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Genetic Structure of *Marchalina hellenica* (Hemiptera: Margarodidae) Populations from Turkey: Preliminary mtDNA Sequencing Data

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Abstract The scale insect *Marchalina hellenica* (Gennadius) (Hemiptera: Margarodidae) contributes to the production of pine honey in Turkey and Greece via the honeydew excreted when it feeds on pine trees. Although it is an insect of prime economic importance, there is no information on its genetic structure. Preliminary data were obtained based on sequencing analysis of 12s rDNA and COI mtDNA gene segments from samples from four areas of Turkey. Sequences of the 12s rDNA gene segment from Greek samples available in GenBank were also included. No variability was detected concerning the COI mtDNA gene segment analysis, although 13 haplotypes were revealed based on the 12s rDNA gene segment. The most distant population was from Mudanya-Bursa Province (Turkey). Further research is necessary on the genetic structure and variability of *M. hellenica* populations from the two neighboring countries.

Keywords *Marchalina hellenica* · Turkey · mtDNA · Genetic structure · Sequencing

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Introduction

Scale insects provide nutritious carbohydrate liquids for honey bees, although it is generally believed that flower nectar is the main source of honey. According to Kunkel (1997), many species of scale insects, belonging to at least six families, produce honeydew as a source for honey worldwide. In Turkey and Greece, honey bees collect honeydew mainly from *Marchalina hellenica* (Gennadius) (Hemiptera: Margarodidae), which feeds on pine trees. This pine honey represents almost 40% (20,000 tons) of the annual honey production in Turkey and 65% (10,000 tons) in Greece, and the world pine honey production comes almost entirely from these two countries (Santas 1983; Thrasyvoulou and Manikis 1996; Sunay et al. 2003; Sunay and Boyacioglu 2008).

Marchalina hellenica is endemic to these countries, but it has been introduced on the island of Ischia in Italy (Kailidis 1965; Nikolopoulos 1965; Santas 1979, 1983; Tranfaglia and Tremblay 1984; Fimiani and Solino 1994; Priore et al. 1996; Pollini 1998). It has also been reported in the Sochi region on the Black Sea coast of the Russian Caucasus (Gavrilov and Kuznetsova 2004). In Greece, it is found mainly on *Pinus halepensis* and *Pinus brutia* (Pinales: Pinaceae) (Bodenheimer 1953; Nikolopoulos 1959, 1964; Kailidis 1965; Gounari 2006) and rarely on other pine species (Nikolopoulos 1964, 1965; Avtzis 1985; Pollini 1998). Bacandritsos et al. (2004) have recently announced the establishment of *M. hellenica* also on *Abies cephalonica* (Pinales: Pinaceae) trees on Helmos Mountain in Greece. The insect is the most prevalent, however, on *P. brutia* in the Mediterrenean region of Turkey and has also been recorded on *P. pinea* and *P. sylvestris* in western Turkey (Selmi 1983; Yesil et al. 2005; Akkuzu et al. 2006). Since 2006, *M. hellenica* has been included in the European and Mediterranean Plant Protection alert list.

Marchalina hellenica belongs to the family Margarodidae (Hemiptera, Coccoidea). It has one generation per year, and the adult females appear on the trees only after mid March. Because the male is rarely found, it has been suggested that the insect is produced mainly parthenogenetically and rarely bisexually (Nikolopoulos 1964, 1965; Pollini 1998; Erlinghagen 2001).

Given that *M. hellenica* is an insect of prime economic importance for honey production, several studies of its biology have been conducted during the past decades. Information concerning the genetic structure of the species, however, is still missing. The reproduction and low migratory ability of the insect must be taken into consideration for any genetic study. An interesting question that arises is whether geographically distant populations of the species are genetically divergent. The results of Margaritopoulos et al. (2003) from the samples of Greece showed a degree of both intra- and inter-population genetic variation. The intra-population variation observed was associated with host type and region of origin.

New tools and resources are making the study of scale insects by scientists more exciting. Mitochondrial DNA (mtDNA) markers have been widely used to address population and evolutionary questions. The mtDNA of eukaryote cells has a relatively fast mutation rate, resulting in significant variance in mtDNA sequences. This kind of analysis provides information on the nature as well as on the extent of differences between sequences in nuclear and mtDNA (Dowling et al. 1990) and can

be used effectively, as well as economically, to analyze geographic variation and hybridization (Hoy 1994).

Recently, DNA barcoding has been developed as a standardized method to identify taxa. The DNA barcode consists of a short mitochondrial genetic marker, which can distinguish individuals of a species based on the principle that genetic variation between species exceeds variation within species (Hebert et al. 2003, 2004). This idea aims at developing the DNA barcode for the identification of species using a standard region of the COI mtDNA gene (Ratnasingham and Hebert 2007).

The aim of this research is to examine the genetic structure in a systematic way and to detect possible genetic variability among *M. hellenica* populations from different areas. Preliminary results, based on sequencing of two mtDNA gene segments of *M. hellenica* populations from Turkey, are presented.

Materials and Methods

Sampling and DNA Sequencing

Samples of *M. hellenica* were collected from non-neighboring pine trees (five adult females/population) from four areas of western Turkey: Mudanya in Bursa Province, Kestel in Bursa Province, Kesan in Edirne Province of northwest Turkey, and Mesudiye in Mugla Province of southwest Turkey (Fig. 1).

Fig. 1 Sampling sites for *Marchalina hellenica* collected from pine trees in western Turkey: *1* Mudanya in Bursa Province, *2* Kestel in Bursa Province, *3* Kesan in Edirne Province, *4* Mesudiye in Mugla Province

The samples were placed alive in absolute ethanol and stored in the laboratory at −20°C until processing. Total DNA extraction was carried out using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol, after minor modifications, and examined through agarose gel electrophoresis. Fragments of two mtDNA gene segments (12s rDNA and COI) were amplified using a PEQLab Thermocycler (Primus 25 Advanced). The primers used for the 12s rDNA gene segment were 5′-AAACTG GGATTAGATACCCCACTAT-3′ and 5′-GAGGGT GACGGGCGGTGTGT-3′ (Palumbi et al. 1991). For the COI segment, the barcode primers 5′-GGTCAACAAATCATAAAGATATTGG-3′ and 5′-TAAACTTCAGG GTGACCAAAAAATCA-3′ were used (Folmer et al. 1994).

The polymerase chain reaction (PCR) (Saiki et al. 1988) for the 12s rDNA gene segment consisted of 2.4 U *Taq* polymerase (Invitrogen), 5 µl 10× reaction buffer (Invitrogen), 5 µl dNTP mix (2 mM; BioLabs), 5 µl $MgCl_2$ (5 mM; Invitrogen), approximately 100 ng DNA, 0.5 µl each primer (20 mM; BioLabs), and sterile water. PCR amplification conditions were 4 min denaturation at 94°C, followed by 35 cycles of 94°C for 1 min, annealing at 44°C for 1 min, and extension at 72°C for 2 min. For COI, PCR consisted of 1.5 U *Taq* polymerase (Invitrogen), 5 µl 10× reaction buffer (Invitrogen), 5 µl dNTP mix (2 mM; BioLabs), 3 µl $MgCl_2$ (3 mM; Invitrogen), approximately 100 ng DNA, 0.5 µl each primer (10 µM; BioLabs), and sterile water. PCR amplification conditions were 1 min at 94°C followed by two groups of cycles. The first group consisted of six cycles at 94°C for 1-min denaturation, annealing at 45°C for 1 min 30 s, and extension at 72°C for 1 min 15 s. The second group included 36 cycles at 94°C for 1-min denaturation, annealing at 51°C for 1 min 30 s, and extension at 72°C for 1 min 15 s. The reaction was concluded with an additional 6-min extension at 72°C at the end of the final amplification cycle.

The PCR products were purified using the Nucleospin Extract II kit (Macherey–Nagel) according to the supplier's protocol.

Individual sequences were determined via automated sequencing provided by Macrogen Company (Seoul, Korea). The primers in the sequencing reactions were the same as in the amplification procedure. The sequences obtained have been deposited to GenBank with accession nos. GU721095–GU721101 for the 12s rDNA segment and HQ225738 for COI.

Data and Phylogenetic Analysis

Multiple-sequence alignments were done with Clustal W2 (Thompson et al. 1994). The computer-generated alignment was further adjusted manually. In addition, the alignment of the 12s rDNA data set was verified against 12s rDNA sequences of *M. hellenica* samples from Greece, available in GenBank (acc. nos. DQ381509–DQ381512 and EF088648–EF088649, corresponding to Greek samples 1–6). Therefore, the authenticity of the mtDNA 12s rDNA sequences produced was verified. The sequences from the Greek samples were included in the statistical process. The sequencing data obtained from the COI gene segment were verified using the Blast program of NCBI.

Pairwise genetic distance, based on 12s rDNA sequencing data, was estimated using Mega version 4.0.2 (Tamura et al. 2007) and the Kimura (1980) two-parameter model. A distance analysis was also performed using the Tamura–Nei plus gamma model (Tamura and Nei 1993; Yang 1994), a model selected using the FindModel tool (Tao 2005), to construct a maximum likelihood tree using the PhyML program (Guindon and Gascuel 2003; HCV sequence data base). Haplotypes were detected using the DnaSP version 5.10.00 package (Librado and Rozas 2009), as well as the number of variable sites, haplotype, and nucleotide diversity. Tajima's (D) test for neutrality (Tajima 1989) was performed using the same package.

The UPGMA and minimum evolution analyses were conducted using Mega version 4.0.2 (Tamura et al. 2007) for the data set. Confidence in the nodes was evaluated by 1,000 bootstrap replicates (Felsenstein 1985). A maximum likelihood tree was constructed using the PhyML program (Guindon and Gascuel 2003).

Results

The sequencing of COI and 12s rDNA mtDNA gene segments produced an alignment of about 654 and 160 bp, respectively. One haplotype based on the COI sequencing data and 13 haplotypes based on 12s rDNA were revealed (Table 1), including the sequences of *M. hellenica* samples from Greece available in GenBank. The number of variable sites was 35, with a higher number of variable sites for the haplotypes from Turkey (Fig. 2). The highest genetic divergence value was observed between Turkey 1a and Turkey 3a (Table 2), and the overall average pairwise genetic distance was 0.059 (Kimura 2-parameter). Tajima's (D) test showed no statistical significance (−1.147). The haplotype diversity was 0.989, and the nucleotide diversity was 0.052. It is noted that the sequence of the 12s rDNA

Table 1 *Marchalina hellenica* populations from Greece and Turkey grouped in 13 haplotypes

Haplotype	Population	Source[a]
Hap 1	Greece 1	GenBank DQ381509
Hap 2	Greece 2	GenBank DQ381510
	Turkey 2a	Kestel
Hap 3	Greece 3	GenBank DQ381511
Hap 4	Greece 4	GenBank DQ381512
Hap 5	Greece 5	GenBank EF088648
Hap 6	Greece 6	GenBank EF088649
Hap 7	Turkey 1a	Mudanya
Hap 8	Turkey 1b	
Hap 9	Turkey 2b	Kestel
Hap 10	Turkey 3a	Kesan
Hap 11	Turkey 3b	
Hap 12	Turkey 4a	Mesudiye
Hap 13	Turkey 4b	

[a] Sampling sites for Turkish populations as in Fig. 1

	1	2	11	13	18	41	67	71	72	75	79	80	81	84	91	109	113	117	119	126	127	128	132	137	138	141	142	145	146	147	153	156	157	158	159
Greece 1	T	G	A	G	G	G	T	C	C	A	C	G	G	C	T	G	G	C	G	T	G	C	T	C	A	G	T	G	G	T	A	C	C	A	G
Greece 2	A														A																				
Greece 3	A																																		
Greece 4	A					A																													
Greece 5	A	A				A																													
Greece 6	A	A																							G										
Turkey 1a	A		T	T			G	G	T	G	A	A	A	G			T	G	C	G		A			G		G	A	A	A		T	A		
Turkey 1b	A																									A									
Turkey 2a	A																																		
Turkey 2b	A						G					A				A	T		C		A	A		T	G	A		A	A	A	T		A		
Turkey 3a	A				C																														T
Turkey 3b	A																T																		T
Turkey 4a	A									G						C	T		C	G	A	A	C		G			A	A	A			A	T	
Turkey 4b	A															A						A													

Fig. 2 Variable sites in the 12s rDNA sequence of 13 haplotypes found in *Marchalina hellenica* populations from Greece and Turkey. Haplotype codes as in Table 1

Table 2 Pairwise genetic distance[a] between 13 12s-rDNA haplotypes found in *Marchalina hellenica* populations from Greece and Turkey

Sample[b]	Greece						Turkey						
	1	2	3	4	5	6	1a	1b	2a	2b	3a	3b	4a
Greece													
2	0.006												
3	0.013	0.006											
4	0.013	0.006	0.013										
5	0.019	0.013	0.019	0.006									
6	0.019	0.013	0.019	0.019	0.013								
Turkey													
1a	0.175	0.166	0.175	0.173	0.181	0.166							
1b	0.013	0.006	0.013	0.013	0.019	0.019	0.173						
2a	0.006	**0.000**	0.006	0.006	0.013	0.013	0.166	0.006					
2b	0.111	0.104	0.111	0.111	0.118	0.104	0.127	0.097	0.104				
3a	0.019	0.013	0.019	0.019	0.025	0.025	**0.184**	0.019	0.013	0.119			
3b	0.019	0.013	0.019	0.019	0.025	0.025	0.166	0.019	0.013	0.104	0.013		
4a	0.105	0.097	0.105	0.104	0.111	0.097	0.120	0.104	0.097	0.070	0.113	0.097	
4b	0.019	0.013	0.019	0.019	0.025	0.025	0.165	0.019	0.013	0.089	0.026	0.026	0.090

[a] Kimura (1980) two-parameter values

[b] Codes as in Table 1

segment obtained from the Turkey 2a population (Table 1) is the same as that from Greece 2. The other Turkish populations are different from the Greek ones. Low intravariability is detected among populations from Greece, but higher variability is found among populations from Turkey.

The trees drawn by minimum evolution and UPGMA analyses, based on the 12s rDNA segment sequencing results, exhibited the same topology; therefore, only the UPGMA tree is presented here (Fig. 3). According to this topology, a population from Mudanya in Bursa Province (Turkey 1a) is in a separate clade. The remaining populations are grouped together. The suggested topology is supported by high bootstrap values. The minimum likelihood tree showed a similar topology (Fig. 3).

Discussion

Marchalina hellenica, an endemic species in Greece and Turkey, is an important source of honeydew honey, given that the world pine honey production comes almost entirely from these two countries. There are no previous data on the genetic structure of this insect and the phylogenetic relationships between *M. hellenica* populations originating from the two countries. Our research presents the preliminary results from the first comprehensive sequencing analysis of *M. hellenica.*

Sequencing data obtained from the COI segment, using barcode primers applied for the identification of species, confirmed that all the populations studied belong to a single species. Nevertheless, intraspecies variability was detected among the populations, using the 12s rDNA mtDNA gene segment. The highest genetic divergence value was observed for the population from Mudanya in Bursa Province (Turkey 1a), a result reinforced by the UPGMA and maximum likelihood trees which clearly place the population in a separate clade from the others. Notably, two other populations from Turkey (2a and 1b) are very close genetically to the Greek populations; this may be attributed to human and resource movements between the two countries during previous centuries. The examination of more samples can be more informative about the relationship between sequencing data and the origin of the population. Further research is needed regarding the environmental parameters affecting honeydew production in these regions.

The generally low genetic divergence observed was expected because of the parthenogenetic reproduction of the insect and its behavioral patterns (e.g., low mobility, feeding preferences). The numbers of haplotypes, however, were interesting: six among the Greek populations and another seven among the Turkish populations. This variability is perhaps due to the sampling procedure, which included insects from non-neighboring trees, taking into account the low mobility of the insect. The variability is more obvious between populations from areas of Turkey, as well as between Turkish and Greek populations. The Greek populations show a very high homogeneity. Tajima's (D) test for neutrality shows low-frequency polymorphism, indicating population size expansion (Tajima 1989).

Parthenogenetic species are often thought to represent general purpose genotypes capable of high fitness across different environments (Lynch 1984), but it is now

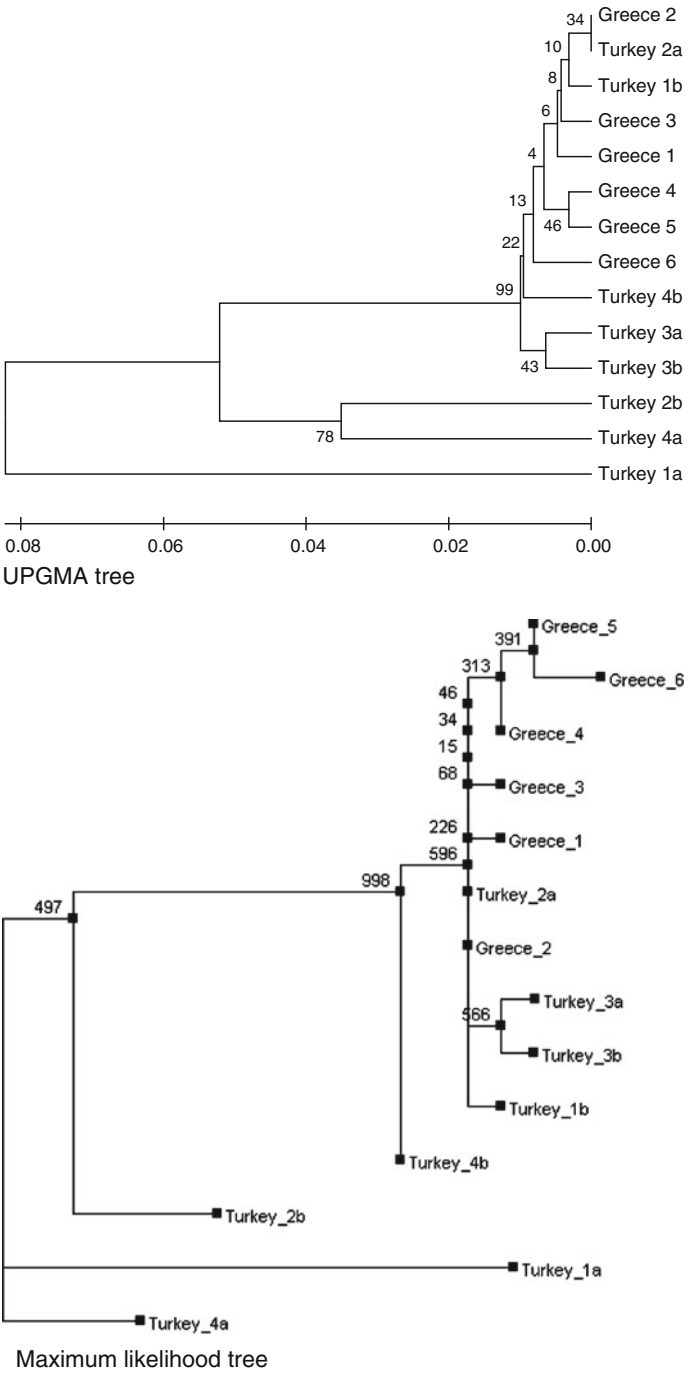

Fig. 3 Phylogenetic relationships of the 13 12s-rDNA haplotypes found in *Marchalina hellenica* populations from Greece and Turkey, based on UPGMA (*top*) and maximum likelihood (*bottom*) analysis. Confidence in the nodes was evaluated by 1,000 bootstrap replicates. Haplotype codes as in Table 1

recognized that they can also contain high levels of genetic variability and comprise specialist genotypes adapted to different conditions (Vrijenhoek 1984; Harshman and Futuyma 1985; Fox et al. 1996; Weeks and Hoffmann 1998). Parthenogenetic lineages also appear capable of exploiting specific niches (Vrijenhoek 1984; Weeks and Hoffmann 1998), and the agricultural environment potentially provides a stable distribution of such niches. Our preliminary data for *M. hellenica* from Turkey agree with that and confirm the results of Margaritopoulos et al. (2003) for *M. hellenica* from Greece.

It is therefore debatable whether geographically distant populations of this species are genetically divergent. Further, detailed investigation is necessary to determine if higher diversity exists between populations of *M. hellenica* from different geographic regions of the two countries. It would also be interesting to perform the test for correlation of the genetic differentiation with productivity and characteristics of honeydew or pine honey produced in the same regions. Information regarding the genetic affinities of *M. hellenica* populations is essential and has to be considered in management programs, especially in cases where the species is artificially introduced in uninfected forests or in localities with existing populations.

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