Szent István University

Veterinary Science Doctoral School

Investigations with certain pathogens of the honeybee (*Apis mellifera* L.) with special respect to the viruses

PhD Thesis

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INTRODUCTION

Honey bees (*Apis mellifera* L.) play an important role in nature and agriculture, they are needed for pollination of different crops. Besides that bee products (honey, royal jelly, wax and bee venom) were used from ancient times until today, for therapeutic purposes, and consumption.

Since the awareness to the importance of bees as producers of food, as well as pollinators and producers of industrial row material have been increased, different bee diseases are regarded with more attention, and scientific research of bee pathogens (*Paenibacillus larvae, Melissococcus pluton*, *Ascosphaera apis, Aspergillus fumigatus*, *Nosema apis, Nosema ceranae, Varroa destructor* and bee viruses) has been continued with increased effort and interest.

The object of the work presented here was to develop diagnostic methods for detection of bee viruses, to carry out a survey on the occurrence of seven honeybee-pathogenic viruses and to develop genetic relationship between selected bee viruses. The survey on the detection of bee viruses and their occurence in Hungarian apiaries is based on using one step RT-PCR assays. With partial sequencing of the genome of selected bee viruses phylogenetic analysis was carreid out.

Besdies the virological surveys the other aim of this study was to detect and identify two parasites of Hungarian *A. mellifera* colonies: the *Nosema apis* and the *Nosema ceranae*. The presence of *N. ceranae* in European honey bee colonies has been reported first time from Spain in 2006. This study revealed, that the Asian *N. ceranae* is more frequent in Europen apiaries, than the European *N. apis*. We developed a discriminative molecular diagnostic assay for the detection of these two Nosema species, and we carried out a survey on the occurence of the two Nosema species in Hungary.

MATERIALS AND METHODS

Prevavlence of pathogenic bee viruses in Hungary

The aim of this survey were to estimate the incidences of the seven economically most important honeybee viruses in Hungarian apiaries: the acute bee paralysis virus (ABPV), the black queen cell virus (BQCV), the chronic bee paralysis virus (CBPV), the deformed wing virus (DWV), the Kashmir bee virus (KBV), the sacbrood virus (SBV), and the israeli acute bee paralysis virus (IAPV). The survey was carried out in two period: between 1999-2004 and

in 2007. Between 1999-2004 honey bee samples were collected from 52, in 2007 from 72 apiaries of volunteer beekeepers from different locations in all parts of Hungary. After the bees were homogenized in glass potters viral RNA was isolated using QIAamp viral RNA mini kit (Qiagen, Düsseldorf, Germany). Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using QIAgen OneStep RT–PCR Kit (Qiagen, Düsseldorf, Germany). Amplifications were performed using virus specific oligonucleotide primer sequences. The oliginucleotide primers detecting ABPV, BQCV, CBPV, DWV, IAPV, KBV and SBV were designed by our research group. These oligonucleotide primers were designed based on the viruses genome deposited in the GenBank databese (National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov/Genbank/index.html). In 2007 we used the primers of Ribiere in detection of CBPV. Following the RT-PCR reactions the amplicons were electrophoreised in 2% Tris-acetate-EDTA agarose gel (containing 0,5µg/ml ethidium-bromid) (OmniPur, Darmstadt, Germany). Reaction mixtures without RNA served as negative controls, and verified positive samples as positive controls. In the case of the IAPV the positiv control served the RNA cloned in a plasmid from Prof. I. Sela (The Hebrew University of Jerusalem, Faculty of Agricultural, Food and Environmental Quality Sciences, Rehovot 76100, Israel).

Phylogentic analysis of selected black queen cell viruses (BQCV)

Bee samples from Hungary, Poland and Austria were used and investigated. The sample processing and the extraction of the viral RNA was the same written above. Two oligonucleotide primer-pairs were designed based on the BQCV genome depositded in the GenBank database (accesion number: AF183905). Primers were designed to amplify partial sequences of the structural polyprotein gene, and the RNA helicase enzyme gene regions. Further sets of primers were also designed to determine the complete genome sequence of selected BQCV genotypes. The RT-PCR and the gel-electrophoresis were carried as written above. The PCR products amplified for sequencing were electrophoretised in 0,8% Standard Low_{mr} Agarose Gel (Bio-Rad, Richmond, CA, USA), and they were excised from the gel and extracted using QIAquick Gel Extraction Kit, (QIAgen, Düsseldorf, Germany). The sequencing PCRs were performed at the Biological Research Center of Hungarian Academy of Sciences in Szeged, and in Agricultural and Biotechnological Research Center (Gödöllő), employing an Abiprisim 310 automated sequencing system. The nucleotide sequences were identified by the Basic Local Alignment Search Tool (NCBI) and they were compiled and aligned with the help of BioEdit 4.7.8 and Align Plus (Scientific and Educational Software)

programs. Multiple nucleotide alignments were created by the ClustalX 1.8 program. The phylogenetic analysis was performed using the Phylogeny Inference Program Package (PHYLIP, version 3,6b, Felsenstein, 2004). Bootstrap resampling analysis of 1000 replicates was performed with the SEQBOOT program to prove the stability of the trees. Distance matrices were generated by the DNADIST/Neighbor-Joining and Fitch programs, using translation/transversion ratio of 2.0. Phylogenetic trees were drawn with the help of TreeView (Win32, version 1.6.6.) software. The differences of the similarity levels of the different genomic regions of selected BQCV genotypes were plotted by the SimPlot program (version 3.5.1., Ray, 2003) using the 2-parameter (Kimura) distance model. The BQCV sequences described in this paper were submitted to GenBank database under accession numbers EF517501-EF517522.

First detection and dominance of *Nosema ceranae* in Hungarian honeybee colonies

38 Hungarian honeybee samples were collected in 2006 and 2007. DNA was extracted from the bee suspension produced by the sample processing with QIAamp DNA Mini Kit (Qiagen, Düsseldorf, Germany) after treatment with a lysis buffer (20mg lysosime/ml, 20mM Tris-HCl [pH 8], 2mM EDTA, 1.2% Triton). Oligonucleotide primers were designed to anneal the LSUrRNA-coding genome region of *N. apis* and *N. ceranae*, according to the sequences deposited in GenBank database (accession numbers: DQ078785 and U97150). After PCR reaction the gel-electrophoresis were carried as written above. For quick differentiation between amplification products of the two nosema species, restriction fragment length polymorphism (RFLP) analysis was applied. The restriction endonuclease *Msl* I cleaves the DNA of the amplicon of *N. ceranae* at the motif CACTA|GTAT**G** resulting a 175 and a 262 nucleotide long fragment, but not that of *N. apis,* due to a nucleotide substitution within the cleavage site (CACTAGTAT**A**). The nucleic acid sequences of the PCR products were determined in subsequent direct sequencing PCRs, and they were compared to the nucleotide sequences deposited in the GenBank to validate the method.

RESULTS, DISCUSSION

Prevalence of pathogenic bee viruses in Hungary

Four honeybee viruses (ABPV, BQCV, DWV, and SBV) were detected in the investigated Hungarian bee samples between 11999-2004. The highest percentage of infection was detected for DWV which occurred in 72% of the investigated apiaries. The second most

frequently detected bee virus in Hungary was BQCV which was found in 54% of the investigated apiaries. ABPV was detected in 37% apiaries, and SBV only in 2% of the apiaries. In 2007 the most frequently virus was the ABPV, it was found in 70,8% of the apiaries. The second frequently virus was the SBV with 62%, the third was DWV 48,6%. The BQCV was present in the 40% of the apiaries. In this year the CBPV was also detected in the 5,5% of the investigated Hungarian apiaries. It is visible, that the presence of the viruses changed during the investigated two periods. The data are demonstrated in Table 1.

Table 1: The occurrence of viruses in adult honeybee samples investigated in surveys between 1999-2004 and 2007.

Virus	**Occurence**	
	between 1999-2004	**in 2007**
Deformed wing virus (DWV)	72%	48,6%
Black queen cell virus (BQCV)	54%	40%
Acute bee paralysis virus (ABPV)	37%	70,8%
Sacbrood virus (SBV)	2%	62%
Chronic bee paralysis virus (CBPV)	0%	5,5%
Kashmir bee virus (KBV)	0%	-
Israeli acute bee paralysis virus (IAPV)	-	0%

In addition, differences were observed regarding simultaneous infections. Between 1999 and 2004 37% of the investigated apiaries were infected with one virus. In 46% there were two viruses, in 11% three viruses. In 2007 16,6% of the apiaries were infected with one virus, 30,5% with two viruses, 34,7% with three viruses, 9,7% with four viruses and 1,4% with five viruses. The data are demonstrated in Figure 1.

There is change in the prevalence of the viruses in this two periods. Two viruses decreased significant, DWV and BQCV are more frequent in 1999-2004 than in 2007. Besides that, the prevalence of two viruses increased in this period. The prevalence of ABPV and SBV is notably higher in 2007 than in 1999-2004. ABPV was in 37% of the apiaries in the first period, and in the second survey it was 70,8%. The SBV had low prevalence in 1999-2004 (2%), and it increased to 62%. The increased SBV prevalence can explain the higher larval death reported by beekeepers during the summer of 2007. Sacbrood is a condition affecting the brood of the honeybee: larvae with sacbrood fail to pupate and death. The increased occurence of the ABPV can explain the narrowed bee colonies during 2007. IAPV was not detected in Hungarian apiaries. In the United States the colony collapse disorder (CCD) was due this virus, and it was detected also in Europe, in France.

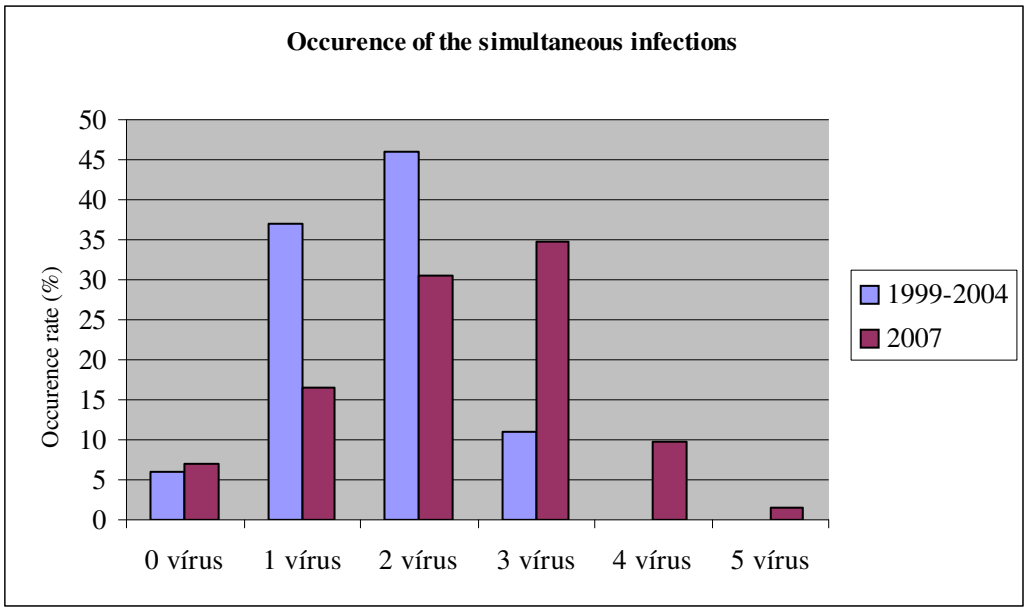

Figure 1: The occurence of the simultaneous infections in 1999-2004 and in 2007.

The number of the simultaneous infected colonies increased. In 1999-2004 the 57% of the investigated colonies were infected with more than one virus, however in 2007 76%. Besides, between 1999-2004 to a maximum three virus was present in one colony at the same time, in 2007 we could find colonies infected with five viruses. On the whole the virus incfection of the bee colonies increased in Hungary, what can weak the colony, in this way the colony will be more sensitive to other infections, and it can lead to decreased tolerance against parasitic mite *Varroa destructor* or nosemosis.

When comparing the prevalence of honeybee-infecting viruses in Hungary to those of the earlier European Union members France and Austria, between 1999 and 2004 the virus infection and the rate of the simultaneous infection was in generally lower. This survey was performed before joining the European Union. The survey in 2007 was performed after joining the European Union. In this year we found increased virus infection in Hungary, similar to that in Austria and France. It is possible, that the joining to European Union is the responsible for it, and the European Union values will be characteristic on Hungary in the future.

Phylogentic analysis of selected black queen cell viruses (BQCV)

Specific amplification products were generated from five Austrian ten Hungarian and seven Polish samples from two regions of the BQCV genome (the helicase enzyme coding region, and the structural polyprotein coding region). The similarity rates are in Table 2. and 3.

2. Table: Similarity rates (in per cent) of the investigated BQCV strains and the reference strain in the helicase enzyme coding region.

	Austrian genotypes	Hungarian genotypes	Polish genotypes	South African reference strian
	Nucleic acid			
Austrian genotypes	95-99 98-100	94-99	82-95	82-83
Hungarian genotypes	97-100	95-99 97-100	81-96	82-84
Polish genotypes	93-98	92-98	82-100 91-100	83-90
South African reference strian	94	93-95	93-99	
	Amino acid			

3. Table: Similarity rates (in per cent) of the investigated BQCV strains and the reference strain in the structural protein coding region.

	Austrian genotypes	Hungarian genotypes	Polish genotypes	South African reference strian
	Nucleic acid v			
Austrian genotypes	96-100 99-100	96-99	89-96	92-93
Hungarian genotypes	98-100	96-99 97-100	89-96	91-93
Polish genotypes	98-100	97-100	89-99 98-100	91-94
South African reference strian	99-100	98-100	99-100	
	Amino acid			

Phylogenetic analyses were performed on the two selected genome regions. The tree based on the partial helicase coding region is demonstrated on the left of Figure 2, and the tree based on the partial structural polyprotein coding region is demonstrated on the right of Figure 2.

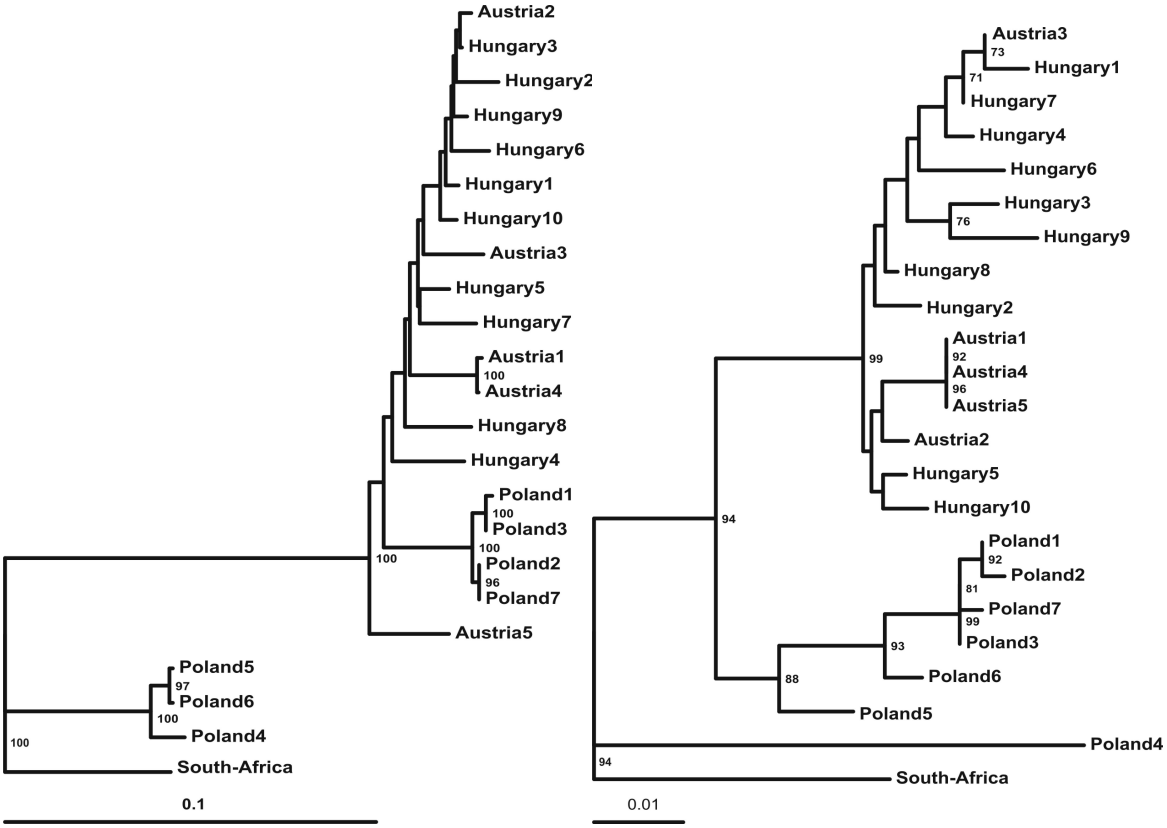

Figure 2: Neighbor-joining tree based on the partial helicase enzyme region (ORF1) (left side) and on the partial structural polyprotein coding region (ORF2) (right side) of BQCV genotypes. Internal labels indicate the percentages of bootstrap values, only values 70% were indicated. Bar represents the genetic distance.

On the tree based on the helicase enzyme coding region all Austrian and Hungarian genotypes, and Polish genotypes 1 to 3 and 7 are clustering within one branch of the tree. Three Polish genotypes (4 to 6) are forming a separate cluster, and the South-African strain represents the third main branch of the tree.

The phylogenetic tree based on the partial structural polyprotein sequences exhibits partly a considerably different topology. The Central European BQCVs form a common cluster; however it is sub-divided into two main groups. One group contains the Austrian and Hungarian genotypes, while the Polish viruses form the second group. Within the Austrian-Hungarian group, four Austrian genotypes form a separate subgroup together with two

Hungarian genotypes, while the other eight Hungarian viruses and one Austrian genotype form a second, more diverse branch. In this tree Poland5 and 6 are clustering together with the other Polish genotypes, while Poland4 remains in seclusion with similar genetic distances from both European genotypes and the South-African strain, the latter representing the third main branch of the tree.

The spatial distance, the limited trade of bee queens, and the existence of ecological barriers (i.e. the Carpathian Mountains) between the Polish and Austrian-Hungarian bee populations are plausible explanations for this sort of genetic separation of BQCV genotypes. The extreme geographical distance explains the divergence of the South-African BQCV from the European ones. One Polish genotype, however, is clustering within a separate group, which is genetically distant from both the other central European viruses and from the South-African strain.

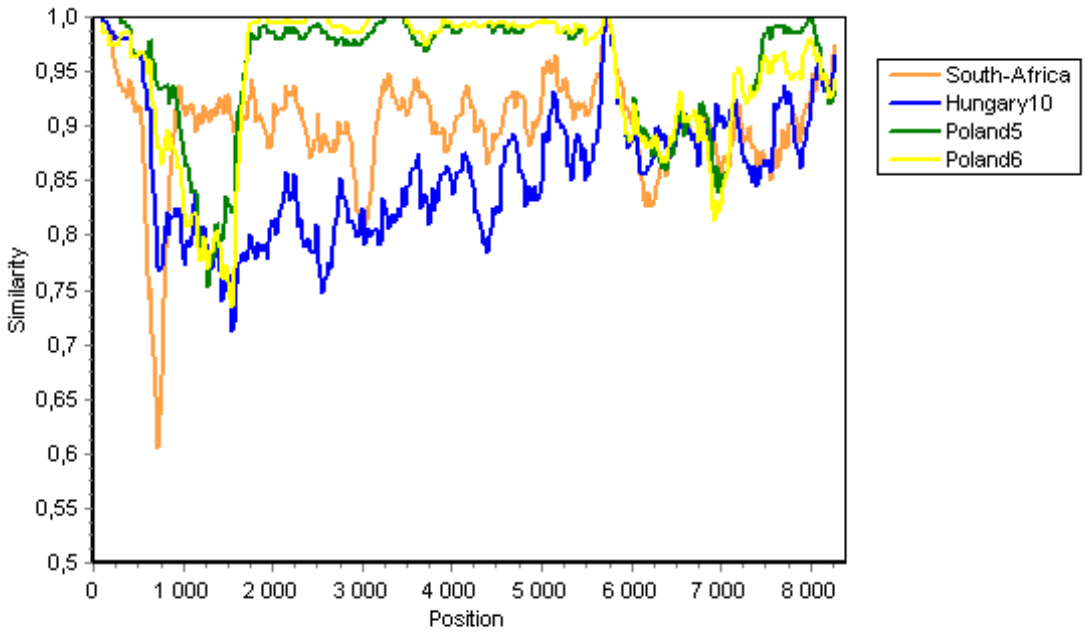

Figure 3: Similarity plot of the South-African reference strain, Polnad5, Poland6 and Hungary10 viruses compared to the Poland4 virus.

To explain the unexpected clustering of the three Polish genotypes a more comprehensive genome analysis was performed on the viruses concerned. Genetic recombination between virus genotypes could explain the different clustering of the same virus strains on the two trees constructed using sequences of the two different genomic regions. To prove this theory the genome sequence of strain Poland4, 5 and 6 were determined nearly on the complete

genome of the viruses. As a representative of the „typical" central European genotypes strain Hungary10 was also sequenced. This region covers the partial 5'UTR, the entire ORF1, intergenic and ORF2 regions, and the partial 3' end of the genome. Multiple sequence alignments were created and the similarity rates within the sequenced regions were demonstrated by SimPlot graphs (Figure 3.).

By the comparison of the BQCV sequences, a typical pattern was found in the diversity of the different genomic regions. Two genome sections (>100 nucleotides) were found where all investigated viruses were homologous. Both areas are non-coding regions close to the beginning of the ORFs. These areas usually play central roles in the transcription-initiation and the expression regulation of the subsequent genes.

The 5'-proximal third of ORF1 proved to be the most variable region of the investigated BQCVs. ORF1 encodes the non-structural proteins but the exact role of this region is still unknown (Leat *et al*., 2000). In the case of several vertebrate viruses non-structural proteins are responsible for the differences in the virulence of the strains. Another observation on the ORF1 is that the unique Polish genotypes are more similar to the South-African strain than to the Hungarian genotype at the 3' side of the frame.

The ORF2 in general exhibited a lower level of sequence divergence than ORF1. However, the similarity rates between the closely related viruses (i.e. the Polish genotypes) were lower in this region than in ORF1. Therefore, ORF2 seems to be a more suitable target region for phylogenetic analysis of BQCV, because the mutation-derived individual sequence changes are more characteristic in this area.

Because the similarities between BQCV genotypes originating from the same geographic regions (i.e. Poland4 versus 5 and 6) suddenly changed within the genome, recombinations of certain genome sections between genotypes is likely, In the current study the parties involved in the recombination events were not accurately identified. Therefore further experiments are necessary to document the exact process of a possible recombination event, and the most frequently affected genomic regions.

First detection and dominance of *Nosema ceranae* in Hungarian honeybee colonies

Nosemosis is considered to be one of the most prevalent and economically damaging of honey bee diseases. It is often associated with diarrhoea, significant reductions in both pollination efficiency and honey production, and even with the collapse of honeybee colonies. *Nosema apis* has been regarded as the causative agent of nosema disease in Europe. The symptoms of nosemosis were most frequently observed in winter and early spring, while in

summer and autumn the disease was rarely reported in Hungary. In recent years, more and more cases were observed between late spring and early autumn in oroginally strong and inetnsively foraging colonies and at warm weather conditions.

Amplification products on the LSUrRNA coding region with an estimated size between 430 and 440 nucleotides were generated from all investigated bee samples. Within the RFLP analysis, the *Msl*I enzyme cleaved the amplification products into two fragments corresponding to the expected molecular sizes (175 and 262 nucleotides long fragments).

Our investigations revealed that 37 of 38 bee samples collected in 2006 and 2007 from Hungarian honeybee colonies were infested with *N. ceranae.*

The nucleotide sequences of four amplification products were determined and compared to the sequences deposited in the GenBank database. Three sequences obtained from the *Nosema*-infested Hungarian honeybees were 100% identical with the *N. ceranae* sequence. in one case the restriction enzyme did not cleave the PCR product, and after sequencing we found that it was 100% similar to *N. apis*.

In recent study we found that *Nosema ceranae* is more frequent in Hungarian apiaries than *Nosema apis* in 2007. Similar results were obtained during the investigations of 12 bee samples from Spanish apiaries, by sequencing the PCR products of the SSUrRNA genes. They found that 11 samples were infested with *N. ceranae*, and only one sample contained *N. apis*. The changes in the seasonality of the disease indicate the recent spread of *N. ceranae* in Europe. As *N. ceranae* originates from the tropical and subtropical regions of Asia, it may prefer warmer climatic conditions, and hence it might play a role in the background of the unusual seasonal appearance of nosemosis in Hungary. It also points out that through the intercontinental trade and traffic of contaminated biological products an exotic parasite may appear and spread in Europe, leading to unexpected effects, even several years following the introduction of the agent.

The PCR-RFLP method described in this study might be a useful tool for the subsequent investigations, and also in diagnostic submissions to differentiate between *N. apis* and *N. ceranae* in honeybee samples. Differentiation of the two species is possible even without sequence analysis.

NEW RESULTS

1. We developed diagnostic methods based on polimerase chain reaction to detect honeybee viruses, ABPV, BQCV, DWV, IAPV are the expansion of our research group.
2. Using these methods we performed first in Hungary a survey in two periods on the incidence of most frequently five honeybee viruses, and based on the changes of these two period we draw conclusion on the infection kinetics of honeybee viruses.
3. We determined the nucleotide sequence of several BQCVs nearly on the complete genome of the virus, and we performed phylogenetic analysis of Central Europen BQCVs.
4. We found that in the case of BQCV (in contrast to the vertebrate viruses) the non-structural polyprotein coding region is more variable than the structural protein coding region.
5. We developed diagnostic method based on PCR to detect Nosema-disease of the honeybee, that gives also a chance to discriminate the two Nosema species (*N. apis* and *N. ceranae*) based on RFLP.
6. We found, that in Hungary *Nosema ceranae* is more frequent than *Nosema apis*.

PUBLICATION LIST

Forgách, Petra, Tamás Bakonyi, **Zsuzsanna Tapaszti**, Norbert Nowotny, Miklós Rusvai. (2007) Prevalence of pathogenic bee viruses in Hungarian apiaries: Situation before joining the European Union. J. Invertebr. Pathol. 98 (2): 235-238. **IF: 2,005**

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