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Distribution and molecular differentiation of *Culex pipiens* complex species in the Middle and Eastern Black Sea Regions of Turkey

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Abstract: *Culex pipiens* species complex is commonly found in tropical and subtropical areas. Identification of species complexes and their spatial distribution patterns are fundamental steps needed to improve our understanding of arboviral disease circulation and their control. The specimens were differentiated using the *Ace-2* gene, and the ecological forms of *Culex pipiens* were identified by analysis of the CQ11 microsatellite locus. Results showed that all sample points comprised of *Culex pipiens* s.s., and seven points contained *Culex torrentium*. Further, we determined that 22 haplotypes from five haplogroups were identified within the *Ace-2* marker, and three haplotypes were contained within the CQ11 microsatellite locus. We determined that there was a high degree of the genetic flow of each marker between populations. In conclusion, *Culex pipiens* complex and sibling species were determined to be distributed throughout the coastal and inland parts of the Black Sea region in Turkey. The occurrence of two distinct ecological forms has the potential to create hybridization zones and a high risk of WNV transmission.

Key words: *Ace-2*, black sea, *Culex pipiens* complex, CQ11, distribution

1. Introduction

Approximately 41 genera and more than 3500 species and subspecies of mosquito are distributed in many parts of the world except the polar circle. Certain mosquito species are well-known efficient vectors of many pathogens including viruses (Becker et al., 2010). The epidemic potential of mosquito-borne diseases, which rank the first among the diseases that cause the most deaths in the world, is an important threat to public health (Marshall, 2000; Weissenböck et al., 2010). The global spreading pattern of vector-borne diseases has produced multiple actions and enhanced surveillance activities require an understanding of the composition and distribution of vector mosquito species (Kollars et al., 2016, Grout et al., 2017). Emerging or reemerging disease outbreaks related to the arboviruses are increasing in Europe; for instance, West Nile (WNV), an important virus for South-eastern Europe and the Mediterranean basin, has caused several epidemics since 1996 in Romania, Italy, Turkey, Greece, and other Balkan countries (Ceianu et al., 2001, Rezza 2014, Martinet et al., 2019). Between 2011 and 2015, 780 West Nile cases were reported in Europe, and 1791 cases were reported in countries neighboring Europe. The last large outbreak in Europe occurred in 2010, which resulted in 35 deaths in Greece (Danis et al., 2011; ECDC, 2017). In that year, 47 human cases were also reported in Turkey, 10 of which resulted in death (Kalaycioglu et al., 2012).

The global strategy to manage arboviral diseases such as dengue, yellow fever, West Nile is highly dependent on mosquito control. Although the use of chemical insecticides is the mainstay of mosquito management in many countries, insecticide resistance is a big problem for controlling mosquito-borne diseases (Liu, 2015). The importance of surveillance activities and mosquito control applications has increased as a result of West Nile epidemics and the spread of invasive mosquito species in the European continent. Furthermore, WNV incidence has reached the highest degree at least twenty years in the European continent.

Culex pipiens (Diptera: Culicidae) species complex is cosmopolitan vectors of diseases that affect wildlife, domestic animals, and humans (Vinogradova, 2000).

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West Nile encephalitis is the most widespread arboviral neurological disease in the world, and it is responsible for large human outbreaks in the Holarctic region as well as in Africa (Chancey et al., 2015). *Cx pipiens* is the most common mosquito in the Holarctic region, and its distribution area covers all temperate regions. In southern Europe, WNV has mostly been detected in the indigenous mosquito species *Cx. pipiens* complex (Engler et al., 2013). This complex plays a primary role in the transmission. Their vectorial capabilities have been confirmed by laboratory studies (Fortuna et al., 2015).

The classification of this complex based on behavioral, physiological, and host preferences (Becker et al., 2010) has produced five taxa (*Cx. pipiens* L, *Cx. quinquefasciatus* Say, *Cx. pipiens pallens* Coquillet, *Cx. australicus* Dobrotworsky and Drummond, *Cx. globocoxitus* Dobrotworsky) (Farajollahi et al., 2011). *Cx. pipiens* s.l. also has two different genetic and ecological biotypes (*Cx. pipiens pipiens, Cx. pipiens molestus*). *Cx. pipiens* s.s. is one of the most common Holarctic mosquito species together with two Palearctic biotypes. The anatougenous form *Cx. pipiens pipiens* mostly prefers blood feeding from birds, and autogenous form *Cx. pipiens* molestus (Forskal) mostly prefers blood from humans. Although *Cx. pipiens pipiens* strongly shows ornithophilic behavior, infrequent feeding pattern has been detected from humans and other mammals (Petric et al., 1999; Gingrich and Casillas, 2004). *Cx. pipiens molestus* shows mostly an anthropophilic behavior, and there is a lack of reliable morphological characters for its identification (Becker et al., 2010). *Culex pipiens molestus* biotype is generally known as an urban mosquito in the northern part of its extensive distribution. Larval distribution is nearly limited to hypogeal aquatic developmental areas and is quite different from *Cx. pipiens pipiens* biotype. *Cx. quinquefasciatus*, *Cx, australiacus* and *Cx. globocoxitus* are non-European species (Farajollahi et al., 2011). Although many studies reported *Cx. quinquefasciatus* as non-European species, recent studies have detected it around Greece, Turkey, and other Mediterranean countries (Shaikevich et al., 2016; Morcicek et al., 2018). The two biotypes coexist in the same natural environment and hybridize in many areas (Reusken et al., 2010; Osario et al., 2014). This situation has increased the risk of viral transmission of WNV (West Nile Virus) from birds to humans (Ciota et al., 2013; Osario et al., 2014). Similarly, molecular analysis has confirmed the hybridization between *Cx. pipiens* s.l and *Cx. quinquefasciatus* where their distribution overlaps (Shaikevich et al., 2016). Some *Culex* species, such as *Culex torrentium* Martini, *Culex vagans* Wiedemann, are morphologically indistinguishable from *Cx. pipiens* complex but are genetically and behaviorally distinct from the species complex (Farajollahi et al., 2011).

Culex pipiens complex is widely distributed throughout Turkey, but researchers have not yet investigated the distribution of this species complex or identified the species present in Turkey. Limited studies regarding mosquito fauna have, however, shown that *Cx. pipiens pipiens*, *Cx. pipiens molestus*, *Culex quinquefasciatus* species, and their hybrids are distributed in Turkey, but their distribution is limited to the eastern, central, and Aegean parts of Turkey (Bedir, 2015; Gunay, 2015; Morcicek et al. 2018). Evaluating the risk of transmission of vector-borne diseases for human populations and updating the geographic distribution of vector species is required. This knowledge regarding *Cx. pipiens* species complex is crucial to enhancing our understanding of vector dynamics in areas in which the implementation of control strategies can effectively limit the transmission of pathogens.

The area in the study has many wetlands that provide primary habitats for birds and are also visited by birds migrating from Africa to Russia (Tsai, et al. 1998). Therefore, the occurrence and distribution pattern of *Cx. pipiens* species complex is essential to understanding likely West Nile virus circulation patterns in the study area and to preventing future epidemics. This study aimed to investigate the spatial occurrence and distribution of *Cx. pipiens* species complex and their habitat type preferences in the middle and eastern Black Sea region of Turkey. In addition, we aimed to reveal which species complexes existed in the middle and eastern Black Sea area by using molecular markers.

2. Materials and methods

2.1. Study area

The middle and Eastern Black Sea region is located in the northeastern part of Turkey and borders Georgia. The area is comprised of the Black Sea coast and its inland area. The 12 cities and many counties of the area have differing climatic and agricultural areas that function as mosquito breeding areas such as rice fields (two large delta areas from Kizilirmak and Yesilirmak rivers), vegetable production fields, and tea, kiwi fruit, and hazelnut plantations. The area also has many small river deltas throughout its coastal zone that spans the middle part of the Black Sea to the Georgian border (Figure 1).

2.2. Mosquito collection and morphological identification

Mosquito larvae and adults were collected from 128 points in 2014 and 92 points in 2015 (12 cities) within the study area. Collected samples were transferred to the laboratory. The immature ones from the collected samples were allowed to develop into adults in rearing cages. Both in vitro grown and adult *Culex* specimens were killed using ethyl acetate and refrigerated until morphologically identified. All samples were morphologically identified using mosquito keys according to the following interactive identification key features (Schaffner et al., 2001).

Figure 1. The study area (study areas are red-lined areas).

2.3. DNA isolations

All mosquito samples were homogenized in 200 µL TE buffer, and genomic DNA was obtained using the GeneJet Genomic DNA purification kit (Thermo-fisher Scientific, Waltham MA, USA). The extracted DNA was dissolved in 85 µL of ddH_2O and kept at –20 °C.

2.4. PCR of Ace-2 gene and microsatellite DNA

PCR procedures were performed according to protocols outlined in the studies of Smith and Fonseca (2004) and Kasai et al. (2008). First, specimens were visually separated using a gel (Smith and Fonseca, 2004; Kasai et al., 2008). B1246s primer and species-specific Acepip, Acepall, Acequin, Acetorr primers were used to amplify the *Ace-2* region of the genome of each specimen to differentiate complex from its sibling species, *Cx. torrentium*. B1246s, F1457 primers were used to sequence the *Ace-2* region (Kasai et al. 2008). The PCR assay used was standardized for a 30 µL reaction volume. Reactions contained 1× PCR buffer, 10 pmol of each dNTP, 2 mM $MgCl_2$, 0.15 mg/mL of bovine serum albumin, one unit of *Taq* DNA polymerase (New England Biolabs), approximately 6 ng genomic DNA, and ddH_2O to 30 µL. The amplification cycle used consisted of one cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 57 °C for 30 s, 72 °C for one minute, and one cycle at 72 °C for five minutes to complete a final extension step (Smith and Fonseca 2004; Kasai et al., 2008). The amplicons were assessed using 2% agarose gel electrophoresis with a 100 bp DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA). Two samples were used for this purpose from 73 collection points.

Sequences of *Cx. pipiens pipiens* and *Cx. pipiens molestus* were differentiated based on partial CQ11 sequences, which were amplified using pip CQ11R, mol CQ11R, and CQ11F2 primers (Bahnck and Fonseca, 2006). The PCR mixture was prepared using 1× Buffer, 2.5 mM $MgCl_2$, 10 pmol of each primer, 0.2 mmol of each dNTP, one unit *Taq* DNA Polymerase (New England Biolabs), 1 µL DNA, and ddH_2O to 25 µL. Cycling parameters were included in an initial 5 min denaturation step at 95 °C, followed by 36 cycles at 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 40 s, and a 5 min final extension step at 72 °C. Amplicons were visualized using 2% agarose gel electrophoresis. Samples were reamplified using universal primers after DNA was separated using agarose gel electrophoresis. Two samples were used for this purpose from 34 collection points. Raw sequences were edited in Mega 7 software using Clustal W version 2.0 section (Bahnck and Fonseca, 2006). Heterozygote individuals were detected according to sequences peaks and complement sequences were verified.

2.5. Data analysis

Population genetics data (haplotypes, polymorphic sites, numbers of haplotypes (N), haplotype diversity (Hd), and nucleotide diversity (π)) of *Cx. pipiens* were assessed using DnaSP 5.0 software (Librado & Rozas 2009). Pairwise FST values among the populations, were calculated to determine whether there were differences between regions by using the software Arlequin 3.5 (Excoffier, & Lischer, 2010). Network analysis was performed using Network 10.2. In addition, Maximum likelihood method based on the Tamura-Nei (1993) phylogenetic tree was performed using MEGA7 software (Kumar et al., 2016).

CORINE Land Cover (CLC) index is a database used for the classification of the land cover according to EEA[1]. It consists of a 3-level hierarchical classification system

[1] European Environment Agency. CORINE Land Cover (CLC) (2006), Version 17. Copenhagen K; 2013. http://www.eea.europa.eu/data-and-maps/data/clc- 2006-vector-data-version-3. Accessed 6 Feb 2016.

with 44 classes at the third and most detailed level. Class 1 includes five classes (artificial surfaces, agricultural areas, forest and seminatural areas, wetlands, water bodies) and they are divided into second (15 groups) and third level (44 groups) ownself. We specified specific habitat types using the CORINE (Co-ordinated information on the environment) land cover database to investigate habitat type preferences of complex species and *Cx. torrentium*. A generalized linear model was used to detect differences in habitat type preferences in land cover classes in IBM SPSS 22.

3. Results

Collected specimens and their ratio and identification types according to years are given in Table 1. A total of 6912 mosquitoes were sampled during the 2014 study year. *Culex* genus represented 52.77% of the whole sampled mosquitoes. A total of 28.88% of the samples collected were *Cx. pipiens* complex and *Culex torrentium*. Although *Culex* genus samples represented up to half of the collected samples in 2014, 2015 *Culex* genus samples represented 33.74%. *Culex pipiens* complex and *Cx. torrentium* were 26.79% of the whole sampled mosquitoes (n = 4232) in 2015. In 2014, specimens of the *Culex* genus were *Cx. pipiens* complex and *Cx. torrentium* (53.59% in *Culex* genus, 28.28% in whole collected mosquitoes), *Cx. mimeticus* (5.61% in *Culex* genus, 2.96% in whole collected mosquitoes), *Cx. hortensis* (36.32% in *Culex* genus, 19.16% in whole collected mosquitoes), *Culex* sp. (4.46% in *Culex* genus, 2.35 % in whole collected mosquitoes). In 2015, specimens of the *Culex* genus were *Cx. pipiens* complex and *Cx. torrentium* (79.41% in *Culex* genus, 26.79% in whole collected mosquitoes), *Cx. mimeticus* (1.12% in *Culex* genus, 0.37% in whole collected mosquitoes), *Cx. hortensis* (14.49% in *Culex* genus, 4.89% in whole collected mosquitoes), *Cx. theileri* (0.84% in *Culex* genus, 0.28% in whole collected mosquitoes), *Culex* sp. (4.13% in *Culex* genus, 1.39% in whole collected mosquitoes). Results were evaluated by collection points base; *Cx. pipiens* complex and *Cx. torrentium* comprised 74.5%, *Cx. mimeticus* comprised 4.38%, *Cx. hortensis* comprised 21.92%, *Culex* sp. comprised 1.75% in 2014. In 2015, *Cx. pipiens* complex and *Cx. torrentium* comprised 58.6%, *Cx. mimeticus* comprised 3.26%, *Cx. hortensis* comprised 15.21%, *Cx. theileri* comprised 2.17%, *Culex* sp. comprised 11.95% (Table 1). The presence of Culex species in sample collection areas is given in Figure 2.

3.1. Occurrence and distribution of *Culex pipiens* complex and *Culex torrentium*

Ace-2 marker molecular results revealed that all samples contained *Cx. pipiens,* and some points also included *Cx. torrentium*. *Cx. torrentium* specimens were identified from only seven collection points (Posof, Galler, Gümüşhane, Ispir Bayburt road, Savsat Artvin, Karakoy, and Sarp). Distribution preferences of *Cx. pipiens* complex and *Cx. torrentium* in habitat types of CORINE level 1 were significantly different (Chi-square = 14.692, df = 3, p = 0.002). Although *Cx. torrentium* is preferred in Agricultural and Forest, seminatural areas, *Cx. pipiens* complex preferred all habitat types (Agriculture, Artificial, Forest and seminatural, water bodies). Significant differences were found in level 2 habitat type distribution for *Cx. pipiens* complex and *Cx. torrentium* (Chi-square = 19.994, df = 8, p = 0.01). Although complex species and *Cx. torrentium* mainly occurred in the heterogeneous agricultural areas in 2014 and 2015, complex species were found in all level-classified areas except mine, dump and construction sites, and maritime wetland areas. In level 3, complex species mainly occurred in fruit trees and berry plantation areas, and *Cx. torrentium* mainly occurred in complex cultivation pattern areas. Statistical analysis showed significant differences (Chi-square = 34.020, df = 14 p = 0.002) in CORINE level 3.

According to the CQ11 marker results, *Cx. pipiens pipiens* and *Cx. pipiens molestus* biotypes were found in the study areas. *Cx. pipiens pipiens* include two haplotypes, and *Cx. pipiens molestus* has one haplotype. The distribution pattern showed significant differences whole 3 CORINE

Table 1. Collected specimens and their ratio (nd: not detect, n: frequency).

% ratio and years / Species	*Culex* genus	Total specimens	*Culex* genus	Total specimens	İdentification
	2014		2015		
Cx. pipiens complex and *Cx. torrentium*	53.59%	28.28%	79.41%	26.79%	Morphological
Cx. hortensis	36.32%	19.16%	14.49%	4.89%	Morphological
Cx. mimeticus	5.61%	2.96%	1.12%	0.37%	Morphological
Cx. theileri	x	x	0.84%	0.28%	Morphological
Others genus	ND	47.23%	ND	73.21%	Morphological
N	6912		4232		5572

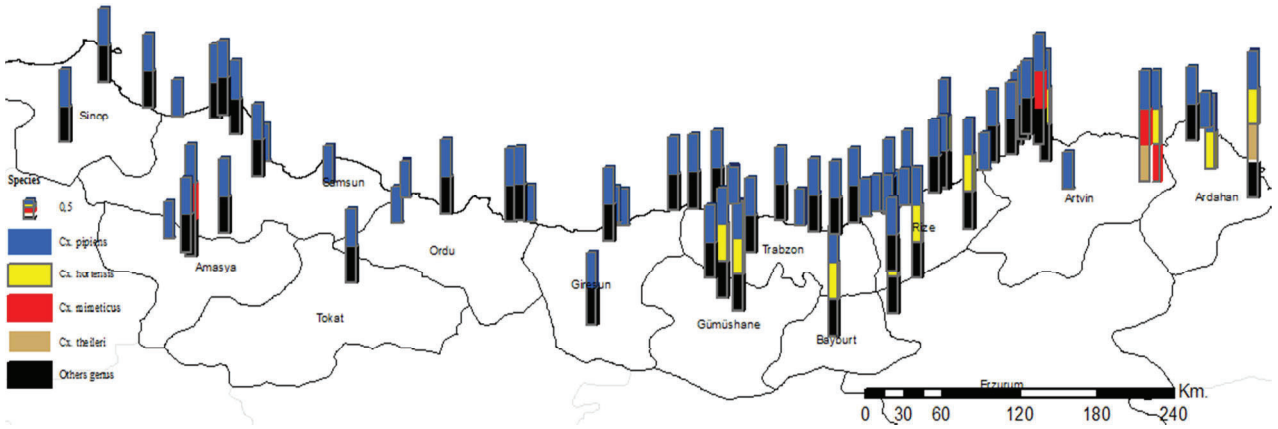

Figure 2. Distribution of Culex species in sample collection areas.

level. *Cx. pipiens pipiens* haplotype 1 and 2 and *Cx. pipiens molestus* were mainly found in agricultural areas for CORINE level 1, but significant differences were found at whole four level 1 habitat types (Chi-square = 26.158, df = 3, p = 0.000). In level 2, *Cx. pipiens pipiens* haplotype 1 and *Cx. pipiens molestus* were found mainly in heterogeneous agricultural areas, while *Cx. pipiens pipiens* haplotype 2 distribution patterns were the same with three habitat types (heterogeneous agricultural areas, arable land, and urban fabric). Although proportional differences were found in the distribution pattern in whole level 2 areas, there were no significant differences (Chi-square = 11.171, df = 7, p = 0.131). In level 3, there are no significant differences between the preferences of the area while differences were found in the distribution in the level three areas (Chi-square = 10.867, df = 12, p = 0.540).

3.2. Sequence analysis, assessment of genetic distance, and phylogenetic tree

A total of 730 samples from 73 collection points (Figure 3) (other collection points not included *Cx. pipiens* complex or *Cx. torrentium* samples according to the morphological data) were morphologically identified as *Cx. pipiens* complex or sibling species *Cx. torrentium*. In total, 146 DNA sequences were used for the molecular analysis of samples. A 651 bp portion of the *Ace-2* gene was obtained for all samples. *Ace-2* sequences consisted of 24 polymorphic and 21 parsimony-informative sites. The average total A+T content of the fragment was 51.73%, and the occurrence of both variable and parsimony-informative sites were 0.032 (21/651). The phylogenetic analysis tree of the *Ace-2* gene from *Cx. pipiens* complex was showed in Figure 4. The ML tree of *Ace-2* sequences of *Cx. pipiens* complex produced five haplogroups under one main lineage when grouped with Tunisian, USA, Iranian and Japanese samples present in GenBank (KU175335.1 for Tunisia, AY196910 for the USA, JF430595.1 for Iran, and AB294405 for Japan). The KM922631.1 sample from Italy was situated as the sister group of *Cx. pipiens*. *Cx. pipiens pallens* (AY497524.1) was placed nearest *Cx. pipiens* complex member. *Cx. australicus* (AY497523.1) and *Cx. quinquefasciatus* (FJ948080.1) samples from GenBank were the farthest from complex members. A total of 22 haplotypes were determined (GenBank accession numbers for *Culex pipiens* complex sequences OL342323-44), and haplotype distributions were grouped based on the city from which they were derived (Table 2). The most frequently occurring haplotypes identified were haplotype 5 (30.28%), haplotype 1 (22.36%), haplotype 4 (9.15%), haplotype 2 (6.33%), and haplotype 10 (5.63%) (Table 2).

An unrooted haplotype network revealed that Hap5 was the main group, and other haplotypes were one to eight mutational steps different from each other. Haplotype relationships are showed in Figure 5. The average nucleotide differences between individuals (k) were 2.07, haplotype diversity (Hd) was 0.848, and nucleotide diversity (π) was 0.00318. Pairwise genetic distances (Fst) between populations are shown in Table 3. According to Fst results, some populations were significantly different than others (e.g., Ordu differs significantly from Ardahan, Artvin, Giresun, Rize, Samsun, Trabzon, Sinop, Bayburt); other areas did not show any significant differences (e.g., Amasya). Although the number of samples varied on the basis of cities, it was found that the regions with a low sample size were located within the main haplotypes except for Sinop and Gümüşhane. Sinop and Gümüşhane samples have own unique haplotypes. The average $_{ST}$ value among populations was 0.18 and the average Nm (reproductive migrants per generation) value was 1.10, which indicated that a low degree of genetic differences between populations was observed and gene flow between the populations of the 12 cities and the Georgian population was high.

CQ11 marker analysis was carried out to determine biotypes of *molestus* or *pipiens* ecological forms. A total of

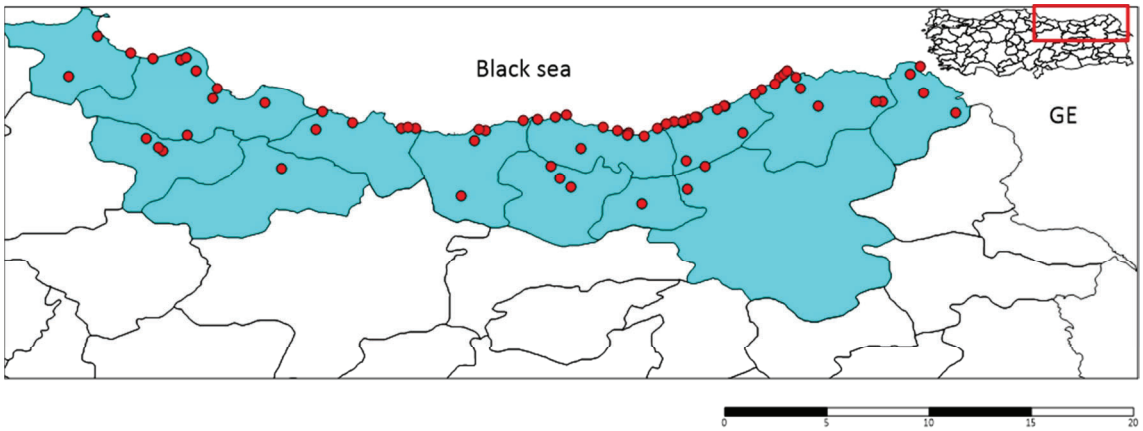

Figure 3. Points of the studied samples for *Ace-2* marker.

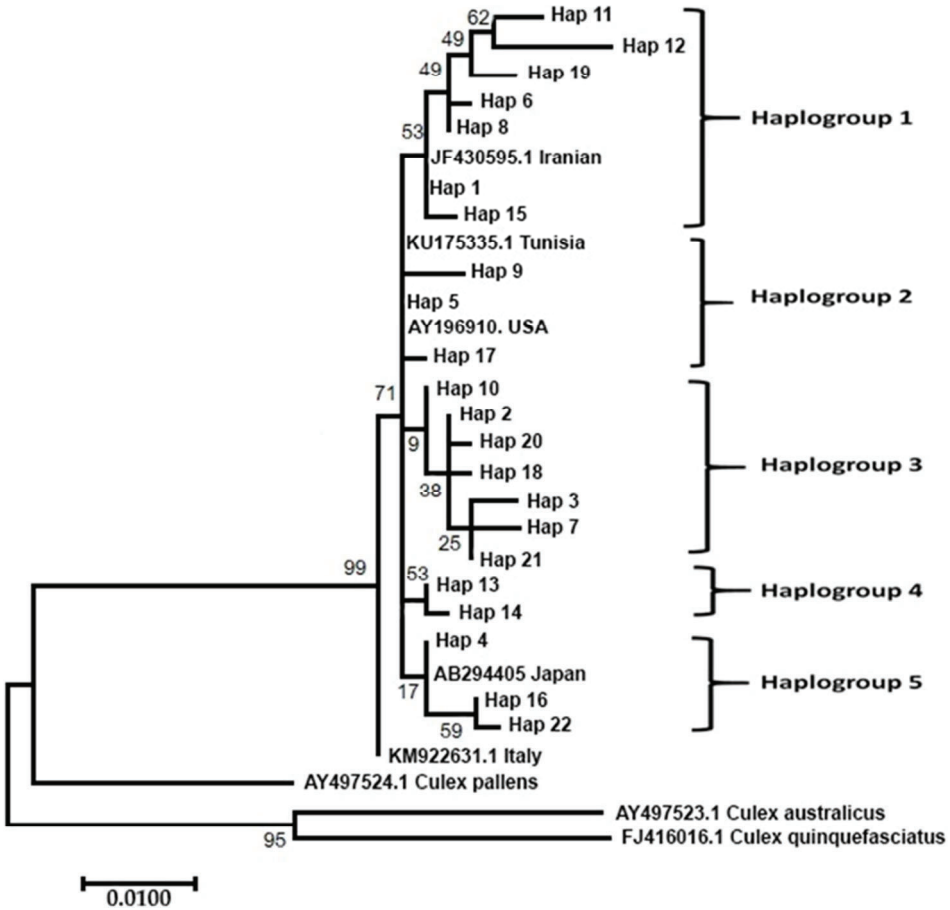

Figure 4. The phylogenetic tree is based on a 651-bp region of the Ace-2 gene from *Culex pipiens.* The tree was constructed using the maximum likelihood method, and bootstrap values are shown as numbers on the tree.

76 samples from 34 collection points were examined (Figure 6). A 151 bp fragment within the CQ11 microsatellite was obtained for all samples. CQ11 sequences consisted of 7 polymorphic and no parsimony-informative sites. The average total A+T content was 54.49%.

The molecular phylogenetic analysis tree of CQ11 gene from *Cx. pipiens* complex is shown in Figure 7. The ML tree of CQ11 sequences of *Cx. pipiens* complex produced three haplotypes under two haplogroups (GenBank accession numbers for *Culex pipiens pipiens* and *Culex pipiens*

Table 2. *Ace-2* haplotype distribution throughout the study area.

Hap	N	Ardahan	Artvin	Rize	Trabzon	Bayburt	Gümüşhane	Giresun	Ordu	Samsun	Sinop	Amasya	Tokat	Gürcistan
N	140	8	19	28	20	3	2	12	13	18	4	9	2	2
Hap1	34	2	2	2	7	0	0	6	4	7	0	2	2	0
Hap2	9	0	4	1	2	0	0	0	0	0	0	2	0	0
Hap3	1	0	0	0	0	0	0	0	0	0	0	1	0	0
Hap4	11	0	1	1	2	2	0	2	0	1	0	2	0	0
Hap5	43	4	10	12	3	1	0	2	2	3	2	2	0	2
Hap6	2	2	0	0	0	0	0	0	0	0	0	0	0	0
Hap7	2	0	2	0	0	0	0	0	0	0	0	0	0	0
Hap8	2	0	0	0	0	0	0	2	0	0	0	0	0	0
Hap9	2	0	0	0	0	0	2	0	0	0	0	0	0	0
Hap10	8	0	0	2	0	0	0	0	6	0	0	0	0	0
Hap11	1	0	0	0	0	0	0	0	1	0	0	0	0	0
Hap12	2	0	0	2	0	0	0	0	0	0	0	0	0	0
Hap13	2	0	0	2	0	0	0	0	0	0	0	0	0	0
Hap14	4	0	0	4	0	0	0	0	0	0	0	0	0	0
Hap15	2	0	0	2	0	0	0	0	0	0	0	0	0	0
Hap16	3	0	0	0	0	0	0	0	0	3	0	0	0	0
Hap17	3	0	0	0	1	0	0	0	0	2	0	0	0	0
Hap18	2	0	0	0	0	0	0	0	0	2	0	0	0	0
Hap19	2	0	0	0	0	0	0	0	0	0	2	0	0	0
Hap20	2	0	0	0	2	0	0	0	0	0	0	0	0	0
Hap21	2	0	0	0	2	0	0	0	0	0	0	0	0	0
Hap22	1	0	0	0	1	0	0	0	0	0	0	0	0	0

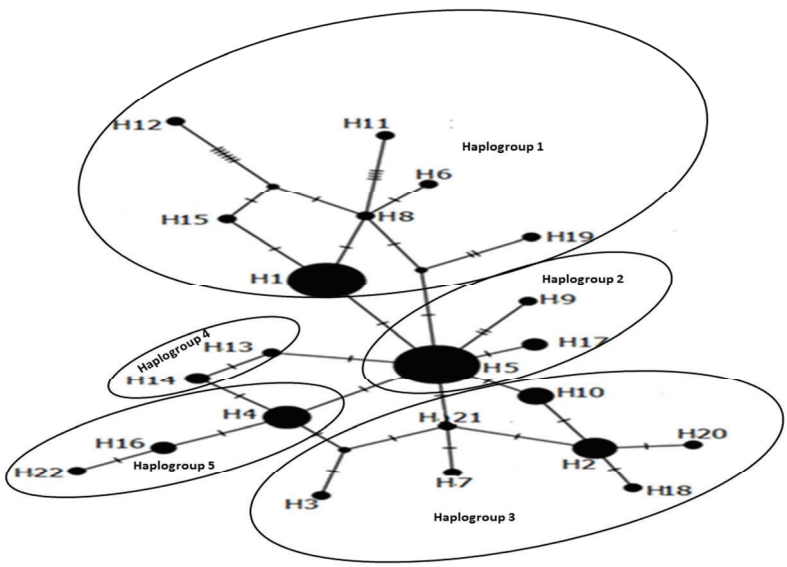

Figure 5. Unrooted haplotype network. Each circle represents a haplotype, and the lines above each link indicate one mutation. Small black dots indicate intermediate, missing, or unsampled haplotypes.

Table 3. Pairwise genetic distance (Fst) between cities according to the *Ace-2* data set.

	N	Amasya	Ardahan	Artvin	Giresun	Ordu	Rize	Samsun	Trabzon	Sinop	Bayburt	Georgia	Tokat
Amasya	9												
Ardahan	8	0.03635											
Artvin	19	0.02306	0.01312										
Giresun	12	0.01131	0.08905	0.16860*									
Ordu	13	0.10990	0.15350*	0.20695*	0.11938*								
Rize	28	0.03381	0.01276	0.02062	0.12970*	0.12667*							
Samsun	18	0.01970	0.06411	0.13652*	-0.00199	0.10384*	0.09986*						
Trabzon	20	−0.0300	0.05631	0.10040*	−0.01100	0.09299*	0.08145*	−0.00469					
Sinop	4	0.10219	0.07438	0.07548	0.23077*	0.24208*	0.04890	0.17161	0.14993*				
Bayburt	3	0.15169	0.44056	0.40221	0.31429	0.41876*	0.31650	0.31323*	0.25156*	0.52941			
Georgia	2	0.15169	−0.0126	−0.0545	0.31429	0.32810	−0.01074	0.23718	0.21531	0.11111	1		
Tokat	2	0.15169	0.27928	0.37199*	−0.04348	0.20394	0.29363	0.02019	0.02673	0.52941	1	1	
Gümüşhane	2	0.31510	0.44056	0.42966*	0.41463*	0.41876	0.33794*	0.34584*	0.31487*	0.52941	1	1	1
$p < 0.05$ was considered significant (*)													

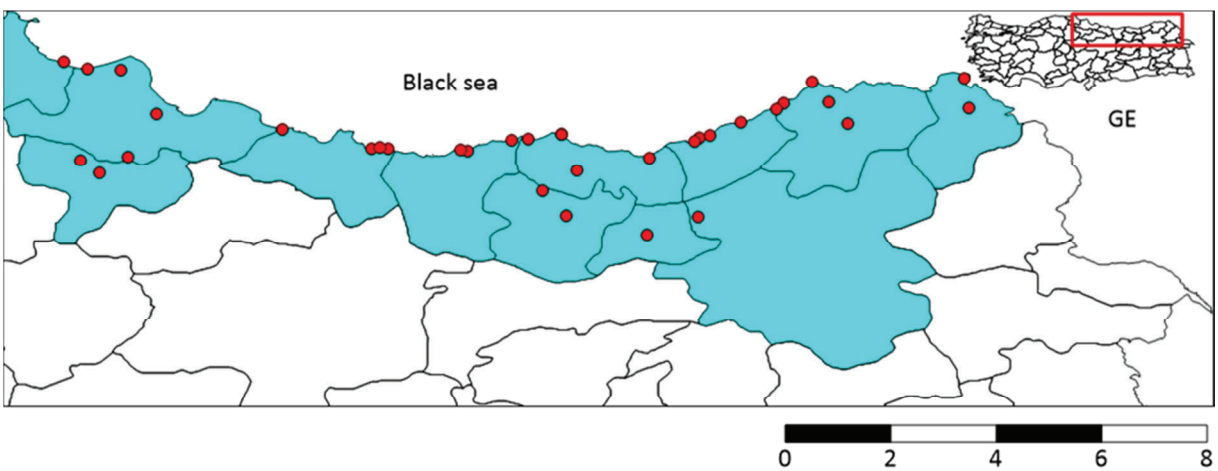

Figure 6. Points of the studied samples for CQ11 marker.

molestus sequences OL342320 -22). Although the sample size in the study was different from the cities, whole cities in the study included pipiens and molestus forms haplotypes except for Ardahan, Bayburt, and Gümüşhane. Ardahan and Gümüşhane samples located molestus form haplotype (haplotype 3). Bayburt samples located main haplotype of pipiens form (haplotype 1). Haplotype 2 was determined to be a sister group to haplotype 1, which consisted of a DQ470146.1 *Cx. pipiens pipiens* sample. The DQ470153.1 *Cx. pipiens quinquefasciatus* sample from GenBank was the nearest neighbor to the samples identified in the study. Haplotype 3 sequences and the DQ470150.1 *Cx. pipiens molestus* GenBank sample was the farthest lineage from haplotypes 1, 2. The AY962873.1 *Cx. australiacus* GenBank sample produced sister branch with hap 3.

A total of three haplotypes were identified, and the distribution of the haplotypes was affected by the city from which samples were collected (Table 4). Frequency of haplotype 1 was determined to be 44.59%, haplotype 2 was 36.48%, and haplotype 3 was 18.93%. Haplotype relationships are shown in Figure 8. The average nucleotide variation among individuals (k) was 2.36, haplotype diversity (Hd) was 0.64, and nucleotide diversity (π) was 0. 015. Pairwise genetic distances (Fst) between populations are shown in Table 5.

The average γst value among populations was 0.050 and the average Nm (reproductive migrants per generation) value was 4.71, which indicates low genetic differences and high degrees of gene flow between the populations of 11 cities in the Black Sea area and the Georgian population.

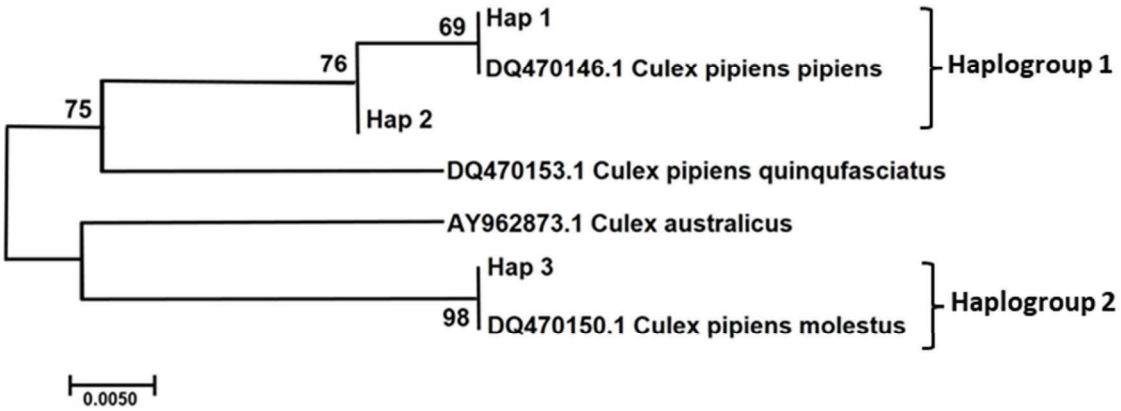

Figure 7. Phylogenetic tree based on a 151-bp region within the CQ11 microsatellite region of *Culex pipiens*. The tree was constructed using the maximum likelihood method, and bootstrap values are shown as numbers on the tree.

Table 4. CQ11 haplotype distribution throughout the study area.

Hap	N	Amasya	Ardahan	Artvin	Giresun	Ordu	Rize	Samsun	Trabzon	Sinop	Bayburt	Gümüşhane	Georgia
N	76	3	2	17	5	9	14	12	6	2	2	2	2
Hap1	34	2		6	3	3	5	7	3	1	2		2
Hap2	14	1		3	1	1	4	3	1		0		
Hap3	28		2	8	1	5	5	2	2	1	0	2	

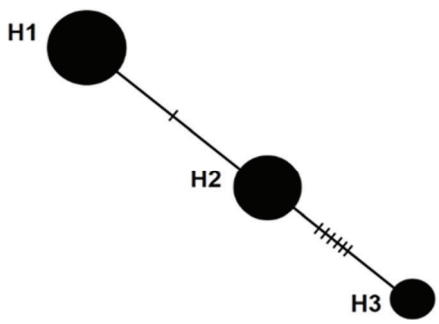

Figure 8. Unrooted haplotype network for CQ11. Each circle represents a haplotype, and lines above each link indicate mutations.

4. Discussion

The incidence of mosquito-borne diseases has rapidly increased over the last 20 years and they continue to threaten the lives of thousands of residents in many areas of the World. Global warming has also enhanced the severity of the situation and resulted in increased mosquito populations yearly. Many factors including transportation, human movement, and the creation of artificial breeding areas affect mosquito distribution. Human movements enhance mutual mating between genetic forms of *Cx. pipiens* complex and increase WNV (West Nile virus) risk.

Our results showed that the *Cx. pipiens* complex is common and distributed in all habitat types in middle and eastern Black Sea areas. Furthermore, sympatric co-occurrence of *Cx. torrentium* and *Cx. pipiens* species complex was found at five out of the seven *Cx. torrentium* sampling sites, solely complex cultivation pattern (CLC3) according to the CORINE classification. Zittra et al. (2016) reported the co-occurrence of the two sibling groups at 14 sampling localities. They also reported that the distribution of *Cx. torrentium* was a discontinuous urban fabric. Although our findings indicated that the rare distribution of the *Cx. torrentium* in our study area, Weitzell et al. (2011) reported that the *Cx. torrentium* is one of the most abundant species in Europe.

Sympatric co-occurrence of *Cx. pipiens* biotypes was found in 14 out of 74 sampling points, which were predominantly agricultural areas. This ratio is half of the *Cx. pipiens molestus* occurrence. The co-existence of the complex forms showed a similar pattern found predominately in heterogeneous agricultural areas (CLC level 2). These forms occur in distinct habitat types related to their ecological demand: *Cx. pipiens pipiens* was found everywhere but *Cx. pipiens molestus* is known to be an underground form (Chevilloon et al., 1995; Gomes et al., 2009; Amraoui et al., 2012). Some studies reported that the co-occurrence of two forms in highly urbanized

Table 5. Pairwise genetic distances (Fst) between cities according to the CQ11 data set.

	N	Amasya	Ardahan	Artvin	Giresun	Ordu	Rize	Samsun	Trabzon	Sinop	Bayburt	Gümüşhane
Amasya	3											
Ardahan	2	–0.06329										
Artvin	17	–0.11275	–0.15996									
Giresun	5	–0.3079	–0.11111	–0.11528								
Ordu	9	–0.05537	–0.2375	–0.07872	–0.11386							
Rize	14	–0.20422	–0.07925	–0.04008	–0.11418	–0.02218						
Samsun	12	–0.23969	–0.03119	–0.03973	–0.15815	–0.02662	–0.06117					
Trabzon	6	–0.23313	–0.16418	–0.11544	–0.21556	–0.13268	–0.09136	–0.12111				
Sinop	2	–0.27397	0	–0.23023	–0.34021	–0.3281	–0.12928	–0.18511	–0.35484			
Bayburt	2	–0.2	1	–0.00427	–0.22449	0.01247	0.02421	–0.1003	–0.16418	0		
Gümüşhane	2	–0.06329	0	–0.15996	–0.11111	–0.2375	–0.07925	–0.03119	–0.16418	0	1	
Georgia	2	–0.2	1	–0.00427	–0.22449	0.01247	0.02421	–0.1003	–0.16418	0	0	1

areas (Rudolf et al., 2013; Osario et al., 2014), and our results are contrary to these studies. Our results showed a cooccurrence pattern mostly in agricultural areas (CLC level 1) with heterogeneous agricultural areas (CLC level 2). Similar observations about the aboveground cooccurrence have also been reported by some studies (Kotera et al., 2010; Amara et al., 2016; Zittra et al., 2016; Beji et al., 2017).

Two *Cx. pipiens* complex forms (*Cx. pipiens pipiens, Cx. pipiens molestus*) along with *Cx. torrentium*, a potent vector for WNV were collected during the two years of the study (Jansen et al., 2019). All samples were identified morphologically as a member of the complex or *Cx. torrentium*. Rapid molecular discrimination of the species complex with *Ace-2* and CQ11 microsatellite markers (Smith and Fonseca, 2004; Bahnck and Fonseca, 2006; Kasai et al., 2008) facilitated the accurate and rapid identification of complex members and forms collected from large sample size and areas. First, we focused on the occurrence of species together with their accurate and rapid identification from large-scale urban, suburban and natural habitats. Most previous surveys have focused generally on urban and suburban environments where human population density is high. These areas generally are at great risk for viral transmission to human populations (Kirkman et al., 2011).

Many studies have reported low genetic variability in the COI region (Fedorova and Shakievich, 2007; Werblow et al., 2013; Werblow et al., 2014). Simonato et al. (2016) reported that a high degree of genetic variability, according to the mtDNA COI and COII markers. Phylogenetic analysis of COI and COII markers by maximum likelihood method revealed three branches and two of them showed a high degree of variability (Simonato et al., 2016). Their results indicated that the low degree of variability of the third branch was observed in areas in which a recent population expansion occurred. They also reported that the *Ace-2* region possessed a high degree of variability. Although our results produced twenty-four haplotypes (based on the different numbers of specimens from the study area), we could not find high genetic differences for the *Ace-2* marker. This may be due to the high gene flow found in our study.

The distribution pattern of *Cx. pipiens* complex along the middle and eastern Black Sea region and the use of the nuclear and microsatellite marker to discriminate the genetic structure between members of the complex and forms were reported in this study. We also reported that a low genetic differentiation and high gene flow was observed between natural and urban/suburban populations of the *Cx. pipiens* complex in the nuclear *Ace-2* marker. *Ace-2* marker analysis revealed the occurrence of a high number of haplotypes under one main lineage in our study. An unrooted haplotype network verified transitions and connections between populations. The diversity index of haplotype values was high, while nucleotide diversity was very low. Pairwise genetic distances (*Ace-2*) between analyzed populations indicated significant differences between some tested populations. Our results indicated that genetic variability of natural and urban/suburban populations varied, which was in contrast to other reports that had assessed for *Ace-2* in urban-suburban area populations (Fonseca et al., 2009; Lee et al., 2012). Though many studies have reported a different degree of genetic variability with mtDNA markers (Fedorova and Shakievich, 2007; Werblow et al., 2013; Werblow et al., 2014; Simonato et al., 2016), Kilic et al. (2019) reported that *Cx. pipiens* populations in the Aegean region of

Turkey were genetically less differentiated when they were assessed using 20 RAPD primers for 25 subpopulations of the species. The researchers also indicated that the results of their studies were consistent with others including Preet and Gupta (2017), Bibi et al. (2015), and Humeres et al. (1998). Our results may be explained by the low geographic distance between populations (maximum of 600 km from east to west) and cities, although pairwise genetic distances between some populations were significant. Furthermore, Cui et al. (2007) also concluded that *Cx. pipiens* populations require considerable distance before genetic isolation occurs. Frequent human movement occurs as a result of agricultural practices around the study area during the active mosquito season. Our study area also has a great quantity of truck traffic that travels from east to west. A low degree of genetic differentiation and a high level of gene flow among populations of the complex species *Cx. pipiens* occurring in the central and eastern Black Sea region indicate that movement occurs either through facilitated such transport or by highly mobile species itself.

Two *Cx. pipiens* biotypes (*pipiens* and *molestus*) were indistinguishable when genetic markers such as *Ace 2* and *ITS 2* were analyzed although other complex species can be distinguished using the Ace marker (Kasai and Komagata, 2008). Some authors reported the reliability of COI-based in the differentiation of the two forms (Shakievich, 2007; Danalaban et al., 2012). First, we aimed to rapidly discriminate between these forms after the analysis of the genetic structure of the species. Therefore, we used the CQ11 region. This method provided a means to accurately differentiate between biotypes, and the separation efficiency was very high when it was assessed using bootstrap values of the ML tree. Our results indicated that the three haplotypes were distributed throughout the study area. The first and second haplotypes formed one main lineage together with the GenBank sample, *Cx. pipiens pipiens* DQ470146, and the third haplotype formed a second lineage together with the GenBank sample, *Cx. pipiens molestus* DQ470150. Our results indicated that the frequency of the *molestus* biotype was around 20%, and the area assessed did not include hybrid populations of the two forms. Many researchers have reported high levels of *pipiens molestus* hybrid mosquitoes in situations in which interbreeding has occurred (Chevillon et al., 1995; Byrne and Nichols, 1999; Vinagrodova, 2000; Weitzel et al., 2009; Rudolf et al., 2013). Our study indicated a lower genetic diversity and a high level of gene flow between populations, despite failing to identify hybrid populations or specimens.

Our study is the first to extensively assess the distribution pattern and perform molecular screening of *Cx. pipiens* complex in the middle and eastern Black Sea region of Turkey. Results indicated that 1) *Culex torrentium* distribution seems to be limited in the study area when compared to *Culex pipiens* complex, 2) *Cx. pipiens* is distributed throughout the middle and eastern Black Sea region, 3) *pipiens* and *molestus* forms cooccur within the same area, often in sympatry, and 4) there is a low degree of genetic differences and a high level of gene flow between *Cx. pipiens* complex populations and forms.

In this study, the occurrence and spatial distribution patterns of *Cx. pipiens* forms and *Cx. torrentium* were identified, and genetic characterization of the mosquitoes is reported for the first time in Turkey. These findings will enhance our understanding of vector–pathogen dynamics in Turkey and inform researchers about the possibility of the occurrence of a possible WNV outbreak. It may also facilitate control efforts in many aspects. Shortly, we need to reevaluate the complex situation and need to perform extensive research studies investigating how the two different forms affect the occurrence of WNV in Turkey.

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