MOLECULAR VARIATION, PHYLOGEOGRAPHY AND PHYLOGENETIC ANALYSIS OF *TRIGONASPIS SYNASPIS* (HYMENOPTERA: CYNIPIDAE) FROM ANATOLIA

GAMZE ATAY

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by
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Approval of the Graduate School of Natural Science

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ABSTRACT

MOLECULAR VARIATION, PHYLOGEOGRAPHY AND PHYLOGENETIC ANALYSES OF *TRIGONASPIS SYNASPIS* (HYMENOPTERA: CYNIPIDAE) FROM ANATOLIA

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Intraspecific molecular variation, phylogenetic and phylogeographic structure of *Trigonaspis synaspis* with wide distribution in Anatolia were investigated in this thesis through sequencing 433 basepairs of mitochondrial cyt b gene and complete ITS2 region of nuclear DNA. For this purpose, 166 individuals representing 21 populations have been studied and 50 unique cyt b and 15 ITS2 haplotypes were identified across the studied area. Genetic diversity estimates indicated the existence of high amount of molecular variation with the average gene diversity of 0.8554 and 0.3119 for the cyt b gene and ITS2 region, respectively. Likewise, nucleotide diversity was 0.0134 for cyt b gene and 0.0015 for the ITS2 region. The divergence between the cyt b haplotypes was ranging from 0.2 to 3.1%, and 0.2 to 2.8% between the ITS2 region haplotypes. Mismatch distribution analysis for both cyt b and ITS2 showed a unimodal profile

indicating population expansion; however the values were not significantly supported by the statistical tests.

Phylogenetic analyses of the cyt b haplotypes conducted through three different approaches produced mostly similar topologies with the best resolution provided by the Bayesian analysis. Overall, all analyses generated similar haplogroup structure and in most cases main clades corresponded the geographic locations of the haplotypes. From the geographic point of view, the grouping of the haplotypes with respect to the eastern or western localities was notorious. On the other hand all analyses conducted on the ITS2 region produced mostly similar topologies with polytomic haplogroup largely without apparent geographic structure.

The conventional insect molecular clock applied to the mtDNA haplotypes signified the effects of the Quaternary climatic oscillations. Most of all, diversification of the outgroup haplotypes of *T. megaptera* from *T. synaspis* haplotypes goes back to 2.3 MYA which corresponds to the late Pliocene. Further, the Beast analysis related to the node ages extrapolated that the Pleistocene fluctuations were influential for shaping the major clade structure in *T. synaspis*. Furthermore, current results of this thesis support the findings of previous studies in the area that geography was also responsible for giving the subsequent phylogeographic structure among the *T. synaspis* lineages.

Key words: Anatolia, Cynipidae, genetic diversity, mitochondrial DNA, nuclear DNA, phylogenetics, phylogeography, *Trigonaspis synaspis*.

ÖZET

TRIGONASPIS SYNASPIS (HYMENOPTERA: CYNIPIDAE) 'İN ANADOLU POPULASYONLARININ MOLEKÜLER VARYASYONU, FİLOCOĞRAFİK VE FİLOGENETİK ANALİZLERİ

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Bu tezde, Anadolu'da geniş bir dağılım gösteren *Trigonaspis synaspis*'in tür içi moleküler varyasyonu, filogenetik ve filocoğrafik yapısı, 433 baz çiftlik mitokondriyal sitokrom b geni ve nüklear DNA'nın ITS2 bölgesi dizi analizi yapılarak araştırılmıştır. Bu amaçla 21 populasyonu temsil eden 166 birey çalışılmış ve 50 özgün sitokrom b ile 15 ITS2 haplotipi belirlenmiştir. Genetik çeşitlilik analizleri Anadolu'da yüksek oranda moleküler varyasyonun olduğunu göstermiştir. Ortalama gen çeşitliliği sitokrom b için 0,8554 ve ITS2 bölgesi için 0,3119'dır. Benzer şekilde nükleotid çeşitliliği cyt b için 0,0134 ve ITS2 bölgesi için ise 0,0015'dir. Sitokrom b haplotipleri arasındaki farklılaşma %0,2 ile %3,1 ve ITS2 bölgesi haplotipleri içinde ise %0,2 ile %2,8 arasında değişmektedir. Uyumsuzluk dağılım analizleri hem sitokrom b için hem de ITS2 için populasyonun genişlediğini işaret eden unimodal bir profil sergilemiştir; ancak değerler istatiksel olarak anlamlı bulunmamıştır.

Sitokrom b geni kullanılarak üç farklı yaklaşımla yapılandırılan filogenetik analizler benzer topolojiler göstermekle birlikte, en iyi ayrışma Bayesian analiziyle sağlanmıştır. Genel olarak bütün analizlerin benzer haplogrupları oluşturduğu ve coğrafik açıdan ele alındığında haplotiplerin doğu ve batı olarak gruplandığı gözlenmiştir. Öte yandan, ITS2 bölgesi ile yürütülen tüm analizler politomik haplogruplar içeren ve belirgin bir coğrafi yapısı olmayan çoğunlukla benzer topolojileri vermiştir.

Trigonaspis synaspis mitokondriyal DNA veri setine uygulanan konvensiyonel böcek moleküler saati Kuaterner dönemindeki iklimsel değişimlerin etkilerine işaret etmektedir. Dışgrup türü olan *T. megaptera* haplotiplerinin *T. synaspis* haplotiplerinden ayrışması Pliyosen'in sonlarına tekabül eden 2,3 milyon yıl öncesine işaret etmektedir. Düğüm yaşlarıyla ilgili olarak yapılan Beast analiz sonuçları *T. synaspis*' in başlıca klad yapılarının ayrışmasında Pleistosen dönemindeki değişimlerin etkili olduğunu destekler niteliktedir. Ayrıca, *T. synaspis* soyhatlarının filocoğrafik yapısının şekillenmesinde Anadolu'daki coğrafik faktörlerin de etkili olduğu ve bu alanda yapılan benzer çalışmaların sonuçlarıyla örtüştüğü belirlenmiştir.

Anahtar kelimeler: Anadolu, Cynipidae, genetik çeşitlilik, mitokondriyal DNA, nüklear DNA, filogenetik, filocoğrafya, *Trigonaspis synaspis*.

To my family…

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CHAPTER I

INTRODUCTION

1.1. Galls: Structure and Formation

Diverse groups of plants show a variety of interactions with other organisms. One of the most fascinating relationships is observed between plants and insects. In this association plants act as a host for parasites providing shelter, food and other necessary materials for the developing larvae in the form of structurally reformed parts or vegetative growths (Ronquist & Liljeblad, 2001). The most complicated plant growths are galls caused by more than 13000 insect species (Stone & Schönrogge, 2003). The major groups of insects with the ability to form galls on plants are gall midges (Cecidomyiidae), aphids (Aphididae) and gallwasps (Cynipidae) (Price, 2005). Among these gall inducing insects gallwasps constitute the primary group from the order Hymenoptera and the family Cynipidae, of which 78% attack oaks (*Quercus*) (Burks, 1979).

Oak gallwasps are accepted as obligatory parasites of their hosts, and highly specialized for different plant parts, tissues and organs of oak trees. Galling site on the host may be leaf, bark, branches or twigs depending on the cynipid species (Stone et al., 2002b). A cynipid gall is composed of larval chamber that is enclosed by a sclerenchyma wall, nutritive tissue for providing food and other materials for the developing larva(e), and an outer layer of parenchyma and sclerenchyma. The number of larval chamber varies between species from one (uni-, monolocular) up to

hundred (multilocular) depending on the gallwasp species. Larval chamber is lined with a thin wall of nutritive tissue developed from the surrounding vacuolated parenchyma that is destined to become nutritive tissue later on during the development of the gall (Stone & Schönrogge, 2003). Outer gall layer is overall responsible for gall diversity observed in cynipids due to modifications in the outermost layer. Species and generation-specific galls exhibit phenotypic variation in gall shape and size as well as galled-site on the host plant which is tied with the extended phenotype of the galler's genes. Gall wall thickness and heavy lignifications, coatings of hairs and spines, length and densities of spines and hairs, presence of sticky resin material and air spaces between larval chamber and outer wall are variations observed in gall morphologies (Stone & Cook, 1998). Variation in gall morphological traits is thought to be related with the defense mechanisms against possible parasitoid attack (Stone et al., 2002b).

Cynipid gall formation is carried on by insect-plant interaction to form novel structure in the plant host. Gall formation starts with adult female laying her eggs down in a specifically chosen place using either chemical clues or volatiles (Eroğlu, 2000). Three stages in gall formation have been distinguished as initiation, growth and maturation. Initiation (gall formation) starts after egg-laying by a female onto meristematic tissue of the host and the process is thought to be related with the ovipositor secretion of the female. Following cell proliferation, differentiation and hypertrophy, cells start forming inner gall chamber lined with inner gall tissue and the surrounding outer gall of cortical parenchyma. Growth is the second stage in gall formation leading gall and inner larval chamber to grow. The parenchyma is transformed into nutritive tissue as the larva inside grows and the nutritive tissue decreases in size. In the last stage gall reaches maturation, cell proliferation stops,

and gall tissue lignifies, the larva inside reaches maturity after pupation and it leaves the gall after making an exit hole to go out as an adult, and finally gall desiccates (Harper et al., 2004).

Galling has been thought to evolve repeatedly in various plant groups and in specific plant parts of the host species. Fossils of insect-induced galls over 300 million years (MY) old are known (Stone et al., 2008), and alone with biogeographic data they suggest that the Cynipidae originated at least at the mid Cretaceous period around 83 million years ago (Ronquist, 1999). Oak gallwasps constitute a species-rich group with nearly 1000 species in 40 genera distributed worldwide, predominantly in the northern hemisphere (Ronquist, 1999). The main oak gallwasp lineages have been argued to diverge in Central America together with their oak hosts (Manos et al., 1999). Species richness is therefore greater in the Nearctic Region with an estimated number of 700 species in 10 genera compared to the Palearctic with nearly 163 species in 10 genera (Rokas et al., 2003).

1.2. The Study Species: *Trigonaspis synaspis* (Hartig, 1843)

The genus *Trigonaspis* is one of the oak gallwasp taxa represented by 25 known species from Holarctic region, mostly from Nearctic. In the western Palearctic area 5 species are found, 3 of them (*T. baetica, T. bruneicornis, T. mendesi*) are endemic to the Iberian Peninsula and 2 of them (*T. megaptera* and *T. synaspis*) are widely distributed in Europe. The genus *Trigonaspis* was first described by Hartig (1840), who chose the type species as *T. crustalis.* Later, Melika & Abrahamson (2002) made *Xanthoteras* Asmead, 1897, *Belizinella* Kovalev, 1965, and *Ussuraspis* Kovalev, 1965 synonymous of *Trigonaspis.*

Trigonaspis synaspis was first named by Hartig (1841) as *Apophyllus synaspis* by using the asexual generation (Melika, 2006). In later years, these asexual

insects were also given as *Biorhiza synaspis* by Taschenberg, 1866 and *Cynips synaspis* by Kaltenbach, 1867. However, Mayr (1881) moved this taxon to the *Trigonaspis* genus (Melika, 2006). On the other hand, *Trigonaspis megapteropsis* is another invalid name which is used for describing the sexual generation of *T. synaspis* by Kieffer (1897-1901) (Melika, 2006).

Like some other gallwasp species, *Trigonaspis synaspis* have two alternative generations. Their spring (sexual) generation matures in May, mostly two years after egg-laying. Asexual generation galls mature in September and their hosts are generally Section Quercus *s. str.*. *Trigonaspis synaspis* uses *Quercus petraea, Q. pubescens, Q. robur, Q. pyrenaica* and *Q. faginea* as host plants. In Transcaucasia and Iran, they also attack *Q. macranthera* and *Q. infectoria* (Melika, 2006).

Asexual generation galls of *Trigonaspis synaspis* are found on *Q. petraea, Q. infectoria* and rarely on other white oak species in Turkey. They are leaf galls attached on the underside of the leaf veins, hardly observed on the upper surface of the leaf and on young shoots. These monolocular small galls, occasionally bigger than a pea, have a dull surface, and they are at first greenish and then reddish brown and spotted brown in coloration (Figure 1). Asexual generation galls have one unattached room in a hard wall when mature, but in early stages this room is attached to the wall with a spongiform tissue which later disappears (Figure 2). Adult leaves the gall after making an exit hole in the gall in late fall (Figure 3).

1.3. Phylogeography and Molecular Markers used in Phylogeography

Phylogeography is a field of research concerned with the principles and processes determining geographical distribution of genetic lineages. It is rather useful in elucidating processes and common patterns of population/ species/ lineage subdivision, speciation, and effects of the past climatic oscillations (Avise, 1998).

Figure 1. Asexual gall of *Trigonaspis synaspis* attached underside of an oak leaf.

Figure 2. Inner gall structure and the larval chamber of *Trigonaspis synaspis.*

Figure 3. Adult *Trigonaspis synaspis* recently emerged from a gall.

Moreover, phylogeography is related with studying dispersal of species/ lineages, origin of a taxon, influences of past environmental factors and physical barriers to shape the current population structure of a taxon and description of cryptic taxa otherwise would not be detected using classical morphological and other conventional characters and methods (Avise, 2000; Challis et al., 2007). Enormous number of studies has been conducted to reveal phylogeographic structure, patterns and processes in various groups of taxa including oak gallwasps (Atkinson et al., 2007; Cook et al., 2002; Liljeblad & Ronquist, 1998; Rokas et al., 2003; Stone et al., 2002a; Stone et al., 2002b; Stone et al., 2007; Stone et al., 2008).

Phylogeography is on one hand quite robust way of assessing the distribution of genetic variation across the range of a species; on the other hand molecular markers have been applied efficiently to test different phylogeographic hypotheses associated with the studied area and species. The development of molecular markers is at the same time directly correlated with the advances in the field of molecular

biology and ended up with great amount of information accumulated in recent years (Ferraris & Palumbi, 1996). In the last few decades a range of molecular techniques have been used to obtain data through the applications of these methods in different animal taxa (Jang-Liaw et al., 2008). In the large body of recent work employing molecular markers in systematics, use of a combination of nuclear and cytoplasmic genomes are predominating the research area since both have specific and unique features that make certain hypotheses to be tested in a more vigorous way (Quenouille et al., 2004). To that end the majority of ongoing researches, genes or segments of nuclear (nDNA) and mitochondrial DNA (mtDNA) are the most commonly sequenced regions in insect systematics (Roehrdanz et al, 2003).

Nuclear genome is biparentally inherited, recombination is apparent and it is possible to test the effects of population size through using specified genes with distinct mutation rates (Hoy, 1994; Zhang & Hewitt, 2003). Among these genes internal transcribed spacer (ITS) regions of nuclear DNA (nDNA) have been commonly used in phylogenetic and phylogeographic studies (Ji et al., 2003). ITS refers to the non-functional RNA placed between structural ribosomal RNAs (rRNA) on a precursor transcript which is read from 5´ to 3´ as polycistron containing 5´ external transcribed sequence (ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA and 3´ ETS (Figure 4). During RNA maturation process ETS and ITS segments are excised and as non-functional maturation products they are rapidly degraded. Either ITS1 or ITS2 region sequences are widely used for taxonomic, molecular phylogeny and phylogeographic studies. In these studies ITS2 is heavily preferred for comparison purposes because of its high copy number which makes it to amplify easily even from small quantities of DNA. In addition, due to relatively low evolutionary pressure acting on ITS segment since it is a non-coding region high

degree of variation is elucidated even among individuals of the same and closely related species. Thus the ITS region currently predominates molecular systematic studies at the species level; for instance to identify geographic structure of populations particularly in Diptera and Hymenoptera. Moreover, specific primer pairs started to accumulate after many studies in insects which allowed testing the efficacy of these primers to amplify the related fragment (Caterino et al., 2000).

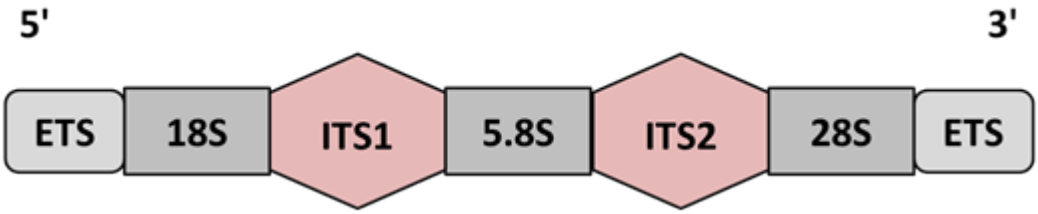

Figure 4. Representation of nuclear ITS (internal transcribed spacer) regions in insect nuclear genome.

Compared to nDNA originated genes, mitochondrial genome in animals have been frequently used in the past decades to asses genetic population structure of species, demographic factors of populations, population expansion and bottleneck events that were experienced by species, recolonization events and invasion routes, phylogeography and phylogenetics of taxa and evolutionary processes that shaped the current structure of taxa (Avise, 2000). Specifically insect mtDNA has been a precious source of genetic variability used in molecular systematics that enhanced our understanding of population and evolutionary biology at both intraspecific and interspecific levels (Arias et al., 2006). MtDNA is maternally inherited circular genome that harbors essential components of eukaryotic genetic material with no known recombination (Lemire, 2005). It is 16 kilobase (Kb) in size and composed of an A+T- rich region in insects and 37 genes, of which 22 codes for tRNAs, 2 codes

for rRNAs and 13 genes for mRNAs encoding protein products (Harrison, 1989) (Figure 5).

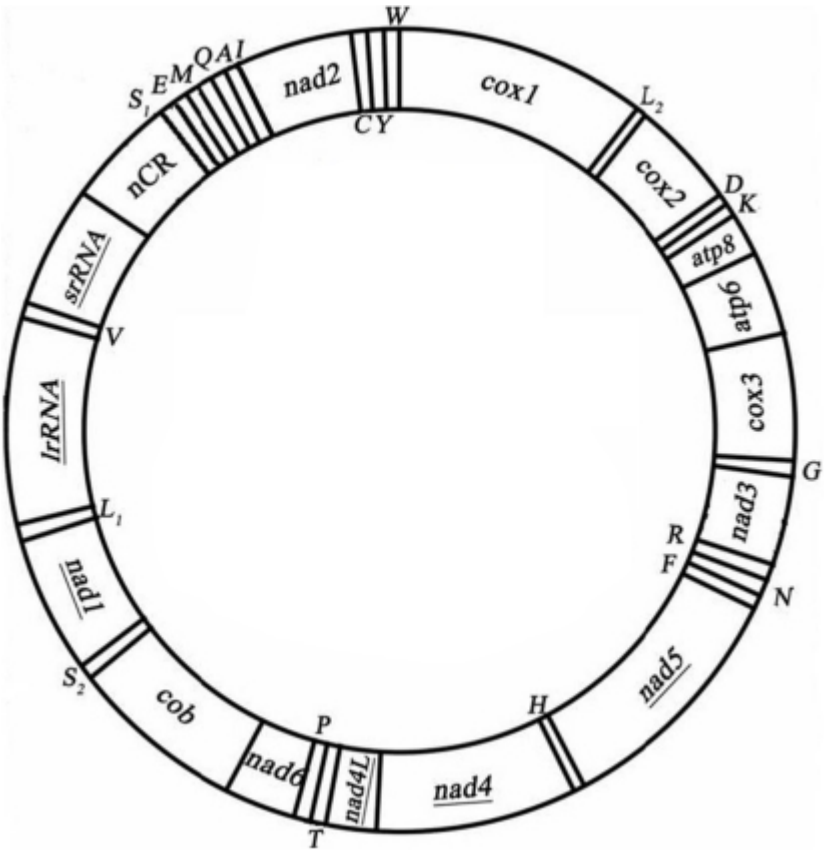

Figure 5. Common circular structure and the gene arrangements of insect mtDNA (from Tan et al., 2011).

Since the discovery of mitochondria evolved from free-living bacteria great deal of information has been accumulated with respect to mitochondrial gene rearrangements, genome structure and nucleotide variations which have been efficiently used at both high and lower levels (Margulis, 1970). Among all mitochondrial genes the presence of different mutation rates in these genes makes mitochondrial genome valuable for any taxonomic category. For mtDNA, the most

frequently studied gene at the intraspecific level is the cytochrome b (cyt b) gene which has been particularly useful for analyzing the historical basis of intraspecific population genetic structure in insects (Jermiin & Crozier, 1994; Loxdale & Lushai, 1998). Because the 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 (Stone & Cook, 1998). Besides, it is the most frequently and extensively used and sequenced as a valuable molecular marker gene for assessing phylogenies and phylogeographic structure of oak gallwasps (Cook et al., 2002; Rokas et al., 2003; Atkinson et al., 2007; Nicholls et al., 2010).

1.4. Importance of Turkey for Gallwasps

Current genetic structure of populations is governed by contemporary constituents such as ecological factors and population demography, and historical aspects and geographic structure of the distribution range of the taxon under study (Hewitt, 1999). Areas with complicated geologic history, the presence of variation in the climatic regimes even within short geographic distances ended up with enriched ecological conditions make an area prone to bear interesting phylogeographic distributional pattern of genetic lineages. Such an example is Turkey which has undergone a transformation of geologic and paleogeomorphological changes. Turkey had passed through a very active neotectonic changes as it is still highly active today (Bozkurt, 2001). It is placed in the derivative of the Aegeid plate or the Irano-Anatolian plate which is proposed to be formed after a tectonic detachment from the north of African landmass in the Cretaceous time. In spite of many other previous changes and modifications, major formation of the Irano-Anatolian plate began in the Late Oligocene with a collision between Indian and Asian Continental masses

followed by Africa and Eurasia. This last collision ended up with the formation of the Taurus Mountains in Anatolia together with the Alps, the Dinaric and the Hellenic Mountains in Yugoslavia, Albania and Greece of today. Associated with these and following regressive and transregressive events a series of faunal exchange occurred recurrently between the Irano-Anatolian plate and continental Europe and between Persian plate and Africa during the Miocene period. In the following period, the Pliocene, flooding events of Mediterranean Sea were responsible for more or less the present form land-sea configuration of Turkey. However, the greatest impact on faunal composition and formation of much or less the current population genetic structure of taxa in Turkey and in the vicinity occurred due to the major effects of the Pleistocene (Hsü, 1978; Rögl, 1999). There are four major glacial periods with interglacials between each resulted in the movement of taxa from north to south, to escape the cooling effects of glacial periods and vice versa during the intergalcials. Thereby, Anatolia acted as a shelter for many plant and animal taxa during the Pleistocene cycles (Hewitt, 1996).

Furthermore, located between Asia, Africa and Europe, Turkey acted as a bridge allowing animal and plant species to be exchanged between these regions, hence having rich fauna and flora in the region and gene flow between populations (Şekercioğlu et al., 2011). Further genetic exchange occurred between populations that played as source of genetic variation later for the European populations (Dubey et al., 2007). One additional explanation for Turkey having high species and genetic diversity is related with topographical structure of Anatolia. As it was sounded up by Davis (1971) the mountain belt running from the north-east part of Turkey near Gümüşhane-Bayburt to the southwards to the Anti-Taurus and making a bifurcation; one leading to the Amanos mountains and the other to the Cilician Taurus. The belt

was later known as the Anatolian Diagonal, and has proven to be important as a break between west and east in Anatolia both with respect to the distribution of taxa and genetic lineages (Avcı, 1993; Ekim & Güner, 1986; Çıplak, 2001; Mutun, 2010).

The effects of the past geologic conditions and physical barriers such as the Anatolian Mountain belt have been of great interest since parasitic groups of organisms are rather exciting organisms and attract particular attention for phylogeographic patterns considering their host dependence. Specially, the traditional model of temperate species anticipates that temperate species survived glacial periods of the Pleistocene in certain shelters known as refuge areas predominantly located in the Iberian Peninsula, Italy, Balkans, and in the more eastern regions covering Anatolia and Iran (Hewitt, 1996; Nieberding et al., 2005; Bohlen et al., 2006). During the interglacial periods, populations are thought to have expanded from shelters with lineages previously isolated in separate refugia often coming into contact (Hewitt, 2001). Besides latest studies have indicated that multiple oak gallwasps lineages diverged prior to the arrival of modern oaks in the western Palearctic and oak gallwasp lineages spread westwards from Anatolia and Iran. Indeed, several western Palearctic oak gallwasps are currently ranging from Iberia to Iran pointed out an eastern origin (Stone et al., 2008; Challis et al., 2007). Besides it has been indicated that Turkey was considered as a Pleistocene refugium and a source for genetic diversity through more ancient westward range expansion into Europe. Infact, researches have shown that Turkish genetic variation was greatest among all examined Palearctic populations and had several otherwise rare alleles, and represented the source for pre-Pleistocene colonization of European gallwasps (Rokas et al., 2003).

1.5. Aims of the Thesis

Historical events experienced by a species and ecological demands of the taxon under investigation shape the current genetic structure of the species, and molecular markers provide worthy information corresponding those factors (Hashiguchi et al., 2006). Ongoing research in Turkey continues to search for if a general pattern is observed in gallwasps to distinguish the factors responsible for the contemporary genetic structure. Moreover, it is important to explore the relationship between gene genealogies and geography and to identify current population genetic structure.

In recent years, there has been more work on oak gallwasps in Turkey, however so far there was no attempt to elucidate the phylogeographic and population genetic structure of *T. synaspis* neither in Turkey nor in other parts of its range. Thus, the major objectives of this thesis are to reveal population genetic structure of *T. synaspis*, to relate the distribution of genetic variation with that of geographic locations of the sampled populations (phylogeography), to infer population demographic factors that might have been somehow shaped by historical factors and physical barriers. Furthermore, obtained results are aimed to be compared with those of other species from the area, in particular with the oak gallwasps. Finally, it is aimed to estimate the divergence times of main lineages of *T. synaspis* obtained with this work and try to elucidate those results with the complex geologic and paleogeographic history of Turkey.

CHAPTER II

MATERIALS AND METHODS

2.1. Collection of *Trigonaspis synaspis*

In this thesis, asexual generation of *Trigonaspis synaspis* was used with large sampling area since the species has wide distribution in Turkey. Thence, asexual generation galls were collected through the field trips from different localities across Turkey between 2011- 2013. Collection sites are shown in Figure 6 and coordinates and other necessary information is given in Table 1. Collected gall samples were brought to Molecular Zoology Laboratory at Abant İzzet Baysal University, Bolu, and kept in jars covered by tulle at room temperature until adult wasps break out the galls. By daily controls, emerged adults were picked in the basis of populations and stored in ultra-deep freezer at -80 ˚C. In total, 166 individuals from 21 populations were included in this study (Table 1).

2.2. DNA Isolation

In order to study both nuclear and cytoplasmic DNA, total DNA was extracted from the frozen samples. A single wasp was homogenized with 1000 µl extraction buffer containing 10 mM Tris (pH 8.0), 2mM EDTA (pH 8.0), 10 mM NaCl and 1% SDS. For breaking the proteins down, 20 µl proteinase K was used for each sample and these homogenates were put in shaking water bath overnight at 55

°C and 65 RPM. Next day, supernatant of each homogenate was taken carefully into another tube, and the same amount of phenol was added to the tube and vortexed. Once more the upper part of the liquid was taken and the same amount of phenol was added. The same amount of chloroform-isoamyl alcohol (24:1) was then added to the supernatant and vortexed. The upper part was taken and sodium acetate and absolute alcohol were added. The solution was vortexed and incubated at -70 °C for 1 hour. It was centrifuged for 10 minutes at 4 °C 5000 RPM. Supernatant of the liquid was spoiled; 1ml of 70% ethanol was added and centrifuged for 5 minutes, at 4 °C at 10000 RPM. The supernatant was spoiled again; the pellet was let to dry at room temperature. After the alcohol was completely vaporized, the pellet was solved with Tris-EDTA buffer (pH 8.0). For ensuring DNA isolation, each sample was run in 1% agarose gel with a molecular marker (Sigma, DO428) at 100 V and 50 mA for 45 minutes with the running buffer of Tris-Boric acid-EDTA (pH 8.0).

2.3. Polymerase Chain Reaction (PCR)

Integrated with polymerase chain reaction (PCR) through using conserved or universal primers polymorphism can be detected as insertions and deletions and changes in nucleotide sequences in the form of point mutations. Amplification of specific genes either coding for protein products or non-coding DNA fragments are ideal for nucleotide sequencing and have been widely exploited for molecular systematic and phylogeographic studies (Roehrdanz & Degrugillier, 1998).

Figure 6. Sampled populations of *Trigonaspis synaspis*. Names, abbreviations and coordinates of the studied populations are shown in Table1.

Table 1. Abbreviations, coordinates and sample sizes of the studied populations of *Trigonaspis synaspis.*

Populations	Abbreviation	Coordinates	Sample Size
1. Adıyaman	ADY	N 37˚ 76' E 37˚ 71'	10
2. Afyon	AFY	N 38˚ 47' E 30˚ 43'	3
3. Bingöl	BNG	N 38˚ 94' E 40˚ 29'	10
4. Bitlis	BTL	N 38˚ 48' E 42˚ 29'	10
5. Bolu	BOL	N 40˚ 71' E 31˚ 51'	10
6. Çankırı	CKR	N 40˚ 48' E 33˚ 19'	2
7. Çorum	COR	N 40˚ 58' E 34˚ 70'	12
8. Elazığ	ELZ	N 38˚ 98' E 38˚ 51'	10
9. Erzincan	ERC	N 39˚ 64' E 38˚ 47'	8
10. Giresun	GRS	N 40˚ 34' E 38˚ 59'	3
11. Karaman	KAR	N 37˚ 39' E 33˚ 16'	6
12. Kastamonu	KAS	N 41˚ 48' E 33˚ 58'	3
13. Kayseri	KAY	N 38˚ 67' E 36˚ 32'	10
14. Kırşehir	KIR	N 39˚ 47' E 33˚ 87'	12
15. Konya	KON	N 37˚ 66' E 31˚ 96'	11
16. Muş	MUS	N 38˚ 64' E 41˚ 75'	3
17. Nevşehir	NEV	N 38˚ 86' E 34˚ 59'	10
18. Sivas	SVS	N 39˚ 80' E 36˚ 06'	8
19. Tokat	TOK	N 39˚ 98' E 36˚ 50'	10
20. Tunceli	TUN	N 39˚ 57' E 39˚ 95'	10
21. Yozgat	YOZ	N 39˚ 76' E 35˚ 93'	5

2. 3. 1. Amplification of Nuclear ITS2 Region

For nucleotide sequencing, ITS2 region of nuclear DNA was amplified from the isolated DNA templates of *T. synaspis* individuals using ITS2 - F – 5´ -ATGAAC ATCGACATTTCGAACGCATAT- 3´ ITS2 - R – 5´ -TTCTTTTCCGCTTAFTAAT ATGCTTAA- 3´ primers (Ji et al., 2003). Since the essential amount for sequencing is about 40 µl, 10 µl 5X buffer, 5 µl $MgCl_2$ (25mM), 3 µl dNTPs (10mM), 1 µl of each primers (20pmol), 0.5 µl template DNA and 0.5 µl *Taq* polymerase (Vivantis) were used and 19 µl of ddH_2O were added to each reactions to complete 40 µl. XP Thermal cycler was used for carrying out the amplification reactions. Temperature rates, times and cycles were set as follows: 5 min at 95 °C for first denaturation; 35 cycles of 40 sec at 95 °C for denaturation, 40 sec at 52.2°C for annealing, 45 sec at 72 °C for elongation and 5 min at 72 °C for last elongation. For ensuring amplification, PCR products were checked in 1% agarose gel with the 1 Kb λ DNA molecular marker (Sigma, DO428). Amplicons were sent to a company and used as template in the following cycle sequencing reaction using Perkin-Elmer BigDye Terminator chemistry on an ABI automated sequencer (Applied Biosystems). For minimizing PCR artifacts, ambiguities and base-calling errors both strands were sequenced.

2. 3 .2. Amplification of Mitochondrial Cytochrome b Gene

With the isolated DNA of each individual, a 433 bp segment of mtDNA cytochrome b (cyt b) gene was amplified using CB1 – 5´ -TATGTACTACCATGAG GACAAATATC- 3´ and CB2 – 5´- ATTACACCTCCTAATTTATTAGGAAT- 3´ primers (Stone et al., 2007). Volume of all components and reagents for 40 µl are as follows: 4 µl 10X *Taq* buffer, 3.2 µl $MgCl_2$ (50mM), 0.8 µl dNTPs (10mM), 0.56 µl

of each primer (20 pmol), 1.6 µl template DNA, 0.56 µl *Taq* polymerase (Vivantis) and 28.72 µl ddH_2O. XP thermal cycler was used for carrying out the reactions. Temperature rates, times and cycles for cyt b region were set as follows: 3 min at 94 °C for first denaturation; 35 cycles of 30 sec at 94 °C for denaturation, 1 min at 50 °C for annealing, 2 min at 72 °C for elongation and lastly 10 min at 72°C for final elongation step. Same procedures were followed for PCR product controls and nucleotide sequencing as has been noted for amplification of nuclear ITS2 region.

2.4. Data Analysis

2.4.1. Sequence Alignment and Haplotype Determination

Sequencing results sent by the company as chromatograms were checked by eye to avoid misreading in both strands. Controlled sequences were transferred to BioEdit 7.0.9 program (Hall, 1999) and trimmed to have only the desired region for both ITS2 and cyt b genes. The alignment of the sequences was performed separately for each sequenced fragment with BioEdit 7.0.9 with default parameters and assembled sequences were collapsed into haplotypes based on the nucleotide substitutions. Coding fragment of mitochondrial cyt b gene was translated into amino acids for each haplotype and none of them contained internal stop codons and non-sense mutations indicating that the haplotypes are original mtDNA sequences, not numts.

Three specimens of *T. megaptera* collected from Giresun and Edirne were also sequenced and detected haplotypes were used as outgroup. Haplotypes determined in this thesis were deposited in GenBank (Acession numbers (KJ716685-KJ716687). Only a single outgroup species from the *Trigonaspis* genus was included

in further analyses due to no other species from the genus shows distribution in Turkey and there is no haplotype submitted to GenBank from other countries.

2. 4. 2. Detection of Genetic Variation and Population Structure

After haplotype determination, data were transferred to DnaSP v.5 (Librado & Rozas, 2009) and MEGA 5.0 (Tamura et al., 2007) programme packages to determine nucleotide and amino acid composition, nucleotide diversity indices and substitution patterns. MEGA program was also used for estimating the transition and transversion ratios of the sequences. Population genetic statistics were estimated using both Arlequin 2001 (Schneider et al., 2000) and DnaSP 4.0 (Rozas et al., 2003). The number of polymorphic sites (S), nucleotide (π) and haplotype (h) diversity (Nei, 1987), the number of pairwise nucleotide differences (k) (Tajima, 1983) were estimated for each population.

Population demographic histories of *Trigonaspis synaspis* were characterized using mismatch distribution as implemented under DnaSP 4.0 (Rozas et al., 2003). Observed number of pairwise differences was used to compare against constant population size and sudden population expansion models. The patterns of pairwise differences between haplotypes often forms a unimodel graph of samples from expanding populations, however samples drawn from populations at demographic equilibrium produce a multimodel pattern of numerous sharp peaks (Rogers & Harpending, 1992). The raggedness index (r) (Harpending, 1994) was calculated to check the deviations from neutrality and to see the smoothness of pairwise mismatch plot. To test deviations from neutrality Tajima's *D* was also calculated (Tajima, 1989a). Although Tajima's *D* is a very commonly used and useful statistics for assessing populations demographic patterns it is less powerful compared to some alternatives because it does not consider departures from neutrality and others can be

more sensitive indicators of population expansion than Tajima's *D*. Thus, for demographic pattern of populations in addition to Tajima's *D* (Tajima, 1989b), Fu's and Li's *D** (Fu and Li, 1993) were also calculated based on the infinite site model without recombination and assumed populations being at equilibrium for mtDNA sequence data.

2.4.3. Phylogenetic and Phylogeographic Analysis

2.4.3.1. Maximum Likelihood, Maximum Parsimony and Bayesian Analysis

The *Trigonaspis synaspis* data matrix was composed of 166 sequences; *T. megaptera* was used as outgroup. The mean transition to transversion ratio and the nucleotide frequencies were provided by the PAUP*4.0 package. The *Trigonaspis synaspis* data were analyzed using maximum likelihood (ML), maximum parsimony (MP) in PAUP*4.0 package, and Bayesian-based inference as implemented in MRBAYES 3.0. MP analysis was performed under the heuristic search options, using TBR branch-swapping algorithm and 100 replicates of random addition of taxa. MP analysis was employed with 1000 bootstrap replicates to assess support of branches (Swofford, 2002). JModeltest 0.1.1 (Posada & Crandal, 1998) was used to find the best model of evolution that fits the data for Bayesian analysis. Based on the arguments presented by Posada and Buckley (2004), the Akaike information criterion (AIC) was used to determine the best-fit substitution model for the data in the ML reconstructions. The substitution models suggested for the analyzed fragments were HKY+I+G for cyt b and GTR+I+G for ITS2. The analysis involved in four simulations Markov Chains, four million generations with trees being sampled every 100 generations and a burn-in of 25%. The software tool TRACER version 1.3.1 (Rambout & Drummond, 2003) was used to observe the parameters and to determine

the number of trees needs to reach stationary (burn-in). After discarding burn-in trees and evaluating convergence, the remaining samples were retained for generating 50% majority rule consensus trees and calculating mean, variance, 95% credibility intervals and posterior probabilities.

2.4.3.2. Network Analysis

Phylogenies are useful tools for organizing genealogical relationships in a group of organisms and used for a variety of researches. Traditional methods like parsimony and maximum-likelihood may be insufficient for a population level study due to their assumptions being inoperative in some cases such that they assume ancestral haplotypes are no longer in the population. But those haplotypes are the most frequent ones sampled in a population level study according to Watterson & Guess (1977). These methods may also assume no recombination and for an accurate reconstruction they need large numbers of variable characters (Clement et al., 2000). Therefore, haplotype networks may be more useful to resolve the relationships within a species as gene flow may lead reticulate rather than hierarchical or treelike structure between populations. Moreover, internal nodes in networks may give more pleasant biological meaning by constant haplotypes (Posada and Crandall, 2001). Networks let one sequence to unite several times without bifurcating and give genealogical information about the divergence of the population. Additionally, reticulations may imply the effects of conflicting phylogenetic signal for mtDNA sequences. Due to all the reasons aforementioned a median joining haplotype network to reveal the relationships among haplotypes were constructed using the program HapStar Version 0.5 (C) (Teacher & Griffiths, 2011).

To test if genetic differentiation is geographically related, populations were clustered into different groupings and analyzed with molecular variance (AMOVA)

available in Arlequin 2.0 program (Excoffier et al., 1992). This method estimates the proportion of genetic variation at different hierarchical levels by using information from the geographical distribution of haplotypes and the pairwise distance between them. AMOVA analysis was performed at the levels: among geographical groups of populations, among populations within each geographical group and within populations. AMOVA with statistical significance was determined by permutation analyses to partition genetic variation at different levels. Statistical significance was determined by 1000 permutations.

2.4.3.3. Estimation of Time of Divergence

Divergence times and their confidence intervals were estimated using a Bayesian Markov Chain Monte Carlo (MCMC) coalescent method implemented in BEAST version 1.5.2 (Drummond & Rambaut, 2007). The data set was calibrated by general substitution rate (2.3% sequence divergence per lineage per million years) as it is commonly applied to insect mitochondrial DNA (Brower, 1994). Dates of the divergence were inferred using HKY model with G and I, a relaxed molecular clock, following the uncorrelated relaxed lognormal clock as implemented in BEAST. Both MRCAs (most recent common ancestors) and MACAs (most ancient common ancestors) were calculated and operators were optimized using BEAUTI. BEAST was run for 20 million generations sampling every 1000 and the convergence to stationary and the effective sample size (ESS) of model parameters were checked by TRACER. The maximum clade credibility tree built with TREEANNOTATOR discarding initial 10% samples as burn-in. FIG-TREE version 1.3.1 (Rambout, 2009) was used to visualize the results.

CHAPTER III

RESULTS

A total of 166 individuals collected from 21 populations were used in this thesis. Whole individual was used to isolate total DNA and extracted DNAs are shown in Figure 7. Isolated DNA of each individual was used for amplifying mitochondrial cytochrome b gene and nuclear ITS2 region and the agarose gel photographs are given in Figure 8 and 9, respectively. Amplified cyt b gene region is 433 basepairs (bp), and the amplicon size of the ITS2 region together with 5.8S and 28S rDNA nucleotides is 487 bp.

PCR products send to the company were sequenced in both directions to reduce the calling errors and results were obtained as chromatograms. Representative chromatograms for cyt b gene and ITS2 region are shown Figure 10 and Figure 11, respectively.

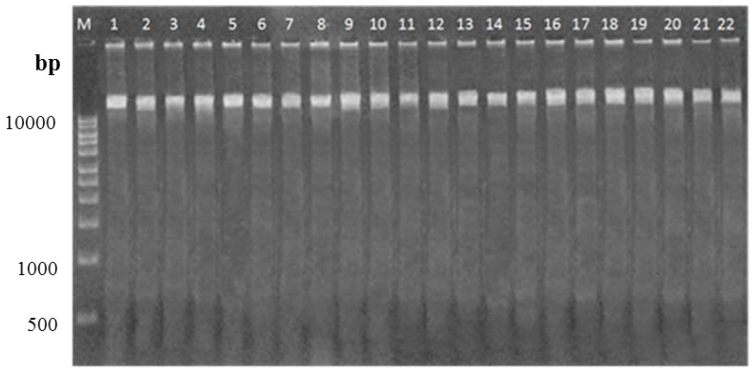

Figure 7. An agarose gel picture of isolated DNA samples of *T. synaspis*. M: 1 Kb λ DNA EcoRI and HindIII digest. 1-10: Kayseri samples, 11-22 Kırşehir samples.

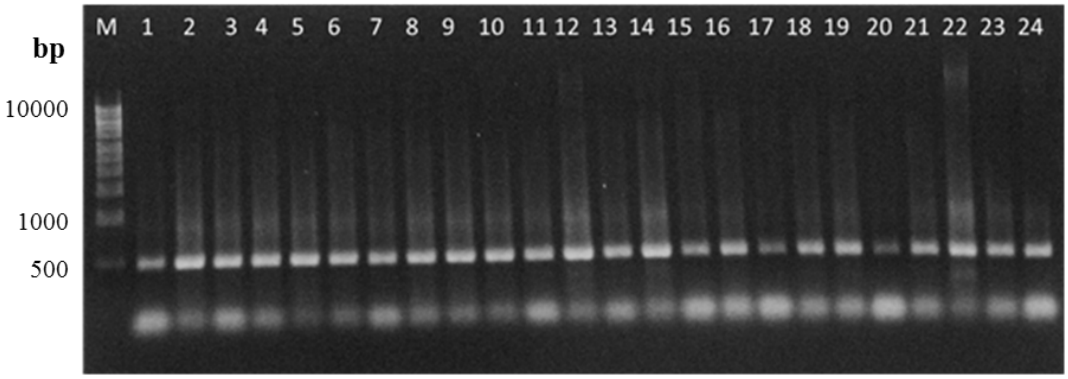

Figure 8. Agarose gel photograph of the amplified cytochrome b gene of *T. synaspis*. M: 1 Kb λ DNA EcoRI and HindIII digest. Samples are as follows: 1-5: Adıyaman, 6-10: Bingöl, 11-15: Bolu, 16-21: Elazığ , and 22-24: Muş.

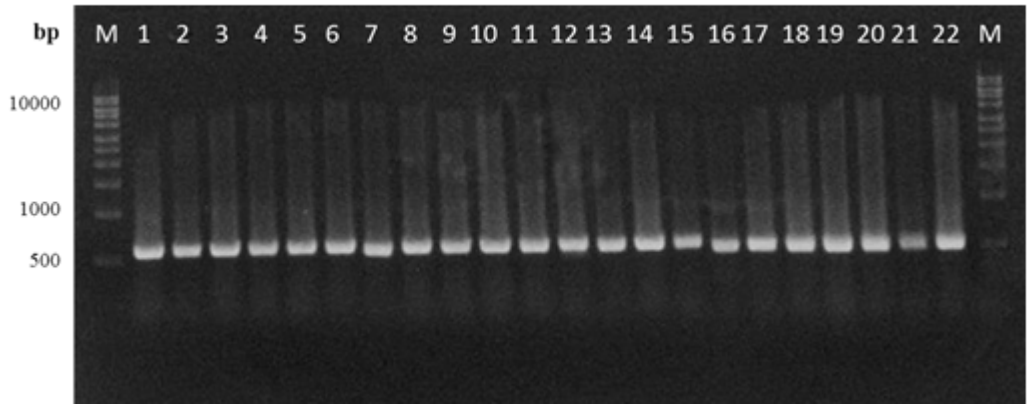

Figure 9. Agarose gel result of ITS2 region amplification. M: 1 Kb λ DNA EcoRI and HindIII digest. Samples are as follows: 1-5: Adıyaman, 6-10: Bingöl, 11-15: Bolu, 16-21: Elazığ, and 22: Muş.

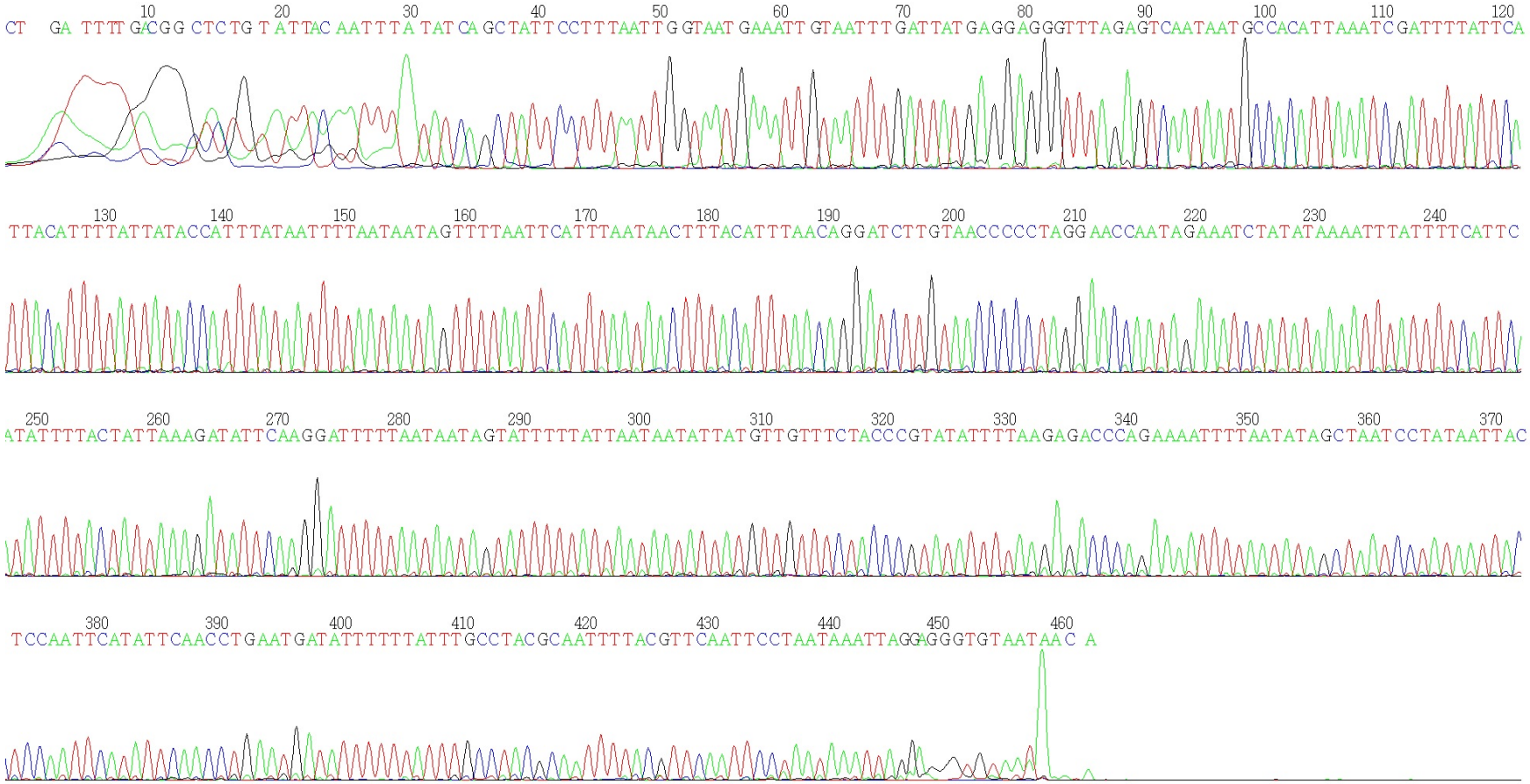

Figure 10. Representative chromatogram of 433 bp cytochrome b gene region of *Trigonaspis synaspis.*

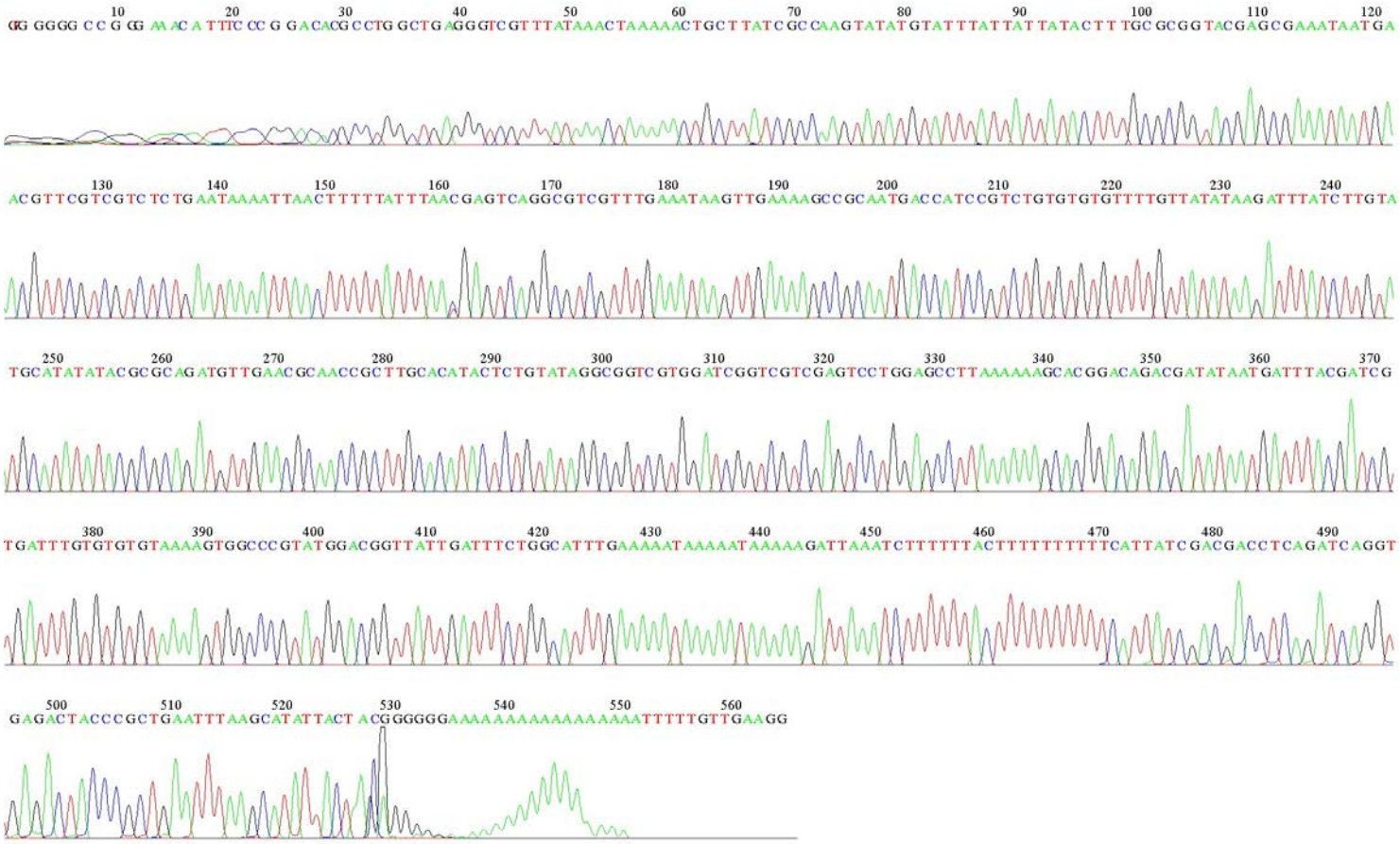

Figure 11. A chromatogram of nuclear ITS2 region of *Trigonaspis synaspis.*

3.1. Genetic Diversity Estimates of *Trigonaspis synaspis*

3. 1. 1. Mitochondrial Marker: Cytochrome b Gene Sequence Variation

A total of 166 individuals representing have been sequenced for both mitochondrial gene and nuclear DNA region. No length variation was detected in any of the mtDNA haplotypes analyzed (Acession number KJ716684 - KJ716685). Of the sequenced 433 bp of mitochondrial cyt b gene of *Trigonaspis synaspis* 391 characters were constant and 42 characters were polymorphic (S= segregating sites= 42). Polymorphic character sites are shown in Table 2. Of these variable sites 17 characters were parsimony uninformative and the remaining 25 were parsimony informative. There was only a single multiple hit at site 103 in nucleotide sequences.

Unique sequences were collapsed into haplotypes and a total of 50 haplotypes have been identified based on the observed substitutions. In the detected haplotypes the nucleotide frequencies are 34, 5 % for A, 42, 2% for T, 13% for C and 10, 3% for G. The transition - transversion ratios are k_1= 211.982 (purines) and k_2= 196.108 (pyrimidines). The transition and transversions ratios are presented in Table 3. In the cyt b haplotypes there was only one transversion (at site 55) and 40 transitions (in all other remaining substitution sites). C/T transition was the most frequently observed one (40.31), followed by G/A (35.68), respectively. Transition/transversion bias in haplotypes was calculated according to the equation provided in Tamura et al. (2004) and was revealed as R= 5.19. Average number of nucleotide differences for all haplotypes was calculated as k= 5.830.

Table 2. Substitutions and their sites in each haplotype. Only polymorphic sites are included in the Table.

Haplotypes	Site	43	55	70	82	85	94	103	109	112	139	151	161	163	176	196	199	205	208	209	214	217	227	232	241	247	253	290	291	319	322	325	326	340	364	365	367	371	388	391	415	418	421
	Base	T	T	T	G	G	C	C	A	T	A	T	G	T	A	A	C	C	C	C	A	T	C	T	T	T	T	G	T	C	C	G	T	C	T	C	T	A	T	A	C	C	A
H1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H2		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
H3		.	.	.	A	.	.	.	.	.	G	.	.	.	.	.	T	.	.	.	G	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
H4		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H5		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.
H6		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.
H7		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H8		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.
H9		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	A	.	.	.	A	.	T	.	.	.	.	.	.	.	.	T	.	.
H10		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
H11		.	.	.	A	A	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	C	T	.	.	.	.	A	.	T	T	A	.	T	.	.	.	.	.	.	.	.	.	.	.
H12		.	.	.	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H13		C	.	.	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H14		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
H15		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.
H16		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.
H17		.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	T	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H18		.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	T	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H19		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.
H20		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
H21		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H22		.	.	.	A	.	.	.	.	.	C	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H23		.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	T	.	T	.	C	.	.	C	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H24		.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	T	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H25		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	A	.	T	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
H26		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	A	.	T	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
H27		.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	T	.	T	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H28		.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	T	.	C	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
H29		.	A	.	A	A	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
H30		.	.	C	A	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	C	.	.	.	.	C	.	.	.	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
H31		.	.	C	A	A	.	.	.	.	G	.	.	C	.	.	.	.	.	.	C	.	.	.	.	C	.	.	T	.	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.
H32		.	.	.	A	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H33		.	.	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H34		.	.	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
H35		.	.	.	A	.	T	.	.	.	.	.	.	.	.	G	T	.	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.
H36		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	T	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	.
H37		.	.	.	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	C	A	.	T	T	A	.	.	C	.	.	.	.	.	.	.	.	.	G
H38		.	.	C	A	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	.	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
H39		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H40		.	.	C	A	A	.	.	.	.	G	.	.	C	.	.	.	.	.	.	C	.	.	.	.	C	.	.	.	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
H41		.	.	.	A	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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H43		.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	T	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
H44		.	.	.	A	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H45		.	.	.	A	.	T	.	G	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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H47		.	.	.	A	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
H48		.	.	.	A	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	A	C	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.
H49		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H50		.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	T	.	.

Table 3. Maximum composite likelihood estimate of the pattern of nucleotide substitutions. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are also shown in the Table.

	A	**T**	**C**	**G**
A	–	0.21	0.06	**10.61**
T	0.17	–	**12.43**	0.05
C	0.17	**40.31**	–	0.05
G	**35.68**	0.21	0.06	–

All sequences of *T. synaspis* were translated into amino acids since cyt b gene is a protein coding gene, and a universal insect amino acid translation list for mitochondrial genome is provided in Table 4. Of the 433 bp cyt b gene translated into amino acids the triplets produced 144 amino acids, of which there were 6 replacement substitutions (Table 5). When the insects' amino acid sequences are compared with those of *T. synaspis* there were no insertion / deletion (in-del) or non-sense amino acids. Thirthy three substitutions occurred at third codon position, one substitution was observed at second codon position and eight were at first codon position. On the other hand, the amino acid replacements occurred at sites 54 (from valine to isoleucine), 59 (from methionine to valine), 97 (from valine to methionine and threonine), 109 (from tyrosine to histidine), 122 (from proline to serine) and 124 (from isoleucine to valine). The only multiple hit in the amino acid substitution were present at amino acid site 97. In the cyt b haplotypes of *T. synaspis* there was a tendency to use leucine (L_{UUA}= 17), followed by isoleucine (I_{AUU}= 16), and phenylalanine and methionine (F_{UUU}=12, M_{AUA}= 12). However, there was no use of some triplets as can be seen in Table 6.

Table 4. Amino acid translation table for insect mitochondrial genome. (T: mRNA codons, AA: amino acids, and A: letter abbreviation for the specified amino acid).

T	AA	A	T	AA	A	T	AA	A	T	AA	A
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**

To detect if there is deviation from neutrality, the mismatch distribution of all pairwise combinations for the cyt b mitochondrial DNA haplotypes was estimated and the result is shown in Figure 12. The mismatch distribution including all samples was indicating a unimodal profile for the *T. synaspis* haplotypes; however the results were not significantly supported. The Harpending's raggedness index was r = 0.30102, but not significant. Overall, Tajima's *D* neutrality test (Tajima's *D* = -1.34298, P > 0.10) and Fu and Li tests (*D** = -1.62363, P > 0.05; *F** = -1.81399, P > 0.10) were not significant for most populations analyzed, indicating that these populations are not deviated significantly from neutrality (Kimura, 1983).

The cyt b haplotype frequencies and the populations that possess those haplotypes are shown in Table 7. Of the 50 ingroup haplotypes there were 22 private

haplotypes (singleton). Of the all singletons, Elazığ had 3, Adıyaman and Sivas had two, and the other populations (Afyon, Bitlis, Çankırı, Çorum, Erzincan, Giresun, Karaman, Kayseri, Kırşehir, Konya, Muş, Tokat, Tunceli and Yozgat) contained a single private haplotype.

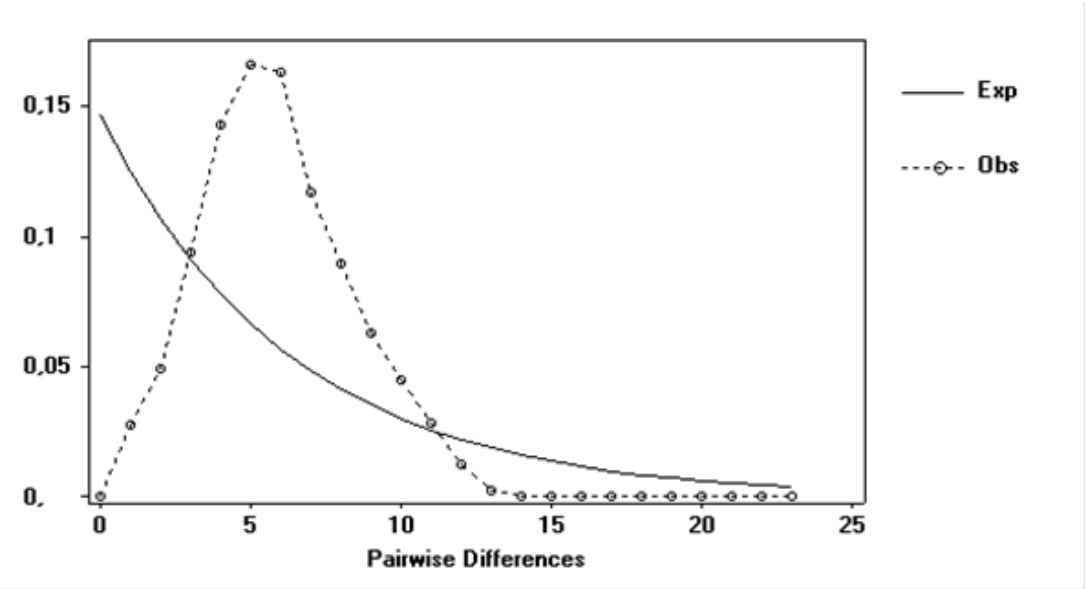

Figure 12. Mismatch distribution of all pairwise combinations for the cyt b mtDNA haplotypes of *T. synaspis*. The observed distributions are represented by dotted line and the expected frequency is depicted by solid line.

Of the remaining, 28 *T. synaspis* cyt b haplotypes are found in more than a single individual representing either a single locality or shared between populations. Of the 50 haplotypes H16 (N= 18) was the most frequent and shared haplotype found in 5 localities as Çankırı (N= 1), Çorum (N= 1), Giresun (N= 2), Kırşehir (N= 11), and Sivas (N= 3). H14 (N= 13) was the second most frequent haplotype found in Bolu (N= 10) and Kastamonu (N=3). The next most frequent haplotype was H10 (N= 12) detected in four populations as following: Bitlis (N= 1), Sivas (N= 2), Tokat (N= 7) and Yozgat (N= 2). On the other hand, when the populations were searched with respect to their haplotype number, Bolu (H14 detected in 10 individuals) and

Kastamonu (H14 found in 3 individuals) had fixed haplotype. All other populations had more than a single type of haplotype. In order to reveal haplotype diversity in number, populations were screened for the presence of diverse haplotypes. In that, Elazığ had the most diverse types of haplotype where 7 distinct haplotypes have been observed out of 10 speciemens. All of the remaining populations showed a range of 2 to 5 haplotypes.

For the determination of sequence difference between each *T. synaspis* haplotype a pairwise comparison was conducted using the MEGA program and the results are shown both in number of differences and percentages separately in Table 8. Pairwise comparisons showed that the haplotype difference ranged between 0.2 % and 3.1 % (1 bp to 13 bp difference, respectively). One base pair nucleotide difference was found between 32 cases (see Table 8). The most divergent haplotypes with 13 bp difference were H37 (from Konya population) and H2 and H3 (both from Adıyaman population).

Molecular variation of *T. synaspis* populations are given in Table 9. Average haplotype diversity was calculated as 0.8554 ± 0.0002, and the average nucleotide diversity was 0.0134 ± 0.0001. Highest haplotype diversity was observed in Çankırı population (h= 1.000 ± 0.5) followed by the Yozgat population (h= 0. 9001 ± 0.161). Bolu and Kastamonu populations did not show any haplotype variation. On the other hand, nucleotide diversity estimates ranged between 0.0000 and 0.0126 ± 0.0081. The highest nucleotide diversity was in the Karaman population (π=0.0126 ± 0.0081) followed by the Kırşehir population (π=0.0125 ± 0.0074).

For revealing the hierarchical distribution of the genetic variation at different levels several grouping schemes with respect to the populations' geographic origins have been conducted and the outcomes of the analyses are given in Table 10 using

the cyt b data set. The first grouping included (Table 10a) in one-group analysis included all 21 populations as distinct groups and highest genetic partitioning belonged to among-groups (56%). There was still high amount of genetic variation present within populations (43%). The second grouping trial (Table10b) in which all populations were divided into two major geographical groupings as ‘Eastern’ and ‘Western’ indicated that among-group variation was only 13% compared to among populations within groups (45%), and within population (40%). Lastly, in the third trial populations were grouped into five groups (Table10c), highest partitioning of genetic variation was observed within population (40%). Several more grouping schemes have been tested and similar results have been obtained in all analyses, thus those results are not included in this thesis here.

Table 5. Translated amino acids of 433 bp cyt b gene region of *T. synaspis* mtDNA. Replacement amino acids are shown in highlighted columns.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
H1	**F**	**W**	**G**	**A**	**T**	**V**	**I**	**T**	**N**	**L**	**M**	**S**	**A**	**I**	**P**	**L**	**I**	**G**	**N**	**E**	**I**	**V**	**I**	**W**	**L**	**W**	**G**	**G**	**F**	**S**	**V**	**N**	**N**	**A**	**T**	**L**	**N**	**R**	**F**	**Y**	**S**	**L**	**H**	**F**	**I**	**M**	**P**	**F**
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Table 5 continued.

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H1	**M**	**I**	**L**	**M**	**M**	**V**	**L**	**I**	**H**	**L**	**M**	**T**	**L**	**H**	**L**	**T**	**G**	**S**	**C**	**N**	**P**	**L**	**G**	**T**	**N**	**S**	**N**	**L**	**Y**	**K**	**I**	**Y**	**F**	**H**	**S**	**Y**	**F**	**T**	**I**	**K**	**D**	**I**	**Q**	**G**	**F**	**L**	**M**	**M**
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Table 5 continued.

	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144
H1	**V**	**F**	**L**	**L**	**M**	**M**	**L**	**C**	**C**	**F**	**Y**	**P**	**Y**	**I**	**L**	**S**	**D**	**P**	**E**	**N**	**F**	**N**	**M**	**A**	**N**	**P**	**M**	**I**	**T**	**P**	**I**	**H**	**I**	**Q**	**P**	**E**	**W**	**Y**	**F**	**L**	**F**	**A**	**Y**	**A**	**I**	**L**	**R**	**S**
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H21	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H22	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H23	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H24	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H25	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H26	**M**	.	.	.	.	.	.	.	.	.	.	.	**H**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H27	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H28	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**S**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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H32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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H37	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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H41	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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H43	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H44	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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H47	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H48	**T**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H49	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Table 6. Amino acid use in *T. synaspis* cyt b gene fragment. Triplets that code for specific amino acids with their universal abbreviations (T – A), frequency of the used amino acids (#) are given in the table. (*) indicates stop codons.

T - A	#	T - A	#	T - A	#	T - A	#
UUU - F	12	UCU - S	0	UAU - Y	6	UGU - C	3
UUC - F	1	UCC - S	1	UAC - Y	2	UGC - C	0
UUA - L	17	UCA - S	4	UAA - *	0	UGA - W	4
UUG - L	0	UCG - S	0	UAG - *	0	UGG - W	0
CUU - L	0	CCU - P	3	CAU - H	5	CGU - R	1
CUC - L	0	CCC - P	1	CAC - H	0	CGC - R	0
CUA - L	2	CCA - P	3	CAA - Q	2	CGA - R	1
CUG - L	0	CCG - P	1	CAG - Q	0	CGG - R	0
AUU - I	16	ACU - T	5	AAU - N	10	AGU - S	0
AUC - I	0	ACC - T	0	AAC - N	1	AGC - S	0
AUA - M	12	ACA - T	3	AAG - K	2	AGA - S	3
AUG - M	0	ACG - T	0	AAA - K	0	AGG - S	0
GUU - V	1	GCU - A	3	GAU - D	1	GGU - G	1
GUC - V	1	GCC - A	2	GAC - D	1	GGC - G	0
GUA - V	3	GCA - A	1	GAA - E	3	GGA - G	3
GUG - V	0	GCG - A	0	GAG - E	0	GGG - G	3

Table 7. Haplotypes and their frequency in each *T. synaspis* population.

Haplotype	Adıyaman	Afyon	Bingöl	Bitlis	Bolu	Çankırı	Çorum	Elazığ	Erzincan	Giresun	Karaman	Kastamonu	Kayseri	Kırşehir	Konya	Muş	Nevşehir	Sivas	Tokat	Tunceli	Yozgat	Total
H1	3																					**3**
H2	1																					**1**
H3	1																					**1**
H4	5							2														**7**
H5		2																				**2**
H6		1																				**1**
H7																	8				1	**9**
H8			8																	1		**9**
H9			2																			**2**
H10				1														2	7		2	**12**
H11				4																		**4**
H12				4												2						**6**
H13				1																		**1**
H14					10							3										**13**
H15						1																**1**
H16						1	1			2				11				3				**18**
H17							7														1	**8**
H18							2															**2**
H19							1															**1**
H20							1						3									**4**
H21								2														**2**
H22								1														**1**
H23								1														**1**
H24								1	5		2											**8**
H25								2														**2**
H26								1														**1**
H27									2									1				**3**
H28									1													**1**
H29										1												**1**
H30											3											**3**
H31											1											**1**
H32													1									**1**
H33													1									**1**
H34													3									**3**
H35													2									**2**
H36														1								**1**
H37															2							**2**
H38															1							**1**
H39															3							**3**
H40															5							**5**
H41																1						**1**
H42																	2					**2**
H43																		1				**1**
H44																		1				**1**
H45																			2			**2**
H46																			1			**1**
H47																				6		**6**
H48																				2		**2**
H49																				1		**1**
H50																					1	**1**
N	**10**	**3**	**10**	**10**	**10**	**2**	**12**	**10**	**8**	**3**	**6**	**3**	**10**	**12**	**11**	**3**	**10**	**8**	**10**	**10**	**5**	**166**
Nhap	**4**	**2**	**2**	**4**	**1**	**2**	**5**	**7**	**3**	**2**	**3**	**1**	**5**	**2**	**4**	**2**	**2**	**5**	**3**	**4**	**4**	**50**

Table 8. Pairwise comparison of 50 *T. synaspis* haplotypes. Net nucleotide differences are shown below diagonal and the percentage of the differences are presented above diagonal.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27	H28	H29	H30	H31	H32	H33	H34	H35	H36	H37	H38	H39	H40	H41	H42	H43	H44	H45	H46	H47	H48	H49	H50
H1		0.009	0.014	0.002	0.007	0.009	0.007	0.014	0.017	0.007	0.026	0.009	0.012	0.009	0.009	0.007	0.009	0.012	0.009	0.007	0.012	0.014	0.017	0.012	0.014	0.014	0.014	0.014	0.014	0.019	0.024	0.009	0.007	0.009	0.017	0.012	0.026	0.019	0.005	0.021	0.014	0.009	0.014	0.012	0.012	0.009	0.014	0.019	0.012	0.009
H2	4		0.005	0.007	0.012	0.009	0.007	0.019	0.017	0.012	0.026	0.014	0.017	0.014	0.009	0.012	0.009	0.012	0.014	0.007	0.017	0.019	0.021	0.017	0.014	0.019	0.019	0.019	0.019	0.024	0.029	0.009	0.012	0.009	0.017	0.012	0.031	0.024	0.009	0.026	0.019	0.009	0.019	0.017	0.012	0.009	0.019	0.024	0.017	0.014
H3	6	2		0.012	0.017	0.014	0.012	0.019	0.017	0.017	0.026	0.014	0.017	0.019	0.014	0.017	0.014	0.017	0.019	0.012	0.017	0.019	0.021	0.017	0.014	0.019	0.019	0.019	0.024	0.029	0.029	0.014	0.017	0.014	0.021	0.017	0.031	0.024	0.014	0.026	0.019	0.014	0.019	0.017	0.017	0.014	0.019	0.024	0.017	0.019
H4	1	3	5		0.005	0.007	0.005	0.012	0.014	0.005	0.024	0.007	0.009	0.007	0.007	0.005	0.007	0.009	0.007	0.005	0.009	0.012	0.014	0.009	0.012	0.012	0.012	0.012	0.012	0.017	0.021	0.007	0.005	0.007	0.014	0.009	0.024	0.017	0.002	0.019	0.012	0.007	0.012	0.009	0.009	0.007	0.012	0.017	0.009	0.007
H5	3	5	7	2		0.002	0.009	0.012	0.014	0.005	0.024	0.012	0.014	0.012	0.012	0.009	0.012	0.014	0.012	0.009	0.014	0.017	0.019	0.014	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.021	0.012	0.009	0.012	0.014	0.014	0.024	0.017	0.002	0.019	0.012	0.012	0.017	0.014	0.014	0.007	0.017	0.021	0.009	0.007
H6	4	4	6	3	1		0.012	0.014	0.017	0.007	0.026	0.014	0.017	0.014	0.014	0.012	0.014	0.017	0.014	0.012	0.017	0.019	0.021	0.017	0.019	0.019	0.019	0.019	0.019	0.019	0.024	0.014	0.012	0.014	0.017	0.017	0.026	0.019	0.005	0.021	0.014	0.014	0.019	0.017	0.017	0.009	0.019	0.024	0.012	0.009
H7	3	3	5	2	4	5		0.012	0.014	0.005	0.024	0.007	0.009	0.007	0.007	0.005	0.002	0.005	0.007	0.005	0.009	0.012	0.014	0.009	0.012	0.012	0.012	0.012	0.012	0.017	0.021	0.002	0.005	0.007	0.009	0.009	0.024	0.017	0.007	0.019	0.012	0.002	0.012	0.009	0.005	0.007	0.012	0.017	0.009	0.007
H8	6	8	8	5	5	6	5		0.002	0.007	0.017	0.009	0.012	0.014	0.014	0.012	0.014	0.017	0.014	0.012	0.007	0.009	0.012	0.007	0.009	0.009	0.009	0.009	0.019	0.019	0.024	0.014	0.012	0.014	0.012	0.017	0.017	0.019	0.009	0.021	0.009	0.014	0.009	0.007	0.017	0.009	0.014	0.019	0.002	0.009
H9	7	7	7	6	6	7	6	1		0.009	0.014	0.012	0.014	0.017	0.012	0.014	0.017	0.019	0.017	0.009	0.009	0.012	0.014	0.009	0.007	0.012	0.012	0.022	0.021	0.026	0.017	0.014	0.012	0.014	0.014	0.019	0.021	0.012	0.024	0.012	0.017	0.012	0.009	0.019	0.007	0.017	0.021	0.005	0.012
H10	3	5	7	2	2	3	2	3	4		0.019	0.007	0.009	0.007	0.007	0.005	0.007	0.009	0.007	0.005	0.009	0.012	0.014	0.009	0.012	0.012	0.012	0.012	0.012	0.012	0.017	0.007	0.005	0.007	0.009	0.009	0.019	0.012	0.002	0.014	0.007	0.007	0.012	0.009	0.009	0.002	0.012	0.017	0.005	0.002
H11	11	11	11	10	10	11	10	7	6	8		0.017	0.019	0.021	0.021	0.024	0.026	0.029	0.026	0.019	0.014	0.017	0.024	0.019	0.012	0.017	0.021	0.021	0.026	0.026	0.026	0.021	0.024	0.021	0.029	0.019	0.014	0.026	0.021	0.029	0.017	0.026	0.021	0.019	0.029	0.017	0.021	0.026	0.014	0.021
H12	4	6	6	3	5	6	3	4	5	3	7		0.002	0.009	0.009	0.007	0.009	0.012	0.009	0.007	0.007	0.009	0.012	0.007	0.009	0.009	0.009	0.009	0.009	0.014	0.019	0.005	0.007	0.009	0.017	0.012	0.017	0.014	0.009	0.017	0.005	0.009	0.009	0.007	0.012	0.009	0.005	0.009	0.007	0.009
H13	5	7	7	4	6	7	4	5	6	4	8	1		0.012	0.012	0.009	0.012	0.014	0.012	0.009	0.009	0.012	0.014	0.009	0.012	0.012	0.012	0.012	0.012	0.017	0.021	0.007	0.009	0.012	0.019	0.014	0.019	0.017	0.012	0.019	0.007	0.012	0.012	0.009	0.014	0.012	0.007	0.012	0.009	0.012
H14	4	6	8	3	5	6	3	6	7	3	9	4	5		0.005	0.002	0.009	0.012	0.005	0.007	0.007	0.009	0.017	0.012	0.009	0.009	0.014	0.014	0.009	0.019	0.019	0.009	0.007	0.009	0.017	0.002	0.017	0.019	0.009	0.021	0.014	0.009	0.014	0.012	0.012	0.009	0.014	0.012	0.009	0.009
H15	4	4	6	3	5	6	3	6	5	3	9	4	5	2		0.002	0.009	0.012	0.005	0.002	0.012	0.014	0.017	0.012	0.009	0.014	0.014	0.014	0.009	0.019	0.024	0.009	0.007	0.005	0.017	0.002	0.021	0.019	0.009	0.021	0.014	0.009	0.014	0.012	0.012	0.005	0.009	0.014	0.012	0.009
H16	3	5	7	2	4	5	2	5	6	2	10	3	4	1	1		0.007	0.009	0.002	0.005	0.009	0.012	0.014	0.009	0.012	0.012	0.012	0.012	0.007	0.017	0.021	0.007	0.005	0.007	0.014	0.005	0.019	0.017	0.007	0.019	0.012	0.007	0.012	0.009	0.009	0.007	0.007	0.012	0.009	0.007
H17	4	4	6	3	5	6	1	6	7	3	11	4	5	4	4	3		0.002	0.009	0.007	0.012	0.014	0.017	0.012	0.014	0.014	0.014	0.014	0.014	0.019	0.024	0.005	0.007	0.009	0.012	0.012	0.026	0.019	0.009	0.021	0.014	0.005	0.014	0.012	0.002	0.009	0.014	0.019	0.012	0.009
H18	5	5	7	4	6	7	2	7	8	4	12	5	6	5	5	4	1		0.012	0.009	0.014	0.017	0.019	0.014	0.017	0.017	0.017	0.017	0.017	0.021	0.026	0.007	0.009	0.012	0.014	0.014	0.029	0.021	0.012	0.024	0.017	0.007	0.017	0.014	0.005	0.012	0.017	0.021	0.014	0.012
H19	4	6	8	3	5	6	3	6	7	3	11	4	5	2	2	1	4	5		0.007	0.012	0.014	0.017	0.012	0.014	0.014	0.014	0.014	0.009	0.019	0.024	0.009	0.007	0.009	0.017	0.007	0.021	0.019	0.009	0.021	0.014	0.009	0.014	0.012	0.012	0.009	0.009	0.014	0.012	0.009
H20	3	3	5	2	4	5	2	5	4	2	8	3	4	3	1	2	3	4	3		0.009	0.012	0.014	0.009	0.007	0.012	0.012	0.012	0.012	0.017	0.021	0.007	0.005	0.002	0.014	0.005	0.024	0.017	0.007	0.019	0.012	0.007	0.012	0.009	0.009	0.002	0.012	0.017	0.009	0.007
H21	5	7	7	4	6	7	4	3	4	4	6	3	4	3	3	4	5	6	5	4		0.002	0.009	0.005	0.002	0.002	0.007	0.007	0.017	0.021	0.021	0.012	0.009	0.012	0.019	0.009	0.014	0.021	0.012	0.024	0.012	0.012	0.007	0.005	0.014	0.012	0.012	0.017	0.005	0.012
H22	6	8	8	5	7	8	5	4	5	5	7	4	5	4	6	5	6	7	6	5	1		0.012	0.007	0.005	0.005	0.009	0.009	0.019	0.024	0.024	0.014	0.012	0.014	0.021	0.012	0.017	0.024	0.014	0.026	0.014	0.014	0.009	0.007	0.017	0.014	0.014	0.019	0.007	0.014
H23	7	9	9	6	8	9	6	5	6	6	10	5	6	7	7	6	7	8	7	6	4	5		0.005	0.012	0.012	0.002	0.007	0.022	0.026	0.031	0.017	0.014	0.017	0.019	0.019	0.024	0.026	0.017	0.029	0.017	0.017	0.007	0.009	0.019	0.017	0.017	0.021	0.009	0.017
H24	5	7	7	4	6	7	4	3	4	4	8	3	4	5	5	4	5	6	5	4	2	3	2		0.007	0.007	0.002	0.002	0.017	0.021	0.026	0.012	0.009	0.012	0.014	0.014	0.019	0.021	0.012	0.024	0.012	0.012	0.002	0.005	0.014	0.012	0.012	0.017	0.005	0.012
H25	6	6	6	5	7	8	5	4	3	5	5	4	5	4	4	5	6	7	6	3	1	2	5	3		0.005	0.009	0.009	0.019	0.024	0.024	0.014	0.012	0.009	0.021	0.007	0.017	0.024	0.014	0.026	0.014	0.014	0.009	0.007	0.017	0.009	0.014	0.019	0.007	0.014
H26	6	8	8	5	7	8	5	4	5	5	7	4	5	4	6	5	6	7	6	5	1	2	5	3	2		0.009	0.009	0.019	0.024	0.024	0.014	0.012	0.014	0.021	0.012	0.017	0.024	0.014	0.026	0.014	0.014	0.009	0.007	0.017	0.014	0.014	0.019	0.007	0.014
H27	6	8	8	5	7	8	5	4	5	5	9	4	5	6	6	5	6	7	6	5	3	4	1	1	4	4		0.005	0.019	0.024	0.029	0.014	0.012	0.014	0.017	0.017	0.021	0.024	0.014	0.026	0.014	0.014	0.005	0.007	0.017	0.014	0.014	0.019	0.007	0.014
H28	6	8	8	5	7	8	5	4	5	5	9	4	5	6	6	5	6	7	6	5	3	4	3	1	4	4	2		0.019	0.024	0.029	0.014	0.012	0.014	0.017	0.017	0.021	0.024	0.014	0.026	0.014	0.014	0.005	0.007	0.017	0.014	0.014	0.019	0.007	0.014
H29	6	8	10	5	7	8	5	8	9	5	11	4	5	4	4	3	6	7	4	5	7	8	9	7	8	8	8	8		0.019	0.024	0.009	0.012	0.014	0.022	0.012	0.022	0.019	0.014	0.022	0.012	0.014	0.019	0.017	0.017	0.014	0.009	0.014	0.017	0.014
H30	8	10	12	7	7	8	7	8	9	5	11	6	7	8	8	7	8	9	8	7	9	10	11	9	10	10	10	10	8		0.005	0.014	0.017	0.019	0.021	0.021	0.021	0.005	0.014	0.002	0.014	0.019	0.024	0.021	0.021	0.014	0.019	0.024	0.017	0.014
H31	10	12	12	9	9	10	9	10	11	7	11	8	9	8	10	9	10	11	10	9	9	10	13	11	10	10	12	12	10	2		0.019	0.021	0.024	0.026	0.021	0.021	0.005	0.019	0.002	0.019	0.024	0.029	0.026	0.026	0.019	0.024	0.029	0.021	0.019
H32	4	4	6	3	5	6	1	6	7	3	9	2	3	4	4	3	2	3	4	3	5	6	7	5	6	6	6	6	4	6	8		0.007	0.009	0.012	0.012	0.021	0.014	0.009	0.017	0.004	0.012	0.012	0.014	0.009	0.009	0.014	0.012	0.009
H33	3	5	7	2	4	5	2	5	6	2	10	3	4	3	3	2	3	4	3	2	4	5	6	4	5	5	5	5	5	7	9	3		0.002	0.014	0.009	0.024	0.017	0.007	0.019	0.012	0.007	0.012	0.009	0.009	0.007	0.012	0.017	0.009	0.007
H34	4	4	6	3	5	6	3	6	5	3	9	4	5	4	2	3	4	5	4	1	5	6	7	5	4	6	6	6	6	8	10	4	1		0.017	0.007	0.026	0.019	0.009	0.021	0.014	0.009	0.014	0.012	0.012	0.005	0.014	0.019	0.012	0.009
H35	7	7	9	6	6	7	4	5	6	4	12	7	8	7	7	6	5	6	7	6	8	9	8	6	9	9	7	7	9	9	11	5	6	7		0.019	0.029	0.021	0.012	0.024	0.017	0.012	0.017	0.019	0.009	0.012	0.021	0.026	0.014	0.012
H36	5	5	7	4	6	7	4	7	6	4	8	5	6	1	1	2	5	6	3	2	4	5	8	6	3	5	7	7	5	9	9	5	4	3	8		0.019	0.021	0.012	0.024	0.017	0.012	0.017	0.014	0.014	0.007	0.012	0.017	0.014	0.012
H37	11	13	13	10	10	11	10	7	8	8	6	7	8	7	9	8	11	12	9	10	6	7	10	8	7	7	9	9	9	9	9	9	10	11	12	8		0.021	0.021	0.024	0.017	0.026	0.021	0.019	0.029	0.021	0.017	0.021	0.014	0.021
H38	8	10	10	7	7	8	7	8	9	5	11	6	7	8	8	7	8	9	8	7	9	10	11	9	10	10	10	10	8	2	2	6	7	8	9	9	9		0.014	0.002	0.014	0.019	0.024	0.021	0.021	0.014	0.019	0.024	0.017	0.014
H39	2	4	6	1	1	2	3	4	5	1	9	4	5	4	4	3	4	5	4	3	5	6	7	5	6	6	6	6	6	6	8	4	3	4	5	5	9	6		0.017	0.009	0.009	0.014	0.012	0.012	0.005	0.014	0.019	0.007	0.005
H40	9	11	11	8	8	9	8	9	10	6	12	7	8	9	9	8	9	10	9	8	10	11	12	10	11	11	11	11	9	1	1	7	8	9	10	10	10	1	7		0.017	0.021	0.026	0.024	0.024	0.017	0.021	0.026	0.019	0.017
H41	6	8	8	5	5	6	5	4	5	3	7	2	3	6	6	5	6	7	6	5	5	6	7	5	6	6	6	6	5	6	8	4	5	6	7	7	7	6	4	7		0.014	0.014	0.012	0.017	0.009	0.009	0.014	0.007	0.009
H42	4	4	6	3	5	6	1	6	7	3	11	4	5	4	4	3	2	3	4	3	5	6	7	5	6	6	6	6	6	8	10	2	3	4	5	5	11	8	4	9	6		0.014	0.012	0.007	0.009	0.014	0.019	0.012	0.005
H43	6	8	8	5	7	8	5	4	5	5	9	4	5	6	6	5	6	7	6	5	3	4	3	1	4	4	2	2	8	10	12	6	5	6	7	7	9	10	6	11	6	6		0.007	0.017	0.014	0.014	0.019	0.007	0.014
H44	5	7	7	4	6	7	4	3	4	4	8	3	4	5	5	4	5	6	5	4	2	3	4	2	3	3	3	3	7	9	11	5	4	5	8	6	8	9	5	10	5	5	3		0.014	0.012	0.012	0.017	0.005	0.012
H45	5	5	7	4	6	7	2	7	8	4	12	5	6	5	5	4	1	2	5	4	6	7	8	6	7	7	7	7	7	9	11	3	4	5	4	6	12	9	5	10	7	3	7	6		0.012	0.017	0.021	0.014	0.012
H46	4	4	6	3	3	4	3	4	3	1	7	4	5	4	2	3	4	5	4	1	5	6	7	5	4	6	6	6	6	6	8	4	3	2	5	3	9	6	2	7	4	4	6	5	5		0.014	0.019	0.007	0.005
H47	6	8	8	5	7	8	5	6	7	5	9	2	3	4	4	3	6	7	4	5	5	6	7	5	6	6	6	6	4	8	10	4	5	6	9	5	7	8	6	9	4	6	6	5	7	6		0.005	0.012	0.014
H48	8	10	10	7	9	10	7	8	9	7	11	4	5	6	6	5	8	9	6	7	7	8	9	7	8	8	8	8	6	10	12	6	7	8	11	7	9	10	8	11	6	8	8	7	9	8	2		0.017	0.019
H49	5	7	7	4	4	5	4	1	2	2	6	3	4	5	5	4	5	6	5	4	2	3	4	2	3	3	3	3	7	7	9	5	4	5	6	6	6	7	3	8	3	5	3	2	6	3	5	7		0.007
H50	4	6	8	3	3	4	3	4	5	1	9	4	5	4	4	3	4	5	4	3	5	6	7	5	6	6	6	6	6	6	8	4	3	4	5	5	9	6	2	7	4	2	6	5	5	2	6	8	3	

Table 9. Number of specimens, haplotype number, haplotype (h) and nucleotide (π) diversity of each *T. synaspis* population using cyt b sequence data.

Population	**N**	**Nh**	**h**	**π**
Adıyaman	10	4	0.7111+/-0.1175	0.0044+/-0.0031
Afyon	3	2	0.6667+/-0.3143	0.0015+/-0.0019
Bingöl	10	2	0.3556+/-0.1591	0.0008+/-0.0009
Bitlis	10	4	0.7333+/-0.1005	0.0101+/-0.0061
Bolu	10	1	0.0000+/-0.0000	0.0000+/-0.0000
Çankırı	2	2	1.0000+/-0.5000	0.0023+/-0.0032
Çorum	12	5	0.6667+/-0.1409	0.0041+/-0.0028
Elazığ	10	7	0.9333+/-0.0620	0.0071+/-0.0046
Erzincan	8	3	0.6071+/-01640	0.0015+/-0.0015
Giresun	3	2	0.6667+/-0.3143	0.0046+/-0.0043
Karaman	6	3	0.7333+/-0.1552	0.0126+/-0.0081
Kastamonu	3	1	0.0000+/-0.0000	0.0000+/-0.0000
Kayseri	10	5	0.8444+/-0.0796	0.0072+/-0.0046
Kırşehir	12	2	0.1667+/-0.1343	0.0007+/-0.0009
Konya	11	4	0.7455+/-0.0978	0.0125+/-0.0074
Muş	3	2	0.6667+/-0.3143	0.0031+/-0.0031
Nevşehir	10	2	0.3556+/-0.1591	0.0008+/-0.0009
Sivas	8	5	0.8575+/-0.1083	0.0074+/-0.0048
Tokat	10	3	0.5111+/-0.1643	0.0037+/-0.0048
Tunceli	10	4	0.6444+/-0.1518	0.0062+/-0.0040
Yozgat	5	4	0.9001+/-0.1610	0.0046+/-0.0036
Average	–	–	**0.8554+/-0.0002**	**0.0134+/-0.0001**

Table 10. AMOVA results of cyt b gene with respect to different groupings.

a) One group: All populations are accepted as distinct group.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation	*F*-index	P-value
Among groups	20	235.131	136.495	56.29	0.56293	<0.001
Within Population	145	153.664	105.975	43.71	0.43707	<0.001

b) Two groups: 1. Eastern populations (BTL, MUS, BNG, ERC, ELZ, TUN, ADY, SVS) and 2. Western populations (Other populations).

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation	*F*-index	P-value
Among groups	1	40.042	0.34727	13.39	0.13391	<0.001
Among populations Within groups	19	195.088	1.18629	45.74	0.52817	<0.001
Within Population	145	153.664	1.05975	40.86	0.59135	<0.001

c) Five groups: 1.BTL, MUS, BNG, ERC, ELZ, TUN, SVS; 2.ADY; 3.KAR, KON; 4.COR, KAY, NEV, TOK, YOZ; and 5.others.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation	*F*-index	P-value
Among groups	4	129.735	0.79625	30.66	0.30660	<0.001
Among populations Within groups	16	105.395	0.74100	28.53	0.41150	<0.001
Within Population	145	153.664	105.975	40.81	0.59193	<0.001

3. 1. 2. Nuclear Marker: Internal Transcribed Spacer 2 (ITS2) Region Variation

The amplified ITS2 fragments of *T. synaspis* after trimming 5.8S and 28S rDNA nucleotides from both ends are 433 bp as it was presented in both Figures 9 and 11. All the following analyses conducted on the ITS2 region included the same 166 specimens used for the cyt b analyses. No length variation was observed for the ITS2 segment of *T. synaspis*. Fifteen haplotypes were observed when all the sequences were collapsed into haplotypes, and they were submitted to GenBank (Acession no. KJ716686).

Of the sequenced ITS2 fragment 414 characters were found to be constant while 19 characters were variable (S= 19). Polymorphic sites are shown in Table 11. Of the variable characters 5 were parsimony informative and the 14 characters were found as parsimony uninformative. There was only one multiple hit at site 309.

In all of the ITS2 haplotypes nucleotide uses were 29.64% for A, 34.84% for T, 20.08% for C and 15.44% for G. The transition and transversion ratios were k_1= 2.917 (purines) and k_2= 2.555 (pyrimidines). The transition and transversion ratios for each nucleotide are shown in Table 12. Eight transversions (at sites 176, 219, 265, 284, 296, 305, 355 and 429) and 10 transitions (in other substitution sites) were detected in ITS2 haplotypes. The most frequently observed transition was C/T (18.8) followed by G/A (18.26). Transition/transversion bias was detected as R= 1.244. Average number of nucleotide differences for all haplotypes was k= 3,581.

Table 11. Substitutions and substitution sites for the nuclear ITS2 segment of *Trigonaspis synaspis*.

Haplotypes	Site	106	118	139	162	176	180	204	216	219	222	248	265	270	284	296	305	309	355	429
	Base	**C**	**T**	**T**	**A**	**G**	**T**	**G**	**G**	**G**	**G**	**C**	**G**	**G**	**G**	**A**	**A**	**G**	**T**	**A**
H1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H2		.	**C**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H3		.	.	.	.	.	.	.	.	.	.	.	**C**	.	.	.	.	.	.	.
H4		.	.	.	.	.	.	.	.	**C**	.	.	.	.	.	.	.	.	.	.
H5		.	**C**	.	.	.	.	.	.	**C**	.	.	.	.	.	.	.	.	.	.
H6		.	.	.	.	.	.	.	.	**C**	.	.	.	.	**C**	.	.	.	.	.
H7		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**C**	.	.
H8		.	.	.	.	.	.	.	.	**C**	**A**	.	.	**A**	.	.	.	**A**	.	.
H9		**T**	.	**C**	**G**	**T**	.	**A**	**A**	.	.	**T**	.	.	.	**C**	.	.	.	.
H10		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**T**
H11		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**A**	.
H12		.	.	.	.	.	**C**	.	.	.	.	.	.	**A**	.	.	.	.	.	.
H13		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**C**	**C**	.	.
H14		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**A**	.	.
H15		.	**C**	.	.	.	.	.	.	.	**A**	.	.	.	.	.	.	.	.	.

Table 12. Maximum composite likelihood estimate of the pattern of nucleotide substitutions for the ITS2 region of *T. synaspis*. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are also shown in the Table.

	A	**T**	**C**	**G**
A	–	7.36	3.26	**12.37**
T	6.26	–	**8.33**	4.24
C	6.26	**18.8**	–	4.24
G	**18.26**	7.36	3.26	–

The mismatch distribution of all pairwise combinations for ITS2 haplotypes was estimated and the resulting diagram is shown in Figure 13. The Harpending's raggedness index was r = 0, 0785. Tajima's *D* neutrality test (Tajima's *D* = -1, 71141, P > 0.05) and Fu and Li tests (*D** = -1, 75230, P > 0.10; *F** = -2, 00210, P > 0.05) were not significant indicating that these populations are not deviated significantly from neutrality.

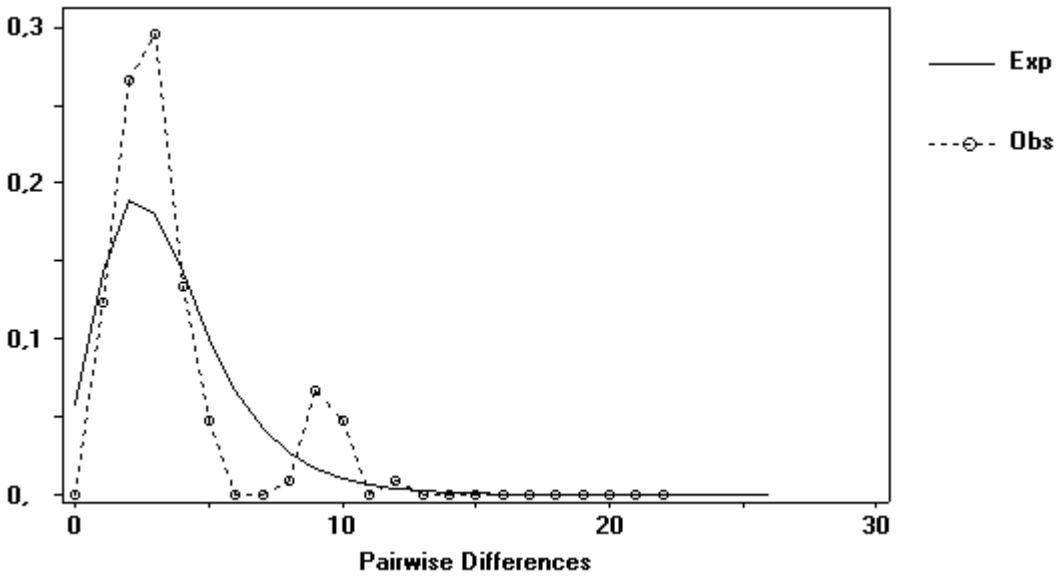

Figure 13. Mismatch distribution of all pairwise combinations for the ITS2 haplotypes. The observed distributions are represented by dotted line and the expected frequency is depicted by solid line.

Haplotype frequencies and the populations having those haplotypes are shown in Table 13. Of the 15 ITS2 haplotypes 8 were found as singleton and the remaining 7 were shared between two or more populations. Of the singleton haplotypes Bingöl had 3 different singletons (H4- H6) and the remaining 5 singleton haplotype were found in other populations (Afyon, Elazığ, Tunceli, Sivas, and Giresun). On the other hand, of the ITS2 haplotypes H1 (N=129) was the most frequent and shared haplotype found in all localities. H2 (N=12) was the second most frequent haplotype found in Adıyaman (N=2), Bingöl (N=3), Elazığ (N=4), and Tunceli (N=3). The next most frequent haplotypes are H7 (N=5) found in Bitlis (N= 2), Çorum (N=3), and H14 (Tokat, N=5).

Furthermore, the populations were investigated with respect to their haplotype numbers. Bolu (N=10), Çankırı (N=2), Erzincan (N=8), Karaman (N=6), Kastamonu (N=3), Kırşehir (N=12), Konya (N=11), Muş (N=3), and Nevşehir (N=10) have only one type of haplotype (H1). All of the other populations possessed more than a single haplotype. When haplotype diversity was investigated with respect to distinct haplotypes, Bingöl had the most diverse types of haplotypes with 5 distinct sequences in 10 examined individuals. All other populations have 2 to 3 haplotypes.

A pairwise comparison to show sequence difference in basepairs and percentage is shown in Table 14. Haplotype difference of ITS2 region ranged between 0.2% and 2.8% (1 bp to 12 bp differences, respectively). One basepair nucleotide difference was found in 13 cases. The highest difference (12 bp) was found between H8 and H9. Haplotype 9 was observed in Giresun population as a singleton, and haplotype 8 is another singleton detected only in one individual from Elazığ population.

Molecular variation of ITS2 region is given in Table 15. Average haplotype and nucleotide diversities are 0.3119 ± 0.0989 and 0.0015 ± 0.0013, respectively. Haplotype diversity estimates ranged between 0.0000 and 0.8000 ± 0.1001. Bingöl population has the highest haplotype diversity (0.8000 ± 0.1001) followed by Tokat population (0.6889 ± 0.1038). Further, nucleotide diversity had a range between 0.0000 and 0.0123 ± 0.0101. The highest nucleotide diversity is found in Giresun population (0.0123 ± 0.0101) followed by Elazığ population (0. 0030 ± 0. 0023).

Several AMOVA analyses were conducted also for the ITS2 region of *T. synaspis*, and only two different grouping schemes were provided in Table 16. One group (Table 16a) in which all populations are included as separate groups indicated that 25, 69% of genetic variation is divided among populations, meanwhile 74, 31% of diversity is observed within populations. In the second trial scheme (Table 16b), there was still very high amount of genetic variation (70, 65%) within population when two groups were formed as ‘Eastern’ and ‘Western’ populations. Other grouping schemes (not shown here) also produced similar outcomes.

Table 13. Haplotypes and their frequency in each *T. synaspis* population for ITS2 region.

Haplotype	ADY	AFY	BNG	BTL	BOL	CKR	COR	ELZ	ERC	GRS	KAR	KAS	KAY	KIR	KON	MUS	NEV	SVS	TOK	TUN	YOZ	Total
H1	8	2	4	8	10	2	9	5	8	2	6	3	8	12	11	3	10	5	3	6	4	**129**
H2	2		3					4												3		**12**
H3		1																				**1**
H4			1																			**1**
H5			1																			**1**
H6			1																			**1**
H7				2			3															**5**
H8								1														**1**
H9										1												**1**
H10													2									**2**
H11																		2				**2**
H12																		1				**1**
H13																			2		1	**3**
H14																			5			**5**
H15																				1		**1**
N	**10**	**3**	**10**	**10**	**10**	**2**	**12**	**10**	**8**	**3**	**6**	**3**	**10**	**12**	**11**	**3**	**10**	**8**	**10**	**10**	**5**	**166**
Nhap	**2**	**2**	**5**	**2**	**1**	**1**	**2**	**3**	**1**	**2**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**3**	**3**	**3**	**2**	**15**

Table 14. Pairwise comparison of the 15 ITS2 haplotypes of *T. synaspis*. Net nucleotide differences are shown below diagonal and the percentage of the differences are presented above diagonal.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15
H1		0.002	0.002	0.002	0.005	0.005	0.002	0.009	0.019	0.002	0.002	0.005	0.005	0.002	0.005
H2	1		0.004	0.005	0.002	0.007	0.005	0.012	0.021	0.005	0.005	0.007	0.007	0.005	0.002
H3	1	2		0.005	0.007	0.007	0.005	0.012	0.021	0.005	0.005	0.007	0.007	0.005	0.007
H4	1	2	2		0.002	0.002	0.005	0.007	0.021	0.005	0.005	0.007	0.007	0.005	0.007
H5	2	1	3	1		0.005	0.007	0.009	0.023	0.007	0.007	0.009	0.009	0.007	0.005
H6	2	3	3	1	2		0.007	0.009	0.023	0.007	0.007	0.009	0.009	0.007	0.009
H7	1	2	2	2	3	3		0.009	0.021	0.005	0.005	0.007	0.002	0.002	0.007
H8	4	5	5	3	4	4	4		0.028	0.012	0.012	0.009	0.012	0.007	0.009
H9	8	9	9	9	10	10	9	12		0.021	0.021	0.023	0.023	0.021	0.023
H10	1	2	2	2	3	3	2	5	9		0.005	0.007	0.007	0.005	0.007
H11	1	2	2	2	3	3	2	5	9	2		0.007	0.007	0.005	0.007
H12	2	3	3	3	4	4	3	4	10	3	3		0.009	0.007	0.009
H13	2	3	3	3	4	4	1	5	10	3	3	4		0.005	0.009
H14	1	2	2	2	3	3	1	3	9	2	2	3	2		0.007
H15	2	1	3	3	2	4	3	4	10	3	3	4	4	3	

Table 15. Number of specimens, haplotype number, haplotype (h) and nucleotide (π) diversity for ITS2 region of each *T. synaspis* population.

Population	**N**	**Nh**	**h**	**π**
Adıyaman	10	2	0.3556+/-01591	0.0008+/-0.0009
Afyon	3	2	0.6667+/-0.3143	0.0015+/-0.0019
Bingöl	10	5	0.8000+/-0.1001	0.0027+/-0.0021
Bitlis	10	2	0.3556+/-0.1591	0.0008+/-0.0009
Bolu	10	1	0.0000+/-0.0000	0.0000+/-0.0000
Çankırı	2	1	0.0000+/-0.0000	0.0000+/-0.0000
Çorum	12	2	0.4091+/-0.1333	0.0009+/-0.0010
Elazığ	10	3	0.6444+/-0.1012	0.0030+/-0.0023
Erzincan	8	1	0.0000+/-0.0000	0.0000+/-0.0000
Giresun	3	2	0.6667+/-0.3143	0.0123+/-0.0101
Karaman	6	1	0.0000+/-0.0000	0.0000+/-0.0000
Kastamonu	3	1	0.0000+/-0.0000	0.0000+/-0.0000
Kayseri	10	2	0.3556+/-0.1591	0.0008+/-0.0009
Kırşehir	12	1	0.0000+/-0.0000	0.0000+/-0.0000
Konya	11	1	0.0000+/-0.0000	0.0000+/-0.0000
Muş	3	1	0.0000+/-0.0000	0.0000+/-0.0000
Nevşehir	10	1	0.0000+/-0.0000	0.0000+/-0.0000
Sivas	8	3	0.6071+/-0.1640	0.0021+/-0.0018
Tokat	10	3	0.6889+/-0.1038	0.0024+/-0.0019
Tunceli	10	3	0.6000+/-0.1305	0.0016+/-0.0015
Yozgat	5	2	0.4000+/-0.2373	0.0018+/-0.0018
Average	–	–	**0.3119+/-0.0989**	**0.0015+/-0.0013**

Table 16. AMOVA results of ITS2 region of *T. synaspis.*

a) One group: All populations are accepted as distinct group.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation	*F*-index	P-value
Among groups	20	19.086	0.08894	25.69	0.25693	<0.001
Within Population	145	37.300	0.25724	74.31	0.74307	<0.001

b) Two groups: 1. Eastern populations (BTL, MUS, BNG, ERC, ELZ, TUN, ADY, SVS), 2. Western populations (All other populations).

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation	*F*-index	P-value
Among groups	1	3.884	0.03695	10.15	0.10147	>0.001
Among populations Within groups	19	15.202	0.06994	19.21	0.21376	<0.001
Within Population	145	37.300	0.25724	70.65	0.29353	<0.001

3.2. Phylogenetic and Phylogeographic Analysis

3.2.1. Phylogenetic Analysis of the Mitochondrial cyt b gene

The obtained cytochrome b gene sequence data were used to analyze the data set through the application of maximum parsimony and maximum likelihood in addition to the Bayesian approach implemented in the programs given in detail in Materials and Methods. In all analyses two haplotypes of *T. megaptera* were used as outgroup to give an evolutionary direction to the ingroup haplotypes.

Both maximum parsimony and maximum likelihood analyses produced similar consensus trees with slightly different bootstrap values. The 50% majority rule tree is a consensus tree of 113 best found trees produced by maximum parsimony with CI= 0,801, RI= 0,123. The maximum likelihood tree was the consensus tree of 237 best found trees with CI= 0,788, RI= 0,220. Due to similar topologies of both analyses a single consensus tree with bootstrap supports on the branches is given in Figure 14. In the combined consensus tree, H11 from the Bitlis locality appears to be the most basally located haplotype (from the node A). A second lineage separated from the node B gives rise to H37 from Konya and a haplogroup is diversified from the same node. From the node C a bifurcation of the haplotypes is observed to form Clade 1 (C1) and Clade 2 (C2). Clade I contains H30, H31, H38 and H40 haplotypes from Konya and Karaman populations originated from C1 node. Clade 2 which is branched out from C2 node and having most of the *T. synaspis* haplotypes contains 6 subclades. The subclade C2a is the largest one containing 13 mostly eastern haplotypes from Bingöl, Elazığ, Erzincan, Tunceli, Karaman, and Sivas. The next group, C2b, contains 7 haplotypes from Çorum, Kayseri, Nevşehir, Tokat and Yozgat populations from Central Anatolia. Group C2c includes Konya population with two monophyletic groups of Adıyaman-Elazığ and

Afyon haplotypes. Groups C2d, C2e and C2f show the monophyletic group of Adıyaman, Kayseri ad Tunceli haplotypes, respectively. Rest of the haplotypes gave rise to a polytomic group under the C2 node.

The cyt b data was also used for obtaining a Bayesian tree, and divergence times have also been estimated with BEAST. The time to the most recent common ancestor (tMRCA) was calculated for all major groups revealed by Bayesian approach using a HKY+I+G model of nucleotide substitutions selected by JMODELTEST and a conventional insect mtDNA relaxed log normal molecular clock with 2.3% divergence/ MYA/ lineage was applied to the age determination. Divergence times are given on the tree with bold numbers and posterior probability values are also shown on the branches. Overall, the Bayesian consensus tree produced more resolution of the phylogenetic relationships among the cyt b haplotypes of *T. synaspis* (Figure 15). Unlike the MP/ML tree the Bayesian tree gives a monophyletic group being at the most basal part on the tree- H11 (Bitlis haplotype) forms a monophyletic group with H37 (Konya haplotype supported with a fairly high support value. Meanwhile, it seems that the separation of all of the ingroup haplotypes diverged around 2.3 million years ago (MYA) from the outgroup haplotypes. The diversification of the most basal monophyletic ingroup haplotypes (H11 and H37) diverged around 2, 1083 MYA from the rest of the *T. synaspis* haplotypes. The remaining haplotypes formed two clades with a separation about 1, 9167 MYA; the first clade covered those haplotypes (H47 and H48, H12, H13, H41) all from the eastern populations (Bitlis, Muş and Tunceli). The divergence time of this haplogroups is approximated around 0,575 MYA. However, the second clade which compromised all other haplotypes further was divided into two haplogroups nearly 1.5333 MYA, of which the first is composed of haplotypes Elazığ, Tunceli,

Bingöl, Erzincan, Sivas and Karaman. The second haplogroups was divided into two subclades (1.3417 MYA): the first one produced a haplogroup (about 0.9583 MYA) composing of eastern haplotypes as basal haplotypes in addition to the Yozgat, Çorum, Tokat, and Nevşehir haplotypes. On the other side, the second haplogroup with two small groups of haplotypes included a combination of east and central Anatolian haplotypes. The separation of this second haplogroup appears to be around 1.15 MYA.

A median joining network analysis to resolve the possible reticulations and to show the evolutionary relationships among haplotypes is shown in Figure 16. The color coded populations and the percentages of the haplotypes shared between/among populations together with the reticulate events in addition to the hypothetical haplotypes in the network. Similar to the findings of all three different sets of phylogenetic analyses but particularly that observed in the BEAST (Bayesian) tree same haplotype groups have been obtained in the network. To be more specific; certain groupings among haplotypes are notorious in the network where Bitlis and Konya haplotypes (H11 and H37, respectively) are connected to the small groups of haplotypes with some hypothetical ones that were either not sampled or they are extinct haplotypes. Besides, H49 found only in Tunceli as a singleton (N=1) seems to be connected to most other eastern haplotypes (Elazığ, Erzincan, Bingöl, Bitlis) with two other haplotypes such as H43 and H44, both singletons found in Sivas.

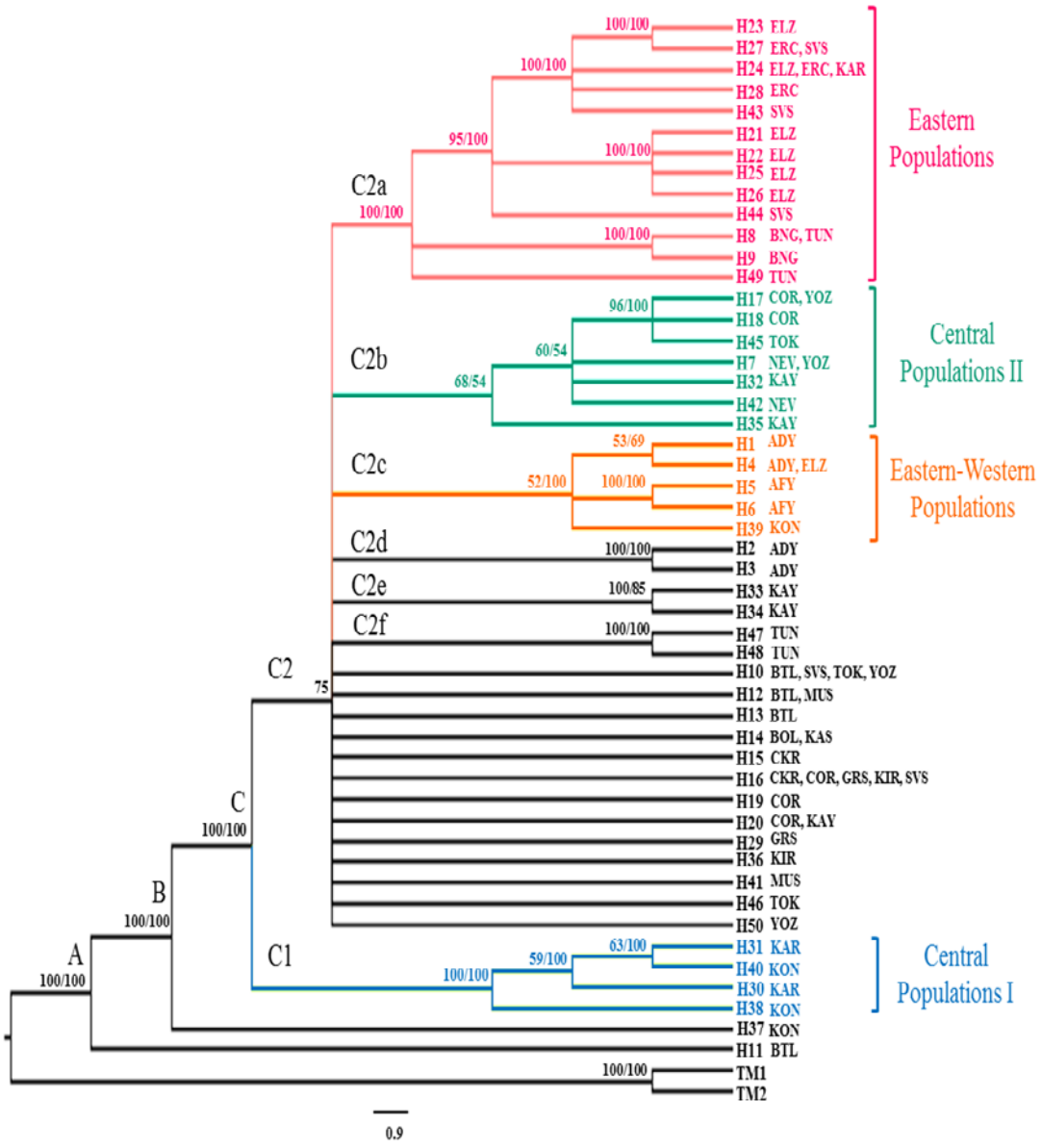

Figure 14. A consensus tree of both maximum parsimony and maximum likelihood of mitochondrial cyt b gene. The bootstrap values are shown on the branches (first is the MP and the second is for the ML).

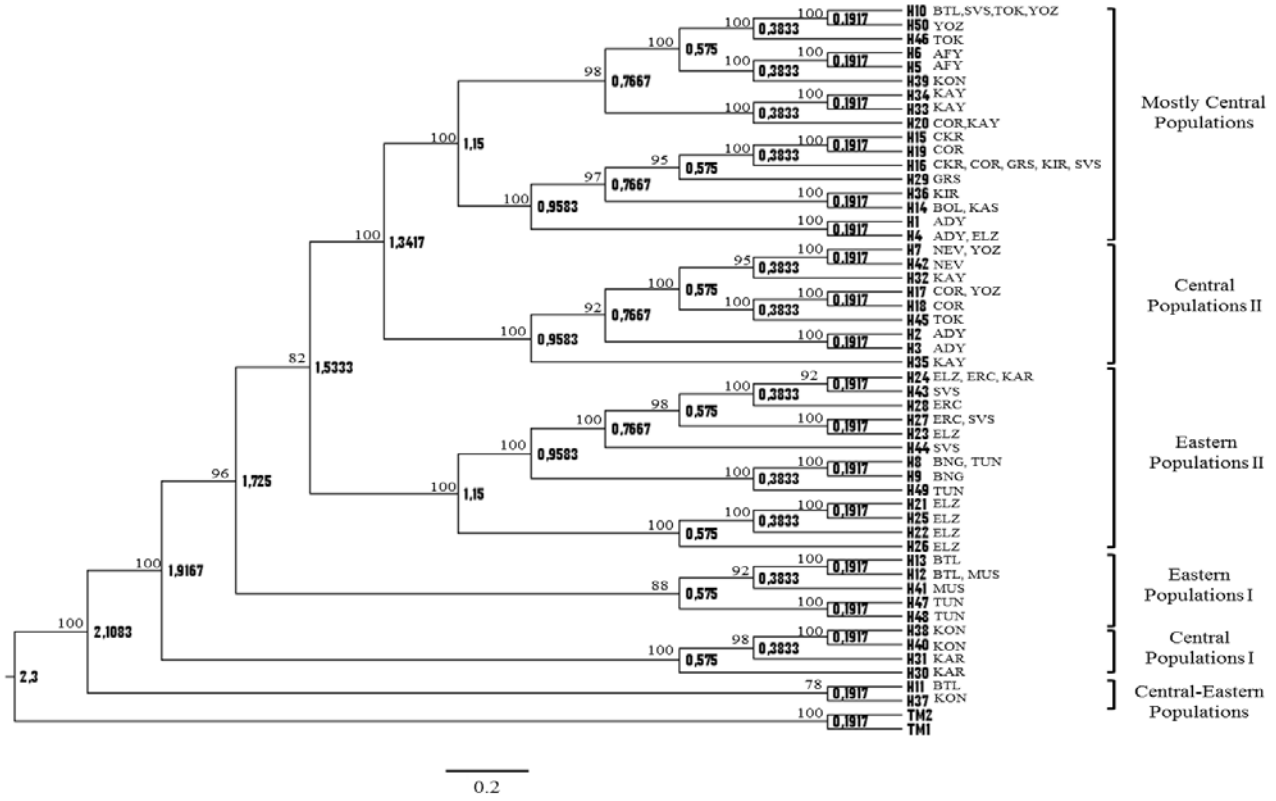

Figure 15. The consensus tree with the node ages (tMRCA) are shown on the interior part of each related node. Posterior probability values are also given at nodes.

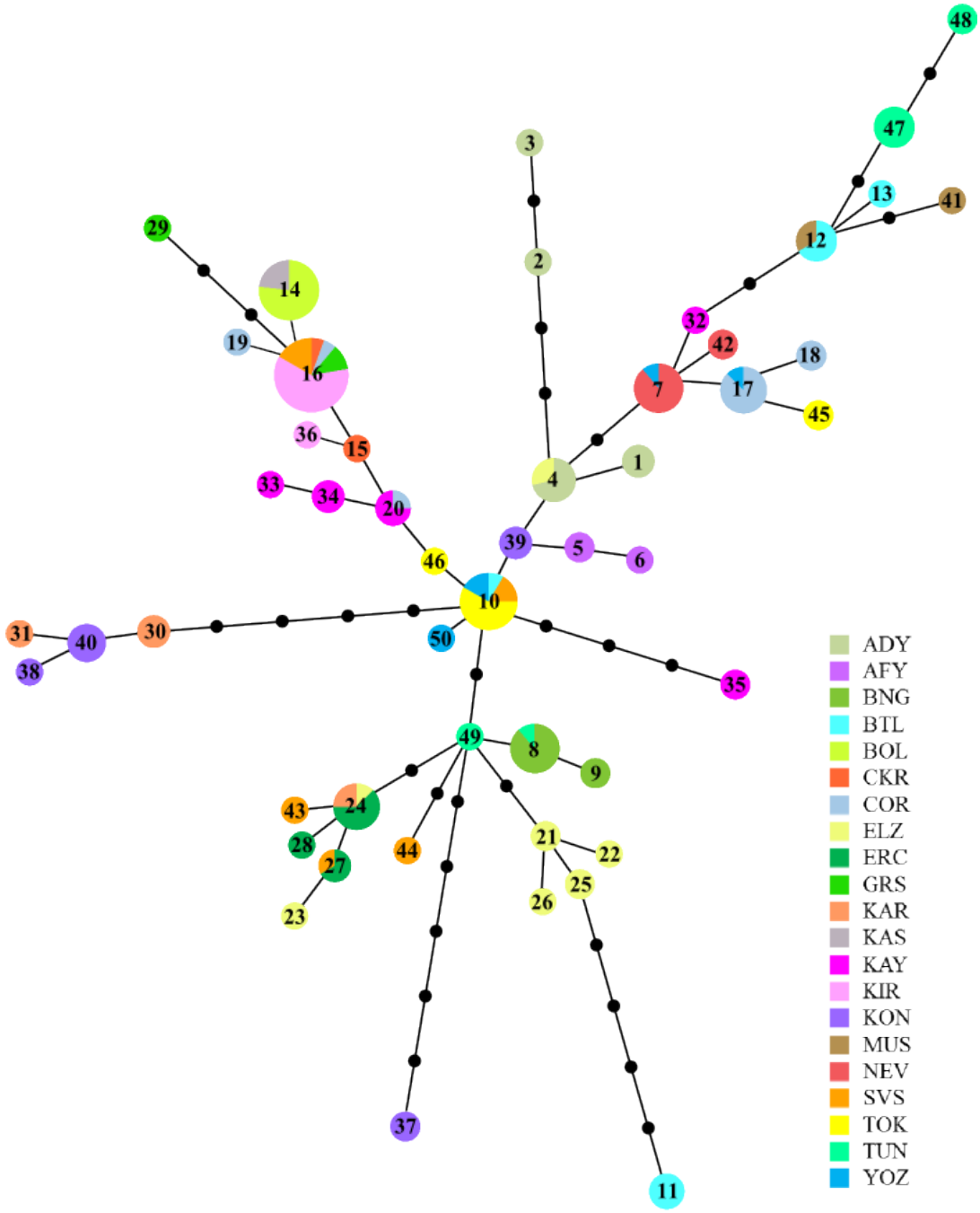

Figure 16. Median joining network of the mitochondrial cyt b haplotypes. Color – coded populations are shown in the boxes.

3.2.2. Phylogenetic Analysis of Nuclear ITS2 Region

Haplotypes of ITS2 region were used for maximum parsimony and maximum likelihood analysis in addition to the Bayesian approach. Two sequences of *T. megaptera* were used as outgroup haplotypes. Results of maximum parsimony and maximum likelihood analyses came out as the same tree with only different bootstrap values. Thus, the 50% majority rule consensus tree of MP and ML analyses is given as a single phylogenetic tree in Figure 17. For ML analysis, mutational model was determined by running JModeltest program and best model for the analysis was found as GTR+I+G.MP tree was a consensus tree of 186 best found trees with CI= 0.57, RI= 0.128, however the ML tree was the consensus tree of 804 trees with CI= 0.78, RI= 0.32. H9 haplotype from Giresun population came out as basal branch from A node. B node contains remaining haplotypes as a polytomic group. Subgroup B1 contains H2, H15 and H5 haplotypes, from Adıyaman, Bingöl, Elazığ and Tunceli populations. Another subgroup bifurcated into monophyletic grouping as B2 (H7-H13; Bitlis-Çorum and Tokat-Yozgat, respectively). Remaining haplotypes (H1, H3, H4, H6, H8, H10, H11, H12, and H14) were placed as single branches in the polytomic group.

The Bayesian consensus tree for ITS2 region was given in Figure 18 which was highly similar to the MP/ML consensus tree with the exception of two monophyletic small clade structure observed between H2 - H15, and H7 - H13. Posterior probability values were relatively lower compared to the bootstrap values of MP/ML trees. Similar to the MP and ML trees the Bayesian tree also supported H9 (from Giresun) as the most basal haplotype which was separated from remaining haplotypes that formed a large polytomic group.

Moreover, the median joining network conducted also for the ITS2 region is given in Figure 19. The haplotype 9 from the Giresun population with the highest nucleotide difference with other haplotypes is the most distantly connected haplotype with seven hypothetical haplotypes connecting H9 to H1. H1 (N=129) being the most frequent and common haplotype is shared among all studied populations of *T. synaspis* produced a star phylogeny observed in the network. It is notorious that H4 (N=1, from Bingöl) is connected to few haplotypes including H6 (from Bingöl) and two other hypothetical haplotypes or the ones that were not sampled in the field trips are connected to a singleton haplotype (H8) found in Elazığ population. In addition, H2 (N=12, from Adıyaman, Bingöl, Elazığ and Tunceli) is connected to two haplotypes as H5 (N=1) from Bingöl, and H15 (N=1) from Tunceli population.

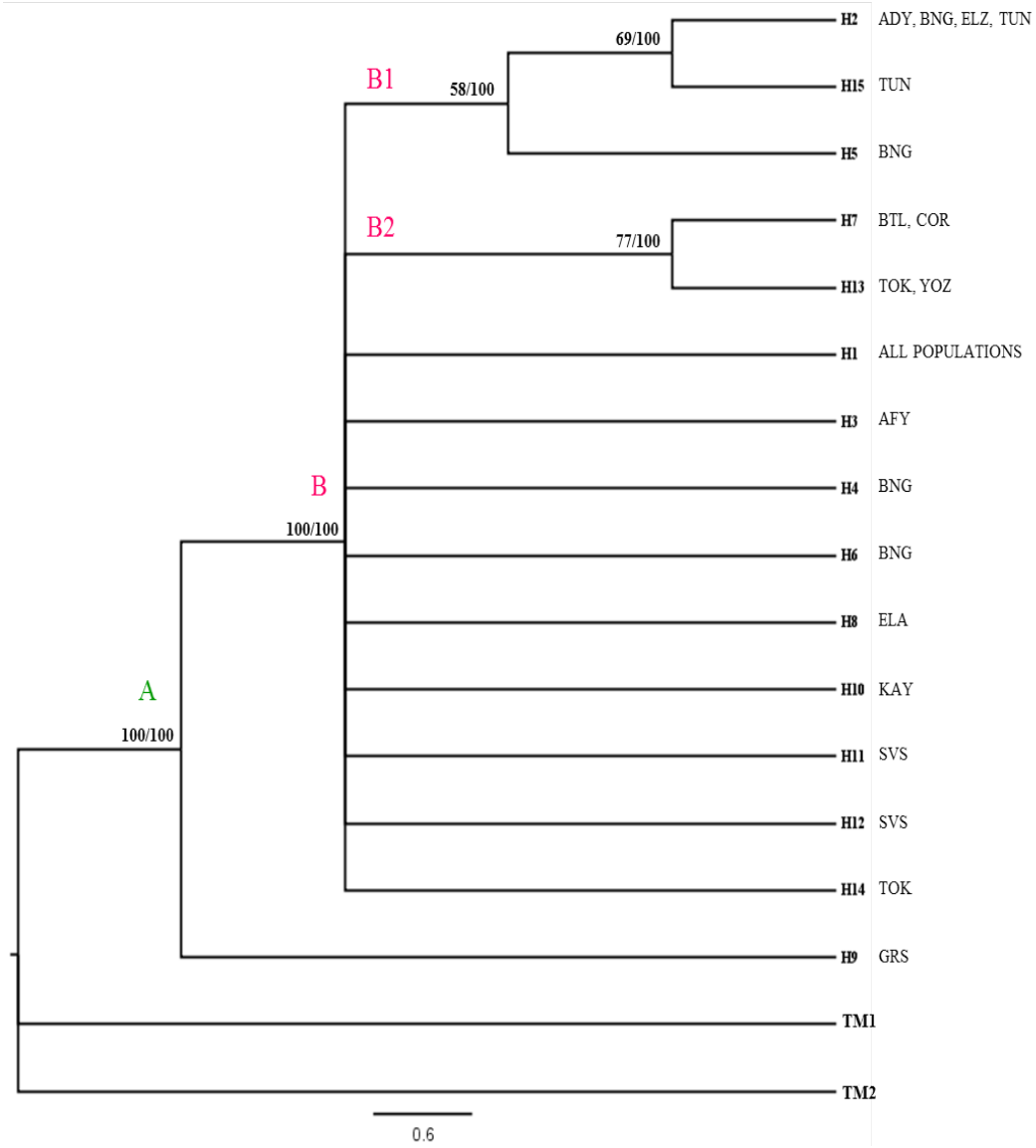

Figure 17. The consensus tree of maximum parsimony and maximum likelihood analysis of ITS2 region. The bootstrap values, first representing MP, and the second one representing ML, are shown on the branches.

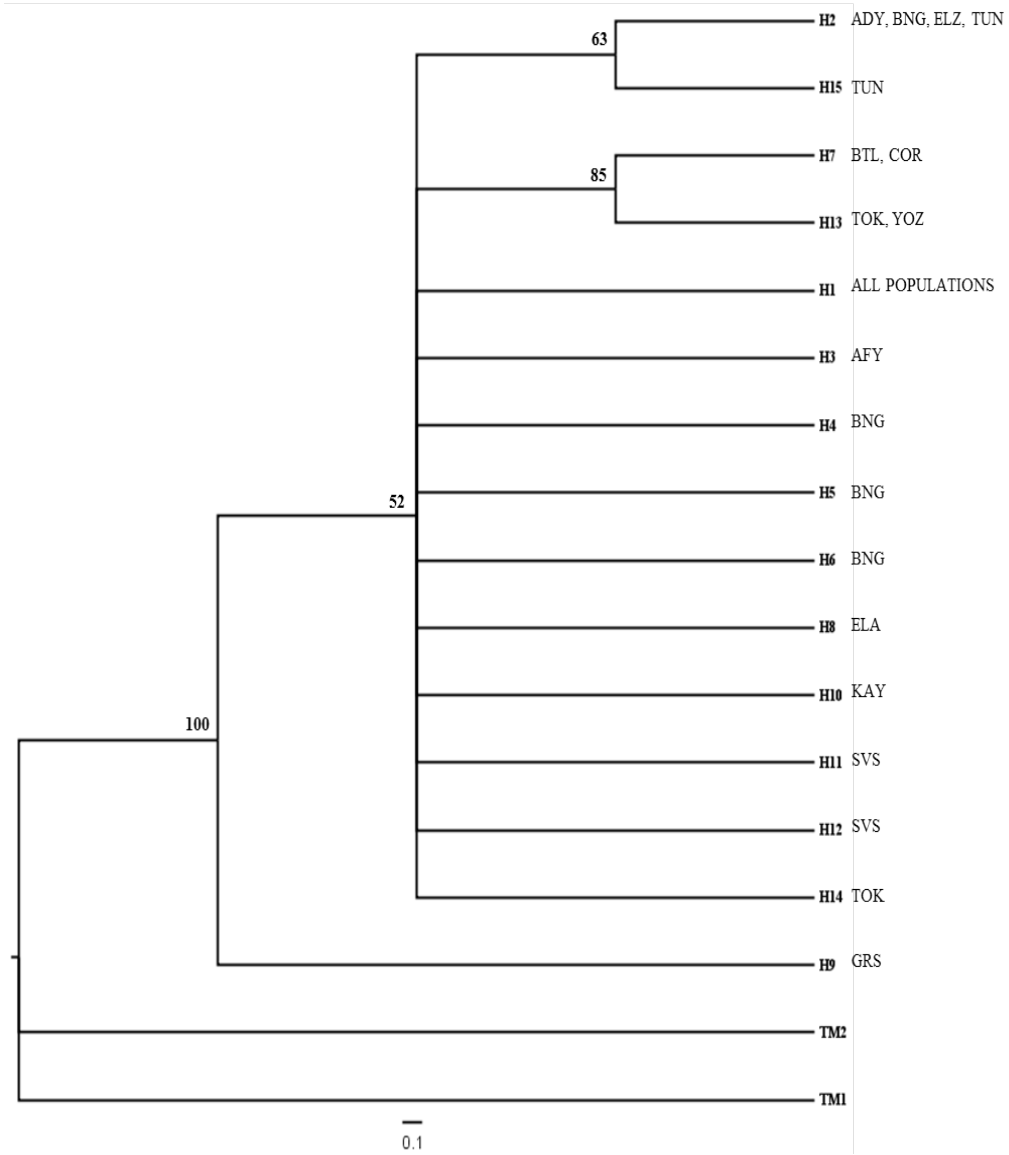

Figure 18. Bayesian consensus tree of the ITS2 sequence data. Posterior probability values are shown on the branches. Because of high polytomy branches were not labeled.

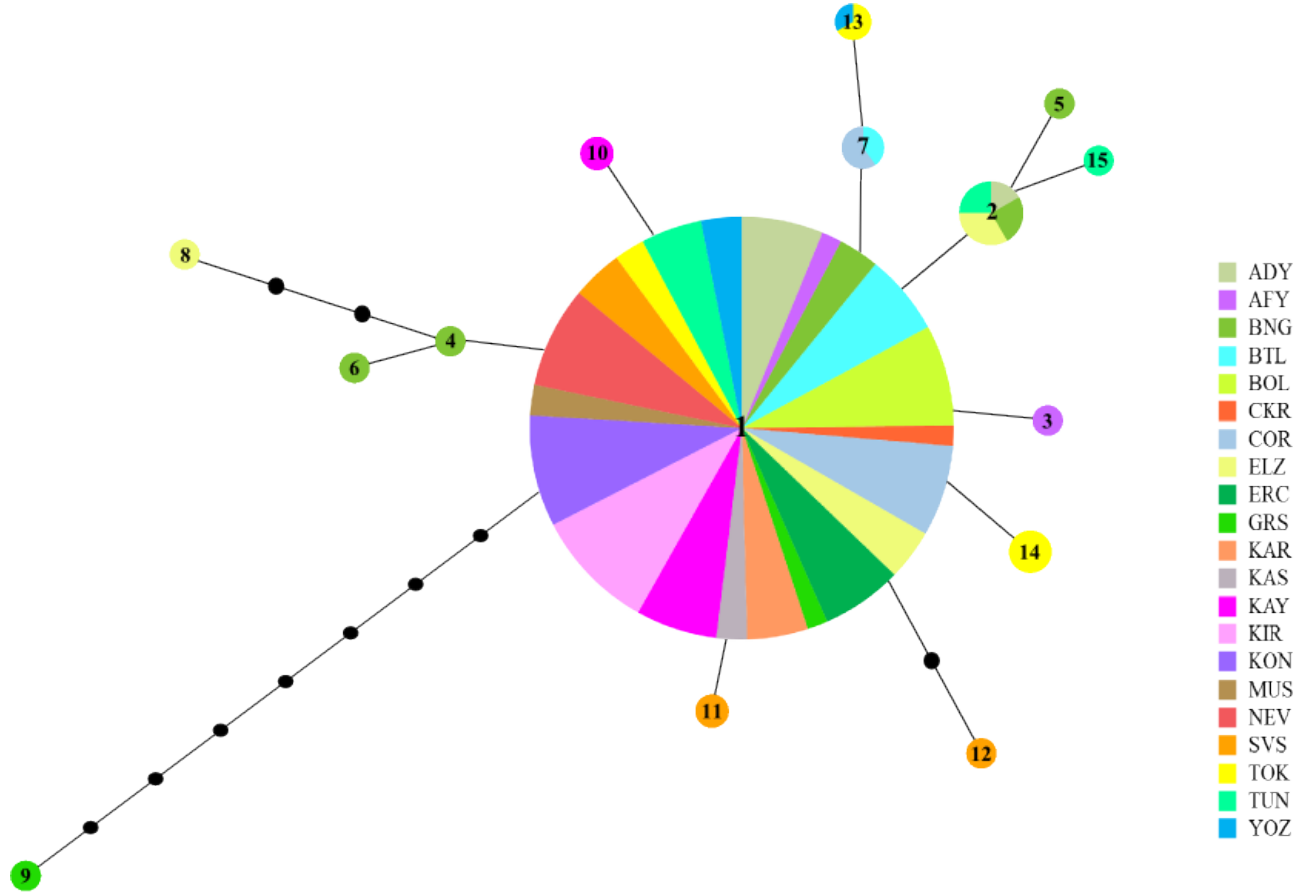

Figure 19. Median Joining network result of the ITS2 haplotypes of *T. synaspis*. Color–coded populations are shown in the boxes.

CHAPTER IV

DISCUSSION

4.1. Genetic Diversity Estimates: mtDNA and nDNA

The base pair composition in *Trigonaspis synaspis* cyt b haplotypes (A= 34, 5, C= 13, G= 10, 3, T= 42, 2) indicates that the collected data is genuine mitochondrial DNA data since anti-G bias is a characteristic of mitochondrial DNA (Zhang & Hewitt, 1996). In *T. synaspis* the amount of A+T is 76, 7% which is well- within the range of those observed in other insect species mtDNA coding genes. For example, mitochondrial genome of honey bee, *Apis mellifera*, contains 84.9% of A+T (Crozier & Crozier, 1993), and *Drosophila yakuba* has 78.6% of A+ T nucleotides (Clary & Wolstenhome, 1985). It is also well-known that protein coding genes of mtDNA such as cytochrome b do not have stop codons or non-sense mutations (Zhang & Hewitt, 1996). The cyt b gene haplotype sequences did not present any stop codons, indels and non-sense mutations when the sequences of *T. synaspis* were translated into amino acids (see Table 5). Sequenced cytochrome b gene of *Trigonaspis synaspis* has one transversion and 40 transitions. It has been reported that insect mitochondrial protein coding genes have higher transition ratios than transversions (Brown et al., 1979).

Revealing 50 cyt b haplotypes across the sampled range of *T. synaspis* from Anatolia indicates the presence of high haplotype diversity in this species. Similarly, high haplotype diversity has been reported for other animal species including oak

gallwasps (Challis et al., 2007; Dinç & Mutun; 2011, Mutun, 2010; Rokas et al., 2003). In fact, almost half of these haplotypes (22 out of 50 haplotypes) are singleton which might signify the importance of several points. Firstly, most of the singletons were found in central/western Anatolian populations (13 singletons in the central/western populations / 9 singletons in the eastern populations). Compared with the singletons, among the 28 haplotypes the most widespread one was found only in 18 individuals (H16) representing 5 central / western populations (Kırşehir, Sivas, Çankırı, Çorum, and Giresun). Phylogeographic studies imply that the presence of more private haplotypes may be young (derived) since they have limited geographic range (Crandal & Templeton, 1993). On the other hand, among all sampled 21 populations Bolu and Kastomonu localities were fixed with respect to a haplotype (H14). Secondly, regarding the diverse number of haplotype determined in populations Elazığ was interesting since 7 distinct haplotypes have been observed, of which 3 were singleton. The presence of high number of singletons may thus indicate that in more recent times there were some new substitutions.

Regarding the nuclear ITS2 segment of *T. synaspis* the size of the region is 433 bp without any length variation within the species. The size of the ITS2 region is congruent with the approximate size of other oak gallwasp species including *Andricus quercustozae* (410 bp), *A. caputmedusae* (415 bp), *A. dentimitratus* (416 bp), and *A. quuercuscalicis* (418 bp) (Nicholls et al., 2012). Among the 15 determined ITS2 haplotypes of *T. synaspis* the number of polymorphic sites was low (S= 19), of which only 5 sites were parsimony informative. Transition/ transversion bias was low (R= 1.244). Of the 15 ITS2 haplotypes 8 were singleton; of these Bingöl had the highest number of singletons (3 distinct singleton haplotypes). One of the interesting finding of the ITS2 region of nDNA in *T. synaspis* is that all

populations shared H1 which was with the highest frequency among all other haplotypes. It is conspicuous that out of 21 populations 9 populations are fixed with H1 (Bolu, Çankırı, Erzincan, Karaman, Kastamonu, Kırşehir, Konya, Muş, and Nevşehir). Similar to the cyt b results the high number of singleton is quite notorious implying once more recent substitutions in derived haplotypes (Crandal & Templeton, 1993).

With respect to the haplotype and nucleotide diversity in each of the examined *T. synaspis* population using cyt b sequence data Çankırı and Yozgat were with the highest haplotype diversity meanwhile Karaman and Kırşehir displayed the highest nucleotide diversity (see Table 9). Overall, within species haplotype diversity was 0.85541, and the average nucleotide diversity was 0.01346, which are fairly high for the oak gallwasps species studied. Nonetheless, the sequences of the nDNA ITS2 sequences also showed that average haplotype and nucleotide diversity estimates as 0.3119 and 0.0015, respectively. The highest haplotype diversity was observed in Bingöl (0.8000 ± 0.1001) and Tokat (0.6889 ± 0.1038) populations. On the other hand, the highest nucleotide diversity was shown by Giresun (0, 0123 ± 0, 0101) and Elazığ (0, 0030 ± 0, 0023) populations. Previous studies on oak gallwasp showed that highest genetic variation is observed in the Turkish populations across Palearctic region. In *A. coriarius* nucleotide diversity was 0. 00529 and 0.00644 in Iranian and Lebanese populations, respectively; however nucleotide diversity was 0.01554 in Turkey (Challis et al., 2007). Likewise, in *A. quercustozae* the greatest genetic diversity was found in Turkey within a range of 0.2- 4.2%, in Italy it ranged between 0.12- 0.17% (Rokas et al., 2003). Other similar investigations on the Anatolian oak gallwasps showed that the genetic diversity across the Turkish populations was quite high. Average haplotype and nucleotide diversity in *A. lucidus* was 0, 8089 and 0,

115542, respectively (Mutun, 2011). Similarly, in *A. caputmedusae* haplotype and nucleotide diversity was 0, 4631 and 0, 101214 (Mutun, 2010), and in *A. quercustozae* haplotype and nucleotide diversity was 0, 45 and 0, 05, respectively (Dinç & Mutun, 2011).

High genetic differentiation between populations within a species is usually coupled with the distinct phylogeographic structure (Avise, 2004). From the phylogeographic point of view, glacial refugia are assumed to harbor high diversity of genotypes and major lineages within a species (Hewitt, 2000). In *T. synaspis*, the existence of high genetic diversity in Turkey can be explained by concurrent results provided by this thesis and other earlier studies to further prove the importance of Anatolia as a possible refuge and hot spot area for oak gallwasps. In fact, Rokas et al. (2003) provided persuasive data that Anatolia was a cradle for oak gallwasp diversity. Moreover, various other studies have also indicated that the eastern distribution ranges of many European plant and animal taxa are assumed to act as source for the European populations. Higher genetic diversity is not only observed in oak gallwasp species but other species also show high genetic variation studied in Turkey including plants (Ansell et al., 2011) and various animals taxa such as bicolored shrew (Dubey et al., 2007), yellow-necked field mouse (Michaux et al., 2004), Anatolian mountain frog (Veith et al., 2003), fishes (Hrbek et al., 2002), ground squirrels (Gündüz et al., 2007) and grasshoppers (Korkmaz et al., 2010). Thus, the current findings of the high genetic diversity in *T. synaspis* seems to provide more concrete data for this statement and shed more light on the existence of great molecular variation in this unique area and its significance for keeping the genetic richness.

4.2. Phylogenetic and Phylogeographic Structure: Implications for Possible Responses to the Climatic Changes

Three separate sets of phylogenetic analyses conducted as maximum parsimony, maximum likelihood and Bayesian analysis using the cyt b sequence data resulted in largely congruent topologies (Figures 14 and 15). Indeed, maximum parsimony and maximum likelihood tree topologies were similar except bootstrap values supporting the branches. With regard to the comparison of the cyt b haplotype tree topologies obtained though MP/ML and the Bayesian, the Bayes tree resolved more polytomies observed in the MP/ML tree (see Figures 14 and 15).

In the MP/ML tree clade C1 compromised Bitlis and Konya haplotypes as the most basal haplotypes similar to that observed in the Bayesian tree with a minor difference where both haplotypes (H11 and H37) were monophyletic and basal at the same time. Other studies on oak gallwasps also indicated that most of the eastern haplotypes were basal compared to the westerly located haplotypes (Mutun, 2010, Dinç & Mutun, 2011). In the MP/ML tree Konya and Karaman haplotypes formed a small clade (C1) at the next basal part on the tree. All other haplotypes fall within the polytomic part of the MP/ML tree while some haplotypes from the eastern and near eastern populations formed (C2a C2d, C2f) well- supported clades. On the other side, near east and central Anatolian grouping of the haplotypes are apparent (C2b). Contrary to the MP/ML tree the Bayesian tree of the cyt b haplotypes resolved more evolutionary relationships with more structuring of the haplotypes with respect to their east and central/west location (Figure 15). Indeed, a well-defined east and west separation of the oak gallwasp haplotypes from *A. caputmedusae* (Mutun, 2010) and *A. quercustozae* (Dinç & Mutun, 2011) have been reported previously, and from that perspective current finding of this thesis on *T. synaspis* seems to support, though

more superficially, an east and west/central distinction. Furthermore, a larger scale study on oak gallwasps by Rokas et al. (2003) made a clear statement of this separation as well. It is also well-known that both distribution of species diversity and genetic lineages across Turkey indicate a separation in different plant and animal taxa (Ekim & Güner, 1986; Çıplak, 2004; Mutun, 2010).

Contrary to all advantages of mtDNA application in phylogeographic studies it is known that the use of only a single mtDNA gene and related phylogenies may fail to provide reliable, well supported trees in phylogenetic analyses (Farias et al., 2001). To overcome such problems the use of both cytoplasmic gene(s) and nuclear DNA regions are preferred over using only a single molecular marker. The results of both molecular markers used in this thesis provided slightly different outcomes. In particular, nDNA ITS2 region sequence analysis conducted in this study produced tree topologies with more polytomies (see Figures 17-18). All the trees produced both by maximum parsimony, maximum likelihood and the Bayesian analysis using the ITS2 region of *T. synaspis* indicated that a haplotype (H9) from the Giresun population was basal to all other haplotypes of the species which was quite interesting since the same individuals' haplotype was not basal in the cyt b trees (H29). This gripping result may be due to the different modes of inheritance of both genomes since mitochondrial genome is free of recombination due to its uniparental inheritance and there is much evolutionary pressure on its protein-coding genes compared to the nuclear genome which is biparentally inherited with recombination particularly in the case of non-coding ITS2 region (Buburuzan et al., 2007). Although through studying multilocus phylogenies new insights are provided into the phylogeny of many organisms in recent years; it is generally accepted that several loci may provide independent estimates of phylogenetic relationships (DeSalle &

Brower, 1997). In fact nuclear DNA, particularly non-coding DNA regions such that ITS2 do not have evolutionary pressure on them compared to the coding genes may enlighten different time zones through sequencing. Since there is no molecular clock used for the non-coding nDNA regions node age estimation was not conducted on ITS2 sequence data, thus it does not allow one to evaluate the time of separation of the lineages although in the case of *T. synaspis* many of the haplotypes fall into polytomies.

The presence of more polytomies among the *T. synaspis* haplotypes in contrast to the cyt b trees may imply that the use of a non-coding nuclear DNA region such as ITS2 seems to be not providing enough resolution at the intraspecific level. In recent years a number of studies have also shown that both mitochondrial and nuclear genes can lead to markedly different phylogenies as a consequence of different inheritance pathways, selection pressures and differences in response to evolutionary processes in both genomes (Shaw, 2002; Zhang & Hewitt, 2003). The slow rate of nuclear DNA evolution is considered to limit its use in intraspecific studies. It is known the fact that the rate of evolution of nuclear DNA sequences in animals is slower than the one of mtDNA (Brown et al., 1979). Moreover, mtDNA becomes monophyletic faster than nuclear genes because its effective population size is one-fourth of to that of nuclear genes (Moritz et al., 1987). Thus, the current results and differences in the resolutionary power of both DNA regions may all be related to the aforementioned natures of mitochondrial and nuclear genomes.

The Quaternary climatic oscillations were resulted in repeated range contraction and expansion of species with an important role in shaping genetic patterns of the current populations (Hewitt, 2000). Such retreats/expansions as well as the following isolation in multiple glacial refugia may maintain high intraspecific

genetic diversity (Weir & Scluter, 2007). These patterns could be well illustrated by phylogeographic analyses of genealogical data within and between the current populations (Avise, 2004). Quaternary climatic oscillations started about 2.4 MYA and resulted in the growth of ice sheets in the Northern hemispheres, at the same time the relevant geological changes in Anatolia had already come to an end (Akçar & Schlüchter, 2005). Rigorous statistical test gives confidence to the interpretation of the association of haplotype diversity and distribution, and allows us to infer scenarios that can be dated to the Pleistocene and the Quaternary with its successive climatic oscillations. Thus, for assessing the effects of the Quaternary climatic changes on the studied oak gallwasp species from Anatolia further analysis on the estimation of the divergence times of the *T. synaspis* haplotypes was estimated, and the results can be explained using several points: first of all the diversification of the outgroup haplotypes from the ingroup species-haplotypes was estimated to be around 2,3 MYA which corresponds the Late Pliocene. Considering the processes in the final formation and minor changes in the topographic structure of Anatolia in the late Pliocene it might be reasonable to imagine that both *T. megaptera* and *T. synaspis* separation might have taken place around the late Pliocene. Secondly, interested in more of the divergence times within *T. synaspis* results related to the node age determination the transitionary time between Pliocene/Pleistocene seem to affect *T. synaspis* linages.

During the Quaternary Period, the climate in Anatolia was milder compared to the northern areas however, species located in Turkey were still influenced greatly by the fluctuations. The glaciation was never observed in Anatolia except that the ice caps formation at the tops of certain mountains indirect effects of glacial cycles seem was not slight in Turkey. Under these conditions, through the application of the

conventional insect mitochondrial DNA clock the possible scenario for the ingroup haplogroup-separation would be as the following: in the beginning of the Pleistocene glacial times it seems that a mtDNA lineage gave rise to the small monophyletic clade including Bitlis and Konya haplotypes (H11 and H37) followed by the separation of the Konya and Karaman haplogroup. From the Figure 15 it is clear that most of the deeper separation of the *T. synaspis* haplotypes occurred during different subglacial times. Among these, around 1, 9167 MYA (Early Pleistocene) some of the eastern haplotype lineages (Bitlis, Muş, and Tunceli) form a distinct subclade and supported with high statistical support (Figures 14 and 15).

Further separation between mitochondrial lineages of *T. synaspis* seem to happen during the Pleistocene, around 1.725 MYA, which gave rise to mainly two clades as eastern/near eastern and central/western haplogroups. The first haplotype group formed two subclades about 1.15 MYA into entirely east and a mixture of east/near east groups. The second group was also recurrently shaped from the middle to the Late Pleistocene. More recent diversification events also took place within the Turkish lineages of the studied species. For instance, around the middle to Late Pleistocene a central/ west grouping was formed about 0.7667 MYA which corresponds the Günz glacial time (790 000-950 000 years BP) of the Pleistocene. Likewise, at the end of the Early Pleistocene it seems that an east based dispersal might have had occurred toward the central to western distribution range of *T. synaspis*. Moreover, in all clades more recent and shallow diversification events are apparent covering Mindel (420 000 to 30 000 years BP), and Riss (140 000 to 80 000 years BP). Beginning with the Pleistocene glacial cycles some diversification events following isolation seem to have reoccurred several times among the *T. synaspis* lineages. Overall current estimates of times of divergence recovered in this thesis

considering the Pleistocene history of Anatolia may support the findings of previous phylogeographic studies on gallwasps from Turkey (Rokas et al., 2003).

Geographic patterns of genetic diversity may reflect recent and contemporary processes in addition to the more drastic effect caused by past events in the studied area. Nevertheless by comparing geographic patterns shown by different species in the same area may shed light into the general influence of common processes and events. Current findings in this thesis on *T. synaspis* may provide some insights into the effects of cyclical climatic changes particularly during the Pleistocene and the heterogeneous topography of Turkey being the two important factors shaping the current spatial distribution of species, and it is believed that more research in different taxa distributed in Anatolia may further provide supporting data to this general conclusion.

REFERENCES

Akçar N., Schlüchter C. (2005). Paleoglaciations in Anatolia: a Schematic Review and First Results. Eiszeitalter und Gegenwart, 55: 102–121.

Ansell S. W., Stenoien H. K., Grundmann M., Russell S. J., Koch M. A., Schneider H., Vogel J. C. (2011). The importance of Anatolian mountains as the cradle of global diversity in *Arabis alpina*, a key arctic-alpine species. Annals of Botany, 108: 241-252.

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

Atkinson R. J., Rokas A., Stone G. N. (2007). Longitudinal patterns in species Richness and genetic diversity in European oaks and oak gallwasps. S. Weiss and N. Ferrand (eds.), Phylogeography of Southern European Refugia, 127-151.

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

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. Harvard University Press, Cambridge, M.A. 447 pp.

Avise J. C. (2004). Molecular Markers, Natural History, and Evolution (Second Edition). Sinauer, Sunderland, MA. 684 pp.

Bohlen J., Perdices A., Doadrio I., Economidis P. S. (2006). Vicariance, colonisation, and fast local speciation in Asia Minor and the Balkans as revealed from the phylogeny of spined loaches (Osteicthyes: Cobitidae). Molecular Phylogenetics and Evolution, 39: 552-561.

Bozkurt E. (2001). Neotectonics of Turkey – a synthesis. Geodynamica Acta, 14: 3-30.

Brower A. V. Z. (1994). Rapid morphological radiation and convergence among races of butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. Proceedings of the National Academy of Sciences, USA, 76: 1967-1971.

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

Buburuzan L., Gorgan L. D., Bara I. I. (2007). Types of DNA used in speciation and phylogeny studies. Analele Ştiintifice ale Universitatii, Alexandru Ioan Cuza, Sectiunea Genetica şi Biologie Moleculera, TOM VIII: 25-30.

Burks B. D. (1979). Cynipidae. Pp.1060-1107. In: Krombein, K.V., Hurd, P.D., Jr., Smith, D.R. and Burks, B.D. (eds.) Catalog of Hymenoptera in America North of Mexico. Vol. 1. Smithsonian Institution Press, Washington, D. C.

Caterino M. S., Cho S., Sperling F. A. (2000). the current state of insect molecular systematics: a thriving Tower of Babel. Annual Review of Entomology, 45: 1-54.

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: 2013-2114.

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., Crandall K. A. (2000). TCS: a computer program to estimate gene genealogies. Molecular Ecology, 9: 1657–1659.

Cook J. M., Rokas A., Pagel M., Stone G. N. (2002). Evolutionary shifts between host oak sections and host-plant organs in *Andricus* gallwasp. Evolution, 56(9): 1821-1830.

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.

Çıplak, B., Mol, A. (2001). Türkiye'de yayılış gösteren *Chorthippus fieber*, 1852 (Acrididae, Gomphocerinae) cinsine ait türler üzerinde sistematik bir çalışma. Akdeniz Üniversitesi, Araştırma Fonu, 20.01.0121.05.

Çıplak B. (2004). Biogeography of Anatolia: the marker group Orthoptera. Memorie della Societa Entomologica Italiana, 82(2): 357-372.

Davis P. H. (1971). Distribution patterns in Anatolia with particular reference to endemism. In: Davis, P. H., Harper, P. C. & Hedge, I. C. (eds.): *Plant life of South-West Asia.* Botanical Society of Edinburgh, Edinburgh. Pp. 15-27.

DeSalle R., Brower.A. V. Z. (1997). Process partitions, congruence and the independence of characters: inferring relationships among closely-related Hawaiian *Drosophila* from multiple gene regions. Systematic Biology, 46: 751-764.

Dubey S., Cosson J.-F., Vohralik V., Krystufek B., Diker E., Vogel P. (2007). Molecular evidence of Pleistocene bidirectional faunal exchange between Europe and the near East: the case of the bicoloured shrew (*Crocidura leucodon*, Soricidae). Journal of Evolutionary Biology, 20: 1799-1808.

Demirsoy A. (1999). Genel ve Türkiye Zoocoğrafyası. Hayvan Coğrafyası, 2. Edition, Meteksan A.Ş. Press, Ankara, Turkey.

Dinç S., Mutun S. (2011). PCR-RFLP variation of the oak gallwasp, *Andricus quercustozae* (Bosc, 1792) (Hymenoptera: Cynipidae) from Turkey. Türkiye Entomoloji Dergisi, 35(1): 47-58.

Drummond A. J., Rambaut A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology, 7: 214.

Ekim T., Güner A., (1986). The Anatolian Diagonal: fact or fiction? Proceedings of the Royal Society of Edinburgh, 89B: 69-77

Eroğlu 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 of Non-Wood Forest Products", Menemen-İzmir (Turkey), 2–8 October 2000, Joint FAO/ECE/ILO Committee on Forest Technology, Management and Training, 10 p.

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.

Farias, I.P., Ottf, G., Sampaio, 1., Schneider, H. and Meyer, A. (2001). The Cytochrome b gene as a phylogenetic marker: The limits of resolution for analyzing relationships among Cichlid fishes. Joumal of Molecular Evolution, 53: 89-103.

Ferraris J. D., Palumbi S. R. (1996). Molecular Zoology Advances, Strategies, and Protocols. Wiley-Liss Inc., 546 pp.

Fu Y.-X., Li W.-H. (1993). Statistical tests of neutrality of mutations. Genetics, 133, 693-709.

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. Trends in Ecology & Evolution, 4(1): 6-11.

Harpending H. (1994). Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. Human Biology, 66: 591-600.

Harper L. J., Schörogge K., Lim K. Y., Francis P., Lichtenstein C. P. (2004). Cynipid galls:insect-induced modifications of plant development create novel plant organs. Plant, Cell & Environment, 27(3):327-335.

Hashiguchi Y., Kado T., Kimura S., Tachida H. (2006). Comparative phylogeography of two bitterlings, *Tanakia lanceolata* and *T. limbata* (Telestei, Cyprinidae), in Kyushu and adjacent districts of western Japan, based on mitochondrial DNA analysis. Zoologica Scripta, 23: 309-322.

Hewitt G. M. (1996). Some genetic consequence 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 recolonization of European biota. Biological Journal of the Linnean Society 68: 87-112.

Hewitt G. M. (2000). The genetic legacy of the Quaternary ice ages. Nature, 405: 907-913.

Hewitt, G. M. (2001). Speciation, hybrid zone and Phylogeography – or seeing genes in space and time. Molecular Ecology, 10: 537-549.

Hoy M. A. (1994). Insect Molecular Genetics An introduction to principles and applications. Academic Press Inc., 546 p.

Hrbek T., Küçük F., Frickey T., Stölting K. N., Wildekamp R. H., Meyer A. (2002). Molecular phylogeny and historical biogeography of the *Apahnius* (Pisces, Cyprinodontiformes) species complex of central Anatolia, Turkey. Molecular Phylogenetics and Evolution, 25: 125-137.

Hsü K. J. (1978). When the Black Sea was drained. Scietific American, 258: 52-63.

Jang-Liaw N.-H., Lee T.-H., Chou W.-H. (2008). Phylogeography of *Sylvirana latouchii* (Anura, Ranidae) in Taiwan. Zoological Science, 25: 68-79.

Jermiin 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.

Ji Y. -J., Zhang D. -X., He L. -J. (2003). Evolutionary conservation and versatility of a new set of primers for amplifying the ribosomal internal transcribed spacer regions in insects and other invertebrates. Molecular Ecology Notes, 3: 581-585.

Gündüz I., Jaarola M., Tez C., Yeniyurt C., Polly P. D. Searle J. B. (2007). Multıgenic and morphometric differentiation of ground squirrels (*Spermaphilus*, Scuiridaem Rodentia) in Turkey, with a description of a new species. Molecular Phylogenetics and Evolution, 43: 916-935.

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

Korkmaz M., Sarı M., Başıbüyük H. H. (2010). Genetic structure of *Chorthippus paralellus* (Orthoptera: Acrididae: Gomphocerinae) populations in Anatolia: a stable

rear edge population. Annals of the Entomological Society of America, 103(4): DOI: 10.1603/An09172.

Lemire B. (2005). Mitochondrial Genetics. WormBook Ed. The *C. elegans* Research Community, WormBook, doi./10.1895/wormbook.1.25.1, http://www.wormbook.org.

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

Liljeblad, J., and F. Ronquist. (1998). A phylogenetic analysis of higher-level gallwasp relationships (Hymenoptera: Cynipidae). Systematic Entomology, 23: 229-252.

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

Manos P. S., Doyle J. J., Nixon K. C. (1999). Phylogeney, biogeography and the processes of molecular differentiation in *Quercus* subgenus *Quercus* (Fagaceae). Molecular Phylogenetics and Evolution, 12: 333-349.

Margulis L. (1970). Origin of eukaryotic cells. Yale University Press, New Heaven C. T.

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

Michaux J. R., Libous R., Paradis E., Filippuccis M. G. (2004). Phylogeographic history of the yellow-necked fieldmouse (*Apodemus flavicollis*) in Europe and in the Near and Middle East. Molecular Phylogenetics and Evolution, 32: 788-792.

Moritz C., Dowling T. E., Brown W. M. (1987). Evolution of animal mitochondrial DNA: relevance for population biolgy and systematics. Annual Review of Ecological Systematics 18: 269-292.

Mutun S. (2010). Intraspecific genetic variation and Phylogeography of the oak gall waps *Andricus caputmedusae* (Hymenoptera: Cynipidae): Effect of the Anatolian Diagonal. Acta Zoologica Acedemiae Scientiarum Hungariacae, 56 (2): 153-172

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

Nei M. (1987). Molecular Evolutionary Genetics. Columbia University Press, New York, NY.

Nicholls J., Fuentes-Utrilla P., Hayward A., Melika G., Csóka G., Nieves-Aldrey J.-L., Pujade-Villar J., Tavakoli M., Schönrogge K., Stone G. N. (2010). Community impact of anthropogenic disturbance: natural enemies exploit multiple routes in pursuit of invaliding herbivore hosts. Evolutionary Biology, 10: 322.

Nicholls J. A., Challis R. J., Mutun S., Stone G. N. (2012). Mitochondrial barcodes are diagnostic of shared refugia but not species in hybridizing oak gallwasps. Molecular Ecology, 21(16): 4051-4062.

Nieberding C., Libois R., Douady C. J., Morand S., Michaux J. R. (2005). Phylogeography of a nematode (*Heligmosomoides polygyrus*) in the western Palearctic region: persistence of northern cryptic populations during ice ages? Molecular Ecology, 14: 765-779.

Quenouille B., Bermingham E., Planes S. (2004). Molecular systematics of the damselfishes (Teleostei: Pomacentridae): Bayesian phylogenetic analyses of mitochondrial and nuclear DNA sequences. Molecular Phylogenetics and Evolution, 31: 66-88.

Price P. W. (2005). Adaptive radiation of gall-inducing insects. Basic and Applied Ecology, 6(5): 413-421.

Posada D., Buckley T. R. (2004). Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Systematic Biology, 53:793–808.

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.

Rambout A., **Drummond A. J. (2003)**. Tracer. Version 1.3.1. Available from http://evolve.zoo.ox.ac.uk/.

Rambout, A. (2009). FigTree. 1.3.1. (http://tree.bio.ed.ac.uk/software/figtree/).

Roehrdanz R. L., Degrugillier M. E. (1998). Long sections of mitochondrial DNA amplified from forteen orders of insects using conserved polymerase chain reaction primers. Annals of the Entomological Society of America, 91(6): 771-778.

Roehrdanz R. L., Szalanski A. L., Levine E. (2003). Mitochondrial DNA and ITS1 differenatiation in geographical populations of northern corn rootworms, *Diabrotica barberi* (Coleoptera: Chrysomelidae): identification of distint genetic populations. Annals of the Entomological Society of America, 96(6): 901-913.

Rogers A. R., Harpending H. (1992). Population growth makes waves in the distribution of pairwise genetic differences. Molecular Biology and Evolution, 9:552-569.

Rokas A., Melika G., Abe Y., Nieves-Aldrey J.-L., Cook J. M., Stone G. N. (2003). 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: 36-45.

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

Ronquist F., Liljeblad J. (2001). Evolution of the gallwasp: host plant association. Evolution, 55: 2503-2522.

Rozas J., Sanchez-DelBarrio J. C., Messeguer X., Rozas R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics Applications Note, 19(18): 2496-2497.

Rögl F. (1999). Mediterranean and Paratethys Paleogeography during the Oligocene and Miocene. In J. Agusti, L. Rook and P. Adrews (eds.). Hominoid Evolution and climatic change in Europe Vol. 1: the evolution of the Neogene terrestrial ecosystems in Europe, pp 8-22. Cambridge University Press, Cambridge U.K.

Schneider S, Roessli D, Excoffier L. (**2000).** Arlequin: a software for population genetics data analysis User manual ver 2.000. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva; Geneva.

Shaw M., Murrell A., Barker S. C. (2002). Low intraspecific variation in the rRNA Internal Transcribed Spacer 2 (ITS2) of the Australian paralysis tick, *Ixodes holocylus*. Parasitology Research, 88(3): 247-252.

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 gallwasp 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 gallwasps (Hymenoptera: Cynipidae). Annual Review of Entomology, 47: 633:68.

Stone G. N., Schönrogge K. (2003). The adaptive significance of insect gall morphology. Trends in Ecology & Evolution, 18: 512 - 521.

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 gallwasps *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* gallwaps. Molecular Ecology, 17: 652-665.

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

Şekercioğlu Ç. H., Anderson S., Akçay E., Bilgin R., Can Ö. E., Semiz G., Tavşanoğlu Ç., Yokeş M. B., Soyumert A., İpekdal K., Sağlam İ. K., Yücel M., Dalfes H. N. (2011). Turkey's globally important biodiversity in crisis. Biological Conservation, 144: 2752-2769.

Tajima F. (1983). Evolutionary relationship of DNA sequences in finite populations. Genetics, 105: 437-460.

Tajima F. (1989a). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics, 123: 585-595.

Tajima F. (1989b). The effect of change in population size on DNA polymorphism. Genetics, 123: 597-601.

Tamura K., Nei M., Kumar S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA), 101: 11030-11035.

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.

Tan H.W., Liu G.H., Dong X., Lin R.Q., Song H.Q., Huang S.Y., Yuan Z.G., Zhao G.H., Zhu X. Q. (2011). The complete mitochondrial genome of the Asiatic cavity-nesting honeybee *Apis cerana* (Hymenoptera: Apidae). PLoS ONE 6(8): e23008. doi:10.1371/journal.pone.0023008.

Teacher A. G. F., Griffiths D. J. (2011). HapStar: Automated haplotype network layout and visualization. Molecular Ecology Resources, 11(1): 151-153.

Veith M., Schmidtler J., Kosuch J., Baran I., Seitz A. (2003). Paleoclimatic changes explain Anatolian mountain frog evolution: a test for alternating vicariance and dispersal events. Molecular Ecology, 12: 185-199.

Watterson G. A., Guess H. A. (1977). Is the most frequent allele the oldest? Theoretical Population Biology, 11: 141–160.

Weir J. T., Schluter D. (2007). The Latitudinal Gradient in Recent Speciation and Extinction Rates of Birds and Mammals. Science, 315(5818): 1574-1576.

Zhang D.-X., Hewitt G. M. (1996). Nuclear integrations: challenges for mitochondrial DNA markers. Trends in Ecology & Evolution, 11: 247-251.

Zhang D.-X., Hewitt G. M. (2003). Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. Molecular Ecology, 12: 563-584.