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The association between ClaI polymorphism and hygienic behavior in *Apis mellifera*

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Abstract: Hygienic behavior represents one of the most prominent disease resistance mechanisms developed by honeybees. This behavior is known to be influenced by the genotype and environmental factors. Therefore, this study aimed to investigate the effect of SNP-8 polymorphism and environmental factors on hygienic behavior in different *Apis mellifera* breeds. A total of fourteen colonies and 148 bees from seven different bee breeds were used for the Pin-killed Brood Assay (PKB) test and PCR-RFLP analysis, respectively. The PKB assay revealed a wide range of hygienic behavior, spanning from 68% to 100% across the fourteen colonies. The frequencies of the H (High) and L (Low) alleles were 0.0709 and 0.9291, and the frequencies of the HH (High-High), HL (High-Low), and LL (Low-Low) genotypes were 0.027, 0.088, and 0.855 (respectively) and the population was not in the Hardy-Weinberg equilibrium. Additionally, the effects of genotype, breed, birth year of queen, and colony power on hygienic behavior were found to be significant ($p < 0.01$). Italian hybrid breeds exhibited the highest levels of hygienic behavior, while Anatolian hybrid breeds demonstrated the lowest. It was also found that colonies with young queens, high colony power, and bees with HL and LL genotypes are more hygienic. A noteworthy finding of this study was the detection of heterozygous individuals (HL), marking the first such observation in this study. Consequently, HL and LL genotypes for hygienic behavior in the apiary could be suggested in the selection program. However, more research with more colonies and genes is needed to increase the effectiveness of selection for hygienic behavioral traits in *A. mellifera*. The widespread of hygienic colonies plays a critical role in preventing the spread of diseases, contributing significantly to the sustainability of beekeeping.

Keywords: Honey bee, hygienic behavior, polymorphism, SNP, *Varroa destructor*

1. Introduction

Honey bees, as social insects, have effectively established themselves in almost all ecosystems worldwide. They play an indispensable role in biodiversity conservation and holistic agriculture, serving as frequent pollinators not only for crops but also for wild plants (Iwasaki and Hogendoorn, 2021; Guzman-Novoa and Morfin, 2019). The ectoparasitic mite (Varroa) is the single most harmful biotic agent of honey bees; it feeds on the hemolymph of larvae, pupae, and adult bees, transmitting some viral infections (Kevan et al., 2006; Genersch and Aubert, 2010; Arechavaleta-Velasco et al., 2012; Emsen et al., 2015; Anguiano-Baez et al., 2016; Chen et al., 2021). However, current research suggest that Varroa feeds fat body tissue rather than hemolymph (Ramsey, 2019). A study in North America and Europe found that Varroa infestation was the largest variable in the causes of high winter mortality (Calovi et al., 2021). There are two protective mechanisms against Varroa; breeder practices and bee grooming behavior. Regarding the first, it has been stated that the cost of controlling parasites and diseases per hive for beekeepers in Gümüşhane province of Türkiye is $0.99 (Dogan and Adanacioglu, 2021). About the second, the significance of this behavior as a crucial resistance trait is noteworthy, particularly among Asian honey bees, which serve as the primary host of *V. jacobsoni* (Arechavaleta-Velasco et al., 2012). Another behavior, known as *V. jacobsoni*-sensitive hygiene behavior, is mite-sensing behavior in infested cells to remove pupae and mites or disruption of mite reproduction (Harbo and Harris, 1999). Hygienic behavior is the best-known example of disease resistance in worker bees (Guzman-Novoa and Morfin, 2019) and this is the ability to recognize and remove diseased or parasitized brood (larvae and pupae) from cells (Arathi et al., 2000).

Few studies have investigated a quantitative trait locus mapping method to identify candidate genes for mite grooming behavior after the relationship between hygienic behavior and genetics has been determined (Arechavaleta-Velasco et al., 2012). Lapidge et al. (2002)

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found that the genetic basis for hygienic behavior according to the quantitative trait locus (QTL) method is more complicatedsince many genes are likely to contribute to the behavior. Oxley et al. (2010) found that the *Hyg*1 locus correlates with hygienic behavior and its function of neurons, receptors, and transcriptions. Harpur et al. (2019) examined the genome-wide sequencing of drones for hygienic behavior and identified 73 candidate genes. Navajas et al. (2008) found 32 genes in four colonies, two from Varroa-susceptible and two from Varroa-tolerant, which varied according to the presence of Varroa. Scannapieco et al. (2017) confirmed that the genes octopamine receptor, smell-impaired, odorant-binding protein 3, and odorant-binding protein 4 defined as candidate genes for hygienic behavior in honey bees were differentially expressed in hygienic and nonhygienic bee colonies.

Beekeepers can take various protective measures against Varroa to reduce colony losses (Kulhanek et al., 2021; Hernandez et al., 2022). One of these measures is synthetic acaricides against mites such as Varroa in beekeeping may cause mite resistance and residue in honey. Using high concentrations of acaricides can be toxic to honeybees. Therefore, one of the best control methods is to select bee colonies with natural mite resistance based on grooming or hygiene behaviors (Kaskinova et al., 2020). There are two methods called Pin-Killed Brood Assay (PKB) and Freeze-Killed Brood Assay (FKB) to determine hygienic behavior (Gramacho et al., 1999; Spivak, 1996; Facchini et al., 2019). PKB is widely used to determine hygienic behavior (Gramacho et al., 1999) because it is a more convenient and less expensive method. With the PKB test, hygienic behavior can be determined and hygienic bees selected according to the test can always successfully deal with Varroa and other bee pests.

The higher colony mortality has been concentrated on Varroa, which remains the most important infestation problem for colony losses among all risk factors (Vandame and Palacio, 2010; Arechavaleta-Velasco et al., 2012). Accordingly, the importance of mite-tolerant honey bee populations seems to be increasing (Arechavaleta-Velasco et al., 2012). Kim et al. (2019) reported that SNP-8 could be used to diagnose Varroa-specific high-hygiene honey bee lines from low-hygiene lines based on base differences in PCR-RFLP method in Korean colonies. Therefore, the SNP-8 gene was chosen for this study. In this context, it is crucial to identify genotypes exhibiting high hygienic behavior within bee populations through molecular biological investigations and subsequently enhance their prevalence. Konya province has the largest agricultural areas and is one of the largest monofloral honey regions in Türkiye (Sari et al., 2020). Bozkır district owns about one-sixth of the apiaries in Konya (TSI, 2021[1]). Despite the intensive beekeeping practices in the region where the study was carried out no genetic studies on hygienic behavior could be found. Also, to our knowledge, the relationships between *Cla*I polymorphism of SNP-8 gene and hygienic behavior in pure Anatolian, pure Carpathian, Caucasian hybrid, and Italian hybrid have not been previously studied. This study hypothesizes that there may be bees with genetically high hygienic behavior in the current population and these bees may have a polymorphism in terms of the SNP-8 gene.

The study aims to investigate the effect of SNP-8/*Cla*I gene and environmental factors on hygienic behavior in honey bees.

2. Materials and methods

2.1. Experimental colonies and data collection

In the present study, seven different breeds of honey bees (pure Anatolian, pure Carpathian, Anatolian hybrid, Carniolan hybrid, Carpathian hybrid, Caucasian hybrid, Italian hybrid) reared in ten-frame Langstroth hives in a private apiary in Bozkır district (latitude: 37.171860 and longitude: 32.216846) in Konya region were used. No genetic or morphometric studies were conducted to determine the breeds of bees used in the study and the colonies were randomly selected. The collection of bee samples and the bee testing techniques used in the experiment complied with the animal welfare guidelines set out in Article 9 of the "Veterinary Services, Plant Health, Food, and Feed Law" of Türkiye. The current colonies are reared in summer (May–September) in Bozkır and winter (October–April) in Antalya with a warmer climate. Except in January and February, the colonies are fed with sugar syrup and bee cakes. Hybrid colonies were obtained by producing new queens from the colonies created by mating pure queens with different drones. For example, the so-called Caucasian hybrid colonies were formed through the production of queens from colonies formed by mating a Pure Caucasian queen and various drones. Pure colonies were obtained by purchasing pure queen bees from beekeepers who produced pure queen bees through artificial insemination. Prior to this study, none of the colonies used in the experiment had undergone any practices related to disease, parasites, or hygienic behavior.

In the present study, in a single apiary, a total of fourteen colonies (pure Anatolian-1, pure Carpathian-1, Anatolian hybrid-2, Carniolan hybrid-1, Carpathian hybrid-4, Caucasian hybrid-4, Italian hybrid-1) were examined and PKB was performed on selected one honeycomb with two trials in 4–5th and 18–19th of April

1 TSI (2021). Turkish Statistical Institute [online]. Website https://data.tuik.gov.tr/Bulten/Index?p=Hayvansal-Uretim-Istatistikleri-Aralik-2021-45593 [accessed 21 April 2022].

(Table 1). A minimum of 6 and a maximum of 12 bee samples (worker bees and drones) from each colony were subjected to molecular analysis. Drone samples were taken to better reflect the queen's genetic structure. Middle-aged worker bees have been selected for molecular studies because they exhibit hygienic behavior, while very young and forager bees have been avoided. However, drones were randomly selected from the colony because drones could not demonstrate hygienic behavior. The bee samples taken were immediately placed in alcohol and stored at +4 °C until DNA isolation.

One of the widely used assays to determine hygienic behavior is the Pin-Killed Brood Assay (PKB) described by Gramacho et al. (1999), another is the Freeze-Killed Brood Assay (FKB) (Spivak, 1996; Facchini et al., 2019). The PKB assay was rigorously performed on a honeycomb containing 100 sealed pupal cells side by side in each colony (Figure 1) and the result could be easily evaluated visually via the PKB assay. After the rhombus-shaped piercing, the results of both tests were easily recorded after 24 h. According to the literature, hygienic behavioral performance can be evaluated as a percentage of cleaned pupal cells (Gramacho et al., 1999; Espinosa-Montaño et al., 2008; Rasolofoarivao et al., 2015). The calculation of the percentage of cells successfully cleared, also referred to as the success rate, was conveniently achieved by dividing the count of cells that underwent clearance by the total number of cells to which PKB was administered.

2.2. DNA extraction and PCR-RFLP method

In performing the PKB assay, 148 bees (53 males, 95 females) from a total of 7 different breeds/ecotypes, 2 pure and 5 hybrids, were sampled in 95% ethanol and stored at +4 °C for molecular analysis. The GeneMATRIX Tissue & Bacterial DNA Purification Kit (EURx, Cat. no. E3551) was used for DNA isolation and all DNAs were stored at –20 °C until PCR. All DNA samples were evaluated by gel electrophoresis and if needed, spectrophotometry was used to determine the quality and yield of the DNAs. The PCR amplifications were performed with the SNP-8 primer (Oligomer) according to the procedure described by Kim et al. (2019) and are presented (Table 2). The PCR reaction mixture consisted of 4.6 µL of water (nuclease-free), 10 µL of the DNA template, 4 µL of ready-made 5x hot mix (FIREPol® Blend Master; DNA Polymerase, proofreading enzyme, master mix buffer, 12.5 mM $MgCl_2$, 2 mM dNTPs (Solis Biodyne)), and 0.35 pmol primers

Table 1. The hygienic behavior test results.

Breeds	N		1. Experiments*			2. Experiments **			ASR %
	♂	♀	NPC (A)	NCC (B) (24h)	SR (%) (B/A)*100	NPC (C)	NCC (D) (24h)	SR (%) (D/C)*100	
Caucasian hybrid	4	7	100	72	72	100	96	96	84.0
Italian hybrid	4	7	100	100	100	100	100	100	100
Caucasian hybrid	5	7	100	94	94	100	99	99	96.5
Pure Carpathian	5	6	100	94	94	100	91	91	92.5
Carpathian hybrid	5	7	100	97	97	100	100	100	98.5
Carpathian hybrid	4	7	100	84	84	100	100	100	92.0
Caucasian hybrid	5	7	100	82	82	100	100	100	91.0
Caucasian hybrid	5	7	100	99	99	100	98	98	98.5
Carpathian hybrid	5	7	100	97	97	100	100	100	98.5
Carniolan hybrid	4	6	100	100	100	100	98	98	99.0
Pure Anatolian	1	7	100	86	86	100	100	100	93.0
Anatolian hybrid	5	7	100	65	65	100	71	71	68.0
Anatolian hybrid	1	7	100	83	83	100	99	99	91.0
Carpathian hybrid	0	6	100	94	94	-	-	-	

*1. Experiments: Average Temperature: 6.99 °C and Humidity: 74.94 on 4–5th April 2020

**2. Experiments: Average Temperature: 10.8 °C and Humidity: 69.00 on 18–19th April 2020

♂: Drone Bee; ♀: Worker Bee

N: Bee samples used for PCR-RFLP analysis

-: There was not second control for the Carpathian hybrid because that colony had a queen change until 18–19th April.

NPC: Number of Pinned Cells (A: 1st control and C: 2nd control); NCC: Number of Cleaned Cells (B: 1st control and D: 2nd control); SR: Success Rates ((B/A: 1st control and D/C: 2nd control); ASR: Average Success Rates.

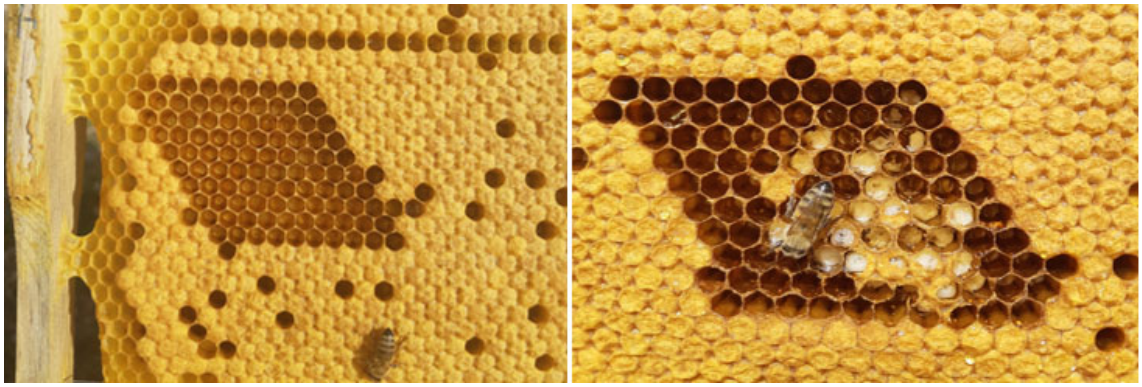

Figure 1. Left image; hygienic colony sample (100% cleaned cells-Carniolan hybrid) and Right image; unhygienic colony sample (71% cleaned cells-Anatolian hybrid).

Table 2. SNP-8 primer sequence and restriction enzyme.

SNP Region	Primers	bp	Restriction Enzymes	Target Line
SNP-8	F: ATTAGGCACGATAATAATCG	~392	*Cla*I	HHB
	R: GTTTTTAAAAATTCTACAG		5′-AT▼CGAT-3′	

HHB: High Hygienic Behavior, bp: Base Pair

in 20 µL final volume. The PCR products were cut by the restriction enzyme *Cla*I (Thermo Scientific™ *Bsu15*I (*Cla*I) (10 U/µL)). Results were shown on 2% agarose gel electrophoresis. The PCR cycling conditions and enzyme protocol were as specified by Kim et al. (2019).

2.3. Statistical analysis

The PopGene Version 1.32 software (Yeh et al., 1997) was used for statistical analysis of allele and genotype frequencies and heterozygosity (Nei, 1973) of the gene region. The chi-square (χ^2) test was performed to determine whether the population was in Hardy-Weinberg equilibrium (Düzgüneş et al., 1983). When determining the environmental factors included in the statistical model, it was considered that they might have an impact on the hygienic behavior of the colonies. The General Linear Model (GLM) was used to assess the effects of genotype and environmental factors on hygiene behavior are shown below.

$$Y_{ijlm} = \mu + \alpha_i + \beta_j + G_l + Cov_{(A,B)} + \varepsilon_{ijlm}$$

Y_{ijlm}: *i*. breed, *j*. birth year of queen, *l*. genotype, and *m*. bee's hygienic behavior success rate,

µ: Mean of traits for population,

α_i: Effect of breed (*i* = breeds; pure Anatolian, pure Carpathian, Anatolian hybrid, Carniolan hybrid, Carpathian hybrid, Caucasian hybrid, Italian hybrid),

β_j: Effect of birth year of queen (*j* = birth year of queen; 2018 and 2019),

G_l: Effect of genotype, (*l* = genotypes; HH, HL and LL),

$Cov_{(A,B)}$: Covariance between colony power (number of frames) and hygienic behavior,

ε_{ijlm}: Random error.

Tukey's multiple comparisons test was performed to assess differences between means that were significant as a result of the analysis of variance. Minitab v16.1.1 software package (Minitab, 2010) was used for statistical analysis.

3. Results

3.1. PKB assay in *A. mellifera*

In the present study, as mentioned earlier, the PKB assay was performed twice in April and the results were recorded. The percentage of cleaned pupae cells was scored for hygienic behavior. As a result of the PKB test, the mean values for the hygiene behavior of the colonies were between 68% and 100%. Accordingly, the 6 colonies (Italian hybrid (100%), Pure Anatolian (99%), Carniolan hybrid (99%), Carpathian hybrid (98.5%), and Caucasian hybrid (96.5%)) showed hygienic behavior (Table 2).

3.2. *ClaI* polymorphism of SNP-8 gene in *A. mellifera*

After the PKB assay, 148 bees were sampled for molecular assay. The SNP-8 primer, PCR, and restriction enzyme conditions used in the study conducted by Kim et al. (2019) were taken into consideration. A 392 bp region of the SNP-8 gene region was amplified. The polymorphisms were observed by digesting the PCR products with the

*Cla*I restriction enzyme. Digestion of the SNP-8 PCR fragment with *Cla*I resulted in fragment lengths of 392 bp for LL; 392, 192, and 180 bp for HL; 192, and 180 bp for HH (Figure 2).

The allele and genotype frequencies for seven different honey bee breeds are given in Table 3.

In our findings, the presence of the HHB gene was observable in the samples containing the primer (SNP-8 in chromosome 4) and subjected to the PKB test. In addition, the allele frequencies were estimated to be 0.0709 for H and 0.9291 for L alleles and genotype frequencies were 0.027, 0.088, and 0.885 for HH, HL, and LL genotypes, respectively. Out of the SNP-8 (*Cla*I) samples, 131 exhibited characteristics of low hygienic behavior (LHB), while 4 demonstrated high hygienic behavior (HHB). Additionally, as a novel contribution to the literature, it was identified 13 honey bees that exhibited heterozygosity for SNP-8 (*Cla*I). The heterozygosity value in the population was determined as 0.132 and the chi-square value was determined as 16.479 (Table 3). Hence, the population was found not to be in Hardy-Weinberg equilibrium ($p < 0.01$).

3.3. Association analysis

Statistical analysis was performed using the Minitab program, first the General Linear Model analysis and then Tukey's test. The effects of breed, birth year of queen, and colony power on hygienic behavior were found to be significant at the $p < 0.01$ level. As a result; the Italian breed showed the highest hygienic behavior and the difference between pure Carpathian and Anatolian hybrids were statistically significant ($p < 0.01$). However, the difference between Italian with Carpathian and Carniolan hybrids with pure Anatolian was statistically insignificant. On the other hand, according to Tukey's test, there has been an improvement in hygienic behavior in the population over the years. According to the Tukey test, it was determined that bees with the HH (79.88% ± 2.83%) genotype exhibited statistically lower hygienic behavior compared to both HL (93.53% ± 1.50%) and LL (91.80% ± 0.68%) genotypes, while no significant difference was found between the HL and LL genotypes. Also, as a result of the regression analysis, it was determined that increasing colony power by one frame would increase the hygienic behavior of bees

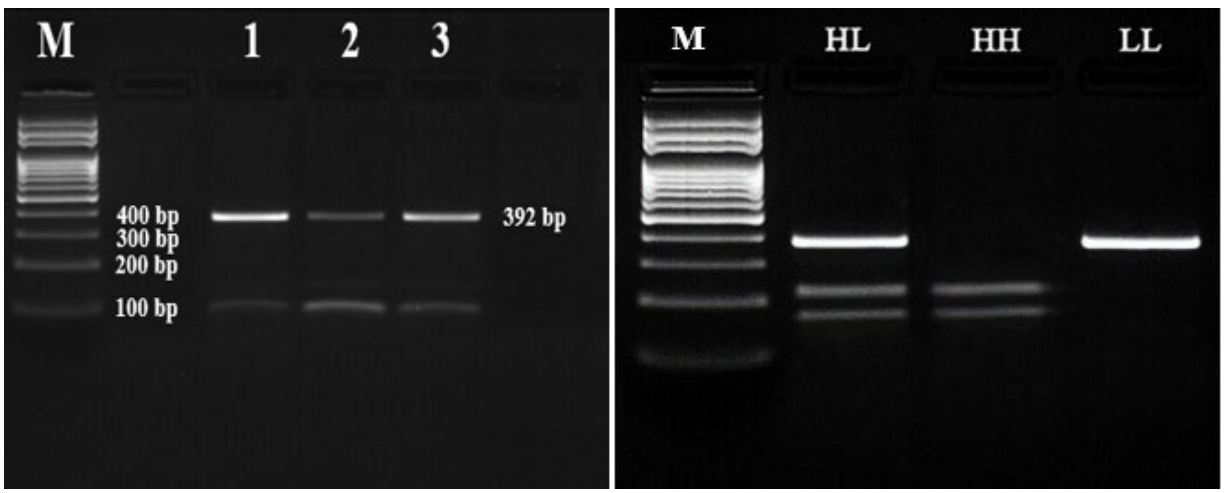

Figure 2. PCR and restriction products picture of the *Cla*I polymorphism.

M: 100 bp Plus DNA Ladder, Left Image Line 1–3: PCR Products and Right Image Line HL: 392, 192 and 180 bp, HH: 192 and 180 bp, LL: 392 bp

Table 3. Analyzed genetic variant (genotype/allele) frequencies of SNP-8 marker.

SNP-8	N	Genotypes			Genotype frequencies			Allele frequencies		χ^2
		HH	HL	LL	HH	HL	LL	H	L	
Observed	148	4	13	131	0.027	0.088	0.855	0.0709	0.9291	16.749**
Expected	148	0.745	19.510	127.745	0.01	0.13	0.86			H_e^2 0.132

N: Number of Bee, χ^2: test of Hardly-Weingberg Equilibrium, ^{2}Heterozygosity, **: $p < 0.005$

by 85.7%. As a result of this study, it was found that breed, genotype, birth year of queen, and colony power explain 65% of the observed variation in hygiene behavior in honey bees (Table 4).

4. Discussion

It is crucial to study the relationship between the polymorphism of gene regions and yield-related traits to improve the effectiveness of selection in beekeeping. This study is designed as a preliminary study to test the genotypes with the hygienic behavior trait and to increase the hygienic worker bees' ratio in the population. QTL studies such as the current study are important to create an accurate selection program for hygienic behavior because the difference between genotypic values in different populations can vary across different environments. Similar to the present study, numerous investigations have been conducted to ascertain the genotypes influencing hygienic behavior (Cornman et al., 2010; Arechavaleta-Velasco et al., 2012; Tsuruda et al., 2012; Kirrane et al., 2015; Eliash and Mikheyev, 2020). The hygienic behavior characteristic is emphasized as the best-known resistance mechanism against diseases (Oskay, 2008; Guzman-Novoa and Morfin, 2019). In the present study, the bee population was not in Hardy-Weinberg equilibrium, possibly due to the small population size. Also, it was found that *Cla*I polymorphism of SNP-8 gene and some environmental factors affect the hygienic behavioral traits of honey bees. In contrast to the present study, Kim et al. (2019) used FKB assay to determine hygienic behavior, but colonies selected by this method always did not successfully cope with mites (Kaskinova et al., 2020; Perrin et al., 2020). Because there is a similar mechanism for Varroa susceptibility and general hygienic behavior (detecting and uncapping diseased brood) in bees (Kaskinova et al., 2020). PKB testing is usually considered easier than FKB testing (Spivak and Downey, 1998; Panasiuk et al., 2008). One of the study's key findings revealed that the percentage of cleaned pupal cells varied from 68% to 100%. According to the literature, a colony is considered hygienic when the removal rate of dead pupae in honey bee colonies is 95% or more after at least two inspections (Spivak and Downey, 1998; Güler and Toy, 2013).

This study was similar to the results of some studies (Kim et al., 2019; Kaskinova et al., 2020; Khan and Ghramh, 2021). Kim et al. (2019) stated that the SNP-8 gene could be used to evaluate the highly hygienic colonies reared in Korea. Kaskinova et al. (2020) pointed out that Varroa resistance of honey bees is a polygenic trait and that establishing gene pathways for this trait may be important for the selection of Varroa-resistant colonies. Khan and Ghramh (2021) observed a significant difference between Italian and Carniolan colonies in their PKB assay results. Similar to the present study, there were some studies on the significance of years for hygienic behavior (Mendizabal, 2004; Bigio et al., 2013).

Several studies have mentioned QTL that influences the likelihood of worker bees exhibiting hygienic behavior against the Varroa (Takeuchi et al., 2001; Behura and Whitfield, 2010; Oxley et al., 2010; Chandrasekaran et al., 2011; Cristino et al., 2014; Boutin et al., 2015; Spötter et al., 2016). The presence of numerous gene regions discussed in previous studies, including the *Mblk*-1 gene and predicted target genes of transcription factors (TFs), suggests the polygenic nature of this trait. This polygenic nature helps elucidate the disparities between PKB and molecular test results observed in the current study in relation to hygiene behaviors. The study by Lapidge et al. (2002) using molecular techniques and QTL link mapping to detect hygienic behavior also supports that conclusion.

Table 4. Relationships between hygienic behavior and SNP-8.

Genotype			**Breed**			**Birth year of queen**			**Regression**	**R^2**
Genotype	**N**	$\bar{X} \pm S_{\bar{x}}$	**Breed**	**N**	$\bar{X} \pm S_{\bar{x}}$	**Year**	**N**	$\bar{X} \pm S_{\bar{x}}$		
			LH	11	96.01 ± 2.21^{A}					
			CH	41	92.12 ± 1.58^{AB}					
HH	4	79.88 ± 2.83^{B}	CARH	10	90.74 ± 2.26^{AB}	2018	34	86.28 ± 1.60^{B}	CP: 0.857±0.294**	0.6500
HL	13	93.53 ± 1.50^{A}	PA	8	89.84 ± 2.32^{AB}	2019	114	90.53 ± 1.15^{A}		
LL	131	91.80 ± 0.68^{A}	CH*	47	87.45 ± 1.48^{B}					
			PC	11	86.55 ± 2.04^{B}					
			AH	20	76.12 ± 1.62^{C}					

A, B: p < 0.01; **: p < 0.01, CP: Colony Power, R^2: Coefficient of Determination, N: Number, $\bar{X}$: Means, $S_{\bar{x}}$: Standard Errors, HH: High Hygienic Behavior, HL: High-Low Heterozygous, LL: Low Hygienic Behavior, IH: Italian Hybrid, CH: Carpathian Hybrid, CARH: Carniolan Hybrid, PA: Pure Anatolian, CH*: Caucasian Hybrid, PC: Pure Carpathian: AH: Anatolian Hybrid

The same researchers emphasized that the genetic basis of hygienic behavior (controlled by a total of 7 loci) is more complicated and the many genes are likely to lend to the behavior. In addition, Arathi et al. (2000) pointed out that the genotypic composition affects the hygienic behavior performance and therefore the result of the hygiene behavior cannot be reached by molecular and pinning tests. This study differed from Kim et al. (2019) concerning the genotyping of high and low hygienic behavior colonies. In the present study, genotyping was carried out according to Kim et al. (2019), the results of the pin test and the molecular test appear to be different in the current study. Nevertheless, when assessed at a population level, the findings of the present study exhibit consistency, indicating that breeders aiming to enhance hygienic colonies within this particular population can achieve this objective by producing queens with HL and LL genotypes. The SNP-8 is the gene region that degrades the peroxidase enzyme and it has probably been predicted to be one of the endogenous enzymes of the insect's protective system (Kim et al., 2019). Viewed from this perspective, it comes to mind that individuals with high hygienic results in terms of SNP-8 may be advantageous in creating a pure breed. Considering the results, it can be said that worker bees with HL and LL genotypes are more hygienic and can be used in selection. To procure worker bees with HL and LL genotypes, it necessitates the determination of both queen and drone genotypes followed by the implementation of artificial insemination techniques. In artificial insemination in beekeeping, it is much easier to determine the genotypic structure of drones compared to queen bees. On the other hand, after cutting the wing of the queen bee and determining its genotypic structure, a way can be followed by using it for artificial insemination. Regarding the contribution of worker bee parents to hygiene behavior, Seltzer et al. (2022) reported that both parents (queen and drone) have a significant impact on the hygienic behavior of worker bees and that selecting highly hygienic drones could spread hygienic traits in local populations. The identification of heterozygous individuals in this study, a novel discovery, requires further study and investigation to explain the association between hygienic behaviors in honey bees. Ultimately, it has been discerned that conducting comprehensive molecular screening and validating the outcomes through field sampling are pivotal components in the advancement of bee lineage development.

5. Conclusion

The present study aimed to correlate the hygienic behavior and the single nucleotide polymorphism of honey bees collected field samples. This is based on the assumption that the negative effects of diseases and pests can be reduced by molecular selection. The study involved the analysis of single nucleotide polymorphism and marked the inaugural molecular-level testing across various breeds in Türkiye. It was seen that colonies with young queens, high colony power, and bees with HL and LL genotypes are more hygienic. Furthermore, heterozygous individuals, previously unreported in the literature, were identified for the first time and hygienic behavior in bees was examined for the first time in Türkiye through both classical and molecular methods. Consequently, it is thought that molecular testing can benefit bee breeding studies by considering more comprehensive or different gene regions. At the same time, it is thought that SNP markers can help a queen and drones to be selected for breeding to establish a bee line. With the potential reduction in drug usage in hygienic bee lines cultivated through molecular selection, the possibility of producing bee products with lowered residue levels emerges, allowing for the integration of these products into the global economy.

Author declarations

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The authors report there are no competing interests to declare.

Ethics approval
This work did not involve the use of animals for laboratory studies. There is no violation of animal right.

Consent to participate
Not applicable.

Consent for publication
Not applicable.

Data availability
Data will be provided by corresponding author on reasonable request.

Code availability
Not applicable.

Authors' contributions
All authors contributed to conception and design of the study. Data and sample collection was performed by Mustafa Kibar. Laboratory analyses were performed by Mustafa Kibar and İnci Şahin Negiş. Statistical analysis and interpretation of data were performed by Mustafa Kibar and İbrahim Aytekin. All authors contributed to and approved the preparation of the final manuscript.

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