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Assessment of Genetic Variation using DNA-Based Molecular Markers and Morphological Traits in Vicia L. Species Growing in Iraq

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ABSTRACT

The genus Vicia L. of the Fabaceae family, commonly known as "vegetables", includes wild and cultivated species that are of great ecological, agronomic, and economic importance. In Iraq, especially in the Kurdistan region, wild Vicia species constitute an important part of the natural flora and play a significant role in increasing biodiversity, agricultural sustainability through biological nitrogen fixation, and forage production. Despite this importance, genetic diversity of these species, especially at the molecular level, have not been comprehensively investigated so far. The present study, in order to more accurately and comprehensively assess the Vicia species in the Kurdistan region of Iraq, 14 wild species were collected from different regions and examined with a combined approach including classical morphological data and modern molecular methods. First, the morphological characteristics of the vegetative and reproductive organs of the samples were quantitatively evaluated and phenotypic relationships between species were investigated using multivariate statistical analysis. The results indicated high morphological diversity between species, but also overlap of traits and phenotypic similarities were observed in some closely related species, which makes classification based solely on morphology a challenge. To solve these challenges, DNA barcoding was used using two gene regions: the ITS region (internal transcribed spacer region in nuclear DNA) and the COI gene (subunit I of the cytochrome oxidase enzyme in mitochondrial DNA). The ITS region was able to show high interspecific diversity and was effective in creating phylogenetic trees. The COI gene, although less variable, also provided useful supplementary information. Phylogenetic trees were created using the Maximum Likelihood method and evaluated using Bootstrap analysis. The PIC index and genetic distance matrices were calculated and phylogenetic trees were constructed based on them. All confirmed sequences were submitted to the Gene Bank database (affiliated with NCBI) and accession numbers were issued for them, which will play an important role in future conservation, taxonomy and plant breeding studies. Overall, this study,

as the first comprehensive study on molecular taxonomy and genetic diversity of Vicia species in Iraq, provides a scientific basis for breeding programs, conservation of genetic resources and future phylogenetic research in arid and semi-arid regions.

***Key words*:** DNA-based markers, Vicia species, Genetic diversity, Morphological attributes, Breeding strategies, Cluster analysis.

INTRODUCTION

Since ancient times, people have turned to plants for their basic needs of sustenance, clothing, medicine, and even energy. Initially, to classify plants, they would focus on most of the obvious, external traits. As knowledge progressed, more complex internal and chemical characters were introduced in classification systems; thus, the taxonomy became more explicit. In recent decades, Angiosperms have been explored with modern tools. More than 350,000 species are known, making this the most diverse plant group. Fabaceae ranks third with around 19,500 species and 750 genera, exhausted Championship, with Orchidaceae and Asteraceae occupying the first two ranks (Willis 2017).

Legumes, members of the Fabaceae family, are appreciated particularly for their nutritional values. They provide proteins and have a high-fat content, with fibers and micronutrients for further benefits. Studies tend to demonstrate that these nutrients decrease the risk of some diseases and, in some instances, might be anti-cancer. Agriculturally, legumes are considered valuable because they increase the fertility of soils by fixing nitrogen, through the mediation of rhizobia bacteria. Legumes find uses beyond the culinary world and agriculture. Their parts come in handy in the production of pharmaceutics and other industrial products, which makes them significant in contemporary society at large (Raveendar *et al*. 2015; Velazquez *et al*. 2010).

Morphological evaluation of genetic diversity of some wild species of Vicia L. in Iraq

Morphological data always play a key role in the classification of plant species based on phylogeny. However, numerous morphological studies do require an intensive knowledge of characters. (Scotland *et al*. 2003). To comprehend fully the evolution of an organism and particularly in the plant community, the following should be considered: phenotypic traits, genomic data, and geographic distribution. By bringing these data sets together, researchers can develop a more holistic view of the evolutionary history of an organism and relate it to how it has adapted and changed (Bouckaert *et al*. 2019).

Throughout history, the taxonomy of the Vicia L. genus has remained relatively stable, with its classification theme remaining unchanged for over a century. While early taxonomists such as Linnaeus (1753) grouped these plant species under two genera (Vicia L. and Ervum), modern classifications have reached a consensus regarding their grouping (Hanelt and Mettin 1989).

Linnaeus, a renowned botanist, recognized two groupings of the genus Vicia in 1753. One of these groupings was equivalent to the subgenus Vicia, while the other consisted of two taxa of sect. Hypechusa, namely V. hybrida and V. lutea. Anops this classification system

provided a valuable framework for botanists to study and understand the genus Vicia (Shehbala 2021).

Mohamad (2010) also studied the genus Vicia in Iraq and conducted a systematic comparative study of 23 species and four varieties of the genus. Studies of macro and micromorphology, cytology, chemistry, ecology and geographical distribution of all taxa of this genus were carried out. Furthermore. Al-Joboury (2017) studied the five species of the Vicia genus in Iraq by describing morphological characters, pollen grains, and geographical and numerical taxonomy, and verifying the interspecific relationships.

Molecular investigation of genetic variation of some wild species of Vicia L. in Iraq

Molecular taxonomy is an essential scientific discipline that helps in the classification and understanding of relationships among plants (Simpson 2019) This discipline relies on molecular comparison of proteins, nucleic acids, and RNA to classify plants into families and clans and also to study the relationships among different orders. Molecular taxonomy is needed because it provides accurate and objective information regarding the genetic constitution and evolutionary history of plants. Introducing the molecular perspective provided by DNA sequence data, it is complementary to traditional morphological classification (Wei *et al*. 2021). In essence, several vital considerations contributed to the emergence of molecular taxonomy.

As this great leap occurred in the history of biology, the door was opened to look into the genetic makeup of organisms, allowing them to dig further into their evolutionary background. Apart from all, the progress in technology and the widespread availability of more advanced devices have accelerated this alternation of the study (Galimberti *et al*. 2012). In plant taxonomy setting. Molecular techniques have gained eminence in this modern era, as they serve to sort out individual organisms within populations and various populations. While morphological characters still play a crucial role in plant systematic studies, such as physical traits and structures they are now accompanied by molecular techniques for taxonomic classification (Verma *et al*. 2022).

Thanks to significant technological developments in genetics during the 1940s and 1950s, a decisive change occurred in the ways botanists practice inferring the evolutionary history of species. These new methods took advantage of the increasing diversity of character sources to consider. Biology has changed dramatically as a result of the invention of Karry Mullis in 1986, the Polymerase Chain Reaction (PCR), which featured the ability to target certain genomic regions for selective DNA amplification (Rouhan and Gaudeul 2021).

Rapid development and evolution have occurred in molecular tools in the past few years, making it easier and more affordable to identify and study the diverse taxa of plants known to exist. Through these tools, scientists can now answer many questions about the evolution and taxonomy of plants, questions that previously could not be addressed by strictly phenotypic means. Among these technologies are random amplified polymorphic DNA (RAPD), DNA barcoding, amplified fragment length polymorphism (AFLP), microsatellites, and single nucleotide polymorphisms (SNP), which may be applied to the Investigating the variety of

plants. (Arif *et al*. 2010). In recent years, the new generation of technologies has developed mainly by hybridizing earlier methodologies (Agarwal *et al*. 2008).

Genetic analysis by DNA Barcoding (ITS) region

Species identification is essential in the ever-changing world. Traditionally, methods of species identification, such as those based on morphometry, were advantageous but quite time consuming and often required specialized knowledge. Molecular biology has thus made the process of species identification quick and easy. (Koima *et al*. 2023). One such approach is DNA barcoding, which involves sequencing a short DNA region specific to the individual species. These DNA barcodes are standardized short sequences of DNA: each is within the range of 400 to 800 base pairs long. DNA barcodes are an invaluable and reputable resource that helps in dealing with the problems faced by botanists in plant systematics, field ecology, evolutionary biology, conservation, and applied forensics. They offer a fast, systematic, precise method for the identification of plant species that have already been identified, described, and named (Kress 2017).

Molecular tools employing DNA barcodes can allow taxonomists to bypass the obstacles that arise in the processes of Identification and delimitation of taxonomic species., and accessing phylogeny (Martínez-Arce *et al*. 2020). DNA barcode is used together with classical morphology, so it is essential that morphological data be concurrently used with DNA sequence data. Morphological and molecular character systems are considered good methods of species identification (Han *et al*., 2021). In the first couple of years this century, DNA barcodes for animals were envisioned and employed based on the use of universally recoverable DNA segments (Wei *et al*. 2021). The generally agreed-upon standard DNA barcodes today for plants are (matK, trnH-psbA, and ITS) (Li *et al*. 2015).

Nuclear ribosomal RNA genes (nrDNA) have become a useful tool In the field of plant taxonomy and phylogenetics over the past years. The reason is that nrDNA contains regions that are differently conserved, rendering them informative at various levels of taxonomic inquiry. In particular, the non-coding regions of nrDNA can be utilized to analyze plants at the species-to-genus levels. (Manzanilla *et al*., 2018). Another argument for supporting nrDNA would be that it is easy to amplify with PCR primers located within the coding regions that have been conserved flanking the spacer region is more flexible. Ribosomal genes are arranged in tandem repeats that undergo concerted evolution in the array sequences at the levels of individuals, populations, and species; thus, there is expected to be low intraspecific variation (relative to interspecific variation), hence necessitating less intraspecific sampling effort (Al-Hemaid *et al*. 2015).

There is no study using molecular genetic techniques DNA Barcoding (ITS) region for genetic diversity assessment and taxonomic wild species of Vicia L growing in Iraq, so we reviewed similar and related literature studies for the last years that concerning to our study and targeted the genus Vicia or some of her species outside Iraq.

Wu *et al*. (2021) conducted a phylogenetic study based on DNA sequence evidence. Their methodology involved sequencing the nrDNA ITS region along with chloroplast markers like

matK, rbcL, and trnL-F. Genetic material, specifically seeds, was sourced from the National Herbage Germplasm Conservation Center of China (Beijing) and the Wild Species Germplasm Repository (Kunming, China). In total, 17 species belonging to the Vicia subgenus Vicilla had their nrDNA ITS and chloroplast regions sequenced for this research. The study aimed to clarify the phylogenetic placement of species within the Vicilla subgenus, thereby providing a theoretical foundation for the accurate classification of both the subgenus Vicilla and the broader genus Vicia.

Wardani *et al*. (2022) conducted a molecular taxonomy study using DNA barcoding to identify Fabaceae species in altered Indonesian landscapes. They evaluated matK and rbcL as DNA barcodes, successfully generating matK barcodes for 51 species (28 genera) and rbcL for 47 species (31 genera). Their findings showed that the combined matK+rbcL barcode achieved 90% accuracy for species identification, while matK alone reached 82.05%. matK proved to be the most diverse marker, with mean interspecific and intraspecific distances of 0.134 and 0.003, respectively.

Molecular evaluation using mitochondrial DNA (mtDNA) cytochrome c oxidase I (COI)

There is no study using molecular genetic techniques DNA Barcoding mitochondrial DNA (mtDNA) cytochrome c oxidase I (COI) for the taxonomy of wild species of Vicia L growing in Iraq. In recent years, mtDNA COI barcoding has been used to identify various species (Shafqat *et al*. 2020). It is more useful to analyze mitochondrial, plastid, and nuclear genomes separately and together when using available techniques and methods for plant phylogenetic analysis. (Barkman *et al*. 2000). We reviewed similar and related literature studies for the last years concerning our study.

In a study by Fazekas *et al*. (2008), eight plastid and one mitochondrial DNA region were compared for their potential as plant barcodes in discriminating 92 species from 32 genera of land plants (251 samples). They found that regions differ in their opportunity to discriminate species and in retrieval difficulty (amplification/sequencing success). Single-locus species resolution was from 7% (23S rDNA) to 59% (trnH-psbA). The plastid loci had highest recovery, from 85 to 100%, with matK being most difficult to amplify, and of course, many loci, including matK, posed significant sequencing problems for complete bidirectional sequencing. Species resolution was improved by combining more variable plastid markers, but this came at diminishing returns; the increase that combinations of four to seven regions gave compared with combinations of two or three regions was negligible (69–71%). The plateauing performance suggests fundamental upper limits on species discrimination precision with moderate numbers of plastid markers. Therefore, solving the plant barcoding conundrum will preferably consider practical matters such as ease of sequence recovery, global alignability, and redundancy of markers in a multilocus system.

Shehbala (2021) used housekeeping genes, nuclear ribosomal internal transcribed spacer (ITS2), and mitochondrial cytochrome c oxidase subunit I (Cox1) to examine the evolutionary relationship among 24 species of Vicia . Plant DNA was extracted and amplified using gene-specific primers, followed by PCR product cleanup with Gene JET. pGEM-T vector was used

to clone and sequence the cleaned DNA fragments. Maximum likelihood phylogenetic trees were constructed using CLUSTAL W and MAGA-X. There were few paraphyletic groups among the species, and most were monophyletic. In section Narbonensis, species were located at the end of the tree, while V. faba was located at the top of the tree. Instead of forming a clade with section Cracca, V. hirsuta formed one with section Narbonensis. The species of section Ervum grouped with those of section Cassubicae and Panduralae instead of being separated. In conclusion, this work showed the molecular phylogenetic tree of 24 selected species of genus Vicia which are able to provide more acceptable details to classify as compared to previous Vicia morphological phylogenetic tree. ITS2 was a more informative tool than Cox1 for dealing with phylogenetic relationships.

MATERIALS AND METHODS

Sampling and Collection of Plant Material

The scientific material for accomplishing the various aspects of this study was directly collected from the field. Fourteen species of wild-growing Vicia were collected in Iraq through field trips. Five field trips were conducted between April 2022 and July 2023, each lasting between 3-5 days. These trips covered most of the accessible provinces in Iraqi Kurdistan where the studied Vicia species are distributed at specific elevations and in different environments (Table 1).

According to Al-Hadeethi *et al*. (2021) and Al-Hadeethi *et al*. (2019), the collected samples were pressed using specialized botanical presses designed for this purpose. In the laboratory, the samples were accurately identified based on the available keys provided to the researcher.

DNA Extraction

The plant DNA extraction protocol was performed according to the modified cetyltrimethylammonium bromide (CTAB)-based method developed by Aboul-Maaty and Oraby (2019). For DNA extraction, the leaf sample was frozen at -25°C, and 250 mg of these leaves were ground with liquid nitrogen using a mortar and pestle until finely ground. CTAB lysis buffer (1 ml) was added to the leaf powder still on the mortar and gently mixed with the pestle. The sample mixture was transferred to microcentrifuge tubes and incubated at 65°C for one h in a water bath, inverting the tubes several times every 20 min. The tubes were then removed from the bath and allowed to cool to room temperature (~25°C). After cooling, chloroform: isoamyl alcohol (24:1 v/v) was added to an equal volume and mixed inverted. The mixture was then centrifuged at 26,500 g for 15 min. transfer the upper aqueous phase carefully to a new microcentrifuge tube. Repeat steps 6–8 if the upper aqueous phase is not clear to ensure DNA purity. Add 6M NaCl to half the resulting volume of the aqueous phase and mix well.

Next, add 1/10th of the initial volume (around 70 µL) of potassium acetate with a concentration of 3M. After that add 500 µL of ice-cold 100% isopropyl alcohol (approximately 2/3 of the aqueous phase volume) Mix gently by inversion to precipitate DNA and incubate the tubes at -20°C for 30 minutes. Then, centrifuge the tubes at 26,500 x g for 5 min, remove the supernatant, and place the tubes upside down on tissue paper to drain any remaining supernatant. Wash the DNA pellet by adding 500 µL of 70% ethanol, invert once to remove residual salts, and centrifuge again at 26,500 x g for 5 min. Discard the 70% ethanol and allow the pellet to dry briefly at room temperature, taking care not to over dry. Finally, add 50 µL of 1X TE buffer, incubate the samples at 50°C for 1–2 h to ensure complete re-suspension, and store the extracted DNA at -20°C until further use. The extracted DNA was assessed for quality and quantity using 1 µl of the sample in the Nano Drop Lite Spectrophotometer, manufactured by Thermo Fisher Scientific Inc. Based in Wilmington, DE, USA.

Polymerase Chain Reaction (PCR) and Sequences

Prepare primers

To amplification the ITS regain, CoxI genes, two primers were used. The primers were prepared according to the instructions provided with the kits, which were imported from Macrogen Company in a lyophilized, freeze-dried form. The primers were dissolved with the nuclease-free water enzyme to give 100 pmol/ml of the stock solution of the primers. The primer solution was prepared by adding 10 µL of alginate stock to 90 µL of nuclease-free water to obtain a ready-to-work primer solution with a concentration of picomoles/microliter. The PCR reaction for ITS and coxI gene was started with a 5 min heating at 94°C, followed by 35 cycles consisting of 94°C denaturation for 30 s, primer annealing at 60 °C for 30 s, and extension at 72°C for 40 s. Reactions ended with a final step of 5 min at 72 °C.

PCR amplification conditions for ITS region and CoxI gene

25 µL of the PCR mix was prepared in a 1.5 ml Eppendorf tube. The master mix was ready to use and contained 10 µL of (Taq polymerase, dNTPs, buffer) 2 µL of reverse and forward primers (Table 2), 2 µL template DNA, 10.5 µL of nuclease-free water, and 0.5 µL of MgCl2.

Table 1. Plant Species used in this study and site of collection, GPS Source

No.	Taxa	Collection site	GPS Source	Date
1	*V.tenuifolia* Roth.	Gulan Mountain, Sulaymaniyah, Iraqi Kurdistan	35°15'23.4"N 45°21'27.5"E	April - 2022
2	*V.angustifolia* L.	Basan, Choman, Erbil, Iraqi Kurdistan	36°36'13.5"N 44°55'59.8"E	July - 2023
3	*V.cracca* L.	Basan, Choman, Erbil, Iraqi Kurdistan	36°36'13.5"N 44°55'59.8"E	July - 2023
4	*V.ervilia* L.	Byara - Tawela Road, Balkha, Sulaymaniyah, Iraqi Kurdistan	35°11'57.8"N 46°09'12.2"E	May - 2022
5	*V.faba* L.	Penjwen Road - Sulaymaniyah, Iraqi Kurdistan	35°26'36.6"N 45°52'36.1"E	April - 2022
6	*V.hybirda* L.	Blkian Road, Penjwen, Sulaymaniyah, Iraqi Kurdistan	35°36'19.2"N 45°57'44.8"E	April - 2022
7	*V.michauxii* Spreng.	Choma-Haji Omaran Road, Erbil, Iraqi Kurdistan	36°40'55.9"N 45°00'07.6"E	May - 2022
8	*V.monantha* Retz.	Chamchamal, Sulaymaniyah, Iraqi Kurdistan	35°32'17.3"N 44°50'52.8"E	March - 2023
9	*V.narbonensis* L.	Kani Panka, Halabja-Sulaymaniyah Road, Iraqi Kurdistan	35°22'51.9"N 45°43'17.6"E	April - 2022
10	*V.palaestina* Boiss.	Ahmad Awa, The Way of Zalm, Halabja, Iraqi Kurdistan	35°18'36.7"N 46°03'57.9"E	March - 2023
11	*V.peregrina* L.	Amedi, Duhok, Iraqi Kurdistan	37°05'44.1"N 43°29'46.1"E	June - 2023
12	*V.sativa* L.	Kani Panka, Halabja-Sulaymaniyah Road, Iraqi Kurdistan	35°22'51.9"N 45°43'17.6"E	April - 2022
		Byara - Tawela Road, Sulaymaniyah, Iraqi Kurdistan	35°11'18.2"N 46°10'41.0"E	March - 2023
13	*V.sericocarpa* Fenzl	Blkian Road, Penjwen, Sulaymaniyah, Iraqi Kurdistan	35°36'19.2"N 45°57'44.8"E	April - 2022
14	*V.villosa* Roth.	Weze, Erbil, Iraqi Kurdistan	36°35'23.4"N 44°59'06.1"E	July - 2023
		Choman, Przha Road, Erbil, Iraqi Kurdistan	36°34'52.0"N 44°59'28.2"E	

Table 2. Primers used for amplification of the ITS region and the Cox I gene

Gene name	Primer name	Sequence 5′ to 3′	Reference
ITS	ITSp3F ITSu4R	YGACTCTCGGCAACGGATA RGTTTCTTTTCCTCCGCTTA	Cheng *et al*., (2016)
CoxI	Coxf CoxR	GGATCTTCTCCACTAACCACAA CCGAAAGAGATGCTGGTATA	Fazekas *et al*., (2008)

RESULTS

Phylogenetic analysis of the combination of the ITS region and COX1

The combined ITS region and COX1 gene sequences show slight variation in length. The average length is 1072 bp, but the sequences range from 1063 bp to 1079 bp with a standard deviation of 6.0 bp and the ratio of G to C was 45.5%. The analysis of nucleotide diversity (□) was (0.0110) and the nucleotide Tajima's test (D) was (-1.0240).

The combined data exhibits a moderate degree of sequence similarity. There are 1,021 identical sites across all 14 sequences, constituting 94.6% of the total sequence length. Additionally, the pairwise identity between sequences is 98.3%. This value falls between the high similarity observed for COX1 alone (99.7%) and the moderate similarity of ITS alone (87.7%). Based on indicators of the combination of the ITS region and COX1, the percentage of genetic similarity and genetic distance between the studied species were calculated.

The highest genetic similarity (100%) appeared between *V. tenuifolia* and *V. monantha*, *V. angustifolia* and *V. sativa*, *V. cracca* and *V. villosa*, and *V. hybrida* with *V. peregrina* and *V. sericocarpa*, whereas the lowest genetic similarity (97%) appeared between *V. ervilia* and species *V.tenuifolia*, *V. cracca*, V.faba, *V.monantha*, and *V.villosa, V.villosa with* species *V. hybrida, V.narbonensis, V.peregrina, and V.sericocarpa. V.cracca with* species *V. hybrida, V.peregrina, and V.sericocarpa.*

Genetic distance values also showed variation among the species studied. The genetic distance value was the highest (0.023) between *V. ervilia* and *V. faba*, whereas the lowest genetic distance (0.000) appeared between *V. tenuifolia* and *V. monantha*, *V. hybrida* with *V. peregrina* .

Phylogenetic analysis of 14 *Vicia* species using maximum likelihood trees revealed clustering patterns, genetic diversity, significance, and deduced connections. The combination of the ITS region and COX1 separated the species into two groups. Cluster (I) is further subdivided into two branches; subgroup (IA) includes only *V. ervilia*, , whereas subgroup (IB) includes *V.angustifolia* and *V. Sativa.* Cluster (II) is further subdivided into two branches; subgroup (II A) is split into two subgroups: subgroup (IIA1) contains *V. hybrida, V.peregrina, V.sericocarpa, V.faba,and V.narbonensis*, , subgroup (IIA2) includes *only V. michauxii* . Subgroup (IIB) is split into two subgroups: subgroup (IIB1) contains *V. tenuifolia* and *V. monantha* and subgroup (IIB2) includes *V. palaestina , V. cracca and V.villosa* (Figure 1).

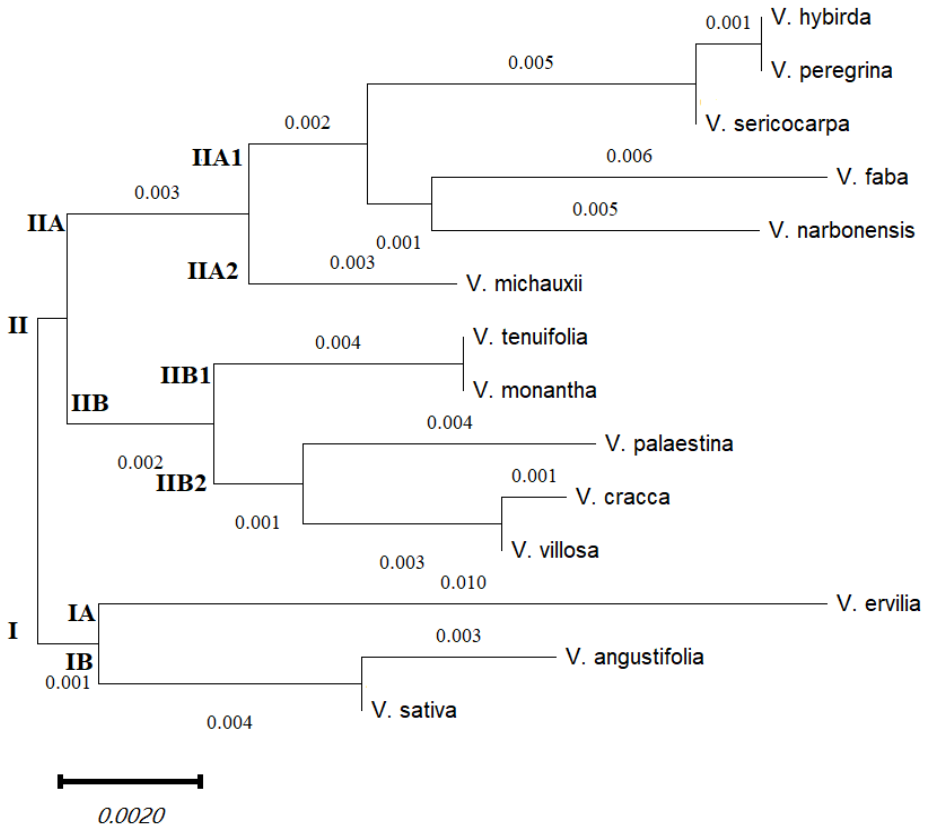

Figure 1. Phylogeny tree of studied species of genus Vicia based on the combination of the ITS region and COX1 between the studied Vicia species

Cluster analysis based on morphological characteristics

The dendrogram reveals distinct clusters and groupings among the Vicia species, suggesting varying degrees of morphological similarity and divergence with an average taxonomical distance coefficient of 0.75 The species were divided into seven groups (Figure 2): The first group includes three species was: V. tenuifolia, V. cracca, and V.villosa, Assessment of morphological characteristics revealed a high degree of similarity between V. tenuifolia and V. cracca, as evidenced by a similarity coefficient of 0.9. These two species shared 17 common characteristics. In contrast, V. villosa exhibited a lower degree of similarity with both V. tenuifolia and V. cracca, with a similarity coefficient of 0.82 and sharing only 11 characteristics with each. This suggests a potentially closer phylogenetic

relationship between V. tenuifolia and V. cracca compared to their relationship with V. villosa. The second group emerged, comprising V. angustifolia, V. sativa, and V. palaestina. Notably, V. angustifolia and V. sativa exhibited a high degree of morphological similarity, as evidenced by a similarity coefficient of 0.85. This close relationship is further supported by the presence of 14 shared characteristics.

Conversely, V. palaestina displayed a lower degree of similarity with both V. angustifolia and V. sativa. Specifically, V. palaestina shared a similarity coefficient of 0.80 with each of the aforementioned species, and only 10 common characteristics were observed. This suggests a potentially greater phylogenetic distance between V. palaestina and the other two species within this group. The third group. Within the third group, comprising V. hybirda, V. michauxii, and V. monantha, a distinct pattern of morphological similarity emerged. V. hybirda and V. michauxii exhibited a high degree of congruence, as evidenced by a similarity coefficient of 0.82. This close relationship is further corroborated by the presence of 13 shared morphological characteristics. Conversely, V. monantha displayed a lower degree of similarity with both V. hybirda and V. michauxii. Specifically, V. monantha shared a similarity coefficient of 0.80 with each of the aforementioned species, and only 9 common characteristics were observed.

This suggests a potentially greater phylogenetic distance between V. monantha and the other two species within this group, warranting further investigation into the evolutionary relationships within this species' complex. The fourth group comprised V. peregrina and V. sericocarpa. These species exhibit a high degree of similarity, sharing 11 common characteristics and demonstrating a similarity coefficient of 0.75. This suggests a close phylogenetic relationship between the two species. Within Group Five, V. ervilia exhibited a distinct taxonomic position. This separation was determined through a quantitative assessment of similarity to other Vicia species included in the study. Specifically, V. ervilia displayed a coefficient of similarity of 0.67, indicating a moderate similarity.

This value and an analysis of shared morphological characters with the remaining species supported its unique placement within the group. V. faba (Group six) exhibited high similarity with V. narbonensis (Group seven), demonstrated by a similarity coefficient of 0.69. These species share nine key characteristics, suggesting a close phylogenetic relationship. This pairing further aligned with V. ervilia at a similarity coefficient of 0.67. These three species collectively formed a clade with eleven other Vicia species at a similarity coefficient of 0.64. This larger clade occupied the most distant position within the dendrogram, indicating a greater degree of divergence from the remaining Vicia species.

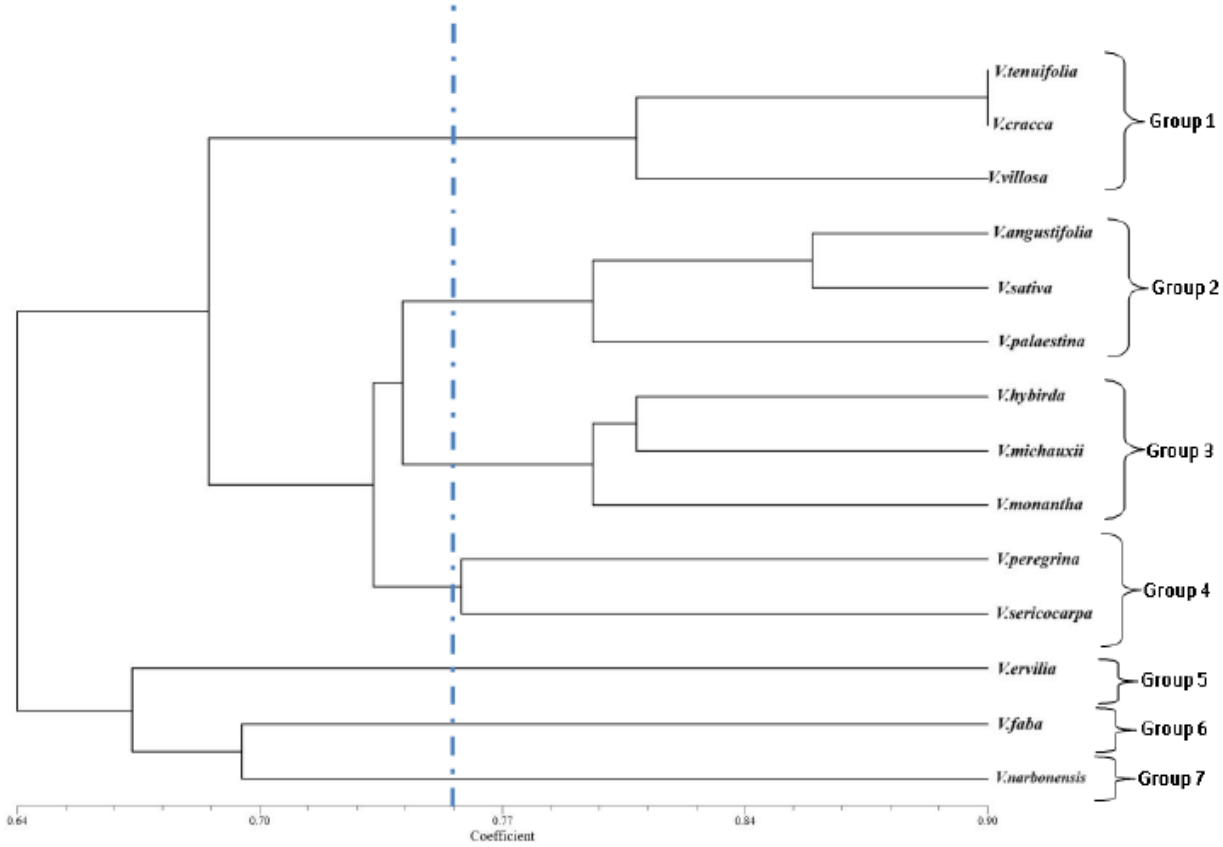

Figure 2. The dendrogram of 14 species of Vicia species generated based on morphological characteristics

DISCUSSION AND CONCLUSION

The present research aligns with previous studies emphasizing the effectiveness of the ITS region for Vicia species discrimination. The successful amplification and sequencing of the ITS region, generating sequences ranging from 378 to 440 bp, and its ability to differentiate between the 14 studied species, echoes the findings of Shiran *et al*. (2014), who also reported easy amplification and phylogenetic utility of ITS sequences in Vicia. The high GC content observed in the ITS region further supports its discriminatory power, as GC-rich regions are often associated with greater sequence variation. The study's findings also resonate with those of Raveendar *et al*. (2015), (2017), who advocated for the use of ITS2 (a component of the ITS region) as a DNA barcode for Vicia species identification. The present research reinforces the notion that ITS, and specifically ITS2, can serve as a valuable tool for resolving taxonomic complexities within this genus. The identification of species-specific markers within the ITS region, as demonstrated by the varying genetic similarities and distances between species, further supports its utility in Vicia taxonomy and aligns with the conclusions of Kaplan *et al*. (2021), who highlighted the importance of such markers for early detection and conservation efforts.

The study's exploration of the COI gene as a potential barcode for Vicia species yielded results that both agree and contrast with previous findings. The observation of high sequence conservation and limited discriminatory power of COI at the species level is consistent with the challenges reported in utilizing COI for plant DNA barcoding due to low mutation rates in mitochondrial genomes. The study's findings echo those of Fazekas *et al*. (2008), who emphasized the importance of considering practical considerations and discriminatory ability when choosing DNA barcodes for plants. The limited variation observed in the COI coding region, despite its successful amplification and sequencing, suggests that it may not be an ideal standalone barcode for Vicia species identification. However, the inclusion of COI data in combination with ITS sequences improved species resolution in phylogenetic analysis, supporting the conclusions of Bosmali *et al*. (2022) and others who advocated for multi-locus approaches in Vicia taxonomy. The study's findings also partially align with those of Shehbala (2021), who reported ITS2 as a more informative tool than COI for phylogenetic relationships in Vicia. While the present research did not directly compare the discriminatory power of ITS and COI, the results suggest that ITS may be more suitable for species-level identification, whereas COI may be more informative at higher taxonomic levels or in combination with other markers.

The study's emphasis on documenting and sharing the obtained sequences in the NCBI database exemplifies the importance of open data sharing in advancing scientific research and facilitating collaborative efforts to understand the biodiversity and evolutionary history of Vicia species globally. this research addresses the knowledge gap in the taxonomic research of Vicia species in Iraq by providing valuable DNA sequence data for native species. This contribution has significant implications for future studies on the genetic diversity, evolutionary relationships, and conservation of Vicia populations in Iraq. The availability of these sequences in international gene banks will enable comparative molecular studies, facilitating global comparisons and unravelling the evolutionary history of Iraqi Vicia species. Moreover, this genetic information will aid in identifying rare or endangered species, enabling the development of effective conservation strategies and the sustainable utilization of Vicia genetic resources for crop improvement and other applications.

Morphological aspects have always been the basic components of plant taxonomy, and this investigation proves their importance for the classification of the Vicia species. According to the study, morphological examination revealed a powerful dissimilarity of the stem height, leaf shape, inflorescence type and flower color which were used in constructing a dendrogram illustrating the interrelationship between the examined species. Concerning the dendrogram, V. tenuifolia, V. cracca, and V. villosa are categorized as one due, to their common morphological characteristics of having a perennial growth habit with prostrating stems. Similarly, Townsend and Guest (1974) have endorsed this categorization since they have previously placed these species into the same Cracca section based on the similar morphometric parameters they have had.

Nonetheless, the analysis of the morphology exposed some differences from previous taxonomic assignments. V. hybrida, V. michauxii, and V. monantha were put together in this study, while Townsend and Guest (1974) separated them into distinct sections. Such

divergence could stem from the use of different morphological characters or the effect of the environmental context on the phenotype expression.

In conclusion, this research contributes to the ongoing dialogue on DNA barcoding for Vicia species identification and phylogenetic analysis. The study's findings support the use of the ITS region as a reliable barcode for Vicia species while highlighting the limitations of the COI gene as a standalone marker. The successful application of a multi-locus approach, combining ITS and COI sequences, underscores the importance of utilizing multiple markers to resolve complex taxonomic relationships within this genus. The documentation and sharing of the obtained sequences in the NCBI database represent a significant contribution to the scientific community, facilitating future research and conservation efforts for Vicia species in Iraq and beyond, which strengthens our understanding of this genus's biodiversity and evolutionary history. This research underscores the importance of continued exploration of molecular markers and techniques to enhance our understanding of plant biodiversity and evolutionary relationships, ultimately contributing to the preservation and sustainable utilization of valuable plant genetic resources. As DNA barcoding technologies and methodologies continue to evolve, future research may explore additional barcoding regions or develop more sensitive techniques to enhance the resolution and accuracy of Vicia species identification and phylogenetic analysis.

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