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Molecular Screening of Varroa-Resistant Trait of Honey Bee Colonies based on *NorpA2* Candidate Gene Polymorphism: A Genetic Case-Control Study

Research Article

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ABSTRACT

In recent years the *Varroa destructor* mite has been a dominant challenge for honey bee colonies, worldwide. Varroa- sensitive hygiene, a behavioral characteristic of honey bee, involves the detection and removal of Varroa larvae from the colony. It has been exploited as a recordable trait for Varroa resistance. The sense of smell is a key element in Varroa sensitive hygiene behavior. In this scenario, *NorpA2* candidate gene, a putative olfactory receptor, is associated with vision and smell in honey bee and is known as a candidate gene for Varroa sensitive hygiene. With this motivation, the main goal of the current work was to determine some single nucleotide polymorphism (SNP) markers of *NorpA2* candidate gene employing PCR sequencing in a case-control approach. To this end, after the identification of resistant (RES) and susceptible (SUS) colonies according to the percentage of infections of the drone pupae to Varroa mite, a total of 10 drone pupae (5 from SUS and 5 from RES) were selected and were subjected for DNA isolation. The polymerase chain reaction (PCR) was performed based on two pairs of specific primers for the amplification of 5′ untranslated region (5′UTR) and promoter regions of *NorpA2* gene. After expected size bands were observed, the purification process and Sanger sequencing of the PCR products were carried out. The sequencing results were quality checked and the alignment and clustering were done using the BLAST and MAFTT software, respectively. Sequences of both UTR and promoter regions displayed multiple variations (SNP, deletion). Interestingly, the results showed the existence of three specific differences in sequence in the form of SNP (C/T) at position 308 and the form of SNP/deletion at positions 504 and 563 of the nucleotide sequence region in the promoter between the SUS and RES groups. To our knowledge, this is the first report on the identification of biomarkers to control host-mite interaction in the honey bee *Apis mellifera*. Further research is required for the reported SNPs to be validated as biomarkers of resistance to Varroa.

KEY WORDS *Apis mellifera*, candidate gene, resistance, single nucleotide polymorphism, *Varroa destructor*.

INTRODUCTION

Research on the genomic perspective of the host-mite interaction in honey bees has a long history (Mondet *et al.* 2020). A challenging problem that arises in this domain is honey bee communication features have to shape reproduction, nurse, and feed larva in contaminated hive environment and presence of large volume of parasites such as the *Varroa destructor* mite (Tantillo *et al.* 2015). *Varroa destructor* represents a significant threat and important parasite in honey bees, which can reproduce in different bee species (Traynor *et al.* 2020). Previous studies have emphasized that *V. destructor* can reproduce on worker and drone larvae. However, because of the longer pupal stage and larger cell size, *V. destructor* prefers drone larvae that facilitate more offspring production (Underwood and Lpez-Uribe, 2019). Beekeeping is extremely dependent on many biotic and abiotic factors, effective strategies to control

Varroa infestation are difficult to quantify, and most are costly and inadequate for control on their own (Jack and Ellis, 2021). Conventional chemical treatments are available for controlling Varroa (Noel *et al*. 2020). However, an increase in beekeeping costs, penetration of pesticides into bee products and increasing resistance of mite are the drawbacks of the chemical treatments and, therefore, are prohibited for long-term use. In large-scale sustainable beekeeping operations, the generation of mite-resistant bees are recommended, and most countries are pursuing this solution as a pest control tool (Jack and Ellis, 2021). Resistance is the host's ability to reduce the amount and number of parasites in the population/pathogenic load, while tolerance is defined as the host's ability to reduce parasite damage (in this case, disease-related mortality) caused by the parasite/pathogen. Varroa mite and mite-borne viruses cause strong damage and loss of bee colonies and also the existence of a complex host-parasite-pathogen relationship creates a scientific puzzle to understand the genetic mechanism of resistance and susceptibility to the Varroa destructor mite (Kurze *et al*. 2016; Thaduri *et al*. 2019; Schmid-Hempel, 2021). The host's ability to prevent the possibility of parasite multiplication and to reduce the parasite population is a successful strategy of the host to diminish the varroa-related damages (Locke *et al*. 2012; Oddie *et al*. 2021). The study of Varroa resistance began as follows: At the degree of Varroa mite spread, the identification of wild honey bee colonies that was resistant to Varroa mite were carried out in various parts of France (Le Conte *et al*. 2007) and the United States (Seeley, 2007). This led to the idea of breeding Varroa mite-infected colonies without the use of treatment. Based on this idea and on *Apis mellifera intermissa*, Kefuss *et al*. (2004) demonstrated the genetic base of Varroa mite resistance. Further identification of resistant colonies in Russian and African honey bees in comparison with *Apis cerana* (Indian honey bee dominant host of Varroa mite), revealed various physiological and behavioral mechanisms of Varroa mite resistance (Rosenkranz *et al*. 1993; Fries *et al*. 1996).

Behavioral mechanisms of resistance to Varroa include hygienic behavior, grooming and varroa sensitive hygienic behavior (Rosenkranz *et al*. 2010). According to these mechanisms, phenotypic breeding for varroa-resistant honey bees were started. Three successful breeding programs were performed in the USA, and resulted in the production of varroa resistant strains.

Hygienic behavior expressing has variation within and between honey bee strains. For example Russian honey bees genetically indicated high heritability of hygienic behavior (Danka *et al*. 2013).

Due to the complexity of the varroa resistance mechanisms in honey bees, these programs have not reached the ideal point (Dietemann *et al*. 2012). According the study by Rinderer *et al*. (2010), honey bees selected based on Varroa sensitive hygienic behavior in the United States were more resistant than those selected based on Hygienic behavior. However, Kirrane *et al*. (2015) claimed that Russian bees show Varroa sensitive hygienic behavior at a high level. Therefore, it can be postulated that the VSH behavior is an important mechanism for the resistance of bees to Varroa which is possible through the detection of infants infected with Varroa mite. It is not clear how to resist the Varroa mite in these bees, and the genomic perspective of the host-mite interaction process for protection was not yet been comprehensively explored (Broeckx *et al*. 2019; Mondet *et al*. 2021). The candidate gene approach is a molecular perspective to look for critical polymorphism primarily associated with a particular phenotype (Dick *et al*. 2015). Several studies have been performed to identify the genetic basis for Varroa mite resistance. Employing microarray technology and examination of both sensitive and resistant bees (which had not been treated for 11 years), Navajas *et al*. (2008) found 148 differentially expressed genes, among which 32 genes were related to the presence of Varroa mite, while 116 genes were related to bee genotype. The presence of Varroa mite altered the expression of genes involved in embryonic development, immunity, and cellular metabolism. Differences in gene expression in resistant bees were related to those genes that regulate nervous system development and neuronal and olfactory sensitivity. Oxley *et al*. (2010) used two groups of bees to determine effective QTLs for Hygienic behavior. The grouping was according to the removal speed of the pupae killed by the cold. They found three loci, one associated with the removal of dead pupae and the other two with uncapping. Four candidate genes related to Hygienic behavior were introduced. These genes were related to olfaction and learning and social behavior and circadian locomotion. Behrens *et al*. (2011) used resistant bees on Gotland Island to identify QTLs affecting reduced mite Reproduction, these bees had not been treated for Varroa mite for 10 years. In the relevant study, a microsatellite marker was used and two candidate genes for this behavior were introduced: the ortholog of the “foxo” gene, effective in insect growth and body size development, immune response, longevity, nutrition, cell death, and energy metabolism and the ortholog to the Drosophila gene “futsch”, involved in phosphorylation and the induction of synaptic plasticity in neurons. Tsuruda *et al*. (2012) found two effective QTLs on chromosomes 9 and 1 in their research to identify QTLs affecting Varroa sensitive hygienic behavior. The two most important candidate genes identified in their study were *NorpA2* and *DOP3* genes. According to the results of research related to Varroa mite resistance in the role of olfactory sense and related genes in Hy-

gienic behavior is emphasized (Navajas *et al*. 2008; Le Conte *et al*. 2011; Tsuruda *et al*. 2012). On the other hand, currently, transcriptome-based evidence pointed out bee antennae play an effective role in shaping the Varroa sensitive hygienic behavior as well as covering Varroa-infected cells (Mondet *et al*. 2015). In this story there is no receptor potential A (*NorpA2*) due to its homologous role in the smell of Drosophila melanogaster, it could play a role in the smell of bees. Although the dopamine receptor has been highlighted in previous studies for cognitive and olfactory learning, this gene could play a role in identifying infants infected with Varroa mite (Pak, 1979; Meyertholen *et al.* 1987; Riesgo-Escovar *et al*. 1995; Pollock *et al*. 2003). In Iran, Elmi (2019) for detection of QTL for Varroa mite resistance traits, correlated the SMR trait criteria, including the criteria for Number of productive mite, Number of offspring, fecundity and Percentage of infection to Varroa mite with six microsatellite sites (UN391, K0429, HQ7622, HQ7691, UN334d, UN086). They performed their studies on a population that had not been treated for Varroa for 3 years. Four loci showed a significant relationship with the measured phenotype; locus HQ7691 with number of productive mite, locus UN334d with number of productive mite and percentage of infection to Varroa mite and locus HQ7622 with all criteria. Locus UN391 between the two candidate genes (GB11764 ortholog Foxo gene, GB13873 ortholog Futsch gene in *Drosophila melanogaster* found in the study of Behrens *et al.* (2011)), showed a significant relationship with several phenotypic criteria. Apis 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase (known NorpA2, Gene ID: 408996), was located LG9 and this gene catalyzes the formation of inositol 1,4,5-trisphosphate and diacylglycerol from phosphatidylinositol 4,5-bisphosphate. This reaction uses calcium as a cofactor and plays an important role in the intracellular transduction of many extracellular signals in the retina. In Human, NorpA2 consisted 22 exons.

With this motivation, the overall purpose of the current work was to investigate the PCR sequence in the case-control application approach in the *NorpA2* gene and to determine the single nucleotide polymorphism in this gene according to this approach.

MATERIALS AND METHODS

The 3-step experimental design of the present study was as below

Step 1. Introducing sensitive and resistant groups of colonies to Varroa mite

We identified the resistant and sensitive colonies in our previous study (Elmi, 2019). The main aim of the mentioned study was to detect QTL associated with Varroa mite-resistance in Azarbaijan honey bee colonies.

As shown in equation 1, the rate of Varroa mite infection was used as the main criterion for determining the resistance of colonies in various regions of East Azerbaijan province, Iran. The criterion was based on the infection of drone pupae with Varroa mite.

$$IP = (NIC/NCC) \times 100 \qquad \text{Equation 1}$$

Where:
IP: percentage of infection.
NIC: number of infected cells.
NCC: number of cells checked.

Thus, having the lowest contamination of Varroa mite, the untreated hybrid colonies of A.M. Carnica × Iranian honey bee that survived for two years were selected as resistant colonies (RES). On the contrary, the highest rate of Varroa mite infection was observed for the colonies of Ajabshir region which were selected as Varroa-mite sensitive colonies (SUS). The Varroa mite reproductive intensity difference between the Ajabshir colonies and Hybrid colonies was statistically significant.

Step 2. Selection of susceptible and resistance individuals

Data on the number of Varroa mites were available for each drone pupae. Five most susceptible pupae to Varroa mite were selected from the SUS colonies and five most resistant pupae to Varroa mite were selected from the RES ones.

Step 3. Molecular PCR-sequencing technique

Molecular identification of candidate gene polymorphism was carried out using PCR-sequencing. The ten selected samples were transferred to a laboratory in a plastic tube containing 96% ethanol, where DNA extraction was carried out using the CTAB method. After the DNA extraction, the DNA quality was assessed using agarose gel electrophoresis and nanodrop. Samples with OD260 to OD280 ratios between 1.8 and 2 were submitted for polymerase chain reaction. The nucleotide sequences of the forward and reverse primers of the UTR and promoter regions of the candidate *NorpA2* gene located on chromosome 9 were then selected. The primer sequence was as follows (Cornelissen, 2015):

For the UTR of *NorpA2* gene:
5'-GGGTAAAGGCAGGTATCGTTTTT-3' ‘and 5'-ATCACCTTCCGTAGCAAAGTTC-3'

For the promoter of *NorpA2* gene:
5'-ACCTTGACGAATTGATATTCACG-3' and 5'-ACGAACGGACAGTGTAGAAGAAG-3'

The solution preparation and the thermal cycler program were optimized using the existing standard methods as were illustrated in Table 1.

Table 1 Temperature program used for PCR reaction

Reaction stage	Temperature ˚C	Time (min)	Number of cycles
Primary denaturation	94	5	1
Secondary denaturation	94	45^{***}	
Annealing	55^{*} 61.5^{**}	45^{***}	30
Extension/elongation	72	1	
Final elongation	72	10	1

* For promoter; ** For untranslated region (UTR) and *** Second.

Amplification reactions were done in volumes of 30 µL and contained on 15 µL master mix 1X (Ampliqon, Denmark), 1 pmol of each primer (Forward and Reverse), and 5.4 µL ddH_2O, and 9 µL of genomic DNA. PCR products were screened on horizontal electrophoresis using 1.5% agarose gel and ethidium bromide staining (the total length of each investigated PCR product is 750-900 bp). Observation of sharp, expected-size bands indicated the region-specific amplification. Thus, the PCR products were sent for Sanger sequencing. After sequencing and quality assessment of the sequences, the alignment was carried out using bioinformatics tools including MAFFT and BLASTn, With the BLAST program, nucleotide sequences were compared with recorded sequence databases in NCBI and the identity of PCR product sequences was assessed and MAFFT was used to align sequences and determine polymorphisms. The polymorphism and the SNP type between the RES and SUS groups were monitored. The phylogenetic tree was plotted using MAFFT and UPGMA methods to show the relationship between individuals.

RESULTS AND DISCUSSION

To genotype- resistant and susceptible individuals by PCR, the specificity of PCR amplification of individual DNAs was checked using agarose gel. According to Figure 1 (1.a; 1.b), the specificity of the two amplified regions of *NorpA2* gene (UTR and promoter) was determined to be optimal. Figure 1 illustrated Electrophoresis results of the PCR product of the *NorpA2* gene in 1.5% Agarose gel.

The resulted sequences from PCR products were aligned with BLAST and, finally the nucleotide differences were examined with the MAFFT software. Nucleotide differences between the SUS and RES groups were determined for UTR (as shown in Figure 2) and promoter (as shown in Figure 4) regions of *NorpA2* gene. Figure 3 and Figure 5 show phylogenetic trees made by UTR and promoter regions of the *NorpA2* gene, respectively, using the MAFFT software (https://mafft.cbrc.jp/alignment/server/). Phylogenetic tree for promoter region showed that individuals susceptible to Varroa mite are in the same group (Figure 5) and but for the UTR region, individuals were not divided into two distinct groups (Figure 3).

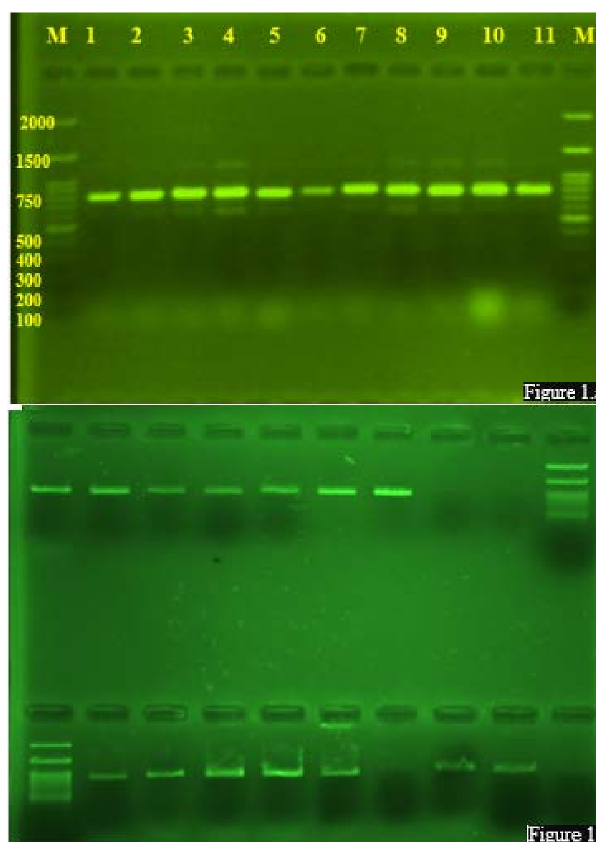

Figure 1 Electrophoresis results of the PCR product of the *NorpA2* gene in 1.5% Agarose gel (1.a: UTR region and 1.b: promotor region)

The examination of the MAFFT software edition showed the existence of differences in the nucleotide sequence of the *NorpA2* gene between the RES and SUS individuals as were reported in Tables 2 and 3. In this report, we examined the pattern and variability of both the promoter region and the UTR region *NorpA2* gene associated with genetic Varroa mite resistance. We hypothesized that the SNP pattern could be different in both resistant and susceptible individuals. The study results provided some interesting insights related to this trait. In this aspect, several polymorphisms, deletion and SNP, were observed in sequences of the *NorpA2* gene as shown in Tables 2 and 3. In the UTR region, despite the nucleotide diversity, more research is needed to obtain a reliable marker.

The results of this study showed Three Specific Difference in single nucleotide polymorphism in regions of the nucleotide sequence between the two groups (resistance and susceptible honey bees). This polymorphism was determined in the three promoter regions of the *NorpA2* gene at positions 308, 504 and 563 of the nucleotide sequence. On the other hand, the phylogenetic tree linked to the promoter region of the *NorpA2* gene showed that Varroa-resistant and Varroa-sensitive honeybees were divided into two separate groups.

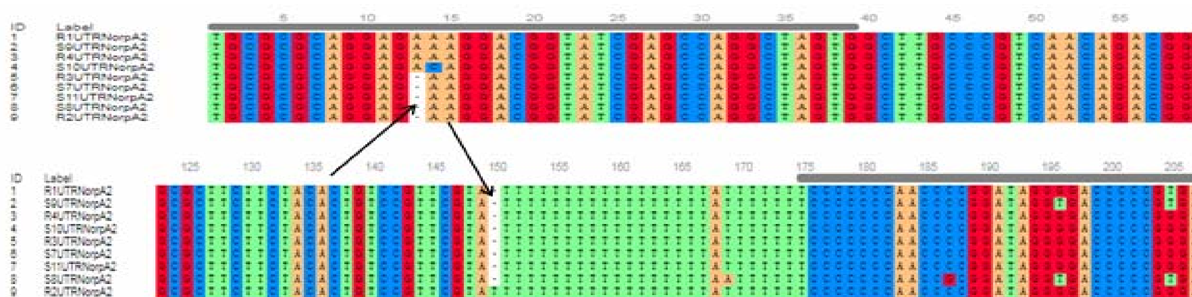

Figure 2 Alignment and nucleotide variation in the UTR region of *NorpA2* gene between SUS and RES honeybees
S: susceptible honeybee and R: resistant honeybee

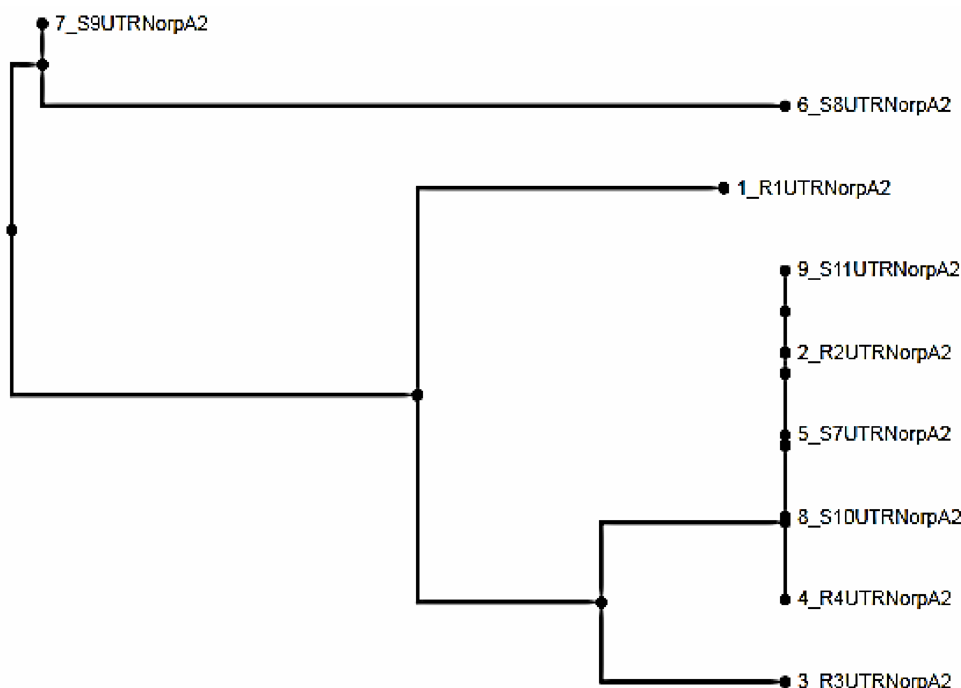

Figure 3 Family tree based on the sequence of UTR region of *NorpA2* gene
S: susceptible honeybee and R: resistant honeybee

Therefore, the result of this achievement can be used as a biomarker to identify honeybees that are resistant to Varroa mites. So far, no such SNPs of this gene have been described.

There are two similar studies on this. In initial research, in Haddad *et al.* (2016) project, the complete genome was sequenced, and all polymorphic regions were compared with the candidate genes found in other studies, and 44 genes were identified that potentially play a role in pathogen resistance in *Apis melliferea syriaca*. *NorpA2* was identified as a gene associated with Varroa-sensitive hygiene behavior. When comparing the genome of *Apis mellifera syriaca* (resistant to Varroa mite) with the reference genome of *Apis mellifera*, they found a SNP in intron 8 that was involved in the pathogen resistance.

In a second study, in the study of Cornelissen (2015), several groups of bees were compared, which are the details of these groups. In one group of Varroa mite-resistant worker bees, originating from Gotland colonies that had been transferred to the Netherlands and no treatment for these colonies. On the other hand, susceptible worker bees were selected from control colonies that were treated with oxalic acid twice a year and also the other group according to the percentage of drones with Varroa-sensitive hygiene behavior in artificial insemination as a resistant group and sensitive group (without artificial insemination with drone bees from colonies with Varroa-sensitive hygiene behavior) were selected. In this study, several candidate genes related to Varroa mite resistance behavior were examined as in the present study.

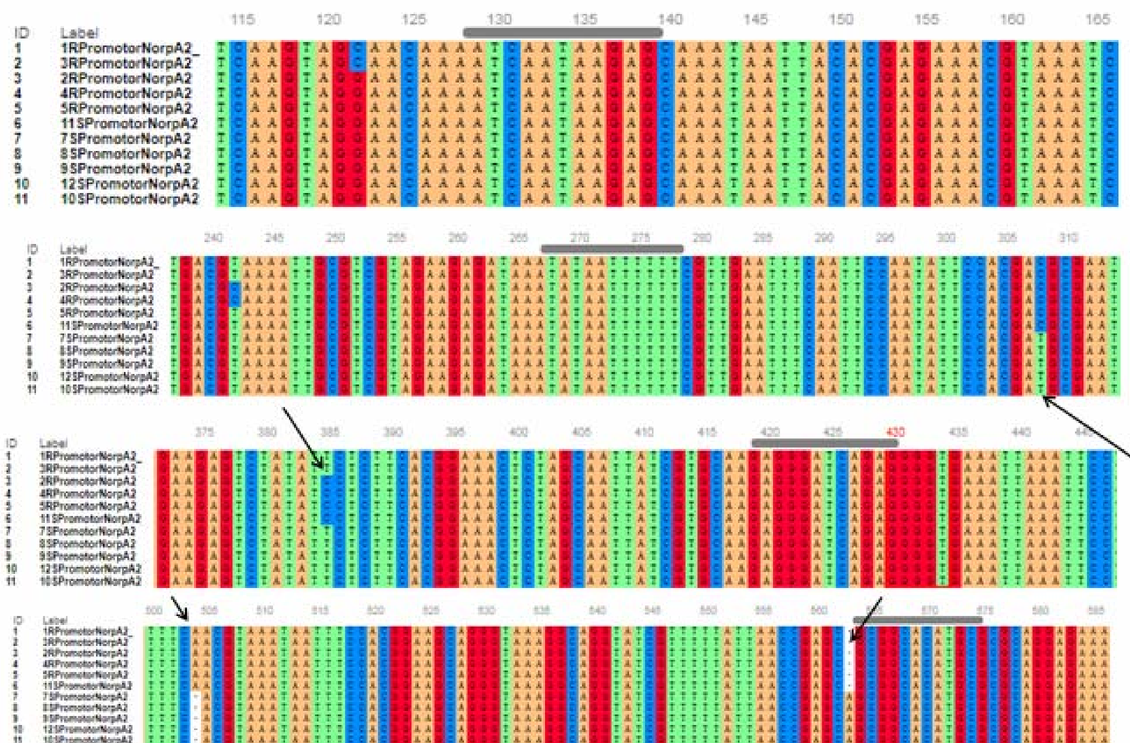

Figure 4 Alignment and nucleotide variation in the promoter region of *NorpA2* gene between SUS and RES honey bees
S: susceptible honeybee and R: resistant honeybee

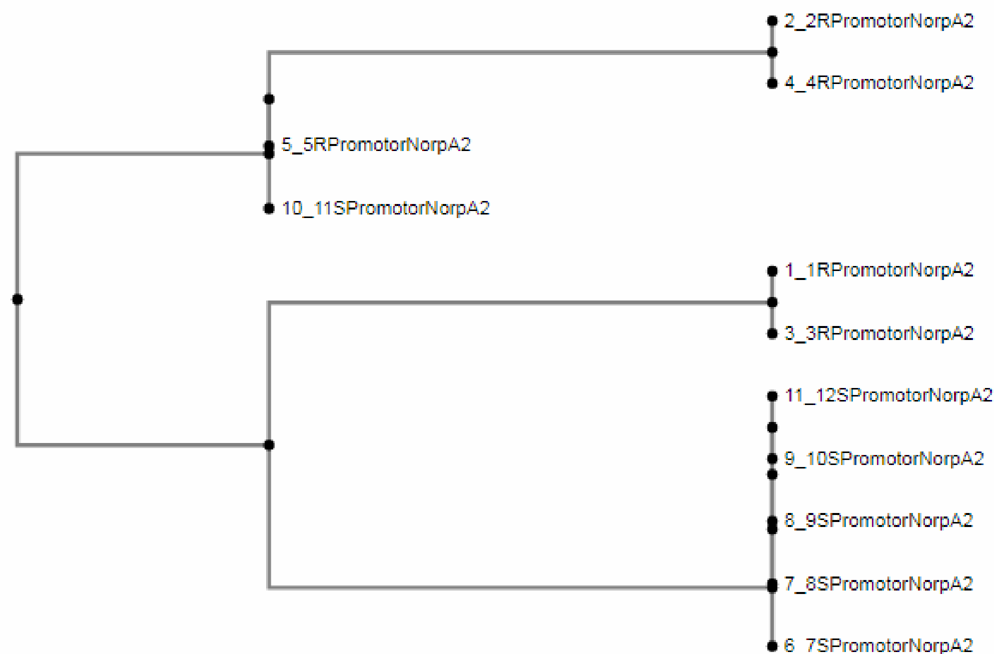

Figure 5 Family tree based on the sequence of promoter region of *NorpA2* gene
S: susceptible honeybee and R: resistant honeybee

Table 2 Sequence variation of the *NorpA2* gene, UTR region

Nucleotide region	Type of variation	Resistant colony	Sensitive colony
13	Deletion	2, 3	7, 8, 11
14	SNP(C)	-	10
150	Deletion	1, 3, 4	7, 8, 9, 10, 11
169	SNP(T)	-	8
187	SNP(G)	-	8
196	SNP(T)	-	8, 9
205	SNP(T)	-	8, 9

SNP: single nucleotide polymorphism.

Table 3 Sequence variation of the promoter region of *NorpA2* gene

Nucleotide region	Type of variation	Resistant colonies	Sensitive colonies
122	SNP(C/G)	C(1,3), G(2,4,5)	G(7,8,9,10,11,12)
242	SNP(C/T)	C(2,4), T(1,3,5)	T(7,8,9,10,11,12)
308	SNP(C/T)	C(1,2,3,4,5)	C(11), T(7,8,9,10,12)
385	SNP(C/T)	C(2,4,5), T(1,3)	C(11), T(7,8,9,10,12)
504	SNP(A)/Deletion	A(1,2,3,4,5)	A(11), Deletion(7,8,9,10,12)
563	SNP(A)/Deletion	Deletion(1,2,3,4,5)	Deletion(11), A(7,8,9,10,12)

SNP: single nucleotide polymorphism.

Cornelissen (2015) showed no change in the nucleotide sequence between the susceptible and resistant Varroa groups for the *NorpA2* gene.

One concern about the outcome of our findings was the small sample size of extremely tolerant and susceptible drones. The results of this study can understand the genetic mechanism of host resistance to mites based on the candidate gene approach. To the best of our knowledge, this is the first report on the identification of biomarkers with a candidate gene to control host mite interaction in the honeybee *Apis mellifera*. This is an interesting topic for future work. Because this experiment was performed with a small number of experimental samples, in order to use the results of this experiment, this experiment should be repeated with a larger number of samples. The main limitation of the current work was the low financial resources for conducting the research. This problem led to the use of fewer experimental samples. Therefore, it is recommended that this study be repeated on a larger scale.

CONCLUSION

On this basis, we conclude that the existence of Specified SNPs in three regions of the *NorpA2* gene promoter between the case-control groups that can be used in the molecular identification of Varroa-resistant colonies and breeding programs to produce Varroa-resistant colonies. Sequencing outputs briefly indicated that the UTR-*NorpA2* gene indicated different variations (SNP, deletion), and the promoter-*NorpA2* gene also indicated SNP in the samples examined. To the best of our knowledge, this is the first report on the identification of biomarkers with a candidate gene to control host mite interaction in the honey bee *Apis mellifera*.

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