Possible effects of Pliocene and Pleistocene on the Anatolian populations of *Andricus tomentosus* (Hymenoptera: Cynipidae)

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Abstract. *Andricus tomentosus* is an oak gall wasp species widely distributed in the Western Palearctic region and is one of the common parasitic species forming its bell-shaped asexual generation galls on white oak species. Despite its widespread distribution in Turkey, little is known about the species. In this study, we investigated 19 populations of *A. tomentosus* using partial sequences of the mitochondrial DNA cytochrome b gene and the nuclear ITS2 region to assess the species' genetic diversity and population genetic structure. Two hundred and forty sequences that generated 47 mitochondrial haplotypes and 28 nuclear alleles revealed intermediate to high genetic variation for both studied regions. Some eastern/southeastern Anatolian populations displayed higher diversity than the central/western localities. Demographic analyses and high haplotype/allele versus low nucleotide diversity implied that *A. tomentosus* populations might have undergone a series of expansion and retraction events in the past, which were correlated with the paleoclimatic and paleoenvironmental changes during Pliocene and Pleistocene. Further, phylogenetic inferences suggested that *A. tomentosus* populations were divided into two well-diverged clades as eastern and western, which offered that geographic barriers were also the main driver of the lineage formation in *A. tomentosus*.

Keywords: oak gall wasp, genetic diversity, geographic barriers, Pliocene, Pleistocene, Turkey.

Introduction

The Turkish landmass, one of the important biodiversity hotspots in the Western Palearctic, was formed by the Tertiary geologic events due to movement and subsequent collision of the African-Arabian plate towards western Eurasia (Rögl 1999). The collision initiated the formation of major mountain ranges in European and Anatolian plates at the end of the Mesozoic era (Okay 2008). Nonetheless, the main geological configuration of the area has taken place through the Oligocene and Miocene epochs (Bozkurt 2001). The latter was characterized by arid environmental conditions known as the Messinian aridity crisis and thought to be responsible for shaping the distribution of many species around the Mediterranean Basin (Karadenizli 2011). With the end of the Messinian stage, ongoing events throughout the Pliocene gave rise to the latest topographic structure of Anatolia (Asian Turkey). Although the subsequent Quaternary period did not drastically change the topography, repeated climatic oscillations during the Pleistocene either promoted speciation events or reshaped the geographic distribution of genetic lineages both in Turkey and in other parts of the Western Palearctic region (Taberlet et al. 1998, Hewitt 2001, Çıplak 2004). In many instances, during the cold periods, populations have either gone extinct in the northern latitudes or retracted to the southern latitudes, and opposite shifts occurred during the warm periods as populations expanded their range towards the northern areas (Hewitt 1996). These expansion and contraction events occurred correspondingly through recurring glacial and interglacial cycles. Rapid expansion of populations resulted in lower genetic diversity in more recently populated areas, whereas higher diversity was kept in refugia during unfavorable conditions of these cycles (Hewitt 1999). There is now a vast amount of literature on the Western Palearctic diversity suggesting Iberia, Italy, and the Balkans as southern refugia and Turkey/Iran as the eastern center of diversity (Hewitt 2004, Schmitt 2007). In this

context, in the Western Palearctic is markedly different from others since, as a major eastern refugium, it played a dual role; firstly, it facilitated the survival of many species during glacial periods by sheltering them so that many northerly distributed taxa shifted their range towards Anatolia; therefore it acted as a key-player for altitudinal shifts of species (Pala et al. 2012, Sağlam et al. 2014), and secondly, it has taken an active role in longitudinal movement of diversity providing genetic source to the European populations (Challis et al. 2007, Bilgin 2011).

Currently, Turkey harbors enriched species and genetic diversity not only due to exhibiting a refuge within a refuge feature with heterogenous structure (Çıplak 2008), but also because of (i) its location at the transect of three phytogeographic regions (the Euro-Siberian, the Mediterranean, and the Irano-Turanian) (Zohary 1973, Médail & Diadema 2009), (ii) its role as a corridor between Asian, European and African faunistic elements, and thus exhibiting a mixture of three continental faunal composition (Demirsoy 2002) and being at the intersection of three of the 36 biodiversity hotspots in the world (the Mediterranean, the Irano-Anatolian, and the Caucasian) (Conservation International 2017), (iii) presence of diverse microhabitats/microrefugia in short geographic distances inducing speciation/ diversification events in different organismal groups (Çıplak 2008, López-Pujo et al. 2016), and (iv) its topographical heterogeneity and existence of significant physical barriers shaping the distribution of species/lineage/genetic diversity (Mutun 2010, Ansell et al. 2002). Among these physical barriers, the northern Anatolian Mountain line separates the northern highlands from the central lowlands. The Anatolian Mountain line, known as the Anatolian Diagonal (Davis 1971), divides the eastern from the central and western part of Anatolia, and the Taurus mountains separates the Mediterranean region from the central Anatolian steps (Demirsoy 2002). These barriers in some parts reaching over altitudinal elevations of 3000 m

have either divided the distribution of temperate species or served as a dispersal corridor for the species preferring lower temperatures (Çıplak et al. 2015, Şekercioğlu et al. 2011, López-Pujo et al. 2016). They also played a key role as ecological barriers for several plant and animal groups (Ekim & Güner 1986), consequently separating species/lineages as eastern/southeastern and central/western as previously reported from animals including oak gall wasps (Çıplak 2003, Dinç & Mutun 2019, Şenol et al. 2019, Külköylüoğlu et al. 2021).

Despite its importance as a major biodiversity hotspot and the origin and diversification center for many Mediterranean and European taxa, Anatolia remains understudied. Although in recent years there has been an increase in the oak gall wasp research in Turkey, there is still little known about the oak gall wasp species, *Andricus tomentosus* (Trotter, 1901) (Hymenoptera: Cynipidae), and therefore it deserves more detailed investigation. Hence, in this study, we investigated only the asexual generation of this obligate parasitic species since there is no record of sexual generation yet (Ionescu 1973). *A. tomentosus* is a widely distributed species in the Western Palearctic, and it is also common in Turkey, forming its bell-shaped galls (Fig. 1) with a single larval chamber mostly on the axillary buds of white oak species, *Quercus infectoria* Oliv. (Melika 2006). In this study, we aimed to answer the following questions: i) Other oak gall wasp species studied so far from Turkey revealed high genetic diversity. Does *A. tomentosus* also display high genetic diversity? ii) Other animal and oak gall wasp species with similar geographic distribution showed a well-structured population structure. Is it also possible that *A. tomentosus* reveals a similar grouping of populations? iii) Did geographic barriers play a significant role in creating a genetic break across the sampled area of the species? and iv) The Pliocene and Pleistocene cycles have played a key role in structuring the contemporary population genetic structure of other oak gall wasps in Turkey. Are there such effects of those factors in *A. tomentosus*?

Material and Methods

Sampling, DNA isolation, and gene amplification
A total of 240 females reared from *A. tomentosus* galls collected between 2010 and 2017 from 19 localities were used in this study (Fig. 2, detailed in Table 1). Genomic DNA from whole specimens was extracted using the DNeasy Tissue Kit (QIAGEN) according to the manufacturer's instructions. We amplified a 433-base pair (bp) fragment of the mitochondrial cytochrome b (cyt b) gene using the primer pair of CB1 5'- TATGTACTACCATGAGGACAAATATC-3' and CB2 5'-ATTACACCTCCTAATTTATTAGGAAT- 3' (Jermiin & Crozier 1994) and the entire nuclear ITS2 region using the primers ITS2 F 5'- ATGAACATCGACATTTCGAACGCATAT-3' and ITS2R 5'-TTCTTTTCCTCCGCTTAGTAATATGCTTAA-3', respectively (Ji et al. 2003). Each 50 μl PCR mixture contained 5 ng template DNA, 0.5 μl 10X reaction buffer, 1.0 μl dNTP mix (10 mM), 2.0 μl MgCl$_2$ (25 mM), 10 pmol of each primer, and 2 U of Taq polymerase (Vivantis), and necessary amount of ddH$_2$O to complete the reaction volume. PCR was performed in a Bioer XP Thermal Cycler (model TC-XP-G, Bioer Technology Co. Ltd.) with the following program for the cyt b gene: one cycle of denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min and extension at 72 °C for 1 min 30 s, followed by 72 °C extension for 10 min and sample storage at 4 °C. For the ITS2 region, 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 final extension (Mutun & Dinç 2019). The purified amplicons of both regions were sent to a company (Macrogen, South Korea) for sequencing in both directions to eliminate base calling errors.

Figure 1. Galls of *A. tomentosus*.

Genetic diversity estimation
Chromatograms were checked, aligned, and trimmed, if necessary, with the BioEdit 7.2.5 program (Hall 1999), and sequences were collapsed into haplotypes/alleles using GenAIEx 6.5 (Peakall & Smouse 2012). The potential presence of numts in haplotypes was checked by searching internal stop codons or non-sense mutations following the translation of the sequences into amino acids using DnaSP 5.10.1 program (Librado & Rozas 2009). All unique sequences were deposited in the GenBank under the accession numbers MT671253 to MT671299 and MT666100 to MT666127 for the cyt b haplotypes and the ITS2 alleles, respectively. The number of polymorphic sites (S), nucleotide (π) and haplotype/allele (h/A$_d$) diversity (Nei 1987), the number of substitutions, pairwise nucleotide differences (k) (Tajima 1989), and population differentiations were calculated using Arlequin 3.5.2.2 (Excoffier & Lischer 2010) and DnaSP 5.10.1 program (Librado & Rozas 2009).

Phylogenetic analysis and population demography
We employed maximum likelihood (ML) and Bayesian analyses to unravel phylogenetic relationships among haplotypes/alleles using PAUP*4.0b10 (Swofford, 2002) and MrBayes 3.2.6 (Ronquist & Huelsenbeck 2003). *Andricus caputmedusae* ve *A. curtisii* sequences were used as outgroups in all phylogenetic analyses (GenBank accession no: JQ228863.1, KT447045.1, JQ229109.1, KT447085.1, for the cyt b and ITS2, respectively). In reconstructing the ML tree, we determined the fittest model to our datasets using JModeltest 2 (Guindon & Gascuel 2003, Darriba et al. 2012). Based on the Akaike information criterion (AIC) (Akaike 1974, Posada & Buckley 2004), we determined that GTR+I+G (-lnL= 989.5164) and HKY+I+G (-lnL= 771.1453) were the best-fit models for the cyt b and the ITS2 region, respectively. We used BEAST version 1.5.2 to estimate divergence times of the cyt b lineages and their confidence intervals using a Bayesian Markov Chain Monte Carlo (MCMC) coalescent method (Drummond & Rambaut 2007) by applying the GTR model with I and Γ following the uncorrelated relaxed lognormal clock. We used a 1.19% divergence/ MYA/ lineage mutation rate for age calibration

(Drummond & Suchard 2010, Papadopoulou et al. 2010). We calculated the most recent and ancient common ancestors using BEAUTY (Hayward & Stone 2006). We ran the analysis for 100 million generations, sampling every 1000 and controlling the convergence to stationary and model parameters' effective sample size (ESS) using TRACER v1.6.0. The maximum clade credibility tree built with TREEANNOTATOR v1.8.4. discarding the initial 25% samples as burn-in. FIG-TREE version 1.3.1 (Rambout 2009) was used to visualize the results. We also computed a pairwise distance matrix between haplotypes/alleles using the Arlequin program for building a minimum spanning tree. We constructed an unrooted 95% probability level network analysis using the program HapStar Version 0.5 (C) (Teacher & Griffiths 2011) and transferred the .svg file to Inkscape 0.91 (www.inkscape.org) to visualize the network.
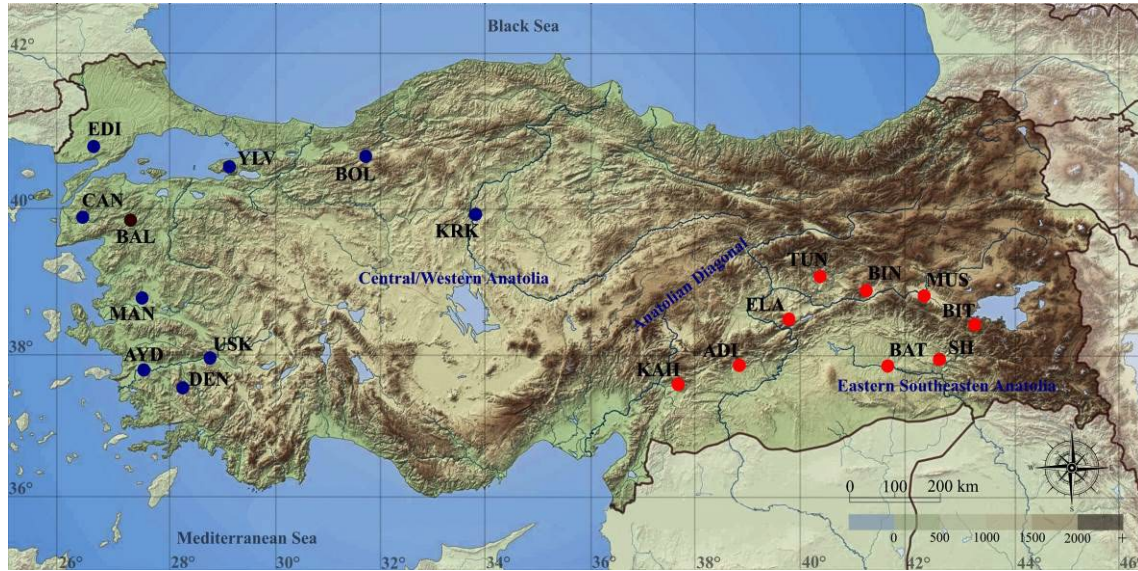

Figure 2. Sampling localities of *A. tomentosus*. Names, abbreviations, and coordinates of populations are given in Table 1. Red coloration indicates eastern/southeastern Anatolian populations and blue-colored localities represent central/western Anatolian populations.

Table 1. Names, abbreviations, coordinates, sample size, number of haplotypes/alleles, haplotype, and nucleotide diversity for each *A. tomentosus* population.

Population	Abbr.	Coordinates	N	CYT B N_{HAP}	ITS2 N_{ALEL}	CYT B H_d	CYT B π	ITS2 A_d	ITS2 π
Adıyaman	ADI	37.7778° N, 37.6847° E	15	5	4	0.8095 +/- 0.0589	0.0234 +/- 0.0127	0.7810 +/- 0.0532	0.0206 +/- 0.0113
Aydın	AYD	37.7653° N, 27.3292° E	15	4	2	0.7048 +/- 0.0742	0.0056 +/- 0.0036	0.5143 +/- 0.0690	0.0037 +/- 0.0026
Balıkesir	BAL	39.9189° N, 27.6310° E	5	4	4	0.9000 +/- 0.1610	0.0175 +/- 0.0115	0.9000 +/- 0.1610	0.0067 +/- 0.0049
Batman	BAT	38.1605° N, 41.6094° E	15	7	8	0.7238 +/- 0.1206	0.0235 +/- 0.0127	0.8952 +/- 0.0525	0.0214 +/- 0.0117
Bitlis	BIT	38.4368° N, 42.5573° E	15	6	3	0.7619 +/- 0.0813	0.0278 +/- 0.0149	0.5619 +/- 0.0954	0.0039 +/- 0.0027
Bingöl	BNG	38.9285° N, 40.3679° E	4	1	3	0.0000 +/- 0.0000	0.0000 +/- 0.0000	0.8333 +/- 0.2224	0.0047 +/- 0.0040
Bolu	BOL	40.9962° N, 32.0624° E	15	4	2	0.4667 +/- 0.1478	0.0061 +/- 0.0038	0.5143 +/- 0.0690	0.0037 +/- 0.0026
Çanakkale	CAN	40.3726° N, 26.7802° E	14	4	4	0.6923 +/- 0.0942	0.0124 +/- 0.0071	0.7582 +/- 0.0601	0.0032 +/- 0.0024
Denizli	DEN	38.3548° N, 29.7400° E	15	8	4	0.9048 +/- 0.0502	0.0225 +/- 0.0122	0.6571 +/- 0.0800	0.0049 +/- 0.0033
Edirne	EDI	41.7984° N, 26.8948° E	15	2	3	0.3429 +/- 0.1278	0.0007 +/- 0.0009	0.6000 +/- 0.0694	0.0015 +/- 0.0014
Elazığ	ELA	38.5799° N, 38.8623° E	15	6	3	0.7905 +/- 0.0785	0.0095 +/- 0.0056	0.6000 +/- 0.0694	0.0015 +/- 0.0014
Kahramanmaraş	KAH	37.4676° N, 37.4410° E	15	5	5	0.7048 +/- 0.0878	0.0021 +/- 0.0017	0.7429 +/- 0.0895	0.0022 +/- 0.0018
Kırıkkale	KRK	40.1019° N, 33.7483° E	2	2	1	1.0000 +/- 0.5000	0.0046 +/- 0.0056	0.0000 +/- 0.0000	0.0000 +/- 0.0000
Manisa	MAN	38.1782° N, 28.5247° E	15	5	3	0.6762 +/- 0.1049	0.0059 +/- 0.0038	0.5619 +/- 0.0954	0.0015 +/- 0.0014
Muş	MUS	38.6334° N, 41.7644° E	15	4	4	0.4667 +/- 0.1478	0.0032 +/- 0.0023	0.6000 +/- 0.1129	0.0081 +/- 0.0049
Siirt	SII	38.1378° N, 41.7579° E	15	8	4	0.9048 +/- 0.0502	0.0194 +/- 0.0107	0.6000 +/- 0.1129	0.0203 +/- 0.0111
Tunceli	TUN	39.5901° N, 39.8625° E	5	4	2	0.9000 +/- 0.1610	0.0078 +/- 0.0056	0.4000 +/- 0.2373	0.0019 +/- 0.0019
Uşak	USK	38.5340° N, 29.6646° E	15	1	4	0.0000 +/- 0.0000	0.0000 +/- 0.0000	0.6190 +/- 0.1196	0.0017 +/- 0.0015
Yalova	YLV	40.5413° N, 29.2726° E	15	7	2	0.8286 +/- 0.0823	0.0180 +/- 0.0099	0.5333 +/- 0.0515	0.0038 +/- 0.0027
Average H_d / Average A_d						0.6620 +/- 0.1120		0.6143 +/- 0.0958	
Average π / Average π						0.0111 +/- 0.0065		0.0061 +/- 0.0039	

For analyzing population demographic structure, we employed mismatch distribution analysis using pairwise differences between haplotypes/alleles using DnaSP 5.10.1 (Librado & Rozas 2009). We also calculated the raggedness index (Hri) (Rogers & Harpending 1992) and the sum of squared deviations (SDD) (Schneider et al. 2000) to compare the observed sample distribution with the expected probability distribution. Moreover, we calculated Tajima's *D* (Tajima 1989) and Fu's *F_S* (Fu 1997) using Arlequin 3.5.2.2 (Excoffier & Lischer

2010) to check the deviations from neutrality. BEAST version 2 software (Drummond et al. 2012) was used for conducting a Bayesian skyline plot model using a coalescent-based estimation of population size changes of the species over time with the MCMC sampling procedure (Ho & Shapiro 2011). To unravel whether there is any correlation between the geographic distribution of haplotypes/alleles and genetic diversity, we performed AMOVA analysis using Arlequin 3.5.2.2 (Excoffier & Lischer 2010) at three different hierarchical levels among groups, among populations within groups, and within each population through testing alternative groupings of populations considering their geographic locations until obtaining the highest and statistically significant differentiation value between the groups.

Results

Genetic diversity estimates

Forty-seven haplotypes comprising 19 singleton and 8 private sequences were identified among 240 *A. tomentosus* cyt b sequences (Appendix 1). The total alignment contained 38 parsimony informative sites with 28 transitions and 10 transversions ($R= 2.471$). Thirty-two substitutions (84%) were at the third, and 6 (16%) were at the first codon position. The highest sequence divergence was 29 bp (7%) detected between an Adıyaman haplotype from southeastern Anatolia and a Denizli haplotype from the western part of Turkey. The most common haplotype (H8, N= 61) was shared among 11 populations, four from eastern/southeastern Anatolia and the remaining from central/western Turkey. Haplotype variation was higher in the eastern/southeastern Anatolian populations than the central/western populations (Appendix 1).

Two hundred forty ITS2 sequences with the size of 417 bp generated 28 distinct alleles, which contained 10 singleton and 7 private sequences. Twenty-five sites were polymorphic and parsimony informative, with 17 transitions and 8 transversions ($R= 33.602$). Nucleotide differences in pairwise comparisons ranged from 1 bp (0.2%) to 22 nucleotides (6%), where the highest difference was between the alleles representing western and eastern Anatolia. Geographically, the most widespread allele (A6, N= 66) was shared among 12 populations across Turkey. Similar to the cyt b, allelic variation was higher in the eastern/southeastern populations; nevertheless, some central/western populations also showed high allelic variation that was compatible with the eastern/southeastern populations (Appendix 2).

Average haplotype/allele (H_d/A_d) and nucleotide diversity (π) were $H_d= 0.662+/- 0.112$, and π$= 0.011+/- 0.006$, and $A_d= 0.614 +/- 0.095$ and π$= 0.006 +/- 0.003$, respectively (Table 1). After excluding the localities with sample sizes lower than 10, an eastern (Siirt) and a western (Denizli) population displayed the highest genetic diversity compared to other localities with similar sample sizes. Most of the eastern/southeastern Anatolian populations displayed higher cyt b diversity, yet relatively high genetic diversity for some central/western Anatolian populations was also well-marked. Allelic diversity was also remarkably high, where most eastern/southeastern populations exhibited higher diversity than the central/western populations. Nevertheless, genetic diversity estimates revealed in the central/western Anatolian populations were intermediate to comparatively high (Table 1).

Demographic history, phylogenetic analysis, and times of divergence of lineages

Signatures of historical population size changes for the species were estimated through Bayesian skyline plot analysis, which indicated a slow but steady decline at the species level throughout the late Pliocene and Pleistocene, followed by rapid population expansion in the recent past (Fig. 3). The mismatch distribution analysis for all samples produced multi/bimodal profile and multimodal profile for the cyt b and the ITS2 region, respectively (Fig. 4 a and b). Neutrality tests generated significant Fu's F_S values (Fu's $F_S=$ -24.652, p≤ 0.000, and Fu's $F_S= -21.488$, p≤ 0.000 for the cyt b and ITS2, respectively), which suggested sudden population expansion (Table 2). Individually analyzed populations using the ITS2 data showed that the Muş population from eastern Anatolia had significant negative Tajima's D (Tajima's $D= -1.838$, p≤ 0.05) with small Hri and SSD values suggesting a historical expansion event. On the other hand, the cyt b data indicated recent population expansion (Tajima's $D= -0.823$, p≤ 0.05, Fu's $F_S= -1.753$, p≤ 0.05, with small Hri and SSD values, and unimodal mismatch distribution profile) for the Kahramanmaraş population, which is located at the junction of eastern, southeastern, and southern Anatolia. Similarly, a western Anatolian population from Aydın generated negative and significant Tajima's D (Tajima's $D= -1.701$, p≤ 0.03), indicating the presence of more rare-nucleotide site variants (Table 2). No other analyses, including mismatch distribution graphs obtained for the cyt b gene, indicated rapid population expansion, except for the Kırıkkale and Edirne localities. In fact, historical, in addition to more recent and rapid population expansion, was suggested for the Edirne population. On the other hand, only historical expansion was implied for the Çanakkale, Elazığ, Kahramanmaraş, Manisa, and Uşak populations, whereas the rest of the populations produced multimodal profiles which indicated demographic equilibrium (Graphs were not presented here).

Population differentiation analysis revealed that Kahramanmaraş was the most differentiated from most other populations (Appendix 3). Specifically, pairwise comparisons of the ITS2 data indicated that Kahramanmaraş was the most differentiated population from the Uşak in western Anatolia ($F_{st}= 0.959$, p≤ 0.05). Moreover, some of the western populations differed significantly from the other western populations, where Balıkesir and Uşak ($F_{st}= 0.759$, p≤ 0.05 for the cyt data) and Balıkesir and Edirne ($F_{st}= 0.719$, p≤ 0.05 for the ITS2 data) were highly differentiated populations. These results were implicitly supported by the AMOVA analysis, where a significant amount of genetic variation was present at the within-population level. The cyt b revealed that 63% of the variation existed within populations, and as expected, population differentiation was low ($F_{st}= 0.37$, p≤ 0.05); however, according to the ITS2 results, 33% of the total variance was present within populations and populations were differentiated from each other significantly ($F_{st}= 0.67$, p≤ 0.05).

ML, BI, and Beast tree topologies were congruent with each other except for the bootstrap/ posterior probability values for different branches; therefore, only the Beast tree was presented here showing ML / BI support values on

related branches (Fig. 5). The molecular dating based on the cyt b gene estimated that after diverging from the outgroups around 7.8 million years ago (MYA), *A. tomentosus* split to two haplogroups (A and B clades) around 3.66 MYA. In clade A, a basal Batman haplotype (H16) from eastern Anatolia, forming a monophyly to the rest of the haplotypes, diverged from its sister group around 1.38 MYA. The sister clade is divided into two subclades, both dominated by the western Anatolian haplotypes; some eastern Anatolian haplotypes are scattered in both subclades. Clade B, with a more structured grouping, is dominated by haplotypes representing mostly eastern/southeastern Anatolian populations. A basal haplogroup, which contained haplotypes only from an eastern population in addition to three haplotypes found only in western Anatolia, diverged from its sister subclade around 2.46 MYA. Subsequent splits occurred consecutively around 1.84 MYA and 1.59 MYA, producing subclades comprising haplotypes from eastern/southeastern and central/western Anatolia. It is worth noting that haplotypes detected only in the eastern/southeastern Anatolian populations formed a separate group, which diverged around 910,000 years ago (YA). Further, a series of diversification events was apparent within the eastern/southeastern haplogroup dating back around 600,000 and 9,000 YA. Besides, the results of the phylogenetic analysis of the cyt b gene were overall supported by the generated minimum spanning network (Fig. 6a). In the network, the most frequent haplotype (H8), shared among 11 populations, of which four are from eastern Anatolia, is placed at the center of a star-like phylogeny. H8 is connected to a haplogroup representing the eastern/southeastern populations, corresponding to the clade B grouping in the phylogenetic tree. H8 is also connected to a larger haplogroup, showing three separate haplogroups, two connected to H8 with another eastern Anatolian haplotype. Several small star-like phylogeny formations are also apparent in this haplogroup, largely congruent with Clade A in the phylogenetic tree.

Phylogenetic analyses of the ITS2 alleles also generated similar topologies, where there were only some minor differences in the polytomic part of the tree (Fig. 7). Here, we presented only the ML tree with the BI posterior probability values on related branches, where all branches are strongly supported. In the tree, there are two major clades, where clade A is dominated by the central/western Anatolian alleles, and clade B is comprised of only eastern/southeastern alleles. The clade formation of the ITS2 data is, in most parts, in agreement with the results obtained from the cyt b data set. Moreover, the minimum spanning network of the ITS2 produced congruent results with the phylogenetic analysis supporting the formation of clade A and B of the trees (Fig. 6b). In the network, four star-like allele groupings are apparent: The most common allele (A6), shared among 12 populations across Turkey, gave rise to a group of alleles found only in western Anatolia. The second allele group, which has A5 (also shared mostly among western Anatolian localities) at its center, gave rise to a second western Anatolian and Thrace (European Turkey) allele cluster. The third allele grouping includes only eastern Anatolian alleles with A1 at its center and connected to the western Anatolian allele groups through A9. A1 allele is connected to A15 with one mutational step, and A15 is connected to A23 (from

Kahramanmaraş) through 16 hypothetical alleles. The fourth allele group, where A23 is placed at the center, is directly or indirectly connected to 8 other alleles, all found in eastern/southeastern Anatolia. Overall, the resulting ITS2 network seems to separate the eastern/southeastern Anatolian samples from the central/western and Thrace alleles.
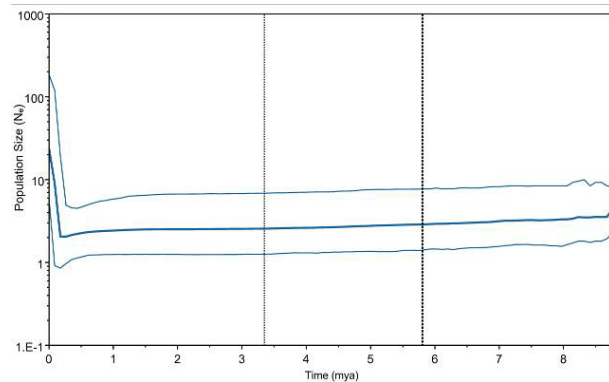
Figure 3. Bayesian skyline plot illustrating changes in effective population size over time for *A. tomentosus*. The y-axis represents the estimated population size, while the x-axis shows time in millions of years before the present. The dotted vertical lines indicate the 95% highest posterior density (HPD) interval, providing a measure of uncertainty in the population size estimates.
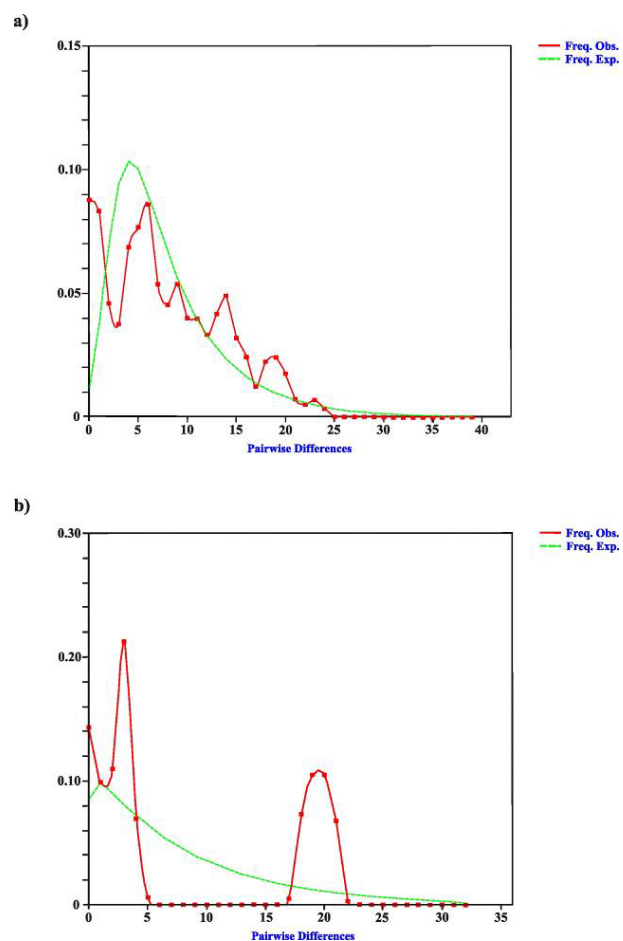
Figure 4. Mismatch distribution graphs including all populations of *A. tomentosus* for a) cyt b gene, and b) ITS2 region. The x-axis represents the number of pairwise differences, and the y-axis shows the frequency of the pairwise comparisons.

Table 2. Population demographic analysis of the cyt b gene and ITS2 region are shown in the columns. The first values indicate the cyt b gene, and the second refers to the ITS2. Hri: Harpending raggedness index, SSD: sum of squared deviation. –: could not be calculated for the population. * p≤0.005 and ** p≤0.001.

| Pop.	Cytb				ITS2			
	Tajima D	Fu F_S	Hri	SSD	Tajima D	Fu F_S	Hri	SSD
ADI	0.2819	6.2762	0.2175	0.0913	1.6500	7.3753	0.2461	0.7844
AYD	-1.7018*	1.8856	0.1619	0.0289	1.9996	3.8695	0.7649	0.2631
BAL	-0.0764	1.1585	0.1700	0.0999	-0.1909	-0.4448	0.0700	0.0181
BAT	1.5800	3.1406	0.1505	0.1283	1.3216	1.5419	0.0679	0.0848
BIT	2.1094	5.3842	0.2662	0.1395	1.0630	2.1263	0.6765	0.2331
BNG	0.0000	0.0000	0.0000	0.0000	-0.7801	0.1335	0.9722	0.2626
BOL	-1.3282	2.1100	0.4377	0.2944	1.9996	3.8695	0.7649	0.2631
CAN	0.5679	4.5375	0.5751	0.2265	1.3963	0.2853	0.0476	0.0022
DEN	1.5675	1.8278	0.0386	0.0229	1.1812	1.4104	0.5609	0.2154
EDI	0.2350	0.5967	0.2163	0.0034	0.2206	0.1055	0.2400	0.0373
ELA	2.5325	1.1771	0.1007	0.0817	0.2206	0.1055	0.2400	0.0373
KAH	-0.8232	-1.7538	0.1891	0.0272	-0.7239	-1.6487	0.2250	0.0315
KRK	0.0000	0.6932	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
MAN	0.1948	0.8745	0.4345	0.1740	0.1392	0.0502	0.1613	0.0171
MUS	-0.2395	0.4717	0.2197	0.3071	-1.8383*	2.9258	0.2572	0.0704
SII	1.0197	1.3612	0.1705	0.0612	1.8380	7.2670	0.3242	0.1838
TUN	-0.8073	-0.1280	0.2300	0.1185	-0.9726	1.0404	0.6800	0.3200
USK	0.0000	0.0000	0.0000	0.0000	-0.6410	-1.0431	0.1965	0.0262
YLV	1.6818	2.1579	0.2721	0.1112	2.1839	3.9884	0.7867	0.2720
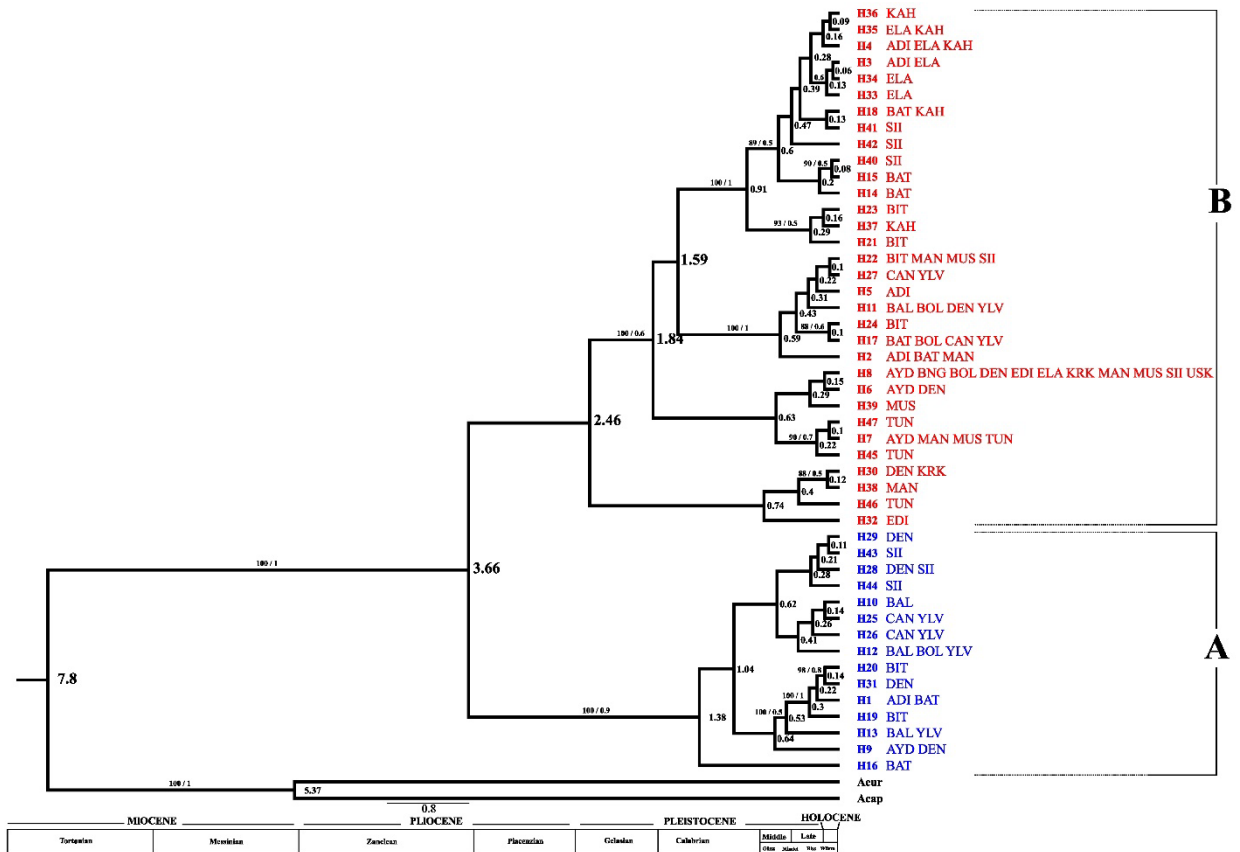
Figure 5. The resulting tree of Beast analysis of the cyt b gene haplotypes with the node ages (tMRCA) shown on the interior part of each related node. Bootstrap/posterior probability values are shown over the branches.

Discussion

Exploring genetic diversity and the geographic distribution of variation existing within species is one of the fundamental works in Biology. In *A. tomentosus*, genetic diversity is higher than the values reported for *A. chodjaii* (Mutun et al. 2019) and *Cynips quercus* (Mutun & Dinç 2019), but it is lower than *Trigonaspis synaspis* (Mutun & Atay 2015), *A. lucidus* (Mutun 2011), *C. quercusfolii* (Dinç & Mutun 2019), and *A. curtisii* (Mutun 2016). Excluding the populations with low sampling size, individual population diversity analysis disclosed that most of the populations harbored intermediate to relatively high variation, which was also strongly supported by the AMOVA analysis. Populations from eastern/southeastern Anatolia revealed high diversity in this study and have also been reported to stand out with their conspicuous level of genetic variation in other oak gall wasp species (Mutun 2010, Dinç & Mutun 2019). On one hand, even though we could not include any samples from other parts of the distribution range of the species other than Turkey and larger sampling is necessary to reach a general conclusion, the presence of higher diversity in the eastern/southeastern Anatolian populations may suggest the importance of the east for providing genetic source to the western populations, which has also been well-supported by other oak gall wasp taxa, where they indicated the importance of eastern localities as source to the European populations (Rokas et al. 2003, Challis et al. 2007). On the other hand, while the eastern/southeastern Anatolian populations showed higher diversity, the western localities cannot be underestimated since they possess relatively high variation, which were also disclosed by other oak gall wasp species (Mutun & Atay 2015, Mutun 2016). Thus, our current results further support the heterogeneous distribution of diversity across Turkey, which may suggest a refuge-within-refuge structure as suggested by other animal groups (Çıplak 2008).
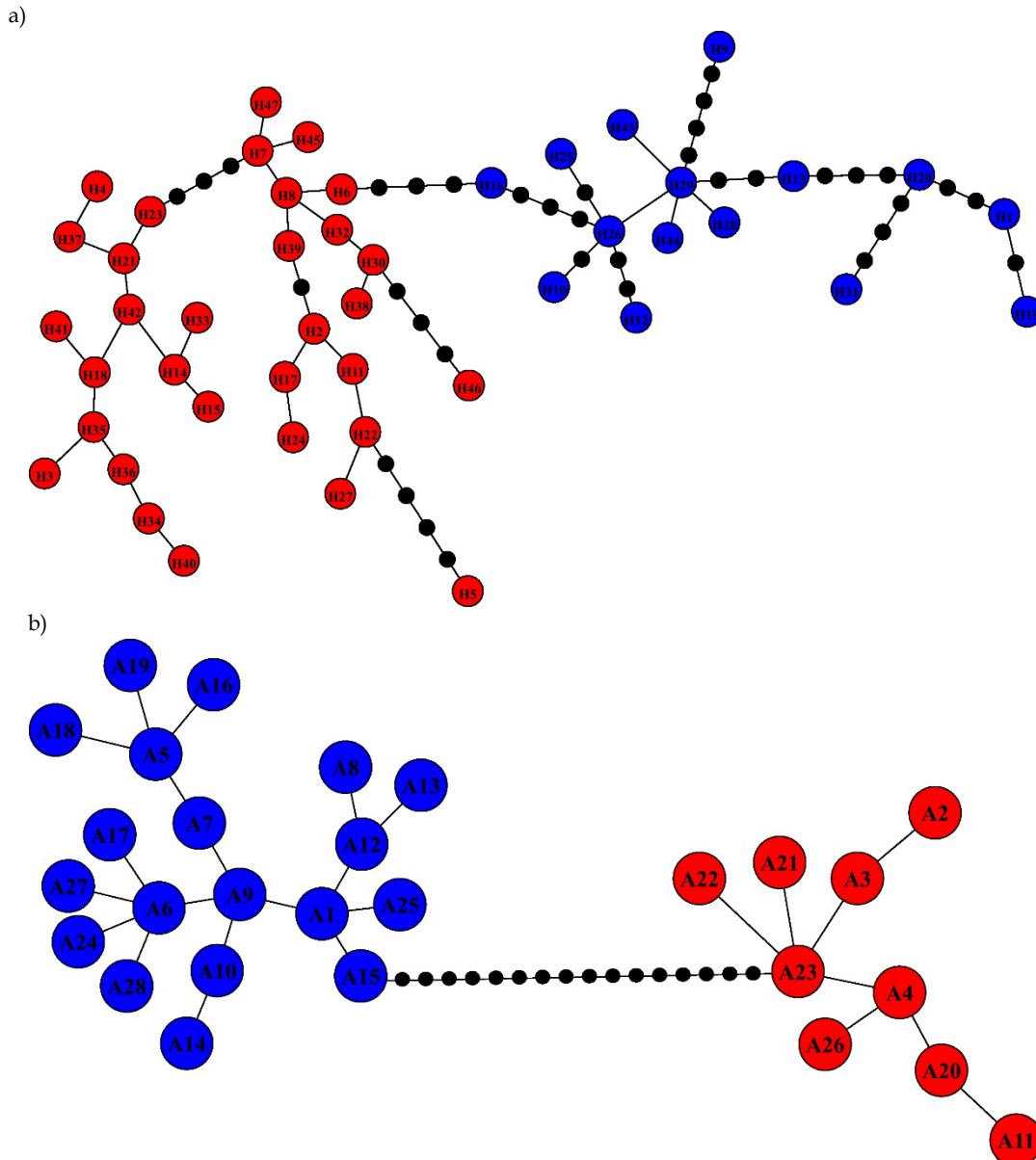

Figure 6. Haplotype network of *A. tomentosus* based on a) cyt b gene, and b) ITS2 sequences. Haplotype numbers are given inside the circles where the circle size is proportional to the frequency of the haplotypes. Hypothetical haplotypes/alleles are represented by small black circles.

It is a well-known statement that past events leave their signatures in the genetic structure of species, and population responses to historical factors can be exploited using demographic analyses (Avise 2004). In *A. tomentosus*, the multi/bimodal mismatch plot for the ITS2 data implies fluctuations in the more historical past, and multimodal shape for the cyt b data may be interpreted as an implication for demographic equilibrium with some changes for the species in the near past since the former is used to explain the more ancient history and the latter implies the relatively recent past events (Zhang & Hewitt 2003). In our analysis, the Bayesian skyline plot supported recent population expansion, implied by higher haplotype versus lower nucleotide diversity and the high number of singleton and private haplotypes/alleles. Neutrality analysis and mismatch distribution of individual populations showed that the Kahramanmaraş population stands out as an expanding population, which was proposed to be a biodiversity hotspot for oak gall wasps as for other animals (Çıplak 2003, Mutun 2010). Nonetheless, several other populations from eastern Anatolia also indicated an expanding population. On the other hand, from the western Anatolia Aydın and Edirne populations appear to be important for western expansion, where negative and significant Tajima's *D* suggests a bias toward rare site mutation haplotypes, which are indicators of rapid demographic expansion (Avise 2004).
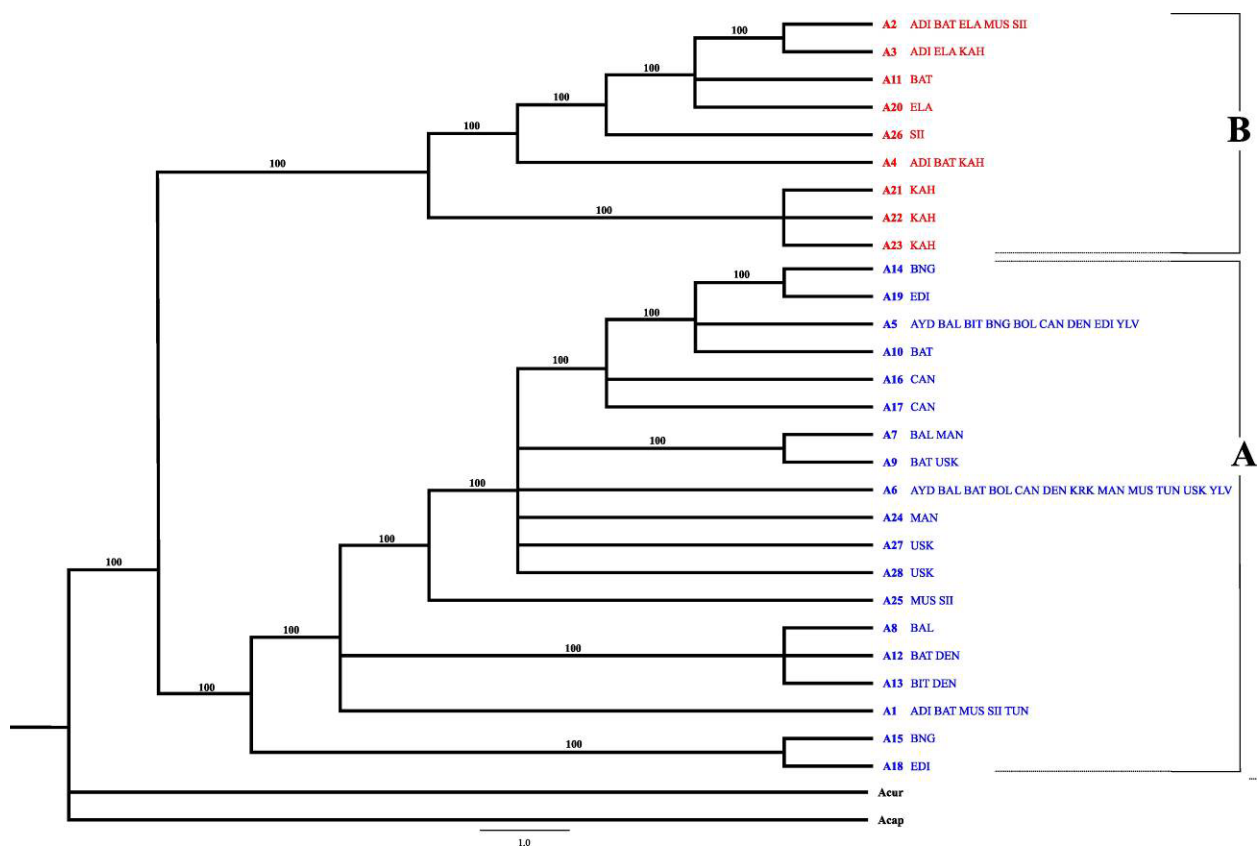

Figure 7. Bayesian consensus tree of ITS2 alleles. Posterior probability and bootstrap values are shown, respectively, on relevant branches.

A great number of studies revealed that the last 5 to 10 million years were crucial for the Turkish biodiversity, where paleogeologic and paleoenvironmental changes were dominating the area and resulting in either speciation or formation of the major lineages in organismal groups (Bilgin 2011, Şekercioğlu et al. 2011). In this study, the approximate times of divergence of *A. tomentosus* from its conspecifics is about 7.8 MYA near the late Miocene. It seems that oak gall wasps, including *A. tomentosus*, have been greatly affected by the ongoing events of the Late Miocene, the period of major geological and climatic changes around the Mediterranean Basin (Gillespie & Roderick 2014). Associated with geological changes, climatic fluctuations occurred around the transition time between Tortonian and Messinian, and during the latter, the climate was drastically changed from wet to more arid conditions (Fortelius et al. 2002). In our current study, the approximate time of divergence estimated for *A. tomentosus* fits well with these events and the split of other oak gall wasp genera, including *Cynips* (Dinç & Mutun 2019); thus, the congruency between the results of two oak gall wasp genera members using the same oak taxa reacted similarly may provide further prove for the importance of the period spanning the last 8 million years for the Turkish oak gall diversity.

After splitting from its conspecifics, lineage diversification within the ingroup species continued through the subsequent Pliocene epoch. Specifically, around 3,66 MYA in early Piacenzian, *A. tomentosus* was divided into two major lineages followed by pre-Pleistocene structuring within the species. A deep split around 1.84 MYA near the transition time between the Gelasian and Calabrian periods appears to cause the division of the western and the eastern lineages.

Similarly, aged splitting events resulted even in the speciation of an oak gall wasp species from the area (Mutun & Atay 2015), suggesting that the Pliocene epoch either initiated speciation or the formation of major lineages in oak gall wasps in Anatolia. Moreover, a series of intermediate and more recent splits between the lineages of the species appear to correlate with the cyclic changes that occurred throughout the Pleistocene. For instance, deep splits in Clade B dividing the eastern/southeastern Anatolian haplogroups from the western/central Anatolian haplotypes, and ~1.59 MYA and 1.04 MYA in Clade A sorting the haplogroups seem to be the major separation events during this period. More structuring also seems to take place during the subsequent events spanning from the middle to late Pleistocene. Further, relatively recent events during the oscillation of the last four glacial periods of Günz (790,000–950,000 years BP), Mindel (420,000 to 30,000 years BP), Riss (140,000 to 80,000 years BP), and Würm (70,000 to 11,700 years BP) appears to form more shallow diversification events in *A. tomentosus* clades. These findings generated congruent results from other animal and gall wasp species (Veith et al. 2003, Bohlen et al. 2006, Bellati et al. 2011, Mutun 2016, Çimen & Mutun 2022). Our results are also supported by pollen analysis of the host taxa from the genus *Quercus,* where Pleistocene cyclic changes have greatly affected the host species currently showing wide distribution in Turkey (Şenkul 2014). Congruent phylogenetic tree constructions and network analysis of the nuclear DNA data also supported lineage groupings for the species, where an eastern versus western grouping was obvious. It is now well-known that east versus west grouping of lineages have been well observed both in other gall wasp species (Rokas et al. 2003, Challis et al. 2007) as well as other animal and plant taxa (Ekim & Güner 1986, Çıplak 2004). This division was explained by the effects of some physical barriers extending from northeast to southwest Anatolia (Mutun 2010, Dinç & Mutun 2019). Overall, our results from mitochondrial and nuclear data with this study provide robust support to draw a general conclusion where oak gall wasps reacted mainly to environmental fluctuations and geography. It is highly likely that oak gall wasps, as obligatory parasites of oak species, react to the same factors correspondingly. However, more thorough studies are still necessary to draw the lines of details in this process both in the host and their obligate parasites.

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+ Appendix 1, Appendix 2, and Appendix 3

Appendix 1. Mitochondrial cyt b haplotypes and their frequencies.

	ADI	AYD	BAL	BAT	BIT	BNG	BOL	CAN	DEN	EDI	ELA	KAH	KRK	MAN	MUS	SII	TUN	USK	YLV	N$_{HAP}$
H1	3			8																11
H2	2			2											1					5
H3	4										4									8
H4	5										1	5								11
H5	1																			1
H6		2							1											3
H7		6												8	1		2			17
H8		6				4	2		2	12	6		1	1	11	1		15		61
H9		1							2											3
H10			1																	1
H11			1				1		1										1	4
H12			1				1												1	3
H13			2																2	4
H14				1																1
H15				1																1
H16				1																1
H17				1			11	4											2	18
H18				1								1								2
H19					6															6
H20					1															1
H21					1															1
H22					5										4	2	1			12
H23					1															1
H24					1															1
H25								1											1	2
H26								2											2	4
H27								7											6	13
H28									1							2				3
H29									2											2
H30									2				1							3
H31									4											4
H32										3										3
H33											1									1
H34											1									1
H35											2	7								9
H36												1								1
H37												1								1
H38														1						1
H39															1					1
H40																1				1
H41																2				2
H42																4				4
H43																2				2
H44																2				2
H45																	1			1
H46																	1			1
H47																	1			1
Total	15	15	5	15	15	4	15	14	15	15	15	15	2	15	15	15	5	15	15	240
N$_{HAP}$	5	4	4	7	6	1	4	4	8	2	6	5	2	5	4	8	4	1	7	

Appendix 2. ITS2 alleles and their frequencies detected in *A. tomentosus*

	ADI	AYD	BAL	BAT	BIT	BNG	BOL	CAN	DEN	EDI	ELA	KAH	KRK	MAN	MUS	SII	TUN	USK	YLV	N$_{ALLEL}$
A1	4			3	1										4	1	1			14
A2	2			2							7				1	1				13
A3	4										7	3								14
A4	5			1							1									7
A5		9	1		9	2	9	4	7	7									8	56
A6		6	2	2			6	4	6				2	9	9		4	9	7	66
A7			1											1						2
A8			1																	1
A9				1														2		3
A10				1																1
A11				1																1
A12				4					1											5
A13					5				1											6
A14						1														1
A15						1														1
A16								5												5
A17								1												1
A18										7										7
A19										1										1
A20												1								1
A21												3								3
A22												1								1
A23												7								7
A24															5					5
A25																1	4			5
A26																9				9
A27																		3		3
A28																		1		1
Total	15	15	5	15	15	4	15	14	15	15	15	15	2	15	15	15	5	15	15	240
N$_{ALL}$	4	2	4	8	3	3	2	4	4	3	3	5	1	3	4	4	2	4	2	

Appendix 3. Population F$_{ST}$ values for *A. tomentosus*. The lower diagonal shows pairwise comparisons of the cyt b, and the upper diagonal gives the F$_{ST}$ values of the ITS2 data. * represent statistical significance at p≤0.05.

	ADI	AYD	BAL	BAT	BIT	BNG	BOL	CAN	DEN	EDI	ELA	KAH	KRK	MAN	MUS	SII	TUN	USK	YLV
ADI		0.659*	0.550*	0.289*	0.650*	0.542*	0.659*	0.661*	0.641*	0.677*	0.223*	0.201*	0.523*	0.691*	0.574*	0.03	0.582*	0.695*	0.658*
AYD	0.294*		0.041	0.264*	0.215*	0.043	-0.071	0.009	-0.036	0.363*	0.943*	0.936*	0.318	0.358*	0.242*	0.645*	0.348*	0.444*	-0.062
BAL	0.272*	0.542*		0.08	0.192	0.067	0.041	0.154	-0.077	0.505*	0.941*	0.930*	-0.049	0.207*	0.003	0.517*	0.043	0.251*	0.002
BAT	0.168*	0.371*	0.040		0.265*	0.128	0.264*	0.292*	0.214*	0.344*	0.670*	0.667*	0.008	0.307*	0.096	0.236*	0.12	0.290*	0.254*
BIT	0.182*	0.291*	0.041	0.023		-0.01	0.215*	0.339*	0.149*	0.153*	0.939*	0.931*	0.575*	0.599*	0.392*	0.655*	0.557*	0.653*	0.252*
BNG	0.187	-0.034	0.513*	0.295	0.221		0.043	0.215	0.026	0.065	0.951*	0.940*	0.529	0.635*	0.265*	0.545*	0.539*	0.666*	0.093
BOL	0.387*	0.416*	0.607*	0.450*	0.307*	0.497*		0.009	-0.036	0.363*	0.943*	0.936*	0.318	0.358*	0.242*	0.645*	0.348*	0.444*	-0.062
CAN	0.334*	0.392*	0.393*	0.340*	0.192*	0.397*	0.123		0.048	0.464*	0.948*	0.942*	0.334	0.355*	0.272*	0.642*	0.393*	0.470*	0.002
DEN	0.263*	0.365*	0.004	0.069	0.078	0.304*	0.479*	0.354*		0.339*	0.929*	0.923*	0.153	0.254*	0.154*	0.621*	0.199	0.335*	-0.044
EDI	0.353*	0.110*	0.720*	0.451*	0.382*	-0.017	0.617*	0.547*	0.454*		0.963*	0.957*	0.830*	0.786*	0.528*	0.690*	0.790*	0.810*	0.414*
ELA	0.043	0.423*	0.578*	0.407*	0.390*	0.360	0.582*	0.536*	0.462*	0.511*		0.512*	0.970*	0.968*	0.891*	0.425*	0.965*	0.966*	0.942*
KAH	0.178*	0.780*	0.808*	0.572*	0.553*	0.886*	0.824*	0.745*	0.629*	0.905*	0.256*		0.957*	0.960*	0.883*	0.410*	0.954*	0.959*	0.935*
KRK	0.061	-0.029	0.253	0.138	0.060	0.385	0.449	0.247	0.115	0.318	0.313*	0.863*		-0.011	-0.208	0.445*	-0.29	-0.236	0.224
MAN	0.338*	0.146*	0.589*	0.426*	0.294*	0.252*	0.172*	0.221*	0.442*	0.399*	0.494*	0.798*	0.216		0.143*	0.649*	0.157	0.192*	0.284*
MUS	0.323*	0.035	0.645*	0.430*	0.337*	-0.047	0.414*	0.409*	0.441*	0.114*	0.463*	0.837*	0.129	0.150*		0.508*	-0.092	0.083*	0.197*
SII	0.058	0.259*	0.174	0.155*	0.154*	0.184	0.420*	0.326*	0.160*	0.339*	0.194*	0.404*	0.011	0.346*	0.324*		0.519*	0.643*	0.638*
TUN	0.206*	0.058	0.439*	0.280*	0.181	0.237*	0.401*	0.296*	0.283*	0.458*	0.398*	0.810*	0.015	0.125	0.245*	0.181*		-0.014	0.272
USK	0.364*	0.122*	0.760*	0.469*	0.398*	0.000	0.645*	0.568*	0.478*	0.143	0.528*	0.929*	0.776	0.429*	0.107	0.361*	0.554*		0.375*
YLV	0.257*	0.297*	0.131	0.192*	0.078	0.265*	0.204*	0.000	0.189*	0.420*	0.458*	0.658*	0.068	0.228*	0.329*	0.202*	0.208*	0.441*	