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Original scientific paper

ISSR MARKER BASED POPULATION GENETIC STUDY OF MEDITERRANEAN FRUIT FLY *Ceratitis capitata* (Diptera: Tephritidae)

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The Mediterranean fruit fly, *Ceratitis capitata*, is a serious pest of agricultural resources. Despite its economic importance, the population genetic structure of this species is still poorly investigated at micro-geographical level, especially from eastern Mediterranean basin. Knowledge about the genetic structure of *C. capitata* populations is a necessary requisite for understanding population history of the species and designing successful regional eradication programs. In the current study, the inter-simple sequences repeat (ISSR) markers were employed to assess the genetic diversity and population structure of seven natural populations of *C. capitata* that were collected from different regions of Turkey. Low to moderate levels of genetic diversity were observed. The estimated values for gene flow (*Nm*) and coefficient of genetic differentiation among populations (G_{ST}) were 3.07 and 0.14, respectively. The results of Principle Component Analysis (PCoA) and Unweighted Pair Group Arithmetic Mean Analysis (UPGMA) tend to be uniform in whole, the Antalya populations was clearly separated from the rest. Local environmental conditions, such as differences in pest control management strategies, agricultural practices, microclimates and human mediated transportations might be important factors in shaping the genetic structure of this species in Antalya. This paper provides useful data for understanding population genetic structure of *C. capitata* populations in eastern Mediterranean basin and development of effective regional pest management strategies.

Keywords: Ceratitis capitata, Genetic variability, ISSR, Colonization process, Turkey

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INTRODUCTION

The invasive Mediterranean fruit fly (also known as medfly), *Ceratitis capitata* (Diptera: Tephritidae), is one of the major economical and agricultural pest species in the tropical and temperate regions of the world. The range of host records for *C. capitata* is relatively large, it infests more than 260 species of fruits and vegetables (WHITE and ELSON-HARRIS, 1992). Medfly is responsible for direct economic loses in fruit production and is the target of costly early detection, control and eradication programs in all areas where it has become established or re-introduced every year (BEROIZ *et al*., 2012). In Turkey, this pest also has important impact on the crop production, it is estimated that it can reduce crop yield between 5-80 percent (AK *et al*., 2008).

Advances in molecular technologies with modern statistical tools are contributing significantly to our knowledge of population structure and dynamics in many insect species. Over the last two decades, the genetic structure of medfly populations, both at the macro and micro geographical levels, and different aspects of the colonization process have been extensively studied (MALACRIDA *et al*., 1992; GOMULISKI *et al*., 1998; MALACRIDA *et al*., 1998; BARUFFI *et al*., 1995; HE and HAYMER, 1999; BONIZONNI *et al*., 2000; GASPERI *et al*., 2002; BONIZONNI *et al*., 2004; REYES and OCHANDO, 2004; KARSTEN *et al*., 2013). The results indicated that under its own dispersal capabilities and human mediated transport *C. capitata* has moved from its putative source area in sub Saharan Africa to Spain probably through the straits of Gibraltar and then spread from Iberian Peninsula to other Northern Mediterranean countries and finally to many areas with temperate and tropical climates such as South and Central America, Hawaiian Islands and Australia in less than two centuries. In addition, in recent years medfly invasions have also been detected in parts of North America, including Florida, and California (BONIZONNI *et al*., 2001).

The general trend of decreasing level of variability from its putative source area towards those areas recently colonized is characteristic for this insect species (MALACRIDA *et al*., 1992; GOMULISKI *et al*., 1998; MALACRIDA *et al*., 1998). Prior studies have also noted that different types of evolutionary forces such as, genetic drift, gene flow, selection and/or bottleneck effect contributed, singly or in concert, to genetic changes during the process of colonization by *C. capitata* (GASPERI *et al*., 1991; MALACRIDA *et al*., 1998; GOMULISKI *et al*., 1998; GASPERI *et al*., 2002).

DNA-based molecular marker systems have been used in wide range of organisms. The inter-simple sequences repeat (ISSR) markers permit detection of variations in inter-microsatellite loci using specific primers synthesized from dinucleotide or trinucleotide repeat structures. The initial studies revealed the universal and multilocus nature of ISSR markers. Later, the potential of this method for population genetic studies have been verified in various insect pests (SOLIANI *et al*., 2010; MENDKI *et al*., 2011; XIE *et al*., 2014) including *Ceratitis* fruit fly species (BEROIZ *et al*., 2012).

Eastern Mediterranean basin seems to have been the putative source area for expansion of this species to other regions of the world (BONIZONNI *et al*., 2004; GÜLER *et al*., 2019). However, there have been very limited studies on the geographic patterns of genetic variation and population structure in *C. capitata* from this region. GÜLER *et al*., (2019) analyzed 7 Turkish populations of Mediterranean fruit fly by sequencing two mitochondrial DNA (mtDNA) regions

within the *cytochrome oxidase I* and *ND4-ND5* genes. These authors reported low level of genetic diversity and no significant variation among the populations studied. However, mtDNA markers are not the most ideal option to resolve contemporary intra-population genetic structures because of its lower evolution rate (comparing with many nuclear markers), single locus nature and selection constraints (MORITZ *et al*., 1987; AVISE, 1994; LUNT *et al*., 1996; GRAPPUTO *et al*., 2005). More comprehensive resolution of population genetic structure needs analysis of nuclear DNA encoded markers. Highly polymorphic markers such as ISSRs have proved to be valuable tool in deep genetic studies of insect species as described above. In this work, we further investigated the population structure of natural *C. capitata* populations, collected from different geographical regions of Turkey which covers large part of Eastern Mediterranean area. Being a multivoltine, highly polyphagous and economically important pest species, the knowledge gained from the genetic studies of natural populations of *C. capitata*, with no doubt, will contribute greatly to our understanding the patterns of invasion and subsequently provide important insights in designing successful strategies for control programs.

MATERIALS AND METHODS

Insect populations

In the present survey, seven wild populations of *C. capitata* from different areas of Turkey were studied. The geographical location of each population was shown in Figure 1. Sampling locations were chosen in fruit producing areas where fruits are severely damaged by medfly. Adult flies were obtained from citrus farms during the period of July- September 2016 by using field traps and then stored at -80°C until DNA extraction was performed.

Figure 1. Map showing the collection sites of *C. capitata* from Turkey

DNA extraction and ISSR amplification

After morphological identification of the collected specimens, genomic DNA was extracted from each single fly according to a standard protocol of BENDER *et al*. (1983).

A total of 10 ISSR primers developed by University of British Colombia, Canada, were initially screened and 6 primers which produced clear, bright and reproducible fragments across the amplifications were selected for further study (Table 1). The DNA amplification mixture of 25 µl contained 50 ng genomic DNA, 2.5 µl 10 x PCR buffer (Fermantas, Lithuania), 4 mM $MgCl_2$, 2 µM primers, 200 mM of each dNTP and 1 U Taq DNA polymerase (Fermantas). To minimize the pipetting error, the PCR components were prepared as master mix for each primer. The amplification reactions were performed in thermal cycler (Eppendorf Master Cycler) with amplification cycle condition programmed for an initial hold of 4 min for strand separation at 94°C followed by 30 cycles of 94°C for 20 sec, 48, or 55°C depending on the primers used for 40 sec, 72°C for 2 min and final extension at 72°C for 8 min. Each amplification reaction was performed at least twice in order to score clearly reproducible bands. Amplified products were separated on 2% agarose gel containing ethidium bromide and digitally photographed by Vilber Lourmat gel documentation system. A 1kb ladder marker (Fermantas) was used as a molecular size standard for evaluation of amplified DNA band sizes and all bands were visually inspected. Amplified bands were scored for their absence (0) or presence (1) to create a matrix dataset with all individuals. Since ISSR markers are dominant markers each band was considered to represent the phenotype at a single biallelic locus. A total of 105 specimens from 7 populations were randomly selected for analysis.

Table 1. Primers for ISSR analysis of Mediterranean fruit fly populations in Turkey.

Primer	Sequence (5′ to 3′)	Annealing temp. (°C)	Range of amplicon (bp)	Number of bands scored	Number of Polymorphic Bands	Percent polymorphic bands
ISSR1	ACC ACC ACC ACC ACC ACC CC	55	210-550	14	12	85.7
ISSR2	CCA TGT GTG TGT GTG TGT	55	500-1300	13	12	92.3
ISSR3	CCA TGA TGA TGA TGA TGA TG	55	500-1500	12	11	91.7
ISSR4	GCA ACA CAC ACA CAC AC	48	480-1100	16	14	87.5
ISSR5	GGG ACA CAC ACA CAC AC	55	400-1050	15	14	93.3
ISSR6	GAG AGA GAG AGA GAG AGG	48	470-1300	17	14	82.3
			Total	87	77	88.51

Statistical analysis

The genetic diversity and variability in the populations were interpreted by using POPGENE software v. 1.32 (YEH *et al*., 1999). The following parameters were calculated for each population, the observed number of alleles (*Na*), effective number of alleles (*Ne*), number of polymorphic loci (*N*), percentage number of polymorphic loci (*P*), Nei's genetic diversity (*H)*

(NEI, 1973), Shannon's diversity information index (*I*), and coefficient of genetic differentiation among populations (G_{ST}). Gene flow was calculated from G_{ST}, expressed as (Nm) = 0.5(1–G_{ST})/G_{ST}. Based on matrices obtained from ISSR data, genetic distances (D_N) among populations were calculated according to NEI (1972). The relations between populations have also been depicted by a dendogram obtained from D_N values. The tree was constructed based on Unweighted Pair Group Arithmetic Mean Analysis (UPGMA). Principle coordinate analysis (PCoA), an effective tool for interpreting population relationships, was performed to ascertain and graphically visualize the genetic relationships among medflies at the population level using Genetix 4.05 software (BELKHIR *et al*., 2004). Hierarchical analysis of molecular variance (AMOVA) was carried out using Arlequin v. 3.5.2 software (EXCOFFIER and LISCHER, 2010) to investigate the genetic structure and variability within and between populations.

RESULTS

The primers amplified a total of 87 distinct bands, 77 polymorphic and 10 monomorphic with an amplicon size ranging from 210bp to 1500bp (Table 1). The total number of bands produced by individual primer ranged from 12 (ISSR-3) to 17 (ISSR-6) with an average of 14.5 bands. Primer ISSR-6 showed the lowest percentage of polymorphic bands (82.3%) whereas the highest polymorphic amplification of 93.3% was found with primer ISSR-5. Overall the mean percentage of polymorphic bands by all primers was 88.51%. Several ISSR fragments were specific for some populations and/or individual flies.

The genetic diversity parameters estimated from 7 geographical populations of *C. capitata* are presented in Table 2. The lowest level of polymorphism was observed in İzmir, Antalya and Mersin populations (33.7%) and the highest in Yalova, Aydın and Muğla populations (44.16%). The average polymorphism for all populations was 38.59%. For each population, the observed number of alleles ranged between 1.34 to 1.44, effective number of alleles from 1.16 to 1.2, the genetic diversity of each site ranged from 0.10 to 0.12. The Shannon's information index values varied from 0.15 to 0.19. Among all populations Muğla and Aydın had the highest level of genetic variability values. The genetic differentiation coefficient (G_{ST}) among the population was 0.14.

Table 2. Genetic diversity parameters in the seven populations of C. capitata detected by ISSR markers.

Population name	*N*	*P*	*Na*	*Ne*	*H*	*I*
Yalova	15	44.16	1.44 ± 0.50	1.16 ± 0.27	0.11 ± 0.16	0.17 ± 0.23
İzmir	15	33.77	1.34 ± 0.48	1.17 ± 0.29	0.10 ± 0.17	0.16 ± 0.24
Aydın	15	44.16	1.44 ± 0.50	1.20 ± 0.32	0.12 ± 0.18	0.19 ± 0.25
Muğla	15	44.16	1.44 ± 0.50	1.20 ± 0.32	0.12 ± 0.17	0.19 ± 0.25
Antalya	15	33.77	1.34 ± 0.48	1.18 ± 0.32	0.10 ± 0.17	0.16 ± 0.25
Mersin	15	33.77	1.34 ± 0.48	1.16 ± 0.32	0.10 ± 0.17	0.15 ± 0.24
Adana	15	36.36	1.36 ± 0.48	1.16 ± 0.27	0.10 ± 0.16	0.16 ± 0.24
Average	15	38.59	1.39 ± 0.49	1.18 ± 0.30	0.11 ± 0.17	0.17 ± 0.24

N, Sample size; P, percentage of polymorphic band; Na, observed number of alleles; Ne, effective number of alleles; H, Nei's gene diversity; I, Shannon's information index.

Genetic distance values were used to quantify the relationships among the populations. The Nei's genetic distances between all pairwise comparisons were, in general, low. The distance values ranged from 0.0471 to 0.1574 (Table 3). These low distance values indicate high genetic similarity among the populations. The lowest level of genetic distance was observed between Mersin and Adana populations whereas the highest one was between İzmir and Antalya populations. The high distance values were seen when Antalya population was compared with other populations which indicates that insects from this region might originate from different sources.

*Table 3. Genetic distance estimated among the seven populations of Mediterranean fruit fly, C. capitata***.**

Population Name	Yalova	İzmir	Aydın	Muğla	Antalya	Mersin	Adana
Yalova	*						
İzmir	0.0672	*					
Aydın	0.0573	0.0924	*				
Muğla	0.0666	0.0561	0.0508	*			
Antalya	0.1566	0.1574	0.1501	0.1375	*		
Mersin	0.0655	0.0767	0.0596	0.0545	0.0889	*	
Adana	0.0559	0.0564	0.0737	0.0644	0.1262	0.0471	*

When a dendogram based on genetic distances was constructed all Turkish populations, except the population collected in Antalya, were clustered together (Figure 2). Consistent with the results of UPGMA, in principle coordinate analysis Antalya population was also clearly separated from all other populations and distributed in the lower left-hand quadrant (Figure 3).

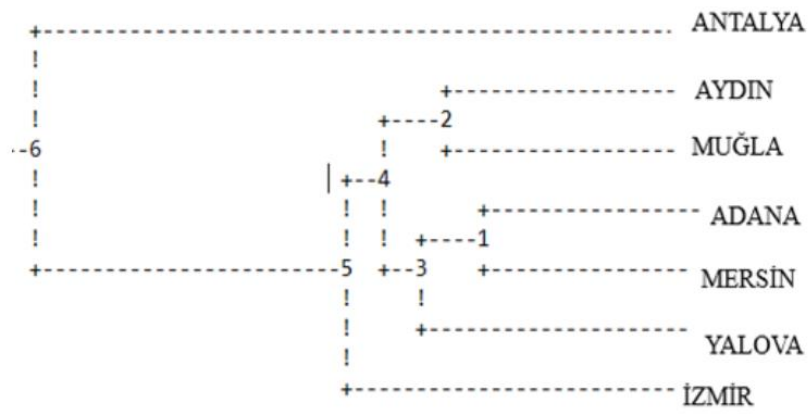

Figure 2. UPGMA dendrogram for the seven populations of *C. capitata*

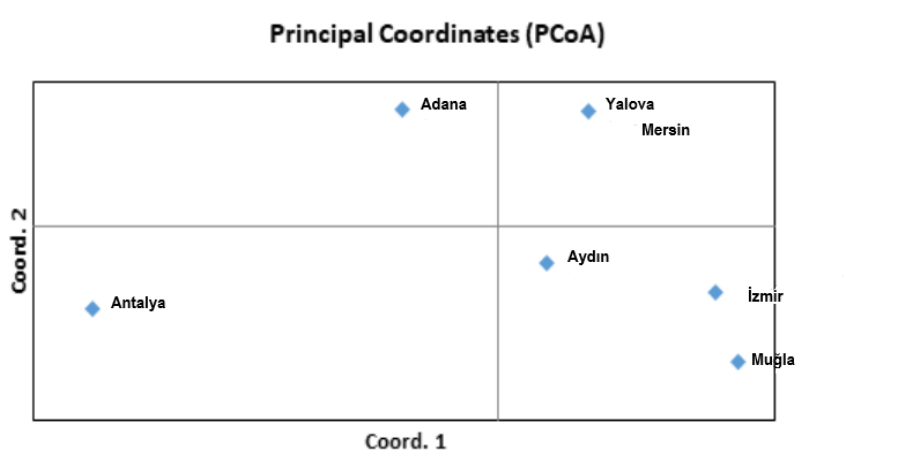

Figure 3. Principle Coordinate Analysis of the seven geographic populations of *C. capitata*

AMOVA quantified the partition of genetic variation among and within 7 different sample groups. The results revealed high levels of within population variability (93%) and low inter-population differentiation (7%).

DISCUSSION

The invasive Mediterranean fruit fly *C. capitata*, is among the world's most dangerous agricultural and economical pest species which limits the access of products to international markets. Deepening our knowledge on fruit flies including that on genetic structure, genetic diversity and dispersal patterns is essential for understanding their invasion risk and damage in agricultural landscapes. In this paper, we examined the genetic diversity and population structure of *C. capitata* samples collected from different geographical areas of Turkey. Several important findings raised from this study, with significant implications for Eastern Mediterranean region where this pest occurs.

Compared to their native ranges alien/invasive species are expected to have reduced genetic variability in their introduced ranges. In general, our results revealed that Turkish *C. capitata* populations are characterized by low to moderate level of genetic diversity and limited population differentiation. The diversity values reported here are lower than those reported from other regions of the world by employing different kind of markers; Central (BONIZONNI *et al*., 2000; GASPERI *et al*., 2002; BONIZONNI *et al*., 2004) and South Africa (KARSTEN *et al*., 2013), and western Mediterranean countries (BONIZONNI *et al*., 2000; GASPERI *et al*., 2002; BONIZONNI *et al*., 2004). Furthermore, the detected the mean percentage of polymorhic loci (38.59%) was lower in comparison with those obtained in other ISSR and RAPD studies. In natural African

populations this value ranged from 80 to 90% (BARUFFI *et al*., 1995; ALAOUI *et al*., 2010). In western Mediterranean populations it varied from 55 to 81% (BARUFFI *et al*., 1995; ALAOUI *et al*., 2010; BEROIZ *et al*., 2012). Several possible scenarios may explain the observed lower level of genetic variability values in our region.

Over the historical aspect of the colonization process some fruit fly species have experienced significant changes in their population structure and range expansion. Chronological records and genetic studies have revealed that *C. capitata* populations are subdivided into 3 main categories, i) ancestral populations in Sub-saharan Africa, ii) ancient populations in the Mediterranean basin, and iii) more recent populations in America and Pacific area (MALACRIDA *et al*., 1992; BARUFFI *et al*., 1995; GASPERI *et al*., 2002). This geographical dispersal from its putative source area, in Sub-Saharan Africa, to Greece is associated with a gradual decrease in genetic variability which is consistent with medfly colonization history probably through subsequent drift, founder effects and bottlenecks (GASPERI *et al*., 1991; MALACRIDA *et al*., 1992; BARUFFI *et al.*, 1995; MALACRIDA *et al*., 1998; GOMULSKI *et al*., 1998; GASPERI *et al*., 2002). Given the relatively lower polymorphism rate seen in samples from Turkey we reasonably propose that our results confirm and support the existence of a decreasing trend in the levels of genetic polymorphism from south Africa to eastern Mediterranean region and agree with the framework for the dispersion history of this pest species (*i.e*. Turkey falls within the ancient range but not an area of natural distribution of *C. capitata*). In Mediterranean basin the presence of *C. capitata* was first recorded in Spain in 1842, then in Italy in 1863 and in Turkey 1904 (FIMIANI, 1989).

Current management of med fly in Turkey mainly depends on the use of insecticides (AK *et al*., 2008). The selection intensity (quantity, time, and techniques of insecticide use, control and eradication practices, pest management strategies, in general) might be strong enough to be responsible, at least in part, for the low variability observed in Turkish populations.

A number of evolutionary factors such as mode of reproduction, mating system, natural selection and gene flow can affect the population genetic structure of a species (HAMRICK and GODT, 1989). High rates of gene flow among populations can reduce differences in their genetic structure. In our study the calculated gene flow among sampling locations was quite high (*Nm*=3.07) consistent with the previous reports for the Mediterranean Spanish populations, *Nm* values were 2.8 and 3.9 for RAPD and ISSR markers, respectively (BEROIZ *et al*., 2012). According to WRIGHT (1951) a value of gene flow greater than one is expected to lower the genetic differentiation of populations. Considering limited dispersal ability of *C. capitata* (KARSTEN *et al*., 2013), the documented level of gene flow in the present study is probably the result of human mediated dispersal (such as fruit trade, the movement of nursery and ornamental plants, tourist industry) and continual distribution of favorable habitats without big rivers and high mountains in the region. Regardless of the precise mode of movement, in long term, the result is potentially enhanced the genetic homogenization, *i.e.*, weakens the genetic differentiation over the widely distributed geographic populations. The AMOVA analysis also revealed that most of the genetic variation resided within populations, as already reported for this species using different kinds of markers (HE and HAYMER, 1999; BONIZONNI *et al*., 2001). Thus, our findings indicate that high gene flow influences the pattern of genetic variation in *C. capitata* populations, particularly its geographic uniformity. Our results will have extensive applicability

to other fruit producing areas and be an important step for the future invasion study of Mediterranean fruit fly.

The results of dendogram and PCoA were almost consistent, two genetically distinct groups were recovered in Turkey. All Turkish populations except the population in Antalya were grouped together. We can propose that the genetic structure of *C. capitata* in Antalya is, probably, the result of complex interactions among different forces at play at local scales (such as differences in pest control management strategies, agricultural practices, microclimates and/or topology, multiple introductions or single introduction of a large number of individuals from different populations). Antalya, the most important tourism center in Mediterranean region, is essentially climatically suitable for medfly and host plants. There are also active international trade routes linking fruit distribution to other regions. Since the Antalya population is genetically distanced from all other populations detailed molecular analysis of this population will clarify the variation that we observed.

In depth knowledge about the population genetic aspects of economically important agricultural pest species is essential for the development of sustainable management strategies. Genetic markers are valuable tools for the analysis of genetic variability, invasion and dispersal patterns and phylogenetic relationships of insects. Given the migrant nature of *C. capitata* environmentally friendly control strategies can only be developed by strengthening regional and international corporation and communication in Mediterranean basin. In future extensive genetic characterization of this important pest species will help to provide valuable information about development of area wide control strategies.

Despite its economic importance, the knowledge of genetic structure of *C. capitata* populations from eastern Mediterranean area is very limited. In the present study we aimed to investigate the current genetic structure and diversity of *C. capitata* populations from different geographic regions of Turkey. Results revealed that Turkish populations showed low to moderate level of genetic polymorphism compared with those seen in other regions of the world. It can be proposed that gene flow in combination with various selection forces (in the form of agricultural practices, pest management strategies etc.) are responsible in shaping the geographic patterns of variation in our region. The information obtained from this study is significant for more comprehensive understanding of the overall genetic structure and history of medfly populations in eastern Mediterranean basin. Our results also proved the validity and suitability of ISSR markers to detect geographic patterns of genetic variation in natural populations of *C. capitata.*

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GENETIČKO PROUČAVANJE POPULACIJE MEDITERANSKE VOĆNE MUVE *Ceratitis capitata* (Diptera: *Tephritidae*) POMOĆU ISSR MARKERA

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Izvod

Mediteranska voćna muva, *Ceratitis capitata*, je ozbiljna štetočina u poljoprivredi. Uprkos svom ekonomskom značaju, populacijska genetska struktura ove vrste je još uvek slabo istražena na mikrogeografskom nivou, posebno iz istočnog mediteranskog basena. Znanje o genetskoj strukturi populacija *C. capitata* neophodan je uslov za razumevanje istorije populacije ove vrste i kreiranje uspešnih regionalnih programa iskorenjivanja. U ovom radu ISSR markeri korišćeni su za procenu genetske raznolikosti i strukture populacije sedam prirodnih populacija *C. capitata* koje su prikupljeni iz različitih regiona Turske. Primećeni su niski do umereni nivoi genetske raznolikosti. Procenjene vrednosti za protok gena (*Nm*) i koeficijent genetske diferencijacije među populacijama (G_{ST}) bile su 3.07 i 0.14, respektivno. Rezultati PcoA i UPGMA analize uglavnom su ujednačeni, populacije Antalije jasno su odvojene od ostalih. Lokalni uslovi životne sredine, kao što su razlike u strategijama suzbijanja štetočina, poljoprivredne prakse, mikroklime i transporta mogu biti važni faktori u oblikovanju genetske strukture ove vrste u Antaliji. Ovaj rad pruža korisne podatke za razumevanje genetske strukture populacije *C. capitata* u basenu istočnog Mediterana, kao i za razvoj efikasnih regionalnih strategija upravljanja štetočinama.

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