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RT-PCR Analysis of Caucasian and Mugla Honey Bees by SNP Markers of Chalkbrood Disease *

Kafkas ve Muğla Bal Arılarının Tebeşir Hastalığının SNP Belirteçleri ile RT-PCR Analizi*

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Abstract: Two different honey bee subspecies' genotypes obtained from Ordu, Apiculture Research Institute were analyzed by SNP markers using Real-Time PCR-HRM. Genomic DNA samples analysed with 10 SNP primers those were used for identification of chalkbrood disease resistance genes and two SNP primers those were obtained from honey bee genom sequencing. Result of SNP analyses, four primers (AMB-00858574, AMB-01151447, AMB00631190, AMB-00686140) amplified in Caucasian honey bee and six (AMB-00858574, AMB-00612262, AMB-01151447, AMB-00631190, AMB-00674355, AMB-00686140) primers amplified in Mugla honey bee. Four amplicons are similar for this subspecies. Result of electrophoresis analyses, five primers (AMB00858574, AMB-00612262, AMB-01151447, AMB-00631190, AMB-00686140) form a band in Caucasian honey bee, seven amplicons (AMB-00858574, AMB00612262, AMB-01151447, AMB-00631190, AMB-00902548, AMB-00674355, AMB-00686140) form a band in Mugla honey bee and four amplicons (AMB00858574, AMB-01151447, AMB-00631190, AMB-00686140) similar for this subspecies. As a result of this study in Caucasian and Muğla honeybee for identification of disease resistance and evaluability as a identification key for this subspecies was emerged the capacity of association of single nucleotide polymorphisms to resistance to chalkbrood in two important honeybee genotypes in country of *Apis mellifera* using RT-PCR for HRM analysis.

Keywords: Honey Bee, SNP, Mugla bee, Caucasian bee, Real-Time PCR, HRM, chalkbrood disease

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Öz: Ordu, Arıcılık Araştırma Enstitüsü'nden sağlanan iki farklı bal arısı alt türüne ait genotipler Real-Time PCR-HRM kullanılarak SNP markörleri ile analiz edilmiştir. Kireç hastalığı direnç genlerinin tanımlanmasında kullanılan 10 SNP primeri ve bal arısı genom dizilemesinden elde edilen iki SNP primeri ile analiz edilen genomik DNA örneklerinde; Kafkas bal arısında amplifiye edilmiş dört primer (AMB-00858574, AMB-01151447, AMB 00631190, AMB-00686140) ve altı (AMB-00858574, AMB-00612262, AMB-01151447, AMB-00631190, AMB-00674355, AMB-00686140) Muğla bal arısında amplifiye edilmiştir. Bu alt tür için dört amplikon benzer olup elektroforez analizleri sonucunda, Kafkas bal arısında beş primer (AMB 00858574, AMB-00612262, AMB-01151447, AMB-00631190, AMB-00686140), yedi amplikon (AMB-00858574, AMB 00612262, AMB-01151447, AMB -00631190, AMB-00902548, AMB-00674355, AMB-00686140) Muğla bal arısında dört amplikon (AMB 00858574, AMB-01151447, AMB-00631190, AMB-00686140) bir bant oluşturmuştur. Bu çalışma sonucunda, Kafkas ve Muğla bal arısı genotiplerinde hastalık direncinin belirlenmesi ve bu alt tür için bir tanımlama anahtarı olarak değerlendirilebileceği, HRM analizi için RT-PCR kullanarak *Apis mellifera*'nın önemli iki bal arısı genotipinde tek nükleotid polimorfizmlerinin kireç direnci ile ilişkilendirme kapasitesi ortaya çıkarılmıştır.

Anahtar Kelimeler: Bal arısı, Mugla arısı, Kafkas arısı, SNP, RT-PCR, HRM, kireç hastalığı

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INTRODUCTION

The honey bee (*Apis mellifera*) is of great economic, agricultural and environmental importance. For this reason, honey bee breeding has spread all over the world, but the natural habitat of *Apis mellifera* is thought to be Europe, North Africa and the Middle East, and phylogenetic analyzes have shown that the origin of *Apis mellifera* is Asia (Han et al., 2012). The honey bee is one of the most important pollinator insect species the world needs. However, unlike other pollinator species, the honey bee has become a part of human life and has become the focus of people's attention. Although the complex social structures of honey bees are of some interest to humans, honey bees are not the only insect that humans have either cultivated or created a social structure. Honey bees arouse people's curiosity. In the civilizations of South East Asia, creation was associated with the honey bee, and the honey bee was frequently mentioned in many Ancient civilizations. The medieval church accepted the honey bee as a symbol of purity. In the Middle Ages, the Holy Roman Emperor Charlemagne established a tax system on honey (Johnson, 2011). Honey bee is seen as a valuable biological material by breeders due to its economic value (Tunca, 2009). The turning point for studies on the honey bee was the sequencing of the genome of *Apis mellifera*. High A+T content, high C+G content, low number of immune-determining genes, absence of transposons, and slow evolutionary rate are the unique features of the honeybee genome. The similarity of daily biological rhythms, RNA interactions and DNA methylation genes with vertebrates makes the honey bee a promising model organism for important biological activities (Gupta, 2012).

Single Nucleotide Polymorphism (SNPs) is the replacement of a single nucleotide in the DNA sequence with one of two different nucleotides. SNPs are generally bi-allelic in practice. This can be explained by the low frequency of single nucleotide polymorphisms occurring at the origin of SNPs. Moreover, the probability of independent nucleotide exchange occurring at the same point is very low. Polymorphism (replacement, deletion, or insertion) of differences at a single nucleotide position occurs. Another reason is due to an effect that occurs in mutations. This effect causes the emergence of two types of SNPs. Mutations can occur as a transition or a transversion; transition is the replacement of purine with purine or pyrimidine with pyrimidine, while transversion is the replacement of purine with pyrimidine or vice versa. SNPs have an advantage over other types of polymorphism in the genetic study of complex traits and diseases. SNPs are also very effective in population gene identification studies. The high frequency of SNPs on the genome gives them a clear advantage in the discovery of trait or disease genes. That is, SNPs can be used in deep gene mapping in gene cloning efforts, or more importantly, as candidate polymorphisms that have been tested as functional or normal mutations for trait or disease. SNPs can be found in any region of the genome, such as exons, introns, intra-gene regions, and promoter regions. SNPs are associated with alleles associated with traits and diseases rather than with alleles associated with yield, function, and physiology. A SNP protein in the coding region can affect gene expression, an SNP in the promoter region, and an SNP in the intron region can affect splice splicing. Due to their simple structure such as base exchange, microarray and other technology techniques can be developed to provide rapid and efficient genotyping of hundreds of individuals using SNP markers. SNPs mutate less than other polymorphisms (Iglesias and Grzelczak, 2020; Komar, 2009). SNPs have been used to scan genetic diversity and resistance to pathogenes in many organisms (Kongchum et al., 2010; Donalds et al., 2017; Elberts et al., 2018; Kosch et al., 2019). Schork et al., (2000), emphasizing the utility of SNPs for genetic epidemiology studies offering an overview of genetic polymorphism and discuss the historical use of polymorphism in the identification of disease-predisposing genes via meiotic mapping, reported that genotyping of hundreds can be done using SNP markers.

Han et al. (2012) investigated the origin of the honey bee with approximately 1000 SNP markers. They used 2 methods to construct the phylogenetic tree: allele affinity and Fst matrix. As a result of their studies, they found that their data was not sufficient to determine the origin of the honey bee. Allelic affinity indicates that the honey bee is of African origin, but the Fst matrix does not. Their analysis showed that the bees in Western and Eastern European regions came from different origins. Chapman et al. (2015) conducted a study of 95 SNP markers to quantify the African honey bee genome that originated in Africa. As a result of their study, they reported that these 95 SNP markers are effectively useful in African honey bee origin

studies. Holloway et al. (2011) investigated the genes that provide resistance to lime disease in honey bees. In their study, they suggested that resistance to lime disease in offspring is almost determined by genes and environmental influence is minimal. They argued that since lime disease has been seen in honey bees for a short period of time, approximately 100 years, very few genes are effective in endurance and chalkbrood infection caused by the fungus *Ascosphaera apis* currently has a significant impact on Australia's apicultural industry (Gerdts et al., 2021). They also reported that SNP markers have important roles in the detection of genes that provide resistance to lime disease. Pinto et al. (2014), in their research; implied that *Apis mellifera mellifera,* named Dark European Honey Bee is increasingly threatened in its native range has led to the establishment of conservation programmes and protected areas throughout western Europe. They applied 1183 SNP markers to assess genetic diversity and introgression levels in several protected populations of A. m. mellifera comparing with samples collected from unprotected populations. SNPs showed different levels of introgression, such as 0% in Norway and 14% in Denmark. Introgression is higher in unprotected breeds (30%) than in preserved breeds (8%). As a result of their studies, they reported that despite controlled breeding studies, some populations still need to be adjusted in the management of breeding studies to clear foreign genes that can be detected by SNPs. Wallberg et al. (2014) investigated 8.3 million SNP regions obtained as a result of sequencing 140 genomes from 14 honey bee populations worldwide. As a result of their studies, they provided information about the genetic evolution and local adaptations of honey bees. They reported that population sizes fluctuate to reflect historical climate waves. In their study, they reported that modern breeds have high genetic diversity and this shows that honey bees do not form bottle noses during cultivation. They reported that the levels of genetic variation are highly shaped by natural selection. They identified genomic traces of local adaptation. They reported that these traces are rich in immune system genes.

Shi et al. (2013), in their study on *A. cerana cerana*, created the first linkage map 17 for *A. cerana cerana* using 1535 SNP markers. In their study, they found 19 linkage groups from 16 chromosomes by comparing the markers with the genome of *A. mellifera*. They reported that the final map obtained contains 16 anchor points with 1535 markers. Chavez-Galarza et al. (2013) investigated the traces of selection to represent both the natural distribution of *Apis mellifera iberiensis* and a wide variety of climates ranging from the semi-arid in south-eastern to oceanic in north-western Iberia by performing genome scanning analysis using SNP markers in their study. They stated that 15 of the 34 results they obtained in total were strongly related to one or more environmental conditions. Henriques et al. (2013) investigated the amount of crossing between Italian and carniolan bees with European black honey bees in their study. In their study, they compared the microsatellite and SNP markers and tried to understand which marker best describes the hybridization level. They stated that the microsatellite data set they used and 2 of the 3 SNP data sets they used in their studies with different data sets on samples from different European countries gave similar results, but the data of 1 SNP data set were different. Shi et al. (2013) performed the first genetic mapping of *Apis cerana cerana*. F_2 worker bees (N=13) were genotyped for 126990 single nucleotide polymorphism. After eliminating the low-quality ones and those who did not pass the Mendelian test, 3000 SNPs were obtained and 1535 of them were used in the creation of the linkage map. They reported that the final map contains 16 link groups containing 1535 markers. The total genetic distance was determined as 3,942.7 cM. They measured the mean marker spacing as 2.6 cM among the 16 linkage groups. They reported that since the map in question is based on SNP markers, easier and faster genotyping methods would be possible compared to the RAPD and microsatellite-based maps used in *A. mellifera*. The study was performed to establish the capacity of association of single nucleotide polymorphisms to resistance to chalkbrood in two important honeybee breds of *Apis mellifera* using RT-PCR for HRM analysis.

MATERIAL AND METHOD

Material

The honeybee samples were enabled from 30 colonies by the experts of Ordu Honeybee Institute which collected from Artvin territory as Caucasian honeybee subspecies and Mugla territory as Mugla ecotype of Anatolia subspecies. The samples were preserved carefully in well-labelled specimen bottles containing

70% alcohol. These samples were transported to the biotechnology laboratory of Ondokuz Mayıs University, Agricultural Faculty, Laboratory of Agricultural Biotechnolgy Department, Samsun in Turkiye.

Methods

Chemicals used DNA master mixes used in the study; Biotium (California, USA), primer sets Centromere DNA Technologies and chemicals required for all other solutions were obtained from Sigma-Aldrich (Taufkirchen, Germany). Genomic DNA isolation was performed according to the CTAB (hexadecyltrimethyl ammonium bromide) isolation method according to Seçgin (2015). Real-Time PCR components used in molecular studies Fast EvaGreen qPCR mastermix was used as master mix Primers: Twelve synthetic oligonucleotide primer pairs were used in SNP analyses. The names and base sequence (5'-3') of the primers used are given in Table 1. In this study, Holloway et al. (2012) investigated the relationship between the resistance to lime disease in honey bees and SNPs, ten SNP regions they found polymorphic and two of the SNP regions on the genome sequence reported in 2011 as a result of the Honey Bee Genome Project conducted by the Human Genome Sequencing Center were used.

Table 1. Used SNP primers used in this study.
Çizelge 1. Bu çalışmada kullanılan SNP primerleri.

No	Primer	Primer Sequence (5′-3′)	GC Oranı
1	rs21784922F	AGCCATTCTATTTTCCGGATCCCGG	52
	rs21784922R	AACTGGGTTATTCTCATTCCCGTTA	40
2	rs21784921F	CTAAGTAAACTTGGGGAAAGATTAG	36
	rs21784921R	GATTAGAATTATTTCAGCCAATTCT	28
3	AMB-00858574F	TCATTGTTCCCACCGATCGAGCAT	50
	AMB-00858574R	CGCTGTTTGGCATTCGACACTTTC	50
4	AMB-00612262F	GAGAGCGCGAAATCACCGATGAAA	50
	AMB-00612262R	TGGCTACAAACTGTTGCTCCATCT	45,8
5	AMB-01118908F	TGTCCTTCGGATTTATCGGGCGAA	50
	AMB-01118908R	CAGATTGCACTGCCAAGCCTTCAA	50,24
6	AMB-01151447F	TGAACCTCAAAGACTACCACGCCA	50
	AMB-01151447R	ATGATTCGCCTTGAACTTGCGACC	50
7	AMB-00902552F	GTTGGTAACGCAAGCTAAATCTACG	47
	AMB-00902552R	TGCGCCAGAATGGTAAACGGATTG	50
8	AMB-00631190F	AACCAGCGTTAAGGGAGGAACAGT	50
	AMB-00631190R	TGTTCGGTGGTGAAACGAGTGGTA	50
9	AMB-00902548F	ACAAGTTGTGTGTACCGGTGGTTG	50
	AMB-00902548R	TCCATTATTGCGACTGCGCAGGAT	50
10	AMB-00674355F	AAGAAAGATCGACGCACGATTGGC	50
	AMB-00674355R	ATTGAATCGCGCGCGGAAAGAGAT	50
11	AMB-00640650F	CCGCGGATTCTTGATAGAGATACCGAA	48,1
	AMB-00640650R	GTTGAGGAAGATGGAGATGTTGAGG	48
12	AMB-00686140F	TCGGTTAATTTACGAACGAAATACATG	35,7
	AMB-00686140R	GCGATAACGTCCGAAATCGCAAATCC	48

The Real-Time PCR and HRM protocol applied in the study are given below. Amplification of the prepared samples was carried out in Biorad brand "Real-Time PCR thermal cycler" device. Step Temperature Time in PCR Cycle ; It was as follows 11 min of initial denaturation at 95 ^{0}C, followed by 40 cycles; each consisting of a denaturation segment 15 s at 94 ^{0}C, an annealing segment of 15 s at 57 ^{0}C, an extension segment of 20 s 72 ^{0}C and the final elongation was applied at 72 ^{0}C for 2 minutes. In the Melting (HRM) Analysis, the results were obtained with 65 ^{0}C > 95 ^{0}C and + 0.2 ^{0}C / s increments.

RESULTS AND DISCUSSION

Comparison of Caucasian bee and Mugla bee using SNP markers was analysed in terms of melting temperature value by RT-PCR. Real-Time PCR and HRM analyses, bee genotypes obtained from ORDU, Apiculture Research Institute were subjected to Real-Time PCR and HRM processes using twelve SNP primers. As a result of Real-Time PCR analysis, six primers did not amplify in either genotypes which performed four primary amplifications in the Caucasian bee genotype and six in the Mugla bee genotype. As a result of the HRM analysis, the temperature of the environment in which the samples were located was increased from 65 °C to 95 °C, with an increase of 0.2 °C per second. Amplicons deteriorated at different temperatures according to their A-T and G-C contents. As a result of this deterioration, the fluorescent dye contained in the amplicons was released and the machine detected the melting temperature of 29 °C when this dye irradiated.

Analysis results are shown in Table 2. The band size of 300 bp at primer 4 (Halloway et al., 2013) is displayed in Figure 1. The band size was similar to primer 9 and primer 10, but the results were not equal to the RT-PCR findings. In Figure 2 and Figure 3, the peaks formed as a result of the HRM analysis of the samples are seen. In the study, two honey bee breeds (Caucasian bee and Muğla bee) were analyzed using Real-Time PCR with twelve SNP primers that were previously evaluated for disease resistance in different breeds. This analysis was evaluated by the presence of both point mutations and electrophoretic bands.

Table 2. HRM value of amplificated primers by RT-PCR analysis.
Çizelge 2. RT-PCR analizi ile çoğaltılan primerlere ait HRM değerleri.

No	Amplificated Primers	Peak Value	Caucasian Honeybee Melting Temperature °C	Mugla Honeybee Peak Value	Mugla Honeybee Melting Temperature °C
1	AMB-00858574	1892,97	79,20	2048,56	79,60
2	AMB-00612262			**1436,55**	**81,20**
3	AMB-01151447	592,37	75,00	653,74	75,40
4	AMB-00631190	463,03	73,80	478,87	74,20
5	AMB-00674355			1046,80	75,40
6	AMB-00686140	783,86	71,20	514,82	71,00

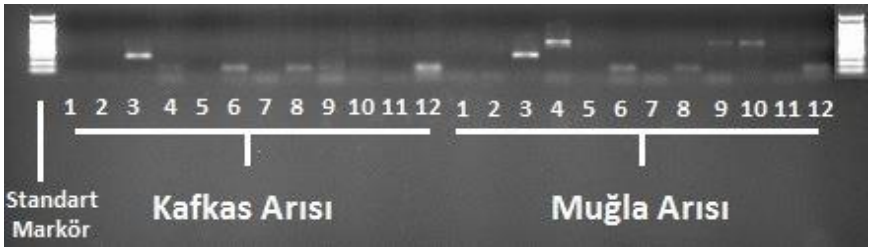

Figure 1. The band sizes of Mugla and Caucasian honeybees runned by twelve SNP primers
Şekil 1. Oniki SNP markörü ile test edilen Muğla ve Kafkas arısında band büyüklükleri

As a result of Real-Time PCR-HRM analysis, four (AMB-00858574, AMB-01151447, AMB-00631190, AMB-00686140) genotypes in Caucasian bees and six (AMB-00858574, AMB-00612262, AMB-01151447, AMB) bees in Mugla AMB-00631190, AMB-00674355, AMB-00686140) generated the primary amplicon. Six of the twelve primers (rs21784922, rs21784921, AMB-01118908, AMB-00902552, AMB-0064 0650, AMB-00902548) did not generate amplicons in either genotype. The peaks formed by the amplicons formed by four primers (AMB-00858574, AMB-01151447, AMB-00631190, AMB-00686140) in Caucasian and Mugla bees were similar, while two amplicons (AMB-00612262, AMB-00674355) were seen only in Mugla bees. This result is also supported by the electrophoresis analysis. In the analysis, twelve PCR products of both genotypes were carried out in 2% gel electrophoresis and results close to Real-Time PCR-HRM analysis were obtained. As a result of the analysis, five amplicons (AMB-00858574, AMB-00612262, AMB-01151447, AMB-00631190, AMB-00686140) formed bands in the Caucasian bee genotype. Seven amplicons (AMB-00858574, AMB-00612262, AMB-01151447, AMB-00631190, AMB-00902548, AMB-00674355, AMB-00686140) formed bands

in Mugla bee genotype. Four of these bands (AMB-00858574, AMB-01151447, AMB-00631190, AMB-00686140) are similar to the Caucasian bee genotype. The three bands (AMB-00612262, AMB-00902548, AMB-00674355) are different. According to the results of HRM performed with AMB-00612262 primer, its absence in Caucasian bees makes Mugla bee advantageous in terms of carrying the chalkbrood disease gene. Aronstein et al (2015) revealed a codominant relationship in the RFLP analysis of bands that appeared with the same primer. Accordingly, while only a single 300 bp RR-dominant genotype was seen in the DNA fragment cut with the EcorV restriction enzyme, the SS and RS genotypes gave 300 bp, 213 bp and 87 bp bands, respectively. Mugla bee genotype strengthens that it carries genes in dominant structure with a single band, and the absence of codominant melting structure also supports this.

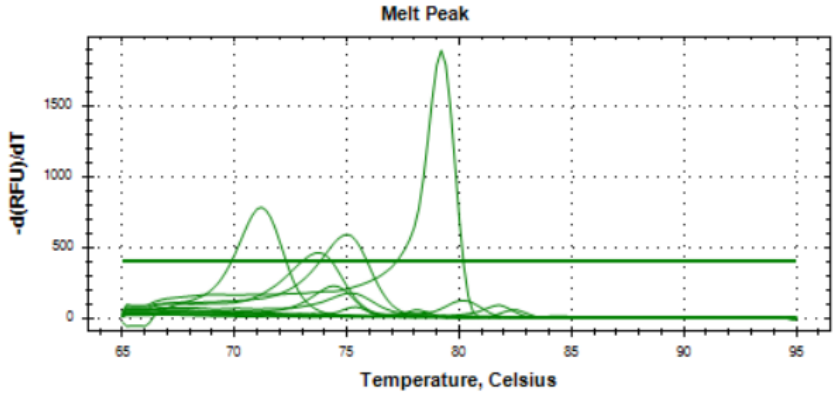

Figure 2a. HRM analysis of Caucasian Bee genotype by RT-PCR
Şekil 2a. RT-PCR ile Kafkas arı genotiplerinin HRM analizi

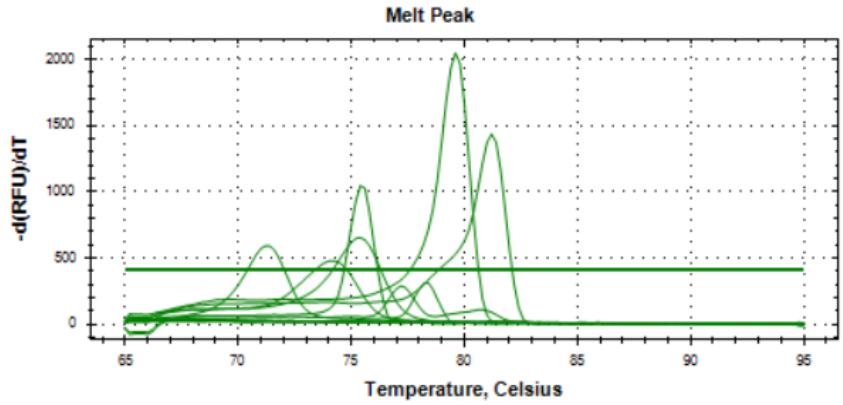

Figure 2b. HRM analysis of Mugla Bee Genotype by RT-PCR
Şekil 2b. RT-PCR ile Muğla arı genotipinin HRM analizi

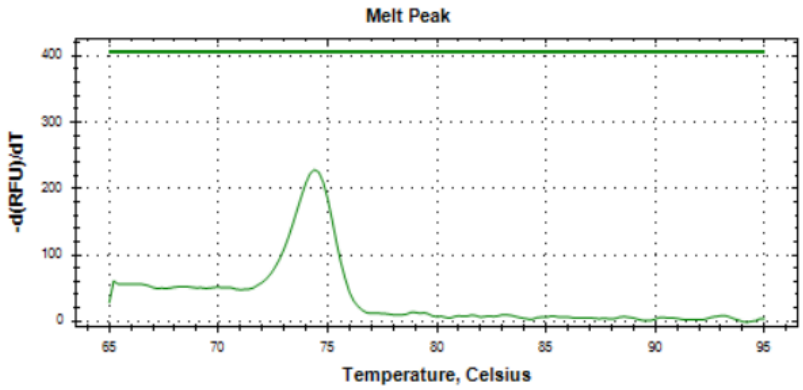

Figure 3a. HRM analysis for AMB-00612262 primers for Caucasian Bee Genotype.
Şekil 3a. Kafkas arı genotipinin AMB-00612262 primerine ait HRM analizi

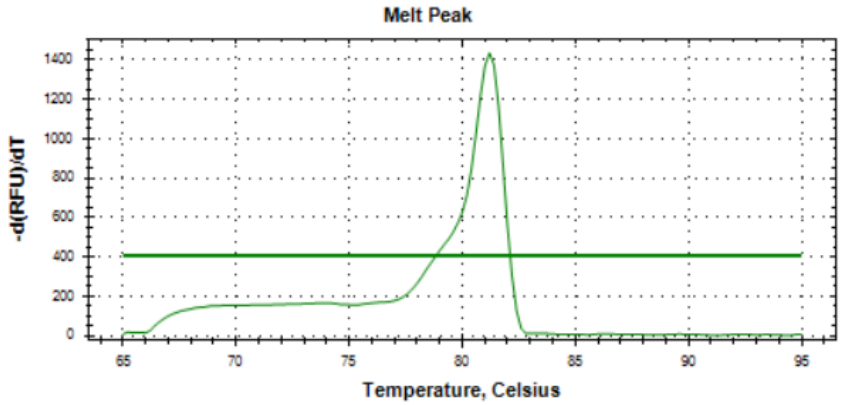

Figure 3b. HRM analysis for AMB-00612262 primers for Mugla bee genotype.
Figure 3b. Muğla arı genotipine ait AMB-00612262 primerine ait HRM analizi

In the study, no point mutations that would create an allele were found with the HRM analysis using the SNP marker. Holloway et al. (2012) investigated the relationship of SNP markers with genes providing resistance to lime disease in their study on various commercial bee populations, and as a result of their study, they found that ten SNPs were associated with these 40 genes and were informative about these genes. In this study; Five of the ten SNP sites were detected in Caucasian bees and seven in Mugla bees. Possibly occurring differences can be considered as deletion or deletion unless proven otherwise.

Chalkbrood disease in honey beee which is created by fungal patogene Ascosphaera apis is an economially damaging larval disease. Although SNP have been used to scan genetic diversity, resistance to pathogenes in many organisms there is no enough study on honey bee (Kongchum et al., 2010; Donalds et al., 2017; Elberts et al., 2018; Kosch et al., 2019). For genotyping immune genes in honeybee, Henriques et al., (2021) developed 91 quality-proved functional SNPs working 89 innate immune genes using the high-sample-thoughput IPLEX MassARRAY system but there was no chalkbrood disease genes.

Schork et al. (2000), as a result of their studies, reported that genotyping of hundreds of individuals can be done using SNP markers. It has been revealed that the SNP markers used in the study are an effective tool in the molecular identification of honey bee breeds. Han (2012) identified 15 honey bee breeds with SNP markers which used 1136 SNP markers in his study and reported that 1029 of these markers showed variation among all races and 40% of SNPs were polymorphic in each race. Pinto et al. (2014) investigated the variation in conserved European populations of *Apis mellifera mellifera* using 1183 SNP markers in their study. As a result of their study, they reported that the variation in protected populations was lower than that in unconserved populations. As a result of this study, it was revealed that three of the twelve primers

used (AMB-00612262, AMB-00902548, AMB-00674355) differ between races. With Real-Time PCR-HRM analysis, without the need for electrophoresis, molecular identification could be made with the help of graphics formed as a result of the analysis. In the light of these results, it was revealed that Real-Time PCR-HRM analysis should be studied in more detail in point mutations in such studies. With this type of differentiation technique, it can be suggested not only to distinguish between races, but also to understand how genetic contamination is from different races in the genetic structure of a population belonging to any honey bee race. Henriques et al. (2013) investigated the amount of crossing between *Apis mellifera luigustica* and *Apis mellifera carnica* honey bee breeds with *Apis mellifera mellifera* honey bee breed.

CONCLUSION

As a result of this study, it was suggested that SNP markers are the ideal marker method for similar studies. These markers can be used for a difference or similarity in the genotypebut their effect may not always manifest itself in the phenotype. However, molecular discrimination is absolutely necessary for the complete differentiation of two different populations or races. High Resolution Melting (HRM) analysis has been used as an accurate tool in disease diagnostics, species identification and SNP genotyping. It may be necessary to increase the number of character-specific SNP markers by sequencing for HRM analysis. In order to develop an effective identification analysis. By increasing the number of SNP markers, race-specific marker sets can be created to make this differentiation much more effective. SNP markers are also used as efficient markers in studies investigating the origin of Apis mellifera. Chapman et al. (2015) conducted a study of 95 SNP markers to quantify the African honey bee genome that originated in Africa. As a result of their studies, they reported that these 95 SNP markers can be used effectively in African honey bee origin studies . In this study, the association of single nucleotide polymorphisms to resistance to chalkbrood disease using RT-PCR for HRM analysis was conducted as two important honeybee breds that are Caucasian and Mugla bees. The results of SNP analysis using RT-PCR and HRM values reveals that there is to carry an potantial for detection of resistance to diseases as well as racial discrimination.

CONFLICT OF INTEREST

The author (s) declared that there are no conflicts of interest under this title.

DECLARATION OF AUTHOR CONTRIBUTION

The authors declared that there are no conflicts of interest under this title.

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