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COMPARISON OF POSSIBLE RECOMBINATION BETWEEN DEFORMED WING VIRUS GENOTYPE A (DWV-A) AND GENOTYPE B (DWV-B) IN THE HONEY BEE *APIS MELLIFERA* AND THE BUMBLE BEE *BOMBUS TERRESTRIS*

Master of Science Thesis

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Melissa Şafak ÇELEBİ'nin hazırladığı **"COMPARISON OF POSSIBLE RECOMBINATION BETWEEN DEFORMED WING VIRUS (DWV-A) AND ITS GENOTYPE (DWV-B) IN THE SPECIES OF THE HONEY BEE APIS MELLIFERA AND THE WILD BEE BOMBUS TERRESTRIS"** adlı bu çalışma aşağıdaki jüri tarafından **MOLEKÜLER BİYOLOJİ VE GENETİK ANA BİLİM DALI**'nda **YÜKSEK LİSANS TEZİ** olarak kabul edilmiştir.

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Melissa Şafak ÇELEBİ

ABSTRACT

COMPARISON OF POSSIBLE RECOMBINATION BETWEEN DEFORMED WING VIRUS GENOTYPE A (DWV-A) AND GENOTYPE B (DWV-B) IN THE HONEY BEE *APIS MELLIFERA* AND THE BUMBLE BEE *BOMBUS TERRESTRIS*

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Honey bees are very important in terms of the economy and our nutritional requirements. In addition to honey bees, many wild bees (e.g. *Bombus* spp.) are also important for pollination and their decreasing numbers are another cause of concern where it occurs across the world. One of the most important emerging infectious diseases (EIDs), not only in *Apis mellifera* but also in *Bombus terrestris*, is *deformed wing virus* (DWV), transmitted by the ectoparasitic mite *Varroa destructor*. DWV is currently found as two prevalent genotypes: DWV genotype A (DWV-A) and DWV genotype B (DWV-B), also known as *Varroa destructor virus-1*. Recombinants between DWV-A and DWV-B have also been detected in field-collected bees. *Apis mellifera* is a very important commercial pollinator so there are many studies on the effects of DWV on it. Indeed, DWV and its parasitic mite vector, *Varroa destructor*, are believed to be important causes for honey bee loss and colony failure. However, although other bee species (e.g. *Bombus* spp.) play a significant role in pollination, research on the impact of viruses on them, including DWV, is limited. When two genotypes of a virus co-infect a host, the possibility for recombination arises, though the dynamics of recombination are unexplored. Here I investigated the extent of recombination in laboratory reared pupae of *Apis mellifera* and *Bombus terrestris*. Ten pupae of *B. terrestris* and 10 of *A. mellifera* were co-infected with DWV-A and DWV-B (10^5 genome equivalents per bee) and incubated for three days for the viruses to

replicate. Virus was extracted from these 3-day-old pupae then used to infect new pupae. This was repeated nine times, producing ten independent lines of virus passaged through naïve *B. terrestris* and *A. mellifera* pupae. RNA was extracted, DNA generated from it by cDNA synthesis, and the DNA used in qPCRs to quantify viral replication in all replicates. To determine if recombination had occurred, primers were used to amplify recombinant DWV-A/DWV-B across the VP3-helicase genes and results compared across the two host species. The IVA (Interactive Virus Assembly) program was also used to see which recombinants dominated in the genome. BLAST analysis allowed me to assign DWV (as DWV-A, DWV-B or DWV-AB-recombinant) sequences within the consensus sequences created by an IVA *de novo* assembly. These results support the idea that recombinants have a growth advantage over parental genotypes.

Keywords: Recombination, Deformed Wing Virus (DWV), RNA viruses, *Bombus terrestris, Apis mellifera*

ÖZET

BAL ARIS *APIS MELLIFERA* VE YABAN ARISI *BOMBUS TERRESTRİS* TÜRLERİNDE DEFORME KANAT VİRÜSÜ (DWV-A) VE GENOTİPİ (DWV-B) ARASINDAKİ OLASI REKOMBİNASYONUN KARŞILAŞTIRILMASI

Melissa Şafak Çelebi

Yüksek Lisans Tezi, Moleküler Biyoloji ve Genetik Ana Bilim Dalı

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Eş Danışman: Prof. Dr. Robert Paxton

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Bal arıları ekonomi ve beslenme gereksinimlerimiz açısından çok önemlidir. Bal arılarına ek olarak, birçok yaban arısı da (örneğin *Bombus* spp.) polinasyon için önemlidir ve azalan sayıları dünya genelindeki bir başka endişe nedendir. Sadece *Apis mellifera*'da değil *Bombus terrestrisi'de* de ortaya çıkan en önemli bulaşıcı hastalıklardan (EID) biri, ektoparazitik akar *Varroa destructor* tarafından bulaşan deforme kanat virüsüdür (DWV). DWV şu anda iki yaygın genotip olarak bulunur: DWV genotip A (DWV-A) ve DWV genotip B (DWV-B), ayrıca *Varroa destructor* virüs-1 olarak da bilinir. Saha çalışmalarından toplanan arılarda DWV-A ve DWV-B arasındaki rekombinantlar da tespit edilmiştir. *Apis mellifera* çok önemli bir ticari polinatördür, bu nedenle DWV'nin bunun üzerindeki etkileri üzerine birçok çalışma vardır. Nitekim, DWV ve parazitik akar vektörü *Varroa destructor*, bal arısı kaybı ve koloni yetmezliğinin önemli nedenleri olduğuna inanılmaktadır. Fakat, diğer arı türlerinde (örneğin *Bombus* spp.), polinasyonda önemli bir rol oynamasına rağmen, virüslerin etkileri -DWV de dahil olmak üzere- üzerine araştırmalar sınırlıdır. Bir virüsün iki genotipi bir konakçıyı birilikte enfekte ettiğinde, rekombinasyon dinamikleri keşfedilmemiş olsa da rekombinasyon olasılığı ortaya çıkar. Burada *Apis mellifera* ve *Bombus terrestris*'in laboratuvar destekli pupalarında rekombinasyonun derecesini araştırdım. On pupa *Bombus terrestris* ve 10 *Apis mellifera,* DWV-A ve DWV-B (arı

başına 10^5 genom eşdeğeri) ile aynı anda enfekte edildi ve virüslerin çoğalması için üç gün boyunca inkübe edildi. Bu üç günlük pupalardan virüs ekstrakt edildi ve ardından yeni pupaları enfekte etmek için kullanıldı. Bu, dokuz kez tekrarlandı ve saf B. terrestris ve A. mellifera pupa'dan geçen on bağımsız virüs hattı (passage) üretildi. RNA ekstrakte edildi, ondan cDNA sentezi ile üretilen DNA tüm kopyalarda viral replikasyonu ölçmek için qPCR'lerde kullanıldı. Rekombinasyonun meydana gelip gelmediğini belirlemek için, primerler, VP3-helikaz genleri boyunca rekombinant DWV-A / DWV-B'yi büyütmek için kullanıldı ve iki konak türünde karşılaştırılan sonuçlar IVA (Interactive Virus Assembly) programı ile analiz edildi. Ayrıca bu analiz genomda hangi rekombinantların egemen olduğunu görmek için kullanılmıştır. BLAST analizi, bir IVA *de novo* düzeneği tarafından oluşturulan konsensüs dizileri içerisinde DWV (DWV-A, DWV-B veya DWV-AB-rekombinant olarak) dizileri atamamı sağladı. Bu sonuçlar, rekombinantların DWV genotiplerine göre büyüme avantajı olduğu fikrini desteklemektedir.

Anahtar Kelimeler: Rekombinasyon, Deforme Kanat Virüsü (DWV), RNA virüsleri, *Bombus terrestris, Apis mellifera*

There is one thing I learned from what I lived

There is one thing I learned from what I lived:
When you live something, you must live it fully
Your lover must be exhausted from your kisses
You must be exhausted from smelling a flower

One can look at the sky for hours
One can look for hours at the sea, at a bird, at a child
Living on this world is being done with it
Growing unbreakable roots into it

When you hug your friend, you must do it with all your power
You must be in a fight with all your muscles, body and passion
And when you lie on the hot sand,
You must rest like a grain of sand, like a leaf, like a stone

One must listen to all the beautiful music
Such that the sounds, the melodies fill inside

One must dive headfirst into this life
Like diving from a rock into an emerald sea

People you don't know must attract you to distant lands
You must live with the desire to read all the books and to know all the lives
You must exchange nothing with the happiness of drinking a glass of water
But for all the happiness there is, you must be filled with the longing to live

And you must also live grief, with honor, with all your presence
Because grief also maturates one, like happiness
Your blood must be intermixed with the large circulation of life
The never ending, fresh blood of life must circulate in your veins

There is one thing I learned from what I lived:
When you live, you must live big, like being one with the rivers, the sky, and the whole universe
Because what we call lifetime is a gift presented to life
And life is a gift presented to you.

Ataol Behramoğlu,1991

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SYMBOLS AND ABBREVIATIONS LIST

µl : microliter
β-actin : beta-actin
A. mellifera : *Apis mellifera*
ABPV : Acute bee paralysis virus
B.C. : Before Christ
BEHR : Compert Biological System
BQCV : Black queen cell virus
B. terrestris : *Bombus terrestris*
bp : basepair
CBPV : Chronic bee paralysis virus
Ct : cycle threshold
DEPC : diethylpyrocarbonate
DNA : Deoxyribonucleic acid
dNTP : deoxynucleotide triphosphates
DWV or DWV-A : Deformed wing virus
EIDs : Emerging infectious diseases
F : Forward
HR : Homologous RNA recombination
IAPV : Israeli acute paralysis virus
IRES : internal ribosome entry site
Kb :kilobase
KBV : Kashmir bee virus
kDa : kiloDalton
K_2PO_4 : Dipotassium phosphate
L : line
LP : leader protein
m : meter
min : minute
mL : millilitres
mM : millimolar
ng : nanogram
NGS : next generation sequencing
IVA : Iterative Virus Assembler
nm : manometer
NS : no sample
nt : nucleotid
ORF : open reading frame
P : passage
PCR : Polymerase chain reaction
pmol : picomole
qRT-PCR : kantitatif Real-Time PCR
R : reverse
R&D : Research & Development
RdRp : RNA-dependent RNA polymerase
RLT buffer : RNeasy Lysis Buffer
RNA : Ribonucleic acid

SBV :Sacbrood virus
Sec : second
Spp : species
T_{A} : the annealing temperature
T_{M} : the melting temperature
VDV-1 or DWV-B : *Varroa destructor* virus-1
V. destructor : *Varroa destructor*
VP : Viral protein
UTR : untranslated region

1. INTRODUCTION

1. 1 The importance of bees in the world

1.1.1 Honey bee

Honey bees are important for the economy and ecology across the worldwide. Thanks to their high adaptability capabilities, they nowadays spread across the whole world except poles (Sheppard & Meixner, 2003); being responsible for economically important honey and beeswax production and playing a very important role in pollination. Honey bees can live in the narrow wood and rock cavities and nest on trees 30 m in height. Their adaptive forces are very high in that they can survive during the seven months of winter and on dry summer months (Koch, 2007). The honey bee is the most studied bee species because of a long-term relationship with human (Moritz, 2006). Honey bees collect nectar and pollen from flowers to meet their carbohydrate and protein requirements, respectively, making honey in the process. The possible reason of domestication of honey bees by the Ancient Egyptians (BC 2600) is the use of honey as a flavouring in Africa, Europe, and the Middle East. Bees were first domesticated by humans in the Ancient Greeks in the 6th century BC and this domestication passed to Medieval Europe. It has now also spread all over the world (Jones & Ransome, 2010; Figure 1).

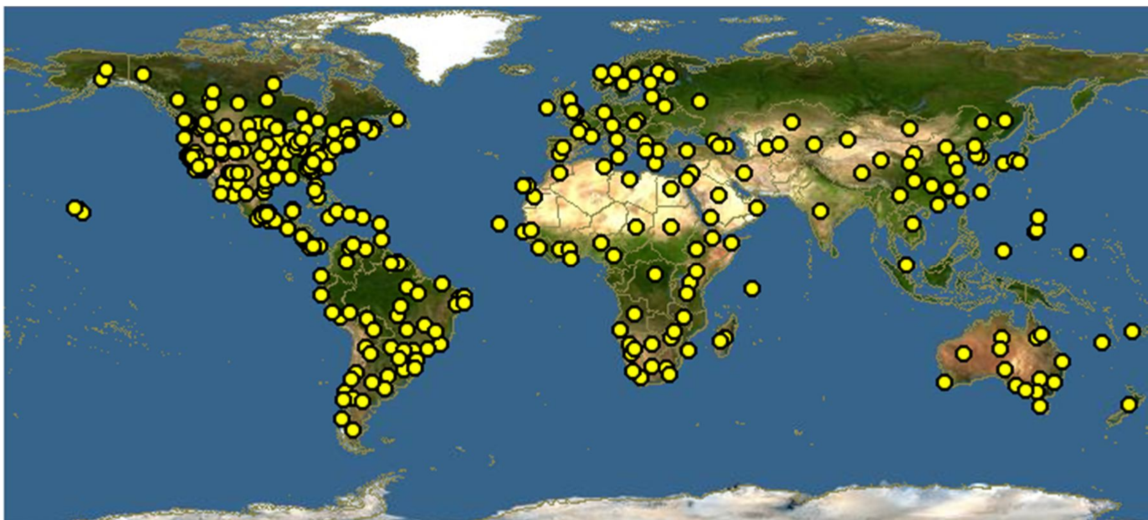


Figure 1. Honey bee (*Apis mellifera*) world species distribution map

1.1.1.1 Western Honey bee: *Apis mellifera*

It is known that there are 27 taxonomically valid honey bee subspecies described so far. Covering these subtypes are lineage O, the Northeast Mediterranean and Middle East subspecies; lineage A, African subspecies; lineage C, the Central and Eastern European subspecies; and lineage M; the West and Northwest European subspecies as four evolutionary lineages (Koch, 2007). The Western honey bee *Apis mellifera* Linnaeus, 1758 belongs to the superfamily Apoidea (currently 'Anthophila') of the order Hymenoptera (Table 1). *A. mellifera* is also an eusocial insect in the subfamily of *Apinae* (Apidae) (Sydney et al., 2002).

A typical *A. mellifera* colony has workers, the queen and drones (Figure 2). *A. mellifera* is accepted as the most important of the economically defined nine members of the genus of *Apis* (Koch, 2007). This species is vital not only for honey production but also as a pollinator. It is considered as the most important commercial pollinator of more than 90% of plant species (Hung et al., 2018; Klein et al., 2007). Pollination services for the production of global agricultural products, in other words, the pollination economic value of bees (IPEV; Table 2), is estimated as € 153 billion worldwide, and € 20 billion for Europe (Gallai et al., 2009). Much of this benefit may be attributed to honey bees. Studies in recent years of different bee species (such as bumble bees) have shown that some are better pollinators than others (Cussans et al., 2010); bee species other than honey bees may therefore also contribute significantly to pollination. In addition to being able to play a role with other pollinators in the pollination of cultivated and wild plants (Breeze et al., 2011; Morse & Fritz, 1983), honey bees are also very important from the ecological point of view because they contribute to biodiversity and the pollination of wild plants (Potts et al., 2010). Therefore, raising healthy honey bees is of great economic importance worldwide.

Table 1. Classification of the genus of *Apis* at the species level

Phylum	Animalia
Branch	Arthropoda
Class	Insecta
Order	Hymenoptera
Family	Apidae
Genus	*Apis*
Species	*A. florea* *A. dorsata* *A. cerena* *A. mellifera* *A. nuluensis* *A. laboriosa* *A. koshevnikovi* *A. nicrocincta* *A. andreniformis*

Table 2. Geographical distribution of the economic value of insect pollination and crop vulnerability[a]. Insect pollination economic value (IPEV) is the proportional contribution of biotic pollination to production multiplied by the total economic value (EV) of the 100 most important commodity crops, summed for all crops in a region. The ratio of IPEV to the EV indicates the economic vulnerability of crops to pollinator loss (Potts et al., 2010)

Geographical region (following FAO)	Insect Pollination Economic Value (IPEV) in 10^9 €	Vulnerability of region (IPEV/EV)[b]
Africa	11.9	8
Central Africa	0.7	7
East Africa	0.9	5
North Africa	4.2	11
South Africa	1.1	6
West Africa	5.0	10
Asia	89.4	10
Central Asia	1.7	14
East Asia	51.5	12
Middle East Asia	9.3	15
Oceania	1.3	7
South Asia	14.0	6
South East Asia	11.6	7
Europe	22.0	11
European Union (25members)	14.2	10
Non EU	7.8	12
North America (Bermuda, Canada and USA)	14.4	11
South and Central America	15.1	6
Central America and Caribbean	3.5	7
South America	11.6	6

a) Based on data from Ref. (Gallai, et al., 2009).

b) Mean vulnerability for region calculated as unweighted mean of vulnerability of sub-regions, where EV = the total economic value of the 100 most important commodity crops

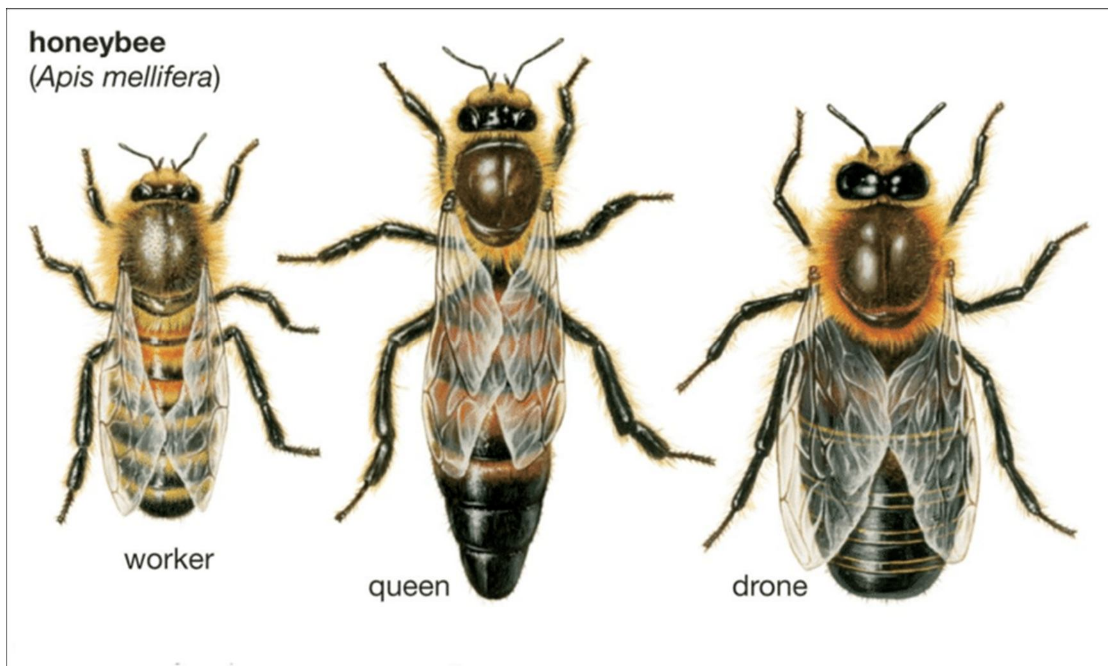


Figure 2. Western honey bee *A. mellifera* worker, queen and drone (by Ketan Bhokray)

1.1.2 Bumble bees

Bumble bees are one of the social bees in the Apoidea superfamily of the order of Hymenoptera, with approximately 250 identified species (Breed, 2014; Cameron et al., 2007). Bumble bees are larger than honey bees in size and have eye-catching colours (Figure 3). They have also an important pollinator function for many plants due to their dense hair and natural vibration movements surrounding their bodies. They may adapt well to different climates, altitudes and habitat conditions: from the Arctic Circle to South America; from sea level to an altitude of 5800 m (Williams & Williams, 1998).

Bumble bees play an important role in pollination by collecting pollen from agricultural crops, meadow-pasture fodder crops, fruit trees, vegetables, medicinal and aromatic plants (Goodwin & Steiner, 1997; Benton, 2000). For this reason, these bees have attracted the attention of scientists since about 100 years ago and studies to produce them commercially have recently met with success. The mass production of bumble bees was achieved by the R & D work of several commercial companies in the Netherlands and Belgium in 1987. As it is understood later, the mass production was accelerated because bumble bees were a good pollinator for greenhouse crops (Velthuis & van Doorn, 2006). Due to their benefits in the aspect of economy and health, the

demand for bumble bee use is increasing in the world and in our country. Today, more than 1 million bumble bee colonies are produced commercially per year in more than 60 countries, where they are used for pollination (Velthuis & van Doorn, 2006). Five species of bumble bees are commercially reared: *B. terrestris, B. lucorum, B. ignitus, B. occidentalis,* and *B. impatiens*.

Figure 3. Commercially cultivated *Bombus* species

1.1.2.1 *Bombus terrestris*

B. terrestris L. is a member of the subfamily of *Apinae* (Hymenoptera: Apidae) (Table 3). The natural life cycle of this species is different from the honey bee. However, like a honey bee colony, individuals of this species live in a colony with a queen bee, drones, and worker bees. The queen is a monandrous. They are social insects because they have a division of labour among themselves (Beekman et al., 1998). In natural habitats, the young queen mates and leaves the colony to find a place of shelter under the soil, which is called diapause (Alford,1975), providing her some ecological flexibility (Dafni, 2015; Estoup et al., 1996). The life cycle of *B. terrestris* is thereby adapted to environmental

factors, and diapause occurs during unfavourable environmental conditions such as low winter temperature, high summer temperature, and periods of drought when bumblebees cannot be obtained necessary nutrients (Beekman et al., 1998; NøRgaard Holm, 1972; Horber,1961).

Table 3. Classification of *Bombus* genus at the species level

Phylum	Animalia
Branch	Arthropoda
Class	Insecta
Team	Hymenoptera
Family	*Apidae*
Genus	*Bombus*
Species	*B. terrestris*

B. terrestris is commonly distributed in Europe and generally found in temperate climates. There are populations in the Near East, Mediterranean Islands, and North Africa because they can live in a wide variety of habitats. *B. terrestris* is reported from many countries and areas where it is not native such as Japan, Chile, Argentina, New Zealand and Tasmania at certain times of the year (Donovan & Wier, 1984; Estoup et al., 1996;Inoue et al., 2008; Montalv et al., 2010; Rasmont et al., 2015; Pierre et al., 2008; Semmens et al., 1993).

1.2 The effect of emergent infectious diseases on pollination

The species *A. mellifera* is important for ecosystems as a pollinator yet has been reported to face serious losses worldwide during the last 20 years (Carreck et al., 2010; Potts et al., 2010; Smith et al., 2013; vanEngelsdorp et al., 2009). The human-induced causes of the decline in pollinators such as honey bees include habitat fragmentation, habitat change, widespread intensification of agriculture, overuse of insecticides, invasive alien species, increased pathogen prevalence and climate change. Insecticides (Figure 2), which are used to obtain more productive crops due to insufficient agricultural area, cause the destruction of many wild insect species. Transfer of agents

causing infectious diseases, e.g. from domesticated livestock to wildlife populations, is termed spill-over whilst a pathogen that increases in prevalence is termed an emerging infectious disease (EID) (Anderson & May, 1979; Cunningham, 1996; Hudson & Greenman, 1998; Tompkins & Begon, 1999; Viggers et al., 1993; Woodford, 1993; Woodroffe, 1999). Managed honey bee populations exhibit elevated mortality, with EIDs implicated as a cause (Daszak et al., 2000; Woolhouse & Gowtage-Sequeria, 2005). Biology of bees, lifestyles, reproductive characteristics and migratory beekeeping programs lead to diseases rapidly spreading among them (Akyol & Korkmaz, 2006). The increase in the impact of diseases on the honey bee also poses a potential threat for many other pollinator species through pathogen spill-over (Fürst et al., 2014a). Furthermore, since wild bees (such as *Bombus* spp.) are also important pollinators, transfer of EIDs from honey bees to wild bee populations has recently been recognised as a serious concern for pollination (McMahon et al., 2015). Colonies with higher worker losses are more prone to overwinter colony losses (Natsopoulou et al., 2017). Recent studies have indicated an interrelation between the prevalence of infectious diseases and colony losses (Cox-Foster et al., 2007; Johnson et al., 2009; Paxton, 2010).

1.2.1 Spread of bee diseases

The bees exhibit coexistence in the colony and any infection transmitted to the larva is therefore easy to spread to all individuals of the colony. Trophallaxis, known as the social diet of bees, allows the individuals in the colony to share the food, which can be delivered orally. Vaccination and seasonal migration are important factors to reduce the intensity and duration of infection. However, when the colonies are unconsciously stressed (inadequate nutrition, insecticide exposure, environmental problems, etc.), they may lose their effective defence ability against infection.

One of the important factors affecting the loss of *A. mellifera* colonies is parasites that are combined with many RNA viruses to infect colonies (Carreck et al., 2010; Cox-Foster et al., 2007; Genersch & Aubert, 2010; Highfield et al., 2009; Johnson et al., 2009; Martin, 2001). These parasites have a compulsory parasitic life in the colony. Recently, there has been a remarkable increase in the geographical distribution of parasitic mites harbouring viruses and, indeed, this interaction mediates the rapid spread of these infectious viral agents (Tsevegmid et al., 2016). Transmission of these viruses

can be horizontal (between the individuals of the same generation) or vertical (between generations) (Chen et al., 2006). Horizontal transfer can take place in many different ways such as through near physical contact, trophallaxis, contaminated food consumption, or oral-faecal transmission. Horizontally transmitted pathogens tend to exhibit high virulence. Vertical transmission is defined as the transmission of viruses to sperm or eggs.

1.2.2 The honey bee ectoparasite: *Varroa destructor*

One of the most common transmission paths of viruses in honey bees is that through a parasitic vector such as *Tropilaelap*s or *Varroa*. *Varroa spp*., ectoparasitic mites, are considered to be the most important parasites of honey bee colonies (Bailey & Ball, 1991; Bailey & Ball, 1991; Ball & Allen, 1988). *Varroa's* proliferation success in honey bees is a direct consequence of the high fit between the life cycle of the mite and the host's life cycle (Chantawannakul & Ramsey, 2018; Figure 4). *Varroa destructor* is thought to cause weakening of the host's immune system by disrupting the hemolymph of the pupa. In addition, RNA viruses transmitted by the mite through feeding on a host can lead to serious damage to the host. As a result, the common occurence of *Varroa* mite in honeybees plays a significant role in honey bee losses across the worldwide.

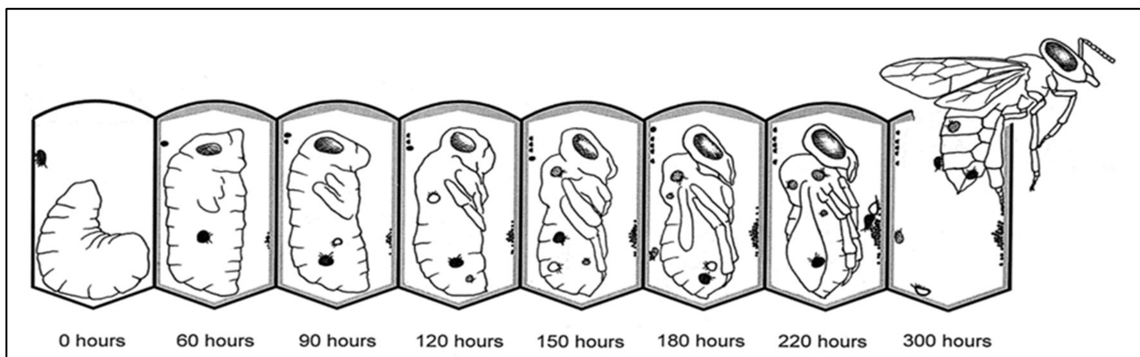


Figure 4. Life cycle of *Varroa destructor* in *A. mellifera* worker bee pupae. *Varroa* are found in host brood cells, where developing pre-pupae and pupae are found, and they are in sync with developmental stages of their host pupae. At 220 hours, the first female and male parasites mate. Thus, the spread of the parasite within the colony is inevitable (visualized by Jenny Bower) (Oldroyd, 1999).

Molecular-based researches on *Varroa jacobsoni* and *V. destructor* populations indicated that they originated from the Asian honey bee *Apis cerena* (Anderson & Trueman, 2000). Today, *V. destructor* infects both *A. cerena* and *A. mellifera* and has been reported to have spread rapidly throughout the world, except Australia and some small islands (Allen et al., 2015; Anderson & Trueman, 2000;Bailey & Ball, 1991; Ball & Allen, 1988). This parasite cannot survive alone, therefore, it spends its entire life in dark bee colonies (Boot et al., 1993). In contrast to *V. jacobsoni*, the main reason for the increase in *V. destructor* populations in colonies of *A. mellifera* is that they can reproduce in both worker and male brood cells (Anderson, 1984; Anderson & Fuchs, 1998; Chantawannakul & Ramsey, 2018). However, since *V. destructor* is a new parasite for *A. mellifera*, a stable host-to-parasite relationship cannot be observed. As a result, the rapid spread of ectoparasite *V. destructor* mites on a global scale has caused the death of millions of *A. mellifera* colonies (Martin, 2001).

Figure 5. Dorsal view of *Varroa destructor* Anderson & Trueman. Photograph by Scott Bauer, USDA.

1.3 RNA viruses of bee

Hitherto, 26 viruses that may associate with- or infect *V. destructor* have also been isolated from *A. mellifera* (Allen & Ball, 1996; Chen & Siede, 2007; Chen et al., 2004; Locke et al., 2012; Martin, 2001). These viruses include Israeli acute paralysis virus (IAPV), Kashmere bee virus (KBV), Black queen cell virus (BQCV), Acute bee paralysis virus (ABPV), *Varroa destructor*-1 virus (VDV-1) and Deformed wing virus (DWV). They are transmitted vertical, horizontal and vector-mediated (Ball & Allen, 1988; Chen et al., 2004; de Miranda & Genersch, 2010; Tentcheva et al., 2004). Furthermore, some viruses (ABPV, BQCV, CBPV, KBV, SBV, DWV and IAPV) have been detected in stored pollen, honey and bee milk (Chen & Siede, 2007). The known honeybee viruses in the families Dicistroviridae (BQCV, KBV, ABPV, IAPV) and Iflaviridae (SBV, DWV and VDV-1) are RNA viruses belonging to the order of Picornavirales (Klee et al., 2007).

Israeli acute paralysis virus (IAPV); Israel acute bee paralysis virus was first described in 2004, and thought to cause significant losses in Israeli apiculture (Maori et al., 2007). In recent years, IAPV has also been associated with Colony Collapse Disorder (CCD) in the US (Cox-Foster et al., 2007), where it has been isolated from several regions such as Florida, California, Maryland, and Pennsylvania, as well as Australia (Chen et al., 2008; Cox-Foster et al., 2007; Maori et al., 2007).

Kashmir bee virus (KBV); Unlike other viruses, it has been reported that there is no (physical) sign of KBV on the host (de Miranda et al., 2013).

Black queen cell virus (BQCV); BQCV, first observed in the queen larval stages in Australia (Anderson, 1993), was later seen in France and Austrian apiaries (Tentcheva et al., 2004; Berényi et al., 2006).

Acute bee paralysis virus (ABPV); ABPV is a common infective agent of bees and it was frequently detected in apparently healthy colonies (Allen & Ball, 1996). This virus was formerly reported as the major contributor to the mortality of honeybees affected by *V. destructor* (Antúnez et al.,2005; Faucon et al., 1992; Nordström et al., 2007).

Varroa destructor virus-1(VDV-1); *V. destructor*-1 virus, is also known as DWV-B, has a wide distribution and is considered as the main variant of deformed wing virus (also

known as DWV-A) (Martin et al., 2012; Mordecai et al., 2016, Ongus et al., 2004; Mordecai et al., 2016).

Deformed wing virus (DWV); DWV was first isolated from the adult *Apis cerena* in Japan and China (Bailey & Ball, 1991). The emergence and distribution of DWV infection has been reported in Europe, North America, South America, Africa, Asia and the Middle East (Allen & Ball, 1996;Antúnez et al., 2012). It is one of the few bee viruses that causes well-defined disease symptoms in DWV-infected bees. Typical disease symptoms of DWV infection are shrunken and wrinkled wings, body size deterioration, and colour change. The same virus has been found to cause infection in various types of bumble bee species including *B. terrestris, B. pascuorum* and *B. huntii* (Genersch et al.,2006; Li et al., 2011; Peng et al., 2011).

1.3.1 Iflaviruses of bee

Iflaviruses are positive single-stranded RNA viruses with a viral capsid with a diameter of about 30 nm and icosahedral structure. The first discovered Iflavirus was SBV (Lee & Furgala, 1965). The most prominent characteristics of these viruses are their non-segmented structure and their positive polarity. Hence, they can be directly translated into protein (Hashimoto et al., 1984). The Iflavirus RNA genome is translated into a large polyprotein, made possible by means of a single open reading frame (ORF). Wu and his colleagues have shown that there is no subgenomic mRNA during translational processing (Wu et al., 2002). Polyprotein translation may initiate at an internal ribosome entry site (IRES) in the 5′ untranslated region (UTR: noncoding regions of viral or mRNAs, usually at both termini. UTRs regulate RNA replication, translation, and stability) of the RNA. The 3′ UTR, highly conserved, is followed by a poly(A) tail (Figure 6) (Isawa et al., 1998; Ongus et al., 2004; Belsham, 2009; Gromeier et al., 1999; Nakashima & Uchiumi, 2009; Roberts & Groppelli, 2009).

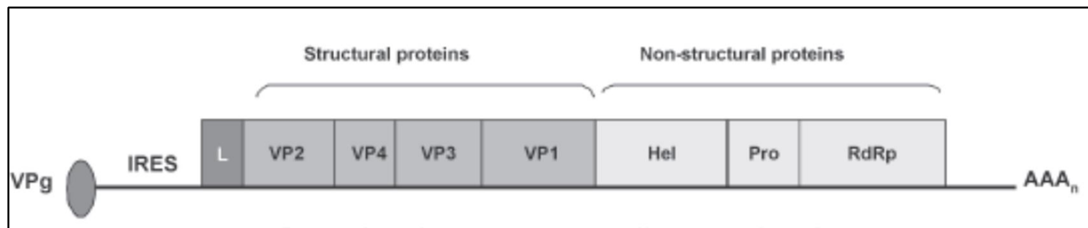


Figure 6. . Iflavirus genomes encode a single polyprotein: VP1-VP4 located in the N-terminal region as the structural proteins and helicase, protease and RNA-dependent RNA polymerase (RdRp: enzymatic complex that synthesizes (replicates) viral RNA) in the C-terminal region as the non-structural proteins. The N-terminal L polypeptide is variable in all iflavirus genomes. The presence of an IRES element in the 5′ UTR has been confirmed for several iflaviruses (Ribeiro et al., 2009).

Iflaviruses consists of a single-stranded undivided RNA genome with a single open reading frame (ORF). The ORF is transformed into processed polyprotein to form structural capsid proteins (occupying the N-terminal and include Vpg proteins) and non-structural proteins (occupying the C-terminal and play a crucial role for processing of polyprotein). The 5' end of the genome contains covalently linked VPg protein involved in RNA replication in the host cell cytoplasm. The VPg protein comprises coding regions for the VP2-VP4-VP3-VP1 regulated capsid proteins, respectively. VP1 is the largest, whereas VP4 is the smallest protein in this protein family (Fauquet, 2005). The VPg protein precedes the protein, commonly referred to as the "short leader protein (LP)", which separates from VP2 before capsid assembly. Non-structural proteins include three functionally important enzymes: RNA helicase, 3C-like cysteine protease and RNA-dependent RNA polymerase (RdRp).

The total genome of 10 Iflaviruses was sequenced in 2009, and their size varied between 9 and 10 kb (without counting the nucleotides in the poly(A) tail at the 3′ end). Their polyprotein sizes are between 2500-3000 amino acids. These viruses include DWV and *Varroa destructor* virus-1 ((Ribeiro et al., 2009); Table 4). In VDV-1, the molecular weights of VP1 and VP2 are estimated as 46 kDa and 32 kDa, respectively. Three bands at–25 kDa are also detected with an anti-capsid antibody (Ongus et al., 2004). The capsid proteins in the related DWV are 44, 32 and 28 kDa in size (Camazine et al., 2006).

1.3.2 The relation between DWV (also known as DWV-A) and *Varroa destructor* virus-1 (also known as DWV-B)

Ongus and his colleagues found virus particles, averaging 27 nm in diameter, from *Varroa* mite found in beehives, which was previously also isolated and observed (Kleespies et al., 2000). As a result of these studies, characterization and taxonomic position of the VDV-1 virus was determined by analysis of the RdRp gene (Ongus et al., 2004). Based on the percentage nucleotide and amino acid sequence analysis, it was observed in the same branch with KV, most familiar with DWV (Figure 7 and Table 4). Nucleic acid sequence similarity of DWV-A and VDV-1 were determined to be 95% at the amino acid level and 84% at the DNA level (Ongus et al., 2004). Therefore, VDV-1 is proposed to be named as DWV "main variant B" taking DWV-A as reference sequence (Mordecai et al., 2016). The most important difference between the two viruses is found at the 5' end of their genome. Furthermore, due to the few base differences in the nucleotides in the coding region, amino acid differences were observed in the encoded proteins, and the DWV-A / DWV-B recombinants were identified as dominant viral strains in *Varroa*-infected honey bees in the United Kingdom (Moore et al., 2011; Ryabov et al., 2014).

Table 4. Percentage of the nucleotide and amino acid sequence similarity based on the homology searches aligning corresponding region of DWV-B and those of other iflaviruses (Ongus et al., 2004)**.**

DWV-B sequence	Sequence identity (similarity) (%)			
	DWV	KV	SBV	IFV
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 nt of ORF*	79	79	40	29
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)	98 (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 of ORF*	90(97)	89(86)	16(32)	11(26)

*Including VP3 and VP4

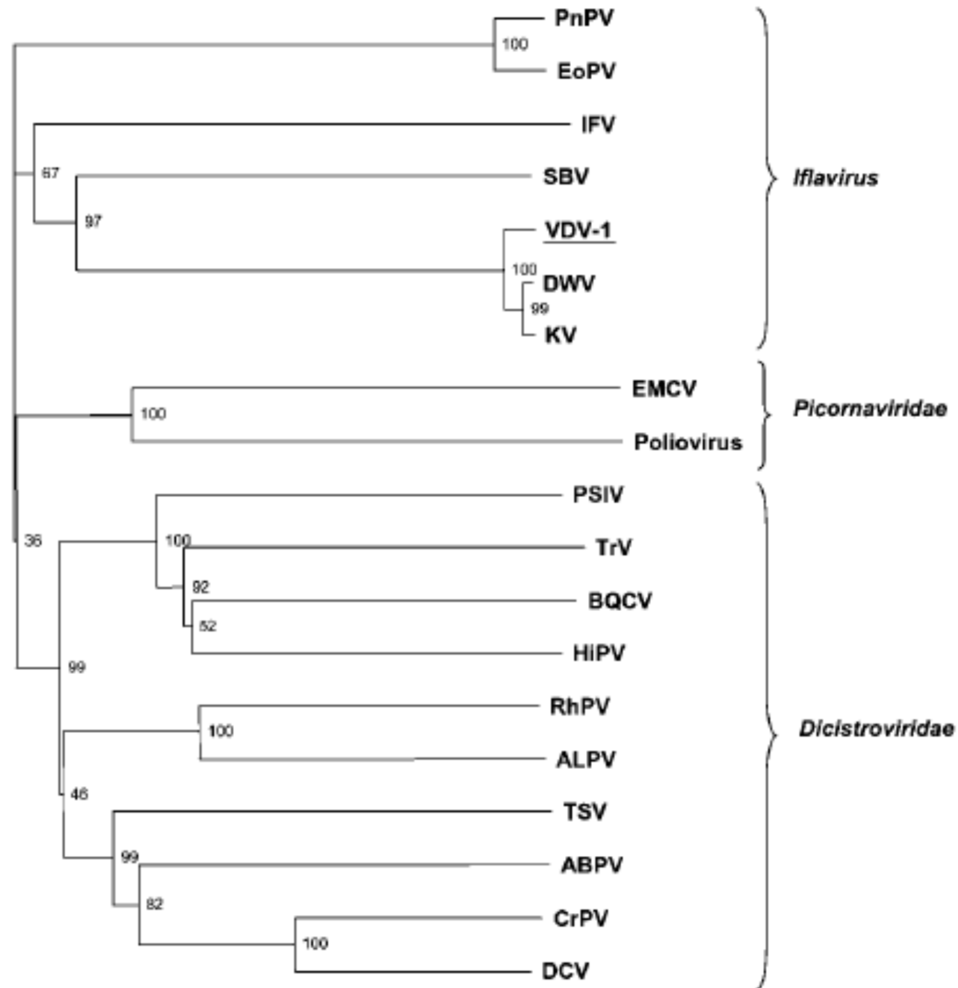


Figure 7. Phylogenetic analysis of the RdRp gene. The tree was constructed from the alignment of 19 RdRp sequences by using the Neighbour Joining (NJ) method (Leat et al., 2000). Numbers at nodes represent bootstrap values as percentages estimated by 1000 replicates. Branch lengths are proportional to relatedness. VDV-1 (underlined) is located in the cluster of Iflavirus . Encephalomyocarditis virus (EMCV) and poliovirus were used as an outgroup.

1.3.2.1 Possible recombination between DWV-A and DWV-B

RNA viruses are thought to have four main evolutionary forces to adapt to changing conditions: selection, mutation, re-assortment, and recombination (Aaziz & Tepfer, 1999; Domingo et al., 1997; Pita et al., 2007). Here I focus on recombination. The RNA-dependent RNA polymerase enzyme, responsible for viral replication in RNA viruses, generates a series of short readings (virus quasi-species) during replication

(Holland & Domingo, 1998). The dynamic mutant populations resulting from this genetic variation are limited to maintaining the continuity of the RNA genome. The propagation of new mutations through re-assortment and recombination may increase genetic diversity and help to create more adaptive populations in the evolutionary process (Aaziz & Tepfer, 1999). RNA recombination, unlike re-assortment (Figure 8), is a hybrid molecule that can occur with co-infection of both viruses in the host, and this phenomenon is thought to occur with the copy-choice model (Holmes, 2008; Lai, 1992). The copy-choice model arises during lack of proofreading in RdRp which is the process of a chain jumping (Aaziz & Tepfer, 1999; Cooper & Poinar, 2000).

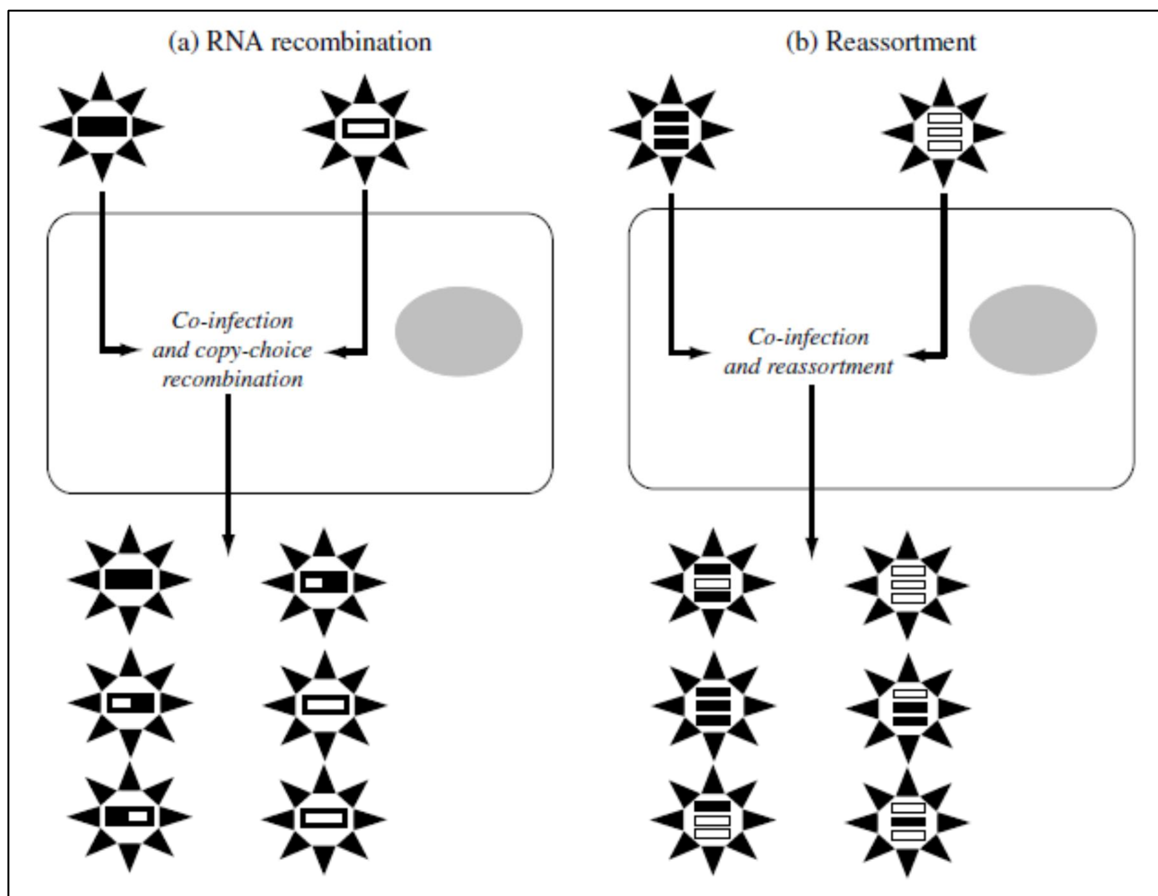


Figure 8. How (a) copy-choice RNA recombination and (b) reassortment create new genetic configurations in RNA viruses. Reassortment only occurs in viruses with segmented genomes.

Two RNA recombination types have been defined (Figure 9). Homologous RNA recombination (HR) occurs between two similar or related RNA molecules with broad sequence homology at precisely comparable (precise HR) or divergent (imprecise or aberrant HR) matched crossover sites (Lai, 1992; this recombination type is shown in the various RNA viruses using both experimental and comparative techniques by Worobey & Holmes, 1999). Cross-linking RNAs occur at sites that are perfectly matched between two RNAs in homologous recombination, so that recombinant RNAs retain the main sequence and structural organization of the parental RNA. The "homologous" term refers both to the sequence homology between the two main RNA and the transitions in the homologous regions on the two molecules. The sequence homology between the two RNAs is not necessary in the crossover regions. RNA recombination including the entire viral genomes, such as Picornavirus recombination, are mostly from this type (Lai, 1992).

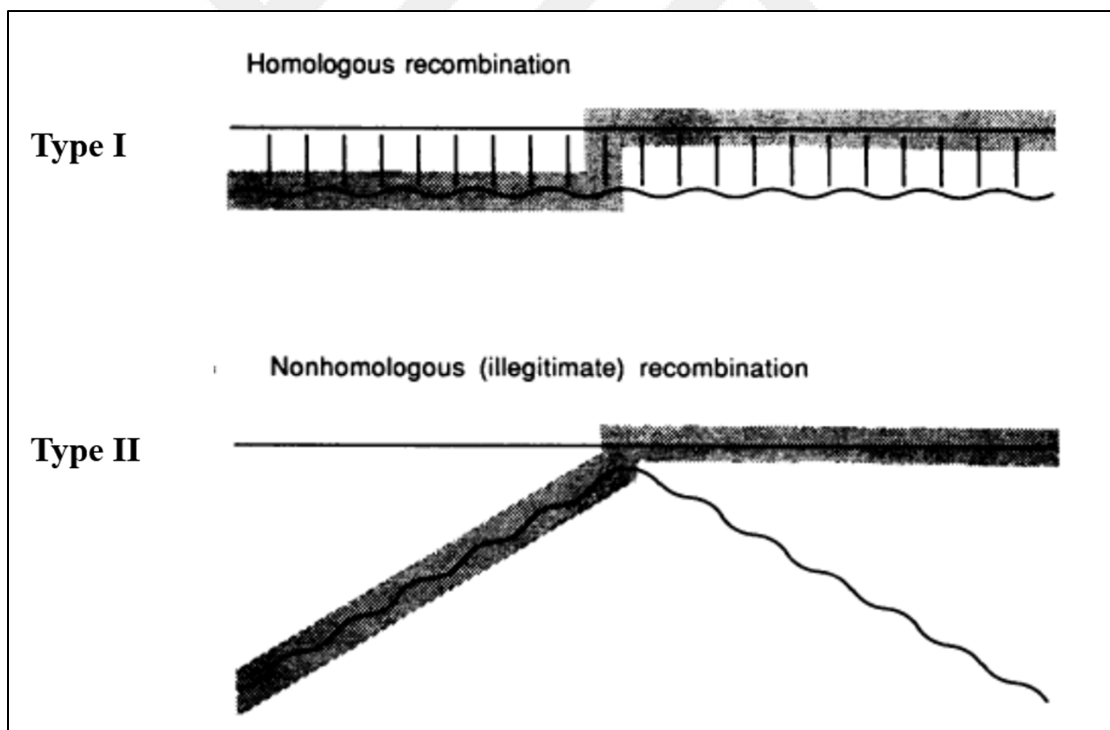


Figure 9. Two defined RNA recombination types. The shaded lines represent recombinant RNA molecules. The vertical lines represent homologous nucleotides

Recombination may occur throughout the entire genome, indicating that recombination is general and not site-specific (Agut et al., 1987; Dalmon et al., 2017a; King et al., 1982). Various recombinant viruses can be obtained in the RNA genome by using two

virus strains with different electrophoretic activity proteins (King et al., 1982; McCahon, 1981; McCahon et al., 1985). There are theoretical explanations on the evolution of recombination (and therefore about some aspects of sexual reproduction): (i) it allows the creation and dissemination of advantageous properties and (ii) the elimination of harmful genes (Hurst & Peck, 1996). The second explanation is often associated with the random loss of individuals in a population with the least harmful alleles, the concept of which is termed "Müller's ratchet" (Worobey & Holmes, 1999; Muller,1964). Müller's ratchet describes the outcome of genetic drift in non-recombining populations. RNA viruses in a handful of organisms in which this hypothesis has actually been tested have generally supported this situation (Müller's ratchet) and showed that the fitness (in the sense of Fisher) of populations in which it occurred has decreased. Although such direct experimental evidence does not suggest an advantage for recombination, it can, in principle, serve to efficiently remove disadvantaged alleles from a population by combining mutant-free portions of different genomes (Chao, Tran, & Tran, 1997).

RNA recombination events are one of the factors affecting the development of viruses. This has been well studied for living organisms belonging to the family of Picornaviridae which infect mammals, but the importance of recombination among viruses transmitted to invertebrate hosts (e.g. *A. mellifera*) has not been fully explored, particularly in honey bee. Moore and his colleagues (2011) tested whether there was a possible recombination between DWV-A and DWV-B in the infected *A. mellifera*. In that study, occurrence of the recombinant DWV-A / DWV-B was reported between the 5' end and the regions encoding structural (capsid) and non-structural viral proteins (Moore et al., 2011). In the same year, recombinant DWV-A / DWV-B was encountered in the head of *A. mellifera* (Zioni et al., 2011). In accordance with this result, recently reported studies support the occurrence of new recombinants between DWV-A and DWV-B (Moore et al., 2011; Ryabov et al., 2017, 2014). In recent years, studies reporting recombination events between DWV-A and DWV-B have been increased in the honey bees. However, studies for bumble bees, particularly in *Bombus* spp., are still insufficient. The aim of this project is to investigate the possible recombination events between DWV-A and DWV-B in the host species *A. mellifera* and the bumble bee *B. terrestris*.

1.4 Aim of the study

Deformed wing virus (DWV) and its parasitic mite vector, *V. destructor*, are known as the important organisms for honey bee loss and colony failure. DWV is also common in wild bees and might play a role in their decline (Fürst et al., 2014b). Recent studies have shown that *V. destructor* alters the dynamics of DWV (McMahon et al., 2016) and a new genotype variant of DVW has been identified and named as genotype-B (also known as DWV-B, *V. destructor* virus-1 or VDV) (Ongus et al., 2004). Co-infection of a host by both genotypes creates an opportunity for recombination and the possible recombinants between DWV-A and DWV-B may have the advantage of greater growth than DWV-A in honey bees (Ryabov et al., 2014). As new recombinants are produced, they can gradually change the genome architecture of the species. Viruses contribute to bee deaths, but their relative role is difficult to distinguish from other variables. However, much of the focus has naturally been on honey bees as this species is the most important commercial pollinator, whereas my project asks whether the bumble bee as another host of DWV has effects on the evolutionary dynamics of this virus. *A. mellifera* species is a very important commercial pollinator so there are many studies on effects of its viruses. However, although other bee species (e.g. *Bombus* spp.) are ecologically important species playing a significant role in pollination, the research on this topic is not sufficient to allow us elucidating all dimensions. Therefore, this study is based on several aims:

(i) Identification of recombinants that can occur between deformed wing virus and its variant in *B. terrestris*, which has an important role in pollination.

(ii) Comparative analysis of rates of recombination in *A. mellifera* and *B. terrestris*.

(iii) Regional detection of recombination through the genome data obtained from next generation sequencing.

(iv) To contribute to the continued efforts to quantitatively track known viruses and discover new virus genomes using next generation sequencing.

This will enable us to better understand the role of viruses in bee health and will facilitate our response to emerging and/or newly identified pathogens.

Further research is needed to evaluate the relevance of my results with respect to bumble bee health and to viral evolution. As a result, this study will shed light on further research which ought to be conducted in this area.

2. MATERIAL-METHODS

2.1 Sampling and verification processes

2.1.1 Sampling

To investigate recombination patterns in the virus genome and their phenotypic effects on *A. mellifera* and *B. terrestris*, specimens of these species were infected with DWV-A and DWV-B. The specimens of *A. mellifera* were provided from the honey bee colonies of "Robert Paxton collection" in the Zoology Department of Martin Luther University. The specimens of *B. terrestris* were obtained from Kopert Biological Systems (BEHR) in Germany. The virus specimens were also provided from "Robert Paxton collection". 15 individuals were selected from each colony of *A. mellifera* and *B. terrestris* and these individuals were firstly confirmed for devoid of virus by RNA isolation and qReal-Time PCR (qRT-PCR). The specimens of the colonies being virus negative were kept alive until the process of virus injection.

2.1.2 Homogenization and RNA extraction and cDNA synthesis with oligo-dT

To extract the total RNA, 15 bee specimens per colony were individually placed in RNase-free bags (Bioreba), containing 7.5 mL of ultra-safe diethylpyrocarbonate (DEPC)-water to aid the RNase enzyme inhibition and were individually crushed by a homogenizer. The lysed tissues (~1 mL) were quickly stored on ice into 1.5 mL microcentrifuge tubes (Greiner). RLT buffer (500 µl) and 1% β-mercaptoethanol were added to each of these lysates. Total RNA was extracted using the RNeasy mini kit (Qiagen) in a QIAcube robot (Qiagen) according to the manufacturer's instructions. The extracted RNA samples were resuspended in 30 µl of RNase-free water and their concentration were quantified by the Nanodrop machine (Epoch).These samples were stored at -80 ° C until cDNA synthesis.

To synthesise cDNA, the extracted RNA samples were initially diluted to 400 ng/µl or 800 ng/µl depending on their concentrations (**Table S1 and S2).** The cDNA was later synthesized from each RNA extract using M-MLV Reverse Transcriptase (Promega). The following components were added to a nuclease-free microcentrifuge tube according to the manufacturer's instructions: 0.8 µl Oligo-dT (Thermo), 3 µl Rt-reaction

buffer (5^x) (Promega), 0.8 µl dNTP (10 mM) (Biozym), 0.4 µl M-MLV Reverse transcriptase (Promega) and 1 µL of RNA template (400 ng). After initial incubation with a heat cycler (Biometra T advanced thermal cycler) at 70 °C for 5 minutes, the reactions were re-incubated for one hour at 42 °C. Inactivation of the reactions was performed for 15 minutes at 72 ° C. The obtained cDNA was stored in -20 ° C until using as a template for amplification in qPCR.

2.1.3 Control PCR reactions

It is essential that colonies are free of viruses in order to accurately predict virus pathogenicity and quantification of DWV viruses in experimental bees. Additionally, colonies must be confirmed to be negative for potential background viruses. For these purposes, the RNA-dependent RNA polymerase gene (RdRp) of DWV-A, DWV-B, CBPV (chronic bee paralysis virus), ABPV (acute bee paralysis virus), IAPV (Israeli acute paralysis virus), BQCV (black queen cell virus), SBPV (slow bee paralysis virus) and (SBV) sac brood virus was amplified with virus specific primers (Table 5). When each colony was confirmed to contain no virus, upstream applications (experiments) were undertaken.

Table 5. All primers and sequences

Viruses	Primers	Primer sequences	Product length	References
BQCV	BQCV-qF7893	AGTGGCGGAGATGTATGC	294	Locke et al., 2012
	BQCV-qB8150	GGAGGTGAAGTGGCTATATC		
KBV	KBV-F6639	CCATACCTGCTGATAACC	200	Locke et al., 2012
	KIABPV-B6707	CTGAATAATACTGTGCGTATC		
SBPV complex	SBPV-F3177	GYGCTTTAGTTCAATTRCC	226	de Miranda et al., 2010; McMahon et al., 2015
	SBPV-B3363	ATTATRGGACGTGARAATATAC		
SBV	SBV-qF3164	GCTCTAACCTCGCATCAAC	335	Locke et al., 2012
	SBV-qB3461	TTGGAACTACGCATTCTCTG		
DWV complex	DWV-F8688	GGTAAGCGATGGTTGTTTG	143	Mondet et al., 2014
	DWV-B8794	CCGTGAATATAGTGTGAGG		
DWV RdRp	DWV-F8668	TTCATTAAAGCCACCTGGAACATC	136	McMahon et al., 2015
	DWV-B8757	TTTCCTCATTAACTGTGTCGTTGA		
VDV-1 RdRp	VDVq-R2a	CTTCCTCATTAACTGAGTTGTTGTC	140	McMahon et al., 2015
	VDVq-F2	TATCTTCATTAAAACCGCCAGGCT		
ABPV	ABPV-F6548	GATACCCCCATGGCTC	197	Locke et al., 2012
	KIABPV-B6707	CTGAATAATACTGTGCGTATC		
CBPV	CBPV1-qF1818	CAACCTGCCTCAACACAG	296	Locke et al., 2012
	CBPV1-qB2077	AATCTGGCAAGGTTGACTGG		

2.2 Injection of viruses into bee specimens

Here, the term "line" was used to indicate the independent "bee samples" preferred for successive infections and the term of "passage" was used to refer the number of "virus" infection within a line. Firstly, viral inoculum mix was prepared, DWV-A and -B viruses were mixed at a genome equivalent concentration of 10^4 ng/µl each (Figure 10a). For the first passage, 10 uninfected white-eyed pupae, named from L1 to L10, were co-infected with a mix of virus or a virus-free extract as control line, through the intersegmental membrane between the third and fourth abdominal segment. Control inoculum was prepared from a parallel batch of uninfected white-eye pupae. After infection, bees must be maintained alive to 72- 75 hours for the development of the pupae and the proliferation of the virus. After 3 days "bee-juice" was prepared with these infected first passage by crushing pupae with 500 µl of K_2PO_4 (10 mM potassium phosphate buffer (pH 7.0)). 10 new uninfected-white-eyed pupal lines were infected with 1 µl of bee-juice. This transfer process was repeated nine times, producing 10 independent lines (L1-L10), with 10 passage of viruses through naive bees (Figure 10). The lines of *B. terrestris* were studied in the winter season of 2018, while the lines of *A. mellifera* were studied in the summer season of the same year. To detect virus infection and demonstrate the presence of DWV-A and –B in each line, 100 µl of the bee-juice and 500 µl of RLT buffer (RNeasy Lysis Buffer) were mixed and total RNA was extracted for each line as described above. The obtained RNA extracts from these lines were also used for cDNA synthesis. The cDNA samples were amplified by quantitative Real-Time PCR (qRT-PCR) to detect the presence of virus and to quantify its concentration.

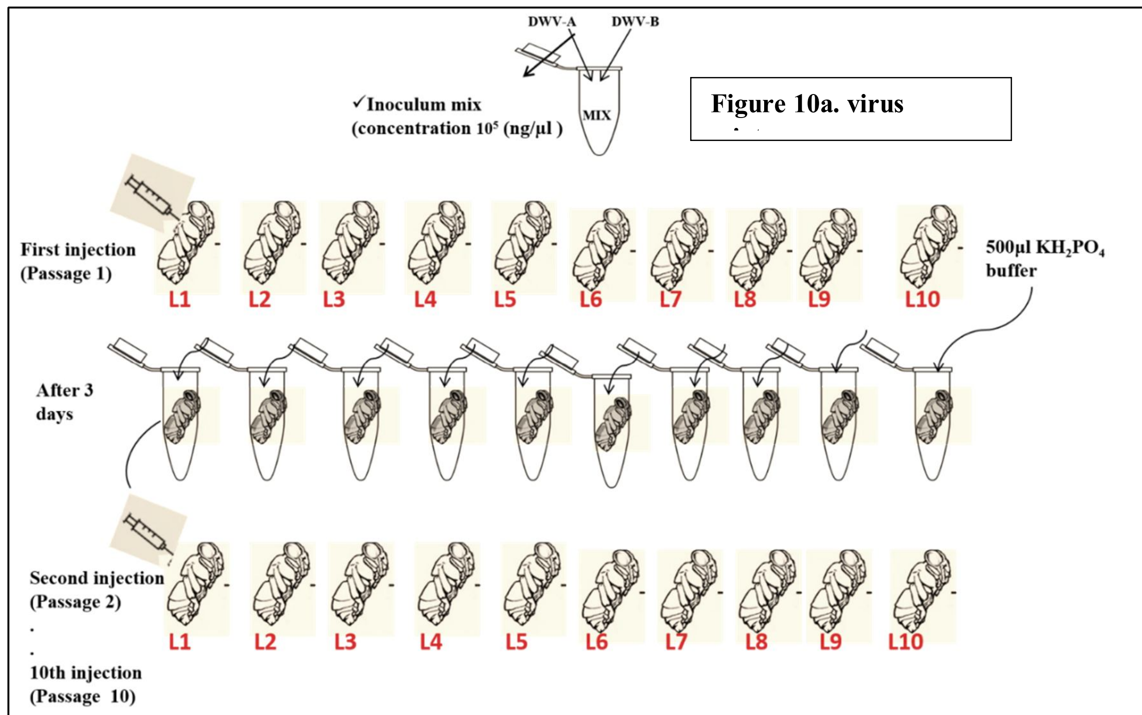


Figure 10b. process of injection

Figure 10. A schematic representation of the injection process applied to bee species.

2.2.1 Quantitative Real-Time PCR

The cDNA samples were diluted 1:10 for 800 ng/µl and 1:5 for 400 ng/µl to use in qRT-PCR. The qRT-PCR reactions were performed in a Bio-Rad C1000 using the SYBR green Sensimix (Bioline). The qRT-PCRs were performed under the following conditions: 5 min at 95 °C, followed by 40 cycles of 10 sec at 95 °C, 57 sec at 37 °C and 30 sec at 72 °C. The reaction products were then denatured at 95 ° C for 1 min and finally cooled at 50 ° C for 1 min. It was then heated from 55 ° C to 95 ° C (0.5 ° C increase per second) to generate the melting profile. To minimize the risk of false positives, an upper loop threshold of 35 (Ct) was applied in positive virus detection. Recently developed primers were used for amplification of the RNA-dependent RNA polymerase gene (RdRp) (Table 6). Amplification of the RdRp gene fragment was carried out in 10 µL volumes containing 5 µl of SYBR Sensimix, 2 pmol of each of the primers (Table 6), 3.6 µl of nuclease-free water and 1 µl of the cDNA template (diluted 1:10 or 1:5, as described above. Also, the host housekeeping gene β-actin was used as the reference (control) gene.

Table 6. Primers and properties of qRT-PCR

Target gene	Primer ID	Nucleotide sequence (5' to 3')	**Primer length (bp)**	**Referans**	Fragment size (bp)
DWV-A RdRp	DWV-F8668	TTCATTAAAGCCACCTGGAACATC	24	McMahon et al., 2015	136
	DWV-B8757	TTTCCTCATTAACTGTGTCGTTGA	24		
DWV-B RdRp	VDVq-R2a	CTTCCTCATTAACTGAGTTGTTGTC	25	McMahon et al., 2015	140
	VDVq-F2	TATCTTCATTAAAACCGCCAGGCT	24		
β-actin (*A.mellifera*)	Am-actin2-qF	CGTGCCGATAGTATTCTTG	19	Martin, 2001	271
	Am-actin2-qB	CTTCGTCACCAACATAGG	18		
β-actin (*B.terrestiris*)	Vd-actin-qF	CGACGGTCAGGTCATCAC	18	Martin, 2001	243
	Vd-actin-qB	GTTGAGGGAGCCAAAGAGG	19		

2.3 PCR amplifications for recombination detection

Four primer sets were designed and synthesised to detect recombination (Figure 11 and Table 7). These primers were to amplify the recombinant DWV-A/DWV-B fragments among the VP3-helicase gene (Primer Set A: 5' DWV-A and DWV-B 3', Primer Set B: 5' DWV-B and DWV-A 3'; please see Ryabov *et al.*, 2017). The currently designed forward and reverse primers approximately correspond to positions 4.9 and 6.5 kb of the DWV-B and DWV-A genomes, respectively. To detect recombination events between these two viral genomes, PCR reaction was carried out in 10 µL volumes containing 6.1 µl Taq Buffer, 2.5 pmol of each of the primers, 0.2 µl dNTP (10 mM) [Biozym], 0.5 U of Taq DNA polymerase and 1 µl of the diluted cDNA product. The PCR reactions were performed on a Biometra T advanced thermal cycler under the following conditions: 2 min at 94 °C, followed by 40 cycles of 30 seconds of 94 ºC, 30 seconds at 54 °C (primer annealing temperature), 30 sec at 72 °C and a final extension at 72 °C for 8 min.

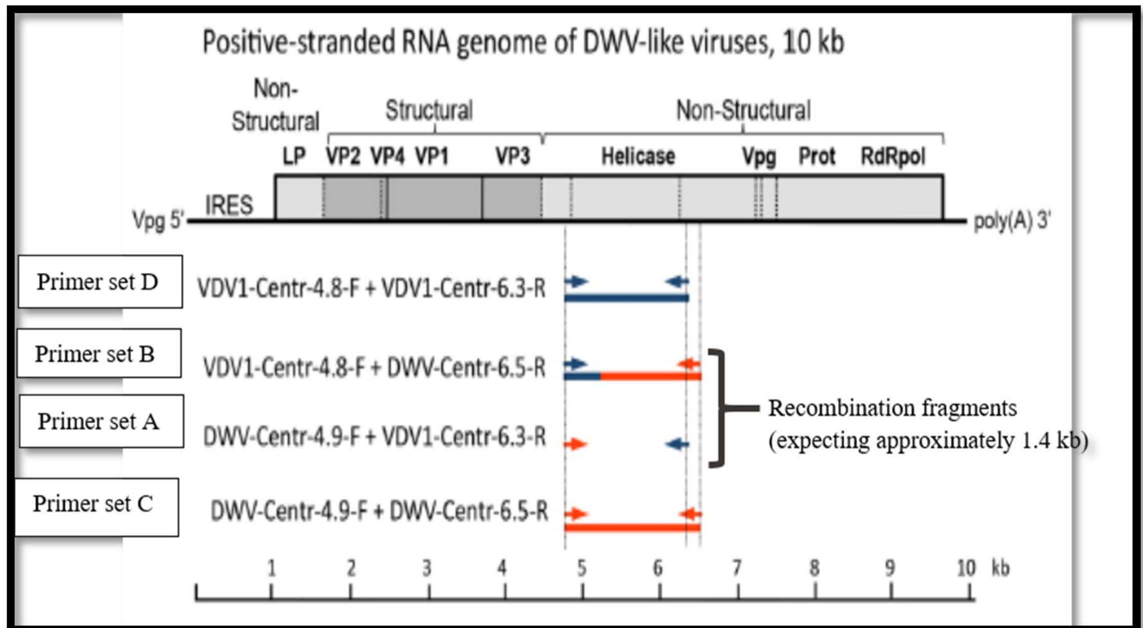


Figure 11. Positions of the specific primers for DWV-B and DWV-A and RT-PCR products of the viral genomic RNA (*Ryabov et al.2017)*

The PCR products were run on a 0.8% agarose gel. During the PCR reaction of each gene region, the primary annealing temperature (T_A) was adjusted by the application of gradient PCR to find the optimum T_M values.

Table 7. Primer sequences used in this study to detect recombination (using the primers described by Ryabov et al. (2017), with slight alterations)

Primer ID	Nucleotide sequence (5' to 3')	Fragment size (bp)	Location (bp)	Target position, polarity, GenBank accession number
DWV-Centr-4920-F	ACTGTAGTCAAGCGGTTACTTGAG		4466	DWV (4911-4934) Forward,AY292384
DWV-Centr-6550-Rev	CTTGGAAGCTTGAGGCTCTGCA	1420	4489	DWV (6521-6541),Reverse,AY29238
VDV-Centr-4890-F	CTAGTAGTTAAGCGGTTATTAGAA		6308	VDV1(4890-4912),Forward,AY251269
VDV-Centr-6310-Rev	CTGAAGTACTAATCTCTGAG	1630	6289	VDV1(6298-6317),Reverse,AY251269

2.3.1 Sequencing and bioinformatics analysis of the recombination points in *A. mellifera* and *B. terrestris*

The datasets from different passages (Passage 6, 7, 8, 9 or 10) of both species were sequenced with the Illumina HiSeq 2000 next generation sequencing (NGS) platform using 150 bp paired end reads, as conducted by FASTERIS (member of the sonic healthcare network). The annotated datasets were analysed using a *de novo* assembler called Iterative Virus Assembler (IVA) (Hunt et al., 2015). IVA was designed specifically for read pairs sequenced at highly variable depth from RNA virus samples. This program also allows assembling 150 bp reads in length, corresponding to the possible sequence lengths of the virus genomes in the NGS datasets. IVA can also distinguish multiple virus populations (e.g. DWV-A and DWV-B as two different populations) within the same dataset and can reconstruct recombinant sequences. The installation of IVA requires the user to install a set of third-party dependencies. IVA version 0.11.0 requires the following programs and versions: (i) kmc version 2.1, (ii) MUMmer version 3.23, (iii) samtools version 0.1.19-44428cd, (iv) SMALT version 0.7.6, and (v) Trimmomatic version 0.32. The below instruction was followed: "After

trimming the reads, the most abundant kmer among the reads is found using kmc (Deorowicz et al., 2013). This short seed kmer is iteratively extended into a contig using reads that have a perfect match to that kmer, treating the reads as unpaired. A list of all possible extension sequences is made (one sequence per overhanging read). IVA identifies the kmer of length *k* among prefixes of the possible extension sequences, for largest possible k, such that the kmer appears at least 10 times and is at least four times as abundant as the next most common kmer of length k. In this way, the seed is iteratively extended until its length reaches the insert size of the read pairs" (Hunt et al., 2015). Using IVA, I was able to determine recombination points between DWV-A and DWV-B in my experimental material.

3. RESULT

3.1 Quantitative analysis of DWV-A and DWV-B viruses

Quantitative measurements of DWV-A and DWV-B were performed using qRT-PCR in all passages and lines of both host species. To quantify the amount of the virus/viruses, a standard dilution curve was used for *B. terrestris*, while Ct values were only used in *A. mellifera*.

3.1.1. Quantitative analysis of DWV-A and DWV-B viruses in *B. Terrestris*

The standard dilution curve was used to calculate the amount of each virus (DWV-A and DWV-B) found in *B. terrestris*, as:

$$\left(SQmean \times 10 \times 15/_{Y}\right) \times 30 \times Z$$

(*Y: RNA volume (the final concentration should be 800 ng/µL) for cDNA synthesis; Z: Bee crushing volume (500 ml for homogenization))*

The above standard dilution curve formula was used for calculating the amount of virus present in the pupae. The results are shown in table 8.

According to the results of quantitative analysis, increasing of the amount of DWV-A found in the pupae was observed in each passage (Table 8a). In contrast to DWV-A was found in the first passage. The results of injections also indicated no significant increasing of the amount of DWV-B for the each passage in the pupae (Table 8b). However, in similar to DWV-A, was as high in the last passage compared with the first passage (Table 8).

Table 8. Quantification value of DWV-A (a) and DWV-B (b) for *B. terrestris*. Each value indicates the amount of ng per µl.

8a. The result of DWV-A*

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
L1	2,57E+09	1,16E+10	4,7E+10	1,7E+10	5,39E+10	7,84E+10	3,34E+11	8,59E+11	2,79E+12	1,39E+12
L2	3,73E+10	2,25E+10	8,5E+10	4E+10	1,24E+11	6,54E+10	3,01E+11	2,55E+12	3,90E+12	1,87E+12
L3	4,83E+10	3,22E+10	1,4E+11	2,2E+10	3,04E+10	8,87E+10	3,56E+11	3,85E+12	3,49E+12	1,39E+12
L4	1,39E+10	6,43E+10	1,5E+11	3,1E+10	4,33E+10	6,01E+10	2,80E+09	1,05E+12	2,03E+12	6,16E+11
L5	4,42E+10	3,29E+10	1,5E+11	3,1E+10	5,69E+10	6,77E+10	6,49E+11	3,01E+12	2,82E+12	1,97E+11
L6	5,09E+09	5,88E+07	8,2E+10	1,9E+10	1,25E+11	5,89E+10	3,78E+11	6,75E+11	2,34E+12	2,02E+12
L7	3,82E+10	5,97E+10	1,8E+11	3,9E+10	4,07E+10	1,06E+11	6,73E+11	1,98E+12	2,25E+12	1,43E+12
L8	2,58E+10	2,52E+10	1,34E+11	2,4E+10	3,63E+10	2,76E+10	3,43E+11	2,49E+12	2,35E+12	1,55E+12
L9	1,76E+10	1,42E+10	1,3E+11	1,3E+10	5,73E+10	6,16E+10	3,51E+11	1,73E+12	2,71E+12	7,11E+11
L10	1,55E+10	6,93E+10	1,3E+11	3,1E+10	3,16E+10	4,73E+10	2,80E+09	1,53E+12	1,81E+12	1,24E+12

8b. The result of DWV-B *

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
L1	1,84E+11	3,86E+11	6,5E+10	5,8E+10	1,76E+11	2,70E+11	4,71E+11	1,60E+12	2,50E+12	1,37E+12
L2	5,80E+11	4,27E+11	1,28E+11	2,33E+11	4,39E+11	3,02E+11	4,21E+11	3,13E+12	5,33E+12	1,79E+12
L3	7,40E+11	1,09E+12	2,22E+11	1,24E+11	1,47E+11	6,26E+11	5,84E+11	4,10E+12	4,23E+12	1,54E+12
L4	3,93E+11	1,07E+11	1,16E+11	2,12E+11	3,05E+11	2,97E+11	8,85E+10	1,89E+12	3,76E+12	9,76E+11
L5	8,67E+11	5,32E+10	1,16E+11	1,44E+11	1,97E+11	5,10E+11	1,09E+12	4,31E+12	4,60E+12	3,11E+11
L6	1,15E+11	1,21E+11	1,28E+11	5,4E+10	4,28E+11	2,37E+11	6,29E+11	1,39E+12	4,04E+12	2,66E+12
L7	6,06E+11	9,13E+10	2,66E+11	1,10E+11	1,48E+11	3,46E+11	8,22E+11	2,68E+12	4,28E+12	1,63E+12
L8	4,46E+11	6,9E+11	7,72E+10	5,8E+10	7,21E+10	1,24E+11	5,52E+11	3,19E+12	2,69E+12	1,23E+12
L9	3,98E+11	8,43E+06	1,79E+11	9,6E+10	1,80E+11	1,95E+11	4,62E+11	1,74E+12	2,96E+12	1,72E+12
L10	3,28E+11	9,26E+10	1,92E+11	1,09E+11	1,30E+11	1,40E+11	6,64E+11	2,03E+12	2,98E+12	1,29E+12

* P: passage; L: Line.

3.1.2 Quantitative analysis of DWV-A and DWV-B viruses in *A. mellifera*

The concentration values based on Ct values of DWV-A and DWV-B viruses in *A. mellifera* are shown in Table 9. The standard curve was not used for quantification. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct levels are inversely

proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample). The real-time assays undergo 35 cycles of amplification. Cts < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample. Table 10 shown that both viruses were expressed (according to Ct value, between 10,39-13,82) into each pupa for every passage.

Table 9. Ct value of DWV-A (a) and DWV-B (b) for *A. mellifera*

9a. DWV-A*

	P1	P2	P3	P4	P5	P6	P7	P8	P9
L1	11,32	12,38	13,11	13,09	11,95	12,65	13,13	12,26	12,34
L2	11,74	19	12,83	12,62	12,91	12,45	12,52	12,15	12,33
L3	11,76	13,57	11,79	12,81	11,61	12,13	11,45	12,28	12,21
L4	10,81	15,2	12,4	12,23	11,57	12,21	12,79	12,63	13,43
L5	10,83	12,16	11,41	12,14	10,8	12,09	11,94	11,17	12,24
L6	11,64	12,09	13,34	12,02	12,24	10,33	13,02	11,37	12,33
L7	11,3	11,74	12,08	12,2	11,27	12,14	11,97	11,96	12,7
L8	12,35	11,75	12,01	12,19	12,03	12,23	12,88	12,31	12,5
L9	10,39	12,91	11,41	12,66	11,62	12,08	13,06	NS	NS
L10	12,08	11,23	11,4	12,33	10,85	12,66	12,85	12,29	12,58

9b. DWV-B*

	P1	P2	P3	P4	P5	P6	P7	P8	P9
L1	11,65	12,59	12,79	12,5	12,43	12,75	14,49	12,65	12,4
L2	12,11	13	12,75	12,06	13,1	12,85	13,56	12,05	12,05
L3	12,2	13,52	12,15	11,89	10,97	12,31	12,9	12,28	12,28
L4	12,66	15,3	11,78	10,78	11,39	12,23	13,42	13,43	13,43
L5	12,15	12,71	12,2	10,64	10,89	12,41	12,75	12,24	12,24
L6	12,51	12,49	13,76	10,48	12,6	11,2	14,01	12,32	12,32
L7	12,26	12,12	12,88	11,72	11,04	12,4	13,49	12,94	12,94
L8	13,01	12,06	12,11	10,74	12,27	12,64	13,53	12,06	12,06
L9	11,21	13,92	11,97	11,72	12,18	12,4	13,82	NS	NS
L10	12,49	12,33	12,95	11,45	11,9	12,41	13,42	13,26	13,05

* P: passage; L: Line., NS: No sample

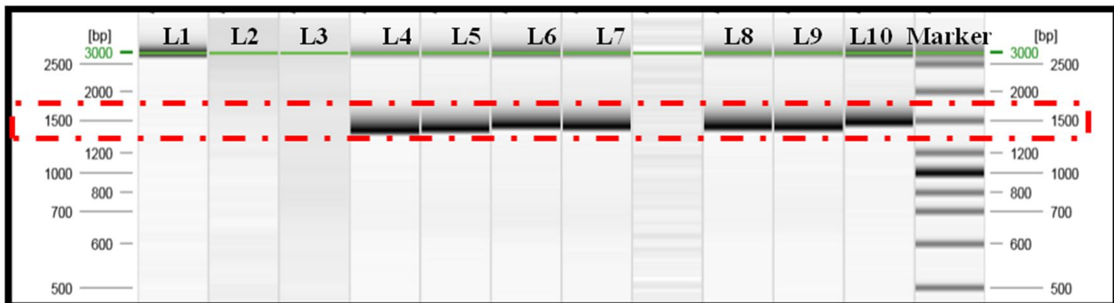
3.2 Possible recombination between DWV-A and DWV-B

3.2.1 Screening of recombination of *A. mellifera*

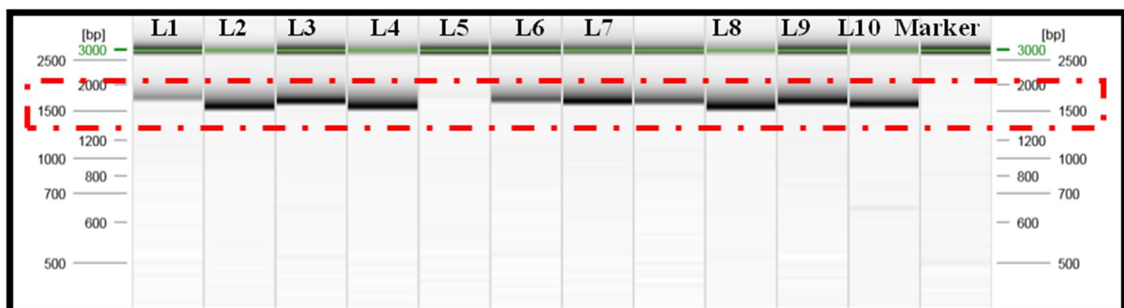
Primer set A and primer set B

Interestingly, the expected sizes (DWVA-F/DWVB-R: 1823bp and DWVA-R/DWVB-F: 1800 bp) of DWV-recombinants were detected using capillary electrophoresis in several of the viral selection lines after the first passage (Figure 12, Figure 14 and Table 10). In the last passage, before the knowledge of NGS data, it was observed that there was almost expected size DWV-recombinants in almost every line (Figure 13, Figure 14 and Table 11).

For first passage, primer set B (DWVB-F/DWVA-R) recombinants; see Figure 12a) revealed more recombinants than primer set A (DWVA-F/DWVB-R). This is similar result with Ryabov *et al.* (2014), who found a predominance of B. However, in the last passage, it was observed not only primer set B, but also primer set A (Figure 12b) and this is in contrast to Ryabov *et al.* (2014), who found a predominance of B-A recombinants in honey bees.



12a. Primer set A for first passage in *Apis mellifera* pupae

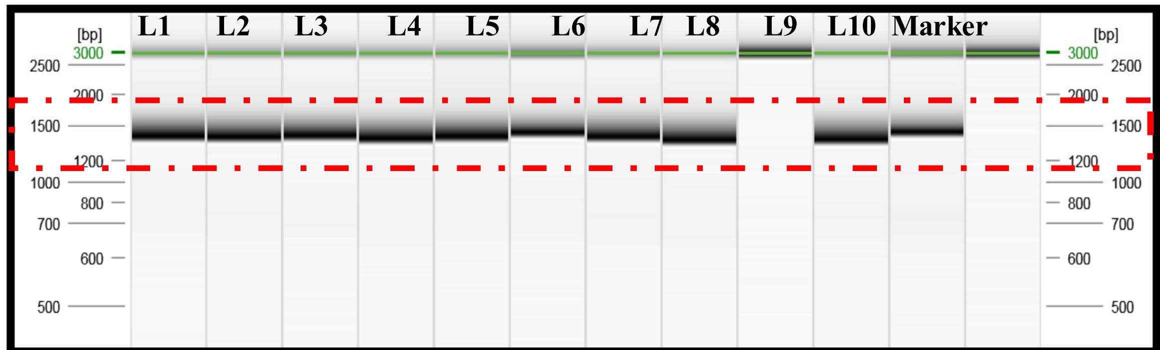


12b. Primer set B for first passage in *Apis mellifera* pupae

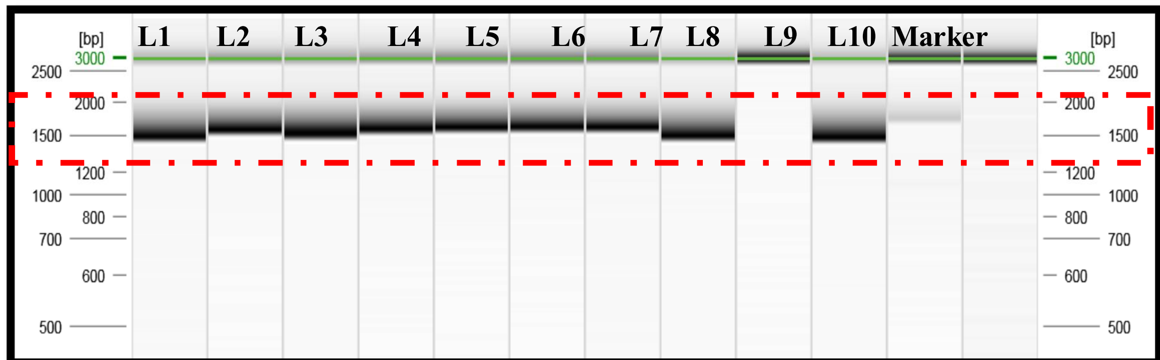
Figure 12. Recombination primer sets capillary electrophoresis results for *A. mellifera*

Table 10. All Primer sets fragment size for first passage were detected by using capillary electrophoresis

	First passage fragments size (bp)			
	Primer set A	Primer set B	Primer set C	Primer set D
P1	50	1765	0	56
P2	375	1596	0	56
P3	NA	1702	58	57
P4	1412	1597	1419	1227
P5	1423	53	1524	56
P6	1460	1728	1494	0
P7	1445	1689	1515	0
P8	386	1695	1484	0
P9	1446	1589	1533	0
P10	1439	1700	1498	0



13a. Primer set A for last passage in *A. mellifera* pupae



13b. Primer set B for last passage in *A. mellifera* pupae

Figure 13. Recombination primer sets capillary electrophoresis results for *A. mellifera*

Table 11. All Primer sets fragment size for last passage were detected by using capillary electrophoresis

Last passage fragments size (bp)				
	Primer set A	Primer set B	Primer set C	Primer set D
P1	1416	1496	1447	0
P2	1409	1594	1439	0
P3	1419	1540	57	56
P4	1396	1609	1493	1319
P5	1412	1629	1533	0
P6	1446	1642	1536	0
P7	1411	1632	1519	0
P8	1382	1502	1474	0
P9	1388	1491	1513	0

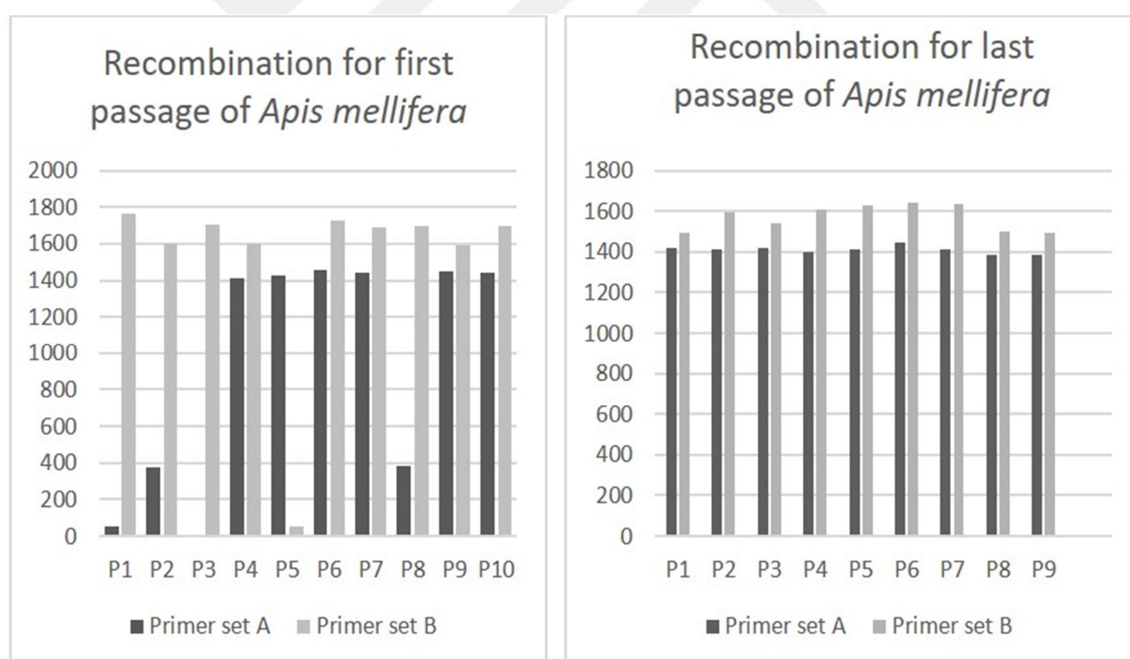


Figure 14. The first passage and the last passage recombination primer sets results were shown as fragment size

Primer set C and primer set D

As seen in the Tables, in the first passage (Table 11) and the last passage (Table 12), only DWV-A was detected (by using Primer set C: DWVA-F/DWVA-R), although in

terms of fragment size I did not obtain the expected size (1420 bp), as revealed by capillary electrophoresis (Figure 15). It is not known why DWV-B (Primer set D: DWVB-F/DWVB-R; expected size: 1630 bp) was not detected.

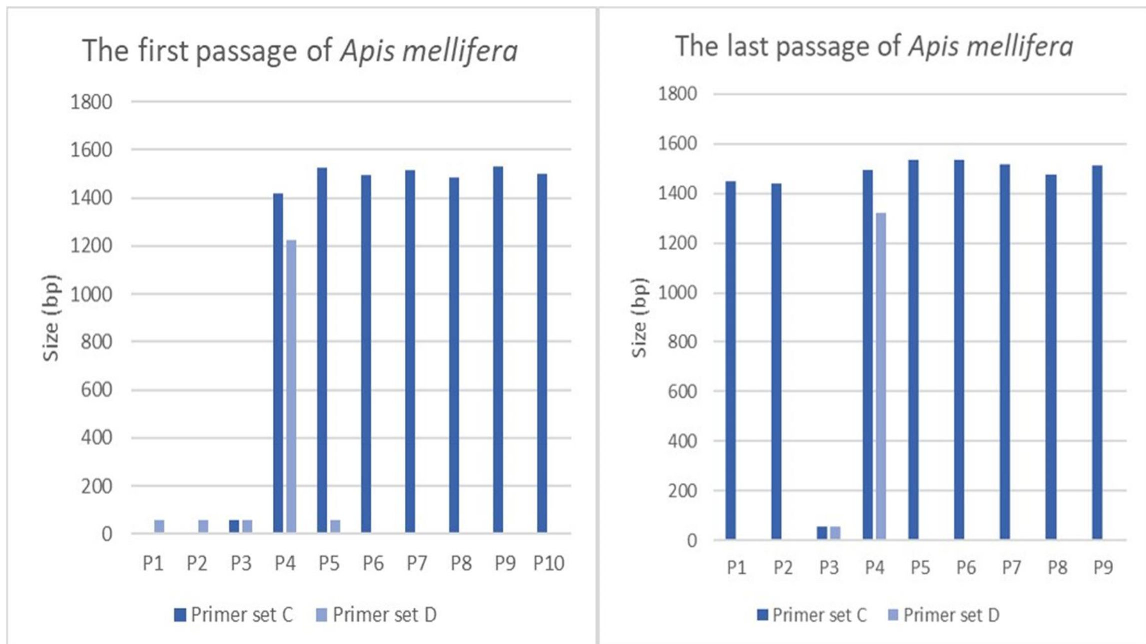


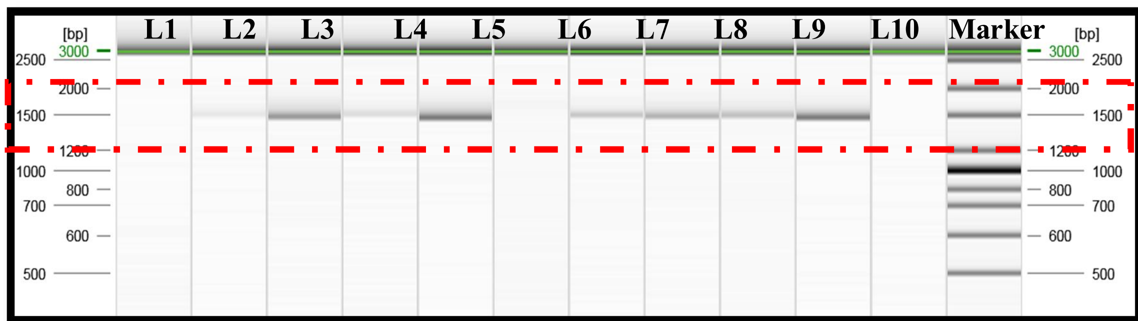
Figure 15. Primer sets of DWV-A and DWV-B

3.2.2 Screening of recombination of *B. Terrestris*

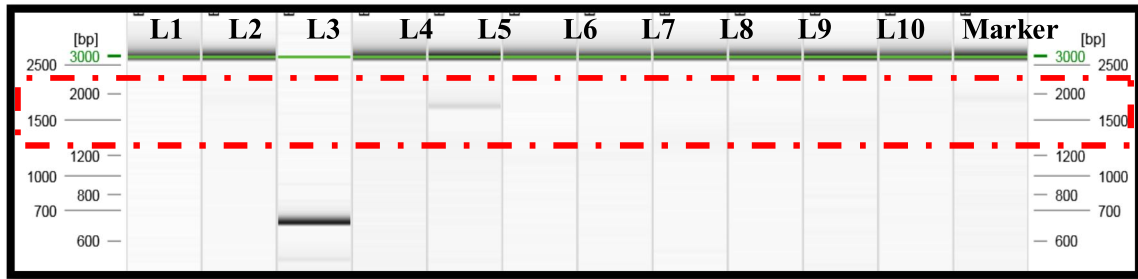
Primer set A and primer set B

The expected size (DWVA-F/DWVB-R: 1823bp and DWVA-R/DWVB-F: 1800 bp) of DWV-recombinants (specially for Primer set A: DWVA-F/DWVB-R) were detected after the first passage in one or two of the viral selection lines by using capillary electrophoresis (Figure 16,Figure 18 and Table 12).). In the last passage, before knowledge of NGS data, it was observed that there were the expected size of DWV-recombinants in almost every line (Figure 17, Figure 18 and Table 13).

In both the first passage and the last passage, primer set A (A-B recombinants; see Figure 17a) revealed more recombinants than primer set B (B-A recombinants; see Figure 17b). This is in contrast to Ryabov *et al.* (2014), who found a predominance of B-A recombinants in honey bees.



16a. Primer set A for first passage in *B. terrestris* pupae

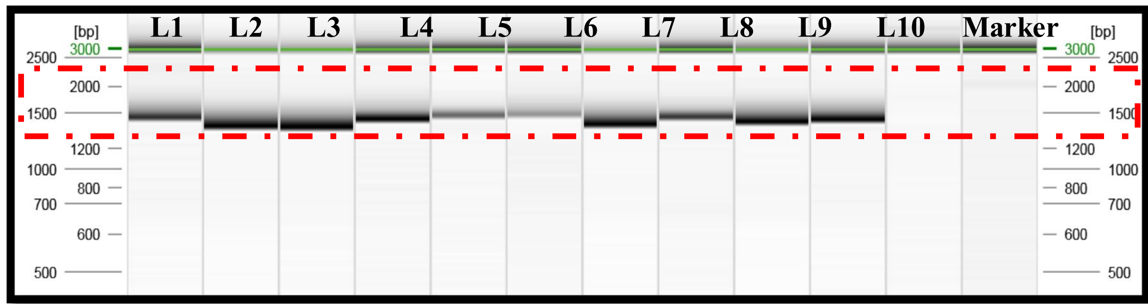


16b. Primer set B for first passage in *B. terrestris* pupae

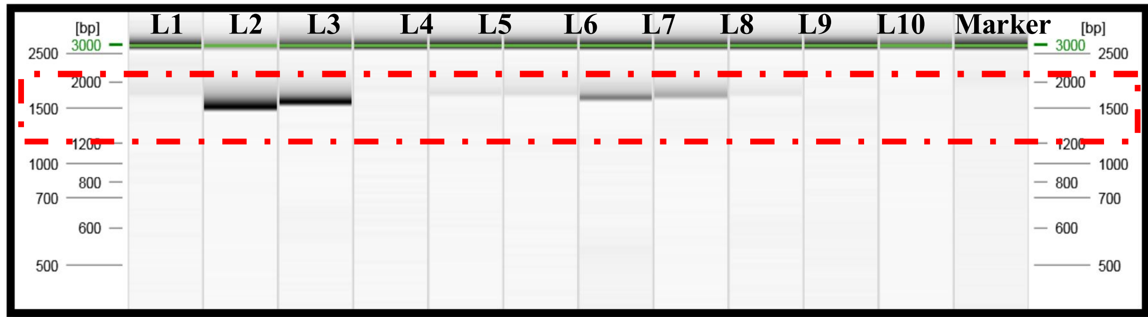
Figure 16. Recombination primer sets capillary electrophoresis results for *Bombus terrestris*

Table 12. All Primer sets fragment size for first passage were detected by using capillary electrophoresis

First passage fragments size (bp)				
	Primer set A	Primer set B	Primer set C	Primer set D
P1	0	0	1324	1407
P2	1525	0	1220	1630
P3	1384	1792	1200	1776
P4	4,57	0	1222	1224
P5	1478	1770	1442	1471
P6	0	0	1460	1382
P7	1500	0	1323	1657
P8	1492	0	1386	1385
P9	1499	0	1446	1308
P10	1479	0	1145	1620



17a. Primer set A for the last passage in *B. terrestris* pupae



17b. Primer set B for the last passage in *B. terrestris* pupae

Figure 17. Recombination primer sets capillary electrophoresis results for *B. terrestris*

Table 13. All Primer sets fragment size for the last passage were detected by using capillary electrophoresis

The last passage fragments size (bp)				
	Primer set A	Primer set B	Primer set C	Primer set D
P1	1466	0	526	0
P2	1392	0	390	66
P3	1384	0	1193	63
P4	1450	0	1473	67
P5	1482	0	1474	1449
P6	1494	0	1885	23
P7	1406	1709	1199	23
P8	1468	1752	1195	23
P9	1428	1795	1476	0
P10	1448	0	1448	1449

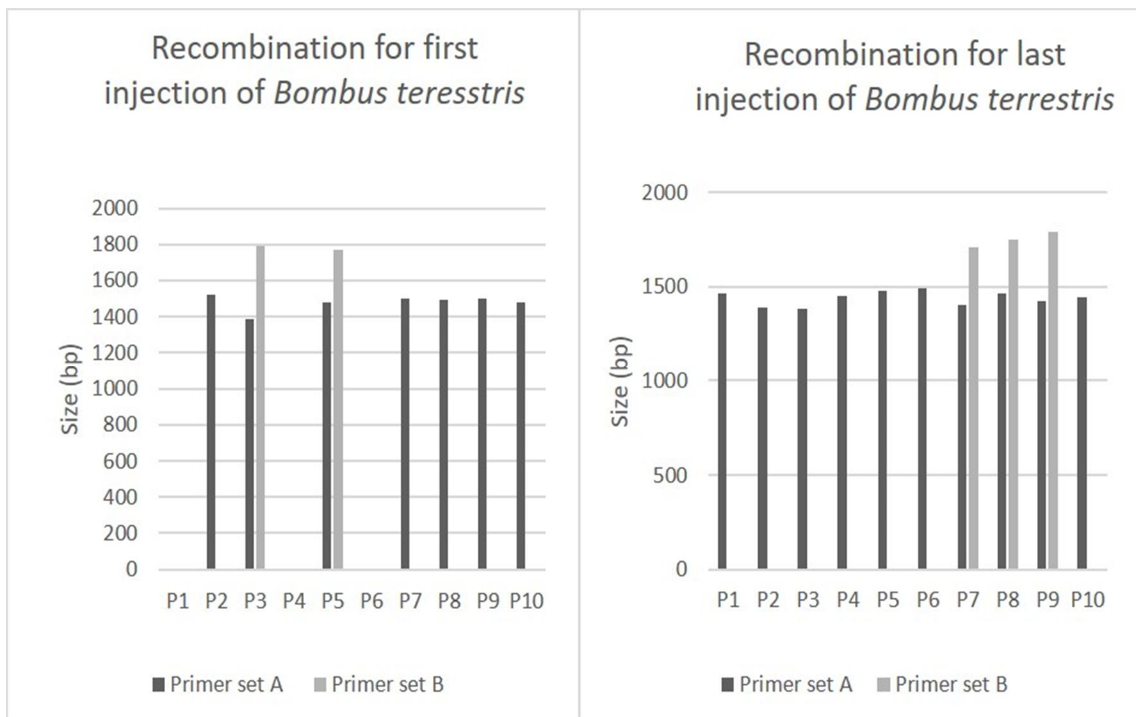


Figure 18. The first passage and the last passage recombination primer sets results were shown as fragment size for *B. terrestris*

Primer set C and primer set D

The nearly expected size of DWV-A (1420 bp) and DWV-B (1630 bp) were found by using capillary electrophoresis in the first passage pupae (Figure 19a). In the other hands, DWV-A more than DWV-B in the last passage of pupae (Figure 19b).

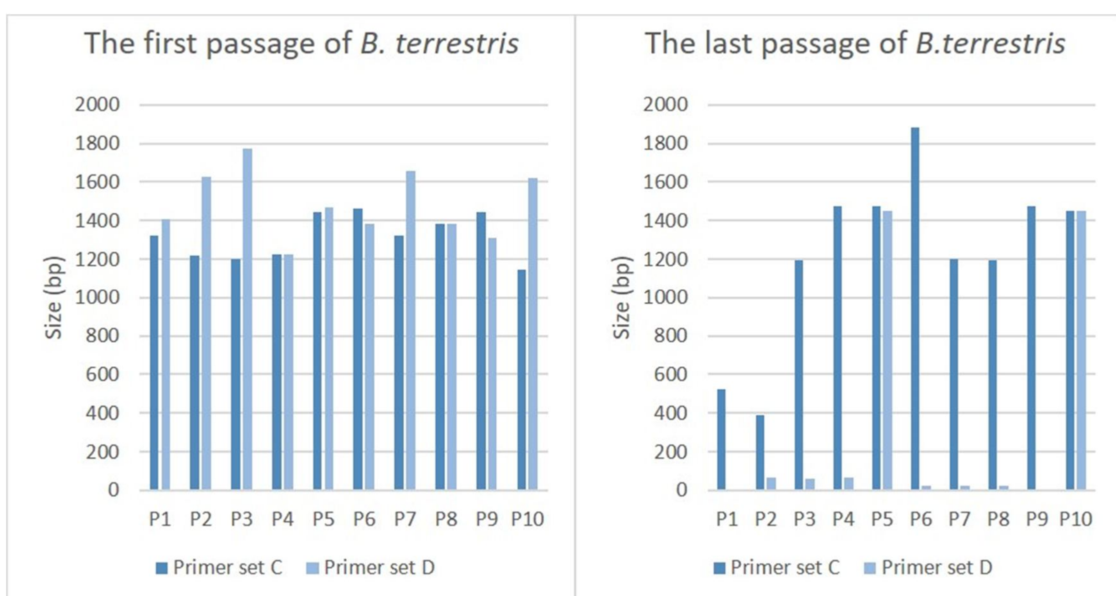


Figure 19. Primer sets of DWV-A and DWV-B of first passage (a) and last passage (b)

3.3 Analysis of recombination points in *A. mellifera* and *B. terrestis* by using IVA

IVA allows us to see what has been selected among the potentially hundreds of recombinants in the genome of DWV through recombination.

Major results:

The BLAST analysis allowed me to assign DWV (as DWV-A, DWV-B or DWV-AB) sequences within the one created by IVA by de novo assembly. The following annotation allowed me to determine whether they were A or B or recombinant contigs. Some contigs remains assigned to the host (mRNA, rRNA or mitochondrial genome) after mapping against the host genome (Apis.meliffera-4.5 and Bombus.terretris-1.0 versions). Some contigs were contamination by Fungus or Bacteria but these are likely artefacts; the libraries were clean of other viruses.

Within *Apis* and *Bombus* we can see a lot of variation in genome content (either complete or partial sequences of DWV-A and/or DWV-B) but in most of cases, at least one genome is complete, or can be complete by combining all partial sequences. Some recombinant contigs can be found but not in all libraries, in *Apis* and *Bombus*. Only two libraries (lib-26 and lib-28 in *Apis*) contain a DWV with a recombinant point within the Helicase part tested by PCR. Other recombinants are located in other parts of the genome.

Table 14. BLAST results of the contig assignation: *A. mellifera* libraries (Fasteris library number / Experimental code)

AECG -21	**AECG -22**	**AECG -23**	**AECG -24**	**AECG -25**	**AECG -26**	**AECG -27**	**AECG -28**	**AECG -29**	**AECG -30**
L1-P9	L2-P9	L3-P9	L4-P9	L5-P9	L6-P6	L7-P9	L8-P9	L9-P8	L10-P8
Contig 1 DWV-A 10,286bp	Contig 1 DWV-A 10,288bp	Contig 1 DWV-A 10,235bp	Contig 1 DWV-A 10,240bp	Contig 1 DWV-A 10,272bp	Contig 1 DWV-AB 10,271bp	Contig 1 DWV-B 10,164bp	Contig 1 DWV-AB 10,073bp	Contig 1 DWV-A 10,287bp	Contig 1 DWV-A 10,242bp
Contig 4 DWV-A 3,202bp	Contig 2; 3,4,5; 6 DWV-B 4,081bp; 2,762bp; 1,950bp; 1,535bp; 1,277bp	Contig 2,3,4,8 DWV-B 5,015bp; 2,189bp; 2,405bp; 1,019bp	Contig 2,3,4,5,6, 12 DWV-B 3,247bp; 2,527bp; 1,999bp; 1,923bp; 916bp; 1,143bp	Contig 2,3,4,5,7 DWV-B 3,950bp; 3,227bp; 1,654bp; 1,488bp; 1,272bp	Contig 2 DWV-A 5,381bp	Contig 2 DWV-A 10,293bp	Contig 2,4, 10 DWV-B 3,115bp; 2,039bp; 908bp	Contig 23 DWV-A 976bp	Contig 2,3 DWV-B 9,013bp; 1,615bp
Contig 2;3;5;6;7;17 DWV-B 2,609bp; 2,024bp; 1,901bp; 1,354bp; 1,323bp; 788bp	Contig 7-18 *Apis* mRNA, rRNA	Contig 5-7,9 *Apis* mRNA, rRNA	Contig 7-11,13 *Apis* mRNA, rRNA	Contig 6, 8-22 *Apis* mRNA, rRNA	Contig 3,5,6,7 DWV-B 1,989bp; 1,623bp; 1,362bp; 1,396bp	Contig 3-17 *Apis* mRNA, rRNA	Contig 3,5,6 DWV-A 3,170bp; 1,483bp; 1,260bp	Contig 2,3,4, 18 DWV-B 4,902bp; 2,692bp; 1,834bp; 785bp	Contig 4-27 *Apis* mRNA, rRNA
Contig 8-16; 18-23 *Apis* mRNA, rRNA,					Contig 4,8-26 *Apis* mRNA, rRNA		Contig 7-9, 11-28, 30-33 *Apis* mRNA, rRNA	Contig 22 DWV-AB 1,130bp	
							Contig 29 Bacteria	Contig 5-17, 19-21, 24-27 *Apis* mRNA, rRNA	

Table 15. BLAST results of the contig assignation: *B.terrestris* libraries (Fasteris library number / Experimental code)

AECG -31	AECG -32	AECG -33	AECG -34	AECG -35	AECG -36	AECG -37	AECG -38	AECG -39	AECG -40
L1-P10	L2-P10	L3-P10	L4-P10	L5-P10	L6-P10	L7-P10	L8-P10	L9-P10	L10-P10
Contig 1 DWV-B 9,198bp	Contig 1 DWV-A 9,879bp	Contig 1 DWV-A 6,492bp	Contig 1 DWV-B 10,282bp	Contig 1 DWV-B 10,255bp	Contig 1 DWV-B 8,274bp	Contig 1 DWV-A 10,289bp	Contig 1 DWV-AB 8,016bp	Contig 1 DWV-A 10,289bp	Contig 1 DWV-B 9,617bp
Contig 19 DWV-B 925bp	Contig 2 DWV-B 5,302bp	Contig 3 DWV-A 4,486bp	Contig 2,3 DWV-A 5,375bp; 2,878bp	Contig 2, 19 DWV-A 9,130bp; 1,085bp	Contig 2,3,4,6 DWV-A 4,220bp; 1,934bp; 1,865bp; 2,534bp	Contig 2,7 DWV-B 2,959bp; 1,084bp	Contig 2,5 DWV-B 3,714bp; 2,446bp	Contig 2,3 DWV-B 5,091bp; 5,316bp	Contig 2 DWV-A 9,912bp
Contig 2 DWV-A 10,282bp	Contig 3,4 DWV-AB 3,653bp; 2,077bp	Contig 2,4 DWV-B 4,984bp; 3,101bp	Contig 4 DWV-AB 2,195bp	Contig 7 DWV-AB 769bp	Contig 8,10 DWV-AB 2,156bp; 1,359bp	Contig 3,4,5,6 DWV-AB 3,078bp; 1,308bp; 1,552bp; 1,240bp	Contig 3,4 DWV-A 4,103bp; 2,902bp	Contig 4-25 *Bombus* mRNA, rRNA, mitochondrial genome	Contig 23 DWV-AB 755bp;
Contig 3-18, 20-26 *Bombus* mRNA, rRNA, mitochondrial genome	Contig 5-29 *Bombus* mRNA, rRNA, mitochondrial genome	Contig 5,19 DWV-AB 1,652bp; 1,221bp	Contig 5-22 *Bombus* mRNA, rRNA, mitochondrial genome	Contig 3-6 , 8-9, 11-17, 19-22 *Bombus* mRNA, rRNA, mitochondrial genome	Contig 14,17, 29, 32, 40-45, 50 *Bombus* mRNA, rRNA, mitochondrial genome	Contig 8-13, 15-27 *Bombus* mRNA, rRNA, mitochondrial genome	Contig 6-21 *Bombus* mRNA, rRNA, mitochondrial genome		Contig 3-22, 24-25 *Bombus* mRNA, rRNA, mitochondrial genome
		Contig 6-18, 20-24 *Bombus* mRNA, rRNA, mitochondrial genome		Contig 10 Fungus Contig 18 Yeast	Contig 5,7,9,11-13, 15-16, 18-28, 30-31, 33-39, 46-49 Bacteria *Aspergillus fumigatus*	Contig 14 Bacteria			

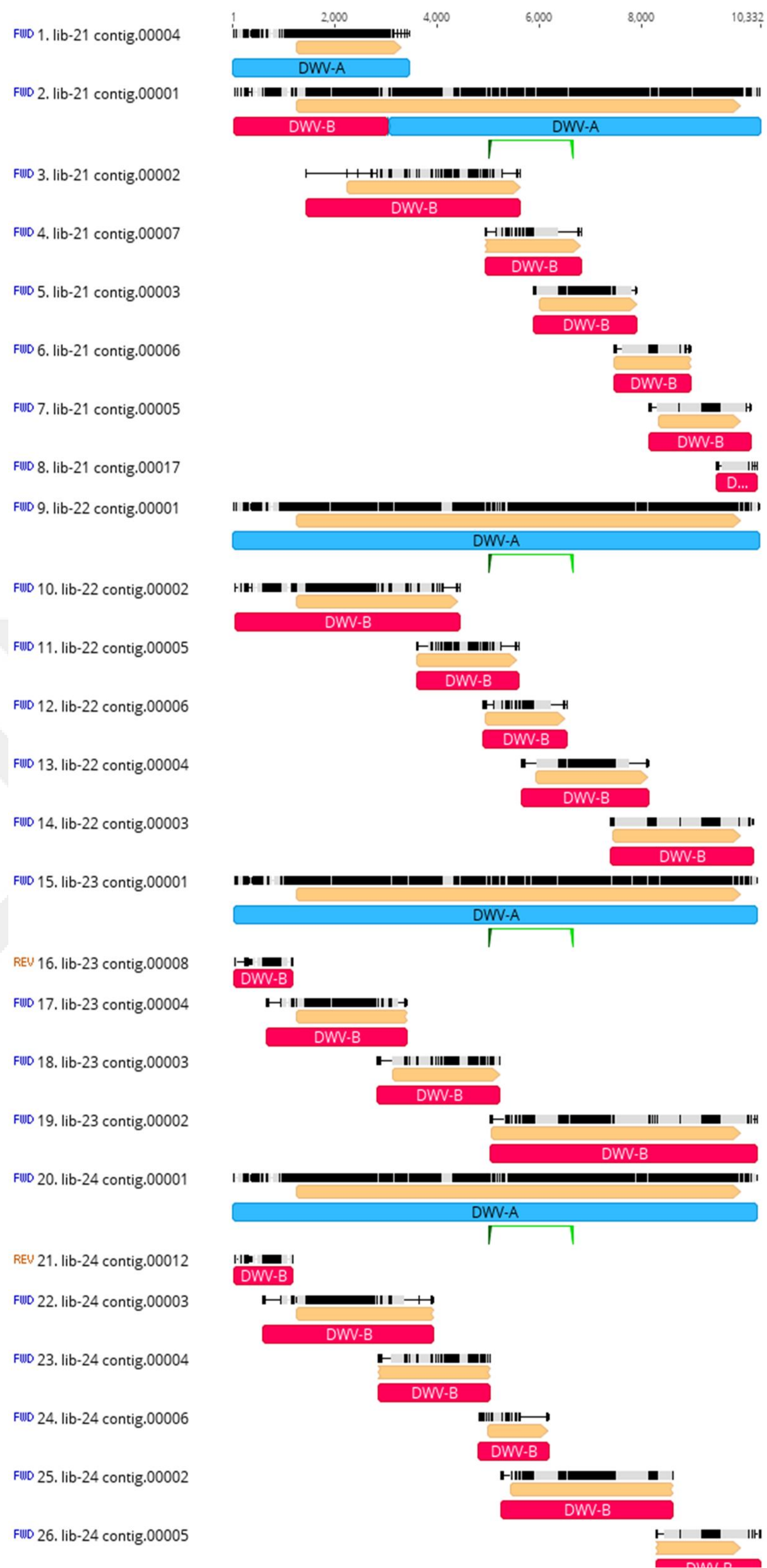
Annotation of all contigs assigned as DWV by BLAST:

Legend of the figures (with an example):

Figure 20. An example of explaining the results of IVA analysis.

- The name is **lib-XX** corresponding the NGS library name, with the **contig number** produced by the analysis with IVA.
- Black/Gray lines correspond to the nucleotide sequence itself. The gray scale is the similarity between all aligned sequences (the more is black, the more is similar).
- Blue and Red rectangles correspond to DWV-A and DWV-B annotation respectively.
- Orange arrows correspond to Open Reading Frames (beginning by an ATG start codon and finishing by a STOP codon when the extremities are clean, when it is not, that means that ORFs are partial in 5' and/or 3' end).
- Green triangles and lines correspond to the position of the Helicase primer used in the PCRs.

In this particular example, contig 1 of the library 26 (*Apis* L6-P6) is a **recombinant** one. It is half DWV-B, half DWV-A. The recombinant point is within the Helicase gene because the **forward primer is within the DWV-B** part and the **reverse primer is within the DWV-A part**. The ORF seems complete (orange arrow across the genome), meaning this virus could be complete and viable, translated a complete polyprotein.


1
2,000
4,000
6,000
8,000
10,332
FWD 1. lib-21 contig.00004
DWV-A
FWD 2. lib-21 contig.00001
DWV-B
DWV-A
FWD 3. lib-21 contig.00002
DWV-B
FWD 4. lib-21 contig.00007
DWV-B
FWD 5. lib-21 contig.00003
DWV-B
FWD 6. lib-21 contig.00006
DWV-B
FWD 7. lib-21 contig.00005
DWV-B
FWD 8. lib-21 contig.00017
D...
FWD 9. lib-22 contig.00001
DWV-A
FWD 10. lib-22 contig.00002
DWV-B
FWD 11. lib-22 contig.00005
DWV-B
FWD 12. lib-22 contig.00006
DWV-B
FWD 13. lib-22 contig.00004
DWV-B
FWD 14. lib-22 contig.00003
DWV-B
FWD 15. lib-23 contig.00001
DWV-A
REV 16. lib-23 contig.00008
DWV-B
FWD 17. lib-23 contig.00004
DWV-B
FWD 18. lib-23 contig.00003
DWV-B
FWD 19. lib-23 contig.00002
DWV-B
FWD 20. lib-24 contig.00001
DWV-A
REV 21. lib-24 contig.00012
DWV-B
FWD 22. lib-24 contig.00003
DWV-B
FWD 23. lib-24 contig.00004
DWV-B
FWD 24. lib-24 contig.00006
DWV-B
FWD 25. lib-24 contig.00002
DWV-B
FWD 26. lib-24 contig.00005

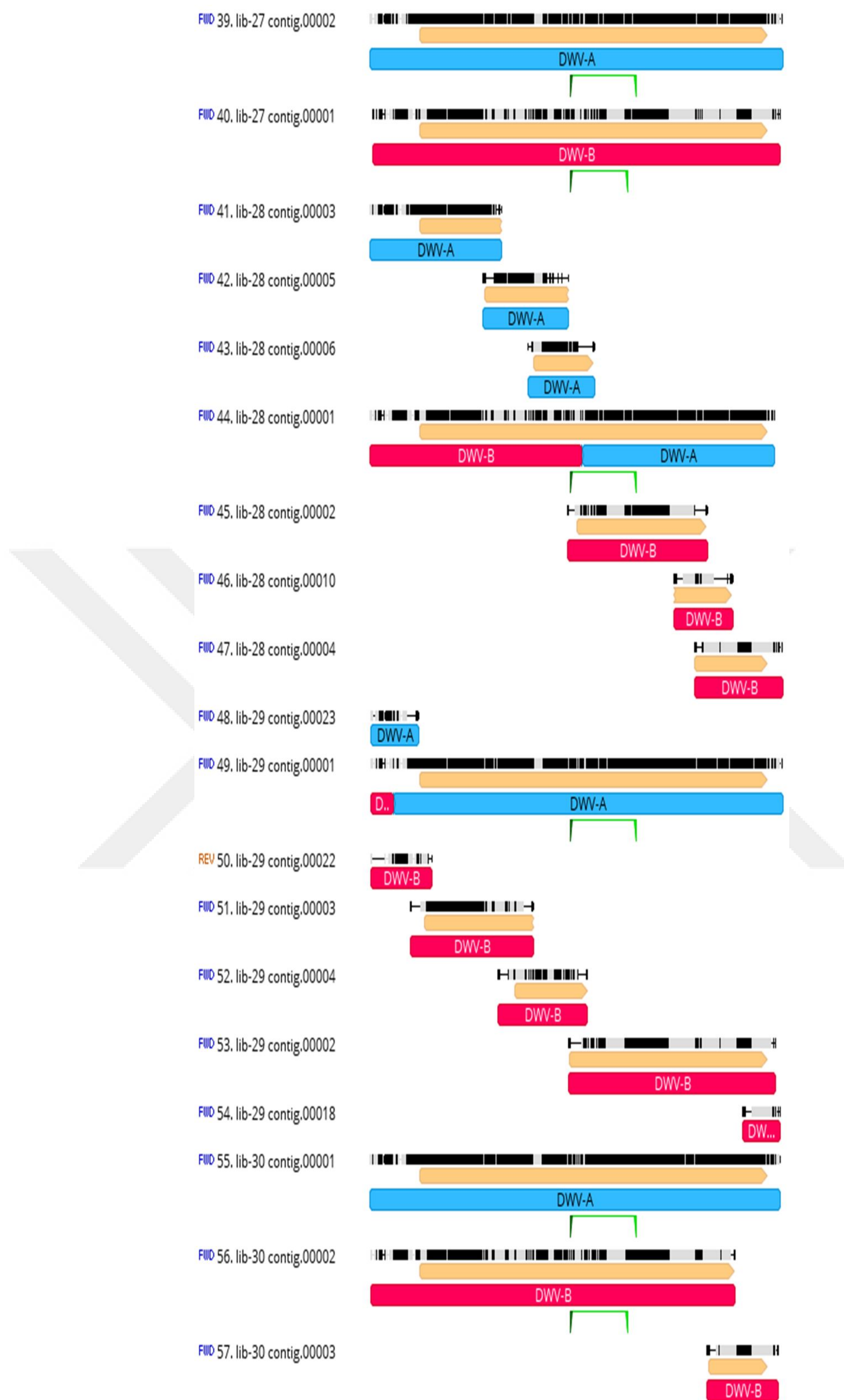
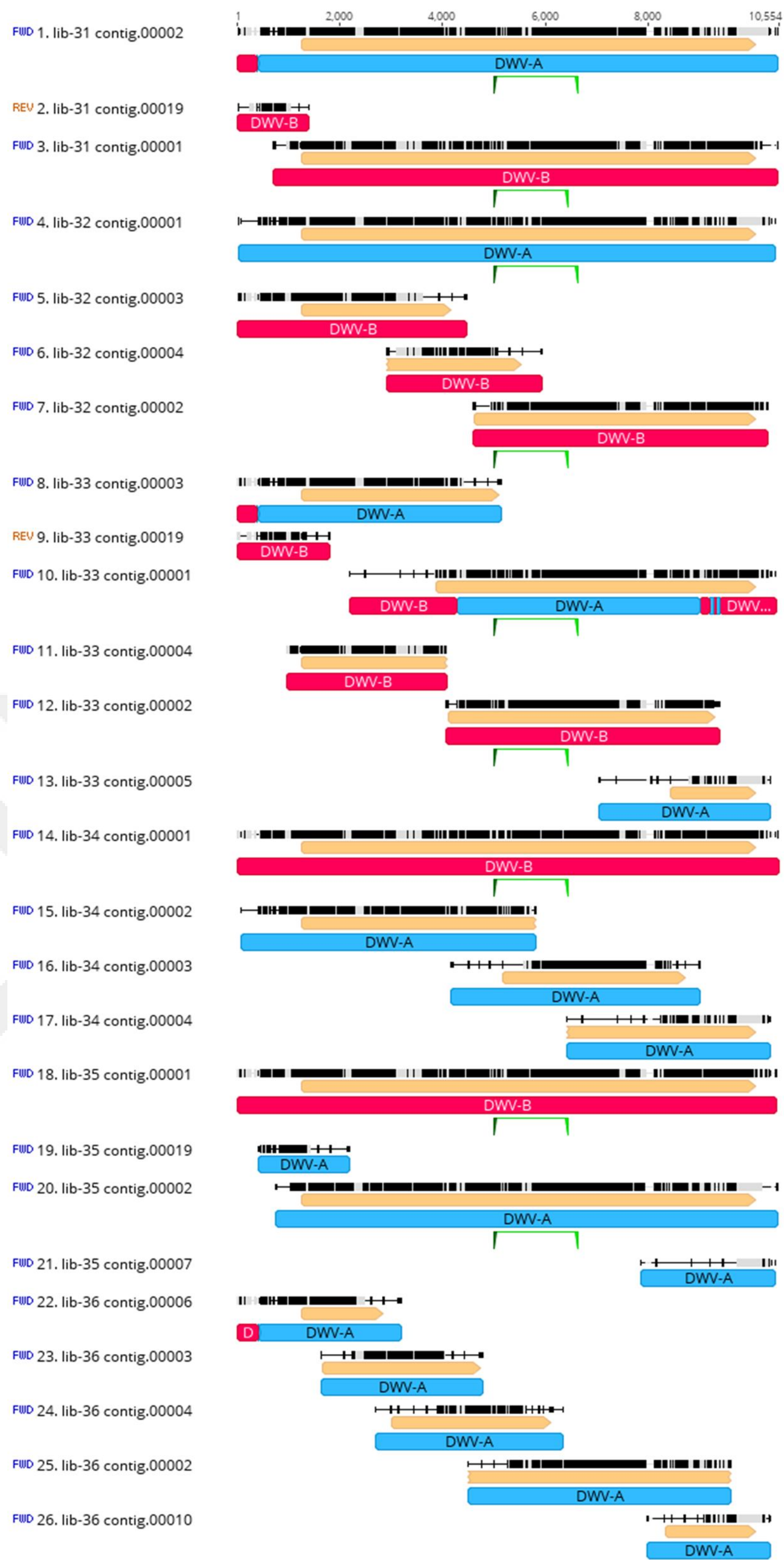


Figure 21. *A. mellifera* recombinant contigs. Only two libraries (lib-26 and lib-28 in *Apis*) contain a DWV with a recombinant point within the Helicase part tested by PCR.


1
2,000
4,000
6,000
8,000
10,554
FWD 1. lib-31 contig.00002
DWV-A
REV 2. lib-31 contig.00019
DWV-B
FWD 3. lib-31 contig.00001
DWV-B
FWD 4. lib-32 contig.00001
DWV-A
FWD 5. lib-32 contig.00003
DWV-B
FWD 6. lib-32 contig.00004
DWV-B
FWD 7. lib-32 contig.00002
DWV-B
FWD 8. lib-33 contig.00003
DWV-A
REV 9. lib-33 contig.00019
DWV-B
FWD 10. lib-33 contig.00001
DWV-B
DWV-A
DWV...
FWD 11. lib-33 contig.00004
DWV-B
FWD 12. lib-33 contig.00002
DWV-B
FWD 13. lib-33 contig.00005
DWV-A
FWD 14. lib-34 contig.00001
DWV-B
FWD 15. lib-34 contig.00002
DWV-A
FWD 16. lib-34 contig.00003
DWV-A
FWD 17. lib-34 contig.00004
DWV-A
FWD 18. lib-35 contig.00001
DWV-B
FWD 19. lib-35 contig.00019
DWV-A
FWD 20. lib-35 contig.00002
DWV-A
FWD 21. lib-35 contig.00007
DWV-A
FWD 22. lib-36 contig.00006
D
DWV-A
FWD 23. lib-36 contig.00003
DWV-A
FWD 24. lib-36 contig.00004
DWV-A
FWD 25. lib-36 contig.00002
DWV-A
FWD 26. lib-36 contig.00010
DWV-A

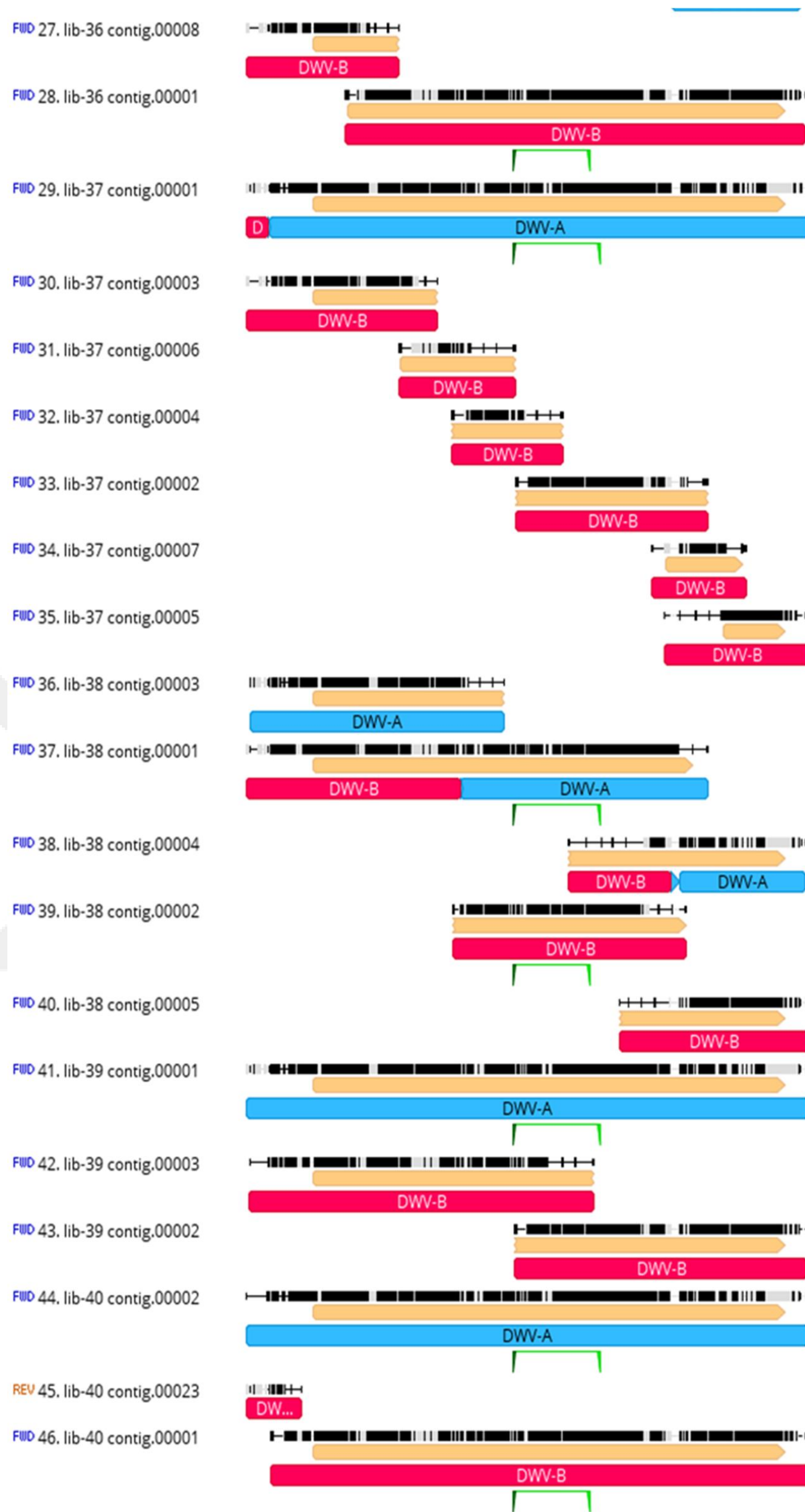


Figure 22. *B. terrestris* recombinant contigs.

Recombinants generated by IVA are in many places, not only in the helicase region but also in the other regions were observed (Table 16).

Table 16. The locations of recombination points

Position of recombinant points				
Host	Library/Contig	Position	Reference genome	Gene
A. mellifera	lib-21.contig1	2,950	NC_004830.2	VP1
A. mellifera	lib-29.contig1	491	NC_004830.2	5' UTR
A. mellifera	lib-26.contig1	5,217	NC_006494.1	Helicase
A. mellifera	lib-28.contig1	5,217	NC_006494.1	Helicase
B. terrestris	lib-31.contig2	342	NC_004830.2	5' UTR
B. terrestris	lib-33.contig3	342	NC_004830.2	5' UTR
B. terrestris	lib-36.contig6	342	NC_004830.2	5' UTR
B. terrestris	lib-37.contig1	342	NC_004830.2	5' UTR
B. terrestris	lib-33.contig1	4,208	NC_004830.2	VP2
B. terrestris	lib-33.contig1	8,761	NC_004830.2	RdRp
B. terrestris	lib-33.contig1	8,957	NC_004830.2	RdRp
B. terrestris	lib-33.contig1	9,020	NC_004830.2	RdRp
B. terrestris	lib-33.contig1	9,071	NC_004830.2	RdRp
B. terrestris	lib-33.contig1	9,135	NC_004830.2	RdRp
B. terrestris	lib-38.contig1	3,944	NC_004830.2	VP2
B. terrestris	lib-38.contig4	7,857	NC_004830.2	3CPro-RdRp

4. DISCUSSION

Honey bees are essential organisms for the production of honey, which has been consumed throughout the world for centuries, as well as other bee products providing significant economic values such as propolis, royal jelly and pollen. Therefore, the presence and protection of honey bees are very important in terms of many economic and nutritional requirements for rapidly increasing human populations. In addition to honey bees, many wild bees are also important for pollination and their decreasing numbers are another cause of concern in the world (Biesmeijer et al., 2006; Vanbergen, 2013; Goulson et al., 2015). Honey bees can also be exposed to infections and pathogens, like other living things. Emerging infectious diseases (EIDs) that cause a decline in bees can either spill over (Daszak, 2000) or spread by mediators such as parasites and parasitoids. Pathogens include protozoans, fungi, bacteria, and viruses (Goulson et al., 2015). EIDs lead to huge losses in the populations of crop-pollinators, such as *A. mellifera* and *B. terrestris* (Evison et al., 2012; Genersch et al 2006; Singh et al 2010; Graystock et al 2013).

There are thought to be increasing effects of pathogens on honey bee populations, which is one of the major crop-pollinating insects, and there is a potential threat of these pathogens spreading to range of wild pollinators worldwide (Furst et al., 2014). In recent years, the deformed wing virus and its ectoparasitic mite *V. destructor* are accepted as one of the most important EIDs both for both *A. mellifera* and *B. terrestris* (Paxton,2010; Fürst et al., 2014, Goulseon et al 2015). *V. destructor*, with its main hosts *A. cerana* or the European honey bee *A. mellifera*, has rapidly spread in Europe, America and Asia from the 1960s to the present (Rosenkranz et al., 2010; Nazzi et al., 2012). *V. destructor* is known to be the vector of DWV-B (Ongus et al., 2004; Martin et al., 2012; Mordecai et al., 2016), and it is also considered to be the main variant of DWV-A (Ongus et al., 2004; de Miranda et al., 2010; Mordecai et al., 2016). Recently reported studies have shown the infection of *B. terrestris* with this virus, which locally visits the same flower as *A. mellifera* (Fürst et al., 2014).

The several reported studies on recombination between the deformed wing virus and its main variant were also supported by an average of 84% nucleotide similarity (Table 4) (Moore et al., 2011; Zioni et al., 2011; Ryabov et al., 2014; McMahon et al., 2016;

Dalmon et al., 2017; Ryabov et al., 2017). The studies on DWV-A / DWV-B recombinants have mostly been reported in *A. mellifera*. However, there is no knowledge about recombination events among the deformed wing viruses detected in *B. terrestris*. Here, I identified the occurrence of recombination in the *B. terrestris* for the first time.

This study tested whether the bumble bee *B. terrestris* influences the evolutionary dynamics of DWV, particularly in relation to recombination, as observed in *A. mellifera*. To determine if recombination had occurred, I used primers designed to amplify recombinant DWV-A / DWV-B at the VP3-helicase genes (Ryabov et al., 2017) and compared results from viral passaging through *B. terrestris* and *A. mellifera*. The recombinant point was detected within the helicase gene using a forward primer that annealed to the DWV-B genome, whereas the reverse primer annealed to the DWV-A genome spanning the helicase gene (Primer set B; Figure 9). According to the study on the designed primers for the helicase gene region in viruses (Ryabov et al., 2014), recombinants were found in the expected region of *A. mellifera* (helicase) and confirmed by IVA results (Figure 19 and Table 15). In particular, it can be said that both recombinant results (lib 26 and lib28), as revealed by IVA, suggest that helicase recombinants have been selected in these two lines. Considering that there may be hundreds of viral recombinants in a host, these results suggest that recombinants have a growth advantage over pure virus (DWV-A), as mentioned in previous studies (Ryabov et al., 2014).

However, contrary to my expectations, the fragments amplified by primer set B in host *B. terrestris* (as revealed by capillary electrophoresis: Figure 14, 15 and Table 14), did not reveal recombination in the helicase region when analysed by IVA. Interestingly, recombinants were found in other viral regions in the host *B. terrestris* (Figure 20 and Table 17). Recombinants were present in different viral regions when coinfecting *B. terrestris* (RdRp, 5'UTR, VP2). Previous studies have shown recombination in the 5'UTR region in *A. mellifera* (Dalmon et al., 2017; Moore et al., 2011), but this study is the first to report viral recombination in *B. terrestris* in the 5'UTR region. Variations occur in viruses due to the fact that RdRp makes false readings during viral replication or integrates another viral strand during RNA replication (either homologous or non-homologous) (Domingo and Holland, 1997; Roosinck, 1997). In particular, the recombination observed in RdRp responsible for viral replication suggests that the virus

may exhibit different variants in species other than *A. mellifera*. Moreover, recombination in the 5'UTR region, which is thought to have important roles during the regulation of replication and translation (Belsham, 2009; Gromeier et al., 1999; Nakashima and Uchiumi, 2009; Roberts and Groppelli, 2009), is quite interesting. More comprehensive studies are needed to interpret the significance of these results. Now that I have identified recombinants, which may occur between deformed wing virus variants, in *B. terrestris*, it will be important to determine whether they impact their host, which plays an important role in pollination. This is important because *Bombus* spp. are more active than honey bees under conditions of lower light intensity and low temperatures (Howlett 2012; Howlett et al. 2013) and interactions between pollinators can improve their efficiency as pollinators by altering their behaviour (Brittain et al. 2013).

The amount of virus I obtained by co-infection of DWV-A and DWV-B in two host species was compared and the effects of the virus on the organism (pupae) were observed. Both viruses were injected simultaneously into the hosts *A. mellifera* and *B. terrestris*. The amount of virus was determined by using qReal-Time PCR. The aim was to observe virus increase in all individuals from the first injection to the last injection. The injection starting from 10^5 increased significantly within the host according to qReal-time PCR results. This increase in the amount of virus suggests that the virulence effect in the host may also increase. The results obtained for *A. mellifera* in Table 10 confirm previous studies (Bowen-Walker, Martin, & Gunn, 1999; Y. Chen et al., 2004; Genersch et al., 2006). The fact that the initial concentrations are the same is important in predicting the virulence of the two virus genotypes. When the "starting quantity (SQ) mean values (The qPCR Standard Curve is a correlation plot generated by running a series of standards of known template concentration and then plotting the known starting quantities against the measured Ct values.) n *B. terrestris* are seen, it is apparent that the increase in the amount for both virus genotypes progressed almost the same. For the first passage; while DWV-A increased from 10^5 to 10^9, it was observed that during the same measurements, DWV-B increased from 10^5 to 10^{11} (Table 9). These results show that both viruses exhibit a very rapid increase from the first initial injection, indicating that their effects within the host can be dangerous for host health. Deformed wing virus in *B. terrestris* was detected by Genersch for the first time (Genersch et al.,

2006). Considering that the relationship of deformed wing virus with *B. terrestris* is recent, the rapid increase in viral titre in the host in my experiment suggests that the virus increases in a range of bee species.

The mechanism of their increased virulence remains unknown. For this reason, the impact of virus recombination on virulence evolution is important to study. Further research is needed to evaluate the relevance of my results with respect to bumble bee and honey bee health and viral evolution.

SUPPLEMENTARY

S1. The value of RNA of *Apis mellifera* (ng/µl)

	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**
L1	408,859	973,005	568,241	155,203	321,772	280,903	151,797	141,914	51,734
L2	567,499	385,612	568,072	504,19	530,427	147,778	201,227	103,512	61,109
L3	267,699	1072,723	597,111	463,806	335,441	71,509	157,944	82,311	51,497
L4	354,956	927,499	279,518	131,151	91,482	103,141	132,319	58,761	89,114
L5	158,353	351,586	777,306	90,859	365,751	90,103	166,001	79,597	79,428
L6	137,864	452,325	613,294	91,824	659,843	103,546	37,667	94,201	36,696
L7	185,849	1034,558	180,055	200,21	153,349	217,846	133,949	83,075	57,191
L8	253,01	264,909	306,168	140,621	144,449	58,954	170,885	85,072	120,544
L9	217,005	603,938	307,164	69,46	112,442	117,713	390,203	NA	NA
L10	217,209	330,546	52,218	202,25	233,326	61,728	88,41	55,438	152,074

S2. The value of RNA of *Bombus terrestris* (ng/µl)

	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**P10**
L1	1425,16	1759,3	1487,51	572,42	843,377	1465,78	1625,32	1816,8	2008,44	1868,45
L2	1453,28	1616,59	2578,42	1299,32	1630,72	1425,14	1454,17	2290,9	2004,91	1747,29
L3	1531,34	1856,73	2443,29	1514,01	1115,6	2059,2	1110,07	1981,33	1911,83	1213,65
L4	1271,34	1156,97	2627,77	1243,63	1183,09	1668,48	1114,52	1779,84	1172,01	866,878
L5	1351,42	1351,33	1638,08	1181,51	973,23	1560,75	1803,29	2292,5	1398,03	632,234
L6	1282,43	1392,23	1948,1	1568,23	1593,97	1168,4	959,439	1607,82	1270,16	1415,21
L7	1376,21	1536,89	1917,11	1212,48	712,324	1089,31	1473,53	1970,78	1071,68	1454,21
L8	1163,21	1539,45	1725,99	962,229	992,684	1155,08	1361,21	1729,39	1623,56	1272,49
L9	844,755	1563,76	2370,93	981,399	918,391	1208,8	965,563	1510,01	1528,28	1293,17
L10	840,414	785,505	2106,73	1356,66	668,393	1166,63	1026,62	1481,38	1017,19	1190,41

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