Insight into the genetic basis of *Varroa destructor* resistance in *Apis mellifera*.

A two way approach

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Insight into the genetic basis of *Varroa destructor* resistance in *Apis mellifera*, a two way approach.

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Abstract

The *Varroa* mite has much impact on *Apis mellifera.* Due to this, breeding honey bees, which are resistant against *Varroa,* becomes more important. The aim of this study was to determine the genetic basis of *Varroa* resistance in European honey bee colonies. Two different research strategies were applied: (1) a candidate gene approach, to see whether there are sequence differences in candidate genes between resistant and non-resistant colonies, but also between the different types of resistance; and (2) a genome wide analysis using Random Amplified Polymorphic DNA (RAPD) PCR, detect new candidate regions in the genome. This will yield more insight into the genetic background of *Varroa* resistance in honey bees, which can help to reduce colony losses due to *Varroa*.

Using a RAPD based genome-wide approach, there was found evidence for the involvement of Prohormone-1. A scan for variation in several candidate genes yielded variation in Dop3 that is likely linked to *Varroa* resistance.

Breeding *Varroa* resistant honey bees is possible. The present study suggests the presence of genetic variation in Dop3 and Prohormone-1, which might be related to *Varroa* resistance in honey bees. This can contribute towards breeding *Varroa* resistant honey bees. Nowadays breeding honey bees is focussing on traits like honey production, gentle temper and low swarming tendency. Future research also can focus on correlation of *Varroa* resistance with these traits. This can prevent beekeepers from breeding *Varroa* resistant lines without production, gentle temper and/or low swarming tendency. Our study already suggests some genes coding for *Varroa* resistance that can be used for a first linkage study.

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1. Introduction

Colony Collapse Disorder is an increasing problem for the European honey bee (*Apis mellifera)* causing many colonies to die during winter (vanEngelsdorp, et al., 2008; 2009). The mite *Varroa destructor* is associated with Colony Collapse Disorder (vanEngelsdorp, et al., 2009). The mite reproduces during the development of pupae and larvae of the honey bee in sealed brood cells (Rosenkranz, et al., 2010). Control of the reproduction of the mite seems to be an important factor to maintain a stable host-parasite, honey bee – *Varroa* mite, relationship (Walter and Proctor, 1999). In addition, *Varroa* feeds on the hemolymph of the honey bee. This causes a decrease of the weight of the honey bee. Besides there is a decrease in water and protein content in the head and the abdomen (Bowen-Walker and Gunn, 2001). Together with these direct effects of *Varroa* mites on *Apis mellifera*, *Varroa* is also a vector for viruses that affect the honey bee, for example the deformed wing virus (Boecking and Genersch, 2008). Despite several studies show that viruses are a minor problem in beekeeping, the combination of viruses with *Varroa* seems to contribute to Colony Collapse Disorder (Rosenkranz, et al., 2010). Another important factor in the winter survival of the colonies seems to be the amount of mites in October, because honey bee colonies with a low *Varroa* mite infestation in autumn are more likely to survive the winter (Genersch, et al., 2010).

Mites are usually controlled using chemical acaricides (Rosenkranz, et al., 2010), but since the mites evolved to be resistant against chemical acaricides, these chemicals became less efficient (Sammataro, et al., 2005). Another consequence of using chemical acaricides is that residues can be found in the honey and pollen (Wallner, 1999). In addition, these chemicals might also have a negative effect on the honey bee health (Johnson, et al., 2009). As an alternative, organic acids and essential oils are used to control *Varroa* (Rosenkranz, et al., 2010). The advantages of these natural compounds are that there is a low risk of residues and accumulation in the wax and honey. Besides there is a low probability that *Varroa* develops resistance (Rosenkranz, et al., 2010). However, treatments with these natural compounds also have disadvantages. Some products are only effective under specific stages in the colony, for example when the colony is without brood. A good knowledge of the honey bee colony is required when using natural compounds. This means that the effectiveness of the natural compounds is variable (Rosenkranz, et al., 2010). Besides treatments with acaricides, biotechnical and biological methods are studied as well, including for example water plunging, heat treatment, ultrasound treatment and reduced brood cell size (Rosenkranz, et al., 2010). However, their results vary and more research is required to increase the effectiveness (Rosenkranz, et al., 2010). So to achieve a healthy honey bee colony and honey without residues of acaricides, a different approach of *Varroa* mite control is needed.

1.1 Breeding for resistance

Such an alternative approach could be breeding *Varroa* resistant honey bee colonies. The original host of *Varroa* is *Apis cerana* (Asian honey bee) and there is a stable host-parasite relationship between *Apis cerana* and *Varroa* (Koeniger, et al., 1983). However, the mite colonized the new host *Apis mellifera* about 30 years ago and since the co-evolution between *Apis mellifera* and *Varroa* has not yet been optimized. This means the *Varroa* mite still contributes to major colony losses (Walter and Proctor, 1999). There were two major experiments which try to reduce the harmful effects of *Varroa* on *Apis mellifera.* The first is a selection experiment performed in the U.S.A., where researchers selected colonies which perform the *Varroa* sensitive hygiene (VSH) behavior. This is a

form of hygienic behavior, which contains two major steps. The first step is the uncapping of the infected brood cell and the second step is the removal of the *Varroa* infected pupae out of the hive, preventing that the mite will reproduce (Harbo and Harris 2005, 2009). However, how the worker bees identify the infected brood cell stays unclear. A second experiment for breeding *Varroa* resistance in honey bees is about the isolated honey bee colonies on the island of Gotland in Sweden. This population has not been treated against *Varroa* for over ten years and hence only resistant colonies will survive (Fries, et al., 2006; Locke and Fries, 2011). This population also shows resistance against *Varroa,* however, the hygienic and grooming behavior of the honey bees has not increased in frequency. It was shown that the reproductive success of the mite was reduced (Locke and Fries, 2011). These two experiments show that possibly there are several mechanisms that can cause resistance against *Varroa* in the honey bee. These mechanisms causing resistance against *Varroa* in honey bees can be roughly divided into two types. The first type of resistance is the VSH behavior. The second type of resistance is the populations that show suppressed mite reproduction, the Gotland population, and for this population the mechanism(s) of resistance is not completely clear.

1.2 Candidate genes for resistance

For both types of resistance the genetic background was already studied through QTL and gene expression studies (Arechavaleta-Velasco, et al., 2012; Behrens, et al., 2011; Le Conte, et al., 2011; Navajas, et al., 2008; Oxley, et al., 2010; Tsuruda, et al., 2012; Zhang, et al., 2010). All data of these studies was put together and compared to see whether there were overlapping genes (Appendix 1). Between all studies four genes overlap, two between a gene expression study and a QTL study and two between different gene expression studies (Table 1).

The first gene that was found in two studies is *poly(ADP-ribose)glycohydrolase,* GB10249. This gene was found in Oxley, et al. (2010) and Navajas, et al. (2008). It was found to be homolog to the *Drosophila blue cheese* gene and is involved in several neurological processes like regulation of synapse organization and axonogenesis (Navajas, et al., 2008; Simonsen, et al., 2007). Furthermore this gene was found to be involved in compound eye development and determination of adult life span (Khodosh, et al., 2006; Simonsen, et al., 2007). *Poly(ADP-ribose)glycohydrolase* was found on chromosome 16 in the honey bee, in a region which successfully predict the level of hygienic performance of individuals (Oxley, et al., 2010). However the QTL for this chromosome was only suggestive and not significant (Oxley, et al., 2010). In Navajas, et al. (2008) this gene was found to be up-regulated in *Varroa* resistant honey bees. It is interesting to see that the gene expression of the *Dynein light intermediate chain (Dlic),* GB16748, the enhancher for *poly(ADP-ribose)glycohydrolase*, is down-regulated with *Varroa* infestation, but up-regulated when the honey bees are resistant, (Navajas, et al., 2008; Simonsen, et al., 2007). However, it might be that *Dlic* is linked to *poly(ADP-ribose)glycohydrolase*, because they are both on chromosome 16 within approximately 520.000 base pairs (bp). *Poly(ADP-ribose)glycohydrolase* might be a gene of interest with respect to *Varroa* resistance in honey bees, because it was found in a region which predicts the level of hygienic performance of individuals, VSH is a kind of hygienic behavior, and the expression of the gene was found to be up-regulated in *Varroa* resistant honey bees (Navajas, et al., 2008; Oxley, et al., 2010).

The second gene that was found is GB14290, uncharacterized, found in Navajas, et al. (2008), Tsuruda, et al. (2012) and Zhang, et al., (2010). The *Drosophila* homolog, *stretchin-myosine light-chain kinase* (*strn*mclk*) is likely involved in visual long-term memory (Jiang, et al., 2011). This gene

was found to be down-regulated in *Varroa* resistant honey bees, but also in honey bees that are infected with *Varroa* (Navajas, et al., 2008). Tsuruda, et al. (2012) found this gene in the region with high LOD-scores. Furthermore this gene is found to be upregulated in *Apis cerana*, Asian honey bee, in prepupae challenged for eight hours with *Varroa* (Zhang, et al., 2010). *Apis cerana* is more hygienic than *Apis mellifera* which may contribute to its resistance against *Varroa* (Peng, et al., 1986). GB14290 might be a gene of interest for VSH behavior.

The third gene, found in two expression studies, was *Down syndrome cell adhesion molecule (Dscam),* GB15141, (Le Conte, et al., 2011; Navajas, et al., 2008). This gene is associated with axon guidance and mushroom body development (Schmucker, et al., 2000; Zhan, et al., 2004). A down regulation of the gene was found in *Varroa* resistance honey bees and in VSH honey bees (Le Conte, et al., 2011; Navajas, et al., 2008). Although this gene was only found in gene expression studies it might be a gene of interest due to its location. The gene is located on chromosome 4 and this chromosome might play a role in honey bees that suppress the *Varroa* reproduction (Behrens, et al., 2011).

As stated in Tsuruda, et al. (2012) no overlapping genes were found between the QTL studies for the VSH population, Tsuruda, et al. (2012) and for the Gotland population, Behrens, et al. (2011). Both honey bee populations are intensively studied and well preserved. For our study promising genes were selected from both, Tsuruda, et al. (2012) and Behrens, et al. (2011). Genes can be promising because they had a high LOD-score or they might have a function that might be associated with *Varroa* resistance.

It is expected that the *Varroa* resistance in the VSH population starts with an olfactorian cue (Tsuruda, et al., 2012). For this reason *no receptor potential A2 (norpA2),* GB14619, was selected for detailed analysis in our study. In *Drosophila* this gene is associated with vision and olfaction (Kim, et al., 2010). The second gene selected for detailed analysis in our study is *D2-like dopamine receptor (Dop3),* GB14561. This gene was located closest to the maximum LOD-score found in the study of Tsuruda, et al. (2012). Dopamine is an important neurotransmitter and is involved in many processes in the central nervous system including control of movement, cognition and affects neuroendocrine secretion (Fumagalli, et al., 1998). It was shown that D2-like dopamine receptor is involved in olfactory memory and learning (Sandoz 2011).

Behrens, et al. (2011), found one gene overlapping with Navajes, et al. (2008). This is the GB11509, uncharacterized, gene which is ortholog to the *Drosophila futsch* gene (Behrens, et al., 2011). The *futsch* gene is found to be involved in the phosphorylation and induction of the plasticity of the synaps in neurons and is down regulated in nonneuronal tissue during development (Hummel, et al., 2000). Another gene that is selected from this study is *forkhead box protein O (foxo),* GB11764. In invertebrates this gene is involved in regulating life span, body size, organ size and cell size (Finch and Ruvkun, 2001; Tatar, et al., 2001).

Table 1. Overlapping genes with name and function.

Honey bee gene ID	Chromosome	Name	Function
GB14561	9	*D2-like dopamine receptor (Dop3)*	D2-like dopamine receptor
GB14619	9	*No receptor potential A2 norpA*	associated with vision and olfaction
GB14290	9	*Stretchin-myosine*	Visual long-term memory

		*light-chain kinase (Strn*mlck)*	
GB15141	4	*Down syndrome cell adhesion molecule (Dscam)*	Axon guidance and mushroom body development
GB11509	7	*Futsch*	Phosphorylation and induction of the plasticity of the synaps
GB11764	7	*Forkhead box protein O (Foxo)*	regulating life span, body size, organ size and cell size
GB10249	16	*Poly(ADP-ribose)glycohydrolase (Bchs)*	Determination of adult life span
GB16748	16	*Dynein light intermediate chain (Dlic2)*	Enhancer for *Bchs*

Table divided into two categories of interest, based on VSH behavior.

The aim of this study was to determine the genetic basis of *Varroa* resistance in European honey bee colonies. Two different research strategies were applied: (1) a candidate gene approach, to see whether there are sequence differences in candidate genes between resistant and non-resistant colonies, but also between the different types of resistance; and (2) a genome wide analysis using Random Amplified Polymorphic DNA (RAPD) PCR, detect new candidate regions in the genome. This will yield more insight into the genetic background of *Varroa* resistance in honey bees, which can help to reduce colony losses due to *Varroa*.

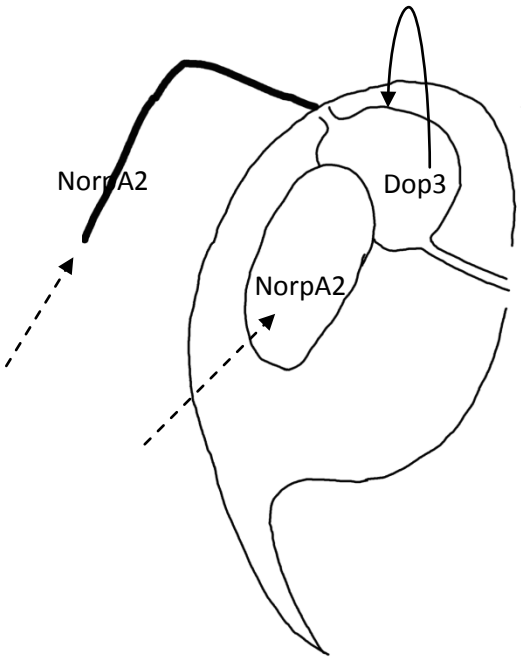

Figure 1. Hypothetic NorpA2 and Dop3 pathways in the honey bee head.
Dotted arrows indicated signal from closed brood cells with a *Varroa* mite in it. Arrow from the brain to the brain the is the signal Dop3 is given to induce VSH behaviour.

An overview of candidate genes is shown in Table 1. The main focus of our study will be on VSH bees. The first gene of interest for these honey bees will be the Dop3 gene. The Dop3 is considered a candidate gene, because of its location in respect to highest LOD-score in the QTL study of Tsuruda et al. (2012). Dop3 encodes for a dopamine receptor (Beggs, et al., 2005). Dopamine is a neurotransmitter and it controls a variety of functions like, locomotor activity, cognition and positive reinforcement (Missale, et al., 1998). These are traits which might be important for performing the VSH behavior (Tsuruda, et al., 2012). Whenever the VSH behavior is approach from the cue onwards NorpA2 might be more important. As mentioned before NorpA2 is associated with vision and olfaction. It might be that the VSH bees see or smell the mites and react on this (Figure 1) (Tsuruda, et al., 2012).

2. Material and Methods

2.1 Collection of Honey bees

For this study three resistant honey bee populations were selected. The first population consists of colonies inseminated with VSH semen. The second population consist of colonies that have a low ratio reproductive mites : non-reproductive mites. The third population consists of colonies that survived over 10 years without treatment against *Varroa* (Table 2).

2.1.1 Collection of VSH bees

No 100% VSH material was available, but artificial single drone insemination did result in 50% and 75% VSH material. Normally a queen mates with multiple drones, so the genetic background of the offspring is unknown. When single drone insemination is applied, all offspring are related to this single drone and the genetic background of the offspring is uniform and known. Because there was 50% and 75% material available, both types were used to see whether there is a genetic difference between these types. These honey bees were sourced from a single beekeeper, Renaud Lavend'homme, and were collected on 9 March 2014 at Braine-le-chateau, Belgium. These samples were collected by opening three brood cells of larval stage worker honey bees and put into 10 ml tubes with 96% ethanol.

2.1.2 Collection of honey bees with reduced reproductive Varroa counts

Besides the VSH bees, samples with reduced reproductive *Varroa* counts were used. The ratio between reproductive mites and non-reproductive mites in the closed brood cells was calculated after opening the brood cells and counting the mites. There was shown that the ratio between reproductive mites and non-reproductive mites is lower for VSH honey bees compared to non-VSH honey bees. However, no studies were performed to see whether this change in ratio also occurs in the unknown phenotypes of resistance compared to non-resistant honey bees as well (Ibrahim and Spivak, 2006). These honey bees were all related (Appendix 3) and the breeding was done by single drone insemination, which makes the genetic background of these honey bees very uniform. These honey bees were sourced from a single beekeeper, Bart-Jan Fernhout, and were collected in August 2015 at Boxmeer. These samples were collected by opening multiple brood cells of almost developed worker honey bees and put into 100 ml tubes with 96% ethanol.

2.1.3 Collection of honey bees with suppressed mite reproduction

This is an experiment started by Bijen@wur, a Dutch beekeeping research station, and mimics the situation on the island of Gotland (T. Blacquiere, personal communication). These honey bees are not treated against *Varroa* and only the surviving colonies are selected for further use in the experiment. In this way they attempt to breed *Varroa* resistance in these honey bee colonies. The honey bees are located at two sites in the Netherlands, one population is at the island of Tiengemeten the other population is located at the Amsterdamse Waterleidingduinen (AWD) in summer and in Lelystad in the winter. The population, which is located at Tiengemeten, likely has some genetic material of the Gotland population, because these colonies are offspring of those from Gotland. However, no control colonies, colonies treated against *Varroa*, are available. From the population, which is located at AWD, a control line is available. These control colonies are treated with oxalicacid twice a year, in December and July when the colony is without brood. From each colony 5 adult worker honey bees were taken from the frames and put into a 10 ml tube filled with 96% ethanol.

The VSH inseminated and reduced reproductive mite counts honey bees were selected for our study, because the genetic background of both groups is uniform due to the single drone inseminations. From the mite suppressed reproduction honey bees a previous BSc thesis study showed ample genetic variation between colonies, which makes it difficult to determine which variation is due to *Varroa* resistance (Lie, 2014).

Table 2. Overview of samples used.

Sample number	Sample type	% of genetic contribution VSH lines	% reduction of reproductive mites
46	VSH inseminated[1]	50	-
47	VSH inseminated[1]	75	-
48	VSH inseminated[1]	50	-
49	VSH inseminated[1]	0	-
50	VSH inseminated[1]	0	-
408	Reduced reproductive mite counts[2]	-	87.5
409	Reduced reproductive mite counts[2]	-	0
412	Reduced reproductive mite counts[2]	-	75
413	Reduced reproductive mite counts[2]	-	0
414	Reduced reproductive mite counts[2]	-	0
420	Reduced reproductive mite counts[2]	-	0
421	Reduced reproductive mite counts[2]	-	0
SMR	Suppressed mite reproduction[3]	-	-

Overview of samples used with estimated percentage of resistance. Estimation of resistance in two ways: VSH inseminated, based on times inseminated with VSH semen, reduced reproductive mite counts, based on the reduction of reproductive mites compared to control lines. 1. Renaud Lavend'homme, Belgium, 2. Bart-Jan Fernhout, the Netherlands, 3. Bijen@wur, the Netherlands.

2.2 DNA extraction

Genomic DNA extraction was based on the DNA extraction using Cetyl trimethylammonium bromide (CTAB) in the Coloss Bee Book (Evans, et al., 2013) with the modifications described below. The abdomen of the honey bee was air dried and put into 1.5 ml safe lock Eppendorf tubes together with 2 mm glass beads. The abdomen was grinded by putting the tubes first in liquid nitrogen and second in the beadbeater for 10 seconds two times. To each tube 500 µl 2x CTAB (Merck; Darmstadt, Germany), 2.5 µl proteinase K (20 mg/ml) (Merck; Darmstadt, Germany), 4 µl RNase cocktail (100 mg/ml) (Sigma Aldrich Chemie b.v.; Zwijndrecht, the Netherlands) and 2 µl 2-mercaptoethanol (0.2%) were added. This content was mixed by vortexing and incubated at 60 °C overnight while shaking.

After incubation samples were centrifuged at maximum speed, 13000 rpm, and the liquid was transferred to a fresh tube. To this solution 500 µl of phenol:chloroform:isoamyl (Sevag 24:1) was added. The content of the tubes was mixed by inverting 20 times. After mixing the tubes were centrifuged for 15 minutes at maximum speed. The upper phase was transferred into a fresh tube and 500 µl cold isopropanol and 50 µl 3M NaAc were added. The content of the tubes was again mixed by inverting 20 times and tubes were placed into -20 °C for incubation of 30 minutes. After incubation samples were centrifuged at maximum speed at 4°C for 30 minutes. The liquid was pipetted of the pellet and 1 ml EtOH (70%, 4°C) was added. The samples were centrifuged for 3 minutes at 4 °C. The pellets were washed with EtOH for a second time and afterwards pellets were air dried. When dry, pellets were resuspended into 75 µl MQ water. The amount and quantity of the DNA was measured by using the Nanodrop 2000 spectrophotometer (Fisher Scientific; Landsmeer, the Netherlands).

2.3 Genome wide approach

2.3.1 RAPD-PCR

RAPD-PCR was performed using a My cycler thermo cycler (Biorad; Veenendaal, the Netherlands) or T gradient thermo cycler (Biometra, Westburg; Leusden, the Netherlands). Amplification reactions were performed in volumes of 50 µl and contained 5x GoTaq buffer, 10 mM dNTPs, RAPD primer (Table 3),Taq Polymerase, 25 mM $MgCl_2$ and 10-20 ng DNA. For the negative control MQ water was used. The used RAPD primers are given in Table 3. The reaction conditions were as follows: denaturation at 94°C for 5 minutes, followed by 94°C for 1 minute, 35°C for 1 minute and 72°C for 2 minutes for 45 cycles and this was followed by an elongation at 72°C for 5 minutes. After PCR, 20 µl of the products were used for electrophoresis at 2% agarose gel prestained with Gelred (VWR international; Amsterdam, the Netherlands). It was photographed under ultraviolet light. Fisher 250 bp scientific ladder (Invitrogen, Fisher Scientific; Landsmeer, the Netherlands) was used as a marker.

Table 3. Used RAPD primers

Primer Name	Sequence (5' – 3')
OPA-05	AGGGGTCTTG
OPA-07	GAAACGGGTG
OPA-09	GGGTAACGCC
OPA-18	AGGTGACCGT

2.3.2 Sequencing

Polymorphic fragments of electrophoresis were cut out when they only appeared in resistant samples. These fragments were purified using the *GenElute™ PCR Clean-Up Kit* (Bioké; Leiden, the Netherlands). In short; the gelpiece containing the fragment of interest was put in binding buffer and heated to 50 °C until the piece was totally dissolved. This was pipetted into an Eppendorf tube with a filter and centrifuged. The filter was washed two times with washing buffer, to remove salts. Finally the DNA was eluted in MQ water and send for sequencing at Eurofins Genomics (Ebersberg, Germany).

2.4 Gene specific approach

2.4.1 Primer design

Primers were designed using Primer3 free software (Untergrasser, et al., 2012). Primer length was set between 20 bp and 25 bp with an optimum of 22 bp, a *Tm* difference of 1 °C, and the size of the amplified fragment was set between 750 – 950 bp. The resulting primers were blasted in NCBI (Ye, et al., 2012) to see the specificity of the products the primers gave. They were checked in the Beacon free software (Premier Biosoft International n.d.) for primer-dimer effects and hairpins.

Table 4. Primers used

Area	Name	Linkage group	Forward primer (5' – 3')	Reverse primer (5' – 3')
GB14561 (~1-800)	UTR Dop3	9	CATCCCTCCCCGATCT CACAC	ATCCGCACCAAGAGAG GATTACT
GB14619 (~1-800)	UTR NorpA2	9	GGGTAAAGGCAGG TATCGTTTT	ATCACCTTCCGTAG CAAAGTTC
GB14561 (~-500-260)	Promotor Dop3	9	CCCAATTAAAACTT CTTCCAAGC	ACACCCTGTTGTTG TTGTTACAGT

GB14619 (~-670-110)	Promotor NorpA2	9	ACCTTGACGAATTG ATATTCACG	ACGAACGGACAGTG TAGAAGAAG
GB14561 (exon 2)	Exon 2 Dop3	9	AGTTCACGGTTCCA TTCAGATT	ATCCAAGGAGCGTT AAAAGTCA
Prohormone-1	Region 1 Prohormone-1	11	GGTTTGTTTTGTTT TCTTTGTTACG	ACATATTGAATACGA AGCGAATCTT
Prohormone-1	Region 2 Prohormone-1	11	GCTCTCTTTCCTTT GTCGTTGTAT	CTCTCTCTCACTTCT CAAATCTCAAG

2.4.2 PCR of candidate genes

PCR was performed using a T gradient thermo cycler (Biometra, Westburg; Leusden, the Netherlands). Amplification reactions were done in volumes of 25 µl and contained on average 5x GoTaq buffer, 10 mM dNTPs, gene specific primer (Table 4), Taq Polymerase and approximately 30 ng DNA. The reaction conditions were as follows, depending on the primer pair used: denaturation at 94°C for 5 minutes, followed by 94°C for 45 seconds, annealing at 54-61.5°C (depending on the primers used) for 45 seconds and 72°C for 1 minute for 30 cycles and this was followed by an elongation at 72°C for 10 minutes. Timing was for all reactions the same, specific PCR conditions are given in Appendix 2. After PCR, 5 µl of the products was used for electrophoresis. The gel used was a 1% agarose gel prestained with Gelred (VWR international; Amsterdam, the Netherlands). It was photographed under ultraviolet light.

2.4.3 Sequencing

PCR samples were purified using the *GenElute™ PCR Clean-Up Kit* (Bioké; Leiden, the Netherlands). In short; the PCR product was bound to the filter with binding buffer. It was washed two times with washing buffer, to remove salts. The PCR product was eluted in MQ water and send for sequencing at Eurofins Genomics. Sequences were aligned using Clustal Omega (School of Medicine and Medical Science, UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Ireland 2013) and analyzed in Mesquite (Maddison and Maddison 2015). When analyzing SNP and indels were relation to *Varroa* resistance was studied. Variations were considered related to *Varroa* resistance when the resistant samples had a SNP or indel compared to the non-resistant samples. Comparison was separated for the VSH inseminated and the reduced mite counts samples.

2.4.4 Cloning of PCR fragments

2.4.4.1 Ligation

Ligation was performed by using the following reaction; 5 µl of 2x Ligase buffer, 1 µl of pGEM-Tvector, 1 µl of T4 DNA Ligase and up to 3 µl purified PCR product. For amount of PCR product added the following formula was used:
$\left(\frac{ng\ of\ vector\ (45) \times kb\ size\ of\ insert\ (0.750)}{kb\ size\ of\ vector\ (3)}\right) \times \left(\frac{insert\ (3)}{vector\ (1)}\right) \approx 33.75\ ng\ of\ insert$. Mixture is placed overnight at 4 °C.

2.4.4.2 Transformation

All steps were performed on ice unless mentioned otherwise. 2 µl of ligation mixture was added to competent cells and the mixture was transferred into the elecrophoration cuvette. The cuvette was placed into the Micro pulser (BioRad; Veenendaal, the Netherlands), room temperature, and a pulse of 1.8 kV was given. Immediately SOC medium, preheated to 37 °C, was added and the cells were placed at 37 °C while gently shaken (150 rpm) to recover for an hour. An undiluted, 1:10 diluted and 1:100 diluted cells were plated on LB + Ampiciline, X-Gal and IPTG and left overnight at 37 °C to grow.

2.4.4.3 Sequencing

5 colonies per transformation from diverse dilutions were selected. From these colonies the plasmid was isolated with the *NucleoSpin® Plasmid/Plasmid (NoLid) protocol* (Bióke; Leiden, the Netherlands). In short; the *E.coli* cells were separated from the LB medium by centrifuging (30 seconds, 11000 rpm). Three buffers were added to separate the plasmid DNA from other *E.coli* cell compartments. After centrifuging (10 minutes, 11000 rpm) the plasmid DNA remains in the supernatant, which is decant in a filter. The plasmid DNA binds on the filter and is washed with two different washing buffers to remove salts. The plasmid DNA is diluted in MQ water and send for sequencing at Eurofins Genomics (Ebersberg, Germany). Sequence analysis was performed as mentioned before.

3. Results

3.1 Genome wide approach

All tested RAPD primers showed polymorphism between, and sometimes within, the colonies, but the degree of polymorphism differs between the primers. OPA5 showed a lot of polymorphism within but also between colonies (Figure 2). OPA7 showed less polymorphism compared to OPA5 and only for the VSH inseminated samples (Figure 3). OPA7 primers did not produce fragments in the samples with reduced reproductive mite counts and was not tested for the samples with suppressed mite reproduction. OPA 9 mainly showed monomorphic bands pattern between colonies and within colonies, however, one polymorphic fragment was found in 50% and 75% VSH resistance (Figure 4). This fragment was not found in the control samples of the VSH colonies (sample 49 and 50). Sequencing analysis showed this fragment to be on chromosome 13 downstream of LOC100578886, odorant receptor 46a. OPA18 showed a polymorphic band pattern. Although some polymorphic fragments were found in both, control lines and resistant lines, or just in a single sample, one polymorphic fragment was found in two resistant samples of the VSH inseminated samples (47 and 48) and in one resistant sample of the reduced reproductive mite counts samples (412), but not in the control samples (Figure 5). After sequencing, the fragment was found to be on chromosome 11 in Prohormone-1 in intron 2 (Figure 6).

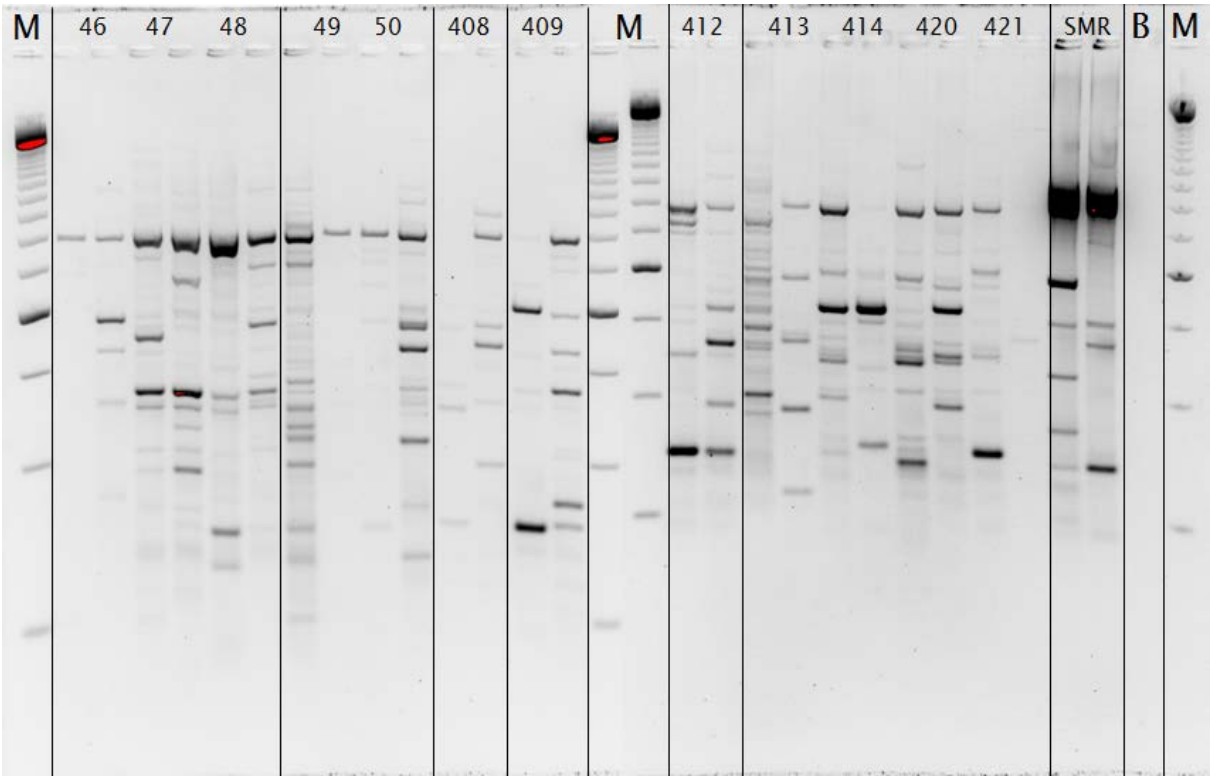

Figure 2. Electrophoresis results OPA5, visualised on 2% agarose gel.
Figure is composed out of 2 separate agarose gels.
M = 250 bp ladder
B = negative control (MQ water)
SMR = Suppressed mite reproduction, samples of bijen@wur
Samples: 46 and 47: 50% VSH, inseminated, 47: 75% VSH, inseminated, 49 and 50: Control of VSH colonies, 408 and 412: 87.5% and 75% reduction in ratio reproductive : non-reproductive mites respectively, 409, 412 – 414, 420 and 421: control of colonies with reduced ratio reproductive : non-reproductive mites.

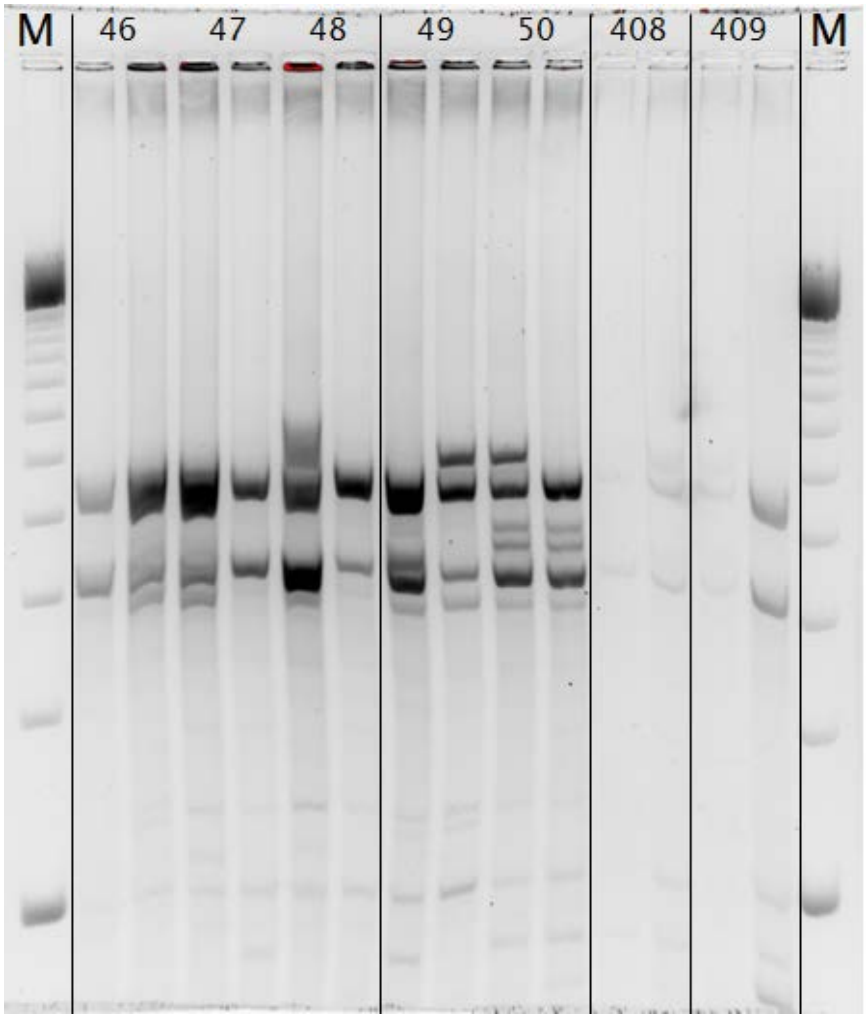

Figure 3. Electrophoresis results OPA7, visualised on 2% agarose gel.
M = 250 bp ladder
Samples: 46 and 47: 50% VSH, inseminated, 47: 75% VSH, inseminated, 49 and 50: Control of VSH colonies, 408: 87.5% reduction in ratio reproductive : non-reproductive mites, 409: control of colonies with reduced ratio reproductive : non-reproductive mites.

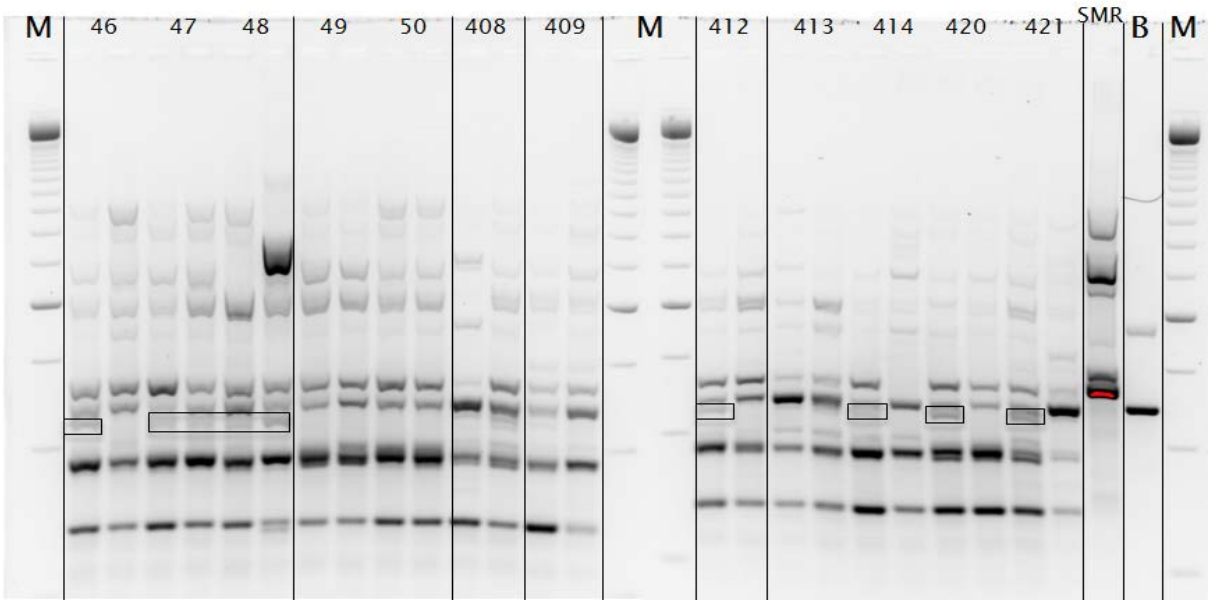

Figure 4. Electrophoresis results OPA9, visualised on 2% agarose gel.
Figure is composed out of 2 separate agarose gels.
M = 250 bp ladder
B = negative control (MQ water)

SMR = Suppressed mite reproduction, samples of bijen@wur
Samples: 46 and 47: 50% VSH, inseminated, 47: 75% VSH, inseminated, 49 and 50: Control of VSH colonies, 408 and 412: 87.5% and 75% reduction in ratio reproductive : non-reproductive mites respectively, 409, 412 – 414, 420 and 421: control of colonies with reduced ratio reproductive : non-reproductive mites.

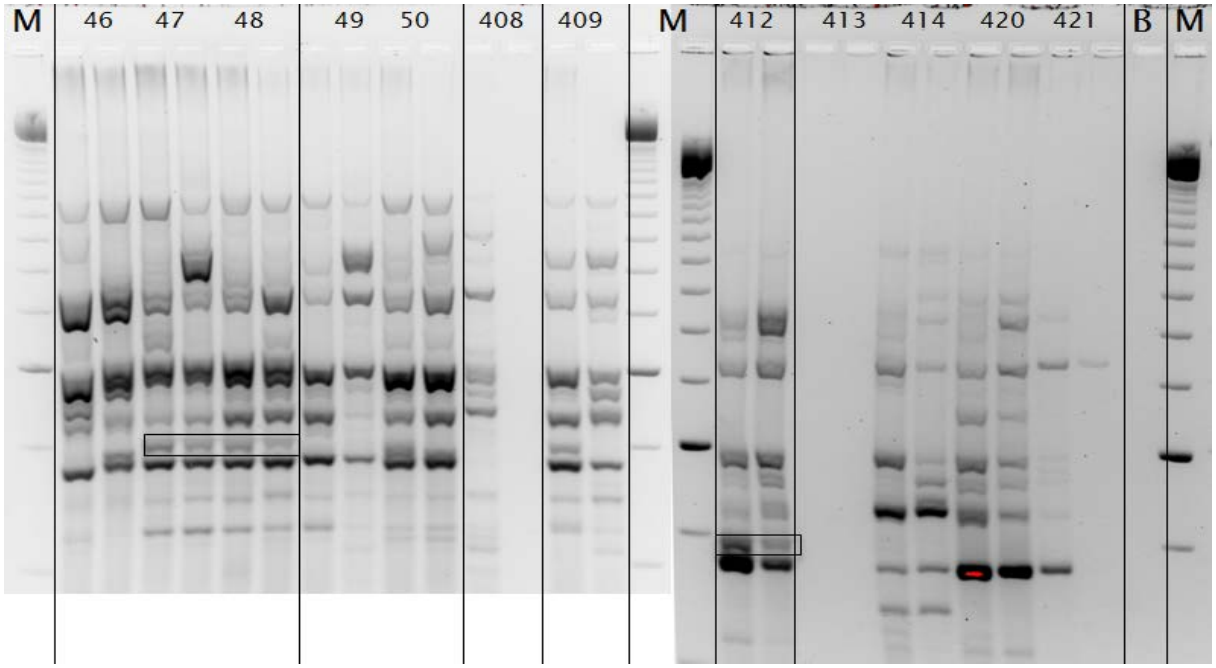

Figure 5. Electrophoresis results OPA18, visualised on 2% agarose gel.
Figure is composed out of 2 separate agarose gels.
M = 250 bp ladder
B = negative control (MQ water)
Samples: 46 and 47: 50% VSH, inseminated, 47: 75% VSH, inseminated, 49 and 50: Control of VSH colonies, 408 and 412: 87.5% and 75% reduction in ratio reproductive : non-reproductive mites respectively, 409, 412 – 414, 420 and 421: control of colonies with reduced ratio reproductive : non-reproductive mites.

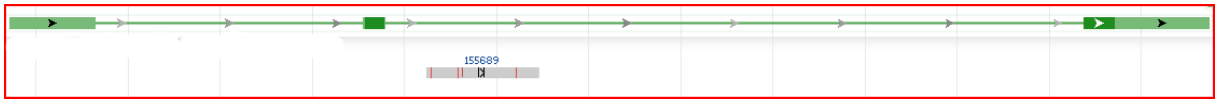

Figure 6. Location of RAPD-PCR products in Prohormone-1.
The green line is Prohomone-1, boxes indicating exons; the grey box is the fragment of the RAPD-PCR.

3.2 Gene specific approach

In total seven regions were sequenced, divided over three candidate genes. In some regions multiple sequence variations were found, but in other regions no sequence variation was found. Sequence variations were related to *Varroa* resistance when the sequence of resistant samples was different from the sequence of non-resistant samples.

3.2.1 Dop3

For Dop3 three regions were sequenced, starting with the UTR region of Dop3. This region showed a double signal that started at different locations (Table 5). A double sequence signal indicates the presence of an insertion or deletion. The first starting point of this double signal was detected at approximately 185 bp downstream of the forward sequence and was found in two of the resistant samples (46 and 47, 50% and 75%, inseminated with VSH semen), while the control samples only gave single signals. The second location where a double signal started was found in the samples with reduced mite counts at approximately 370 bp downstream of the forward sequence. This double

signal was found in sample 408 (resistant), 409 (non-resistant) and 420 (non-resistant). The second Dop3 region sequenced was the 500 bp upstream of the UTR region (Table 6). Also here a double signal was found, indicating an insertion or deletion. After cloning this fragment, a single sequencing signal was found in all samples with sequence differences at several points (Table 6), indicating genetic differences at multiple sites. At 313 bp downstream of the reverse sequence, length variation was found in a Thymine/Cytosine repeat between all samples. The two control samples of the VSH inseminated group showed the longest repeat which was approximately 8 bp longer than the repeat found in the other samples. This 8 bp elongation in the control samples was also an elongation with respect to the *Apis mellifera* reference sequence (NCBI, 2014; Weinstock, et al., 2007). Variation also was found in a polyT region at approximately 425 bp downstream of the reverse sequence. Two *Varroa* resistant samples of the VSH inseminated group (46 and 47, respectively 50% and 75%) have mainly the same deletions. 455 bp downstream of the reverse sequence is another Thymine/Cytosine repeat and here the 50% and 75% samples, 46 and 47, appear to have a deletion. Also after 705 bp downstream of the reverse sequence an insert of Adenine-Guanine in two of the resistant samples (46 and 47) was found. Further no variations were found between the *Varroa* resistant and control samples. The last region sequenced for Dop3 was within exon 2. No difference in the DNA sequences was found between the resistant and the control samples.

Table 5. Double signals found in the UTR region of Dop3.

		VSH inseminated colonies		**Reduced mite counts**	
Downstream location from UTR Dop3 forward sequence	**Type of variation**	**Resistant colonies**	**Sensitive colonies**	**Resistant colonies**	**Sensitive colonies**
~180 bp	Double signal	Yes (46, 47), No (48)	No	No	No
~370 bp	Double signal	No	No	Yes (408), No (412)	Yes (409, 420) and No (413, 414, 421)

Table 6. Sequence variation found in 500 bp upstream from the Dop3 gene

		VSH inseminated colonies		**Reduced mite counts**	
Downstream of reverse sequence	**Type of variation**	**Resistant colonies**	**Sensitive colonies**	**Resistant colonies**	**Sensitive colonies**
90	TCG repeat	Elongation (47)	-	-	-
240	A/T SNP	T	T	T	A (420), T (409, 413, 414, 421)
275	A/G SNP	A	G	A	G (409), A (413, 414, 420, 421)
310	CT repeat	-	Elongation	-	-
425-450	polyT area		-	-	-
510	T/C SNP	T (46,47), C (48)	C	T (408), C (412)	T (409, 413), C (414,420,421)
550	G/T SNP	T (48), G (46, 47)	G	T (408), G (412)	T (409, 413), G (414, 420, 421)
590	TTC repeat	Elongation (48)	Elongation	Elongation (408)	Elongation (409, 413, 414, 420)
640	PolyA	-	-	Deletion GAA (412)	Deletion GAA (421)
670	C/T SNP	T	T	C (408), T (412)	T
705	Insertion	AG (46, 47)	-	-	-
710	A/G SNP	A (46, 47), G (48)	G	A (412), G (408)	A (421), G (409, 413,414, 420)

A = Adenine, C = Cytosine, G = Guanine and T = Thymine

3.2.2 NorpA2

For NorpA2 two regions were sequenced. The first region sequenced was the UTR region in which two polyT regions were found. The first one is located 170 bp downstream of the forward sequenced and the second one 690 bp downstream of the forward sequence. The second region that was sequenced was approximately the 600 bp upstream of the UTR region. In this region differences were found at four locations. These differences were found in the samples with the reduced mite counts, but could not be related to *Varroa* resistance (Table 7).

Table 7. Sequence variation found in 500 bp upstream from the NorpA2 gene

		VSH inseminated colonies		Reduced mite counts	
Downstream of forward sequence	**Type of variation**	**Resistant colonies**	**Sensitive colonies**	**Resistant colonies**	**Sensitive colonies**
130	C/G SNP	C (46, 48), G (47)	C	C (408), G (412)	C (413, 421), G (409, 414, 420)
320	C/T SNP	C	C	C	T (409), C (413, 414, 420, 421)
400	C/T SNP	C	C	C	T (414), C (409, 413, 420, 421)
415	C/T SNP	T (46,48), C (47)	T (49), C (50)	C (408), T (412)	C (409, 414, 420), T (413, 421)

C = Cytosine, G = Guanine and T = Thymine

3.2.3 Prohormone-1

For Prohormone-1, two regions were sequenced, including the annealing points of the RAPD primers. In the first region, region 1, at seven locations SNPs were found. However these SNPs were only found in a single sample, but this was not the same sample every time. Therefore the differences could not be related to *Varroa* resistance. Six locations in the second region (region 2) showed single nucleotide differences (Table 8). Three of these differences could not be related to *Varroa* resistance, because these three variations were found in both control samples and resistant samples. At the other three locations these variations might be related to *Varroa* resistance. They were located approximately 105, 360 and 425 bp downstream of the reverse sequence. These differences were only found between the VSH inseminated colonies and its controls. At 105 and 360 bp from the reverse sequence it was an Adenine/Guanine SNP, at 105 bp downstream of the reverse sequence Guanine occurs in the three resistant colonies, while Adenine was found in the non-resistant samples. At 360 bp downstream of the reverse sequence Adenine only occurs in two resistant samples, 47 and 48, while there is a Guanine base in the other samples. At 425 bp downstream of the reverse sequence a Thymine/Cytosine SNP was found, Cytosine was found in two resistant colonies, 47 and 48 while there is a Thymine base in the others. This last one only for the VSH inseminated colonies and not for the colonies with reduced mite counts, because in the colonies with reduced mite counts Thymine is found in the resistant colonies and Cytosine is found in sensitive colonies.

Table 8. Sequence variation found in the second intron of Prohormone-1.

		VSH inseminated colonies		Reduced mite counts	
Downstream of reverse sequence	**Type of variation**	**Resistant colonies**	**Sensitive colonies**	**Resistant colonies**	**Sensitive colonies**
105	A/G SNP	G	A	G (408), A (412)	G (413, 420), A (409, 414, 421)
116	A/G SNP	G	G	G (408), A (412)	G (413, 420), A (409, 414, 421)
200	A/G SNP	G	G	G (408), A (412)	G (413, 420), A (409, 414, 421)
230	T/C SNP	C (46), T (47,48)	C	C (408), T (412)	C (420), T (409, 413, 414, 421)
360	A/G SNP	A (47, 48), G (46)	G	G	G
425	T/C SNP	C (47, 48), T (46)	T	T	C (413, 421), T (409, 414, 420)

A = Adenine, C = Cytosine, G = Guanine and T = Thymine

4. Discussion

In this thesis, the genetic basis of *Varroa* resistance in *Apis mellifera* was studied using two different methods. Using a RAPD based genome-wide approach, there was found evidence for the involvement of Prohormone-1. A scan for variation in several candidate genes yielded variation in Dop3 that is likely linked to *Varroa* resistance.

Prohormone-1 is associated with resistance against another honey bee disease called chalkbrood (Holloway, et al., 2012). Chalkbrood is a disease that is caused by the fungus *Ascosphaera apis*. Larvae between three and four days old ingest the spores of the fungus by contact with worker honey bees (Flores et al. 1996). In the gut of the larvae the spores start to grow and eventually grow through the outer surface of the larvae (Flores et al. 1996; Holloway et al. 2012). The larvae die in this process and seem to by mummified because they are covered in a thick layer of white mycelium. This layer of mycelium dries in time and gets a chalky appearance, hence chalkbrood (Holloway et al. 2012). Hygienic behavior is not preferred in chalkbrood resistance, because it might increase the chance of spreading spores through the hive. Removing infected larvae might contribute to resistance against chalkbrood, though (Invernizzi, et al., 2009; Invernizzi, et al., 2011). Furthermore, in other species, e.g. remipedia, prohormone-1 is associated with the neuropeptide allatostatin-C (Christie, 2014; Ons, et al., 2011), which is an inhibitor for juvenile hormone (Woodhead, et al., 1989). Juvenile hormone plays two important roles in honey bees. Firstly, it regulates the development of pupae and larvae (Wigglesworth, 1965). Secondly, it also regulates the distribution of social tasks with ageing in worker honey bees (Robinson, 1987; Sullivan, et al., 2000). Both roles might also be linked to the regulation of hygienic behavior in response to *Varroa*. The development of pupae and larvae can be disturbed in presence of *Varroa*, which might cause the worker honey bees to start hygienic behavior and remove the pupae. Similarly, when more worker honey bees will be cleaning, the distribution of social tasks in the worker bees might need to be changed. More research is needed to see whether this gene is really contributing to *Varroa* resistance in honey bees and in what way.

A fragment downstream from a gene coding for an odorant receptor (Odorant receptor 46a, LOC100578886) on chromosome 13 was isolated from the electrophoresis using OPA9. This might be an interesting fragment, because there is a possible connection between olfactory sensitivity and VSH suggested (Tsuruda, et al., 2012). Whether there is an influence of variations downstream of the Odorant receptor 46a gene on the gene itself needs to be further studied. Gene expression studies also suggested the involvement of genes on chromosome 13 (Le Conte et al. 2011; Navajas et al. 2008). Although the gene found in our study was not the same compared to the genes found in the gene expression studies, the regions, in which the genes are located, are approximately 300 kb – 1.000 kb apart. In this 1000 kb region also other genes were found in a sequencing study for *Varroa* resistance by Haddad, et al. (2015), which suggests this 1000 kb region on chromosome 13, in which odorant receptor 46a is located, might be of importance for breeding *Varroa* resistant honey bees.

Although our study showed no sequence variation for the NorpA2 gene in relation to *Varroa* resistance, this does not mean this gene should not be studied further. A study of the national centre for agricultural research and extension of Jordan found a SNP in intron 8 of the NorpA2 gene between the reference genome of *Apis mellifera* and *Apis mellifera syriaca*, which was related to pathogen resistance. *Apis mellifera syriaca* is, like *Apis cerana,* resistant against *Varroa* (Haddad, et

al., 2015) In Haddad, et al. (2015) there were identified 44 genes which potentially are involved in pathogen resistance in *Apis melliferea syriaca*. There were three major differences between our study and the study of Haddad, et al. (2015). The first difference is the method used for identifying polymorphisms, next generation sequences versus Sanger sequencing. In our study first candidate regions for sequences were selected whereas in Haddad, et al., 2015 the full genome was sequenced and all regions with polymorphisms were compared with candidate genes found in other studies. This also indicates the second difference namely, the studies used for candidate gene selection. The studies used were different in our study compard to Haddad, et al. (2015). The third difference between our study and the study of Haddad, et al. (2015) is the honey bee subspecies that was studied. In our study family lines of *Apis mellifera mellifera* were compared and these family lines had different degrees of *Varroa* resistance (0%, 50%, 75% or 87.5%), whereas in Haddad, et al. (2015) *Apis melliferea syriaca* was compared to the NCBI *Apis mellifera.* This may also causes that our study found sequence variation in Dop3, which might related to *Varroa* resistance, while there were no SNPs found in Dop3 related to involvement on pathogen resistance in *Apis melliferea syriaca* (Haddad, et al., 2015).

Prohormone-1 and Odorant receptor 46a were identified with the RAPD method. One of the most common problems with RAPD primers is the replication of experiments (Cushwa and Medrano 1996; Pérez, et al., 1998). It is difficult to replicate results from different laboratory while the same method was used (Cushwa and Medrano 1996). It is not only difficult to reproduce the results of different laboratory also within laboratory reproducibility of RAPD is not equal to 1 (Pérez, et al., 1998). However, a previous BSc thesis study also found polymorphism for the OPA18 primer for a 750 bp fragment (Lie, 2014). This suggests this variation is not due to randomness, but to genomic variation.

Genomic variation is not the same as sequence variation. PolyT parts or repetitive sequences can cause sequence variations which are not genomic variations. When polyT parts or repetitive sequences are in the template DNA mistakes occur during PCR. These mistakes from the PCR can result in variations in sequence. The sequence variations in these cases are not due to genomic variation (B. Koopmanschap, personal communication). Only sequence variations due to genomic variation should be taken into account, because these might contribute to *Varroa* resistance in honey bees.

Breeding *Varroa* resistant honey bees already happens in the U.S.A., 100% VSH colonies (Tsuruda, et al., 2012). With drones of these colonies, queens of European colonies were inseminated (sample 46-48). The drones selected for insemination were collected directly from the frames of the 100% VSH colonies (B.J. Fernhout, personal communication). However, drones can fly freely from one hive to another. This not only accounts for 100% VSH drones, but also for non-resistant drones. By collecting the drones directly from the frames, it can happen that the drones selected for insemination were non-resistant drones through this free-flying. A better way of collecting the drones is to collect them when they are emerging out of the brood cells.

Breeding *Varroa* resistant honey bees is already possible (Behrens, et al., 2011; Tsuruda, et al., 2012); however, the genetics behind it still stays unclear (Zakar, et al., 2014). This study suggests the resistance is based on other genes and chromosomes than suggested in previous studies (Behrens, et al., 2011; Tsuruda, et al., 2012), which was also suggested in the study by Haddad, et al. (2015). The present study suggests the presence of genetic variation in Dop3 and Prohormone-1, which might be

related to *Varroa* resistance in honey bees. Although the results were not consistent over all samples, indicating the observed variations need to be studied further, the current MSc thesis study gained more insight in the genetics behind *Varroa* resistance. This can contribute towards breeding *Varroa* resistant honey bees. Nowadays breeding honey bees is focussing on traits like honey production, gentle temper and low swarming tendency (Buchler, et al., 2010). Future research also can focus on correlation of *Varroa* resistance with these traits. This can prevent beekeepers from breeding *Varroa* resistant lines without production, gentle temper and/or low swarming tendency. Our study already suggests some genes coding for *Varroa* resistance that can be used for a first linkage study.

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Appendix

Appendix 1: Summary of QTL studies

Chromosome number	Honey Bee gene ID	Reference
5	GB14853	Arechavaleta-Velasco, et al., 2012
5	GB17462	Arechavaleta-Velasco, et al., 2012
5	GB16526	Arechavaleta-Velasco, et al., 2012
5	GB12705	Arechavaleta-Velasco, et al., 2012
5	GB10440	Arechavaleta-Velasco, et al., 2012
5	GB11239	Arechavaleta-Velasco, et al., 2012
5	GB18754	Arechavaleta-Velasco, et al., 2012
5	GB16719	Arechavaleta-Velasco, et al., 2012
5	GB19109	Arechavaleta-Velasco, et al., 2012
5	GB10140	Arechavaleta-Velasco, et al., 2012
5	GB10034	Arechavaleta-Velasco, et al., 2012
5	GB14672	Arechavaleta-Velasco, et al., 2012
5	GB17256	Arechavaleta-Velasco, et al., 2012
5	GB20007	Arechavaleta-Velasco, et al., 2012
5	GB17376	Arechavaleta-Velasco, et al., 2012
5	GB17810	Arechavaleta-Velasco, et al., 2012
5	GB18337	Arechavaleta-Velasco, et al., 2012
5	GB10743	Arechavaleta-Velasco, et al., 2012
5	GB11559	Arechavaleta-Velasco, et al., 2012
5	GB12154	Arechavaleta-Velasco, et al., 2012
5	GB13244	Arechavaleta-Velasco, et al., 2012
5	GB15435	Arechavaleta-Velasco, et al., 2012
5	GB16547	Arechavaleta-Velasco, et al., 2012
5	GB18506	Arechavaleta-Velasco, et al., 2012
5	GB19804	Arechavaleta-Velasco, et al., 2012
7	GB16050	Behrens, et al., 2011
7	GB16379	Behrens, et al., 2011
7	GB15230	Behrens, et al., 2011
7	GB16879	Behrens, et al., 2011
7	GB14497	Behrens, et al., 2011
7	GB13873	Behrens, et al., 2011
7	GB10033	Behrens, et al., 2011
7	GB11764	Behrens, et al., 2011
7	GB12612	Behrens, et al., 2011
7	GB13746	Behrens, et al., 2011
7	GB13854	Behrens, et al., 2011
7	GB14642	Behrens, et al., 2011
7	GB15635	Behrens, et al., 2011
7	GB18884	Behrens, et al., 2011
7	GB19142	Behrens, et al., 2011
7	GB30232	Behrens, et al., 2011

7	GB30367	Behrens, et al., 2011
9	LOC100577079	Behrens, et al., 2011
9	LOC100578536	Behrens, et al., 2011
9	LOC100578904	Behrens, et al., 2011
9	GB15121	Behrens, et al., 2011
9	GB11613	Behrens, et al., 2011
9	LOC100576261	Behrens, et al., 2011
9	GB11598	Behrens, et al., 2011
9	GB14509	Behrens, et al., 2011
9	GB12325	Behrens, et al., 2011
9	GB12300	Behrens, et al., 2011
9	GB11250	Behrens, et al., 2011
9	GB12689	Behrens, et al., 2011
9	GB10150	Behrens, et al., 2011
9	GB14644	Behrens, et al., 2011
9	LOC100576158	Behrens, et al., 2011
9	LOC100576352	Behrens, et al., 2011
9	GB13397	Behrens, et al., 2011
9	LOC100576220	Behrens, et al., 2011
9	GB10780	Behrens, et al., 2011
9	GB10498	Behrens, et al., 2011
9	GB14121	Behrens, et al., 2011
9	GB10091	Behrens, et al., 2011
9	GB16222	Behrens, et al., 2011
9	LOC100577524	Behrens, et al., 2011
9	GB14327	Behrens, et al., 2011
9	GB17311	Behrens, et al., 2011
9	GB16541	Behrens, et al., 2011
9	GB17438	Behrens, et al., 2011
9	GB15983	Behrens, et al., 2011
9	GB18729	Behrens, et al., 2011
9	LOC100577133	Behrens, et al., 2011
9	GB13720	Behrens, et al., 2011
9	LOC100577010	Behrens, et al., 2011
9	LOC100576813	Behrens, et al., 2011
9	GB15181	Behrens, et al., 2011
9	LOC100576864	Behrens, et al., 2011
9	GB10088	Behrens, et al., 2011
9	GB17597	Behrens, et al., 2011
2	GB12301	Oxley, et al., 2010
2	GB18005	Oxley, et al., 2010
2	GB13500	Oxley, et al., 2010
2	GB19512	Oxley, et al., 2010
2	GB12722	Oxley, et al., 2010
2	GB17360	Oxley, et al., 2010
2	GB19509	Oxley, et al., 2010

2	GB11204	Oxley, et al., 2010
2	GB14982	Oxley, et al., 2010
2	GB19476	Oxley, et al., 2010
2	GB13839	Oxley, et al., 2010
2	GB17042	Oxley, et al., 2010
2	GB10700	Oxley, et al., 2010
2	GB19838	Oxley, et al., 2010
2	GB19351	Oxley, et al., 2010
2	GB12228	Oxley, et al., 2010
2	GB11135	Oxley, et al., 2010
2	GB18541	Oxley, et al., 2010
2	GB15412	Oxley, et al., 2010
5	GB16989	Oxley, et al., 2010
5	GB12487	Oxley, et al., 2010
5	GB11114	Oxley, et al., 2010
5	GB11846	Oxley, et al., 2010
5	GB17582	Oxley, et al., 2010
16	GB18029	Oxley, et al., 2010
16	GB19738	Oxley, et al., 2010
16	GB10249	Oxley, et al., 2010
16	GB11352	Oxley, et al., 2010
16	LOC413529	Oxley, et al., 2010
16	GB17099	Oxley, et al., 2010
16	GB18285	Oxley, et al., 2010
1	GB16023	Tsuruda, et al., 2012
1	GB11694	Tsuruda, et al., 2012
1	GB19573	Tsuruda, et al., 2012
1	GB16181	Tsuruda, et al., 2012
1	GB11311	Tsuruda, et al., 2012
1	GB14379	Tsuruda, et al., 2012
1	GB13163	Tsuruda, et al., 2012
1	GB17990	Tsuruda, et al., 2012
1	GB10049	Tsuruda, et al., 2012
1	GB19123	Tsuruda, et al., 2012
1	GB13413	Tsuruda, et al., 2012
1	GB12299	Tsuruda, et al., 2012
1	GB10077	Tsuruda, et al., 2012
1	GB17666	Tsuruda, et al., 2012
1	GB11344	Tsuruda, et al., 2012
1	GB16999	Tsuruda, et al., 2012
1	GB12314	Tsuruda, et al., 2012
1	GB14157	Tsuruda, et al., 2012
1	GB17865	Tsuruda, et al., 2012
1	GB18179	Tsuruda, et al., 2012
1	GB15278	Tsuruda, et al., 2012
1	GB14179	Tsuruda, et al., 2012

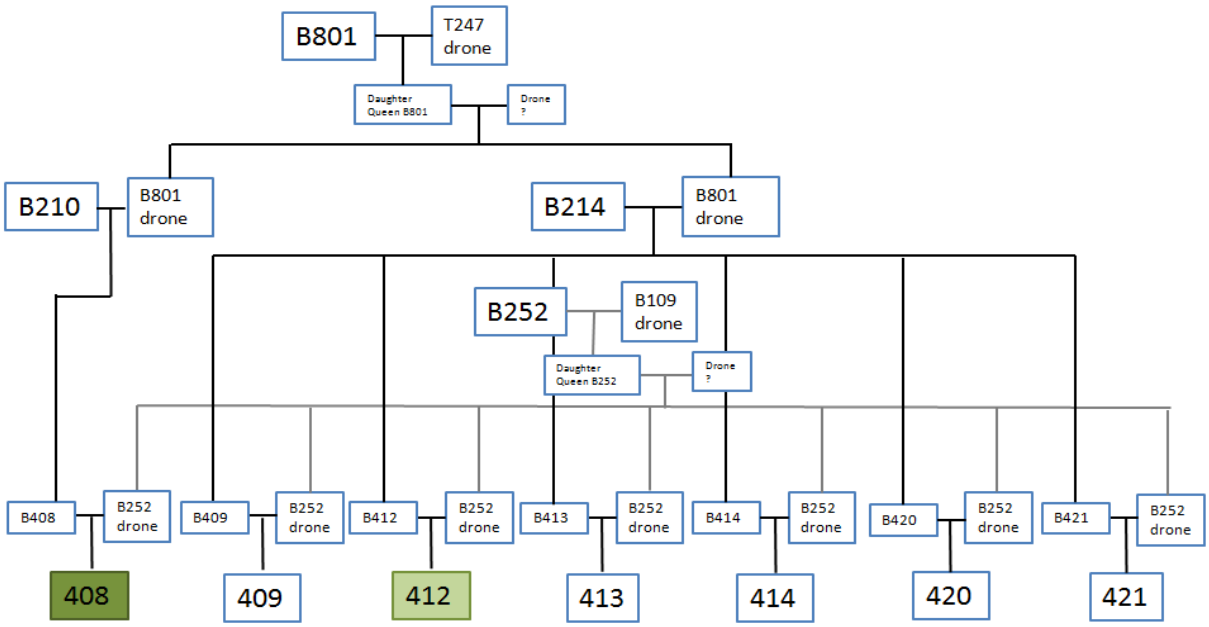
1	GB19383	Tsuruda, et al., 2012
1	GB18087	Tsuruda, et al., 2012
1	GB15566	Tsuruda, et al., 2012
1	GB13779	Tsuruda, et al., 2012
1	GB17608	Tsuruda, et al., 2012
1	GB17608	Tsuruda, et al., 2012
1	GB17608	Tsuruda, et al., 2012
1	GB11992	Tsuruda, et al., 2012
1	GB18990	Tsuruda, et al., 2012
1	GB18958	Tsuruda, et al., 2012
1	GB10333	Tsuruda, et al., 2012
1	GB11159	Tsuruda, et al., 2012
1	GB12769	Tsuruda, et al., 2012
1	GB15897	Tsuruda, et al., 2012
1	GB19011	Tsuruda, et al., 2012
9	GB12094	Tsuruda, et al., 2012
9	GB17677	Tsuruda, et al., 2012
9	GB11986	Tsuruda, et al., 2012
9	GB15650	Tsuruda, et al., 2012
9	GB14561	Tsuruda, et al., 2012
9	GB14619	Tsuruda, et al., 2012
9	GB30420	Tsuruda, et al., 2012
9	GB12836	Tsuruda, et al., 2012
9	GB18182	Tsuruda, et al., 2012
9	GB17322	Tsuruda, et al., 2012
9	GB17322	Tsuruda, et al., 2012
9	GB10653	Tsuruda, et al., 2012
9	GB16013	Tsuruda, et al., 2012
9	GB18280	Tsuruda, et al., 2012
9	GB17640	Tsuruda, et al., 2012
9	GB16277	Tsuruda, et al., 2012
9	GB16097	Tsuruda, et al., 2012
9	GB11859	Tsuruda, et al., 2012
9	GB13209	Tsuruda, et al., 2012
9	GB11883	Tsuruda, et al., 2012
9	GB12719	Tsuruda, et al., 2012
9	GB10237	Tsuruda, et al., 2012
9	GB14749	Tsuruda, et al., 2012
9	GB14991	Tsuruda, et al., 2012
9	GB16408	Tsuruda, et al., 2012
9	GB10808	Tsuruda, et al., 2012
9	GB10568	Tsuruda, et al., 2012
9	GB12681	Tsuruda, et al., 2012
9	GB14290	Tsuruda, et al., 2012
9	GB19672	Tsuruda, et al., 2012
9	GB15720	Tsuruda, et al., 2012

9	GB11386	Tsuruda, et al., 2012
9	GB12219	Tsuruda, et al., 2012
9	GB16984	Tsuruda, et al., 2012
9	GB12006	Tsuruda, et al., 2012
9	GB17383	Tsuruda, et al., 2012
9	GB12634	Tsuruda, et al., 2012
9	GB14091	Tsuruda, et al., 2012
9	GB16093	Tsuruda, et al., 2012
9	GB19520	Tsuruda, et al., 2012
9	GB14706	Tsuruda, et al., 2012
9	GB18583	Tsuruda, et al., 2012
9	GB10793	Tsuruda, et al., 2012
9	GB14763	Tsuruda, et al., 2012
9	GB10458	Tsuruda, et al., 2012
9	GB10410	Tsuruda, et al., 2012
9	GB10996	Tsuruda, et al., 2012
9	GB11073	Tsuruda, et al., 2012
9	GB12004	Tsuruda, et al., 2012
9	GB12494	Tsuruda, et al., 2012
9	GB13523	Tsuruda, et al., 2012
9	GB13565	Tsuruda, et al., 2012
9	GB14020	Tsuruda, et al., 2012
9	GB14580	Tsuruda, et al., 2012
9	GB14905	Tsuruda, et al., 2012
9	GB15003	Tsuruda, et al., 2012
9	GB15048	Tsuruda, et al., 2012
9	GB15156	Tsuruda, et al., 2012
9	GB16925	Tsuruda, et al., 2012
9	GB19232	Tsuruda, et al., 2012
9	GB30249	Tsuruda, et al., 2012
9	GB30250	Tsuruda, et al., 2012
9	GB30419	Tsuruda, et al., 2012

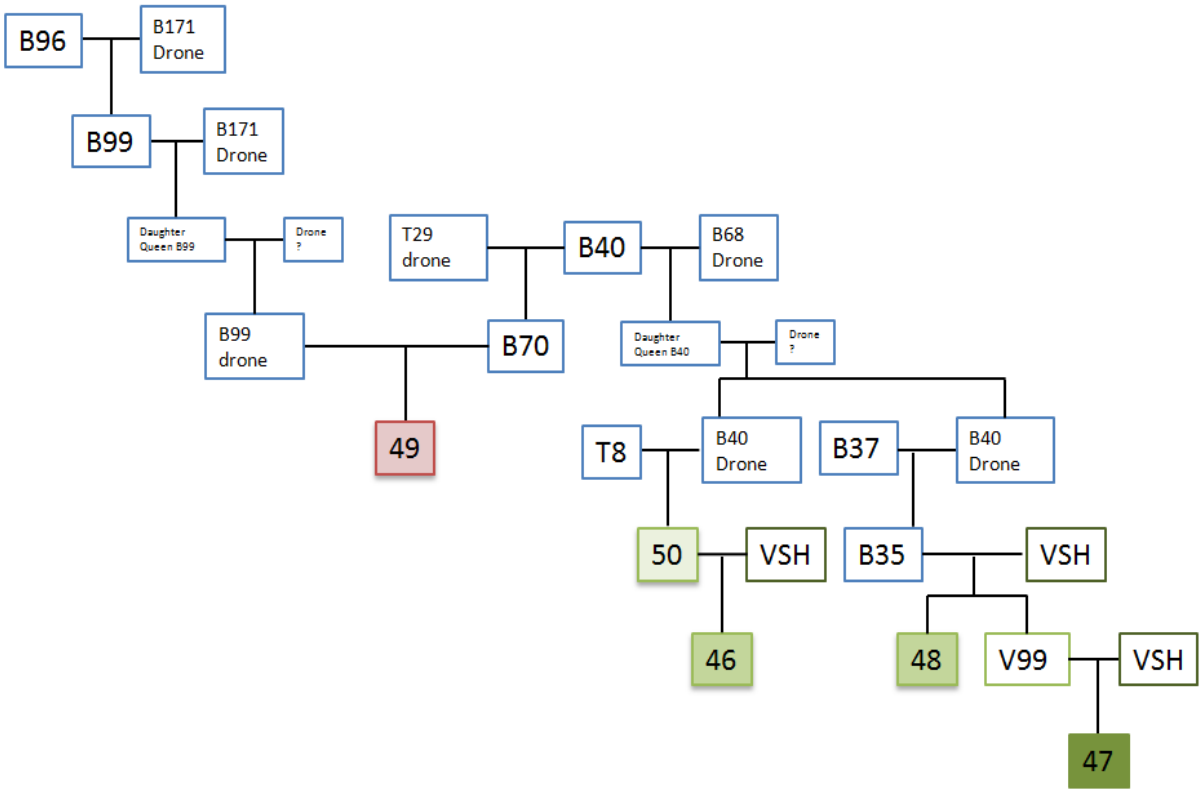
Appendix 2: Specific PCR reaction conditions per primer pair

Primer pair	Denaturation (°C)	Annealing (°C)	Elongation (°C)
Promotor Dop3	94	55	72
UTR Dop3	94	58	72
Exon 2 Dop3	94	54.5	72
Promotor NorpA2	94	55	72
UTR NorpA2	94	61.5	72
Region 1 Prohormone-1	94	53.5	72
Region 2	94	55	72

Appendix 3: Pedigree of samples



Pedigree of reduced reproductive mite counts (B.J. Fernhout, personal communication)



Pedigree of VSH inseminated (B.J. Fernhout, personal communication)