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Virus isolate from carp: genetic characterization reveals a novel picornavirus with two aphthovirus 2A-like sequences

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Picornaviruses have been isolated from a variety of hosts, mainly mammals and birds. Here, we describe the sequence analysis of carp picornavirus 1 (CPV-1) F37/06 that was isolated from an organ pool (heart, brain, liver) of a common carp (*Cyprinus carpio*). This carp perished after an accidental discharge of liquid manure into a fish pond and presented without obvious clinical symptoms. Experimental intraperitoneal infection of young carp with CPV-1 revealed no clinical signs, but the virus was re-isolated from various organs. Sequence analysis of almost the complete genome (7632 nt excluding the poly-A tract) revealed a novel picornavirus clade. In phylogenetic trees, the polymerase sequence clusters with parechoviruses, duck hepatitis A virus, eel picornavirus and aquamavirus A. The ORF includes 6807 nt and encodes a polyprotein of 2269 amino acids. CPV-1 has a genome layout like that of picornaviruses except for the presence of two aphthovirus 2A-like NPGP sequence motifs: VPg+5′UTR[1AB-1C-1D-2A1^{npgp}/2A2^{npgp}-2B-2C^{ATPase}/3A-3B^{VPg}-3C^{pro}-3D^{pol}]3′UTR-poly-A. 2A1^{npgp} and 2A2^{npgp} are separated by 133 amino acids. The proteins 2A2^{npgp}, 2B, 3A and 3B^{VPg} have no significant similarity to the corresponding proteins of other picornaviruses. Amino acid identities of the orthologous proteins P1, 2C, 3C^{pro} and 3D^{pol} range from 16.4 to 40.8 % in the eel picornavirus/CPV-1 comparison. 3D^{pol} shows the closest similarity to eel picornavirus, with an amino acid identity of 40.8 %, followed by human parechovirus (36.5 %), duck hepatitis A virus (32.7 %) and swine pasivirus (29.3 %). Both the unique genome organization and low sequence similarity support the assignment of CPV-1 to a novel picornavirus species within a novel genus.

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INTRODUCTION

All members of the family *Picornaviridae* are small, non-enveloped, icosahedral viruses (Knowles *et al.*, 2012). Their positive-strand RNA genomes range in size from 7000 to 9100 nt. A virus-encoded peptide (3B^{VPg}) is linked covalently to the 5′-end of the RNA. The structure of the 5′-untranslated region (UTR) of RNA is an important signal for the initiation of positive-strand RNA synthesis and cap-independent translation. Most picornavirus genomes have one ORF encoding a polyprotein that is processed co- and post-translationally into 10–12 mature polypeptides. The canine *Dicipivirus*, however, is the only known picornavirus that has two ORFs and two internal ribosome entry sites (IRES) that drive independent translation of the capsid precursor and the nonstructural protein precursor (Woo *et al.*, 2012). The picornavirus 3′-UTR has also RNA

The GenBank/EMBL/DDBJ accession number for the carp picornavirus genome sequence is KF306267.

structures necessary for RNA synthesis; the viral RNA is polyadenylated (Knowles *et al.*, 2012).

The family *Picornaviridae* is currently comprised of 37 species grouped in 17 genera (*Aphthovirus*, *Aquamavirus*, *Avihepatovirus*, *Cardiovirus*, *Cosavirus*, *Dicipivirus*, *Enterovirus*, *Erbovirus*, *Hepatovirus*, *Kobuvirus*, *Megrivirus*, *Parechovirus*, *Salivirus*, *Sapelovirus*, *Senecavirus*, *Teschovirus*, *Tremovirus*; http://ictvonline.org/; Adams *et al.*, 2013). In addition, the genomes of numerous yet unclassified picornavirus candidates have been sequenced, most of which were without prior virus isolation (an updated list may be found at http://www.picornaviridae.com/). The gene regions coding for the capsid proteins 1AB, 1C, 1D and the nonstructural proteins 2C, $3C^{pro}$ and $3D^{pol}$ are common to all known picornaviruses whereas the sequences of other nonstructural proteins (leader protein, 2A, 2B, 3A, $3B^{VPg}$) are not conserved between members of different picornavirus genera and may be unique for some picornavirus genera (Knowles *et al.*, 2012).

Recently, we completed the sequence of eel picornavirus 1 (EPV-1), which was isolated from the common eel (Fichtner *et al.*, 2013). Here, we describe another fish picornavirus, which further substantiates the importance of limnic ecosystems for picornavirus ecology. Carp picornavirus 1 (CPV-1) was isolated from a common carp (*Cyprinus carpio*). The viral genome contains an ORF of 6807 nt [2269 amino acids (aa)]. The predicted polyprotein displays the typical organization of a picornavirus. According to the Picornavirus Study Group criteria (www.picornastudygroup.com), the unique genome organization of CPV-1 and low similarity between the conserved P1, 2C and 3CD precursor proteins of CPV-1 and the orthologous proteins of the other picornaviruses suggest a novel species in the family *Picornaviridae*.

RESULTS

Virus isolation and experimental infection of carp

An accidental discharge of liquid manure into a fish-rearing pond in Lower Saxony, Germany, caused a fish kill with high losses (almost 100 %) of carp (*Cyprinus carpio*) and tench (*Tinca tinca*) in 2006. Dead fish showed no external clinical signs. Several dead carp specimens were sent to the National Reference Laboratory for Fish Diseases at the Friedrich Loeffler Institute and investigated for the presence of infectious agents. Using fat head minnow (FHM) cells, a virus was recovered from an organ pool of heart, brain and liver samples. Carp picornavirus 1 (CPV-1) isolate F37/06 induced a cytopathic effect 5–7 days post-infection (p.i.) in FHM cells. Chloroform treatment did not reduce the infectivity of the virus, and treatment with 5-iodo-2-deoxyuridin did not impair virus replication, indicating a chloroform-resistant RNA virus. This virus was characterized as picorna-like by electron microscopy (Fig. 1a).

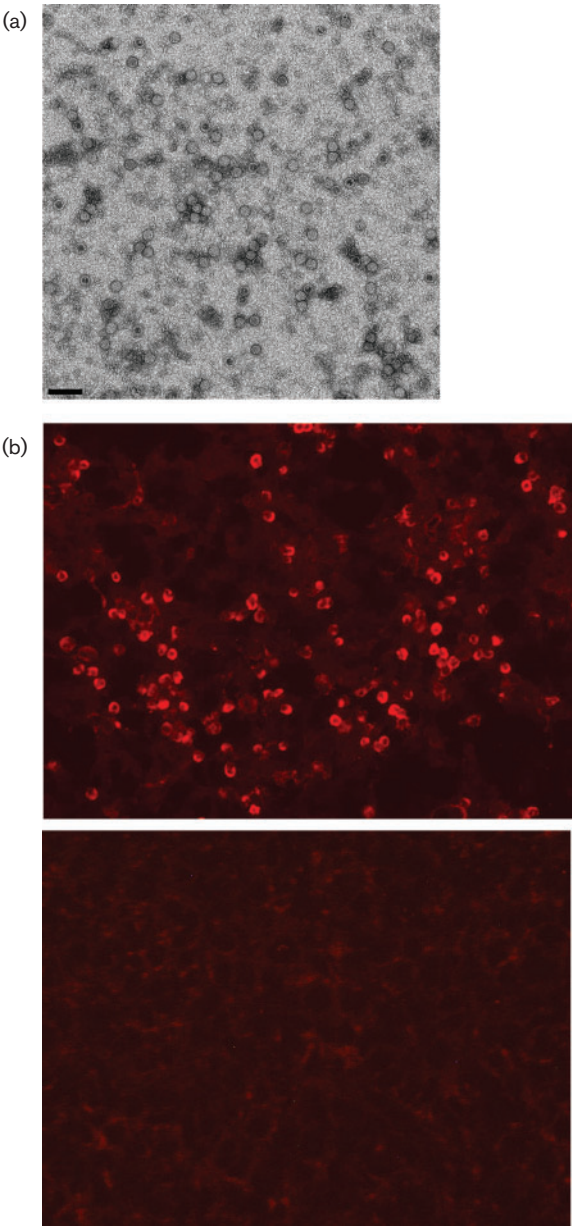

Fig. 1. (a) Electron micrograph of CPV-1. The bar represents 100 nm. (b) Detection of CPV-1 by indirect immunofluorescence assay using anti-5C9 monoclonal antibody and Cy3-conjugated goat anti-mouse IgG in infected (upper panel) and uninfected (lower panel) FHM cells.

In order to investigate the pathogenicity of CPV-1, groups of five specific pathogen-free carp (approx. 25 g) were infected by either intraperitoneal (i.p.) injection of 10^6 50 % tissue culture infective dose ($TCID_{50}$) CPV-1 F37/06 or by exposure of fish to virus-containing aquarium water (5 l with 10^6 $TCID_{50}$ virus ml^{-1}) for 1 h. Subsequently, the carp were kept in 400 l aquaria for 21 days at a water temperature of 20 °C. Two control groups of five carp each were treated and kept under the same conditions; the same

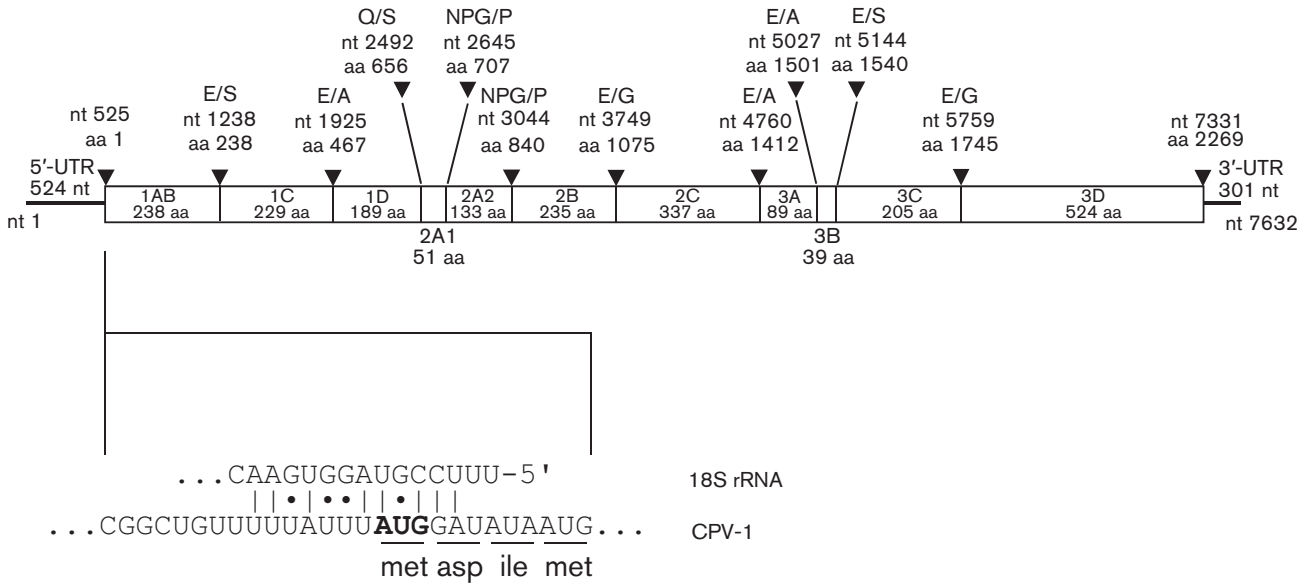

Fig. 2. Schematic depiction of the CPV-1 genome. The ORF is indicated by a box. Positions of putative nt and aa cleavage sites and the lengths of the deduced proteins are shown. The Kozak consensus sequence at the start codon is presented in the lower panel. It allows formation of up to 12 bp at the 5′-end of 18S rRNA.

volume of analogous cell culture medium without virus was administered or added to the aquarium water. Carp that were i.p.-infected with CPV-1 showed no clinical signs, but virus could be re-isolated (2 days p.i. from brain, 10 days p.i. from liver and heart, 21 days p.i. from brain and heart) and identified by indirect immunofluorescence using 5C9, a monoclonal antibody against CPV-1 and Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) (Fig. 1b).

Genus	Species	1AB	1C	1D
unassigned	CPV-1	MDIM...230aa...NVLE	SPPG...221aa...VGFE	AGSD...181aa...YFLQ
unassigned	EPV-1	MATT...240aa...PRPQ	GPLR...224aa...LNFQ	MKDE...295aa...GEIL
Aquamavirus	SeAV-1	MESF...258aa...PYRE	GFVK...224aa...FTDQ	GNFE...174aa...SKLQ
Avihepatovirus	DHAV-1	MDTL...248aa...FDNQ	GKRK...229aa...TNNQ	GDSN...230aa...LEIE
Parechovirus	HPeV-1	METI...281aa...IYDN	APNG...245aa...VTFQ	NSWG...226aa...QSPY
unassigned	SPaV-1	MDVV...245aa...SDSQ	GPKR...236aa...VKFH	GWGS...282aa...GSKE
unassigned	R.a. PV-1	>219aa...KSTH	ATNF...226aa...MAVQ	ISWG...263aa...SFTQ

	2A1	2A2	2A3	2B	2C
CPV-1	SPPA... 43aa...SNPG	PWFV...125aa...QNPG		PRSQ...227aa...MFTE	GLFD...329aa...LQAE
EPV-1	TDKM... 14aa...PNPG	PHAE...132aa...DMVT		GMGK...118aa...REPQ	APPF...365aa...VMKQ
SePV-1	SGCF... 21aa...SNPG	PLYV... 92aa...VEEQ		GFMD...102aa...PTQQ	GFAS...293aa...MCRQ
DHAV-1	SDQI... 12aa...PNPG	PILV...153aa...EFVS	HLPR...116aa...TTDQ	SFPG...111aa...LEDQ	SGKA...325aa...FMNQ
HPeV-1		GQQP...139aa...GDEQ		GLSL...114aa...LSNQ	GPFK...321aa...LENQ
SPaV-1	ELQN... 9aa...QNPG	PVLM...104aa...FDSQ		GVDD...117aa...FESQ	SLAA...323aa...FISQ
R.a. PV-1	ELRR... 15aa...SNPG	PVEL.....			

	3A	3B1	3B2	3C	3D
CPV-1	AGED... 81aa...NKEE	APYD... 31aa...LTKE		SPVT...197aa...MVLE	GIVV...516aa...DRLA*
EPV-1	GAEE...138aa...MEPE	RAYD... 12aa...RMPQ		GGFN...191aa...RIAQ	GVVV...476aa...KIFY*
SePV-1	NNEL... 96aa...VVGE	GAYD... 16aa...PSEQ	SAYE... 14aa...VVEG	GPSD...194aa...NDVE	GVVV...470aa...CWPW*
DHAV-1	SKVR... 85aa...RFAQ	SIYS... 26aa...LEDQ		SGRV...173aa...VFNQ	GKVV...445aa...CMMI*
HPeV-1	TLDD...109aa...SKDE	RAYN... 12aa...PVSQ		REFK...192aa...MSDQ	GIVT...461aa...MVFD*
SPaV-1	APET... 82aa...LDSQ	RPYN... 17aa...LVSQ		GPYN...183aa...LESQ	GLVT...447aa...SMLS*
R.a. PV-1	.....				

Fig. 3. Comparison of predicted polyprotein cleavage sites. P4–P1 aa, cleavage sites (/), P1'–P4' aa and lengths of the deduced proteins are shown. Sequences used are CPV-1 and picornaviruses with the closest phylogenetic relation (in each case, one representative member). GenBank accession numbers and abbreviations are given in Table 1. The upper panel displays P1, the middle panel P2 and the lower panel P3 proteins.

No external clinical signs were observed in the carp exposed to virus in the aquarium water over the entire duration of the experiment, and virus re-isolation was not successful. In the indirect immunofluorescence assay, 5C9 showed no cross-reactivity with a picornavirus isolated from eels (data not shown).

Virus sequencing and genetic characterization

For molecular characterization, CPV-1 RNA was purified from CPV-infected FHM cells and used for library preparation. Sequencing was performed with the Illumina/Solexa methodology on a GAIIx (Illumina) instrument. Reads with lengths of 76 nt were created. Approximately 90 000 picornavirus-similar reads were used for a *de novo* assembly. A large contig of 7616 nt was obtained with missing nucleotides at the 5′- and 3′-ends. Sixteen missing nucleotides at the 3′-end were determined by conventional Sanger sequencing as described previously (Philipps *et al.*, 2012). Attempts to extend sequence information by Rapid Amplification of cDNA Ends (5′-RACE) failed. Our dataset was checked for potential contamination by mapping all reads to the nucleotide collection database nr/nt at the National Center for Biotechnology Information (NCBI) but showed no indication of microbial contaminants. The majority of non-CPV-1 reads were of fish rRNA origin.

The genome organization of CPV-1 is depicted in Fig. 2. The viral RNA comprises at least 7632 nt except for poly-A, with a 5′-UTR of at least 524 nt, an ORF of 6807 nt and an exceptionally long 3′-UTR of 301 nt. No similarity to any of the established picornavirus IRES types was detected. Neither sequences fitting the cGNRAg and cUNCGg stable tetraloop consensus sequences nor modifications of these motifs that could serve to identify possible stem–loop structures were observed. Mfold (http://mfold.rna.albany.edu/) proposed several possible structures of the 5′-UTR sequences but their significance is not clear (data not shown). The viral RNA exhibits a typical Kozak consensus sequence (Kozak, 1987) around the start codon ($A_{525}UG$) that allows binding of the 5′-end of the 18S rRNA (12); up to 12 bp are formed at this site (Fig. 2). However, a short oligopyrimidine tract supposed to bind the 3′-end of the 18S rRNA is less evident.

The deduced polyprotein precursor has three capsid proteins (1AB, 1C, 1D) and eight nonstructural proteins ($2A1^{npgp}$, $2A2^{npgp}$, 2B, 2C, 3A, $3B^{VPg}$, $3C^{pro}$, $3D^{pol}$). It lacks both an obvious leader polypeptide and a myristoylation signal found in many picornaviruses. The capsid proteins 1AB and 1C have conserved rhv domains (http://pfam.sanger.ac.uk/). The 1D protein is relatively short (189 aa versus 295 aa in the bluegill BGPV-1 picornavirus, M. Hoffman, personal communication; Fig. 3). In order to exclude an assembly artefact, the correctness of the CPV-1 1D gene region was verified by conventional Sanger sequencing (data not shown). Interestingly, the CPV-1 genome exhibits two 2A regions with an NPGP ribosome-skipping sequence motif (Luke *et al.*, 2008). Presuming a potential 3C cleavage site at $Q_{656}S$, 2A1 has a length of 51 aa. However, it is unclear whether 2A1 is cleaved off the 1D protein. 2A2 is a unique protein of 133 aa that shares no similarity to any available picornavirus sequence. Only the short 16 aa C-terminal sequence shows similarity to the known aphthovirus 2A-like polypeptides. The greatest similarity was shared with the 2A protein of the human cosavirus. The 2B protein is unique. The 2C protein has a D_{1203}PIxIHFxGxxGxGKT motif (Hel-A; refs. 5, 10) that could serve as a NTP-binding site. Conserved amino acids of the Hel-B and Hel-C motifs include Q_{1252}xVHYIDD and K_{1291}GxxYxxxVVVxTxN. The 3A and $3B^{VPg}$ proteins are also unique. The $3B^{VPg}$ N-terminus was identified by assumption of a tyrosine residue at the third position (Y_{1504}). $3B^{VPg}$ is a rather long polypeptide with 39 residues. The deduced 3C protease has an H-D-C catalytic triad with a $GxC_{1702}G$ active site and a G_{1718}xH substrate-binding pocket. It is remarkable that the P1 position of all but one $3C^{pro}$ cleavage site is occupied by a glutamic acid rather than a glutamine residue; serine, alanine and glycine residues are observed at the P1' site (Fig. 3). Conserved motifs of the 3D polymerase are K_{1914}DELR (motif I) (Koonin & Dolja, 1993), I_{1931}xxxxLxxxVxxRMYxxxxxxxxY (motif II), A_{1964}VG (motif III), D_{1990}YSxFDG (motif IV), G_{2043}GMPSG (motif V), Y_{2093}GDD (motif VI) and F_{2142}LKR (motif VII). Unique features of $3D^{pol}$ are an insertion of 9 aa at position 2075 (adjacent to the YGDD motif) and a C-terminal extension.

Table 2 presents the amino acid identities of the orthologous picornavirus proteins (1AB, 1C, 1D, 2C, $3C^{pro}$, $3D^{pol}$) of all acknowledged picornavirus genera (one representative member each) and nine tentative picornavirus species compared with CPV-1. The data reveal a low similarity of CPV-1 to the known picornaviruses. The closest relative is eel picornavirus with amino acid identities ranging from 16.4 % ($3C^{pro}$) to 40.8 % ($3D^{pol}$).

Viral RNA genomes exhibit characteristic abundances of dinucleotides. Therefore, a nucleotide composition analysis (NCA) and subsequent discriminant analysis may be useful for host inference of an RNA virus (Boros *et al.*, 2011; Kapoor *et al.*, 2010; Ng *et al.*, 2012; Shan *et al.*, 2011). In order to analyse whether fish picornaviruses match this hypothesis, a compilation of 468 nearly complete picornavirus genomes was used for an NCA comprising 445 mammalian, 21 avian and two fish viruses. As observed previously for vertebrate RNA viruses, our data demonstrate that picornaviruses exhibit a characteristic underrepresentation of CpG dinucleotides that is more distinct in genomes with low G + C content (Fig. 4a). In addition to CPV-1 and EPV-1 sequences, we also analysed a collection of 49 genomes and gene segments, respectively, of fish RNA viruses. The latter collection comprised sequences of several virus taxa (*Alphavirus*, *Aquabirnavirus*, *Rhabdovirus*, *Isavirus*, *Betanodavirus*, *Hepevirus*, and two novel yet unclassified RNA viruses from carp, i.e. a proposed seadornavirus and a picorna-like dicistronic + ssRNA virus). Fig. 4(b) shows the scores of the two canonical

Table 1. Sequences used for phylogenetic analyses

Sequence	Genus	Species and type	Strain	GenBank
1	*Aphthovirus*	Foot-and-mouth disease virus (FMDV) O1	Kaufbeuren/FRG/66	X00871
2		Bovine rhinitis A virus (BRAV) 2	H-1	JN936206
3		Bovine rhinitis B virus (BRBV) 1	EC11	EU236594
4		Equine rhinitis A virus (ERAV) 1	PERV	X96870
5	*Aquamavirus*	Aquamavirus A (SeAV-A) 1	HO.02.21	EU142040
6	*Avihepatovirus*	Duck hepatitis A virus (DHAV) 1	O3D	DQ249299
7	*Cardiovirus*	Encephalomyocarditis virus (EMCV) 1	R (Rueckert)	M81861
8		Theilovirus (ThV) TMEV	GDVII	M20562
9	*Cosavirus*	Human cosavirus A (HCoSV-A) 1	0553	FJ438902
10		Human cosavirus B (HCoSV-B) 1	2263	FJ438907
11		Human cosavirus D (HCoSV-D) 1	5004	FJ438908
12		Human cosavirus E (HCoSV-E) 1	Australia/81	FJ555055
13		Human cosavirus F (HCoSV-F) 1	PK5006	JN867758
14	*Dicipivirus*	Cadicivirus A (CaPdV) 1	Canine picodicistrovirus 209/Hong Kong/2008	JN819202
15	*Enterovirus*	Enterovirus A (EV-A) 71	BrCr	U22521
16		Enterovirus B (EV-B) CV-B3	Nancy	M33854
17		Enterovirus C (EV-C) PV-1	Mahoney	V01149
18		Enterovirus D (EV-D) 70	670/71	D00820
19		Enterovirus E (EV-E) 1	LCR4	DQ092769
20		Enterovirus F (EV-F) 1	BEV-261 RM2	DQ092770
21		Enterovirus G (EV-G) 1	UKG/410/73	AF363453
22		Enterovirus H (EV-H) 1	A-2 plaque virus	AF201894
23		Enterovirus J (EV-J) SV6	1631	AF326766
24		Human rhinovirus A (HRV-A) 16	11757	L24917
25		Human rhinovirus B (HRV-B) 14	1959	K02121
26		Human rhinovirus C (HRV-C) 3	QPM	EF186077
27	*Erbovirus*	Equine rhinitis B virus (ERBV) 1	P1436/71	X96871
28	*Hepatovirus*	Hepatitis A virus (HAV)	HM-175	M14707
29	*Kobuvirus*	Aichivirus A (AiV-A) 1	A846/88	AB010145
30		Aichivirus B (AiV-B) 1	U-1	AB084788
31		Aichivirus C (AiV-C) 1	Swine/S-1/HUN	EU787590
32		Unassigned	Murine kobuvirus M/5/USA/2009	JF755427
33		Unassigned	Canine kobuvirus dog/AN211D/USA/2009	JN387133
34	*Megrivirus*	Melegrivirus A	Turkey hepatitis virus 2993D	HM751199
35	*Parechovirus*	Human parechovirus (HPeV) 1	Harris	L02971
36		Ljungan virus (LjV) 1	87/012	AF327920
37	*Salivirus*	Salivirus A (SaV-A) 1	NG-J1	GQ179640
38	*Sapelovirus*	Avian sapelovirus (ASV) 1	TW90A	AY563023
39		Porcine sapelovirus (PSV) 1	V13	AF406813
40		Simian sapelovirus (SSV) 1	SV2-2383	AY064708
41		Unassigned	California sealion sapelovirus 1 1162	JN420368
42	*Senecavirus*	Seneca Valley virus (SVV) 1	SVV-001	DQ641257
43	*Teschovirus*	Porcine teschovirus (PTV) 1	Talfan	AF231769
44	*Tremovirus*	Avian encephalitis virus (AEV)	Calnek vaccine strain	AJ225173
45	Unassigned	Unassigned	Quail picornavirus 1 HUN/2010	JN674502
46	Unassigned	Unassigned	Pigeon picornavirus A 03/603-7/2003	FR727145*
47	Unassigned	Unassigned	Pigeon picornavirus B 03/641/2003	FR727144
48	Unassigned	Unassigned	Canine picornavirus 1 325F	JN831356
49	Unassigned	Unassigned	Feline picornavirus 1 073F	JN572115
50	Unassigned	Unassigned	Miniopterus schreibersii picornavirus 1	JQ814851
51	Unassigned	Unassigned	Ia io picornavirus 1	JQ814852
52	Unassigned	Unassigned	Rhinolophus affinis picornavirus 1 (R.a. PV-1)	JQ814853†
53	Unassigned	Unassigned	Bat picornavirus 1 NC16A	HQ595340
54	Unassigned	Unassigned	Bat picornavirus 2 MH9F	HQ595342

Table 1. cont.

Sequence	Genus	Species and type	Strain	GenBank
55	Unassigned	Unassigned	Bat picornavirus 3 TLC5F	HQ595344
56	Unassigned	Unassigned	Bovine hungarovirus 1 cattle/2008/HUN	JQ941880
57	Unassigned	Unassigned	Murine mosavirus 1 M-7	JF973687
58	Unassigned	Unassigned	Murine rosavirus 1 M-7	JF973686
59	Unassigned	Unassigned	Turdivirus 1 00356	GU182406
60	Unassigned	Unassigned	Turdivirus 2 10707	GU182408
61	Unassigned	Unassigned	Turdivirus 3 10878	GU182410
62	Unassigned	Unassigned	Gallivirus turkey/M1/76/2011/HUN	JQ691613
63	Unassigned	Unassigned	Swine pasivirus (SPa-V) 1	JQ316470
64	Unassigned	Unassigned	Eel picornavirus 1 F15/05	KC843627
65	Unassigned	Unassigned	Bluegill picornavirus 1 bluegill/USA/04-032/2003	JX134222

*Partial sequence (2C + P3 + 3′-UTR).
†Partial sequence (P1 + 2A).

factors that were calculated by the discriminant analysis using a stepwise selection procedure. As a result of this analysis, the frequencies of GA, UC, UG, CC, CG, AU and GG possess the main discriminatory meaning. Fig. 4(b) displays overlapping scattering of the avian and mammalian picornaviruses but a distinctive dispersion of most fish RNA viruses. However, this difference is not distinctive for the CPV-1 and EPV-1 fish picornaviruses in this study.

Phylogenetic analyses

Phylogenetic analyses were conducted to investigate the relationship of CPV-1 to EPV-1 and other picornaviruses. For this, nucleotide sequences of two conserved gene regions of CPV-1 (encoding the P1 and the 3CD precursor proteins) were compared to the corresponding gene regions of 64 picornaviruses representing all approved and the available tentative picornavirus species. The sequence of BGPV-1 (M. Hoffman, personal communication) was included in these analyses. Both phylogenetic trees (Fig. 5) revealed that BGPV-1, EPV-1, *Parechovirus*, *Avihepatovirus*, *Aquamavirus*, the swine pasivirus, and the *Rhinolaphis affinis* picornavirus are the closest relatives of CPV-1 and comprise a subgroup within the *Picornaviridae*.

DISCUSSION

Recently, we characterized EPV-1, which was proposed to constitute a novel picornavirus species (Fichtner *et al.*, 2013). Here, we describe CPV-1, another fish picornavirus that was isolated from organs of a dead carp and genetically analysed. The genome of CPV-1 exhibits a number of characteristic features. (i) Although the 5′-UTR sequence is still incomplete, it is distinct from that of all known picornaviruses and presumably constitutes a distinct IRES type. (ii) Compared with the VP1 of BGPV-1, the 1D protein is rather short. The significance of this finding is unknown and was verified by conventional Sanger sequencing. (iii) One exceptional feature is the presence of two aphthovirus 2A-like polypeptides with large unique aa sequences. It is presently unknown whether 2A1 is indeed cleaved off the capsid protein precursor by $3C^{pro}$. The capsid proteins of many picorna-like viruses and double-stranded RNA viruses that are released by ribosomal skipping at NPGP sequence motifs are not processed by 3C-like proteinases (e.g. ifla- and totiviruses). Another example is the PX protein of hepatitis A virus (the 1D/2A precursor) which is assumed to be processed by an unspecified host proteinase rather than $3C^{pro}$ (Graff *et al.*, 1999). (iv) CPV-1 has a 9 aa insertion N-terminal to the active site of $3C^{pro}$. A similar 10 aa insertion is also seen in the EPV-1 sequence. (v) Another unique 9 aa insertion is found N-terminal to the YGDD motif of the 3D polymerase. In addition, there is a stretch of hydrophobic and negatively charged aa at the C-terminus of $3D^{pol}$. The sequences of 2B, 3A and $3B^{VPg}$ are unique. Due to the extended unique genome regions of the different picornavirus genera, only sequence comparisons of the P1, $2C^{ATPase}$ and $3C^{pro}$-$3D^{pol}$ sequences which are orthologous, yield meaningful results as presented in Table 2. The data in this table reveal a low similarity of the CPV-1 proteins to the orthologous picornavirus proteins. This finding is supported by the phylogenetic analyses, which reveal long distinct branches at the root of the trees (Fig. 5).

The NCA confirmed previous results regarding underrepresentation of CpG dinucleotides and overrepresentation of CpA and UpG dinucleotides (Boros *et al.*, 2011; Kapoor *et al.*, 2010). The discriminant analysis indicates a differing scattering of some of the 52 RNA genomes and gene segments of fish viruses compared to mammalian and avian picornaviruses. Whether this finding could be useful in host inference remains to be investigated. For CPV-1 and EPV-1, a clear host inference by this approach failed due to insufficiently distinct scores of the discriminant analysis (Fig. 4b).

The sequence data presented here reveal a novel picornavirus that is distinct from all known picornaviruses because of two features. Firstly, CPV-1 has a unique genome organization with two aphthovirus 2A-like sequence motifs (Fig. 1). Secondly, we observed a very low similarity between the sequences of CPV-1 and the other picornaviruses except BGPV-1. Both features, the unique genome organization and low sequence similarity support the assignment of CPV-1 to a novel picornavirus species within a novel genus. We propose the species name 'Cyprovirus' (from Latin *Cyprinus*=carp) and the genus name 'Limnipivirus' (from Greek *limne* [λιμνε]=lake and picornavirus).

Metagenomic research in recent years has extended our knowledge of picornaviruses and revealed their astounding diversity. In 2005, the Eighth ICTV report listed just nine picornavirus genera and 22 species. The 2013 update in the Ninth ICTV report announced a total of 17 acknowledged genera and 37 species (Adams *et al.*, 2013; Stanway *et al.*, 2005). In addition, several further picornavirus candidates await ratification (for a comprehensive compilation, see www.picornaviridae.com).

Most of the known picornavirus species have mammalian hosts, but the number of viruses identified in avian samples is increasing. In addition, there are several candidate picornaviruses of lower vertebrates as listed in the Ninth ICTV report (Knowles *et al.*, 2012). As long as no sequence information is available, concerns whether these viruses indeed are picornaviruses may be justified. Therefore, the recent isolation and the genetic analysis of several fish picornaviruses (from carp, the common eel, bluegill and fathead minnow) represents a considerable advance in the field. Nevertheless, many questions remain to be answered. (i) The pathogenicity and means of transmission of CPV-1 are unclear. Based on the limited experimental evidence, the virus may be considered an orphan with no apparent pathogenicity in carp, but this view may change when more data become available. Low pathogenicity might explain the concealed circulation of CPV-1 in economically important carp populations over many years. Experimental exposure of young carp to water with very high virus titres in fish tanks was insufficient for a successful infection, but i.p. infection

Table 2. Amino acid identities of picornavirus proteins compared with CPV-1

The results are given as percentages of identical pairs.

		Protein*					
Genus	**GenBank**	**1AB**	**1C**	**1D**	**2C**	**3C**	**3D**
Aphthovirus	FMDV-O1 (X00871)	17.0	12.5	10.9	18.0	11.7	23.2
Aquamavirus	SeAV-A1 (EU142040)	16.8	14.9	23.1	23.1	12.6	25.8
Avihepatovirus	DHAV-1 (DQ249299)	24.0	16.2	22.8	20.2	14.2	32.7
Cardiovirus	EMCV-1 (M81861)	15.3	19.0	9.7	15.5	12.5	23.1
Cosavirus	HCoSV-A1 (FJ438902	17.0	19.2	15.2	17.5	10.5	23.8
Dicipivirus	*Cadicivirus* A1 (JN819202)	17.0	19.5	10.4	14.8	10.4	22.8
Enterovirus	EV-C PV-1 (V01149)	21.0	17.1	8.0	18.5	13.2	26.6
Erbovirus	ERBV-1 (X96871)	16.7	18.9	12.1	17.5	11.1	24.5
Hepatovirus	HAV (M14707)	14.6	15.2	10.9	15.9	9.1	25.5
Kobuvirus	AiV-A1 (AB010145)	19.6	16.5	11.6	19.0	13.6	24.5
Megrivirus	*Melegrivirus* A1 (HM751199)	13.9	20.1	10.3	21.3	11.7	25.5
Parechovirus	HPeV-1 (L02971)	23.7	18.6	21.5	21.8	10.4	36.5
Salivirus	SaV-A1 (GQ179640)	21.5	21.0	11.3	17.8	14.5	24.3
Sapelovirus	PSV-1 (AF406813)	18.8	18.6	10.3	15.5	14.5	25.6
Senecavirus	SVV-1 (DQ641257)	16.5	17.5	8.0	15.0	13.5	23.0
Teschovirus	PTV-1 (AF231769)	18.9	13.9	8.4	17.1	12.7	23.3
Tremovirus	AEV-1 (AJ225173)	15.0	13.3	11.2	16.3	12.1	22.8
Unassigned	BHUV-1/2008/HUN (JQ941880)	18.2	16.1	6.6	18.3	13.5	22.8
Unassigned	EPV-1 (KC843627)	21.0	20.9	23.8	25.1	16.4	40.8
Unassigned	*M. schreibersii* PV-1 (JQ814851)	16.0	16.8	10.0	17.0	10.8	24.3
Unassigned	Murine Mosavirus M-7 (JF973687)	17.7	17.1	13.0	16.2	10.0	25.9
Unassigned	Murine Rosavirus M-7 (JF973686)	18.6	17.7	9.0	16.9	12.5	28.3
Unassigned	*R. affinis* PV-1 (JQ814853)†	18.1	19.4	14.7	–	–	–
Unassigned	SPaV-1 (JQ316470)	19.3	17.4	16.8	22.1	18.3	29.3
Unassigned	*Turdivirus* 1 (GU182406)	16.5	17.9	11.1	18.5	14.8	26.6
Unassigned	*Turdivirus* 2 (GU182408)	16.2	19.7	8.3	21.2	10.6	25.1

*Predicted proteins, only orthologous proteins were included.
†Partial sequence comprising 1AB, 1C, 1D and 2A1.

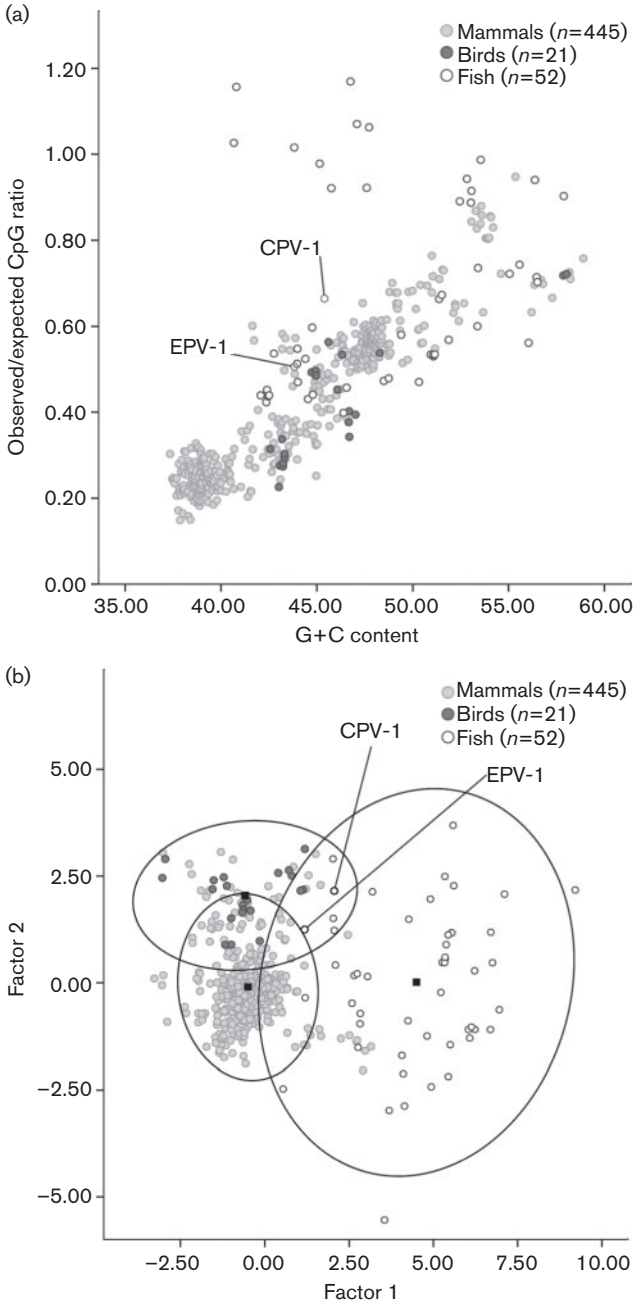

Fig. 4. (a) Nucleotide composition analysis. Frequencies of CpG dinucleotides in picornaviruses from mammals, birds and fish are expressed as observed-to-expected ratios versus G+C content. CPV-1 and EPV-1 are indicated. (b) Canonical score plot. The two most significant contributory factors of mononucleotide and dinucleotide frequencies were calculated in discriminant analysis and are displayed together with 95% confidence ellipses positioned around the centroid (black square) of each group. The dots represent values for the 468 picornaviruses (445 mammalian, 21 avian, 2 fish viruses) and 49 RNA sequences of fish viruses and other virus families. The values of CPV-1 and EPV-1 are indicated.

was. This indicates that the natural infection route is not an obvious one. In contrast, elvers were readily infected by swimming in eel picornavirus-containing water (Fichtner *et al.*, 2013). Picornaviruses in terrestrial ecosystems spread by droplet infection or a faecal–oral infection route via contaminated surfaces and water. Novel means of distribution remain to be identified for picornaviruses that circulate in limnic and marine ecosystems. (ii) The host range of CPV-1 is unknown, and other species may host CPV-1 beside carp. (iii) Both the prevalence and geographical distribution of CPV-1 are also presently unclear. The availability of sequence data, however, will allow the use of sensitive diagnostic assays in future studies.

It is tempting and promising to further explore the host range of *Picornaviridae* and to look for these viruses in other lower vertebrate species.

METHODS

Viruses and cells. CPV-1 strain F37/06 was isolated from an organ pool (heart, brain, liver) of a dead common carp (*Cyprinus carpio*) and propagated in FHM cells (catalogue no. CCLV-RIE 57, Collection of Cell Lines in Veterinary medicine of the Friedrich Loeffler Institute, Insel Riems). Cells were propagated in Eagle's Minimum Eessential Medium supplemented with Earle's salts, 10% fetal bovine serum, 100 000 IU l^{-1} penicillin, and 100 mg l^{-1} streptomycin.

Indirect immunofluorescence assay. Indirect immunofluorescence assay was carried out as described previously (Dauber et al. 2001). Briefly, infected monolayers of FHM cells were incubated for 24 h, washed with isotonic buffer (NaCl 8.28 g l^{-1}, Na_2HPO_4 x 2 H_2O 1.186 g l^{-1}, KH_2PO_4 0.2 g l^{-1}) and fixed in acetone. After rehydration in isotonic buffer, 25 µl of 5C9 hybridoma supernatant was added, followed by incubation at room temperature for 1 h. Then, indocarbocyanin conjugated goat anti-mouse IgG antibody was used as secondary antibody. The fluorescence intensity was rated from not detectable (–) to strong (+++) according to microscopical examination. To determine cross-reactivity of monoclonal antibody 5C9, positive reagents were tested on cells infected with other fish viruses including eel picornavirus. Hybridoma 5C9 was prepared after immunization of female BALB/c mice with concentrated and partially purified CPV suspended in complete Freund's adjuvant and screened as described previously (Dauber *et al.* 2001).

Electron microscopy and biochemical characterization of CPV-1. *Electron microscopy.* Cell culture supernatants of infected cell cultures were adsorbed to formvar-coated copper grids for 7 min. The grids were drained with filter paper, stained with phosphotungstic acid (pH 6.0) for 7 min. and examined with a transmission electron microscope (Tecnai 12, Philips).

Inactivation studies. Lipid solvent sensitivity was determined by addition of 10% chloroform (Roth) for 4 h at 4 °C to suspensions of: CPV-1 strain F37/06, the birnavirus infectious pancreatic necrosis virus (IPNV) European reference Sp strain (Vestergård Jorgensen & Grauballe, 1971) as a resistant control virus, and the rhabdovirus spring viraemia of carp virus (SVCV) RC 56/70 strain (Fijan *et al.*, 1971), as a sensitive control virus. After centrifugation at 2000 ***g***, the supernatant was titrated on cell cultures and compared to untreated samples.

Nucleic acid inhibition assay. Cell cultures were infected with CPV-1 strain F37/06, an iridovirus (Epizootic haematopoietic necrosis virus,

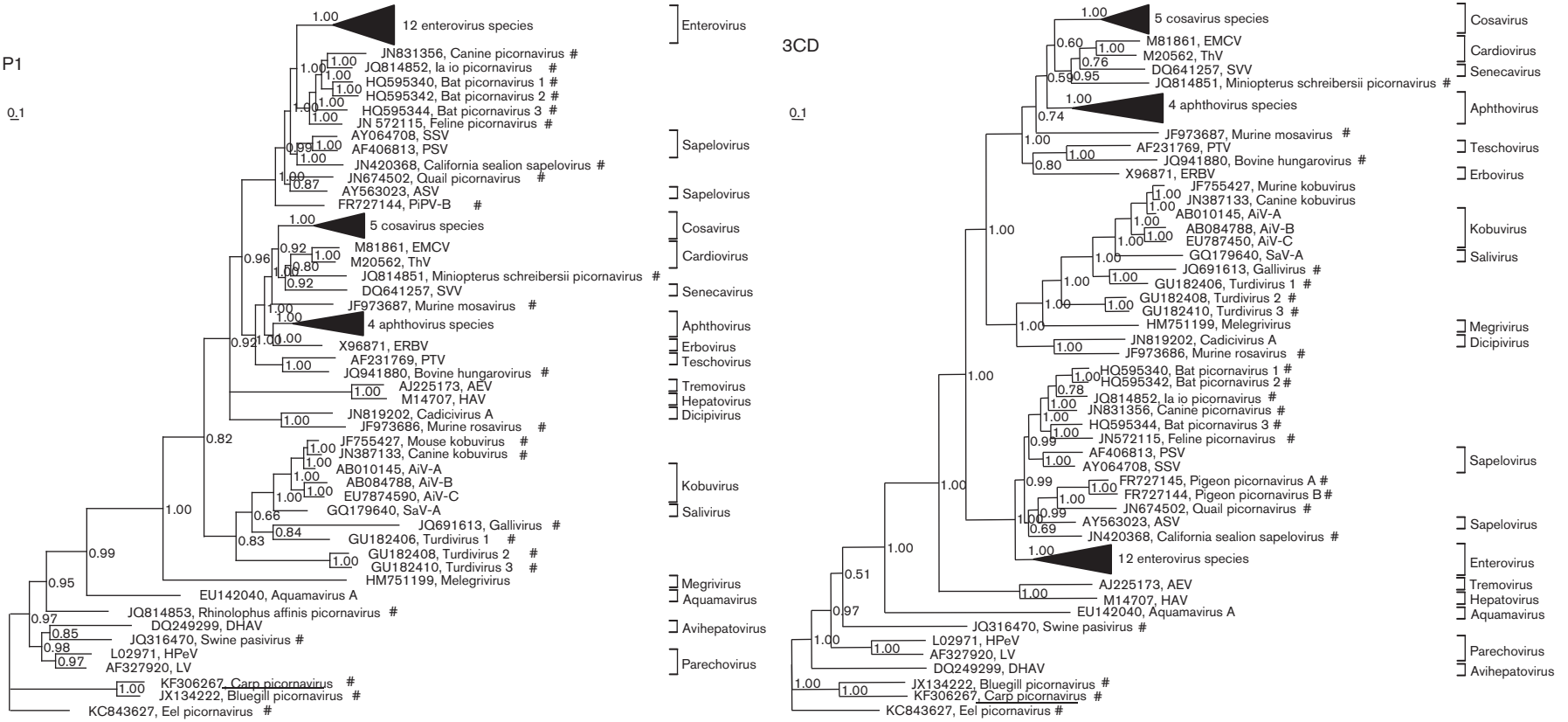

Fig. 5. Phylogenetic analysis of picornavirus P1 (upper panel) and 3CD (lower panel) gene regions. Sixty-four sequences obtained from the GenBank (see Table 1) and the CPV-1 sequence (underlined) were included. The tree was inferred with MrBayes 3.1.2 using the GTR substitution model assuming gamma distribution and invariant sites. GenBank accession numbers, species names and acknowledged genus names or strain designations of tentative picornaviruses (indicated by #) are shown. For clarity, branches of enteroviruses, aphthoviruses and cosaviruses were condensed. The scale bars indicate substitutions per site.

EHNV Isolate 86/8774 (Langdon *et al.*, 1988), DNA genome), and a birnavirus (IPNV European reference Sp strain, RNA genome) at an m.o.i. of 0.1 and grown in cell culture medium with 1000 or 100 μg ml^{-1} 5-iodo-2′-deoxyuridine (IDU, Serva). Infectivity was determined 4 days p.i. by titration.

RNA isolation. Virus RNA was purified from virus-infected FHM cells. Petri dishes with confluent cell monolayers were infected at low m.o.i. Three to four days post-infection, infected cells showing cytopathic effect were freeze–thawed three times to release virus. The lysate was transferred to 15 ml tubes and centrifuged at 4000 ***g*** for 20 min. Then, the lysate was decanted and virus was precipitated from the supernatant by ultracentrifugation at 100 000 ***g*** for 3 h at 4–8 °C. Viral RNA was extracted from the pellets using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and stored at −80 °C.

Sample preparation, sequencing and assembly. Sample preparation was done with 500 ng of purified RNA using Illumina's RNA sample preparation kit (RS-100-0801) and Multiplexing Sample Preparation Oligo kit (PE-400-1001) as described in the manufacturer's description, except for poly-A purification. The RNA was isolated as described above and used for library preparation. Sequencing was performed on a GAIIx instrument (Illumina) running in 76-cycle mode to create reads with lengths of 76 nt using sequencing chemistry v5. To exclude low-quality reads, all reads having at least 1 nt with a Phred quality score below 3 were discarded. For assembly, the remaining reads were mapped to the NCBI nucleotide sequence database to identify picornavirus-similar reads. These reads, together with reads that did not map to the nr/nt database (201 144 total reads), were used for a *de novo* assembly using the CLC Genomics Workbench v4.7.1 (CLCbio, http://www.clcbio.com). The assembly resulted in 51 contigs. Then, the reads were mapped back to the contigs using the CLC Genomics Workbench. All contigs were removed with a base coverage lower than 30-fold in order to remove contigs originating from reads of potential co-isolated RNA molecules that passed the initial nr/nt database mapping approach. This resulted in one contig with a length of 7616 nt and a coverage of 174.14-fold.

Fragment amplification and cycle sequencing. For reverse transcription, 20 pmol T-RACE primer (50-CCGATCGCTCGAGA-ATAGCCCTTTTTTTTTTTTTTTTTTTT-30) were hybridized to 5 μg viral RNA. Then 40 U RevertAid Premium reverse transcriptase (Thermo Fisher Scientific/Fermentas) and a nucleotide mix were added in a final reaction volume of 20 μl. Two microlitres of cDNA were subjected to PCR amplification using specific oligonucleotide primer pairs. Standard PCR cycling conditions were: one cycle of 94 °C for 5 min; 40 cycles of 94 °C for 30 s, 55 °C for 50 s, 72 °C for 1 : 30 min; and a final cycle of 72 °C for 7 min followed by holding at 4 °C. PCR products were analysed by gel electrophoresis and gel-extracted employing the QIAquick Gel Extraction kit (Qiagen). Purified amplification products were sequenced with Sanger sequencing using the CEQ DTCS Quick Start kit (Beckman Coulter) and analysed on a CEQ8000 sequencer (Beckman Coulter).

Phylogenetic and molecular genetic analysis. Picornavirus sequences used for comparisons and phylogenetic analyses were retrieved from GenBank (Table 1). Nucleotide sequences were aligned with CLUSTAL Omega (www.ebi.ac.uk) and adjusted manually with the help of MEGA5 (Tamura *et al.*, 2011). For phylogenetic tree reconstruction of nucleotide alignments, four Bayesian Metropolis-coupled Markov chains were calculated with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) using an optimal substitution model. The substitution model was selected on the basis of the Bayesian information criterion (BIC) and the corrected Akaike information criterion (AICc). Modeltest implemented in MEGA5 suggested the GTR substitution model assuming gamma-distributed rates among sites and invariant sites. Convergence was reached after 829 000 generations (P1 region) and 1 389 000 generations (3CD region), respectively. Nucleotide and amino acid identities between aligned sequences were calculated with CLUSTAL Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and presented as percentage. An NCA was conducted with SSE.1 (Simmonds, 2012). The discriminant analysis with stepwise selection was performed with SPSS 21 for Windows using default settings.

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