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# Determination of insecticide resistance based on the Kdr mutation in *Anopheles maculipennis* complex from mediterranean and Aegean regions

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**Abstract**
The purpose of this study is to determine the level of insecticide resistance against DDT, Deltamethrin and Permethrin insecticides within the populations of *Anopheles maculipennis* complex in the region of Aegean and Mediterranean. By using the adult female specimens of *An. maculipennis* complex samples obtained from the populations, World Health Organization's standard insecticide susceptibility tests were carried out in the laboratory. After the insecticide susceptibility tests, genomic DNA isolation were done for 145 females from different *Anopheles* population. By using genomic DNA of 97 specimens, Vssc1 gene region sequences which cause *kdr* resistance were obtained and polymorphisms in proteins were determined. For Vssc1 gene region, TTG (leucine) in 59, TCG (serine) in 2 and T(T/C)G (leucine/serine) in 36 of 97 samples was determined at 1014$^{th}$ position. Moreover, RFLP analysis was carried out on Asetilkolin esterase 1 gene by using *Alu1* restriction endonuclease for 107 samples. As a result of the analyses, no mutation correlated with insecticide resistance was found in *Ace-1* gene. In order to confirm the validity of these results at species level, molecular species identification studies were done. By using molecular DNAs, ITS 2 region sequences were obtained for 75 samples and based on comparisons of the ITS 2 sequences with those in GenBank, three species as *An. maculipennis s.s., An. melanoon* and *An. sacharovi* were determined.

**Keywords:** *An. maculipennis* complex, insecticide resistance, *kdr*, *Vssc1*, *Ace-1*, *Alu*, Turkey.

## 1. Introduction

The *Anopheles maculipennis* complex formally comprised 12 Palearctic members including *Anopheles atroparvus, Anopheles beklemishevi, Anopheles labranchiae, Anopheles maculipennis, Anopheles martinus, Anopheles melanoon, Anopheles messeae, Anopheles sacharovi, Anopheles persiensis, An. daciae, An. lewisi* and *An. artemievi* [1-5]. *An. atroparvus, An. labranchiae* and *An. sacharovi* are known to be efficient current or historical vectors of malaria [6, 7] and three species of this complex, *An. sacharovi, An. melanoon* and *An. maculipennis* found in Turkey [1, 8-10]. *Anopheles sacharovi* is main vector in Turkey and has wide spread distribution in many parts of Turkey, but is generally found in sympatrik populations with *An. maculipennis* and *An. melanoon*. Malaria has prevailed as the most important vector-borne disease for many years in Turkey. Therefore, the most important target species of malaria eradication program is *An. maculipennis* complex including *Anopheles sacharovi.* Chemical insecticides (i.e. pyrethroids, organochlorines, organophosphates and carbamates) were used extensively for this purpose which led to the emergence of insecticide resistance in Anopheles populations in Turkey [11-17] as in other countries [18, 19]. Although development of resistance to chemical insecticides has been reported by many of the authors, chemical insecticides are still being heavily used in control operations in many areas of the World [20-22].

The world health organization (WHO) has defined insecticide resistance as "the biggest single obstacle in the struggle against vector-borne diseases" and it is estimated that the annual cost of resistance may be US 1.4 billion dollars in the United States [23, 24].

The major mechanisms by which insects acquire resistance to insecticides are elevated levels of detoxifying enzymes (metabolic resistance) and target-site insensitivity [25, 26].

The primary target sites of pyrethroids are the voltage-gated sodium channels (Vssc1) in nevre membranes. A mutation of leucine at pozition 1014 to phenylalanine residue (L1014F) in Vssc1 an autosome B in *Anopheles* results in insensitivity of the target site to pyrethroids and is termed the mutation. Knock-down resistance (Kdr) due to a point mutation in the Vssc1 at L1014F residue (position) is a common mechanism of resistance to DDT and pyrethroid group insecticides. The selection of this resistance may pose a serious threat to the success of the pyrethroid- impregnated bednet and malaria control programs [27].

Screening *Anopheles* populations for the *kdr* mutation has become one of the mainstays of programmes that monitor the development of insecticide resistance [28]. Different regions of the gene also confer *kdr* resistance in some other insects, but in *Anopheles* this is the only locus where point mutations have been reported to date conferring resistance [29, 30]. One allele commonly associated with resistance to DDT and pyrethroid is the *kdr* resistance or *kdr* allele. Only two different point mutations have been found in Anophelines at this locus where point mutations have been reported to date conferring resistance.

The importance of these mutations for the control of *Anopheles* mosquitoes is not yet fully understood. However, monitoring its frequency, as a rapid indicator of the development of resistance, should be an integral component of insecticide resistance management programmes.

To date, resistance among *Anopheles* species to at least one of the four commonly used insecticide classes has been reported in 64 malaria-endemic countries worldwide, the vast majority reporting resistance to pyrethroids [31, 32]. WHO standard tests performed with different mosquito populations in many regions reveal the development of resistance to carbamate, organophosphate (OP) pyrethroids (PY) and organochlorine insecticides due to the fact that chemical insecticides were used extensively in malaria eradication programs in Turkey as well [33-35]. Kasap *et al*. [36] (1999) conducted a study using samples from *An. sacharovi* population in Çukurova region in Adana and showed the presence of *kdr* mutation. Studies on insecticide resistance of *Anopheles* populations in Turkey are not up-to-date and there are no comprehensive studies which reveal the mechanism of the resistance. Thus, new studies are needed to find out the current resistance status of the *Anopheles maculipennis* complex. The objective of this study was to evaluate the kdr and *ace* resistances which are responsible for the insecticide resistance in the *An. maculipennis* populations in the Mediterranean and Aegean regions of Turkey.

## Material and Methods

### Mosquito collection and WHO susceptibility test

Adult female *Anopheles* mosquitoes were collected by the indoor-resting catch technique (WHO,1975) [37] in July-October 2011-2012 from Aydın, Izmir, Burdur, Muğla and Isparta populations for susceptibility tests and molecular characterization of insecticide resistance (Figure 1.)

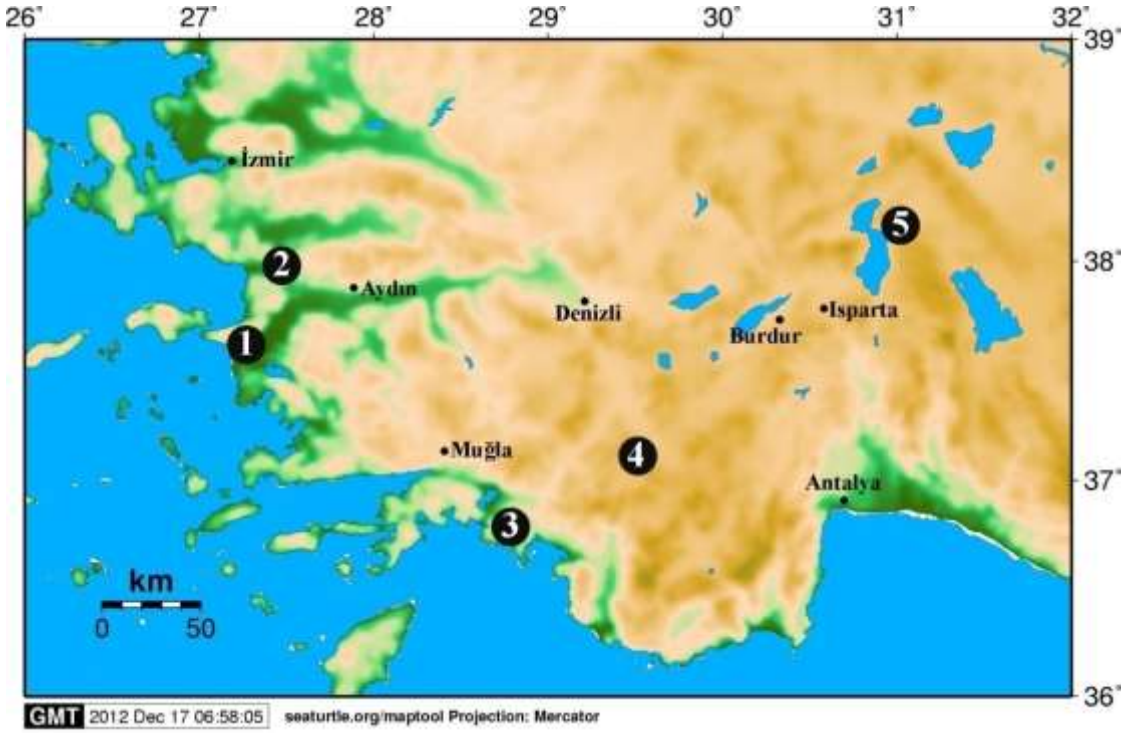


**Fig 1:** Geographical locations of the sampling sites

In laboratory, all collected bloodfed female mosquitoes were placed one by one in 200 mL containers 100 ml of which consisted of distilled water and kept until they laid eggs. Larvaes were obtained after hatching and F1 generation was produced and reared to adult stage for each population. Using 2-4 year-old non-blood-fed females from F1 generation samples, WHO susceptibility test (provided from Malaysia, WHO) carried out with DDT (4%), deltamethrin (0.025%), permethrin (0.25%); on *An. maculipennis* complex specimens based on recommended procedure (WHO, 1981) [38] and percent of death was recorded after 24 hours recovery. The exposure tubes were held vertically, and each test included controls, where mosquitoes from the same population were 'treated' the same way, but without exposure to insecticide.

Surviving mosquitoes from susceptibility tests were killed by y freezing at -20 °C prior to molecular species identification and *kdr* analysis.

### Molecular analysis

Genomic DNA was extracted from individual females using the method described by Invitrogen PureLink genomic DNA isolation kit. The *An. maculipennis* complex species identification carried out ITS2 region gene was amplified using the 5.8 SF and 28SR universal primers listed by Collins and Pastewitz [39]. Amplification programme consisted of an initial denaturation at 94 °C, for 5 minutes followed by thirty cycles of denaturation at 94 °C for 45 seconds, annealing 53 °C for 45 seconds and extension 72 °C for 45seconds, followed by an additional 10 min at 72 °C. After this procedure approximately 450 bp bands were obtained.

Vssc1 regions of rDNA was also amplified using the An. kdr R2, AgR_kdr and AgF_kdr universal primers listed by Syafruddin *et al*., [40]. Polymerase chain reaction reactions carried out in 2 levels. Reaction cycles involved in initial denaturation at 94 °C 5 min followed by one cycle of 94 °C 30s, 47 °C for 30s and 72 °C 1.5 min, followed by 29 cycles of 94 °C 30s, 47 °C 30s, 72 °C 1 min, followed by an additional 1 min at 72 °C.

The *kdr* amplicons were approximately 260 bp in size. They were then prepared for DNA sequencing. As expected, the DNA sequence of the VGSC gene varied significantly among

the Anopheles species analyzed, but the deduced amino acid sequences indicated a high level of sequence conservation.

The *Alu 1* restriction enzyme (10 Unit/µl, BIORON, Cat No: 250101S) was used in the study for the determination of the G119S mutation in the *Ace–1* gene region. *Ace–1* gene region was amplified using the Ex3Ag F and Ex3Ag R universal primers listed by Ahoua Alou *et al*., [41]. The band size for *Ace-1* gene region is 555–560 bp. The PCR products obtained by the amplification of the *Ace–1* gene region have two cutting sites (5' AG↓CT 3') for the *Ace–1* enzyme. For the cutting process with the restriction enzyme, *Ace–1* gene region PCR products were subjected to incubation at 37 °C for 16 h and then subjected to reactions at 65 °C for 20 min to terminate the enzyme activity. As a result of the cutting of the *Alu-1* enzyme had 2 cutting sites in the sequences about 560 bp in length. If there is no mutation in the cut product, 3 band profiles 236, 89 and 182 bp in length appears on the agarose gel image. PCR–RFLP products were separated in 1.5% agarose gel electrophoresis and the genotypes of the individuals were determined on the basis of the number of bands in the resulting gel profile.

Sequencing were conducted under BigDye terminator cycling conditions and the reacted products were purified using ethanol precipitation and run using Applied Biosystem 3730Xl DNA ANALYZER by Macrogen Inc. Later on, each DNA sequence was converted in the FASTA format and compared with the sequences in the GenBank on http://www.ncbi.nlm.nih.gov.

## Results

### Insecticide susceptibility tests

WHO [42] insecticide susceptibility tests were performed in the laboratory using 510 female samples of the Anopheles maculipennis complex populations collected in İzmir, Aydın, Burdur, Isparta and Muğla locations. Insecticide resistance status was evaluated by using the classification determined by the WHO [43] in which 98–100% mortality indicates susceptibility, 80–97% mortality suggests possible resistance requiring confirmation, and ≤80% mortality suggests resistance.

In İzmir populations, 90% mortality of the susceptible strain was obtained with 0.025% deltamethrin, 4% DDT and 0.25% permethrin. Current state showed that this population is susceptible for DDT, deltamethrin and permethrin. In Aydın populations, mortality rate is 40% for DDT; so this population is resistant for DDT. On the contrary, mortality rates for deltamethrin and permethrin was 80%. In Isparta populations, mortality rate is % 70 for DDT, full susceptibility (100% mortality) was obtained with deltamethrin and permethrin after 24 hours. In Burdur populations, mortality rate is 57% for DDT, 97% for deltamethrin, 87% for permethrin. In Muğla populations mortality rate is 65% for DDT but full susceptibility (100% mortality) was obtained with deltamethrin and permethrin after 24 hours. In general, the Aydın, Burdur and Mugla populations was more resistant than the İzmir and Isparta population for DDT. Nevertheless results showed that insecticide resistance status changes between locations and populations.

### Identification of the species of Anopheles maculipennis complex

After the insecticide susceptibility tests, a method based on the differences between the ITS 2 sequences was used to identify the species of the samples whose DNAs were isolated. The sequences of the ITS 2 regions obtained from the female samples were compared with the sequences already present for the *An. maculipennis* complex species in the GenBank and species identification was carried out for each sequence. Of the sequences obtained in this study, 49 (66%) of them were determined to be 100% compatible with HQ878035 [44] and AY8422515 [45] recorded for *An. sacharovi* in the GenBank while 14 (18.42%) of them were compatible with HQ877939 [44] and FJ210877 [46] sequences belonging to *An. maculipennis* s.s. species and 12 (15.87%) of them were compatible with AJ224330 [47] and AF452389 [48] sequences recorded for *An. melanoon* species.

### *Kdr* mutation

As a result of the susceptibility tests, a total of 106 samples was selected from each and every location to carry out PCR reactions to determine the *kdr* mutation in the Vssc1 gene. Sequences were obtained in order to determine the *kdr* mutation in the Vssc1 gene of the 106 PCR products. However, 9 of the sequences were not suitable for the mutation screening; therefore a total of 97 sequences of the Vssc1 gene were used for the evaluation and the amino acid genotype distribution of the sequences was determined (Table 1)

**Table 1:** Genotype distribution based on *kdr* mutation in the Vssc1 gen

| Locality | Genotype | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | TTG | | | T(T/C)G | | | TCG | | |
| | *An. sacharovi* | *An. maculipennis* | *An. melanoon* | *An. sacharovi* | *An. maculipennis* | *An. melanoon* | *An. sacharovi* | *An. maculipennis* | *An. melanoon* |
| IZMİR | 3 | 8 | | 4 | | | | | |
| AYDIN | 3 | | 5 | 5 | 1 | | 1 | | |
| ISPARTA | 2 | | | 8 | 3 | 2 | | | |
| BURDUR | 10 | 3 | 5 | 6 | 2 | | 1 | | |
| MUĞLA | 10 | 7 | 3 | 5 | | | | | |
| TOPLAM | 28 | 18 | 13 | 28 | 6 | 2 | 2 | | |

Analysis of DNA sequences of 97 amplicons representing 3 *Anopheles* species indicated that the L1014F polymorphism of the Vssc1 gene, known as L1014F allele was detected in two *An. sacharovi* specimen from Aydın and from Burdur populations. This allele was found in either homozygous or heterozygous form. At 1014$^{th}$ position, most of the 59 sequences which carry the Leucine amino acid were found in the samples from Muğla and Burdur populations. The sequences showing heterozygous polymorphisms were mostly obtained from the samples from Isparta location.

***Ace*-1 mutation**

*Ace–1* gene region of a total of 111 samples from 5 populations were amplified by PCR reactions in order to determine the mutation which causes the development of resistance in the *Ace–1* gene and products in 550-600 bp size were obtained and agarose gel electrophoresis screenings were carried out. Then, the PCR products were prepared for cutting with *Alu 1* restriction enzyme. A total of 107 samples from the PCR products size 555/560 bp of the *Ace–1* gene region was cut with *Alu 1* restriction enzyme which has the recognition sequence in this region in order to determine G119S mutation and their band size was determined by running them in 1.5% agarose gel electrophoresis In the partially amplified sequences of the *Ace-1* gene regions of the An. maculipennis complex samples, there are two regions where *Alu 1* enzyme results in cutting. As a result of the cutting of the 107 samples 560 bp in length with *Alu 1* enzyme, 3 band profiles were detected about the size of 240, 85 and 100 bp in one sample which no mutation was observed. Therefore, samples with mutations in the *Ace–1* gene region were found in none of the localities.

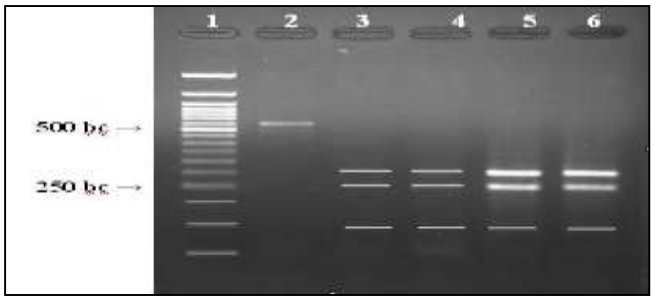


**Fig 2:** Results of the *Alu 1* enzyme cutting reaction

**Discussion**

In this day and time, pesticide resistance is the most important issue in the pest control, therefore, the investigation of resistance mechanisms in pest population and of adaptive differences in resistant-sensitive genotypes is of paramount importance. Characterization of insecticide resistance will allow obtaining data about the evolutionary process through which the target insect goes during its adaptation to environmental changes. *Kdr* and *Ace–1* mutations can confer the formation of resistance to organochlorine and pyrethroid group insecticides used heavily in the past or still used for various reasons in our country. In this direction, the study aimed, using molecular methods, to determine the presence of kdr and *Ace–1* mutations in the specimens of *An. maculipennis* complex populations sampled from different areas of Turkey's Mediterranean and Aegean regions. WHO insecticide susceptibility tests were performed for DDT, permethrin and deltamethrin insecticides on a total of 510 female *Anopheles* collected in Burdur (Uylupınar), Isparta (Gelendost), Muğla (Kapıkargın), İzmir (Belevi) and Aydın (Tuzburgazı) localities. The susceptibility test results were assessed on the basis of population sampling and different conclusions were reached; however, all populations in general were found to still have resistance to DDT. In contrast, development of resistance to permethrin and deltamethrin was not detected in the populations for certain because the mortality rate for permethrin in Burdur locality was found to be 87% while 100% in Isparta locality. Similarly, the mortality rate for permethrin was found to be 90% in Aydın and İzmir localities and 95% in Muğla locality.

On the other hand, the mortality percentage rates for deltamethrin differed from population to population. The lowest mortality rate (90%) was obtained in İzmir locality while the highest mortality rate (100%) was obtained in Muğla locality. Although these results fail to explain the mechanism of resistance, they show that the resistance to organochlorine insecticides in the populations is still effective and that the existing mechanism of resistance has started being effective against pyrethroid insecticides such as DDT commonly used for various purposes in recent years. Therefore, it has been established that the resistance which is present and effective in the sampling populations can only be based on a genetic factor. Akıner [49] reports that the resistance, which still exists due to the use of DDT and its derivatives for many years, could limit the use of insecticides whose target regions are the same as organochlorine insecticides even though the former were developed later than the latter. This is due to the fact that populations can develop cross-resistance even though the use of DDT has been discontinued.

Similar results were obtained in many studies for DDT and for other insecticides from the same group. It is reported that pyrethroid resistance has been developed in *An. albimanus, An. stephensi* and *An. gambiae* [50-53]. In the control of malaria in our country, insecticides such as DDT, lindane, Malathion, pirimiphos-methyl and bendiocarb were used [35] and *An. sacharovi* populations were exposed to insecticide pressure due to the intense use of DDT both for agricultural and public health purposes in the following years of 1952. *An. sacharovi* developed resistance to DDT and lindane in 1959 although they were used in the 1950s and 60s. Zulueta [19] reports the variability of resistance or susceptibility developing to insecticides in *An. sacharovi* samples in Greece, Iran, Italy, Romania and Turkey depending on the populations. *An. sacharovi* samples from Turkey, Tarsus revealed high physiological resistance to DDT while the samples from Greece revealed high physiological resistance to dieldrin. Hemingway *et al*. [54] reports resistance to DDT in *An. sacharovi* populations in 1984 although malathion replaced DDT in malaria control. They also point out the presence in Çukurova populations of AChE mechanism of resistance which also confers the formation of resistance to organophosphate and carbamate insecticides in *An. sacharovi* populations. Kasap *et al*. [37] applied WHO susceptibility tests to *An. sacharovi*, samples collected in Adana-Tuzla (Karataş) region and reported mortality rates for DDT in *An. sacharovi*. Kasap *et al*., [35]'s study on An. sacharovi determined low mortality rate for DDT in Adana, Adıyaman and Antalya populations and high mortality rates (95%) in Aydın (67%) and Muğla (77%) populations. Adana population was identified as the most resistant population for deltamethrin and permethrin and the other populations showed resistance in varying proportions. Lak *et al*. [55] applied WHO susceptibility tests to the *An. sacharovi* samples collected in the border line regions of Iran, Armenia, Nakhichevan and Turkey, and subjected them to various insecticides. The results indicate that the *An. sacharovi* samples are resistant to DDT, tolerant to dieldrin and susceptible to insecticides such as bendiocarb, propoxur and malathion. As revealed in the results obtained from the research outlined above, there has long been a significant resistance to DDT in *Anopheles* in both Turkey and in nearby countries. On

the other hand, the recent studies show that resistance to deltamethrin and permethrin insecticides has developed due to cross-development of resistance. Similarly, although the sample size of this study is not large enough to draw any definite conclusions, the presence of DDT resistance in the sampled populations can be stated according to the results of the tests. Apart from the number of samples; climate, season, year or period in which samples are collected and season-based population movements can also affect the test results. Therefore, WHO susceptibility tests applied to a limited number of samples in a single period of the year are not sufficient to demonstrate the resistance or susceptibility of the populations. In these cases, results of molecular studies on mechanisms which confer development of resistance are needed.

There is a positive correlation between the phenotype of resistance to pyrethroids and DDT in *Anopheles* and the *kdr* genotype [56-58]. Therefore, after WHO susceptibility tests carried out on the samples of *An. maculipennis* complex collected from the localities, molecular studies were conducted on the same samples for the detection of the mutations which confer the *kdr* resistance. There is only one study conducted in Turkey for the determination of the insecticide resistance occurring depending on the gene mutation at the molecular level in *Anopheles* populations [59]. In this study, researchers made screening for the mutation for 12 samples of Adana population of *An. sacharovi* which were identified as resistant to pyrethroids as a result of WHO susceptibility tests. 3 different amino acids; serine (TCG), leucine (TTG) and phenylalanine (TTT) were found at 1014th position in 4 of 12 amplified samples the region of which is associated with voltage- gated sodium channel gene and *kdr* mutation. This way, the researchers demonstrated the relationship between the gene mutation and the pyrethroid resistance in Adana population of *An. sacharovi.*

The genotype distribution of the Vssc1 was determined to be leucine amino acid in 57 samples, heterozygous polymorphism in terms of leucine/serine amino acid in 36 samples and serine amino acid in 2 samples at 1014th position, the region associated with the *kdr* resistance of the Vssc1. A significant part of the 59 sequences determined to carry the leucine amino acid at 1014th position was found in the samples collected in Muğla and Burdur. The sequences showing heterozygous polymorphism were obtained from the samples collected in Isparta locality. Although amino acid change which confers the *kdr* resistance was not detected in most of the samples, serine amino acid was detected in one sample of *An. sacharovi* species from each Burdur and Aydın locality. This situation reveals the presence of the mutation which confers the formation of resistance in some individuals in the populations rather than showing the resistance of Burdur and Aydın populations. In addition, 2 individuals which contain serine at 1014th position show that the mutation which confers the *kdr* resistance is transferred to the next generations and that the *kdr* mutation, which confers resistance to DDT and to deltamethrin, permethrin as cross-resistance, is only partially recessive in *An. sacharovi* populations even though DDT is not used for many years. The data reveal that if DDT or insecticides with a similar mechanism are used for control of *Anopheles* populations, it may become dominant in the populations due to the selection of resistant alleles in the populations. This study reveals different results from or similar results to those of the study which was conducted on other key *Anopheles* species and which investigated similar mechanisms in other countries. Verhaeghen *et al.* [60] conducted a study on *Anopheles* species in Mekong in China using FRET/MCA and PCR-RFLP methods. However, they were not able to detect the *kdr* mutation in any species. The researchers claimed that the absence of the *kdr* mutation was due to the fact that insecticides were not used in the region for a long time. To explain this phenomenon, they put forth various opinions in that they suggested that with the disappearance of insecticide pressure, resistance can be conferred by metabolic enzyme systems different from the resistance to DDT and pyrethroids, that each species may react differently against different insecticide or that it might be caused by the presence of a different insecticide pressure or of genetic pressure.

Awolola *et al.* [61] carried out a study on *Anopheles gambiae* s.s in Nigeria and detected the *kdr* mutation only in the molecular S form of the species. They tried to explain the absence of the *kdr* mutation in the M form of the species by arguing that *An. gambiae* s.s. might have a different pyrethroid resistance mechanism. Although the *kdr* mutation was found in the molecular S form of *An. gambiae* species in different studies, mutation was not observed in the sympatric M form [62, 63]. These results suggest that although the genetic forms of this vector are subjected to similar insecticide pressures in the same region, they show variations in terms of resistance and that there is no gene flow between the molecular forms of the species [64]. A similar situation was also determined in this study. There are signs that resistance mechanisms might exist between the species in *An. maculipennis* sympatric populations in Burdur and Isparta localities or that there might also be significant differences regarding the conservation of the resistance mutations. Kang *et al*., (2012) carried out a study on *An. sinensis* complex and detected the mutation at 1014. codon for *kdr* in *An. sirensis* s.s. while they did not observe the *kdr* mutation in the other 5 species they examined (*An. pullus, An. kleini, An. sineroides, An. lesteri, An. belenrae*). They attributed this situation to *An. sirensis* s.s species having a larger population and a more diffuse spread than other species of *An. sinensis* complex and reported that the biochemical and specific gene expression studies should also be conducted.

In this study we also made analyses for the determination of the mutation which confers the development of resistance in *Ace–1* gene. Cutting reactions with *Alu-1* enzyme of the products belonging to the gene region obtained from PCR reactions of a total of 144 samples collected from 5 localities were carried out. As a result of the cutting with *Alu-1* enzyme, all the samples exhibited three band profiles and the *Ace* mutation which confers the formation of insensitive AchE was not observed in any of the samples. Although there are no studies conducted on mosquitos in this context in this country, Başkurt *et al*., [66] showed that 13 different combinations occurred at 3 amino acid positions in the *Ace* gene obtained from the *Musca domestica* samples collected from the Aegean and Central Anatolia.

This is the first study which has obtained data for the *Anopheles maculipennis* complex. Of all the samples in the study, serine amino acid, which confers the *kdr* mutations at 1014$^{th}$ position, was found only in two samples. The fact that this mutation was observed only in 2 out of 97 samples on which mutation screening was carried out indicates that these two individuals are resistant or different in terms of their genetic traits rather than the population being resistant.

Although the test results do not show the susceptibility of the populations for pyrethroids, they reveal that more extensive studies should be carried out. For example, the populations in question should be sampled more frequently or every season and resistance studies should be conducted on these samples regularly. In addition, migrations from insecticide-free areas to insecticide-applied areas have an important role in the change in both resistance and adaptation. Therefore, migration of individuals from resistant populations to susceptible populations is one of the subjects that has to be studied in this area [67].

In addition, studies should be carried out by choosing larger geographic regions, collecting more individuals and colleting almost all the samples belonging to a complex or a species in case a complex or a species is to be studied on. More comprehensive molecular and biochemical studies should be conducted on the origin of the resistance by forming insecticide resistant colonies in laboratory conditions. Metabolic resistance mechanisms should also be examined in order to see if different enzyme systems confer the formation of resistance.

**Conclusion**

Finally, insecticide applications should be implemented in limited areas rather than large areas to reduce the development of insecticide resistance in populations and should be implemented during the spawning periods of insects and the reproductive periods of adults instead of all seasons. Non-persistent and fast-acting insecticides should be used in appropriate doses instead of permanent insecticides. Different insecticides should be preferred for control measures of adult and larvae of insects and different insecticide applications should be implemented alternately.

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