V. sativa* accessions grouped into a robustly supported clade (BS = 99) encompassing local and reference samples. Similarly, *V. sericocarpa* clustered tightly with Iranian accessions (BS = 99), demonstrating high congruence with GenBank data.

Taken together, the ML analysis confirmed that most Iraqi accessions reliably clustered with their conspecific sequences from adjacent geographic regions, reinforcing their molecular identification. However, the lack of strong separation between certain lineages—particularly *V. tenuifolia* and *V. villosa*—is consistent with the absence of barcode gaps, underscoring the limited discriminatory power of ITS for resolving closely related *Vicia* taxa.

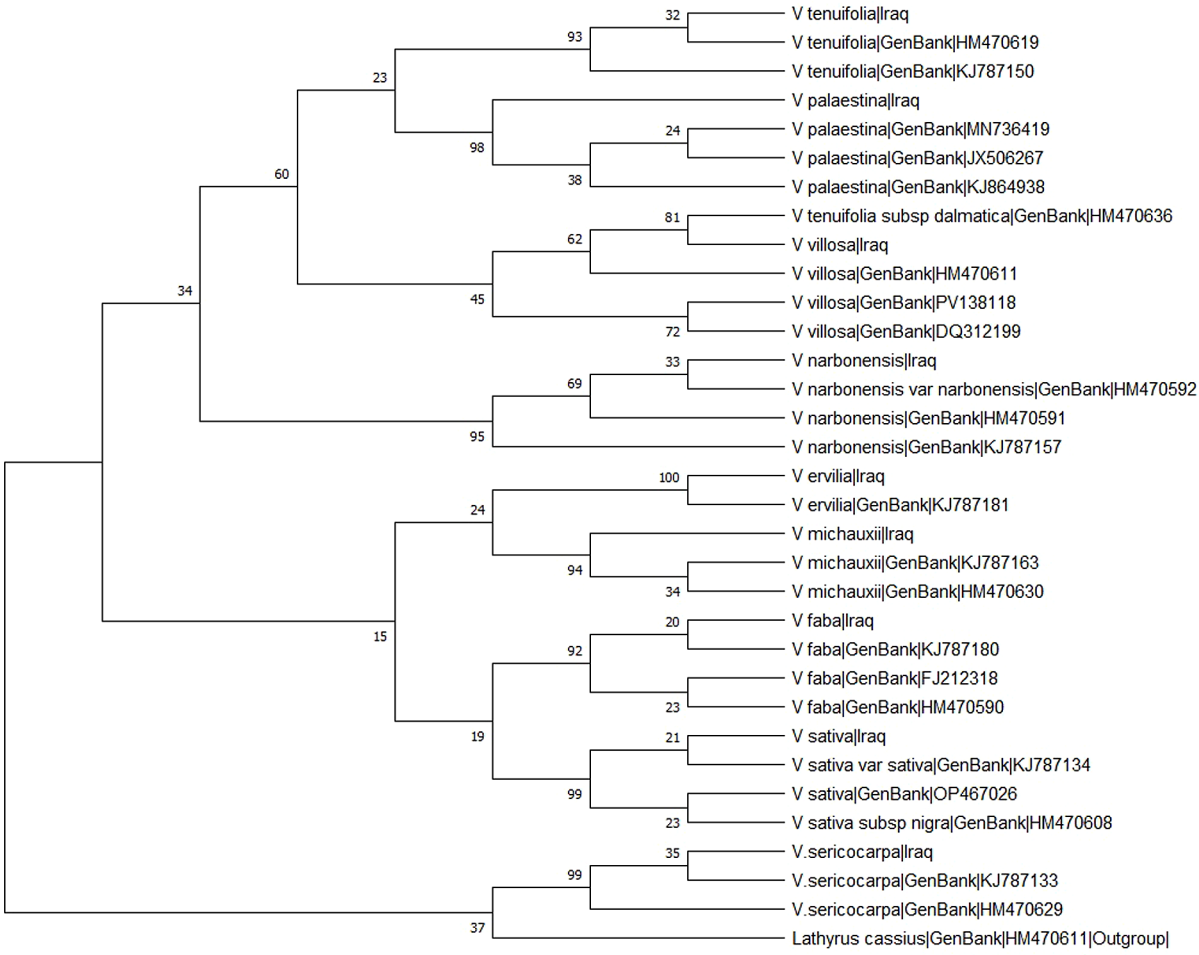

FIGURE 4. Maximum Likelihood (ML) tree of *Vicia* species based on ITS sequences (K2P model, 1,000 bootstrap replicates). Bootstrap support values >20% are shown at the nodes. Iraqi accessions are indicated alongside GenBank references, with *Lathyrus cassius* designated as the outgroup.

Discussion

This study represents the first molecular evaluation of nine *Vicia* species from Iraqi Kurdistan using the nuclear ITS region. The results highlight both the strengths and limitations of ITS as a DNA barcode: it provides useful resolution for several taxa but fails to discriminate among closely related or recently diverged lineages.

Sequence Variation and GC Content

The ITS dataset showed a moderate level of sequence variation, including 41 parsimony-informative sites, along with GC content that varied between 48.6 and 50.0 percent. This compositional stability reduces the risk of bias in phylogenetic analyses and affirms the dependable use of ITS for comparative analyses. Comparable values have been noted in other legume studies (Cheng *et al.*, 2016; Besse, 2014), further supporting its effectiveness as a universal barcode.

Intra- and Interspecific Divergence

Barcode gap analysis demonstrated variable discriminatory power of ITS among the studied taxa. Clear barcode gaps were observed in *V. ervilia*, *V. faba*, *V. narbonensis*, and *V. sericocarpa*, where interspecific divergence exceeded intraspecific variation, supporting accurate delimitation. Conversely, *V. tenuifolia*, *V. villosa*, *V. palaestina*, *V. michauxii*, and *V. sativa* lacked a barcode gap, indicating partial overlap between intra- and interspecific distances. Notably, *V. sativa* exhibited the highest intraspecific divergence (mean = 0.018, max = 0.049), suggestive of cryptic diversity, hybridization, or incomplete lineage sorting. Comparable findings were reported by Wu *et al.* (2020) in extensive datasets of *Vicia*, where closely related species displayed overlapping divergences, complicating species delimitation.

Species identification success

TaxonDNA analyses yielded ~90% correct identifications under BM and BCM criteria, but the All-Species Barcodes test achieved only 56% success, with high ambiguity in *V. tenuifolia* and *V. villosa*. This discrepancy reflects the limited discriminatory power of ITS in certain complexes, in agreement with earlier studies on Fabaceae (Raveendar *et al.* 2017; Bosmali *et al.* 2022). Misassignments between *V. tenuifolia* and *V. villosa* mirror their morphological similarity and suspected shared ancestry, also noted by Shiran *et al.* (2014) and Kaplan *et al.* (2021).

Phylogenetic Inference and Regional Affinities

The Maximum Likelihood tree largely confirmed the molecular identity of Iraqi accessions, which clustered with conspecific sequences from adjacent regions. Strong bootstrap support (>90%) characterized clades of *V. ervilia*, *V. faba*, *V. michauxii*, *V. sativa*, and *V. sericocarpa*, corroborating their clear taxonomic boundaries. In contrast, weak to moderate support values in *V. tenuifolia*, *V. villosa*, and *V. palaestina* reflected the absence of barcode gaps and highlighted the difficulty of resolving recently diverged or hybridizing taxa. These results are consistent with the phylogenetic ambiguities previously documented by Van de Wouw *et al.* (2001) and Wu *et al.* (2021), which emphasized the need for multilocus or genomic-scale data to untangle complex lineages within *Vicia*.

Our findings corroborate the conclusions of Raveendar *et al.* (2017), who found ITS moderately effective but insufficient as a sole barcode for *Vicia*. Similarly, Bosmali *et al.* (2022) and Wu *et al.* (2020, 2021) emphasized that the combination of ITS with plastid markers such as *matK* and *rbcL* significantly enhances resolution. The congruence between our results and regional studies (Omar *et al.*, 2019; Han *et al.*, 2021) suggests that while ITS can confirm species boundaries in divergent taxa, it is inadequate for species complexes were recent radiation and introgression blur genetic boundaries.

Comparison with previous studies

Our results align with those of Raveendar *et al.* (2017), who found ITS moderately effective but insufficient as a standalone barcode for *Vicia*. Bosmali *et al.* (2022) and Wu *et al.* (2020, 2021) further showed that combining ITS with plastid markers (matK, rbcL) significantly enhances resolution. Similarly, regional studies (Omar *et al.* 2019; Han

et al. 2021) confirmed ITS utility for boundary confirmation but highlighted its inadequacy in complexes shaped by recent radiation or introgression.

Implications and Future Perspectives

Despite its limitations, ITS was intentionally selected here due to its cost-effectiveness, ease of amplification, and suitability in regions where molecular data are lacking. This study therefore provides the first molecular framework for *Vicia* in Iraq and supplies GenBank reference sequences that can aid future identification and comparative analyses.

ITS should be regarded as a preliminary but insufficient marker for robust taxonomy. Future studies should integrate multilocus barcoding and next-generation sequencing approaches, such as the Angiosperms353 probe set (Johnson *et al.* 2019), which has shown high potential for resolving both deep and shallow phylogenetic relationships. Expanding sampling across the heterogeneous landscapes of Iraqi Kurdistan will also improve phylogenetic resolution and conservation planning, as some species may harbor cryptic diversity or hybrid lineages.

Conclusion

In summary, the ITS region provides valuable support for species identification and phylogenetic reconstruction in Iraqi *Vicia* but lacks sufficient discriminatory power for closely related taxa. Multilocus or genomic-scale approaches are required to achieve robust species delimitation and to guide the conservation of plant genetic resources in this biodiversity-rich region.

Authors' contributions

All authors contributed equally to the design, execution, and analysis of the study. All authors reviewed and approved the final version of the manuscript.

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