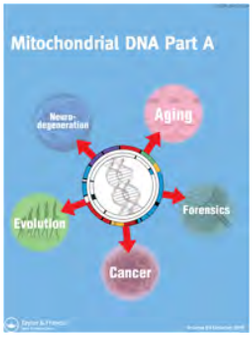

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RESEARCH ARTICLE

Population structure and patterns of geographic differentiation of *Bactrocera oleae* (Diptera: Tephritidae) in Eastern Mediterranean Basin

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ABSTRACT

The olive fly (*Bactrocera oleae*) is the most destructive pest of olives in most commercial olive-growing regions worldwide. Significant economic damage to olive production is caused by the larvae of this fly, which feed on the pulp of *Olea* fruits. Studying the genetic structure of insect pest populations is essential for the success of pest management strategies. Our primary goal in the present study was to examine the population structures of olive flies collected over a wide geographic area from Turkey, a representative of eastern Mediterranean region, using two mitochondrial DNA sequences as genetic markers. The data revealed a high level of genetic variability in olive fly populations and a moderate level of genetic differentiation between Mediterranean and Aegean populations in Turkey. We also merged the sequences obtained in the present study with previously published sequences from across the world into the data matrix. Strong population substructure and a significant correlation between genetic and geographic distances were detected in northern Mediterranean basin populations of *B. oleae,* indicating the possibility of a westward expansion of the species in the continent. In addition, our results revealed a very close genetic relationship between the Aegean and Iranian populations, which suggests that *B. oleae* was introduced to Iran from western parts of Turkey. However, additional markers and analytical approaches are required to determine the exact colonization route of olive fly.

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KEYWORDS
Bactrocera oleae; population structure; mitochondrial variation; colonizing species; gene flow; olive

Introduction

Olive, the most emblematic tree crop of the Mediterranean basin, is one of the oldest agricultural tree crops in the region, with remarkable historic, cultural, nutritional, and economic significance to the people of the area for many millennia. Besides its important socio-economic role, its hardiness and longevity also represent the values that the Mediterranean cultures hold central. The olive fly, *Bactrocera oleae* (Diptera: Tephritidae) is the most destructive pest of olive trees worldwide, and causes significant production losses of olives and its derivatives in the Mediterranean area, where 95% of the world's cultivated olive trees are grown. The extent of loss varies from 5% to 30%, depending on the environmental conditions (Mazomenos 1989; Katsoyannos 1992), and the annual economic production loss for olive industry has been estimated to be in excess of one billion USD in this region (Van Asch et al. 2015).

In addition to an inherent scientific interest, a good knowledge of the biology, population structures, and geographical variability of insect pest species is critical for designing effective control or eradication strategies, i.e. eliminating their populations or reducing them to subeconomic damage levels (Roderick and Navajas 2003; Segura et al. 2008). Despite the agricultural and economic impact of olive fly, our knowledge about the population structure, genetic diversity, and geographical limits of its subpopulations in the Mediterranean basin is still far from complete (Augustinos et al. 2005; Nardi et al. 2005, 2010; Segura et al. 2008; Zygouridis et al. 2009; Van Asch et al. 2012, 2015; Dogac et al. 2013; Matallanas et al. 2013; Ramezani et al. 2015).

Initial genetic analyses of natural olive fly populations have tried investigating the population structures and colonization route of olive fly populations in several regions worldwide, using different molecular markers. Studies (Nardi et al. 2005, 2010) are consistent in postulating an African origin for the species, followed by a spread into the Mediterranean basin and more recently, because of human intervention, into the American region, i.e. spread through a series of range expansion events. Multiple genetic studies (Augustinos et al. 2005; Nardi et al. 2005; Zygouridis et al. 2009; Dogac et al. 2013) have also indicated that the eastern Mediterranean region could have played a key role in the colonization and movement of olive fly populations to the Mediterranean basin, which is presumably the original source of American olive fly populations. However, the debate persists about the number of genetic groups present in Mediterranean region. Specifically, one (Nardi et al. 2005; Segura et al. 2008), two (Nardi et al. 2010), or three (Augustinos et al. 2005; Zygouridis et al. 2009; Van Asch et al. 2012, 2015) local genetic groups have been identified

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Supplemental data for this article can be accessed here.

in the region so far, characterized by medium-high levels of gene flow.

The olive fly invasion deserves attention for designing effective management and control strategies as well. So far, mainly two different invasion scenarios have been proposed to explain the colonization route of olive fly populations in the northern part of Mediterranean basin. First, eastern Mediterranean was inferred as the source region of *B. oleae* associating with gradual decrease of heterozygosity in east to west cline, which advocates that the species migrated westward to Iberia, probably coupled with the introduction of cultivated olives from its Levantine center of domestication (Augustinos et al. 2005). An alternative interpretation suggested an older origin, associated with the fragmentation of wild olive host in different glacial refugia on this continent (Nardi et al. 2010).

It is now generally accepted that the mitochondrial genome of animals is a highly informative tool for genetic analysis because of peculiarities such as strictly maternal inheritance, high copy number, absence of recombination, and relatively high evolutionary rate (Hu et al. 2008; Wan et al. 2011). Besides these characteristics, the wealth of comparative data available in literature greatly simplifies the reconstruction of phylogenetic relationships and conducting phylogeographic studies.

Considering the economic impact of olive in Mediterranean agriculture, knowledge of the population genetic architecture of olive fly is an essential prerequisite to develop large scale and improved management programs for the region. Keeping in view the importance of this pest and the peculiarities of mitochondrial DNA, the fundamental aim of the present study was to obtain new information about the population structure, genetic diversity, and contemporary route of olive fly invasions in the northern Mediterranean basin. The genetic variability was determined by sequencing two mitochondrial-genome segments (2052 bp in total) of olive fly in field-collected samples from Turkey, a representative of eastern Mediterranean region and the putative source of the observed olive fly invasion. Obtained mtDNA haplotypes were integrated and comparatively analysed together with previously reported sequences from across the species worldwide range. Understanding the genetic structure and differentiation of the olive fly populations should benefit the control programs in Mediterranean region and help develop more efficient control strategies.

Materials and methods

Collection of olive fly samples

Olive fly samples were collected from 38 different sampling sites in 12 provinces, which cover all the major olive-producing areas in Turkey in 2009–2010. Collection sites and number of flies used in the study are presented in Table 1 and Figure 1. Infested olive fruits were kept at room temperature until larvae emerged and developed to adulthood. Adult samples were frozen and stored at −80 °C until further analysis.

Selection of polymorphic mtDNA regions

Briefly, our analyses centred on two highly variable sections of mtDNA that have been previously used to obtain a recent discrimination among Mediterranean samples (Van Asch et al. 2012, 2015; Matallanas et al. 2013; Ramezani et al. 2015). The first polymorphic region selected for amplification and sequencing in our survey, segment I, includes *cytochrome oxidase I (COI)* gene (86% coverage), while the second polymorphic region, segment II, includes the *COI* (11% coverage), *tRNA-Leu,* and *COII* (95% coverage) genes. The total length of these sections, segment III (2052 bp), represents approximately 13% of the complete mitochondrial genome of *B. oleae*. This fine-scale population genetic study was designed to complement the previous studies based on mtDNA markers by Matallanas et al. (2013), Van Asch et al. (2015), and Ramezani et al. (2015).

DNA extraction, PCR amplification, and sequencing of mtDNA segments

Genomic DNA was extracted from individual flies following the protocol of Bender et al. (1983). For each segment, 9–15 specimens per province (>123 flies) were analysed (Table 1).

A 1151 bp fragment of mtDNA region, segment I, from all genomic DNA samples was amplified by PCR using the newly designed primer pair PF1 (5′-TCAGCCATTTAATCGCGA CAATGGC-3′) and PR1 (5′-ATCGGCGTGGTATTCCCGCTAATCC-3′) (primers were designed according to the mitochondrial genomic sequence of *B. oleae* AY210702). PCR amplification was carried out in 20 µl reaction volume containing 50–100 ng of genomic DNA, 2 µl of 10 × buffer (Thermo Scientific, Vilnius, Lithuania), 3.2 µl of $MgCl_2$ (2.5 mM), 1 µl dNTP (10 mM each), 2 µl (0.1 µM) of each primer (Thermo Scientific), and 0.5 U *Taq* DNA polymerase (Thermo Scientific). PCR amplifications were performed using the following temperature cycling profile: initial denaturation period of 5 min at 94 °C, followed by 35 amplification cycles of 94 °C for 45 s, 60 °C for 1 min, and 72 °C for 1 min. PCR ended with a final extension step of 72 °C for 8 min.

We amplified another portion of the mitochondrial DNA region, segment II (901 bp in length), with primers, designed in the current study, PF2 (5′-ACGCCTATACAACATGAAATGTA-3′) and PR2 (5′-CAATACTTGCTTTCAGTCATCTAATG-3′). The reaction mix for segment II was the same as for segment I, but with lower $MgCl_2$ (1.5 µl) concentration. The thermal cycling conditions were: initial denaturation step of 5 min at 95 °C, followed by 35 cycles at 95 °C for 45 s, 52 °C for 30 s, and 72 °C for 1 min, and a final elongation step at 72 °C for 7 min. All amplified products were purified using QIAquick Gel Extraction kit (Qiagen, Hannover, Germany) and sequenced using PCR primers in both directions to increase accuracy, using an ABI 3100 DNA genetic analyser.

Data analysis

DNA sequences obtained from the studied populations were edited and verified as follows: first, electropherograms were inspected using Geospiza FinchTV program (available at

Table 1. *B. oleae* sampling locations and the number of flies analysed per provinces. N_{segI}, N_{segII} and $N_{segI+II}$ the number of flies used for segment I, II and I + II, respectively.

Regions	Provinces	Sub-locations and their numbers	Coordinates	N_{segI}	N_{segII}	$N_{segI+II}$
Aegean	Çanakkale	1 Eceabat	40° 10.8′ N 26° 19.2′ E	15	11	11
		2 Geyikli	39° 48.0′ N 26° 10.8′ E			
		3 Gökçeada	40° 12 0′N 25° 52 5′ E			
		4 İntepe	40° 00.0′ N 26° 18.0′ E			
	Bursa	5 Yalova	40° 39.0′ N 29° 16.2′ E	15	15	15
		6 Erdek	40° 25.2′ N 27° 46.8′ E			
		7 Mudanya	40° 22.2′ N 28° 22.8′ E			
		8 Gemlik	40° 25.8′ N 29° 09.0′ E			
	Balıkesir	9 Küçükkuyu	39° 33.0′ N 26° 34.8′ E	10	10	10
		10 Zeytinli	39° 34.2′ N 26° 43.2′ E			
		11 Edremit	39° 33.0′ N 26° 34.8′ E			
	Manisa	12 Turgutlu	38° 30.0′ N 27° 42.0′ E	10	10	10
		13 Salihli	38° 28.2′ N 28° 09.0′ E			
		14 Saruhanlı	38° 43.8′ N 27° 34.2′ E			
	İzmir	15 Bornova	38° 27.0′ N 27° 13.2′ E	10	10	10
		16 Kemalpaşa	38° 25.2′ N 27° 25.2′ E			
		17 Menemen	38° 36.0′ N 27° 03.0′ E			
	Aydın	18 Çine	37° 37.2′ N 28° 03.0′ E	10	10	10
		19 Germencik	37° 52.2′ N 27° 34.8′ E			
		20 İncirliova	37° 49.8′ N 27° 42.0′ E			
	Muğla	21 Gökova	40° 46.2′ N 43° 37.8′ E	10	9	9
		22 Yerkesik	37° 07.8′ N 28° 16.2′ E			
		23 Bayır	37° 19.8′ N 28° 06.0′ E			
Mediterranean	Mersin	24 Silifke	39° 34.2′ N 26° 43.2′ E	10	10	10
		25 Tarsus	36° 55.8′ N 34° 55.8′ E			
		26 Mezitli	36° 49.2′ N 34° 46.2′ E			
	Adana	27 Kozan	37° 27.0′ N 35° 48.0′ E	10	10	10
		28 Kürkçüler	37° 16.2′ N 35° 37.8′ E			
		29 Karaisalı	37° 13.8′ N 35° 03.0′ E			
	Osmaniye	30 Cevdetiye	37° 07.2′ N 36° 22.2′ E	14	9	9
		31 Kadirli	37° 22.2′ N 36° 04.2′ E			
		32 Toprakkale	37° 04.2′ N 36° 07.8′ E			
	Hatay	33 Samandağ	36° 04.8′ N 35° 58.8′ E	10	10	10
		34 Altınözü	36° 06.0′ N 36° 13.8′ E			
		35 Antakya	36° 13.2′ N 39° 09.0′ E			
	Gaziantep	36 Nurdağı	37° 10.1′ N 36° 44 2′ E	10	10	10
		37 Zincirli	37° 07.2′ N 36° 39.0′ E			
		38 Islahiye	36° 13.2′ N 39° 09.0′ E			

Figure 1. Sampling locations for olive fly (*B. oleae*) in Turkey. The numbers indicate the sub-locations (see Table 1).

http://www.geospiza.com/ftvdlinfo.html) and the consensus sequence of two mtDNA fragments combined from one specimen of each olive fly was constructed using the program SeqMan (DNAstar, Lasegene). Multiple alignments of the sequences were carried out as implemented in CLUSTAL W in Mega 6.1 (Tamura et al. 2013). All edited mtDNA sequences were then checked to confirm whether they were all appropriately translated into the predicted amino acid sequences using invertebrate mitochondrial genetic codes, with the same program package.

DnaSP v 5.10.01 (Librado and Rozas 2009) was used to calculate the standard genetic diversity estimates (haplotype diversity, *Hd*; nucleotide diversity, π; average number of nucleotide differences, *k*) for each population. The same software was also used to determine the number and proportion of haplotypes, nucleotide composition, variable sites, and gene flow, as calculated by N_m value, among populations. Differences in genetic diversity, using the values of *Hd*, π, and *k*, in the Mediterranean and Aegean regions were tested using the Mann–Whitney *U* test (Mendenhall and Beaver 1991) in SPSS 15.0 (SPSS Inc., Chicago, IL). The levels of genetic differentiation between pairs of populations, in terms of pairwise F_{ST} values, were calculated using Arlequin 3.5.2 (Excoffier and Lischer 2010). To investigate the population genetic structure within and between populations of *B. oleae*, we carried out analysis of molecular variance (AMOVA) test (Excoffier and Lischer 2010) that partitions total variance into its components, among groups, among populations within groups, and within populations, based on the variance in haplotype frequencies and the number of mutations between haplotypes. We grouped the Turkey populations as Aegean and Mediterranean, according to their geographic locations (these two regions are separated by >700 km). To identify the phylogenetic relationships among the mitochondrial haplotypes, a Median Joining haplotype network was constructed using the software, Network (ver. 4.6) (Bandelt et al. 1999; Polzin and Daneschmand 2003). In order to detect isolation by distance, the correlation between genetic ($F_{ST}/(1 - F_{ST})$) and geographic distance matrices were compared using the Mantel test (Mantel 1967). Google Earth ver. 4.2 (http://earth.google.com/download-earth.html) was used to estimate the geographical distance between each pair of populations.

To further test the population genetic structure and diversity on a wider geographical scale, our data was added to additional mtDNA sequences obtained from GenBank (for sequence accession numbers, see Tables S1, S2, and S3). The dataset were partitioned according to country of origin, covering five countries (Iran, Italy, France, and Spain plus Portugal, here named as Iberia) that constitute a reasonably complete coverage of the distributional range of this species in the Mediterranean basin. To lower the sample bias and obtain accurate information about the genetic characterization of olive fly populations, we included only the collection localities with haplotype sequence variants of 10 or higher in population structuring analysis. Sequences of newly determined haplotypes in the present study, for both mitochondrial segments, were deposited in GenBank NCBI databases under accession numbers (Segment I Accession Nos. KY111478–KY111512 and Segment II Accession Nos. KY111513–KY111527).

Results

Nucleotide information

In the present study, two mtDNA segments were used as genetic markers to examine the population genetic structure and diversity of *B. oleae* populations within Turkey and the Mediterranean basin. For this purpose, segments I and II were separately obtained and sequenced from more than 123 specimens, collected from 38 sampling sites in Turkey; no characteristics of heteroplasmy or insertion/deletion events were detected. The A + T content was about 65% for each segment. The informative non-synonymous substitutions resulted in 13 and 6 amino acid replacements at segments I and II, respectively.

For segment I, of the 1151 variable positions, 44 polymorphic sites were observed (3.8% variation), which were characterized by 25 singleton and 19 parsimony-informative sites. On the basis of sequence information, a total of 40 sequence variants (haplotypes) were identified in 134 olive fly specimens, and the mean haplotype and nucleotide diversity indices were found to be 0.8612 and 0.0025, respectively. The segment II was 901 bp long, which contained 17 polymorphic sites (1.9% variation) and 13 parsimony-informative sites. In total, we detected 19 novel haplotypes for this segment out of 124 olive fly specimens, and the average haplotype and nucleotide diversities were 0.7759 and 0.0024, respectively. We also analysed the combined mtDNA segments I and II regions simultaneously; overall 61 haplotypes were observed in 124 olive fly specimens, and the mean haplotype and nucleotide diversities were high, more than 0.9269 and 0.0023, respectively. Fifty-seven polymorphic sites were observed, 29 of them were parsimony-informative. Comparison with previously published sequences led to the identification of 34 and 14 unique haplotypes for segment I and segment II, respectively, and 56 haplotypes after concatenation of two markers for each individual, which indicates that these two regions are informative for olive fly populations. Almost half of the haplotypes detected from each mtDNA segments were found at low frequencies, in only one fly in each population.

The relative frequencies and distribution of different mitochondrial DNA haplotypes in each olive fly population based on both the single segment and combined datasets are presented in Tables S1–3. For segment I, the average number of haplotypes per collection site was 3.3, ranging between 5 and 10 per population. In Mediterranean region, the average number of haplotypes per province was 4.4; however, for the Aegean region, this number was lower, at 3.7. There were eight haplotypes shared by all olive fly populations, and 14 and 18 haplotypes were specific to Mediterranean and Aegean regions, respectively. For segment II, the average number of haplotypes per collection site was 1.6, ranging between 3 and 7 per population. In Mediterranean region, the average number of haplotypes per province was 2.2; however, for the Aegean region, this number was 2.

There were six haplotypes shared by all the olive fly populations. Five and eight haplotypes were unique to Mediterranean and Aegean regions, respectively. For combined datasets, in the Mediterranean region, the average number of haplotypes per province was 6.6; however, for the Aegean region, this number was lower, at 5. Twenty-six and 28 haplotypes were specific to Mediterranean and Aegean regions, respectively. Seven of the haplotypes were shared by both regions.

The list of identical haplotypes, based on both single segment and combined datasets, from previous studies are presented in Table 2a–c. The results, for both single and combined segments, showed that the majority of haplotypes from Iran and some haplotypes from Europe (specifically from Italy) were primarily grouped with the predominant haplotypes found in Aegean region. However, the specific haplotypes from Levant and USA were grouped with the haplotypes observed in both Mediterranean and Aegean regions of Turkey. Consistent with the findings of Nardi et al. (2005) and Dogac et al. (2013), this result supports an eastern Mediterranean origin of the populations that invaded USA. Two specific Aegean haplotypes (H18 from segment I and H42 from combined dataset) were also detected in Iranian populations. It is also interesting to note that specific haplotypes from Africa, the source of Mediterranean populations (Nardi et al. 2005), were observed for both segments in the Aegean region populations.

Table 2. List of identical haplotypes from previous studies for comparison.

Haplotype location	Source	Identical haplotypes in our study
(a)		
Turkey	(Nardi et al. 2010)	H10
USA	(Nardi et al. 2010)	
USA	(Nardi et al. 2010)	
Italy	(Nardi et al. 2010)	H11
Spain	(Matallanas et al. 2013)	
Iran	(Ramezani et al. 2015)	H12
Iran	(Ramezani et al. 2015)	
Iran	(Ramezani et al. 2015)	
Iran	(Ramezani et al. 2015)	
Iran	(Ramezani et al. 2015)	
Spain	(Matallanas et al. 2013)	
Italy	(Matallanas et al. 2013)	
Tunisia	(Matallanas et al. 2013)	
Italy	(Nardi et al. 2010)	
Italy	(Nardi et al. 2010)	
Iran	(Ramezani et al. 2015)	H18
Turkey	(Nardi et al. 2010)	
Israel	(Nardi et al. 2010)	H22
Tunisia	(Matallanas et al. 2013)	
Kenya	(Nardi et al. 2010)	H31
Kenya	(Nardi et al. 2010)	
S. Africa	(Nardi et al. 2010)	
S. Africa	(Nardi et al. 2010)	
(b)		
Turkey	(Nardi et al. 2010)	H1
Israel	(Nardi et al. 2010)	
USA	(Nardi et al. 2010)	
USA	(Nardi et al. 2010)	
Iran	(Ramezani et al. 2015)	
Turkey	(Nardi et al. 2010)	H3
Iran	(Ramezani et al. 2015)	H10
Iran	(Ramezani et al. 2015)	
Iran	(Ramezani et al. 2015)	
Iran	(Ramezani et al. 2015)	
Iran	(Ramezani et al. 2015)	
Iran	(Ramezani et al. 2015)	
Iran	(Ramezani et al. 2015)	
Italy	(Nardi et al. 2010)	
Italy	(Nardi et al. 2010)	
Italy	(Nardi et al. 2010)	
Italy	(Nardi et al. 2010)	
Morocco	(Nardi et al. 2010)	
Portugal	(Van Asch et al. 2015)	
Portugal	(Van Asch et al. 2015)	
Portugal	(Van Asch et al. 2015)	H11
Portugal	(Van Asch et al. 2015)	
Portugal	(Van Asch et al. 2015)	
France	(Van Asch et al. 2015)	
Kenya	(Nardi et al. 2010)	
S. Africa	(Nardi et al. 2010)	H15
(c)		
Turkey	(Nardi et al. 2010)	H4
USA	(Nardi et al. 2010)	
USA	(Nardi et al. 2010)	
Turkey	(Nardi et al. 2010)	H7
Iran	(Ramezani et al. 2015)	H24
Iran	(Ramezani et al. 2015)	
Iran	(Ramezani et al. 2015)	
Iran	(Ramezani et al. 2015)	
Iran	(Ramezani et al. 2015)	
Italy	(Nardi et al. 2010)	
Italy	(Nardi et al. 2010)	
Italy	(Nardi et al. 2010)	H29
Iran	(Ramezani et al. 2015)	H42

(a) Segment I, (b) Segment II, and (c) Segment I + Segment II.

Genetic diversity

Basic descriptive indices of genetic diversity for each mtDNA segments for each population are summarized in Table 3a–c; all populations exhibited fairly high levels of genetic diversity in Turkey. For segment I, among the 12 populations, we observed the highest haplotype diversities in Mugla and Mersin populations (0.9555) and the highest nucleotide diversity was detected in the Çanakkale population (0.0038). For segment II, the Mersin and Mugla populations had the highest haplotype (0.9111) and nucleotide (0.0033) diversities, respectively. Whereas, for the segment I + segment II sequences, the haplotype diversity ranged from 0.733 (Bursa) to 1.000 (Muğla), and the nucleotide diversity ranged from 0.0016 (Gaziantep) to 0.0035 (Çanakkale). The Aegean and Mediterranean regions did not have significantly different levels of genetic diversity for any of the analysed mtDNA sequences (Mann–Whitney *U*-test, $p > .05$).

Population genetic structure and haplotype network

For segments I and II, the mean pairwise estimates of genetic differentiation between population groups, measured by the fixation index F_{ST}, revealed strong population structuring at a large scale. The genetic differentiation among most of the populations within Aegean and Mediterranean regions of Turkey was not significant. However, we obtained significant results while comparing populations from the two regions (Tables S4a–c). Comparisons of genetic variability were performed with the global dataset as well, and genetic differentiation values were found as follows (Table 4a,b). For both segments, among the populations of Mediterranean basin, we observed the highest genetic differentiation between populations of eastern Mediterranean region of Turkey and Iberia (a distance of almost 4100 km). Considering

Table 3. Results obtained from genetic diversity analysis among the 12 geographic populations of *B. oleae* in Turkey.

Regions	Populations	*N*	*H*	*Hd*	π	*K*
(a)						
Aegean	Çanakkale	15	10	0.9333	0.0038	4.3428
	Bursa	15	8	0.7333	0.0020	2.3428
	Balıkesir	10	7	0.8667	0.0023	2.6000
	Manisa	10	6	0.8444	0.0022	2.4667
	İzmir	10	5	0.7556	0.0019	2.1778
	Aydın	10	6	0.7778	0.0018	2.0666
	Muğla	10	8	0.9555	0.0028	3.2222
	Mean	**11.43**	**7.14**	**0.8380**	**0.0024**	**2.7456**
Mediterranean	Mersin	10	8	0.9555	0.0024	2.8000
	Adana	10	6	0.7778	0.0021	2.4888
	Osmaniye	14	9	0.9121	0.0027	3.1428
	Hatay	10	7	0.9111	0.0016	1.8222
	Gaziantep	10	7	0.9111	0.0017	2.0000
	Mean	**10.8**	**7.4**	**0.89352**	**0.002**	**2.4508**
(b)						
Aegean	Çanakkale	11	5	0.7818	0.0032	2.8364
	Bursa	15	4	0.4667	0.0012	1.0857
	Balıkesir	10	6	0.8667	0.0026	2.3111
	Manisa	10	5	0.8444	0.0030	2.6889
	İzmir	10	5	0.8444	0.0022	2.0000
	Aydın	10	6	0.8889	0.0024	2.1778
	Muğla	9	5	0.8333	0.0033	3.0000
	Mean	**10.71**	**5.14**	**0.7895**	**0.0026**	**2.3000**
Mediterranean	Mersin	10	7	0.9111	0.0029	2.6444
	Adana	10	5	0.7556	0.0021	1.8667
	Osmaniye	9	4	0.6944	0.0022	2.0000
	Hatay	10	6	0.7778	0.0019	1.6667
	Gaziantep	10	3	0.6444	0.0015	1.3111
	Ortalama	**9.8**	**5**	**0.7567**	**0.0021**	**1.8978**
(c)						
Aegean	Çanakkale	11	9	0.9455	0.0035	7.0910
	Bursa	15	8	0.7333	0.0017	3.4286
	Balıkesir	10	9	0.9778	0.0024	4.9111
	Manisa	10	8	0.9556	0.0025	5.1556
	İzmir	10	7	0.9111	0.0020	4.1778
	Aydın	10	8	0.9556	0.0021	4.2444
	Muğla	9	9	1.0000	0.0031	6.4444
	Mean	**10.71**	**8.29**	**0.9256**	**0.0025**	**5.0647**
Mediterranean	Mersin	10	9	0.9778	0.0027	5.4444
	Adana	10	6	0.7778	0.0021	4.3556
	Osmaniye	9	8	0.9722	0.0027	5.5556
	Hatay	10	8	0.9333	0.0017	3.4889
	Gaziantep	10	9	0.9778	0.0016	3.3111
	Ortalama	**9.8**	**8**	**0.9278**	**0.0022**	**4.4311**

(a) For segment I, (b) segment II, (c) segment I + segment II. *N*: number of sequences; *H*: number of haplotypes; *Hd*: haplotype diversity; π: nucleotide diversity; *k:* average number of nucleotide differences per population.

Table 4. Pairwise genetic differentiation (F_{ST}) values between olive fly populations (below diagonal) in Mediterranean basin and approximate geographic distances (km) (above diagonal) for (a) Segment I, (b) Segment II.

	E_Turkey	W_Turkey	Spain
(a)			
E_Turkey	–	800	4100
W_Turkey	0.29518*	–	3300
Spain	0.58687*	0.28516*	–

	Iran	E_Turkey	W_Turkey	Italy	France	Iberia
(b)						
Iran	–	1600	2400	3500	4500	5500
E_Turkey	0.51598*	–	800	2300	3300	4100
W_Turkey	0.09469*	0.25698*	–	1500	2500	3300
Italy	0.28970*	0.55428*	0.29802*	–	1200	2000
France	0.56214*	0.63758*	0.47191*	0.24496*	–	1500
Iberia	0.64569*	0.66637*	0.54979*	0.43901*	0.05706	–

*Statistical significance at $p < .05$.

this distance, the level of gene flow can be considered an important factor that influences the shape of genetic structure of olive fly populations in the Mediterranean basin. Significant genetic differentiations were observed for segment II, among most of the olive fly populations in Mediterranean basin ($p < .05$), with the exception of French and Iberian populations ($F_{ST} = 0.05706$, $p > .05$). Results revealed that the F_{ST} values for both segments showed a trend of increasing genetic differentiation when moving from east coast of Turkey to Iberia in the Mediterranean basin (i.e. geographically related olive fly population substructures in the region). Even though a high level of gene flow has been reported in Mediterranean olive fly populations earlier ($N_m = 6.16$) (Augustinos et al. 2005), it seems this level of gene flow does not seem to be enough to homogenize the populations on a continental scale. The second lowest level of genetic differentiation of F_{ST} value ($F_{ST} = 0.09469$), observed between the Aegean region and Iran, indicates a small level of genetic differentiation (Wright 1978) between these two regions, suggesting that samples from the Aegean region were more similar to Iranian samples compared with the samples from eastern part of Turkey. The F_{ST} value of the segment II dataset showed a higher and significant F_{ST} value ($F_{ST} = 0.2897$, $p < .05$) between Iran and Italy.

Based on the sequence information from both the mtDNA segments, the AMOVA of populations, consistent with the F_{ST} values presented above, revealed that a significant proportion of genetic variation originated among geographical regions in the Mediterranean basin ($p \leq .05$) (Table 5a,b). For both mtDNA segments, for the Turkish samples, grouping collections according to Aegean and Mediterranean regions resulted in a moderate (< 30%) but significant variation among regions in Turkey ($p < .05$). AMOVA of the segment I dataset revealed the presence of significant structure between Turkish and Spanish olive fly populations ($p < .05$). For segment II, the results showed that the percentage of molecular variance between western Turkey and Iran was lower (8.83%) than the western Turkey–Italy pair (29.41%), even though both results were statistically significant.

In order to further understand the genetic relationships among these new haplotype data, within the context of previously published data, we constructed haplotype networks (Figure 2(a–c)). Analyses demonstrated a high level of haplotype richness among sampled populations and exhibited only a low number of unobserved haplotypes, which are unlikely to have greatly affected the interpretation of our results. Taking into account the differential distribution and frequency of haplotypes for each mtDNA segments, network analysis recovered similar topologies characterized by the presence of several predominant haplotypes located in the centre of the networks, surrounded by many low frequency derived haplotypes, which connected to these high frequency haplotypes through several mutation steps. For segment I, considering the geographic distribution, five predominant haplotypes (H5, H22, H10, H12, and H11) were found. Haplotype H5 was specific to the olive fly populations in Turkey. Haplotype H12 is the most common and widely distributed haplotype in Aegean region. H11 is composed of haplotypes that are mainly found in western Mediterranean

Table 5. Analysis of molecular variance (AMOVA) based on segment I (a) and segment II (b) of mtDNA used to compare Turkish (east and west) olive fly populations and those belonging to indicated groups.

	W_ vs. E_Turkey	W_Turkey vs. Spain	E_Turkey vs. Spain
(a)			
Between groups	29.13	28.8	58.06
Among populations/within groups	0.0002	1.05	0.49
Within populations	70.86	70.15	41.46

	W vs. E_Turkey E_Turkey vs. Iran	W_Turkey vs. Iran E_Turkey vs. Italy	W_Turkey vs. Italy E_Turkey vs. France	W_Turkey vs. France East Turkey vs. Iberia	W_Turkey vs. Iberia
(b)					
Between groups	25.17	8.83	29.41	46.98	53.75
Among populations/within groups	1.66	2.41	1.36	0.85	2.49
Within populations	73.17	88.76	69.23	52.17	43.77
Between groups	51.07	55.10	63.44	65.29	
Among populations/within groups	0.3	–0.15	–0.25	1.82	
Within populations	48.63	45.05	36.81	32.89	

E_Turkey: Eastern Turkey; W_Turkey: Western Turkey.
Values represent the percentage of variance attributable to each source.

basin (Italy and Spain), and most of the haplotypes from this area were one or two mutations away from this haplotype. The segment I haplotypes for the populations from Turkey and Spain were resolved into two clusters. For segment II, haplotype H3 predominated the Mediterranean region. On the other hand, H10 was the most common haplotype in Aegean region, and could be an intermediate group between Italy and eastern Mediterranean region. Haplotypes H20 and H23 were mainly present in French + Iberian and Italian samples, respectively. H20 seems to be a western European cluster. Haplotype 11 of Turkey, which is one mutation away from a predominant H20, is grouped with the other western Mediterranean samples. For segment II, a certain level of association was observed between the geographical origin of the sequences and the haplotype information. The haplotypes H6, H7, H24, and H4 were four dominant haplotypes for the combined dataset. H6 was a specific haplotype and observed in both regions of Turkey. H7 was a common haplotype in the olive fly samples from the Mediterranean populations of Turkey. Most of the Aegean region samples, together with most of the Iranian and several Italian samples were grouped in haplotype H24. Network analysis for this dataset revealed no evidence for clear geographical clustering, probably because of the weak sample size of populations from most localities. In all analyses, most of the Iranian haplotypes were grouped with the dominant haplotypes in Aegean region, or connected to specific haplotypes from this region through one or two mutation steps, indicating that the populations from these areas have similar population histories. In contrast to earlier findings (Van Asch et al. 2012; Van Asch et al. 2015; Ramezani et al. 2015), the samples from France and Iberia were not well-structured and clearly different from the other Mediterranean haplotypes.

As other analyses provided a signal for isolation by distance at a large spatial scale, for mtDNA segments I and II, the Mantel test showed significant correlation between the geographic and genetic distances between populations in Mediterranean basin (Mantel statistics $r_{segI} = 0.7904$, $p < .05$; $r_{segII} = 0.8798$, $p < .05$), indicating that the genetic differentiation increased when moving from the east coast of Turkey to Spain (Figure 3(a,b)) (to eliminate the potential impact of the peripheral Iranian populations, we excluded these samples from the analysis). When we included the Iranian data for segment I, Mantel tests showed no significant association between geographic distance and genetic distance ($r = 0.2684$; $p > .05$) (Fig. S1a,b). However, for segment II, after including the Iranian dataset, the Mantel test revealed a weak but still significant correlation between these two variables ($r = 0.6506$; $p < .05$). Consistent with isolation by distance on a broad geographical scale, for both segments, we also observed the same correlation on a smaller spatial scale within Turkey ($r_{segI} = 0.6418$; $p < .05$; $r_{segII} = 0.49$; $p < .05$) (Fig. S2a,b).

Discussion

Variability in Turkey

Results revealed that all populations of *B. oleae* in Turkey are highly polymorphic for the two analysed mtDNA fragments. This high level of genetic variability is characteristic of olive fly populations (Augustinos et al. 2005; Nardi et al. 2005; Segura et al. 2008; Zygouridis et al. 2009; Van Asch et al. 2012, 2015; Dogac et al. 2013; Matallanas et al. 2013; Ramezani et al. 2015). It was previously suggested (Segura et al. 2008; Dogac et al. 2013; Matallanas et al. 2013) that there are several possible factors that influence the genetic variability of *B. oleae* populations in the Mediterranean basin: (i) the time elapsed since the establishment of the population, (ii) continuity and wide extensions of olive groves, (iii) effective high population densities, and (iv) high rates of gene flow.

Although we observed a high level of gene flow among the olive fly populations in Turkey ($Nm_{seg\ I} = 8.7$; $Nm_{seg\ II} = 7.5$; $Nm_{seg\ I+II} = 13.5$), significant genetic differentiation between the Aegean and Mediterranean regions were apparent in all analysed datasets. This result corroborates the findings of a previous study where Turkish olive flies were analysed using different molecular markers (Dogac et al. 2013). In that report, the authors had suggested three possible factors to explain the genetic heterogeneity among the

populations. First, the presence of a natural route for olive fly dispersal through a continuous resource-rich region, the absence of natural barriers to gene flow, and appropriate climatic conditions from Middle East (specifically from Syrian border) to the Mediterranean region of Turkey. High dispersal capacity in olive fly has been reported previously (Fletcher 1989a, 1989b; Rice et al. 2003). Second, less continuous cover of olive plantations from east to west (i.e. fragmented habitats). Third, in Turkey, the olive fly management has involved heavy use of chemical pesticides. Therefore, the local variation in selection intensity due to agricultural purposes might be an important factor to maintain variation between regions.

Comparing east and west

Mitochondrial cytochrome oxidase I (mtCOI) gene has been extensively used for studying population genetic structures, and in phylogeographic studies of insect populations

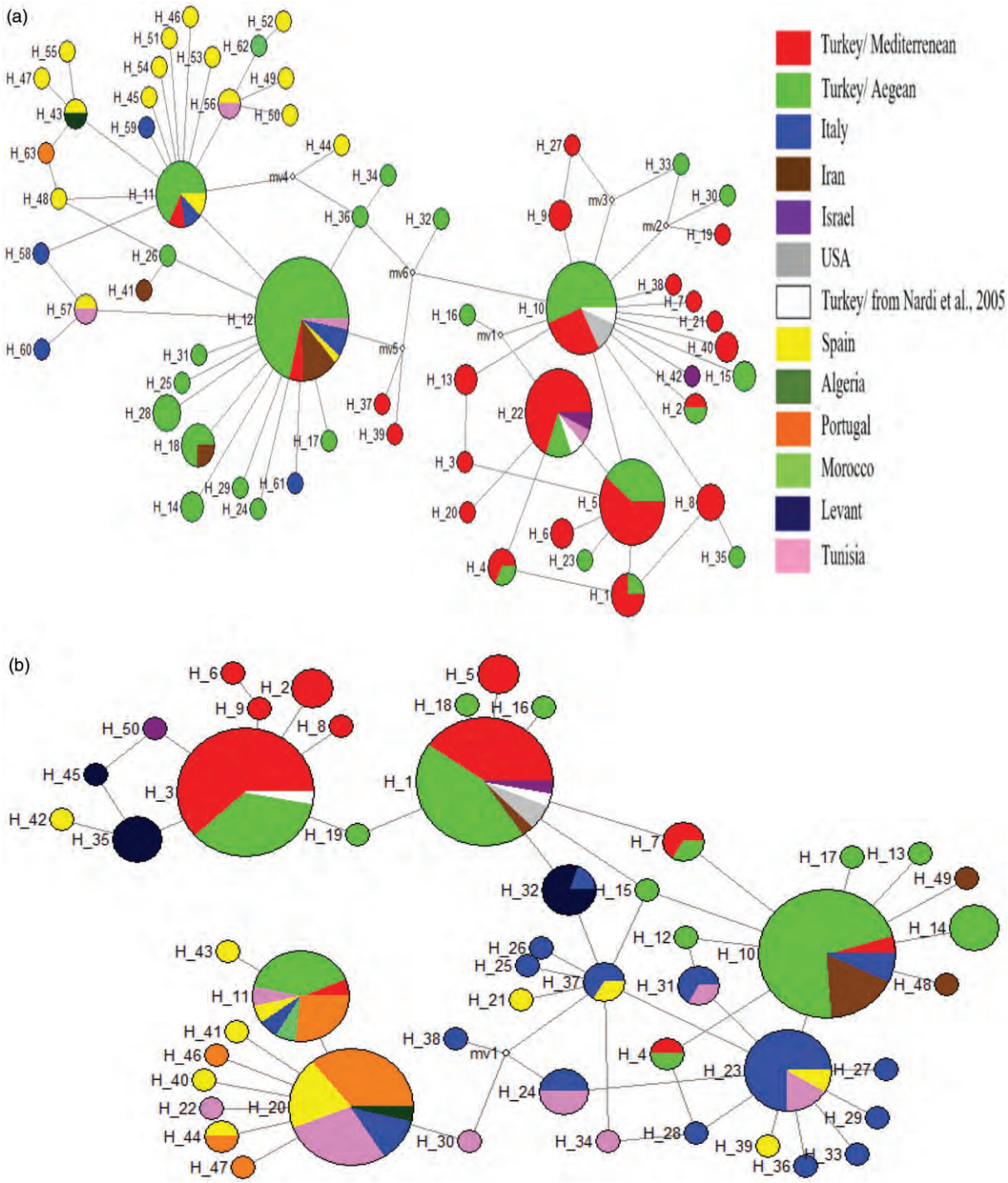

Figure 2. Mitochondrial haplotype networks for segment I (a), segment II (b) and segment I + II (c) of olive fly populations. Haplotype numbers and their distribution by region are listed in Tables S1, S2 and S3, respectively. The size of circle corresponds to haplotype frequency in the data set. Empty circle represents unobserved intermediate haplotypes. A full colour version of this figure is available online.

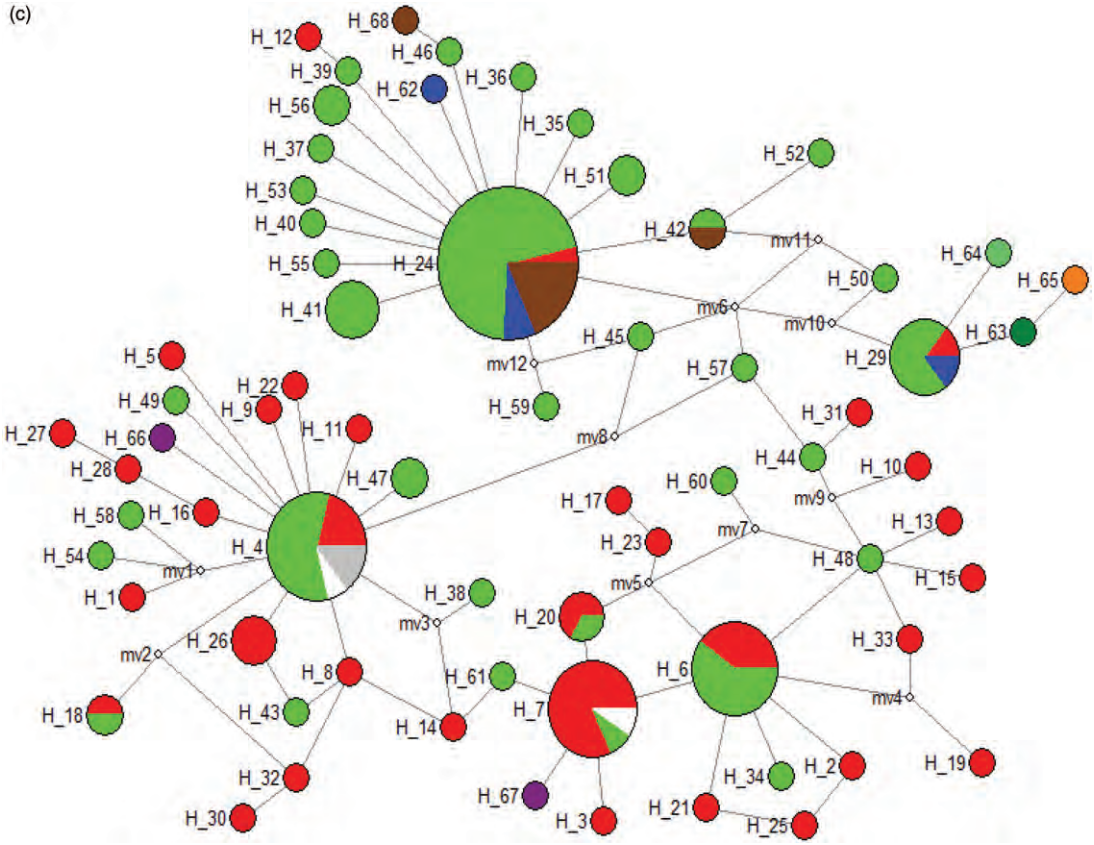

Figure 2. Continued.

belonging to genera such as *Bactrocera* and *Ceratitis* (Li et al. 2012; Karsten et al. 2013). In the olive fly, using the mtCOI marker, significant genetic differentiation was reported in the populations of western Mediterranean region, between samples from Spain, Tunisia, and Italy (Matallanas et al. 2013). One of our primary goals in the present study was to examine the population genetic structures and variability of olive fly populations collected from widely separated localities in Turkey, a representative of eastern Mediterranean basin, and compare them with the western Mediterranean basin populations by sequencing identical mtDNA regions. Low number of common haplotypes, significant levels of genetic differentiation, and high genetic variation between the Turkey and western Mediterranean populations indicate that the two regions probably have different population histories. Mantel test also revealed a significant correlation between genetics and linear straight geographical distances within segment I, which indicates that the geographic distance is probably responsible for this partitioning of genetic variation in the Mediterranean basin. However, it is important to bear in mind that lower number of samples ($n = 70$) were used by Matallanas et al. (2013).

Given that the Turkish and Spanish populations show a high haplotype diversity, the average values for Turkish and Spanish populations are 0.861 and 0.844, respectively (Spanish data from Matallanas et al. 2013), the assessment of genetic diversity is based on nucleotide diversity, which is defined as the mean number of base mutations per site between two randomly drawn sequences from a population (Nei and Li 1979). It is a useful indicator for assessing the degree of variation in nucleotide sites between populations and can be used to measure the genetic diversity. Ancestral populations generally possess significantly higher levels of genetic diversity than recently established populations because of the founder effect in new populations (Templeton 1980; Grant and Bowen 1998). For identical mtDNA regions, a notable difference in nucleotide diversity values has been detected between Turkish and Spanish samples, the mean values were 0.0025 and 0.0013, respectively (Spanish data from Matallanas et al. 2013). These values possibly imply that Turkish samples might be closer to ancestral populations than the less diverse samples from Spain, suggesting a trend of expansion from east to west.

Structure of Mediterranean populations

A high level of genetic structuring was identified among the Mediterranean basin populations of *B. oleae* for segment II (Table 4). All the analysed populations (from eastern Mediterranean and Aegean regions of Turkey, Italy, France, and Iberia) were significantly different. The findings are consistent with previous population genetic studies on olive flies that reported different levels of genetic structuring in the Mediterranean basin (Augustinos et al. 2005; Nardi et al. 2005, 2010; Van Asch et al. 2012; Dogac et al. 2013), even the geographic limits of the population ranges are poorly defined. However, unlike previous studies (Augustinos et al. 2005; Nardi et al. 2005; Dogac et al. 2013), we could

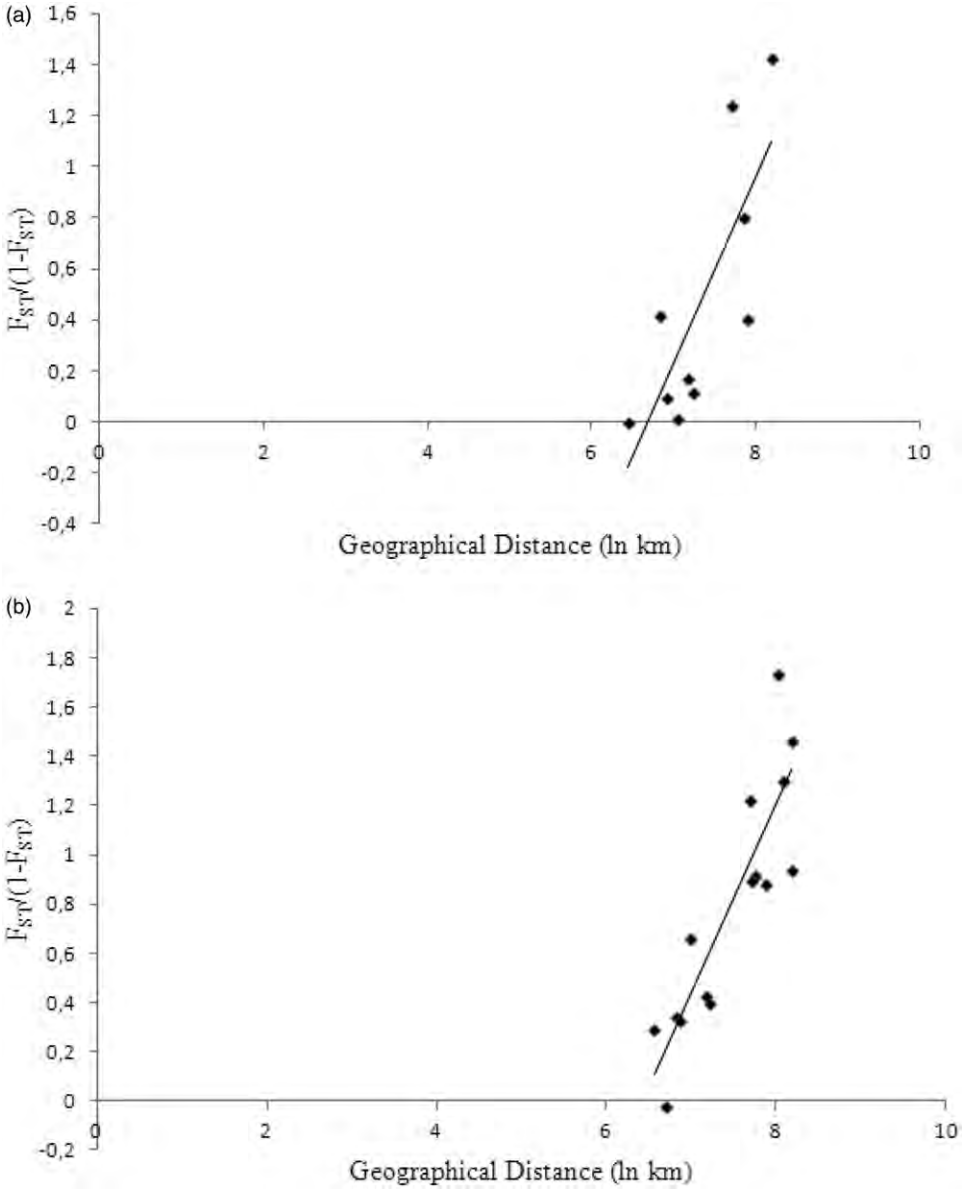

Figure 3. The correlation between matrices of genetic and geographic distances (ln km) among populations of *B. oleae* in Mediterranean basin (a) for segment I and (b) for segment II.

differentiate the Aegean and Italian populations using this segment. Similar to the result of segment I, Mantel test results for segment II also revealed significant relationships between genetic and geographic distances in the area. The observed increase in F_{ST} values (from eastern coast of Turkey to Spain) in the Mediterranean basin, together with the increase in the geographic distances, supports the notion that this species might be colonizing westwards in southern Europe; a similar result has been reported previously using microsatellite data (Augustinos et al. 2005).

Origin of Iranian populations

There are government-established olive research institutions in Aegean region, which is the biggest olive-producing region and an intensive olive-trading area in Turkey. These centres produce and distribute olive seedlings of various varieties and clones to farmers who are willing to establish new olive grove plantations anywhere in Turkey. The number of olive-producing areas has increased in the Mediterranean region during the last 20 years. Many olive seedlings of different varieties and clones have been transferred and planted in this region during olive tree-plantation campaigns. The role of anthropogenic disturbances in structuring the current genetic variability in fruit flies, which have the capacity to expand to new areas quickly, cannot be ignored. In order to determine the origin of Iranian olive fly populations, mtDNA markers were used in a recent study, which made an interesting claim that central Mediterranean populations (specifically Italy) were the main source of Iranian olive fly populations, primarily as a result of human intervention (Ramezani et al. 2015). This is the second case of human-mediated introduction of olive fly in history. However, we believe that in order to precisely understand the genetic characterization and historical patterns of olive fly movement, more DNA-based sequence analyses should be carried out from potentially

critical areas in eastern Mediterranean (Aegean and Mediterranean regions of Turkey), which could help us make stronger inferences. Our results suggested the presence of a weak genetic structuring due to a low level of differentiation among Aegean and Iranian populations. It is possible that the current olive fly populations in Iran originated from western Turkey. Considering the historical, cultural, and demographic ties between Turkey and Iran, we hypothesize that the introduction of olive fly to Iran could have happened via trade/transportation of seedlings, cultivars, clones, and/or infested fruits (i.e. accidental human mediated introduction) or intensive trading activities, either from Aegean or Mediterranean region, which are very close to the Iranian border. Such examples are available for other pest species from different regions, such as *Ceratitis capitata* (Malacrida et al. 2007) and *B. cucurbitae* (Virgilio et al. 2010). However, based on the available data, we cannot entirely rule out the hypothesis proposed by Ramezani et al. (2015), multiple independent introductions, or large number of founders from multiple sources. Although the exact origin of Iranian olive fly populations is not clear, based on the data of Ramezani et al. (2015) olive fly appears to have medium to high levels of genetic diversity in this country.

Regardless of its dispersal route, if *B. oleae* had a relatively recent arrival to Iran, it could still retain the genetic signature of a founder event (i.e. reduced genetic variability). A single marker, mtDNA, might not be representative of the genome as a whole. Therefore, in order to test the above hypotheses, we need to have broad genetic data, together with ecological investigations, from the potential source populations and native range of olive fly. Employing nuclear loci would also help interpret the demographic history and invasive processes in insect species. Polymorphic molecular marker microsatellites are distributed throughout the nuclear genome. They show high levels of variability and are generally neutral unless linked to loci under strong selection. They have been used frequently for genetic differentiation in evolutionary studies (Nardi et al. 2005; Aketarawong et al. 2007). Thus, in order to obtain a comprehensive phylogeographic understanding of the olive fly populations in the Mediterranean basin, we should use different kind of markers to reach general conclusions and reconstruct patterns of migration. In addition to molecular markers, some morphological characters of olive fly have also been used to separate the populations in Turkey (Doğac et al. 2015). However, the results did not reveal a clear separation among populations.

In conclusion, within the context of previously published datasets, the overall profile of population genetic structures in Turkish olive fly populations provided insights into the genetic variability and colonization process of *B. oleae* in Mediterranean basin and Iran. However, the reservation must be kept in mind that a limited number of sequences are available for analysis from the worldwide geographic ranges of olive fly. A larger-scale study and a bigger sample size per population from potentially critical areas, such as Africa and North America, need to be genotyped to precisely understand and confirm the genetic structures and colonization route of this species around the world. However, apart from its purely scientific value, we believe that this information could also contribute to the development and design of more efficient and safe methods for management and control strategies for a major pest of world agriculture.

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