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Short communication

A novel defective recombinant porcine enterovirus G virus carrying a porcine torovirus papain-like cysteine protease gene and a putative anti-apoptosis gene in place of viral structural protein genes

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ABSTRACT

Enterovirus G (EV-G) belongs to the family of *Picornaviridae*. Two types of recombinant porcine EV-Gs carrying papain-like cysteine protease (PLCP) gene of porcine torovirus, a virus in *Coronaviridae,* are reported. Type 1 recombinant EV-Gs are detected in pig feces in Japan, USA, and Belgium and carry the PLPC gene at the junction site of 2C/3A genes, while PLPC gene replaces the viral structural genes in type 2 recombinant EV-G detected in pig feces in a Chinese farm. We identified a novel type 2 recombinant EV-G carrying the PLCP gene with flanking sequences in place of the viral structural genes in pig feces in Japan. The ~0.3 kb-long upstream flanking sequence had no sequence homology with any proteins deposited in GenBank, while the downstream ~0.9 kb-long flanking sequence included a domain having high amino acid sequence homology with a baculoviral inhibitor of apoptosis repeat superfamily. The pig feces, where the novel type 2 recombinant EV-G was detected, also carried type 1 recombinant EV-G. The amount of type 1 and type 2 recombinant EV-G genomes was almost same in the pig feces. Although the phylogenetic analysis suggested that these two recombinant EV-Gs have independently evolved, type 1 recombinant EV-G might have served as a helper virus by providing viral structural proteins for dissemination of the type 2 recombinant EV-G.

Enterovirus G (EV-G) is a non-enveloped RNA virus, belonging to the family of *Picornaviridae*. Many types of EV-G have been identified (Anbalagan et al., 2014; Bunke et al., 2018; Zell et al., 2017) and the unique properties for reclassification of these viruses were reported (Krumbholz et al., 2002). The viral genome is single-stranded, positive-sensed RNA and consisted of the 5′ untranslated regions (5′ UTR), one open reading frame (ORF), 3′ UTR and the 3′ end poly(A) sequence. In infected cells, the polyprotein is translated from the ORF and then processed to 4 structural proteins (VP1, VP2, VP3, VP4) and 7 non-structural proteins (2Apro, 2B, 2C, 3A, 3B, 3Cpro, 3Dpol) via viral proteinases, 2A and 3CD.

By using a metagenomics approach, we have detected the genomes of EV-G1, 2, 3, 4, 6, 9, 10 and a new type of EV-Gs in feces of pigs with or without diarrhea in Japan (Tsuchiaka et al., 2017). Among them, 16 sequences of EV-G1 and one isolate of EV-G2 show an insertion of a papain-like cysteine protease (PLCP) gene from porcine torovirus at the 2C-3A junction sites (Tsuchiaka et al., 2017) (Fig. 1). We call them type 1 recombinant EV-G in this study. Our previous data demonstrated high prevalence of type 1 recombinant EV-G in the EV-G population (Tsuchiaka et al., 2018). Our previous data demonstrate high

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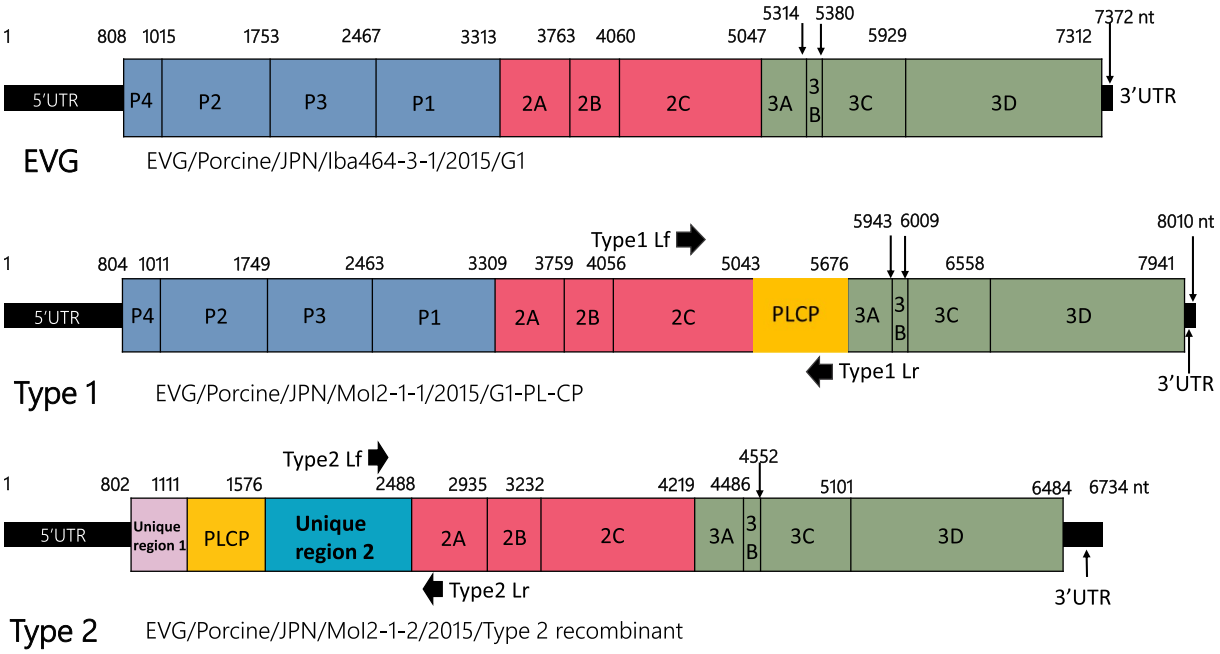

Fig. 1. Schematic diagram of the genome organization of EV-G, type 1 and type 2 recombinant EVGs. Genome order of EV-G, type 1 and type 2 recombinant EV-G came from the EVG/Porcine/JPN/Iba464-3-1/2015/G1 (Tsuchiaka et al., 2017), EVG/Porcine/JPN/MoI2-1-1/2015/G1-PL-CP (Tsuchiaka et al., 2018), and a newly identified type 2 EV-G (EVG/Porcine/JPN/MoI2-1-2/2015/type 2) in this study, respectively.

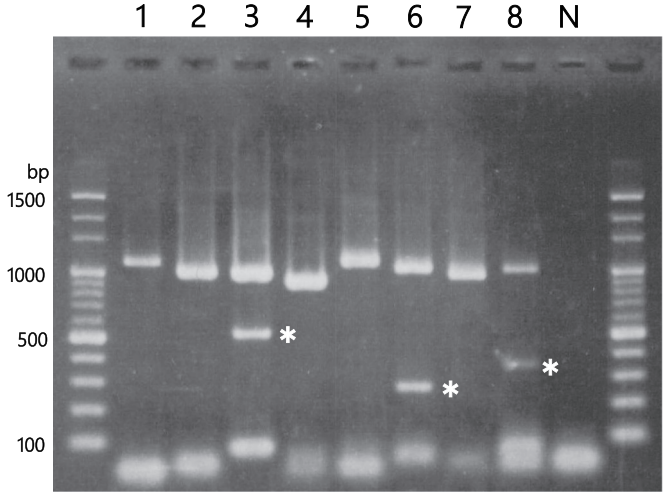

Fig. 2. Overlapping PCR of type 2 recombinant EV-G. RNAs were re-extracted in this study from the pig feces using High Pure Viral Nucleic Acid Kit (Roche), and cDNA was synthesized with random primers using SuperScript III First-Strand Synthesis System (Invitrogen), as described previously (Tsuchiaka et al., 2018). Primers for PCR (Table 1) were designed to amplify the viral genome at approximately every 1 kb with overlap regions. PCR was performed using the Premix Taq (Takara Bio) with the following conditions: an initial denaturation at 95 °C for 2 min; followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. Lane 1, 31F and 1191R primers; lane 2, 930F and 1978R primers; lane 3, 1725F and 2760R primers; lane 4, 2500F and 3447R primers; lane 5, 3001F and 4188R primers; lane 6, 3921F and 4961R primers; lane 7, 4835F and 5852R primers; lane 8, 5619F and 6694R primers in Table 1. The PCR products were electrophoresed using agarose gel and nucleotide sequences were confirmed by direct sequence of the PCR products, which had been purified by agarose gel electrophoresis. The three bands marked as asterisk were confirmed no relation to EV-G by direct sequencing.

Table 1
Primers used in this study.

Primers for overlapping PCR		
Primer name	Nucleotide sequences (5′ to 3′)	position
31F	CAACCTGGCGCTAGTACAC	31–49
1191R	TGCACAGATTGTTCTTTGGTAG	1170–1191
930F	CCACTCTAACTAAAGAAGGCGA	930–951
1978R	ATTGCCACTGGTGAGAGAC	1960–1978
1725F	ACACTCTTCCCTCAGGTTCTAA	1725–1746
2760R	GTGGAATAGTGTGCATCAGC	2741–2760
2500F	GCGGTCCAGCACTATTCAT	2500–2518
3447R	TGCTCTGTGGTTGGACAAG	3429–3447
3001F	CAACTATGCCAGTCAGCTTTC	3001–3021
4188R	TGGTTTCTCCTCTTCCATTCTG	4167–4188
3921F	CCCACTATATCCAATGGCAAAG	3921–3941
4961R	TCATAGTACGGTGGGTTGGA	4942–4961
4835F	AGTGGAACCAATGAGGCTG	4835–4853
5852R	TCTGCAGTGCCTGAAACATTA	5832–5852
5619F	GCAGATTGATTGAAGCCTCC	5619–5638
6694R	AGTTTAGGCCAATCCGGATAAT	6673–6694

SYBR real-time PCR		
Primer name	Nucleotide sequences (5′ to 3′)	position
Type1 Lf	TGATGTGGACATTGAAGTAGC	4778–4808
Type1 Lr	ACTGCATCATGTTGTTCTCC	5209–5306
Type2 Lf	CCTAAAAGGATGGCTAGTGC	2173–2174
Type2 Lr	TTACACCTGGCAAGTTTGTC	2635–2655

For type 1 amplification: type 1 Lf and type 1 Lr.
For type 2 amplification: type 2 Lf and type 2 Lr.

prevalence of type 1 recombinant EV-G in the EV-G population (Tsuchiaka et al., 2018). Type 1 recombinant EV-Gs have been discovered from feces of neonatal pigs showing clinical symptoms, such as diarrhea, in the US, Belgium, Germany and South Korea (Bunke et al., 2018; Conceição-Lee and Lee, 2019; Neto et al., 2017; Knutson et al., 2017; Shang et al., 2017), while type 1 recombinant EV-Gs identified in Japan are detected from normal as well as diarrhea pig feces (Tsuchiaka

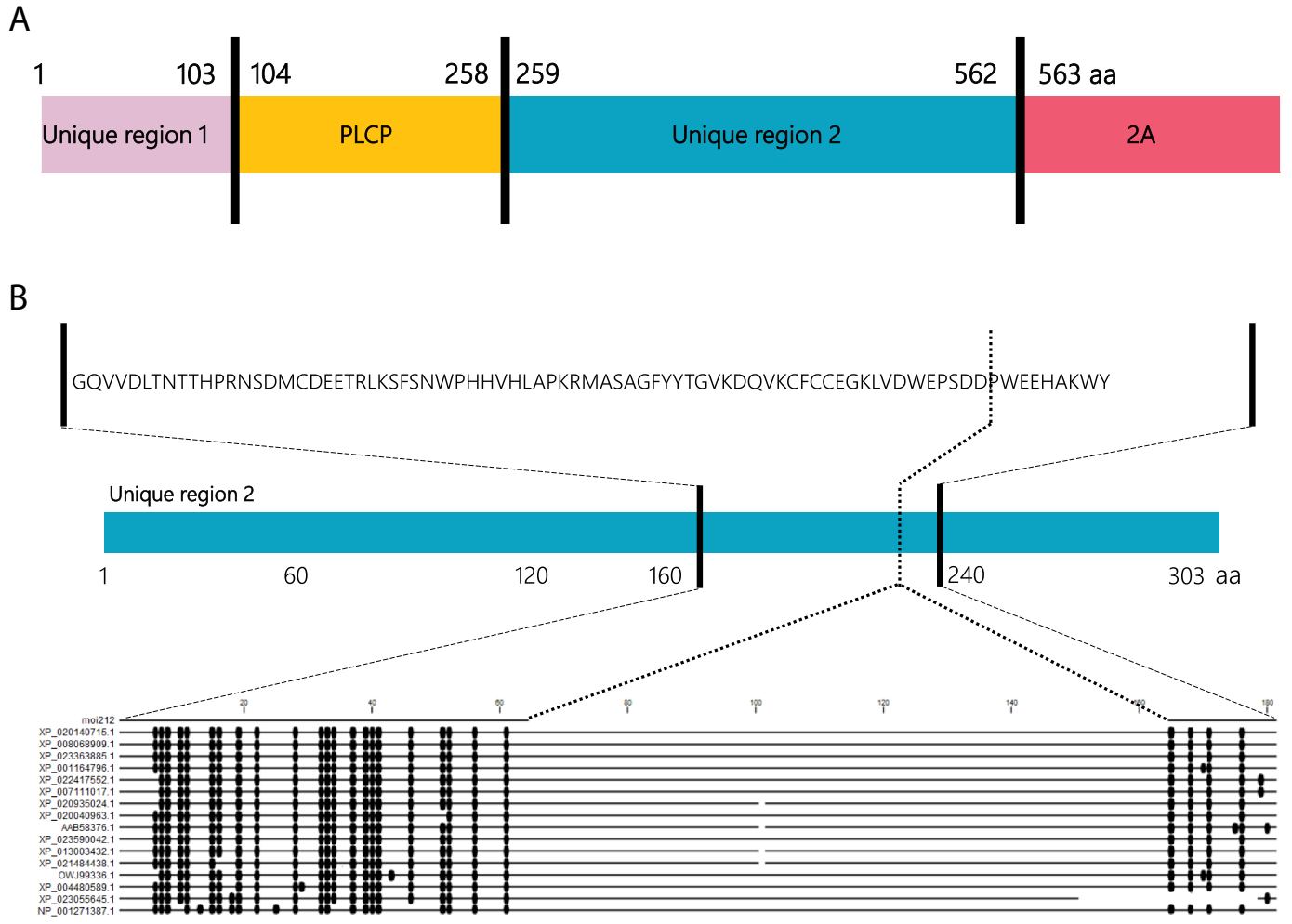

Fig. 3. Amino acid alignment of unique region 2 and BIR family.
(A) Junction sites of unique region 1-PLCP-unique region 2-N-terminal truncated 2A of EVG/Porcine/JPN/MoI2-1-2/2015/type 2. (B) Amino acid sequence of amino acids at 180-240 of unique region 2 of the newly identified type 2 EV-G is shown in top and is aligned with other BIR family protein. moi212uniq2 represents the newly identified type 2 EV-G. The accession number of protein sequences from GenBank used in this figure is indicated as XP_020140715.1: E3 ubiquitin-protein ligase XIAP isoform X3 of *Microcebus murinus*, XP_008068909.1: E3 ubiquitin-protein ligase XIAP of *Carlito syrichta*, XP_023363885.1: E3 ubiquitin-protein ligase XIAP of *Otolemur garnettii*, NP_001164796.1: E3 ubiquitin-protein ligase XIAP of *Oryctolagus cuniculus*, XP_022417552.1: E3 ubiquitin-protein ligase XIAP isoform X1 of *Delphinapterus leucas*, XP_007111017.1: E3 ubiquitin-protein ligase XIAP isoform X1 of *Physeter catodon*, XP_020935024.1: E3 ubiquitin-protein ligase XIAP isoform X1 of *Sus scrofa*, XP_020040963.1: E3 ubiquitin-protein ligase XIAP of *Castor canadensis*, AAB58376.1: X-linked inhibitor of apoptosis of *Mus musculus*, XP_023590042.1: E3 ubiquitin-protein ligase XIAP isoform X2 of *Trichechus manatus latirostris*, XP_013003432.1: E3 ubiquitin-protein ligase XIAP of *Cavia porcellus*, XP_021484438.1: E3 ubiquitin-protein ligase XIAP of *Meriones unguiculatus*, OWJ99336.1: XIAP of *Cervus elaphus* hippelaphus, XP_004480589.1: E3 ubiquitin-protein ligase XIAP of *Dasypus novemcinctus*, XP_023055645.1: E3 ubiquitin-protein ligase XIAP of Piliocolobus tephrosceles, NP_001271387.1: E3 ubiquitin-protein ligase XIAP of *Canis lupus familiaris*. Dots indicates different amino acids from moi212uniq2.

et al., 2018). In addition to type 1 recombinant EV-G, second type of recombinant EV-G (we call type 2 recombinant EV-G in this study), which carried the PLCP gene in place of viral structural genes, has been identified in a Chinese pig farm (Wang et al., 2018). The PLCP gene is encoded in the ORF1 of the genome of torovirus, a member of family *Coronaviridae*, and the order *Nidovirales* (Conceição-Neto et al., 2017; Knutson et al., 2017; Shang et al., 2017; Tsuchiaka et al., 2017; Wang et al., 2018). The PLCP of nidoviruses serves as a protease to cleave viral gene 1 polyproteins to mature proteins (Mielech et al., 2014). It also has functions of deubiquitinating and deISGylating (interferon-stimulated gene15-removing) enzymes, which plays important roles in viral pathogenesis by acting as an innate immunity antagonist (Shang et al., 2017).

In the present study, we identified a novel type 2 recombinant EV-G in pig feces in Japan. In contrast to type 2 recombinant EV-G detected with short flanking genes in the Chinese pig farm (Wang et al., 2018), the novel type 2 recombinant EV-G had an insertion of PLCP with long flanking genes, one of which had a region showing high homology with a baculovirus gene having anti-apoptotic function.

Our previous metagenomics analyses of RNAs extracted from pig feces obtained from Japanese pig farms have identified various types of EV-Gs and type 1 recombinant EV-G (Tsuchiaka et al., 2018). We performed further analyses using the same metagenomics data and RNA samples. Subsequent metagenomics analysis of the RNAs of the non-diarrheal pig feces obtained from a pig farm in Tottori prefecture, Japan, led to discovery of a novel type 2 recombinant EV-G genome (EVG/Porcine/JPN/MoI2-1-2/2015: GenBank with accession number LC316774). This fecal sample also contains type 1 recombinant EV-G (EVG/Porcine/JPN/MoI2-1-1/2015/G1-Type 1) (Tsuchiaka et al., 2018).

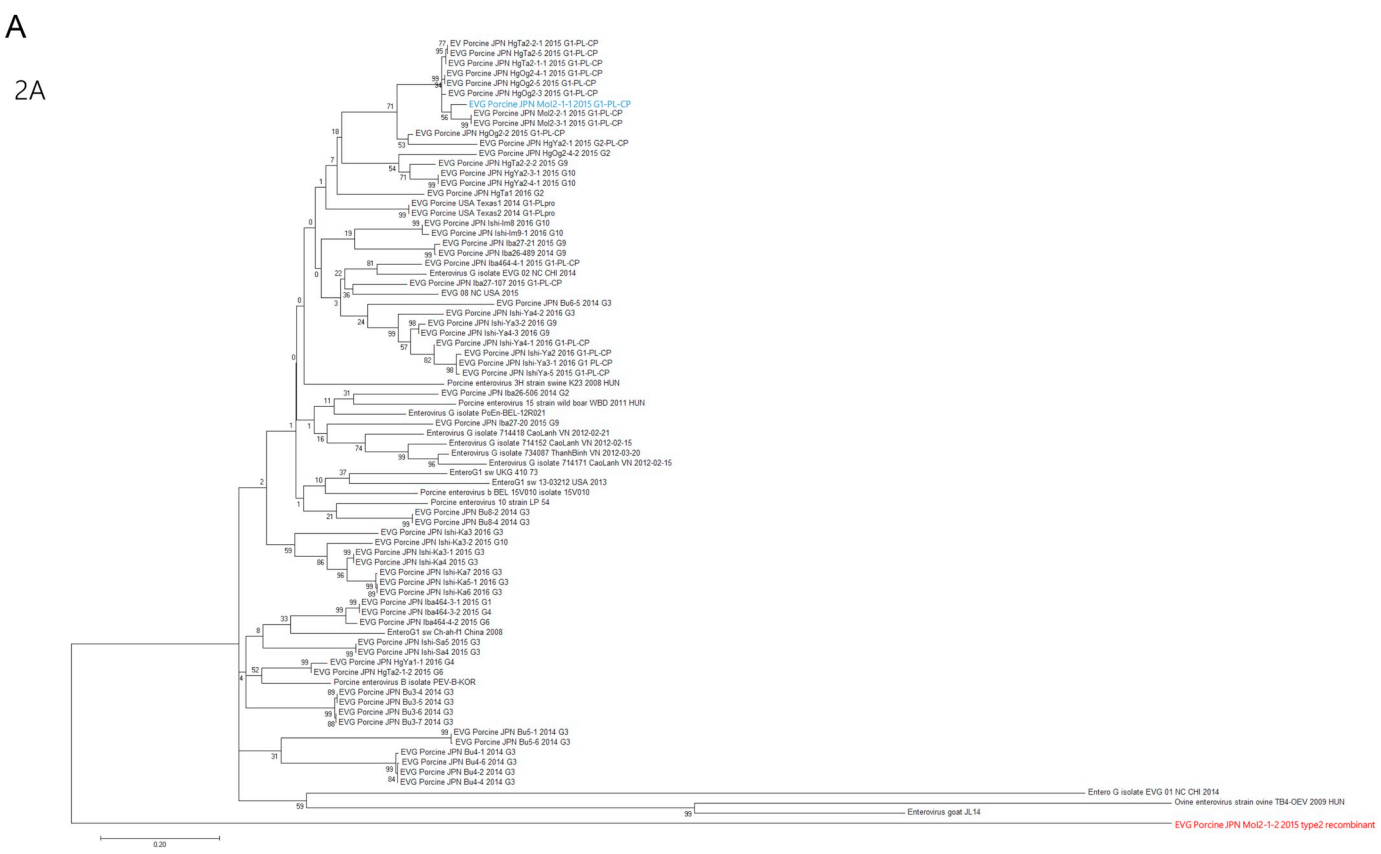

Fig. 4. Phylogenetic trees of EVG/Porcine/JPN/MoI2-1-2/2015/type 2 with enterovirus G strains from GenBank database based on nucleotide sequences of PLCP (A), 2A (B), 2B (C), 2C (D), 3C (E) and 3D (F). The trees were constructed using Neighbor-joining method in MEGA 7.0.14 and bootstrap test (n-1000). The genetic distance was calculated using Kimura's two parameter model. The scale bar indicates nucleotide substitutions per site.

The metagenomic reads were assembled without gaps, revealing the presence of full-length type 1 and type 2 recombinant EV-G RNAs in the pig fecal sample (Supple Fig. 1A and B). We performed long RT-PCR to amplify the viral genome approximately every 1 kb with overlapping regions. Overlapping PCR products were successfully obtained and direct sequencing analysis of the gel-purified PCR products showed that the nucleotide sequences of the PCR products were the same as those obtained in metagenomics analysis (Fig. 2). By the results of conventional RT-PCR (data not shown) and SYBR-green real-time RT-PCR using primers in Table 1 (average Ct-value 25.0066 for type 1, 26.5738 for type 2), we also found that the fecal sample contained similar amounts of the type 1 and type 2 recombinant viruses, which implying that type 2 recombinant EV-G was not a minor population compared with type 1 recombinant EV-G in the sample. These results strongly suggested that virus particles carrying type 2 recombinant genome indeed existed in the fecal sample and our data were not an artifact of metagenomics analysis.

The new type 2 recombinant EV-G genome was approximately 6.7 kb-long and had the following gene order: 5′ UTR - unique region 1 - PLCP – unique region 2 – 2A – 2B – 2C – 3A – 3B – 3C – 3D – 3′ UTR (Fig. 1). The new type 2 recombinant EV-G genome carried a single ORF, suggesting that translation of the viral polyprotein starts at the translational start codon (ATG) at the 5′ end of the unique region 1 and ends at the stop codon (TAA) of 3D gene. In contrast to the reported type 2 recombinant EV-G genome in China, which carries the PLCP gene of pig torovirus in place of viral structural genes (Wang et al., 2018), the newly identified type 2 recombinant EV-G carried the PLCP gene from pig torovirus plus ~0.3 kb-long upstream and ~0.9 kb-long downstream flanking sequences (Fig. 3A). In addition, the newly identified type 2 recombinant EV-G lacked 11 amino acids at the N-terminal of 2A protein. We noted that the same 11 amino acids are also deleted in type 2 recombinant EV-G detected in the pig farm in China (Wang et al., 2018). In contrast, the N-terminal truncation of the 2A proteinase gene did not occur in type 1 recombinant and non-recombinant EV-Gs, implying that the truncated 2A may be a specific characteristic feature among type 2 recombinant EV-Gs. Furthermore, type 2 recombinant EV-Gs lacked GxCG motif in 2A, which is considered to form part of the active site of the protease and is conserved among enteroviruses (Gorbalenya et al., 1989). In contrast all type 1 recombinant EV-Gs carry this motif in 2A (data not shown). An internal region (amino acids 180–240) of the 304 amino acid-long unique region 2 of the newly identified type 2 recombinant EV-G showed high amino acid homology with genes of baculoviral inhibitor of apoptosis repeat (BIR) superfamily by a multiple sequence alignment using Clustal W (Thompson et al., 1994) in MEGA 7 (Kumar et al., 2016) (Fig. 3B). In contrast, unique region 1 and other regions in unique region 2 showed no significant amino acid homologies with any known proteins.

To clarify the close relationship between type 1 and type 2

B

2B

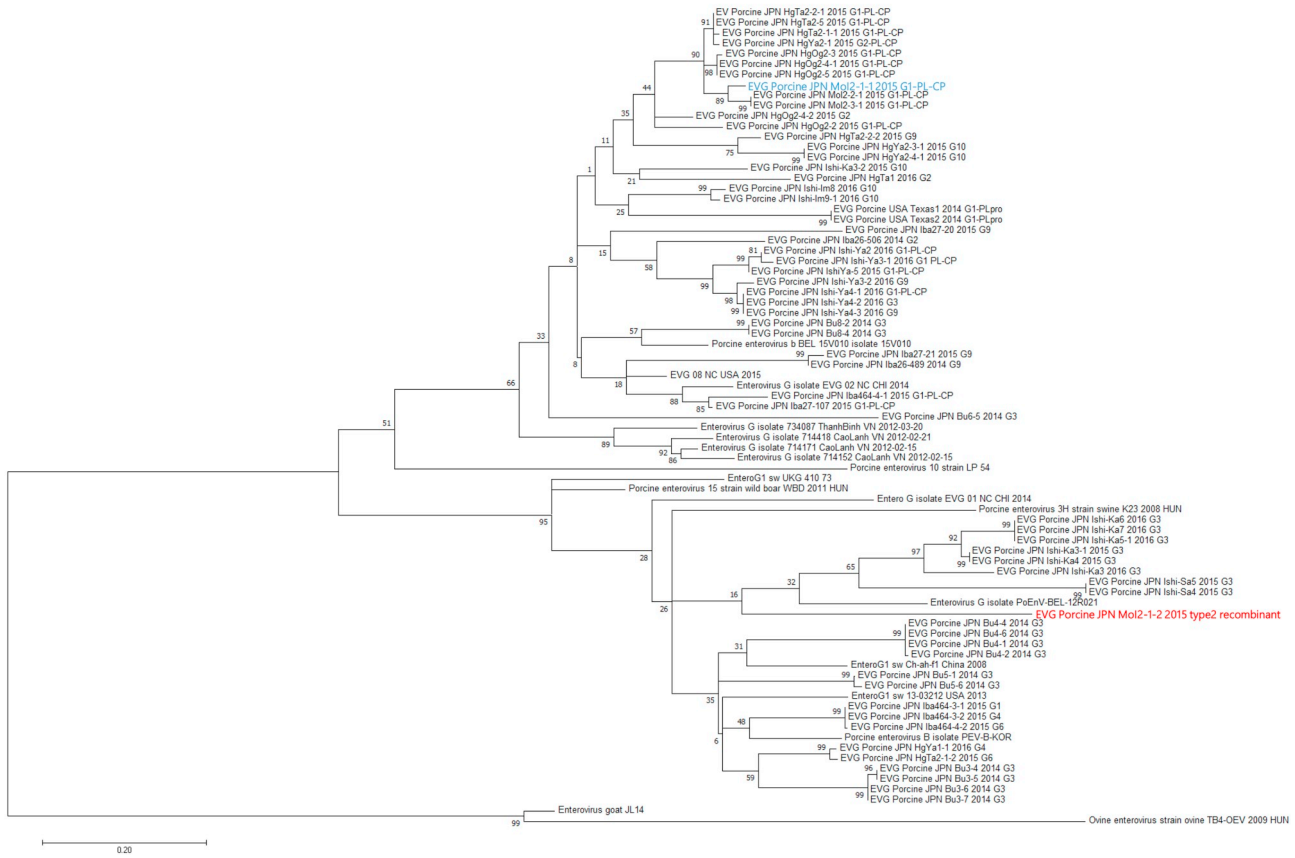

Fig. 4. (*continued*)

C

2C

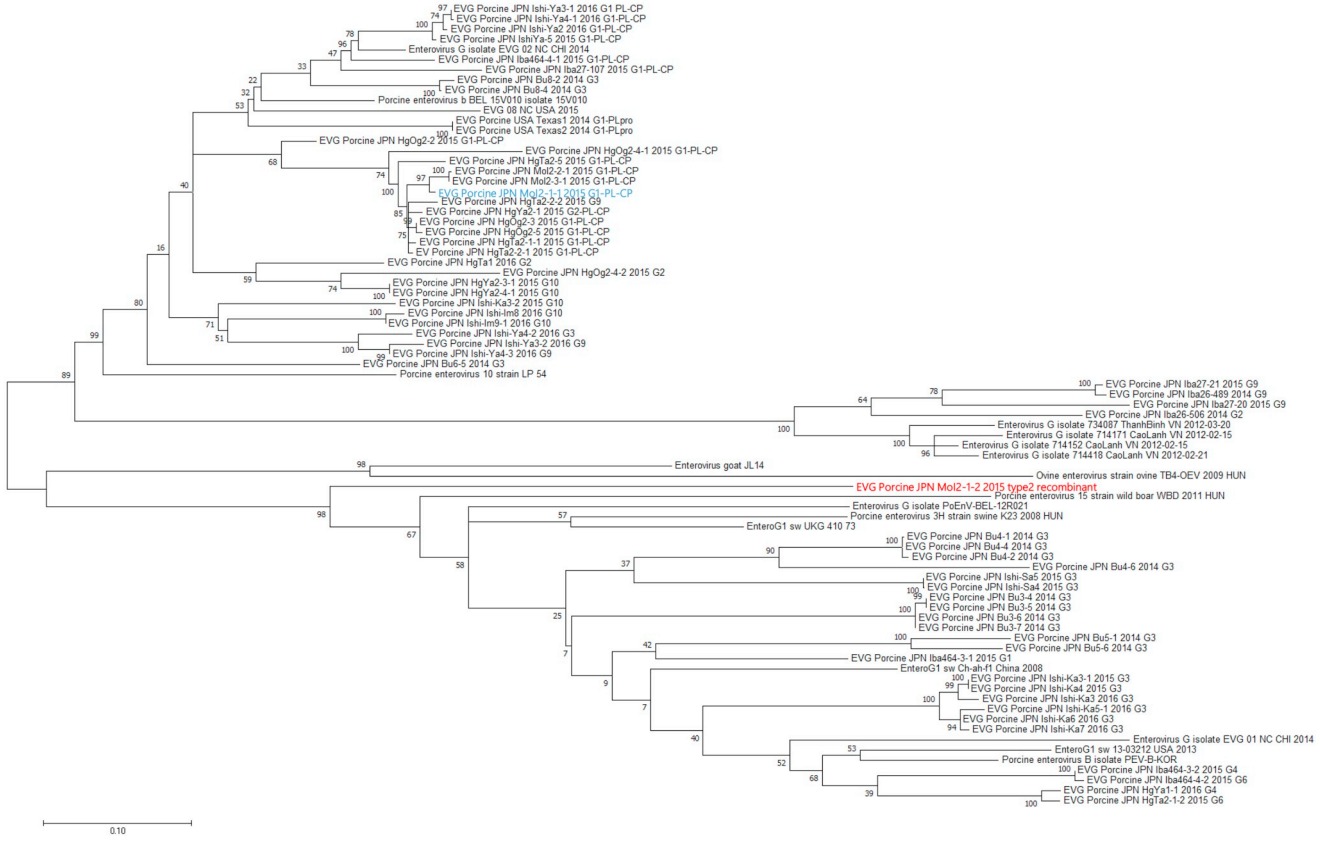

Fig. 4. (*continued*)

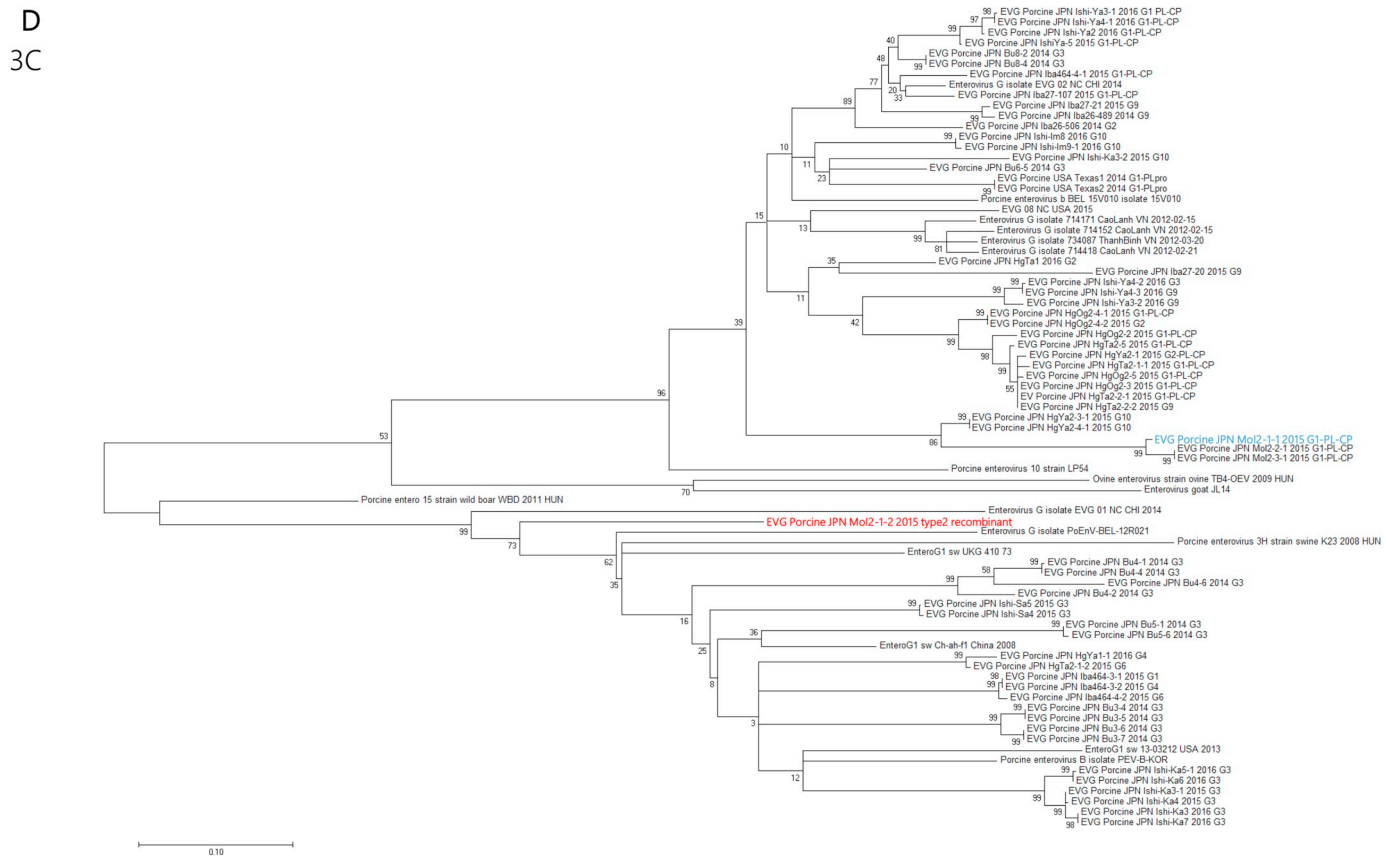

Fig. 4. (*continued*)

recombinant EV-Gs, Nucleotide sequences were first subjected to a multiple sequence alignment using Clustal W (Thompson et al., 1994) in Mega 7 (Kumar et al., 2016), and then, evolutionary distance was estimated with Tamura-Nei model (PLCP) (Tamura and Nei, 1993) and General Time Reversible model (2A, 2B, 2C, 3C, 3D) (Nei and Kumar, 2000). Phylogenetic trees were generated by the maximum-likehood method (T92 + G model or GTR + G + I model) and analyzed with 1000 replicates for bootstrap using MEGA 7. Phylogenetic analysis showed that 2A, 2B, 2C, 3C, 3D genes of the newly identified type 2 EV-G made different clusters from known non-recombination EV-Gs and type 1 recombinant EV-Gs (Fig. 4A to E), including type 1 recombinant EV-G, which co-existed with the new type 2 recombinant type 2 in the same feces; type 1 (shown in blue) and type 2 (shown in red) were found in the same feces collected in this study. Interestingly, PLCP of type 1 and type 2 recombinant EV-Gs belong to same cluster (Fig. 4F). These data suggest that the type 2 recombinant EV-G was not directly derived from known type 1 recombinant EV-Gs nor non-recombinant EV-Gs, and the origin of PLCP of each type was same torovirus. We also noted that the new type 2 recombinant EV-G had the 247 nt-long 3′ UTR (Fig. 1), which was substantially longer than 40 to 165 nt-long 3′ UTR of most of picornaviruses, supporting the notion that the new type 2 recombinant EV-G has evolved independently from type 1 recombinant EV-Gs or non-recombinant EV-Gs.

In the present study, we reported the genome of a novel type 2 recombinant EV-G from pig feces in Japan. The newly identified type 2 recombinant EV-G lacked the genes for structural proteins, while it carried most of the genes encoding viral nonstructural proteins. Accordingly, this defective recombinant EV-G required helper virus, which should provide viral structural proteins for dissemination, and underwent RNA replication in the absence of helper virus. Because the type 1 recombinant EV-G was detected in the same feces sample as the new type 2 recombinant EV-G, this type 1 recombinant EV-G, which belongs to different subtype, might have served as the helper virus. Otherwise, in this study, we might detect genome of type 2 recombinant EV-G in the dead host cells, not in the viral particles. In this case, it is possibility that toroviruses infected persistently in the porcine cells which facilitates type 2 recombination frequently with circulating EV-Gs and genome of type 2 recombinant EV-Gs was not packaged into viral particle.

Some RNA viruses acquire a new function, e.g., inhibition of the host immune functions, by gaining a new gene via RNA-RNA recombination (Simon-Loriere and Holmes, 2011). Enteroviruses are genetically and antigenically highly variable due to recombination within as well as between serotypes (Perez-Losada et al., 2015; Simmonds and Welch, 2006). Poliovirus and coxsackievirus undergo RNA recombination, with higher efficiency for non-homologous RNA recombination than homologous RNA recombination, in cell cultures, suggesting that non-homologous RNA recombination may be a transient and intermediate step for the generation and selection of the fittest homologous recombinants (Lukashev, 2010). Identification of two different types of recombinant EV-G implied that non-homologous recombination between picornavirus RNA and non-picornavirus genes drives evolution of

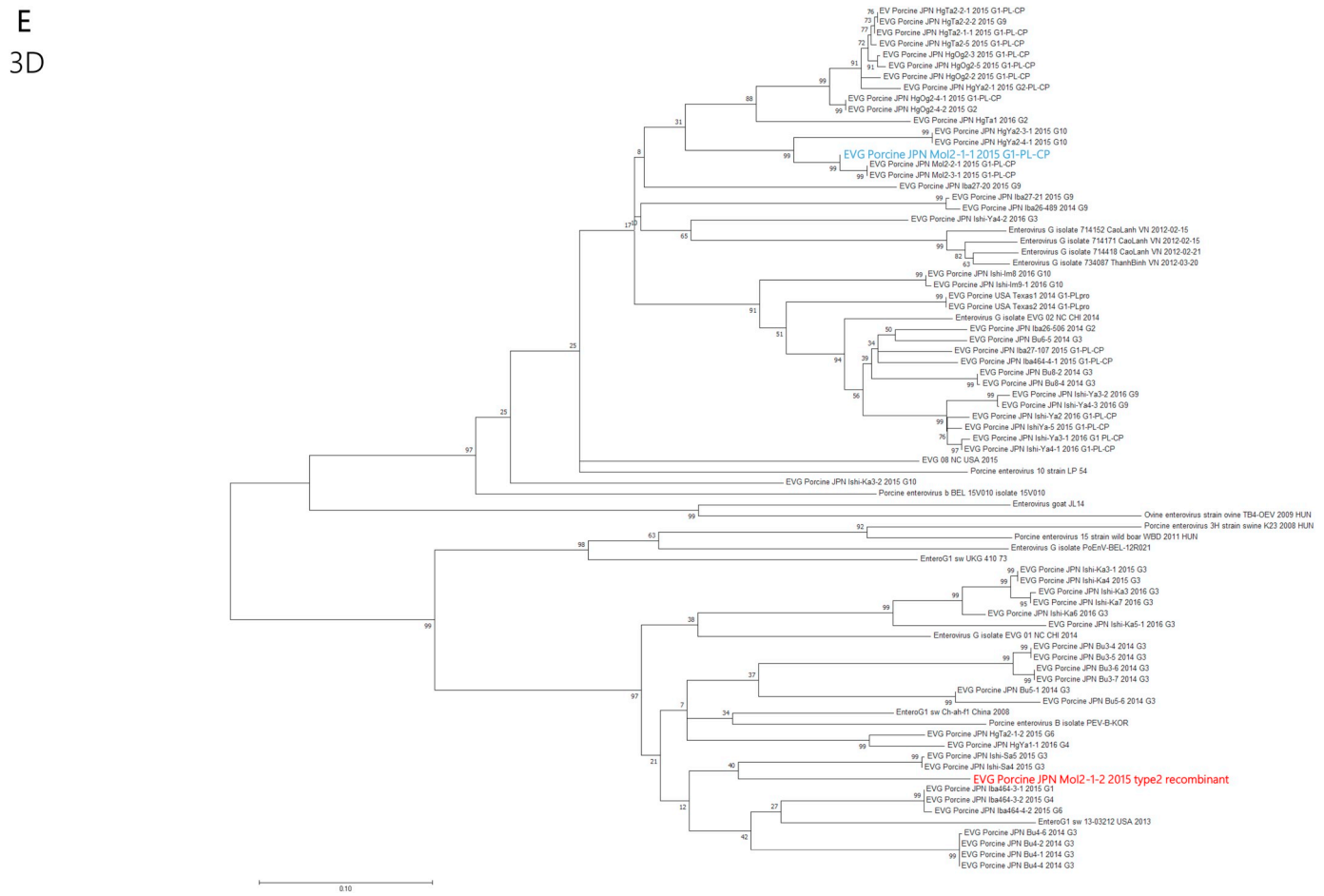

Fig. 4. (*continued*)

picornaviruses.

Although both type 1 and type 2 recombinant EV-Gs carry the PLCP gene derived from torovirus, location of the PLCP gene in the genome of the two recombinant EV-Gs differ (see Fig. 1A). As PLCP is known to have deISGylation activity (Shang et al., 2017), retention of torovirus PLCP gene in the two different recombinant EV-G types imply that the PLCP gene may suppress host innate immune functions, facilitating survival of these recombinant EV-Gs. The cluster of PLCP was same between recombinant EV-G type 1 and the newly identified recombinant EV-G type 2 (Fig. 4F), suggesting that these two different types of EV-Gs acquired the PLCP by RNA recombination from same subtypes of toroviruses.

Unlike most of recombinant EV-Gs, the newly identified type 2 recombinant EV-G carried the unique region 2, which had a domain showing extensive amino acid homology with the BIR superfamily. The baculoviral inhibitor of apoptosis (IAP) protein facilitates viral replication by preventing apoptosis (Clem and Miller, 1994; Holmblat et al., 2014). Generally, IAP proteins contain one to three BIR domains, which play an important role in the anti-apoptotic function. IAPs have a RING domain, which relates E3 ubiquitin ligase activity for ubiquitination of target proteins degradation, at the C-terminal region (Crook and Clem, 1993; Morizane et al., 2005; Ni et al., 2015; Vaux and Silke, 2005). Possibly, the BIR-like domain and the PLCP gene inhibited apoptosis and host innate immune function, respectively, leading to efficient replication of the newly identified type 2 recombinant EV-G.

One unanswered question in this study was whether the protein region translated from the non-picornaviral genes of the newly identified type 2 recombinant EV-G undergoes protein processing. Coronavirus PLCP cleaves the viral gene 1 polyproteins through recognition of a LXGG motif, while PLCP of arterivirus, another nidovirus, recognizes sequence LIGG, TTGG or PSGG (Mielech et al., 2014). However, cleavage specificity of torovirus PLCP has not need identified. Accordingly, it is unclear whether the PLCP in the newly identified type 2 recombinant EV-G cleaves any regions in the protein region translated from the inserted foreign genes. Enterovirus 2A and 3CD cleave Y/G pair and Q/G pair, respectively (Earle et al., 1988), while these pairs were absent at junctions at unique region 1/PLCP, PLCP/unique region 2 and unique region2/truncated 2A as well as within the putative polyprotein translated from the non-picornavirus sequences. Moreover, the absence of GxCG motif conserved in chymotrypsin-like protease in 2A of type 2 recombinant EV-Gs suggests that the 2A proteinase function of the type 2 recombinant EV-G 2A was most probably defective. As enterovirus 2A induces cleavage in host proteins, absence of biologically active 2A potentially affect host environment, including translational status, and possibly affect viral gene expression. Taken together, it is unlikely that the protein region translated from the inserted foreign genes undergo processing by the truncated 2A and 3CD. If the torovirus PLCP does not induce a cleavage(s) into the polyprotein of the newly identified type 2 recombinant EV-G, a protein consisted of unique region 1, PLPC, unique region 2 and N-terminal truncated 2A,

PLCP F

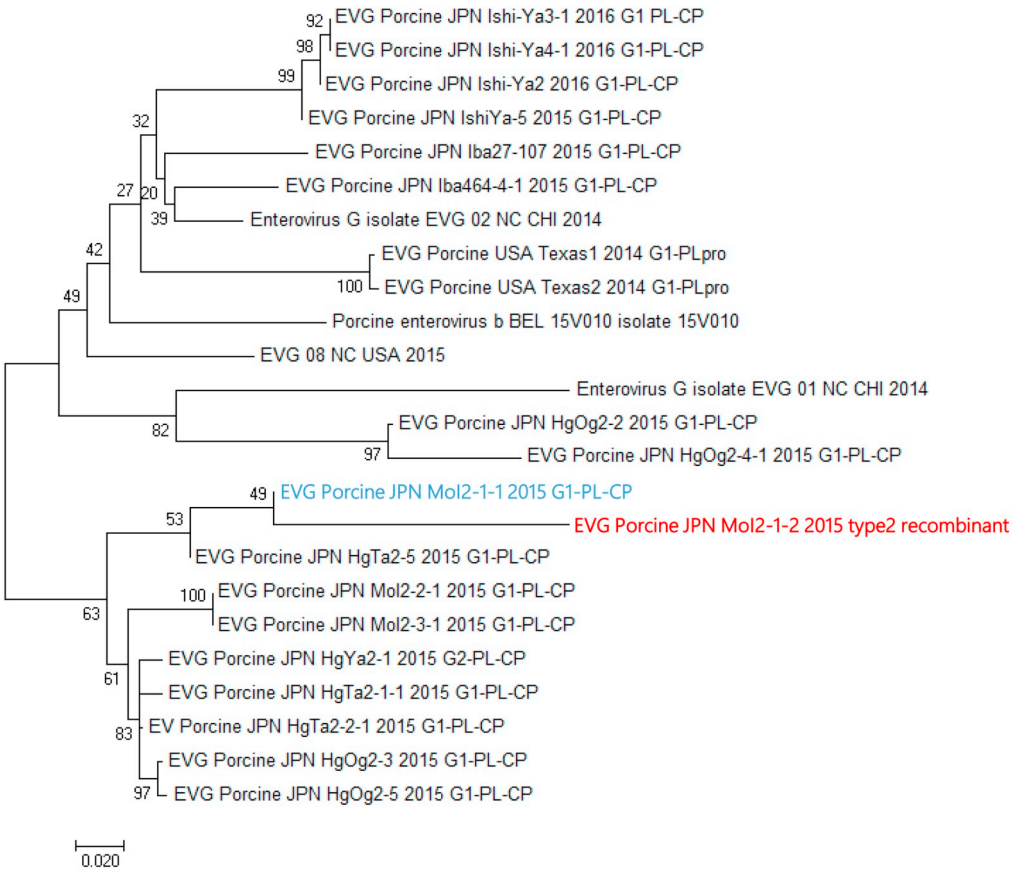

Fig. 4. (*continued*)

might have been accumulated in cells infected with the newly identified type 2 recombinant EV-G. If so, testing the deISGylation function and anti-apoptotic function of this putative protein would provide a clue as to a possible reason(s) for retention of these nonpicornavirus sequences in the type 2 recombinant EV-G. Alternatively, the putative polyprotein with the torovirus PLCP and the BIR domain may have another biological function that is important for replication of the newly identified type 2 recombinant EV-G.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103975.

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