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Characterization of a Novel Picornavirus Isolate from a Diseased European Eel (*Anguilla anguilla*)

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A novel picornavirus was isolated from specimens of a diseased European eel (*Anguilla anguilla*). This virus induced a cytopathic effect in eel embryonic kidney cells and high mortality in a controlled transmission study using elvers. Eel picornavirus has a genome of 7,496 nucleotides that encodes a polyprotein of 2,259 amino acids. It has a typical picornavirus genome layout, but its low similarity to known viral proteins suggests a novel species in the family *Picornaviridae*.

All members of the family *Picornaviridae* are small, nonenveloped, icosahedral viruses with positive-strand RNA genomes. Genome sizes range from 7,000 to 9,100 nucleotides. The 5′ end of the RNA is covalently linked to a small virus-encoded peptide (3B); the 3′ end is polyadenylated. The genome encodes one or two polyproteins that are processed co- and posttranslationally into 10 to 14 mature polypeptides. Cap-independent translation initiation is stimulated by one or two internal ribosome entry site (IRES) elements. Common to all picornaviruses are homologous capsid proteins and the nonstructural proteins 2C, $3C^{pro}$, and $3D^{pol}$. The sequences of other nonstructural proteins (leader protein, 2A, 2B, 3A, and 3B) are not conserved and may be unique to some picornavirus genera (1, 2).

The family *Picornaviridae* is currently comprised of 37 species grouped into 17 genera (*Aphthovirus*, *Aquamavirus*, *Avihepatovirus*, *Cardiovirus*, *Cosavirus*, *Dicipivirus*, *Enterovirus*, *Erbovirus*, *Hepatovirus*, *Kobuvirus*, *Megrivirus*, *Parechovirus*, *Salivirus*, *Sapelovirus*, *Senecavirus*, *Teschovirus*, and *Tremovirus*; http://ictvonline.org/). However, picornavirus taxonomy is still in flux, as next-generation sequencing technologies facilitate metagenomics studies using viral nucleic acid-containing specimens. Analysis of viral genomes without prior virus isolation led to the identification of more than 30 approved or tentative picornavirus species from humans (cosavirus [3], salivirus [4], saffold virus [5], and rhinovirus-C [6]), rodents (mosavirus, rosavirus, and murine kobuvirus [7]), pinnipeds (seal picornavirus [8] and California sea lion sapelovirus [9]), domestic cats and dogs (feline picornavirus [10], canine picornavirus [11], canine kobuvirus [12], and canine picodicistrovirus [2]), ungulates (bovine hungarovirus, ovine hungarovirus [13], swine pasivirus [14], and porcine kobuvirus [15]), birds (turdiviruses 1 to 3 [16], turkey hepatitis virus [17], pigeon picornaviruses A and B [18], quail picornavirus [19], and gallivirus [20]), and bats (bat picornaviruses 1 to 3 [21], bat kobuvirus-like virus [22], *Rhinolophus affinis* picornavirus, *Ia io* picornavirus, and *Miniopterus schreibersii* picornavirus [23]).

Although all published picornaviruses were identified in mammals and birds, there are reports of picornavirus-like viruses of reptiles (24, 25), fish (26–31), and marine invertebrates (32–35), indicating circulation of such viruses in aquatic ecosystems. Here, we describe a fish picornavirus which proves that picornavirus ecology indeed includes lower vertebrates of freshwater and probably marine ecosystems. Eel picornavirus 1 (EPV-1) was isolated from a European eel (*Anguilla anguilla*) from Lake Constance on the Rhine River. The viral genome contains an open reading frame (ORF) of 6,777 nucleotides (2,259 amino acids [aa]), and the predicted polyprotein displays the typical organization of a picornavirus. According to the Picornavirus Study Group criteria (www.picornastudygroup.com/definitions/genus_definition.htm), low similarity to the conserved P1 and 3CD precursor proteins of EPV-1 suggests a novel genus in the family *Picornaviridae*.

Starting in May 2005, an increased number of diseased or dead eels were observed in fish traps and aquacultures in the Lake Constance area. Diseased fish presented with increased mucus production, mucous ulcers, and reddened skin but no hemorrhages. One sample (F15/05) that was sent to the National Reference Laboratory for Fish Diseases, Insel Riems, Germany, included specimens of heart, spleen, kidney, liver, and brain. In routine bacteriology by a regional laboratory, only *Aeromonas hydrophila* was detected. By PCR, the genome of the anguillid herpesvirus (*Herpesvirus anguillae* [HVA]) was detected in the kidney. A cytopathogenic, chloroform-resistant virus could be isolated from all four organ samples using eel embryonic kidney (EK-1) cells [catalogue no. CCLV-RIE 809, Collection of Cell Lines in Veterinary Medicine (CCLV) of the Friedrich Loeffler Institut]. This virus exhibited a picornavirus-like appearance in electron microscopy (Fig. 1A) and was designated EPV-1 F15/05. After passaging of the virus, HVA-specific PCR was negative. EPV-1 F15/05 was propagated in EK-1 cells. Infected cell cultures were incubated at 26°C. Three to 4 days postinfection (p.i.), a cytopathic effect was visible (Fig. 1B).

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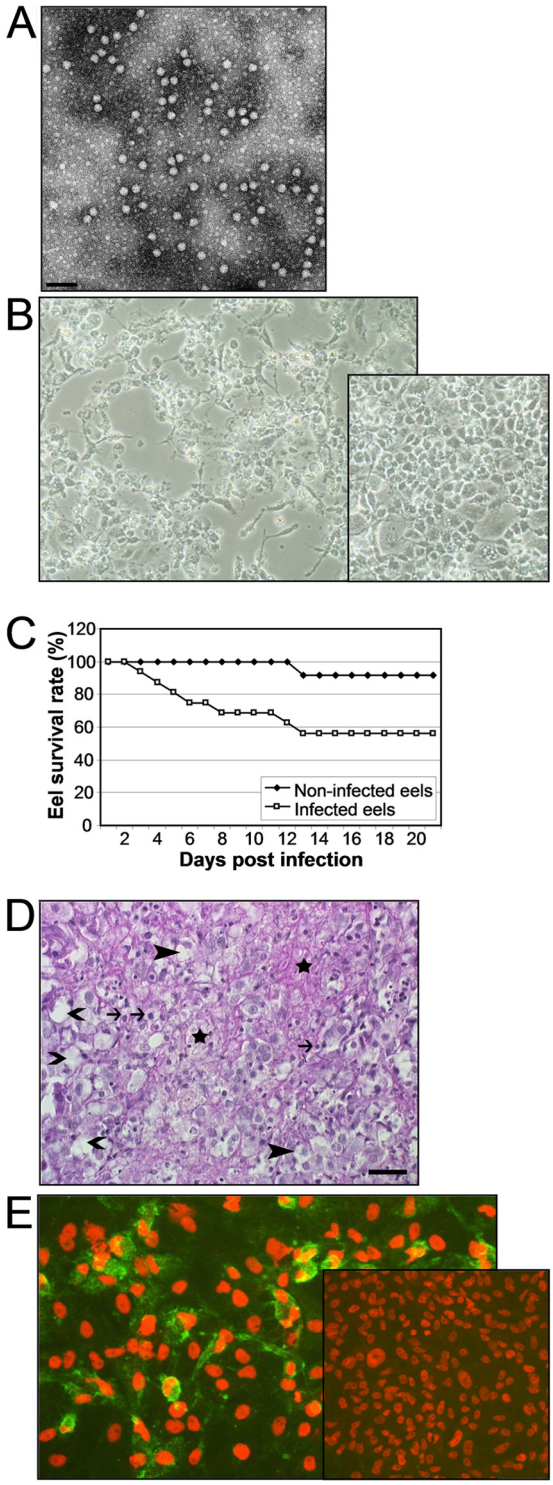

FIG 1 Characterization of eel picornavirus. (A) Electron micrograph of EPV-1 from supernatant of infected EK-1 cells. Bar, 100 nm. (B) Cytopathic effect of EK-1 cells induced by EPV-1 after 3 days p.i. The inset shows uninfected cells. (C) Survival rates of infected and control eels. Glass eels ($n = 16$) were infected with EPV-1 by bathing for 1 h in virus-containing water. A control group of 12 glass eels was mock infected. The eels were kept in aquaria for 21 days. One glass eel of the control group died after an injury. (D) Histopathological changes in the liver of an experimentally infected glass eel. Hepatocytes are irregularly vacuolated (closed arrowheads). Multiple cell membranes are disrupted (open arrowheads). There are karyopyknosis (arrows) and small areas of necrosis and fibrin exudation (stars). Bar = 25 μm. Hematoxylin-eosin stain. (E) Detection of EPV-1 by indirect immunofluorescence using the rabbit antiserum T51 (in-house production) and goat anti-rabbit IgG–FITC fluorescein isothiocyanate (Sigma), with counterstaining of nuclei with propidium iodide. The inset shows uninfected cells.

As EPV-1 was isolated from an eel with a mixed infection, its virulence was examined in a controlled infection experiment. A group of glass eels (length, approximately 10 cm; $n = 16$) was infected by bathing in virus-containing water (10^6 50% tissue culture infective doses [$TCID_{50}$]/ml water) for 1 h. Subsequently, the eels were kept in 400-liter aquaria at a water temperature of 20°C for 21 days. A control group of eels ($n = 12$) was treated and kept under the same conditions and the same volume of an analogous cell culture medium without adding virus to the bath. Beginning 3 days p.i., infected eels died without external signs; at the end of the experiment, 7 of the 16 infected eels had died (43%) (Fig. 1C). In the untreated control group, one eel died in consequence of an injury. Histological examination of all organs, performed at the Centre for Fish and Wildlife Health, Bern, Switzerland, showed decreased numbers of fat vacuoles in hepatocytes of infected euthanized and dead eels compared to uninfected controls, increased single-cell necrosis, scattered small necrotic foci, and mild infiltrations of lymphocytes and macrophages in the liver parenchyma (Fig. 1D). In the kidneys, vacuolization and hyaline droplet degeneration of renal tubular epithelium and multiple small necroses in the interstitium were observed. Virus could be reisolated from 10 of 10 infected euthanized or dead eels and was identified as picornavirus by means of indirect immunofluorescence assay (Fig. 1E) and electron microscopy. No virus could be isolated from the control group.

The genome of EPV-1 was sequenced using the Illumina/Solexa method. Viral RNA was purified from EPV-infected EK-1 cells. Virus was released from infected cells showing cytopathic effect by three freeze-thaw steps. After pelleting of cell detritus (centrifugation at 4,000 × *g* for 20 min), virus was sedimented by ultracentrifugation (100,000 × *g*, 3 h, 4°C). RNA extraction from the pellets and sample preparation for Illumina sequencing were done as described previously (36). Sequencing was performed on a GAIIx (Illumina) instrument to create reads with lengths of 76 nucleotides (nt). For assembly, the reads were mapped to the NCBI nucleotide sequence database to identify similar picornavirus reads. These were used for *de novo* assembly using ABySS (37) with a *k*-mer length of 60. Four internal gaps of 73, 67, 60, and 34 nucleotides as well as the 3′ end (approximately 180 nt) and the very 5′ end were not covered by this approach (Fig. 2). The gaps and the 3′ end were sequenced by conventional Sanger sequencing as described previously (36). All attempts to amplify the very 5′ end by rapid amplification of 5′ cDNA ends (5′ RACE) failed.

As depicted in Fig. 2, the genome of EPV-1 exhibits a typical picornaviral genome organization and is comprised of 7,496 nucleotides with a 5′ untranslated region (UTR) of at least 488 nt, a large ORF of 6,777 nt, and a long 3′ UTR of 231 nt. The genome of EPV-1 has no obvious leader peptide-encoding gene region. Conserved Rhv domains (ranging from aa 130 to 245 and 485 to 630) were found in the capsid proteins 1AB and 1C (http://pfam.sanger.ac.uk/). The P1 precursor is most likely released by an aphthovirus-like 2A1 peptide. The 2A2 gene region exhibits the H-box/NC sequence motifs (H_{797}YGV and N_{865}CE), but the ALTXKAXXX

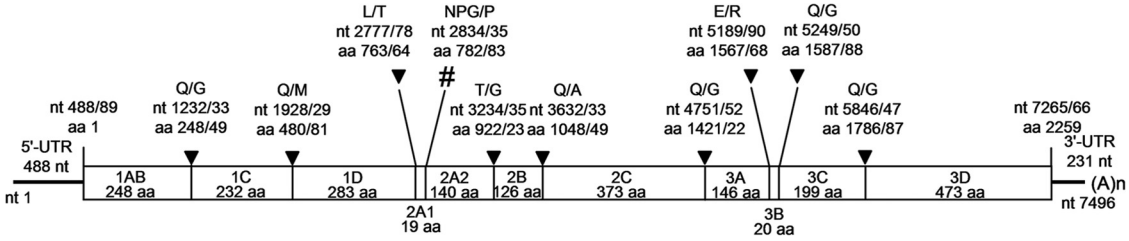

FIG 2 Schematic depiction of the eel picornavirus genome. The 5′ and 3′ UTRs and the ORF (box) are displayed. Filled triangles show the $3C^{pro}$ processing sites; the ribosomal skip sequence is indicated by a pound sign. (A)n, poly(A) tail. Nucleotide (nt) and amino acid (aa) positions of putative cleavage sites are given as well as the amino acid positions of the P1/P1′ sites.

KXXL motif, which is common to the 2A proteins of parechoviruses, is modified (A_{818}TTXKVXXXKXXD). The 2B protein has homology to the 2B protein of parechoviruses (38), duck hepatitis A virus (DHAV-1) (39), and swine pasivirus (14) (Table 1). The 2C gene region exhibits three conserved sequence motifs (A, G_{1189}XXGXGKS; B, Q_{1244}YFHVIDDLAQ; C, K_{1283}GMSYTSRVVIATTN) (reference 40 and literature cited therein). 3A shares some similarity with Ljungan virus (LV) (41) but no obvious homology to any other known picornavirus (Table 1). The N terminus of 3B (RAY_{1570}) is homologous to that of the parechovirus 3B. The putative 3C protease region encodes a $GXC_{1749}G$ active site and a G_{1765}XH substrate-binding pocket (40). Conserved motives of the 3D polymerase are the K_{1954}DELR, G_{2081}GMPSG, Y_{2122}GDD, and F_{2170}LKR sequences (40). Table 1 compares the predicted processing products of EPV-1 with those of its closest relatives. In general, EPV-1 exhibits low similarity to all known picornaviruses.

For phylogenetic analyses, the P1 gene region and the 3CD-encoding gene region of EPV-1 were compared to the corresponding gene regions of 64 picornaviruses representing all approved or tentative picornavirus species. Reference picornavirus sequences were retrieved from GenBank and used for comparisons and phylogenetic analyses. Nucleotide sequences were aligned with MEGA5 (42) and adjusted manually. For phylogenetic tree reconstruction, four Bayesian Metropolis-coupled Markov chains were calculated with MrBayes 3.1.2 (43) using an optimal substitution model. Modeltest implemented in MEGA5 suggested GTR+G+I for both analyses. Convergence was reached after 674,000 generations (P1 region) and 322,000 generations (3CD region). Both phylogenetic trees revealed that *Parechovirus*, *Avihepatovirus*, *Aquamavirus*, and the swine pasivirus are the closest relatives of EPV-1. In addition, the P1 gene region of *Rhinolaphis affinis* picornavirus is also related to EPV-1 (Fig. 3). Low amino acid identity as shown in Table 1 and Fig. 3 suggests that EPV-1 constitutes a novel picornavirus species and probably a novel picornavirus genus. Therefore, we propose the species name "*Anguavirus*."

So far, few aspects of picornavirus ecology have been elucidated. Due to the fecal-oral transmission route, picornaviruses can be considered environmental viruses, and detection of enteroviruses, hepatitis A virus, and Aichi virus in shellfish (44, 45) as well as the identification of seal picornavirus (8) and an enterovirus from the bottlenose dolphin (46) confirms this view. However, PCR detection of picornaviruses does not necessarily indicate their replication in bivalve molluscs but suggests contamination with sewage and bioaccumulation. Viruses with picornavirus-like morphology in electron micrographs have been observed repeatedly in fish, reptiles, and invertebrates, but virus isolation and propagation in cultured cells have often failed (for example, see references, 24, 25, 28, 31, 32, 33, 35, 47, and 48). Therefore, isolation of EPV-1 from eels, experimental infection with induction of clinical signs, and reisolation constitute proof that picornaviruses indeed infect lower vertebrates and raise the question of how sta-

TABLE 1 Pairwise amino acid identities[a]

EPV-1 protein[b]	% Identity with protein from:					
	Parechovirus HPeV-1 (L02971)	*Parechovirus* LV-1 (AF327920)	*Avihepatovirus* DHAV-1 (DQ249299)	*Aquamavirus* SePV-1 (EU142040)	Unassigned SPaV-1 (JQ316470)	Unassigned *R. affinis* PV-1 (JQ814853)[c]
1AB	30.1	33.6	25.3	27.7	24.5	17.1
1C	26.6	31.2	22.7	31.1	30.8	29.2
1D	22.8	22.7	18.7	15.9	21.5	21.5
2A1[d]		24.0	52.2	20.7	36.4	40.0
2A2[e]	27.9	33.8	30.0			
2B	28.6	25.8	21.3	13.3	19.7	
2C	27.6	27.3	29.9	26.5	27.3	
3A	8.8	20.3	12.9	11.0	12.2	
3C	18.3	19.1	21.0	26.1	19.1	
3D	34.6	41.6	38.6	29.0	31.3	

[a] EPV, eel picornavirus; HPeV, human parechovirus; LV, Ljungan virus; DHAV, duck hepatitis A virus; SePV, seal picornavirus; SPaV, swine pasivirus; R. affinis PV, Rhinolophus affinis picornavirus.
[b] Predicted proteins. 3B was excluded.
[c] Partial sequence comprising 1AB, 1C, 1D, and 2A1.
[d] Aphthovirus-like 2A peptide with cleavage at the NPG/P sequence.
[e] 2A protein with H-box/NC motif.

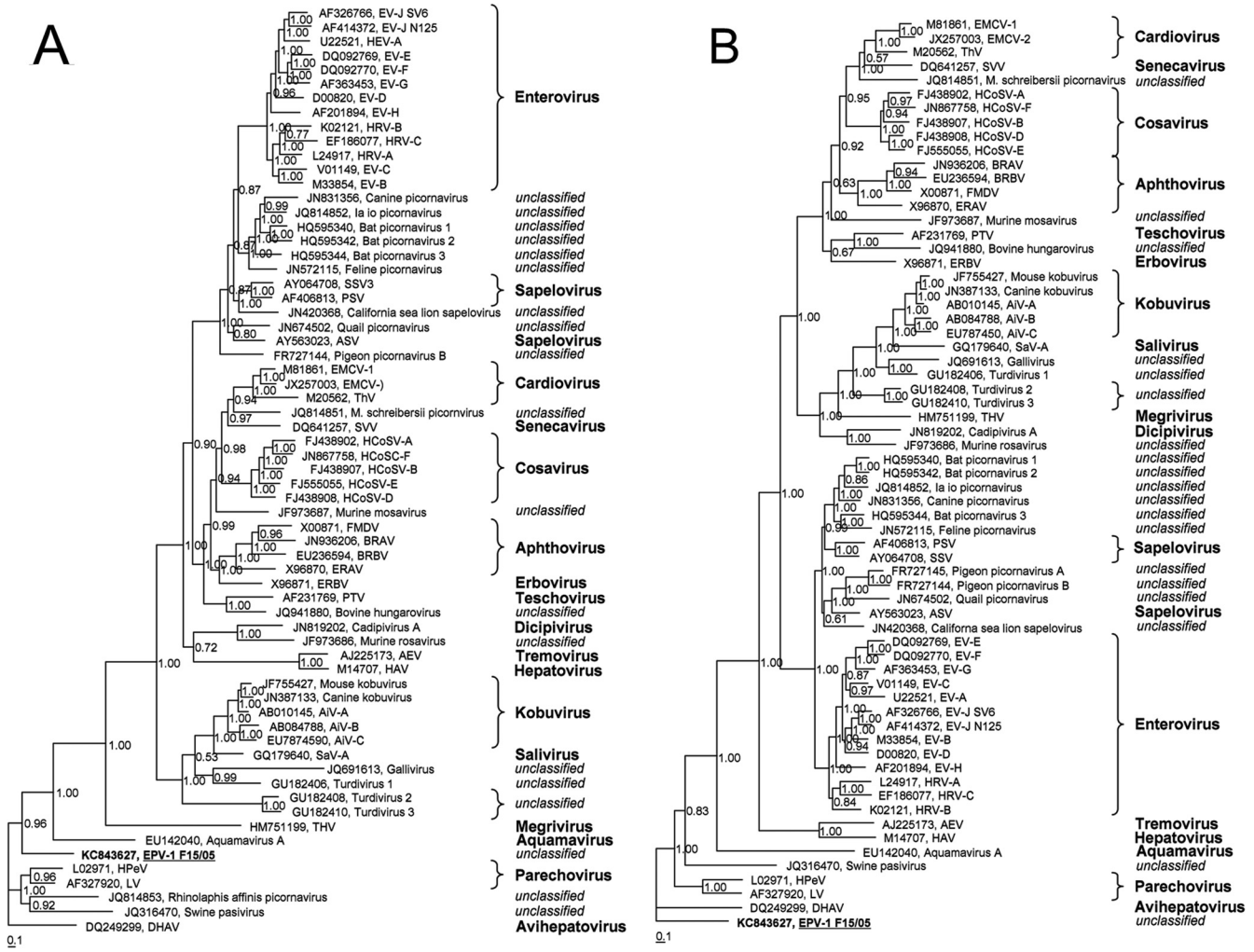

FIG 3 Phylogenetic analyses of the P1-encoding (1AB-1D) (A) and the 3CD-encoding (B) gene regions of 65 picornavirus strains. Sixty-four sequences obtained from the GenBank and the EPV-1 sequence were included. The tree was inferred with MrBayes 3.1.2 using the GTR substitution model assuming gamma distribution and invariant sites. GenBank accession numbers, (tentative) species names, and strain designations are presented. The scale bar indicates substitutions per site. Numbers at nodes indicate posterior probabilities.

ble infection chains are maintained in aquatic ecosystems with high dilution and/or continuous outflow.

Nucleotide sequence accession number. The RNA sequence of EPV-1 F15/05 was submitted to GenBank with accession no. KC843627.

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