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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Relationship of SNPs in Octopamine and Tyramine Receptor Genes with Hygienic Behavior in Honey Bees and Their Effects on Breeding Process*

Bal Arılarında Oktopamin ve Tyramine Reseptör Genlerindeki SNP'lerin Hijyenik Davranışla İlişkisi ve Islah Sürecine Etkisi

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Abstract

Hygienic behavior in honey bees is a critical for maintaining colony health, preventing the spread of diseases, and providing resistance to harmful parasites. This behavior is defined as the worker bees detecting diseased, dead or parasitized brood cells and removing and cleaning them from the honeycomb cells. This feature, which is the defense mechanism of the colony, has developed on a genetic basis in natural selection and controlled breeding processes. Therefore, understanding the genetic mechanisms of hygienic behavior has become an important research subject in honey bee breeding studies. Recent studies have shown that neurotransmitter systems and receptor genes in honeybees affect various social and cognitive processes, including hygienic behavior. It is known that biogenic amines, especially octopamine and tyramine, regulate the learning, memory, olfactory perception, decision-making mechanisms and social behaviors of bees. Octopamine receptor and tyramine receptor can have a direct effect on stress response, flight activity, foraging behavior and hygienic behavior by acting as stimulants in the nervous system. In this study, in order to understand the genetic basis of hygienic behavior in Efe ecotype honey bees (*Apis mellifera anatoliaca*), colonies showing hygienic and non-hygienic behavior were determined according to the pin-killed test. Then, single nucleotide polymorphisms (SNPs) were detected in the AmOA1 and AmTYR1 gene regions of worker bees belonging to these colonies. As a result of the sequence analysis, 10 polymorphisms were determined in the AmOA1 receptor gene and 11 polymorphisms were determined in the AmTYR1 receptor gene. However, no significant difference was observed in the distribution of these SNPs between colonies showing hygienic and non-hygienic behavior. These results indicate that there is no direct relationship between hygienic behavior in Efe Bees and SNPs in AmOA1 and AmTYR1 genes. Therefore, no differences were detected between colonies in terms of SNPs. The polymorphisms have not been reported before in Efe Bee and this contributes to the originality of the study. More comprehensive studies to be conducted in the future will increase the knowledge in this field and contribute to the development of new strategies for selecting colonies in terms of hygienic behavior in the beekeeping industry.

Keywords: *Apis mellifera*, Biogenic amines, Colony resistance, Learning and memory, Neurotransmitters

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*This study was summarized from the Neslihan Özsoy's PhD thesis.

Öz

Bal arılarında hijyenik davranış, koloni sağlığının sürdürülmesi, hastalıkların yayılmasının önlenmesi ve zararlı parazitlere karşı direnç sağlanması açısından kritik bir öneme sahiptir. Bu davranış, işçi arıların hastalıklı, ölü veya parazitlenmiş yavru hücrelerini tespit ederek petek gözlerinden çıkarması ve temizlemesi olarak tanımlanmaktadır. Zararlılara karşı koloninin savunma mekanizmasını oluşturan bu özellik, doğal seçilim ve kontrollü ıslah süreçlerinde genetik bir temele dayanarak gelişmiştir. Bu nedenle, hijyenik davranışın genetik mekanizmalarının anlaşılması, bal arısı ıslah çalışmalarında önemli bir araştırma konusu haline gelmiştir. Son yıllarda yapılan çalışmalar, bal arılarındaki nörotransmitter sistemlerinin ve reseptör genlerinin, hijyenik davranış da dahil olmak üzere çeşitli sosyal ve bilişsel süreçleri etkilediğini göstermektedir. Özellikle oktopamin ve tyramin gibi biyojenik aminlerin, arıların öğrenme, hafıza, koku algısı, karar verme mekanizmaları ve sosyal davranışlarını düzenlediği bilinmektedir. Oktopamin reseptörü ve tyramine reseptörü, sinir sisteminde uyarıcı işlev görerek stres yanıtı, uçuş aktivitesi, besin arama davranışı ve hijyenik davranış üzerinde doğrudan etkiye sahip olabilmektedir. Bu çalışmada, Efe ekotipi bal arılarında (*Apis mellifera anatoliaca*) hijyenik davranışın genetik temellerini anlamak amacıyla pin-killed testi uygulanarak hijyenik davranış gösteren ve hijyenik davranış göstermeyen koloniler belirlenmiştir. Ardından, bu kolonilere ait işçi arıların AmOA1 ve AmTYR1 gen bölgelerinde tek nükleotid polimorfizmleri (SNP) tespit edilmeye çalışılmıştır. Dizi analizi sonucunda, AmOA1 reseptör geninde toplam 10 polimorfizm ve AmTYR1 reseptör geninde 11 polimorfizm belirlenmiştir. Ancak, yüksek ve düşük hijyenik davranış gösteren koloniler arasında bu SNP'lerin dağılımında belirgin bir fark gözlenmemiştir. Bu sonuçlar, Efe Arısı’nda hijyenik davranış ile AmOA1 ve AmTYR1 genlerindeki SNP’ler arasında doğrudan bir ilişki olmadığına işaret etmektedir. Daha önce Efe Arısı'nda bu genlerde herhangi bir polimorfizm rapor edilmemiş olması, bu çalışmanın özgün bir katkı sunduğunu göstermektedir. Gelecekte gerçekleştirilecek daha kapsamlı genomik çalışmalar, bu alandaki bilgi birikimini artırarak arıcılık sektöründe hijyenik davranışı yönünden kolonilerin seçilmesi için yeni stratejiler geliştirilmesine katkı sağlayacaktır.

Anahtar Kelimeler: *Apis mellifera*, Biyojenik aminler, Koloni direnci, Öğrenme ve hafıza, Nörotransmitterler

1. Introduction

Insect sensory organs convert mechanical, chemical and light energy coming from the environment into electrical energy of nerve impulses in sensory neurons, and from there the information is transmitted to the brain or ventral nerve cord and causes behavioral responses (Farooqui, 2007). Therefore, a stimulus must be present in the colony for hygienic behavior to occur and that worker bees that are genetically predisposed to hygienic behavior can perform this behavior in the presence of the stimulus (Arathi et al., 2006). For this purpose, the neural processes that occur in worker bees will perceive and respond to stimuli from the environment (Morfin et al., 2019). Therefore, a stimulus must be present in the colony for hygienic behavior to occur and worker bees that are genetically predisposed to hygienic behavior can perform this behavior in the presence of a stimulus (Arathi et al., 2006). For this purpose, the neural processes that occur in worker bees will perceive and respond to stimuli from the environment (Morfin et al., 2019). In this regard, the antennas of honey bees play a very important role in hygienic behavior (McAfee et al., 2017). The olfactory system in honeybees consists of antennae, antennal lobes, and mushroom bodies (MBs), and that ~60,000 olfactory sensory neurons (OSNs) are located within cuticle-covered sensilla along the antennae. Honey bees detect odors and tastes using their antennas and the olfactory receptors located in the OSNs on their antennas (Spivak and Danka, 2021) and worker bees showing hygienic behavior also identify diseased or dead larvae and pupae using olfactory cues (Masterman et al., 2000; Gramacho and Spivak, 2003; Spivak et al., 2003; Arathi et al., 2006; Goode et al., 2006; Bienefeld et al., 2015; Rasolofoarivao et al., 2015; Scannapieco et al., 2017; McAfee et al., 2018; Morfin et al., 2019; Wagoner et al., 2020; Lee et al., 2020; Spivak and Danka, 2021). Hygienic behavior has been defined as the ability of worker bees to detect diseased and dead brood, open these brood and remove them from the cells (Rothenbuhler, 1964; Arechavaleta-Velasco et al., 2011). Worker bees showing hygienic behavior remove diseased brood from the colony before the pathogen reaches the infectious stage (Arathi et al., 2006; Swansan et al., 2009). Bacterial, viral, fungal and parasitic factors are the most important causes of colony losses (Bozdeveci et al., 2024), and colonies with hygienic behavior are resistant to many brood and bee diseases such as American foulbrood (Spivak and Reuter, 2001; Gramacho and Spivak, 2003; Arathi et al., 2006; Lazarov et al., 2020; Lee et al., 2020), chalk brood (Cause: *Ascosphaera apis*) (Gramacho and Spivak, 2003; Arathi et al., 2006; Lazarov et al., 2020; Invernizzi et al., 2011), Varroa (Spivak, 1996; Gramacho and Spivak, 2003; Arathi et al., 2006; Pinto et al., 2012; Ji et al., 2014; Lazarov et al., 2020; Wielewski et al., 2012).

Honey bee behaviors are either under the control or modulation of biogenic amines (Thamm et al., 2017). Biogenic amine receptors are densely expressed in different parts of the bee brain, especially in mushroom bodies (Peng et al., 2021). Octopamine and Tyramine are biogenic monoamines that function as neurohormones, neuromodulators, and neurotransmitters in invertebrates (Ali et al., 2012; Kastner et al., 2014; Braza et al., 2019) and modulate many important physiological processes (Kastner et al., 2014).

Octopamine receptors have been classified as α1-adrenergic-like receptors (Octα1-R, or OA1), α2-adrenergic-like receptors (Octα2R, OA3), β-adrenergic-like receptors (Octβ-R, OA2: Octβ1-R, Octβ2-R, Octβ3-R) (Finetti et al., 2021). It has been reported that octopamine effective in in increasing arousal levels (Sinakevitch et al., 2011), stimuli-increased food consumption behavior (Scheiner et al., 2006), in regulation of forage behavior (Aonuma and Watanabe, 2012), aggression (Sinakevitch et al., 2011), control of locomotion (Sinakevitch et al., 2011; Li et al., 2016), preparing the insect for high-energy actions (Schendzielorz et al., 2015), recognizing nestmates (Barron et al., 2007; Duportets et al., 2010; Sinakevitch et al., 2011; Dimić et al., 2020), walking, mating (Ali et al., 2012), laying eggs (Li et al., 2016; Xu et al., 2020), stinging behavior (Molaei et al., 2005; Farooqui and Farooqui, 2010; Ali et al., 2012), memory formation (Pflüger and Stevenson, 2005; Duportets et al., 2010; Farooqui and Farooqui 2010; Schendzielorz et al., 2015; Blenau and Baumann 2016; Wallberg et al., 2017), tongue (proboscis) extension, juvenile hormone release from corpora allata (Molaei et al., 2005), muscular systems, endocrine tissues (Bischof and Enan, 2004), and learning (Bischof and Enan, 2004; Pflüger and Stevenson, 2005; Scheiner et al., 2006; Schendzielorz et al., 2015; Blenau and Baumann, 2016; Dimić et al., 2020).

Octopamine also plays a role in hygienic behavior (Barron et al., 2007; Sinakevitch et al., 2011). In hygienic honey bees, the brain protocerebral neurons (cluster-3 cells) have significantly higher levels of Octopamine immunoreactivity, which is linked to the ability to detect diseased brood odors (Goode et al., 2006). Hygienic bees that detect diseased or dead brood open the cells and remove the affected larvae or pupae from the colony under the influence of neuromodulators such as Octopamine (Spivak et al., 2003; Morfin et al., 2019).

Tyramine receptors are classified as TAR1 (or TYR1), TAR2 (or TYR2) and TAR3 (or TYR3) (Finetti et al., 2021). Tyramine has been shown to play a role in learning (Mustard et al., 2005; Guo et al., 2018; Braza et al., 2019; Wang et al., 2020), memory (Guo et al., 2018; Braza et al., 2019; Wang et al., 2020), behavior, energy metabolism (Guo et al., 2018; Braza et al., 2019), sucrose response (Guo et al., 2018; Thamm et al., 2021), reproductive physiology (Lange, 2009; Guo et al., 2018), olfaction (Lange, 2009; Ishida and Ozaki, 2011; Gainetdinov et al., 2018), muscle contraction in insects. (Enan, 2005; Ishida and Ozaki, 2011), insect movement (Lange, 2009; Gainetdinov et. al., 2018; Braza et. al., 2019), pheromone response (Braza et. al., 2019), brain development (Ji et. al., 2016), flight behavior, food search and taste perception (Gainetdinov et. al., 2018). It has been emphasized that Tyramine affects general odor-sensitive ORNs (Olfactory Receptor Neurons) (Zhukovskaya and Polyanovsky, 2017). Tyramine is involved in appetite regulation (Brigaud et al., 2009) and increases behavioral responses to antennal stimulation using taste stimuli (Brigaud et al., 2009; Lange, 2009) and sugar sensitivity (Behrends and Scheiner, 2012), and stimulates ovary development in worker bees (Lange, 2009).

Genetic studies have determined that 73 genes are related to hygienic behavior and these genes are generally related to neuronal development and sensory perception in insects (Harpur et al., 2019). Octopamine receptor, smell-impaired, odorant binding protein (OBP) 3 and OBP 4 genes are expressed differently between colonies that show hygienic and non-hygienic behavior and may be effective in the perception of diseased odors by worker bees that show hygienic behavior (Scannapieco et al., 2017). It has been determined that 4 SNPs (Adenosine receptor and Cyclin-dependent kinase 5 activator, Octopamine receptor β-2R and OBP 1) are responsible for the detection and opening of Varroa-infected brood cells by honey bees (Spötter et al., 2016).

The goal of the present study was to determine the SNPs of "Octopamine Receptor" and planned to investigate the possibilities of these receptor on use in hygienic behavior breeding in breeder Efe Bee colonies in Aegean Agricultural Research Institute (AARI).

2. Materials and Methods

2.1. Honey bee colonies

The decision regarding the registration of the Efe Bee, which was registered as a result of the breeding studies carried out by the AARI-Beekeeping Research Center between 2008-2018, was published in the Official Gazette dated 16 May 2019 and numbered 30776 in Türkiye. Among 102 breeder Efe Bee colonies, bees were collected from the 5 colonies with the hygienic behavior and the 5 colonies with the non-hygienic behavior and analyzed. Analyses were performed on a total of 60 bee samples.

2.2. Rearing of queen bees

Queen bees were produced from the breeding hives located in AARI apiary in İzmir by the larvae transfer (doolittle) method reported by Laidlaw (1979). The main breeding colonies of Efe Bee are located in Bozcaada and the region is the protection zone of Efe Bee and no other bees are allowed from outside. In order to create breeding queens, the produced queens were taken from the nucleus hives and taken to the AARI apiary in Bozcaada to be mated. After the matings were completed, the nucleus hives were brought to AARI (İzmir) and the fertile breeder queens were transferred to production hives consisting of Langstroth type hives.

2.3. Hygienic behavior tests

Hygienic behavior tests were performed 2 months after the queens were transferred to the Langstroth type hives (between August 10 and September 22). Tests were performed adapted to the pin-killed method reported by Büchler et al., (2013). Accordingly, a pupal area was determined in one frame in each hive and 100 brood (containing pupa) cells in this determined area were punctured with a needle and the cleaning status of these cells at 24 hours was treatment and the average was calculated. Tests were performed 3 times with a 21-day interval and 5 colonies with the hygienic behavior (≥95%; Guzman et al., 2002) and 5 colonies with the non-hygienic behavior were determined. The percentage of hygienic behavior was calculated according to the method reported by Palacio et al., (2000).

Kim et al., (2019) reported that hygienic behavior is generally performed by adult worker bees that are 15-17 days old. In this regard, as Boutin et al., (2015) stated, worker honey bee samples were taken the day following the hygienic test and only the bees in the brood frame where the hygienic test was performed were sampled. The samples were collected in tubes and placed in a liquid nitrogen tank in the field where the colonies were located until they were stored at -80 °C and immediately taken to the -80 °C freezer in the AARI - Apiculture Research Center after the study in the field was completed.

2.4. Genetic analysis

RNA isolation, cDNA synthesis and PCR analyses were performed at Afyon Kocatepe University - Faculty of Veterinary Medicine, and SNP analyses were performed in a private laboratory in Eskişehir. Worker bee heads were used for RNA isolation. RNA isolation was performed using a commercial kit (TRIzol™ Reagent, Thermo Fisher Scientific, 15596026) according to the protocol recommended by the company. The isolated total RNAs were measured in terms of quality and quantity using both a Nanodrop spectrophotometer (Thermo Fisher Scientific) and a Qubit 2.0 Fluorometer (Invitrogen) using the Qubit RNA Analysis Kit. Those with an RNA DNA^{-1} ratio of 1.8 and above were used in the study.

The primers for the Octopamine receptor gene (AmOA1) and Tyramine receptor gene (AmTYR1) used in the study were designed using the reference mRNA sequence (NM_001011565.1 and NM_001011594.1) obtained from NCBI and the FastPCR Professional 6.1.2 package program (Kalendar et al., 2009). The formation of dimer and hairpin between the primers was checked with the same program and primers that did not form dimer/hairpin were selected. The primers used in the study and the length of the amplified regions are given in the table below (*Table 1*).

Table 1. Forward and reverse primers of octopamine receptor gene and tyramine receptor gene

Gene	Forward primer (5'→3')		Reverse primer (5'→3')		Product size	Tm
Octopamine receptor gene (AmOA1)	F1	taattggacaggacgagggga	R1	ctatccaggctgatggcgcac	1536	60
	F2	tttgtgcgccatcagcctgga	R2	cgcactcgattctcgtcgtgc	1428	60
Tyramine receptor gene (AmTYR1)	F3	gctcgaacagagaagactgga	R3	gccgagtagtgtcccgatgac	1367	59
	F4	gggaaacaagatttgccgccga	R4	tgacggacactctgcccggaa	1452	61
	F5	ttgatgtcggctgcttgcttcc	R5	ctctccttccctcaaacgcga	1379	60

Gradient PCR was applied to determine the annealing temperatures of the primers. For this purpose, a PCR mixture was prepared consisting of 1xPCR buffer, 2mM MgCl2, 0.2 mM dNTPs, 0.3 pmol forward primer, 0.3 pmol reverse primer, 1 U Platinium Taq polymerase (ThermoFisher, 10966034) and 20 ng μL^{-1} cDNA. 10 PCR product tubes representing 10 colonies obtained as a result of PCR were taken to the agarose gel electrophoresis stage. After the running process was completed, the gel was visualized with the VisionCapt (Bio-Vision, Vilber Lourmat) gel imaging system.

DNA sequence analysis was performed to determine the base sequence and SNPs of the PCR products. After DNA sequence analysis, the results were rearranged according to the reference sequence using the Sequencher 5.6.1 package program. Then, all samples were aligned using the BioEdit program (Hall, 1999) and polymorphic SNPs and inserted and deleted regions were determined.

3. Results and Discussion

3.1. Hygienic behavior tests

A total of 3 hygienic behavior tests were performed on 102 breeder Efe Bee colonies every 21 days. According to the test results, a total of 5 hygienic behavior colonies and 5 non-hygienic behavior colonies were determined (*Table 2*).

3.2. Genetic analysis

The bands of the primers octopamine F2-R2 (1428bp) and tyramine F3-R3 (1367bp) were detected, but the bands of the primers octopamine F1-R1 (1536bp), tyramine F4-R4 (1452bp) and tyramine F5-R5 (1379bp) were not detected. The gel images obtained as a result of PCR are shown below (*Figure 1* and *2*). In octopamine F2-R2 and tyramine F3-R3 the bands which the image was detected were evaluated as positive, and the bands which the image was not detected were evaluated as negative. Accordingly, all images (10 images) were obtained as positive in F2-R2 (*Figure 1*) and 9 images were obtained as positive in tyramine F3-R3 (*Figure 2*).

Table 2. Selected hives based on hygienic behavior measurement results

Hive number	Percentage of hygienic behavior	Number of bees taken for genetic analysis	Hive number	Percentage of hygienic behavior	Number of bees taken for genetic analysis
Hygienic behavior			**Non-hygienic behavior**		
101-1-Y	100%	6	123-2-D	73%	6
128-2-Y	100%	6	232-3-D	69%	6
218-2-Y	100%	6	160-2-D	68%	6
172-2-Y	99%	6	185-1-D	67%	6
156-2-Y	98%	6	245-1-D	63%	6
Total		**30**	**Total**		**30**

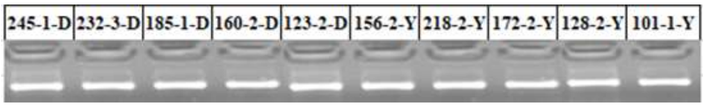


Figure 1. PCR image of octopamine F2-R2

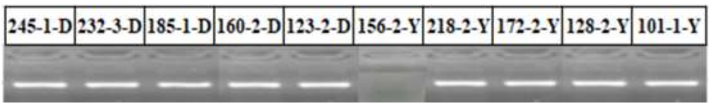


Figure 2. PCR image of tyramine F3-R3

Figure 3 shows the graphic sequence of the AmOA1 (F2-R2 primer) gene. The grey area in the figure is the 5'UTR region. The yellow part is the region where the exons are located, and the brown bases represent the 3'UTR region. Those written in red indicate that there is a mutation or polymorphism. When the figure is examined, it is seen that there are 10 polymorphisms and in 1 sample showing hygienic behavior, a 40-base region was added at base 2291.

The first polymorphism occurred at base 1725 of exon 4 and adenine converted to guanine. This resulted in an amino acid change, caused the conversion of threonine to alanine. Guanine converted to adenine in exon 6, at base 1961 and cytosine converted to thymine in exon 6, at base 2126 and guanine converted to adenine in exon 6, at base 2153 and guanine converted to adenine in exon 7, at base 2351 and thymine converted to cytosine in exon 8, at base 2552 and guanine converted to adenine in exon 8, at base 2606 and cytosine converted to thymine in exon 9, at base 2734. These created a silent SNP. Adenine converted to guanine at base 2843, but this occurred in the 3'UTR region. A 40-base section (CCCAGAAGACCCTAGCAGGAGGAACAGCTGCGAGAGTCCA) was added at base 2291 in exon 7 in one sample showing hygienic behavior. Since the addition of this section can cause a change in the protein sequence, it is thought that it can cause phenotypic changes (*Figure 4* and *5*).

Figure 6 shows the graphic sequence of the AmTYR1 (F3-R3) gene. In the tyramine receptor, 1 exon could be sequenced and all SNPs are located in exon 1. The grey area in the figure is the 5'UTR region. The yellow part is the region where the exons are located, and the brown bases represent the 3'UTR region. Those written in red indicate that there is a mutation or polymorphism. When the figure is examined, it is seen that there are 11 polymorphisms. There is a 3-base deletion starting from base 103 in the 5'UTR region.

The first polymorphism occurred at base103 with a thymine-guanine-adenine deletion, but this deletion is located in the 5'UTR region. At the 158 base, the cytosine is converted to guanine. This resulted in an amino acid change, caused the conversion of aspartic acid to glutamic acid. At the 207 base, the guanine base is converted to adenine. This resulted in an amino acid change, caused the conversion of Valine to Isoleucine. Cytosine converted to guanine at base 218, guanine converted to cytosine at base 248, cytosine converted to thymine at base 467, cytosine converted to thymine at base 956, guanine converted to adenine at base 1133, cytosine converted to thymine at base 1187, cytosine converted to thymine at base 1063, adenine converted to guanine at base 1133, cytosine converted to thymine at base 1187, guanine converted to adenine at base1211 and guanine converted to adenine at base 1277. These created a silent SNP (*Figure 7*).

GAAATAATTGGACAGGACGAGGGGAGGGGCGGTAATAGAAATTCAATCCCGGAACTCGAAAATTTCTATCT
ATCCTCGACGGATAATAATTTCGAGGAATTTATCAACACGCTCTTCGAAGCGTGAAAACGGGAGGGATTCTT
GTATTCCTTTCGGCAGGAAATGAAGAAACTTTTCTTCTGGAAAACCGGTTATCGAATACTGATATTTCCAGG
TGTATCTATCCCCAGTGCTTTCTATTCTCGAAACAAAGAAAAAGGGGTTCTCGGATCGAGAATATTTCTCCA
TTTCGTATGGAGAACGTTCTTGGACACGTGATATATCCTGAATCAAGTGGACGAGTCAAATTTGTGTTTAAG
ACAGTGTGAAATATTGTTTAAATTTTAAGGACGATGCGAGGAAGAGGACGGTGAAAATAATTTTTTGGTGA
TGATAAATAAAGGAAGGTTGAGATAATTATAGACCGATTTCGAATTGAATGAGGAGTGAACGAGACAATAA
GGAAAATCAGACGACGAAAGTGAAAAAGAAAAGGAAAGGATGGCGTTTGTCGAGGATGAATGGTAAATCT
CGAAGGTGCTAATAGTTTTAATTGTTCTTTTCTGGAACGTTTTGGAGGAGGATTGAACGAGGTGCATGATTC
GAAGCTAATTTTATCATTCATCATTTAAAAATTTCCTCTGAACATCGATCAGTTGTTTTATAATTCTCTTAAT
CTCCGATTATAAGCTGAATCCAAAAAGAAAAAATCAAGAAGAACCTAACCTCTAAATCTAACCTCAAAAAA
CTTGCATGAAACAATCGAGCAAAAATAATTGATCAAACACGTGTAAAAATAAGGAAGCTGCGAAACGAAC
CTAACCTCAAAATAAAAAGAACAGTATCTTCCTCCAAAGACCAAACAAAACGAATAGAACCTAACCTCAAA
AACCTAATTAAAAATTCCTGAACGAACAAAGATATACAAAGAAGAGAGAAAAGAATCGATCAACGATGAT
CTCAGGATTTGCAAAGGATCGATGATCGAATCGTTGCCGTGATGGAGGCGATCCATGCGATCCGTATTCGTC
GCTTTTCTTCCAGTGAAATCGTTTCGCGAGAGTCGTTGTTCGGTGCGTTGTTCGGCAGCAAGCGGTCTGAGA
TGGTTCGAGATTTGGCGCGACAGCTTGCCAACTAAAATGCGAGAGCTGAACGCGACCGCATGTGCTGCCCT
TTACGAGCGCGTCGAGTGGTCCGGACCGTGGATCCTGGTTACCCTGATCGTGCTCGCGATAGTGAACGTGAT
GGTGGTGCTGGGCAACGTGCTCGTGATACTCGCTGTCTATCACACGAGCAAGCTGCGAAACGTGACGAATA
TGTTCATCGTTAGCCTCGCGGTGGCGGATCTGATGGTCGGCCTGGCTGTCCTTCCGTTTAGCGCTACGTGGG
AGGTTTTCAAGGTGTGGATATTCGGTGATCTATGGTGCTCCATCTGGCTGGCGGTCGACGTATGGATGTGCA
CCGCGTCGATATTGAATTTGTGCGCCATCAGCCTGGATAGATACTTAGCTGTGACCAGGCCAGTCAGTTATC
CTCAGATCATGTCGCCGAGGAGAGCAAGGCTGCTGGTTGCAACCGTGTGGATCTTGAGCTTCGTCATCTGCT
TCCCACCCCTGGTCGGCTGGAAAGACAAACGGTCTCATCCCGCGTACAACATGACGTTTGCTCAAAACGGA
CCGTTCAAC**[c.1725A>G]**CCACCACCATCTTCGTCCCCGTGAAACCGTGTCCTTGGATCTGCGAGCTGACCAA
CGATGCCGGCTACGTCGTTTATAGCGCTCTTGGTTCTTTCTATATACCGATGTTGGTCATGCTGTTTTTCTACT
GGAGGATCTATAACGCGGCCGTCTCCACCACAAAGGCTATTAATCAAGGCTTCCGCACGACAAAGAGTTCG
AAAATGTTTGGCTCTAGATTCGACGAAGAGAGGTT**[c.1961G>A]**ACCTTGAGGATCCATCGTGGCCGAGGAA
GCGTGCACAATGGGAGCAACAACGGAAGTCCGAGGAGCCCCGAGTCGAACAGTCGGTGCTCGGTGAAAAG
GGAGAAGATAAAAATCTCGGTGTCGTATCCGAGCACGGAGACGTTGAATACAAAGTGTAACACCCT**[c.2126**
C>T]GAAAGAACGCCATCCAAGTGTTCTCA**[c.2153G>A]**ACCTCTGTGCATTACAGCAATGGGCAGACGCACA
GCCAATTGTGTCCAACCCCCAGAAGCACTCATTTAAAGGTGAGCGGCATCAACAGGGTTGGAAGCACCAGA
AGACCTAGCAGGAGGAACAGCTGCGAGAGTCA**[c.2291Ins>40bp]**ATGATGGGTGATGAGATGTCGTTGCGGG
AGCTCACTCAAGTCACCGAGGAGAAGCCAAG**[c.2351G>A]**GTGATGAAGATGGGGAAGAGAAATATTAAAG
CTCAGGTGAAGAGATTTCGAATGGAGACGAAGGCGGCGAAGACACTGGGCATCATCGTCGGCGGGTTCATC
CTCTGCTGGCTTCCTTTCTTCACAATGTACTTGGTGCGCGCCTTTTGCCGCAACTGCATCCACCCCACTGTGT
TTAGCGTACTGTTTTGGCTAGGCTA**[c.2552T>C]**TGCAACTCCGCAATAAATCCGTGTATCTACGCGCTGTTTA
GCAAGGACTTCCG**[c.2606G>A]**TTCGCGTTCAAAAGTATCATCTGCAAGTGCTTCTGCAAACGGCGGACGAA
CACTTTGAGACGCGGCAGCGATGGAAGTCAATTAGCGATGAGAAACGATCGGAGCCCAAGCTACTCGATGC
AAGTTC**[c.2734C>T]**CCAACAGGGGGCGTCCATCGACGACTCGGACCCGGACCCAAGCTCAGAACCGACTGT
GCATTCGCAGAGCGAGTCGAGATGACTGTAGCGTGCCAGGTGTGGCGCTCA**[c.2843A>G]**CGCGTCACCTTC
GATCCTAGAACCTCGAAAGTGAGGATCCAATCTTTCTAAGGATTTCACGTTTCGTGCCGCGAAATCGAGCAC
GACGAGAATCGAGTGCGAAAGGATGGACGTGGAATTCGCGACAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAACTCGAGGGG

Figure 3. Graphic sequence of the AmOA1 (F2-R2 primer) gene

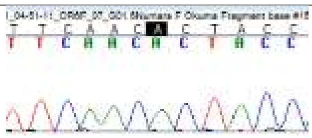
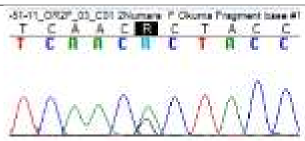
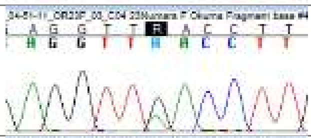
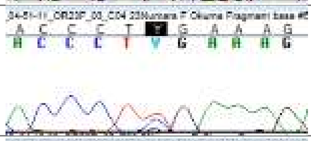
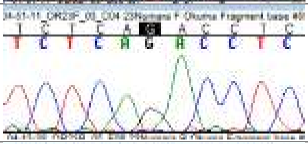
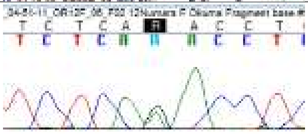
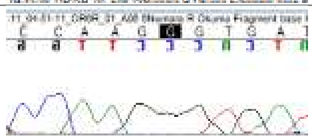
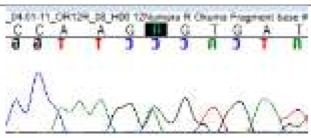
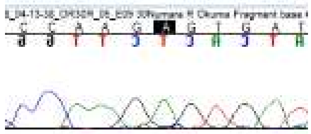
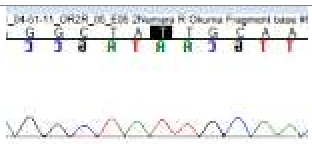
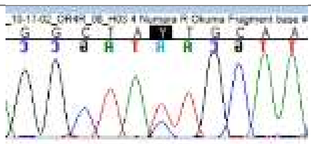
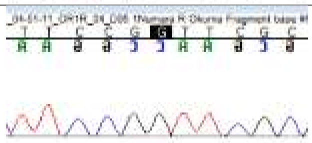
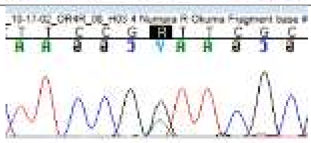
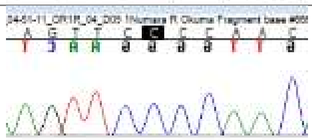
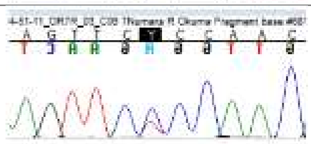
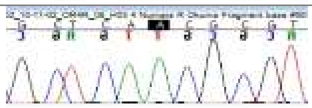
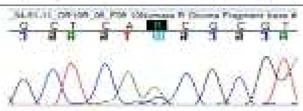
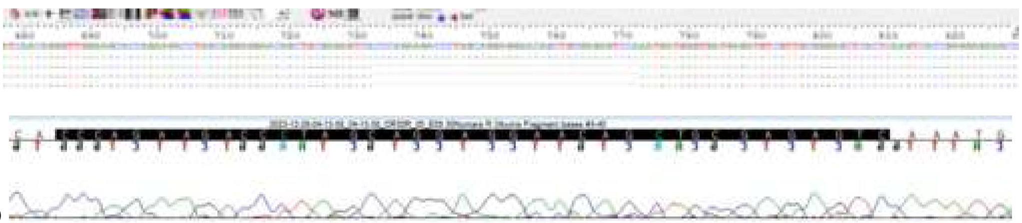
SNP	Chromatogram	Chromatogram	Chromatogram	Amino Acid
c.1725 A>G				Treonin > Alanin
c.1961 G>A				Silent
c.2126 C>T				Silent
c.2153 G>A				Silent
c. 2351 G>A				Silent
c.2552 T>C				Silent
c.2606 G>A				Silent
c.2734 C>T				Silent
c.2843 A>G				3'UTR

Figure 4. Chromatogram image of SNPs in the AmOA1 (F2-R2 primer) gene

(a)



(b)

Figure 5. Added bases in one sample but not in the other (a) and chromatogram image (b) (c.2291 Ins >40bp)

CAAAAATGTGACGCTCGGCGTGCGCTCGACGGGCCGGGGCTCGAACAGAGAAGACTGGACGACTATTGGGAGCGTGACG
TTGGATTGACGAGAGCGGGCTCG**[c.103-105TGA>del]** ACAACATGAACTCGAGCGGGGAA TCAG GC GGGA CGATGAC
CGAGGACTA CGA**[c.158C>G]**ATGACAGGCTGCGGCCCGCCGGAAGAGGAA ACGGGC TCGAAC
TTGCCG**[c.207G>A]**TTTGGGAAGC**[c.218C>G]**GCGGCCGCCTCATTGACGCTTGGCTTC
CT**[c.248G>C]**GTTCTCGCGACGGTGTTGGGGAACGCGCTCGTGATCCTGAGCGTGTTCACGTACAGGCCCCTTCGTATCGTT
CAGAACTTCTTTATCGTGTCGCTAGCTGTCGCCGATCTCGCGGTCGCGATCCTCGTGATGCCGTTCAACGTCGCCTACCTCC
TCCTCGGCAAGTGGATCTTCGGCATACACCTGTGCAAATTGTGGCTAACCTGCGACGTGCTCTG**[c.467C>T]**TGCACGGCCA
GCATACTGAATCTGTGCGCCATCGCCCTCGACCGTTACTGGGCGATCACCGATCCCATCAATTACGCCCAGAAACGTACCC
TGAAGAGAGTACTGGCCACGATAGCCGGGGTGTGGATCCTGTCGGGGGCGATCAGCTCGCCCCCCCTGGCCGGTTGGAAC
GACTGGCCCGAGGAGTTGGAACCGGGCACACCCTGCCAATTGACCAGGAGGCAGGGATACGTCATCTACTCGTCGCTCGG
CTCCTTCTTCATCCCGTTGCTGTTGATGAGCCTCGTCTACCTCGAGATATATCTGGCCACGAGGAGACGGTTGAGGGAGCG
GGCCAGGCAGAGCAGGATAAACGCTGTCCAGTCGACGAGGCATCGCGAGGCGGACGACGCCGAGGAGTCGGTCAGCTCG
GAGACGAATCATAACGAGAGGTCGACCCCTCGGTCGCACGCCAAGCCGTCGTTGATCGACGACGAGCCGACCGAGGT**[c.95**
6C>T]ACGATAGGGGGTGGCGGTACCACGTCCTCGAGGCGGACGACGGGGAGCAGGGCAGCCGCGACCACGACAACGGTT
TACCAATTCATCGAGGAGAGGCAGAGGATCT**[c.1063C>T]**GTTGTCGAAGGAGAGACGGGCCGCGAGGACATTGGGCGTGA
TAATGGGCGTGTTCGTCGTATGCTGGTT**[c.1133A>G]**CCATTCTTTCTGATGTACGTGATCGTCCCGTTCTGCCCCGATTGTT
GCCCCTC**[c.1187C>T]**GATCGTATGGTCTACTTCATCAC**[c.1211G>A]**TGGCTCGGTTACGTGAACAGCGCCCTCAACCCGCTC
ATCTACACCATCTTCAACCTCGACTACAG**[c.1277G>A]**AGGGCATTCAGACGGTTGTTGCGCATTCGTTGAACACTTGTTGTT
GGCAACGCTTGTTTATTGCATCTATCGCGAAATTGTGGGAAACAAGATTTGCCGCCGAAATCGTCATCGGGACACTACTCG
GCCCCGCTTGGGATTGGTCGGCGAGGAAGGAAAGAGGATTGGAGTTCGGCTGAGTTTTTCGAGGAAGCGGCACCGAGCTT
GGCACGATTCAAATTCTTTCGAAGGAGGAAACGAGAGGGAAAAAATATGAAGAGTTTATTTTCGCGGAACGAAGAAGAA
GCGTCTAAAATATGGCGGTGGATTATTCGTAGAAACAAAGAGAGAGTTCGATGGAAGATTAAGCGATCAAAGGTCGAATA
CACGATAGTCTAATTTAGCGACGACGATGTTCACTCCCGGAAGAGTCGTTCCATTAAACTTGTTCCATTAATCCTGCTAGG
GAAGCGAGGGAGCGCGGATAAGGAATTTTAAGGAAATGAAGTCGCCGCCCCTGCTCGATTGTCCTATACCTCATTTCATCC
GTCGTTACGCGTTGAGTTCAGCTGAACGATAAATTCACCGCTATCTTCGATCGGTCATGAATAATGGAAATCGGTGGATTC
GATCGTCGCTACTCGGTCGTCTCGACTCGAACAAGACCGTAAACCGAAGACCATCGAATGAATTGAGCGGAGGGGAGGGG
AGGGTTGTAGTGGAAGAGGCGTCGTGTAAAAATTCGATTTCGACGCCGAGTCGATCCGAAGTCGTTCCATTTCCTAAGGA
GCCTTGTAAAATATTGCTCTCCCGAGAATTCGAATATTCTTGATGAATCGATATCGAATATGGCGAGCCTTTTTTAAATTTC
TTTCGCCCTTGCTTCCACCTCTTTCTTTTTATCCATAAGAGATAGGATGGGGTTAGGTTAGGTTAAGCTGTTGCGTATGAAA
TGCGCCAAATTTCCTGAAAATTCACTGGAATTATTAATTTGACATTTTAGAAAGGAAAAAAAGAATCTGTCTCATTGGTAC
AAAAAAATTTAAGTTACAAAGCTCGATCGAGAATTCGATAATTTCATTTGCAACAGCCTAATTCAAACATAAGATGGAAA
AGAGAAAAAAGGAAAAAGAAGAACTCTTCTCGTCGCCAATCTCAAAAAAGCCGAGCAGAGAAATTCACGTTTACGAATA
GGAATTTTCTACGAAACCGACCAAGACTCCAGTTAAGATGTTTTAGAATATAGAATTTGCGTATTCTAGGTGGTATACAAG
CACGTGTGTACGTTGTATATTTCACCTAAATGTCGGTGGCGCACAAGGGGTGATTACAACCCGTTCGACATCACGTGACGG
TCTTTTAATAGAGAAAAAATCGAGCGGGTGTTTTATTCGCTGAACAATGGGGACAGTTACCCTCCTCTTGGATGGCCGATG
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TTCTTCAAAGAATCCTTCCGGGCAGAGTGTCCGTCAGATTTACGAATTCATGGCCATAGAGAATTGGAAATCTCGACAGAT
TCGATCGATCGTAACCGAGTCGAGATTGTTTTTTTTCTCTTTTGTTATTTTCGAAATTTATGGAATCCTATGAAAATATGAC
ACGATGTTTTTCAATTATTAATTACGGTTTTTGCATAGTAATTCGCACAGTTTTCTATTTATCATGTTATTTTTCGTATCATT
AAATGATATCATAATTTTATTTATAATTAAGTATTTTCCAAAATTTTATTTATTTAAAATTAATTAGAATATATATATTATG
ATTTATAAGGCGATCAAAAATAAAAAATATGATAAAGAACATCCGTGCACACAGAGCATATAATGAAAGCAAAGAAAGG
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TTTATTTTAAGATTCTATAAAAAATGCACAAGATATCGACCGATTTGAAAAATTTCAAATCGACGGACAAGACGATTTTCT
ACACCGAGTATTCCCTCTTGCGCTCCCACGAGCACGATCACGGTGTAATGGACAAGTAGACCTCGTTACGTGGCGTAACGT
GGTTGGTCGATTTTTCTTGGAAAGCGTGGCGTGACCGGTCGGTCCCGAGGAAAAACTTTCTCAGGAGAAAGAGCATTGAT
CATCGACTCGGAGGAGGGGAAACTAAAGAATTCCGTTTTCTTTTCTTTCCCCCCTTTTTTTTTTTTTTTTCTTCTTAGCAACT
TAGACCGTGAGACATCGCGTGATACGATGATAATATCCCTAAATGTGCGATTAGACGTTAAACCTAATCCCTAATTTACAC
GAACAATCGCAAATCGTGAATATCGAATAACAAAAGGGAAATCCAAAGAGTGCGAGGTGAAGGCGTAAGAAAAGGGCG
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TGACATTTAAAGCGCACACGAATCGTGTACAGGGCGCGTTCACGCGCGAGCCGAATACAAAAACCTTGGCTCCTCGTGCA
TCGGCTCGTTCGTCGATGCGTCGCGATCGCGTTTGAGGGAAGGAGAGGAAAAATCGTTCTCTCTAATGGAAAAAAAAAAA
AGAAAAAAAAAGAGAAAAAAGAAAAAAACGTATAAAAAAAAAAAAAAAA

Figure 6. Graphic sequence of the AmTYR1 (F3-R3 primer) gene

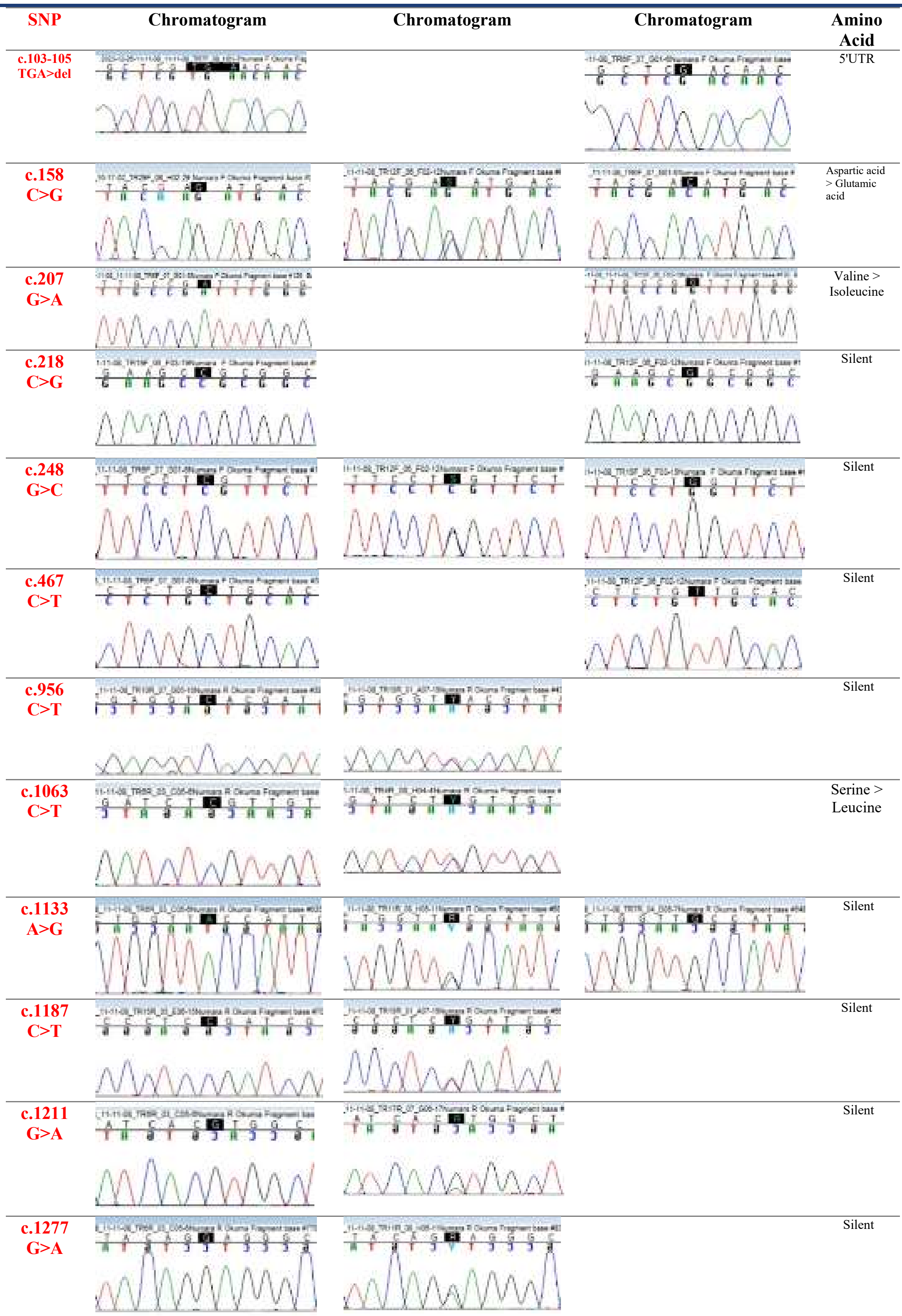

Figure 7. Chromatogram image of SNPs in the AmTYR1 (F3-R3 primer) gene

4. Conclusions

In this study, hygienic tests were carried out a total of 3 times at 21-day intervals according to the pin-killed method on 102 breeder Efe Bee colonies created from breeder queen bees produced in AARI. As stated by Guzman et al., (2002), colonies showing more than 95% hygienic behavior were determined as hygienic behavior colonies, and colonies showing less than 95% hygienic behavior were determined as non-hygienic behavior colonies. The pin-killed method has also been used in many scientific studies on hygienic behavior (Palacio et al., 2000; Nicodemo et al., 2013; Oskay et al., 2014; Kekeçoğlu et al., 2015). In this study, the pin-killed method was used to determine colonies that showed hygienic and non-hygienic behavior, and in all pin-killed measurements, bee samples were collected by putting them in tubes and placed in liquid nitrogen, then stored in a -80°C. As Goode et al., (2006) stated, since 15-20 day old worker bees exhibit hygienic behavior, worker bees in the brood area were collected for analysis in this study.

According to all measurement results, it was decided to use 5 hygienic behavior colonies and 5 non-hygienic behavior colonies in SNP analyses.

Studies show that the honey bee octopamine receptor plays a role in processing sensory inputs, modulating behavior and learning, and hormone biosynthesis, and also plays a role in hygienic behavior by contributing to cellular signaling processes. Despite this, there are limited studies on the determination of SNPs in the Octopamine receptor in honey bees. Therefore, the focus was on Octopamine receptors in hygienic behavior and SNPs in the AmOA1 receptor gene were investigated.

Since Farooqui (2007) stated that information about environmental stimuli is transmitted to the insect brain and causes certain behavioral responses, and hygienic behavior occurs with these responses, and Peng et al., (2021) also stated that biogenic amine receptors are expressed in different parts of the bee brain, worker bee heads were used for RNA analysis. SNPs in the Octopamine receptor gene were tried to be determined in the samples sent for sequence analysis.

We tried to compare our groups with hygienic behavior and non-hygienic behavior in order to determine the importance in hygienic behavior breeding. As a result of the sequence analysis, 10 polymorphisms were determined in the AmOA1 (F2-R2 primer) gene in all of the breeder Efe Bees showing hygienic and non-hygienic behavior. An additional gene region was detected in only one sample. In the AmTYR1 (F3-R3 primer) gene, 1 exon could be sequenced and 11 polymorphisms were determined.

Göze and Özdil (2023) reported the need to describe the behavioral characteristics of honey bee races in detail. All the polymorphisms identified and how some of the polymorphisms cause changes in amino acids have not been reported before in Efe Bee. It is thought that the identified SNPs can be evaluated as a breeding criterion, but it is beneficial to study with more samples in future studies to determine the relationship of polymorphisms with hygienic behavior.

No specific article was found that directly relates of the AmOA1 and AmTYR1 receptor genes to hygienic behavior in honey bees.

Classical breeding methods have been used in beekeeping for many years and therefore there is a wide knowledge and experience on this subject. These methods are based on natural selection and the natural behavior of bees, which can help protect genetic diversity. Classical methods are generally less costly, do not require special laboratory equipment, are usually long-term, and require observation and selection over several generations. Selecting targeted features may be less precise and there is a risk of spreading undesirable features. Classical methods may be less effective when genetic information is limited. Molecular breeding methods allow for rapid and precise identification and modification of genetic traits. Genetic modifications enable the effective development of specific traits, such as resistance to certain diseases or pests. Advanced molecular methods enable more conscious and targeted breeding studies using genomic information. Molecular methods are advantageous when specific genetic traits need to be obtained quickly and precisely.

Octopamine receptors and tyramine receptors are proteins that mediate the effects of this neurotransmitter in cells. Octopamine receptor genes and tyramine receptor genes play a key role in regulating the nervous system functions and behaviors of honey bees. Research on these genes can help us better understand bee biology and develop more effective methods to protect the health of bees. Manipulating the functioning of octopamine receptors and the function of octopamine receptor genes may increase the resistance of bees to certain diseases or stress factors. This may allow for the development of new strategies in beekeeping practices.

Octopamine and tyramine are one of the bee's neurotransmitters, and this molecule functions similarly to adrenaline and noradrenaline in invertebrates. Several different octopamine receptor genes and tyramine receptor genes have been identified in honeybees. These genes are expressed in different tissues and cell types and regulate a variety of behaviors and physiological responses in bees. The functioning and regulation of octopamine receptor genes is also critical to understanding bee responses to environmental stimuli. Breeding studies conducted using molecular methods towards hygienic behavior in honey bees both increase the health and productivity of bee colonies and provide environmental and economic benefits. Such breeding studies play an important role in increasing the sustainability and success of beekeeping. Beekeepers can protect the health of their colonies and reduce chemical use by choosing colonies that exhibit hygienic behavior. This has positive consequences for both bee populations and ecosystem health.

The results obtained from this study have made significant contributions in terms of guiding breeding studies. More comprehensive studies to be conducted in the future will increase the knowledge base on this subject and it will contribute to the development of application issues.

Acknowledgment

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Ethical Statement

Since this study was conducted on an invertebrate species, *Apis mellifera*, approval from ethics committees was not required.

Conflicts of Interest

We declare that there is no conflict of interest between us as the article authors.

Authorship Contribution Statement

Concept: Özsoy, N., Yücel, B., Erdoğan, M.; Design: Özsoy, N., Yücel, B., Erdoğan, M.; Data Collection or Processing: Özsoy, N., Yücel, B., Erdoğan, M.; Literature Search: Özsoy, N.; Writing, Review and Editing: Özsoy, N., Yücel, B., Erdoğan, M.

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