Molecular phylogenetics of Aedes aegypti (L., 1762) (Diptera: Culicidae) in Eastern Black Sea area of Turkey and possible relations with the Caucasian invasion

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Abstract: *Aedes aegypti* (L., 1762) (Diptera: Culicidae) is an important pest for human health. It vectors many diseases, including yellow fever (YF), dengue (DENV), and chikungunya (CHIKV). This species has invaded numerous countries including Balkan and Caucasian countries few past decades. The established populations of species were found in Turkey in 2015. We investigated the genetic variation, molecular phylogenetics, and differences between samples from Turkey and Georgia using four mitochondrial and one genomic DNA markers. The possible origin of the species was determined separately for each gene region using maximum likelihood trees. The ML analysis showed a close relation with the Caucasian samples, and some haplotypes are specific to this region. Our results suggest that Turkish *Ae. aegypti* strains might have been a mix of Asian and American strains. The differences between Turkish and Georgian samples were statistically insignificant and values of difference were very low according to AMOVA. Pairwise difference values between the two countries indicated that populations may have the same origin and variation value between two countries is very low. As a conclusion, our results revealed that our region (Turkey and Georgia) samples were most probably a new invasion rather than an ancient one.

Key words: *Aedes aegypti*, mitochondrial and genomic marker, molecular phylogenetics, Black Sea region

1. Introduction

Aedes aegypti (L., 1762) (Diptera: Culicidae) transmits several arboviruses such as dengue, yellow fever, zika, and chikungunya. These viral diseases are considerable health threats in many parts of the World (Kraemer et al., 2015). Dengue is caused by the dengue virus (DENV); it affects around 400 million people every year and over four billion people are at risk (Bhatt et al., 2013). Dengue risk is affected by mosquito genotypes, climatic conditions, environmental changes, growth and movement of human populations, and immunity (Morrison et al., 2008). Similar risks can be considered for yellow fever, zika, and chikungunya. Despite there being an effective vaccine, yellow fever has not been eradicated and more than thousands of new cases reported annually from Africa and Amazonia. There are reports of imported yellow fever cases in Asia and Europe (Jácome et al., 2019). Zika is another disease that has been circulating in some regions in Africa, Asia, Americas, and Pacific. This disease was declared as public health emergence by WHO[1] in 2015. Transmission has decreased over the years (Masmejan et al., 2020). Chikungunya virus was first reported in 1952-53 in Tanzania. Since then numerous

epidemics of chikungunya have reported in more than 60 countries in Asia, America, Pacific, and Europe including India, Malaysia and Indonesia (Manzoor et al., 2022).

Epidemic of mosquito-borne diseases has been observed in Turkey. Dengue occurred eleven times between 1889 and 1945 in İzmir (four times), Manisa (one time), Antalya (one time), Çanakkale (one time), İstanbul (two times), Trabzon (two times). These outbreaks have coincided with those in the other Mediterranean country. The largest epidemics in the Mediterranean basin (Turkey and Greece) were between 1927and 1928 and the last one occurred in 1945 in Israel and other Middle East countries (Schaffner and Mathis, 2014). There are no specific antiviral therapy or vaccines to treat or control diseases transmitted by *Ae. aegypti*, except for yellow fever. The only applicable disease prevention strategy is vector control.

Aedes aegypti is a native species from Africa that has now widely spread to almost every continent. It was an abundant species during the first part of the 20th century in Southern European and Mediterranean countries such as Turkey, Greece, Yugoslavia, Corsica (France), Spain, Syria, and Lebanon (Curtin, 1967). *Aedes aegypti* was

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[1] http://www.who.int/mediacentre/factsheets/fs117/en/ [accessed 23 April 2021].

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found in eastern Mediterranean ports in the 1950s but has since disappeared as a result of malaria control campaigns held in the Mediterranean basin; area management and lower winter climate conditions have also contributed to its disappearance (Toma et al., 2011).

Aedes aegypti was reported from Italy (one time), Israel (one time), and Turkey (several times) since 1950. In Turkey sporadic records of this species were found around the Mediterranean coast city where historically dengue outbreak areas also occurred. Schaffner and Mathis (2014) indicated that there are persistent small populations in Turkey, even though they could not find any established populations in that area. The established population of the species in Madeira in 2004 has raised a public health alarm about the risk of reinvasion to continental Europe (Almeida et al., 2007). Findings of established populations on Northern coast of Black Sea (Russia and Georgia) in 2008 (Yunicheva et al., 2008) and north-eastern Black Sea region in Turkey in 2015 show that it can become an even more dangerous for the continental Europe (Akıner et al., 2016).

COI (cytochrome oxidase subunit I) and ND4 (NADH dehydrogenase subunit 4) and ND5 (NADH dehydrogenase subunit 5) are mitochondrial gene markers used throughout the world for identification, population genetics, phylogenetics, and population diversity studies on *Aedes* species. (Bosio et al., 2005; Bracco et al., 2007; Elnour et al., 2020; Naim et al., 2020). These studies preferred mitochondrial gene regions over the ITS2 (internal transcribed spacer 2) gene regions even though both are frequently assessed gene regions (Gupta et al., 2016; Zé-Zé et al., 2020; Khater et al., 2021).

The main objective of this study was to identify the molecular phylogenetics of *Ae. aegypti* in Turkey and its genetic relationships to populations from other Caucasian countries and other geographic regions of the world. We used four mitochondrial and one genomic DNA gene regions to determine the genetic variation of sampled mosquito populations from the eastern part of the Black Sea region in Turkey.

2. Materials and methods

2.1. Mosquito collections, identification

Mosquitoes were collected by larval dipping and BG sentinel trap. Larvae were collected from Georgia and Turkey and only adults from Turkey. Larvae collection sites were used tires, concrete pools, and discarded plastic containers. The adults were collected from near the larval habitat (Table 1). Larvae are kept under laboratory conditions until adult emergence for reliable identification of the mosquito species. Morphological identification of mosquitoes was performed using a computer-assisted Leica Microsystem EZ4 stereomicroscope according to Schaffner et al. (2001).

Aedes aegypti samples were separated from other *Aedes* specimens after morphological identification and keep at –20 °C for molecular studies. In total 30 individuals were selected randomly from five separate populations in Rize and Artvin provinces of Turkey. Three samples selected randomly from Batumi, Keda, Tbilisi, and Khobi provinces in Georgia were also included. Totally 14 populations are selected for the study (Figure 1).

2.2. DNA extraction, amplification, and sequencing

For the datasets, total DNA extraction of individuals belonging to each population was performed separately using GeneJET DNA Extraction Kit (Thermo Scientific) according to the manufacturer's instructions. Extracted total DNA was stored at –20 °C until PCR amplification.

Four mitochondrial gene regions including two COI and two NADH dehydrogenase (ND4 and ND5) described by Folmer et al. (1994), Simon et al. (1994), Bracco et al. (2007), Birungi and Munstermann (2002), and one genomic gene region (ITS, I2) that was described by Porter and Colin (1991) were used for molecular studies. Another ITS gene region (I1) that described by Higa et al. (2010) was used for confirmation of the species that described morphologically according to the gel band size. PCR master mix was prepared using Biolabs Taq polymerase (New England BioLabs, USA) according to the manufacturer's instructions to be the final volume of 30 μL for each tube. The PCR reactions were performed using a T100 Thermal Cycler (Bio-Rad, Hercules, CA). The used primers sequence and PCR amplification conditions were given in Table 2. The PCR products were visualized in 1.5% agarose gel (Figure 2). Finally, the positive samples were sequenced by Macrogen, Amsterdam, the Netherlands.

2.3. Data analysis

The raw sequences were processed using MEGA 7 software (Kumar et al., 2016), and all sequences were purified according to high-quality read peaks for each gene region in the same program. The sequences were blasted using the National Center for Biotechnology website[2] and the sequences with 97% or more similarity with *Ae. aegypti* were used in the study. Multiple sequence alignment of sequences was performed using the Clustal W algorithm in MEGA7 software (Kumar et al., 2016), and each sequence data was clipped separately for each gene region, with the same length. For *Ae. aegypti* base lengths, polymorphic sites (S), numbers of haplotypes (H), haplotype diversity (Hd), and nucleotide diversity (π) were calculated using DnaSP 5.0 (Librado and Rozas, 2009) for each gene region. Sequence data for each gene region for population analysis were combined using Sequence Matrix v1.7 (Vaidya et al., 2011). The partition-homogeneity test function of PAUP 4.0 (Swofford, 1998) was used to test congruence between gene regions (Farris et al., 1995). Phylogenetic

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[2] http://blast.ncbi.nlm.nih.gov [accessed 10 September 2022].

Table 1. Mosquito collection sites and general information about the collection locations.

Province	Collection Site	Sample code	Latitude	Longitude	Stage	Years	Habitat
Artvin	TrA1	Arh1, Arh2, Arh3	41.3586	41.3181	Larvae and adult	2019	Used tires, near larval habitat
	TrA2	Ort1, Ort2, Ort3	41.3611	41.3272	Larvae	2020	Used tires
	TrA3	Hop1, Hop2, Hop 3	41.3876	41.4378	Larvae and adult	2015–19	Used tires, near larval habitat
	TrA4	Kp1, Kp2, Kp3	41.5173	41.5505	Larvae	2015–19	Concrete pool
	TrA5	Ub1, Ub2, Ub3	41.3213	41.3602	Larvae	2017–19	Used tires and plastic cube
Rize	TrR1	Fın1, Fın2, Fın3	41.2712	41.1668	Larvae and adult	2015–19	Used tires, near larval habitat
	TrR2	Gy1, Gy2, Gy3	41.1759	40.9176	Larvae	2018–19	Plastic cube
	TrR3	Ham1, Ham2, Ham3	41.1763	40.9336	Larvae	2015–19	Used tires
	TrR4	Paz1, Paz2, Paz3	41.1823	40.8933	Larvae and adult	2015–20	Used tires, near larval habitat
	TrR5	Ard1, Ard2, Ard3	41.1875	40.9668	Larvae and adult	2015–19	Used tires, near larval habitat
Batumi	GeoB1	Bat1, Bat2, Bat3	41.6390	41.6230	Larvae	2015	Used tires
Keda	GeoK1	Ked1, Ked2, Ked3	41.6010	41.9430	Larvae	2015	Used tires
Tbilisi	GeoT1	Tbl1, Tbl2, Tbl3	41.7067	41.7067	Larvae	2015	Used tires
Khobi	GeoP1	Pot1, Pot2, Pot3	42.1470	41.6800	Larvae	2015	Used tires
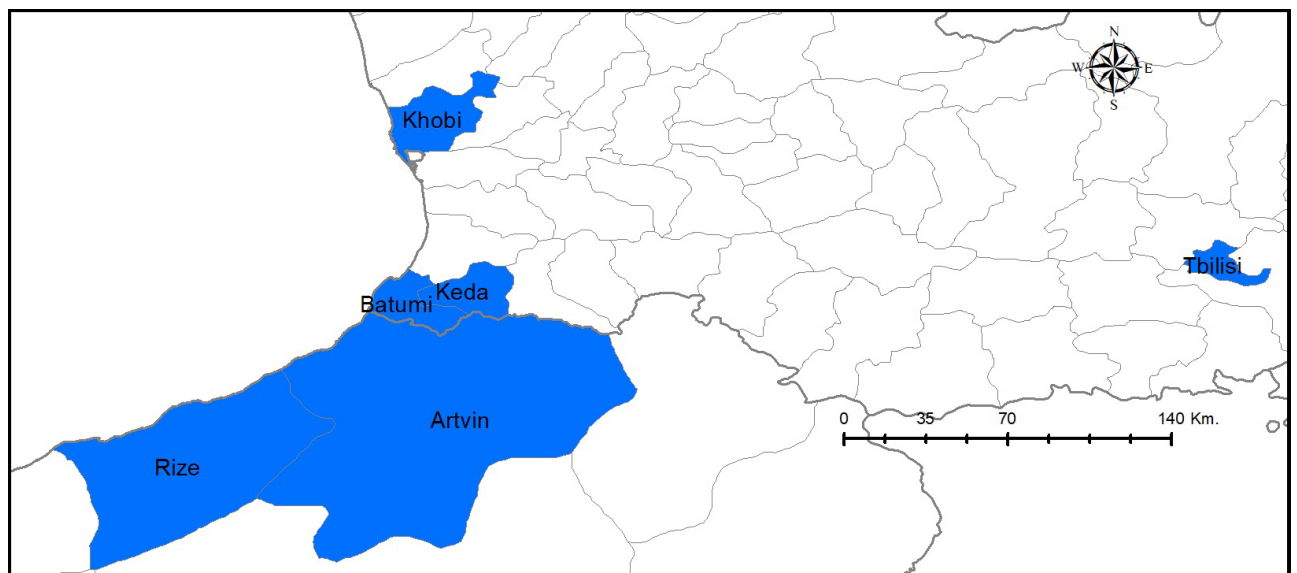
Figure 1. Mosquito collection areas.

Table 2. Used primers sequence and PCR conditions.

Gene region	Primer name	Sequence (5' to 3'	Cycle condition	Reference	Expected band size
COI (C1)	LCO- 1490	GGTCAACAAATCATAAAGATATTG	95 °C, 5 min, 1 cycle; 95 °C, 30 s, 50 °C, 30 s, 72 °C, 1 min, 35 cycles; 72 °C, 5 min, 1 cycle	Folmer et al. (1994)	Approx. 650 bp
	HCO- 2198	TAAACTTCAGGGTGACCAAAAAATCA			
COI (C2)	C1-J-1718	GGAGGATTTGGAAATTGATTAGTTC	95 °C, 5 min, 1 cycle; 97 °C, 30 s, 50 °C, 45 s, 72 °C, 1 min, 35 cycles; 72 °C, 5 min, 1 cycle	Simon et al. (1994)	Approx. 550 bp
	C1-N-2191	CCCGGTAAAATTAAAATATAAACTTC			
ND4 (N4)	ND4F	ATTGCCTAAGGCTCATGTAG	96 °C, 1 min, 1 cycle; 96 °C, 30 s, 56 °C, 30 s, 72 °C, 1 min, 35 cycles; 72 °C, 7 min, 1 cycle	Bracco et al. (2007)	Approx. 500 bp
	ND4R	TCGGCTTCCTAGTCGTTCAT			
ND5 (N5)	ND5F	TCCTTAGAATAAAATCCCGC	98 °C, 2 min, 1 cycle; 95 °C, 30 s, 45 °C, 30 s, 72 °C, 45s, 30 cycles; 72 °C, 5 min, 1 cycle	Birungi and Munstermann (2002)	Approx. 450 bp
	ND5R	GTTTCTGCTTTAGTTCATTCTTC			
ITS (I1)	18S-FHIN	GTAAGCTTCCTTTGTACACACCGCCCGT	97 °C, 4 min, 1 cycle; 96 °C, 30 s, 48 °C, 30 s, 72 °C, 2 min, 30 cycles; 72 °C, 4 min, 1 cycle	Higa et al. (2010)	Approx. 450 bp
	aeg.r1	TAACGGACACCGTTCTAGGCCCT			
ITS (I2)	5.8 s	TGTGAACTGCAGGACACATG	95°C, 5 min, 1 cycle; 95 °C, 30 s, 58 °C, 30 s, 72 °C, 1 min, 35 cycles; 72 °C, 5 min, 1 cycle	Porter and Colin (1991)	Approx. 400 bp
	28 s	ATGCTTAAATTTAGGGGGTA			
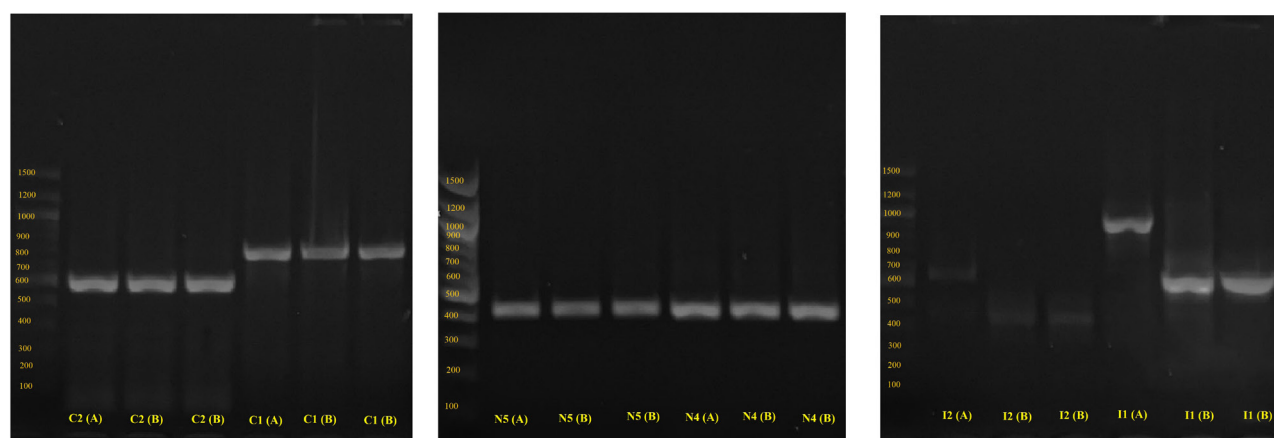
Figure 2. PCR products of band sizes on 1.5% agarose gel for six gen regions (Used gene codes was given downer of figure and the species was given in parenthesis A=*Aedes albopictus*, B= *Aedes aegypti*).

relationships of the specimens were determined using the maximum likelihood (ML) method using the MEGA7 software. In the topology of all phylogenetic trees, single female specimens selected from the *Ae. albopictus* (Skuse, 1895) KRD1 strain that has colonized the Black Sea region in 2015 were used as an outgroup. The analysis run on 1000 replicates for inferred bootstrap consensus. The best-

fit model was selected using MODELTEST 3.0 software (Posada and Crandall, 1998).

Pairwise differences between populations and AMOVA analysis were made using the Arlequin v. 3.5.1.2 software (Excoffier et al., 1992; Excoffier and Lischer, 2010) and p values were corrected using Holm's correction method and median-joining network analysis was performed using

Network 10.2 (Bandel et al., 1999) to show the relationship between haplotypes (combined sequence data were used).

2.4. Data collection

The data previously recorded in the NCBI database were compiled and used in this study. Compilation processing was performed separately for each gene region. Genbank samples used in the study and general information on these samples and haplotypes with which these samples match are given in supplementary file 1.

3. Results

3.1. Sequence variation and phylogenetic relation analysis of *Aedes aegypti*

From mtDNA COI (C1) gene region, samples produced 632 bp nucleotide sequences for *Ae. aegypti*. COI (C1) gene region was represented by four haplotypes associated with three segregating sites. Calculated Hd and Pi values were 0.573 and 0.00114, respectively (Tables 3 and 4).

Maximum likelihood phylogenetic tree of *Ae. aegypti* mtCOI (C1) from Turkey and Georgia revealed that they diverged as four molecular groups in a single clade. The haplotypes of clustering had low bootstrap support. According to the results, Hap_1 clustered with the Asia (Cambodia), Latin America (Peru), Caribbean (Puerto Rico (laboratory strain), and Europa (the United Kingdom and Germany) samples, while Hap_3 was not clustered with any samples. Hap_2 and Hap_4 clustered with Caucasian (Russia) samples under the two separate branches (Figure 3).

For mtDNA COI (C2) gene region, samples produced 483 bp nucleotide sequences. The C2 gene region consisted of four haplotypes associated with three segregating sites. Hd and Pi values were 0.573 and 0.00150, respectively (Tables 3 and 4).

Maximum likelihood (ML) phylogenetic tree revealed that the haplotypes located two different branches under the single clade. The grouping of haplotypes on the clade determined that Hap_1 was clustered with Asia (Japan, India, Viet Nam, Cambodia), South-West Indian Ocean island (Sri Lanka), Middle America (Panama), South Pacific Ocean islands (French Polynesia, New Caledonia), Latin America (Colombia, Bolivia), Europa (Portugal), Caucasia (Russia), and West Africa (Cape Verde) samples, Hap_3 and Hap_4 did not cluster any other samples and (Figure 4). Hap_2 did not clustered with any sample and was grouped in an independent branch different from the other samples.

For mtDNA ND4 gene region, samples produced 410 bp nucleotide sequences. The obtained Hd and Pi values were 0.483 and 0.00118, respectively. The ND4 gene region consisted of two haplotypes, and these haplotypes were associated with one segregating site (Tables 3 and Table 4). Maximum likelihood (ML) phylogenetic tree revealed the two haplotypes located two different branches under one clade. Hap_1 was clustered with Asian (China), Middle America (Mexico City), and Latin American (Colombia). Also, KRD1 sample belonging to *Ae. albopictus* species was clustered with Hap_1 samples. Therefore, *Ae. albopictus* specimen with EF153761 Genbank number was used as the outgroup. Hap_2 is associated with Hap_1 and clustered next branches with Southwest Indian Ocean Island (Sri Lanka), Latin America (Brazil), and Middle America (Mexico City) samples (Figure 5).

For mtDNA ND5 gene region, samples produced 41 bp nucleotide sequences. The obtained Hd was 0.483 and Pi values were 0.00117. The ND5 gene region consisted of two haplotypes in total, and these haplotypes were associated with one segregating site (Tables 3 and 4).

Two haplotypes obtained from ND5 gene sequences were located on two different branches under one clade. Hap_1 clustered with the west Africa (Cape Verda) sample, while Hap_2 occurred in a new subgroup within Hap_1 and clustered with Asia (Vietnam), Caucasia (Russia), Indian ocean island (Malaysia) and south pacific islands (French Polynesia) samples (Figure 6).

For Nuclear DNA ITS2 (I2) gene region has deletion areas in the same sequences. Two hundred and sixty-five bp nucleotide sequences were obtained from samples with

Table 3. Summary of population genetics (Haplotype and nucleotide diversity (Hd and π), number of haplotypes (h) segregating sites (S) of *Ae. aegyptii* collected from Turkey and Georgia).

Marker type	Gene code	Pi	h	Hd	bp	S	N
Mitochondrial	C1	0.00114	4	0.573	632	3	42
Mitochondrial	C2	0.00150	4	0.573	483	3	42
Mitochondrial	N4	0.00118	2	0.483	410	1	42
Mitochondrial	N5	0.00117	2	0.483	414	1	42
Nuclear	I2	0.00227	6	0.466	267	3	42
Mix	Total	0.00420	6	0.7224	2206	15	42

Table 4. Polymorphic sites of populations for five gen regions.

	48	189	450		245	310	478		150		396		102	125	182	183	260
	C1				C2				N4		N5		I2				
Ard1	C	T	G		C	C	C		T		C		C	A	-	-	A
Ard2	.	.	.		.	.	.		.		.		.	.	-	-	.
Ard3	.	.	.		.	.	.		.		.		.	.	-	-	.
Arh1	.	.	.		.	.	.		.		.		.	.	G	T	.
Arh2	.	.	.		.	.	.		.		.		.	.	-	-	.
Arh3	.	.	.		.	.	.		.		.		.	.	-	-	.
Hop1	T	.	.		T	.	.		C		T		.	G	G	T	.
Hop2	.	.	A		.	T	.		C		T		.	.	A	T	.
Hop3	.	.	A		.	T	.		C		T		.	.	A	T	.
Paz1	.	.	.		.	.	.		.		.		.	G	-	-	C
Paz2	.	.	.		.	.	.		.		.		.	.	-	-	.
Paz3	.	.	.		.	.	.		.		.		.	.	-	-	.
Ort1	T	.	.		T	.	.		C		T		.	G	G	T	.
Ort2	.	.	.		.	.	.		.		.		.	.	-	-	.
Ort3	.	.	.		.	.	.		.		.		.	.	-	-	.
Fın1	.	.	.		.	.	.		.		.		.	G	-	-	C
Fın2	.	.	.		.	.	.		.		.		.	.	-	-	.
Fın3	.	.	.		.	.	.		.		.		.	.	-	-	.
Gy1	.	.	.		.	.	.		.		.		.	.	-	-	.
Gy2	.	.	.		.	.	.		.		.		.	.	-	-	.
Gy3	.	.	.		.	.	.		.		.		.	.	-	-	.
Ham1	.	.	.		.	.	.		.		.		.	.	-	-	.
Ham2	.	.	.		.	.	.		.		.		.	.	-	-	.
Ham3	.	.	.		.	.	.		.		.		.	.	-	-	.
Ub1	.	.	.		.	.	.		.		.		.	.	-	-	.
Ub2	.	.	.		.	.	.		.		.		.	.	-	-	.
Ub3	.	.	.		.	.	.		.		.		.	.	-	-	.
Kp1	T	C	.		T	.	A		C		T		T	.	-	-	.
Kp2	.	.	.		.	.	.		.		.		.	.	G	T	.
Kp3	.	.	A		.	T	.		C		T		.	.	A	T	.
Ked1	T	.	.		T	.	.		C		T		.	G	G	T	.
Ked2	.	.	.		.	.	.		.		.		.	.	G	T	.
Ked3	.	.	A		.	T	.		C		T		.	.	A	T	.
Bat1	T	C	.		T	.	A		C		T		T	.	-	-	.
Bat2	.	.	.		.	.	.		.		.		.	G	-	-	C
Bat3	.	.	A		.	T	.		C		T		.	.	A	T	.
Pot1	T	.	.		T	.	.		C		T		.	G	G	T	.
Pot2	T	.	.		T	.	.		C		T		.	G	G	T	.
Pot3	.	.	A		.	T	.		C		T		.	.	A	T	.
Tbl1	T	.	.		T	.	.		C		T		.	G	G	T	.
Tbl2	T	.	.		T	.	.		C		T		.	G	G	T	.
Tbl3	.	.	A		.	T	.		C		T		.	.	A	T	.
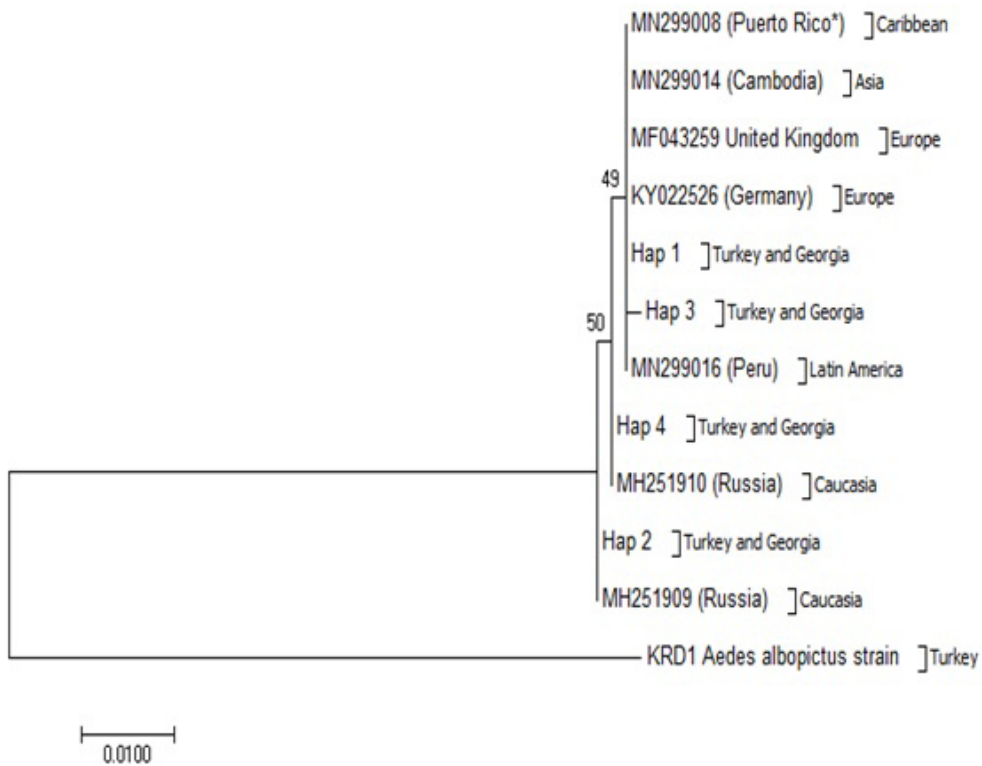
Figure 3. Maximum likelihood tree based on the GTR (General Time Reversible) model for COI (C1).

areas deletion while 267 bp nucleotide sequences were obtained from without the areas deletion. Hd and Pi values of sequences were 0.466 and 0.00227, respectively (Tables 3 and 4). Sequences produced four haplotypes when deletions are not considered as differences associated with three segregating sites while sequences produced 6 haplotypes when deletions are considered as differences associated with 6 segregating sites.

Haplotypes occurred in one clade on the hierarchical three branches on the maximum likelihood tree. Hap_6 clustered with east Africa (Saudi Arabian, Sudan) and South Pacific Ocean islands (New Caledonia) samples and lab strain from the USA. First, Hap_1 and Hap_5 clustered with Caucasia (Russia, Georgia), lab strain from the USA, South Pacific Ocean islands (New Caledonia), and Asia (Indian). Hap2 located in one small branch with Caucasian sample under the second branch. Hap_4 stated alone on the third branch, finally hap 3 stated a subgroup on the third branch with Asian (Indian, China), East Africa (Sudan) and Indian Ocean island (Indonesia) (Figure 7).

3.2. Combined data analysis of *Aedes aegypti*
For *Ae. aegypti*, we detected five informative character genes including four mitochondrial and one nuclear gene region. The partition-homogeneity test showed that all the sequences could be used together (p < 005).

Therefore, we used combined sequence data to calculate genetic relationships among geographical strains and the demographic history of strains to identify differences between countries. Combined data produced 6 haplotypes. The Hap_1 was dominant and found in Artvin and Rize populations. Hap_3 was infrequent and found in Artvin and Batumi populations (Table 5). The relationship between haplotypes is given in Figure 8.

3.3. Genetic relationships among populations
Genetic relationships among the populations from six provinces in Georgia and Turkey were determined using pairwise distances. The distance values ranged between 0.8428, and –0.5000 (Table 6). Rize population was significantly the most different from the others. The difference between Artvin-Khobi and Artvin-Tbilisi pairs was the same and the difference was found statistically significant. The difference between the other pairs was negative (this is also considered zero) and however not statistically significant (Table 5).

3.4. AMOVA analysis
We performed the AMOVA test to determine whether there is a difference between the populations of Turkey and Georgia (Table 7). Variance component proportion of within populations is large (59.27%) while among
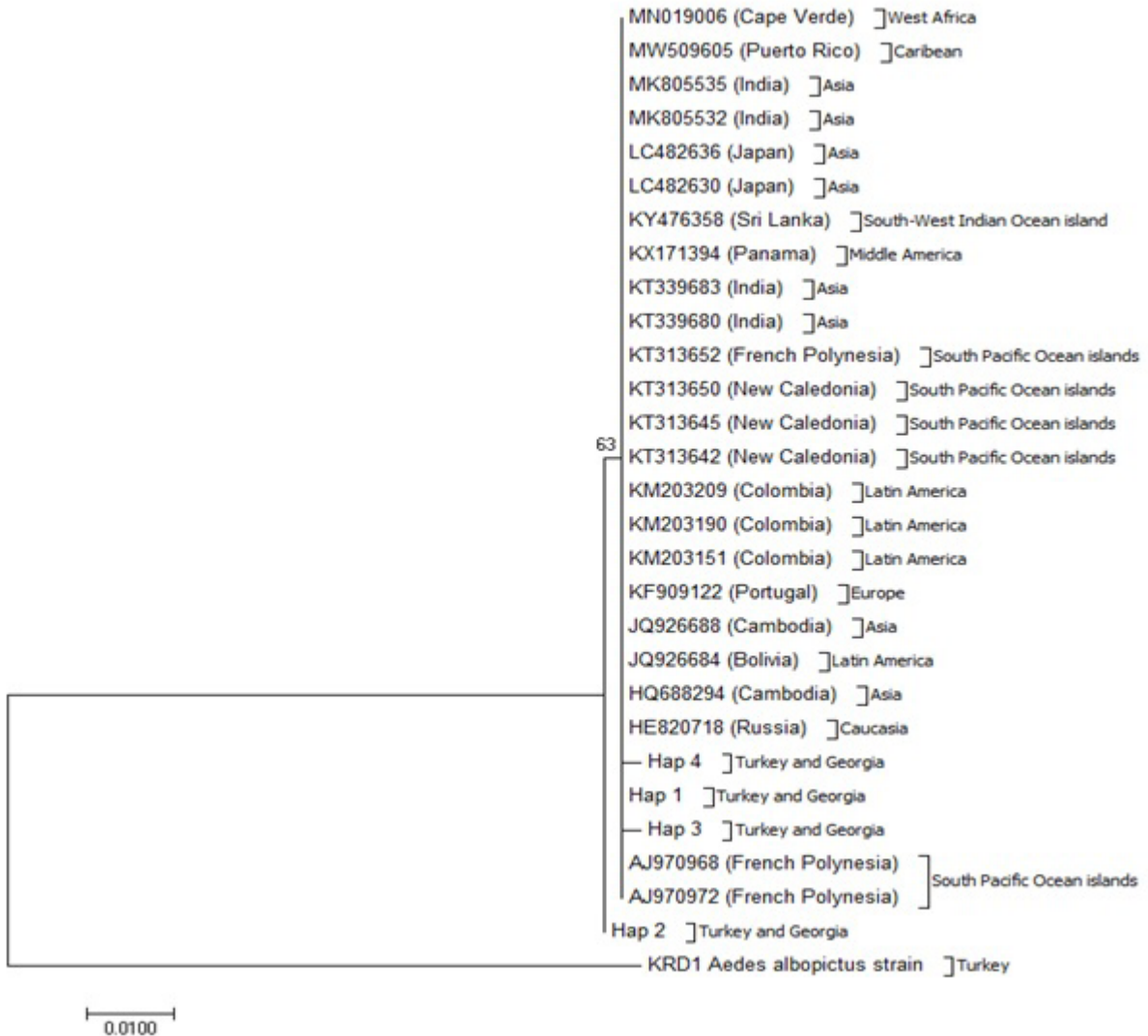
Figure 4. Maximum likelihood tree based on the GTR model for COI (C2).

geographical strains within groups proportion is low (8.47%). F-statistics, among groups, among populations within groups, and within populations were FSC = 0.12508, FST = 0.40734, and FCT = 0.32262, respectively, and were found statistically nonsignificant (Table 7).

4. Discussion
Aedes aegypti has expanded its distribution range to other countries since last century. Climate change, improved transportation, poor sanitary conditions, uncontrolled urbanization have favored this range expansion of *Ae. aegypti* or *Ae. albopictus* (Cruz et al., 2015; Serra et al., 2016).

In the last few decades, *Ae. aegypti* species has been recorded from different parts of Asia, Africa and America (Kraemer et al., 2015). First European record of an established population was in Madeira Island. In 2001, the second record of an established populations was in Caucasian region (Sochi, Russia) (Ganushkina et al., 2012; Ganushkina et al., 2016). Recently, *Ae. aegypti* populations have been reported in Georgia (Tbilisi, Kutaisi, Kobuleti, Batumi, Keda) and Eastern Black Sea coast of Turkey (Artvin, Rize, and Trabzon) in 2015 (Akıner et al., 2016). The Turkish populations in the eastern Black Sea area have been persistent in the region since.

It is not known whether records of this species' origin is a new invasion into these regions or an expansion of the ancient species in the region. If the populations from an ancient origin, it is likely that existence will continue in the region. But, if the species is a new invasion, we should know the origin of the species in order to predict of future scenarios of the species existence related to the ecological
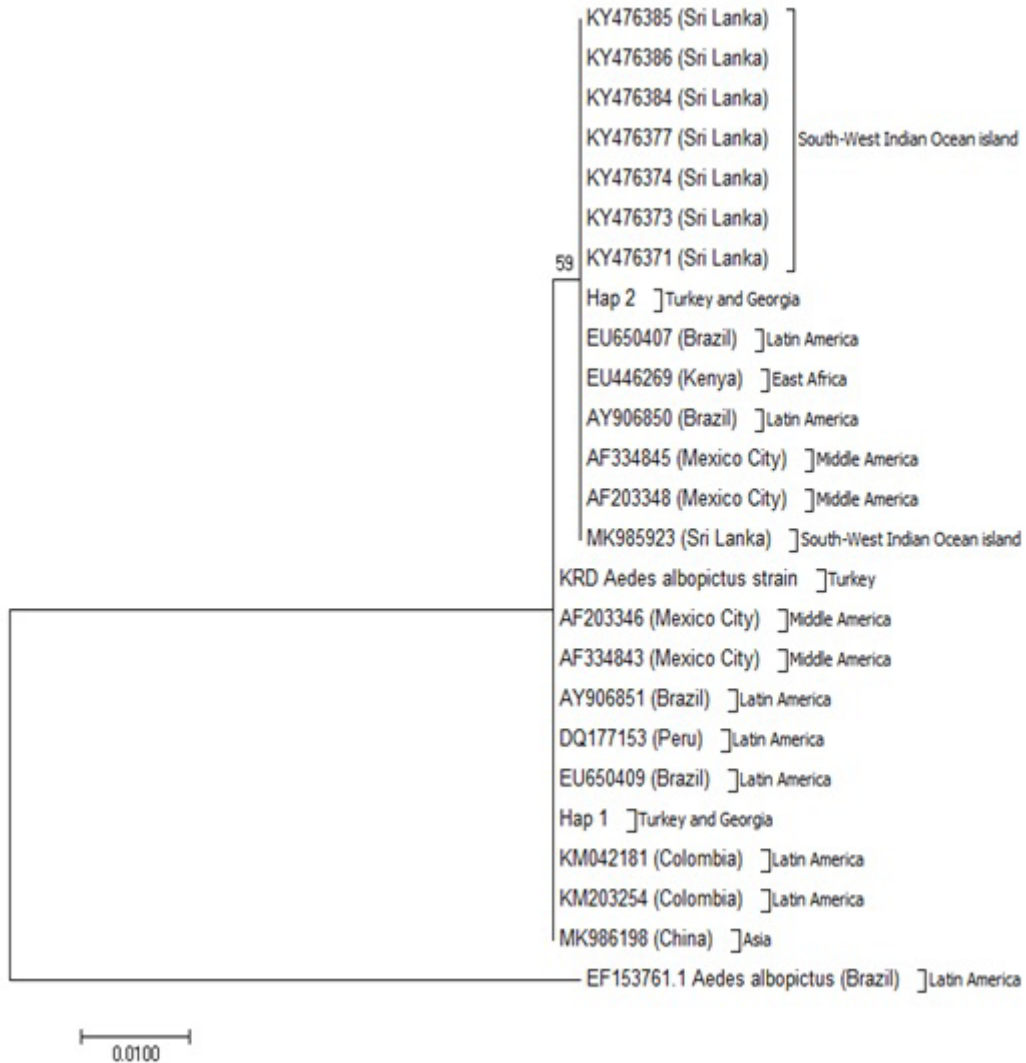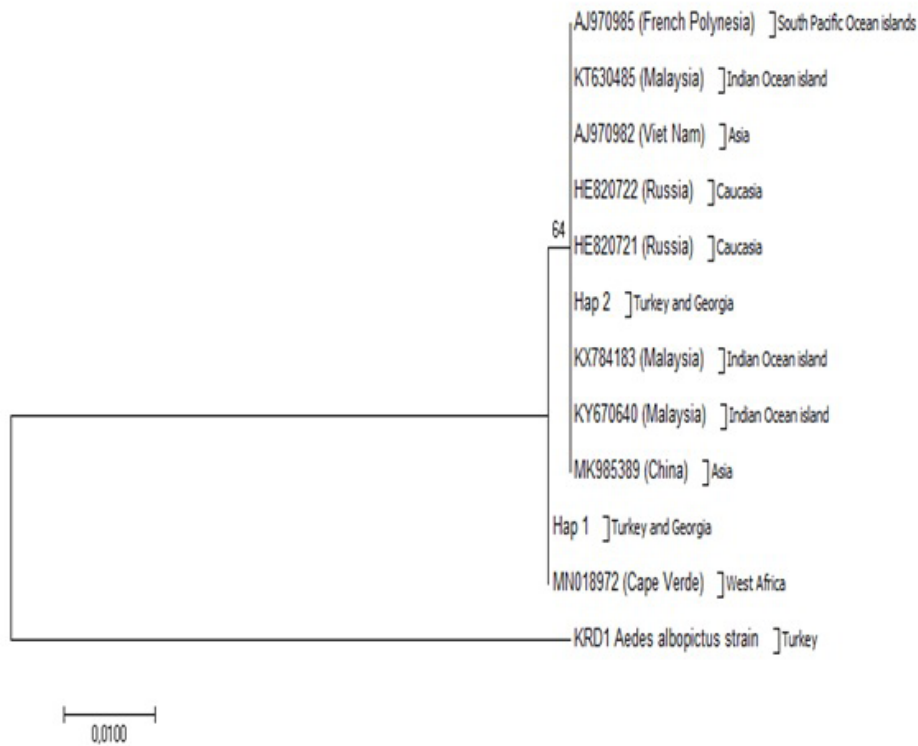
Figure 5. Maximum likelihood tree based on the GTR model for ND4 (N4).

demands of the species (Khater et al., 2021). It is unknown whether the origins of these population are from a single or separate invasions. If separate invasion occurred in the one area, there will be an increase in the genetic diversity of the species. In parallel, this may be contribute to both ecological (e.g., climatic adaptation) and biological (e.g., pesticide resistance) tolerance of the species (Gao et al., 2021). In this study, we investigated genetic variation of *Ae. aegypti* populations from the eastern Black Sea region of Turkey and Georgia using four mitochondrial and one genomic DNA gene regions. We also investigated whether or not separate invasion occurred in the study area for Turkey and Georgia and possible origin of the species.

The haplotypes obtained from the sequence data and the phylogenetic trees derived from them are generally closely related to the Caucasian (Russia, Georgia) samples.

Some haplotypes are related to Oceania, Asia, Europe, and American samples but there was no particular clustering with these samples. Moreover, Hap_2 and Hap_4 for the COI (C1) gene region and Hap_1 and Hap_2 haplotypes for the ITS2 (I2) gene region were represented only by the Caucasian region. Two-nucleotide deletion observed in the sequence data obtained from the ITS2 (I2) gene region, and this deletion was specific to the Turkey and Caucasian region. Shaikevich et al. (2018) determined the presence of the same deletion in the ITS2 gene region similarly on the Black Sea coasts in Russia. They also found similarity of these ITS2 gene regions with the Rockfeller strain in the Caucasian region. Our results of Hap_1 of the COI (C1) gene region and two haplotypes (Hap_1 and Hap_6) of the ITS2 gene region showed the similarity as the laboratory strains (Puerto Rico, Rockfeller strain) that colonized in

Figure 6. Maximum likelihood tree based on the GTR model for ND5 (N5).

the 1950s in America and Caribbean (Kuno, 2014). *Aedes albopictus* (KRD strain) strain used as an outgroup for the ND4 gene region and *Ae. aegypti* samples overlapped. This situation reveals that the ND4 gene region is not distinctive for *Ae. albopictus*. Our results showed that some haplotypes of COI markers (C1 and C2) and ITS gene region (Hap_3 for C1, Hap_2, Hap_3, and Hap_4 for C2, and Hap_4 for I2) were missing. This situation can only be explained by the insufficient sequence data from the gene regions of the species or there may be a unique strain of the species in this Caucasian area. The gene regions we used are frequently preferred for molecular studies for *Ae. aegypti* worldwide; therefore, second probability of our regions strains is more possible. Kotsakiozi et al. (2018) predicted five different scenario for possible origin of the Black Sea *Ae. aegypti* populations. The scenarios were generally based on new introduction to Black Sea area or continuation of the previously strains reported before 1950s. They implied that the Black Sea populations can be ancient strains although the specimens are closer to Asian specimens.

The haplotype numbers, nucleotide, and haplotype diversity of *Ae. aegypti* samples in the Black Sea region were found to be quite low. Our results consisted with Shaikevich et al. (2018) results. They stated that the COI and ITS

gene regions in the Caucasian region have low variation. Generally, low haplotype and nucleotide diversity are considered characteristic of invasive populations. Similarly, the repeated bottleneck effect negatively affects genetic variation such as unsuitable environmental conditions (Mousson et al., 2005). Paduan and Ribolla (2008) found 24 haplotypes (π = 0.01740) and haplotype (gene) diversity (Hd = 0.787) in Brazil for the ND4 gene region in their study. They found that the nucleotide diversity (π) of the COI gene region was greater than that of ND4. Similarly, Damal et al. (2013) determined 42 haplotypes (Hd = 0.817) for the ND4 gene region and 12 haplotypes (Hd = 0.713) for the ND5 gene region in Florida. Yohan et al. (2018) determined seven haplotypes in four cities in Indonesia for COI. Khater et al. (2021) determined the presence of 8 haplotypes for COI, 14 for ND4, and 4 for ITS2 using the Arabian Peninsula ITS, COI, and ND4 primers. Our results showed low number of haplotypes, nucleotide diversity and haplotype diversity for al tested gene regions. This results may be interpreted to our strains newly invaded strains in our area and in contrast to more possible scenario of Kotsakiozi's et al. (2018).

We found significant differences between the Rize population and other populations (Turkey and Georgian) after combining the obtained sequences. Difference ratios

Figure 7. Maximum likelihood tree based on the Kimura 2-parameter model for ITS2 (I2).

Table 5. Combined data's haplotype frequency and distribution.

Haplotype	Rize	Artvin	Batumi	Keda	Khobi	Tbilisi	Frequency
Hap_1	13	7	0	0	0	0	20
Hap_2	0	2	0	1	0	0	3
Hap_3	0	1	1	0	0	0	2
Hap_4	2	0	1	0	0	0	3
Hap_5	0	3	1	1	1	1	7
Hap_6	0	2	0	1	2	2	7
Frequency	15	15	3	3	3	3	42
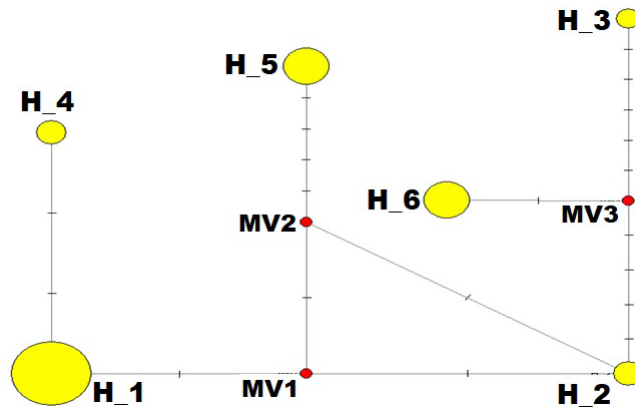
Figure 8. Relationships of the six haplotypes according to combined sequences. (In the figure, the parts shown as dashes (–) between the connections represent mutations, the sizes of the circles related to the frequencies of the haplotypes, and the areas shown with a red circle represent possible haplotypes).

Table 6. Pairwise differences between populations (*: statistically significant, NS: nonsignificant).

	Rize	Artvin	Batumi	Keda	Khobi	Tbilisi
Rize		*	*	*	*	*
Artvin	0.23755		NS	NS	*	*
Batumi	0.52339	−0.06877		NS	NS	NS
Keda	0.72832	−0.02131	−0.16667		NS	NS
Khobi	0.84281	0.21707	−0.03636	−0.27273		NS
Tbilisi	0.84281	0.21707	−0.03636	−0.27273	−0.5000	

Table 7. Analysis of molecular variance (AMOVA) between Turkey and Georgia populations.

Source of variation	d.f	Sum of squares	Variance components (% of variation)	F-statistics	
Among groups	1	17.210	0.83266 (32.26%)	FSC	0.12508 (NS)
Among populations within groups	4	11.367	0.21867 (8.47%)	FST	0.40734 (NS)
Within populations	36	55.067	1.52963 (59.27%)	FCT	0.32262 (NS)
Total	41	83.643	2.58096		

increased with geographical distance. Hap_1 unique for Turkish strains and the other haplotypes frequency gradually changed West to East direction from Rize to Tbilisi. Significant differences were determined between Artvin-Tbilisi pairs and Artvin-Khobi pairs. The reason for the difference between Artvin-Tbilisi pairs may be explained as having different climatic structures, but this does not apply in Artvin-Khobi pairs. Haplotype differences and distributions were decreased east to west and this

implied that the decreasing of genetic variation. This may be related to the recent and rapid expansion of the species. Furthermore, this may be related to the population size in that areas. Genetic variation is affected by population size. Davies et al. (1999) indicated that this situation and they implied that a low population size generates low levels of mtDNA variability. Birungi and Munsterman (2002) indicated that the recent and rapid expansion via modern transport, passive dispersal may be generating

new mtDNA haplotypes. A rapid expansion has been seen in the species since their first report (Ganushkina et al., 2012; Akıner et al., 2015; Demirci et al., 2021).

As a conclusion, our results obtained revealed that there is a high probability that the species newly invaded from the mix sources since 1990s via the increased activities of ports around the Black Sea coast (Sochi, Batumi).

References

Akiner MM, Demirci B, Babuadze G, Robert V, Schaffner F (2016). Spread of the invasive mosquitoes *Aedes aegypti* and *Aedes albopictus* in the Black Sea region increases risk of chikungunya, dengue, and Zika outbreaks in Europe. PLoS Neglected Tropical Diseases 10 (4): e0004664. https://doi.org/10.1371/journal.pntd.0004664

Almeida APG, Gonçalves YM, Novo MT, Sousa CA, Melim M et al. (2007). Vector monitoring of *Aedes aegypti* in the Autonomous Region of Madeira, Portugal. Euro Surveill 12 (46): 3311. https://doi.org/10.2807/esw.12.46.03311-en

Bandel HJ, Forster P, Röhl A (1999). Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution 16 (1): 37-48. https://doi.org/10.1093/oxfordjournals.molbev.a026036

Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW et al. (2013). The global distribution and burden of dengue. Nature 496 (7446): 504-507. https://doi.org/10.1038/nature12060

Birungi J, Munstermann LE (2002). Genetic structure of *Aedes albopictus* (Diptera: Culicidae) populations based on mitochondrial ND5 sequences: evidence for an independent invasion into Brazil and United States. Annals of the Entomological Society of America 95 (1): 125-132. https://doi.org/10.1603/0013

Bosio CF, Harrington LC, Jones JW, Sithiprasasna R, Norris DE et al. (2005). Genetic structure of *Aedes aegypti* populations in Thailand using mitochondrial DNA. The American Journal of Tropical Medicine and Hygiene 72 (4): 434-442. https://doi.org/10.4269/ajtmh.2005.72.434

Bracco JE, Capurro ML, Lourenço-de-Oliveira R, Sallum MAM (2007). Genetic variability of *Aedes aegypti* in the Americas using a mitochondrial gene: evidence of multiple introductions. Memorias do Instituto Oswaldo Cruz 102 (5): 573-580. https://doi.org/10.1590/S0074-02762007005000062

Cruz LCTA, Serra OP, Leal-santos FA, Ribeiro ALM, Slhessarenko RD et al. (2015). Natural transovarial transmission of dengue virus 4 in *Aedes aegypti* from Cuiabá, state of Mato Grosso, Brazil. Revista da Sociedade Brasileira de Medicina Tropical 48 (1): 18-25. https://doi.org/10.1590/0037-8682-0264-2014

Curtin TJ (1967). Status of *Aedes aegypti* in the Eastern Mediterranean. Journal of Medical Entomology 4 (1): 48-50. https://doi.org/10.1093/jmedent/4.1.48

Damal K, Murrell EG, Julian SA, Conn JE, Loew SS (2013). Phylogeography of *Aedes aegypti* (yellow fever mosquito) in South Florida: mtDNA evidence for human-aided dispersal. The American Journal of Tropical Medicine and Hygiene 89 (3): 482. https://doi.org/10.4269/ajtmh.13-0102

Davies N, Villablanca FX, Roderick GK (1999). Determining the source of newly founded populations: multilocus genotyping in nonequilibrium population genetics. Trends in Ecology and Evolution 14 (1): 17-21. https://doi.org/10.1016/S0169-5347(98)01530-4

Demirci B, Bedir H, Öztürk M, Akiner MM (2021). Status of the invasive mosquito species Aedes aegypti (L., 1762) and Aedes albopictus (Skuse, 1895) (Diptera: Culicidae) in Turkey. Turkish Journal of Entomology 45 (2): 279-292. https://doi.org/10.16970/entoted.879297

Elnour MAB, Moustafa MAM, Khogali R, Azrag RS, Alanazi AD et al. (2020). Distinct haplotypes and free movement of *Aedes aegypti* in Port Sudan, Sudan. Journal of Applied Entomology 144 (9): 817-823. https://doi.org/10.1111/jen.12816

Excoffier L, Lischer HE (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular ecology resources 10 (3): 564-567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Excoffier L, Smouse PE, Quattro J (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics, 131 (2): 479-491. https://doi.org/10.1093/genetics/131.2.479

Farris JS, Kallersjo M, Kluge AG, Bult C (1995). Constructing a significance test for incongruence. Systematic Biology 44 (4): 570-572. https://doi.org/10.2307/2413663

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3 (1): 294-299.

Ganushkina LA, Patraman IV, Rezza G, Migliorini L, Litvinov SK et al. (2016). Detection of *Aedes aegypti*, *Aedes albopictus*, and *Aedes koreicus* in the area of Sochi, Russia. Vector-Borne and Zoonotic Diseases 16 (1): 58-60. https://doi.org/10.1089/vbz.2014.1761

Ganushkina LA, Tanygina EY, Bezzhonova OV, Sergiev VP (2012). Detection of *Aedes Stegomyia) albopictus* Skuse mosquitoes in the Russian Federation. Meditsinskaia Parazitologiia i Parazitarnye Bolezni 1 (1): 3-4.

Gao J, Zhang HD, Guo XX, Xing D, Dong YD et al. (2021). Dispersal patterns and population genetic structure of *Aedes albopictus* (Diptera: Culicidae) in three different climatic regions of China. Parasites and Vectors, 14 (1): 1-13. https://doi.org/10.21203/rs.3.rs-38761/v3

Gupta K, Dhawan R, Kajla M. Kumar S. Jnanasiddhy B et al. (2016). Molecular identification of Aedes aegypti mosquitoes from Pilani region of Rajasthan, India. Journal of Vector Borne Diseases, 53 (2): 149-155. https://doi.org/10.4103/0972-9062.331413

Higa Y, Toma T, Tsuda Y, Miyagi I (2010). A multiplex PCR-based molecular identification of five morphologically related, medically important subgenus Stegomyia mosquitoes from the genus Aedes (Diptera: Culicidae) found in the Ryukyu Archipelago, Japan. Japanese Journal of Infectious Diseases 63 (5): 312-316. https://doi.org/10.7883/yoken.63.312

Jácome R, Carrasco-Hernández R, Campillo-Balderas JA, López-Vidal Y, Lazcano A et al. (2019). A yellow flag on the horizon: the looming threat of yellow fever to North America. International Journal of Infectious Diseases 87 (1): 143-150. https://doi.org/10.1016/j.ijid.2019.07.033

Khater EI, Baig F, Kamal HA, Powell JR, Saleh AA (2021). Molecular phylogenetics and population genetics of the dengue vector *Aedes aegypti* from the Arabian Peninsula. Journal of Medical Entomology 58 (6): 2161-2176. https://doi.org/10.1093/jme/tjab112

Kotsakiozi P, Gloria-Soria A, Schaffner F, Robert V, Powell JR (2018). *Aedes aegypti* in the Black Sea: recent introduction or ancient remnant? Parasites and Vectors 11 (1): 1-13. https://doi.org/10.1186/s13071-018-2933-2

Kraemer MU, Sinka ME, Duda KA, Mylne AQ, Shearer FM et al. (2015). The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. Elife 4 (1): e08347. https://doi.org/10.7554/eLife.08347

Kumar S, Stecher G, Tamura K (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33 (7): 1870-1874. https://doi.org/10.1093/molbev/msw054

Kuno G (2014). Early history of laboratory breeding of *Aedes aegypti* (Diptera: Culicidae) focusing on the origins and use of selected strains. Journal of Medical Entomology 47 (6): 957-971. https://doi.org/10.1603/ME10152

Librado P, Rozas J (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25 (11): 1451-1452. https://doi.org/10.1093/bioinformatics/btp187

Manzoor KN, Javed F, Ejaz M, Ali M, Mujaddadi N et al. (2022). The global emergence of Chikungunya infection: An integrated view. Reviews in Medical Virology 32 (3): e2287. https://doi.org/10.1002/rmv.2287

Masmejan S, Musso D, Vouga M, Pomar L, Dashraath P et al. (2020). Zika virus. Pathogens 9 (11): 898. https://doi.org/10.3390/pathogens9110898

Morrison AC, Zielinski-Gutierrez E, Scott TW, Rosenberg R (2008). Defining challenges and proposing solutions for control of the virus vector *Aedes aegypti*. PLoS Medicine 5 (3): e68. https://doi.org/10.1371/journal.pmed.0050068

Mousson L, Dauga C, Garrigues T, Schaffner F, Vazeille M et al. (2005). Phylogeography of *Aedes (Stegomyia) aegypti* (L.) and *Aedes (Stegomyia) albopictus* (Skuse)(Diptera: Culicidae) based on mitochondrial DNA variations. Genetics Research 86 (1): 1-11. https://doi.org/10.1017/S0016672305007627

Naim DM, Kamal NZM, Mahboob S (2020). Population structure and genetic diversity of *Aedes aegypti* and *Aedes albopictus* in Penang as revealed by mitochondrial DNA cytochrome oxidase I. Saudi Journal of Biological Sciences 27 (3): 953-967. https://doi.org/10.1016/j.sjbs.2020.01.021

Paduan KDS, Ribolla PEM (2008). Mitochondrial DNA polymorphism and heteroplasmy in populations of *Aedes aegypti* in Brazil. Journal of Medical Entomology 45 (1): 59-67. https://doi.org/10.1093/jmedent/45.1.59

Porter C, Collins FH (1991). Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culcidae). The American Journal of Tropical Medicine and Hygiene 45 (2): 271-279. https://doi.org/10.4269/ajtmh.1991.45.271

Posada D, Crandall KA (1998). MODELTEST: testing the model of DNA substitution. Bioinformatics (Oxford, England) 14 (9): 817-818. https://doi.org/10.1093/bioinformatics/14.9.817

Schaffner F, Guy A, Bernard G, Jean-Paul H, Rhaiem A et al. (2001). Les moustiques d'Europe : Logiciel d'identification et d'enseignement = The Mosquitoes of Europe : an İdentification and Training Programme. Paris (FRA); Montpellier : IRD ; EID, 1 CD ROM (Didactiques). ISBN 2-7099-1485-9.

Schaffner F, Mathis A (2014). Dengue and dengue vectors in the WHO European region: past, present, and scenarios for the future. The Lancet Infectious Diseases 14 (12): 1271-1280. https://doi: 10.1016/S1473-3099(14)70834-5

Serra OP, Cardoso BF, Ribeiro ALM, Santos FAL, Slhessarenko RD (2016) Mayaro virus and dengue virus 1 and 4 natural infection in culicids from Cuiabá, state of Mato Grosso, Brazil. Memórias do Instituto Oswaldo Cruz 111 (1) 20-29. https://doi.org/10.1590/0074-02760150270

Shaikevich E, Patraman I, Bogacheva AS, Rakova VM, Zelya OP et al. (2018). Invasive mosquito species *Aedes albopictus* and *Aedes aegypti* on the Black Sea coast of the Caucasus: genetics (COI, ITS2), Wolbachia and Dirofilaria infections. Vavilovskii Zhurnal Genetiki i Selektsii 22 (5): 574-585. https://doi.org/10.18699/VJ18.397

Simon C, Frati F, Beckenback A, Crepsi B, Liu H et al. (1994). Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 87 (1): 651-701.

Swofford DL (1998). PAUP*(version 4.0). Phylogenetic analysis using parsimony (* and other method). Sunderland MA, (beta version).

Toma L, Di Luca M, Severini F, Boccolini D, Romi R (2011). *Aedes aegypti*: risk of introduction in Italy and strategy to detect the possible re-introduction. Pest Management e salute pubblica. Veterinaria Italiana Collana Monografia 23 (1): 18-26.

Vaidya G, Lohman DJ, Meier R (2011). SequenceMatrix: concatenation software for the fast assembly of multigene datasets with character set and codon information. Cladistics 27 (2): 171-180. https://doi.org/10.1111/j.1096-0031.2010.00329.x

Yohan B, Fauziah Y, Sayono S, Trimarsanto H, Sasmono RT (2018). Genetic diversity of *Aedes aegypti* (Diptera: Culicidae) isolated from five cities in north coast area of Central Java, Indonesia. Southeast Asian Journal of Tropical Medicine and Public Health 49 (2): 217-226.

Yunicheva YU, Ryabova TE, Markovich NY, Bezzhonova OV, Ganushkina LA et al. (2008). First data on the presence of breeding populations of the *Aedes aegypti* L. mosquito in Greater Sochi and various cities of Abkhazia. Meditsinskaia Parazitologiia I Parazitarnye Bolezni 3 (1): 40-43.

Zé -Zé L, Borges V, Osório HC, Machado J, Gomes JP, Alves MJ (2020). Mitogenome diversity of *Aedes (Stegomyia) albopictus*: Detection of multiple introduction events in Portugal. PLoS Neglected Tropical Diseases 14 (9): e0008657. https://doi.org/10.1371/journal.pntd.0008657