Research article

Detection of *Mblk-1* Gene Polymorphisms in Honey Bees and their Influence on Resistance to Varroa Destructor Mites

Kristina Morkūnienė*, Rūta Insodaitė, Laimutis Kučinskas, Renata Bižienė

Lithuanian University of Health Sciences, Institute of Biology Systems and Genetic Research, LT- 47181 Tilžės st. 18, Kaunas, Lithuania.

Abstract | The *Varroa destructor* mite is a widespread major pest of honey bees *Apis mellifera*. The rapid spread of *Varroa* mites among bee colonies may be due to several factors, including drifting of infested bees, movement of bee swarms, robbing of weakened colonies. However, some bees' colonies are resistant to *V. destructor* and that may be related to changes in the amino acid sequence of the Mblk1 protein leading to higher brain functions in bees. In this study, we aimed to test the contribution of three *Mblk-1* gene polymorphisms to the resistance to *V. destructor* mites. This case–control study involved 117 DNA samples that were genotyped for three single nucleotide polymorphisms (SNP) using the real-time polymerase chain reaction method. Statistical analysis was performed with SPSS Statistics 20 and PLINK software. SNP at position 7454459 (Asn → Thr) of *Mblk-1* gene mutant allele A was more common in untreated domestic and wild honey bees (18.90% and 8.14% respectively; p=0.001) compared to treated domestic bees which persistently infected with the disease. Regression analysis showed that recessive AA genotype of this polymorphism significantly reduced the odds for varroosis (odds ratio=0.166, 95% confidence interval = 0,049-0,562, p=0.004). SNP at position 7454459 (Asn → Thr) of the *Mblk-1* gene has a prominent interface with resistance to varroosis.

Keywords: honey bees, *Mblk-1* gene, mite, *Varroa destructor*, polymorphism.

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***Correspondence** | Kristina Morkūnienė, Lithuanian University of Health Sciences, Institute of Biology Systems and Genetic Research, LT- 47181 Tilžės st. 18, Kaunas, Lithuania; **Email:** kristina.morkuniene@lsmuni.lt
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INTRODUCTION

Varroosis is considered a major pest of honey bees *Apis mellifera*. It is an invasive disease caused by *Varroa destructor* mites that infect bees at any stage of their development (Bokaie et al., 2013). Varroa mites are obligatory ectoparasites which feed on fat bodies of developing larvae/pupae and adult honey bees (Ramsey et al., 2019) and reproduce in the brood (Rosenkranz et al., 2010). Parasites feed on the hemolymph of honey bee brood and adult bees and reduce their viability and productivity, disrupting normal development. The mite is responsible for low brood emergence rates and decreased adult life expectancy, which finally leads to loss of colonies. The main reasons that make honey bees susceptible to pathogenic infections are: high social behavior, genetic homogeneity, and close physical contact (Chen et al., 2007, van Dooremalen et al., 2012, Guichard et al., 2020). Several authors have studied the effects of Varroa feeding on the honey bee (De Grandi-Hoffman et al., 2004, Nazzi et al., 2016, Traynor et al., 2020) however, given the frequent concurrent presence of viruses with Varroa, such effects could well be related to the combined action of the parasite and the pathogens than to the mite alone. The prevalence of Varroa mites has increased the frequency of viral infections such as: Acute Bee Paralysis Virus (ABPV), Israeli Acute Paralysis Virus (IAPV), Kashmir Bee Virus (KBV), Sacbrood Virus (SBV), and Deformed Wing Virus (DWV) among bees' colonies. For that reason, *Varroa destructor* mites can act as transfer vectors for different bee viruses (Yue et al., 2005, Boecking et al., 2013). The spread of varroosis and its relation with viral infections causes significant economic losses to beekeepers (Clermont et al., 2014). In addition, Varroa mite may intensify the problems of pollination in the future.

Various chemical substances, application techniques, and methods are currently used to control the mite's population. However intensive use of many chemical substances against the *V. destructor* mites increased of resistance and decrease their efficiency and contamination of products such as honey and beeswax (Milani 1999, Wallner, 1999). Natural products – essential oils and formic acid, are also used against *V. destructor* mites however, their effectiveness is not as good as chemical substances (Bokaie et al., 2013). It has been observed that some bees' colonies are able to defend themselves against *V. destructor* mites without additional chemical or natural substances. Several studies have shown that *V. destructor* parasitism alters the expression pattern of immune-related (Yang et al., 2005, Navajas et al., 2008, Hamiduzzaman et al., 2012) and behavioral-related genes in honey bees (Le Conte et al., 2011). The resistance of honey bees is linked to genetic factors that determine some of their behavior to protect against parasites. Mushroom body large-type Kenyon cell-specific protein-1 (*Mblk-1*) was identified as a novel transcription factor that may play important roles in higher bee brain functions. The *Mblk-1* gene is specifically expressed in a subtype of mushroom bodies neurons called large-type Kenyon cells (Park et al., 2002, Menzel et al., 2006). In honey bees, the mushroom bodies receive multimodal information and play important roles in higher-order learning and social behavior (Takayanagi-Kiya et al., 2017). A study by Conlon and coauthors (2018) demonstrate that changes in the *Mblk-1* gene may be related to parasite resistance. In *V. destructor* resistant bees' family's genome, they identified three single nucleotide polymorphisms at 7454459, 7454648, and 7454648 positions of the *Mblk-1* gene. These three polymorphisms change the amino acid sequence of the *Mblk-1* protein and might be the answer to why some bees' colonies are more resistant to *V. destructor* compared to others (Conlon et al., 2019).

In this study we aimed to reveal the relationship of polymorphisms at positions 7454459, 7454648 and 7454648 of the *Mblk-1* gene on the risk of varroosis.

MATERIALS AND METHODS

Study population

A group of 43 treated for the *Varroa destructor* and 59 untreated domestic workers honey bees were collected from 4 apiaries located in different regions of Lithuania. The group of untreated domestic bees was also included with 15 wild workers honey bees.

Mblk-1 genotyping

Genotyping of *Mblk-1* gene polymorphisms was carried out at the Laboratory of Genetics of the Institute of Biology Systems and Genetic Research of LUHS. Genomic DNA was extracted from workers honey bees body tissues using a genomic DNA purification kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. For the study were selected three *Mblk-1* gene polymorphisms located in chromosome 15: 7454459 (Asn → Thr), 7454648 (Gln → Arg), and 7454648 (Leu → Pro). SNPs in *Mblk-1* gene were estimated by using genotyping kits which were constructed according to Table 1 by Applied Biosystems. Applied Biosystems 7900HT Real-Time Polymerase Chain Reaction System (Applied Biosystems, Foster City, CA, USA) was used for SNPs detection. The cycling program started with heating at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. Finally, allelic discrimination was performed using SDS 2.3 software provided by Applied Biosystems.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20 software (IMB Corp., Armonk, NY, USA). The results are presented as total numbers, percentages, mean, and standard deviation (SD). The distribution of SNP genotypes in treated and untreated for *Varroa destructor* was evaluated by Hardy-Weinberg equilibrium (HWE) using the chi-square test. The homogeneity of the distribution of polymorphism genotypes between honey bee groups was compared using χ^2 and Fisher one-tailed and two-tailed tests. The association between the *Mblk-1* gene polymorphisms and *Varroa destructor* was estimated by computing odds ratios (ORs) and their 95% confidence intervals (Cls) from logistic regression in five inheritance models: Recessive (wild-type homozygous with heterozygous vs. minor allele homozygous), dominant (wild-type homozygous vs. heterozygous with minor allele homozygous), overdominant (wild-type homozygous with minor allele homozygous vs. heterozygous) and additive inheritance model.

Polymorphisms located at 7454459 (Asn → Thr), 7454648 (Gln → Arg), and 7454648 (Leu → Pro) positions of the *Mblk-1* gene are on chromosome 15, therefore haplotype analysis was carried out. Estimation of haplotype frequencies and haplotype association with frequencies of at least 5% were carried out using PLINK software version 1.07 (Purcell et al., 2007). Results were considered statistically significant when the p-value was less than 0.05.

RESULTS

All analyzed *Mblk-1* gene polymorphisms genotypes and allelic in treated for *Varroa destructor* mites and untreated honey bees are presented in Table 2**.** The distribution of all SNPs in both groups was consistent with the HWE. Statistically significant differences were revealed in the distribution of the *Mblk-1* gene polymorphism at position 7454459 (Asn → Thr) among analyzed honey bee groups.

Table 1: Primer and probes sequences designed for genotyping single-nucleotide poly-morphisms in *Mblk-1* gene

Mblk-1 gene SNP location	Forward primer	Reverse primer	VIC marked probe	FAM marked probe
7454459 Asn → Thr	CAGAGGATGTC-TACAACATTCT-TTTAAAAAATCA	GCCGATATAT-TTCTTTCAT-TTAACATTTAAT-GAATTATAA	ATTTTAATTATAT-GAAATAGAAA-CAT	ATTATATGAAATG-GAAACAT
7454648 Gln → Arg	ACGAAAAAT-TTTGTTATTGTAT-TCGAAATACATA-GATGT	TCCCCATCT-TATGTGTT-GAAAGCAT	ACTTGT-CAAAATAAAGT-TAAAT	CTTGTCAAAATAAG-GTTAAAT
7454648 Leu → Pro	GAGGAAATAAAG-CATAAGGGATAAT-GTCGATAT	CACACAAT-CAAATTTAAAT-GATCAGT-GACGAT	TTGTATCATC-CGATCTGTTTT	ATCATCCGACCTGT-TTT

Table 2: Distribution of genotypic and allelic frequencies for single nucleotide polymorphisms at *Mblk-1* gene positions 7454459 (Asn → Thr), 7454648 (Gln → Arg), and 7454648 (Leu → Pro) in the treated for *Varroa destructor* domestic honey bees (n=43) and untreated domestic and wild honey bees (n=74) groups

Mblk-1 SNP position			Not treated bees, n (%)	*p*-Value HWE	Treated bees, n (%)	*p*-Value HWE	*p*-Value
7454459 (Asn → Thr)	Genotype	GG	50 (67.57)	0.091	37 (86.05)	0.056	0.017
		GA	20 (27.03)		5 (11.63)		
		AA	4 (5.40)		1 (2.35)		
	Allele	G	120 (81.10)	-	79 (91.86)	-	0.001
		A	28 (18.90)		7 (8.14)		
7454648 (Gln → Arg)	Genotype	CC	67 (90.54)	0.811	41 (95.35)	0.687	0.223
		CT	6 (8.12)		2 (5.65)		
		TT	1 (1.35)		0 (0.0)		
	Allele	G	140 (94.59)	-	87 (97.67)	-	0.721
		A	8 (5.41)		2 (2.33)		
7454648 (Leu → Pro)	Genotype	GG	70 (94.59)	0.482	42 (97.67)	0.210	0.855
		GA	4 (5.41)		1 (2.33)		
		AA	0 (0.00)		0 (0.00)		
	Allele	G	144 (97.30)	-	85 (98.84)	-	0.465
		A	4 (2.70)		1 (1.16)		

Polymorphism, at position 7454459 (Asn → Thr) mutant allele A was more common in untreated domestic and wild honey bees (18.90% and 8.14% respectively; p=0.001) compared to treated domestic bees which are persistently infected with the disease (Table 2). The distribution of *Mblk-1* gene polymorphism at position 7454459 (Asn → Thr) genotypes among the studied honey bee groups was also statistically significant. Wild type homozygote GG genotype was more common between treated for *Varroa destructor* domestic bees, and heterozygote GA genotype was more common between untreated domestic and wild honey bees' group (7454459 (Asn → Thr) genotypes GG, GA: 86.05% and 11.63 % vs. 67.57% and 27.03% respectively; p=0,017). Other analyzed polymorphisms at positions 7454648 (Gln → Arg) and 7454648 (Leu → Pro) of the *Mblk-1* gene did not show any statistically significant distribution of genotypes and alleles between analyzed honey bee groups (Table 2).

Associations between *Mblk-1* gene polymorphisms at positions 7454459 (Asn → Thr), 7454648 (Gln → Arg),

7454648 (Leu → Pro), and varroosis according to the inheritance models are presented in Table 3**.** Binomial logistic regression analysis showed that the recessive (p=0.004), overdominant (p=0.009), and additive (p=0.010) variables were significant of *Mblk-1* gene polymorphism at position 7454459 (Asn → Thr) (Table 3). The lowest Akaike information criterion (144.295) was for the recessive model (OR=0.166, 95% CI=0.05-0.56, p=0.004) of *Mblk-1* gene

Table 3: Model selection according to Akaike information criteria (AIC) for *Mblk-1* gene polymorphisms at positions 7454459 (Asn → Thr), 7454648 (Gln → Arg), and 7454648 (Leu → Pro)

Mblk-1 gene SNP position	Model	OR (95% Cl)	*p*-Value	AIC
7454459 (Asn → Thr)	Dominant (GG *vs.* GA+AA)	3.561 (0.313-1.479)	0.306	152.752
	Recessive (AA *vs.* GA+GG)	0.166 (0.049-0.562)	0.004	144.295
	Overdominant (GA *vs.* GG+AA)	0.165 (1.593-4.631)	0.009	145.735
	Additive	0.118 (0.920-0.726)	0.010	145.719
7454648 (Gln → Arg)	Recessive (AA *vs.* GA+GG)	0.303 (0.583-9.087)	0.234	152.460
	Overdominant (GA *vs.* GG+AA)	0.213 (0.583-1.714)	0.412	152.460
	Additive	0.157 (0.110-1.548)	0.079	153.450
7454648 (Leu → Pro)	Recessive (AA *vs.* GA+GG)	0.738 (0.108-2.516)	0.699	153.736
	Overdominant (GA *vs.* GG+AA)	0.738 (0.183-1.516)	0.649	153.136
	Additive	0.714 (0.202-2.528)	0.412	153.614

Table 4: Haplotype association of single nucleotide polymorphisms at *Mblk-1* gene at positions 7454459 (Asn → Thr), 7454648 (Gln → Arg) and 7454648 (Leu → Pro) with varroosis

SNPs positions	Frequency		Chi-square	Degrees of freedom	*p*-Value
7454459 (Asn → Thr) - 7454648 (Gln → Arg) - 7454648 (Leu → Pro)					
Haplotype	Treated	Not treated			
G-G-G	0.986	0.973	1.041	1	0.059
A-A-G	0.003	0.006	0.578	1	0.893
A-G-G	0.009	0.014	0.084	1	0.841
G-A-G	0.001	0.005	0.072	1	0.874
G-G-A	0.001	0.002	0.978	1	0.894

polymorphism at position 7454459 (Asn → Thr) (Table 3). Other analyzed *Mblk-1* gene polymorphisms inheritance models did not show any statistically significant results.

Association analysis between the risk of varroosis and haplotypes for *Mblk-1* gene polymorphism at positions 7454459 (Asn → Thr), 7454648 (Gln → Arg), and 7454648 (Leu → Pro) are shown in Table 4**.** The linkage disequilibrium between these three polymorphisms (D' value) was 0.662. However, the analysis did not show any statistically significant results.

DISCUSSION

The honey bee (*Apis melifera*) is one of the most valuable pollinators worldwide. Over the last few decades, increased honey bee colony losses have been reported possibly as a result of a growing number of interacting threats, such as habitat losses, nutritional deficiencies, pesticides, pests, and pathogens (Guichard et al., 2020). Among the parasitic threats, the invasive mite *V. destructor* is often identified as the main macrobiotic cause of colony losses of honey bees. In contrast to the original host (*A. cerana*) *V. destructor* is lethal to *A. melifera* due to unlimited reproduction in both the drone and worker brood, which subsequently leads to high infection levels, threatens colony survival and reproduction (Amdam et al., 2004, Zaobidna et al., 2017).

In recent years it has been reported that some colonies in Europe, Africa, and America become resistant to *V. destructor*. It was observed that this phenomenon could be related to brood cell size, smaller colony sizes, alterations of brood volatile compounds, and behavioral defense such as mite-infested brood removal (Hawkins et al., 2021). Bees' tolerance to *V. destructor* is also characterized by differences in the expression of genes related to embryonic development, cell metabolism, immune response, regulation of neuronal development, neuronal sensitivity, and olfaction. These insights were obtained by comparing two groups of bees: Varroa-susceptible and Varroa-resistant (Navajas et al., 2008).

Grooming behavior which is one of the behavioral resistance mechanisms based on the genetic basis in honey bees is a defense response against parasitic mites that may be directly related to transcription factor *Mblk-1* expression changes (Yildis et al., 2020, Kaskinova et al., 2020). But exact causes that lead to *Mblk-1* expression changes in dif-

ferent bees' phenotypes are not known yet.

Conlon and coauthors (Park et al., 2002) in the study of *Mblk-1* polymorphisms among bee colonies in which mite reproduction was successful vs unsuccessful separate three SNPs which segregate better between these two phenotypes. In our study, only one SNP, at position 7454459 (Asn → Thr) of the *Mblk-1* gene showed statistically significant separation between two different bees' phenotypes. According to that, our findings suggest that *Mblk-1* gene polymorphism at 7454459 (Asn → Thr) position may have an impact on honey bee's resistance to *V. destructor* mites by affecting *Mblk-1* expression. Whereas *Mblk-1* is preferentially expressed in the neuronal circuits of mushroom bodies, it may play an important role in grooming and hygienic behaviors (Ji et al., 2014).

CONCLUSIONS

No other pathogen or parasite has had a comparable impact on honey bees, in part because varroa only recently adapted from its original host, the Asian honey bee (*Apis cerana*) to exploit a naïve host with inadequate innate defenses (Traynor *et al.,* 2020). *V. destructor* is one of the greatest threats to the honey bees, *Apis mellifera*, worldwide, breeding varroa-tolerant honey bees is an ideal strategy, as it either reduces or eliminates the need for acaricides with-out requiring additional Varroa control measures. In this study, we identified *Mblk-1* SNP, which may be involved in resistance to *V. destructor.* Our finding confirmed that changes in gene, effects on grooming behavior may play important roles in the resistance to *V. destructor*. However, expression changes among this gene due to SNP at 7454459 (Asn → Thr) position may be investigated in future studies. Nevertheless, farmers are recommended to breed those honey bees' colonies that are not infected with *V. destructor* mites, thus spreading the *Mblk-1* polymorphism at position 7454459 (Asn → Thr) of the gene and reducing the use of chemical substances required for the treatment of the disease.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

NOVELTY STATEMENT

All authors confirm that the manuscript has not been published elsewhere and is not currently being reviewed by another journal.

AUTHORS CONTRIBUTION

Author's contribution to the concept, assumptions and methodology used int the article (%): Kristina Morkūnienė 30%, Rūta Insodaitė 30%, Renata Bižienė 30%, Laimutis Kučinskas 10%.

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