An-Najah National University

Faculty of Graduate Studies

Molecular Phylogenetic Relationship among Closely Related Species of the Clover Genus (*Trifolium* – Leguminosae) in Palestine / West Bank

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Dedication

I dedicate my thesis to my family and friends.

Mom and Dad,

I could never have done this without your faith, support and constant encouragement. Thank you for teaching me to believe in Allah, in myself and in my dreams.

To my sister and brothers (Haya, Mohammad and Munther) for cheering me until the end

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الاقرار

أنا الموقعة أدناه، مقدمة الرسالة التي تحمل العنوان :

Molecular Phylogenetic Relationship among Closely Related Species of the Clover Genus (*Trifolium* – Leguminosae) in Palestine /West Bank.

أقر بأن ما اشتملت عليه هذه الرسالة إنما هو نتاج جهدي الخاص، بإستثناء ما تمت الإشارة إليه حيثما ورد، و أن هذه الرسالة ككل، أو أي جزء منها لم يقدم من قبل لنيل أي درجة أو لقب علمي لدى أي مؤسسة أو بحثية أخرى .

Declaration

The work provided in this thesis, unless otherwise referenced. Is the researcher`s own work and has not been submitted from anywhere else, for any other degree or qualification.

Student's name: **إسم الطالبة:**

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List of Abbreviations

DNA	Deoxyribonucleic acid
cpDNA	Chloroplast deoxyribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RNase	Ribonuclease
ITS	Internal Transcribed Spacer
PCR	Polymerase Chain Reaction
Taq	Thermusaquaticus
Mgcl2	Magnesium Chloride
NH4cl	Ammonium Chloride
Hgcl	Mercuric Chloride
CTAB	Cetyltrimethyl Ammonium Bromide
SDS	Sodium dodecyl sulfate
PVP	Polyvinylpyrrolidone
EDTA	Ethylenediaminetetraacetic acid
Nacl	Sodium chloride
Tris-Hcl	Tris(hydroxymethyl)aminomethane-hydrochloric acid
TE	Tris -EDTA
g	Gram
mg	Milligram
µg	Microgram
ng	Nanogram
L	Litter
ml	Milliliter
µl	Microliter
µM	Micromolar
nm	Nanometre
pH	potential of hydrogen
w/v	Weight /volume percentage concentration
v/v	Volume/ volume percentage
rpm	Revolutions per minute
h	Hour
sec	Second
min	Minute
bp	base pair
BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide Basic Local Alignment Search Tool
MEGA6	Molecular Evolutionary Genetics Analysis version 6
K2P	kimura 2- parameter model
V. no.	Voucher number

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Abstract

Leguminosae family is the third largest family of angiosperms; which comprises three sub-families: Caesalpinioideae, Mimosoideae and Papilionoideae. Legumes are flowering plants in Leguminosae family that include a large number of species. *Trifolium* L. (Clover genus, True Clover) is one of the most important genera in legumes with agricultural values. A Mediterranean region contains the highest biological diversity due to the variation in Mediterranean climate. The native distribution of Clover genus is found there.

To resolve the conflicts in the morphological characters in some of the closely related species of *Trifolium*, it was necessary to use molecular markers.

Internal Transcribed Spacer (ITS) regions of the nuclear ribosomal DNA (nrDNA) are the most widely used marker in plant research. They have

been proved to be useful source of information for phylogenetic studies in many angiosperm families including Leguminosae.

Fresh specimens of different species of *Trifolium* were collected during the flowering period (March – May 2017) from different locations in Palestine/West Bank. Then a representative plant specimen of each species was deposited at An-Najah National University Herbarium. Depending on Flora Palaestina, and using of Stereo microscope, the collected samples were identified and classified based on their morphological characteristics.

CTAB extraction protocol was followed for plant genomic DNA extraction. The ITS regions (ITS-1 spacer, 5.8S rDNA and ITS-2 spacer) were amplified using universal primers. Phylogenetic tree was constructed for the 12 classified samples that belong to Clover genus (*Trifolium* L.) using Neighbor-Joining method and *Salvia candidissima* was used as the out-group. Kimura's 2-parameter method was used to compute Pair-wise distances. All DNA sequences that were used in the study were deposited at the GeneBank database under accession numbers.

Molecular ITS sequence analysis of both *T.purpurem* and *T.dasyurum* showed that they are identical species, since they have identical ITS sequences (0% bp differences) and were clustered together in the same clade I/ sub- clade Ia. This coincides with the high resemblance of their morphological characters.

T.campestre and *T.grandiflorum* could be regarded taxonomically as one species, since they also have identical ITS sequence (0% bp differences) and were clustered together in the same clade II/ sub-clade IIb in the constructed phylogenetic tree.

Based on this study, it is recommended to identify both *T.purpurem* and *T.dasyurum* as same species. Accordingly depending on the nomenclature rules *T.dasyurum* (1832) can be considered as synonym to *T.purpurem* (1807), which can be referred as the valid name of that species. Similarly this can be applied to both *T.campestre* (1804) and *T.grandiflorum* (1767). The former one could be regarded as synonym to *T.grandiflorum* which could be taken as the valid name.

In addition, the current conducted traditional morphological and molecular taxonomical study is the first of its type that was able to highlight the phylogenetic relationship among closely related species of the genus *Trifolium* in Palestine/West Bank. This paves the way for future in-depth researches on different more species of the same genus. In addition, using other molecular markers could reveal more clarification of the taxonomy of *Trifolium* different species.

Chapter one

Introduction

1.1 General Background

The Mediterranean climatic region was mainly delimited based on the bioclimatic criteria in spite of other possible categories as biogeography and floristic ones (Akman, 1982; 1999). Hot, arid summers and moist, cool winters characterize the Mediterranean climatic (Poluuin and Huxley, 1967). Arid lands, portraying a typical transition zone between the Saharo-Arabin desert biome and temperate climates which are represented by most of the eastern Mediterranean region including Turkey, Syria, Lebanon, Palestine and Jordan (Heywood, 1995a; 1995b; 2003). It is characterized by rich plant diversity due to its distinguishing position as a meeting point of the phytogeographical regions of the Mediterranean, Irano-Turanian, Saharo- Sindian and Sudano-Decanian; each of which have their own flora along with a large number of bioregional and pluriregional species (Davis and Heywood, 1994). The flora and vegetation of the eastern Mediterranean is of enormous variability which is represented by nearly 15,000 different plant species belonging to 1000 genera and 500 families (Heywood, 1995b). Accordingly, the Mediterranean Basin is one of 34 world biodiversity hotspots (Myers, 2003; Myers et al., 2000). Moreover, the Mediterranean region retains its biological significance due to a high level of plant endemism and the occurrence of many relict species (Greuter, 1991).

Palestine locates at a meeting point between Europe, Asia and Africa in the southeastern of the Mediterranean Sea, which contributed to the diversity of phytogeographic zone, which in turn caused a large diversity in the Flora

of Palestine. Despite its small size, the West Bank which is located in the Palestinian Territories (PT) comprises approximately 3 percent of the world's biodiversity and contains high density of species as well as a large number of endemic species (endemic are only found in restricted regions and therefore unique genetic information) (ARIJ,1997). Distribution Atlas of plants in the Flora Palaestina area (Danin, 2004), comprises updated nomenclature, distribution and habitat data for the species in the area covered by Flora Palaestina (Zohary, 1966a; 1966b; 1972a; 1972b; Dothan, 1978a; 1978b; 1986a; 1986b; Dothan and Danin, 1991). Total number of 2750 species in 138 families listed in the Atlas and about 276 were not previously recorded in the Flora Palaestina. About 27 of them are found new to science, described after the publication of Flora Palaestina; 105 are new adventive species, 34 of them escaped from cultivation and established through spontaneous germination. The marked aliens in the study area were 160 species (5.8% of entire flora).The previous recorded data is still under updating in Flora of Israel on line recoding so far 2866 plant species of flowering seed vascular plants (Danin, 2006+).

Several studies revealed that most dominant families in Palestine were the Compositae, Gramineae, Leguminosaes, Cruciferae, Labiatae and Liliaceae (ARIJ, 1997; Ali-Shtayeh and Jamous, 2002; 2003). Approximately 800 of these plants are rarely found and around 140 are endemic (Zohary, 1966a; 1966b). A total number of 334 plant species were recorded to be threatened in the West Bank and Gaza Strip (Ali-Shtayeh and Jamous, 2002).

There is an obvious association between legumes distribution in nature and their morpho-physiological differences with the soil characteristics, related to the nature of parent rock, soil chemical properties and soil texture (Piano and Francis, 1993). Legumes in the Mediterranean semiarid areas play a main role as a pasture plants for their forage quality and assisting nitrogen fixation, which are increase soil fertility and giving the nutritional requirements for other plant species (Crespo, 1997).

1.2 Leguminosae Family

Leguminosae, (the Fabaceae family) is the third largest family of angiosperms; which comprises three sub-subfamilies: Caesalpinioideae, Mimosoideae and Papilionoideae, with approximately 727 Genera and 19,330 species. Legumes are flowering plants in Leguminosae family that include a large number of species. They are harvested as crops for human and animal consumption as well as for their oils, fibers, fuel and fertilizers (Lewis et al., 2005). Legumes vary in habit from annual, perennial herbs to shrubs and trees, ranging from the smallest plants of deserts to the tallest of rain forest trees (Rundel, 1989). Many species in Leguminosae family have the ability to fix atmospheric nitrogen via a symbiotic association with root-nodulating bacteria (McKey, 1994; Sprent, 2001). Based on morphological characterization, leaves of the Leguminosae family are usually of pinnate compound form, arranged alternately (one leaf per node). Their flowers are hermaphroditic (containing both the stamen and pistil), which makes the plants self-fertile. Moreover they have zygomorphic flowers with a five merous corolla. Flowers of the

Leguminosae family are composed of one large petal, known as standard, which folds over the rest petals for protection. Also, there are in front of the standard, two horizontal petals are known as wings, while, the other two petals known as the keel that are united by their margins. The fruit (pod) of Leguminosae family is often one celled. That is usually dehisces along two opposite longitudinal splits which is known by the legume fruit type. Pod of legumes may contain one or few seeds with large embryo and little endosperm for each seed. Irregular nodules on roots of these plants can absorb nitrogen, which is needed for plants growth (Zohary, 1987).

The genus *Trifolium* L. (Clover) is one of the most important genera of the family Leguminosae, subfamily Papilionoideae with agricultural value (Zohary, 1972). *Trifolium* includes 250-300 species, including some of the most economically important species (Williams, 1987). However, according to the International Legume Database and Information System (ILDIS), only 217 species were accepted (Zohary and Heller, 1984).The name of the clover genus refers to the distinctive leaves that are usually composed of three leaflets (trifoliolate).

Due to the higher species diversity found in the Mediterranean regions, the native distribution of *Trifolium* is generally found there, with a secondary center of distribution in north-eastern America, and now is widely distributed throughout the temperate and subtropical regions of the world (Zohary and Heller, 1984; Caradus, 1995). Flowers of *Trifolium* are usually small to medium-size (0.3 - 2.5 cm) arranged in capitate to spicate heads. Wing and keel (lower petals) are partially connate and their claws are

adnate to the staminal tube; banner (the upper petal) may also be connat to the lower petals, and sometimes to the free stamens (Hossain, 1961; Zohary and Heller, 1984). Corolla is persistent, marcescent or caducos with white, yellow, purple and fresh-colored or even pink to lilac color. Calyx is mostly tubular or campanulate that is generally persistent after anthesis (Zohary, 1972). Fruits are usually 1–2 seeded, but may contain up to nine seeds. The pods may be regularly and irregularly dehiscent. In later cases, pods are often of papery texture and wholly contained within the persistent corolla or calyx (Zohary, 1972).

1.3 Taxonomic History of the Clover Genus (*Trifolium)*

Trifolium as an important genus in the Leguminosae family, subfamily Papilionoideae, was found to be closely related to the genera *Medicago* and *Trigonella* causing their classification in the same tribe Trifolieae (Heyn, 1981). Their pods characters are the main criteria to distinguish this genus among the three genera. The *Medicago* pods are spiral and coiled, but in *Trigonella* are usually straight or arcute. However *Trifolium* pods are more ovate or oblong (Hossain, 1961; Zohary and Heller, 1984).

The clover genus has been subjected to various different taxonomic studies revealed in its classification into different sub taxa. The first of all was conducted by Linnaeus (1753) as he divided the genus into five groups, while, it was divided into seven ones by Seringe (1825). However, in 1832, Presl splitted the genus into nine new sub taxa. But Boissier in 1873 reduced the number of the sections to seven. Later on, Lojacono (1883)

distinguished two subgenera within the genus, and their subsequent classifying in to 11 sections and the second into two. Although, Hossain (1961) divided the genus into eight subgenera, another approach was adopted by Zohary and Heller in (1984), as they classified the genus into eight sections. These sections were referred to Lotoidea, Paramesus, Mystillus, Vesicaria, Chronosemium, Trifolium, Tricocephalum, and Involucrarium. Six sections out of them were restricted to the Old World or Eurasia and some extend to Africa. Only Involucrarium and Trifolium were distributed in the New World, in North and South America (Zohary and Heller, 1984; Steiner et al., 1997).

1.4 Molecular Phylogenic Relationships

In Plant cells, the suitable sources of DNA to study genetic diversity and phylogenetic relationships are nuclear genome (DNA), mitochondrial genome (mt DNA) and chloroplast genome (cp DNA). However ribosomal DNA (rDNA) genes are specific genes that can be used for genetic diversity of nuclear genome (Zhang et al., 1999).

As molecular methods are direct tools for vital genetic information, they are used in research on plant diversity. Moreover, choosing the appropriate molecular markers enables the detection of phylogenetic relationship among different plant taxa (Ghariani et al., 2003; Poyraz et al., 2012; Taşkın et al., 2012).

Internal Transcribed Spacer (ITS) regions of the nuclear ribosomal DNA (nrDNA) are the most widely used marker in plants research (Patwardhan

et al., 2014). They have been proved to be useful source of information for phylogenetic studies in many angiosperm families including Leguminosae (Alvarez and Webdel, 2003). Regions between 18S (small rRNA subunit) and 28S (large rRNA subunit) in nuclear ribosomal DNA are exploited in several diversity studies (Planco and Perez, 1997; Penteado et al., 1996; Nickrent and Patrick, 1998).

Trifolium is a member of a large monophyletic clade of 45 genera, which are commonly referred to Temperate, Herbaceous Clade (THC) (Polhill, 1981; Lavin et al., 1990; Doyle, 1995). However, more recently they are known as Internal Repeat Lacking Clade (IRLC) (Hu et al., 1999). The IRLC is composed of the tribes Trifolieae and Fabeae, and the genera *Cicer*, *Galega*, and *Parochetus* (Liston and Wheeler, 1994; Sanderson et al., 1996; Wojciechowski et al., 2004). Molecular phylogenetic studies have proved strongly that *Trifolium* is embedded within *"*vicioid clade*"* (Sanderson and Liston, 1995; Sanderson et al., 1996). Within the vicioid clade, Fabeae and Trifolieae comprise a monophyletic group. Similarly Steele and Wojciechowski in (2003) conducted a phylogenetic analysis of the Trifolieae and Fabeae based on cpDNA *matK*, upon strong support for this monophyly of *Trifolium* was provided. Moreover, this monophyly has been demonstrated by the results obtained from nuclear ITS and chloroplast *trnL* molecular analysis among 218 *Trifolium* species (Ellison et al., 2006). On the contrary, Watson (2000) contradicted the monophyly of most *Trifolium* sections which were recognized by Zohary and Heller (1984).

His study was conducted on 59 Old World *Trifolium* species based on nrDNA ITS sequences and restriction site analysis of PCR-amplified DNA.

In addition the utility of nrDNA ITS sequence in resolving the phylogenetic relationship among closely related species of three subspecies of *Trifolium nigrescens* was performed (Williams et al., 2001).

As no previous molecular phylogenetic analysis of *Trifolium* has sampled the taxonomic breadth of its identified species in Zohary (1966). Therefore, they were the aim of this study to highlight the taxonomical issue in respect to their identification and classification.

1.5 Aim of the Study

To resolve the taxonomic conflicts in morphological dependent classification of closely related species of *Trifolium* in Palestine / West Bank based on the molecular analysis of Internal Transcribed Spacer (ITS) regions.

Chapter Two

Materials and Methods

2.1 Plant Materials

Fresh specimens of different species of *Trifolium* were collected during the flowering period (March – May 2017) from different locations in Palestine/West Bank. Representative samples of the species under the study were identified depending on taxonomical characteristics: shape of flowering heads, color of petals, arrangement of leaves, form of stipules, form of calyx, and number of nerves on calyx. Specimens were pressed till drying in appropriate conditions at An-Najah National University Herbarium. Table 2.1 shows scientific and common names of the studied species with their locations and Lifespan.

The samples were chemically poisoned using a mixture of mercuric chloride and ammonium chloride (150 g of mercuric chloride (HgCl) and ammonium chloride (NH4Cl) were dissolved in as little water as possible, then 10 L of 96% ethanol was added to the previous mixture). The poisoned specimens were labeled with the date of collection, location, name of the collector, then mounted on herbarium sheets and given a herbarium voucher number. Table 2.2 shows the Voucher numbers of the studied species and their scientific names. The sheets were deposited at the herbarium, Department Biology and Biotechnology, Faculty of Science at An – Najah National University.

Table 2.1: Scientific and Common names of studied species of *Trifolium* with their locations and Lifespan .

No. of samples	Scientific name	Locations	Common name	Lifespan
1.	*Trifolium purpureum* Loisel.	Tulkrem / Far'oun	Purple clover	Annual
2.	*Trifolium purpureum* Loisel.	Tulkrem / Far'oun	Purple clover	Annual
3.	*Trifolium purpureum* Loisel.	Tulkrem / Far'oun	Purple clover	Annual
4.	*Trifolium purpureum* Loisel.	Jenin / near Arab American University	Purple clover	Annual
5.	*Trifolium purpureum* Loisel.	Jenin / near Arab American University	Purple clover	Annual
6.	*Trifolium campestre* Schreb.	Nablus / Beit -wazan	Low Hop Clover	Annual
7.	*Trifolium campestre* Schreb.	Nablus / Beit -wazan	Low Hop Clover	Annual
8.	*Trifolium stellatum* L.	Jenin / near Arab American University	Star Clover	Annual
9.	*Trifolium stellatum* L.	Jenin / near Arab American University	Star Clover	Annual
10.	*Trifolium stellatum* L.	Jenin / near Arab American University	Star Clover	Annual
11.	*Trifolium dasyurum* C.	Jericho/Wadi Al Qalt	Eastern star clover	Annual
12.	*Trifolium dasyurum* C.	Jericho/Wadi Al Qalt	Eastern star clover	Annual

Table 2.2: Voucher numbers, GenBank accession numbers of the studied species and their scientific names.

No. of sample	Scientific name	Voucher no.	GenBank no.
1.	*Trifolium purpureum* Loisel.	1863	MF589956
2.	*Trifolium purpureum* Loisel.	1864	MF589957
3.	*Trifolium purpureum* Loisel.	1865	MF589958
4.	*Trifolium purpureum* Loisel.	1883	MF589959
5.	*Trifolium purpureum* Loisel.	1884	MF589960
6.	*Trifolium campestre* Schreb.	1878	MF589961
7.	*Trifolium campestre* Schreb.	1879	MF589962
8.	*Trifolium stellatum* L.	1886	MF589963
9.	*Trifolium stellatum* L.	1887	MF589964
10.	*Trifolium stellatum* L.	1888	MF589965
11.	*Trifolium dasyurum* C.	1856	MF589966
12.	*Trifolium dasyurum* C.	1857	MF589967

2.2 Identification, Classification and Taxonomy of the Collected Samples

Depending on Flora Palaestina (Zohary, 1966a), and using of Stereo microscope, the collected samples were identified and classified based on morphological characteristics:

- Habit: annual or perennial species.
- Leaves: if trifoliolate, leaflets entire or dentate, leaf arrangement alternate or opposite.
- Inflorescence: capitate or spicate heads.
- Calyx: tubular or campanulate, number of nerves, equal or unequal teeth, throat of calyx open or closed, with the presence of callosity or by a ring of hairs.

- Corolla color: colored with pink, white, lilac, violet, or 2-colored.

2.3 Plant DNA Extraction

Genomic DNA was extracted from fresh plant material of each plant species under the study (100 mg fresh leaves weight) using Cetyltrimethyl Ammonium Bromide (CTAB) extraction protocol from Gawel and Jarret (1991) with some modifications.

Fresh leaves were ground to a fine powder in mortar and pestle in the presence of liquid nitrogen. Fifty mg of the powder was transferred to 1.5 ml Eppendorf® Safe-Lock microcentrifuge tube. A total of 900 µl of extraction buffer was added to each tube [100 mM Tris-HCL, 20mM of EDTA pH 8.0, 1.4 M of NaCl , 4% w/v CTAB , 2% w/v PVP K90 and 1% final volume of β-mercaptoethanol (added just before use)]. The mixture was homogenized using Hand Held Homogenizer (MRC Ltd). The mixture was vortexed for 1 min and then incubated for 45 min at 65 ºC in a water bath. After incubation tubes were centrifuged at a maximum speed of (14.000 rpm), the lysate from each tube was transferred into new 1.5 ml eppendorf tube, then 400 µl of chloroform-isoamylalcohol (24:1, v/v) was added and tubes were centrifuged for 15 min at 3000 rpm. After centrifugation the upper aqueous phase was transferred to QIAshredder spin columns to remove precipitate and cell debris, then tubes were centrifuged for 5 min at 14.000 rpm , equal volume of Isopropanol was added to the lysate and incubated over night at (-20 ºC). The supernatant was discarded after centrifugation for 5 min at 14.000 rpm, and then DNA

pellets were rinsed with 50 µl of 70% cold ethanol and centrifuged for 3 min at 14.000 rpm. The supernatant was discarded and the pellets allowed to dry for 45 min, then resuspended in 50 µl TE buffer. RNase digestion was followed to remove RNAs from the solution, by addition 2 µl of RNase A and incubation for 1 h at 37 ºC.

2.4 PCR Amplification and Gel Electrophoresis

The nuclear ribosomal DNA including the ITS regions (ITS-1 spacer, 5.8S rDNA and ITS-2 spacer) were amplified using universal primers (Fior et al., 2006). Primer sequences were ITS-1F (5'-TCC GTA GGT GAA CCT GCG GAA GGA TCA TTG-3') and ITS-4R (5'-TCC TCC GCT TAT TGA TAT GC-3'). The amplified PCR product was approximately 700 bp. Briefly, the PCR reaction was performed with a final volume of 25 µl containing 12.5 µl of PCR premix with $MgCl_2$ (ReadyMixTM Taq PCR Reaction Mix with $MgCl_2$, Sigma), 0.4 µM of each primer, 3µl (10.25 - 31.6 ng/ µl) of DNA template. The DNA amplification was performed with a thermal cycler (Mastercycler Personal, Eppendorf) using the following conditions: initial denaturation for 3 min at 94 ºC was followed by 35 cycles, each cycle consisting of denaturation at 94 ºC for 50 sec, annealing at 50 ºC for 50 sec and extension at 72 ºC for 2 min, with a final extension step at 72 ºC for 10 min. The PCR products were resolved by electrophoresis through 1.5 % agarose gel to determine the size of amplified fragment after ethidium bromide staining (0.5 µg/ml).

2.5 DNA Cleaning and Sequencing

The amplified PCR products were cleaned using ChargeSwitch®-Pro PCR Clean-Up Kit (Invitrogen, USA), following the manufacturer's protocol. DNA PCR products were sequenced by dideoxynucleotide chain termination method using 3130 Genetic Analyzer (Applied Biosystems®, USA), Bethlehem University, Bethlehem, Palestine. The sequencing of PCR product was carried out with ITS-1F and ITS-4R primers used singly in forward and reverse reactions, respectively, and BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems®, USA). Sequence information was submitted for accession number in primary bioinformatics web servers.

2.6 Sequence Alignment and Analysis

The comparison of the continuous sequences of ITS region of *Trifolium* studied species was carried out with previously available sequences in GenBank using Blastn (Nucleotide Basic Local Alignment Search Tool) system. Multiple alignments were done using ClustalW of the computer program MEGA software (version 6). Phylogenetic analysis was based on alignments obtained from ClustalW of a 627 bp sequence. Phylogenetic tree was constructed using the Neighbor-Joining method in the same software. The robustness of the groupings in the Neighbor Joining analysis was assessed with 1000 bootstrap resembling. Reference sequences were retrieved from GenBank used for phylogenetic analysis and *Salvia*

candidissima was used as an out-group. Pair-wise distances were computed using the Kimura's 2-parameter method (K2-P).

Chapter Three

Results

3.1 Morphological Characterization of Leguminosae Family

3.1.1. Classification of Leguminosae Family

Kingdom: Plantae – Plants

Subkingdom: Tracheobionta – Vascular plants

Superdivision: Spermatophyta – Seed plants

Division: Magnoliophyta – Flowering plants

Class: Magnoliopsida – Dicotyledons

Subclass: Rosidae

Order: Fabales

Family: Leguminosae

The following description template was obtained from Flora Palaestina (Zohary, 1966a).

3.1.2. Description of the Leguminosae Family

Herbaceous or woody plants, rarely creeping or climbing, sometimes spiny. Leaves alternate, rarely opposite, usually pinnate, 3-foliolate or digitate, sometimes terminating in or reduced to a tendril, rarely leaves simple; stipules usually present. Flowers hermaphrodite, in axillary or terminal racemes, panicles, heads or spikes, rarely solitary, zygomorphic. Calyx 5-, rarely 4-merous, with sepals more or less united at base, often 2-lipped. Corolla 5-merous, papilionaceous, consisting of a posterior, outermost petal (the standard), 2 lateral, often horizontal, petals (the wings) and 2 anterior and innermost ones usually united by their margins (the keel). Fruit usually

a 1-celled, many seeded pod (legume), dehiscing by 2 valves along the ventral and dorsal sutures. Seeds usually fairly large sometimes arillate.

3.1.3. Key the Studied *Trifolium* Species

1. Flowers yellow to pale yellow, standard flattish or spoon-shaped all along; calyx tube 5- nerved, calyx upper teeth much shorter than the three lower, throat open……1. *T.campestre*

1. Flowers white, pink or purple, standard oblong limbed, somewhat longer than wings; calyx tube 10- nerved, calyx teeth equal in length, throat open or not.

2. Flowers whitish or pink; calyx campanulate, densely subappresed-hispid, throat densely fleecy but not closed by a callosity. Stipules not membranous, dentate, not ending with cuspidate apex……..2. *T.stellatum*

2. Flowers pink or purple; calyx cylindrical, subappresed hirsute or appresed to patulous-hairy, throat closed by callosity or provided with hair ring. Stipules membranous, entire, ending with cuspidate apex.

3. Heads cylindrical. All leaves including upper most ones alternate. Flowers corolla longer than calyx; calyx subappresed hirsute, throat completely closed by a callosity ……3.*T.purpureum*

3. Heads ovoid. All leaves alternate except upper ones opposite. Flowers corolla as long as calyx; calyx appresed to patulous-hairy, throat provided by a hair ring…….....4.*T.dasyurum*

3.1.4. Description of the Clover Genus (*Trifolium* L.) and the Studied Species

Annual or perennial herbs with erect, ascending or procumbent stems, sometimes with creeping rootstocks. Leaves with 3, rarely with 5 (or 7) dentate or entire leaflets; stipules partly connate and adnate to petiole, entire or rarely dentate. Racemes axillary or terminal, often head-like or spike-like or umbellate, pedunculate or sessile, many- rarely few-flowered, sometimes involucrate by free or connate bracts. Flowers pedicellate or sessile, bracteate or not, all or rarely only the outer (lower) flowers fertile. Calyx mostly tubular or campanulate, 5, 10-20(rarely30-36) - nerved, with 5 equal or unequal teeth; sometimes calyx 2-lipped, with upper teeth often shorter or longer and partly connate at base; throat of calyx open or closed by a callosity or by a ring of hairs; tube of calyx sometimes inflated in fruit. Corolla persistent, marcescent or caduceus, white, yellow, purple; pink, fresh -coloured, lilac, violet or 2-coloured; standard free or connate at base with wings and keel; the latter two sometimes connate with one another and mostly adnate to the stamens; wings often longer than keel. Pod indehiscent, mostly 1-2 (rarely 4-8)-seeded, enclosed in the persistent calyx and sometimes also in the persistent corolla, rarely exserted, usually

membranous, rarely leathery, ovoid to oblong or linear. Seeds globular to ovoid and oblong, sometimes reniform or lenticular.

3.1.4.1 ***Trifolium campestre*** Schreb. In Sturm, Deutschl. F1.1, 16: t.253 (1804). *T.agrarium* L., Sp.P1772 (1753) p.p.; Boiss., F1. 2: 153(1872) p.p *T. procumbens* L., F1. Suec.ed. 2, 261 no. 673 (1755) p.p. non L., Sp. P1. 772 (1753); Boiss., 1.c. 154 p.p.(Zohary, 1966a) [Plate 1].

Annual, hairy or almost glabrous, 10-30 cm. Stems erect, ascending or prostrate, simple or branched. Leaves petiolate; stipules herbaceous, ovate to oblong, long- acuminate; leaflet 0.8-1.6 x 0.4-0.8 cm., ovate to oblong – elliptical, often with cuneate base, truncate or retuse at apex, denticulate in upper half; terminal leaflet long- petioluate. Peduncles as long as or longer or shorter then leaves. Heads 0.8-1.3 X0.7-1 cm., many flowered, often globular. Pedicels shorter than calyx, becoming deflexed early. Flowers rather dense, later becoming imbricated. Calyx white, 5- nerved, glabrous or rarely slightly hairy; tube membranous; the 2 upper teeth very short, triangular or lanceolate, the others about twice as long as tube or longer, long-subulate. Corolla (4-) 5- 6 (-7)mm., yellow to pale yellow, turning brown in fruit; standard 4-5mm., with orbicular limb, flat or spoon-shaped, denticulate at margin, many-nerved. FI. February-April (-October).

Area: Mediterranean, with extensions into the Euro-Siberian and Irano-Turanian regions.

Habit: Fields, Al-bitaha' roadsides. Al-Jalīl, Akka Plain, The Western Central Plains of Palestine, Palestinian Plain, Al Jaleel Al A'alaa Mt.,

Kurmul Mt., Marj Ibn Amir Plain, Mt. Faqqua, Nablus, Hebron Mts., An-Naqab, Tell el-Qadi, Al-Hula Plain, Al-Qhor, Beesan Valley, Gilead, Ammon, Mo'ab. Common.

Distribution: Tulkarem, Kufur Qud, Road to Ramallah, Nablus –bit wazn [Plate3].

3.1.4.2 ***Trifolium stellatum*** L. Sp. P1.769 (1753); Boiss., F1. 2:121 (1872). (Zohary, 1966a) [Plate1].

Annual, patulous and soft-villose, 10-20 cm. Stem few, mostly erect or ascending, sparsely branched or unbranched. Leaves long-petioled; stipules membranous, ovate, obtuse, dentate, green at margin; leaflets mostly 5-8 X 4-8 mm., obcordate with cuneate base, dentate in upper part . Heads 1.5-2 cm., long-peduncled, many- flowered, broadly obovoid to globular. Flowers 1.5-1.8 cm., loose. Calyx campannulate, densely subappressed-hispid, 10-nerved; tube shorter than the lanceolate- subulate teeth, the latter somewhat connate at base; throat densely fleecy but not closed by a callosity. Corolla mostly shorter than or as long as, rarely somewhat longer than calyx teeth, white or pink; standard with ovate to oblong limb, slightly longer than wings. Fruiting calyx with a long and sharp-pointed base; teeth spreading, broadened at base, with bristles directed upwards. Pod short-stipitate, membranous, lanceolate to pear-shaped. Fl. February-April

Area: Mediterranean, with slight extensions into adjacent regions.

Habit: Roadsides**,** fields and Al-bitaha'., Akka Plain, The Western Central Plains of Palestine, Palestinian Plain, Al Jaleel Al A'alaa Mt., Kurmul Mt., Marj Ibn Amir Plain, Mt. Faqqua, Nablus, Wadi Al- Khalil, Hebron Mts., Tell el-Qadi, Al-Hula Plain, Al-Qhor, Gilead, Ammon, Mo'ab.

Distribution: Jenin, Road to Ramallah [Plate3].

3.1.4.3 ***Trifolium purpureum*** Loisel., F1. Gall 484, t.14 (1807). (Zohary, 1966a) [Plate2].

Annual, mostly appressed- to subappressed- hirsute, 10- 30 (-50) cm. Stems few or many, erect, ascending or rarely procumbent, branching above, striate. Leaves with petioles shortening towards apex of stem, stipules 1-1.5cm., membranous between nerves, oblong- lanceolate, with upper portion subulate; leaflets 2-4(-6) X 0.2-1 cm., oblong- lanceolate to linear or ellipictal, acute, mucronulate, obscurely toothed above, mostly hairy only at margins. Heads (1-) 1.5-6 cm., at the end of dichotomous or simple branches, conical or ovoid in flowers, ovoid- oblong to cylindrical in fruit. Flowers 1.5-2 cm. Calyx up to 1 cm., subappressed- hirsute; tube 3mm. or more, almost cylindrical; teeth subulat, the lowermost tooth longer than calyx, slightly longer than to twice as long as the rest. Corolla distinctly longer than calyx, purple above, whitish- lilac below, sometimes lilac or whitish all over ; standard with oblong limb distinctly longer than wings. Fruiting calyx tube obconical, prominently nerved; teeth blunt, divergent or spreading, plumose, with bristles arising from tubercle; throat completely closed by callosity. Seeds about 1mm., ovoid, brown. F1. February-May

Area: Mainly Mediterranean, with extensions into adjacent W. Irano-Turanian and Euro-Siberian regions.

Habit: Fields, Al-bitaha' and roadsides. Al-Jalīl, Akka Plain, The Western Central Plains of Palestine, Palestinian Plain, Al Jaleel Al A'alaa Mt., Kurmul Mt., Marj Ibn Amir Plain, Mt. Faqqua, Nablus, Wadi Al –Khalil , Hebron Mts., An-Naqab, Tell el-Qadi, Al- Hula Plain, Al-Qhor, Beesan Valley, Gilead, Ammon. Common.

Distribution: Jenin, Kur Qud, Road to Ramallah, Tulkrem. [Plate3]

3.1.4.4 ***Trifolium dasyurum*** C. Presl, Sumb. Bot. 1:53 t.33 (1832). *T.formosun* Urv., Mem. Soc. Linn. Paris 1: 350 (1822) non Savi, Obs. Trif. 102 (1810) nec Curt. Ex DG., Prodr. 2: 200 (1825); Boiss., F1. 2: 124 (1872). *T.formosun* Uvar. Var. *minus* Post, F1 Syr. Pal. Sin. 236 (1883-1896) et ed. 2, 1: 338 (1932). *T. velivolum* Paine, Palest. Explor. Soc. Statement 3: 103 (1875). (Zohary, 1966a) [Plate2].

Annual, appressed- or antrorsely pubescent, 10-30(-40) cm. Stems few or many, ascending, diffuse, often dichotomously branching above, striate. Both branches of each fork, or only one of them, developed and ending with a head. Leaves opposite (when only one branch of the fork develops),petiolate, uppermost leaves subsessile; stipules membranous, inflated, with arcuate nerves and a long, subulate to cuspidate tip; leaflets 1-305 X0.2-1 cm., elliptical to oblong, acute, not dentate or obscurely so. Heads 1.5-3 (-4) cm., many-flowered, ovoid. Calyx appressed- to patulous-hairy; tube cylindrical to obconical; teeth about twice as long as tube,

equal, subulate, truncate, with lanceolate base. Corolla about as long as calyx, purple above, whitish or pink below; standard with oblong limb, somewhat longer than wings. Fruiting calyx with top-shaped tube, divergent or spreading teeth and closed throat. Seeds smooth about 1.8mm. F1.March-May

Area: E. Mediterranean, with extensions into adjacent territories of the Irano-Turanian region.

Habit: Roadsides and fallow fields. Al-Jalīl, The Western Central Plains of Palestine, Palestinian Plain, Al Jaleel Al A'alaa Mt. Kurmul Mt., Marj Ibn Amir Plain, Mt. Faqqua, Nablus, Wadi Al-Khalil, Hebron Mts., An-Naqab, Tell el-Qadi, Al- Hula Plain, Al-Ghor, Gilead, Ammon, Common.

Distribution: Jericho /Wdi Al-Qalt. [Plate3].

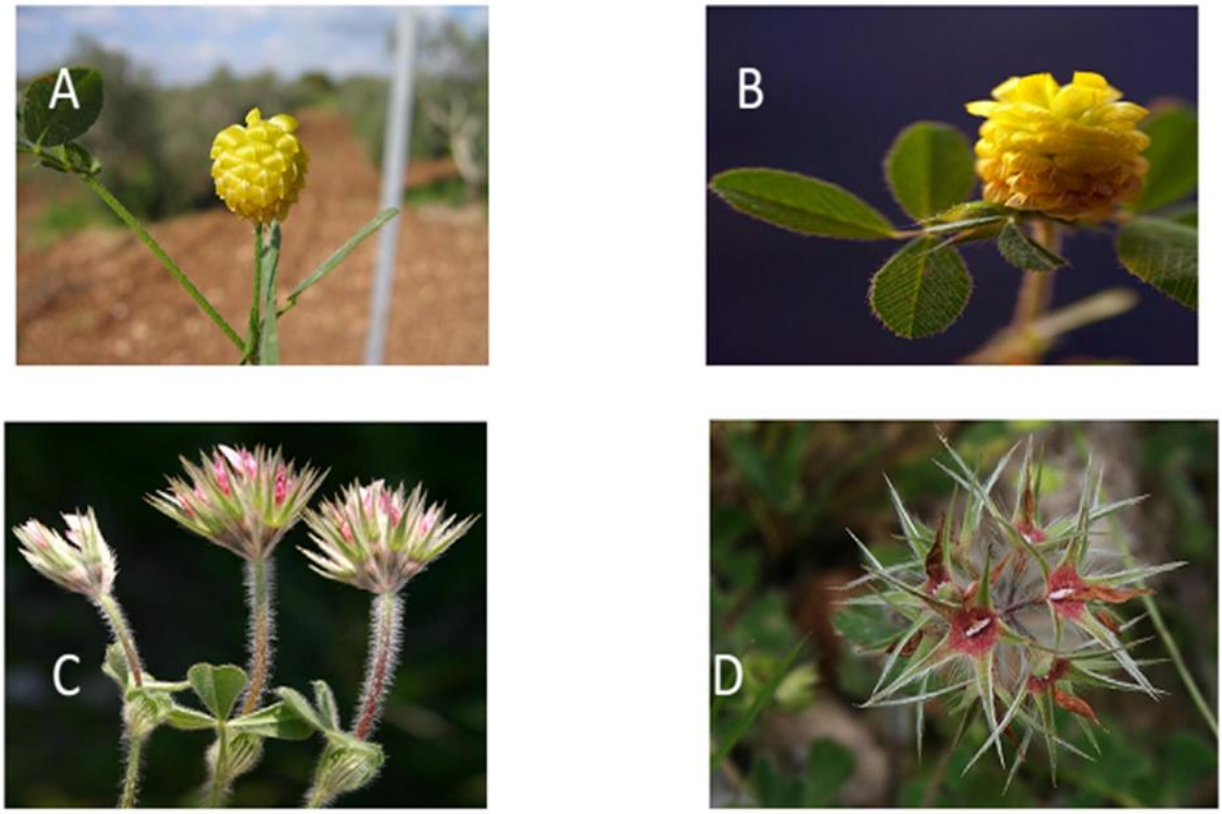

Plate 1: Colored photographs of the studied species

A. ***Trifolium campestre*** **B.** ***Trifolium campestre***

C. ***Trifolium stellatum*** **D.** ***Trifolium stellatum***

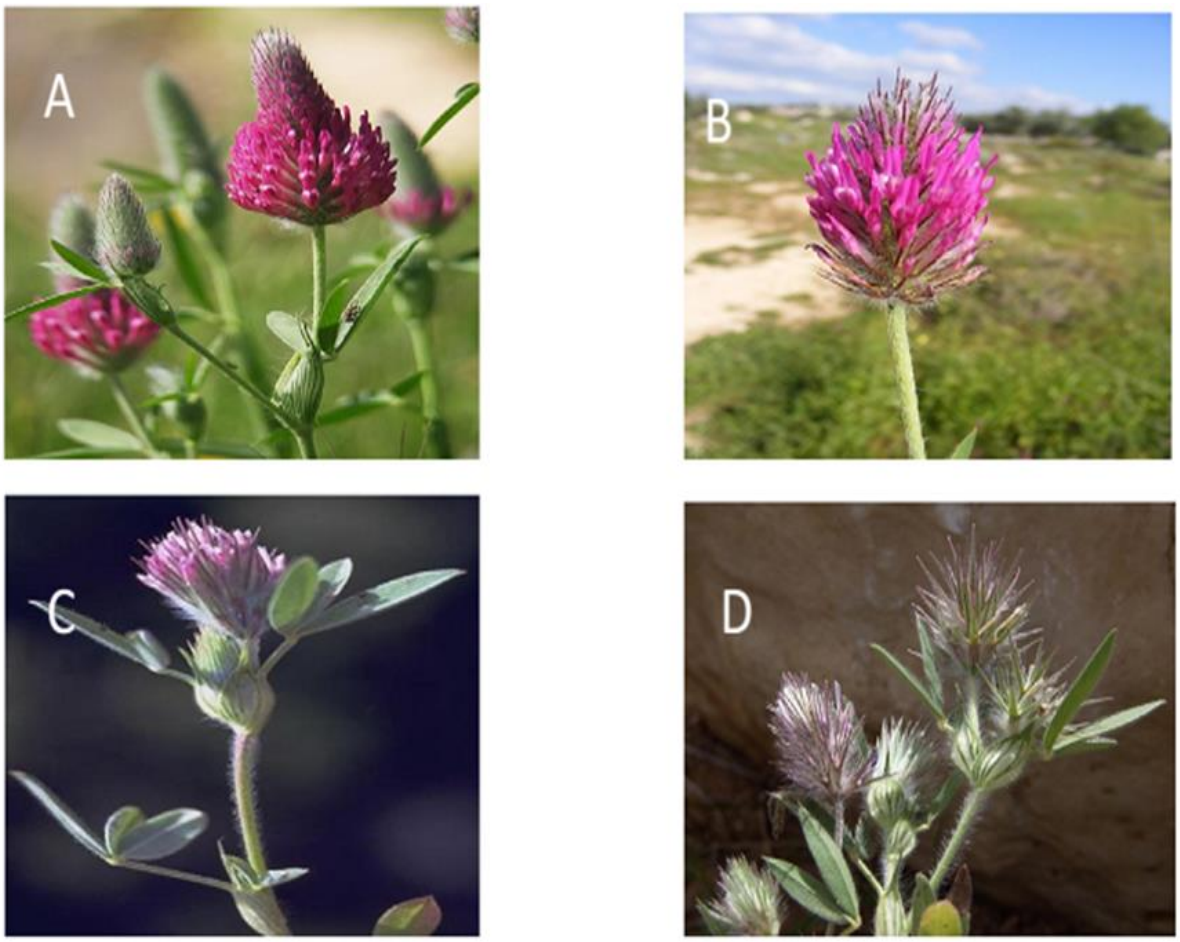

Plate 2: Colored photographs of the studied species

A. ***Trifolium purpureum*** **B.** ***Trifolium purpureum***

C. ***Trifolium dasyurum*** **D.** ***Trifolium dasyurum***

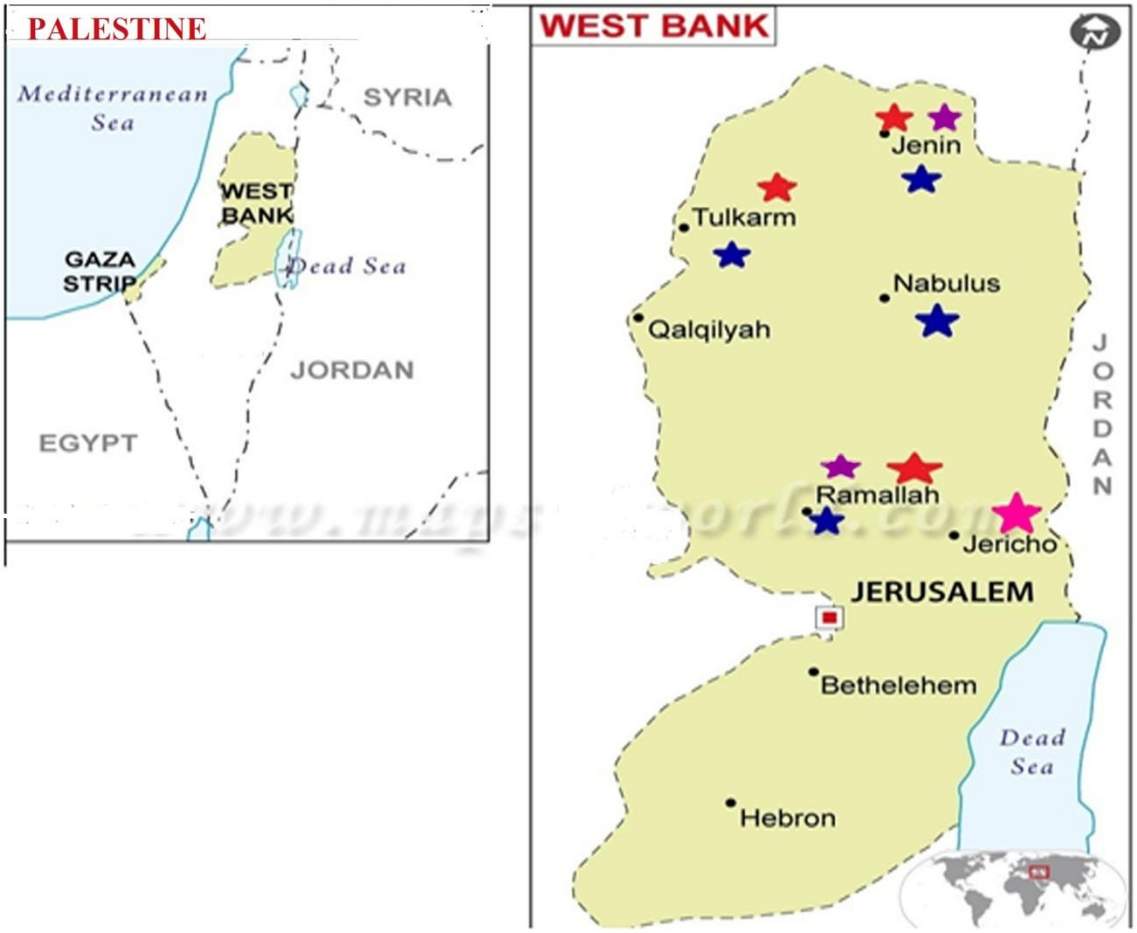

Plate 3: Map of Palestine showing the distribution of the studied species

Trifolium campestre ***Trifolium purputeum***

Trifolium dasyurum ***Trifolium stellatum***

3.2. Molecular Characterization of the Studied *Trifolium* Species

3.2.1 Obtained DNA Extract Quantity and Quality of Investigated *Trifolium* Species.

Table 3.1: DNA Extract Quantity and Quality of the studied species.

No.of sample	Plant Species sample	OD260	260/280 (Purity)	ng/ µl (DNA concentration)
1.	*T.purpureum*	0.344	1.442	17.2
2.	*T.purpureum*	0.289	1.323	14.45
3.	*T.purpureum*	0.408	1.65	20.4
4.	*T.purpureum*	0.216	1.126	10.8
5.	*T.purpureum*	0.326	1.4	16.3
6.	*T.campestre*	0.208	1.134	10.25
7.	*T.campestre*	0.205	1.064	10.25
8.	*T. stellatum*	0.398	1.75	20
9.	*T. stellatum*	0.316	1.414	15.8
10.	*T. stellatum*	0.218	1.276	11
11.	*T. dasyurum*	0.632	1.47	31.6
12.	*T. dasyurum*	0.419	1.6	21

3.2.2 Agarose Gel Electrophoresis Analysis for the Amplified ITS Sequences

Specific sites of nuclear ribosomal DNA (ITS-1 spacer, 5.8S rDNA, ITS-2 spacer) of the studied species were amplified using ITS-1F and ITS-4R primers. The amplified PCR products for all studied species gave a single band with approximately 700 bp (Figure 3.1).

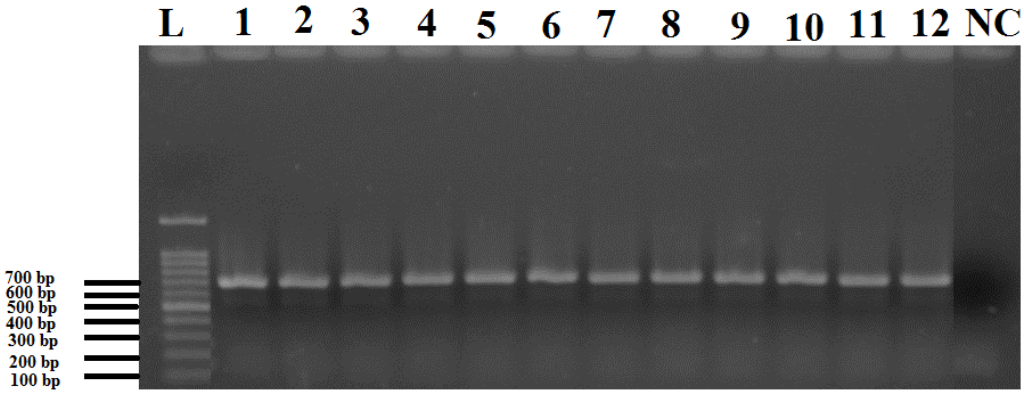

Figure 3.1: Agarose gel electrophoresis analysis showing detection of amplified ITS regions of different species of *Trifolium.* Lane L: 100-bp DNA ladder; lanes1-5: *T.Purpurem*; lanes 6-7: *T.campestre*; lanes, 8-10: *T.stellatum*; lanes, 11-12: *T.dasyurum*; lane NC: negative control.

3.2.3 Genetic Relationship among Closely Related Species of the Clover Genus (*Trifolium*- Leguminosae)

Sequences of nrDNA ITS (≈ 700 bp) were obtained from 12 classified plant leaf samples that belong to the Clover genus (*Trifolium*). Afterwards phylogenetic tree was constructed according to the similarity among *Trifolium* species, where the bootstrap was inferred 1000 replicates. All DNA ITS sequences of the examined species were deposited at the GeneBank database under the accession numbers (MF589956-MF589967). Table 2.2 shows the Voucher number and GeneBank no. for all studied species samples.

The resulted phylogenetic tree demonstrates the relationship among the studied closely related species and their related species of clover genus retrieved from the GeneBank. Whereas, evolutionary DNA distances between ITS region sequences were computed using the K2-P method for the studied closely related *Trifolium* Species and other species retrieved from the GeneBank (Figure 3.2, Table 3.2 respectively).

Two main clades (I, II) appeared in the phylogenetic tree according to ITS sequences analysis. Clade I contains two sub-clades (Ia, Ib). Sub-clade (Ia) includes sequences of ITS regions for *T.purpurem* and *T.dysurum* and their closely related species. Whereas, clade II contains two sub-clades (IIa, IIb). Sub-clade (IIa) include *T.stellatum* and its closely related species, but, sub-clade (IIb) includes *T.campestre* and its closely related species.

The presence of *T.purpurem* (MF589956, MF589957, MF589958, MF589959, MF589960), *T.daysurum* (MF589966, MF589967) and the species that were retrieved form GeneBank *T.purpurem* (DQ312140), *T.daysurum* (DQ312040) in the same clade I (sub-clade Ia), showed that they are identical species, since they have 0% bp differences (Table 3.2). Moreover, *T.purpurem* and *T. dasyurum* form a sister group to *T.angustifolium*, *T.dichroanthum* and *T.prophetarum* (DQ312200.1, DQ312044.1 and DQ312206.1 respectively) (Figure 3.2).

In clade II, sub-clade (IIa) shows that *T.stellatum* (MF589963, MF589964, MF589965) are closely related to *T.incarnatum* which was retrieved GeneBank (AF053160.1). In the phylogenetic tree, sub-clade (IIb) shows

that *T.campestre* (MF589961, MF589962) and *T.grandiflorum* which was retrieved from GeneBank (DQ312062.1) are identical species, since they have identical ITS sequence (0% bp differences) (Table 3.2). In addition, based on the phylogenetic tree and on the resulted base pair difference data (Table 3.2), *T.boissieri* (DQ312017.1) is related to both *T. campestre* and *T.grandiflorum*, since they have 1% bp differences (Table 3.2). Those three species form a sister group to *T. glanduliferum* (DQ312056) as illustrated in the phylogenetic tree. However, *S. candidissima* was found to be quite divergent and did not fall in any of the major clusters as illustrated (Figure 3.2).

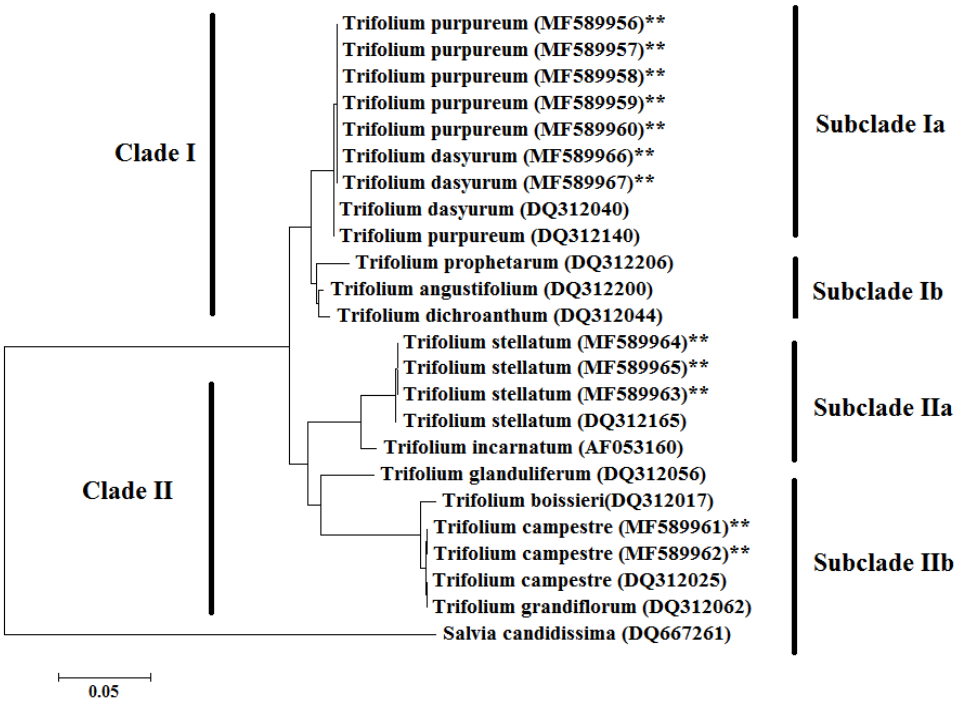

Fig 3.2: Phylogenetic analysis by Neighbor-Joining method based on ITS region sequences. Sequences of *Trifolium* L. species (*T.purpurem*, *T.dasyurum*, *T.campestre* and *T.stellatum*) denoted by asterisk represent the studied samples. Other reference sequences species used for phylogenetic analysis were retrieved from GeneBank. *Salvia candidissima* was used as an out - group. Bootstrap consensus tree was inferred from 1000 replicates.

Table 3.2. Genetic differences between ITS region sequences obtained from the studied *Trifloium* species (donated by asterisk) and those retrieved from the GeneBank. The evolutionary DNA distances were compound using K2P method.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.
1.Trifolium_purpureum_(MF589956)**																							
2.Trifolium_purpureum_(MF589957)**	0.00																						
3.Trifolium_purpureum_(MF589958)**	0.00	0.00																					
4.Trifolium_purpureum_(MF589959)**	0.00	0.00	0.00																				
5.Trifolium_purpureum_(MF589960)**	0.00	0.00	0.00	0.00																			
6.Trifolium_campestre_(MF589961)**	0.10	0.10	0.10	0.10	0.10																		
7.Trifolium_campestre_(MF589962)**	0.10	0.10	0.10	0.10	0.10	0.00																	
8.Trifolium_stellatum_(MF589963)**	0.08	0.08	0.08	0.08	0.08	0.11	0.11																
9.Trifolium_stellatum_(MF589964)**	0.08	0.08	0.08	0.08	0.08	0.11	0.11	0.00															
10.Trifolium_stellatum_(MF589965)**	0.08	0.08	0.08	0.08	0.08	0.11	0.11	0.00	0.00														
11.Trifolium_dasyurum_(MF589966)**	0.00	0.00	0.00	0.00	0.00	0.10	0.10	0.08	0.08	0.08													
12.Trifolium_dasyurum_(MF589967)**	0.00	0.00	0.00	0.00	0.00	0.10	0.10	0.08	0.08	0.08	0.00												
13.Trifolium_glanduliferum_(DQ312056)	0.07	0.07	0.07	0.07	0.07	0.09	0.09	0.08	0.08	0.08	0.07	0.07											
14.Trifolium_incarnatum_(AF053160)	0.08	0.08	0.08	0.08	0.08	0.10	0.10	0.03	0.03	0.03	0.08	0.08	0.07										
15.Trifolium_campestre_(DQ312025)	0.10	0.10	0.10	0.10	0.10	0.00	0.00	0.11	0.11	0.11	0.10	0.10	0.09	0.10									
16.Trifolium_stellatum_(DQ312165)	0.09	0.09	0.09	0.09	0.09	0.11	0.11	0.00	0.00	0.00	0.09	0.09	0.08	0.03	0.11								
17.Trifolium_boissieri(DQ312017)	0.10	0.10	0.10	0.10	0.10	0.01	0.01	0.12	0.12	0.12	0.10	0.10	0.09	0.11	0.01	0.12							
18.Trifolium_grandiflorum_(DQ312062)	0.10	0.10	0.10	0.10	0.10	0.00	0.00	0.11	0.11	0.11	0.10	0.10	0.09	0.10	0.00	0.11	0.01						
19.Trifolium_dasyurum_(DQ312040)	0.00	0.00	0.00	0.00	0.00	0.10	0.10	0.09	0.09	0.09	0.00	0.00	0.07	0.07	0.10	0.08	0.10	0.10					
20.Trifolium_prophetarum_(DQ312206)	0.04	0.04	0.04	0.04	0.04	0.11	0.11	0.09	0.09	0.09	0.04	0.04	0.07	0.07	0.11	0.08	0.12	0.11	0.03				
21.Trifolium_angustifolium_(DQ312200)	0.02	0.02	0.02	0.02	0.02	0.10	0.10	0.08	0.08	0.08	0.02	0.02	0.06	0.06	0.10	0.07	0.10	0.10	0.02	0.02			
22.Trifolium_purpureum_(DQ312140)	0.00	0.00	0.00	0.00	0.00	0.10	0.10	0.09	0.09	0.09	0.00	0.00	0.07	0.07	0.10	0.08	0.10	0.10	0.00	0.03	0.02		
23.Trifolium_dichroanthum_(DQ312044)	0.02	0.02	0.02	0.02	0.02	0.10	0.10	0.08	0.08	0.08	0.02	0.02	0.07	0.07	0.10	0.08	0.11	0.10	0.02	0.03	0.01	0.02	
24.Salvia_candidissima_(DQ667261)	0.42	0.42	0.42	0.42	0.42	0.47	0.47	0.45	0.45	0.45	0.42	0.42	0.44	0.44	0.46	0.45	0.46	0.46	0.41	0.42	0.42	0.41	0.42

Chapter Four

Discussion

4.1 Taxonomy of *Trifolium*

Classification of *Trifolium* depends on essential morphological characters such as calyx, corolla, and others. Even in spite of the fairly unmodified pods in the species of that genus, sometimes they can be reliable for their identification and classification. An elaborated taxonomical review on the genus *Trifolium* was carried out by Hossain on 1961. In his review he subdivided this genus in to eight subgenera. Nevertheless, an important comprehensive study on the genus *Trifolium* revealed in the nomenclature of these eight subgenera by sections (Zohary and Heller, 1984). Those sections are: Lotoidea, Paramesus, Mystillus, Vesicaria, Chronosemium, Trifolium, Tricocephalum and Involucrarium. Although many differences are recognized among the members of the considered sections, the only character that they shared in, is the adnate claws of wings and keel to the staminal bundle (Hossain, 1961).

Species of the section Chronosemium (*T.campestre*, *T.boissieri*, and *T.grandiflorum*) are characterized by some common and uncommon morphological features to those in section Paramesus (*T.glanduliferum*). In the current study, they were shown to be sister groups in the same clade II/ sub-clade IIb in the constructed phylogenetic tree (Figure 3.1). However, a unique feature to the species of the section Paramesus in having glands on their stipules tips may be considered the reason for their classification to separate sections rather than one. This molecular ITS evidence in this study coincides with the taxonomical debate of Hossain (1961) regarding those taxa.

Morphological characters of *T.campestre* illustrate that it is closely related to *T.boissieri*. The only different character between these two species is the shape of the corolla standard. In *T.campestre* the standard is usually flattish or spoon-shaped all along, whereas it is spoon-shaped from above only in *T.boissieri*. The resulted 1% bp difference (Table 3.2) and their possion in the same clade II/ sub-clade IIb in the constructed phylogenetic tree (Figure 3.1) support that both *T.campestre* and *T.boissieri* could be actually considered related two species.

An outstanding information was obtained from the alignment of *T.campestre* ITS sequence in the GeneBank using Blastn, that *T.grandiflorum* has an identical ITS sequence, as they both have 0% bp difference (Table 3.2). Based on the observed morphological characters of *T.grandiflorum* (Flora of Israel Online, 2006+), the only difference was the petals color as being pink in *T.grandiflorum* and yellow in *T.campestre*. Since, different phenotypes, for example different flower colors can't be considered as different genotypes (Zohary, 1987). This can be confirmed by that the genetic mechanisms controlling floral features are apparently unstable, resulting in fluctuating asymmetry (Friesen et al., 1997). So a direct relation between the phonetic and genetic variation indeed can't be easily established (Treu et al., 2001). Therefore, *T.campestre* and *T.grandiflorum* could be regarded taxonomically as one species.

The section Trifolium (*T.purpurem*, *T.dasyurum*, *T.stellatum*, *T.angustifolium* and *T.dichroanthum*… etc.) contains the largest species number of the genus *Trifolium*. The ITS sequence analysis of *T.purpurem*

and *T.dasyurum* showed that they are identical species, since they have 0% bp difference (Table 3.2), and were clustered together in clade I/ sub-clade Ia in the constructed phylogenetic tree (Figure 3.2). This coincides with the high resemblance of their morphological characters. However, the arrangement of their leaves was the reason after distinguishing them as two different species. All leaves including the uppermost ones are alternate in *T.purpurem*, while opposite in *T.dasyurum.* This is consistent with a previously conducted study on *T.purpurem* and *T.dasyurum*, using ITS and other marker (chloroplast *trnL*). The recorded results revealed the same out finding that *T.purpurem* and *T.dasyurum* are identical ones (Ellison et al., 2006). Subsequently, from the previous taxonomical and bioinformatics evidences, *T.purpurem* and *T.dasyurum* could be classified as the same species.

Another three closely related species of the section Trifolium *T.angustifolium*, *T.dichroanthum* and *T.prophetarum* (sub-clade Ib) can be considered as sister group to the other closely related species *T.purpurem* and *T.dasyurum* (sub-clade Ia) since they all were grouped in the same clade I. This goes along with similarities and some differences among them in their morphological characters. For example *T.purpurem* and *T.angustifolium* differ in the exertion of corolla as it is highly exerted in *T.purpurem*, while hardly exerted in *T.angustifolium*. Another morphological difference is observed in *T.dichroanthum* from *T.purpurem*, in that the former has two colored petals, but uniform color in *T.purpurem*. Moreover, *T.prophetarum* (sub-clade Ib) differs from *T.dasyurum* (sub-

clade Ia) in that the petals in the former are longer than calyx but as long as calyx in the latter.

In addition, the constructed phylogenetic tree (Figure 3.2) revealed the close relationship between *T.stellatum* (sub-clade IIa) of the section Trifolium to *T.glanduliferum* which is in the section Paramesus (sub-clade IIb) as they were positioned in the same clade II. Their separation in two sub-clades may probably refer to the presence of glands on the stipules in *T.glanduliferum* while their absence in *T.stellatum*. This ITS sequence analysis result confirms the morphological discrimination between these two species by Zohary and Heller (1984).

4.2 Optimization of Plant DNA Extraction

Having high quality of DNA is mandatory for all DNA experimental procedures. All plant DNA extraction protocols include the same basic steps of disruption of the cell wall, cell membrane and nuclear membrane to release the DNA, followed by its precipitation (Moreira and Oliveira, 2011). Biomolecules such as proteins, polysaccharides, phenols and other secondary metabolites have to be removed from the samples to guarantee that the extracted DNA is pure.

Basic protocols for DNA extraction include using Sodium Dodecyl Sulfate (SDS), Cetyltrimethyl Ammonium Bromide (CTAB) and commercially plant DNA extraction Kits.

Firstly standard Qiagen DNeasy plant mini kit (Qiagen, Crawley, UK) protocol was used. The DNA quantity and quality were evaluated using spectrophotometer NanoDrop (Genova Nano Spectrophotometer). The DNA quantity was investigated by recording the absorbance at 260 nm. The DNA concentration in most samples was in the range of 18.9 - 52.8 ng/µl. However, the quality of DNA was detected by the ratio of the absorbance at 260 and 280 nm (A260/A280 ratio) (Sambrook et al., 1989).The obtained DNA quality of the examined samples was in the range of 0.027 - 0.109. Therefore, due to that obtained low DNA quality and quantity, slight modification on Qiagen DNeasy plant mini kit protocol was carried out. The lysis incubation time was prolonged from 10 min to 1 h, to increase the DNA quantity. However, the conducted previous modification did not achieve any pronounced enhancement in that.

Therefore, Lefort and Douglas (1999) manual CTAB DNA extraction protocol was followed with minor modifications in increasing lysis time and reducing the final TE buffer volume in which the DNA was dissolved. For example, the extracted DNA of *T.stellatum* was of good quantity (56.1 ng/ µl) as well as good quality (260/280 ratio = 1.79). However, still no PCR products were obtained.

As a result, it was concluded that the presence of polysaccharides or polyphenols, could be a major inhibitor of having PCR products. So to overcome this conflict, increasing the concentration of both CTAB and NaCl salt was necessary to precipitate pure nucleic acids (Barnwell et al., 1998). In addition, to have efficient plant cell lysis, they were homogenized

with CTAB lysis buffer using Hand Held Homogenizer (MRC Ltd). Adding to that, QIAshredder spin columns were used to ensure extracted DNA more purified from polysaccharides or polyphenols.

Those DNA extracts resulted in positive PCR products, which were subjected to further analysis in this study.

Conclusions and Recommendations

Reliable evolutionary relationships construction among different organisms could depend on the DNA sequence analysis rather than the traditional taxonomic tools. Moreover, the molecular markers could be beneficial for identifying the specimens that don't have one or more essential parts like flowers, fruits…. etc, as in the case of plants.

The out findings in this study have proved that ITS sequencing is an effective method for identifying different *Trifolium* species and the construction of phylogenetic tree illustrating the relationship among them. Therefore, some of the conflicts in the traditional taxonomy of *Trifolium* were resolved in this study.

Based on this study, it is recommended to identify both *T.purpurem* and *T.dasyurum* as same species. Accordingly depending on the nomenclature rules *T.dasyurum* (1832) can be considered as synonym to *T.purpurem* (1807), which can be referred as the valid name of that species. Similarly this can be applied to both *T.campestre* (1804) and *T.grandiflorum* (1767).

The former could be regarded as synonym to *T.grandiflorum* which could be taken as the valid name.

In addition, the current conducted traditional morphological and molecular taxonomical study is the first of its type that was able to highlight the phylogenetic relationship among some closely related species of the genus *Trifolium* in Palestine/West Bank. This paves the way for future in-depth researches on more different species of the same genus. In addition, using other molecular markers could reveal more clarification of the taxonomy of *Trifolium* different species.

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Appendixe

Appendix A

ITS sequences of the studied *Trifolium* species

MF589956*Trifolium purpureum*
TCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTTACATGCAGACA
AACATGTGAATCAGTTTCAACACATAGGGCTGGTTCGAGGTGTTCCCCACCT
CGGCTTGCCACTGGTTCGGAGGTGGACGATGCCTTGCGCGTTCCCCTTTGTG
CCAAAACACAAACCCCGGCGCTGAATGCGTCAAGGAATTTAAAATTTGCTC
TAAGCGCACCTGCATGGCACCGGAGACGGTTTTCGTGCGGGTTGTGTTCTGA
CACATAATATAGAATGACTCTCGGCAACGGATATCTAGGCTCTTGCATCGAT
GAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
CCATCGAGTCTTTGAACGCAAGTTGCGCCCGATGCCATTAGGTTGAGGGCA
CGTCTGCCTGGGCGTCACATGTCGAAGCTTCTTGCCAATTTCCTAATGATAG
GTATTGTGCAGGGTGAATGTTGGCCTCCCGTGAGCTCCATCGTCTCATGGTT
GGTTGAAAATTGAGACCTTGGTAGTTTGTGCCATGATAGATGGTGGTTGTGT
TACGCACGAGCCAAAATAAATCATGTGCTGCTCTATCGAATTTTAGCCTCTT
TTACCCACATGTGTTTCTAAACGCTCGTGATGAGACCTCAGGTCAGGCGGGG
CTACCCGCTGAATTTAAGCATATCAATAAGCGGAGGA
MF589957*Trifolium purpureum*
TCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTTACATGCAGACA
AACATGTGAATCAGTTTCAACACATAGGGCTGGTTCGAGGTGTTCCCCACCT
CGGCTTGCCACTGGTTCGGAGGTGGACGATGCCTTGCGCGTTCCCCTTTGTG
CCAAAACACAAACCCCGGCGCTGAATGCGTCAAGGAATTTAAAATTTGCTC
TAAGCGCACCTGCATGGCACCGGAGACGGTTTTCGTGCGGGTTGTGTTCTGA
CACATAATATAGAATGACTCTCGGCAACGGATATCTAGGCTCTTGCATCGAT
GAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
CCATCGAGTCTTTGAACGCAAGTTGCGCCCGATGCCATTAGGTTGAGGGCA
CGTCTGCCTGGGCGTCACATGTCGAAGCTTCTTGCCAATTTCCTAATGATAG
GTATTGTGCAGGGTGAATGTTGGCCTCCCGTGAGCTCCATCGTCTCATGGTT
GGTTGAAAATTGAGACCTTGGTAGTTTGTGCCATGATAGATGGTGGTTGTGT
TACGCACGAGCCAAAATAAATCATGTGCTGCTCTATCGAATTTTAGCCTCTT
TTACCCACATGTGTTTCTAAACGCTCGTGATGAGACCTCAGGTCAGGCGGGG
CTACCCGCTGAATTTAAGCATATCAATAAGCGGAGGA
MF589958*Trifolium purpureum*
TCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTTACATGCAGACA
AACATGTGAATCAGTTTCAACACATAGGGCTGGTTCGAGGTGTTCCCCACCT
CGGCTTGCCACTGGTTCGGAGGTGGACGATGCCTTGCGCGTTCCCCTTTGTG
CCAAAACACAAACCCCGGCGCTGAATGCGTCAAGGAATTTAAAATTTGCTC
TAAGCGCACCTGCATGGCACCGGAGACGGTTTTCGTGCGGGTTGTGTTCTGA
CACATAATATAGAATGACTCTCGGCAACGGATATCTAGGCTCTTGCATCGAT
GAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
CCATCGAGTCTTTGAACGCAAGTTGCGCCCGATGCCATTAGGTTGAGGGCA
CGTCTGCCTGGGCGTCACATGTCGAAGCTTCTTGCCAATTTCCTAATGATAG
GTATTGTGCAGGGTGAATGTTGGCCTCCCGTGAGCTCCATCGTCTCATGGTT
GGTTGAAAATTGAGACCTTGGTAGTTTGTGCCATGATAGATGGTGGTTGTGT
TACSCACGAGCCAAAATAAATCATGTGCTGCTCTATCGAATTTTAGCCTCTT

TTACCCACATGTGTTTCTAAACGCTCGTGATGAGACCTCAGGTCAGGCGGGG
CTACCCGCTGAATTTAAGCATATCAATAAGCGGAGGA
MF589959*Trifolium purpureum*
TCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTTACATGCAGACA
AACATGTGAATCAGTTTCAACACATAGGGCTGGTTCGAGGTGTTCCCCACCT
CGGCTTGCCACTGGTTCGGAGGTGGACGATGCCTTGCGCGTTCCCCTTTGTG
CCAAAACACAAACCCCGGCGCTGAATGCGTCAAGGAATTTAAAATTTGCTC
TAAGCGCACCTGCATGGCACCGGAGACGGTTTTCGTGCGGGTTGTGTTCTGA
CACATAATATAGAATGACTCTCGGCAACGGATATCTAGGCTCTTGCATCGAT
GAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
CCATCGAGTCTTTGAACGCAAGTTGCGCCCGATGCCATTAGGTTGAGGGCA
CGTCTGCCTGGGCGTCACATGTCGAAGCTTCTTGCCAATTTCCTAATGATAG
GTATTGTGCAGGGTGAATGTTGGCCTCCCGTGAGCTCCATCGTCTCATGGTT
GGTTGAAAATTGAGACCTTGGTAGTTTGTGCCATGATAGATGGTGGTTGTGT
TACGCACGWGCCAAAATAAATCATGTGCTGCTCTATCGAATTTTAGCCTCTT
TTACCCACATGTGTTTCTAAACGCTCGTGATGAGACCTCAGGTCAGGCGGGG
CTACCCGCTGAATTTAAGCATATCAATAAGCGGAGGA
MF589960*Trifolium purpureum*
TCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTTACATGCAGACA
AACATGTGAATCAGTTTCAACACATAGGGCTGGTTCGAGGTGTTCCCCACCT
CGGCTTGCCACTGGTTCGGAGGTGGACGATGCCTTGCGCGTTCCCCTTTGTG
CCAAAACACAAACCCCGGCGCTGAATGCGTCAAGGAATTTAAAATTTGCTC
TAAGCGCACCTGCATGGCACCGGAGACGGTTTTCGTGCGGGTTGTGTTCTGA
CACATAATATAGAATGACTCTCGGCAACGGATATCTAGGCTCTTGCATCGAT
GAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
CCATCGAGTCTTTGAACGCAAGTTGCGCCCGATGCCATTAGGTTGAGGGCA
CGTCTGCCTGGGCGTCACATGTCGAAGCTTCTTGCCAATTTCCTAATGATAG
GTATTGTGCAGGGTGAATGTTGGCCTCCCGTGAGCTCCATCGTCTCATGGTT
GGTTGAAAATTGAGACCTTGGTAGTTTGTGCCATGATAGATGGTGGTTGTGT
TACGCACGWGCCAAAATAAATCATGTGCTGCTCTATCGAATTTTAGCCTCTT
TTACCCACATGTGTTTCTAAACGCTCGTGATGAGACCTCAGGTCAGGCGGGG
CTACCCGCTGAATTTAAGCATATCAATAAGCGGAGGA
MF589961*Trifolium campestre*
TCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTTACATATCTAACA
CGTGAATAAGTTTGGACACATAAGGTTGGCTTGAGATGTTCAACACCTCGG
CTGACCTCTGGTTTGGAGGTGGATGTTGAAATGCGTTCTCCTTTATTGCCAA
AACTCAAACCCCGGCGCTGAATGCGTCAAGGAATTTAAAAGTTGGTTTGAG
CGCACCTGCATGCATCCGGAGACGGTTTTGCTTGTGGGTTGTGTTTTTACAC
ATGTTATAGAATGACTCTCGGCAACGGATATCTAGGCTCTTGCATCGATGAA
GAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCA
TCGAGTCTTTGAACGCAAGTTGCGCCCAATGCCATTAGGTTGAGGGCACGTC
TGCCTGGGTGTCACATATCGAAGCCTTTTGCCAATTTCCTATTCTTAGGTGTT
GTGCAGGGTGAATGTTGGCCTCCCGTGAGCTCTCTTGTCCCATGGTTGGTTG
AAAATTGATGCCTCGGTAGCGTGAGCCATGATAGATGGTGGTTGTGCGACC
CACAAAACCAAATCATGTGCCAGCTCTATCGAATTTTACCCACATGCGTTAA
TAAACGCTCGTGATGAGACCTCAGGTCAGGCGGGGCTACCCGCTGAATTTA
AGCATATCAATAAGCGGAGGA
MF589962*Trifolium campestre*
TCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTTACATATCTAACA
CRTGAATAAGTTTGGACACATAAGGTTGGCTTGAGATGTTCAACACCTCGGC

TGACCTCTGGTTTGGAGGTGGATGTTGAAATGCGTTCTCCTTTATTGCCAAA
ACTCAAACCCCGGCGCTGAATGCGTCAAGGAATTTAAAAGTTGGTTTGAGC
GCACCTGCATGCATCCGGAGACGGTTTTGCTTGTGGGTTGTGTTTTTACACA
TGTTATAGAATGACTCTCGGCAACGGATATCTAGGCTCTTGCATCGATGAAG
AACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCAT
CGAGTCTTTGAACGCAAGTTGCGCCCAATGCCATTAGGTTGAGGGCACGTCT
GCCTGGGTGTCACATATCGAAGCCTTTTGCCAATTTCCTATTCTTAGGTGTTG
TGCAGGGTGAATGTTGGCCTCCCGTGAGCTCTCTTGTCCCATGGTTGGTTGA
AAATTGATGCCTCGGTAGCGTGAGCCATGATAGATGGTGGTTGTGCGACCC
ACAAAACCAAATCATGTGCCAGCTCTATCGAATTTTACCCACATGCGTTAAT
AAACGCTCGTGATGAGACCTCAGGTCAGGCGGGGCTACCCGCTGAATTTAA
GCATATCAATAAGCGGAGGA
MF589963*Trifolium stellatum*
TCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTTACATGCAGACC
AACACGTGAATTAGTTTGAACACATAGGGTTGGTTTGAGGTGTTCAACACCT
TGGCTTGCCTTTGGTTTGGAGGATGACCACTTGTGCGTCCTCCTTTGTGCCA
AAACAAAACCCCGGCGCTAAATGCGTCAAGGAATTTAAATTTTGATCTGAG
CGCGCACTTGCATGCCACCGGAGTCGGTGTTCGTGCGGTTTGTGTTCTGACA
CATAATATAGAATGACTCTCGACAACGGATATCTAGGCTCTTGCATCGATGA
AGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACC
ATCGAGTCTTTGAACGCAAGTTGCGCCCGATGCCATTCGGTTGAGGGCACGT
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MF589964*Trifolium stellatum*
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MF589965*Trifolium stellatum*
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جامعة النجاح الوطنية

كلية الدراسات العليا

دراسة العلاقة الوراثية التطورية بين الأنواع المتقاربة التابعة لجنس البرسيم (*Trifolium* – عائلة البقوليات) في فلسطين/ الضفة الغربية.

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قدمت هذه الأطروحة استكمالا لمتطلبات الحصول على درجة الماجستير في العلوم الحياتية بكلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس– فلسطين.

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الملخص

تعد عائلة البقوليات أو القرنيات ثالث أكبر عائلة في النباتات المزهرة (كاسيات البذور) التي تشمل ثلاث عوائل : (البقموات , السنطوات , فولاوات) . البقوليات هي نباتات مزهرة داخل عائلة القرنيات التي تضم عدد كبير من الأصناف المختلفة. جنس البرسيم (*Trifolium* L.) هو واحد من أكبر الأجناس التابعة لعائلة البقوليات ذات أهمية زراعيه كبيرة . منطقة البحر الأبيض المتوسط تضم أعلى تنوع حيوي وذلك بسب المناخ، لذلك إنتشار البرسيم الأصلي يعود إلى تلك المنطقة .

لحل العقبات في التصنيف المعتمد على الصفات الشكلية لبعض الأنواع المتقاربة التابعة لجنس البرسيم (*Trifolium*) كان من الضروري إستخدام التقنيات الجزيئية . مناطق (Internal Transcribed Spacer /ITS) هي من أكثر العلامات استخداما في البحوث النباتية لقدرتها على أن تكون مصدر مفيد للمعلومات في دراسة النشوء و التطور خاصة في عائلة البقوليات .

تم تجميع عينات نباتية من بعض الأنواع التابعة لجنس البرسيم خلال فترة الإزدهار (مارس– مايو 2017) من مواقع مختلفة في فلسطين / الضفة الغربية و من ثم تم تصنيف هذه العينات اعتمادا على الصفات الشكلية و رجوعا إلى (Flora Palaestina) ، وتم حفظ عينة لكل الأنواع المدروسة في معشبة جامعة النجاح الوطنية/ نابلس .

تم استخلاص الحمض النووي (DNA) من الأوراق باستخدام (CTAB) . ثم تم تحديد الخصائص الجينية باستخدام البادئات (ITS1, 5.8S, ITS2) لتكثير و معرفة تسلسل منطقة (ITS) . قدر الأختلاف بين تسلسل الجينات و أنشأت شجرة النشوء و التطور باستخدام نماذج Kimura's 2- parameter و Neighbor-joining method على التوالي . أودعت كل من الإثناَ عَشَرَ عينة التابعة لجنس البرسيم (*Trifolium*) في قاعدة بيانات في بنك الجينات تحت أرقام الإنضمام .

أثبت تحليل تسلسل ITS الجزيئية بأن كل من *T.purpureum* و *T.daysurum* هي أنواع متطابقة . لأن لديهم تسلسل ITS متطابقة . و كما تجمعت في فرع واحد I (مجوعة Ia) في شجرة النشوء و التطور. هذا يتزامن مع وجود تشابه كبير في الصفات الشكلية لديهم. أيضا *T.campestre* و *T.grandiflorum* (DQ312062.1) يمكن إعتبارهما تصنيفيا كنوع واحد و ذلك لأن لديهم تسلسل ITS متطابقة، كما و تجمعت في فرع واحد II (مجموعة IIb) في شجرة النشوء و التطور .

بناء على هذة الدراسة، فأنه يوصى بالتعريف عن *T.purpureum* و *T.daysurum* كنوع واحد وتبعا لقواعد نظام التسميات العالمي، فأن *T.daysurum* (1832) هو مرادف *T.purpureum*(1807) . أيضا كما و يتم تطبيق قاعدة التسميات على(1804) *T.campestre* و(1767) *T.grandiflorum* على أن يكون *T.campestre* مرادف *T.grandiflorum* .

تعتبر هذه الدراسة الجامعة بين التصنيف الشكلي التقليدي و التصنيف الجزيئي الأولى من نوعها لتسليطها الضوء على العلاقة التطورية بين بعض الأنواع المتقاربة التابعة لجنس البرسيم في فلسطين/ الضفة الغربية. وهذا يمهد الطريق لبحوت مستقبلية أخرى متعمقة في مختلف الأنواع التابعة لنفس الجنس . بالأضافة إلى ذلك، استخدام علامات جزيئية أخرى يمكن أن يكشف عن المزيد من التوضيح لتصنيف الأنواع الأخرى من جنس البرسيم (*Trifolium*) .