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Phylogenetic Relationship among Some species of the Genera *Lens*, *Vicia*, *Lathyrus* and *Pisum* (Leguminosae) in Palestine

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Abstract

Leguminosae or Fabaceae is the third largest flowering plant family. It is economically important for food production and soil fertility. The molecular phylogenetic analysis of closely related species of Leguminosae family: *Lens culinaris, Vicia sativa, V. palaestina, V. peregrina, V. faba, V. narbonensis, Lathyrus aphaca, Pisum fulvum* and *P. sativum* aids in the discrimination among these closely related species. In this study, 18S and 28S as universal primers were used for amplifying and sequencing the internal transcribed spacer (ITS) region of the studied species. This was conducted on around two-five plant individual samples of each one of the species under investigation. The phylogenetic tree construction was carried out using Unweighted Pair Group method. The phylogenetic analysis among the studied species revealed that *V. peregrina, V. faba, V. narbonensis, V. palaestina, Lens culinaris,* and *V. sativa* were grouped into one clade (clade I). However, *V. sativa* occupied the farthest position in clade I, and was separated from other species of *Vicia* genus. *P. sativum, P. fulvum* and *Lathyrus aphaca* were grouped into another clade (clade II). On the other hand, *Lens culinaris* occupied a position within the core of *Vicia* near to *V. palaestina*. Accordingly, it is recommended to transfer *Lens culinaris* into *Vicia* genus based on its morphological and molecular characterization. Therefore, the new classification of *Lens culinaris* is recommended to be *Vicia culinaris.*

Keywords: Fabaceae, Internal Transcribed Spacer, (ITS), *Lathyrus*, *Lens*, Palestine, *Pisum*, rDNA, *Vicia.*

1. Introduction

The genetic diversity via the evolutionary process is very important for the survival of the species as it assists the species to adapt to environmental changes through the natural selection process and reduces their extinction risk (Grassi *et al*., 2006).

Legumes are flowering plants which belong to the Fabaceae (Leguminosae**)** family. They are different in size and in habit varying from herbaceous to woody plants, which are widely distributed worldwide. Legumes are used as an important source of food for humans and animals; in addition to their synthesis of many secondary compounds for medical principles, coloring, etc. (Andrea, 2011).

Each genus of the four Fabaceae genera (*Lens, Vicia*, *Lathyrus* and *Pisum*) has at least one unique character, which aids in the discrimination among them. Although legumes' classification depends on the morphological characters, many conflicts are encountered in this process (Andrea, 2011). Moreover, morphological continuum is observed, in particular between *Lens* and *Vicia*. Hence, *Lens* is a *Vicia* with a *Lathyrus* style characters. Moreover, *V. sativa* var. *platysperma* and *V. lunata* have an intermediate form between *Vicia* and *Lens* (Erskine *et al*., 2009). Therefore, molecular tools may provide better classification and identification discrimination.

The rDNA (ribosomal DNA) genes are specific genes of the nuclear genome that are used for genetic diversity (Zhang *et al*., 1990). The high degree of variation of the internal transcribed spacers (ITS), even between closely-related species, has been helpful to many biodiversity topic studies (Nickrent and Patrick, 1998; Penteado *et al*., 1996; Polanco and Perez, 1995; 1997).

Morphological and cytological studies of *Lens montbretti* recommended its transfer from the genus *Lens* to the genus *Vicia* classifying it as *Vicia montbretti* (Ladizinsky and Sakar, 1982). Moreover, the molecular analysis of *Lens montbretti* and *V. montbretti* helped in the reclassification of *Lens montbretti* in the genus *Vicia*, in spite of its lentoid calyx, style, and flattened seed characteristics (Mayer and Bagga, 2002). Similarly, the prominence of molecular information correlation with the morphological information was shown by the fact that *P. sativum* is sister to the monophyletic *Lathyrus* species. Moreover, *Lens* species created a clade near to the *Vicia* species, indicating that *Lens* is a close genus to *Vicia* (Steele and Wojciechowski, 2003).

Therefore, the Phylogenetic relationship among different species (*Lens culinaris, V. sativa, V. palaestina, V. peregrina, V. faba, V. narbonensis, Lathyrus aphaca,*

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Pisum fulvum and *P. sativum*) was studied. In addition, the taxonomic classification conflict of *Lens culinaris* and its phylogenetic relationship to the morphological closely related species *Vicia palaestina* was investigated. Data considering the classifications of these plant species based on molecular techniques and sequence information seem to be the first of their kind in Palestine.

2. Materials and Methods

2.1. Plant Material Collection

Two to five fresh plant specimens of the species under investigation were collected from different localities in Palestine via intensive field trips during the period of study (December-April, 2014). Voucher herbarium specimens were deposited at herbarium of the Department of Biology and Biotechnology, Faculty of Science, An-Najah National University (Table 1).

2.2. Taxonomical Analysis and Identification of the Collected Plant Species

The collected plant species of the legume genera under investigation were classified and identified relying on their morphological characters according to Flora Palaestina (Zohary 1987).

Table 1. Scientific names of the studied species with their: common names, wild or cultivated states, the locations from where they were collected, and voucher numbers.

Scientific name	Common name	Wild vs.Cultivated	Place	Accession No.	Voucher numbers
Lathyrus aphaca L.	Yellow pea, Yellow vetch	Wild	Tubas	KJ864924	1606a
		Wild	Tubas	KJ864925	1606b
P. fulvum Sm.	Tawny pea	Wild	Salfit	KJ864933	1598
		Wild	Taluza	KJ864934	1607a
		Wild	Taluza	KJ864935	1607b
		Wild	Taluza	KJ864936	1607d
P. sativum L.	Garden pea	Cultivated	Aqqaba	KJ864943	1579
		Cultivated	Yasid	KJ864945	1580
		Cultivated	Qalqilia	KJ864942	1584
		Cultivated	Maithaloun	KJ864944	1586
		Cultivated	Salfit	KJ864946	1609
V. palaestina Boiss.	Palestine vetch	Wild	Beit-Wazan	KJ864940	1603
		Wild	Beit-Leed	KJ864937	1610a
		Wild	Beit-Leed	KJ864941	1610b
		Wild	Al-Ameryah	KJ864938	1611
		Wild	Tubas	KJ864939	1570
V. peregrine L.	Rambling vetch	Wild	Salfit	KJ864952	1600a
		Wild	Salfit	KJ864955	1600b
		Wild	Beit-Wazan	KJ864953	1613a
		Wild	Beit-Wazan	KJ864956	1613b
		Wild	Al-Ameryah	KJ864954	1614
V. sativa L.	Common vetch	Cultivated	Yamun	KJ864947	1601a
		Cultivated	Yamun	KJ864950	1601b
		Cultivated	Tamun	KJ864948	1602a
		Cultivated	Tamun	KJ864951	1602b
		Wild	Beit-Wazan	KJ864949	1612
V. faba L.	Broad bean, fava bean	Cultivated	Maithaloun	KJ864957	1578
		Cultivated	Salfit	KJ864958	1582
V. narbonensis L.	Narbon bean	Wild	Tubas	KJ864959	1571a
		Wild	Tubas	KJ864960	1571b
Lens culinaris Medik.	Lentil	Cultivated	Yasid	KJ864928	1581a
		Cultivated	Yasid	KJ864929	1581b
		Cultivated	Qalqilia	KJ864930	1585a
		Cultivated	Qalqelia	KJ864931	1585b
		Cultivated	Maithaloun	KJ864932	1599

2.3. Genomic DNA Extraction

The total genomic DNA from previously frozen leaf samples of the studied plant species was extracted. *Salvia dominica* (Labiatae) was included as an outgroup. The frozen leaf samples were ground into a fine powder using mortar and pestle in the presence of liquid nitrogen. After that, the genomic DNA was extracted from a total of 0.1 g of the leaf tissue powder using PureLink™ Plant Total DNA Purification Kit (Invitrogen, USA), following the manufacturers protocol for the isolation of total genomic DNA.

2.4. PCR Amplification and Gel Electrophoresis

The nuclear ribosomal DNA encompassing the ITS regions (ITS-1 spacer, 5.8S rDNA and ITS-2 spacer) using

universal primers. Here the primer sequences were 5′-CCT TMT CAT YTA GAG GAA GGA G-3′ for 18S and 5′-CCG CTT ATT KAT ATG CTT AAA-3′ for 28S. The PCR reaction mix with a final volume of 25 µL, was performed with 12.5 µL of PCR premix (ReadyMix^{TM}Taq PCR Reaction Mix with $MgCl_2$, Sigma, USA), 0.4 µM of each primer and 2 µL of DNA template. The amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation for three minutes at 94 ºC was followed by thirty-five cycles of denaturation at 94 ºC for forty-five seconds, annealing at 56 ºC for one minute and extension at 72 ºC for two minutes, with a final extension step at 72 ºC for five minutes. The PCR products were resolved by electrophoresis through 1.5 % agarose gel to determine the size of the amplified fragment after ethidium bromide staining (Muir *et al*., 2001).

2.5. DNA Cleaning and Sequencing

The obtained PCR products were cleaned with ChargeSwitch®-Pro PCR Clean-Up Kit (Invitrogen, USA), following the manufacturer's protocol PCR product clean up. The DNA PCR products were sequenced by dideoxynucleotide chain termination method using the 3130 Genetic Analyzer (Applied Biosystems®, USA), Bethlehem University, Bethlehem, Palestine. The sequencing PCR reaction was performed with 18S and 28S primers used singly in forward and reverse reactions and BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems®, USA). Sequences were further submitted for accession numbers in primary bioinformatics web servers.

2.6. Sequence Alignment and Phylogenetic Analysis

The sequences of ITS region of the examined nine Leguminosae species were compared with previously available sequences in NCBI (National Center for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool) system. Multiple alignments were done using ClustalW of the computer program CLC Main Workbench software (version 5.6.1, 2009, CLC bio, Aarhus, Denmark). Pairwise distances were generated using the Kimura 2-parameter method. Phylogenetic analyses were based on alignments obtained from ClustalW of a 600 bp sequence. After that, a phylogenetic tree was constructed using the program Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in the same software.

The robustness of the groupings in the UPGMA analysis was assessed with 1000 bootstrap (bs) re-samplings. Reference sequences were retrieved from GenBank and were used for the phylogenetic analyses. *Salvia dominica* was used as an outgroup.

3. Results

3.1. Description of the Studied Plant Species

3.1.1. Lathyrus aphaca L.

Annual, glaucescent, subglabrous, 10-75 cm. Stems: usually erect or ascending, branched mainly at base, flexuous, angular. Leaves: abortive, reduced to stipules and tendrils; stipules: 0.5-4 cm., sessile, simple, leaf-like, ovate, sagitate-hastate or truncate at base, apiculate; tendrils: 1-6 cm., simple, filiform. Peduncles: as long as tendrils and longer than stipules, muticous or short-aristate. Racemes axillary, 1(-2)-flowered. Pedicles: as long as the calyx tube, erect or slightly curved, often hairy. Flowers: 1-1.5 cm. Calyx: about 1 cm.; teeth much longer than tube, almost equal, lanceolate, acute. Corolla: longer than calyx, yellow; standard longer than the wings and the incurved, whitish and pink-veined keel. Pod 3(-4-7)-seeded, 2-3 x 0.4-0.6 cm., erect, compressed, oblong-linear, sometimes falcate, slightly torulose, beaked, reticulately veined. Seeds: 2-4 mm., subglobular, brown-black, smooth. Fl. February-April.

3.1.2. Pisum fulvum Sm.

Annual, glabrous, 15-70 cm. Stems: ascending or procumbent, rarely erect, slender. Leaves: 3-12 cm., spreading; stipules: 1-4 cm., ovate, semicordate, dentate or incised all around or up to middle; leaflets 1 (-2)-paired, 1.5-2.5 x 1-1.5 cm., ovate, mostly dentate. Racemes 1 (-2-3)-flowered, with peduncles longer than stipules. Flowers: about 1 cm. or less. Corolla: rusty-yellow or riddish-brown, pale in subterranean flowers; standard broad, ovate to orbicular, retuse to two-lobed. Pod 2.5-3 (-4) x 0.7-1 cm., short-beaked, net-veined. Seeds: about 4 mm., black, velvety, punctulate. Fl. February-April.

3.1.3. Pisum sativum Tackholm

Annual, glabrous, 40-150 cm. Stems: angular or roundish, hollow, covered with a waxy bloom. Leaves: 6-15 cm., spreading; stipules: about 8 cm., ovate, semisagittate; leaflets (0-) 1-2 (-3)-paired, 1-5 x 1-4 cm., broad, elliptic to oblong, entire to coarsely-toothed. Racemes 1-4-flowered, with peduncles shorter than stipules. Flowers: about 3 cm. Corolla: white to pink or purple; standard broad, ovate to orbicular, retuse to two-lobed. Pod 4-15 x 1.5-2.5 cm., short-beaked, net-veined. Seeds: about 5 mm., whitish, gray, green or brownish, smooth or wrinkled punctulate. Fl. February-April.

3.1.4. Vicia palaestina Boiss.

Annual, sparingly appressed-hairy, 15-80 cm. or more. Stems: climbing, simple to branched, slender. Leaves: 2-7.5 cm., subglabrous to pubescent; stipules: 2-4 mm., semihastate, those of the uppermost leaves lanceolate to oblanceolate; tendrils often branched; leaflets (5-) 6-10-paired, 0.5-3 x 0.05-0.3 (-0.5) cm., subsessile, narrowly linear to narrowly oblanceolate, acute to obtuse, mucronulate. Peduncles: long but shorter than subtending leaves, muticous. Racemes (2-) 3-8 (-9)-flowered, generally one-sided. Pedicels: about as long as calyx, pubescent. Flowers: (5-) 6-9 mm., deflexed. Calyx: about 2 mm., somewhat hairy; rim of tube: slightly oblique; teeth: a little shorter than tube, the lower teeth: longer, lanceolate-triangular. Corolla: about three times as long as calyx; standard longer than wings, blue, slightly retuse at apex; wings: white-blue or cream-blue; keel dark blue at apex. Style: subcompressed, hairy at apex. Pod: (1.3-) 2-2.5 x (0.4-) 0.5-0.8 cm., stipitate, 1-4-seeded, compressed, rhombic-elliptical to oblong, more or less torulose, short-beaked, glabrous, somewhat net-veined. Seeds: 3-6 mm., globular to compressed-ovoid, brown to blackish-brown, smooth; hilum short, linear. F1. February-May.

3.1.5. Vicia peregrina L.

Annual, appressed-puberulent or pubescent, 15-60 (-75) cm. Stems: procumbent to erect, usually branched,

angular. Leaves: 1.5-6.5 cm.; stipules: 2-4 mm., narrow, semihastate or semisagittate, free portion subulate or lanceolate, pilose; tendrils: simple to branched; leaflets: 2-) 3-7-paired, 1-3 x 0.1-0.6 cm., subsessile, narrowly linear to oblanceolate, tapering at base, retuse, rarely acuminate, mucronulate. Racemes: axillary, mostly 1-flowered. Pedicels: about as long as to a little longer than calyx, hairy. Flowers: 1.1-2 cm. Calyx: 6-7 mm., slightly gibbous, with an oblique limb; teeth: almost as long as tube, the upper teeth shorter, connivent, lanceolate, acuminate. Corolla: about twice as long as clayx, purple or blue-violet, paler at base, sometimes white; standard longer than wings, notched. Style: hairy at apex. Pod: 2-4 x 0.4-1.2 cm., short-stipitate, 3-7-seeded, deflexed, more or less compressed, oblong-linear, shorter-beaked, appressed-hairy to subglabrous, sometimes with violet-purple spots. Seeds: about 4 mm., subglobular, sometimes subangular, mostly dark brown or mottled with black; hilum oblong, dark. Fl. February-May.

3.1.6. Vicia sativa L.

Annual, hairy to subglabrous, 20-80 cm. Stems: erect to procumbent, branching from base. Leaves: 3-11 cm.; stipules varying in length, semihastate, dentate, usually with a purple nectary spot beneath; tendrils: usually branched; leaflets: 4-10-paired, varying in size and shape, 1-3 x 0.4-1 cm., linear or lanceolate to oblong or obovate, sometimes elliptical, obcordate or cuneate, acutish or obtuse to truncate or retuse, mucronate, mostly entire. Racemes: axillary, almost sessile, 1-3-flowered. Flowers 1-3 cm., short-pedicelled. Calyx 1-3 cm., campanulate, hairy, rim of calyx tube even (not oblique); calyx teeth 0.3-1 x 0.1-0.2 cm., subequal, linear-subulate or lanceolate, acute-mucronate. Corolla: one and a half to two and a half times as long as calyx, sometimes two-coloured; standard 0.7-1.3 cm., broad, obvate-orbicular, notched, whitish-pink to purplish-violet, claw about as long as limb; wings shorter than standard, bluish-pink to purplish-violet; keel shorter than wings, paler, usually darker at apex. Pod: 3-6.5 x 0.4-1 cm., 2-10-seeded, pods compressed to turgid, linear, torulose or not, more or less pubescent, net-veined, yellowish to brown or black, rarely whitish. Seeds: 3-6 mm., rarely larger, subglobular, sometimes compressed, plain or variegated, greenish-grey or brown-yellow or black; hilum short, linear. Fl. (February-) March-May (-June).

3.1.7. Vicia faba L.

Annual, glabrous, 30-160 cm. Stems: erect, unbranched. Leaves: 6-12 cm.; stipules 1-2 mm., sagittate, base toothed; tendrils 0 or rudimentary; leaflets: 2-6-paired, 5-8.5 x 2.2-3.5 cm., ovate to elliptic, obtuse to acute. Racemes: axillary, subsessile, mostly 2-6-flowered. Floweres: 2-3 cm., dull white. Calyx: 7-15 mm., campanulate, unequal toothed; calyx teeth: 0.5 x 0.3 mm., Corolla: about twice as long as clayx, white wings with dark blotches; standard 1.5 cm., broad, wings: shorter than standard, keel: shorter than wings. Pod: 5-15 x 1-2 cm., 2-5-seeded, cylindrical or flattened, glabrous or pubescent. Seeds: 10-30 mm., flat, green. Fl. February-May.

3.1.8. Vicia narbonensis L.

Annual, subglabrous to sparingly pubescent or hirsute, 15-50 cm. Stems: ascending, procumbent to erect, branched, thick, angular. Leaves: 1.5-9 cm.; stipules: semihastate to semiorbicular, entire or dentate to incised, with a dark nectary spot beneath; tendrils: simple or somewhat branched, lacking in the lower leaves; leaflets: (1-) 2-3 (-4)-paired, 1-6x1-3 cm., subsessile, elliptical or oblong-lanceolate or ovate to obovate, obtuse or rarely acute, rounded or truncate to retuse, mucronulate, entire or dentate-serrate at margin or denticulate near apex, sparingly to densely hairy, especially along nerves, ciliate at margin. Peduncles: very short and thick. Racemes: (1-) 3-6-flowered. Flowers: 1.8-3.2 cm., short-pedicelled, deflexed to erect. Calyx: 0.7-1.3 cm., sparingly hairy; tube with oblique rim; teeth: unequal, the lower teeth longer, as long as tube, mostly ciliate. Corolla: 2-2.5 times as long as calyx, purple-violet; standard longer and keel shorter than wings. Style: hairy at apex. Pod: 3.5-6.5x0.8-1.5 cm., short-stipitate, flattened, linear to oblong-rhomboidal, curved and beaked, hairy or glabrescent, ciliate and tuberculate-denticulate at margin, often nerved. Seeds: 4-6 mm., subglobular, brown-black, more or less smooth; hilum: oblong-elliptical to oblong-ovate, whitish. Fl. February-June.

3.1.9. Lens culinaris Medik.

Annual, 16-20 cm., hairy. Stems: few to many, erect, sparingly branching, angular. Leaves: paripinnate, at least part of them terminating in a branched tendril; stipules: small, lanceolate, entire; leaflets: 3-7-paired, 0.8-1.5 x 0.4-0.6 cm., oblong-linear to linear. Peduncles: shorter than leaves, ending in an awn up to 1 cm. Racemes: 1 (-2)-flowered. Flowers: 4-6 mm. Calyx: short-campanulate; teeth: much longer than tube, nearly as long as or longer than corolla, almost equal, filiform-subulate. Corolla: white, rarely pink or violet. Staminal tube: oblique. Pod: 0.7-1.2 x 0.3-0.5 cm., deflexed, ovate-rhombic. Seeds: 1-2, lenticular, rarely almost globular. Fl. April.

3.2. Molecular Characterization of The Studied Species

Specific sites of DNA; ITS-1 spacer, 5.8S rDNA and ITS-2 spacer in four genera of Leguminosae family as well as the outgroup, *S. dominica* (labiatae), were amplifed using universal primers 18S and 28S. On an agarose gel, the PCR products obtained from genomic DNA, yielded a single band of approximately 720 bp for all of the tested species including *S. dominica* (Figure 1).

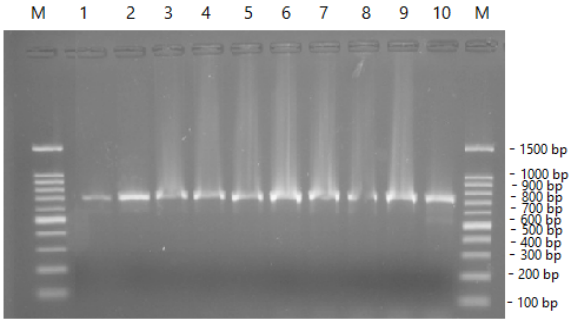

Figure 1. Agarose gel electrophoresis analysis showing the detection of amplified ITS region of different species of Leguminosae family as well as *S. dominica* as an outgroup. Lanes: M, 50 bp DNA marker; 1, *Lathyrus aphaca*; 2, *P. fulvum*; 3, *P. sativum*; 4, *V. palaestina*; 5, *V. peregrina*; 6, *V. sativa*; 7, *V. faba*; 8, *V. narbonensis*; 9; *Lens culinaris* and 10, *S. dominica*.Thirty-five classified samples of Leguminosae species belonging to *Lathyrus*, *Pisum*, *Vicia* and *Lens* genera were sequenced.

The distance matrix and phylogenetic tree of the amplified ITS region were established among the nine

Leguminosae species under investigation (Figure 2 and Table 2). The phylogenetic tree was constructed according to the similarity among the resulted sequences of the studied Leguminosae species; where the bootstrap consensus tree was inferred from 1000 replicates. The sequence information was between 663–683 bp. The obtained sequences were further registered in the GenBank database (https://www.ncbi.nlm.nih.gov) under the accession numbers (KJ864924, KJ864925 and KJ864928-KJ864961). Phylogenetic analysis in the current work revealed that the ITS sequences of Leguminosae species of interest with the highest average of intraspecies genetic divergence (9.9 %) was recorded between *V. palaestina* (KJ864941) and *P. sativum* (KJ864944) and *P. fulvum* (KJ864933, KJ864933 and KJ864933). While, the low average of intraspecies genetic divergence (1.9 %) was recorded between *V. faba* (KJ864957, KJ864958) and *V. peregrina* (KJ864954) (Table 2).

Based on the obtained phylogenetic tree, two main clades were revealed. Clade I included five species of *Vicia* genus: *V. peregrina*, *V. faba*, *V. narbonensis*, *V. palaestina* and *V. sativa*, in the same order in the phylogenetic tree respectively, as well as *Lens culinaris*. Clade II included *P. sativum*, *P. fulvum* and *Lathyrus aphaca*. Furthermore, clade I was subdivided into two subclades: a and b. Subclade Ia had five species, four of them belong to the genus *Vicia* and the fifth one was *Lens culinaris*. However, *V. sativa* occupied a basal position in the clade I. This may confirm that *V. sativa* represents a taxon distantly related to all other species of *Vicia*. *Lens culinaris* belongs to the subclade Ia. It was close to the species of *Vicia*, and appeared basally as sister to many *Vicia* species, in particular, *V. palaestina.*

The results of this research showed that all of the studied cultivated (KJ864947, KJ864948, KJ864950, and KJ864951) and the wild type (KJ864949) samples of *V. sativa* were clustered close to each other in the same group (100 % bs). Moreover, all of *Vicia* species could be considered as sisters to each other according to their botanical and molecular properties.

However, clade II was subdivided into two subclades: c and d, which were represented by the two *Pisum* studied species and *Lathyrus aphaca*, respectively (68.9 % bs). A high molecular similarity between them was observed, which was more than their resemblance to other genera. The two *Pisum sativum* and *P. fulvum* species were near to each other in the constructed phylogenetic tree in this study (100 % bs).

Finally, the current work data confirm that the species of *Pisum* genus formed a monophyletic group as all species of *Pisum* genus were clustered into the same group and had the same ancestor. On the other hand, *V. sativa* clade splits from the other closely- related species in the same genus. As a result, the genus *Vicia* was considered as a paraphyletic one. However, *S. dominica* was found to be quite divergent, and did not fall in any of the major clusters (Figure 2).

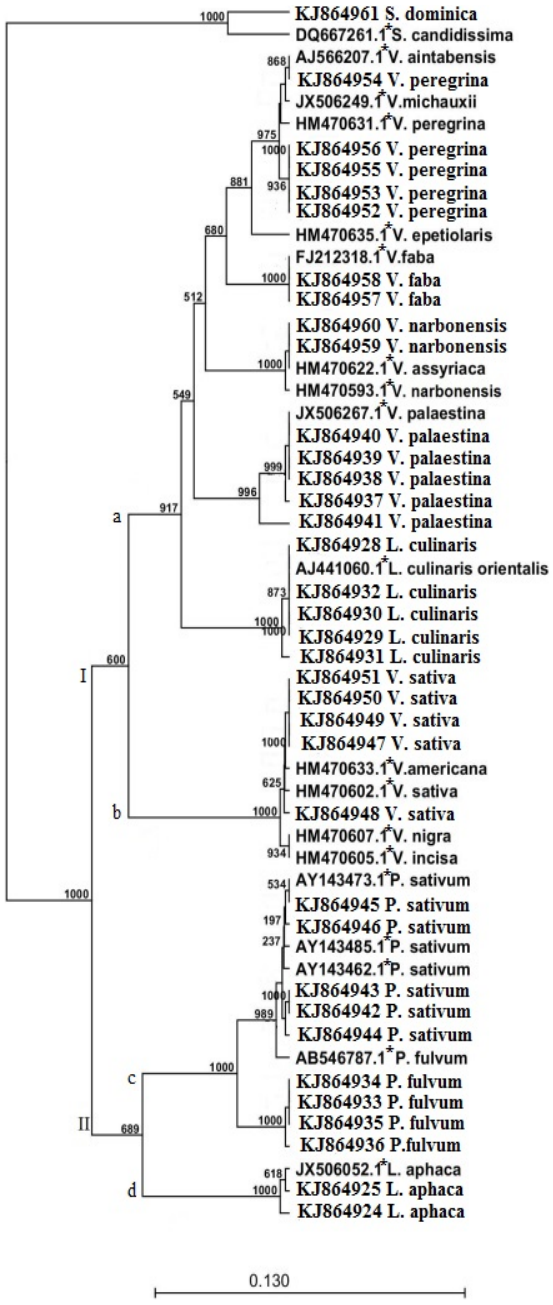

Figure 2. Phylogenetic analysis by UPGMA method based on ITS site. Sequence of some Palestinian Leguminosae species (*Lathyrus aphaca, P. fulvum, P. sativum, V. palaestina, V. peregrina, V. sativa, V. faba, V. narbonensis* and *Lens culinaris*) as well as *S. dominica* as an outgroup were used for phylogenetic analysis. Reference sequences belonging to species of Leguminosae family (denoted by asterisk) were retrieved from GenBank. The bootstrap consensus tree was inferred from 1000 replicates. The latin numbers (I, II) represent the clades, where the following letters (a, b, c and d) represent the subclades of the phylogenetic tree.

Table 2. Genetic differences between ITS region sequences derived from studied Palestinian Leguminosae species. DNA distances were created by K2P model using MEGA software version 5.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1. L._aphaca KJ864924																																		
2. L._aphaca KJ864925	0.005																																	
3. L. culinaris KJ864928	0.060	0.059																																
4. L._culinaris KJ864929	0.060	0.059	0.000																															
5. L. culinaris KJ864930	0.060	0.059	0.000	0.000																														
6. L. culinaris KJ864931	0.064	0.062	0.003	0.003	0.003																													
7. L. culinaris KJ864932	0.060	0.059	0.000	0.000	0.000	0.003																												
8. P. fulvum KJ864933	0.059	0.057	0.070	0.070	0.070	0.074	0.070																											
9. P._fulvum KJ864934	0.059	0.057	0.070	0.070	0.070	0.074	0.070	0.000																										
10. P. fulvum KJ864935	0.059	0.057	0.070	0.070	0.070	0.074	0.070	0.000	0.000																									
11. P. fulvum KJ864936	0.060	0.059	0.072	0.072	0.072	0.076	0.072	0.002	0.002	0.002																								
12. V. palaestina KJ864937	0.066	0.064	0.044	0.044	0.044	0.047	0.044	0.081	0.081	0.081	0.081																							
13. V. palaestina KJ864938	0.064	0.062	0.042	0.042	0.042	0.046	0.042	0.079	0.079	0.079	0.079	0.002																						
14. V. palaestina KJ864939	0.064	0.062	0.042	0.042	0.042	0.046	0.042	0.079	0.079	0.079	0.079	0.002	0.000																					
15. V. palaestina KJ864940	0.064	0.062	0.042	0.042	0.042	0.046	0.042	0.079	0.079	0.079	0.079	0.002	0.000	0.000																				
16. V. palaestina KJ864941	0.079	0.077	0.057	0.057	0.057	0.060	0.057	0.095	0.095	0.095	0.094	0.015	0.014	0.014	0.014																			
17. P. sativum KJ864942	0.062	0.061	0.070	0.070	0.070	0.074	0.070	0.026	0.026	0.026	0.028	0.079	0.078	0.078	0.078	0.093																		
18. P. sativum KJ864943	0.062	0.061	0.070	0.070	0.070	0.074	0.070	0.026	0.026	0.026	0.028	0.079	0.078	0.078	0.078	0.093	0.000																	
19. P. sativum KJ864944	0.064	0.062	0.070	0.070	0.070	0.074	0.070	0.028	0.028	0.028	0.030	0.081	0.079	0.079	0.079	0.095	0.092	0.092																
20. P. sativum KJ864945	0.061	0.059	0.068	0.068	0.068	0.072	0.068	0.024	0.024	0.024	0.026	0.078	0.076	0.076	0.076	0.091	0.092	0.092	0.093															
21. P. sativum KJ864946	0.062	0.061	0.070	0.070	0.070	0.074	0.070	0.026	0.026	0.026	0.028	0.080	0.078	0.078	0.078	0.093	0.093	0.093	0.095	0.092														
22. P. sativum KJ864947	0.060	0.059	0.046	0.046	0.046	0.049	0.046	0.070	0.070	0.070	0.072	0.049	0.048	0.048	0.048	0.062	0.074	0.074	0.076	0.072	0.074													
23. P. sativum KJ861948	0.061	0.062	0.049	0.049	0.049	0.053	0.049	0.073	0.073	0.073	0.075	0.053	0.051	0.051	0.051	0.062	0.077	0.077	0.079	0.076	0.078	0.003												
24. P. sativum KJ864949	0.060	0.059	0.046	0.046	0.046	0.049	0.046	0.070	0.070	0.070	0.072	0.049	0.048	0.048	0.048	0.062	0.074	0.074	0.076	0.072	0.074	0.000	0.003											
25. P. sativum KJ864950	0.060	0.059	0.046	0.046	0.046	0.049	0.046	0.070	0.070	0.070	0.072	0.049	0.048	0.048	0.048	0.062	0.074	0.074	0.076	0.072	0.074	0.000	0.003	0.000										
26. P. sativum KJ864951	0.060	0.059	0.046	0.046	0.046	0.049	0.046	0.070	0.070	0.070	0.072	0.049	0.048	0.048	0.048	0.062	0.074	0.074	0.076	0.072	0.074	0.000	0.003	0.000	0.000									
27. V. peregrine KJ864952	0.057	0.055	0.037	0.037	0.037	0.040	0.037	0.066	0.066	0.066	0.068	0.031	0.030	0.030	0.030	0.044	0.066	0.066	0.068	0.065	0.066	0.031	0.035	0.031	0.031	0.031	0.000							
28. V. peregrine KJ864953	0.057	0.055	0.037	0.037	0.037	0.040	0.037	0.066	0.066	0.066	0.068	0.031	0.030	0.030	0.030	0.044	0.066	0.066	0.068	0.065	0.066	0.031	0.035	0.031	0.031	0.031	0.000	0.000						
29. V. peregrine KJ864954	0.059	0.057	0.035	0.035	0.035	0.039	0.035	0.064	0.064	0.064	0.066	0.030	0.028	0.028	0.028	0.042	0.064	0.064	0.066	0.063	0.065	0.030	0.033	0.030	0.030	0.030	0.002	0.002	0.002					
30. V. peregrine KJ864955	0.057	0.055	0.037	0.037	0.037	0.040	0.037	0.066	0.066	0.066	0.068	0.031	0.030	0.030	0.030	0.044	0.066	0.066	0.068	0.065	0.066	0.031	0.035	0.031	0.031	0.031	0.000	0.000	0.000	0.002				
31. V. peregrine KJ864956	0.057	0.055	0.037	0.037	0.037	0.040	0.037	0.066	0.066	0.066	0.068	0.031	0.030	0.030	0.030	0.044	0.066	0.066	0.068	0.065	0.066	0.031	0.035	0.031	0.031	0.031	0.000	0.000	0.000	0.002	0.000			
32. V. faba KJ864957	0.064	0.062	0.040	0.040	0.040	0.044	0.040	0.070	0.070	0.070	0.072	0.042	0.040	0.040	0.040	0.055	0.070	0.070	0.072	0.068	0.070	0.033	0.037	0.033	0.033	0.033	0.021	0.021	0.021	0.019	0.021	0.021		
33. V. faba KJ864958	0.064	0.062	0.040	0.040	0.040	0.044	0.040	0.070	0.070	0.070	0.072	0.042	0.040	0.040	0.040	0.055	0.070	0.070	0.072	0.068	0.070	0.033	0.037	0.033	0.033	0.033	0.021	0.021	0.021	0.019	0.021	0.021	0.000	
34. V. narbonensis_KJ864959	0.068	0.066	0.048	0.048	0.048	0.051	0.048	0.083	0.083	0.083	0.085	0.047	0.046	0.046	0.046	0.060	0.080	0.080	0.080	0.078	0.080	0.049	0.049	0.049	0.049	0.049	0.039	0.039	0.039	0.037	0.039	0.039	0.038	0.038
35. V._narbonensis_KJ864960	0.068	0.066	0.048	0.048	0.048	0.051	0.048	0.083	0.083	0.083	0.085	0.047	0.046	0.046	0.046	0.060	0.080	0.080	0.080	0.080	0.080	0.049	0.049	0.049	0.049	0.049	0.039	0.039	0.039	0.037	0.039	0.039	0.038	0.038	0.000

4. Discussion

Evolutionary relationship illustration and reconstruction among different organisms have been among the hot topics of research lately. The phylogenetic relationship construction was based mainly on the accumulation of DNA sequence data in GeneBank targeting a new genetic classification that often conflicts with the traditional taxonomical tools. Nuclear ribosomal ITS sequence data have great potential to resolve plant phylogenies at different taxa levels. Upon that they were used to detect the phylogenetic relationship among several wild and cultivated plant species as in *Allium* species (Gurushidze *et al*., 2007) as well as among different legume species (Mayer and Bagga, 2002; Sonnante *et al*., 2003; Steele and Wojciechowski, 2003). Nevertheless, until now no similar analysis on the phylogenetic relationship for Leguminosae species has been reported in Palestine using molecular techniques such as the ITS region analysis.

The constructed phylogenetic tree in this study showed that some reference species retrieved from GenBank database (NCBI) clustered together such as *V. assyriaca* and *V. narbonensis* or *V. michauxii, V. aintabensis* and *V. peregrina* or *V. american* and *V. sativa*. This may indicate that these species, which clustered together, have a close phylogenetic relationship.

In this research, *Lens culinaris* formed a cluster within *Vicia* genus, where this species was closest to *V. palaestina* more than any other species of *Vicia* (91.7 % bs). The continuum morphological properties between *Vicia palaestina* and *Lens culinaris* is a main conflict in their discrimination from each other. Since *Vicia palaestina* and *Lens culinaris* are characterized by overlapping in their morphological characters, branched stems, branched tendrils and the number of seeds, as well as the number of leaflets, the number of raceme flowers and the size and shape of the stipules were observed. The molecular out-finding coincides with the resemblance of their morphological properties. These two species are similar to each other, except in the hairy style and the seed shape. Style is hairy all around or only on the lower side in *V. palaestina*. On the other hand, style is hairy on the upper side in *Lens culinaris*. In addition, they varied in having round and compressed seeds respectively. Therefore, in spite of this morphological discrimination, the recorded molecular data strongly supported the close relationship between them, which lead to the recommendation of their reclassification in the same genus.

The results of the current research agreed with previous studies in that *Lens* is a close genus to *Vicia*, as they formed a clade near each other based on ITS sequences (Foladi *et al*., 2013; Steele and Wojciechowski, 2003). Another study indicated that *V. faba* and *V. narbonensis* are sisters to each other, as the obtained bootstrap supports the replacement of *V. faba* into the *V. narbonensis* group (Leht, 2009). The previous study result supports those obtained in this work, as *V. peregrina, V. faba* and *V. narbonensis* were clustered with each other in the phylogenetic tree confirming their relationship to each other. On the other hand, results showed that *V. sativa* was farther similar to the other *Vicia* species due to its separation from other species of *Vicia* genus.

Furthermore, the location of *V. sativa* and *Lens culinaris* in different subclades in the resulted phylogenetic tree in this study agrees with their different morphological properties. In addition, the results pointed out that the cultivated and wild samples of *V. sativa* were clustered close to each other in the same group. This indicates the genetic closeness between wild and cultivated species, revealing the ability to consider the wild species as the origin of the cultivated ones. Nevertheless, this can be taken into account in the absence of hybridization occurrence or human intervention.

The subclade represented by *Lathyrus aphaca* is sister to the one comprised of *P. sativum* and *P. fulvum* as they formed a monophyletic group in the phylogenetic tree. This molecular analysis is in harmony with the common feature between them as both have large stipules, which are missing in *Vicia* and *Lens* genera. The obtained results are compatible with previous studies (Steele and Wojciechowski, 2003).

Two species of *Pisum* genus; *P. sativum* and *P. fulvum* formed one subclade reflecting the close relationship between them estimating that the wild *P. fulvum* is the origin of the cultivated *P. sativum* as was reported previously by (Schaefer *et al*., 2012). However, other studies showed a contradiction as *P. fulvum* diverges from other *Pisum* genus species (Palmer *et al*., 1985; Polans and Saar, 2002; Saar and Polans, 2000). Therefore, more studies considering a wider spectrum of *Pisum* species are still required to clarify this taxonomical issue. Nevertheless, the purple flowered sample of *P. sativum* was farther from the other white flowered samples. As a result, different phenotypes as different flower colors can't be considered as different species (Zohary, 1987). This complex relationship between phenotypes and genotypes confirmed by the genetic mechanisms controlling the floral number and shape is apparently unstable, resulting in a fluctuating asymmetry (Friesen *et al*., 1997). As a result, it is not easy to establish a direct relation between the phenetic variations and the genetic ones (Treu *et al*., 2001). Accordingly, further studies related to that aspect could be conducted to provide a more elaborate clear view.

The genus *Vicia* did not form a monophyletic group; instead it formed a paraphyletic one with *Lens*. This result is consistent with a previous report indicating that genus *Vicia* formed a paraphyletic group with *Lens*, *Pisum*, and *Lathyrus* (Steele and Wojciechowski, 2003) and was confirmed by obtaining a monophyletic *Vicia* by transferring *Lens* and *Lathyrus saxatilis* to *Vicia* genus (Schaefer *et al*., 2012).

Therefore, the phylogenetic relationship among plant species using ITS sequencing is an effective method for identifying unknown plant specimens that don’t have one or more essential parts such as flowers, fruits, etc., without which, the accurate identification and classification would be sometimes impossible. However, the classification of a new species according to the resulted clusters in the phylogenetic tree at family, genus and species levels is possible accurately by referring to the GenBank data via the use of small amounts of leaves.

5. Conclusion

Morphological and molecular properties of Palestinian *Lens culinaris* and *V. palaestina* revealed the close relationship between these two species. This indicates that the shape of the seed and the hairy characters of style are not enough to separate these two species into two different genera. Therefore, it is recommended to transfer *Lens culinaris* into *Vicia* genus based on the morphological and molecular characterization of this species. Accordingly, the new classification of *Lens culinaris* is proposed to be *Vicia culinaris*. However, further studies on other different species of the Leguminosae family are needed to provide more information on the relationship among the Leguminosae closely-related genera and species.

Conflict of Interest

No conflicts of interest have been declared by the authors

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