INVESTIGATING GENETIC VARIATION AND POPULATION STRUCTURE OF *ANDRICUS LIGNICOLUS* (HARTIG, 1840) USING MITOCHONDRIAL DNA

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by
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I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Sciences.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

INVESTIGATING GENETIC VARIATION AND POPULATION STRUCTURE OF *ANDRICUS LIGNICOLUS* (HARTIG, 1840) USING MITOCHONDRIAL DNA

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Oak taxa and gall-forming insect species, *Andricus lignicolus,* are parts of the Anatolian species diversity. Parthenogenetic generation galls of *A. lignicolus* are induced on the oak host as *Q. robur, Q. petraea* and *Q. pubescens.* In this thesis genetic variation, phylogenetic and phylogeographic structure and genetic differentiation among populations of *A. lignicolus* were investigated. For this purpose, mtDNA was isolated from 117 individuals from 15 populations, and a 433-bp region of mtDNA cytochrome b (cyt b) gene was amplified using PCR. After sequencing amplicons 18 specific haplotypes have been detected. Of the 58 characters showing variation, 21 were parsimony uninformative and 37 characters were parsimony informative, and there were several multiple hits. The nucleotide frequencies of *A. lignicolus* haplotypes were 34%, 11%, 9% and 44% for A, C, G and

T, respectively. Among the variable sites, there were 30 transitions and 20 transversions (ti/ tv= 1.5). Average haplotype and nucleotide diversity were 0.32519 and 0.0087955, respectively. The highest haplotype diversity (h= 0.7778) was found in Kütahya population and the highest nucleotide diversity (π=0.042494) was estimated in Uşak population.

Mismatch distribution analyses indicated that the studied *A. lignicolus* populations were in genetic equilibrium. Hierarchical F-statistics (AMOVA) were conducted for detecting hierarchical partitioning of molecular variation into researcher predefined–groups. Among several trials AMOVA analysis indicated highest and statistically the most significant variation being partitioned in each population (71.43% between each locality).

Phylogenetic analyses were conducted through the application of maximum likelihood and maximum parsimony provided consensus trees that were similar in their topologies with two major clades supported with high bootstrap values. Bayesian analysis gave more polytomies in the obtained tree without major basal groupings of the haplotypes. Parsimony network analysis produced four haplogroups, of which the largest one covered half of the *A. lignicolus* haplotypes. Evaluated overall, findings of this thesis shed light into the genetic variation and population structure together with the intraspecific phylogeny of *A. lignicolus* species in Turkey.

Keywords: Cynipidae, oak gall wasp, *Andricus lignicolus*, mitochondrial DNA, intraspecific genetic diversity, phylogeography.

ÖZET

GENETİK VARYASYON VE POPULASYON YAPISININ *ANDRICUS LIGNICOLUS* (HARTİG, 1840)' DA MİTOKONDRİYAL DNA KULLANILARAK ARAŞTIRILMASI

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Meşe taksonları ile gal arısı *Andricus lignicolus* Anadolu tür çeşitliliğinin birer parçasıdır. *Andricus lignicolus* türünün eşeysiz nesil galleri *Q. Robur*, *Q. Petraea* ve *Q. Pubescens* türü meşelerde oluşturulur. Bu tezde *A. lignicolus*'un genetik çeşitliliği, filogenetik ve filocoğrafik yapısı ve populasyonlar arası genetik farklılaşması araştırılmıştır. Bu amaçla, 15 populasyondan 117 bireyin mitokondri DNA'sı izole edilmiş ve mitokondriyal sitokrom b geninin 433 baz çiftlik bölgesi çoğaltılmıştır. Dizilime işleminden sonra 18 haplotip belirlenmiştir. Haplotiplerde varyasyon gösterirken 58 karakterden 21'I bilgi sağlayıcı değildir, ancak 37 karakter filogenetik olarak bilgilendirici bulunmuştur. *Andricus lignicolus* haplotiplerinde, nükleotid frekansları sırasıyla A, C, G, T bazları için %34, %11, %9 ve %44 olarak

bulunmuştur. Substitüsyonlar arasında 30 transisyon ve 20 transversiyon tespit edilmiştir (ti/tv= 1.5). Ortalama haplotip ve nükleotid çeşitliliği 0,32519 ve 0,0087955 olarak hesaplanmıştır. En yüksek haplotip çeşitliliği Kütahya populasyonunda (h= 0,7778) ve en yüksek nükleotid çeşitliliği Uşak populasyonunda (π=0,042494) bulunmuştur.

Uyumsuzluk dağılım analizi ile çalışılan *A. lignicolus* populasyonlarının genetik dengede olduğu gözlenmiştir. AMOVA analizi araştırıcının daha önceden belirlediği gruplara moleküler varyasyonun hiyerarşik olarak dağılımını belirler. Yapılan farklı gruplandırma denemelerinden istatistiksel olarak en anlamlı ve yüksek değerin populasyonlarca paylaşıldığı bulunmuştur (% 71.43 herbir populasyon karşılaştırıldığında).

Filogenetik analizler maksimum olasılık ve maksimum tutumluluk uygulamaları doğrultusunda yürütülmüştür. Elde edilen sonuç ağaçları yüksek bootstrap değerleriyle desteklenen iki temel klad ayrımı gösteren aynı topografiyi vermiştir. Bayesian analizi önemli bazal gruplaşmaların olmadığı ve daha çok politomi içeren ağaçla sonuçlanmıştır. Parsimoni network analizi en büyüğü *A. lignicolus* haplotiplerinin yarısını içeren 4 haplogrup üretmiştir. Genel olarak ele alındığında bu tezde elde edilen veriler Türkiye'deki *A. lignicolus* türünün tür içi filogenisi ile beraber genetik çeşitlilik ve populasyon yapısına da ışık tutmaktadır.

Anahtar Kelimeler: Cynipidae, meşe mazı arısı, *Andricus lignicolus*, mitokondriyal DNA, tür içi genetik çeşitlilik, filocoğrafya.

To my parents for believing in me…

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CHAPTER 1

INTRODUCTION

Turkey as a center of genetic diversity and origin of many European species holds its importance for the world's biota (Rokas et al., 2003a). Due partly to connecting three continents and its unique location between Asia, Europe and Africa, bearing different topographical zones with diverse climatic regions Turkey is thought to be one of the most species rich areas (Demirsoy, 2008; Kekeçoğlu & Soysal, 2010). It is estimated to host around 10.000 plant and 80.000 animal species (Avcı, 1993; Demirsoy, 2008).

High species and genetic diversity in Turkey is further associated with the presence of different topographical characteristics and complicated geologic history of the area (Demirsoy, 2008). Especially during the last 20 million years (MY) there has been more alteration in the region leading to the more current structure of Anatolian land mass. Volcanic eruptions, refraction and folding bound to fault activity, migrations of species and anthropological effects are known to shape contemporary species diversity and genetic structure of Turkish fauna and flora (Demirsoy, 2008). Moreover, periodic glacial and inter glacial periods such as Pleistocene cycles are important factors to have an impact on animal and plants distributed in Turkey (Çıplak, 2008).

In the last glacial maximum, northern parts of the land mass where Anatolia placed was mostly covered by ice sheets. During the glacial periods of Pleistocene, species distributed in the more northern areas migrated to the south where mild climatic conditions were dominating the area (Hewitt, 1999, 2004). While Mediterranean Sea was a strong barrier for terrestrial taxa, Anatolia became a refugium as the most southern point allowing most animal and plant species surviving in Turkey because of its mild climate during the harsh times in the vicinity (Hewitt, 1996, 1999). However during interglacial periods, Anatolia acted as a passageway for many species allowing them to expand their range from east to the west and from the south to the more northern parts (Rokas et al., 2003a). Therefore, it is concluded that Anatolia played significant role as the source population for European species including oak gall wasps (Stone et al., 2002a).

1. 1. Oak Gall Wasps

A variety of organisms such as bacteria, fungi, nematods and insects have the ability to induce galls in different parts of host plants. Of the gall inducing organisms, insects are represented by 13000 species containing aphids (Aphididae, Homoptera), midges (Cecidomyiidae, Diptera), sawflies (Tenthredinidae, Hymenoptera) and cynipids (Cynipidae, Hymenoptera) (Shorthouse et al., 2005). Among all others, the most complex gall structures are formed by gall midges and oak gall wasps (Stone & Schönrogge, 2003). Galls of oak gall wasps from the tribus Cynipini show complicated relationships with other insects particularly from the same family, therefore the microcosmos is examined under three trophic levels: Gall tissue, oak gall wasps - inquilines, parasitoid and predators (Hayward & Stone, 2005; Stone et al., 2002b). Inquilines with 145 species from 7 genera under Synergini

tribus lost their gall-making ability secondarily and used to host other gall inducers' galls as host (Melika, 2006). Some members of Ichneumonidae, Braconidae and Chalcidoidea are known as parasitoid. They cause high mortality of oak gall wasps where parasitoids fed either as ectobionts (externally) or endobionts (internally) (Askew, 1984; Stone et al., 2002b; Csóka et al., 2004).

Oak gall wasps are grouped under the order Hymenoptera which is one of the largest insect orders comprising approximately 300.000 species (Mason & Huber, 1993). From the order Hymenoptera, the superfamily Cynipoidea containing 3000 species (Fergusson, 1995; Ronquist, 1999) is composed of seven families, of which two families are extinct and five are extant. These seven families are examined under macrocynipids and microcynipids. Macrocynipids are larger (4.0 to 20 mm long) than microcynipids (1.0 to 10.00 mm long); Austrocynipidae, Ibaliidae and Liopteridae are macrocynipids and Cynipidae and Figitidae are microcynipids with respect to their size (Ronquist, 1995; 1999).

The microcynipid family Cynipidae comprises 6 tribus as Aylacini (the herb cynipid gall wasp), Diplolepidini (the rose cynipid gall wasp), Eschatocerini (the acacia cynipid gall wasp), Pediaspidini (the sycamore cynipid gall wasp), Synergini (the inquilines) and Cynipini (the oak gall wasp) (Csóka et al., 2004). The most specious and diverse gall forming insects on oaks are gall wasps of Cynipini that induce galls on several plant groups preferably on oaks from the family Fagaceae (Askew, 1984; Pujade-Villar et al., 2001).

Oak gall wasps are known with great diversity in gall shape and size. Each species induces species specific galls on particular organs of certain host plants. Some gall making cynipid species further acquired capability of inducing generation

specific galls on unique host plant parts such as branch, acorn and leaf surface. Although mechanism of gall induction is not known yet in detail, evidence suggests that inducers cause some alteration in plant tissues (Shorthouse & Rohfritsch, 1992). Several hypotheses have been put forward to explain gall formation (Vårdal, 2004), nevertheless the most accepted one stated important role displayed by larva during the whole process of gall development. Larval secretion of Dipteran larva initiates gall formation or female ovipositor secretions composing also symbiotic viruses injected by the ovipositing female may ease gall induction (Cornell, 1983).

Oak gall wasps generally prefer definite plant organ as the galling site (Csóka, 1997; Shorthouse & Rohfritsch, 1992). Independent of the galling location in the host plant, each gall is composed of two major parts; larval chamber and the outer gall part. Gall tissues which are developed outside the larval chamber causes morphologic diversity of cynipid galls. Despite wide range of phenotypic variation observed among different galls such that being surrounded by woody or spongy tissue, complex air spaces within the gall and surface coatings of sticky resins, spines or hairs (Stone & Cook, 1998; Eroğlu, 2000), inner part of galls including the larval chamber is the same in its structure where nutritive plant tissue and sclerenchyma are the characterizing materials of this part of the gall (Bronner, 1992; Hartley, 1997). Galls can be either unilocular or multilocular. Multilocular galls having up to hundred larval chambers are formed to protect developing larvae from parasitoid attacks and decrease the rate of larval death (Stone et al., 2002b).

Many oak gall wasps are known with lifecycle involving two generations where sexual generation galls are formed in spring and early summer however, asexual or parthenogenetic generation galls are formed in late summer/ autumn

(heterogony) (Stone et al., 2009). As oaks being woody plants mostly found as tree or long shrub their wood anatomy, maturation time of fruits, leaf and bark construction, they are classified under three sections as white oaks, black (red) oaks, and evergreen oaks. In Turkey, oaks are represented by 23 taxa with hybrids (Yaltırık, 1982).

Two generations of oak gall wasps shift their oak hosts; asexual forms prefer only white oaks of section *Quercus* sensu stricto such as *Q. infectoria*, *Q. pubescens*, *Q. petraea*, *Q. robur*, *Q. hartwissiana*, *Q. vulcanica*, *Q. pontica*, and sexual forms use either white and black (red) oaks like *Q. cerris var. cerris*, *Q. trojana*, *Q. libani*, *Q. brantii*, but never both (Cook et al., 2002). A single generation mostly asexual generation is known for many oak cynipids (Melika et al., 2000). Therefore it is believed that they lost their sexual generations and have become obligately parthenogenetic (Stone et al., 2002b).

1. 2. Turkish Cynipidae Fauna and the Study Species: *Andricus lignicolus* (Hartig, 1840)

Cynipidae as one of the largest families of the Cynipoidea is represented by 1400 species showing worldwide distribution. Oak gall wasps with nearly 1000 species classified under 40 genera found predominantly in Northern Hemisphere (Ronquist, 1999). Major lineage diversification thought to have occurred in Central America and the greatest species richness is found in the Nearctic region with an estimated number of 700 species under 29 genera. However, the Palearctic region is less species rich with around 160 species under 10 genera (Stone et al., 2002a).

Studies on the Turkish Cynipidae fauna are very limited. Alkan (1952) reported 24 species from five genera, Baş (1973) reported 34 species from five genera and Kıyak et al. (2008) recorded 30 species from 7 genera. Melika and Stone (2001) described a new Cynipidae species from Iran and Turkey, and Melika et al. (2004) gave a new species description from Turkey, Greece and Iran. The checklist of Cynipidae fauna of Turkey was published by Katılmış and Kıyak (2008) stated the presence of 81 species in Turkey. Kemal and Koçak (2010) studied Cynipidae fauna of Van and the vicinity, and a recent study by Mutun and Dinç (2011) added a new Cynipid species to the Turkish Cynipid fauna increasing the number to 90. A new species from Turkey was recently described by Dinç et al. (in press) and the exact number of Cynipid species has yet to be discovered in Turkey and the actual Cynipid species richness might be much higher than these numbers.

One of the Turkish cynipid species is *Andricus lignicolus* (Hymenoptera, Cynipidae) which has two generations per year; sexual spring generation and asexual autumn generation. Sexual form is very similar with *Andricus burgundus* and *A. kollari* (Melika, 2006). It is placed in *Kollari* clade (Stone et al., 2008). Asexual forms are seen on white oaks especially *Quercus petraea, Q. robur, Q. pubescens.* Sexual forms are only known from Turkish oaks, *Q. cerris* and they are spread to Europe through *Q. cerris* (Walker et al., 2002). Asexual galls of *A. lignicolus* complete their development in autumn (Askew & Neill, 1993). Unilocular galls of *A. lignicolus* are spherical in their shape with nearly 8 mm in diameter. Four or five galls can often be found in the same shoot. Galls are dark greenish in the beginning and they become brown later during their development and they are rough and have cracks. Larval chamber is located near to gall's basal part (Askew & Neill, 1993).

Andricus lignicolus shows distribution throughout southern Europe from north-eastern Spain to the Balkans and Turkey (Melika, 2006).

1. 3. Mitochondrial DNA in Phylogeographic and Phylogenetic Studies

In the last several decades with the fascinating advances in the application of novel molecular methods made these new approaches powerful tools for estimating genetic variation and revealing evolutionary relationships at both interspecific and intraspecific levels (Chapco, 1997). Molecular tools have often been employed to expose population genetic structure within a species range, phylogenetic and phylogeographic structure of taxa, gene flow between and among populations, hybridization events, to check the presence of refuge areas, and population demographic changes of organisms (Arias et al., 2006; Loxdale & Lushai, 1998; Gündüz et al., 2005; Kyriazi et al., 2008; Von Dohlen et al., 2002).

Mitochondrial DNA (mtDNA) is a matrilineally inherited circular genome showing no recombination and there is no known repair mechanism. It has reduced effective population size compared to that of the nuclear genome, and universal primers are readily used in variety of organisms. Due to these features mtDNA is subject to high substitution rate and thus it is preferred for population level studies in animals (Harrison, 1989; Hoy, 1994). Mitochondrial genome is around 16 Kilobases (Kb) in its size in arthropods. It is composed of a control region in varying size in different groups of insects which is the reason of size differences among insect species. The control region is also known as A+T rich region in insects due to the higher percentage of adenine and thymine nucleotides. The whole genome is composed of 37 genes, of which 22 tRNA, 2 rRNA and 13 protein coding genes are observed (Lemire, 2005; Sun et al., 2009).

Mitochondrial DNA has been used in diverse studies including phylogeography which is comparatively a novel field. As a new-fangled area, phylogeography refers to the field of study that seeks patterns of geographical distribution of genealogical lineages through mtDNA haplotypes (Avise et al., 1987). Large bodies of literature in the field of phylogeography in different animal taxa have been accumulated in the last two decades (Avise, 1998; Bermingham & Moritz, 1998; Hewitt, 2004; Hickerson et al., 2010) proving it as a powerful tool in diverse groups of organisms including oak gall wasps (Cook et al., 2002; Kyriazi et al., 2008; Rokas et al., 2003b; Rubinoff & Sperling, 2004; Stone et al., 2002a).

Within the phylogeographic framework of very diverse insect taxa, mitochondrial cytochrome b gene has been utilized for estimating genetic variability of sampled populations, population genetic structure and genetic differentiation among populations (Cook et al., 2002). It has also been clearly shown that multiple haplotypes of the cyt b gene have been ordered phylogenetically with respect to geographic location of oak gall wasp species (Rokas et al., 2003b).

1. 4. Aim of the Thesis

The gall wasp species, *Andricus lignicolus*, from Turkey was chosen as the study material of this thesis. The main objectives of this study are

- to investigate intraspecific genetic variation of the species showing distribution in Turkey,

- to examine phylogenetic and phylogeographic structure of the species,

- to reveal genetic differentiation among populations of the species,

- to evaluate findings of the thesis within the phylogenetic and phylogeographic framework and to compare the results with those of other oak gall wasp species.

CHAPTER 2

MATERIALS AND METHODS

2. 1. Sample Collection

A total of 117 individuals from the distribution range of *Andricus lignicolus* in Turkey representing 15 populations have been collected during the fall of 2010-2012 (Figure 1). Collected galls were brought to the Molecular Zoology Laboratory, Abant Izzet Baysal University, Bolu. For rearing adults, galls were put in jars covered by tulle and kept at room temperature and checked daily for the emergence of adults (Figures 2 and 3). Hatched adults were kept at -80 °C until DNA isolation. The number of individuals used from each population, coordinates of the localities and the sampled populations are shown in Table 1.

2. 2. DNA Isolation

Total DNA was isolated via standard DNA isolation method using the following steps:

1. Adult gall wasp was homogenized with 200 µl of extraction buffer (10 mM Tris pH 8.0, 2 mM EDTA pH 8.0, 10 mM NaCl and 1% SDS) and 800 µl of extraction buffer was added to this homogenate.

2. 20 µl of proteinase K was added to each homogenate and this mixture was put into water bath at 55 °C and 65 rpm for overnight.

3. Supernatant was taken from tubes and the same amount of phenol was added to the sample. They were mixed well through vortexing. After taking supernatant, this step was repeated twice.

4. Chloroform-isoamyl alcohol (24:1) was added to the sample and supernatant was taken.
5. After the adjustment of the amount of solution, sodium acetate (3 M, pH 5.2) was added.
6. Absolute ethanol was added to the solution, vortexed and incubated at -70°C for 1 hour. It was centrifuged at 4°C, 16000 RPM for 15 minutes.
7. After washing the pellet with 1 ml of 70% ethanol, it was solved with appropriate amount of ddH_2O.

For ensuring DNA isolation, samples were run in 1% agarose gel using 1X TBE running buffer with a proper molecular weight marker (Figure 4). The running conditions of the agarose gel were at 100 V and 50 mA for an hour.

2. 3. Mitochondrial DNA Gene Amplification

After DNA isolation, 433 base pair (bp) of mitochondrial cytochrome b gene was amplified with CB1- 5'- TATGTACTACCATGAGGACAAATATC- 3' and CB2- 5'- ATTACACCTCCTAATTTATTAGGAAT- 3' primers (Challis et al., 2007; Simon et al., 1994; Stone et al., 2007). Cytochrome b (cyt b) gene has been particularly useful for analyzing the historical basis of intraspecific population genetic structure (Jermiin & Crozier, 1994). Because cyt b gene (1) is the best known mitochondrially encoded polypeptide chain with respect to its structure and function, (2) it is relatively large protein-coding gene, (3) insect specific oligonucleotide primers

encompassing the gene are well-known, (4) it is the most frequently and extensively used and sequenced gene for different and closely related gall wasp species (Jermiin & Crozier, 1994; Acs et al., 2010). To amplify the required cyt b gene fragment, each PCR reaction contained 0.77 µl of dNTP, 0, 54 µl of CB1 and CB2 primers (each of them 20mM), 1.54 µl of DNA, 3.84 µl of 10X reaction buffer, 3.07 µl of $MgCl_2$ and 0.5 µl of Taq DNA polymerase enzyme. Proper amount of double distilled sterile H_2O was used to complete the volume of samples to 40 µl. Amplification was performed under the following conditions: 3 min at 94°C, 35 cycles of 30 sec at 94 °C, 1 min at 50 °C, 2 min at 72 °C, and final extension of 10 min at 72°C. PCR products were visualized on 1 % agarose gel buffered with Tris-Boric acid-EDTA (TBE) with 1 Kb DNA ladder. The gel was stained with ethidium bromide, and checked under UV light and photographed (Figure 5).

2. 4. Sequencing of the Cytochrome b Gene

Mitochondrial DNA sequencing emerged as a method with the finest resolution for population level studies in animals including insects (Arias et al., 2006; Loxdale & Lushai, 1998). A usual automated sequencing protocol of the amplified gene segment was employed using fluorescent markers in the form of dideoxynucleotides. As a general procedure, following cycle sequencing, products are purified, and samples are loaded for sequencing and information is transferred into computer under optic sensor laser light (Sambrook et al., 1989).

In this study, PCR products were prepared for sequencing and send to a company. Samples were sequenced by Applied Biosystems 3730XL model machine. A representative chromatogram of *A. lignicolus* is given in Figure 6.

2. 5. Data Analysis

2. 5. 1. Sequence Alignment, Genetic Variation and Population Structure

Chromatograms were controlled by eye, after trimming the sequences to the proper length; sequences were transferred to BioEdit 6.2. program for comparison purposes and for the determination of unique DNA sequences (haplotypes) (Hall, 1999). After editing and aligning the sequences nucleotide and amino acid composition, nucleotide diversity indices and substitution patterns were calculated using DnaSP v.5 (Librado & Rozas, 2009) and Mega 5.0 computer packages (Tamura et al., 2007). Transitions and transversion ratios were estimated by MEGA program. Population genetic statistics, the number of polymorphic sites (S), nucleotide (π) and haplotype (h) diversity, the number of pairwise nucleotide differences (k), population differentiations in the form of F_{st}, population demographic factors were determined using the computer programs such as Arlequin (Excoffier et al., 1992) and DnaSP 4.0 (Librado & Rozas, 2009).

2. 5. 2. Phylogenetic and Phylogeographic Analysis

Multiple methods to infer phylogenetic relationships among *A. lignicolus* haplotypes have been employed: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). MP and ML analyses were performed in PAUP*4.0b10 (Swofford, 2002). Bootstrap support for the MP trees was calculated using the 10,000 replicates. For ML analyses a model of evolution was determined through the Akaike information criterion (AIC) implemented in JModeltest 3.7 (Posada & Crandal, 1998). ML analysis was performed using the PAUP software with a heuristic search and bootstrap support was calculated using 100 replicates (heuristic search, stepwise addition, 10 random-sequence addition replicates, TBR

branch swapping). BI was performed to employ a metropolis coupled, Markov chain Monte Carlo (MCMC) sampling approach using the software MrBayes 3.1 (Huelsenbeck & Ronquist, 2001). In all phylogenetic analyses, *Andricus kollari* and *A. caliciformis*, two outgroup species from the Kollari clade, have been used to root the phylogenetic trees to give a direction to the evolutionary changes in the ingroup species. The genealogical relationships among haplotypes by means of a statistical parsimony network were also analyzed (Templeton, 2008). Haplotype networks are often better suited than phylogenetic trees to depict relationships within species as gene flow may lead to reticulate, rather than hierarchical, or treelike structure between populations. Moreover, in networks internal nodes have clear biological meaning as persistent ancestral haplotypes (Posada and Crandal, 2001). In the case of mitochondrial DNA gene sequences, reticulations may imply the effects of conflicting phylogenetic signal. Therefore, a network analysis was conducted to further check the evolutionary relationships of the obtained haplotypes through TCS computer program to reveal any reticulation in the data set (Clement et al., 2000).

An analysis of molecular variance (AMOVA) was performed based on the Euclidian distances between haplotypes using Arlequin (Excoffier et al., 1992). The total genetic variation was partitioned among the groups of populations based on their geographical locations and the second test was entirely based on the clustering of the populations obtained through the haplotype network analysis results. The significance of the variance components was calculated using 1000 random permutation of the data set.

Figure 1. Sampled populations of *A. lignicolus*. Locality abbreviations are shown in Table 1.

Table 1. The sampled populations of *Andricus lignicolus* with their abbreviations, coordinates and population sizes.

Populations	Abbreviation	Coordinates	Sample Size
Düzce	DUZ	N 40° 541.472’ E 31° 103.794’	8
Denizli	DEN	N 38° 3406399’ E 29° 7409433’	7
Çanakkale	CAN	N 40° 24.005’ E 26° 56.024’	10
Manisa	MAN	N 39° 16414’ E 28° 63154’	4
İstanbul	IST	N 41° 09.402’ E 29° 75.722’	4
Balıkesir	BAL	N 39° 55.715’ E 27° 19.989’	3
Eskişehir	ESK	N 39° 353.67’ E 30° 660.88’	9
Afyon	AFY	N 39° 058.83’ E 30° 545.17’	6
Antalya	ANT	N 36° 413.42’ E 29° 692.70’	7
Konya	KON	N 37° 455.63’ E 31° 541.86’	10
Kayseri	KAY	N 38° 370.63’ E 36° 231.43’	9
Kırıkkale	KIR	N 40° 101.99’ E 33° 718.34’	10
Kahramanmaraş	KAH	N 38° 179.73’ E 36° 779.15’	10
Uşak	USK	N 38° 733.75’ E 29° 217.66’	10
Kütahya	KUT	N 38° 957.59’ E 29° 329.71’	10

CHAPTER 3

RESULTS

3. 1. Genetic Variation in *Andricus lignicolus* populations

Galls of *A. lignicolus* have been collected from 15 localities (Figure 1 and Table 1) and adults have been reared from galls (Figure 2 and 3). Total DNA was extracted from 117 individuals (Table 1). Whole specimens were used in DNA extraction and agarose gel photograph is shown in Figure 4. From the isolated DNA a 433 base pair (bp) region of the mitochondrial cytochrome b gene was amplified and the PCR products are shown in Figure 5. Amplicons have been sequenced and the representative chromatogram of an *A. lignicolus* individual is shown in Figure 6.

In the sequences of 433 bp of cyt b gene segment, 365 sites were constant and 58 characters were showing variation (Table 2). Of the total variable sites, 21 characters were parsimony uninformative and 37 characters were parsimony informative. A total of 18 haplotypes were determined through considering these variations in nucleotides. The nucleotide frequencies of *A. lignicolus* haplotypes were 34%, 11%, 9% and 44% for A, C, G and T, respectively. There were multiple hits at eight sites (127, 142, 146, 272, 304, 376, 415 and 421). Among other variable sites, there were 30 transitions and 20 transversions (ti/ tv= 1.5). As it was reported in other insect species, there was high amount of transition substitutions compared to transversions substitutions, and in insect's mtDNA there is tendency to be rich in A and T nucleotides (Brower, 1994).

Figure 2. Galls of *A. lignicolus.*

Figure 3. Adult specimen of *A. lignicolus*.

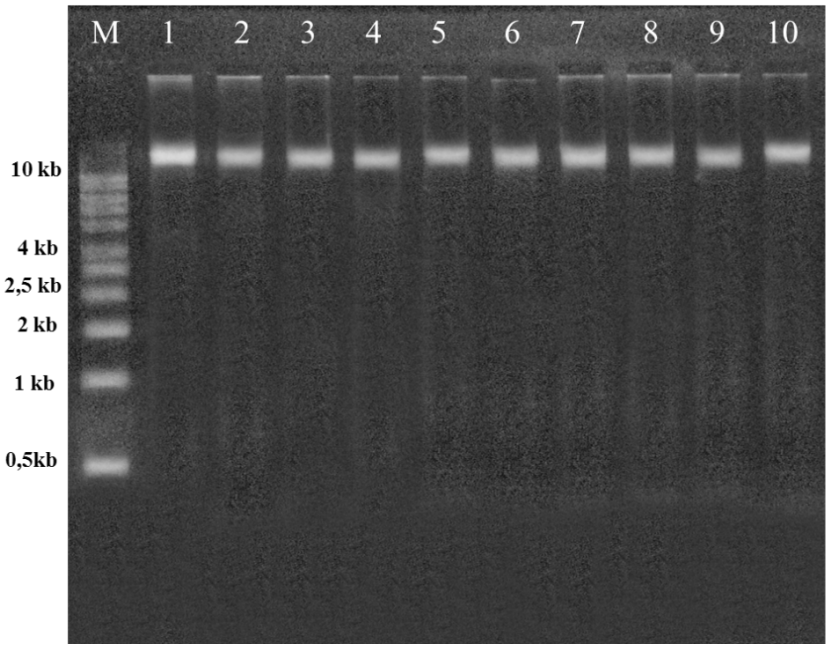

Figure 4. Isolated DNA samples of *A. lignicolus* specimens. M: 1 Kb λ DNA marker. 1-4 Düzce, 5-7 Eskişehir, 8-10 Balıkesir.

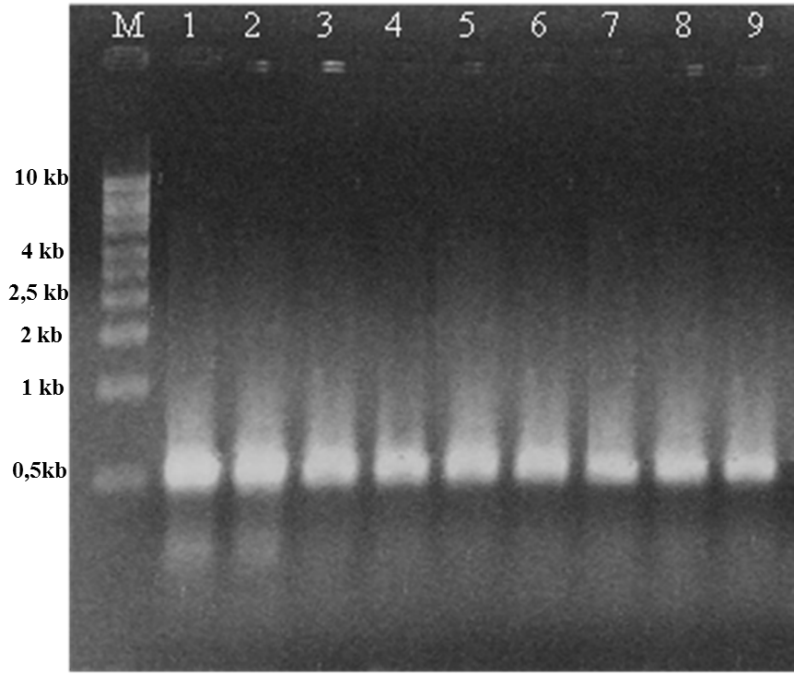

Figure 5. Amplified 433 bp cyt b fragment of *A. lignicolus* mtDNA. M: 1 Kb λ DNA marker. 1-3 Kırıkkale, 4-6 Eskişehir, 7-9 Kayseri.

Table 2. Substitution sites in *A. lignicolus* haplotypes are shown with the specific site numbers given at the top row.

Haplotypes	Site	74	76	85	97	100	106	112	127	137	139	142	146	148	163	166	178	182	184	190	196	202	205	214	215	217	224	229	232	239	262	268	272	273	295	296	301	304	305	306	316	319	322	328	331	340	343	352	358	364	373	376	388	394	415	419	421	424	433
	Base	T	A	A	T	T	T	C	T	G	A	A	A	T	A	T	A	C	T	A	T	T	T	T	A	G	T	A	T	C	A	T	C	G	T	A	T	A	A	T	T	T	T	T	T	T	A	C	T	T	C	T	T	A	A	G	A	T	A
H1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H2		.	.	.	.	.	A	T	.	A	T	T	G	.	T	.	G	T	A	C	.	.	.	.	A	.	G	A	.	.	.	T	A	.	.	.	.	.	C	.	.	.	C	.	.	.	T	.	.	C	T	A	C	.	.	.	T	C	.	.	.
H3		.	.	.	C	.	.	.	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	T	.	.	.	.	.	.	.	.	.	.
H4		.	.	.	C	.	.	.	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	T	.	.	.	.	.	.	.	.	.	.
H5		.	.	.	C	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
H6		.	.	.	C	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	.	.
H7		.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	.	.
H8		.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H9		.	.	.	.	.	A	T	.	A	.	T	.	.	T	.	.	.	.	.	.	C	.	.	A	A	G	A	.	.	C	T	A	.	.	.	.	T	C	.	.	.	C	A	.	T	T	C	C	T	C	.	.	.	.	T	.	.	.	.	.
H10		.	.	.	.	.	A	T	.	A	.	T	.	.	T	.	.	.	.	.	.	C	.	.	A	A	G	A	.	.	.	T	A	.	.	.	.	C	C	.	.	.	.	C	A	.	T	T	C	C	T	C	.	.	.	.	T	.	.	.	.	.
H11		.	.	.	.	.	A	T	.	A	.	T	.	.	T	.	.	.	.	.	.	C	.	.	A	A	G	A	.	.	C	T	A	.	.	.	.	T	C	.	C	.	.	C	A	.	T	T	C	C	T	C	.	.	.	.	T	.	.	.	.	.
H12		.	.	.	.	.	A	T	.	A	.	T	.	.	T	.	.	.	.	.	.	C	.	T	A	A	G	A	.	.	C	T	A	.	.	.	.	T	C	.	.	.	.	C	A	.	T	T	C	C	T	C	.	.	.	.	T	.	.	.	.	.
H13		.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	.	.
H14		C	T	.	.	.	A	T	C	A	.	C	.	C	T	A	.	T	A	.	A	C	.	.	A	.	.	A	T	.	.	T	C	A	T	A	T	.	.	.	.	C	C	.	.	C	T	T	.	C	T	.	.	T	C	.	C	.	T	.	.	.	.
H15		C	T	.	.	.	A	T	C	A	.	C	.	C	T	A	.	T	A	.	A	C	.	.	A	.	.	A	T	.	.	T	C	A	T	A	T	.	.	.	.	C	C	.	.	C	T	T	.	C	T	.	.	T	T	A	C	.	T	.	.	.	.
H16		.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H17		C	T	.	.	C	A	T	C	A	.	C	.	.	T	A	.	T	A	.	A	C	.	.	A	.	.	A	T	.	.	T	C	A	T	A	T	.	.	.	.	C	C	.	.	C	T	T	.	C	T	.	.	T	C	.	C	.	T	.	.	.	.
H18		C	T	G	.	.	A	T	C	A	.	C	C	.	T	A	.	T	A	.	A	C	C	.	A	.	.	A	T	.	.	T	C	A	T	A	T	.	.	.	.	C	C	.	.	C	T	T	.	C	T	.	.	T	C	.	C	.	T	.	.	.	.

Drosophila mtDNA was used for amino acid translation of the studied cyt b fragment and it was shown in Table 3. The translated amino acid sequences of the protein coding mitochondrial cyt b gene region contained 16 amino acid replacements, and they were shown in Table 4. When the amino acid translation of the sequences was compared to that of other insects, there were no indels or non-sense mutations.

Haplotypes and their frequencies in each of the studied locality were shown in Table 5. The most abundant haplotype (H1) was found in 36 individuals from 8 populations (Afyon, Denizli, Düzce, Eskişehir, İstanbul, Kahramanmaraş, Kütahya and Manisa). The second most common haplotype (H14) was found in 12 individuals from 3 populations (Konya, Kütahya and Uşak). Three haplotypes (H4, H5 and H6) were private haplotypes (singletons) found only in a single individual in a population. Interestingly, Çanakkale population had two of the private haplotypes (H5 and H6). H4 was another private haplotype found in Balıkesir. When the populations were examined with respect to their haplotype number Kütahya population had the highest number of haplotype (N= 4) followed by Çanakkale, Kahramanmaraş, Uşak and Kayseri (N= 3). The Balıkesir, İstanbul, Konya and Manisa populations each had two distinct haplotypes. The remaining populations (Afyon, Antalya, Denizli, Düzce, Eskişehir and Kırıkkale) had only a single type of haplotype shared among their specimens (Table 5).

A pairwise comparison among *A. lignicolus* haplotypes was conducted to determine sequence differences. Among all pairwise comparisons, the sequence divergence varied from 0.02 % to 10.6 % (1 to 46 bp, respectively) (Table 6). The most divergent haplotypes H5 (N= 1, Çanakkale population) and H18 (N= 2, Uşak population) were separated from each other by 46 nucleotides. The least divergence

Table 3. Triplets that code for insect amino acid based on *Drosophila*. Abbreviations are also shown in Table.

UUU Phe	**F**	UCU Ser	**S**	UAU Tyr	**Y**	UGU Cys	**C**
UUC Phe	**F**	UCC Ser	**S**	UAC Tyr	**Y**	UGC Cys	**C**
UUA Leu	**L**	UCA Ser	**S**	UAA Stop	**Stop**	UGA Trp	**W**
UUG Leu	**L**	UCG Ser	**S**	UAG Stop	**Stop**	UGG Trp	**W**
CUU Leu	**L**	CCU Pro	**P**	CAU His	**H**	CGU Arg	**R**
CUC Leu	**L**	CCC Pro	**P**	CAC His	**H**	CGC Arg	**R**
CUA Leu	**L**	CCA Pro	**P**	CAA Gln	**Q**	CGA Arg	**R**
CUG Leu	**L**	CCG Pro	**P**	CAG Gln	**Q**	CGG Arg	**R**
AUU Ile	**I**	ACU Thr	**T**	AAU Asn	**N**	AGU Ser	**S**
AUC Ile	**I**	ACC Thr	**T**	AAC Asn	**N**	AGC Ser	**S**
AUA Met	**M**	ACA Thr	**T**	AAG Lys	**K**	AGA Ser	**S**
AUG Met	**M**	ACG Thr	**T**	AAA LYS	**K**	AGG Ser	**S**
GUU Val	**V**	GCU Ala	**A**	GAU Asp	**D**	GGU Gly	**G**
GUC Val	**V**	GCC Ala	**A**	GAC Asp	**D**	GGC Gly	**G**
GUA Val	**V**	GCA Ala	**A**	GAA Glu	**E**	GGA Gly	**G**
GUG Val	**V**	GCG Ala	**A**	GAG Glu	**E**	GGG Gly	**G**

Table 4. Amino acid translation of the 433 bp cyt b gene region of *Andricus lignicolus*. Replacement substitutions were shown in marked columns.

	1 … 48
H1	F W G A T V I T N L M S A I P F I G N N I V I W L W G G F S I N N A T L N R F Y S F H F I V P F
H2	. . . . I . .
H3	. . . . L . . . M . .
H4	. . . . L . . . M . .
H5	. . . . M . .
H6	. . . . M . .
H7	. . . . M . .
H8	. . . .
H9	. . . . M . .
H10	. . . . M . .
H11	. . . . M . .
H12	. . . . M . .
H13	. . . . M . .
H14	. . . . M . .
H15	. . . . M . .
H16	. . . .
H17	. . . . M . .
H18	. . . . M . .
Ac	. . . . L . . . E . . . . V . . . . L . . . M . .
Ak	. . . . L . . . E . . . . V . . . . L . . . M . .

	49 … 96
H1	I I L M M V I I H L M F L H S T G S N N P L G V N S N F Y K I T F H Y Y F T I K D I V G F L I I
H2	V . . . . I . . D L . . . . M . . . . .
H3	. . . . S . . . .
H4	. . . .
H5	V . . . .
H6	V . . . .
H7	. . . .
H8	. . . .
H9	. . . . M . . D L . . . . M . . . . .
H10	. . . . M . . D L . . . . M . . . . .
H11	. . . . M . . D L . . . . M . . . . .
H12	. . . . M . . D L . . . . M . . . . .
H13	. . . . M . . . . .
H14	. . . . . . M . . . . I . . . L . . . . Q . . . . .
H15	. . . . . . M . . . . I . . . L . . . . Q . . . . .
H16	. . . .
H17	. . . . . . M . . . . I . . . L . . . . Q . . . . .
H18	. . . . . . M . . . . I . . . L . . . . Q . . . . .
Ac	V . . . . I L . . . . T . . L . . . . T . . . L . . . P . . I . . . V . . M Q . . . F M
Ak	V . . . . I L . . . . T . . L . . . . T . . . L . . . P . . I . . . V . . M Q . . . F M

Table 4 (continued).

	97 … 144
H1	F M L M M F M C C Y Y P Y I L S D P E N F N F S N P M I T P I H I Q P E W Y F L F A Y A L R S
H2	. . . . . L . . . .
H3	. . . .
H4	. . . .
H5	. . . .
H6	. . . .
H7	. . . .
H8	. . . .
H9	. . . . I L . . . . M . . . .
H10	. . . . I L . . . . M . . . .
H11	. . . . I L . . . . M . . . .
H12	. . . . I L . . . . M . . . .
H13	. . . . . S . . . .
H14	. I M I . . . .
H15	. I M I . . . . T . . .
H16	. . . .
H17	. I M I . . . .
H18	. I M I . . . .
Ac	I I G L L L L . . F V . . M . G . . . . . . M A . . . .
Ak	I I G L L L L . . F V . . M . G . . . . . . M A . . . .

with 1 bp difference were determined between H3 (N= 2 Balıkesir) and H4 (N=1 Balıkesir), H1 (common haplotype) and H8- (N= 2 İstanbul) H16 (N= 4 Kütahya, N= 3 Manisa), H6 (N= 1 Çanakkale) and H7 (N= 8 Çanakkale), H9 (N= 6 Kahramanmaraş, N= 2 Kayseri, N= 1 Kütahya) and H10 N= 3 Kahramanmaraş- H11 N= 5 Kayseri- H12 (N= 2 Kayseri), H14 (N= 4 Konya, N= 3 Kütahya, N= 5 Uşak) and H17 (N= 3 Uşak) haplotypes.

The genetic diversity estimates of *A. lignicolus* populations are given in Table 7. Values of haplotype diversity varied from 0.00 to 0.7778 with an average of 0.32519. The highest haplotype diversity (*h*= 0.7778) was found in Kütahya population, followed by the Uşak (*h*= 0.6889) and Kayseri, Balıkesir and İstanbul populations (*h*= 0. 6667). Six of the remaining populations showed no haplotype diversity due to the occurrence of only a single type of haplotype in those populations (see Table 5 and 7). Nucleotide diversity ranged between 0.00 and 0.042494. The average nucleotide diversity was calculated as π= 0.0087955. The Uşak population displayed the highest nucleotide diversity estimate π= 0.042494 followed by the Konya population (π= 0.039415) and the Kütahya population (π= 0. 030793). The lowest nucleotide diversity was found in those populations with no haplotype diversity (Afyon, Antalya, Denizli, Düzce, Eskişehir and Kırıkkale populations).

The pairwise F_{st} calculations showed significant genetic differentiation among some populations (Table 8). In particular, two locations showed complete differentiation from some other populations; Antalya was significantly different from

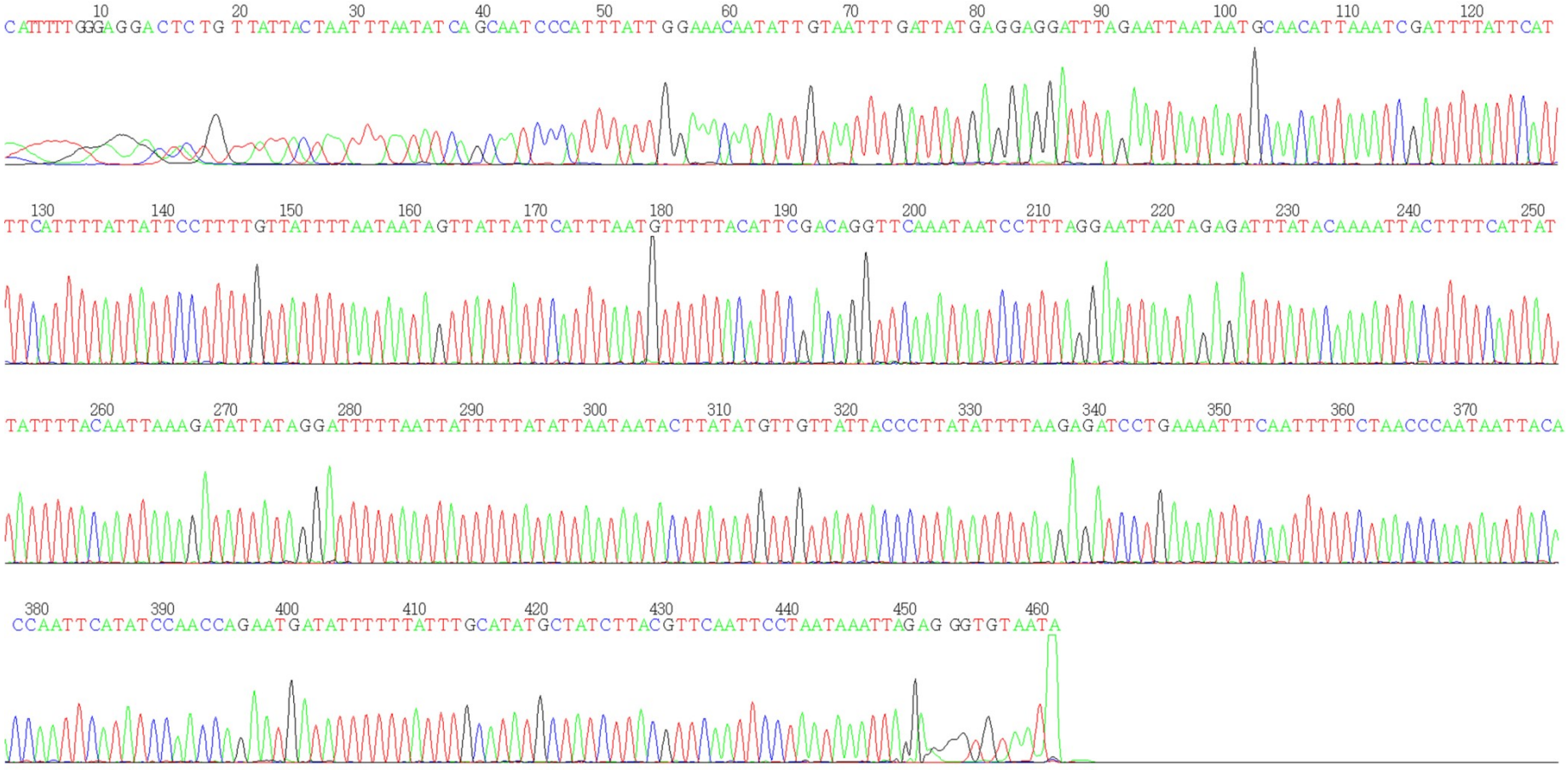

Figure 6. Chromatogram of the 433 bp region of cytb gene of *A.lignicolus.*

Table 5. Haplotypes and their frequencies determined in each *Andricus lignicolus* population. N_{hap}: Number of that specific haplotype in the population.

Haplotype	Afyon	Antalya	Balıkesir	Çanakkale	Denizli	Düzce	Eskişehir	İstanbul	K.Maraş	Kayseri	Kırıkklale	Konya	Kütahya	Manisa	Uşak	Nhap
H1	6				7	8	9	2	1				2	1		**36**
H2		7														**7**
H3			2													**2**
H4			1													**1**
H5				1												**1**
H6				1												**1**
H7				8												**8**
H8								2								**2**
H9									6	2			1			**9**
H10									3							**3**
H11										5						**5**
H12										2						**2**
H13											10					**10**
H14												4	3		5	**12**
H15												6				**6**
H16													4	3		**7**
H17															3	**3**
H18															2	**2**
Total	**6**	**7**	**3**	**10**	**7**	**8**	**9**	**4**	**10**	**9**	**10**	**10**	**10**	**4**	**10**	**117**

Table 6. Pairwise comparisons among eighteen haplotypes of *Andricus lignicolus* and the outgroup haplotypes (Ac: *Andricus caliciformis* and Ak: *Andricus kollari*). Percent differences between haplotypes are shown in the upper part of the diagonal and the number of base pair differences is shown in the lower part of the diagonal.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	Ac	Ak
H1		0.057	0.016	0.013	0.041	0.011	0.009	0.002	0.055	0.053	0.057	0.057	0.013	0.078	0.080	0.002	0.080	0.083	0.173	0.154
H2	25		0.060	0.057	0.076	0.050	0.053	0.060	0.041	0.039	0.043	0.004	0.053	0.069	0.071	0.060	0.071	0.073	0.182	0.163
H3	7	26		0.002	0.039	0.009	0.006	0.018	0.053	0.050	0.055	0.055	0.011	0.073	0.076	0.016	0.076	0.078	0.168	0.150
H4	6	25	1		0.036	0.006	0.004	0.016	0.050	0.048	0.053	0.053	0.009	0.071	0.073	0.013	0.073	0.076	0.168	0.150
H5	18	33	17	16		0.030	0.032	0.043	0.080	0.078	0.083	0.083	0.036	0.101	0.103	0.043	0.103	0.106	0.196	0.177
H6	5	22	4	3	13		0.002	0.013	0.053	0.050	0.055	0.055	0.006	0.076	0.078	0.013	0.078	0.080	0.170	0.152
H7	4	23	3	2	14	1		0.011	0.050	0.048	0.053	0.053	0.004	0.073	0.076	0.011	0.076	0.078	0.173	0.154
H8	1	26	8	7	19	6	5		0.057	0.055	0.060	0.060	0.016	0.076	0.078	0.004	0.078	0.080	0.170	0.157
H9	24	18	23	22	35	23	22	25		0.002	0.002	0.002	0.050	0.071	0.073	0.057	0.073	0.076	0.180	0.161
H10	23	17	22	21	34	22	21	24	1		0.004	0.004	0.048	0.069	0.071	0.055	0.071	0.073	0.180	0.161
H11	25	19	24	23	36	24	23	26	1	2		0.004	0.053	0.073	0.076	0.060	0.076	0.078	0.182	0.163
H12	25	19	24	23	36	24	23	26	1	2	2		0.053	0.073	0.076	0.060	0.076	0.078	0.180	0.163
H13	6	23	5	4	16	3	2	7	22	21	23	23		0.076	0.078	0.016	0.078	0.080	0.175	0.157
H14	34	30	32	31	44	33	32	33	31	30	32	32	33		0.004	0.076	0.002	0.004	0.166	0.152
H15	35	31	33	32	45	34	33	34	32	31	33	33	34	2		0.078	0.006	0.009	0.168	0.154
H16	1	26	7	6	19	6	5	2	25	24	26	26	7	33	34		0.078	0.080	0.173	0.154
H17	35	31	33	32	45	34	33	34	32	31	33	33	34	1	3	34		0.006	0.168	0.154
H18	36	32	34	33	46	35	34	35	33	32	34	34	35	2	4	35	3		0.166	0.157
Ac	75	79	73	73	73	74	75	74	78	78	79	78	76	72	73	75	73	72		0.023
Ak	67	71	65	65	65	66	67	68	70	70	71	71	68	66	67	67	67	68	10	

Table 7. Genetic diversity estimates of *Andricus lignicolus* populations. N: number of samples per population, N_h: number of haplotypes, h: haplotype diversity, π: nucleotide diversity.

Population	**N**	**Nh**	**h**	**π**
Afyon	6	1	0.0000 +/- 0.0000	0.000000 +/- 0.000000
Antalya	7	1	0.0000 +/- 0.0000	0.000000 +/- 0.000000
Balıkesir	3	2	0.6667 +/- 0.3143	0.001540 +/- 0.001920
Çanakkale	10	3	0.3778 +/- 0.1813	0.001283 +/- 0.001291
Denizli	7	1	0.0000 +/- 0.0000	0.000000 +/- 0.000000
Düzce	8	1	0.0000 +/- 0.0000	0.000000 +/- 0.000000
Eskişehir	9	1	0.0000 +/- 0.0000	0.000000 +/- 0.000000
İstanbul	4	2	0.6667 +/- 0.2041	0.001540 +/- 0.001728
K. Maraş	10	3	0.6000 +/- 0.1305	0.011855 +/- 0.007097
Kayseri	9	3	0.6667 +/- 0.1318	0.002181 +/- 0.001861
Kırıkkale	10	1	0.0000 +/- 0.0000	0.000000 +/- 0.000000
Konya	10	2	0.5333 +/- 0.0947	0.039415 +/- 0.021692
Kütahya	10	4	0.7778 +/- 0.0907	0.030793 +/- 0.017133
Manisa	4	2	0.5000 +/- 0.2652	0.001155 +/- 0.001432
Uşak	10	3	0.6889 +/- 0.1038	0.042494 +/- 0.023320

Afyon, Denizli, Düzce, Eskişehir and Kırıkkale (F_{st} = 1), and Kırıkkale was different from Afyon, Antalya, Denizli, Düzce and Eskişehir (F_{st} = 1). Some of the populations had no genetic differentiation (F_{st}= 0) from each other (Afyon and Denizli, Düzce and Eskişehir population) due to these populations sharing a single haplotype (H1) (see Table 5). Other populations displayed genetic differentiation values in a scale of 0 and 1 with statistically significant support ($p< 0.001$) indicating some degrees of differentiation from each other.

The mismatch distribution of all pairwise combinations for the cyt b gene of mitochondrial DNA haplotypes is shown in Figure 7. The mismatch distribution including all samples was indicating a bimodal profile. The Harpending's raggedness index was low (r= 0.0044) but not significant. Overall, Tajima's *D* neutrality test (Tajima's *D*= 0.47873 P> 0.10) and Fu and Li tests *D** = 0.41064 P> 0.10; *F**= 0.50013 P> 0.10) were not significant for most populations examined indicating that these populations are in genetic equilibrium (Kimura, 1983).

An AMOVA analysis for revealing population structuring was conducted through forming "two categories" AMOVA tests. Several trials in grouping the populations have been tested for significant clustering and the partitioning of the genetic variation at two/ three levels, however only two of the trial schemes were included in Table 8. In the first trial scheme each population was considered singly and it was determined that variation was partitioned "among population" with a percentage of 71.43, and the remaining variation (28.57%) was at "within population" level. These results were statistically significant ($p<0.001$) (Table 9a). In the contrary, 27.31% of variance components were found at "among groups", 48.71% at "among populations within groups", and 23.98% at "within population"

Table 8. Fst representation calculated in a pairwise manner to compare fifteen *Andricus lignicolus* populations.

	Afyon	Antalya	Balıkesir	Çanakkale	Denizli	Düzce	Eskişehir	İstanbul	K.Maraş	Kayseri	Kırıkkale	Konya	Kütahya	Manisa
Afyon														
Antalya	1													
Balıkesir	0.971	0.993												
Çanakkale	0.914	0.985	0.804											
Denizli	0	1	0.974	0.919										
Düzce	0	1	0.977	0.924	0									
Eskişehir	0	1	0.979	0.928	0	0								
İstanbul	0.441	0.991	0.906	0.872	0.481	0.515	0.544							
K.Maraş	0.848	0.836	0.8	0.859	0.858	0.866	0.873	0.821						
Kayseri	0.976	0.971	0.962	0.967	0.978	0.979	0.98	0.965	0.115					
Kırıkkale	1	1	0.973	0.879	1	1	1	0.973	0.872	0.98				
Konya	0.661	0.622	0.557	0.695	0.679	0.694	0.708	0.61	0.452	0.534	0.707			
Kütahya	0.25	0.661	0.287	0.41	0.274	0.296	0.315	0.195	0.352	0.527	0.479	0.414		
Manisa	0.736	0.993	0.914	0.888	0.76	0.78	0.797	0.533	0.825	0.967	0.981	0.611	0.177	
Uşak	0.626	0.577	0.514	0.661	0.645	0.661	0.676	0.572	0.329	0.414	0.673	0.037	0.347	0.574

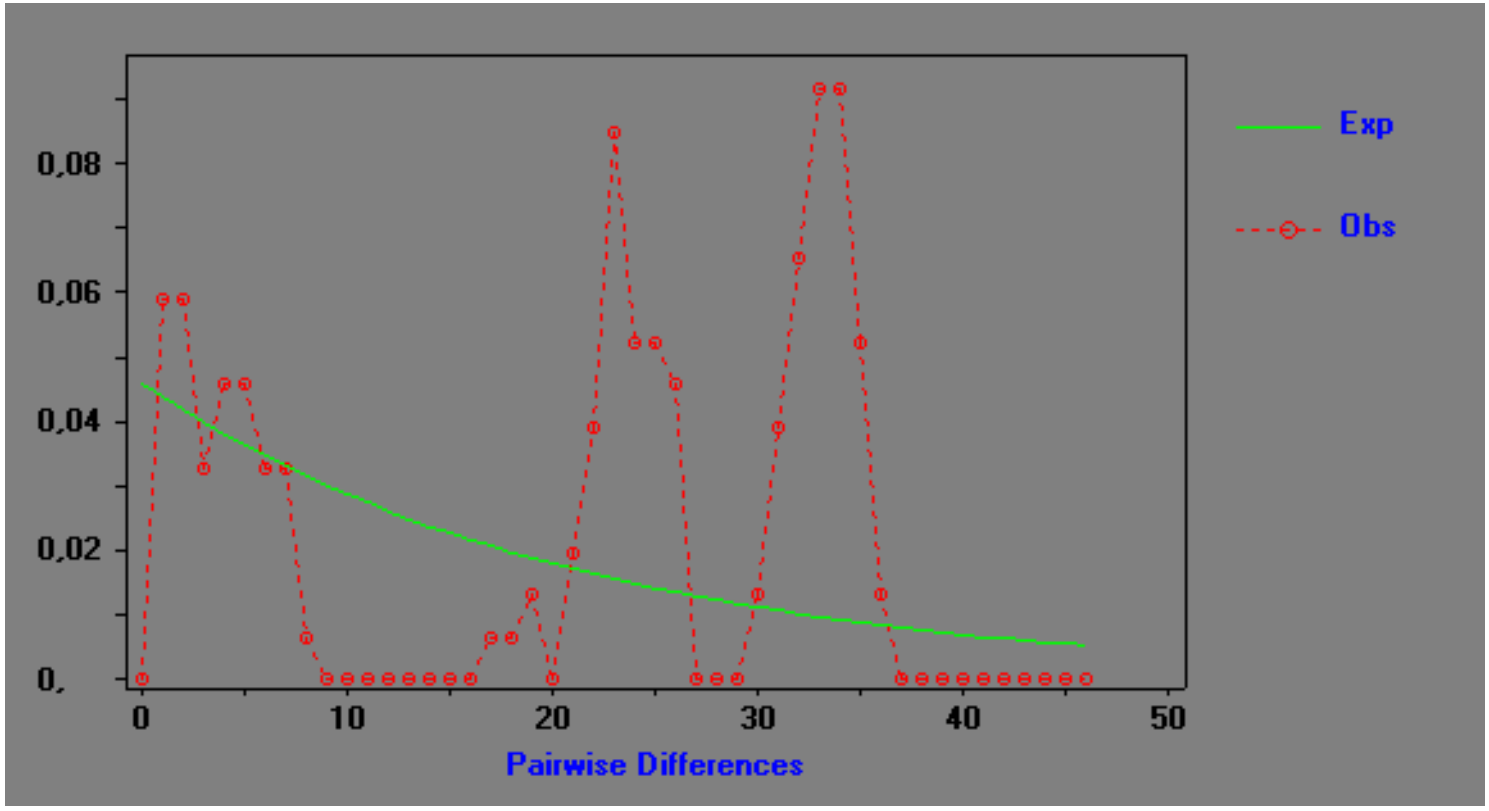

Figure 7. Mismatch distribution of all pairwise combinations for the cyt b mitochondrial DNA haplotypes. The observed and expected distributions are shown in graph obtained from DnaSP program.

Table 9. AMOVA results of *Andricus lignicolus* populations obtained from the Arlequin program (d. f. degrees of freedom).

a) Groups: Each population is taken individually.

Source of Variation	d. f.	Sum of Squares	Variance Components	Percentage of Variation	*F*-index	P- value
Among groups	14	702.614	6.16084	71.43	0.43080	<0.001
Within populations	102	251.394	2.46465	28.57	0.17114	<0.001

Table 9 (continued).

b) Two groups as is following: Group 1: Afyon, Denizli, Düzce, Eskişehir Group 2: All other populations.

Source of Variation	d. f.	Sum of Squares	Variance Components	Percentage of Variation	*F*-index	P- value
Among groups	1	167.583	2.80626 Va	27.31	0.67010	<0.001
Among populations within groups	13	535.031	5.00631 Vb	48.71	0.76018	<0.001
Within populations	102	251.394	2.46465 Vc	23.98	0.27306	<0.001

level when all the populations were divided into two groups based on the presence of a haplotype (H1) in the analyzed populations (for groupings see Table 9b).

3.2. Phylogenetic Relationships among *Andricus lignicolus* haplotypes

Phylogenetic relationships among 18 *A. lignicolus* haplotypes were estimated both by MP and ML through using PAUP 4.0b program. Since both algorithms estimated similar tree topologies with different bootstrap values, thus only a single phylogenetic tree was shown in Figure 8 with bootstrap values on the branches separated by a slash; first indicating MP and the second showing ML bootstrap values, respectively. MP is a consensus tree of 123 shortest trees and a single consensus tree was produced with a CI= 0.691 with 139 steps in length. For ML analysis, Modeltest program was run to determine the mutational model that best approximated the sequence evolution of the data set and to calculate the transition and transversions ratios. Modeltest identified HKY+I (Hasegawa et al., 1985) as the best fit model to the data set (I= 0.3778), therefore this substitution model was utilized in ML and further in BI analysis.

ML and MP trees produced two well supported clades: Clade A is composed of a basally located haplotype (H15) from Konya population and a small polytomic group of three haplotypes (H14 from Konya, Kütahya, Uşak, H17 from Uşak, and H18 from Konya population). However, Clade B is larger and composed of further two sub-clades. The first sub-clade contains H2 from Antalya population as the most basal haplotype, H12 from Kayseri population and a polytomic group of haplotypes; H9 shared among Kahramanmaraş, Kayseri and Kütahya populations, H11 from Kayseri population and H12 from Kayseri population. The second sub-clade is composed of a large polytomic group of haplotypes: H13 (Kırıkkale) H5-H6-H7

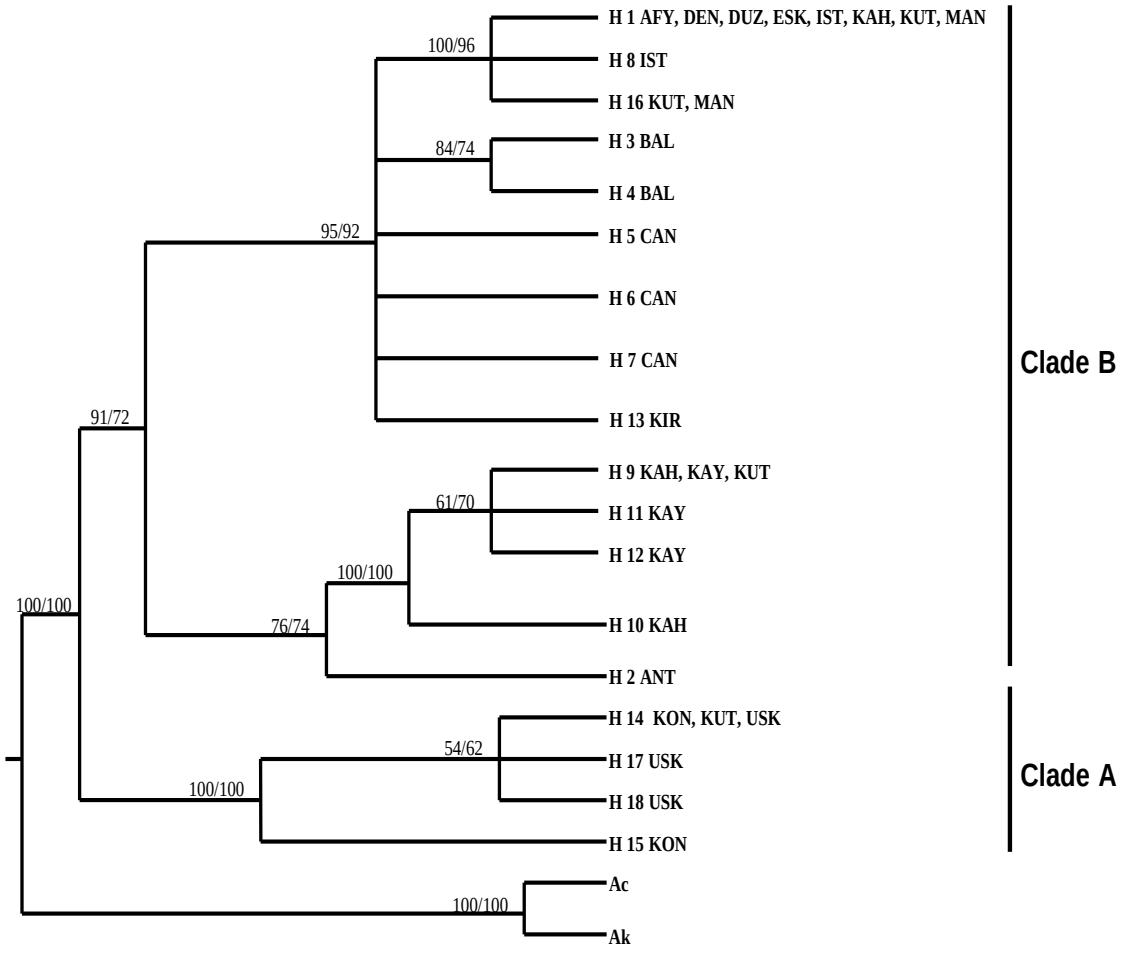

Figure 8. Results of ML and MP analyses combined on a phylogenetic tree for the cyt b gene region of mtDNA sequence of *A. lignicolus*. Boostrap values are shown for both MP and ML, respectively.

from Çanakkale population with some additional haplotypes. Of these, H3 and H4 from Balıkesir population make a monophyletic grouping. The most common haplotype (H1) found in eight locations, H8 from İstanbul population and H16 shared between Kütahya and Manisa form a small polytomic group with high boostrap values.

An alternative analysis, Bayesian Analysis (BI) was performed using MrBayes 3.1 program. Bayesian tree is given in Figure 9 with the posterior probabilities on the branches. In the Bayesian tree there is polytomy at the basal part which is composing H16 (from Kütahya and Manisa), H8 (from İstanbul), H1 (shared common haplotype from Afyon, Denizli, Düzce, Eskişehir. İstanbul, Kahramanmaraş, Kütahya, Manisa), and a large clade which covers rest of the haplotypes. In the large clade there is also polytomy which is consisted from H13 (KIR) and H7 (CAN), a monophyletic group including H5 and H6 from Çanakkale population, another monophyletic group including H3 and H4 from Balıkesir, and the third lineage making polytomy which includes the remaining haplotypes. Within this lineage two haplogroups are observed to be monophyletic. Of this, the first subclade is composed of a small polyphyletic group which includes H9 (from Kahramanmaraş, Kayseri, Kütahya), H10 (from Kahramanmaraş), H11 and H12 (both from Kayseri population). Another polytomic group has a basally located haplotype H2 from Antalya, and the small polytomic group composed of H14 (Konya, Kütahya, Uşak), H15, H16 and H17 (Uşak).

For a better illustration of haplotype relationships a network analysis of the 18 haplotypes was conducted and the result yielded by TCS 1.18 is shown in Figure 10. TCS analysis produced three main haplogroups with an additional single haplotype (H2 from Antalya) that could not be connected under the 95% connection limit. H2 with the uppermost substitution value could not be grouped with other haplotypes. In

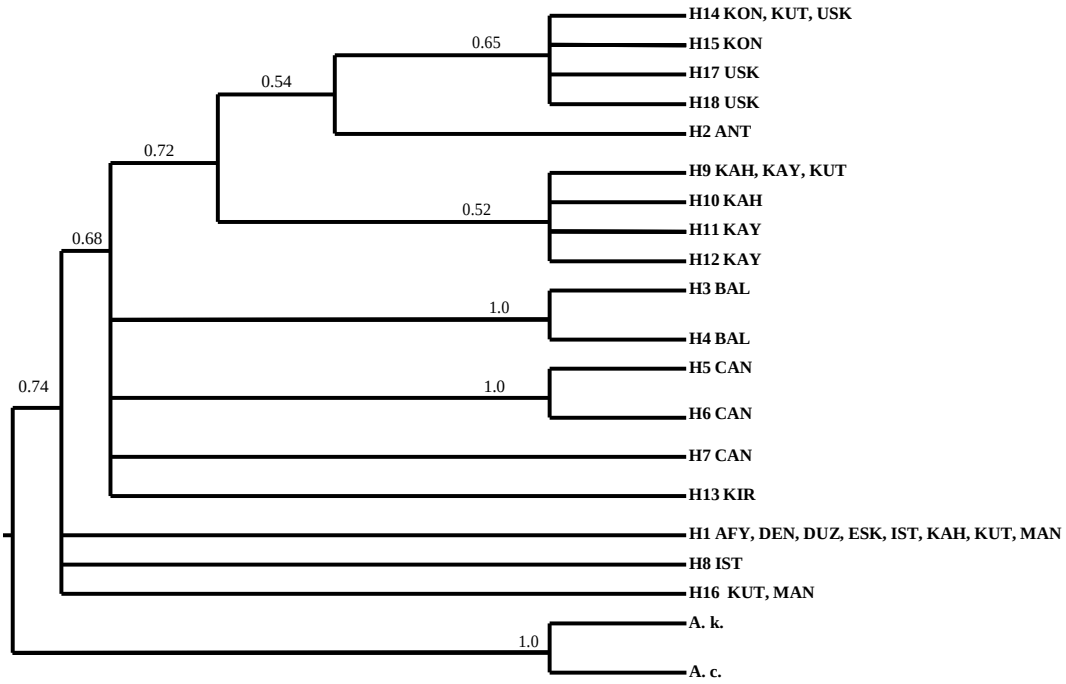

Figure 9. Resulting tree of MRBayes analysis for the cyt b gene region of mtDNA sequence of *A. lignicolus*. Posterior probabilities are shown on the branches.

the obtained TCS network haplotypes of the largest cluster seemed to be derived from H1 which was the most common and geographically the most widely distributed haplotype. In the large cluster H6 and H8 were not connected to any other haplotypes other than H1. However, H1 was connected to three hypothetical haplotypes that were giving a connection to several other haplotypes such that H5 and H7. As can be seen in Figure 10 in the large group of haplotypes of the network in addition to H5, H6 and H7, H4 H3 and H13 are clustered within this group. The third haplotype cluster was forming a small grouping with the uppermost substitution value belonged to H9. Haplotype 9 seemed to be connected to H12, H10 and H11. The last haplogroup contains H14 with the uppermost value which was connected to two hypothetical haplotypes with overall three detected haplotypes, namely H15, H17 and H18.

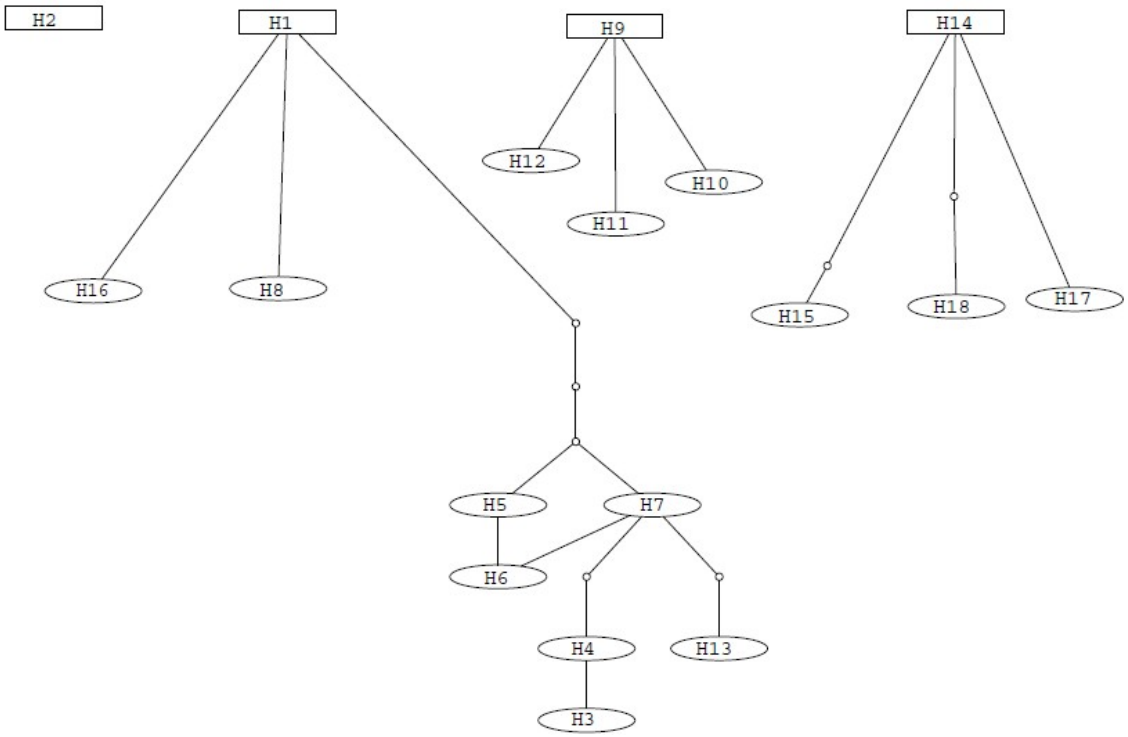

Figure 10. Parsimony network analysis of *A. lignicolus* haplotypes. The haplogroups can not be connected under the 95% limit. Haplotype numbers are shown in circles. Line length and circles are proportional to the number of mutational steps and frequency of the haplotype, respectively. The central haplotypes with the highest outgroup weight are depicted in squares. Small circles represent steps between observed haplotypes.

CHAPTER 4

DISCUSSION

4. 1. Genetic Variation of *Andricus lignicolus* populations

Amplification results of all specimens of *A. lignicolus* were 433 bp and there were no indels, non-sense mutations and stop codons. It is well known that protein coding sequences do not have premature stop codons (Zhang & Hewitt, 1996). Multiple cytochrome b-like fragments, indel and stop codons have been determined in such cynipid species as *A. aries*, *A. theophrasteus*, *A. tomentosus* and *N. lanuginosus* (Rokas et al., 2003b; Bensasson et al., 2001). Neither pseudogenes, intramitochondrial duplications nor heteroplasmy was detected when *A. lignicolus* haplotypes were compared to the corresponding cyt b region of other insect species.

The nucleotide composition of *A. lignicolus* haplotypes were %34, 11%, 9% and 44% for A, C, G and T. It has been reported that there is a tendency for A+T biased nucleotide sequence in insect mitochondrial genome (Chapco, 1997; Flook et al., 2000; Loxdale & Lushai, 1998); in other words there is bias against guanine on light strand of the protein coding sequences of mtDNA. For instance, *D. yakuba* mitochondrial genome has 78.6% A+ T nucleotides (Clary & Wolstenhome, 1985) whereas honey bee has 84.9% A+ T (Crozier & Crozier, 1993). The nucleotide composition is thus concordant with both of the insect species used quite often for comparisons. In addition to the tendency within insect orders toward having A+T bias particularly in derived taxa (Zucker, 1989) G is the rarest nucleotide bias

particularly in insects (Crozier & Crozier, 1993) as it was also observed in *A. lignicolus* haplotypes.

In insect mitochondrial protein coding gene segments transitions are observed more compared to transversions (Brown et al., 1979) because of poor or deficient mtDNA repair mechanisms and tautomeric base pairing (Wilson et al., 1985). In the sequenced region, there were 30 transitions and 20 transversion substitutions (ti/ tv= 1.5) which is well within the range of transition and transversion ratios of other insect species (Jermiin & Crozier, 1994).

A total of 18 haplotypes were detected out of the 117 individuals of *A. lignicolus*. Haplotype 1 (H1) is the most common haplotype found in 36 individuals representing eight populations (Afyon, Denizli, Düzce, Eskişehir, İstanbul, Kahramanmaraş, Kütahya and Manisa). The frequency of the wide distribution area across populations of H1 may imply that this haplotype might be older than other haplotypes detected in this species. Indeed, phylogeographic studies revealed that more common haplotypes might have had longer time to disperse through the distribution area compared to geographically more restricted haplotypes which might be derived (Crandal & Templeton, 1993). Moreover, sharing haplotypes between/ among populations may entail that *A. lignicolus* had a widespread natural distribution in the region as it was proposed by distribution data of an insect species (Esa et al., 2006).

In *A. lignicolus* average nucleotide diversity was 0.0087955 (0.8%). In other gall wasp species genetic diversity shows wide range of variation. In *A. coriarius* nucleotide diversity was 0.00529 (0.5%) in Iranian populations, 0.00644 (0.6%) in Lebanese populations, however in Turkey as it was placed in the main clade,

nucleotide diversity was 0.01554 (1.5%) (Challis et al., 2007). In *A. quercustozae* the greatest genetic diversity was detected in Turkey within a range of 0.2- 4.2%, in Italy it ranged between 0.12- 0.17%, in Iberian Peninsula it was 0.07. RFLP based haplotype and nucleotide diversity for *A. caputmedusae* were 0.4631 and 0.3204 (Mutun, 2010), for *A. lucidus* 0.8089 and 0.115542 (Mutun, 2011), 0.45 and 0.054 for *A. quercustozae* (Dinç & Mutun, 2011). Overall assessment of the findings confirm that even with low level of genetic diversity detected in this study variation is still well within the range of European oak gall wasp species and other Turkish gallwasp species.

Besides detection of comparatively high nucleotide diversity, *A. lignicolus* showed low levels of haplotypic variation whose average was 0.32519. One explanation for the detection of low haplotype variation might be related to the sampling size or as an alternative, species specific demographic factors might have possibly influenced *A. lignicolus* populations. It is known that galls of oak gall wasps can be parasitized by some inquilines leading some of the individuals not to develop inside the gall (Bailey et al., 2009). Likewise, parasitoid infection may cause gall inducing cynipids to be parasitized. Such an example is *Synophrus politus* which infects galls of some cynipid species (Washburn & Cornell, 1981). *Synophrus politus*, a member of the tribe Synergini, is known to infect the gall just after the gall begins its development and affects the wasp larva itself (Hayward & Stone, 2006; Pujade-Villar et al., 2001). Infected galls are quite similar in coloration pattern and other phenotypic features when compared with the non-infected galls of *A. lignicolus*. Similar effects have been reported for *Andricus burgundus* gall wasps (Pujade-Villar et al., 2001), however there is no report for *A. lignicolus* to be infected by *S. politus* (except personal observations). Thus, *S. politus* may be responsible for

the observance of low amount of genetic variation in *A. lignicolus* populations. In addition to inquiline and parasitoid attacks, there have been several reports on certain alpha proteobacteria, such as *Wolbachia* spp., and fungi infecting and causing high mortality in gall wasps during the developmental processes of larvae (Rokas et al. 2002). More interestingly, parasitic attacks may even skew the sex ratio in oak gall wasps (Atkinson et al., 2003).

On the other hand, if each locality is examined individually highest haplotype diversity was detected in Kütahya followed by the Uşak population (Table 8). Likewise, highest nucleotide diversity was estimated for the Uşak population then for Konya and Kütahya populations (Table 7). However, in six populations (Afyon, Antalya, Denizli, Düzce, Eskişehir and Kırıkkale) both haplotype and nucleotide diversity could not be observed (both values are 0) which is due to detection of only a single haplotype. One way of explaning this observation would be insufficient sampling. However, observing only a single haplotype with balanced sampling across the distribution range may well also be correlated with parasitoid attacks being common in these populations and it might be highly possible that some of the lineages have been swapped out of those localities (Hayward & Stone, 2006).

Pairwise comparisons among eighteen *A. lignicolus* haplotypes revealed that the highest number of base differences in mere counting was between haplotype 5 (from the Çanakkale population) and haplotype 18 (from the Uşak population) with 46 nucleotide differences. The lowest number of difference occurs between several populations such that Balıkesir and İstanbul population (H1 and H8) and several other pairwise comparisons (see Table 6). The presence of such high amount of sequence difference may indicate the presence of a cryptic species, however there is no apparent data to attest this hypothesis yet. It is known that morphologically

indistinguishable species, in other words cryptic species may lie within another taxonomically defined species. Cryptic species may be more common than it is once thought (Williams et al., 2012). A new cryptic oak gall wasp species from Turkey and Iran has been described recently which was previously classified under *A. coriarius* (Challis et al., 2007). Further research is definitely necessary to draw such results to conclude that there may be such a situation for *A. lignicolus* as well.

4. 2. Phylogenetic and Phylogeographic Structure of *Andricus lignicolus*

Phylogenetic analysis of *A. lignicolus* haplotypes using both MP and ML analyses gave the same tree topology. In the MP and ML phylogenetic tree formation of two major clades is obvious and was significantly supported with high boostrap value (Figure 8). Evolutionary relationships were not clearly resolved in small subclades in both Clade A and B, however in Clade B in the large polytomic part in addition to the presence of a commonly shared haplotype (H1), all other haplotypes were geographically restricted to the western populations. In spite of a monophyletic grouping of two Balıkesir haplotypes (H3 and H4) relationships of all other haplotypes showed polytomy which may be due to several possible reasons as not enough time being passed since divergence between lineages and incomplete lineage sorting (Avise, 2000). While some of the haplotypes representing westerly populations such that H14, H17, H18, H15 (from Konya, Kütahya and Uşak) seems to be well separated from other haplotypes.

Unlike MP and ML trees, BI tree is composed of more polytomic groups at the basal part. H1 (common haplotype), H8 (from İstanbul) and H16 (Kütahya and Manisa) all are polytomic and another polytomic lineage gives rise to the maing clade structure. In the main clade there are some polytomic structuring as well

although two monophyletic grouping drawed the attention. In the mentioned groupings haplotypes from Balıkesir and Çanakkale were sister to each other. Some discrepancies are also obvious between the resulting trees of both MP-ML and BI. This may be due to using only a single type of genomic data and further additional data set can be included to get over this incongruity observed between analyses methods. Under certain conditions phylogenetic and phylogeographic inferences can be partially misleading by introgression and retention of the ancestral polymorphism seen in the taxon. It may be also due to the tendency of the interpretations based solely on mtDNA genes which may have tendency to reach to the incomplete conclusions (Kyriazi et al., 2008).

In conjunction with the phylogenetic analyses dealt previously for *A. lignicolus* further analysis covering a network analysis indicated the presence of three major groupings with an additional single haplotype that could not be connected with 95% confidence limit (see Figure 10). One advantage of the application of a network analysis is that hybridization and any other types of reticulations can be revealed by this nested clade and network analysis (Bandelt et al. 1999, Posada et al., 2006; Templeton, 1998). As it can be seen in TCS network result shown in Figure 10 there is an apparent reticulation event.

Hierarchical F-statistics (AMOVA) utilized for hierarchical partitioning of variance into researcher predefined-groups indicated highest and statistically significant grouping into two groups (see Table 9). Of the forementioned grouping in Table 9, ~ 72% of variation was present among groups and ~28% was within populations of each group. The observance of considerable part of the total genetic variability within species was allocated between groups. The highly significant differentiation detected among groups is quite interesting implying the presence of

high differentiation among some of the sampled localities which may be an indication of either physical barrier to gene flow (Meglecz et al., 1999) or the presence of cryptic species that was not be able to be determined yet by the scientists within an already defined species (Challis et al., 2007; Williams et al., 2012). Indeed, pairwise comparisons of populations through calculating F_{st} values demonstrated that several populations were differentiated from each other with the uppermost value. In particular, a complete differentiation (F_{st}= 1) between Kırıkkale and Antalya, Denizli, Afyon, Düzce, Eskişehir is conspicuous (see Table 8). Moreover, Antalya is highly differentiated from Afyon, Denizli, Düzce and Eskişehir. Because high F_{st} values indicate little or no migration between populations due to separation of the populations by large geographic distances (Nguyen et al., 2006) can be applied to some of the *A. lignicolus* populations. However, no differentiation (F_{st}= 0) observed between Afyon, Denizli, Düzce and Eskişehir is quite noticeable. This is because of these populations sharing the same haplotype. In this study, the paradoxical results in differentiation between populations may be partially explained by the swapped out lineages since some *A. lignicolus* populations have only a single haplotype. Such heavy attacks by parasitoid or other predators have been reported from other gall wasp species including *A. lignicolus*, *A. kollari* and *A. corruptrix* (Walker et al., 2002).

On the other hand, the result of the mismatch analysis of *A. lignicolus* has a multi-peaked pattern characterizing much or less a stable population structure. The species is in equilibrium genetically as the Harpending's raggedness index was low but non - significant. Similarly, Tajimas's D neutrality test (Tajima's D= 0.47873 P>0.10) and Fu and Li tests *D** = 0.41064 P> 0.10; *F**= 0.50013 P> 0.10) were not significant. The main reason is that some populations have only one type of

haplotype. In *A. lignicolus* within population variation was highly varying from no variation to 0.7778 (haplotypic diversity). Furthermore, the large mtDNA differences currently found between some ingroup haplotypes could be explained by one or several factors including small population size, however some populations with small population size such as Balıkesir and Manisa had high haplotypic variation, thus it might not be directly related to the small sampling size. In addition to population size, past bottleneck events or the presence of geographic barriers to gene flow among populations may also the reason of low genetic diversity detected in populations (Nguyen et al., 2006).

Further research is still necessary in *A. lignicolus* with more sampling from Turkey and in the nearby area to solve if a cryptic species is present within the distribution of the species. Furthermore, additional molecular markers (nuclear gene) can further be used to examine the species in much greater detail. However, I believe that the current study will give some lights on oak gall wasps in Turkey.

REFERENCES

Ács Z., Challis J. R., Bihari P., Blaxter M., Hayward A., Melika G., Csóka G., Pénzes Z., Pujade-Villar J., Nieves-Aldrey J-L., Schönrogge K., Stone G. N. (2010). Phylogeny and DNA barcoding of inquiline oak gallwasps (Hymenoptera: Cynipidae) of the Western Palaearctic. Molecular Phylogenetics and Evolution, 55: 210-255.

Alkan B. (1952). Türkiye'nin Zoosesid (Zoocecid) leri (kökeni hayvansal bitki urları) üzerinde çalışmalar II. A. Ü. Ziraat Fakültesi 1952 yıllığı, Ankara. 17: 259-279.

Arias C. M., Brito M. R., Francisco O. F., Moretto G., De Oliveira F. F., Silvestre D., Sheppard S. (2006). Molecular markers as a tool for population and evolutionary studies of stingless bees. Apidologie, 37: 259-274.

Askew R. R. (1984). The biology of gall wasps. Pp. 223-271 in: Biology of gall insects (ed.Ananthakrishan, T.N.). Oxford & IBH Publishing Company New Delhi, Bombay, Calcutta.

Askew R. R., Neill M. P. (1993). Parasitoids and inquilines of the agamic generation of Andricus lignicolus (Hymenoptera: Cynipidae) in Britain. The Entomologist, 112: 43-48.

Atkinson R. J., Brown G. S., Stone G. N. (2003). Skewed sex ratios and multiple founding in galls of the oak apple gallwasp *Biorhiza pallida*. Ecological Entomology, 28: 14-24.

Avcı M. (1993). Türkiye' nin flora bölgeleri ve Anadolu Diagonal'ine coğrafi bir yaklaşım. Türk Coğrafya Dergisi, 28: 225-248.

Avise J. C., Arnold J., Ball R. M., Bermingham E., Lamb T., Neigel J. E., Reeb C. A., Saunders N.C. (1987). Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annual Review of Ecology Evolution and Systematics, 18: 489-522.

Avise J. C. (1998). The history and purview of phylogeography: a personal reflection. Molecular Ecology, 7: 371-379.

Avise J. C. (2000). Phylogeography: The History and Formation of Species. Cambridge, MA: Harvard Univ. Press.

Bailey R., Schönrogge K., Cook J. M., Melika G., Csoka G., Thuroczy C., Stone G. N. (2009). Host niches and defensive extended phenotypes structure parasitoid wasp communities. PLoS Biology, 7(8): 1-12.

Bandelt H. J., Forster P., Rohl A. (1999). Median-joining for inferring intraspecific phylogenies. Molecular Biology and Evolution, 16 (1): 37-48.

Baş R. (1973). Türkiye'de orman ağaçlarında zarar yapan zar kanatlılar (Hymenoptera) üzerine araştırmalar. OAE Yayınları, Tek. Bült. Serisi No: 66 Ankara, 155p**.**

Bensasson D., Zhang X., Hartl D. L., Hewitt G. M. (2001). Mitochondrial pseudogenes: evolution's misplaced witnesses. Trends in Ecology and Evolution, 16: 314–21.

Bermingham E., Moritz C. (1998). Comparitive phylogeography: concepts and applications. Molecular Ecology, 7: 367-369.

Bronner R. (1992). The role of nutritive cells in the nutrition of cynipids and cecidomyiids. In: Shorthouse J. D., Rohfritsch O. (eds) Biology of insect-induced galls. Oxford University Press, New York, pp: 118-140.

Brower A. V. Z. (1994). Rapid morphological radiation and convergence among races of butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. Procedings of the National Academy of Sciences USA, 91: 6491-6495.

Brown W. M., George M. JR., and Wilson A. C. (1979). Rapid evolution of animal mitochondrialDNA. Proceedings of the National Academy of Sciences, USA 76: 1967- 1971.

Challis R. J., Mutun S., Nieves-Aldrey J-L., Preuss S., Rokas A., Aebi A., Sadeghi E., Tavakoli M., Stone G. N. (2007). Longitudinal range expansion and cryptic eastern species in the western Palaearctic oak gallwasp *Andricus coriarius*. Molecular Ecology, 16: 2103-2114.

Chapco W. (1997). Molecular evolutinary genetics in orthopteroid insects. Pp. 337–354. In: GANGWARES K., MURALIDHARAN M. C. and MURALIDHARAN M. (eds): Bionomics of grasshoppers, kathydids and their kins. CAB International Press.

Clary D. O., Wolstenholme D. R. (1985). The mitochondrial DNA molecule of Drosophila yakuba: nucleotide sequence, gene organization, and genetic code. Journal of Molecular Evolution, 22: 252-271.

Clement M., Posada D., Crandal K. A. (2000). TCS: a computer program to estimate gene genealogies. Molecular Ecology, 9(10): 1657-1660.

Cook J. M., Rokas A., Pagel M. & Stone G. N. (2002). Evolutionary shifts between host oak sections and host-plant organs in *Andricus* gallwasps. Evolution, 56(9): 18

Cornell H. V. (1983). The secondary chemistry and complex morphology of galls formed by the Cynipinae. Why and how? American Midland Naturalist, 110: 225-234.

Crandall K.A., Templeton A.R. (1993). Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. Genetics 134(3): 959-969.

Crozier R., H., Crozier Y. C. (1993). The mitochondrial genome of the honeybee *Apis mellifera*: Complete sequence and genome organization. Genetics, 133: 97-117.

Csóka G. (1997). Gubacsok-Plant galls. Agroinform, Budapest.

Csóka G., Stone G. N. & Melika G. (2004). Biology, Ecology and Evolution of gall inducing Cynipidae. Pp. 569-636 in: Biology, ecology and evolution of gall-inducing arthropods (eds. Raman, A., C.W. Schaefer & T.M. Withers). Science Publishers, Inc. Enfield, New Hampshire, USA.

Çıplak B. (2008). The analogy between interglacial and global warming for the glacial relicts in a refugium: A biogeographic perspective for conservation of Anatolian Orthoptera. In: Fattorini, S. (Ed.) Insect Ecology and Conservation, Pp.135-163. Research Sign Post , Kerela, India.

Demirsoy A. (2008). Genel Zoocoğrafya ve Türkiye Zoocoğrafyası 'Hayvan Coğrafyası'. Ed: Demirsoy, yedinci baskı, Meteksan, Ankara.

Dinç S. Mutun S., Melika G. (in press). A new species of *Andricus* Hartig oak gallwasp from Turkey (Hymeoptera: Cynipidae, Cynipini). North western Journal of Zoology.

Dinç S., Mutun S. (2011). PCR-RFLP variation of the oak galwasp, *Andricus quercustozae* (Bosc, 1792) (Hymenoptera: Cynipidae) from Turkey. Turkish Journal of Entomology, 35(1): 47-58.

Esa Y., Siraj S. S., Daud S. K., Rahim K. A. A., Abdullah M. T., Japning J. R. R., Tan S. G. (2006). Mitochondrial DNA diversity of *Tor douronensis* Valenciennes (Cyprinidae) in Malaysian Borneo. Pertanika J. Trop. Agric. Sci., 29(1&2): 47-55.

Eroğlu M. M. (2000). Gall production strategy that is interfering with life cycles of two important gall-maker species, *Andricus gallaetinctoriae* (Oliv) and *A. kollari* Htg. (Hymenoptera: Cynipidae) in Turkey. Seminar on 'Harvesting on Non-wood Forest Products', Menemen-İzmir (Turkey), Joint FAO/ECE/ILO Committee on Forest Technology, Management and Training, p.10.

Excoffier L., Smouse P. E., Quattro J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes application to human mitochondrial DNA restriction data. Genetics, 131: 479-491.

Fergusson N. D. M. (1995). The cynipoid families. Pp. 247-265 in: The Hymenoptera of Costa Rica (eds. Hanson, P.E. & Gauld, I.D.). Oxford University Press. Oxford, NewYork, Tokyo.

Flook P. K., Klee S., Rowell C. H. F. (2000). Molecular phylogenetic analysis of the Pneumoroidea (Orthoptera, Caelifera): Molecular data resolve morphological character conflicts in the basal Acridomorpha. Molecular Phylogenetics and Evolution, 15 (3): 345-354.

Gündüz İ., Rambau R. V., Tez C., Searle J. B. (2005). Mitochondrial DNA variation in the western house mouse (*Mus musculus domesticus*) close to its site of origin: studies in Turkey. Biological Journal of the Linnean Society, 84: 473-485.

Hall T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41: 95-98.

Harrison R. G. (1989). Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. TREE, 4(1): 6-11.

Hartley S. E. (1997). The chemical composition of plant galls: are levels of nutrients and secondary compounds controlled by the gall- former? Oecologia, 113: 492-501.

Hasegawa M., Kishino H., Yano T. (1985). "Dating of human-ape splitting by a molecular clock of mitochondrial DNA". Journal of Molecular Evolution, 22: 160-174.

Hayward A., **Stone G. N. (2005).** Oak gall wasp communities: Evolution and ecology. Basic and Applied Ecology, 6: 435-443.

Hayward A., **Stone G. N. (2006).** Comparative phylogeography across two trophic levels: the oak gall wasp *Andricus kollari* and its chalcid parasitoid *Megastigmus stigmatizans.* Molecular Ecology, 15: 479-489.

Hewitt G. M. (1996). Some genetic consequences of ice ages, and their role in divergence and speciation. Biological Journal of the Linnean Society, 58: 247-276.

Hewitt G. M. (1999). Post-glacial re-colonization of European biota. Biological Journal of the Linnean Society, 68: 87-112.

Hewitt G. M. (2004). The structure of biodiversity-insights from molecular phylogeography. Frontiers in Zoology, 1 (4): 1-16.

Hickerson M. J., Carstens B. C., Cavender-Bares J., Crandall K. A., Graham C. H., Johnson J. B., Rissler L., Victoriano P. F., Yoder A. D. (2010). Phylogeography's past, present, and future: 10 years after Avise, 2000. Molecular Phylogenetics and Evolution, 54: 291-301.

Hoy M. A. (1994). Insect molecular genetics: An introduction to principles and applications. Academic Press Inc. CA, USA. p. 546.

Huelsenbeck J. P., and Ronquist F. (2001). MrBayes: Bayesian inference of phylogeny. Bioinformatics, 17: 754-755.

Jermin L. S., Crozier R. H. (1994). The cytochrome b region in the mitochondrial DNA of the ant *Tetraponera rufoniger*-a sequence divergence in Hymenoptera may be associated with nucleotide content. Journal of Molecular Evolution, 38: 282-294.

Katılmış Y., Kıyak S. (2008). Checklist of Cynipidae of Turkey with a new genus record. Journal of Natural History, 42(31-32): 2161-2167.

Kekeçoğlu M., Soysal İ. M. (2010). Genetic Diversity of Bee Ecotypes in Turkey and Evidence for Geographical Differences. Romanian Biotechnological Letters, 15: 5.

Kemal M., Kocak A. Ö. (2010). Winter trips to south Van Lake and the cynipid galls on oaks (Hymenoptera, Cynipidae). Cesa News, 56: 1-66.

Kıyak S., Kılıç T., Katılmış Y. (2008). A contribution to the knowledge of the Cynipidae fauna of Turkey (Hymenoptera: Cynipidae). Munis Entomology & Zoology, 3(1): 523-535.

Kimura M. (1983). The Neutral Theory of Molecular Evolution. Cambridge University Press, Cambridge.

Kyriazi P., Poulakakis N., Parmakelis A., Crochet P. A., Moravec J., Rastegar-Pouyani N., Trsigenopoulos C. S., Magoulas A., Mylonas M., Lymberakis P. (2008). Mitochondrial DNA reveals the genealogical history of the snake-eyed lizards (*Ophisops elegans* and *O. occidentalis*) (Sauria: Lacertidae). Molecular Phylogenetics and Evolution, 49: 795-805.

Lemire B. (2005). Mitochondrial Genetics in The *C. elegans* Research Community, WormBook ed. J. Hodgkin & P. Anderson.

Librado P., Rozas J. (2009). DnaSP v5: A Software for Comprehensive Analysis of DNA Polymorphism Data. Bioinformatics, 25: 1451-1452.

Loxdale H. D., Lushai G. (1998). Molecular markers in entomology. Bulletin of Entomological Research, 88: 577-600.

Mason W. R. M., Huber J. T. (1993). Order Hymneoptera in Hymneoptera of the World: An identification guide to families. Eds. H. Goulet & J. T. Huber. Canada Communication Group Publishing.

Meglecz E., Neve G., Katalin P., Varga Z. (1999). Genetic variation in space and time in *Parnassius mnemosyne* (L.) (Lepidoptera) populations in north-east Hungary: implications for conservation. Biological Conservation, 89: 251-259.

Melika G., Csoka G., Pujade-Villar J. (2000). Check-list of the oak gall wasps of Hungary, with some taxonomic notes (Hymenoptera: Cynipidae, Cynipinae, Cynipini). Annales Historico-Naturales Musei Nationalis Hungarici, 92: 265-296.

Melika G., Stone G. N. (2001). A new species of cynipid gall wasp from Turkey (Hymenoptera:Cynipidae). Folia Entemolica Hungarica, 62: 127-131.

Melika G., Ross-Farre P., Penzes Z. S., Pujade-Villar J. (2004). UFO Abei Melika et Pujade-Villar (Hymenoptera:Cynipidae:Synergini) new genus and new species from japan. Acta Zoologica Academiae Scientiarum Hungaricae, 51(4): 313-327.

Melika G. (2006*).* Gall wasps of Ukraine: Cynipidae. Schmalhausen Institute of Zoology, National Academy of Sciences of Ukraine, (2 vols).

Mutun S. (2010). Intraspecific genetic variation and the phylogeography of the oak gallwasp *Andricus caputmedusae* (Hymenoptera: Cynipidae): Effects of the Anatolian Diagonal. Acta Zoologica Academiae Scientarium Hungaricae, 56(2): 153-172.

Mutun S. (2011). Intraspecific genetic diversity and phylogeography of the oak gall wasp *Andricus lucidus* (Hymenoptera: Cynipidae) populations in Anatolia. Turkish Journal of Zoology, 35(4): 559-570.

Mutun S., Dinç S. (2011). Contributions to the gallwasp (Hymenoptera:Cynipidae) fauna of Turkey with one new record. Journal of Applied Biological Science, 5(3): 83-85.

Nguyen T. T. T., Ingram B., Sungan S., Gooley G., Sim S. Y., Tinggi D., De Silva S. S. (2006). Mitochondrial DNA diversity of brood stock of two indigenous mahseer species, *Tor tambroides* and *Tor dourenensis* (Cyprinidae) cultured in Sarawak. Aquaculture, 253: 259-269.

Posada D., Crandall K.A. (1998). MODELTEST: Testing the model of DNA substitution. Bioinformatics, 14: 817-818.

Posada D., Crandall K. A. (2001). Intraspecific gene genealogies: trees grafting into networks. Trends in Ecology and Evolution, 16: 37-45.

Posada D., Crandall K. A., Templeton A. R. (2006). Nested clade analysis statistics. Molecular Ecology, 6: 590-593.

Pujade-Villar J., Bellido D., Segú G. & Melika G. (2001). Current state of knowledgeof heterogeny in Cynipidae (Hymenoptera, Cynipoidea). Sessió Conjunta d'Entomologia de l'Institució Catalana d' História Natural i la Societat Catalana deLepidopterologia, 11(1999): 97-107.

Rokas A., Atkinson J. R., Nieves- Aldrey J-L., West A., Stone G. N. (2002). The incidence and diversity of *Wolbachia* in gallwasps (Hymenoptera; Cynipidae) on oak. Molecular Ecology, 11: 1815-1829.

Rokas A., Atkinson R. J., Webster L. M. I., Csöka G., Stone G. N. (2003a). Out of Anatolia: longitidunal gradients in genetic diversity support an eastern origin for a circum-Mediterranean oak gallwasp *Andricus quercustozae*. Molecular Ecology, 12: 2153-2174.

Rokas A., Melika G., Abe Y., Nieves- Aldrey J-L., Cook J. M., Stone G. N. (2003b). Lifecycle closure, lineage sorting, and hybridization revealed in a phylogenetic analysis of European oak gallwasps (Hymenoptera: Cynipidae: Cynipini) using mitochondrial sequence data. Molecular phylogenetics and Evolution, 26(1): 36-45.

Ronquist F. (1995). Phylogeny and early evolution of Cynipoidea (Hymenoptera). Systematic Entomology, 20(4): 309-335.

Ronquist F. (1999). Phylogeny, classification and evolution of the Cynipoidea. Zoologica Scripta, 28(1-2): 139-164.

Rubinoff D., Sternling F. A. H. (2004). Mitochondrial DNA sequence, morphology and ecology yield contrasting conservation implications for two threatened buck moths (Hemileuca: Saturniidae). Biological Conservation, 118: 341-351.

Sambrook J., Fritsch E. F., and Maniatis T. (1989). Molecular Cloning: A Laboratory Manual, vol. I. 2nd edition. Cold Spring Harbor Laboratory Press, ISBN 0-87969-309-6.

Shorthouse J. D., Rohfritsch O. (1992). Biology of insect-induced galls.Oxford University press New York.

Shorthouse J. D., Wool D., Raman A. (2005). Gall-inducing insects- Nature's most sophisticated herbivores. Basic and Applied Ecology, 6: 407-411.

Simon C., Frati F., Beckenbach A., Crespi B., Liu H., Flook P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America, 87(6): 651-701.

Stone G. N., Cook J. M. (1998). The structure of cynipid oak galls: patterns in the evolution of an extended phenotype. Proceedings of the Royal Society of London, 265: 979-988.

Stone G. N., Atkinson R. J., Brown G., Rokas A. (2002a). The population genetic consequences of range expansion: a review of pattern and process, and the value of oak gallwasps as a model system. Biodiversity Science, 10(1): 80-97.

Stone G. N., Schönrogge K., Atkinson J. R., Bellido D., Pujade-Villar J. (2002b). The population biology of oak gall wasps (Hymenoptera: Cynipidae). Annual Review of Entomology, 47: 633-668.

Stone G. N., Schönrogge K. (2003). The adaptive significance of insect gall morphology. Trends in Ecology and Evolution, 18(10): 512-522.

Stone G. N., Challis R. J., Atkinson R. J., Csöka G., Hayward A., Melika G., Mutun S., Preuss S., Rokas A., Sadeghi E. and Schröngrogge K. (2007). The phylogeographical clade trade: tracing the impact of human-mediated dispersal on the colonization of northern Europe by the oak gall wasp *Andricus kollari*. Molecular Ecology, 16: 2768-2781.

Stone G. N., Atkinson R. J., Rokas A., Nieves-Aldrey J-S., Melika G., Acs Z., Csoka G., Hayward A., Bailey R., Buckee C., McVean G. A. T. (2008). Evidence for widespread cryptic sexual generations in apparently purely asexual *Andricus* gallwasp. Molecular Ecology, 17: 652-665.

Stone G. N., Hernandez-Lopez A., Nicholls J. A., di Pierro E., Pujade-Villar J., Melika G., Cook J. A. (2009). Extreme host plant conservatism during at least 20 million years of host plant pursuit by oak gall wasps. Evolution, 63(4): 854-869.

Sun Z., Wan D.-G., Murphy R. W., Ma L., Zhang X.-S., Huang D.-W. (2009). Comparison of base composition and codon usage in insect mitochondrial genomes. Genes and Genomics, 31(1): 65-71.

Swofford D. L. (2002). PAUP*. Phylogenetic Analysis Using Parsimony. Sinauer Associates, Sunderland, Massachusetts.

Tamura K., Dudley J., Nei M., Kumar S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution, 24: 1596-1599.

Templeton A. R. (1998). Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. Molecular Ecology, 7: 381-397.

Templeton A. R. (2008). Nested Clade Analysis: an extensively validated method for strong phylogeographic inference. Molecular Ecology, 17: 1877-1880.

Vårdal H. (2004). From Insect Parasitoid to Gall Inducers and Inquilines. Morphological Evolution in Cynipoid Wasps. Acta Universitatis Upsaliensis. Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology, 932. 41 pp. Uppsala.

Von Dohlen C. D., Kurosu U., Aoki S. (2002). Phylogenetic and evolution of the eastern Asian-eastern North American disjunct aphid tribe, Hormaphidini (Hemiptera: Aphididae). Molecular Phylogenetics and Evolution, 23: 257-267.

Walker P., Leather S. R., Crawley M. J. (2002). Differential rates of invasion in three related alien oak gall wasps (Cynipidae: Hymenoptera)**.** Diversity and Distribution, 8: 335-349.

Washburn J. O. & Cornell H. V. (1981). Parasitoids, patches, and phenology: their possible role in the local extinction of a cynipid gall wasp population. Ecology, 62(6): 1597-1602.

Williams P. H., An J., Brown M. J. F., Carolan J. C., Goulson D., Jiaxing H., Masao I. (2012). Cryptic Bumblebee Species: Consequences for Conservation and the Trade in Greenhouse Pollinators. PLoSONE, 7(3): e32992. doi: 10.1371.

Wilson A. C., Cann R. L., Carr S. M., George M., Gyllensten U. B., Helmbychowsiur K. M., Higuchi G., Palumbi S. R., Prager E. M., Sager D., Stoneking M. (1985). Mitochondrial DNA and two perspectives on evolutionary genetics. Biological Journal of the Linnean Society. 26: 375-400.

Yaltırık F. (1982). Türkiye meşeleri teşhis kılavuzu. Yenilik basımevi, İstanbul.

Zhang X. D., & Hewitt M. G. (1996). Assessment of the universality and utility of a set of conserved mitochondrial COI primers in insects. Insect Molecular Biology, 6(2): 143-150.

Zucker M. (1989). Computer prediction of RNA structure. Methods in Enzymology, 180: 262-288.