

## PALEOENVIRONMENT PLAYED KEY ROLE FOR THE ANATOLIAN POPULATIONS OF *CYNIPS DIVISA* (HYMENOPTERA: CYNIPIDAE)

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*(Received 31<sup>st</sup> March 2021; accepted 18<sup>th</sup> May 2021)*

**ABSTRACT.** Red-pea gall wasp, *Cynips divisa*, is an oak-dependent species with a wide distribution range in the Western Palearctic. In this study, we aimed to investigate the genetic diversity of the species across Anatolia and reveal possible factors that governed its contemporary phylogeographic pattern. For this purpose, we sequenced 433 base pairs of mitochondrial cytochrome b gene and the entire nuclear ITS2 region of 278 individuals collected from 22 localities. Our sequence data generated 115 cyt b haplotypes and 15 ITS2 alleles. Estimated genetic diversity for the species was well within the limits of other gall wasp species. Phylogenetic analysis pointed to a separation of *C. divisa* from outgroups around the Pliocene. Diversification estimates of main haplogroups show signals of major lineage divergences through the Quaternary period. Moreover, splits resulted in more shallow structuring during the last 780,000 years appear to play a key role in the geographic distribution of genetic diversity of red-pea gall wasp species in Anatolia. Our current results support the general view that phylogeography of the Anatolian cynipids has been mainly shaped in a period spanning the last few million years due mostly to changing paleoenvironmental conditions of the area.

**Keywords:** *Red-pea gall wasp, cyt b, ITS2, phylogeography, Turkey*

### INTRODUCTION

In the last few decades, paleoenvironmental changes and paleogeography have been well documented as key factors for either triggering speciation events or creating isolated populations with distinct genetic lineages within species [1]. In particular, a time period spanning the last few million years has been proven to shape the Palearctic fauna and flora [2]. Located in the Western Palearctic, Anatolia is known to play a significant role as a shelter and a corridor route between Asian and European diversity during the glacial periods of Quaternary [3]. As one of the refugia after the Balkans, Italy, Iberia, and North Africa, the Turkish refugium in the Western Palearctic allowed temperate species to survive the harsh environmental conditions of Pliocene and Pleistocene [4]. Moreover, studies conducted on both plants and animals have suggested that Anatolia had significant effects on many of the European taxa particularly for oak gall wasps through acting as a source of genetic variation [5].

Oak gall wasps (Hymenoptera: Cynipidae) with nearly 1,400 described species are the most known and well-studied gall inducer insect group distributed across the Holarctic [6]. Anatolia is considered as one of the most important hotspots for the biodiversity of

oak gall wasps and houses 150 species classified under 25 genera [6]. The red-pea gall wasp species, *Cynips divisa*, is a heteroecious and heterogonic oak gall wasp species with a sexual and an asexual generation. Sexual generation galls are pubescent with conical shape with hairy surface, and located on the margins of leaves, sometimes occurring on catkins. Sexual generation galls develop in the spring; they are initially green, and later in the development, they turn into reddish coloration. However, monolocular globular asexual generation galls located underside of leaves of *Quercus petraea*, *Q. infectoria* and other white oak species appear in late summer [7]. In this study, only adults reared from asexual generation galls were used to obtain sequence data of partial mtDNA cytochrome b gene (cyt b) and complete nuclear ITS2 region. Major aims of this study are i) to disclose genetic diversity and its geographical distribution across the sampled area, and ii) to reveal possible factors that might have been responsible for the contemporary phylogeographic structure in the Anatolian populations of the species.

## MATERIALS AND METHODS

A total of 278 asexual generation galls of *C. divisa* were collected from 22 localities during the late summer and early fall season between 2010-2017 (Table 1). Total genomic DNA was extracted from adults using the DNeasy Tissue Kit (Qiagen) following the manufacturer's instructions. A 433-base pair (bp) of mitochondrial cyt b gene was amplified using CB1- 5'- TATGTACTACCATGAGGACAAATATC- 3' and CB2- 5'- ATTACACCTCCTAATTTATTAGGAAT- 3' primers [8] following the protocols used for oak gall wasps [4]. The nuclear ITS2 region was amplified using MutITSintF 5'- GTTCGTCGCGTCTCTG-3' and MutITSintR 5'-CCGTCCATAA TGGCCAC-3' primers [9]. Amplification reactions were carried out in a volume of 35  $\mu$ l volumes containing 10  $\mu$ l 5X buffer, 5  $\mu$ l MgCl<sub>2</sub> (25 mM), 3  $\mu$ l dNTPs (10 mM), 1  $\mu$ l of each primer (20 pmol), 0.5  $\mu$ l template DNA, 0.5  $\mu$ l Taq polymerase (Vivantis) and 15  $\mu$ l ddH<sub>2</sub>O. Amplification conditions were 5 min at 95 °C for the first denaturation; 35 cycles of 30 seconds at 94 °C for denaturation, 30 seconds at 52°C for annealing, 30 seconds at 72 °C for elongation, and 5 minutes at 72 °C for the last elongation step. Both mtDNA and nDNA amplicons were sent to a company (MACROGEN, South Korea) for sequencing both strands.

All cyt b and ITS2 sequences were aligned, and haplotypes/alleles were determined using GenAIEx 6.5 [10]. Mitochondrial DNA sequences were checked for the presence of numts (nuclear mitochondrial DNA sequences) to ensure that there are no stop codons nor non-sense mutations after translating the cyt b sequences into amino acids using DnaSP 5.10.1 program [11]. Number of polymorphic sites (S), nucleotide ( $\pi$ ) and haplotype (h) diversity [12], number of substitutions and pairwise nucleotide differences (k) [13] were estimated separately both for the cyt b gene and the ITS2 region. To disclose the signs of historical events, we conducted population demographic analysis through mismatch distribution analysis as implemented in DnaSP 5.10.1 [11]. The raggedness index (Hri) [14], the sum of squared deviations (SSD), Tajima's *D* [15], and Fu's *F<sub>s</sub>* [16, 17] were calculated to determine neutrality and the smoothness of the pairwise mismatch plot.

Haplotype and allele data sets were analysed separately for revealing phylogenetic relationships of sequences. *Cynips quercus* Fourcroy (1785) and *Cynips longiventris* Hartig (1840) were used as outgroups in all phylogenetic analysis (GenBank Accession number for *C. quercus*: MH361234-MH361235 and MH361286, and DQ218010-

AF539582 and MN853888 for *Cynips longiventris* for the cyt b haplotypes and the ITS2 alleles, respectively). We conducted a Maximum Likelihood (ML) and a Bayesian analyses using PAUP\*4.0b10 [18] and MrBayes 3.2.6 programs, respectively [19]. For the ML tree, Akaike information criterion (AICc) was used to determine the best-fit substitution model using JModeltest 2 [20] and the analysis suggested GTR+I+G as the substitution model for both data sets. We estimated the geographic separation of the major *C. divisa* cyt b genetic lineages and their confidence intervals using a Bayesian Markov Chain Monte Carlo (MCMC) coalescent method implemented in BEAST ver. 1.5.2 following the uncorrelated relaxed lognormal clock [21]. A 1.19% sequence divergence per lineage (2.39% pairwise) per million years mutation rate was used for the calibration of the molecular clock [22]. BEAUTI v1.8.0 was used for operator optimizations [23]. BEAST analysis was run for 100 million generations sampling every 1000 and convergence was examined using the program Tracer v1.6.0. We built the maximum clade credibility tree using TreeAnnotator v1.8.4. after discarding the initial 25% samples as burn-in. We employed FigTree ver. 1.3.1 [24] to visualize the resulting chronograms. Since phylogenetic analyses may not be adequate for revealing genealogical relationships at the population level [25], a haplotype network was employed to *C. divisa* data sets. For this purpose, HapStar Version 0.5 (C) [26] was used to draw networks of haplotypes and alleles.

## RESULTS AND DISCUSSION

### *Genetic diversity estimates and demographic dynamics of Cynips divisa*

In the 433 bp cyt b sequences of *C. divisa*, there was only 45 parsimony informative sites, which yielded 115 cyt b haplotypes (GenBank Accession numbers: MH230991-MH231105). In sequences, there were neither stop codons nor non-sense mutations, and A+T nucleotides (76.64%) were higher than G+C nucleotides (23.35%). The absence of non-sense/stop codons and the presence of anti-G bias suggested that the sequences were genuine mitochondrial DNA in their origin [27]. Pairwise comparisons showed that sequence differences between cyt haplotypes ranged from 0.2% (1 base) to 7.3% (28 bases). On the other hand, a 395 base pairs (bp) ITS2 sequences of 278 specimens generated 15 distinct alleles (GenBank Accession No: MH231106- MH231120). There were 16 polymorphic sites, of which 3 were parsimony informative. Nucleotide frequencies of ITS2 alleles were 26.08%, 31.03%, 18.88%, and 24.02% for A, T, C, and G, respectively. Sequence variation ranged from 1 base (0.4%) to 7 bases (2.9%). The number of singleton haplotypes and alleles were 84 (73% of haplotypes) and 11 (73% of alleles), respectively. Eleven percent of the cyt b haplotypes (13 out of 115 haplotypes) were private sequences. Similar findings considering a high number of singletons and private sequences were reported from other gall wasp species from Turkey indicating recency of those rare sequences [28].

Haplotype and nucleotide diversity are good estimators of the contemporary genetic variation of a species [12]. In *C. divisa*, both data sets produced high haplotype versus low nucleotide diversity with a mean gene diversity of  $\sim 0.818$  and  $0.316$ , and the mean nucleotide diversity of  $\sim 0.011$  and  $0.001$  for cyt b and ITS2, respectively. Most of the *C. divisa* populations displayed high genetic diversity estimates where the highest haplotype diversity was in Siirt ( $h = 1.000 \pm 0.126$ ). Except for İzmir and Bolu, all other remaining localities displayed a substantial amount of genetic diversity estimates. Accordingly,

nucleotide diversity was striking for many *C. divisa* populations where the highest nucleotide diversity was in one of the eastern localities (Bingöl,  $\pi = 0.024$ ) (Table 1). Likewise, average allele ( $A_d$ ) and nucleotide diversity for the ITS2 region were  $A_d = 0.316$  and  $\pi = 0.001$ , respectively. Most populations demonstrated high allelic diversity where Malatya was with the highest value ( $h = 0.800 \pm 0.172$ ) followed by Elazığ ( $h = 0.743 \pm 0.086$ ) and Bingöl ( $h = 0.704 \pm 0.113$ ). Likewise, nucleotide diversity was highest in Bingöl ( $\pi = 0.007 \pm 0.005$ ), and Elazığ ( $\pi = 0.004 \pm 0.003$ ). While mean genetic diversity estimates are, on one hand, well-within the limits of other oak gall wasp taxa, on the other hand, estimates for those populations with genetic diversity estimates greater than 0.500 are highly similar to those of other oak gall wasp species studied from Turkey [9]. High genetic diversity was not only reported for oak gall wasps but also some other animal and plant species from Turkey and was interpreted as a result of factors such as the unique location of Turkey at the junction of three phylogeographic regions, recurrent fluctuations between the glacial and interglacial cycles of Quaternary, and variation in topography [29]. Our current findings in *C. divisa* regarding high haplotype versus low nucleotide diversity and a high number of singleton and private sequences is often interpreted as a sign of population expansion after population declines in the past [19].

In *C. divisa*, mismatch analyses using all pairwise combinations of both cyt b (Fig. 1a) and ITS2 data (Fig. 1b) sign multimodal profile with several picks. The cyt b data with multimodal profiles for almost all populations indicated structured populations (mismatch graphs of individually analysed populations are not shown here). Among all populations, it appears that Bingöl is the most structured population. This result is also supported by large SSD and negative, but non-significant Tajima's  $D$  and Fu's  $F_s$  values (Table 1). In addition, some other populations such as Elazığ, Cankiri, Isparta, Malatya, Kırşehir, Siirt, Uşak, and Çorum show significant negative values suggesting population expansion. However, some differences between Tajima's  $D$  and Fu's  $F_s$  of few populations may be due to either different resolving power of both tests, or as an alternative severe and concurrent fluctuations in those populations over time might have had occurred [30]. Overall historical demography assessment of *C. divisa* populations proposes severe recurrent fluctuations resulted in the structured population demographies for the species. In fact, population size changes as a response to oscillations in the environment have long been known to affect the geographic distribution of genetic diversity [2], and common for the Anatolian species [31].

On the other hand, the ITS2 graph with two shallow and one drastic pick can be interpreted as more ancient, but a non-drastic population decline and subsequent expansion, followed by severe population decline near the more recent time. While the ITS2 data implied population expansion for all populations except for Bingöl where it showed a multimodal profile indicating equilibrium (graph not shown here). It was also supported by its relatively high SSD value suggesting a presently non-expanding population structure (Table 1). All other populations of *C. divisa* showing unimodal profile (graph not shown here) are also supported by low SSD and Hri indices. Mostly negative Tajima's  $D$  and Fu's  $F_s$  values may also support these findings even though some of the values were not statistically significant. Discrepancies between Tajima's  $D$  and Fu's  $F_s$  may be explained by Hri and SSD being more sensitive indicators as compared to Tajima's  $D$  and Fu's  $F_s$  [30]. As a general pattern unimodal pattern in mismatch distribution analysis, significant negative Tajima's  $D$  and Fu's  $F_s$ , smaller SSD and Hri values indicate population expansion, however multimodal graph, significant

positive Tajima's  $D$  and Fu's  $F_s$ , larger SSD and Hri imply either declining or non-expanding population [32]. It seems that both data sets used in *C. divisa* provided resolution for different time zones as would be expected since the ITS2 region provides information for the more ancient events and the cyt b for the more recent demographic processes.

### ***Phylogenetic analysis: history left its footprint***

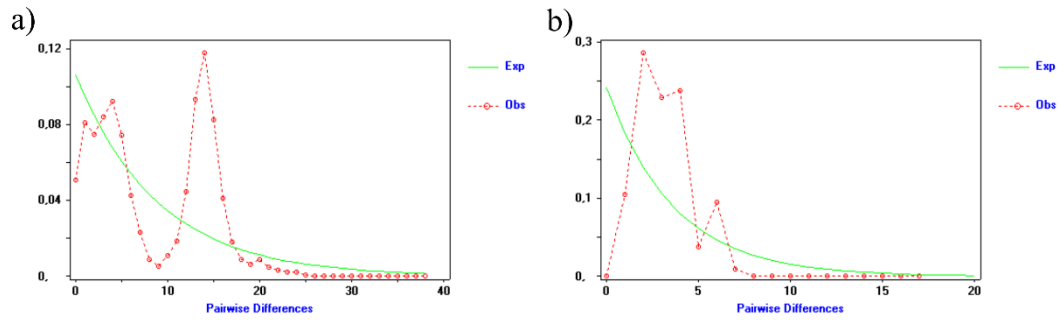
ML analysis of the cyt b haplotypes produced a consensus tree with the same tree topology generated by the Beast analysis, therefore only the latter is provided here (Fig. 2). The footmarks of the historical events particularly climatic and environmental fluctuations are observed in the current structuring of populations [1]. Responses given by the species/populations/lineages as contraction and expansions result in the more and either deep or shallow structured phylogenies [32]. In *C. divisa*, the first separation of the species from its congeners in the middle Pliocene around 3.34 MYA is thought as a period of drastic environmental fluctuations [2]. In *C. divisa* there are two clades where Clade A is dominated by the haplotypes mainly from eastern populations, and the Clade B is dominated by central and western haplotypes. Such groupings revealed in *C. divisa* produced compatible results with other gall wasps species from Anatolia [33].

**Table 1.** Sampling localities, coordinates, sampling size, number of haplotypes/alleles, genetic diversity indices ( $h$ : haplotype diversity,  $\pi$ : nucleotide diversity,  $Ad$ : allele diversity) and neutrality tests of *C. divisa*. \*:  $P \leq 0,005$ , \*\*:  $P \leq 0,001$ .

Population	Abb.	Coord.	Size	$N_{HAP/ALLEL}$	CYT B ( $h/\pi$ )	ITS2 ( $Ad/\pi$ )	Hri <sub>(cytb/ITS2)</sub>	SSD <sub>(cytb/ITS2)</sub>	Tajima's $D$ (cytb/ITS2)	Fu's $F_s$ (cytb/ITS2)
AFYON	AFY	N 38° 79' E 30° 05'	13	9 / 2	0.8718 +/- 0.0913 0.0113 +/- 0.0066	0.1538 +/- 0.1261 0.0006 +/- 0.0010	0.13445 0.50296	0.07019 0.02811	-131.594 -1.14915	-181.538 -0.53714
AYDIN	AYD	N 37° 56' E 28° 35'	13	9 / 2	0.9359 +/- 0.0507 0.0093 +/- 0.0056	0.3846 +/- 0.1321 0.0015 +/- 0.0017	0.05177 0.20118	0.01729 0.00596	-0.42324 0.42560	-244.396 0.68913
BALIKESIR	BAL	N 40° 17' E 28° 08'	14	10 / 3	0.9451 +/- 0.0451 0.0196 +/- 0.0108	0.2747 +/- 0.1484 0.0016 +/- 0.0017	0.04190 0.35672	0.03633 0.00913	-0.35012 -0.95919	-0.89417 -0.85452
BINGOL	BIN	N 38° 92' E 40° 36'	15	11 / 6	0.9333 +/- 0.0538 0.0242 +/- 0.4725	0.7048 +/- 0.1139 0.0075 +/- 0.0051	0.05070 0.03673	0.89016 0.40243*	-0.43070 -1.19511	-0.79783 -0.97962
BOLU	BOL	N 40° 57' E 35° 48'	14	3 / 3	0.4725 +/- 0.1358 0.0018 +/- 0.0015	0.4725 +/- 0.1358 0.0023 +/- 0.0022	0.16688 0.10313	0.01921 0.00088	-122.200 -0.20057	0.37544 -0.20707
CANAKKALE	CAN	N 39° 58' E 26° 52'	15	8 / 1	0.7905 +/- 0.1049 0.0085 +/- 0.0051	0.0000 +/- 0.0000 0.0000 +/- 0.0000	0.08435 0.00000	0.03185 0.00000	-0.79437 0.00000	-105.590 0.00000
CANKIRI	CNK	N 40° 54' E 29° 27'	15	12 / 1	0.9619 +/- 0.0399 0.0081 +/- 0.0049	0.0000 +/- 0.0000 0.0000 +/- 0.0000	0.02775 0.00000	0.00416 0.00000	-0.70224 0.00000	-6.64000** 0.00000
CORUM	COR	N 40° 20' E 35° 21'	14	10 / 2	0.8901 +/- 0.0807 0.0087 +/- 0.0052	0.1429 +/- 0.1188 0.0006 +/- 0.0009	0.09781 0.53061	0.02830 0.00009	-1.66756 -1.15524	-3.58416 0.59478
DENIZLI	DEN	N 38° 04' E 28° 78'	13	8 / 2	0.8077 +/- 0.1131 0.0068 +/- 0.0042	0.1538 +/- 0.1261 0.0006 +/- 0.0010	0.04815 0.50296	0.01513 0.02811	-143.731 -1.14915	-227.640 -0.53714
ELAZIG	ELA	N 38° 53' E 38° 75'	13	6 / 5	0.7179 +/- 0.1279 0.0079 +/- 0.0048	0.7436 +/- 0.0866 0.0046 +/- 0.0036	0.07133 0.11834	0.01553 0.01020	-1.99717* -1.01207	0.26359 -1.39819
ESKISEHIR	ESK	N 39° 64' E 31° 52'	5	4 / 1	0.9000 +/- 0.1610 0.0097 +/- 0.0067	0.0000 +/- 0.0000 0.0000 +/- 0.0000	0.43000 0.43802	0.15852 0.01756	0.66055 0.00000	0.21161 0.00000
GIRESUN	GIR	N 40° 28' E 38° 81'	11	9 / 2	0.9636 +/- 0.0510 0.0198 +/- 0.0112	0.1818 +/- 0.1436 0.0007 +/- 0.0011	0.07272 0.43802	0.07970 0.01756	116.343 -1.12850	-125.283 -0.40988
ISPARTA	ISP	N 37° 74' E 31° 18'	7	3 / 2	0.5238 +/- 0.2086 0.0096 +/- 0.0062	0.4762 +/- 0.1713 0.0019 +/- 0.0021	0.20861 0.22902	0.07915 0.01718	-1.46766 0.55902	303.724 0.58867
IZMIR	IZM	N 38° 05' E 28° 11'	5	2 / 2	0.4000 +/- 0.2373 0.0046 +/- 0.0036	0.6000 +/- 0.1753 0.0024 +/- 0.0026	0.68000 0.40000	0.21713 0.05428	-112.397 1.22474	263.906 0.62615

**Table 1. (Continued).**

Population	Abb.	Coord.	Size	N <sub>HAP/ALLEL</sub>	CYT B (h/π)	ITS2 (A <sub>d</sub> /π)	Hri <sub>(cytb/ITS2)</sub>	SSD <sub>(cytb/ITS2)</sub>	Tajima's D <sub>(cytb/ITS2)</sub>	Fu's F <sub>s</sub> <sub>(cytb/ITS2)</sub>
KAYSERI	KAY	N 38° 58' E 36° 42'	20	9 / 3	0.9053 +/-0.0351 0.0179 +/-0.0097	0.4684 +/-0.1045 0.0021 +/-0.0021	0.04033 0.12723	0.03792 0.00447	200.602 -0.08998	142.862 -0.06013
KIRSEHIR	KIR	N 39° 47' E 33° 87'	16	8 / 2	0.8000 +/-0.0916 0.0058 +/-0.0037	0.3250 +/-0.1251 0.0013 +/-0.0015	0.03263 0.22813	0.32960* 0.00265	-136.280 0.15575	-203.103 0.55122
KONYA	KON	N 37° 54' E 31° 46'	20	13 / 2	0.8842 +/-0.0666 0.0168 +/-0.0091	0.1000 +/-0.0880 0.0004 +/-0.0008	0.69931 0.65000	0.01772 0.00006	-0.53832 -1.16439	-208.758 -0.87930
MALATYA	MAL	N 38° 99' E 37° 81'	6	5 / 3	0.9333 +/-0.1217 0.0173 +/-0.0109	0.8000 +/-0.1721 0.0040 +/-0.0036	0.06666 0.36000	0.04717 0.06645	-1.36789 -1.23311	0.34704 -1.81280*
SIIRT	SII	N 38° 12' E 41° 67'	5	5 / 1	1.0000 +/-0.1265 0.0078 +/-0.0056	0.0000 +/-0.0000 0.0000 +/-0.0000	0.16000 0.00000	0.06432 0.00000	0.08298 0.00000	-2.00426 0.00000
SIVAS	SIV	N 39° 93' E 37° 84'	14	7 / 3	0.8242 +/-0.0781 0.0193 +/-0.0107	0.3846 +/-0.1494 0.0020 +/-0.0020	0.07462 0.15868	0.05696 0.00072	139.288 -0.53247	207.179 -0.46544
TOKAT	TOK	N 40° 44' E 37° 31'	15	10 / 2	0.9333 +/-0.0449 0.0177 +/-0.0098	0.3429 +/-0.1278 0.0013 +/-0.0016	0.04126 0.21633	0.04508 0.00342	0.77525 0.23502	-0.76545 0.59667
USAK	USK	N 38° 53' E 29° 66'	15	11 / 2	0.9524 +/-0.0403 0.0099 +/-0.0058	0.2476 +/-0.1307 0.0009 +/-0.0013	0.04780 0.31601	0.02268 0.28138	-0.50288 -0.39883	-3.96493 0.13336

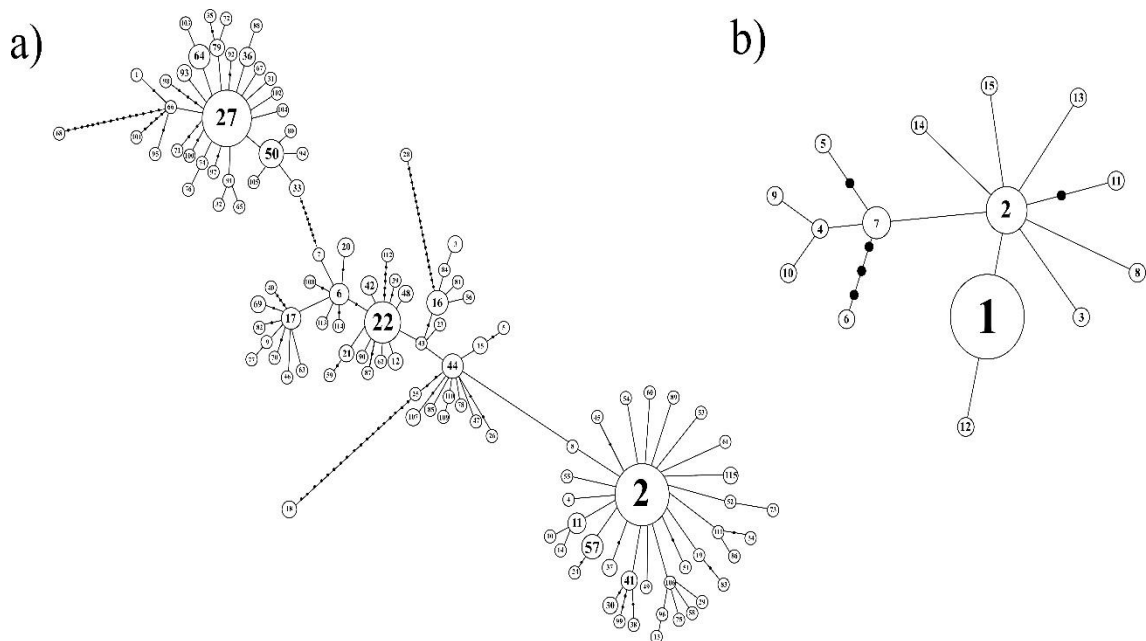


**Fig. 1.** Mismatch distribution of all pairwise combinations for a) the *cytb* haplotypes. b) the *ITS2* alleles. The observed distribution represented by red dotted line, and the expected frequencies depicted by green line.

Main clade and subclade separation predated the Pleistocene period. The estimated age of the basal haplotypes ranging from 2.99 MY to 2.38 million years (MY) indicate the end of the Pliocene and early Pleistocene. Likewise, the A and B clades separation nearly 1.79 MYA corresponds to the early Pleistocene. In recent years, a great deal of evidence has accumulated regarding the effects of the Pleistocene fluctuations shaping both geographic distributions of species and as well as genetic diversity and lineages [2]. It seems that the ongoing series of glacial and interglacial cycles have also left their effects on the structured phylogeny of the *C. divisa* lineages. Although Turkey as a place that was never covered by large ice sheets, where only mountain tops were with enlarged glaciers, and temperature drop was pronounced, and many species either shifted their range or contracted their populations [33]. Continuing oscillations during the last few glacial periods also affected greatly the vegetation of Turkey [34]. A recent study showed that vegetation including oak host taxa together with climate in Anatolia and adjacent regions oscillated concurrently during the last glacial periods [35]. Analysis on *C. divisa* denotes that lineages were shaped thoroughly from the beginning to the end of the

Pleistocene. Divergence of Clade A and B occurred around 1.50 MYA ended up with a separation of some eastern and western lineages creating at the same time some haplogroup formations. Splitting between the lineages of *C. divisa* continued through the more recent history of the species in Anatolia particularly during the Mid-Pleistocene. But the dominating cycles resulting in the more recent structuring were during the last four glacial times. These diversification estimates are also concordant with the previous findings of other species [5, 26, 9].

Minimum spanning network for better resolving evolutionary relationships among haplotypes and visualize possible reticulations was conducted, and the result was shown in Fig. 2a. The network provided similar structuring with phylogenetic trees. There are several main haplogroups forming star like phylogeny in the network where three major groupings are apparent each with common haplotypes at its centre. The first group includes H27 at the centre, which is a shared and common haplotype found in 9 localities mainly from the eastern part of the sampling area. Moreover, 17 haplotypes directly and 15 haplotypes indirectly connected to H27 at this haplogroup. When evaluated altogether they are dominated by the haplotypes from the easterly located populations.



**Fig. 2.** Minimum spanning tree of a) the *cyt b* haplotypes and b) the *ITS2* alleles of *C. divisa*. Circle size is proportional to the frequency of alleles across the sampling area. ● indicates hypothetical haplotypes/alleles.

The first haplogroup relates with the second via H33 from Bingöl. More structured appearance is obvious in the second haplogroup, where there are several small star-like phylogenies. One of the most striking features of this haplogroup is the presence of haplotypes from the western and central part of Turkey that dominate the entire group.

The second haplogroup is related with the third haplogroup through H44 and H8, and then to the most frequent haplotype H2 (found in 14 different localities). Except for Bingöl, Sivas, Tokat, and Kayseri, H2 was detected in all central and western localities.

Overall, it is also apparent that the third haplogroup is governed by the west and central haplotypes.

Maximum likelihood analysis for the ITS2 alleles did not provide high resolution where a large polytomy was present. The only large clade was formed by the alleles representing only the eastern localities (Bingöl, Elazığ, Malatya, and Siirt), and all other alleles were placed in the polytomic part, and no relationship would be resolved, therefore it was not presented here. The time frame unveiled by the ITS2 falls into more ancient times, and more recent times can be resolved by the *cyt b* data. Some differences between the results of the two markers in the case of *C. divisa* may be due possibly to different inheritance modes, mutation rates, and selection pressure of the two marker genes [27]. Minimum spanning tree of the ITS2 alleles is shown in Fig. 2b. The most common allele (A1), shared among 20 populations out of 22 localities, is connected directly to A2 which is also a shared allele among 16 populations. Two-star phylogeny formations are observed in the network. The first includes A2 at the centre, and it is connected to 8 other alleles including A1. In this first group Malatya (A14), Sivas (A15), Kayseri (A13), Elazığ (A11), Balıkesir (A3), and Bolu (A8) alleles are placed. The second star phylogeny has A7 at its centre. A7 was detected as another common allele shared among Bingöl, Elazığ, Malatya, and Siirt. This second allele group is connected to alleles all of which are found only in east part of the sampled area of *C. divisa*. The minimum spanning network overall supports the phylogenetic analysis of *C. divisa* alleles.

As a general assessment, the geographic distribution of genetic variation in *C. divisa* points out the importance of climatic changes in shaping the genetic structure of the species. The findings of this study provide further evidence for Quaternary oscillations in those places that were not even covered by glaciers and show how they markedly shaped the phylogeography of a species. While much more work is still necessary to reveal the outcomes of climatic fluctuations in the biological world, data obtained from *C. divisa* will be helpful to enlighten the past and it will facilitate making inferences about past factors that resulted in the present Anatolian biodiversity.

## CONCLUSION

In this study, an oak gall wasp species, *C. divisa*, was explored for the purpose of revealing genetic diversity, and the possible reasons that resulted in the current pattern. Our results point to i) *C. divisa* has a substantial amount of genetic diversity in the Anatolian populations as compared to the previously studied Turkish and European gall wasp taxa, ii) High haplotype versus low nucleotide diversity apparent for both regions designate population size changes over time, possibly bottleneck event(s) followed by population expansion, iii) Phylogenetic and statistical analyses employed on both regions indicated that there was a geographic structuring of the population up to a certain level. Not as robust as some other previously examined oak gall wasp species, *C. divisa* displays haplogroup formations that are geographically significant, iv) *Cynips divisa* seems to have diverged around Pliocene from its congeners incorporated into the analysis as outgroups, v) Formation of major clades and shallow subclades appear to be associated with climatic oscillations of Pleistocene. In particular, the last 780.000 years seem to play a key role in shaping the major diversification events of the species in Turkey.

Current findings of this study emphasize the importance of historical events that produced the contemporary structure of a species. Among other possible historical



**Acknowledgement.** This study was supported by the Bolu Abant İzzet Baysal University, Grant No: 2016.03.01. 1069.

**Conflict of Interest.** The authors declared that there is no conflict of interest.

**Authorship Contributions.** Concept: E. C., S. M., Design: E. C., S. M., Data Collection or Processing: E. C., S. M., Analysis or Interpretation: E. C., S. M., Literature Search: E. C., S. M., Writing: E. C., S. M.

**Financial Disclosure.** This research was financially supported by Bolu Abant İzzet Baysal University with the Grant No: 2016.03.01.1069.

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