BOLU ABANT IZZET BAYSAL UNIVERSITY

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

INVESTIGATING PHYLOGEOGRAPHIC STRUCTURE OF THE OAK GALL WASP *ANDRICUS TOMENTOSUS* IN TURKEY USING CYT B GENE AND ITS2 REGION SEQUENCES

MASTER OF SCIENCE

OMAR A. DANSO

BOLU, AUGUST 2019

BOLU ABANT IZZET BAYSAL UNIVERSITY

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DEPARTMENT OF BIOLOGY

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APPROVAL OF THE THESIS

INVESTIGATING PHYLOGEOGRAPHIC STRUCTURE OF THE OAK GALL WASP *ANDRICUS TOMENTOSUS* IN TURKEY USING CYT B GENE AND ITS2 REGION SEQUENCES submitted by **OMAR A. DANSO** and defended before the below named jury in partial fulfillment of the requirements for the degree of **Master of Science** in **Department of Biology, The Graduate School of Natural and Applied Sciences of Bolu Abant Izzet Baysal University in 7.08.2019** by

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To my Father and Mother.

DECLARATION

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

OMAR A. DANSO

ABSTRACT

INVESTIGATING PHYLOGEOGRAPHIC STRUCTURE OF THE OAK GALL WASP *ANDRICUS TOMENTOSUS* IN TURKEY USING CYT B GENE AND ITS2 REGION SEQUENCES

MSC THESIS
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BOLU ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: PROF. DR. SERAP MUTUN)

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Many species have evolved the ability to redirect plant growth and development to result a unique structure called galls, which provide gall inducing organisms with unique protection and food. *Andricus tomentosus* as an obligate parasitic gall wasp species forming its asexual generation galls in the section Quercus oaks were collected from 12 localities across Turkey. This thesis is designed to investigate and reveal genetic variation and its geographic distribution, population changes through expansion and contractions, divergence times between major lineages, as well as the possible reasons for the current distribution of genetic diversity. For these purposes, a 433 base pairs of mitochondrial DNA cytochrome b gene and the entire ITS2 region from nuclear genome were amplified and sequenced. All sequences generated 36 mtDNA haplotypes and 11 ITS2 alleles. Intermediate to high genetic variation was detected in *A. tomentosus* populations in Turkey. In addition, the higher diversity of haplotypes and lower diversity of nucleotide is an indication of population expansion. ML, MP, and Bayesian inference to reveal phylogenetic and phylogeographic structure of the species in Turkey also revealed that there is a geographical haplotype grouping. Application of molecular clock showed that the splitting of *A. tomentosus* from the outgroup species took place during the Miocene Epoch. Subsequent diversifications seem to have taken place within the species creating deep to intermediate to relatively shallow splits in *A. tomentosus* species in Turkey. The overall findings of this thesis revealed that factors such as paleoenvironmental changes and paleoclimatic oscillations have impacted their imprints on *A. tomentosus* in Turkey.

KEYWORDS: *Andricus tomentosus,* Genetic diversity, mtDNA, nDNA Paleoclimatic changes.

ÖZET

GAL ARISI *ANDRICUS TOMENTOSUS*'UN TÜRKIYE'DEKİ FİLOCOĞRAFİK YAPILANMASININ SİTOKROM B GENİ VE ITS2 BÖLGESİNİN DİZİLENMESİ YOLUYLA ARAŞTIRILMASI
YÜKSEK LİSANS TEZİ
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Pek çok tür bitkilerde gal adı verilen yapıların oluşumunu sağlayarak gallerden besin temin ederek ve korunaklı bir yerde gelişimini sürdürme yeteneği kazanmıştır. Meşelere zorunlu parazit bir tür olarak kabul edilen *Andricus tomentosus* eşeysiz jenerasyon gallerini Quercus seksiyonundan meşelerde oluşturur. Çalışma materyali olan türün galleri Türkiye'den 12 farklı lokasyondan toplandı. Bu tez çalışmasında *A. tomentosus* türünün genetik çeşitliliği ve mevcut çeşitliliğin coğrafik dağılımı, populasyonların geçirmiş olduğu muhtemel genişleme ve daralma olayları, ana soy hatlarının ayrışma zamanları ve genetik çeşitliliğin bugünkü dağılımına neden olan muhtemel olayların araştırılması amaçlanmıştır. Bu amaçla, mitokondriyal DNA sitokrom b geninin 433 baz çiftlik bir kısmı ile nüklear genomdan ITS2 bölgesinin tamamı çoğaltılmış ve dizilenmiştir. Dizilenen 120 bireyden 36 haplotip ve 11 alel belirlenmiştir. Veriler *A. tomentosus* türünün Türkiye'deki popülasyonlarında orta, hatta yüksek sayılabilecek bir genetik çeşitliliğin olduğunu ortaya koymuştur. Ayrıca, nükleotid çeşitliğiyle karşılaştırıldığında yüksek haplotip çeşitliliği türün geçmişte genişleme gösterdiğine işaret etmiştir. Türün filogenetik ve filocoğrafik yapılanmasını araştırmak için yapılan ML, MP ve Bayesian analizleri haplotiplerin bazı gruplamalar oluşturduğu ortaya koymuştur. Moleküler saat uygulamasının da yapıldığı analizler *A. tomentosus* türünün dış grup türlerden Miyosen döneminde ayrıldığına işaret etmiştir. Bu ayrışmayı takip eden bazı olaylar derin ayrışmalara ilave olarak daha orta ve yakın zaman farklılaşmaları olduğunu da göstermektedir. Genel olarak bu tez çalışması ile elde edilen bulgular *A. tomentosus* türünün geçmişteki dalgalanmalardan özellikle de son dönem olaylarından etkilendiğine işaret etmektedir.

ANAHTAR KELİMELER: *Andricus tomentosus,* Genetik çeşitlilik, mtDNA, nDNA Paleoklimatik değişiklikler.

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LIST OF ABBREVIATIONS AND SYMBOLS

A : Adenine
AIC : Akaike information criteria
AMOVA : Analysis of molecular variance
BEAST : Bayesian Evolutionary Analysis Sampling Trees
bp : Base pair
^{0}C : Celsius degree
CI : Consistency index
Cyt b : Cytochrome b
C : Cytosine
ddH2O : Double-distilled water
dNTP : Deoxynucleotide triphosphate
EtOH : Ethanol
EDTA : Ethylenediaminetetraacetic acid
GTR : General Time Reversible
G : Guanine
h : Haplotype diversity
HI : Homoplasy index
Hri : Harpending's raggedness index
ITS2 : Internal transcribed spacer 2
Kb : Kilobase
$MgCl_2$: Magnesium chloride
ML : Maximum likelihood
MP : Maximum parsimony
µl : Microliter
mg : Milligram
mm : Millimeter
mM : Millimolar
MRCA : Most recent common ancestor
mtDNA : Mitochondrial DNA
MYA : Million years ago
nDNA : Nuclear DNA

PAUP : Phylogenetic analysis using parsimony program

PCR : Polymerase chain reaction

rpm : Revolutions per minute

NaAce : Sodium acetate

NaCl : Sodium chloride

SDS : Sodium Dodecyl Sulfate

SP **:** Species

UV : Ultraviolet

Vol : Volume

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1. INTRODUCTION

1.1 Galls and Gall Formation

Gall was given a variety of definitions by different researchers and have been mentioned in many publications, nonetheless, gall as a term was first coined by an early scientist, Theophrastus (371-286 BC), in one of his publications (Senn, 1942; Thanos, 2005). Galls were first believed to be just an ordinary impotent feature of the plants. Even without a proper definition, utilization of galls by man was documented several centuries ago. A lot of important dates and calendar marks were printed by gall either as an ink extract or in other forms (Kolar et al., 2006). Since then, galls were used for printing and writing notes, Diplomatic scripts, as well as scientific documents. Researchers and artists like, Leonardo da Vinci, Rembrandt and Van Gogh have used gall inks for painting their arts. Thomas Jefferson also documented the first declaration of Independence with gall ink (Kolar et al., 2006). Moreover, galls have been used as medicine, dying, tattooing, tanning, fuel and food in certain parts of the world (Fagan, 1918). The people of Sumer and Egypt used galls to cure sicknesses such as dysentery and malaria (Ekici, 1975). Literally the Greeks were using galls as fuel. The early Chinese society utilized galls for worshipping and spiritual uses to serve their Gods. Animals such as sheep, cows and many other herbivores feed on plant galls (Weis et al.,1985).

After their primary definitions as simple plant structures, galls have been defined more thoroughly. Their most recent description explained galls as abnormal plant growths in different shapes and forms which emerge in response to a parasitic

activity caused by either insects, mites, midges, wasps, aphids, bacteria, fungi or nematodes (Schultz et al., 2019). Gall formers can induce their taxon-specific galls in different host plant species (Lijleblad and Ronquist, 1998; Ronquist, 1998).

Among other gall inducing organisms, insects evolved as the dominating group with nearly 13000 identified species (Lijleblad and Ronquist, 1998; Ronquist, 1999). Insect galls are phenotypically extremely diverse, and it is thought that genes of the developing larva inside galls are the determinants of gall's external phenotype (Schultz, 2019). Insect galls share some traits making the galls even more diverse and raised the possible questions such as, how far does these insects diverge? (Stone and Crook, 1998). Hence, insect galls display highly complex morphological features and diversity. This phenomenon of galls is directly amalgamated to the diversity of host (Stone, 2002).

Gall inducing insects choose their hosts based on tannins, secondary metabolites, and volatiles (Price et al., 1987). The biological mechanisms used by insects to facilitate gall induction is not fully understood yet, however many researchers believe that it involves physical, chemical and mechanical processes (Stone, 2003). Nonetheless, typical gall formation process includes three phases; initiation, growth, and maturation . At initiation stage, female selects proper host plant using the clues and the specific part to begin oviposition. Evidences mapped a series of secretion at this stage (Stone, 2002). In addition, an interaction between the meristematic tissue and the mechanical process by the ovipositor of wasps controls this process (Stone, 2003).

Studies indicated that meristematic tissue is needed to initiate gall induction (Stone, 2003). Subsequently, the growth phase follows, and the larval chambers are formed and vascular linings to form the chambers. The last stage is the maturation

phase which involves a decrease in the total number of cell division. The dependency ratio of the larva on the host plant decreases immensely. Feeding by the larva continues to the gall nutritive tissue and extended further to the parenchyma cells. In this stage pupation starts, and lignification of the gall is extended further. Subsequently, due to the feeding activity on the parenchyma cells by the wasps, pores are created that serve as the exit route for the adult (Stone, 2002).

Insect galls that are formed in a variety of host plants developed great deal of variation. In fact, most of the galls are similar in their internal structure where there is one or more larval chamber for housing the developing larva inside the gall. A typical gall develops in either monocular (with a single larval chamber) and multilocular (with more than one larval chamber) forms, and the number of larval chambers is species specific feature (Stone, 2002). In the course of evolutionary history, an increase in the complexity of galls, reduction in the number of larval chambers per gall were developed (Cornell, 1983).

Despite the similarity in the internal gall structure among species, external gall phenotype shows great diversity, and varies from species to species. Such evolutionary features as nectar secretion, gall toughness, gall wall thickness, larval chambers per gall, and coatings of sticky resins are some of the characters that show variation among galls of different species (Stone et al., 2000). It is believed that outer gall phenotype such as hairy or spiny projections, being sticky versus non-sticky feature, thicker gall wall, and the presence of resin material at the outer surface of galls are evolutionarily derived characters. These evolutionarily newer features are thought to have evolved for defense against other parasites or predators (Stone et al., 2000).

For many years, formation, development and evolution of such highly complex insect galls have taken great attention by the researchers. Evolution of galling is

currently explained by three main hypotheses: enemy hypothesis, nutrition hypothesis, and microclimate hypothesis. According to the first hypothesis, galls provide larva a room for safe environment from predators. Secretion on gall surface as a way of modification to prevent the known specialist predators from attack is used to support this hypothesis. Structural modifications through secretion such as nectars, increased gall thickness, gall hardness, and recruitment of ant guards gives a minimal predators' attack. Thus, many researchers agreed on enemy hypothesis. Nutrients hypothesis suggested that in order to make food available to their developing larvae, gallers must do gall induction (Stone, 2003). Exterior to the larval chamber(s) lies a nutritive tissue that the larvae feed on during their internal life span. Superior to the nutritive tissue, there is outer layer of parenchyma and sclerenchyma cells (Stone and Schönrogge, 2003). The internal nutritive tissue of galls provides enough food to the developing larvae. Lastly, the so-called microclimatic hypothesis proposes the gallers strategy in avoiding harsh environmental conditions such as humidity, desiccation, and other harmful substances from the environment (Stone, 2003).

1.2 Oak Gall Wasps (Hymenoptera: Cynipidae)

Among other gall forming insects, oak gall wasps is one of the most successful groups that began to produce one of the most complicated gall phenotypes over the long evolutionary history of gall-making process (Dreger-Jauffer and Shorthouse, 1992). Gall wasps, from the order Hymenoptera, family Cynipidae and the subfamily Cynipoidea, makes the second largest gall-making insect groups after gall midges from Cecidomyiidae family (Schönrogge, 2003). A recent study revealed that such large group of gall wasps are composed of twelve tribes including Diplopedini, Eschatocerini, Pediaspidini, Aylacini, and Cynipini (Penzes et al., 2018). Parasitism is

a common phenomenon among the cynipids where an attack of gall wasp larva is possible by other species of parasitoids and inquilines. The so-called parasitoids and inquiline do not have the gall making ability (Askew, 1965). While members of few tribes do not have the ability to induce their own galls, oak gall wasps, are gall inducers (Cornell, 1983).

Since gall wasps depend upon Castanea and oak trees (*Quercus*) from the family Fagaceae as their host plants for survival oak gall wasps are accepted as obligate parasites of these plant species (Abe et al., 2007). Due to the necessity of the host plants cynipid evolution is paralleled along with their host taxa. The global pattern of the diversity of oak gall wasps goes back as early as Paleocene (65-56 MYA) or Eocene (56-35 MYA) (Cannon and Manos, 2003). They are thought to have diverged from Asia where one of the lineages migrated to North America through Beringian Land bridge (Thorne, 1993; White et al., 1997). It was suggested that the North American oak trees evolved around the Eocene (White et al., 1997). Oak sections of Cerris and Quercus is proposed to disperse into the Western Palearctic during the Pliocene era (5MYA) through Himalaya Mountain and Central Asian highlands (Manos and Stanford, 2001). However, one cannot conclude the above dispersal route and origination of the oak taxa since there are ongoing arguments about the actual origin and diversity of oak taxa (Abe et al., 2007).

Nonetheless, the first gall fossils are dated as ca. 300 million years (MY) as primitive and simple structures. Recent studies have proposed that first and relatively simple herb galls were already present ca. 80 MYA (Stone vd., 2008). Along with that, the diversification of *Quercus* is believed to have already taken place in the Eastern Asia around these era (Acs et al., 2007). This brought the possible origination of oak gall wasps from this region (Acs et al., 2007). Thus, Paleocene and Eocene are two

possible skeptical periods in which oak gall wasps are believed to have originated from the Eastern Asia and moved to North America and continued its diversification over there resulting in a highly diverse group (Throne, 1993; White et al., 1997).

Gall wasps are very specific in the use of host plant and the time of the attack. They show a series of relationships with their hosts including geography, evolution and shifting along time (Ronquist and Liljeblad, 2001). Ancestral gall wasp is believed to have shifted (heteroecy) from one host to the other in different generations. Few gall wasp genera including *Andricus* there is an alternation of generation between a sexual and asexual generation each year (heterogony). Gall wasps with such complex life cycle produce their sexual generation in spring and their asexual generation in late summer and autumn (Askew, 1984). While asexual generation females lay down their eggs on *Cerris* hosts, the sexual generation prefers the *Quercus* section as the oviposition site (Abrahamson and Weir, 1997).

Gall wasp growth and development are directed by the chemical signals (hormones) from the cynipid (Schultz 2019). These processes are transcriptionally encoded by a set of co-regulated genes (Schultz 2019). This makes it possible for galling organisms to modify the growth and development of their host plants (Ambrus, 1974; Rohfritsch, 1992). Each species induces a distinct gall morphology such as spongy, woody, sticky resins, hairy or spiny external features. Adults also greatly varies in size (Askew, 1987; Price et al., 1987). However, closely related species often induce similar galls (Price et al., 1987). Recently, researchers' interest in galls increases as a model in fields of molecular phylogenetics, systematics, and evolutionary biology to understand and answer some evolutionary questions. This is attributed to complicatedness, important phenotypic variation and diversity they showed among galls and gall inducing wasp species.

1.3 Oak Gall Wasps: Species and Genetic Diversity in Turkey

Gall wasps in the Nearctic region are represented by 700 species under 30 genera (Csoka et al., 2005). In the contrary, around 200 species have been reported from the Palearctic area where Turkey, Caucasus and Iran are the centers of the oak gall wasp diversity in the Western Palearctic (Rokas et al., 2003).

Oak gall wasp species diversity is thought to be closely correlated with their oak host species diversity. Although there is ongoing debate about the classification and origin of oaks, they are taxonomically grouped under three sections in Turkey: white oaks (Quercus L.), red oaks (Cerris Loudon), and evergreen oaks (Ilex Loudon) (Yaltırık, 1984). About 28% of the land area is composed of forestry areas, and oaks are one of the most important forest trees in Turkey. Eighteen species of oaks and their hybrids covers almost 6476 277 hectare-area in Turkey (OGM, 2014). Since oak taxa are the obligate hosts of oak gall wasps and possessing hosts for both asexual generation host taxa from the section Quercus and sexual generation hosts from the section Cerris make Turkey a haven for oak gall wasps. Due to the presence of both host taxa in relatively large area oak gall wasp species diversity and richness of this group is conspicuously high.

Even though there were several studies in the past, the first checklist study published by Katılmış and Kıyak (2008) reported 81 gall wasp species from Turkey. A new species was described from Turkey by Melika and Stone (2001), and following studies added few more species to the Turkish oak gall wasp fauna (Katılmış and Kıyak, 2009). Kemal and Koçak (2010) reported three more oak gall wasp species from Van and vicinity. Mutun and Dinç (2011) reported *Cynips korsakovi* as a new record to the Turkish fauna. Mete and Demirsoy (2012) reported 24 oak gall wasp species representing 5 genera from Kemaliye, Erzincan, and Dinç et al. (2013)

described a new *Andricus* species from Turkey. An oak gall wasp species, *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae), was accepted by EPPO (European and Mediterranean Plant Protection Organization) as the first gall wasp species as pest. The pest species was reported from Turkey by İpekdal et al. (2014) and emphasized its importance due particularly to its damage to the products and its economic effects in the world (İpekdal et al., 2014). In the same year, four new oak gall wasp species were described by Mutun et al. (2014). Lastly, the most recent study reported 110 oak gall wasp species from Turkey (Azmaz and Katılmış, 2017). It is believed that the number of species present in Turkey is higher than the reported number.

Besides faunistic studies revealing relatively high species diversity in Turkey first molecular-based investigations started in early 2000s and indicated the existence of high genetic diversity in oak gall wasps. These studies revealed that Turkey is the genetic source for the European oak gall wasps and constitutes the center of diversity. Therefore, Anatolia was referred as the 'cradle for oaks gall wasps' (Rokas et al., 2003). Furthermore, a study conducted in *Andricus coriarius* sampled from the Western Palearctic from Spain, Italy, Croatia, Hungary, Slovakia, Greece, Lebanon, Turkey to Iran revealed that the highest genetic diversity was present in the Turkish and Iranian populations. In this species, mitochondrial DNA cytochrome b gene region and the nuclear ITS region showed a diversity of nucleotide of 0.005 and 0.006 in Iran and Lebanese populations while Turkish populations showed, 0.015 (Challis et al., 2007). A new cryptic species was also disclosed from Iran and Turkey with the same study.

High level of genetic diversity was also reported from another oak gall wasp species from Turkey and analysis indicated that the eastern localities including the

Anatolian populations provided genetic source to the European *A. kollari* populations (Stone et al., 2007). Phylogeographic studies (Dinç and Mutun, 2011) also revealed the existence of high level of genetic diversity in the Turkish populations of *A. lucidus* as 0.808 haplotype and 0.115 nucleotide diversity was present (Mutun, 2011). Likewise, intermediate to high level of genetic variation was observed in *A. caputmedusae* as haplotype diversity (0.463) and nucleotide diversity (0.101) was comparable to other oak gall wasp taxa (Mutun, 2010). Additional studies revealed that *A. quercustozae* possessed 0.450 and 0.450 in haplotype and nucleotide diversity, respectively (Dinç and Mutun, 2011).

Subsequent studies indicated that some of the geographic barriers played significant role as a physical barrier to gene flow and shaped intraspecific phylogeographic structure of oak gall wasps in Turkey. As it was well documented geographic barriers shape distribution of genetic variation and lineages across the range of a species (Avise, 2000). In Turkey, the Taurus Mountains located in the south separates the Mediterranean region from the Central Anatolia, the northern and the western Anatolian mountains delineates the Black Sea coastal area from the central steppe environments (Demirsoy, 2002). Another such high altitudinal barrier is the mountain zone extending from the northeast toward the southeast is the Anatolian Diagonal (AD), which was first described by Davis (1971). The western side of the AD is generally below 1500 m with hotter and arid conditions; however, the eastern part is about 2000m on average and is with more humid and colder temperatures (Ekim and Güner, 1986). It was suggested that the difference in environmental conditions on both sides of the AD were important to create a break in several taxa extending their distribution across Turkey (Gür, 2016).

Geographically, Turkey is located at the junction of three phytogeographical regions as the Euro-Siberian in the north, the Mediterranean in the south and the western coastal part of Turkey, and the Irano-Turanian region covering the east, southeast and Central Anatolia (Figure 1.1) (Zohary, 1973). The AD divides the Irano-Turanian phytogeographic region into eastern and western sides, where in both subregions a variety of oak and oak gall wasp taxa show distribution. Such break was detected between the major lineages of *A. caputmedusae* (Mutun, 2010), *A. lucidus* (Mutun, 2011), *A. quercustozae* (Dinç and Mutun, 2011), and *A. gallaetinctoriae* (Mutun et al., 2013). In these oak gall wasp species, an apparent genetic break between eastern and the central-western part of the Turkish populations were congruent with the location of the AD. Similar genetic break was also detected in the oak gall wasp species *Trigonaspis synaspis* resulting in the formation of two major clade structure as east and west (Mutun and Atay, 2015). Some more recent studies further produced congruent and robust results indicating eastern versus western-central separation of main clades across Turkey in *Cynips quercus* (Mutun and Dinç, 2019), *C. quercusfolii* (Dinç and Mutun, 2019), and *A. chodjaii* (Mutun et al., 2019).

Figure 1.1. Map of Turkey showing phytogeographic regions and the Anatolian Diagonal. Eur-SIB: Euro-Siberian, IR-TUR: Irano-Turanian, C.A.: Central Anatolian, E.A.: Eastern Anatolian, MED: Mediterranean.

1.4 Paleogeography of Turkey

Turkey, today located as a bridge between Asia and Europe was originally formed from three main land plates as the Pontides, the Anatolides-Taurides and the Arabian plate (Bozkurt, 2001). After the first formation of the initial land masses, a series of transgression and regressions and orogenetic and tectonic events have resulted in the formation of Turkey (Okay, 2008).

At the end of the Mesozoic era African continent collided with the northern land mass and later gave rise to Europe and Asia. With this collision, major altitudinal differences became apparent initiating a series of orogenesis. Associated with these orogenetic events several mountain ranges in Europe and the Taurus Mountains in south and North Anatolian mountain zone in the north of Turkey were formed near the

end of the Mesozoic time (65- 70 MYA). Consequently, major formation of Turkey occurred through the following epoch as the Oligocene (33,9-23 MYA) and Miocene epoch (23-5,3 MYA). Ongoing reformation and final shaping continued through the Miocene (Aquitanian (23-20.4 MYA), Burdigalian (20.4-16 MYA), Langhian (16-13.8 MYA), Serravallian (13.8- 11.6 MYA), Tortonian (11.6-7.2 MYA), and Messinian (7.2-5.3 MYA), and this stage was ended with the Messinian aridity crisis (Karadenizli, 2011). Repeated faunal exchanges during the Miocene epoch between Turkey and Europe, and Africa resulted in complex faunal composition currently characterizing some faunal elements in Turkey (Çıplak, 2004).

Ongoing events continued throughout the following epoch, the Pliocene (5-2.6 MYA), which gave the latest paleogeographic and topographic structure of today's Turkey. Moreover, the next Quaternary period (1.8 MYA-current) was the time of shaping the contemporary Turkish diversity particularly at the species and subspecies level in many animal taxa (Demirsoy, 1999). Specifically, the first stage of the Quaternary period, the Pleistocene (1.8 MYA-10.000 YA), was divided into at least four subsequent glacial and interglacial cycles between each glacial. The Pleistocene with these four major glacial ages (Günz (800.000- 600.000 YA), Mindel (500.000-200.000 YA), Riss (200.000-130.000 YA), Würm (115.000- 11.700 YA) operated on the Turkish faunal elements as well as in the genetic composition of taxa distributed in the area (Çıplak 2003).

The consequences of the Quaternary glacial cycles were extensively studied in the Western part of the Palearctic region (Hewitt, 1999; Schmitt, 2007) including Turkey (Çıplak, 2008). As the glacial sheets expanded, temperate organisms remained in the southern refugia such as Mediterranean Europe, Italy, primarily Iberia, the

Balkans and Turkey (Hewitt, 1999). Except those populations used refugia as shelters others particularly that lacked sufficient dispersal rates have perished due to the loss of suitable habitat (Thomas et al., 2004). Prior to the last glacial period, refugia provided source for the recolonization on the rest of Europe (Hewitt, 1999; Schmitt, 2007). Further evidence proclaimed that, apart from the well-known southern regions, the so-called cryptic refugia existed at the north where microclimatic conditions were favorable (Deffontaine et al., 2005; Ursenbacher et al., 2006). Similarly, the patterns of Quaternary expansion in the southern refugia were well-recorded in the gall-inducing cynipids, and the evidences are (1) populations of Iberian refugium are mostly genetically distinct from those in southern Central Europe, (2) that colonization of northern Europe is primarily from Central Europe rather than Iberia (Rokas et al., 2001; Stone et al., 2007). However, the most ancient pattern evidence (the 'Out of Anatolia' hypothesis; Rokas et al., 2003; Challis et al., 2007; Stone et al., 2007) has it that the longitudinal range expansion is from the older regions such as Anatolia and Iran as their source of genetic diversity. Because most parts of Turkey were never under the direct effect of glaciers, large number of plant and animal species escaped and migrated into Turkey from the northern latitudes and formed a complex system (Demirsoy, 1996). Thus, these climatic fluctuations of the Pleistocene epoch played an important role not only for the European oak gall wasp populations (Challis et al. 2007) but also for the Turkish oak gall wasp populations through creating structured phylogeographic and phylogenetic pattern (Dinç and Mutun, 2019).

1.5 Molecular Markers: Mitochondrial and Nuclear DNA Markers

With the great advances in 20th Century both in laboratory techniques and computer analysis, molecular methods became one of the most preferred tools to study

geographic distribution of genetic lineages, divergence, and many more contemporary issues. The use of these markers increases as interest in the science and researches increases not because of the lack of character free invariability, rather due to the large number of cumulative information revealed (Grechko, 2001). In addition to other developments in Biology, mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) markers have become powerful molecular tools and are widely employed in systematics and phylogenetics (Boore, 1999) to distinguish common ancestry-homology and homoplasy -convergence, ancestry-analogy, parallelisms, and other evolutionary processes (Grechko, 2001).

Mitochondrial DNA is characterized by small size of about 16-20 Kb in arthropods, closed circular shape with homologous sequences, lacks repair mechanisms, showing variation among populations, low or no recombination, and it is maternally inherited (Harrison, 1989). Majority of the regions are exons (protein coding) except at the control region (Harrison, 1989). The whole genome constitutes 37 genes; 22 tRNA, 13 protein coding regions that coordinate the ATP synthesis and 2 rRNA (Lemire, 2005). One of the protein coding genes is the cytochrome b (cyt b) gene which is a conserved region and provides large amount of information particularly at the species and population level (Kartavsev and Lee, 2006). Each group of taxa and gene show differences in their mutation rate, and gene order may change from Order to Order (Grechko, 2001). In fact, these features made it novel for molecular and evolutionary studies. Tracing recent maternal genealogies as far back as about 2-5 million years ago (MYA), reconstructing population structures, revealing phylogeny and phylogeography are powerful qualities of mtDNA that also make many novel research areas including entomologists to rely on it for the best resolution (Lemire, 2005).

In molecular systematics field, nuclear genome is another widely used molecular marker. It is linear genome with larger size, (Parker et al., 1998), and is self-regulated, high mutation rate, biparentally inherited, quality resolution at any level in the hierarchy. Consequently, entomologists rely on it on their studies due to these features (Avise, 2004). Additionally, its ITS2 region is also known for greater resolution at the population and species level studies (Loxdale and Lushai, 1998). In recent years, ITS2 region of different taxa has been utilized for DNA barcoding (Ji et al., 2003). Often a times, these two vital markers are used in combination for greater resolution, thus for better resolution. In this thesis, both markers were used as a combination across the populations of the sampled area.

1.6 Study Material: *Andricus tomentosus* (Trotter, 1901)

Andricus tomentosus (Trotter, 1901) is a widespread oak gall wasp species recorded from Iran, Syria, Italy (Ionescu, 1973), Ukraine, Hungary, Bulgaria, Greece (Trotter, 1901; Pujade-Villar et al., 2002), and Turkey (Azmaz and Katılmış, 2017). Its host plants include *Quercus petraea*, *Q. pubescens*, *Q. robur*, *Q. frainetto* (Pujade-Villar et al.,2002), and in Turkey and Iran it is commonly found on *Q. infectoria*. The species is grouped in the Hartigi clade (Melika and Bechtold, 2001). Like many oak gall wasps, *Andricus tomentosus* has only the asexual generation known currently and no record of sexual generation yet (Ionescu, 1973). The galls are monolocular with born-hard and brown gall walls, bell-shaped, develops mostly from the axillary buds (Melika, 2003) and its older galls persist on the tree (Rohwer and Fagan, 1917). Asexual *A. tomentosus* female have its body shaped like an amber alongside with some black spots with special brown veins on the wings (Torre and Kieffer, 1910). Adult

female size ranges from 4.0-5.6mm. The larva emerges in the month of March-April (Ionescu, 1973).

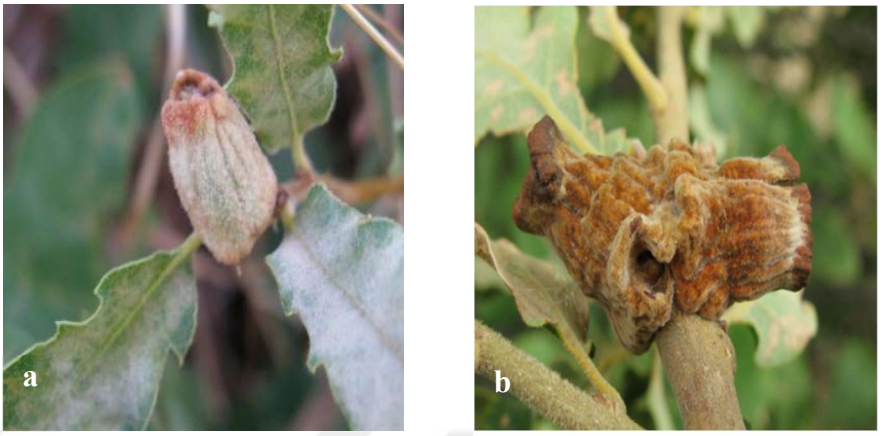

Figure 1.2. Typical *A. tomentosus* galls collected during this study. a) showing locations near leaves, b) showing the location on the stem.

2. AIMS AND SCOPE OF THE THESIS

In this thesis the oak gall wasp species, *Andricus tomentosus* (Trotter, 1901), collected from different localities across its distribution range in Turkey was used to answer the following questions:

1. Other oak gall wasp species studied so far in Turkey revealed high genetic diversity. Does *A. tomentosus* also display high genetic diversity?
2. Other animal and oak gall wasp species with similar geographic distribution showed a structured population genetic structure. Is it also possible that *A. tomentosus* reveal a similar grouping of populations?
3. The Quaternary cycles have played a key role for structuring the contemporary population genetic structure of other oak gall wasps in Turkey. Are there such effects of those factors on *A. tomentosus*?
4. Lastly, if the species reacted to past climatic changes how did its populations reacted to the fluctuations?

3. MATERIALS AND METHODS

3.1 Sampling for *Andricus tomentosus*

A total of 120 asexual generation individuals were collected from 12 locations within the distribution range of the species in Turkey in the summer of 2017. The collected samples were kept in the Molecular Zoology Laboratory, Bolu Abant İzzet Baysal University for rearing the adults. The total number of individuals used from each population, coordinates of the collection sites and locations are shown Figure 3.1 and Table 3.1.

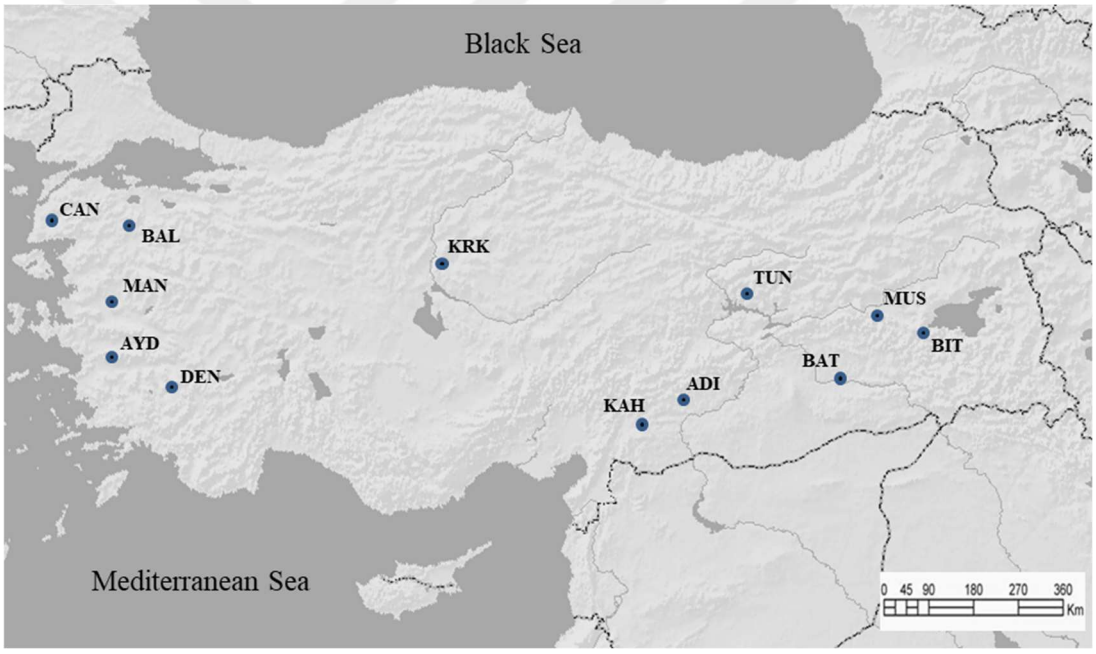

Figure 3.1. Sampling sites of *Andricus tomentosus*.

Table 3.1. Sampled locations for *A. tomentosus*, size of each population and coordinates of each locality.

Location	Abbreviation	Coordinates	N
Adıyaman	ADI	N 37° 77.782′ E 37° 68.476′	10
Aydın	AYD	N 37° 76.535′ E 27° 32.922′	10
Balıkesir	BAL	N 39° 91.898′ E 27° 63.106′	10
Batman	BAT	N 38° 16.050′ E 41° 60.946′	10
Bitlis	BIT	N 38° 43.688′ E 42° 55.733′	10
Çanakkale	CAN	N 40° 37.262′ E 26° 78.025′	10
Denizli	DEN	N 38° 35.487′ E 29° 74.005′	10
Kahramanmaraş	KAH	N 37° 46.763′ E 37° 44.109′	10
Kırıkkale	KRK	N 40° 10.199′ E 33° 74.834′	10
Manisa	MAN	N 38° 17.821′ E 28° 52.471′	10
Muş	MUS	N 38° 63.347′ E 41° 76.446′	10
Tunceli	TUN	N 39° 59.013′ E 39° 86.258′	10
Total	**12**		**120**

3.2 Preservation of the Samples

The galls collected from the field were filled in jars and covered with a tulle at the Molecular Zoology Lab in Bolu Abant İzzet Baysal University. The jars were checked daily to notice the emerging of adults from the galls. Emerged adults were collected, put in the tube where 90% ethanol was added for preservation. At the same time, some of the adults were kept in the temperature of -80°C freezer until the time of DNA extraction.

3.3 DNA Isolation Protocol

Routinely used total genomic DNA isolation method was applied using the following steps:

1. 200 µL of extraction buffer (10 Mm Tris Ph 8.0, 10 Mm NaCL, 2 Mm EDTA and 1% SDS) and additional 800 µL of extraction buffer were used in homogenizing the adult.
2. 20 µL of Proteinase K was added into each homogenate and left to incubate in water bath for overnight at 56°C, 65 RPM.
3. 600 µL of the supernatant was taken from the incubated tubes and mixed with 600 µL of phenol for two consecutive steps.
4. 400/500 µL supernatant from step 3 mixture was transferred into new tubes and 400 µL of chloro-isoamyl-alcohol (24:1) was added to tubes.
5. 3 M, pH 5.2 of sodium acetate was added (33.3 µL) to the sample tubes.

   (Volume of liquid ÷ 2.25) ÷4= volume of sodium acetate

6. 66.7 µL of absolute alcohol was added. The mixture was vortexed and allowed to wait for 1 hour at -70°C, after which it was centrifuged for 15 minutes at 1400-16000 RPM, at 4°C.

   Alcohol + (volume of sodium acetate × 2)

7. The supernatant was discarded and 1000 µL of 70% alcohol was added, centrifuged for 5 minutes.
8. Alcohol was carefully poured out, and the tubes were then taken to the drying chamber (Nüve N 500) at 3°C for about 5 minutes to precipitate the alcohol.

9. 30 µL of ultrapure water was added to dilute the DNA and the sample was kept at -20°C until the following step.

Extracted DNA samples were checked via agarose gel electrophoresis. For this step, 5 µl of extracted DNA was mixed with 3 µl of DNA loading dye, and the sample was run on 1% agarose gel including ethidium bromide (Sigma E5134). In the tank buffer, 1X TBE (0,089 M Tris (Sigma T1503), 0,089 M Boric acid (Sigma B6768), 0,001 M disodium EDTA (Sigma E5134)) was used, and the gel was electrophoresed for 1 hour at 100 volts (V) and 50 mA. 1 Kb λ molecular marker (Sigma D3937) was used as molecular ruler to check the size of the isolated DNA. Ultra-Lum Ultra Cam A650model UV transilluminator system was used for visualization of the DNA samples and subsequently they were photographed with Canon Powershot A650 model camera. An agarose gel photograph of DNA isolated from some *A. tomentosus* samples is shown in Figure 3.2.

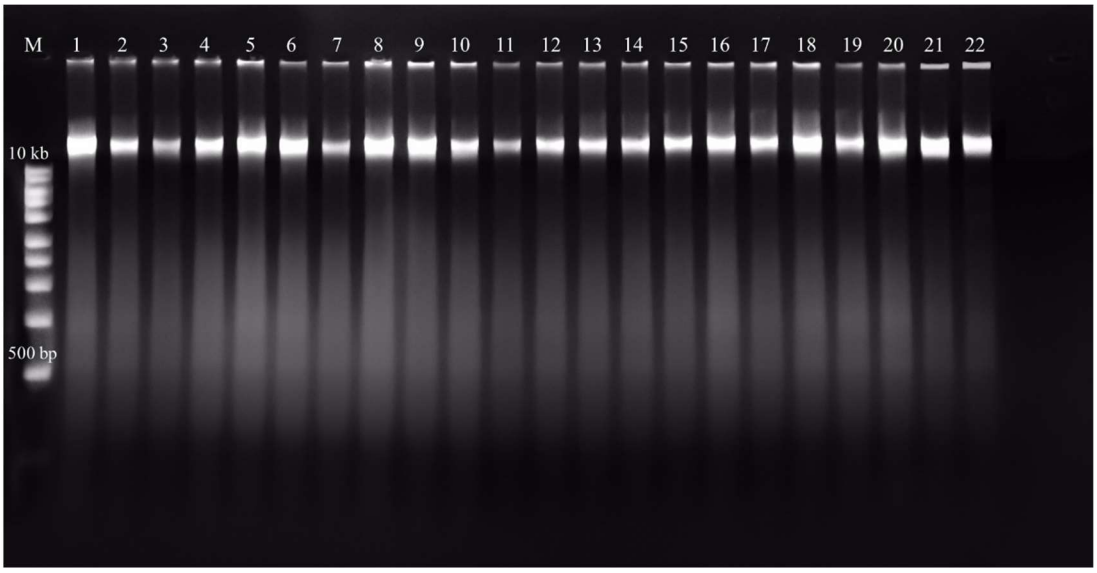

Figure 3.2. Agarose gel photograph of some of the isolated DNA samples from *A. tomentosus*. M: 10 Kb marker, 1-10: Aydın, 11-20: Manisa, 21-22: K.Maraş.

3.4 Amplification of the Mitochondrial DNA Gene

Amplification of a 433-base pair (bp) region of the mitochondrial cyt b gene was accomplished using the primer pair of CB1 – 5'- TATGTACTACCATGAGGA CAAATATC 3' and CB2 5'- ATTACACCTCCTAATTTATTAGGAAT- 3' (Simon et al. 1994, Stone et al. 2007). PCR mixture contained 0.44 µL primer set, 4.40 µL of 10X buffer, 2 µL of $MgCl_2$, 2 µL, 0.7µL dNTPs, 0.35µL Vitaq (Vivantis), 0.35µL of total DNA and enough amount of distilled autoclaved water was added for making the total volume to 35µL. All amplifications were carried out in a Bioer model thermal cycler. Amplification of the cyt b gene region was carried out with the following thermal cycling conditions.

1.	94°C – 4 min.	1 cycle	Initial Denaturation
2.	94°C – 30 sec.	X 35 cycles	Denaturation
	50°C – 1 min.		Annealing
	72°C – 2 min.		Extension
3.	72°C –10 min.	1 cycle	Final Elongation

Amplicons were controlled using 1% agarose gel electrophoresis and a representative gel photograph of the amplified cyt b gene segment was provided in Figure 3.3.

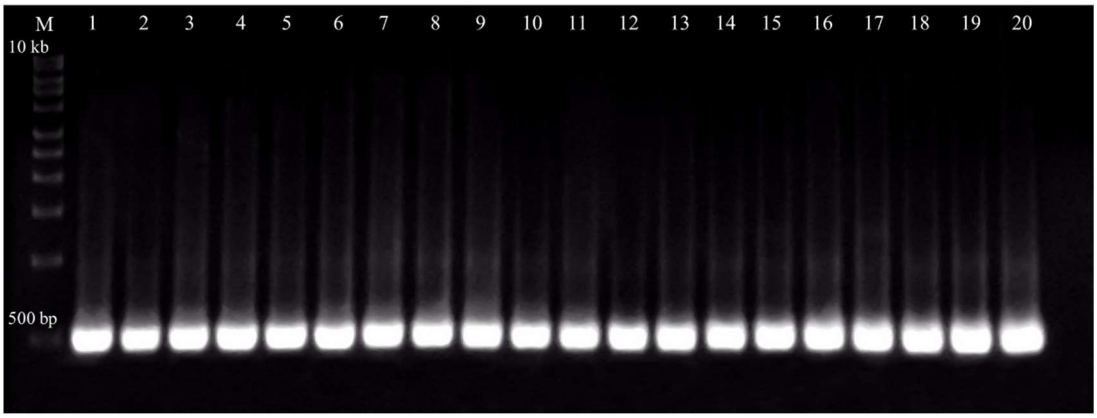

Figure 3.3. Amplified cyt b gene segment of mtDNA samples from *A. tomentosus* individuals. M: 10 Kb marker, 1-10: Aydın, 11-20: Manisa.

3.5 Amplification of Nuclear ITS2 Region

Amplification of the ITS2 region was conducted using the primer set ITS2F 5'-ATGAACATCGACATTTCGAACGCATAT-3' and ITS2R 5'-TTCTTTTCCTCC GCTTAGTAATATGCTTAA-3' (Ji et al., 2003). Each reaction mixture is composed of 10 µl of 5X buffer, 5 µl of $MgCl_2$ (25mM), 3 µl of dNTPs (10mM), 1 µl of each primer (20pmol), 0.5 µl of template DNA and 0.5 µl of Taq polymerase (Vivantis). Finally, necessary amount of autoclaved ddH_2O was added to complete final volume of 35 µl. Amplification conditions in the thermal cycler was as following:

1.	94°C – 3 min.	1 cycle	Initial Denaturation
2.	94°C – 40 sec.		Denaturation
	52°C – 40 sec.	X 28 cycles	Annealing
	72°C – 5 min.		Extension
3.	72°C –5 min.	1 cycle	Final Elongation

Amplicons of the ITS2 region were controlled using 1% agarose gel through also loading a 1 Kb λ DNA molecular marker (Sigma, DO428) to determine the size of the ITS2 region. A photograph of the agarose gel electrophoresis for the ITS2 region is shown in Figure 3.4.

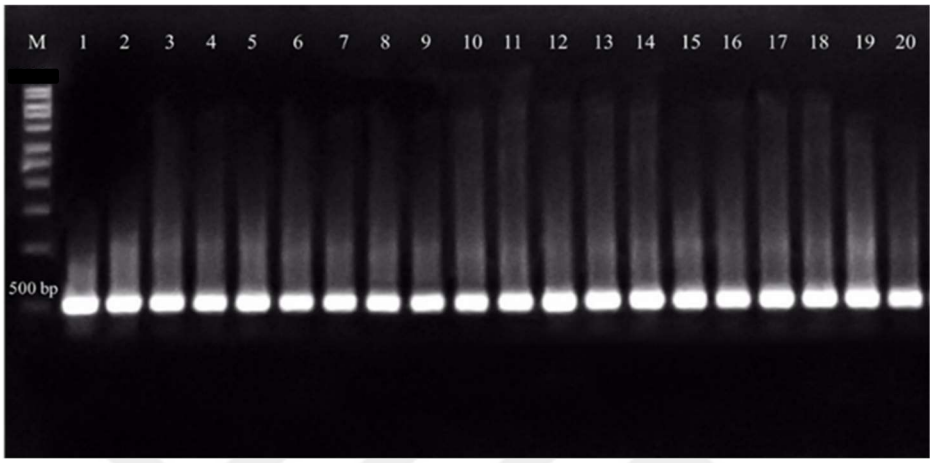

Figure 3.4. ITS2 amplified DNA samples from *A. tomentosus* individuals. M: 10 Kb marker, 1-10: Aydın, 11-20: Manisa.

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3.6 DNA Sequencing

One of the greatest advancements in the molecular studies is probably the invention of conventional sequencers that can be used to sequence even the entire genomes within a minimal period by the sequencing companies. Using the Biosystems 3730XL sequencer, PCR products were sent to a company (MACROGEN, South Korea) to sequence the samples using the standard automated sequencing protocol with the help of fluorescence dye. PCR products were also purified by the company, loaded for sequencing, subsequently the information data are transferred into the computerized system (Sambrook et al., 1989). The outcomes of the sequencing procedures were obtained in the form of chromatograms and used in the following step.

3.7 Data Analysis

3.7.1 Sequence Alignment and Haplotype/Allele Determination

Chromatograms of the strands that were sent by the company were foremost investigated by naked eye to see the existence of any misreading, then with the power of the program package Geneious 10.2 (Kearse et al., 2012). Subsequently, the sequences were aligned and if necessary, they were trimmed on both ends. Using the program DnaSP 5.10.1 (Librado and Rozas, 2009), the unique sequences with the tendency of collapsing into haplotypes (unique mitochondrial DNA sequences) and alleles (unique nuclear ITS2 sequences) were determined and different files were constructed in order for analyzing the data. Furthermore, the determined cyt b haplotypes were assigned to explain the amino acid after the translation using DnaSP 5.10.1 program (Librado and Rozas, 2009). Consequently, presence of any internal stop codons and the non-sense mutations were also searched to ensure that the obtained sequences are original mitochondrial copies, not numpts.

3.7.2 Estimation of Genetic Diversity Indices

In the process of estimation the genetic diversity present in *A. tomentosus*, monomorphic and polymorphic sites (S), informative characters, nucleotide (π), haplotype (h) and allele (A_d) diversity (Nei, 1987), frequency and number of substitutions sites and pairwise nucleotide differences (k) (Tajima, 1983) were separately estimated for the cyt b gene and the nuclear ITS2 region, respectively. Nucleotide frequencies and transition to transversion ratios were obtained using PAUP*4.0 package (Swofford, 2002). Population differentiations were determined

using the Arlequin 3.5.2.2 (Excoffier and Lischer, 2010) and DnaSP 5.10.1 program (Librado and Rozas, 2009).

Haplotype/allele or gene diversity is the probability of two randomly sampled sequences being different whereas nucleotide diversity is the average number of nucleotide differences per site determined after pairwise comparisons of sequences (Nei, 1987). Thus, genetic diversity in the form of haplotype and nucleotide/allelic diversity was determined for each population using the program Arlequin 3.5.2.2 (Excoffier et al., 2010).

3.7.3 Neutrality Tests and Demographic History Analysis

Past events leave their signatures on the gene pool of populations, and those historical events can shape the contemporary population genetic structure and responses of populations to the environmental changes are investigated (Avise, 2000). Those changes can also be observed in mitochondrial genes of populations although they are accepted as neutrally evolving markers, however there may be a possibility to deviate from neutrality (Ramos-Onsins and Rozas, 2002). In fact, any departures from neutrality under non-neutral processes may be caused by either balancing or directional selection and demographic contraction or expansion of populations (Nei, 1987).

Thus, determination of departures from neutrality due to selection or other factors are tested though applying several statistical tests in this thesis. All analyses were run as two sets including population expansion versus constant population size models to detect whether there was any deviation from neutrality and if they were expanding or declining or they are currently at demographic equilibrium. For these

purposes, two different classes of statistics were used: Class I statistics which is based on mutation frequency, and Class II which is based on haplotype/allele distribution-based statistics (Ramos-Onsins and Rozas, 2002).

Furthermore, Tajima's *D* (Tajima, 1989), was calculated for Class I and Fu's F_S was also calculated for Class II in addition to the raggedness index (Hri) and SSD values (Harpending, 1994). Populations produce statistically significant negative results when they expanded in the past or underwent a selective sweep (Ray et al., 2003), while positive values are generated if populations were under the effect of balancing selection or recent population decline (Ramirez-Soriano et al., 2008). In recent years, these programs are used to test population parameters such as deviations from neutrality. However, since analysis such as Tajima's *D* is not always a strong indicator as Fu's *Fs*, they are commonly combined to have trustworthy and stronger results.

Moreover, mismatch distribution analysis based on pairwise differences of DNA sequences were used for analyzing population demographic structure of *A. tomentosus* through applying DnaSP 5.10.1 (Librado and Rozas, 2009). Since mismatch analysis and the number of pairwise differences are based on the population expansion model and the population size, it becomes possible to investigate the mismatch analyses. In mismatch distribution graph, a unimodal profile indicates population expansion whereas multimodal graphs are generated when populations were stable (Rogers and Harpending, 1992).

3.7.4 Analysis of Population Differentiation

Andricus tomentosus populations were compared in a pairwise manner to reveal how they are differentiated from each other through calculating F_{ST} values. Arlequin 3.5.2.2 program (Excoffier and Lischer, 2010) was used for the calculation of F_{ST} values. Their statistical significance were checked at the level of $p \leq 0.05$. In addition, AMOVA (Analysis of Molecular Variance) analysis using the same program was calculated to detect the partitioning of genetic variation at different hierarchical levels as within groups, within group-among populations and within populations. Different grouping schemes were tested to reveal the highest value. Thus, the highest value produced by the specific grouping-scheme was given in this thesis. Statistical significance of AMOVA analysis was tested by 1000 permutations.

3.7.5 Phylogenetic and Phylogeographic Analysis

Three methods as maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) were used to analyze both cyt b and ITS2 data separately for phylogenetic and phylogeographic analysis. In all analyses, *A. caputmedusae* (GenBank Accession number: JQ228863.1), *A. kollari* (GenBank Accession number: DQ925341.1), and *A. curtisii* (GenBank Accession number: KT447045.1) sequences were used as outgroups. A heuristic search using TBR branch-swapping model was applied for the MP analysis (Swofford, 2002). JModeltest 0.1.1 (Posada and Crandall, 1998) was used for searching the best fit evolutionary model to the data set obtained in this thesis. Akaike information criterion (AICc) was used in this analysis, and the result was later used for the reconstruction of ML analysis using PAUP*4.0b10 program (Swofford, 2002).

Divergence times of the main lineages of *A. tomentosus* and the confidence intervals were estimated using the program BEAST version 1.5.2 (Drummond and Rambaut, 2007) through utilizing a Bayesian Markov Chain Monte Carlo (MCMC) coalescent method for cyt b gene. The molecular clock for this insect species was calibrated with the standard protocol in calculating the divergence times using 2.3% sequence divergence/ lineage/ MY (Papadopoulou et al., 2010). To calculate for the dates in BEAST analysis GTR model where G and I are used as a relaxed molecular clock.

Since phylogenetic analyses may usually not guarantee to reveal genealogical patterns and relationships of sequences at the level of populations (Posada and Crandall, 2001), a haplotype network analysis is commonly applied in most researches. For network analysis, Hap Star Version 0.5 (C) (Teacher and Griffiths, 2011) was used to sketch networks of haplotypes and alleles, separately. For this purpose, haplotype/allele data matrices were transferred to Arlequin program to obtain a distance matrix for calculation of pairwise differences between haplotypes/alleles for reconstructing a minimum spanning network. The obtained .svg file was transferred to Inc scape 0.91 (www.incscape.org) program for the visualization of the network.

4. RESULTS

4.1 Diversity indices of *A. tomentosus*

4.1.1 Cytochrome b Gene Diversity

A total of 120 individuals were used in this dissertation across the 12 sampled populations. With regards to mutations, there is no record of either none-sense mutations or stop codons within the haplotypes which confirms how unique the mitochondrial DNA sequences are. The number of polymorphic and monomorphic sites recorded are 55 and 378, respectively. The number of parsimony informative character is 50 (Table 4.1). There are 5 multiple hits recorded in the haplotypes at the site 123, 127, 188, 298, and 376. Based on the variable sites present in the cyt b sequences of *A. tomentosus*, a total of 36 haplotypes (unique mtDNA sequences) were determined among all studied 120 specimens.

In all haplotypes the rate of mutations of transition and transversions ratio is R = 2.024 with k1 = 3.265 for purine and k2 = 7.259 for pyrimidines. Estimation of nucleotides substitution in the maximum likelihood is recorded between C/T = 41.1% and G/A = 14.98% (Table 4.2). The frequencies of nucleotides in the determined haplotypes were 34.10% for A, 42.10% for T, 12.21% for C, and 11.59% for G. The highest substitution occurred at the 3rd codon position (36 substitutions, 65,46%), followed by the first codon positional changes (12 substitutions, 21,82%), and the second codon positional changes (7 substitutions, 12.72 %). Overall, the transition and transversion rate for cytochrome b gene region for *A. tomentosus* is 37, 23.

Table 4.1. Total 36 cyt b haplotypes of the mitochondrial cyt b gene *of A. tomentosus*. Only polymorphic character sites are shown.

Haplotype	49	79	80	85	94	108	109	116	121	122	123	124	126	127	188	189	190	196	202	205	207	208	209	213	214	232	238	241	244	247	248	253	257	262	277	279	286	298	316	323	328	332	337	340	343	351	361	364	376	379	397	406	418
H1	A	A	G	G	T	T	T	T	C	T	C	T	T	A	C	T	G	G	T	T	C	T	C	T	T	A	T	T	A	G	T	T	G	G	C	C	C	A	T	G	C	T	G	T	T	C	A	G	C	T
H2	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	T	.	T	.	.	A	T	.	.	.	.	T	.	.	.
H3	.	.	.	A	A	.	.	.	.	.	.	.	.	.	T	.	A	A	.	C	.	.	.	.	.	C	.	.	.	.	.	.	.	A	.	A	.	.	.	T	T	.	T	.	.	A	T	.	.	.	T	T	A	T	.
H4	.	.	.	A	.	C	.	C	T	.	.	.	C	C	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	G	.	A	T	.	T	.	.	A	T	.	.	.	C	T	T	A	T	.
H5	.	.	.	A	A	.	.	.	.	.	.	.	.	.	T	.	A	A	.	C	.	A	.	.	.	C	.	T	.	.	.	.	A	.	A	.	.	.	T	T	.	T	G	.	A	T	A	.	.	.	T	T	A	T	.
H6	.	.	C	A	.	C	.	C	T	.	G	.	C	C	T	.	A	.	.	.	T	.	C	A	A	.	.	T	C	C	T	C	A	.	A	.	G	.	A	T	T	T	G	G	A	T	.	T	A	C	A	T	A	T	C
H7	.	.	.	A	.	C	.	C	T	.	.	.	C	C	T	.	A	.	.	.	T	.	C	A	A	.	.	T	C	C	T	C	A	.	A	.	G	.	A	T	T	T	G	G	A	T	.	T	A	C	A	T	A	T	C
H8	.	.	.	A	.	C	.	C	T	.	T	.	C	C	T	.	A	.	.	.	T	.	C	A	A	.	.	T	C	C	T	C	A	.	A	.	G	.	A	T	T	T	G	G	A	T	.	T	A	C	A	T	A	T	C
H9	.	.	.	A	.	.	A	.	T	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	T	G	.	A	T	.	.	.	.	.	.	.	.	.
H10	.	.	.	A	.	C	.	C	T	A	.	.	C	.	T	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	T	G	.	A	T	.	.	.	C	.	.	.	.	.
H11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.
H12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
H14	.	.	.	A	.	.	.	.	T	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.	.	A	T	.	T	.	.	A	T	.	T	A	C	T	T	A	T	.
H15	.	.	C	A	.	C	.	C	T	A	T	.	C	C	T	.	A	.	A	.	T	.	C	A	A	.	.	T	C	C	T	C	A	.	A	A	G	C	A	T	T	T	G	G	A	T	.	T	A	C	A	T	A	T	C
H16	.	.	.	A	A	.	.	.	.	.	.	.	.	.	T	.	A	A	.	C	.	.	.	.	.	C	.	T	.	.	.	.	A	.	A	.	.	C	T	T	T	T	G	G	A	T	.	T	A	C	A	T	A	T	.
H17	.	.	.	A	.	C	.	C	T	A	T	.	C	C	T	.	A	.	A	.	T	.	C	A	A	.	.	T	C	C	T	C	A	.	A	A	G	C	A	T	T	T	G	G	A	T	.	T	A	C	A	T	A	T	C
H18	.	.	.	A	.	C	A	C	T	.	.	.	C	C	T	.	A	.	.	.	.	.	.	.	A	.	.	T	C	C	T	C	A	.	A	A	G	C	A	T	T	T	G	G	A	T	.	T	A	C	A	T	A	T	C
H19	.	.	.	A	.	C	.	C	T	.	.	.	C	C	A	.	A	.	.	.	T	.	C	A	A	.	.	T	C	C	T	C	A	.	A	.	G	.	A	T	T	T	G	G	A	T	.	T	A	C	A	T	A	T	C
H20	.	.	.	A	.	C	A	C	T	.	.	.	C	.	T	.	A	.	.	.	T	.	C	A	A	.	.	T	C	C	T	C	A	.	A	.	G	.	A	T	T	T	G	G	A	T	.	T	A	C	A	T	A	T	C
H21	.	.	.	A	.	C	A	C	T	.	T	.	C	C	T	.	A	.	.	.	T	.	C	A	A	.	.	T	C	C	T	C	A	.	A	.	G	.	A	T	T	T	G	.	A	T	.	T	A	C	A	T	A	T	C
H22	.	.	.	A	.	.	.	.	T	.	.	.	C	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	T	G	.	A	T	.	.	.	.	.	.	.	.	.
H23	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H24	.	.	.	A	.	.	.	.	T	.	.	.	.	.	T	.	A	A	.	C	.	A	.	.	.	.	.	T	.	.	.	.	A	.	A	.	.	.	A	T	.	T	G	.	A	T	.	.	.	.	T	T	A	T	.
H25	.	.	C	A	.	.	.	.	T	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	T	C	.	.	.	.	.	A	.	G	.	A	T	T	T	G	G	A	T	.	T	A	C	A	T	A	T	C
H26	.	.	C	A	.	C	.	C	T	A	T	.	C	C	T	.	A	.	.	.	T	.	C	A	A	.	.	T	C	C	T	C	A	.	A	.	G	.	A	T	T	T	G	G	A	T	.	T	A	C	A	T	A	T	C
H27	.	.	C	A	.	.	.	C	T	.	.	.	C	.	T	.	A	.	.	.	T	.	C	A	A	.	.	T	C	C	T	C	A	.	A	.	G	.	A	T	T	T	G	G	A	T	.	T	A	C	A	T	A	T	C
H28	.	.	C	A	.	.	.	C	T	A	.	.	C	.	T	.	A	.	.	.	T	.	C	A	A	.	.	T	C	C	T	C	A	.	A	.	G	.	A	T	T	T	G	G	A	T	.	T	A	C	A	T	A	T	C
H29	.	.	.	A	.	.	.	.	T	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	G	.	A	T	.	T	G	.	A	T	.	.	.	C	T	T	A	T	.
H30	.	.	.	A	.	C	.	C	T	A	.	.	C	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	G	.	A	T	.	T	G	.	A	T	.	T	.	C	T	T	A	T	C
H31	.	.	.	A	.	C	.	C	T	.	.	.	C	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	G	.	A	T	.	T	G	.	A	T	.	.	.	C	T	T	A	T	.
H32	T	.	.	A	.	.	.	.	T	.	.	A	.	T	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	G	.	A	T	.	T	G	.	A	T	.	T	.	C	T	T	A	T	.
H33	.	.	.	A	.	.	.	.	T	.	.	.	C	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	G	.	A	T	T	T	G	.	A	T	.	.	.	C	T	T	A	T	.
H34	.	.	.	A	.	.	.	.	T	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	G	.	A	T	T	T	G	.	A	T	.	.	.	C	T	T	A	T	.
H35	.	.	.	A	.	C	.	C	T	.	.	.	C	C	T	.	A	.	.	.	.	.	.	.	.	.	.	.	C	C	T	C	A	.	A	.	G	.	A	T	T	T	G	.	A	T	.	T	.	C	T	T	A	T	C
H36	.	.	.	A	.	.	.	C	T	.	.	.	C	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	G	.	A	T	T	T	G	.	A	T	.	.	.	C	T	T	A	T	.

Table 4.2. Maximum composite likelihood estimates of the pattern of nucleotide substitutions for the cyt b region of *A. tomentosus*. Rates of different transitional substitutions are shown in bold and those of transversion substitutions are shown in italics.

	A	**T**	**C**	**G**
A	–	*5.66*	*1.64*	**5.09**
T	*4.59*	–	**11.93**	*1.56*
C	*4.59*	**41.1**	–	*1.56*
G	**14.98**	*5.66*	*1.64*	–

since cyt b gene fragment being a protein coding segment, *A. tomentosus* haplotypes were translated into amino acids through using insect mitochondrial amino acid features. The 433 bp fragment of *A. tomentosus* 36 haplotypes, there are 144 amino acids, and out of these amino acids 19 (13.19%) of them show polymorphism (Table 4.3). There is a definite tendency to use some amino acids more than others (Table 4.4). The most frequently used amino acids are Leucine (L) (N= 20) and Isoleucine (I) (N= 16). There are no non-used amino acids in the haplotypes.

Tablo 4.2. Polymorphic amino acid sites of *A. tomentosus* cyt b 36 haplotypes.

	16	**27**	**36**	**39**	**41**	**42**	**63**	**67**	**69**	**71**	**83**	**86**	**93**	**95**	**99**	**108**	**110**	**111**	**120**
H1	L	G	L	F	S	L	L	N	P	G	I	S	F	F	G	P	M	L	A
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H3	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
H4	.	.	P	L	.	S	.	.	.	.	.	T	.	L	.	.	.	.	.
H5	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	V	.	.
H6	.	R	P	L	C	S	.	.	L	E	F	T	.	L	.	S	V	V	S
H7	.	.	P	L	.	S	.	.	L	E	F	T	.	L	.	S	V	V	S
H8	.	.	P	L	F	S	.	.	L	E	F	T	.	L	.	S	V	V	S
H9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.
H10	.	.	P	L	T	S	S	.	.	.	.	.	.	.	.	.	V	.	.
H11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H14	.	.	.	.	.	S	.	.	.	.	.	T	.	.	.	.	.	.	S
H15	.	R	P	L	I	S	.	K	L	E	F	T	Y	L	R	S	V	V	S
H16	.	.	.	.	.	.	.	.	.	.	.	T	.	.	R	S	V	V	S
H17	.	.	P	L	I	S	.	K	L	E	F	T	Y	L	R	S	V	V	S
H18	.	.	P	L	.	S	.	.	.	.	F	T	Y	L	R	S	V	V	S
H19	.	.	P	L	.	S	M	.	L	E	F	T	.	L	.	S	V	V	S
H20	.	.	P	L	.	S	.	.	L	E	F	T	.	L	.	S	V	V	S
H21	.	.	P	L	F	S	.	.	L	E	F	T	.	L	.	S	V	.	S
H22	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	V	.	.
H23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H24	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	V	.	.
H25	.	R	.	.	.	.	.	.	.	.	.	.	.	L	.	S	V	V	S
H26	.	R	P	L	I	S	.	.	L	E	F	T	.	L	.	S	V	V	S
H27	.	R	.	L	.	S	.	.	L	E	F	T	.	L	.	S	V	V	S
H28	.	R	.	L	T	S	.	.	L	E	F	T	.	L	.	S	V	V	S
H29	.	.	.	.	.	.	.	.	.	.	.	T	.	L	.	.	V	.	.
H30	.	.	P	L	T	S	.	.	.	.	.	T	.	L	.	.	V	.	S
H31	.	.	P	L	.	S	.	.	.	.	.	T	.	L	.	.	V	.	.
H32	F	.	.	.	.	F	.	.	.	.	.	T	.	L	.	.	V	.	S
H33	.	.	.	.	.	S	.	.	.	.	.	T	.	L	.	S	V	.	.
H34	.	.	.	.	.	.	.	.	.	.	.	T	.	L	.	S	V	.	.
H35	.	.	P	L	.	S	.	.	.	.	F	T	.	L	.	S	V	.	S
H36	.	.	.	L	.	S	.	.	.	.	.	T	.	L	.	S	V	.	.

Table 4.3. Frequency of amino acids used in *A. tomentosu* cyt b haplotypes.

UUU-F	12 (1.85)	UCU-S	2 (2.29)	UAU-Y	4 (1.33)	UGU-C	1 (1.00)
UUC-F	1 (0.15)	UCC-S	0 (0.00)	UAC-Y	2 (0.67)	UGC-C	1 (1.00)
UUA-L	18 (5.40)	UCA-S	3 (3.43)	UAA-*	0 (0.00)	UGA-W	3 (1.50)
UUG-L	0 (0.00)	UCG-S	0 (0.00)	UAG-*	0 (0.00)	UGG-W	1 (0.50)
CUU-L	1 (0.30)	CCU-P	4 (1.78)	CAU-H	5 (2.00)	CGU-R	0 (0.00)
CUC-L	0 (0.00)	CCC-P	1 (0.44)	CAC-H	0 (0.00)	CGC-R	0 (0.00)
CUA-L	0 (0.00)	CCA-P	4 (1.78)	CAA-Q	2 (2.00)	CGA-R	2 (4.00)
CUG-L	1 (0.30)	CCG-P	0 (0.00)	CAG-Q	0 (0.00)	CGG-R	0 (0.00)
AUU-I	16 (2.00)	ACU-T	5 (2.50)	AAU-N	12 (2.00)	AGU-S	0 (0.00)
AUC-I	0 (0.00)	ACC-T	1 (0.50)	AAC-N	0 (0.00)	AGC-S	0 (0.00)
AUA-M	10 (2.00)	ACA-T	2 (1.00)	AAA-K	2 (2.00)	AGA-S	2 (2.29)
AUG-M	0 (0.00)	ACG-T	0 (0.00)	AAG-K	0 (0.00)	AGG-S	0 (0.00)
GUU-V	2 (1.33)	GCU-A	2 (1.33)	GAU-D	1 (1.00)	GGU-G	0 (0.00)
GUC-V	0 (0.00)	GCC-A	0 (0.00)	GAC-D	1 (1.00)	GGC-G	0 (0.00)
GUA-V	4 (2.67)	GCA-A	3 (2.00)	GAA-E	2 (1.33)	GGA-G	3 (1.33)
GUG-V	0 (0.00)	GCG-A	1 (0.67)	GAG-E	1 (0.67)	GGG-G	6 (2.67)

The frequency of the 36 haplotypes changes from population to population and from haplotype to haplotype. Out of 36 haplotypes there are 9 singleton haplotypes (H16, H18, H19, H20, H23, H25, 30, H32, and H35) (Table 4.5). The number of private haplotypes is also high where there are 16 of them. Only 11 haplotypes are shared between localities. H1-H7, H9- H11 are the shared between different populations. The interesting point is that these common haplotypes are shared between only two populations. The most frequent haplotype (H5) was detected in 13 individuals from Adıyaman and Kahramanmaraş localities. The next frequent haplotype was H24 (N= 10) detected as a private haplotype only in Kırıkkale. The third most common haplotypes found is 6 in each of the two localities (H1 and H29). H1 is shared between Adıyaman and Batman while H29 is found only in Muş as a private haplotype. With regards to haplotype richness per locality Aydın (NHAP = 6, N= 10) is the richest locality followed by Adıyaman, Batman, Çanakkale, Denizli, and Manisa. Each of these localities possessed 5 haplotypes out of 10 individuals sequenced.

Table 4.4. Cyt b haplotypes and their frequencies in *A. tomentosus* populations.

	ADI	AYD	BAL	BAT	BIT	CAN	DEN	KAH	KRK	MAN	MUS	TUN	Total
H1	2			4									6
H2	1			1									2
H3	1			2									3
H4	3			1									4
H5	3							10					13
H6		3								2			5
H7		1				3							4
H8		2				3							5
H9		2					2						4
H10		1					2						3
H11		1					2						3
H12			5										5
H13			5										5
H14				2									2
H15					5								5
H16					1								1
H17					3								3
H18					1								1
H19						1							1
H20						1							1
H21						2							2
H22							3						3
H23							1						1
H24									10				10
H25										1			1
H26										3			3
H27										2			2
H28										2			2
H29											6		6
H30											1		1
H31											2		2
H32											1		1
H33												2	2
H34												5	5
H35												1	1
H36												2	2
N	10	10	10	10	10	10	10	10	10	10	10	10	120
N_{Hap}	5	6	2	5	4	5	5	1	1	5	4	4	

The studied populations were also investigated with regards to their genetic diversity estimates and the results are given in Table 4.6. First, considering the genetic diversity estimates *A. tomentosus* displays relatively high level of diversity where average haplotype diversity is h= 0.647+/- 0.077 and the nucleotide diversity is π= 0.013+/- 0.007. While Kahramanmaraş and Kırıkkale localities did not show any genetic diversity due to lack of variation in the haplotypes Adıyaman was with the highest haplotype diversity (h= 0.888+/- 0.075). The Denizli and Manisa localities are also with high haplotype diversity with h= 0.866+/- 0.071. All the remaining populations also displayed considerably high haplotype diversity. Considering nucleotide diversity Adıyaman was with the highest value (π= 0.040+/- 0.022) followed by Aydın (π= 0.030+/- 0.016) and Batman (π= 0.027+/- 0.015).

Table 4.6. Sample size, haplotype number, haplotype (h) and nucleotide (π) diversities for the cyt b gene and ITS2 region.

Populations	Abb.	Size	CYT B	ITS2	CYT B		ITS2	
			N_{HAP}	N_{ALEL}	h	π	A_d	π
AYDIN	AYD	10	5	2	0.844 +/- 0.079	0.030 +/- 0.016	0.466 +/- 0.131	0.003 +/- 0.001
ADIYAMAN	ADI	10	6	2	0.888 +/- 0.075	0.040 +/- 0.022	0.533 +/- 0.094	0.003 +/- 0.002
BALIKESİR	BAL	10	2	2	0.555 +/- 0.074	0.005 +/- 0.003	0.533 +/- 0.094	0.004 +/- 0.002
BATMAN	BAT	10	5	2	0.822 +/- 0.096	0.027 +/- 0.015	0.467 +/- 0.131	0.020 +/- 0.011
BİTLİS	BIT	10	4	3	0.711 +/- 0.117	0.014 +/- 0.008	0.711 +/- 0.086	0.003 +/- 0.002
ÇANAKKALE	CAN	10	5	2	0.844 +/- 0.079	0.004 +/- 0.002	0.356 +/- 0.159	0.002 +/- 0.001
DENİZLİ	DEN	10	5	2	0.866 +/- 0.071	0.019 +/- 0.011	0.533 +/- 0.094	0.003 +/- 0.002
K.MARAŞ	KAH	10	1	3	0.000 +/- 0.000	0.000 +/- 0.000	0.622 +/- 0.138	0.003 +/- 0.002
KIRIKKALE	KRK	10	1	1	0.000 +/- 0.000	0.000 +/- 0.000	0.000 +/- 0.000	0.000 +/- 0.000
MANİSA	MAN	10	5	2	0.866 +/- 0.071	0.010 +/- 0.006	0.467 +/- 0.131	0.001 +/- 0.001
MUŞ	MUS	10	5	2	0.644 +/- 0.151	0.006 +/- 0.004	0.356 +/- 0.159	0.000 +/- 0.001
TUNCELİ	TUN	10	4	3	0.733 +/- 0.119	0.006 +/- 0.003	0.711 +/- 0.086	0.004 +/- 0.002
Average					0.647 +/- 0.077	0.013 +/- 0.007	0.480 +/- 0.109	0.004 +/- 0.002

All detected haplotypes were compared with each other and the pairwise comparison results are presented in Table 4.7. The results showed that *A. tomentosus* haplotypes indicated sequence differences ranging from 1 bp (0.2%) to 45 bp (10.4%). The highest nucleotide difference is between H23 from Denizli and H15 from Bitlis population. A pairwise comparison was also conducted between localities through calculating F_{ST} values and the results are presented in Table 4.8. According to the results the most differentiated populations are Kahramanmaraş and Kırıkkale with an F_{ST} value of F_{ST} = 0.098 and it is statistically significant ($p \leq 0.05$). Most other populations are also highly differentiated from each other. For instance, Kahramanmaraş population is differentiated from Çanakkale (F_{ST} = 0.096), and Balıkesir (F_{ST} = 0.0945). Balıkesir and Çanakkale populations seem to be differentiated from each other with an F_{ST} = 0.042, however this differentiation is not statistically supported. Overall, it appears that most of the populations are differentiated from each other in a degree, and some of the geographically closer populations are also differentiated however, those differentiations are not statistically significant.

Table 4.7. Pairwise comparison of 36 *A. tomentosus* haplotypes. Net nucleotide differences are shown below diagonal and the percentage of the differences are presented above diagonal.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27	H28	H29	H30	H31	H32	H33	H34	H35	H36
H1		0.021	0.042	0.046	0.051	0.088	0.083	0.085	0.025	0.037	0.009	0.007	0.002	0.042	0.097	0.060	0.095	0.083	0.083	0.083	0.085	0.025	0.007	0.046	0.058	0.090	0.081	0.083	0.042	0.055	0.048	0.051	0.046	0.044	0.067	0.048
H2	9		0.023	0.025	0.032	0.069	0.065	0.067	0.014	0.025	0.025	0.014	0.023	0.021	0.079	0.044	0.076	0.065	0.065	0.065	0.067	0.014	0.028	0.025	0.039	0.072	0.062	0.065	0.021	0.035	0.028	0.030	0.025	0.023	0.046	0.028
H3	18	10		0.030	0.009	0.069	0.065	0.067	0.032	0.044	0.046	0.037	0.039	0.025	0.079	0.021	0.076	0.065	0.067	0.065	0.067	0.032	0.048	0.016	0.044	0.072	0.062	0.065	0.021	0.035	0.028	0.030	0.025	0.023	0.046	0.028
H4	20	11	13		0.039	0.044	0.039	0.042	0.035	0.028	0.046	0.039	0.044	0.014	0.053	0.046	0.051	0.039	0.039	0.044	0.042	0.030	0.053	0.028	0.037	0.046	0.046	0.048	0.014	0.014	0.007	0.021	0.014	0.016	0.021	0.012
H5	22	14	4	17		0.069	0.065	0.067	0.037	0.048	0.055	0.046	0.048	0.035	0.079	0.021	0.076	0.065	0.067	0.065	0.067	0.037	0.058	0.012	0.044	0.072	0.062	0.065	0.025	0.039	0.032	0.035	0.030	0.028	0.051	0.032
H6	38	30	30	19	30		0.005	0.005	0.067	0.060	0.088	0.081	0.085	0.048	0.012	0.053	0.014	0.018	0.007	0.009	0.009	0.062	0.095	0.058	0.030	0.005	0.007	0.009	0.048	0.039	0.042	0.051	0.044	0.046	0.023	0.042
H7	36	28	28	17	28	2		0.002	0.062	0.055	0.083	0.076	0.081	0.044	0.014	0.048	0.012	0.014	0.002	0.005	0.007	0.058	0.090	0.053	0.030	0.007	0.007	0.009	0.044	0.035	0.037	0.046	0.039	0.042	0.018	0.037
H8	37	29	29	18	29	2	1		0.065	0.058	0.085	0.079	0.083	0.046	0.012	0.051	0.009	0.016	0.005	0.007	0.005	0.060	0.092	0.055	0.032	0.005	0.009	0.012	0.046	0.037	0.039	0.048	0.042	0.044	0.021	0.039
H9	11	6	14	15	16	29	27	28		0.016	0.030	0.018	0.028	0.030	0.076	0.046	0.074	0.058	0.065	0.058	0.060	0.005	0.032	0.025	0.037	0.069	0.060	0.062	0.021	0.035	0.028	0.030	0.025	0.023	0.046	0.028
H10	16	11	19	12	21	26	24	25	7		0.037	0.030	0.039	0.032	0.065	0.053	0.062	0.055	0.058	0.055	0.058	0.012	0.044	0.037	0.044	0.058	0.058	0.055	0.028	0.023	0.021	0.037	0.028	0.030	0.039	0.025
H11	4	11	20	20	24	38	36	37	13	16		0.016	0.012	0.042	0.097	0.060	0.095	0.083	0.083	0.083	0.085	0.030	0.007	0.051	0.058	0.090	0.081	0.083	0.042	0.055	0.048	0.051	0.046	0.044	0.067	0.048
H12	3	6	16	17	20	35	33	34	8	13	7		0.009	0.035	0.090	0.055	0.088	0.076	0.076	0.076	0.079	0.018	0.014	0.039	0.051	0.083	0.074	0.076	0.035	0.048	0.042	0.044	0.039	0.037	0.060	0.042
H13	1	10	17	19	21	37	35	36	12	17	5	4		0.039	0.095	0.058	0.092	0.081	0.081	0.081	0.083	0.028	0.009	0.044	0.055	0.088	0.079	0.081	0.039	0.053	0.046	0.048	0.044	0.042	0.065	0.046
H14	18	9	11	6	15	21	19	20	13	14	18	15	17		0.058	0.032	0.055	0.044	0.044	0.044	0.046	0.025	0.048	0.023	0.028	0.051	0.042	0.044	0.014	0.018	0.016	0.018	0.014	0.016	0.030	0.016
H15	42	34	34	23	34	5	6	5	33	28	42	39	41	25		0.058	0.002	0.018	0.016	0.018	0.016	0.072	0.104	0.067	0.039	0.007	0.016	0.014	0.058	0.044	0.051	0.060	0.053	0.055	0.032	0.051
H16	26	19	9	20	9	23	21	22	20	23	26	24	25	14	25		0.055	0.044	0.051	0.048	0.055	0.046	0.067	0.028	0.028	0.055	0.046	0.048	0.032	0.042	0.039	0.037	0.032	0.030	0.048	0.035
H17	41	33	33	22	33	6	5	4	32	27	41	38	40	24	1	24		0.016	0.014	0.016	0.014	0.069	0.102	0.065	0.042	0.009	0.018	0.016	0.055	0.042	0.048	0.058	0.051	0.053	0.030	0.048
H18	36	28	28	17	28	8	6	7	25	24	36	33	35	19	8	19	7		0.016	0.014	0.016	0.058	0.090	0.053	0.030	0.021	0.021	0.023	0.044	0.035	0.037	0.046	0.039	0.042	0.018	0.037
H19	36	28	29	17	29	3	1	2	28	25	36	33	35	19	7	22	6	7		0.007	0.009	0.060	0.090	0.055	0.032	0.009	0.009	0.012	0.046	0.037	0.039	0.048	0.042	0.044	0.021	0.039
H20	36	28	28	19	28	4	2	3	25	24	36	33	35	19	8	21	7	6	3		0.007	0.058	0.090	0.053	0.030	0.012	0.007	0.009	0.044	0.035	0.037	0.048	0.039	0.042	0.023	0.037
H21	37	29	29	18	29	4	3	2	26	25	37	34	36	20	7	24	6	7	4	3		0.060	0.092	0.055	0.037	0.009	0.014	0.016	0.046	0.037	0.039	0.048	0.042	0.044	0.021	0.039
H22	11	6	14	13	16	27	25	26	2	5	13	8	12	11	31	20	30	25	26	25	26		0.032	0.025	0.037	0.065	0.055	0.058	0.021	0.030	0.023	0.030	0.021	0.023	0.042	0.023
H23	3	12	21	23	25	41	39	40	14	19	3	6	4	21	45	29	44	39	39	39	40	14		0.053	0.065	0.097	0.088	0.090	0.048	0.062	0.055	0.058	0.053	0.051	0.074	0.055
H24	20	11	7	12	5	25	23	24	11	16	22	17	19	10	29	12	28	23	24	23	24	11	23		0.032	0.060	0.051	0.053	0.014	0.028	0.021	0.023	0.018	0.016	0.039	0.021
H25	25	17	19	16	19	13	13	14	16	19	25	22	24	12	17	12	18	13	14	13	16	16	28	14		0.032	0.023	0.025	0.023	0.028	0.030	0.028	0.023	0.021	0.030	0.025
H26	39	31	31	20	31	2	3	2	30	25	39	36	38	22	3	24	4	9	4	5	4	28	42	26	14		0.009	0.007	0.051	0.037	0.044	0.053	0.046	0.048	0.025	0.044
H27	35	27	27	20	27	3	3	4	26	25	35	32	34	18	7	20	8	9	4	3	6	24	38	22	10	4		0.002	0.042	0.037	0.039	0.046	0.037	0.039	0.025	0.035
H28	36	28	28	21	28	4	4	5	27	24	36	33	35	19	6	21	7	10	5	4	7	25	39	23	11	3	1		0.044	0.035	0.042	0.048	0.039	0.042	0.028	0.037
H29	18	9	9	6	11	21	19	20	9	12	18	15	17	6	25	14	24	19	20	19	20	9	21	6	10	22	18	19		0.014	0.007	0.009	0.005	0.002	0.025	0.007
H30	24	15	15	6	17	17	15	16	15	10	24	21	23	8	19	18	18	15	16	15	16	13	27	12	12	16	16	15	6		0.007	0.018	0.014	0.016	0.016	0.012
H31	21	12	12	3	14	18	16	17	12	9	21	18	20	7	22	17	21	16	17	16	17	10	24	9	13	19	17	18	3	3		0.016	0.007	0.009	0.018	0.005
H32	22	13	13	9	15	22	20	21	13	16	22	19	21	8	26	16	25	20	21	21	21	13	25	10	12	23	20	21	4	8	7		0.014	0.012	0.028	0.016
H33	20	11	11	6	13	19	17	18	11	12	20	17	19	6	23	14	22	17	18	17	18	9	23	8	10	20	16	17	2	6	3	6		0.002	0.021	0.002
H34	19	10	10	7	12	20	18	19	10	13	19	16	18	7	24	13	23	18	19	18	19	10	22	7	9	21	17	18	1	7	4	5	1		0.023	0.005
H35	29	20	20	9	22	10	8	9	20	17	29	26	28	13	14	21	13	8	9	10	9	18	32	17	13	11	11	12	11	7	8	12	9	10		0.018
H36	21	12	12	5	14	18	16	17	12	11	21	18	20	7	22	15	21	16	17	16	17	10	24	9	11	19	15	16	3	5	2	7	1	2	8	

Table 4.8. Cyt b region F_{ST} analysis results showing pairwise genetic variations between populations. *: p= 0.001.

	Adıyaman	Aydın	Balıkesir	Batman	Bitlis	Çanakkale	Denizli	K. Maraş	Kırıkkale	Manisa	Muş	Tunceli
Adıyaman			*		*	*	*	*	*	*	*	*
Aydın	0.31809			*	*		*	*	*	*	*	*
Balıkesir	0.46373	0.60826			*	*	*	*	*	*	*	*
Batman	0.00511	0.3567	0.28732			*	*	*	*	*	*	*
Bitlis	0.66972	0.29192	0.88401	0.71159			*	*	*	*	*	*
Çanakkale	0.72119	0.23589	0.94203	0.76306	0.45035			*	*	*	*	*
Denizli	0.28118	0.3833	0.45246	0.19489	0.76974	0.82602			*	*	*	*
K. Maraş	0.41495	0.65217	0.9458	0.62137	0.89397	0.96892	0.78462			*	*	*
Kırıkkale	0.39348	0.57282	0.93827	0.5661	0.8771	0.96234	0.72727	1.00000			*	*
Manisa	0.6764	0.19694	0.90327	0.7168	0.34829	0.34752	0.77962	0.92277	0.90613			*
Muş	0.30712	0.39776	0.85859	0.43283	0.79132	0.87885	0.59047	0.89065	0.81871	0.80956		
Tunceli	0.36622	0.37969	0.86977	0.47651	0.77992	0.87074	0.62097	0.90359	0.84755	0.79501	0.25101	

4.1.2 Assessment of the ITS2 Region Genetic Diversity

All *A. tomentosus* samples were used for amplifying the entire nuclear ITS2 region. The amplification produced a 417 bp length for this region after 5.8S and 28S trimming process. Sequences of *A. tomentosus* ITS2 region yielded 11 distinct alleles.

Eleven alleles displayed 392 monomorphic characters, 25 polymorphic characters, 22 parsimony informative characters (Table 4.9). In addition, frequencies of the nucleotides for A, T, C, and G are 29.58%, 33.81%, 15.87%, 20.73%, respectively. Estimation of nucleotides substitution in the maximum likelihood is between C/T = 39.51% and G/A = 9.52% (Table 4.10). K1 = 2.500 (purines), k2 = 9.074 (pyrimidines) were recorded as the transition and transversion ratios respectively. Overall transition/transversion bias is R = 2.561. There are no multiple hits in the alleles.

Considering the frequency of the alleles across the sampled area A1 is the most common allele (N = 62), which is shared among 10 localities (Table 4.11). The second most common allele is the A2 detected in 28 individuals from 6 populations. All other alleles are found in fewer localities with lower number of individuals. None of the alleles are singletons, however 8 alleles are private alleles. Bitlis, Kahramanmaraş and

Tunceli populations possess 3 different alleles meanwhile all other remaining localities are represented by only two separate alleles. Only Kırıkkale population is fixed with a single type of allele (A1).

Table 4.9. Eleven alleles of the ITS2 region of *A. tomentosus*. Only polymorphic character sites were shown.

Allel	27	29	83	88	98	106	108	117	144	157	158	160	174	312	341	356	374	383	384	387	390	391	394	395	396
A1	T	A	C	G	A	A	C	C	A	C	G	C	G	T	C	T	T	A	T	T	C	T	T	T	C
A2	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
A3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	T
A4	C	G	T	.	C	G	T	T	C	T	C	T	A	G	G	C	A	T	.	.	T	.	.	.	.
A5	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	T
A6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.
A7	C	G	T	A	C	G	T	T	C	T	C	T	A	G	G	C	.	.	.	.	T	.	.	C	T
A8	C	G	T	A	C	G	T	T	C	T	C	T	A	G	G	C	.	.	.	C	T	.	C	.	T
A9	C	G	T	A	C	G	T	T	C	T	C	T	A	G	G	C	.	.	.	C	T	.	.	.	T
A10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
A11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.

Table 4.10. Maximum composite likelihood estimates of the pattern of nucleotide substitutions for the ITS2 region of *A. tomentosus*.

	A	**T**	**C**	**G**
A	–	*4.35*	*2.04*	**6.67**
T	*3.81*	–	**18.54**	*2.67*
C	*3.81*	**39.51**	–	*2.67*
G	**9.52**	*4.35*	*2.04*	–

Table 4.11. ITS2 allele frequencies in *A. tomentosus* populations.

	ADI	AYD	BAL	BAT	BIT	CAN	DEN	KAH	KRK	MAN	MUS	TUN	Total
A1	4	7	6		4	8	6		10	7	8	2	62
A2	6	3		7	4		4					4	28
A3			4										4
A4				3									3
A5					2								2
A6						2							2
A7								6					6
A8								2					2
A9								2					2
A10										3		4	7
A11											2		2
N	10	10	10	10	10	10	10	10	10	10	10	10	120
N_{ALEL}	2	2	2	2	3	2	2	3	1	2	2	3	

Genetic diversity estimates also calculated for the ITS2 data set are presented given in Table 4.5. Average allele diversity is A_d = 0.480 +/- 0.109 and the nucleotide diversity is π= 0.004 +/- 0.002. The highest allele diversity is estimated for the Tunceli and Bitlis with similar genetic estimates (A_d = 0.711 +/- 0.086) population followed by Kahramanmaraş (A_d = 0.622 +/- 0.138), Adıyaman and Balıkesir (A_d = 0.533 +/- 0.094 for each). Except Kırıkkale locality other remaining populations displayed moderate level of allelic diversity. Furthermore, when populations studied for their nucleotide diversity both Batman locality showed the highest diversity (π= 0.020 +/- 0.002). The next highest nucleotide diversity was found in Tunceli and Balıkesir populations (π= 0.004 +/- 0.002) while other localities with lower estimates. Similar to allele diversity Kırıkkale population did not show any nucleotide diversity.

Pairwise comparison of alleles indicated that differences between alleles range between 1 bp (0.02%) and 22 bp (5.3%). The highest differentiation was between A8 (from Kahramanmaraş) and A6 (from Çanakkale) (Table 4.12). Alleles A3 (from Balıkesir) and A4 (from Batman) were also differed from each other with 21 bp. Among all comparisons only five of them differed by one bp from each other. A pairwise comparisons were also conducted to compare *A. tomentosus* populations (Table 4.13). The results showed that Kahramanmaraş population is highly differentiated from most other populations. For example, it differed from Adıyaman, Aydın, Balıkesir, Bitlis, Çanakkale, Kırıkkale and Manisa localities (see Table 4.13). Most of the differentiation values for the comparisons were statistically significant.

Table 4.12. Pairwise comparison of 11 *A. tomentosus* alleles. Net nucleotide differences are shown below diagonal and the percentage of the differences are presented above diagonal.

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
A1		2	3	18	3	2	19	20	19	1	1
A2	0.005		3	18	1	4	17	18	17	3	3
A3	0.007	0.007		21	4	3	20	19	20	2	4
A4	0.043	0.043	0.050		17	20	5	6	5	19	17
A5	0.007	0.002	0.010	0.041		5	18	19	18	4	4
A6	0.005	0.010	0.007	0.048	0.012		21	22	21	1	3
A7	0.046	0.041	0.048	0.012	0.043	0.050		3	2	20	18
A8	0.048	0.043	0.046	0.014	0.046	0.053	0.007		1	21	19
A9	0.046	0.041	0.048	0.012	0.043	0.050	0.005	0.002		20	18
A10	0.002	0.007	0.005	0.046	0.010	0.002	0.048	0.050	0.048		2
A11	0.002	0.007	0.010	0.041	0.010	0.007	0.043	0.046	0.043	0.005	

Table 4.13. ITS2 region F_{ST} analysis results showing pairwise genetic variations between populations. + : p= 0.001.

	Adıyaman	Aydın	Balıkesir	Batman	Bitlis	Çanakkale	Denizli	K. Maraş	Kırıkkale	Manisa	Muş	Tunceli
Adıyaman				*				*		*		
Aydın	0.0741		*			*		*	*	*	*	
Balıkesir	0.1880	0.3056		*	*			*			*	
Batman	0.2686	0.2058	0.3280			*	*	*	*	*	*	*
Bitlis	0.0799	-0.0728	0.2872	0.1869		*		*	*	*	*	
Çanakkale	0.1778	0.4444	0.1975	0.3673	0.4074		*	*				*
Denizli	-0.0870	-0.0256	0.2064	0.2415	-0.0036	0.2593		*		*	*	
K. Maraş	0.9367	0.9309	0.9223	0.6389	0.9219	0.9456	0.9324		*	*	*	*
Kırıkkale	0.2222	0.5556	0.3333	0.3824	0.4921	0.1111	0.3333	0.9630				*
Manisa	0.2222	0.4889	0.1799	0.3756	0.4444	-0.0153	0.3030	0.9516	0.2222			
Muş	0.1944	0.4921	0.3016	0.3637	0.4444	0.1111	0.2889	0.9532	0.1111	0.1778		*
Tunceli	0.0404	0.0741	0.0909	0.2470	0.0786	0.1975	0.0196	0.9196	0.3333	0.1799	0.3016	

Both the cy b and the ITS2 data sets were analyzed for revealing the hierarchical distribution of the genetic variation at different levels. Although several grouping schemes have been tested only the trial with the highest values were presented here (Table 4.14a and b). Among all other trials the highest among population variation was obtained when each population is considered as a distinct group, therefore 12 different groups were introduced into the analyzes. Genetic variation revealed by the cyt b data indicated that 68.49% of variation is present at the among population- level, and 31.51% of variation is still kept at the within population-

level. (Table 4.14a). Like the cyt b data, AMOVA analysis revealed that 67.60% of genetic variation is also present at the among population-level, and 32.40% of variation is existed at the within population-level (Table 4.14b).

Table 4.14. AMOVA analysis of a) the cyt b and b) the ITS2 data of *A. tomentosus*.

a)

Source Variation	d.f.	Sum of Squares	Variance components	% of Variation	P-value
Among Pops.	11	743.275	6.45982	68.49	<0.001
Within Pops.	108	321.000	2.97222	31.51	<0.001
Total	119	1064.275	9.43205		
F_{ST}: 0.6848					

b)

Source Variation	d.f.	Sum of Squares	Variance components	% of Variation	P-value
Among Pops.	11	190.875	1.65588	67.60	<0.001
Within Pops.	108	85.700	0.79352	32.40	<0.001
Total	119	276.575	2.44939		
Fixation Indices: F_{ST}: 0.6760					

4.2 Population History of *A. tomentosus*

In analyzing the demography of the populations, different models were used to search for the changes of the population size and the neutrality test have also been conducted on both cyt b and ITS2 data sets separately. Deviations from the neutrality was tested through several analyses and the results are given in Table 4.15. Average Fu's *Fs* for all samples are negative and statistically significant (Fu's *Fs* = -22.6677, $p \leq 0.001$), and the Tajima's *D* value was Tajima's *D* = 1.240, $p \leq 0.09$). The estimations for Harpending's raggedness index (Hri = 0.193) and the SSD value (SSD = 0.080) were supporting Tajima's *D* and Fu's *Fs*. Populations were also tested individually, and the results indicated that none of the estimations for Tajima's *D* and Fu's *Fs* were statistically significant. Despite the absence of statistical support Çanakkale

population produced negative Fu's *Fs*, and Bitlis, Manisa, Muş and Tunceli populations generated negative Tajima's *D* values.

Table 4.15. Fu's F_S, Tajima's *D*, Hri and SSD values calculated for each *A. tomentosus* population for cyt b and ITS2. * P ≤ 0.01, ** P ≤ 0.001.

Population	Fu's F_S		Tajima's *D*		Hri		SSD	
	CYT B	ITS2	CYT B	ITS2	CYT B	ITS2	CYT B	ITS2
Adıyaman	4.661	2.052	1.389	1.032	0.188*	0.720	0.072*	0.175
Aydın	3.851	2.338	0.901	1.641	0.107	0.787	0.069	0.204
Balıkesir	4.324	3.338	2.194	1.831	0.814*	0.786*	0.322*	0.272
Batman	4.262	10.383	1.174	1.495	0.350**	0.720	0.149**	0.290*
Bitlis	3.865	1.194	-1.402	1.227	0.151	0.107	0.082	0.027
Çanakkale	-0.871	1.523	0.023	0.019	0.063	0.668	0.009	0.127
Denizli	3.068	2.338	0.884	1.641	0.190	0.786*	0.078	0.204
K. Maraş	0.000	1.194	0.000	1.227	0.000	0.186	0.000	0.053
Kırıkkale	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Manisa	1.215	0.818	-0.549	0.820	0.072	0.222	0.038	0.016
Muş	1.337	0.417	-0.586	0.015	0.299	0.210	0.118	0.004
Tunceli	1.216	1.453	-1.144	1.831	0.078	0.170	0.028	0.045

The mismatch distribution including all samples are shown in Figure 4.1a. The multimodal profile of all *A. tomentosus* samples indicate a stable population size or no deviation from neutrality. Results of each of the individually analyzed population is also given in Figure 4.2. With regards to the mismatch analysis, it appears that except Çanakkale population all others are much or less stable populations. The existence of unimodal profile for the Çanakkale locality may indicate a population expansion. Meanwhile, all other localities represent demographically stable population structure with multimodal graphs.

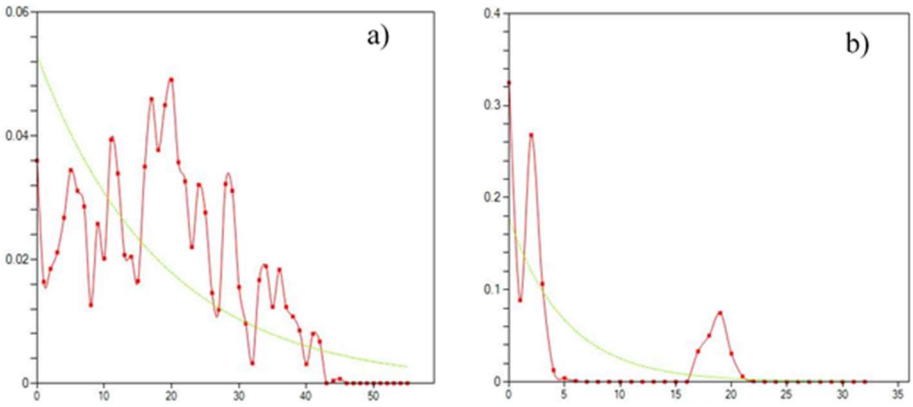

Figure 4.1. Mismatch distribution of all pairwise combinations for a) cyt b haplotypes and b) the ITS alleles of *A. tomentosus*. The observed distribution is represented by red line, and the expected frequencies are depicted by green dotted line.

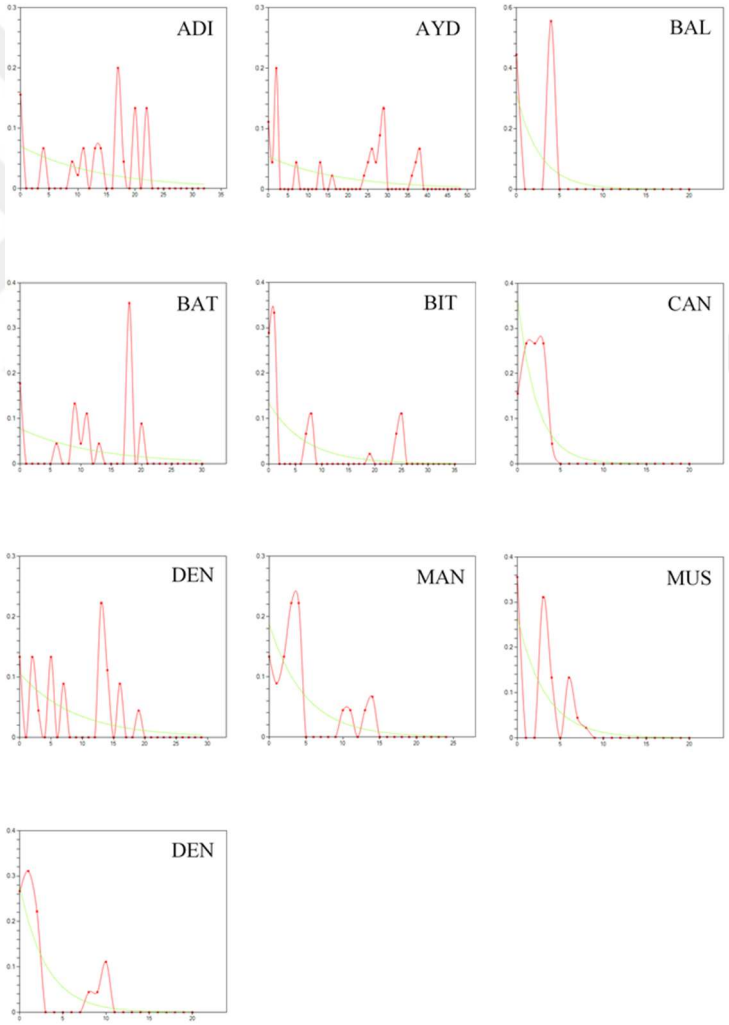

Figure 4.2. Mismatch distribution of populations with their own haplotypes for cyt b data set. The observed distribution represented by red line, and the expected frequencies depicted by green line.

On the other hand, analysis of the ITS2 data produced non-significant positive Tajima's *D* and Fu's *Fs* values together with relatively higher Hri and lower SSD value for all *A. tomentosus* samples. When each population was tested separately all of them produced positive Tajima's *D* (Tajima's *D* = 0.00963, $p \leq 0.56$) and Fu's *Fs* (Fu's *Fs* = 3.00812, $p \leq 0.85$) values, however none of them were statistically supported (Table 4.15). The mismatch distribution analysis for all population also generated a multimodal profile implying a stable population structure for the species (Figure 4.1b). Each population was also analyzed separately, and the results showed that Manisa and Muş populations underwent population expansion however, all other populations were much or less stable over time (Figure 4.3).

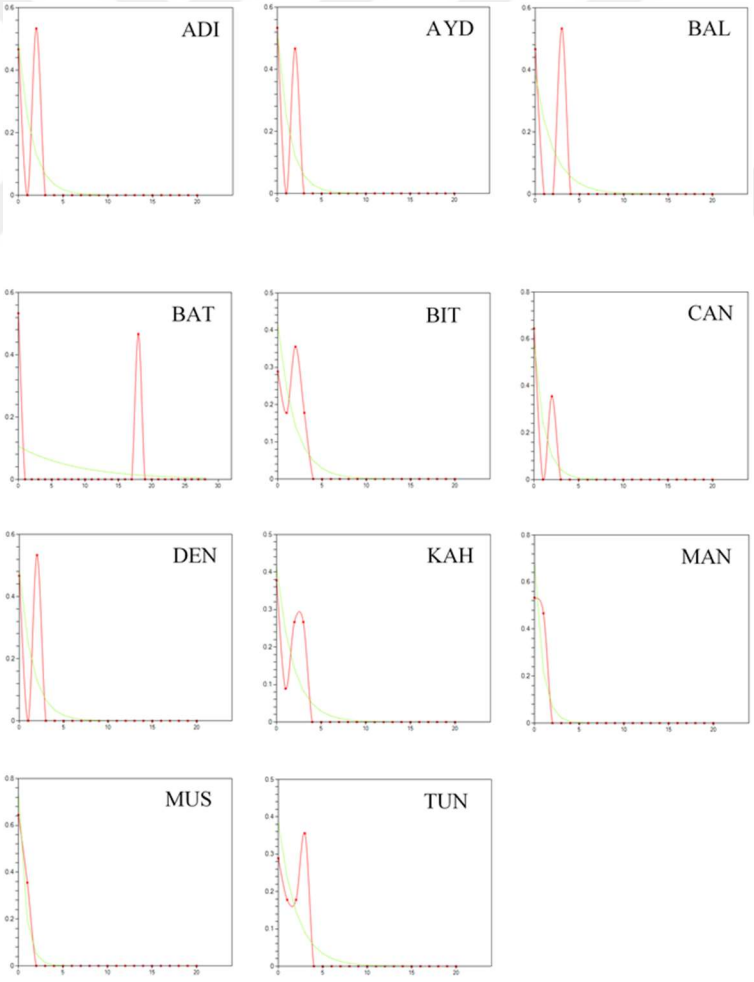

Figure 4.3. Mismatch distribution of populations with their own haplotypes for ITS2 data set. The observed distribution represented by red line, and the expected frequencies depicted by green line.

4.3 Phylogenetic and Phylogeographic Analysis of *A. tomentosus*

Maximum likelihood (ML), Maximum parsimony (MP) and Bayesian inference (BI) were used in revealing the genetic diversity of *A. tomentosus* Turkish populations on both the ITS2 and cyt b gene. In the MP analysis for the cyt b, a 50% resultant majority rule consensus tree of 54 equally likely trees was produced and shown in Fig. 4.4. The obtained values of the consensus tree are CI (consistency index) = 0.549383, R (retention index) = 0.834842, and HI (homoplasy index) = 0.450617, respectively. Statically supported bootstrap value was attained along all the tree branches. In the MP tree, the presence of a polytomic structure at the base of the tree is apparent where H16 (from Bitlis population), H5 (a shared haplotype between Adıyaman and Kahramanmaraş), H3 (another shared haplotype between Adıyaman and Batman) and lastly a large clade are the parts of the polytomy. The large clade is divided into two lineages as H24 from Kırıkkale and the rest of the haplotype group. The clade is further composed of two subclades as the first one is dominated by the haplotypes from the western part of the sampling area. The western haplotypes are the representatives of the populations specifically from Aydın, Denizli, and Balıkesir. In addition to the western localities there are two haplotypes representing the eastern populations (H1 and H2 shared haplotypes between Adıyaman and Batman). The second subclade is more structured where a Muş haplotype (H29) is grouped with a small clade which is formed from three haplotypes. The tree haplotypes are from Muş (H32 as the most basal one), and a monophyletic clade consisted of H14 from Batman and H25 from Manisa. The sister subclade to the small clade is a mixed

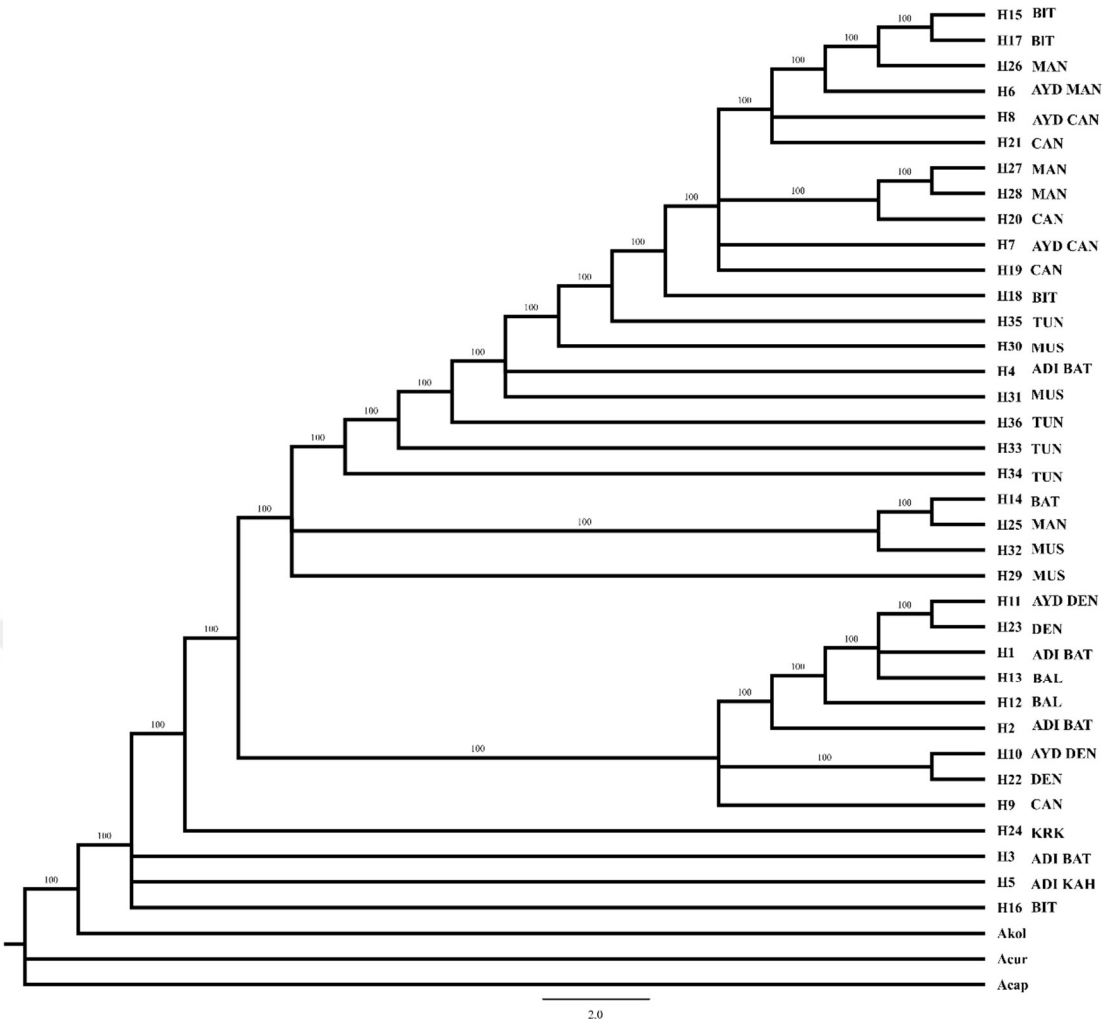

Figure 4.4. Maximum parsimony 50% majority rule consensus tree of the cyt b haplotypes of *A. tomentosus*. The consensus tree is a resulting tree of 54. Supporting bootstrap values are shown over relevant branches.

subclade which is composed of mainly easterly sampled populations in addition to some of the western localities.

Maximum likelihood analysis was run using the resulting evolutionary model suggested by JModeltest program. The program proposed GTR+ I model with the -lnL1478.55970647 for the cyt b data set, and all the parameters were set considering the suggested model. The ML analysis produced 11 equally likely tree. A consensus tree was generated using the trees and the result is shown in Figure 4.5. The CI value

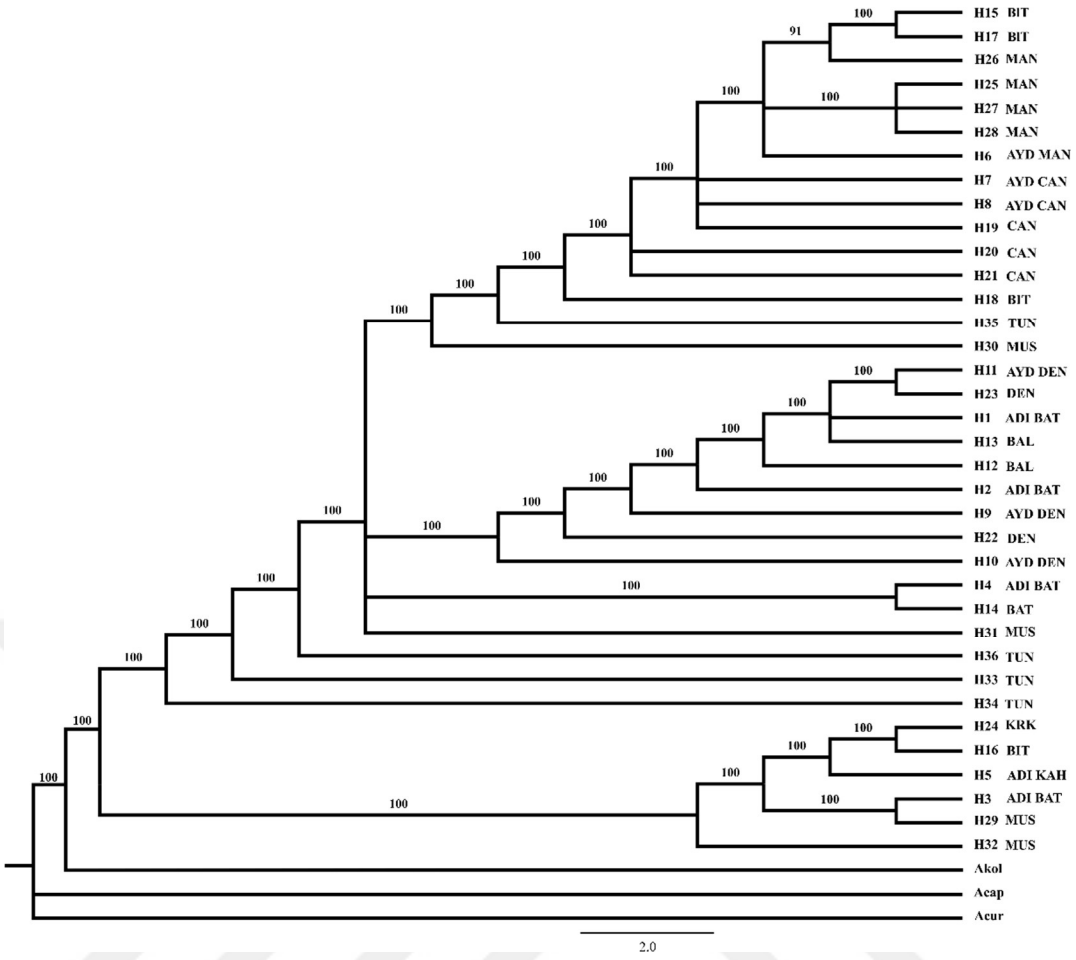

Figure 4.5. Maximum Likelihood 50% majority rule consensus tree of the cyt b haplotypes of *A. tomentosus* (-lnL= 1478.5597). The consensus tree is a resulting tree of 11 subtrees. The bootstrap values over 50% support are shown on relevant branches.

for the consensus tree is CI = 0.533, RI = 0.824, HI = 0.467, respectively. All the branches of the tree are well-supported. Compared the to the MP tree there are some minor differences in the ML tree where the major structuring show similarities. The presence of a two main-clade structure is apparent. The first clade is dominated by the haplotypes sampled from Muş, Adıyaman, Batman, Kahramanmaraş, Bitlis and a haplotype detected only in Kırıkkale locality. The second clade possess a haplotype found in the Tunceli population (H34) is basal to all other haplotypes that formed a sister clade to H34. Another Tunceli haplotype (H33) is also a basal haplotype to the rest. Likewise, H36 from Tunceli is further kept in the basal part of a structurally

polytomic sister clade. In this sister clade H31 is the first haplotype of the polytomy in addition to a monophyletic small clade formed by the haplotypes from Adıyaman and Batman, a clade that is composed of mainly western locality haplotypes, and a larger clade that is consisted of 15 haplotypes both from the western and eastern populations.

Phylogenetic trees may not always resolve all the evolutionary relationships among haplotypes for better visualization and solving probable reticulations particularly at the species level. Therefore, a minimum spanning network analysis was conducted, and it is presented in Figure 4.6. An obvious pattern was generated by the network supporting the phylogenetic tree analyses. Like the phylogenetic trees, haplotypes sampled from the eastern localities are at the junction of three main haplotype-groupings. A Bitlis haplotype (H18), a Tunceli haplotype (H35), a Muş haplotype (H30) connect the three haplogroups. These haplotypes are connected to each other through some hypothetical haplotypes that are supposedly either not sampled by chance or they are extinct. Out of the three-haplotype group, the first one is dominated mainly by the eastern haplotypes (from Tunceli, Muş, Adıyaman-Kahramanmaraş and Bitlis). The second is formed by a large group of haplotypes that represent both eastern and western localities, lastly the third haplogroup is composed of mainly by the haplotypes sampled from the western localities (Çanakkale, Aydın, Manisa).

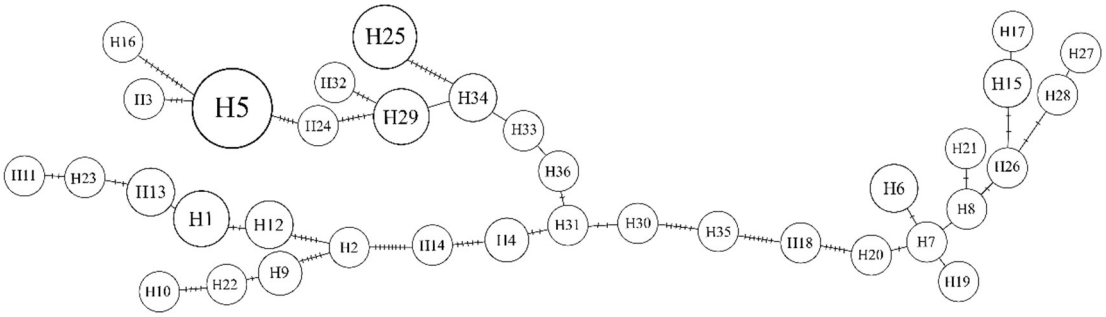

Figure 4.6. Results of the network analysis that was produced using the cyt b haplotypes by Hapstar 0.7. Size of the circles are proportional to the haplotype frequency.

The second data set, the ITS2, was also analyzed through the application of MP, ML and BI analyses. All analyses produced the same tree topology, therefore only a single tree is presented here in Figure 4.7. Bootstrap values and posterior probability for MP/ML and BI trees were highly supporting the allele relationships. In the tree, there are two main clades. The first clade is composed of the alleles only from the eastern localities (from Batman and Kahramanmaraş). The second clade possess A11 sampled from an eastern population (from Muş) in its basal part. The sister clade to A11 lineage forms a polytomic subclade where one lineage, the A1, includes the most common allele. The other alleles in the polytomic part includes a monophyletic clade formed from A2 (from Adıyaman, Aydın, Batman, Bitlis, Denizli and Tunceli) and A5 (from Bitlis), and the second is composed of A10 (from Manisa, Tunceli), A6 (from Çanakkale), and A3 (from Balıkesir).

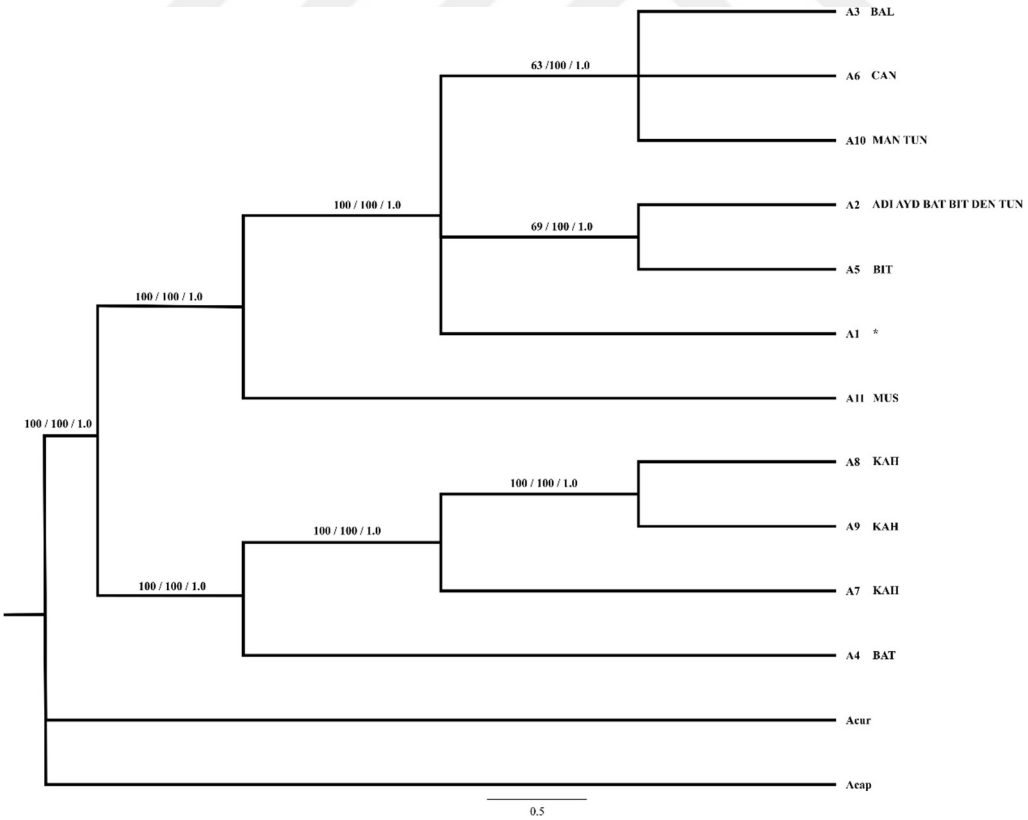

Figure 4.7. The consensus tree of both MP, ML and the BI analysis of the ITS2 region. The values on the branches represent the bootstrap values for MP, ML and branch posterior probabilities of the BI tree, respectively. *: Common allele.

The minimum spanning network resolved the evolutionary relationships among alleles (Figure 4.8). In the network, a clear separation is obvious between the alleles where a group of alleles sampled only from the eastern populations (Kahramanmaraş and Batman) formed a smaller group. The second group is dominated by the alleles mainly from the western localities including the most common allele (A1). The western and the eastern allele groups are connected to each other through many hypothetical alleles, and an allele, the A2. The A2 is a shared allele among mostly eastern localities (Adıyaman, Batman, Bitlis, and Tunceli in addition to two western localities (Aydın and Denizli).

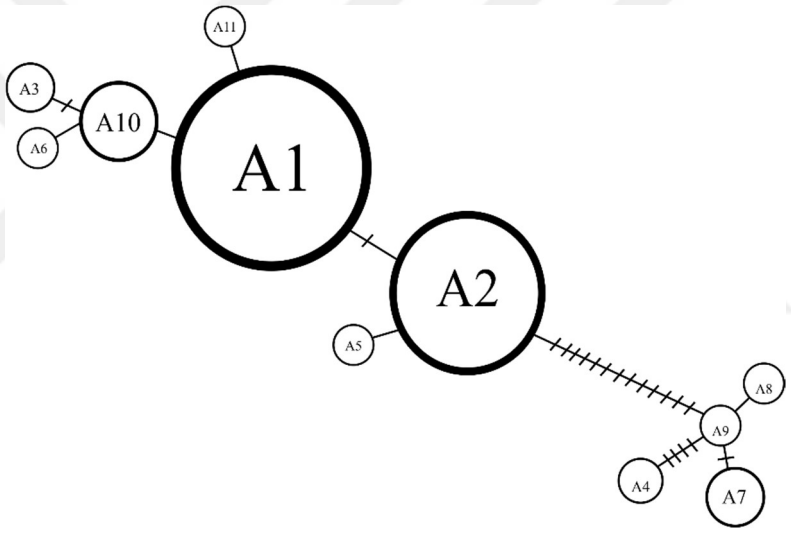

Figure 4.8. Results of the network analysis that was produced using the ITS2 alleles by Hapstar 0.7. Size of the circles are proportional to the haplotype frequency.

4.4 Divergence Times of the *A. tomentosus* Lineages

Bayesian based-BEAST tree is shown in Figure 4.9 with the posterior probabilities and divergence times at the tree branches. The resultant tree is consistent with the MP and ML trees. The result revealed that the ingroup haplotype

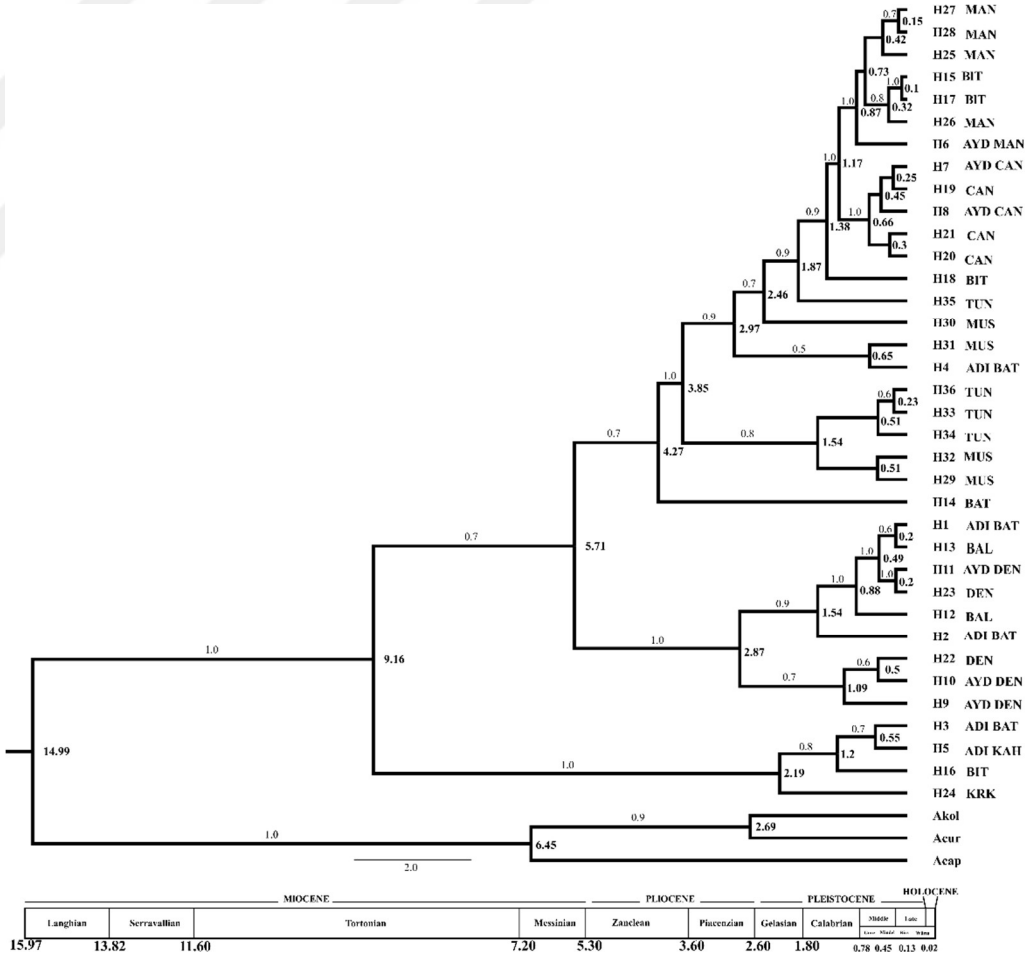

Figure 4.9. Beast consensus tree of cytb haplotypes with the node ages (tMRCA) and posterior probabilities are shown the interior part of each related node. Posterior probability values are also given at nodes.

diverged from the outgroup haplotype in the Miocene era about 15 million years ago (MYA) (95% HPD interval 3.5778- 57.8692).

The *A. tomentosus* haplotypes were divided into two clades about 9.16 MYA between the Tortonian (11.60-7.25 MYA) and the Messinian age (7.20- 5.30 MYA) of the Miocene. The basal clade of the two sister clades composed of Adıyaman, Batman, Kahramanmaraş, Bitlis and a Kırıkkale haplotype. In this small clade the first separation seems to have started 2.16 MYA around the pre-Pleistocene time and subsequent diversification events continued during the Pleistocene between 1.2 MYA and 550.000 years ago (YA). The second main clade of *A. tomentosus* appears to be diverged around 5.71 MYA in the Late Messinian age generating two major subclades. The first of these subclades composed of a mixed group of haplotypes both from eastern and western part of the sampling area. In this subclade further divergences have taken place separating two haplogroups from each other around 2.87 MYA. This diversification results in the formation of a haplotype group including haplotypes from Denizli and Aydın from the second haplotype group with the haplotypes from Adıyaman, Batman, Balıkesir, Denizli, and Aydın.

The second main clade is much more structured than the first clade. The clade has a Batman haplotype at its basal part (H14 from Batman). Application of the molecular clock implied that H14 from Batman diverged from the about 4.77 MYA around the Zanclean age (5.3- 3.6 MYA) of the Pliocene Epoch (5.3- 2.6 MYA). Around the 3.85 MYA in the Zanclean age further diversification also took place separating an eastern-based haplogroup consisting of Muş and Tunceli haplotypes. The other haplogroup with a monophyletic haplotype clade (H31 from Muş and H4 from Adıyaman-Batman) is diverged from rest of the haplotypes around 2.97 MYA (Piacenzian age of the Pliocene). A Muş haplotype (H30) diverged nearly 2.30 MYA,

similarly a Tunceli haplotype (H35) about 1.87 MYA in the Gelasian stage of the Early Pleistocene (2.6 MYA- 10.000 YA). With the start of the Pleistocene intermediate splitting events seem to occur resulting in the shallow diversifications in *A. tomentosus* in Turkey. During these ongoing and relatively more intermediate to current splitting events seem to have greatly affected *A. tomentosus* in Turkey. Around 1.17 MYA a split of two subclades occurred separating Aydın and Çanakkale haplotypes (H7, H19, H8, H21, and H20) from the remaining haplotypes. The second small subclade with the haplotypes from Aydın-Manisa and mainly from Bitlis and Manisa. The ongoing final diversification events seem to have continued through the glacial and interglacial cycles of the Pleistocene and the last split is coincided with the Holocene (ca. 10.000 YA).

5. DISCUSSION

5.1 Genetic Diversity of *A. tomentosus*

Distribution of genetic variation across the range of a species is governed by historical and contemporary factors (Avise, 2000). Establishing the factors that shaped the current distribution of genetic variation through utilizing sequences originated from mitochondrial and nuclear genome is one of the most important part of the research. Specifically, in the last two decades, in systematics and phylogenetics, many researchers used them in order to assign groupings and classify the study populations per the data and this thesis is not an exception. However, there is a need to distinguish the two genomes used in this research. Insect's mitochondrial genome is A+T rich in addition to have a 1) biasness in having more transition than transversion (Brown et al., 1979), 2) it has no stop codons or non-sense mutations in the protein coding genes (Zhang and Hewitt, 1996). These features are useful criteria to distinguish this genome from the nuclear region.

In this study, genetic variation of a 433 bp-fragment of mitochondrial DNA and 417 bp ITS2 region were utilized to test the formulated hypothesis on *A. tomentosus* ranging from revealing its genetic diversity to its phylogeographic structure in Turkey. Hence, it is intended to reveal genetic diversity at the intraspecific and population level and to reconstruct the phylogenetic relationships among the haplotypes and alleles. In this thesis, a total of 120 *A. tomentosus* individuals reared from the asexual gall of the species revealed 36 cyt b haplotypes and 11 ITS2 alleles. Aside the high haplotype number in the cyt b gene, the number of private and singleton haplotype is also very high. Pair wise comparison revealed 9 singletons that have only a single substitution of nucleotide together with 16 private haplotypes in cytochrome

b gene data. The possible explanation to these higher number of both the singleton and private haplotype may be an indication of recent derivation of these sequences. Because it is known that more recently derived sequences are not geographically widespread across the distribution range of a species (Crandall and Templeton, 1993), it is reasonable to assume that the higher number of singleton and private haplotypes imply a recent derivation of these *A. tomentosus* haplotypes in Turkey.

Haplotype diversity is defined as the chance of any two samples that are randomly selected from the population carry different sequences. Differences in the average nucleotides per site among sequences defines their nucleotides diversity (Nei, 1987). In this study, *A. tomentosus* populations indicated a haplotypic diversity of h=0.647 for cyt b gene, and h= 0.480 for the ITS2 region. Comparisons within individual populations, Adıyaman showed the highest diversity with h= 0.888 for the cyt b followed by Denizli and Manisa with h=0.866 for cyt b. The rest of the populations also showed a similar pattern of diversity. It appears that Adıyaman is a very vital geographic location for *A. tomentosus* genetic variation. Similar findings for the Adıyaman locality was also reported for another oak gall wasp species, *A. caputmedusae* (Mutun, 2010). Two populations from the western part of the sampling area, Denizli and Manisa, are with the second greatest haplotypic diversity. It seems that western Turkish populations of this species also bear high diversity in Turkey.

With regards to the allelic diversity of the populations, Tunceli and Bitlis standout to be having the highest genetic diversity. The higher allelic diversity in these areas is also supported by the previous researches conducted on the other oak gall wasp species such as *A. lucidus* (Mutun, 2011), *T. synaspis* (Mutun and Atay, 2015), *A. curtisii* (Mutun, 2016), *A. chodjaii* (Mutun et al. 2019), *C. quercus* (Mutun and Dinç, 2019), and *C. quercusfolii* (Dinç and Mutun, 2019). Furthermore, in the light of the

nucleotide diversity estimates for the ITS2 region, an eastern population, Batman, showed the highest nucleotide diversity. Similar researches conducted on different species of oak gall wasp taxa in the vicinity of Batman also revealed similar nucleotide diversity (Mutun and Atay, 2015; Mutun, 2016). Results of *A. tomentosus* also indicate that there is a higher haplotype diversity and a low nucleotide diversity values based on both cyt b gene and ITS2 regions which are π = 0.013 and 0.004 respectively. Higher haplotype diversity over nucleotide diversity is suggested as an indication of past expansions (Avise, 2000; Zhang et al., 2006). Thus, based on the generalization of the findings for the area, some past events might have taken place there that acted on the genetics of the species of oak gall wasps. However, there will be a need to do more studies on other species to be able to draw such a strong conclusion.

In addition, the genetic diversity estimates of *A. tomentosus* revealed to be higher than many oak galls wasp species found in the continental Europe and either higher or equilibrate the Turkish species. For example, the studies conducted on other *Andricus* species showed a lower diversity than *A. tomentosus*. Palearctic populations of *A. coriarius* showed π= 0.00529 in Iran, π= 0.00644 in Lebanon while π= 0.01554 in Turkey (Challis et al., 2006) which is slightly above the diversity showed by *A. tomentosus*. In the light of its haplotype diversity of *A. tomentosus* compared to the Turkish oak gall wasp species has shown that, there is a great arm race of diversity among them. Some species are higher in the haplotype diversity while lower than *A. tomentosus* in allelic diversity. A study on *A. quercustozae* revealed that genetic diversity was 0.2 in Turkey and 0.12 in Italy (Rokas et al., 2003). Another Turkish species, *A. lucidus*, also showed a very high diversity in terms of both the haplotype and allele 0.808 and 0.115 (Mutun, 2011) while *A. caputmedusae* in Turkey showed 0.101 and 0.463 revealing the balance between it and *A. tomentosus* (Mutun, 2010).

Interestingly, not only the Turkish oak gall wasp species shows this great diversity but equally has been reported in many plants (Ansell et al., 2011), fishes (Hrberk et al., 2004), Anatolian mountain frog (Veith et al., 2003) and grasshoppers (Korkmaz et al., 2002). These results indicated that, *A. tomentosus* having higher genetic diversity in Turkey cannot be coincident considering the genetic diversity of both plant and animal species in Turkey and explained mostly by a very complicated paleogeographic and paleoecological history of Turkey and the vicinity area, the presence of great diversity in habitats even in short geographic distances, its topographic diversity, and its unique placement among three continents (Demirsoy, 1999).

5.2 Population Demographic Analysis of *A. tomentosus*

Factors such as the changes of the population size either expansions or contractions can all be traced on the genomes of the study organism. For instance, habitat or host destruction can cause population bottlenecks by reducing the genetic variation within the population. Likewise, habitat expansion causes the host organisms to expand their distribution range and eventually leads to higher genetic variation over time **(**Ramos and Rozas, 2002). Hence, through DNA sequence analysis, researchers can detect the footprints of the past events on the DNA sequences and consequently can get an idea of the population demography of a species such as the historical in population changes, possible effects of biogeographic and climatic changes over the populations, and nature of habitats of the past. Haplotype differences through unimodal graphs signify the expanding population while demographic equilibrium populations usually produce sharp peaks forming multimodal graph (Rogers and Harpending, 1992). In *A. tomentosus* the cyt b gene average value for Fu's F_S test of all the populations indicated a significant negative value indicating expansion of the

species in the past. Unlike Fu's F_S test, the Tajima's D index was positive however, it was not statistically significant. Therefore, in *A. tomentosus* an overall expansion is suggested since the significant values indicate expanding populations in the sampled area of the species (Kimura, 1983). On the other hand, individual population analysis revealed that Çanakkale Muş, Manisa, Bitlis and Tunceli produced negative Tajima's D and Fu's F_S test values but they were insignificant. It may be possible that inefficient sampling was responsible for their nonsignificant results.

Further demographic analysis regarding pairwise mismatch distribution, all *A. tomentosus* populations showed multimodal graph indicating a stable and structured populations. However, Çanakkale population standout to be producing a unimodal graph indicating an expansion process of this population. With regards to the ragged distribution for lineage spread test, the value is also statistically insignificant. On the other hand, the mismatch distribution graph of all the ITS2 alleles across all populations also produced a multimodal profile supporting the cyt b mismatch results. Hence, both data sets imply ragged but stable population structure for the species in the past in Turkey. Nonetheless, analysis of the ITS2 data on the individual populations indicated that Manisa and Muş populations underwent population expansion. This differences between the results of the cyt b and ITS2 is a normal phenomenon in the populations since organisms can show differences since both data sets provide information on different time zones of the species (Avise, 2000). However, overall results of *A. tomentosus* imply that the species has long standing history in Turkey and it has underwent a series of events which lest their signs on the genome of the species as it was concluded in other oak gall wasp species studied from Turkey (Dinç and Mutun, 2019).

5.3 Divergence of *A. tomentosus* Lineages: Possible Effects Paleoenvironmental and Paleoclimatic Changes

The effects of climate and topology on a species may be inferred from the results of the analysis based on the application of a conventional insect molecular clock on the mtDNA cyt b gene sequences. Consequently, estimations of major diversification events and an understanding of approximate ages of lineage separations may be possible (Kuchta and Meyer, 2001; Loxadale and Lushai, 1998; Paker et al., 1998).

In *A. tomentosus*, the data sets revealed a similar revelation with that of the other species of gall wasps studied in Turkey which is a strong evidence of genetics, lineage distributions and geographical realities in Turkey. In that, it also showed that around the last 10-15 million years were specifically important for the species. The cyt b gene has been revealed that the ingroup haplotype diverged from the outgroup haplotype in the Miocene era about 15 million years ago (MYA). It is known that the Miocene era (23- 5.3 MYA) was the time of great changes during which Turkish landmass and the vicinity were geologically very active due to ongoing orogenesis which resulted in the formation of major mountain zones (Karadenizli, 2011). It seems that the ancestral stock species of *Andricus* that gave rise to *A. tomentosus* was already distributed in the area. Subsequent to the diversification of the first diversification of *A. tomentosus* from the outgroup, its lineage was divided into two main clades around the end of the Tortonian stage (11.6-7.2 MYA) nearly 9.16 MYA. This separation seems to produce the basal clade composed of Adıyaman, Batman, Kahramanmaraş, Bitlis and a Kırıkkale haplotype from its sister clade. The basal clade underwent some further shallower diversification events over time. Similar splitting events dating back to the Miocene was also reported for *C. quercusfolii* where ancestral deep splits were

detected in the Turkish clade (Dinç and Mutun, 2019). Thus, a pattern seems to cover at least some of the oak gall wasp species affected by the ancient events of Miocene.

Further divergences starting around Late Miocene during the Messinian age (7.20- 5.30 MYA) was effective in the second clade where around 5.71 MYA a deep split occurred generating a structured clade form. This split may be explained by the changes of the Messinian aridity crisis which was characterized by the changes between wetter to more arid environmental conditions (Fortelius et al., 2002). Wide-scale studies conducted on a variety of oak gall wasp taxa in the Western Palearctic suggested similar species splits in other oak gall wasp taxa around the same time period (Stone et al., 2009).

It is now well-known that in the Western Palearctic the Quaternary glacial cycles begun with the Pleistocene epoch played a key role to shape the phylogeographic structuring of species (Hewitt, 1996). Located in the southern part of the Western Palearctic, Turkey was never under the direct effect of the massive ice sheets, however recurrent glaciations in the northern area had their indirect impact on the Turkish taxa and left their footprints on the genome. For instance, associated fluctuation of the Quaternary induced speciation in some species with low dispersal ability in Turkey (Demirsoy, 2000) and created fragmentation and within the distribution range of some other species (Çıplak, 2008). In *A. tomentosus*, a well-structured clade formation may well fit with the effect Quaternary fluctuations. Specifically, formation of several clade structures seems to support such effects. In fact, the second clade of *A. tomentosus*, is divided into two subclades. The first subclade consisted of the haplotypes both from western and eastern sampling localities, however the second subclade is much more structured. Molecular clock also revealed that H14 from Batman diverged from the about 4.77 MYA around the

Zanclean age (5.3- 3.6 MYA) of the Pliocene Epoch (5.3- 2.6 MYA). Around 3.85 MYA in the Zanclean age, further diversification also took place separating an eastern-based haplogroup consisting of Muş and Tunceli haplotypes. Although a series of splitting events observed in the first clade that started around early Pleistocene appear to cause some divergences. These splits have resulted in the separation of major haplogroups around 1.54 MYA in the Pleistocene through deriving Muş and Tunceli haplotypes to diverge from each other and form separate small groups. Such splits were also reported from other oak gall wasp species from Turkey (Mutun and Dinç, 2019).

Further diversifications have shaped the *A. tomentosus* lineages in Turkey. A haplogroup with a monophyletic haplotype clade (H31 from Muş and H4 from Adıyaman-Batman) was diverged from rest of the haplotypes around 2.97 MYA (Piacenzian age of the Pliocene). The Muş haplotype (H30) diverged nearly 2.30 MYA, so a Tunceli haplotype (H35) splits around 1.87 MYA in the Gelasian stage of the Early Pleistocene (2.6 MYA). At the beginning of Pleistocene, semi splitting events occurred which resulted in shallow diversifications in *A. tomentosus*. Likewise, around 1.17 MYA, two subclade-splitting occurred to separate Aydın and Çanakkale haplotypes (H7, H19, H8, H21, and H20) from the remaining haplotypes. Last but not the least, a subclade with the haplotypes from Aydın-Manisa and mainly from Bitlis and Manisa were separated from each other. Subsequently, the final diversification events seem to have continued through the glacial and interglacial cycles of the Pleistocene and the last split is coincided with the Holocene (ca. 10.000 YA). Similar diversification events have also been reported for *T. synaspis* (Mutun and Atay, 2015), *C. quercus* (Mutun and Dinç, 2019), and *C. divisa* (Çimen, 2018).

As a consequence, the current findings of this study may pinpoint the importance of the historical factors and how past changes shape grouping of the genetic lineages of species. The findings obtained with this thesis also designate that a general pattern may be drawn for the Turkish oak gall wasp taxa where they reacted almost similarly, even though each taxon respond in its specific way in detail.

6. CONCLUSION

From the analysis of the sequences of mitochondrial DNA and nuclear genome, the following conclusions are drawn:

1. *A. tomentosus* has intermediate to high level of genetic diversity estimates in the sampled area. In fact, its diversity compared to the other oak gall wasp species in Turkey is either at per or higher than some.
2. The higher diversity of haplotypes/alleles and over nucleotide diversity indicated an overall expansion of the species in the past.
3. Results from the ITS2 region and the cyt b gene regarding phylogenetic and phylogeographic analysis revealed structured genetic lineages of the species.
4. The ingroup haplotypes of *A. tomentosus* splits from the outgroup haplotype in the Miocene era about 15 MYA.
5. Reconstruction of the evolutionary relationships of alleles and haplotypes revealed a call-on-call result with that of the network analysis.
6. Finally, compiling the above results clearly indicated that, the genetic lineages of *A. tomentosus* were significantly affected by the past climatic fluctuations and other environmental factors.

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