



Seasonal resistance status of two main malaria vectors *Anopheles superpictus* and *Anopheles sacharovi* (Diptera: Culicidae) populations from Turkey

Rumeysa Yesim Manap¹ · Nazli Pinar² · Evin Gunenc² · Taylan Dogaroglu³ · Vatan Taskin⁴ · İrfan Kandemir⁵ · Ersin Dogac²

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Abstract

Anopheles species are vectors for malaria. To date, insecticide application has been the primary method for controlling mosquito disease vectors. Chemical interventions to control vectors may occasionally prove ineffective, due to the development of insecticide resistance. Target-site insensitivity is one of the primary mechanisms that contribute to resistance. This study aims to determine the G119S (mutation of glycine to serine) and L1014S (mutation of leucine to phenylalanine) mutation rates of *Anopheles superpictus* Grassi, 1899 (Culicidae: Anophelinae) and *Anopheles sacharovi* Favre, 1903 (Culicidae: Anophelinae) populations and their seasonal variations in the Aegean Region. For both *A. superpictus* and *A. sacharovi*, the G119S mutation was observed at a low frequency during all three periods. The mean L1014S frequency for *A. sacharovi* populations in the spring 2018, fall 2018, and spring 2019 periods was 0.063, 0.156, and 0.196, respectively. For *A. superpictus* populations, the frequencies were 0.025, 0.013, and 0.024, respectively. Pyrethroids, the most widely utilized insecticide in recent years, which are presumed to be effective, will ultimately exhibit reduced efficacy in some of these populations.

Keywords *A. superpictus* · *A. sacharovi* · Insecticide resistance · Target site insensitivity · Turkey

Introduction

The genus *Anopheles* (Culicidae: Anophelinae) includes 465 species worldwide (Harbach 2013), of which 50 species have been reported from Turkey (Alten et al. 2007; Kasap and Alptekin 1997; Özbilgin et al. 2011; Ramsdale et al. 2001). Although it has now become almost zero due to intense efforts, malaria remains the most important vectorial disease

in Turkey for a long time, and studies have mainly focused on the *Anopheles* species (Dogan et al. 2010; Özbilgin et al. 2011). Among *Anopheles* species in Turkey, *A. subalpinus*, *A. superpictus*, and *A. sacharovi* are among the most important malaria vector species (Alten et al. 2007; Kasap and Alptekin 1997; Özbilgin et al. 2011; Ramsdale et al. 2001). The use of insecticides is the principal method for managing vectors, thus restraining the effects of related vector-borne diseases (N'Guessan et al. 2007). Resistance, which is a genetically based and evolutionary process, can also be defined as the response of individuals in a population to insecticide selection pressures (Çağlar and Skavdis 2008).

The intense use of pyrethroids (PYs) and DDT has resulted in the characteristic “knock-down resistance” or *kdr* phenotype in many insects, including *Anopheles* species. (Hemingway et al. 2004; Martinez-Torres et al. 1998). Organophosphates (OPs) are among the most frequently used insecticides worldwide. This insecticide inhibits the activity of acetylcholinesterase (AChE) (Aldridge 1950). The G119S mutation has been correlated with high levels of resistance in mosquito vector species including *Anopheles* (Djogbénou et al. 2008a; Edi et al. 2014; Essandoh

✉ Ersin Dogac
ersindogac@mu.edu.tr; ersindogac@hotmail.com

¹ Department of Pathology Laboratory Techniques, Vocational School, Dogus University, Istanbul, Turkey

² Department of Molecular Biology and Genetics, Faculty of Science, Mugla Sitki Kocman University, Mugla, Turkey

³ Department of Bee Breeding, Ula Vocational School, Mugla Sitki Kocman University, Mugla, Turkey

⁴ Department of Biology, Faculty of Science, Mugla Sitki Kocman University, Mugla, Turkey

⁵ Department of Biology, Faculty of Science, Ankara University, Ankara, Turkey

et al. 2013; Weill et al. 2003, 2004), and *Culex* species (Cui et al. 2006; Essandoh et al. 2013; Glick 1992; Weill et al. 2003, 2004). The mutation often causes fitness cost effects, which include an enhanced risk of predation, shorter overwintering, smaller adult body size, and a longer developmental period for both male and female mosquitoes (Brito et al. 2013; Bourguet et al. 2004; Djogbénou et al. 2008a, b; Kliot and Ghanim 2012; Lynd et al. 2010; Nkahe et al. 2020; Osoro et al. 2021). Nonetheless, the fitness cost can be reduced in field populations because of duplication of the *ace-1* gene, which has been reported in *Anopheles* and *Culex* species (Djogbénou et al. 2009; Edi et al. 2014; Essandoh et al. 2013; Labbé et al. 2007).

This study aims to determine the seasonal changes in the genetic basis of the insecticide resistance mechanism of the target sites (*ace-1* and *vgsc*) in populations of two important malaria vector species, *A. sacharovi* and *A. superpictus*, collected in three different periods from the locations under OPs and PYs insecticide selection pressure in the Aegean Region. Afterwards, we tried to specify if, and how swiftly, resistance became connected with the mutations for target site insensitivity in the *ace-1* and *vgsc* genes, and how genotype frequencies were altered in these field populations in different seasons.

Materials and methods

Mosquito sampling and DNA extraction

Field studies were conducted between June 2018 and June 2019 for spring 2018, fall 2018, and spring 2019 to collect *A. superpictus* and *A. sacharovi* populations from Muğla, Aydın, İzmir, Balıkesir, Denizli, and Çanakkale provinces. A total of 1440 single mosquitoes of *A. superpictus* and *A. sacharovi* were sampled from 24 different locations across six provinces of the Aegean region of Turkey (Fig. 1, Table S1). Mosquito larvae were collected by the dipping method before being transferred to the laboratory in plastic bottles and sampled from urban, peri-urban, and rural settings, including irrigation channels, drainage channels, puddles, ponds, swamps, and streams. The larval samples transported to the insectarium were placed in containers with their water and transferred to net cages to obtain adult specimens. Environmental conditions were maintained at 70–80% relative humidity and 26–28 °C, with a 12:12 h photoperiod. The containers were monitored periodically for biofilm formation, and fish food was supplemented at regular intervals to nourish the larvae. Cotton pads saturated with tap water and 10% sucrose solution were placed on the net cages regularly to meet the hydration and nutritional requirements of adult mosquitoes. The emergence of adult mosquitoes was observed daily, and the emerged adults were transferred



Fig. 1 Sampling locations

to 96% ethyl alcohol using an oral aspirator and stored at -80°C in a freezer for subsequent analysis. Genomic DNA extractions of 1440 individuals for three collection periods were conducted using the Zymo Research Quick DNA Mini-prep Plus Kit (Catalog number: D4068 and D406) in accordance with the manufacturer's protocol.

Morphological and molecular identification of *Anopheles* samples

Morphological identification of the *A. sacharovi* and *A. superpictus* samples was examined under a binocular microscope using adult identification keys (Becker et al. 2010; Glick 1992) and MosKeyTool (Gunay et al. 2017). The ITS2 region of ribosomal DNA (rDNA) has demonstrated efficacy for DNA-based species identification and has been successfully utilized for the identification of *Anopheles* species (Djadid et al. 2007; Manouchehri et al. 1992; Oshaghi et al. 2008; Proft et al. 1999; Sevgili and Şimşek 2012). Because *A. sacharovi* is a member of the *A. maculipennis* complex, in which members are morphologically similar, *A. sacharovi* samples were verified by ITS2 sequencing of eight individuals from each province (a total of 48 samples). The rDNA ITS2 region was amplified and directly sequenced following the method described by Djadid et al. (2007).

Identification of mutations in *ace-1* and *vgsc* gene

Genotyping of *ace-1* and *vgsc* gene regions was conducted in individual mosquito specimens to detect mutations associated with resistance to insecticides and any possible seasonal alteration of their frequencies. For both species (*A. sacharovi* and *A. superpictus*), from each location ten individual mosquitoes (in total 240/season) were sequenced to identify the frequencies of mutations in *ace-1* and *vgsc* genes. The G119S mutation in the *ace-1* gene has been determined based on the protocols implemented by Weill et al. (2004) and Ahoua Alou et al. (2010). The *kdr* mutations L1014F/C/S in the *vgsc* gene mutations in the *kdr* mutation region of the *vgsc* gene encoding voltage-sensitive sodium channel protein and contributing significantly to resistance to PY group insecticides in these organisms were determined by applying the method developed by Martinez-Torres et al. (1998).

The genotypic frequencies of resistance mutations in natural mosquito populations were compared to Hardy–Weinberg expectations using the exact test methods applied in POPGENE (ver.1.32) software (Yeh et al. 1999). Statistical differences in frequencies of resistance-associated mutations between the populations and seasons were investigated using the Kruskal–Wallis test. The non-parametric Tukey test was applied when a significant difference was observed. The significance level was set at $\alpha=0.05$ for all analyses. All statistical analyses were conducted using the Statistica ver. 10.0 software (StatSoft Inc. 2011).

Results

Mutation and genotype frequency of *vgsc* gene

In the spring 2018 collection period, the L1014S mutation in the *vgsc* gene was examined in 239 samples (number of samples with sequence data obtained) for *A. sacharovi*, and 237 samples (number of samples obtained from the sequence data) for *A. superpictus*. For *A. sacharovi*, the mean L1014S frequency was 0.063, with the highest mutation frequency observed in Aydın (0.154). For *A. superpictus*, the highest mutation frequency was observed in Denizli (0.075), with a mean L1014S frequency of 0.025.

During the fall 2018 collection period, mutation screening was conducted on 240 *A. sacharovi* and 238 *A. superpictus* samples. For *A. sacharovi*, the mean L1014S frequency during this period was 0.156, with the highest mutation frequency observed in Muğla (0.475). The mean L1014S frequency for *A. superpictus* was 0.013, with the highest mutation frequency observed in Balıkesir (0.050).

In spring 2019, mutation screening was performed on 235 individuals for *A. sacharovi* and 234 samples for *A.*

superpictus. For *A. sacharovi*, the mean L1014S frequency observed in spring 2019 was 0.196, with the highest mutation frequency observed in Denizli (0.275). For *A. superpictus*, the mean L1014S frequency was 0.024, with the highest frequency observed in Denizli (0.041) (Tables 1 and 2).

The Kruskal–Wallis test revealed a similar trend in *A. sacharovi* populations of Çanakkale, İzmir, and Denizli. In these provinces, no statistical difference was observed between the 2018 spring and 2018 fall periods in terms of mutation frequency. In the spring of 2019, the L1014S mutation frequency increased significantly ($p<0.05$) compared to that in the other two periods. A significant difference was found between the two spring periods in Balıkesir ($p<0.05$). A significant difference was observed between the spring and fall terms in 2018 in the Muğla province ($p<0.05$). No significant difference was found among the three periods in terms of L1014S mutation frequency in Aydın ($p>0.05$). While no difference was observed between Spring 2018 and Fall 2018, Spring 2019 was significantly different. In provinces where *A. superpictus* populations were sampled, no statistically significant differences were found among the periods ($p>0.05$).

Mutation and genotype frequency of the *ace-1* gene

During the spring 2018 collection period, 227 samples of *A. sacharovi* were examined, and no mutations were detected. For *A. superpictus*, 221 samples were analyzed, with only one individual exhibiting a heterozygote genotype. The SS (G119G) genotype frequency was 100% for *A. sacharovi* populations from all provinces and *A. superpictus* populations in all provinces except Çanakkale province, where the RS (G119S) genotype frequency was 2.56%. In the *A. superpictus* populations, the mean G119S mutation frequency was 0.002, with the highest observed in Çanakkale province at 0.013.

During the fall of 2018, 207 samples of *A. sacharovi* and 213 samples of *A. superpictus* were examined. In *A. sacharovi* populations, the mean G119S mutation frequency was 0.012, with the highest observed in the Muğla population (0.056). In *A. superpictus* populations, the mean G119S mutation frequency was 0.002, with the highest frequency of 0.013 observed in the Aydın population.

In the spring of 2019, 224 samples of *A. sacharovi* and 234 samples of *A. superpictus* were examined. No mutations were detected in the samples during this period for both species, resulting in an SS genotype frequency of 100% in both species. Individuals with RS and RR genotypes were not observed (Tables S2 and S3). The Kruskal–Wallis test revealed no statistically significant difference for the *ace-1* mutation in either species across the three periods ($p>0.05$).

Table 1 Three collection periods frequencies of *vgsc* mutations and genotypes concerning the six *A. sacharovi* populations from the Aegean region, (a) Spring 2018; (b) Fall 2018; (c) Spring 2019

(a) Spring 2018

I. Period (Spring 2018)

Populations	N	Mutation Frequency		Number of Genotypes			Genotype Frequency (%)			HW
		1014L	1014S	SS (LL)	RS (LS)	RR (SS)	SS (LL)	RS (LS)	RR (SS)	
Çanakkale	40	1.000	0.000	40	0	0	100.00	0.00	0.00	0.000
Balıkesir	40	0.987	0.013	39	1	0	97.50	2.50	0.00	1.000
İzmir	40	0.950	0.050	38	0	2	95.00	0.00	5.00	0.000
Aydın	39	0.846	0.154	33	0	6	84.62	0.00	15.38	0.000
Muğla	40	0.875	0.125	34	2	4	85.00	5.00	10.00	0.000
Denizli	40	0.962	0.038	38	1	1	95.00	2.50	2.50	0.000
Total	239			222	4	13				
Mean		0.937	0.063				92.85	1.67	5.48	

(b) Fall 2018

II. Period (Fall 2018)

Populations	N	Mutation frequency		Number of genotypes			Genotype frequency (%)			HW
		1014L	1014S	SS (LL)	RS (LS)	RR (SS)	SS (LL)	RS (LS)	RR (SS)	
Çanakkale	40	1.000	0.000	40	0	0	100.00	0.00	0.00	0.000
Balıkesir	40	0.825	0.175	33	0	7	82.50	0.00	17.50	0.000
İzmir	40	1.000	0.000	40	0	0	100.00	0.00	0.00	0.000
Aydın	40	0.712	0.288	28	1	11	70.00	2.50	27.50	0.000
Muğla	40	0.525	0.475	18	6	16	45.00	15.00	40.00	0.000
Denizli	40	1.000	0.000	40	0	0	100.00	0.00	0.00	0.000
Total	240			199	7	34				
Mean		0.844	0.156				82.92	2.92	14.17	

(c) Spring 2019

III. Period (Spring 2019)

Populations	N	Mutation frequency		Number of genotypes			Genotype frequency (%)			HW
		1014L	1014S	SS (LL)	RS (LS)	RR (SS)	SS (LL)	RS (LS)	RR (SS)	
Çanakkale	39	0.885	0.115	31	7	1	79.49	17.95	2.56	0.384
Balıkesir	39	0.795	0.205	23	16	0	58.97	41.03	0.00	0.121
İzmir	40	0.762	0.238	21	19	0	52.50	47.50	0.00	0.056
Aydın	40	0.887	0.113	33	5	2	82.50	12.50	5.00	0.011
Muğla	37	0.770	0.230	21	15	1	56.76	40.54	2.70	0.490
Denizli	40	0.725	0.275	18	22	0	45.00	55.00	0.00	0.020
Total	235			147	84	4				
Mean		0.804	0.196				62.54	35.75	1.71	

HW indicates the *p* values for deviation from the Hardy–Weinberg equilibrium

Discussion

In Turkey, studies have predominantly focused on *A. sacharovi* species, and the number of investigations conducted appears to correlate with the incidence of malaria cases in the country. Consequently, it has not been feasible to monitor the historical development of resistance in Turkey at the

species level or to evaluate the current situation. Nevertheless, interest in these significant vector species has persisted globally, and research on vector control has continued in parallel with scientific advancements. Recent studies on the molecular basis of OP/carbamate (CA) (Ahoua Alou et al. 2010; Dabiré et al. 2009; Djogbénou et al. 2008c; Edi et al. 2012; Essandoh et al. 2013; Weill et al. 2003, 2004) and PY/

Table 2 Three collection periods frequencies of *vgsc* mutations and genotypes concerning the six *A. superpictus* populations from the Aegean region, (a) Spring 2018; (b) Fall 2018; (c) Spring 2019

(a) Spring 2018

I. Period (Spring 2018)

Populations	N	Mutation frequency		Number of genotypes			Genotype frequency (%)			HW
		1014L	1014S	SS (LL)	RS (LS)	RR (SS)	SS (LL)	RS (LS)	RR (SS)	
Çanakkale	39	1.000	0.000	39	0	0	100.00	0.00	0.00	0.000
Balıkesir	40	0.975	0.025	39	0	1	97.50	0.00	2.50	0.000
İzmir	38	0.961	0.039	36	1	1	94.74	2.63	2.63	0.000
Aydın	40	1.000	0.000	40	0	0	100.00	0.00	0.00	0.000
Muğla	40	0.987	0.013	39	1	0	97.50	2.50	0.00	1.000
Denizli	40	0.925	0.075	37	0	3	92.50	0.00	7.50	0.000
Total	237			230	2	5				
Mean		0.975	0.025				97.04	0.86	2.11	

(b) Fall 2018

II. Period (Fall 2018)

Populations	N	Mutation frequency		Number of genotypes			Genotype frequency (%)			HW
		1014L	1014S	SS (LL)	RS (LS)	RR (SS)	SS (LL)	RS (LS)	RR (SS)	
Çanakkale	40	1.000	0.000	40	0	0	100.00	0.00	0.00	0.000
Balıkesir	40	0.950	0.050	37	2	1	92.50	5.00	2.50	0.001
İzmir	40	1.000	0.000	40	0	0	100.00	0.00	0.00	0.000
Aydın	40	1.000	0.000	40	0	0	100.00	0.00	0.00	0.000
Muğla	38	0.987	0.013	37	1	0	97.37	2.63	0.00	1.000
Denizli	40	0.987	0.013	39	1	0	97.50	2.50	0.00	1.000
Total	238			233	4	1				
Mean		0.987	0.013				97.89	1.69	0.42	

(c) Spring 2019

III. Period (Spring 2019)

Populations	N	Mutation frequency		Number of genotypes			Genotype frequency (%)			HW
		1014L	1014S	SS (LL)	RS (LS)	RR (SS)	SS (LL)	RS (LS)	RR (SS)	
Çanakkale	40	0.987	0.013	39	1	0	97.50	2.50	0.00	1.000
Balıkesir	40	0.962	0.038	37	3	0	92.50	7.50	0.00	0.842
İzmir	39	0.974	0.026	38	0	1	97.44	0.00	2.56	1.000
Aydın	40	0.975	0.025	38	2	0	95.00	5.00	0.00	0.909
Muğla	38	1.000	0.000	38	0	0	100.00	0.00	0.00	0.000
Denizli	37	0.959	0.041	34	3	0	91.89	8.11	0.00	0.835
Total	234			224	9	1				
Mean		0.976	0.024				95.72	3.85	0.43	

HW indicates the *p* values for deviation from the Hardy–Weinberg equilibrium

DDT (Enayati et al. 2003; Lol et al. 2013; Martinez-Torres et al. 1998; Ranson et al. 2000; Syafruddin et al. 2010), insecticide resistance in *Anopheles* populations have been observed in various regions of the world. To date, a limited number of Turkish studies on mosquito species have characterized insecticide resistance and its mechanisms using both biochemical (Akıner et al. 2013; Akıner 2014; Akıner

and Ekşi 2015; Çağlar and Skavdis 2008; Hemingway et al. 1992; Kasap et al. 1981, 1992, 2000; Lüleyp and Kasap 2000; Yavaşoglu et al. 2019; Yaylagül et al. 2015) and molecular (Bursalı 2013; Lüleyp et al. 2002; Taşkın et al. 2016; Yavaşoglu et al. 2021) techniques.

In our study, mutations in *vgsc* and *ace-1* gene regions were detected in different populations of *A. sacharovi* and

A. superpictus species. While *ace-1* G119S mutation was not observed homozygously in both species studied, *vgsc* *kdr* mutation was observed homozygously and/or heterozygously in both species. When examined on a species basis, a higher frequency of *kdr* mutation was observed in the *A. sacharovi* species (spring 2018=0.063, fall 2018=0.156, and spring 2019=0.196) compared to the *A. superpictus* species (spring 2018=0.025, fall 2018=0.013, and spring 2019=0.024), and this situation revealed the necessity of determining species-specific resistance levels and applying species-specific insecticides. According to the mutation rates determined at the population level, the mutation rate in *A. superpictus* populations is quite low.

The results indicated that the G119S mutation was not observed in either *A. sacharovi* and *A. superpictus* populations sampled from the Aegean region, and the resistance allele frequency was low. Similarly, the presence of the *kdr* and *ace-1R* alleles in *A. gambiae* samples collected from Uganda was examined using a diagnostic test, and it was reported that the *kdr* L1014S ('*kdr east*') allele was present, while the *ace-1R* and *kdr* L1014F ('*kdr west*') alleles were absent (Ramphul et al. 2009). *Kdr* and *ace-1* mutations were screened in *A. gambiae*, *A. funestus*, and *A. mascarensis* collected from Madagascar, and no mutations were observed in any of these three species (Rakotoson et al. 2017). An *ace-1* mutation was also identified in *A. albimanus*, *A. darlingi*, *A. coluzzii*, *A. gambiae* sensu stricto (*s.s.*), *A. melas*, and *A. arabiensis* species by different researchers; homozygous mutations were not detected in either species, and heterozygous mutation frequencies were found to be relatively low (Assogba et al. 2020; Chukwuekezie et al. 2020; Lol et al. 2019; Sy et al. 2021). Furthermore, Yavasoglu et al. (2021) conducted a study in the Aegean and Mediterranean regions of Turkey using *A. sacharovi* samples, and *ace-1* G119S mutations were not detected in the studied populations (Yavasoglu et al. 2021). In *Anopheles* species, glycine is naturally encoded by GGG, and its transformation to AGC results in serine (Weill et al. 2004). This two-step change in codon preference might explain why no G119S homozygous mutation in the AChE enzyme was observed in the studied populations.

Due to their resistance to organophosphates (OPs) and their potential environmental toxicity, OPs were gradually replaced by pyrethroids (PYs) in the 2000s. However, a study conducted in Turkey demonstrated that populations of *Anopheles* species developed resistance to PY (Yavasoglu et al. 2021). Persistent insecticide resistance can be attributed to the uncontrolled use of PYs or fixation of knockdown resistance (*kdr*) alleles in populations, which may cause cross-resistance and contribute to the spread of resistant alleles (Yavasoglu et al. 2021). The *kdr* allele frequencies obtained in our study are informative for the continuation or discontinuation of DDT/PY use in the

future. For instance, the relatively high L1014S mutation in the Aydın and Muğla *A. sacharovi* species indicates that PYs resistance could be a potential issue for these populations in the future. A relatively high mutation frequency was observed in the fall of 2018 (Aydın=0.288, Muğla=0.475) period samples, which was not observed in the spring of 2018 and spring of 2019 (Aydın=0.113, Muğla=0.230) periods in these populations, and where insecticide pressure was intense, exhibited a slight seasonal variation in insecticide resistance alleles. However, this was not the case for the *A. superpictus* populations and has a lower mutation frequency than *A. sacharovi* species. The high number of heterozygous individuals and resistance mutation frequency in the Aydın, Muğla, and Balıkesir populations of *A. sacharovi* species indicates that these populations require effective resistance management as soon as possible. In parallel with the results obtained by Yavasoglu et al. (2021), in our study, the L1014S allele was almost fixed in the Aegean Region, which indicates the necessity of developing new resistance management strategies for PYs insecticides. In Turkey, there is only one study at the molecular level (Lüleyap et al. 2002) that showed that the *kdr* mutation has existed in the past and plays a role in insecticide resistance. Another study was carried out by Yavasoglu et al. (2021), who detected both *ace-1* and *kdr* mutations. These results suggest that the L1014S mutation is specific to the Aegean population. These results also indicate that the L1014S mutation frequencies are important for PY-based vector control programs because of their preservation and transmission to the next generation (Chen et al. 2019; Tan et al. 2014).

The common point of these studies is that the *kdr* mutation is a significant mechanism in the development of resistance; however, the mutation rate and variety were observed to differ according to the species and in different locations of the species. Studies on *Anopheles* species have focused on *A. gambiae*, which is the primary malaria vector, reported that the *kdr* mutation in *A. gambiae* *ss.* populations were found only in the molecular S form of the species, but not in the M form, which may be attributed to a different resistance mechanism (Awolola et al. 2003; Diabate et al. 2004). Research has demonstrated that different forms of the same species may exhibit varying levels of resistance, even when subjected to identical insecticide selection pressure (Corbel et al. 2004; Diabate et al. 2002; Etang et al. 2006). Consequently, even *A. sacharovi* and *A. superpictus* samples collected from the same environment may display different mutation frequencies under the same insecticide selection pressure due to their distinct species classification. Considering the aforementioned studies, it is postulated that the difference between the mutation rates might be attributed to the involvement of diverse resistance mechanisms in each species. Further investigation into the involvement of

different resistance mechanisms is warranted in future studies on these species.

Numerous *Anopheles* species develop resistance to various insecticide groups, with both metabolic detoxification mechanisms and target-site insensitivity mechanisms contributing to this resistance. It is posited that the *kdr* mechanism, which is crucial for the acquisition of resistance to PY group insecticides in *Anopheles* species, may be significant; however, the presence of metabolic resistance should not be disregarded. Furthermore, the *ace-1* mutation, which contributes to resistance to OP and CA group insecticides, was not observed in homozygous form in either species in this study, suggesting that alternative mechanisms, such as metabolic detoxification processes, may be implicated in resistance to this group of insecticides. Comparable findings were reported by Yavasoglu et al. (2021).

The results of this study indicated that the RR frequencies were higher than the RS frequencies for both the spring and fall of 2018. A study conducted by Yavasoglu et al. (2021) yielded similar findings; they also observed that the populations they investigated exhibited a higher RR frequency than RS frequency. Upon examination of the number of insecticides used in 2017, 2018, and 2019 (Table S4), a correlation was identified between the number of insecticides used annually per province and resistance allele frequencies. In all provinces, the amount of insecticide used increased substantially between 2017 and 2018, except Aydın province. The increase in the number of resistant individuals in the spring of 2018 suggests that the resistance allele was in the initial stages of the spreading process, which may explain the increase in RR frequencies between the spring and fall of 2018. Furthermore, this increase may indicate that selection pressure was favoring homozygous resistant individuals between spring and fall in 2018. *A. sacharovi* is recognized as one of the most thermophilic species (Becker et al. 2010); consequently, the RR genotype may confer an advantage during summer, or resistant individuals may possess a reproductive advantage. In the spring of 2019, an increase in RS frequency was observed, while the RR frequency decreased across all populations. This observation suggests that the RR genotype may be disadvantageous for overwinter survival. However, individuals with the RS genotype appear to have a higher survival rate compared to their RR-genotyped counterparts. It is proposed that the homozygous L1014F mutation may influence the winter survival of *A. sacharovi*, and it appears that when the mutation is heterozygous, individuals may be able to compensate for these effects. Comparable results regarding winter survival were observed in studies on the survival effect of pyrethroids conducted by Taşkın et al. (2016) and Gazave et al. (2001) in *C. pipiens*, Rinkevich et al. (2013) in *M. domestica*, and Osoro et al. (2021) in *A. gambiae* (Gazave et al. 2001; Rinkevich et al. 2013; Taşkın et al. 2016). As the 2019 fall populations deviated from the

Hardy–Weinberg equilibrium, it is hypothesized that the initial spread phase of this resistance allele has concluded, and that this allele may pose challenges for vector control efforts in the future.

Conclusion

In this study, resistance acquired through the target-site insensitivity mechanism was investigated. Detection of the L1014S allele frequencies contributed to the determination of measures that should be implemented before these alleles reach their peak, and the efficacy of the utilized insecticide diminishes. The observed seasonal variation in resistance alleles, particularly in *A. Sacharovi* populations (Muğla, Aydın, and Balıkesir), is noteworthy. As pyrethroids (PYs) are the primary chemical components of mosquito control agents, novel insecticide resistance management strategies are urgently required before resistance levels in populations escalate significantly. Given that resistance development is evident in the studied populations, it is imperative to determine the resistance levels of the target species and apply insecticides accordingly in control program studies. Future research should examine changes in mosquito populations over time, particularly as insecticide use and coverage evolve, new interventions emerge, and climate and land use changes occur.

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Data availability The datasets used or analysed during the current study are available from the corresponding author upon justified request.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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