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Original article (Orijinal araştırma)

Monitoring and distribution of *kdr* and *ace-1* mutation variations in *Culex pipiens* L., 1758 (Diptera: Culicidae) in artificial sites and agricultural fields in the central and eastern Black Sea Region of Türkiye[1]

Türkiye'nin Orta ve Doğu Karadeniz Bölgesi tarımsal ve yapay alanlarda yayılım gösteren *Culex pipiens* L., 1758 (Diptera: Culicidae)'te *kdr* ve *ace-1* mutasyon varyasyonlarının izlenmesi ve dağılımı

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Abstract

Culex pipiens L., 1758 (Diptera: Culicidae) is one of the most important pests and disease vectors in the world. It is of major importance to monitor the development of insecticide resistance in order to effectively control. This study investigated the presence of mutations in specific loci of the *Vgsc* (*kdr* L1014F/C) and *ace-1* (G119S, F290V) gene, associated with insecticide resistance in *Culex pipiens* collected from nine provinces in central and eastern Black Sea Region of Türkiye in the 2020 active season. For *kdr*, L1014F mutation was determined for each region with three different silent mutations for wild and resistant type alleles, while L1014C was not recorded in any of the analyzed populations. For *ace-1*, substitution F290V was detected at a low frequency in heterozygosity, while G119S was more widespread, in the analyzed populations. For *ace-1*, G119I (6 populations) and G119A (5 populations) substitution was firstly described. Types of mutations differences related to the resistance between artificial sites and agricultural fields were not significantly different.

Keywords: *ace-1* resistance, common house mosquito, insecticide resistance, *kdr* resistance

Öz

Culex pipiens L., 1758 (Diptera: Culicidae) dünyadaki en önemli ve hastalık vektörü olan türlerden biridir. Efektif bir kontrol yapılabilmesi için insektisitlere karşı gelişen direnci takip etmek büyük öneme sahiptir. Bu çalışmada Türkiye Orta ve Doğu Karadeniz Bölgesi'nde 2020 aktif sezonunda dokuz ilden toplanan *Cx. pipiens* örneklerinde *vgsc* (*kdr* L1014F/C) ve *ace-1* (G119S, F290V) spesifik bölgelerinde direnç ile ilgili mutasyonların varlığı araştırılmıştır. *kdr* için, her bölgede L1014F mutasyonu belirlenirken, yabanıl ve dirençli tip aleller için üç farklı sessiz mutasyon tespit edilirken çalışılan popülasyonların hiçbirinde L1014C mutasyonu saptanmamıştır. *ace-1* bölgesi için, çalışılan popülasyonlarda F290V değişimi heterozigot ve düşük oranlarda saptanırken, G119S değişimi daha yaygın bulunmuştur. *ace-1* bölgesi için G119I (6 popülasyon) ve G119A (5 popülasyon) değişimleri ilk defa tespit edilmiştir. Dirence neden olan mutasyon tiplerinde yapay ve tarımsal alanlar arasında anlamlı fark bulunamamıştır.

Anahtar sözcükler: *ace-1* direnci, ev sivrisineği, insektisit direnci, *kdr* direnci

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Introduction

Mosquito-borne diseases pose a significant threat to public health, as they affect half of the population around the globe, leading to millions of fatal outcomes. Those caused by arboviruses are increasingly emerging or re-emerging in Europe. For example, West Nile virus disease cases have been reported from southeastern Europe and the Mediterranean Basin since the first large epidemic in 1996 in Romania (Ceianu et al., 2001; Rezza, 2014; Martinet et al., 2019). The epidemic potential of such diseases has been enhanced in the Palearctic Region by the spread of invasive mosquitoes (Marshall, 2000). *Culex pipiens* L., 1758 (Diptera: Culicidae) species complex, commonly known the house mosquito, are a pest and can also serve as vector for several arboviruses like West Nile virus (WNV), Rift Valley fever, and *Dirofilaria immitis* (Leidy, 1856) (Spirurida: Onchocercidae) (Diaz-Badillo et al., 2011; Akıner & Eksi, 2015; Grigoraki et al., 2018; Zakhia et al., 2018). WNV was first detected in Türkiye in the 1970s and has since spread to different areas of the country (Ari, 1972; Ozkul et al., 2006; Kalaycioglu et al., 2012; Ergunay et al., 2014; Akıner et al., 2019). There is no cure or efficacious vaccine for most vector-borne diseases. Therefore, the main control method to prevent these diseases is vector control. In the past organochlorine insecticides were used, but now pyrethroids, organophosphates and carbamates insecticides are most commonly used for mosquito control in Türkiye. However, the overuse of chemical insecticides imposes selection pressure for resistance genes, leading to mosquitoes becoming resistant to insecticides over time.

Two main insecticide resistance mechanisms are important in mosquitoes: (1) metabolic resistance arising from an increase in detoxification activity of enzyme families, namely glutathione S-transferases, mixed-function oxygenases, and carboxyl-esterase (Kasai et al., 1998; Hemingway et al., 2004; Whalon et al., 2008; Akıner & Ekşi, 2015); and (2) target-site insensitivity deriving from point mutations related to the nervous system proteins (Hemingway et al., 2004; Shi et al., 2015).

Pyrethroid and DDT insecticides affect the voltage-gated sodium channels *(vgsc)* of insects (Donnely et al., 2009). However, single nucleotide polymorphisms [SNP] or multiple substitutions occurring in the *Vgsc* genes reduce or eliminate the binding affinity of these insecticides to the sodium channel protein. There are more than 30 resistance-associated single nucleotide polymorphisms in sodium channel protein encoding genes (Wang et al., 2012; Lol et al., 2013). Knockdown resistance (*kdr*) resistance, the most important and well-known single nucleotide polymorphisms, involves the replacement of leucine (TTA) to phenylalanine (TTT) (L-to-F) at codon 1014 in domain II (Shi et al., 2015). This genetic locus in homozygosity (1014F/1014F) combined with the P450 metabolic resistance could produce highly resistant phenotype (Edi et al., 2012). In addition, *kdr*-type resistance mutations, such as L1014H/C/S/W have been identified in previous studies (Rinkevich et al., 2013; Scott et al., 2015; Taskin et al., 2016).

Organophosphates and carbamates target the acetylcholinesterase (AChE) which hydrolyzes the neurotransmitter acetylcholine (ACh) to acyl-enzyme and free choline to terminate nerve impulses of insects (Colovic et al., 2013). These insecticide groups are structurally similar to ACh, which is the substrate of the AChE enzyme, so inhibit AChE irreversibly by competing with ACh (Alout et al., 2008). Three different point mutations in the *ace-1* gene are responsible for resistance to organophosphates and carbamates. However, in *ace-1*, only Gly-to-Ser at codon 119 (G119S) and Phe to Val (F290V) at codon 290 were detected in *Cx. pipiens* species complex (Alout et al., 2008).

This study investigated of the target site mutations of *Cx. pipiens* species complex, which are widespread in parts of the Black Sea Region. For this purpose, L1014F, G119S and F290V mutations related to the insecticide resistance were screened in *Cx. pipiens* species complex and the variation of the mutation types in artificial sites and agricultural fields were also investigated.

Materials and Method

Mosquito collection and field classification

Thirty-three locations from nine provinces in the Black Sea Region were selected as the study areas. Mosquito collection was performed according to field sampling methods for mosquitoes described by European Center of Disease Control (Medlock et al., 2018). Briefly, larval mosquito samples were collected using 250 ml standard larval dipper and adult mosquito samples were collected using EVS trap with CO_2. The collections were performed in the active season of 2020 (May to October). Collection sites were classified artificial (man-made containers, inside usage tires, discarded metal and plastic containers, buckets, basement water puddles, marble, irrigation canals and ponds) and agricultural fields. Coordinates of sampling areas were recorded in decimal degrees with the help of GPS device (eTrex Vista HCx, Garmin, Olathe, KS, USA).

Sampling sites classification was performed by embedding the coordinates obtained in the field studies into CORINE (coordinated information on the environment) land cover (obtained from EEA, 2018) with a resolution of 1 km, and the CORINE equivalents of the coordinates were determined on the ArcGIS 10.5. The first level CORINE was used as the class of the samples (Table 1).

Table 1. Mosquito collection locations and features

Province	Location	Latitude (°N)	Longitude (°E)	CORINE code (class level 1)	Stage	Habitat
Amasya	Amasya	40.6674	35.8462	112 (artificial)	Larvae	Used tires
	Merzifon	40.8710	35.4639	111 (artificial)	Larvae	Puddle
	Saluca	40.7841	35.6817	242 (agricultural)	Larvae	Irrigation canal
Artvin	Arhavi	41.3586	41.3184	112 (artificial)	Larvae	Roadside puddle
	Artvin	41.1810	41.8308	112 (artificial)	Larvae	Used tires
	Borçka	41.3832	41.6909	222 (agricultural)	Larvae	Used tires
	Hopa	41.3876	41.4378	121 (artificial)	Larvae and adults	Used tires
Çorum	Osmancık	40.9691	34.8042	112 (artificial)	Larvae	Pond
Giresun	Görele	41.0374	38.9839	222 (agricultural)	Larvae	Metal container
Ordu	Gülyalı	40.9668	38.0572	112 (artificial)	Larvae	Roadside puddle
	Turnasuyu	40.9803	38.0019	222 (agricultural)	Larvae	Puddle
	Ünye	41.1229	37.2947	111 (artificial)	Larvae	Used tires
Rize	Ardeşen	41.1893	40.9701	112 (artificial)	Larvae	Used tires
	Çayeli	41.0720	40.7152	121 (artificial)	Larvae	Roadside puddle
	Fındıklı	41.2801	41.1527	112 (artificial)	Larvae	Used tires
	Hamidiye	41.1832	40.9535	222 (agricultural)	Larvae	Marble
	Ikizdere	40.7740	40.5577	242 (agricultural)	Larvae	Used tires
	Iyidere	40.9880	40.3309	112 (artificial)	Larvae	Used tires
	Pazar	41.1820	40.8932	112 (artificial)	Larvae and adults	Used tires. Near the larval habitat
	Rize	41.0416	40.5771	121 (artificial)	Larvae	Puddle

Table 1. Continued

Province	Location	Latitude (°N)	Longitude (°E)	CORINE code (class level 1)	Stage	Habitat
Samsun	Bafra	41.6177	35.8746	212 (agricultural)	Larvae and adults	Irrigation canal
	Çarşamba	41.2052	36.7417	242 (agricultural)	Larvae and adults	Puddle and tunnel
	Engiz	41.4941	36.0854	212 (agricultural)	Larvae	Irrigation canal
Sinop	Boyabat	41.4654	34.8217	213 (agricultural)	Larvae	Irrigation canal
	Dikmen	41.6508	35.2678	242 (agricultural)	Larvae	Irrigation canal
	Laçin	40.7751	34.8870	112 (artificial)	Larvae	Puddle
Trabzon	Akçaabat	41.0122	39.5935	111 (artificial)	Larvae	Used tires
	Arsin	40.9631	39.9889	112 (artificial)	Larvae	Metal container
	Çarşıbaşı	41.0877	39.3859	112 (artificial)	Larvae	Used tires
	Sümela	40.7307	39.6374	243 (agricultural)	Larvae	Plastic container
	Sürmene	40.9086	40.1078	112 (artificial)	Larvae	Roadside puddle
	Trabzon	40.9766	39.7480	121 (artificial)	Larvae	Used tires
	Vakfıkebir	41.0402	39.2802	112 (artificial)	Larvae and adults	Marble. Near the larval habitat

Species identification

Identification of *Cx. pipiens* species complex specimens was conducted using a computer-assisted Leica Microsystem EZ4 stereomicroscope (Leica Microsystems, Wetzlar, Germany) and a mosquito identification key prepared by Schaffner et al. (2001).

Molecular studies

DNA isolation

DNA isolation from *Cx. pipiens* samples individually was performed with the Gene JET genomic DNA isolation Kit (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania). Isolation was performed according to the manufacturer's instructions. Obtained DNA was labeled and stored at -20°C until the PCR was performed.

Identification of species complex members

The *ace-2* gene second intron region was amplified according to Smith & Fonseca (2004) to detect *Cx. pipiens*. The primers of ACEquin (5'-CCTTCTTGAATGGCTGTGGCA-3'), ACEpip (5'-GGAAACAACGACGTATGTACT-3'), ACEtorr (5′-TGCCTGTGCTACCAGTGATGTT-3′) and B1246 (5'-TGGAGCCTCCTCTTCACGGC-3') were used for species complex identification. The PCR conditions were as described by Smith and Fonseca (2004). Amplified DNA regions were visualized using 1.5% agarose gel.

Molecular assays

***kdr* mutations (L1014) diagnostic assays**

kdr mutation assays were performed according to the PCR method described by Martinez-Torres et al. (1998). PCR process was performed in two parallel reactions; the first reaction included forward-Cgd1 (5'-GTGGAACTTCACCGACTTC-3') reverse-Cgd2 (5'-GCAAGGCTAAGAAAAGGTTAAG-3') and forward-Cgd3 (5'-CCACCGTAGTGATAGGAAATTTA-3') primers, and the second reaction included forward-Cgd1, reverse-Cgd2 and forward-Cgd4 (5'-CCACCGTAGTGATAGGAAATTTT-3') primers. Amplified PCR products were run on the 1.5% agarose. The samples were classified according to base size using PCR

amplification of specific alleles (PASA). The sample subset consisting of 330 samples was sequenced using primers Cgd1 and Cgd2 (Macrogen Europe, Amsterdam, Netherlands).

Ace-1 mutations (F290V and G119S) diagnostic assays

Ace-1 mutations assays were performed using two different primer sets. For F290V mutation, Valdir 5'-ACGCTGGGGATCTGCGAGG-3', Valrev 5'-TCCACAACCGGAACGAACGGAAA-3', CxEx5dir 5'-GTCTGGCCGAGGCCGTCA-3', CxKrev2 5'-TGCTTCTGTGCGTGTACAGG-3' primers described by Weill et al. (2004) were used. PCR was performed according to the Weill et al. (2004). Amplified PCR products were run on 1.5% agarose gel, the samples were classified according to base size using PCR PASA method. Three hundred and thirty-three samples arbitrarily selected from the sample subset were amplified and sequenced using the CxEx5 and CxKrev2 primers covering the entire region (Macrogen).

For G119S mutation, molecular assays were performed using CxEx3dir (5'-CGACTCGGACCCACT GGT-3') and CxEx3rev (5'-GTTCTGATCAAACAGCCCCGC-3') primer set described by Weill et al. (2004). PCR was performed according to the Weill et al. (2004). The amplicons were sequenced (Macrogen). After the obtained sequences were aligned, each complementary sequence was cut virtually from the Alu-1 restriction site by using ClustalX2 program.

Data analysis

The frequencies were determined by the PASA method (L1014F and F290V) and sequencing (G119S) results were compared with Hardy-Weinberg expectations in the GenAlEx (ver 6.5) software. Differences between frequencies from the artificial sites and agricultural fields were examined using the AMOVA test (calculated in the Arlequin program using resistance codons and obtained frequencies). Raw sequence data were processed with Mega 7 (Kumar et al., 2016). Single nucleotide polymorphism points in the gene regions were determined according to the methods determined by Martinez-Torres et al. (1998), Alout et al. (2007a), and Weill et al. (2004), and the frequencies of the SNPs were calculated. Discrimination of species belonging to the *Cx. pipiens* species complex was based on different bands size upon PCR amplification of an *ace-2* region.

Results

Species identification

One thousand six hundred and fifty *Cx. pipiens* species complex field samples were analyzed. *Culex pipiens* species complex specimens were collected as larvae from all sampling points and as adults in some areas. The majority of the larvae were collected from the insides of used tires (~39%).

After morphological and molecular identification, the samples were determined to belong to the *Cx. pipiens* species complex. All samples produced approximately 600 bp bands on the agarose gel.

DDT and Pyrethroids resistance mutations

The most common L1014F mutation in *Cx. pipiens* species complex were screened using the PASA method. The scans included different frequencies for all three alleles, wild-type, heterozygous, and homozygous, of 33 populations from nine provinces. The frequency of the *kdr* wild-type allele (1014L) in the populations ranged up to 0.7 and the highest frequency denoted in Arsin population. Dikmen population was showed lowest degree of the wild-type allele frequency. The heterozygous frequency ranged up to 0.75, while the Iyidere population had the highest heterozygous allele frequency. Heterozygous genotypes were not observed in the Fındıklı population. The resistance allele frequency was zero in some populations (i.e., Borçka, Arhavi, Ardeşen, Pazar, Iyidere, Ikizdere, Arsin, Vakıfkebir, Boyabat and Laçin populations). Most of the population genotype frequencies were not suitable for Hardy-Weinberg equilibrium ($P < 0.05$) (Table 2).

Table 2. Genotype and variant allele frequencies (VAF) Hardy-Weinberg equilibrium ($\chi 2$) and P-values for L1014 *kdr* mutation in *Culex pipiens* according to PASA method (n = 50)

Provinces	Location	Genotype frequency				$\chi 2$	P
		L1014L	L1014L/F	L1014F	VAF		
Amasya	Amasya	0.545	0.364	0.091	0.273	0.347	0.556
	Merzifon	0.411	0.214	0.375	0.482	16.3	0.000*
	Saluca	0.333	0.222	0.444	0.556	15.1	0.000*
Artvin	Artvin	0.368	0.474	0.158	0.395	0.004	0.951
	Arhavi	0.471	0.529	0.000	0.265	6.48	0.011*
	Borçka	0.385	0.615	0.000	0.308	9.88	0.002*
	Hopa	0.264	0.415	0.321	0.528	1.40	0.237
Çorum	Osmancık	0.250	0.250	0.500	0.625	10.9	0.001*
Giresun	Görele	0.625	0.250	0.125	0.250	5.56	0.018*
Ordu	Gülyalı	0.275	0.275	0.450	0.588	9.36	0.002*
	Turnasuyu	0.448	0.172	0.379	0.466	21.4	0.000*
	Ünye	0.308	0.500	0.192	0.442	0.009	0.924
Rize	Ardeşen	0.556	0.444	0.000	0.222	4.08	0.043*
	Çayeli	0.176	0.765	0.059	0.441	15.2	0.000*
	Fındıklı	0.286	0.000	0.714	0.714	50.0	0.000*
	Hamidiye	0.333	0.375	0.292	0.479	3.09	0.079
	Ikizdere	0.667	0.333	0.000	0.167	2.00	0.157
	Iyidere	0.250	0.750	0.000	0.375	18.0	0.000*
	Pazar	0.600	0.400	0.000	0.200	3.12	0.077
	Rize	0.275	0.319	0.406	0.565	6.17	0.013*
Samsun	Bafra	0.484	0.226	0.290	0.403	14.1	0.000*
	Çarşamba	0.111	0.111	0.778	0.833	18.0	0.000*
	Engiz	0.286	0.429	0.286	0.500	1.02	0.312
Sinop	Boyabat	0.500	0.500	0.000	0.250	5.56	0.018*
	Dikmen	0.000	0.250	0.750	0.875	1.02	0.312
	Laçin	0.600	0.400	0.000	0.200	3.12	0.077
Trabzon	Akçaabat	0.417	0.333	0.250	0.417	4.94	0.026*
	Arsin	0.700	0.300	0.000	0.150	1.56	0.212
	Çarşıbaşı	0.444	0.333	0.222	0.389	4.46	0.035*
	Sümela	0.250	0.500	0.250	0.500	0.00	1.000
	Sürmene	0.514	0.257	0.229	0.357	9.68	0.002*
	Trabzon	0.556	0.222	0.222	0.333	12.5	0.000*
	Vakfıkebir	0.333	0.667	0.000	0.333	12.5	0.000*

* significant at $P < 0.05$.

Organophosphate/carbamate resistance mutations

Screening for the F290V mutation, which causes organophosphate/carbamate resistance, was performed using the PASA method. The frequency of the wild-type genotype frequency was high, while the frequencies of the resistant alleles were quite low. Wild-type genotype frequencies varied between 0.6 and 1 (except Amasya, Fındıklı, Sürmene populations) and the highest allele frequency was observed for four populations (Ardeşen, Çayeli, Ünye and Merzifon). The resistant allele frequency ranged up to 0.05 and the highest allele frequency was observed in the Arsin population. The variant allele was found in most of the populations except in Ardeşen, Çayeli, Ünye and Merzifon populations. All population genotype frequencies were suitable for Hardy-Weinberg equilibrium except for Fındıklı, Arsin and Sürmene populations (Table 3).

Table 3. Genotype and variant allele frequencies (VAF) Hardy-Weinberg equilibrium (χ2) and P-values for F290 a*ce-1* mutation in *Culex pipiens* according to PASA method (n = 50)

Provinces	Location	Genotype frequency				χ2	P
		F290F	F290F/V	F290V	VAF		
Amasya	Amasya	0.660	0.300	0.040	0.190	0.032	0.858
	Merzifon	1.000	0.000	0.000	0.000	-	-
	Saluca	0.840	0.160	0.000	0.080	0.378	0.539
Artvin	Arhavi	0.840	0.160	0.000	0.080	0.378	0.539
	Artvin	0.700	0.280	0.020	0.160	0.087	0.768
	Borçka	0.640	0.340	0.020	0.190	0.547	0.459
	Hopa	0.860	0.140	0.000	0.070	0.283	0.595
Çorum	Dikmen	0.780	0.200	0.020	0.120	0.140	0.708
	Osmancık	0.820	0.160	0.020	0.100	0.617	0.432
Giresun	Görele	0.800	0.180	0.020	0.110	0.326	0.568
Ordu	Gülyalı	0.840	0.140	0.020	0.090	1.05	0.304
	Turnasuyu	0.860	0.140	0.000	0.070	0.283	0.595
	Ünye	1.000	0.000	0.000	0.000	-	-
Rize	Ardeşen	1.000	0.000	0.000	0.000	-	-
	Çayeli	1.000	0.000	0.000	0.000	-	-
	Fındıklı	0.020	0.960	0.020	0.500	42.3	0.000*
	Hamidiye	0.94	0.060	0.000	0.030	0.047	0.827
	Ikizdere	0.920	0.080	0.000	0.040	0.087	0.768
	Iyidere	0.660	0.340	0.000	0.170	2.10	0.148
	Pazar	0.920	0.080	0.000	0.040	0.087	0.768
	Rize	0.860	0.140	0.000	0.070	0.283	0.595
Samsun	Bafra	0.920	0.080	0.000	0.040	0.086	0.768
	Çarşamba	0.620	0.340	0.040	0.210	0.030	0.861
	Engiz	0.820	0.160	0.020	0.100	0.617	0.432

Table 3. Continued

Provinces	Location	Genotype frequency				χ2	P
		F290F	F290F/V	F290V	VAF		
Sinop	Boyabat	0.760	0.200	0.040	0.140	1.44	0.231
	Laçin	0.720	0.260	0.020	0.150	0.019	0.890
Trabzon	Akçaabat	0.720	0.260	0.020	0.150	0.019	0.890
	Arsin	0.800	0.150	0.050	0.130	7.26	0.007*
	Çarşıbaşı	0.780	0.200	0.020	0.120	0.140	0.708
	Sümela	0.680	0.280	0.040	0.180	0.132	0.716
	Sürmene	0.380	0.620	0.000	0.310	10.1	0.001*
	Trabzon	0.86	0.14	0.000	0.070	0.283	0.595
	Vakfıkebir	0.840	0.160	0.000	0.080	0.378	0.539

* significant at P < 0.05.

The G119S mutation screening was performed by sequence analysis. The frequency of the wild-type genotype was high, while the frequencies of the resistant alleles were quite low. Wild-type genotype frequencies varied between 0.3 and 0.8 and the highest allele frequency was observed in Rize, Iyidere, Arsin, Çarşıbaşı, Ünye. The resistant genotype frequency ranged up to 0.5 and the highest allele frequency was observed in the Merzifon population. The variant allele was found in all of the populations. Population genotype frequencies were suitable for Hardy-Weinberg equilibrium except for Bafra, Dikmen, Osmancık and Amasya populations (Table 4).

Table 4. Genotype and variant allele frequencies (VAF) Hardy-Weinberg equilibrium (χ2) and P-values for G119 *ace-1* mutation in *Culex pipiens* according to sequence data (n = 10 unless noted)

Province	Location	Genotype frequency				χ2	P
		G119G	G119G/S	G119S	VAF:		
Amasya	Amasya	0.600	0.100	0.300	0.350	6.09	0.014*
	Merzifon	0.300	0.200	0.500	0.600	3.40	0.065
	Saluca	0.600	0.200	0.200	0.300	2.74	0.098
Artvin	Arhavi	0.500	0.300	0.200	0.350	1.16	0.281
	Artvin	0.700	0.300	0.000	0.150	0.311	0.577
	Borçka	0.600	0.400	0.000	0.200	0.625	0.429
	Hopa	0.600	0.400	0.000	0.200	0.625	0.429
Çorum	Osmancık	0.700	0.000	0.300	0.300	10.0	0.002*
Giresun	Görele	0.600	0.300	0.100	0.250	0.400	0.527
Ordu	Gülyalı	0.600	0.200	0.200	0.300	2.74	0.098
	Turnasuyu	0.600	0.400	0.000	0.200	0.625	0.429
	Ünye	0.800	0.200	0.000	0.100	0.123	0.725
Rize	Ardeşen	0.500	0.300	0.200	0.350	1.16	0.281
	Çayeli	0.600	0.300	0.100	0.250	0.400	0.527
	Fındıklı	0.600	0.200	0.200	0.300	2.74	0.098

Table 4. Continued

Province	Location	Genotype frequency			VAF:	χ2	P
		G119G	G119G/S	G119S			
	Hamidiye	0.600	0.200	0.200	0.300	2.74	0.098
	Ikizdere	0.700	0.300	0.000	0.150	0.311	0.577
Rize	Iyidere	0.800	0.200	0.000	0.100	0.123	0.725
	Pazar	0.500	0.400	0.100	0.300	0.023	0.880
	Rize	0.800	0.200	0.000	0.100	0.123	0.725
	Bafra	0.700	0.000	0.300	0.300	10.0	0.002*
Samsun	Çarşamba	0.600	0.200	0.200	0.300	2.74	0.098
	Engiz	0.500	0.300	0.200	0.350	1.16	0.281
	Boyabat (n = 9)	0.667	0.222	0.000	0.222	1.15	0.284
Sinop	Dikmen	0.700	0.100	0.200	0.250	5.38	0.020*
	Laçin	0.500	0.400	0.100	0.300	0.023	0.880
	Akçaabat	0.700	0.200	0.100	0.200	1.41	0.236
	Arsin	0.800	0.200	0.000	0.100	0.123	0.725
	Çarşıbaşı	0.800	0.200	0.000	0.100	0.123	0.725
Trabzon	Sümela	0.600	0.200	0.200	0.300	2.74	0.098
	Sürmene (n = 9)	0.667	0.333	0.000	0.167	0.360	0.549
	Trabzon	0.600	0.200	0.200	0.300	2.74	0.098
	Vakfıkebir	0.600	0.200	0.200	0.300	2.74	0.098

* significant at P < 0.05.

Mutation combinations

We examined the different codon combinations in the loci of interest associated with insecticide resistance, in particular *kdr* L1014 and *ace-1* G119, F290. We identified six different genotype combinations at the L1014F mutation point. For all locations, the TTA (leucine) codon had a highest frequency (0.679). Frequencies of TTA/C (leucine/phenylalanine), TTG (leucine), TTT/G (phenylalanine/leucine) codons were quite low and their values were 0.009, 0.006, and 0.009, respectively. In addition, the TTG codon encoding the amino acid leucine was a silent mutation. The frequencies of the determined gene combinations are given in Table 5.

We identified four different codons in the *ace-1* gene locus G119S. Among all the sequences, the GGC (glycine) codon and AGC (serine) codon frequencies followed, and their values were 0.709 and 0.079, respectively. Heterozygote frequency of the point mutation (RGC) was 0.176. The frequencies of ATC and ARC mutations were quite low in the population and their values were 0.018 and 0.015 respectively. Glycine/isoleucine (6 populations) and glycine/asparagine (5 populations) substitutions frequencies quite low and found around 0.1 for all determined populations. These types of substitutions were firstly described in G119 locus. TTT (phenylalanine) and G/TTT (valine/phenylalanine) codon combinations were determined at the F290V mutation point, which is the other *ace-1* resistance mutation point, and the frequency values were 0.842 and 0.158, respectively (Table 5).

Table 5. Codon combinations in *kdr* L1014F and *ace-1* G119S, F290V mutation sites and their frequencies (W:A or T, M:A or C, K:G or T, and R:A or G; n = 10 per location)

Location	L1014F						G119S					F290V	
	TTA	TTW	TTT	TTM	TTG	TTK	GGC	AGC	RGC	ATC	ARC	TTT	KTT
Amasya	0.8	0.1	0.1	-	-	-	0.6	0.3	0.1	-	-	0.9	0.1
Merzifon	1.0	-	-	-	-	-	0.5	0.3	0.1	0.1	-	1.0	-
Saluca	0.9	-	0.1	-	-	-	0.6	0.1	0.2	0.1	-	1.0	-
Arhavi	0.7	0.3	-	-	-	-	0.6	0.1	0.2	0.1	-	0.9	0.1
Artvin	0.4	0.3	0.1	-	0.1	0.1	0.8	-	0.1	0.1	-	0.8	0.2
Borçka	0.5	0.3	0.1	-	-	0.1	0.8	-	0.2	-	-	0.9	0.1
Hopa	0.7	0.3	-	-	-	-	0.7	-	0.2	-	0.1	0.9	0.1
Osmancık	0.5	0.3	0.2	-	-	-	0.8	0.2	-	-	-	0.9	0.1
Görele	0.7	0.3	-	-	-	-	0.8	-	0.2	-	-	0.7	0.3
Gülyalı	0.7	0.2	0.1	-	-	-	0.7	-	0.3	-	-	0.9	0.1
Turnasuyu	0.7	0.2	0.1	-	-	-	0.8	-	0.1	-	0.1	0.8	0.2
Ünye	0.9	0.1	-	-	-	-	0.8	-	0.2	-	-	1.0	-
Ardeşen	0.9	0.1	-	-	-	-	0.5	0.2	0.3	-	-	1.0	-
Çayeli	0.9	0.1	-	-	-	-	0.6	0.1	0.3	-	-	1.0	-
Fındıklı	0.2	0.7	0.1	-	-	-	0.8	-	0.2	-	-	0.8	0.2
Hamidiye	0.8	0.1	0.1	-	-	-	0.7	0.1	0.2	-	-	0.8	0.2
Ikizdere	1.0	-	-	-	-	-	0.8	-	0.2	-	-	0.8	0.2
Iyidere	0.5	0.2	0.2	0.1	-	-	0.8	-	0.2	-	-	0.7	0.3
Pazar	0.8	0.2	-	-	-	-	0.6	0.1	0.3	-	-	0.9	0.1
Rize	0.7	0.2	-	0.1	-	-	0.8	-	0.2	-	-	0.8	0.2
Bafra	0.6	0.2	-	-	0.1	0.1	0.8	0.1	-	-	0.1	1.0	-
Çarşamba	0.5	0.4	0.1	-	-	-	0.6	0.2	0.2	-	-	0.7	0.3
Engiz	0.7	0.2	0.1	-	-	-	0.6	0.1	0.3	-	-	0.9	0.1
Boyabat	0.5	0.4	0.1	-	-	-	0.8	-	0.1	-	-	0.7	0.3
Dikmen	0.6	0.2	0.2	-	-	-	0.7	0.2	0.1	-	-	0.8	0.2
Laçin	0.6	0.3	0.1	-	-	-	0.5	0.1	0.3	0.1	-	0.8	0.2
Akçaabat	0.6	0.3	0.1	-	-	-	0.8	-	0.1	-	0.1	0.7	0.3
Arsin	0.8	0.1	0.1	-	-	-	0.8	-	0.2	-	-	0.9	0.1
Çarşıbaşı	0.7	0.2	0.1	-	-	-	0.9	-	0.1	-	-	0.8	0.2
Sümela	0.6	0.2	0.2	-	-	-	0.7	0.1	0.1	0.1	-	0.7	0.3
Sürmene	0.4	0.5	-	0.1	-	-	0.7	-	0.2	-	0.1	0.6	0.4
Trabzon	0.7	0.2	0.1	-	-	-	0.7	0.2	0.1	-	-	0.9	0.1
Vakfıkebir	0.8	0.2	-	-	-	-	0.7	0.1	0.2	-	-	0.8	0.2
Total	0.679	0.224	0.073	0.009	0.006	0.009	0.709	0.079	0.176	0.018	0.015	0.842	0.158

Amova analyses

We conducted AMOVA analysis to determine the *kdr* and *ace-1* resistance among the CORINE land cover in level 1. The analysis of the *kdr* resistance variance component among groups was found to be low (0.61%) and FCT distance was 0.242 (P > 0.05). The difference between the populations was high and the FST value revealed a low distance of 75.3% (P < 0.005). The AMOVA analysis by *ace-1* F290V region resistance among the CORINE land cover in level 1 results was similar to *kdr* resistance analysis. Variance between groups -0.98% and FCT value was 0.218 (P > 0.05). Variance component among populations within groups and within populations was 0.003 (21.0%) and 0.012 (79.0%) respectively and FSC and FST values was 0.210 and -0.010, respectively (P > 0.05). For G119S region analysis did not revealed any significant differences among groups and populations. FCT, FSC and FST statistics were the lowest of the tested locations and no significant differences (Table 6).

Table 6. Analysis of Molecular Variance (AMOVA) of the two groups (artificial sites and agricultural fields) of the *Culex pipiens* L1014F and F290V, G119S mutations

Cross comparison	Variance components (% of variation)			F-statistics		
	Among groups	Among populations within groups	Within populations	FCT	FSC	FST
L1014F	0.00057 (0.61%)	0.02269 (24.9%)	0.07090 (75.3%)	0.24242	0.24705*	0.00610*
F290V	-0.00015 (-0.98%)	0.00343 (21.0%)	0.01229 (79.0%)	0.21798	0.21032*	-0.00980*
G119S	-0.00053 (-0.64%)	0.00024 (0.29%)	0.08260 (100%)	-0.00650	0.00294	-0.00354

* significant at P < 0.05.

Discussion

Culex pipiens are biting pests and are vectors of many pathogens important to human and animal health. Therefore, vector control studies generally target this species in many areas. However, vector control studies restrict to the development of insecticide resistance. Rapid identification of target-site resistance mutations in *Cx. pipiens* wild populations can improve control operations through effective resistance management. In this study, mutations in the *vgsc* and *ace-1* genes, related to insecticide resistance, were monitored in *Cx. pipiens* populations in the central and eastern Black Sea Region of Türkiye. Insecticide application related to the agricultural purposes supported the selection pressure in many agricultural areas for mosquito species (Awolola et al., 2007; Akıner et al., 2013). Therefore, different areas *vgsc* and *ace-1* genes frequencies may be different according to the insecticide selection pressure from different areas. Secondly, we investigated frequencies of different alleles that are related to insecticide resistance and possible differences in artificial (constructed or changed by human) sites and agricultural fields. In addition, we screened the presence of new mutation types in the genetic loci related to the insecticide resistance.

Voltage-gated sodium channels are an important for membrane exitability and are responsible for the depolarization phase of action potential in all types of exitable cells (Yu & Catterall, 2003). It is important to get basic data about frequencies of different allele combinations related to the pyrethroid and DDT resistance on local and regional scales (Wang et al., 2012). The L1014F mutation was firstly discovered in *Musca domestica* L related to the Pyrethroid group insecticides and DDT (Chandrasiri et al., 2020). The *Vgsc* L1014 (TTA) codon which encodes leucine and is known as a wild type, was present in all populations, and varied various degrees. The TTT codon encodes phenylalanine and has been reported by many sources to cause resistance (Martinez-Torres et al., 1998; Scott et al., 2015; Shi et al., 2015; Wang et al., 2015; Bkhache et al., 2016; Taskin et al., 2016). Our results revealed three different silent mutations for wild-type and resistant genotypes. While one of them encodes leucine, other genotypes displayed heterozygote

properties (L/F). Our study identified genotypes with TTA homozygote susceptible, TTW (A/T) heterozygote resistance, TTT homozygote resistance, TTM (A/C) heterozygote resistance with silent mutations, TTG homozygote susceptibility with silent mutations and TTK (G/T) heterozygote resistance with silent mutations. Ponce et al. (2016) reported several substitutions with cysteine, histidine, serine or tryptophan in *Culex quinquefasciatus* (Say, 1823) (Diptera: Culicidae) populations. Although our results revealed six mutation types, only one amino acid substitution was observed. The substitution of A to T and A to C and A to G was found in our study and incidence of the A to T was found at a high rate. Although Roberts & Andre (1994) reported the predominance A to C mutations in Sri Lankan *Cx. quinquefasciatus*, Chandrasiri et al. (2020) found A to T mutations predominance in *Cx. quinquefasciatus* populations of Sri Lanka in subsequent years. These results indicated that mutation frequencies can change over time and insecticide resistance dynamic process. A to T or A to C mutation types in third position of codon (1014) is also described in Turkish *Cx. pipiens* populations (Taskin et al., 2016). They also reported predominance of A to T mutations like our study. TTG codon mutation encoding leucine was determined as homozygosity and heterozygosity in some populations of the study (Artvin, Borcka and Bafra populations). It has also been detected in *Anopheles sinensis* (Wiedemann, 1828) (Diptera: Culicidae) in China in previous studies (Zhong et al., 2013; Wang et al., 2015). However, there has been no previous report of this mutation type in *Cx. pipiens* species complex. The average frequency of the wild-type, susceptible allele (L1014) was comparably high (0.2-0.9) in our study. Heterozygote and homozygote resistance type substitutions frequency may be supported moderate or low level of resistance in the middle and eastern Black Sea populations in Türkiye. Taskin et al. (2016) reported high frequencies of two types substitutions (L1014F and L1014C) in *Cx. pipiens* Aegean Region populations in Türkiye. Although they found high frequency of L1014C substitution, we did not observe this type of substitutions. They indicated the possibility of *kdr* as an important mechanism of insecticide resistance in Türkiye *Cx. pipiens* species complex (Taskin et al., 2016). Many factors affect pyrethroid and DDT resistance such as P450 mediated enhanced metabolism. Therefore, real resistance ratio should estimate all types of mechanisms together when using this type of insecticides.

The *kdr* allele frequencies in the studied populations were generally not suitable for Hardy-Weinberg expectation and the difference was statistically significant ($P < 0.05$). This situation can be associated with resistance selection pressure in these areas. In Borçka, Arhavi, Ardeşen, Iyidere, Vakfıkebir, Boyabat and Çayeli populations, homozygous resistance genotype frequencies were zero or quite low, and the frequencies of heterozygous genotypes were high. This may indicate that there is a balance selection in these locations. High resistant genotype frequencies (Rize, Gülyalı, Fındıklı, Çarşamba and Osmancık populations) may explain selection pressure continues in these areas. Low or moderate rate of L1014F mutations may explain long term use of DDT in Türkiye for malaria eradication campaign and agriculture in many areas. Taskin et al. (2016) reported the same situation in Aegean Region of Türkiye where they found another mutation, L1014C, in high frequency. They associated these results with permethrin and another novel insecticide (pyrethroid) usage in those areas. They additionally highlighted the prolonged and excessive use of pyrethroids and the imposing selection pressure against *Cx. pipiens* in that region.

Three distinct mutations in *ace-1* region were described related to the organophosphate and carbamate resistance (Massouli et al., 1992). Most common resistance mutation type in mosquitoes (including *Cx. pipiens*) was identified G119S in the *ace-1* gene region around the catalytic site (Weill et al., 2003). F290V was described in *Cx. pipiens* strain collected in Cyprus (Wirth & Georghiou, 1996) and has been found around in Mediterranean areas several times (Alout et al., 2009; Osta et al., 2012; Taskin et al., 2016) Another point mutation (F331W) related to the resistance was described in East Asian *Culex tritaeniorhynchus* (Giles, 1901) (Diptera: Culicidae) populations (Nabeshima et al., 2004; Alout et al., 2007a, b). Four combinations were found in *ace-1* G119 position in our study: GGC (glycine) homozygote susceptible, AGC (serine) homozygote resistant, RGC (GGC/AGC) heterozygote resistant, and different codon combinations encoding isoleucine and serine, asparagine ARC (AGC/AAC). Although four

combinations were found, homozygote susceptible frequencies were dominant (0.709). Homozygote resistant frequencies were found around half of the tested populations but frequencies were quite low (0.079). Heterozygote resistant genotypes were detected across all analyzed populations except Artvin, Hopa, Borçka, Bafra and Osmancık. The other types frequencies were quite low, encoding for isoleucine (ATC) or asparagine/serine (AAC/AGC). Many studies on *Cx. pipiens* and other mosquito species have reported that the frequency of the GGC codon is high, and the frequency of the AGC codon is low (Alout et al., 2007b; Dabire et al., 2014; Taskin et al., 2016; Bkhache at al., 2019; Major et al., 2020). Our results are consistent with the findings of studies conducted in Mediterranean countries (Osta et al., 2012; Kioulos et al., 2014). The high mutation frequencies found could reflect the history of insecticidal interventions around the Mediterranean Basin, possibly implying that selection pressure still occurs. Taskin et al. (2016) described the same situation in Aegean Region of Türkiye. In addition, no records were found for the ATC and AAC codons determined in other studies of *Cx. pipiens* and other mosquito species. The effect of the new codon type on the species needs to be determined. In this regard, their contribution to insecticide resistance should be investigated. Second mutation in the *ace-1* gene combination F290V was found in the study area. These mutation types were described in Cyprus *Cx. pipiens* populations (Alout et al., 2007b; Alout et al., 2009). Although G119S mutation were described around the world, F290V was rarely recorded in Mediterranean countries such as Greece, Morocco, Tunisia, and Türkiye (Alout et al., 2007b; Kioulous et al., 2014; Ben Cheikh et al., 2009; Taskin et al., 2016; Arich et al., 2021). Two different codon combinations were identified at the F290 mutation point causing organophosphate/carbamate resistance in this study, and these combinations were TTT codon (wild type) and GTT codon (encoding valine). The frequency of the TTT codon in all populations was high and its value was 0.842. The GTT codon was found only as heterozygous in the populations. The results obtained in the study correlated with the results obtained in the Aegean Region of Türkiye (Taskin et al., 2016). For all AChE mutations, studied populations showed tendencies towards an excess of heterozygotes. Similar results were obtained by some authors (Taskin et al., 2016; Arich et al., 2021).

Our results revealed different degrees of the target-site mutation related to the insecticide resistance. Target-site mutations showed heterozygosity in the field the degree of the mutations still low in the field. This situation will be problematic for the future control application in the field due to the nature of the insecticide resistance. In the AMOVA analyses made with the resistance frequencies obtained, the resistance differences between artificial sites and agricultural fields were determined to be low. However, there was substantial variation within populations. This situation shows that insecticide resistance did not differ in terms of area (constructed/modified or temporary growing areas by changing human and agricultural areas) and that insecticide resistance could be related to whether or not insecticide was used in the areas.

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