Propositions (Stellingen)

1. *Varroa destructor virus* 1 does not kill the *Varroa destructor* mite.
   This thesis

2. Deformed wing symptoms in bees may result from a combination of factors other than a mere infection with *Deformed wing virus*.
   This thesis

3. The genome organisation of *Infectious flacherie virus* in the 8th Report of the International Committee on Taxonomy of Viruses gives a mispresentation of the report by Isawa *et al.* that may have consequences for the taxonomy of the genus *Iflavirus*.
   Fauquet *et al.* (2005). Virus Taxonomy. pp 780
   Isawa *et al.* (1998). *Arch Virol* 143: 127-143.

4. Reference to the plasmid pUltraBac-1 (derived from pFastBacDUAL) as a bicistronic vector is misleading since the genes of interest are expressed from two separate promoters.
   Philipps *et al.* (2005). *Protein Expr Purif* 42: 211-218

5. PCR-based methods are more accurate than antibody-based methods in identifying infecting viruses.
   Denholm (1999). PhD thesis. Dept of Entomology & Nematology, Keele University, UK.
   Chen *et al.* (2004). *J Invert Pathol* 87: 84-93.

6. Malaria-related complications can be reduced significantly in a cost effective manner by the simple use of insecticide-treated bed nets.
   Ter Kuile *et al.* (2003). *Am J Trop Med Hyg* 68: 68-77.

7. In order to breed a beautiful dog that meets the breed standards, the maternal line is just as important as the paternal line.

8. Whether the weather is good or bad is subjective but whichever way it can offer an opportunity for innovation and enjoyment.

Stellingen behorende bij het proefschrift:

***Varroa destructor virus* 1: A new picorna-like virus in *Varroa* mites as well as honey bees**

Wageningen, Wednesday 12th April 2006 — Juliette R. Ongus

Varroa destructor virus 1: A new picorna-like virus in *Varroa* mites as well as honey bees

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Varroa destructor virus 1: A new picorna-like virus in *Varroa* mites as well as honey bees

Juliette R. Ongus

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Contents

Chapter 1

General introduction

Honey bees are important insects that have benefited mankind for medicinal and nutritional purposes for thousands of years. They have also provided an important balance in the environment allowing many plant species to pollinate and proliferate. Honey bees have naturally occurring parasites and pathogens which usually do not destroy the bees. The development of long-distance travel among human beings introduced global trade in plants and animals introducing new species and diseases to regions where they did not exist previously. New diseases were introduced into bees and affected them severely. Among the most serious diseases are those caused by viruses and blood-sucking parasites. This chapter introduces the honey bee to the reader and also mentions some diseases suffered by the bee. Important parasites will be mentioned along with the effects they have on the bee. The chapter also defines the problem caused by an important parasitic mite and the discovery of virus in the mite body which was the basis of the research carried out. The scope of the study done will be summarised.

The biology of the honey bee

The family *Apidae* has seven species of honey bees and several subspecies or geographical races. The western honey bee, *Apis mellifera*, is distributed widely throughout Europe, Africa, and parts of western Asia, as well as in the Americas (Winston, 1987). *Apis cerana* is the major Asian bee species. All honey bees are eusocial insects, meaning that they engage in favourable social activity. A typical *A. mellifera* colony consists of a reproductive queen (a fertile female), 20,000-60,000 adult workers (non-reproductive females), 10,000-30,000 worker brood (eggs, larvae and pupae) and several hundred drones (males) (Bailey & Ball, 1991). On average, workers take 21 days to develop into adults, drones 24 days, and queens 16 days (Winston, 1987). The drones are present only during the early summer in Europe and their sole function is to mate with queens. The queen only leaves the colony to mate, during swarming (colony division) or when a colony deserts a nest site. Her main task is to lay eggs (Denholm, 1999). Adult workers are involved in basic cleaning and comb-building, nursing of brood and tending to the queen. They are also involved in outside tasks, such as colony ventilation, guarding and foraging. Figure 1-1 shows a graphic presentation of the queen, worker and drone.

Figure 1-1: A schematic diagram of a queen, a worker and a drone bee (not proportional to real size). (Dade, 1977).

Fertilised (diploid) eggs develop into non-reproductive female workers, while unfertilised (haploid) eggs develop into drones. The diet of the diploid female larva determines whether the emerging adult will be a queen or worker. All larvae are fed on 'royal jelly', which is a protein-rich secretion from the mandibular and hypopharyngeal glands of nurse bees. Larvae destined to become queens are fed larger quantities of this royal jelly for a longer period. Pollen collected by foraging worker bees is the main source of protein. Bees also forage for nectar, which is converted into honey by salivary enzymes and stored, along with pollen, to provide a food source during winter and in periods of food shortage. Wax secreted from glands in the abdomen of young bees is used to construct the hexagonal combs. In addition to honey, wax and propolis (a resinous secretion collected by bees from trees) are useful hive products (Winston, 1987).

History and taxonomy of *Varroa* mites

In 1904 in Java, Indonesia, Oudemans described the *Varroa jacobsoni* mite in brood cells of drone larvae of *A. cerana* (Oudemans, 1904). The mite was later found in European honey bee colonies in Japan. *A. mellifera* had been imported to Japan in 1876 (Tanabe & Tamaki, 1986). The Asian honey bee, *A. cerana*, is less susceptible to this mite compared to *A. mellifera*. Mites can attack only a few *A. cerana* bees as the adult workers kill and remove most of them from the brood (Bailey & Ball, 1991). Studies made on the genotypic, phenotypic and reproductive variation showed that *Varroa* forms a complex of 18 different genetic variants that belong to 2 different species *Varroa jacobsoni* (infesting *A. cerana*) and *Varroa destructor* (infesting *A. mellifera*). There are no morphological differences to distinguish the genetic types (Anderson & Trueman, 2000). *V. destructor* can cause widespread losses in colonies in relatively short periods of time. When untreated, colonies might die within 3 to 4 years (de Jong *et al.*, 1982a).

Worldwide distribution of *Varroa* mites

The *Varroa* mite has been found on honey bees in Asia, Europe, the Americas, New Zealand, North and South Africa. Figure 1-2 shows the global distribution of the mite as per data available in 2000. The mite has spread worldwide in the last few decades due to the commercial transport of bees, the migratory activities of beekeepers, drifting bees and swarms that may fly long distances (Sumpter & Martin, 2004). The mite was initially observed in Indonesia (Oudemans, 1904). Its occurrence has since been reported in Singapore (Gunther, 1951), USSR (Breguetova, 1953), Hong Kong (Delfinado, 1963), Philippines (Delfinado, 1963), The Peoples Republic of China (Tzien-He, 1965), India (Phadke *et al.*, 1966), North Korea (Tian, 1967), Cambodia (Ehara, 1968), Japan (Ehara, 1968), Vietnam (Stephen, 1968), Thailand (Laigo & Morse, 1969), Czechoslovakia (Samsinak & Haragsim, 1972), Bulgaria (Velitchkov & Natchev, 1973), South Korea (Delfinado & Baker, 1974), Paraguay (Orosi-Pal, 1975), Romania (Orosi-Pal, 1975), Taiwan (Akratanakul & Burgett, 1975), Argentina (Montiel & Piola, 1976), Poland (Koivulehto, 1976), Uruguay (Grobov, 1976), Germany (Ruttner, 1977), Bangladesh (Marin, 1978), Myanmar (Marin, 1978), Brazil (Alves *et al.*, 1975), Hungary (Buza, 1978), Tunisia (Hicheri, 1978), Greece (Santas, 1979), Yugoslavia (Santas, 1979), Iran (Crane, 1979), Libya (Crane, 1979), Turkey (Crane, 1979), Lebanon (Popa, 1980), USA (anonymous, 1987), (Wenner & Bushing, 1996) South Africa (Allsopp *et al.*, 1997), and New Zealand (Anonymous, 2000). Apart from their common appearance on living bees, *Varroa* mites have also been found on the flower-feeding insects *Bombus pennsylvanicus* (Hymenoptera: *Apidae*), *Palpada vinetorum* (Diptera: *Syrphidae*) and on *Phanaeus vindex* (Coleoptera: *Scarabaeidae*) (Kevan *et al.*, 1991).

The only regions of the world where the *Varroa* mite has not yet spread to are Australia and the central part of Africa (Oldroyd, 1999).

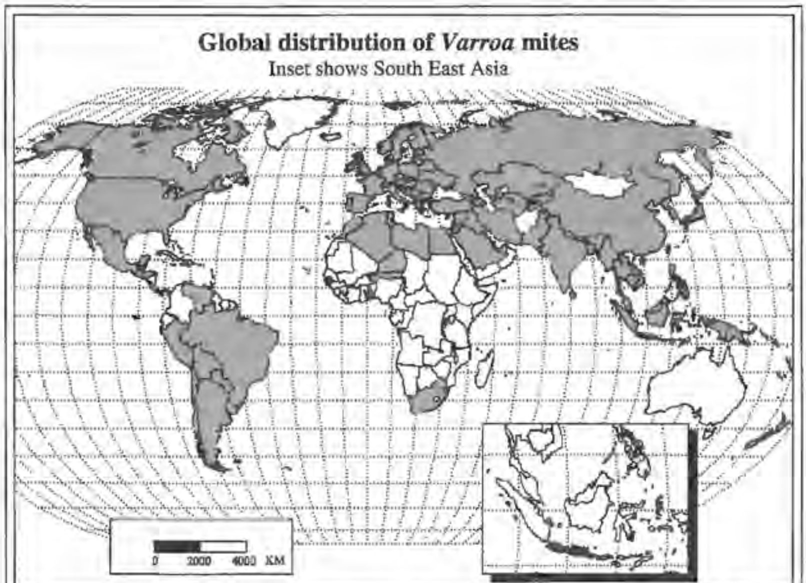

Figure 1-2: The global distribution of *Varroa* mites. The shaded regions are those where documentary evidence of the mite's presence was recorded by the year 2000. Adapted (with added updates) from Arlinghaus *et al.*, 1998 (permission granted). The mite was first observed in Java Indonesia in 1904 (highlighted in the inset) and has since been identified in most areas of the world.

Honey bee pathogens

Various agents such as bacteria, fungi, viruses, parasitic mites, microspora and amoeba cause disease in the honey bee (Bailey & Ball, 1991). The bacterium *Melissococcus plutonius* causes European foulbrood (EFB) disease in the honey bee brood. The dead larvae are watery and pasty in appearance, and are yellow to black in colour. American foulbrood is caused by a spore forming bacterium *Paenibacillus larvae*. It is characterised by sticky larval remains. Chalkbrood disease is caused by the spores of the fungus *Ascosphaera apis* when ingested with food. The larvae are covered with white fungal mycelial growth which causes them to dry out and die. The tracheal mite (*Acarapis woodi*) is an internal parasite that lives in honey bee breathing tubes in the thorax, abdomen and head. The mites pierce the breathing tube walls with their mouth parts and feed on the haemolymph of the bees. The mite *V. destructor* is an external parasite that feeds on the haemolymph of adult bees, larvae and pupae (Bailey & Ball, 1991).

Most viruses infecting bees are insect picorna-like viruses. They were first observed in *A. mellifera* in the early 1960s (Bailey *et al.*, 1963; Bailey *et al.*, 1964). Picorna-like viruses have invertebrate hosts unlike picornaviruses which have vertebrate hosts. The viruses are referred to as picorna-like because of their morphological and physico-chemical resemblance to picornaviruses (Fauquet *et al.*, 2005). They are small, non-enveloped and have positive-sense single-stranded RNA genomes (Fig. 1-3). The viruses currently fall into two taxonomical groups: the family *Dicistroviridae* with members whose genomes have two non-overlapping open reading frames, and the unassigned genus *Iflavirus*, characterized by a genome with a single open reading frame and similarity to vertebrate picornaviruses (Christian *et al.*, 2005). Dicistroviruses affecting bees include *Kashmir bee virus* (KBV; de Miranda *et al.*, 2004), *Black queen cell virus* (BQCV; Leat *et al.*, 2000), and *Acute paralysis virus* (APV; Govan *et al.*, 2000). Iflaviruses reported in bees are *Sacbrood virus* (SBV; Ghosh *et al.*, 1999), *Deformed wing virus* (DWV; de Miranda *et al.*, 2003; Lanzi & Rossi, 2003) and *Kakugo virus* (Fujiyuki *et al.*, 2004).

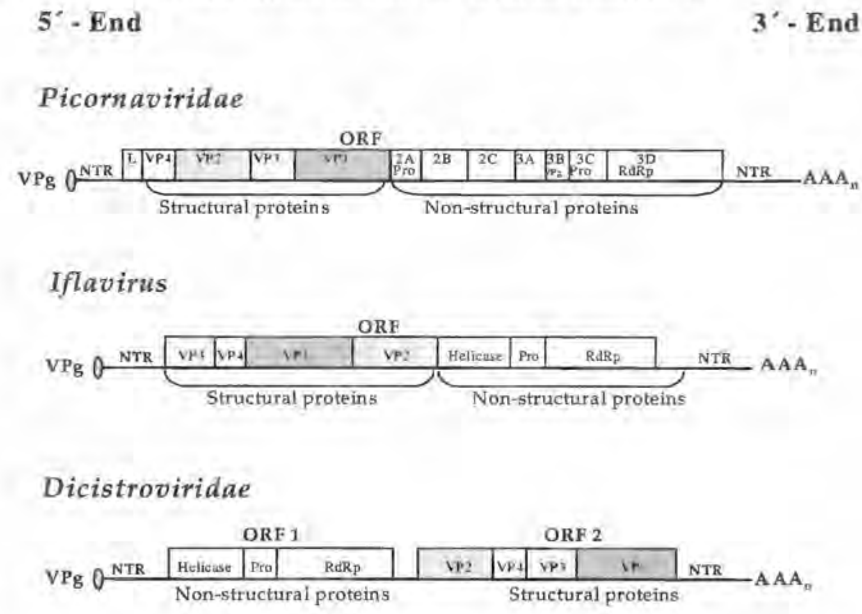

Figure 1-3: Schematic diagram of the genome organisation of *Picornaviridae*, *Iflavirus* and *Dicistroviridae*. The genome-linked protein (VPg) on the 5′ end has been shown to be present in picorna-like viruses such as *Cricket paralysis virus* and *Drosophila C virus* (King & Moore, 1988). The structural proteins (VP1-VP4), shaded in the diagram, play a role in virus attachment to the host cell. Some picornaviruses (cardioviruses and aphthoviruses) encode a leader protein (L) at the beginning of the ORF. The 5′ a nontranslated region (NTR) contains an internal ribosome entry site (IRES) in the three groups. *Dicistroviridae* have a second IRES in the intergenic region. The viruses belonging to these groups have a polyadenylated tail on the 3′ end of the genome.

Other viruses associated with bees include: *Apis Iridescent Virus*, the *Iridovirus* which was isolated and characterised from *A. cerana* (Bailey & Ball, 1991). The filamentous virus and bee virus Y are intimately associated with the parasitic microsporidian organism *Nosema apis*, a parasite infecting the midgut of adult bees (Allen & Ball, 1996). Bee virus X is found in association with the amoeba *Malpighamoeba mellificae*, a parasite infecting the Malphigian tubules of adult bees (Allen & Ball, 1996).

***Varroa destructor*: a honey bee ectoparasite**

The ectoparasitic mite *V. destructor* is one of the most serious pests of the honey bee *A. mellifera*. Figure 1-4 shows an adult female (in the left panel) and male (in the right panel) mite. The adult female mite is reddish-brown in colour and has an oval and flat body measuring about 1.1 to 1.2 mm long and 1.5 to 1.6 mm wide. Male mites are smaller, measuring about 0.7 mm by 0.7 mm, and are pale to light tan in colour (Bailey & Ball, 1991).

Figure 1-4: Photographs of adult *Varroa destructor* mites. The left panel shows an adult female mite feeding on bee pupa and the right a male mite. Photographs taken by Ms Rosalinde M. Keijzer.

An adult female mite lives either attached to an adult bee (called the phoretic phase) or within a sealed brood cell in which it reproduces. During the phoretic phase the mites will remain attached, occasionally moving between adult bees. The mites reproduce in the brood cell on pupae of worker bees and drones (de Jong, 1988), of which the drone is distinctly preferred (Bailey & Ball, 1991). The reproduction cycle starts when a female mite enters a brood cell just before it is sealed. The female mite lays one unfertilised and a number of fertilised eggs in the upper part of the cell (Fig. 1-5).

A male hatches from the unfertilised egg and the females from the fertilised eggs (Rehm & Ritter, 1989). Mating occurs within the sealed cells. The development of the mite is synchronised to that of the bee (Fig. 1-6). Adult female mites are then released in the colony, either when the developed bee emerges from the cell or when workers remove the dead brood (Martin, 2001). Several of the female mite offspring will have reached adulthood by the time the adult bee emerges from the cell. As the host bee emerges, the female mites move out to parasitize other bees or larvae. Most of the female mites move to a new adult worker bee soon after being released from the cell. Males are only found inside the brood cells of the honey bee and die at emergence together with any immature daughters.

Figure 1-5: The life cycle of *Varroa destructor* on an *Apis mellifera* worker. At 60 hours an unfertilised egg is laid near the top of the cell (top left). The egg develops into a male which mates with its mother. Fertilised eggs are laid from 90 hours onwards and develop into daughters. A faecal pile develops towards the bottom of the cell (bottom right) as more mite daughters hatch and grow. All the mites within the cell feed on the developing bee and mature females leave the cell when the bee emerges. The male mite and any immature daughters left in the cell, die (from Oldroyd, 1999).

All mite stages feed on bee haemolymph, which is obtained by piercing the cuticle of the developing or adult bee (Oldroyd, 1999). The *Varroa* female punctures the host pupae, in particular drone brood, only at the second abdominal sternite (Kanbar & Engels, 2005). The repeated feeding of the mite and its offspring at the fistula (the piercing site in the cuticle) prevents it from healing thus ensuring a constant supply of haemolymph (Gelbe *et al.*, 1987; Donze & Guerin, 1994).

Individual bees infested by *Varroa* mites show noticeable reduction in weight at emergence, which is correlated to an increasing number of mites (De Jong *et al.*, 1982a). One or two mites will cause a decrease in vitality of the emerging bee. Higher amounts of mites parasitizing per brood cell results in malformations like shorter abdomens, deformed wings and legs (Duay *et al.*, 2003). Drifting workers and drones within an apiary spread the mites from colony to colony.

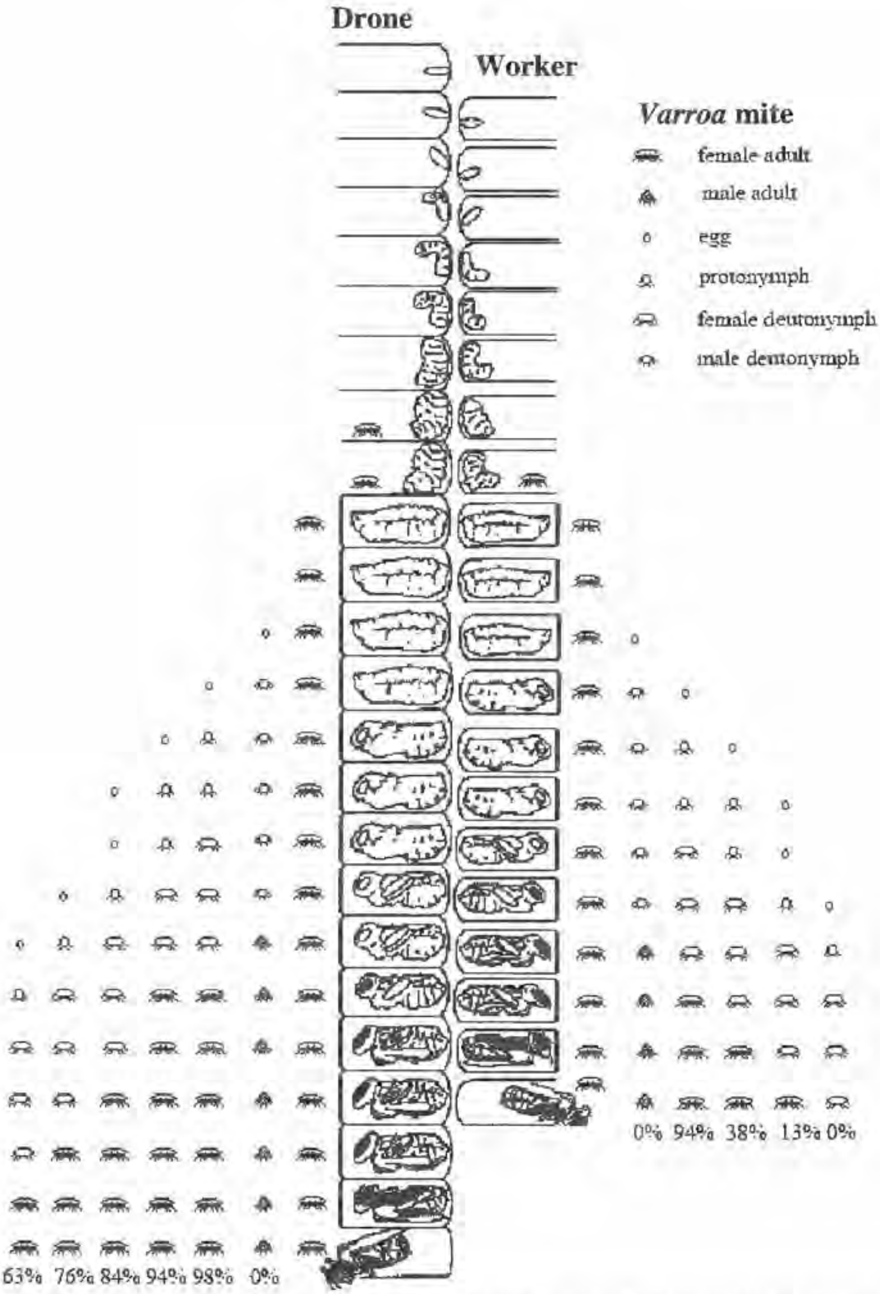

Figure 1-6: The daily development (from top to bottom) of *Varroa* offspring in both worker (right) and drone (left) bee cells. The development of the mite is synchronised to that of the bee. The percentages at the bottom give the survival rate of each mite offspring at the time the bee emerges. Adapted from Martin (1997).

***Varroa destructor*: a vector of bee viruses**

Relationships between honey bee parasites and viruses have been formed over time. Important examples are the microsporidian *Nosema apis* which is associated with BQCV (Bailey *et al.*, 1983). The *V. destructor* mite has been associated with the transmission of a number of bee viruses including APV (Ball, 1989; Batuev, 1979), *Slow paralysis virus* (SPV) (Denholm, 1999) and DWV (Bowen-Walker *et al.*, 1999). Much of the pathology and mortality is seen in severely mite-infested bee colonies and linked to the mite-mediated transmission of viruses (Martin *et al.*, 1998). Viruses are transmitted during feeding when the mite injects a fluid, possibly salivary in origin into the haemolymph (Gelbe *et al.*, 1987). Alternatively, the feeding site could act as the location where the mite can acquire a virus. The open wound could also offer a perfect setting for opportunistic infections by other pathogens to set in. The term 'bee parasitic mite syndrome' has been used to describe a disease complex in which colonies are heavily infested with mites and infected with viruses. When the mite population becomes too dense, the balance of the mite, bee and virus and is lost resulting in colony mortality.

The majority of honey bee viruses persist at low levels as inapparent, sub-lethal infections and occasionally cause outbreaks of mortality, for example SBV (Ball & Bailey, 1991). This has been shown to change when the bees become infested with the *Varroa* mite, which may act as a vector to some of these viruses (Sumpter & Martin, 2004) and induces the accumulation of viruses as a result of enhanced virus replication in the bee which often leads to high mortality as seen in APV (Ball, 1989; Batuev, 1979), SPV, KBV (Denholm, 1999) and DWV (Bowen-Walker *et al.*, 1999). These four viruses are also known to multiply rapidly when injected into bee pupae (Denholm, 1999).

Recently, picorna-like virus particles (27 nm in diameter) were isolated from a population of *Varroa* mites in beehives at Wageningen University. Examination of mite tissue by electron microscopy revealed aggregates of virus-like particles of the same size located in the cytoplasm (see Chapter 2 of this thesis for more details). Similar observations were made by Kleespies *et al.* (2000) from a sample of mites in Germany, but the virus could not be recovered. The purified particles from the Wageningen mites contained RNA as genetic material. This suggested that the picorna-like virus could replicate in the mite, and formed the motivation to carry out the study described in this thesis.

Economic and ecological significance

The impact of *Varroa* infestation is felt directly by the beekeeping sector. Due to weakening caused by the constant feeding of the *Varroa* mite on the haemolymph of developing honey bee larvae, pupae and adults, a bee colony once attacked by the mite is at risk of being lost if left untreated. The mite is of great concern to the beekeeping industry, being very costly when considering both colony losses and the costs of prevention or control. This would mean that the income that beekeepers derive from the sale of bee products would be reduced or lost altogether. Besides their economic importance, bees also have a crucial ecological value and honey, bee wax and propolis comprise only a part of the benefits derived from them. *Varroa* infestation can impact indirectly on sectors that benefit from honey bee pollination. Bees play an important role in the pollination of crops producing fruits and berries around the world. Without their help, the propagation of many species of plants in nature and in agriculture would be threatened.

In central Africa where the mites have not yet been reported, bee viruses are rare, but the spread of the *Varroa* mite is an emerging threat to many small holder farms where beekeeping is an important source of income.

Biological control of the *Varroa* mite

Biological control agents of pests are naturally occurring predators or parasites that will normally attack and kill a pest whilst sparing desirable organisms. Ongoing studies on the application of entomopathogenic fungi *Hirsutella thompsonii* and *Metarhizium anisopliae* (Kanga *et al.*, 2002), showed that they are pathogenic to *Varroa* mites at controlled conditions similar to those in a colony. Studies have also been performed to measure the pathogenicity of isolates of mitosporic fungi (Shaw *et al.*, 2002) and bacteria, such as *Bacillus thuringiensis*, to which the mites may be susceptible. The identification of pathogens harmful to *Varroa* but not to bees may open new avenues of controlling the mite in specific and environmentally friendly ways. In addition, the identification of biological control agents may have the potential for an effective, long-term and specific control method against *Varroa* mites.

The problem

The feeding habits of *Varroa* mites cause bees to suffer weight loss and physiological damage. In addition, the mite is also a vector of a number of pathogens, including bacteria, viruses and fungi, some of which also contribute to the killing of the bee populations (Bailey & Ball, 1991). To date, no known biological control method is effectively able to eliminate any lethal pests in bee populations. The identification of a specific biological control agent against the mite will save bees and the livelihood of many beekeepers around the world. Therefore, it is crucial that potential agents are identified and investigated for their potential to control *Varroa* mites. Transportation of infested bee stocks and the beekeeping practices, including long distance transport of bees, contributed to the rapid spread of the mite within Europe (Anonymous, 1997) and is a major threat to regions where the mite has not yet been seen.

Scope of the thesis

At the beginning of the research presented in this thesis, high amounts of picorna-like virus particles were detected in the tissue of *V. destructor* from apiaries of Wageningen University in The Netherlands. Aggregates of virus particles were observed in the cytoplasm of mite tissue apparently going through an infection cycle. Although picorna-like virus particles had been detected in *Varroa* mites previously (Kleespies *et al.*, 2000), there was no molecular data available to definitely identify these viruses. The aim of this research was to isolate this virus from the mite, characterise it in detail and study its behaviour in mite and bee populations with the intention to determine if it had potential as a biological control agent against the *Varroa* mite. *V. destructor* has been implicated in the spread of bee viruses (Denholm, 1999; Sumpter & Martin, 2004), but it was not known whether bee viruses could replicate in mites or whether they were pathogenic or not.

To further characterise this virus, a large amount of virus was isolated and purified from the Wageningen mites and was used to generate antiserum in rabbits. This antiserum would subsequently be used for ELISA and blot analyses to asses the extent of spread of the virus in the bee colonies. There was need to generate data on cytopathology of the virus therefore electron microscopy and immunohistochemistry was used to locate the virus particles and to identify the infected tissues **(Chapter 2)**.

Since the molecular identity of this tentatively new virus was unknown, the entire genome of the virus was sequenced and analysed using immunocapture RT-PCR followed by sequencing through genome walking (**Chapter 3**). The information obtained was used to study the taxonomic position of this virus, which was called *Varroa destructor virus* 1 (VDV-1), in a phylogenetic analysis along with members of the families *Picornaviridae* and *Dicistroviridae* and genus *Iflavirus*. All of these taxa represent small non-enveloped viruses with single-stranded positive-sense RNA genomes. The latter two groups contain various known bee viruses.

The visualization of aggregates of picorna-like virus particles in mite tissues by electron microscopy indicated that these viruses were going through an infection cycle. To confirm the ability of the virus to replicate in mites an RT-PCR-based method was designed to detect the complementary negative RNA strand in mite tissue. This strand only exists during viral replication where it provides a template for the synthesis of the viral RNA genome of positive polarity. A similar test was set-up for DWV, in order to be able to discriminate between these viruses since both were detected in the mites (**Chapter 4**).

In **Chapter 5** the viral proteins are analysed in more detail. Picorna(-like) viruses translate their proteins first as polyproteins which are cotranslationally cleaved into functional polypeptides which are involved in replication of the virus (non-structural proteins) or form the viral capsid (structural proteins). Capsid proteins are the point of primary contact between the virus and its host and play a vital role in enabling the virus to infect the host tissue. Investigations were made to determine how the capsid proteins are processed by sequencing the N-terminal of the proteins. The constituent structural proteins were expressed separately and analysed for their antigenic properties and response to the antiserum generated in rabbit against the purified virus, so as to determine the role each protein plays in generating antibodies from the virus particle. The protease was expressed separately to make antibodies that could possibly be used to detect viral replication using immunological methods.

In the genome of *Picornaviridae*, the 5'-nontranslated region (5' NTR) contains the 5' internal ribosome entry site (IRES) which is required for cap-independent initiation of translation of the viral polyprotein and also plays an important role in determining the host range of the virus. The secondary structures of *Iflavirus* 5' NTRs had not been previously determined but can be predicted to locate regions with potential importance for IRES activity. A bee or mite tissue or cell culture system that could facilitate the study of VDV-1 replication is not available. As an alternative it was investigated whether the virus could replicate in lepidopteran or *Drosophila* cell lines. The 5' NTR of VDV-1 was investigated to determine if it contained a putative IRES that would be active in these cell types. The IRES activity in the 5' NTR was investigated using bicistronic reporter constructs via plasmids transfected into the cell lines. In this way a compatible cell system might be identified to facilitate the study of the virus (**Chapter 6**).

An ecological survey was carried out (**Chapter 7**) to investigate the extent to which VDV-1 had spread in the mite and/or bee populations, to identify whether the mite or the bee must be regarded as the primary host and to get clues about its transmission routes. This study involved the use of ELISA techniques to detect the virus in mites and bees, and was further qualified using RT-PCR with virus-specific primer sets. To study the prevalence of VDV-1 in Europe and to analyse its geographical variation mites from various regions were analysed. Bee samples from Kenya (a *Varroa*-free region) were investigated for the presence of this virus.

In **Chapter 8** the results of the whole study will be discussed in a broader context, taking into account the literature data from other researchers and other viral systems. The taxonomic position of VDV-1 will be discussed. Novel research questions are formulated and suggestions on how these should be addressed presented, as well as the expected applicability of the pathogen for biological control of the *Varroa* mite.

Chapter 2

Detection and localisation of picorna-like virus particles in tissues of *Varroa destructor*, an ectoparasite of the honey bee, *Apis mellifera*

Virus-like particles, 27-nm in diameter, were observed in extracts of individual *Varroa destructor* mites and in sections of mite tissue. Antiserum was prepared against these particles to detect the virus in individual mites. Immunohistology studies showed that the gastric caecae were heavily infected, whereas no immunostaining could be detected in other mite tissues or organs, like the salivary glands, brain, rectum or reproductive organs. By electron microscopy large aggregates of virus-like particles in para-crystalline lattices were found in cells of the gastric caecae. The particles, reminiscent to picorna-like viruses occurred mainly in the cytoplasm, whereas some virus particles were sparsely scattered in vacuoles. Occasionally, the particles were also observed in membrane-bound vesicles or in long tubular membrane structures. The accumulation of the picorna-like virus particles in the cytoplasm and the presence of the virus in membrane structures give a stong indication that the virus replicates in the mite. The nature of the virus needs to be determined using molecular methodology.

This chapter will be published in a slightly modified form as:
Quiansong, Z., Ongus, J. R., Bengsch, E. & Peters, D. (2006). Detection and localisation of picorna-like virus particles in tissues of *Varroa destructor*, an ectoparasite of the honey bee, *Apis mellifera*. In preparation

INTRODUCTION

The ectoparasite mite (*Varroa destructor* Anderson & Trueman) feeds on the body fluids of the larvae, pupae and adults of the western honey bee (*Apis mellifera* L.), causing serious damage to bee colonies. The parasitized bees lose weight, have a shortened life span and the colony may perish (Beetsma *et al.*, 1989). The extent of this damage depends on the mite density in the colony (Garedew *et al.*, 2004). The density can be controlled by the application of several rather effective acaricides (Ritter, 1990). However, the use of these agents has important disadvantages as their residues contaminate bee products like honey, wax, propolis and royal jelly (de Greef *et al.*, 1994). Application of technical methods, like the use of trap-combs (Fries & Hansen, 1993), is usually labour intensive (Calis, 2001). Control of the mite with biological agents like fungi and viruses pathogenic to the mites will not have these disadvantages.

Besides the damage caused to the bees, the mite is also involved in the transmission of bee pathogens like bacteria, fungi and viruses (Gliñski & Jarosz, 1990; Liu, 1996; Chen *et al.*, 2004a). The *V. destructor* mite has been associated with the transmission of invertebrate picorna-like viruses that cause paralysis in infected bees such as *Acute paralysis virus* (APV; Ball, 1989; Batuev, 1979) and *Slow paralysis virus* (SPV) (Denholm, 1999). The appearance of *Deformed wing virus*, which causes severe deformity and malformations in bees, was closely associated with the arrival of the *V. destructor* mite (Bowen-Walker *et al.*, 1999). In severely mite-infested bee colonies, the pathology and mortality is linked to the mite-mediated transmission of viruses which occurs mainly during feeding (Martin *et al.*, 1998).

Viruses, in particular baculoviruses, can often be applied successfully to control insect pests (Cory, 2003; Moscardi, 1999). A non-occluded baculo-like virus, which may be pathogenic to tetranychid mites *Panonychus citri*, was observed by Reed *et al.* (1972). This virus replicates in the midgut of the mite and is transmitted when healthy mites ingest faeces from infected mites (Reed *et al.*, 1975). A virus resembling the *P. citri* virus was detected in the European red mite *P. ulmi* (Putnam & Herne, 1966). Both viruses only cause natural epizootics in dense populations of their respective hosts (Reed, 1981; Putnam, 1970). Aggregates of unidentified particles have been detected in the gravid females of the phytoseiid mite *Neoseiulus cucumeris* (Steiner, 1993) and *Phytoseiulus persimilis* (Bjørnson *et al*, 1997). Besides these viruses infecting mites, a few reports exist on the occurrence of viruses in mites associated with bees. Liu (1991) found virus-like particles in the body cavity of the mite *Acarapis woodi* living in the trachea of the honey bee. These particles, 27-30 nm in size, were observed in the cytoplasm of infected tissue in para-crystalline arrays forming hexagonal patterns. A putative iridovirus was isolated from the *Varroa* mite in the USA by Camazine & Liu (1998). This virus was discovered after a case of high mortality in bees. Picorna-like virus particles have previously been observed in *Varroa* mites (Kleespies *et al.*, 2000). The particles detected occurred as irregularly-shaped aggregates and were reported to be present in the vacuoles and nuclei of the fat body and muscle tissue, but overall pathology was not detected in the mite.

Some eriophyoid and tetranychid mite species are known to transmit plant viruses. However, these viruses might not have harmful effects on the mite, although the *High plains virus* and *Pigeonpea sterility mosaic virus* (Kumar *et al.*, 2002) may replicate in their respective vectors. The large amounts of the beetle-transmitted *Brome mosaic virus* detected in the nonvector *Aceria tulipa* feeding on infected plants seem to cause severe cytopathological effects in the midgut cells in which the virus replicates (Paliwal, 1972).

Considering the knowledge that the use of various chemical applications may affect the quality of the bee products, and the cumbersome techniques like trap-comb method, searching for viruses which may be pathogenic to the *Varroa* mite may offer new ways to combat this mite. To this end a search was made on the occurrence of viruses in mites collected in beehives by light and electron microscopy. For the detection of viral particles, extracts were prepared from mite samples for the production of antiserum; the occurrence of virus-like particles in mite tissue was studied by light microscopy using this antiserum and by electron microscopy.

MATERIALS AND METHODS

Sampling of mites from beehives

Mites were collected from heavily infested beehives at the Wageningen University apiary. Dead and living mites, which dropped down from the bees, were collected on retractable trays at the bottom of the hives. The honey, wax, faecal and other debris was separated from the mites by suspending the samples in water containing 2% ethanol, followed by several washes over sieves with different gauzes.

Purification of virus particles from mites

One gram of washed mite sample was macerated and suspended in 1 ml 0.01 M potassium phosphate (K_2HPO_4/KH_2PO_4) buffer, pH 7.3. Large debris was removed by centrifugation at 5,000 rpm for 10 min at 4°C in a Sorvall B21 centrifuge. The supernatant was centrifuged through a 30% sucrose solution at 27,000 rpm for 6 h in a Beckman ultracentrifuge to collect the virus particles. The pellet with partially purified virus particles was re-suspended in 1 ml 0.01 M potassium phosphate buffer and centrifuged at 40,000 rpm for 3 h at 4°C in a discontinuous 10-40% sucrose gradient. The fraction (band) with virus particles was removed and centrifuged at 30,000 rpm for 3 h. The pellet was re-suspended in 1 ml 0.01 M potassium phosphate buffer and stored at 4°C.

Antibody production and immunodetection

The virus, used for the production of antiserum in a rabbit, was further purified from the partial purified preparation by equilibrium centrifugation in a CsCl gradient with an initial density of 1.37 g/cm^3 (= 42% CsCl w/v). Polyclonal antiserum was prepared by administering an immunisation injection to the rabbit followed by a booster injection two weeks later. Blood was collected two and four weeks after the booster injection.

The polyclonal antiserum was checked by detecting virus infections in individual mites by enzyme-linked immunosorbent assays (ELISA). The collected mites were ground in liquid nitrogen, incubated in 0.2 ml of 0.01 M potassium phosphate buffer and assayed by ELISA as described by Clark & Adams (1977).

Detection of virus particles in mites

Dead mites were collected from retractable trays at the bottom of hives at the Wageningen University apiary. They were sorted out into four groups according to their cuticle colour (light ochre, dark ochre to an orange, brown, and brown with black spots). They were individually tested by ELISA for virus infection.

Electron microscopy of viruses in mite tissue

The mite bodies were fixed for 1.5 h at room temperature with 3% paraformaldehyde in 0.1 M phosphate buffer (K_2HPO_4/KH_2PO_4), pH 7.2, with 2.5% sucrose (w/v). Immediately after fixation, the mites were washed three times with 0.1 M phosphate buffer, pH 7.2, at 4°C and kept overnight in the same buffer. The mites were dehydrated in five steps using 50% ethanol at 0°C for 30 min, 50% ethanol at 20°C for 30 min, 70% ethanol at 20°C for 30 min, 90% ethanol at 20°C for 45 min and 100% ethanol at 20°C for 60 min. The dehydrated mites were transferred to a 1:1 mixture of 100% ethanol and LR gold resin and held at -20°C for 1 h, then to a pure LR gold solution and kept overnight at -20°C. The mites in LR gold were embedded by low temperature UV-mediated polymerisation of the resin for 48 h at -20°C. The embedded mites were sliced into sections of 60 nm thickness.

Immunohistochemistry and light microscopy

The appendages of the living mites were removed prior to fixation of the bodies for 2 h at 4°C in 0.01M phosphate buffer (K_2HPO_4/KH_2PO_4, pH 7.5) containing 2% formalin. The fixative was washed out overnight using PBS (140 mM NaCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4) containing 6% sucrose at 4°C. The specimens were dehydrated in acetone for 1 h at 4°C and refreshed with acetone until the liquid remained clear. The bodies were then infiltrated with a solution in 100 ml of Technovit® 8100 resin 2-hydroxyethyl methacrylate [GMA]) (Electron Microscopy Sciences) containing 0.6 g of Hardener I (benzoyl peroxide), with mild agitation in vacuum for 7 h at 4°C. The embedding solution was freshly prepared by adding 1 ml of Hardener II to 30 ml of the infiltration solution. The mites were embedded in this solution in a mould at 4°C overnight.

Tissue sections, 4 μm thick, were prepared by slicing the embedded material using a microtome. Each section was mounted onto a drop of water on a glass slide. The mounted slides were dried at 60°C for 5 min, washed with PBS for 15 min at room temperature and then incubated with 1% BSA in PBS solution for 30 min. Antiserum was applied in a 1:200 dilution in 1% BSA in PBS at room temperature for 2 h. After incubation the sections were washed in PBS for 6 x 5 min and 3 x 10 min. The following steps were carried out in the dark. The sections were incubated with horse radish peroxidase conjugated IgG at a 1:200 dilution in 1% BSA in PBS at room temperature for 1 h. After washing three times for 5 min, the sections were incubated with substrate solution (0.05% diaminobenzidine chromogen, 0.01% hydrogen peroxide in 0.05M Tris/HCl, pH 7.5). The reaction was stopped by washing the sections three times with distilled water. The tissues were counterstained with Mayer's hematoxylin solution (Canemco Inc & Marivac Inc.) for 15 min. After washing briefly with tap water, the sections were serially dehydrated in 70% ethanol for 1 sec, 90% ethanol for 1 sec, 100% ethanol for 1 sec and then air-dried. The sections were mounted with a cover slip using DPX mounting medium and observed using a light microscope. Control specimens were prepared from virus-free female mites. These were taken from mite families of which the nymphs were negative for infection as shown with ELISA (Chapter 7).

RESULTS

Extraction of virus from mites

A few isometric virus particles measuring 27 nm in diameter were occasionally found in extracts of individual mites. Numerous particles with this diameter were observed following an extraction and purification procedure on mites collected from the Wageningen University apiary. From their size and morphology, these particles are reminiscent to picorna-like viruses of invertebrates, either from the family *Dicistroviridae* of from the genus *Iflavirus* (Christian *et al.*, 2005). A highly purified preparation (Fig. 2-1) was used to raise antibody in a rabbit.

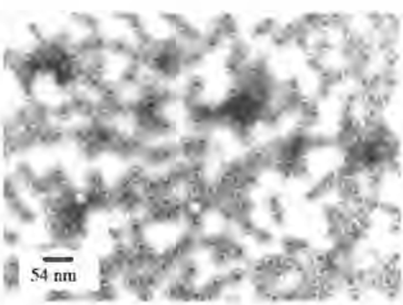

Figure 2-1: Purified picorna-like virus particles of a *V. destructor* preparation used to prepare antiserum in a rabbit. Bar represents 54 nm.

Detection of virus in individual mites

Dead mites found on the retractable trays were individually tested by ELISA for virus occurrence. The collected mites were divided in four groups according to their colour (Fig. 2-2). The four colour groups established were light ochre, dark ochre to an orange, brown and brown with black spots. The latter group contained often black spots.

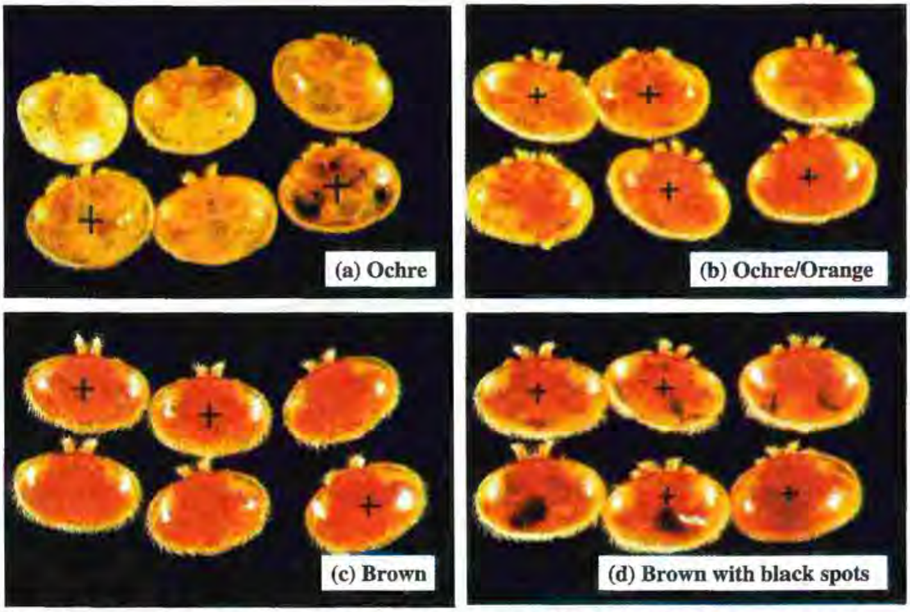

Figure 2-2: *Varroa* mites sorted out into four colour groups: (a) light ochre, (b) dark ochre to an orange, (c) brown and (d) brown with black spots. In all the groups, mites that tested positive in ELISA for virus infection have been marked by the plus sign.

Analysis of the individuals of each group revealed that infected individuals as well as non-infected individuals occurred in each group (Table 2-1). The virus could be detected in approximately 50% of the mites in all colour groups. These results show that differences in colour could not be used to differentiate non-infected mites from healthy mites. Statistical differences were also not found in the average ELISA values between the four groups (data not shown).

Table 2-1: The number of ELISA positive *Varroa mites* in the groups with different colours.

Appearance in colour	Total	Positive	Negative
Ochre	31	15	16
Ochre/orange	52	21	31
Brown	50	26	24
Brown with black spots	42	24	18
Total	**175**	**86**	**89**

Analysis of mite families living on a bee emerging from a comb cell showed that all members of a mite family were either all positive or all negative for the presence of virus in ELISA. This may suggest that the virus is highly infectious for mites and can be transmitted either horizontally or vertically or both (Chapter 7).

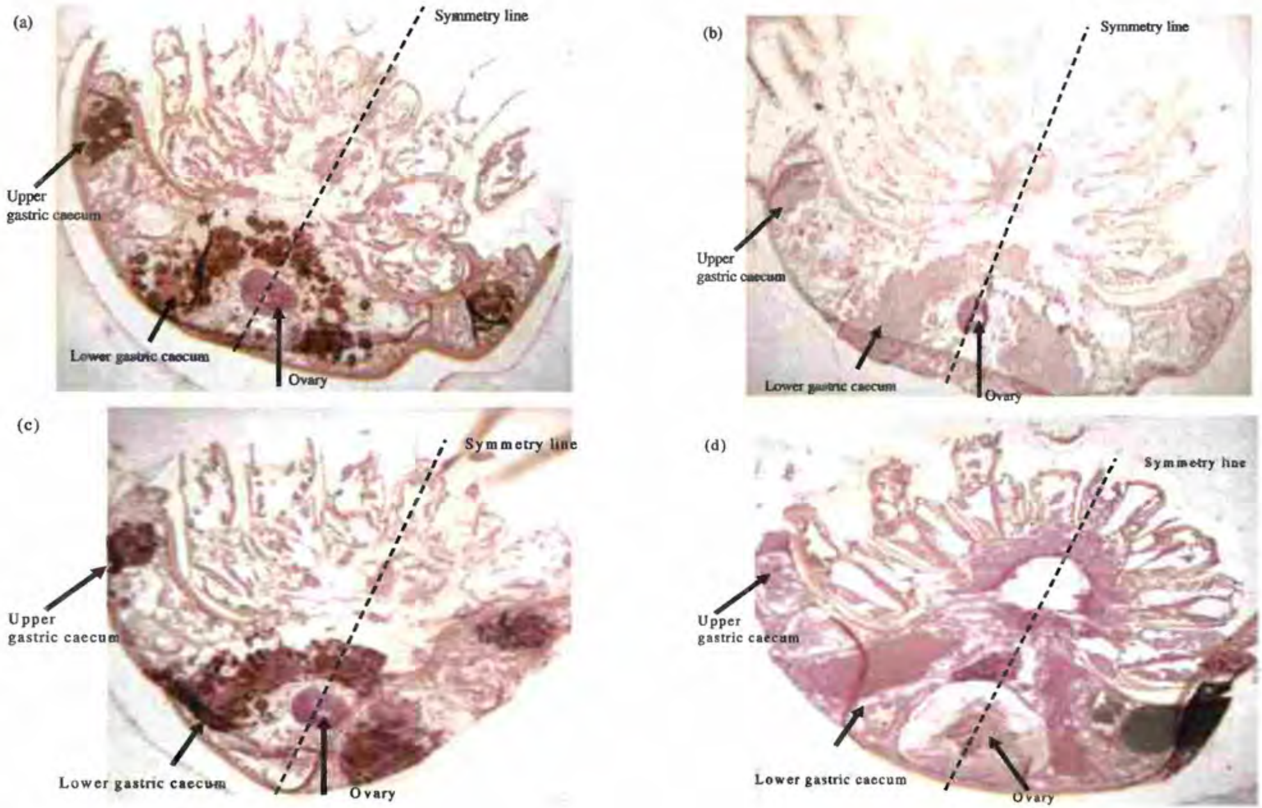

Figure 2-3: Immunohistology of *Varroa* tissue. Panels (a) and (c) show sections of infected tissue from two mites and panels (b) and (d) show negative control sections from non-infected mite tissue. The sections were stained as described in Material and Methods. The diagonal lines indicate the symmetry position between the right and left halves of the bodies. The infected tissues of the upper and lower gastric caecae are visible as dark coloured spots.

Distribution of the virus within the mite body

Varroa mites have a hard protective exoskeleton that protects the vital organs in the body. Figure 2-4 shows a schematic representation of the dorsal view of the *Varroa* mite. The brain, muscle, mouth parts and salivary glands are located in the anterior part of the body (De Ruiter & Kaas, 1983). The reproductive organs, Malphigian tubules and most of the digestive system are located in the posterior part.

Virus infection could be detected in mites by chromogen deposition on the infected tissues as a result of an enzymatic reaction between the conjugate on the secondary antibody and the substrate. Virus could prominently be found in both the upper and lower gastric caecae lobes of the alimentary tract (Fig. 2-3). The virus was notably absent in the muscle tissue, brain, and rectum. The technique used did not detect any virus in the salivary glands or the reproductive organs.

Examination of ultra thin sections by electron microscopy revealed high concentrations of icosahedral particles resembling in size those which were extracted from mites. These particles could only be detected throughout the gastric caecae. Figure 2-5 shows that gastric caecum tissue in the posterior lobe, close to the fourth leg, was heavily infected. No virus particles were found in the fat body cells neighbouring the gastric caecum tissues. Virus particles could also not be detected in the other organs such as the salivary glands, brains and in muscle tissues.

The virus particles in infected cells occurred either scattered throughout in irregular arrangements or clustered in para-crystalline arrays in the cytoplasm of cells (Fig. 2-6a). Virus particles also occurred in vacuoles (Fig. 2-6b). Occasionally, infected cells were encountered in which the virus particles were enclosed in apparently closed membranous sacs (Fig. 2-6c), or were aligned between membranes or enclosed in tubular membranous structures (Fig. 2-6d).

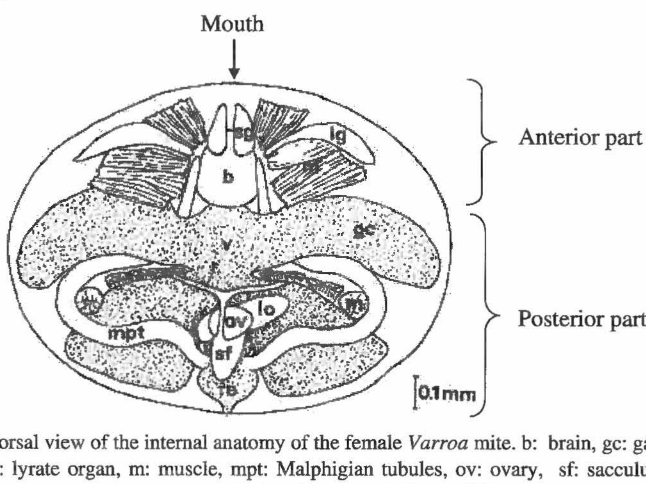

Figure 2-4: A dorsal view of the internal anatomy of the female *Varroa* mite. b: brain, gc: gastric caecum, lg: lateral gland, lo: lyrate organ, m: muscle, mpt: Malphigian tubules, ov: ovary, sf: sacculus foemineus, sg: salivary gland, r: ramus, re: rectum, v: ventriculus (de Ruiter & Kaas, 1983).

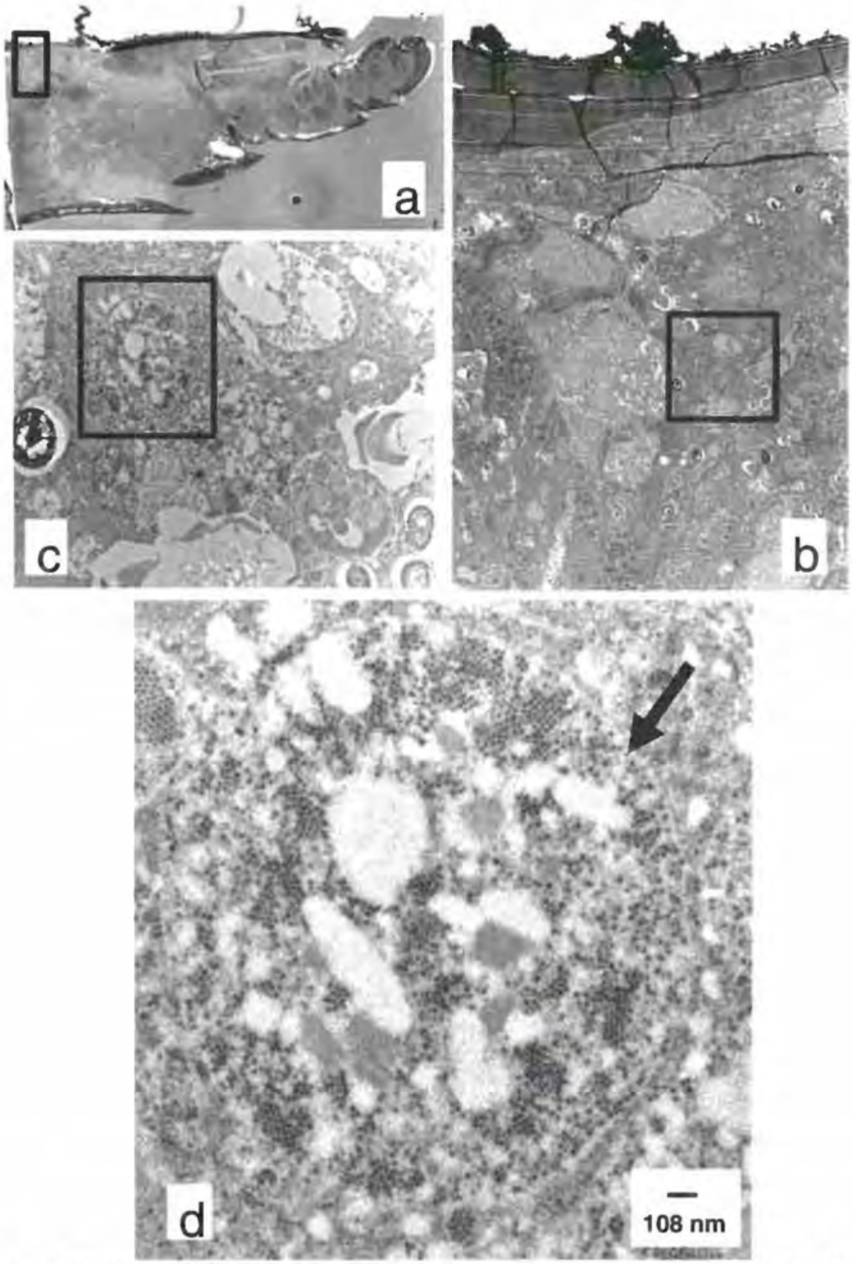

Figure 2-5: Heavily infected gastric caecum tissue in the posterior lobe, close to the fourth leg (a). The infected tissue in panel (a) highlighted by the rectangle is enlarged in (b). Infected cells with virus-like particles scattered throughout the cytoplasm are pictured in (c) which is enlarged from marked section in (b). Panel (d) shows a heavily infected cell with virus-like particles scattered in the cytoplasm. Size bar in (d) is equal to 108 nm.

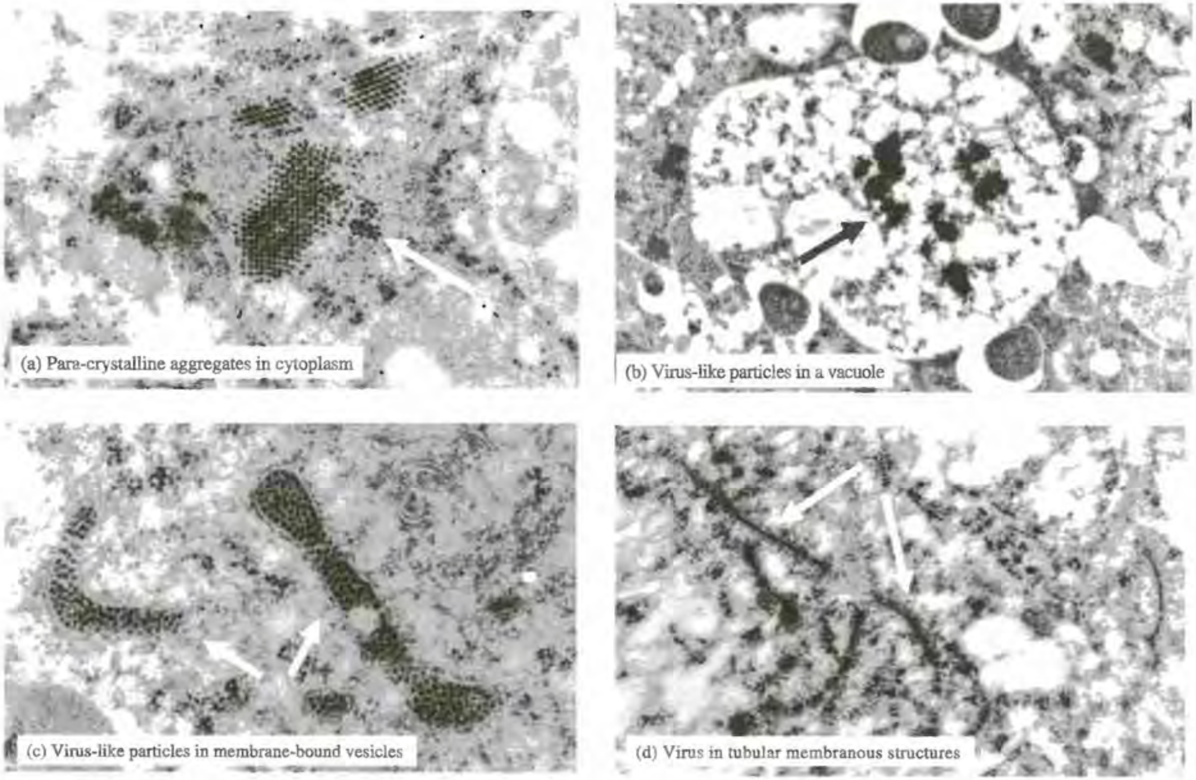

Figure 2-6: Electron micrographs of *Varroa* mite tissue showing 27 nm virus-like particles indicated by arrows. (a) Accumulation of virus-like particles in a vacuole. (b) The virus particles observed as para-crystalline structures in the cytoplasm of a *Varroa* mite cell. (c) Virus-like particles tightly packed in membrane-bound vesicles. (d) virus particles between membranes or arranged in tubular membranous structures

DISCUSSION

Virus-like particles, approximately 27 nm in diameter, were isolated from *Varroa* mites infesting honey bees or from dead mites collected from trays below hives. These particles very much resemble picorna-like viruses from invertebrates (Christian *et al.*, 2005). The techniques used to observe and detect the infection can not differentiate between viruses with a similar morphology and size. Electron microscopy cannot differentiate between morphologically similar picorna-like viruses so it would not be possible to tell if the infecting virus contains a mixture of similar virus types. So, molecular methodology needs to be applied to identify and characterize this virus further (Chapter 3).

Immunohistochemistry very much depends on the quality of the antiserum raised. If it was prepared from a mixture of viruses then it could detect all viruses that would have been present during isolation from infected mites. The virus particles isolated might be specific to the mite, but could also have the ability to infect both mites and bees. If the latter is the case, it cannot be excluded that the antiserum prepared could detect both mite and bee viruses in mite extracts either as a result of ingestion and/or replication of these viruses in mites.

Using light microscopy and immunohistochemistry virus occurrence was detected in the gastric caecae, whereas no obvious infections were found in any other tissue or organ. Electron microscopic examination revealed the presence of the virus in the cytoplasm of infected cells either organised in crystalline structures or unarranged. These may be cells / tissues in different stages of virus infection. The accumulation of the virus in large para-crystalline lattices (Fig. 2-6a), and the dispersal of numerous particles in the cytoplasm (Fig. 2-5d) in the gastric caecae cells strongly suggest that the virus replicates in the *Varroa* mite. Virus particles were also observed in vacuoles (Fig. 2-6b), usually in unarranged order and at much lower density than in the cytoplasm. Since this is most likely an RNA virus, replication in vacuoles is not likely to occur, therefore, the virus might have leaked from the cytoplasm into the vacuoles.

No virus particles were observed in nuclei. Kleespies *et al.* (2000) reported the presence of picorna-like virus particles in the nuclei. Replication and accumulation of an RNA virus in the nucleus is unlikely. The virus found by these authors might be another virus likely with a DNA genome or the vacuoles were mistaken to be nuclei.

The virus could also be found associated with membrane structures. It occurs in apparent sacs (Fig. 2-6c) or between two parallel running membranes (Fig. 2-6d). The viruses of some plant virus families can be found in tubules which have similar appearance as the mite virus in the parallel running membranes. The function of the viruses in the tubules in plants and between the membranes in mites differs. The viruses in plants moves through these tubular structures from one cell to another (Zambryski & Crawford, 2000), whereas the membrane associated viruses in mites are probably not in a process of transmission or spread from one cell to another since picorna-like viruses exit the infected cell by lysis (Rueckert, 1996). Luteovirus particles (plant infecting positive-sense single stranded RNA viruses), transmitted by aphids, are enclosed in smooth vesicular structures when they pass the gut epithelial cells during their passage from the hindgut lumen to the haemocoel (Gildow, 1993; Reinhold *et al.*, 2003). Subsequently, these viruses pass the accessory salivary gland cells in tubular structures in their transport from the haemocoel to the salivary ducts (Gildow & Gray, 1993). In both cases the membranous structures serve as a transport vehicle for these viruses which have to pass a one cell-layer-thick membrane. The membrane-bound viruses in the mite might be involved in some sort of process without virus trafficking from one cell to another. Such trafficking has been observed without any uncoating and multiplication of human immunodeficiency viruses when these viruses pass through gastro-intestinal cells (Bomsel, 1997).

The mite virus is apparently restricted to the gastric caecum of the *Varroa* mite. Restriction of viruses to the intestinal tract is not an uncommon phenomenon in mites (Reed & Hall, 1972; Paliwal, 1972). This tissue restriction and the apparent absence of the virus in the salivary glands will affect the mechanism by which these viruses might infect the mites. It has been speculated that the virus particles may be brought back to the mouth parts by regurgitation of gut contents as observed in the transmission of some plant viruses by flea beetles (Paliwal, 1972; Gergerich *et al.,* 1983). Alternatively the virus may be excreted with the faeces and ingested by healthy mites as has been suggested for the apparent non-occluded viruses infecting the digestive tracts of *P. citri* and *P. ulmi* (Reed *et al.,* 1975; Putnam, 1970). A limited possibility of missing the virus particles in ovarian tissue does not eliminate the possibility that the virus is transovarially transmitted.

ACKNOWLEDGEMENTS

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Chapter 3

Complete genome sequence of a picorna-like virus of the genus *Iflavirus* isolated from the mite *Varroa destructor*

Invertebrate picorna-like viruses are small non-enveloped viruses with single-stranded, positive-sense RNA genomes. Sequence analysis of the RNA of a 27 nm picorna-like virus isolated from the *Varroa destructor* mite revealed a genome organisation with high similarity to members of the genus *Iflavirus*. Phylogenetic analysis of the polymerase showed that the virus was most closely related to *Deformed wing virus* (DWV) and *Kakugo virus* (KV) of the honey bee. The virus has a genome of 10,112 nucleotides (without the poly-A tail) with an overall RNA sequence identity of 84% to DWV and KV. The genome has one large open reading frame translated into a 2893 aa polyprotein with an amino acid identity of 95% to DWV and KV. This virus was named *Varroa destructor virus* 1 (VDV-1).

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Ongus, J. R., Peters, D., Bonmatin, J-M., Bengsch, E., Vlak, J. M. & van Oers, M. M. (2004). Complete sequence of a picorna-like virus of the genus *Iflavirus* replicating in the mite *Varroa destructor*. *Journal of General Virology* 85: 3747-3755.

INTRODUCTION

Invertebrate picorna-like viruses are small, non-enveloped viruses that have an isometric particle. The RNA genome is single-stranded, and of positive sense and is either mono- or dicistronic (van Regenmortel *et al.*, 2000).

The monocistronic viruses have been grouped in the genus *Iflavirus* (Christian *et al.*, 2002), which has not been placed in a virus family yet. The type species of this genus is *Infectious flacherie virus* (IFV), which was isolated from the silkworm *Bombyx mori* (Isawa *et al.*, 1998). Two species have officially been assigned to this genus, *Sacbrood virus* (SBV) of bees (Ghosh *et al.*, 1999) and *Perina nuda picorna-like virus* (PnPV) (Wu *et al.*, 2002). *Deformed wing virus* (DWV) (de Miranda *et al.*, 2003; Lanzi & Rossi, 2003) of honey bees and the recently analysed *Kakugo virus* (KV) (Fujiyuki *et al.*, 2004), which was isolated from the brains of aggressive worker honey bees are closely related to each other and have the characteristics of iflaviruses. Another potential member of the genus *Iflavirus* is *Ectropis obliqua picorna-like virus* (EoPV) (Wang *et al.*, 2004) isolated from the moth *Ectropis obliqua*. Iflaviruses have a single large open reading frame (ORF), which encodes both the structural and non-structural proteins. Like in picornaviruses of vertebrates, the capsid proteins are encoded by the 5′-part of the genome and the proteins involved in virus replication by the 3′-part. Unlike vertebrate picornaviruses, iflaviruses lack the 2A-protease/2B/2C region (Fauquet *et al.*, 2005) as shown in Figure 3-1.

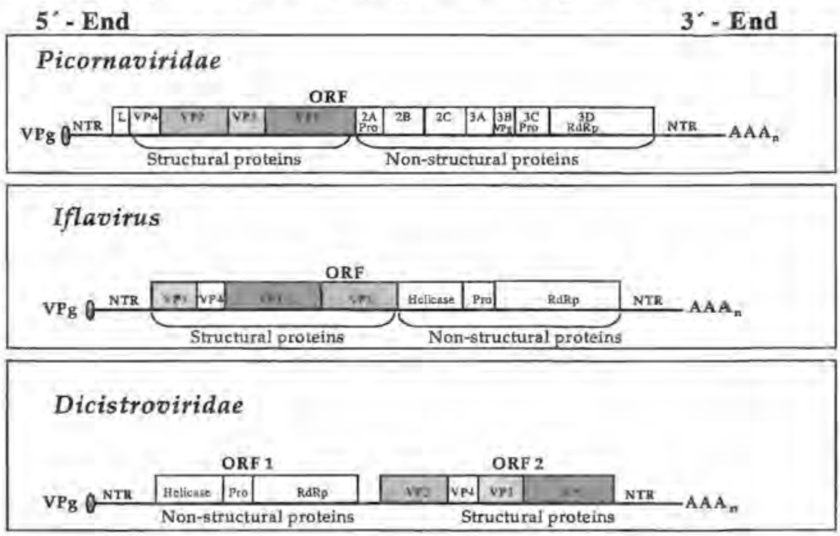

Figure 3-1: Schematic diagram of the genome organisation of *Picornaviridae*, *Iflavirus* and *Dicistroviridae*. The genome-linked protein (VPg) on the 5′ end has been shown to be present in picorna-like viruses such as *Cricket paralysis virus* and *Drosophila C virus* (King & Moore, 1988). The structural proteins (VP1-VP4) are shaded. Some picornaviruses (cardioviruses and aphthoviruses) encode a leader protein (L) at the beginning of the ORF. The viruses belonging to these groups have a polyadenylated tail on the 3′ end of the genome.

The dicistronic viruses have recently been accommodated in the new family *Dicistroviridae*, containing the single genus *Cripavirus* (Christian *et al.*, 2005), with the type species *Cricket paralysis virus* (CrPV) (Wilson *et al.*, 2000). Other species within this genus include: *Acute bee paralysis virus* (ABPV) (Govan *et al.*, 2000), *Aphid lethal paralysis virus* (ALPV) (van Munster *et al.*, 2002), *Black-queen cell virus* (BQCV) (Leat *et al.*, 2000), *Drosophila C virus* (DCV) (Johnson & Christian, 1998), *Rhopalosiphum padi virus* (RhPV) (Moon *et al.*, 1998), *Taura syndrome virus* (TSV) of shrimp (Mari *et al.*, 2002), *Triatoma virus* (TrV) (Czibener *et al.*, 2000), *Himetobi P virus* (HiPV) (Nakashima *et al.*, 1999) and *Plautia stali intestine virus* (PSIV) (Sasaki *et al.*, 1998). Members of this family are characterised by having two non-overlapping ORFs encoding either the structural or non-structural proteins. An intergenic region, which functions as an internal ribosome entry site (IRES), separates the two ORFs. In dicistroviruses, the proteins involved in RNA replication and polyprotein processing are encoded by the 5′-proximal ORF, whereas the 3′-proximal ORF encodes the capsid proteins (Leat *et al.*, 2000).

In the search for pathogens of the mite *Varroa destructor*, we isolated virus particles with an average diameter of 27 nm resembling picorna-like viruses from mites in beehives at Wageningen University (Chapter 2). Picorna-like virus particles have previously been observed in *Varroa* mites (Kleespies *et al.*, 2000). In order to identify this virus, the complete sequence of the viral genome was determined. Phylogenetic analysis was used to estimate the taxonomical position of the virus.

MATERIALS AND METHODS

Viral RNA isolation

Virus particles were isolated from *Varroa destructor* mites according to the method described in Chapter 2. RNA used as template for cloning and sequencing the virus genome was isolated from 20 µl virus suspension. RNA extraction was done using 800 µl TRIZOL® reagent (Invitrogen) and 160 µl of chloroform according to the manufacturer's instructions. To facilitate RNA precipitation 200 µg glycogen was added to the sample. The extracted RNA was precipitated from the aqueous phase by adding an equal volume of 2-propanol and washed once with 70% ethanol. RNA was re-suspended in sterile distilled water.

Cloning and sequencing of the VDV-1 genome

Initially cDNA was synthesised using the immunocapture RT-PCR method with some modification to the protocol described by Kokko *et al.* (1996) and combined with 3′ rapid amplification of cDNA ends (3′ RACE) methodology.

For the immunocapture, eppendorf tubes were coated with 100 µl antibody solution containing 1 µg IgG (Chapter 2) in phosphate buffered saline (PBS) (140 mM NaCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4), for 2 h at 37°C, washed three times with PBS and incubated overnight at 4°C with a few ground mites in PBS in order to trap the virus. The unbound material was removed and the tubes washed three times with PBS finally removing all excess buffer by pipetting after a brief centrifugation to collect the liquid at the bottom of the tube. First strand cDNA was directly synthesised in the tube containing the trapped virus using the enzyme SuperScript™ reverse transcriptase (Invitrogen) and a commercially available oligo-dT anchor primer (5′ GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTTV 3′) (Roche), assuming that if the isolated virus was indeed an insect picorna-like virus a poly-A tail would be present. The first strand cDNA obtained was used in a PCR reaction using a forward degenerate primer (5′ ATIGTIIIITAYGGIGAYGA 3′) for a conserved region around the GDD motif (IVXYGDD) which is highly conserved in the RNA-dependent RNA polymerase (RdRp) amino acid sequences of picorna(-like) viruses (Fig. 3-3a region VI) in combination with the oligo-dT primer mentioned above. The PCR was run for 35 cycles with an annealing temperature of 40°C for 3 min and elongation at 72°C for 3 min and final a elongation of 7 min at 72°C.

In order to walk towards the 5′-end of the viral genome, 5′ RACE was used repeatedly using the 5′RACE kit (Roche) in combination with the Expand long-template PCR system (Roche) essentially as described by van Oers *et al.* (2003). This strategy involves the use of specific primers to synthesise cDNA from RNA isolated from virus particles, and a nested primer in PCR. PCR products were cloned in pGEM-T easy (Promega) and analysed by automated sequencing. Prior to the cloning of the 5′-terminal region of the viral genome, the isolated RNA was treated with proteinase K in order to ensure that a genome-linked protein (VPg) was removed. Two microgram of total RNA was digested with 100 μg/ml proteinase K in 50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$, 0.2% SDS at 60°C for 1 h. The proteinase was subsequently removed by phenol-chloroform/isoamyl alcohol extraction. RNA was precipitated from the aqueous phase with an equal volume of 2-propanol and washed once with 70% ethanol.

Computer-assisted sequence analysis

The software SeqMan in the programme DNASTAR™ was used to build contiguous data of overlapping clones from which a consensus nucleotide sequence of the virus was derived using information from at least six clones for every nucleotide. The consensus nucleotide sequence was compared to related sequences in GenBank using the BLAST tool on the National Centre for Biotechnology Information (NCBI) site and the Fasta tool for similarity searches on the European Molecular Biology Laboratory (EMBL) site. Phylogenetic trees were constructed with the predicted RdRp amino acid sequences from data available for related viruses in GenBank. ClustalX software (Thompson *et al.*, 1997) was used to create an alignment of the related sequences and the GeneDoc programme (Nicholas *et al.*, 1997) to edit the alignment. Phylogenetic trees were plotted using the neighbour-joining method. The confidence levels as percentages were estimated by 1000 replicates in a bootstrap analysis using ClustalX. Other virus sequences used in this paper and their accession numbers are listed in Table 3-1.

Table 3-1: Nucleotide sequence accession numbers and general classification of the picorna(-like) viruses discussed in this paper.

Virus	Accession number	Classification
DCV	AF014388	Family: *Dicistroviridae*
CrPV	AF218039	
PSIV	AB006531	
BQCV	AF183905	
ABPV	AF150629	
RhPV	AF022937	
HiPV	AB017037	
TrV	AF178440	
TSV	AF277675	
ALPV	AF536531	
IFV	AB000906	Genus: *Iflavirus*
DWV	AY292384	
KV	AB070959	
SBV	AF092924	
PnPV	AF323747	
EoPV	AY365064	
EMCV	M81861	Family: *Picornaviridae*
Human poliovirus 2	X00595	

RESULTS

Genome sequence

Cloning and sequencing of the virus genome yielded a continuous sequence of 10,112 nucleotides, excluding the polyadenylated tail [GenBank accession number AY251269]. The nucleotide base composition of the genome is 29.21% A, 32.20% U, 22.61% G and 15.98% C. The use of an oligo-dT reverse primer in RT-PCR in combination with a forward degenerate primer annealing to a putative conserved YGDD motif in the polymerase region (Fig. 3-3a) amplified a product of approximately 800 bases, indicating that a poly-A tail was present at the 3′-end of the genome. The sequence of this PCR product revealed part of the coding sequence homologous to RdRp sequences of picorna-like viruses. The virus genome (Fig. 3-2) has one large ORF (nucleotides 1118-9799), which translates into a single polyprotein. The structural proteins are encoded by the 5′ part of the coding sequence, while the 3′ part codes for the non-structural proteins. The 5′-non-translated region (5′-NTR) is 1117 bases long, and the 3′ non-translated region (3′-NTR) has a length of 313 nt.

1 GCAUAGCGAAUUACGGUGCAACUAACAAUUUUAGAUAGUAGCCAUGAACAAACAUUAUGA
61 UUACUCACUACGUAUUGAUCAUUUUUACAAUGACUUGCGUAGCAUGAAGCGCAUGCUUGU
121 AGUUAUAACUAUGUUAUUUUGCAAGUUGGAGAUAAUUGUAUUGGAUUAUGGAUGCGUGCA
181 CUAAGUGUCUACAUCUAUAGUCGUUUGUGGUUCAAGUUUUUGUGUUAGUAGUACAAUCUU
241 GAAGAAUGUAAGUAUCGUAUGAAUGAUAUUUGAAUGACAACACUGAAGUAUAAAAUAUAU
301 AAAAUCCAAAAAUAUUUUUAAUCUUAUUCAGUGUAGUGUUUGAUAGAGUAGAAUGCCAUG
361 UGACCGCUCAAAGGAGUCCAUUAUGGUAUAUCAUUCGAAGUCGAAUACUUGUGUAUAGUU
421 AUUGUAUUUUAUUAGUAAUAUUAGCAGUCCGUAACUAUCAUAAUCCUAUUAUAGUUUGAU
481 UAUAUGAUAGACCACUGCAGUAUCGAGUAGAGUUUAGAAAGAGUAGUGCAAUAGUAAGAU
541 CACUGUCACCGACCACUCAUUGUAAUAGUGAGGUUUGUCGGAAACCAGUUAUUGUGCAGC
601 GACUAGCAAUCGUGAAUCAAUAUAGUUGGUAUUCUAAAUAUGAGACGAUUCGGCGAUUUU
661 AUUGCGACUGAAAUUUCAUAUUUAGCAUGUCAGGUCUUAUUAUGAAUGCUCGAGUAUUUA
721 UUUUUGCGGUAGAGUAGGGACCCCUCUAUCUCUCAGGUACUGUAUGAGGCGAAAGUGUGA
781 AAGUAAUUUAUGUCUCUAUACAUAAGUGACUGUAUCGGGAUUUCCUUUGGCAAGAAUCCU
841 UUUAAUUCAGUAUAAUUUAUGCUACGGUACGUUACGUUCGCAGGGCACCCGUUAAUGUCA
901 CAUAGCCCAGACGAUGACGAAUGGAAAGACAUUACUUUUUAUUUUAAUGCUACGAUUAUU
961 GCUGUUUUAUUUUGCUGUUUUUAUUUGUUAUUAUAUUUUGCUAUUUUAUUAUUGCUAAAU
1021 AUAUUUCUUUACUAUUUUUGCUUUAUAUAUUAGAUUCAAUUCUUUUUAUUUUAUAUUUUC
1081 AAUUUGAUUUUGAUUUUGAAGGUAAAUAUAUAUAAAA**AUG**GCAUUUAGUUGUGGAACUCU
5′ NTR region ← ORF start
1141 UUCUUAUGCUGCUGUUGCCCAAGCUCCCUCUGUAGCUCAUGCUCCCCGUAGUUGGGAGAU
1201 UGAUGAAGCUAGGCGUCGACGCGUUAUCAAGCGUUUGGCGUUGGAACAGGAACGGAUUCG
1261 AAACGUUCUUGAUGUCACUGUGUAUGAUCAUACAACGUGGGAGCAAGAGGAUGCGCGUGA
1321 UAAUGAGUUCCUUAUGGAACAAUUGAAUAAUUUAUAUACGAUAUAUUCUAUAGCUGAAAG
1381 AUGUACCCGCCGCCCUGUUCAAGAACAUGUCCCCAUUUCAAUCAGUAAUAGAUAUUCCCC
1441 UUUAGAAUCCCUUAAGAUUGAGGUAGGAAAAGACGCGGGUGAGUUCGUAUUUAAGAAACC
1501 CAAAUAUACAAAGAUUUGUAAGAAAGUGAAACGGGUGGCAUCAAAAUUUGUGCGCGAGAA
1561 AGUUGUUAGGCCCGUUUGUAAUCGAUCGCCCAUGUUAUUAUUUAAAAUUAAGAAAGUAAU
1621 AUAUGAUCUACAUUUGUAUCGGUUACGGAAACAAGUUCGGCUUCUCAGACGCGAAAAACA
1681 GCGUGAAUACGAGUUAGAGUGUGUUACUAGUUUGCUACAGCUAUCUAAUCCUGUUUCAGC
N P G / P
1741 UAAACCUGAGAUGGACAAUCCUAAUCCUGGUCCAGAUGGUGAAGGUGAAGUUGAAUUAGA
1801 AAAGGAUAGUAAUGUAGUAUUAACUACACAACGUGAUCCUAGCACCUCUAUUCCUGCUCC
1861 AACUAGUGUGAAGUGGAGUAGAUGGACUAGUAAUGAUGUUGUGGAUGAUUAUGCCACUAU
1921 AACUUCGCGUUGGUAUCAGAUUGCCGAAUUUGUGUGGUCAAAGGAUGAUCCAUUUGAUAA
1981 GGAAUUGGCGCGCUUAAUUUUACCUCGAGCUUUGUUAUCUAGUAUUGAGGCUAAUUCUGA
2041 CGCUAUUUGUGAUGUACCUAAUACUAUUCCGUUUAAGGUACAUGCAUAUUGGCGUGGAGA
2101 UAUGGAAGUUCGAGUGCAGAUUAACUCGAAUAAAUUCCAGGUUGGUCAAUUACAGGCAAC
2161 UUGGUACUAUUCGGAUCAUGAAAAUUUGAAUAUCCAGACGAAGCGAAGUGUGUAUGGUUU
2221 UUCGCAUAUGGAUCAUGCUUUGAUUAGCGCAUCAGCGAGUAAUGAAGCAAAAUUAGUGAU
2281 ACCUUUUAAACACGUAUAUCCAUUCUUACCAACGCGUGUCGUUCCUGAUUGGACAACUGG
2341 UAUUCUUGAUAUGGGUACCUUAAAUAUUCGUGUAAUUGCUCCACUACGUAUGAGUGCGAC
2401 GGGACCAACCACUUGUAAUGUUGUAGUAUUUAUUAAGUUAAAUAAUAGUGAAUUCACUGG
2461 CACUUCUUCUGGUAAGUUUUACGCGAAUCAAAUCAGGGCAAAACCUGAAAUGGACCGUGU
G C N M / D N P
2521 GUUAAAUUUGGCAGAAGGAUUACUAAAUAAUACCGUAGGUGGUUGUAAUAUGGAUAAUCC
S Y Q Q S → <u>VP1</u>
2581 GUCAUAUCAGCAAUCUCCGCGUCAUUUUGUUCCUACUGGUAUGCAUAGUUUAGCUUUAGG
2641 CACUAAUUUAGUAGAGCCUUUGCAUGCAUUACGAUUAGAUGCAUCAGGUACAACACAACA
2701 UCCAGUUGGGUGUGCGCCUGAUGAAGAUAUGACUGUAUCUUCCAUUGCAUCACGAUAUGG
2761 UUUAAUUCGCCAAGUGCAAUGGAAGAAAGACCAUGCGAAAGGAUCAUUAUUAUUACAACU
2821 UGAUGCUGAUCCUUUCGUUGAACAGAAAAUUGAGGGAACCAAUCCAAUUUCUUUGUAUUG
2881 GUUUGCUCCGGUUGGAGUCGUAUCUAGUAUGUUUAUGCAAUGGAGAGGUUCUUUAGAAUA
2941 UAGAUUUGAUAUUAUAGCUUCCCAAUUUCAUACGGGUAGGUUAAUUGUAGGUUAUGUUCC
3001 UGGACUGACUGCUUCUUUACAACGUCAAAUGGACUAUAUGAAAUUGAAGUCAUCUAGUUA
3061 UGUGGUGUUUGAUUUACAGGAAAGUAAUAGUUUUACGUUUGAAGUGCCCUAUGUGUCAUA
3121 CAGACCGUGGUGGGUGCGUAAGUAUGGUGGUAAUUAUCUGCCAUCUUCUACUGAUGCGCC
3181 UAGCACACUGUUUAUGUAUGUACAAGUACCAUUGAUACCUAUGGAAGCUGUUUCUGAUAC
3241 UAUAGAUAUCAAUGUGUAUGUGCGUGGUGGCAGUUCGUUUGAGGUUUGUGUUCCAGUCCA

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3301 ACCUAGUUUAGGUUUGAACUGGAAUACAGAUUUCAUAUUACGUAAUGAUGAGGAGUACCG
3361 CGCAAAGAAUGGAUAUGCACCGUAUUAUGCUGGUGUGUGGCAUAGCUUCAAUAAUAGUAA
3421 UUCGCUUGUUUUUAGAUGGGGUUCGGCUUCAGAUCAAAUUGCUCAAUGGCCAACAAUAAC
3481 AGUGCCUCGAGGAGAGUUGGCAUUCUUGCGUAUCCGCGAUGCUAAGCAAGCUGCUGUAGG
3541 AACGCAACCUUGGCGUACUAUGGUCGUUUGGCCUUCAGGUCAUGGAUAUAAUAUUGGAAU
3601 ACCAACUUAUAAUGCUGAACGAGCAAGACAACUUGCUCAGCAUUUGUAUGGUGGUGGGUC
3661 UUUGACAGAUGAAAAGGCUAAGCAAUUAUUUGUGCCUGCUAACCAGCAAGGACCCGGCAA
3721 AGUAAGUAAUGGUAACCCCGUCUGGGAAGUAAUGCGCGCGCCUCUUGCAACUCAGCAAGC
                                     E   A   V   P   E / G   E   E   S   R   N T
3781 GCAUAUACAAGAUUUUGAAUUGUUGAAGCUGUUCCAGAAGGCGAAGAAUCACGCAACAC
     T   V   L   D   T   T   T   T   L   Q   S   →  VP2
3841 UACGGUGUUAGACACGACAACAACGUUACAGUCUAGCGGAUUUGGUCGCGCUUUCUUCGG
3901 UGAGGCAUUUAACGAUCUUAAGACGUUAAUGCGCCGAUACCAAUUAUAUGGUCAAUUAUU
3961 GUUAUCCGUUACUACGGAUAAGGAUAUUGAUCAUUGUAUGUUUACCUUCCCUUGUUUACC
4021 UCAAGGGUUAGCGUUAGAUAUAGGUUCGGCUGGAUCUCCUCAUGAAAUAUUUAAUCGCUG
4081 CCGUGAUGGUAUCAUUCCAUUGAUAGCGUCAGGGUAUCGGUUUUAUCGAGGCGAUUUACG
4141 GUUCAAAAUUGUUUUCCCAAGUAACGUUAAUAGCAAUAUUUGGGUACAACAUCGACCAGA
4201 UCGUAGACUGAAAGGAUGGUCUGAAGCGAAAAUAGUAAACUGUGAUGCUGUAUCUACUGG
4261 ACAAGGUGUUUAUAAUCAUGGAUAUGCUAGUCAUAUUCAGAUUACGCGUGUAAAUAAUGU
4321 UAUAGAAUUGGAAGUCCCGUUUUAUAACGCUACGUGCUAUAAUUAUUUGCAAGCGUUUAA
4381 CCCAUCUAGUGCAGCGUCGAGUUAUGCCGUUUCGCUCGGAGAGAUUUCGGUUGGUUUUCA
4441 GGCUACUAGUGAUGACAUUGCAGCCAUAGUUAAUAAACCUGUAACUAUAUAUUACAGUAU
4501 UGGCGAUGGUAUGCAGUUUUCGCAGUGGGUUGGUUAUCAACCAAUGAUGAUUCUAGAUCA
4561 AUUGCCAGCACCAGUAGUUAGGGCUGUGCCUGAGGGCCCUAUAGCGAAGAUAAAGAAUUU
4621 UUUCCACCAAACGGCAGAUGAAGUUCGAGAAGCUCAGGCCGCAAAGAUGCGUGAAGAUAU
4681 GGGUAUAGUAGUCCAGGACGUUAUAGGAGAGUUAAGUCAGGCUAUACCCGAUCUUCAACA
4741 ACCGGAAGUUCAAGCGAAUGUUUUUUCUCUGGUGUCACAGUUAGUGCAUGCUAUCAUCGG
4801 UACUAGCCUUAAGACAGUUGCUUGGGCGAUUGUUUCGAUUUUUGUAACUUUAGGUUUGAU
4861 UGGACGUGAAAUGAUGCAUUCAGUCAUAACUGUAGUUAAGCGGUUAUUAGAAAAAUAUCA
4921 CUUGGCGACGCAACCCCAGGAAUCCGCCAAUUCAGGUACGGUUAUUUCCGCUAUUCCAGA
4981 AGCACCCAAUGCUGAAGCAGAGGAGGCCAGUGCCUGGGUAUCCAUUAUUUAUAAUGGUGU
5041 GUGUAAUAUGUUGAAUGUAGCCGCUCAAAAACCGAAACAAUUUAAAGAUUGGGUAAAAUU
5101 AGCUACCGUAGAUUUUAGUAAUAAUUGUAGAGGUAGUAAUCAGGUAUUUGUGUUUUUCAA
5161 GAAUACGUUUGAAGUGUUGAAGAAAAUGUGGGGUUAUGUGUUUUGUCAGAGUAAUCCUGC
5221 AGCGCGACUCUUGAAAGCAGUGAAUGAUGAACCUGAGAUUUUAAAAGCGUGGGUUAAAGA
5281 AUGUCUGUAUUUAGAUGAUCCUAAAUUUAGAAUGCGACGUGCGCAUGAUCAAGAGUAUAU
5341 UGAGAGAGUGUUUGCGGCCCAUUCGUAUGGACAAAUUUUAUUGCAUGACUUAACGGCUGA
5401 AAUGAAUCAAUCGCGUAAUUUAAGUGUGUUUACGAGAGUGUAUGAUCAAAUAUCUAAAUU
5461 GAAGACGGAUCUCAUGGAAAUGGGAUCAAACCCAUAUAUCAGGCGUGAAUGCUUUACGAU
                              G   I   G   K   S
5521 UUGUAUGUGUGGUGCAUCUGGAAUUGGUAAGUCUUAUUUAACUGAUUCUUUAUGCAGCGA
5581 GCUCUUACGUGCGAGUCGUACUCCAGUGACAACGGGCAUUAAGUGUGUCGUGAACCCUUU
                 Y   W   D   [Helicase]
5641 GUCUGAUUAUUGGGAUCAGUGUGAUUUUCAGCCCGUUUUAUGUGUUGAUGACAUGUGGAG
5701 UGUUGAAACGUCUACUACGCUCGAUAAACAGUUAAAUAUGCUAUUUCAGGUUCAUUCACC
                                  A   D   L   E   G   K
5761 AAUUGUACUUUCACCUCCUAAAGCUGAUUUAGAAGGUAAGAAAAUGCGUUAUAAUCCUGA
5821 AAUAUUCAUAUAUAAUACGAAUAAACCUUUUCCGAGGUUUGAUCGUAUAGCUAUGGAAGC
5881 UAUUUAUCGACGUAGAAACGUUUUAAUUGAAUGUAAGGCUAAUGAAGAGAAGAAGCGUGG
5941 AUGUAAACAUUGUGAGAAUAAUAUACCCAUUGCUGAAUGUAGUCCAAAAAUUUUGAAAGA
6001 UUUUCAUCACAUUAAAUUUCGUUAUGCUCAUGAUGUGUGUAAUUCUGAAACUACGUGGUC
6061 UGAGUGGAUGUCGUAUAAUGAAUUUUUGGAAUGGAUUACUCCUGUAUAUAUGGCUAAUCG
6121 ACGUAAAGCAAAUGAAUCGUUUAAGAUGCGUGUUGAUGAAAUGCAAAUGUUGCGUAUGGA
6181 UGAGCCCUUGGAAGGCGAUAAUAUUUUAAAUAAGUAUGUUGAAGUUAAUCAGCGCUUAGU
6241 UGAGGAAAUGAAAGCUUUUAAAGAGCGAACCCUCUGGGCUGAUUUACAACGUGUUGGCUC
6301 AGAGAUUAGUACUUCAGUUAAGAAAGCAUUACCAACUAUUUCCAUUACUGAGAAGCUACC
6361 ACAUUGGACUAUCCAAUGUGGCAUAGCUAAGCCUGAAAUGGAUCAUGCUUAUGAAGUUAU
6421 GAGUUCAUAUGCAGCAGGAAUGAACGCAGAAAUUGAAGCGCAUGAACAAGUUCGUCGUUC
6481 UUCUUUGGAAUGUCAGUAUAUUGAGCCUUCAACUUCAAGACCUCUGGAUGAAGAGGGUCC
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6541 UACUAUCGACGAGGAAUUACUUGGCGAAGUAGAAUUUACUUCUUCAGCUUUGGAGCGUUU
6601 GGUUGAUGAGGGGUAUAUUACUGGUAAACAAAAGAAGUACAUGGCAACUUGGUGUACGAA
6661 ACGAAGAGAGCAUGUAUCCGAUUUUGAUUUAGUAUGGACGGAUAAUUUGCGUGUUUUGAG
6721 UGCGUAUGUCCACGAGCGUUCUACAUCUACGCGUUUAUCUACCGAUGAUGUUAAAUUAUU
6781 UAAGACGAUUAGUAUGUUACAUCAGAGGUAUGAUACCACUGAUUGUGCAAAAUGCCAACA
6841 UUGGUAUGCACCAUUAACAGCUAUUUAUGUUGAUGAUAGAAAGCUAUUUUGGUGCCAGAA
6901 GGAGACUAAGACUUUGAUAGAUGUUCGUAAAUUGUCGAAAGAGGACGUUACAGUCCAAUC
6961 GAAAUUAAUUAACUUAUCGGUUCCGUGCGGUGAUGUAUGUAUGUUACAUUCUAAGUACUU
7021 UAAUUAUUUAUUCCAUAAAGCGUGGUUGUUUGAAAAUCCAACAUGGCGUUUAAUAUAUAA
7081 UGGUACUAAGAAAGGUAUGCCUGAGUAUUUCAUGAAUUGCGUGGAUGAAAUUUCAUUAGA
7141 UUCAAAAUUUUGUAAAGUAAAGGUUUGGCUUCAAGCAAUUAUUGAUAAAUAUUUGACUCG
7201 UCCAGUGAAAAUGAUUCGUGACUUUCUAUUUAAAUGGUGGCCGCAAGUAGCAUACGUGUU
7261 AAGUUUGUUAGGUAUAAUUGGUAUAACUGCGUAUGAGAUGCGUAAUCCUAAAUCAACAGC
7321 AGAAGACUUGGCUGAGCACUAUGUUAAUAGGCAUUGUAGUUCAGAUUUUUGGUCACCAGG
7381 UAUGGCGACUCCUCAGGGAUUAAAAUAUAGUGAAGCGAUAACAGCUAAAGCGCCUAGAAU
7441 CCAUAGAUUGCCCGUUACUACUAGACCUCAGGGAUCAACGCAACAAGUUGACGCCGCUGU
7501 GAAUAAGAUUUUGCAGAAUAUGGUGUAUAUCGGUGUUGUGUUUCCGAAAGUGCCUGGUAG
7561 UAAGUGGCGAGAUAUUAAUUUUAGAUGUCUUAUGCUUCAUAAUCGGCAAUGUUUGAUGUU
7621 GCGGCAUUACAUUGAGUCGACGGCUGCUUUUCCGGAGGGUACCAAAUACUAUUUUAAGUA
7681 UAUUCAUAAUCAAGAAACUCGAAUGUCAGGUGAUAUAUCUGGUAUUGAGAUUGAUUUAUU
7741 GAGUUUACCUAGAUUGUAUUAUGGUGGCUUAGCUGGGGAAGAGUCGUUCGAUAGCAAUAU
7801 AGUGUUAGUAACUAUGCCGAAUAGAAUUCCUGAGUGUAAGAGUAUUGUGAAGUUUAUAGC
7861 UUCACAUGCUGAACAUGCUCGUGCUCAAAAUGAUGGUGUGUUAGUUACUGGUGAACAUAC
7921 UCAGUUAUUGGCAUUCGAGAAUAAUAAUAAAACACCUAUAAGUAUUAAUGCUGAUGGUUU
                                                         G  V  C
7981 GUAUGAGGUUAUACUUCAAGGAGUAUACACUUAUCCAUACCAUGGUGAUGGUGUUUGUGG
       G  S  I  L  L     [Protease]     I  I  G  I  H  V  A  G
8041 GUCUAUAUUAUUGUCUCGUAAUUUACAACGACCGAUUAUAGGAUCCAUGUAGCUGGUAC
8101 UGAAGGAUUACAUGGCUUUGGUGUUGCUGAACCUCUUGUUCAUGAGAUGUUCACUGGGAA
8161 AGCAAUAGAGAGUGAAAGGGAACCGUAUGAUCGUGUGUAUGAAUUACCUUUGCGUGAAUU
8221 AGAUGAAUCUGAUAUAGGUUUAGAUACUGAUUUAUAUCCUAUAGGAAGAGUUGAUGCGAA
8281 AUUAGCUCAUGCCCAAAGUCCUUCAACAGGAAUUAAAAAGACGCUUAUUCAUGGUACUUU
8341 UGAUGUUUGGACUGAACCGAAUCCGAUGUCAUCACGAGACCCAAGAAUAGCACCACAUGA
8401 UCCGUUGAAGUUAGGGUGUGAGAAACAUGGUAUGCCAUGUUCUCCAUUUAAUCGAAAACA
8461 UUUGGAAUUAGCAACGACUCAUUUAAAGGAGAAGUUAAUUUCCGUAGUUAAACCUAUAAA
8521 CGGAUGCAAGAUUAGAAGUUUGCAAGAUGCUGUGUGUGGUGUACCAGGUUUGGAUGGCUU
8581 UGAUUCAAUAUCCUGGAAUACUAGUGCUGGUUUUCCUUUAUCUUCAUUAAAACCGCCAGG
8641 CUCUUCUGGUAAGCGAUGGUUGUUUGAUAUUGAAUUACAAGAUUCAGGAUGUUAUCUUUU
8701 GAGAGGGAUGAGACCUGAACUUGAGAUACAGUUGACAACAACUCAGUUAAUGAGGAAGAA
                                   D  C  L  K  D
8761 GGGAAUGAAGCCUCACACUAUAUUCACGGAUUGUUUGAAAGAUACAUGUUUGCCUGUGGA
8821 AAAAUGCAGAAUACCUGGUAAGACUAGAAUAUUUAGUAUAAGUCCCGUCCAAUUUACGAU
8881 UCCAUUCCGACAAUACUAUCUCGAUUUUAUGGCGUCGUACCGUGCCGCUAGACUUAAUGC
8941 UGAGCAUGGAAUAGGUAUAGACGUGAACAGCUUGGAAUGGACAAACUUGGCAACAAGUCU
9001 GUCGAAGUAUGGCACGCAUAUUGUGACAGGAGAUUACAAGAAUUUUGGUCCUGGGUUAGA
9061 UUCUGAUGUUGCCGCUUCAGCUUUCGAAAUUAUCAUUGAUUGGGUGUUAAAUUACACUGA
9121 AGAAGAUGAUAAAGACGAAAUGAAGCGUGUAAUGUGGACUAUGGCUCAGGAAAUCUUAGC
                                    [Polymerase]          P  S  G
9181 UCCUAGUCACUUAUGUCGUGAUUUAGUAUAUCGCGUACCAUGCGGUAUUCCUUCUGGAUC
9241 ACCAAUUACGGACAUUUUGAAUACUAUUUCGAAUUGUUUGUUAAUUCGAUUGGCUUGGCA
                                                            Y  G
9301 AGGUAUUACUGAUUUGCCUUUAUCCGAAUUUUCUAGACAUGUCGUGCUAGUUUGUUACGG
      D  D
9361 UGAUGAUCUCAUCAUGAAUGUAAGUGAUGAGAUGAUAGAUAAAUUCAACGCUGUAACAAU
9421 UGGCGAUUUCUUUUCGCGAUAUAAGAUGGAAUUUACGGAUCAGGAUAAAUCUGGAAAUAC
9481 AGUGCGGUGGCGAACUUUACAAACUGCCACGUUUUUGAAGCAUGGGUUCUUGAAACAUCC
9541 AACAAGACCCGUGUUUCUAGCCAAUCUGGAUAAGGUUUCUAUAGAAGGAACAACCAAUUG
9601 GACACAUGCUCGAGGAUUGGGUCGUCGAGUAGCAACCAUUGAGAAUGCUAAACAAGCGCU
9661 AGAGUUGGCAUUCGGAUGGGGUCCCGAAUACUUUAAUCAUGUUCGGAAUACCAUUAAAAU
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9721 GGCAUUCGACAAGUUAGGUAUUUAUGAGGAUCUCAUCACAUGGGAAGAAAUGGAUGUUAG
9781 AUGUUAUGCUAGCGCGUAAUUUUAAGAUUUUAAUACUCAUUAAAAUUAAUUUAUAUUUAG
Stop signal → 3′ NTR region
9841 GUUAUUGGAAUUGAGGGAAGUACCACCCCCCAAGACCUUCGUUUUAAAUCUACUAAGAGG
9901 AGUGAACUUGCAUAUAAGAGUCUAAAAGCAGAGUGGAUUAGACCACCACUUUUAGCUUAU
9961 AUGUGAGGAAGGUUGAGUUGCCUCUAAAGACUCAGCUCCGUAGUAGAGUAGUUUUAGUUA
10021 CGAUUAAAGUGGUACUCUAGGUUAGGUGUUACUCGCGUAUUGUCGCAUAACGGCAAUGCG
10081 UCCUAAUUUUAGUAUAGUUUAACCAUAAUAGG-**Poly A tail**

Figure 3-2: The nucleotide sequence of VDV-1 genomic RNA. The numbers on the left indicate the position of nucleotide sequence. The initiation and termination codons are denoted as reverse-type letters. Putative cleavage sites at the N-terminus of VP1 and VP2 structural proteins (Chapter 5) have been indicated by a forward slash and a potential cleavage NPGP site (picornaviral 2A protease type) has been indicated. Some conserved domains of the helicase, protease and polymerase have been included. The 5′ and 3′ non-translated regions (NTR) have been indicated on either side of the open reading frame.

The isolated virus RNA sequence has 84% nucleotide identity (without the poly-A tail) to the DWV (de Miranda *et al.*, 2003; Lanzi & Rossi, 2003) and KV (Fujiyuki *et al.*, 2004) sequences. In the 5′-NTR there is a deletion of 11 nucleotides (compared to the DWV and KV sequences) occurring 2 nt before the AUG translational start codon. Four single-nucleotide and two double-nucleotide deletions as well as two single-nucleotide insertions are spread out in this region. Overall, the 5′-NTR of VDV-1 is shorter than the corresponding regions of DWV (de Miranda *et al.*, 2003) (by 22 nt) and KV (by 39 nt).

Table 3-2: Percentage nucleotide and amino acid sequence identity [%I] and similarity [%S] in an alignment between corresponding regions of VDV-1 and other iflaviruses.

VDV-1		**DWV** %I	**KV** %I	**SBV** %I	**IFV** %I
Nucleotide sequence	Genome (without poly-A tail)	84	84	40	33
	3′ NTR	89	88	8	29
	RdRp	84	85	48	39
	Protease	88	88	44	31
	Helicase	85	85	44	33
	VP1	84	84	43	33
	VP2	84	84	45	31
	First 1455 bases from ORF start including VP3 and VP4	79	79	40	29
	5′ NTR (IRES)	81	80	8	5

	DWV		**KV**		**SBV**		**IFV**	
	%I	%S	%I	%S	%I	%S	%I	%S
Predicted VDV-1 polyprotein	95	98	95	98	18	35	12	28

Table 3-2 shows the percentage nucleotide identities between the isolated virus and the most closely related viruses generated from an alignment of the sequences. The RdRp nucleotide sequence of this virus has about 48% identity to that of SBV. Since the nucleotide sequence of our virus was not present in the online databases (GenBank and EMBL) and is sufficiently different from that of DWV and KV, we named this virus *Varroa destructor virus* 1 (VDV-1) because it was isolated from the mite *V. destructor.*

Predicted amino acid sequences

The structural proteins are predicted to be on the 5′ part of the genome. A comparative search of similar sequences in the NCBI database identifies structural proteins in the N-terminal half of the polyprotein but there are no strongly conserved motifs. The VDV-1 polyprotein has one NPGP motif (amino acid position 216-219 from the ORF start), which is a conserved picornavirus motif for cleavage at the 2A/2B site (Ryan & Flint 1997).

Based on the RNA sequence, the VDV-1 non-structural proteins were located on the 3′ half of the genome. They have conserved functional motifs characteristic to RdRps, proteases or helicases of viruses in the picorna-like super family (Koonin & Dolja, 1993). Eight conserved domains have been identified in RdRp amino acid sequences (Koonin & Dolja, 1993) and these are also present in VDV-1 (Fig. 3-3a). The three conserved domains in helicase sequences of picorna-like viruses were present in the VDV-1 helicase (Fig. 3-3b). The protease of VDV-1 has GXCG and GXHXXG motifs, which are also conserved in the 3C-like proteases of other picorna (-like) viruses included in the alignment (Fig. 3-3c). These conserved regions in the protease are consistent with the putative catalytic residues and substrate binding sites reported by Koonin & Dolja, 1993.

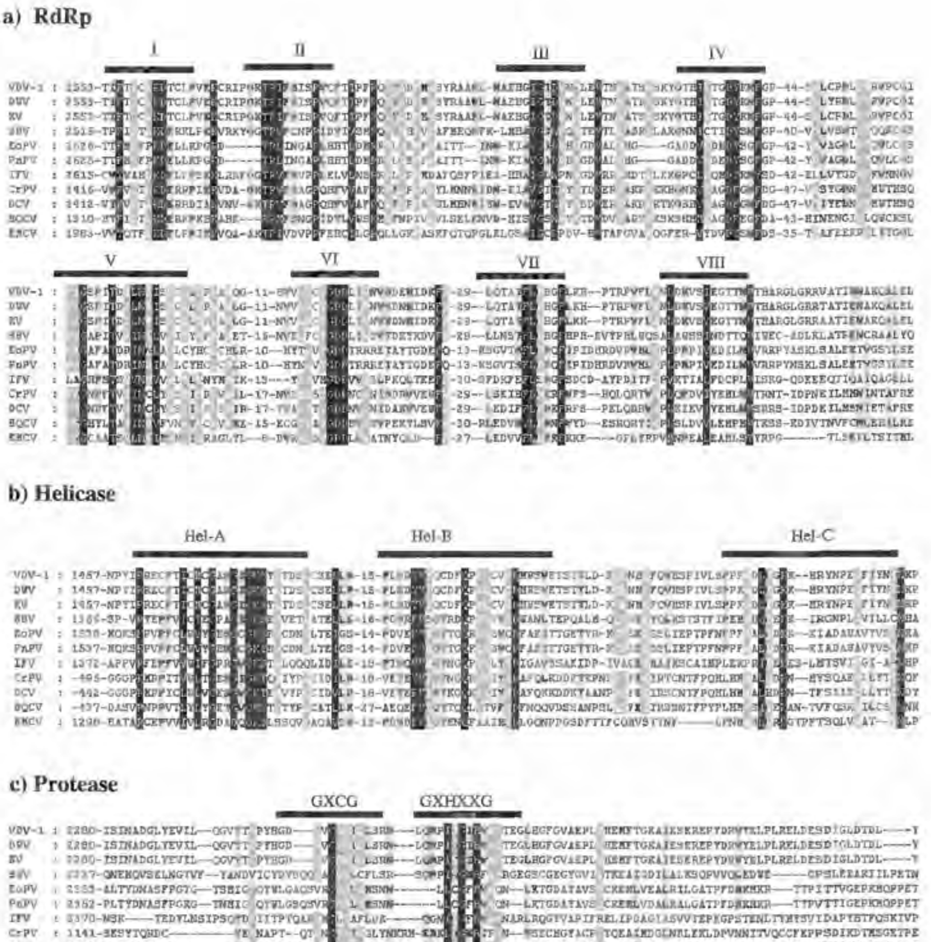

Figure 3-3: Multiple sequence alignment of VDV-1 with picorna-like viruses for (a) RdRp sequences (b) helicase and (c) protease domains. Conserved regions corresponding to those recognised by Koonin & Dolja (1993) are indicated with bars above the protein alignment. The black shading indicates 100% sequence identity and the dark grey shading indicates 80% identity. The light grey shading shows 60% identity. The numbers at the beginning of the sequences represent the amino acid position from the ORF start. The numbers within sequences represent the number of omitted amino acids. The amino acid sequences were deduced from nucleotide sequences for which accession numbers are given in Table 3-1.

A phylogenetic tree was constructed for the predicted amino acid sequence of the RdRp domain of VDV-1 and eighteen related viruses in GenBank. The RdRp tree (Fig. 3-4) segregated the viruses into their (assigned or proposed) taxonomic groups (*Picornaviridae*, *Iflavirus* and *Dicistroviridae*). The phylogenetic tree also showed that VDV-1 is most closely related to DWV and KV and clusters together with more distantly related members of the genus *Iflavirus*. VDV-1, DWV, KV and SBV appeared to be more closely related to each other than to IFV, EoPV and PnPV. The functional domains of the helicase and protease regions of VDV-1, DWV and KV are identical except for variations in the flanking regions and are therefore not suitable for the phylogenetic analysis.

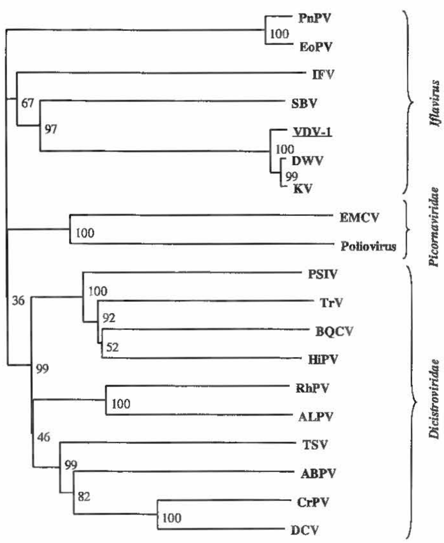

Figure 3-4: Phylogenetic analysis of the RdRp domains. Trees were constructed from the alignment of RdRp sequences using the neighbour-joining method as reported by Leat *et al.*, 2000. The internal labels at the nodes represent bootstrap values as percentages estimated by 1000 replicates. The branch lengths are proportional to relatedness. VDV-1, which is located in the *Iflavirus* cluster, is underlined. EMCV and *Poliovirus* were used as an outgroup.

DISCUSSION

VDV-1 has morphological characteristics similar to invertebrate picorna-like viruses and has a positive-sense RNA genome with an organisation which matches that of members of the genus *Iflavirus*. The VDV-1 genome is comparatively longer than picornaviral genomes which range from about 7200-8500 nt (Rueckert, 1996). The 1117 nt 5′ NTR is similar in length to those of mammalian picornaviruses (600-1200 nt) (Rueckert, 1996). Other iflaviruses, except DWV and KV, have sequences shorter than 500 nt in this region (Chapter 6).

The polyprotein translated from the single large ORF (2893 aa) has the same length as that of DWV and KV. Pairwise alignment of the entire VDV-1 and DWV/KV polyproteins resulted in parallel conservation from the N to the C terminal end. This was also observed in a similar alignment of the 2987 aa of EoPV (Wang *et al.,* 2004) and 2986 aa PnPV (Wu *et al.,* 2002) polyprotein sequences. Like picornaviruses, VDV-1 polyprotein encodes a 3C-like protease which cleaves the polyprotein into smaller and functionally active proteins. RdRp which is located on the C terminal of the polyprotein is used by the virus to replicate its genome. It is has been used as a reliable protein for the construction of phylogenetic trees for the classification of RNA viruses because it tends to be highly conserved among related groups (Zanotto *et al.,* 1996; Culley *et al.,* 2003). The closest relation to VDV-1 was found with two honey bee viruses, DWV and KV. Since *Varroa* mites live in close parasitic proximity with bees, the high homology observed with two bee viruses is not surprising as they have probably evolved from a common ancestor.

Despite the fact that DWV and KV are closely related to each other [having RNA identities of 97% (Fujiyuki *et al.,* 2004) and polyprotein identities of 98%], much closer than to VDV-1, they are accompanied by clear pathological differences. DWV causes deformity in the wing of bees and KV manifests aggressiveness in infected worker bees. KV infection does not result in symptoms of deformation of the wings and the RNA has been detected almost exclusively in the brains of aggressive worker honey bees (Fujiyuki *et al.,* 2004). The bees at the Wageningen University hives so far do not exhibit deformed wings neither are the workers unduly aggressive. The biological properties of VDV-1 in bees will be studied in Chapter 7.

DWV isolates from Pennsylvania USA (GenBank AY292384), Italy (GenBank AJ489744) and France (GenBank AY224602) have either been completely or partially sequenced and show 98-99% nucleotide identity to each other and 96-97% identity to KV. VDV-1 nucleotide sequence displays 83-84% identity to the DWV isolates and KV. Considering the statistics of the molecular data and the absence of the symptoms attributed to DWV and KV infection in the Wageningen bees, we conclude that despite VDV-1 being closely related to the two it is different. The extent of the difference remains to be determined.

ACKNOWLEDGEMENTS

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Chapter 4

Varroa destructor virus 1 (VDV-1) and *Deformed wing virus* (DWV) replicate in the mite *Varroa destructor*

Previous electron microscopy studies of *Varroa destructor* mite tissue showed accumulations of para-crystalline structures of 27 nm picorna-like virus particles scattered in the cytoplasm giving the apparent indication of a virus replicating in the mite (Chapter 2). The virus was isolated from mite, the RNA completely sequenced and the virus was named *Varroa destructor virus* 1 (VDV-1) (Chapter 3). To determine whether VDV-1 indeed replicated in these mites, a selective RT-PCR test was developed to detect negative-sense RNA strands, known to function as replication intermediates in picorna(-like) viruses. VDV-1 was discriminated from the closely related *Deformed wing virus* (DWV) (84% overall genome identity) by using two primers sets, each specific to one virus. Both viruses were found in the population of *Varroa* mites studied and both viruses were shown to replicate in this mite.

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INTRODUCTION

Picorna-like virus particles were isolated from a population of *Varroa destructor* mites at the Wageningen University apiary (Chapter 2). The virus was sequenced and named *Varroa destructor virus* 1 (VDV-1). VDV-1 belongs to the Genus *Iflavirus* and has 84% nucleotide sequence identity to *Deformed wing virus* (DWV; de Miranda *et al.*, 2003) of the honey bee (Chapter 3). VDV-1 and DWV have single-stranded, positive-sense RNA (ssRNA[+]) genomes which contain one large open reading frame. Iflaviruses resemble picornaviruses morphologically, physico-chemically and in genome organisation (Fauquet *et al.*, 2005).

Conclusive studies about *Iflavirus* replication have not yet been carried out, but it was assumed that because of their similarity, iflaviruses go through similar steps in their infection cycle as picornaviruses. The initial event in the infection process involves the attachment of the picornavirus to specific receptors on the cell membrane and the release of the naked RNA into the cytoplasm where translation occurs. Replication of picornaviruses is entirely cytoplasmic (Rueckert, 1996) (Fig. 4-1). Replication complexes are usually closely associated with membrane complexes derived from the endoplasmic reticulum, and free negative sense RNA is not found (Rueckert, 1996).

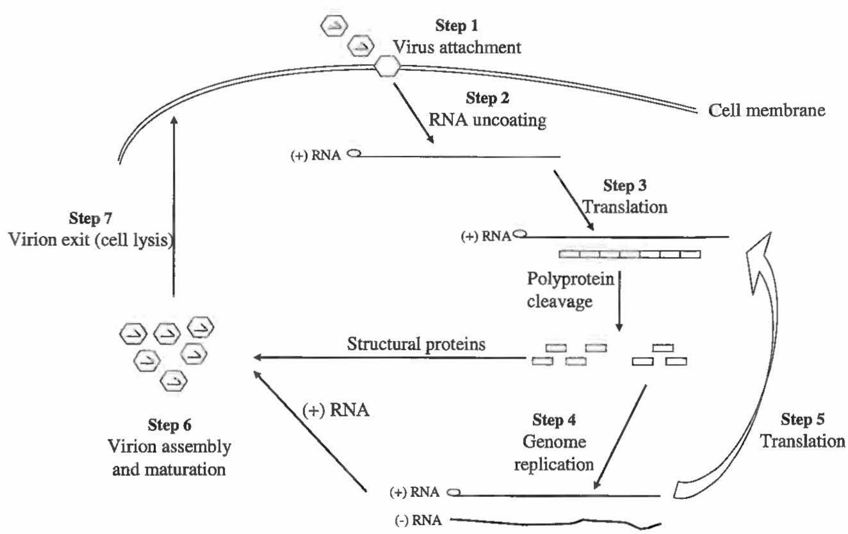


Figure 4-1: A schematic overview of the picornavirus infection cycle. The virus adheres to receptors on the cell surface (step 1) and following conformational changes in the virus capsid, the RNA genome is injected into the host cell (step 2). The genome acts like mRNA and is translated (step 3). The RNA-dependent RNA polymerase (RdRp) drives genome replication (step 4). Some of the positive sense strands generated during replication are used to translate yet more polyprotein (step 5), while the rest is assembled together with capsid proteins to form virions (step 6). Finally, the mature virions exit the cell and the infection cycle begins afresh.

The picornavirus genomes can be translated directly once introduced into the cytoplasm and the naked viral RNA is usually infectious. The linear RNA genomes of the *Picornaviridae* are translated into a viral polyprotein precursor (Fauquet *et al.,* 2005). The host cell does not contain a RNA-dependent RNA polymerase (RdRp) to allow viral replication. To overcome this constraint, the majority of RNA viruses encode their own RNA-dependent RNA polymerase. Picornavirus-encoded proteinases autocatalytically cleave the polyprotein into functional individual viral polypeptides, one of which is the RdRp (Rueckert, 1996). The release of the RdRp allows the process of viral genome replication to proceed. Using the genome strand as template the RdRp transcribes a complementary negative-sense RNA strand (RNA[-]), which in turn can act as a template for the production of nascent positive-sense RNA (RNA[+]) that is translated to produce yet more viral proteins. Virions are assembled in the cytoplasm where the positive-strand RNA is packaged into capsid proteins. The virions mature and finally exit from the cell by cell lysis once large numbers have accumulated in the cytoplasm (Rueckert, 1996).

V. destructor is an obligate parasite of the honey bee (Bailey & Ball, 1991). The mite sucks the haemolymph of the bee for nutrients and might take up or transmit viruses to bees as it feeds (Denholm, 1999). Bowen-Walker *et al.*, (1999) demonstrated the presence of DWV in *Varroa* mites using enzyme-linked immunosorbent assay (ELISA). Electron microscopy studies of *V. destructor* mite tissue showed accumulations of para-crystalline structures of 27 nm picorna-like virus particles scattered in the cytoplasm giving the apparent indication of a virus replicating in the mite (Chapter 2). To determine whether VDV-1 replicates in the mites, a PCR test was designed to detect the complementary (negative) strand of the viral genome, which serves as a replication intermediate. The close relationship between VDV-1 and DWV and the close proximity of the hosts from which either was isolated made it necessary to design primer sets to distinguish between these two viral sequences.

MATERIALS AND METHODS

Isolation of RNA from *Varroa destructor* mites

Live mites that had fallen onto retractable trays at the bottom of the hives at the Wageningen University apiary were collected and used directly for analysis. Total RNA was isolated from about 50 live mites. The mite bodies were macerated in the presence of RNasin® (Promega) prior to RNA extraction with 800 μl TRIZOL® reagent (Invitrogen) and 160 μl of chloroform according to the manufacturer's instructions. To facilitate RNA precipitation 200 μg glycogen was added to the sample. Finally, the RNA was precipitated and re-suspended in sterile distilled water.

Reverse transcription PCR (RT-PCR)

The VDV-1 and DWV RNA sequences were aligned and primer sequences were designed from corresponding regions in the two sequences that were not highly conserved. Primers used in the RT-PCR analysis are listed in Table 4-1.

Table 4-1: Primers used for selective RT-PCR of VDV-1 and DWV.

Primer name [#]	Primer sequence	Nucleotide position [$]	PCR product
VDV-1 F_{RT}	GCGAAGTAGAATTTACTTCTTCA	6564-6586	
VDV-1 F_{RTPCR}	CGAAACGAAGAGAGCATGTAT	6657-6677	**1130 bp**
VDV-1 R_{RTPCR}	CGACTCTTCCCCAGCTAAG	7786-7768	
VDV-1 R	AGCACGAGCATGTTCAGC	7885-7868	
DWV F_{RT}	GACACTGAATTCACATCACAG	6588-6608	
DWV F_{RTPCR}	GTAAGCGTCGTGAACATACTG	6679-6699	**1129 bp**
DWV R_{RTPCR}	GACTCCTCTCCCGCGAGA	7807-7790	
DWV-R	TCTGAGCACGTATATGTTCATT	7911-7890	

[#] F stands for forward, R is for reverse

[$] Nucleotide positions for VDV-1 correspond to the submitted sequence under the GenBank accession number AY251269 and for DWV the sequence under the accession number AY292384.

Two micrograms of total RNA isolated from live mites were used to make cDNA from viral RNA of negative polarity using the forward primer VDV-1 F_{RT}. The incubation temperature of the reaction mixture was raised to 55°C for 2 min prior to the addition of *Avian myoblastosis virus* reverse transcriptase (AMV RT) (Roche) and the mixture was further incubated at the same temperature for 60 min after adding the enzyme. The transcriptase was inactivated at 70°C for 10 min. AMV RT and the primer were not added to the negative control mix during reverse transcription. The cDNA was purified with the 'High Pure PCR Product Purification' kit (Roche) and PCR amplification was performed with *Taq* DNA Polymerase (Promega) using the forward primer VDV-1 F_{RTPCR} and the reverse primer VDV-1 R_{RTPCR}. Following a 5 min denaturing step at 94°C the PCR was done for 30 cycles with denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and 2 min elongation at 72°C. The final elongation step was extended to 7 min at 72°C. A positive PCR control was performed with a plasmid clone of the region of the virus to be amplified. The same plasmid was used as template in a PCR with corresponding DWV primers based on the sequence of de Miranda *et al.*, (2003), DWV F_{RTPCR} and DWV R_{RTPCR}, to establish the specificity of the PCR primers used.

In order to confirm that the VDV-1 virus was present in the live mites used for RNA extraction, cDNA was synthesised against the positive-sense, viral RNA genome using the reverse primer VDV-1 R, and PCR amplification was done using the same PCR primers as for the negative sense detection, VDV-1 F_{RTPCR} and VDV-1 R_{RTPCR}.

In a comparable way, the DWV reverse primer DWV-R was used to detect the DWV genome and the forward primer DWV F_{RT} to detect negative strand, and hence replication of DWV in the mites following the same procedure as for VDV-1. The PCR products of the replication analysis of both viruses were run in 1% agarose gels and fragments with the correct size were sequenced with the respective reverse primers VDV-1 R_{RTPCR} and DWV R_{RTPCR}.

RESULTS AND DISCUSSION

During the replication of RNA viruses with a single-stranded, positive-sense genome, a full-length complementary RNA of negative polarity is normally transcribed in the host. This serves as the template for the multiplication of the genome to be packaged into the capsid to form new virion progeny. A selective RT-PCR was set up to detect the presence of either VDV-1 or DWV RNA[-] strands in infected *V. destructor* mites in an effort to determine whether these viruses replicate in this mite species. The primers used to make cDNA were specific for either the negative or the positive strand (Table 4-1). Once specific cDNA was synthesized, a PCR was performed with the same pair of primers for both positive and negative strand detection, for PCR cannot be sense specific. This primer pair was also used for the controls, which are valid for both positive and negative sense detection.

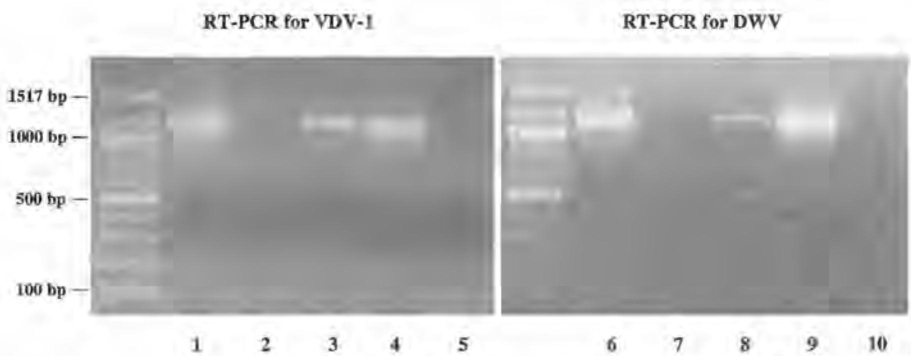


Figure 4-2: Reverse transcription PCR with selective primers for VDV-1 (left panel) and DWV (right panel). The amplified fragments have a length of 1130 bp for VDV-1 and 1129 bp for DWV. In the RT-PCR for VDV-1, PCRs were performed to amplify the positive sense viral RNA genome (lane 1), as well as the negative sense RNA (lane 3). A negative control without a RT step (lane 2) and a positive PCR control using a plasmid clone as template (lane 4) were also performed. The specificity of the primers was checked by PCR using a DWV plasmid clone with VDV-1 primers (lane 5). The replication analysis for DWV was carried out in a similar way (right panel) and lanes 6-10 were loaded in the same order as lanes 1-5.

The RNA sequences of VDV-1 and DWV have high nucleotide sequence similarity, so primer sets specific to each of the two viruses were designed from regions in the genome that showed divergence. This would make it possible to discriminate between these two viruses in a replication assay. The specificity of the PCR primers selected was ascertained by PCRs on plasmid templates bearing either a cDNA clone of the VDV-1 or DWV genome region to be amplified. A PCR product of 1130 bp was amplified when VDV-1 primers were used in combination with a VDV-1 (Fig. 4-2, lane 4), but not a DWV-1 template (lane 5), and vice versa for DWV primers (lanes 9 and 10) which amplified a product of 1129 bp. Both VDV-1 (lane 3) and DWV (lane 8) replicated in *V. destructor* mites, as seen by RT-PCR amplification of the negative strand. VDV-1 and DWV positive strand genomes were also detected (lanes 1 and 6) showing that the mite extract contained both viruses. No product was observed when the RT step was omitted (lanes 2 and 7) indicating that the amplification is RNA-dependent. The RT-PCR product from the negative-sense RNA template was sequenced and the results were confirmed.

In this study, an RT-PCR test was developed to discriminate between VDV-1 and DWV and to selectively detect the presence of either positive or negative RNA strands. With these tests it was demonstrated that both VDV-1 as well as DWV replicate in the *V. destructor* mites. The presence of *Kakugo virus* (Fujiyuki *et al.*, 2004) in these mites was not studied. Although the replication of VDV-1 and DWV within one mite population was demonstrated, this PCR test is not suitable to establish their co-existence of both viruses within a single mite in one reaction since the amplified products are of the same size. Co-existence could however be tested with these primers in separate PCR reactions. The presence of DWV in *Varroa* mites was previously shown by a positive reaction in ELISA with anti-serum raised against DWV isolated from bees with deformed wings symptoms (Bowen-Walker *et al.*, 1999), but this technique is not adequate to show virus replication.

The detection of both ssRNA[+] and RNA[-] strands of both VDV-1 and DWV in the mite body cannot be explained simply to be a result of ingestion of these RNAs from the haemolymph of the bees on which they parasitize. In Chapter 2, picorna-like virus particles were observed in the tissue of the mite in para-crystalline aggregates which are usually observed in the final stages of an infection cycle, which involves virus assembly. The determination of replication of the two viruses involved detecting the RNA[-] strand which only exists as naked RNA in the cytoplasm in the region where replication occurs. If naked RNA would have been ingested by the mite from an infected bee, it would be highly unlikely that the RNA would survive in the gut of the mite where the digestive juices/enzymes would be very active. A study of the digestive system of the ectoparasitic sheep scab mite, *Psoroptes ovis* (Acari: *Psoroptidae*) revealed the presence of a number of digestive enzymes and symbiotic bacteria (Hamilton *et al*, 2003), which not only provide additional nutrition to the mites but also play a role in digestion by secreting digestive enzymes (Douglas *et al*, 2001).

Allen & Ball (1996) described that infestation of bees with the *Varroa* mite in some cases led to the rapid increase of virus infection in bees. The spread of DWV, in particular, in apiaries around Europe has been closely linked to the presence of this mite (Korpela *et al.*, 1992). The virus could merely be transmitted to the bee by the mite during feeding, but the ability of DWV to multiply in the mite could offer a clear explanation why the mite strongly triggers the increment in virus load of infested bees leading to their death.

In order to improve the detection method for VDV-1 and DWV and to establishing their co-existence in an individual mite, a different set of specific primers yielding PCR products of different sizes for each virus is required. This would allow the simultaneous detection of both viruses in PCR reactions for individual mites.

ACKNOWLEDGEMENTS

We thank Dr. Johan Calis and Dr. Willem-Jan Boot for providing the mites. This research was performed in co-operation with CNRS, Orléans, France (contract 081.0870.00).

Chapter 5

Analysis of *Varroa destructor virus* 1 proteins

Varroa destructor virus 1 (VDV-1) has a genome organisation typical for the genus *Iflavirus*. The genome of iflaviruses has one large open reading frame encoding a polyprotein which is processed to release smaller polypeptides that are functionally active as structural or non-structural proteins. The *Iflavirus* capsid is made up of four structural proteins named VP1 to VP4 in reducing molecular mass. Mapping of the structural proteins VP1 and VP2 on the VDV-1 genome by N-terminal sequencing supports the N to C terminal arrangement VP3-VP4-VP1-VP2 found in *Infectious flacherie virus* (IFV), the type species of this genus. Iflaviruses therefore have a different arrangement of structural proteins in the polyprotein compared to picornaviruses. The mapping identified at least two proteolytic cleavage sites in the region of the structural proteins. Polyclonal antibodies prepared against virus purified from *Varroa destructor* mites recognised VP1 strongly and detected only this protein when tested against bacterially expressed fusion proteins of the individual structural peptides. VP1 appeared to be the immunodominant protein also in a Western blot analysis of purified virus. In addition, polyclonal antibodies were successfully prepared for the viral protease and could potentially be used as an alternative to PCR-based methods in monitoring VDV-1 replication. The size of structural protein encoding region and the identification of a potential protease cleavage sequence NPGP in the beginning of this region led to the conclusion that VDV-1 may code for a leader peptide of about 25 kDa at the N-terminus of the polyprotein.

INTRODUCTION

Invertebrate picorna-like viruses of the genus *Iflavirus* have a genome arrangement similar to that of true mammalian picornaviruses (Fauquet *et al.,* 2005). Picornaviruses are positive-stranded RNA viruses. The genome contains a single large open reading frame (ORF) which is translated into a polyprotein from which the mature structural and non-structural proteins are released by proteolytic cleavage. The structural proteins are located on the N-terminal half and the non-structural proteins on the C-terminal side of the polyprotein. In picornaviruses, the serine-like protease 3C accounts for most of the cleavages while protease 2A accounts for some of the proteolytic activity. The leader protein (L) of aphthoviruses also has some proteolytic activity (Fauquet *et al.,* 2005). Iflaviruses have a 3C-like protease and may also have a leader protein at the N-terminus of the polyprotein (Isawa *et al.,* 1998), but do not have the 2A protease (Chapter 3; Fig. 3-1).

Picornaviral virions have a capsid which comprises four structural proteins named VP1 to VP4 in decreasing molecular mass (Fauquet *et al.,* 2005). Mature virions of iflaviruses also contain four structural proteins. Within the polyprotein, the structural proteins of *Infectious flacherie virus* (IFV), the type species of the genus *Iflavirus*, are arranged in the order VP3 – VP4 – VP1 – VP2 in the N to C terminal direction (Isawa *et al.,* 1998; Chapter 3, Fig. 3-1). The picornaviral structural proteins are arranged differently in the order VP4-VP2-VP3-VP1. Other members of the genus *Iflavirus* are; *Sacbrood virus* (SBV; Ghosh *et al.,* 1999), *Perina nuda picorna-like virus* (PnPV; Wu *et al.,* 2002), *Deformed wing virus* (DWV; de Miranda *et al.,* 2003), *Kakugo virus* (KV; Fujiyuki *et al.,* 2004), *Ectropis obliqua picorna-like virus* (EoPV; Wang *et al.,* 2004) and *Varroa destructor virus* 1 (VDV-1). The structural proteins of PnPV have been sequenced N-terminally (Wu *et al.,* 2002) and agree with the arrangement in IFV, but no information is available on the structural proteins of the other viruses.

There is very limited information available about the iflaviral non-structural proteins. However, polyprotein sequence predictions showed conserved motifs identifying a helicase, protease and RNA-dependent RNA polymerase (Chapter 3; Fig. 3-3).

This study aims at investigating the location of VDV-1 structural proteins in the polyprotein through N-terminal sequencing to identify cleavage sites. The predicted individual structural proteins are expressed in bacteria as fusion proteins to establish their antigenicity by Western blot analysis. Antibodies are raised against the protease for possible use for rapid and cost-effective detection of viral replication through methods such as ELISA.

MATERIALS AND METHODS

SDS-PAGE, Western blot analysis of VDV-1 structural proteins

A sample of virus purified from *Varroa destructor* mites (Chapter 2) was used to resolve the structural proteins on a 12% polyacrylamide gel in an SDS-PAGE analysis as described by Laemmli, (1970). The proteins were stained using coomassie brilliant blue G250 (Merck). For Western blot analysis the proteins were blotted onto a nitrocellulose membrane (Millipore) and detected using antiserum raised in rabbits against the purified virus (Chapter 2). The blot was incubated with goat anti-rabbit antibody conjugated to alkaline phosphatase (Invitrogen) and was developed in alkaline phosphatase buffer (0.1 M Tris/HCl, 0.1 M NaCl, pH 9.5) containing 200 µl BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidine salt)/NBT (nitro blue tetrazolium chloride) solution (Roche).

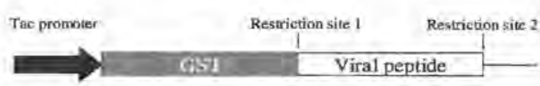
N-terminal sequencing

Structural proteins were resolved on a 12% polyacrylamide gel and blotted onto a PVDF membrane (Millipore). The proteins were stained using coomassie brilliant Blue R-250. The two large structural proteins (VP1 and VP2) were sequenced at Centre de Biophysique Moléculaire, CNRS, France using Edman degradation.

Cloning of VDV-1 genome sequences

The coding regions of the VDV-1 structural proteins and protease were amplified by PCR from a plasmid template and cloned downstream of the *Tac* promoter and in frame with Glutathione S-transferase (GST) in a pGEX-2T vector (Amersham Biosciences) (Fig. 5-1a) or Maltose binding protein (MBP) from a pMAL-c2 vector (New England Biolabs) (Fig. 5-1b). The PCR primers designed for the amplification including the added restriction sites for cloning are listed in Table 5-1. The estimated N- and C-terminals of the putative leader peptide were trimmed and replaced by 6 x His tag coding sequence on both ends to stabilise the protein so that it would not cleave itself off. The amplified products were digested with the restriction enzymes indicated and cloned into the respective vector. The coding regions of the structural protein VP1 and VP2 were determined from the N-terminal sequences. The C-terminal end of VP3 was approximated to give a protein whose theoretical size is similar to that seen on gel. The leader peptide was assumed to be cleaved at the NPGP site (Chapter 3) and VP3 and VP4 were cloned in one peptide.

a) pGEX-2T clones



b) pMAL-c2 clones

Figure 5-1: A representation of the cloned vectors for fusion protein expression. GST-fused proteins were expressed from pGEX-2T clones (a) and MBP-fused proteins from pMAL-c2 clones (b). The viral peptides were cloned in frame downstream of either GST or MBP.

Table 5-1: Primers for the PCR amplification of coding regions for VDV-1 peptides.

Primer name	Primer sequence[§]	Location in VDV-1 genome[#]	Restriction site added
GST-VP1$_F$	GCGT**GGATCC**GATAATCCGTCATATCAGCAATC	2573-2595	***Bam*HI**
GST-VP1$_R$	ACGAT**GAATTC**CTTCTGGAACAGCTTCAACAAA	3820-3800	***Eco*RI**
GST-VP2$_F$	GCGT**GGATCC**GGCGAAGAATCACGCAAC	3821-3838	***Bam*HI**
GST-VP2$_R$	ACGAT**GAATTC**CTCGAACTTCATCTGCCGT	4648-4631	***Eco*RI**
GST-VP3/4$_F$	GCGT**GGATCC**CCAGATGGTGAAGGTGAAGT	1772-1791	***Bam*HI**
GST-VP3/4$_R$	AATT**CCCGGG**TCCATATTACAACCACCTACGGT	2574-2552	***Sma*I**
GST-L$_F$	GCGT**GGATCC**CATCACCATCACCATCACTCTTATGCTG CTGTTGCCC	1142-1160	***Bam*HI** 6 x His tag underlined
GST-L$_R$	ACGAT**GAATTC**CGTGATGGTGATGGTGATGTTTAGCT GAAACAGGATTAGATAG	1744-1721	***Eco*RI** 6 x His tag underlined
GST-PROTEASE$_F$	GCGT**GGATCC**TTGCGGCATTACATTGAGTCG	7619-7639	***Bam*HI**
GST-PROTEASE$_R$	CGAT**GAATTC**CTGGTGCTATTCTTGGGTCTCGTGA	8395-8372	***Eco*RI**
MBP-PROTEASE$_F$	ATTC**GGATCC**TTGCGGCATTACATTGAGTCG	7619-7639	***Bam*HI**
MBP-PROTEASE$_R$	TTGC**CTGCAG**TGGTGCTATTCTTGGGTCTCGTGA	8395-8372	***Pst*I**

[†] The subscript in the primer name F stands for forward and R for reverse. L stands for the putative leader peptide.
[§] Restriction sites in primer sequence are indicated in bold letters. 6 x His codes are underlined.
[#] VDV-1 nucleotide positions correspond to the submitted sequence with accession number AY251269.

Expression and purification of fusion proteins

E. coli BL21 cells (Stratagene) were transformed with the expression vectors for fusion proteins. Colonies screened to contain the correct constructs were grown overnight at 37°C in LB medium (Peptone:Yeast extract:NaCl at a ratio of 2:1:1) with ampicillin to maintain the plasmids. The overnight culture was diluted 1:100 into fresh pre-warmed medium with ampicillin and grown at 37°C with agitation to an optical density of between 0.5 - 1.0 at 600 nm. IPTG was added to a final concentration of 0.1 mM to induce the expression. Incubation was continued for an additional 2-6 h at 28°C. The GST-fused structural proteins served to raise antibodies and the MBP-fused structural proteins were designed to test the specificity of the antisera against the VDV1 structural proteins. In the case of the protease, the MBP-fused protease was used for antibody production and the GST-fused protease as control.

For purification of the GST fusion proteins, the culture was chilled on ice for 30 min prior to the pelleting of cells by centrifugation at 5000 rpm for 10 min at 4°C. The pellet was resuspended in ice-cold PBS (140 mM NaCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4) to 1/100 of the original volume. The cells were frozen overnight and thawed on ice followed by disruption using ultrasonication in short bursts. The total soluble protein was clarified by centrifugation at 10,000 r.p.m. for 10 min at 4°C. Glutathione Sepharose 4B (Amersham Biosciences) was used to bind the GST portion of the fusion proteins in a batch purification process using the manufacturer's instructions. The unbound material was washed away with PBS and the GST-fusion proteins were eluted with 10 mM reduced glutathione in 50 mM Tris/HCl, pH 8.0.

For purification of MBP-protease, the culture was chilled on ice followed by centrifugation at 5000 rpm for 10 min at 4°C. The pellet was resuspended in ice-cold column buffer (20 mM Tris/HCl pH7.4, 200 mM NaCl, 1 mM EDTA). After freezing, thawing and ultrasonication, the total soluble protein was clarified by centrifugation. MBP was bound to amylose resin (New England Biolabs) and unbound matter was removed by washing with column buffer. The MBP-protease fusion protein was then eluted using column buffer containing 10 mM maltose.

Analysis of fusion proteins by SDS-PAGE and Western blot analysis

The expression of the GST and MBP fused proteins was analysed in a 12% SDS-PAGE gel. The proteins were stained using coomassie brilliant blue G250 (Merck). For Western blot analysis, proteins resolved in a 12% polyacrylamide gel were blotted onto a nitrocellulose membrane and detected using antiserum raised against purified virus (Chapter 2). The blot was incubated with goat anti-rabbit antibody conjugated to alkaline phosphatase (Invitrogen) and was developed in alkaline phosphatase buffer (0.1 M Tris/HCl, 0.1 M NaCl, pH 9.5) containing 200 μl BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidine salt)/NBT (nitro blue tetrazolium chloride) solution (Roche).

RESULTS

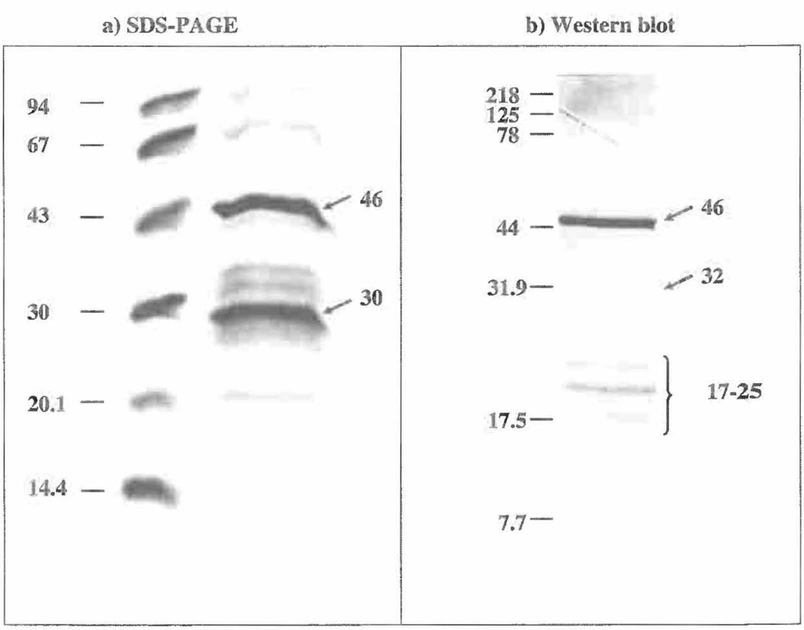


Figure 5-2: SDS-PAGE (a) and Western blot analysis (b) of purified virus from mites. The numbers on the left of each panel indicate the protein size marker in kilo Daltons. The approximate sizes of the proteins on gel or blot and indicated on the right side.

Detection of VDV-1 proteins in virus samples from mites

SDS-PAGE of virus sample purified from *Varroa* mites showed two strong bands of about 46 kDa and 30 kDa and weaker bands ranging from 20 to 94 kDa (Fig. 5-2a). Western blot analysis of the virus sample using IgG raised against purified virus particles revealed five proteins. The antibodies reacted strongly with a protein of approximately 46 kDa (Fig. 5-2b). Three reactive proteins were in the 17–25 kDa range and one protein had an estimated size of 32 kDa.

Mapping of the structural proteins on the VDV-1 genome

The two prominent structural proteins, with molecular masses of approximately 46 and 30 kDa (indicated in Fig. 5-2a), were excised from the PVDF membrane and N-terminally sequenced using Edman degradation. Analysis of the 46 kDa protein yielded the sequence XNPSYQQS (aa 486–493 in the VDV-1 polyprotein) and the sequence XEESXNTTVLDXTTXLQS (aa 902–919) was obtained for the 30 kDa protein. These positions are in agreement with the arrangement of the structural proteins in the IFV genome (Isawa *et al.*, 1998; Chapter 3, Fig. 3-1), the type species of the genus *Iflavirus*: VP1 is positioned N-terminally from VP2, which is located upstream of the helicase (Chapter 3, Fig. 3-2). Both proteins appeared to be N-terminally blocked so the first amino acid could not be identified. This blocking may have been caused by reactions with chemical contaminants such as cyanate or formic acid during sample preparation or storage.

SDS-PAGE and Western blot analysis of fusion proteins

Segments of the VDV-1 structural proteins and protease were individually expressed as fusion proteins. The boundaries of the structural proteins were estimated from N-terminal sequencing or predicted cleavage sites. The protease was cloned to include the entire conserved amino acid sequence region (Chapter 3; Fig. 3-3c) (nucleotide position 7619 to 8395). The purpose for expressing these proteins was to raise antibodies that could be used to determine the antigenicity and approximate sizes of the structural proteins in the VDV-1 capsid and to generate an antiserum against the protease that could be used to detect viral replication.

The crude bacterial extract and the purified GST fusion proteins were analysed by SDS-PAGE (Fig. 5-3a) and Western blot analysis (Fig. 5-3b). Antiserum prepared from purified virus was used to develop the blot. Only the GST-VP1 fusion protein gave a signal in both the purified and crude bacterial protein samples. The MBP fusion proteins were also checked using the same antibodies raised against purified virus and only the MBP-VP1 fusion protein was recognised. The purified GST fusion proteins were used to raise antibodies without success. They failed to recognise the corresponding MBP fusion proteins used as control and neither did they react with the protein that was used to make them (data not shown). The expected sizes of the expressed fusion proteins are listed in Table 5-2.

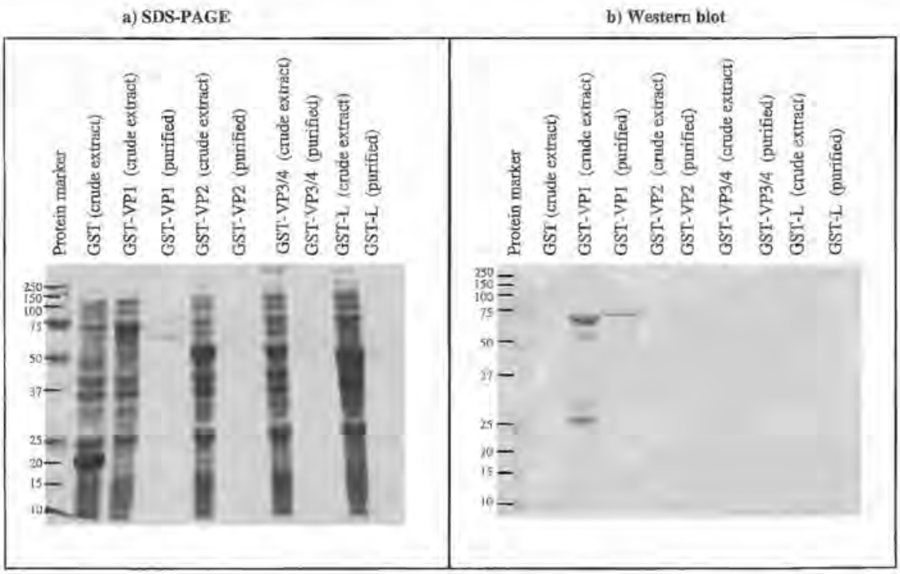


Figure 5-3: SDS-PAGE (a) and Western blot analysis (b) of BL21 *E. coli*-expressed GST fusion proteins. Antiserum against purified virus was used for Western blot analysis. The sizes of the bands (in kilo Daltons) of the protein marker are indicated on the left of the gel or blot.

Table 5-2: Theoretical sizes of the GST and MBP fusion proteins. The structural proteins VP1, VP2 and the putative leader peptide (L) were expressed separately while VP3 and VP4 were expressed as one protein. The only non-structural protein expressed was the protease.

Protein	kDa
GST	27.0
GST-VP1	73.6
GST-VP2	57.7
GST-VP3/4	57.0
GST-L	52.3
GST-protease	55.7
MBP	42.5
MBP-protease	79.0

Specificity of the protease antibodies

The VDV-1 protease fused to MBP was used to produce specific protease antibodies in rats. The antiserum obtained was able to detect the MBP-protease fusion protein. The serum contained antibodies against MBP and against the protease part, as shown by recognition of MBP alone and of a GST-protease fusion protein (Fig. 5-4). No detection was observed with GST alone. The antibodies have not yet been applied to test the presence of VDV-1 protease in infected mite or bee tissues, but could be used to locate virus replication in mite or bee tissue by immunohistology.

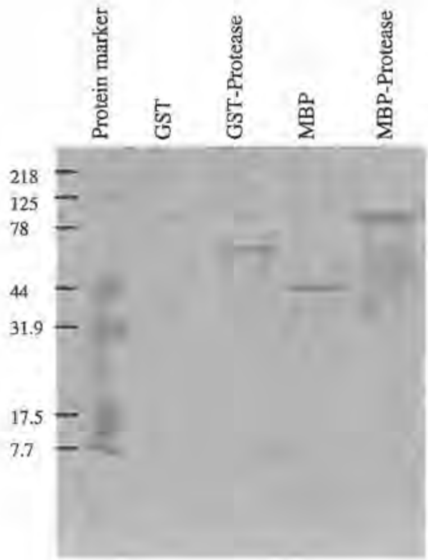


Figure 5-4: Analysis of the MBP-protease antiserum by Western blotting. Purified protein was analysed and detected with antiserum raised in rats against the MBP-protease fusion protein. The sizes of the protein marker are on the left of the panel.

DISCUSSION

The amino acid sequence of the entire VDV-1 polyprotein predicted from the nucleotide sequence (Chapter 3), and the various segments were compared to polyprotein sequences of the most closely related viruses (Table 5-3). VDV-1 and DWV polyproteins are 95% identical. The least conserved region is the N-terminal part.

Two structural proteins of VDV-1 were defined and their location in the genome determined. VP1 (46 kDa), the largest capsid protein, appears to be the only protein responsible for the antigenic response of the virion. In the SDS-PAGE gel (Fig. 5-2a), VP2 (30 kDa) is almost equally abundant in the virion capsid as VP1, but does not respond to the antiserum raised against purified virus (Fig. 5-3), suggesting that it may be hidden from the surface or is less antigenic. Some extra protein bands can be seen on the SDS-PAGE gel. The higher molecular weight bands are most likely of mite origin and the lower molecular mass bands could be of virus origin. On the Western blot for the purified virus, a protein similar in size to VP1 was recognised prominently but some weak signals are also detected. All these are probably of mite origin since it has been determined that the antiserum against virus raised in rabbit only recognises VP1 and the other structural proteins of the virus are not recognised by it.

Table 5-3: Comparison of VDV-1 proteins to those of related iflaviruses. %I = percentage identity and %S = percentage similarity

VDV-1		DWV		KV		SBV		IFV	
		%I	%S	%I	%S	%I	%S	%I	%S
Amino acid sequence	Entire polyprotein	95	98	95	98	18	35	12	28
	RdRp	95	98	95	98	32	52	18	35
	Protease	97	98	97	98	24	42	15	28
	Helicase	95	98	95	98	14	32	11	26
	VP1	97	99	96	98	16	31	13	28
	VP2	98	99	98	99	8	28	8	25
	First 485 aa from ORF start including VP3 and VP4	90	97	89	96	16	32	11	26

N-terminal sequencing of VP1 and VP2 identified their location in the polyprotein (Chapter 3; Fig. 3-2). This was found to be in agreement with the determined arrangement in IFV (Isawa *et al.*, 1998). N-terminal sequencing of the structural proteins of IFV and PnPV indicated that the region encoding the coat proteins did not start at the beginning of the ORF. For IFV the coding region (for VP3) started at amino acid position 149 (Isawa *et al.*, 1998) and for PnPV at position 320 (Wu *et al.*, 2002), leading to the speculation that these two viruses may encode a leader protein just like some vertebrate picornaviruses do (Chapter 3; Fig. 3-1). A similar situation may exist for VDV-1 (and DWV). Table 5-4 gives a summary of the cleavage junctions in the structural proteins of VDV-1 compared to those of IFV and PnPV.

Table 5-4: Cleavage sites of *Iflavirus* structural proteins. Only two cleavage sites of VDV-1 structural proteins were determined. The branch defining the L/VP3 junction is speculated to be active. The first peptide in the polyprotein has not yet been established to have characteristics of the leader peptide (L).

	L?/VP3	VP3/VP4	VP4/VP1	VP1/VP2
IFV	TQPQ/GPPE	VQGQ/GPEF	MRNK/DKPV	TVPQ/GPVQ
PnPV	VTAQ/GDED	SNPG/PFLS	KKDM/DRPQ	VTAM/GDER
VDV-1	PNPG/PDGE ?	-	GCNM/DNPS	AVPE/GEES

Cardioviruses and aphthoviruses have polyproteins, which are somewhat different from the other picornaviruses in that their ORFs encode an additional N-terminal leader peptide, L, which precedes the P1 capsid region (Rueckert, 1996). The picornavirus L protein is an autocatalytic papain-like protease that releases itself from the nascent polyprotein after translation of the virus genome (Ryan & Flint, 1997). It cleaves translation initiation factor eIF-4G so as to severely restrict or shut off cap-dependent host protein synthesis during infection therefore giving the virus genome a greater chance of being translated via cap-independent initiation from an upstream IRES in the genomic RNA (Dvorak *et al.*, 2001). Gorbalenya *et al.* (1991) identified a cysteine-tryptophan pair and a histidine as the active residues in the *Aphthovirus* L protease. The polypeptide sequences preceding the capsid region of IFV and PnPV and the comparable region in VDV-1 do not have these defined critical functional motifs to render them active as proteases of the L type and unless proven otherwise there is not sufficient information to indicate that these N-terminal parts function as proteases (see also Wang *et al.*, 2004; Ghosh *et al.*, 1999). However, the size of the first 485 aa (55.5 kDa) which includes the VP3 and VP4 proteins appears to be much larger than the possible weight of the two proteins combined. Apparently there is an extra undefined peptide in this region, which may be a protease but of a different kind to that of aphthoviruses.

The VDV-1 polyprotein has one NPGP motif (aa 216–219 from the N-terminus), which is a conserved picornavirus motif for cleavage at the 2A/2B site (Ryan & Flint, 1997). PnPV has two NPGP sites, one of which has been demonstrated to be active and to define the break between the first and second coat proteins, whereas the other is assumed to be the C-terminal site of the fourth coat protein relative to the ORF start (Wu *et al.*, 2002). If we assume that this site is also active in the VDV-1 polyprotein, a protein of approximately 25 kDa would be released. This 25 kDa protein alone would have the lowest identity to homologous proteins of DWV and KV, 83 and 81 %, respectively, and could be a target for specific immunodetection of these viruses, as the antiserum used in this study which was raised against virus purified from mites was probably raised from a mixed population of viruses, since both VDV1- and DWV were shown to replicate in the mites (Chapter 4).

In addition, antisera were raised successfully against the VDV-1 protease. The conserved motifs of the protease are almost identical in DWV and VDV-1 (Chapter 3; Fig. 3-3c) so antiserum raised against the VDV-1 protease will probably recognise both viruses with equal efficiency. Since the protease is a non-structural protein only expressed upon active virus replication, these antibodies can now be used to develop a rapid ELISA method to determine viral replication in the mite and bee. This would provide an more cost effective alternative to minus strand specific RT-PCR (Chapters 4 and 7) for detecting replication of VDV-1 or DWV in mites and bees. The antiserum also has potential to determine in which tissues the virus replicates in the organisms using an immunohistology assay.

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Chapter 6

The 5' nontranslated region of *Varroa destructor virus* 1: Structure prediction and IRES activity in *Lymantria dispar* cells

Structure prediction of the 5' nontranslated region (5' NTR) of four *Iflavirus* RNAs revealed two types of potential internal ribosome entry sites (IRES) in this group of viruses, which are discriminated by size and level of complexity. In contrast to the intergenic IRES of Dicistroviruses, the potential 5' IRES structures of iflaviruses do not have pseudoknots. To test the activity of one of these, a bicistronic construct was made in which the 5' NTR of *Varroa destructor virus* 1 (VDV-1) containing a putative IRES was cloned between two reporter genes, enhanced green fluorescent protein (EGFP) and firefly luciferase (Fluc). The presence of the 5' NTR of VDV-1 greatly enhanced the expression levels of the second reporter gene (Fluc) in *Lymantria dispar* Ld652Y cells, showing that the 5' NTR of VDV-1 contains a functional IRES element. This IRES element was active in a host specific manner since it showed lower activity in *Spodoptera frugiperda* Sf21 cells and no activity in *Drosophila melanogaster* S2 cells. This is the first *Iflavirus* 5' NTR for which IRES activity has been experimentally confirmed.

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INTRODUCTION

Viruses within the genus *Iflavirus* resemble viruses in the families *Picornaviridae* and *Dicistroviridae* in genome structure and organisation. They all contain single-stranded positive-sense RNA which is active as messenger and is polyadenylated at the 3' terminus. These viruses have a genome-linked viral protein (VPg) on the 5' end. *Picornaviridae* and *Iflaviruses* have a single large open reading frame (ORF) encoding a single polyprotein, while in the *Dicistroviridae*, two non-overlapping ORFs are separated by an intergenic region (Fig. 3-1 of Chapter 3; Christian *et al.*, 2005). In all these viruses, the ORFs are flanked by 5' and 3' nontranslated regions (NTRs), which contain functionally active RNA structures that are utilised at various stages of the infection process, including polyprotein synthesis and viral replication.

Translation of most cellular mRNAs normally starts with the recognition of the 5' methylated cap followed by scanning along the molecule to find the AUG initiation codon (Hershey & Merrick 2000). In this process, a binary complex of eIF-2 and GTP binds activated met-tRNA to form a ternary complex that binds to the 40S subunit forming the 43S pre-initiation complex. This complex associates with the mRNA cap structure through the translation initiation factor eIF-4F, a complex of eIF4A, eIF4B and eIF4G. The eIF4A component exhibits RNA helicase activity to unwind mRNA secondary structure allowing access of the AUG start codon to the ribosomal subunits. Finally, the 60S subunit associates with the pre-initiation complex to form the 80S initiation complex facilitated by the hydrolysis of the GTP bound to eIF-2 (reviewed by Merrick, 2004).

Instead, *Picornaviridae, Dicistroviridae* and probably also *Iflavirus* RNAs are translated via cap-independent mechanisms. They have 5' NTRs that form complex secondary structures which constitute an internal ribosome entry site (IRES) (Witwer, 2001; Hellen & Sarnow, 2001). The best studied IRES structures are those of the *Picornaviridae*, of which two types have been distinguished: the *Entero-/Rhinovirus* group and the *Aphtho-/Cardio-/Hepatovirus* group (Witwer, 2001). *Dicistroviridae* use an IRES element in the 5′ NTR to direct the translation of the first ORF (Masoumi *et al.*, 2003; Wilson *et al.*, 2000), while a second IRES element located between the two ORFs drives expression of the latter ORF (Masoumi *et al.*, 2003; Wilson *et al.*, 2000). IRES elements in the latter intergenic region of dicistroviruses are the ones that have been most characterised (Jan & Sarnow, 2002; Domier & McCoppin, 2003).

The 5′ NTRs of a few virus species in the family *Dicistroviridae* have been experimentally tested for IRES activity. *Cricket paralysis virus* (CrPV; Wilson *et al.*, 2000) has an IRES element within the 709 nt long 5' NTR which is active in a variety of lepidopteran and dipteran cell lines including *Drosophila melanogaster* (DL2 cells) and *Trichoplusia ni* (TN368 cells) (Masoumi *et al*, 2003; Scotti *et al.*, 1996). This is in line with the host range of CrPV in nature which includes insects of the order *Diptera*, *Lepidoptera*, *Orthoptera*, and *Heteroptera* (Christian & Scotti, 1998; Wilson *et al.*, 2000). *Rhopalosiphum padi virus* (RhPV; Moon *et al.*, 1998), an aphid-borne virus, has a 5' NTR of 579 nt with an IRES, which also functions in *Drosophila* embryo extracts and a variety of lepidopteran insect cells including *Spodoptera frugiperda Sf9* cells and *Sf21* cells, in rabbit reticulocyte lysate and in wheat germ translation systems (Woolaway *et al.*, 2001; Domier & McCoppin, 2003; Pijlman *et al.*, 2006 in press; Royall *et al.*, 2004). *Triatoma virus* (TrV; Czibener *et al.*, 2000) isolated from the trypanosome *Triatoma infestans*, which transmits Chagas' disease in Argentina, has a 5' NTR of 549 nt which contains an IRES element which is active in *Xenopus* oocytes (Czibener *et al.*, 2005).

Viruses belonging to the genus *Iflavirus* include *Infectious flacherie virus* (IFV; Isawa *et al.*, 1998), *Sacbrood virus* (SBV; Ghosh *et al.*, 1999), *Varroa destructor virus* 1 (VDV-1; Chapter 3), *Deformed Wing virus* (DWV; de Miranda *et al.*, 2003), *Kakugo virus* (KV; Fujiyuki *et al.*, 2004), *Perina nuda picorna-like virus* (PnPV; Wu *et al.*, 2002) and *Ectropis obliqua picorna-like virus* (EoPV; Wang *et al.*, 2004). In the present study, computer-assisted analysis was performed to predict the secondary structure of four *Iflavirus* 5' NTRs so as to identify possible IRES motifs. For this analysis the relatively long 5' NTR sequences of VDV-1 and DWV were chosen, and compared to PnPV and EoPV, which have shorter 5' NTR sequences in the *Iflavirus* group (Table 6-1). IFV and SBV have very short 5′ NTR sequences.

The functionality of the 5' NTR of VDV-1 was tested experimentally in various cultured insect cell lines. This would also identify possible permissive cell culture systems for VDV-1. VDV-1 was originally isolated from the *Varroa destructor* mite, an ectoparasite of the honey bee *Apis mellifera*. The virus is able to replicate both in the mite (Chapter 4)) and in the honey bee (will be presented in Chapter 7) but currently there are no cell culture systems available that would facilitate the study of this virus.

Table 6-1: Nucleotide sequence length of the 5′ NTRs of iflaviruses and the G+C content in this region.

Iflavirus	5' NTR	G+C content
PnPV	473 nt	36.79%
EoPV	390 nt	33.85%
IFV	156 nt	39.10%
SBV	178 nt	34.27%
KV	1156 nt	31.92%
DWV	1139 nt	32.31%
VDV-1	1117 nt	32.05%

MATERIALS AND METHODS

Prediction of the secondary structure of *Iflavirus* RNA 5' NTR

Predictions for RNA secondary structures were obtained using two different algorithms. The 'Zuker' Mfold program predicts the overall minimum free energy of the RNA molecule (Zuker, 2003), while in the genetic algorithm (Gultyaev *et al.*, 1995) the RNA is sequentially folded from the 5' to 3' end, thereby simulating the folding process during replication. In the latter program stems are added to the growing structure by stepwise selection from a list containing only those stems that are compatible with those already incorporated. The selection of the stem to be added depends, among others, on the free energy of the stem. As the folding progresses, less stable structures are removed in favour of more stable motifs, also ensuring that short-range interactions prevail over long-range pairings (Gultyaev *et al.*, 1995). Another advantage of the genetic algorithm is the possibility to predict RNA pseudoknot structures, which influence translation initiation by frameshifting or ribosome read-through (Giedroc *et al.*, 2000).

The secondary structures predicted in this way are analysed by comparison with homologous sequences, in order to find support for the proposed secondary structures from co-variations or compensated base changes in stem regions. The preferred sequence requirement for an optimal comparison is an identity ranging from approximately 60-80% between the homologous RNA molecules being compared (Gardner, *et al.*, 2005). The pairs VDV-1/DWV and PnPV/EoPV were selected because they show high conservation in the respective pairwise alignments (see also Table 6-3).

Cloning of reporter plasmids

To test for IRES function in the 5' NTR of VDV-1, a series of reporter plasmids were constructed. The backbone of these reporter plasmids was the pIZ/V5-His plasmid (Invitrogen). The reporter genes, enhanced green fluorescent protein (EGFP) and firefly luciferase (Fluc), were cloned into this plasmid under the control of the *Orgyia pseudotsugata* multiple capsid nucleopolyhedrovirus (OpMNPV) immediate-early 2 (OpIE2) promoter (Theilmann & Stewart, 1992) (Fig. 6-1).

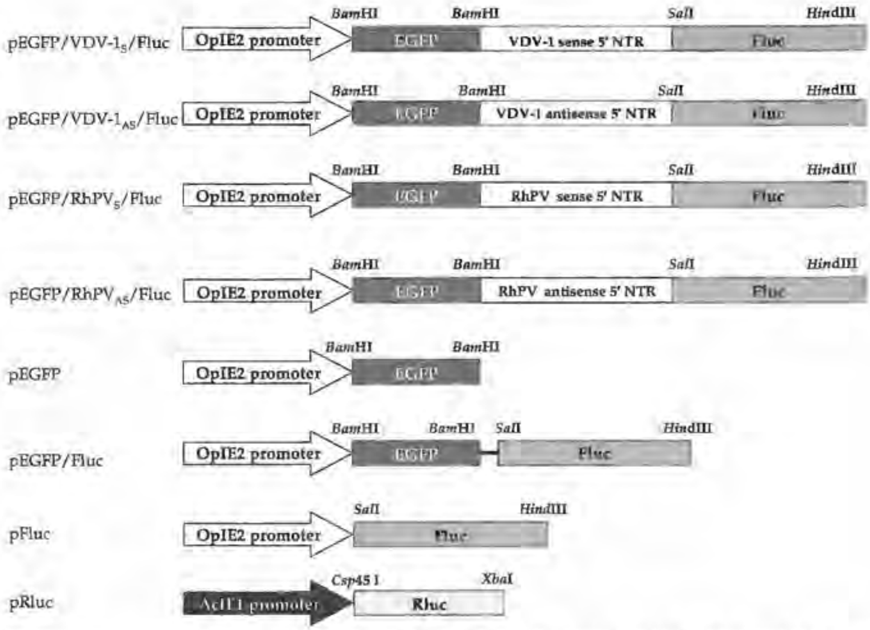

Figure 6-1: Reporter plasmids used to study IRES activity of the VDV-1 5' NTR. The restriction sites used in cloning are indicated at the top of each bar. The names of the plasmids are indicated on the left margin.

In order to make these constructs, the PCR products were amplified and cloned into pGEM-T Easy (Promega). The primers used contained extensions to introduce the appropriate restriction sites (Table 6-2). The sequences of the PCR products were verified by automated sequence analysis (Greenomics, Wageningen, NL). Firefly luciferase (Fluc) was amplified from the plasmid pGL3-control (Promega) and cloned via pGEM-T easy into pET 28a(+) (Novagen) as a *SalI*/*Hin*dIII fragment. The Fluc fragment was then recloned into the pIZ/V5 plasmid as a *Bam*HI/*Not*I fragment generating pFluc. EGFP with an added eukaryote Kozak consensus ribosome binding site (GCCACC) (Kozak, 1986) before the start codon was amplified by PCR from the plasmid pEGFP (Clontech Laboratories) and cloned as a *Bam*HI fragment into pFluc to give pEGFP/Fluc. The 5' NTR of VDV-1, extended with the first six codons of the polyprotein, was amplified from a plasmid clone containing a 5' segment of the virus genome (GenBank accession number AY251269). It was cloned as a *Bam*HI/*Sal*I insert into pFluc either in the sense orientation, and in frame giving a fusion with Fluc, or in the antisense orientation. EGFP was cloned into these two constructs as a *Bam*HI fragment to generate the plasmids pEGFP/VDV-1_S/Fluc and pEGFP/VDV-1_{AS}/Fluc respectively.

Three control plasmids were used in these assays: one with EGFP only (pEGFP), another with EGFP and Fluc not fused (pEGFP/Fluc) and a third with Fluc directly downstream of the OpIE2 promoter (pFluc). As a positive control, the RhPV 5' NTR with the first six codons of the flanking polyprotein were amplified from the pGEM-CAT/RhPVΔ1/LUC plasmid (Woolaway *et al.*, 2001) and cloned as a *Bam*HI/*Sal*I fragment between EGFP and Fluc in the sense or antisense orientation (pEGFP/RhPV$_S$/Fluc and pEGFP/RhPV$_{AS}$/Fluc). An internal transfection control plasmid containing *Renilla* luciferase (pRluc) under control of the *Autographa californica* MNPV immediate-early 1 (AcIE1) promoter (Jarvis *et al.*, 1996) was used to correct for variations in transfection efficiency (Nalcacioglu, *et al.*, 2003).

Table 6-2: PCR primers used for cloning reporter plasmids.

Primer name[#]	Primer sequence (5' to 3' end)[*]	Added sequence
EGFP F	CGCG**GGATCC**GCCACCATGGTGAGCAAGGGCGAGG	Kozak consensus ribosome binding site (underlined) *Bam*HI site
EGFP R	CGCG**GGATCC**TTACTTGTACAGCTC	*Bam*HI site
Fluc F	CGCG**GTCGAC**ATGGAAGACGCCAAAAACATAAAG	*Sal*I site
Fluc R	CGCG**AAGCTT**TTACACGGCGATCTTTCCGC	*Hin*dIII site
VDV-1 5' NTR sense F	CGCG**GGATCC**GCATAGCGAATTACGGTGCA	*Bam*HI site
VDV-1 5' NTR sense R	CGCG**GTCGAC**AAGAGTTCCACAACTAAATGCCAT	*Sal*I site
VDV-1 5' NTR antisense F	CGCG**GTCGAC**GCATAGCGAATTACGGTGCA	*Sal*I site
VDV-1 5' NTR antisense R	CGCG**GGATCC**AAGAGTTCCACAACTAAATGCCAT	*Bam*HI site
RhPV 5' NTR sense F	CGCG**GGATCC**GATAAAAGAACCTAT	*Bam*HI site
RhPV 5' NTR sense R	CGCG**GTCGAC**ATTGGTGCAAGACATCGTAGACAT TATAAATAGATAAAGCTAATGTTATA	*Sal*I site
RhPV 5' NTR antisense F	CGCG**GTCGAC**GATAAAAGAACCTAT	*Sal*I site
RhPV 5' NTR antisense R	CGCG**GGATCC**ATTGGTGCAAGACATCGTAGACAT TATAAATAGATAAAGCTAATGTTATA	*Bam*HI site

[#] The forward primers have a name ending with F and the reverse primers end with R.
[*] The restriction sites are indicated in bold letters.

Transfection of reporter vectors into insect cells

The insect cells used in this study were: *D. melanogaster* S2 cells (Schneider, 1972), *S. frugiperda* Sf21 cells (Vaughn *et al.*, 1977) and *Lymantria dispar* Ld652Y cells (Goodwin *et al.*, 1978). Sf21 and Ld652Y cells were grown in Grace's supplemented insect medium (Invitrogen) with 10% foetal bovine serum (FBS) and the S2 cells in Schneider's *Drosophila* medium (Invitrogen) supplemented with 20% FBS. One million cells were seeded into 35 mm dishes and incubated overnight at 27°C. Following overnight incubation, the medium over Sf21 and Ld652Y cells was replaced with serum-free Grace's insect medium (SFM) (SIGMA) and S2 cells with serum-free Schneider's *Drosophila* medium two hours before transfection. Five micrograms of reporter plasmid and 5 µg of the pRluc internal control plasmid were diluted into sterile deionised water to a final volume of 25 µl. Ten µl cellfectin® reagent (Invitrogen) was diluted with 15 µl sterile water and added to the DNA mix. After 15 min incubation, 500 µl of SFM was added to the DNA-transfection mix, which was then used to cover the cells. After 1 h incubation at 27°C with gentle rocking, 500 µl SFM was added to the cells which were further incubated for 3 hr at 27°C. One ml of Grace's insect medium supplemented with 20% FBS was added to the

Sf21 and Ld652Y cells, and medium with 40% FBS to S2 cells. Incubation was continued at 27°C for 48 h. The transfections were performed in duplicate.

Luciferase assay

Cells were examined under a fluorescent microscope for EGPF expression and harvested 48 h post transfection, washed once with phosphate buffered saline pH 7.4 and suspended in 250 µl passive lysis buffer (Promega's Dual-Luciferase® Reporter Assay System). Lysis was allowed to proceed at room temperature for 15 min followed by two freeze-thaw cycles and vortexing. The lysates were clarified by centrifugation at 14,000 r.p.m for 30 sec in an Eppendorf centrifuge 5417C. Firefly luciferase and *Renilla* luciferase expression were sequentially measured using reagents and protocol from the Dual-Luciferase® Reporter Assay System and the readings made using a TD-20/20 single-tube luminometer (Turner BioSystems), designed to run Promega's genetic reporter assays.

RESULTS

The *Iflavirus* RNA 5' NTR structures

VDV-1 and DWV have 81% nucleotide identity in the 5' NTR region. The 5' NTR of VDV-1 is 1117 nt long, while that of DWV is 22 nucleotides longer as a result of nucleotide insertions (Table 6-1). PnPV and EoPV have shorter 5' NTRs of 473 and 390 nt, respectively. A pairwise alignment of the entire 5' NTR of PnPV and EoPV shows that they have 64% overall nucleotide identity in this region. The PnPV sequence has an extension of 83 nt at the 5' end. If the extra 5' sequence is not considered in the alignment then the identity level rises to 78%. The entire 5' NTR of these four viruses was used for structure prediction.

In the VDV-1/DWV structure (Fig. 6-2), seven dominant and conserved structural elements are proposed, five of which are hairpins and two (I and V) form a branched, cloverleaf-like structure. It is interesting to note that the branched structure labelled V has two identical palindromic domains in the VDV-1 sequence at the bulges, highlighted by the broken rectangles, and parallel sequences which are highlighted by the broken arrows. In hairpin structure III, the VDV-1 and DWV sequences were quite different but the overall configuration was maintained in both. No conserved secondary structure could be proposed so far for the regions in between structures II and III and between III and IV and including that just before the underlined AUG start codon, because no co-variation support for the predicted structures could be obtained in the homologous sequence. It may well be that the secondary structure for these regions can be determined more easily when other, homologous, *Iflavirus* RNA sequences can be included in the analysis.

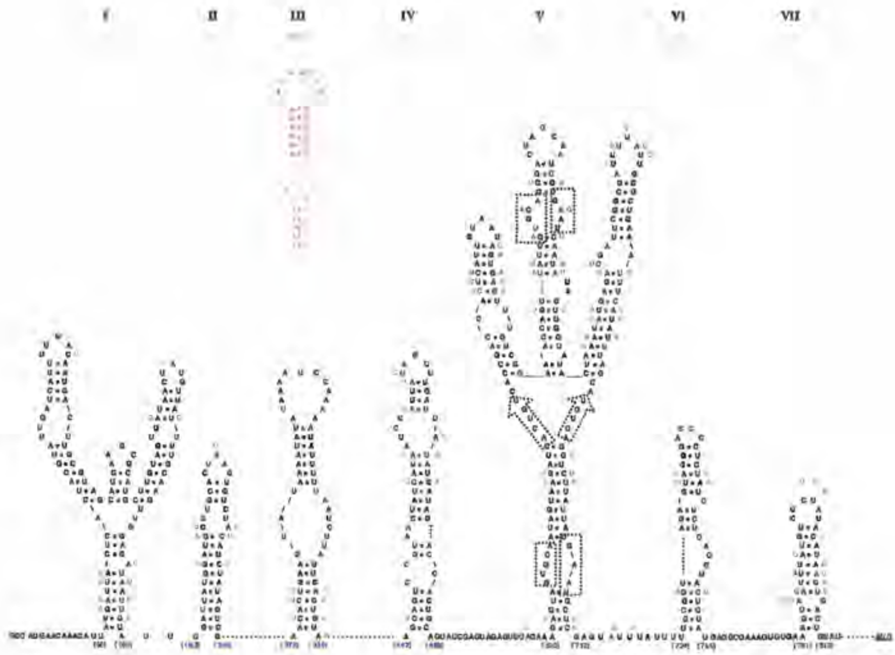

Figure 6-2: The predicted conserved secondary structure of VDV-1 and DWV 5' NTR based on the VDV-1 sequence. The letters in the periphery indicate the differences in the DWV sequence and thereby showing the occurrence of the co-variations in the stem regions. The sequence of DWV in hairpin III is different from that of VDV-1 but the structure is maintained. The numbers at the bottom indicate the nucleotide sequence position in the VDV-1 genome from the 5' end. The lines in between the sequence have been added to give continuity to the two-dimensional structure, except for the dotted breaks at the bottom between structures II and III and between III and IV and including that just before the underlined AUG start codon which indicate the omission of unsupported structures (Table 6-3).

The conserved structural features predicted for VDV-1 and DWV were located approximately within the first 800 nucleotides which have a slightly higher G+C content compared to the remaining portion before the AUG start codon (Table 6-3) for which no structure could be predicted with high probability.

Table 6-3: The nucleotide composition of the 5' NTR of VDV-1 and DWV.

	5' NTR of VDV-1		5' NTR of DWV	
nucleotide position	1-810 (structured)	811-1117	1-818 (structured)	819-1139
A+U	65.80 %	73.62 %	66.01 %	71.96 %
G+C	34.20 %	26.38 %	33.99 %	28.04 %

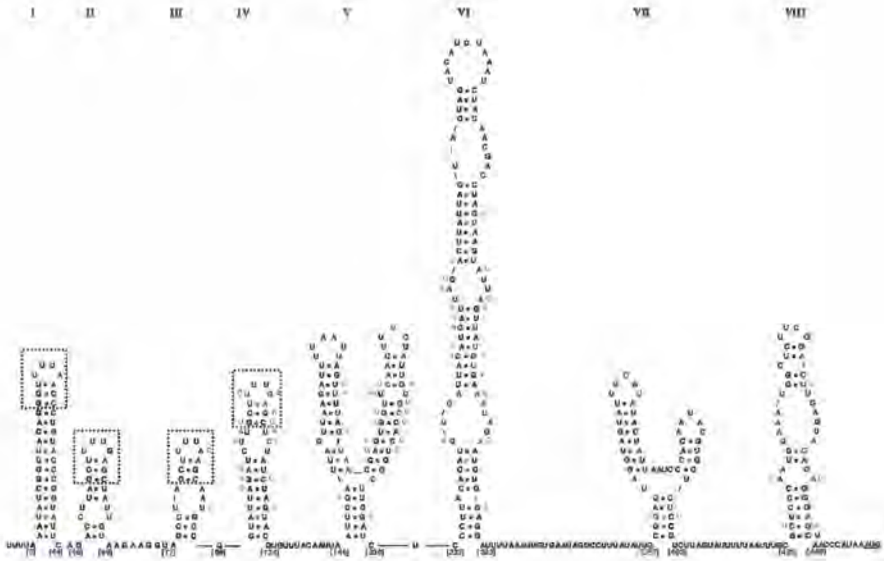

Figure 6-3: The predicted conserved secondary structure of PnPV and EoPV based on the PnPV sequence. The letters on the periphery indicate the differences in the EoPV sequence. The numbers at the bottom indicate the nucleotide sequence position in the PnPV genome from the 5' end. The lines in between the sequence have been added to give prevent overlap in a two-dimensional drawing. The broken rectangles indicate conserved stem and tetraloop motifs at the tips of the hairpins I to IV.

The PnPV/EoPV 5' NTRs have eight dominant structural features most of which are hairpins of which two (V and VII) are branched. The hairpins labelled I to IV have a conserved stem and tetraloop at their tips (highlighted in the diagram using broken rectangles). The predicted structures are spread out almost evenly along the entire 5' NTR.

Compensatory mutations are the basis of co-variations that support the existence of stems in the two sequences being compared (Witwer, 2001). Element I, III and V of the VDV-1/DWV type structure and element V of the PnPV/EoPV type have good examples of such support. Many other variations between the sequences do not alter the structure, for example when a G-U pair is changed to a G-C pair and *vice versa* or similarly upon an A-U to G-U pair change.

At least two types of conserved structural features in the 5' NTR of iflaviruses are predicted; the longer VDV-1/DWV type which was based on the VDV-1 sequence (Fig. 6-2), which is probably also conserved in the closely related *Kakugo virus* (Fujiyuki *et al.*, 2004), and the shorter PnPV/EoPV type based on the PnPV sequence (Fig. 6-3). Extension of the secondary structures proposed here may well be possible if other *Iflavirus* RNA sequences become available in the near future.

Firefly luciferase expression due to IRES activity in the 5' NTR of VDV-1
The conserved structural features predicted in the 5' NTR of these iflaviruses point to the presence of putative IRES elements in these regions. An IRES element functions by binding to cellular translation initiation factors leading to the recruitment of ribosomes for the translation of a downstream ORF. The ability of the 5' NTR of VDV-1 to function as an IRES in cell culture was examined. For this analysis, bicistronic reporter plasmids were designed (Fig. 6-1). The translation of the first ORF (EGFP) located directly downstream of the promoter will proceed via a 5' methylated cap-dependent mechanism. The stop signal at the end of this ORF prevents the ribosomes from reading through to the second ORF. The expression of the second ORF (Fluc) will only occur if there is an intervening IRES element which will recruit ribosomes, allowing translation of the latter ORF to occur (Royall *et al.*, 2004).

Following transfection of the bicistronic constructs into *Drosophila* S2, or the lepidopteran Sf21 and Ld652Y cells, EGFP expression was observed in all cultured cell types. Firefly luciferase activity was measured to check for IRES activity in the 5' NTR of VDV-1. The construct in which the 5' NTR of VDV-1 was present in the sense orientation (pEGFP/VDV-1_S/Fluc) gave significantly higher luciferase activity, about ten times more, compared to the anti-sense construct, in Ld652Y cells (Fig. 6-4). In Sf21 cells, there was a slight increase in the luciferase reading when the 5′ NTR was placed in a sense orientation between the reporter genes compared to the anti-sense construct, but the activity remained lower than that in Ld652Y cells. There was minimal activity detected in S2 cells but there was no significant difference in the luminescence between the sense and antisense orientations of the 5′ NTR of VDV-1. In the absence of an intervening 5' NTR sequence between EGFP and Fluc, there was limited expression of Fluc, in the same order of magnitude as that measured for the antisense orientation constructs. Mock-transfected cells and the monocistronic EGFP construct registered no luciferase activity, as expected. The control clone with Fluc directly behind the OpIE2 promoter, as expected, gave very high activity, and had to be diluted one hundred times to obtain a range of luciferase activity in the same scale as that obtained from the reporter plasmids.

The 5' IRES of the *Dicistrovirus* RhPV has been shown to be functional in Sf21 cells (Royall *et al.*, 2004) and *Drosophila* embryo extracts (Woolaway *et al.*, 2001) and was included here as a positive control to estimate the relative strength of the VDV-1 IRES. The 5' IRES of VDV-1 was stronger than that of RhPV in Ld652Y cells, but the 5' IRES of RhPV was slightly stronger in Sf21 cells than that of VDV-1. In general, Ld652Y cells gave the best results for both VDV-1 and RhPV. The performance of the IRES of both was poor in the *Drosophila* S2 cells.

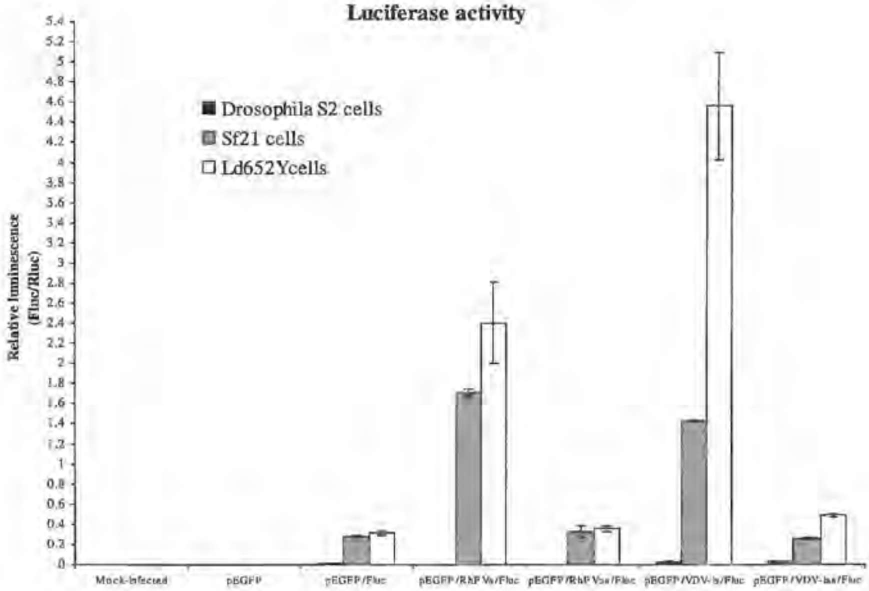

Figure 6-4: Luciferase activity obtained from the analysis of IRES activity in the 5' NTR of VDV-1 and RhPV in S2 cells (*D. melanogaster*), Sf21 cells (*S. frugiperda*) and Ld652Y cells (*L. dispar*). The relative luminescence (Fluc/Rluc) was used to plot the histogram. Each bar represents the mean of an experiment done in duplicate and the variation lines show the standard deviation from the mean.

DISCUSSION

The nucleotide sequence of single-stranded RNA plays a significant role in how the molecule will be folded. The secondary structure is composed of short-range interactions involving the base-pairing of neighbouring nucleotides to form hairpin and loop structures. The structural elements predicted in this study for the 5' NTRs of four *Iflavirus* species are mainly simple hairpins, in which the VDV-1/DWV type structure shows more complexity than that of PnPV/EoPV, which might be related to the difference in size of the 5' NTR. So far, no pseudoknot structures were detected in either *Iflavirus* structure type. IRES structures in the intergenic region of *Dicistroviridae* have been shown to have pseudoknot formations which influence translation initiation, for example, the translation of the second ORF of CrPV (Jan & Sarnow, 2003) and RhPV (Domier & McCoppin, 2003) start from the codon CCU (for the amino acid proline).

Within the *Picornaviridae*, 5' NTR structures have been divided into two types based on structural similarities; the *Entero-/Rhinovirus* group and the *Aphtho-/Cardio-/Hepatovirus* group. However, the IRES structure can vary even within a genus (Witwer, 2001). The VDV-1/DWV type structure shows some resemblance to the *Entero-/Rhinovirus* group, which also starts with a three-branched or cloverleaf-like element. In contrast, the

PnPV/EoPV structure starts with a very stable hairpin on the 5' end which has eight supporting G-C pairs. The EoPV sequence is 83nt shorter on the 5' end where the stable hairpin is located in the PnPV sequence. The hairpin appears to be stable (ΔG = -28.5 kcal/mole) such that it was assumed to exist even without supporting substitutions from the EoPV sequence. The PnPV and EoPV have shorter 5' NTRs with simpler structures that are unique and do not resemble any 5' NTR structures among picornaviruses.

The conserved structural features in the 5' NTR of VDV-1 were mainly confined to the first 810 nucleotides indicating that the essential elements for the putative IRES are probably located in this region. The three-branched element V (Fig. 6-2) could have a vital role for the IRES. The structure is the most prominent and contains parallel and palindromic features which could be important recognition signals for translation initiation and binding of translation factors. There was no sufficient evidence based on co-variations or stable structure formations between structures II and III and between III and IV and in the remaining A+U rich region (the 300 unstructured bases before the AUG start codon) of the 5' NTR to support the prediction of conserved structures between VDV-1 and DWV. These regions could remain mostly single stranded or could form an elaborate fold at the 3' end of the 5' NTR like that seen in enteroviruses (Witwer, 2001).

Conserved features are also present in the PnPV/EoPV structure (Fig. 6-3) such as the repeated stem and loop at the tip of hairpins I to IV which could form important recognition points for translation initiation factors. Lopez de Quinto & Martinez-Salas (1997) compared picornavirus IRES secondary structures and revealed the existence of conserved motifs in the loops. A GNRA motif in the loop of stem 2 and RAAA on stem 4 are required for IRES activity. Similar conserved motifs were identified in the intergenic IRES of some *Dicistroviridae* (Domier *et al.*, 2000). The PnPV/EoPV motif in loops I to IV is YUUV (Y stands for C or U in that position and V is for A, C or G) VDV-1 and DWV do not appear to have such conserved loop motifs.

The structure predictions of the 5' NTR of VDV-1 support the existence of an IRES in this sequence. IRES activity was investigated using bicistronic reporter plasmids in cell culture systems. Due to the absence of mite and bee cell systems, alternative cell lines were used. VDV-1 infects both the *V. destructor* mite and the honey bee, and is able to replicate in both organisms (Chapter 4 and 7). Bees are insects and mites are arachnids. The fact that the virus is able to replicate in very different organisms justified the attempt to find an alternative cell system where the 5' NTR could be assayed for IRES activity. Ultimately, this may lead to a cell system supporting viral genome replication, since IRES activity enables the translation of viral proteins needed to drive replication.

The bicistronic reporter plasmids were constructed in the PIZ/V5 plasmid under the control of the OpIE2 promoter which uses the host cell transcription machinery and does not need viral factors for activation (Theilmann & Stewart, 1992). The expression of the first ORF in the reporter plasmids is mediated by cap-dependent translation. The observation of EGFP expression in the cells following transfection shows that the transcripts are formed and that the first cistron is expressed. Since both ORFs are located on the same transcript, the expression of the second ORF will occur only when preceded by an active IRES element. The very low Fluc expression with the EGFP/Fluc construct, where the IRES element was absent, indicated that there was hardly any read-through by ribosomes once they reached the stop codon of the EGFP ORF (Fig. 6-4). The ten-fold enhancement of the Fluc signal in the Ld652Y cells when the 5' NTR of VDV-1 was incorporated between the two ORFs, demonstrated that this region contains a functional IRES element. This IRES appears to be cell type specific since it was not active in the S2 cells. Host factors interact with the IRES element to control its function and it is known that tissue-specific expression of these essential RNA-binding proteins may account for distinct tropisms (Pilipenko, 2001). The predicted secondary structure of the VDV-1 5' NTR (Fig. 6-2) should facilitate targeted mutation studies to identify crucial motifs for 5' IRES activity.

A comparison of the 5' IRES activity of VDV-1 to that of RhPV shows that their relative strengths are similar but very dependent on the cell culture system. The 5' RhPV IRES has been shown to be stronger if the extra codons of the virus polyprotein were omitted from the construct when cloning the 5' NTR (Woolaway *et al.*, 2001). This would probably enhance the expression of Fluc in the reporter plasmids. Even though 5' RhPV IRES activity has been demonstrated in *Drosophila* embryo extracts (Woolaway *et al.*, 2001), no activity was observed in the S2 cells in the current study.

The 5' and intergenic IRES elements of CrPV also display different levels of activity in different insect cells (Masoumi *et al.*, 2003). CrPV 5' IRES activity in a cell line was a prerequisite to support genome replication, but did not guarantee that the cell line could support replication. This finding suggested that additional cellular and/or viral factors may be involved in processing of viral products and packaging of viral particles. In addition, the strength of the 5' IRES activity was not directly correlated to the ability to support CrPV replication, as moderate IRES activity could also support CrPV replication.

This is the first demonstration of the activity of an *Iflavirus* IRES. The observation that Ld652Y cells are permissive to the 5' IRES of VDV-1 may be an important step towards identifying a cell system that could support VDV-1 replication. To investigate whether VDV-1 is able to replicate in the Ld652Y cell line, these cells may be transfected with VDV-1 genomic RNA. Viral gene expression can then be tested with specific antibodies (Chapter 5) to detect the structural proteins and the detection of the complementary negative sense RNA strand using RT-PCR can confirm virus replication.

ACKNOWLEDGEMENTS

Dr. Lisa Roberts (University of Surrey, UK), is acknowledged for sharing the pGEM-CAT/RhPVΔ1/LUC plasmid.

Chapter 7

Transmission and incidence of *Varroa destructor virus* 1 in the mite *Varroa destructor* and the honey bee *Apis mellifera*:
A comparison with *Deformed wing virus*

Varroa destructor virus 1 (VDV-1) and *Deformed wing virus* (DWV) are closely related with 84% genome sequence identity. Infections due to VDV-1 or DWV could not be distinguished using immunological techniques due to the high amino acid similarity (97%) of the immunodiminant protein VP1, but could be distinguished using specific PCR methods. Immunoassays showed that both adult male and female mites were infected with VDV-1 and/or DWV. When one member of a mite family was infected, all other members were infected. ELISA analysis showed that the proportion of *Varroa* mites carrying VDV-1 and/or DWV was 71%, slightly higher than the proportion of infected bees (65%). Virus was detected in mite eggs but not in bee eggs using a dot-blot immunoassay, indicating that vertical transmission might occur in the mite population but not in the bees. A discriminative PCR demonstrated co-existence of VDV-1 and DWV in the same individual mite or bee. Eighty eight percent of the mites had VDV-1, 19% were co-infected with VDV-1 and DWV, and no mites with only DWV infection were detected. Seventy nine percent of the adult bees had VDV-1, 26% co-infected with VDV-1 and DWV and there was no DWV only infection in the adult bees, in the specimens tested. It was concluded that VDV-1 and possibly DWV are primarily mite viruses which found a suitable host in the bee, and this is underscored by the replication of VDV-1 in the bee just as well as in the mite. An investigation of the viruses among European mites indicated that the two viruses exist together in hives across different regions of mainland Europe.

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INTRODUCTION

Most viruses infecting bees are RNA viruses with an isometric shape and often belong to the picorna-like viruses. The invertebrate picorna-like viruses are small isometric particles with a diameter of around 30 nm, which are non-enveloped. Their single stranded RNA genomes are of positive sense and can be mono- or dicistronic (Christian *et al.*, 2005). The majority of honey bee viruses persist at low levels as inapparent, sub-lethal infections and occasionally cause lethal outbreaks, for example *Sacbrood virus* (SBV; Ball & Bailey, 1991). The ectoparasitic *Varroa destructor* mite has been identified as a vector of several bee viruses including *Acute paralysis virus* (APV) (Ball, 1989; Batuev, 1979), *Slow paralysis virus* (SPV), *Kashmir bee virus* (KBV) (Denholm, 1999) and *Deformed wing virus* (DWV; Bowen-Walker *et al.*, 1999). Picorna-like virus particles were detected in extracts of *V. destructor* mites collected from the Wageningen University apiary and the presence of virus-like particles were confirmed in mite tissues by electron microscopy (Chapter 2). Similar observations were made by Kleespies *et al.*, (2000) from a sample of mites in Germany. The *Varroa* mite is an obligate parasite of the honey bee (Denholm, 1999) and its development is well synchronised to that of the bee (Fig. 7-1).

Previously, honey bee viruses were detected in extracts from bees and mainly characterized by serology (Denholm, 1999). The methods used in purification did not separate different, but morphologically similar viruses. Therefore preparations used to produce antisera may have contained virus mixtures (Denholm, 1999). The current advances in nucleotide sequence technologies have enabled the development of specific PCR-based detection methods that indisputably identify the different viruses. Recently, the virus observed in the Wageningen mites was isolated, sequenced and named *Varroa destructor virus* 1 (VDV-1) (Chapter 3). This virus is closely related to DWV isolated from honey bees, with 84% genome sequence and 95% polyprotein identity.

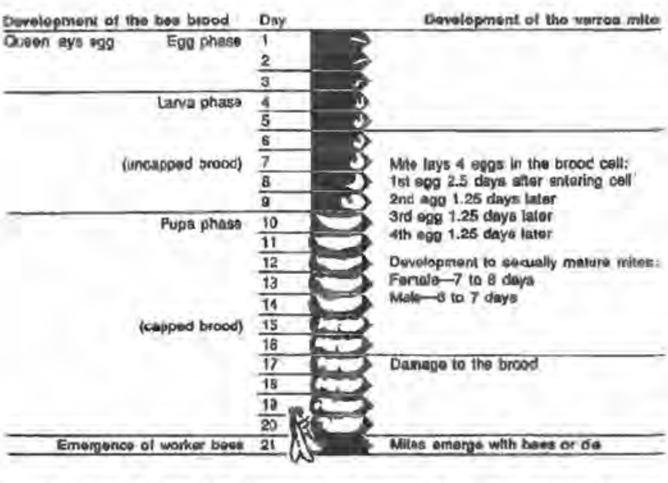


Figure 7-1: Graphic showing how the *Varroa* development in a bee colony is synchronised to the development of the bee (figure from Denholm, 1999).

VDV-1 and DWV both replicate in the mite (Chapter 4). DWV has been reported in the honey bee, whereas this information is lacking for VDV-1. A close correlation exists between *V. destructor* infestation and DWV infection in honey bee colonies (Ball 1989; Bailey & Ball 1991). Because of the cohabitation of mites and bees, and the close genetic relationship between VDV-1 and DWV, the possibility that VDV-1 can infect honey bees can not be excluded. In chapter 4, a specific PCR test was developed to distinguish between these two viruses and also detect their replication.

The objective of the study is this chapter is to investigate the incidence and transmission pathways of VDV-1 in the mite, the bee, and between the mite and bee. For this purpose, specific RT-PCR methods will be applied to detect this virus and to demonstrate its ability to replicate in the honey bee. This investigation also aims to study the geographic distribution of VDV-1 in mainland Europe. DWV is investigated in parallel for comparison.

MATERIALS AND METHODS

Sampling of mites and bees

Samples were collected from two heavily-infested colonies at the Wageningen University apiary from July to September 2004 during the *Varroa* peak. The colonies were maintained without controlling the mites during this period. Honeycombs were selected for sampling eggs, pupae, larvae and adults of mostly worker bees, as well as *Varroa* mite eggs, nymphs and adults (Fig. 7-2). The mites were carefully separated from uncapped and capped brood cells and stored individually at -70°C until use. Bee specimens were carefully checked to avoid inclusion of infesting mites. Adult bees were collected when they emerged from the cell (Fig. 7-1). The bee specimens were separated into two groups; those that were infested with mites and those that were not. The mites from the infested bees were individually stored and were analysed for the presence of VDV-1 and/or DWV in comparison to the bees they infested.

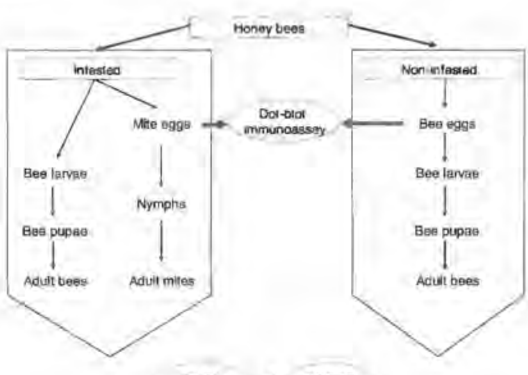


Figure 7-2: Sampling plan for bees and mites from untreated hives in Wageningen. The samples were analysed by ELISA, RT-PCR and dot-blot assay to detect infection by VDV-1 and/or DWV.

Purification of virus from *Varroa* mites

One gram of mites was gently stirred in 2% ethanol overnight. The mites were collected by centrifugation at 5000 rpm for 5 min in a Sorvall B21 centrifuge and macerated in 10 ml 0.01 M potassium phosphate (K_2HPO_4/KH_2PO_4) buffer, pH 7.3. Large debris was removed by centrifugation at 5000 rpm for 10 min at 4°C. The supernatant was centrifuged through 30% sucrose cushion at 27,000 rpm for 6 h in a Beckman ultracentrifuge to collect the particles. The pellet was re-suspended in 1 ml 0.01 M potassium phosphate buffer and centrifuged at 40,000 rpm for 3 h at 4°C in a discontinuous 10-40% sucrose gradient. The fraction with the virus particles was removed and the particles collected in a pellet, which was dissolved in 1 ml of potassium phosphate buffer, and stored at 4°C.

Enzyme-linked immunosorbent assay (ELISA)

Cliniplate polystyrene microtitre plates (Labsystem) were coated with antiserum (prepared against purified virus particles isolated from *Varroa* mites, Chapter 2) diluted at a ratio of 1:1000 in coating buffer (0.1 M $NaCO_3$, pH 9.6) and incubated for 2 h at 37°C. The wells of the microtitre plate were washed three times with distilled water to remove unbound immunoglobulin. Samples of individual honey bees were prepared by grinding in 1 ml PBS (140 mM NaCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4) in 1.5 ml Eppendorf tubes. Individual mites were macerated in liquid nitrogen and suspended in 300 µl PBS. An aliquot of 150 µl bee or mite suspension was added to separate wells and incubated for 2 h at 37°C. The bee and mite material was removed and the wells were washed 3 times with distilled water. An aliquot of 150 µl IgG conjugated to alkaline phosphatase (1 µg/ml) was added and incubated for 2 h at 37°C. The conjugate was removed and the wells rinsed 3 times with distilled water. An amount of 150 µl substrate buffer containing 1 mg/ml P-nitrophenyl phosphate disodium was added and incubated at room temperature to allow adequate colour development. The absorbance was measured at 405 nm.

Total RNA isolation from individual mites and bees

Total RNA was isolated from individual *Varroa* mites and bees. The samples were ground in 800 µl TRIZOL® reagent (Invitrogen) and 200 µg glycogen was added to the sample to facilitate the precipitation of RNA. One hundred and sixty microlitres of chloroform was added followed by vortexing for 30 sec. Centrifugation was done at 14,000 rpm for 5 min and the aqueous phase transferred to a new tube. The extracted RNA was precipitated from the aqueous phase by adding an equal volume 2-propanol and incubated for 1 h at -20°C. The sample was centrifuged at 14,000 rpm for 15 min the RNA pellet was washed once with 70% ethanol, dried, re-suspended in sterile distilled water and quantified by spectrophotometry. This RNA was used for specific RT-PCR detection of VDV-1 and/or DWV genomes and for the presence of their respective complementary negative strands which exist as replication intermediates.

PCR primer design

Two sets of RT-PCR primers were designed to be used at the melting temperature of 55°C. The first set of primers detects VDV-1 and DWV in separate reactions (Fig. 7-3a and Table

7-1). The PCR primers used for the specific individual detection of VDV-1 or DWV genomes have been described in Chapter 4, and give expected products of similar size (1130 and 1129 bp, respectively).

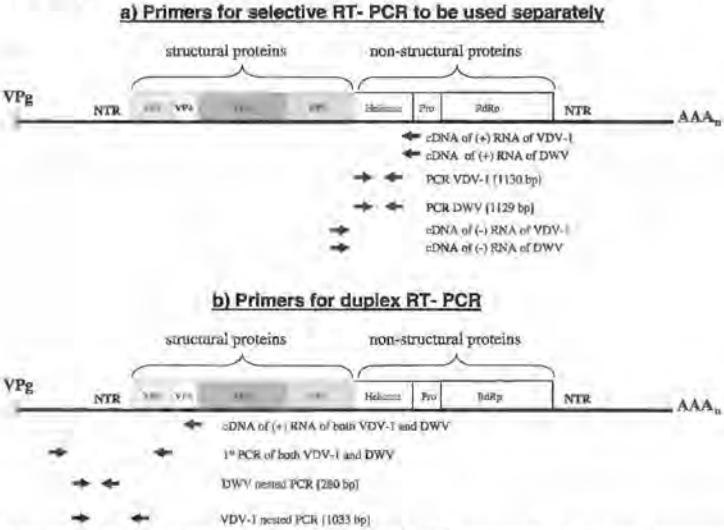


Figure 7-3: Primers for RT-PCR for detecting VDV-1 and DWV separately (a) or simultaneously within one PCR reaction (b). The arrows indicate the location of the primers in the virus genome. See Tables 7-1 and 7-2 for primer sequences.

Table 7-1: Primers used for selective RT-PCR of VDV-1 or DWV. Only one set of primers (either VDV-1 or DWV) can be used in a single PCR reaction because the product size is about the same for either virus sequence (Chapter 4). These primers have been mapped to the virus genome in Fig. 7-3a.

Primer name [#]	Primer sequence	Nucleotide position [§]	Product
VDV-1 R	AGCACGAGCATGTTCAGC	7885-7868	cDNA of VDV-1 (+) RNA
VDV-1 F_{RT}	GCGAAGTAGAATTTACTTCTTCA	6564-6586	cDNA of VDV-1 (-) RNA
VDV-1 F_{RTPCR}	CGAAACGAAGAGAGCATGTAT	6657-6677	1130 bp VDV-1 PCR
VDV-1 R_{RTPCR}	CGACTCTTCCCCAGCTAAG	7786-7768	
DWV-R	TCTGAGCACGTATATGTTCATT	7911-7890	cDNA of DWV (+) RNA
DWV F_{RT}	GACACTGAATTCACATCACAG	6588-6608	cDNA of DWV (-) RNA
DWV F_{RTPCR}	GTAAGCGTCGTGAACATACTG	6679-6699	1129 bp DWV PCR
DWV R_{RTPCR}	GACTCCTCTCCCGCGAGA	7807-7790	

[#] F stands for forward, R is for reverse

[§] Nucleotide positions for VDV-1 correspond to the submitted sequence under the accession number AY251269 and for DWV the sequence under the accession number AY292384.

The second set of primers is used to detect both viruses simultaneously in a nested duplex RT-PCR (Fig. 7-3b and Table 7-2). Their locations in the virus genome are illustrated in Fig 7-3b. In the chosen set-up, a PCR product of 1033 bp would indicate that VDV-1 was present and a 280 bp band indicates the presence of DWV. The sensitivity of the duplex RT-PCR primers was determined in a PCR with serially diluted plasmid templates. The amount of template used was 50, 25, 5, 2.5, 0.5, 0.25, 0.05 and 0.025 ng. The primer sets were tested singly with their respective plasmid templates, and also in a duplex PCR with both the VDV-1 and DWV plasmid templates added, each plasmid in the amounts given.

Table 7-2: Primers used for selective duplex RT-PCR of VDV-1 and DWV.

Primer name [#]	Primer sequence	Nucleotide position [§]	Product
VDV-1/DWV R_{cDNA}	CATTACTAGTCCATCTACTCC	[VDV-1] 1875-1895 [DWV] 1897-1917	cDNA of (+) RNA of both VDV-1 and DWV
VDV-1/DWV F_{PCR}	TATAGTCGTTTGTGGTTCAAG	[VDV-1] 196-216 [DWV] 204-224	1st PCR 1608 bp VDV-1 1622 bp DWV
VDV-1/DWV R_{PCR}	TTTTCTAATTCAACTTCACC	[VDV-1] 1803-1784 [DWV] 1825-1806	
VDV-1 F_{PCR}	GAAGTCGAATACTTGTGTATAGT	397-419	2nd PCR (nested) 1033 bp VDV-1
VDV-1 R_{PCR}	ATTACTGATTGAAATGGGGACA	1429-1408	
DWV F_{PCR}	CGAAGTTGAATGTATTTATAAGAA	406-429	2nd PCR (nested) 280 bp DWV
DWV R_{PCR}	GAAGTTTCAGTCGCACCGC	685-667	

[#] F stands for forward, R is for reverse

[§] Nucleotide positions for VDV-1 correspond to the submitted sequence under the accession number AY251269 and for DWV the sequence under the accession number AY292384.

Reverse transcription PCR (RT-PCR)

An amount of 2 µg of total RNA was used to synthesise VDV-1 or DWV cDNA. To show the presence of VDV-1 in the samples from which RNA was extracted, the cDNA of the positive sense RNA strand (viral genome) was synthesised using the reverse primer VDV-1 R (Table 7-1). The incubation temperature of the reverse transcription reaction mixture (RNA template, primer, buffer and deoxynucleotide triphosphate) was raised to 55°C for 2 min prior to the addition of AMV reverse transcriptase (Roche). This mixture was further incubated at the same temperature for 60 min. The transcriptase was then inactivated at 70°C for 10 min. PCR amplification was performed with *Taq* DNA polymerase (Promega) in a 50 µl reaction mixture with 2 µl of the cDNA mix as template, using the forward primer VDV-1 F_{RTPCR} and the reverse primer VDV-1 R_{RTPCR} (Table 7-1). In a similar way, the DWV reverse primer DWV-R was used to make cDNA of the DWV genome and PCR was performed with DWV F_{RTPCR} and DWV R_{RTPCR} primers (Table 7-1). Following a 5 min denaturing step at 94°C the PCR was done for 30 cycles with denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and 2 min elongation at 72°C. The final elongation was done at 72°C for 7 min. The specificity of the primers is described in Chapter 4.

In order to demonstrate replication of both viruses in mites and bees, cDNA of the negative RNA strand (complementary replication intermediate) was synthesised by using the forward primers VDV-1 F_{RT} and DWV F_{RT} (Table 7-1), respectively. PCR was done using primers and conditions described in the previous paragraph.

Dot-Blot immunoassay

The presence of VDV-1 and/or DWV in mite and bee eggs was studied using a dot-blot immunoassay on a nitrocellulose membrane (Millipore). A virus sample isolated from mites was used as positive control and *Trichoplusia ni* eggs, PBS and water were used as negative control. The membranes were cut into suitable sizes for the number of samples to be blotted and pre-marked to identify individual samples. Individual mite and bee eggs were macerated and suspended in a droplet of PBS. The material was spotted onto the marked positions on the membranes and allowed to bind. The membranes were blocked by incubation in 1% skimmed milk powder in PBS for 15 min with gentle shaking in a petri dish at room temperature. The membranes were washed three times shaking by gently with 0.25% skimmed milk powder in PBS, then incubated for 30 min in 0.25% skimmed milk powder in PBS containing a 1:1000 dilution of the antiserum raised in rabbit against virus isolated from mites. After incubation, the membranes were washed three times with 0.25% skimmed milk powder in PBS and incubated for 30 min with goat anti-rabbit antibody conjugated to alkaline phosphatase (Invitrogen). After three washes with 0.25% skimmed milk powder in PBS, the membranes were washed two times in alkaline phosphatase buffer (0.1 M Tris/HCl, 0.1 M NaCl, pH 9.5). The membranes were incubated in 10ml alkaline phosphatase buffer containing 200 μl BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidine salt)/NBT (nitro blue tetrazolium chloride) solution (Roche) for 5 to 20 min. The reaction was stopped by washing the membranes in distilled water.

Analysis of *Varroa* mite infection in various European countries

Total RNA was extracted from samples of *Varroa* mites obtained from a number of European countries (Avignon in France, Oberürsel and Kirchhain in Germany, Grugliasco and Udine in Italy, and Evora in Portugal). These mites were also stored at -70°C until use. A duplex detection system was designed to detect both VDV-1 and DWV in a single PCR. cDNA of the positive sense RNA strand (viral genome) was synthesised using a reverse primer VDV-1/DWV R_{cDNA} (Table 7-2) that could anneal to both virus genomes. The first PCR was done to amplify both VDV-1 and DWV using the primer pair VDV-1/DWV F_{PCR} and VDV-1/DWV R_{PCR} (Table 7-2). The subsequent duplex PCR was nested and carried out in the presence of primer pairs that were specific to each virus. The VDV-1 forward and reverse PCR primers used were VDV-1 F_{PCR} and VDV-1 R_{PCR}. The DWV forward and reverse primers used were DWV F_{PCR} and DWV R_{PCR} (Table 7-2). The VDV-1 and DWV primer sets could be used together or separately to improve the sensitivity of the detection (Fig. 7-12).

RESULTS

Detection of virus by ELISA

Mites infesting honey bees in the Wageningen University Apiary contained VDV-1 as well as DWV, as shown in previous RT-PCR experiments (Chapter 4). Thus a positive serological reaction using the antiserum prepared from purified virus, which recognizes both viruses, is indicative of an infection with either virus or with both viruses. Analysis of adult mites and their offspring showed that invasion of a brood cell by an infected female mite resulted in the infection of all mite offspring (Table 7-3). In case the mother mite was not infected the whole mite family appeared to be free of infection. The infection of the mite family in most cases also resulted in the presence of virus in the emerging bee.

Table 7-3: ELISA on mite families and the infested bee pupae per brood cell.

Cell	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Bee	(+)	(-)	(+)	(+)	(+)	(-)	(+)	(+)	(-)	(+)	(+)	(-)	(-)	(+)	(+)
Mite family member	(+) MM♀	(+) MM♀	(+) DN ♂	(+) MM♀	(+) MM♀	(-) MM♀	(+) MM♀	(+) MM♀	(-) MM♀	(+) MM♀	(+) MM♀	(-) MM♀	(-) MM♀	(+) MM♀	(+) MM♀
	(+) AM♂	(+) DN ♂	(+) DN ♀	(+) AM ♂	(+) AM ♂	(-) PN ♀	(+) MM♀	(+) AM♂	(-) ♀	(+) AM ♂	(+) MM♀	(-) AM ♂	(-) AM ♂	(+) AM ♂	(+) PN♀
	(+) DN♀	(+) DN ♀	(+) DN ♀	(+) ♀	(+) ♀	(-) Faeces	(+) AM ♂	(-) Egg	(-) ♀	(+) DN ♀	(+) AM ♂	(-) DN ♀	(-) DN ♀	(+) ♀	
	(+) DN♀		(-) Faeces	(+) DN ♀	(+) DN ♀		(+) AM ♂		(-) DN ♀	(+) DN ♀	(+) AM ♂	(-) DN ♀	(-) DN ♀	(+) DN ♀	
	(+) PN ♀			(+) DN ♀	(+) DN ♀		(+) DN ♀		(-) PN ♀	(+) Faeces	(+) DN ♀	(-) PN ♀	(-) DN ♀	(+) DN ♀	
	(+) PN ♀			(-) Faeces	(-) PN ♀		(+) DN ♀		(-) Egg		(+) DN ♀	(-) Faeces	(-) PN ♀	(+) DN ♀	
	(-) Faeces				(+) PN ♀		(+) DN ♀		(-) Faeces		(-) Faeces		(-) Faeces	(+) DN ♀	
					(-) Faeces		(+) DN ♀							(+) DN ♀	
							(+) PN ♀							(+) PN ♀	
							(+) PN ♀							(+) PN ♀	
							(+) Egg							(-) Egg	
							(-) Faeces							(-) Faeces	

MM♀: mother mite, AM ♂: adult male, ♀ : female daughter, DN ♀: female deutonymph, PN ♀: female protonymph
(+) shows positive ELISA results and (-) is for negative ELISA signal

These results show that analysis of a single mite in a family could be used to determine whether a mite family and the bee on which the mites parasitized had VDV-1 and/or DWV. Infection of a whole family enabled the separation of infected mite families and bees from non-infected mite families and bees, just by analysing one mite. The rest of the mites and bees could then be used in other studies to evaluate the identity of virus infecting the mites and bees by PCR, and the dynamics of the infection process.

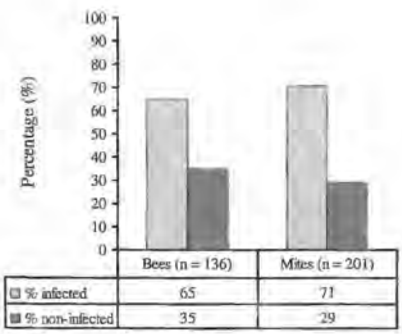


Figure 7-4: ELISA analysis to detect the overall infection rate in mites (adults and nymphs) and bees (larvae, pupae and adults).

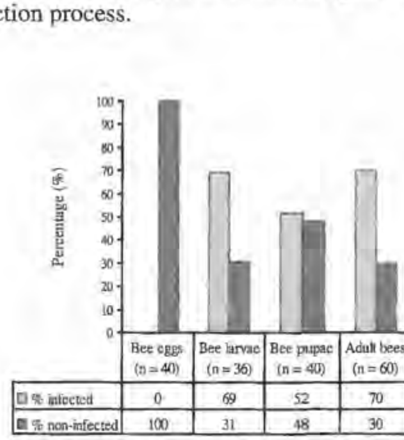


Figure 7-5: ELISA on bee eggs and mite-infested bee larvae, pupae and adults. The eggs were free of virus whereas more that 50% of the larvae, pupae and adults were infected.

Upon analysis of the mite families and bees, it appeared that 71% of the mites and 65% of the mite-infested bees (larvae, pupae and adults) were ELISA positive (Fig. 7-4).The proportion of virus-infected bee larvae, pupae and adults was 69%, 52%, and 70% respectively (Fig. 7-5). The bee eggs (which are never infested by *Varroa* mites) did not give any positive ELISA reading indicating that they did not contain VDV-1 and/or DWV. No virus was detected in mite faeces, excluding contamination by faeces as a virus transmission route.

Mite-free bee samples (larvae, pupae and adults) were analysed by ELISA to determine if the virus could be transmitted between bees without the intervention of the mite. Out of the 41 samples tested, only one gave a positive result. This positive result could be due to an escaped infesting mite which was infected or could indicate a low incidence of infection independent of mite infestation.

Specific detection of VDV-1 and DWV in mites and bees

VDV-1 and DWV are closely related viruses with an RNA identity of 84% (Chapter 3). The immunodominant protein VP1 is 97% conserved (Chapter 5). Because of the high similarity in their protein profiles and the ability of VDV-1 and DWV to infect mites (Chapter 4), it was imperative that the ELISA data has to be validated by a PCR-based method to determine whether positive ELISA reactions were due to VDV-1 and/or DWV. To achieve this aim, primer sets specific for each virus were designed (Table 7-1 and Fig. 7-3a). These primer sets are used separately to amplify either VDV-1 or DWV cDNA products, respectively in a single PCR reaction.

Sixteen randomly-selected adult female mites from infested bees were individually tested for infection by RT-PCR. The mites could not be analysed for the presence of virus by ELISA first before PCR because of their small size. Fourteen of them were infected with VDV-1 (Fig. 6) and three mites (4, 6 and 12), infected with VDV-1 were co-infected with DWV (Fig. 7-6b). Attempts to detect the viruses in individual mite eggs or in pools of 10 eggs were unsuccessful.

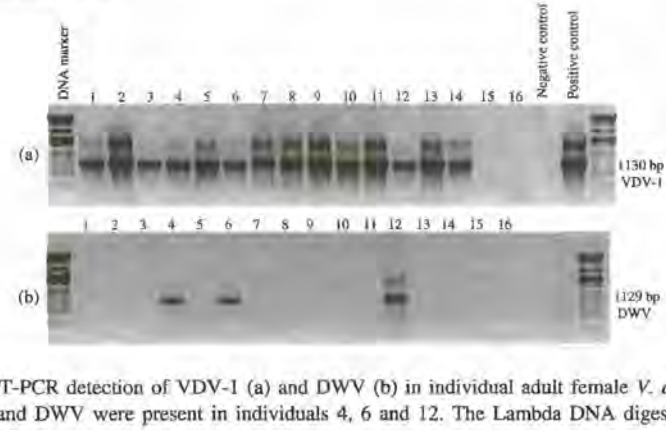
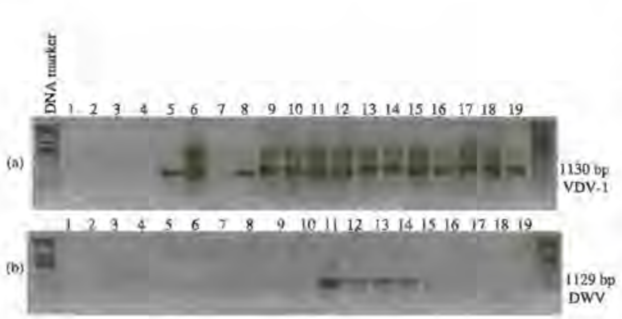


Figure 7-6: RT-PCR detection of VDV-1 (a) and DWV (b) in individual adult female *V. destructor* mites. Both VDV-1 and DWV were present in individuals 4, 6 and 12. The Lambda DNA digested with *Eco*RI, *Hind*III and *Bam*HI is used as DNA marker.

Bees were selected after the mites infesting them had been analysed by ELISA. Some of the mites gave positive and others negative readings (Table in Fig. 7-7). Both VDV-1 and DWV were detected in the adult bees that had been infested with the ELISA-positive *Varroa* mites. The samples labelled 11 to 15 (Fig. 7-7) indicate that both viruses are able to co-exist within the same individual bee as was also the case in the mites (Fig. 7-6). The bees gave positive results only when the infesting mite was infected. Bees gave negative PCR results when they were infested by ELISA-negative mites (for example bees 4 and 7 in Fig. 7-7). In a few cases, no virus could be detected in the bee even though the infesting mite was positive in ELISA (bees 1, 2 and 3).



Mite sample	ELISA (405 nm)	ELISA Result
1	1.274	+
2	1.097	+
3	0.300	+
4	0.086	-
5	0.563	+
6	0.955	+
7	0.114	-
8	0.303	+
9	0.873	+
10	1.220	+
11	1.152	+
12	1.070	+
13	1.033	+
14	0.938	+
15	0.470	+
16	1.151	+
17	1.131	+
18	1.310	+
19	1.045	+
Blank	0.065	-
Positive control	0.866	+

Figure 7-7: RT-PCR detection of VDV-1 and DWV in individual mite-infested adult honey bees (on the left). Both viruses were detected in the bees. The DNA marker is lambda DNA digested with *Eco*RI, *Hin*dIII and *Bam*HI. The table on the right gives the ELISA readings of the infesting mites. The number assigned to each mite corresponds to that given to the bee it infested.

In Chapter 4 it was demonstrated that VDV-1 is able to replicate in *Varroa* mites. This conclusion was based on the finding that the viral complementary RNA strand was detected by RT-PCR using the primer VDV-1 F_{RT} to make cDNA and VDV-1 F_{RTPCR} and VDV-1 R_{RTPCR} for PCR (Table 7-1). Detection of this RNA strand in bees showed unequivocally that VDV-1 also replicates in bee pupae and adults (Fig. 7-8). The presence of the virus genomic RNA in the samples had been established first to be sure that the bees were infected. The bees analysed were infested with mites that gave positive ELISA signals (Table in Fig. 7-7).

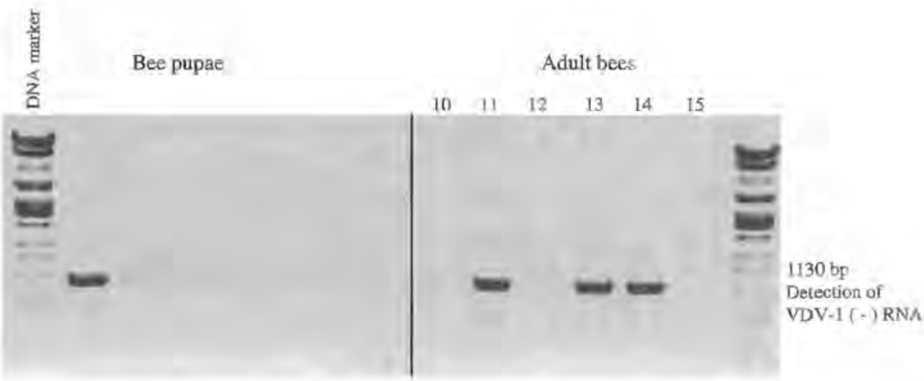


Figure 7-8: Detection of the complementary RNA strand of VDV-1 in mite-infested pupae and adults of honey bees. The numbers assigned to the adult bees represent the same individual bees with identical numbers in Fig. 7-7. The DNA marker is lambda DNA digested with *Eco*RI, *Hind*III and *Bam*HI.

In an attempt to locate where VDV-1 is present in an infected bee and detect where the virus replicates, five adult honey bees were sectioned into three parts (head, thorax and abdomen). Total RNA was isolated from each part separately and cDNA synthesised either from the genome or the complementary RNA strands. VDV-1 was present and replicated in head, thorax and abdomen of infected bees as shown by the presence of the genomic and complementary strand, respectively (Fig. 7-9).

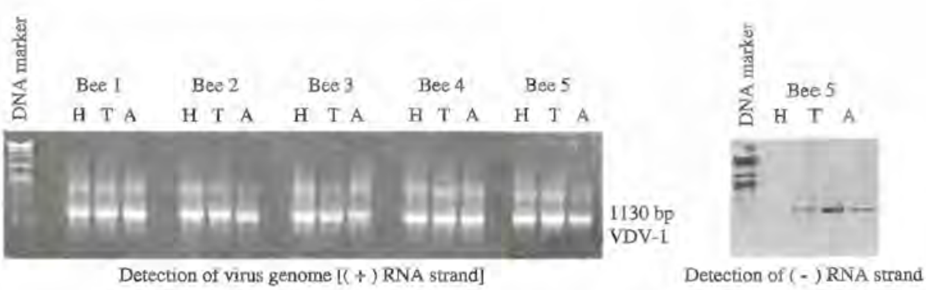


Figure 7-9: Localisation of VDV-1 in infected honey bees using RT-PCR. VDV-1 was detected in the head (H), thorax (T) and abdomen (A) of five infected adult honey bees. On the right, detection of the negative-sense RNA in bee 5 shows that replication occurs wherever the virus is located. The DNA marker is lambda DNA digested with *Eco*RI, *Hind*III and *Bam*HI.

All the data from mites and bees (larvae, pupae and adults) has not been presented here but prevalence rates of VDV-1 and DWV infection, as determined by RT-PCR, show that VDV-1 was the predominant virus. Eighty eight percent of the adult female mites had

VDV-1 while 19% of the mites were co-infected with VDV-1 and DWV, and no mites with only DWV infection were detected. Seventy nine percent of the adult bees had VDV-1, 26% of the adult bees were co-infected with VDV-1 and DWV and there was no DWV only infection in the adult bees either, in the specimens tested. Both viruses were not detected in individual bee eggs or ten pooled eggs by RT-PCR. This observation confirms observations made by ELISA on bee eggs (Fig. 7-5).

To determine whether VDV-1 and DWV could be detected in bees from a region where the *Varroa* mite has not yet been encountered (Oldroyd, 1999), samples of forty five bees from five different hives in Nairobi (Kenya) were tested. The samples proved to be negative for VDV-1 and/or DWV.

Transmission of virus within mite and bee populations

Unsuccessful attempts were made to detect infected mite and bee eggs individually using ELISA and RT-PCR. It was thought that because of their small size, mite eggs could not be individually tested using these methods. The sample would be diluted very much resulting in a poor ELISA signal or RNA could not be isolated in sufficient amounts to make cDNA at a detectable level that would give a positive PCR signal.

The dot-blot immunoassay is an alternative method where the sample is analysed in a concentrated form with almost no wastage. Detection of the presence of virus in eggs is positive demonstration of vertical transmission. Individual mite and bee eggs and some bee larvae and pupae were macerated in a small amount of PBS and spotted onto a nitrocellulose membrane. Different dilutions of the purified virus were added as a positive control. Water, PBS, and *T. ni* eggs were used as negative control. In total, 16 out of 46 mite eggs analysed (all data not shown), gave a strong signal indicating that they were infected with virus (VDV-1 and/or DWV). On immuno dot blot membranes, mite eggs give high colour intensity when they are infected with virus (Fig. 7-10). In some cases they show even a higher signal than the undiluted virus sample. Detection of virus in mite eggs is a strong indicator that the virus(es) might be vertically transmitted among the mites. Individual members of an infected mite family were also included (Fig. 7-10; top row) showing that the females and the males can be infected.

A total of 27 bee eggs were analysed on various blots and all appeared to be non-infected when compared to the negative controls. These observations suggested that VDV-1 and DWV are not transmitted vertically in bees. Some infected bee larvae and pupae were also analysed on the blot (Fig 7-10).

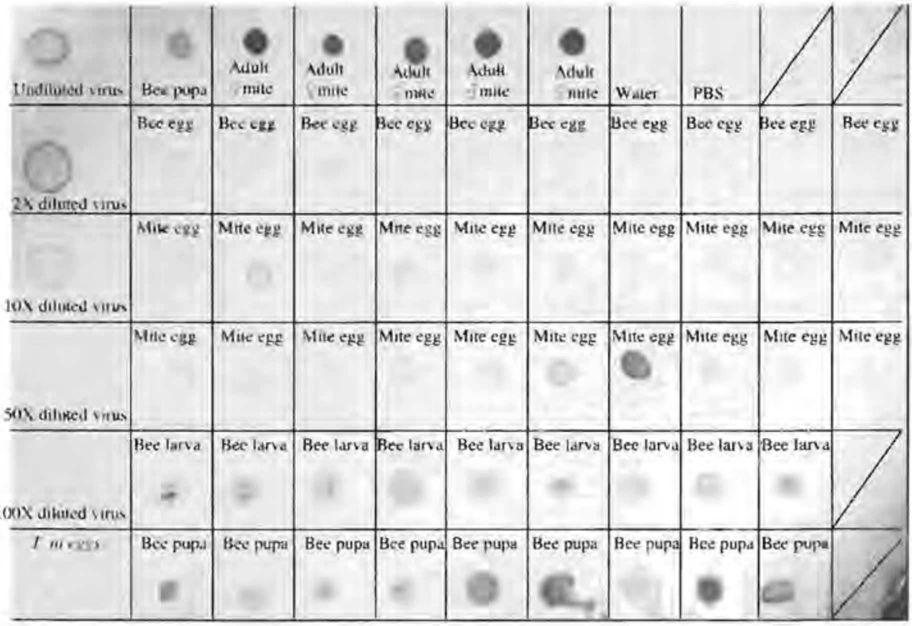


Figure 7-10: Dot blot assay of mite eggs and adult mites and, eggs, larvae and pupae of honey bees. A series of increasing dilutions of a purified virus preparation was used as positive control (left column). *T. ni* eggs, PBS and water were used as negative controls.

Virus infections in mites from different European countries

In order to investigate the geographic distribution of VDV-1 and DWV in mainland Europe, a different set of primers (Table 7-2; Fig 7-3b) was used to detect and differentiate the two viruses in a single PCR reaction. Mite samples obtained from apiaries from other European countries were monitored for the presence of VDV-1 and DWV.

The specificity of PCR primers (Table 7-2 and Fig. 7-3b) used to detect the two viruses simultaneously in a duplex PCR or individually was tested (Fig. 7-11) to ensure that both VDV-1 and DWV could be detected at the same time without one primer set interfering with the other. The lanes labelled 1-3 had a plasmid template of VDV-1 and PCR was done with either VDV-1 or DWV primers or both primer sets in the same PCR reaction. In this case only the VDV-1 sequence was amplified and gave the size expected for the VDV-1 product, showing that the DWV primers did not recognise the VDV-1 sequence. In a reverse experiment it was shown that DWV was only recognised by DWV primers (lanes 4-6). The lanes labelled 7-9 had both VDV-1 and DWV plasmid templates. PCR was done with either VDV-1 or DWV primers or both primer sets in the same reaction. The primer sets proved to be specific for either VDV-1 or DWV and both sequences were amplified where both primer sets were present showing that the primers could be used successfully to detect VDV-1 and DWV simultaneously.

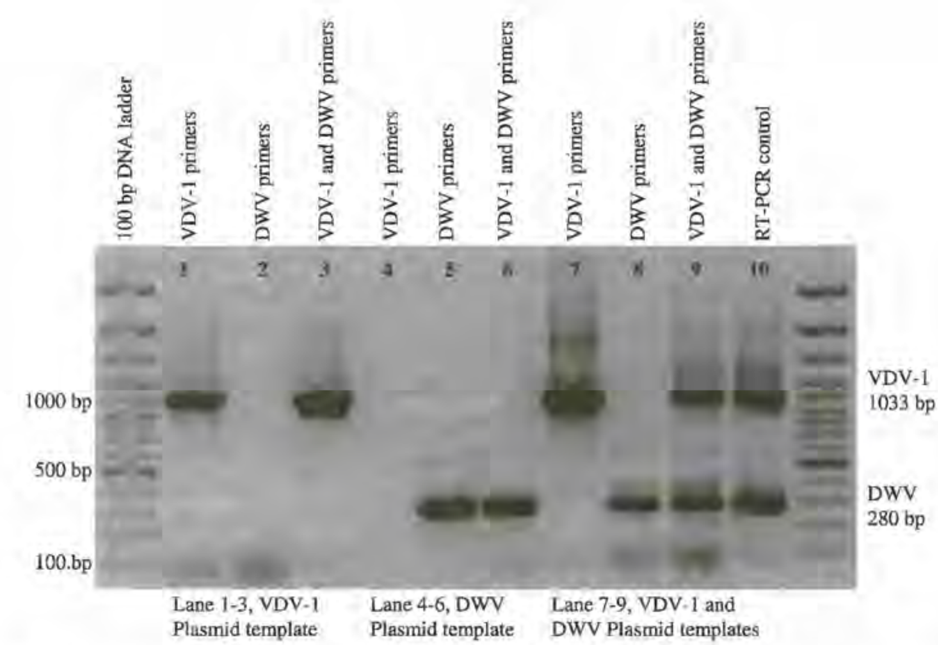


Figure 7-11: Specificity of two primer sets designed for use in a duplex PCR for the simultaneous detection of VDV-1 and DWV. The lanes marked 1 to 9 show the specificity of the reaction in the detection of either VDV-1 or DWV or both templates. RNA from a number of infected mites was used for the RT-PCR control (lane 10). The DNA size marker is the 100 bp DNA ladder (Fermentas).

Having confirmed that the primers designed were specific, a dilution series of VDV-1 and/or DWV plasmid templates was used in a PCR test to determine the minimum detectable concentration of the template (Fig. 7-12). From the dilution series used in this test, the minimum amount of template detected in the duplex PCR was 0.25 ng (Fig. 7-12, panel A), whereas the single PCR reactions are 5 times more sensitive, since 0.05 ng template can be amplified when only one primer set was used at a time (Fig. 7-12, Panels B and C). These results suggest that in order to improve the sensitivity of detecting either virus, two separate reactions should be performed where only one primer set is included per reaction.

A nested duplex RT-PCR was used to detect VDV-1 and DWV simultaneously in the mites from different regions in Europe. VDV-1 was detected in mites from Germany, Italy and France but not from Portugal. DWV was detected in every sample analysed.

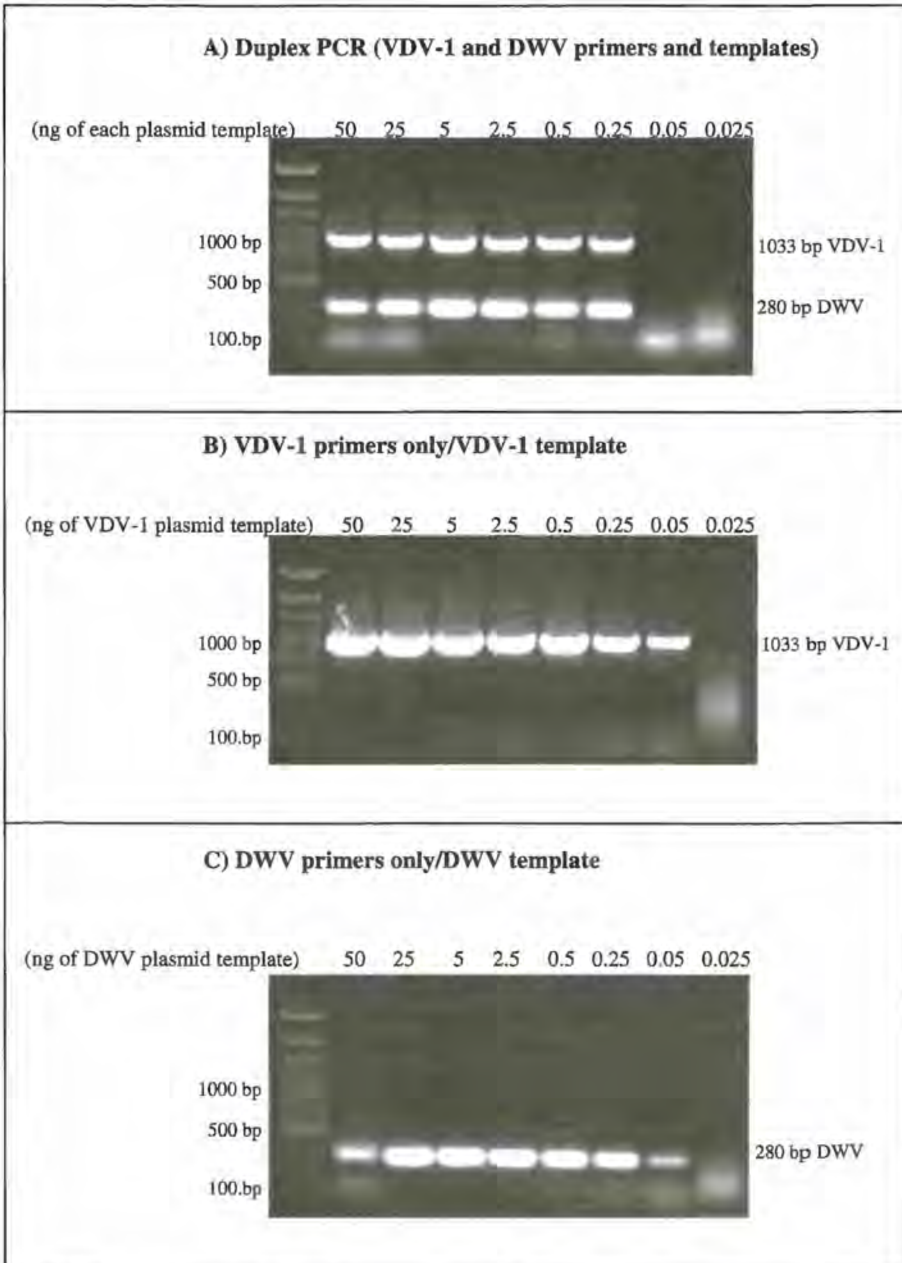


Figure 7-12: Sensitivity test for PCR primers used in the simultaneous detection of VDV-1 and DWV in a single PCR mix. Panel A shows the results of a duplex PCR in the presence of the plasmid templates of both viruses. Each template is present in amounts indicated at the top of the lane. Panel B shows the test of the VDV-1 primers and panel C the DWV primers tested individually in the presence of their respective templates alone. The DNA size marker is the 100 bp DNA ladder (Fermentas).

DISCUSSION

The mite and bee populations in two untreated bee hives at the Wageningen University apiary were heavily infected with VDV-1 and to a lesser extent DWV. The proportion of infected mites (71%) as determined by ELISA was slightly higher than that of bees (65%). Analysis of individual mites and bees by RT-PCR showed that VDV-1 was the predominant virus which was detected at higher frequency than DWV, the latter being present in less than 30% of infected individuals.

VDV-1 and DWV share 95% of their polyprotein sequence (Chapter 3). Only the coat proteins determine the serological reaction especially VP1 (Chapter 5). This immunodominant protein of VDV-1 and DWV showed slightly higher sequence identity (97%) than the polyprotein (Chapter 5), thus ELISA or dot-blot immunoassay would not be appropriate to distinguish between the two viruses as they will show strong cross reactivity in their antisera. It is very likely that the antiserum raised using virus extracted and purified from mites was produced against both viruses. ELISA and blot immunoassay showed that when a single member of a mite family was infected, all other members were also infected and that both adult male and female mites can be infected. These results suggest that the virus could either be transmitted horizontally to the nymphs through feeding on the bee's haemolymph or vertically from the mother mite through her eggs. Virus was detected in mite eggs using a blot immunoassay. This strongly suggested that vertical transmission does occur in the mite population. Bee eggs analysed by ELISA or blot immunoassay did not yield positive results. This observation suggests that either the virus is not vertically transmitted by the queen bee or she was not infected. The queens were not tested for infection in this study. However, studies by Shen *et al.* (2005) showed that some bee viruses (KBV and SBV) can be transmitted vertically by the queen.

Among the bees that were not infested by mites, virus (VDV-1 and/or DWV) was detected in only one out of 41 samples tested implying that either an infesting mite escaped without detection or that the virus was spread by worker bees feeding the brood. Nordström *et al.* (1999) showed that DWV can be transmitted by nurse bees to the larvae. All food for this brood including honey, pollen and royal jelly is processed using secretions from the hypopharyngeal and mandibular glands of the workers (Shen *et al.*, 2005). This would suggest that limited horizontal transmission occurs within the bee population but this alone cannot result in the high levels of infection recorded in this study. There was strong relationship between the number of infected mites (71% infection rate) and infected bees (65% infection rate), with the mites bearing the higher burden of infection. The mite appears to be the main vehicle of transmission.

The ELISA and RT-PCR data from adult bees and the infesting mites indicated that bees were only infected with VDV-1 and/or DWV if the mite was infected (ELISA positive), but virus could not be detected in all bees infested by infected mites (Fig 7-7; bees 1, 2 and 3). In this study it was observed that if the bee was infected, the infesting mite was also infected. The reverse was not observed. These results are supported by the study done by

Nordström (2003) which indicated that the mother mites may contain DWV while the pupal host remained free from overt infection. However, Nordström (2003) demonstrated that the adult mite and its progeny can acquire DWV from an infected bee. Bowen-Walker *et al.* (1999) used ELISA-based methods to show that mites were able to acquire DWV from infected bees and to transmit the virus to non-infected bees. Horizontal transmission does occur between the bee and mite and the virus is able to move from an infected mite to a bee and possibly in the reverse direction (not demonstrated in this study) as the mite feeds on the bee's haemolymph.

The use of specific primers in RT-PCR made it possible to distinguish between the closely related VDV-1 and DWV (84% genome sequence identity; Chapter 3), and to even detect both simultaneously in the same individual mite or bee using a duplex PCR. In this study it has been established that VDV-1 can also replicate in the bee just as it was previously established that it does in the mite (Chapter 4). This could indicate that the honey bee is used by VDV-1 as a reservoir or as a secondary host from where other mites (the primary hosts) can pick up the infection. Virus could be detected in mite eggs on a dot blot immunoassay, but because of the small size of mite eggs, extraction of total RNA from an individual for the purpose of performing RT-PCR did not yield any positive result. It is thought that the amount of RNA available for synthesising cDNA was very little such that the template was not detectable in PCR. It was therefore not possible to conclude whether the infecting virus was VDV-1 and/or DWV. Better results could be obtained if the batch size is much larger, therefore, increasing the chances that more mite eggs selected could be infected. These can be analysed by nested PCR to improve the sensitivity of detection. Bee eggs are comparatively much bigger in size and were analysed by RT-PCR individually or in batches of 10 eggs, but did not give any positive result confirming earlier findings that indicated that vertical transmission of VDV-1 or DWV might not occur in the bees.

The data gathered so far in this study and information from literature that strongly associates DWV with *Varroa* mites (Ball 1989; Bailey & Ball 1991) suggests that VDV-1 and DWV are mite viruses. This idea is further supported by the absence of either VDV-1 or DWV in bees from Kenya (a region with no mite infestation; Oldroyd 1999). The absence of DWV in bees from a mite-free region in northern Sweden (Yue & Genersch, 2005), strengthens the argument that the primary host of VDV-1 and DWV is the *Varroa* mite and these viruses found a convenient host in the bee.

In this study, VDV-1 was detected in the head, thorax and abdomen of infected bees and was able to replicate in the three locations. This observation suggests that VDV-1 replicates in various tissues. Yue & Genersch (2005) detected DWV by RT-PCR in the head, thorax and abdomen of infected bees and demonstrated that this virus could also replicate in these three body zones.

Mixed virus infections in bees are widespread in nature. RT-PCR studies by Shen *et al.* (2005) indicated that co-infections of KBV and SBV did occur in the bee populations investigated. These viruses were detected in food sources and in faecal matter. KBV was detected in infesting mites, and most importantly, in the mite saliva. This finding suggests that the mite may be able to vector this particular virus. Individual bees can harbour multiple virus infections simultaneously. They often tend to be dual infections. Up to four viruses (DWV, KBV, SBV and *Black queen cell virus*) were detected simultaneously in a multiple RT-PCR from an individual bee (Chen *et al.*, 2004b). This study demonstrated that VDV-1 and DWV can co-exist in an individual mite or bee and both viruses were detected in mites from different European countries showing that it is not an unusual occurrence.

Little is known about the pathogenicity of VDV-1 within the mite or bee populations. Other investigators observed that bees displaying deformed wings had usually large amounts of DWV, but high titres were not necessarily linked to wing deformity (Nordström *et al.*, 1999; Bowen-Walker et al., 1999). Morphological differences were not observed during sample collection between VDV-1 infected or non-infected mites or bees.

Investigation of the mite samples from various regions of mainland Europe revealed that VDV-1 was as widely spread as DWV and that the two viruses apparently co-exist. The pathobiology of VDV-1 and DWV in the mite and bee still needs to be investigated.

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Chapter 8

General discussion

Varroa destructor is a parasitic mite that feeds on the haemolymph of honey bees. Within the last three decades, *V. destructor* invaded honey bee colonies in Europe and many other regions in the world creating a new and serious threat to the survival of the honey bee *Apis mellifera* (de Jong, 1982b). The appearance of *V. destructor* was particularly associated with lethargy, malformations and deformities in bees some of which were attributed to the appearance of new viruses such as *Deformed wing virus* (DWV) (Bowen-Walker *et al.*, 1999). In this research, a new virus named *Varroa destructor virus* 1 (VDV-1) was isolated from this mite and sequenced (Chapters 2 and 3). Analysis of sequence data showed that VDV-1 has an 84% genome sequence identity to DWV. The VDV-1 genome has one large open reading frame (ORF) which is translated into a 2893 amino acid-long polyprotein with an amino acid identity of 95% relative to DWV.

Genetic variation in VDV-1 and DWV

The genomes of RNA viruses exist as a distribution of sequence variants based on a consensus sequence (Matthews, 1991). RNA-dependent RNA polymerases drive the rapid rate of viral replication resulting in a high error rate that causes changes in viral RNA sequences (Domingo & Holland, 1988). This forms the basis of the 'quasi species' concept of viruses (Domingo & Holland, 1988), where a species consists of a mixture of near isogenic genomes. Single nucleotide changes in the genome of some RNA viruses have been demonstrated either to be silent or to affect characteristics such as symptom expression, transmission, virulence, viral movement and host mediated resistance (Blanchard & Anderson, 1998). This variation is responsible for the rapid adaptation of RNA viruses to new environments and hosts (for example influenza virus). This concept prompted the investigation of the genetic variations in VDV-1 and DWV in mite populations from bee hives in various European countries. To this aim, specific primers were designed to amplify 1033 bp of VDV-1 and 280 bp of DWV (Chapter 7). The PCR amplified products were cloned and sequenced to establish the relationship between the sequences of VDV-1 isolated from Wageningen in The Netherlands, Avignon in France, Oberürsel and Kirchhain in Germany, Grugliasco and Udine in Italy, and Evora in Portugal. The obtained sequences were aligned using the ClustalX program (Thompson *et al.*, 1997) and viewed in GeneDoc (Nicholas, 1997). The neighbour joining trees were plotted in ClustalX and bootstrap values estimated from 1000 replicates. The trees were observed in TreeView software (Page, 1996).

VDV-1 sequences could be amplified from mites collected from all the regions except from Evora (Portugal). These sequences showed 96 – 99% mutual identity (or 1-4% single nucleotide polymorphisms) and confirm that they all belong to one virus species, VDV-1. At certain nucleotide positions (indicated with an asterisk) in the amplified genome region, the same nucleotide change was observed in more than one sequence in both VDV-1 (Fig.

8-1a) and DWV (Fig. 8-1b) sequences. Real hot spots with more than 2 nucleotide possibilities at a particular position were not observed, neither were longer stretches of variable nucleotides. The virus sampled from Avignon (France) was most closely related to the VDV-1 sequence from Wageningen (The Netherlands) (Fig. 8-2a).

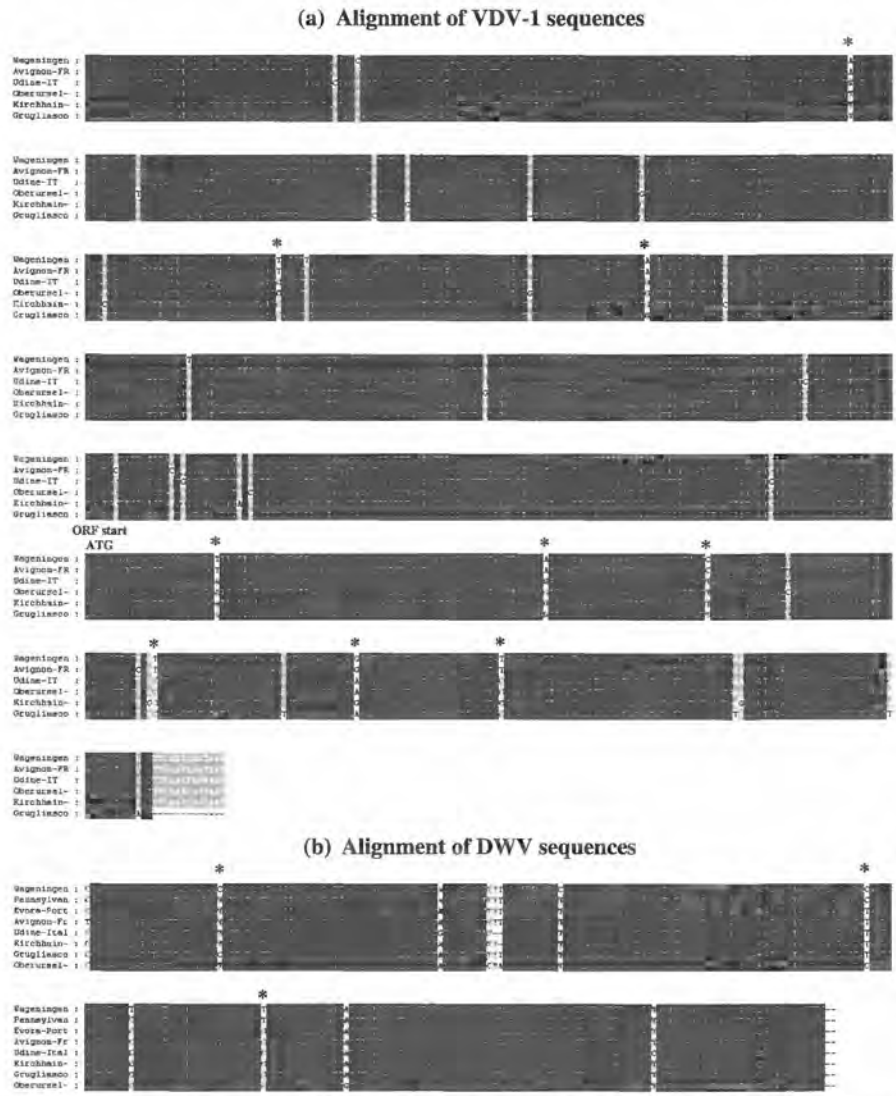

Figure 8-1: Alignment of European VDV-1 mapping to the nucleotide position 397-1429 in the virus genome (GenBank accession number AY251269) (a) and DWV mapping to the nucleotide position 406-685 in the DWV genome (b) sequences. The DWV sequence from Pennsylvania, USA (GenBank accession number AY292384) used as the basis for comparison. The DWV Brescia Italy sequence (GenBank accession number AJ489744) was included in the comparison.

A comparison of the 280 bp from the 5′ non translated region of DWV sequences amplified from mite samples from the various locations in Europe to sequences in GenBank showed 96-99% nucleotide identity (Fig. 8-1b). The Wageningen DWV is most related to the Pennsylvania DWV sequence (Fig. 8-2b). The degree of polymorphism is similar to that seen in the VDV-1 sequences, although in the latter case the 5′ end of the coding region was included. The variations in the VDV-1 sequences are silent in the region containing the beginning part of the polyprotein in that they do not change the amino acid sequence. Changes in the 5′ NTR could affect viral replication and translation for example a C-to-U mutation at base 472 in the poliovirus IRES was demonstrated to alter its tissue tropism and attenuation (Kauder & Racaniello, 2004).

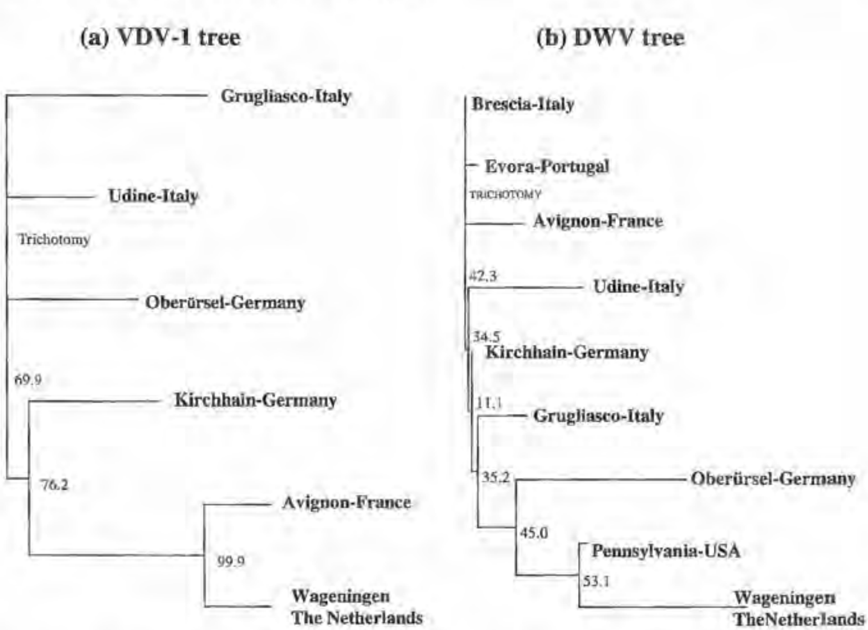

Figure 8-2: Phylogenetic tree for VDV-1 (a) and DWV (b) plotted from the aligned sequences in Fig. 8-1. The numbers at the nodes of the tree are the bootstrap values as percentages estimated from 1000 replicates.

Kakugo virus (KV; Fujiyuki *et al.*, 2004), isolated from the brains of aggressive worker honey bees in Japan, has a 97% genome identity to DWV (de Miranda *et al.*, 2003). Considering that the genome variation falls within the range seen in the DWV isolates analysed here suggests that KV and DWV are variants of the same virus species only differing in tissue tropism and/or virulence.

Bee or mite viruses?

VDV-1 and DWV have both been detected and found to replicate in *V. destructor* and *A. mellifera* (Chapters 4 and 7). The possibility of vertical transmission of either or both viruses was demonstrated in the mite population by the detection of virus in mite eggs using a dot-blot immunoassay. There was no indication that vertical transmission did occur in the bee population studied (Chapter 7). Because of the unspecific nature of the antiserum used in the immunoassay, it is not known with certainty which of the two viruses was detected in the mite eggs, but since both viruses were present in the populations studied, and neither was detected in bee eggs, both are thought to have the ability to be vertically transmitted via mite eggs.

The current hypothesis for the transmission of VDV-1 and DWV between mite and bee populations based upon results recorded from research in this thesis, with support from published literature (Nordström, 2003; Bowen-Walker *et al.*, 1999), is illustrated in Figure 8-3.

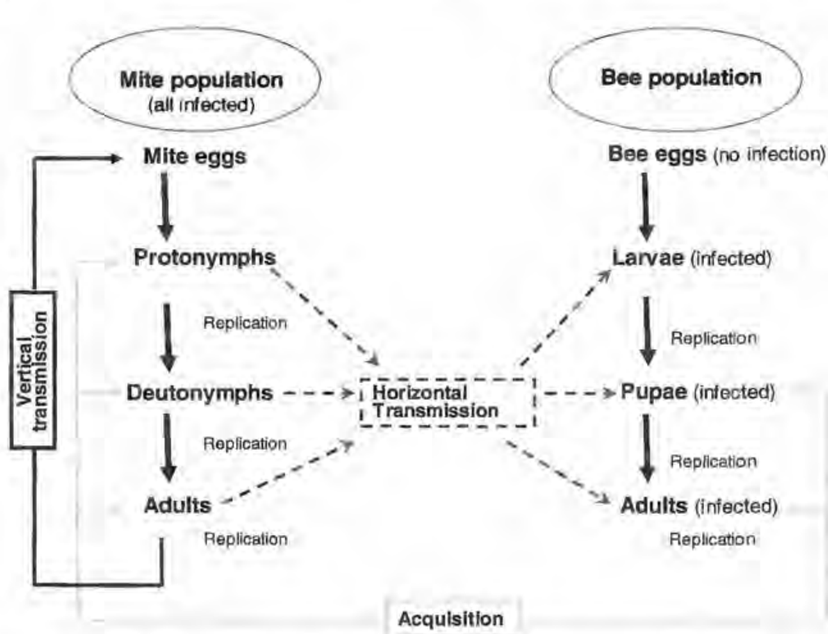

Figure 8-3: A schematic representation of the transmission of VDV-1 and DWV between the mite and bee populations. The downward facing arrows indicate the direction of growth of either the mite or bee. VDV-1 replication was detected in both the mite and bee stages indicated. Nordström (2003) and Bowen-Walker *et al.* (1999) reported that the mother mite and its progeny could acquire DWV from the honey bee pupae and adults. Mites infest bees from the larval stage and transmit VDV-1 and DWV horizontally to the bee as they feed (indicated by the broken lines). The infecting viruses could be detected in the mite eggs suggesting the occurrence of vertical transmission in mites.

Since VDV-1 and/or DWV go through a full transmission cycle in the mite population, the current thought is that these viruses are primarily mite viruses. The research presented in this thesis demonstrated that bees contained these viruses only when infested by infected mites suggesting that the virus was moving from the mite to the bee. Published literature has recorded that DWV can also be acquired by mites from infected bees (Nordström, 2003; Bowen-Walker *et al.*, 1999). The transmission flow shows that these viruses are able to replicate in bees and appear to have adapted to the bee as a secondary host. Additional evidence that these are mite viruses is the fact that VDV-1 and DWV were not detected in bees from regions in Kenya and the northern parts of Sweden where the *Varroa* mite is absent (Oldroyd, 1999; Yue & Genersch, 2005). This suggests that VDV-1 and DWV are strongly associated with the mite. The spread of DWV in *A. mellifera* has been attributed to the introduction of *Varroa* mite infestation in the colonies (Bailey & Ball, 1991; Bowen-Walker *et al.*, 1999). Thus the prevention of mites going to non-infested areas is of utmost importance to avoid disease.

It has been reported that deformed wing symptoms in honey bees could be brought about by physiological factors in the development of the bee other than an infection with DWV, such as the detrimental effects evoked by heavy infestation with *Varroa* mites (de Jong *et al.*, 1982a) (though these authors did not consider the effect of viral infection in their study) or microbial septicaemia, due to infection with microorganisms transmitted by *Varroa* (Shabanov, 1984; Gliñski & Jarosz, 1992). *Varroa* infestation is reported to have a general immunosuppression effect on honey bees, which enhances microbial infections (Yang & Cox-Foster, 2005). Bacterial factors combined with the mite infestation were thought to enhance the replication of DWV.

DWV appears to have been prematurely named since infection with this virus does not always cause a deformed wing phenotype (Nordström *et al.*, 1999; Bowen-Walker et al., 1999; Chapter 2). Most of the bees in the Wageningen hives were infected with VDV-1 and some with DWV but the deformed symptoms were rarely found among bees that were later found to be infected with DWV and VDV-1. When VDV-1 and DWV co-exist in a host there are chances that recombination could occur only if they infect the same cell as is known for poliovirus (Wimmer *et al.*, 1993). It is not known if VDV-1 and DWV can infect the same cell at the same time. If they could, the most likely form of recombination would be cross packaging during virion assembly where for example the genome of VDV-1 could be encapsidated in the structural proteins of DWV. Genetic recombination involving the cross over of single-stranded RNA viral genomes can also occur as demonstrated by Duggal *et al.* (1997) for poliovirus in a cell-free system. No VDV-1/DWV crossover recombinants were detected in this study, although, the number of VDV-1/DWV isolates investigated was limited. The predominant reason for genetic variation in RNA viruses remains the absence of proofreading and editing functions in the RNA-dependent RNA polymerase (Domingo & Holland, 1988).

VDV-1 IRES structure and the location of the virus in mite tissue

The secondary structure of the 5' NTR of VDV-1 was predicted in Chapter 6 and was found to resemble internal ribosome entry site (IRES) structures of picornaviruses of the *Rhino-/Enterovirus* type (Witwer, 2001). The VDV-1 IRES especially resembles that of poliovirus (Wimmer *et al.*, 1993). The availability of a predicted secondary structure for the 5' NTR of VDV-1 should facilitate targeted mutation studies to identify crucial motifs for 5' IRES activity. Disruptions caused by mutations that alter key elements in the structure can be applied to map the boundaries and most important elements for IRES activity. Mutations may be in the form of deletions that will melt a prominent structural element, for example, the branched clover-leaf structure labelled V in the VDV-1 5' NTR structure (Chapter 6), or nucleotide substitutions that will either undo a structural element or disrupt a conserved motif, for example the conserved palindrome and parallel domains in the structure labelled V in the VDV-1 structure, which are thought to be important recognition sites for translation initiation factors and could be targeted for mutation. By extrapolation from the poliovirus 5' NTR structure and mapping of the IRES (Wimmer *et al.*, 1993), the VDV-1 IRES would include the prominent clover-leaf element V and the two hairpins immediately flanking it on either side.

IRES elements also play an important role in some protein expression systems, where two proteins can be expressed under the control of one promoter from a bicistronic expression vector. An example of an available commercial plasmid that is based on such a system is pIRES1*neo* (Clontech Laboratories). The advantages of such systems are: they save time and resources while developing stable cell lines (Rees *et al.*, 1996), they can achieve high-level expression of recombinant proteins, they minimize or eliminate the dependence on clonal selection and continuous protein expression can be carried out over time in culture due to the enhanced accumulation of stable RNA transcripts (Huang & Gorman, 1990). Recently, the *Rhopalosiphum padi virus* 5' IRES was used for continued dominant selection of bacmid-derived recombinant baculoviruses in insect cell culture (Pijlman *et al.*, 2006).

The VDV-1 IRES was active in a host-specific manner showing high activity in *Lymantria dispar* Ld652Y cells, but lower activity in *Spodoptera frugiperda* Sf21 cells and no activity in *Drosophila melanogaster* S2 cells (Chapter 6). Picornaviruses exhibit a variation in host range as well as differences in the tissues that they infect (Pilipenko *et al.*, 2001); this tropism is associated with the manifestation of pathogenic properties (Rueckert, 1996). Tissue specificity depends on the ability of a picornavirus to bind and enter particular cells, and on host-specific factors that interact with the viral genome and that support for instance the function of the IRES element (Rueckert, 1996). Different host cell RNA-binding proteins are required by IRES elements of different viruses for their function (Pilipenko *et al.*, 2000). The tissue-specific expression of these factors may explain the distinct tropisms of the viruses (Pilipenko *et al.*, 2001). Poliovirus replication for instance occurs in a few organs, including the brain, spinal cord, and alimentary tract (Bodian, 1959). VDV-1 and/or DWV were located in the digestive system of *V. destructor* mites using immunohistology (Chapter 2). The secondary structure of the IRES of VDV-1 resembles that of poliovirus and since IRES structure appears to influence tissue tropism, VDV-1 and DWV may behave like "invertebrate enteroviruses".

Results presented in this thesis demonstrated that VDV-1 could also be found in the head, thorax and abdomen of the honey bee. Yue & Genersch (2005) positively detected DWV by RT-PCR in the head, thorax and abdomen of infected bees. Both viruses could thus replicate in these three parts of the body. These viruses may be restricted to similar organs in the honey bee as in humans, but this needs more in depth analysis. *In situ* hybridisation of various organs of the mite and bee with VDV-1/DVW cDNA can identify the infected tissue. In view of this, KV (which appears to be a variant of DWV) was positively connected to infection of the brain of honey bees (Fujiyuki *et al.*, 2004). KV may contain non-silent mutations that affect its neurotropism, resulting in higher viral load in the brain. It is also possible that the Japan bees' aggressiveness was caused by other reasons besides just the presence of KV.

There is now need to investigate if VDV-1 can replicate in the Ld652Y cell line. The use of intact virus to test replication will depend on the ability of the virus to attach and penetrate the cell to release the genome RNA into the cytoplasm. The absence of suitable receptors on the surface of this cell line will mean that this approach may not be feasible. In that case, genomic RNA isolated from purified virus should be transfected into these cells. For a pure isolate, RNA transcripts can be synthesised using a strong promoter in a plasmid clone of full-length genome cDNA and transfected into the cells. Infectious cDNA clones of another picorna-like virus from bees, *Black queen cell virus*, have been constructed (Benjeddou *et al.*, 2002) suggesting that this is a feasible strategy. The ability of the virus to replicate would then be tested by looking for the complementary RNA strand. The permissiveness of the cell line to support proper assembly of infectious particles can be assessed using antibodies to detect the structural proteins such as those described in Chapter 5. The amount of infectious virus produced will ultimately depend on the permissiveness of the cells. The virus could be isolated from the cells and used to infect mites or bees to confirm their infectivity.

Taxonomy

The 7th International Committee on Taxonomy of Viruses (ICTV) report formalised the concept of virus species for the first time (Van Regenmortel *et al.*, 2000). ICTV uses different viral properties to describe and characterise viral taxa, which results in names of orders, families, subfamilies, genera and species. Species is universally accepted as the lowest taxonomic clustering of living organisms. In 1991, the virus species definition by ICTV stated that: "A virus species is a polythetic class of viruses that constitute a replicating lineage and occupy a particular ecological niche". A polythetic class comprises members with several properties in common but no single common attribute is present in all of its members, such that no single property can be used to define the species (Francki *et al.*, 1991). A single character, such as host response or a certain degree of genome sequence dissimilarity cannot be used to absolutely differentiate two virus species within the same genus. The 7th ICTV report therefore concludes that the use of a single discriminating character to distinguish species is deemed to be inappropriate because of the inherent variability amongst members of the species.

The ICTV 7th report continues to state that a virus species is considered to be a replicating lineage with members that descend from a common ancestor and represent an evolving lineage whose membership varies over time. Shared descent also links different species, genera and determines phylogeny. Sequence data indicate the extent to which a virus has diverged over time. As variations accumulate, the importance of genotypic and phenotypic differences will lead to the conclusion that a separate entity has been formed. Characters useful in discriminating between virus species within the same genus are: genome sequence relatedness, natural host range, cell and tissue tropism, pathogenicity and cytopathology, mode of transmission, physico-chemical properties of virions, and antigenic properties of viral proteins (Van Regenmortel *et al.*, 2000).

The research presented in this thesis raises the question about the classification of VDV-1 and DWV. ICTV rules state the insufficiency of using sequence differences alone in classification. Whereas DWV has been associated with deformities in developing bees, VDV-1 has not presented any clear symptoms in the bees or mites it infected. There is also the observation that DWV infections do not automatically cause deformity at lower titres, but rather the infection that results in very high titres of DWV brings about the deformed symptoms (Nordström *et al.*, 1999). No study has so far been done to demonstrate conclusively that mite-free bee larvae and pupae inoculated with DWV end up as adults with deformed wing symptoms. More research needs to be done to investigate the pathobiology of VDV-1 and DWV in bees. This will require the use of pure isolates of each virus, which could be obtained in tissue culture by making transcripts from a cDNA clone in a cell line that is permissive to viral replication (Benjeddou *et al.*, 2002).

According to the ICTV 7th report, characters for species demarcation vary in different genera. No single criterion has absolute supremacy over others, but it is the total of the information gathered that enables reliable species demarcation. This is ambiguous since boundaries are not clearly defined and the threshold can be subjective. VDV-1 and DWV are iflaviruses sharing 84% of their genomes. They are both able to infect the honey bee *A. mellifera* and the mite *V. destructor*. *Ectropis obliqua picorna-like virus* (EoPV; Wang *et al.*, 2004) and *Perina nuda picorna-like virus* (PnPV; Wu *et al.*, 2002) both named after the moth species from which they were isolated are also iflaviruses and have 81% mutual genome identity and share 87% of their amino acid sequence. That the situation is far from clear is exemplified by a case study among baculoviruses of the genus *Nucleopolyhedrovirus* identified two individuals, *Helicoverpa zea* single-nucleocapsid nucleopolyhedrovirus (HzSNPV) and *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus (HearSNPV) whose genomes are very similar in their nucleotide (97% identity) composition, and which encode highly identical proteins (99% identity) (Chen *et al.*, 2002). These viruses were named after the lepidopteran hosts from which they were isolated, but may represent one species with two hosts. However, in the 8th report of ICTV (Fauquet *et al.*, 2005) they are still recorded as separate species.

The question now is, can each of the four mentioned iflaviruses be considered as individual species or should some be grouped? Sequence data shows that VDV-1 and DWV appear to have split from a common ancestor and formed two fairly stable populations. So far, no intermediates have been found; however, each of the two could be referred to as variants of the same species. The structures of the 5′ NTR (Chapter 6) suggest that coupling of these species would place VDV-1 and DWV into one group and PnPV and EoPV into another. The second question to be asked is how far should they move apart to be considered as independent species? There is sufficient difference in their genetic makeup that has been maintained and cannot allow both viruses to be referred to as identical. These questions can only be answered once all the characteristics useful in discriminating between virus species are fully investigated for these viruses.

Detection methods

The use of antibodies raised in rabbits against purified virus from mites enabled the detection of both VDV-1 and DWV infections (Chapter 2) since the antiserum was very likely prepared from a mixture of the two viruses. This antiserum proved to be useful in ELISA for the screening of a large number of specimens and also in blot immunoassays to check for infection with both viruses. The results reported in Chapter 5 show that the major structural protein VP1 was responsible for the immunogenic response of the viruses in rabbits, since it was the only protein recognised by this antiserum. The VP1 proteins of VDV-1 and DWV are 97% identical; it is therefore not possible to differentiate an immunogenic response in rabbits due to VDV-1 or DWV.

The research described in this thesis (Chapter 4) also showed that VDV-1 and DWV can be distinguished using specific primers in RT-PCR to detect either virus sequence, also when the individual mite or bee is co-infected by both viruses. The current trend in identifying virus infections in mites and bees is moving away from the use of serology alone towards qualifying the observed results using specific PCR-based methods (Tentcheva, 2004; Genersch *et al.*, 2006). This has led to the finding that multiple viruses are able to infect an individual bee (Chen *et al.*, 2004b; Shen *et al.*, 2005).

The infecting viruses could be present in an individual at low levels. In order to improve VDV-1 and DWV detection, a nested PCR was developed where the first round of PCR with primers that anneal to both sequences is followed by a second round of PCR using specific primers to discriminate between the two sequences (Chapter 7).

Relative load of VDV-1 and DWV in mites and bees

In analysing VDV-1 and DWV infections in mites and bees from Wageningen, VDV-1 infection was detected in most of the samples and gave stronger signals than DWV (Chapter 7). The stronger VDV-1 signal could be attributed to the performance of the primers selected in PCR or could imply that the DWV loads were lower. When the selected primers were used to test the specificity and sensitivity of detection using plasmid templates bearing either VDV-1 or DWV sequences, apparently the primer sets for both viruses performed the same in this control experiment (Chapter 7). There was no visible indication that either set was better than the other. Normal PCR is not quantitative since reactions can become saturated and the product is only checked at the end of a run. A more reliable method to detect the absolute and relative load of DWV and VDV-1 would be quantitative PCR (Q-PCR) (Heid *et al.*, 1996; Genersch *et al.*, 2006). This would give more accurate information on the individual viral load especially where co-existence occurs.

Management of *Varroa* infestation

Since some bee viruses are associated with *Varroa* mites and mites play a role in bacterial infections in bees, control of mites is not only important to prevent direct effects of their feeding behaviour, but also in controlling or combating these viruses and other opportunistic infections. The current options for the control of *V. destructor* include the application of various chemicals, organic acids and manipulative treatments. A variety of chemicals have been used to control *Varroa* mite populations in bee colonies. Among the chemicals commonly used are approved synthetic acaricides which specifically affect mites, such as, Tau-fluvalinate (Apistan®), flumethrin and coumaphos (Perizin) (Koeniger & Fuchs, 1988; Ritter, 1990). Organic acids (formic, lactic and oxalic acid vapour), essential oils and even simple compounds like sulphur are also in use (Eguaras *et al.*, 1996). The chemicals are applied as vapours or by spraying. Thymol is a natural chemical found among many plant species, most notably in thyme, which is toxic to *Varroa* mites at doses relatively safe for honey bees (Charriere & Imdorf, 2002). Two European products that use thymol as active ingredient are Apiguard®, produced by Vita Europe (England) and Apilife VAR® produced by Chemicals LAF (Italy). The higher surface area to volume ratio of the mites compared to that of the bees is thought to be one of the reasons why non-specific toxic chemicals kill the mites faster than the bees (Bailey & Ball, 1991). The continuous treatment of the beehives with these chemicals and compounds over a long period of time may reduce the mite populations, but several of these chemicals have clear disadvantages. Formic acid threatens the survival of the brood, young bees and the queens (Fries, 1989) and acaricides fail to reach mites in capped brood. A way to circumvent these failures is to replace old queens when no capped brood is present in the colony (Bailey & Ball, 1991). Mites have been found to develop resistance to the acaracide fluvalinate (Milani, 1994; Lodesani *et al.*, 1995) and if not used properly, the residues of the synthetic acaricides can accumulate and persist in honey and other bee products (Lodesani *et al.*, 1992). In addition, some of the chemicals applied pose a health risk to the beekeepers.

Technical methods for the control of the *Varroa* mite are mainly used by small-scale beekeepers to minimise the use of non-specific acaricides. Most of the methods work by trapping mites in brood combs (Fries & Hansen, 1993), which are then separated from the colony, or by causing mites to drop off adult bees by mechanical means. Some of these methods include the removal of infested drone brood before emergence, comb trapping of mites followed by destruction of the comb, and the use of open mesh floors, which prevent live mites that drop from the hive from returning (Calis, 2001). These methods are inexpensive and do not require the use of chemicals. Nevertheless, they are not sufficient when used alone. They are time-consuming, only effective in the case of moderate infestation and need high level of beekeeping skills.

Biological control

The development of biological control of *Varroa* would provide an alternative to the use of acaricides, and could form part of an integrated pest management system. An effective pathogen would spread rapidly to maintain *Varroa* populations below a damage threshold giving long term or permanent control. A variety of microorganisms have been isolated from *V. destructor* mites that had apparent pathological symptoms, in an attempt to verify their pathogenicity. These include bacteria, viruses, *Rickettsiae*, fungi and parasitoids. Some like the bacteria *Bacillus thuringiensis* showed some disease symptoms, and like the rest of the microorganisms studied, it could affect other organisms besides the mites (Chandler *et al.*, 2001). No effective natural enemies able to cause wide population decline of *Varroa*, without harming other organisms in the process, have yet been identified.

Entomopathogenic fungi provide prime candidates for *Varroa* control since many species are active against acarines (mites and ticks) in nature. The potential of *Hirsutella thompsonii* and *Metarhizium anisopliae* as biological control agents of *Varroa* were evaluated in a laboratory setting and in observation hives (Kanga *et al.*, 2002). In the laboratory, the time needed to kill 90% of the mites (LT_{90}) was 4.16 days for *H. thompsonii* and 5.85 days for *M. anisopliae* at 1.1×10^3 conidia mm^{-2}, at a temperature (34 ± 1^oC) similar to conditions in a brood nest of a honey bee colony. The disadvantage is that the treatments did not significantly affect the mite population in sealed brood. *H. thompsonii* was apparently harmless to honey bee workers and brood, but Shaw *et al.* (2002) reported that four isolates of *M. anisopliae* caused significant mortality to honey bees.

The use of VDV-1 as a biological control agent against the mite does not seem feasible even though the virus apparently originated from the mite. This position is taken because VDV-1 and also DWV seem to have successfully found an alternative host in the honey bee and DWV and the closely related KV have been implicated in causing severe disease symptoms in infected bees (Bowen-Walker *et al.*, 1999; Fujiyuki *et al.*, 2004). To date it is unclear whether VDV-1 is pathogenic to bees either directly or indirectly by making them more vulnerable to other pathogens. It remains unclear whether VDV-1 has a negative effect on the mites either at the individual or the population level.

Although only a limited number of bees from Central Africa have been tested, neither DWV nor VDV-1 could be detected. Since the mite *V. destructor* has not yet been identified in this area and since it might be involved in the spread of DWV and VDV-1, it is of utmost importance to keep the mite out of Central Africa by preventing the importation of bee stocks from infested hives. However, drifting bees and swarms of infested bees from other regions on the continent cannot be controlled. This means that it is just a matter of time before the mite finds its way to Central Africa. Methods are are available to detect iflaviruses such as DWV or VDV-1 (Chapter 4).

Conclusion

In this research VDV-1 was characterised and compared to DWV. Both viruses have diverged to form separate and fairly stable populations. VDV-1 (and DWV) can infect and replicate in the *Varroa* mite as well as in the honey bee, and they co-exist in apiaries found around mainland Europe. The study also showed that there is potential to find alternative cell systems in which VDV-1 could replicate and these could be used for further investigations of its behaviour. VDV-1 cannot be considered to be a suitable candidate to be used as a biological control agent against the *V. destructor* mite because of its ability to infect and replicate in honey bees and the absence of clear pathogenic effects in mites. The virus has apparently broadly spread in Europe but has not reduced the *Varroa* mite problem.

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Summary

Varroa destructor mite is an ectoparasite of the honey bee *Apis mellifera*. This species was recently differentiated from *Varroa jacobsoni* species which infests the Asian bee *Apis cerana*. *Varroa* mites feed entirely on the bee's haemolymph and have been associated with the spread of a number of viruses. Since the mites were first observed in Java, Indonesia in 1904, they have been reported in most regions of the world except Australia and the equatorial regions of Africa. *V. destructor* severely affects and threatens the survival of *A. mellifera*. The mite was spread to *A. mellifera* colonies in other areas by migratory practices of bee keepers and by drifting swarms. The survival of bee colonies attacked by these mites is threatened since the mites weaken the colony if left untreated. Honey bees are important pollinators in nature and in the agricultural and horticultural industries. Bee keepers can also earn from the sale of honey, bee wax and propolis. At this moment, there is no absolute method available for controlling the mite. The methods currently employed use chemicals which contaminate honey and other bee products, and mites are developing resistance to some of the chemicals used. The economic impact of mite infestation makes it necessary to investigate alternative options for control.

At the onset of the research described in this thesis, 27 nm picorna-like virus particles were observed in mite tissue apparently going through an infection cycle. The identity of this virus was unknown and it was unclear if it was infectious to the mite only or to the bee as well. The aim of this research was to isolate this virus from the mite, characterise its genome in detail and study its behaviour in mite and bee populations with the intention to determine its potential as a biological control agent against the *Varroa* mite.

Electron microscopic examinations showed para-crystalline aggregates of virus particles in the cytoplasm of mite tissue. The virus particles were purified from mites collected from the Wageningen University apiary and used to raise rabbit polyclonal antibodies. The antibody was applied to locate the virus in tissue sections of mites in an immunohistology examination which revealed that the virus was abundantly located in the tissues of the lower digestive tract (Chapter 2). The virus was not detected in salivary glands, indicating that this virus is not transmitted via these glands.

In the next phase RNA was isolated from these virus particles and the viral genome was fully sequenced (Chapter 3). The virus has a single-stranded, positive-sense genome which is polyadenylated at the 3′ terminus and can serve directly as messenger RNA. The genome has a length of 10,112 nucleotides (without the poly-A tail) and one large open reading frame (ORF) encoding a polyprotein of 2893 amino acids. The structural proteins are located in the N-terminal half and the non-structural proteins on the C-terminal half of the polyprotein, and are produced by autoproteolytic cleavage. The ORF is flanked on either side by nontranslated regions (NTRs). Phylogenetic analysis of its RNA-dependent RNA polymerase in a comparison with those of related viruses in the GenBank database revealed that this virus shows high sequence similarity to members of the genus *Iflavirus*. The

genome organisation is also highly comparable to iflaviruses and clearly distinct from that of dicistroviruses. Since the genome sequence of this virus had not been previously reported, the virus was named *Varroa destructor virus* 1 (VDV-1) after the mite from which it was first isolated. VDV-1 is most closely related to *Deformed wing virus* (DWV) which was isolated from honey bees with wing abnormalities. VDV-1 and DWV have 84% genome and 95% polyprotein identity.

To confirm that VDV-1 is able to replicate in the mite, primers were designed to detect specifically the negative-sense RNA strand, which only occurs as replication intermediate, and hence provides evidence for viral replication. With these primers cDNA was synthesised and was further amplified by PCR. Two sets of specific primers were made to distinguish between and detect either VDV-1 or DWV. In a similar way, primers, specific for the positive-sense strand, were used to detect the genomes of both VDV-1 and DWV in the mites. These experiments showed that both VDV-1 and DWV were replicating in the *Varroa* mite (Chapter 4). These findings are in good agreement with the observation of para-crystalline aggregates in electron microscopy images, which also supports the replication of both viruses in the cytoplasm of mite cells (Chapter 2).

In Chapter 5, the structural proteins of the isolated virus were examined by SDS-PAGE and it was observed that the two largest proteins (VP1 and VP2) were present in relatively equal amounts in the virus. These proteins were N-terminally sequenced to reveal the amino acids that determined the proteolytic cleavage sites. VP2 was mapped directly N-terminal to the non-structural helicase, while VP1 was located immediately upstream of VP2. Through Western blot analysis it was demonstrated that only VP1 reacted very strongly to the antiserum prepared against the virus. In a next experiment the structural proteins were expressed individually fused to glutathione S-transferase. The resulting fusion proteins were tested in Western blot analysis using the antiserum against purified virus. This study revealed that VP1 was the only structural protein which was recognised, implying that this protein probably covered the entire surface of the virus particle, hiding the other structural proteins beneath, or that the other structural proteins were not immunogenic. The viral 3C-like protease was also expressed as a fusion protein and used to raise antibodies, which efficiently detected the protease polypeptide in a control experiment. This antiserum might be used to detect viral replication using an antibody-based method such as ELISA, or to localise replication in the mite body using histochemical methods in addition to molecular techniques.

So far there is no report on the structure and function of the 5′ nontranslated region (5′ NTR) of the RNA of iflaviruses. One of the aims of this investigation was to predict whether conserved secondary structures occurred in the 5′ NTR of four iflaviruses (Chapter 6). The predictions revealed two types of structures. VDV-1 and DWV have long 5′ NTRs with complex structures that resemble those of enteroviruses (*Picornaviridae*), particularly that of *Poliovirus*. *Perina nuda picorna-like virus* (PnPV) and *Ectropis obliqua picorna-like virus* (EoPV) have shorter 5′ NTRs with simpler structures that are unique and do not resemble any 5′ NTR structures among picornaviruses. The translation of the ORF of picorna(-like) viruses in general is initiated by the recognition of one or two internal ribosome entry sites (IRES). The cap-dependent mechanism of translation initiation employed by most cellular mRNAs is not used by these viruses. The IRES activity in the 5′ NTRs of iflaviruses had not yet been determined experimentally. Therefore, IRES activity in the 5′ NTR of VDV-1 was examined. This research was also aimed at identifying a permissive cell line with the ability to support VDV-1 IRES function, which is a prerequisite for the translation of the polyprotein ORF, and which might potentially support viral replication. The activity of the IRES of VDV-1 was investigated by cloning the 5′ NTR between two reporter genes: enhanced green fluorescent protein (EGFP) and firefly luciferase (Fluc). EGFP was cloned directly downstream of the OpIE2 promoter, which is activated by cellular factors, and is translated via a cap-dependent mechanism. The translation of Fluc was dependent on IRES activity in the 5′ NTR of VDV-1 in the respective cell lines. The presence of the 5' NTR of VDV-1 greatly improved the expression levels of the second reporter gene (Fluc) in *Lymantria dispar* Ld652Y cells, showing that the 5' NTR of VDV-1 contains a functional IRES element. This IRES element was active in a host specific manner since it showed much lower activity in *Spodoptera frugiperda* Sf21 cells and no activity in *Drosophila melanogaster* S2 cells.

The transmission of VDV-1 between the mite *V. destructor* and the honey bee *A. mellifera* was surveyed in comparison with DWV (Chapter 7). To determine the spread of these viruses in mites (adults, nymphs and eggs) and bees (eggs, larvae, pupae and adults), the antiserum raised against purified virus was used in ELISA analyses. Infections by VDV-1 or DWV could not be distinguished using this immunology technique due to the high similarity (97%) in the immunodominant protein VP1. The proportion of *Varroa* mites (71%) infected with VDV-1 and/or DWV at the Wageningen University apiary was slightly higher than that of bees (65%). Vertical transmission to the next generation was established in the mite population since virus was detected in mite eggs using a dot-blot immunoassay. Virus could not be detected in bee eggs, implying that no vertical transmission occurred in this species. Subsequently, nested RT-PCR was used to distinguish VDV-1 from DWV and with this technique both viruses could be detected even in the same individual. Eighty eight percent of the mites had VDV-1, 19% of the mites were co-infected with VDV-1 and DWV, and no mites with only DWV infection were detected. Seventy nine percent of the adult bees had VDV-1, 26% of the adult bees were co-infected with VDV-1 and DWV and there was no DWV only infection in the adult bees either, in the specimens tested. The conclusion from this experiment is that VDV-1 and DWV are able to co-exist in an individual mite or bee and can replicate in both organisms. A limited survey of these viruses among mites from different regions of mainland Europe indicated that the two viruses exist together in hives across this part of the world.

The research described in this thesis compared two closely related viruses able to infect both the mite and bee. One of these viruses (VDV-1) is new. In this study the pathogenicity of VDV-1 in mites and bees was not analysed in detail, but so far clear symptoms have not been found in the mite or the bee that could be attributed to VDV-1 infection. In these

studies, DWV also did not result in clear symptoms in mites and bees. Due to the results obtained, VDV-1 is not a prime candidate for biological control of the *V. destructor* mite.

Samenvatting

De mijt *Varroa destructor* is een ectoparasiet van de honingbij, *Apis mellifera*. De soortnaam *V. destructor* is onlangs ingevoerd om deze mijt te onderscheiden van *Varroa jacobsoni*, die parasiteert op de Aziatische bij *Apis cerana*. *Varroa*-mijten voeden zich uitsluitend met hemolymf van bijen en zijn betrokken bij de verspreiding van een aantal virussen. Sinds hun ontdekking op het Indonesische eiland Java in 1904 worden *Varroa*-mijten nu bijna overal in de wereld gevonden, behalve in Australië en in gebieden in Centraal Afrika. *V. destructor* vormt een ernstige bedreiging voor *A. mellifera*. De mijt werd geïntroduceerd in *A. mellifera*-kolonies in nieuwe gebieden via bijentransport door imkers en door uitzwermende bijen. De overlevingskansen van bijenkolonies, die door deze mijt worden geparasiteerd, nemen drastisch af wanneer er geen maatregelen genomen worden, omdat de bijen bij een grote dichtheid van deze mijt ernstig verzwakt worden. Honingbijen spelen een belangrijke rol bij de bestuiving van planten, zowel in de natuur als in de land- en tuinbouw. Imkers verdienen ook aan de verkoop van honing, bijenwas en propolis. Op dit moment zijn er geen afdoende methoden beschikbaar om de mijt te bestrijden. De chemische middelen die op dit moment worden gebruikt leiden tot verontreiniging van honing en andere bijenproducten, en de mijten hebben inmiddels resistentie ontwikkeld tegen een aantal van deze bestrijdingsmiddelen. De economische gevolgen van de aantasting van bijen door mijten maken het noodzakelijk om naar alternatieve bestrijdingsmethodes te zoeken.

Bij het begin van het onderzoek, beschreven in dit proefschrift, werden in mijten picornavirus-achtige virusdeeltjes gevonden met een diameter van 27 nm. De identiteit van het virus was onbekend en het was ook niet duidelijk of dit een virus was dat alleen mijten infecteert of ook bijen. Het doel van de studie was om dit virus te isoleren, het grondig te karakteriseren en om het gedrag van dit virus in mijten- en bijenpopulaties te onderzoeken. Dit alles geschiedde om te bepalen of dit virus zou kunnen worden ontwikkeld tot biologisch bestrijdingsmiddel van de *Varroa* mijt.

Op elektronenmicroscopische foto's waren in het cytoplasma van mijtenweefsel parakristallijnen aggregaten van virusdeeltjes te zien. Virusdeeltjes werden geïsoleerd uit mijten die verzameld waren in de bijenkasten van Wageningen Universiteit en vervolgens gebruikt om antiserum op te wekken in konijnen. Het antiserum werd gebruikt voor een immunohistochemische detectie van het virus in weefsel van mijten en dit liet zien dat het virus overvloedig aanwezig was in cellen van het abdominale deel van het spijsverteringskanaal, de gastric caccae, maar niet in speekselklieren (Hoofdstuk 2). Hetgeen aangeeft dat de speekselklieren waarschijnlijk geen rol spelen in virusoverdracht van dit virus,

In de volgende fase van het onderzoek werd uit deze virusdeeltjes RNA geïsoleerd en werd de basenvolgorde van het virale genoom bepaald (Hoofdstuk 3). Het virus heeft een enkelstrengs RNA genoom met een positieve polariteit en een polyA staart aan het 3'einde,

zodat het direct als boodschapper RNA kan fungeren. Het genoom heeft een lengte van 10.112 nucleotiden (zonder de poly-A staart) en bezit één groot 'open reading frame' (ORF) dat codeert voor een polyproteïne van 2893 aminozuren. De structurele eiwitten bevinden zich in het N-terminale en de niet-structurele eiwitten in het C-terminale deel van het polyproteïne en worden door autoproteolytische activiteit geproduceerd. Het ORF wordt aan beide zijden geflankeerd door RNA-segmenten die niet vertaald worden in eiwit, de zogenaamde 'niet-vertaalde regio's (NTRs). Uit fylogenetische analyse van het RNA-afhankelijke RNA polymerase (RdRp) van dit virus bleek dat het nauw verwant was aan de RdRps van virussen van het genus *Iflavirus*. Ook de genoomorganisatie is gelijk aan die van iflavirussen. Omdat de gevonden sequentie nog niet eerder gerapporteerd was, werd het virus *V. destructor virus* 1 (VDV-1) genoemd, naar de mijt waaruit het geïsoleerd was. VDV-1 is nauw verwant aan *Deformed wing virus* (DWV), dat geïsoleerd is uit honingbijen en morfologische afwijkingen aan de vleugels veroorzaakt. De genoomsequenties van VDV-1 en DWV zijn voor 84% identiek, de polyproteïnes voor 95%.

Om te bevestigen dat VDV-1 in staat is te repliceren in de mijt werden oligonucleotiden ('primers') ontworpen om specifiek de negatieve streng van het RNA aan te tonen. Deze negatieve streng komt alleen voor als replicatie-intermediair en is derhalve bewijs voor virusvermenigvuldiging. Voor dit experiment werden twee primerparen gebruikt, opdat onderscheid gemaakt kon worden tussen VDV-1 en DWV. Met deze primers werd eerst cDNA gemaakt, dat vervolgens werd vermeerderd met behulp van PCR. Op vergelijkbare wijze werden primers specifiek voor de 'positieve' streng gebruikt om het genomisch RNA van VDV-1 en DWV te detecteren in de mijt. Deze experimenten lieten zien dat zowel VDV-1 als DWV repliceert in de mijten. (Hoofdstuk 4). Deze resultaten komen overeen met de eerdere elektronenmicroscopische waarneming van de grote accumulatie van virusdeeltjes in parakristallijnen aggregaten, hetgeen al een sterke aanwijzing was voor replicatie van het virus in het cytoplasma van mijtencellen (Hoofdstuk 2).

In hoofdstuk 5 werden de structurele eiwitten van het geïsoleerde virus bestudeerd met behulp van SDS-PAGE en dit gaf aan dat de twee grootste virale eiwitten (VP1 en VP2) in relatief gelijke hoeveelheden aanwezig waren in het virusdeeltje. De N-terminale aminozuurvolgorde van VP1 en VP2 werd bepaald om vast te stellen waar het polyproteïne geknipt wordt. VP2 bevindt zich in het polyproteïne meteen N-terminaal van het helicase, het eerste niet-structurele viruseiwit. In het polyproteïne ligt VP1 direct voor VP2. In Western blot analyses bleek het antiserum, opgewekt tegen het gezuiverde virus, alleen heel sterk met VP1 te reageren. In een vervolgexperiment werden de structurele eiwitten één voor één tot expressie gebracht in *Escherichia coli*, als fusie met glutathion S-transferase. De fusie-eiwitten werden vervolgens getest in een Western blot analyse met antiserum tegen het gezuiverde virus en dit bevestigde dat VP1 het enige structurele eiwit is, dat door dit antiserum herkend wordt. Dit suggereert dat VP1 het oppervlak van het virusdeeltje zodanig bedekt dat de andere structurele eiwitten worden afgeschermd, of dat de andere structurele eiwitten niet-immunogeen zijn. Het virale 3C-achtige protease werd ook als een fusie-eiwit tot expressie gebracht en hiertegen werden antilichamen opgewekt. Met deze antilichamen kon het protease efficiënt worden gedetecteerd in een controle-experiment.

Dit antiserum kan in de toekomst toegepast worden om replicerend VDV-1 virus aan te tonen met bijvoorbeeld ELISA, in aanvulling op de ontwikkelde RT-PCR techniek (Hoofdstuk 4), of om replicatie van het virus in de mijt te lokaliseren met behulp van immunohistologische technieken.

Tot dusver is over de structuur en de functie van de 5′ niet-vertaalde regio's (5′ NTR) van het RNA van iflavirussen nog niets bekend. In Hoofdstuk 6 van dit onderzoek wordt de geconserveerde secundaire structuren voorspeld in de 5′ NTR van het RNA van vier iflavirussen. Hierbij werden twee verschillende 5′ NTR types gevonden: VDV-1 en DWV hebben lange 5′ NTRs met complexe secundaire structuren, die lijken op die van enterovirussen (*Picornaviridae*), met name op die van poliovirus. *Perina nuda picorna-like virus* (PnPV) en *Ectropis obliqua picorna-like virus* (EoPV) hebben kortere 5′ NTRs met eenvoudigere structuren. Dergelijke 5'NTRs zijn niet gevonden in picornavirussen. Picorna-achtige virussen maken geen gebruik van het 'cap'-afhankelijke translatie mechanisme, dat gebruikt wordt om de meeste cellulaire mRNAs te vertalen. Bij deze virussen start de vertaling van het ORF met de herkenning van een 'internal ribosome entry sites' (IRES). VDV-1 is het eerst iflavirus waarvan de IRES activiteit van de 5'NTR ook experimenteel werd onderzocht. Dit onderzoek was ook bedoeld om een cellijn te identificeren, die de functie van de VDV-1 IRES kon ondersteunen, hetgeen een absolute voorwaarde is voor de translatie van het ORF, en die mogelijk virale replicatie zou toelaten. De activiteit van de VDV-1 IRES werd bestudeerd met behulp van een bicistronisch construct waarbij de 5′ NTR gekloneerd werd tussen twee 'reporter' genen: groen fluorescerend proteïne (EGFP) en luciferase (Fluc). EGFP werd direct achter een OpIE2 promoter geplaatst, en wordt via het cap-afhankelijk translatie-mechanisme in de cel vertaald in eiwit. In dit experiment, is de translatie van Fluc, afhankelijk van IRES activiteit in de 5' NTR van VDV-1. De aanwezigheid van de VDV-1 5'NTR verhoogde het expressieniveau van Fluc aanzienlijk en dit betekent dat de 5' NTR van VDV-1 een functioneel IRES element bevat. De activiteit van deze IRES werd gevonden in *Lymantria dispar* Ld652Y cellen, en is gastheer-specifiek omdat de activiteit veel lager was in *Spodoptera frugiperda* Sf21 cellen, en omdat de IRES inactief was in *Drosophila melanogaster* S2 cellen.

De overdracht van VDV-1 tussen de mijt *V. destructor* en de honingbij *A. mellifera* in vergelijking met DWV werd bestudeerd in Hoofdstuk 7. Om de verspreiding van deze virussen in mijten (eieren, nymfen en adulten) en bijen (eieren, larven, poppen en adulten) aan te tonen werd het antiserum tegen gezuiverd virus gebruikt in ELISA analyses. Infecties van VDV-1 en DWV konden niet uit elkaar gehouden worden met deze immunologische techniek vanwege de grote overeenkomst in het immunodominante structurele eiwit VP1 (97% identiteit). In de bijenkasten van de Wageningen Universiteit was het percentage van de *Varroa* mijten (71%) met VDV-1 en/of DWV iets hoger dan dat van de bijen (65%). Verticale overdracht van het virus naar de volgende generatie mijten werd aangetoond met een immunologische 'dot blot' methode. Het virus werd niet gevonden in bijeneieren, wat betekent dat transovariale overdracht van VDV-1 en DWV in bijen niet plaats lijkt te vinden. Via een 'nested RT-PCR' methode werd onderscheid gemaakt tussen VDV-1 en DWV en met deze techniek konden beide virussen in mijten en bijen worden aangetoond, zelfs in één individu. Achtentachtig procent van de mijten bevatte VDV-1, 19% had een co-infectie met DWV ondergaan. Mijten met alleen DWV werden niet gevonden. Van de volwassen bijen had 79% VDV-1, 26% had een co-infectie met DWV, en in de geteste monsters werden ook geen bijen gevonden met alleen een DWV-infectie. De conclusie van dit hoofdstuk is dat VDV-1 en DWV samen kunnen voorkomen in een individuele mijt of bij en dat beide zich zowel in bijen als in mijten kunnen vermeerderen. Bij een beperkt onderzoek aan mijten in bijenkolonies uit verschillende delen van het Europese vaste land bleek dat beide virussen naast elkaar voorkomen.

Het onderzoek, beschreven in dit proefschrift, vergelijkt twee nauwverwante virussen die zowel mijten als bijen infecteren. Een ervan (VDV-1), is nieuw. In deze studie is het ziektebeeld dat VDV-1 veroorzaakt in mijten en bijen niet in detail onderzocht, maar tot dusverre zijn noch in mijten noch in bijen duidelijke symptomen waargenomen, die toegeschreven konden worden aan een VDV-1 infectie. Ook bij een DWV infectie werden in deze studie geen duidelijke symptomen waargenomen (bij bijen noch mijten). Gezien de gevonden resultaten lijkt VDV-1 geen primaire kandidaat te zijn voor de biologische bestrijding van de *V. destructor* mijt.

Curriculum vitae

Juliette Rose Ongus was born on 19th January 1974 in Mombasa, Kenya. In December 1991 she graduated from Moi Girls' High school in Eldoret, Kenya with a Kenya Secondary Certificate of Education (KSCE). In December 1997, she obtained a Bachelor of Science degree in Food Science and Technology from The University of Nairobi, Kenya, and graduated with a first class honours. From May 1998 to June 1999 she worked as a management trainee in UNGA Ltd. (Eldoret, Kenya).

In July 1999 she was awarded a scholarship administered by The Netherlands organisation for co-operation in higher education (NUFFIC), and in January 2001 she graduated with a Master of Science degree in Biotechnology from Wageningen University, The Netherlands. From March 2002 to April 2006 she was involved in a 4-year PhD research program at the Laboratory of Virology of Wageningen University in co-operation with CNSR, Orleans, France.

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PE&RC PhD Education Statement Form

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 22 credits (= 32 ECTS = 22 weeks of activities)

Review of Literature (4 credits)
- Picorna-like viruses of invertebrates (2002)

Writing of Project Proposal (5 credits)
- Characterisation of a Picorna-like virus isolated from the *Varroa destructor* mite for its possible use as a biological control agent against the mite (2002)

Post-Graduate Courses (1 credit)
- Molecular phylogenies: the reconstruction and interpretation (2003)

Deficiency, Refresh, Brush-up and General Courses (1.4 credits)
- Teaching and supervising thesis students (2005)
- Career Perspectives (2005)

PhD Discussion Groups (5 credits)
- In the tracks of evolution (2004/2005)
- Weekly work discussion meetings and literature survey meetings attended by PhD students, undergraduate students and staff of the Laboratory of Virology (2002-2005)

PE&RC Annual Meetings, Seminars and Introduction Days (0.5 credits)
- PE&RC annual meeting: "Biological disasters"(2004)
- PE&RC annual meeting: "The truth of science"(2005)

International Symposia, Workshops and Conferences (6 credits)
- 34th Annual Meeting of the Society for Invertebrate Pathology, The Netherlands (2001)
- 12th International Congress of Virology, France (2002)
- 37th Annual Meeting of the Society for Invertebrate Pathology, Finland (2004)

Laboratory Training and Working Visits (1 credit)
- RNA folding. Leiden University, The Netherlands (2005)

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