EVOLUTIONARY RELATIONSHIPS AMONG *ASTRAGALUS* SPECIES NATIVE TO TURKEY

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ABSTRACT

EVOLUTIONARY RELATIONSHIPS AMONG *ASTRAGALUS* SPECIES NATIVE TO TURKEY

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Evolutionary relationships within and among three *Astragalus* sections (*Incani* DC., *Hypoglottidei* DC., and *Dissitiflori* DC.) that were native to Turkey were inferred from variations of nucleotide sequences of both chloroplast and nuclear genome regions.

In the current study, Fifty-six species included in the three *Astragalus* sections were utilized to figure out phylogenetic relationships and estimate evolutionary divergence time based on DNA sequence of *trnL* intron (*trnL5'-L3'*) , *trnL3'-F*$^{(GAA)}$ (*trnL-F* intergenic spacer), *trnV* intron, *matK* (*maturase kinase*) cpDNA (chloroplast) and *ITS* (*internal transcribed spacer*) nDNA (nuclear) regions.

Fifty-six *Astragalus* species with their replicas and one *Cicer* species as outgroup were analyzed by polymerase chain reaction amplification and DNA sequencing methods. Eleven unknown samples were also used in the current study to understand their section and species name. The results of the study indicated that unknown A35 and A52 samples could be named as *A. dasycarpus*, while unknown A65 and A66 samples as *A. ovatus* and lastly unknown A2 sample as *A. nitens* or *A. aucheri*. Section of unknown A3, A16, A20, A108, A109 and A110 samples were determined as *Incani*, but the exact species identification of these samples

were not possible because of their close phylogenetic associations with more than one species.

Highest genetic diversity was observed when the DNA sequences of *ITS* nrDNA (nuclear ribosomal) region comprising three subregions as *ITS1*, *5.8S* and *ITS2* was used, while the lowest one was calculated when DNA sequence of *trnL-F* cpDNA region was analyzed. The genetic divergence between *Incani* and *Dissitiflori* sections was highest whereas between *Hypoglottidei* and *Dissitiflori* was lowest based on all used regions.

To figure out phylogenetic relationships among *Astragalus* species distributed in Turkey and in other regions of the World, DNA sequences of studied regions of foreign samples were collected from the NCBI database and were evaluated with DNA sequence of Turkish species used in the curent study. The Iranian samples either scattered in the phylogenetic tree or attached to our samples externally. South and North American samples (New World *Astragalus* or Neo *Astragalus* group) were nested within a different subcluster, which was located in the main cluster produced by samples of Old World *Astragalus* group (Turkish samples). With these results, we can say that New World *Astragalus* group is monophyletic and diverged from Old World *Astragalus* group.

Evolutionary divergence time for *Astragalus* genus was estimated as about 12.5 - 14.5 million years (Ma), and that of New World *Astragalus* group as 5.0 - 4.0 Ma when rates of nucleotide substitutions of *trnL* intron and *matK* cpDNA regions were analyzed. In addition to evolutionary divergence time estimation for *Astragalus* and New World *Astragalus* group, divergence times among used three sections of the genus were also calculated by using DNA sequences of *trnL*, *trnV* intron and *matK* cpDNA regions and results indicated that *Hypoglottidei* and *Dissitiflori* sections diverged about 5.0-7.0 million years later than *Incani* section.

Key words: *Astragalus*, Phylogeny, *trnL* intron, *trnL3'-F*$^{(GAA)}$, *trnV* intron, *matK, ITS*

ÖZ

TÜRKİYE'DE DOĞAL OLARAK BULUNAN *ASTRAGALUS* TÜRLERİ ARASINDAKİ EVRİMSEL İLİŞKİLER

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Türkiye'de doğal olarak yayılış gösteren *Astragalus* cinsine ait üç seksiyonun (*Incani* DC., *Hypoglottidei* DC. ve *Dissitiflori* DC.) kendi içlerinde ve aralarında var olan evrimsel ilişki kloroplast ve nükleer genomda bulunan birkaç bölgedeki nükleotit dizi varyasyonlarıyla açığa kavuşturulmuştur.

Bu çalışmada, kloroplast DNA'sında bulunan *trnL* intron (*trnL5'-L3'*), *trnL3'-F*$^{(GAA)}$ (*trnL-F* intergenic spacer), *trnV* intron, *matK* (*matüraz kinaz*) bölgeleri ve nükleer DNA'da bulunan *ITS* (*internal transcribed spacer*) bölgesinin DNA dizileri, üç *Astragalus* seksiyonunda bulunan Elli altı türün filogenetik ilişkilerini anlamak ve farklılaşma zamanını belirlemek için kullanılmıştır.

Kopyaları ile birlikte Elli altı *Astragalus* türü ve dış grup olarak kullanılan *Cicer* cinsine ait bir tür, polimeraz zincir reaksiyonu ve DNA sekanslama metodu kullanılarak analiz edilmiştir. Ayrıca On bir tane tayin edilememiş örnek, her birinin seksiyonlarını anlamak ve isimlendirmesini yapmak için çalışmada kullanılmıştır. Çalışma sonunda tanımlanmamış A35 ve A52 örneklerinin *A. dasycarpus,* A65 ve A66 örneklerinin *A. ovatus* ve son olarak A2 örneğinin *A. nitens* veya *A. aucheri* olarak isimlendirilmesine karar verilmiştir. Tanımlanmamış A3, A16, A20, A108, A109 ve A110 örneklerinin seksiyonu *Incani* olarak

saptanmıştır. Fakat kesin isimlendirilmeleri birden fazla türle olan yakın filogenetik ilişkilerinden dolayı yapılamamıştır.

En yüksek varyasyon *ITS1*, *5.8S* ve *ITS2* alt bölgelerini kapsayan *ITS* nrDNA bölgesinin DNA dizisi kullanıldığında görülürken, en az varyasyon *trnL-F* cpDNA bölgesinin DNA dizisi analiz edildiğinde saptanmıştır. Analizi yapılan tüm bölgelerde genetik uzaklık *Incani* ve *Dissitiflori* seksiyonları arasında en fazlayken, *Hypoglottidei* ve *Dissitiflori* seksiyonları arasında en azdır.

Türkiye'de ve dünyanın diğer bölgelerinde dağılım gösteren *Astragalus* türlerinin filogenetik ilişkisini anlamak için yabancı örneklerin bu çalışmada kullanılan bölgelerinin DNA dizileri NCBI veri tabanından toplanmış ve çalışmada kullanılan Türkiye örneklerinin DNA dizileriyle beraber değerlendirilmiştir. İran örnekleri filogenetik ağaçta ya dağılım göstermiş yada bizim örneklerimize dışarıdan bağlanmıştır, Kuzey ve Güney Amerika'dan alınan örnekler (Yeni Dünya *Astragalus* veya Neo-*Astragalus*) Eski Dünya *Astragalus* örneklerinin oluşturduğu (Türkiye örnekleri) ana grupta alt grup olarak bir araya gelmişlerdir. Bu sonucu kullanarak Yeni Dünya *Astragalus* grubunun monofiletik olduğunu ve Eski Dünya *Astragalus* grubundan ayrıldığını söyleyebiliriz.

trnL intron ve *matK* cpDNA bölgelerindeki nükleotit değişimleri analiz edildiğinde *Astragalus* cinsinin evrimsel farklılaşma zamanı yaklaşık 12.5 - 14.5 milyon yıl (Ma), Yeni Dünya *Astragalus* grubunun evrimsel farklılaşma zamanı ise 5.0 – 4.0 Ma olarak tahmin edilmiştir. *Astragalus* ve Yeni Dünya *Astragalus* grubunun farklılaşma zamanın tahmin edilmesine ek olarak, kullanılan üç *Astragalus* seksiyonlarının farklılaşma zamanı da *trnL*, *trnV* intron ve *matK* cpDNA bölgeleri kullanılarak hesaplanmış ve sonuçlar *Hypoglottidei* ve *Dissitiflori* seksiyonlarının ayrışma zamanının *Incani* seksiyonunun ayrışma zamanından 5.0-7.0 milyon yıl sonra olduğunu göstermiştir.

Anahtar kelimeler: *Astragalus*, Filogeni, *trnL* intron, *trnL3'-F*$^{(GAA)}$, *trnV* intron, *matK, ITS*

To my love……

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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	Polyvinylpyrrolidone
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

Turkey has very special geographical, geological, topographical structures and different climatic types, which resulted three main phytogeographical regions as Euro-Siberian, Mediterranean and Irano-Turanian. The Irano-Turanian region is the largest one and covers Central and Eastern Anatolia of Turkey. Although this region is so wide and rich in herbaceous and perennial species, understanding of this region's structure is incomplete due to the difficulties of identification in some genera. *Acantholimon, Artemisia, Rosacea, Quercus* and *Astragalus* are among the several problematic genera. Understanding phylogenetic structures and relations of these complicated genera is not only invaluable for understanding plant diversity of the phytogeographic region, but also useful for revising Flora of Turkey, which has missing knowledge in some part.

Floristic structure of Turkey is always very attractive to botanists because of its high plant diversity. Studies performed up to now have shown that there are about 9000 species of vascular plants in Turkey and 2763 of them are endemic (Kaya and Raynal, 2001). Turkey has not only rich plant diversity, but also has two important genetic diversity centers (Middle East and Mediterranean) described by Vavilov (1951). The richness of Turkish flora is invaluable source for us to use these plants for different purposes. Many of them can be preferred as food for humans and animals as well as used as raw material to produce different types of drugs (Kaya et al., 1997).

The first study about floristic structure of Turkey is 'Flora Orientalis' published by Swiss botanist E. Boissier at 1867-88. The most important achievement showing floristic constitution of Turkey is *'Flora of Turkey and the East Aegean Island'* published by P.H. Davis at 1965-88. After publication of this book, it was realized that there are some difficulties in describing one species even genus during taxonomical studies. These difficulties were emerged during preparation efforts of *Flora of Turkey* because of limited time and materials. Sometimes these problems were expressed before publication, but no solution was found at that time. To get more reliable and better resources for botanist, these suspicious samples must be selected out or revised before they are used in further studies.

Taxonomists sometimes cannot agree with their colleagues about delimitation of a species. Morphological and/or anatomical traits are used to understand the level of one sample at the taxonomical hierarchy. However, sometimes these characters may not be useful because of their complex or similar structures. One of the problematic genera is *Astragalus* with the highest number of species in Leguminosae (Fabaceae) family. Turkey has the highest number of *Astragalus* species (425) after Russia (1000) and Iran (700) throughout the world. Therefore, botanist and other scientist need to revise this genus in Turkey. Recently, application of molecular techniques to clarify some unresolved issues within plant systematic studies become a very common procedure in biological sciences. One of the main purposes of this thesis is to understand and delimit molecular phylogenetic relationships between and within three main sections (*Incani* DC., *Hypoglottidei* DC., and *Dissitiflori* DC.) of *Astragalus* L. genus by using different genomic and chloroplast DNA regions.

1.1 Description and Morphology of *Astragalus* Genus

Astragalus is included in Legume family which is the third largest family of flowering plants (Mabberley, 1997) with about 730 genera and more than 19400 species (Lewis et al., 2005). This family comprises many species and varieties that are consumed as food and used to produce fiber, oil, timber, various chemicals

and medicines. Moreover, species included in this genus fulfill an important function; that is, nitrogen fixation by using symbiotic bacteria.

Astragalus is a large genus of flowering plants with more than 2,500 species of herbs and small shrubs and more than 250 sections in the family Leguminosae. They can be annual or perennial, up to 200 cm, with stemmed or stemless structure. Leguminous flowers with banner, wing, keel structures and alternate, pinnately compound leaves are other characteristics of the genus. They have bladder-like pods with various sizes and shapes as fruit with kidney-shaped seeds. Some species have so hard seed coat that they can survive in the soil for 40 years or longer. (Figure 1.1, 1.2 and 1.3).

Figure 1.1 Leguminous flower (banner, wing, keel) structure of *Astragalus sp*.
(http://science.halleyhosting.com/nature/basin/5petal/pea/astragalus/succumbens.html)

Figure 1.2 Leaves of *Astragalus cicer*, which are arranged alternately on the stem and are pinnately compound.
(http://www.uwyo.edu/plantsciences/UWplant/Forages/Legume/cicer-milkvetch.html)

Figure 1.3 Fruits, bladder-like pod structure, of *Astragalus cicer*.
(http://www.uwyo.edu/plantsciences/UWplant/Forages/Legume/cicer-milkvetch.html)

1.2 Taxonomy of *Astragalus* Genus

Although there are several studies about taxonomical structure of the genus, a complete monograph is not available. This may be due to rich spices diversity of the genus, diversity of habitat, size of the plants, structure of stipules, leaf rachis, inflorescence, length and structure of sepal and petals (Chaudhary et al., 2008).

Some *Astragalus* species possessing different specializations or unique characteristics have been separated from others and produced new genera. Tournefort in 1700 named genus *Tragacantha* that included about 300 morphologically diverse species distributed throughout southwest and southcentral Asia and qualified by even-pinnate leaves and unilocular pods with 1-2 seeded fruits (Podlech, 1983; Engel, 1991) (Figure 1.4). However, species of *Tragacantha* were moved into *Astragalus* genus by Zarre and Podlech (1997).

One of the classical problems of *Astragalus* taxonomy is the relations between *Astragalus* and *Phaca* genus that was distinguished from *Astragalus* species by its inflated unilocular pod structure. Unilocular pod is an uncommon character within *Astragalus* species so species with this character state were separated and called as genus *Phaca* by Linnaeus (1753). Earlier, Tournefort named *Phaca* species as *Astragaloides* and a century later Bunge (1868 and 1869) claimed *Phaca* as a

subgenus of *Astragalus*. Moreover, Gray (1864) quitted the distinction between these two genera completely due to extreme variations in the septum characters of the pod structures. Lastly, Barneby (1964) showed taxonomical history of *Phaca* genus and separated this genus from *Astragalus* (Figure 1.4).

As seen in Figure 1.4, different nomenclatures were used for each taxon by several plant systematists at different times. This complex relationships among samples can be an indicator how *Astragalus* and other close genera are morphologically very similar to each other.

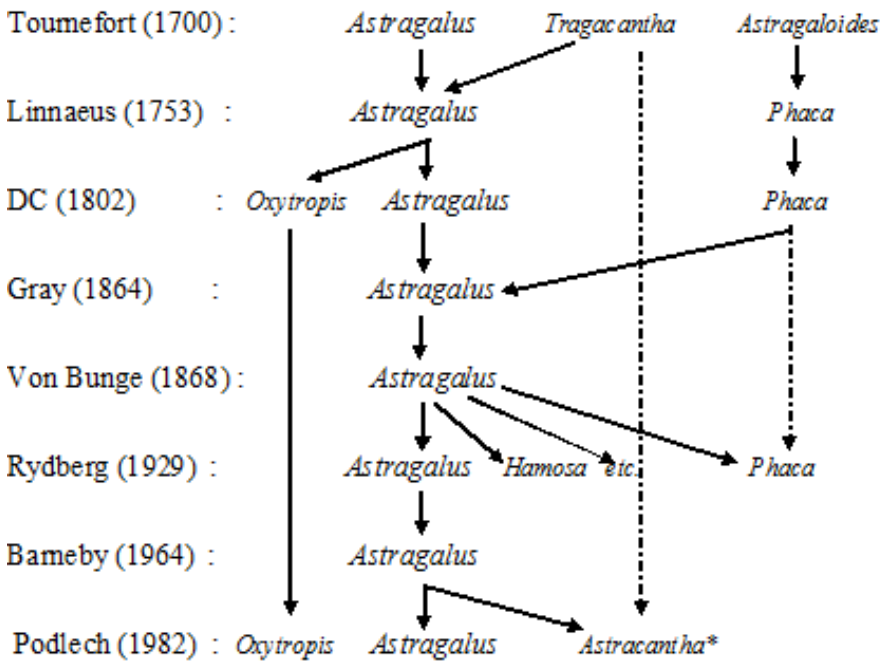

Figure 1.4 Taxonomical history of *Astragalus* genus and related genera.
Conservation of a taxon during history is indicated by solid arrows while rearragement is shown by dashed-arrows. * Zarre and Podlech (1997) combined *Astracantha* samples with *Astragalus* taxon (Wojciechowski et al., 1999).

A summary for the taxonomic status of *Astragalus* 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

Like most of the close relatives in the tribe Galegeae (Polhill, 1981a and 1981b), this genus was originated from Eurasia and mountains, steppes of southwestern Asia and Himalayan plateau. Species of this genus are generally distributed in arid and semi-arid mountainous regions of the northern Hemisphere and Andes of South America (100 spp.) and East Africa with limited numbers. This genus has the highest diversity in the Irano-Turkish region of Southwestern Asia, and the Sino-Himalayan Plateau of southcentral Asia (1500-2500 spp.), the Great Basin and Colorado Plateau of western North America (400-450 spp.) (Podlech, 1986).

1.4 Chemical Composition and Significance of *Astragalus* Genus

Some of the *Astragalus* species, especially non-toxic ones, are used for medical purposes. Chemical substances are generally isolated from their roots. To get these chemicals efficiently, these plants are allowed to mature for about four to seven years. Samples of this genus contain many secondary metabolites comprising amino acids, polysaccharides, triterpene glycosides, flavonoids, isoflavones, saponins and trace minerals (Yin et al., 2009). Many of these chemicals are used for medical purposes. In addition to non-toxic and handy

chemicals, many species of *Astragalus* also contain toxic compounds such as swainsonine (Davis et al., 1984), 3-nitropropanol (Tunez et al., 2010) and selenium (Somer and Caliskan, 2007). If these substances were taken in high amount by animals during grazing time, they could suffer from crazy illness and even die.

Especially in the U.S.A locoweed term is used to call some samples of this genus. Locoweed gets its name from loco (crazy) term that is coming from Spanish. Locoweed samples have poisonous chemicals (high amount of indolizidine alkaloid swainsonine, 3-nitropropanol etc.) causing neurological damage and depression in animals. However, not all species of *Astragalus* are toxic. Some of them are very useful to feed animals. The most common one preferred as livestock forage, is *Astragalus cicer* known as soil builder.

1.5 Uses of *Astragalus* Genus

Most of the species of *Astragalus* genus have considerable value in medicine especially in China. The main part of plant used for medicinal purposes is root. Traditional Chinese medicine has used some of *Astragalus* genus to remedy some illnesses such as weakness, respiratory problems, diabetes, AIDS (Liu et al., 2003), high blood pressure etc. Some chemicals extracted from this genus are also useful to inhibit the spread and growth of cancer cells (Tin et al., 2007).
Certain *Astragalus* species such as *A. bisulcatus* (Quinn et al., 2008) and *pattersoni* (Cannon, 1971) accumulate relatively large amount of selenium from soil so these species are called as indicator species for selenium and can be used to indicate which soil is candidate for selenium poisoning.

"Gum tragacanth" is extracted from some *Astragalus* species such as *A. gummifer* distributed around southcentral Asia. This substance is obtained from root and stem parts of plants and used for fabrication of lotions, ice creams,

pharmaceuticals due to colloidal and hydrophilic properties. In Turkey, especially *A.microcephalus* is used to produce gum tragacanth (Dogan et al., 1985).

1.6 The Genus *Astragalus* in Turkey

Astragalus L. with 425 species in 62 sections is the largest genus in Turkey (Davis et al., 1988; Duran and Aytac, 2005; Ekici et al., 2008). The distributions of the genus scatter at mountains and steppes in Irano-Turanian region of Turkey and comprising about 210 endemics (Duman and Akan, 2003; Martin et al., 2008).

After revision of the genus by Chamberlain and Matthews in 1970, Davis et al., (1988) and Aytac (2000) identified fourty-one additional species which were native to Turkey. Moreover, Podlech (1999; 2001) and Podlech and Sytin (2002) described thirty-three new species. In addition to these studies, various scientists (Akan and Civelek, 2001; Aytac et al., 2001; Ekici and Aytac, 2001; Aytac and Ekici, 2002; Hamzaoglu 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) contributed to improve the taxonomy of *Astragalus* genus.

1.7 Molecular Phylogeny

Astragalus genus has not only high number of species, but also it is considered as one of the most taxonomically complex and diverse genus in Leguminosae family. It has been recently realized that the use of only morphological characters for some species of this genus may not be enough to decide systematic relations of the sample within the genus. Therefore, molecular markers are preferred to solve taxonomical problems faced during systematical studies of the genus.

New introduced techniques in the molecular field and gene studies are very helpful for rapid and certain decision of phylogeny among plant species (Sareela et al., 2007). Many molecular studies have been carried out in the *Astragalus* genus. Different coding and non-coding regions from both nuclear and chloroplast DNAs were studied. For example, nuclear ribosomal DNA internal transcribed spacer (*ITS*) (Wojciechowski et al., 1993), Expressed Sequence Tags (*EST*), *matK* (Hilu et al., 2003), *trnL-F* (Walker and Metcalf, 2008), *rpoC1* and *rpoC2* (Liston and Wheeler, 1994), Amplified Fragment Length Polymorphism (*AFLP*) (Kingston and Rosel, 2004) and Restriction Fragment Length Polymorphism (*RFLP*) (Tansksley et al., 1989), were some of the molecular systems which were extensively used for molecular systematic studies.

Astracantha Podl. genus with 214 species, having less amount of inflorescence and unilocular pods, was separated from *Astragalus* genus by Podlech (1983). After that, Zarre and Podlech (1997) combined *Astracantha* with *Astragalus* genus by using morphological and anatomical properties. The systematic of *Astracantha* has been further improved by a molecular study based on cpDNA region, *trnL* intron, and nrDNA *ITS* region (Wojciechowski et al., 1999).

Oxytropis genus is one of the very close relative to *Astragalus* genus. Firstly, *Oxytropis* species were thought within *Astragalus* genus by Linnaeus. After that, De Candolle in 1802 separated *Oxytropis* samples from *Astragalus* by using keel structure (pointed) of the flower and pod septum (arising from adaxial suture) (Chaudhary et al., 2008). Wojciechowski and his coworkers (1999) and Wojciechowski (2005) proved that *Oxytropis* is monophyletic and not included in *Astragalus* genus by using again sequences of *trn*L intron and *ITS* regions.

Diversity within *Astragalus* genus was also studied by using molecular techniques (Sanderson and Wojciechowski, 1996; Wojciechowski et al., 1999; Dong et al., 2003; Wojciechowski, 2005). These studies indicated that species of *Astragalus* genus have more variation rates compared with other flowering species. These authors used sequences of nuclear and chloroplast DNA regions to depict

phylogenetic relationships among *Astragalus* species. Results obtained based on sequences of nuclear genome regions may be more useful to understand evolutionary structure of this genus because nuclear genome has more sequence variation with respect to DNA sequences of chloroplast genome. However, sequence of each DNA region whether it is located on nuclear or chloroplast genome has importance to figure out phylogenetic relations within *Astragalus* genus.

1.7.1 Studied Chloroplast and Genomic DNA Regions During the Study

Numerous regions of nuclear and chloroplast DNA are available to construct reliable phylogenetic trees for plant species. Therefore, the scientists focused on phylogeny of angiosperms have preferred to reclassify all families of flowering plants with the use of the DNA-based phylogenetic trees. There are some reports indicating that DNA sequences of each region evolve differently. For instance, nuclear genome evolution rate is the fastest one while evolution of plastid DNA is the slowest (Savolainen and Chase, 2003).

Genome sequences of an individual have been evolving by the influences of mutation and fixation in the population. The rate of substitution between homologous sequences often demonstrates the evolutionary distinctiveness of an organism with respect to the other; so this information can be useful to construct phylogenetic trees. To construct a phylogenetic tree for many eukaryotes and particularly for plants, analyses of multigenes (combined molecular data) are very functional. Therefore during the study not only chloroplast DNAs, but also genomic DNAs were used to figure out phylogenetic relationships among species of three main sections of *Astragalus* genus.

1.7.1.1 Chloroplast DNA (cpDNA) Regions

To study phylogenetic and evolutionary relations between species or higher/lower taxa, DNA sequence data have been preferred because this data provide confidential knowledge about evolutionary relationships at different taxonomical levels. Regions located on chloroplast DNA are very useful for evolutionary and phylogenetic studies, because these regions have some advantages in plant systematics. These advantages are:

1- Chloroplast genome is a relatively abundant component (many identical copies) compared with total DNA providing simple amplification during PCR application.
2- It is haploid and does not have allelic variation because generally uniparentally inherited and, so intra-individual (allelic) variation is absent (Small et al., 2004).
3- Composition and order of chloroplast genes are highly conserved especially in flowering plants.

Because of these advantages, use of chloroplast DNA to find out phylogenetic relationships and elucidate questions about the phylogenetic history of major groups is becoming a flourishing field (Clegg and Zurawski, 1992).

To amplify non-coding regions of the cpDNA, many universal primers were designed by using evolutionary conservation feature of a gene sequence (Taberlet et al., 1991; Shaw et al., 2007) so diverse introns and/or intergenic spacers of the chloroplast DNA are being preferred to clarify evolutionary structure of numerous species or taxa. In addition to several advantages, few disadvantages are also there. One of them is the carrying of chloroplast from both parents for some species (Corriveau and Coleman, 1988) and the other is the deficiency of knowledge to understand polyploidy and hybridization events if chloroplast is also inherited uniparently (Small et al., 2004). Several conflictions about inheritance pathway of cpDNA in *Astragalus* species are there; Corriveau and Coleman (1988) stated that this genus has maternal transmission, while Zhang et al., (2003)

indicated that biparental cytoplasmic inheritance was recognized in two *Astragalus* species. In the current study, four different areas, *trnL5'-L3'* (*trnL* intron)*, trnL3'-F* $^{(GAA)}$ (*trnL-F intergenic spacer*), *trnV* intron and *matK* (*maturase Kinase*) of chloroplast genome were used. More information about them has been provided below.

1.7.1.1.1 *trnL5'-L3'* (*trnL* intron) and *trnL3'-F* $^{(GAA)}$ (*trnL-F* intergenic spacer) Regions

The non-coding regions demonstrate highest mutation frequency so they can be chosen for evolutionary-relationship analysis (Taberlet et al., 1991). One of them is t-RNA (*trn*T-*trn*F) region which is the most prevalently examined cpDNA part because of its wide-ranging use to address phylogenetic relationships especially under the family level (Taberlet et al.,1991; Kelchner 2000). This region consists of *trn*L $^{(UAA)}$ gene and two flanking intergenic spacers (IGS); *trnT-L* and *trnL-F*.

Anticodon loop of $tRNA^{Leu(UAA)}$ is encoded by a single copy region of the chloroplast genome and this region is interfered by *trnL* intron. The *trnL* gene comprises group I intron positioned between the U and the A of the UAA anticodon loop. The area of this intron, interrupting the anticodon of the $tRNA^{Leu}$ $^{(UAA)}$ gene (U-intron-AA), is conserved from cyanobacteria to plant chloroplasts (Kuhsel et al., 1990). Firstly, Taberlet et al., (1991) introduced this region's markers and since then the *trnL* intron and *trnL-F* intergenic spacer regions have been preferred frequently for investigations at various taxonomic levels. After introduction of these regions for phylogenetic studies, lots of scientist such as Gielly and Taberlet (1996), Ohsako and Ohnishi (2000), and Mummenhoff et al., (2001) have used these genetic markers especially to elucidate interspecific relationship.

trnL intron (*trnL5'-trnL3'),* and *trnL-F* intergenic spacer [(*trnL3'-trnF* $^{(GAA)}$] regions are called as *trnL-F* region. This region is very useful because conserved

trnL genes, a group I intron, and the *trnL-F* intergenic spacer are located in it. In plants, *trnL* intron region generally contains conserved sequence in the regions flanking both *trnL* exons [*trnL* $^{(UAA)}$ *5'* exon and *trnL* $^{(UAA)}$ *3'* exon], while the central part is highly variable (*trnL* intron) compared with exonic regions (Bakker et al., 2000). The *trnL-F* intergenic spacer is highly variable non-coding region in respect to composition and length of region between and within plant species (Bohle et al., 1994; Gielly and Taberlet, 1994; Ham et al., 1994; Mes and Hart, 1994). Both *trnL* intron and the *trnL-F* spacer have tandem repeats that form stem–loop structures, and sequences of *trnL-F* spacer contain promoter elements for the *trnF* gene (Hao et al., 2009).

Although the *trnT-trnF* region contains high substitution rate in many plant species (Bayer and Starr 1998; Bakker et al., 2000; Mansion and Struwe 2004), this region in some holoparasitic plants was lost partly or completely so *trnT-L* and *trnL-F* IGS regions show great sequence divergence and large deletions (Freyer et al., 1995; dePamphilis et al., 1997; Lohan and Wolfe, 1998).

1.7.1.1.2 *trnV* intron Region

Deno et al., (1982) first sequenced a gene of chloroplast encoding tRNA^{UAC} (*trnV*) from the chloroplast DNA of *Nicotiana tabacum* (tobacco). It was proven that this region includes a group II intron (Keller and Michel, 1985). After that, Clegg et al., (1986) studied this region with four plant species (tobacco, maize, barley and garden pea) to compare *trnV* introns of them. They found 12 sequence blocks that differed in levels of substitutions. Substitutions and indels (deletion/insertion) are used to evaluate phylogenetic relationships between and/or within plant species.

Non-coding sequences tend to evolve faster compared with coding sequences so they may provide useful information to get a tree showing phylogenetic relationships. Thus, the *trnV* intron was selected to realize phylogenetic relations between species of *Astragalus*.

Most scientist compared the DNA sequences of *trnL* intron and *trnV* intron regions by using different species of angiosperms, and concluded that mainly six different domains evolving with different rates were included in their DNA sequences. These special domains are thought to be responsible for the formation of secondary structure of the regions that are needed for the functions of intronic regions (Clegg et al., 1986; Learn et al., 1992; Fangan et al., 1994). As like non-coding intergenic spacer regions, intronic regions evolve faster due to the affects of nucleotide substitutions and indel mutations, which are very useful to understand phylogenetic structures between species. However, mentioned regions may not be useful to depict phylogenetic relations between higher taxa because of too many nucleotide substitutions in the sequences. (Olmstead and Palmer, 1994).

1.7.1.1.3 *matK* (*maturase Kinase*) Gene Region

The *trnK* intron includes *matK* gene encoding a maturase-like protein (Neuhaus and Link, 1987), and two non-coding regions flanking both sides of the *matK* gene (*trnK5'-matK-trnK3'*). This gene was first discovered in tobacco (Sugita et al., 1985), and then it was named as *matK* in mustard based on its similar composition and structure to mitochondrial Group II intron encoded maturases in yeast (Neuhaus and Link, 1987). Except one of chloroplast introns, all others are found in group II and III subclasses (Michel et al., 1989; Sugiura, 1992). As mentioned above in *trnL-F* region section, the only exception is $tRNA^{Leu}$ intron which is included in group I intron coming ancient origin dated back to cyanobacteria (Kuhsel et al., 1990; Besendahl et al., 2002).

The *matK* gene has two exclusive properties, which emphasize its value in molecular biology and evolution. One of them is its fast evolutionary rate and the other is its function as a group II intron maturase. It has about 1500 bp length with its flanking regions of few hundred base pairs of DNA on each side, but in the current study, partial part (about 1200 bp) of the gene, which is near to 5' end was used. The sequence close to the 3' end of this gene is highly conserved; number of

substitutions and indels are lower with respect to 5' end of the gene (Hilu and Liang, 1997), so the left sequence near to the 3' end was not sequenced. *MatK* gene comprises high substitution rates within species so it is a potential candidate to study evolution and plant systematics. This gene has also high amount of variation at nucleic acid level of codon position, and low transition/transversion ratio. Because of these convenient properties, the *matK* gene can be used to work out family and species level relationships (Selvaraj at al., 2008).

The mode and speed of *matK* evolution are different from other genes of chloroplast genome. Substitution rate of this gene is three times higher than that of the large subunit of Rubisco (*rbcL*) (Johnson and Soltis, 1994; Olmstead and Palmer, 1994), expressing it as a rapidly evolving gene. This substitution rate supplies high signal to realize evolutionary relationships between plants at all taxonomic levels (Soltis and Soltis, 1998; Hilu et al., 2003). Huli and his colleagues (2003) reported that getting an idea with sequences of *matK* gene is equal to using about eleven other combined gene regions. The same group also confesses that there are changes in number and size of insertion/deletions on this region in addition to substitutions.

1.7.1.2 Genomic DNA Region

Molecular systematists have begun to use non-coding spacers located on nuclear ribosomal DNA (nrDNA) when they recognized a wide range of phylogenetic trees constructed from regions of cpDNA. The nrDNA involves abundant copies, which are facilitating amplification of the region and has high evolution rate that is very useful to see relations between lower taxonomic levels (Small et al., 2004).

1.7.1.2.1 *ITS* (*Internal transcribed spacer*) Region

Eukaryotic ribosomal RNA genes are called as ribosomal DNA (rDNA) and are located on nucleolar organizing regions (NORs) of the chromosomal structure. Each gene unit contains a transcribed region including gene for *18S* (small subunit of ribosome), *5.8S* and *26S* (large subunit of ribosome) rRNAs and external transcribed spacers (*ETS*). This unit also has another structure that is located on either side of *5.8S* rRNA gene. They are named as *ITS1* and *ITS2*. Young rRNA transcript comprises *5' ETS, 18S rRNA, ITS1* (~230 bp), *5.8S rRNA* (~180 bp), *ITS2* (~220 bp), *26S rRNA* and *3'ETS*. At maturation stage of rRNA, *ETS* and *ITS* pieces are removed and degraded. The *18S* and *26S* coding regions have been preferred to show phylogenetic relations at the family or higher taxonomic levels in plants (Zimmer et al., 1989). However, DNA sequences of *ITS* region seem to be proper for assigning relations at lower taxonomic levels such as genera or species because evolving rates of spacer regions are higher compared that of coding regions (Suh et al., 1993).

DNA sequences of both *ITS1* and *ITS2* subregions are widely preferred for taxonomical and phylogenetical studies of plant species because this region can be easily amplified due to the high copy number of rRNA genes. Because of this feature, interested regions (*ITS1* and *2*) are smoothly amplified even low quantity of DNA is available. The other advantage of this region is the level of variation. The DNA sequences of this region are fairly conserved within species, but variable between them. This property is very convenient to understand taxonomical relationships between species (Hillis and Dixon, 1991). Although some researchers have hesitations about the utility of the region for phylogenetic studies due to paralogous regions of rDNA (Li et al., 2004). Nevertheless, this region still can be very useful when combined with sequences of nuclear or chloroplast regions (Kenicer et al., 2005).

CHAPTER 2

JUSTIFICATION OF THE STUDY

'Flora of Turkey and *the East Aegean Islands'* was published by P.H. Davis in nine volumes from 1965 to 1988 (Davis, 1965-1988). Because of special geographic and climatic structure, Turkey has high biodiversity compared with neighbouring countries. Therefore, biodiversity of Turkey attracts many local and foreign scientists. New taxa have been added or decided taxonomical levels of them have been changed since publishing of *Flora of Turkey*. Almost all of these studies were completed by using morphological and sometimes anatomical characters. Actually, these methods can be very useful and sufficient to understand taxonomical level of a studied sample. However, some taxa are not suitable as morphologically to understand their level in the taxonomy since morphological characters are easily affected by environmental factors (Cai et al., 1999). These morphological differentiations between plant samples can cause paralogism during classification studies. To solve this problem and construct reliable phylogentic trees, taxonomists should carry out new classification systems and further revision studies.

Different molecular techniques provide encouraging approaches for taxonomic and evolutionary studies of both plant and animal species. For phylogenetic studies, not only molecular markers such as RFLP and AFLPs (Tansksley et al., 1989), but also sequencing of several genomic (Zhang et al, 2008; Blattner, 2004) and chloroplast (Petersen, 1997) DNA regions can be used. Among these, chloroplast genome regions have been preferred to others because of their wide-ranging use to address phylogenetic relationships especially under the family level

(Taberlet et al., 1991; Kelchner, 2000). Noncoding regions of chloroplast genome extend usefulness of molecular techniques at lower taxonomic levels because of rapid evolution of these region sequences with respect to that of coding sequences. High insertion/deletion rates and nucleotide substitutions observed through noncoding chloroplast DNA regions have caused high evolutionary rates (Clegg and Zurawski, 1992). The *matK* region, even if it has coding sequence, was also useful for the phylogenetic studies because it is the most rapidly evolving plastid gene which provides valuable information to figure out phylogenetic relationships at the intrageneric level (Yang et al., 2004). The *ITS* region, located within nuclear genome, is also helpful to understand phylogenetic relationships among species since the sequence of the region carries long and tandem repeating units in higher plants (Appels and Dvorak, 1982) and contains highly conserved sequences, which are also very useful for evolutionary studies.

Taxonomic uncertainties regarding to *Astragalus* species and insufficient morphological cladistic analysis of the genus indicated need for additional molecular studies for this genus (Wojciechowski, 1993). Therefore, in the current study, both chloroplast (*trnL* intron, *trnL-F* intergenic spacer, *trnV, matK*) and genomic DNA regions (*ITS*) were chosen to be studied inorder to shed light on phylogenetic and evolutionary relationships among species included in three main *Astragalus* sections; *Incani* DC., *Hypoglottidei* DC., and *Dissitiflori* DC. Combined molecular data-set to understand phylogenetic relationships among *Astragalus* genus are rare. Therefore, in the current study, five different genomic and chloroplast regions (large multi-gene/sequence) were preferred to construct phylogenetic trees of *Astragalus* genus.

CHAPTER 3

OBJECTIVES OF THE STUDY

1- To elucidate interspecific phylogenetic and evolutionary relationships among and within three sections (*Incani* DC., *Hypoglottidei* DC., and *Dissitiflori* DC.) of *Astragalus* genus that are naturally distributed in Turkey by using *trnL5'-L3' (trnL* intron*), trnL3'-F* intergenic spacer, *trnV* and *matK* cpDNA regions and *ITS* (*internal transcribed spacers*) genomic DNA regions.

2- To compare and test usefulness of sequence analysis, which were obtained from chloroplast and nuclear genome regions of *Astragalus* species.

3- To provide new information and perspectives for taxonomists and systematists by using molecular diversity data to evaluate taxonomic categories of *Astragalus* species that are closely related to each other and with some taxonomic problems.

CHAPTER 4

MATERIALS AND METHODS

4.1 Plant Materials

All of the studied samples included *Incani* DC., *Hypoglottidei* DC., and *Dissitiflori* DC., sections were collected by Prof. Dr. Murat Ekici during field studies from 2006 to 2009 of the research project (TBAG-106T284) supported by the Scientific and Technical Research Council of Turkey (TUBITAK). In addition to collected fresh specimens, several missing samples were also supported by him through herbarium of Gazi University.

For each species, at least three samples from different areas were utilized to have adequate representation of the species and to provide more informative results at the species level. These samples were picked not only from the provinces in which the occurrence of the species recorded in *Flora of Turkey,* but also were collected from new locations in Turkey. To keep the leaf samples fresh, collected samples were kept in a small sandwich bag containing dry silica gel pellets during field trip and until they were brought to the laboratory.

The genus *Astragalus* has about 425 species in Turkey, which is the largest species number for genus *Astragalus* after Russia (1000) and Iran (700). Moreover, 201 species (48.20%) of the total *Astragalus* species are endemic to Turkey. Due to endemism or other special factors, if a specimen was not collected for a species, the leaf samples were provided by the Gazi University Herbarium. All the collected species with their sections, and voucher numbers were provided in Table 4.1.

Table 4.1 Studied species of three sections of *Astragalus* genus collected from different locations of Turkey. The names in parenthesis after the species name indicate synonymous.

Section	Code	Species name	Location (Province/Town)
	97/ME3835	*A.achundovii*	Hakkari /Cukurca
	109 (B)	*A.achundovii*	Hakkari /Cukurca
	142/3877	*A.achundovii*	Hakkari/Cukurca
	89/H5849	*A.ancistrocarpus*	Sanlıurfa/Viransehir
	143/5784	*A.ancistrocarpus*	Sanlıurfa/Birecik
	143B/5784	*A.ancistrocarpus*	Sanlıurfa/Birecik
	21/ME3679	A.*brachycarpus*	Van/Ercek
	34/ME3682	*A.brachycarpus*	Van/Ercek
	115/3765	*A.brachycarpus*	Erzurum /Tortum
	43/ME3594	*A.brevidentatus (geocyamus)*	Kastamonu /Tosya
	46/ME3594	*A.brevidentatus (geocyamus)*	Kastamonu /Tosya
	113/3751	*A.brevidentatus(geocyamus)*	Ankara/Sereflikochisar
	96/ME3836	*A.campylosema (fodinarum, pendulus)*	Elazıg /Maden/ Degirmendere
	31/ME 3648	*A.campylosema (fodinarum, pendulus)*	Erzurum/ Tortun
	15/ME3607	*A.campylosema (fodinarum, pendulus)*	Sivas /Zara/ Divrigi
	32/ME3657	*A.campylosema (fodinarum, pendulus)*	Agri /Homur /Tutak
	74/ME3772	*A.campylosema (fodinarum, pendulus)*	Antalya /Korkuteli /Mamatlı
	73/ME3771	*A.cariensis*	Mugla /Yilanli mtn.
	126/3821	*A.cariensis*	Mugla /Yilanli mtn.
	73B/ME3771	*A.cariensis*	Mugla/Yilanli mtn.
	23/ME3647	*A.cinereus*	Erzincan /Tercan /Askale
	56/ME3646	*A.cinereus*	Erzincan/ Tercan /Askale
	59/ME3650	*A.cinereus*	Erzurum /Tortun
	95/HA5853	*A.clavatus*	Mardin/ Bakirkiri
	95B/HA5853	*A.clavatus*	Mardin / Bakirkiri
	95C/HA5853	*A.clavatus*	Mardin / Bakirkiri
	7/SK2073	*A.czorochensis*	Erzurum /Hınıs
	26/ME3719	*A.czorochensis*	Artvin prison
	29/ME3714	*A.czorochensis*	Artvin/Ortakoy /Perta
	133/3869	*A.czorochensis*	Artvin prison
	4/SK2050	*A.elongatus*	Sivas /Akkısla/Alaman
	68H5654	*A.elongatus*	Sanliurfa/Birecik
	116/3779	*A.elongatus*	Kastomonu /Tosya
	24/ME3717	*A.frickii*	Artvin/Kafkosor
	112/3767	*A.frickii*	Artvin /Kafkosor
	112B/3767	*A.frickii*	Artvin/Kafkosor

INCANI

Table 4.1 continued

	134/3813	*A.germanicopolitanus (barbarae)*	Cankiri/Ilgaz
	18/ME3592	*A.germanicopolitanus (barbarae)*	Cankiri /Ilgaz
	75/ME3773	*A.germanicopolitanus (barbarae)*	Cankiri /Ilgaz
	19/ME3606	*A.glaucophyllus*	Sivas/Hafik
	38/ME3605	*A.glaucophyllus*	Sivas /Hafik
	144/3863	*A.glaucophyllus*	Sivas /Hafik
	77/ME3780	*A.humillimus*	Kastamonu/Tosya
	131/3849	*A.humillimus*	Kastamonu /Tosya
	145/3816	*A.humillimus*	Kastamonu /Tosya
	10/SK2091	*A.latifolius*	Agrı/Dogu Beyazit
	62/ME3688	*A.latifolius*	Van/Ozalp
	120/3709	*A.latifolius*	Erzurum /Oltu/Senkaya
	44/ME3617	*A.longisubulatus*	Erzincan /Kemaliye/Arapgir
	146/3747	*A.longisubulatus*	Erzincan /Kemaliye/Arapgir
INCANI	146B/3747	*A.longisubulatus*	Erzincan /Kemaliye/Arapgir
	108(A)	*A.micrancistrus*	Van/Baskale
	135/3830	*A.micrancistrus*	Van/Baskale
	147/3758	*A.micrancistrus*	Van/Baskale
	138	*A.nezaketae*	Erzincan/Uzumlu
	138B	*A.nezaketae*	Erzincan/Uzumlu
	138C	*A.nezaketae*	Erzincan/Uzumlu
	8/SK2087	*A.olurensis*	Artvin/Ishan
	79/ME3794	*A.olurensis*	Artvin/Yusufeli
	148/3716	*A.olurensis*	Artvin/Yusufeli
	25/ME3685	*A.polhillii*	Van/ Ercek
	122/3683	*A.polhillii*	Van /Ercek
	124/3683	*A.polhillii*	Van/ Ercek
	45/ME3601	*A.sanguinolentus*	Sivas /Zara/Divrigi
	71/ME3766	*A.sanguinolentus*	Artvin
	149/3850	*A.sanguinolentus*	Yozgat/Aydincik
	80/ME3781	*A.scabrifolius*	Malatya/Darande
	83/ME3782	*A.scabrifolius*	Malatya/Darende
	98	*A.scabrifolius*	Malatya/Darende
	127/3837	*A.scabrifolius*	Malatya/Darende
INCANI	136/3824	*A.scabrifolius*	Malatya/Darende
	87/ME3819	*A.schizopterus*	Denizli/Derbent
	150/AD2058	*A.schizopterus*	Antalya/Akseki
	87B/ME3819	*A.schizopterus*	Denizli/Derbent
	42/ME3593	*A.sigmoideus*	Kastamonu/Tosya
	121/3775	*A.sigmoideus*	Cankiri /Ilgaz
	151/3847	*A.sigmoideus*	Cankiri /Ilgaz
	41/ME3598	*A.spruneri*	Sivas/ Zara/ Divrigi
	78/ME3777	*A.spruneri*	Cankırı/Ilgaz/Tosya
	132/3817	*A.spruneri*	Kastamonu/ Ilgaz
	27/ME3675	*A.robustus*	Agrı/Tutak
	49/ME3651	*A.robustus*	Erzurum/Tortum
	53/ME3662	*A.robustus*	Erzurum /Meymenser
	55/ME3674	*A.robustus*	Agrı/Tutak
	92/ME3829	*A.robustus*	Van/Baskale

Table 4.1 continued

	17/ME3622	*A.tigridis*	Erzincan/Ilic/Divrigi
	54/ME3644	*A.tigridis*	Erzincan /Tercan/Askale
	30/ME3643	*A.tigridis*	Erzincan/ Tercan/Askale
	111	*A.turkmenensis*	Konya/Karapinar
	111B	*A.turkmenensis*	Konya/Karapinar
	111C	*A.turkmenensis*	Konya/Karapinar
	12/SK2108	*A.yildirimlii*	Ankara
	12B/SK2108	*A.yildirimlii*	Ankara
	12C/SK2108	*A.yildirimlii*	Ankara
	1/SK2042	*A.zaraensis*	Sivas/Sarkisla/Pinarbasi
	13/ME3596	*A.zaraensis*	Sivas/Zara/Divrigi
	14/ME3595	*A.zaraensis*	Sivas/Zara/Divrigi
	39/ME3597	*A.zaraensis*	Sivas /Yildizeli
HYPOGLOTTIDEI	101/ME3852	*A.akmanii*	Kahramanmaras /Ahir mtn.
	101B/ME3852	*A.akmanii*	Kahramanmaras/Ahir mtn.
	152/ME3853	*A.akmanii*	Kahramanmaras/Ahir mtn.
	28/ME3715	*A.bachmarensis*	Artvin/Ortakoy/Perta
	128/ME3718	*A.bachmarensis*	Artvin prison
	153/ME3802	*A.bachmarensis*	Artvin/Ortakoy/Perta
	104/AD8047	*A.cedreticola*	Antalya/Akseki/Pinarbası
	104B/AD8047	*A.cedreticola*	Antalya/Akseki/Pinarbası
	154/AD3090	*A.cedreticola*	Antalya/Akseki/Pinarbası
	72/ME3761	*A.cicer*	Erzurum/Tortum
	72B/ME3761	*A.cicer*	Erzurum/Tortum
	72C/ME3761	*A.cicer*	Erzurum/Tortum
	64/ME3759	*A.dasycarpus (sachanewii)*	Van/Artos mtn.
	67/ME3751	*A.dasycarpus (sachanewii)*	Van/Artos mtn.
	69/ME3752	*A.dasycarpus (sachanewii)*	Van/Artos mtn.
	119/3799	*A.viciaefolius (flaccidus)*	Artvin/Ortakoy
	140/3769	*A.viciaefolius (flaccidus)*	Trabzon
	117/3057	*A.viciaefolius (flaccidus)*	Trabzon/Arakli
	102/ME3864	*A.hartvigii*	Antalya/Fethiye
	155/3867	*A.hartvigii*	Antalya/Fethiye
	155B/3867	*A.hartvigii*	Antalya/Fethiye
	33/ME 3716	*A.lasioglottis*	Artvin/Ortakoy
	114	*A.lasioglottis*	Artvin
	156/3875	*A.lasioglottis*	Artvin
	125/3875	*A.lasioglottis*	Artvin/Borckaya
	100/ME3854	*A.melanocarpus*	Kahramanmaras/Goksun
	157/3884	*A.melanocarpus*	Kahramanmaras/Goksun
HYPOGLOTTIDEI	100B/ME3854	*A.melanocarpus*	Kahramanmaras/Goksun
	70/ME3768	*A.oreades*	Rize/Camlihemsin
	70B/ME3768	*A.oreades*	Rize/Camlihemsin
	70C/ME3768	*A.oreades*	Rize/Camlihemsin
	51/ME3723	*A.ovatus*	Trabzon/Maska
	85/ME3809	*A.ovatus*	Trabzon /Torul
	85B/ME3809	*A.ovatus*	Trabzon /Torul
	63/ME3760	*A.saganlugensis*	Agri/Caldiran/ Muradiye
	94/ME3831	*A.saganlugensis*	Van/Baskale
	158/3693	*A.saganlugensis*	Agri/Caldiran/Muradiye
	103/ME3866	*A.scholerianus*	Konya/Aksehir/Sultan mtn.
	103B/ME3866	*A.scholerianus*	Konya/Aksehir/Sultan mtn.
	103C/ME3866	*A.scholerianus*	Konya/Aksehir/Sultan mtn.
	88/H5850	*A.vexillaris*	Sanliurfa /Hilvan/Siverek
	88B/H5850	*A.vexillaris*	Sanliurfa/Hilvan/Siverek
	88C/H5850	*A.vexillaris*	Sanliurfa/Hilvan/Siverek

Table 4.1 continued

	47/ME3722	*A.viridissimus*	Trabzon/Macka
	48/ME3721	*A.viridissimus*	Trabzon /Macka
	61/ME3720	*A.viridissimus*	Trabzon/Macka
	81/ME3807	*A.viridissimus*	Trabzon /Macka/Sumela
DISSITIFLORI	11/SK2098	*A.argyroides*	Agrı/Dogu Beyazit
	163/3724	*A.argyroides*	Agrı/Dogu Beyazit
	163B/3724	*A.argyroides*	Agrı/Dogu Beyazit
	5/SK2052	*A.aucheri (leptothamnus)*	Sivas/Sincan/Kangal
	40/ME3634	*A.aucheri (leptothamnus)*	Erzincan /Kemaliye
	57/ME3645	*A.aucheri (leptothamnus)*	Erzincan/Tercan /Askale
	107/H5040	*A.aucheri (leptothamnus)*	Kirikkale/Cerikli
	123/3599	*A.aucheri (leptothamnus)*	Sivas/Zara/Divrigi
	139	*A.beypazaricus*	Beypazari
	139B	*A.beypazaricus*	Beypazari
	139C	*A.beypazaricus*	Beypazari
	60/ME3694	*A.cornutus*	Agri/Kagizman
	159/3754	*A.cornutus*	Agri/Kagizman
	159/3754	*A.cornutus*	Agri/Kagizman
	86/ME3818	*A.gladiatus*	Denizli/Derbent/Buldan
	90/ME3845	*A.gladiatus*	Denizli/Derbent/Buldan
	86B/ME3818	*A.gladiatus*	Denizli/Derbent/Buldan
	76/ME3774	*A.kastamonuensis*	Kastamonu/Ilgaz
	141/3812	*A.kastomunuensis*	Kastamonu /Ilgaz
	160/3846	*A.kastamonuensis*	Kastamonu/Ilgaz
	91/ME3840	*A.nigrifructus*	Konya/Eregli
	137/3841	*A.nigrifructus*	Konya/Eregli
	91B/ME3840	*A.nigrifructus*	Konya/Eregli
	36/ME3599	*A.nitens*	Sivas /Zara/Divrigi
	93/ME3838	*A.nitens*	Malatya/Darende/Gurun
	99/ME3827	*A.nitens*	Malatya/Darende/Akcaoren
	50/ME3664	*A.subulatus*	Erzurum/Horasan /Karaurgan
	82/ME3789	*A.subulatus*	Erzurum/Horasan /Karaurgan
	82B/ME3789	*A.subulatus*	Erzurum/Horasan /Karaurgan
	9/SK2088	*A.taochius*	Artvin/Ishan
	58/ME3713	*A.taochius*	Erzurum/ Oltu/Senkaya
	84/ME3792	*A.taochius*	Artvin/Yusufeli
	22/ME3702	*A.viridis*	Kars/Kagizman/Tuzluca
	161/3120	*A.viridis*	Kars/Kagizman /Tuzluca
	162/3755	*A.viridis*	Kars/Kagizman/Tuzluca
UNIDENTIFIED SAMPLES-SPECIES	U-*Astragalus*A52	-	-
	U-*Astragalus*A66	-	-
	U-*Astragalus*A35	-	-
	U-*Astragalus*A65	-	-
	U-*Astragalus*A2	-	-
	U-*Astragalus*A110	-	-
	U-*Astragalus*A16	-	-
	U-*Astragalus*A20	-	-
	U-*Astragalus*A108	-	-
	U-*Astragalus*A109	-	-
	U-*Astragalus*A3	-	-

4.1.1 DNA Extraction from *Astragalus* Samples

Total DNA was extracted from young leaf tissues of each sample with a slightly modified CTAB (cetyl trimethylammonium bromide) protocol (Doyle and Doyle, 1987). The modifed procedure was as follow:

1. For each sample, about 100 mg fresh leaf tissue was put in autoclave-sterilized mortar and grounded by adding 500 µl extraction buffer (2XCTAB). Prior to starting extraction, 0.8 gr polyvinylpyrrolidone (PVP) and 100 µl β-mercaptoethanol were added to extraction buffer. Before starting isolation, extraction buffer with other added substances was incubated in water bath at 60 ºC for 10-15 minutes to dissolve the PVP.

2. Liquid mixture was poured into 1.5 mL eppondorf tubes. After adding about 7 µl proteinaz K, tubes were incubated at 65°C for at least one hour, mixing once after each 30 minutes.

3. After incubation, 500 µl of chloroform: isoamyl alcohol (24:1) were added and mixed by gently shaking tubes under fume hood, mixture is centrifuged at 13000 rpm for 15 minutes.

4. The aqueous phase (top part) of the mixture was transferred into new eppendorf tubes and 500 µl of cold isopropanol was added.

5. Tubes were incubated at -80 ºC for at least half an hour. After cold incubation, they were again centrifuged at 13000 rpm for 10 minutes. Total DNA settled down at the bottom of the tubes.

6. To get DNA pellet, supernatant was discarded very carefully and 70% and 95% EtOH were used to clean and remove remnant matter from DNA. Then, tubes were inverted on a clean tissue paper and allowed to dry for 15-20 minutes upside down, or until pellet looked dry.

7. The DNA pellets were hydrated with 100 µl TE (Tris EDTA) and resuspended overnight at room temperature. Qantification of total DNA amount was carried out by using spectrophotometer (Thermo Fisher Scientific Inc. NanoDrop 2000 Spectrophotometer Version 1.4.1).

8. Dissolved DNA was diluted to 10 ng/µl for PCR reactions. The diluted DNAs were stored at +4ºC for short term while for long term storage, they were kept in -20 ºC or -80 ºC.

The compositions of buffers and solutions used during DNA isolation protocol were given in Table 4.2.

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

Buffer and Solutions	Contents and concentrations
2 X CTAB	2 gr CTAB 10 ml (pH : 8.0) Tris HCl 4 ml (pH:8.0) 0.5M Ethylenediaminetetraaceticacid disodium salt (EDTA) 28 ml 5M NaCl is completed upto 100 ml with dH_2O
Chloroform-Isoamyl alcohol	(24:1)
β-Mercaptoethanol	17.5 ml β-Mercaptoethanol is completed upto 250 ml with dH_2O
Isopropanol	Pure isopropanol, ice cold
Ethanol	70% and 95% in dH_2O
TE solution	10 mM Tris HCl (pH : 7) 10 mM EDTA

4.2 Structures of Studied Chloroplast and Genomic DNA Regions with Their Used Primer Pairs

4.2.1 Chloroplast DNA (cpDNA) Regions

4.2.1.1 *trnL5'-L3'* (*trnL* intron) and *trnL3'-F* $^{(GAA)}$ (*trnL-F* intergenic spacer) Region

To amplify *trnL* intron and *trnL-F* intergenic spacer regions, two primer pairs were used. The information on their names, sequences and lengths of amplified regions with figurative representation can be seen in Table 4.3 and Figure 4.1, respectively.

Table 4.3 Names 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).

Region name	Primer name	Sequence	Length of Region
trnL intron	trnc (F) trnd (R)	5' CGA AAT CGG TAG ACG CTA CG 3' 5' GGG GAT AGA GG ACT TGA AC 3'	~580 bp
trnL-F IGS	trne (F) trnf (R)	5' GGT TCA AGT CCC TCT ATC CC 3' 5' ATT TGA ACT GGT GAC ACG AG 3'	~170 bp

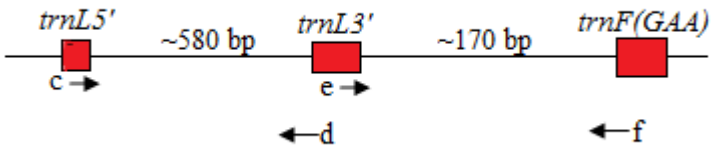

Figure 4.1 Positions and directions of universal primers (c-d and e-f) used to amplify the chloroplast *trnL* $^{(UAA)}$ intron (*trnL5'-trnL3'),* and *trnL-F* intergenic spacer [(*trnL3'-trnF* $^{(GAA)}$] regions (Taberlet et al., 1991).
(c and e = Forward primers, d and f =Reverse primers).

4.2.1.2 *trnV* intron Region

To amplify this region, a pair of primers were used. Detail information on the used primers, their names, length of amplified regions and figurative representation were given in Table 4.4 and Figure 4.2, respectively.

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

Primer name	Sequence	Length of Region
trnVF (F)	5'GTA GAG CAC CTC GTT TAC AC3'	~ 600 bp
trnVR (R)	5'CTC GAA CCG TAG ACC TTC TC 3'	

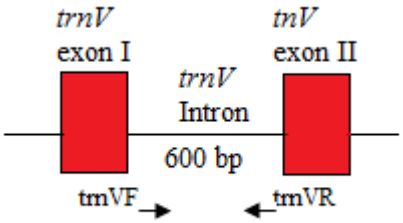

Figure 4.2 *trnV* genes and amplified *trnV* intron. Intronic region was amplified by using trnVF (Forward) and trnVR (reverse) primers.

4.2.1.3 *matK* (*maturase Kinase*) Gene Region

To amplify the focused region, one primer pair was used. Information on primer names, their sequences, approximate length of the region and figurative representation were given in Table 4.5 and Figure 4.3, respectively.

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

Primer name	Sequence	Length of Region
F1 (F)	5' ACT GTA TCG CAC TAT GTA TCA 3'	(yellow part in Fig.4.3)
R3 (R)	5' GAT CCG CTG TGA TAA TGA GA 3'	~ 1200 bp

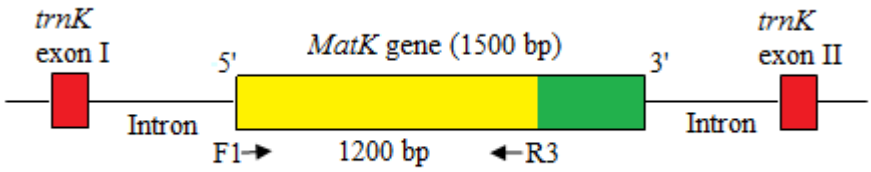

Figure 4.3 Sketch of the *trnK* gene and its *matK*-containing intron. Yellow part was amplified by F1 (Forward) and R3 (reverse) primers.

4.2.2 Genomic DNA Region

4.2.2.1 *ITS* (*Internal transcribed spacer*) Region

In the current study, up to now four different regions (*trnL* intron, *trnL-F* intergenic spacer, *trnV* intron and *matK*) located on chloroplast genome were used to understand phylogenetic relations within *Astragalus* genus. To strengthen and enrich the results of the cpDNA regions, one more area located on genomic DNA, *ITS*, was selected.

DNA sequences and length of the region have been evolving very fast so universal primers were designed by using protected regions flanking the interested region. Most of the scientists amplify *ITS1* and *ITS2* subregions separately by using two

primer pairs (Suh et al., 1993; Gernandt and Liston, 1999; Beltrame-Botelho et al., 2005). However, in this study to reduce labor and costs, only one primer pair was used to amplify these two regions with 5.8S at the same time. ITSL (forward) and ITS4 (reverse) primers were preferred to amplify the total region (*ITS1+5.8S+ITS2*) (Hsiao et al., 1995).

Detailed information on sequences of primers and approximate length of amplified region and figurative representation were given in Table 4.6 and Figure 4.4, respectively.

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

Primer name	**Sequence**	**Length of Region** (*ITS1-5.8S-ITS2*)
ITSL (F)	5'TCG TAA CAA GGT TTC CGT AGG TG3'	(~230 - ~180 - ~220
ITS4 (R)	5' TCC TCC GCT TAT TGA TAT GC 3'	bp)

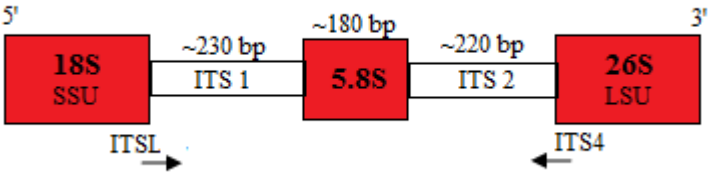

Figure 4.4 Schematic organization of the *ITS* locus indicating *18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S rRNA*. The *ITS1* separates the coding region of the *18S* subunit and the *5.8S* rDNA, and the *ITS2* separates *5.8S* rDNA region from the *26S* rDNA. ITSL and ITS4 are the Forward and Reverse primers,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

4.3 Polymerase Chain Reaction (PCR)

The first step to receive sequences from interested region is to amplify the target DNA region. There are many studies describing optimized PCR conditions for each primer pairs. However, these specified conditions sometimes may not be useful for given samples due to sample type, DNA structure, or used materials or procedures. Because of these reasons, for each primer pairs or DNA region, PCR optimization must be done. Table 4.7 illustrates optimized PCR mixture conditions for each region or primer pairs. Volume or amount of each ingredient for one region was different, but total volume was always 50µl. Only optimization of PCR mixture composition may not be enough to amplify interested region properly, so in addition to PCR mixture optimization, a PCR amplification programme was also optimized for each primer pair (Table 4.8).

Table 4.7 Composition of PCR reaction mixtures for each cp and nuclear DNA region/primer pairs.

Ingredients of PCR mixture	***trnL* intron/** trn c-d	***trnL3'-F (GAA)/*** trn e-f	***trnV* intron/** trnVF-VR	***MatK/*** F1-R3	***ITS/*** itsL-4
H_2O	26.8 µl	28.8 µl	29.8 µl	25.7 µl	34.8 µl
$MgCl_2$ (25 mM)	4 µl	4 µl	4 µl	4 µl	3 µl
10X PCR Buffer	4 µl	5 µl	4 µl	5 µl	3 µl
dNTP (10 mM)	4 µl	3 µl	3 µl	3 µl	2 µl
Forward primer (10 µM)	4 µl	3 µl	3 µl	4 µl	2 µl
Reverse primer (10 µM)	4 µl	3 µl	3 µl	4 µl	2 µl
Taq DNA poly. (5u/µl)	0.2 µl	0.2 µl	0.2 µl	0.3 µl	0.2 µl
DNA (10 ng/ µl)	3 µl	3 µl	3 µl	4 µl	3 µl
Total	50 µl	50 µl	50 µl	50 µl	50 µl

Table 4.8 Optimized PCR amplification programme for amplification of interested regions of chloroplast and nuclear genomes.

Region (Primers)	Step	Temperature	Time	Cycle #	Description
***trnL* intron** (trn c-d)	1	95°C	5'	1	Denaturation
	2	94 °C	30"	35	Denaturation
		58°C	30"		Annealing
		72 °C	50"		Extension
	3	72 °C	10'	1	Final Extension
trnL3'-F(GAA) (trn e-f)	1	95°C	5'	1	Denaturation
	2	94 °C	30"	30	Denaturation
		60°C	25"		Annealing
		72 °C	30"		Extension
	3	72 °C	10'	1	Final Extension
***trnV* intron** (trnVF-VR)	1	95°C	3'	1	Denaturation
	2	94 °C	30"	35	Denaturation
		58°C	30"		Annealing
		72 °C	50"		Extension
	3	72 °C	10'	1	Final Extension
MatK (F1-R3)	1	95°C	5'	1	Denaturation
	2	94 °C	60"	35	Denaturation
		58°C	60"		Annealing
		72 °C	90"		Extension
	3	72 °C	10'	1	Final Extension
ITS 1*and *2 (trnL-4)	1	97°C	5'	1	Denaturation
	2	94 °C	45"	30	Denaturation
		49°C	30"		Annealing
		72 °C	45"		Extension
	3	72 °C	7'	1	Final Extension

4.4 Agarose Gel Electrophoresis

Percentage of agarose gels was decided with respect to length of amplified region and prepared by dissolving and boiling of calculated amount of agarose with 1X TBE (Tris-Borate-EDTA) or TAE (Tris-Acetate-EDTA) buffer by using a microwave oven. The solution was poured into a horizontal gel tray that had inserted combs and was left for polymerization. After polymerization of the gel, 1X TBE or TAE buffer was poured into the electrophoresis apparatus and combs were gently removed from the gel to form wells for loading the samples. All samples were mixed with 6X DNA loading dye (Fermentas) and loaded into each well using a micropipette. When electric power is applied, DNA runs towards the anode. Generally, agarose gels were run at 80-100 V for 1 hour. When electrophoresis was completed, DNA fragments were stained with ethidium bromide in a gel tray. After staining, the bands were visualized by direct examination of the gel under UV light. If interested bands were amplified successfully, they were used for DNA sequencing. Amplified DNA bands were run in different concentrations of agarose gels such as 1% or 2%. Suitable concentration of the gel is decided according to the size of the DNA strands that is run in it.

4.5 Data Collection

After optimization step, each of the studied regions [(*trnL* intron*, trnL-F, trnV* intron, *matK* and *ITS (ITS1+5.8S+ITS2* subregions)] was amplified and PCR products were stored at -20°C until sequence analysis was done. Before sequencing procedure, DNA purification process was carried out. Both of purification and sequencing steps were done by the REFGEN Biotechnology Company, Teknokent of METU, Ankara. An ABI 310 Genetic Analyzer (PE applied Biosystem) automatic sequencer was used for sequencing of amplified DNA products. To get accurate DNA sequences for further analysis, both forward and reverse DNA sequences were obtained for the studied chloroplast and nuclear regions. Then, they were aligned by CLUSTAL X software. Finch TV (Version

1.4.0) manufactured by the Geopiza Research Team was used to view and edit the chromatogram data (Patterson et al., 2004-2006). The DNA sequence data obtained for the studied regions were in good quality so that there were no difficulties during identification of the bases according to the chromatogram data (see Appendix A).

4.6 Analysis of Data

All DNA sequences for each chloroplast and nuclear regions of *Astragalus* samples were collected and arranged according to the format of MEGA software so that they could be analyzed by using MEGA (Molecular Evolutionary Genetics Analysis) 5.0 software (Tamura et al., 2007). The DNA sequences obtained from *trn* and *matK* regions of cpDNA and *ITS* region of nDNA were evaluated in each section separately. The sequences prepared for further analysis were generally shorter than the unprocessed sequences since the starting and terminal parts of the sequence were removed from the raw data. The reason why these two parts were cut off from the original sequence is the unreliability of those parts due to unclean nucleotide peak structures at the chromatogram.

By using the MEGA program (Tamura et al., 2007), total nucleotide length (bp), GC content (%), nucleotide deletion and insertion, conserved and variable sites, parsimony informative sites, transition/transversion (tr/tv) ratio and nucleotide diversity of the sequences were calculated for *Astragalus* species with regard to *trn* and *mat*K regions of cpDNA and *ITS* region of nDNA.

Number of nucleotide substitutions taking place between two or more DNA sequences determines the evolutionary distance. Phylogenetic divergences are essential to obtain knowledge about molecular evolution and are very helpful for phylogenetic reconstructions of upper and lower species level. The genetic divergences between species or samples were calculated by using Kimura (1980) 2-parameter model in MEGA. Kimura (1980)'s two parameter-model corrects for

multiple hits, taking into account transversional and transitional substitution rates, and assume that frequencies of the four nucleotides are the same and that substitution rates do not change between sites.

To construct phylogenetic trees, neighbor-joining (NJ) method (Saitou and Nei 1987) was used together with bootstrap test analysis. This method is a simple version of the minimum evolution (ME) method. In the preferred method, the *S* value (smallest value of the sum of all branches) is not computed for all or many topologies, but the examination of different topologies is embedded in an algorithm, so that only one final tree is produced (Nei and Kumar; 2000). Normally, NJ method constructs an unrooted tree since it does not need the assumption of a continuous rate of evolution. However if an outgroup taxon is added to the input data rooted tree is constructed, in the current study *Cicer* sp. was used as an outgroup sample. Felsenstein's (1985) bootstrap test is commonly preferred one to show reliability of the constructed tree. If there are *m* DNA sequences, each with *n* nucleotides, by using some tree building method a phylogenetic tree can be reconstructed. During analysis, *n* nucleotides are randomly chosen with replacements from each sequence, giving rise to *m* rows of *n* columns. Each tree is produced by a new set of sequences. By using the same tree building method and produced data, a tree is constructed and then the topology of this tree is compared to that of the original tree. If an interior branch of the original tree is different from that of bootstrap tree, a value of 0 is given while all other branches are given score 1. Resampling the sites and reconstruction of tree are repeated several hundred times. The percentage of times each interior branch is given a value of 1 is noted. This is known as the bootstrap value. When this value is 95% or higher, it is safe to say that, the topology at that branch is "correct". Moreover, if the value is greater than 50%, the topology is considered as informative (Nei and Kumar, 2000).

4.7 Molecular Clock Estimation

The 'molecular evolutionary clock' term was first used and described by Zuckerkandl and Pauling (1965). The clock provides opportunity to estimate times of species divergence by using nucleotide differences in DNA sequences. If two or more lineages evolved at the same, constant rate, the number of variations among two species or samples would be straightforward index of the time since they diverged from their common ancestor (Futuyma, 2005). Therefore, we can estimate the phylogeny by the rate of nucleotide variations between DNA sequences of two taxa. To calculate the rate of molecular evolution, the number of parsimony informative sites in the sequenced DNA region is used. The following equation was used to estimate molecular clock for *Astragalus* genus.

$$\text{Molecular Clock} = \frac{\text{k}}{\text{mutation rate}}$$

Where k is equal to:

$$\text{k} = -\left(\frac{3}{4}\right)\ln\left(1 - \frac{4}{3}d\right)$$

The d in the above equation was calculated as:

$$d = \frac{Variable\ Site}{Total\ Number\ of\ Base\ Pairs\ Sequenced}$$

In the equation, *d* means the number of substitutions per base pair while *k* is the substitutions since divergence time. In the current study, DNA sequences of three regions (*trnL*, *trnV* and *matK*) in chloroplast DNA were evaluated. For each region, this value was estimated separately. As the mutation rate, $2x10^{-9}$ of plant cpDNA as a constant value, was used (Pevsner, 2009).

DNA sequences of different species within Astragalean clade [*Lessertia herbacea, Colutea arborescens*, and *Sutherlandia frutescens* (Wojciechowski et al., 1999)] gathered from NCBI database were also used to understand divergence time of *Astragalus* genus from different genus in this clade. Each of these species has DNA sequences of *trnL* intron and *matK* regions so age of *Astragalus* genus was estimated by using these two regions.

In the current study, not only DNA sequences of Turkish samples (Old World *Astragalus* group), but also those of North and South American samples (New World *Astragalus* group) were used to figure out phylogenetic relationships between these species. Estimation of divergence time for *Astragalus* genus, New World *Astragalus* group and for each used section was estimated separately by using DNA sequences of studied regions. During calculation of these values at least three representative species for each section or group were selected.

CHAPTER 5

RESULTS

5.1 Amplification of *trn* and *matK* Regions of Chloroplast and *ITS* Region of Genomic DNA

By using optimized PCR mixture and cycle conditions (provided in Table 4.7 and Table 4.8) for each studied region, the regions of cpDNA and nDNA were amplified and sequenced for each of the 191 samples coming from three different sections of genus *Astragalus*. Figures 5.1-5.5 depict running DNAs of each species in agarose gel and respective DNA ladder, which was used to estimate the length of the amplified DNA. The proper DNA ladder was selected with respect to the expected length of the amplified bands. The GeneRuler™ 50 and 100 bp ladders were appropriate for all the studied regions. To check whether there is contamination from the environment or other studied samples, negative control was also used. The main difference between negative control and studied samples is the loading of DNA sample to the amplification mixture. For control group, DNA sample was not loaded to the PCR tube, so no band was expected in agorose gel electrophoresis. The concentration of agarose gel was determined according to the length of amplified DNA band which was extrapolated from the marker DNA. For the smaller sized bands, e.g. 200-300 bp, more concentrated agarose gels (2-2.5%) were prepared while for larger sized ones, lower concentrations gel (1%) were used.

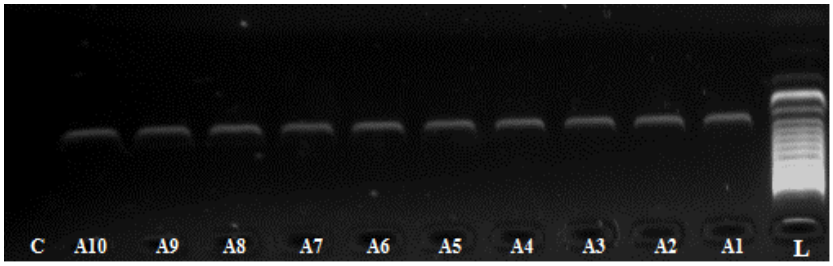

Figure 5.1 PCR products of *trnL* intron region run in 1.5% agarose gel electrophoresis.

Each *Astragalus* species was designated by the abbreviations between A1 and A10 (C; Negative control, L; GeneRuler™ 100 bp Plus DNA Ladder).

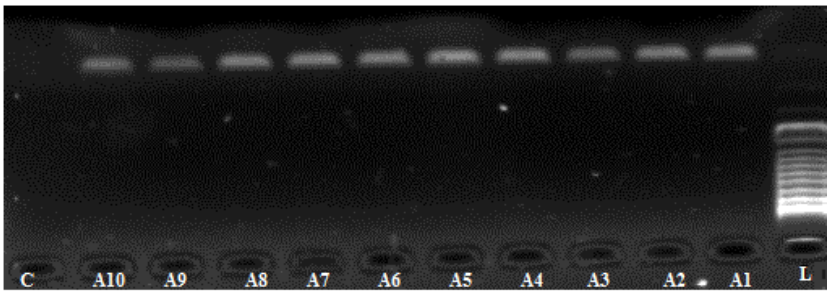

Figure 5.2 PCR products of *trnL*-F region that was run in 2% agarose gel electrophoresis.

Each *Astragalus* species was designated by the abbreviations between A1 and A10 (C; Negative control, L; GeneRuler™ 100 bp Plus DNA Ladder).

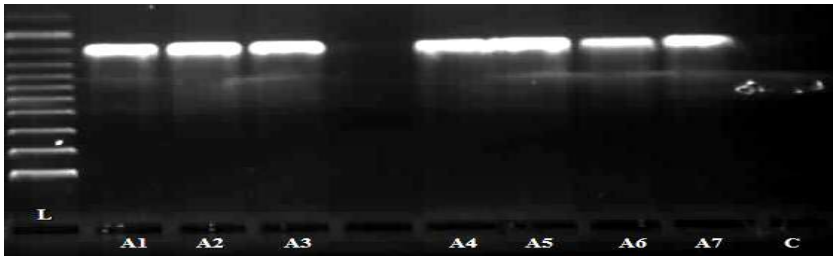

Figure 5.3 PCR products of *trnV* intron region in 1.5% agarose gel electrophoresis.

Each *Astragalus* species was designated by the abbreviations between A1 and A7 (C; Negative control, L; GeneRuler™ 100 bp Plus DNA Ladder).

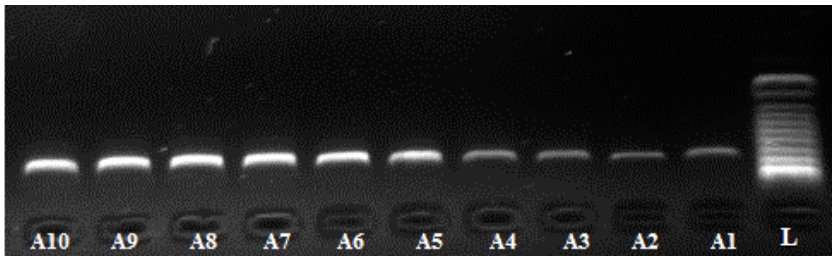

Figure 5.4 PCR products of *matK* region run in 1% agarose gel electrophoresis.

Each *Astragalus* species was designated by the abbreviations between A1 and A10 (L; GeneRuler™ 100 bp Plus DNA Ladder).

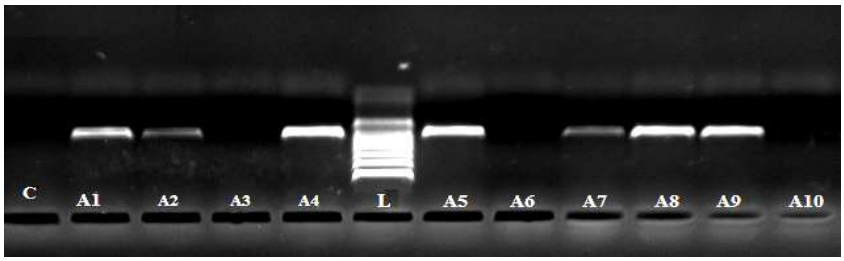

Figure 5.5 PCR products of *ITS* region that was run in 1.5% agarose gel electrophoresis.

Each *Astragalus* species was designated by the abbreviations between A1 and A10; not enough amplification for A3, A6 and A10 samples (C; Negative control, L; GeneRuler™ 50 bp DNA Ladder)

5.2 Molecular Diversity and Molecular Phylogeny in *Astragalus*

5.2.1 Molecular Diversity Statistics of *Astragalus* Species based on Each Region

After obtaining the DNA sequences for each studied cpDNA and nDNA regions of the species; 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 genus *Astragalus* were calculated by using the MEGA program. All of these mentioned parameters were calculated for three sections (*Incani* DC., *Hypoglottidei* DC., and *Dissitiflori* DC.) of the genus as well as for whole species by combining data from three sections.

5.2.1.1 *trnL5'-L3'* (*trnL* intron) and *trnL3'-F*$^{(GAA)}$ Regions of cpDNA

Although the length of the *trnL* intron region ranged from 549 to 575 bp, after the alignment of all samples of *Astragalus* in MEGA software, the length of the region was recognized as 578 bp because of indels on the DNA sequences (Table 5.1). Especially two species, A. *viciaefolius* and A. *viridissimus*, included in *Hypoglottidei* section, had two large-scale deletions, the first one was located between 125th and 160th bp (thirty-four nucleotides were missing) and the second one was observed between 414th and 421st bp (six nucleotides were missing).

GC content (%) of each section and the total sample were almost the same which was about 32% (Table 5.1). The GC% content of any DNA sequence is important since generally this statistic is higher in effectively used parts of the DNA (Mishra et al., 2009). Therefore, this content could be useful in assessing the gene potential of a region. In addition, this value is also significant in phylogenetic analyses where different frequencies of nucleotides cause phylogenetic reconstruction quite difficult.

Table 5.1 Estimated molecular diversity parameters based on *trnL5'-L3' (trnL* intron) chloroplast DNA region for each section and whole *Astragalus* data.

	Incani	*Hypoglottidei*	*Dissitiflori*	Unknown	Total
Number of species	30	15	11	?*	56
Number of sequence	98	47	35	11	191
Total length (bp)	549	569	575	569	578
GC content (%)	31.8	31.6	31.6	31.8	31.7
Conserved sites	542	563	572	547	548
Variable sites	7	6	3	22	30
Parsimony informative sites	7	6	3	22	30
Transitional pairs	54.1	0.0	44.8	46.7	53.2
Transversional pairs	45.9	100.0	55.2	53.3	46.8
Transition/Transversion (tr/tv) (R) ratio	0.97	0.00	0.70	0.74	0.96
Number of deletion	0	42	6	38	52
Number of insertion	0	42	6	38	52
Nucleotide diversity	0.030	0.020	0.003	0.039	0.039

* Since these samples were not classified taxonomically, the number of species is unknown

A parsimony informative site means that at least two types of nucleotides with a minimum frequency of two must be located on the different DNA sequences of samples. For *trnL* intronic region, this value was same with that of variable site for each of section and whole *Astragalus* data (Table 5.1). It means that for each variable site, at least two varied nucleotides were located on at least two different sequences.

Table 5.2 Pattern estimations of nucleotide substitutions for each section and whole samples of *Astragalus* genus with regard to *trnL* intronic region.
Rates of different transitional substitutions were indicated in bold and those of transversionsal substitutions were indicated in italics.

Nucleotide	A	T	C	G	Section
A	-	*6.6*	*3.6*	**0**	*Incani*
T	*9.07*	-	**19.1**	*3.69*	
C	*9.07*	**34.97**	-	*3.69*	
G	**0**	*6.6*	*3.6*	-	
A	-	*14.05*	*7.78*	**0**	*Hypoglottidei*
T	*20.11*	-	**0**	*8.06*	
C	*20.11*	**0**	-	*8.06*	
G	**0**	*14.05*	*7.78*	-	
A	-	*7.67*	*4.26*	**4.71**	*Dissitiflori*
T	*11.19*	-	**10.11**	*4.48*	
C	*11.19*	**18.19**	-	*4.48*	
G	**11.77**	*7.67*	*4.26*	-	
A	-	*6.63*	*3.65*	**3.95**	Total
T	*9.32*	-	**14.03**	*3.77*	
C	*9.32*	**25.51**	-	*3.77*	
G	**9.77**	*6.63*	*3.65*	-	

If pyrimidine is substituted by a pyrimidine [T (Thymine) / C (Cytosine)], or a purine by a purine [A (Adenine) / G (Guanine)], this situation is called as transition. Transversion means conversion a purine to a pyrimidine, or vice versa [A (Adenine) / T (Thymine) or G (Guanine) / C (Cytosine)]. Percentage of transition and transversion of whole *Astragalus* data were estimated as 53.2 and 46.8, respectively (Table 5.1 and Table 5.2). The Transition / Transversion (R) ratio indicates the proportion between the numbers of transitions to the number of transversions for a pair of sequences. If these numbers are equal to each other, R value becomes 0.5. This value should not be confused with the ratio of the transition and transversion rates ($k = \alpha/\beta$). The overall transition/transversion bias, R, is 0.96 (Table 5.1), where $R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$. ($k_1$: transition/transversion rate ratios for purines and k_2; for pyrimidines). In Table

5.2, each entry indicates the possibility of substitution from one base to the other. Transitions and transversions were shown in bold and italic characters, respectively.

For *Incani* section k_1 was 0 because no transition from A to G or vice versa was appeared in the analysis involving nucleotide sequences of 98 samples. Therefore, it is for sure to say that transition value (54.1) was only caused by transition from T to C or vice versa (Table 5.1 and Table 5.2). For *Hypoglottidei* section, transition value was 0 because there was no substitution between G/A or T/C nucleotides. Therefore, transversion value was calculated as 100 and R was 0 for this section. Variations within the section were only caused by transversion (Table 5.1 and Table 5.2). For the last section (*Dissitiflori*), these three parameters (transition, transversion and R) had intermediate values, which were between estimations of *Incani* and *Hypoglottidei* (Table 5.1 and Table 5.2). Transitions and transversions were estimated as 44.8 and 55.2 for this section, respectively.

Range of deletion was very variable among sections. While *Dissitiflori* section had a few deleted nucleotides, two species (A. *viciaefolius* and *viridissimus*) within *Hypoglottidei* section had very large missing parts compared with species of the other two sections. Size of deleted regions was very divergent within *Hypoglottidei* section because of these two mentioned species. There was no deleted or inserted nucleotides among DNA sequences of *Incani* species. Therefore, phylogenetic relationships among species of this section were evaluated by using nucleotide substitutions located through the sequences (Table 5.1).

Not only deleted, but also inserted DNA sequences were located in the sequences. Especially, *Hypoglottidei* and *Dissitiflori* sections had large inserted DNA sequences. Both of substitutions and indels have invaluable importance to depict phylogenic relationships among species and sections. When all sequences of used species were aligned together, most of inserted nucleotides were observed in DNA sequences of *Dissitiflori* species meaning that these nucleotides of the inserted

sequences have not been deleted during evolutionary time, while the other sections lost these nucleotides.

All of these genetic parameters were also calculated by using sequences of *trnL3'-F* $^{(GAA)}$ cpDNA region. These values were provided in Table 5.3.

Table 5.3 Estimated molecular diversity parameters regarding *trnL3'-F* $^{(GAA)}$ region of cpDNA for each section and whole *Astragalus* data.

	Incani	*Hypoglottidei*	*Dissitiflori*	Unknown	Total
Number of species	30	15	11	?*	56
Number of sequence	98	47	35	11	191
Total length (bp)	167	166	166	167	167
GC content (%)	37.2	37.4	38	37.3	37.3
Conserved sites	167	166	166	166	166
Variable sites	0	0	0	1	1
Parsimony informative sites	0	0	0	0	0
Transitional pairs	0	0	0	0	0
Transversional pairs	0	0	0	100	100
Transition/Transversion (tr/tv) ratio	0	0	0	0	0
Number of deletion	2	0	0	1	3
Number of insertion	2	0	0	1	3
Nucleotide diversity	0	0	0	0.004	0.004

* Since these samples were not classified taxonomically, the number of species is unknown

The length of the *trnL3'-F* $^{(GAA)}$ region was almost the same within the *Astragalus* genus, which was about 167 bp. Not only their length, but also nucleotide contents were very similar among sections. The only difference was Guanine (G) nucleotide located at 57th base in DNA sequences of *Dissitiflori* species. Thymine (T) nucleotide was observed instead of Guanine at the same base position of the DNA sequences of *Incani* and *Hypoglottidei* species. This substitution could be very useful for phylogenetic separation of *Dissitiflori* section from the other two sections within genus *Astragalus.* In addition to substitution, indels were also recognized among sections (Table 5.3). Within each section, there were no nucleotide variations between sequences of species. Therefore, both of transition and transversion values were 0 within each section. However, when the data were combined from all sections, the values of transition and transversion parameters were estimated as 0 and 100, respectively. This result was expected because the only difference among sequences was G that replaced T. As understood from former sentence, only transversion was seen in this data (no transition). GC contents were similar among sections and within genus. There were T base repeats in the sequence of each species, but the numbers of repeat showed variations, which were caused by indels among sequences of *Astragalus* species. *Incani* section carried ten T bases, while the remaining two sections had one missing T base, so they were carrying nine T bases. Insertion within *Incani* and deletion within and between *Hypoglottidei* and *Dissitiflori* sections can be very useful to understand phylogenetic relations between and within sections of *Astragalus* genus.

5.2.1.2 *trnV* intron Region of cpDNA

In addition to *trnL-F* region, another chloroplast DNA site, *trnV* intron, was used for phylogenetic studies of *Astragalus* genus. The molecular diversity parameters were also estimated by using the obtained sequence data from this region (Table 5.4).

Table 5.4 Estimated molecular diversity parameters based on *trnV* intron region of chloroplast DNA for each section and whole *Astragalus* data.

	Incani	*Hypoglottidei*	*Dissitiflori*	Unknown	Total
Number of species	30	15	11	?*	56
Number of sequence	98	47	35	11	191
Total length (bp)	589	602	608	608	608
GC content (%)	31.2	31.2	31.1	31.2	31.1
Conserved sites	586	593	608	597	590
Variable sites	3	9	0	11	18
Parsimony informative sites	3	9	0	10	18
Transitional pairs	18.0	43.0	0	34.3	38.6
Transversional pairs	82.0	57.0	0	65.7	61.4
Transition/Transversion (tr/tv) ratio	0.18	0.64	0	0.44	0.53
Number of deletion	0	6	0	19	25
Number of insertion	0	6	0	19	25
Nucleotide diversity	0.002	0.004	0	0.021	0.022

* Since these samples were not classified taxonomically, the number of species is unknown

The length of the region with indels was ranged from 589 to 608 bp. GC content was almost the same among sections (31%). The most variable section was *Hypoglottidei* while the most conservative one was *Dissitiflori* among three sections of *Astragalus*. Main part of substitution rate was contributed by transversion for *Incani* section while for *Hypoglottidei* the values of transition and transversion were close to each other. There was no substitution within *Dissitiflori* section. Therefore, R value was 0 for *Dissitiflori* and very low for *Incani* section.

Particularly *Incani* section shows morphological differences compared with the other two sections by its infinitesimal stem structure. This morphological differentiation may be demonstrated by using sequences of species of each section. Each section was separated from the other based on variable sites and indels.

5.2.1.3 *matK* (*maturase Kinase*) Region of cpDNA

In the sequence analysis, the *matK* gene began with the start codon ATG and had around 1200 bp in length. The remaining 300 bp of the interested region could not be completed because of the limited funding. However, almost complete length of the *mat*K region was sequenced. When specimens of each section were aligned together, total length of the region ranged from 1229 to 1238 due to indels along the sequence. This region had both indels and substitutions like reported in the sequence of previous studied chloroplast DNA regions, but there was an important difference in the DNA sequence of *matK* region. So far, the studied regions had both insertions and deletions that showed compatibility within sections. For example, if a species carried a deletion or insertion, the other species within the same section also carried same or similar indels. However, only one species, *A. cicer*, found in *Hypoglottidei* section harbored the nine bp-inserted multinucleotides. Therefore, *A. cicer* would be separated from other studied species in the constructed tree that depicts phylogenetic relations between species and sections. The GC content was the same between sections (Table 5.5). The most variable section was *Hypoglottidei* with 11 variable sites. The other two sections had equal number of variable nucleotides. All of these variable sites were parsimony informative, meaning that these variations were recognized at least two different DNA sequences of samples. The values of transition, transversion and R demonstrated notable differences. The *Incani* section exhibited lower transitions so its R value (the overall Transition/Transversion bias) was 0.28 while for *Dissitiflori* it was calculated as 2.04 (Table 5.5). This crucial difference was caused by missing transitions involved in pyrimidines (T/C). Therefore, the

transition/transversion rate ratios for pyrimidines (k_2) was zero for *Incani* section. This transition value originated completely from transition between A and G (purines) bases. The R value of *Dissitiflori* section was greater because transition was caused by both purines and pyrimidines. The transition/transversion rate for purines (k_1) was 7.29 and 2.70 for pyrimidines (k_2) so R value was greater, 2.04, compared with the value in *Incani* section.

Table 5.5 Estimated molecular diversity parameters regarding the sequence of *matK* region of cpDNA for each section and whole *Astragalus* data.

	Incani	*Hypoglottidei*	*Dissitiflori*	Unknown	Total
Number of species	30	15	11	?*	56
Number of sequence	98	47	35	11	191
Total length (bp)	1229	1238	1229	1229	1238
GC content (%)	31.3	31.1	31.1	31.2	31.3
Conserved sites	1224	1227	1224	1214	1211
Variable sites	5	11	5	15	27
Parsimony informative sites	5	11	5	9	27
Transitional pairs	25.6	48.1	70.7	76.1	62.3
Transversional pairs	74.4	51.9	29.3	23.9	37.7
Transition/Transversion (tr/tv) ratio	0.28	0.79	2.04	2.86	1.45
Number of deletion	0	9	0	0	9
Number of insertion	0	9	0	0	9
Nucleotide diversity	0.002	0.003	0.002	0.005	0.005

* Since these samples were not classified taxonomically, the number of species is unknown

5.2.1.4 *ITS* (*Internal transcribed spacer*) Region of nDNA

In addition to studied regions of cpDNA, another region located on nuclear genome was used to explore molecular diversity, distance and phylogenetic relationships among *Astragalus* sections. The nDNA sequence of *ITS* region is one of widely used sequence of DNA to understand taxonomical and phylogenic structures. In this study, *ITS1* and *ITS2* subregions were not amplified separately. Some researchers prefer to amplify these two regions separately by using two primer pairs. However, in the current study, only one primer pair was used and *ITS1* and *ITS2* with *5.8S* were amplified together. The approximate sequence length for each subregion was decided by using diverse sequences of *Astragalus* species that were accessible in NCBI database (Wojciechowski et al., 1999; Kazempour Osaloo et al., 2003; Scherson et al., 2008). First, all molecular diversity parameters were estimated separately for each section of *Astragalus* (Table 5.6). Then, the same parameters were estimated for the whole *Astragalus* data. Additionaly, to understand which subregion of *ITS* was more diverse, molecular diversity parameters were also calculated for each subregion separately by using whole *Astragalus* data (Table 5.7).

Among the studied sections of *Astragalus*, the most conserved one was *Incani* (Table 5.6). It contained only nine variable sites while the other two had at least eighteen variable positions. These variable sites are the reasons for separation of *Incani* from other sections in the constructed phylogenetic tree. Fifty-six variable nucleotides were included within combined data. This number was very high when compared with sequences of studied chloroplast DNA regions. Therefore, we can conclude that *ITS* region as a nuclear genome part was more diverse with respect to the studied regions of cpDNA (*trn* and *matK*). Number and position of deleted and inserted nucleotides within both *Hypoglottidei* and *Dissitiflori* sections were almost the same. Variable sites for each section were also parsimony informative. There were no remarkable differences among sections with respect to values of GC content (Table 5.6). The pyrimidines (T/C) greatly

contributed to the transition values for all three sections so it can be safe to say that remarkable part of variable sites were caused by C or T base substitution.

Table 5.6 Estimated molecular diversity parameters based on *ITS* region of genomic DNA for each section and whole *Astragalus* data.

	Incani	*Hypoglottidei*	*Dissitiflori*	Unknown	Total
Number of species	30	15	11	?*	56
Number of sequence	98	47	35	11	191
Total length (bp)	634	634	634	639	642
GC content (%)	53.3	53.8	53.9	53.6	53.6
Conserved sites	625	616	612	608	586
Variable sites	9	18	22	31	56
Parsimony informative sites	9	18	22	26	56
Transitional pairs	52.3	61.0	47.5	63.8	62.7
Transversional pairs	47.7	39	52.5	36.2	37.3
Transition/Transversion (tr/tv) ratio	1.09	1.55	0.72	1.75	1.67
Number of deletion	1	1	1	7	9
Number of insertion	1	1	1	7	9
Nucleotide diversity	0.002	0.006	0.008	0.024	0.026

* Since these samples were not classified taxonomically, the number of species is unknown

Table 5.7 Estimated molecular diversity parameters for each subregions of *ITS* region.

	ITS1	*5.8S*	*ITS2*
Number of species	56	56	56
Number of sequence	191	191	191
Total length (bp)	236	180	226
GC content (%)	55.6	52.2	52.6
Conserved sites	211	175	200
Variable sites	25	5	26
Parsimony informative sites	25	5	26
Transitional pairs	55.5	33.0	80.2
Transversional pairs	44.5	77.0	19.8
Transition/Transversion (tr/tv) ratio	1.24	0.48	4.01
Number of deletion	8	0	1
Number of insertion	8	0	1
Nucleotide diversity	0.042	0.009	0.024

Length of the *ITS1* and *ITS2* subregions were almost equal and the region between them, 5.8S, was shorter. When diversity parameters for each subregion were calculated by using combined *Astragalus* data, the GC content of each subregion was close to each other (Table 5.7). The *ITS1* had slightly higher value than *ITS2* and *5.8S* subregions. Main difference between subregions was in variable/conserved sites. The *5.8S* region was more conserved compared with the other two sites, which were located at each side of the *5.8S* region. Actually, this result was expected because *5.8S* region is transcribed region, which means this region is responsible for production of 5.8S rRNA so it is more protected than intronic DNA regions. In addition to substitutions, deleted or inserted nucleotides

are also used to reveal the evolutionary relationships among species. Both intronic subregions (*ITS1* and *ITS2*) had inserted and deleted nucleotides while there was none in *5.8S* exonic region (Table 5.7). There were important differences between R values of these three subregions. For *5.8S*, most of substitutions were caused by transversion so R value was very low (0.48) wheras transition value dominates in both *ITS1* and *ITS2* subregions. Especially, for *ITS2* subregion this rate was so high that R value was calculated as about 4.00 (Table 5.7).

5.2.2 Genetic Divergence among Sections of *Astragalus* Genus

To reduce statistical error, at least three specimens were included for each species. As expected, identical sequences were received from each three samples (replicas) originating from a given species. Genetic divergence among species as well as among sections were calculated by using sequences of four cpDNA regions (*trnL* intron, *trnL-F* IGS, *trnV* intron and *matK*) and that of one region (*ITS*) of nDNA. Genetic divergence data among sections was provided in Table 5.8.

Genetic divergence among sections was estimated by using number of substitutions per site from averaging over all sequence pairs among sections. To calculate overall divergence, whole *Astragalus* data was used; sections were not taken into consideration. Kimura 2-parameter (Kimura M., 1980) model was preferred to analyze these two parameters.

Table 5.8 Genetic divergence among sections based on both chloroplast and genomic DNA regions.

	Incani	***Hypoglottidei***	***Dissitiflori***	**Overall**	**Regions**
Incani					
Hypoglottidei	0.068			0.040	*trnL5'-L3'*
Dissitiflori	**0.071**	0.028			
Incani					
Hypoglottidei	0.006			0.005	*trnL3'-F*
Dissitiflori	**0.012**	0.006			
Incani					
Hypoglottidei	0.038			0.023	*trnV*
Dissitiflori	**0.045**	0.015			
Incani					
Hypoglottidei	0.007			0.005	*matK*
Dissitiflori	**0.008**	0.006			
Incani					
Hypoglottidei	0.047			0.027	*ITS*
Dissitiflori	**0.049**	0.018			

When DNA sequences of *trnL5'-L3'* region was considered, genetic divergence between *Incani* and *Dissitiflori* sections was greater (0.071) than those values between other section combinations. Genetic divergence among *Hypoglottidei* and *Dissitiflori* sections was very low (0.028) as expected from conserved sequences of species found in these two sections (Table 5.8). Separation of *Incani* section from other two is anticipated when morphological similarities between *Hypoglottidei* and *Dissitiflori* sections are considered. The *Incani* section is known as stemless which means their upper parts are so close to the ground that stem structure could not be seen without a close and careful look. The other two

sections, *Hypoglottidei* and *Dissitiflori,* have distinct stem structures, which they share.

trnL3'-F region was less informative compared with former region. Actually, both of these regions are located side by side on the chloroplast DNA, but their lengths are different. The *trnL* intronic region is almost two times longer in length than that of *trnL3'-F* region. Although the length of the *trnL3'-F* region was shorter and genetic divergence between species and sections was low, it was still informative. Genetic divergence between *Incani* and *Dissitiflori* was higher (0.012) than that is between *Incani* and *Hypoglottidei* as well as between *Dissitiflori and Hypoglottidei* (0.006) (Table 5.8).

The sequence analysis of *trnV* intron region revealed similar results with those of *trnL* intron and *trnL-F* regions. The *Incani* section was the most divergent (0.038 and 0.045) from other two sections, so separation of *Incani* from *Hypoglottidei* and *Dissitiflori* sections was expected in the phylogenetic tree. Genetic divergence between *Dissitiflori* and *Hypoglottidei* was lower (0.015) as seen from the results of *trnL* intron and *trnL-F* regions, so close relationship between these two sections were expected in the constructed phylogenetic tree. As seen in Table 5.8, the divergence among sections calculated by using DNA sequences of *trnV* region was lower than values that were estimated by using sequences of *trnL* intron region, but higher than the results of *trnL-F* region.

MatK, last studied cpDNA region, showed similar results to *trn*, but the values were lower than those obtained from the other three studied chloroplast DNA regions (*trn*) though this region had longer DNA sequences compared with others. Therefore, it can be safe to say that length of the region is not always enough to elucidate phylogenetic relationships between species. Although getting more informative molecular diversity data from longer DNA sequence is more probable, the main factors affecting the outcomes are nucleotide substitution and indels located in the DNA sequence. The *Incani* section was slightly further from other two sections (0.007 and 0.008) and distance between *Dissitiflori* and

Hypoglottidei was lower (0.006, Table 5.8). The divergence values among sections were nearly the same compared with results of *trnL-F* region though length of this region was very short (~170 bp).

Results obtained by using DNA sequences of *ITS* genomic region were almost the same with conclusions of the studied chloroplast DNA regions. As seen in Table 5.8, *Incani* was the most distant section within three sections of *Astragalus* genus. Genetic distance between *Incani* and *Dissitiflori* (0.049) was nearly the same with the value between *Incani* and *Hypoglottidei* (0.047).

Both results from genomic and chloroplast DNA regions revealed similar results when genetic divergence among sections was estimated. *Incani* section was detached from other two sections regardless of the cpDNA or nDNA region was used. Moreover, the molecular divergence between *Incani* and *Dissitiflori* was always higher than divergence between other section combinations. Therefore, in the phylogenetic tree, position of the *Dissitiflori* was expected to be located further apart from *Incani* section. Because of short distance appeared between *Dissitiflori* and *Hypoglottidei,* close location of them would be again expected in the constructed tree.

In addition to genetic divergence among sections, divergence between species of each section was also explored (Table 5.9). The sample numbers per section used in evaluation were variable because each section had different number of species. Furthermore, there were three or more specimens available for each species. In the Table 5.9, only the most divergent species for each section and whole *Astragalus* data with their divergence within parenthesis and overall mean divergence were given.

Table 5.9 Genetic divergence between species within each section and whole *Astragalus* data by using five different DNA regions.

Sections	The most divergent species within sections and combined data	Overall mean divergence	Region
Incani	*A.brevidentatus-A.achundovii* (0.010) *A.brevidentatus-A.olurensis* (0.010)	0.003	*trnL5'-L3'*
Hypoglottidei	Several combinations* (0.076)	0.022	
Dissitiflori	*A.subulatus-A.aucheri* (0.014) *A.subulatus-A.nigrifructus* (0.014) *A.subulatus-A.nitens* (0.014) *A.subulatus-A.taochius* (0.014)	0.003	
Overall	*A.brevidentatus-A.viciaefolius* (0.096) *A.brevidentatus-A.viridissimus* (0.096)	0.040	
Incani	*A.brevidentatus*-all other species (0.012)	0.001	*trnL3'-F*
Hypoglottidei	-	-	
Dissitiflori	-	-	
Overall	Several combinations* (0.024)	0.005	
Incani	*A.tigridis-A.yildirimlii* (0.005)	0.002	*trnV*
Hypoglottidei	*A.cicer-A.dasycarpus* (0.018) *A.cicer-A.bachmarensis* (0.018) *A.cicer-A.lasioglottis* (0.018)	0.004	
Dissitiflori	-	-	
Overall	*A.tigridis-A.cicer* (0.053)	0.023	
Incani	Several combinations* (0.004)	0.002	*matK*
Hypoglottidei	*A.cedreticola-A.cicer* (0.013)	0.003	
Dissitiflori	Several combinations* (0.003)	0.001	
Overall	Several combinations* (0.015)	0.005	
Incani	Several combinations* (0.008)	0.002	*ITS*
Hypoglottidei	*A.cicer-A.viciaefolius* (0.021) *A.cicer-A.viridissimus* (0.021)	0.006	
Dissitiflori	*A.beypazaricus-A.gladiatus* (0.021)	0.008	
Overall	*A.germanicopolitanus-A.gladiatus* (0.058)	0.027	

* The highest genetic divergence was observed among several species, to reduce complexity identity of these species were not given in the table.

After compiling DNA sequences of each studied region, genetic divergence among species within a section was estimated to find out which species is closer or more distant to the other species that is found within the same section. DNA sequences of each studied region indicated similar results when divergence among sections was estimated; *Incani* section was always more distant to the *Dissitiflori* section and distances between *Hypoglottidei* and *Dissitiflori* was always shorter. However, the most divergent species within a section or whole *Astragalus* data were altered based on studied DNA region. This result demonstrated that none of the handled region was enough to show exact phylogenetic relationships at the species level.

According to *trnL* intron region, the most conservative section was *Incani*. Although this section had more than half of used specimens, genetic divergence between species of this section was close to each other. Therefore, most of *Incani* species were expected to be clustered together within one group in a constructed phylogenetic tree. Genetic divergence between *A. brevidentatus - A. achundovii* and *A. brevidentatus - A.olurensis* were the highest (0.010) compared with other species combinations (Table 5.9). This result was expected because most of variable sites (Table 5.1) within *Incani* section were caused by these three species. Overall mean distance for this section was too low (0.003) compared with distance between *A. brevidentatus - A. achundovii* and *A. brevidentatus - A .olurensis* (0.010). This high value (0.010) was decreased to a low overall mean value (0.003) by effects of short or no distance among other species of the section.

Genetic divergences among species of *Hypoglottidei* section were very diverse so overall mean distance was very high (0.022) compared with value of 0.003 for *Incani* section. The highest value of genetic divergence between species was 0.076. This was observed in several species combinations such as *A. akmani - A. viridissimus, A. cicer - A. viridissimus, A. cicer - A. viciaefolius,* and *A. ovatus - A. viciaefolius* (Table 5.9).

Species of *Dissitiflori* section had similar genetic divergence values with species of *Incani* section; overall mean was equal to mean (0.003) of section *Incani*, while the highest genetic divergence values (0.014) among *Dissitiflori* species were slightly higher than that of *Incani* species (Table 5.9). The highest genetic divergence values were observed in species combinations of *A. subulatus* - *A. aucheri, A. subulatus* - *A. nigrifructus*, *A. subulatus* - *A. nitens,* and *A. subulatus* - *A. taochius* (Table 5.9)

The highest genetic distances between species were observed when whole *Astragalus* data was used as an input data. In this data, fifty-six species with at least three sequences were included. *A. brevidentatus* (*Incani*) has the highest (0.096) genetic divergence from two species (*A. viciaefolius* and *A.viridissimus*) appeared in *Hypoglottidei* section (Table 5.9).

TrnL-F cpDNA region showed too low genetic divergence within and between sections in contrast to the neighboring region (*trnL* intron). There was no genetic divergence between species of both *Hypoglottidei* and *Dissitiflori* sections. Moreover, only *A. brevidentatus* was genetically diverged from other species of *Incani* section. In other words, all species of *Incani* section except *A. brevidentatus* had the same DNA sequence. Thus, genetic divergences estimated between them were zero. For this region, the highest divergence value was found between *A. brevidentatus* and species of *Hypoglottidei* or *Dissitiflori* sections (0.024, Table 5.9).

If DNA sequence of *trnV* region was taken into consideration, short genetic divergence was observed between species of *Incani* section. Actually, this result was caused by few nucleotide differences within the section. The divergence between *A.tigridis* and *A. yildirimlii* was the furthest (0.005). Most of the species had no divergence between them so overall mean divergence was 0.002 for *Incani* section (Table 5.9). *A.cicer* harbored diverse nucleotides with regard to the DNA sequence of *trnV* region of other species within the *Hypoglottidei* section. Therefore, it was located distantly to the others. The highest divergence was

observed with species *A. dasycarpus, A. bachmarensis* and *A. lasioglottis* (0.018). This value was decreased to 0.004 as overall mean because there were very little genetic divergences among the most of the remaining species of the section (Table 5.9).There were no nucleotide substitutions and/or indels in the DNA sequence of *Dissitiflori* species, so no genetic divergence between species of this section was observed. When all used species were combined and used as an input data, genetic divergence between *A. tigridis* (*Incani)* and *A. cicer* (*Hypoglottidei*) was the highest (0.053, Table 5.9).

The last studied chloroplast region, *matK*, showed low genetic divergence both within and between sections. Genetic divergence within *Incani* (0.004) and *Dissitiflori* (0.003) sections were low while *Hypoglottidei* (0.013) had higher divergence among its species. The low value was induced by few substitutions in the DNA sequences of species within the same section. Higher divergence within *Hypoglottidei* section was caused by the DNA sequence of *A. cicer* that had several substitutions and insertions in the sequence of *matK* region. The highest divergence within this section was recognized between *A. cicer* and *A. cedreticola* (0.013, Table 5.9).

As a genomic DNA region, only *ITS* nrDNA region was studied. This region showed more divergence compared with regions of cpDNA except for *trnL* intron region. Within *Incani* section, the highest genetic divergence was calculated as 0.008. The other two sections, *Hypoglottidei* and *Dissitiflori*, possessed more divergence (0.021) within them with almost similar overall mean values (0.006-0.008). Within *Hypoglottidei* section, the highest genetic divergences (0.021) were estimated between *A. cicer* - *A. viciaefolius* and *A. cicer* - *A. viridissimus* species. *A.cicer* was also the most distant species to the other species within the same section when *trnV* and *matK* chloroplast regions were evaluated. For *Dissitiflori* section, the most remoteness (0.021) was observed between *A.beypazaricus* and *A.gladiatus* species. When whole *Astragalus* data were used, the highest genetic divergence was found between *A. germanicopolitanus (Incani)* and *A. gladiatus (Dissitiflori)* (0.058).

5.2.3 The Phylogenetic Trees Construction by using the Sequence Data from Regions of cpDNA and nDNA

After computation of genetic divergence and parameters by MEGA 5.0 software for each section with their species and repeats, by choosing just one representative individual for each species, a phylogenetic tree was constructed. Only one representative was enough to construct phylogenetic tree because each repeat of one species had exactly the same DNA sequence with sequences of its other repeats. If all used samples were included to construct a phylogenetic tree, there would be complexity to understand relationships between species because trying to see all studied samples in one tree reduces the resolution of the tree. Each studied region showed different divergence and diversity values so phylogenetic trees constructed by using DNA sequences of these regions were expected to be different at least at the species level. Therefore, for each studied DNA region, firstly, a tree comprising all sections was constructed and then to understand the phylogenetic relationships among species of one section further, other trees were constructed for each section.

5.2.3.1 Phylogenetic Tree Construction by using Sequence Data from *trnL5'-L3'* Region

Different deleted fractions and several substitutions were recognized in the aligned DNA sequences of *trnL* intron region of *Astragalus* species. Some patterns indicating indels and substitutions for each section were provided in Figure 5.6. Nucleotide absence was shown as a dot, inserted (deleted) base as blue and substitution as red color. Numbers above each column depict the position of the corresponding nucleotide in the whole alignment (Figure 5.6).

Nucleotide Positions in the Aligned DNA sequences of *trnL* Intron Region

Section	Species name	113	114	119	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162
INCANI	*A. achundovii*	-	A	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	T	T	C	A	G	A	A	A	G	T	T	C	A	A	A	T	T	A	A	A	C	A	A	A
	A. ancistrocarpus	-	A	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. humillimus	-	A	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. latifolius	-	A	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. nezaketae	-	A	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	T	T	C	A	G	A	A	A	G	T	T	C	A	A	A	T	T	A	A	A	C	A	A	A
	A. polhillii	-	A	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. sanguinolentus	-	A	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	T	T	C	A	G	A	A	A	G	T	T	C	A	A	A	T	T	A	A	A	C	A	A	A
	A. scabrifolius	-	A	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. zaraensis	-	A	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
HYPOGLOTTIDEI	*A. akmanii*	C	G	C	A	A	A	A	-	-	-	-	-	-	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. bachmarensis	C	G	C	A	A	A	A	-	-	-	-	-	-	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. cicer	C	G	C	A	A	A	A	-	-	-	-	-	-	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. dasycarpus	C	G	C	A	A	A	A	-	-	-	-	-	-	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	T	T	T	A	A	A	C	A	A	A
	A. viciaefolius	C	G	C	A	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	A. hartvigii	C	G	C	A	A	A	A	-	-	-	-	-	-	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. lasioglottis	C	G	C	A	A	A	A	-	-	-	-	-	-	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. ovatus	C	G	C	A	A	A	A	-	-	-	-	-	-	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. saganlugensis	C	G	C	A	A	A	A	-	-	-	-	-	-	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	T	T	T	A	A	A	C	A	A	A
	A. scholerianus	C	G	C	A	A	A	A	-	-	-	-	-	-	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. vexillaris	C	G	C	A	A	A	A	-	-	-	-	-	-	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	T	T	T	A	A	A	C	A	A	A
	A. viridissimus	C	G	C	A	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DISSITIFLORI	*A. argyroides*	C	G	C	A	A	A	A	T	A	A	A	A	G	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. aucheri	C	G	C	A	A	A	A	G	A	A	A	A	G	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. beypazaricus	C	G	C	A	A	A	A	T	A	A	A	A	G	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. nigrifructus	C	G	C	A	A	A	A	G	A	A	A	A	G	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. subulatus	C	G	C	A	A	A	A	T	A	A	A	A	G	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A
	A. viridis	C	G	C	A	A	A	A	T	A	A	A	A	G	T	A	A	A	A	G	T	T	C	A	G	A	A	A	G	T	T	A	A	A	A	T	T	A	A	A	C	A	A	A

Figure 5.6 The regions of indels and nucleotide substitutions in the DNA sequences of *trnL* intron cpDNA region.

Section	Species name	163	164	165	166	167	221	222	224	250	267	294	295	296	297	298	299	300	301	302	303	304	306	321	366	395	396	401	405	414	415	416	417	418	419	420	421
		Nucleotide Positions in the Aligned DNA sequences of *trnL* Intron Region																																			
INCANI	*A. achundovii*	T	A	A	A	G	T	A	C	C	T	A	-	-	-	-	T	-	-	-	-	G	T	T	A	G	T	C	T	A	-	-	-	-	-	-	T
	A. ancistrocarpus	T	A	A	A	G	T	A	C	C	T	A	-	-	-	-	T	-	-	-	-	G	T	T	A	T	T	C	C	A	-	-	-	-	-	-	C
	A. humillimus	T	A	A	A	G	T	A	C	C	T	A	-	-	-	-	T	-	-	-	-	G	T	T	A	G	T	C	T	A	-	-	-	-	-	-	C
	A. latifolius	T	A	A	A	G	T	A	C	C	T	A	-	-	-	-	T	-	-	-	-	G	T	T	A	T	T	C	C	A	-	-	-	-	-	-	C
	A. nezaketae	T	A	A	A	G	T	A	C	C	T	A	-	-	-	-	T	-	-	-	-	G	T	T	A	G	T	C	T	A	-	-	-	-	-	-	C
	A. polhillii	T	A	A	A	G	T	A	C	C	T	A	-	-	-	-	T	-	-	-	-	G	T	T	A	T	T	C	C	A	-	-	-	-	-	-	C
	A. sanguinolentus	T	A	A	A	G	T	A	C	C	T	A	-	-	-	-	T	-	-	-	-	G	T	T	A	G	T	C	T	A	-	-	-	-	-	-	C
	A. scabrifolius	T	A	A	A	G	T	A	C	C	T	A	-	-	-	-	T	-	-	-	-	G	T	T	A	T	T	C	C	A	-	-	-	-	-	-	C
	A. zaraensis	T	A	A	A	G	T	A	C	C	T	A	-	-	-	-	T	-	-	-	-	G	T	T	A	T	T	C	C	A	-	-	-	-	-	-	C
HYPOGLOTTIDEI	*A. akmanii*	-	A	A	A	G	A	A	A	T	C	A	-	-	T	T	T	A	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C
	A. bachmarensis	-	A	A	A	G	T	C	A	T	C	A	-	-	T	T	T	A	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C
	A. cicer	-	A	A	A	G	T	A	A	T	C	A	T	A	T	T	T	T	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C
	A. dasycarpus	-	A	A	A	G	T	A	A	T	C	A	-	-	T	T	T	A	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C
	A. viciaefolius	-	-	-	-	G	T	A	A	T	C	A	-	-	T	T	T	T	T	A	T	A	A	A	A	G	G	G	T	A	-	-	-	-	-	-	C
	A. hartvigii	-	A	A	A	G	A	A	A	T	C	A	-	-	T	T	T	A	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C
	A. lasioglottis	-	A	A	A	G	T	C	A	T	C	A	-	-	T	T	T	A	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C
	A. ovatus	-	A	A	A	G	T	A	A	T	C	A	-	-	T	T	T	A	T	A	T	A	A	A	C	G	G	G	T	A	T	T	C	C	A	A	C
	A. saganlugensis	-	A	A	A	G	T	A	A	T	C	A	-	-	T	T	T	A	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C
	A. scholerianus	-	A	A	A	G	T	A	A	T	C	A	-	-	T	T	T	T	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C
	A. vexillaris	-	A	A	A	G	T	A	A	T	C	A	-	-	T	T	T	A	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C
	A. viridissimus	-	-	-	-	G	T	A	A	T	C	A	-	-	T	T	T	T	T	A	T	A	A	A	A	G	G	G	T	A	-	-	-	-	-	-	C
DISSITIFLORI	*A. argyroides*	-	A	A	A	G	T	A	C	T	C	A	-	-	T	G	T	T	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C
	A. aucheri	-	A	A	A	G	T	A	C	T	C	A	-	-	T	G	T	T	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C
	A. beypazaricus	-	A	A	A	G	T	A	C	T	C	A	-	-	T	G	T	T	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C
	A. nigrifructus	-	A	A	A	G	T	A	C	T	C	A	-	-	T	G	T	T	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C
	A. subulatus	-	A	A	A	G	T	A	C	T	C	A	-	-	T	G	T	T	T	A	T	A	A	A	A	G	G	G	T	A	-	-	-	-	-	-	C
	A. viridis	-	A	A	A	G	T	A	C	T	C	A	-	-	T	G	T	T	T	A	T	A	A	A	A	G	G	G	T	A	T	T	C	C	A	A	C

Figure 5.6 continued

There were nine specific substitutions in the sequences of *Astragalus* species that allowed separation of *Incani* from *Hypoglottidei* and *Dissitiflori* species (Figure 5.6, positions 114A-114G, 119T-119C, 250C-250T, 267T-267C, 304G-304A, 306T-306A, 321T-321A, 396T-396G, 401C-401G). In addition to substitutions, deleted or inserted nucleotides with different numbers caused separation of *Incani* species from the others [113 (C deletion), 123-138 (AAAT/GAAAAGTAAAA deletion), 163 (T insertion), 295-303 (except 299th base, TATTA/TTAT deletion)]. Separation of *Dissitiflori* from *Hypoglottidei* species was also caused by both nucleotide substitutions and indels [127 (T/G insertion), 224 (C-A substitution), 298 (G-T substitution), Figure 5.6].

While phylogenetic tree was constructed, evolutionary distance was calculated via the Kimura 2-parameter method with a bootstrap replication of 1000. The name of a few specimens could not be assigned so they were named as U (Unknown)-*Astragalus* and used during analyses to understand their relations to the identified species. Figure 5.7 depicts phylogenetic relationships between species and sections of *Astragalus* genus based on sequence of *trnL* intron region. This phylogenetic tree was also used to understand relatedness or connections among unknown samples (U-*Astragalus*) and identified species.

Species *A. viridissimus* and *A. viciaefolius* were not grouped with other species of the *Hypoglottidei* section due to a large deleted nucleotide region in their sequences (Figure 5.6) and were joined to the *Incani* cluster from outer. All species found in *Incani* section carried a big deleted area between 123th and 138th nucleotide sites. This mentioned deleted region of *A. viridissimus* and *A. viciaefolius* coincided with these deleted sites (Figure 5.6), so they attached to *Incani* cluster externally instead of *Hypoglottidei* cluster (Figure 5.7).

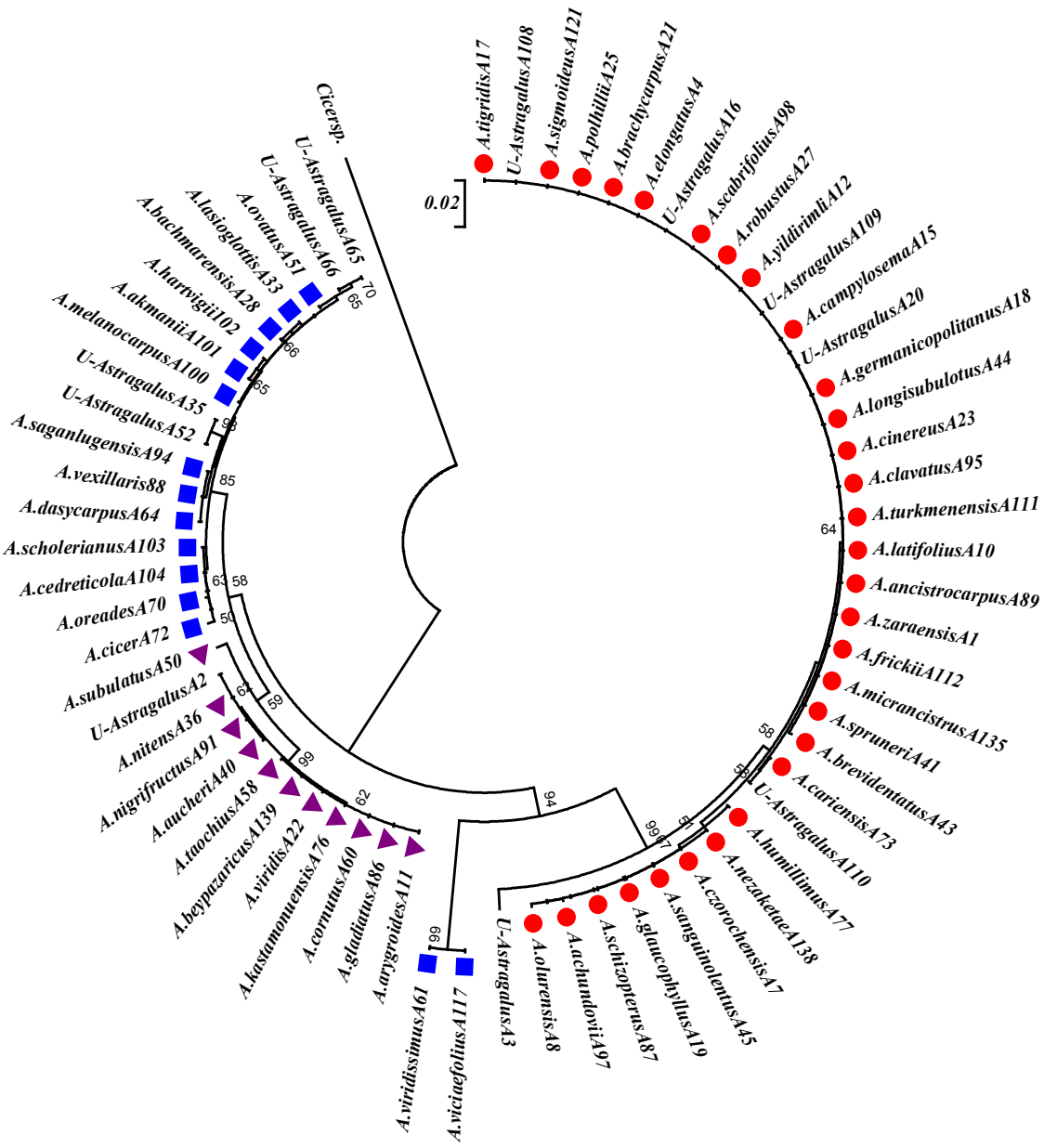

Figure 5.7 Phylogenetic tree, constructed by using the sequences from *trnL5'-L3'* region of cpDNA.

(Numbers on tree branches indicate bootstrap values, Circles: *Incani*, Squares: *Hypoglottidei* and Triangles*: Dissitiflori* section. U: Unknown *Astragalus* sample).

After observing releations of all used samples in the constructed phylogenetic tree (Figure 5.7), species of each section and unknown *Astragalus* samples were gathered together in a data to see relationship with this section and their phylogenetic relationships with unknown samples (Figure 5.8, Figure 5.9, Figure 5.10).

All studied species of *Incani* section were ended up within one cluster indicated by 'I' in Figure 5.8. Most of *Incani* species formed a subcluster with a bootstrap value of 65 while a few of them were grouped in another subcluster with a bootstrap value of 63. The species located in the first cluster had the same DNA sequences (Except U-*Astragalus* A3 and *A. brevidentatus*) so that they could not be separated from each other. The species such as *A. humillimus, A. olurensis, A. achundovii, A. schizopterus, A. czorochensis, A. glaucophyllus, A. nezaketae,* and *A. sanguinolentus* formed together the second cluster (Figure 5.8). One of the unidentified species (U-*Astragalus* A3) attached to the first subcluster with further genetic distance due to deleted nucleotides in its sequence. Actually, the sequence of this unknown species was considerably different from all other studied specimens of the *Incani* section (Figure 5.7 and Figure 5.8). Five of unknown samples of *Astragalus* were grouped in another cluster. From this result, it can be understood that they were not included in *Incani* section (Figure 5.8, Figure 5.9 and Figure 5.10). Bootstrap values were quite high for each cluster and subclusters, although these values do not show how accurate our tree, they only give knowledge about the stability of the tree topology. These values are used to inspect whether the sequence is sufficient to validate the topology (Berry and Gascuel, 1996). The second main cluster contained unidentified samples from both *Dissitiflori* (D) and *Hypoglottidei* (H) sections. The unidentified U-*Astragalus* A2 sample could belong to *Dissitiflori* section while other four samples (U-*Astragalus* A35, A52, A65, and A66) may belong to *Hypoglottidei* section.

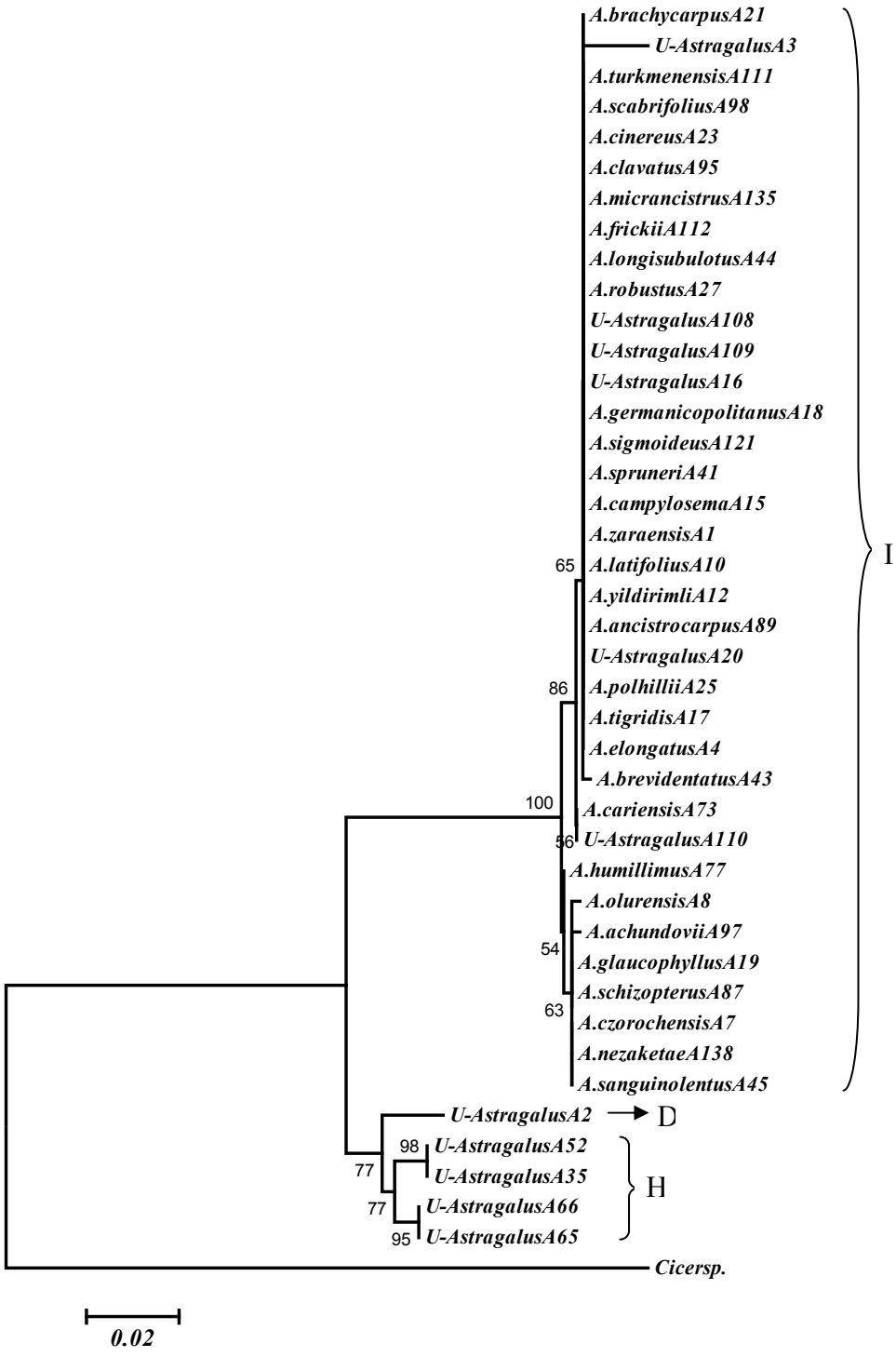

Figure 5.8 Phylogenetic tree, constructed using the sequences of *trnL5'-L3'* region of cpDNA, depicting relationships among the species of *Incani* section.
(Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori*. U: Unknown *Astragalus* samples).

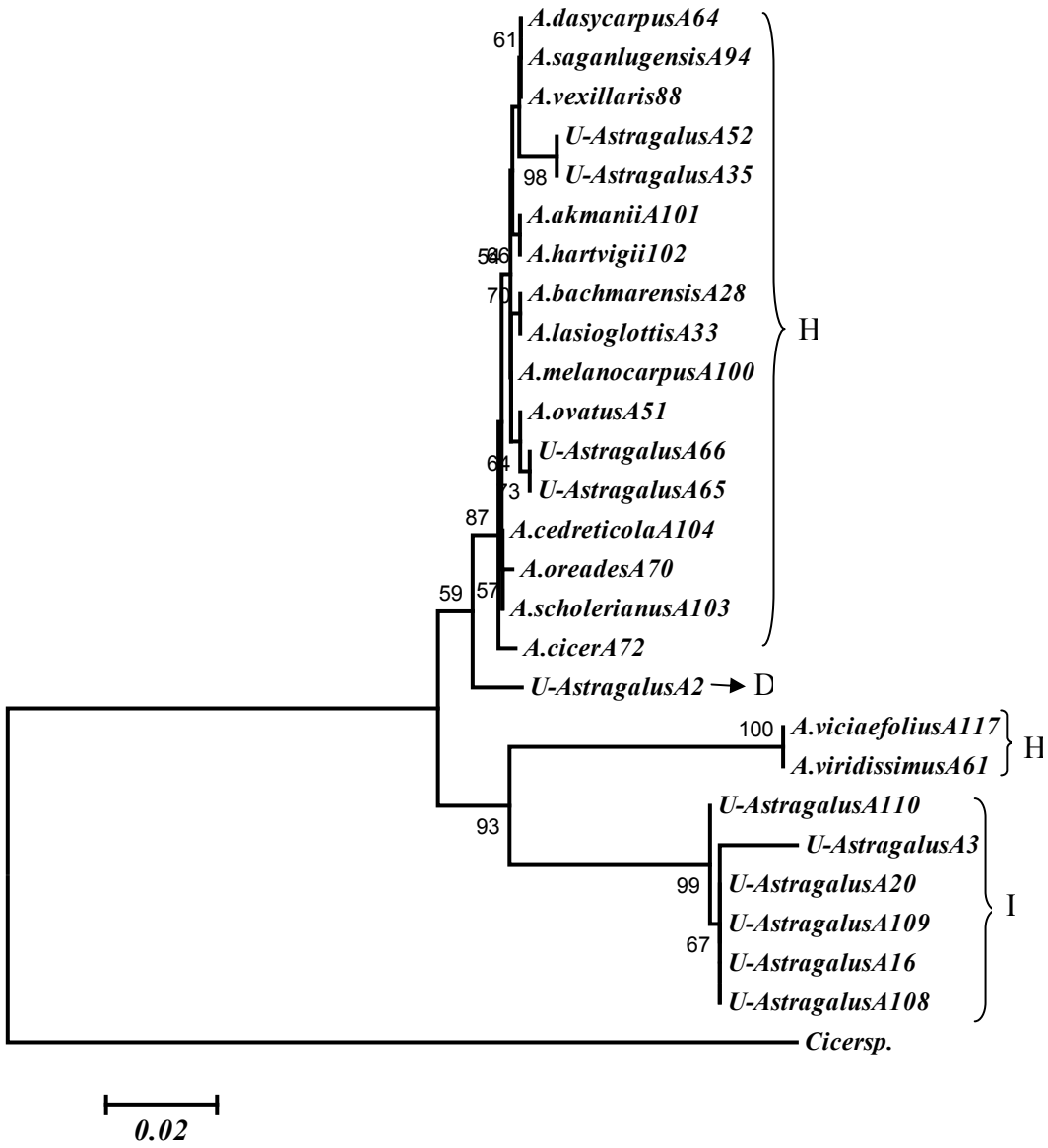

Figure 5.9 Phylogenetic tree, constructed using the sequences of *trnL5'-L3'* region of cpDNA, depicting relationships among the species of *Hypoglottidei* section. (Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori*. U: Unknown *Astragalus* samples).

Species of *Hypoglottidei* section formed several different small clusters. This is caused by high nucleotide variations and indels in sequence of trnL5'-L3' region among species of the section. *A.cicer* attached to the other species of the same section externally, and *A. viridissimus* and *A. viciaefolius* species were not included in *Hypoglottidei* cluster (Figure 5.9). Four of unidentified samples, U-*Astragalus* A35, A52, A65 and A66, were included in the cluster formed by most of *Hypoglottidei* species. U-*Astragalus* A35 and A52 were found in the same subcluster, which was expected since they had completely the same DNA sequences. These two samples were phylogenetically nearer to A. *saganlugensis, A.vexillaris,* and *A. dasycarpus* with respect to the other *Hypoglottidei* species. The other two samples, U-*Astragalus* A65 and A66, were clustered together and close to *A. ovatus.* Based on these outcomes section of four unknown samples can be easily determined, but species definition of them cannot be made (Figure 5.9).

DNA sequences of *trnL* intron region was also used to analyze the relationships among species of *Dissitiflori* section (Figure 5.10). Only U-*Astragalus* A2 specimen was grouped within main *Dissitiflori* cluster while other unkown samples formed two smaller clusters. With the help from Figure 5.7, which embraced all species of each section, it was understood that one of the small clusters comprised only *Incani* samples (A110, A109, A108, A16, A20, and A3) whereas the other included samples (A35, A52, A65, and A66) of *Hypoglottidei* section (Figure 5.10). Although U-*Astragalus* A3 sample was located with other unknown samples of *Incani*, it joined to the cluster, externally. Similar result was also observed at Figure 5.8 when only species of *Incani* section and all unknown samples were used for analyses.

At Figure 5.10, one of the unanticipated results was the distance of *A. subulatus* to the other species of the same section. Normally, all species of *Dissitiflori* was expected to be within the *Dissitiflori* (D) cluster, but this species joined to the cluster externally. Within the section, the most distant species was *A. subulatus* (Table 5.9) so it joined to the D cluster from outer. This result might be deu to the

substitutions and multinucleotide deletions, which were appeared between 414th and 421st bases in the DNA sequence of *A.subulatus*.

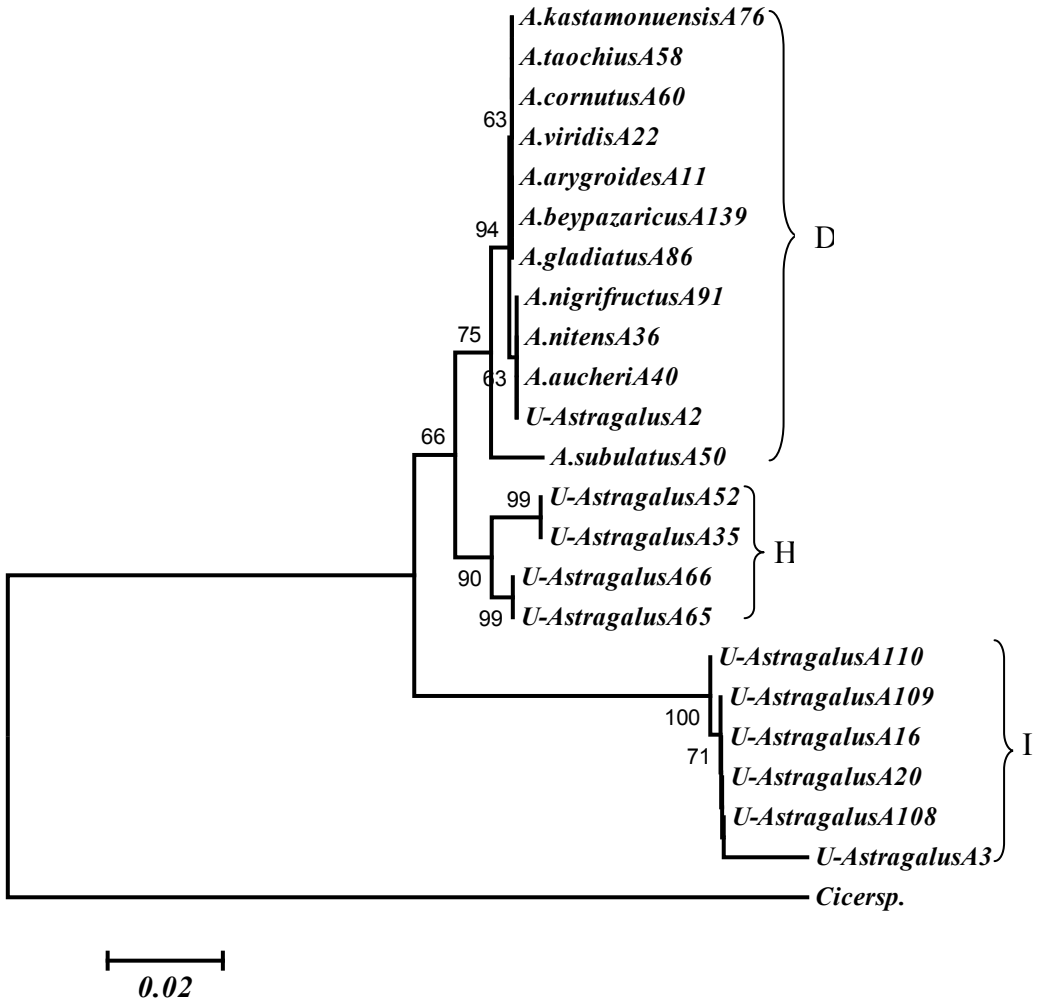

Figure 5.10 Phylogenetic tree, constructed using the sequences from *trnL5'-L3'* Region of cpDNA, depicting relationships among the species of *Dissitiflori* section.

(Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori.* U: Unknown *Astragalus* samples).

5.2.3.2 Phylogenetic Tree Construction by using *trnL3'-F* $^{(GAA)}$ Region of cpDNA

DNA sequences of *trnL-F* region was shorter and more conservative (Table 5.3) compared with neighboring region (*trnL* intron) of chloroplast DNA. Low variation may be caused because of shortness of the region. The length of the region was about 170 bp and only one substitution and three deleted nucleotides determined the phylogenetic relationships among *Astragalus* sections and species (Figure 5.11).

		Nucleotide Positions in the Aligned DNA sequences of *trnL-F* Region																				
Section	**Species name**	**56**	**57**	**58**	**59**	**60**	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**	**71**	**72**	**73**	**74**	**75**	**76**	**77**
INCANI	*A. achundovii*	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	T	A	T	T	T
	A. ancistrocarpus	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	T	A	T	T	T
	A. brevidentatus	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	T	-	-	T	T
	A. latifolius	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	T	A	T	T	T
	A. nezaketae	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	T	A	T	T	T
	A. polhillii	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	T	A	T	T	T
	A. sanguinolentus	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	T	A	T	T	T
	A. scabrifolius	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	T	A	T	T	T
	A. zaraensis	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	T	A	T	T	T
HYPOGLOTTIDEI	*A. akmanii*	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. bachmarensis	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. cicer	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. dasycarpus	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. viciaefolius	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. hartvigii	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. lasioglottis	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. ovatus	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. saganlugensis	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. scholerianus	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. vexillaris	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. viridissimus	A	T	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
DISSITIFLORI	*A. argyroides*	A	G	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. aucheri	A	G	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. beypazaricus	A	G	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. nigrifructus	A	G	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. subulatus	A	G	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T
	A. viridis	A	G	A	T	C	C	T	C	T	T	T	T	T	T	T	T	T	-	A	T	T	T

Figure 5.11 Substitutions and indels in the DNA sequences of *trnL-F* cpDNA region for each section.

[Nucleotide absence was shown as a dot, inserted (deleted) base as blue and substitution as red color. Numbers above each column depict the position of the corresponding nucleotide in the whole alignment (5' to 3')].

As it was evident from Figure 5.11, a substitution, which occurred between thymine (T) and Guanine (G) at 57th bp is the main reason for separation of *Dissitiflori* species from the species of other sections (*Incani* and *Hypoglottidei).* Within each section, there were almost no differences among species except for *A. brevidentatus* in *Incani* section. This species had two missing nucleotides, Adenine and Thymine, which were located between 73rd and 76th bases.

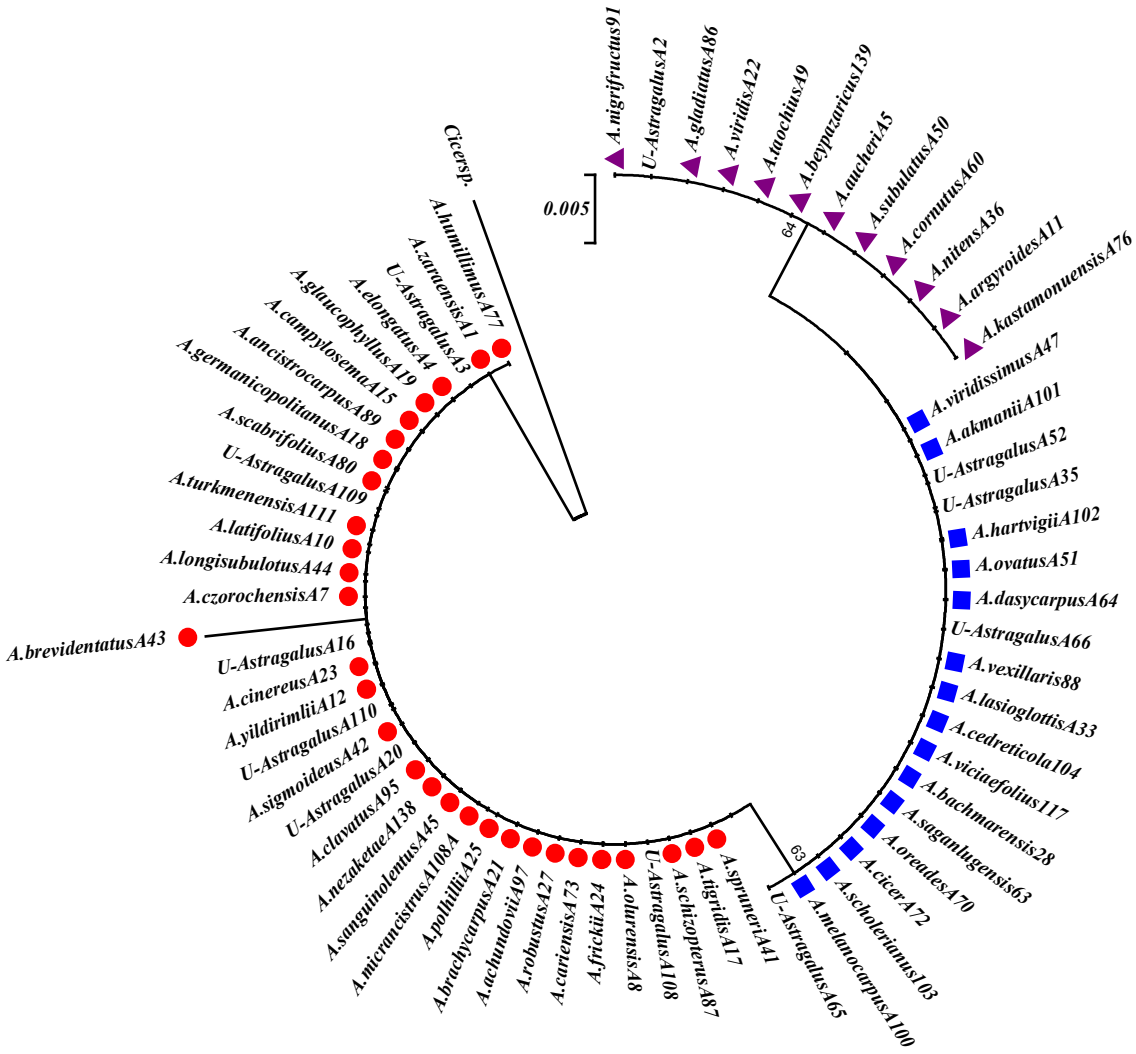

Figure 5.12 Phylogenetic tree, constructed using the sequences of *trnL-F* region of cpDNA depicting relationships among the species of three sections of *Astragalus*. (Numbers on branches of the tree indicate bootstrap values, Circles: *Incani*, Squares: *Hypoglottidei* and Triangles*: Dissitiflori* section. U: Unknown *Astragalus* sample).

Constructed phylogenetic tree (Figure 5.12) including all sections with their representative species depicted similar results with the tree (Figure 5.7) constructed by using DNA sequences of *trnL* intron region. As it is in the prevously-constructed tree (Figure 5.7), *Incani* was clustered distantly to the *Dissitiflori* section in the phylogenetic tree that was constructed via the sequences of *trnL-F* region (Figure 5.12). This interaction was expected due to genetic divergences among sections (Table 5.8). Genetic divergence between these two sections was the highest when both DNA sequences of *trnL* intron (0.071) and *trnL-F* (0.012) region were analyzed. The main difference between the trees was branching structure within each section.

Figure 5.13 signifies phylogenetic relationships among species of *Incani* section and unknown samples coming from different sections. Each of *Incani* species with several unidentified species of *Astragalus* were grouped together and constituted *Incani* (I) cluster. Unidentifed species within 'I' cluster were U-*Astragalus* A3, A16, A20, A108, A109, and A110 samples. These samples were the same with individuals, which were grouped within *Incani* section in the phylogentic tree constructed according to the DNA sequences of *trnL* intron chloroplast region. Because of the low variation in *trnL-F* region of cpDNA among sequences of specimens, all unidentified samples were joined to the tree directly. This means there were no genetic differences between sequences of unidentified samples and those of *Incani* species except for *A. brevidentatus,* which was separated from other *Incani* species because of two missing nucleotides. Other unknown samples, A2, A35, A52, A65, A66, gave rise to the other cluster at the top region of the tree (Figure 5.13). Except A2, all other four samples were regarded as species of *Hypoglottidei* section.

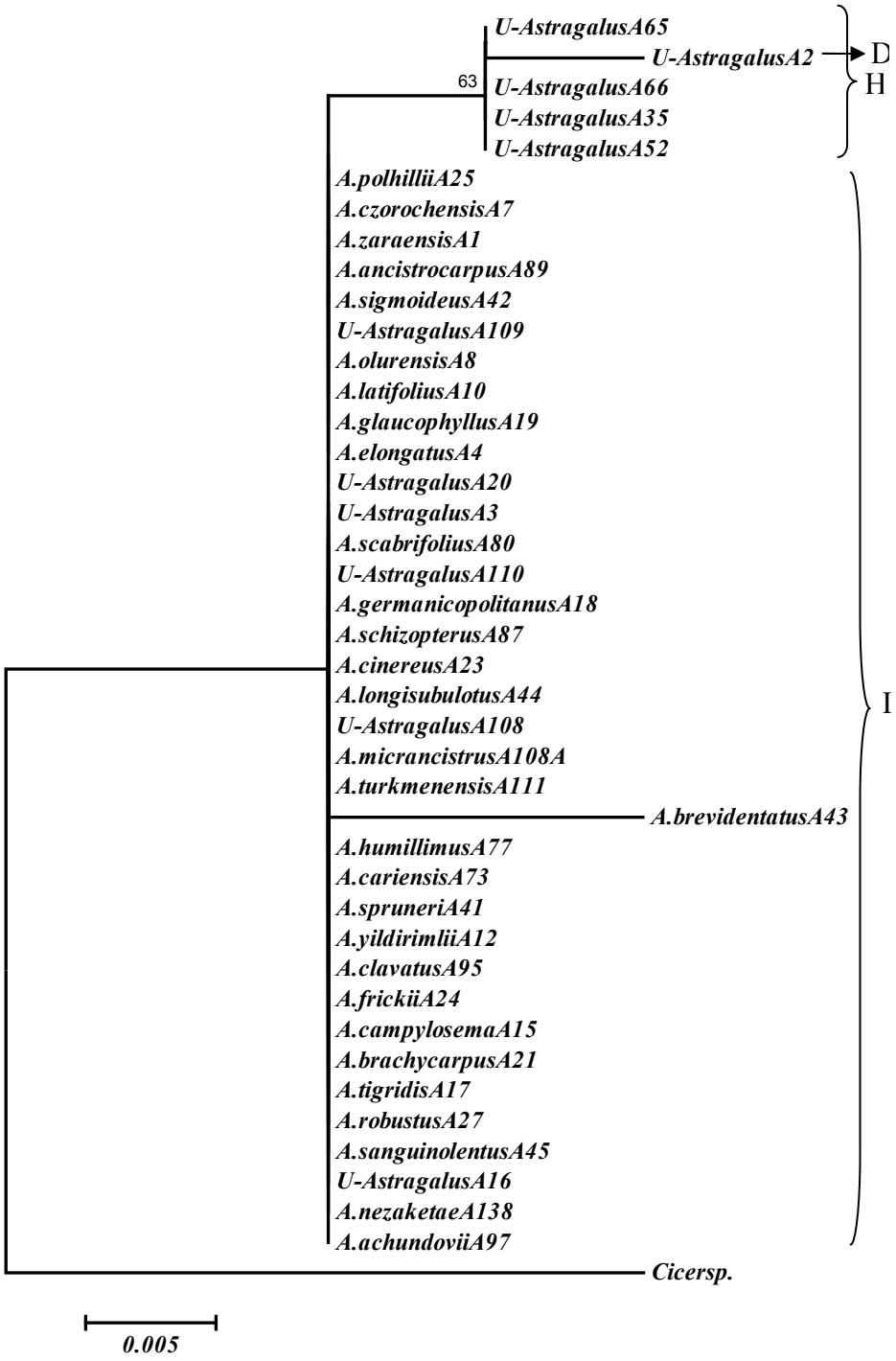

Figure 5.13 Phylogenetic tree, constructed using the sequences of *trnL-F* region of cpDNA, depicting relationships among the species of *Incani* section.
(Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori.* U: Unknown *Astragalus* samples).

Phylogenetic relationships among species of *Hypoglottidei* section were provided in Figure 5.14 with all unidentified species. Unknown samples coming from different sections of *Astragalus* formed a new cluster. All species of *Hypoglottidei* section were clustered together with several unknown samples such as A2, A35, A52, A65, and A66. Four of them were expected to be clustered with species of *Hypoglottidei* section. A2 individual was included in the H cluster; this result was normally not expected because A2 sample was found in *Dissitiflori* section. However, *Dissitiflori* species were genetically closer to the species of *Hypoglottidei* section so this sample was grouped with species of *Hypoglottidei.*

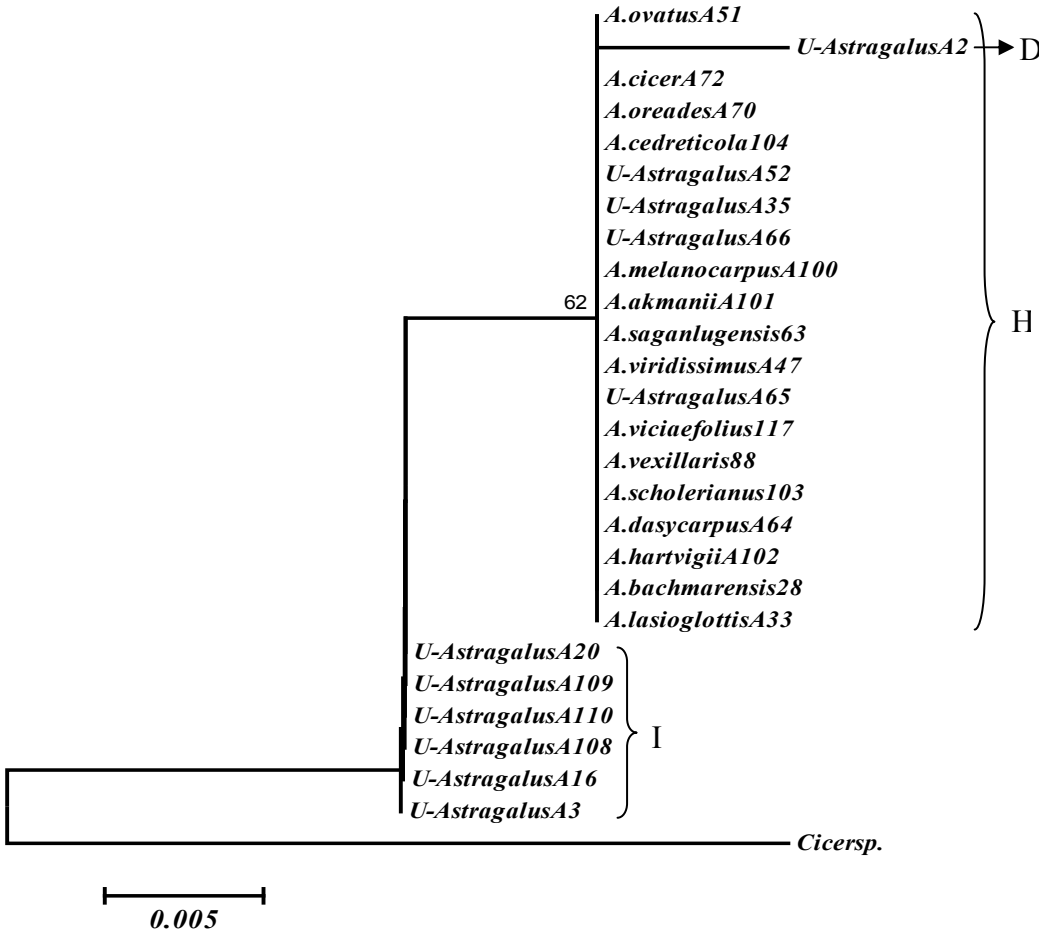

Figure 5.14 Phylogenetic tree, constructed using the sequences of *trnL-F* region of cpDNA, depicting relationships among the species of *Hypoglottidei* section.
(Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori*).

For *Dissitiflori* section, eleven species were used and their phylogenetic relationships were indicated in Figure 5.15. At this tree, three main clusters were constructed; first one comprised only *Dissitiflori* species and unknown A2 sample, which is expected to be in *Dissitiflori* section based on outcomes of former region. The DNA sequences of each *Dissitiflori* species were completely the same to each other so they were grouped together without any genetic distance. Other used unknown samples were divided into two clusters that comprised samples from different sections. U-*Astragalus* A35, A52, A5 and A66 were included in 'H' cluster whereas U-*Astragalus* A3, A16, A20, A108, A109, A110 were included in 'I' cluster.

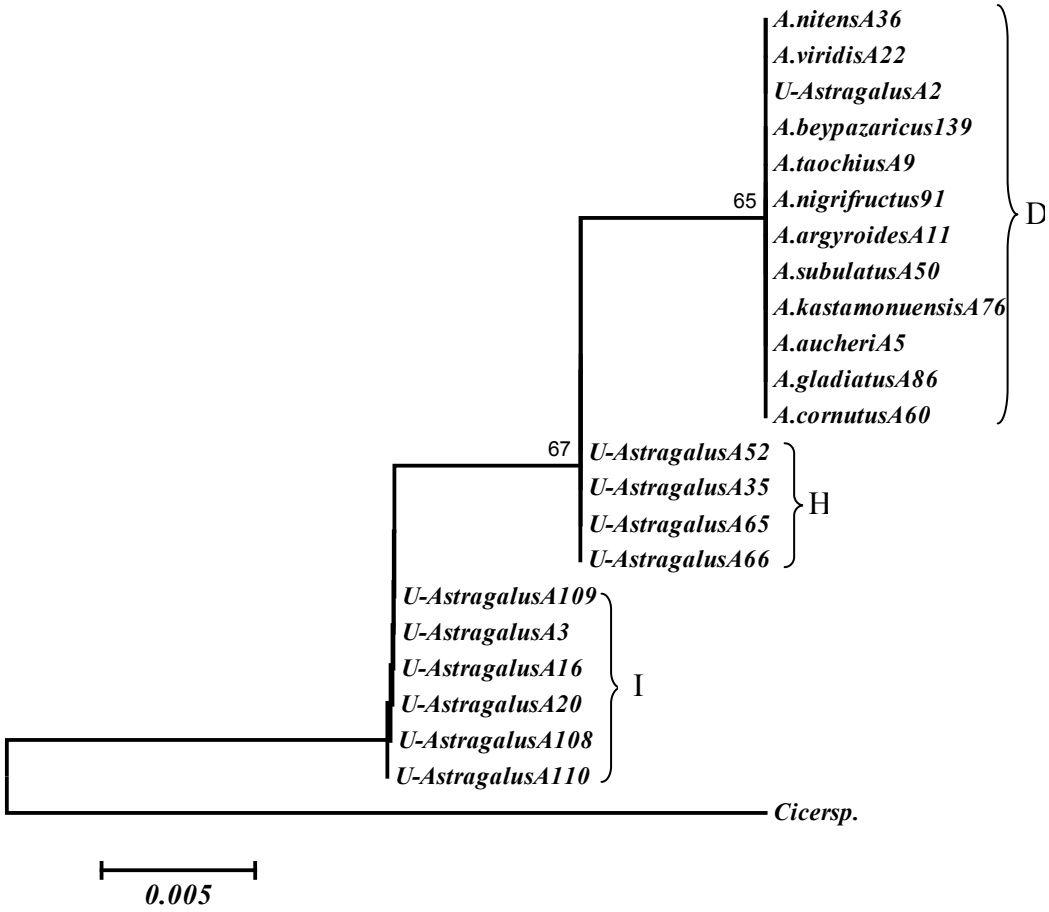

Figure 5.15 Phylogenetic tree, constructed using the sequences of *trnL-F* region of cpDNA, depicting relationships among the species of *Dissitiflori* section.
(Numbers indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori*).

5.2.3.3 Phylogenetic Tree Construction by Using Sequence Data from *trnV* Region of cpDNA

TrnL intron and *trnL-F* region were located side by side on the chloroplast DNA. Therefore, *trnV* region, which resided distantly to them was selected as a third region. Although the total length of the region was about 608 bp, low sequence diversity was observed among and within sections. Nevertheless, these variations were useful to distinguish sections from each other. One of the crucial variations among sections was inserted and/or deleted nucleotides. Almost all deleted nucleotides were found within *Incani* section and inserted regions resided through DNA sequences of *Dissitiflori* and *Hypoglottidei* species. Figure 5.16 depicts indels and substitutions among and within sections of *Astragalus.* The substitution rate was more in *Hypoglottidei* section (Table 5.4) compared with those of other sections so more branching was expected at the phylogenetic tree for this section (Figure 5.17).

		Nucleotide Positions in the Aligned DNA sequences of *trnV* Region																						
Section	**Species name**	**60**	**66**	**119**	**120**	**121**	**122**	**123**	**124**	**125**	**126**	**127**	**128**	**129**	**130**	**131**	**132**	**246**	**257**	**283**	**284**	**285**	**286**	**287**	**288**
I	*A. achundovii*	T	G	G	-	-	-	-	-	-	-	-	-	-	-	-	A	C	T	A	T	C	T	T	A
	A. brevidentatus	T	G	G	-		-	-	-	-	-	-	-	-	-	-	A	C	T	A	T	C	T	T	A
	A. latifolius	T	G	G	-	-	-	-	-	-	-	-	-	-	-	-	A	C	T	A	T	C	T	T	A
	A. nezaketae	T	G	G	-	-	-	-	-	-	-	-	-	-	-	-	A	C	T	A	T	C	T	T	A
	A. sanguinolentus	T	G	G	-	-	-	-	-	-	-	-	-	-	-	-	A	C	T	A	T	C	T	T	A
	A. zaraensis	T	G	G	-	-	-	-	-	-	-	-	-	-	-	-	A	C	T	A	T	C	T	T	A
H	*A. akmanii*	G	T	G	C	A	A	A	G	G	A	T	T	T	A	G	A	C	T	T	A	T	T	T	A
	A. cicer	G	T	G	C	A	A	A	G	G	A	T	T	T	A	G	A	T	G	T	A	T	T	-	-
	A. dasycarpus	G	T	G	C	A	A	A	G	G	A	T	T	T	A	G	A	C	T	T	A	T	T	T	A
	A. viciaefolius	G	T	G	C	A	A	A	G	G	A	T	T	T	A	G	A	C	T	T	A	T	T	G	A
	A. ovatus	G	T	G	C	A	A	A	G	G	A	T	T	T	A	G	A	C	T	T	A	T	T	T	A
D	*A. aucheri*	G	G	G	C	A	A	A	G	G	A	T	T	T	A	G	A	C	T	T	A	T	T	T	A
	A. beypazaricus	G	G	G	C	A	A	A	G	G	A	T	T	T	A	G	A	C	T	T	A	T	T	T	A
	A. nigrifructus	G	G	G	C	A	A	A	G	G	A	T	T	T	A	G	A	C	T	T	A	T	T	T	A
	A. viridis	G	G	G	C	A	A	A	G	G	A	T	T	T	A	G	A	C	T	T	A	T	T	T	A

Figure 5.16 Substitutions and indels in the DNA sequences of *trnV* region for each section. (I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori*).

[Nucleotide absence was shown as a dot, inserted (deleted) base as blue and substitution as red color. Numbers above each column depict the position of the corresponding nucleotide in the whole alignment (5' to 3')].

Section	Species name	Nucleotide Positions in the Aligned DNA sequences of *trnV* Region																			
		289	290	291	292	293	294	304	424	425	426	427	428	429	430	431	436	464	487	546	559
I	*A. achundovii*	T	C	T	T	T	T	A	T	-	-	-	-	-	-	C	T	C	G	-	T
	A. brevidentatus	T	A	T	T	T	T	A	T	-	-	-	-	-	-	C	T	A	G	-	T
	A. latifolius	T	A	T	T	T	T	A	T	-	-	-	-	-	-	C	T	A	G	-	T
	A. nezaketae	T	C	T	T	T	T	A	T	-	-	-	-	-	-	C	T	C	G	-	T
	A. sanguinolentus	T	C	T	T	T	T	A	T	-	-	-	-	-	-	C	T	C	G	-	T
	A. zaraensis	T	A	T	T	T	T	A	T	-	-	-	-	-	-	C	T	A	G	-	T
H	*A. akmanii*	T	A	T	T	T	T	A	T	-	-	-	-	-	-	C	T	C	A	A	C
	A. cicer	-	-	-	-	T	C	A	T	-	-	-	-	-	-	C	T	C	A	A	C
	A. dasycarpus	T	A	T	T	T	T	T	T	-	-	-	-	-	-	C	T	C	A	A	C
	A. viciaefolius	T	A	T	T	T	T	A	T	-	-	-	-	-	-	C	T	C	A	A	C
	A. ovatus	T	A	T	T	T	T	A	T	-	-	-	-	-	-	C	T	C	A	A	C
D	*A. aucheri*	T	A	T	T	T	T	A	T	T	C	T	A	A	T	C	A	C	A	A	C
	A. beypazaricus	T	A	T	T	T	T	A	T	T	C	T	A	A	T	C	A	C	A	A	C
	A. nigrifructus	T	A	T	T	T	T	A	T	T	C	T	A	A	T	C	A	C	A	A	C
	A. viridis	T	A	T	T	T	T	A	T	T	C	T	A	A	T	C	A	C	A	A	C

Figure 5.16 continued

Incani section was separated from *Hypoglottidei* and *Dissitiflori* sections via nucleotide substitutions [(60T-60G), (283A-283T), (284T-284A), (285C-285T), (487G-487A), (559T-559C)] and a large deleted region located between 119th and 132nd bases (CAAAGGATTTAG) in addition to the Adenine nucleotide deletion at 546th base (Figure 5.16). Moreover, *Hypoglottidei* was separated from *Dissitiflori* species via two nucleotide substitutions [(66T-66G), and (436T-436A)] and a small deleted region observed between 424th and 431st bases (TCTAAT, Figure 5.16). The highest diversity was noticed among *Hypoglottidei* species while no variation was observed among *Dissitiflori* species.

Figure 5.17 indicates phylogenetic relationships between studied sections and their species. At the section level, the tree was quite informative because sequences of the region were enough to separate sections from each other. As seen at the tree, *Incani* section had two large clusters, while there were several small clusters and branching types within *Hypoglottidei* section. Since the DNA sequences had identical matches among species within *Dissitiflori* section, no different clustering was observed in this section. All species of the section were included in a big cluster (Figure 5.17).

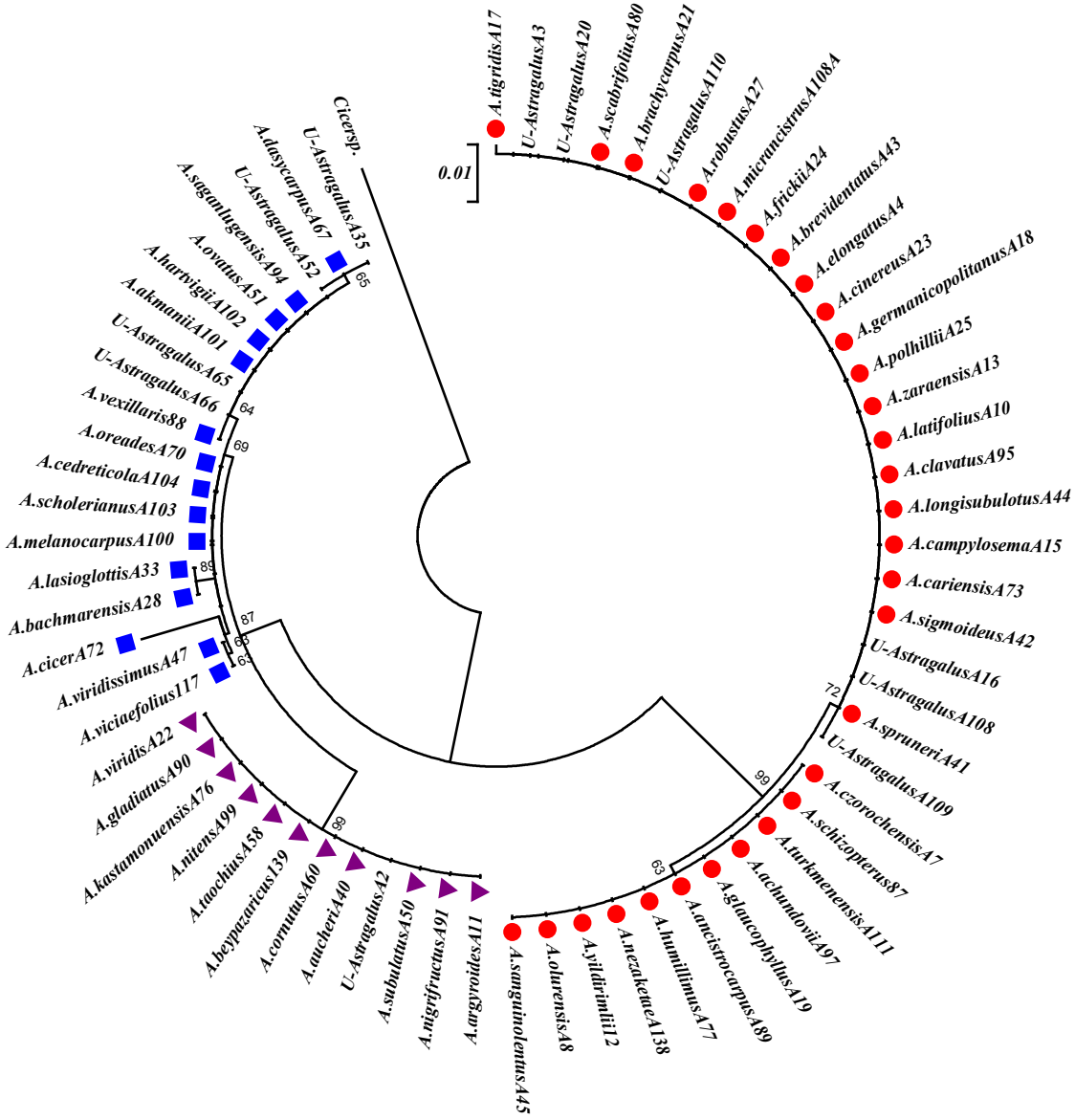

Figure 5.17 Phylogenetic tree, constructed using the sequences of *trnV* region of cpDNA, depicting relationships among the species of *Astragalus*.

(Numbers on branches of the tree indicate bootstrap values, Circles: *Incani*, Squares: *Hypoglottidei* and Triangles*: Dissitiflori* section. U: Unknown *Astragalus* sample).

Each species of *Incani* sections and unknown samples were analyzed together to shed light on relationships among species of this section and to figure out positions and sections of unidentified samples (Figure 5.18). Two main clusters with high bootstrap values were apparent in the constructed dendrogram. The first one, which was constituted by two subclusters, comprised only species of *Incani* section so this cluster was named as 'I'. Most of the species of *Incani* section were located in the first subcluster with all unknown species (A3, A16, A20, A108, A109, and A110). All species bound the cluster directly while *A.tigridis* was distantly located in the cluster. Separation of this species was due to by a substitution occurred between Cytosine and Thymine at 466th bp position of the region. Eleven species *(A. humillimus, A. olurensis, A. achundovii, A. schizopterus, A. czorochensis, A. glaucophyllus, A. nezaketae, A. sanguinolentus, A. ancistrocarpus, A.turkmenensis,* and *A. yildirimlii)* were separated from other *Incani* species and accounted for formation of second subcluster within 'I' cluster (Figure 5.18). The second main cluster was composed by unidentified samples, which were thought to be *Dissitiflori* and/or *Hypoglottidei* species. A2 sample joined to the cluster distantly, because this sample was included in different section (*Dissitiflori*) from where other samples (A35, A52, A65, A66) were found (*Hypoglottidei*).

Phylogenetic relationships of *Hypoglottidei* species and unidentified samples were given in Figure 5.19. The species of this section had high nucleotide variation among them (Table 5.4, Figure 5.16). Number of substitutions were high so different branching formations were observed in the constructed tree (Figure 5.19). There were two main clusters; the first one, H cluster, had mainly species of *Hypoglottidei* section with a few unidentified samples. This cluster contained several small subclusters with different branching structures. One of the subcluster composed of *A. dasycapus* and two unidentified samples (A35, A52). These two unidentified samples joined to the subcluster directly, so we can say that A35 and A52 samples may be *A.dasycarpus,* but still this identification cannot be accepted as exactly accurate without supports of other used regions. Moreover, *A. viciaefolius, A. viridissimus* and *A. cicer* were grouped in a different subcluster.

Although *A. cicer* grouped with *A. viciaefolius* and *A. viridissimus*, it joined distantly to the subcluster since the species had more substitutions and indels with respect to that of other *Hypoglottidei* species.

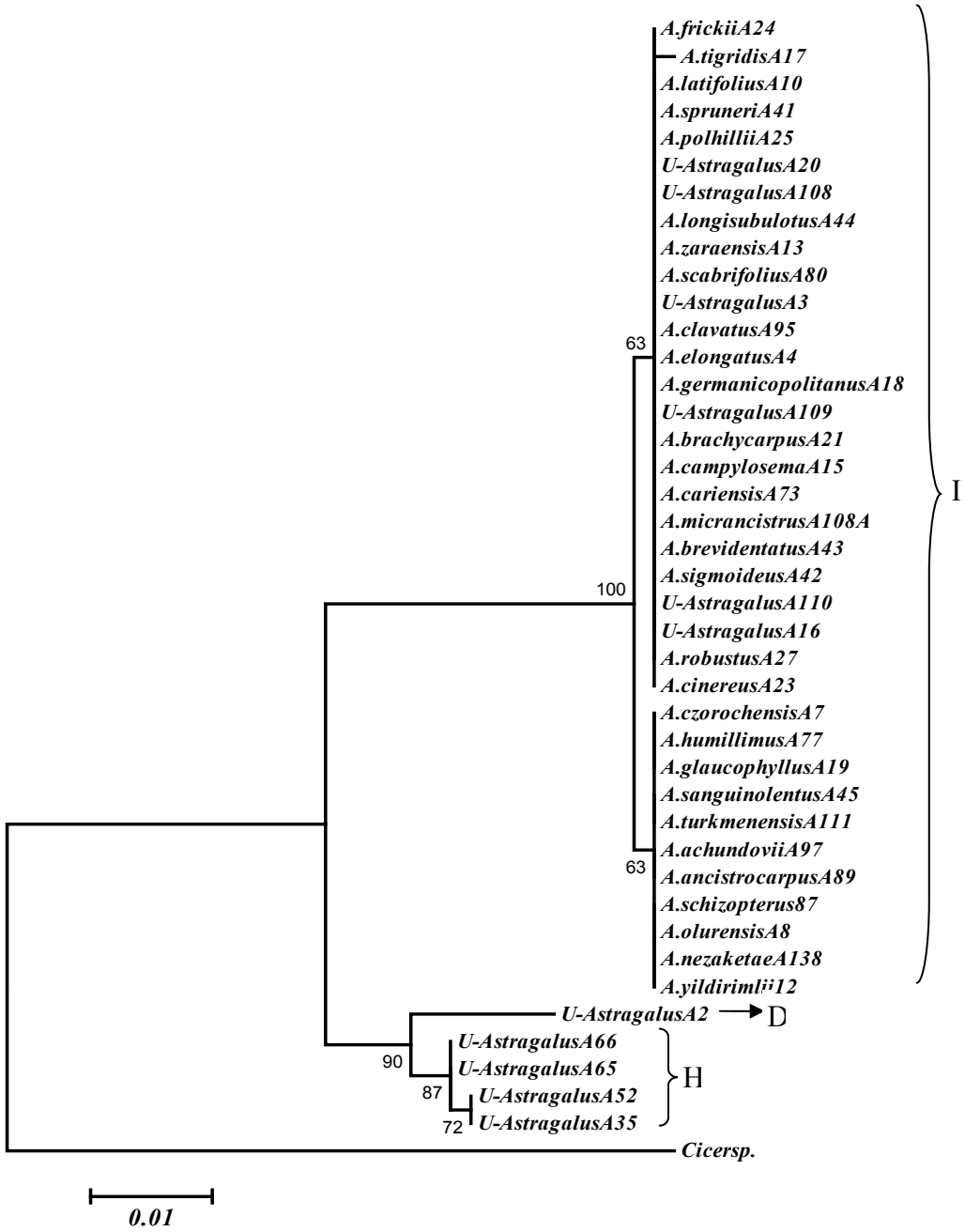

Figure 5.18 Phylogenetic tree, constructed using the sequences of *trnV* region of cpDNA, depicting relationships among the species of *Incani* section.
(Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori*).

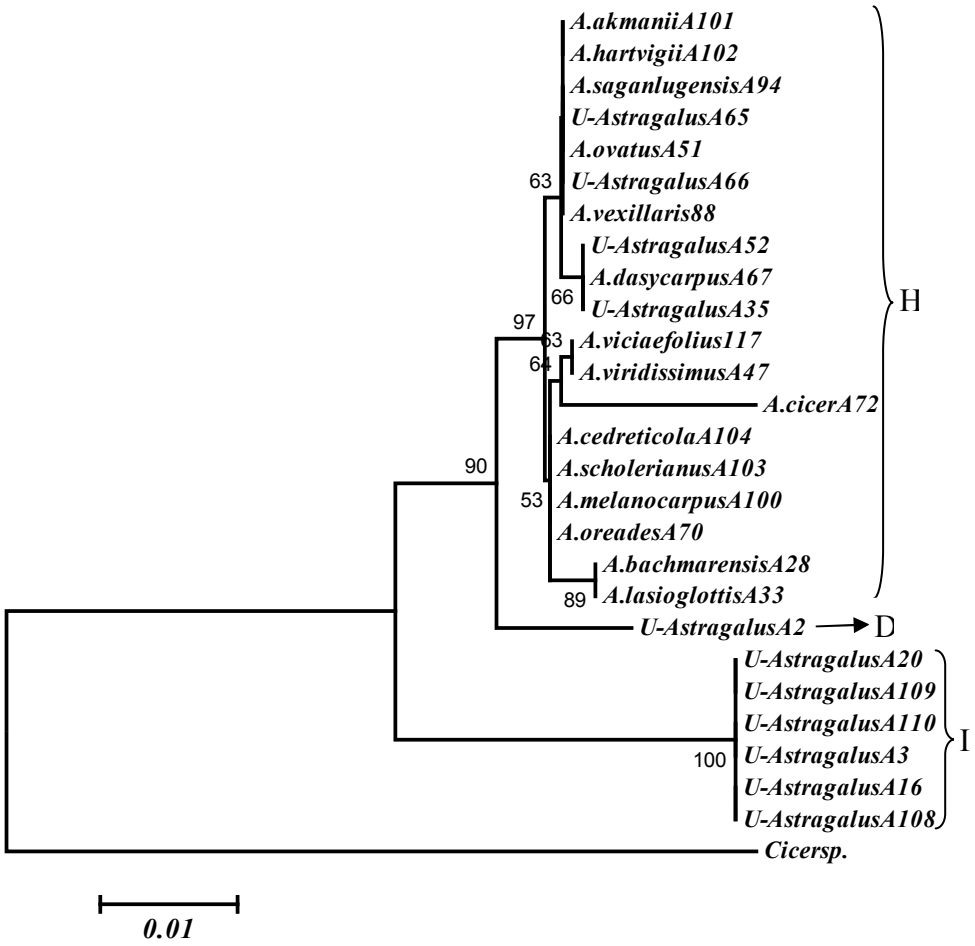

Figure 5.19 Phylogenetic tree, constructed using the sequences of *trnV* region of cpDNA, depicting relationships among the species of *Hypoglottidei* section. (Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori).*

Figure 5.20 indicates genetic relationships among species of *Dissitiflori* section and unidentified samples, which were decided by using nucleotide variations and indels of DNA sequences of *trnV* cpDNA region. As mentioned in the above sections, the DNA sequences of each *Dissitiflori* species were completely the same (Figure 5.17) so all of candidate *Dissitiflori* species were located in 'D' cluster without any divergence. In addition to D cluster, two other main clusters (I and H) with high bootstrap values (100%) were present in the constructed tree. As

expected, 'I' cluster joined to the tree after binding of H cluster because *Hypoglottidei* species genetically were more similar to the species of *Dissitiflori* section.

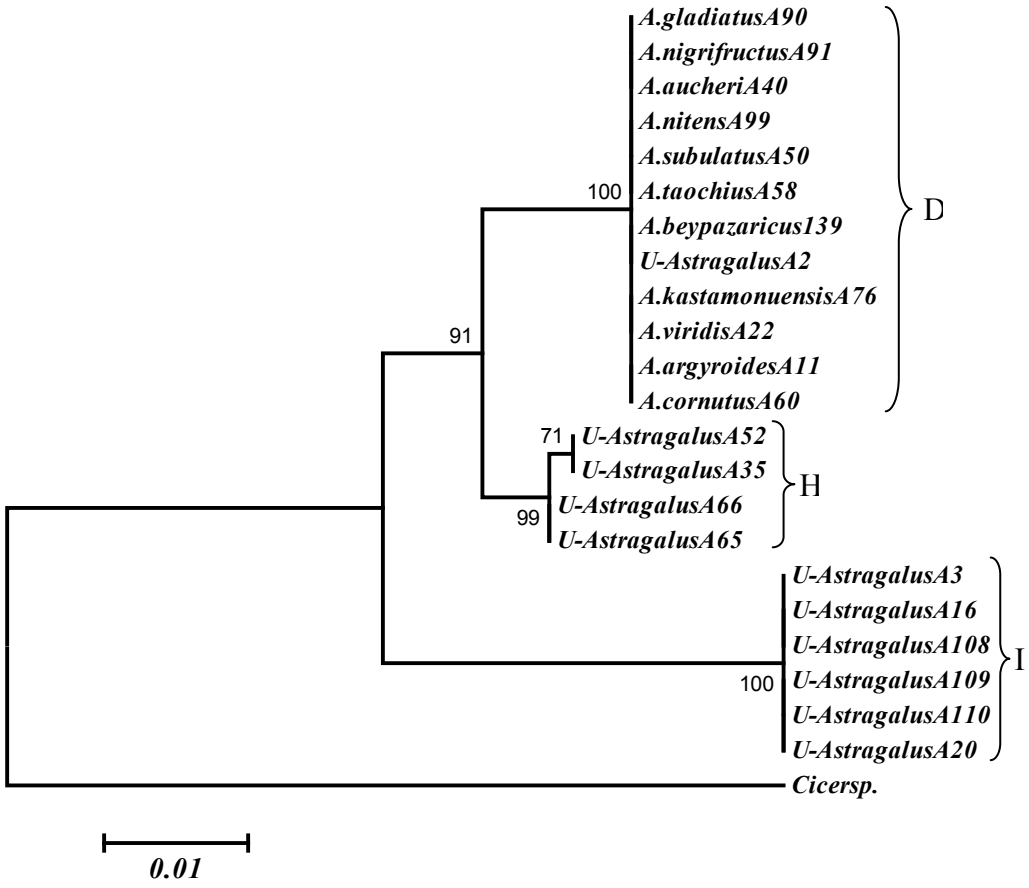

Figure 5.20 Phylogenetic tree, constructed using the sequences of *trnV* region of cpDNA, depicting relationships among the species of *Dissitiflori* section.
(Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori).*

5.2.3.4 Phylogenetic Tree Construction by Using Sequence Data of *matK* cpDNA Region

To understand phylogenetic structure of the three *Astragalus* sections, *matK* region was also chosen. The sequence of this region begins with a start codon 'ATG' and terminates with a stop codon 'TGA' after about 1500 bp from starting base. Generally, whole region is amplified by using two primer pairs and combined DNA sequence is utilized for analyses. The first primer pair is capable to amplify nearly complete sequence of the region (~1200bp). Therefore, remaining part of the region, which is about 300 bp was not amplified to reduce labour and costs.

Both nucleotide substitutions and indels were recognized in the sequenced region, but only one (*A.cicer*) of fifty-six species had inserted nucleotides between 565th and 575th bp positions. This species possessed repeated nucleotide series at the mentioned positions. All remaining studied *Astragalus* species had 'ATAGGAATA' sequence only once while *A.cicer* had tandem repeat of this sequence (Figure 5.21). Due to the length of the *matK* region, high number of substitutions was expected. However, there were about twenty-seven positions, which indicated nucleotide substitutions. Three of the substitutions (209th, 247th, and 973rd) were only observed in the DNA sequence of *A. cedreticola* (Figure 5.21). Generally, there was compatibility within section when a substitution was considered. If one nucleotide variation was occurred in DNA sequence of one species, same substitution was generally observed in that of each species within the same section. However, most of the substitutions in *matK* region were only detected in one or few species. Three nucleotide substitutions (168C-168T, 303C-303T, 1054C-1054G) caused separation of *Incani* form the other sections. Two substitutions (183T-183C, 999C-999T) caused separation of *Dissitiflori* from the other two sections. *Hypoglottidei s*pecies carried Cytosin at 930nd base in the aligned DNA while *Incani* and *Dissitiflori* species carried Thymine base, which caused separation of this section from the others (Figure 5.21).

Section	Species name	30	168	183	209	213	247	303	350	351	453	565	566	567	568	569	570	571	572	573	574	575	587	634	691	807	815	828	879	894	930	955	959	969	973	999	1031	1054
I	*A. achundovii*	C	C	C	T	C	T	C	C	A	A	A	-	-	-	-	-	-	-	-	-	G	A	T	A	A	T	G	G	T	T	G	C	A	C	T	G	C
	A. ancistrocarpus	A	C	C	T	C	T	C	C	A	A	A	-	-	-	-	-	-	-	-	-	G	C	G	C	A	T	G	A	T	T	G	C	A	C	T	G	C
	A. nezaketae	C	C	C	T	C	T	C	C	A	A	A	-	-	-	-	-	-	-	-	-	G	A	T	A	A	T	G	G	T	T	G	C	A	C	T	G	C
	A. polhillii	A	C	C	T	C	T	C	C	A	A	A	-	-	-	-	-	-	-	-	-	G	C	G	C	A	T	G	A	T	T	G	C	A	C	T	G	C
	A. scabrifolius	A	C	C	T	C	T	C	C	A	A	A	-	-	-	-	-	-	-	-	-	G	C	G	C	A	T	G	A	T	T	G	C	A	C	T	G	C
H	*A. akmanii*	A	T	C	T	C	T	T	C	A	A	A	-	-	-	-	-	-	-	-	-	G	C	T	A	A	T	G	G	T	C	G	C	A	C	T	G	G
	A. cedreticola	A	T	C	C	A	G	T	C	C	A	A	-	-	-	-	-	-	-	-	-	G	C	T	A	A	T	G	G	T	C	G	C	A	G	T	G	G
	A. cicer	A	T	C	T	C	T	T	G	A	C	A	A	T	A	G	G	A	A	T	A	G	C	T	A	A	T	G	G	T	C	G	C	A	C	T	G	G
	A. dasycarpus	A	T	C	T	C	T	T	C	A	A	A	-	-	-	-	-	-	-	-	-	G	C	T	A	A	T	G	G	T	C	G	T	A	C	T	G	G
	A. viciaefolius	A	T	C	T	C	T	T	C	A	A	A	-	-	-	-	-	-	-	-	-	G	C	T	A	A	T	G	G	T	C	G	C	C	C	T	A	G
	A. hartvigii	A	T	C	T	C	T	T	C	A	A	A	-	-	-	-	-	-	-	-	-	G	C	T	A	A	T	A	G	T	C	G	C	A	C	T	G	G
	A. ovatus	A	T	C	T	C	T	T	C	A	A	A	-	-	-	-	-	-	-	-	-	G	C	T	A	A	T	A	G	T	C	G	T	A	C	T	G	G
	A. scholerianus	A	T	C	T	A	T	T	C	C	A	A	-	-	-	-	-	-	-	-	-	G	C	T	A	A	T	G	G	T	C	G	C	A	C	T	G	G
	A. viridissimus	A	T	C	T	C	T	T	C	A	A	A	-	-	-	-	-	-	-	-	-	G	C	T	A	A	T	G	G	T	C	G	C	C	C	T	A	G
D	*A. argyroides*	A	T	T	T	C	T	T	C	A	A	A	-	-	-	-	-	-	-	-	-	G	C	T	A	A	T	G	G	T	T	G	C	A	C	C	G	G
	A. aucheri	A	T	T	T	C	T	T	C	A	A	A	-	-	-	-	-	-	-	-	-	G	C	T	A	G	C	G	G	A	T	A	C	A	C	C	G	G
	A. beypazaricus	A	T	T	T	C	T	T	C	A	A	A	-	-	-	-	-	-	-	-	-	G	C	T	A	A	T	G	G	T	T	G	C	A	C	C	G	G
	A. nigrifructus	A	T	T	T	C	T	T	C	A	A	A	-	-	-	-	-	-	-	-	-	G	C	T	A	G	C	G	G	A	T	A	C	A	C	C	G	G

Nucleotide Positions in the Aligned DNA sequences of *matK* Region

Figure 5.21 Substitutions and indels in the DNA sequences of *matK* region for each section.

(I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori*). Nucleotide absence was shown as a dot, inserted (deleted) base as blue and substitution as red color. Numbers above each column depict the position of the corresponding nucleotide in the whole alignment (5' to 3').

Relationhips of sections in the phylogenetic tree constructed by using variations and indels of *matK* region were similar to those results obtained previously by using the data from other chloroplast regions. Figure 5.22 depicts relationships among three sections *Astragalus* and the species of the sections based on *matK* region. Unidentified samples were located within the same section where they were clustered based on the sequences of former chloroplast DNA regions. Although unknown samples were not clearly identified, sections and phylogenetic relationships of them with other species can be easly understood. After constructing the phylogenetic tree comprising all species of the three used sections, trees involving species of only one section was also constructed (Figure 5.23, Figure 5.24, and Figure 5.25) to realize close relationships among species within the section.

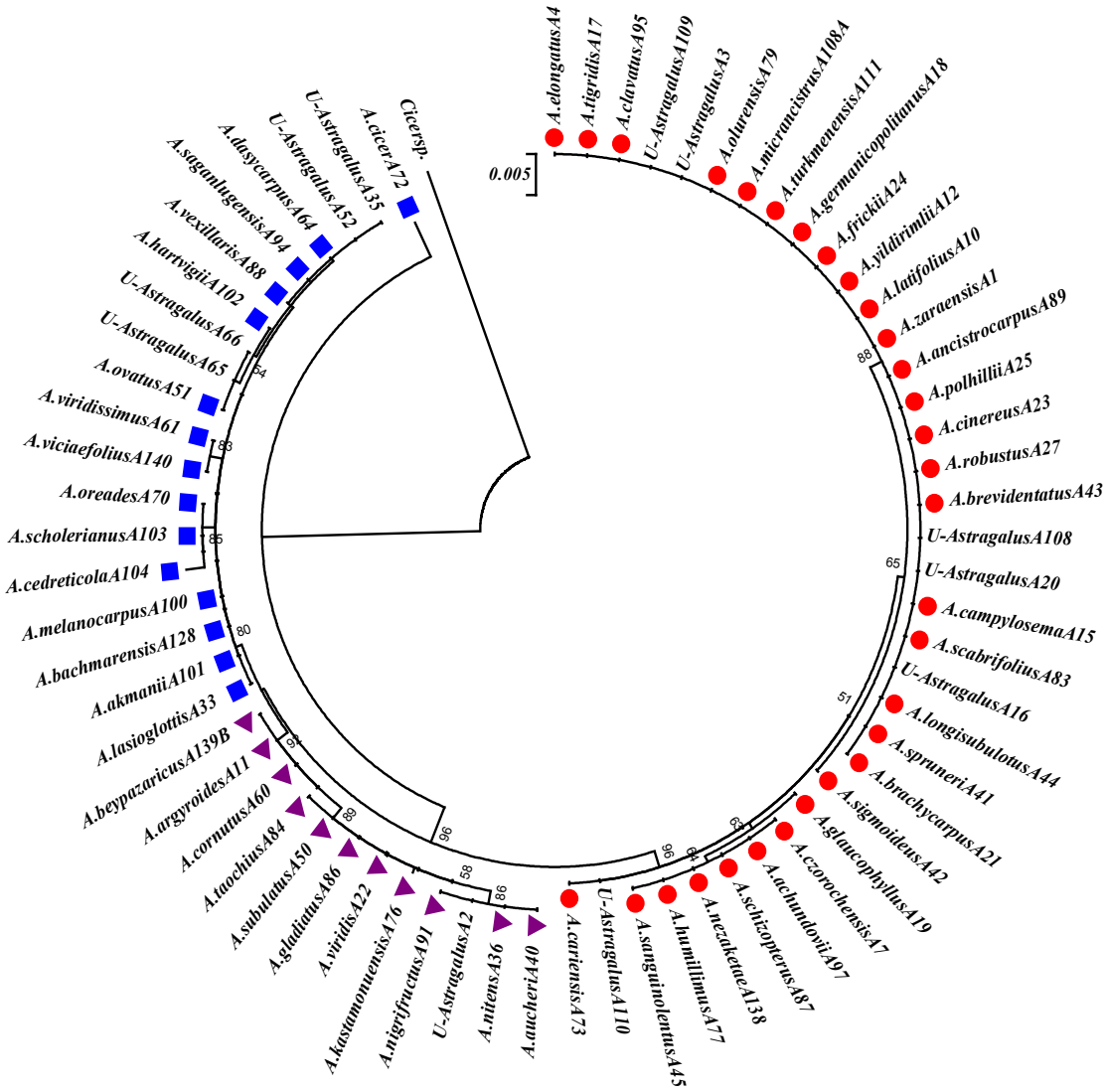

Figure 5.22 Phylogenetic tree, constructed using the sequences of *matK* region of cpDNA, depicting relationships among the species of *Astragalus*.

(Numbers on branches of the tree indicate bootstrap values, Circles: *Incani*, Squares: *Hypoglottidei* and Triangles*: Dissitiflori* section. U: Unknown *Astragalus* sample).

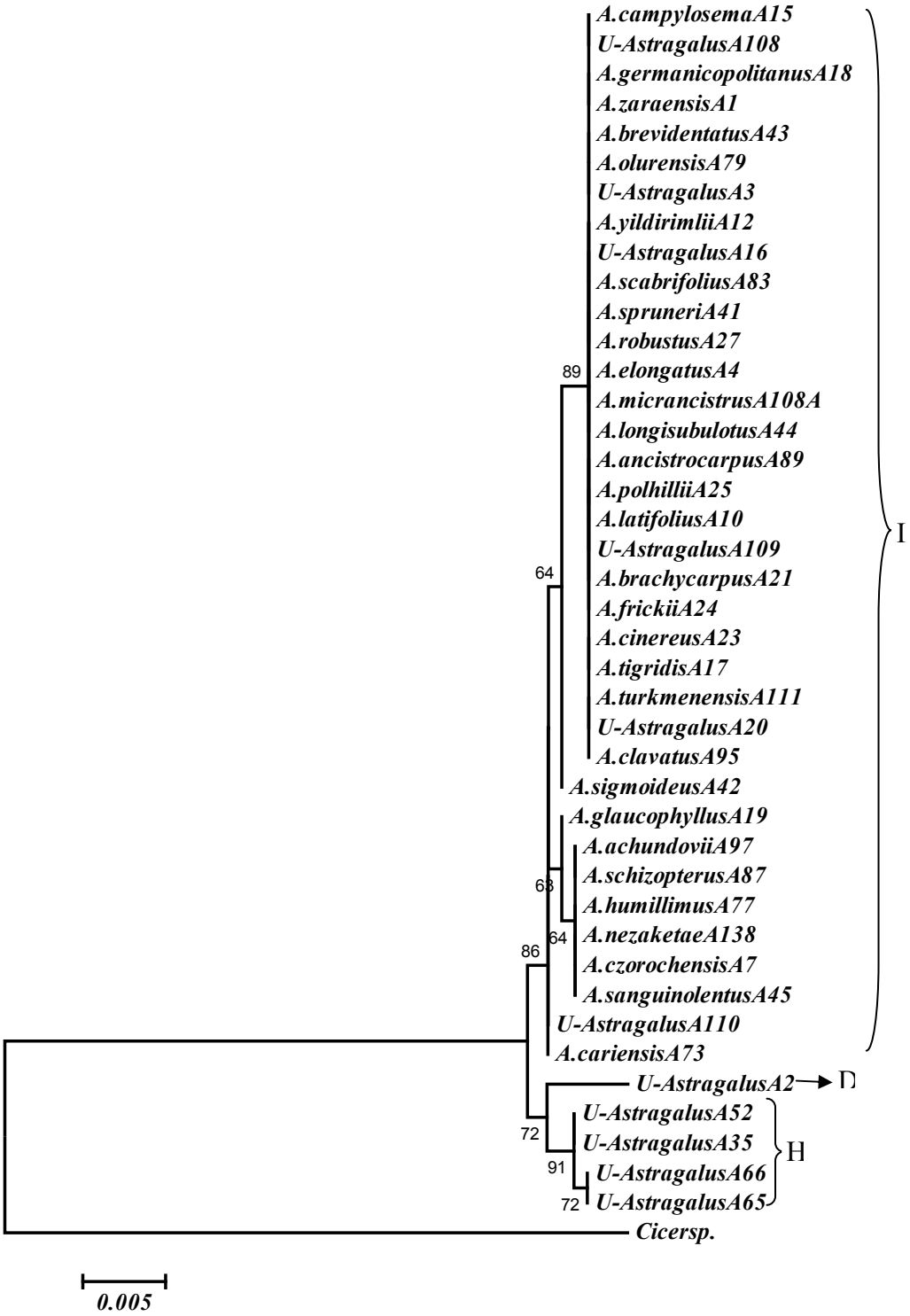

Figure 5.23 Phylogenetic tree, constructed using the sequences of *matK* region of cpDNA, depicting relationships among the species of *Incani* section.

(Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori).*

The phylogenetic tree given in Figure 5.23 describes the phylogenetic relationship among species of *Incani* section and unidentified species samples. As in previous analyses based on *trn* regions, again *Cicer* species was used as an outgroup sample. Although two main clusters were recognized in the phylogenetic tree (Figure 5.23), the first one (I cluster) had a few branching and subclusters. All used *Incani* species were grouped in this cluster with quite high bootstrap values. In addition to identified *Incani* species, six of unidentified but thought as *Incani* species (A3, A16, A20, A108, A109, A110) were also joined to this cluster. Except the unidentified *Astragalus* A110 sample, others were included in the same subcluster without any distance since they carried identical *matK* DNA sequences. Therefore, the exact characterization of these samples was not possible. However, based on the constructed tree, it can be said that A110 sample had very close relation with *A. cariensis* (Figure 5.23).

More than half of the used *Incani* species were clustered together in a subcluster located top part of the tree. There was no branching or junction point in this subcluster and all species and/or unknown samples joined directly to the subcluster. *A. achundovii, A. schizopterus, A. czorochensis, A. glaucophyllus, A. nezaketae, A. humillimus* and *A. sanguinolentus* species were grouped in the second subcluster of I cluster. Except *A. glaucophyllus*, all of other species found in the second subcluster were joined to the subcluster directly. This suggests that the DNA sequences of these species were identical while *A. glaucophyllus* had some nucleotide variations.

Unknown samples, which were not included in the 'I' cluster, formed the second main, but a smaller cluster. This cluster contains samples from both the species of *Hypoglottidei* and *Dissitiflori* sections. At least, they could be considered as the species, which belong to these two sections. Within this cluster, one of the unknown sample (A2) joined to the cluster externally. This binding position was expected because A2 sample was thought to be in *Dissitiflori* section while A35, A52, A65 and A66 samples were expected to be included by *Hypoglottidei*. Unknown A35 and 52 samples were separated from A65 and 66 even if all of

them were found in *Hypoglottidei* section. This separation was also observed in the phylogenetic trees constructed by using sequences of both *trnL* and *trnV* intron regions.

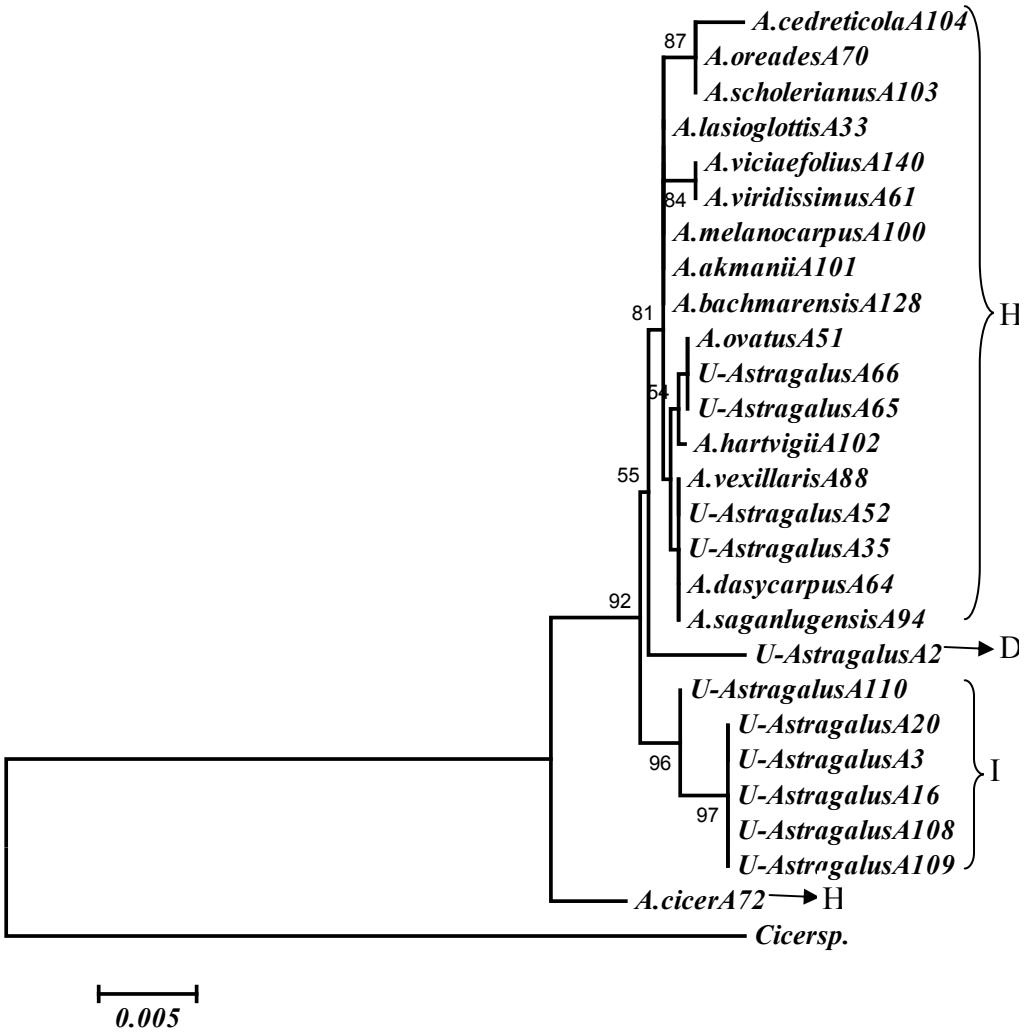

Figure 5.24 Phylogenetic tree, constructed using the sequences of *matK* region of cpDNA, depicting relationships among the species of *Hypoglottidei* section.
(Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori).*

Phylogenetic relationships between species of *Hypoglottidei* and unknown samples were depicted in Figure 5.24. The tree had two main clusters; one of them (H) included species of *Hypoglottidei* section while the other contained some of unidentified samples. The H cluster had branching and subclusters. *A. viridissimus* and *viciaefolius* were separated from other species of the same section and were located in a small subcluster with a high bootstrap value (%84).

Astragalus cedreticola, A. oreades and *A. scholerianus* formed another subcluster within the H cluster. Unknown but thought as *Hypoglottidei* species dispersed in the constructed tree. The samples A65 and A66 grouped with *A. ovatus* without any genetic distance between them. Therefore, it is likely that unidentified A65 and A66 samples could be *A. ovatus.* The sample A35 and A52 were included in a subcluster with *A. saganlugensis, A. dasycarpus*, and *A. vexillaris* without any genetic divergence among them. Thus, A35 and A52 may be identified as one of these three species.

Unindentified A2 sample, thought as *Dissitiflori* species, joined to the 'H' cluster, externally. Its separation from other unknown samples (I cluster) was expected, because genetic divergence between *Hypoglottidei* and *Dissitiflori* section was less compared with that of *Incani* section. The second cluster contained only unidentified samples that were considered as *Incani* species. Except for A110, all of others had identical DNA sequences so they were located without genetic divergence among them, while A110 was separated from this subgroup with high bootstrap value (%96, Figure 5.24).

Interestingly *A. cicer* was separated from both close and unidentified species-samples whether they were found in *Hypoglottidei* section or not. This divergence was due to inserted nucleotides in sequences of the studied region (Figure 5.21 and Figure 5.24).

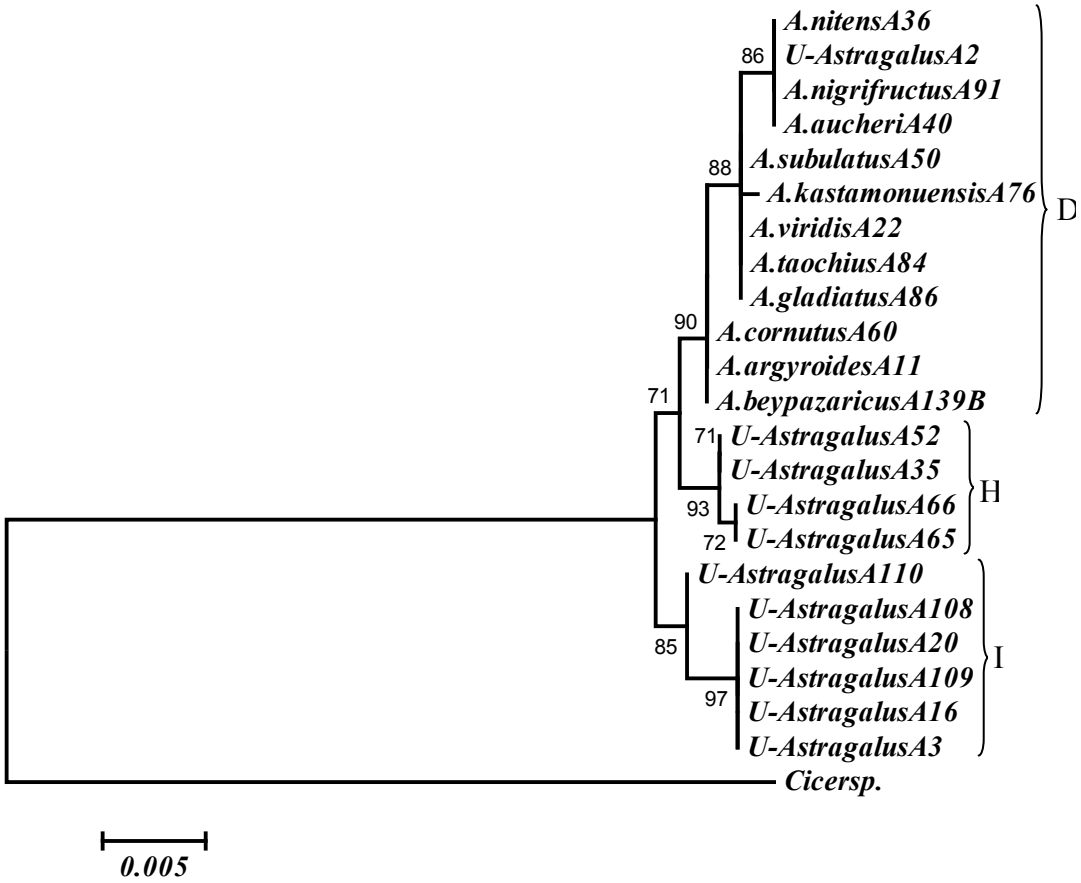

Figure 5.25 Phylogenetic tree, constructed using the sequences of *matK* region of cpDNA, depicting relationships among the species of *Dissitiflori* section.
(Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori).*

Phylogenetic relationships between species of *Dissitiflori* section were depicted in Figure 5.25. To show separation of sections, unidentified samples were also included in the analyses. Three subclusters with high bootstrap values were observed in the 'D' cluster. There were no genetic divergence between *A. aucheri*, A. *nigrifructus A.nitens* and unidentified A2 sample so they joined to the subcluster directly. Within *Hypoglottidei* (H) cluster, A35 with A52 and A65 with A66 were grouped. The third main cluster contained A3, A16, A20, A108, A109, and A110 samples (distantly), which belong to *Incani* section (Figure 5.25).

5.2.3.5 Phylogenetic Tree Construction by Using Sequence Data of *ITS* nrDNA Region

In addition to the regions of chloroplast DNA, genomic DNA region (*ITS*) was used to understand the phylogenetic relationships among species of *Astragalus* genus. This region was intentionally selected to see whether there was any difference between phylogenetic trees, which were constructed by using both DNA sequences of chloroplast and genomic DNA regions.

The length of the region was about 640 bp and amount of molecular diversity in each subregion (*ITS1, 5.8S*, and *ITS2*) varied considerably. Firstly, by using the complete sequence, phylogenetic trees were constructed for each section of *Astragalus.* Then, to see which subregion was more informative and appropriate for evolutionary studies, trees were constructed by using each subregion separately as well.

Both indels and nucleotide substitutions were observed in the DNA sequences of the region (Figure 5.26a, 5.26b). Most of deletions were observed through DNA sequence of both *Hypoglottidei* and *Dissitiflori* species. The separation of *Incani* species from those of two other sections was due to insertion of 'CGCACA' sequences located between 107th and 114th bases, base deletion at 75th base, and several nucleotide substitutions (67A-67G, 97C-97T, 106C-106G, 107A-107T, 118T-118C, 131C-131T, Figure 5.26a). All *Incani* species carried Cytosin base at 73rd base and it was converted to Thymine base in the DNA sequences of species included in the other two sections except for *A. viciaefolius* and *A.viridissimus*. *Hypoglottidei* species were separated from other sections by Adenine base located at 84th base, which was substituted by Guanine in the sequence of *Incani* and *Dissitiflori* species. Moreover, *Dissitiflori* species were separated by Cytosin at 192nd base that was changed to Guanine base in the DNA sequences of other section species. Three used sections could be separated from each other based on 209th base since each section carried different base type (T in *Incani*; C in *Hypoglottidei*; and G in *Dissitiflori*, Figure 5.26a).

Section	Species name	Nucleotide Positions in the Aligned DNA sequences of *ITS1* Subregion																				
		67	73	75	84	97	106	107	108	109	110	111	112	113	114	118	122	123	126	131	192	209
I	*A. achundovii*	A	C	-	G	C	C	A	C	G	C	A	C	A	G	T	C	-	A	C	G	T
	A. brevidentatus	A	C	-	G	C	C	A	C	G	C	A	C	A	G	T	C	-	A	C	G	T
	A.germanicopolitanus	A	C	-	G	C	C	A	C	G	C	A	C	A	G	T	-	-	A	C	G	T
	A. nezaketae	A	C	-	G	C	C	A	C	G	C	A	C	A	G	T	C	-	A	C	G	T
	A. sanguinolentus	A	C	-	G	C	C	A	C	G	C	A	C	A	G	T	C	-	A	C	G	T
	A. zaraensis	A	C	-	G	C	C	A	C	G	C	A	C	A	G	T	C	-	A	C	G	T
H	*A. akmanii*	G	T	G	A	T	G	T	-	-	-	-	-	-	G	C	C	-	T	T	G	C
	A. cicer	G	T	G	A	T	G	T	-	-	-	-	-	-	G	C	C	C	T	T	G	C
	A. dasycarpus	G	T	G	A	T	G	T	-	-	-	-	-	-	G	C	C	-	T	T	G	C
	A. viciaefolius	G	A	G	A	T	G	T	-	-	-	-	-	-	G	C	C	-	T	T	G	C
	A. ovatus	G	T	G	A	T	G	T	-	-	-	-	-	-	G	C	C	-	G	T	G	C
D	*A. aucheri*	G	T	G	G	T	G	T	-	-	-	-	-	-	G	C	C	-	T	T	C	G
	A. beypazaricus	G	T	G	G	T	G	T	-	-	-	-	-	-	G	C	C	-	T	T	C	G
	A. nigrifructus	G	T	G	G	T	G	T	-	-	-	-	-	-	G	C	C	-	T	T	C	G
	A. viridis	G	T	G	G	T	G	T	-	-	-	-	-	-	G	C	C	-	T	T	C	G

Figure 5.26a: Substitutions and indels in the DNA sequences of *ITS1* subregion for each section.

[Nucleotide absence was shown as a dot, inserted (deleted) base as blue and substitution as red color. Numbers above each column depict the position of the corresponding nucleotide in the whole alignment (5' to 3')].

DNA sequence of *5.8S* subregion was more conservative than that of *ITS1* and *ITS2*. Only five nucleotide substitutions (367T-367A, 368T-368G, 374A-374C 415T-415C, 421C-421A) were observed. There was no inserted and/or deleted bases. Three of nucleotide substitutions caused separation of *Incani* species from species of the other two sections. The other two substitutions (374A-374C and 421C-421A) were only seen in the DNA sequence of *A. kastomonuensis* species located within *Dissitiflori* section. In addition to *ITS1*, substitutions occurred at 549th, 572nd, 574th, 602nd and 604th bases through DNA sequence of *ITS2* subregion were enough for phylogenetic separation of sections in the constructed tree (Figure 5.26b).

Section	Species name	Nucleotide Positions in the Aligned DNA sequences of *5.8S* and *ITS2* Regions																				
		5.8S					*ITS2*															
		367	368	374	415	421	434	451	479	487	498	504	529	549	555	572	574	591	592	593	602	604
I	*A. achundovii*	T	T	A	T	C	G	A	T	T	G	T	A	C	A	C	C	T	A	T	T	T
	A. brevidentatus	T	T	A	T	C	T	A	T	T	G	T	A	C	A	C	C	T	A	T	T	T
	A.germanicopolitanus	T	T	A	T	C	T	A	T	T	G	T	A	C	A	C	C	T	A	T	T	T
	A. nezaketae	T	T	A	T	C	G	A	T	T	C	T	A	C	A	C	C	T	A	T	T	T
	A. sanguinolentus	T	T	A	T	C	G	A	T	T	G	T	A	C	A	C	C	T	A	T	T	T
	A. zaraensis	T	T	A	T	C	T	A	T	T	G	T	A	C	A	C	C	T	A	C	T	T
H	*A. akmanii*	A	G	A	C	C	G	A	T	T	G	C	A	C	A	T	T	T	A	T	C	C
	A. cicer	A	G	A	C	C	G	A	T	T	G	C	A	T	A	T	T	T	A	T	C	C
	A. dasycarpus	A	G	A	C	C	G	A	T	T	G	C	A	C	A	T	T	T	A	T	C	C
	A. viciaefolius	A	G	A	C	C	G	C	T	T	G	T	A	C	G	T	T	T	A	T	C	C
	A. ovatus	A	G	A	C	C	G	G	T	T	G	C	A	C	A	T	T	T	T	T	C	C
D	*A. aucheri*	A	G	A	C	C	G	A	A	A	G	C	G	A	A	T	C	T	A	C	C	C
	A. beypazaricus	A	G	A	C	C	T	A	T	T	G	T	A	A	A	C	C	T	A	T	T	T
	A. kastamonuensis	A	G	C	C	A	G	A	T	T	G	C	G	A	A	T	C	A	A	T	C	C
	A. nigrifructus	A	G	A	C	C	G	A	T	A	G	C	G	A	A	T	C	T	A	T	C	C
	A. viridis	A	G	A	C	C	G	A	A	A	G	C	G	A	A	T	C	T	A	C	C	C

Figure 5.26b: Substitutions and indels in the DNA sequences of *5.8S* and *ITS2* subregions for each section.

[Nucleotide absence was shown as a dot, inserted (deleted) base as blue and substitution as red color. Numbers above each column depict the position of the corresponding nucleotide in the whole alignment. (I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori*)].

Phylogenetic tree (Figure 5.27), which was constructed without any section separation, was similar to the trees that were constructed by using sequences of each studied cpDNA regions. The highest genetic divergence was observed between sections *Incani* and *Dissitiflori* when both regions of chloroplast and genomic DNA were used (Table 5.8). Therefore, it can be said that, there was no crucial differences between results of cpDNA and genomic DNA regions. When sequences of *ITS* region were used, as expected the highest genetic divergence was found between *A.germanicopolitanus* (*Incani*) and *A.gladiatus* (*Dissitiflori*) (0.058, Table 5.9).

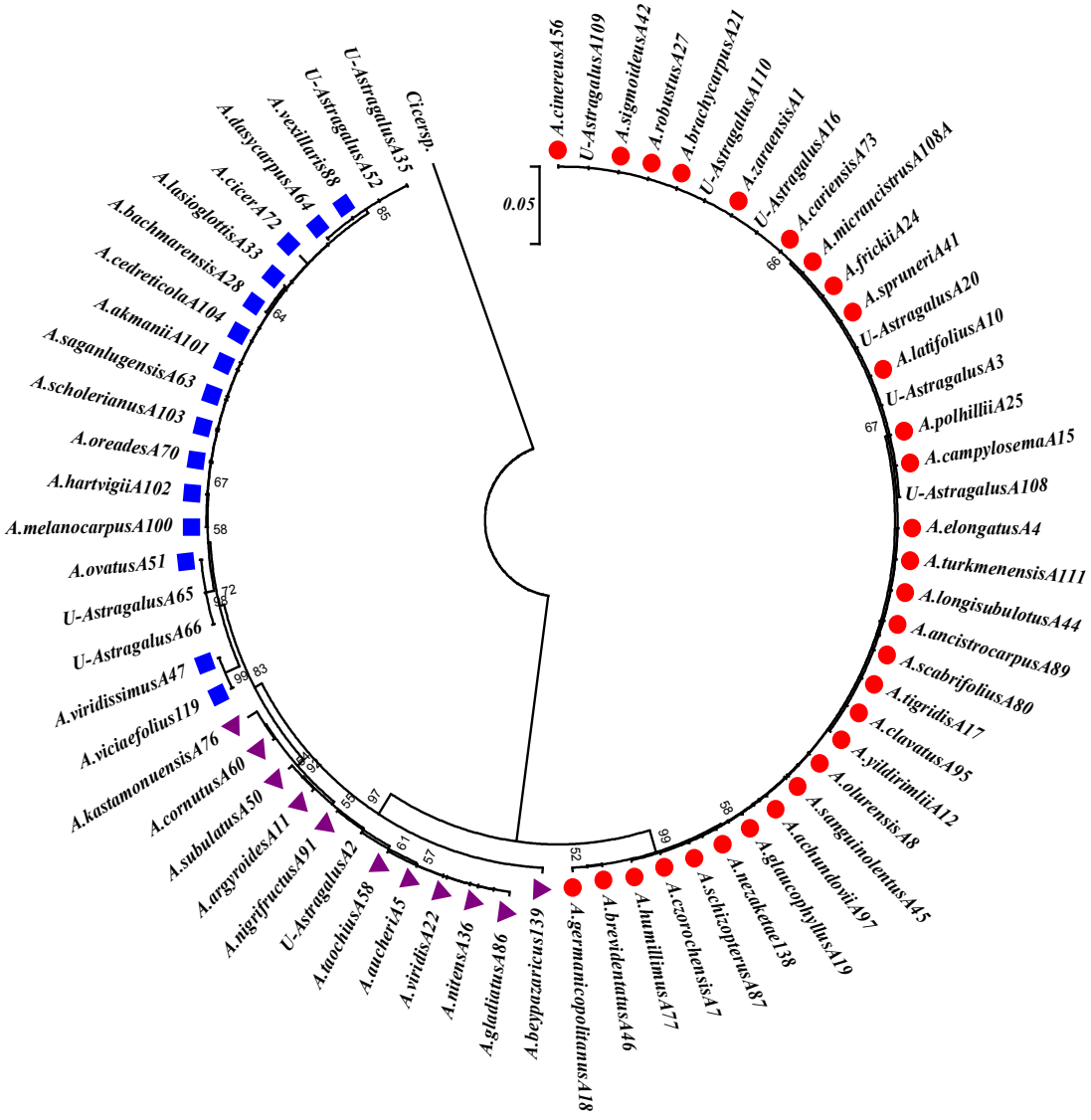

Figure 5.27 Phylogenetic tree, constructed using the sequences of *ITS* region of genomic DNA, depicting relationships among the species of *Astragalus*.
(Numbers on branches of the tree indicate bootstrap values, Circles: *Incani*, Squares: *Hypoglottidei* and Triangles*: Dissitiflori* section. U: Unknown *Astragalus* sample).

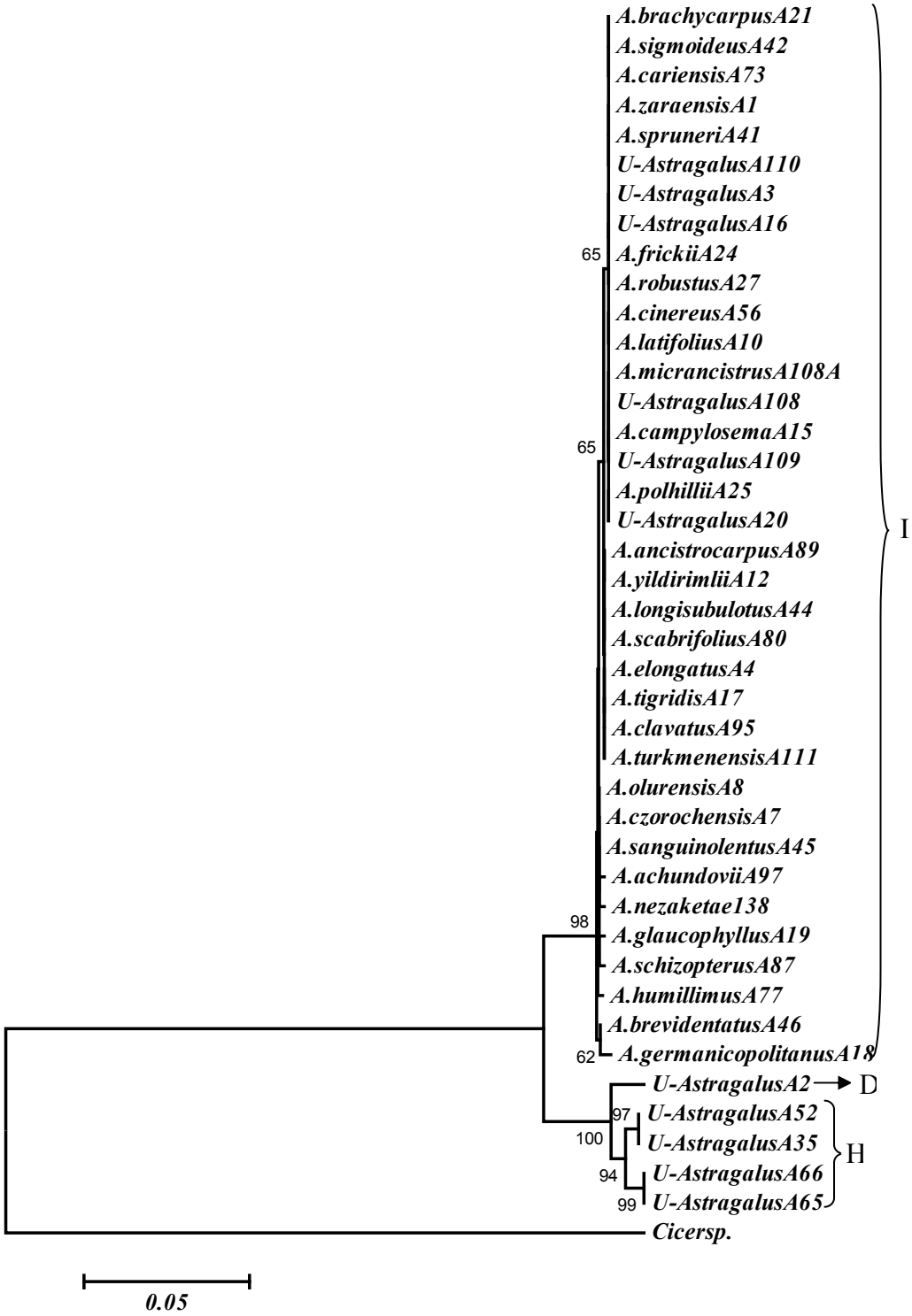

Figure 5.28 Phylogenetic tree constructed by sequence of *ITS* region (*ITS1, ITS2* and *5.8S*) depicts relationships among species of *Incani* section.

(Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori*).

Figure 5.28 depicts phylogenetic relationships among *Incani* species. *A. olurensis, A. czorochensis, A. sanguinolentus, A. achundovii, A. nezaketae, A. glaucophyllus, A. schizopterus, A. humillimus, A. brevidentatus,* and *A.germanicopolitanus* species were separated from other species of same section. As like former analyses, unknown samples were also included for each tree construction studies. Unidentified species-samples A2, 35, 52, 65, and 66 were clustered together within the second main cluster and A2, which was considered as *Dissitiflori* species, was separated from the other four unknown, but suspected as *Hypoglottidei* samples. The close genetic relationships between *Dissitiflori* and *Hypoglottidei* sections were also obvious from the figure (Figure 5.28). The unidentified A2 sample did not group with any species of *Incani* section, because there was high genetic divergence between *Incani* and *Dissitiflori* sections (Table 5.8).

Species of *Hypoglottidei* sections and unknown samples were analyzed together to understand phylogenetic relations between and within sections. Variable sites within the section was high so different binding patterns were seen at the constructed tree (Figure 5.29). As seen at the top part of the dendrogram, *A. bachmarensis* and *A. lasioglottis* were separated from their relative species and their close relation was also supported by *trnL*, *trnV* and *matK* cpDNA regions. Unidentified samples A35 and 52 grouped with *A. dasycarpus* and *A. vexillaris* so these unknown samples could be identified as either *A. dasycarpus* or *A. vexillaris*. For exact identification, constructed phylogenetic trees of other studied regions can be used. *A.cicer* attached to the tree distantly like it was in previously studied cpDNA regions. Unknown A2 sample joined to the 'H' instead of 'I' cluster, this connection was expected because this sample was estimated to be *Dissitiflori* species, and genetically *Incani* was distant to *Dissitiflori* section with respect to used chloroplast and genomic DNA regions. Unknown but thought as *Incani* species (A3, 16, 20, 108, 109, and 110) were located within the second main cluster (I) without any genetic divergence among them.

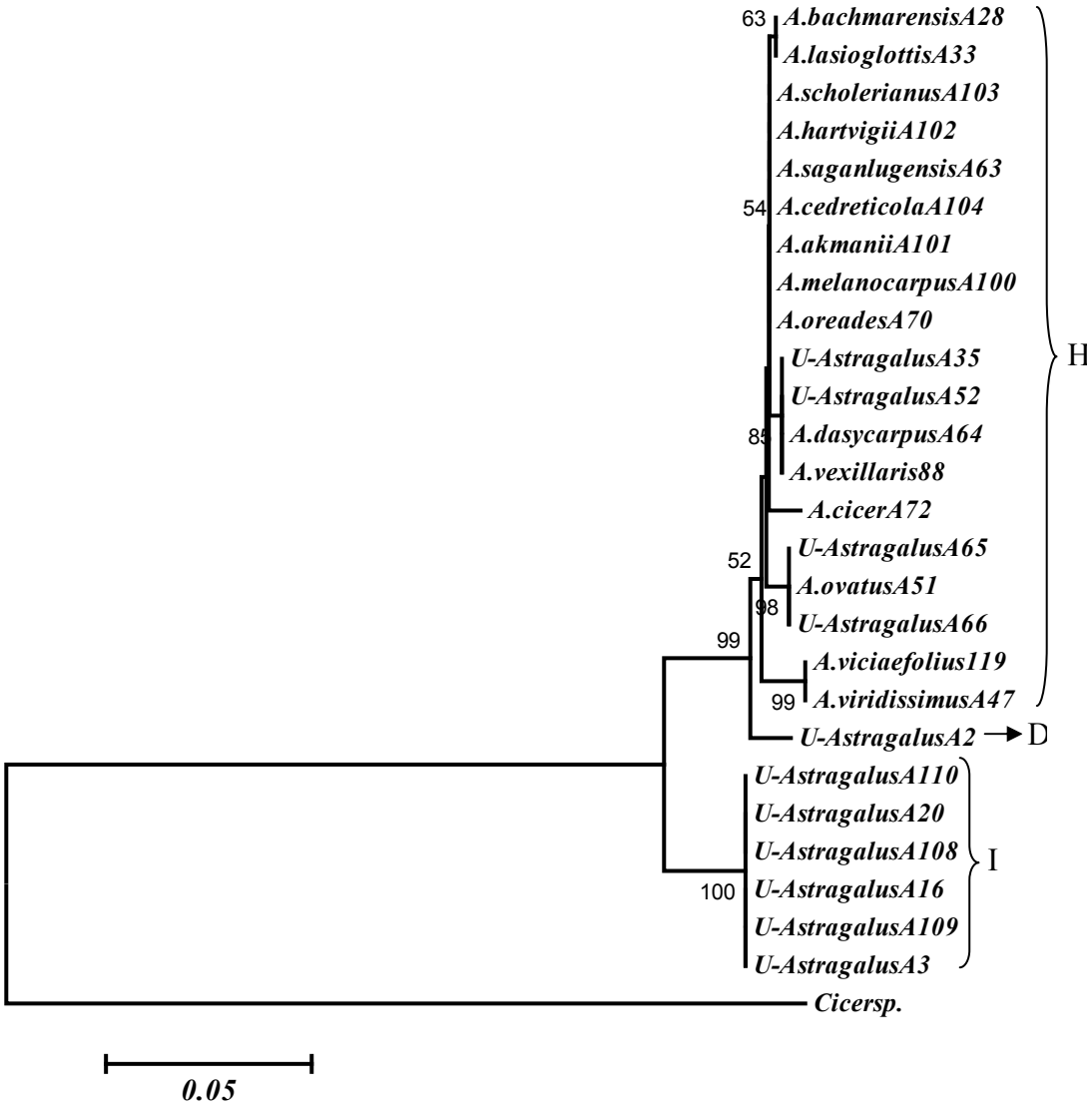

Figure 5.29 Phylogenetic tree constructed by DNA sequence of *ITS* region (*ITS1, ITS2* and *5.8S*) depicts relationships among species of *Hypoglottidei* section. (Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori*).

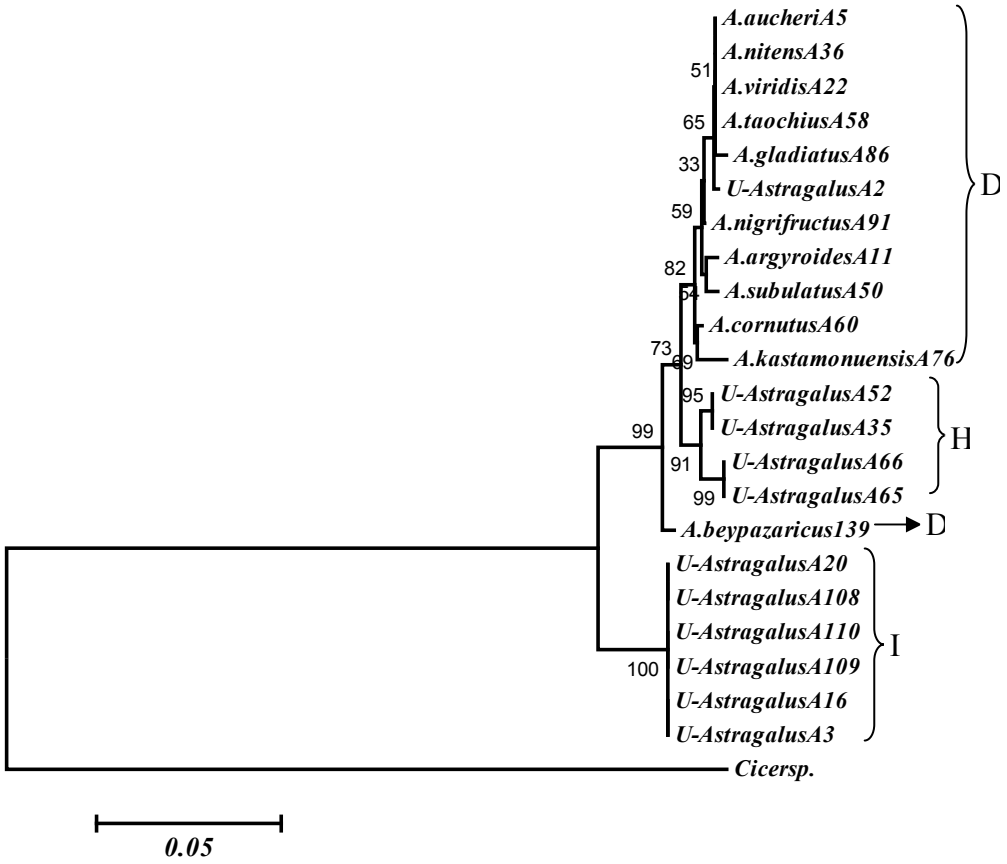

Figure 5.30 Phylogenetic tree constructed by DNA sequence of *ITS* region (*ITS1, ITS2* and *5.8S*) depicts relationships among species of *Dissitiflori* section.
(Numbers on branches of the tree indicate bootstrap values, I: *Incani*, H: *Hypoglottidei* and D: *Dissitiflori*).

Eleven species of *Dissitiflori* section and all of unknown samples were also analyzed together. The constructed tree was provided in Figure 5.30. Several branches in this tree were present because of high nucleotide variations among species as well as sections. *A. nitens, A. viridis, A. aucheri,* and *A. taochis* samples grouped together without any genetic divergence among them while *A. gladiatus* and unknown A2 sample joined to the subcluster externally. The most interesting connection was the attachment point of species *A. beypazaricus*. Although this species was included in *Dissitiflori* section, it joined to both 'D' and 'H' clusters,

externally (Figure 5.30). This means that *A. beypazaricus* was considerably distant to the all of the other species included in the same section. Separation of this species was caused by few substitutions located in DNA sequence of *ITS2* subregion (Figure 5.26b). The DNA sequence of unknown A35 sample was identical to that of A52, and A65 with A66, so two subclusters were observed within the 'H' cluster. Within 'I' cluster, all unknown samples joined to the cluster directly because there was no genetic divergence among them.

When the DNA sequences of *ITS1* and *ITS2* subregions were evaluated separately, almost the same phylogenetic tree was obtained (trees were not shown). However, the DNA sequences of *5.8S* subregion generated quite a different phylogenetic tree (Figure 5.31), compared with the trees obtained from *ITS1* and *ITS2* data. There were only five nucleotide substitution sites and no insertion and/or deletion through DNA sequnce of 5.8S subregion. Nucleotide substitutions were always found between *Incani* and the other two sections (*Hypoglottidei* and *Dissitiflori)*.

All species of both *Hypoglottidei* and *Dissitiflori* sections except *A. kastomunuensis* had identical DNA sequences, so they were located in the second main cluster without any genetic distance between them (Figure 5.31). *A. kastomunuensis* had two substitutions, which caused to separation of it from other species. *TrnL* intron*, trnL-F, trnV* intron*, matK* and *ITS* regions separated *Hypoglottidei* from *Dissitiflori* section; eventhough genetic distance among them was low. As a summary, it can be said that *5.8S* subregion was more conservative than all other used regions so it was only enough to separate *Incani* section from other used two sections.

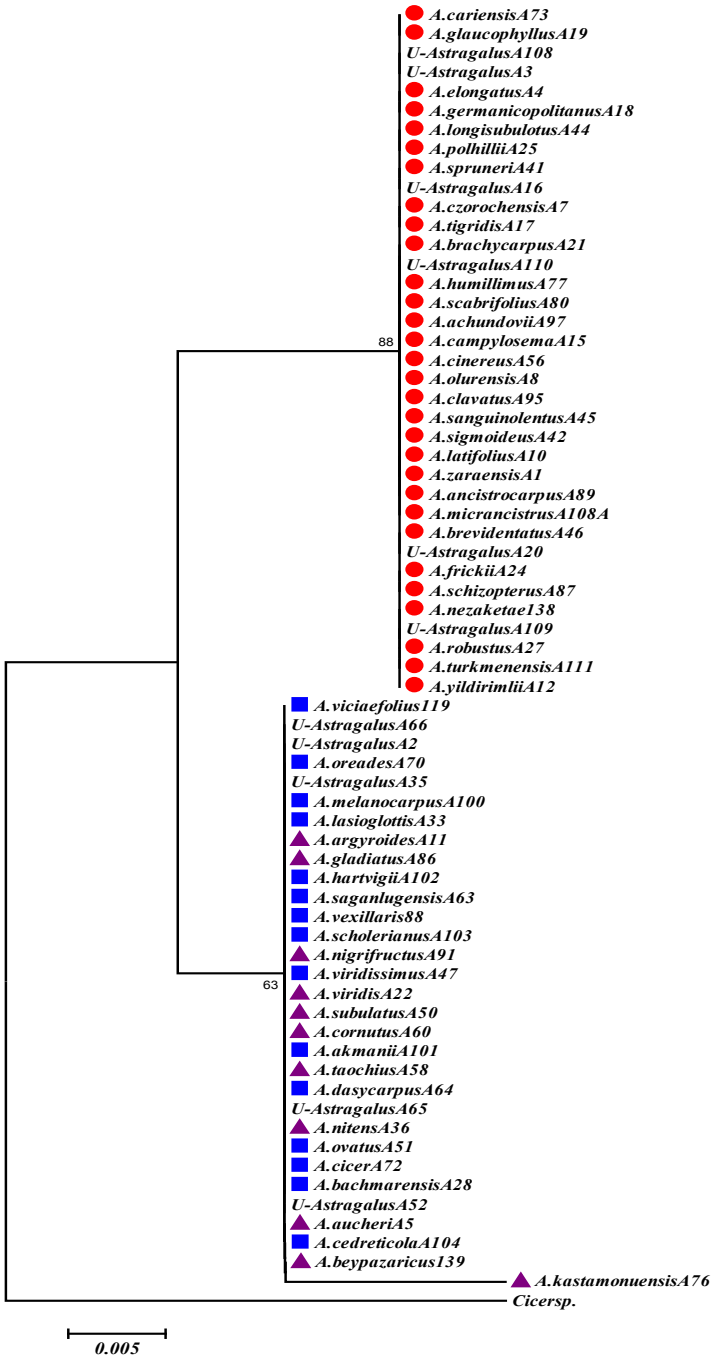

Figure 5.31 Phylogenetic tree, constructed by sequence of *5.8S* subregion of nrDNA, depicting relationships among the species of *Astragalus*.

(Numbers on branches of the tree indicate bootstrap values, Circles: *Incani*, Squares: *Hypoglottidei* and Triangles*: Dissitiflori* section. U: Unknown *Astragalus* sample).

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

In the current study, only three sections of *Astragalus* genus, which are native to Turkey were studied. All species from each section except for A. *latistipulatus* within *Hypoglottidei* section were sequenced by using four cpDNA and one nDNA regions. Therefore, results gathered up to now were revealing the phylogenetic relationships among *Astragalus* species native to Turkey. To find out evolutionary relationship and divergence between Turkish and foreign *Astragalus* species, additional DNA sequences for each studied DNA region were obtained from the NCBI (National Center for Biotechnology Information) database, and analyzed with DNA sequences of species from the current study.

Each studied region's DNA sequences of *Astragalus* genus that were studied by different researchers were taken from NCBI website and grouped with respect to countries or main geographic regions to understand phylogenetic relationships between Turkish and foreign *Astragalus* species. At the following tables (Tables 5.10-5.14), values of parameters calculated for each region or country by using sequences of studied regions were provided. There was no available DNA sequence of *trnV* region of any *Astragalus* species from the NCBI database, so comparison of studied species with foreign *Astragalus* species based on *trnV* region could not be made. On the contrary, great number of sequences for *trnL* intron and *ITS* regions were available from NCBI databases.

In addition to DNA sequences of *trnL* intron region obtained from *Astragalus* species in Turkey, sequences of different *Astragalus* species were included from different regions or countries to understand phylogenetic relationships between native and foreign *Astragalus* species (Table 5.10).

Table 5.10 Genetic diversity parameters of *Astragalus* genus from different parts of the World. Values were estimated by using sequences of *trnL* intron region.

	Turkey	N.America	S.America	Morocco	Iran	Eurasia	Total
Number of species	56	11	2	2	22	2	95
Number of sequence	56	11	2	2	22	2	95
GC content (%)	31.7	31.8	31	32.1	31.7	31.6	31.7
Conserved sites	548	545	544	522	502	542	528
Variable sites	30	18	1	19	84	9	102
Parsimony informative sites	30	8	-	-	58	-	76
Transitional pairs	53.2	40.7	-	-	41.6	-	40.2
Transversional pairs	46.8	59.3	-		58.4	-	59.8
Transition/Transversion (tr/tv) ratio	0.96	0.63	-	-	0.63	-	0.57
Number of deletion	52	29	1	21	81	16	115
Number of insertion	52	29	1	21	81	16	115

Most of the *trnL* intron sequences were taken from *Astragalus* species native to Iran (22 species, Table 5.10). Although number of Iranian samples were less than half the number of studied Turkish samples, they showed high molecular diversity in DNA sequence of the *trnL* intron region. This result can be expected because one of the main centers of *Astragalus* genus exists in Iran, which contains more than 800 species (Maassoumi, 2005). Number of parsimony informative sites (58) was lower with respect to that of variable sites (84) among samples coming from Iran. Less informativeness may be cased by anticoincidence of variations between two sequences. Variable sites of species, which were included in North America, South America, Eurasia and Morocco, were less compared with those of Iranian and Turkish samples. This could be due to both low number of sequences and/or less molecular diversity among species. Transition/Transversion rate and GC

content of each country were almost similar with others. Number of deleted nucleotide (81) was high in Iranian samples.

Three big clusters were seen at the tree (Appendix B.1) that was constructed by using sequences of *trnL* intron region. Samples of Iranian species dispersed, so each section (*Incani, Hypoglottidei,* and *Dissitiflori*) connected with one or more Iranian samples. Dispersion of the Iranian samples throughout the phylogenetic tree was expected because of the presence of high genetic diversity within it.

All used *Incani* samples were clustered with most of Iranian and all of North/South American samples (Appendix B.1). This indicates that the DNA sequences of North/South American samples were quite similar with those of *Incani* species. No North/South American samples formed clusters with samples of *Hypoglottidei,* and/or *Dissitiflori* section. A few samples from Iran and one sample from Morocco joined the main cluster of the tree externally which was due to nucleotide substitution and indels that were found in the DNA sequences (Appendix B.1).

Table 5.11 Genetic divergence of *Astragalus* genus throughout the World. Estimations were done by using DNA sequences of *trnL* intron cpDNA region.

	1	2	3	4	5	6
Turkey						
N.America	0.015					
Iran	0.024	0.024				
Morocco	**0.027**	0.023	**0.034**			
Eurasia	0.015	0.012	0.022	**0.025**		
S.America	0.014	0.006	0.022	0.021	0.010	

Table 5.11 depicts genetic divergence of *Astragalus* species taken from several locations of the world. The highest divergence (0.034) was observed between Morocco and Iranian samples. Turkey was also genetically the most distant location to Morocco (0.027). In addition to this result, genetic divergence between Iran and Turkey was quite high eventhough Iran geographically located next to Turkey. This high genetic divergence may be caused by utilization of DNA sequences of different (foreign) *Astragalus* species.

In addition to DNA sequences of *trnL* intron region, *trnL-F* region's sequences were collected from NCBI database and analyzed with samples of the current study. All new DNA sequences were taken from Iranian species, so only Turkish and Iranian samples were compared to each other based on sequences of this region. As like results of *trnL* intron region (Table 5.10), variation sites of *trnL-F* region were higher in DNA sequences of Iranian samples (Table 5.12). Iranian samples had 24 variable sites in the sequences with eight parsimony informative sites while only one nucleotide variation was observed in the DNA sequences of Turkish species. Number of nucleotide deletions and/or insertions was the other difference between Iranian and Turkish samples. Although only 18 Iranian samples, which were less than half of used Turkish species, were there in the analyses, variable sites, nucleotide deletions and insertions were more than those of Turkish samples (Table 5.12).

Phylogenetic tree was also constructed to see relations between and within Turkish and Iranian samples (Appendix B.2). When species of Turkish samples were used, only *A.brevidentatus* was separated from other samples due to two nucleotide deletions (Figure 5.12 and 5.13). Moreover, all species of one section were separated from those of other sections at the tree (Figure 5.12). When Iranian samples were included in the analyses and a new tree was constructed, most of the Iranian samples were separated from Turkish samples and joined the tree externally (Appendix B.2). This distinction was caused because of nucleotide variations and indels that were seen in the DNA sequences of Iranian samples. Interestingly, when Iranian samples were included in the tree, *A.brevidentatus*

species was separated from its related species and grouped with several Iranian samples. This clustering pattern was caused two nucleotide deletions, which was seen in the DNA sequences of *A.brevidentatus*. Most of Iranian samples had deleted nucleotides that coincided with these deleted parts of DNA sequences of *A.brevidentatus,* so this species was separated from Turkish samples and grouped with samples of Iran. As a summary, we can say that both *trnL* intron and *trnL-F* chloroplast DNA regions showed high genetic diversity between species of Turkey and other countries that was caused by indels and nucleotide substitutions.

Table 5.12 Genetic diversity parameters of *Astragalus* genus in different parts of the World. Values were estimated by using DNA sequences of *trnL-F* cpDNA region.

	Turkey	Iran	Total
Number of secies	56	18	74
Number of sequence	56	18	74
GC content (%)	37.3	37.3	37.4
Conserved sites	166	149	154
Variable sites	1	24	25
Parsimony informative sites	1	8	9
Transitional pairs	0	41.8	40.0
Transversional pairs	100	58.2	60.0
Transition/Transversion (tr/tv) ratio	0	0.69	0.54
Number of deletion	3	31	40
Number of insertion	3	31	40

Table 5.13 depicts diversity parameters, which was estimated via partial DNA sequences of *matK* chloroplast region, between *Astragalus* samples of Turkey and those of other countries. All of the foreign sequences were obtained from the NCBI database. Number of variable sites and deleted nucleotides were higher in North American samples compared with those of Turkish and Chinese samples (Table 5.13). Although North American samples had more variable sites, almost all of these samples formed the cluster with samples of *Incani* section in the phylogenetic tree (Appendix B.3). This type of connection between *Incani* samples of Turkish and American samples was also seen in the tree that was constructed by using DNA sequences of *trnL* intron region (Appendix B.1). Most of Chinese samples were separated from both North American and Turkish samples, and they joined to the main cluster externally in the constructed tree. This distinct attachment of the Chinese samples was due to nucleotide insertion that was located between 179^{th} and 184^{th} base positions (dat was not shown). *A. cicer* was separated from all other samples because of nucleotide insertions (Figure 5.21, 5.22 and 5.24) when only Turkish samples were used. However, when DNA sequences of foreign samples were included in the analyses, *A. cicer* clustered with related species within *Hypoglottidei* section.

Table 5.13 Genetic diversity parameters of *Astragalus* genus in the different parts of the World. Values were estimated by using partial DNA sequences of *matK* region.

	Turkey	America	China	Total
Number of species	56	19	11	86
Number of sequence	56	19	18	93
GC content (%)	31.3	30.9	31.3	31.1
Conserved sites	1211	1141	1206	1102
Variable sites	27	103	38	142
Parsimony informative sites	27	25	18	59
Transitional pairs	62.3	46.4	47.6	54.1
Transversional pairs	37.7	53.6	52.4	45.9
Transition/Transversion (tr/tv) ratio	1.45	0.75	0.79	1.03
Number of deletion	9	21	15	30
Number of insertion	9	21	15	30

DNA sequences of *ITS* region from *Astragalus* species of Turkey and the other countries were evaluated to see genetic diversity and relationships. There were more DNA sequences available for this region in the NCBI database, compared with the sequences of other studied cpDNA regions. The value of GC content was one of the crucial differences between outcomes of chloroplast and genomic DNA regions. This value was around 35% when DNA sequences of Chloroplast DNA region (*trnL* intron*, trnL-F* and *matK*) were used. However, as seen in Table 5.14, GC content of *ITS* region was more than 50% for all geographic regions or countries. Nucleotide substitutions generally caused transitions for each studied countries, so R value was greater than 1. A few DNA sequences of *ITS* region were located in the NCBI database for Iran and France, which did not have any

nucleotide variations. The highest nucleotide diversity was observed among the *Astragalus* species of China though only seven samples were available from the NCBI database. Similar situation was encountered in Eurasian group; only five samples were found in the NCBI database but the number of nucleotide variation was observed as 81. The variation found in both Chinese and Eurasian species was more than expected. This high value can be explained by the usage of DNA sequences of different species that are located within different and/or distinct sections.

Both nucleotide substitutions and indels were used to construct a phylogenetic tree (Appendix B.4). Three of Eurasian samples clustered with species of *Hypoglottidei* sections, while two of them attached to the tree externally. One of the samples that grouped with *Hypoglottidei* species was AB231110, which joined to *A. cicer*. The other two Eurasian samples (AB051922 and 051917) were included in *Sesamei* section (Badr and Sharawy, 2007) in which no species were used in the current study. However, still these two samples clustered with samples of *Hypoglottidei* section with a high bootstrap value. Therefore, it can be said that section *Sesamei* was genetically near to *Hypoglottidei* section. AB051911 and AB051910 samples attached to the tree externally and they were identified as *A. vogelii* and *A. epiglottis*, respectively. Separation of them was expected because *A. vogelii* is found in *Herpocaulos* while *A. epiglottis* in *Epiglottis* section. FJ613404 sample clustered with *A. viridissimus* and A. *viciaefolius* with bootstrap values of 100. The sample was identified as *A. viridissimus* and was collected from Turkey by Uzun et al., (unpublished) so this close relationship was meaningful.

Table 5.14 Genetic diversity parameters of *Astragalus* genus in different parts of the World. Values were estimated by using sequences of *ITS* genomic region.

	Turkey	N.America	S.America	China	France	Iran	Eurasian	Russia	Total
Number of species	56	6	8	7	1	2	5	3	88
Number of sequence	56	6	8	7	3	2	5	3	90
GC content (%)	53.6	54.1	54.2	54.1	54.6	54.8	54.4	54.4	53.9
Conserved sites	586	620	619	539	637	633	564	621	471
Variable sites	56	17	17	101	-	4	81	-	182
Parsimony informative sites	56	14	8	39	-	-	26	-	125
Transitional pairs	62.7	69.5	84.3	63.8	-	-	70.2	-	64.3
Transversional pairs	37.3	30.5	15.7	36.2	-	-	29.8	-	35.7
Transition/Transversion (tr/tv) ratio	1.67	2.25	5.31	1.75	-	-	2.33	-	1.79
Number of deletion	9	1	1	16	-	2	15	-	34
Number of insertion	9	1	1	16	-	2	15	-	34

All of South and North American samples clustered together and joined to the *Hypoglottidei* cluster with a high bootstrap value. Samples coming from America also clustered with those of *Incani* section when regions of Chloroplast DNA (*trnL* intron, and *matK*) were used. Contrary to this result, American samples located distantly to the samples of *Incani* section when sequences of *ITS* region were analyzed. Based on these results, we can say that American samples were genetically close to *Incani* section when regions of Chloroplast DNA were used whereas close to *Hypoglottidei* and *Dissitiflori* sections when *ITS* genomic DNA region was utilized.

Samples of France, Russia, China, and Iran joined to the constructed phylogenetic tree, outwardly. Similar outcomes were also observed when regions of chloroplast

DNA were analyzed. Iranian and Chinese samples also separated from Turkish species when sequences of *trnL-F* (Appendix B.2) and *matK* (Appendix B.3) regions were used, respectively. With respect to whole results, it can be safe to say that North and South American samples are genetically closer to Turkish samples, while Chinese, Russian, and French samples are further apart.

5.2.5 Molecular Clock Estimation

New World *Astragalus* species were diverged from Old World *Astragalus* ones (Wojciechowski et al., 1999; Wojciechowski, 2005). To support and prove this opinion, divergence time of both Old and New World *Astragalus* samples were calculated separately (Table 5.15). Different species from Astragalean clade were used to estimate the evolutionary divergence time of *Astragalus* (Old) genus based on sequences of *trnL* intron and *matK* regions. The *trnL-F* and *trnV* intron cpDNA regions were not used for molecular clock estimation because of the absence or poor DNA sequences of these regions in NCBA database. After age estimation for New and Old World *Astragalus* group, it was also estimated for each used *Astragalus* section, *Incani, Hypoglottidei* and *Dissitiflori,* to see times between appearing of these sections. The *trnL-F* cpDNA region was not prefered to calculate molecular clock for each section because the number of variable and so parsimony informative sites were too low.

Table 5.15 indicates the number of parsimony informative sites, total length of the region, *d* and *k* values, and lastly molecular clock times for *trnL* intron*, trnV* intron and *matK* regions, separately. While estimation of molecular clock was done, the number of variation site (parsimony informative) was used. The values of estimated divergence time were almost similar when DNA sequences of chloroplast DNA regions were used to eastimate molecular clock for Old/New World *Astragalus* group and sections (Table 5.15).

Old World *Astragalus* group diverged from other genus within Astragalean clade about 12.5, and 14.5 million years ago based on DNA sequences of *trnL* intron, and *matK* cpDNA regions. When cpDNA regions were considered, it can be said that Old World *Astragalus* group, which comprises ancestor species diverged about 12-14 million years ago. This value was estimated as 12.4 for *Astragalus* species (Old World group) in the study, which was done by Wojciechowski (2005), so there was compatibility between the results.

The *trnL* intron and *matK* regions were used to calculate divergence time of New World *Astragalus* species. As expected, these estimated values were lower with respect to that of Old World group because North and South American species emerged after the origination of Old World *Astragalus* species in Eurasia (Polhill, 1981a). Divergence time of New World *Astragalus* group was calculated as 5 and 4 million years ago when DNA sequences of *trnL* and *matK* regions were used (Table 5.15). This value was estimated as 4.4 for Neo-*Astragalus* species (New World group) in the study, which was done by Wojciechowski (2005).

Molecular clock was lastly estimated for *Incani, Hypoglottidei* and *Dissitiflori* sections separately by using DNA sequences of *trnL*, *trnV* intron and *matK* regions. Divergence time of *Hypoglottidei* and *Dissitiflori* sections from *Incani* section was calculated as 5-7 million years when DNA sequences of cpDNA regions were used. By using these values and the constructed phylogenetic trees based on each studied region, it may be safe to say that *Hypoglottidei* and *Dissitiflori* sections were younger than the *Incani* section. However, different regions would be also needed to prove and get more precise results. Divergence time between *Hypoglottidei* and *Dissitiflori* sections was very low (1.0-3.5 Ma, Table 5.15) when cpDNA regions were analyzed and this means *Hypoglottidei* and *Dissitiflori* sections diverged at almost the same time.

Table 5.15 Molecular Clock Estimations for New (NWA) and Old World *Astragalus* (OWA) species and Three Used *Astragalus* Sections via DNA Sequences of Differeent Regions.

***Astragalus* Genus**	**Regions**	**# of Parsimony Informative Sites**	**Length of the regions (bp)**	**d**	**k**	**MCE (mya)***
OWA-Astragalean Clade	***trnL***	14	~575	2.4×10^{-2}	2.4×10^{-2}	**12.5**
	matK	35	~1230	2.8×10^{-2}	2.9×10^{-2}	**14.5**
NWA-OWA	***trnL***	6	~630	1.0×10^{-2}	1.0×10^{-2}	**5.0**
	matK	10	~1214	0.8×10^{-2}	0.8×10^{-2}	**4.0**
Incani-Hypoglottidei	***trnL***	8	~549	1.5×10^{-2}	1.5×10^{-2}	**7.5**
	trnV	8	~589	1.4×10^{-2}	1.4×10^{-2}	**7.0**
	matK	14	~1229	1.1×10^{-2}	1.1×10^{-2}	**5.5**
Incani- Dissitiflori	***trnL***	8	~560	1.4×10^{-2}	1.4×10^{-2}	**7.0**
	trnV	8	~600	1.3×10^{-2}	1.3×10^{-2}	**6.5**
	matK	14	~1229	1.1×10^{-2}	1.1×10^{-2}	**5.5**
Hypoglottidei-Dissitiflori	***trnL***	3	570	0.5×10^{-2}	0.5×10^{-2}	**2.5**
	trnV	2	~608	0.3×10^{-2}	0.3×10^{-2}	**1**
	matK	9	~1229	0.7×10^{-2}	0.7×10^{-2}	**3.5**

* Molecular Clock Estimation (Million Years Ago)

CHAPTER 6

DISCUSSION

The DNA sequence data from chloroplast and genomic DNA regions of *Astragalus* genus were evaluated to understand phylogenetic relationships among sections and species of the genus. The results from each studied chloroplast and nuclear DNA regions were considered separately to reduce complexity in discussions.

6.1 Molecular Diversity in *Astragalus* Species based on cpDNA and nDNA Regions

In the current study, most of the used regions were selected from chloroplast genome. Regions located in the DNA of chloroplast are commonly used for evolutionary and phylogenetic studies since large amount of cpDNA are obtained from plant cells. Because of this, studying DNA sequences of chloroplast genome is a rapidly developing area (Clegg and Zurawski, 1992).

6.1.1 *trn* cpDNA Regions [*L5'-L3'* (*trnL* intron), *trnL3'-F* $^{(GAA)}$ (*trnL-F* intergenic spacer), and *trnV* intron]

DNA sequences of non-coding regions tend to evolve faster with respect to sequences of coding regions, so these regions have introduced more information for phylogenetic studies. The DNA sequences of *trnL* intron, *trnL-F* and *trnV* non-

coding cpDNA regions have been used prevalently to understand phylogenetic and evolutionary structures of species especially for discrimination of interspecific relationships (Gielly and Taberlet 1996; Wang et al., 1999; Ohsako and Ohnishi 2000; Mummenhoff et al., 2001).

Almost all informative sites (30) were located in the DNA sequences of *trnL* intron region while that of *trnL-F* region had only one informative site. Although *trnL-F* region is located next to *trnL* intron, the number of polymorphic sites was too low compared with that of *trnL* intron. Not only shorter length of the *trnL-F* region, but also similar sequences among different species caused this crucial difference. DNA sequences of *trnV* intron region almost gave similar results with those of *trnL* intron and *trnL-F*. The variation in the length of *trnV* intron sequence is minor compared with sequence of *trnL* intron because *trnV* intron region carried less indels in respect to DNA sequences of *trnL* intron. There was no genetic variation among species of *Dissitiflori* section, which displayed almost similar results when DNA sequences of *trnL* intron and *trnL-F* cpDNA regions were used. Therefore, it can be safe to say that within three sections, *Dissitiflori* section had minimum genetic variation. The number of used species (11) for this section may also have a role in the results of observing low genetic variation.

The highest number of variable sites was found in *Incani* section when *trnL* intron region was taken into consideration. The high number of variable sites indicated that this section had more variation within it and relatively higher mutation rates with respect to *Hypoglottidei* and *Dissitiflori* sections. In addition to this, higher genetic variation can be caused by used number of samples or species. There were 30 species available for *Incani* section whereas it was 15, and 11 species for *Hypoglottidei* and *Dissitiflori* sections, respectively. After *Incani* section, *Hypoglottidei* showed the second highest genetic variation. During evolutionary time, variations and/or mutations occurred in a part of DNA sequence could lead the sections or populations to separate more and become new taxonomic entities so knowing evolutionary structure of a sample is important for conservation studies of species.

Some authors (Shaw et al., 2005; Kress et al., 2005) pointed out some challenges concerning the suitability of *trnL* intron region. They stated that the resolution of this region is low compared to some of other non-coding regions of chloroplast DNA. According to them, the data from this region have low separation capacity for especially closely related species, which makes this region be less useful for phylogenetic studies. The low discrimination of the region is originated from lower intraspecific variation with respect to resolution strength of other non-coding regions of chloroplast DNA. However, in the current study, there was no problem of using this region since species of different sections of *Astragalus* were the subjects of the study.

Indels observed in the DNA sequences of *trnL* intron region was used to understand phylogenetic relations between species or section of *Astragalus* genus (Bayer and Starr, 1998; Wojciechowski et al., 1999; Kazemi et al., 2009). Utility of multibase insertion and deletion to find out phylogenetic relationships of different plant species was proven by several studies (Ham et al., 1994, Bohle et al., 1994). Gielly and Taberlet (1996) mentioned that the frequency of insertions and deletions occurred less in *trnL* intron region than in the sequence of intergenic spacer such as *trnL-F*. However, in the present study, many deleted or inserted nucleotide sites, most of which were phylogenetically informative, were observed in the DNA sequence of *trnL, trnV* intron and *matK* regions. There were only three indels in the sequence of *trnL-F* region.

Most of mutations in the chloroplast DNA were the results of indels, which have a length-change in DNA sequence between 1 and 10 bp (Vijverberg and Bachmann 1999). Indels that occupy large areas are only located in non-coding regions of the genome while smaller ones may show diversity within groups (Kelchner 2000). As mentioned in section 1.7.1.1.1 (introduction to *trnL* intronic region), this region's sequence can be deleted partly or completely (Freyer et al., 1995; dePamphilis et al., 1997; Lohan and Wolfe, 1998). The sequence length of samples coming from *Hypoglottidei* section ranged from 530 to 569 bp, meaning that deleted sequence part was very long in this section. In the current study, high

number of indels in DNA sequence of *trnL* intron region was observed for each studied section of *Astragalus.* The *Incani* section had quite high deleted sequences with only one inserted nucleotide site. However, because of large deleted region observed in sequence of *A. viridissimus* and *A. viciaefolius*, *Hypoglottidei* section had the most deleted sites compared with those of *Incani* and *Dissitiflori* sections. Unlike results of Gielly and Taberlet (1996), the number of insertion/deletion was higher in *trnL* region compared to that of *trnL-F* region. This outcome was meaningful because the length of the *trnL* region was longer so the number of indels can be expected to be high in this region. Moreover, the sequence of *trnL* intron showed more nucleotide variations among sections and their species so more deleted or inserted parts might be expected in this region with respect to that of *trnL-F*.

Wang and his colleagues (1999) studied *trnV* intron region to understand phylogenetic relationships between Eurasian Pines, and concluded that used species had low number of variable sites. Furthermore, some of them were not seen as informative. Thus, the tree constructed by this region was poorly resolved at the section level. In the current study, even though the number of variable sites was low, the constructed phylogentic tree was capable of separating the studied sections from each other. Therefore, it can be said that *trnV* intron region is highly suitable to understand phylogenetic relationships among the species of *Astragalus* genus at the section level, and is partially useful within a section.

Clegg (Clegg, 1993) indicated that substitutions between purines (A-G) or pyrimidines (C-T) are generally biased, and frequency of indels was observed more compared with that of substitutions (Bakker et al., 2000). *Incani* section showed a similar situation which was described by Clegg (1993); transition was slightly higher than transversion in sequence of *trnL* intron region, while for *Hypoglottidei,* and *Dissitiflori* sections, the value of transversion was higher. This difference was also useful to separate *Incani* section from others. Higher transversion was also observed when DNA sequence of *trnL-F* region was used. Higher transversion value is consistent with the results of Baker et al., (Bakker et

al., 2000), where nucleotide substitutions are biased towards transversions rather than transitions. If there was transition bias in the DNA sequence, ratio of transition/transversion (ti/tv) would change between 2 and 10 (Bakker et al., 2000). However, for *trnL* intron and *trnL-F* regions, these values were less than 1 because values of transition and transversion of *trnL* intron region were almost the same. Furthermore, for *trnL-F* region all substitutions caused transversion rather than transitions (Table 5.1 and Table 5.3).

There are some ambiguities about ratio of nucleotide substitutions and indels, which are observed in the DNA sequences of noncoding regions of chloroplast DNA. The frequency of indels are more when compared with substitution ratio (Clegg, 1993) while some researchers insist that frequency of substitution was almost the same that of indels (Gielly and Taberlet, 1994). Results of *trnL* and *trnV* intron regions are compatible with the opinion of Clegg (1993). The number of indels was more than that of substitutions in DNA sequences of these two regions. Almost similar results were observed when DNA sequences of *trnL-F* region were considered, but the difference between the values of indels and substitutions was very low.

Wojciechowski et al., (1999) indicated that DNA sequences of *trnL* intron region are less informative than that of *ITS* genomic DNA region. Similar result was also found in the study of Gielly and Taberlet (1996) who studied the genus *Gentiana* to understand phylogenetic relationships. However, for the current study the situation was different because each *trn* cpDNA region (*trnL* intron, *trnL-F* and *trnV*) were very useful to discriminate species at the section level. In addition to these cpDNA regions, *ITS* region was also very useful to see relations of species within a section.

GC content (%) of DNA sequences of *trnL, trnL-F* and *trnV* regions were close to each other and not very high (32, 37, and 31%, Table 5.1, 5.3, and 5.4). The richness of GC content demonstrates a bias to the substitution likelihoods. This content also has effectiveness on the stability of DNA and adaptation to

environmental conditions (Torres, 1990 and Baldwin et al., 1995). If this content is low in the DNA sequence, it means that AT content is higher which is thought to be because of crucial amount of transversion (Bakker et al., 2000). Although there is a high AT amount in sequences of the *trnL, trnV* intron and *trnL-F* regions, transversion occurring between A and T was observed less frequently than transversion that is observed between G and C nucleotides in angiosperms (Yang, 1994; Bakker et al., 2000). AT content was higher than the GC content in the current study and these results support the observation of Bakker et al., (2000).

6.1.2 *matK* cpDNA Region

The *mat*K region is present in every species, and its sequence is one of the least protected among the plastid genes (Olmstead and Palmer, 1994; Soltis and Soltis, 1998), so it is commonly preferred for phylogenetic studies at the interspecific level (Johnson et al., 1996).

Although length of this region was the longest within studied four chloroplast DNA regions, it had less variable sites with respect to *trnL* intron region. This region contains a gene encoding a maturase-like protein (Neuhaus and Link, 1987) so the presence of low variation is expected. Nevertheless, existing nucleotide variation were high enough to separate clearly *Astragalus* species at the section level.

GC content value was almost the same with values that were calculated by using *trnL*, *trnV* intron and *trnL-F* regions. Nucleotide substitutions gave rise to transitions within *Dissitiflori* section, while caused transversions within *Incani* and *Hypoglottidei* sections. This difference caused the separation of *Dissitiflori* section from *Incani* and *Hypoglottidei.* Nucleotide insertion was only observed in the DNA sequences of *A. cicer* that was included in *Hypoglottidei* section. Because of this insertion, *A. cicer* was separated from not only species of different sections, but also related species of the same section.

When all used chloroplast DNA regions are taken into consideration, it is safe to say that; all preferred regions are very useful to separate species of *Astragalus* genus at the section level. Phylogenetic relationships among some unknown *Astragalus* and identified samples were stable or almost the same when DNA sequence of *trnL* intron, *trnV* intron and *matK* cpDNA regions were analyzed. For instance, unknown A35 and A52 samples were clustered with *A. dasycarpus, A. vexillaris* and *A.* s*aganlugensis* species when *trnL, matK* regions were analyzed. Moreover, same unknown samples were joined to *A. dasycarpus* species without any genetic distance when *trnV* intron region was analyzed. Therefore, these two samples could be identified as *A. dasycarpus.* Unknown A65 and A66 samples were also grouped with *A. ovatus* without any genetic distance when *trnL* intron, *trnV* intron and *matK* cpDNA regions were analyzed, so identification of these two samples could be *A. ovatus*. *A.cicer* was located distanly to the related species in the tree constructed by using *trnL* intron, *trnV* intron and *matK* cpDNA regions. However, this genetic divergence was not enough to move this species to the another section. The close relation between unknown A2 and *A. nitens*, *A. nigrifructus* and *A. aucheri* species was obvious when *trnL* and *matK* regions were utilized. This relation was sufficent to think A2 sample may be coming from one of these species.

6.1.3 *ITS* nDNA Region

The DNA sequences of *ITS1* subregion of *Astragalus* had a length of about 236 bp while that of *ITS2* was 10 bp shorter than *ITS1*. For most species, the length of *ITS1* subregion is generally shorter than that of *ITS2* (Dong et al., 2003; Baldwin, 1993). The difference within the current study may be due to the addition of about 20 bp of DNA sequence from *18S* region, which is located next to *ITS1* subregion. This unconsciously added DNA sequence from the beginning part of the studied region did not affect the results of genetic diversity and distance parameters since there were no differences among the added DNA sequences of *Astragalus* species. Wojciechowski et al., (1993) indicated results that are quite similar with our

results. They used DNA sequences of *ITS* region to depict evolutionary relationships among species of *Astragalus* genus and denoted the length of *ITS1* and *ITS2* subregions as about 230 and 217, respectively. Therefore, the result taken from present study is compatible with the results of the study done by Wojciechowski et al., (1993).

Fifty-six variable sites, the highest observed number within the all studied regions, were observed in the DNA sequences of *ITS* region. Evolutionary rate of nuclear DNA was higher than that of DNA sequences of mitochondrial and plastid DNA (Savolainen and Case, 2003). Therefore, higher number of variable sites can be expected for this region. *Dissitiflori* (22 variable sites) and *Hypoglottidei* (18 variable sites) sections were more diverse ones among three sections of *Astragalus* genus. The *ITS* (*ITS1+5.8S+ITS2*) sequence data clearly separated *Incani* section from *Dissitiflori* and *Hypoglottidei* sections by the number of nucleotide substitutions and indels. This separation was also based on stem structures of sections. Species of *Incani* section have very short stem length (stemless) with respect to those of other two sections, which have distinctive stem structures. It is clear that total DNA sequence of *ITS* region data was able to separate each section phylogenetically from the others. However, only DNA sequence of *5.8S* subregion was not enough to distinguish *Dissitiflori* from *Hypoglottidei* while it was capable to separate *Incani* (stemless) from *Dissitiflori* and *Hypoglottidei* (distinct stem) sections.

Unknown A35 and A52 samples were grouped with *A. dasycarpus* and *A.vexillaris,* and A65 and A66 samples were clustered with species *A. ovatus* in the constructed phylogenetic tree without any genetic distance as it was the same in the previous results that were produced by the data from *trnL* intron and *matK* cpDNA regions. Therefore, identifications of these samples were also done similarly by the data of this region.

GC content of internal transcribed spacer region was about 56% in *ITS1* and 52% in *ITS2*. Similar results were also observed in different species such as zucchini,

cucumber (Torres et al., 1990), melon (Kavanagh and Timmis, 1988), tomato (Kiss et al., 1988) and rice (Takaiwa et al., 1985). This result also supports the results of Wojciechowski et al., (1993) who reported the GC content as 54% - 60% for *ITS1* and 50% - 54% for *ITS2* subregion in *Astragalus* genus.

ITS1 and *ITS2* subregions had similar values when GC content and variable sites were taken into consideration, but they were separated from each other by the number of deleted (inserted) nucleotides and transition/transversion ratio. The eight deleted nucleotides were located in DNA sequence of *ITS1* whereas only one indel was observed in that of *ITS2* subregion. Although, for both subregions, transition value was higher, there was a big difference between values. The transition value was quite high in *ITS2* subregion. As it was expected, the *5.8S* subregion was discriminated from both *ITS1* and *ITS2* subregions with less variable sites and no deletion or insertion since it is an exonic region.

Wojciechowski et al., (1993) introduced 28 small indels (1 or 2 bp length) scattered in *ITS1* and *ITS2* subregions, and two larger indels with 7 bp in *ITS1* (at positions 101-107) and 6 bp in *ITS2* (at positions 275-280). In the present study, one large indel with 7 bp (between 107 and 114 bp) was also noticed in *ITS1* subregion in addition to 1 bp indel in the same region. The position of the large indel almost coincided with the position of indel that was reported by Wojciechowski et al., (1993). However, a large indel was not observed in the sequence of *ITS2* in contrary to the observations of Wojciechowski (1993). The large indel in *ITS1* was omitted from phylogenetic analyses because Wojciechowski thought this region caused major impact on the inferred phylogeny in the group. In the present study, the mentioned region was included in the analyses and New World *Astragalus* species (Neo-*Astragalus*), which carried the same deleted regions in DNA sequence of *ITS1* subregion showed a close association with *Dissitiflori* and *Hypoglottidei* species in the phylogenetic tree.

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

The species of *Incani* section always located distanly to that of *Dissitiflori* in the phylogenetic trees whichever chloroplast or nuclear DNA regions were used for analyses. This result appeared to be due to different number of variable and indel sites observed among the sections. *Incani* section showed always closer association to *Hypoglottidei* than *Dissitiflori* section because less genetic variation was observed among sequences of *Incani* and *Hypoglottidei* species. Moreover, within the studied three sections, the lowest average genetic distance was observed between *Hypoglottidei* and *Dissitiflori*. These results which were yielded by DNA sequences of used regions were meaningful. Phylogenetically, *Incani* was always distant to the others, while *Hypoglottidei* and *Dissitiflori* sections were closer to each other. Actually, the same situation was also observed when morphological structures of these three sections were used. That is, *Incani* is distinctively different (stemless) compared to the other two sections.

In addition to genetic divergence among sections, genetic divergences among species within a section were also estimated. The highest genetic divergence among the species of *Hypoglottidei* section was observed for each analyzed region except for *trnL-F*. The high genetic divergence within this section was caused by the high number of variable nucleotide sites which were scattered in the DNA sequences of *Hypoglottidei* species. Genetic divergences between *A. brevidentatus* - *A. viciaefolius* and *A. brevidentatus* - *A. viridissimus* were maximum due to high number of substitution and large indels in sequence of *A. viciaefolius* and *A. viridissimus* when DNA sequences of *trnL* intron region was analyzed.

A. viciaefolius and *A. viridissimus* species did not join to the closely related species of the same section due to a large deleted sequence in the sequence of *trnL* intron region, which was also described by different scientists (Freyer et al., 1995; dePamphilis et al., 1997; Lohan and Wolfe, 1998). Instead of *Hypoglottidei* clade, they attached to the *Incani* cluster externally, because all species of *Incani* had a

large deleted region that coincided with deleted nucleotide sites of *A. viciaefolius* and *A. viridissimus*. However, there were no certain results to move these two species into *Incani* section at this point. Only deletion part in the sequence was not enough to change the taxonomical level of one species. Nucleotide substitutions of DNA sequences of *A. viciaefolius* and *A. viridissimus* were compatible with that of *Hypoglottidei* species. Therefore, phylogenetically, these two species were still accepted within the *Hypoglottidei* section.

Within the *Hypoglottidei* section, *A. cicer* was grouped distantly to the other species of the same section when *trnV* intron, *matK* and *ITS* regions were analyzed. Again, this separation was originated from indels and nucleotide substitutions, which were observed between DNA sequences of *A. cicer* and the other species of *Hypoglottidei.* This species was also included in the studies that were done by different scientists (Wojciechowski et al., 1993; Sanderson and Wojciechowski, 1996). This species was also separated from the other species because in the mentioned studies major parts of the specimens were taken from New World (Neo-*Astragalus*) group. The separation of this species was meaningful because *A.cicer* is from Old world *Astragalus* group. The Old World Clade generally contains species with euploid chromosome numbers while Neo-*Astragalus* Clade includes species with aneuploid chromosome numbers (Kazempour Osaloo et al., 2005). In the current study, all used species came from Old World group, but still there are several mutations (indels and substitution) causing phylogenetically separation of species at the different taxonomical levels. This species was separated from not only the species of the same section, but also all other species included from two different sections when the phylogenetic tree was constructed by using DNA sequences of *matK* region. However, this time mainly one large multibase nucleotide insertion caused to the diversity instead of deletion. We can not exactly say whether these multibase-nucleotides were deleted from the DNA sequences of all other species or were inserted in that of *A.cicer.*

Wojciechowski et al., (1999) studied DNA sequences of *ITS* and *trnL* intron regions to understand phylogenetic relationship between Old and New World *Astragalus* species, and concluded that all New World *Astragalus* species with aneuploid chromosome numbers form a monophyletic clade within Old World clade that was constructed by species with euploid chromosome numbers. Both multiple and single base indels were observed in the DNA sequences of both *trnL* intron and *ITS* regions in addition to nucleotide base substitutions. However, only sequence data of *ITS* region revealed well-supported and meaningful clades in their study. Kazemi et al., (2009) also indicated that the DNA sequences of *trnL* intron were less informative than that of *ITS* region because low substitution was seen in the DNA sequences of *trnL* intron region. However, in the current study, the DNA sequences of both *trnL* and *ITS* regions were substantially informative to understand phylogenetic relationships among *Incani, Hypoglottidei,* and *Dissitiflori* sections. The section discrimination was also observed when unknown samples and described species of one section were analyzed together and phylogenetic tree was constructed.

Kazemi and his colleagues (2009) studied 27 species of *Astragalus,* and 3 species of relatives of genus as outgroup. The length of aligned *trnL* intron and *trnL-F* region was 685 and 403 bp, respectively. The numbers of parsimony informative site were 22 for *trnL-F* and 88 for *trnL* intron region. These numbers were very high with respect to the values of present study where only one variable site was observed in the DNA sequences of *trnL-F* intergenic spacer region. Several authors (Kenicer et al., 2005; Oskoeiyan et al., unpublished) also supported our result that shorter length and lower number of parsimony informative sites has been observed in the DNA sequence of *trnL-F* region in the Fabaceae family. However, even though the length of studied regions in our study was shorter, and the numbers of variable and informative sites were lower, both of *trnL-F* and *trnL* intron regions were capable to construct a meaningful phylogenetic tree.

Shaw and his coworkers (2005) pointed out interesting results where they used 21 non-coding regions within Angiosperms to understand heterogeneity of the

preffered regions, and concluded that the *trnL-F* intergenic spacer region was much more diverse, despite its shorter length, than the *trnL* intron. This result is clearly different from outcomes of most of the studies in addition to findings of the present study. The length of *trnL-F* region can be variable between species and higher level. We observed its length between 165 and 167 bp, while Kazemi et al., (2009) reported the length ranging from 98 to 115 bp in *Astragalus* genus.

The various scientists extensively used *ITS* region to depict phylogenetic relationships between species of Angiosperms (Delgado-Salinas et al, 1999; Nickrent and Doyle, 1995, Wojciechowski et al., 1999; Baldwin et al., 1995; Wen et al., 1996; Scherson et al., 2008; Haque et al., 2009; Ali et al., 2011). Almost all studies proved the usefulness of this region to understand phylogenetic relations of diverse species. As it is like in all of the used regions, each section was separated from the others with high bootstrap values when DNA sequence of *ITS* region of nuclear genomic DNA was analyzed. The *ITS1* and *ITS2* subregions were highly variable among closely related species of used three sections. They were evolutionary informative at the section level. The *Incani* section was separated from the other two sections via one deleted nucleotide and a large multibase insertion in *ITS1* subregion. The *Hypoglottidei* section was separated from *Dissitiflori* section via few substitutions in *ITS1* and *ITS2* subregion. Kazempour Osaloo et al., (2005) used *ITS* region to understand phylogenetic relations of 212 species that was found in the different sections of Old World *Astragalus* group and species of *Incani, Hypoglottidei,* and *Dissitiflori* sections were separated from each other as well. In addition to Old world samples, two aneuploid New World *Astragalus* species were also included in the same study to see phylogenetic relationships of them with Old World samples. These two species located within Old World clade and closer to *Hypoglottidei,* and *Dissitiflori* sections whereas further to *Incani* section as it was like in the current study.

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

There are no phylogenetic studies indicating phylogenetic relationships between species of *Astragalus* genus that are native to Turkey. Therefore, this study will be unique to understand not only evolutionary relationships between species of the genus that are native to Turkey, but also phylogenetic relationships among *Astragalu*s species which are natural in Turkey and other countries of the World.

As it was mentioned before, all used species in the present study were included in the Old World *Astragalus* group; there was no Neo-*Astragalus* (New World) species. To see phylogenetic relations between Old and New World *Astragalus* species, many DNA sequences of both New and Old World *Astragalus* species were collected from NCBI database. All diversity parameters were computed by using DNA sequences of *Astragalus* species that are distributed in different parts of the World.

When the GC content of *trnL* intron region was calculated, almost similar values were found among species from different regions of the World. This result proved the consistent estimation of the GC content through the World. The main difference was observed between the number of variable sites and indels. This result can be expected because the number of nucleotide substitution and inserted/deleted sites of *trnL* intron region is quite variable between species of the same genus. Especially the number of variable and indel sites in sequence of *trnL* intron region was considerably higher in Iranian and Turkish species than those of North and South America. The same results were also reported in the study of Wojciechowski and his coworkers (1999); who found that nucleotide divergence was less than 0.03 substitution/site when both Old and New World samples were analyzed, but it was under 0.01 substitution/site when only New World species were used. Therefore, we can say that New World *Astragalus* species have less diversity with respect to the diversity of Old World *Astragalus* species. The phylogenetic tree was also constructed to see relationships among species that

were taken from different regions of the World (Appendix B.1). The Iranian species of *Astragalus* were scattered in the tree and attached to the different sections of Turkish species. This result indicated the similarity between DNA sequences of Iranian and Turkish samples. The North and South American samples were clustered together within Old World cluster as expected, since almost all of the American samples are aneuploid and were separated from euploid Old World samples.

Additional DNA sequences of *trnL-F* region obtained from Iran showed the presence of high number of variable sites and indels. This result was not unexpected because there was only one variable site and three indels in the DNA sequences of Turkish species. Iran is one of the diversity centers for *Astragalus* genus, so high diversity is expected. The other effect is the sections of preferred species; if species was selected from different sections, more diversity would be found. Most of Iranian samples were phylogenetically separated from Turkish species in the tree (Appendix B.2). Separation was caused by high nucleotide substitutions that were seen between Iranian and Turkish samples.

To compare the DNA sequences of *matK* region, Chinese and American species were used in addition to Turkish species. There were no crucial differences between the number of variable and indel sites of both Turkish and Chinese samples. However, these values were very high in the American samples. This result may be caused by DNA sequences of diverse species, which were taken from different parts of the American continent. Most of American samples were again nested together in a different subcluster, which was located near to *Incani* cluster (Appendix B.3). The same close relation was also noticed when DNA sequences of *trnL* intron region was used. Although there was less diversity between Chinese and Turkish samples, they connected to the tree externally because of large insertion that was found in the sequences of the Chinese samples.

ITS region was the commonly studied region so the DNA sequence of the region was obtained from different parts of the World. Higher values for variable and

indels sites were observed in samples of Turkey, China and Eurasia as expected. Both North and South American samples had lower values of variation because these samples were found in New World group. The North and South American samples were nested together within a different subcluster in the tree. The same results were also observed when *trnL* and *matK* regions were used. The Iranian and Chinese samples joined the tree externally as it was the case when sequences of *trnL-F* (Appendix B.2) and *matK* (Appendix B.3) regions were used, respectively. Species type and section can cause the separation of Chinese and Iranian samples from ours. As it was mentioned before, all used regions were very efficient to separate species at the section level. Therefore, if foreign samples from different sections were included in the study, separation of them could be easily done.

DNA sequence of nrDNA *ITS* region within the aneuploids (New World *Astragalus* samples) is very low that causes poor resolutions and weak supports to figure out relationships between species within the clades of the constructed trees (Wojciechowski et al., 1993). The present study supported this result when genetic diversity parameters of *Astragalus* genus from different parts of the World were calculated based on the DNA sequences of *ITS* region. The American samples had lower number of genetic divergence than those of Turkish, Chinese and Eurasian samples. American samples were genetically close to *Incani* section when DNA sequences of cpDNA regions were used, but close to *Hypoglottidei* and *Dissitiflori* sections when that of *ITS* region was utilized. The reason why New World samples were phylogenetically closer to *Hypoglottidei* and *Dissitiflori* species than *Incani* was the presence of a large indel region observed in DNA sequence of *ITS1* subregion. This large region was coincided with indel of DNA sequences of *Hypoglottidei* and *Dissitiflori* species. Interestingly, New World samples were close to species of *Incani* section when DNA sequences of cpDNA regions were analyzed. Biparental effects on the DNA sequence of *ITS* region may cause this contradiction because only maternal transmission is occurred in cytoplasmic inheritance within *Astragalus* genus.

Ledingham (1957 and 1960) has recommended that the North and South American species (New World *Astragalus*) should be separated from the euploid *Astragalus* species (Old World *Astragalus*) and proposed to call them by using a new genus name. However, in the current study, North and South American samples located closely to the Turkish species in the phylogenetic trees, which were constructed by using DNA sequences of *trnL* intron, *matK* and *ITS* regions. Therefore, aneuploid *Astragalus* species should be called by using a formal name such as New World *Astragalus* instead of separating them from *Astragalus* genus and naming with a new genus name.

6.4 Estimation of Molecular Clock

Lastly, evolutionary divergence time of the genus was estimated by using DNA sequences of each studied region. Both length of the DNA region and parsimony informative sites were used to estimate divergence time of *Astragalus* genus from its sister groups. Wojciechowski (2005) estimated the age of *Astragalus* genus as about 12.4 million years (Ma) while Lavin et al., (2005) estimated as 16.1 Ma by using DNA sequences of *MatK* region.

In the current study, for *Astragalus* genus, New World *Astragalus* group and *Incani, Hypoglottidei*, *Dissitiflori* sections, estimation of evolutionary divergence time was done based on DNA sequences of different regions. The *trnL-F* region was not useful to estimate evolutionary divergence time of the genus since only one region was informative in the sequence, so estimation of this region was ignored. Number of informative sites was very high for *ITS* region, and proper mutation rate for nuclear genome was not found from literature so estimated divergence time went up for *Astragalus* genus (data was not shown). Therefore, this region was ignored, and the data from *trnL* and *matK* regions were taken into consideration to calculate evolutionary divergence time in *Astragalus* genus. The estimated divergence time of this genus was between 12.5 and 14.5 Ma that were almost the same with the estimations of Wojciechowski (2005) and Lavin et al.,

(2005). The divergence time of New World species was calculated as 4.0-5.0 Ma which is compatible with the results of Wojciechowski (2005) and Wojciechowski et al., (1999). Moreover, divergence time was estimated for each used section separately. Based on the results, we can say that *Incani* section was older than *Hypoglottidei* and *Dissitiflori* sections, and the divergence of the *Hypoglottidei* and *Dissitiflori* sections from the *Incani* section is proposed to take place approximately 5-7 million years later.

CHAPTER 7

CONCLUSION

The goal of the current study was to detect the phylogenetic relationships among species of *Astragalus* genus that are native to Turkey by using both nuclear and chloroplast DNA regions.

Phylogenetic relationships among species of a selected section showed differences based on used DNA region. However, A. *glaucophyllus, A. schizopterus, A.czorochensis, A.nezaketae* and *A.sanguinolentus* species of *Incani* section clustered together without almost any genetic distance when t*rnL*, *trnV* intron and *matK* cpDNA regions were used. Moreover, within the same section, close relationships among *A. turkmenensis*, *A. yildirimlii* and *A. ancistrocarpus* species were proven to exist by *trnV* intron and *ITS* regions.

From *Hypoglottidei* section, *A. akmanii* and *A. hartvigii* formed a subcluster in the constructed tree based on *trnL* intron, *trnV* intron and *ITS* regions, so a close relationship was expected between them. Similar relations were also observed between *A. viridissimus* - *viciaefolius,* and *A. bachmarensis* - *A. lasioglottis* species either region was used to construct the tree. *A.cicer* included in *Hypoglottidei* section attached to the *Hypoglottidei* (H) cluster distantly either region was used for analyses. This distant relation was caused by both indels and nucleotide substitutions through the sequenced DNA. However, this outcome was not enough to separate and move this species to another section. Lastly, close relationship between *A. nitens* and *A. aucheri* included in *Dissitiflori* section was

proven by the constructed trees using the DNA sequences of *trnL* intron, *matK* and *ITS* regions.

Unknown A35 and A52 samples could be named as *A. dasycarpus* because these two samples were clustered together with *A. dasycarpus* whatever region was used to construct pyhlogenetic tree (except for *trnL-F* region in which almost no variation was observed). Unknown A65 and A66 could be named as *A. ovatus* since these two samples were clustered with it in the tree constructed by each region and lastly unknown A2 sample could be called as *A. nitens* or *A. aucheri* based on each region used except for *trnL-F* and *trnV* intron. The section of unknown A3, A16, A20, A108, A109 and A110 samples was determined based on each used region, but their exact species identifications were not possible because of their close phylogenetic relationships with more than one species.

The highest genetic diversity was observed in the DNA sequences of *ITS* region, which is located in the nuclear genome. Therefore, it can be said that molecular diversity was higher in the region of nuclear DNA than that of chloroplast DNA. This region consists of three subregions, *ITS1*, *5.8S* and *ITS2*. Genetic diversity in each subregion varied considerably. The DNA sequence of *5.8S* subregion had few variable sites among sections so *ITS1* and *ITS2* subregions were sufficient to construct a phylogenetic tree that depicted evolutionary relationships among the sections of *Astragalus*.

The lowest molecular diversity was observed when DNA sequence of *trnL-F* chloroplast DNA region was analyzed. This low diversity could be explained with the short length of this region. On the other hand, the cpDNA region with the longest length, *matK* chloroplast DNA region, had also low genetic diversity among the sections of *Astragalus.* However, this result could be expected since *matK* region is an exonic area encoding a maturase-like protein.

In addition to nucleotide substitution, indels are also important to depict phylogenetic relations among species of *Astragalus*. In the current study, indels

were observed in the DNA sequence of each used region with different frequency. There was a consistency between the numbers of inserted or deleted nucleotides especially at the section level so these cpDNA regions could be used for separation of one section from the others. Therefore, each of the used regions whether genetic diversity was low or high was useful to construct phylogenetic trees, which had high quality of resolution at the section level.

The highest genetic divergence was found between *Incani* and *Dissitiflori* sections whereas the lowest one was observed between *Hypoglottidei* and *Dissitiflori* sections. The separation of *Incani* from the other used sections was also supported by morphological structures of them. The species of *Incani* section do not have distinctive stem structure while the other two sections have.

To understand evolutionary relationships between *Astragalus* species of Turkey and the species from other regions of the world, the DNA sequences of studied regions of cpDNA and nDNA were gathered from the NCBI database and were evaluated together. The Iranian samples either scattered in the phylogenetic tree or attached to Turkish *Astragalus* species externally. This different arrangement may be caused by the sections of selected samples. South and North American samples (New World *Astragalus* group) were nested within a different subcluster, which was located in the main clade produced by samples of Old World *Astragalus* samples. By using this result, we can say that New World *Astragalus* group is monophyletic and diverged from Old world *Astragalus* samples about 4.0-5.0 million years ago.

The outcomes of the current study are very important to understand evolutionary relationships among three large sections of *Astragalus* genus. Additional taxa and sequences from other useful DNA regions such as *ndhF, rbcL, rps16* intron, *psbA-trnH* intergenic spacer cpDNA regions may provide further insights to understand phylogenetic relationships among *Astragalus* species not only at the section, but also at the species level.

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APPENDIX A

CHROMATOGRAM STRUCTURES FOR EACH STUDIED REGIONS

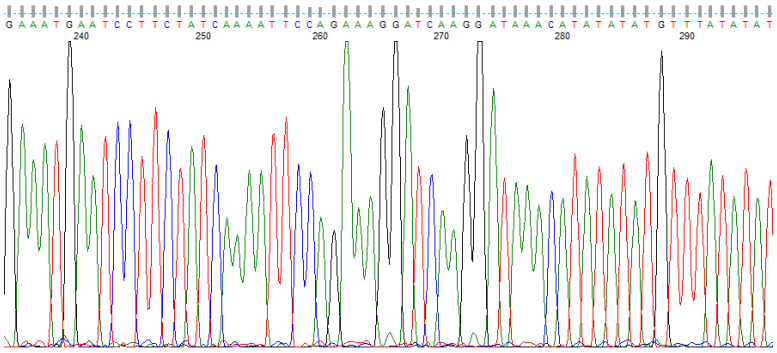

Figure A.1 An example of chromatogram for *trnL5'-L3'* (*trnL* intron)

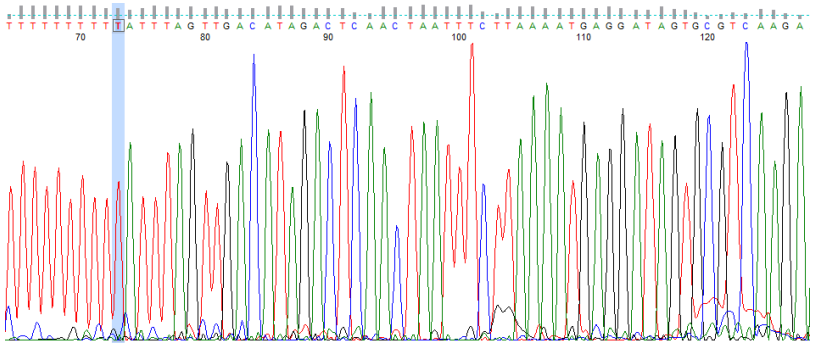

Figure A.2 An example of chromatogram for *trnL3'-F*$^{(GAA)}$ (*trnL-F* intergenic spacer)

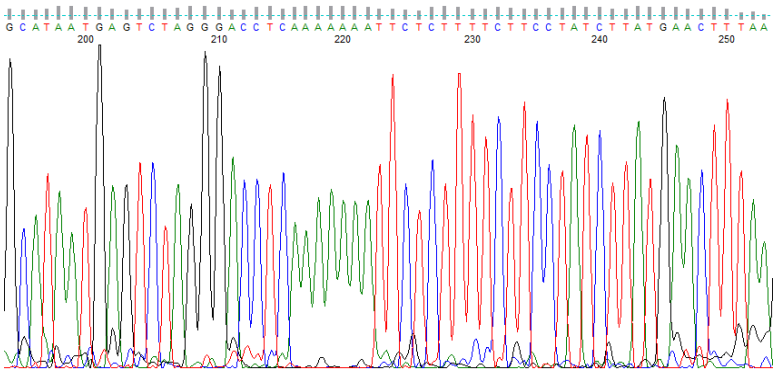

Figure A.3 An example of chromatogram for *trnV* intron

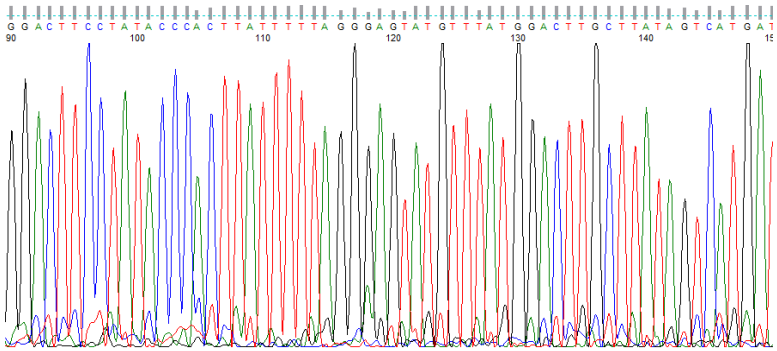

Figure A.4 An example of chromatogram for *matK* (*maturase Kinase*)

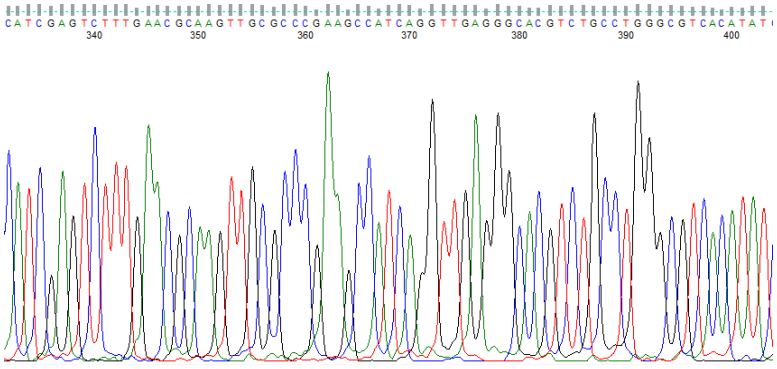

Figure A.5 An example of chromatogram for *ITS (Internal transcribed spacer)*

APPENDIX B

PHYLOGENETIC TREE CONSTRUCTIONS WITH OLD AND NEW WORLD *ASTRAGALUS* SPECIES

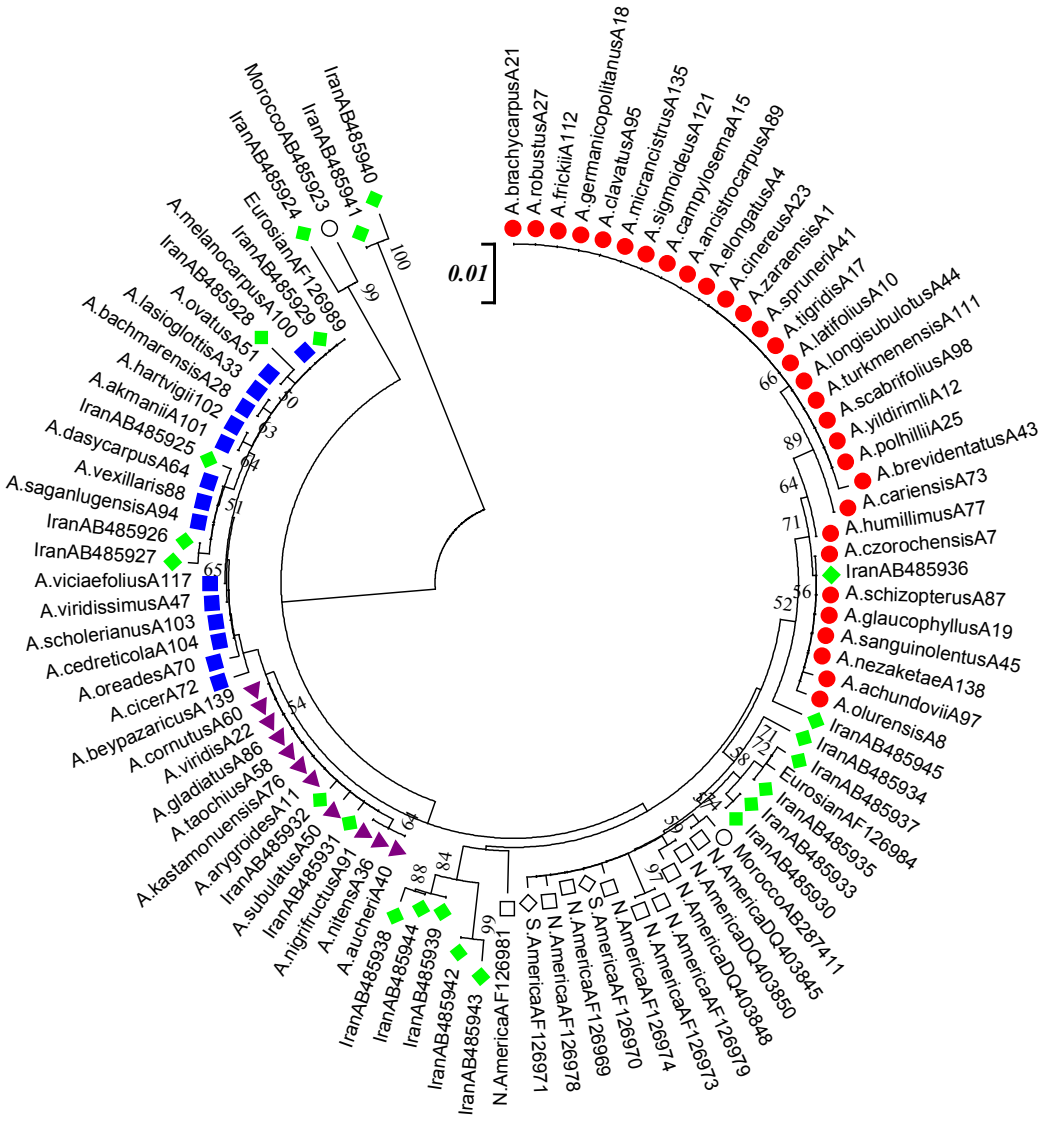

Figure B.1 Phylogenetic tree (*trnL*) depicting relations of Turkey species with other species that live outside of Turkey (Filled circles: *Incani*, Filled Squares: *Hypoglottidei,* Filled Triangles*: Dissitiflori* section, Circles: Morocco, Squares: North and South America, and Green Squares: Iran).

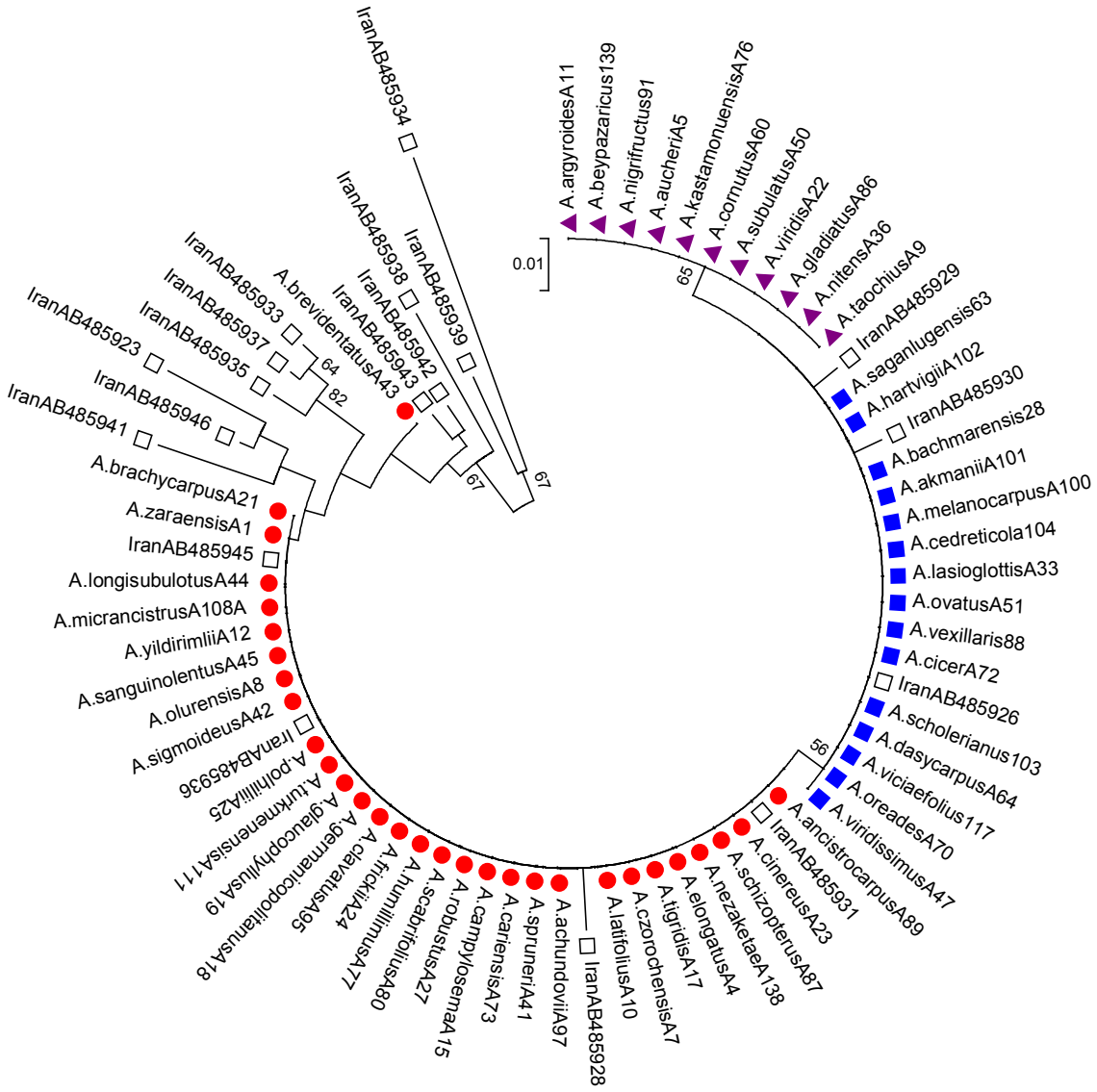

Figure B.2 Phylogenetic tree (*trnL-F*) depicting relations of Turkey species with other species that live outside of Turkey (Filled circles: *Incani*, Filled Squares: *Hypoglottidei,* Filled Triangles*: Dissitiflori* section, Squares: Iran).

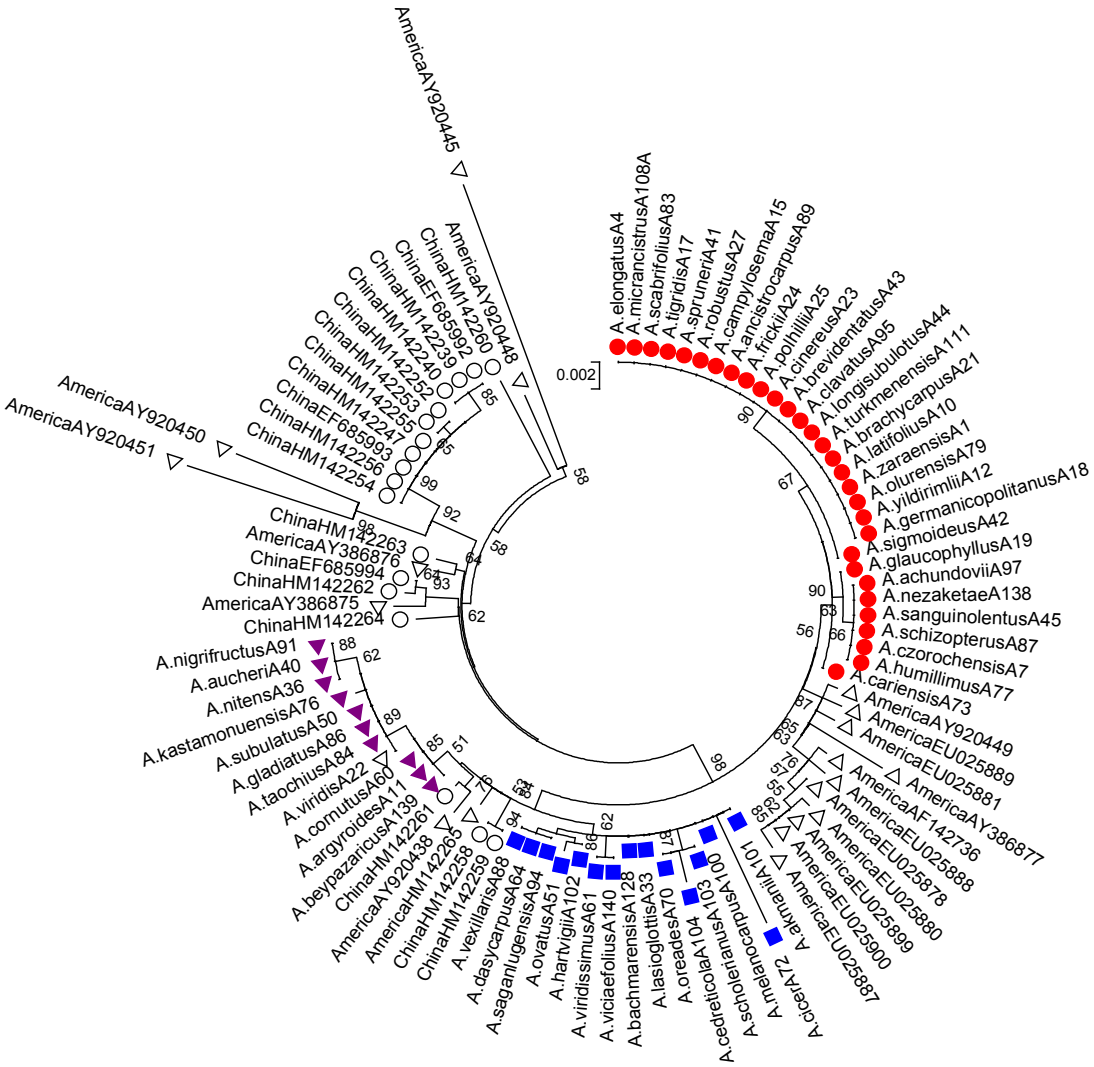

Figure B.3 Phylogenetic tree (*matK*) depicting relations of Turkey species with other species that live outside of Turkey (Filled circles: *Incani*, Filled Squares: *Hypoglottidei,* Filled Triangles*: Dissitiflori* section, Triangles: America, Circles: China).

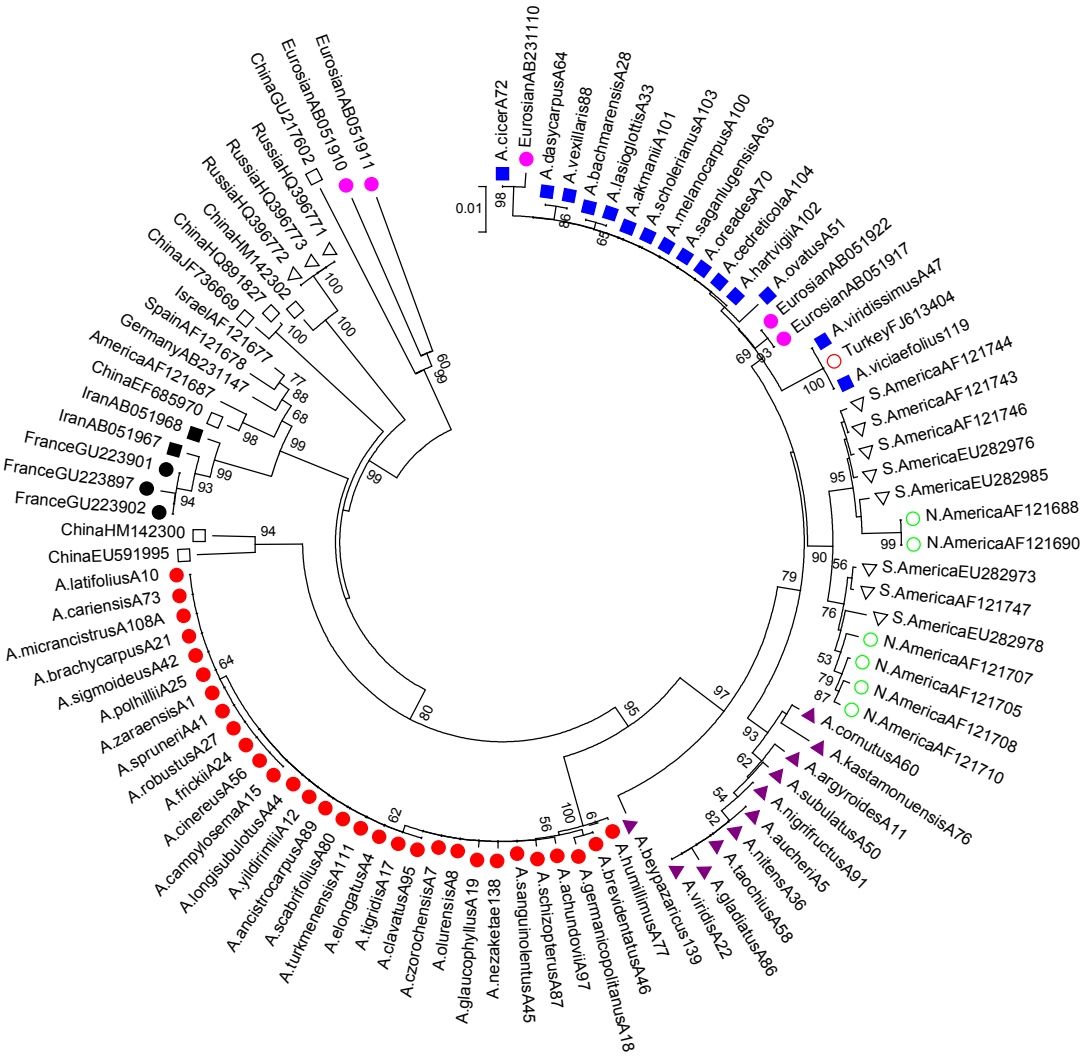

Figure B.4 Phylogenetic tree (*ITS*) depicting relations of Turkey species with other species that live outside of Turkey (Red Filled circles: *Incani*, Blue Filled Squares: *Hypoglottidei,* Purple Filled Triangles*: Dissitiflori* section, Pink Filled Circles: Eurasian, Green Circles: N.America, Upside down Triangles: S.America, Squares: China, Black Filled Squares: Iran, Black Filled Circles: France, Triangles: Russia).

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Publications and Preprints

Dizkırıcı, A., Ekici M., Kaya, Z., 2012. Phylogenetics of three *Astragalus* sections in Turkey (*Incani* DC., *Hypoglottidei* DC., and *Dissitiflori* DC.) and their comparisons with New World *Astragalus* group based on *ITS* nrDNA region. (In Preparation)

Dizkırıcı, A., Cetiner, C., Birsin, M., Kaya, Z., Onde, S. and Ozgen, M. 2012. Phylogenetic relationships of *Triticum* and *Aegilops* genera based on DNA sequences of *trnT-F* chloroplast DNA non-coding region. (In Preparation)

Dizkırıcı, A., Cetiner, C., Birsin, M., Kaya, Z., Onde, S. and Ozgen, M. 2012. Molecular phylogeny of *Triticum* and *Aegilops* genera based on partial sequences of chloroplast DNA *matK* gene. (In Preparation)

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Awards and Honors

TUBITAK (The Scientific and Technical Research Council of Turkey) International Research Fellowship Programme, Plant Genome Mapping Lab, University of Georgia, Athens/Atlanta, USA. June-December 2010

Service

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