

**EMERGING
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3 **Novel circovirus in European catfish (*Silurus glanis*)**
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47 Newly identified circovirus of European catfish showed close relationship with barbel
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Abstract

PCR amplification and sequencing were used to identify a novel circular DNA virus in European catfish (*Silurus glanis*). Full genome characterization and phylogenetic analysis showed that the virus belonged to the *Circoviridae* family and it was closely related to the previously described barbel circovirus.

Members of the *Circoviridae* family are among the smallest known viruses and have a single stranded circular DNA genome of 1.7-2.3 kilobases. They are present in several avian species and pigs, and have also been detected most recently in fish (barbel, *Barbus barbus*). Besides the members of the *Circovirus* genus of this family new circular genomes of similar nature were described in different environmental and fecal samples (1-5) showing that they were more widespread than previously thought and the establishment of a new genus (*Cyclovirus*) for a group of these viruses was proposed (4).

An extensive research was initiated after the first detection of a fish circovirus (6) in order to assess the presence and importance of these viruses in other fish species. The present study describes the identification and genetic analysis of a new circovirus identified in European catfish (*Silurus glanis*).

The Study

During the spawning season of 2011 the number of fully developed European catfish found dead in Lake Balaton of Hungary was unexpectedly higher than in previous decades. Dead and moribund catfish of 6-50 kg body weight were collected and examined for possible causes of the unusual phenomenon. The routine bacteriological, parasitological, virological examinations and toxicological tests did not show the presence of any known cause to explain

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3 the excessive fish death. Pathology and histopathology revealed skin lesions, vascular
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5 dilations in the skin, inflammation of the gastrointestinal tract and nuclear fragmentation in
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7 the hematopoietic cells raising the suspicion of some kind of undetected toxicosis.
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10 Tissue samples of 6 European catfish were used for the detection of circoviruses in the same
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12 approach as it was previously done for the barbel circovirus (BaCV) (6). Liver, spleen, gills,
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14 kidneys and gonads (approximately 0.1 g of each) were processed individually and viral DNA
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16 was extracted using the Viral Gene-spin™ DNA/RNA Extraction Kit (Intron Biotechnology
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18 Inc., Korea) as recommended by the manufacturer. The nested PCR system of Halami et al.
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20 (7) was used for the detection of circovirus DNA and 3 out of the 6 examined fish were found
21
22 positive. The amplicons were sequenced and a primer pair (HT-F: 5'-
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24 CAGACCATGCTTCCGGTACT-3', HT-R: 5'-GGGCTTCCTCGAAGGTTATC -3') was
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26 designed using the Primer3 program (8) for the amplification of the remaining part of the
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28 circular genome. Positive samples from 2 fish were selected for full circovirus genome
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30 amplification and analysis. DNA was multiplied with the TempliPhi™ 100 Amplification Kit
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32 (GE Healthcare, UK) with Exo-Resistant Random Primers (Fermentas, Lithuania) and
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34 amplified by PCR with the newly designed primer pair. The amplicons were sequenced and
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36 aligned with the sequences obtained from the initial PCR amplicons with the CAP3 program
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38 (9). The full genome nucleotide sequences (CfCV-H5 and CfCV-H6, GenBank no.:
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40 JQ011377 and JQ011378) were analyzed by the BioEdit Sequence Alignment Editor version
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42 7.0.5.3 (10) and by NCBI tools (www.ncbi.nlm.nih.gov). Phylogenetic trees were
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44 constructed with the neighbor-joining method of the MEGA 5 software (11) using 1000
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46 bootstrap values.
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51 The size of both CfCV genomes was 1966 nucleotides (nt), forming a covalently closed
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53 circular molecule. The sequences were 99.4% identical to each other. Two major open
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55 reading frames (ORF) of opposite orientations, separated by short intergenic sequences (282
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3 bases at the 5' end and 60 bases at the 3' end) were predicted. ORF1 is located between 251
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5 and 1193 nt and has a potential coding capacity of 314 amino acids (aa), whereas ORF2 spans
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7 from 1934 to 1253 nt encoding 217 aa. Besides ORF1 and 2, two additional ORFs of smaller
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9 sizes, ORF3 (158 aa, 472 bases, from nt 473 to 1) and ORF4 (136 aa, 408 bases, from nt 27 to
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11 435) were identified in the genome. The sequence allowing for a stem loop structure
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13 characteristic to circoviruses was predicted by Mfold (12) and was located within the 5'
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15 intergenic region. The conserved nonanucleotide sequence (5'-TAGTATTAC-3') is identical
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17 to that of several members of the *Circovirus* genus including BaCV, porcine circovirus type 1
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19 and some avian circoviruses. The nonamer of the NG13 virus of human origin reported by Li
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21 et al. (4) was also the characteristic circovirus sequence and not the 5'-TAATACTAT-3'
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23 typical for cycloviruses. The stem length of CfCV is 9 basepairs, the same as for NG13 and
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25 shorter than the stem of most circoviruses.
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29 Blast analysis of ORF1 indicated homology with circovirus replication associated protein
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31 (Rep) sequences both at nucleotide and amino acid levels, and the putative protein is a
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33 member of the circovirus Rep family according to the Pfam database search. Based on ORF1
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35 derived aa Blast comparisons the closest relative of CfCV is NG13 with 55% identity,
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37 whereas the rest of known circovirus Reps including BaCV showed lower similarities (49-52
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39 %). The amino-terminal part of the potential capsid (Cap) protein (coded by ORF2) carries a
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41 31 aa long arginine rich stretch (RRRTFRRPIRRRMHRRTRGRRMIRRRSRRSR) starting at
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43 residue 4, homologous to the circovirus Cap nuclear localization signal, and the closest
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45 relative of CfCV based on Cap aa comparison is also NG13 (35% similarity). The barbel and
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47 swan (7) circovirus Caps together with the RW-C sequence from reclaimed water (2) showed
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49 27-28% similarity, but the value for other circoviruses was below that. GenBank search of
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51 ORF3 and 4 sequences did not reveal any homology with known viral genes.
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3 Phylogenetic analysis of the newly identified CfCV-H5 and -H6 was performed for complete
4 genome sequences and also for the Rep and Cap proteins using available GenBank data,
5 including selected sequences from the *Circovirus* and the *Cyclovirus* genera. The full genome
6 comparisons indicated that CfCV was a new virus within the *Circoviridae* family (Figure 1)
7 closer related to the *Circovirus* genus than to the cycloviruses. Similar results were obtained
8 when the tree was generated based on Rep protein sequences (not shown). The Cap sequence
9 analysis indicated that CfCV-H5 and -H6 formed a separate group together with the recently
10 identified BaCV and NG13 (Figure 2).
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14 The similarity to the other only known fish circovirus (BaCV) was not surprising but NG13
15 was detected in human stool samples of children with acute flaccid paralysis (4). NG13
16 together with the partial Rep sequence of NG24 (4) were the only ones that could not be
17 grouped in that study either to the *Circovirus* or the *Cyclovirus* genera. Although the
18 similarity of NG13 to CfCV and BaCV was low, it was detected not only in the case of the
19 Cap sequence but also for the entire genome, indicating a closer relation among these viruses.
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36 **Conclusions**

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38 The results of the study showed that circoviruses were present in European catfish. The role
39 of the virus as a pathogen is still to be determined, but circoviruses are generally considered to
40 be immune-suppressive, so CfCV may play such role in the development of diseases,
41 especially during the exhaustive spawning season. The similarity of CfCV (or BaCV) to the
42 NG13 of human origin raises several possibilities, namely, that NG13 may be a food born
43 contamination from fish source or that these viruses may have a common but so far unknown
44 origin.
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12 Dr. Lőrincz is a PhD student of the Szent István University, Faculty of Veterinary Science.

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14 Her research interest is circovirus evolution, focusing on new hosts of known circoviruses and
15 viral discovery.
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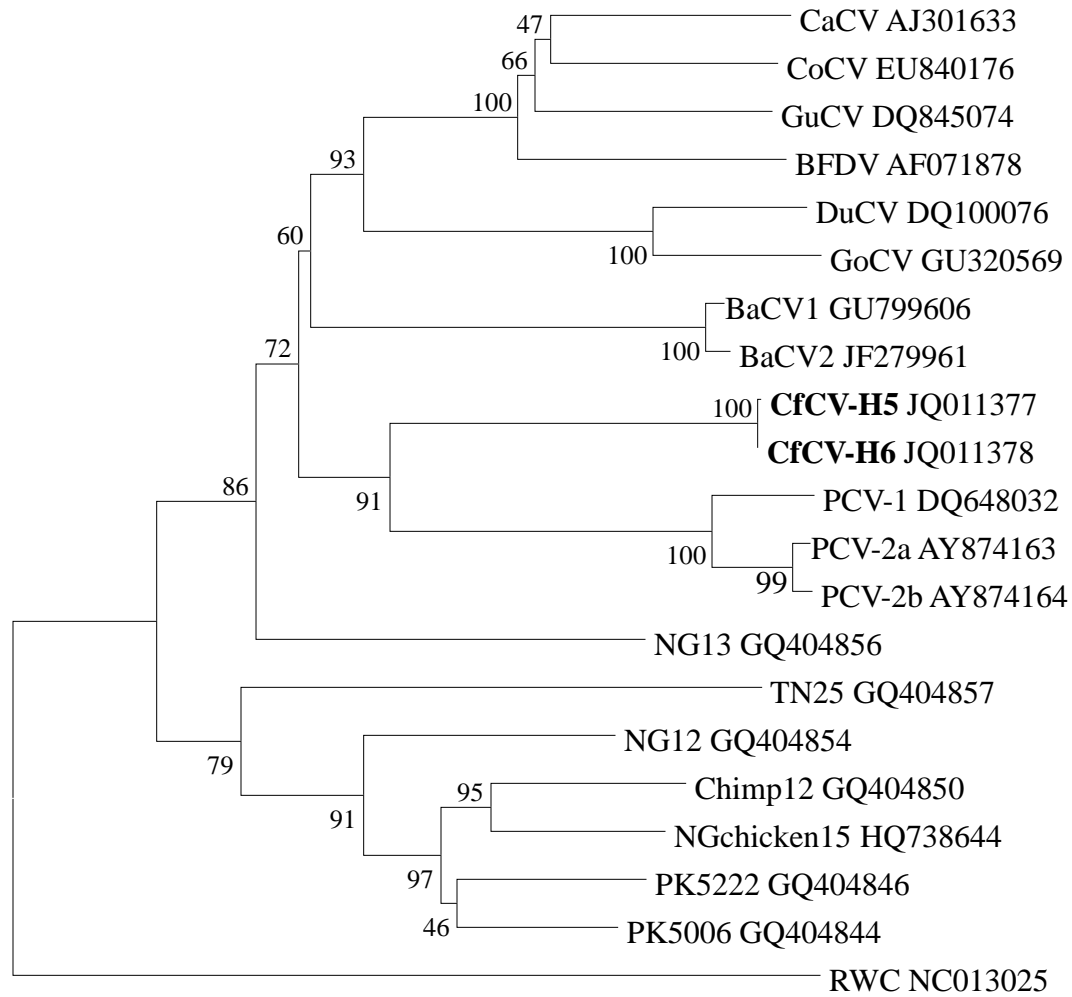
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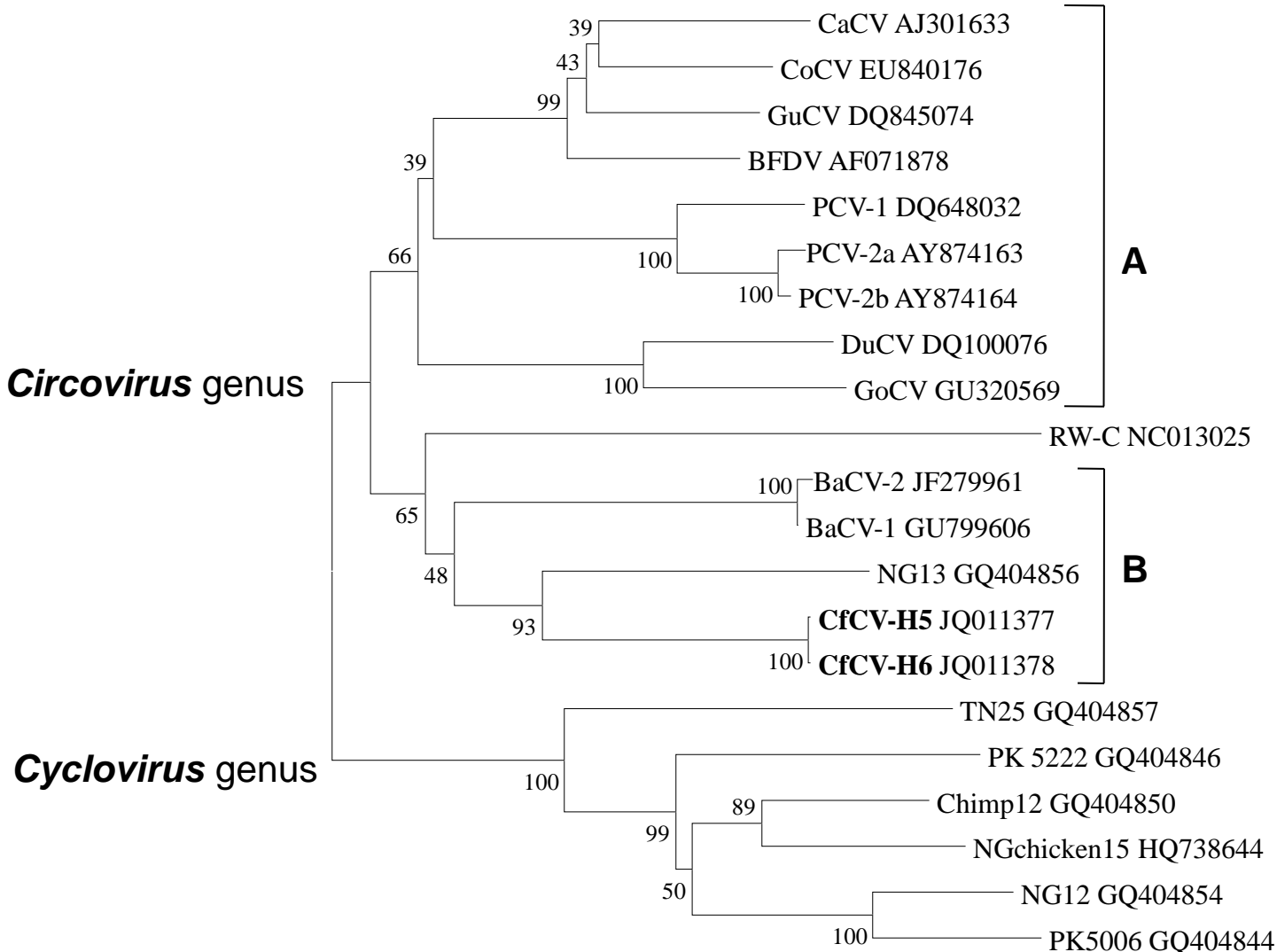
Figure 1. Phylogenetic analysis of the European catfish circovirus (CfCV-H5 and -H6) based on complete genomic DNA sequences. The viruses included are: canary circovirus (CaCV), columbid circovirus (CoCV), gull circovirus (GuCV), beak and feather disease virus (BFDV), duck circovirus (DuCV), goose circovirus (GoCV), barbel circovirus (BaCV1, BaCV2), porcine circovirus (PCV1, PCV2a, PCV2b), human stool-associated circular virus (NG13), cycloviruses (TN25, Chimp 12, NG 12, NGchicken15, PK 5222, PK5006), circovirus-like genome from reclaimed water (RW-C). Scale represents estimated phylogenetic distance. GenBank accession numbers are shown on the tree.

Figure 2. Phylogenetic analysis of the predicted Cap protein amino acid sequences. Names are as in Figure 1. The *Circovirus* genus is divided into 2 groups, A and B. **A** representing the traditional circoviruses and **B** including the fish viruses and NG13.



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