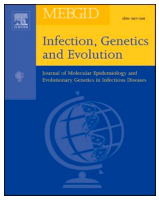
Infection, Genetics and Evolution 118 (2024) 105548

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid



Detection of circovirus in free-ranging brown rats *(Rattus norvegicus)*

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ARTICLE INFO

Keywords:
Free-ranging brown rats
PCR
Circovirus
Evolutionary relations

ABSTRACT

Accidentally found, two poisoned brown rats from Hungary were surveyed for presence of circoviral DNA, using specific nested primers, designed against the *rep* gene of the virus. Both specimens were positive. The whole genomes were amplified using inverse PCR based on the Rep sequence parts and sequenced by the primer walking method. Genomic analyses revealed that these novel rat viruses, together with tawny owl-associated circovirus reported by Italian researchers in 2022, are sequence variations of the same virus from genus *Circovirus*. In phylogenetic reconstructions, these circovirus strains detected from brown rats clustered closest to circoviruses derived from faeces samples of various predatory mammals. Molecular data as well as the phylogenetic analyses of the complete derived replication-associated protein and the capsid protein, as well as the prey preference of the host species of the recently described tawny owl-associated virus suggest that brown rat could be the evolutionary adapted host of the viruses described in this paper (brown rat circovirus types 1 and 2) and the previously reported tawny owl-associated virus. Possible pathogenic and zoonotic role of these viruses need further studies.

1. Introduction

Circovirus is a genus of viruses, part of the *Circoviridae* family, which contains ubiquiter microorganisms, widespread among animal species worldwide, although have veterinary and economic importance only in pigs (postweaning multisystemic wasting syndrome, Opriessnig et al., 2020) and in birds (the beak and feather disease, Raidal et al., 2015). The virus consists of non-enveloped, icosahedral capsid of 15-25 nm in diameter, with single-stranded not-segmented circular DNA as genome. These viruses are associated with lymphoid depletion and immunosuppressive conditions in infected animals, leading to systemic illness. The virus encodes two major proteins: the capsid-associated protein (Cap), and the replication-associated protein (Rep) (Nath et al., 2021). Cap is the only structural protein component of the virion and plays a crucial role throughout the virus replication cycle, including viral attachment, cell entry, genome uncoating, and packaging of the newly formed viral particles. Rep mediates recognition of replication origin motifs in the viral genome sequence and is responsible for endonuclease activity and initiation of replication of the virus (Rosario et al., 2017).

Circoviruses are characterised by a high rate of nucleotide substitution and recombination, as well as horizontal gene transfer (Liu et al., 2011; Martin et al., 2011; Rosario et al., 2012). These features can cause the rapid evolution of circoviruses, contributing to their spread, environmental adaptation and various pathogenicity. (Lefeuvre et al., 2009; Rosario et al., 2012).

As circoviruses lack DNA polymerase enzyme in their genome, they depend on the enzyme pool of rapidly dividing S-phase host cell nucleus for replication. This feature can result in dysfunction of haematopoietic organs due to viral replication and in the development of immune system deficiency, which may also lead to secondary infections or coinfections caused by apathogenic or pathogenic microorganisms (Todd, 2000; Todd et al., 2001; Faurez et al., 2009; Ouvang et al., 2019).

A large-scale microbiome study of 3055 free-ranging rodent individuals for mammalian viruses in China revealed 1675–2151 bp long circovirus sequences, from individuals of South China field mouse (*Apodemus draco)*, Chevrier's field mouse (*Apodemus chevrieri*), smokebellied rat (*Niviventer eha*), Clarke's vole (*Neodon clarkei*) and Mongolian five-toed jerboa (*Allactaga sibirica*) (Wu et al., 2018). Further rodent complete circovirus genome was also deposited in GenBank (MF497827) from bamboo rat (*Rhizomys pruinosus*).

Circoviruses tend to jump species barriers, as porcine circoviruses have been detected serologically and by PCR from local free-ranging

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https://doi.org/10.1016/j.meegid.2023.105548
Received 31 October 2023; Received in revised form 28 December 2023; Accepted 29 December 2023
Available online 2 January 2024

rodent populations of several pig farms (Lőrincz et al., 2010; Pinheiro et al., 2013; Truong et al., 2013; Zhai et al., 2016) even their reservoir role was suspected (Zhao et al., 2023). A rodent-like circovirus (ToCV) was also found by PCR in the spleen of a tawny owl (*Strix aluco*). The sequence was clustered among mammalian and non-avian circoviruses in the phylogenetic tree, making the source of this virus questionable (Legnardi et al., 2022), i.e., whether it is an authentic virus of the host, the owl, or whether it originated from a rodent that was eaten as prey by the owl. The source of circoviruses in infections is questionable, as the host range of circoviruses is quite wide (Zhai et al., 2019). Mammalian circoviruses were detected from several mammal species like bats, elks, minks, foxes, chimpanzees (Li, 2010; Franzo, 2021; Vidovszky et al., 2023) and from a wide variety of bird species (Tran, 2022) but also from mosquitoes.

Our aim with the present study was to investigate the presence of circovirus infections in free-ranging brown rats (*Rattus norvegicus*).

2. Methods

2.1. Sample collection and preparation

Fresh carcasses (>24 h) of two free-ranging brown rats were collected in Hungary. The two adult brown rats (*Rattus norvegicus*) individuals were found dead in gardens of residential areas. One of the carcasses was found in a northern district of Budapest, the other from Hajdúböszörmény at 186 air kilometers east of Budapest. Both animals were healthy adults, a female in Budapest (April 2019) and a male in Hajdúböszörmény (September 2021).

The main visceral organs (spleen, liver, kidneys, lungs, heart, brain) were aseptically resected. Data of the body, body size (grams, cm), gender, site and time of death were recorded. Cell suspensions in distilled water were made mixing the resected organs (50 mm^3 each) listed above. From this suspensions DNA was extracted by Genomic DNA Mini Kit (Geneaid, Biotech Ltd. New Taipei City, Taiwan). One µl from this DNA suspension was used as a template in further PCR reactions.

2.2. PCR assays

To detect circoviral DNA, a broad-spectrum, consensus nested PCR assay was used, described by Halami et al. (2008) which amplified an approximately 350-bp fragment of the Rep protein-coding gene. For the amplification of whole circular genomes, an inverse nested PCR assay was designed by selecting specific primers on the base of sequences of PCR products of the diagnostic Halami PCR. The selected primers were ratCVF1 5'-CACCCACGTTCCGTAAAACAGC-3′ and ratCVR1 5'-AAGAGTTCGAAGCTGACGGAGG-3′ for the first round, primers ratCVF2 5'-TTCCACATGGGCACGCGTAAAG-3′ and ratCVR2 5'-CTGTTGTTG CCAAGACGATGCC-3′ were used in the second round. The reactions were performed in 50 µl final volume as follows: 21 µl nuclease-free water (Thermo Scientific), 25 µl 2 × PrimeSTAR® Max DNA Polymerase enzyme premix (TaKaRa), 1 µl of each primer (15 pmol/ µl) and 2 µl target DNA. The thermocycling parameters were identical in both rounds: initial denaturation at 98 °C for 3 min, 35 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 5 s and elongation at 72 °C for 1 min, following a final elongation step at 72 °C for 2 min. The yield of PCR amplicons were checked by agarose gel electrophoresis. The sequences of the two whole genomes were determined by primer walking.

2.3. Sequencing

PCR amplicons were purified with the application of NucleoSpin® Extract II kit (Macherey-Nagel). Depending on the PCR results, the Sanger sequencing was performed with the use of the outer or the inner oligos (10 pmol/µl) on both strand applying primer walking approach. Reactions were performed with BigDye™ Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems). The capillary electrophoresis of the sequencing reactions were run on an ABI 3500xL Genetic Analyzer (Applied Biosystems) by a commercial supplier.

2.4. Bioinformatics and phylogenetic analyses

The identity of the newly obtained sequences was confirmed using the BLAST algorithms run at the NCBI GenBank database. The sequenced fragments were quality controlled and assembled applying the Staden package (Staden et al., 1998). Deduced amino acid (aa) sequences were generated with the JavaScript DNA Translator 1.1 program (Perry 3rd, 2002). From the Rep and Cap sequences of the representatives of *Circovirus* genus, multiple aa sequence alignments were generated online using the T-Coffee server at Cedric Notredame's Lab with Mcoffee mode applying Mmafft pair and Mmafft msa alignment computational methods. Sequence alignments were edited manually using BioEdit v7.0.5.3. (Hall, 1999). Selection of best fit evolutionary models according to Bayesian Information Criterion (BIC) was performed by the ProtTest v3.4.2 package (Darriba et al., 2011).

For the phylogenetic tree reconstructions of both proteins, we performed maximum likelihood analysis on the PhyML 3.0 server (Guindon et al., 2010) and Bayesian phylogenetic inference using the MrBayes v3.2.7 program (Ronquist et al., 2012), respectively. The parameters were the follows: LG + I + G model (α = 1.1, p-inv = 0.078) in case of Rep and VT + I + G + F (α = 2.508, p-inv = 0.005) for Cap. Bayesian analyses were run with the following settings: ngen = 1,000,000 (or until the value of standard deviation of split frequencies fell below 0.01), 4 runs, 4 chains, samplefreq = 100, burn-in = 40%. The topologies of the phylogenetic trees were tested by Shimodaira–Hasegawa-like approximate likelihood-ratio test (PhyML) and posterior probability (MrBayes). Phylogenetic trees were edited and visualised in FigTree v1.4.3.

3. Results

3.1. The sampled animals

Necropsy revealed no visible lesions, or obvious clinical signs of any diseases, pathogens or parasites.

3.2. PCR analyses, sequencing and genome annotation

During the BLASTn search, both newly determined genome sequences showed the highest score with 100% coverage (98.11% and 98.22% nt identity) with the corresponding genome of a tawny owl-associated circovirus (OL411978) described by Legnardi et al. (2022). The genome size of both circoviruses: brown rat circovirus 1 (BrCV1) and brown rat circovirus 2 (BrCV2) was 1745 nt. The balanced G + C content of the genomes was 47.11% for BrCV1 and 47.56% for BrCV2. Genome-wide pairwise analysis showed 98.5% identity between the two strains. The annotation of the complete genomes revealed the characteristic genome organisation of the genus *Circovirus*. The replication origin is on the Rep encoding strand. The conserved nonamer nucleotide sequence (AAGTATTAC), the RC endonuclease domain motifs I (CFTVNN), II (PHLQG) and III (ENQKYCSK) as well as the SF3 helicase domain motifs Walker A (GPPGVGKTKYAV), Walker B (IFDDF), motif C (ITSN) and arginine-finger (ALFRRI) of the Rep were identical in both strains. The Rep is 294 aa in length (nt 171–1055), whereas the Cap is 214 aa (nt 1735–1091). 25 nucleotide differences were identified between the two genomes, from which six resulted in aa changes, one in the Rep gene, and five in the Cap sequences. The arginine rich stretch at the amino-terminal part of the Cap were slightly different, MRTYYG**K**RPFKP**K**RYYAQRRPYAPRKRAYRRRR**I**WIRRPR for BrCV1 and MRTYYG**R**RPFKP**R**RYYAQRRPYAPRKRAYRRRR**L**WIRRPR for BrCV2. The nucleotide sequences of the stem-loop structures were identical.

The whole genome sequences have been submitted to GenBank under accession numbers OR553090 (brown rat circovirus 1) and

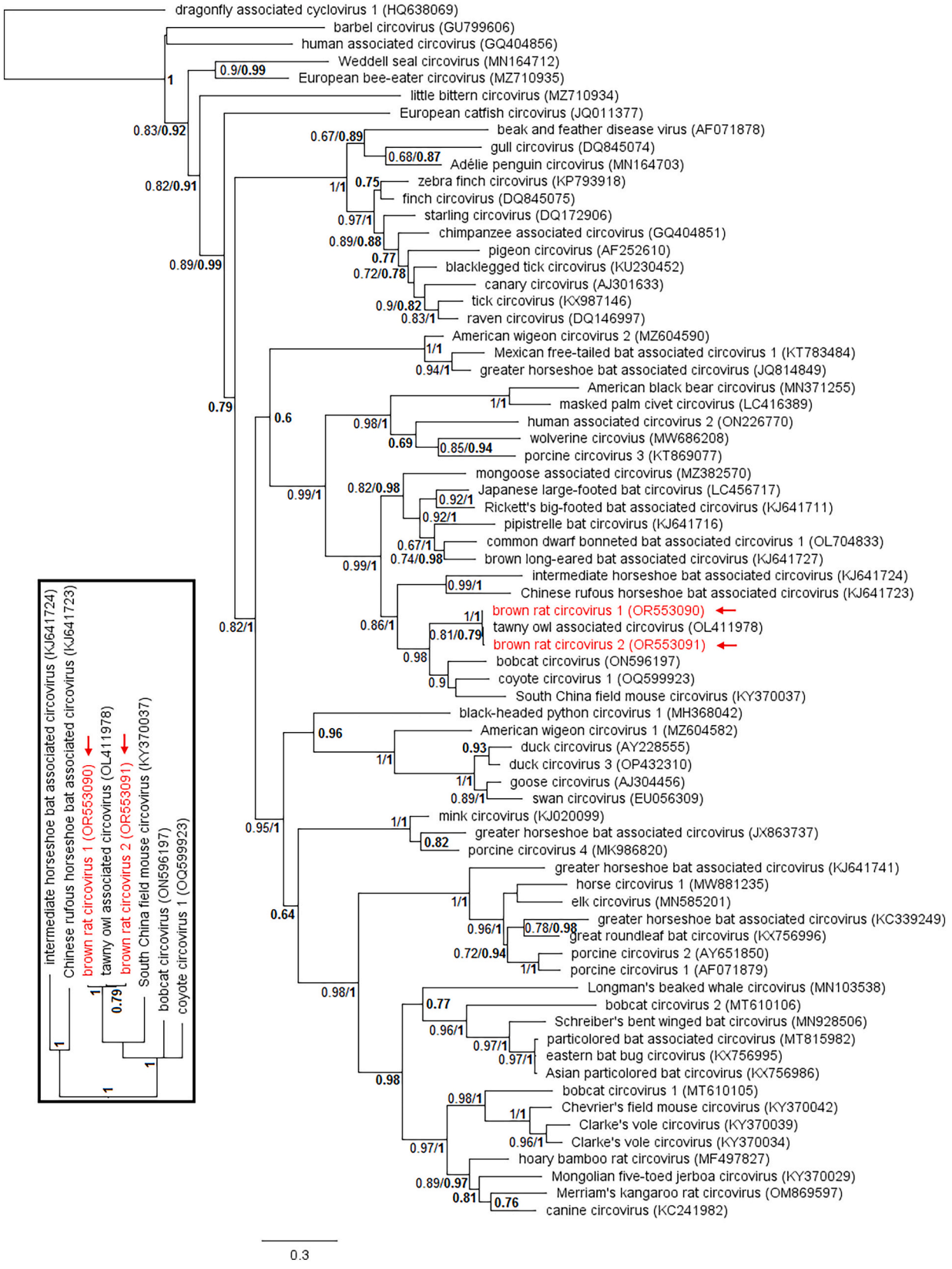

Fig. 1. Maximum likelihood tree of the genus *Circovirus* based on the amino acid sequence alignment of the complete Rep proteins. The maximum likelihood SH-like aLRT probability support values and Bayesian posterior probability values (bold) that are higher than 60% are shown at the nodes. The newly discovered viruses are indicated with red arrows. The viruses are marked with their hosts names. Scale bar represents 0.3 amino acid substitutions per site. The Bayesian tree structure of brown rat circoviruses type 1 and 2 and their closest relatives, which differs slightly from the topology of the ML reconstruction, is shown in the framed figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

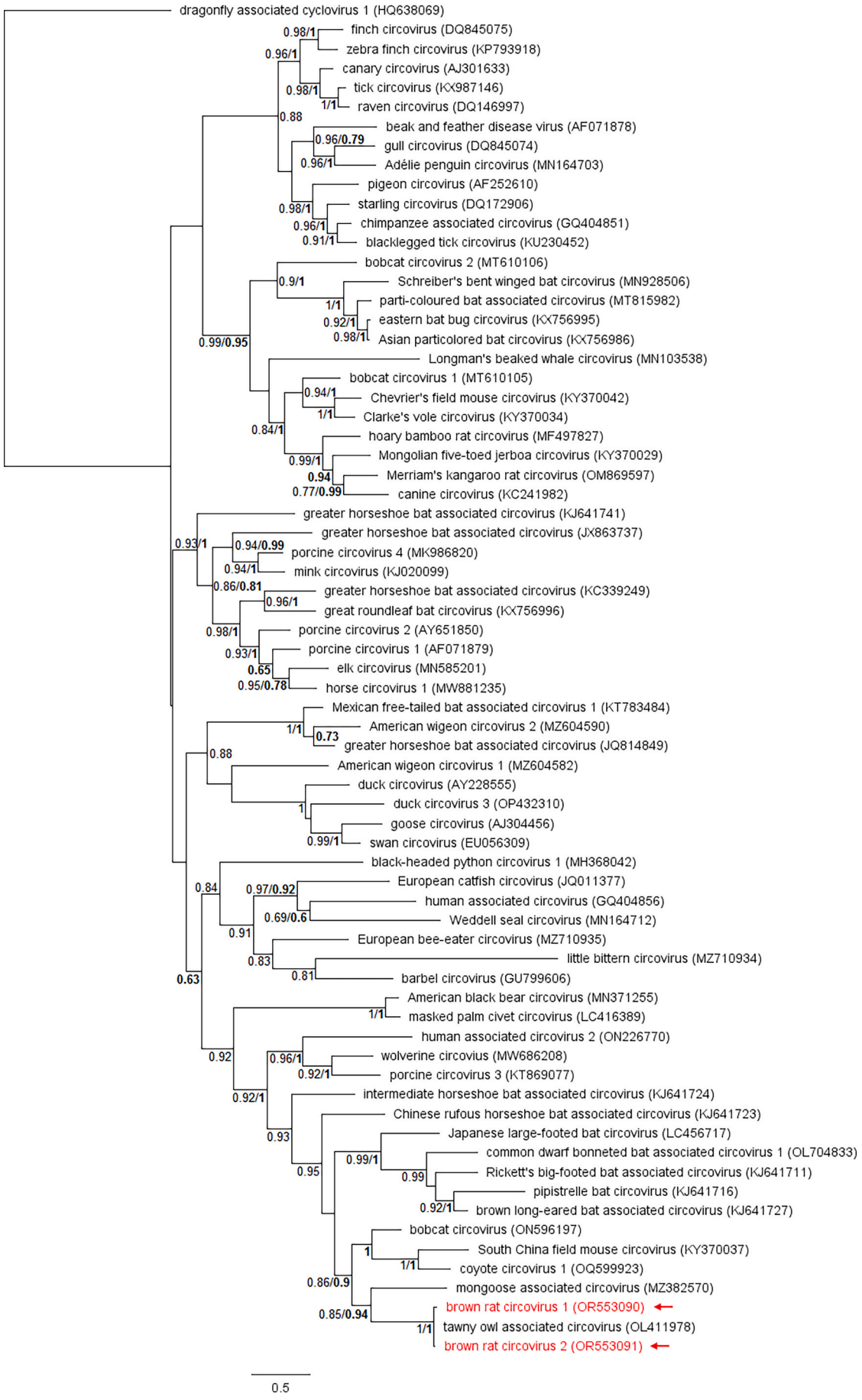

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Fig. 2. Maximum likelihood tree of the genus *Circovirus* based on the amino acid sequence alignment of the complete Cap proteins. The maximum likelihood SH-like aLRT probability support values and Bayesian posterior probability values (bold) that are higher than 60% are shown at the nodes. The newly discovered viruses are indicated by red arrows. The viruses are labelled with their host names. The scale bar represents 0.5 amino acid substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

OR553091 (brown rat circovirus 2).

3.3. Phylogenetic analyses

The Rep and Cap were used to study the evolutionary relationships of the described putative rat circoviruses (Figs. 1 and 2). The topology of the phylogenetic trees provided clear evidence that the novel circoviruses belong to the genus *Circovirus* within the family *Circoviridae*. Together with the recently described tawny owl-associated circovirus, rat circoviruses form a distinct clade supported by high confidence values. While the topologies of the trees differ slightly, the species compositions of the closest sister groups of our rat circoviruses are almost identical in both cases. The brown rat circoviruses were positioned between certain circoviruses originating from faeces of predatory mammalian species.

4. Discussion

In this paper we report the detection of two, practically identical (98,5%) circoviruses from parenchymal organs of two non-related brown rat individuals. In both phylogenetic figures the two viral sequences situated side by side close to each other with a circovirus sequence detected by Italian authors (Legnardi et al., 2022) from spleen of a tawny owl (*Strix aluco*).

The double amino acid substitution from lysine to arginine at position 7 and 13 of the arginine-rich part of the capsid protein may provide to BrCV2 capsid a presumably stronger nucleic acid binding effect than in case of BrCV1. This may result in a more stable linkage between the genomic DNA of BrCV2 and its capsid than BrCV1, which is an important feature for virion stability. The ToCV capsid also have the same amino acid sequence of the arginine-rich part like in BrCV2.

The genome-wide nucleotide identity between the two rat circovirus strains and ToCV indicates that these three viruses are sequence variants of the same virus species. The other neighbouring sequences originated from faeces samples of coyote (*Canis latrans*), bobcat, red lynx (*Lynx rufus*) (Cerna et al., 2023; Payne et al., 2020), small Indian mongoose (*Urva auropunctata*) (Gainor et al., 2021) and South China field mouse (*Apodemus draco*) (Wu et al., 2018). These data do not contradict the rodent-origin of our sequences, even indirectly support it, as all of these species are carnivorous and all of them feed on small rodents. These circoviral sequences were detected from faeces, which further support their rodent/prey origin. Some circoviruses might temporarily replicate in not related host species as Legnardi et al., 2022 detected a mammal circovirus in spleen of an owl, or this PCR positivity in an avian spleen might be the sign of an ongoing immune response against a virus absorbed from intestinal lumen. On both phylogenetic trees (Rep and Cap) our sequences were situated close to a circovirus, detected from a small rodent, the South-China field mouse (*Apodemius draco*). This further confirms the rodent origin of our viruses as this virus is not from faeces of a carnivorous species, but tissues of a small rodent individual. Additionally, this small rodent lives in the Chinese Far-east, which is just the region which considered the original area of evolution of brown rat, as species (Hulme-Beaman et al., 2021). Brown rats are still living in nature far from human settlements in the temperate zone of the Far-East (Manchuria, Korea, and the Southern Russian Far-East).

Our circovirus-positive rats showed no signs of any disease, which may suggest that these viruses have a long-term evolutionary relationship with their rodent hosts, resulting in an immune adaptation. As similar rodent circovirus sequences were detected in North America (coyote, bobcat) East-Asia (Chinese field mouse) and Europe (tawny owl in Italy and brown rats in Hungary) we might suppose that one, or some closely related rodent circoviruses are present in various small rodent species of several continents.

In conclusion, comparing the tawny owl-associated circovirus with the entire genomes of brown rat circovirus types 1 and 2, showed 98.1% and 98.2% identity. Moreover, the amino acid sequence patterns of both the RC endonuclease and SF 3 helicase domains, and the stem-loop structure, were identical. The genetic characterizations confirm the findings of the phylogenetic reconstructions, as brown rat circovirus types 1 and 2 described in this paper, as well as the tawny owl-associated circovirus, are different variants of the same virus. Based on our findings and the species identification criteria in the *Circovirus* genus, we suggest ICTV to approve the use of the rat circovirus species term for these viruses.

Funding

The work was financed by the grant K137798 of the National Office for Research, Development and Innovation (NKFI).

Ethics statements

We did not work with living animals.

CRediT authorship contribution statement

Z.L. Tarján: Writing – original draft, Methodology. **S. Szekeres:** Investigation, Formal analysis. **M.Z. Vidovszky:** Methodology, Investigation. **L. Egyed:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing interest.

Data availability

All kind of data are avaiable upon request from the corresponding author, László Egyed egyed.laszlo@vmri.hun-ren.hu

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