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BRIEF REPORT

Genetic characterization of a second novel picornavirus from an amphibian host, smooth newt (*Lissotriton vulgaris*)

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Abstract In this study, a novel picornavirus was identified in faecal samples from smooth newts (*Lissotriton vulgaris*). The complete genome of picornavirus strain newt/II-5-Pilis/2014/HUN (KX463670) is 7755 nt long with type-IV IRES and has 39.6% aa sequence identity in the protein P1 to the corresponding protein of bat picornavirus (KJ641686, unassigned) and 42.7% and 53.5% aa sequence identity in the 2C and 3CD protein, respectively, to oscivirus (GU182410, genus *Oscivirus*). Interestingly, the L-protein of newt/II-5-Pilis/2014/HUN has conserved aa motifs that are similar to those found in phosphatase-1 catalytic (PP1C) subunit binding region (pfam10488) proteins. This second amphibian-origin picornavirus could represent a novel species and could be a founding member of a potential novel picornavirus genus.

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Since 1934, adenoviruses, alloherpesviruses, parvovirus-like viruses, retroviruses and caliciviruses have been reported in amphibians, mostly in frogs [1, 2]. Members of the family *Iridoviridae* are the most frequent viruses in the common frog (*Rana temporaria*) and federally listed Sonora tiger salamanders (*Ambystoma tigrinum stebbinsi*) that cause mass mortality [3–6]. The smooth newt (*Lissotriton vulgaris*) is one of the most common amphibians in the forest zone of the temperate belt [5, 6]; however, there is limited information available about the viruses that infect it.

Picornaviruses (family *Picornaviridae*) have been found in various vertebrate host species, including reptiles [7–10], fish [11–13], birds [14] and mammals, including humans (http://www.picornaviridae.com). The first picornavirus from an amphibian (proposed genus "*Ampivirus*") was recently identified in a smooth newt [15]. Picornaviruses are small, non-enveloped viruses with a positive-sense, single-stranded RNA genome [16http://www.picornaviridae.com]. The genomic 5′ untranslated region (UTR) with its secondary RNA structures (including the IRES) is required for translation and RNA replication [17, 18]. Generally, the viral genome encodes a single polyprotein transcribed from a single open reading frame (ORF). The polyprotein is cleaved into smaller functionally active proteins: P1 encodes the viral capsid proteins; P2 and P3 encode the non-structural proteins [19, 20]. In addition, many picornaviruses encode a leader (L) protein before the P1 region with different types of functions [21]. At the 3′ end of the genome, there is a 3′UTR and a poly(A) tail. Picornavirus genomes contain further essential and additional secondary RNA structures (e.g., cis-acting replication element (*CRE*), "barbell"-like structures) [22–25].

In this report, we describe the molecular identification and complete genome characterization of a novel picornavirus from a smooth newt using viral metagenomics.

Clinically healthy smooth newts were collected in 2013 and 2014 from five natural ponds and three meadows located in or in the vicinity of the Pilis Mountains in Hungary. The faecal samples (N = 24) from male smooth newts were collected in 2013 as described previously [15]. In 2014, a total of 16 female smooth newts were caught in the lakes Katlan (N = 2) and Ilona (N = 3) and the meadows Alsóhosszúrét (N = 6) (47°42′55″N/19°01′23″E) and Felsőhosszúrét-elülső (N = 5) (47°43′36″N/19°00′58″E) between 24 March and 14 April and were kept individually in plastic containers filled with ~3 L of reconstituted soft water [26]. Faecal samples were stored at -20 °C. Unlike in 2013, animals were not fed with sludge worms (*Tubifex tubifex*) prior to the sample collection.

Permission to capture and conduct experiments on the animals was issued by the national authority of the Middle-Danube-Valley Inspectorate for Environmental Protection, Nature Conservation and Water Management (KTF: 5192-7/2013 and KTF: 603-3/2014, 603-4/2014).

Four faecal samples from four different animals, representatives of the four sampling areas (lakes Katlan and Ilona; meadows Alsóhosszúrét and Felsőhosszúrét-elülső) in 2014 were randomly selected and pooled for viral metagenomic analysis. Briefly, specimens were diluted in phosphate-buffered saline (PBS), passed through a 0.45-m sterile filter (Millipore), and centrifuged at 6,000×*g* for 5 min. The filtrate was then treated with a mixture of DNases and RNases to digest unprotected nucleic acids [27]. Viral-particle-protected nucleic acids were then extracted using the QIAamp spin-column technique (QIAGEN) and subjected to a viral metagenomic analysis using sequence-independent random RT-PCR amplification [28]. A viral cDNA library was constructed using a ScriptSeqTM v2 RNA-Seq Library Preparation Kit (Epicentre), and the library was then sequenced using the MiSeq Illumina platform according to the manufacturer's instructions, and as described previously [27]. Reads and assembled contigs greater than 100 bp were compared to the GenBank protein database (BlastX), using a cutoff value (E-value) of 10^{-10}.

RNA was extracted from 300 µl of faecal suspension using TRI Reagent (Molecular Research Centre, Cincinnati, OH, USA) according to the manufacturer's instruction. Specific primers were designed based on the reads/contigs to obtain the complete viral genome sequence, and for the verification of the metagenomic contigs by primer-walking, 5′/3′RACE, dsRNA-RACE and TAIL-PCR methods were used [25, 29, 30]. PCR products were sequenced directly and then run on an automated sequencer (ABI Prism 310, Applied Biosystems, Stafford, USA).

The screening primer pairs S12-Livupi-3DSCR-R (5′-ATCACCRTCMCKSACCCACAT-3′, corresponding to nt position 7487 to 7507 of II-5-Pilis/2014/HUN) and S12-Livupi-3DSCR-F (5′-TTMTYACACCACMTTCTTGAA-3′, corresponding to nt position 6729 to nt 6750 of II-5-Pilis/2014/HUN) were designed based on the partial nt sequences of the $3D^{pol}$ regions of newt/II-5-Pilis/2014/HUN and representative oscivirus strains, respectively.

The probable AUG initiation start codon was analysed by ATG^{Pr} prediction (http://atgpr.dbcls.jp/), and the N-terminal myristoylation signal was searched by NMT - The MYR Predictor (http://mendel.imp.ac.at/myristate/SUPLpredictor.htm) web applications.

The nt/aa sequence analysis was performed using NCBI BLASTn/BLASTp, GeneDoc ver. 2.7 and BioEdit ver. 7.2.5 [31]. The multiple nt sequences were aligned based on codons, using MEGA 6.06 [32]. The aa sequences of representative picornaviruses and the study strain were compared using the SIAS web server (http://imed.med.ucm.es/Tools/sias), using the "Length of Multiple Sequence Alignment" option. The hypothetical cleavage map of the picornavirus polyprotein was derived from alignments with other picornaviruses and NetPicoRNA prediction (http://www.cbs.dtu.dk/services/NetPicoRNA/).

All evolutionary analyses were conducted in MEGA 6.06 [32]. The nt sequences of representative picornaviruses and the study strain were aligned based on codons and pre-tested using a best nt/aa model (ML) search. Dendrograms were constructed by the ML method based on the general time-reversible model with gamma distribution (+G) and invariable sites (+I).

The secondary RNA structures of the 5′UTR, including the IRES and the CRE, were predicted using RNAstructure (http://rna.urmc.rochester.edu/RNAstructureWeb/index.html) and ViennaRNA Web Service (http://rna.tbi.univie.ac.at/) with manual correction as described previously [18].

The complete picornavirus sequence of newt/II-5-Pilis/2014/HUN and the partial 3D sequence of newt/II-6-Pilis/2014/HUN were submitted to GenBank under accession numbers KX463670 and KX463671.

Specimen pools (containing four faecal samples) from smooth newts captured from two lakes and two meadows in the Pilis Mountains of Hungary were subjected to viral metagenomics analysis. After *de novo* assembly, 1041 sequence reads were obtained showing similarity to viruses (BLASTx cutoff E score $\leq 10^{-10}$) from this sample pool. The detected sequences containing more than 10 reads were from viruses of an unclassified virus family (N = 261), *Podoviridae* (N = 192), *Marseilleviridae* (N = 159), *Mimiviridae* (N = 156), *Picornaviridae* (N = 86), *Rhabdoviridae* (N = 70), *Tombusviridae*

(N = 17), *Circoviridae* (N = 14), and *Microviridae* (N = 13). The reads corresponding to the family *Picornaviridae* were selected for further analysis. The 32 *in silico*-assembled picornavirus contigs (11 related to *Aichivirus A* and 21 related to *Oscivirus*) could be assembled, and ~3% of the picornavirus genome was covered (data not shown). Sequence-specific primer pairs (S12-3CD-R: 5′-GGAGGGTCAGAGTTCGAAT-3′ and S12-3CD-F: 5′-TTGGAAGGCTACACCAGTA-3′) were designed based on the 3CD sequence contig to identify the picornaviral RNA from the specimen pool. Only one sample – II/5 from the meadow Felsőhosszúrét-elülső – was positive by PCR. Further sequence-specific primer pairs were designed to fill the gaps between the regions by RT-PCR.

The complete RNA genome of strain newt/II-5-Pilis/2014/HUN (KX463670) is 7755 nt long, excluding the poly(A)-tail, and it has the common picornaviral genome organization 5UTR$^{\text{IRES-IV}}$/L/P1(VP0-VP3-VP1)/P2(2A$^{\text{Hbox/NC}}$-2B-2C)/P3(3A-3B$^{\text{VPg}}$-3C$^{\text{Pro}}$-3D$^{\text{Pol}}$)/3UTR-poly(A) (Fig. 1A). The study strain has 46.85% G+C content, and it has a nucleotide distribution of 26.1% A, 27.1% U, 21.5% G and 25.4% C.

The exact length of the 5′UTR of the study strain is uncertain (Fig. 1A, Fig. 2A). The first in-frame AUG initiation codon is at nt position 611-613 (CUCA**A$_{611}$UG**C). However, there is a potential alternative initiation start codon downstream at nt position 644-646 (CGCA**A$_{644}$UG**G) with an optimal Kozak context (RxxAUGG) [33] (Fig. 1A, Fig. 2A). The AUG codon at nt position 471-473 is a non-in-frame start codon.

In the GenBank database, 84% sequence identity was found between the study strain (from nt 388 to nt 428) and the 5′UTR of kobuvirus S-1-HUN/2007 (EU787450) (from nt 496 to nt 543). Based upon this sequence similarity and the predicted secondary RNA structure, newt/II-5-Pilis/2014/HUN has a potential type-IV IRES (Fig. 2A). Domain II contains a loop E motif (GAA and AGUA) in the inner loop. Domains IIId and IIIe have GGG(A) and GAUA tetraloop nucleotide motifs and an A-A mismatch at the base of hairpin III [34, 35]. Upstream of the initiation codon of newt/II-5-Pilis/2014/HUN, a 178-nt-long unidentified nt sequence was found starting with a long, significant polypurine (A)-rich sequence (PARS) tract (Fig. 2A).

The 3′UTR of newt/II-5-Pilis/2014/HUN is 96-nt long and has no significant nt sequence identity to any viral sequences in GenBank.

A total of 14 possible *CRE* motifs (10 of 5′-AAACA-3′ and 4 of 5′-AAACG-3′) were found in the genome of newt/II-5-Pilis/2014/HUN. Based on the RNA secondary structure prediction, the thermodynamically most favoured site (A$_{3666}$AACG in the top loop of the stem-loop structure, with more than 90% structural probability and ΔG = -41.7 kcal/mol) is located between nt 3666 and 3670 in the junction of the 2A and 2B regions (Fig. 2B).

The newt/II-5-Pilis/2014/HUN genome encodes a 2261-aa-long polyprotein (Fig. 1A). The potential polyprotein cleavage sites were predicted based on a comparison to the prototype oscivirus A2 strain thrush/Hong Kong/10878/2006 (GU182410) (Fig. 1A). The putative L protein is 279 nt (93 aa) long, and using a BlastX search, 46% aa identity was revealed to the closest match, a CNPV231 MyD116-like domain of canarypox virus (NP_925254). Putative conserved domains (V$_{30}$TFS and R$_{49}$xGPWExxARDRxRFxRRI aa motifs) were detected with high probabilities (E-value: 6.59e^{-10}) with the help of the conserved domain search (Fig. 1B). The P1, P2 and P3 regions are 2409 nt (803 aa), 2046 nt (682 aa) and 2328 nt (776 aa) long. A potential N-terminal myristoylation motif (GxxxT/S, **G$_{83}$QSIT**) was reliably predicted at the N-terminal end of VP0 hypothetical protein. The conserved **H**-box/**NC** aa motifs (**H$_{911}$**WAI and **N$_{969}$C**THF) in 2A; the conserved Walker A (GxxGxGKS, **G$_{1365}$DPGIGKS**), Walker B (DDLxQ, **D$_{1415}$DLGQ**) and Walker C (KGxxxxSxxxxx(S/T)(S/T)N, **K$_{1448}$GTPYTSRVIIV PAN**) motifs in 2C; the conserved picornavirus H-D-C catalytic triad (TD**H$_{1726}$**Y, **D$_{1764}$**MTK and GL**C$_{1826}$**G, where cysteine is the active-site of the cysteine protease), the histidine (**H$_{1844}$**YAG) residue in the trypsin-like protease domain in 3C; and well-conserved aa motifs (**K$_{2036}$**DELR, **G$_{2163}$**NPSG, **Y$_{2204}$**GDD and **F$_{2253}$**LKR) in 3D [36] are present in newt/II-5-Pilis/2014/HUN.

All P1, P2 and P3 protein products of the study strain have low aa sequence similarity to picornaviruses. In a BlastX search, using the complete polyprotein, P1, P2 and P3 showed 36% aa sequence identity to oscivirus A2 (thrush/Hong Kong/10878/2006, GU182410), 37% sequence aa identity to bat picornavirus (BtMr-PicoV/JX2010, KJ641686), 34% aa sequence identity to Aichi virus (kvgh99012632/2010, JX564249), and 51% aa sequence identity to oscivirus A1 (robin/Hong Kong/007167/2007, GU182409). The aa sequence similarity of the study strain to the corresponding regions (P1, 2C and 3CD) of representative picornaviruses, determined using the SIAS web server, is shown in Table S1.

For phylogenetic analysis, the P1, 2C and 3CD regions were also used to do a BLAST search of the GenBank database, and these protein products showed 37% aa sequence identity to bat picornavirus (as mentioned above), 43% aa sequence identity to oscivirus A2 (thrush/Hong Kong/10878/2006, GU182410), and 52% aa sequence identity to oscivirus A1 (robin/Hong Kong/007167/2007, GU182409), respectively. The phylogenetic trees based on the P1, 2C and 3CD nt sequence of newt/II-5-Pilis/2014/

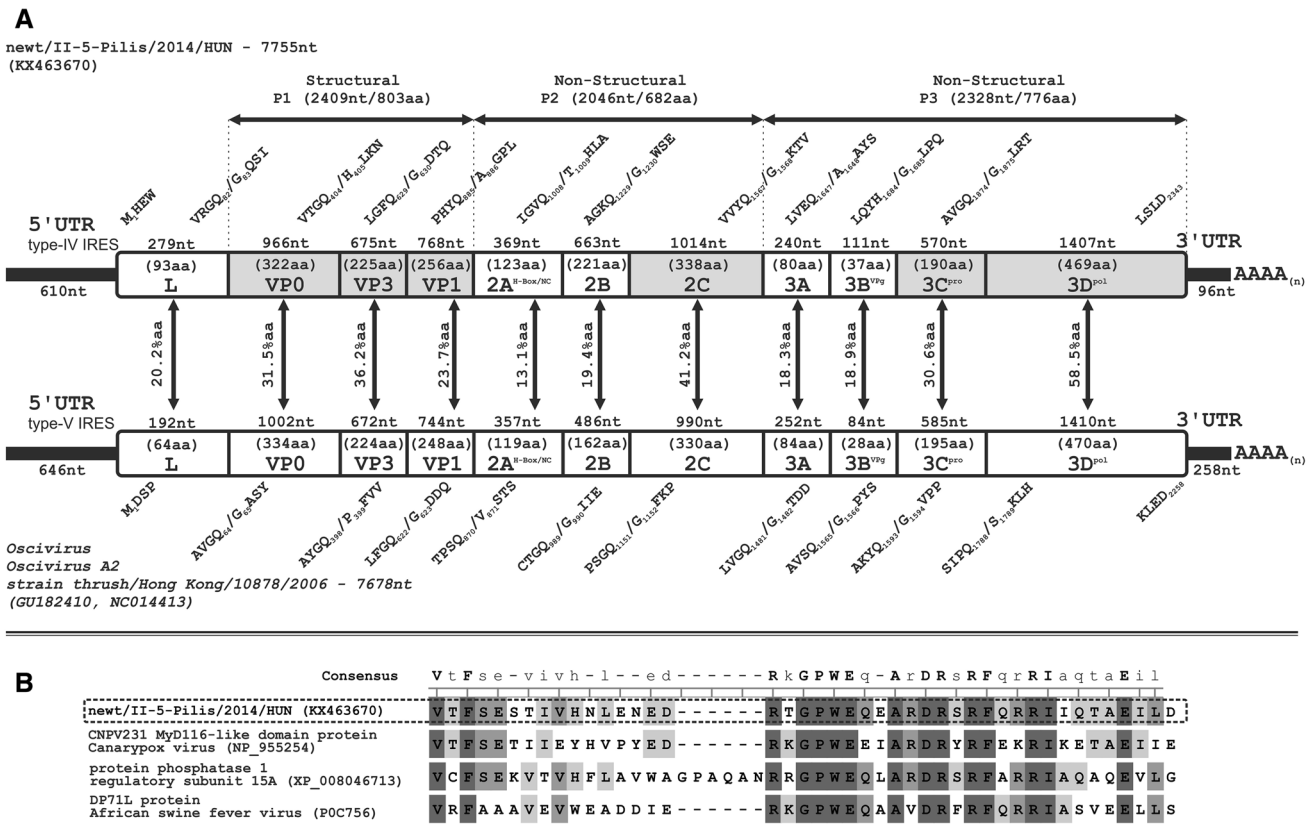

Fig. 1 **A)** Comparison of the genome organization of picornavirus strain newt/II-5-Pilis/2014/HUN (KX463670) from smooth newt and the closest relative oscivirus A2 strain thrush/Hong Kong/10878/2006 (GU182410) a member of the genus *Oscivirus*. The lengths of the complete genomes excluding the poly(A) tail are indicated next to the strain name; the nucleotide (nt) and amino acid (aa) lengths of the hypothetical genes are indicated above and below the gene boxes. The predicted aa cleavage sites are shown on the border of each region. The pairwise aa identity scores are presented between the related protein products. **B)** Sequence alignment of the leader (L) protein of the study strain marked with dotted line with the closest match in the protein database of GenBank. The L protein of the study strain has 46% aa sequence identity (E-value: 2e-07) to the CNPV231 MyD116-like domain protein (NP_955254) of canarypox virus (NC_005309), 49% aa sequence identity (E-value: 6e-07) to protein phosphate 1 regulatory subunit of Philippine tarsier (*Carlito syrichta*) (XP_008046713), and 43% aa sequence identity to the DP71 protein of African swine fever virus (P0C756). Conserved amino acids are shown in bold in the consensus sequence

HUN and representative members of the family *Picornaviridae* could indicate that the study strain may be a novel picornavirus related to osciviruses, but distant from the previously identified newt picornavirus "ampivirus" (Fig. 3A-C).

Using the screening primer pair Livupi-3DSCR-R/F, two (5%) faecal samples (II-5 and II-6) out of 40 were PCR positive, both of which were collected from the same territory (meadow Felsőhosszúrét-elülső) in the Pilis Mountains in 2014. Sequence analysis showed that the picornavirus sequence of strain newt/II-6-Pilis/2014/HUN (KX463671) from sample II-6 was 99% nt identical to the study strain, and only five nt differences were revealed in the analysed $3D^{pol}$ region.

Amphibians have existed on Earth for over 300 million years, with more than 7500 existing species. A large number of amphibians are threatened worldwide, and approximately 43% of the species have become extinct [37, 38]. The first picornavirus ("ampivirus") from newts (also the first picornavirus in amphibians) was discovered recently [15]. Here, we report the detection and characterization of a second novel amphibian-origin picornavirus, which is more similar to the bird oscivirus than to the newt-origin ampivirus. The two known picornaviruses from smooth newts (newt/II-5-Pilis/2014/HUN and ampivirus) have different genome organisations (type-? IRES-VP0-VP3… versus type-IV IRES-L-VP0…) and are situated in two different branches of the picornavirus phylogeny. According to the taxonomic genus and species demarcation criteria of the International Committee on Taxonomy of Viruses (ICTV) *Picornaviridae* Study Group (http://www.picornastudygroup.com/definition/genus_definition.htm), different species of a given genus have at least 58% aa sequence identity. In the complete polyprotein, the P1, P2, P3, 2C and 3CD regions of this novel newt picornavirus showed low (less than 58%) aa identity to the corresponding proteins of the known picornaviruses. Based on

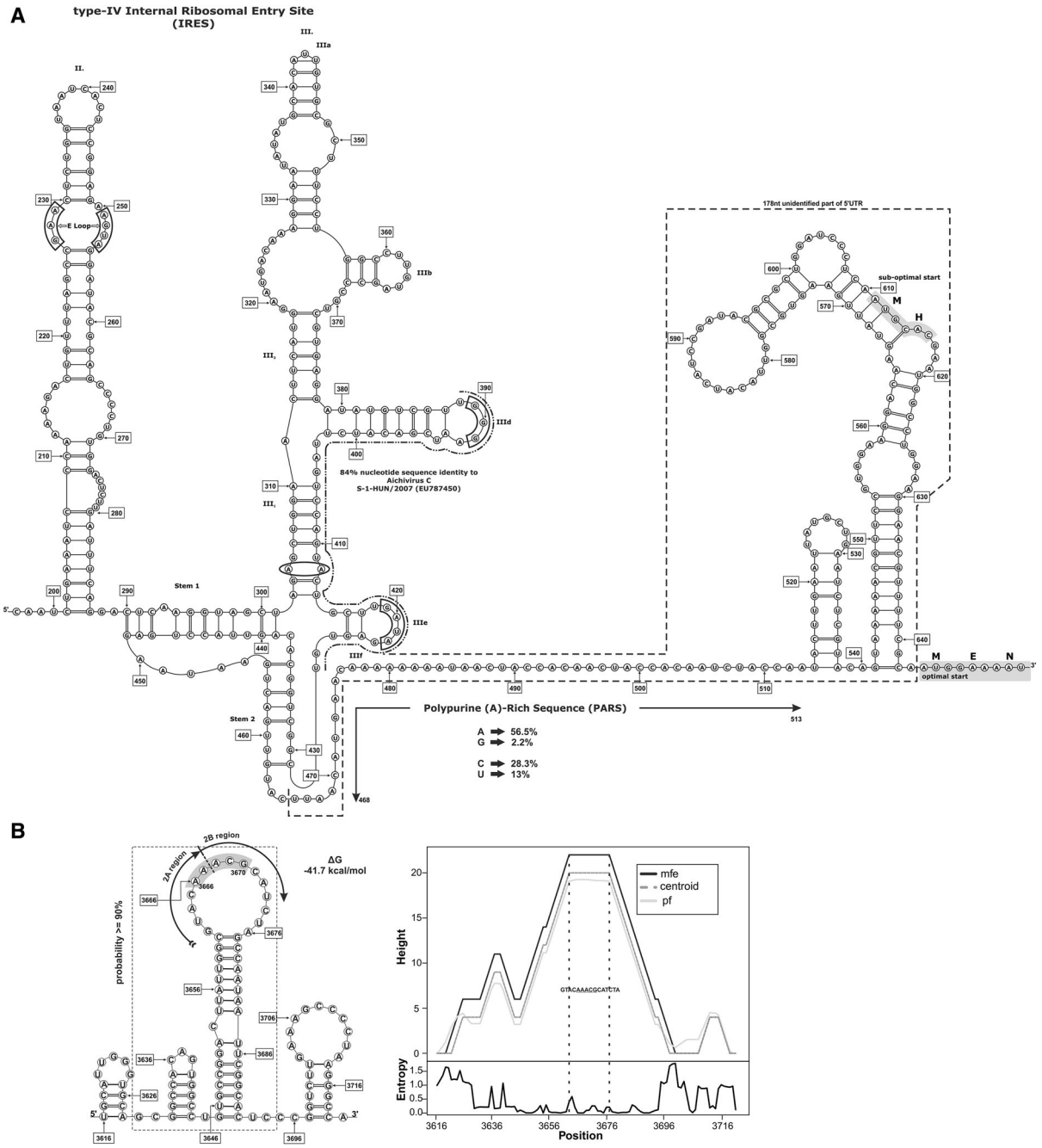

Fig. 2 **A**) Predicted RNA secondary structure of the 5′UTR and internal ribosomal entry site (IRES) of picornavirus strain newt/II-5-Pilis/2014/HUN (KX463670) from smooth newt. The type-IV IRES is annotated as proposed previously [18, 35]. Domains/hairpins are labelled as II and III, maintaining the current nomenclature. The conserved nucleotides GAA and AGUA of loop E in domain II, the GGG(A) and GAUA tetraloops in the apical portion of domains IIId and IIIe, and the A-A mismatch in the basal region of hairpin III are indicated by a thick black line. The positions of nucleotides that are conserved between the study strain and porcine kobuvirus (EU787450) (genus *Kobuvirus*) are indicated by a dotted line. The putative in-frame initiation AUG start codons (from nt 611 and 644) are marked with grey boxes, and the second one is indicated by a grey background and black lines. **B**) Predicted secondary RNA structure of a *cis*-acting replication element (*CRE*) in the cleavage border of the 2A/2B region. Bases contributing to the AAACG motif within the loop sequence are indicated by a grey background. The mountain plot of the *CRE* represents the secondary structure in the plot of height versus position, where the height m(k) is given by the number of base pairs enclosing the base at that position. The loops correspond to plateaus, hairpin loops to peaks, and helices to slopes. mfe, minimum free energy; centroid, centroid structure of the RNA sequence, a secondary structure with minimal base pair distance to all other secondary structures in the Boltzmann ensemble; pf, partition function, this sums all Boltzmann weighted free energies of each secondary structure that is possible given the RNA sequence. This provides the possibility to calculate base pairing probabilities for each possible pair of bases. The positional minimum free energy (entropy: ΔG) is shown under the mountain plot. The position of the mountain plot and the entropy correspond to the nt position of the secondary structure of *CRE*

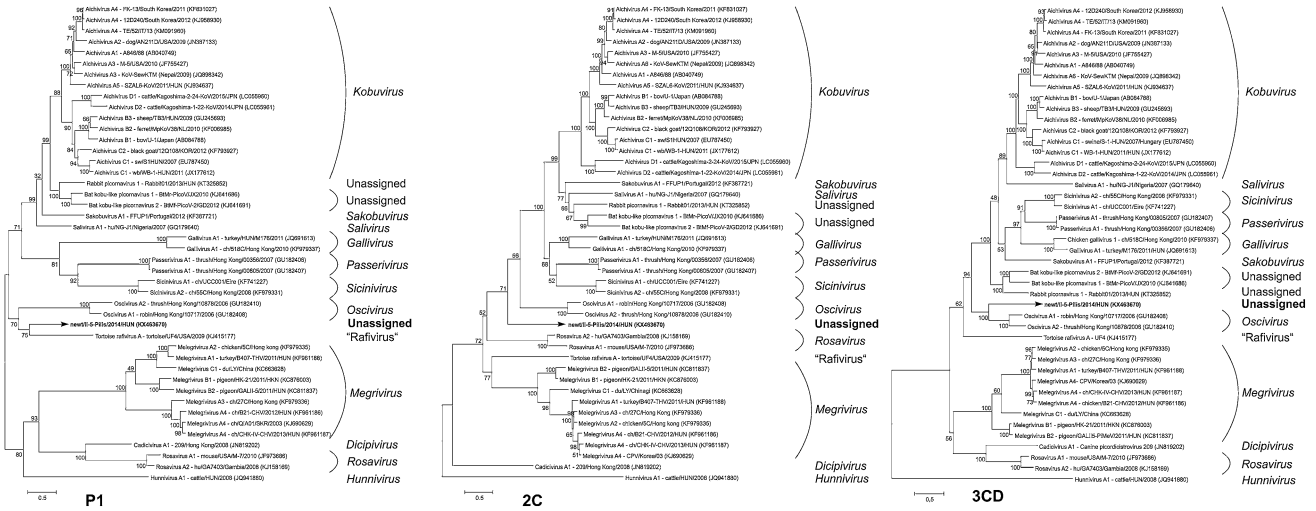

Fig. 3 Phylogenetic analysis of smooth newt picornavirus strain newt/II-5-Pilis/2014/HUN (KX463670 in **bold**) and representative picornaviruses based on the complete P1 (**A**), 2C (**B**), and 3CD (**C**) nt sequences. The strain cattle/HUN/2008 (JQ941880, genus *Hunnivirus*) is used as an outgroup in each phylogenetic tree. The evolutionary analyses were conducted in MEGA6, and evolution history was inferred using the maximum-likelihood method based on the general time-reversible model with discrete Gamma distribution (+G), allowing evolutionarily invariable sites (+I), and bootstrap values were determined with 1000 replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site

these data, newt/II-5-Pilis/2014/HUN should be considered a member of a new picornavirus species in the proposed genus "*Livupivirus*" (originated from *Lissotriton vulgaris* picornavirus). However, the formal classification of the study strain is subject to further discussion by the ICTV.

Considering its sequence similarities and predicted RNA secondary structure, the 5′UTR of newt/II-5-Pilis/2014/HUN has a type-IV IRES. Interestingly, between the IRES structure and the putative initiation start codon, an unidentified 178-nt-long sequence was found in newt/II-5-Pilis/2014/HUN, starting with a 46-nt-long polypurine (A)-rich sequence (PARS) instead of a polypyrimidine tract. These elements potentially have the capability to overcome kingdom-specific barriers in transcription and translation and make a virus viable in different types of cells (plant or animal) [39]. Further experimental studies are necessary to determine whether this is the situation in newt/II-5-Pilis/2014/HUN. The cis-acting replication element (*CRE*) plays a part in picornavirus RNA replication and synthesis [24]. The function of this high-priority secondary structure has been verified, but its location in the picornavirus genome varies [24]. The stem-loop structure of *CRE* in newt picornavirus is potentially located in the 2A/2B junction region, but this requires experimental evidence.

The L protein shows the greatest diversity, both in function and in sequence size, among picornaviruses [40]. More than 55% of known members of picornavirus genera encode an L protein, but only a few of them have a well-known function. For example, aphthovirus, erbovirus and mosavirus L proteins have a papain-like protease function, while avian sapelovirus L protein could be a trypsin-like protease [41]. Instead of protease activity, the cardiovirus L protein is a putative zinc-binding metalloprotein, which is involved in counteracting host antidefenses [42, 43]. The L protein of Aichi virus genus (*Kobuvirus*) is involved in viral RNA replication and encapsidation [17]. Interestingly, several conserved aa motifs are found in the L protein of newt/II-5-Pilis/2014/HUN that are present in phosphatase-1 catalytic (PP1C) subunit binding region (pfam10488) proteins, in Gadd-34 apoptosis-associated proteins, in MyD116-like domain of canarypox virus, and the NL-S gene product of African swine fever virus [44–47], respectively. The PP1C proteins (formerly known as Gadd34 or MyD116) are co-ordinately overexpressed in cells inducing genotoxic stress and certain other growth arrest signals to cause apoptosis [48, 49]. The function and biological activity of the L protein in the strain identified in this study are still unknown, but aa sequence similarities and conserved aa motifs suggest that it is a virulence factor with a phosphatase function that could be involved in the regulation of a variety of cellular processes [44–46].

A large number of picornaviruses have already been described in higher vertebrates (mammals and birds) with the help of newly available molecular techniques, but the picornavirus diversity is less known in lower vertebrates (fish, amphibians, and reptiles). According to the IUCN Red List of Threatened Species (2014), the estimated numbers of fish, amphibian and reptile species that are extant in nature are 32,900, 7,302, and 10,038, respectively (http://www.iucnredlist.org). Despite this taxonomic diversity, to date, picornaviruses have been reported in a handful of species of fish, including common eel (*Anguilla anguilla*) [11], bluegill

fish (*Lepomis macrochirus*) [12, 50], common carp (*Cyprinus carpio*) [51], fathead minnow (*Pimephales promelas*) [13], brassy minnow (*Hybognathus hankinsoni*) [13], amphibians, including smooth newt (*Lissotriton vulgaris*); [15] and reptiles, including several terrestrial tortoises (*Testudo hermanni*, *T. graeca*, *Pyxis arachnoides*, *Geochelone sulcate*, *Indotestudo forstenii*) [7–10]. Comparing the estimated number of species of birds that are hosts of diverse picornaviruses [14], it is conceivable that the lower vertebrates, including newts can also be infected by several, genetically divergent picornaviruses. It should be also noted that detection of a virus in faeces does not necessarily imply that it infects the animal from which the faecal sample was obtained. While this novel picornavirus was detected in faecal samples collected from more than one newt, further studies are necessary to exclude the possibility of a dietary origin and a viral "innocent bystander" effect. The lower vertebrates – fish, amphibians, and reptiles – might be reservoirs of unknown viral pathogens including picornaviruses with a wide range of virus diversity, and therefore, further viral metagenomic analyses are needed to explore their real diversity, which could also help to understand picornavirus evolution.

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