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Molecular Phylogeny of Some *Cinara* Species (Hemiptera: Aphidoidae) Feeding on Cupressaceae Species in Turkey

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ABSTRACT

Cinara species feed on conifers of the families Cupressaceae and Pinaceae and it has been shown that host plant plays crucial role on diversity of this genus. *Cinara* (*Cinara*) *juniperensis, C.* (*Cupressobium*) *cupressi* and *C.* (*Cupressobium*) *tujafilina* species infesting *Juniperus* sp., *Cupressus* sp. and *Plathycladus* sp., respectively were studied. It is difficult to distinguish these species properly based on morphological identification key due to high amount of morphological similarities. In this study, partial sequences of mitochondrial cytochrome oxidase subunit I (COI) gene were used to identify and to reveal phylogenetic relationships of determined *Cinara* species. Intraspecific and interspecific distinctions were 0.2% -2.2% and 2.5%-7.8% for three species of *Cinara*, respectively. The topology of the tree showed closer relationship between *C. tujafilina* and *C. cupressi* (95-96 % bootstrap) while *C. juniperensis* showed lower similarity with them. Phylogenetic tree inferred from both Maximum parsimony and Neighbour joining analyses revealed that *C. tujafilina* and *C. cupressi* were monophyletic. Findings revealed the host plant effectiveness in phylogeny of the determined *Cinara* species.

Key words: Cinara, Cupressaceae, Cytochrome oxidase subunit I, *Juniperus,* phylogeny, Turkey.

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INTRODUCTION

The genus *Cinara* has four subgenera *Cedrobium, Cinara*, *Schizolachnus* and *Cupressobium,* including about 200 species (Manzano-Marine, Szabo, Simon, Horn, & Latorre, 2016; Blackman & Eastop, 2019). Of these species, about 150 species are native of North America, 30 of Europe and 20 of the Far East, respectively. Most of the determined *Cinara* (Hemiptera, Lachnidae) species classified in the subgenus *Cinara* are associated with Pinaceae (Blackman & Eastop, 2019), and they infest lignified parts, branches, trunks, roots and leaves of coniferous trees, not showing a host alternation. *Cinara* species have specific morphological characters according to the parts of plants they feed (Favret & Voegtlin, 2004a; 2004c; Durak, Lachowska-Cierlik, & Bartoszewski, 2014). Favret & Voegtlin (2004a; 2004b) revealed a closer phylogenetic relationship between species colonizing similar feeding parts than between those infesting the same host plants. *Cinara* species (Hemiptera: Aphidoidea) originated from Asia about 50 million years ago and the ancestors of these aphids fed on the Angiosperm species before they migrated to the conifers. Thus, it has been thought that Lachninae aphids are interesting group due to their distribution on coniferous host plants, cypress family (Meseguer, Coeur d'acier, Genson, & Jousselin, 2015). The cypress family (Cupressaceae) includes nearly 150 species in 30 genera, occurs mainly in warm climate (Blackman & Eastop, 2019).

The *Cinara* species are monophyletic in the family Aphididae (Heie, 1987; Normark, 2000), identification of the *Cinara* species is quite difficult due to their unspecific morphological characters (Foottit & Mackauer, 1990; Watson, Voegtlin, Murphy, & Foottit, 1999). which give rise to some identification problems (Favret, 2004a). For example, they can be classified into subgenera according to the length of dorsal HT I and the number of subapical hairs on processus terminalis (Durak et al, 2014), which are open to make mistake easily during measurements.

The dispersal ability of the *Cinara* species is limited because of the high weight to wing length ratio. Some species of genus are recorded even without winged morphs and therefore they are susceptible to geographical isolation. These features make *Cinara* to study ecological speciation basically driven by preferred host plant and parts of the host plant preferred by species (Jousselin, Cruaud, Genson, Chevenet, Foottit, & Cœur d'acier, 2013; Meseguer et al, 2015; Chen, Favret, Jiang, Wang, & Qiao, 2016). To define these species and explore the diversity based on morphology resulted in some difficulties and unexpected confusions. Molecular studies have become popular tool in inventory of biodiversity to overcome these difficulties (Foottit, Maw, Von Dohlen, & Hebert, 2008) including *Cinara* genus.

Mitochondrial cytochrome oxidase subunit I gene is used commonly to identify insects belonging to various genera, and especially aphids (Milankov, Stamenkovic, Ludoski, Stahls, & Vujic, 2005; Foottit et al, 2008). It was also used to determine genetic variations and reveal phylogenetic relationships within the genus Cinara (Favret & Voegtlin, 2004b; Durak, Sadowska-Woda, Machordom, & Borowiak-Sobkowiak, 2008; El Mujtar, Covelli, Delfino & Grau, 2009). Findings of the mitochondrial phylogenetic studies are generally compatible with results derived from other studies such as

morphology and nuclear genes (Cameron, 2014) even there are still less studies conducted in aphids. Although there have been numerous taxonomic studies conducted on aphids around the World (Eastop, 1972; Heie, 1987; Blackman & Eastop, 2019), combination of the morphological and molecular studies are insufficient that might play important role to determine phylogenetic relationships among non-host alternating aphid species like Cinara. Although, to date, some faunistic studies have been done in Turkey (Görür, Akyildirim, Olcabey, & Akyurek, 2012; Şenol, Beğen, Görür, & Gezici, 2014), no investigation has been conducted on phylogenetic relationships among Cupressaceae-feeding species in Turkey. The aim of the present study was to determine genetic variation and to reveal phylogenetic relationships among the Cinara species infesting Cupressaceae, using partial sequences of mitochondrial DNA cytochrome oxidase subunit I (COI) gene.

MATERIAL AND METHODS

Cinara specimens were collected in Afyonkarahisar, Kütahya, Uşak and Niğde provinces in Turkey (Fig.1) during the summer period of 2012-2014 from Cupressaceae plants and preserved in 95% ethanol during field study and some were preserved in -80°C freezers for long-term storage. Notes about aphid morphological features (coloration and patterning) and photos of aphids were recorded. About 50 *Cinara* specimens were collected from leaves and shoot apices on *Cupressus* spp., *Plathycladus* spp. and *Juniperus* spp. Host trees were identified by botanist who study in botany department. Specimens were identified following online based identification key by Blackman & Eastop (2019) and confirmed with other resources (Eastop, 1972; Heie, 1987). DNA was extracted from 10 specimens and only one individual of *Cinara* aphid was used for DNA extraction and rest of the sampled individuals processed for permanent slide. Permanent slides were examined under the microscope and initial identification was performed. Voucher specimens were deposited in Biology department laboratory at Niğde Ömer Halisdemir University. We obtained COI sequences available from GenBank for *Cinara (Cinara)* and both *Adelges japonicus* (FJ50241) and *Adelges laricis* (FJ502446), belonging to Aphididae as outgroups. All aphid species covered in this study are presented in Table 1.

DNA extraction, polymerase chain reaction amplification and sequencing

The DNA was extracted from single aphids with kit procedure (Invitrogen, PureLink Genomic DNA kits) according to the manufacturer's protocol. DNA fragment was amplified by using COIS (5-GGAGGATTTGGAAATTGATTAGTTCC-3)/COIA (5_GCTAATCATC TAAAAATTTTAATTCCTGTTGG-3) primers (El Mujtar et al, 2009), which give about 397 bp of the COI gene from the mitochondrial genome. PCR reactions were carried out in 50 µl reaction aliquots containing 2 µl DNA, 2 µl of each primer (10 uM), 0.3 µl of Taq DNA polymerase (2.5u/µl Fermantes), 5 µl of 10X Taq buffer, 1 µl of 10mM dNTPs, 4 µl BSA, 4 µl $MgCl_2$ and ultra-pure water. The temperature profile for the amplification of the COI gene fragment included an pre-denaturation step of 94 °C for 6 min followed by 35 cycles of 94 °C for 1 min, 56

°C for 1.30 min, 72 °C for 1.30 min and a final extension period of 72 °C for 5 min, then storaged at + 4 °C. The PCR products were resolved in 1 % agarose gel by electrophoresis at 80 volt, if a single band was observed, PCR product (50-250 ng/ul) was cleaned and then sequenced both forward and reverse direction by the ABI 3100 Genetic Analyzer (Macrogen).

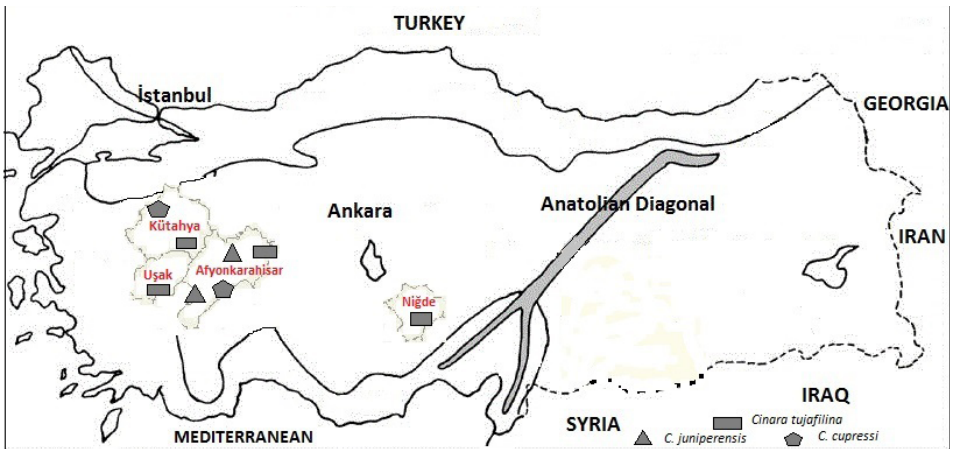

Fig. 1. Map of *Cinara* specimens collected from inner western Anatolia and Niğde, Turkey.

Table1. A list of sampling localities and host plants.

No	Species	Location	District	Host Plant	Date	Haplotype
HABA1 (GB: MN526020)	*Cinara tujafilina*	Uşak	Eşme	*Plathycladus* spp.	12.6.2013	**HAP5-E**
HABA3 (GB: MN526012)	*C. tujafilina*	Kütahya	Gediz	*Plathycladus* spp.	23.8.2012	**HAP6-F**
HABA4 (GB: MN526015)	*C. tujafilina*	Uşak	Gediz	*Plathycladus* spp.	14.8.2012	**HAP7-G**
HABB4 (GB: MN526013)	*C. tujafilina*	Niğde	Merkez	*Plathycladus* spp.	03.7.2013	**HAP4-D**
HABC1 (GB: MN526021)	*C. tujafilina*	Niğde	Merkez	*Plathycladus* spp.	15.7.2014	**HAP9-I**
HABF1 (GB: MN526014)	*C. tujafilina*	Kütahya	Domaniç	*Plathycladus* spp.	22.8.2014	**HAP12-L**
EU151496.1 (Durak et al, 2008)	*C. tujafilina*	Poland	--------	*Plathycladus* spp.	-------	**HAP4-D**
HAB10 (GB: MN526016)	*C.cupressi*	Afyonkarahisar	Döğen	*Cupressus* spp.	02.6.2014	**HAP1-A**
EU881687.1 (El Mujtar et al, 2009)	*C.cupressi*	Poland	--------	*Cupressus* spp.	-------	**HAP2-B**
JQ247997.1 (Durak, 2011)	*C.cupressi*	Poland	--------	*Plathycladus* spp.	-------	**HAP2-B**
KR033001.1 (Gwiazdowski et al, 2015)	*C.cupressi*	Canada	--------	*Cupressus* spp.	-------	**HAP2-B**
LT600422.1 (Manzano-Marin et al, 2016)	*C.cupressi*	Spain	-------	*Cupressus* spp.	-------	**HAP3-C**
HABB3 (GB: MN526017)	*C. juniperensis*	Kütahya	Gediz	*Juniperus* spp.	08.8.2014	**HAP8-H**
HABD2	*C. juniperensis*	Kütahya	Çavdarhisar	*Juniperus* spp.	29.7.2012	**HAP10-J**
HABD4	*C. juniperensis*	Kütahya	Gediz	*Juniperus* spp.	17.6.2013	**HAP11-K**

*GB:GenBank Accession Numbers

Phylogenetic analysis

COI sequences were aligned in Geneious v.R6.1.6 (Genious, 2017) and DnaSP v.5.10 (Rozas & Librado, 2009). This programs were used to determine haplotypes and to estimate haplotype and nucleotide diversities within each species. The alignment contained 397 bp and this region was aligned both reverse and forward direction. We used MEGA 7.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) to calculate the genetic distances among sequences of the *Cinara* species, based on the Kimura 2- parameter (K2P) model of DNA substitution (Kimura, 1980) and their reliability has been tested with 10,000 bootstrap replications (Felsenstein, 1985).

Phylogenetic trees were constructed using tree analyses: neighbour joining (NJ) and maximum parsimony (MP). JModelTest 2.0 was used to determine the best fit substitution model of nucleotide evolution. Aphid species, *Adelges japonicus* (FJ502415) and *A. laricis* (FJ502446) were used as an outgroup in the phylogenetic analysis. List of *Cinara* samples and host plants were given in Table1.

RESULTS

Fifteen mitochondrial COI sequences (397 bp) of *Cinara* species sampled on Cupressaceae from both Turkey and other countries were analyzed and 12 haplotypes were determined (Table 1). The overall transition/transversion ratio (R) was 3.685. A sequence analysis for 397 bp lengths of mitochondrial COI-coding DNA emphasized an abundance of A-T nucleotides. The nucleotide composition of *Cinara* haplotypes were determined (A= 37.70%, T/U=35.45%, C=11.81% and G=15.04%). The proportion of A+T in *Cinara* haplotypes was 73.15% and G+C was 26.85%.

The range of the interspecific pairwise sample divergences (K2P model) was 2.5-7.8%, while intraspecific pairwise sample divergences between three species of *Cinara* ranged from 0.2 to 2.2 % (Table 2).

All phylogenetic trees distinguished clearly separated three major clades of haplotypes according to the host plant. *Cinara* species feeding on *Cupressus* sp. and *Plathycladus* sp. were formed sister clade. Third clade is composed of sequences collected from *Juniperus* sp. They were compared with the sequences obtained from the GenBank database (Table 2). Three COI haplotypes were found among three sequences of *Cinara juniperensis*, three haplotypes of *C. cupressi*, six COI haplotypes were found among seven sequences of *Cinara tujafilina*. Haplotype diversity (Hd): 0.962, nucleotide diversity (Pi): 0.03730 and variance of haplotype diversity: 0.00159

Twere determined. The genetic distance between these haplotypes is very low (0.0015) based on the K2P substitution model. Almost all haplotypes were clustered according to a specific host plant based on the overall NJ and MP analysis by COI region of the distance among the 12 haplotypes (Fig. 2). When comparing COI sequences of different studies obtained from GenBank (Table 1), we found that a total of six haplotypes from *Cinara* sampled on *Plathycladus* sp., tree haplotypes on *Cupressus* sp. and tree haplotypes on *Juniperus* sp.

Table 2. Mitochondrial DNA pairwise distance of *Cinara* species

		1	2	3	4	5	6	7	8	9	10	11	12
1	**C. cupressi_HABB10**												
2	**C. cupressi_EU881687.1**	**0.017**											
3	**C. cupressi_LT600422.1**	**0.022**	**0.005**										
4	**C. tujafilina_HABA1**	0,038	0,056	0,056									
5	**C. tujafilina_HABA3**	0,030	0,048	0,048	**0.017**								
6	**C. tujafilina_HABA4**	0,033	0,051	0,051	**0.015**	**0.012**							
7	**C. tujafilina_HABB4**	0,025	0,043	0,043	**0.012**	**0.005**	**0.007**						
8	**C. tujafilina_HABC1**	0,027	0,045	0,045	**0.015**	**0.007**	**0.005**	**0.002**					
9	**C. tujafilina_HABF1**	0,030	0,048	0,048	**0.017**	**0.010**	**0.007**	**0.005**	**0.007**				
10	**C. juniperensis_HABD2**	0,054	0,070	0,076	0,070	0,067	0,070	0,062	0,064	0,067			
11	**C. juniperensis_HABD4**	0,054	0,070	0,075	0,072	0,064	0,067	0,059	0,061	0,064	**0.020**		
12	**C. juniperensis_HABB3**	0,057	0,073	**0,078**	**0,072**	0,070	0,073	0,064	0,067	0,070	**0.002**	**0.022**	

J and MP trees showed that *Cinara* sequences obtained from GenBank and this study created three distinct clusters. *Cinara juniperensis* showed a deep divergence from other *Cinara* species. *C. tujafilina* and *C. cupressi* were at same cluster. *C.*

N*cupressi* haplotype from Turkey showed separate cluster from Poland and Canadian haplotypes (Fig. 2).

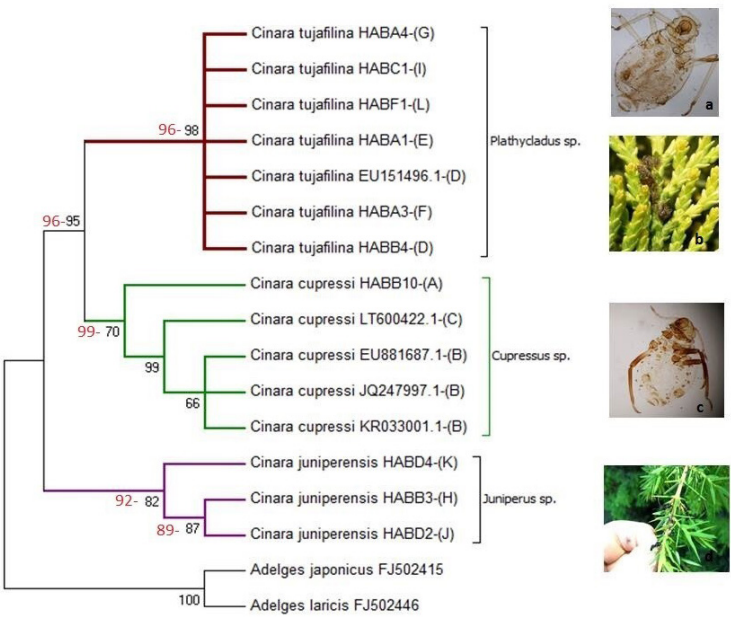

Fig. 2. Maximum Parsinomy (MP) and Neighbour-joining (NJ) trees for phylogenetic clustering of three aphids species in relation to partial COI mitochondrial gen a. *C. tujafilina* b. *C. tujafilina* on *Plathycladus* sp. c. *C. cupressi* d. *C. juniperensis* on *Juniperus* sp.

DISCUSSION AND CONCLUSION

The *Cinara* species are connected with conifers, trees and shrubs, also ornamental shrubs in urban green areas. Selection by the host plant better explains genetic differences among clonal lineages of *Cinara* than geographical distances due to their lower flight capabilities. Recent studies conducted by several researchers revealed that aphids, particularly *Cinara,* showed a rapid radiation related with the host plant shift (Ortiz-Rivas, Moya, & Martinez-Torres, 2004; Ortiz-Rivas & Martinez-Torres, 2010). Genetic differentiation within clonal lineages of *Cinara* could be related to the adaptation to the host-plant (Silva, Ruilova, & Urrutia, 2005). Despite many morphological similarities, an analysis of the mitochondrial DNA clearly indicates genetic distinction of the species. Previous studies show that low genetic diversity was observed by mtDNA COI analysis on *Cinara* species within inter species used *Juniperus* as a host and no differences were found within intra species, it could be caused by same microhabitat shared by these species and some species within the genus are very close relative

species (Durak, 2011). Findings of the presented study showed similarity with Durak (2011) and El Mujtar et al (2009). Genetic divergence of *Cinara* species was 2.5%-7.8% collected on Cupressaceae. Analyzes on sequencing of the COI gene showed that genetic divergence between *C. juniperensis* and *C. tujafilina* was 5.9% -7.3%, while between *C. tujafilina* and *C. cupressi* was 2.5% -5.6%.

As a result of these studies, *Cinara* (*Cupressobium*) genus has been shown to be a monophyletic group like other studies (Durak et al, 2014). Furthermore, Favret & Voegtlin (2004a; 2004c) revealed the strong host plant effect on *Cinara* aphids on Cupressaceae. In accordance with previous results, species are clearly separated on the phylogenetic tree relative to the host plant and same groups have an important amount of differences that can be explained with the influence of localities.

Sequences of *Cinara* specimens from Turkey were used to compare sequences obtained from GenBank by El Mujtar et al (2009). Sequences from *C. tujafilina* had 99% nucleotide identity with *C. tujafilina* reported in Poland and *C. cupressi* showed 85-90% nucleotide identity with *C. tujafilina.* Foottit et al (2008), using a region of the CO-I gene from 300 species from 130 genera of aphids, detected low intraspecific variation and showed that molecular methods are useful for identification of aphid species. Recent studies pointed out that how strongly mitochondrial genome sequence studies reveal branching in aphids (Chen, Wang, Jiang, & Qiao, 2017). Verified mitochondrial COI sequences have been amplified using different primer combinations by different researchers and some intraspecific variation shown in the overlapping regions (Favret & Voegtlin, 2004a; Durak et al, 2008; Foottit et al, 2008). El Mujtar et al. (2009) used mtDNA COI gene region to determine two morphologically similar species (*C. cupressi* and *C. tujafilina*) on the same host and combined molecular and morphological findings. Findings of the mitochondrial phylogenetic studies are generally compatible with results derived from other studies such as morphology and nuclear genes (Cameron, 2014) even there are still less studies conducted in aphids. It was clearly shown that phylogenetic data and morphological distinctions derived in this study were in coincidence and supported each other. Overall evaluation of the findings indicated lower genetic diversity among species, they basically showed a distribution related with host plant. Despite accordance between morphological distinctions and phylogenetic data obtained in this study, study conducted on *Cinara* species feed on Cupressaceae were insufficient in Turkey, findings presented there are preliminary study to determine phylogeny of the Turkish *Cinara* population. Molecular identification of species belonging to *Cinara* will certainly enable to learn and understand their phylogenetic relations. Turkey is a very large country and common host plants of the *Cinara* widely distributed in Turkey, thus to understand general pattern, more studies should be carried out with larger sample sizes and different gene regions.

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