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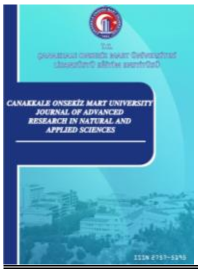

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The role of Anatolia between Asia and Europe: A case study of oak gall wasp species, *Cynips quercus (Hymenoptera, Cynipidae)*

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Research Article

Abstract – In this study, we used DNA sequence data of a western Palearctic oak gall wasp species, *Cynips quercus*, showing distribution from Europe to Iran to answer the following questions: i) Do the eastern *C. quercus* populations have higher genetic diversity than the European populations?, ii) Are the eastern populations source for the European populations?, iii) What is the possible date of divergence between the Asian and European populations? For these purposes, we analysed 88 cytochrome b sequences representing Iran, Türkiye, Hungary, and Spain. Our analyses revealed that the highest genetic diversity was in Türkiye followed by Iran, Hungary, and Spain, respectively. Iranian samples were most divergent from the Spain and Hungary sequences. Pairwise comparisons showed that the highest gene flow was between Türkiye and Hungary, and between Türkiye and Iran, respectively. Phylogenetic analysis grouped the Turkish haplotypes with the Iranian sequences, and Spain was grouped with Hungary. Network analysis revealed that the western Turkish samples were separated from eastern sequences, and all of the eastern Turkish sequences were clustered with the Iranian samples meanwhile a western Turkish lineage provided a source to Hungary and then to Spain. Times of divergence analysis estimated that the Turkish lineage split from the Iranian lineage around 4,67 million years ago, and Hungarian and Spanish lineages diverged from each other about 3 million years ago. Correspondingly, our analyses suggested that Anatolia played a key role as a bridge between the Asian and European populations of *C. quercus*.

***Keywords** – Asia, Cynips quercus, cyt b, Europe, Oak gall wasp*

1.Introduction

Anatolia as a natural bridge connecting the three continents was formed around the late Oligocene and middle Miocene because of the collision of the Arabian and African plates (Şengör & Yılmaz, 1981). After the collision, the eastern Anatolia uplifted while the western part was still lowland. By the end of the subsequent Pliocene epoch, Anatolian geomorphology was almost completed generating the current topological structure of the Turkish landmass (Erol, 1983). Associated palaeogeologic and palaeotectonic events accompanied by paleoclimatic fluctuations during the Tertiary and the Pleistocene epoch of the Quaternary have had a profound effect on the Anatolian biodiversity (Demirsoy, 2002). In many instances, these ongoing changes have either promoted speciation events or caused substantial range shifts in many plant and animal species that already inhabited Anatolia (Bilgin, 2011). The Anatolian Peninsula is located at the junction of three phytogeographic regions as the Irano-Turanian, the Mediterranean and the Euro-Siberian, and Asian, Mediterranean, Caucasian, and African faunistic elements are currently the inhabitants of Anatolia (Şekercioğlu et al., 2011). With over 10.000 plant and 80.000 animal species/subspecies constituting one of the 35 biodiversity hotspots Anatolia

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has been reported as a biodiversity corridor between Asia and Europe (Stone et al., 2007; Ansell et al., 2011; Bilgin, 2011). In particular, during the recurring changes of Pleistocene most of the northern latitudes of Asia and Europe were inhabitable for many taxa, thus populations of those species retreated to southern latitudes (Hewitt, 1999). In recent years, there is an increasing number of studies addressing Iran and Anatolia as the eastern, and the Balkans, Spain, and Italy as the southern source populations for post-Pleistocene colonization of Europe (Ansell et al., 2011; Koch et al., 2017). Specifically, in oak gall wasps Iranian and Anatolian populations have been proposed as the origin for the European populations (Rokas et al., 2003).

Oak gall wasps from the family Cynipidae are obligate parasites of the oak taxa, and nearly 150 species have been recorded from Türkiye (Bayrak & Avcı, 2019). *Cynips quercus* Fourcroy, 1785 (Hymenoptera, Cynipidae, Cynipini) is an oak gall wasp species with alternation of generation between sexual and asexual generations and shows widespread distribution in the western Palearctic (Melika, 2006). The species forms its asexual generation galls under the leaves of white oak taxa from the Quercus section of the family Fagaceae. In this study, we used 88 sequences of the mitochondrial DNA cytochrome b gene (cyt b) covering a 433 base pairs of *C. quercus* downloaded from NCBI GenBank representing Iran, Türkiye, Hungary, and Spain to answer the following questions: i) Do the eastern *C. quercus* populations have higher genetic diversity than the European populations?, ii) Are the eastern populations source for the European populations?, iii) What is the possible date of divergence between the Asian and European populations?

2.Materials and Methods

Eighty-eight cyt b sequences of *C. quercus* currently present in GenBank were downloaded and used in this study to estimate genetic diversity among the Turkish, Iranian, Hungarian, and Spanish populations (Table 1). We used Arlequin 3.5.2.2 (Excoffier & Lischer, 2010) and DnaSP 5.10.1 programs (Librado & Rozas, 2009) to determine the number of polymorphic sites (S), nucleotide (π) and haplotype/allele (h) diversity (Nei, 1987), gene flow (N_m) and population differentiation (F_{ST}). Demographic analyses and any deviations from neutrality were analysed by calculating the raggedness index (Hri) (Harpending, 1994), the sum of squared deviations (SDD) (Schneider & Excoffier, 1999), Tajima's *D* (Tajima, 1989) and Fu's F_S (Fu, 1997) using Arlequin 3.5.2.2 (Excoffier & Lischer, 2010).

For revealing phylogenetic relationships among haplotypes, we performed maximum parsimony (MP) and maximum likelihood (ML) analyses using PAUP*4.0b10 (Swofford, 2002). In all tree reconstruction analyses, we used equivalent sequences for the cyt b gene of *C. divisa*, *C. disticha,* and *C. korsakovi* as outgroups (GenBank Accession numbers are given in Figure 1). For the MP analysis, the TBR branch-swapping algorithm with 1000 replicates of random addition of taxa under the heuristic search options and 1000 bootstrap replicates were employed to assess support for branches (Swofford, 2002). We used JModeltest 2 (Darriba et al., 2012) to determine the best fit model for our dataset and detected the GTR+G model (ln= -198.5792), and we applied this model in ML and other analyses. To estimate divergence times of the *C. quercus* lineages we performed Bayesian Inference (BI) using the software package BEAST version 1.5.2 (Drummond et al., 2012) by applying 1.19% sequence divergence per lineage (2.39% pairwise) per million years of mutation rate for the age calibration (Papadopoulou et al., 2010). Both MRCAs (most recent common ancestors) and MACAs (most ancient common ancestors) calculations and operator optimizations (Hayward & Stone, 2006) were performed with BEAUti ver. 1.8.0. The BEAST analysis was run for 100 million generations sampling every 1000 and we controlled the convergence to stationary and the effective sample size (ESS) of model parameters using Tracer ver. 1.6.0. The maximum clade credibility tree was built with Treeannotator ver. 1.8.4. through discarding the initial 25% samples as burn-in. For visualization of the results, we used Fig-Tree ver. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). Further, an unrooted parsimony haplotype network with 95% probability levels was constructed using HapStar Version 0.5 (C) to better understand the evolutionary relationships of haplotypes (Teacher & Griffiths, 2011).

3.Results and Discussion

A 433 base-pair of the mitochondrial cyt b gene of 88 sequences representing 52 Turkish, 17 Hungarian, 10 Iranian, and 9 Spanish haplotypes possessed 325 constant and 108 polymorphic characters (Table 1, GenBank Accession Number for each haplotype is shown on the phylogenetic tree in Figure 1). Among polymorphic sites, only 78 characters were parsimony informative. There were no shared haplotypes among Türkiye, Iran, Hungary, and Spain, thus haplotype diversity was calculated as 1.000 for each of these localities. Nucleotide diversity was highest in Türkiye (π= 0.0258) followed by Iran (π= 0.0233), Hungary (π= 0.0166), and Spain (π= 0.0083) (Table 1). A recent study conducted on only the Turkish populations of *C. quercus* revealed the presence of high genetic variation in the species particularly in the eastern/southeastern Anatolian populations (Mutun & Dinç, 2019). In this current study, the presence of high genetic diversity in each of the *C. quercus* populations is not unexpected since the Iberian Peninsula and the Balkans have been used as shelter areas by many taxa during the harsh environmental conditions of the last few ice ages, thus constituting the two important diversity centres for Europe (Hewitt, 2004). Nonetheless, Iran and Anatolia are also well-proven refugia and eastern centres of diversity for the western Palearctic which are known to combine Caucasian and Asian diversity (Bilgin, 2011). Sequences used in this study representing four of the diversity centres harboured diversity in oak gall wasps were generated congruent results with other oak gall wasp species (Rokas et al., 2003; Stone et al., 2007). For instance, in *Andricus kollari* the nucleotide diversity was greatest in the Anatolian populations, it was intermediate in Hungary, and the lowest diversity was determined in Spain (Stone et al., 2007). Further, in *Andricus quercustozae* Anatolia was with the highest genetic richness as compared to Morocco, Spain, France, Italy, Hungary, and Greece (Rokas et al., 2003). In another oak gall wasp species with similar geographic distribution, *Andricus coriarius*, the Turkish populations had the highest genetic variation when compared to the Iranian and Lebanon populations (Challis et al., 2007). Population studies conducted on gall wasp species revealed a decline in genetic diversity from east to west in the refugia in the Mediterranean Basin (Atkinson, 2007). In the case of *C. quercus*, higher genetic diversity revealed in Anatolia in comparison with the Iranian population may be either due to the unequal number of sequences representing the Turkish and the Iranian localities, or Anatolia, indeed, harbours much higher diversity than Iran. Nevertheless, more samples from Iran are necessary for the clarification of Iran and its role in oak gall wasp species.

Population demographic analysis of *C. quercus* produced negative but non-significant Tajima's *D* values for Türkiye, Iran and Spain while Hungary was with positive and insignificant value (Table 1). However, all four populations generated statistically significant negative Fu's F_S values indicating population expansion expected under neutrality in the population growth model (Ramos-Onsins & Rozas, 2002). We also calculated low and non-significant Hri and SSD values for each locality of *C. quercus* which suggest expanding populations.

Table 1.
C. quercus populations, GenBank Accession numbers of haplotypes, N_{hap}: haplotype number, h: haplotype diversity, π: nucleotide diversity, and resulting values calculated for demographic analyses of each population. (*=$P \leq 0.05$).

Pop.	GenBank Acc. No.	N_{hap}	h/π	Tajima's *D*	Fu's *Fs*	Hri	SSD
Türkiye	MH361234-MH361285	52	1.000 +/- 0.0038 / 0.0258 +/- 0.0132	-0.9364	-245.120*	0.021	0.032
Hungary	JQ416460-JQ416478, DQ218011	17	1.000 +/- 0.0202 / 0.0166 +/- 0.0091	0.2281	-117.324*	0.041	0.021
Iran	JQ416448-JQ416459	10	1.000 +/- 0.0447 / 0.0233 +/- 0.0131	-0.5110	-34.451*	0.065	0.042
Spain	JQ416436-JQ416445	9	1.000 +/- 0.0524 / 0.0083 +/- 0.0052	-11.690	-612.711*	0.037	0.005

It is known that higher and non-significant Hri and SSD values are observed in stable and non-expanding populations while lower and non-significant values are predicted under suddenly expanding populations (Rogers & Harpending, 1992). Population expansion has been reported, for instance, for *Andricus kollari* where the European population of the species originated after the expansion of two genetically divergent eastern Mediterranean diversity centres including the Turkish source population in the east (Stone et al., 2007). Although more sequences, in particular, from Iran and three other localities, are necessary for drawing a more general conclusion on *C. quercus*, it seems that Anatolia as one of the eastern populations provided a genetic source to the European localities, yet each of the European locality implied recent population expansion as well.

Gene flow (N_m) estimates revealed that the highest gene flow was between Türkiye and Hungary (N_m= 0.3486), and between Türkiye and Iran (N_m= 0.2845), respectively. However, the least amount of genetic exchange was between Iran and Spain (N_m= 0.1391) (Table 2). Correspondingly, pairwise genetic differentiation (F_{ST}) analysis revealed that Iran was the most diverged population from Spain (F_{ST}= 0.7823), and Hungary (F_{ST}= 0.7202), respectively, and as expected it was least differentiated from Türkiye (F_{ST}= 0.6374). Thus, genetic differentiation analysis produced supporting evidence for the gene flow between populations of *C. quercus*. Under isolation by distance model of population, gene flow or genetic exchange between geographically distant populations is lower than the geographically proximate populations (Stone et al., 2002). In *C. quercus*, the lowest and statistically significant F_{ST} values between Iran and Spain, and between Türkiye and Spain support the isolation by distance. Expectedly, the lowest F_{ST} calculated for the pairwise comparisons of Türkiye and Hungary may propose that the Turkish lineages might have provided a source to the Hungarian population.

Table 2.
Genetic differentiation and gene flow between populations. The lower diagonal shows F_{ST}, and the upper diagonal indicates gene flow (N_m), where all values are significant, $p \leq 0.001$.

	Türkiye	**Hungary**	**Iran**	**Spain**
Türkiye		0.3486	0.2845	0.2119
Hungary	0.5892		0.1942	0.2567
Iran	0.6374	0.7202		0.1391
Spain	0.7024	0.6608	0.7823	

Maximum parsimony, maximum likelihood, Bayesian inference and Beast analysis were conducted to reveal evolutionary relationships of haplotypes and times of divergence of *C. quercus* lineages. Since all phylogenetic analyses generated a similar topology, we presented here only a single tree (Figure 2). Our ingroup species, *C. quercus*, seems to diverge from the outgroup taxa around 7,85 million years ago (MYA). The sorting of the ingroup haplotypes from the outgroup sequences dates back to the Tortonian age (11.6- 7.2 MYA) of the Late Miocene epoch, which is characterized by important geological, climatic, and environmental changes altering the world and its biodiversity (Pound et al., 2011). After its first divergence, the *C. quercus* lineage split into two distinct haplogroups ~6.34 MYA during the Messinian age (7.2- 5.3 MYA) of the Late Miocene. This first separation generated two main clades where the first was comprised of the Iranian and all of the Turkish sequences, and the second clade was constituted by the Hungarian and the Spanish sequences. Therefore, hereafter we termed the former clade as the Asian and the latter as the European clade. Recent studies have well proved that the Messinian age was characterised by great palaeogeological and temperature changes which are associated very closely with climatic fluctuations, particularly in the Mediterranean Basin (Krijgsman et al., 1999). It seems that at least the ancestral basal lineage of *C. quercus* spread around the Irano-Anatolian landmass was divided into haplogroups which coincides with the Messinian environmental crisis. The Messinian stage appears to have further promoted the diversification of this clade into a Turkish haplogroup of only in the eastern Anatolian sequences and Iran, the other clade including all other sequences from the rest of Türkiye. Subsequent diversification events occurred ~4.67 MYA around the early Pliocene resulting the formation of the Iranian lineages from the Turkish lineage. Similar deep splitting events during the early to mid-Pliocene also ended up with inner clade formation within the Turkish *C. quercus* lineages. Our analysis related to the eastern part of the West Palearctic is strongly supported by the time estimation for the speciation of other gall wasp species including *Andricus coriarius* and its cryptic species formed as a result of ongoing events spanning the same period around Anatolia, Iran, and the Levant region (Challis et al., 2007). In *A. coriarius*, the main lineage formation occurred in Pliocene and further splits have occurred in subsequent periods. In correlation with the ongoing changes, it, most probably, caused a pre-Pleistocene separation of the Hungarian and Spanish lineages around ~ 3 MYA. Along the recurrent cyclic changes of Pleistocene, a series of intermediate to relatively shallow divergences of *C. quercus* lineages within each of the Anatolian, Iranian, Hungarian, and Spanish subclades seem to generate the current polygenetic lineages of the species. Our current results are overall in agreement with the findings from other western Palearctic species (Hewitt, 2004; Poulakakis et al., 2005).

Past geologic and environmental changes and accompanied climatic oscillations have been shown to correlate with the phylogenetic structuring of species by leaving a profound effect on the organismal groups in the western Palearctic (Nichols & Hewitt, 1994) and these signals can be revealed through network analysis. Our haplotype network analysis revealed the same structuring of the *C. quercus* sequences (Figure 2). A clear separation of four distinct clusters representing the Turkish, Iranian, Hungarian, and Spanish haplotypes is well-defined and the Turkish samples were further divided into the western and eastern Anatolian haplogroups. Moreover, the eastern Turkish haplogroup is directly connected to the Iranian cluster through many hypothetical haplotypes that were not either sampled or are extinct. While all the Turkish sequences related to the Iranian samples a haplotype from a western Turkish lineage connected directly to a Hungarian haplotype implies that Anatolia might have provided a genetic source to Hungary and then to Spain. Eventually, both Spain and Hungary sequences formed separate haplogroups. It is important to note here that Spain haplotypes are directly connected to the Hungary haplotypes via several hypothetical haplotypes. All phylogenetic inferences in addition to the network analysis signal the great effect of the relatively ancient to intermediate splits being well-correlated with the past changes spanning from 8 MY to the last few more recent glaciation periods. Thus, our current analysis of *C. quercus* representing the two Asian and two European glacial refugia produced congruent results with other animal groups from the western Palearctic (Rokas et al., 2003; Challis et al., 2007).

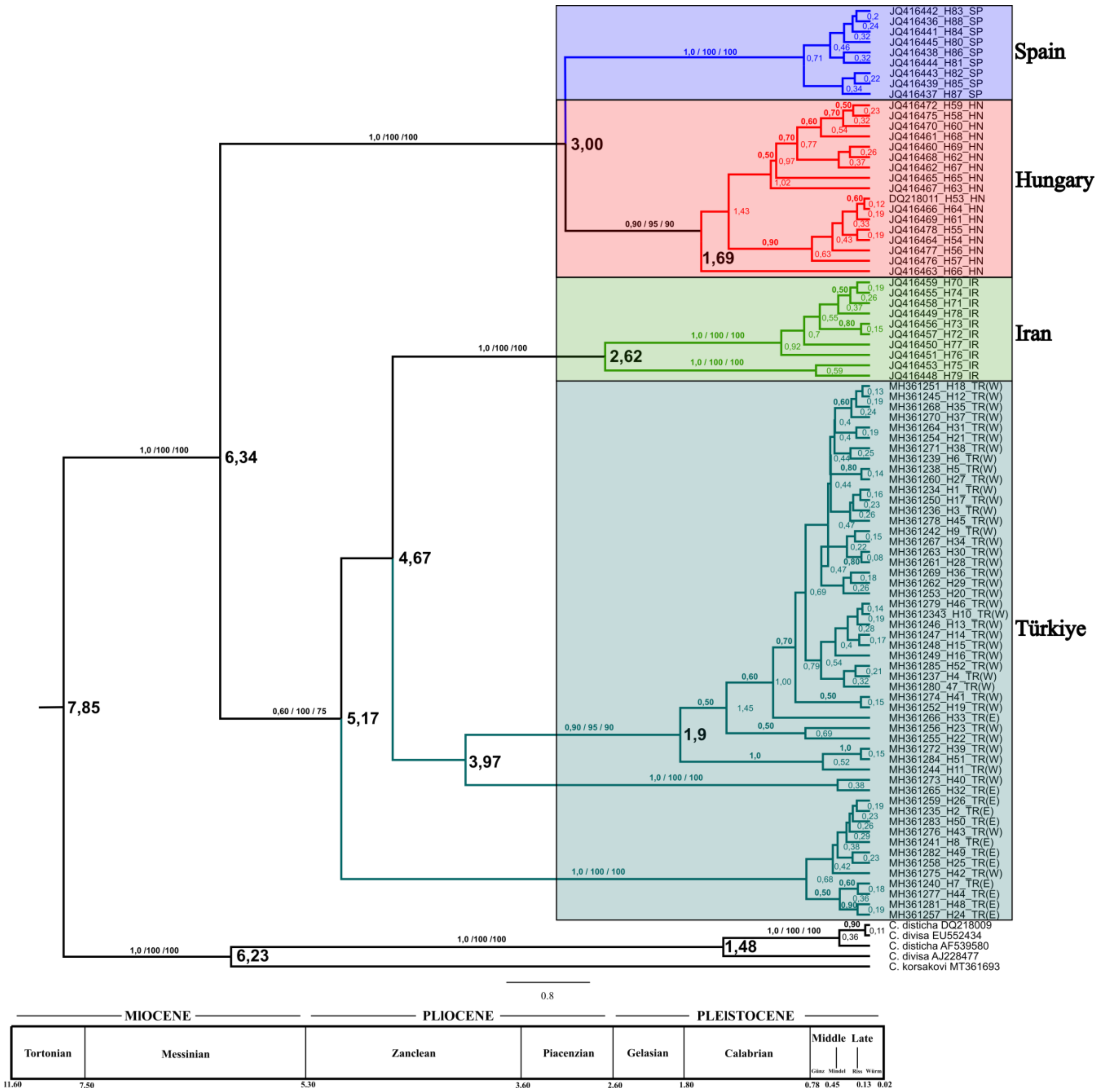

Figure 1. The resulting Beast tree of *C. quercus*. Values at each node represent times of divergence, and numbers on the branches show bootstrap values for the BI posterior probability, and MP/ML, respectively. (E= the east side and W= the west side of Türkiye in reference to major physical barriers in Anatolia).

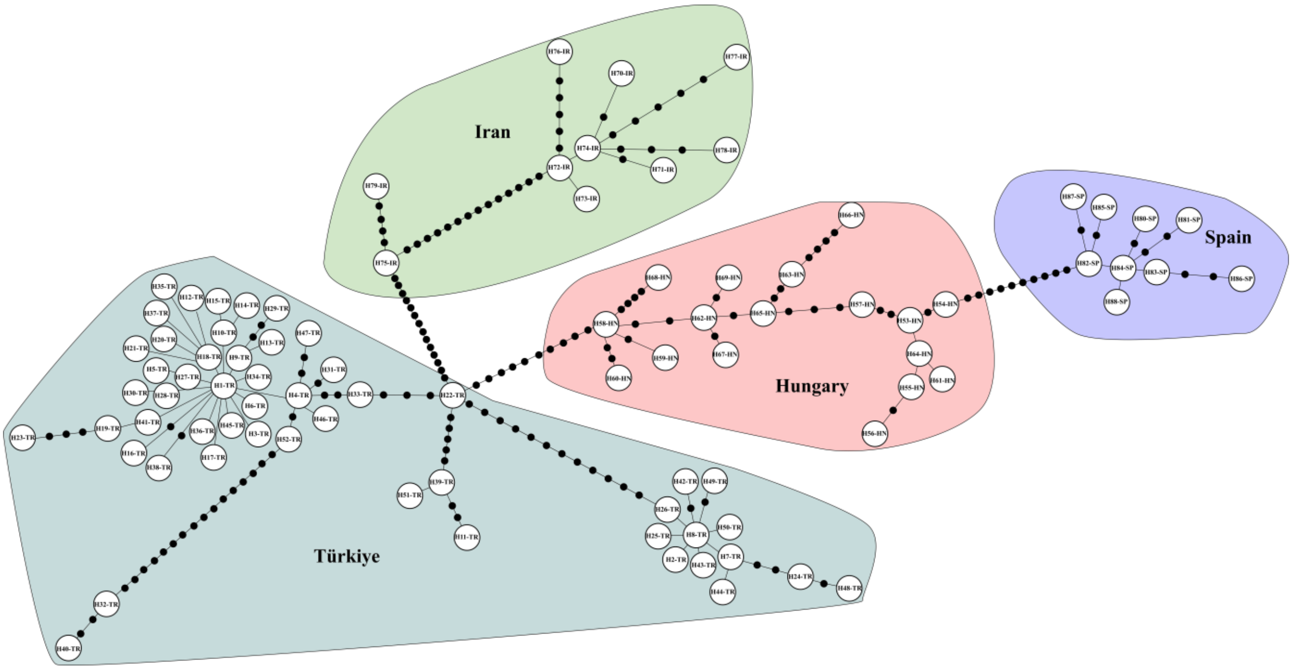

Figure 2. Haplotype network of *C. quercus* sequences. Each circle represents a haplotype which is shown by numbers within the relevant circle. Hypothetical haplotypes are represented by black circles.

4.Conclusion

Genetic diversity analysis revealed that Türkiye harboured the highest level of genetic diversity. Iran, Spain, and Hungary localities also harbour relatively high diversity which is not unexpected since each played a significant role as a distinct glacial refugium in the past. Our phylogenetic tree proposed that the Turkish samples were separated from the eastern sequences, and all the eastern Turkish sequences were clustered with the Iranian samples. Further, a western Turkish lineage provided a source to Hungary and then to Spain eventually these two European localities formed separate haplogroups. Gene flow and genetic differentiation estimates supported an east-to-west dispersal of the species. Moreover, times of divergence analysis implied that past drastic paleogeologic and paleoclimatic changes have been the main drivers of the species phylogenetic structuring since the late Miocene. Our overall analysis indicates that *C. quercus* is currently represented by two Asian and two European major lineages and Anatolia played a key role as a natural bridge between Asia and Europe.

Author Contributions

Serap Mutun: Conceptualization, Methodology, Data Curation, Writing - Original Draft, Writing - Review & Editing.

Serdar Dinç: Formal analysis, Data Curation, Writing - Review & Editing.

Conflicts of Interest

The authors declare no conflict of interest.

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