

Original article (Orijinal araştırma)

Genetic diversity of Turkish populations of *Planococcus citri* Risso, 1813 (Hemiptera: Pseudococcidae)¹

Türkiye *Planococcus citri* Risso, 1813 (Hemiptera: Pseudococcidae) popülasyonlarının genetik çeşitliliği

Mehmet KARACAOĞLU² Gül SATAR³ James J. SMITH⁴ Serdar SATAR^{5*}

Abstract

The genetic diversity and population genetics of the citrus mealybug, *Planococcus citri* Risso, 1813 (Hemiptera: Pseudococcidae), were investigated based on sequencing of mitochondrial DNA. A total of 108 individuals were collected from different host plants in Turkey during 2011-2015. Partial sequences of the cytochrome c oxidase subunit 1 gene revealed five haplotypes in Turkey, with one of these (Hap 1) as the common haplotype, being present in 102 individuals. Additionally, 90 homologous nucleotide sequences retrieved from GenBank were incorporated into the analyses and compared with sequences of isolates from Turkey. Molecular diversity indices revealed overall high mitochondrial DNA diversity for these populations. Further, Tajima's D test and Fu's Fs test showed negative values, except in South Africa, indicating deviations from neutrality and suggesting recent population expansion for the populations. Pairwise comparisons of the different populations using the pairwise fixation index, was significant for some comparisons, indicating genetic differentiation among the *P. citri* populations studied. Based on these findings and those from earlier studies, it was hypothesized that demographic expansion has occurred in *P. citri* via the introduction of mealybugs by anthropogenic movements.

Keywords: Citrus mealybug, haplotype diversity, Mediterranean, mtDNA, molecular phylogenetic

Öz

Turunçgil unlubiti, *Planococcus citri* Risso, 1813 (Hemiptera: Pseudococcidae)'nin genetik çeşitliliği ve popülasyon genetiği, mitokondriyal DNA bölgesine göre araştırılmıştır. Türkiye'de 2011-2015 yıllarında farklı konukçu bitkilerden toplam 108 birey toplanmıştır. Sitokrom C oksidaz alt birim geninin kısmi gen dizileri Türkiye'de beş haplotip olduğunu ortaya çıkarmıştır, bunlardan biri (Hap 1) yaygın haplotip olarak 102 örnekte saptanmıştır. Ayrıca, 90 homolog nükleotit *P. citri* sekansları analizlerimize dahil edilmiş ve Türkiye izolatlarına ait diziler ile karşılaştırılmıştır. Moleküler çeşitlilik indeksleri, bu popülasyonlar için genel olarak yüksek mitokondriyal DNA çeşitliliğini ortaya çıkarmıştır. Ayrıca, Tajima'nın D testi ve Fu'nun Fs testi Güney Asya hariç popülasyonların nötral değişim endeksinde sapmaları ve son popülasyon yayılmasını ifade eden negatif değerler göstermiştir. Çalışılan *P. citri* popülasyonları arasında genetik farklılaşmayı gösteren, ikili sabitleme endeksi kullanan farklı popülasyonların ikili karşılaştırmaları bazı bölgeler için önemli bulunmuştur. Bu çalışma ve daha önceki çalışmalardan elde edilen bulgulara dayanarak, antropojenik hareketlerle *P. citri*'de demografik genişlemenin meydana geldiği varsayılmaktadır.

Anahtar sözcükler: Turunçgil unlubiti, haplotip çeşitliliği, Akdeniz, mtDNA, moleküler filogenetik

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² İnönü University, Faculty of Agriculture, Department of Plant Protection, 44000, Malatya, Turkey

³ Çukurova University, Biotechnology Research and Application Center, 01100, Adana, Turkey

⁴ Michigan State University, Department of Entomology 244 Farm Lane, Room 243, East Lansing, MI 48825-1115, USA

⁵ Çukurova University, Faculty of Agriculture, Department of Plant Protection, 01100, Adana, Turkey

* Corresponding author (Sorumlu yazar) e-mail: hserhat@cu.edu.tr

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Introduction

Citrus mealybug, *Planococcus citri* Risso, 1813 (Hemiptera: Pseudococcidae), is an important pest on cultivated and agricultural plants such as banana, citrus, cotton, grapes, and ornamentals, such as chrysanthemum (Ben-Dov, 1994; García Morales et al., 2016; Karacaoğlu & Satar, 2017a). Citrus mealybug can feed directly on the phloem tissue of the plants and cause early dropping of flowers and fruits, and the honeydew from mealybugs leads indirectly to the development of sooty mold on leaves and fruit surfaces, which negatively affects photosynthesis (Lodos, 1986; Uygun, 2001; Douglas & Kruger, 2008; Meyer et al., 2008; Nakaune et al., 2008; Satar et al., 2013). The citrus mealybug is a well-known pest in agricultural crops around the world, and over the past two decades there has been increased damage by citrus mealybug, causing serious yield losses in the East Mediterranean Region of Turkey (Karacaoğlu & Satar, 2017b).

Comprehensive control methods to suppress of the pest are chemical and biological control in citrus production areas of Turkey which spreads along the Mediterranean Region and westward to İzmir in the Aegean Region. However, these methods could lead to yield different results in the application areas. In the west part of the Mediterranean Region generally is applying biological control and getting positive result (Yayla & Satar, 2012; Karacaoğlu & Satar, 2017b; Telli & Yiğit, 2019). However, the eastern regions had some problem with biological control and even chemical control has not enough successful in some zone of this area. Especially, no positive results have been obtained after releasing of predators and parasitoids at the lower Seyhan District in the East Mediterranean in the last two decades (Karacaoğlu, 2016; Karacaoğlu & Satar, 2017b). The reasons for these differences might be the development of resistance to the insecticides, effect of the host plants, geographic or climatic differences factors. Moreover, introducing the subspecies or invasive form of *P. citri* into new geographical areas by importing or exporting fruit or ornamental plants may also explain these observed differences. This may have resulted in the *P. citri* group or complex in the region.

Reliable techniques for correct identification of taxa are essential for establishing accurate control programs (Lourenco et al., 2006). The mealybug species on citrus in all the Mediterranean Region of Turkey was identified as *P. citri* by taxonomy since 1950s (Bodenheimer, 1951; Soylu & Ürel, 1977; Kansu & Uygun, 1980; Karacaoğlu et al., 2016). However, morphological identification is needed to be done by professional expertise, even when insects are provided at the right stage and as adult females, one cannot distinguish *P. citri* individuals from those of closely related species (Cox, 1989; Malausa et al., 2011). Nowadays, the most commonly used tools to detect differences among individuals and populations are molecular techniques. Many researchers have aimed at using DNA markers to the taxa concerned are closely related species, biology, mealybug-symbiont coevolution, and epigenetic mechanisms of mealybugs (Baumann & Baumann, 2005; Khosla et al., 2006; Hardy et al., 2008; Rugman-Jones et al., 2009; Daane et al., 2011; Beltrà et al., 2012; Roda et al., 2013; Wang et al., 2016a, b; Huang et al., 2017; Poveda-Martinez et al., 2019). Mitochondrial DNA (mtDNA) has been used extensively in population studies of many insect species because it evolves rapidly and, unlike nuclear DNA, it lacks recombination and many regions are conserved (Roderick, 1996; Malausa et al., 2011).

This study aimed to reveal whether the differences arising in the struggle of mealybug control are based on a genetic differentiation and compare the Turkish population with world population. Therefore, phylogenetic relationships among different *P. citri* populations from Turkey and other countries from different continents were investigated. Mealybugs were collected from widely spread major citrus production areas and other host plants such as vegetables, shrubs and ornamental plants in Turkey, and a partial of the mitochondrial cytochrome c oxidase subunit 1 (COI) region was sequenced from each individual insect. The results obtained yield information about the genetic variation of citrus mealybug populations in the citrus production regions of Turkey and the presence of subspecies or cryptic species are discussed. Also, the geographic origin of *P. citri* were traced with population genetic analyses with specimens from Turkey and other locations in GenBank.

Materials and methods

Sampling of *Planococcus citri* populations and identification

The survey was conducted to collect *P. citri* samples between July and October from the Aegean Region in 2011-2013, and June from the Mediterranean Region of Turkey in 2011-2015 (Figure 1 & Table 1). Mealybugs were mainly sampled from citrus orchards but collections were also made from the fruits, leaves and branches of weeds, ornamental plants and vegetables infested with mealybugs. The infected plant materials were brought to the Citrus Pest Laboratory, Department of Plant Protection, Çukurova University and inspected under a Leica S8APO (Leica Microsystems Ltd., Wetzlar, Germany) binocular microscope. Mealybug samples were stored in 1.5-mL Eppendorf tubes containing 99% ethanol at -80°C . Preparations of mealybugs were made for morphological identification according to Kozar & Kosztarab (1988), then identified by Dr. Bora Kaydan (Çukurova University, Vocational School of Imamoğlu, Adana, Turkey) and deposited in the collection of Citrus Research Laboratory at Çukurova University.

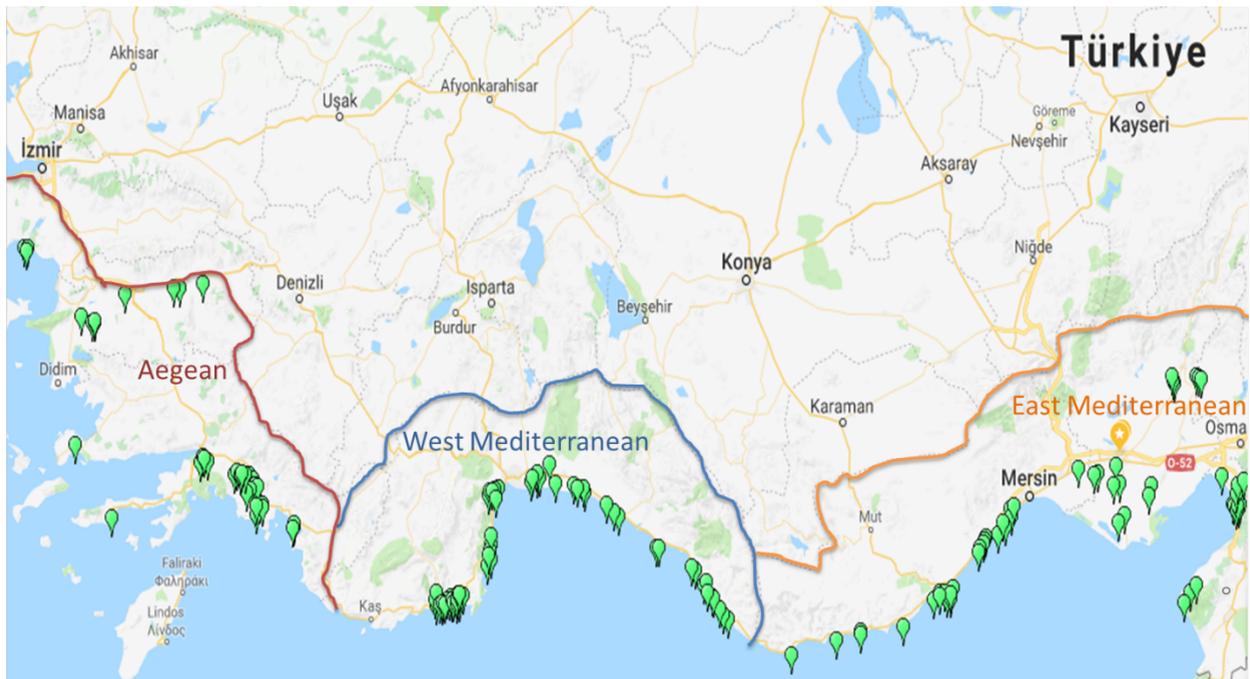


Figure 1. Sampling site for *Planococcus citri* populations in three regions of Turkey.

DNA isolation and PCR amplification

Genomic DNA of 108 specimens was extracted from an adult using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacture's protocol. The primer pairs, C1-J-2183 (5'-CAACATTTAT TTTGATTTTTGG-3') and C1-N-2568 (5'-GCWACWACRTAATAKGTATCATG-3') were used for PCR amplification of mealybug mitochondrial DNA as described by Malausa et al. (2011). The reaction mixture was prepared with a total volume of 50 μl containing Taq buffer (10X), 2.5 mM MgCl_2 , 250 μM dNTPs, 1 μM primer, 0.5 U Taq, and 5 μl DNA template. The PCR conditions were: 1 min at 95°C for predenaturation, followed by 35 cycles of 95°C for 15 s, 52°C for 15 s, 72°C for 1 min, and last extension of 72°C for 7 min. The PCR products were run on 1% agarose gel, and viewed over a UV light. The PCR products were subjected to Sanger sequencing by Molgentek®, Istanbul, Turkey.

Table 1. List of insect specimens examined in this study from three regions of Turkey, including collection date, location, and host plant

No	Date	Province	District	Host plant	Latitude (E)	Longitude (N)
East Mediterranean						
1	07.06.2011	Adana	Seyhan	<i>Capparis spinosa</i> L. (1753)	35°01'66.13"	36°09'85.19"
2	09.06.2011	Adana	Yüreğir	<i>Kalanchoe blossfeldiana</i> Poelln (1934)	35°03'41.17"	37°00'12.20"
3	12.06.2011	Adana	Kadirli	<i>Solanum muricatum</i> Aiton, (1789)	36°05'23.87"	37°02'21.00"
4	24.06.2011	Adana	Yüreğir	<i>Kalanchoe blossfeldiana</i>	35°03'41.17"	37°00'12.20"
5	24.06.2011	Adana	Yüreğir	unknown	35°03'41.17"	37°00'12.20"
6	01.07.2011	Adana	Ceyhan	<i>Citrus aurantium</i> L. (1754)	35°04'84.60"	37°01'57.66"
11	13.07.2011	Mersin	Tarsus	<i>Lycopersicon esculentum</i> L. (1753)	35°02'38.85"	36°05'83.13"
12	13.07.2011	Mersin	Tarsus	<i>Schefflera</i> spp.	35°02'38.85"	36°05'83.13"
13	13.07.2011	Mersin	Tarsus	unknown	35°02'38.85"	36°05'83.13"
14	13.07.2011	Mersin	Tarsus	<i>Ficus benjamina</i> L. (1767)	35°02'38.85"	36°05'83.13"
15	14.10.2011	Mersin	Tarsus	<i>Cydonia oblonga</i> Mill. (1768)	34°05'33.92"	36°05'50.55"
16	17.11.2011	Mersin	Anamur	<i>Musa paradisiaca</i> L. (1753)	32°05'24.20"	36°05'46.00"
17	18.11.2011	Mersin	Silifke	<i>Citrullus lanatus</i> (Thunb.) Matsum. & Nakai, (1916)	34°00'15.85"	36°02'02.41"
18	15.12.2011	Adana	Yüreğir	<i>Punica granatum</i> L. (1753)	35°03'41.17"	37°00'12.20"
19	19.04.2012	Adana	Çukurova	<i>Begonia</i> sp.	35°01'74.21"	37°02'09.51"
20	04.06.2012	Adana	Seyhan	<i>Malva sylvestris</i> L. (1753)	35°01'66.13"	36°09'85.19"
24	11.07.2012	Adana	Yüreğir	<i>Citrus paradisi</i> Macfad. (1830)	35°06'08.00"	37°00'29.53"
26	11.09.2012	Adana	Yüreğir	<i>Vitis</i> sp.	35°03'41.17"	37°00'12.00"
27	17.10.2012	Adana	Yüreğir	<i>Diospyros kaki</i> L.F., 1782	35°02'09.14"	36°05'81.02"
29	16.02.2013	Mersin	Anamur	<i>Musa paradisiaca</i>	32°05'21.62"	36°06'12.96"
45	12.06.2013	Mersin	Tarsus	<i>Citrus sinensis</i> (L.) Osbeck 1765	35°00'17.52"	36°09'94.42"
46	12.06.2013	Mersin	Silifke	<i>Citrus limon</i> (L.) Osbeck 1765	33°06'35.00"	36°38'27.83"
47	14.06.2013	Adana	Seyhan	<i>Ceratonia siliqua</i> L. (1753)	35°01'83.45"	37°00'40.87"
48	16.06.2013	Adana	Yüreğir	<i>Solanum muricatum</i> Aiton 1789	35°03'41.17"	37°00'12.20"
49	18.06.2013	Adana	Seyhan	<i>Citrus sinensis</i>	35°01'84.24"	6°05'54.41"
57	26.06.2013	Adana	Sarıçam	<i>Citrus paradisi</i>	35°06'08.00"	37°00'29.53"
58	27.06.2013	Adana	Seyhan	<i>Citrus paradisi</i>	35°01'66.13"	36°09'85.19"
59	27.06.2013	Mersin	Erdemli	<i>Citrus paradisi</i>	34°07'83.00"	36°06'24.98"
70	06.07.2013	Mersin	Aydıncık	<i>Citrus sinensis</i>	33°03'58.78"	36°01'83.97"
71	06.07.2013	Mersin	Aydıncık	<i>Citrus limon</i>	33°00'17.00"	36°02'05.33"
73	01.08.2013	Adana	Çukurova	<i>Morus alba</i> L. (1753)	35°01'80.08"	37°02'58.59"
74	23.08.2013	Hatay	Samandağ	<i>Citrus sinensis</i>	38°03'15.00"	37°05'80.22"
75	03.09.2013	Adana	Çukurova	<i>Rosa rugosa</i> Thunb. (1784)	35°01'74.66"	37°02'12.73"
87	01.10.2013	Hatay	Samandağ	<i>Citrus sinensis</i>	35°07'18.00"	36°01'29.03"
88	04.11.2013	Adana	Yüreğir	<i>Punica granatum</i>	35°03'41.17"	37°00'12.20"
89	30.07.2013	Mersin	Erdemli	<i>Citrus medica</i> var. <i>sarcodactylis</i> (Siebold ex Hoola van Nooten) Swingle	34°03'38.78"	36°06'24.98"
91	17.06.2014	Mersin	Erdemli	<i>Citrus limon</i>	34°02'96.00"	36°06'24.20"
92	17.06.2014	Mersin	Erdemli	<i>Citrus limon</i>	34°02'68.63"	36°06'03.13"

Table 1. Continued

93	18.06.2014	Adana	Kozan	<i>Citrus sinensis</i>	35°07'30.02"	37°04'29.20"
94	26.06.2014	Mersin	Anamur	<i>Citrus sinensis</i>	32°01'45.00"	36°09'06.10"
99	01.07.2014	Adana	Seyhan	<i>Citrus sinensis</i>	35°01'32.77"	36°09'64.12"
100	05.09.2014	Hatay	İskenderun	<i>Cupressus sempervirens</i> L. (1753)	35°08'81.38"	36°04'05.53"
101	05.09.2014	Hatay	İskenderun	<i>Citrus sinensis</i>	35°08'68.03"	36°03'81.20"
102	05.09.2014	Hatay	İskenderun	<i>Punica granatum</i>	35°08'68.03"	36°03'81.12"
103	07.09.2014	Hatay	Dörtyol	<i>Citrus paradisi</i>	36°01'88.13"	36°08'19.22"
104	07.09.2014	Hatay	Erzin	<i>Citrus reticulata</i> Blanco (1837)	36°02'22.22"	36°09'14.47"
105	31.05.2015	Mersin	Silifke	<i>Citrus limon</i>	33°04'55.00"	36°08'13.11"
106	31.05.2015	Mersin	Mezitli	<i>Citrus limon</i>	34°04'15.00"	36°07'54.77"
107	11.06.2015	Mersin	Mezitli	<i>Citrus sinensis</i>	34°04'54.93"	36°00'79.20"
108	12.06.2015	Mersin	Akdeniz	<i>Citrus sinensis</i>	34°07'55.08"	36°08'55.87"
West Mediterranean						
7	05.07.2011	Antalya	Finike	<i>Cupressus sempervirens</i>	30°08'00.43"	36°02'15.14"
8	06.07.2011	Antalya	Finike	<i>Citrus sinensis</i>	30°08'02.43"	36°02'15.14"
9	08.07.2011	Antalya	Finike	<i>Begonia</i> sp.	30°09'44.90"	36°01'83.82"
10	08.07.2011	Antalya	Finike	<i>Hibiscus rosa-sinensis</i> L. (1753)	30°09'44.90"	36°01'83.82"
21	12.06.2012	Antalya	Finike	<i>Citrus sinensis</i>	30°01'33.33"	36°01'94.96"
22	12.06.2012	Antalya	Finike	<i>Citrus sinensis</i>	30°01'33.33"	36°01'94.96"
25	13.07.2012	Antalya	Alanya	<i>Ceratonia siliqua</i> L. (1753)	31°05'34.14"	36°03'45.70"
30	09.03.2013	Antalya	Alanaya	<i>Rosa rugosa</i>	31°05'32.56"	36°03'55.85"
39	07.06.2013	Antalya	Finike	<i>Citrus sinensis</i>	30°12'24.36"	34°01'67.16"
40	07.06.2013	Antalya	Kumluca	<i>Citrus sinensis</i>	30°01'46.17"	36°03'48.38"
41	07.06.2013	Antalya	Kumluca	<i>Citrus sinensis</i>	30°03'15.13"	36°03'24.78"
42	07.06.2013	Antalya	Kumluca	<i>Citrus sinensis</i>	30°03'42.37"	36°02'75.53"
43	07.06.2013	Antalya	Kemer	<i>Ficus benjamina</i>	30°05'51.03"	36°05'45.38"
44	07.06.2013	Antalya	Serik	<i>Citrus paradisi</i>	35°03'03.93"	37°02'85.44"
50	19.06.2013	Antalya	Gazipaşa	<i>Citrus sinensis</i>	32°01'81.00"	36°02'65.03"
51	19.06.2013	Antalya	Gazipaşa	<i>Citrus sinensis</i>	32°07'35.00"	36°09'56.11"
52	19.06.2013	Antalya	Alanya	<i>Citrus sinensis</i>	32°01'82.08"	36°04'29.02"
53	19.06.2013	Antalya	Manavgat	<i>Citrus sinensis</i>	31°05'18.73"	36°07'58.62"
54	20.06.2013	Antalya	Serik	<i>Citrus limon</i>	30°09'24.73"	36°09'46.72"
55	20.06.2013	Antalya	Serik	<i>Citrus sinensis</i>	30°09'95.28"	37°00'15.02"
56	20.06.2013	Antalya	Aksu	<i>Citrus sinensis</i>	30°08'57.72"	36°09'31.58"
63	04.07.2013	Antalya	Finike	<i>Citrus sinensis</i>	30°09'45.00"	36°04'75.30"
64	04.07.2013	Antalya	Finike	<i>Citrus sinensis</i>	30°01'60.12"	36°01'85.00"
65	04.07.2013	Antalya	Kumluca	<i>Citrus sinensis</i>	30°02'51.42"	36°04'13.00"
66	05.07.2013	Antalya	Konyaaltı	<i>Citrus sinensis</i>	30°06'07.48"	36°08'95.53"
67	05.07.2013	Antalya	Konyaaltı	<i>Punica granatum</i>	30°05'49.48"	36°05'15.52"
68	05.07.2013	Antalya	Konyaaltı	<i>Citrus limon</i>	30°05'39.73"	36°08'84.72"

Table 1. Continued

69	05.07.2013	Antalya	Konyaaltı	<i>Citrus sinensis</i>	30°05'57.88"	36°08'81.73"
72	12.07.2013	Antalya	Gazipaşa	<i>Schinus molle</i> L. (1753)	32°01'84.86"	36°01'61.41"
90	24.05.2014	Antalya	Muratpaşa	<i>Cupressus sempervirens</i>	30°03'84.29"	36°05'43.14"
95	26.06.2014	Antalya	Gazipaşa	<i>Citrus sinensis</i>	32°06'55.00"	36°00'25.10"
96	26.06.2014	Antalya	Manavgat	<i>Citrus sinensis</i>	31°00'85.00"	36°09'55.30"
97	27.06.2014	Antalya	Kemer	<i>Citrus sinensis</i>	30°00'25.00"	36°05'63.52"
98	28.06.2014	Antalya	Aksu	<i>Citrus sinensis</i>	30°08'63.88"	36°09'46.77"
Aegean						
23	26.06.2012	Muğla	Bozburun	<i>Pinus brutia</i> Ten (1815)	28°01'33.69"	36°04'44.04"
28	02.11.2012	Muğla	Bodrum	unknown	28°07'45.92"	37°09'58.78"
31	03.06.2013	İzmir	Menderes	<i>Citrus reticulata</i>	27°08'05.00"	37°03'15.23"
32	04.06.2013	Aydın	Söke	<i>Citrus sinensis</i>	27°32'22.17"	37°42'47.12"
33	06.06.2013	Muğla	Köyceğiz	<i>Citrus sinensis</i>	28°08'15.00"	37°00'33.08"
34	06.06.2013	Muğla	Köyceğiz	<i>Citrus sinensis</i>	28°07'38.13"	36°04'31.90"
35	06.06.2013	Muğla	Ortaca	<i>Citrus sinensis</i>	28°07'96.58"	36°08'19.92"
36	06.06.2013	Muğla	Dalaman	<i>Citrus sinensis</i>	35°02'94.12"	37°00'41.18"
37	06.06.2013	Muğla	Dalaman	<i>Citrus sinensis</i>	28°06'85.00"	36°07'63.52"
38	06.06.2013	Muğla	Fethiye	<i>Citrus sinensis</i>	28°09'63.58"	36°07'41.28"
60	03.07.2013	Muğla	Köyceğiz	<i>Citrus reticulata</i>	28°06'23.88"	36°09'80.57"
61	03.07.2013	Muğla	Köyceğiz	<i>Citrus sinensis</i>	28°07'18.62"	36°08'90.98"
62	04.07.2013	Muğla	Fethiye	<i>Citrus sinensis</i>	29°01'55.00"	36°07'18.43"
76	09.09.2013	Aydın	Sultanhisar	<i>Citrus sinensis</i>	28°02'85.00"	37°08'80.37"
77	10.09.2013	İzmir	Menderes	<i>Citrus reticulata</i>	27°01'38.46"	38°05'05.05"
78	10.09.2013	Aydın	Söke	<i>Citrus sinensis</i>	27°05'40.93"	37°07'14.77"
79	10.09.2013	Aydın	Söke	<i>Portulaca oleracea</i> L. (1753)	27°05'40.93"	37°07'14.77"
80	10.09.2013	Aydın	Söke	<i>Citrus sinensis</i>	27°03'45.00"	37°07'55.17"
81	10.09.2013	Aydın	Söke	<i>Portulaca oleracea</i>	27°03'45.00"	37°07'55.17"
82	10.09.2013	Aydın	Söke	<i>Citrus sinensis</i>	27°05'35.62"	37°03'05.17"
83	11.09.2013	Muğla	Datça	<i>Citrus reticulata</i>	27°06'71.07"	36°05'43.24"
84	12.09.2013	Muğla	Ortaca	<i>Punica granatum</i>	28°07'93.47"	36°05'04.39"
85	12.09.2013	Muğla	Dalaman	<i>Citrus paradisi</i>	28°08'10.02"	36°08'04.62"
86	12.09.2013	Muğla	Fethiye	<i>Citrus sinensis</i>	29°00'50.07"	36°06'99.52"

Phylogenetic analyses

DNA sequences for each of the 108 insects collected in Turkey were obtained in both directions and final base calls were made using FinchTV (FinchTV, 2019). The alignment of sequences was done using Mega 6.0 (Tamura et al., 2013). Both direction sequences were contiged for each individual. DNA sequences from an additional 90 *P. citri* individuals from different countries around the world were obtained for the same COI gene region from NCBI GenBank. Phylogenetic analysis was carried out using the neighbor-joining method (bootstrap 1000) as implemented in MEGA 6.0 (Felsenstein, 1985; Saitou & Nei, 1987) using the Kimura two-parameter model (Kimura, 1980; Tamura et al., 2013), which was the most appropriate model for the datasets as determined in MEGA. A COI sequence from *Planococcus minor* (Maskell, 1897) (Hemiptera: Pseudococcidae) (GenBank accession KY373094) was used as the outgroup.

The haplotype networks of *Planococcus citri* mtDNA COI gene

Mitochondrial COI gene-specific haplotype networks were constructed from DNA sequences from the specimens in this study and reference genes using the median-joining method (Bandelt et al., 1999) contained within the software program PopArt (Leigh & Bryant, 2015). Specimens were color-coded according to geographic region (Table 1) to enable display of the proportion of each haplotype from each region.

Population genetic analysis

The specimens in the research and reference genes were grouped geographically as the Americas (Brazil and the USA), the Middle East (Iran and Turkey), Europe (France and Spain), North Africa (Egypt and Tunisia), South Africa (South Africa) and the Far East (China, Philippines, South Korea and Vietnam) for population genetic analysis. One hundred and ninety-eight specimens were analyzed (Table 2). Population diversity indices: haplotypes number, nucleotide diversity (π), haplotype diversity (Hd), numbers of segregating sites and the average number of pairwise nucleotide differences within the population (K), were calculated using DnaSP 4.5 software. The neutrality indices of Tajima's D and Fu's Fs in each population were also calculated using DnaSP (Librado & Rozas, 2009). The total values were calculated for Turkish specimens and Turkish specimens + reference genes, separately. Arlequin 3.1 was used to calculate pairwise genetic difference (F_{ST}) between all populations (Sharma et al., 2013).

Results

Planococcus citri specimens from the three geographic regions yielded 230 bp COI fragments from 108 samples. The obtained haplotypes sequences in this study have been submitted to GenBank with accessions MN930633 to MN930637. Searching GenBank with these sequences allowed us to obtain an additional 90 COI sequences from *P. citri* individuals from across the globe (Figure 1, Tables 1 & 2).

The Turkish populations have five haplotypes (Hap 1-5). Hap 1 (n=102) was dominant for three subregions (94%) followed by Hap 2 (3%). Besides, two haplotypes from the Aegean Region (Hap 1 and 3), three haplotypes from the East (Hap 1, 2 and 5) and West (Hap 1, 2 and 4) Mediterranean Regions were determined (Tables 1 & 2). Hap 1 on different plants such as weeds, vegetables, shrubs, and trees, Hap 2 on *Schefflera* spp. and *Ficus benjamini* were collected. However, Hap 3 and 4 on *Citrus sinensis*, Hap 5 on *Citrus limon* were detected (Table 1). When the Turkish haplotypes were compared to each other, they were found to have a few bases difference (Table 3).

In addition to the five haplotypes detected in Turkey, an additional nine haplotypes were detected worldwide, giving 14 haplotypes in total. With respect to geographic distribution, there were three haplotypes from the Americas, six from the Middle East, five from Europe, four from the Far East and three from Africa (Table 2). Among the world samples, out of Turkey specimens, Hap 1 (n=44) was again the most common (49%) followed by Hap 2 (n=19; 21%). Hap 1 was the most frequent haplotype present in China and South Korea, while Hap 2 was present in Brazil, South Korea and Philippines.

The phylogenetic relationships of the 14 haplotypes are shown in the phylogenetic tree presented in Figure 2. The tree shows that the 14 haplotypes detected from 198 specimens form two major groups (Figure 2). One of the branches contains Hap 3 and 13, the other branch was two forked and has rest of the other haplotypes except Hap 12. Hap 10, 2, 6 and 14 are grouped on the same subbranch, while Hap 4, 5, 7 and 8 were on the other branch and this branch is also including the most common Hap 1. Only one branch had bootstrap values >50. Hap 12 was grouped in the tree on the same branch with the outgroup sequence from *Planococcus minor*.

Table 2. Accessions of nucleotide sequenced of COI gene of *Planococcus citri* collected in Turkey and from different countries obtained from published databases (n, specimen number)

Country	n	Accession numbers	Number of Haplotype	Group
Brazil	6	KJ530615, KJ530616	1	America
		KJ530612, KJ530613, KJ530614	2	
		KJ530611	6	
USA	2	MJMB463, MJMB373	1	
France	6	JQ085542, GU134705	1	Europe (EU)
		JQ085543, GU134706	2	
		GU134707	10	
		GU168801	11	
Spain	4	JF714200, JF714201	1	
		JF714199	2	
		JF714198	14	
China	34	KY372821, KY373047, KY372583, KY373077, KY372860, KY372516, KY373009, KY372671, KY373012, KY372899, KY372610, KY372545, KY372866, KY372939, KY372979, KY373108, KY373051, KY372496, KY372602, KY372871, KY373081, KP692646, KP692637, KP692648, KP692640, KP692644, KP692641, KP692643, KP692647, KP692639, KY372651	1	Far East
		KY373030, KP692645	2	
		KY372472	7	
Vietnam	4	DSPKJ267, DSPKJ268	1	
		DSPKJ167, DSPKJ166	2	
India	1	KU296034	12	
Philippines	3	DSPKJ110, DSPKJ112, DSPKJ111	2	
South Korea	12	HM474285, HM474286, HM474287, HM474278, HM474284, HM474279	1	
		HM474288, GU936938, HM474280, HM474281, HM474282, HM474283	2	
Turkey	108	<u>East Mediterranean</u> 1, 2, 3, 4, 5, 6, 11, 13, 15, 16, 17, 18, 19, 20, 24, 26, 27, 29, 45, 47, 48, 49, 57, 58, 59, 70, 71, 73, 74, 75, 87, 88, 89, 91, 92, 93, 94, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108 <u>West Mediterranean</u> 7, 8, 9, 10, 21, 22, 25, 30, 40, 41, 42, 44, 50, 51, 52, 53, 54, 55, 56, 63, 64, 65, 66, 67, 68, 69, 72, 90, 95, 96, 97, 98 <u>Aegean</u> 23, 28, 31, 32, 33, 34, 35, 36, 38, 60, 61, 62, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86	1	Middle East
		<u>East Mediterranean</u> 12, 14 <u>West Mediterranean</u> 43	2	
		<u>Aegean</u> 37	3	
		<u>West Mediterranean</u> 39	4	
		<u>East Mediterranean</u> 46	5	
		JF905464	13	
Tunisia	2	MJMB301, MJMB303	1	
		JQ085540	1	
Egypt	3	JQ085544	8	North Africa
		JQ085545	9	
South Africa	12	GBMH1664	1	South Africa
		SIBI435, SIBI436, SIBI43, SIBI438, SIBI439	11	
		SIBI196, SIBI197, SIBI198, SIBI199, SIBI200, SIBI213	13	

Hap 1 and 2 are the two main groups according to haplotype network (Figure 3) and both haplotypes distributed all over the geographic groups except Hap 2, which was not detected in South Africa. Hap 11 and 13 were recorded as common haplotypes in South Africa and are separately localized on the haplotype network (Figure 2). The single nucleotide differences from Hap 1 generally detected in the network.

High mitochondrial DNA diversity was revealed overall for the populations examined according to molecular diversity indices (Table 4). Aegean has higher K (0.182) and π (0.00076) values and the lowest Hd (0.019) value, while West Mediterranean Region has highest Hd (0.127) value among the regions of Turkey. However, the highest Hd (0.800) values for Europe, the highest K (1.200) and π (0.00525) values were for North Africa in comparison of world populations. Further, Tajima's D test and Fu's FS test showed negative values, except in South Africa, indicating deviations from neutrality. Pairwise comparisons of the different populations using the F_{ST} was significant for all comparisons except those between the Americas and the Far East, the Americas and Europe, North Africa and the Far East, and lastly Europe and the Far East (Table 5).

Table 3. Sequences Differences between the five haplotypes of the COI gene of *Planococcus citri* from Turkey

Haplotype number	(position in alignment) BP				
	50	107	114	125	146
Haplotype 1	T	T	A	C	A
Haplotype 2	T	T	A	C	T
Haplotype 3	T	C	A	T	A
Haplotype 4	C	T	A	C	A
Haplotype 5	T	T	G	C	A

Table 4. Diversity and neutrality indices of *Planococcus citri* populations calculated from nucleotide sequence of mitochondrial COI gene

Geographic origin	n	S	K	H	Hd±S.D.	π	D	Fu's Fs
E. Med_TR	54	2	0.110	3	0.108±0.057	0.00056	-1.31	-2.42
W. Med_TR	31	2	0.129	3	0.127±0.080	0.00054	-1.51	-2.40
Aegean_TR	22	2	0.182	2	0.019±0.081	0.00076	-1.51	-0.11
Turkey	108	5	0.129	5	0.108±0.041	0.00084	-1.82*	-5.63**
America	8	2	0.821	3	0.679±0.122	0.00364	0.24	-0.15
Europe	10	4	1.156	5	0.800±0.100	0.00506	-0.72	-1.90
Far East	54	8	0.651	4	0.445±0.061	0.00284	-1.69	-0.16***
South Africa	12	2	1.076	3	0.621±0.087	0.00471	1.82	0.84
Middle East	109	5	0.145	6	0.124±0.043	0.00063	-1.78	-7.31*
North Africa	5	3	1.200	3	0.700±0.218	0.00525	-1.05	-0.19
Worldwide	198	15	0.526	14	0.397±0.042	0.00232	-4.74*	-13.40***

Statistical differences *, P<0.05; **, P<0.02; ***, P<0.01;

East Mediterranean Region: Adana (n=29), Mersin (n=20), Hatay (n=6); West Mediterranean Region: Antalya (n=31); Aegean Region: İzmir (n=2), Aydın (7), Muğla (13); Turkey: Adana, Antalya, Mersin, Hatay, Muğla, Aydın, İzmir, Artvin (1); the Americas (Brazil and USA); Europe (France and Spain); the Far East (China, Philippines, South Korea and Vietnam); South Africa; the Middle East (Iran and Turkey); North Africa (Egypt and Tunisia); Worldwide: Americas, Europe, the Far East, South Africa, the Middle East and North Africa.

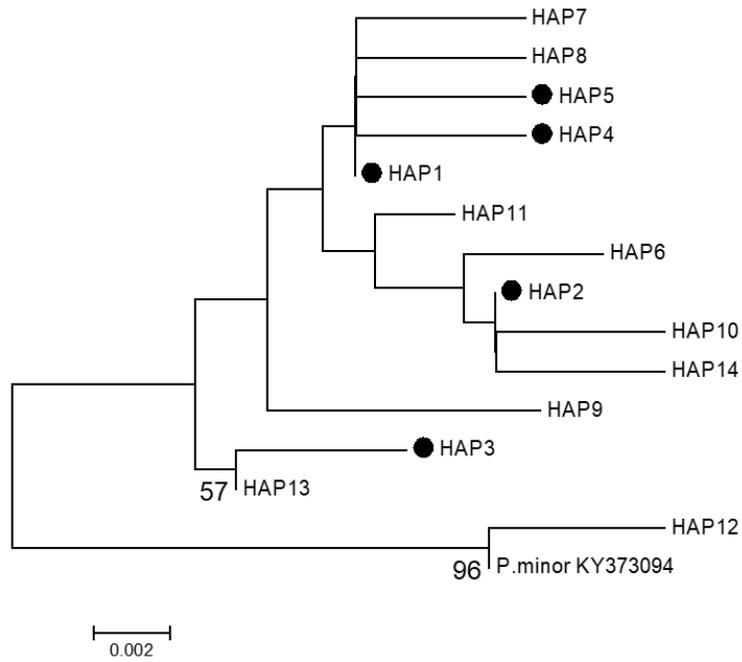


Figure 2. Dendrogram constructed by neighbor-joining method from the COI sequences of *Planococcus citri* analyzed in the present study along with those retrieved from GenBank belong to the Americas, Europe, the Far East, the Middle East, North Africa, and South Africa. Bootstrap values greater than 50% are indicated at branch nodes. Outgroup, *Planococcus minor*. Dots indicate the Turkish haplotypes.

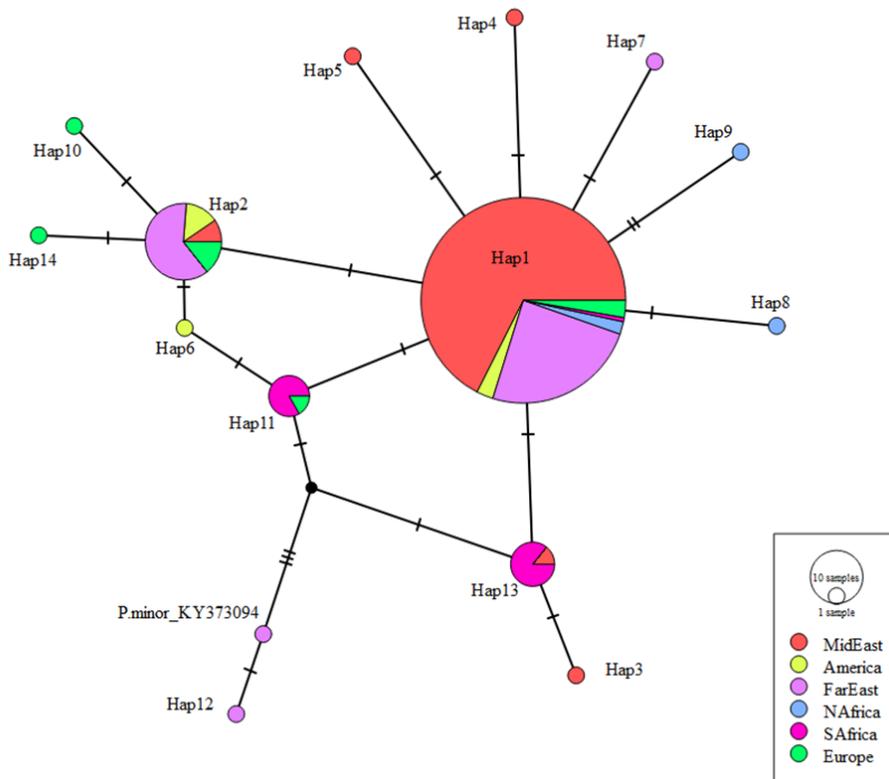


Figure 3. Haplotype network of the 14 haplotypes identified in *Planococcus citri*. Tick marks show base differences between haplotypes.

Table 5. Pairwise genetic distance (F_{st}) between different world samples of *Planococcus citri* calculated from nucleotide sequence of mitochondrial COI gene

	America	Far East	Middle East	North Africa	Europe
Far East	0.034				
Middle East	0.549*	0.142*			
North Africa	0.193*	0.134	0.358*		
Europe	0.000	0.057	0.520*	0.161*	
South Africa	0.331*	0.369*	0.631*	0.258*	0.318*

* The asterisk indicates statistical difference between the geographical regions ($p < 0.05$).

Discussion

The research revealed that *P. citri* has significant genetic differentiation with a few base shifts based on the used COI molecular marker. *Planococcus citri* has two predominant haplotypes Hap 1 and 2 both Turkey and over the world and other haplotypes modified from these two haplotypes are apparent in the haplotype network. Haplotype networks are an intuitive method for visualizing relationships between individual genotypes at the population level (Leigh & Bryant, 2015). The differences between the sequences generally located on the different point of the sequences and with limited nucleotide substitutions. Detected fourteen haplotypes all around the world were close to each other according to the phylogenetic tree. Hap 1 clustered with Hap 4 and 5 from Turkish haplotypes, Hap 7 from China and Hap 8 from Egypt. All these are connected each other via an historical trade route (the Silk Road). Moreover, Hap 2 clustered with Hap 10 and 14 from Europe, Hap 6 from Brazil and Hap 11 from South of Africa this also another trade route (the Spice Road). Along this road, Brazil is out the group but it has connection with Spain and Portugal culture. America and Europe have totally same haplotypes, likely due to transportation of the pest by plant trade.

Hap 12 (GenBank accession KU296034) from India was more closely related to the outgroup haplotype from *P. minor* than it was to *P. citri*. These two-mealybug species are very difficult to distinguish from each other morphologically (Cox & Wetton, 1988) and, the identification technique to separate these two species is based on Cox Score. Cox score uses point system and if the score less than 35 meaning *P. minor*, if higher it is *P. citri*. (Cox, 1989). Nagalakshmi (2019) reported that the separation of these two species is based on variation in numbers of ventral oral collar tubular ducts. Wang et al. (2016b) detected the nucleotide sequence identity of 5' and 3' COI gens of between *P. citri* and *P. minor* were 97-98% and 96-98%, respectively. And the sequences have stable species-specific identification sited on 5' and 3' for both species. Moreover, *P. citri* and *P. minor* clustered on the same branch for COI region among 54 mealybug species as a monophyletic group, the genetic distance between two species was 1.96% for nearest neighbor analyses (Wang et al., 2016a).

East and West Mediterranean Regions of Turkey have similar H_d , π , and K values and the values were higher than Aegean Region. Tajima's D and F_u 's F_s values of Turkey which calculated from three subregions were negative and statically significant show that the population have rare alleles and expecting a new population expansion in the region. The Middle East has more haplotypes than the other regions, however, the Far East has more segregating sites, Europe has the highest H_d value, even low sample number. Far East and Middle East also have statistically significant F_u 's F_s values. If the expansion of *P. citri* had started from the Far East throughout the world, occurring a high of number of rare alleles from the region might be considered normal. Europe and the Americas specimens were genetically totally same according to F_{ST} . The Middle East and South Africa are further apart.

Planococcus citri has wide morphological variation indicating that it is a complex of different ecological, biological and geographical races (Ferris, 1950; de Lotto, 1964; Padi, 1990) or possibly contains cryptic species (Rung et al., 2009). For example, *P. citri* on roots of coffee is considered to be a different

race from mealybugs feeding on aerial parts of coffee from East Africa (de Lotto, 1964). Both races have different morphological characters such as size, shorter antenna and legs and dermal suture number. Moreover, the race living on roots feeds on fungi. However, research on chromosomal patterns and symbiotic organisms of the two races did not show them to be different from *P. citri* (de Lotto, 1964). So, the single haplotypes, for example only one individual from Hap 3, 4 and 5 obtained in Turkey, should be investigated in this respect.

While Hap 2 was found on *Ficus* sp. in our research, it was characterized generally on the ornamental plants and other trees in the different researches such as *Ficus* sp. and *Kalanchoe* sp. (Abd-Rabou et al., 2012), *Mackaya bella* Harv., 1859 (Acanthaceae) and *Clerodendrum* sp. (Malausa et al., 2011), *Bischofia javanica* Blume, 1827 (Wang et al., 2014). These findings may support that Hap 2 generally does not prefer *Citrus* sp. and might represent a race or cryptic species.

Increasing International trade and intercontinental transportation activities of ornamental plants, especially from the Far East, may be causing a breakdown of biogeographic barriers within and between species. Quarantine measures generally target prevention of entry of new species to a region. However, the movement of cryptic species or haplotypes to a new region is difficult to control, because we lack information on the population genetic history of the species. Citrus mealybug probably originated from China and spread from there throughout the world (Barlet, 1978). Given its invasive dispersal potential, it now occurs all around the world including Africa, America, Asia and Europe (García Morales et al., 2016). As the Far East including China, India is considered the center of origin of citrus (Gmitter & Hu, 1990; Nicolosi, 2007), therefore transportation of the fruit and propagating material from this region may explain the spread of the citrus mealybug. Another dispersal mechanism is long distance transport of ornamental plants. As a result of this trade, over the last two decades two important pests have established well away from their origin, *Bemisia tabaci* (Gennadius, 1889) (Hemiptera: Aleyrodidae) subspecies (de Moraes et al., 2018) and *Thrips hawaiiensis* (Morgan, 1913) (Thysanoptera: Thripidae) (Reynaud et al., 2008; Atakan et al., 2015). The Far East is the likely origin of these pests, paralleling to the distribution of citrus plants throughout the world (Bartlett, 1978).

The study indicates that Hap 1 and 2 are possibility different cryptic species based on few base changes, because of different host plant range. Different cryptic species might not be so significant for chemical control tactics. However, they might be important for biological control strategies, because of their effect on fitness cost of parasitoids (Forbes et al., 2012; He et al., 2019). Insecticide pressure on the pest directly affects its survival ability and might cause genetic modification of the insect. The data from lower Seyhan revealed no genetic differentiation of *P. citri* populations in that district. However, this does not mean that the district does not have variation, as the small dataset is inadequate to support that conclusion. The information is insufficient to ascertain the biotype of the citrus mealybug so further research with different genomic regions or techniques is needed to provide more specific information about the *P. citri* complex.

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