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BRIEF REPORT

Dicipivirus (family *Picornaviridae*) in wild Northern white-breasted hedgehog (*Erinaceus roumanicus*)

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Abstract Using random amplification and high-throughput sequencing technology a novel picornavirus with dicistronic genome organization and genetically related to canine picodicistrovirus (genus *Dicipivirus*, family *Picornaviridae*) was identified and characterized in Northern white-breasted hedgehogs. Hedgehog dicipivirus (hedgehog/H14/2015/HUN, MF188967) was detected in 15 (75%) of 20 faecal specimens by RT-PCR with high viral loads (up to $8.2x10^8$ genomic copies/ml faeces). Hedgehog dicipivirus RNA was also identified in blood, ear skin, abdominal muscle and liver tissues. While the general dicistronic genome organization of hedgehog/H14/2015/HUN is similar to canine picodicistrovirus (5'UTR-P1-IGR-P2/P3-3UTR) there are some unique genome characteristics within the untranslated regions, especially in the functional IRES elements. This study reports the putative second member of the genus *Dicipivirus*, in a novel host species.

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Main text

Picornaviruses are genetically diverse group of viruses (taxonomically classified within the family *Picornaviridae*) that have been found in various vertebrate host species. At present, the family is divided into 35 genera and 80 species – as of May 2017 [1]. In general, picornaviruses have ~6.7–9.9 kb long +ssRNA genome and traditionally encode a single polyprotein transcribed from a single open reading frame (ORF) flanked by a 5' and 3' untranslated region (UTR) and a poly(A)-tail [2]. The P1 region, encoding the capsid proteins, is located at the 5' end of the picornavirus genome. In 2012, a novel picornavirus – called canine picodicistrovirus 1 (CaPdV-1) - with a naturally occurring, unique dicistronic genome was described for the first time [3]. Until now, this canine picodicistrovirus is the sole representative of species *Cadicivirus A*, genus *Dicipivirus* (*Picornaviridae*), viruses with a picornavirus-like genome organization (gene order). Importantly, however, the P1 (capsid proteins) and P2/P3 (non-structural proteins) regions of canine picodicistrovirus 1 are encoded in two ORFs separated by a functionally active intergenic region (IGR) containing an internal ribosomal entry site (IRES), a phenomenon present in members of the family *Dicistroviridae*. Canine picodicistroviruses were originally detected from faecal samples of dogs collected in 2007/2009, in Hong Kong [3]. Until now, there have been no further reports or additional identification/confirmation of dicipivirus in dogs or any other animal hosts.

The Northern white-breasted hedgehog (*Erinaceus roumanicus*) is a medium-sized mammal in the genus *Erinaceus,* family Erinaceidae (Mammalia: Eulipotyphla). It can

be found in Central Europe, Balkan Peninsula, Adriatic Islands, Ukraine and Russia (as far as the Ob River in Siberia). The Northern white-breasted hedgehog inhabits farmland, parks and gardens in rural and urban areas, scrubby habitats at the edge of forests, and shrubby vegetation [4]. They feed on earthworms, insects (larvae, pupae and imagoes as well), snails and slugs, small vertebrates (amphibians, lizards and occasionally young rodents), chicks and eggs of birds and even some berries and fruits [5]. Like its congener the European hedgehog (*E. europaeus*), it is more abundant in artificial than in natural habitats [4].

Using random amplification and high-throughput sequencing technology a novel picornavirus with dicistronic genome organization and genetically related to canine picodicistrovirus was identified and characterized in Northern white-breasted hedgehog. This study reports the second putative member of the genus *Dicipivirus* identified in a novel host species.

A total of 20 faecal specimens were collected from Northern white-breasted hedgehog (*Erinaceus roumanicus*). Eight faecal samples (H1, H2, H4, H9-H11, H13 and H14) were collected from carcasses of road-hit hedgehogs collected from April to June in 2015, from urban environments, mainly (N = 7) from the streets of the capital Budapest, and one from the suburb Szentendre, Hungary. Twelve faecal samples (MR1-MR9 and MR11-MR13) were collected from wild-living hedgehogs (collected from April to July in 2015) from natural wild areas near the town of Pécs, Baranya County, Southwest Hungary. Except H10 and H11 which were juvenile animals (< 1-year-old) the other animals were adults (> 1-year-old) based on the animal size and – if it was possible to investigate - the skull bone ossification. The distance between the two sampling areas is 170 km. Samples were collected by qualified biologists with valid permissions (the National Inspectorate for Environment, Nature and Water: 4018-4/2015). The collected faecal samples were stored at -80 °C. A specimen pool containing 3 faecal samples (H9, H13 and H14) collected from 3 Northern white-breasted hedgehogs (from dead animals which were hit by cars whilst on roads) was randomly selected for viral metagenomics analysis. Briefly, the PBS-diluted specimen was passed through a 0.45-µm sterile filter (Millipore) and centrifuged at 6,000X*g* for 5 min. Subsequently the filtrate was treated with a mixture of DNases and RNases to digest unprotected nucleic acids [6]. Viral-particle protected nucleic acids were extracted using the QIAamp spin-column technique (Qiagen) and subjected to a viral metagenomic analysis using sequence independent random amplification [7]. A viral cDNA library was constructed by Nextera XT DNA Library Preparation Kit (Illumina) and then the library was sequenced on the HiSeq Illumina platform according to the manufacturer's instruction, and also as described previously [6]. The acquired reads were trimmed; *de-novo* assembled and analyzed using an in-house pipeline [6]. The reads and contigs greater than 100-bp were compared to the GenBank protein database (BLASTx). Virus family-level categorization of viral metagenomic reads was based on the best BLASTx-scores (E-value $\leq 10^{-10}$). Sequence specific screening primer pairs (dicipi-F: 5'-TCA ATCCCGGATGGAAGAAAC-3' corresponding to nt positions 3639-3659 and dicipi-R: 5'-ATAGAAACACGGAAC ACCGAAA-3' corresponding to nt positions 4002-3981 of the study strain) were designed based on the IGR-IRES sequence contig to identify the picornavirus RNA from the specimen pool. Different sets of specific primers were designed to the metagenomic reads/contigs to obtain the complete viral genome (hedgehog/H14/2015/HUN) and for verification of the metagenomic contigs by primer-walking, 5'/3'RACE and TAIL-PCR methods. PCR-products were sequenced directly and then run on an automated sequencer (ABI Prism 310, Applied Biosystems, Stafford, USA). All faecal specimens (N = 20) from Northern white-breasted hedgehogs were tested by RT-PCR using dicipi-F/dicipi-R as primer-pairs for screening. In addition, available coagulated blood (H14), ear skin (H1, H4 and H14), abdominal muscle (H4, H13 and H14), spleen (H4 and H14) and liver (H1 and H14) tissues were also tested by RT-PCR using the same dicipi-F/dicipi-R primers.

All evolutionary analyses were conducted in MEGA 6.06 [8]. The complete structural (P1) and non-structural (3CD) amino acid sequences of the study strain and representative picornaviruses were aligned by MEGA 6.06 and pre-tested using a best aa model (ML) search. Dendrograms were constructed by the Maximum-likelihood method based on the Poisson model with gamma distribution (+ G) and invariable sites (+ I). Assessing the reliability of the trees bootstrap values were provided and determined with 1000 replicates.

The RNA secondary structure of the 5'UTR and IGR IRES were predicted using Mfold [9] (Zucker 2003) with manual correction in order to locate the analogous structures found in canine picodicistrovirus [3, 10, 11]. Possible polyprotein cleavage sites were predicted from alignments with canine picodicistrovirus (JN819202) polyproteins.

Quantification reactions are based on picornavirus cDNA transcribed using the reverse primer H14R 5'-TCCCACAAG ACTGGTCGTTCC-3' (corresponding to nt positions 3739-3719 of the study strain) and amplified by primers (H14R/dicipi-F) designed against the IGR region using a real-time PCR assay (LightCycler FastStart DNA Master SYBR Green I, Roche, Mannheim, Germany). For absolute quantification and the generation of a standard curve, a hundred-fold dilution series of a silica-column (Qiagen, Hilden, Germany) purified, and spectrophotometrically quantified, single PCR amplicon of the picornavirus was used. In the qPCR analysis three technical repeats were included.

A specimen pool containing three faecal samples from Northern white-breasted hedgehogs was subjected to viral metagenomics analysis. After *de novo* assembly of the 23.004.466 total number of reads, 315.105 sequence reads were obtained showing similarity to viruses from this pool. The detected sequences containing more than 50 reads were from viruses taxonomically classifiable within the *Picornaviridae* (N = 151.949), unclassified viruses (N = 83.300), *Tombusviridae* (N = 54.383), *Virgaviridae* (N = 10.829), *Microviridae* (N = 8.810), *Alphaflexiviridae* (N = 2.250), *Circoviridae* (N = 1.536), *Permutotetraviridae* (N = 441), *Luteoviridae* (N = 398), *Parvoviridae* (N = 370), *Podoviridae* (N = 225), *Tymoviridae* (N = 99), *Carmotetraviridae* (N = 79), *Iflaviridae* (N = 71), and *Dicistroviridae* (N = 67). The 93.943 reads and the 5 sequence contigs (between 1060 and 6620 nts in length) corresponding to canine picodicistrovirus (JN819202) (genus *Dicipivirus*, family *Picornaviridae*) were selected for further analysis. Using the screening primer (dicipi-F/dicipi-R), 2 (H13 and H14) of the 3 specimens from the sample pool were RT-PCR-positive for the dicipivirus. To characterize the complete picornavirus genome from sample H14 different sets of specific primers were designed on the basis of the metagenomic sequence reads and amplicons were sequenced directly by Sanger sequencing. The complete genome of the dicipivirus strain hedgehog/H14/2015/HUN (MF188967) is 8,838 nt long including a 776-nt 5' end, a 588-nt IGR and a 130-nt 3' end excluding the poly(A)-tail (Figure 1). The G+C content of the genome is 46.7%. The P1 region of hedgehog/H14/2015/HUN encodes a capsid polyprotein of 919 aa (2,760 nt) that is 75aa longer than the corresponding P1 of canine picodicistrovirus (JN819202). Dicipiviruses including hedgehog/H14/2015/HUN do not have a putative L-protein preceding the capsid region. Compared to the aa sequence of canine picodicistrovirus (JN819202), the predicted length of the capsid proteins of hedgehog/H14/2015/HUN were 53aa (VP4), 248aa (VP2), 251aa (VP3) and 367aa (VP1). These proteins (VP4, VP2, VP3 and VP1) had 73%, 66%, 65% and 57% aa identity to the corresponding proteins from canine picodicistrovirus (JN819202), respectively.

The P2/P3 region of hedgehog/H14/2015/HUN encodes a nonstructural protein polycursor of 1527aa (4,584 nt), which is 121aa longer than the corresponding P2/P3 of canine picodicistrovirus (JN819202). The P2 region in the genome encodes the nonstructural proteins of 2A, 2B and 2C; however, the cleavage sites and lengths of these proteins could not be predicted (Figure 1). The predicted 2A does not possess the characteristic catalytic aa residues of chymotrypsin-like proteases, i.e. the NPGP or H-box/NC motifs. The 2C possesses the GxxGxGKS (G_{357}PPGIGKS) motif for NTP binding and the D_{408}DLGQ motif for putative helicase activity. The P3 region encodes the 3A, 3B (small viral protein, genome-linked = VPg), 3C (protease) and 3D (RNA-dependent RNA polymerase) proteins (Figure 1). Following sequence analysis the main difference in coding sequence length between hedgehog/H14/2015/HUN and canine picodicistrovirus was found to be between the 2C and 3B regions. The potential 3A is significantly longer (~ 3 times) in hedgehog/H14/2015/HUN than in canine picodicistrovirus. The predicted 3B is 27aa in length and has 44% aa identity to the 3B of canine picodicistrovirus. The 3C contains the catalytic triad of His-Glu-Cys (H_{879}-E_{888}-C_{991}) including the conserved GxCG motif (G_{989}YCG). The 3D contains the conserved picornaviral motifs of K_{1199}DELR, Y_{1370}GDD, and F_{1419}LKR, although the GGxPSG motif is replaced by the

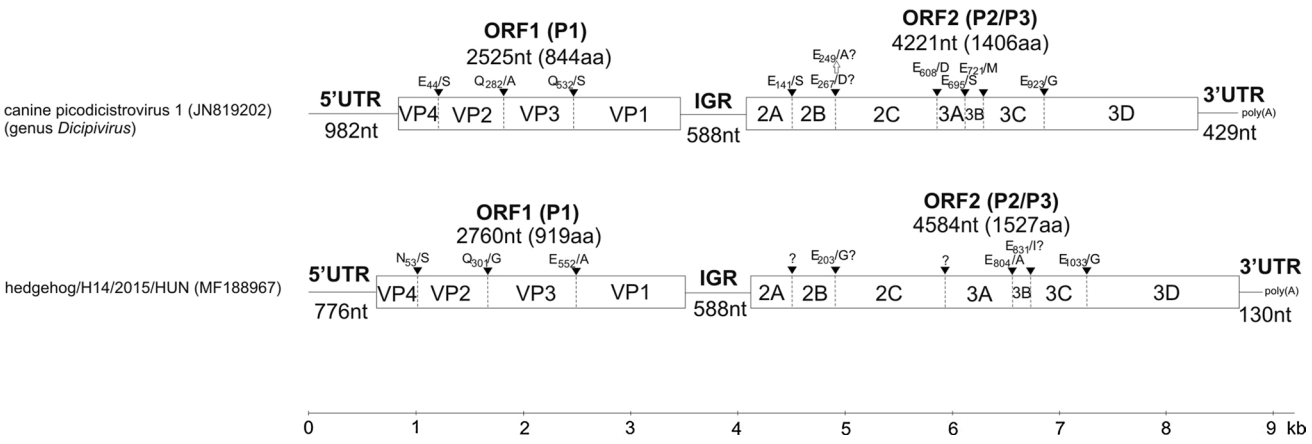

Fig. 1 A schematic illustrating the genome organization and predicted protein cleavage sites of the dicipivirus strain hedgehog/H14/2015/HUN (MF188967), when compared to canine picodicistrovirus (JN819202). The genome map and individual ORFs are drawn to scale. The P1 protein is translated in a similar reading frame as P2/P3. aa: amino acid; IGR: intergenic region; nt: nucleotide; ORF: open reading frame; UTR: untranslated region; VP: viral protein

G_{1328}AMPSG motif in hedgehog/H14/2015/HUN, similar to canine picodicistrovirus. The 3C and 3D had 76% and 71% aa identity to the corresponding proteins from canine picodicistrovirus (JN819202), respectively.

Phylogenetic analysis based on the complete aa sequences of the P1 and 3CD proteins showed that hedgehog/H14/2015/HUN clustered together with canine picodicistrovirus, viruses classifiable within the genus *Dicipivirus* (Figure 2).

The 5'UTR of the hedgehog/H14/2015/HUN is 776nt long, which is 206nt shorter than the 5'UTR of canine picodicistrovirus (Figure 1). By sequence comparison the nt identity of the two 5'UTR regions was 38%, but this was higher (57%) at the 5' end of the 5'UTR (from nt position 647 to 776 of hedgehog/H14/2015/HUN; within the IRES). The canine picodicistrovirus IRES comprises 12 domains (d1-d12) [10]. It diverges structurally from canonical type-I IRESs but contains essential motifs (including GNRA tetraloop motif) in subdomains d10c

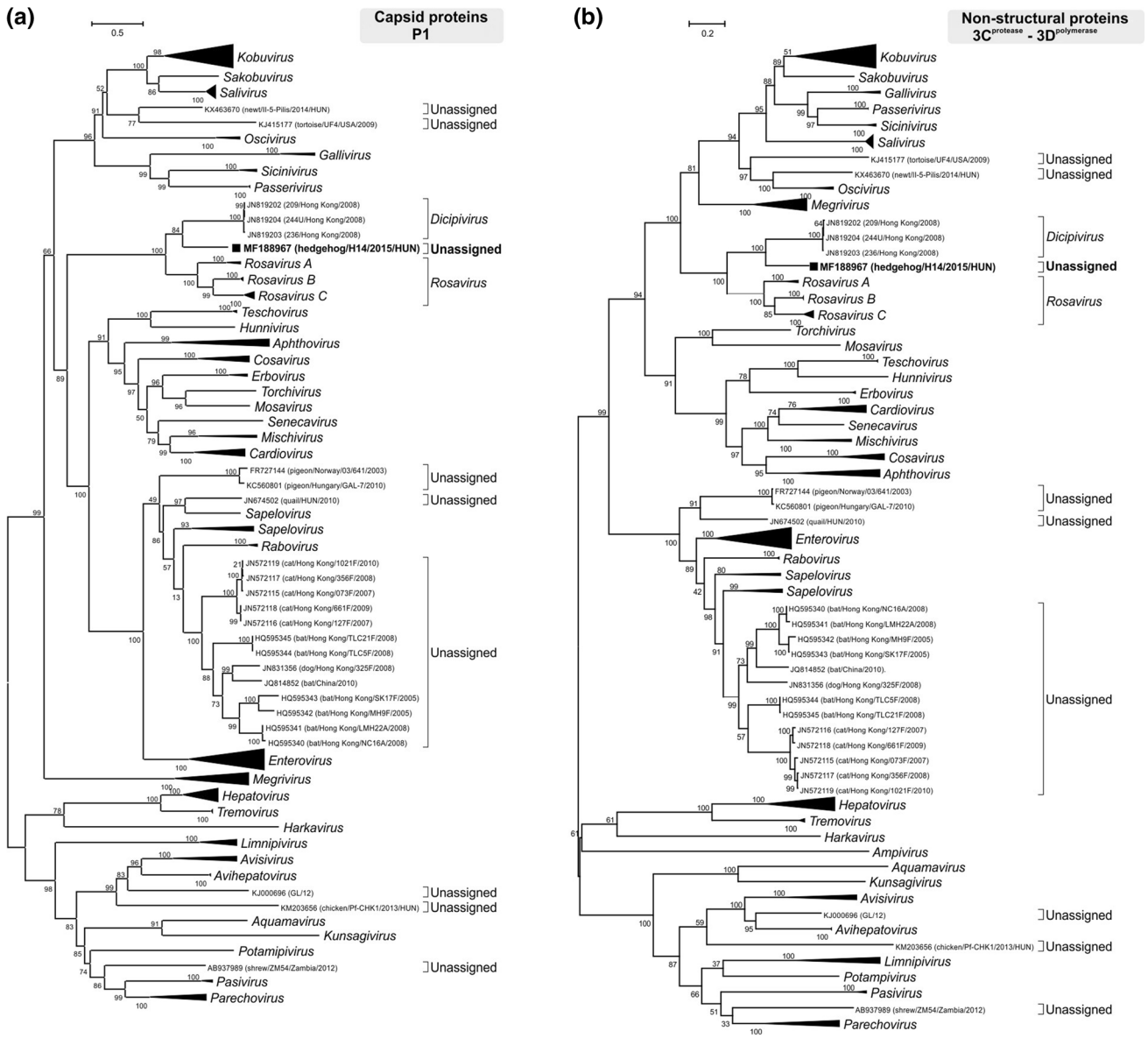

Fig. 2 Phylogenetic analysis of strain hedgehog/H14/2015/HUN (MF188967) (bold letter) and representative picornaviruses based on the complete A) P1 structural and B) 3CD non-structural protein sequences. These evolutionary analyses were conducted in MEGA6 [8], and evolutionary history was inferred using the Maximum-likelihood method based on the Poisson correction model with discrete Gamma distribution (+G), allowing evolutionarily invariable sites (+I). Altogether, 172 sequences were involved in the analysis using all sites; bootstrap values were determined with 1000 replicates. The tree was drawn to scale, with branch lengths representing the number of substitutions per site

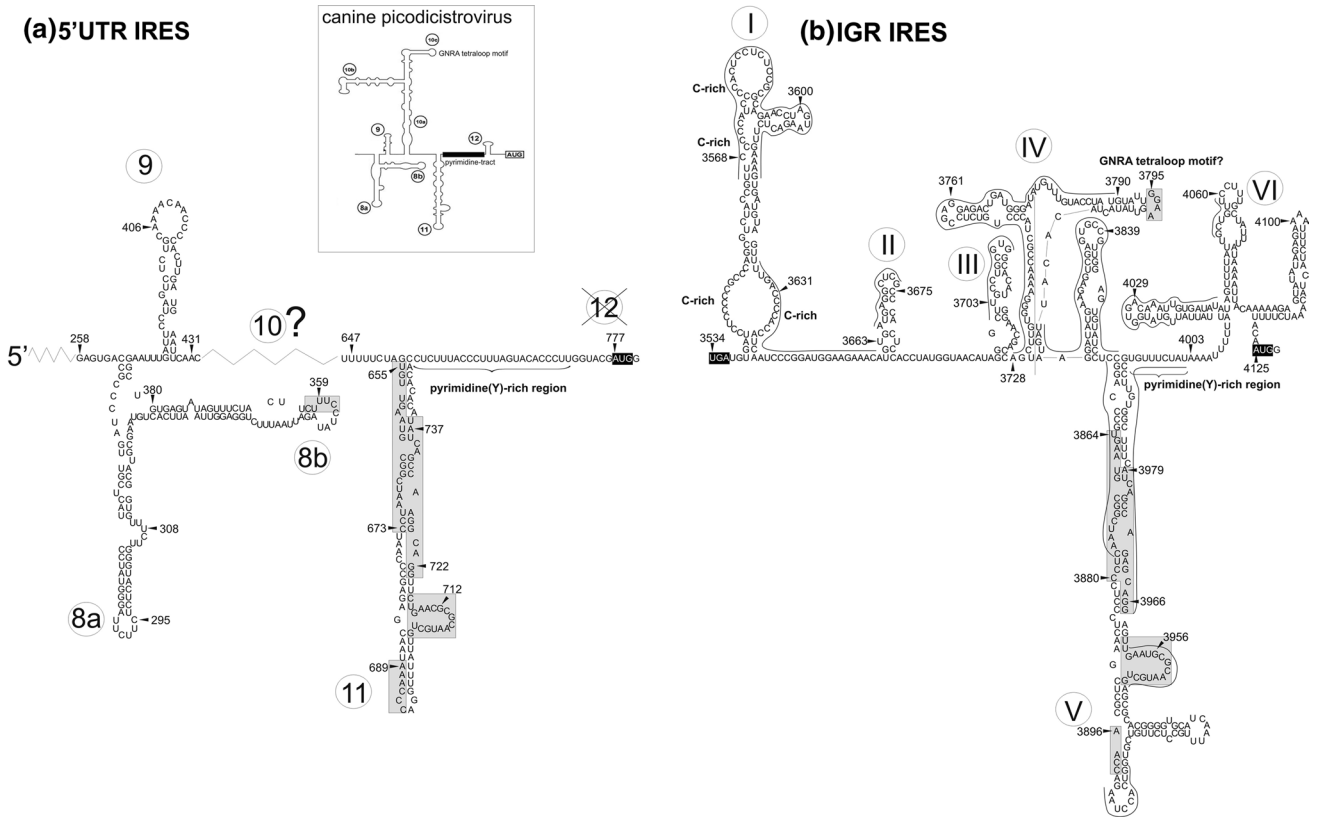

Fig. 3 The predicted secondary RNA structure of (a) the partial 5'UTR IRES and (b) IGR IRES of the dicipivirus strain hedgehog/H14/2015/HUN (MF188967). The RNA structures, domains and conserved motifs (shaded boxes) have been annotated, as proposed previously [3, 10, 11]. The 5'UTR IRES of hedgehog/H14/2015/HUN domain d10 is unpredictable; it also lacks the d12 domain. The AUG initiation and the stop codons are indicated by black boxes. The continuous line represents the conserved nucleotides between canine picodicistrovirus (JN819202) and hedgehog/H14/2015/HUN in IGR IRESes

and d11 (Figure 3) that is homologous to poliovirus dIVc and dV and requires the same IRES *trans*-acting factor, the poly(C)-binding protein 2 (PCBP2) [10, 11]. Structural homology is apparent between IRES elements (d8-d12) of canine picodicistrovirus and the 5'UTR IRES of hedgehog/H14/2015/HUN, such as within the domains d8, d9 and d11 as well as the pyrimidine(Y)-tract (Figure 3). There are also major differences: the structure of the d10 of hedgehog/H14/2015/HUN lacks equivalent sequences (including the GNRA tetraloop) found in d10 of canine picodicistrovirus and is presently unpredictable; it also lacks the d12 domain (Figure 3).

The 588nt long IGR of the hedgehog/H14/2015/HUN is similar in length and shows 64% nt identity to the IGR of canine picodicistrovirus (Figure 1). Structural homology is apparent between IGR IRES elements (I-VI) of canine picodicistrovirus [3] and the corresponding IGR region of hedgehog/H14/2015/HUN, such as the domains I, II, III, V and the pyrimidine(Y)-tract (Figure 3). However, structural differences are also identifiable in domains IV and VI. The nt sequence and the secondary RNA structure of hedgehog/H14/2015/HUN domain d11 (5'UTR IRES) and domain V (IGR IRES) are highly similar to each other (Figure 3).

The 3'UTR is 130nt long and 299nt shorter than the 3'UTR of canine picodicistrovirus (Figure 1). Similar nt sequence was not found in GenBank.

Using the dicipi-F/dicipi-R screening primer-pairs a total of 15 (75%) of the 20 faecal specimens collected from Northern white-breasted hedgehogs from two geographic regions were RT-PCR-positive for dicipivirus; all were confirmed by direct nucleotide sequencing. Four (50%) of the 8 and 10 (83%) of the 12 faecal specimens contained dicipivirus originated from urban/suburban (Budapest and Szentendre) and wild natural (Pécs) areas, respectively. The result of the real-time PCR quantification of hedgehog/H14/2015/HUN reveals the presence of 8.2×10^8 genomic copies/ml faeces (SD: $\pm 2.02 \times 10^8$) in sample H14. Available tissues samples collected from dead hedgehogs from urban/suburban (Budapest and Szentendre) areas with dicipivirus-positive faecal sample were also tested for dicipivirus by RT-PCR using the dicipi-F/dicipi-R primers. A total of 6 (55%) of the 11 tissue samples were RT-PCR-positive; all were confirmed

as dicipivirus sequences using direct nucleotide sequencing. In detail, dicipivirus RNA was detected in blood (H14), ear skin (H1), abdominal muscle (H4, H13 and H14) and liver (H14) specimens. Based on the amplified 321-bp-long IGR there was 100% nucleotide sequence identity between the H14 sequence that originated from faeces, muscle, blood and liver. The nucleotide sequence identity was 99% between sequences from different individuals in the same genome region. The results of the real-time PCR quantification of hedgehog/H14/2015/HUN revealed the presence of 1.03 x 10^7, 1.03 x 10^5 and 7.73 x 10^5 genomic copies/ml in abdominal muscle, blood and liver tissues from H14, respectively.

Dicipiviruses are a novel and genetically unique group of viruses, classifiable within the family *Picornaviridae*. Until now, only the prototype canine picodicistrovirus was known, which was discovered from faecal samples in dogs [3]. The genetic diversity, origin and host species spectrum of this group of viruses remains open to further investigation. This study reports the identification, quantification and complete genome characterization of a second member of this genus in faecal samples and - for the first time – in different tissue samples collected from wild Northern white-breasted hedgehogs in two geographic areas in Hungary. Using RT-PCR and qPCR this dicipivirus was detected at a high frequency (three fourth of the specimens were RT-PCR-positive) in these animal faeces; a high viral load in the faecal and tissue samples was also determined. The highest number of metagenomic reads related to picornaviruses, by viral metagenomics. Dicipivirus RNA was also identified in blood, ear skin, abdominal muscle and liver tissues. Identical nucleotide sequences were detected in the different specimens collected from the same animal. These results indicate that dicipiviruses are common in the tested hedgehog population and may replicate in this potential host species.

Hedgehog/H14/2015/HUN VP1 and 3D proteins have 43% and 29% aa sequence divergence when compared to the corresponding proteins of canine picodicistrovirus, respectively. Based on sequence- and phylogenetic analysis hedgehog/H14/2015/HUN represents, taxonomically, a potential novel species within the genus *Dicipivirus*. While the general dicistronic genome organization of hedgehog/H14/2015/HUN is similar to canine picodicistrovirus (5'UTR-P1-IGR-P2/P3-3UTR) there are some unique genome characteristics, especially within the untranslated regions and the two functional IRES elements. Similar to the 5'UTR-IRES of canine picodicistrovirus the 5'UTR-IRES of hedgehog/H14/2015/HUN has a type-I-like IRES but differences are present in sequence length as well as the possible RNA secondary structures of the IRES domains d10 and d12. Domain d10 is not predictable by comparison with d10 of canine picodicistrovirus while d12 is probably missing in hedgehog/H14/2015/HUN. Indeed, experimental evidence suggests that domain d12 is not essential for IRES function in canine picodicisitrovirus [10]. Similarly, structural differences were also identified in domains IV and VI of IGR-IRES between these two dicipiviruses.

Exotic pets, including hedgehogs, have become popular in recent years among pet owners, especially in North America [12]. All pets have a flora of microbes, and it should be known which are potentially zoonotic. Hedgehogs pose a risk for a number of potential zoonotic viral infections including bunyaviruses (Tahyna virus, CCHF), flavivirus (tick-borne encephalitis) and rabies. In addition they could impact on susceptible livestock, e.g. through Foot-and-mouth (picorna)virus transmission [12]. A recent study has also reported a novel hepatovirus (family *Picornaviridae*) in a faecal sample from hedgehog [13]; however, most virological studies target *E. europaeus* and as a result our current knowledge of viruses in *E. roumanicus* is limited. In this study, faecal samples collected from *E. roumanicus* hedgehogs in Hungary potentially contained a minimum of 14 different (bacterial, plant, fungal, insect and mammal) virus families. Interestingly, the picornavirus, and especially the dicipivirus sequence, reads outnumbered all other known viral sequence reads using our viral metagenomics platform.

Detection and characterization of further members of the genus *Dicipivirus* will help researchers to understand the origin, evolution, genetics and host species diversity of picorna- and picorna-like viruses with dicistronic genome organizations.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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