INTRASPECIFIC MTDNA VARIATION AND PHYLOGENETIC STRUCTURE OF THE GALL WASP *ANDRICUS QUERCUSTOZAE* (HYMENOPTERA: CYNIPIDAE)

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by
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ABSTRACT

INTRASPECIFIC MTDNA VARIATION AND PHYLOGENETIC STRUCTURE OF THE GALL WASP *ANDRICUS QUERCUSTOZAE* (HYMENOPTERA: CYNIPIDAE)

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Intraspecific mitochondrial DNA variation and phylogenetic relationships of *Andricus quercustozae* haplotypes from Turkey was studied using PCR-RFLP method. An 2540 base pair mitochondrial DNA region covering ND4, ND4L, $tRNA^{Thr}$, $tRNA^{Pro}$, ND6, cyt b gene and a second 1800 base pair fragment comprising the ATPases (6, 8) and COIII amplified by PCR were digested with restriction enzymes including HindIII and HinfI, HaeIII, HindIII, EcoRI, respectively. A total of 28 haplotypes were detected among 95 individuals collected from 16 populations. The estimated average haplotype and nucleotide diversity within populations were 0.45 and 0.05, respectively. The highest nucleotide divergence estimate among the analyzed oak gall wasp populations was 9.0% indicating an ancient split between *A. quercustozae* haplotypes.

Dendrogram obtained using PHYLIP program depicting the phylogenetic relationships of haplotypes indicated that there was a significant relationship between geographical distribution of haplotypes and their clustering. AMOVA analysis for estimation of the partitioning of genetic differentiation at different

hierarchical levels was statistically significant. Analysis of variance revealed the highest genetic variance (38%) was present within groups, a significant partitioning of variance (25%) was found among groups and an intermediate level of variance was present within populations (35%).

Overall, the present study indicates that the oak gall wasp haplotypes found in different populations of *A. quercustozae* have significant amount of genetic diversity and they form geographically significant groupings. It can also be concluded that varied topography of Turkey plays an important role in shaping the current population genetic structure of the oak gall wasp species.

Keywords: Cynipidae, Oak Gall Wasp, *Andricus quercustozae*, Mitochondrial DNA, PCR-FRLP, intraspecific genetic diversity, phylogeography.

ÖZET

MAZI ARISI *ANDRICUS QUERCUSTOZAE* (HYMENOPTERA: CYNIPIDAE)' NİN TÜR İÇİ MTDNA VARYASYONU VE FİLOGENETİK YAPISI

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Bu çalışmada ülkemizde bulunan *Andricus quercustezae*'nin tür içi mitokondrial DNA varyasyonu ve bu türün haplotipleri arasındaki filogenetik ilişki PCR-RFLP yöntemi kullanılarak çalışılmıştır. Mitokondriyal DNAnın 2540 baz çiftlik ND4, ND4L, $tRNA^{Thr}$, $tRNA^{Pro}$, ND6, cyt b gen bölgelerini içeren bir kısmı ile 1800 bazlık ATPases (6, 8) ve COIII bölgeleri çoğaltılarak elde edilen PCR ürünleri sırasıyla HindIII ve HinfI, HaeIII, HindIII, EcoRI ile kesilmiştir. Onaltı populasyondan toplanan 95 bireyde toplam 28 haplotip bulunmuştur. Çalışılan türün populasyon içi ortalama haplotip ve nükleotid çeşitliliği sırasıyla 0.45 ve 0.05 olarak hesaplanmıştır. Çalışılan mazı arısı populasyonlarında *A.quercustozae* haplotipleri arasındaki en yüksek nükleotid farklılaşması %9.0 olarak bulunmuştur.

Haplotipler arasındaki filogenetik ilişkileri belirlemek için kullanılan PHYLIP programı sonucu elde edilen dendrogram haplotiplerin coğrafi dağılımları ile bu haplotiplerin gruplanmaları arasında anlamlı bir korelasyonun olduğunu ortaya koymuştur. Genetik farklılaşmanın farklı hiyerarşik düzeylerdeki dağılımını

belirlemek için uygulanan AMOVA analizleri istatistiksel olarak oldukça yüksek oranda desteklenmiştir. Varyans analizi en fazla çeşitliliğin grup içinde (%38), buna takiben populasyonlar içinde (%35) ve gruplar arasında (%25) olduğunu ortaya çıkarmıştır. Bu çalışma genel olarak ülkemizde farklı populasyonlarda bulunan meşe mazı arısı haplotiplerinin oldukça fazla bir genetik çeşitliliğe sahip olduğunu ve belirgin coğrafik bir şekilde grup oluşturduklarını ortaya koymuştur. Ayrıca meşe mazı arısı populasyonlarının mevcut populasyon genetik yapılarının şekillenmesinde ülkemiz topoğrafyasının önemli bir rol oynadığı sonucuna varılmıştır.

Anahtar Kelimeler: Cynipidae, Meşe Mazı Arısı, *Andricus quercustozae*, mitokondriyal DNA, PCR-FRLP, tür içi genetik çeşitlilik, filocoğrafya.

To my beloved uncle…

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CHAPTER 1

INTRODUCTION

Galls are abnormal growths composed of plant tissues within which gall inducing organisms continue its development until they reach maturity. They are formed as a result of complex interactions between gall-causing organisms and host plants. Various groups of organisms including roundworms, fungi, bacteria and insects have the ability to induce galls on different plant groups (Eroğlu, 2000). An estimated number of 13.000 insect species can initiate gall formation on such plants as Rosaceae, Compositae and Fagaceae family members and ninety percent of gall forming species forms galls on dicotyledonous plants (Oğurlu and Avcı, 1998).

Four insect taxa have ability to induce galls on various host plants. These are sawflies (Hymenoptera: Tenthredinidae), aphids (Homoptera: Aphididae), gall midges (Diptera: Cecidomyiidae) and oak gall wasps (Hymenoptera: Cynipidae) (Price, 2005). Oak gall wasps are grouped under family Cynipidae which is placed in the super family Cynipoidea. Oak gall wasps are from the tribe Cynipini which is one of the six tribes in the subfamily Cynipinae. Members of this subfamily include gall forming phytophagous wasps and some other gall wasps that their larvae have lost the ability to induce gall formation. An estimated 200 Cynipid species can not induce

galls, instead of gall induction their larva feed on galls of other cynipids. In other words, inquilines live commensally in the home of other species and they act as additional host resources for parasitoids. They generally attack rose and oak gallers but can also be seen in other galls on woody host plants (Ronquist and Liljeblad, 2001). Therefore at least three trophic levels of relationships can be observed among cynipids: cynipid galls provide supplies for rich and closed communities characterised by natural enemies, predominantly chalcid Hymenopteran parasitoids, and a distinct set of inquilines, hyperparasitoids and superparasitoids (Hayward and Stone, 2006).

1.1. Gall Structure and Diversity

Cynipini is the largest tribe in the subfamily Cynipinae with approximately 1360 defined species under 27 genera but the actual number is estimated to be around 6000 (Liljeblad and Ronquist, 1998). Gall wasps mainly distribute in the temperate regions of the Northern Hemisphere predominantly in the Holarctic area. Oak cynipids show highest diversity in the Nearctic region with around 700 species followed by Europe with 200 species (Hayward and Stone, 2006). Some gall forming Cynipid genera are very large such as 300 species in *Andricus*, 150 species in *Callirhytis*, and 100 species in *Neuroterus* genus have been described so far. Oak gall wasps show enormous radiation among other cynipids forming the second largest insect group with 1300 identified species (Price, 2005).

Galls of oak gall wasps contain tissue types that are very different from ungalled host plant tissue and they represent the extended phenotypes of gall-inducer genes (Stone *et al*., 2002). Galls show extremely various phenotypes from open pits to totally enclosed structures. The most complex gall structures and the greatest

diversity of cynipid galls is a result of variation in gall tissues in the forms of woody or spongy tissue, extrafloral nectarines, complex air spaces within the gall, and surface coating or sticky resin materials, hairs and spines. Cynipid galls can be formed on all plant organs such as flowers, leaves, buds, twigs and roots. They include from one to more than a hundred larval chambers inside a gall (Ronquist and Liljeblad, 2001).

Gall induction begins with the insects laying their eggs on the surface of plant. Although the molecular basis of gall induction remains unknown either mechanical damage or salivary secretions of gall inducing organism initiates increased production of normal plant hormones which result in increase in cell size and / or cell number. Cynipid galls can be composed of two parts: the larval chamber and the outer gall. The larval chamber is similar in all cynipid galls and can include either a single chamber (unilocular) or up to hundred larval chambers (multilocular). The larval chamber is surrounded with a thin layer of nutritive tissue; continue with the vacuolated parenchyma which is destined to become nutritive tissue further in development. A hard layer of sclerenchyma encloses the nutritive tissue for serving protection and also vascular bundles connect the gall to the plant vascular system (Stone and Cook, 1998; Stone *et al*., 2003).

Galls provide nutrients, shelter and protection to the inducer (Shorthouse *et al.*, 2005). They are developed as a result of interactions between the inducing insect and host plant and the insect gains the control of growth and physiology of attacked organs to its advantage. Therefore galls are accepted as the most complicated association between insects and plant species. Several hypotheses were used to explain gall diversity and the evolution of gall formation. Of the hypotheses that

have been advanced for the adaptive significance of gall induction three of them were frequently accepted as relevant to explain gall morphology and its evolution: the enemy hypothesis, the nutritive hypothesis and the microenvironment hypothesis. The enemy hypothesis states that galls are used as shelters by developing larvae against natural enemies such as nonspecialist predators and pathogens. In entirely closed galls, all attacks take place through the gall tissues and selection favors any modifications of gall morphology that increase the galler survival. Increased thickness of gall tissue, gall hardness and external coatings of hairs significantly diminish the enemy attacks and increase the galler survival chance. On the other hand, the nutrition hypothesis explains that galls provide nutrients and fluids necessary for developing larvae inside the larval chamber. The last hypothesis states that gall tissues play an important role to protect the galling insect from unfavorable environmental conditions such as desiccation (Stone *et al*., 2002).

1.2. The Study Species: *Andricus quercustozae* (Bosc, 1792)

Andricus (Hartig, 1840) is a Holartic genus of oak cynipid gall wasps with representatives in Eurasia and in North America. Around 80 species are known from the western Palearctic, particularly from Europe and Turkey (Melika *et al.*, 2004). A recent study reported the presence of 21 *Andricus* species from Turkey (Kıyak *et al*. 2008). This genus shows significant diversity in both host-plant use and gall location along variation in gall form. Different *Andricus* species induce highly characteristic and complex gall structure, and the genus is known to form greatly diversified galls on their host plants primarily on *Quercus* and related genera in the family Fagaceae (Cook *et al.*, 2002).

Most of the members of the genus *Andricus* include lifecycles with two generations each year which may be associated with host alternation (heteroecy) and cyclical parthenogenesis (heterogony): a sexual generation in the spring and a parthenogenetic generation in the summer / autumn. In host alternating *Andricus* species, asexual generations of *Andricus* species can be seen on white oaks such as *Q. infectoria, Q. petraea*, *Q. pubescens and Q. robur*. In contrast, sexual generations prefer either white or black oaks including *Q. cerris* and *Q. suber*. Sexual generations of most *Andricus* species attack buds or catkins and parthenogenetic generations form gall on buds, with very few on acorns, catkins or roots (Stone *et al.*, 2002).

Andricus quercustozae (Bosc, 1792) is a widely distributed oak gall wasp species extending from Morocco through Turkey to Iran (Rokas *et al.*, 2003). Hard-shelled and unilocular asexual generation galls are induced by the members of this species on the buds of oak trees in the late summer and early fall. The asexual galls of this species in Turkey are bright reddish brown and very sticky when mature, however the galls of the same species formed by the western populations have matt brown and non-sticky surface. Becuase of such differences in gall morphological characteristics, members of the eastern and western populations of the species across its range is sometimes called as insana form although reared adults from the two gall forms do not significantly differ from each other (Melika *et al.*, 2000; Rokas *et al*., 2003).

1.3. Mitochondrial DNA and PCR-RFLP Method

Mitochondrial DNA (mtDNA) is maternally inherited circular genome ranging from 15.0 to 17.0 kilobases (Kbp), although sizes of 14-26 Kbp have been

found in different insect species due to differences in the copy number of short, tandemly repeated sequences or the duplication or deletion of sequences (Crozier and Crozier, 1993). In animals, the mitochondrial genome contains 37 genes and a control region that is, in insects, called as the A+T- rich region (Harrison, 1989; Zhang *et al.*, 1995). Among the 37 genes, there are 13 protein encoding genes, 22 tRNA genes and 2 ribosomal RNA genes (Moritz *et al.* 1987; Harrsion, 1989). Gene order variation occurs both between and within animal phyla but within insects, tRNA genes are known to vary in position both between the orders Diptera and Hymenoptera (Crozier and Crozier, 1993).

MtDNA has been extensively used as a molecular marker in various animal groups (Reeb and Avise, 1990; Perez-Suarez *et al.*, 1994; Verspoor *et al.*, 1999; Duran and Rützler 2006; Gündüz *et al.* 2005; Hayward and Stone, 2006; Horn *et al.*, 2006; Jang-Liaw *et al.*, 2008). Small size, relatively rapid rate of evolutionary change, maternal inheritance and the absence of recombination make mtDNA an ideal marker for diverse evolutionary studies including phylogenetic inference, identification of species origin, phylogeography, analysis of population history and structure among closely related insect taxa at both intraspecific and interspecific levels (Avise, 1994; 2000). Thus, analysis of mtDNA divergence can provide detailed information about phylogenetic relationships at different levels in the evolutionary hierarchy using polymorphism present in this molecule (Schiff *et al.*, 1994; Seddon *et al.*, 2002; Tzeng, 2007). The interregional variation in the evolutionary rate of mtDNA is particularly useful because it facilitates the choice of appropriate genes and techniques for phylogeographic or phylogenetic studies (Avise *et al.*, 1987).

Polymorphisms in mtDNA molecule can be detected as insertions and deletions, presence or absence of restriction sites, and changes of nucleotide sequence (Chapco, 1997). Examining mtDNA genome variation was facilitated by the use of polymerase chain reaction (PCR). Combined with the use of conserved or universal mtDNA primers (primers that amplify the same region from different species) has greatly increased the speed at which data can be obtained and the number of individuals or groups that can be sampled (Simon *et al.*, 1994).

Polymerase chain reaction products of specific genes have been widely applied in phylogenetic and phylogeographic studies in diverse groups of animals. Associated with PCR amplification of gene fragments, DNA restriction fragment length polymorphism (RFLP) is a frequently used molecular method for detecting any base substitutions or insertion / deletion events. Each specific restriction enzyme recognizes a unique sequence of nucleotides of DNA strand (Loxdale and Lushai, 1998). Therefore, the use of restriction endonucleases are preferred to identify individuals in a population or a species based on unique patterns of restriction enzyme cutting in specific regions of DNA. Any mutations that are present between individuals can create or destroy the sites recognized by specific restriction enzymes causing variation between individuals in the length of restriction fragments. The fragments produced by digestion of amplified PCR product by different restriction enzymes are sorted according to their size and / or conformation by gel electrophoresis (Hwang *et al.*, 2001). Although PCR-RFLP technique is not certainly as good as sequencing, it is much cheaper and quicker, especially for a large sample size.

Since restriction endonucleases cleave double stranded DNA at specific recognition sites producing a series of fragments, the number and sizes of which vary depending on where within the DNA recognition sequence occurs. Thus banding profile creates different patterns that can be conveniently served as markers of genetically distinct lineages. Simple measures of genetic distance can be derived from comparison of restriction fragment patterns. Each fragment is considered as a character having two states as present or absent thereby providing a data set appropriate for phylogenetic and phylogeographic analysis (Loxdale and Lushai, 1998). Thus, the analysis of restriction length polymorphisms of mtDNA has been extensively used for revealing extensive intraspecific variation in a number of species including insects (Martin and Simon, 1990; Fransisco *et al.*, 2001; Gross *et al.*, 2002; Alifakiotis *et al.*, 2003; Weinlich *et al.*, 2004; Cheng and Lu, 2005; Bardakçı et al., 2006).

1.4. Phylogeography and Application of mtDNA in Phylogeographic Studies

Animal populations are subject to being genetically structured by isolating factors such as the rise of a mountain range, the splitting of land masses and glaciations which result in vicariance. Both historical events experienced by a species and its ecological requirements shape the current genetic structure (Avise *et al.*, 1987). Populations separated by vicariant events may become genetically differentiated through the evolutionary time. The outcome of this separation is a function of rate of evolution and the duration of the population isolation.

The evolutionary subdivision of a species is most likely to be resolved by studying mtDNA variation. Since mtDNA has a high mutation rate compared to single-copy nuclear DNA and under equilibrium conditions its maternal inheritance gives it an effective population size one quarter that of nuclear DNA. Therefore, population differentiation is expected to evolve more rapidly for mtDNA than nuclear DNA making it to be more sensitive indicator of population structuring. Moreover, mtDNA is not subject to recombination and variants are inherited as haplotype clones which can be ordered phylogenetically in a way which reflects the evolutionary genealogy of the variants (Avise, 1994).

Combined with the observed distribution of haplotype variation, these characteristics of mtDNA can provide important insight into the patterns and processes associated with the development of current population structuring which is termed as phylogeography (Avise, 2000). Phylogeography is a field of study concerned with the principles and underlining processes determining the geographical distribution of genealogical lineages, including those within species (Avise, 2006). It is useful in understanding processes such as population subdivision, population expansion and contraction and for revealing the signature left on the genetic structure of populations by the major historical factors (Avise *et al.*, 1987; Bermingham and Moritz ,1998).

Mitochondrial DNA has been efficiently used as a molecular marker for phylogenetic and phylogeographic analysis of both populations and species. Many studies have been conducted on phylogeography of various groups of animals including oak gall wasps showed that mtDNA haplotypes were geographically clustered into different groupings depicting general patterns of their distribution in a

given area (Clemente *et al.*, 2000; Sert *et al.*, 2004; Horn *et al.*, 2006; Challis *et al.*, 2007; Stone *et al.*, 2007; Stone *et al.*, 2008).

1.5. Geologic History of Turkey

Turkey is one of the most important places in the world due to not only its plant and animal species richness but also its genetic diversity present in these species. The reasons of Turkey having such great species and genetic diversity are because of heterogenous topography, varied climates, and the presence of diverse phytogeographical regions, its geologic location between Europe and Asia and very complex geologic history of the area which is the result of the collision between these major continents (Avcı,1993).

The current structure of Turkey was shaped by several geologic events. These geologic processes include the signatures of active continental margins and subduction which date back the Paleozoic, the closure of Tethyan oceans, and a sequence of continent collisions before a period of extension which began in the late Cenozoic (Rögl, 1998; Görür and Tüysüz, 2001). The collision of the Arabian plate with Eurasia and the terminal closure of Neothetyan Ocean lasted in the time interval of late Pliocene-Quaternary and did not change much in land-sea configuration and topography after the Pliocene (Bozkurt, 2001). The most important consequences of these events for Turkey were the fusion of the seven Anatolian subplates, upfolding of the Taurus mountains and the uplift of the Central Anatolian highlands (Kosswig, 1955). Furthermore, Anatolia was repeatedly affected by fauna and flora exchanges because of its specific location at the interface of the European, Asian and African

biomes from where it experienced repeated invasions since the late Oligocene (Weith *et al.*, 2003). Anatolia is therefore accepted as one of the most important areas for the colonization of Europe by animals and plants served primarily as a land bridge for African mammals during late Oligocene and the early Miocene.

In addition to these previous events, cyclical cooling processes of glacial periods that have started in the Pliocene and continued through Pleistocene had enormous effects on creating the contemporary population genetic structure of many species currently present in Turkey. About 2.6 million years ago (MYA) the arctic ice sheets formed and Quaternary climatic fluctuations were associated with enormous temperature shifts of 7-14 ºC (Hewitt 1996; 1999). With each oscillation species either went extinct over most of their distribution range, dispersed into new locations or separated and isolated into refugia (Hewitt, 2000). The primary effects of such climatic changes on a species are range shifts including contraction of the distribution range of the species. It is well known that species contracted their range during the cold periods of glacial cycles into suitable environments and used some areas as shelter (refuge) in order to escape from the harsh conditions of environment and expand their range during the interglacials (Hewitt, 1996; 2000; Bennett and Provan, 2008). The impact of Pleistocene climatic fluctuations on European biota is well known and it was documented that Turkey provided both animal and plant taxa at both species and genetic level. A number of phylogeographic studies have revealed that Anatolia acted as a refuge and source area for recolonization of Europe after the Pleistocene glaciations (Hewitt, 2000; Seddon *et al.*, 2002; Rokas *et al.*, 2003).

During the last glacial cycles of Pleistocene many species which currently show distribution across Europe had their refuges in the southern and southeastern parts including the Balkans and Turkey. When the climate warmed these species that used Anatolia as a shelter expanded from this area and spread in to north and west rapidly (Petit *et al.*, 2002). Thus recent studies on European biota clearly indicate the importance of Turkey and it is thus accepted as a source area for genetic variation for many species that are currently distributed in Europe.

An additional factor that shaped the current species composition and genetic diversity in many plants and animals in Turkey is the Anatolian Diagonal. This mountain belt was proposed as a significant geographic barrier shaping the comtemporary species diversity across Turkey and dividing species / lineage distribution into east and west (Davis, 1971; Çıplak *et al.*, 1993; Çıplak, 2004). The Anatolian Diagonal as a line of mountain ranges runs from the southern part of Gümüşhane - Bayburt toward the southwest of Turkey to the Taurus Mountains (Davis, 1971; Ekim and Güner, 1986). A number of previous studies of various taxa based on the species distribution and composition in the area suggested that together with the Tertiary history of Turkey, the Anatolian Diagonal might have influenced the species distributed in Turkey creating a break at specific and generic level (Çıplak *et al.*, 1993; Çıplak, 2003). As for the Diagonal several other altitudinal belts in Anatolia were suggested either to fragment species / lineage distribution or to provide distribution for their east-west or north-south expansion (Çıplak, 2008). Thus, defining range distributions of lineages or genetic structuring of individual species has special importance in understanding biogeography of Anatolia.

1. 6. Objectives

The species used in this thesis is the oak gall wasp *A. quercustozae* which shows a large distribution range in Turkey. Since this species is found on either sides of the Diagonal it can be a good model species i) to reveal the genetic diversity present across the species range in Turkey, ii) to explore phylogenetic structure of *A. quercustozae* in Anatolia, iii) to reveal the possible influences of the major historical events on the distribution patterns of genetic variation of the oak gallwasp species across Turkey, iv) to test the effects of the Anatolian Diagonal or other altitudinal belts whether they in fact acted as geographical barriers to shape the phylogeographic structure as it was suggested by many previous works using the distribution patterns of both plant and animal species in Anatolia (Davis, 1971; Ekim and Güner, 1986; Çıplak *et al.*, 1993). For these purposes, a mitochondrial fragment length variation using PCR-RFLP method was used to draw conclusion on the aspects given above for oak gallwasp species *A. quercustozae*.

CHAPTER 2

MATERIAL AND METHODS

2.1 Sample Collection

A total of 95 specimens of *Andricus quercustozae* were collected in the summer of 2006-2008 from 16 locations covering most of the distribution range of the species in Turkey. Sampled populations and their coordinates are given in Figure 1 and Table 1, respectively. All specimens were stored at -80 ºC until DNA isolation.

2.2. DNA Isolation

Single gall wasp individuals were used for extraction of total genomic DNA using the DNeasy Tissue Kit (QIAGEN). Individual gall wasps kept at -80 ºC freezer were placed in 1.5 ml microcentrifuge tubes. For macerating the insect into smaller pieces each specimen was overlayed with 180 µl ATL buffer and 20 µl of proteinase K was added to the mixture. The tubes were vortexed and the samples were incubated overnight at 56 ºC in a shaking water bath until the tissue was completely lysed. The incubated samples were vortexed for 15 seconds and 200 µl of AL buffer

was added to the sample and mixed thoroughly. After adding 200 µl of 100% ethanol the tubes were mixed again thoroughly. The sample was transferred into a DNeasy Mini spin column in a 2 ml collection tube and it was centrifuged at 8000 rpm for 1 minute. After discarding the flow-through the spin column was placed in a new 2 ml collection tube and 500 µl of buffer AW1 was added to each tube and they were centrifuged for 1 minute at 14000 rpm and the same steps were repeated for obtaining clean sample. After the final centrifugation step the spin column was transferred to a new microcentrifuge tube. For elution 200 µl of buffer AE was added to the sample and incubated for 5 minutes at room temperature. In the final stage for obtaining DNA the samples were centrifuged for 1 minute at 8000 rpm. The extracted DNA samples were checked by gel electrophoresis using % 1 agarose (Sigma) containing ethidium bromide with 1x TBE running buffer (0,089 M Tris, 0,089 M Boric acid, 0,001 M disodium EDTA), visualized under UV light and photographed.

2.3. Mitochondrial DNA Gene Amplification

The size of the amplified PCR- products for two regions is approximately 2540 and 1800 base pairs (bp), respectively. A c. a. 2.5 Kbp mitochondrial fragment spanning the ND4, ND4L, $tRNA^{Thr}$, $tRNA^{Pro}$, ND6 regions and a part of the cytochrome b gene was amplified using primers ND4 and CBN (Simon *et al.*, 1994) and the mt19 and mt22 (Moretto and Arias, 2005). The primer pairs for amplification of these genes were used previously in other Hymenoptera species (Fransisco *et al.*, 2001; Moretto and Arias, 2005). Primer sequences for both mtDNA fragments are as follows: CBN 5′- ATTAC ACCTCCTAATTTATTAGGAAT-3′, ND4 5′-GGAGCTTCAACATGAGCCT-3′ (Simon *et al.*, 1994) and mt19 5′- GAAA

TTTGTGGAGCAAATCATAG-3′, mt22 5′-TCAACAAAGTGTCAGTATCA -3′ (Moretto and Arias, 2005). All PCR reactions were carried out in 25 µl volumes containing 0.5 µl of the total DNA extraction, 2.5 µl 10X PCR buffer (Promega), 2.0 µl $MgCl_2$ (25mM), 1.0 µl dNTPs (2mM each), 0.75 µl of each primer (20µM) and 1.25 U of Taq DNA Polymerase (Promega). Thermocycling was performed in a thermal cycler (Techne, UK) using the following program: 5 min at 94 ºC, 35 cycles of 1 min at 94 ºC, 1 min 20 s at 44 ºC, 2 min at 64 ºC, and a final extension step of 10 min at 64 ºC. PCR products were visualized on 1% agarose gels buffered with Tris-Boric acid-EDTA (TBE), stained with ethidium bromide, and visualized under UV light.

2.4. Digestion with Restriction Enzymes

Restriction fragment length polymorphism (PCR- RFLP) analysis was performed on two polymerase chain reaction amplified mtDNA fragments encompassing c.a.2.5 Kbp and 1.8 Kbp regions. Restriction digestion of the amplified mtDNA regions were performed with restriction enzymes (HinfI, HindIII, EcoRI and Hae III: MBI Fermentas and TAKARA) according to the manufacturers instructions. The same restriction endonucleases were also used in previous works in other insects (e.g. Fransisco *et al.*, 2001; Moretto and Arias, 2005). Restriction fragments were separated by electrophoresis in 1% agarose gels containing ethidium bromide with 1x TBE running buffer, visualized under UV light and photographed.

2.5. Data Analysis

Different restriction patterns for each restriction enzyme were assigned a letter as they were observed. The presence or absence of restriction sites was inferred

for each enzyme from completely additive fragment patterns, and each individual insect was designated a composite haplotype based on the observed RFLP patterns. Two outgroup species, *A. caputmedusae* and *A. lucidus*, were used in all the following analyses to give a direction to the evolutionary changes in the ingroup species.

Haplotype diversity for each population was calculated from haplotype frequencies (Nei, 1987). Nucleotide diversity within each population was calculated based on restriction sites between all haplotypes (Nei and Li, 1979). Divergence among populations was estimated according to Nei and Tajima (1981) using the DA program contained in the software REAP (McElroy *et al.*, 1991). Nucleotide-sequence divergence among haplotypes (d_{ij}) and the number of net nucleotide substitutions between all pairs of populations (d_A) were calculated for restriction site data according to Nei (1987). The matrix of pair wise d_A values was used to construct a UPGMA dendrogram showing relationships among populations, using the NEIGHBOR program from the PHYLIP, ver. 3.5c package (Felsenstein, 1992). From the basic presence-absence matrix of restriction sites for each haplotype generated by the program REAP GENERATE, the data were bootstrapped with 1000 replicates using the PHYLIP SEQBOOT program. Unrooted DOLLO parsimony trees were constructed using the PHYLIP DOLLOP program. From these trees, the majority rule consensus tree was obtained using the PHYLIP CONSENSE program. The average number of nucleotide substitutions per site between haplotypes was used to obtain a neighbour joining tree using the PHYLIP NEIGHBOR program. The degree of geographic heterogeneity of mtDNA haplotype distributions was assessed

using χ^2 statistics (Roff and Bentzen, 1989). The significance level was tested by Monte Carlo simulations using the Monte routine from the REAP package.

The partitioning of molecular variation was revealed by analysis of molecular variance (AMOVA) implemented in the program ARLEQUIN 3.1 (Schneider *et al.*, 1996). AMOVA analysis allows the user to test for genetic structure by comparing results from various *a priori* groupings to be assigned and choosing the structure that maximizes the among group variance. A hierarchical analysis of variance partitions the total variance into covariance components due to intra-individual differences, inter-individual differences, and/or inter-population level differences. Thus, AMOVA estimates genetic distances based on pair wise F_{ST} indices between all pairs of populations (Excoffier *et al.*, 1992). The F_{ST} statistics was tested for significance using 10000 permutations. Related F_{ST} statistics are defined as follows: F_{CT} is the correlation of random haplotypes within a population group, relative to that of random pairs of haplotypes from the entire data set; F_{SC} is the correlation of molecular diversity of randomly selected haplotypes within populations, relative to that of random pairs of haplotypes from within the region; F_{ST} is the correlation of randomly selected haplotypes within populations, relative to that of random pairs of haplotypes from the entire data set. AMOVA conducted on the whole data set was performed using the groupings obtained by UPGMA dendrogram of populations. The partitioning of the variation was tested for the i) among groups, ii) among populations within groups, and iii) within populations (Excoffier *et al.*, 2005).

CHAPTER 3

RESULTS

A total of 95 specimens collected from 16 localities were used in this study (Figure 1 and Table 1). Number of specimens varied between 2 to 11 individuals for each population. During the field trips conducted between 2006-2008 *Andricus quercustozae* was observed to prefer mostly *Q. infectoria* and *Q. petraea*, and less frequently *Q. robur* and *Q. pubescens* oaks as possible hosts to induce its species and generation specific galls. The asexual generation galls collected between Augusts – September were dark brown indicating the time of the larva being close to maturation (Figure 2 and 3). When the galls brought to the laboratory specimens were taken out of the gall and they were checked for identification. Most galls had single specimen inside the larval chamber but rarely two individuals were observed within the chamber. The galls with more than one individual inside the chamber were eliminated from further work in an effort to prevent parasitoid or other contaminating factor.

From each gall total DNA was extracted and kept at -20ºC until DNA use in the amplification procedure. The total size of the isolated mtDNA of *A. quercustozae*

is approximately 16 Kbp and the photographs of isolated mtDNA are shown in Figure 4a and 4b. In this study, the two mtDNA gene fragments are 2.5 Kbp and 1.8 Kbp in their size, respectively. The first segment used for PCR-RFLP analysis covering the amplified ND4, ND4L, $tRNA^{Thr}$, $tRNA^{Pro}$, ND6 region is shown in Figure 5a and 5b. The amplified PCR products of the second region including cytochrome b and ATPases (6, 8), COIII genes are given in Figure 6.

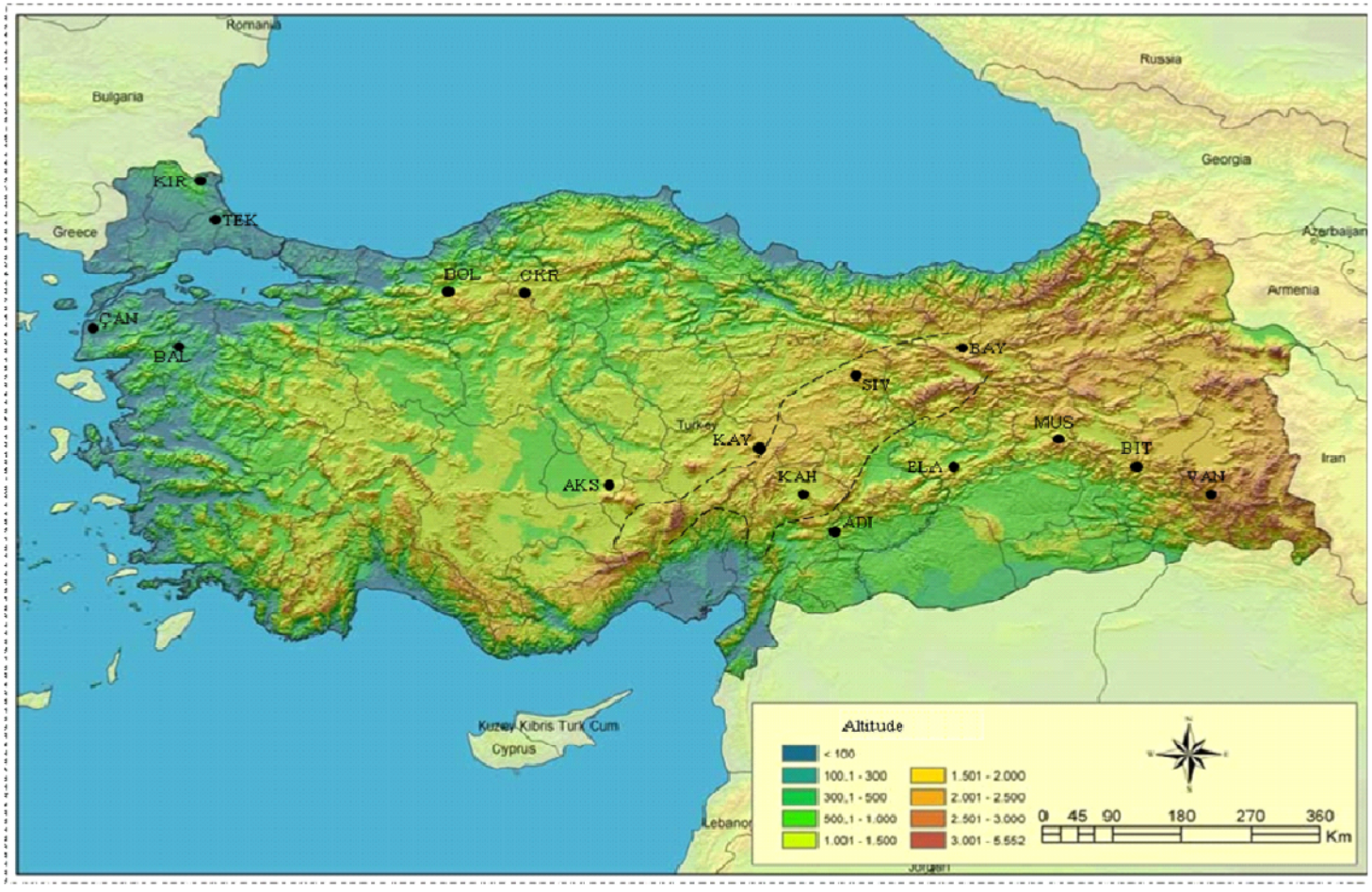


Figure 1. Sampling sites for *Andricus quercustozae*. Population abbreviations and coordinates are shown in Table 1.

Table 1. Localities of sampled populations of *A. quercustozae* with their abbreviations and coordinates.

Population	**Abbreviation**	**Locality**	**Coordinates**
1. Adıyaman	ADI	Besni	N 37^0 45.869' E 37^0 43.136'
2. Aksaray	AKS	Hasan Mountain	N 38^0 09.931' E 34^0 11.463'
3. Balıkesir	BAL	Edremit	N 39^0 35.623' E 27^0 04.082'
4. Bitlis	BIT	Baykan	N 38^0 21.019' E 42^0 02.412'
5. Bolu	BOL	Gölköy	N 40^0 40.380' E 31^0 25.991'
6. Çanakkale	CAN	Üvecik	N 39^0 53.213' E 26^0 11.747'
7. Çankırı	CKR	Çerkeş	N 40^0 32.138' E 32^0 38.418'
8. Elazığ	ELA	near the Hazar Lake	N 38^0 29.886' E 39^0 22.599'
9. Kırklareli	KIR	İğneada	N 41^0 56.657' E 27^0 40.765'
10. Muş	MUS	Güroymak yolu	N 38^0 36.793' E 41^0 56.529'
11.Tekirdağ	TEK	Saray	N 41^0 25.829' E 28^0 05.673'
12.Van	VAN	Çatak	N 37^0 55.015' E 42^0 57.828'
13. Kahramanmaraş	KAH	Göksun	N 37^0 43.514' E 36^0 40.038'
14. Bayburt	BAY	Aşkale yolu	N 40^0 05.385' E 40^0 25.327'
15. Sivas	SIV	Zara	N 39^0 56.082' E 37^0 51.828'
16. Kayseri	KAY	Pınarbaşı	N 38^0 40.512' E 36^0 19.973'

Figure 2. *Andricus quercustozae* asexual gall formed on a host tree.

Fıgure 3. *Andricus quercustozae* asexual gall.

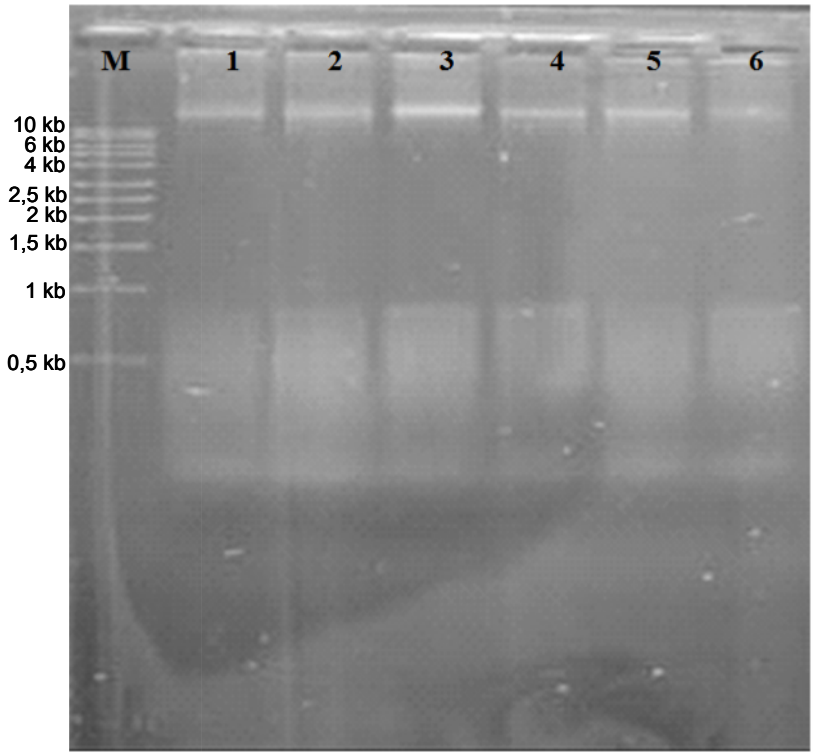


Figure 4a. Isolated mtDNA samples from different oak galls wasp individuals. M: 10 Kb DNA marker.

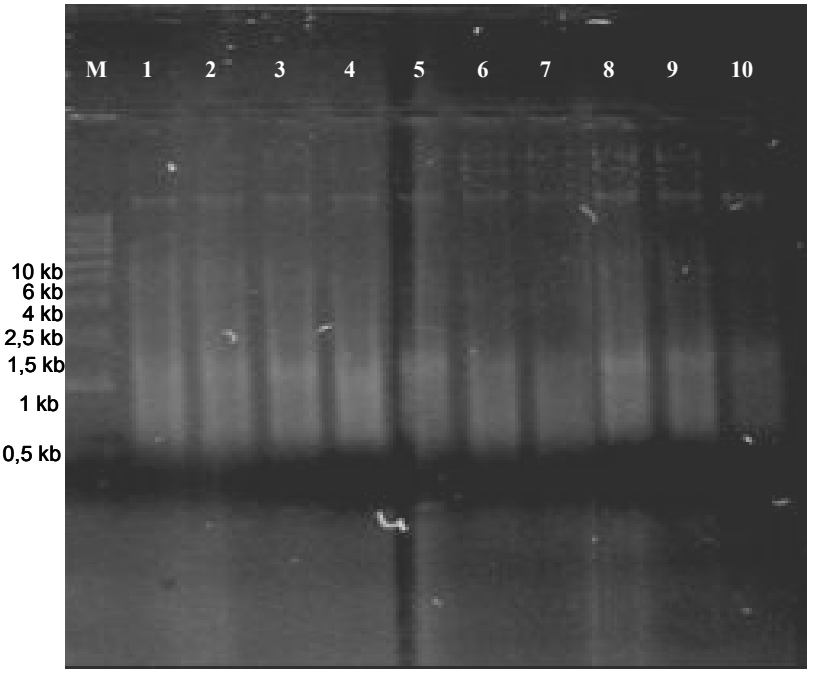


Figure 4b. Isolated mtDNA samples from different oak galls wasp individuals M: 10 Kb DNA marker.

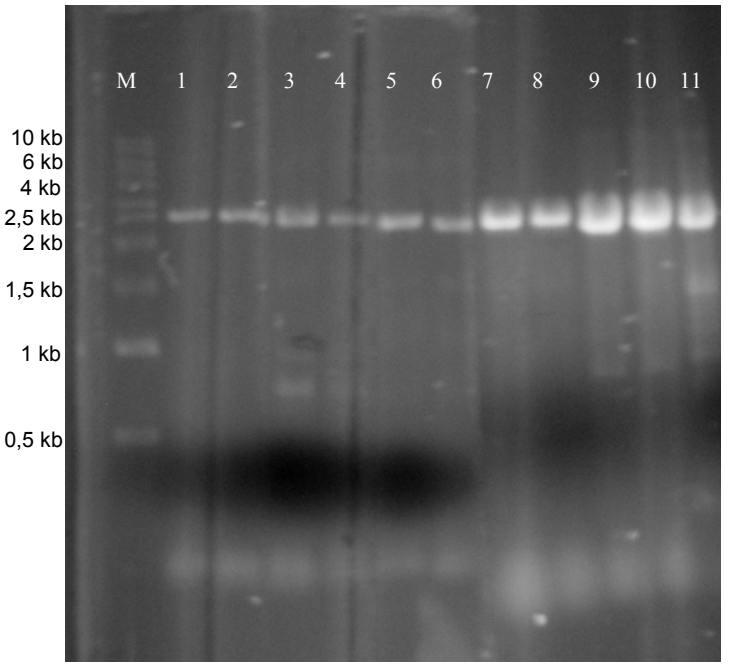


Figure 5a. Agarose gel photograph of the amplified 2.5 Kbp region of *A. quercustozae* mtDNA. M: 1 Kb λ DNA marker, Samples: 1: Balıkesir, 2: Kırklareli, 3: Çanakkale, 4-6: Çankırı, 7-8: Sivas, 9-10: Bayburt, 11: Bitlis.

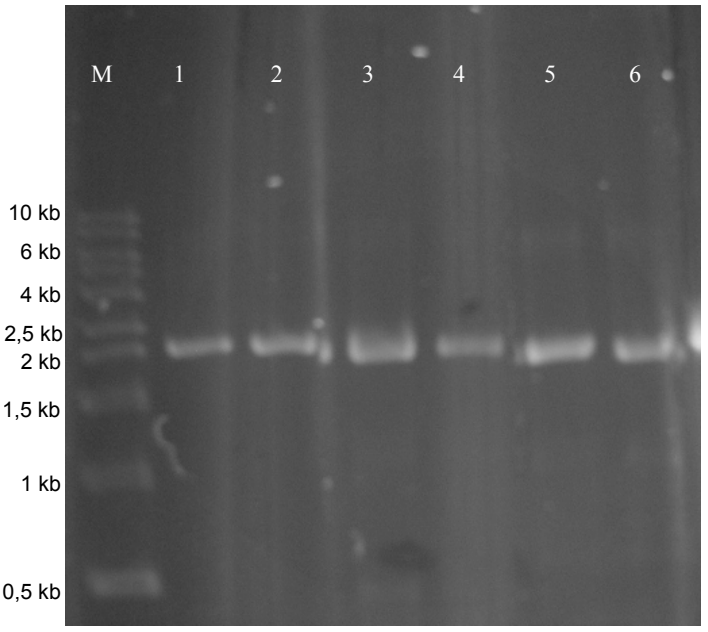


Figure 5b. Agarose gel photograph of the amplified 2.5 Kbp region of *A. quercustozae* mtDNA. M: 1 Kb λ DNA marker, Samples: 1-2: Kayseri, 3: Elazığ, 4: Van, 5: Malatya, 6: Bolu.

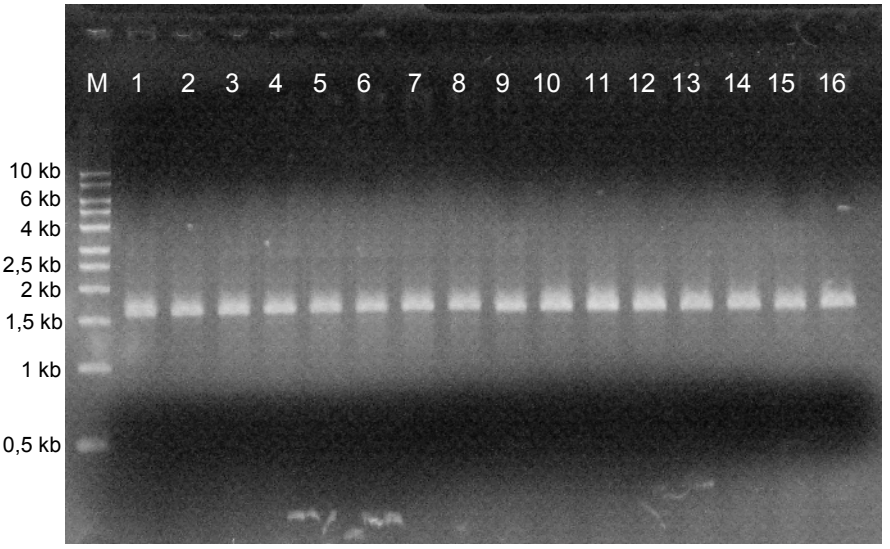


Figure 6. Agarose gel image of the amplified 1800 bp region of *A. quercustozae* mtDNA. M: 1 Kb λ DNA marker, Samples: 1: Adıyaman, 2: Aksaray, 3: Balıkesir, 4-5: Bolu, 6: Elazığ, 7-8: Van, 9: Bayburt, 10: Sivas, 11: Kayseri.

3.1. Mitochondrial DNA Variation within and among *Andricus quercustozae* Populations

A total of 28 composite haplotypes were detected from all specimens of *A. quercustozae* collected from 16 populations sampled across most of the distribution range of the species in Turkey. The mtDNA fragments from 95 individuals had 67 recognition sites for the restriction enzymes used in this study representing 270 nucleotides. The first analyzed mtDNA fragment possesed three recognition sites with Hind III restriction enzyme. Two haplotype profiles as A and B was obtained with this enzyme (Figure 7 and Figure 15). The second analyzed mtDNA gene region of *A. quercustozae* digested with Hinf I produced twelve distinct haplotype pattern

from A to K (Figures 8-9 and Figure 16). The same region had eight different haplotype patterns when the PCR products cut by Hae III enzyme. The photographs of digested PCR products of some *A. quercustozae* samples are shown in Figures 10-12 and the haplotype patterns are given in Figure 17. The other enzymes used to cut the amplified PCR products are Hind III generated three haplotype pattern as A, B and C (Figure 13 and 18), and EcoR I that produced only a single haplotype pattern (Figure 14 and 18).

The composite haplotypes and their frequencies in each studied population are shown in Table 2. The most common haplotype was Type 6 detected in 21 individuals from 5 populations (Aksaray, Bitlis, Bolu, Kırklareli and Tekirdağ). Based on frequency of haplotypes, Type 8 was found in 8 individuals from 2 populations (Tekirdağ and Kayseri). Among the twenty eight detected haplotypes, Type 9, 17 and 20 were found to be the third common haplotypes, each of which were present in 6 individuals. Type 9 was detected in 2 populations (Bayburt and Sivas). However Type 17 and 20 were fixed to a single population, Çanakkale and Çankırı populations, respectively. Among all determined haplotypes eleven were private (Type 1, 3-5, 11, 19 and 24-28), that is to say observed in only one individual. On the other hand, based on haplotype number observed in sixteen examined populations Adıyaman and Muş had four haplotypes, Aksaray, Balıkesir Bolu and Tekirdağ had three haplotypes. Only one or two haplotypes were detected in each of the remaining populations.

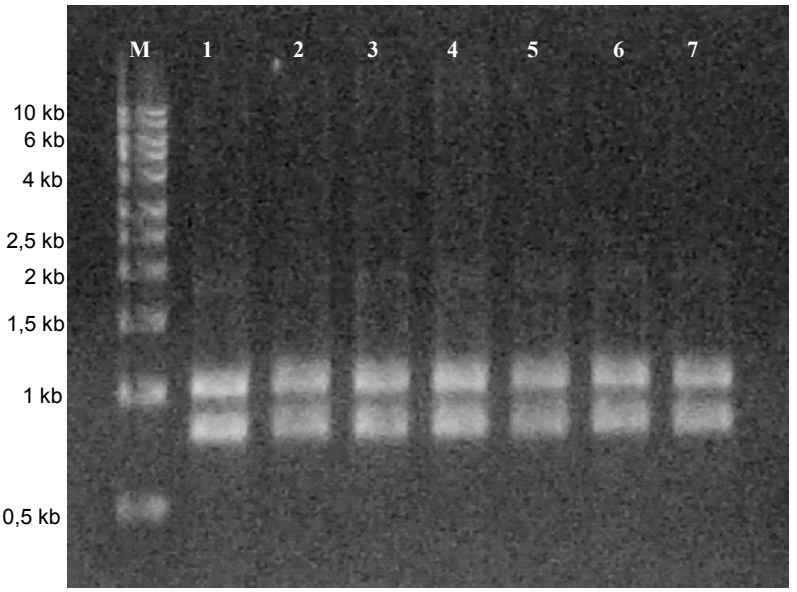


Figure 7. Hind III restriction digestion of some PCR samples. M: 1 Kb λ DNA marker. Samples 1-3: Çanakkale speciemens, 4-7: Balıkesir speciemens.

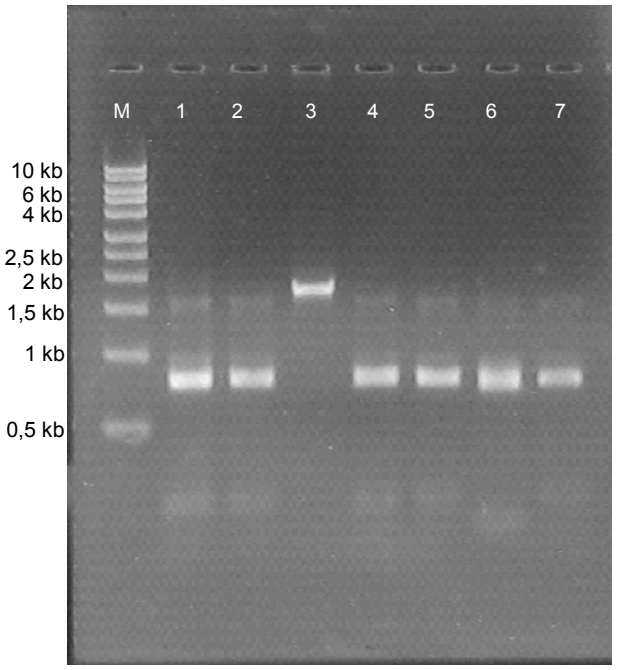


Figure 8. Hinf I from the Muş population. M: 1 Kb λ DNA marker, 1-7: Muş1-Muş 7 samples.

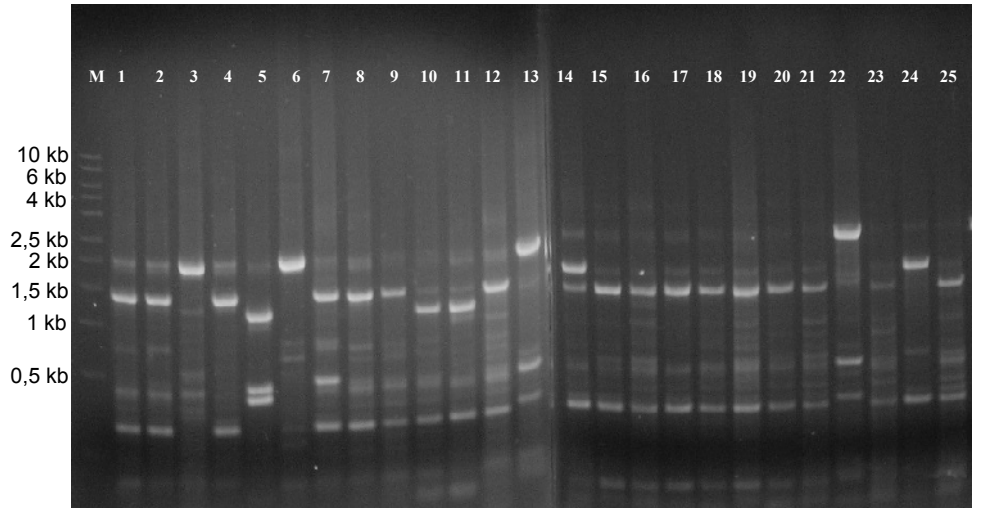


Figure 9. Hinf I cut samples. M: 1 Kb λ DNA marker. Samples: 1-4: Adıyaman, 5-9: Aksaray, 10-14: Balıkesir, 15-17: Bitlis, 18-20: Elazığ, 21-23: Kayseri, 24-25: Van.

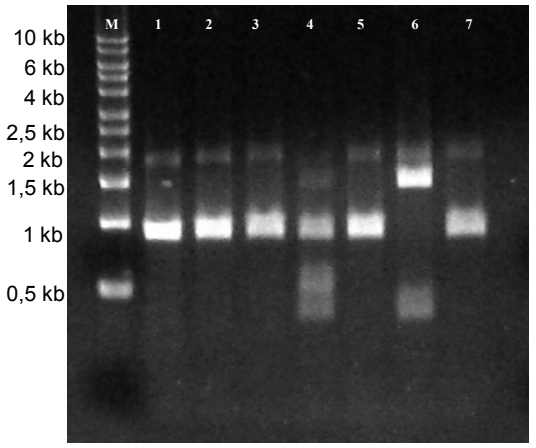


Figure 10. Hae III from Muş population. M: 1 Kb λ DNA marker, 1-7: Muş1-Muş 7 samples.

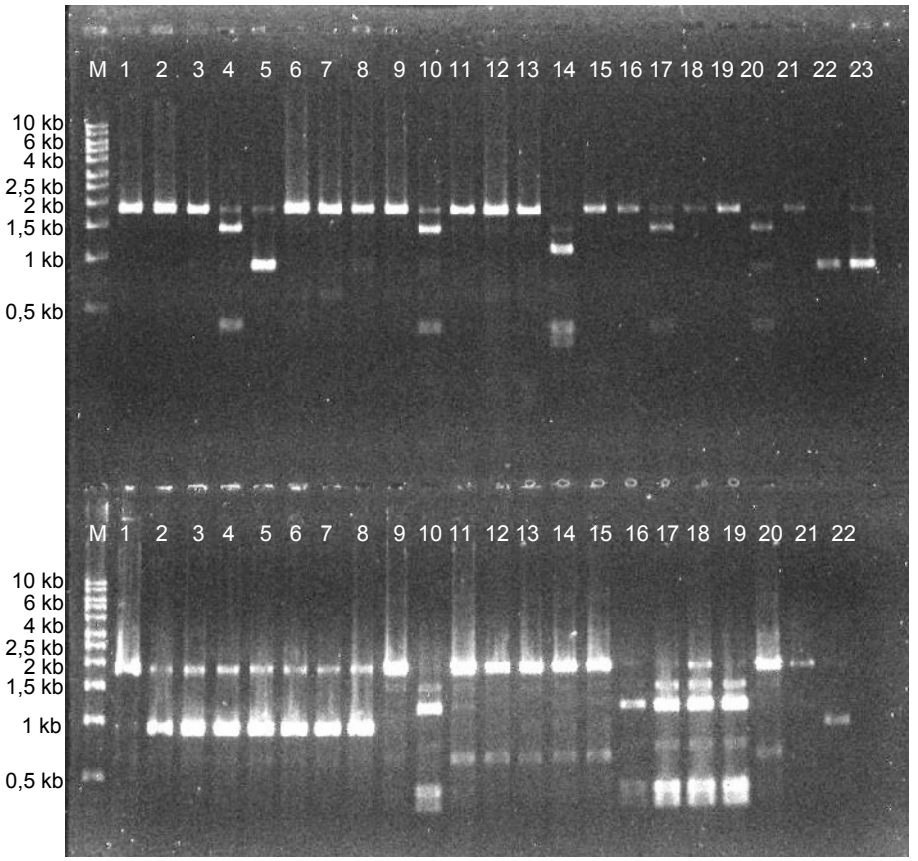


Figure 11. Hae III restriction digestion pattern of some *A. quercustozae* samples. M: 1 Kb λ DNA marker. Samples (in the upper part of the figure): 1-5: Adıyaman, 6-13: Aksaray, 14-19: Balıkesir, 20-21: Bayburt, 22-23: Van. Samples (at the bottom of the figure): 1-9: Bitlis, 10-20: Bolu, 21-22: K.Maraş.

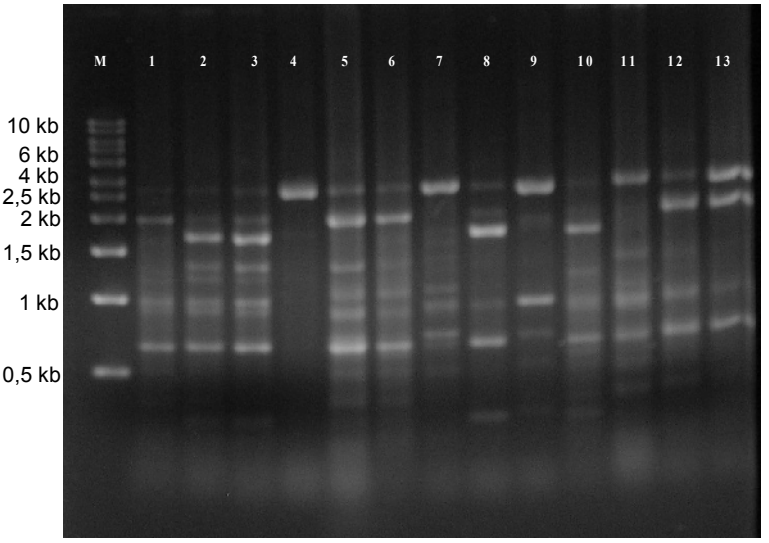


Figure 12. Some *A. quecustozae* samples cut by Hae III restriction endonuclease. M: 1 Kb λ DNA marker. Samples: 1-3: Çanakkale, 4-5: Çankırı, 6-8: Kırklareli, 9-12: Bolu, 13: Sivas.

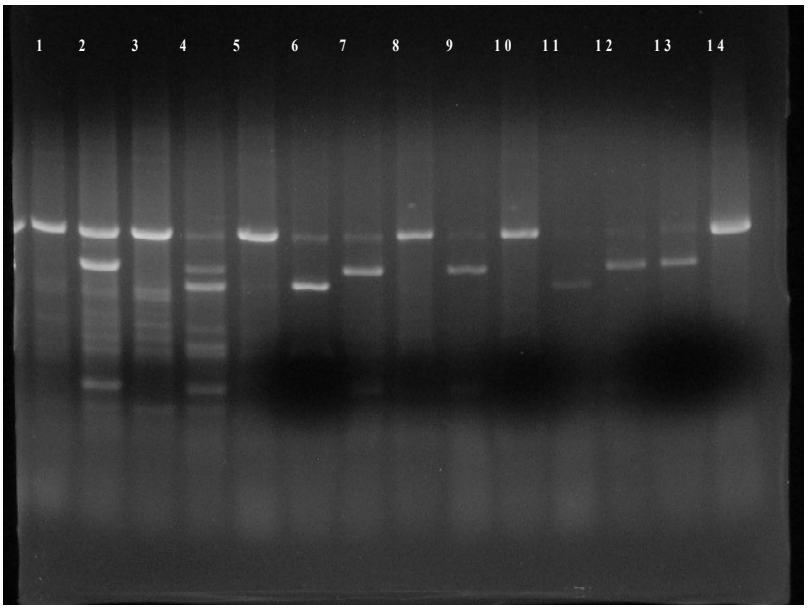


Figure 13. Hind III digestion pattern of some *A. quercustozae* individuals. Samples: 1-3: Bolu, 4-5: Çankırı, 6-9: Muş, 10-11: Bayburt, 12-14: Tekirdağ.

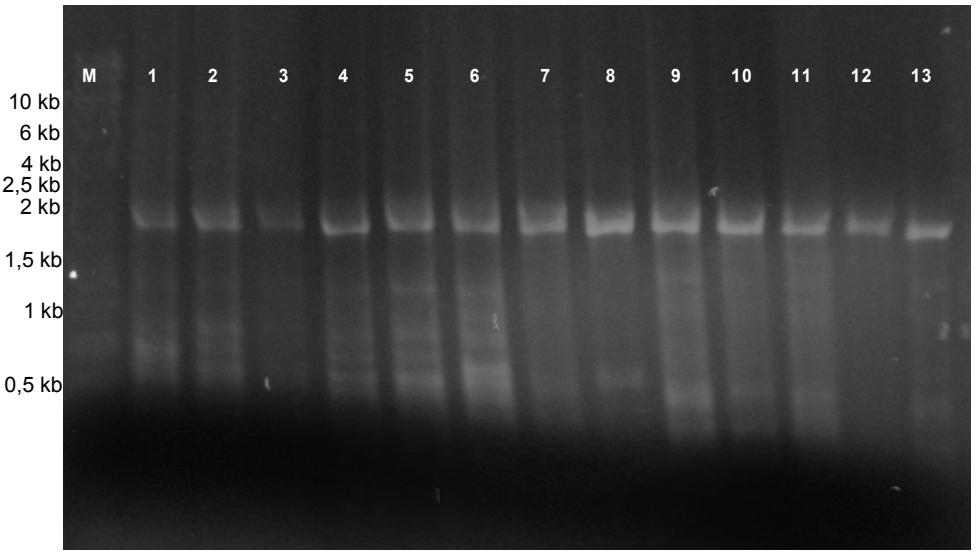


Figure 14. EcoR I restriction enzyme digested PCR samples. M: 1 Kb λ DNA marker. Samples 1-2: Tekirdağ, 3-8: Bolu, 9-10: Van, 11-13: Kahramanmaraş.

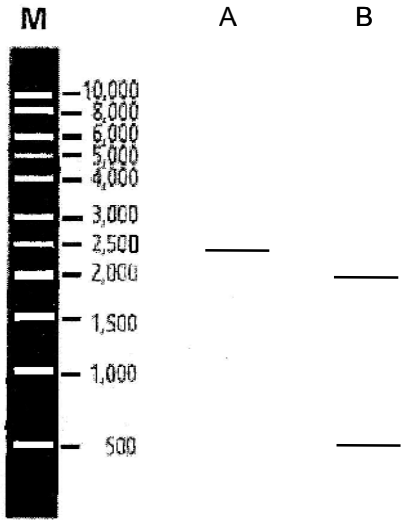


Figure 15. Hind III restriction digestion pattern of the ND4-CBN mtDNA fragment

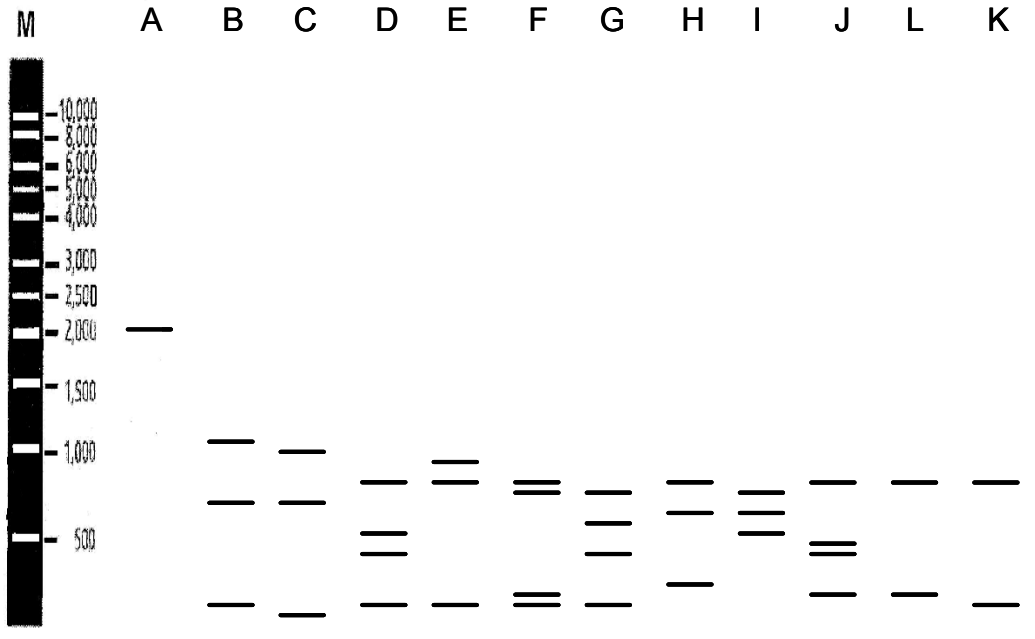


Figure 16. Hinf I restriction enzyme digestion haplotype pattern of the second mtDNA fragment.

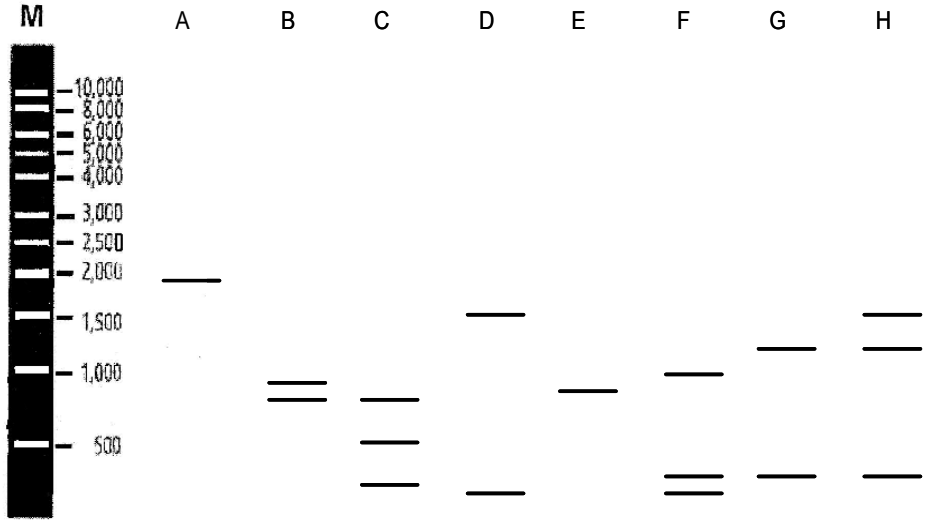


Figure 17. Hae III restriction digestion pattern of the ATPases (6, 8) and COIII mtDNA fragment of *A. quercustozae*.

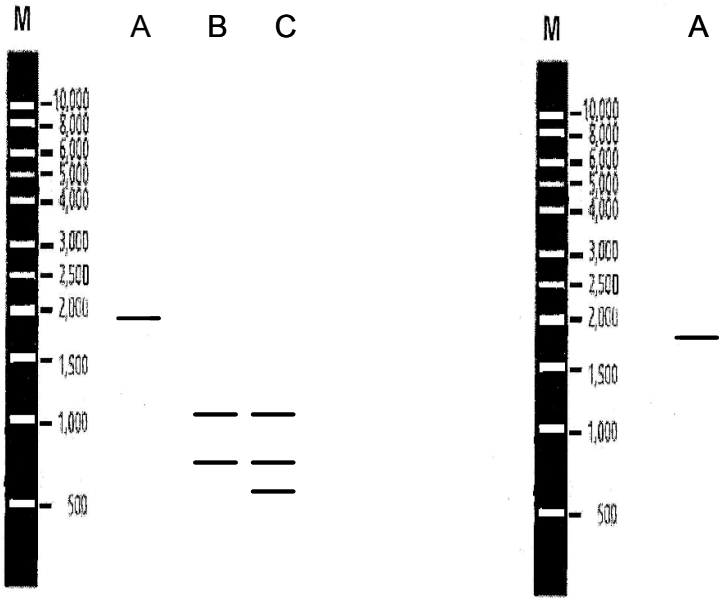


Figure 18. Hind III and EcoR I restriction digestion patterns of the ATPases (6, 8) and COIII mtDNA fragment of *A. quercustozae*.

Haplotype and nucleotide diversity of each *A. quercustozae* population calculated using the REAP program is given in Table 3. The average haplotype diversity for the studied populations was 0.45. Among the 16 analyzed populations the highest haplotype diversity (1.00) was estimated for the Kahramanmaraş and Bayburt populations followed by the Adıyaman population (0.90). On the other hand, the lowest haplotype diversity was calculated for the Çankırı, Çanakkale, Kırklareli, Van and Sivas populations (0.00). Overall, haplotype diversity was significantly high for most of the studied populations. The average nucleotide diversity for the analyzed populations was 0.054. Among all examined *A. quercustozae* populations the highest nucleotide diversity (0.152) was observed in the Kahramanmaraş population followed by the Adıyaman (0.131) and Balıkesir (0.129) populations. The lowest nucleotide diversity (0.000) was found in the Çanakkale, Çankrı, Kırklareli,Van and Sivas populations. Overall, most of the *A. quercustozae* populations showed significantly high amount of nucleotide diversity.

Table 2. Composite haplotypes and their frequencies among *A. quercustozae* populations. Composite haplotypes detected in outgroups are also given as A. c. H (*A. caputmedusae* haplotype) and A. L. H. (*A. lucidus* haplotype). Composite haplotypes based on digestion pattern of restriction enzymes shown by capital letters are given in the following order for both mtDNA fargment: HindIII and HinfI, HaeIII, HindIII, EcoRI.

Haplotype	Composite Haplotype	ADI	AKS	BAL	BIT	BOL	CAN	CKR	ELA	KIR	MUS	TEK	VAN	KAH	BAY	SIV	KAY	Total
Type 1	AABBA										1							1
Type 2	ABBBA										4							4
Type 3	ABCBA										1							1
Type 4	ACDBA										1							1
Type 5	ADABA		1															1
Type 6	AEABA		6		1	6				7		1						21
Type 7	AAAAA																2	2
Type 8	ALABA											4					4	8
Type 9	AGABA														1	5		6
Type 10	AFABA			4										1				5
Type 11	AKBBA						1											1
Type 12	AKEBA												2					2
Type 13	AEEBA				7													7
Type 14	AEFBA											2						2
Type 15	AEGBA					2												2
Type 16	AEHBA					3												3
Type 17	AFBBA						6											6
Type 18	AFDBA		1	1														2
Type 19	AFFBA			1														1
Type 20	AHABA							6										6
Type 21	AHACA								4									4
Type 22	AIAAA								2									2
Type 23	AJAAA	2																2
Type 24	BKABA	1																1
Type 25	BKDBA	1																1
Type 26	BKEBA	1																1
Type 27	BGEBA													1				1
Type 28	AGDBA														1			1
A.C.H	BBBBB																	2
A.L.H	BBBBB																	2
Sample Size		5	8	6	8	11	7	6	6	7	7	7	2	2	2	5	6	95

Table 3. Mean +/- S.E. haplotype and nucleotide diversity for the *A. quercustozae* populations.

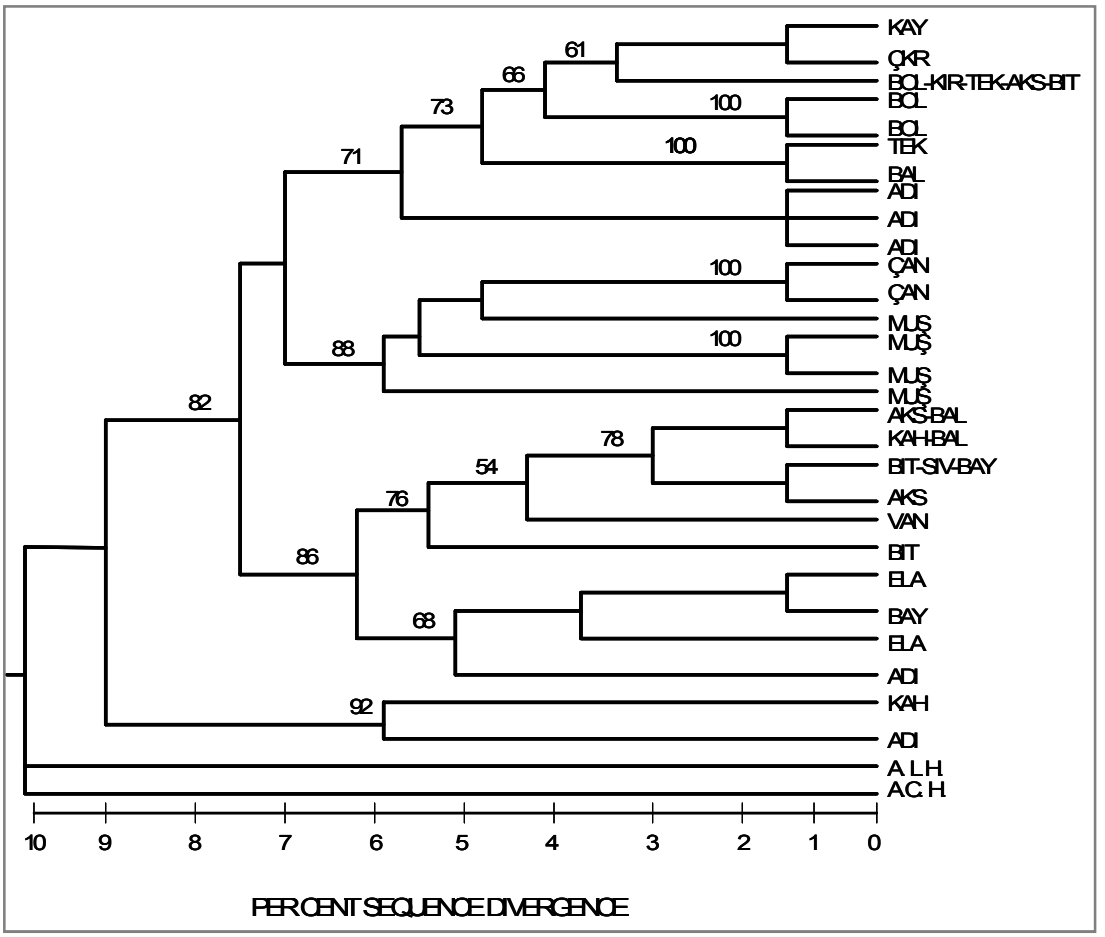
Population	**Haplotype Diversity**	**Nucleotide Diversity**
Adıyaman	0.9000+/- 0.16100	0.131257
Aksaray	0.4643+/- 0.20001	0.043322
Balıkesir	0.6000+/- 0.21517	0.129451
Bitlis	0.2500+/- 0.18020	0.013416
Bolu	0.6545+/-0.11148	0.059503
Çanakkale	0.0000+/- 0.00000	0.000000
Çankırı	0.0000+/- 0.00000	0.000000
Elazığ	0.5333+/- 0.17213	0.077950
Kırklareli	0.0000+/- 0.00000	0.000000
Muş	0.7143+/- 0.18090	0.095328
Tekirdağ	0.6667+/- 0.15983	0.072608
Van	0.0000+/- 0.00000	0.000000
Kahramanmaraş	1.0000+/- 0.50000	0.152941
Bayburt	1.0000+/- 0.50000	0.070948
Sivas	0.0000+/- 0.00000	0.000000
Kayseri	0.5333+/- 0.17213	0.032437
Average	0.4543+/- 0.00856	0.054948+/- 0.0001716

3.2. Phylogenetic Relationships among Haplotypes

Haplotype data matrix based on percent sequence divergence was used to reconstruct a UPGMA dendrogram using the twenty eight haplotypes from sixteen *A. quercustozae* populations with two outgroup species, *A. caputmedusae* and *A. lucidus*, used to root the tree (Figure 19). The percent sequence divergence values are also given in a scale on the dendrogram to show the genetic differentiation of the haplotypes. In the dendrogram haplotypes found in the Kahramanmaraş and Adıyaman populations were grouped at the most basal part of the dendrogram and clearly separated from all other haplotypes with a high bootstrap support. The remaining haplotypes formed two clusters: The first cluster is composed of two subclusters of which the first cluster includes haplotypes from the Adıyaman, Elazığ and Bayburt populations. The second subcluster comprises two haplotypes found in the Aksaray, Balıkesir, Kahramanmaraş, Bitlis, Sivas, Bayburt and Van populations. Most of the lineageas are supported by high bootstrap values on this cluster. On the other hand, the second main cluster is composed of two groupings. The first subgroup of this cluster comprises haplotypes from Çanakkale and Muş. The second subgroup covers haplotypes from the Adıyaman, Kayseri, Çankırı, Tekirdağ, Bolu, Balıkesir, Kırklareli, Aksaray and Bitlis localities.

A neighbor joining tree resulting from the distance matrix among all mtDNA haplotypes is presented in Figure 20 with associated bootstrap values (>50 are shown on the branches). As shown on the dendrogram three distinct groupings with respect to the geographic location of populations can be distinguished and named as A, B and C. Because Group C is composed of haplotypes found in both easterly and westerly located populations this cluster is named as Eastern / Western Clade.

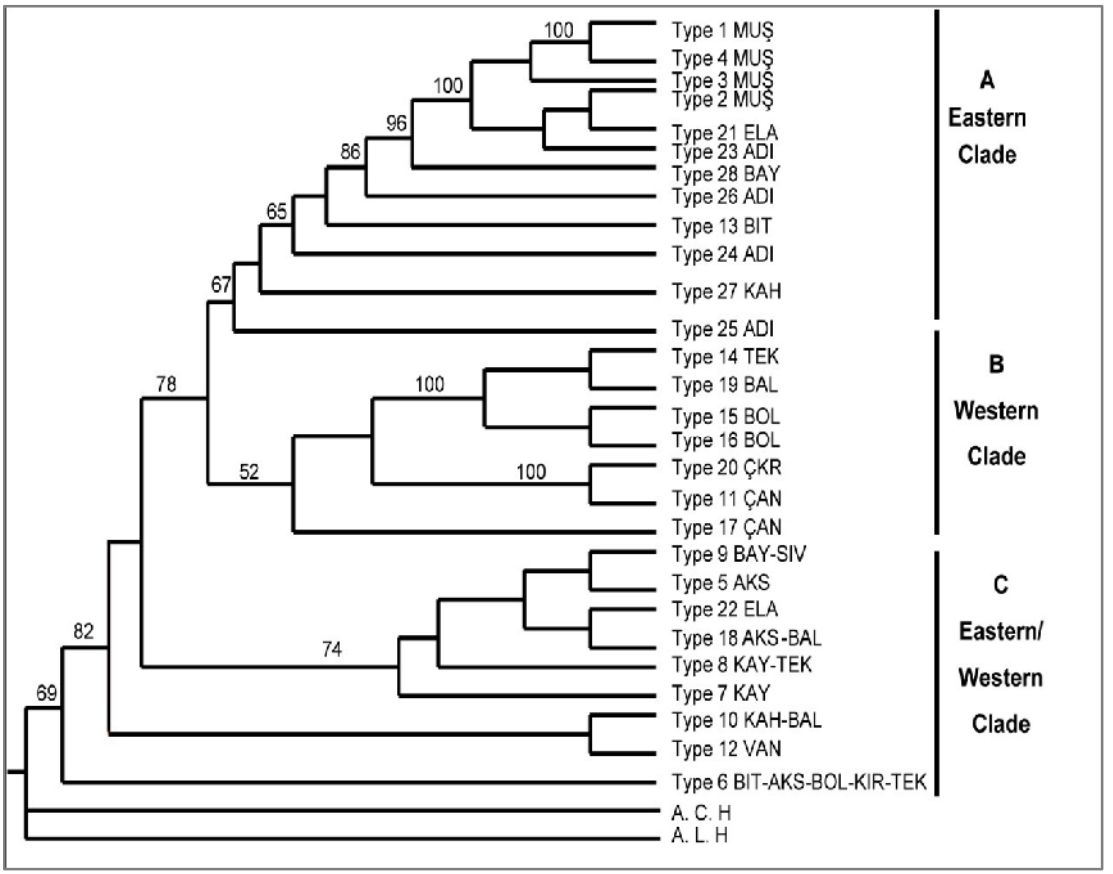
Figure 19. UPGMA dendrogram of *A. quercustozae* populations based on pairwise estimates of percentage sequence divergence. Numbers above branches represent the bootstrap values obtained from 1000 replicates of the restriction fragment data between the haplotypes. Support values <50% are not given.



The C clade includes Type 6 at the most basal position and found as the most common haplotype in five different locations. Furthermore, Type 10 and 12 are grouped together represented by the individuals from Kahramanmaraş-Balıkesir and Van populations, respectively. Type 9 shared between the Bayburt and Sivas populations are grouped with Type 5 from the Aksaray population. Furthermore, Type 22 from Elazığ and Type 18 shared between Aksaray and Balıkesir also make a small group. Haplotype 8 from Kayseri and Tekirdağ is basal to this small group and further Type 7 from the Kayseri population is another basal haplotype in this cluster. The B group named as Western Clade comprises haplotypes only from westerly located populations. Haploptypes Type 14 from Tekirdağ, Type 19 from Balıkesir, Types 15 and 16 from Bolu, Type 20 from Çankırı and Type 11 from Çanakkale are grouped in this cluster. On the other hand, A cluster named as Eastern clade due to the presence of those haplotypes represented only by easterly located populations. Haplotype Type 1-4 from Muş, Type 21 from Elazığ, Types 23-26 from Adıyaman, Type 28 from Bayburt and finally Type 13 from Bitlis and Type 27 from Kahramanmaraş are all found in this cluster.

An unrooted Dollo parsimony majority-rule consensus tree of *A. quercustozae* mtDNA haplotypes with supporting bootstrap values of >50 out of 1000 replicates is represented in Figure 21. The dendrogram depicting the main groupings of the analyzed haplotypes show similar results with the neighbor-joining dendrogram given in Figure 20. Haplotypes from different populations constituted three main groupings into eastern, western and eastern/western which is formed by the mixed haplotypes in this cluster. The most common haplotype (Type 6) found in five distinct populations were grouped under the eastern/western grouping.

Figure 20. Neighbor-joining dendrogram of the 28 haplotypes of *A. quercustozae.* Numbers above the branches represent the bootstrap values 1000 replicates of the restriction fragment data between the haplotypes. Support values <50% are not represented.



Other haplotypes included in the mixed cluster are from Kayseri, Tekirdağ, Adıyaman, Çanakkale, Elazığ, Aksaray, Bayburt, Sivas, Kahramanmaraş, Balıkesir and Van. Western cluster covered haplotypes from Çanakkale, Bolu, Tekirdağ, Balıkesir and Çankırı. On the other hand, haplotypes found in the Muş, Elazığ, Adıyaman, Bayburt, Bitlis and Kahramanmaraş populations were grouped under the eastern cluster.

The nucleotide divergence between the populations calculated using the DA program of REAP ranged from 0.001 to 9.0% (Table 4). Based on pair-wise comparisons of nucleotide divergence of *A. quercustozae* populations, Kırklareli and Bayburt populations are the most diverged populations (9.0%). Likewise, Muş and Çanakkale populations are diverged with 8.3% and Balıkesir is diverged from Kayseri population with 8.2%. The Bayburt and Sivas populations are the least diverged ones (0.001%).

For estimating the extent of genetic differentiation at different hierarchical levels, an AMOVA analysis was performed with alternative grouping schemes. For all grouping options, analysis of variance using AMOVA showed that all component of variance partitioning (among groups, among populations within groups and within populations) were statistically significant. Analysis of variance using AMOVA revealed highly significant amount of genetic variation (35.62%, $P < 0.001$) within populations indicating that each population has high levels of genetic variation (Table 5). It also revealed that a significant partitioning of variance (25.50%) was found among groups. On the other hand, 38.88% of genetic variance was present within groups. When several different grouping schemes were analyzed using

Figure 21. Unrooted Dollo parsimony majority-rule consensus tree of *A. quercustozae* mtDNA haplotypes. Numbers at nodes indicates bootstrap values. Support values <50% are not represented.

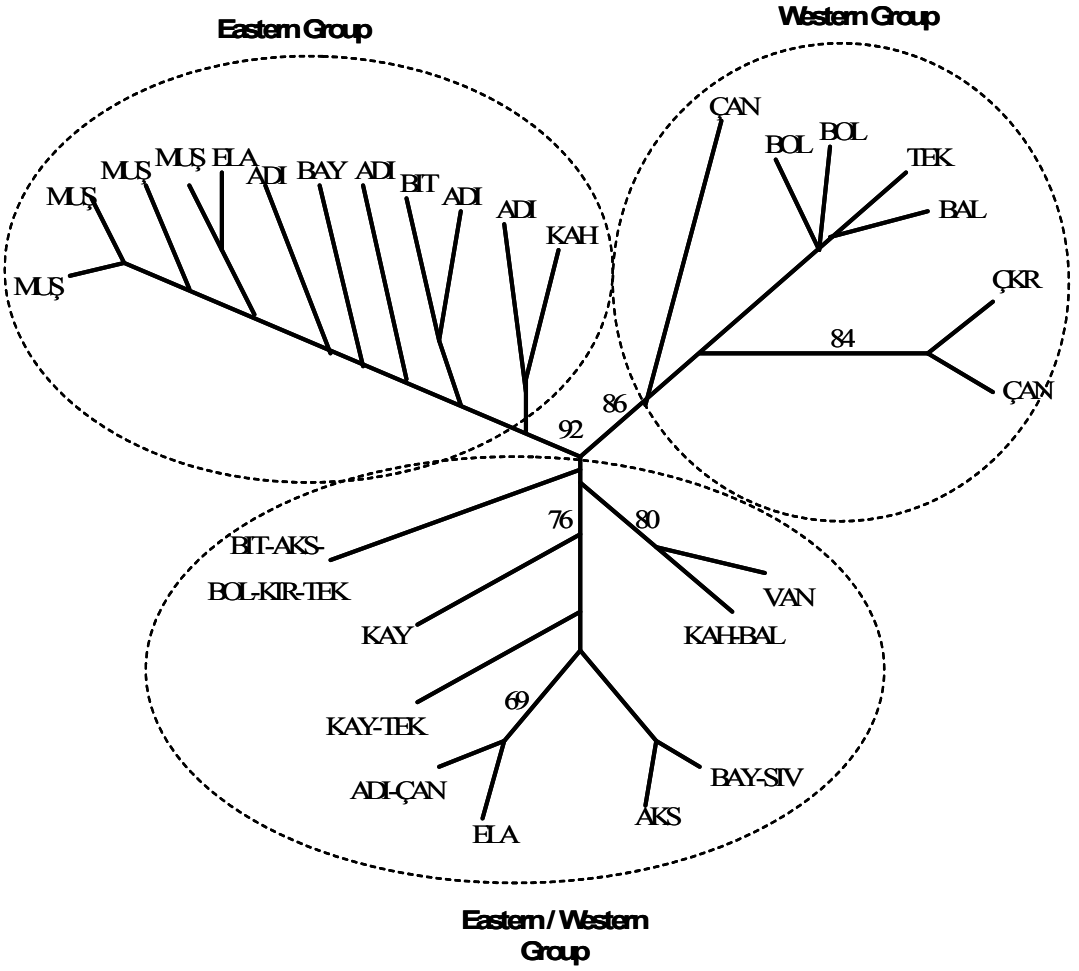


Table 4. Pair-wise nucleotide diversity (above diagonal) and net nucleotide divergence (below diagonal) among the populations of *A. quercustozae* obtained from the REAP program package.

	Adıyaman	Aksaray	Balıkesir	Bitlis	Bolu	Çanakkale	Çankırı	Elazığ	Kırklareli	Muş	Tekirdağ	Van	K.Maraş	Bayburt	Sivas	Kayseri
Adıyaman		0,112817	0,13754	0,12325	0,11607	0,15191	0,17746	0,15183	0,10031	0,16077	0,12656	0,16667	0,15866	0,14813	0,11913	0,12956
Aksaray	0,02552		0,10188	0,08147	0,06667	0,10576	0,19734	0,16672	0,02166	0,11314	0,11964	0,10062	0,14883	0,12398	0,06037	0,14557
Balıkesir	0,00719	0,01549		0,11068	0,10977	0,15679	0,20929	0,18314	0,08293	0,16661	0,14577	0,18219	0,13798	0,16308	0,11209	0,16391
Bitlis	0,05092	0,05311	0,03925		0,08036	0,19541	0,22047	0,18097	0,04695	0,15132	0,07106	0,20901	0,15381	0,16926	0,11806	0,11444
Bolu	0,02069	0,01526	0,01529	0,04391		0,13503	0,13917	0,13137	0,04695	0,12361	0,10727	0,12878	0,14239	0,12287	0,06692	0,12103
Çanakkale	0,05477	0,05261	0,06056	0,05719	0,07374		0,19641	0,17423	0,10576	0,16268	0,16869	0,12688	0,21957	0,14451	0,08088	0,15297
Çankırı	0,06818	0,06946	0,06456	0,06376	0,06994	0,03091		0,06295	0,19906	0,22471	0,24588	0,22353	0,22892	0,22353	0,22353	0,25368
Elazığ	0,04722	0,06068	0,07944	0,01359	0,06264	0,01037	0,02394		0,16665	0,20811	0,19717	0,18302	0,19557	0,18302	0,18302	0,20225
Kırklareli	0,03466	0,00025	0,01821	0,04024	0,01701	0,07426	0,06991	0,06276		0,10262	0,09899	0,10732	0,13698	0,12636	0,05366	0,13338
Muş	0,04747	0,04382	0,05421	0,06915	0,04619	0,08351	0,06773	0,06014	0,05995		0,16087	0,15859	0,14515	0,15511	0,10901	0,16361
Tekirdağ	0,02463	0,06116	0,04474	0,02805	0,04121	0,06088	0,06212	0,06218	0,06269	0,07691		0,13861	0,14107	0,11524	0,07878	0,06256
Van	0,00103	0,07896	0,06177	0,02029	0,09003	0,00953	0,05235	0,06144	0,00507	0,05193	0,01023		0,13928	0,03099	0,03099	0,09296
K.Maraş	0,01656	0,05071	0,00321	0,07063	0,03616	0,06115	0,06524	0,06022	0,06051	0,02101	0,03422	0,06282		0,12565	0,11201	0,13393
Bayburt	0,04702	0,06685	0,06288	0,06208	0,05764	0,07757	0,06885	0,05085	0,09089	0,07196	0,04347	0,00482	0,01372		0,03547	0,06526
Sivas	0,05351	0,03871	0,04736	0,06135	0,03717	0,04938	0,06635	0,05144	0,05366	0,06134	0,04277	0,03199	0,03555	0,00001		0,05087
Kayseri	0,04771	0,05076	0,08296	0,00951	0,07506	0,05052	0,06274	0,05176	0,05171	0,09073	0,01044	0,07675	0,04125	0,01357	0,03466	

Arlequin program, AMOVA analysis produced similar results in which within groups and within population variation were higher compared to among groups. Due to shared common haplotypes among populations AMOVA results may not show highest value of among group difference. Current data indicate that each population developed its own genetic variation through time in addition to having an obvious differentiation among groups.

The F_{ST} values between each population pair is given in Table 6. Pairwise differences among populations indicate that some populations are highly differentiated from each other. Van population is different from Aksaray, Balıkesir and Bolu population (1.000). Likewise, Sivas is highly differentiated from Aksaray, Balıkesir, Bolu and Van population (1.000). Moreover, Bolu is greatly different from Aksaray and Balıkesir population (1.000). Although some F_{ST} values are very high the Bayburt and Adıyaman populations show the least amount of divergence (-0.00585) indicating that these populations have some similar cut sites in their haplotypes. However, most of the pairwise F_{ST} estimates are significantly high among the *A. quercustozae* populations. Overall, different degrees of differenation are observed within the range of the oak gall wasp species implying the possibility of several factors acting on the species population structure.

Table 5. Analysis of molecular variance (AMOVA) among the studied oak gall wasp populations grouped into five groupings obtained with the unrooted Dollo parsimony majority-rule consensus tree of mtDNA haplotypes. * $P < 0.001$ after 1000 permutations. Va, Vb, and Vc are the associate covariance components.

Source of Variation	**d.f.**	**Sum of Squares**	**Variance Components**	**Percentage of Variation**	**Fixation indices**
Among groups	4	89.093	0.82780Va	25.50*	F_{CT}=0.25500
Within groups	11	92.877	1.26201Vb	38.88*	F_{ST}=0.64377
Within populations	78	90.201	1.15642Vc	35.62*	F_{SC}=0.52183

Table 6. Pair wise F_{ST} values among the *A. quercustozae* populations obtaiıned from Arlequin program. Population abbreviations are given Table 1.

Distance method: Pairwise F_{ST} difference among poplations

	ADI	AKS	BAL	BIT	BOL	ÇAN	ÇKR	ELA	KIR	MUŞ	TEK	VAN	KAH	BAY	SIV	KAY
ADI	0.00000															
AKS	0.40476	0.00000														
BAL	0.64407	1.00000	0.00000													
BIT	0.34468	0.77086	0.70476	0.00000												
BOL	0.62956	1.00000	1.00000	0.83243	0.00000											
ÇAN	0.36536	0.29528	0.80229	0.60621	0.76572	0.00000										
ÇKR	0.22262	0.01243	0.78845	0.47876	0.73287	0.17721	0.00000									
ELA	0.26687	0.49776	0.77143	0.49032	0.63077	0.45517	0.26909	0.00000								
KIR	0.46276	0.68693	0.77931	0.59574	0.28889	0.63549	0.54258	0.35385	0.00000							
MUŞ	0.46154	0.70513	0.64407	0.62227	0.73447	0.62209	0.58141	0.48844	0.58879	0.00000						
TEK	0.51882	0.84741	0.95163	0.77993	0.94954	0.47243	0.49580	0.64566	0.73653	0.68685	0.00000					
VAN	0.49072	1.00000	1.00000	0.64023	1.00000	0.69174	0.65230	0.53846	0.55140	0.55966	0.86870	0.00000				
KAH	0.03562	0.74900	0.71429	0.26271	0.79310	0.57732	0.43666	0.34498	0.44904	0.45337	0.69396	0.30769	0.00000			
BAY	−0.00585	0.92905	0.92941	0.61039	0.93814	0.70193	0.62696	0.53846	0.58086	0.51975	0.88334	0.82353	−0.09091	0.00000		
SIV	0.20904	1.00000	1.00000	0.78102	1.00000	0.78726	0.74004	0.67391	0.70944	0.69887	0.95319	1.00000	0.47368	0.47368	0.00000	
KAY	0.43510	0.64069	0.66497	0.44334	0.61176	0.59237	0.49196	0.34635	0.42644	0.54320	0.65670	0.17397	0.13785	0.47179	0.67000	0.00000

CHAPTER 4

DISCUSSION AND CONCLUSION

Molecular methods have been frequently used to identify geographic variation and to reconstruct phylogeographic relationships among populations of a species (Avise, 1994). Analysis of animal mtDNA is most favored for providing suitably variable genetic signature of the past historical events. The levels of discontinuity observed among groups of mtDNA lineages depends on the effectiveness of barriers to gene flow and the rate of extinction of intermediate haplotypes. Therefore, geographic subdivisions in the distribution of mtDNA haplotypes can reflect historical events that are important for a species to obtain its current phylogenetic and phylogeographic structure (Avise, 2000).

In this thesis mtDNA PCR-RFLP method was used to assess intraspecific genetic variation of *A. quercustozae* and the possible effects of major geographic barriers shaped the current population structure of this species. The present analysis of PCR-RFLP site variation at the ND4, ND6, Cyt B and ATPases (6, 8), COIII regions of the mtDNA of the Turkish oak gall wasps was motivated by evidence that these regions were variable in other oak gall wasp species and thus could reveal genetic

differences among the examined populations. The existence of variation in the analyzed segments of *A.quercustozae* is in accordance with other Hymenopteran species restriction enzyme recognition sites (Fransisco *et al.*, 2001; Moretto and Arias, 2005; Mutun, in press). Thus, the results presented here reveal the presence of several variable restriction sites in these fragments and allowed identification of distinct haplotypes in *A. quercustozae* oak gall wasp species.

4. 1. Intraspecific mtDNA Variation of *Andricus quercustozae*

In this study a total of 28 composite haplotypes were detected out of the 95 analyzed individuals across most of the range of the species from Turkey. In another Turkish oak gall wasp species, *A. caputmedusae*, 31 haplotypes out of 180 individuals were determined covering the entire distribution range of the species in Turkey. The average haplotype and nucleotide diversity within *A. caputmedusae* populations were 0.46 and 0.102, respectively (Mutun, in press). On the other hand, 26 haplotypes were detected among 144 *A. lucidus* individuals collected from 9 populations from Turkey. The estimated average haplotype and nucleotide diversity within the populations of *A. lucidus* were 0.80 and 0.115, respectively (unpublished data). In *A. quercustozae* the estimated haplotype diversity is 0.45 and the average nucleotide diversity is 0.054. The haplotype and nucleotide diversity of *A. quercustozae* seems to be the lowest among the three oak gall wasp species from Turkey but this may be related to the lower number of specimens used in this study.

Among the sixteen analyzed *A. quercustozae* populations the highest haplotype diversity was estimated for the Kahramanmaraş and Bayburt populations (1.00) followed by the Adıyaman (0.90) and Muş (0.71) populations. Likewise studies of

two other oak gall wasp species, *A. caputmedusae* and *A. lucidus*, the Kahramanmaraş and Adıyaman populations showed strikingly high levels of haplotype diversity. Moreover, nucleotide diversity is highest in the Kahramanmaraş population (0.152) followed by the Adıyaman population (0.131). Similar results found in *A. caputmedusae* and *A. lucidus* is evident. Thus, the current findings of *A. quercustozae* reported in this thesis support the presence of high genetic diversity both in Kahramanmaraş and Adıyaman localities. This may be a general pattern in Turkey for the oak gall wasp species and requires further research.

In the south of the sampling area for *A. quercustozae* Amonos Mountains are located and in the eastern part Southeastern Taurus extends from the connection section of Southern, Southeastern and Eastern Taurus where this area is known as the Maraş Triangle (Çıplak, 2008). In both Kahramanmaraş and Adıyaman populations, the observed high level of genetic diversity may imply several points: the area where the Kahramanmaraş and Adıyaman populations sampled is known with its high species/lineage diversity (Çıplak, 2008). The presence of conspicously high levels of genetic variation found for *A. quercustozae* populations may support the proposed idea of the Maraş Triangle. This area may not only be important for species diversity but also for the genetic diversity. Moreover, parallel results obtained for *A. caputmedusae* and *A. lucidus* for the Kahramanmaraş and Adıyaman populations coincide with the Maraş Triangle also is supported with the results of this study. Current findings on *A. quercustozae* provide supporting evidence for the area to be treated as a hotspot.

Moreover, the highest haplotype and nucleotide diversity observed in Kahramanmaraş and Adıyaman populations (See Table 3) may suggest a historical expansion process from these populations to other areas. Populations with high

haplotype and nucleotide diversity may imply the presence of a shelter area and the lower amount of genetic variation present in other sampled populations in the periphery shows gene flow from the source population to other populations (Bennett and Provan, 2008; Provan and Bennett, 2008).

With this study higher genetic variation was found for *A. quercustozae* species is concordant with the results of a study conducted on the same species (Rokas *et al.*, 2003). In this study of *A. quercustozae* covered the Palearctic region applied allozyme and sequencing of an mtDNA gene and revealed that the European gall wasp populations possibly derived from Turkey. The Turkish populations overall showed not only greater genetic variation but also represented the center of genetic diversity for the species. Moreover, phylogenetic and population structuring suggested that the oak gall wasp species is now widespread in Europe diverged in Anatolia and expanded its range westward prior to Pleistocene (Rokas et. al., 2003). Furthermore, studies on another oak gall wasp species, *A. coriarius,* revealed the presence of higher genetic diversity with a westward expansion of about 1.6 million years before present might have possibly took place with a distinct origin from Turkey and Iran (Challis *et al.*, 2007). Similar results showing a signature of westward colonization with greater genetic variation was reported for the oak gall wasp species, *A. kollari* (Stone *et al.*, 2007). Likewise, high genetic diversity was also reported for other animal species from Turkey (Gündüz *et al.*, 2007; Bardakçı *et al.*, 2006).

4.2. Phylogenetic Relationships among *A. quercustozae* Haplotypes

The nucleotide divergence between the populations of *A. quercustozae* calculated using the DA program of REAP ranged from 0.001 to 9.0%. Based on

pair-wise comparisons of nucleotide divergence of *A. quercustozae* populations, Kırklareli and Bayburt populations are the most diverged populations (9.0%). Bolu and Van populations (9.0%) are also similarly diverged from each other. The next level of divergence occurs between Çanakkale and Muş (8.3%), Elazığ and Balıkesir (7.9%) followed by Aksaray and Van (7.8%) (Table 4). The least diverged populations are Bayburt and Sivas populations (0.001%). The divergence estimates between pairs of populations may well correlate with the geographic distances of the populations (Neigel and Avise, 1993). On the other hand, neighbor-joining dendrogram and the unrooted Dollo parsimony majority-rule consensus tree (see Figures 20 and 21) generated some geographically significant groupings. The formation of three major clades as eastern, western and eastern/western mixed cluster is striking and implies geographic partitioning of some haplotypes. Under a commonly accepted phylogeographic approach in geographically significant groupings more commonly distributed haplotypes are ancestral haplotypes when compared to the derived haplotypes that show more restriction in their distribution (Slatkin, 1991; Crandal and Templeton, 1993). Of the 28 haplotypes Type 6 is found in 5 populations including Aksaray, Bitlis, Bolu, Kırklareli and Tekirdağ may suggest that this haplotype is more ancestral one compared to other haplotypes. The absence of this haplotype from more easterly located populations except the Bitlis population may be due to sampling. The presence of eleven private haplotypes in different populations may be due to each population developing its own genetic variation through time (Crandal and Templeton, 1993).

For many species it has been shown that the phylogeny of mtDNA haplotypes corresponds well to the geographical distribution of populations and geographical barriers have been found to shape current distribution of the lineages (Avise, 2000).

Present study indicates the presence of an obvious correlation between the genetic relationships among mtDNA types to their geographical distribution into roughly east and west with an additional mixed groups of haplotypes. Complex geologic history of Turkey results in both local and larger scale isolation between faunal elements. Moreover, the Anatolian Diagonal acts as a major geographic barrier dividing species/lineage distribution into east/west (Çıplak *et al.*, 1993; Çıplak, 2004). Current results indicating an east-west separation (Figure 20 and 21) may support a significant geographic pattern considering the Anatolian Diagonal. Specifically in Clade A, all Muş haplotypes are grouped with Elazığ, Adıyaman, Bayburt, Bitlis and Kahramamaraş haplotypes. In Clade B haplotypes from Tekirdağ, Balıkesir, Bolu, Çankırı and Çanakkale are grouped together. Finally, the Clade C is composed of haplotypes in both easterly and westerly located populations with respect to the location of the Anatolian Diagonal. Overall the clustering of all *A. quercustozae* haplotypes with respect to the location of the Anatolian Diagonal fits well in most parts with the present results of this study.

The contemporary genetic structure of populations has also been enormously influenced both by ice ages and climatic fluctuations during the Quaternary periods (Hewitt, 1996; 1999). These oscillations are thought to be important factors to shape the distribution of most species all over the world leading to genetic consequences and population structuring (Hewitt, 2000). For estimating the extent of genetic differentiation at different hierarchical levels, an AMOVA analysis was performed with distinct groupings based on the geographic origins of the haplotypes. For all grouping options, analysis of variance using AMOVA showed that all component of variance partitioning (among groups, among populations within groups and within populations) were statistically significant (see Table 5). Several different grouping

schemes of the populations with respect to their locations to the Anatolian Diagonal, AMOVA analysis produced lower lever of among group variance component among groups and higher amount of genetic partitioning was found within populations with high statisctical support. Current data may also indicate that each population developed its own genetic variation through time (Avise, 2006) in addition to having differentiation among groups which may be due to the effects of the environmental fluctuations occurred in the area although Turkey was never covered by ice sheets.

Mitochondrial DNA divergence can be used to estimate the divergence time of the analyzed mtDNA lineages. According to the general application of a conventional mtDNA clock of 2.3% per million years of a mutation rate for insect mtDNA (Brower, 1994), the divergence between the most divergent haplotypes (9.0%) took place about 3.9 million years ago (MYA) which predates to the Pleistocene glacial events. However, the other divergence estimates may well coincides with the last glacial fluctuations. The lesser amount of divergence seen among some of the *A. quercustozae* populations may indicate higher levels of gene flow or more recent genetic divergence.

Current results obtained with this study indicate that coupled with the environmental fluctations of the Pleistocene the Anatolian Diagonal have acted as a barrier to the genetic structure of the populations of the oak gall wasp species. Analysis of mtDNA PCR-RFLP was useful in inferring phylogeographic relationships of *A. quercustozae* from Turkey. The oak gall wasp lineages were well defined according to their geographical origin, and data provided evidences that their contemporary distribution was greatly influenced by major geological events which occurred in Anatolia. More phylogeographic studies of different species from Turkey will facilitate to identify a general pattern in the area with ancient lineages and

possible refugia and the proposed conclusion drawn with this study can be proven by future and more detailed work in Turkey.

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