MOLECULAR PHYLOGENETIC RELATIONSHIPS OF SIX *ASTRAGALUS* L. SECTIONS (*Halicacabus, Megalocystis, Macrophyllium, Hymenostegis, Hymenocoleous, Poterion*) NATIVE TO TURKEY BASED ON cpDNA AND nDNA REGIONS

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submitted by **MEVLÜDE ALEV ATEŞ** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Department of Biological Sciences, Middle East Technical University** by,

Prof.Dr.Gülbin Dural Ünver _____
Dean, Graduate School of Natural and Applied Sciences

Prof. Dr. Orhan Adalı _____
Head of the Department, Biological Sciences

Prof. Dr. Zeki Kaya _____
Supervisor, Dept. of Biological Sciences METU

Examining Committee Members

Prof. Dr. Musa Doğan _____
Dept. Biological Sciences, METU

Prof. Dr. Zeki Kaya _____
Dept. Biological Sciences, METU

Prof.Dr. Hayri Duman _____
Biology Dept., Gazi University

Prof. Dr. Sertaç Önde _____
Dept. Biological Sciences, METU

Assoc.Prof.Dr. Seher Karaman Erkul _____
Biology Dept., Aksaray University

Date: 30.11.2017

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name : Mevlüde Alev ATEŞ

Signature :

ABSTRACT

MOLECULAR PHYLOGENETIC RELATIONSHIPS OF SIX *ASTRAGALUS* L. SECTIONS (*Halicacabus, Megalocystis, Macrophyllium, Hymenostegis, Hymenocoleous, Poterion*) NATIVE TO TURKEY BASED ON cpDNA AND nDNA REGIONS

ATEŞ, Mevlüde Alev

Ph.D., Department of Biological Sciences

Supervisor: Prof.Dr.Zeki KAYA

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The thirty species involved in six *Astragalus* sections were studied not only to understand phylogenetic relationships and also to estimate evolutionary divergence times by using the DNA sequences of cpDNA (chloroplast) and nDNA (nuclear) regions.

The results showed that the DNA sequences of *ITS* nr (nuclear ribosomal) region had with the highest genetic diversity. Based on all studied regions, *Poterion* section was the most diverge section than others while *Megalocystis* and *Halicacabus* sections were the closest ones. Moreover, *A.vaginans* from *Hymenocoleous* section was located also close to *Hymenostegis* section but always in different branch. It is notable that, *A.dipodurus* and *A.oleaefolius* species from *Macrophyllium* section placed in a different sub-branch in all phylogenetic trees constructed by both studied cpDNA and nDNA regions.

To understand the phylogenetic relationships among the Turkish *Astragalus* species and in other regions of the world, DNA sequences of studied regions and foreign samples obtained from NCBI database were analyzed together. Turkish species formed a separated cluster, but close to Old world species in the phylogenetic trees of both *trnL, matK* and *ITS* regions. Moreover, some Old world species like Chinese, Australian and Korean were in close association with New world species. From all these results, it can be said that although Turkish species were derived from an independent diversity cluster.

Evolutionary divergence time for *Astragalus* genus was estimated. All analysis depicts that studied sections of *Astragalus* genus that are native to Turkey were diverged from other New and Old world ones at Pleistocene period. According to two different cpDNA regions (*trn*L and *mat*K), results indicated that *Poterion* section appeared to be newly diverged from other sections of Turkish *Astragalus* sections.

Key Words: *Astragalus*, Phylogeny, cpDNA,nDNA

ÖZ

TÜRKİYE'DE DOĞAL YAYILIŞ GÖSTEREN *ASTRAGALUS* L. CİNSİNE AİT ALTI SEKSİYONUN (*Halicacabus, Megalocystis, Macrophyllium, Hymenostegis, Hymenocoleous, Poterion*) ARASINDAKİ KLOROPLAST VE NÜKLEER DNA BÖLGELERİNE DAYALI MOLEKÜLER FİLOGENETİK ANALİZLERİ

ATEŞ, Mevlüde Alev
Doktora, Biyolojik Bilimler Bölümü
Tez Danışmanı: Prof.Dr.Zeki KAYA
Aralık 2017,142 Sayfa

Çalışmada, kloroplast DNA'sına ve nükleer genom bölgelerine ait DNA dizileri kullanılarak altı adet *Astragalus* seksiyonuna ait toplam otuz türün filogenetik ilişkileri ve türleşme zamanları belirlenmeye çalışılmıştır.

Çalışılan kloroplast ve çekirdek DNA bölgeleri içinde, çekirdek genomdaki ITS gen bölgesinin en yüksek genetik farklılığa sahip olduğu bulunmuşturTüm bölgelerden elde edilen sonuçlara göre, *Poterion* seksiyonunun diğer çalışılan *Astragalus* seksiyonlarından oldukça farklı olduğu görülmüştür. Çalışılan *Astragalus* seksiyonları içerisinde *Halicacabus* ve *Megalocystis* seksiyonları genetik açıdan birbirlerine en yakın seksiyonlar olduğu belirlenmiştir. Bunlara ek olarak *Hymenocoleous* seksiyonun ait olan *A.vaginans* türünün her zaman *Hymenostegis* seksiyonuna ait türlere yakın fakat onlardan ayrı bir dalda konumlandığı görülmüştür. Ayrıca *A.trifoliastrum* türü de ait olduğu *Hymenostegis* seksiyonunun diğer türlerinden farklı alt dalda yer aldığı belirlenmiştir. Bunun yanısıra *Macrophyllium* seksiyonuna ait

A.dipodurus ve *A.oleaefolius* türleri de seksiyonun diğer türlerinden tüm çalışılan cpDNA ve nDNA bölgelerine göre elde edilen filogenetik ağaçlarda, farklı dallarda yer almaktadırlar.

Dünyanın diğer bölgelerinde yayılış gösteren bazı *Astragalus* türlerinin Türkiye'deki çalışılan türlerle olan filogenetik ilişkilerini anlamak amacıyla, bu çalışmada kullanılan bölgelerin DNA dizileri NCBI veri tabanından alınmış ve çalışmada kullanılan Türkiye örnekleri ile birlikte analiz edilmiştir. Tüm çalışılan bölgelere göre, Türkiye örnekleri eski dünya türlerine yakın pozisyon alsalar da, yeni dünya türlerine genetik açıdan daha yakın olduğu görülmektedir. Çin, Kore, Avusturalya'dan alınan bazı eski dünya türleri ise yeni dünya türlerine daha yakın pozisyon almışlardır. Bu veriler ışığında Türkiye türlerinin genetik açıdan farklı oldukları söylenebilir.

Evrimsel farklılaşma zamanlarının analizleri incelendiğinde Türkiye *Astragalus* türleri, yeni ve eski dünya türlerinden Pleistosen döneminde ayrılmış ve çalışılan türler içerisinde ise özellikle *Poterion* seksiyonu Türkiye *Astragalus* seksiyonlarına göre daha yakın dönemde farklılaşmış olduğu düşünülmektedir.

Anahtar Kelimeler: *Astragalus*, Filogeini, cpDNA , nDNA

To My Family…

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This thesis is dedicated to my future.

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment length Polymorphism
cpDNA	Chloroplast DNA
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraaceticacid disodium salt
ETOH	Ethanol
ETS	Expressed Sequence Tags
IGS	Intergenic Spacer
ITS	Internal Transcribed Spacer Region
matK	The maturase Kinase
MEGA	Molecular Evolutionary Genetic Analysis
NCBI	National Center for Biotechnology Information
NJ	Neighbour-joining
NORs	Nucleolar organizing regions
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrolidone
RAPD	Random Amplification of Polymorphic DNA
rbcL	Large subunit of Rubisco
rRNA	Ribosomal ribonucleic acid

TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris EDTA
t-RNA	Transfer Ribonucleic Acid
rDNA	Ribosomal DNA
nDNA	Nuclear DNA
nrDNA	Nuclear Ribosomal DNA

CHAPTER 1

INTRODUCTION

Legume (Fabaceae) family, with almost 730 genera and over 19.400 species worldwide, is the 3rd largest family of flowering plants (Mabberley, 1997). The family involves different horticultural varieties and species including harvested crops, fiber, fuel, timber, oil, and medicinal plants. All members of this family prefer temperate and tropical regions of the world by symbolizing as trees to annual herbs (Rundel, 1989). Actually, by forming root nodules, all legumes have significant role in the terrestrial nitrogen cycle (Sprent, 2001). Fabaceae has a plentiful and constant fossil record proved that speciation was derived from the Early-Tertiary (Crepet and Taylor, 1985, Herendeen *et al.*, 1992).

Fabaceae family is quite wide spread in Turkey. There are three main phytogeographical regions including Euro-Siberian, Mediterranean and Irano-Turanian phytogeographic regions (Zohary, 1973). By means of its high plant diversity, floristic structure of Turkey has fascinated many botanists. Until now, studies indicated that there are approximately 9753 species of vascular plants of which 3035 of them are endemic (Kaya and Raynal, 2001; Güner *et al*.,2012). In addition to its richness, Turkey has two significant genetic diversity centers; Middle Eastern and Mediterranean (Vavilov, 1951).

Fabaceae is mostly found in tropical rainforests and dry forests of North & South America and Africa. There are many genera in this family, but the largest ones are

Astragalus L., *Acacia* Mill, *Indigofera* L., *Crotalaria* L. and *Mimosa* L. which make up of approximately quarter of legume family. *Astragalus* L., with 3000 species of herbaceous perennial and annual species is the largest genus of legume family (Podlech & Zarre, 2013). Moreover, after Iran and Russia, Turkey has the highest number of native *Astragalus* species (478 species), 202 of them are endemic (Aytac and Ekici, 2012). Due not only high species diversity but also common economical uses of this genus, many scientific studies focus on different species of *Astragalus* genus. Furthermore, by the reason of taxonomic challenges of *Astragalus*, botanists need to revise the genus. As a result of it, molecular techniques become widely used tool for taxonomic revision. In order to interpret some complex issues within plant systematic studies, molecular data was commonly use to bring new aspect.

1.1.Morphology of *Astragalus* genus

With more than 3000 species of herbs, small shrubs and more than 250 sections, *Astragalus* is one of the largest genera in flowering plants. The most of them are annual species but also there are perennial species. Height of the plants is up to 200 cm with stems or stemless form and with thorns or thornless. The legume family is characterized with its flower forms such that flowers are banner, wing, keel structures (Figure 1.1) and alternate, pinnately compound leaves (Figure 1.2). Typically they are entomophilous plants and the flowers are usually interesting for pollinators such as bees, insects, birds. The stamens are diadelphous, so they are also self pollinated plants. They have bladder-like pods with different sizes and shapes of fruits (Figure 1.3). The seeds are kidney-shaped (Figure 1.4). Many species have very hard seed coat, therefore they can maintain in viability under the soil for years.

Figure 1.1: ***Astragalus ermineus*** **flower with specific structures (Photo : S.Erkul, 2014)**

Figure 1.2 ***Astragalus zederbaueri*** **with pinnately compound leaves. (Photo: S.Erkul, 2014)**

Figure 1.3: *Astragalus anthylloides* fruits with its bladder-like pod structure (Photo: S.Erkul, 2014)

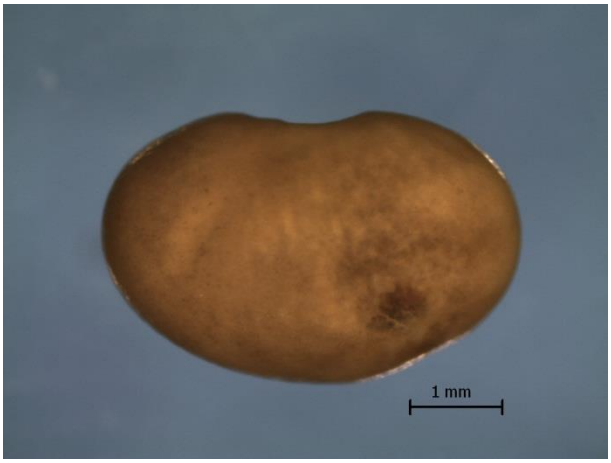

Figure1.4 *Astragalus oleaeifolius* seed under light microscope (Photo: S.Erkul, 2014)

1.2. Taxonomy of *Astragalus* Genus

Taxonomy of *Astragalus* genus is still in question. Especially, taxonomic relationship between *Phaca* and *Astragalus* genera is one of the most important problem in classical taxonomy. Pod structure of the species is the basic point of their separation. *Phaca* genus has inflated unilocular pod structure, but this feature is not common in species of *Astragalus* genus. Therefore, Linnaeus (1753) claimed that species with unilocular pod structure were clarified as member of *Phaca* genus. Also Tournefort claimed that *Phaca* species as the member of Astragaloides before Linnaeus. Moreover, in 1700, Tournefort called genus *Tragacantha* species by using different morphological characters of approximately 300 species that dispensed throughout southwest and southcentral Asia. Even-pinnate leaves and unilocular pods with 1-2 seeded fruits were used for this separation (Podlech, 1983; Engel, 1991). On the other hand, because of insufficient differences, Zarre and Podlech (1997) put all these *Tragacantha* into *Astragalus* genus. In 1868 and 1869 Bunge called that *Phaca* was a subgenus of *Astragalus* and furthermore, in 1864, Gray renounced the differences between two genera upon the septum characters of pod structures. After all, in 20th century, Barneby (1964) indicated taxonomical history of *Phaca* genus and he completely separated two genera from each other (Figure 1.5).

According to all these taxonomical history of *Astragalus* genus, it can be easily understood that *Astragalus* and other close genera are very similar to each other. Therefore, these morphological similarities cause too many problems on classical systematics of this genus. Since, problems of taxonomy of the genus are not resolved, there are many studies addressing to these issues (Chaudhary *et al*. 2008). However, a complete monograph is still not available. Both species richness, due to diversity of the genus and structures including stipules, leaf rachis, inflorescence, length and size of sepal and petals may be the main reasons of this complexity (Chaudhary *et al*. 2008).

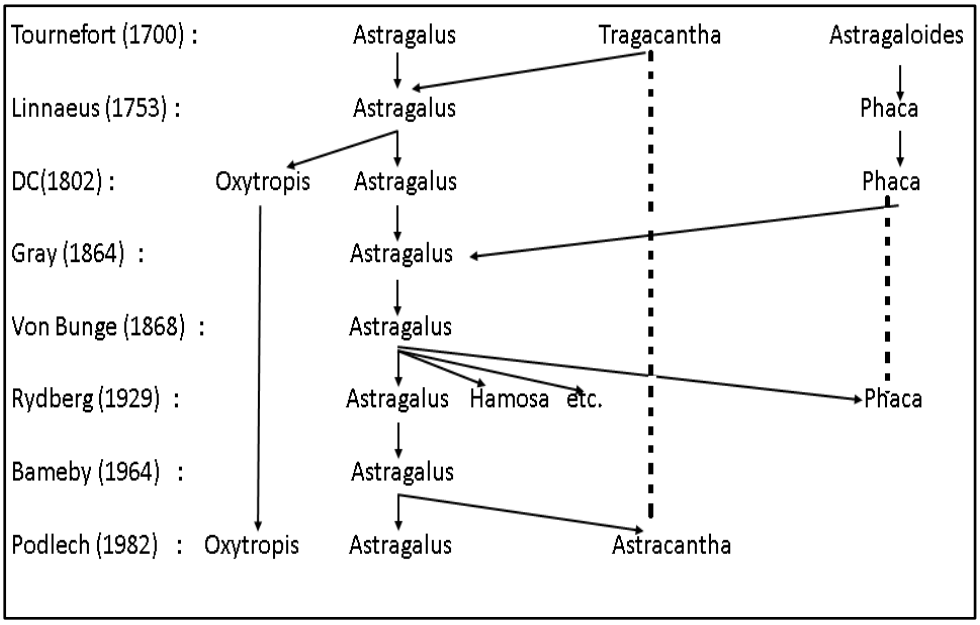

Figure 1.5: Taxonomical history of *Astragalus* and closed genera. Solid lines show unchanged taxonomy of genus, and dashed ones show displacement (Wojciechowski *et al.*, 1999)

As a consequence of all these studies on *Astragalus* genus, the current taxonomical position of the genus has been provided in Table 1.1.

Table 1.1 Scientific classification of *Astragalus* genus

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Fabales
Family	Fabaceae(Leguminosae)
Subfamily	Faboideae
Tribe	Galegeae
Genus	*Astragalus*

1.3.Origin and distribution of *Astragalus* Genus

Endemism ratio among *Astragalus* species is very high. There are narrow endemics and usually found in marginal habitats or required edaphic specializations (Wojciechowski *et al.*, 1999). The genus *Astragalus* was originated from mountains, steppes of southwestern Asia and Himalayan Plateau and Eurasia same as tribe *Galegeae* (Polhill, 1981a, 1981b). *Astragalus* species prefer arid and semi-arid mountainous regions of northern Hemisphere, Andes of South America and East Africa. Moreover, this genus is also naturally found in the Mediterranean climatic regions of Pasific coasts of North and South America and southern Europe and northern Africa (Wojciechowski *et al.*, 1999). In Irano-Turanion region of southwestern Asia and Sino-Himalayan Plateau of southcentral Asia are the main biodiversity centers for the genus. Furthermore, the genus *Astragalus* has high diversity in great basin and Colorado plateau of western North America (Podlech, 1986)(Figure 1.6).

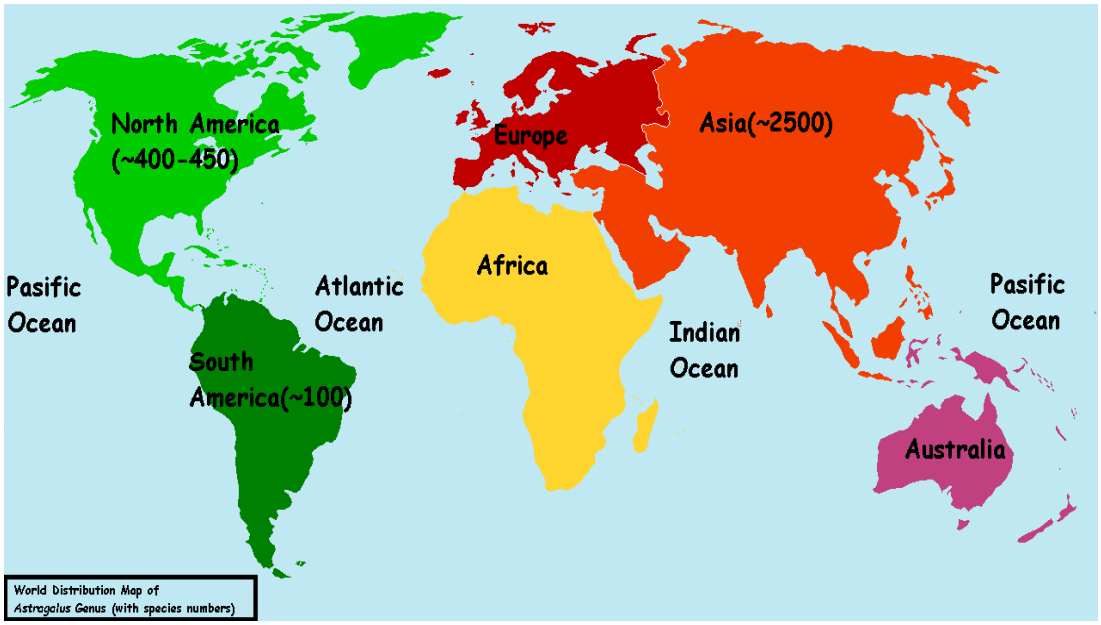

Figure 1.6: The natural distribution of *Astragalus*. The numbers in the parenthesis next to continents are the number of species representing the genus.

1.4. The genus *Astragalus* L. in Turkey

Astragalus L. is the largest genus with 475 species and 64 sections in Turkey (Davis *et.al*, 1988; Duran and Aytac, 2005; Ekici *et al.* 2008; Aytac and Ekici 2012; Karaman Erkul and Aytac , 2013). Steppes and mountainous regions are the general habitats of these species in Irano-Turanian regions of Turkey about %42 of them are endemics to Turkey (202 species) (Karaman Erkul, *et al.*, 2015)(Figure 1.7).

In 1970, Chamberlain and Matthews made a revision of *Astragalus* genus. After that, Davis *et al*., (1988) and Aytac (2000) found 41 new species that were native to Turkey. In addition to these studies, Podlech (1999; 2000) and Podlech and Sytin (2002) identified 31 new species. Until recent years, many scientists studied to improve the taxonomy of *Astragalus* genus (Akan and Civelek, 2001; Aytac *et al.*, 2001; Ekici and Aytac, 2001; Aytac and Ekici, 2002; Hamzaoğlu and Kurt, 2002; Akan and Duman, 2003; Gokturk *et al.*, 2003; Hamzaoğlu, 2003; Ketenoglu and Menemen, 2003; Ghahremani-nejad and Behcet, 2003; Akan and Aytac, 2004; Duran and Aytac, 2005; Podlech and Ekici, 2008; Uzun *et al.*, 2009; Ozudogru *et al.*, 2011; Aytac and Ekici,2012; Karaman Erkul *et al.*, 2013, Dinç *et al.*,2013; Podlech and Zarre , 2013; Karaman Erkul *et al.*, 2015; Bagheri *et al.*,2015; İlçim and Behçet , 2016).

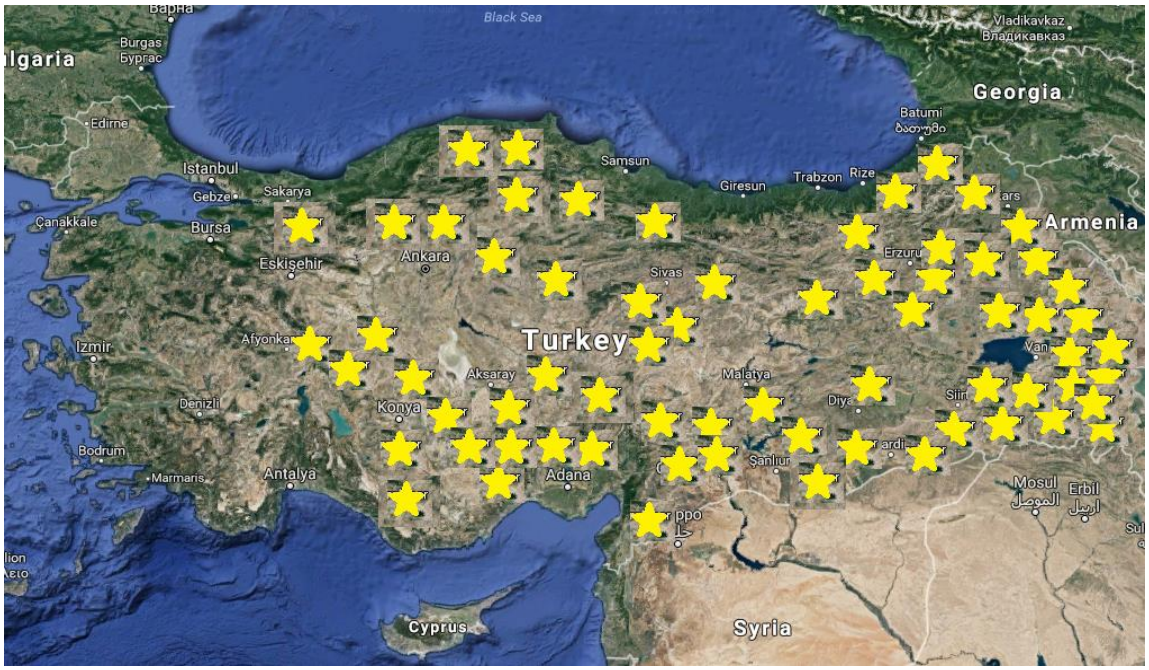

Figure 1.7: Map showing the distribution of studied *Astragalus* sections and species in Turkey

1.5. Chemical composition, significance and uses of *Astragalus* genus

Species of the genus include varieties of secondary metabolites like aminoacids, polysaccharides, triterpene glycosides, flavonoids, isoflavones, and saponins and trace minerals (Yin *et al.*, 2009). These metabolites are used for medicinal purposes, except for toxic-ones. Generally, these chemicals are obtained from roots. Some of *Astragalus* species have been used by traditional Chinese medicine for weakness, diabetes, respiratory problems, high blood pressure and AIDS (Liu *et al.,* 2003). Moreover, some extracts are used to prevent the growth of cancer cells (Tin *et al.*, 2007). On the other hand, many species have toxic admixtures that causes crazy illness in animals by getting high amount during grazing time, such as swainsonine (Davis *et al.*, 1984), 3-nitropropanol (Tunez *et al.*, 2010) and selenium (Somer and Caliskan, 2007). These kinds of toxic species that causes neurological damage in animals are called locoweed in USA. However, non-toxic species of this genus are very useful for feeding animals.

Roots of *Astragalus gummifer* (which is located around southcentral Asia) is used for extracting "gum tragacanth". It is the basic structure of lotions, ice-creams, pharmaceuticals because of its colloidal and hydrophilic features. In Turkey, *Astragalus microcephalus* is also used for producing gum tragacanth (Baytop, 1971).

One of the most significant property of some *Astragalus* species being an indicator of high selenium in soils, like *Astragalus bisulcatus* (Quinn *et al.*, 2008) and *Astragalus pattersoni* (Cannon, 1971). Selenium-rich soils are the habitats for these species.

1.6. Molecular Phylogeny

Molecular phylogeny is the branch of phylogeny which use DNA sequence to get the information about evolutionary relationships of interested organisms. The consequences of molecular phylogenetic data are very useful for solving and understanding taxonomic relationships of the organisms.

DNA barcoding is a taxonomical method to figure out if a specimen belongs to that it is a particular species or not by using small DNA fragments (Hebert *et al.*, 2003). This method is helpful to gain perspective in conservation studies when traditional taxonomic identification is not sufficient. In this method, intra and interspecific genetic differences are estimated by the help of pairwise calculations (Meyer and Paulay, 2005) and monophyletic species are the key point for DNA barcoding studies (Lahaye *et al.*, 2008).

Molecular phylogenetic systematics are favorable because;

(1) DNA sequences are certain; so instinctive decisions are eliminated
(2) Many studies can be done by using different parts of DNA on the same organism; therefore, results could be interpreted via many data
(3) with new data from genes, many morphological features could be understood clearly and thus, taxonomic revision of the species is done easily (Graham and Wilcox, 2000).

In recent years, molecular data have been widely used for revision and classification in many organisms. Especially in plants, many regions of nuclear and chloroplast DNA regions are applicable to construct phylogenetic trees. Many researchers concentrated on phylogenetic analysis of flowering plants to revise families of angiosperms by looking at DNA-based data. According to some studies, DNA sequences of different region evolve differently (Wojciechowski *et al.*, 1993; Baldwin, 1995). For example, evolution rate of nuclear DNA is faster than that is in plastid DNA (Savolainen and Chase, 2003).

Consequences of mutations and stabilizing of this changes in the population are the key points of evolutions in genome sequences. The base substitution rate among homologous sequences indicates the evolutionary distinctiveness of an organism against to others. Therefore, phylogenetic trees are constructed by using molecular data. Particularly for plants, analyses of associated molecular data are very useful.

Recent molecular studies prove the fact that Fabaceae is a single monophyletic family (Doyle *et al.*, 2000). Moreover, *Astragalus* genus is recognized as the most taxonomically complex genus in this family due to high of species diversity. Therefore, systematic relationships among some species of this genus could not be done by using only morphological characters. In this situation, molecular markers are properly useful to understand the problems of classical taxonomy. Especially, in recent years new techniques which are found in genetic studies, are valuable for valid agreement on morphology and genetic of plant systematics (Sareela *et al.*, 2007). For instance, *Astracantha* Podl. genus was separated from *Astragalus* genus according to its unilocular pods and inflorescence amounts by Podlech (1983). However, Zarre and Podlech (1997) integrated *Astracantha* and *Astragalus* genus due to their anatomical features. After that, Wojciechowski (1999) developed the systematics of *Astracantha* via cpDNA(*trn* L intron) and nDNA (ITS region) and he reported that rate of species diversification in *Astragalus* higher than others often cited, continental and insular adaptive radiations in angiosperms. Also, *Oxytropis* genus is the closest relative to *Astragalus* genus. At the beginning, Linnaeus reported that *Oxytropis* genus involved in *Astragalus* genus. After that, De Candolle segregated *Oxytropis* species from

Astragalus species according to their keel structure (Chaudhary *et al.*, 2008). Lastly, Wojciechowski (2005) supported that *Oxytropis* is a monophyletic group that it could be separated from *Astragalus* genus according to the information of *trn*L and ITS regions in chloroplast DNA and genomic DNA respectively.

Diversity within the genus *Astragalus* was also studied by using molecular data (Sanderson and Wojciechowski, 1996; Wojciechowski *et al.*, 1999; Dong *et al.*, 2003; Wojciechowski, 2005). These studies reported that species of this genus have great variations compared to other flowering plants. Whether it is nuclear or chloroplast DNA sequences, molecular data contributed significantly to obtain phylogenetic relations within the genus *Astragalus*. Moreover, based on the results from these studies, nuclear genome regions may be more effective to figure out evolutionary features of this genus due to its great variation compared to the gene region of chloroplast genome.

1.6.1. Chloroplast DNA (cpDNA) Regions

To understand phylogenetic and evolutionary relationships among species or higher/lower taxa, molecular data have been selected due to its informativeness. Especially in plants, cpDNA regions are favorable for this kind of studies because they have some advantages such as;

(1) Chloroplast genome is in multiple copies comparing to total DNA therefore it provides simple amplification during PCR practice.
(2) It is uniparentally inherited and as a result of being haploid, allelic variation is absent (Small *et al.*, 2004).
(3) Particularly in plants, it is highly conserved.

Using cpDNA also creates some problems like being biparental for some species (Corriveau and Coleman, 1988). Moreover, the other disadvantage is occur when the chloroplast is inherited uniparentally. There will be a lack of informayiveness to figure out polyploidy and hybridization events in these kind of situations (Small *et al.*, 2004). Corriveau and Coleman (1988) reported that the genus *Astragalus* has maternal

transmission. In addition to this confliction about inheritance pattern of cpDNA in *Astragalus* genus, Zhang *et al.* (2003), report that biparental cytoplasmic inheritance was found in two *Astragalus* species.

1.6.1.1. *trn*L5'-L3'(*trn*L intron) and *trn*L3'-F$^{(GAA)}$ (*trn* L-F intergenic spacer) Regions

Chloroplast DNA regions are not only the most widely used regions, but also it is the key point of molecular data in phylogenetic studies (Baldwin, 1992; Baldwin *et al.*,1995; Alvarez and Wendel, 2003). Particularly, non-coding regions reveal the highest mutation frequency because of their less functional features than coding ones. Therefore, for the evolutionary-relationship analysis below family level, these regions are widely preferred (Taberlet *et al.*, 1991; Gielly and Taberlet, 1994).

One of the generally used noncoding cpDNA region is t-RNA. It consists of *trn*L gene and two flanking intergenic spacers; *trn*T-L and *trn* L-F. *trn*-L gene includes of two highly conserved exons that are separated by a group I intron. Anticodon loop of tRNA is encrypted by a simple copy region of chloroplast genome. This region is intervened by *trn*L intron. The position of this intron which is cut off the anticodon of the tRNA gene (U-intron-AA), is protected from cyanobacteria to plant chloroplasts (Kuhsel *et al.*, 1990). Taberlet *et al.*, (1991) studied this region and provided as markers to the botanists. Then, scientists used *trn*L intron and *trn*L-F intergenic spacer regions for studying different taxonomic levels. After invention of the region, many scientists like Gielly and Taberlet (1996), Ohsako and Ohnishi (2000) and Mummenhoff *et al.*, (2001) used region in order to clarify interspecific relationship.

Due to the position of both *trn*L genes and *trn* L-F intergenic spacers, the *trn* L-F region became very valuable. In plants, *trn*L intron region includes not only conserved sequence in the regions flanking *trn*L exons, but also its highly variable parts compare exonic regions in the center (Bakker *et al.*, 2000). This central part is highly used because of including conservative flanking regions (Bogler and Ortega, 2004).

Moreover, *trn* L-F intergenic spacer is a highly variable non-coding region due to its configuration between and within plant species (Bohle *et al.*, 1994; Gielly and Taberlet, 1994; Ham *et al.*, 1994; Mes and Hart,1994). *trn*L intron and *trn* L-F spacer have tandem repeats that figure out stem-loop structures. Also the sequences of *trn* L-F spacer involve promotor parts for the *trn*F gene (Hao *et al.*,2009).

Despite *trn* T-F region have high substitution rate (Bayer and Starr 1998; Bakker *et al.*, 2000; Mansion and Struwe, 2004), in some holoparasitic plants, this part was not found. As a consequence of this situation, *trn*T-L and *trn* L-F intergenic spacer regions display divergence and great deletions (Freyer *et al.*, 1995; dePamphilis *et al.*, 1997; Lohan and Wolfe, 1998).

1.6.1.2. *trn*V intron region

The tRNA (*trn*V) region was studied in first time by Deno *et al.*, (1982) in cpDNA of tobacco (*Nicotiana tabacum*) plant. It consists of a group II intron (Keller and Michel, 1985). Then, Clegg *et al.* (1986) found that there were 12 sequence blocks at variable substitution levels in *trn*V intron of four plant species (maize, barley, tobacco and pea). The substitutions and indels in the region are generally studied to figure out phylogenetic relationships between and/or within plant species.

Due to having a high substitution rate, the *trn* L-F and *trn*V regions are widely used in phylogenetic studies (Bayer and Starr, 1998; Bakker *et al.*, 2000; Mansion and Struwe, 2004). By comparing *trn*L-F and *trn* V intron regions from different species, researchers found special domains which are responsible for the configuration of secondary structure of the regions, evolved in different rates in the sequences (Clegg *et al.*, 1986; Learn *et al.*, 1992; Fangan *et al.*, 1994). Not only the noncoding intergenic spacers, but also intronic regions evolve faster because of the effects of substitutions and indels in the sequences. Although these changes are favorable to figure out phylogenetic structures between species, they are not very useful for phylogenetic studies between higher taxa (Olmstead and Palmer, 1994).

1.6.1.3. *mat*K (maturase Kinase) gene region

Many researchers have pointed out that using slowly evolve genes in cpDNA due to the number of multiple hits and level of homoplasy is useful in phylogenetic studies (Farris, 1977; Swofford *et al.* 1996; Olmstead *et al.*, 1998; Graham *et al.*, 2000) However, using of slowly evolved genes has many challenges, like taxon sampling because of the needs for large number of nucleotides per species to get different characters. This situation gives rise many restrictions for researchers. In order to eliminate these challenges, regions that can contribute useful data in significant level of phylogenetic studies without compromising taxon representation are crucial for certain analyses of evolutionary histories. The rapid evolving *mat*K gene supports all these preconditions (Hilu *et al.*, 2003).

The *mat*K gene, which was firstly studied in tobacco (Sugita *et al.*, 1985), is an open reading frame (ORF) encoding a protein, maturase that is used in RNA splicing (Neuhaus and Link, 1987; Wolfe *et al.*, 1992). This gene is located between two introns of *trn*K gene which involve in group II intron that encrypts the *mat*K. Unlike other group II introns, *trn*K gene has encoding function (Hausner *et al.*, 2006). Because of these kinds of useful features of *mat*K gene, it is widely used in plant phylogenies as an indicator.

*mat*K gene has beneficial properties for phylogenetic studies at family and species levels due to high amount of nucleotide variation and low transition/transversion ratio (Tanaka *et al.*,1997; Selvaraj *et al.*,2008). One of the most significant properties of this gene was reported by Hilu *et al.* (2003) that combination of approximately 11 gene region sequences could not be useful like *mat*K gene sequence. Moreover, they revealed that many different size of insertion/deletion (indels) and nonsynonymous mutations could be found in addition to substutions (Johnson and Soltis, 1994, 1995; Olmstead and Palmer, 1994; Hilu and Liang, 1997; Soltis and Soltis, 1998; Hilu *et al.*, 2003).

Typically, the *mat*K is approximately 1500 base pairs in length with its flanking regions, but the sequence near to the 3' end is highly conserved in where indels and substitution numbers are less than 5' end of the gene (Hilu and Liang, 1997).

1.6.2. Genomic DNA region

The basic problems of studying nucleotide characters for lower taxonomic levels is the identification of amplifiable and rapidly evolving DNA regions which can support adequate variations within short sequence alignments (Baldwin *et al.*, 1995). In order to solve these problems, noncoding regions of nuclear ribosomal DNA (nDNA) has been widely studied by molecular systematists. Because of its high evolutionary rate and the amount of copy number which can be easily amplified, nDNA is usually used to show relationships between lower taxonomic levels (Small *et al.*, 2004).

1.6.2.1. ITS (internal transcribed spacer) Region

For lower – level phylogenetic challenges in angiosperms, internal transcribed spacer (ITS) region has been used in many study in recent years (Zimmer *et al.*, 1989; Suh *et al.*, 1993). ITS composes of 3 regions; 5.8S subunit, ITS-1 and ITS-2 spacers. Although 5.8S subunit is highly conserved part of this gene region, ITS-1 and ITS-2 are the most preferable spacers in plant molecular phylogenetic studies due to mutations, deletions, or point mutations in the regions (Baldwin *et al.*, 1995).

Many different features of ITS region support its usage for phylogenetic studies on angiosperms. These are;

(1) The ITS region is highly repeated in number in plant genomes (Rogers and Bendich, 1987; Hamby and Zimmer, 1992). Large copy numbers strongly support amplification and sequencing of nDNA.
(2) Due to its genomic properties, this region withstands fast concerted evolution.

(3) Although it is small in size (<700bp), it is highly conserved spacers, and also easy to amplify even with herbarium materials (Wojciechowski *et al.*, 1993; Baldwin, 1995).

Although, there are some debates about ITS regions for phylogenetic studies such as its fairly conserved within species, many researchers still prefer this region by combining data from other nDNA or cpDNA regions (Li *et al.*,2004; Kenicer *et al.*, 2005).

CHAPTER 2

JUSTIFICATION OF THE STUDY

In contrast to neighbouring countries, Turkey has a high valuable plant biodiversity due to its special biogeographic features. Therefore, every year new taxa have been added or separated to different taxonomic levels of plant systematics. Typically, these studies were carried out via morphological or anatomical characters. Although these characters are adequate for some cases, some taxa do not have special morphological characters for taxonomic classification because many of these traits are simply changed by physical environmental effects. With regard to getting over this problem, taxonomists should find new methods to be used their revision studies. In recent years, especially molecular techniques have became useful to support taxonomical studies. Both molecular markers like RFLP and AFLPs (Tansksley *et al.*, 1989) and amplifying cpDNA or nr DNA regions (Zhang *et al.*, 2008) (Petersen, 1997) have been used by researchers. Combined of analysis of genes from various subcellular parts are recognized to be a successful methods to assess organismal phylogeny (Qui *et.*al., 1999, Soltis *et al.*, 2000, 2003; Zanis *et.* al., 2002, Hilu *et al.*, 2003).

Astragalus genus has many taxonomic problems due to many similarities among the member species with respect to morphological features. Therefore, taxonomy of the genus needs to be evaluated by molecular studies (Wojciechowski, 1993). To eloborate this problem, gene regions from both cpDNA and nDNA genomes which were underlized in Angiosperm phylogenetic studies, were selected in order to shed light on phylogenetic and evolutionary relationships among species consist of six different sections (*Macrophyllium*, *Hymenostegis*, *Poterion*, *Megalocystis*,

Halicacabus and *Hymenocoleus)* of the genus *Astragalus* in Turkey. Both coding or noncoding regions (*trn* and *mat*K) of cpDNA are useful for molecular studies due to their rapid evolutionary rates. Moreover, ITS spacer of nDNA is also highly useful to figure out phylogenetic relationships among species since the region has highly conserved sequences. Combined sequence data from both cpDNA and nDNA regions for understanding phylogenetic relationships among these sections of *Astragalus* are very rare. Therefore, molecular data from five different genomic and choloroplast regions in the current study are expected to fill the gap via constructing a phylogenetic tree among different sections of the genus *Astragalus*.

CHAPTER 3

OBJECTIVES OF THE STUDY

The objectives of the study were;

1) To clarify interspecific phylogenetic and evolutionary relationships among and within naturally found six sections (*Macrophyllium*, *Hymenostegis*, *Poterion*, *Megalocystis*, *Halicacabus* and *Hymenocoleus)* of *Astragalus* in Turkey via *trn*L5'-L3' (*trn* L intron), *trn*L3'-F intergenic spacer, *trn* V and *mat*K cpDNA regions and ITS (internal transcribed spacers) genomic DNA regions.

2) To analyze the efficiency of sequence analysis based on the data from cpDNA and nDNA regions of the studied sections of the genus *Astragalus*.

3) To contribute new information and aspects to the taxonomists by using molecular data so that they could reassess taxonomic studies of *Astragalus* species which are known with their taxonomic problems.

CHAPTER 4

MATERIALS AND METHODS

4.1 Plant Materials

Turkey, is very rich in regard to number of *Astragalus* genus after Russia and Iran. It has approximately 475 species dispersed to 64 sections. Due to special geographical locations and climatic factors of Turkey about 42% (202 species) of the species of the genus is endemic to the country.

Samples which belong to the studied sections of *Astragalus* (*Macrophyllium*, *Hymenostegis*, *Poterion*, *Megalocystis*, *Halicacabus* and *Hymenocoleus*) were collected in collaborate with Assos. Prof. Dr. Seher Karaman Erkul during the field studies from 2011-2014. As a part of the research project (TUBİTAK 110T911). All samples were freshly collected leaves of species in the field.

Samples were acquired from not only the exact locations Flora of Turkey (Davis) but also new locations in Turkey. For molecular studies, fresh leaves are necessary to obtain a qualify DNA. For this reason, fresh leaves of samples ,which were collected at field trips, were held in small transparent labelled plastic bags with dry silica jels for protecting samples from contaminations due to humidity until they were arrived at laboratory. Moreover, at least 3 samples from different locations were collected to have sufficient representations for a given species. Moreover, for each species, at least one sample were kept at the collections of Gazi and Aksu Herbariums.

Both names, sections of studied species and topographic informations were provided in the Table 4.1.

Table 4.1: Studied species of six sections of the genus *Astragalus* collected fromdifferent locations of Turkey.

Section	Sample Code	Species Name	Location(Town/Provinence)
Halicacabus	2541a	*A.anthylloides*	Ankara/Ayaş
	2541b	*A.anthylloides*	Ankara/Ayaş
	2558a	*A.anthylloides*	Sivas/Divriği
	2558b	*A.anthylloides*	Sivas/Divriği
	2617b	*A.anthylloides*	Ankara/Ayaş
	2541a	*A.anthylloides*	Ankara/Ayaş
	2558a	*A.anthylloides*	Sivas/Divriği
	2567a	*A.chardini*	Erzurum/Oltu
	2567a	*A.chardini*	Erzurum/Oltu
	2567b	*A.chardini*	Erzurum/Oltu
	2571	*A.chardini*	Erzurum/Horasan
	2578a	*A.halicacabus*	Van/Muradiye
	2578b	*A.halicacabus*	Van/Muradiye
	2585	*A.halicacabus*	Van/Pirreşit Dağı
	2578a	*A.halicacabus*	Van/Muradiye
	2701	*A.mesites*	Ağrı/Murat Vadisi
	2704	*A.mesites*	Muş/Malazgirt
	2706	*A.mesites*	Van/Bahçesaray
	2709	*A.mesites*	Van/Muradiye
	2713	*A.mesites*	Muş/Malazgirt
	2572a	*A.wagneri*	Ağrı/Doğubeyazıt
	2572b	*A.wagneri*	Ağrı/Doğubeyazıt
	2572	*A.wagneri*	Ağrı/Doğubeyazıt
	2543a	*A.zederbaueri*	Konya/Altınapa Barajı
	2543b	*A.zederbaueri*	Konya/Altınapa Barajı
	2547	*A.zederbaueri*	Konya/Beyşehir Yolu
	2548	*A.zederbaueri*	Konya/Ermenek
	2697b	*A.surugensis*	Ş.Urfa/Hilvan Yolu
	2697a	*A.surugensis*	Ş.Urfa/Hilvan Yolu
	2697c	*A.surugensis*	Ş.Urfa/Hilvan Yolu

Table 4.1(Cont'd)

Megalocystis	2627a	*A.ermineus*	Van/Gevaş
	2627b	*A.ermineus*	Van/Gevaş
	2627a	*A.ermineus*	Van/Gevaş
	2698b	*A.micracme*	Hakkari/Çukurca
	2698a	*A.micracme*	Hakkari Çukurca
	2597	*A.micracme*	Hakkari Çukurca
	2734a	*A.szowitsii*	Ağrı/Doğubeyazıt
	2734b	*A.szowitsii*	Ağrı/Doğubeyazıt
	2734c	*A.szowitsii*	Ağrı/Doğubeyazıt
Poterion	2519a	*A.russelli*	Ş.Urfa/Ceylanpınarı
	2519b	*A.russelli*	Ş.Urfa/Ceylanpınarı
	2536	*A.russelli*	Ş.Urfa/Hilvan
	2538	*A.russelli*	Ş.Urfa/Hilvan
	2702	*A.brugueri*	Şırnak/Cizre
	2705	*A.brugueri*	Şırnak/Kumçatı
Hymenostegis	2710	*A.brugueri*	Şırnak/Nusaybin
	2589	*A.zohrabi*	Van/Erçek
	2626	*A.zohrabi*	Van/Bahçesaray
	2590	*A.zohrabi*	Ağrı/Gevaş
	2712	*A.velenowskyii*	Van/Çaldıran
	2632	*A.velenowskyii*	Van/Erciş
	2635	*A.hymenocystis*	Van/Tendürek
	2591	*A.hymenocystis*	Van/Muradiye
	2752a	*A.hymenocystis*	Van/Gürpınar
	2752b	*A.hymenocystis*	Van/Gürpınar
	2752c	*A.hymenocystis*	Van/Gürpınar
	2765	*A.hymenocystis*	Van/Gürpınar
	2591	*A.hymenocystis*	Van/Özalp
	2599	*A.hirticalyx*	Van/Hoşap
	2600	*A.hirticalyx*	Van/Hoşap
	2601	*A.hirticalyx*	Van/Erciş
	2635	*A.lagopoides*	Van/Erciş
	2569	*A.lagopoides*	Van/Erciş
	2569	*A.lagopoides*	Van/Muradiye
	2576	*A.lagopoides*	Van/Bendimahi
	2581	*A.lagopoides*	Van/Bendimahi
	2618	*A.lagopoides*	Nevşehir/Ürgüp

Table 4.1(Cont'd)

Hymenostegis	2654	*A.lagopoides*	Van/Horasan
	2588	*A.lagopoides*	Van/Altındere
	2622	*A.lagopoides*	Van/Yukarınarlı
	2646	*A.lagopoides*	Ağrı/Çaldıran
	2761	*A.lagopoides*	Van/Sugeçer Köyü
	2779	*A.lagopoides*	Sivas/Gürün
	2783a	*A.lagopoides*	Ağrı/Doğubeyazıt
	2783b	*A.lagopoides*	Ağrı/Doğubeyazıt
	2794	*A.lagopoides*	Ardahan
	2798	*A.lagopoides*	Erzurum/Oltu
	2616b	*A.brachypodus*	Van/Güzeldere
	2616c	*A.brachypodus*	Van/Güzeldere
	2616a	*A.brachypodus*	Van/Güzeldere
	2616b	*A.brachypodus*	Van/Güzeldere
	2584	*A.sosnowskii*	Van/Adaklı Köyü
	2588	*A.sosnowskii*	Van/Erciş
	2592	*A.sosnowskii*	Van/Erçek
	2593	*A.sosnowskii*	Van/Özalp
	2633	*A.sosnowskii*	Van/Erciş
	2632	*A.sosnowskii*	Van/Erciş
	2660	*A.sosnowskii*	Erzurum/Oltu
	2667	*A.sosnowskii*	Erzurum/Oltu
	2609a	*A.trifoliastrum*	Van/Güzeldere
	2609b	*A.trifoliastrum*	Van/Güzeldere
	2650	*A.trifoliastrum*	Van/Güzeldere
	2781	*A.trifoliastrum*	Van/Kurubaş
	2609a	*A.trifolisotrum*	Van/Kurubaş
	2652a	*A.ciloensis*	Van/Gürpınar
	2652b	*A.ciloensis*	Van/Gürpınar
	2652c	*A.ciloensis*	Van/Gürpınar
	2652d	*A.ciloensis*	Van/Gürpınar
	2622	*A.gueruenensis*	Sivas/Gürün
	2659	*A.gueruenensis*	Sivas/Gürün
	2527	*A.gueruenensis*	Sivas/Gürün
	2575	*A.gueruenensis*	Sivas/Gürün
	2577a	*A.uraniolimneus*	Van/Muradiye
	2577b	*A.uraniolimneus*	Van/Muradiye
	2577c	*A.uraniolimneus*	Van/Muradiye

Table 4.1(Cont'd)

Hymenostegis	2582	*A.uraniolimneus*	Van/Muradiye
	2599	*A.uraniolimneus*	Van/Muradiye
	2747	*A.uraniolimneus*	Van/Muradiye
	2749	*A.uraniolimneus*	Van/Hoşap
	2797	*A.uraniolimneus*	Van/Hoşap
Macrophyllium	2625e	*A.dipodurus*	Konya/Karasınır
	2625d	*A.dipodurus*	Konya/Karasınır
	2625a	*A.dipodurus*	Konya/Karasınır
	2625b	*A.dipodurus*	Konya/Karasınır
	2625c	*A.dipodurus*	Konya/Karasınır
	2736	*A.dipodurus*	G.Antep/Arat Dağı
	2620a	*A.yukselii*	Konya/Hadim
	2620b	*A.yukselii*	Konya/Hadim
	2620c	*A.yukselii*	Konya/Hadim
	2542a	*A.oleaefolius*	Aksaray/Taptuk emre Köyü
	2542b	*A.oleaefolius*	Aksaray
	2542c	*A.oleaefolius*	Aksaray
	2557b	*A.oleaefolius*	Sivas/Şarkışla
	2557c	*A.oleaefolius*	Sivas/Şarkışla
	2557a	*A.oleaefolius*	Sivas/Şarkışla
	2657	*A.oleaefolius*	Erzurum/İspir
	2735	*A.oleaefolius*	G.Antep/Nizip
	2738	*A.oleaefolius*	Sivas/Gemerek
	2598c	*A.longifolius*	Hakkari/Çukurca
	2598a	*A.longifolius*	Hakkari/Çukurca
	2598b	*A.longifolius*	Hakkari/Çukurca
	2587b	*A.longifolius*	Van/Hasanabdal
	2647	*A.longifolius*	Van/Hasanabdal
	2587a	*A.longifolius*	Van/Hasanabdal
	2659	*A.longifolius*	Van/Hasanabdal
	2661	*A.longifolius*	Van/Hasanabdal
	2549d	*A.isauricus*	Konya/Taşkent
	2549c	*A.isauricus*	Konya/Taşkent
	2549a	*A.isauricus*	Konya/Taşkent
	2549b	*A.isauricus*	Konya/Taşkent

Table 4.1(Cont'd)

Macrophyllium	2527	*A.isauricus*	Konya/Hadim
	2665	*A.cephalotes*	Erzurum/Oltu
	2669	*A.cephalotes*	Artvin/Şavşat
	2670	*A.cephalotes*	Artvin/Şavşat
	2670a	*A.cephalotes*	Artvin/Şavşat
	2733	*A.cephalotes*	Artvin/Yusufeli
	2793	*A.cephalotes*	Artvin/Şavşat
	2795	*A.cephalotes*	Artvin/Yusufeli
Hymenocoleus	2672c	*A.vaginans*	K.Maraş/Çağlayancerit
	2672a	*A.vaginans*	K.Maraş/Çağlayancerit
	2672b	*A.vaginans*	K.Maraş/Çağlayancerit
	2776	*A.vaginans*	Niğde/Ulukışla

4.1.1 DNA extraction from samples

Fresh leaves of samples were used to extract total DNA from leaves dried in silica gels in the study. For DNA extraction, a modified CTAB (cetyl trimethylammonium bromide) protocol (Doyle and Doyle, 1987) was applied. The details of the procedure was as follows;

1) For each sample, about 200 mg leaves was put in autoclaved mortar and ground in liquid -80^{o} C nitrogen to increase the surface area.

2) Before starting the first step of isolation, 0.8gr polyvinylpyrrolidone (PVP) was added to extraction buffer (2XCTAB) (Table 4.2). Then it was incubated in water bath at 60^{o}C. Approximately 100 mg fresh leaves were ground by adding 500 µl extraction buffer.

3) Mixture of leaf tissue and buffer were poured into 2ml eppendorf tubes. Then, 5 µl proteinase K and 200 µl βmercaptoethanol were added to each tubes. After adding these chemicals, tubes were incubated at 65°C for at least one hour.

4) At the end of incubation period, all tubes were put into centrifugation which were set at 14000 rpm for 20 minutes at +4ºC for centrifigating. After that, the supernatant phase of the mixture (top green aqueous part) was taken out to a fresh tube.

5) 500 µl of chloroform octanol (24:1) was added to the tubes with supernatant at step 4 above. Then, they were mixed gently via vortex. All tubes were centrifuged at 14000 rpm for 15 minutes at +4ºC again.

6) After the centrifugation period, the aqueous phase (top part) of the mixture was transferred into a fresh tube and 500 µl of cold isopropanol was added.

7) Tubes were incubated at -80 ºC for at least one hour. After cold incubation, they were again centrifuged at 14000 rpm for 20 minutes at +4ºC. So that total DNA could precipitate down at the bottom of the tubes.

8) To obtain DNA pellet, supernatant in the tubes from the step 7 was poured very carefully and 70% EtOH were used to clean and remove residue materials from DNA. Then, tubes were inverted on a clean tissue paper and kept for drying for 15-20 minutes.

9) The dry DNA pellets were hydrated with 100 µl TE (Tris EDTA) and kept overnight at room temperature.

At the end of isolation protocol, DNA quantifications were made by using a spectrophotometer (Thermo Fisher Scientific Inc. NanoDrop 2000 Spectrophotometer Version 1.4.1). Dissolved DNA was diluted to 10 ng/µl for PCR reactions by adding double distilled H_2O. The diluted DNAs were stored at +4ºC for short term while for long term storage, they were kept of -20 ºC or -80 ºC.

The compositions of buffers and reaction mixture solutions that were used in DNA isolation protocol were given in the table 4.2 in detail.

Table 4.2 Buffers and solutions used during DNA isolation from fresh leaf tissue

Buffer/Solutions	Concentration/Content
2XCTAB	2gr CTAB (Cetyl trimethyammonium bromide)
	10 ml (pH:8.0) Tris HCl (Tris(hyroxymethl)aminomethane hydrochloride)
	4ml (pH:8.0) 0.5M EDTA(Ethylenediaminetetraaceticacid disodium salt)
	28ml 5M NaCl is completed up to 100 ml with dH_2O
β - Mercaptoethanol	25 ml β – Mercaptoethano is completed up to 100 ml with dH_2O
Chloroform – octanol	24:1
Ethanol	70 % in dH2O
TE Buffer	10 M Tris HCl
	10 EDTA

4.2 Studied Chloroplast and Genomic DNA Regions

4.2.1 Chloroplast DNA (cpDNA) Regions

4.2.1.1 *trnL*5'-L3' (*trn*L intron) and *trn*L3'-F $^{(GAA)}$ (*trn*L-F intergenic spacer) Region

Two primer pairs were used in order to amplify *trn*L and *trn*L-F intergenic spacer regions of cpDNA. The primers of these regions were adapted from the study of Taberlet *et al.* (1991). The sequences of these primers were given in the Table 4.3. Moreover, schematic drawings for position and direction of primers were also given in Figure 4.1.

Table 4.3 The codes and sequences of the two universal primer pairs used to amplify the *trnL* (*UAA*) intron and *trnL*-F intergenic spacer region and their aproximate lengths (Taberlet *et al.*, 1991).

Name	Primer	Sequence	Length
*trn*L	trn c (forward)	5' CGA AAT CGG TAG ACG CTA CG 3'	~520bp
intron	trn d (reverse)	5' GGG GAT AGA GG ACT TGA AC 3'	
trn L-F	trn e (forward)	5' GGT TCA AGT CCC TCT ATC CC 3'	~160bp
IGS	trn f (reverse)	5' ATT TGA ACT GGT GAC ACG AG 3'	

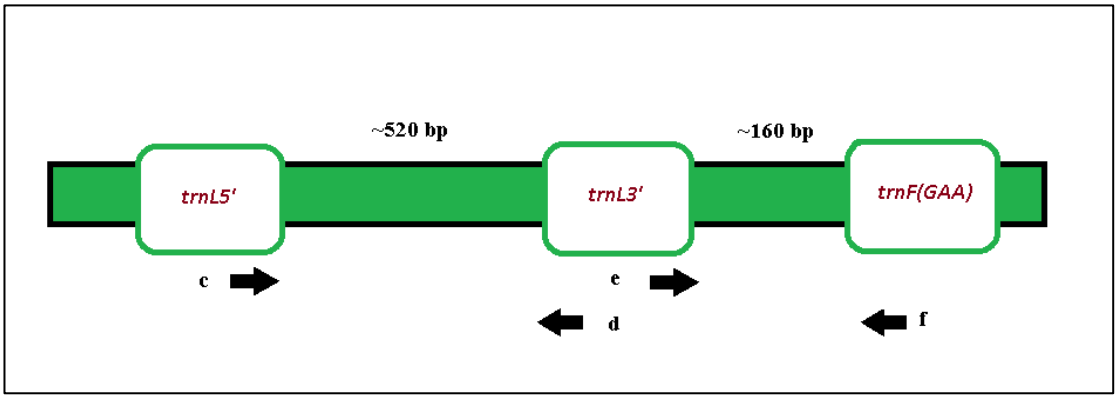

Figure 4.1 Positions and directions of universal primers (c-d and e-f) used to amplify the chloroplast *trnL* (*UAA*) intron (*trnL5'-trnL3'*), and *trnL*-F intergenic spacer [(*trnL3'-trnF* (*GAA*)] regions (Taberlet *et al.*, 1991). (c and e = Forward primers, d and f =Reverse primers).

4.2.1.2 *trn* V intron region

Two primer pairs were designed to amplify *trn* V intron regions of cpDNA. The primers of this region were optimized from the study of Wang *et al.*(1999). The sequences of these primers were given in the Table 4.4. Schematic drawings of position and direction of primers were also provided in Figure 4.2.

Table 4.4 Codes and sequences of primer pair amplifying the *trnV* intron region and its lenght (Wang *et al.*, 1999).

Name	Primer	Sequence	Length
*trn*V intron	trnVF (forward) trnVR (reverse)	5'GTA GAG CAC CTC GTT TAC AC 3' 5'CTC GAA CCG TAG ACC TTC TC 3'	~550bp

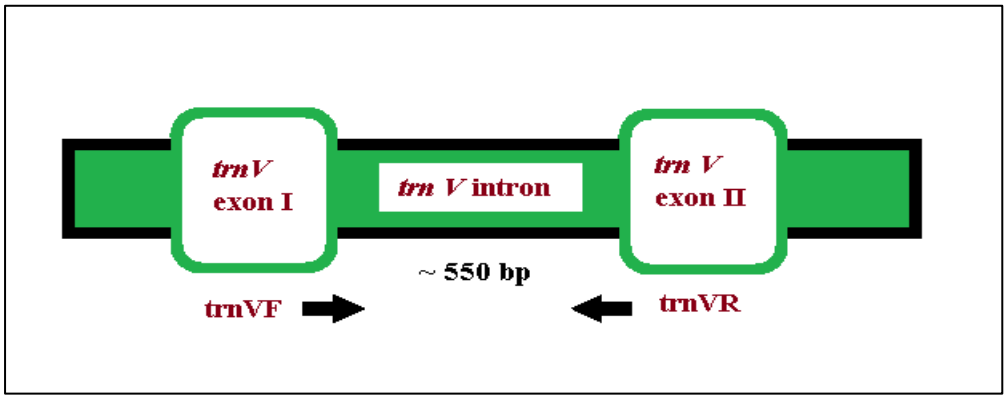

Figure 4.2 *trnV* genes and amplified *trn*V intron. Intronic region was amplified by using *trn*VF (Forward) and *trn*VR (reverse) primers

4.2.1.3 *mat*K (maturase Kinase) gene region

By using one primer set, *mat*K region was amplified in this study. The primers of the region were adapted from the study of Li *et al.* (1999). The sequences of these primers were given in the Table 4.5. Schematic drawings of position and direction of primers were also provided in Figure 4.3.

Table 4.5 Codes and sequences of the primer pair amplifying partial matK region and its approximate lenght (Li *et al.*, 1997).

Name	Primer	Sequence	Length
*mat*K gene region	F1 (forward) R3 (reverse)	5' ACT GTA TCG CAC TAT GTA TCA 3' 5' GAT CCG CTG TGA TAA TGA GA 3'	~1200bp

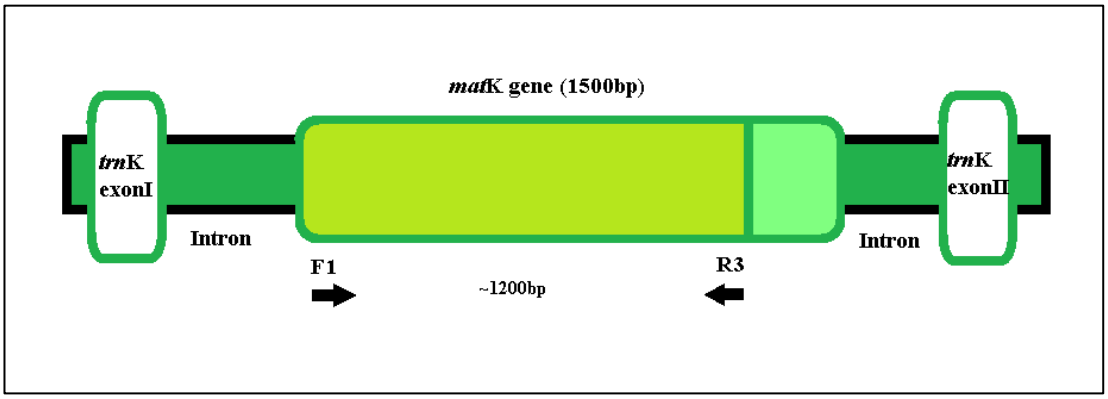

Figure 4.3 Sketch of the *trnK* gene and its *matK*-containing intron. Light green part was amplified by F1 (Forward) and R3 (reverse) primers.

4.2.2 Genomic DNA region

4.2.2.1 Internal transcribed spacer (ITS) Region of nDNA

With regard to strengthen and improve the results of the cpDNA regions, additionally ITS region was chosen from nDNA. Universal primers were selected to amplify this region. Although some researchers amplify ITS1 and ITS2 sub regions separately (Suh *et al.*, 1993; Gernanth and Liston, 1999; Beltame-Botelho *et al.*, 2005), in this study one primer set was used to amplify both sub regions along 5.8S gene region. As a result of this approach, cost, labor and time were reduced for this study. The primers of the region were adapted from the study of Hsiao *et al.* (1995). The sequences of

these primer were given in the Table 4.6. Schematic drawings of position and direction of primers were also provided in Figure 4.4.

Table 4.6 Names and sequences of the primers used for amplification of ITS1+5.8S+ITS2 region and their approximate lengths as base pair (Hsiao *et al.*, 1995)

Name	Primer	Sequence	Length
ITS region	ITSL (forward)	5'TCG TAA CAA GGT TTC CGT AGG TG3'	~630bp (ITS1~230 5.8S~180 ITS2~220bp)
	ITS4 (reverse)	5' TCC TCC GCT TAT TGA TAT GC 3'	

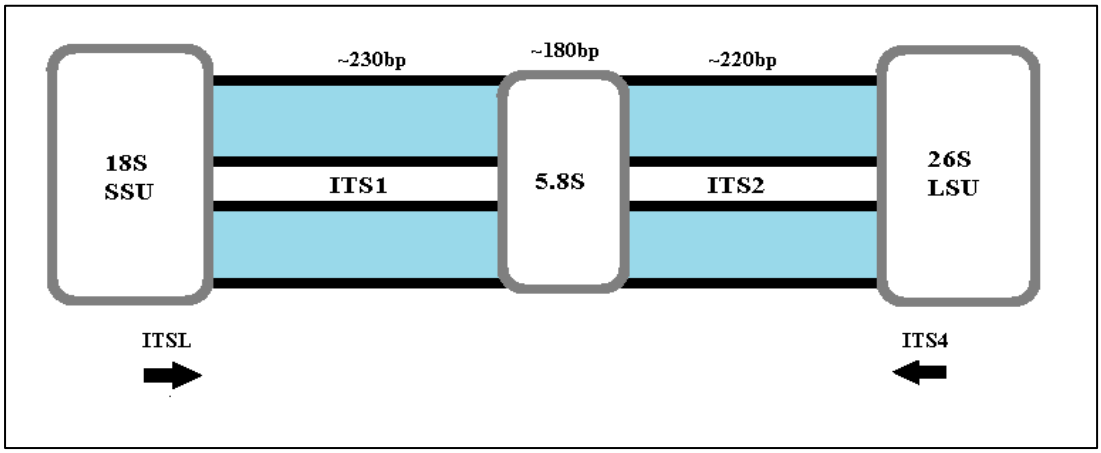

Figure 4.4 Schematic organization of the ITS region indicating 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S rRNA. The ITS1 separates the coding region of the 18S subunit from the 5.8S rDNA, and the ITS2 separates 5.8S rDNA region from the 26S rDNA. ITSL and ITS4 are the Forward and Reverse primers, respectively.

4.3 Polymerase Chain Reaction (PCR)

To amplify the interested cp and nr DNA regions, PCR amplification conditions for each primers of each region need to be optimized. Although, each primer sets have specific conditions, it could be also changed according to nature of samples in each study. Therefore, PCR condition optimization is an important step for molecular studies. In Table 4.7, optimized PCR conditions for the study were given. Each primer sets require different mixture that needs to be optimized for amplification. Not only mixture volume is important, but also PCR amplification program steps are significant for a successful DNA amplification. These program steps which were optimized for each primer sets were provided Table 4.8.

Table 4.7 Components and their concentrations in PCR reaction mixtures for each cp and nuclear DNA regions

	Component amount in studied regions/primer pairs				
Components and concentrations of PCR mixtures	*trnL* intron/ trn c-d	*trnL3'-F* (GAA)/ trn e-f	*trnV* intron/ trnVF-VR	*matK*/ F1-R3	ITS/ ITSL-4
H_2O	34.5 µl	33.5 µl	31.4 µl	21.9 µl	34.5 µl
$MgCl_2$ (25 mM)	5 µl	4 µl	5 µl	6 µl	3 µl
10X PCR Buffer	5 µl	6 µl	5 µl	6 µl	3 µl
dNTP (10 mM)	2 µl	2 µl	1 µl	3.5 µl	2 µl
Forward primer (10 µM)	0.5 µl	1 µl	2 µl	4 µl	2 µl
Reverse primer (10 µM)	0.5 µl	1 µl	2 µl	4 µl	2 µl
Taq DNA poly. (5u/µl)	0.5 µl	0.5 µl	0.6 µl	0.6 µl	0.5 µl
DNA (10 ng/ µl)	2 µl	2 µl	3 µl	4 µl	3 µl
Total	50 µl	50 µl	50 µl	50 µl	50 µl

Table 4.8 Optimized PCR amplification programs studied regions of chloroplast and nuclear genomes

Region (Primers)	Step	Temperature (˚C)	Time Minute(') & Second(")	# of cycles	Description
***trnL* intron (trn c-d)**	1	95˚C	5'	1	Denaturation
	2	94 ˚C	30"	35	Denaturation
		52.3 ˚C	30"		Annealing
		72 ˚C	30"		Extension
	3	72 ˚C	10'	1	Final Extension
***trnL3'-F* (GAA) (trn e-f)**	1	95 ˚C	5'	1	Denaturation
	2	94 ˚C	30"	35	Denaturation
		60 ˚C	30"		Annealing
		72 ˚C	30"		Extension
	3	72 ˚C	10'	1	Final Extension
***trnV* intron (trnVF-VR)**	1	95 ˚C	5'	1	Denaturation
	2	94 ˚C	30"	35	Denaturation
		56 ˚C	30"		Annealing
		72 ˚C	40"		Extension
	3	72 ˚C	10'	1	Final Extension
***mat*K (F1-R3)**	1	95 ˚C	5'	1	Denaturation
	2	94 ˚C	40"	35	Denaturation
		52 ˚C	45"		Annealing
		72 ˚C	90"		Extension
	3	72 ˚C	10'	1	Final Extension
ITS1&2 (ITSL-ITS4)	1	95 ˚C	5'	1	Denaturation
	2	95 ˚C	40"	35	Denaturation
		50.7 ˚C	40"		Annealing
		72 ˚C	50"		Extension
	3	72 ˚C	10'	1	Final Extension

4.4 Agarose Gel Electrophoresis

While preparing the agarose gels different percentages were optimized according to the length and size of interested region. 1X TBE (Tris-Borate-EDTA - 1 liter of 5X stock solution: 54 g of Tris base – 27.5 g of Boric acid – 20 ml of 0.5 M EDTA pH 8.0) buffer was used for dissolving agarose. Dissolved solution was prepared in a microwave oven and liquid gel was poured into horizontal gel tray in which combs were inserted before for loading PCR products. After polymerization of the gel, 1X TBE buffer was again prepared for running procedure of gells and poured into electrophoresis apparatus. All combs were took off from gel gently. 6X DNA loading dyes (Fermentas) for samples and dye + product combinations were loaded into fresh wells separately. Usually, gels were run under 90-100 V for at least for an hour. After electrophoresis, EtBr (Ethidium Bromide) was used to stain DNA fragments. The bands were evaluated under UV light. If the bands were clear enough and gave the expected size of the studied region, they were stored at -20C until sequence analysis, they were sent to the company for sequencing.

4.5 Data Collection

All purification and sequencing reactions of studied regions (*trn*L intron, *trn* L-F, *trn* V intron, *mat*K and ITS (ITS1+5.8S+ITS2 subregions) were performed in the REFGEN BIOTECHNOLOGY facilities (Ankara University, Technocity, Gölbaşı, Ankara). An ABI 310 Genetic Analyzer (PE applied Biosystem) automatic sequencer was used to examine for sequencing of amplified products. Sequencing results were examined by Finch TV software (Version 1.4.0-manufactured by Geopiza Research Team) (Patterson *et al.*, 2004-2006). Both forward and reverse DNA sequences were obtained to get proper sequences of a given region and they were aligned by MUSCLE (Multiple Sequence Comparison by Log Expectation) tool (Edgar, 2004) of MEGA 7.0.9 (Molecular Evolutionary Genetics Analysis) software (Tamura *et al.*, 2017). This tool has many advantageous over Clustal W and T-coffee because it uses pairwise alignment clarifying the tree (combination of sequences, separate nodes). Moreover, MUSCLE repeats the iterations until maximum iterations are obtained. All the

sequences were checked for miscalled bases. Therefore there were no challenges in identification of nucleotide bases (Appendix A).

In order to show the differentiation between *Astragalus* species in Turkey and in the world, relevant sequences were retrieved from NCBI by BLAST (Basic Local Alignment Search Tool) for *trn*, *mat*K and *ITS* regions. Although, *trn*L intron and *trn* L-F spacer were often studied in the world, studies on *trn* V intron were less frequently than other 2 *trn* regions. Therefore, GenBank data for *trn* V intron were not adequate to make in comparison between studied *Astragalus* species in Turkey and other *Astragalus* species in the world.

4.6 Analysis of Data

Obtained DNA sequences for the studied regions of cpDNA (*trn* and *mat*K) and nDNA (ITS) were assessed independently for each region. All the sequences were formed for the analysis by editing null bases composed of unsuccessful nucleotide peaks from raw data. After preparing of sequences, all the data for *Astragalus* samples were organized so that if could be analyzed with the MEGA 7.09 software. Important phylogenetic and molecular evolutionary statistics such as total nucleotide length(base pairs), GC contents (%), nucleotide deletions and insertions, conserved and variable sites, parsimony informative sites, transition/transversion(tr/tv) ratio and nucleotide diversity were calculated for *Astragalus* species with respect to *trn*, *mat*K, ITS regions of both cpDNA and nDNA via MEGA 7.09 software.

Generally, deletions and insertions are symbolized by gaps that are acquired during the alignment of homologous regions of sequences. Complete deletion method was used for calculating the distances among species. This method is advantageous in most situations whereas various DNA sequences evolve with the different evolutionary rates. Evolutionary distance pursues to consider the mean number of alterations per site during two sequences separated from each other. Phylogenetic divergences are very important to get information about molecular evolution since they are used for

phylogenetic reconstructions of upper and lower taxonomic level. Furthermore, Kimura (1980) – 2parameter model was used to calculate the genetic divergences among species. This model corrects all the multiple hits by taking into account transversional and transitional substitution rates, and estimate the same frequency for all nucleotide bases (A, T, G, and C).

The ratio of transition/transversion (R) demonstrates the proportion between the numbers of transition to the number of transversion for a pair of sequences. This ratio was calculated by using the formula below;

$$R = [A \times G \times k1 + T \times C \times k2] / [(A + G) \times (T + C)]$$

Where *k1* is transition/transversion rate ratios for purine and *k2* is same for pyrimidines.

Phylogenetic trees are the most useful tool to evaluate the evolutionary relationships between various samples and their ancestors. In some situations, these trees can be rooted according to different parameter methods that are used for constructing the trees, or not. In the current study neighbor-joining (NJ) method (Saitou and Nei, 1987) was used with bootstrap test analysis. The NJ is the basic type of minimum evolutionary (ME) method. In NJ method, S value, which is the smallest value of the sum of all branches, is not calculated for all topologies. However, the algorithm is consumed for the analysis of different topologies. As a consequence of this method, only one final tree is assembled (Nei and Kumar, 2000). Generally, NJ method gives an unrooted tree however, if there is an outgroup taxon for distinguishing evolutionary rate, rooted tree could be obtained. In the current study, *Cicer* sp. was selected as an outgroup sample in the studied regions. Furthermore, bootstrap test (Felsenstein, 1985) was consucted to indicate the reliability of the phylogenetic trees. In the large number of trees, the acceptance of the branch is verified by criticizing the frequency of the occurrence of the branch. This is bootstrap value and assuming that the value is

higher than 95%, the topology of that branch is “correct”. In contrast, if the value is between 50 and 95% the topology is consider as informative (Nei and Kumar, 2000).

4.7 Molecular Clock Estimation

Zuckerkandl and Pauling (1965) were the first researchers who depicted the term ‘Molecular Evolutionary Clock’. According to the nucleotide differences in DNA sequences, the clock determines the times of species divergence. When the evolution time of the two or more lineages is the same, constant rate, which figures out the number of variations among two samples, would be unambiguous index of the time untill their separation from common ancestor (Futuyma, 2005). For that reason, the phylogeny can be calculated by looking at the rate of nucleotide variations between sequences of two taxa. In order to estimate the rate of molecular evolution, the number of parsimony informative sites are utilized in the studied DNA region. The equation for calculating the molecular clock of *Astragalus* genus is;

$$Molecular\ Clock = \frac{k}{Mutation\ Rate}$$

Where k is equal to; $k = -\left(\frac{3}{4}\right)\ln\left(1 - \frac{4}{3}d\right)$

d is the number of substitutions per base pair whereas k is the substitutions since divergent time.

The d is calculated as; $d = \frac{Variable\ Site}{Total\ Number\ of\ Base\ Pairs\ Sequenced}$

In the current study, cpDNA regions (*trn*L and *mat*K) were assessed to get molecular clock divergence time. For every region, the numbers calculated independently.

Moreover, mutation rate was obtained as 2×10^{-3} of plant cpDNA as a constant value (Pevsner, 2009).

Some *Astragalean* clade sequences were retrieved from NCBI database in order to interpret divergence time of *Astragalus* genus from others. Furthermore, new world *Astragalus* group species were included to understand the phylogenetic relationships between Turkish samples (Old World). For calculation of divergence time for *Astragalus* genus, studied regions which are common to both new and old world *Astragalus* species were utilized. For estimating the values, at least 2 representative species for each section were selected.

CHAPTER 5

RESULTS

5.1 Amplification of *trn* and *mat*K Regions of cpDNA and ITS regions of nDNA

PCR amplifications of *trn* and *mat*K regions of chloroplast DNA and ITS regions of genomic DNA of studied samples were completed by using the conditions that were given at the tables in the previous chapter. Total of 152 samples coming from six different sections of *Astragalus* genus were examined for each regions. Figure 5.1 indicate the PCR products in agarose gels that were clearly obtained. Furthermore, to comprehend the approximate size of the products, DNA ladder was selected and used according to the predicted size of them. The GeneRuler^{TM} 50 and 100bp ladders were chosen for all the studied regions. Generally, concentration of agarose gel is decided with respect to product size which was extrapolated from the marker DNA. Lower concentrated gels (%1) were used for larger size products (over 600bp); however, high concentrated ones (%2-2.5) were used for smaller sized (up to 200-300bp).

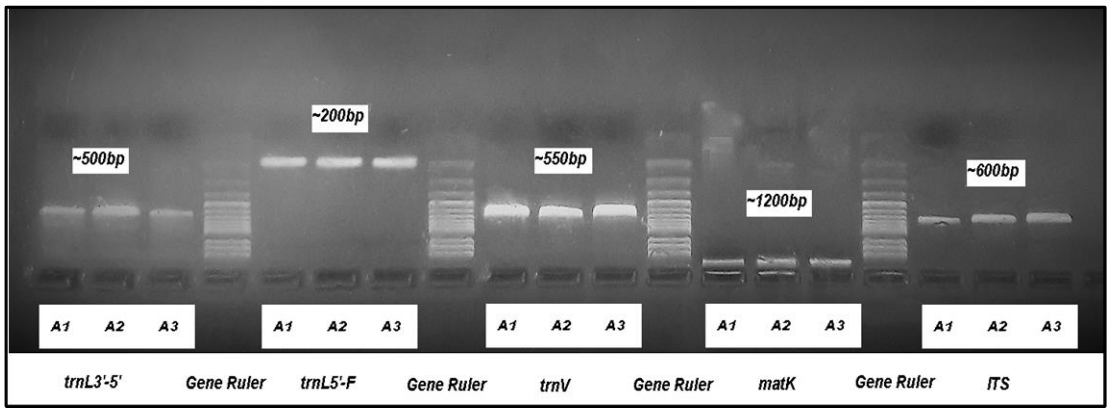

Figure 5.1. PCR products of all studied regions and their lengths that run in%2 agarose gel electrophoresis (A1: *A.russelli,* A2: *A.halicacabus,* A3: *A.ciloensis*).

5.2 Molecular Diversity and Molecular Phylogeny in *Astragalus*

In the current study, 152 individuals from 30 *Astragalus* species including six sections that are native to Turkey, were selected and identified. Data which were based on four cpDNA and one nDNA regions were recorded.

All the data that were acquired from samples as total nucleotide length (bp), conserved and variable sites, parsimony informative sites, nucleotide diversity, transition/transversion (tr/tv) ratio, GC content (%), number of deleted/inserted nucleotides and number of sequences used for each section of *Astragalus* were computed by using MEGA 7.09 program. All these parameters were estimated for all 6 sections of the genus at each studied regions separately. Moreover, combination of 3 *trn* regions were also added to simplify some complex results. In the shed light on this combination, comparison between studied non-coding regions and coding regions became more plausible. Furthermore, to get more refined results, the number of observed dissimilarity was calculated as proportion of the sequence length. Therefore, for instance, if there is 5% dissimilarity, it is only little alteration like 5.17% in large regions such as *mat*K. On the other hand, this proportion would be altered in large scales at shorter sequences, for instance, from 50% to 87% like *trn* L3'-F (Jukes and Cantor, 1969).

5.2.1 Molecular Diversity Statistics of *Astragalus* Species based on studied cpDNA and nDNA regions

5.2.1.1 *trn*L5'-L3' (*trn*L intron) and *trn* L3'-F$^{(GAA)}$ Regions of cpDNA

After the sequence alignment of all samples for *trn*L introns in MEGA program, the length of the region was found as 520bp because of the uncertainity of peaks at the end and at the beginning of sequences of the region. Moreover, because of indels on the DNA sequences, the length of this region varied among sections as well as species. Particularly, between 106th and 118th base position there were Adenin repeats. The changes in the number of this repetitions were given diversity among sections and

species. Furthermore, between 229th and 235th base position there was a deletion. This deletion was specific to the species which are included in *Poterion* section. Additionally, in 143th and 178th base position there were single insertions in *A.brugueri*. Also, *A.mesites* had 2 insertions, one of them was in between 245th-250th base positions. The other was in between 451st – 456th base positions.

Average GC (%) content of the studied *Astragalus* sections were 30, 5 % (Table5.1). GC content value, which gives variety of frequencies of nucleotides, is meaningful for creating phylogenetic reconstruction difficulties.

Table 5.1 Estimated molecular diversity parameters of *trn*L5'-L3' (*trnL* intron) chloroplast DNA region in studied *Astragalus* sections

	Astragalus sections						
	Halicacabus	*Megalocyctis*	*Hymenocoleous*	*Hymenostegis*	*Macrophyllium*	*Poterion*	Total
Number of species	7	3	1	11	6	2	30
Total length(bp)	520	520	520	520	520	520	520
GC content(%)	30.6	30.6	30.5	30.4	30.6	30.9	30.5
Conserved sites	485	476	480	478	478	466	471
Variable sites	3	3	0	3	3	9	20
Parsimony informative sites	3	3	0	3	3	9	20
Transitional pairs	68.79	35.7	33.33	46.38	38.45	36.38	40.14
Transversional pairs	31.21	64.3	66.67	53.62	61.55	63.62	59.86
Transition/transversion (tr/tv)(R)ratio	2.01	0.50	0.43	0.69	0.50	0.50	0.58
Number of deletion	9	0	0	1	2	1	13
Number of insertion	1	1	0	0	0	1	3

Variable sites were different in each sections, but total number of variable sites in all species considered together were 20. All the variable sites were parsimony informative in each section. A parsimony informative site represents that at least 2 types of nucleotides with a minimum frequencies of them ought to be present in distinct DNA sequence of samples. This means that at least 2 different nucleotides were placed on at least 2 different distinct sequences.

Dealing with transition and transversion sites, when a purine is substituted by another purine (Adenine vs Guanine) or a visa versa (Thymine vs Cytosine), it is named transition. On the other hand, in transversion conversion, a purine to pyrimidine or visa versa (Adenine vs Tymine or Guanine vs Cytosine) takes place. Percentage of transition and transversion of studied *Astragalus* species were calculated as 40.14 and 59.86 respectively. Moreover, R (the ratio of transition/transversion) value was calculated as 0.58 for *trn*L intron region.

Among studied *Astragalus* sections, the deletions were very variable. Maximum number of deletion were observed in *Halicacabu*s section while there were a few deletions in *Hymenostegis* and *Megalocystis*. Species of *Poterion* section had 7 missing bases. Although there were deletions in DNA sequences, also insertions among *Astragalus* sections were presented with the similar magnitude. Especially in *Halicacabus* section, *A.mesites* had 10 nucleotides more than other species of sections. *A.brugueri* from *Poterion* section had extra 2 nucleotides compared to other *Poterion* section species. Not only substitutions, but also indels have precious value to illustrate phylogenetic relationships among species and sections. When all the sequences of studied samples were evaluated together, most of the inserted and deleted nucleotides were observed in *Halicacabus* section. This means that these nucleotides have been conserved during evolutionary process of the past.

Nucleotide diversity parameters were also computed for *trn*L3'-F$^{(GAA)}$ cpDNA region (Table 5.2). The length of the region was approximately 145 bp. The region is short. Nucleotide sequences did not vary much among sections of *Astragalus*. There were Tymine repetations between 54th and 68th base positions. This repetition seems to be

the cause of divergence among species and sections. Moreover, there was insertions in 121st base position in Poterion, Hymenocoleous and *Macrophyllium* sections. Substitutions in 17th (Guanin instead of Cytosin) and 110th (Tymine instead of Guanin) base positions *A.trifoliastrum* species were observed. This substitutions seem to be responsible for phylogenetic diversity within *Hymenostegis* section. Furthermore, *Hymenocoleous* section was separated from other sections by substitutions at 52nd (Cytosin instead of Tymine) and 47th (Adenin instead of Tymine) base positions (Table 5.2).

GC (%) contents of this region were closed among studied *Astragalus* sections, with an average of 34.1%. Although the size of the region was very short, variable sites were high (15) and all of them were parsimony informative. Transition and transversion values were estimated as 43.87 and 56.13, respectively. The proportion transition to transversion (R) was founded to be 0.95. This means that substitutions between purines and also pyrimidines were the main source of variations among species. Furthermore, insertions and deletions among sections were low, but very useful to establish phylogenetic relations between and within *Astragalus* sections.

Table 5.2 Estimated molecular diversity parameters regarding trnL3'-F $^{(GAA)}$ region of cpDNA *Astragalus* sections.

	Astragalus sections						
	Halicacabus	*Megalocyctis*	*Hymenocoleous*	*Hymenostegis*	*Macrophyllium*	*Poterion*	Total
Number of species	7	3	1	11	6	2	30
Total length(bp)	145	145	145	145	145	145	145
GC content(%)	33.8	34.1	35.4	33.7	34.3	35.3	34.1
Conserved sites	131	132	133	128	129	131	119
Variable sites	2	0	0	5	5	0	15
Parsimony informative sites	2	0	0	5	5	0	15
Transitional pairs	99.68	33.34	33.33	1.54	25.47	33.33	43.87
Transversional pairs	0.32	66.66	66.67	98.46	74.53	66.67	56.13
Transition/transversion (tr/tv)(R)ratio	388.5	0.51	0.50	0.02	0.42	0.49	0.95
Number of deletion	0	0	0	0	0	6	6
Number of insertion	0	0	0	0	0	0	0

5.2.1.2 *trn* V intron Region of cpDNA

Trn V intron region was examined for studied *Astragalus* sections as well. Molecular diversity parameters were also calculated by using MEGA. The parameters were given in Table 5.3.

Total length of the region was approximately 540bp. Overall GC content of the region was 31.8%. Number of variable sites were 12 and half of them were found in *Halicacabus* section. All of these variable sites were informative. Although it was highly variable, there were no substitutions within *Halicacabus* section. All the substitutions were due to transversions. Moreover, estimated R value of all sequences was lower than other trn regions (0.237). This means that while estimating phylogenetic relationships among sections, indels could be useful.

Table 5.3 Estimated molecular diversity parameters based on trnV intron region of chloroplast DNA for *Astragalus* sections.

	Astragalus sections						
	Halicacabus	*Megalocyctis*	*Hymenocoleous*	*Hymenostegis*	*Macrophyllium*	*Poterion*	Total
Number of species	7	3	1	11	6	2	30
Total length(bp)	540	540	540	540	540	540	540
GC content(%)	31.8	31.8	31.9	31.8	31.7	31.6	31.8
Conserved sites	510	515	515	514	514	510	504
Variable sites	6	1	0	2	2	0	12
Parsimony informative sites	6	1	0	2	2	0	12
Transitional pairs	0	0	33.33	99.58	0	33.34	22.11
Transversional pairs	100	100	66.67	0.42	100	66.66	77.89
Transition/transversion (tr/tv)(R)ratio	0	0	0.44	201.43	0	0.43	0.24
Number of deletion	7	1	0	0	0	6	14
Number of insertion	0	0	0	0	0	0	0

5.2.1.3 Combination of *trn* Regions

In order to determine the consistency of the results in each studied *trn* regions, combinated data from the sequences of all 3 regions were analyzed with the MEGA program. Parameters were obtained as in the previous sections. The combination revealed 1215 bp in length. There were 43 variable sites. 22 of these sites were parsimony informative. Overall transition/transversion bias were 47.35 and 52.65, respectively. According to these values, R value was 0.77. Results from combined trn data indicated that *Hymenostegis* section was the most divergent section among studied *Astragalus* sections. Moreover, *Poterion* section was very distinct from other sections. However, *Megalocystis* and *Halicacabus* sections were found to be the closest each other. These results were found to be useful constructing overall phylogenetic relationships among studied *Astragalus* sections.

5.2.1.4 *mat*K (maturase kinase) Region of cpDNA

In the current study, the length of matK gene region was found to be approximately 1225 bp. Since the region had indels and substitutions, the proportion of the variable sites (variable site / total length) was less than those in trn regions. Although the variable sites were a few, all of them were parsimony informative (Table5.4). In total, there were 23 variable sites. Most variable sites were formed in *Hymenostegis* section. Maximum indels were seen in this section (8 bases). The insertion and deletions were not found among studied sections of *Astragalus*, but there were different insertions at species level. For instance; *A.trifoliastrum* had missing bases at 513th, 514th, 515th base positions which were difficult compared to other species of *Hymenostegis* section. Moreover, *A.hirticalyx* had insertion at 527th (Tymine) and 532nd (Guanine) base positions. *A.vaginans* from *Hymenocoleous* section had substitutions at 478th (Guanine instead of Adenine), 651st (tymine instead of Guanine), 1164th (tymine instead of Adenine), and 1204th (Tymine instead of Cytosine) base positions. Addition to these substitution there was an indel at 532nd base (Guanine). Although generally the informative data on substitutions provided phylogenetic information within species, all these were also useful to determine phylogenetic relationships among sections; because *A. vaginans* was the only studied species in *Hymenocoleous* section.

Furthermore, *A. wagneri, A. zederbaueri, A. velenowskyii* had substitution at 1156th base position (Adenine instead of Guanine), 531st -535th base position (Guanine), 596th -602nd base position (Tymine) and between 746th-752nd base positions (Adenine).

Although there were substitutions in the *mat*K gene region at species level, there were also insertions and deletions at *Astragalus* section level. For example, species of *Poterion* had deletions at 553rd base position and substitutions at 388th (Cytosine instead of Tymine), 531st (Cytosine instead of Adenine), and 1156th (Adenine instead of Guanine) base positions.

Overall GC content was 31% while transition and transversion were 42.27 and 57.73 respectively. Although total length of the sequences of the *mat*K gene region were higher than *trn* regions, R value was 0.625. As results of transitions in both pyrimidines and purines, the R value of *Hymenostegis* section was 8.86 (highest one). On the other hand, due to no substitution in *Megalocystis* section, R value was the lowest (0).

Table 5.4 Estimated molecular diversity parameters regarding the *matK* region of cpDNA in *Astragalus* sections.

	Astragalus sections						
	Halicacabus	*Megalocyctis*	*Hymenocoleous*	*Hymenostegis*	*Macrophyllium*	*Poterion*	Total
Number of species	7	3	1	11	6	2	30
Total length(bp)	1225	1225	1225	1225	1225	1225	1225
GC content(%)	31	31	31.2	31	30.9	30.8	31
Conserved sites	1202	1205	1206	1200	1201	1205	1188
Variable sites	6	1	0	9	4	0	23
Parsimony informative sites	6	1	0	9	4	0	23
Transitional pairs	53.7	0	33.33	91.49	34.44	33.33	42.27
Transversional pairs	46.28	100	66.66	8.51	65.56	66.66	57.73
Transition/transversion (tr/tv)(R)ratio	1.00	0	0.43	8.86	0.43	0.43	0.63
Number of deletion	1	0	0	5	1	1	8
Number of insertion	2	1	0	3	2	0	8

5.2.1.5 ITS (Internal transcribed spacer) region of genomic DNA

In addition to cpDNA regions, ITS region of nDNA was also examined in the current study. ITS1, 5.8S and ITS2 were amplified together by using one primer pair. The approximate length of region was determined by comparing with two ITS region of other *Astragalus* species that were available in NCBI database. At the beginning, all parameters were calculated for each studied section and then they were calculated for whole studied samples. After all, to comprehend that which sub region of ITS region was more diverse, all molecular diversity parameters were calculated for each sub region separately (Table 5.5).

According to studied sections of *Astragalus* in the current study, total length of combined region of ITS was found to be as 615 bp. Overall variable sites were 49 which were parsimony informative. The most variable section was *Halicacabus* section. These variable sites were the main reason causing to phylogenetic differentiation among sections. Comparing to cpDNA regions, the variable sites were very high in this region. Although this region was more variable than cpDNA regions, variation was mostly at species level. For example, *A.trifoliastrum* was quite distinct to other *Hymenostegis* section species. At the 137th base position, there was a substitution on Adenine instead of Tymine. Moreover, *A.zederbaueri and A.surugensis* had a substitution, at 66th base position and an insertion (Cystosine) at the 534th base position. There were also substitutions at section level. For instance, species of *Poterion* section had substitutions at the 136th (Cytosine instead of Adenine), 401st (Tymine instead of Adenine), 412th (Adenine instead of Guanine), 465th (Cytosine instead of Tymine) and 479th (Guanine instead of Tymine) base positions. Also, species of *Hymenocoleous* had substitutions at the 480th (Guanine instead of Adenine) and 553rd (Adenine instead of Guanine) base positions.

Moreover, there were not so much differences among sections regarding to amount of GC content. Overall GC content was found as 53. 9%. Transition and transversion bias values were 60 and 30 respectively. The R value was 1.48 which was higher than the studied cpDNA regions due to richness of Guanine and Cytosine bases.

Table 5.5 Estimated overall molecular diversity parameters of ITS regions of genomic DNA in studied *Astragalus* sections

	Astragalus sections						
	Halicacabus	*Megalocyctis*	*Hymenocoleous*	*Hymenostegis*	*Macrophyllium*	*Poterion*	Total
Number of species	7	3	1	11	6	2	30
Total length(bp)	615	615	615	615	615	615	615
GC content(%)	53.9	54	54	53.9	53.9	53.6	53.9
Conserved sites	575	586	600	583	584	600	553
Variable sites	25	14	0	17	16	0	49
Parsimony informative sites	9	0	0	8	6	0	49
Transitional pairs	49.69	57.73	33.33	69.84	67.82	33.34	60
Transversional pairs	50.31	42.27	66.67	30.16	32.18	66.66	30
Transition/transversion (tr/tv)(R)ratio	0.98	1.35	0.50	2.28	2.07	0.50	1.48
Number of deletion	0	0	0	0	0	0	0
Number of insertion	0	0	0	0	0	0	0

Length of ITS 1 and ITS 2 regions were closed to each other, but as it is expected, and it was longer than 5.8S region (Table5.5). When the parameters were calculated separately for each region of ITS, it could be easily seen that the 5.8S region was much more conserved compared to the other regions of ITS. There were only 1 variable site. It could be safe to say that 5.8S region is a transcribed region that is responsible for producing 5.8S rRNA. Therefore, it is more conserved than other intronic regions. Intronic sub regions had large amount of insertions and deletions. Generally, these variations become highly useful for phylogenetic analysis. However, in this study, there were no indels in studied species of *Astragalus* genus.

Table 5.6 Estimated molecular diversity parameters based on ITS sub regions of genomic DNA in studied *Astragalus* sections

	***Astragalus* sections**																				
	Halicacabus			***Megalocyctis***			***Hymenocoleous***			***Hymenostegis***			***Macrophyllium***			***Poterion***			**Total**		
	ITS1	**5.8S**	**ITS2**	**ITS1**	**5.8S**	**ITS2**	**ITS1**	**5.8S**	**ITS2**	**ITS1**	**5.8S**	**ITS2**	**ITS1**	**5.8S**	**ITS2**	**ITS1**	**5.8S**	**ITS2**	**ITS1**	**5.8S**	**ITS2**
Number of species	7	**7**	7	3	**3**	3	1	**1**	1	11	**11**	11	6	**6**	6	2	**2**	2	30	**30**	30
Total length(bp)	213	**164**	234	213	**164**	234	213	**164**	234	213	**164**	234	213	**164**	234	213	**164**	234	213	**164**	234
GC content(%)	55.9	**51.8**	52.4	57.1	**51.6**	53.1	57.8	**51.8**	52.2	57.2	**51.8**	52.8	56.9	**51.8**	52.8	56.3	**51.8**	52.6	57.1	**51.7**	52.7
Conserved sites	194	**164**	217	199	**163**	224	206	**164**	230	193	**164**	227	196	**164**	224	206	**164**	230	179	**163**	209
Variable sites	12	**0**	13	7	**1**	6	0	**0**	0	13	**0**	3	10	**0**	6	0	**0**	0	27	**1**	21
Parsimony informative sites	5	**0**	4	0	**0**	0	0	**0**	0	4	**0**	3	1	**0**	5	0	**0**	0	27	**1**	21
Transitional pairs	57.9	**33.3**	41.8	57.8	**99.6**	87.0	33.3	**33.3**	33.3	62.4	**33.3**	66.5	73.0	**33.3**	69.7	33.3	**33.3**	33.3	63.48	**99.52**	54.74
Transversional pairs	42.1	**66.7**	58.2	42.2	**0.4**	13.0	66.7	**66.7**	66.7	37.6	**66.7**	33.5	27.0	**66.7**	30.3	66.7	**66.7**	66.7	36.52	**0.38**	45.26
Transition/transversion (tr/tv)(R)ratio	1.37	**0.51**	0.72	1.38	**289.3**	6.62	0.49	**0.51**	0.49	1.67	**0.51**	1.97	2.72	**0.51**	2.52	0.51	**0.51**	0.51	1.73	**289.2**	1.19
Number of deletion	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0
Number of insertion	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0

5.2.2 Genetic Divergence Among studied *Astragalus* Sections and Species

In the current study, minimum 3 specimens for each species were used in the analyses. Not surprisingly, identical sequences were obtained from each replicas. Both genetic divergence among species and among sections were obtained for studied cp and nr DNA regions. (Table 5.7). Kimura 2-parameter (Kimura M., 1980) model was used to get genetic divergence parameters (genetic divergence among sections and overall genetic divergence between species).

According to sequences of *trn*L5'-L3' region, studied sections were so closed to each other and parameters were too small. However, results clearly indicated genetic divergence pattern among studied *Astragalus* sections. *Poterion* section was the most divergent section among studied sections. Particularly, genetic divergence was greater between *Megalocystis* and *Poterion* sections than (0.0135) others. Generally, genetic divergence parameters values between *Halicacabus* and *Hymenocoleous* sections were lower than other studied sections. Therefore, it can be safe to say that these sections were closed to each other. However, other studied *Astragalus* sections did not divergence much. It could be said that morphological divergences among sections were not supported by the sequence diversity of the non-coding DNA region.

*trn*L3'-F region was the extensition part of the trn L5'-3' region. It was shorter than all other studied regions. However, it was higly informative. Genetic divergence values among sections were found to be high for *trn* L5'-3' region. Genetically, *Hymenocoleous* section was the most diverged section among 6 *Astragalus* sections. Moreover, *Poterion* section was again one of the most divergent sections to other studied sections. On the contrary to most divergent sections, the closest sections were *Halicacabus* and *Megalocystis* sections. It appears that, the thorny stem structure of *Halicacabus* was not useful to determine phylogenetic relationship among these two sections.

The sequence analysis of *trn*V region did not yield results which are similar to previous *trn* regions. Only again, *Poterion* section was the most divergent section among studied sections. Especially between *Poterion* and *Halicacabus* sections, the genetic divergence was the highest (0. 0091). On the other hand, the closest sections were found to be as *Hymenostegis*, *Megalocystis* and *Hymenocoleous* sections. Consequently, while determining the phylogenetic relationships among sections according to *trn* V region, the results were not parallel to those obtained from other studied *trn* regions.

When data from, all *trn* regions were combined and analyzed, results indicated that most divergent sections was *Poterion* section again. Moreover, genetic divergence between *Poterion* and *Megalocystis* sections was the highest (0. 0206). On the other hand, the closest sections were *Halicacabus* and *Megalocystis* sections (0. 0045).

The last studied cpDNA region was *mat*K, which is a coding region. Although *mat*K was the longest region in the current study, the genetic divergence power among sections were not so high. The most divergent section was again *Poterion* section. The genetic divergence value between *Poterion* and *Hymenocoleous* sections was 0. 0059. Contrarily, the closest sections were *Hymenostegis*, *Macrophyllium* and *Halicacabus*. Although, *mat*K was the longest and coding region in the current study, this region did not allow a clear separation among sections. Therefore; when the results compared with t*rn*L-F region divetsity parameters, the length of the region did not seem to be important for the phylogenetic separations.

Lastly, the results of ITS region indicated that most divergent section was again *Poterion* section. Especially, the divergence between *Halicacabus* and *Poterion* section was high (0.0344). Moreover, *Hymenocoleous* and *Hymenostegis* sections were the closest sections according to studied genomic DNA region (0.0101). Other values were also compatible with cpDNA regions, so genetic divergence among sections were clearly estimated by using ITS region.

Consequently, genetic divergence results of both chloroplast and genomic DNA regions revealed compatible results. *Poterion* section was the most divergent section regardless of cp and nDNA regions. Moreover, even though *Hymenocoleous* section was close to *Hymenostegis* section, two section was diverged from each others. On the other hand, *Halicacabus* and *Megalocystis* sections were found to be close ones. For that reason, in the phylogenetic tree, position of these 2 sections was expected to be placed closed to each other based on these genetic divergence parameters.

In addition to estimation of genetic divergence among sections, genetic divergence among species of the genus *Astragalus* were calculated as well. In the table 5.8, only the most divergent species for each section and for each studied region were given. Moreover, overall mean divergences were shown for each regions as well.

Table 5.7 Genetic divergence among sections based on both chloroplast and genomic DNA regions

	Halicacabus	*Megalocystis*	*Poterion*	*Macrophyllium*	*Hymenostegis*	*Hymenocoleous*	Region
Halicacabus							*trncd*
Megalocystis	0.0045						
Poterion	0.0108	0.0135					
Macrophyllium	0.0024	0.0052	0.0115				
Hymenostegis	0.0024	0.0036	0.0115	0.0031			
Hymenocoleous	0.0009	0.0036	0.0099	0.0016	0.0015		
Halicacabus							*trnef*
Megalocystis	0.0042						
Poterion	0.0399	0.0399					
Macrophyllium	0.0227	0.0227	0.0260				
Hymenostegis	0.0475	0.0475	0.0282	0.0344			
Hymenocoleous	0.0481	0.0481	0.0078	0.0341	0.0350		
Halicacabus							*trnv*
Hymenocoleous	0.0013						
Hymenostegis	0.0015	0.0002					
Macrophyllium	0.0019	0.0005	0.0007				
Megalocystis	0.0013	0	0.0002	0.0005			
Poterion	0.0091	0.0080	0.0082	0.0086	0.0080		

Table 5.7 (Cont'd): Genetic divergence among sections based on both chloroplast and genomic DNA regions

	Halicacabus	*Megalocystis*	*Poterion*	*Macrophyllium*	*Hymenostegis*	*Hymenocoleous*	Region
Halicacabus							*matK*
Megalocystis	0.0017						
Poterion	0.0029	0.0036					
Macrophyllium	0.0016	0.0019	0.0033				
Hymenostegis	0.0013	0.0016	0.0029	0.0013			
Hymenocoleous	0.0040	0.0028	0.0059	0.0041	0.0037		
Halicacabus							ITS
Megalocystis	0.0152						
Poterion	0.0344	0.0278					
Macrophyllium	0.0170	0.0139	0.0269				
Hymenostegis	0.0141	0.0116	0.0302	0.0124			
Hymenocoleous	0.0142	0.0146	0.0342	0.0155	0.0101		

Table 5.8 Genetic divergence between species within each section of *Astragalus* genus using five different DNA regions

Sections	The most divergent species within sections and combined data			Overall mean divergence	Region
Halicacabus	*A.mesites*	*A.chardini*	0.0063	0.0017	*trn cd*
Macrophyllium	*A.longifolious-*	*A.isauricus*	0.0063	0.0025	
		A.yukselii	0.0063		
Megalocyctis	*A.ermenious-*	*A.szowitsii*	0.0063	0.0034	
Hymenostegis	*Several combinations*		0.0063	0.0019	
Poterion	*A.russelii*	*A.brugieri*	0.0193	0.0116	
Hymenocoleous					
overall	*A.brugueri*	*A.szowitsii*	0.0260	0.0040	
		A.micracme	0.0260		
		A.isauricus	0.0260		
		A.yukselii	0.0260		
		A.mesites	0.0260		
Halicacabus	*A.chardini-*	*A.halicacabus*	0.0153	0.0064	*trn ef*
		A.zederbaueri	0.0153		
		A.anthylloidei	0.0153		
Macrophyllium	*A.cephalotes*	*A.yukselii*	0.0305	0.0114	
Megalocyctis	-			-	
Hymenostegis	*A.trifoliastrum*	*Several species*	0.0386	0.0082	
Poterion	-			-	
Hymenocoleous	-			-	
overall	*Several combinations*		0.0481	0.0282	

Table 5.8 (Cont'd) Genetic divergence between species within each section of *Astragalus* genus using five different DNA regions

Sections	The most divergent species within sections and combined data			Overall mean divergence	Region
Halicacabus	*A.anthylloides*	*A.wagneri*	0.0099	0.0029	*trnV*
		A.zederbaueri	0.0099		
Macrophyllium	Several combinations		0.0039	0,0018	
Megalocyctis	*A.ermenious* -	*A.micracme*	0.0019	0.0008	
		A.szowitsii	0.0019		
Hymenostegis	Several combinations		0.0019	0.003	
Poterion	-			-	
Hymenocoleous	-			-	
overall	*A.anthylloides*	*A.brugueri*	0.0161	0.0018	
		A.russelii	0.0161		
Halicacabus	Several combinations		0.0025	0.0014	*matK*
Macrophyllium	*A.yukselii*	*A.diphodurus*	0.0020	0.0007	
Megalocyctis	*A.ermenious-* -	*A.micracme*	0.0008	0.0004	
		A.szowitsii	0.0008		
Hymenostegis	*A.hirticalyx*	*A.velenowski*	0.0068	0.0010	
Poterion	-			-	
Hymenocoleous	-			-	
overall	*A.hirticalyx*	*A.diphodurus*	0.0084	0.0017	
		A.brugueri-	0.0084		
		A.russelii-	0.0084		

Table 5.8 (Cont'd) Genetic divergence between species within each section of *Astragalus* genus using five different DNA regions

Sections	The most divergent species within sections and combined data			Overall mean divergence	Region
Halicacabus	*A.mesites*	*A.surugensis*	0.0272	0.0148	ITS
Macrophyllium	*A.cephalotes*	*A.diphodurus*	0.0221	0.0109	
		A.oleaefolious	0.0221		
Megalocyctis	*A.ermenious*	*A.micracme*	0.0237	0.0126	
Hymenostegis	*A.guruensis*	*A.sosnowsyki*	0.0204	0.0088	
Poterion	-			-	
Hymenocoleous	-			-	
overall	*A.chardini*	*Arusselii*	0.0376	0.0152	
		Abrugueri	0.0376		

After assembling all sequences of studied samples, genetic divergence among species within each section was analyzed. Even if the genetic divergence among sections were obtained similarly in every studied regions, the most distant species or closer species were changed based on studied cp and nr DNA regions. These results suggest that, for determining phylogenetic relationship at species level, none of the studied region alone was fully sufficient to describe the exact placemant of species in phylogenetic tree.

According to *trnL* intron region, overall genetic diversity which also means that nucleotide diversity between species is a measure of polymorphism in a sample of gene sequences. Also it is a summary statistic used to represent patterns of molecular diversity within a sample of gene copies (Nei, 1987) and estimated as 0.0040. Based on genetic divergence between species, *A.brugueri-A.szowitsii, A.brugueri-A.micracme, A.brugueri-A.isauricus, A.brugueri-A.yukselii,* and *A.brugueri-A.mesites* were the most divergent combinations (0.0260). Also, *A.brugueri* is a species of *Poterion* section. These results were similar to the section based results regarding *Poterion* section which was the most divergent section. Therefore, it is expected that *Poterion* section will be located in different clade in the phylogenetic tree.

According to *trn* L-F region of cpDNA, overall mean divergence was 0.0282. Although this region was the shortest one among studied regions, high genetic divergences between species were found. For example, *A.trifoliastrum* from *Hymenostegis* section was the most diverged species (0.0386). Species of *Poterion* section were diverged away from other species of studied *Astragalus* section.

When the other studied *trn* region (*trn*V) was analyzed, overall mean divergence was estimated as 0.0018 which is lower than in other *trn* regions. However, like other *trn* diversity parameters, species of *Poterion* section (*A.brugueri* and *A.russelii*) were the most divergent species among all studied species. Furthermore, *A.anthylloides* and *A.brugueri* -A.*russelii* (0.0161) were the most divergent combinations between studied samples.

The combination data from all 3 *trn* regions were analyzed to get further insight in the genetic divergence among species. The overall mean divergence was calculated as 0.0060. It was found that most divergent species were *A.trifoliastrum* and *A.brugueri* (0.0220). This means that species *A.brugueri* from *Poterion* section and species *A.trifoliastrum* from *Hymenostegis* section are expected to be positioned in a different clades in constructed phylogenetic tree.

The last cpDNA region was *mat*K region. Overall mean divergence was calculated as 0.0017. The most divergent species combinations were *A.diphodurus-A.hirticalyx* (0.0084), *A.brugueri-A.hirticalyx* (0.0084) and *A.russelii-A.hirticalyx* (0.0084). *A.hirticalyx* was the most divergent species among the studied species. Moreover, species of *Poterion* section were again diverged and expected to be in a separated clade in phylogenetic tree constructed with the data from *mat*K region.

Based on studied genomic DNA region, ITS, overall mean divergence was estimated as 0.0152. *A.chardini* from *Halicacabus* section was the most diverged species. The genetic divergence between *A.chardini-A.russelii* (0.0376) and *A.chardini-A.brugueri* (0.0376) were the highest regarding to this region.

According to the results from chloroplast and genomic DNA regions, genetic divergence between species were variable depending on studied regions. Although the divergence values were different among species level, overall result indicated that species of *Poterion* section had the most divergent species. Moreover, A.*trifoliastrum, A.chardini and A.hirticalyx* species had also highly diverged from species of their sections. Based on these divergence results, it is expected that these distantly related species will be located at different subclades in the constructed phylogenetic trees.

5.2.3 The phylogenetic tree construction by using the sequence data from regions of cpDNA and nDNA

In addition to estimation of genetic divergence and parameters, phylogenetic trees were constructed using data from each region for all studied sections with the MEGA 7.0 software. For each tree, only one sample from each species was used for constructing phylogenetic trees since sequences within speces did not vary.

5.2.3.1 Phylogenetic tree construction by using sequence data from studied cpDNA regions

Several substitutions and deletions/insertions were found in the align sequences of *trn*L intron, *trn* L-F intergenic specer, *trn* V and *matK* gene region of studied *Astragalus* species. In the Tables 5.9, 5.10, 5.11, 5.12 nucleotide deletions, nucleotide insertions were given respectively.

In *trn*L intron, there was a specific deletion with 7 base pairs in length at the positions from 230th to 236th bases in *Poterion* section species (Table5.9). *A.mesites* species had 2 different insertions with 4 base pairs in length at the position between 247th - 250th and 453rd -456th bases. At the 110th base position there was a missing nucleotide in *Megalocystis* section and that caused separation of this section from other sections.

Table 5.9 : The position of indels and nucleotide substitutions in *trn*L intron region of cpDNA in *Astragalus* species

Nucleotide Positions in Aligned DNA sequences of trnL Intron Region																																	
Astragalus		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	3	4	4	4	4
Sections	Species	0	0	1	4	5	5	5	6	6	6	6	7	7	7	8	3	3	3	3	3	3	3	4	4	4	5	6	5	5	5	5
		8	9	0	4	7	8	9	5	6	7	8	7	8	9	4	0	1	2	3	4	5	6	7	8	9	0	0	3	4	5	6
Halicacabus	*A.zederbaueri*	-	-	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.wagneri	-	-	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.surugensis	-	-	-	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.anthylloides	-	-	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.chardini	-	-	-	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.mesites	-	A	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	A	T	A	T	G	A	A	A	T
	A.halicacabus	-	-	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
Megalocystis	*A.szowitsii*	-	-	-	-	A	A	T	T	G	A	C	A	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.ermenicus	-	-	-	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	A	-	-	-	-
	A.micracme	-	-	-	-	A	A	T	T	G	A	C	A	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
Poterion	*A.bruguieri*	-	A	A	G	T	G	-	G	A	C	C	C	A	T	T	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-
	A.russelli	-	A	A	-	A	A	T	T	G	A	C	C	A	-	C	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-
Macrophyllium	*A.cephalotes*	-	A	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.dipodurus	-	-	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.isauricus	-	A	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.longifolius	A	A	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.oleaefolius	-	-	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.yukselii	-	A	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
Hymenostegis	*A.lagopoides*	-	A	A	-	A	A	T	T	G	A	C	A	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.brachypodus	A	A	A	-	A	A	T	T	G	A	C	A	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.ciloensis	-	A	A	-	A	A	T	T	G	A	C	A	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.hymenocystis	-	A	A	-	A	A	T	T	G	A	C	A	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.hirticalyx	-	A	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.sosnowskyi	A	A	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.trifoliastrum	-	A	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.gueruenensis	-	A	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.uraniolimneus	-	A	A	-	A	A	T	T	G	A	C	A	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.velenovskyi	A	A	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
	A.zohrabi	A	A	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-
Hymenocoleous	*A.vaginans*	-	A	A	-	A	A	T	T	G	A	C	C	A	-	C	A	A	G	G	A	T	C	-	-	-	-	G	-	-	-	-

Although the sequences of *trn*L-F region was the shortest region in the current study, it has relatively high informative sites compared to the other longer cpDNA regions. It was approximately 145bp in length. The deletions between 56th and 59th base positions were present that could be the reason for the separation of *Poterion* section from the other sections. There were additional substitutions from 15th to 152nd base positions contributed in the formation of the clades in the phylogenetic tree (Table 5.10). *Megalocystis* and *Halicacabus* sections were separated by the substitution at the 15th base position (C/A).

Table 5.10 : The position of indels and nucleotide substitutions in *trn*L-F region of cpDNA in *Astaragalus* species

Nucleotide Positions in Aligned DNA sequences of trnL-F Region																		
Astragalus																	1	1
Sections	Species	1 5	1 9	2 4	2 8	3 1	3 9	4 3	4 7	4 9	5 4	5 5	5 6	5 7	5 8	5 9	4 1	5 2
Halicacabus	*A.halicacabus*	C	A	C	A	C	T	A	T	T	T	C	T	T	T	T	G	-
	A.chardini	C	A	C	A	C	T	A	C	T	T	T	T	T	T	T	G	-
	A.surugensis	C	A	C	A	C	T	A	T	T	T	C	T	T	T	T	G	-
	A.mesites	C	A	C	A	C	T	A	T	T	T	T	T	T	T	T	G	-
	A.wagneri	C	A	C	A	C	T	A	C	T	T	C	T	T	T	T	G	-
	A.anthylloides	C	A	C	A	C	T	A	T	T	T	C	T	T	T	T	G	-
Hymenostegis	*A.zohrabi*	A	C	T	A	T	T	A	C	A	T	C	C	T	T	T	G	-
	A.lagopoides	A	C	T	A	T	T	A	C	A	T	C	C	T	T	T	G	-
	A.velenovskyi	A	C	T	A	T	T	A	C	A	T	C	C	T	T	T	G	-
	A.sosnowskyi	A	C	T	A	T	T	A	C	A	T	C	C	T	T	T	G	-
	A.uraniolimneus	A	C	T	A	T	T	A	C	A	T	C	C	T	T	T	G	-
	A.gueruenensis	A	C	T	A	T	T	A	C	A	T	C	C	T	T	T	G	-
	A.trifoliastrum	A	G	T	T	T	T	A	C	T	T	C	C	T	T	T	T	-
	A.hymenocyctis	A	C	T	A	T	T	A	C	T	T	C	C	T	T	T	G	-
	A. hirticalyx	A	C	T	A	T	T	A	C	T	T	C	C	T	T	T	G	-
	A.ciloensis	A	C	T	A	T	T	A	C	T	T	C	C	T	T	T	G	-
	A.brachypodus	A	C	T	A	T	T	A	C	A	T	C	C	T	T	T	G	-
Macrophyllium	*A.oleaefolius*	A	A	C	A	C	T	T	C	T	T	C	T	T	T	T	G	G
	A.dipodurus	A	A	C	A	C	T	T	C	T	T	C	T	T	T	T	G	G
	A.yukselii	A	A	C	A	T	A	A	C	T	T	C	C	T	T	T	G	G
	A.isauricus	A	A	C	A	C	T	A	C	A	T	C	T	T	T	T	G	G
	A.cephalotes	A	A	C	A	C	T	T	C	T	T	C	T	T	T	T	G	G
	A.longifolius	A	A	C	A	C	T	A	C	T	T	C	T	T	T	T	G	G
Megalocystis	*A.micracme*	C	A	C	A	C	T	A	T	T	T	C	-	T	T	T	G	-
	A.ermenicus	C	A	C	A	C	T	A	T	T	T	C	-	T	T	T	G	-
	A.szowitzii	C	A	C	A	C	T	A	T	T	T	C	-	T	T	T	G	-
Poterion	*A.russelli*	A	A	C	A	T	T	A	C	A	C	C	-	-	-	-	G	G
	A.bruguieri	A	A	C	A	T	T	A	C	A	C	C	-	-	-	T	G	G
Hymenocoleous	*A.vaginans*	A	A	C	T	T	T	A	C	A	C	C	-	C	T	T	G	G

Even though *trn* V region size was long, the genetic diversity within sections was quite low and it was nearly same between species of studied sections. Table5.11 indicates the indels and substitions among species of studied *Astragalus* sections. Particularly, only there were deletions between 396th and 401st base positions on the species of *Poterion* section. Moreover, at the base position 386, there was a substitution between A/T. Also, *A.dipodurus* and *A.oleaefolius* from *Macrophyllium* section had substitution at the 22nd base position.

Table 5.11: The position of indels and nucleotide substitutions in trnV region of cpDNA in *Astragalus* species

Nucleotide Positions in Aligned DNA sequences of trnV Intron Region																				
Astragalus			2	2	2	2	2	2	2	2	3	3	3	3	3	3	4	4	4	5
Sections	Species	2	4	4	4	4	4	4	4	5	8	9	9	9	9	9	0	0	3	0
		2	1	2	3	4	5	6	7	5	6	5	6	7	8	9	0	1	2	6
Halicacabus	*A.anthylloides*	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
	A.chardini	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
	A.halicacabus	G	-	-	-	-	-	-	-	A	A	T	A	A	T	C	T	A	C	A
	A.mesites	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
	A.surugensis	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
	A.wagneri	G	T	A	T	T	T	T	A	-	A	T	A	A	T	C	A	A	C	A
	A.zederbaueri	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
Hymenostegis	*A.cephalotes*	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
	A.ciloensis	G	T	A	T	T	T	T	A	T	A	T	A	A	T	C	T	A	C	A
	A.hirticalyx	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
	A.hymenocystis	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
	A.lagopoides	G	T	A	T	T	T	T	A	T	A	T	A	A	T	C	T	A	C	A
	A.brachypodus	G	T	A	T	T	T	T	A	T	A	T	A	A	T	C	T	A	C	A
	A.sosnowskyi	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
	A.trifoliastrum	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
	A.uraniolimneus	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
	A.velenovskyi	G	T	A	T	T	T	T	A	-	A	T	A	A	T	C	T	A	C	A
	A.zohrabi	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
	A.gueruenensis	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
Macrophyllium	*A.dipodurus*	T	T	A	T	T	T	T	A	T	A	T	A	A	T	C	T	A	C	A
	A.isauricus	G	T	A	T	T	T	T	A	T	A	T	A	A	T	C	T	A	C	A
	A.longifolius	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
	A. oleifolious	T	T	A	T	T	T	T	A	T	A	T	A	A	T	C	T	A	C	A
	A.yukselii	G	T	A	T	T	T	T	A	T	A	T	A	A	T	C	T	A	C	A
Megalocyctis	*A.ermineus*	G	T	A	T	T	T	T	A	-	A	T	A	A	T	C	A	A	C	A
	A..micracme	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
	A.szowitsii	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	A
Poterion	*A.russelii*	G	T	A	T	T	T	T	A	A	T	T	-	-	-	-	-	-	T	A
	A.bruguieri	G	T	A	T	T	T	T	A	A	T	T	-	-	-	-	-	-	T	A
Hymenocoleous	*A.vaginans*	G	T	A	T	T	T	T	A	A	A	T	A	A	T	C	T	A	C	-

In the current study, not only non-coding regions but also coding regions of cpDNA was studied in order to comprehend molecular phylogenetic relationships within sections and between species of *Astragalus* genus. The *mat*K gene region is one of the coding region located in cpDNA which starts with a 'ATG' start codon and finishes with 'TGA' stop codon. Generally, it is approximately 1500bp in length. However, in the current study, it was amplified as 1225 bp. Remaining part of the region could not be amplified due to challenges and low variations in sequences.

Because of being the longest studied region in the current study, it was expected that *mat*K region had more substitutions than other regions. However, the number of nucleotide substitutions were not higher than the other studied regions. Insertions at the 392nd, 452nd, 534th base positions were more found in *A.chardini*. Also, *A.hirticalyx* had both insertions at 515th, 529th and 534th base positions, and deletions at 581st and 599th base positions. Substitutions at 142nd, 292nd, 480th, 516th, 555th, 557th, 635th, 751st, 973rd and 1198th base positions could be useful for the separations of sections in the current study (Table5.12)

Table 5.12: The position of indels and nucleotide substitutions in *matK* region of cpDNA in *Astragalus* species

Nucleotide Positions in Aligned DNA sequences ofmatK gene Region																								
Astragalus		109	142	292	392	452	480	515	516	517	529	534	551	555	557	581	599	627	653	749	751	973	1198
Sections	Species																							
Halicacabus	*A.halicacabus*	A	G	G	-	-	G	-	T	T	-	G	T	T	T	A	T	T	T	-	A	C	T
	A.mesites	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	T
	A.zederbaueri	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	T
	A.chardini	-	G	G	T	C	G	-	T	T	-	G	T	T	T	A	-	-	T	-	A	T	T
	A.anthylloides	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	-	T	-	C	T	T
	A.surugensis	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	G
	A.wagneri	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	T
Macrophyllium	*A.cephalotes*	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	T
	A.dipodurus	A	G	T	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	T
	A.oleaefolius	A	G	T	-	-	G	-	T	T	-	-	T	T	T	A	-	T	T	-	A	T	T
	A.isauricus	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	T
	A.yukselii	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	T
	A.longifolious	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	T
Megalocystis	*A.micracme*	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	G
	A.szowitsii	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	G
	A.ermineus	A	T	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	G
Hymenostegis	*A.lagopoides*	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	T
	A.brachypodus	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	T
	Ahymenocystis	A	G	G	-	-	G	-	C	T	-	-	T	T	T	-	T	T	T	-	A	T	T
	A.velenovskyi	A	G	G	-	-	G	-	C	T	-	-	T	T	T	A	T	T	T	-	A	T	T
	A.trifoliastrum	A	G	G	-	-	G	-	-	-	-	-	T	T	T	A	T	T	T	A	A	T	T
	A.zohrabi	A	G	G	-	-	G	-	C	T	-	-	T	T	T	A	T	T	T	A	A	T	T
	A.sosnowskyi	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	A	A	T	T
	A.uraniolimneus	A	G	G	-	-	G	-	C	T	-	-	T	T	T	A	T	T	T	A	A	T	T
	A.hirticalyx	A	G	G	-	-	G	T	T	T	T	G	T	G	A	-	-	T	G	A	A	T	T
	A.ciloensis	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	T
	A.gueruenensis	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	T
Poterion	*A.bruguieri*	A	G	G	-	-	G	-	T	T	-	-	-	T	T	A	T	T	T	-	A	T	T
	A.russelii	A	G	G	-	-	G	-	T	T	-	-	T	T	T	A	T	T	T	-	A	T	T
Hymenocoleous	*A.vaginans*	A	G	G	-	-	A	-	T	T	-	G	T	T	T	A	T	T	G	-	A	T	G

As a result of these analysis phylogenetic trees were constructed with bootstrap values by using the MEGA software. The Kimura 2-parameter method was used. Figure 5.2 indicates the phylogenetic relationships among sections and between species of the genus *Astragalus*, by using the combined data from cpDNA regions. The sequences of *Cicer anatolicum* was obtained from the NCBI databank and used as outgroup to estimate the genetic similarities among studied *Astragalus* species. As indicated in Figure 5.2, there were two main clades in constructed phylogenetic tree. One of these clades composed of *Poterion*, *Hymenocoleous* and Hymenostegis sections while *Macrophyllium*, *Halicacabus* and *Megalocystis* sections formed the other main clade in the phylogenetic tree. *Hymenostegis* section formed a main branch under the one of the main clades. However, *A.trifoliastrum* positioned in different sub-branch than other species of Hymenostegis section with bootstrap value of 51. *A.hymenostegis*, *A.hirticalyx* and *A.ciloensis* formed a sub-clade together and separated from the other species of this section. *Poterion* section and *Hymenocoleous* sections together formed a different branch through *Hymenocoleous* section represented with one species separated from *Poterion* section and formed different sub-branch with a high bootstrap value.

The other main clade composed of three sections. One of the sub-clade composed of *A.isauricus* and *A.yukselii* from *Macrophyllium* section which were separated from other species of this section. Except *A.cephalotes*, *A.dipodurus* and *A.oleaeifolius*, the other species of *Macrophyllium* section dispersed in different sub-branches. Other than *A.wagneri*, all species of *Halicacabus* and *Megalocystis* section located in a single sub-branch. Although, *A.szowitzii* and *A. micracme* positioned in a different sub-branch, they closely positioned in the same branch with the species of *Halicacabus* section. Even though *A.chardini* and *A.mesites* formed a single sub-branch, they positioned close to *A.wagneri* from *Halicacabus* section and formed a single-sub branch together.

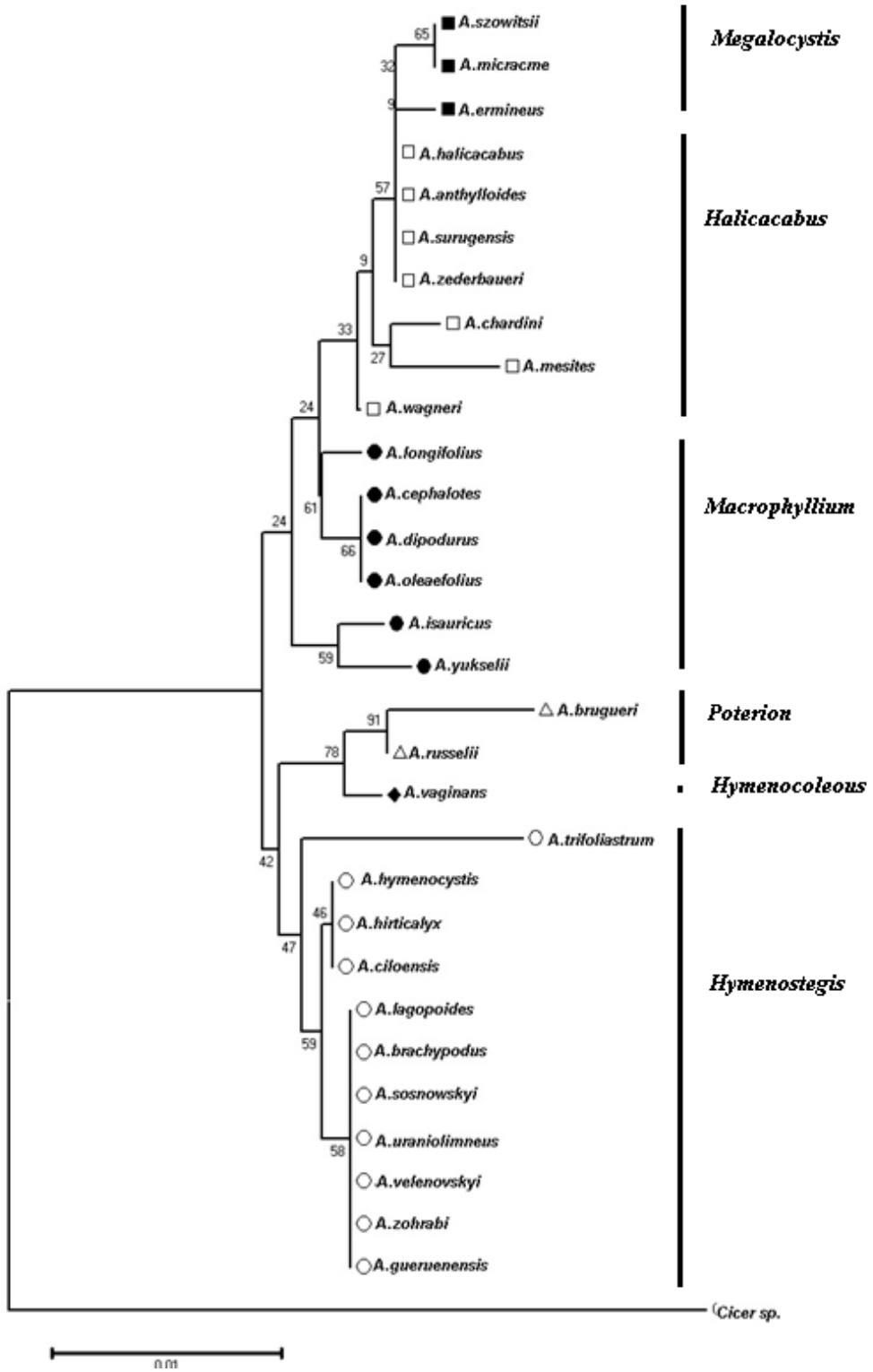

Figure 5.2. Phylogenetic tree, constructed using the sequences of studied cpDNA region, depicting relationships among the species of *Astragalus* (● Macrophyllium, □ Halicacabus, ○ Hymenostegis, ■ Megalocystis, ◊ Hymenocoleous, Δ Poterion sections).

5.2.3.2 Phylogenetic Tree Construction by Using Sequence Data of ITS (Internal Transcribe Spacer) Region

In order to compare the phylogenetic data from cpDNA regions, the ITS region of the nDNA was chosen and studied due to its high evolutionary rate, the amount of copy number in plant genomes.

The total length of ITS region was about 640bp. Variable sites in each sub regions (ITS-1, 5.8S, and ITS-2) varied considerably. In ITS-1 sub region there were no indels and deletions. However, *Poterion* section had several substitutions at the 31st (T/G), 33rd (G/A), 38th (G/A), 69th A/G), 89th C/T), 140th(C-T/A), 163rd(C/T) base positions. Also, some species of *Halicacabus* section had substitutions at the 64th (C), 68th (C) and 148th (A) base positions. The 5.8S sub region was very conservative. There was only one nucleotide substitution at the 4th base position. For example in some species of *Hymenostegis* section, Adenine was replaced Guanine base at this position (Table 5.13).

Table 5.13a Substitutions and indels in the DNA sequences of ITS1 subregion for each *Astragalus* species

Nucleotide Positions in Aligned DNA sequences of ITS -1 Region																									
Astragalus														1	1	1	1	1	1	1	1	1	1	1	2
Sections	Species	3 1	3 3	3 6	3 8	6 4	6 8	6 9	8 0	8 1	8 3	8 9	9 6	0 5	1 8	4 0	4 1	4 6	4 8	6 3	6 5	6 6	7 4	8 1	9 5	1 3
Halicacabus	*A.anthylloides*	T	G	C	G	C	T	A	A	G	T	C	G	C	C	C	T	T	C	C	T	C	C	G	T	C
Halicacabus	*A.chardini*	T	G	C	G	T	T	A	A	T	T	C	C	C	C	C	T	T	A	C	T	C	C	G	C	C
Halicacabus	*A.halicacabus*	T	G	C	G	T	T	A	A	G	T	C	G	C	C	C	A	T	C	C	T	C	C	G	T	C
Halicacabus	*A.mesites*	T	G	C	G	T	T	A	A	G	T	C	C	C	C	C	T	T	A	C	T	C	T	A	C	C
Halicacabus	*A.surugensis*	T	G	C	G	C	C	A	A	G	T	C	G	C	C	C	T	C	C	C	T	C	C	G	T	C
Halicacabus	*A.wagneri*	T	G	C	G	T	T	A	A	G	T	C	C	C	C	C	T	T	A	C	T	C	C	G	C	C
Halicacabus	*A.zederbaueri*	T	G	T	G	T	C	A	A	G	T	C	C	C	C	C	T	T	A	C	T	C	C	G	C	C
Hymenostegis	*A.brachypodus*	T	G	C	G	T	T	A	A	G	T	C	C	C	C	C	T	T	C	C	T	C	C	G	C	T
Hymenostegis	*A.ciloensis*	T	G	C	G	T	T	A	A	G	T	C	G	C	G	C	T	T	T	C	T	C	C	G	T	C
Hymenostegis	*A.hirticalyx*	T	G	C	G	T	T	A	A	G	T	C	C	C	C	C	T	T	C	C	T	C	C	G	C	T
Hymenostegis	*A.hymenocystis*	T	G	C	G	T	T	A	A	G	T	C	G	C	C	C	T	T	T	C	T	C	C	G	T	C
Hymenostegis	*A.lagopoides*	T	G	C	G	T	T	A	A	G	T	C	G	C	C	C	T	T	T	C	T	C	C	G	T	C
Hymenostegis	*A.sosnowskyi*	T	G	C	G	T	T	A	A	G	T	C	C	C	C	C	T	T	C	C	T	C	C	G	C	T
Hymenostegis	*A.trifoliastrum*	T	G	C	G	T	T	A	A	G	T	C	C	C	C	C	A	T	C	C	T	C	C	G	C	T
Hymenostegis	*A.gueruenensis*	T	G	C	G	T	T	A	C	G	G	C	C	T	C	T	T	T	C	T	C	C	C	G	T	C
Hymenostegis	*A.uraniolimneus*	T	G	C	G	T	T	A	A	G	T	C	G	C	C	C	T	T	C	C	T	C	C	G	C	C
Hymenostegis	*A.velenovski*	T	G	C	G	T	T	A	A	G	T	C	G	C	C	C	T	T	T	C	T	C	C	G	T	C
Hymenostegis	*A.zohrabi*	T	G	C	G	T	T	A	A	G	T	C	C	C	C	C	T	T	C	C	T	C	C	G	C	T

Table 5.13a(Cont'd): Substitutions and indels in the DNA sequences of ITS1 subregion for each Astragalus species.

Macrophyllium	*A.cephalotes*	T	G	C	G	T	T	A	C	G	G	T	C	T	C	T	T	T	C	T	C	C	C	G	T	T
Macrophyllium	*A.dipodurus*	T	G	C	G	T	T	A	A	G	T	C	G	C	C	C	T	T	T	C	T	C	C	G	T	C
Macrophyllium	*A.isauricus*	T	G	C	G	T	T	A	A	G	T	C	G	C	C	C	T	T	C	C	T	C	C	G	T	C
Macrophyllium	*A.longifolius*	T	G	C	G	T	T	A	A	G	T	C	G	C	C	C	T	T	C	C	T	C	C	G	T	C
Macrophyllium	*A.oleaefolius*	T	G	C	G	T	T	A	A	G	T	C	G	C	C	C	T	T	T	C	T	C	C	G	T	C
Macrophyllium	*A.yukselii*	T	G	C	G	T	T	A	A	G	T	C	G	C	C	C	T	T	C	C	T	C	C	G	T	C
Megalocystis	*A.ermineus*	T	G	C	G	T	T	A	A	G	T	C	C	C	C	C	T	T	A	C	T	C	C	G	C	C
Megalocystis	*A.micracme*	T	G	C	G	T	T	A	C	G	G	C	C	T	C	C	T	T	C	C	T	T	C	G	T	T
Megalocystis	*A.szowitsii*	T	G	C	G	T	T	A	A	G	T	C	C	C	C	C	T	T	C	C	T	C	C	G	T	C
Poterion	*A.brugueri*	G	A	C	A	T	T	G	C	G	G	T	C	T	C	A	T	T	C	T	T	C	C	G	T	C
Poterion	*A.russelii*	G	A	C	A	T	T	G	C	G	G	T	C	T	C	A	T	T	C	T	T	C	C	G	T	C
Hymenocoleous	*A.vaginans*	T	G	C	G	T	T	A	A	G	T	C	G	C	C	C	T	T	C	C	T	C	C	G	C	C

Table 5.13b: Substitutions and indels in the DNA sequences of 5.8S and ITS2 subregions for each *Astragalus* species.

Nucleotide Positions in Aligned DNA sequences of 5.8S& ITS -2 Regions																		
Astragalus			1	1	1	2	2	2	2	2	2	3	3	3	3	3	3	3
Sections	Species		8	9	9	0	0	1	3	6	7	2	3	3	3	5	5	6
		4	1	0	3	7	9	3	5	2	6	9	0	1	2	1	2	1
Halicacabus	*A.anthylloides*	G	-	A	G	C	T	C	G	C	G	G	C	T	-	A	T	C
Halicacabus	*A.chardini*	G	C	A	G	T	A	T	G	C	G	T	C	C	-	A	T	C
Halicacabus	*A.halicacabus*	G	-	A	G	C	T	C	G	C	G	G	C	T	-	A	T	C
Halicacabus	*A.mesites*	G	-	A	T	T	A	C	G	C	G	T	C	T	-	A	T	T
Halicacabus	*A.surugensis*	G	-	A	G	C	T	C	G	C	G	G	C	T	-	A	T	C
Halicacabus	*A.wagneri*	G	-	A	G	T	T	C	G	C	G	T	C	T	-	A	A	C
Halicacabus	*A.zederbaueri*	A	-	A	G	T	A	C	T	C	G	T	C	T	C	A	T	C
Hymenostegis	*A.brachypodus*	A	-	A	G	T	A	C	G	C	G	T	C	T	-	A	T	C
Hymenostegis	*A.ciloensis*	G	-	A	G	C	A	C	G	C	G	G	C	C	-	A	T	C
Hymenostegis	*A.hirticalyx*	A	-	A	G	T	A	C	G	C	G	T	C	T	-	A	T	C
Hymenostegis	*A.hymenocystis*	G	-	A	G	C	A	C	G	C	G	G	C	C	-	A	T	C
Hymenostegis	*A.lagopoides*	G	-	A	G	C	A	C	G	C	G	G	C	C	-	A	T	C
Hymenostegis	*A.sosnowskyi*	A	-	A	G	T	A	C	G	C	G	G	C	C	-	A	T	C
Hymenostegis	*A.trifoliastrum*	A	-	A	G	T	A	C	G	C	G	T	C	T	-	A	T	C
Hymenostegis	*A.gueruenensis*	G	-	A	G	C	A	C	G	C	G	G	C	T	-	A	T	C
Hymenostegis	*A.uraniolimneus*	G	-	A	G	T	A	C	G	C	G	T	C	T	-	A	T	C
Hymenostegis	*A.velenovski*	G	-	A	G	C	A	C	G	C	G	G	C	T	-	A	T	C
Hymenostegis	*A.zohrabi*		-	A	G	T	A	C	G	C	G	T	C	T	-	A	T	C

Table5.13b (Cont'd) : Substitutions and indels in the DNA sequences of 5.8S and ITS2 subregions for each *Astragalus* species

Macrophyllium	*A.cephalotes*	G	-	A	G	C	A	T	G	C	G	G	C	T	-	A	T	C
Macrophyllium	*A.dipodurus*	G	-	A	G	C	A	C	G	C	G	G	C	C	-	A	T	C
Macrophyllium	*A.isauricus*	G	-	A	G	C	T	C	G	C	G	G	C	T	-	A	A	C
Macrophyllium	*A.longifolius*	G	-	A	G	C	A	C	G	C	G	G	C	T	-	A	T	C
Macrophyllium	*A.oleaefolius*	G	-	A	G	C	A	C	G	C	G	G	C	C	-	A	T	C
Macrophyllium	*A.yukselii*	G	-	A	G	C	T	C	G	C	G	G	C	T	-	A	A	C
Megalocystis	*A.ermineus*	G	-	G	G	T	T	C	G	C	G	T	C	T	-	A	T	C
Megalocystis	*A.micracme*	A	-	A	G	C	A	C	G	C	G	G	C	C	-	A	T	C
Megalocystis	*A.szowitsii*	G	-	A	G	C	A	C	G	C	G	G	C	T	-	A	T	C
Poterion	*A.brugueri*	G	-	A	G	C	G	C	G	T	T	G	C	T	-	A	T	C
Poterion	*A.russelii*	G	-	A	G	C	G	C	G	T	T	G	C	T	-	A	T	C
Hymenocoleous	*A.vaginans*	G	-	A	G	T	A	C	G	C	G	T	C	T	-	G	T	C

As in ITS-1 sub-region, *Poterion* section had specific substitution at the 209th (A-T/G), 262nd (C/T) and 276th (G/T) base positions. Moreover, the substitutions at the 207th, 209th, 329th and 331st base positions were informative for the constructions of the phylogenetic tree. (Table5.13b)

According to the ITS region, the constructed phylogenetic tree yielded two main clades (Figure 5.3). *Poterion* section formed a branch under one of the main clades with a bootstrap value of 71. In other main clade, there were many sub-clades and branches. However, most of the *Hymenostegis* section species ended up in the same sub-clade. Interestingly, but not surprisingly, *A.oleaefolius* and *A.dipodurus* from *Macrophyllium* section located as close to *Hymenostegis* section and formed a branch under the same sub-clade like cpDNA regions. Furthermore, again like results from cpDNA regions, *Megalocystis* and *Halicacabus* sections were positioned close to each other. In addition to these results, also *Hymenocoleous* section formed close relationship with *Hymenostegis* section species, but it formed a single branch with *A.uraniolimneus*. Although, *A.trifoliastrum* from *Hymenostegis* section is generally located in a different branch from other species of *Hymenostegis* section, it was in the same branch with some of the *Hymenostegis* section species with small divergence in the phylogenetic tree. Furthermore, *A.cephalotes* and *A.gueruenensis* from different sections located in a single branch far away from their sections.

Figure 5.3 Phylogenetic tree, constructed using the sequences of ITS region of genomic DNA, depicting relationships among the species of *Astragalus* (● *Macrophyllium,* □ *Halicacabus,* ○ *Hymenostegis* , ■ *Megalocystis,* ◊ *Hymenocoleous,* ∆*Poterion sections)*

As a summary, ITS region of nDNA supported the results of cpDNA regions. Moreover, even though the genetic divergence among species within sections was high compared to cpDNA regions, phylogenetic tree didn't separate clearly sections from each other, but it provided informative results.

5.2.3.3 Comparison of Astragalus Species that are Native to Turkey with Those Distributed through the World

Six different sections (*Hymenostegis, Halicacabus, Megalocystis, Macrophyllium, Poterion and Hymenocoleus*) of *Astragalus* genus native to Turkey were examined with cp and nDNA sequence data in the current study. The results were useful to understand phylogenetic relationships among *Astragalus* species of six different sections native to Turkey. In order to figure out evolutionary divergence of *Astragalus* species of Turkey from the species of other regions of the world, additional cp and nDNA sequence data for each studied region were retrieved from the NCBI database and comparatively analyzed.

Molecular diversity parameters were calculated for *trn* L and ITS regions by comparing Turkish *Astragalus* species with the species from other regions of the world (Table 5.14). There was no trnV sequence data available from the NCBI data base, so it was excluded from the analysis.

Table 5.14 Genetic diversity parameters of *Astragalus* genus from different parts of the World. Parameters were estimated by using sequences of *trnL* intron region

	Turkey	Iran	East Asia	Europe	Africa	New world	Total
Number of species	30	10	3	4	2	10	60
Total length(bp)	538	538	538	538	538	538	538
GC content(%)	30.5	30.8	31.8	30.8	30.8	31.2	30.9
Conserved sites	471	431		466	459	458	411
Variable sites	20	59	16	15	9	19	93
Parsimony informative sites	20	44	-	2	-	8	76
Transitional pairs	40.14	49.09	42.75	21.47	-	17.24	45.06
Transversional pairs	59.86	50.91	57.25	78.53	-	82.76	54.94
Transition/transversion (tr/tv)(R)ratio	0.58	0.87	0.72	0.26	-	0.21	0.79
Number of deletion	13	54	35	15	19	17	153
Number of insertion	3	20	5	5	-	16	49

Generally, Iran is accepted as the main genetic diversity center for *Astragalus* genus in the world where approximately over 800 species are naturally found (Maassoumi, 2005). In the current study, Iranian *Astragalus* genus with 10 different species depict high diversity (59 variable sites). Most of them (44 sites) were parsimony informative. After Iran, Turkish samples and New world (North and South America) species were the second with respect to variable sites. Species from other regions had low variable sites. The reason might be due to inclusion of the low number of species into the analysis.

Table 5.15 Genetic divergence of Astragalus genus throughout the World. Estimations were done by using DNA sequences of *trnL* intron region of cpDNA.

	Iran	Turkey	Korea	Australia	China	Europe	Africa	New world
Iran								
Turkey	0.0238							
Korea	0.0246	0.0107						
Australia	0.0346	0.0150	0.0221					
China	0.0427	0.0283	0.0312	0.0356				
Europe	0.0264	0.0074	0.0143	0.0188	0.0300			
Africa	0.0261	0.0062	0.0132	0.0176	0.0312	0.0088		
New world	0.0268	0.0080	0.0141	0.0168	0.0311	0.0114	0.0101	

The highest divergence was observed between East Asian species (Korea, Australia and China) and Iranian species (ranged from 0.0246 to 0.0427). Moreover, although the highest divergence was found between Iranian and East Asian species from, the lowest divergence was observed between Iranian and Turkish species (0.0238). Interestingly, the lowest genetic diversity was also observed between Turkish samples and European samples, African samples and New World samples (ranging from 0.0074 to 0.0080).

In the phylogenetic tree constructed by using *trn*L intron data from *Astragalus* species of the World, it was clear that most of the Iranian species were located in a different clade. However some of them included into which consist of mainly Turkish species. In general, Turkish species, were separated from the rest of species from the world.

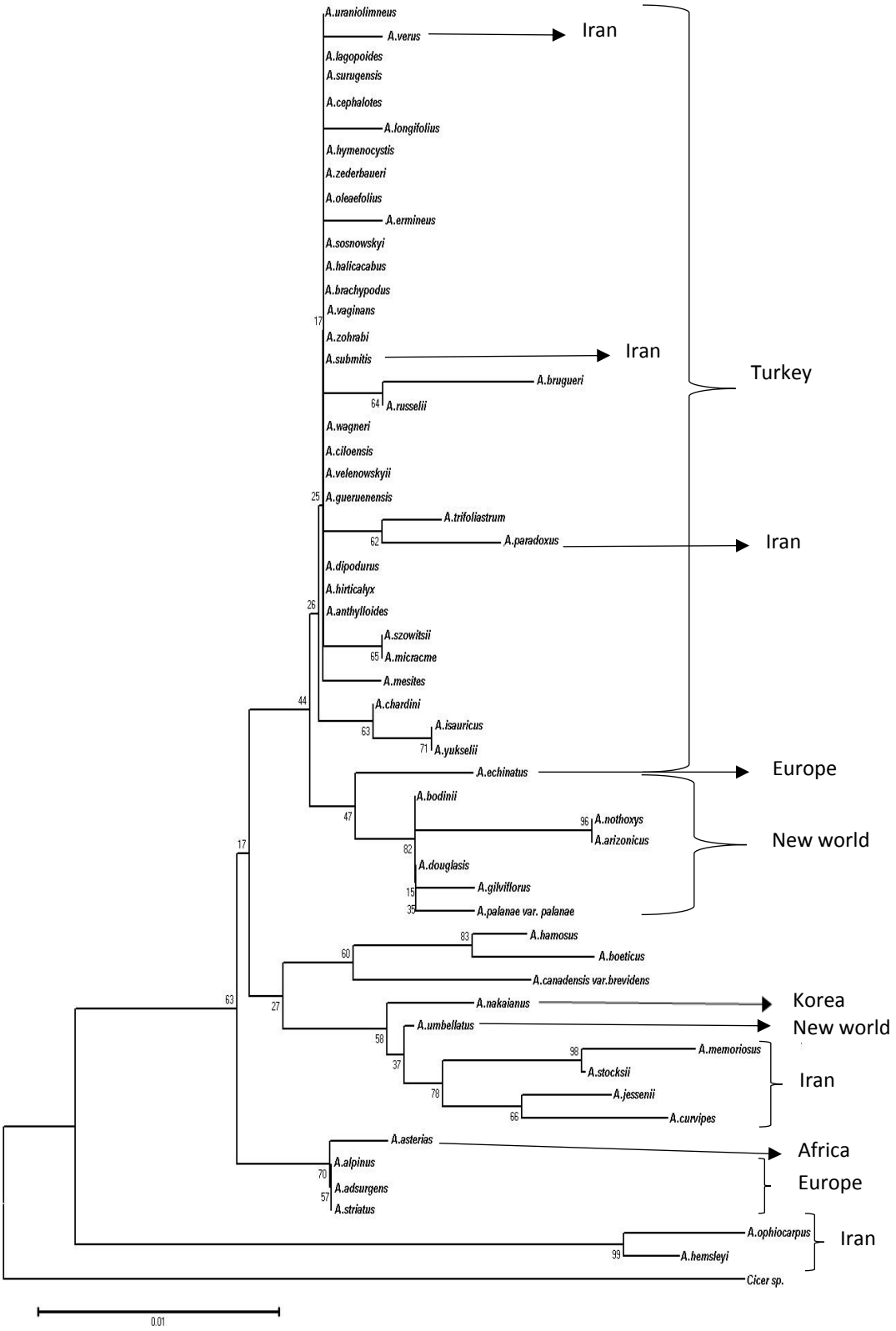

Figure 5.4: Phylogenetic tree (*trnL*) depicting relations of Turkey species with other *Astragalus* species from different regions of the world.

Table 5.16 Genetic diversity parameters of *Astragalus* species from different regions of the World. Values were estimated by using DNA sequences of *matK* region of cpDNA

	Turkey	Old world	New world	Total
Number of species	30	7	4	41
GC content(%)	31	30.4	30.4	30.8
Conserved sites	1188	1111	1142	1071
Variable sites	23	60	23	106
Parsimony informative sites	23	16	1	51
Transitional pairs	42.27	40.2	64.8	50.07
Transversional pairs	57.73	59.8	35.2	49.93
Transition/transversion (tr/tv)(R)ratio	0.63	0.57	1.58	0.86
Number of deletion	8	2	-	10
Number of insertion	8	5	-	13

The *mat*K region of cpDNA from Astragalus species of the world was also evaluated for a world wide comparison of species. There were not many DNA sequence data for this region available from the NCBI database. The sequences from Old world species were mainly from East Asia while those from New world species were from North America.

Molecular diversity parameters calculated for using matK region of *Astaragalus* species were given in Table 5.16. Both number of variable sites and parsimony informative sites were low compared to trnL region. Even though the matK is the longest studied region, variable site, informative site and deletion nucleotide numbers were relatively low, but Old world species seem to be highly variable. Transversition/ Transition bias ratios varied among species of the world, but the overall ratio was 0.86.

Table 5.17 Genetic divergence in *matK* region of cpDNA of the genus *Astragalus* throughout the World.

	Turkey	New world	Old world
Turkey			
New world	0.0185		
Old world	0.0249	0.0145	

Table 5.17 depicts the group mean distance among Turkish, New World and Old world *Astragalus* species. Turkish samples and New world samples appeared to be genetically quite similar to each other (0.0185). Also, genetic distance between Old and New World species is low (0. 0145). The highest genetic divergence was seen between old world and Turkey based on *matK* region (0.0249).

Phylogenetic tree (Figure 5.4) depicts that all Turkish samples are clustered in a single clade. Old world species which were studied from China were grouped with some New world species in a different sub clade under this main clade. Moreover, Old world species from Australia were in another subclade with some New world species. Based on the results of *mat*K region of cpDNA it can clearly said that Turkish samples were genetically distinct and formed a different clade indicating that Turkey is one of the diversity centers of *Astragalus* in the world.

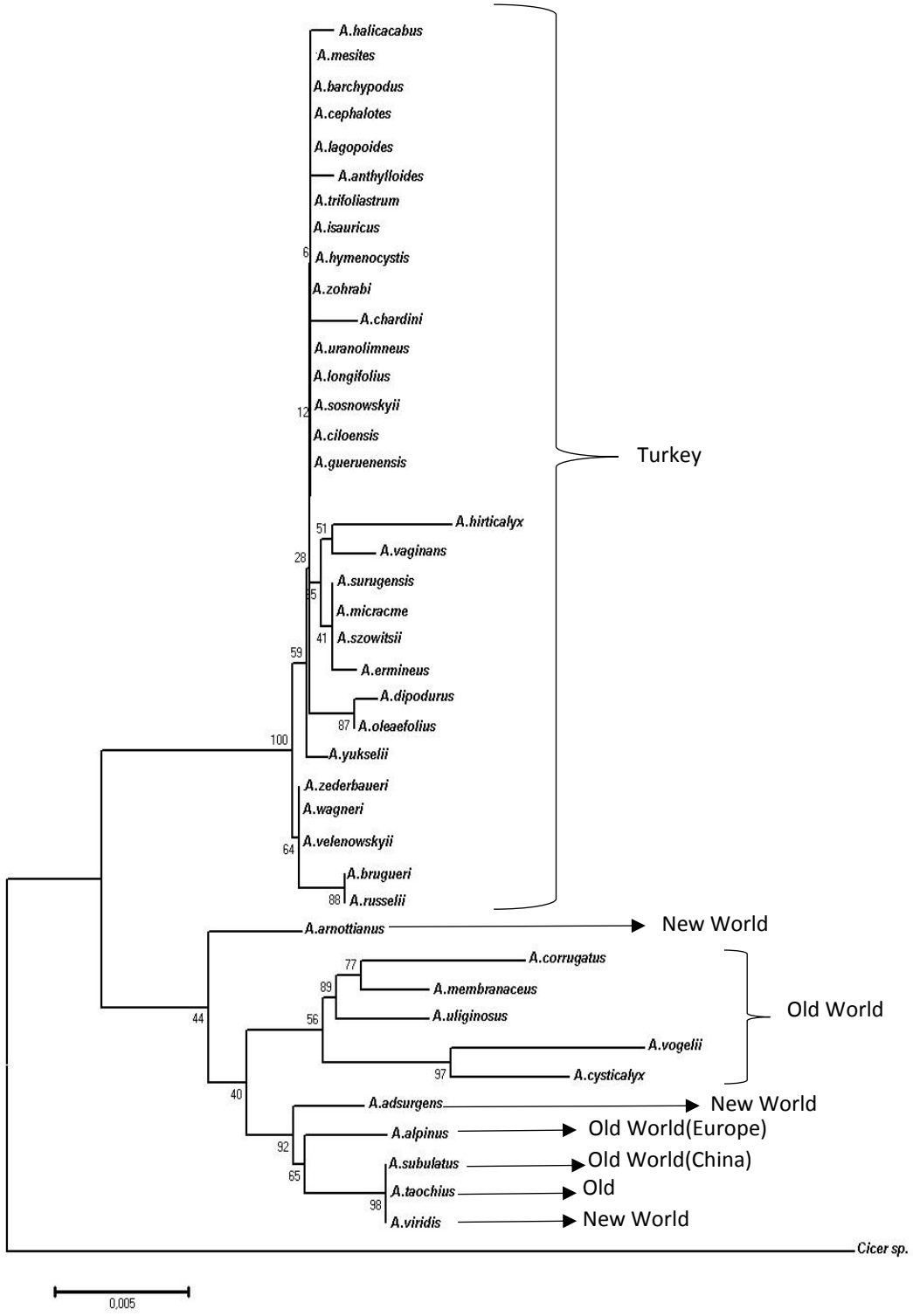

Figure 5.5: Phylogenetic tree (*matK*) depicting relations of Turkish *Astragalus* species with the other species from different regions of The World.

Table 5.18 Genetic diversity parameters of ITS region in the genus *Astragalus* from different parts of the World.

	Turkey	Old world	New world	Total
Number of species	30	8	12	50
GC content(%)	53.9	53.7	53.8	53.5
Conserved sites	553	493	551	455
Variable sites	49	89	24	131
Parsimony informative sites	49	38	9	91
Transitional pairs	61.14	67.08	66.98	61.23
Transversional pairs	38.86	32.95	33.02	38.77
Transition/transversion (tr/tv)(R)ratio	1.55	2.01	2.00	1.56
Number of deletion	0	5	20	25
Number of insertion	0	14	2	16

The number of GC content varried significantly between cpDNA (~33%) and nDNA (~53%). Moreover, due to nucleotide substitutions, transversion/transition bias (R) values were higher in this region than cpDNA region (over 1). Variable sites were ranged from 24 in New World to 89 in Old World species. High percentages of variable sites were parsimony informative (Table5.18).

Table 5.19 Genetic divergence of the genus *Astragalus* throughout the World based on ITS sequence data.

	Turkey	Old world	New world
Turkey			
Old world	0.0529		
New world	0.0398	0.0537	

When average genetic distance were compared among geographical group of the species with Old World species (mostly from Iran, Iraq) were quite diverged from Turkish species. However, again as in the case for cpDNA regions, New world and Turkish species seem to be genetically close to each other(Table 5.19).

Since, most of the sequences of ITS region from Old World species were obtained from Iraq and Iran, six species from Iran and Iraq located in a one of the main clade. Other main clade in the phylogenetic tree (Figure 5.5) composed of New world and Turkish samples. Especially Old World species from Australia were positioned with New world species in the sub clade. Furthermore, *A.gummifer* from Iraq, was placed with Turkish species. All Turkish species formed a single sub clade.

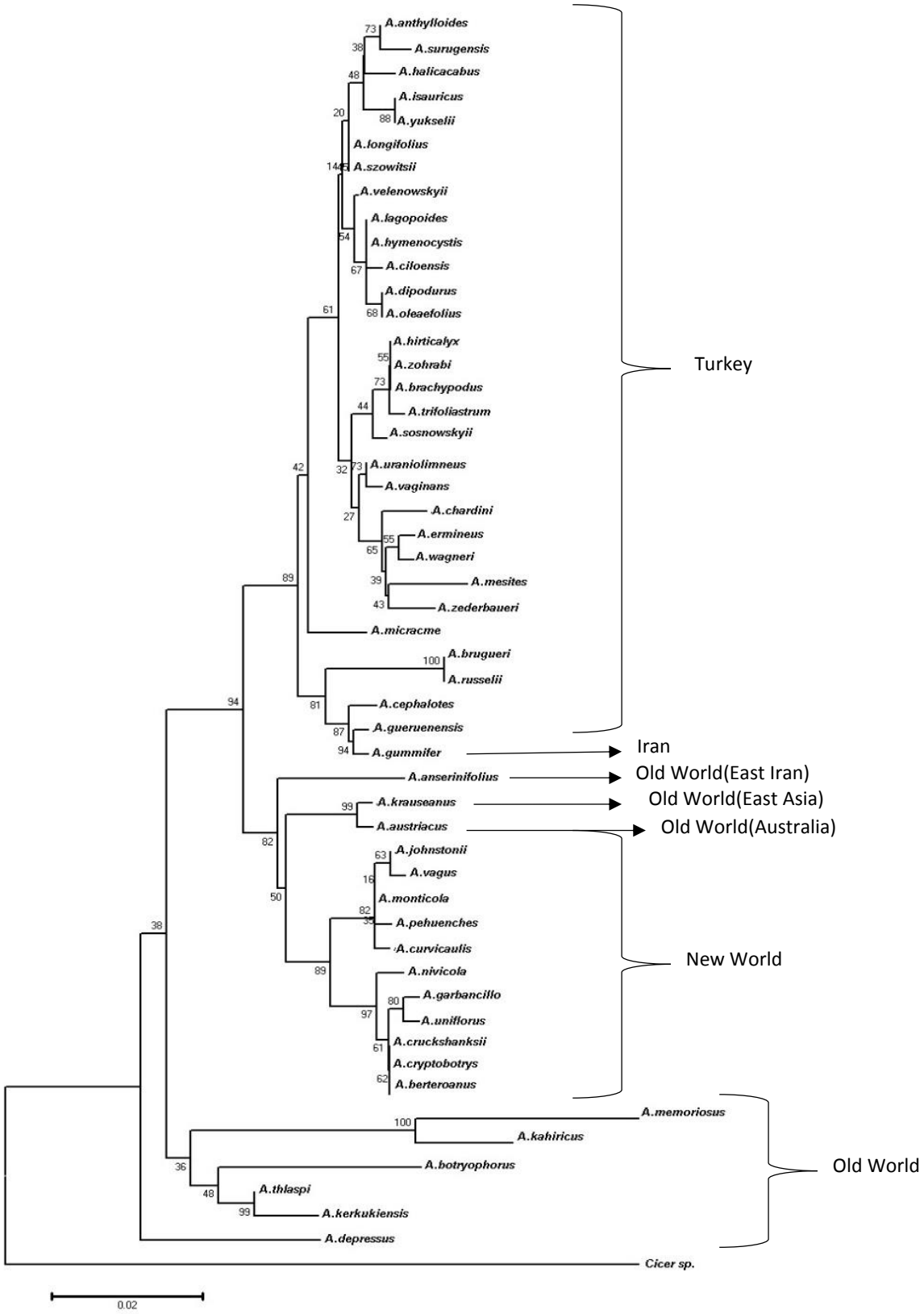

Figure 5.6 : Phylogenetic tree (ITS) depicting relations of Turkey species with other species that live outside of Turkey

5.2.4 Molecular Clock Estimation

Divergence times among the sections of the genus *Astragalus* from Turkish *Astragalus* species, Old World, New World species were estimated separately (Table 5.20). According to Wojciechowski *et al.* (1999 and 2005), New world *Astragalus* species were diverged from the Old world species. Therefore, to support this information, cpDNA regions of the genus were used. All calculations were done by using the formula that was given in the Materials and Methods section. However, only *trn*L intron and *mat*K regions could be used to make these comparisons because their sequence data of other cpDNA regions were not available in the NCBI database.

Parsimony informative sites, total length of the sequenced region, *d* and *k* values and estimation of molecular clock times for *trn*L intron and *mat*K regions were given in tht Table 5.20 and 5.21 , respectively. The results indicate that estimated divergence time varied among geographic regions. Old World *Astragalus* diverged from other genus within Astragalean clade about 12.5 million years ago with respect to *trn*L intron and 14.5 million years ago regarding *mat*K regions of cpDNA.

When the divergence times were estimated between available New world and Old world *Astragalus* species in the current study, the results indicated that New and Old world Astragalus species were diverged from each other about 7.3 million years ago with respect to *mat*K region. This divergence time is much earlier than it is estimated for *trn*L intron (5.4 million years ago). This could be explained that *trn*L intron may be more conserved than *mat*K region.

In addition to all these comparisons, the studied *Astragalus* sections were also compared to each other. According to *trn*L and *mat*K regions, values of all six different sections were highly variable. Divergent time of *trn*L intron generally was later than it was *mat*K region. Based on *trn* L intron, *Poterion* section appears to be newly diverged from other sections (0.12 million years ago)(Table 5.20 and 5.21).When the data of *mat*K region was used for the estimations, results depicted that *Megalocystis*

section seems to be diverged from other sections (1.5-1.9 million years ago) which is more recent compared to the other sections.

Table 5.20 Molecular Clock Estimations for New (NWA) and Old World Astragalus (OWA) species and six Turkish *Astragalus* sections using DNA sequences of *trnL* Region.

	Pi-*trnL*	Total	d	K	MC(MYA)	
Halicacabus-Megalocystis	6	538	0.011	0.011	5.6	
Halicacabus-Macrophyllium	5	538	0.009	0.009	4.7	
Halicacabus-Hymenocoloeus	3	538	0.006	0.006	2.8	
Halicacabus-Hymenostegis	6	538	0.011	0.011	5.6	
Halicacabus-Poterion	13	538	0.024	0.025	1.2	
Megalocystis-Macrophyllium	6	538	0.011	0.011	5.6	
Megalocystis-Hymenostegis	5	538	0.009	0.009	4.7	
Megalocystis-Poterion	13	538	0.024	0.025	1.2	*trn*L
Megalocystis-Hymenocoloeus	3	538	0.006	0.006	2.8	
Hymenocoloeus-Poterion	10	538	0.019	0.019	9.4	
Hymenocoloeus-Macrophyllium	3	538	0.006	0.006	2.8	
Hymenocoloeus -Hymenostegis	3	538	0.006	0.006	2.8	
Poterion-Macrophyllium	13	538	0.024	0.025	0.12	
Poterion-Hymenostegis	13	538	0.024	0.025	0.12	
Macrophyllium-Hymenostegis	6	538	0.011	0.011	5.6	
Old World-New World	54	538	0.100	0.108	5.4	
OWA-Astragalean Clade	14	575	0.024	0.024	12.5	

Table 5.21 Molecular Clock Estimations for New (NWA) and Old World *Astragalus* (OWA) species and six Turkish Astragalus Sections using DNA sequences of *matK* Region.

	Pi-*matK*	Total	d	K	MC(MYA)	
Halicacabus-Macrophyllium	10	1322	0.008	0.008	3.8	
Halicacabus-Hymenocoloeus	9	1322	0.007	0.007	3.4	
Halicacabus-Hymenostegis	14	1322	0.011	0.011	5.3	
Halicacabus-Poterion	8	1322	0.006	0.006	3.0	
Megalocystis-Macrophyllium	5	1322	0.004	0.004	1.9	
Megalocystis-Hymenostegis	10	1322	0.008	0.008	3.8	
Megalocystis-Poterion	5	1322	0.004	0.004	1.9	
Megalocystis-Hymenocoloeus	4	1322	0.003	0.003	1.5	*matK*
Hymenocoloeus-Poterion	7	1322	0.005	0.005	2.7	
Hymenocoloeus-Macrophyllium	8	1322	0.006	0.006	3.0	
Hymenocoloeus -Hymenostegis	12	1322	0.009	0.009	4.6	
Poterion-Macrophyllium	7	1322	0.005	0.005	2.7	
Poterion-Hymenostegis	11	1322	0.008	0.008	4.2	
Macrophyllium-Hymenostegis	13	1322	0.010	0.010	4.9	
Old World-New World	17	1173	0.014	0.015	7.3	
OWA-Astragalean Clade	35	1230	0.028	0.029	14.5	

CHAPTER 6

DISCUSSION

In the current study, to understand phylogenetic relationships among sections and species of *Astragalus* genus, both cpDNA and nDNA regions were studied. The results of studied regions were assessed. The results were discussed separately in this section.

6.1 Molecular Diversity Analysis in *Astragalus* Species depend on cpDNA and nDNA regions

Due to huge number of copies in plant cells, chloroplast DNAs are frequently studied in evolutionary and phylogenetic analysis. In previous years, as a result of this property, many molecular studies are done by using cpDNA regions (Clegg and Zurawski, 1992). Moreover, cpDNA genes generally have small rates of sequence divergence due to formation of secondary structures (Kushel *et al.*., 1990). For that reasons, cpDNA regions are advantageous for phylogenetic and evolutionary studies at higher taxonomic level (Taberlet, 1991).

Addition to cpDNA regions, nDNA regions are also used to understand evolutionary and phylogenetic relationships of taxa. In the current study, ITS region were chosen due to its high evolutionary rate, easy amplification and high repeated numbers in plant genomes.

6.1.1 Non-coding *trn* cpDNA Regions [L5'-L3' (*trn*L intron), *trn*L3'-F $^{(GAA)}$ (*trn* L-F intergenic spacer), and *trn* V intron]

Non-coding regions maintain fast evolution rate against coding regions. Therefore these regions provide extensive data for phylogenetic studies. Basically *trn* L intron, *trn*L-F and *trn*V regions have been studied in non-coding parts of cpDNA in order to comprehend interspecific relationships of species and their evolutionary and phylogenetic relationships at different levels (Gielly and Taberlet 1996; Wang *et al.* , 1999, Ohsako and Ohnishi 2000; Mummenhoff *et al.*, 2001).

In the current study, the highest variable sites were found in *trn* L-F region (10%) compared with other studied *trn* regions. Even though *trn* L intron and *trn* L-F region are placed consecutively in cpDNA, the number variable sites were more in *trn* L-F region than previous part. Both the short length of the region and differences in these short sequences based on sections caused this kind of difference among regions. Kazemi *et al.* (2009) also obtained percentage of variable sites approximately 10% in their studies. Also, Shaw and his colleagues (2005) pointed out that *trn*L-F region was much more diverse even if it was shorter in length. In addition to these regions, *trn* V region was also studied. This region had the variable sites among studied non-coding regions due to presence lowest number of indels with respect to others. *Trn* L intron and *trn* V region results were not given clear separations within sections alone. In previous studies some researchers (Shaw *et al.*, 2005; Kress *et al.*, 2005; Kazemi *et al.*2009) claimed that *trn* L region have low capacity to separate particularly closely related species. Moreover, they stated that resolution of this region is very low compared to some other non-coding regions of cpDNA. All these makes this region be less appreciated for phylogenetic studies. Likewise these researchers, in the current study, there was a problem of usage of *trn*L intron alone because of some difficulties in the separation of studied *Astragalus* sections. Furthermore, these difficulties may also vary from high genetic similarity of studied samples sequences. Only *Poterion* section was separated clearly from other sections due to involvement of large insertions in the region.

In spite of estimating phylogenetic relationships of different plant species at different levels, multi base deletions and insertions were widely used in molecular studies. Large number of mutations in cpDNA were generated by indels that have a size change in the sequence in different numbers (Vijverberg and Bachman, 1999). Gielly and Taberlet (1996) claimed that the percentages of insertions and deletions occured less in *trn*L intron than intergenic spacer like *trn*L-F regions in cpDNA. Like these studies, in the current study, most informative and clear substitutions and indels were seen in *trn*L-F intergenic spacer which were resulted a clear section separations. Moreover, specific Tymine repetitions in the sequence also result in section divergence.

In 1999 Wang and his colleagues used *trn* V intronic region to figure out phylogenetic relationships among Pines and claimed that variable sites of this region were very low and most of them were not informative. Somewhat at species level, this region does not seem to be very useful. Even if the number was low, *trn*V was useful to separate the samples at section levels. In the current study, also, *trn* V intron resulted in low variation and only *Poterion* section had deletions and this deletion gave the only clear separation of *Poterion* sections from other studied sections.

As mentioned in the definition of *trn*L intron region, generally even if it is informative or not there are many large deletions or insertions partly or completely in the sequences (Freyer *et al.*, 1995; dePamphilis *et al.*, 1997; Lohan and Wolfe, 1998). Sometimes the sequence length became more in some species contrast to others. In the current study, in *trn*L intron, *A.mesites* had specific insertions different than other samples. Also, there were large deletions in *Poterion* section. *A.brugueri* had specific deletions rather than *A.russelii* in the same section. This results were significant, meaning that the length of the *trn* L intron became longer and gave informations about section divergence with nucleotide substitution rates. However, this kind of single deletions at species levels might not provide informative data for phylogenetic analysis among sections.

The percentage between transition/transversion values would change between 2 to 10 when there was transition bias in the sequences (Bakker *et al.*, 2000). This value is a measure of nucleotide polymorphism in a sample of gene sequence and also it is a summary statistic used to indicate patterns of molecular diversity within a sample of gene copies (Nei, 1987). As seen in the previous chapter, some regions within species of different sections there were some doubts on R value which were greater than 1. However, in the average of each non-coding region R values were not over 1 as expected in the current study.

The reason of stability of DNA was linked with GC (%) content in the sequence. Also adaptation to the environmental conditions is based on GC (%) content richness (Torres, 1990 and Baldwin *et al.*, 1995). When this percentage is low, it is claimed that there are significant amount of transversion (Bakker *et al.*, 2000). Even though the amount of AT is high in the sequences of non-coding regions, transversion building between Adenine and Thymine was formed to be less than transversion between Guanine and Cytosine nucleotides in Angiosperms (Yang, 1994; Bakker *et al.*, 2000). In the current study GC (%) contents in each studied *trn* region were not so high (*trn*L: 30, 5%; *trn* L-F: 34%; *trn*V: 31, 8%). It means these results support the previous studies based on transversion/ transition ratios.

6.1.2 *matK* gene region of cpDNA

Due to the presence in every plant, *mat*K region is generally chosen for studying molecular phylogeny at interspecific level (Johnson *et al.*, 1996). However, this sequence is the least conserved ones in among plastid gene sequences (Olmstead and Palmer, 1994; Soltis and Soltis, 1998). Sometimes this characteristics become more preferable for molecular phylogenetic studies.

In the current study, *mat*K was the longest studied region in among other cpDNA regions. Surprisingly, *mat*K region had low variable sites compared to other short regions like *trn*L-F intergenic spacer. The *mat*K region includes a specific gene

encoding maturase-like protein (Neuhaus and Link, 1987). Therefore low genetic divergence is expected due to its coding property. In the current study, since there were low number of variable sites in this region, there were no clear separation at section level. Only *Poterion* section had got specific indels which caused in separation of the section from other sections. Moreover, *A.vaginans* from *Hymenocoleous* section had got crucial substitutions that caused separation at section level. Nevertheless all these insertions and substitutions were not adequate to separate all studied *Astragalus* sections. Furthermore, GC content of the region was approximately close to other studied non-coding regions of cpDNA.

When all studied cpDNA regions are considered together, it can be said that these regions alone are not adequate enough to separate species of *Astragalus* genus at section level. Furthermore, although they separate some sections of *Astragalus* from others, genetic divergence within sections are too low. Only some very genetically divergent species can be separated from others by using these cpDNA regions. However, in order to eliminate some statistical errors, when all cpDNA regions combined and added consecutively, results are obtained more reliable. Variable sites, which are more informative than separated ones, gave specific separations at section levels. All *Hymenostegis* sections have the same variable sites except for *A.trifoliastrum*. Moreover, *Poterion* section has specific insertions and deletions. In contrast to these results all *Megalocytis* and *Halicacabus* species have approximately same substitutions compared to the others. Also, *Hymenocoleous* and *Macrophyllium* section species have particular sequence divergence. In the light of these outcomes, it can be safe to say that using only one non-coding region is not enough to figure out phylogenetic relationships among studied *Astragalus* sections. Furthermore, non-coding regions are more conservative than studied coding region *mat*K. Although it is the longest studied region, it is not the reliable one. Reliability is depend on not the size, it depends on mutational changes occurring while speciation takes place.

6.1.3 ITS nDNA region

The main obstacle for lower-level phylogenetic studies is the identification of easily amplifiable and rapidly evolving, but actually alignable DNA regions which can support applicable and adequate variation in short sequence fragments. Particularly, there is such necessity from nuclear genome which support the affluence of cpDNA sequence data from previous studies (Baldwin *et al.*, 1995). Moreover, evolution rate of nuclear DNA is higher than cpDNA (Savolainen and Case, 2003). All these features do not destroy the wealth of cpDNA regions, but then clearly indicate the potential needs of any assessment of organismal phylogeny depend on a single region (Doyle, 1992).

In the current study, ITS regions were used to figure out phylogenetic relationships of studied *Astragalus* genus. Total length of the region was about 611 bp in length. Bagheri *et al.* (2015; 2016) also amplified this region in some species of *Hymenostegis* section of *Astragalus* genus and reported that it was about 606 bp in length. ITS1 sub-region was about 213 bp in length while ITS2 sub-region was 234 bp. For many species, ITS1 is shorter than ITS2 region because of the elimination of 18S region sequences that is located at the beginning of ITS1 sub-region (Dong *et al.*, 2003; Baldwin, 1995). Our results are compatible with these information.

The highest variable sites were obtained from all the ITS regions compared to cpDNA regions in the current study. Due to the characteristics of this region, this high value was expected. Although all these variable sites were informative, none of them was due to insertion or deletion. All of them resulted from nucleotide substitutions. Wojciechowski *et al.*, (1993) claimed that there were large deletions in ITS1 sub-region in *Astragalus* species that were examined on their study and they generalized it. However, our results do not support this generalization in the studied *Astragalus* section.

Based on these substitutions, R values of sub regions were higher than cpDNA regions. This was expected because of the high number of nucleotide substitutions in this regions. As it was expected that 5.8S sub region was different than other sub-regions due to presence of single substitution since it is an exonic region.

GC (%) content of ITS regions was about 54% (57.1% in ITS1 and 52% in ITS2). In different species like zucchini and cucumber (Torres *et al.*, 1990), melon (Kavanagh and Timmis, 1988), tomato (Kiss *et al.*, 1988) and rice (Takaiwa *et al.*, 1985) similar values were reported. According to Wojciechowski *et al.* (1993), GC content was changed as 54-60% for ITS1 and 50-54% for ITS2 sub regions in *Astragalus* genus like some other close families. Our results supported Wojciechowski *et al.* (1993) which means that stability of nDNA region is higher than it is in cpDNA.

Wojciechowski *et al.* (1999) examined ITS and *trn*L intron regions to estimate phylogenetic relationship between Old and New world *Astragalus* species and claimed that New world *Astragalus* species with aneuploidy chromosome number form a monophyletic clade within Old world clade that was represented by species with euploid chromosome number. Furthermore, they revealed many substitutions and indels in studied regions. Also, Kazemi *et al.* (2009) depicted that *trn*L intron was less informative than ITS region. Furthermore, Dizkırıcı (2012) examined that total ITS region data was capable to separate each studied sections (*Incani*, *Hypoglottidei* and *Dissitiflori*) of the genus *Astragalus,* and was more diverge than other cpDNA regions. Like all these studies, our data from ITS region was more informative than *trn*L intron data. The section discrimination was observed clearly with ITS sequence data.

6.2 Genetic Divergence among Sections of *Astragalus* Genus and Constructed Phylogenetic Trees

The subject of current study was to understand phylogenetic relationships of six *Astragalus* sections (*Halicacabus, Megalocystis, Macrophyllium, Poterion, Hymenostegis and Hymenocoleous*) native to Turkey. All these studied sections were genetically close to each other. Therefore, genetic divergence among sections were

low, but diversity parameters were enough to support or reject previous taxonomical study of sections.

Whether studied region was cpDNA or nDNA region, generally *Poterion* section was the most distinct section among these sections. Due to different numbers of indels and substitutions, *Poterion* section was always located in a different clade in phylogenetic trees which were constructed by studied cp and nDNA regions. Moreover, whether the region was from cpDNA or nDNA, *Halicacabus* and *Megalocystis* sections were the closest sections phylogenetically. These section species repeatedly located together at the same branches or sub-clades in the phylogenetic tree. Furthermore, whether species of *Macrophyllium* section were sometimes separated into *Halicacabus* section species, members of the section were located close to each other in phylogenetic tree of all studied regions. Addition to these results, *Hymenostegis* section species usually formed a sub-clade, but only *A.trifoliastrum* always located within sub-clade, but it was distantly located to other members of this section. Lastly, *A.vaginans* which is the only one sample of *Hymenocoleous* section, always located at different branches from others, but close to *Hymenostegis* section samples.

After De Candolle's classification (1825), Bunge (1868) announced two sections *Halicacabus* and *Megalocystis* and he got *Anthylloidei* section in section *Halicacabus*. Molecular and taxonomic studies were claimed that morphological character could not explicate infrageneric relationships in spiny and hairy *Astragalus* species (Nadari Safar *et al.*, 2014). Under the light of these morphological characteristics, *Halicacabus* and *Megalocystis* sections merged into *Anthylloidei* section (Maassoumi, 1995; Podlech *et al.*, 2001; Podlech and Zarre, 2013). Analyzed characters had evolved in years in section *Anthylloidei* and geographical distribution of members of the section matches more or less certain geographic characters in large areas (Nadari Safar *et al.*, 2014). Under the light of these taxonomic revision, it can be safe to say that our phylogenetic results support this revision whether based on cp or nr DNA regions.

About *Poterion* section, Tietze (1988) had a detailed study that, this section had many different morphological characters like *Microphysa* section rather than other sections. The hairs are entirely white and short, even on the calyx. The shape and type of hairs are different than other sections. In addition to all these taxonomic separations, our phylogenetic results also separated *Poterion* section from other studied sections very distinctly. In all phylogenetic trees based on studied regions, *Poterion* section located in a single clade or sub clade.

Hymenocoleous section was identified as a single section in Flora of Turkey by Chamberlain and Matthews (1970). They reported that *A.vaginans* which is the only member of *Hymenocoleous* section was separated from *Hymenostegis* section based on its imparipinnate leaves and forming wide cushions. However, Zarre and Podlech's study (1996) on *A.vaginans* claimed that leaves forms were not enough to separate this section from *Hymenostegis* section. Therefore, they eliminated *Hymenocoleous* section and put *A.vaginans* into *Hymenostegis* section. After Podlech and Zarre (1996), Karaman Erkul *et al.*(2015) reported that they agreed on all previous taxonomic studies that *Hymenocoleous* as a species section (Maassoumi, 1995; Podlech *et al.*, 2001; Bageri *et al.*, 2011; Podlech and Zarre, 2013). Moreover, Bageri (2016) studied molecular phylogeny on different *Astragalus* species of *Hymenostegis* section and pointed out that *A.vaginans* is varied from the species of *Hymenostegis* section based on ITS region of nDNA. In the current study *A.vaginans* always located at close to *Hymenostegis* section samples in the phylogenetic tree but in a single branch or sub-clade. As a result of our molecular data, phylogenetically we support Bageri's molecular and Karaman Erkul's taxonomic revision studies that *A.vaginans* should be kept in *Hymenocoleous* section.

In our phylogenetic analysis, *A.trifoliastrum* did not join into *Hymenostegis* section samples. Especially in phylogenetic trees based on combination of all cpDNA regions, this species located at different position from other *Hymenostegis* samples. Bageri *et al.* (2015) claimed in their revision study that based on molecular, morphological and chromosomal data, *A.trifoliastrum* was a different species like *A.vaginans* from other *Hymenostegis* section species.

A.oleaefolius and *A.dipodurus* from *Macrophyllium* section were located very close to each other at same branch under same sub-clade with other *Macrophyllium* samples based on both nDNA region (ITS) and cpDNA regions (*trnV* and *matK*) with high genetic similarities. Zarre (2003) reported that morphologically these two species have strongly appressed leaf hairs in the former and spreading in the latter different than other *Macrophyllium* species. Under this taxonomic features, it can be said that our phylogenetic data supported the conclusion from morphological characters. However, phylogenetically, these two species were still accepted within *Macrophyllium* section due to their genetic similarities with other samples of *Macrophyllium* section.

6.3 *Astragalus* Species that are Native to Turkey and Those Distributed throughout the World

There were previous molecular study about some *Astragalus* species native to Turkey (Dizkırıcı, 2012). However, there are no molecular studies to data showing phylogenetic relationships within species of *Halicacabus*, *Megalocystis*, *Poterion*, *Macrophyllium*, *Hymenocoleous* and *Hymenostegis* sections of *Astragalus* genus that are native to Turkey. For that reason, this study will have a significance contribution to figure out both evolutionary relationships between species of these sections and also phylogenetic relationships among *Astragalus* species that are native to Turkey and other regions of the World.

All sections in the current study are native to Turkey and considered as Old world *Astragalus* species. To figure out the phylogenetic relationships between New and Old world *Astragalus* species, sequences of New world and Old world *Astragalus* from other regions of the World were obtained from NCBI databank.

When the studied regions of cp and nDNA are compared with all the World studies, many scientists mostly studied *trn*L and ITS regions and in recent year's *mat*K region is also become popular in some species. Even if they are old or new world species, GC content of *trn* L intron region were found similar like our samples. This outcome

supported the stability of the GC content of *Astragalus* species throughout the World species. In phylogenetic analysis, the divergence between samples were the result of the numbers of substitutions and indels in the sequences. Based on *trn*L intronic regions, Old world species that were obtained from Iran, were distinct than New world species. However, they were close to Turkish *Astragalus* species than others. This means that, due to the geographical positions of Turkey and Iran, they locate in Iranian –Turanian geographic region, and although they are separated with geographic barriers, they are closer to each other than New world ones.Nevertheless, distinct cluster of Tukish *Astragalus* species in phylogenetic trees suggest that Turkey appears to be diversity center of *Astragalus*. Moreover, Iranian species were distinct from other Old world species that were taken from China, Korea and Australia. Based on the results, it was seen that these Old world samples were very close to New World species. Because of the World position of these countries, this result did not surprise us. This means that ancient years during the continent separation occurred, these species were separated from their main genetic center (Iran and Turkey) together. Furthermore, interestingly, the genetic divergence between Turkish samples and New world samples were less than between Turkish and Iranian samples. It is clearly seen that Turkish species are speciated as a link between Old world and New world. The phylogenetic tree that was constructed by using *trn*L intron depicted that, not only all Iranian samples located different than other countries, but also some of them positioned inside of Turkish sample based clades. Additionally, European and African species were located at the same branch with Turkish samples and New world samples. This means that genetic similarity was seen between these species rather than Iranian ones, so that Turkish Astragalus diversity center is important for spread of the species to these regions of the World.

To figure out the comparison of Turkish species with the World ones, *mat*K region was also used. Mostly Chinese and American samples were obtained from databank and as a result it was seen that all Turkish samples clustered together and formed a main clade than others due to different substitutions and indels in their DNA sequences. New world and Old world samples located at the same main clade. Because the samples from Old world were taken from China, this clustering was not surprised us. As it mentioned in the section dealing with the *trn*L region, Chinese and American

samples are genetically similar to each other due to the position of these continentals before the continental separation period of the World.

It was seen that in recent years, nDNA regions have been commonly studied in the world. Many sequences of ITS region were found from Old and New world countries. When Turkish samples and World samples compared to each other, only one *Astragalus* species *A.gummifer* were located with Turkish samples in the same clade. Other main clade composed of 2 different sub clades and all Iranian samples were located in same sub clade. Wojciechowski *et al.*(1993) claimed that DNA sequences of ITS region of New World samples had low variable sites than Old world ones. This causes weak supports to understand phylogenetic relationships between species among clades of the constructed phylogenetic trees. In the current study as it was formed like in the phylogenetic trees of *trn*L and *mat*K regions, New world species and some Old world species such as Chinese, Australian and Korean ones were nested in the same sub-clade and closer to Turkish samples than Iranian ones.

Dizkırıcı (2012) also studied Turkish *Astragalus* species belonging to *Incani, Hypoglottidei* and *Dissitiflori* sections native to Turkey. Dizkırıcı found that American samples were genetically closer to *Incani* sections based on cpDNA regions, but close to *Hypoglottidei* and *Dissitiflori* species according to ITS regions. Results of the current study also yielded parallel results to her study. Dizkırıcı (2012) reported that biparental effects of ITS region might cause this contradiction due to maternal transmission is formed in cytoplasmic inheritance within *Astragalus* genus.

Ledingham (1957 and 1960) recommended that the new world species should be distinguished from Old world *Astragalus* species and introduced as with a new genus name. Nonetheless, in the current study, not only American species and Turkish species have different chromosome number, but also they located close to each other based on cpDNA and nDNA regions. As a consequence, giving a new genus name should be supported with further data.

6.4 Estimation of Molecular Clock

For estimating evolutionary divergence time of studied *Astragalus* genus in the current study, *trn*L and *mat*K regions were used. Although parsimony informative sites were higher in ITS region, this region was not used due to not having calculated mutation rate of nDNA on this genus. For cpDNA, mutation rate was obtained as 2×10^{-3} as a constant value (Pevsner, 2009). Moreover, cpDNA is maternally inherited and is affected from mutations lower than nDNA. Therefore, cpDNA regions were used to estimate molecular clock values.

Wojciechowski (2005) estimated that based on *mat*K gene region, the *Astragalus* species evolved about 12.4 million years ago, while Lavin *et al.* (2005) estimated as 16.1 million years old. In the current study, it was found parallel to these studies that Old world *Astragalus* group diverged from other genus within Astragalean clade about 12.5 million years ago according to *trn*L intron and 14.5 million years ago based on *mat*K regions of cpDNA.

In the current study, based on the chosen species from Old and New world *Astragalus* species which were taken from NCBI database for comparisons, New world *Astragalus* species were diverged from Old world ones from 7.3 million years ago with respect to the *mat*K sequence data. This timing corresponce to the Miocene geologic time period. Furthermore, by using *trn*L non-coding region, this value was 0.54 million years ago in the Pleistocene. Also, *Poterion* section was found to be diverged from other studied sections in same geological period as late Pleistocene based on *trn* L intron and *mat*K gene regions. Under the light of these results, it could be safe to say that *Poterion* section was younger than the other sections of *Astragalus* genus in evolutionary time.

CHAPTER 7

CONCLUSION

Regarding the goal of the current study, tree non-coding regions of *trn* and *mat*K region from cpDNA and ITS region from nDNA have been studied. Furthermore, molecular diversity of six *Astragalus* sections (*Halicacabus, Megalocytis, Macrophyllium, Hymenostegis, Poterion and Hymenocoleous*) native to Turkey and their evolutionary relationships with other *Astragalus* species from Old and New world species have been examined.

Among tree non-coding *trn* regions (*trn*L intron, *trn*L-F intergenic spacer and *trn*V) and *mat*K gene region from cp DNA and ITS region from nDNA, lowest genetic diversity was observed in the longest region *mat*K. However, even if *trn* L-F region was the shortest studied region, it had the highest diversity (approximately %10). This results could be explained that *mat*K region is an exonic area encoding a maturase-like protein.

Besides nucleotide substitutions, indels and deletions are significant to show phylogenetic relationships among species of *Astragalus*. In this study, indels were observed in varying frequencies in each regions. Particularly, at section levels, nucleotide deletions and insertions were useful in cpDNA regions. Although there were no deletion and insertion in ITS region, genetic divergence both in cpDNA regions and nDNA regions were helpful to construct phylogenetic trees whether low or high.

All the constructed phylogenetic trees shed light on the molecular phylogenetic relationships among studied *Astragalus* sections of Turkey. Based on the studied regions, *Poterion* section was always separated from other sections. The members of the section always created a separate cluster or sub cluster. Moreover, members of this section had the highest genetic divergence compared to other species. Interestingly, this section appears to be newly separated from other sections according to estimated molecular clock values (0.12 mya).

Members of *Megalocystis* and *Halicacabus* sections were always clustered together in phylogenetic trees under same branch or sub branches. Also, the lowest genetic divergence was found between these sections. These molecular outcomes supported that these sections could be combined under *Anthylloidei* section. The presence of thorny stem should not be the only reason for the separation of these two sections.

In the current study, species of *Hymenostegis* section were mostly located closed to each other under sub-clades. However *A. trifoliastrum* was always located distantly to other members of the section with high bootstrap values in the phylogenetic tree. This outcome was supported by the morphological analysis, so that this species could be examined separately from this section.

A.vaginans which is the only member of *Hymenocoleous* section was formed a different, but close branch to species of *Hymenostegis* section in both cpDNA and nDNA region based sequence data. According to current molecular study, it could be said that although *Hymenostegis* section and *Hynenocoleous* section have similar morphological characteristics, they appeared to be different sections based on molecular data. Thus, *Hymenocoleous* section should continue to be treated as one of the section of *Astragalus* genus.

To figure out evolutionary relationships between Turkish *Astragalus* and the species of Old and New world, sequences of studied regions were obtained from NCBI databank and were assessed together by using different cp and nr DNA regions.

According to results, Old world species separated from New world ones in all phylogenetic trees. Even though Turkish species were generally formed a separate groupings, they were suprisingly close to New world ones. Moreover, some Old world species like Chinese, Australian and Korean were closer to New world species. This results support that separate groupings of Turkish species which may indicate evolvement of another diversity center diverged from Iranian diversity center. The geographic barriers like high East Anatolian Mountains between Turkey and Iran were the reason of this separations. Moreover, Old world species were also separated by geographic forms in Asia so that some Old world species were closer to the New world ones. It is clear that Iran and Asia Minor are the main genetic pool of *Astragalus* species and all the others derived from these regions.

Molecular clock analysis depicts that studied sections of *Astragalus* genus native to Turkey was diverged from other *Astragalus* species at mostly about Pleistocene period. According to two different cpDNA region, outcomes indicated that although non-coding regions were highly conserved, coding region was the least conserved one. Therefore, the data from non-coding regions indicated that the Old world and New world species derived from each other in recent periods. However they were separated much earlier times based on coding region.

This study has yielded very important information to figure out evolutionary and phylogenetic relationships among six different *Astragalus* genus native to Turkey. To understand clear relationships of all *Astragalus* sections, additional taxa and sequences from other discriminatory DNA regions like rbcl, ETS, rps16 intron, ndhF, psbA-trnH intergenic spacer of both cp and nr DNA may be included at either section and species levels in the future.

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APPENDIX A

CHROMATOGRAM STRUCTURES FOR EACH STUDIED REGIONS

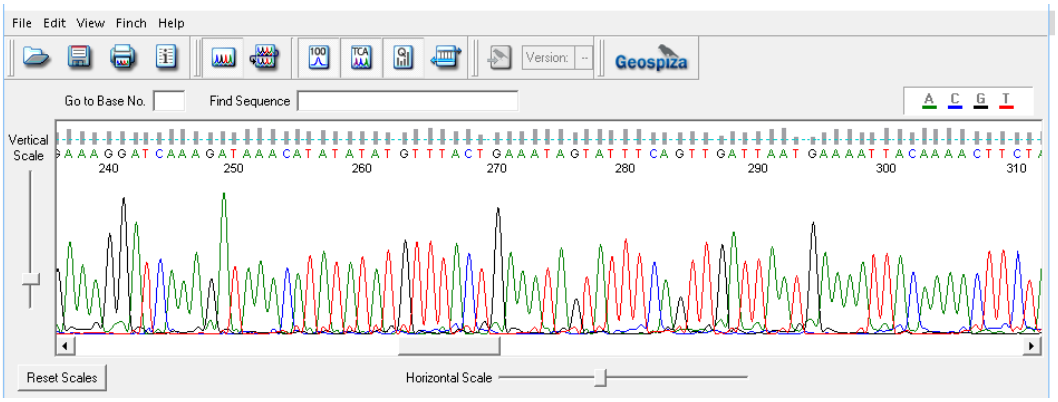

Figure A1: An example of chromatogram for trnL5'-L3' (trnL intron)

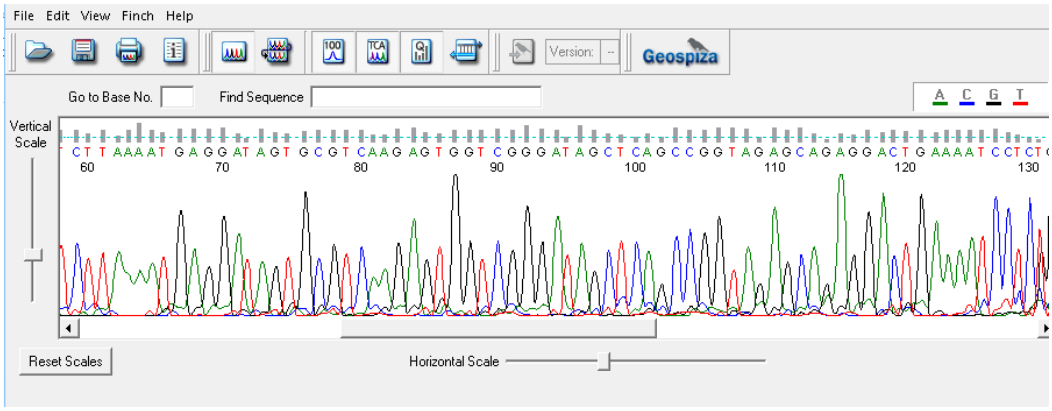

Figure A2: An example of chromatogram for trnL3'-F(GAA) (trnL-F intergenic spacer)

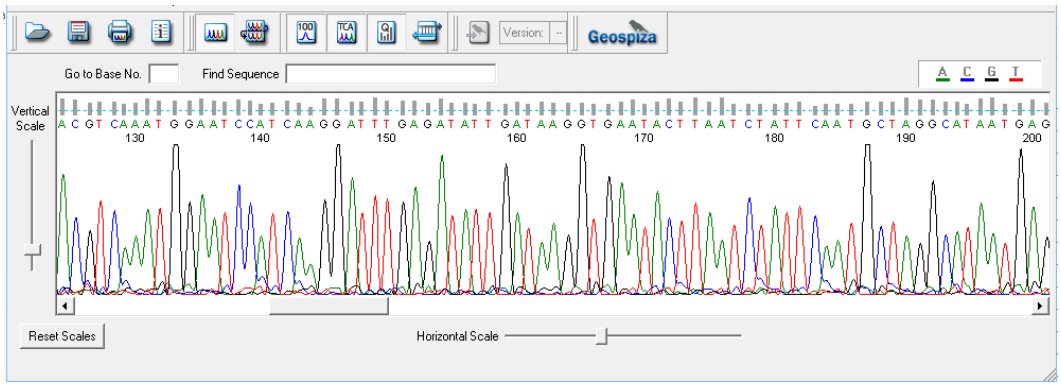

Figure A3: An example of chromatogram for trnV intron

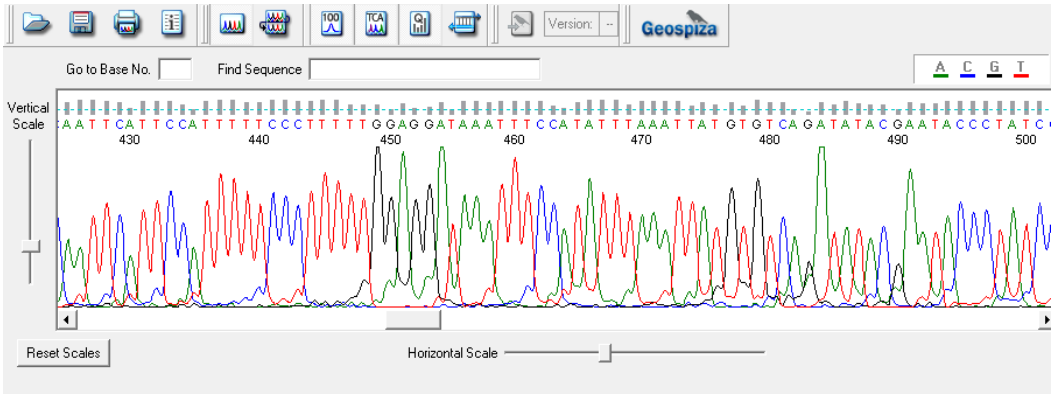

Figure A4: An example of chromatogram for matK (maturase Kinase)

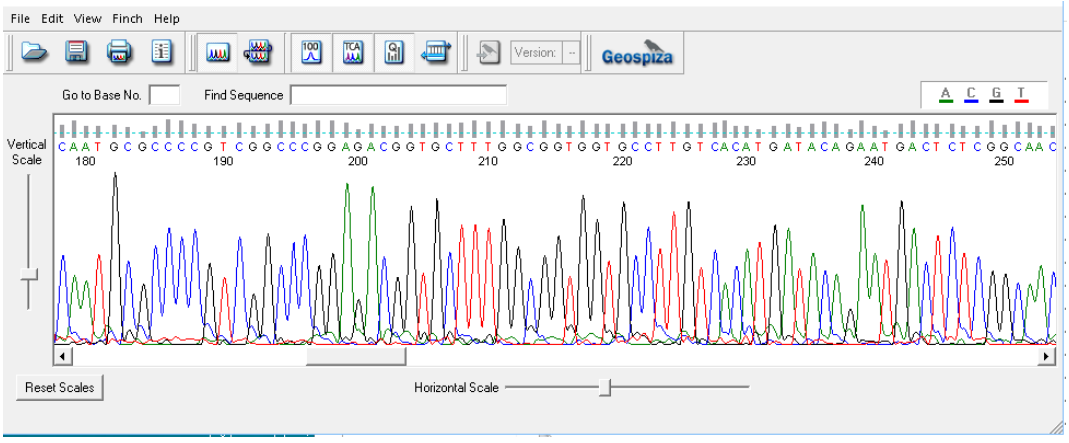

Figure A5 An example of chromatogram for ITS (Internal transcribed spacer)

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Ateş, Mevlüde Alev
Nationality: Turkish(TC)
ID nummber: 16189506196
Birth Date/Place: 17.02.1984, Antalya
Marital Status: Single
Phone: +903122105160/+905422906744
e-mail: ates.alev@gmail.com
aates@metu.edu.tr

EDUCATION

Degree	Institution	Year of Graduation
Ph.D.	METU Department of Biological Sciences	2017
M.Sc.	METU Department of Biological Sciences	2011
B.Sc	GAZİ UNV.Department of Biological Science Education	2007
High School	Metin Nuran Çakallıklı Anatolian High School	2002

WORK EXPERIENCE

Year	Place	Enrollment
2007-Present	METU Department of Biological Sciences	Research Assistant
2007-Present	METU Department of Biological Sciences	Teaching Assistant

FOREIGN lANGUAGES

Advanced English, Fluent German

PUBLICATIONS

1. Ozel Ç.A., Khawar K.M., Karaman S., **Ateş M.A**. and Arslan O., 2007.Efficient *in vitro* multiplication in *Ornithogalum ulophyllum* Hand.-Mazz. from twin scale explants. Scientia Horticulturae, Hortı 1684.

2. Atici T., Khawar K.M., Ozel Ç.A., Katircioglu H. and **Ates M.A**. 2008 Use of psyllium (isubgol) husk as an alternative gelling agent for the culture of prokaryotic microalgae (Cyanobacteria) *Chroococcus limneticus* Lemmermann and eukaryotic green microalgae (Chlorophyta) *Scenedesmus quadricauda* (Turpin) Brebisson. African Journal of Biotechnology Vol. 7 (8), pp. 1163-1167.

ORGANIZATIONS

Organizing Committee & Helper

a. Internatıonal Symposıum On Bıology Of Rare And Endemıc Plant Specıes 19-23 April 2014 Antalya, Turkey

b. Internatıonal Symposıum On Bıology Of Rare And Endemıc Plant Specıes 24-27 April 2012 Fethiye, Turkey

c. Internatıonal Symposıum On Bıology Of Rare And Endemıc Plant Specıes 26-29 May 2010 Fethiye, Turkey

Projects (researcher)

a. Revealing of Gentic Diversity of wild type Turkish Hazelnut (*CORYLUS COLURNA* L.) by using SSR microsatellite markers- Project of Western Black Sea Forestry Research Institute- Project number: 08.1602/2014-2017(2014-2017)

b. Revision of *Scrophularia* L. (Scrophulariaceae) in Turkey – TUBİTAK (The Scıentıfic And Technologıcal Research Councıl Of Turkey)Project number: 112T140 (2013-2017)

c. Revision of the sections *Macrophyllium* Bunge, *Hymenostegis* Bunge, *Poterium* Bunge, *Megalocystis* Bunge, *Halicacabus* Bunge and *Hymenocoleus* Bunge of *Astragalus* L. (Leguminosae) genus in Turkey-TUBİTAK (The Scıentıfıc And Technologıcal Research Councıl Of Turkey)Project number: 110T911 (2011-2014)

d. Molecular Phlogenetic Relationships and Genetic Variations of Turkish Firs(Aies spp.) Repuclic of Turkey General Directorate of Forestry, Forest Tree Breeding and Seeds Research Institute TÜBİTAK, Tarım Orman ve Gıda Teknolojileri Araştırma Grubu Proje No: TOVAG-109O717

PRESENTATIONS

Oral Presentations:

1. Phylogenetic relationship of six *Astragalus* sections based on ITS (Internal Transcribed Spacer) regions of genomic DNA- **Mevlüde Alev ATEŞ**, Seher KARAMAN ERKUL, Sertaç ÖNDE, Zeki KAYA- International Symposium on Biodiversity and Edible Wild Species (BEWS2017) 3-5 April 2017 Antalya, Turkey
2. Phylogenetic relationship of six Astragalus sections based on non-coding trn regions of chloroplast genome- **Mevlüde Alev ATEŞ**, Seher KARAMAN ERKUL, Sertaç ÖNDE, Zeki KAYA -Internatıonal Symposıum On Bıology Of Rare And Endemıc Plant Specıes 19-23 April 2014 Antalya, Turkey

3. The sequence data on non-coding trn region of chloroplast genomes of Turkish firs indicating the speciation from a single ancestral fir - **Mevlüde Alev ATEŞ**, Funda ÖZDEMIR DEĞIRMENCI, Yasemin TAYANÇ, Burcu ÇENGEL, Gaye KANDEMIR, Ercan VELIOĞLU, and Zeki KAYA - Internatıonal Symposıum On Bıology Of Rare And Endemıc Plant Specıes 26-29 May 2010 Fethiye, Turkey

Poster Presentations:

1. Taxonomic Revision of six Astragalus Sections That Native to Turkey based on three non-coding *trn* regions of cpDNA- **Mevlüde Alev ATEŞ**, Seher KARAMAN ERKUL, Sertaç ÖNDE, Zeki KAYA- SEAB 2017 The 3rd International Symposium on EuroAsian Biodiversity-5-8July-2017.

2. Phylogenetic Relatıonship Of *Scrophularia* Species Based On Non-Coding *trn* Regions and *mat*K Gene Region Of Chloroplast Genome - **Mevlüde Alev ATEŞ**, M. Erkan UZUNHİSARCIKLI, Ebru DOĞAN GÜNER, Sertaç ÖNDE, Murat EKİCİ , Zeki KAYA-Plant Biology Europe EPSO/FESPB 2016 Congress Prague, Czech Republic-June 26-30,2016.

3. Phylogenetıc Analyses Of Six Astragalus Sectıons Based On Noncoding Trn L'3-L'5 Region Of Chloroplast Genome- **Mevlüde ATEŞ**, Funda ÖZDEMİR DEĞİRMENCİ, Seher KARAMAN ERKUL2, Sertaç ÖNDE, Zeki KAYA - Internatıonal Symposıum On Bıology Of Rare And Endemıc Plant Specıes 24-27 April 2012 Fethiye, Turkey

REWARDS

- TÜBA(Turkish Science Academy) Translation- Mansition- Bioinformatics for Biologists- Chapter 5-2015.

GENERALRESEARCH INTERESTS

- Plant Molecular Genetics
- Plant Biology
- Plant Systematics
- Plant Tissue Culture
- Conservation Biology in Plants
- General Biology
- Teaching techniques in Biology

COMPUTER SKILLS

- Office Programs
- Internet
- MEGA (Molecular Evolutionary Genetics Analysis) Program versions
- Peak Scanner Program
- Finch TV sequence viewer Program
- R-package
- SPSS
- PAUP
- BEAST