

# A diarrheic chicken simultaneously co-infected with multiple picornaviruses: Complete genome analysis of avian picornaviruses representing up to six genera

Ákos Boros<sup>a</sup>, Péter Pankovics<sup>a</sup>, Ádám Adonyi<sup>a</sup>, Hajnalka Fenyvesi<sup>a</sup>, J. Michael Day<sup>b</sup>, Tung Gia Phan<sup>c,d</sup>, Eric Delwart<sup>c,d</sup>, Gábor Reuter<sup>a,\*</sup>

<sup>a</sup> Regional Laboratory of Virology, National Reference Laboratory of Gastroenteric Viruses, ÁNTSZ Regional Institute of State Public Health Service, Pécs, Hungary and University of Pécs, Pécs, Hungary

<sup>b</sup> United States Department of Agriculture, Agricultural Research Service, U. S. National Poultry Research Center, Southeast Poultry Research Laboratory, Athens, GA, USA

<sup>c</sup> Blood Systems Research Institute, San Francisco, CA, USA

<sup>d</sup> University of California, San Francisco, CA, USA

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## ABSTRACT

In this study all currently known chicken picornaviruses including a novel one (chicken phacovirus 1, KT880670) were identified by viral metagenomic and RT-PCR methods from a single specimen of a diarrheic chicken suffering from a total of eight picornavirus co-infections, in Hungary. The complete genomes of six picornaviruses were determined and their genomic and phylogenetic characteristics and UTR RNA structural models analyzed in details. Picornaviruses belonged to genera *Sicinivirus* (the first complete genome), *Gallivirus*, *Tremovirus*, *Avisivirus* and "Orivirus" (two potential genotypes). In addition, the unassigned phacoviruses were also detected in multiple samples of chickens in the USA. Multiple co-infections promote and facilitate the recombination and evolution of picornaviruses and eventually could contribute to the severity of the diarrhea in chicken, in one of the most important food sources of humans.

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## Introduction

Members of family Picornaviridae are small viruses with positive sense, single-stranded RNA genomes whose length range between 7.2 and 9.7 kb with the predominant presence of a single Open Reading Frame (ORF). The general picornavirus genome layout contain the 5'UTR-[ORF: L (not always present)-VP0 (or cleaved into VP4 and VP2)-VP3-VP1-2A-2B-2C-3A-3B-3C-3D]-3'UTR-polyA-tail (Palmenberg et al., 2010). A few picornaviruses have slightly modified genome plans such as the cadiciviruses (genus *Dicipivirus*) with two ORFs or the unassigned aaliviruses with up to six 2A regions (Woo et al., 2012; Wang et al., 2014). The 5' and 3' un-translated regions (UTRs) contain several structurally conserved motifs, generally one of the five different types of internal ribosomal entry sites (IRES) and the apical "8"

structure at the 5' end and s2m, "barbell-like" structure or repeated sequences similar to the 'Unit As' of megriviruses at the 3' end (Palmenberg et al., 2010; Boros et al., 2014c). The family Picornaviridae currently consists of 50 species grouped into 29 genera and a continuously increasing number of novel, currently unassigned picornaviruses (Knowles et al., 2012; Adams et al., 2015; [www.picornaviridae.com](http://www.picornaviridae.com)). The picornaviruses have been identified from various vertebrate species, including birds. The majority of the currently known avian picornaviruses belong to five different phylogenetic clusters: the megrivirus-, the passerivirus-, the avihepatovirus-, the tremovirus-, and the avian sapelovirus clusters of which the last cluster is not known to contain chicken picornaviruses (Boros et al., 2014c). At least six different picornaviruses: the chicken megrivirus (genus *Megrivirus*, megrivirus cluster), sicinivirus A1 (*Sicinivirus*, passerivirus cluster), chicken gallivirus 1 (*Gallivirus*, passerivirus cluster), chicken picornavirus 2, 3 (Unassigned, avihepatovirus cluster), Orivirus A1 ("Orivirus", avihepatovirus cluster) and avian encephalomyelitis virus 1 (*Tremovirus*, tremovirus cluster) have been identified from chickens. Among these the complete genome of sicinivirus has not been reported before (Farkas et al., 2012; Boros et al., 2014a, 2014b; Bullman et al., 2014; Lau

\* Correspondence to: Regional Laboratory of Virology, National Reference Laboratory of Gastroenteric Viruses, ÁNTSZ Regional Institute of State Public Health Service, Szabadság út 7, H-7623 Pécs, Hungary. Tel.: +36 72 514 979; fax: +36 72 514 949.

E-mail address: [reuter.gabor@gmail.com](mailto:reuter.gabor@gmail.com) (G. Reuter).

et al., 2014; Tannock and Shafren, 1994). These avian picornaviruses have been frequently detected in cloacal specimens collected from both healthy and affected chickens (Lau et al., 2014; Bullman et al., 2014; Farkas et al., 2012; Boros et al., 2014a), but little information is available with regard to co-infections with these viruses. There are only a few studies describing the presence of galli-, and avisiviruses; as well as galli-, and siciniviruses present as co-infections in turkeys (Boros et al., 2013) and chickens (Bullman et al., 2014). Poultry, including chickens are one of the most important food sources of humans, and gastro-intestinal infections of these birds are known to negatively impact meat and egg production and cause veterinary, economic, and even human health concerns (Guy, 1998; Chan et al., 2015). Therefore, the discovery of further avian viruses which could be involved in gastro-intestinal infections potentially bears on veterinary care and medical importance. Metagenomic approaches give us the opportunity to simultaneously detect large number of sequences from different microbes including viruses which could contribute to the development of diarrheic syndromes (Day et al., 2015; Finkbeiner et al., 2008). Although due to the short sequence reads and sometimes poor coverage, the assembled metagenomic sequences of field samples usually hold the risk of containing artificial chimeric contigs and unnoticed sequence variants especially when closely related species or multiple genotypes with uneven copies are present simultaneously (Teeling and Glöckner, 2012; Vázquez-Castellanos et al., 2014). Therefore in this study the complete genomes of members of the enteric picornavirome identified by viral metagenomic approach of a single, diarrheic chicken were determined and analyzed. Using different RT-PCR types and metagenomic sequences, the complete genomes of six different picornaviruses were determined, which belong to 4 known (*Sicinivirus*, *Gallivirus*, *Tremovirus*, *Avisivirus*) and one novel ("Orivirus") genera. The taxonomic position of one picornavirus, chicken phacovirus 1 strain Pf-CHK1/PhV cannot be resolve unambiguously. Furthermore, the prototype strain chicken/Pf-CHK1/2013/HUN (GenBank Accession number: KM203656) of Orivirus A1 (genus "Orivirus") previously described by our research group (Boros et al., 2014a) was also identified from the same cloacal sample.

## Results

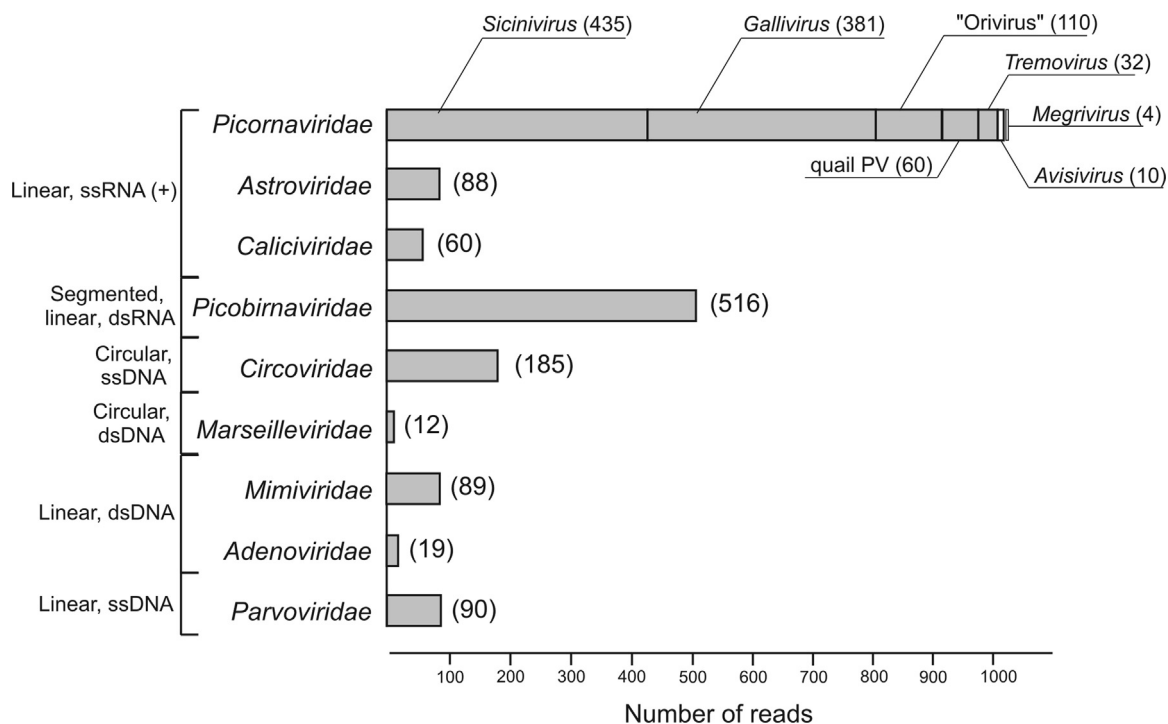
### Viral metagenomic overview

The *in silico* analysis of viral metagenomic sequences of the single specimen Pf-CHK1 identified a total of 13,016 unique viral reads of which 10,906 likely originated from viruses infecting bacteria (*Podoviridae*:  $N=10$ , *Siphoviridae*:  $N=43$  and *Microviridae*:  $N=7178$ ), plants (*Phycodnaviridae*:  $N=40$ ) and unclassified ( $N=3635$ ) virus families based on BLASTx  $E$  scores  $< 10^{-5}$ . The rest of the sequence reads ( $N=2110$ ) belong to different eukaryotic viruses, with reads from RNA virus families predominating ( $N=1715$ ,  $\approx 80\%$  of the eukaryotic viral reads). The largest group of RNA viruses represented are picornaviruses ( $N=1032$  reads), followed by the group of picobirnavirus-related sequences with considerably fewer reads (Fig. 1). The detailed BLASTx analyses of the picornavirus-related sequences suggested the presence of at least seven different picornaviruses in the analyzed sample (Fig. 1).

A total of six complete genomes from the seven picornaviruses identified by BLASTx analyses of metagenomic reads were verified and determined in this study using RT-PCR methods. The 4 sequence reads related to megriviruses were not detectable by RT-PCR, which could suggest the initially low copy number of megriviruses in the sample.

### Genome analysis of the *Sicinivirus*-related picornavirus

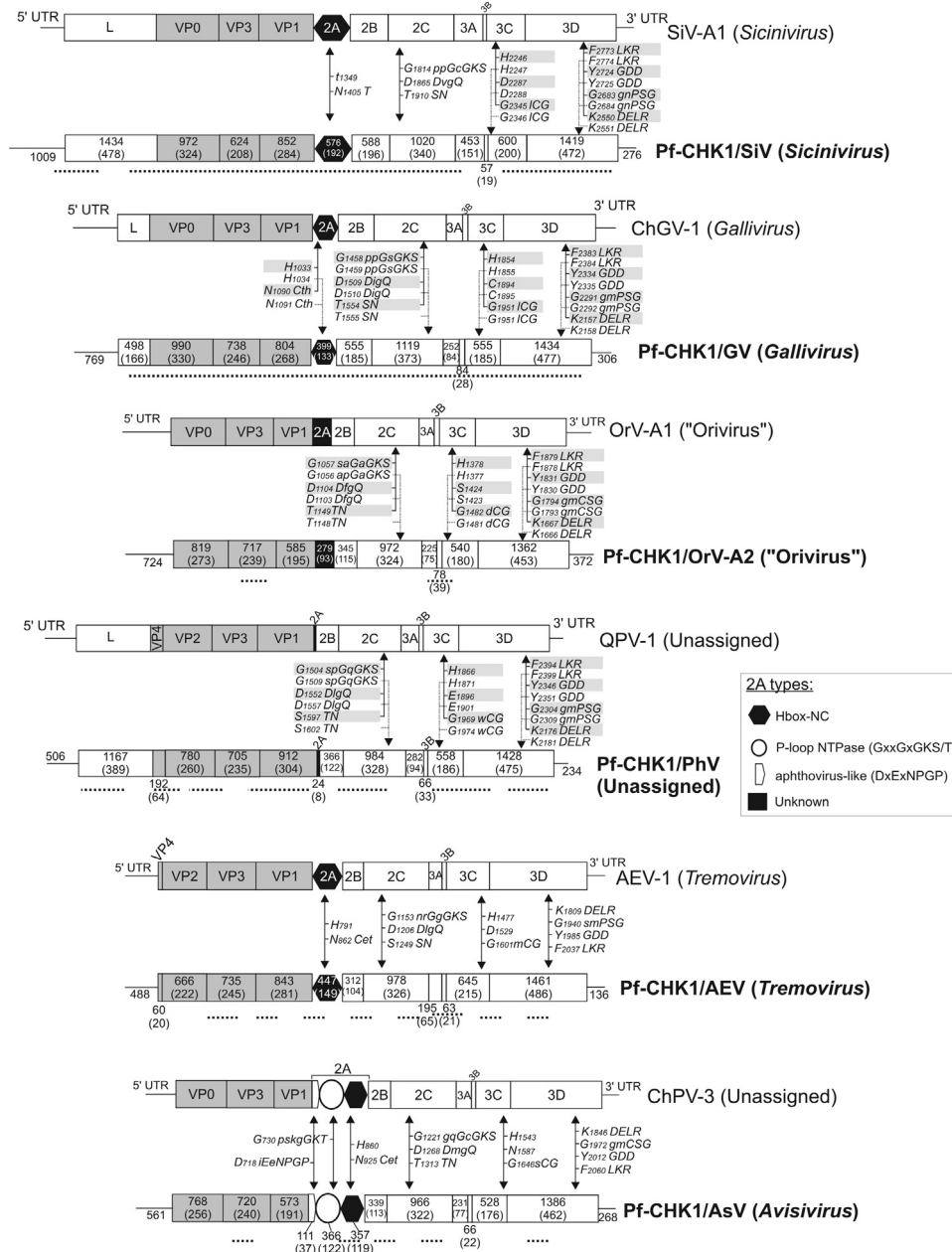
The sicinivirus-related reads covered  $\approx 74\%$  of the 9243-nt-long genome of *Sicinivirus* A1 (SiV-A1) strain UCC001 (KF741227) with variable depth of coverage ranging 1–17 (Fig. 2). Due to the variable depth of coverage and the unknown 5' and 3' ends, multiple RT-PCR reactions were used to verify all of the covered genome regions and to acquire the complete genome. The 9883-nt-long picornavirus genome of strain Pf-CHK1/SiV (KT880665) is the longest picornavirus genome reported to date. The Pf-CHK1/SiV show an overall 82% nt identity to the partial genome (5' end is



**Fig. 1.** Virus family-level categorization of eukaryotic viral metagenomic reads is based on the best BLASTx-scores ( $E$ -value  $\leq 10^{-5}$ ). The picornavirus-related reads are further characterized based on the highest sequence identity to the members of the different genera. quail PV: quail picornavirus 1 (currently unassigned). Numbers in brackets represent the number of reads.

missing) of SiV-A1 strain UCC001 of genus *Sicivirus* as the closest match using BLASTn. The two viruses share the same genome layout of L-3-3-4, identical conserved aa motifs of 2A<sup>Hbox/NC</sup>, 2C<sup>Hel</sup>, 3C<sup>Pro</sup> and 3D<sup>Pol</sup> and similar predicted cleavage sites (Fig. 2, Table 1). The 1009-nt-long 5' UTR of Pf-CHK1/SiV is 636-nt-longer than the incomplete, 373-nt-long 5' UTR of prototype strain UCC001 of genus *Sicivirus*. Pf-CHK1/SiV was predicted to have a type-II-like IRES where all the characteristic domains (A–L) and motifs are recognizable, including the binding sites of pyrimidine-tract binding protein (PTB) and eIF4G proteins, as well as the conserved sequence motifs of GNRA, RAAA (Duke et al., 1992; Belsham and Jackson, 2000) (Fig. 3A). The characteristic domains of J, K and L of type-II IRESes located at the partial 5' UTR of SiV-A1

were also predicted by Bullman et al. (2014). Although while the J and K domains of Pf-CHK1/SiV showed 97% nt identity to the corresponding domains of SiV-A1 strain UCC001, the domain L contains a 46-nt-long insertion and showed only 28% nt identity to the same domain of strain UCC001 (Fig. 3A). Interestingly, the spacer sequence between the L domain and the predicted start codon is located in an optimal Kozak context (tt/aCAaCA<sub>1010/374</sub>TGG, conserved nts in uppercase, start codon was underlined) of Pf-CHK1/SiV and SiV-A1 strain UCC001 and is unusually long (~170 nt). The length of spacer sequences of picornaviruses with type-II IRESes range between 16 and 50 nt (Yu et al., 2011; Boros et al., 2012a, 2013). Despite the low sequence identity between the long spacers of Pf-CHK1/SiV and SiV-A1 strain UCC001, multiple



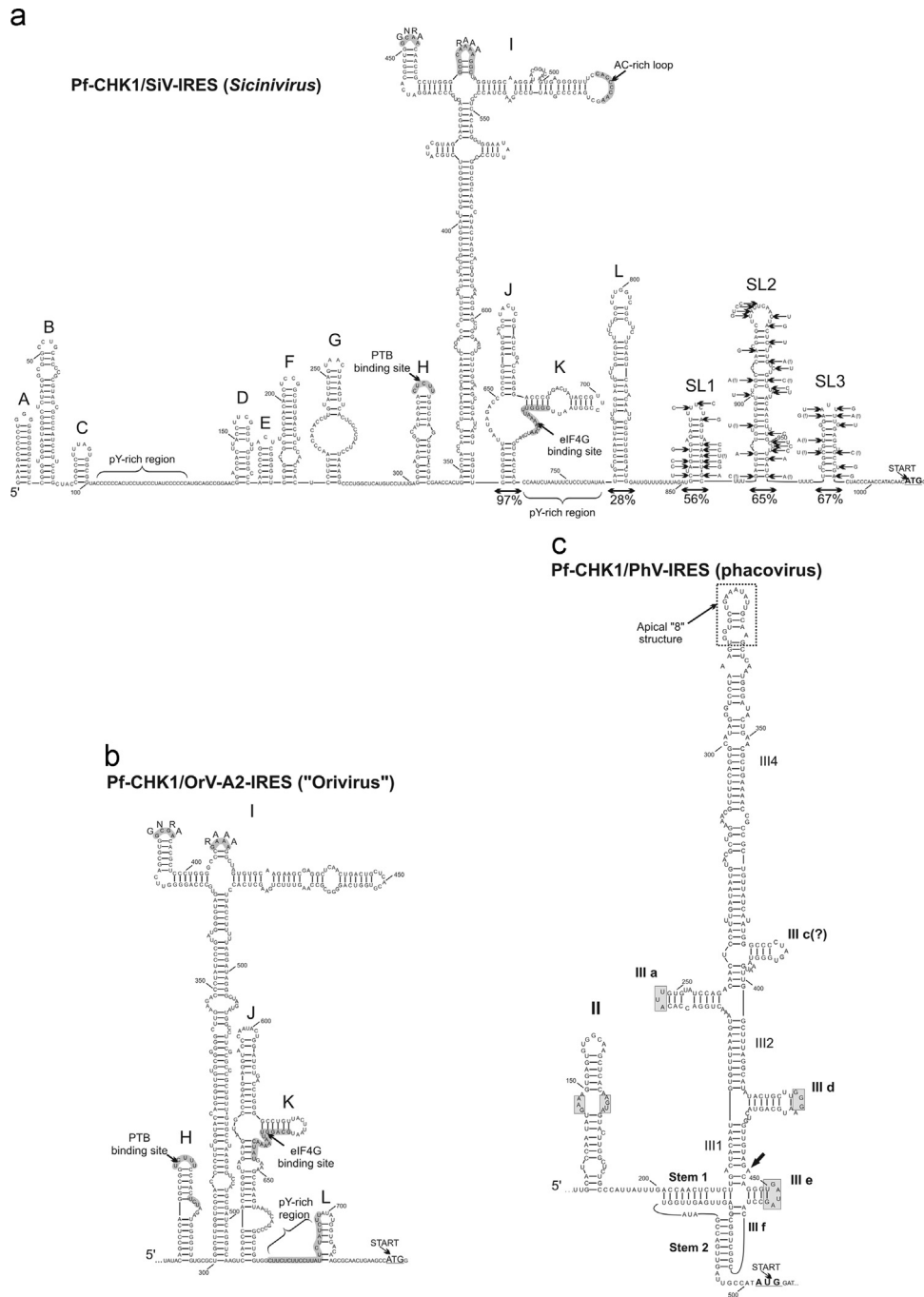
**Fig. 2.** Genome maps and conserved amino acid motifs of the study picornaviruses together with their closest relatives. The gene boxes corresponding to the viral capsid proteins are depicted with gray background. The genome maps are drawn to scale and aligned to the VP1/2 A1 junction. The nucleotide (upper numbers) and amino acid (lower numbers in brackets) lengths of the corresponding genomic regions are shown in each gene box. Dashed lines under the study sequence maps represents the length and localization of genome regions covered by the viral metagenomic reads. The position and sequence of amino acid motifs of different 2A types (depicted with different shapes found in a frame), 2C<sup>Hel</sup> (Gorbalenya et al., 1990), 3C<sup>Pro</sup> (Gorbalenya et al., 1989), and 3D<sup>Pol</sup> (Argos et al., 1984) are shown between the genome maps. The conserved and variable amino acids are represented by uppercase and lowercase letters respectively. SiV-A1: Sicivirus A1 (KF741227), ChGV-1: chicken gallivirus 1 (KF979337), OrV-A1: Orivirus A1 (KM203656), QPV-1: quail picornavirus 1 (JN674502), AEV-1: avian encephalomyelitis virus 1 (KF979338), ChPV-3: chicken picornavirus 3 (KF979334).

**Table 1**  
Nucleotide and amino acid (in brackets) identity values in percent and predicted proteolytic cleavage sites (P6–P2') of the study viruses (top sequences) and the closest relatives (bottom sequences).

Genome regions↓	PfCHK1/SiV vs. SiV-A1		PfCHK1/GV vs. ChGV-1		PfCHK1/OrV-A2 vs. OrV-A1		PfCHK1/PhV vs. QPV-1		PfCHK1/AsV vs. ChPV-3		PfCHK1/AEV vs. AEV-1	
	nt (aa) % IDs	Cleavage sites (P6–P2')	nt (aa) % IDs	Cleavage sites (P6–P2')	nt (aa) % IDs	Cleavage sites (P6–P2')	nt (aa) % IDs	Cleavage sites (P6–P2')	nt (aa) % IDs	Cleavage sites (P6–P2')	nt (aa) % IDs	Cleavage sites (P6–P2')
<b>5' UTR</b>	62*	–	90*	–	69	–	52	–	89	–	90	–
<b>L</b>	79 (91)	WVTSEQ-GA WVTSEQ-GA	84 (92)	SFDTSQ-GL SFDTSQ-GL	–	–	59 (55)	CDGLLQ-GQ CDGQFQ-GQ	–	–	–	–
<b>VP4</b>	–	–	–	–	–	–	66 (60)	MIPDLQ-SP FVPDLQ-SP	–	–	91 (100)	VLSVLN-DE VLSVLN-DE
<b>VP2 [VP0]</b>	[87 (95)]	NIPQNG-GV NIPQNG-GV	[82 (89)]	ANPSSQ-GL ANPSSQ-GL	[75 (91)]	LNLTAQ-GL LNLNAQ-GL	70 (75)	AAASLN-GV SAAALE-GV	[79 (90)]	RPYQSQ-GQ RPYRVQ-GQ	86 (98)	SPLVAQ-MM SPLVAQ-MM
<b>VP3</b>	87 (98)	ASIQIQ-GD ASIQIQ-GD	85 (94)	AVAQPQ-GD AAVSRQ-GD	75 (91)	SDLKLG-GI SDLKLG-GI	65 (75)	DNPYYQ-GP DNAYFQ-GP	79 (92)	GDYHIE-GP GDYHIE-GP	85 (99)	TSVFAQ-GK TSVFAQ-GK
<b>VP1</b>	86 (94)	DFSEAQ-AS DFSEAQ-AS	82 (89)	IPAKRQ-NG IPAKRQ-NG	71 (86)	PTVSNQ-GP AALSDQ-GP	66 (69)	KAGIMQ-GP RQGEKQ-GP	79 (89)	KLPCPR-YT KLPCPR-YT	86 (98)	FDELEE-AQ FDELEE-AQ
<b>2A1</b>	84 (93)	AQJISQ-AV AQJISQ-AV	90 (94)	HLPKRQ-GP QIPKRQ-GP	59 (65)	YTCSAQ-AP YTCSAQ-AP	55 (50)	LSSEKQ-GV PCARKQ-GL	65 (75)	IEENPG-PV IEENPG-PV	83 (96)	TKLPQK-VG TKLPQK-VG
<b>2A2</b>	–	–	–	–	–	–	–	–	82 (98)	LPYNTR-GT LPYNTR-GT	–	–
<b>2A3</b>	–	–	–	–	–	–	–	–	85 (99)	CLPHDQ-SG CLPHDQ-SG	–	–
<b>2B</b>	84 (98)	EVPQPQ-GP EVPQPQ-GP	85 (90)	PAPEPQ-GV PAPEPQ-GV	75 (93)	DAIGDQ-GP EAMGDQ-AP	67 (70)	CLNPKQ-GP KQ-GP	87 (100)	PIFRAE-GP PIFRAE-GP	85 (97)	TTPKAQ-GL TTPKAQ-GL
<b>2C</b>	80 (92)	NAPKKQ-GL STPKKQ-GR	91 (98)	PPPKPQ-GK PPPKPQ-GK	76 (87)	INMMDQ-GD VNIMDQ-GD	73 (84)	VDGLFQ-GP VDGLFQ-GP	85 (99)	ASLFNQ-GA ASLFNQ-GA	84 (95)	IQYWAQ-SN IQYWAQ-SN
<b>3A</b>	77 (88)	LISKPQ-AA LISKPQ-AA	89 (92)	FKERRQ-GA FKERRQ-GA	74 (76)	KYKEDE-GA KFKEDE-GA	70 (77)	HKFESHQ-GP HKFVPQ-GP	89 (97)	YRAIQN-QL YRAIQN-QL	84 (95)	DQQHDQ-SA DQQHDQ-SA
<b>3B</b>	73 (63)	EETKVQ-AP KEPETQ-AP	88 (100)	PRARRQ-GI PRARRQ-GI	75 (88)	APVSNQ-NL APVSNQ-NL	71 (77)	RTIQIQ-GP RTVNVQ-GP	86 (100)	RDFKPE-GP RDFKPE-GP	85 (95)	EQADAQ-SV EQADAQ-SV
<b>3C</b>	83 (93)	TPQSQ-SE VPQSQ-SE	91 (97)	RQANPQ-SV RQANPQ-SV	71 (85)	YGVVQ-GL YGVVQ-GL	65 (74)	YFSQKQ-GL YFVQKQ-GK	87 (100)	YPCFAQ-GV YPCFAQ-GV	85 (99)	LKTRAQ-CS LKTRAQ-CS
<b>3D</b>	84 (94)	–	93 (99)	–	71 (83)	–	70 (81)	–	86 (98)	–	84 (95)	–
<b>3' UTR</b>	97	–	95	–	81	–	47	–	97	–	87	–

Abbreviations: SiV-A1: Sicinivirus A1 (KF741227), ChGV-1: chicken gallivirus 1 (KF979337), OrV-A1: Orivirus A1 (KM203656), QPV-1: quail picornavirus 1 (JN674502), ChPV-3: chicken picornavirus 3 (KF979334), AEV-1: avian encephalomyelitis virus 1 (KF979338).

\* Due to the absence of complete 5'UTRs of reference strains only the available partial 5'UTR sequences were used for comparisons.

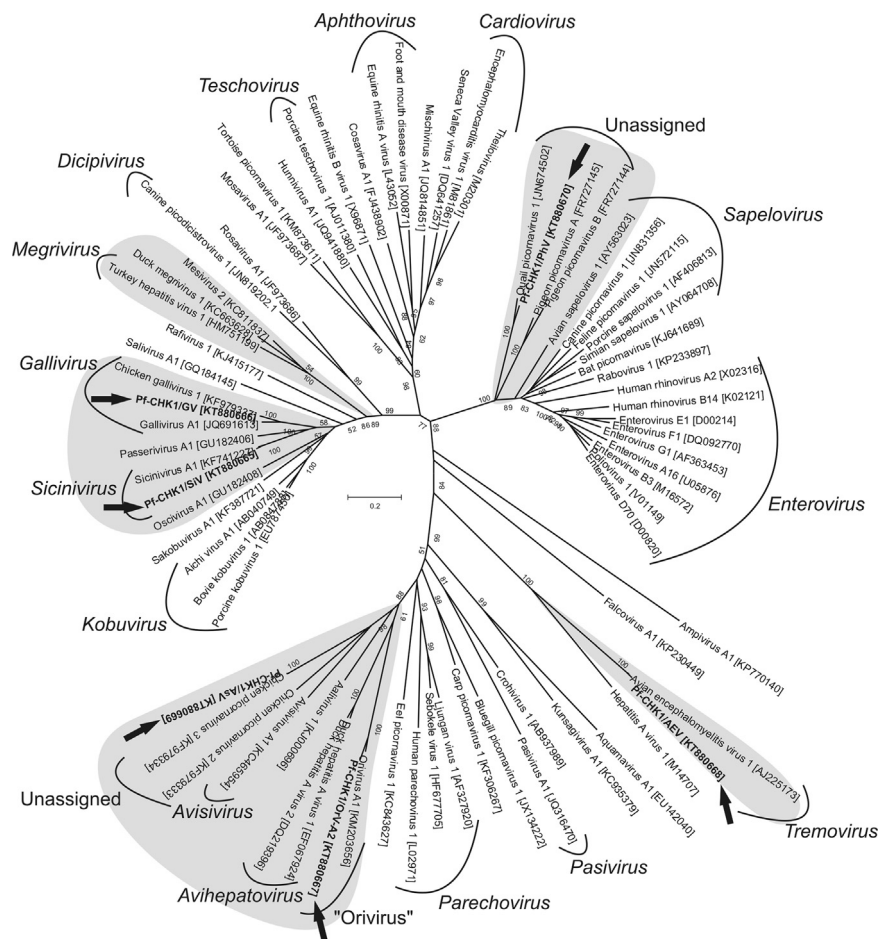


**Fig. 3.** Secondary RNA structures of internal ribosomal entry sites (IRESes) of Pf-CHK1/SiV (a), Pf-CHK1/OrV-A2 (b) and Pf-CHK1/PhV (c). (a) The main domains of the type-II IRES of Sicinivirus Pf-CHK1/SiV are annotated from A to L in figure part (a), from H–L in figure part (b) as previously proposed (Duke et al., 1992). The three stem-loop structures (SL1–3) are identified using covariance analysis of the corresponding regions of Pf-CHK1/SiV and SiV-A1 strain UCC001 (KF741227) where black arrows show either the covariant or structurally irrelevant (silent) nucleotides of SiV-A1. (!)-signs show the different nucleotides of SiV-A1 causing the loss of Watson–Crick and U–G/G–U “Wobble” base pairing. Percentage values indicate the nt identities of the corresponding domains of Pf-CHK1/SiV and SiV-A1 strain UCC001. The positions of conserved type-II IRES motifs are highlighted by gray. PTB: pyrimidine tract binding protein, pY: pyrimidine bases. (c) The main domains (II, III) and hairpins (IIIa, IIIb, etc.) of Pf-CHK1/PhV-IRES are labeled according to the corresponding regions of type-IV IRES of hepatitis C virus (Hellen and de Breyne, 2007). Stem 1 and 2 are parts of the pseudoknot. Gray boxes indicate conserved sequence regions of type-IV IRESes (Hellen and de Breyne, 2007). Black arrow: conserved A–A misspair of domain III. The conserved apical “8” structure is shown with a dashed box.

stem-loop structures (SL1–3) with unknown functions could be predicted using covariance analysis (Fig. 3A). The 3'UTR of Pf-CHK1/SiV also contain the conserved “barbell-like” motif (data not shown) similar to those found in siciniviruses and several other picornaviruses (Bullman et al., 2014; Boros et al., 2014c). The Pf-CHK1/SiV was clustered together with the other siciniviruses in the 3D<sup>pol</sup> and VP1 phylogenetic trees where the Irish prototype strain of SiV-A1 (UCC001) was the closest relative (Figs. 4 and 5A).

#### Genome analysis of the Gallivirus-related picornavirus

The gallivirus-related metagenomic reads could be aligned to the 8432-nt-long genome of chicken gallivirus 1 strain 518C (ChGV-1, KF979337) covering nearly the full length of the genome from nt position 491–8136 (Fig. 2) with variable depth of coverage ranging from 1 to 20. Using specific primer pairs based upon the gallivirus-related metagenomic sequences the 8506-



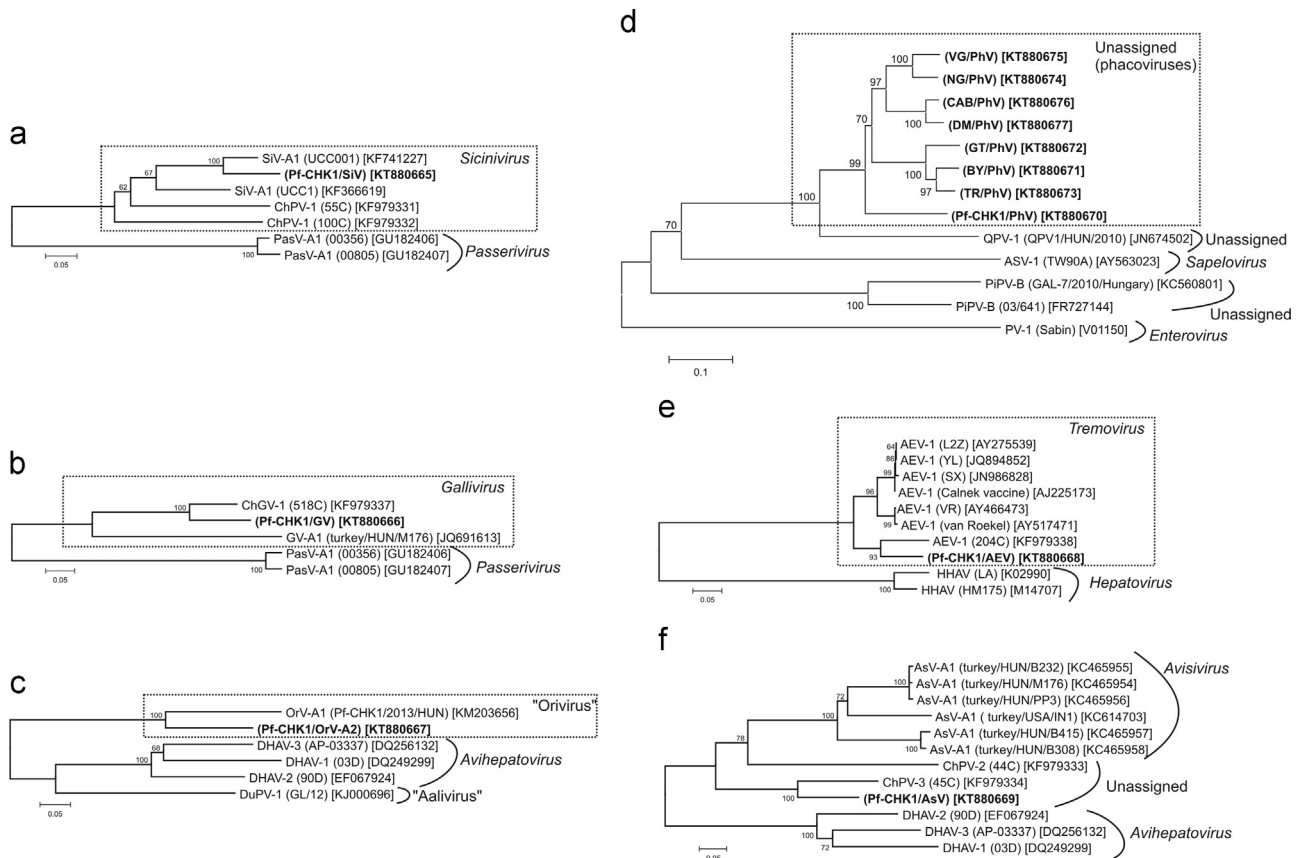
**Fig. 4.** Phylogenetic positions of the study viruses (in **bold** and indicated with black arrows). The Neighbor-Joining tree is constructed from the 3D polymerase amino acid sequences of the representative members of family Picornaviridae. Positions of the previously identified five avian picornavirus phylogenetic clusters (Boros et al., 2014c) are highlighted with gray background. Some of the picornavirus genera are shown with italics to help the orientation.

nt-long complete genome of Pf-CHK1/GV (KT880666) was determined, which shows an overall 88% nt identity to ChGV-1 strain 518C, the closest match using BLASTn. The two viruses share the same genome layout of L-3-3-4, similar predicted cleavage sites, and identical conserved aa motifs of 2A<sup>Hbox</sup>/NC, 2C<sup>Hel</sup>, 3C<sup>Pro</sup> and 3D<sup>PoI</sup> (Fig. 2, Table 1). The transcription of Pf-CHK1/GV was predicted to start from the first in-frame AUG codon found in optimal Kozak context (gagAaa<sub>770</sub>TGG, conserved nts in uppercase, start codon was underlined), although the predicted start codon of ChGV-1 strain 518C, which was 49 nt upstream (ttgctgA<sub>722</sub>TGt), was also identifiable in the study strain but not in Kozak context. The 769-nt-long 5' UTR of Pf-CHK1/GV show significant sequence identity (Table 1) to the 5' UTR ChGV-1 strain 518C and is predicted to contain the same type-II-like IRES (data not shown) described previously by Lau et al. (2014). The 3'UTR of Pf-CHK1/GV also contains the conserved "barbell-like" structure which is a characteristic genome feature of galliviruses and could also be found in the 3'UTR of ChGV-1 (data not shown). The Pf-CHK1/GV was clustered together with the other galliviruses in the 3D<sup>PoI</sup> and VP1 phylogenetic trees where the ChGV-1 strain chicken/518C/Hong Kong/2010 was the closest relative (Figs. 4 and 5B).

#### Genome analysis of the "Orivirus"-related picornavirus

The majority of the "Orivirus"-related reads belonged to the prototype strain chicken/Pf-CHK1/2013/HUN (KM203656) of Orivirus A1, which was originally identified from the same study

sample Pf-CHK1 (Boros et al., 2014a). However, there were two, a 204-, and a 201-nt-long orivirus-related contigs showing only 75 and 78% sequence identities to the corresponding VP3 and 3B regions of prototype strain of Orivirus A1 (Fig. 2), suggesting the presence of a different, but Orivirus A1-related virus in the same sample. Using primers specific to these two contigs, the complete genome of Pf-CHK1/OrV-A2 (KT880667) was determined. The 7018-nt-long genome of Pf-CHK1/OrV-A2 showed overall 73% nucleotide (nt) identity to Orivirus A1 strain chicken/Pf-CHK1/2013/HUN (KM203656), the closest match using BLASTn search. The two viruses share the same genome layout of 3-3-4, similar predicted cleavage sites and similar conserved aa motifs of 2C<sup>Hel</sup>, 3C<sup>Pro</sup> and 3D<sup>PoI</sup> (Fig. 2, Table 1). In spite of the relatively low (69%) sequence identity between their 5'UTRs, the Pf-CHK1/OrV-A2 have the same predicted type-II-like IRES as OrV-A1, where all of the characteristic motifs of the binding sites of PTB, translation initiation factor eIF4G, and conserved sequence motifs of GNRA, RAAA were identifiable (Duke et al., 1992; Belsham and Jackson, 2000) (Fig. 3B). The compact 3'UTR structure of Pf-CHK1/OrV-A2 with the three tandem repeated sequence motifs similar to those of "Unit A"s originally identified among megriviruses and a pseudoknot also similar to the 3'UTR organization of OrV-A1 (Fig. 6A and C) (Boros et al., 2014a, 2014c). Based on the similar genomic properties and their considerable sequence identity the close relationship of Pf-CHK1/OrV-A2 and OrV-A1 was supported by the close phylogenetic position in the 3D<sup>PoI</sup> phylogenetic tree (Fig. 4). The genetic distance between their VP1 and 2A regions



**Fig. 5.** Phylogenetic relationship of Pf-CHK1/SiV (a), Pf-CHK1/GV (b), Pf-CHK1/OrV-A2 (c), Pf-CHK1/PhV and related viruses from the USA designated as VG/PhV, NG/PhV, CAB/PhV, DM/PhV, GT/PhV, BY/PhV and TR/PhV (d), Pf-CHK1/AEV (e), Pf-CHK1/AsV (f) and their closest relatives (strain names in brackets, accession numbers in square brackets) based on Neighbor-Joining trees of complete VP1 nucleotide sequences. Abbreviations: SiV-A1: Sincivirus A1, ChGV-1: chicken gallivirus 1, GV-A1: Gallivirus A1, PasV-A1: Passerivirus A1, OrV-A1: Orivirus A1, DHAV: Duck hepatitis A virus, DuPV: duck picornavirus, QPV-1: quail picornavirus 1, PiPV-B: pigeon picornavirus B, ASV-1: Avian sapelovirus 1, AEV-1: avian encephalomyelitis virus 1, HHAV: human hepatitis A virus, AsV-A1: Avisivirus A1, ChPV: chicken picornavirus.

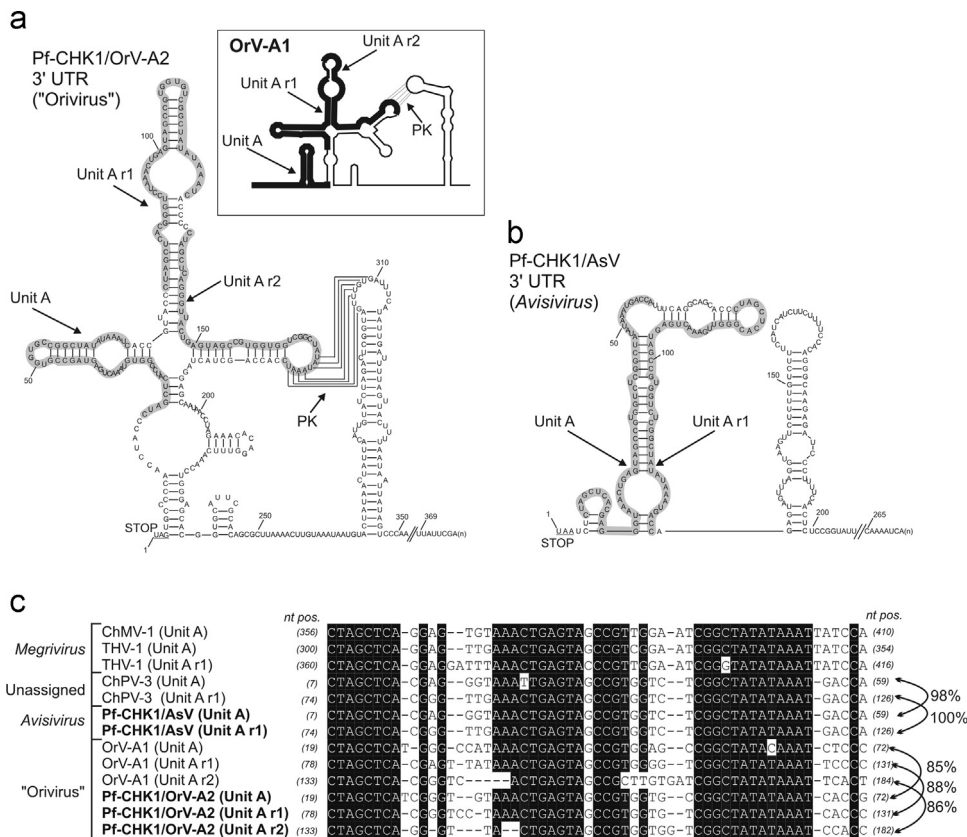
indicates that the Pf-CHK1/OrV-A2 and OrV-A1 are separate viruses (Figs. 5C and 7, Table 1).

*Genome analysis of the quail picornavirus 1-related picornavirus (phacovirus)*

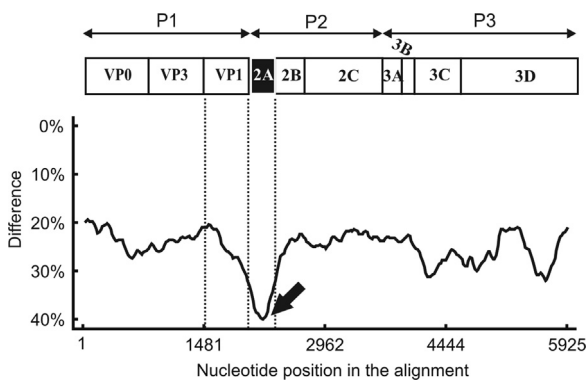
The 8204-nt-long complete genome of Pf-CHK1/PhV (KT880670) shows an overall 65% nt (73% aa) and 73%, 79% and 79% aa identity to the P1, P2 and P3 genome regions of the currently unassigned quail picornavirus 1 (QPV-1) strain QPV1/HUN/2010 (JN674502), the most similar sequence found in GenBank. The Pf-CHK1/PhV show 43%, 40% and 46% aa identity to the avian sapelovirus 1 (AY563023) of genus *Sapelovirus*. The Pf-CHK1/PhV shares the same genome layout of L-4-3-4 and identical aa motifs of 2C<sup>Hel</sup>, 3C<sup>Pro</sup> and 3D<sup>Pol</sup> as QPV-1 and similar predicted proteolytic cleavage sites with the exception of VP2↓VP3 where an atypical AAASLN<sub>713</sub>↓GV is found instead of SAAALE<sub>713</sub>↓GV (identical aas are underlined) and VP1↓2A where the predicted cleavage site of KAGIMQ<sub>1252</sub>↓GP is 5 aa downstream of the VP1↓2A junction (aa pos. 1247) of QPV-1, which suggests the presence of a small, only 8-aa-long 2A (Fig. 2, Table 1). The N-terminal part of VP4 contains a potential myristoylation motif of GxxxS (G<sub>390</sub>qitS where the lowercase letters are non-conserved amino acids) (Chow et al., 1987). The conserved GxCG motif of chymotrypsin-like proteases was not identifiable in the Leader of Pf-CHK1/PhV but contains three regions where four cysteine (Cys) residues are present in a row. Furthermore a 31-aa-long repeated aa motif of PCYGRKTQCIGTNGLHWQCEGHEGMDECCCC (identical aas

are underlined) was found at the C-terminal part at aa position 299-329 and 352-384. Beside the relatively low (53%) sequence identity the Cys-rich islands as well as the repeated motifs at the C-terminal end terminated with four Cys were also identified in the Leader of QPV-1 (Pankovics et al., 2012). The 506-nt-long 5'UTR of Pf-CHK1/PhV is 12-nt-longer than the 5'UTR of QPV-1 and share only 52% nt identity. Beside the relatively low identity the Pf-CHK1/PhV shares a structurally similar *Hepacivirus/Pestivirus*-like type-IV IRES as QPV-1 with all of structurally conserved domains and motifs identified in several type-IV IRESes (Hellen and de Breyne, 2007; Pankovics et al., 2012) (Fig. 3C). Furthermore the extension of domain III with the highly conserved apical “8” structure, which is a characteristic feature of QPV-1 and several other avian picornaviruses with type-IV IRES is also present in the IRES of Pf-CHK1/PhV (Pankovics et al., 2012; Boros et al., 2014c) (Fig. 3C).

For the detection of Pf-CHK1/PhV-like viruses in other chicken samples collected from different geographical region screening RT-PCR reactions with generic VP3/2B primer pair were made in eight pooled intestinal samples of “sentinel” broiler chickens collected from different poultry flocks in the USA (Day et al., 2015). These samples were chosen for RT-PCR screening based on the presence of Pf-CHK1/PhV-related reads in the viral metagenomic records of the pooled intestinal samples, which was found in the SRA database of NCBI. Seven from the eight samples collected from chickens of separate chicken flocks in the USA were RT-PCR positive for Pf-CHK1/PhV with the exception of SPF control group (“MSPP”). The USA strains grouped together phylogenetically (Fig. 5D).



**Fig. 6.** Predicted secondary RNA structures of the 3'UTRs of Orivirus PF-CHK1/OrV-A2 (a) and Avisivirus PF-CHK1/AsV (b) and the nt alignment of 3'UTR regions containing the "Unit A" sequences (c). The locations of repeated Unit A motifs (Unit A, Unit A r1, Unit A r2) are depicted with gray highlights. The line drawing of the predicted 3'UTR structure of OrV-A1 is shown in frame for comparison (Boros et al., 2014a). PK: pseudoknot. The nts with black background in the alignment are highly conserved. Percentage values indicate the nt identities of the corresponding Unit A sequences indicated with black arrows. The study viruses in the alignment are labeled in **bold**. The nt positions are counted from the stop codon of the given picornavirus. ChMV-1: chicken megrivirus 1 (KF961186), THV-1: turkey hepatitis virus 1 (KF961188), OrV-A1: Orivirus A1 (KM203656), ChPV-3: chicken picornavirus 3 (KF979334).



**Fig. 7.** Distance plot analysis is based on the comparison of the complete coding genome region of Pf-CHK1/OrV-A2 and Orivirus A1 (KM203656). The genome map of Pf-CHK1/OrV-A2 is drawn to scale. Dashed lines show the borders of VP1 and 2A. Black arrow indicates the location of the most diverse genome region in the middle of 2A.

#### Genome analysis of the Tremovirus-related picornavirus

The 7031-nt-long complete genome of Pf-CHK1/AEV (KT880668) shows 85% nt identity to the avian encephalomyelitis virus 1 (AEV-1) strain 204C (KF979338) of genus *Tremovirus*, the most similar sequence found in GenBank. The two viruses share the same genome layout of 4-3-4 identical conserved aa motifs of 2A<sup>Hbox/NC</sup>, 2C<sup>Hel</sup>, 3C<sup>Pro</sup> and 3D<sup>Pol</sup> and similar predicted cleavage sites (Fig. 2, Table 1). The 488-nt-long 5' UTR is predicted to contain a type-IV IRES (data not

shown) as found among AEVs (Bakhshesh et al., 2008). In the phylogenetic tree of immunodominant VP1 the Pf-CHK1/AEV was separated from the historic AEV vaccine strains of Calnek and Van Roekel and grouped together with a novel field strain 204C identified in China (Fig. 5E).

#### Genome analysis of the Avisivirus-related picornavirus

The 7270-nt-long complete genome of Pf-CHK1/AsV (KT880669) shows 83% nt (96% aa) identity to the currently unassigned chicken picornavirus 3 (ChPV-3) strain 45C (KF979334), the closest match found in GenBank using BLASTn search. The Pf-CHK1/AsV shows 43%, 45% and 54% aa identity to the P1, P2 and P3 genome regions of Avisivirus A1 strain USA-IN1 (KC614703) of genus *Avisivirus*, respectively. The Pf-CHK1/AsV and ChPV-3 share the similar predicted cleavage sites with the exception of VP1↓2A site where an atypical cleavage site of KLPCPR↓YT was found instead of KLPCPQ↓YT (identical aas were underlined) predicted in ChPV-3, which could question the active nature of this cleavage site, although there was no potential 3C<sup>Pro</sup> cleavage motif identifiable in the given region. The Pf-CHK1/AsV and ChPV-3 share the same genome layout of 3-5-4, with potentially three 2A and identical conserved aa motifs of 2A1-3, 2C<sup>Hel</sup>, 3C<sup>Pro</sup> and 3D<sup>Pol</sup> (Boros et al., 2013; Ng et al., 2013) (Fig. 2, Table 1). The three 2A regions of Pf-CHK1/AsV contain the Aphthovirus-2A-like NPGP-type/P-loop NTPase-type/Hbox-NC-type peptides, which 2A-triad is predominant among the avian picornaviruses of genera *Avihepatovirus*, *Avisivirus*, as well as ChPV-2 and 3 (Lau et al., 2014; Boros et al., 2014c) (Fig. 2). The 561-nt-long 5' UTR of Pf-CHK1/AsV is 103-nt-longer than the 5' UTR of ChPV-3 strain 45C



and shares high (89%) sequence identity as well as the same predicted type-II-like IRES (data not shown) similar to the avisiviruses (Boros et al., 2013; Lau et al., 2014). The 268-nt-long 3'UTR shows high sequence identity to the corresponding genome region of ChPV-3 and both contain two tandem repeated sequence motifs similar to the “Unit A” sequences identified in the 3'UTR of chicken and turkey megriviruses (genus *Megrivirus*) and Orivirus A1 (Boros et al., 2014c) (Fig. 6B and C). The Unit A sequences were predicted to fold into a compact secondary RNA structure, which resemble the complex 3'UTR structure of oriviruses with three repeated “Unit A” motifs (Fig. 6B). The similar genomic properties and the high sequence identity as well as the clustering of Pf-CHK1/AsV to the ChPV-3 in the 3D<sup>pol</sup> and VP1 phylogenetic trees suggest the close relationship of these two viruses (Figs. 4 and 5F).

## Discussion

In this study, the picornavirus diversity of a single cloacal sample collected from a diarrheic chicken from a “back yard” family farm a few days before the onset of the diarrhea of the sampled animal was examined using viral metagenomics followed by conventional RT-PCR techniques. Based on the *in silico* results of metagenomic data and the full length genome analyses 8 different picornaviruses were identifiable. However, the megrivirus-related sequences could not be confirmed by RT-PCR likely indicating a very low viral load and the presence of a divergent Orivirus A1-related virus (Pf-CHK1/OrV-A2) could not be readily distinguished by metagenomic analysis. The discrepancies in the number of identifiable picornaviruses using the viral metagenomic and RT-PCR techniques suggest that using both methods enhances insight into the viral diversity. One of the eight picornaviruses (Orivirus A1 strain chicken/Pf-CHK1/2013/HUN – KM203656) was previously described (Boros et al., 2014a) from the study sample, therefore a total of further six different picornavirus genomes were completely determined and analyzed in this study. Based on the results of sequence comparisons, genomic and phylogenetic analyses, Pf-CHK1/SiV, Pf-CHK1/GV, Pf-CHK1/OrV-A2, Pf-CHK1/AEV most likely belong to the genera *Sicivirus*, *Gallivirus*, “Orivirus” and *Tremovirus* respectively. The relatively low sequence identity and the phylogenetic separation in the VP1 and 2A genome regions between OrV-A1 and Pf-CHK1/OrV-A2 could suggest that Pf-CHK1/OrV-A2 belongs to a different genotype (Orivirus A2) of the candidate species “Orivirus A”. The phylogenetic separation of Pf-CHK1/AEV from the historic AEV vaccine strains of Calnek and Van Roekel (Tannock and Shafren, 1994) suggests that there could be several different wild types of AEV strains with unknown pathogenic potential circulating among birds with sequence differences from the vaccine strains.

The Pf-CHK1/PhV showed the highest sequence identity and similar genomic features to a currently unassigned quail picornavirus 1 (Pankovics et al., 2012). Based on the phylogenetic clustering and moderate aa sequence identity values at the viral polyprotein P1, 2C, 3C and 3D regions; QPV-1 and Pf-CHK1/PhV probably belong to the same species. Therefore the proposed virus name (phacovirus) of Pf-CHK1/PhV refers to the family name (*Phasianidae*) of the common quail and chicken from which the QPV-1 (Pankovics et al., 2012) and Pf-CHK1/PhV were identified. More than 40% aa identity (43% and 43%) at the P1 genome region between the QPV-1 and ASV-1 of genus *Sapelovirus* (Pankovics et al., 2012) as well as between the Pf-CHK1/PhV and ASV-1 do not fulfill the currently accepted genus demarcation criteria where the aa sequence identity between the P1, P2 and P3 of viruses in different genera should be less than 40%, 40% and 50% ([http://www.picornastudygroup.com/definitions/genus\\_definition.htm](http://www.picornastudygroup.com/definitions/genus_definition.htm)). However, the phylogenetic separation of the two viruses from the

sapeloviruses, certain genomic features like the presence of apical “8” structure in the type-IV IRES-es, the long Leader with unidentifiable chymotrypsin-like protease function, which is presented in the Leader of ASV-1 and in the 2A of other sapeloviruses (Tseng and Tsai, 2007), and the low sequence identity at the P2 and P3 genome regions might support the taxonomic separation of QPV-1 and chicken phacovirus 1 strain Pf-CHK1/PhV to a novel picornavirus genus or at least to a novel species in the genus *Sapelovirus*. For the support of chickens as the natural hosts of phacovirus and to gain insight into the genotype-, and geographic distribution of chicken phacovirus 1-like viruses, RT-PCR screening using samples collected from different poultry flocks in the USA was performed. The phylogenetic analysis of VP1 genome regions of the USA strains revealed the presence of multiple, potentially different genotypes and could suggest the worldwide distribution of chicken phacoviruses among chicken flocks.

The highest sequence identity, similar genomic features, and closest phylogenetic relationship of Pf-CHK1/AsV to the currently unassigned chicken picornavirus 3 suggest that these two viruses might belong to a same species. Based on the currently accepted criteria of sequence-based genus demarcation and the similar genomic features of Pf-CHK1/AsV, ChPV-3 and the members of genus *Avisivirus*, which includes the same IRES-type, same 2A types, suggest that Pf-CHK1/AsV together with ChPV-3 belong to a novel species of genus *Avisivirus* rather than a novel genus.

The complete genome analysis of the study viruses revealed the presence of sequence motifs, currently present only among chicken picornaviruses, like (i) the repeated “Unit A” sequences located at the 3'UTR of Avisivirus Pf-CHK1/AsV, Orivirus Pf-CHK1/OrV-A2 which is also present at the 3'UTR's of chicken megriviruses, Orivirus A1 and chicken picornavirus 2 and 3; (ii) and the unusually long, highly structured spacer in the 5'UTR of siciniviruses, which suggest an essential role of these motifs in the replication cycle of this viruses in chicken hosts (Boros et al., 2014a, 2014b, 2014c; Bullman et al., 2014). The unexpectedly low sequence identity at the 2A genome region of OrV-A1 and Pf-CHK1/OrV-A2 could suggest the accelerated pace of evolutionary changes affecting this genome region and supports the modular evolution of picornavirus genomes, although this level of 2A difference is not unprecedented among picornaviruses: the aa sequence difference of the 2A of HRV-45 and HRV-80 of *Rhinovirus A* is greater than 45% (Laine et al., 2005).

There is an increasing number of studies analyzing the picornavirus diversity in poultry birds, including chickens (Farkas et al., 2012; Lau et al., 2014; Day et al., 2015; Bullman et al., 2014). These studies which include samples collected from different birds focused on the examination of overall picornavirus diversity of poultry, rather than the co-infecting picornaviruses presented in a single specimen. The results of our analysis revealed that all of the currently known chicken picornaviruses including a novel one (chicken phacovirus 1 strain Pf-CHK1/PhV) were simultaneously shed by a single bird. These phylogenetically distant viruses belong to four currently known avian picornavirus clusters: the passerivirus-, avihepatovirus-, tremovirus- and the first chicken picornavirus (chicken phacovirus 1 strain Pf-CHK1/PhV), which belongs to the avian sapelovirus cluster (Boros et al., 2014c).

The insufficient cleaning of the concrete floor of the back yard farm where the sampled animal was kept may contribute to the presence of high picornavirus diversity in this bird, which was recently introduced into that environment. Co-infection with different picornaviruses could facilitate the picornavirus evolution and enable the emergence of recombinant picornaviruses, similar to the possible recombinant chicken megriviruses and chicken picornavirus 5 (Boros et al., 2014b; Lau et al., 2014), although little is known about the host cell spectra and the true recombination

capabilities of the known avian picornaviruses (Tannock and Shafren, 1994; Honkavuori et al., 2011).

Multiple picornaviruses together with several other eukaryotic viruses including picobirna-, calici-, astro- and parvovirus-related viruses were identifiable from the studied diarrheic chicken, although any contribution of one or more (picorna)viruses to the development of diarrhea without the analysis of possible co-infecting bacteria, parasites and without the known genetic background of the host should be treated with caution. All of the identified picornaviruses were also found in apparently healthy birds (Tannock and Shafren, 1994; Bullman et al., 2014; Lau et al., 2014; Day et al., 2015). Nevertheless the presence of such a large number of co-infecting (picorna)viruses most likely could contribute to at least the severity of the diarrhea.

## Materials and methods

### Background data of the study birds

The single cloacal sample (sample ID: Pf-CHK1) from which all picornavirus complete genomes were determined was collected from a 4-week-old diarrheic broiler chicken (*Gallus gallus domesticus*). The sampled chicken was held with 10 other, apparently healthy chickens in a family “back yard” farm in Orosháza, Hungary, in April 2013. The chickens were introduced into the farm a few days before the onset of the diarrhea. The poultry enclosure had an open top and a concrete floor and it was regularly cleaned only with water wash. The poultry were kept in close proximity to a cattle-grid and swinery.

For the epidemiological investigation of one study virus, chicken phacovirus (Pf-CHK1/PhV), eight ( $N=8$ ) pooled intestinal samples were used. The intestinal samples were collected from “sentinel” birds which were placed in contact for five days with six commercial and one privately owned “back-yard” broiler chicken flocks in the USA with past problems of respiratory and enteric maladies (Day et al., 2015). Each of the pooled intestinal samples contained intestinal homogenates collected from five two-week-old broiler chickens. The sentinel birds were kept in commercial specific-pathogen free (SPF) conditions before the placement. The pooled samples from sentinel birds were labeled as GT, DM, TR, CAB, BY, VG, NG. The intestinal content from SPF control group was labeled as MSPF (Day et al., 2015).

### Sample preparation, viral metagenomics and in silico NGS data analysis

The Pf-CHK1 cloacal sample was subjected to a viral metagenomic analysis using random RT-PCR amplification of viral-particle protected nucleic acids as previously described (Phan et al., 2013). Briefly, viral cDNA library was constructed by ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicentre) and sequenced using the MiSeq Illumina platform. The sequence reads were assembled to contigs and sequences longer than 100-bp were compared to the GenBank protein database using BLASTx. The Expected value ( $E$ ) cut-off rate was set up  $10^{-5}$  for the categorization of the reads.

The detailed sample preparation and enteric virome analysis of the 8 pooled intestinal samples were previously published (Day et al., 2015). The viral metagenomic data of the pooled intestinal samples are available in the NCBI Sequence Read Archive (SRA) under the accession numbers of SRX380376, SRX380436–SRX380441 (Day et al., 2015).

### Genome acquisition and analysis of picornavirus genomes

The sequence reads related to different picornaviruses served as targets for sequence specific oligonucleotide primer design. Sequence specific primers were used to amplify the overlapping genome regions by different RT-PCR and 5'/3' rapid amplification of cDNA ends (RACE) reactions (Boros et al., 2011, 2012b). The PCR products were directly sequenced from both directions using dye-terminator sequencing method and run on an automated sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Stafford, Texas, USA) in Hungary. The sequences of the RT-PCR products were used for the verification of the reads and for the analysis of the unknown parts of the genome not covered by metagenomic sequences, including the complete 5' and 3' ends. The oligonucleotide primer pairs used in this study were available on request.

A generic VP3/2B primer pair (PhaV-VP3-Fgen: 5' ATA CWG CWA TTT CTG TGT GTG G 3'; PhaV-2B-Rgen: 5' TCA GAA CCC ATY TGA TTT GCA 3') was designed to determine the complete VP1 and 2A genome regions of chicken phacovirus 1 strain Pf-CHK1/PhV-related viruses from the pooled intestinal samples collected from sentinel birds in the USA. The experimental conditions of screening and sequencing reactions were previously described (Day and Zsak, 2015) and used with minor modifications: RT incubation of 50 °C for 30 min followed by 95 °C for 15 min; PCR cycling consisted of 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, followed by a final incubation at 72 °C for 10 min. The RT-PCR amplicons of PhaV were cloned into the pMiniT vector (New England Biolabs) and sequenced in both directions with Big Dye Terminator v1.1 cycle sequencing kit (ThermoFisher Scientific) and run on the Applied Biosystems 3730 DNA Analyzer at the USDA/ARS Southeast Poultry Research Laboratory (SEPR) sequencing facility.

### Sequence and phylogenetic analyses

The picornavirus sequences were aligned using CLUSTAL\_X software v. 2.0.3 (Thompson et al., 1997). The pairwise nucleotide (nt) and amino acid (aa) identity calculations of the aligned sequences were performed using GeneDoc v. 2.7 (Nicholas and Nicholas, 1997). Phylogenetic trees were constructed using MEGA software ver. 6.06 (Tamura et al., 2013). The Neighbor-Joining method with the Jones–Taylor–Thornton matrix-based model was used to construct the 3D tree of amino acid sequences, while the Jukes–Cantor model was used to create the phylogenetic tree of VP1 nucleotide sequences. Bootstrap values were set up to 1000 replicates and only likelihood percentages of more than 50% were indicated in phylogenetic trees.

The possible proteolytic cleavage sites were predicted primarily based on the aa alignments of the closest relatives and verified by the NetPicoRNA web tool (Blom et al., 1996).

Distance plot analysis was performed with the RDP software ver. 4.16 using the Similarities model with a window size of 300 nt and a step size of 20 nt (Martin et al., 2015). The secondary structure of the 5' and 3' UTRs were predicted using the Mfold program Zucker (2003) and a two-dimensional model was drawn using the CorelDraw Graphics Suite v. 12.

The consensus RNA secondary structures based on covariance analysis were predicted using the RNAalifold web server (Lorenz et al., 2011).

The complete genome sequences of Sicinivirus Pf-CHK1/SiV, Gallivirus Pf-CHK1/GV, Orivirus Pf-CHK1/OrV-A2, chicken phacovirus 1 Pf-CHK1/PhV, Avian encephalomyelitis virus Pf-CHK1/AEV and Avisivirus Pf-CHK1/AsV and partial VP3-2A sequences of chicken phacovirus 1 strain Pf-CHK1/PhV-related viruses were

submitted to GenBank and assigned the accession numbers of KT880665–KT880677.

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