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UBIQUITER CIRCOVIRUS SEQUENCES RAISE CHALLENGES IN LABORATORY DIAGNOSIS: THE CASE OF HONEY BEE AND BEE MITE, REPTILES, AND FREE LIVING AMOEBAE

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Circoviruses of pigs and birds are established pathogens, however, the exact role of other, recently described circoviruses and circovirus-like viruses remains to be elucidated. The aim of this study was the detection of circoviruses in neglected host species, including honey bees, exotic reptiles and free-living amoebae by widely used broad-spectrum polymerase chain reaction (PCR) assays specific for the replication initiation protein coding gene of these viruses. The majority of sequences obtained from honey bees were highly similar to canine and porcine circoviruses, or, were distantly related to dragonfly cycloviruses. Other *rep* sequences detected in some honey bees, reptiles and amoebae showed similarities to various *rep* sequences deposited in the GenBank. Back-to-back PCR primers designed for the amplification of whole viral genomes failed to work that suggested the existence of integrated *rep*-like elements in many samples. Rolling circle amplification and exonuclease treatment confirmed the absence of small circular DNA genomes in the specimens analysed. In case of honey bees *Varroa* mite DNA contamination might be a source of the identified endogenous *rep*-like elements. The reptile and amoebae *rep*-like sequences were nearly identical with each other and with sequences detected in chimpanzee feces raising the possibility that detection of novel or unusual *rep*-like elements in some host species might originate from the microbial community of the host. Our results indicate that attention is needed when broad-spectrum *rep* gene specific polymerase chain reaction is chosen for laboratory diagnosis of circovirus infections.

Keywords: circovirus, integrated, *rep,* sequencing

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Introduction

The amount of sequence information about viruses with single-stranded circular DNA genome increased sharply over the past few years in line with the development of high-throughput sequencing techniques that are widely used in virological investigations of eukaryotic organisms and environmental samples [1, 2]. Much has been learnt about the veterinary importance of some members of the *Circoviridae* family (including *Porcine circovirus-2* and *Beak and feather disease virus* within the *Circovirus* genus and *Chicken anaemia virus* within the *Gyrovirus* genus), affecting worldwide a handful of animal host species, including pigs and poultry [3–6]. Recently, novel circoviruses (CV) and cycloviruses (CyV) have been described in various tissues and excretes (e.g., stool, skin, cerebrospinal fluid, blood and nasopharyngeal aspirate samples) of mammals (e.g., beef, goat, camel, sheep, chimpanzee, dog, mink, bat and humans) [7–14], birds (e.g., swan, pigeon, gull, duck, goose, raven, canary, finch, and starling) [5, 7, 10, 15–22], fish (e.g., barbel, European catfish, European eel) [23–25], and insects (e.g., dragonfly and cockroach) [7, 26, 27]. Metagenomic analyses have revealed that circovirus-like viruses with a different genome length and structure are also prevalent in the environmental specimens (e.g., water, sewage, soil), although, the source and the origin of these viruses has remained unknown [1, 2, 28].

CVs are amongst the smallest animal viruses with a genome length of 1700–2000 nt, which encode two main open reading frames (ORF); one ORF encodes the capsid protein and one supports the virus replication [1, 7]. The ORF encoding the replication initiation protein is referred to as *rep* gene and contains well conserved sequences that are common among the corresponding circo-, gemini- and nanovirus sequences [1, 7]. The encoded Rep protein of these viruses plays a pivotal role in the initiation and progress of viral replication [1]. Furthermore, *rep*-like sequences were detected not only in the genome of exogenous viruses but were also described as integrated genomic elements in plasmids, transposons, phages, bacteria, and a wide variety of eukaryotic organisms, including unicellular organisms, plants, fungi and animals [29, 30].

Recently, we have participated in the survey of viruses affecting Hungarian honey bees *(Apis mellifera)* that covered diagnostic testing of known bee-viruses and some other broadly distributed viruses, including CVs. Also, we investigated the role of various viruses in the deaths of exotic reptiles perished in captivity by screening viral agents in tissue samples. A third study aimed at investigating the role of free-living amoebae (FLA) in the carriage of infectious agents, including CVs. When relevant data from these independent studies were merged we ob-

served some intriguing phenomena about CVs. Because we felt that our findings have implications of CV diagnostics we undertook to summarize our results in the following report.

Materials and methods

Samples

Honey bee and *Varroa destructor* mite samples were collected for a pilot study in Hungary. Honey bee specimens, each including eight or nine animals, were homogenized with the TissueLyser (Qiagen) high-throughput disruption instrument according to the manufacturer's recommendations. Two hundred µl of the supernatants were used for nucleic acid extraction by the MagNA Pure LC Total Nucleic Acid Isolation Kit on the MagNA Pure LC Instrument (Roche Diagnostics).

Reptile carcasses were sent to our laboratory in 2013 for virus diagnostics. Pooled samples of kidney, heart, lung, spleen, liver, intestine, stomach, trachea, oesophagus and tongue of five animals belonging to chelonian species (*Testudines* and *Trachemys* species), two Schneider's skink *(Eumeces schneideri),* one leopard gecko *(Eublepharis macularius)*, and two ball pythons *(Python regius)* were used for virus detection. DNA was isolated with DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions.

FLA species were isolated from surface water samples including the river Danube, Lake Balaton, and the Lake of City Park, Budapest. Non-nutrient agar plates (1.5% agar in Page's saline) coated with heat inactivated (60 °C, 1 hour) *Escherichia coli* were used for isolation of FLA. Amoebic trophozoites were scraped from the agar surface and resuspended in PBS. DNA was extracted with DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. The amoebae were identified by PCR specific for FLA and by sequencing of the resulting amplicons [31].

Purified DNA of CV isolates as well as clinical samples from pig (*Porcine circovirus-2,* PCV-2), pigeon, duck, goose and fish one each positive for CV were applied as positive control in the molecular biological analyses.

Laboratory methods

Broad-range PCR assays specific for the conservative *rep* region of CVs and CyVs were utilized using published oligonucleotides and protocols [10, 15].

Twenty-five µl reaction mixtures were used for both PCR systems that contained 2.5 mM $MgCl_2$, 200 µM dNTP mix, 400 nM of each primer, 1X Taq Buffer with KCl, 1 U Taq DNA polymerase (Thermo Fisher Scientific) and 1–3 µl of nucleic acid samples. The cycling protocols used for the PCR with CV-F1/CV-R1 and the nested CV-F2/CV-R2 primers were as follows: 95 °C for 3 min denaturation, 40 cycles of 95 °C for 30 s, 52 °C (first round of the PCR) or 56 °C (second round of PCR) for 30 s and 72 °C extension for 1 min, and a final extension step at 72 °C for 10 min [10]. The cycling protocols used for the PCR with Cv-s/ Cv-as and the nested Cn-s/Cv-as primers were as follows: 95 °C for 3 min denaturation, 45 cycles of 95 °C for 30 s, 46 °C (first round of PCR) or 56 °C (second round of PCR) for 1 min and 72 °C extension for 1 min, and a final extension step at 72 °C for 10 min [15].

PCR products were purified by the Geneaid Gel/PCR DNA Fragments Extraction kit (Geneaid Biotech) after gel electrophoresis and directly sequenced using an ABI PRISM 3100-Avant Genetic Analyzer and BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific).

For the amplification of whole CV genomes, back-to-back primers were designed based on the sequences obtained by diagnostic PCR and Sanger sequencing. Back-to-back PCRs were carried out in 20 µl reaction volume containing 400 nM of each primer, 250 mM dNTP mix, 1X Phusion Green HF Buffer and 0.2 U Phusion DNA polymerase (Thermo Fisher Scientific) and 1–2 µl of nucleic acid samples. The cycling protocols were as follows: 98 °C for 30 s denaturation, 45 cycles of denaturation at 98 °C for 10 s, annealing at the melting temperature of the primers for 30 s and extension at 72 °C for 1 min, and the final extension step at 72 °C for 10 min.

Rolling circle amplification (RCA) was performed with phi29 DNA polymerase from the purified nucleic acid samples. 2.5 µl of nucleic acid was denatured at 95 °C for 3 minutes and immediately placed on ice slurry. The RCA reaction mix of 25 µl contained 200 µM of dNTP mix, 30 µM of random hexamer primer mix, 1X BSA, 1X reaction buffer and 2.5 U phi29 DNA polymerase (New England Biolabs). Mixtures were incubated at 30 °C for 16 hours followed by the inactivation of the enzyme at 65 °C for 10 minutes. Results of the RCA were visualized by agarose gel electrophoresis.

Exonuclease digestion of the linear DNA molecules was achieved in a total volume of 20 µl containing 5 U of Lambda Exonuclease (New England Biolabs) and 1X Lambda Exonuclease Reaction Buffer and it was incubated at 37 °C for 1 hour and heat inactivated at 75 °C for 10 minutes. As a second step, 5 U of Exonuclease I (New England Biolabs) was added to the reaction mixture above and was incubated at 37 °C for 1 hour and then inactivated at 80 °C for 20 minutes.

Various amplification and digestion products were visualized by agarose gel electrophoresis using 1% agarose gel prepared with 0.5X TBE buffer and GelRed Nucleic Acid Gel Stain (Biotium) and the electrophoresis occurred at 100 V for 30–60 min.

Computer analysis

The newly generated sequences are available in Table I. Phylogenetic analysis was performed using the MEGA 6 software (http://www.megasoftware.net/) and phylogenetic trees were constructed by the neighbour-joining statistical method with p-distance substitution model and 500 bootstrap replicates (Fig. 1). The scale bar represents the nt substitution per site.

For comparison, *rep* and *rep*-like sequences were retrieved through blast search of the Basic Local Alignment Search Tool (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

GenBank reference sequence accession numbers were as follows: *Porcine circovirus-2,* AY424404; *Porcine circovirus-1*, AY660574; Canine circovirus, KC241983, JQ821392; Dragonfly cyclovirus 6, KC512918; Dragonfly cyclovirus 3, JX185424; TN4 cyclovirus from human stool, GQ404902; PK5006 cyclovirus from human stool, GQ404844; NG12 cyclovirus from human stool, GQ404854; Chimp162 from chimpanzee stool, GQ404883; Chimp17 from chimpanzee stool, GQ404851; *Beak and feather disease virus,* AF071878; *Duck circovirus,* DQ100076; *Swan circovirus* EU056310; Barbel circovirus, GU799606; Barbel circovirus isolate BaCV2, JF279961; Catfish circovirus isolate H5, JQ011377; Catfish circovirus isolate H6, JQ011378; *rep*-like sequence of *Catla catla,* KF051799; *rep*-like sequence of *Labeo rohita,* KF051798.

Results

CV-like sequences detected in honey bee samples

Putative CV *rep*-like sequences were detected by PCR in 30 of the 186 (16.1%) honey bee and in six (100%) *Varroa* mite samples. Eight honey bee specimens were excluded from further analysis due to the low quantity of the PCR products; 22 honey bee and all six *Varroa* PCR products were subjected to Sanger sequencing. Sequence ambiguities were observed for four honey bee and the six *Varroa* samples. Altogether, 18 honey bee sequences were determined and used for phylogenetic analyses.

Table I. Circovirus *rep*-like sequences detected in honey bees, reptiles (chelonian species, Schneider's skink, ball python) and free-living amoebae (FLA)

>hb6
CGTGAACCTTAAGAAGACCAGCCGTATGGGTGCCTTGAAGGCACGGTTGGGTGGTCGCG
GCCATTTTGAGCCTGCTCGGGGGGATGATTGCAGCAATAGAGATTATTGCTCAAAGGGGG
GCGACATATTGATTGAATCGGGCGAAGTGTCTAAGCAAGGAAAACGTAATGACTTACATG
ATGCAGTGGTGACCTTGAAGGAGACGAAGAGCCTTGCTGCGGTGGCTGCTGCTCACCCCG
AGACTTACGTGAAGTTCTCGCGAGGTCTGCGAGAGCTCCTGCTTATAAGCCCGGAGATGG
CCACGCCCCGGAAGTGGAAGACGGAAGTGCACGTCCTCGTTGGACCTCCGGGCTGTGGC
AAAAGTCGGTACTGCCTAGAGACTGCACCGGAAGCATATTGGAAGCCCCGTGG

>hb10
ATTTTAAGTTCAAAACGAAGCAACGATTCAACACTGTACGGGCGATCCTTGGAGGCCGTG
GACACCTTGAAGGTTCAAAAGGCTCTCCTAAGCAGAATCATGACTATTGTGCGAAGTCCG
GAGATTACGAAACGGTGGGGGACCTCGTTATTGCGTCACAGTCATCTCTTGAACTGGTGT
GCCAAATGCTTAAAGATGGAGTACGACTGGCTCAAGTCGCAGCCGATTACCCCTCAATTT
TTGTCCGTCACCACAGAGGCCTTAGAGAGCTTAGCCTTATTCTTGGAGTCGTCAAACCCAG
AGATTTTAAGACTGGTAAGTGGGCCGCTGCGCGGAGCATACCGGAATCGCTCTCGCGATT
CCGGGCCAATCTTTTATTAACAGTCAGGTGGATGATTCTAAGTCTTCTGGTTCTTGTTTCA
GAGGTGCTTGTGTTCTATGGAGCCTCTGGCACCGGCAAGTCTCGTCGCGCGGCAGAG

>hb13
CGTGAACCTTAAGAAGACCAGCCGTATGGGTGCCTTGAAGGCACGGTTGGGTGGTCGCG
GCCATTTTGAGCCTGCTCGGGGGGATGACTGCAGCAATAGAGATTATTGCTCAAAGGGGG
GCGATATATTGATTGAATCGGGCGAAGTGTCTAAGCAAGGGAAACGTAATGACTTACATG
ATGCAGTGGTGACCTTGAAGGAGACGAAGAGCCTTGCTGCGGTGGCTGCTGCTCACCCCG
AGACCTACGTGAAGTTCTCGCGAGGTCTGCGAGAGCTCCTGCTTATAAGCCCGGAGATGG
CCACGCCCCGGAAGTGGAAGACGGAAGTGCACGTCCTCGTTGGACCTCCGGGCTGTGGC
AAAAGTCGGTACTGCCTAGAGACTGCACCGGAAGCATATTGGAAG

>hb15
TGCGCTTTAGTACCATCAAACAGCATCTCGATAACCGAATCCATATTGAGAAGGCAAATG
GATCCGACGAAGACAACCAAAAATACTGTTCAAAAGCAGGCGAGTTTTTTGAAACGGGT
GTGCCTCACACCCAAGGGAAGAGAAACGATTTGGCGGCCGTCGTGGATACCATATCGAGT
GGAGCGGACATCTCATCAGTGGCTAGGACTCACCCCACCAGTTTTATCAAATACCATCGTG
GAATCAAAGAATACATCAAAACCATCAGACCTATTCCCGTGCGAGACTTCAAAACTGAGG
TCCGATATTACTACGGAGAACCAGGGACGGGCAAATCCAGAACAGCATTGGAAGAGGCA
AAAGAAAGAGCCG

>hb19
GACTATGACGCCGCGCTAAGCGCTCGCTTCGCTCGCTTATGTAATAAGTTTTATGTCAAAT
ATAGGCTTTATCAACTTCAAATCCAAGCGGGAGTTTAGTGTCATCAAACAAATTATCGGA
GACTCTGCTCATATCGAGCCAAGCAATGGCAACGACCAACAAAATAAAGAAATATTGCTCT
AAAAGCGATAACTTCTGGGAATTCGGAGTTCCGGTTACGCAGGGCCAACGTACAGACCTT
GAAGCTGTTGTTGAAAGAATTAGATCCTCTGATAGAATCGAACAAGTGGTGTCCGAGTAC
CCTGAGACTTATATTCGTTACCACAGGGGAATTGAGAAATTGTTTGGATATATATCTAAGC
GAGCTAAAAGGGACTGGAAAACTGAAACGTTCGTCTACTATGGCGAACCTGGTAGTGGG
AAGTCCAGAACCGCCTATGAAATTGCCCTCGCCAGAGAGGGAGGT

Table I. (cont.)

>hb25
CGTGAACCTTAAGAAGACCACCCGTATGGGTGCTCTGAAGGCACGCTTGGGAGGTCGCGG
CCATTTTGAGCCTGCTCGGGGGGACGACTGTAGCAATCAGGCATATTGCTCAAAGGGGGG
CGACATCTTGGTTGAATCGGGAGCAGTTTCTAAGCAAGGCAAGCGTAATGACTTACATGA
TGCAGTGTCGACGCTGAAGGAGACGAAGAGCCTTGCCGCGGTCGCTGCTGCTCACCCCGA
GACTTACGTCAAGTTCTCGCGAGGACTGCGCGAGCTTCTGCTTATAAGCCCGGAGATGGC
CACGCCCCGGAAGTGGAAGACGGAAGTGAATGTCCTCGTTGGACCCCCGGGCTGTGGCA
AAAGTCGGTACTGCCTAGAGACTGCACCGGATGCATATTGGAAGCCCCGTGGGAAAT

>hb26
CGTGAACCTTAAGAAGACCACCCGTATGGGTGCTCTGAAGGCACGCTTGGGTGGTCGCGG
CCATTTTGAGCCTGCTCGGGGGGACGACTGTAGCAATCAGGCATATTGCTCAAAGGGGGG
CGACATCTTGATTGAATCGGGAGTAGTTTCTAAGCAAGGCAAGCGTAATGACTTACATGAT
GCAGTGTCGACGCTGAAGGAGACGAAGAGCCTTGCCGCGGTCGCTGCTGCTCACCCCGAG
ACTTACGTCAAGTTCTCGCGAGGACTGCGCGAGCTTCTGCTTATAAGCCCGGAGATGGCC
ACGCCCCGGAAGTGGAAGACGGAAGTGAACGTCCTCGTTGGACCCCCGGGCTGTGGCAA
AAGTCGGTACTGCCTAGAGACTGCACCGGATGCATAT

>hb34
TGCTAGCCGTGAATTACCAACATCATTGGACCGTTTCAAAGCACTAATTAGTGAACGTGCA
CACATCGAAATTGCTAAAGCAAGTGAAGAAGAAGAAAAAAACCGTCAAAAAAGGCGGT
CATTATGAGGAACACGGGCGTTTAAATTCGCACGGTCAGCGAAATGACTTATACAACGTT
GTAGATACCCTTTTGAATAATCAGGACGATCCCATTGGCGCTGTGGCTGAATCGCATCCAG
TTGCATTTATAAAATTTCGAAGAGGCATACGTGATCTTGCAACTACCCTTAAATTAGGAGC
TAAACGAAACTATCGCACAAAATTGTGTATTGTTTATGGATACCCAGGGACCGGCAAAAG
TTTTGGTGTCAGACAGATATGTGATAAACTATTTGGTGAAGAT

>hb45
CGTGAACCTTAAGAAGACCAGCCGTATGGGTGCCTTGAAGGCACGCTTGGGTGGTCGCGG
CCATTTTGAGCCTGCTCGGGGGGATGATTGCAGCAATAGAGATTATTGCTCAAAGGGGGG
CGACATATTGATTGAATCGGGCGAAGTGTCTAAGCAAGGTAAACGCAATGACTTACATGA
TGCAGTGGCGACCTTGAAGGACACGAAGAGCCTTGCTGCGGTGGCTGCTGCTCACCCCGA
GACTTACGTGAAGTTCTCGCGAGGTCTGCGAGAGCTCCTGCTTATAAGCCCGGAGATGGC
CACGCCCCGGAAGTGGAAGACGGAAGTGCACGTCCTCGTTGGACCTCCGGGCTGTGGCA
AAAGTCGGTACTGCCTAGAGACTGCACCGGATGCCTATTGGAAG

>hb46
CGTGAACCTTAAGAAGACCAGTCGTATGGGTGCCTTGAAGGCACGGTTGGGTGGTCGCGG
CCATTTTGAGCCTGCTCGGGGGGATGATTGCAGCAATAGAGATTATTGCTCAAAGGGGGG
CGACATATTGATTGAATCGGGCGAAGTGTCTAAGCAAGGGAAACGTAATGACTTACATGA
TGCAGTGGCGACCTTGAAGGATACGAAGAGCCTTGCTGCGGTGGCTGCTGCTCACCCCGA
GACTTACGTGAAGTTCTCGCGAGGTCTGCGAGAGCTCCTGCTTATAAGCCCGGAGATGGC
CACGCCCCGGA

>hb53
CGTGAACCTCAAGAAGACCACCCGTATGGGTGCCTTGAAGTCACGCTTGGGTGGTCGCGG
CCATTTTGAGCCTGCTCGGGGGGACGATTGCAGCAATAGAGATTATTGCTCAAAGGGGGG
CGACATCTTGATTGAATGTGGCGAAGTGTCTAAACAAGGAAAACGCAATGACTTACATGA
TGCAGTGACGACCTTGAAGGAGTCGAGAAGCCTTGCTGCCGTGGCTGCTGCTCACCCCGA
GACTTACGTGAAGTTCTCGCGAGGACTGCGGGAGCTGCTGCTTATAAGCCCGGAGATGAC
CACGCCCCGGAAGTGGAAGACGGAAGTGCACGTCCTCTGTGGACCTCCGGGCTGTGGCA
AAAGTCGGTACTGCCTAGATACTGCACCGGATGCATATTGGAAGCCCC

Table I. (cont.)

>hb76
CGTGAACCTTAAGAAGACCACCCGTATGGGTGCTCTGAAGGCACGCTTGGGTGGTCGCGG
CCATTTTGAGCCTGCTCGGGGGGACGACTGTAGCAATCAGGCATATTGCTCAAAGGGGGG
CGATATCTTGATTGAATCGGGAGCAGTTTCTAAGCAAGGCAAACGGAATGACTTACATGA
TGCAGTGTCGACGCTGAAGGTAACCAAGAGCCTTGCCGCGGTCGCTGCTGCTCACCCCGA
GACTTACGTCAAGTTCTCGCGAGGACTGCGAGAGCTCCTGCTTATAAGCCCGGAGATGGC
CACGCCCCGGAAGTGGAAGACGGAAGTGAACGTCCTCGTTGGACCCCCGGGCTGTGGCA
AAAGTCGGTACTGCCTAGAGACTGCACCGGATGCATATTGGAAG

>hb77
CGTGAACCTTAAGAAGACCACCCGTATGGGTGCTCTGAAGGCACGCTTGGGTGGTCGCGG
CCATTTTGAGCCTGCTCGGGGGGACGACTGTAGCAATCAGGCATATTGCTCAAAGGGGGG
CGATATCTTGATTGAATCGGGAGCAGTTTCTAAGCAAGGCAAACGGAATGACTTACATGA
TGCAGTGTCGACGCTGAAGGTAACCAAGAGCCTTGCCGCGGTCGCTGCTGCTCACCCCGA
GACTTACGTCAAGTTCTCGCGAGGACTGCGAGAGCTCCTGCTTATAAGCCCGGAGATGGC
CACGCCCCGGAAGTGGAAGACGGAAGTGAACGTCCTCGTTGGACCCCCGGGCTGTGGCA
AAAGTCGGTACTGCCTAGAGACTGCACCGGATGCATATTGGAAGCCCCGTGGGAAATGGT
GGGACGGCT

>hb79
TGCTAGCCGTGAATTACCAACATCATTGGACCGTTTCAAAGCACTAATTAGTGAACGTGCA
CACATCGAAATTGCTAAAGCAAGTGAAGAAGAAGAAAAAAAACCGTCAAAAAAGGCGGT
CATTATGAGGAACACGGGCGTTTAAATTCGCACGGTCAGCGAAATGACTTATACAACGTT
GTAGATACCCTTTTGAATAATCAGGACGATCCCATTGGCGCTGTGGCTGAATCGCATCCAG
TTGCATTTATAAAATTTCGAAGAGGCATACGTGATCTTGCAACTACCCTTAAATTAGGAGC
TAAACGAAACTATCGCACAAAATTGTGTATTGTTTATGGATACCCAGGGACCGGCAAAAG
TTTTGGTGTCAGACAGATATGTGATAAACTATTTGGTGAAGATCT

>hb85
GCAGCTCGAGGAATACCAACGACCATAGACAGATTCAAAGCCTTAATCAGCTCTCGTGCA
CATGTAGAAATAGCAAGACCAACAGAAGAACAGAATCGTGCATATTGTGTAAAAGGAGGT
AATTACGAAGAATATGGAAGAATCAATTCTCAAGGACAACGAAATGACCTGCACAACGTC
GTGGATACTTTACTGGATGCTACAGATGACCCAATCAACGCTGTTGCTACGAATCACCCTG
TTGCCTTCATTAAATTCGGACGTGGCATCAGAGATTTGGCATGTCAGTTACGCCTGGGAAC
TAAGAGAACACACAGAACCAA-ACTGT-GTATTGTATGGGGTTACCCAGGTACAGGCAAAA
GCTTCGGTGTTCGTCAATGTGCCGAAAAATTGGTTGGCGAAGAGAAATATATTATAA

>hb91
TGCTAGCCGTGAATTACCAACATCATTGGACCGTTTCAAAGCACTAATTAGTGAACGTGCA
CACATCGAAATTGCTAAAGCAAGTGAAGAAGAAGAAAAAAAACCGTCAAAAAAGGCGGT
CATTATGAGGAACACGGGCGTTTAAATTCGCACGGTCAGCGAAATGACTTATACAACGTT
GTAGATACCCTTTTGAATAATCAGGACGATCCCATTGGCGCTGTGGCTGAATCGCATCCAG
TTGCATTTATAAAATTTCGAAGAGGCATACGTGATCTTGCAACTATCCTTAAATTAGGAGC
TAAACGAAACTATCGCACAAAATTGTGTATTGTTTATGGATACCCAGGGACCGGCAAAAG
TTTTGGTGTCAGACAGATATGTGATAAACTATTTGGTGAAGATCTGACATTTTATAAA

>hb95
CGTGAACCTTAAGAAGACCAGCCGTATGGGTGCCTTGAAGGCACGGTTGGGTGGTCGCG
GCCATTTTGAGCCTGCTCGGGGGGATGATTGCAGCAATAGAGATTATTGCTCAAAGGGGG
GCGACATATTGATTGAATCGGGCGAAGTGTCTAAGCAAGGGAAACGTAATGACTTACATG
ATGCAGTGGCGACCTTGAAGGAGACGAAGAGCCTTGCTGCGGTGGCTGCTGCTCACCCCG
AGACTTACGTGAAGTTCTCGCGAGGTCTGCGAGAGCTCCTGCTTATAAGCCCGGAGATGG
CCACGCCCCGGAAGTGGAAGACGGAAGTGCACGTCCTCGTTGGACCTCCGGGCTGTGGC
AAAAGTCGGTACTGCCTAGAGACTGCACCGGATGCATATTGGAAG

Table I. (cont.)

>hb109 GTTCGCTAATTTTGTGAAGAAGCAGACTTTTAATAAAGTGAAGTGGTATTTGGGTGCCCG CTGCCACATCGAGAAAGCGAAAGGAACAGATCAGCAGAATAAAGAATACTGCAGTAAAG AAGGCAACTTACTGATTGAGTGTGGAGCTCCTAGATCTCAGGGACAACGGAGTGACCTGT CTACTGCTGTGAGTACCTTGTTGGAGAGCGGGAGTCTGGTGACCGTTGCAGAGCAGCACC CTGTAACGTTTGTCAGAAATTTCCGCGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAA ATGCAGAAGCGTGA
>FLA2 TTGGGTACACAACGCCTCCATCTAGAGGTACGTCGAGGTACATCAGTAGAAGCTATAGAGT ATTGCCAGAAAGAAGGTACCCGTATAGATGGACCATGGACAGCAGGTACGCCTTCCGAGG GTCAGGGAGAGCGAACAGATATAACCAAAGTGAAAGAGATGATAGATCGTGGAGCGTCTG AGATAGAAGTAGCAGAGGCAAGCTTCGCCCTATGGTGTGTTCACCATAGGGCTTTCTTACG GTACAAAGTACTAAAAACACCTGATAGGGATTTTCAAACTTTAGTCACTGTAGTTGTAGGT CCACCTGGGACAGGG
>FLA3 TTGGGTACACAACGCCTCCATCTAGAGGTACGTCGAGGTACATCAGTAGAAGCTATAGAGT ATTGCCAGAAAGAAGGTACCCGTATAGATGGACCATGGACAGCAGGTACGCCTTCCGAGG GTCAGGGAGAGCGAACAGATATAACCAAAGTGAAAGAGATGATAGATCGTGGAGCGTCTG AGATAGAAGTAGCAGAGGCAAGCTTCGCCCTATGGTGTGTTCACCATAGGGCTTTCTTACG GTACAAAGTACTAAAAACACCTGATAGGGATTTTCAAACTTTAGTCACTGTAGTTGTAGGT CCACCTGGGACAGGGAAATCACGGTACGCTAACGAGAGAGATAAAGGGGCTTTCTGGAA GCAGCATGGTACTTGG
>FLA4 TTGGGTACACAACGCCTCCATCTAGAGGTACGTCGAGGTACATCAGTAGAAGCTATAGAGT ATTGCCAGAAAGAAGGTACCCGTATAGATGGACCATGGACAGCAGGTACGCCTTCCGAGG GTCAGGGAGAGCGAACAGATATAACCAAAGTGAAAGAGATGATAGATCGTGGAGCGTCTG AGATAGAAGTAGCAGAGGCAAGCTTCGCCCTATGGTGTGTTCACCATAGGGCTTTCTTACG GTACAAAGTACTAAAAACACCTGATAGGGATTTTCAAACTTTAGTCACTGTAGTTGTAGGT CCACCTGGGACAGGGAAATCACGGTACGCTAACGAGAGAGATAAAGGGGCTTTCTGGAA GCAGCATGGTACTTGG
>FLA5 GAGGTACATCAGTAGAAGCTATAGAATATTGCCAGAAAGAAGGTACCCGTATAGATGGAC CATGGACAGCAGGTACTCCTTCCGAGGGGCAGGGAGAGCGAACAGATATAACCAAAGTG AAAGAGATGATAGATCGTGGAGCATCTGAGATAGAAGTAGCAGAGGCAAGCTTCGCCCTA TGGTGTGTTCACCATAGGGCTTTCTTACGGTACAAAGTACTAAAAACACCTGATAGGGATT TTCAAACTTTAGTCACTGTAGTTGTAGGTCCACCTGGGACAGGGAAATCACGGTACGCTAA CGAGAGAGATAAAGGGGCTTTCTGGAAGCAGCATGGTACTTGG
>FLA6 TTGGGTACACAACGCCTCCATCTCGAGGTACGTCGAGGTACATCAGTAGAAGCTATAGAAT ATTGCCAGAAAGAAGGTACCCGTATAGATGGACCATGGACAGCAGGTACTCCTTCCGAGG GGCAGGGAGAGCGAACAGATATAACCAAAGTGAAAGAGATGATAGATCGTGGAGCATCT GAGATAGAAGTAGCAGAGGCAAGCTTCGCCCTATGGTGTGTTCACCATAGGGCTTTCTTAC GGTACAAAGTACTAAAAACACCTGATAGGGATTTTCAAACTTTAGTCACTGTAGTTGTAGG TCCACCTGGGACAGGGAAATCACGGTACGCTAACGAGAGAGATAAAGGGGCTTTCTGGA AGCAGCATGGTACTTGG

Table I. (cont.)

>T1_Chelonian CGTAGAGTTCAAGCAGAAACGGACCATGAATCAAGTGAAGACTCAATTGGGTACACAACG CCTCCATCTAGAGGTACGTCGAGGTACATCAGTAGAAGCTATAGAGTATTGCCAGAAAGAA GGTACCCGTATAGATGGACCATGGACAGCAGGTACGCCTTCCGAGGGTCAGGGAGAGCGA ACAGATATAACCAAAGTGAAAGAGATGATAGATCGTGGAGCGTCTGAGATAGAAGTAGCA GAGGCAAGCTTCGCCCTTTGGTGTGTTCACCATAGGGCTTTCTTACGGTACAAAGTACTAA AAACACCTGATAGGGATTTTCAAACTTTAGTTACTGTAGTTGTAGGTCCACCTGGGACAGG GAAATCACGGTACGCTAACGAGAGAGATAAAGGGGCT
>T2_Chelonian CGTAGAGTTCAAGCAGAAACGGACAATGAATCAAGTGAAGAATCAGTTGGGTACACAACG CCTCCATCTAGAGGTACGTCGAGGTACATCAGTAGAAGCTATAGAATATTGCCAGAAAGAA GGTACCCGTATAGATGGACCATGGACAGCAGGTACTCCTTCCGAGGGGCAGGGAGAGCGA ACAGATATAACCAAAGTGAAAGAGATGATAGATCGTGGAGCATCTGAGATAGAAGTAGCA GAGGCAAGCTTCGCCCTATGGTGTGTTCACCATAGGGCTTTCTTACGGTACAAAGTACTAA AAACACCTGATAGGGATTTTCAAACTTTAGTCACTGTAGTTGTAGGTCCACCTGGGACAGG GAAATCACGGTACGCTAACGAGAGAGATAAAGGGGCT
>T4_Chelonian CGTAGAGTTCAAGCAGAAACGGACCATGAATCAAGTGAAGACTCAATTGGGTACACAACG CCTCCATCTAGAGGTACGTCGAGGTACATCAGTAGAAGCTATAGAGTATTGCCAGAAAGAA GGTACCCGTATAGATGGACCATGGACAGCAGGTACGCCTTCCGAGGGTCAGGGAGAGCGA ACAGATATAACCAAAGTGAAAGAGATGATAGATCGTGGAGCGTCTGAGATAGAAGTAGCA GAGGCAAGCTTCGCCCTTTGGTGTGTTCACCATAGGGCTTTCTTACGGTACAAAGTACTAA AAACACCTGATAGGGATTTTCAAACTTTAGTCACTGTAGTTGTAGGTCCACCTGGGACAGG GAAATCACGGTACGCTAACGAGAGAGATAAAGGGGCT
>T5_Chelonian AAGGTACCCGTATAGATGGACCATGGACAGCAGGTACGCCTTCCGAGGGTCAGGGAGAGC GAACAGATATAACCAAAGTGAAAGAGATGATAGATCGTGGAGCGTCTGAGATAGAAGTAG CAGAGGCAAGCTTCGCCCTTTGGTGTGTTCACCATAGGGCTTTCTTACGGTACAAAGTACT AAAAACACCTGATAGGGATTTTCAAACTTTAGTTACTGTAGTTGTAGGTCCACCTGGGACA GGGAAATCACGGTACGCTAACGAGAGAGATAAAGGGGCT
>R10_Ball_pithon CGTAGAGTTCAAGCAGAAACGGACCATGAATCAAGTGAAGACTCAATTGGGTACACAACG CCTCCATCTAGAGGTACGTCGAGGTACATCAGTAGAAGCTATAGAATATTGCCAGAAAGAA GGTACCCGTATAGATGGACCATGGACAGCAGGTACGCCTTCCGAGGGTCAGGGAGAGCGA ACAGATATAACCAAAGTAAAAGAGATGATAGATCGTGGAGCGTCTGAGATAGAAGTAGCA GAGGCAAGCTTCGCCCTATGGTGTGTTCACCATAGGGCTTTCTTACGGTACAAAGTACTAA AAACACCTGATAGGGATTTTCAAACTTTAGTCACTGTAGTTGTAGGTCCACCTGGGACAGG GAAATCACGGTACGCTAACGAGAGAGACAAAGGGGCT
>R12_Skink CGTAGAGTTCAAGCAGAAACGGACAATGAATCAAGTGAAGAATCAGTTGGGTACACAACG CCTCCATCTCGAGGTACGTCGAGGTACATCAGTAGAAGCTATAGAATATTGCCAGAAAGAA GGTACCCGTATAGATGGACCATGGACAGCAGGTACTCCTTCCGAGGGGCAGGGAGAGCGA ACAGATATAACCAAAGTGAAAGAGATGATAGATCGTGGAGCATCTGAGATAGAAGTAGCA GAGGCAAGCTTCGCCCTTTGGTGTGTTCACCATAGGGCTTTCTTACGGTACAAAGTACTAA AAACACCTGATAGGGATTTTCAAACTTTAGTCACTGTAGTTGTAGGTCCACCTGGGACAGG GAAATCACGGTACGCTAACGAGAGAGATAAAGGGGCT

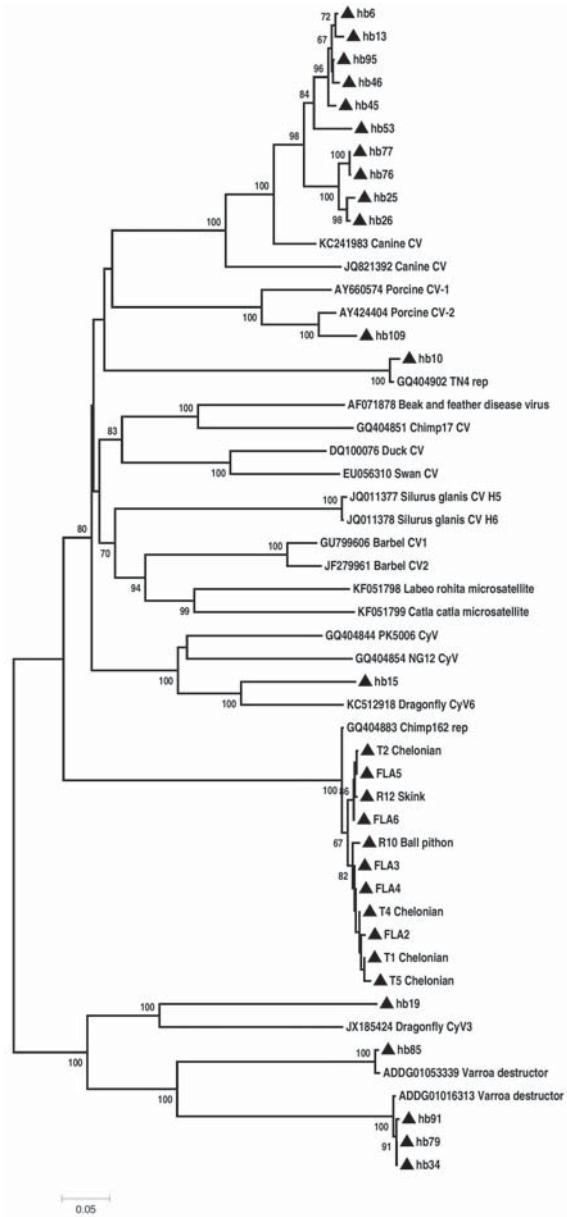

Figure 1. Phylogenetic analysis of circovirus (CV) *rep*-like sequences detected by PCR in honey bee (hb), Varroa destructor, reptile and free-living amoebae (FLA) samples using the neighbor-joining statistical method and p-distance substitution model of the MEGA 6 software (http://www.megasoftware.net/). The analysis based on 500 bootstrap replicates and values >60% are represented. Hungarian sequences are labelled by triangles

One honey bee gave amplicons with the primers designed by Halami et al. [15]. The resulting *rep*-like sequence (hb109) perfectly matched with PCV-2 sequences deposited in the GenBank (Fig. 1). The remaining 21 (and the eight samples that was not sequenced due to the low quantity of PCR products) honey bee and the six *Varroa* samples tested positive for CV exclusively with the primer set of Li et al. [10].

Ten sequences amplified from honey bees (hb6, hb13, hb25, hb26, hb45, hb46, hb53, hb76, hb77, hb95) were highly similar (90–100% nt identity) to each other and were related (90–94% nt identity) to the *rep* of a canine CV (CanCV; KC241983) (Fig. 1). The deduced aa sequences showed 93–97% identity with this reference. Three pairs of honey bee samples (hb25 and hb26, hb45 and hb46, hb76 and hb77) originated from the same hive, but only the hb76 and hb77 nt sequences were identical. The hb10 sequence was highly similar (99% nt and aa identity) to a partial *rep*-like sequence (GQ404902) from human stool sample and distantly related to *rep*-like sequences detected in sewage (<41% nt identity).

The hb15 and hb19 sequences showed 83% and 63% nt identity with to the *rep* of dragonfly cyclovirus (DfCyV) 6 (KC512918) and DfCyV3 (JX185424), respectively. The aa identity was 80% between the hb15 and DfCyV6; the hb19 *rep*-like sequence contained internal stop codons.

The sequences generated from the PCR amplicons of hb34, hb79, hb85 and hb91 samples were distantly related (43–45% nt identity) to the *rep* of DfCyV3, but the nt composition of the hb85 sequence greatly differed (63% nt identity) from the nearly identical hb34, hb79 and hb91 CV-like sequences. All these divergent sequences showed 99% nt identity with respective contigs of *Varroa* mite whole genome shotgun sequences deposited in the GenBank (ADDG01016313, ADDG01053339). The deduced aa sequence of the hb85 *rep*-like sequence showed 38% aa identity with the DfCyV3 but aa sequences could not be predicted for hb34, hb39 and hb91 nt due to internal stop codons.

CV-like sequences detected in reptiles

CV *rep*-like sequences were detected by PCR primers of Li et al. [10] in the pooled organ samples of six reptiles (6/10, 60%), in particular, in four chelonian species, one skink and one ball python specimens. The primer sets of Halami *et al*. [15] could not amplify any CV-like sequences. The six sequences showed 98–100% nt similarity to each other without any significant differences regarding the host species. The closest relative of the sequences was a CV *rep*-like sequence (GQ404854) detected in chimpanzee stool; other CV *rep* or *rep*-like sequences

were only distantly related (<45% nt identity) to the reptile origin *rep*-like sequences. The deduced aa sequences of the reptile sequences showed 99–100% similarity among each other and with the reference sequence as well.

CV-like sequences detected in free-living amoebae

Platyamoeba and *Acanthamoeba* species were isolated from environmental samples. *rep*-like sequences were detected in all of the six amoebae isolates by using the primer set of Li et al. [10]. The FLA-origin CV-like sequences were highly similar to each other (98–100%), to the *rep*-like sequences of the reptile samples (98–100%) and to the *rep*-like sequence detected in chimpanzee stool (98–100%), and showed low similarity (<45% nt identity) to other CV *rep* or *rep*-like sequences. One amoebae *rep*-like sequence was too short and was not suitable for inclusion in phylogenetic analysis. The deduced aa sequences of the FLA-origin *rep*-like sequences showed 100% similarity with each other, with the reptile and the reference aa sequences.

Results of the back-to-back PCRs, RCA and exonuclease digestion

Fourteen back-to-back primer sets were designed on the basis of the sequencing results of the diagnostic PCRs and attempts were made to amplify the whole genomes of putative CVs in the honey bee samples. The high similarity among CV-like sequences originating from the reptiles and amoebae allowed the use of two common back-to-back primer sets for the whole genome PCRs. However, none of our attempts with the specific back-to-back PCRs were successful and RCA assays have also failed.

The efficacy of the exonuclease treatment using the Lambda Exonuclease alone or in combination with the Exonuclease I was verified with the diagnostic primer sets [10, 15]. PCV-2, fish, pigeon, duck and goose CV positive samples were used as positive control samples in the experiment. The honey bee, reptile and amoebae DNA specimens treated with the exonucleases tested negative for the diagnostic PCRs, while the samples used as positive controls remained positive for CV-specific PCR after the DNA digestion steps. This confirmed that, at least majority of, pig, fish, pigeon, duck and goose origin CVs used in the control reaction were present in circular form, and suggested that the CV-like DNA in the test samples were in linear form.

Discussion

Some members of the *Circoviridae* virus family are extensively studied since the immunosuppressive effect of these viruses contribute to the development of serious syndromes causing significant economic losses [3–6]. In the present study we aimed at the detection and characterization of CVs from various hosts to broaden the knowledge about their host specificity and pathogenic potential. CV *rep*-specific PCR was carried out by two, widely used universal PCR primer sets [10, 15]. The primers designed by Li et al. [10], which are highly degenerate primer pairs, amplified *rep*-like sequences very efficiently; 29 of the 30 honey bee (17/18 confirmed by sequencing), six mite, six reptile and six amoebae samples (47/48) tested positive by these primers, whereas the other primer set [15] detected *rep* sequence only in one (1/48 tested PCR positive) honey bee specimen.

In a recent review, eukaryotic genome databases were searched systematically for small circular ssDNA viral sequences [30]. The analyses revealed that CV-like sequences may be integrated into a number of animal genomes and may represent germline infections. According to this comprehensive analysis endogenous *rep*-like sequences clustered with known viruses but in distinct branches and deduced aa sequences often contained inframe stop codons. In our study direct sequencing of the PCR products amplified from honey bee samples gave ambiguous results. A series of sequences was related to CanCV, DfCyV6 and PCV-2 sequences and the deduced aa sequences were not interrupted by stop codons in the amplified fragment. We suspected that these sequences may have been parts of whole viral genomes; however, back-to-back PCR, RCA and exonuclease treatment implied this hypothesis was erroneous. Of interest, the hb10 honey bee sample, along with the reptile and the amoebae *rep*-like sequences showed high identity with corresponding GenBank reference sequences (≥98% at nt and aa level), although these references did not represent whole viral genomes. Based on the PCR and sequencing results these closely related sequences were also thought to originate from exogenous viruses, but additional laboratory methods excluded this possibility.

Five honey bee *rep*-like sequences (hb19, hb34, hb79, hb85 and hb91) were distantly related to DfCyV3 *rep* gene and four of them showed near perfect sequence match to sequences obtained by *Varroa* whole genome shotgun sequences [30]. Liu et al. identified multiple integrated elements in the genome of *Varroa* mite and according to their hypothesis this may be the consequence of multiple integration events resulting in high copy number of endogenous sequences [30].

These integrated *rep*-like sequences can be fairly diverse at various regions of the *Varroa* genome. In our study six *Varroa* mite samples were tested and *rep*-like sequences were detected in all six samples by PCR; however, these (and some honey bee) sequences could not be analysed by direct sequencing due to sequence ambiguities probably caused by multiple different *rep*-like sequences in the host genome. Thus, our findings were consistent with the report by Liu et al. [30]. In addition, the hb19, hb34, hb79, and hb91 sequences contained multiple stop codons that are very typical in integrated *rep*-like sequences [30]. Collectively, sequence analyses, as well as, results of back-to-back PCR, RCA and exonuclease treatment strengthened the presumption that these five divergent sequences may be endogenous *rep*-like elements. Although, the honey bees were carefully handled and decontaminated before nucleic acid extraction, it cannot be excluded that some residual DNA on the surface of the animals resulted in contamination and false positive results when testing honey bee samples. Thus the integrated elements detected in honey bees in this study may have been originated from *Varroa* mites or another organism colonizing the bee or the mite.

The *rep*-like sequences found in reptiles and amoebae were highly similar to *rep* sequences detected in chimpanzee feces but the whole genomic sequence of the reference virus encoding for that is not available. Our results indicate that the *rep*-like sequences of the reptiles and amoebae may be integrated elements; it is conceivable that ingested amoebae or another microbe may be the source of *rep*-sequences in reptiles and chimpanzee. This would explain why the obtained *rep*-like sequences are so conserved irrespectively of the geographical region and the host species origin. Of note, integrated *rep*-like elements were also detected in the unicellular parasites *Giardia intestinalis* and *Entamoeba hystolitica* [30].

PCR is a simple, time-saving and cost effective method and *rep* is a conservative region making broad range *rep*-specific primers as the choice of laboratory diagnosis of CVs and CyVs. However, the presented data together with previously published results indicate that these primers successfully amplify not only exogenous CVs and CyVs, but also endogenous *rep*-like genomic elements of reptiles, insects, and fish, and/or the microbial community colonizing these animals [29]. Widespread presence of endogenous *rep*-like elements in eukaryotic organisms and the environment draws attention on the importance of careful selection of the applied diagnostic methods. Detection and sequencing of only partial genome fragments that is routine for CV diagnosis may result in oversight of integrated genomic *rep*-like elements. In light of these findings, published data about CV and CyV diversity in various host species may require reconsideration.

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