SZENT ISTVÁN UNIVERSITY

Doctoral Training Programme in Veterinary Science

Occurrence of the honey bee viruses in Hungary, investigations of the molecular structure of certain viruses

Ph.D. Dissertation

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I. LIST OF ABBREVIATIONS

aa:	Amino acid
ABPV:	Acute Bee Paralysis Virus
AGID:	Agar-gel immunodiffusion
APV:	Acute paralysis virus
bp:	Base pair
BQCV:	Black queen cell virus
BVY:	Bee virus Y
cDNA:	Complementary DNA
CrPV:	Cricket paralysis virus
DCV:	Drosophila C virus
dNTP:	Deoxy-nucleotide triphosphate
dsDNA:	Double stranded deoxyribonucleic acid
dT:	Deoxytymidine
ELISA:	Enzyme linked immunosorbent assay
EM:	Electron microscopy
FV:	Filamentous virus
HiPV:	Himetobi P virus
ID:	Immunodiffusion
KBV:	Kashmir bee virus
NCBI:	National Center for Biotechnology Information
nt:	Nucleotide
ORF:	Open reading frame
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PHYLIP:	Phylogeny interference program package
PSIV:	*Plauti stali* intestine virus
RdRp:	RNA dependant RNA polymerase
RhPV:	*Rhopalosiphum padi* virus
RT-PCR:	Reverse transcription – polymerase chain reaction
SBV:	Sacbrood virus
ssRNA:	Single stranded ribonucleic acid
UV:	Ultraviolet

II. ABSTRACT

Viruses of the honey bee (*Apis mellifera* Linneaus) have been known for a long time. However, recently the attention of researchers and beekeepers has turned towards the relationship between these viruses and the parasitic mite *Varroa destructor* (former name *V. jacobsoni*). Although clinical symptoms indicated the presence of some of the bee specific viruses in Hungary, none has previously been isolated or identified in our country. In July, 1997 unusual adult bee and brood mortality was observed in some colonies of an apiary in Budapest known to be infested with *Varroa destructor*. Large amounts of virion particles were detected in honey bee pupae experimentally inoculated with bacterium-free extracts of diseased adult bees. Crystalline arrays of 30 nm particles were seen in ultrathin sections of the tissues of injected pupae and naturally infected adult bees. The virus was purified by gradient ultracentrifugation and was identified as acute bee paralysis virus (ABPV) by agar-gel immunodiffusion (AGID) tests.

Since ABPV is considered to be a common infectious agent of the honey bee, and it is present in high proportions of bee colonies worldwide, a two years survey was undertaken to determine its occurrence in field samples of adult bees and the parasitic mite *Varroa destructor* in Hungary. Considering the difficulties in the isolation of ABPV, we used polymerase chain reaction following reverse transcription (RT-PCR) to detect the viral nucleic acid in bee samples. We demonstrated the presence of ABPV RNA in 14 of 114 seemingly healthy colonies collected from eight apiaries. The investigation revealed that two third of the apiaries were infected with ABPV at a 12.2 % infection rate. In seven other apiaries out of eight investigated (87.5 %) the presence of the virus was also detected from colonies following a sudden collapse; these colonies were simultaneously infected with *Nosema apis* or infested with *Varroa destructor*. Virus specific nucleic acid was also identified in the mites collected from two apiaries falling into the latter category. The amplicon of RT-PCR was sequenced and the nucleic acid sequence was aligned to the complete ABPV sequence deposited in the GenBank database revealing a 93 % identity.

Regarding the wide distribution of ABPV in Central Europe with various clinical manifestations, phylogenetic analysis was performed on isolates to reveal the variability of the ABPV genome, and the molecular relationship between virus strains of different geographic origin. A 3071 nt fragment of the ABPV genome including the entire structural protein gene region, has been amplified from one Austrian, three German, three Polish and four Hungarian

bee samples employing six different RT-PCR assays. The amplicons were sequenced, and the nucleotide sequences were compiled and aligned. The sequences showed identity rates of 94% to 95% compared to the reference strain. The phylogenetic analysis revealed three distinct genotypes: the ABPV samples from Austria and Germany were grouped together in one branch, while the Polish and the Hungarian strains formed two other distinct clusters. Another comparative and phylogenetic analysis was carried out on a shorter (401 nt) fragment of the ABPV structural protein gene; in this analysis, all ABPV sequences available to date have been included (eleven sequences of probable UK origin deposited in the GenBank database, partial sequences of the samples mentioned above, and additional ten sequences amplified from nine Hungarian and one Polish ABPV specimens). The nucleotide sequences of these virus strains showed identity rates between 89% and 96%, respectively. In the phylogenetic tree constructed with these sequences, the ABPV strains were separated into at least two major branches. One is composed of the British sequences deposited in GenBank, while the other branch comprised the isolates from continental Europe; however, every branch could be sub-divided into several distinct clusters. The RT-PCR assays represent the methodical basis for phylogenetic analysis and classification of new ABPV isolates.

To reveal the genetic variability of the non-structural protein genes, the helicase and protease regions of one Hungarian and one Polish ABPV isolates were analyzed. A 4338 nt long sequence was determined, which covers 45.7% of the genome. The sequences were aligned to the reference complete ABPV genome. Sequence analysis revealed 93% identity to the reference strains, while the two Central European strains have shown 97% identity to each other. By the comparison of the deduced amino acid sequences 96% identity to the reference strain and 99% identity within the Central European strains were observed. The investigations supported that the helicase and protease genes are conserved genomic regions of ABPV, with similary low level of sequence divergence as it was observed in the structural protein gene regions of the investigated strains.

Within the survey on the occurrence of ABPV in Hungary a virus designated as Hu-B1/97 was isolated from an acute disease outbreak in an apiary causing high mortality among adult bees. In the identification procedure of the virus with ABPV specific immune-serum in AGID test, interestingly, a double precipitation line occured, indicating the presence of two antigenically related but not identical viruses. Discriminating primer pairs designed in the structural protein-coding region of ABPV were used in RT-PCR investigations. Sequencing of the amplicons proved that the virus suspension contains indeed a mixture of two genetically distinct viruses. Homology search demonstrated a new variant of Kashmir bee

virus (KBV) as one of the components (nucleic acid identity 83.6 %) while the other virus was closely related to the prototype ABPV strain (93.6 % nucleic acid identity).

The sensitivity and the easy application of RT-PCR proved to be extremely useful in the diagnostics of the viral infections of bees. Therefore a diagnostic RT-PCR method was developed and tested for the detection of four important bee viruses in field samples. Specific primer pairs were selected for the amplification of SBV, BQCV, ABPV and KBV genomic fragment in one amplification panel. The amplified products are well-distinguishable by their sizes. The described method is useful for the quick and reliable detection of bee viruses from field samples.

III. INTRODUCTION

Honey bees have remarkable ecological impact and also have great importance in agricultural economy, as they play the most significant role in the pollination of field crops. Bee products (i.e. honey, wax, propolis, royal jelly and bee venom) have been used since the prehistoric times for consumption and for therapeutic purposes. Honey bees exist all over the world at different climates. The habitat of *Apis mellifera* ranges from the tip of southern Africa to southern Scandinavia, and from continental Europe to western Asia. Since bees are highly adaptable insects, they are able to adjust to a wide variety of climates and geographic regions.

The vital role of bee pollination in ecology and in agriculture is hardly realized by the general public due to the lack of adequate information in most countries. Bees are essential for pollination of nearly 40 different crops, of which most are self-incompatible (i.e. apples, pears), because they need cross pollination for crop production. Bees are also important for partially self-incompatible crops (i.e. field beans) and are beneficial for self-fertile crops but not for self-pollinating crops (i.e. oil seed rape). The benefit of pollination is seen in increased fruit yields (i.e. apples, clover), improved fruit quality (i.e. strawberry), synchronized seed ripening (i.e. oilseed rape), improved oil content (i.e. sunflower) or increased hybrid vigor in seed crops due to an increased germination and establishment. Since over the years huge amounts of insecticides and pesticides have been used in the agricultural industry, which has killed or decimated lot of insect species, extincting them as pollinators, nowadays bees are the most valuable pollinators.

Hungary has advantageous geographic and environmental conditions for beekeeping. Approximately 850 000 bee colonies of 30 000 beekeepers produce honey at the moment in the country. Besides other very important nectar producing plants, the two third of the locust tree (*Robinia pseudoacacia*) population of Europe is located in Hungary. Locust honey is of high quality, popular and it is in demand in the international trade.

The appearance of the parasitic mite *Varroa destructor* in the country in the early '80s caused serious losses and made profitable beekeeping more difficult. Although within the last twenty years more and more effective drugs and sophisticated treatments were developed against varroosis and the beekeepers learned to coexist with the mite infestation, novel consequences of the presence of the Varroa mites in bee colonies have been observed

recently. Varroosis is a severe problem worldwide, and the scientific interest is increasing in the mite-induced diseases, in particular the viral infections.

Several viruses have been isolated from honey bees, most of them belong to the *Picornaviridae* family. Hence they are difficult to identify by their morphology, since most of them have a size of about 30 nm. The exceptions are the filamentous virus, which is much bigger (150 × 450 nm), and the *Apis iridescent* virus with a size of about 150 nm, while chronic paralysis associated and the cloudy wing viruses belong to the smaller sized bee viruses with a diameter of 17 nm. Besides being similar in size and shape most of them posses ssRNA except the filamentous virus which is the only known bee virus with a dsDNA genom.

The virological diagnostic methods have limited value in the case of bee viruses. The similarities in the clinical symptoms and in virion morphology make the identification of the viruses rather complicated. The virus isolation based on the serial passages of the viruses in cell cultures and serological characterization using serumneutralization is not possible on bee viruses, due to the lack of bee specific cell cultures. Therefore the novel molecular techniques in the nucleic acid investigations (i.e. polymerase chain reaction) are extremely promising in this field, since they created the opportunity of genetic identification and characterization of bee viruses.

This study presents the results of four years' investigations on the honey bee viruses. The first isolation of an acute bee paralysis virus strain from a case of increased bee mortality focused our interest to the occurrence of viral infections of honey bees in Hungary and also the molecular characterization of the later isolates. The investigations were performed at the Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest; and at the Institute of Virology, University of Veterinary Science, Vienna. The results of the studies have been published in international scientific journals. In this dissertation I have compiled the publications in a chronological and logical order, which presentation method has advantages and disadvantages as well. One of the main handicaps is, that some parts (i.e. introductions, materials and methods) unavoidably contain repetitions. The six chapters of the "Investigations" part represent six scientific reports which were already published or are submitted for publication. Since these articles all deal with viral infections of the honey bee and in each article we had to introduce our work and the background of the certain viral infections, occasional repetitions were unavoidable. On the other hand, each report was written on independent series of investigations, even if based on the results of the previous ones. Therefore in each case new aspects of the same facts are emphasized depending on the actual investigations and the aim of the study. Wishing to save the original text, I did not change the articles already published, I simply standardized their

format. This led to some unavoidable inconsistencies in the nomenclature and in the conclusions. I would like to ask the reader to take into consideration, for example, that the first article was published in 1999, when the parasitic mite *Varroa destructor* had its former name (*V. jacobsoni*) having been reclassified as *V. destructor* later (Anderson et al., 2000), or that complete genome sequences of the bee viruses were not available in the beginning of our studies. Several diagnostic methods (i.e. diagnostic RT-PCR assays to certain bee viruses) have been developed by us and other research groups simultaneously. The statements of the different chapters are often based upon the previous ones. I hope, that the abstract and the general conclusions help to integrate the chapters into a coherent work in the readers' mind.

IV. INVESTIGATIONS

Chapter 1

Occurrence of acute paralysis virus of the honey bee (*Apis mellifera*) in a Hungarian apiary infested with the parasitic mite *Varroa jacobsoni*

1.1. INTRODUCTION

Viruses can replicate in all types of living cells ranging from bacteria to cells of invertebrates and various cell types of higher mammals. The first non-occluded insect virus, sacbrood, was first recognized by White in 1917 in honey bee larvae and later isolated and characterized by Bailey et al. (1964). Intensive study since that time has shown the honey bee to be the primary source of picorna-like viruses in insects, with 18 viruses detected so far (Allen and Ball, 1996). Viruses persist in the bee population at a low level of inapparent infection: clinical symptoms appear only when virus replication is initiated and infection becomes systemic. Infected cells can no longer perform their essential function and their mass destruction leads to disturbances in the function of vital organs. Outbreaks of severe disease due to virus infection are relatively uncommon because transmission is limited by the death of infected individuals away from the colony, by the short life span of bees during summer and by various defense mechanisms (e.g. hygienic behaviour). Conversely, virus spread can be facilitated by range of other factors such as dysentery, infection with *Nosema apis* and overcrowded conditions. In recent years the spread of *Varroa jacobsoni* almost world wide has focused attention on the viruses of bees and their association with colony mortality (Ball and Allen, 1988; Kulincevic et al., 1990; Hung et al., 1995).

1.2. MATERIALS AND METHODS

From late July 1997, a Budapest beekeeper observed sporadic adult bee mortality in his colonies. Eight to ten-days-old bees undertaking orientation flights were the first to show symptoms of crawling and paralysis and some individuals had distended abdomens and appeared dysenteric.

An aqueous homogenate of 30 dead bees collected from the apiary was heat fixed and tested for *N. apis* infection by staining first with 0.4% methylene blue for 15 min and then with 0.6% fuchsine solution for several seconds.

Subsequently, bacterium-free extracts were prepared from diseased living bees according to the method described by Bruce et al. (1995). The bees were collected from three affected colonies (112, 93, and 121 living bees, respectively) and exterminated with an overdose of CO_2. At the end of the process the three samples were resuspended in 1 ml phosphate buffered saline (PBS) and 0.5 ml of each was united (combined stock suspension). Tenfold serial dilutions of the combined suspension were filtered through 200 nm pore size Nalgene filters and three groups of 40, eight to ten-days-old, white or light brown eyed pupae from symptomless colonies of the same apiary were injected intra-abdominally with 10 µl of the stock suspension, the 1:10 and the 1:100 dilutions. The pupae were maintained in an incubator at 35 °C and each day three of them were fixed for histological examination. The samples were pre-fixed with 4% paraformaldehyde, post-fixed with 1% osmium tetroxide, embedded in Durcupan, and ultrathin sections were made.

On the fourth day after inoculation 20 pupae from each group were homogenized in PBS, and a suspension was prepared according to the method described above. The samples were ultracentrifuged at 130,000 g for 3 hour in a Sorvall Combi Plus ultracentrifuge, and the pellets were resuspended in 2 ml PBS. This suspension was layered onto a caesium chloride gradient (1.2-1.5 g/ml), and centrifuged at the same velocity for 24 hours. At the end of this period two well-visible bands were formed in the gradient (1.32-1.33 and 1.37-1.38 g/ml, respectively). The bands were separated by fractioning and dialysed overnight against PBS. The purified virus suspensions were tested by agar-gel immundiffusion (AGID) against antisera to six different 30 nm honey bee viruses (Allen and Ball, 1996). Samples taken from the above two bands were counterstained with uranyl acetate and lead citrate and examined in a JEM-JEOL 100S transmission electron microscope.

1. 3. RESULTS

On dissection of naturally diseased bees, the only pathological finding was the distension of the honey sac and large intestine. No *N. apis* infection could be detected in these bees. During detailed colony health inspection carried out at the end of August many dead larvae were found and numerous adult female *V. jacobsoni* were seen on the adult bees. By the end of September the bee population in the affected colonies had dramatically declined. Despite the application of an acaricide treatment, 16 out of the 45 colonies were in poor condition before wintering.

Ultrathin sections of various organs of the affected bees and experimentally infected pupae revealed the presence of virus particles 30 nm in diameter, in crystalline arrays, in the cytoplasm of cells (Figure.1).

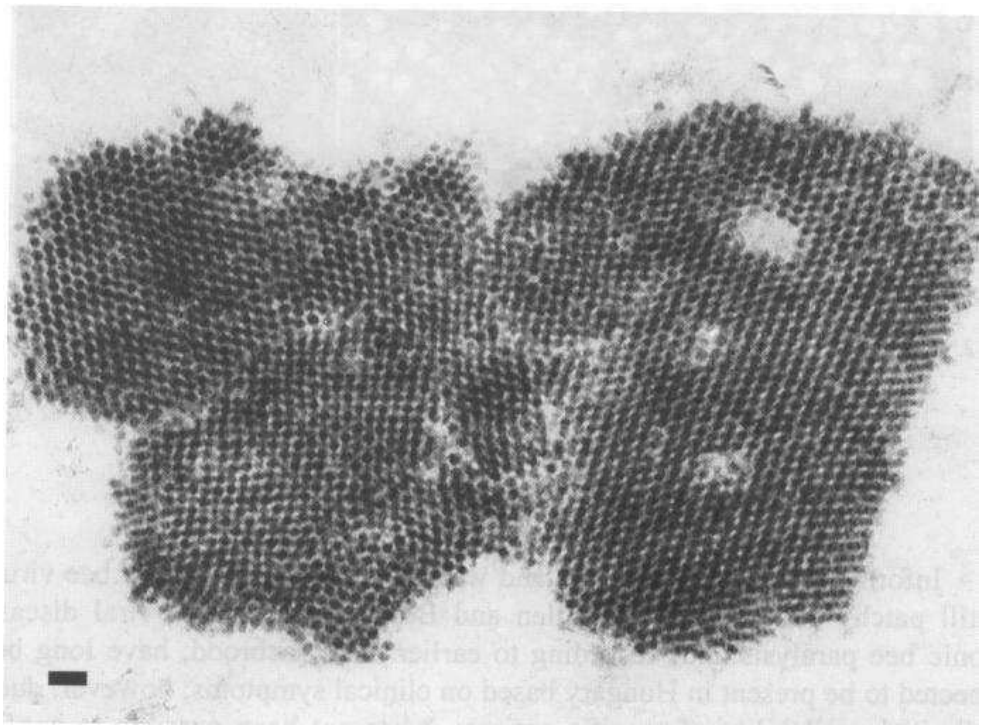

Figure 1.: Group of 30 nm virus particles in crystalline array in the cytoplasm of a cell an experimentally infected pupa. Bar = 100nm

Masses of virus particles of similar size were observed in the extracts of experimentally infected pupae purified by caesium chloride gradient centrifugation and negatively stained (Figure 2). The upper band of the caesium chloride gradient (band A: 1.32-1.33 g/ml) gave no reaction when tested by immunodiffusion against six different honey bee virus antisera and no virus particles were visualized by electron microscopy. The material was probably of host

origin. The lower band (band B: 1.37-1.38 g/ml) gave a strong positive reaction only against acute bee paralysis virus (ABPV) antiserum by immunodiffusion.

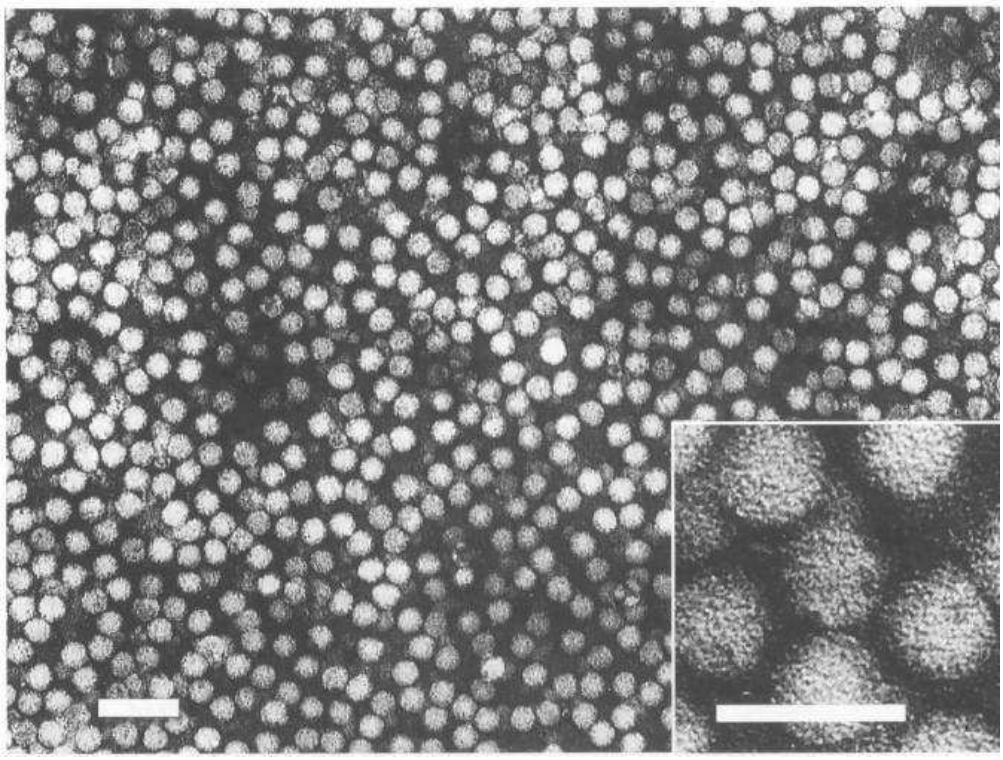

Figure 2.: Acute bee paralysis virus (ABPV) particles extracted from experimentally infected pupae purified in a caesium chloride gradient. Bar = 100 nm (Insert bar = 50 nm)

1.4. DISCUSSION

Information on the incidence and world distribution of honey bee viruses is still patchy and incomplete (Allen and Ball, 1996). Of the viral diseases, chronic bee paralysis and, according to earlier data, sacbrood, have long been suspected to be present in Hungary based on clinical symptoms, however, due to the limited availability of specific antisera it has not been possible to confirm their presence serologically (Szücs, 1973; Koltai, 1985; Békési and Rusvai, 1998).

Acute bee paralysis virus (ABPV) was originally discovered during laboratory infection experiments (Bailey et al., 1963) and, until recently, was never associated with disease or mortality of bees in nature. However, the virus is commonly present in small amounts in apparently healthy bees, especially in the summer, but it may normally only be detected indirectly, by sensitive infectivity tests (Bailey et al., 1981). By this means ABPV has been detected in live adult bees in France, Italy, Canada, New Zealand and Australia. In contrast,

large amounts of ABPV have been detected directly, by serology, in individual dead adult bees and brood from colonies in several countries in Europe and in the USA severely infested with *V. jacobsoni*. The evidence from a number of different sources suggest that ABPV infection is linked to the mortality of mite-infested colonies (Kulincevic et al., 1990). Laboratory experiments have also demonstrated that the mite acts as a virus vector and can transmit infection from severely infected individuals to healthy bees and brood.

In the investigations reported here ABPV was not detected directly by serology in dead or diseased adult bees or brood from affected colonies and unequivocal proof of the cause of the observed mortality has not been established. However, ultrathin sections of the tissues of naturally infected bees revealed the presence of crystalline arrays of virus particles of the same size as ABPV. The field symptoms also suggested a paralytic disease.

Like most virus diseases, the virus diseases of bees cannot be controlled by medication. Treatment of the underlaying problem may bring improvement in viral infections that occur in close association with specific pathogens or syndromes. Thus, the control of *N. apis* diminishes the severity of infection caused by black queen cell virus (BQCV), bee virus Y (BVY) and filamentosus virus (FV), and the prevention of dysentery or elimination of its cause has a similar effect on infection by bee virus X (Bailey and Ball, 1991; Allen and Ball, 1996) In laboratory experiments the mite *V. jacobsoni* has been shown to transmit a number of unrelated honey bee viruses (Ball, 1989), but it is likely that those which predominate and which are economically important in nature are infective for both adult bees and pupae by introduction into the haemolymph. Therefore, effective control of the parasitic mite is essential to reduce colony mortality due to associated virus infections (Békési and Rusvai, 1998).

As *V. jacobsoni* is regarded as a source of major economic losses in Hungary, it is important that the factors aggravating these losses and potentially contributing to severe bee mortality to be elucidated. An effective control strategy can be developed only by establishing a precise diagnosis and by ruling out other causative agents in all cases. Further studies are needed to determine the incidence and prevalence of bee viruses in Hungary and their contribution to the mortality of colonies infested with the mite.

Chapter 2

Detection of acute bee paralysis virus by RT-PCR in honey bee and *Varroa destructor* field samples: Rapid screening of representative Hungarian apiaries

2.1. INTRODUCTION

Acute bee paralysis was diagnosed first by Bailey et al. (1963) as an inapparent infection of adult honey bees. Since that time the presence of the virus has been detected in several countries throughout Europe, including Hungary (Békési et al., 1999). ABPV is considered to be a common infective agent present in a high proportion of apiaries, causing hidden infections (Hung et al., 1996c) but resulting in losses only in colonies heavily infested with the parasitic mite *Varroa destructor* (Ball 1985, Ritter et al., 1984). This mite had been previously identified as *Varroa jacobsoni*, but the type infesting *A. mellifera* was recently taxonomically changed to *V. destructor* (Anderson, 2000; Anderson and Trueman, 2000). The mite is considered to act as an activator of the inapparent infection and also as a virus vector transmitting ABPV (Ball and Allen 1988, Bowen-Walker et al., 1999). This supposition was supported by the detection of the virus in the mites by the use of indirect ELISA (Allen et al., 1986). The role of *Varroa destructor* as a predisposing factor and vector was also reported in the case of other honey bee pathogens (Abrol 1996, Brødsgaard et al., 2000). The term “bee parasitic mite syndrome” has been used for the disease complex, that is observed in colonies infested with mites and infected with viruses simultaneously (Shimanuki et al., 1994, Hung et al., 1995) and accompanied with high mortality.

Several hypothesises has been formed to explain which effects are responsible for causing the symptoms. The feeding activities of *V. destructor* can reduce the protein content of the hemolymph (Glinski and Jarosz, 1985), cause weight loss, and reduce longevity in the parasitized bee (De Jong and De Jong, 1983). Furthermore there are hypotheses directly involving the ABPV. Faucon et al. (1992) showed that *V. destructor* could transmit ABPV into a bee’s haemolymph when the mite feeds. Ball (1989) showed that *V. destructor* collected from naturally infested colonies transmitted ABPV and other viruses to healthy test pupae. Adult bees in which the virus has been activated or injected by *V. destructor* are probably able to infect young larvae by secreting the virus in gland secretions that are fed to the larvae before the adult bee succumbs (Ball and Allen, 1988).

Studies on inapparent infections of ABPV (and Kashmir Bee Virus) by Shimanuki et al. (1994) suggest that the impact of the mite is mainly activation and not transmission of the virus. Their work also indicated that mere piercing by the mite did not activate an infection. Referring to the laboratory experiments by Ball (1989) and Shimanuki et al. (1994) opens for the possibility that the detection of ABPV in the control pupae could have been the result of an activation of an inapparent infection, elicited by the feeding of the mite and not a transmission.

Besides the role of the mite (whether *V. destructor* transmits the virus or just activates an inapparent infection), the pathogenicity of the virus and its relationship to the mite infestation seems to be far from being understood. The importance and consequences of the viral infections of the honey bee, among which ABPV is one of the most frequent one in many countries (Vecchi et al., 1990, Ruzicka 1991), is also not fully appreciated. For example in Britain where the parasitic mite *Varroa destructor* had not occurred in the time of their investigations, Bailey and Ball reported (1991) that ABPV had never been associated with disease or mortality in nature. The virus appeared to be contained within the tissues that are not directly essential to the life of the bee. Infectivity tests made by Bailey and Gibbs (1964) estimated that live adult bees in the summer could contain as much as 10^6 virus particles without showing signs of paralysis and without any increase in mortality. In such an inapparent infection the virus must be contained in non-vital tissues i.e. fat-body cells, and the replication of the virus must be suppressed. This statement is supported by the fact that in other tests, where the virus is injected directly into the blood, as few as 10^3 virus particles can cause acute paralysis (Ball, 1985). Activation of ABPV may happen by piercing the body wall of the bee, which then soon after will become systemically infected and succumb. Alternatively, when the mite pierces the tissues it causes damages which might enhance the release of the virus and allow it to replicate. Another hypothesis is that the mite activates the virus by the introduction of foreign proteins such as the mite's digestive enzymes released into the blood while sucking.

Studies from Eastern Europe and America, reveal that ABPV may be a major cause of death in bee colonies infested with *Varroa destructor* (Batuev, 1979, Carpana et al., 1991, Österlund, 1998). What is more, according to records from Belize and Nicaragua ABPV was detected in large amounts in dead adult bees and diseased brood and yet it is reported that *Varroa destructor* is absent from both countries (Allen and Ball, 1996). In Hungary large amounts of ABPV were detected in 1998 during an outbreak when characteristic symptoms of paralysis were observed and other causes leading to increased mortality were excluded (Békési et al. 1999).

The difficulties of the diagnosis of ABPV may also contribute to the contradictory opinions on the significance of the infection. The conventional diagnosis of ABPV infection, like in the case of the other bee viruses, is based on the detection of the virus from homogenates of bees using electron microscopy directly or from homogenates of pupae following inoculation with the test material (Vecchi et al., 1990). The latter diagnostic method is labour and time consuming and also season dependent, since pupae can be collected only in spring and summer. Electron microscopy generally is complemented by agar-gel immunodiffusion (AGID) test, since several bee pathogen viruses are morphologically similar to ABPV. This procedure has a low sensitivity, requires the costly development of immune-reactive sera, and is not suitable for large-scale screening. The method of indirect ELISA worked out by Allen et al. (1986) was very sensitive but also immune-serum dependent.

Recently the use of PCR in the direct diagnosis of bee virus infections was shown to be a very appropriate tool, to overcome the aforementioned difficulties of the diagnosis of bee virus infections: it is not dependant on immune-serum, there are no cross-reactions and the diagnosis can be supported by genetic identification using the amplicons. These advantages were utilized by Benjeddou et al. (2001) when they developed an RT-PCR method, which was used for testing laboratory specimens containing ABPV in high concentration. Field samples were not included in their investigations and the sensitivity of the system was not compared to any other classical method of virus identification and/or diagnosis.

To collect information on the connection between virus infection, mite infestation and clinical symptoms observed in a colony or among an apiary, it is necessary to trace the spread and circulation of ABPV. For this work a sensitive, reliable and high throughput approach is needed. The polymerase chain reaction following reverse transcription (RT-PCR) has been successfully applied for the diagnostics of sacbrood virus (Grabensteiner et al., 2001), Kashmir bee virus (Hung and Shimanuki, 1999), and recently on black queen cell virus (BQCV) and ABPV as well (Benjeddou et al., 2001). In this latter report stock virus from artificially infected pupae was tested. In the present study, we report on a RT-PCR method for the detection of the ABPV genome in field specimens.

2.2. MATERIALS AND METHODS

2.2.1. Samples and sampling

Three categories of samples were investigated:

A: Apparently healthy adult bees, *A mellifera* were sent by twelve volunteers from the five regions of Hungary. The volunteering beekeepers had 30 to 150 colonies, and sent samples during the test period from three randomly selected colonies. The same three colonies of the apiaries were tested in the spring (March-May) and in the autumn (August-October) of 1999 and 2000. All together 114 colonies were sampled. These samples contained 100-500 adult bees.

B: In addition, samples sent by beekeepers following a sudden collapse of several colonies (six apiaries: five pooled samples from five apiaries each collected from four colonies, and ten individual colony-samples sent by one apiary), and following unusually high winter mortality (further two apiaries, one pooled sample from four colonies of each apiary) were analysed. The amount of dead and moribund bees sent for investigation varied between 0.5-2.5 kg per apiary.

C: Four further symptomless apiaries not participating in the survey were also sampled (pooled samples, each collected from four randomly selected colonies). These samples also contained 100–500 adult bees.

All samples were personally transported or sent by express mail in carefully wrapped paper sacks or boxes.

Cesium chloride gradient purified virus suspension from 100 white or light brown eyed pupae artificially infected with 10 µl of the ABPV isolated by our group in 1998 (Békési et al., 1999) served as positive control.

2.2.2. Parasitological investigations

The samples were checked for the presence of Varroa mites, and mites from the different samples were collected separately for PCR testing.

2.2.3. Preparation of specimens for the PCR

Following parasitological investigations 50 adult bees were homogenized in 10 ml phosphate buffered saline (PBS), centrifuged at 1500 g for 10 min. Supernatants were transferred into sterile tubes and centrifuged again at 12 500 g for 15 min to clean them from cell debris and bacteria. Mite homogenates (if mites could be collected from the samples) were tested by PCR, and prepared according to the same protocol using 0.5 ml PBS. The number of mites collected from one sample varied between 1 and 300.

The viral RNA was isolated from the clear supernatants using QIAamp viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions, and reverse-transcribed into cDNA using oligo(dT) primer method with RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania).

2.2.4. Titration

The sensitivity of the RT-PCR was tested on tenfold dilutions of the gradient purified virus suspension. The same suspension was also tested in agar-gel immuinodiffusion (AGID), but in the latter test twofold dilutions of the same virus suspension were reacted with the ABPV specific rabbit serum.

2.2.5. Agar-gel immunodiffusion

Bee homogenates were measured into the 32 µl wells of 0.8% agar-gel produced according to standard methods (Hoskins, 1967) and reacted with ABPV specific antisera raised in rabbits and kindly provided by dr. G. Topolska (Warsaw Agricultural University, Warsaw, Poland). Results were read after 48 hours incubation at 37 ^{o}C.

2.2.6. Primers

A pair of oligonucleotide primers were designed from the partial sequence of ABPV genome published by Ghosh et al. (1999) in GenBank (NCBI, http://www.ncbi.nlm.nih.gov, accession number AF126050), using Primer 2.0 software (Scientific and Educational Software, Serial No. 50178). The code and the nucleotide sequences of the selected primers were: ABPV1 (5'-CATATTGGCGAGCCACTATG-3') and ABPV2 (5'-CCACTTCCACACAACTATCG-3').

2.2.7. Amplification conditions

Amplification was performed in 50 µl reaction mixture containing 10 pmol deoxynucleozide triphosphate (dNTP) mix, 1.5 mM $MgCl_2$, 50 pmol of the appropriate primers, 2 µl cDNA and 1.5 U Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania). This reaction mixture was subjected to 40 cycles with an initial incubation at 94°C for 3 min, followed by heat denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and DNA extension at 72°C for 1 min. Thereafter the samples were maintained at 72°C for 2 min for the final extension.

We used the cDNA of the purified Hungarian isolate as positive control (the strain was propagated by inoculation of PCR-negative pupae as described above) and a reaction mixture without cDNA as negative control.

2.2.8. Identification of the PCR product

Following the RT-PCR reaction, 10 µl of the amplicon was electrophoresed in a 1% Tris borate-EDTA buffered agarose gel containing 0.5 µg/ml ethidium bromide, at 80 V for 1 hour. The bands were visualized by UV translumination at 312 nm and photographed by a Kodak DS Electrophoresis Documentation and Analysis System using the Kodak Digital Science 1D software. Product sizes were determined with the reference to λ phage DNA cleaved with *Pst*I restriction enzyme.

2.2.9. Nucleotide sequencing and computer analyses

The PCR product amplified by ABPV1 and ABPV2 primers from inoculated pupae was electrophoresed in a 0.8% Standard Low-m_r Agarose Gel (Bio-Rad, Richmond, CA, USA) at 80 V for 2 hours. The position of the amplicon was checked with short translumination, and than it was excised from the gel and extracted using QIAquick Gel Extraction Kit (Qiagen, Germany). Fluorescence-based sequencing PCR was performed at the Biological Research Centre of the Hungarian Academy of Sciences in Szeged, employing an AbiPrism 2.1.0 automated sequencing system. The primers used for sequencing were identical to those in the RT-PCR reaction.

The nucleotide sequences were compared using FASTA (NCBI) and BioEdit 4.7.8 software programs and verified by visual inspection. The multiple alignments were performed using BioEdit 4.7.8 and Clustal W 5.a software programs.

2.3. RESULTS

2.3.1. Electrophoresis of the PCR product

Following the RT-PCR reaction with the ABPV1 and ABPV2 primers on the isolated RNA of the purified ABPV suspension an approximately 400 bp product was detected. By the amplification the virus signal was always detected in the artificially infected pupae but not in the non-infected ones (Figure 1.).

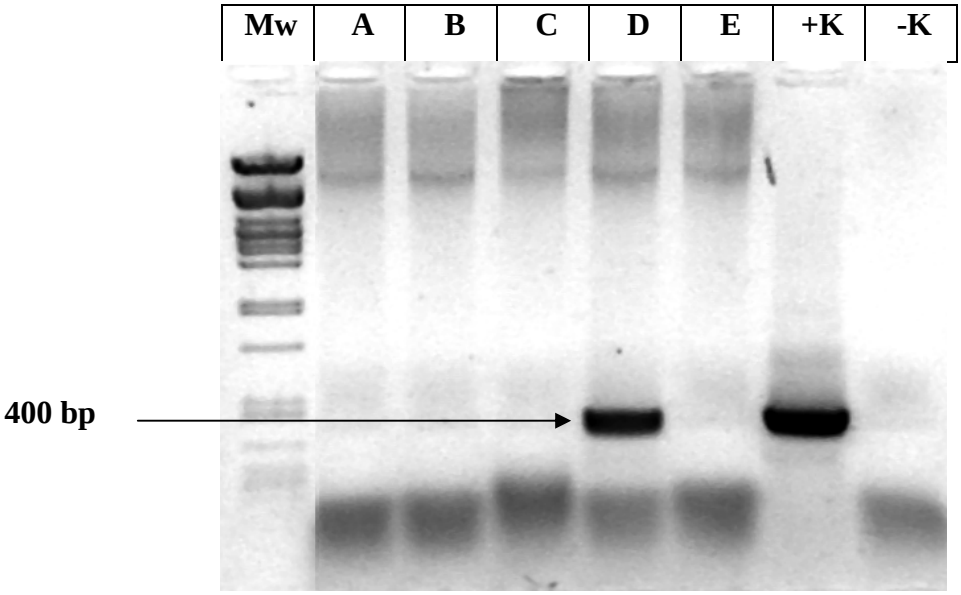

Figure 1.: Diagnostic RT-PCR in agarose gel electrophoresis. Mw standard (*Pst*I cleaved λ-phage DNA), A, B, C, E: negative field samples; D: positive field sample, +K: positive control (ABPV inoculated into pupae), -K: negative control.

2.3.2. Titration

The results of the titration by RT-PCR are shown by Figures 2/A and 2/B. Shortly: in the AGID tests the suspension of artificially infected pupae gave positive reaction only with the concentrated and with the 1:2 to 1:16 dilutions of the homogenate. With the 1:32 diluted homogenate no visible precipitation line could be detected. The same virus suspension gave a clear, well visible band by PCR even if it was diluted 10^4 times (Figure 2/B). Two strongly

positive samples were also titrated (Figure 2/A) for sake of testing the sensitivity on field samples too: neither of them was positive with AGID. Even the sample No155. giving the strongest signal in RT-PCR and showing a well visible band in 1:100 dilution was negative by AGID, as all field samples tested by AGID for comparison. The majority of these positive samples (like No80, KATKI/G, May 2000) when titrated with RT-PCR were positive in the not diluted and in the tenfold dilution.

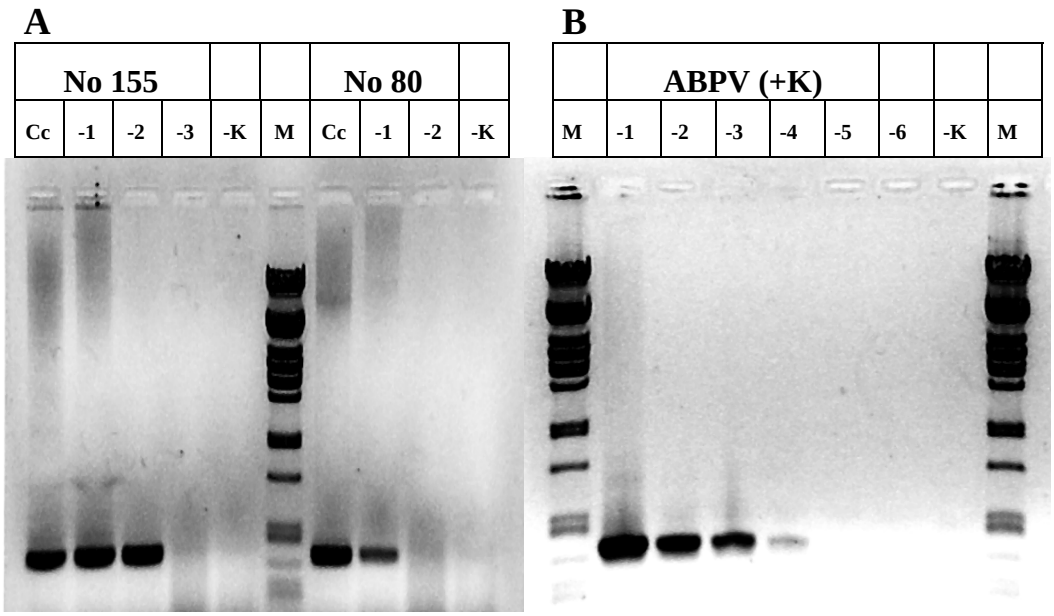

Figure 2.: RT-PCR titration A: positive field samples: No155 (strong positivity) No80 (lower positivity), B: positive control: ABPV inoculated into pupae, Cc: undiluted homogenate, -1: tenfold dilution, -2: 100 fold dilution, -3: 1000 fold dilution, etc., M: standard (*Pst*I cleaved λ-phage DNA) -K: negative control

2.3.3. Nucleotide sequencing and computer analysis

The fragment amplified with the ABPV1-ABPV2 primer-pair was sequenced and a 398 base long sequence was identified. The sequence was aligned to the GenBank database and the highest identity (93%) was found with the ABPV complete genome (Govan et al., 2000; AF150629) (Figure 3). The sequence was deposited in the GenBank database under accession number AY059372.

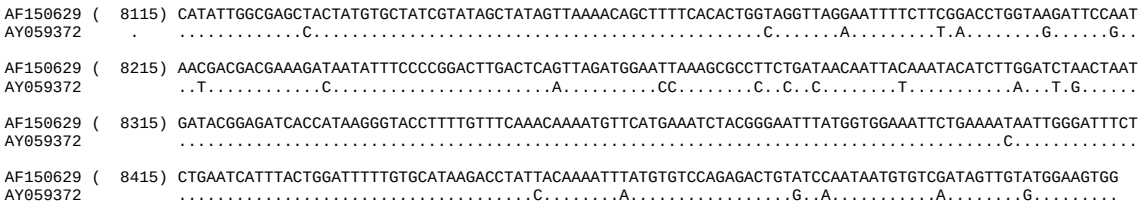

Figure 3.: Nucleotide sequence of the amplicon of the diagnostic PCR product (AY059372) aligned with the complete sequence deposited in the GenBank (AF150629, Govan et al., 2000)

2.3.4. Survey on the occurrence of ABPV in Hungarian apiaries

Besides investigations on artificially infected pupae using this diagnostic primer-pair, a survey was started on field samples collected from volunteering apiculturists living at different locations in Hungary. The samples were checked for the presence of *Varroa destructor*, and were tested by RT-PCR to detect ABPV specific nucleic acid, first only in the bees but later also in the mites.

Twelve apiaries had sent samples on a regular basis (Category A, no clinical symptoms or losses) and eight of them (66.6 %) proved to be infected at least once within the test period of 2 years (Table I). Considering the individual colonies, the infection rate was less: 14 from 114 colonies (12.2 %). Since not all colonies sampled in an apiary in a certain season were positive, the infected and non-infected colonies may be present simultaneously. Furthermore, presence of the virus in a certain apiary was inconsistent, some colonies and apiaries were found negative in one season and became positive in the next. For example apiary PT/B sent bees from colony No 62, 63 and 98 throughout the investigation period, of which No 98 was infected in spring and autumn of 1999, but not in 2000. Contrary to that SzL/Zs was sending samples from colonies No 24, 33 and 43 and in the spring of 1999 colonies No 33 and 43 were infected, but in the spring of 2000, No 33 was negative. In the autumn of 1999 and 2000 all three colonies were negative.

The eight apiaries sending samples for aetiological investigations (Category B) were infected in a much higher ratio. The virus-specific nucleic acid was detected in samples from seven apiaries (either in the bees, or in the mites, or in both), what means a positive rate of 87.5 % (Table II.). In one apiary colonies were tested individually: five out of ten (50 %) were positive, a rate higher than the 12.2 % found among the colonies of symptomless, regularly tested apiaries. The samples sent from these apiaries struggling with high mortality and clinical symptoms had been pooled by the bee keepers from four colonies (see Methods) and

arrived for aetiological investigations (including nosema infection, insecticide-intoxication, which latter was excluded by parallel investigations in each case). In the “problematic” cases seven of eight apiaries proved to be infected (87.5 %) with ABPV, and the viral nucleic acid was also detected in mite homogenates. Altough the sampling was different in Category A and B (apparently healthy bees and low number of mites on one side, dead and moribund bees, high number of mites on the other), the regular presence of the virus in the “problematic” apiaries is remarkable. The randomly selected colonies of the four symptomless apiaries not participating in the regular sampling, but also tested on request within the period (Category C) were negative.

Table I.: Results of the survey of the RT-PCR test on samples sent by volunteering bee keepers. Numerators indicate how many samples proved to be positive (infected/infested) from a total indicated by the denominators. N = not tested (For various reasons: i.e. samples were not sent, one volunteer had to give up bee-keeping due to health problems, etc.). Samples from which mites were collected and tested with RT-PCR are signed with asterisk.

Code of the apiary	**Samples**							
	1999 spring		1999 autumn		2000 spring		2000 autumn	
	Virus	Mite	Virus	Mite	Virus	Mite	Virus	Mite
PT/B	**1**/3	0/3	**1**/3	1/3	0/3	0/3	0/3	0/3
KF/K	0/3	2/3	0/3	2/3	**1**/3	1/3	**1**/3	2/3*
PL/D	0/3	2/3	0/3	2/3	**2**/3	3/3	N	N
PF/B	0/3	1/3	0/3	0/3	0/3	0/3	N	N
BI/L	0/3	1/3	0/3	2/3	N	N	0/3	0/3
HD/K	0/3	2/3	0/3	1/3	N	N	0/3	0/3
CsP/K	**1**/3	3/3	0/3	0/3	N	N	N	N
SzL/B	0/3	0/3	0/3	1/3	0/3	0/3	N	N
SzL/Zs	**2**/3	2/3	0/3	3/3	**1**/3	2/3	0/3	3/3*
SzB/M	N	0/3	0/3	3/3	0/3	3/3	**1**/3	3/3*
PJ/K	N	2/3	**1**/3	3/3	0/3	1/3	0/3	3/3*
HM/K	N	3/3	**2**/3	2/3	0/3	0/3	0/3	0/3

Table II.: Results of the aetiological investigations by ABPV RT-PCR test on samples sent by bee keepers struggling with problems due to unknown reason, but not participating in the survey (Category B). Samples from which mites were collected and tested with RT-PCR are signed with asterisk.

Code of the apiary	Sampling	Cause of investigation	Result of RT-PCR	Auxilliary diagnosis
CsSzM/K	Apr.1999	Depopulation	Positive	Nosemosis, no Varroa infestation
KATKI/G	May 2000	Depopulation	5 positive in 10	Heavy varroosis in 6 from 10*
HTGy/T	Febr. 2001	Poor wintering	Positive	Heavy varroosis, Varroa PCR positive*
GA/H	Febr. 2001	Poor wintering	Negative	Heavy varroosis, Varroa PCR positive*
SzI/Kh	May 2001	Paralysis, depopulation	Positive	Nosemosis, no Varroa infestation
KI/K	May 2001	Paralysis, depopulation	Positive	Nosemosis, no Varroa infestation
MI/Kh	May 2001	Depopulation	Negative	
SzGy/Kh	May 2001	Paralysis, depopulation	Positive	Nosemosis, no Varroa infestation

From 6 samples, *Varroa destructor* were analysed for the presence of the virus. In the last phase of the survey (2000 autumn, 2001 spring) Varroa mites were also tested for the presence of ABPV. From the "regular" adult bee samples (Category A) containing 200-500 bees sent by the volunteers, a rather low number of mites could be collected (1-15), and the virus was not detected in their homogenates. In the "problematic" cases (Category B) a mass of dead bees sometimes swept from the bottom of the hives and weighing between 0.2 – 2.5 kg was sent, from which 200-300 mites could easily be collected. Although the presumed role of *Varroa destructor* mite as a virus carrier and a possible vector has been supported by the demonstration of the virus in the mites by our RT-PCR method and previously by ELISA (Allen et al., 1986), the presence of virus specific nucleic acid could not be demonstrated in some of the mite homogenates by our RT-PCR investigations, even if the colonies were heavily infested with mites, and simultaneously heavily infected with ABPV.

Only in 2 samples (pooled samples of 4 colonies of which all bees had died during the winter) was the virus detected. In the negative samples (Table I.) the number of the mites collected varied between 1 and 15, while from each of the 2 positive samples (Table II.) 300 mites were retrieved. This indicates that not all the mites are carrying the virus, and the rearing number of mites tested will increase the probability of detection.

In addition to *Varroa destructor* infestation, nosema disease was also regularly observed in the "problematic" apiaries (Category B) together with ABPV infection. Heavy varroosis was observed in two while *Nosema apis* Zander was detected in four of the eight apiaries that sent samples for aetiological investigations and were found ABPV positive (Table II.).

2.4. DISCUSSION

The diagnosis of viral infections in the honey bee has been rather complicated compared to other fields of veterinary virology. The lack of characteristic clinical symptoms and pathological alterations makes the recognition of most diseases difficult. Since cell cultures of bee origin are not available, the only way of isolation and artificial propagation of viruses is the experimental infection of pupae. Furthermore, as bees do not produce antibodies against pathogens, the indirect determination of viral infections (widely used in other fields of veterinary praxis) is not possible. Electron microscopy and serological methods to detect sometimes very low amounts of viral antigen in field samples contribute to the difficulties described in the introduction. Therefore the RT-PCR method worked out to amplify unique regions of the viral nucleic acid present in the samples seems to be very promising in the diagnosis of bee virus infections.

The RT-PCR method worked out by our group is based on a primer pair (ABPV1 and ABPV2) designed within the structural protein region of the viral genome, producing an amplicon between base pairs 8107 and 8504. To test the reliability of our RT-PCR in the diagnostic work, the sensitivity of our system was compared to AGID, the only other widespread diagnostic method. It was not surprising, that the same virus suspension gained by artificial infection of pupae and giving a positive result up to 1:16 in the AGID test, proved to be positive up to $1:10^4$ dilution in RT-PCR.

Using this very sensitive, fast and specific method in a survey we have detected ABPV infection in apparently healthy bee colonies as well as in colonies with high mortality. Furthermore the virus was also detected in *Varroa destructor* samples collected from the mite infested colonies.

Besides varroosis, nosema infection was also frequently detected in the apiaries struggling with severe losses (Category B). This fact raises the possibility that virus infections may be activated or the losses caused by these infections may be enhanced by other predisposing factors in insects too. This phenomenon is frequently observed among the virus infections of vertebrates, where predisposing factors (i.e. shipping, immunosuppression, crowding, etc.) or co-infections (chlamydia, mycoplasma) activate virus infections followed

by bacterial secondary infections (Yates, 1982, Nordengrahn et al., 1996). None of the factors alone will lead to severe disease or economic losses, but the cumulative effect of the factors is frequently fatal. It seems, that the existence of these “polyfactorial” disease complexes may not be excluded in the case of invertebrates either. Our survey does not help to find an answer to the question whether the virus or the cofactor (varroosis, nosema disease) is more important, and which of them may be considered as primary agent.

Chapter 3

Phylogenetic analysis of acute bee paralysis virus strains

3.1. INTRODUCTION

The acute bee paralysis virus (ABPV) was first described as inapparent infection of the honey bee (*Apis mellifera*) (Bailey et al., 1963). The presence of the virus has been reported from several countries worldwide (Carpana et al., 1991, Faucon et al., 1992, Hung et al., 1995, Nordstrom et al., 1999, Topolska et al., 1995). ABPV is considered to be a common infective agent of bees, which is frequently detected in apparently healthy colonies. However, it has been presumed that this virus plays a role in cases of sudden collapse *Apis mellifera* colonies infested with parasitic mite *Varroa destructor* (Békési et al., 1999, Nordstrom et al., 1999) (former name: *Varroa jacobsoni*). ABPV was suggested to be a primary cause of bee mortality in such colonies in Germany (Ball and Allen, 1988), Yugoslavia (Kulincevic et al., 1990), France (Faucon et al., 1992), and the United States of America (Hung et al., 1996a), respectively.The world wide spread of *Varroa destructor* in honey bee colonies has significant influence on virus infection of bees. On the one hand the Varroa mite is a possible vector for the virus (Ball and Allen, 1988, Bowen-Walker et al., 1999), on the other hand, the mite weakens the bees and activates the viral infection, leading to clinical symptoms and severe losses in the apiaries (Ball and Allen, 1988, Brødsgaard et al., 2000, Ritter et al., 1984). Some scientists however, doubt the essential role of both the mites (Allen et al., 1986, Hung et al., 1995) and the viruses (Hung and Shimanuki, 1999, Hung et al., 1999) in the so-called "bee parasitic mite syndrome" (joint infection of viruses, *Acarapis woodi* and *Varroa destructor*) (Shimanuki et al., 1994). In the UK, whereas, not ABPV but slow paralysis virus (SPV) was found as an agent responsible for the rapid decline and death of many Varroa mite infested colonies (Ball, 1997). The contrary findings on the role of ABPV in the mortality of honey bee colonies might be, to some extent, explained by the presence of genetically diverse virus strains with different virulence.

ABPV has a single stranded, positive sense, polyadenylated RNA genome comprising of 9,491 nucleotides (nt). The complete nucleotide sequence was determined recently (Govan et al., 2000). The genome encodes for two open reading frames (ORFs). ORF1 encodes the non-structural proteins (RNA-dependent RNA polymerase, helicase, protease), while ORF2 encodes the three major structural proteins (35, 33, and 24 kDa) and a minor protein (9.4 kDa) transcribed together in a capsid polyprotein (Govan et al., 2000). ABPV belongs to the

Picornaviridae family, although its genomic RNA is considerably longer than that of picornaviruses (approx. 9,500 nt vs. 7,500 nt), and it differs also in its genome organization compared to other picornaviruses. It was therefore suggested to classify ABPV together with some other picorna-like viruses infecting insects into a novel taxonomically group called cricket paralysis-like viruses (Govan et al., 2000). Antigenic relationship (Allen and Ball, 1996) and sequence similarities can be observed between ABPV and the Kashmir bee virus, another picorna-like virus infecting honey bees.

Several studies are discussing the importance of viruses in diseases of bees. To date already 18 different honey bee viruses have been described (Allen et al., 1986, Grabensteiner and Nowotny, 2001). Most of them often cause inapparent infections. Such infections are sometimes exacerbated and activated by subservient environmental factors. Besides mite infestation and bacterial infections, pollution and comprehensive use of chemicals and insecticides in agricultural technology triggers environmental stress in bees (Bromenshenk et al., 1991, Fleche et al., 1997, Kevan, 1999). In certain cases even the acaricides used against Varroa mites have to be blamed for the suppression of the bee's immune system (Brødsgaard et al., 2000). In addition, the notable decrease of natural pollinator species - also due to environmental pollution - emphasizes the significance of honey bees in the pollination of plants, thus the importance of healthy bees is far beyond honey production (Spira, 2001).

The evaluation of the significance of bee viruses is hampered by diagnostic problems. It is difficult to isolate bee viruses due to the lack of permanent cell lines of bee origin. The only way for propagation of honey bee viruses is the experimental infection of bee pupae or newly emerged bees. Since honey bee virus infections are widespread, often without causing symptoms, an experimental infection may activate persistent infections with other viruses present in the apparently healthy pupae resulting in a mixture of different viruses in the pupae-extracts. Furthermore, the morphological appearance and physico-chemical features of most of the honey bee viruses are quite similar; the use of some classical virological methods such as electron microscopy is therefore difficult for the identification of bee viruses. Several methods have been developed to detect viral antigens in clinical samples, such as immunodiffusion, enzyme-linked immunosorbent assay (ELISA), chemiluminescent Western blotting, and radioimmunassays (Allen and Ball, 1995, Allen et al., 1986, Stoltz et al., 1995). The disadvantage of these techniques is that specific antisera are required. Raising specific antisera is complicated regarding to the difficulties in the production of large amounts of pure virus suspension.

Reverse transcription-polymerase chain reaction (RT-PCR) assays have been developed for the detection of virus-specific RNA of certain honey bee viruses such as SBV (Grabensteiner et al., 2001), Kashmir bee virus (Hung et al., 1996c), black queen cell virus and ABPV (Benjeddou et al., 2001), respectively. RT-PCR proved to be a quick, specific, sensitive and reliable technique for the detection of honey bee virus infections. The method can be easily established in independent laboratories and standardized using identical primers and protocols. A further advantage of RT-PCR is that genetic comparison and classification of different virus strains can be rapidly carried out by sequencing of the appropriate PCR products (Grabensteiner et al., 2001).

The aim of this study was to establish RT-PCR assays for the sensitive direct detection of ABPV in clinical samples, to reveal and compare the nucleotide sequence of the ABPV capsid polyprotein gene region of different European isolates, and to assess the genetic relationship between ABPV strains of distinct geographic origin.

3.2. MATERIALS AND METHODS

3.2.1. Samples

The ABPV isolates originated from infected honey bees collected in four different European countries. The samples from Austria (one), and Germany (three) were collected from outbreaks of acute bee paralysis, the Hungarian and Polish samples were taken from colonies showing clinical symptoms (Hungary – six, Poland – one), but also from obviously healthy colonies from apiaries participating in an ABPV survey (seven and three, respectively). The strains were isolated within a five-years period (1996 - 2000). Virus identification was carried out by agarose gel immundiffusion (AGID) test and electron microscopy (EM). The samples were also tested for other picorna-like honey bee viruses such as sacbrood and black queen cell virus. Each sample contained 50 to 60 dead honey bees, which were transported at -20°C and were stored at -80°C until investigated.

3.2.2. Isolation of RNA

The bees were homogenized either in liquid nitrogen or in sterile glass potters using sterile phosphate buffered saline (PBS). The homogenates were centrifuged at 20,000 × g for 10 min, and the supernatant was used for RNA extraction. In some cases the virus-containing samples were propagated by inoculation of virus-free pupae, and purified by cesium chloride

gradient ultracentrifugation followed by dialysis against PBS. RNA was extracted from 140 µl virus suspension using the QIAamp viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

3.2.3. Primer design

Six different primer pairs were selected based on the complete ABPV sequence (accession number AF150629) deposited at the GenBank database with the help of a Primer Designer program (Scientific and Educational Software, version 3.0). The oligonucleotides were designed in order to amplify overlapping PCR products comprising the entire structural protein gene region of ABPV (Figure 1). The sequences, orientations, locations and product sizes are shown in Table I. Nucleotide positions are referring to the ABPV sequence deposited under the accession no. AF150629. The oligonucleotides were synthesized by GibcoBRL Life Technologies, Ltd. (Paisley, Scotland, UK).

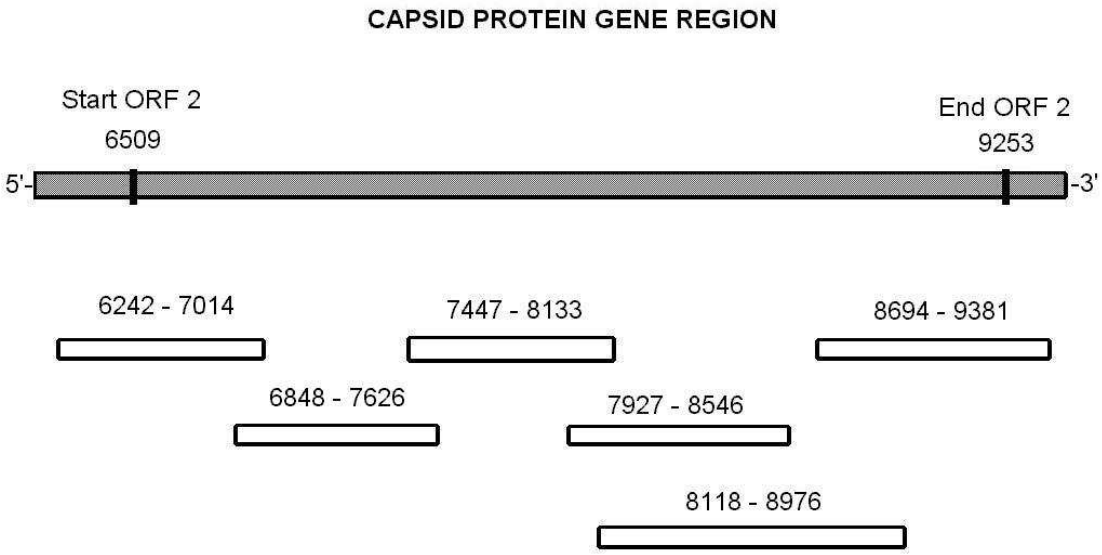

Figure 1.: Locations of the amplified RT-PCT products within the capsid polyprotein region (ORF 2) of the ABPV. Amplicons APV17f-18r, APV21f-22r, APV25f-26r, APV19f-20r, APV 23nf-24nr, and APV27f-28r, respectively.

Table I. Oligonucleotide primer pairs selected for ABPV RT-PCR

Primers[a]	Sequence (5´ to 3´)	Nucleotide positions[b]	Length of the amplified product (bp)
APV 17 f	TAT CAG AAG GCC ACT GGA GA	6242 - 6261	722
APV 18 r	TCC ACT CGG TCA TCA TAA GG	6995 - 7014	
APV 19 f	TCT TGG ACA TTG CCT TCA GT	6848 - 6867	778
APV 20 r	ATA CCA TTC GCC ACC TTG TT	7607 - 7626	
APV 21 f	TGC AGT TCC AGA AGT TAA GA	7447 - 7466	686
APV 22 r	ATA GTR GCT CGC CAA TAT GA	8114 - 8133	
APV 23n f	GTG CTA TCT TGG AAT ACT AC	7928 - 7947	618
APV 24n r	AAG GYT TAG GTT CTA CTA CT	8527 - 8546	
APV 25 f	GGA ACA TGG AAG CAT TAT TG	8694 - 8713	687
APV 26 r	AAT GTC TTC TCG AAC CAT AG	9362 - 9381	
APV 27 f	ATT GGC GAG CYA CTA TGT GC	8118 - 8137	858
APV 28 r	CGC GGT AYT AAG AAG CTA CG	8957 - 8976	

[a] f, forward; r, reverse

[b] Nucleotide positions refer to the published ABPV sequence (GenBank accession no. AF150629).

3.2.4. RT-PCR

Reverse transcription and amplifications were performed in a continuous RT-PCR method by employing the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany). Each 25 µl reaction mixture contained 5 µl of 5 × buffer (final $MgCl_2$ concentration 1.5 mM), 0.4 mM of each deoxynucleozide triphosphate (dNTP), 10 U rRNasin™ RNase Inhibitor (Promega, USA), 0.8 µM of the appropriate forward and reverse primers, 1 µl of enzyme mix (containing Omniscript™ and Sensiscript™ Reverse Transcriptases and HotStarTaq™ DNA polymerase) and 2.5 µl of template RNA. Reverse transcription was carried out at 50°C for 30 min. Following an initial denaturation at 95°C for 15 min, the reaction mixture was subjected to 40 cycles of heat denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and DNA extension at 72°C for 1 min, completed by a final extension of further 10 min at 72°C. The samples were kept at 4°C until electrophoresis was carried out. The reactions were performed in a Perkin Elmer GeneAmp PCR System 2400 thermocycler.

3.2.5. Gel electrophoresis

Following RT-PCR 3 µl of the amplicons were electrophoresed in a 1.2% Tris acetate-EDTA-agarose gel at 6 V/cm for 80 min. The gel was stained with ethidium bromide and the bands were visualized by UV translumination at 312 nm using a TFX 35M UV transluminator (Life Technologies, UK) and photographed with a Kodak DS Electrophoresis Documentation and Analysis System using the Kodak Digital Science 1D software program. Product sizes were determined with reference to a 100-bp molecular weight ladder (Amersham Pharmacia Biotech).

3.2.6. Nucleotide sequencing and computer analyses

The total amount of the amplicons was electrophoresed in agarose gel (as described above), the fragments were excised from the gel, and DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen, Germany) according to the supplier's instructions. To control the extraction efficiency and for estimation of the DNA content, 2 µl of the extracts were electrophoresed in agarose gel. Fluorescence-based direct sequencing was performed on the PCR products. The sequencing PCR was carried out using the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer) with AmpliTaq DNA polymerase. The reaction mixture contained 5 µl of Big Dye Terminator Reaction Mix, (comprising the necessary components in an appropriate buffer solution), 4 pmol of oligonucleotides (the same as for RT-PCR), 10 - 15 ng of template DNA (5-10 µl) and distilled water to a final volume of 20 µl. Sequencing PCR was performed in 30 amplification cycles of 96°C for 30 s (denaturation), 50°C for 10 s (primer annealing), and 60°C for 4 min (DNA extension). Thereafter, the products were precipitated with 70% ethanol containing 0.5 mM $MgCl_2$ by incubation at room temperature for 10 min, and the precipitates were centrifuged at 20,000 × g for 25 min. Each pellet was resuspended in 30 µl of ABI Prism template suppression reagent denaturing buffer (Perkin Elmer), and shortly before sequencing the samples were heated to 100°C for 2 min and quickly cooled by ice. The products were sequenced in both directions by the ABI Prism 310 genetic analyzer (Perkin Elmer) automated sequencing system.

The nucleotide and deduced amino acid sequences were compiled and aligned with the help of the Align Plus program (Scientific and Educational software, version 3.0, serial no. 43071). Discrepancies were revised by visual inspection. Phylogenetic analysis was performed using the Phylogeny Inference Program Package (PHYLIP) version 3.57c.

Bootstrap resampling analysis of 100 replicates were generated with the SEQBOOT program to prove the stability of the trees. Distance matrices were generated by the DNADIST/Neighbor-Joining and Fitch programs, using a translation/transversion ratio of 2.0.

3.2.7. Nucleotide sequence accession numbers

The ABPV sequences described in this paper were submitted to the GenBank database under accession numbers AY053366 - AY053385.

3.3. RESULTS

3.3.1. Analysis of ABPV samples by RT-PCR

Six different RT-PCR assays have been developed, employing primer pairs designed for the amplification of overlapping fragments, which cover the entire capsid protein gene region of ABPV (Table 1, Figure 1). To ensure that the RT-PCR assays detect nucleic acid from diverse ABPV strains, the reactions were performed on eleven samples, which originated from different European countries: besides one Austrian and three German samples three samples from Poland and four from Hungary were tested. All samples proved to be positive in all RT-PCR assays. The amplicons could be observed as clear and distinct bands of the expected molecular weight (Figure 2), even in the case of one German sample, which contained a mixture of ABPV and black queen cell virus. Amplification products never occured in the negative controls. For the amplification of the ABPV genomic region from nucleotide pos. 8044 to pos. 8512, the initially designed forward (APV 23 f) and reverse (APV 24 r) primers gave only weak signals in RT-PCR (Figure 2) and failed at sequencing. Therefore a new primer pair (APV 23 nf and APV 24 nr) was designed covering the same region. The new oligonucleotides operated properly (Figure 2), thus they were applied instead of the initially designed primer pair. The RT-PCR assay employing this newly designed primer pair (APV 23 nf and APV 24 nr) amplifies an ABPV genome region, for which sequence data of eleven (probably UK) isolates have been deposited in the GenBank database. In order to make the comparison of ABPV strains as comprehensive as possible, we used this RT-PCR assay for the amplification of the remaining ABPV samples, which we have received for analysis, i.e. one additional Polish and nine additional Hungarian samples. The RT-PCRs resulted again in pure products with identical size.

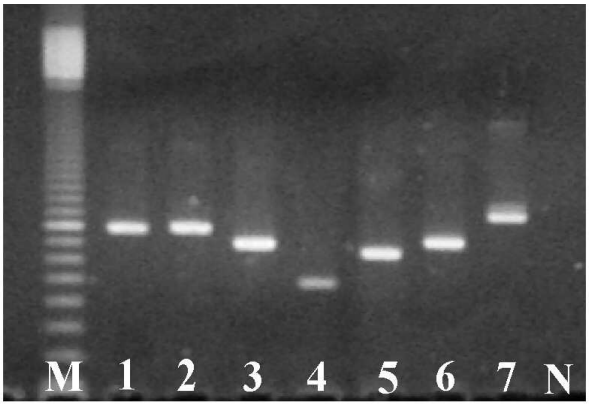

Figure 2.: Gel electrophoresis of RT-PCR products on the capsid polyprotein region of ABPV (Lane 1: APV 17f-18r, 2: APV 19f-20r, 3: APV 21f-22r, 4: APV 23f-24r, 5: APV 23nf-24nr, 6: APV 25f-26r, and 7: APV 27f-28r). Lane M: DNA size marker (100-bp ladder), lane N: negative control.

3.3.2. Sequence analysis and comparison

In total, 72 RT-PCR amplification products were sequenced in both directions. The sequences were identified as ABPV sequences by BLAST search (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA). The sequences derived from the six overlapping PCR products of all analysed ABPV samples were compiled and aligned, using the published complete ABPV sequence (Govan et al., 2000) as a reference. In total, sequence information of a 3071 nt fragment was available from every investigated isolate, which covers 32.4% of the ABPV genome including the entire capsid protein gene region. The isolates showed 94 - 95% identity compared with the reference strain. When analysing the sequences of the strains, two German isolates proved to be fully identical, thus only one of them was used in the further studies. In Figure 3 the nucleotide alignment is presented (nt pos. 6283 to pos. 9353 of the ABPV reference strain).

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AF150629 (6283) TAATGGTTATGCTCAGGAGAGATTCCTAAATTACTACTTGTAATTTGGGAATCGCAACACAACATGGTTACCCATAGATTGAGGAAATTTCCAATAAACT
Germany1        .......................T.........T.........AA....T.......................G.................G....
Germany2        .......................T.........T.........AA....T.......................G.................G....
Austria         .......................T.........T.........AA....T.......................G.................G....
Poland1         .......................T.........T.........AA....T.......................G.................G....
Poland2         .......................T.........T.........AA....T.......................G.................G....
Poland3         .......................T.........T.........AA....T.......................................................
Hungary1        .......................T.........T.........AA....T.......................A.......................
Hungary2        ....................G...T.........T.........AA....T.......................A.......................
Hungary3        .......................T.........T.........AA....T.......................A.......................
Hungary4        .......................T.........T.........AA....T.......................A.......................

AF150629 (6383) CAGTATTAAGGCTTGTTGTGTTGGACAAGGTGCCCTATTTAGGGTGAGGAGCCTTACTGGCAGCCCCAGTGAATCCTCCATTGGATAGGAACAGCTATAT
Germany1        ..A.C.....................................................G.....................T.....................
Germany2        ..A.C.....................................................G.....................T.....................
Austria         ..A.C.....................................................G.....................T.....................
Poland1         ..A.C.....................................................G.....................T.....................
Poland2         ..A.C.....................................................G.....................T.....................
Poland3         ....T.....................................................................T.....................
Hungary1        .GA.T.....................................................................T.....................
Hungary2        ..A.T.....................................................................T.....................
Hungary3        .GA.T.....................................................................T.....................
Hungary4        .GA.T.....................................................................T.....................
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AF150629 (6483) TGGGTAGTTGTAGCAGTTGTATTCAAATGAATGCAGCGTTCCGAAATATCATACCTGCCGATCAAGAAACAAATACTTCCAACGTACATAATACGCAACT
Germany1        ..............................................C.............................................
Germany2        ..............................................C.............................................
Austria         ..............................................C.............................................
Poland1         ..............................................C.............................................
Poland2         ..............................................C.............................................
Poland3         ......................T.G....................C.............................................
Hungary1        ......................T.....................CT.............................................
Hungary2        ......................T.....................CT.............................................
Hungary3        ......................T.....................CT.............................................
Hungary4        ......................T.....................CT.............................................

AF150629 (6583) CGCGTCGACCTCTGAAGAAAACTCAGTTGAAACGGAACAAATCACCACCTTTCATGATGTGGAAACTCCAAATAGGATCAATACCCCCATGGCTCAAGAC
Germany1        ..................................................................G...................T
Germany2        ..................................................................G...................T
Austria         ..................................................................G...................T
Poland1         ....................T.............................................G...................T
Poland2         ..................................................................G...................T
Poland3         ..................................................................G...................T
Hungary1        ..................................................................G...................T
Hungary2        ..................................................................G...................T
Hungary3        ..................................................................G...................T
Hungary4        ..................G.............................................G...................T

AF150629 (6683) ACTTCATCGGCTCGGAGCATGGATGATACGCACAGTATTATTCAGTTTTTACAACGCCCCGTACTCATTGACCACATTGAGGTCATTGCTGGATCAACAG
Germany1        ..................................................................A...................
Germany2        ..................................................................A...................
Austria         ..................................................................A...................
Poland1         ..................................................................A..............G....
Poland2         ..................................................................A...................
Poland3         ..............................................C....................................
Hungary1        ..............................................C....................................
Hungary2        ..............................................C....................................
Hungary3        ..............................................C....................................
Hungary4        ..............................................C....................................

AF150629 (6783) CAGATGATAACAAACCCCTCAATAGATATGTGTTAAATCGACAGAATCCACAACCATTTGTTAAATCTTGGACATTGCCTTCAGTAGTTTTAAGTGCTGG
Germany1        ..........................................A.................G...................T..................
Germany2        ..........................................A.................G...................T..................
Austria         ..........................................A.................G...................T..................
Poland1         ................................G.......A.................G...................T..................
Poland2         ..........................................A.................G...................T..................
Poland3         ..........................................A.................G...................T........G........
Hungary1        ..........................................A.................G...................T........G........
Hungary2        ..........................................A.................G...................T........G........
Hungary3        ..........................................A.................G...................T..G.....G........
Hungary4        ..........................................A.................G...................T........G........

AF150629 (6883) AGGTAAGGGACAAAAATTAGCTAATTTTAAATATTTACGATGTGATGTTAAAGTCAAAATTGTTCTAAACGCAAATCCTTTCATAGCTGGAAGATTATAC
Germany1        .........C.......................C.................G.............T.......................C............
Germany2        .........C.......................C.................G.............T.......................C............
Austria         .........C.......................C.................G.............T.......................C............
Poland1         .........T.......................C.....................................................C............
Poland2         .........C.......................C.....................................................C........G...
Poland3         ......A..C.......................C.............................T.............G.................G...
Hungary1        ......A..C.......................C..G.................T.....................................G...
Hungary2        ......A..C.......................C..G.................T.....................................G...
Hungary3        ......A..C.......................C..G.................T.....................................G...
Hungary4        ......A..C.......................C..G.................T.....................................G...

AF150629 (6983) TTAGCATATTCACCTTATGATGACCGAGTGGACCCAGCGCGTTCAATTTTAAACACCTCACGAGCTGGAGTTACGGGATACCCTGGAATAGAGATTGATT
Germany1        ....................C.................A.........C.....................T.....A.............G....A.....C.
Germany2        ....................C...........T.....A.....................T.....A...........GG....A..A..C.
Austria         ....................C.................A.........C.....................T.....A.............G....A.....C.
Poland1         ....................C.................A.........C....T................T.....A.............G....A.....C.
Poland2         ....................C..T..............A.........C.....................T.....A.............G....A.....C.
Poland3         ....................C..T..............A.....................................C.....A.................A.......
Hungary1        ....................C..T..............A.....................................A.................A.......
Hungary2        ....................C..T..............A...........G.........................A.................A.......
Hungary3        ....................C..T..............A.....................................A.................A.......
Hungary4        ....................C..T..............A.....................................A..............C..A.......

AF150629 (7083) TTCAATTAGATAATTCCGTAGAAATGACCATACCATACGCTTCTTTTCAAGAAGCATATGATTTAGTCACAGGTACTGAAGATTTTGTCAAGCTATATCT
Germany1        .C..............T.....................T.....................................................T..........
Germany2        .C..............T.....................T.....................................................T..........
Austria         .C..............T.....................T.....................................................T..........
Poland1         .C..............T.....................T..................G..................................T..........
Poland2         .C.....G........T.....................T.....................................................T.....T.
Poland3         .C.....G........T.....................T.....................................C.....................T.
Hungary1        .C.....G........T.....................T.....................................................T..........
Hungary2        .C.....G........T.....................T.....................................................T.....T.
Hungary3        .C.....G........T.....................T.....................................................T..........
Hungary4        .C.....G........T.....................T.....................................................T..........

AF150629 (7183) ATTTACAATAACGCCCATATTATCCCCGACTAGCACATCTGCTAGTTCAAAGGTGGACCTTTCAGTTTATATGTGGTTGGATAATATTTCCTTAGTCATA
Germany1        ...C........A..............A.....T..G.................A........G.............................G......
Germany2        ...C........A..............A.....T..G.................A........G.............................G......
Austria         ...C........A..T...........A.....T.....................A........G.............................G......
Poland1         ...C........A..............A.....T.....................A........G.............................G......
Poland2         ............A...........T..A.....T.....................A........G.............................G......
Poland3         ............A...........T..........G..............A..A........G..............A.....................
Hungary1        ............A...........T........T................A..A........C..............A.....................
Hungary2        ............A...........T.............................A........G..............A.....................
Hungary3        ............A...........T........T................A..A........G..............A.....................
Hungary4        ............A...........T.........................A..A........G..............A..................T...

AF150629 (7283) CCCACGTATCGCGTTAACACGAGCATCGTACCAAATGTGGGGACTGTTGTTCAGACAGTACAAAATATGACTACACGAGACAGTGAAACAATTAGGAAAG
Germany1        ................T.....................A.A.............................C...A..........G.............
Germany2        ................T.....................A.A............G....C.........A..C...A..........G.............
Austria         ................T..A..............C...A.A.............................C...AA.........G.............
Poland1         ................T.....................A.A..CA.........................C...A..........G.............
Poland2         ................T.....................A.A.................T...........C...A..........G...........G.
Poland3         ................T.....................TAA.................T...........C...A.....................G.
Hungary1        ..G..A...........T..............C.....TAA.............A.....T..............A.G.....................
Hungary2        ..G..A...........T..............C.....TAA.............A.....T..............A.............T.........
Hungary3        ..G..A...........T..............C.....TAA.............A.....T..............A.....................
Hungary4        ..G..A...........T.....T........C.....TAA.............A.....T..............A.....................
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AF150629 (7383) CAATGGTTGCATTAAGGAAAAATAATAAATCAACTTACGACTATATAGTGCAAGCTTTATCTTCTGCAGTTCCAGAAGTTAAGAATGTAACTATGCAGAT
Germany1        ............G.....................................A.....................................................A..
Germany2        ............G.....................................A.....................................................A..
Austria         ............G.....................T...............A.....................................................A..
Poland1         .......G..G..G.....................................A.....................................................A..
Poland2         .........G..G.....................................A.....................................................A..
Poland3         .....A....G.....................T.................A.....................................A..............A..
Hungary1        .T...A....G.....................T.................A.................C...................................A..
Hungary2        .T...A....G.....................T.................A.................A...................................A..
Hungary3        .T...A....G.....................T.................A.................C...................................A..
Hungary4        .....A....G.....................T.................A.....................................................A..

AF150629 (7483) CAATTCCAAGAAAAATAATTCCAATAAAATGGCAACACCTGTTAAGGAAAAAACAAAAAATATACCCAAACCAAAGACTGAAAATCCGAAGATTGGACCA
Germany1        ......T............C.....................A......C....G.GC..G..T.....................A.............
Germany2        ......T............C.................T.....A......C....G.GC....TT.....G..............A.............
Austria         ......T............C.....................A......T....G.G.....T.....................A.............
Poland1         ......T............C.....................A......C......GC.....T..G.................A.............
Poland2         ......T............C.....................A......C......GC.....T..G.................A.............
Poland3         T.....T............C....C......A....G.....A..............C.....T..G.................A.............
Hungary1        ......T............C.....A....G.....A.............G.....T..G.................A.............
Hungary2        ......T..A.........C.....A....G....C...A.............G.....T..G.................A.............
Hungary3        ......T............C.....A....G.....A.............G.....T..G.................A.............
Hungary4        ......T............C.....A....G.....A.............G.....T..G.................A..A..........

AF150629 (7583) ATATCAGAACTAGCCACAGGAGTCAACAAGGTGGCGAATGGTATTGAGAGAATACCTGTGATTGGAGAGATGGCAAAACCTGTAACTTCAACAATTAAAT
Germany1        ........GT.G...........T..............C........A.....................................T..............
Germany2        ........GT.G...........T..............C........A.....................................T..............
Austria         ........GT.G...........T..............C........A.............G.....................T..............
Poland1         ........GT.G..T........T..............C........A.....................................T..............
Poland2         ........GT.G...........T..............C........A.....................................T..............
Poland3         .........T.G...........T.....................................G.....................C..............
Hungary1        .........T.G...........T.....................................................................T..............
Hungary2        .........T.G...........T.....................................A.....................T..............
Hungary3        .........T.G...........T.....................................................................T..............
Hungary4        .........T.G...........T.....................................................................T..............

AF150629 (7683) GGGTTGCTGACAAGATTGGATCTGTGGCAGCAATTTTTGGATGGTCGAAACCCAGAAATCTAGAACAAGTTAATTTATATCAGAATGTTCCTGGATGGGG
Germany1        ..................................................A............T.....................................................
Germany2        ..................................................A.....A......T.....................................................
Austria         ..................................................A............T.....................................................
Poland1         ..................................................A............T.G...................................................
Poland2         ..................................................A............T.....................................C.......
Poland3         ........................G.............A.....T......T.................C...........C.......
Hungary1        ........................G.....C.........A.....T......T.................C.....................
Hungary2        ........................G.....C.........A.....T......T.................C.....................
Hungary3        ........................G.....C.........A.....T......T.................C.....................
Hungary4        ........................G.....C.........A.....T......T.................C.....................

AF150629 (7783) TTATTCACTCTATAAGGGAATAGATAATAGTGTGCCATTGGCTTTTGACCCCAATAACGAACTAGGTGATTTGAGAGATGTATTTCCTTCTGGAGTTGAT
Germany1        .........T.............C.....C................T..G.....................................................C
Germany2        .........T.............C.....C................T..G.....................................................C
Austria         .........T.............C.....C..T.............T..G.....................................................C
Poland1         ......................C.....C................T..G.....T.............................G...........C
Poland2         ......................C.....C................T..G.....T.....................................................C
Poland3         ......................C.....C..................G.....T..T.....................................................C
Hungary1        ......................C.....C..................G.................C.....................................C
Hungary2        ......................C.....C..................G.....................................................C
Hungary3        ......................C.....C..................G.....................................................C
Hungary4        ......................C.....C..................G.....................................................C

AF150629 (7883) GAAATGGCGATAGGATATGTTTGTGGCAATCCTGCTGTTAAACATGTGCTATCTTGGAATACTACGGATAAAGTTCAAGCACCAATAAGTAATGGAGATG
Germany1        ........A..............T.....................................................T..................
Germany2        ........A..............T.....................................................T..................
Austria         ........A..............T.....................................................T..................
Poland1         ........A..............T................C....................................T..................
Poland2         ........A..............T.....................................................T..................
Poland3         ..................................................G..........A..............TG..................
Hungary1        ......................T.......C..C..........G..........A..............T..................
Hungary2        ......................T.......C..C..........G..........A..............T..................
Hungary3        ..............G..........T.......C..C..........G..........A..........C....T..................
Hungary4        ......................T.......C..C..........G..........A..............T..................

AF150629 (7983) ACTGGGGAGGAGTGATACCTGTTGGTATGCCATGTTATTCTAAAATCATACGGACAACAGAGAACGATACGACACGGACTAATACTGAAATTATGGATCC
Germany1        .........G.............................G..A..............T..A..A....AA.....G......G.........
Germany2        .........G........C....................G..A..............T..A..A....AA.....G......G.........
Austria         .........G.............................G..A.........T......T..A..A....AA.....G......G.........
Poland1         .....................................G..AT.............T..A..A....AA.....G......G.........
Poland2         .....................................G..A..............T..A..A....AA.....G......G.........
Poland3         ..............................C.......G..A.............T..T..C..A....AA.....G......G.........
Hungary1        ..............................C.......G..A.......A.........T.....A....AA.....C......G..T.......
Hungary2        ..............................C.......G..A.......A.............A....AA.....G......G..T.......
Hungary3        ..............................C.......G..A.......A.........T.....A....AA.....G......G..T.......
Hungary4        ..............................C.......G..A.......A.........T.....A....AA.....G......G..T.......

AF150629 (8083) TGCCCCTTGTGAATATGTTTGCAATATGTTTTCATATTGGCGAGCTACTATGTGCTATCGTATAGCTATAGTTAAAACAGCTTTTCACACTGGTAGGTTA
Germany1        .....................................C.....................................................................
Germany2        .....................................C.....................................T...............................
Austria         ..................................T..C.....................................................................
Poland1         .....................................C.....................................................................
Poland2         .....................................C.....................................................................
Poland3         .....................................................................................................
Hungary1        .....................................C.....................................................C......
Hungary2        .....................................................................................C......
Hungary3        .....................................................................................C......
Hungary4        .....................................................................................C......

AF150629 (8183) GGAATTTTCTTCGGACCTGGTAAGATTCCAATAACGACGACGAAAGATAATATTTCCCCGGACTTGACTCAGTTAGATGGAATTAAAGCGCCTTCTGATA
Germany1        .A..........A...A....GA.....................C........A...........A...................C..C..C.
Germany2        .A..........A...A....GA.....................C........A...........A...................C..C..C.
Austria         .A..........A...A....GA.........T...........C........A...........A...................C..C..C.
Poland1         .A..........A...A....GA........G........A..G.....C........A...........A...................C..C..C.
Poland2         .A..........AG..A....GA.........T...........C........A...........A...................C..C..C.
Poland3         .A.........T.A.......G..........T...........C........A...........A.....................C.
Hungary1        .A.........T.A.......G......G....T...........C....................A..........CC........C..C..C.
Hungary2        .A.........T.A.......G..........T...........C....................A..........CC........C..C..C.
Hungary3        .A.........T.A.......G......G....T...........C...........A...C.......A..........CC........C..C..C.
Hungary4        .A.........T.A.......G..........T...........C....................A..........CC........C..C..C.
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AF150629 (8283) ACAATTACAAATACATCTTGGATCTAACTAATGATACGGAGATCACCATAAGGGTACCTTTTGTTTCAAACAAAATGTTCATGAAATCTACGGGAATTTA
Germany1        .......T.............................A.............C.....................................
Germany2        .......T.............................A.............C.....................................
Austria         .......T..G..........................A....T.........C.....................................
Poland1         .......T..........A..................................C.....................T...............
Poland2         .......T..........A..................................C.....................................
Poland3         .......T..........A.....................................................................
Hungary1        .......T..........A...T.G..............................................................
Hungary2        .......T..........A...T.G.........................A.....................................
Hungary3        .......T..........A...T.G.........................A.....................................
Hungary4        .......T..........A...T.G.........................A..G..................................C...

AF150629 (8383) TGGTGGAAATTCTGAAAATAATTGGGATTTCTCTGAATCATTTACTGGATTTTTGTGCATAAGACCTATTACAAAATTTATGTGTCCAGAGACTGTATCC
Germany1        ..........................................................T.........G.C........A..................T
Germany2        ..........................................................T.........G.C........A..................T
Austria         ..........................................................T.........G.C........A..................T
Poland1         ..........................................................T.........G.C........A..................T
Poland2         ..........................................................T.........G.C........A..................T
Poland3         ..........................................................C.........A.................G..A
Hungary1        ................C.........................................C.........A.................G..A
Hungary2        ......G..........C.........................................C.........A.................G..A
Hungary3        ................C.........................................C.........A.................G..A
Hungary4        ................C.........................................C.........A.................G..A

AF150629 (8483) AATAATGTGTCGATAGTTGTATGGAAGTGGGCTGAAGATGTGGTAGTAGTAGAACCTAAGCCTTTACTTTCAGGACCAACGCAAGTGTTTCAACCACCTG
Germany1        ........A.........................G....A.................A.................G.....................
Germany2        ........A.........................G....A.................A.................G.....................
Austria         ........A.........................G....A.................A.................G.....................
Poland1         ....G...A.........................G....A.................A.................G.....................
Poland2         ..................................G....A.................A.................G.....................
Poland3         ........A..........G..................A..C..............A.....G.....................................
Hungary1        ...........A........G.....................A..............A.................G.....................
Hungary2        ...........A........G.....A..............A..T..............A.....G.....................................
Hungary3        ...........A........G.....................A..T..............A.....................................
Hungary4        ...........A........G.....................A..T..............A.....G.....................................

AF150629 (8583) TAACATCTGCAGATTCTATCAATACCATAGATGCTTCAATGCAAATTAACTTAGCAAATAAAGCTGATGAAAATGTAGTTACATTCTTTGATTCTGATGA
Germany1        ....T..C.............TT.....................T.....G.............G.....GA.............C.....
Germany2        ....T..C.............TT.....................T.....G.............G.....CA.................A.
Austria         ....T..C.............TT.....................T.....G.............G.....GA.....................
Poland1         ....T.............T....TT...................T.....G.............G.....GA.C...................
Poland2         ....T..C.............TT.....................T.....G.............G.....GA.....................
Poland3         ....T..C.............T.....................C.....G.............G.....G......................
Hungary1        ....T..C.............TT.....................T.....C.............G.....GA.....................
Hungary2        ....T..C..........A....TT.....................G.....G.....G.....GA.....................C..
Hungary3        ....T..C..........A....TT.....................G.....G.....G.....GA.....................
Hungary4        ....T..C..........A....TT.....................G.....G.....G.....GA.....................

AF150629 (8683) TGCTGAAGAAAGGAACATGGAAGCATTATTGAAAGGAAGTGGTGAACAAATCATGAATTTGAGATCCTTACTAAGAACGTTTAGGACCATATCAGAAAAT
Germany1        C.....G.....................................................................T.......A.....................
Germany2        C.....G.....................................................................T.......A.....................
Austria         C.....G.....................................G...............................T.......A.....................
Poland1         C.....................................................................T.......A.....................
Poland2         C.....G.....................................................T...T.......A.....................
Poland3         C.....G.....................................................T...T.......A........T..............
Hungary1        C.....G.....................................C.....................T...T.......A........T..............
Hungary2        C.....G.....................................C.....................T...T.......A........T..............
Hungary3        C.....G.................G...................C.....................T...T.......A........T..............
Hungary4        C.....G.....................................C.....C.....T...T.......A........T..............

AF150629 (8783) TGGAATTTACCACCTAACACAAAAACAGCAATAACAGATTTGACTGACGTTGCGGATAAAGAAGGTAGGGATTATATGTCTTATTTATCATACATCTATA
Germany1        ...........G.....................C..A.....T.....A.....G........A.....................T....
Germany2        ...........G..C.............G........C..A.....T.....A.....G........A...........C.............T....
Austria         ...........G.....................C..A.....T.....A.....G........A.....................T....
Poland1         ...........G.....T........G.............A.....T.....A..C..G........A.....................
Poland2         ...........G.....................A.....T.....A.....G........A.....................
Poland3         ..........................................T...........G........A.....................
Hungary1        ................T.....................T...........G........A.....................T....
Hungary2        ......C..........T.....................T...........G........A.....................
Hungary3        ................T.....................T...........G........A.....................
Hungary4        ................T..G..................T...........G........A.....................

AF150629 (8883) GATTCTATAGAGGAGGGAGAAGATATAAGTTTTTCAATACAACAGCTTTGAAACAATCTCAAACTTGCTATGTTCGTAGCTTCTTAATACCGCGATATTA
Germany1        ..............A..............T...........C.....................A.........T...G.............
Germany2        ..............A..............T...........C.....................A.........T...G.............
Austria         ..............A..............T...........C.....................A.........T...G.............
Poland1         ..............A..............T...........C.....................A.........T...G.............
Poland2         ..............A..............T...........C.....................A....C.........G....A........
Poland3         ..............A.....................C.....................A....C.........G....A........
Hungary1        ..............A.....................C.....................A....C........GG.G..A........
Hungary2        ..............A.....................C..A..................A....C........GG.G..A........
Hungary3        ..............A.....................C.....................A....C........GG.G..A........
Hungary4        ..............A.....................C.....................T...A....C........GG.G..A........

AF150629 (8983) TACTGCTGATAACACAAACAACGATGGACCTTCACATATAACATATCCAGTTCTTAATCCAGTTCATGAAGTAGAAGTACCATACTATTGTCAATATAGG
Germany1        ....A..............T.....................................................................
Germany2        ....A..............T.....................................C...............T...............
Austria         ....A..............T.....................................................................
Poland1         ....A..............T.....................................................................
Poland2         ....A..............T.....................................................G...............
Poland3         ....A..............T.....................................................G..............A
Hungary1        ....A.......T.....T..T..................................................................A
Hungary2        ....A............T..T..................................................................A
Hungary3        ....A............T..T..................................................................A
Hungary4        ....A............T..T..................................................................A

AF150629 (9083) AAATTACCAGTAGCATCTACAACCGACAAAGGGTATGATGCATCTTTGATGTATTATTCTAATGTTGGTACCAATCAAATTGTTGCTCGAGCTGGTAATG
Germany1        .....G.....G...........T.........................C.....................................C....
Germany2        .....G.....G...........T.........................C.....................................C....
Austria         .....G.....G...........T.........................C.....................................C....
Poland1         .....G.....G........T..T.........................C.....................................C....
Poland2         .....G.....G........T.............................C.....C...............................C....
Poland3         ..................G.....................C.....C...........T.....................C....
Hungary1        ...............................A.....................C.................T.....G.............C..C....
Hungary2        ...............................A.....................C.....................T...............C..C....
Hungary3        ...............................A.....................C.................T...................C..C....
Hungary4        ...............................A.....................C.................T...................C..C....
```

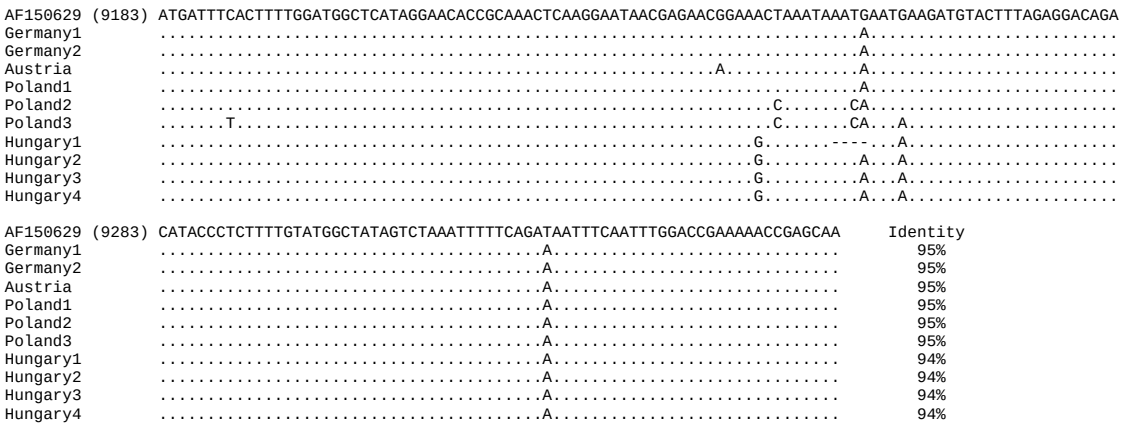
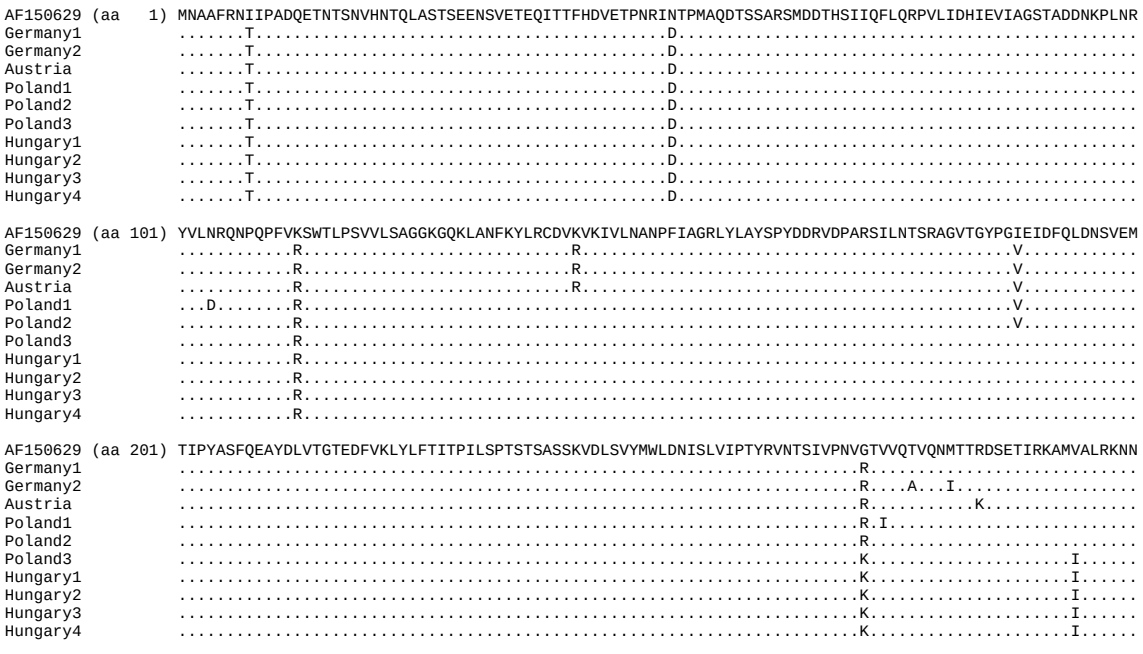


Figure 3.: Multiple alignment of the nucleotide sequences of the capsid polyprotein region of ABPV isolates with different geographic origin (nucleotide positions 6283 to 9353 according to the reference strain AF150629). The sequences are deposited in GenBank database under accession numbers AY053366 - AY053375.

Deduced amino acid sequences were generated in the capsid protein gene region (nt 6509 to nt 9253), resulting in 914 amino acid (aa) long polypeptide sequences. The aa sequences were also aligned and compared to the reference strain. They exhibited an identity rate of 97 to 98% (Figure 4).

Phylogenetic trees were constructed using the nucleotide sequences of the various isolates, and the stability of the tree was tested by bootstrap analysis of 100 replicates. The optimizing tree reconstruction method (Fitch) proposed the phylogenetic tree presented in Figure 5.



```
AF150629 (aa 301) KSTYDYIVQALSSAVPEVKNVTMQINSKKNNSNKMATPVKEKTKNIPKPKTENPKIGPISELATGVNKVANGIERIPVIGEMAKPVTSTIKWVADKIGSV
Germany1          ............................P.........P.SM.....................................................
Germany2          ............................P.....S...P.S.L.....................................................
Austria           ............................P.........S.S.....................................V...............
Poland1           ............................P.........P.S.....................................................
Poland2           ............................P.........P.S.....................................................
Poland3           ............................P...T.....................................................
Hungary1          ............................P...T........S.....................................................
Hungary2          ............................P...T..A.....S.....................................................
Hungary3          ............................P...T........S.....................................................
Hungary4          ............................P...T........S.....................................................

AF150629 (aa 401) AAIFGWSKPRNLEQVNLYQNVPGWGYSLYKGIDNSVPLAFDPNNELGDLRDVFPSGVDEMAIGYVCGNPAVKHVLSWNTTDKVQAPISNGDDWGGVIPVG
Germany1          ..................................................................................V.............
Germany2          ..................................................................................V.............
Austria           ..................................................................................V.............
Poland1           ..................................................................................V.............
Poland2           ..................................................................................V.............
Poland3           ..................................................................................V.............
Hungary1          ..................................................................................V.............
Hungary2          ..................................................................................V.............
Hungary3          ..................................................................................V.............
Hungary4          ..................................................................................V.............

AF150629 (aa 501) MPCYSKIIRTTENDTTRTNTEIMDPAPCEYVCNMFSYWRATMCYRIAIVKTAFHTGRLGIFFGPGKIPITTTKDNISPDLTQLDGIKAPSDNNYKYILDL
Germany1          ............E..Q.K..V.................................E...E..R.....................................
Germany2          ............E..Q.K..V.................................E...E..R.....................................
Austria           .........I..E..Q.K..V.................................E...E..R...M.................................
Poland1           .......L.....E..Q.K..V.................................E...E..R..M.................................
Poland2           ............E..Q.K..V.................................E...E..R...M.................................
Poland3           ...........D....Q.K..V.................................E...E..R...M.................................
Hungary1          .........K......Q....VL................................E...E..R...M..............T..............
Hungary2          .........K......Q.K..VL................................E...E..R...M..............T..............
Hungary3          .........K......Q.K..VL................................E...E..R...M..............T..............
Hungary4          .........K......Q.K..VL................................E...E..R...M..............T..............

AF150629 (aa 601) TNDTEITIRVPFVSNKMFMKSTGIYGGNSENNWDFSESFTGFLCIRPITKFMCPETVSNNVSIVVWKWAEDVVVVEPKPLLSGPTQVFQPPVTSADSINT
Germany1          ..............................................V..L.....................................................I
Germany2          ..............................................V..L.....................................................I
Austria           ..............................................V..L.....................................................I
Poland1           ..............................................V..L........S............................................I
Poland2           ..............................................V..L.....................................................I
Poland3           ..............................................L.....................................................
Hungary1          ..............................................L.....................................................I
Hungary2          ..............................................L.....................................................I
Hungary3          ..............................................L.....................................................I
Hungary4          ..............................................L.....................................................I

AF150629 (aa 701) IDASMQINLANKADENVVTFFDSDDAEERNMEALLKGSGEQIMNLRSLLRTFRTISENWNLPPNTKTAITDLTDVADKEGRDYMSYLSYIYRFYRGGRRY
Germany1          ................I.....................................................................................
Germany2          ................I.....N...............................................................................
Austria           ................I.....................................................................................
Poland1           ................I.....................................................................................
Poland2           ................I.....................................................................................
Poland3           ................................................................................................
Hungary1          ................I.....................................................................................
Hungary2          ................I.....................................................................................
Hungary3          ................I.....................................................................................
Hungary4          ................I.....................................................................................

AF150629 (aa 801) KFFNTTALKQSQTCYVRSFLIPRYYTADNTNNDGPSHITYPVLNPVHEVEVPYYCQYRKLPVASTTDKGYDASLMYYSNVGTNQIVARAGNDDFTFGWLI
Germany1          ...............I....V.....T.....................................................................
Germany2          ...............I....V.....T.....................................................................
Austria           ...............I....V.....T.....................................................................
Poland1           ...............I....V.....T.....................................................................
Poland2           ...............I....V.....T.....................................................................
Poland3           ...............I....V.....T.....................................................................
Hungary1          ...............I....V.....T.....................................................................
Hungary2          ...............I....V.....T.....................................................................
Hungary3          ...............I....V.....T.....................................................................
Hungary4          ...............I....V.....T.....................................................................

AF150629 (aa 901) GTPQTQGITRTETK     Identity
Germany1          ..............        97%
Germany2          ..............        97%
Austria           ..............        97%
Poland1           ..............        97%
Poland2           ..............        97%
Poland3           ..............        98%
Hungary1          ............A.        97%
Hungary2          ............A.        97%
Hungary3          ............A.        97%
Hungary4          ............A.        97%
```

Figure 4.: Multiple alignment of amino acid sequences deduced from the nucleotide sequences of the capsid polyprotein gene of ABPV isolates with different geographic origin (amino acid positions 1 to 914 according to the reference strain AF150629, protein_id AAG13119.1).

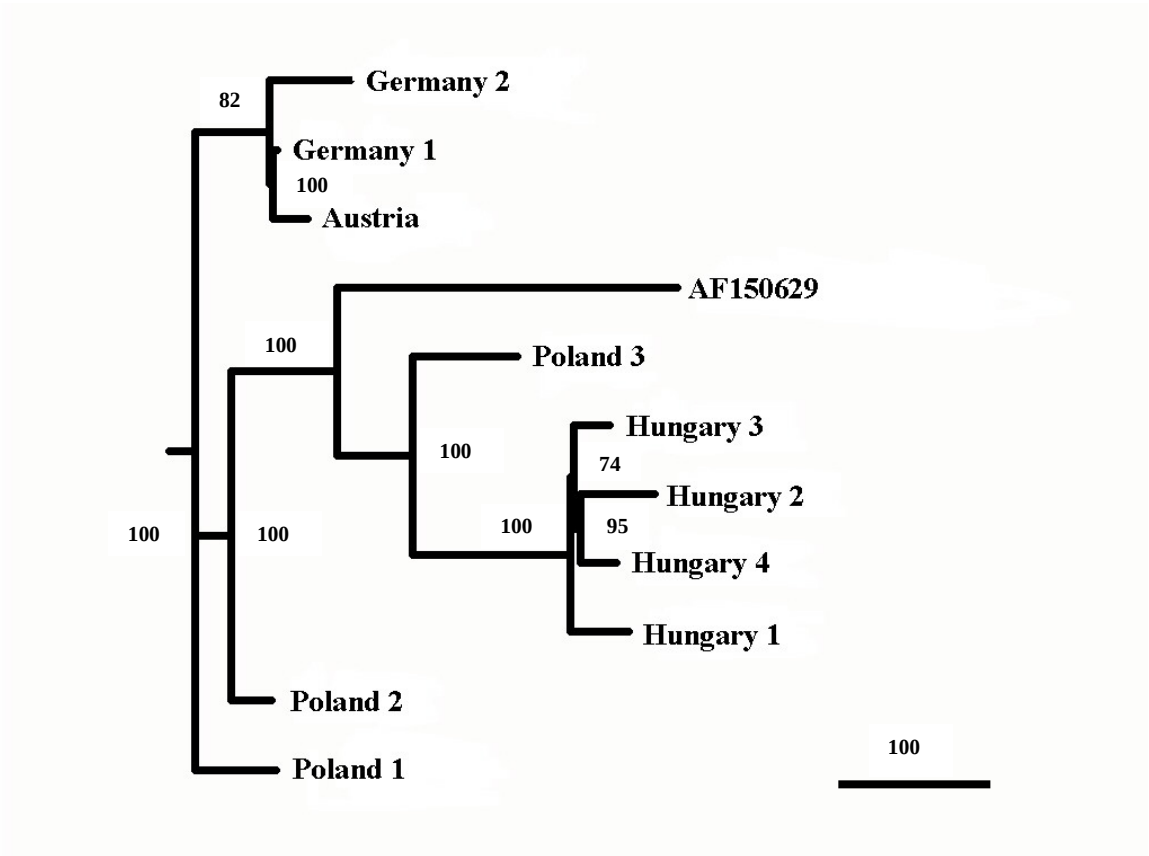
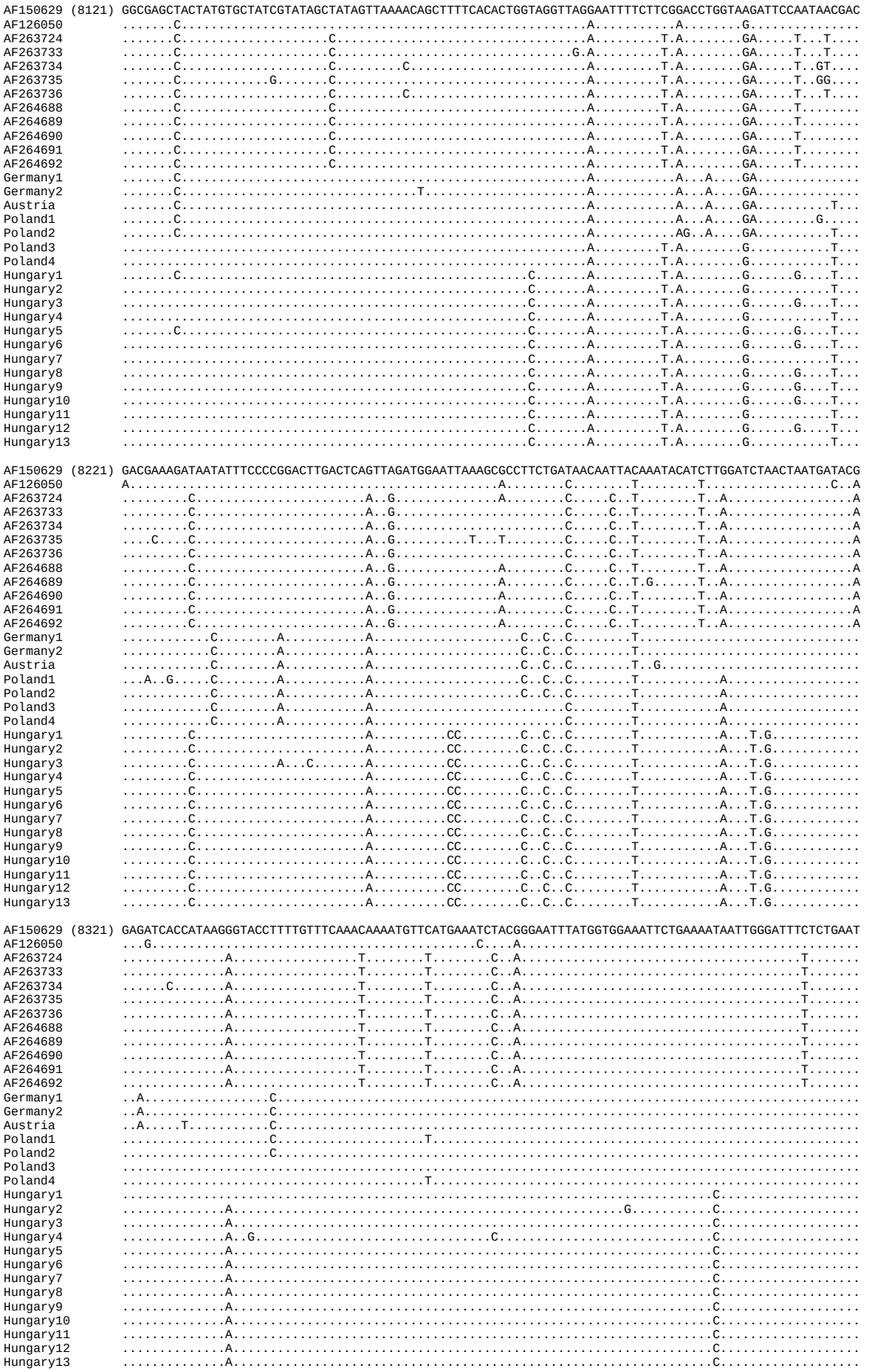


Figure 5.: Phylogenetic tree illustrating the genetic relationship among ABPV strains based on the genomic region 6283 to 9353, generated by the DNADIST/Fitch program. Numbers at nodes represent the percentage of 100 bootstrap pseudoreplicates that contained the cluster distal to the node. Genetic distance is indicated by the bar at lower right.

The sequence analysis of the capsid protein gene region disclosed a slightly higher degree of sequence divergence of the ABPV isolates approx. between nucleotide positions 8000 and 8600. Since additional (shorter) ABPV sequences in this genomic region have been deposited in the GenBank database by other research groups (AF126050, Ghosh et al., 1999; AF263724, AF263733 - AF263736, AF264688 - AF264692, Hung), the remaining eleven ABPV isolates were sequenced also in this region to compose a shorter, but more comprehensive comparison of the different isolates. These shorter nucleotide sequences were evaluated by the same principles as described before. In total, 32 isolates were aligned using a 401 nt stretch from nt position 8121 to 8521. The isolates exhibited identity rates between 89% and 96% compared to the reference strain (Figure 6).

Deduced amino acid sequences were also compiled and aligned. The 133 aa long polypeptide sequences showed identity rates of 90% to 96% in comparison with the reference strain (Figure 7).

Phylogenetic trees were constructed and bootstrap analysis was performed. A representative tree generated by the Neighbor-Joining method is shown in Figure 8.



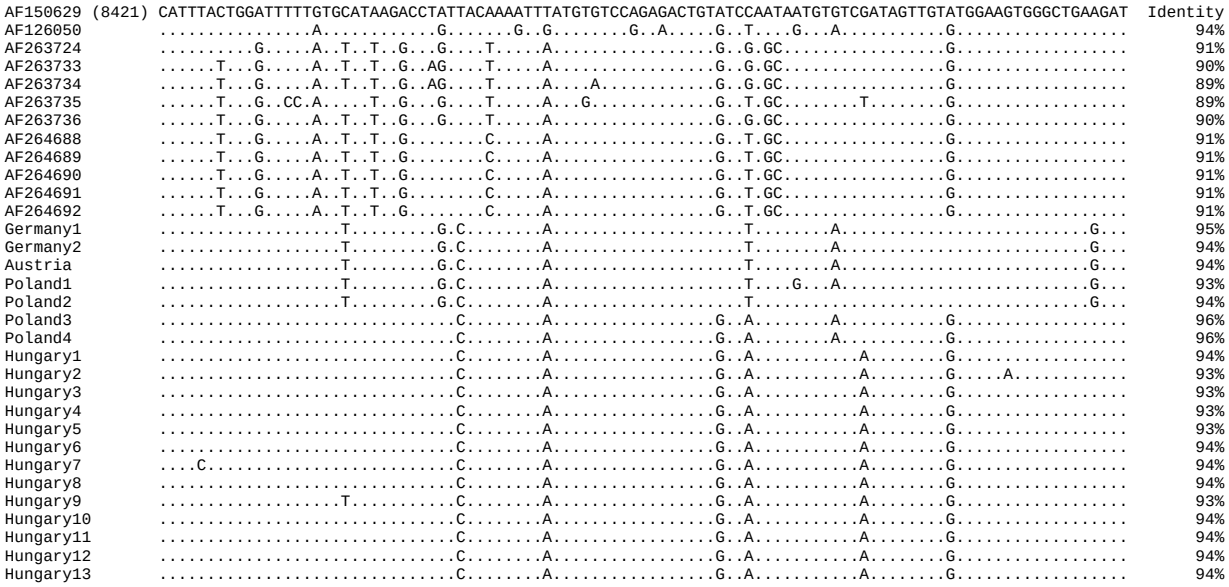
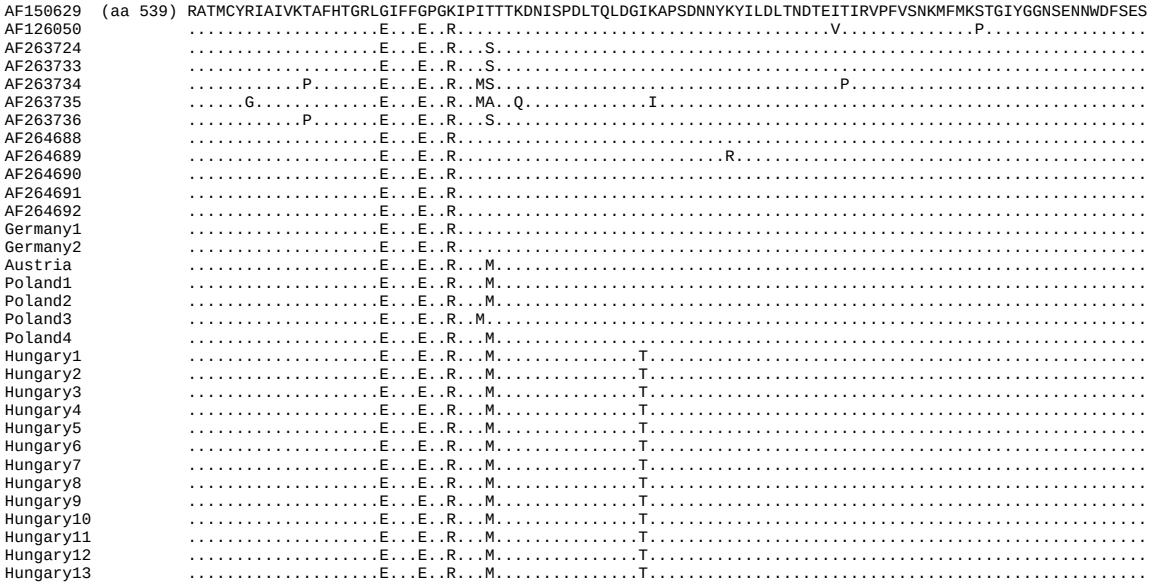


Figure 6.: Multiple alignment of the nucleotide sequences of ABPV isolates with different geographic origin obtained with primer pair APV 23nf-24nr and originated from GenBank (nucleotide positions 8121 to 8521 according to the reference strain AF150629). The sequences are deposited in GenBank database under accession numbers AY053366 - AY053385.



```
AF150629  (aa 639) FTGFLCIRPITKFMCPETVSNNVSIVVWKWAED     Identity
AF126050           .........V..L........S...........        94%
AF263724           .........V..L.......S...........        95%
AF263733           .I.......V..L.......S...........        94%
AF263734           .I.......V..L.Y.....S...........        91%
AF263735           .I.......V..L.G.....S...........        90%
AF263736           .I.......V..L.......S...........        93%
AF264688           .I.........L.......S...........        95%
AF264689           .I.........L.......S...........        95%
AF264690           .I.........L.......S...........        95%
AF264691           .I.........L.......S...........        95%
AF264692           .I.........L.......S...........        95%
Germany1           .........V..L..................        96%
Germany2           .........V..L..................        96%
Austria            .........V..L..................        95%
Poland1            .........V..L..................        95%
Poland2            ...........L..................        96%
Poland3            .........V..L........S...........        95%
Poland4            ...........L..................        96%
Hungary1           ...........L..................        95%
Hungary2           ...........L..................        95%
Hungary3           ...........L..................        95%
Hungary4           ...........L..................        95%
Hungary5           ...........L..................        95%
Hungary6           ...........L..................        95%
Hungary7           ...........L..................        95%
Hungary8           ...........L..................        95%
Hungary9           ...........L..................        95%
Hungary10          ...........L..................        95%
Hungary11          ...........L..................        95%
Hungary12          ...........L..................        95%
Hungary13          ...........L..................        95%
```

Figure 7.: Multiple alignment of amino acid sequences deduced from the nucleotide sequences obtained with primer pair APV 23nf-24nr and originated from GenBank (amino acid positions 1 to 133 according to the reference strain AF150629, protein_id AAG13119.1).

3.4. DISCUSSION

The scientific interest in honey bee viruses is increasing. The majority of bee viruses have been formerly characterized by classical virological methods (Allen et al., 1986, Newman et al., 1973), and their presence was reported worldwide; our knowledge of the molecular properties and genome organization of some of these viruses is the result of recent studies (Ghosh et al., 1999, Govan et al., 2000). There is still very limited information available on the taxonomic classification of honey bee viruses, on the existence of serotypes or virulence-variants within one species, as well as on the role of the viruses in bee diseases. The molecular comparison of various isolates is an exact and reliable method to study the relationships between and within different species, since it detects directly the changes of the genetic information in the course of evolution, and employs statistical evaluation of the data.

This study presents a phylogenetic analysis of the structural protein gene region of ABPV strains. Eleven European ABPV isolates were aligned and compared to the reference strain of probable UK origin deposited in the GenBank database. We focused our investigations on the structural protein gene region, because this genomic region shows usually a higher divergence than non-structural sections.

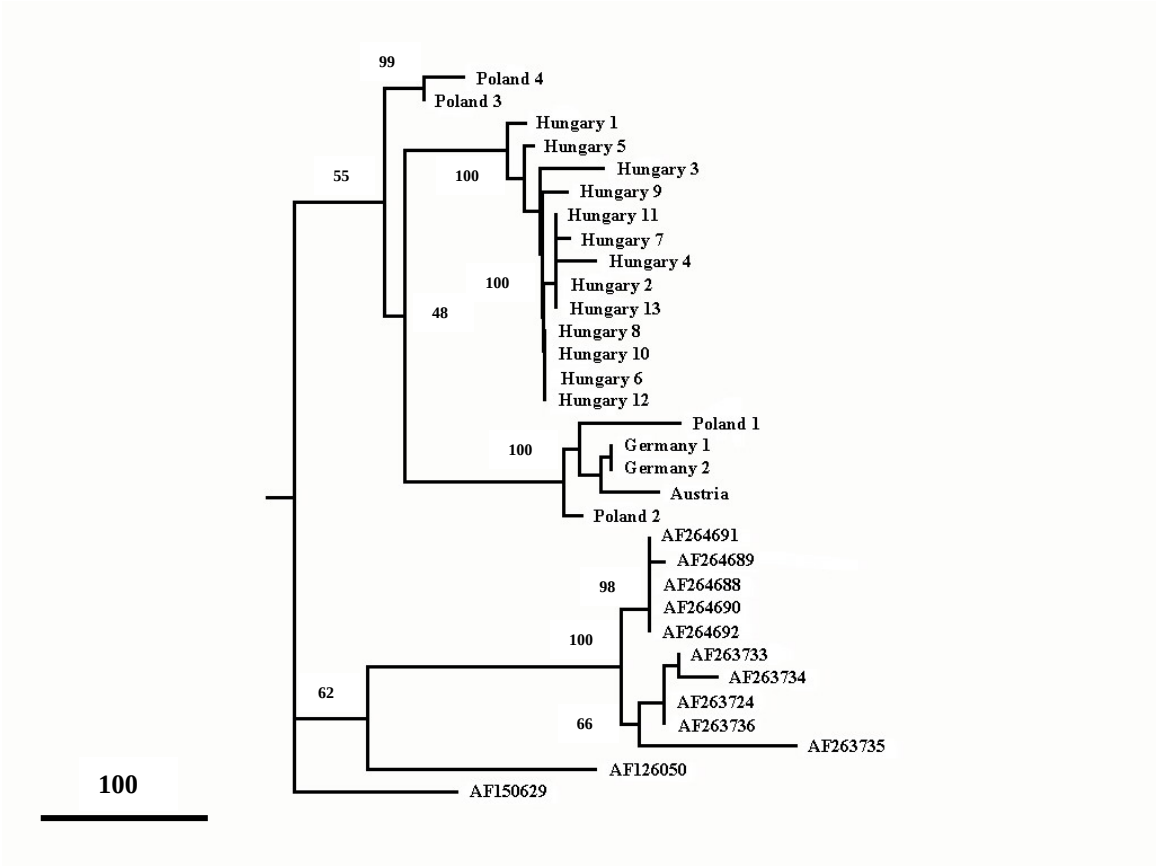


Figure 8.: Phylogenetic tree illustrating the genetic relationship among ABPV strains based on the genomic region 8121 to 8521, generated by the DNADIST/Neighbor-Joining program. Numbers at nodes represent the percentage of 100 bootstrap pseudoreplicates that contained the cluster distal to the node. Genetic distance is indicated by the bar at lower left.

The analysed isolates showed 94% to 95% sequence identity compared to the UK reference strain. The nucleotide exchanges were distributed fairly evenly over the entire region that we have sequenced, in some parts, however, (e.g. from nt position 7000 to 8000, 8100 to 8300, and 8400 to 9000) a slightly higher sequence divergence was observed between the isolates.

The probable relationship between the isolates is illustrated by a phylogenetic tree (Figure 5). The strains are forming at least three groups. The Austrian and German isolates are clustering together in one branch; the Hungarian strains belong to a second distinct cluster, while the Polish ABPV samples exhibit a higher diversity. The reference strain from the UK shows a relatively lower genetic relationship to the Central-European ABPV strains.

In the comparison of the partial capsid protein gene region (nt pos. 8121 to 8521) we included partial sequences of the above-mentioned ABPV strains as well as the sequences of

one additional Polish and nine additional Hungarian strains, and also eleven sequences of viruses of probable UK origin, which had been deposited in the GenBank database. On the 401 nucleotide long overlapping part of the sequences the strains have shown identity rates between 89% and 96%, respectively, in comparison with the reference UK strain.

The phylogenetic tree comprising a total of 32 isolates suggests the separation of at least two distinct genetic linkages of ABPV (Figure 8). One major branch is composed of the British strains' sequences originated from the GenBank, while the other contains the continental European isolates. Within the British genotype the isolates sequenced by different research groups are detached to separate clusters. The continental European genotype also subdivided into at least three distinct subtypes. The Austrian and German isolates are showing the similar, low extent of divergence as in the previous tree, but two Polish strains are showing a relative closer relationship with them, than with the other isolates. The Hungarian strains are forming a distinct, homogeneous cluster; while two, closely related Polish isolates are in seclusion.

The deduced amino acid sequences have shown higher identity in both analyses (97-98%, and 90-96%, respectively) and within the genotypes the degree of amino acid variability was even lower. Nonsense mutations were not observed, and a four nucleotides deletion detected in one Hungarian strain was downstream from the end of the coding region, so did not affect the transcribed polyprotein.

The results of this study revealed, that although the ABPV capsid polyprotein gene is a relatively conserved genomic region in the investigated isolates, the observed diversity is a convenient indicator for the identification and classification of ABPV isolates with different geographic origin. The molecular comparison gave an insight of the variability of the ABPV isolates and exhibited genomic relationships between the strains. Similar genetic divergence was observed in the case of sacbrood virus (Grabensteiner et al., 2001), supporting the geographical segregation of bee virus strains.

Since the ABPV is a worldwide distributed honey bee virus, the genomic analysis of isolates from other continents may supplement our knowledge on the diversity of this virus. The RT-PCR assays described in this paper are proven to be sensitive and reliable methods for the detection and classification of ABPV isolates; they provide an appropriate manner for further investigations.

Chapter 4

Nucleotide sequence analysis of the non-structural protein gene region of ABPV strains

4.1. INTRODUCTION

Acute bee paralysis virus (ABPV, Bailey et al., 1963) is a common infective agent of honey bees (*Apis mellifera*), which is frequently present world-wide in apparently healthy colonies (Allen and Ball, 1996), but in several cases it causes sudden collapse of bee colonies. In these cases the colonies are usually infested with the mite *Varroa destructor* (Ball and Allen, 1988), which may act as a virus vector and is therefore an important predisposing factor in the clinical manifestation of the disease (Abrol, 1996).

ABPV has a single stranded, positive sense, polyadenylated RNA genome comprising of 9,491 nucleotides. The complete nucleotide sequence of the virus has been determined (Govan et al., 2000). The genome encodes for two open reading frames (ORFs). ORF 1 starts at nucleotide 605 and extends to 6325 and ORF 2 starts at nucleotide 6509 and ends at 9253. By comparison of the deduced amino acid sequence of the ABPV ORF 1 to related viruses of *Picornaviridae, Caliciviridae, Comoviridae* and *Sequiviridae*, it was found to contain three conserved domains. These non-structural protein domains are the RNA helicase, the cysteine protease and the RNA-dependant RNA polymerase (RdRp). The putative helicase domain of ABPV starts at amino acid position 533 and extends to position 654. The putative cystein protease domain is located between amino acid positions 1163 and 1327. The putative RdRp domain starts at amino acid position 1565 and extends to position 1840 (Govan et al. 2000). ORF2 encodes three major structural proteins (35, 33, and 24 kDa) and a minor protein (9.4 kDa) transcribed together in a capsid polyprotein.

The ABPV was grouped to the insect-infecting picorna-like viruses of *Picornaviridae* (Moore et al., 1985). Insect viruses belonging to the same group of *Picornaviridae* in general show only minor sequence similarities. The ABPV shows 29 % identity to Drosophila C virus (DCV), 28 % to Cricket paralysis virus (CrPV), 29 % to *Plauti stali* intestine virus (PSIV) and 31 % to the Himetobi P virus (HiPV) on amino acid level (Evans and Hung, 2000).

More recently it has been recognized that many of the insect infecting RNA viruses have genome structures different from the ones of picornaviruses and similar to those of caliciviruses. Picornaviruses possess monocistronic genomes with the structural genes at the

5' end, and the replicase proteins at the 3' end of the genome. Caliciviruses have bicistronic genomes with replicase proteins at the 5' end and the structural proteins at the 3' end of the genome. Superficial genomic similarity to the caliciviruses has been found after sequencing of the CrPV, DCV, PSIV, HiPV and *Rhopalosiphum padi* virus (RhPV) which all have bicistronic genomes with replicase proteins at the 5' part, and structural proteins at the 3' part of the genome. However, although they share the same genomic structure, the insect infecting viruses, contrary to the caliciviruses, do not produce subgenomic RNA. The translation initiation in these viruses is found to be facilitated by an internal ribosomal entry site. This difference accounts for the CrPV, PSIV, HiPV, DCV, RhPV which at present are classified into a novel group of "Cricket paralysis-like viruses" distinct from the family *Picornaviridae* (van Regenmortel et al., 1999). Analysis of the overall genomic structure of ABPV showed similarities to those of DCV, PSIV, RhPV and HiPV, therefore it was suggested that the ABPV belongs to the group of “Cricket paralysis-like viruses” (Govan et al., 2000).

Concerning the distribution of the virus, it has been detected in Britain, France, Italy, Canada, New Zealand and probably Australia without any associated disease or mortality (Allen and Ball, 1996). In central part of Europe and in America on the other hand, the findings are different (Österlund et al., 1998, Bakonyi et al., 2002). In these areas ABPV has been found in large numbers of diseased brood and dead adult bees from colonies infested with *Acarapis woodi* and *Varroa destructor* and the virus has also been detected within the Varroa mite itself (Allen et al., 1986). Regarding the interactions between *A. woodi*, *V. destructor* and bee viruses including ABPV, and potentially also other microorganisms, the term "Bee–Parasitic Mite Syndrome" was established (Hung et al., 1995). This term is pertaining to the unknown mechanisms by which these organisms cause large colony losses, especially in Central Europe and America.

The observations support the premise of the presence of virulence-variants amongst ABPV strains. Since the conventional virus classification and characterization methods (i.e. isolation in cell cultures, serotyping) are unadapted to bee viruses, novel and reliable methods are necessary for the identification of distinct strains within ABPV. Investigations of the viral nucleic acid provide the opportunity of genetic characterization of the isolates and genotyping. Polymerase chain reactions following reverse transcription (RT-PCR) have been developed recently for the identification of ABPV in purified virus suspension (Benjeddou et al., 2001), for the detection of ABPV from bee samples (Bakonyi et al., 2002) and also for phylogenetic analysis. Genotyping is in accordance with the virulence, antigenic properties and geographical distribution of the different strains (Lomniczi et al., 1998). These findings have led to increased interest for the phylogenetic relationship within ABPV isolates. The

complete structural protein gene region of ten Central-European strains was investigated recently. The isolates showed 94% to 95% identity rates compared to the reference strain, and the phylogenetic analysis revealed three distinct genotypes within the Central-European strains. A comprehensive analysis on a partial sequence within the structural protein region of several ABPV isolates found identity rates between 89% and 96%, and the isolates from Britain and from continental Europe were separated into two major branches, which were sub-divided into distinct clusters.

The relative high level of identity of the different strains in the structural protein region prompted us to investigate other parts of the ABPV genome. This paper presents the partial sequencing and sequence analysis of the non-structural protein gene region of a Hungarian and a Polish ABPV isolate.

4.2. MATERIALS AND METHODS

4.2.1.Viruses

The two investigated ABPV strains were isolated from cases of increased bee mortality in Hungary (Békési et al., 1999) and in Poland (Topolska et al., 1995). The viruses were propagated in bee pupae, purified by gradient ultracentrifugation and identified by agarose gel immundiffusion (AGID) test and electron microscopy (EM).

4.2.2. RNA purification and reverse transcription

Viral RNA was isolated from the ultracentrifuge grade purified and dialyzed virus suspensions employing QIAamp viral RNA Mini Kit (Qiagen, Germany). The RNA was reverse-transcribed into cDNA (complementary DNA) using oligo (dT) primer method with RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania), following the manufacturer's instructions. RNA and cDNA was stored at -80 °C.

4.2.3. Primer design

Amplifications were performed using ABPV specific oligonucleotide primer sequences. Six pairs of primers were selected based on the published complete genome sequence of ABPV (accession number: AF150629) in GenBank database (http://www.ncbi.nlm.nih.gov) with the help of Primer 2.0 and Oligo 3.4 software programmes. The primers were designed

to produce overlapping amplicons for sequencing (Figure 1.). The sequences, orientations, locations and product sizes are shown in Table I. Nucleotide positions are referring to the ABPV complete sequence (AF150629). The oligonucleotides were synthesized by Creative Labor Ltd. (Szeged, Hungary).

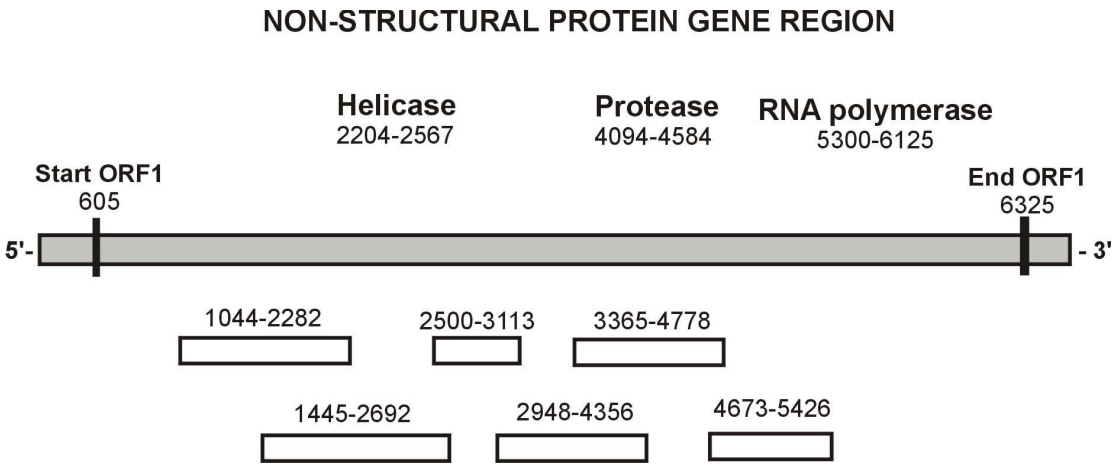


Figure 1.: Schematic representation of the non-structural protein gene region of ABPV (ORF1). The amplified RT-PCR products are represented with open boxes. Numbers indicate the nucleotide positions of the three enzyme encoding regions and the six amplicons.

4.2.4. Polymerase chain reaction

The reaction mixture contained 5 µl of 10 × reaction buffer, 1.5 mM $MgCl_2$, 10 pmol deoxynucleozide triphosphate mix (dNTP), 20 pmol from the appropriate primers, 2µl cDNA, 1.5 U Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania) and distilled water up to the final volume of 50 µl. After an initial incubation at 94 °C for 3 min, the PCR mixture was subjected to 40 cycles. One replication cycle consisted of heat denaturation step at 94 °C for 1 min, primer annealing at 55 °C for 1 min and DNA extension at 72 °C for 1 min. Thereafter the samples were maintained at 72 °C for 2 minutes for the final extension.

Table I. Oligonucleotide primer pairs selected for ABPV RT-PCR

Primer[a]	Sequence (5´ to 3´)	Nucleotide positions[b]	Length of the amplified product (bp)
ABPV 1044f	GTG GCT CAT GGA CTG ACA TA	1044 - 1063	1238
ABPV 2282r	CTA TTG CTA GAG GCC AAG TC	2263 - 2282	
ABPV 1445f	TGT GTA GAT TTG ATT AGT GC	1445 - 1464	1247
ABPV 2692r	TTT TCC TGC TGA TTT AGA CC	2673 - 2692	
ABPV 2500f	CAT GGC TCA CCT CGA AGA TA	2500 - 2519	613
ABPV 3113r	CAG CTA TTC CTT CAT ACG TG	3094 - 3113	
ABPV 2948f	GCA CGT ACT ATG GAT GAA GC	2948 - 2967	1408
ABPV 4356r	ACT GGA AGA GTG ACC TCA CA	4337 - 4356	
ABPV 3365f	TGG AAA TGG TTA TGT AGT GG	3365 - 3384	1413
ABPV 4778r	GTG AAG GCA AAT CGC AAA AA	4759 - 4778	
ABPV 4673f	CAA TTG GAC CTA GAT CAG AC	4673 - 4692	753
ABPV 5426r	CCA TAA GGT GTG CTA TGA AG	5407 - 5426	

[a] f: forward; r: reverse

[b] Nucleotide positions refer to the published complete ABPV sequence (GenBank accession No. AF150629).

4.2.5. Gel electrophoresis

Following the RT-PCR reaction, 10 µl of the amplicons was electrophoresed in a 1% Tris borate–EDTA-agarose gel (Seakem FCM BioProducts, Rockland Maine, USA) containing 0.5 µg/ml ethidium bromide, at 80 V for 1 hour. The bands were visualized by UV translumination at 312 nm and photographed by a Kodak DS Electrophoresis Documentation and Analysis System using the Kodak Digital Science 1D software. Product sizes were determined with the references to λ phage DNA cleaved with *Pst*I restriction enzyme.

4.2.6. Nucleotide sequencing and computer analysis

The PCR products amplified from Hungarian and Polish ABPV strains were electrophoresed in a 0.8 % Standard Low-mr Agarose Gel (Bio-Rad, Richmond, CA, USA) at 80 V for 2 hours. The amplicons were excised from the gel and extracted using QIAquick Gel Extraction Kit (Qiagen, Germany). Fluorescence-based direct sequencing PCRs were performed at the Biological Research Centre of the Hungarian Academy of Sciences in Szeged, and the products were sequenced in both directions employing an AbiPrism 2.1.0

automated sequencing system. The primers used for sequencing were identical to those in the RT-PCR reaction.

The nucleotide sequences were complied and compared using FASTA (NCBI) and BioEdit 4.7.8. softwares, and were verified by visual inspection. Deduced amino acid sequences were generated and multiple alignments were performed using BioEdit 4.7.8, DNASIS 7.04 and Clustal W 5.a softwares.

Nucleotide sequence accession numbers: The ABPV sequences described in this paper were submitted to GenBank database under accession numbers AF486072 and AF486073.

4.3. RESULTS

4.3.1. Amplification of selected genomic regions

Specific oligonucleotide primers were applied for the amplification of the helicase and protease domain region of a Hungarian (Hungary 1) and a Polish (Poland 1) ABPV strain by RT-PCR. The amplifications yielded clear and distinct products of the expected molecular weight with both strains. Products and product sizes are demonstrated in Figure 2.

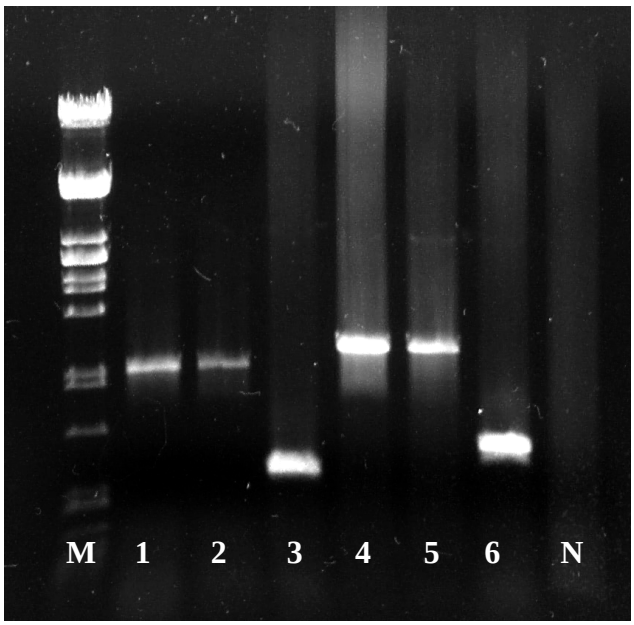


Figure 2.: Gel electrophoresis of RT-PCR products on the non-structural gene region of ABPV (Lane 1: ABPV1044f – ABPV 2282r, 2: ABPV 1445f – ABPV 2692r, 3: ABPV 2500f – ABPV 3113r, 4: ABPV 2948f – ABPV 4356r , 5: ABPV 3365f – ABPV 4778r, 6: ABPV 4673f – ABPV 5426r). Lane M: DNA size marker (*PstI* cleaved λ-phage DNA), N: Negative control

4.3.2. Sequence analysis and comparison

The amplicons produced by different primer pairs were sequenced in both directions. In total, 24 sequences were determined and identified as ABPV by using FASTA search against the GenBank database. The sequences of the six overlapping PCR products were complied and aligned to the published complete ABPV sequence (AF150629, Govan et. al., 2000.). Continuous nucleotides sequences were determined between nt positions 1063 and 5406 of the Hungary 1 (AF486072) and Poland 1 (AF486073) strains. The 4338 nt long sequence covers 45.7% of the genome and contains the helicase and protease gene regions. Multiple alignments of the sequences are shown in Figure 3. The Hungary 1 strain has 93.1% identity, while the Poland 1 strain has 93.0% identity to the reference strain. The two Central-European strains show 97.3% sequence identity. The differences are mainly separate point mutations and lower level of sequence divergence was observed within the helicase and protease domain regions than the other parts of the reading frame (Hungary 1–Ref. strain: 96% identity, Poland 1 – Ref. strain: 94-96% identity). Nucleotide deletions in the Central-European strains (or insertions in the reference strain) were observed in nt positions 1730, 1751, 1752, 1851, 1852 and 1855, respectively.

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AF150629 (1063) ATTGTTGTTGTTGTCTGGAGATGTAGAAACCAATCCAGGACCTGTTGAAACGTATAAAGACTTATGCAGAAGGAAAAATATTCGTAAGAGAAAATCTCGA
AF486072        T.....A....G.................T.....T.........................G..T.............................A...
AF486073        ......A.....................T.....T......A.................G..T.............C.............A...

AF150629 (1163) ATTCGAGAAGAAATCAAAATGCAACAACATATTGATAAGATAATAGGGCAGGAGAATGAAGAGTATAAGATTATAAACGTCAATATGCAGGGAATCTTTA
AF486072        .CC.............................C.....................................................................
AF486073        .CC...........T.................C.........A.............................T..T.................

AF150629 (1263) GTTTTAATGAAGAAAAAGAAATAATTAAAAGTACGGCTTGGAAATTCAATAGCACTCTAGACAAAACAAATTCTATAATAGACAATTTAATACCCCAATT
AF486072        .......C...........G...........C.........................G..T.....G...........G..T.....G.....T.....
AF486073        ............................C.........................G..T.....G...........G..T.....G.....T.....

AF150629 (1363) AGAAGAAACTTTAGCAGGTTTCAGAAAAACATATTCAAAATGTGAATCAAAAATTTTTGGTACTATAAGTGTGGTTGATGTGTGTGTAGATTTGATTAGT
AF486072        G...................T.......................G.........................A..A..C.............G.....
AF486073        G...................T.........................G.....................A.....................G.....

AF150629 (1463) GCTTTATTACAAGTTAGTTTTGCTAAACCAGCAATGAAGATAGCTTCATTAGCAGTAGAAGTTTTTAGATTAATTAAGAAATATGTTAGCAATATTAATA
AF486072        ..G...C....G.................................A.................C........G.................T.........G
AF486073        ..GC..C....G.................................A.................C........G.................T........CG

AF150629 (1536) TCAATATCGATAAGATTAAAGAATTGTTATCTTATGGTAAGGTAGCTCTTAACAATAATAATCCAATTATTCATGTAACAATGCAATCTAATTCTCCCAT
AF486072        .T..............G.........G............A.....................................................C.....C.....
AF486073        .T.....................................A.....................................................C.....C.....

AF150629 (1663) TCTTGAAGTTTTATTACAACCCAACATTATAGTTAGCGCCATATTTATAGCATTAAGCGTAGTGTTCCACAAAAAATTTACCTACAAAAAACTGGGAATT
AF486072        .T.....................T...........T..T.................T.........-.......................--CA......
AF486073        .TA....................................T.................T.........-.......................--CA......

AF150629 (1763) GAAGCAATGATTAAAAGACTTGGAGATTTGGGAAGAGCAGCCAAAGGCTGTTCAGATTTGAATGTGGTGCTCAACCAGGCTATAACCAACCACATGTTAG
AF486072        ..............................................G........G...........A.....T..T..A..........--..-.......
AF486073        ......................G...................G.....................A........T.............--.C-........

AF150629 (1863) AACATTTTGGGAAGAATGTTCTTGGTTTAAAACAAGAAGATGAACTCAAAGTCCTAGTGGAAGGATATAGAAATTGGTGTGATGAAGTCAGAGATTTAGT
AF486072        .C.........................................G......A.TT..........T.......................T..G........
AF486073        .C.....................................G............A.TT..........T.......G..............T...........

AF150629 (1963) AGGACATAAAATCAATTCAGATGGGGAATTAGACAGCAAAAGTATCGTTGAAAATATAATGAAGGATGTTTATGAGATACAAAGAATTGAGAATATGTAT
AF486072        ............T..C.................T...........T.........................................A.........
AF486073        ............T..C.........A.....G..T..T.........T...........T.....................................A.........

AF150629 (2063) AAGAAAGGTTTGGAGATTTCTCGTAACATAGCTGAATTAAAGTTACCAACAAAATTAACAATTAGTTTTAATACGCACATGAGATACTTGACTGAAGTTT
AF486072        ............A..A.....C.....T.....................G........G..............C.....T.........C..........C.
AF486073        ..............A.....C.....T.....................................................T...................C.

AF150629 (2163) TCAAATCAGTTGACACATCTGGAGCTTTTGGAAACAAACCTCGAACGCAACCAATAGTTATTTGGCTTTTTGGAGAATCAGGAAGAGGAAAATCGGGGAT
AF486072        .T.....................................................................................................
AF486073        .T.....................................................C........C...................G.................
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AF150629 (2263) GACTTGGCCTCTAGCAATAGATCTAAATAACAGCTTACTTGATAATGTAGATGAAATGAGGAATTTTTCAAAGAACATCTATATGAGAAATGTTGAACAG
AF486072        .........T...................T.....................................................C......
AF486073        .........T...................T.....................G.....................T.....G.....C......

AF150629 (2363) GAATTCTGGGATAATTATCAAGGACAAAATATTGTATGTYATGATGATTTTGGACAAATGAGGGATTCATCATCTAACCCTAACCCTGAATTTATGGAGT
AF486072        ....................................T....C.................................T.....................
AF486073        .....T.........................A.....T....C.................A.............T.....................

AF150629 (2463) TAATACGTACAGCTAACATAGCTCCATACCCCTTACACATGGCTCACCTCGAAGATAAAAGGAAAACAAAATTCACATCAAAAGTAATCATTATGACATC
AF486072        ............C...........G..T..T.......................C.................T...........T..T........G..
AF486073        ............................T..T.....................................T...........G.....C.....G..

AF150629 (2563) TAATGTTTTTGAACAGGATGTTAATTCACTAACATTTCCAGATGCATTTAGGAGAAGAGTAGATTTGTGCGCTGAGGTAAAGAATAAAGATGAATTTACA
AF486072        ...........................................................T.....................C....
AF486073        ...........................................................T...................C.....C...

AF150629 (2663) AAAATGTGCTGGTCTAAATCAGCAGGAAAAATGGTTCAGAGATTAGATAAGGGAAAGGTTAAGAAAATAACAGGAGATATTCATTCAACAGTACCATACA
AF486072        ..........................................G.......A...A.....................C.........A.........
AF486073        ..........................................G.......A...A..C..................C.........A.......T.

AF150629 (2763) TTGTTGATCTGATTGATCCAGAAAGCGGGGAAGTGTATAAGACTGGATTAGAATATGAAGAATTTCTCGATATGTGTCTAGAAAAGACTAGTCAATGTAG
AF486072        ..............................C.....C.....A.....................T.........T..................G.....
AF486073        ....A.........................T.....C.....A.....................T.........T..................G.....

AF150629 (2863) AGATGATTCTGCAAAATTAAATGATTTTTTAATGGATTATGCAGAGAAAAGAGCAAATAGAAGCAGAGAAATTGATGAAGTTTGCGCACGTACTATGGAT
AF486072        ...C..C.................G.............C.....................G..T....................T.............
AF486073        ...C..C.................G.............C.....................G..T.A..................................

AF150629 (2963) GAAGCTTTTGTTGACGCATATGATGACGTCATCGATGTAAACATGCAAATTGAAACAGTTGATGAAATGGAACTGATTGAACCCAACAAATTAAGAGAGA
AF486072        ........C.....................A..T.............G........G..C.....................T....T.......A.....
AF486073        ..............................A..T.......G......G........G..............................T.......A.....

AF150629 (3063) TGATAGAACAATGCTCAAATAAGATAGTATACACGTATGAAGGAATAGCTGTTAAAATAACATCATTAGCATTTAAACTAGCCACACTAAACTACGAAGA
AF486072        ..........G......G...T......T.........C.........................G.....G...........T..C...G.T..T.....
AF486073        ..........G......G.....................................................G...........T..C.....T..T.....

AF150629 (3163) ACAATGGGAACAGATTAAGGAAATGAAATATTATGTTAAAGTGAGTTCAGGTGTGAACTATCTTAAAAGAGTGTTAAGTCAGGGTATGAAAGTGTGTGAA
AF486072        ..G................A..........C...C...................T.G...............A..G.........C..............
AF486073        ...................A..........C...C...................TGG...............A..G.........T..............

AF150629 (3263) GAATGGATGAAAGAAATGATTAACTACGTTAAGGAACATCCATGGATGACCGTTAGTTTAATACTGGGCACTCTCATTGGAATACTTACTGTTGTAGGGT
AF486072        ..............G.....................A.........................G.....A........T..C.....................A.
AF486073        ..............G...........T.....A.................T........G.....A........T..C.....................A.

AF150629 (3363) TTTGGAAATGGTTATGTAGTGGCGATAAAAAGAAGAATCCGATAAAAAGACATTTTATTAATACAGGCAATGTTCTAATCTTACCCGATAGAGAATTAAA
AF486072        .............G........G.....G..A........AG.T..............................T.G........T..............
AF486073        .............G........G.....G..A........AG.T..............................G........T...........G..

AF150629 (3463) CACCTTTTGGAAAAATCAAGAATCATTGGACTTAAGGGATATGTATATTAATCGAGTTGAAGAACACATAATTAGCCTATTGAAATTGCAACATAAAGTA
AF486072        T..T..C.......G...G.....G........G..AA.....C.........A............T.......A.........G..............
AF486073        T..T.........................G........G..AA.....C.........A............T.......A.........G..............

AF150629 (3563) GTCTTAGTTCCAAAAGTTACTAAGTATATTCTTACAACTGTTGAGAACCATGCTAAAATATCAGATAAAATCATTTTGATTACAAGAAATAGATATTTGA
AF486072        ..T.....A..G.....C.....AC.....T.A.................C......C.....................................
AF486073        ..T.....A..G.....C.....AC.....T.A.................C......C.....................................

AF150629 (3663) ATTACCAGGGCAAGTTTGTTGAATTGATTTGTGGAGAAATTAATCAATTCTTCATTGACCCGGAAACATTAGATACCAACGTGGAAGCTTTTGCTTCAGC
AF486072        .......A..T......A.....................C.................T..A.................T..................
AF486073        .......A..T......A.....................C.................T..A.................T..................

AF150629 (3763) TGATCTTAAGACATTTGTACAAAGGAAACCTATTGTCATTGAAGGACCTGAATTTGTAGAGGCACAAACATCAGGAGATCAGATTACATTAAGGAAACAA
AF486072        .........A...........G.....................................T.....................G.......C.
AF486073        .........A...........G.....G.............................T.............C..G........A.....G.......C.

AF150629 (3863) ACACAAAAAGTCATAGAGGCATTCGCTAGTTCAGATGCAATCACAATGGCTCGCAAAACACCAAAGTTTGTTGAGAGTGATGATGTGGTCGAAGTTTCAA
AF486072        .........A....T........T.....................................T....A...........C........A..T.........
AF486073        .........A....T........T.....................................T....A...........C........A..T.........

AF150629 (3963) TGCAAATGTGGAAAGATCAAGTTGCGCAAAAGTTGATAACTAACCGAGTTTTAACAAACCTGTATAAAATTTGTTTAGTTAAGGAGAATGGTGATATGGT
AF486072        ............G.....G.....A.................................TT.............C.......G.................
AF486073        ...................G.....................................T.............C.......G...........C.....

AF150629 (4063) ACCGCTGCTAAATGGTTTGTTTGTTCGTTCAAACATAATGTTAGCCCCTGGACACTTAGTGGGTTTCTTATCAGATAGTGATACAATTGAAATAAGAAAT
AF486072        ...A.....................................................A.....................................
AF486073        ...A.....G.....................................................C........C.....................

AF150629 (4163) CTATTTGATGTAGTTTTCAGAGTACCATGGAAAGACGTGAAGAAAGTTGACGTAGTAAACGCCTTCGGTGAGAGCAAAGAAGCGGTTTTGTTATGTTTTC
AF486072        ..................................T.............T........T.....T.................A.................
AF486073        ..................................T.............T........T.....T.......T.........A.............C.

AF150629 (4263) CGAAATTTGTATGTCAACACACGGATTTGGTGAAGCATTTTCAAGATTCAGAATCTATGTCAAAATTCAAAAGATGTGAGGTCACTCTTCCAGTGTTGAG
AF486072        .A.................T..A..C.............................A.....................................
AF486073        .A.................T..A.................................A.....................................

AF150629 (4363) ATATTCAGATAAAATGAATAGATTTTTAGCAACATTAATAGAATGTGACAAAGTTGAAGCTTATGATAGACCATACACTTTAAATGACTCATCAAAAGGA
AF486072        ...........................................................C...........T.....................
AF486073        ...........................................................C...........T.....................

AF150629 (4463) CAATATATATTAAGACAAGGATTGGAATACACCATGCCAACAACAAATGGGGATTGTGGTGCACCATTAGTAATTAATGAAACCCAAGTTATACGTAAAA
AF486072        ...........G..........A.............G.....G.....................C.................T.................
AF486073        ...........G..........A.............G.....G.....................C.................T.................

AF150629 (4563) TAGCTGGAATTCATGTTGCTGGTGATGCCCGAGGAAAAGCTTATGCAGAATCAATAAGTCAAAAAGATTTAATAAGAGCTTTTTCTAAAATTGACGTTAG
AF486072        ..............................C..G.............G.............G.................C...........T.....
AF486073        ..............................C..G.............G.............G.................C...........T.....

AF150629 (4663) TATGCAGATTCAATTGGACCTAGATCAGACATTGAATTTTAACCAACAGCAGAVAATAATACCACCAAACGCAGAATTCGGACCTGAGGATTTAGATTTT
AF486072        ......A.....................................A....A.............T.............................
AF486073        ......A.....................................C........A....G.........C.....T.....................

AF150629 (4763) TGCGATTTGCCTTCACTTAAAATGATACCAGTGGGTAGATTGAGTGAACCTTTGTTCGAACCTGGTAAGACAGACATACGACCTTCCCTGGTATATGGAA
AF486072        ..T.....A.....................G.....C...........G........T..............G..............T.....C....G.
AF486073        ..T.....A.....................G.....C.....................T.....................................T.....C....G.

AF150629 (4863) AAATCTCAGAAATTAAGACAAAACCCGCCATCCTAAGAAATGTGATAGTGGATGGAAAAATTGTTAATATAAAACATAAAAACTTGAAGAAATGCGCCAT
AF486072        ....T..G.................T..T...T.G..G......G.G.....................................................T..
AF486073        ....T..G.................T...T.G..G......G.G.....................................................T..T..
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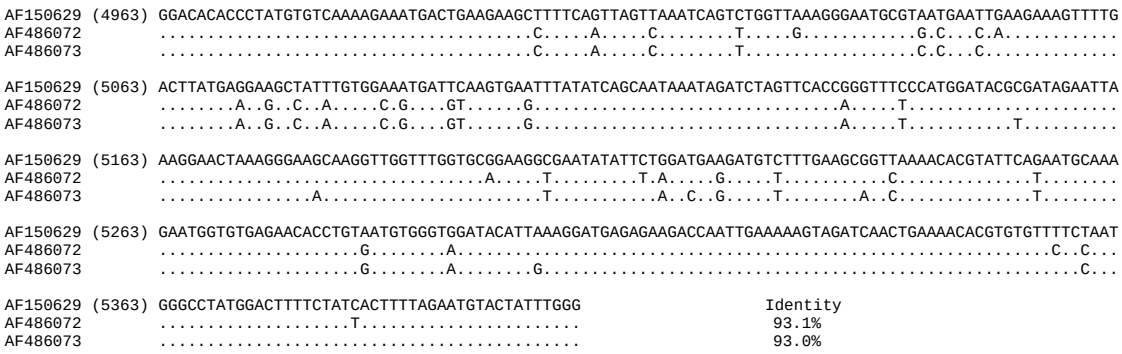

Figure 3.: Multiple alignments of the nucleotide sequences of the helicase and protease protein regions of ABPV isolates Hungary 1 (AF486072) and Poland 1 (AF486073). Nucleotide positions 1063 to 5406 are according to the reference strain (AF150629). Helicase and protease domains are underlined.

Deduced amino acid sequences were generated from the nucleotide sequences of the Hungarian and Polish strains. The 1447 amino acid (aa) long polypeptide was also aligned to the replicase polyprotein region of the reference strain revealing 96.3% sequence identity between the Hungary 1 and reference strain, and 96.4% identity between the Poland 1 and reference strain (Figure 4). The Hungarian and Polish isolates showed 98.7% aa identity. The aa sequences of the helicase domain region are practically identical in the three analyzed sequences. (In the case of the reference strain in aa position 600 the amino acid could not been determined, because in the nucleotide sequence in position 2402 a pyrimidine base indicated. In the Hungarian and Polish isolates a thymidine could be found in this position.) Within the protease domain region one amino acid divergence was observed in the Polish strain in aa position 1211. Amino acid deletions were found in the Central-European isolates in aa positions 381 and 416.

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AF150629 (aa  954) TFWKNQESLDLRDMYINRVEEHIISLLKLQHKVVLVPKVTKYILTTVENHAKISDKIILITRNRYLNYQGKFVELICGEINQFFIDPETLDTNVEAFASA
AF486072           ....S.......N.H..Q......N.................H.........L...................I.........................
AF486073           ............N.H..Q......N.................H.........L...................I.........................

AF150629 (aa 1054) DLKTFVQRKPIVIEGPEFVEAQTSGDQITLRKQTQKVIEAFASSDAITMARKTPKFVESDDVVEVSMQMWKDQVAQKLITNRVLTNLYKICLVKENGDMV
AF486072           ................................P...I................S.....................................R......
AF486073           ................................P...I................S.....................................R......

AF150629 (aa 1154) PLLNGLFVRSNIMLAPGHLVGFLSDSDTIEIRNLFDVVFRVPWKDVKKVDVVNAFGESKEAVLLCFPKFVCQHTDLVKHFQDSESMSKFKRCEVTLPVLR
AF486072           ..................................................................................................
AF486073           ..........................................................I.......................................

AF150629 (aa 1254) YSDKMNRFLATLIECDKVEAYDRPYTLNDSSKGQYILRQGLEYTMPTTNGDCGAPLVINETQVIRKIAGIHVAGDARGKAYAESISQKDLIRAFSKIDVS
AF486072           ..................................................................................................
AF486073           ..................................................................................................

AF150629 (aa 1354) MQIQLDLDQTLNFNQQQXIIPPNAEFGPEDLDFCDLPSLKMIPVGRLSEPLFEPGKTDIRPSLVYGKISEIKTKPAILRNVIVDGKIVNIKHKNLKKCAM
AF486072           ................K.............................................H................V.................
AF486073           ................R.............................................H................V.................

AF150629 (aa 1454) DTPYVSKEMTEEAFQLVKSVWLKGMRNELKKVLTYEEAICGNDSSEFISAINRSSSPGFPWIRDRIKGTKGKQGWFGAEGEYILDEDVFEAVKTRIQNAK
AF486072           ........................D.............S.V.....................................................H...
AF486073           ........................H.............S.V.....................................................H...

AF150629 (aa 1554) NGVRTPVMWVDTLKDERRPIEKVDQLKTRVFSNGPMDFSITFRMYYL                    Identity
AF486072           ..........................................                      96.3%
AF486073           ..........................................                      96.4%
```

Figure 4.: Multiple alignments of the deduced amino acid sequences of the helicase and protease protein regions of ABPV isolates Hungary 1 (AF486072) and Poland 1 (AF486073). Amino acid positions 154 to 1601 are according to the ORF1 of the reference strain (AF150629). Helicase and protease domains are underlined.

4.5. DISCUSSION

Several factors contribute in the development of clinical symptoms of ABPV infection. Besides the susceptibility of the host species, the environmental predisposing factors and the virulence of the pathogen also influence the course of the disease. The honey bee is not the only host of ABPV. The virus has been detected also in bumblebees (*Bombus spp*.) in Britain, and it is the only bee virus known so far to have an alternative host in the nature (Bailey and Ball, 1991). On the other hand, there are no data available on the differences in the susceptibility and sensitivity to ABPV in the different subspecies or breeds of the honey bee. The individual biological status of certain bees or colonies (i.e. age, immune-response, colony population) may also be important in the course of the disease. Weakening factors, such as intoxication or infestation with parasites (especially the presence of *V. destructor*) facilitates the development of virus-induced diseases.

Natural virulence-variants have been observed within several virus genera (i.e. porcine enterovirus, avian paramyxovirus...). The differences in the virulence are the consequences of mutations resulting in changes of the viral proteins. In some cases the changes of the structural proteins lead to modified surface receptors of the virions, and therefore modified tissue specificity in the host organism. In other cases non-structural proteins change, influencing the multiplication of the virus in the host cell and also the effect of the virus

infection on the cell, on the organ or on the host organism. Virulence is an inherited property of the virus with genetic background, and its changes are the results of virus evolution. Therefore the differences in the viral genome are useful indicators helping the detection and characterization of virus strains with diverse virulence.

The aim of our investigations was to detect variable regions in the ABPV genome and describe the differences in virus strains with various geographic origin. An earlier study revealed a relatively low level of sequence divergence of ABPV strains in the structural protein gene region (Bakonyi et al., unpublished). This time we focused on the non-structural protein region of two ABPV isolates. Since the RNA dependant RNA polymerase is usually a very conserved genomic region in RNA viruses, the genes of the other two enzymes, helicase and protease were analysed in this work.

The investigations revealed a sequence divergence in the non-structural protein gene region similar to the one observed in the structural protein genes (93%). The Central-European strains share higher identity to each other (97%) than to the reference strain (most probable of British origin). Most part of the nucleotide changes result "silent mutations", they do not influence the amino acid sequence. Particularly the amino acid sequences of the putative helicase and protease domains are practically unchanged coding for identical enzymes.

Although our findings do not prove the presence of virulence-variants within ABPV strains, they do not refute it either. On one hand the virus strains investigated in this study are originated from clinical cases of outbreaks of the disease, so both of them may be “more virulent” strains. The results described in this paper support the exact differentiation within the diverse strains and provides the methodical base of the phylogenetic analysis of the non-structural protein gene region of new ABPV isolates.

Chapter 5

Detection of a new variant of Kashmir bee virus in Hungary

5.1. INTRODUCTION

Kashmir bee virus (KBV) was first isolated from *Apis cerana* (Bailey and Woods, 1977) but later was also detected in *Apis mellifera*. The virus seems to cause inapparent infections throughout Australasia, and is also present on the American continent (Bruce et al., 1995). However, in Europe it has only been detected in Spain so far (Allen and Ball, 1995).

In laboratory experiments, KBV appears to be the most pathogenic of the honeybee viruses, killing brood and adult bees within three days following injection of only a few infective particles (Bailey et al., 1979).

Employing the AGID test, which is the most widespread method used in bee virus identification, KBV cross-reacts with acute bee paralysis virus (ABPV); thus it was suggested, that KBV and ABPV are variants of the same virus, and different names for these viruses might be inappropriate (Allen and Ball, 1995). This antigenic relationship may also lead to misdiagnosis, especially in the case of inapparent infections, which demonstrates the importance of more specific assays.

The recently introduced genetic methods of virus demonstration (RT-PCR) are useful not only in the diagnosis but also in the identification and classification of viruses. The polymerase chain reaction (PCR) enables the exact identification of viruses even if they are closely related to each other, supposing that at least partial nucleotide sequences are known from a certain virus. (RT-) PCR is relatively fast, sparing virus propagation and purification, and thus avoids the difficulties in honeybee virus isolation, which are caused by the lack of species- specific cell cultures. This method is also extremely sensitive and specific compared to AGID. PCR also provides the opportunity to perform phylogenetic analysis by sequencing the amplification products and comparing them to sequences of different other viruses deposited in the GenBank database. This allows to recognize the relatedness of viruses, which is important for viral epidemiology and taxonomy. Therefore nowadays the taxonomical unit “serotype” is frequently complemented by “genotype”, referring to the characteristic changes in the viral genome, which are important for virus evolution, but may not reflected the antigenic properties in all cases (Harrach and Benkő, 1998). Mostly, however, are “genotype”

and “serotype” not contradictory, but complete and confirm each other (Harrach and Benkő, 1998).

This paper presents the partial sequencing and sequence comparison of two honeybee viruses isolated in Hungary from a disease outbreak accompanied with high mortality of adult bees.

5.2. MATERIALS AND METHODS

5.2.1. Specimens

A virus strain designated Hu-B1/97 was isolated from honeybee samples. The virus was propagated in pupae and purified by caesium-chloride gradient ultracentrifugation (Békési et al., 1999, Bakonyi et al., 2002). The pupae used for virus propagation were previously tested and have been found negative for the presence of four bee pathogenic viruses: ABPV, KBV, black queen cell virus (BQCV) and sacbrood virus (SBV). The virus was identified as ABPV by AGID test.

5.2.2. Agar gel immunodiffusion test

The purified virus suspension was tested against anti-ABPV reference immune-serum in wells punctured into 0.9 % (w/v) agar plates. Reference ABPV virus suspension and reference serum applied as controls originated from the collection of the Faculty of Veterinary Medicine, Warsaw Agricultural University. Twofold dilutions of the antigen were made in PBS.

5.2.3. Nucleic acid isolation and reverse transcription

The viral RNA was extracted from 140 µl of the gradient purified and dialysed virus solution by employing the QIAamp viral RNA purification kit, following the manufacturer´s instructions. Pure RNA was eluted in 40 µl of appropriate buffer. The reverse-transcription was performed on 11 µl of viral RNA extract using RevertAid™ First Strand cDNA Synthesis Kit (MBA Fermentas, Vilnius, Lithuania).

5.2.4. Oligonucleotides

Primer pairs were designed for the amplification of distinct genomic regions of ABPV and KBV. The oligonucleotides were selected from the available and most conservative parts of the nucleotide sequences of the aforementioned viruses deposited in GenBank database (ABPV: accession numbers AF126050, AF150629; KBV: AF263732, AF034541) (Hung et al., 2000). The oligonucleotides were designed using the Primer 2.0 and Oligo 3.4 programs. The primers used in the RT-PCR reactions are presented in Table I., and their location on the viral genome is illustrated in Figure 1.

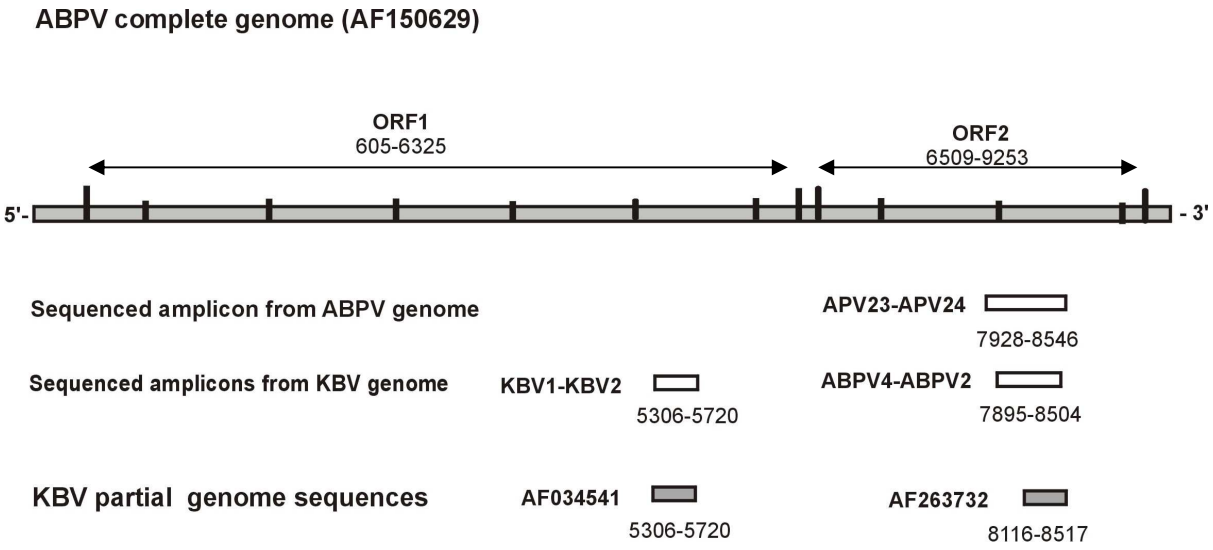

Figure 1.: The location of the sequenced products amplified by the different primer pairs from the ABPV and the KBV like component of the virus suspension. Shaded boxes represent the ABPV complete genome and the partial KBV sequences available in the GenBank. Open reading frame 1 (ORF1) encodes the non structural proteins, and ORF2 the structural proteins of the virion. Open boxes represent the amplicons, numbers refer to the nucleotide positions on the complete ABPV genome (AF150629).

5.2.5. PCR, nucleotide sequencing and sequence analyses

The nucleic acid multiplications, sequencing and sequence analysis procedures were performed as described previously (Bakonyi et al., 2002).

Table I.: Primers selected for RT-PCR of ABPV and KBV

Oligo[a]	Sequence (5' to 3')	Nucleotide positions	Product
ABPV2r	CCA CTT CCA CAC AAC TAT CG	7895-7914 [b]	609
ABPV4f	TGC AAG TGT TAT CAC CGT TA	8485-8504 [b]	
APV23f	GTG CTA TCT TGG AAT ACT AC	7928-7947 [b]	618
APV24r	AAG GYT TAG GTT CTA CTA CT	8527-8546 [b]	
KBV1f	GAT GAA CGT CGA CCT ATT GA	5305-5324 [c]	415
KBV2r	TGT GGG TTG GCT ATG AGT CA	5701-5720 [c]	

[a] f: forward; r: reverse primers

Nucleotide positions refer to the published [b] complete ABPV sequence (GenBank accession number: AF150629), [c] partial KBV sequence (AF034541).

5.3. RESULTS

5.3.1. AGID test

Well visible double precipitation lines were seen between the wells containing the ABPV-specific immune-serum and 1:2 - 1:8 dilutions of the purified virus suspension (Figure 2).

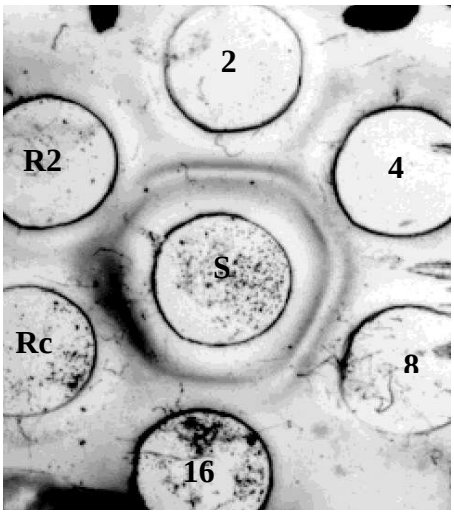

Figure 2.: The agarose gel precipitation of the isolate containing two viruses that react with anti-ABPV rabbit serum. Serum was applied into the central reservoir (S); the serial twofold dilutions of the antigen were measured clockwise (2-16), two wells contain reference ABPV virus (Rc) and its twofold dilution (R2).

5.3.2. Electrophoresis of the PCR products

Following RT-PCR with the APV23f - APV24r primer pair on the isolated RNA of the purified virus suspension, a 618 bp product was detected. Using the ABPV4f and ABPV2r oligonucleotide primers, a 609 bp band was visible in the gels after electrophoresis. After PCR amplifications, signals were only detected in the artificially infected pupae and never in the non-infected controls (Figure 3). The Kashmir bee virus specific primers (KBVf and KBVr) produced an amplicon of 415 bp following RT-PCR performed on the isolated viral RNA (Figure 3). None of the other ABPV strains isolated so far by our group (Bakonyi et al., 2002) gave positive results with the KBV-specific primer pair.

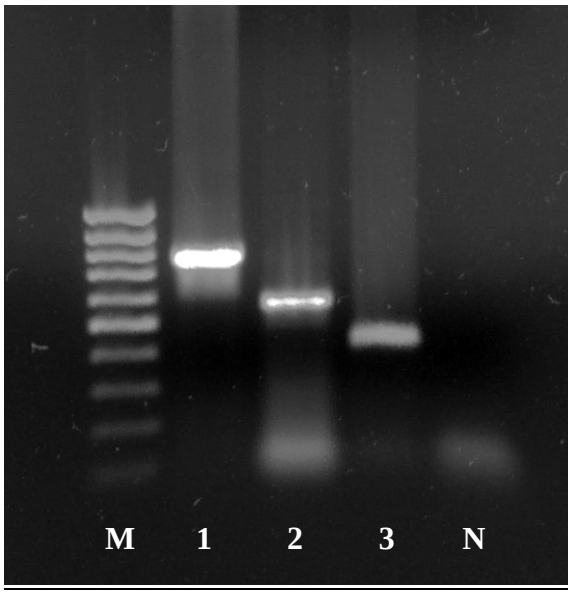

Figure 3.: The agarose gel electrophoresis of the amplicons produced by different primer pairs. M: 100 bp ladder, 1: APV23f-APV24r, 2: ABPV4f-ABPV2r, 3: KBV1f-KBV2r, N: negative control.

5.3.3. Nucleotide sequencing and computer analysis

The PCR products amplified by different primer pairs were sequenced, and the nucleotide sequences were compared with the sequences deposited in the GenBank database. The amplicon produced by the primer pair APV23f - APV24r (AY053377) showed the highest identity with the ABPV genome (Govan et al., 2000; AF150629). In case of the PCR products amplified by the oligonucleotide primers ABPV4f and ABPV2r (AF346301) the highest identity was found with a partial sequence of Kashmir bee virus structural protein

gene (AF263732) followed by the sequence of the ABPV complete genome (Govan et al., 2000; AF150629).

The KBV1f - KBV2r amplification product exhibited highest identity with a partial sequence of the polymerase gene region of a KBV sequence (AF034541) deposited in the GenBank database.

In the structural protein gene region two amplicons (AY053377 and AF346301) were aligned to the ABPV complete genome sequence. The two amplicons overlap in a 394 bp region, and they also overlap with a partial KBV structural protein sequence (AF263732) (Figure 4.). In this overlapping region the product generated with primers APV23f-APV24r shows 93.6 % identity to ABPV and 72.4 % identity to KBV. These data prove that one of the components present in our virus suspension is an ABPV variant. The amplicon produced by ABPV4f-ABPV2r shows 79.0 % identity to KBV and only 71.9 % to ABPV. This indicates that the other virus isolate is closer to KBV than to ABPV. (The reference ABPV and KBV sequences have 69.4 % identity to each other in this region.).

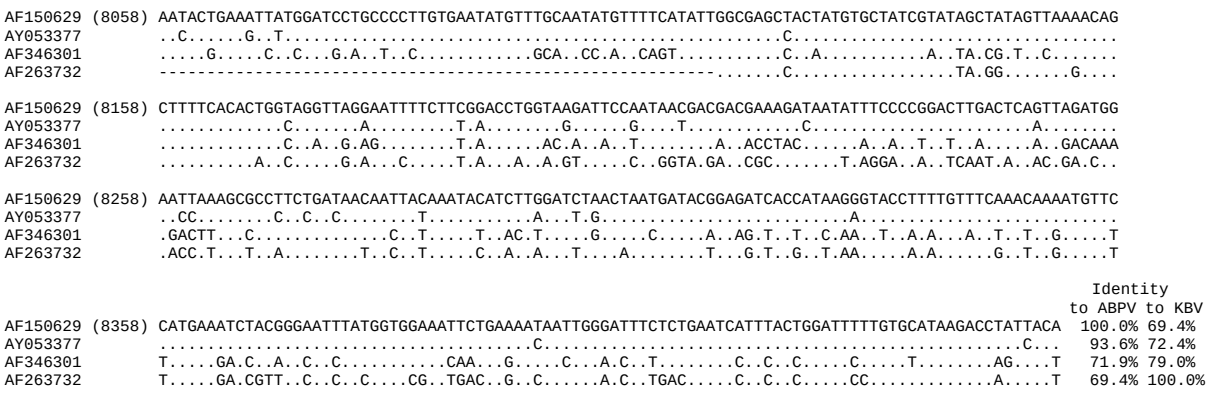

Figure 4.: The nucleotide sequences of amplicons aligned to reference ABPV (AF150629) and KBV (AF263732) sequences. The two amplicons were produced by oligos APV23f and APV24r (AY053377) and ABPV4f and ABPV2r (AF376301). AY053377 is the ABPV like component of the isolate, AF376301 is the one amplified from the KBV like component. The nucleotide identity rates are shown at the end of the alignment.

In the non-structural protein gene region, the nucleotide sequence of the amplicon produced by the primer pair KBV1f - KBV2r (AF468967) was also aligned to reference KBV and ABPV sequences (Figure 5). It shows an 83.6 % identity to the partial KBV sequence, and 78.6 % identity to ABPV. (The reference ABPV and KBV sequences exhibit 76.6 % identity to each other in this region.) This is an additional proof that one component of the isolate is genetically closer to KBV.

```
AF150629 (5336) CAACTGAAAACACGTGTGTTTTCTAATGGGCCTATGGACTTTTCTATCACTTTTAGAATGTACTATTTGGGCTTCATAGCACACCTTATGGAAAATCGAA
AF468967        AC.T.......T.....A.....A.....A..A.....T..C..A..T......C.G.....T........T..T.....C...T.A.....G..C....
AF034541        ...T..........A..A..C..A.....A..A.....T..C.....AG.....C.......T...........T.....T..TT.A............

AF150629 (5436) TAACCAATGAAGTATCCATAGGAACTAATGTTTATTCCCAAGATTGGAATAAGACAGTTAGAAAACTTAAAACTATGGGACCCAAGGTTATTGCAGGAGA
AF468967        .T..T........T..T..T.....G..C..G.....T..G.......G.................T.G..GCAA......AGT..A..A..C..T..T..
AF034541        .T..T.....G.....T..T.....G.....G.....T..G..C....G...A..T...C.C...T.G.CC.AAT.T...AAT..A........G..T..

AF150629 (5536) TTTCTCAACCTTTGATGGATCTTTGAATGTTTGCATTATGGAAAAATTTGCTGACCTAGCGAATGAATTTTATGATGACGGATCAGAGAATGCATTAATT
AF468967        ...T.....T...........AC.T..C.......................A..T..T..T.....G......A....T...C.C..A............
AF034541        ...T.....T...........AC.......A..T.................G..TT..........G.....C.....T..TG.T......TGCC.G...

                                                                                                                Identity
                                                                                                              to ABPV   to KBV
AF150629 (5636) CGACATGTTTTGCTTATGGATGTATATAACTCAACACACATTTGTGGTGATTCCGTATATATGATGACACACAGTCAACCCTCT   100.0%      76.6%
AF468967        ........A..AT.G........G..C..T...GT......C.....A.....A..T...........T..T..C......AAA    78.6%      83.6%
AF034541        A.G.....G..AT.G........G.....T..TGT.........CAA...C..T..C...........T..T..C......A.A    76.6%     100.0%
```

Figure 5.: The nucleotide sequence of the amplicon produced by KBV1f and KBV2r oligos (AF468967) aligned to reference ABPV (AF150629) and KBV (AF034541) sequences. The aligned product is amplified from the KBV like component. The nucleotide identity rates are shown at the end of the alignment.

The deduced amino acid sequences are shown aligned in Figure 6 (structural protein gene region) and Figure 7 (non-structural protein gene region), respectively. The differences in the amino acid sequence identities are less prominent; the identity of the HuB1/97 variant in the structural protein gene region (ORF2) was 82.8 % to KBV and 83.8 % to ABPV. The ABPV variant shows in the same region 95.5 % identity to ABPV and only 79.3 % to KBV. The KBV and ABPV reference sequences share 76.6 % identity at the amino acid level.

```
AF150629 (aa  539) RATMCYRIAIVKTAFHTGRLGIFFGPGKIPITTTKDNISPDLTQLDGIKAPSDNNYKYILDLTNDTEITIRVPFVSNKMFMKSTGIYGGNSENNWDFSES
AY053377           ....................E...E..R...M.................................T..............................
AF346301           ........TV..........E...E..Q......PT.........TKDL..................V..K..Y........T.......N....N....
AF263732           ........TV..........E...E..S..MVR.AD.LG..QS..N.TI..................V..K..Y........TV....A.D....N.D..

                                      Identity %
                                    to ABPV     to KBV
AF150629 (aa  639) FTGFLCIRPIT      100.0        76.6
AY053377           ...........       95.5        79.3
AF346301           .........V.       83.8        82.8
AF263732           ...........       76.6       100.0
```

Figure 6.: The alignment of the deduced amino acid sequences of the corresponding amplicons in the structural protein genome region. The amino acid identity rates are shown at the end of the alignment.

```
AF150629 (aa 1779) QLKTRVFSNGPMDFSITFRMYYLGFIAHLMENRITNEVSIGTNVYSQDWNKTVRKLKTMGPKVIAGDFSTFDGSLNVCIMEKFADLANEFYDDGSENALI
AF468967           T.....................................................S.......Q..S.............................N..P.....
AF034541           ................A.............................S......TKF.N.............................A..C..

                                                 Identity %
                                               to ABPV     to KBV
AF150629 (aa 1879) RHVLLMDVYNSTHICGDSVYMMTHSQPS     100.0        91.4
AF468967           ...........V..............K      93.7        91.4
AF034541           ...........V...N..........T      91.4       100.0
```

Figure 7.: The alignment of the deduced amino acid sequences of the corresponding amplicons in the non structural protein genome region. The amino acid identity rates are shown at the end of the alignment.

In the investigated ORF1 region, the HuB1/97 isolate shares 91.4 % amino acid identity with KBV and 93.7 % with ABPV; the ABPV and KBV reference sequences also exhibit 91.4 % identity to each other.

5.4. DISCUSSION

Our results suggest, that ABPV and KBV seem to have a common ancestor, and have developed since then through serial point mutations in different directions. Whether they now may be considered to be different viruses or only virus variants will be a matter of international discussions and agreement. The close antigenic relationship of ABPV and KBV is also demonstrated by their serological cross-reactivity in AGID. The traditional classification of viruses at the species level is often based on antigenic properties of the viruses resulting in the proposal of different "serotypes". Serotyping, however, seems to be inappropriate method to classify honeybee viruses due to several reasons. The most widespread method of serotyping, the virus-neutralization is not possible in the case of honeybee viruses due to the lack of cell cultures of honey bee origin. Hemagglutination inhibition is not possible in the case of non hemagglutinating viruses to which group most bee viruses belong. The specificity of AGID, complement fixation and immunofluorescence is insufficient to rely on when exact taxation of viruses is the aim. Therefore, in case of honeybee viruses, genotyping seems to be a very effective and appropriate method for virus classification. The characteristics of genomic organization and the ratio of identity between different viruses can be the basis of classification, to determine whether a virus is only a variant among several others, or a different pathogen.

Since in our investigations, the alignment of the nucleotide sequence has revealed an identity of 79.0 % to the KBV and only 71.9 % to the prototype ABPV in the first sequenced part within ORF2, we concluded, that our isolate is rather a variant of KBV rather than of ABPV. The results prompted us to check our sample with KBV specific primers, which were designed against the sequences deposited in the GenBank database. The discriminating primer pair (KBV1f and KBV2r) annealed to our new variant, but not to the ABPV related component of our virus suspension or to any other ABPV strain from our collection. The amplicon was also sequenced, and the sequences were aligned.

Sequence alignment and analysis revealed that the differences are mainly point mutations in both, the structural and in the non-structural regions. Some of the nucleotides present in Hu-B1/97, and not identical in the three viruses, are common with ABPV and others with KBV, so most probably our new virus is rather an intermediate variant between

ABPV and KBV and not a result of recombination (although this possibility may also be given by the simultaneous presence of the two viruses in bee populations). Furthermore our finding supports the theory, that ABPV and KBV should not be considered to be separate viruses, but variants of the same pathogen. This supposition is strengthened further by the results of the alignment of the deduced amino acid sequences. Despite of the higher identity of the HuB1/97 with KBV at the nucleic acid level, our new variant's deduced amino acid sequence shows higher identity to ABPV due to the silent mutations.

Unfortunately the further sequencing of Hu-B1/97 was hindered up to now by the inability of the primers designed against the prototype ABPV genome to anneal to the Hu-B1/97 genome. KBV partial sequences from regions different from the sequenced ones are not deposited in the GenBank database; therefore, we could not design further KBV specific primers. Though we are aware of the fact that the Hu-B1/97 sequence is too short to make final conclusions, this low level of homology would mean a high diversity in the genome of different ABPV/KBV strains. This diversity is difficult to prove by AGID due to the strong cross reaction caused by the similar protein structure (indicated by the high identity of the amino acid sequence). It is also difficult to reveal by sequencing of PCR products, because primers may not attach to the different variants' genomes due to the relatively higher diversity at the nucleic acid sequence level. If this varying degree of identity is valid for the whole genome, the possibility of variation in the biological properties (pathogenicity, virulence, etc.) of the virus cannot be excluded. This premise may explain the contradiction, that the ABPV usually causes inapparent infection, but sometimes is reported to be a major cause of mortality especially in mite-infested colonies (Ball and Allen 1988, Bakonyi et al. 2002).

To summarise, our results show, that at least two rather different genotypes of ABPV/KBV are present simultaneously in the Hungarian apiaries, and the later seems to be genetically closer to the partially sequenced KBV strain than to ABPV. The differences in the nucleotide sequence of the ABPV reference strain and Hu-B1/97 prompted us to start a thorough and detailed sequence analysis on our other PCR positive samples. Further studies should reveal the occurrence rate of the Hu-B1/97 strain in Hungary, and help to decide whether the different genotype is connected with differences in pathogenicity or any other characteristics of an infection caused by the different virus strains.

Chapter 6

Development of reverse transcription-polymerase chain reactions for the detection of four honey bee viruses.

6.1. INTRODUCTION

The scientific and practical impact of the research on honey bee viruses is emerging worldwide. Although 18 different viruses have been isolated from honey bees so far (Allen and Ball, 1996), our knowledge on the distribution and economical or animal health importance of these viruses is still incomplete. Most of them do not cause clinical symptoms, but in several cases viral infections induce severe diseases (Bailey and Ball, 1991). Pathognomical symptoms of the disease can be observed on worker bee larvae, for example, in the case of sacbrood virus (SBV, White, 1917; Bailey et al., 1964; Bailey and Ball, 1991), or on queen-cells infected with black queen-cell virus (BQCV, Bailey and Woods, 1977; Bailely and Ball, 1996). Other viruses, like acute paralysis virus (ABPV, Bailey et al., 1963; Ball et al., 1985) or Kashmir bee virus (KBV, Bailey and Woods, 1977; Hung et al., 1996b) may induce sudden collapse of bee colonies infested with the parasitic mite *Varroa destructor*. Since cell cultures of bee origin are not available at the moment, the detection of bee viruses is based on the propagation of viruses by experimental infection of bee pupae or newly emerged bees followed by electron microscopy (EM) and agargel-immunodiffusion (AGID) or ELISA using specific sera (Allen et al., 1986; Vecchi et al., 1990).

The development of the new molecular diagnostic techniques gave the opportunity for the detailed investigation of the nucleic acid structure in several honey bee viruses (Evans and Hung, 2000). Complete genome sequences have been determined in the case of SBV (Ghosh et al., 1999), BQCV (Leat et al., 2000) and ABPV (Govan et al., 2000), while from KBV partial genome sequences were determined (Hung et al., 2000). Sensitive and reliable diagnostic methods were developed for the detection of viral nucleic acid of KBV (Stoltz et al., 1995), SBV (Grabensteiner et al., 2001), ABPV and BQCV (Benjeddou et al., 2001) applying reverse transcription polymerase chain reaction (RT-PCR).

This scientific note presents a diagnostic method for the detection and identification of four RNA viruses of the honey bee using RT-PCR in one amplification panel.

6.2. MATERIALS AND METHODS

6.2.1. Viruses

The viruses tested by RT-PCR were isolated in Hungary (ABPV, KBV) and in Poland (SBV, BQCV, Topolska et al., 1995) from field samples. Viruses were propagated in honey bee pupae, purified by gradient ultracentrifugation and identified using EM investigations and AGID tests (Békési et al., 1999; Topolska et al., 1995).

6.2.2. RNA purification and reverse transcription

Viral RNA was isolated with QIAamp viral RNA Mini Kit and reverse transcription was performed using oligo(dT) primers applying RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) following the manufacturer's instructions.

6.2.3. Oligonucleotides

Four primer pairs were designed upon from the published genome sequences of BQCV (accession number: AF183905), KBV (AF034541), SBV (AF092924) and ABPV (AF150629) for the amplification of distinct genomic regions of the viral RNA with distinct size in the different viruses. Primer 2.0 software (Scientific and Educational Software, Serial No. 50178) was used for the design. The primer sequences, orientations, locations and product sizes are shown in Table I. The oligonucleotides were synthesized by Creative Labor Ltd. (Szeged, Hungary).

6.2.4. PCR

Amplifications were performed in 50 µl reaction mixtures containing 1.5 mM $MgCl_2$, 10 pmol deoxynucleozide triphosphate (dNTP) mix, 50 pmol of the appropriate primers, 2 µl cDNA and 1.5 U Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania). After the initial incubation step at 94°C for 3 min, the reaction mixture was subjected to 40 cycles of heat denaturation step at 94°C for 1 min, followed by primer annealing step at 55°C for 1 min, and DNA extension step at 72°C for 1 min. The reactions were terminated with a final extension step at 72°C for 5 min for the. The amplifications were performed in MJ Research MiniCycler (MJ Research, Inc. Watertown, Massachusetts, USA).

Table I.: Primers selected for RT-PCR of BQCV, KBV, SBV and ABPV

Primer[a]	Sequence (5´ to 3´)	Nucleotide positions	Length of the amplified product (bp)
BQCV76f	GTA TGC TTG GAG ACC AGG TT	76 - 95 [b]	223
BQCV299r	CAT GCT TCA GGT AGT ACA GG	280 - 299 [b]	
KBV1f	GAT GAA CGT CGA CCT ATT GA	5305 - 5324 [c]	415
KBV2r	TGT GGG TTG GCT ATG AGT CA	5701 - 5720 [c]	
SBV1f	ATA CCA ACC GAT TCC TCA GT	218 - 237 [d]	645
SBV2r	TCA TTC CTT TTA CCA TTT AC	844 - 863 [d]	
ABPV 4673f	CAA TTG GAC CTA GAT CAG AC	4673 - 4692 [c]	753
ABPV 5426r	CCA TAA GGT GTG CTA TGA AG	5407 - 5426 [c]	

[a] f: forward; r: reverse primers

Nucleotide positions refer to the published complete [b]BQCV (AF183905), [c]ABPV (AF150629) and [d]SBV (AF150629) sequences.

6.2.5. Gel electrophoresis

The products (10 µl) were electrophoresed in 1% Tris borate-EDTA buffered agarose gel containing 0.5 µg/ml ethidium bromide, at 6 V/cm for 1 hour. The bands were visualized by UV translumination at 312 nm and photographed by a Kodak DS Electrophoresis Documentation and Analysis System using the Kodak Digital Science 1D software. Product sizes were determined with the reference to GenRuler 100 bp DNA Ladder (MBI Fermentas, Vilnius, Lithuania).

6.3. RESULTS AND DISCUSSION

The genomic RNAs of four bee viruses were detected by specific primer pairs in four separate RT-PCR reactions using the same amplification panel. The amplifications resulted distinct products appearing as definite bands of the expected molecular weights (Figure 1). The BQCV specific primers amplified a product about 230 bp in size from the BQCV strain and also in the case of 7 out of 8 field samples (queen pupae from blackened cells) from Poland. Queen cells showing clinical symptoms were not available from Hungary so far, and the tested apparently healthy pupae were negative by RT-PCR. A Hungarian bee virus isolate (Hu-B1/97) was tested with the KBV specific primers. The oligonucleotides amplified an about 410 bp long product from this isolate, but not from other genetically related isolates.

The SBV specific primers generated an approximately 650 bp long product with the Polish SBV virus strain, but not with the other templates. Five Hungarian field samples (gondola-shaped pupae) have been also tested, but they were found to be negative by RT-PCR and by electron microscopy as well. Amplifications with the ABPV specific primer pair resulted products with approximately 750 bp in size from a purified Hungarian ABPV isolate, from 10 tested Hungarian field samples and also from a Polish ABPV isolate as well. In the case of the KBV and ABPV primers, the specificity of the amplifications were also verified by the sequencing of the PCR products (accession numbers AF468967, AF486072 and AF486073).

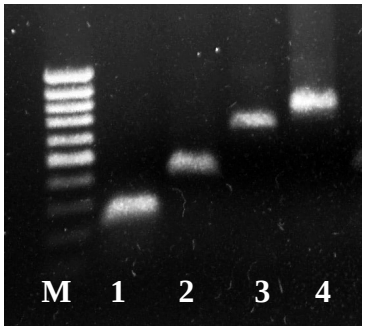

Figure 1.: The agarose gel electrophoresis of the RT-PCR products of BQCV, KBV, SBV and APPV isolates with the corresponding specific primer pairs. M: 100 bp ladder, 1: BQCV76f-BQCV299r (223 bp), 2: KBV1f-KBV2r (415 bp), 3: SBV1f-SBV2r (645 bp) 4: ABPV4673f-ABPV5426r (753 bp)

The RT-PCR methods described in this paper provide a quick and easy technique for the detection of the four most important bee viruses. This system exceeds the previous ones in the practical point of view. Since the amplification conditions are the same for all eight primers, it is possible to perform the reactions with different primers using the same amplification programme, sparing time and working-power. The primers were selected to provide well distinguishable products with different molecular weight (BQCV: 223 bp, KBV: 415 bp, SBV: 645 bp, ABPV: 753 bp), which makes the identification easier. Further attempts are being performed to establish the optimal combination of the different primer concentrations, to establish a multiplex RT-PCR system based on the aforementioned specific primers.

V. CONCLUSIONS AND NEW RESULTS

This work represents the results of a four years long work on the occurrence of honey bee viruses in Hungary and also reveals the relationship and divergence of acute bee paralysis virus (ABPV) strains. Although honey bee viruses are distributed world-wide, and the presence of some viruses was also suspected in Hungary, investigations focused on these viruses have not been performed before.

A clinical case with sudden collapse of bee colonies turned our interest towards the virus infections of the honey bees. The first isolation of the ABPV in Hungary was the beginning of diverse and comprehensive series of investigations. Besides epidemiological studies, development of new diagnostic methods and comparative molecular and phylogenetic investigations on certain bee viruses were carried out within the frames of our research programme. Taking the specialities of the host species into consideration, we had to broaden our knowledge to beekeeping, bee pathology and parasitology as well. In the last few years remarkable international scientific development could be observed in the field of bee virus research. For example, all the complete genome sequences and molecular investigation methods on bee viruses have been described in the last three years. These new results rendered significant help to our research, and also prompted us to do all efforts to save our position in the vanguard of bee virus research.

Our investigations resulted the following new scientific results:

1. We have detected, propagated, identified and described for the first time the acute bee paralysis virus in diseased bees collected from a Hungarian apiary.
2. We have developed rapid and sensitive diagnostic methods based on RT-PCR for the detection of ABPV, KBV, SBV and BQCV in honey bee samples.
3. We had started a survey on the occurrence of the virus in Hungary, and demonstrated the presence of ABPV in two third of the investigated apiaries. The comparison of the occurrence rate of the virus in healthy colonies and in colonies with symptoms of the disease supported the impact of the virus in bee diseases.

4. We have demonstrated the presence of the viral nucleic acid of the ABPV in the parasitic mite *Varroa destructor*, supporting the theories on the role of the mite as a vector of the virus.
5. We have analyzed the genetic relationship between Central European ABPV strains. Phylogenetic trees based on the alignments of the structural protein gene region revealed at least two distinct genetic linkages of ABPV, which are sub-divided into several genotypes reflecting the geographic origin of the isolates. The methods provide a basis of phylogenetic analysis of new ABPV isolates.
6. We have determined the partial nucleotide sequence and the sequence divergence of the non structural protein gene region of a Hungarian and a Polish ABPV isolate.
7. We have detected and isolated a new virus variant in a honey bee sample from Hungary. The phylogenetic analysis of the new variant indicated close relationship with KBV and ABPV strains.

Since all of our new results raise several further questions, we plan to carry on the research on bee viruses. We would like to sequence longer parts from the new Kashmir-like virus variant and also would like to continue the sequencing of the ABPV isolates. We would like to develop new diagnostic methods for the detection of other bee viruses and with the help of these new techniques, we would like to isolate further viruses from Hungarian honey bee samples.

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