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RESEARCH ARTICLE

Genetic Structure of *Anopheles sacharovi* (Diptera: Culicidae) Populations from Türkiye

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Abstract

Objective: The *Anopheles maculupennis* complex is known to be a vector of malaria in Türkiye. *Anopheles sacharovi* is a member of the *Anopheles maculipennis* complex and is a major vector of malaria in Europe and the Middle East capable of transmitting both *Plasmodium vivax* and *P. falciparum*. The genetic diversity and population structure of the six populations of *Anopheles sacharovi* from Türkiye are studied here using the random amplified polymorphic DNA (RAPD) marker.

Materials and Methods: *Anopheles sacharovi* samples were collected from Muğla, Aydın, İzmir, Çanakkale, Balıkesir, and Denizli. Total DNA was extracted using the Lifton method, and a total of 120 individuals were screened using six RAPD primers.

Results: A total of 300 loci were observed in the six primers. Very close genetic diversity was observed in the studied populations. The number of alleles (n_a) observed for all populations was 1.55 ± 0.49, and the mean number of effective alleles (n_e) was 1.23 ± 0.31. The ratio of polymorphic loci for all populations ranged between 50.33%-60.33%, and Nei's genetic diversity (h) ranged between 0.1253-0.1576. The gene flow level (N_m) was 2.08, and the genetic differentiation value (G_{st}) was 0.19. The conclusions from the unweighted pair group arithmetic mean analysis (UPGMA) was inclined to be homogeneous on the whole, with the İzmir population being clearly separated from the rest.

Conclusion: Regional environmental conditions such as human-mediated transport, agricultural implementations, and discrepancies in vector control strategies might be considered agents in forming the genetic structure of this species in İzmir. Understanding the gene flow rates and phylogenetic relationships between vector species are very important for applying sustainable and effective pest management. This study ensures helpful knowledge for better understanding the population genetic structure of *An. sacharovi* populations in Türkiye.

Keywords: *Anopheles sacharovi*, RAPD, Population genetics, Genetic diversity

Introduction

Mosquitoes (Diptera: Culicidae) are organisms with serious importance for human health due to being vectors of many diseases such as yellow fever, dengue fever, West Nile virus, encephalitis, lymphatic filariasis, and most importantly malaria (Çağlar *et al*., 2008; Becker *et al*., 2010). The existence of 50 species belonging to eight genera has been reported in Türkiye. Under the genus *Anopheles* (*An.*), *An. algeriensis*, *An. clavier*, *An. hyrcanus*, *An. maculipennis*, *An. marteri*, *An. melanoon*, *An. pulcherrimus*, *An. messeae*, *An. plumbeus*, *An. superpictus*, and *An. sacharovi* species constitute the *Anopheles* fauna of Türkiye. Among these, *An. superpictus* and *An. sacharovi* are the most significant malaria vector species (Ramsdale *et al*., 2001; Çağlar *et al*., 2008). *Anopheles sacharovi*, a member of the *Anopheles maculipennis* complex, is one of the main malaria vectors in Türkiye and can be found in Europe and the Middle East (Sedaghat *et al*., 2003). Even though malaria cases have significantly been reduced in Türkiye, because of the irregular migrations through malaria-endemic countries such as Afghanistan, Pakistan, Iraq, and Syria, malaria still continues to be an endemic health threat. To control

mosquitoes in Türkiye, strategies are usually designed to target larvae and adults (Yavaşoğlu *et al*., 2019). The adults of this species can be distinguished from other members of the complex by the lighter color of their mesonotum and wing characteristics. Larvae can be found in shallow sun-exposed fresh or saline water bodies. They are more tolerant to saline water than other members of the complex and also the most thermophilic (Becker *et al*., 2010).

The battle against mosquitoes goes back to the beginning of the battle against the diseases they cause. Since the early 1950s, humanity has used chemical methods against agricultural and medical pests to control arthropods (Taskin *et al*., 2016). Chemical methods of control grew with the discovery of DDT, and malaria cases during World War II dramatically decreased. After the discovery of DDT, the development of organochlorine insecticides and other groups of insecticides such as organophosphates, carbamates, and pyrethroids accelerated the processes of chemical control (Akıner, 2009). Since the 1980s after the introduction of biological methods of control, integrated control treatments have been carried out with the use of mechanical, chemical, biological, and sterilized insect techniques, and these have been widely applied throughout Türkiye and around the world (Alten & Çağlar, 1998).

Genetic markers based on nucleic acids can be used to study species characterization, genetic diversity, phylogenetic relationships, and insect identification (Rita *et al*., 2014). Random amplified polymorphic DNA–polymerase chain reaction (RAPD-PCR) and other nonspecific amplification methods are quite useful for analyzing genetic variation within species due to the help they provide in quickly obtaining vast genetic information (Posso *et al*., 2003). RAPD-PCR is a method that allows one to amplify the template genomic DNA using random primers. The RAPD method has some advantages over gene mapping and gene tagging such as being inexpensive and easy to use, able to use universal primer sets and quickly scan genomes, able to produce or commercially acquire unlimited synthetic oligonucleotides, able to isolate the cloned DNA probes, able to eliminate the need to prepare hybridization filters. RAPD-PCR is also suitable for automation and highly polymorphic and only requires a small amount of DNA (Kelly, 1995).

Genetic diversity studies have been performed on *Anopheles* species using RAPD-PCR, internal transcribed spacer 2 (ITS2) markers, microsatellite loci, mitochondrial DNA, single nucleotide polymorphism genotyping, and isoenzyme analysis (Pinedo-Cancino *et al*., 2006). Many studies on *Anopheles* species are found to have been conducted with RAPD around the world in order to better understand the population genetic structure of this species. These species involve *An. gambiae*, *An. arabiensis*, *An. minimus*, *An. aconitus*, *An. pampanai*, *An. varuna*, *An. nuneztovari*, *An. darlingi*, *An. marajoara*, *An. triannulatus*, *An. halophylus*, *An. campestris*, *An. peditaeniatus*, *An. jamesii*, *An. maculatus*, *An. philippinensis*, *An. annularis*, *An. nivipes*, *An. sinensis*, *An. jeyporiensis*, *An. Vagus*, and *An. culucifacies* (Wilkerson *et al*., 1993; Favia *et al*., 1994; Dimopoulos *et al*., 1996; Kengne *et al*., 2001; Elisa Posso *et al*., 2003, 2006; Pinedo-Cancino *et al*., 2006; Silva-Do-Nascimento *et al*., 2006; Gonzalez *et al*., 2007; Rita *et al*., 2014; Tyagi *et al*., 2015). However, no study is found in

Figure 1. Sample locations of *An. sacharovi* from Turkey.

Table 1. The sequences of primers used for RAPD-PCR.

Number	Primer	Length of oligonucleotide	Nucleotide sequence	GC content %
1	OPL-02	10	TGGGCGTCAA	60
2	OPB-10	10	CTGCTGGGAC	70
3	OPE-03	10	CCAGATGCAC	60
4	OPC-07	10	GTCCCGACGA	70
5	OPN-08	10	ACCTCAGCTC	60
6	OPD-01	10	ACCGCGAAGG	70

the literature to have been conducted on the *An. sacharovi* species.

This study investigates the intra- and inter-population genetic diversity of natural *An. sacharovi* populations sampled from diverse geographical regions of Türkiye, which covers a large portion of the Eastern Mediterranean area.

Materials and Methods

Sample Collection

The study has collected *An. sacharovi* samples from the provinces of Muğla, Aydın, İzmir, Çanakkale, Balıkesir, and Denizli as the selected study area between 2018-2019 within the scope of the Scientific and Technological Research Council of Türkiye (TÜBİTAK) 1001 project. Larval and pupal collections were performed in six provinces in Western Türkiye, with the *Anopheles* specimens' species being identified using standard morphological keys (Glick, 1992; Becker *et al*., 2010). The *An. sacharovi* samples were also verified using the ITS2 sequencing on eight individuals for each province (a total of 48 samples). Analysis of the rDNA ITS2 region was amplified and directly sequenced following Djadid *et al*.'s (2007) method. The provinces from which all the samples were collected are presented in Figure1.

DNA Isolation and Amplification of the RAPD Loci

DNA isolation of the collected samples was carried out by modifying the Lifton method as used by Bender *et al*. (1983). This study uses the 10-base RAPD oligonucleotide primer

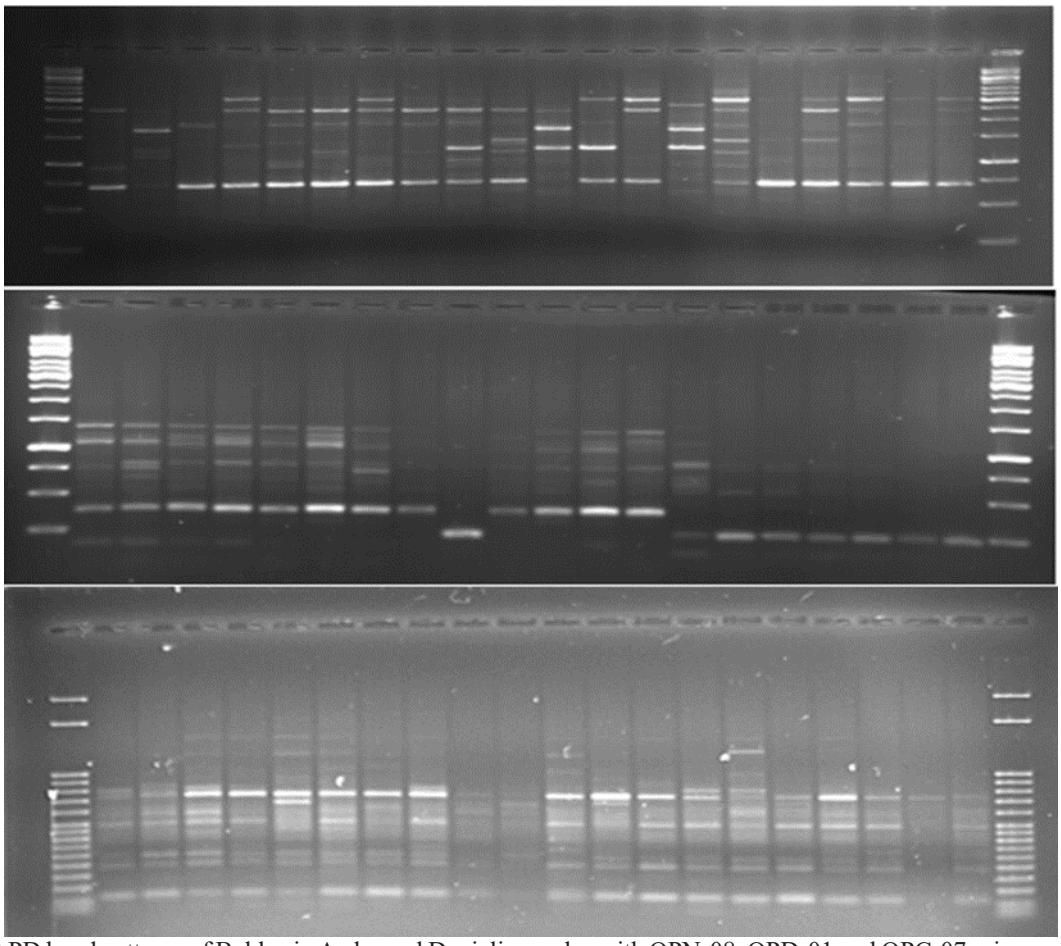

Figure 2. RAPD band patterns of Balıkesir, Aydın and Denizli samples with OPN-08, OPD-01 and OPC-07 primers, respectively.

Table 2. Genetic diversity parameters of *An. sacharovi*. Sample size (N), Observed Number of Alleles (n_a), Effective Number of Alleles (n_e), Gene Diversity (h), Percentage of Polymorphic Band ($P\%$).

Populations	N	n_a	n_e	h	$P\%$
Muğla	17	1.5033±0.5008	1.1991±0.2995	0.1253	50.33 %
Denizli	17	1.5467±0.4986	1.2581±0.3345	0.1576	54.67 %
Aydın	19	1.5333±0.4997	1.2447±0.3159	0.1525	53.33 %
İzmir	17	1.5100±0.5007	1.2344±0.3188	0.1450	51.00 %
Balıkesir	19	1.5933±0.4920	1.2249±0.2941	0.1444	59.33 %
Çanakkale	17	1.6033±0.4900	1.2100±0.2875	0.1361	60.33 %
Average	**17.66**	**1.55±0.50**	**1.23±0.31**	**0.14**	**54.83 %**

from OPERON Technologies (Alameda, California). These primers are OPL-02, OPB-10, OPE-03, OPC-07, OPN-08, and OPD-01. All of these primers are composed of random sequences and have a G + C content between 60%-70%. The PCR conditions used in this study were applied to the studied species as per Rita *et al*. (2014). Accordingly, the optimum PCR mix involve the 8 ng template DNA for a 25 µl reaction volume, 3 mM MgCl2, 11.5 pmol primer, 200 µM dNTP (for each dNTP), 1unit Taq DNA Polymerase, 2.5 µl PCR buffer (10x), and 0.6 mg/ml BSA. The optimum number of cycles for the PCR is 40, and after four minutes of pre-denaturing at 94°C, each cycle was run for 1 minute at 94°C (denaturation), 2 minutes at 37°C (binding), and 2 minutes at 72°C (extension). The PCR products were clarified by post-cycle stretching at 72°C for 10 minutes. The products were run at a constant current of 70 V in a 1% agarose gel and viewed on a Vilber Lourmat imaging device.

Data Analysis

The RAPD band patterns of the populations were visualized with the help of the Vilber Lourmat gel imaging device, and these photographs were read in a computer environment. When evaluating the segregation data at this single locus, the presence of the band is measured as "1", its absence as "0", and no detection of the PCR product as ".". The analyses of the data file created with these values were performed using computer-based programs such as POPGENE Version 1.32 (Yeh *et al*., 1999) and GenAlex (Peakall & Smouse, 2006).

Results

Genetic Structures and Population Genetic Diversity

As a result of the analysis of the six primers (i.e., OPL-02, OPB-10, OPE-03, OPC-07, OPN-08, and OPD-01) applied to a total of 120 individuals, a total of 300 loci were detected, all of which were polymorphic. The highest number of loci was obtained from the OPL-02 primer with 74, and the least number of loci were obtained from the OPE-03 primer with 35. The overall mean polymorphism rate was 40.93% (Table 1), with Figure 2 presenting the RAPD band patterns of the individuals belonging to the Balıkesir, Aydın, and Denizli populations.

The mean observed allele number (n_a), which is one of the components of genetic diversity, was found to be 1.55 ± 0.50 for all populations, with the mean effective allele number (n_e) being 1.23 ± 0.31. In the studied populations, Muğla was the population with the lowest n_a value (n_a = 1.5033 ± 0.5008), while the population with the highest n_a value was Çanakkale (n_a = 1.6033 ± 0.4900. In the studied populations, Muğla again was the population with the lowest n_e value (n_e = 1.1991 ± 0.2995), while the population with the highest n_e value was Aydın (n_e = 1.2447 ± 0.3159). The mean rate of polymorphic loci in the studied populations was found to be 54.83%, and the population with the lowest polymorphic locus ratio was Muğla (50.33%). Meanwhile the Çanakkale population had the highest rate of polymorphic loci (60.33%). In this study, the average Nei's genetic diversity value (h) was found to be 0.14, with the Denizli population having the highest (h = 0.1576), and the Muğla population having the lowest (h = 0.1253; Table 2).

According to the G-statistic, the total genetic diversity (H_T) was found to be 0.18 ± 0.02 at 300 loci. Of this, 0.14 is intra-population genetic diversity (H_S), and 0.04 is inter-population genetic diversity (D_{st}). Also, the mean genetic differentiation coefficient (G_{st}) was found to be 0.19. The gene flow level (N_m), obtained as the average of the 300 loci in this study, was found to be 2.08.

The level of genetic variation between populations was determined by calculating Nei's (1978) standard genetic distance (DN) values for the studied population pairs. DN values based on the 300 loci are given in Table 2. When considering the values, although all the populations had low genetic differentiation values, the lowest was found between Aydın and Muğla (DN = 0.0307) and the highest between İzmir and Çanakkale (DN = 0.0579; Table 3).

The visual expression of genetic distances between populations is given in the unweighted pair-group method with arithmetic average (UPGMA) dendrogram

Table 3. Genetic distance values between studied populations (Nei 1978).

Populations	Muğla	Denizli	Aydın	İzmir	Balıkesir	Çanakkale
Muğla	*****					
Denizli	0.0507	*****				
Aydın	0.0307	0.0569	*****			
İzmir	0.0539	0.0450	0.0568	*****		
Balıkesir	0.0400	0.0358	0.0432	0.0502	*****	
Çanakkale	0.0383	0.0410	0.0321	0.0579	0.0357	*****

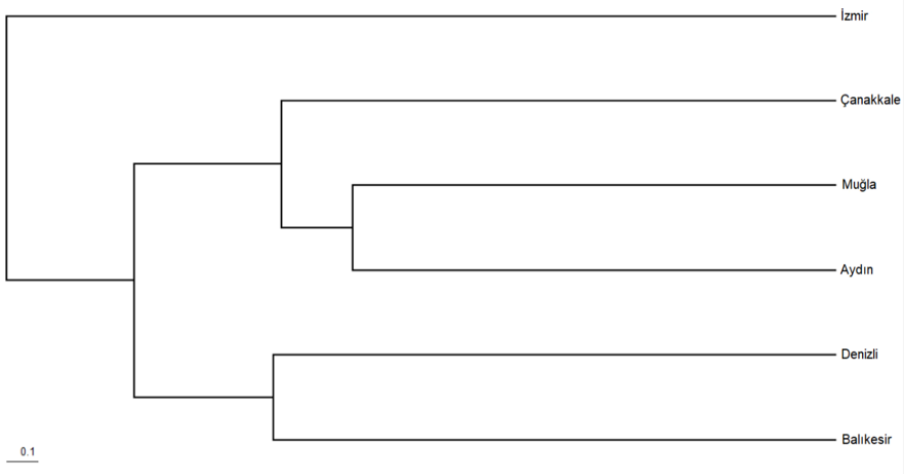

Figure 3. Dendrogram based on Nei 1972 genetic distance matrix.

and principal coordinates (PCoA) diagram (Figs. 3 and 4, respectively). When constructing the dendrogram based on genetic distances, the İzmir population was most clearly differentiated, while the Çanakkale, Muğla, Aydın, Denizli, and Balıkesir populations were clustered together. Consistent with the results of the UPGMA, the İzmir population was also separated from all other populations in the PCoA analysis. According to the results of the analysis of molecular variance (AMOVA), 83% of the genetic variation calculated for the 300 loci was observed to originate from within the population and 17% to originate from among the populations.

Discussion

When looking at mosquitoes as the carriers of various diseases that have great effects on human beings such as malaria in particular, the *Anopheles* genus has been the most interesting group in ecological, physiological, systematic, biochemical, genetic, and other fields. The

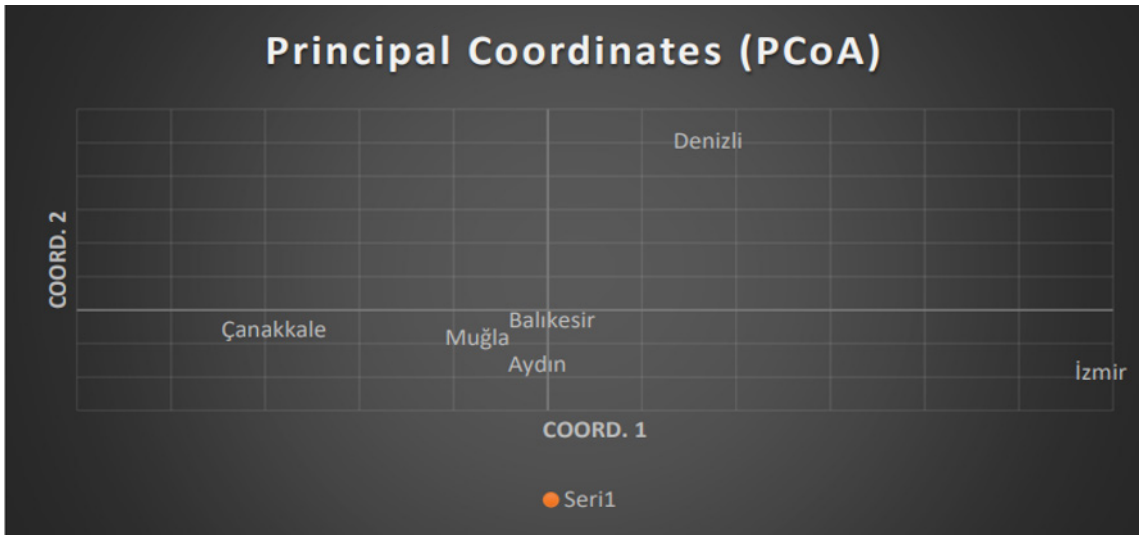

Figure 4. Principal coordinates (PCoA) diagram for studied populations.

Anopheles genus has 485 species, 80 of which are vectors of malaria, which causes the death of many people in various parts of the world, and is one of the species that people control the most (Harbach & Kitching, 2015). When considering the studies that have been conducted on the *Anopheles* species, these species have been shown to not only be vectors for malaria but also vectors for arboviruses such as the Rift Valley fever virus, the West Nile virus, and the O'nyong'nyong virus (Özbilgin *et al*., 2011). Although malaria has reached almost zero cases as a result of intense controls, it has remained the most important vectorial disease in Türkiye for a long time. Therefore, *Anopheles* species have been the main target organisms in control studies (Dogan *et al*., 2010; Özbilgin *et al*., 2011). Genetic methods for assessing the structure and differentiation of *An. sacharovi* populations have been used to provide information on population differentiation, diversity, and gene flow. Understanding these properties enables better adaptation of vector control methods. This study has determined the genetic diversity, population genetic structure, and inter-population relationships of *An. sacharovi* as an important vector pest in the Aegean regions of Türkiye using RAPD markers.

The number of studies aimed at understanding the genetic structure of *An. sacharovi* populations as an important vector species are very limited. No study other than the one conducted with the help of microsatellite markers is found in Türkiye (unpublished). The current study has determined the genetic diversity, population genetic structure, and inter-population relations of *An. sacharovi* in the western populations of Türkiye. In all the samples, which compared six provinces, genetic similarities were observed in terms of both the number of alleles as well as in the allele frequencies. The study used RAPD markers to elucidate the genetic structures of the *An. sacharovi* populations.

A total of 300 loci were identified based on the six primers scanned, all of which were determined to be polymorphic loci as a result of the analysis. The total genetic diversity (H_T) was found to be 0.18 ± 0.02 for the 300 detected RAPD loci. While 0.14 ± 0.01 of the genetic diversity constituting the total genetic diversity is due to intra-population genetic diversity (H_S), 0.04 is due to inter-population genetic diversity (D_{st}). The contribution of intra-population genetic diversity in total genetic diversity was calculated higher than the inter-population genetic diversity (77.8% vs. 22.2%). This situation might be a consequence of connected populations. Using the D_{st} and H_T values, the genetic differentiation coefficient (G_{st}) for all populations was calculated as 0.19. When considering the polymorphic loci rates for all analyzed populations, the location with the lowest polymorphic loci number is Muğla with 151 polymorphic loci and a polymorphism percentage of 50.33%. The location with the highest number of polymorphic loci is Çanakkale with 181 polymorphic loci and a polymorphism percentage of 60.33%. According to Nei's genetic diversity (h) values, the lowest genetic diversity is in Muğla (h = 0.13), while the highest genetic diversity was found in Çanakkale (h = 0.16).

Inter-population genetic diversity levels were analyzed for the *An. sacharovi* samples that were collected from the Denizli, Aydın, İzmir, Balıkesir, Çanakkale, and Muğla locations in the Aegean Region using six different primers. According to the obtained results in consideration of the genetic distances, İzmir is seen to have a different genetic structure than the other five locations. The reason for this may be that the *Anopheles* there were overexposed to environmental stress factors due to İzmir both being a gulf province and having a relatively metropolitan structure. These stress factors could involve insecticides, which are used extensively in the agricultural, tourism, and industrial sectors. This suggests that another factor might affect the change in the genetic pool of the *Anopheles* species from other geographies on the Aegean Sea coasts, one that originates from the gulf.

When examining the RAPD studies in the literature on *Anopheles* species, differences are seen in terms of various values. Posso *et al*. (2003) found a genetic diversity value of 0.34 in the results they obtained from the RAPD markers used to analyze the genetic diversity and structure of 119 samples from three *An. nuneztovari* populations they sampled from Colombia. They stated the gene flow values (G_{st} = 0.035, N_m = 6.8) to be higher than the genetic differentiation and gene flow values (G_{st} = 0.08, N_m = 2.8) among Northeastern populations. Posso *et al*. (2006) also reported the heterozygosity ratios of the *An. darlingi*, *An. nuneztoveri*, and *An. marajoara* species to range between 0.28-0.34 according to the results they obtained from RAPD markers. The current study saw the mean heterozygosity value to be 0.14, the genetic differentiation level (G_{st}) to be 0.19, and the gene flow value (N_m) to be 2.08. Pinedo-Cancino *et al*. (2006) investigated the genetic diversity of *An. darlingi*, an important malaria vector, using 5 RAPD primers on 270 samples collected from nine different locations around the city of Iquitos in Peru. They stated the intra-population genetic diversity values in *An. darlingi* to vary between 0.27-0.32, and their average genetic differentiation value to be 0.0017. Gonzalez *et al*. (2007) reported the genetic diversity value of the three *An. darlingi* populations they studied to be

0.374, with a low geographic separation present between the eastern and western populations. Rita *et al*. (2014) reported the population of 10 different *Anopheles* species (i.e., *An. campestris*, *An. peditaeniatus*, *An. jamesii*, *An. maculatus*, *An. philippinensis*, *An. annularis*, *An. nivipes*, *An. sinensi*, *An. jeyporiensis*, and *An. vagus*) to have a polymorphism rate of 65% and a genetic diversity value of 0.22. Although the results from the current show parallels to the results obtained in the literature, differences in genetic diversity were also able to be observed between different populations of the same species as well as between different species. This study used six RAPD primers for 120 samples in the *An. sacharovi* species and determined the genetic diversity value within the population to be 0.14 and the genetic differentiation value to be 0.19, which is due to the differences between the species.

The molecular analysis of variance (AMOVA) showed that most of the genetic variation (83% for *An. sacharovi*) was conserved within populations rather than between populations. The AMOVA and UPGMA dendrograms indicated no genetic structure variations to have occurred in the *An. sacharovi* species populations. A mechanism for mixing these mosquito populations from different geographical areas as well as a probable gene flow rate are evident from these observations, despite the high geographical distance present among the study sites. For this reason, the sites in the present study are attached by mosquito habitats, which permits gene flow among these locations. High intra-population genetic variation and migration rates can ensure the occasion for novel phenotypes to arise in pest populations (Zhou *et al*., 2000). In addition, the area, climatic alterations, and resistance to ecological factors (Willi *et al*., 2006) also have the ability to produce selection pressure on insects. Population genetic research ensures beneficial information concerning the potential for wide-ranging pest control, especially in species with a wide geographical spread (Alphey & Bonsall, 2018). Comprehending the phylogenetic relationships between insect populations is extremely significant for implementing a sustainable and effective *An. sacharovi* management. Nonetheless, information regarding the demographic history and population genetics of *An. sacharovi* in Türkiye remains limited.

The UPGMA dendrogram and PCoA diagram showed the *An. sacharovi* populations in Türkiye to not exhibit clustering. The possible reason for this situation is due to the rate of gene flow over time being considered suitable for estimating gene exchange between populations (Slatkin, 1985). The gene flow (N_m) values obtained by microsatellite studies for different *Anopheles* species have been reported to vary between 4-483 (Rongnoparut *et al*., 1999; Braginets *et al*., 2003; Michel *et al*., 2005; Ogola *et al*., 2019; Kaddumukosa *et al*., 2020). This current study was performed with RAPD markers and found a gene flow (N_m) value of $N_m = 2.08$. The likely reason for this low gene flow level is thought to be a result of the geographical distances between the populations. In addition, the intense control of these vector organisms may be another explanation for the low level of gene flow. The observed gene flow ($N_m = 2.08$) between populations appears to have been sufficient for homogenizing the mosquito populations in Türkiye. Variable effective population sizes, climate, and different collection times may also be some of the factors influencing differentiation.

In conclusion, the present study has characterized the *An. sacharovi* populations for the first time by using the RAPD marker. Future studies should investigate changes in mosquito populations over time, particularly as insecticide use and coverage evolve, new interventions emerge, and climate and land use changes. As a result, the examination of *Anopheles* populations in Türkiye from more locations, with more samples, and base sequence analyses from different parts of the genome or analysis of loci without any selection pressure will clearly make an important contribution to future studies in this field.

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