Short Communication

Molecular evolutionary analysis of ORF3 and M genes of porcine epidemic diarrhea virus in HeiLongJiang province of China

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Abstract

In this present study, 11 porcine epidemic diarrhea viruses (PEDVs) were isolated from the fecal samples of piglets infected with PEDV in HeiLongJiang province of China. In order to analyze the molecular evolution of these re-emerging PEDVs, the ORF3 and M genes of 11 PEDVs were amplified by reverse transcriptase polymerase chain reaction, cloned, sequenced, and analyzed with each other as well as other PEDV reference strains. The complete nucleotide sequence comparison was performed with Bioedit software, and the results showed that the similarity of 11 ORF3 and M genes ranged from 97% to 100%. Nucleotides and amino acids were changed at some sites in the ORF3 and M genes of the 11 PEDV isolates when compared with CV777 reference strain, indicating that high level of variation may have occurred in the ORF3 and M genes of PEDV strains. To establish genetic relationships of the fully sequenced ORF3 and M genes, multiple sequence alignments were carried and phylogenetic analysis was performed. The results indicated that these PEDV isolates belonged to genotype 3.4 that is dominant subgroup in PEDV Group, showed a close relationship with some recent reported Chinese PEDV strains, and exhibited comparatively rapid genetic evolution, as revealed by these viruses responsible for the recent PEDV outbreak in HeiLongJiang province clustered closely together with other Chinese strains with rapid evolution rate. This study might be not only crucial to understand the currently prevailing PEDV strains in northeast of China, but also provides valuable data for preventing and controlling PEDV.

Keywords: Porcine epidemic diarrhea virus, Molecular evolution, Phylogenetic analysis, ORF3 gene, M gene

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Porcine epidemic diarrhea (PED) is a devastating swine disease that is characterized by acute enteritis and lethal watery diarrhea followed by dehydration leading to death with a high mortality rate in piglets (Debouck and Pensaert, 1980). The disease is caused by the PED virus (PEDV), which belongs to family *Coronaviridae*, genus *Alphacoronavirus*, and has an envelope surrounding a single-stranded positive-sense RNA genome (Pensaert and de Bouck, 1978). PEDV's genome is approximately 28 k (nt) length including a 5′untranslated region (UTR), a 3′ UTR and at least seven open reading frames (ORFs): ORF1a, ORF1b, and ORF2-6 (Song and Park, 2012). The remaining ORFs in the 3′ terminal region code for four major structural proteins, the 150-220 kDa glycosylated spike (S) protein, 20-30 kDa membrane (M) protein, 7 kDa envelop (E) protein, and 58 kDa nucleocapsid (N) protein. The two large ORF1a and 1b cover the 5′ two-third of the genome and encode the non-structural replicase genes (Rep) (Duarte and Laude, 1994). By the phylogenetic analysis of the partial spike glycoprotein genes, the PEDV isolates were divided into three groups (G1, G2, G3), which had three subgroups (G1-1, G1-2, G1-3) (Chen *et al*., 2008).

Since firstly reported in Belgium and the United Kingdom in 1971, PEDV has been reported in the worldwide pig industry (Song *et al*., 2015). In Asia, PEDV first occurred in 1982 in Japan and since then, PEDV caused severe epidemics in adjacent Asian countries, particularly in China and South Korea (Lee *et al*., 2010; Pan *et al*., 2012). The presence of PED in China was confirmed by the immunofluorescence assay and serum neutralization test in 1984 (Lee, 2015). It is reported that outbreak of PED with increased severity of diarrhoea, vomiting, and dehydration have occurred in China since 2010 (Ge *et al*., 2013; Yang *et al*., 2013). The disease approached a mobility rate as high as 100% and a mortality rate of 80-100% in piglets less than 10 days old, and has been recognized as a devastating illness causing death in neonatal piglets (Fan *et al*., 2017; Sun *et al*., 2012). New outbreaks associated with a novel PEDV strain that is genetically distant from the prototype PEDV strain, CV777, have been reported in China (Wang *et al*., 2016). Therefore, it is important to better understand the genetic variations and relationships between different strains, which would be benefit for finding out the reason of the continuously outbreak of PEDV and develop new strategy to control and prevent PEDV infection. The pigs of HeiLongJiang provinces located in northeast of China, were seriously affected and sustained great losses. In this study, porcine intestinal tissues and fecal specimens were collected from several swine farms in the HeiLongJiang province, China. Among those samples, we confirmed 11 positive PEDV using RT-PCR and sequence analysis. To our knowledge, PEDV M (Chen *et al*., 2008) and ORF3 (Wang *et al*., 2016) genes were widely used to investigate the genetic diversity and molecular epidemiology. Thus, molecular epidemiology and gene variation of these 11 positive PEDV were investigated using sequence analyses of the M and ORF3 genes to determine the reason for the recurrence of this disease and provide insights into the genetic evolution of PEDV strains circulating in HeiLongJiang province of China.

The collection of samples from the diseased pigs used in this study was approved by the Animal Care and Use Committee of the HeiLongJiang BaYi Agricultural University in China. In this study, intestinal samples were obtained from 3-day-old piglets with diarrhea from several farms in HeiLongJiang province, China. Extraneous transmissible gastroenteritis virus, rotavirus A, kobuvirus, porcine circovirus-2, and suid herpesvirus 1 were excluded, and 11 positive PEDV strains (Fig. 1A) were determined by RT-PCR method using the first primers of S/ORF3 (Wu *et al*., 2016). To obtain the ORF3 (Fig. 1B) and M (Fig. 1C) genes of PEDV, the other two pairs of specific primers were designed to amplify the sequence (Table 1), based on the genome of PEDV CV777 strain (GenBank No. AF353511). The strain CV777, initially characterized in 1978 (Debouck and Pensaert, 1980), has been widely propagated both within pigs and in cell culture and comprehensively accepted as the reference sequence in molecular evolutionary analysis. ORF3 gene and M gene of PEDV isolates used for nucleotide sequence analysis, amino acid sequence analysis and phylogenetic analysis were represented in supplemental Table 1 and supplemental Table 2, respectively. Nucleotide and the deduced amino acid sequences of ORF3 and M genes of 11 isolates were aligned and analyzed by the Bioedit software (Version 7.0.0) (Hall, 1999). The sequence data showed that the ORF3 genes of 11 PEDV isolates consisted of 675 nucleotides (nt) in length, which encoded 225 amino acid residues. M genes of 11 PEDV isolates consisted of 681 nt in length, which encoded 227 amino acid residues. The full-length of ORF3 and M genes were same long as other strains, such CV777, LZC and QH strains. The complete nucleotide sequence comparison showed the similarity ranged from 98.1% to 100% among 11 ORF3 genes. Nucleotide identities between CV777 and 11 ORF3 genes varied from 96.1 to 97%. At the same time, the complete nucleotide sequence similarity among the M genes was determined also as 98.7%-100%. Comparison of nucleotide sequences showed variable similarities between the 11 M genes and CV777, ranging from 97.5 % to 98.5% identity.

Based on the sequence analysis and comparison with reference strains, the nucleotide changes in ORF3 genes and M genes were represented in supplemental Fig. 1 and supplemental Fig. 2. Compared with CV777 strain, as showed in Table2, we observed the following nucleotide changes in all 11 ORF3 genes at: G54A, G160A, G235A and G301A; T62C, T162C, T360C and T393C; C63T, C237T, C243T, C264T, C274T and C450T; T238G and T546G; A497G. The additional nucleotide changes at different position in some isolates were observed. The nucleotide changes would lead to changes in the predict amino acid sequences of PEDV isolates. These ORF3 protein of 11 PEDV isolates had amino acids changes at poison V21A, V54I, V79I, F80V, L92F, A101T, N166S and H182Q. Furthermore, there were additional amino acid changes at different position in some isolates. Similarly, the nucleotide changes and deduced amino acids changes based on the complete M gene of all PEDV strains were analyzed. The results showed the nucleotide changes in 11 M genes as followings at: G37C; G180A; C198T,

C213T, C222T and C234T; T285C and T618C; T348A; A603C; G640T. These specific nucleotide changes of M genes led to amino acid changes at position E13Q, A214S. For the HLJ/HG-1/16, HLJ/HG-2/16, HLJ/JMS-1/16, HLJ/MDJ-1/16, HLJ/MDJ-2/16 and HLJ/QQHE-1/16 isolates, the amino acid changes were observed at position A79S and V129I. These data indicate that a comparatively high level of variation may have occurred in the ORF3 and M genes of PEDV strains which have prevailed in China in recent years.

In order to establish genetic relationships of the fully sequenced ORF3 and M genes, multiple sequence alignments were carried out using ClustalX 2.1 (Thompson *et al*., 1997), and phylogenetic analysis was performed using the MEGA 4 program (Tamura *et al*., 2007). Phylogenetic trees based on the nucleotide sequences of the ORF3 and M genes were generated using the neighbor-joining method, with bootstrapping over 1, 000 replicates. The evolutionary distances were computed using the Jukes-Cantor method and were presented as the number of base substitutions per site. The phylogenetic tree based on the ORF3 gene of 52 PEDV strains indicated that PEDV strains could be catalogued into three groups designated G1, G2 and G3 (Fig. 2). Group 3 can be divided into four subgroups. All the 11 PEDV strains identified in China belonged to Group 3.4, together with other strains isolated from 2011 to 2015, suggesting the majority of PEDV strains identified from 2011 to 2016 were in subgroup 3.4. Phylogenetic analysis based on the ORF3 gene shows that 11 PEDV strains have a close relationship with the other subgroup 3.4 members, such as recent reported isolates from Chinese and USA strains. At the same time, a total of 52 available M genes were used for further phylogenetic analysis. As shown in Fig. 3, the phylogenetic tree of the M genes indicated that the PEDV strains also fell into three groups. All the 11 PEDV strains in this study were also classified into subgroup 3.4. The phylogenetic tree based on M genes showed the similar characteristics with that based on the ORF3 genes, implying that group 3.4 was the dominant subgroup in the PEDV Group. The results suggested that the virus responsible for the recent PEDV outbreak in HeiLongJiang province clustered closely together with other Chinese domestic strains, including PEDV-F-J-Fuzhou, CH/XCYL/11, JXYC1302, and CH/HeN/PY-2015 strains. In addition, we performed Bayesian Markov chain Monte Carlo (MCMC, the BEAST package, version 1.5.1) analysis of the M and ORF3 genes to investigate the diversity of evolutionary rates. The results showed that mean evolutional rates of ORF3 and M genes obtained from 11 isolates exhibited 2.52×10^{-3} and 1.78×10^{-3} substitutions per site per year (subs/site/year), respectively, which were much higher than those in previous strains and demonstrated comparatively high level of variation. Taken together, these results suggested that PEDV strains were still widely prevailing in northeast of China, which belong to the genotype 3.4, and exhibits comparatively rapid variation and genetic evolution.

In conclusion, our study has determined the 11 complete ORF3 and M sequences of PEDV strains which are still prevalent in pig farms of HeiLongJiang province of China. The results revealed that nucleotides and amino acids were changed at some sites in the ORF3 and M genes of the 11 PEDV isolates. Phylogenetic analysis indicated that these PEDV isolates primarily belonged to genotype 3.4, showed a close relationship with some recent reported Chinese strains, and exhibits comparatively rapid variation and genetic evolution. This study might be not only crucial to understand the currently prevailing PEDV strains in northeast of China, but also provide valuable data for preventing and controlling PEDV.

Conflicts of interest: These authors declare that there is no conflict of interests regarding the publication of this article.

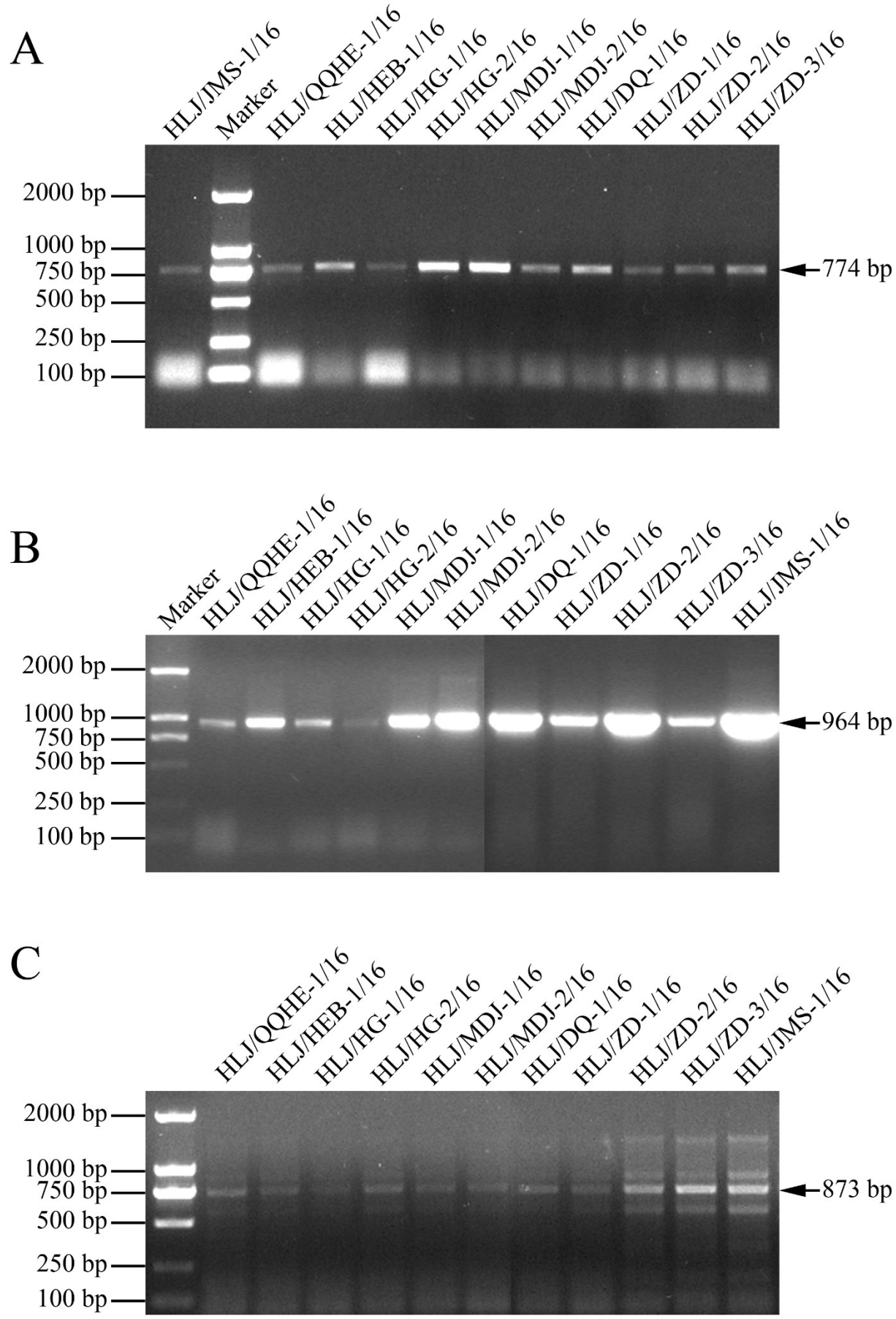


Figure 1 (A) Detection of 11 PEDV isolates using RT-PCR method to amplify the DNA fragments of the S/ORF3 genes. (B) Amplification of 11 ORF3 genes using RT-PCR. (C) Amplification of 11 M genes using RT-PCR.

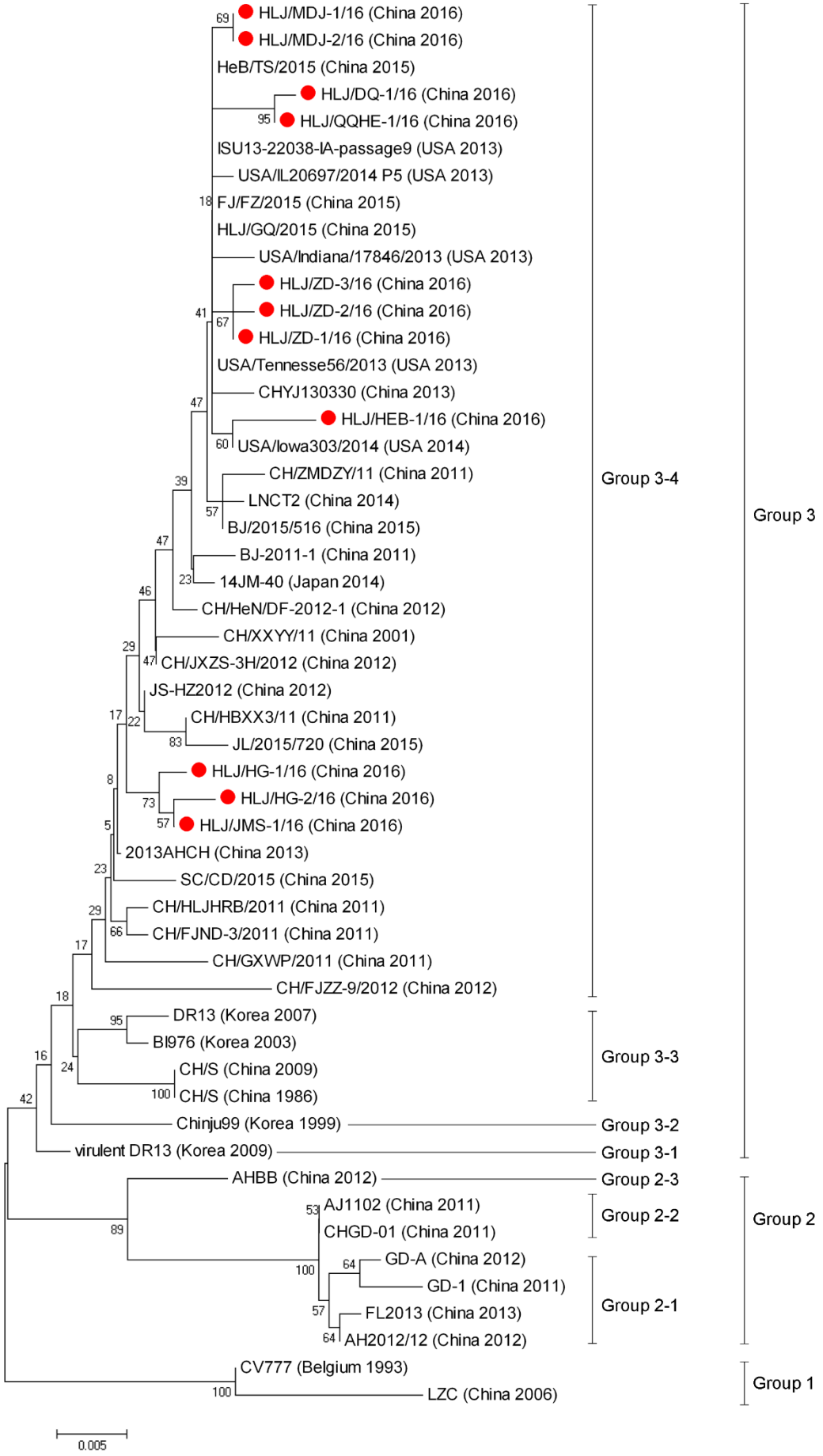


Figure 2 Phylogenetic analysis based on the nucleotide sequences corresponding to 11 ORF3 genes and reference strains. The phylogenetic trees were constructed by the neighbour-joining method. The 11 PEDV isolates evaluated in this study are labeled with red closed circle symbols "●".

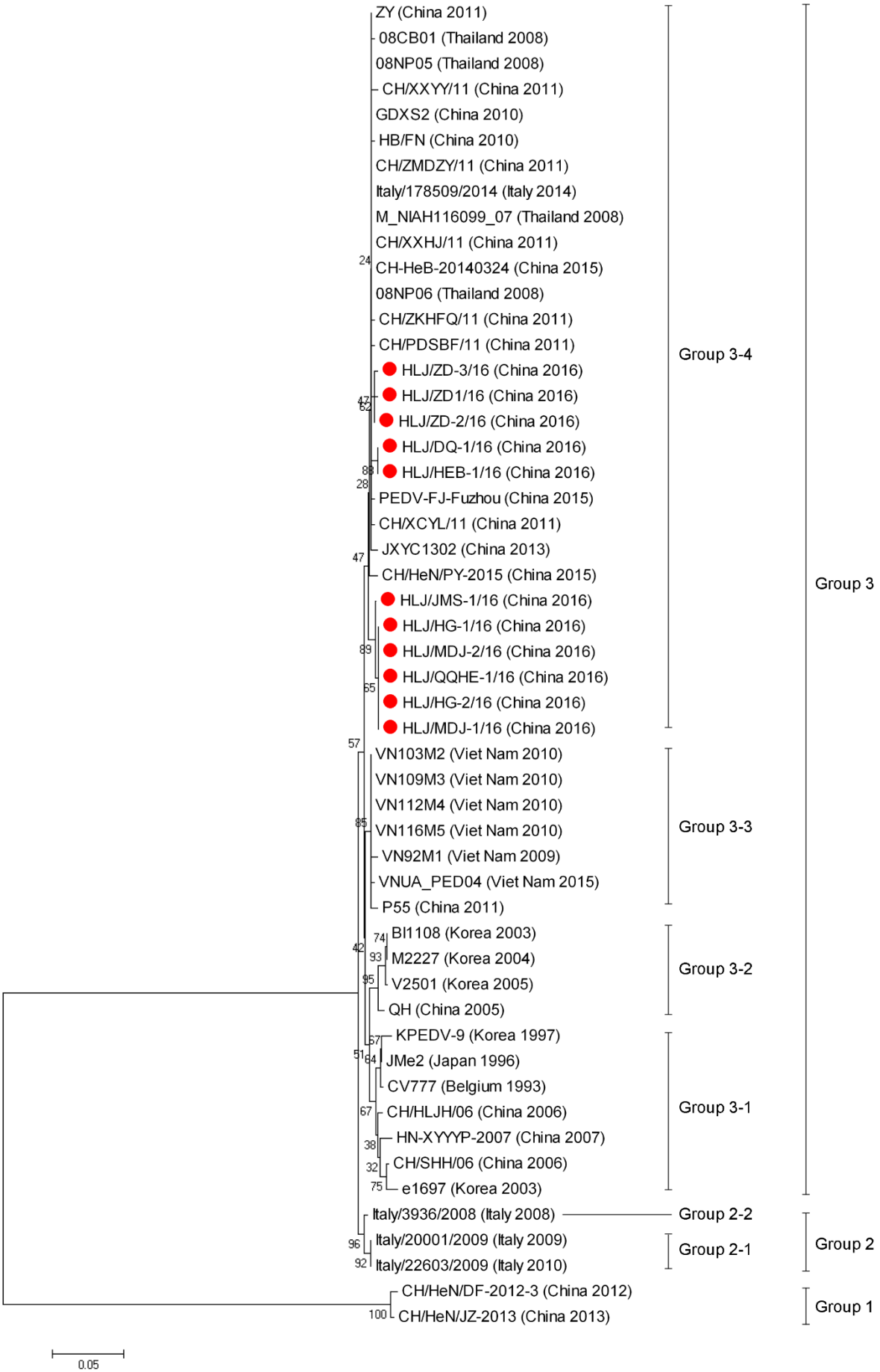
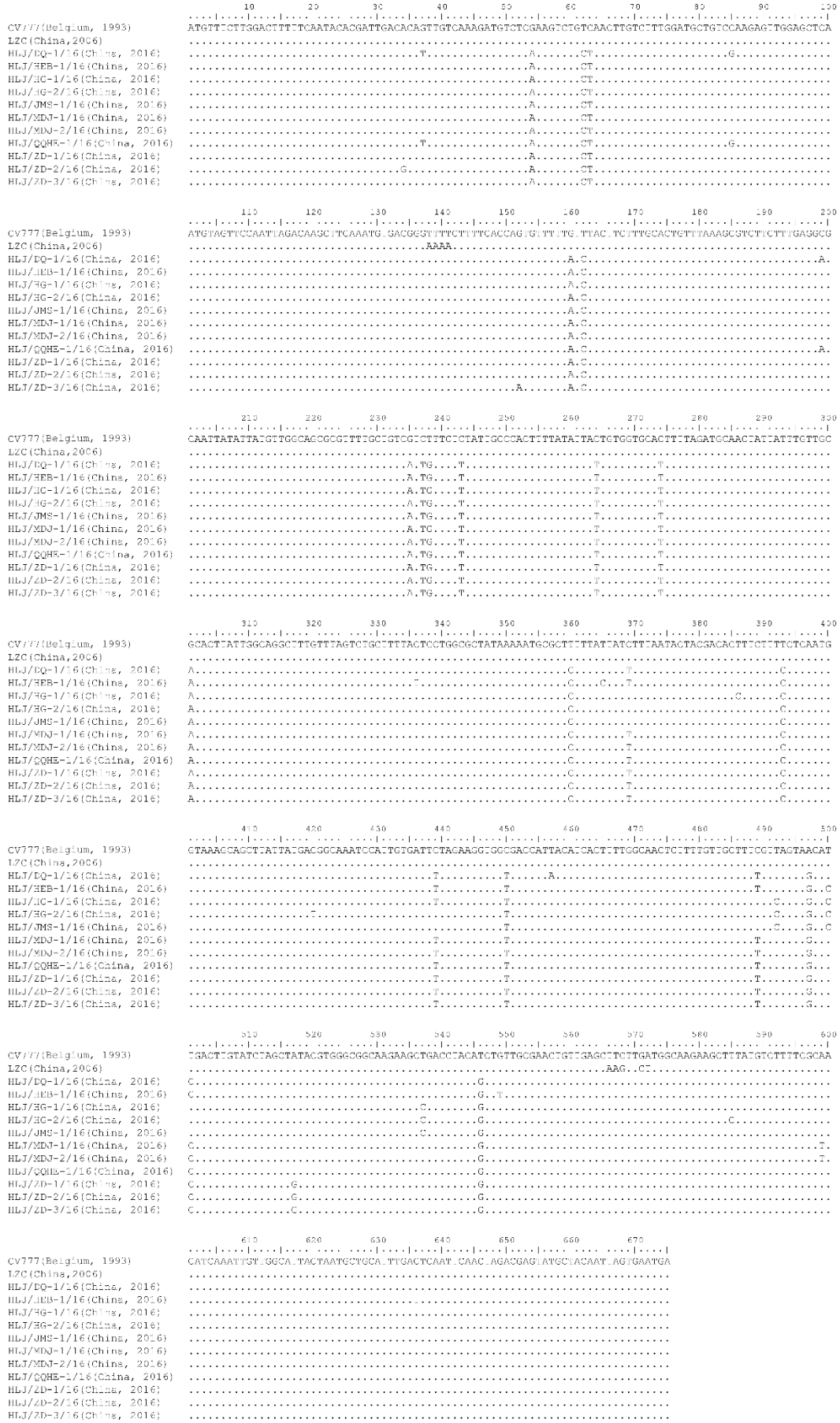
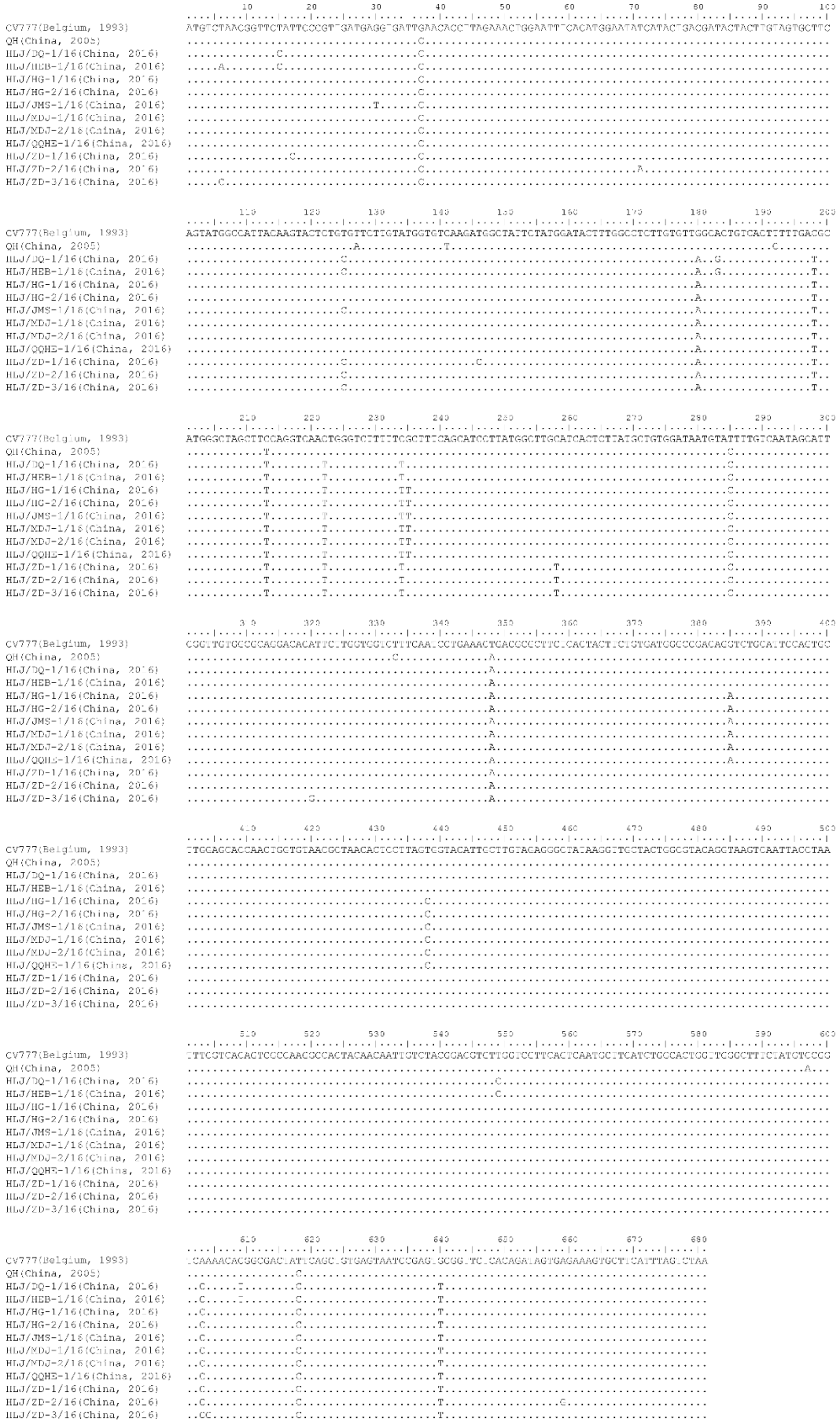


Figure 3 Phylogenetic analysis based on the nucleotide sequences corresponding to 11 M genes and reference strains. The phylogenetic trees were constructed by the neighbour-joining method. The 11 PEDV isolates evaluated in this study are labeled with red closed circle symbols "●".



Supplemental Figure 1 Comparison of the nucleotide sequences of the ORF3 genes of 11 PEDV isolates and PEDV reference strains by BioEdit software. The numbers indicate the location of nucleotides from the start codon of ORF3 gene. The dots (·) indicate regions where the sequences are identical to those of CV777 strain. Changed nucleotides are indicated with the letters (A, T, C, and G).



Supplemental Figure 2 Comparison of the nucleotide sequences of the M genes of 11 PEDV isolates and PEDV reference strains by BioEdit software. The numbers indicate the location of nucleotides from the start codon of M gene. The dots (·) indicate regions where the sequences are identical to those of CV777 strain. Changed nucleotides are indicated with the letters (A, T, C, and G).

Table 1 Primers used for detecting positive PEDV as well as amplifying ORF3 and M genes of PEDV.

Primers name	Orientation	Primer sequences	Target genes	Fragment sizes (bp)
S/ORF3-F	Forward	5′-CCTAGACTTCAACCTTACGA-3′	*S/ORF3*	774
S/ORF3-R	Reverse	5′-CAGGAAAAAGAGTACGAAAA-3′		
ORF3-F	Forward	5′-CGTGGTGGGTTTGGTTGAT-3′	*ORF3*	964
ORF3-R	Reverse	5′- CGGTGACAAGTGAAGCACAGAT-3′		
M-F	Forward	5′-TACATGCGAATTGACCCCCT-3′	*M*	873
M-R	Reverse	5′- ATCCTTGTTAGTGGGTACAGCG-3′		

Table 2 Sequence analysis of ORF3 gene and M gene.

Genes	nucleotide changes	amino acids changes
ORF3	G54A, G160A, G235A, G301A; T62C, T162C, T360C, T393C; C63T, C237T, C243T, C264T, C274T, C450T; T238G, T546G; A497G	V21A, V54I, V79I, F80V, L92F, A101T, N166S, H182Q
M	G37C; G180A; C198T, C213T, C222T, C234T; T285C, T618C; T348A; A603C; G640T	E13Q, A214S

Supplemental Table 1 The ORF3 genes of PEDV isolates used for sequence alignment, sequence analysis, and phylogenetic analysis.

Isolates	Accession number	Origin	Isolates	Accession number	Origin
HLJ/DQ-1/16	KX852326	China, 2016	CHGD-01	JX261936	China, 2011
HLJ/HEB-1/16	KX852336	China, 2016	GD-1	JX647847	China, 2011
HLJ/HG-1/16	KX852338	China, 2016	AHBB	JX910244	China, 2012
HLJ/HG-2/16	KX852322	China, 2016	CH/FJZZ-9/2012	KC140102	China, 2012
HLJ/JMS-1/16	KX852333	China, 2016	CH/ZMDZY/11	KC196276	China, 2011
HLJ/MDJ-1/16	KX852330	China, 2016	JS-HZ2012	KC210147	China, 2012
HLJ/MDJ-2/16	KX852329	China, 2016	USA/Indiana/17846/2013	KF452323	USA, 2013
HLJ/QQHE-1/16	KX852327	China, 2016	2013AHCH	KF495657	China, 2013
HLJ/ZD1/16	KX852337	China, 2016	ISU13-22038-IA-passage9	KF650375	USA, 2013
HLJ/ZD-2/16	KX852325	China, 2016	CH/JXZS-3H/2012	KF840541	China, 2012
HLJ/ZD-3/16	KX852324	China, 2016	CHYJ130330	KJ020932	China, 2013
CV777	AF353511	Belgium, 1993	USA/Tennesse56/2013	KJ645654	USA, 2013
LZC	EF185992	China, 2006	CH/HeN/DF-2012-1	KM048300	China, 2012
DR13	EU054929	Korea, 2007	FL2013	KP765609	China, 2013
Chinju99	EU792474	Korea, 1999	USA/Iowa303/2014	KR265827	USA, 2014
CH/S	GU372733	China, 2009	LNCT2	KT323980	China, 2014
BI976	HQ537433	Korea, 2003	USA/IL20697/2014 P5	KT860508	USA, 2013
CH/S	JN547228	China, 1986	14JM-40	KT968512	Japan, 2014
CH/XXYY/11	JN547401	China, 2001	SC/CD/2015	KU641644	China, 2015
BJ-2011-1	JN825712	China, 2011	HeB/TS/2015	KU641666	China, 2015
virulent DR13	JQ023161	Korea, 2009	FJ/FZ/2015	KU641669	China, 2015
CH/HLJHRB/2011	JQ027026	China, 2011	HLJ/GQ/2015	KU641671	China, 2015
CH/FJND-3/2011	JQ282909	China, 2011	JL/2015/720	KU641675	China, 2015
CH/GXWP/2011	JQ664298	China, 2011	BJ/2015/516	KU641677	China, 2015
CH/HBXX3/11	JQ664731	China, 2011	AH2012/12	KU646831	China, 2012
GD-A	JX112709	China, 2012	AJ1102	JX188454	China, 2011

The bold text in the table indicates that ORF3 gene of 11 strains were sequenced in our lab and submitted to GenBank.

Supplemental Table 2 The M genes of PEDV isolates used for sequence alignment, sequence analysis, and phylogenetic analysis

Isolates	Accession number	Origin	Isolates	Accession number	Origin
HLJ/DQ-1/16	KX852347	China, 2016	VN109M3	HQ883481	Viet Nam, 2010
HLJ/HEB-1/16	KX852340	China, 2016	VN112M4	HQ883482	Viet Nam, 2010
HLJ/HG-1/16	KX852343	China, 2016	VN116M5	HQ883483	Viet Nam, 2010
HLJ/HG-2/16	KX852344	China, 2016	HB/FN	JF508465	China, 2010
HLJ/JMS-1/16	KX852353	China, 2016	GDXS2	JN089723	China, 2010
HLJ/MDJ-1/16	KX852345	China, 2016	CH/ZMDZY/11	JN400898	China, 2011
HLJ/MDJ-2/16	KX852346	China, 2016	CH/XXHJ/11	JN400899	China, 2011
HLJ/QQHE-1/16	KX852339	China, 2016	CH/XCYL/11	JN400900	China, 2011
HLJ/ZD1/16	KX852349	China, 2016	CH/ZKHFQ/11	JN400901	China, 2011
HLJ/ZD-2/16	KX852350	China, 2016	CH/PDSBF/11	JN400903	China, 2011
HLJ/ZD-3/16	KY010201	China, 2016	CH/XXYY/11	JN400908	China, 2011
KPEDV-9	AF015888	Korea,1997	VN92M1	HQ883479	Viet Nam, 2009
CV777	AF353511	Belgium, 1993	P55	JQ723736	China, 2011
QH	AY974335	China, 2005	ZY	KF150217	China, 2011
JMe2	D89752	Japan, 1996	JXYC1302	KJ526112	China, 2013
CH/HLJH/06	EU033964	China, 2006	CH/HeN/DF-2012-3	KM048301	China, 2012
CH/SHH/06	EU033966	China, 2006	CH/HeN/JZ-2013	KM048303	China, 2013
HN-XYYYP-2007	EU287429	China, 2007	CH/HeN/PY-2015	KR078317	China, 2015
M_NIAH116099_07	EU542417	Thailand, 2008	Italy/3936/2008	KT027417	Italy, 2008
08CB01	FJ196166	Thailand, 2008	Italy/20001/2009	KT027419	Italy, 2009
08NP05	FJ196176	Thailand, 2008	Italy/22603/2009	KT027420	Italy, 2009
08NP06	FJ196177	Thailand, 2008	Italy/178509/2014	KT027429	Italy, 2014
BI1108	FJ687451	Korea, 2003	CH-HeB-20140324	KT799996	China, 2015
e1697	FJ687454	Korea, 2003	PEDV-FJ-Fuzhou	KX253991	China, 2015
M2227	FJ687456	Korea, 2004	VNUA_PED04	LC101732	Viet Nam, 2015
V2501	FJ687458	Korea, 2005	VN103M2	HQ883480	Viet Nam, 2010

The bold text in the table indicates that M gene of 11 strains were sequenced in our lab and submitted to GenBank.

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