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Characterization of a novel porcine enterovirus in wild boars in Hungary

Ákos Boros[1], **Csaba Nemes**[2], **Péter Pankovics**[1], **Hunor Bíró**[3], **Beatrix Kapusinszky**[4], **Eric Delwart**[4,5], and **Gábor Reuter**[1,4,*]

[1]Regional Laboratory of Virology, National Reference Laboratory of Gastroenteric Viruses, ÁNTSZ Regional Institute of State Public Health Service, Pécs, Szabadság út 7. Hungary, H-7623

[2]Veterinary Diagnostic Directorate of the Central Agricultural Office, Kaposvár, Cseri utca 18. Hungary, H-7400

[3]SHP Ltd. Kaposvár, Nagyváthy köz 2. Hungary, H-7400

[4]Blood Systems Research Institute, 270 Masonic Avenue, San Francisco, CA 94118, USA

[5]University of California, San Francisco, 55 Laguna Street, San Francisco, CA 94102-6232, USA

Abstract

Porcine enteroviruses (PEVs) are members of the family *Picornaviridae*, genus *Enterovirus*. Until now, only three different PEV genotypes (PEV-9 and -10, and PEV-3H/PEV-14) have been detected in domestic pigs, and there is no information about the presence of PEVs in wild animals. Here, we identify and characterize the complete genomes of PEV originated from 5 of 10 (50%) of wild boar (*Sus scrofa*) piglets by RT-PCR and pyrosequencing. Wild boar/WBD/2011/HUN (JN807387) PEV showed only 67% amino acid identity in VP1 compared to the most closely related prototype PEV-3H/PEV-14. Wild boar enterovirus represents a novel PEV genotype, provisionally called PEV-15.

Keywords

picornavirus; porcine enterovirus; swine; wild boar; epidemiology; Hungary

The porcine enteroviruses (PEVs) are members of the family *Picornaviridae* and were originally divided into 13 serotypes (PEV-1 to -13) [1, 10]. In the past few years, due to increasing knowledge, PEVs have been divided into three genera within the family *Picornaviridae* based on genome analysis. The genus *Teschovirus* (species *Porcine teschovirus*, PTV) currently comprises 12 serotypes, the previously identified PEV serotypes 1–7 and 11–13 and a recently found possible new serotype (PTV-12) [3, 7, 21]. PEV-8 (a member of the former PEV-A group) has recently been reclassified as a member of the genus *Sapelovirus* [11]. The last two serotypes (PEV-9 and PEV-10) remain in the PEV-B group. Recently, a candidate novel PEV-B sero/genotype from 10-day-old piglets in Hungary was characterized [2]. This new Hungarian sero/genotype was provisionally named

*Address for correspondence: Dr. Gábor Reuter, Regional Laboratory of Virology, ÁNTSZ Regional Institute of State Public Health Service, H-7623 Szabadság út 7. Pécs, Hungary, Telephone: 0036 06 (72) 514 979, Fax: 0036 06 (72) 514 949, reuter.gabor@ddr.antsz.hu.

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PEV-3H [2] and also temporarily referred to as PEV-14, in agreement with the suggestion of Nick J. Knowles, the chairman of the *Picornaviridae* Study Group.

Porcine enteroviruses are small, non-enveloped viruses with a single-stranded RNA genome of positive polarity, approximately 7.4 kb in length and share the same genomic organization as the human enteroviruses [8, 11, 17]. PEVs are present in porcine fecal samples, but the role of these viruses as enteric pathogens has not yet been demonstrated [2, 8, 10]. PEVs have been reported in domestic pigs in only a few countries, including Italy, the United Kingdom, Japan and Hungary [2, 4, 10, 18]. The occurrence of PEVs in wild animals such as wild boars has not been reported. These free-living animals could serve as reservoirs for enteroviruses (e.g., the human coxsackie virus B5-related swine vesicular disease virus), as they do for several other viruses, including hepatitis E virus, swine influenza virus and, food-and-mouth disease virus, which is infectious to other domestic animals and humans, sometimes causing serious diseases [14].

In this study, we provide data on the presence, genome organization and molecular epidemiology of PEV in wild boars and perform genetic analysis to understand the diversity and genetic relationships of this enterovirus group.

Fecal samples from wild boars (*Sus scrofa*) of two different age groups (6-week-old and 8-week-old, n=5–5) were collected from an animal park located in southwestern Hungary in April 2011. None of the sampled boars showed any clinical symptoms at the time of sample collection. The boars were in captive breeding but had no contact with domestic pigs.

Fecal samples were processed and tested for enterovirus (EV) by reverse transcription PCR (RT-PCR) targeting the conserved 5′ untranslated region (5′UTR). RNA extraction and the RT-PCR procedures, including the 5′UTR screening primers, were the same as described previously [2].

One of the EV 5′UTR RT-PCR-positive fecal samples (WBD) was selected for viral metagenomic analysis: 0.1 M phosphate-buffered saline (PBS)-diluted specimens were passed through a 0.45-μm filter and centrifuged at 6,000 × *g* for 5 min. The pellet was mixed with a mixture of nucleases to enrich for particle-protected nucleic acids. Nucleic acids were extracted using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Viral RNA and DNA nucleic acid libraries were constructed by sequence-independent random RT-PCR amplification as described previously [20]. 454 pyrosequencing using 454 GS FLX technology was then performed as described previously [6, 20]. The pyrosequencing reads were assembled *de novo*, and sequence contigs and singletons were compared to the GenBank nucleotide and protein databases using BLASTn.

Specific primer pairs were designed based on the sequence contigs from the pyrosequencing reads to determine the complete nucleotide sequence of a selected EV-positive sample, wild boar/WBD/2011/HUN. The 5′ and the 3′ ends of the genome were determined using a 5′/3′ RACE PCR kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. For the analysis of VP1 genome part from all of the 5′UTR RT-PCR-positive samples, two primers (WBev-3394-R: 3′-TCT TCA TGG GTT GCA AGG TGT-5′ and WBev-2626-F: 3′-GCA TGC TGG AAA CTA GAC ATG -5′) were designed to recognize the conserved sequences in the 5′ part of VP1 and 2A (corresponding to nt 2626–3394 of the PEV-9 UKG/410/73, NC_004441), resulting in 768-nt-long PCR products.

PCR products were sequenced directly in both directions using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK) with the specific primers and run on an automated sequencer (ABI PRISM 310 Genetic Analyzer; Applied

Biosystems, Stafford, USA). For phylogenetic analysis, EV sequences obtained from the GenBank database and the study sequences were aligned using Clusal X, and similarity calculations were performed using the GeneDoc 2.7 software [15]. Phylogenetic trees of the deduced amino acid alignments of complete VP1 and P1 were created using the maximum-likelihood method based on the Jones-Taylor-Thornton matrix-based model in MEGA software (version 5) [19]. Bootstrap values (based on 1000 replicates) for each node are given if >50%. Recombination analysis was performed using SimPlot ver. 3.5.1 and RDP ver. 3.44 [12, 13]. Partial VP1 sequences and the complete genome sequence of wild boar/WBD/2011/HUN were submitted to GenBank under accession numbers: JN807387-JN807389.

Two (40%) and three (60%) of the five fecal samples from 6- and 8-week-old clinically healthy wild boar piglets were RT-PCR positive for EVs using generic 5′UTR and VP1 primer pairs. Three of the five 5′UTR and VP1 PCR products were sequenced and identified as porcine enterovirus (wild boar/WBA/2011/HUN, wild boar/WBC/2011/HUN and wild boar/WBD/2011/HUN), with the highest nt sequence identity (5′UTR: 86%; VP1: 66%) to PEV-3H/PEV-14 (swine/K23/2008/HUN, HQ702854). The 5′UTR and partial VP1 study sequences showed ≥99% nt sequence identity and in case of VP1, complete aa pairwise identity. The phylogenetic trees based on the deduced aa sequences of VP1 revealed that wild boar PEV sequences were separated from the currently known PEV strains and form a distinct lineage (Fig. 1). One of the EV-positive samples (WBD) was selected for complete genome sequencing and phylogenetic analysis.

The complete genome length of the wild boar/WBD/2011/HUN was 7387 nt excluding the poly(A) tail. A large ORF of 6507 nt, which encodes a potential 2168-aa-long viral polyprotein precursor, was flanked by an 811-nt-long 5′UTR and a 68-nt-long 3′UTR. The P1, P2 and P3 regions were 2505 nt (835 aa), 1734 nt (578 aa) and 2268 nt (755 aa) long, respectively. The percent differences calculated from pairwise comparisons revealed that the wild boar/WBD/2011/HUN differed from the PEV-9 (UKG/410/73), PEV-10 (LP54/UK/75), PEV-3H/PEV-14 (swine/K23/2008/HUN) prototype strains by an average 35% aa and 36% nt difference in the VP1 region (Fig. 2.). The non-structural region of the wild boar/WBD/2011/HUN genome consistently shows closer similarity to PEV-9 and PEV-3H/PEV-14 (nt/aa differences, 18%/7%) than to PEV-10 (22%/11%). At the 2B and 3A regions, the aa difference is 18% and 13%, respectively, between the study sequence and PEV-10, but at the same genome regions, the difference between the study sequence and PEV-9 and PEV-3H/PEV-14 was only 5% and 4%, respectively. This contrasts with the other non-structural genome regions (e.g., 2A and 3B), where the PEV-10 strain was the closest relative of the study sequence (Fig. 2.). There was no clear evidence of possible recombination events between the available sequences using bootscan and SimPlot analysis (data not shown). Fig. 3 shows the phylogenetic relationship of the wild boar/WBD/2011/HUN and the other PEVs to the human, simian and bovine enteroviruses based on the deduced aa sequences of the complete structural region (P1) (Fig. 3). Based on the results of sequence and phylogenetic analysis, wild boar/WBD/2011/HUN represents a potential novel porcine enterovirus genotype/serotype.

In the present study, we have identified porcine enteroviruses in a group of wild boar piglets for the first time at relatively high incidence (40–60%). The presence of PEVs in 6- and 8-week-old wild boars was in contrast to a previous study where PEVs were not detectable in fecal samples from 4-week-old or older domestic swine [2]. This could indicate a different course of PEV infection in wild boars within the group examined. The relatively high abundance of these viruses and the complete amino acid identity in the VP1 region between the study sequences suggest the endemic circulation of a closely related virus among wild boar piglets at the animal park where the samples were collected. This means that wild boar

could serve as a reservoir for PEVs. The samples were taken from clinically healthy wild boars. This could indicate that PEVs generally do not cause serious illness in wild boars, similar to what is generally reported for domestic pigs, where only a minority of studies have reported PEV-associated disease, such as the first detection of PEV-9 and -10 from dermal lesions [2, 10, 18].

Beside some partial PEV sequences, there are only three complete PEV genome sequences (UKG/410/73, LP 54/UK/75, and swine/K23/2008/HUN) available in the GenBank database. To analyze its genomic organization and genetic properties as well as its relationship to the available porcine PEV genotypes, the complete nucleotide sequence of the wild boar PEV was determined. Based on the P1 protein phylogenetic tree, wild boar/WBD/2011/HUN belongs to the PEVs, but it is separate from the three known PEV genotypes. The non-structural genome part (P2 and P3) of wild boar/WBD/2011/HUN shows higher amino acid sequence similarity to PEV-9 and PEV-3H/PEV-14 than to PEV-10, although the distribution of percent differences in distinct parts of the genome in the pairwise comparisons between the study sequence and the three PEVs shows a mosaic pattern. In the 2A and 3B regions, the closest relative was PEV-10, but in the 2B and 3A regions, the difference between the study sequence and PEV-10 was at least three times higher than the difference between the study sequence and PEV-9 or PEV-3H/PEV-14. Although Simplot and bootscan analysis did not support recombination events as a cause of the mosaic pattern, recombination was not excluded due to the low number of available parental sequences. Further PEV strains from wild boars living in geographical different regions should be detected and analyzed to investigate the diversity and the transmission of PEVs between wild boars and domestic pigs.

Sequence similarity and phylogenetic relationships suggest that VP1 contains serotype-specific information that can be used for virus identification and sero-/genotype determination. Enteroviruses of the same serotype could be distinguished from those of heterologous serotypes, and the limits of intraserotypic nucleotide and amino acid sequence divergence appeared to be about 25% and 12%, respectively [16, 17]. The complete VP1 nucleotide and deduced amino acid sequence difference was 35%/33% between wild boar/WBD/2011/HUN and the closest PEV strain, swine/K23/2008/HUN. Sequence and phylogenetic analysis indicated that wild boar/WBD/2011/HUN is probably a new genotype/serotype of PEV. The present difficulties with PEV nomenclature (the disappearance of the PEV-A group, only two serotypes belonging to the PEV-B group with the names PEV-9 and PEV-10 without the existence of PEV-B sero/genotypes 1–8) and the proposed changes in enterovirus taxonomy in the future (i.e., it has been proposed to rename the PEV-B group “Enterovirus G”) [9] will probably also affect the current PEV-B sero/genotype names. Therefore, wild boar/WBD/2011/HUN is provisionally named porcine enterovirus 15 (PEV-15) in accordance with the guidance given by Nick J. Knowles, the chairman of the *Picornaviridae* Study Group.

In contrast to the bovine, simian or human enteroviruses, the PEVs are a poorly studied group, and therefore little is known about the diversity, distribution or medical importance (including veterinary or human concerns) of these viruses. Specific molecular methods are now available for investigation of the prevalence and molecular epidemiology of PEV infections in domestic and free-living swine to understand PEV heterogeneity (genotype/serotype) and circulation between domestic and wild animals.

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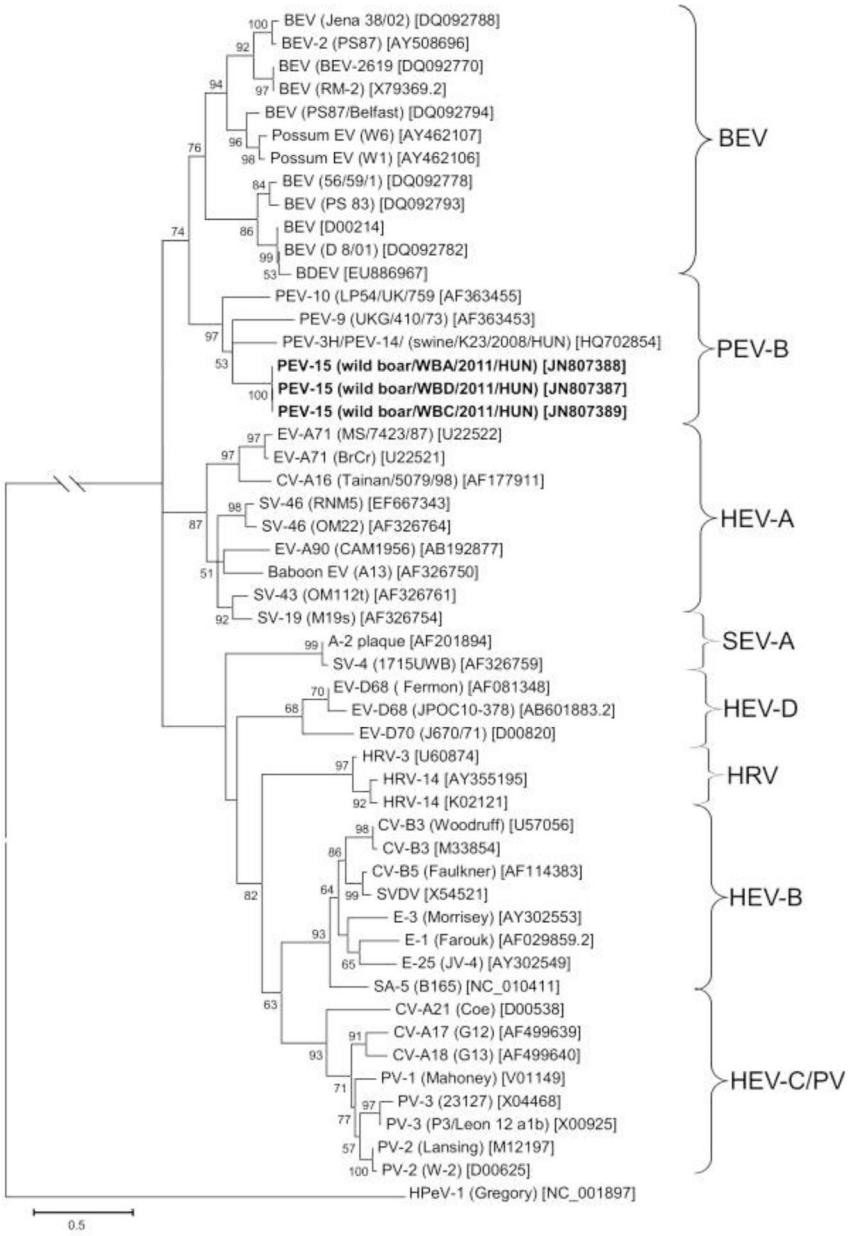

Fig. 1.

Relationship between enteroviruses – including the study sequences **in bold** – based on the currently available deduced amino acid sequences of the complete VP1 of the representative members of the genus *Enterovirus*. Every major enterovirus group contained homologous and heterologous serotypes. Labels are as follows: sero/genotype name, strain name in brackets and accession numbers in square brackets. CV, coxsackie virus; SVDV, swine vesicular disease virus; E, echovirus; EV, human enterovirus; HRV, human rhinovirus; PV, polio virus; SV, simian enterovirus; BDEV, bottlenose dolphin enterovirus; BEV, bovine enterovirus. Human parechovirus type 1 (HPeV-1) was used as an outgroup.

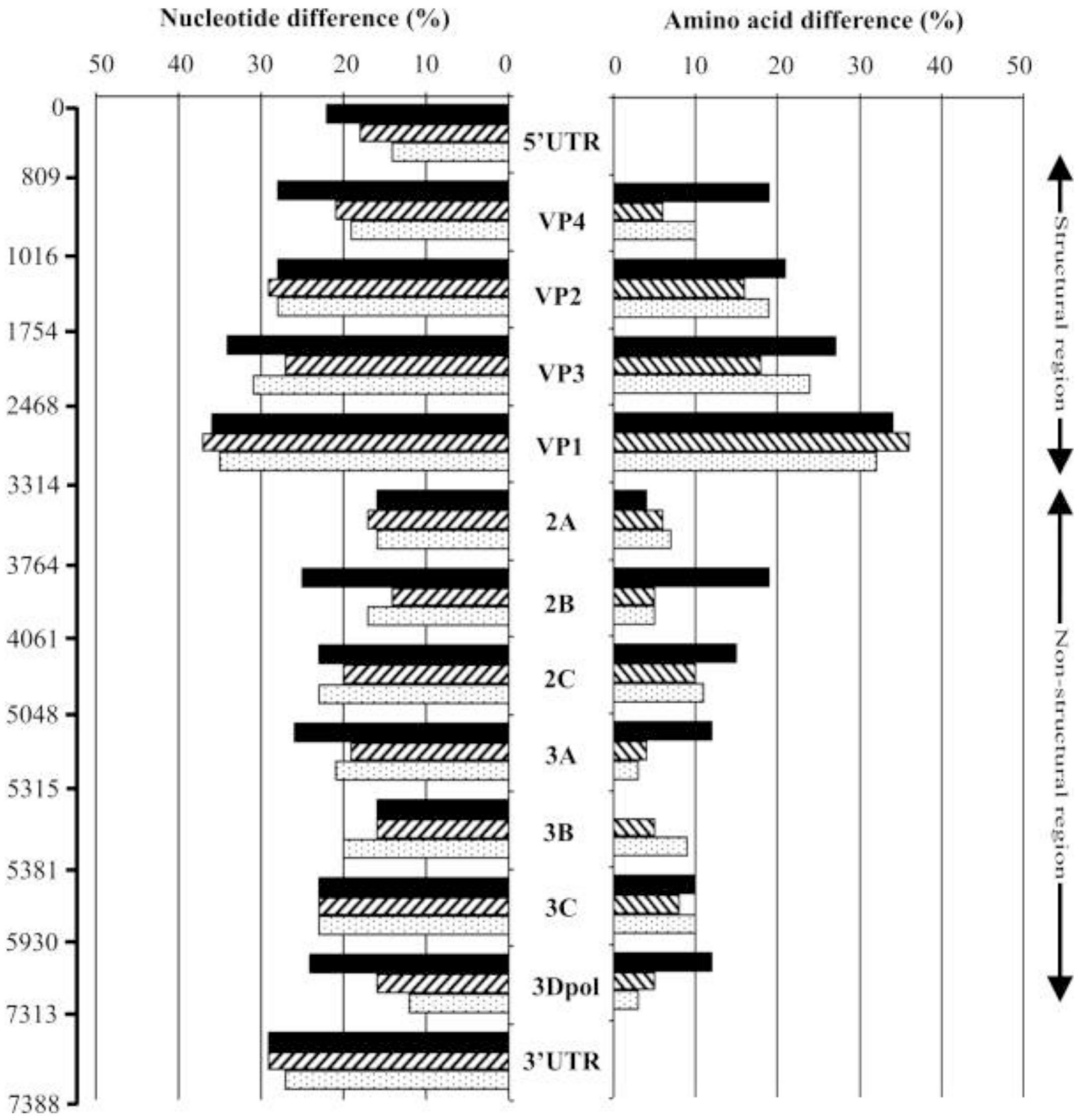

Fig. 2.

Percent differences calculated from pairwise comparisons of nucleotide and amino acid sequences between wild boar/WBD/2011/HUN and PEV-9 UKG/410/73 (hatched bars), PEV-10 LP54/UK/75 (black bars) and PEV-3H/PEV-14 swine/K23/2008/HUN (dotted bars) illustrated in dual graph. Numbers at the left side of the y-axes represent the last nucleotide position of the corresponding genome region (labeled at the center of the graph) of PEV-9 (UKG/410/73).

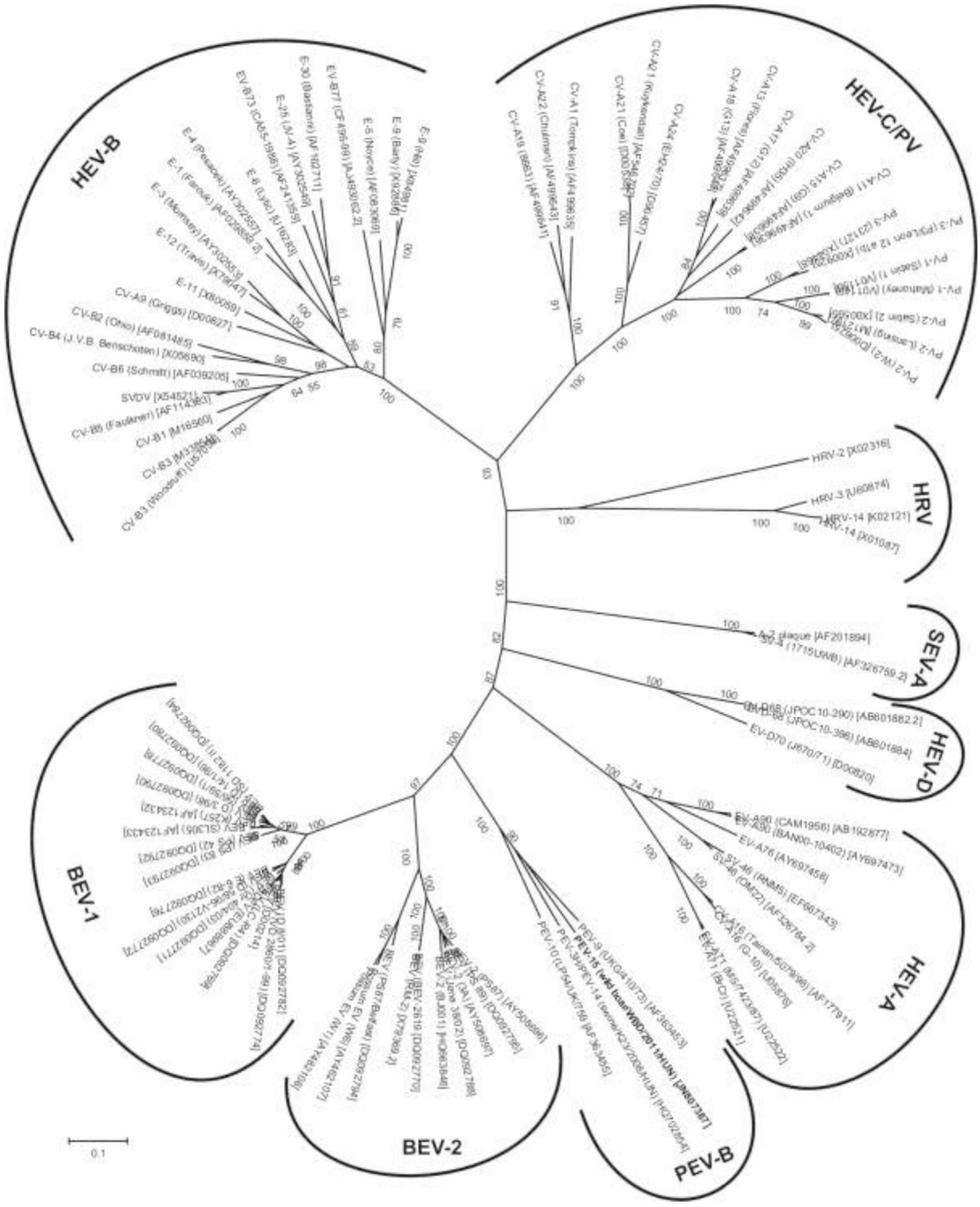

Fig. 3.

Phylogenetic analysis based on the deduced amino acid sequences of the complete structural region (P1) of the study sequence (**in bold**) and the representative members of the genus *Enterovirus* obtained from GenBank. Labels are the same as in Fig. 1.