博士論文

Molecular Evolution of Human Enterovirus D68 in the Philippines

フィリピンにおけるエンテロウイルス D68 の遺伝子進化

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1. SUMMARY

Increased detections of enterovirus D68 (EV-D68) have been reported from many parts of the world, which pose a significant health threat since the virus is reported to be associated with severe respiratory illness and neurological complications such as acute flaccid paralysis (AFP). However, the reasons for increasing detection of EV-D68 are still unclear.

In the Philippines, the increased number of cases with EV-D68 among patients with acute respiratory infections had been described in previous reports during 2008–2011[2,3]. To monitor the continuous evolution of EV-D68 in the country, I followed up the study conducted among pediatric patients hospitalized with severe acute respiratory infection (sARI) from August 2012 to February 2014, which included the cases in areas affected by the typhoon Haiyan (Yolanda) in November 2013. EV-D68 was screened by polymerase chain reaction (PCR) using 5'untranslated region (UTR)-specific primers. The genetic evolution was further analyzed by nucleotide sequencing based on partial viral capsid protein (VP) 1. I identified 20 EV-D68-positive cases among 1,854 hospitalized patients with sARI. The detection rate of EV-D68 in this study was 1.0% (20/1,854). Interestingly, thirteen EV-D68-positive cases were detected after the typhoon. Next, phylogenetic tree was constructed to compare the VP1 gene sequences of the EV-D68 strains, which showed that the samples were divided into two distinct sublineages: A11 (in lineage 3) and PL13 (in lineage 2). All EV-D68-positive samples collected before May 2013 were classified into the A11 sublineage, which also consisted of strains from Thailand and China collected in 2011 and 2012. However, EV-D68-positive samples after October 2013 formed a distinct sublineage, PL13. Although the strains in the PL13 sublineage were closely related to the strains collected in

Thailand in 2011. This sublineage was clearly distinct. This study showed that EV-D68 has been circulating in the population, and more positive cases were found in the areas devastated by typhoon.

When combined with the previous studies, four outbreaks of EV-D68 had been identified in the Philippines during 2008–2015. This study also aimed to gain insight into molecular characteristics of EV-D68 that have been circulating in the Philippines since 2008 and to infer its genetic relationship with viruses from other countries in Asia and other regions. I conducted molecular analyses of 442 sequences of VP1 collected from the Philippines and other countries in Asia, and other regions from 1962 to 2015. Phylogenetic analysis of these sequences revealed that most of recent large outbreaks occurred from 2012 to 2015 in North America, Europe, and Asia were caused by viruses in lineage 2. Most of Asian viruses in lineage 2 were clustered separately from North American and European viruses. EV-D68 sequences identified in the Philippines were similar to sequences from other countries mainly in Asian (e.g. China, Hong Kong, Japan, and Taiwan). However, the lineage 3 includes sequences from different countries in different regions during 2007–2015. Significant clustering by continent was observed in lineage 2 than in lineage 3. Whole genome analysis indicated that capsid VP1 of the viruses showed the most variable protein in which particular residues on antigenic BC and DE loops of the recent lineage 2 viruses were influenced by positive selection without statistical significance. The viruses in lineage 2 had genetic changes during its expansion in recent years. This study revealed changing trends of virus population size for each lineage in Asia from 2005 to 2015. Lineage 1 viruses exhibited increasing trend in genetic diversity since 2005. Lineage 2 viruses showed fluctuating pattern in genetic diversity while lineages 3 viruses maintained its high genetic diversity throughout the period.

2. **INTRODUCTION**

Acute respiratory infection (ARI) is one of the most common illnesses throughout the world and presents the major cause of morbidity and mortality of all ages. The ARI can be caused by a variety of pathogens, including bacteria and viruses. Among respiratory RNA viruses, a significant increase of enterovirus D68 (EV-D68) have been reported from many parts of the world, which pose a significant health threat since the virus is reported to be associated with severe respiratory illness and neurological complications such as acute flaccid paralysis (AFP). With the recent improvement of molecular techniques, several studies have revealed a high genetic diversity of the viral genes. However, it is still unknown if any virological factors are involved in increased detection and association with more severe illness. Therefore, it is necessary to understand molecular mechanism that might be responsible for changing epidemiology and pathogenesis of the virus.

Virological characteristics

Virion structure and genome organization

Enterovirus D68 (EV-D68) strains (designed as the Fermon, Rhyne, Franklin, and Robinson strains) were first isolated from four pediatric patients hospitalized with lower respiratory tract infections in California, United States in 1962. All patients had clinical and x-ray evidences of pneumonia and bronchiolitis. Four viruses were then investigated for their characteristics and the virus, represented by Fermon virus, was found to be antigenically distinct from currently known enteroviruses and other respiratory viruses. The novel picornavirus associated with respiratory infection was subsequently proposed in 1967[5].

EV-D68 particle is a spherical, non-enveloped with the size of about 30 nanometers in diameters as observed under an electron microscope (Figure 1A and 1B). Since the virus particles lack a lipid envelope, its infectivity is insensitive to organic solvents[6]. In general, enteroviruses (EVs) carry a positive single-stranded ribonucleic acid (RNA) genome, approximately 7,500 nucleotides in length that encodes structural proteins VP1 to VP4 and non-structural proteins 2A, 2B, 2C, 3A, 3B, 3C, and 3D (Figure 2). Genomic RNA is covalently linked at the 5'end to a protein called VPg (virion protein, genome linked)[7,8]. In poliovirus, the VPg was reported as a primer for RNA synthesis[1,9]. The 5' untranslated region (UTR) of EVs is long (approximately 600 to 1,200 nucleotides depending on the virus) and highly structured which contains sequences that control genome replication and translation[7]. The 5'UTR contains an internal ribosomal entry site (IRES) that directs translation of the messenger RNA (mRNA) by internal ribosome binding[6]. In addition, the 5'UTR is relatively conserved genomic region among EVs so that is used as a target region for molecular screening by reverse transcription-polymerase chain reaction (RT-PCR). The majority of the EV-D68 detections reported in the recent years, which also included EV-D68 reported in the Philippines, were based on positive results from RT-PCR and DNA sequencing by using primers specific for the 5'UTR of the virus[8,10-15]. The P1 region encodes the viral capsid proteins. The capsid of EV-D68 is comprised of four structural proteins of which the shell is formed by VP1, VP2, and VP3 while VP4 lies on an internal surface (Figure 3). The VP1 capsid is generally considered as the most variable protein of the enterovirus genome[6]. It forms an eight stranded, antiparallel β-barrel (also known as a β-barrel jelly roll) of B, C, D, E, F, G, H, and I (Figure 4). The variable BC and DE loops of VP1 protein are recognized as the neutralized antigenic sites of the virus which can be exposed to the host immune system and influence pathogenesis or immune respond to infection[6]. The P2 and P3 regions encode

proteins involved in protein processing (2Apro, 3Cpro, 3CDpro) and genome replication (2B, 2C, 3AB, 3CDpro, 3Dpol)[6] (Figure 2).

Classification

EV-D68 is a member of the *Enterovirus* genus of the *Picornaviridae* family. The genus *Enterovirus* is mainly divided into 12 species, including EV species A (EV-A), EV-B, EV-C, EV-D, EV-E, EV-F, EV-G, EV-H, EV-J, Rhinovirus A (RV-A), RV-B and RV-C by Picornaviridae study group (available at http://www.picornaviridae.com/enterovirus/enterovirus.htm, accessed by October, 2016). At present, EV-D consists of 5 serotypes, including EV-D68, EV-D70, EV-D94, EV-D111 (from both humans and chimpanzees), and EV-D120 (from gorillas), as shown in Table 1. Moreover, human rhinovirus 87 (HRV87) was confirmed to share both biological and molecular properties to EV-D68[16] and was then reclassified as a strain of EV-D68 by Picornaviridae study group (available at http://www.picornaviridae.com/enterovirus/ev-d/ev-d.htm). Due to the fact that EVs are genetically and antigenically diverse, serotypes have been commonly determined by neutralization tests using polyclonal antibodies. More recently, the RT-PCR and genomic sequencing is used as a common method to determine serotypes and further genetic characterization. Especially, VP1 region is commonly used to determine serotypes and genotypes of EVs[17,18]. Molecular analyses based on VP1 nucleotide sequences among the more recently circulating EV-D68 strains revealed 3 major genetic groups. The genetic groups of EV-D68 in this study are designated as lineages 1, 2, and 3 based on the previously published classification[19]. Multiple nucleotide alignment of VP1 region showed that all the lineage 3 strains exclusively had a deletion of 3 nucleotides coding for an

amino acid (Asparagine; N) at the nucleotide positions 2806–2808 in VP1 region (Figure 5B), corresponding to the Fermon strain (Figure 5A).

Regarding to the 5'UTR of the EV-D68, the genetic variation has been reported between the end of IRES and the polyprotein open reading frame (ORF) among viruses in 3 genetic groups[1,10,20,21]. Viruses belonged to lineages 1 and 2 (Figure 5C) were reported to have nucleotides deletions at positions 681−704 (24-nucleotides deletion) and 721−731 (11-nucleotides deletion) while lineage 3 (Figure 5B) had 24-nucleotides deletion only at positions 681−704 comparing to the Fermon strain (Figure 5A).

Acid sensitivity

EV-D68 was initially reported as an acid stable virus[5]. However, the virus was later reported with acid sensitive feature which was confirmed by similar experiments[16]. Accordingly, a study conducted by Oberste et al.[13] in 2004 shown that EV-D68 isolates (Fermon; the prototype strain, MN89; Minnesota 1989, MN98; Minnesota 1998, MD02-1; Maryland 2002, TX01; Texas 2001, and TX02; Texas 2002) grew poorly at 37°C relative to growth at 33°C and the virus titres were reduced by incubation at pH3.0. In contrast, the control virus (Echovirus 11) grew equally well at 33°C and 37°C and the virus titre was not affected by treatment at pH3.0. Likewise, these characteristics are found in common among Cardioviruses, enteroviruses (except rhinoviruses) hepatoviruses, and parechoviruses of the Picornaviridae family which are acid stable and maintain infectivity at pH values of 3.0 and lower[6]. The acid lability and temperature sensitivity of EV-D68 shared common biological characteristics with rhinovirus, which is a well-known cause of respiratory infection[9,13,16]. Differences in pH stability influence the sites of replication of the virus[6]. These shared features might underlie mechanisms for their respiratory tract tropism[1].

Neutralizing antigenic site

Basically, the surface of viral capsid contains the major neutralization antigenic sites which consisted of the amino acid residues that are recognized by antibodies that block viral infectivity[6]. Several reports have identified various amino acid sequences located in BC and DE loops which are associated with antigenic epitopes among enteroviruses[22-26]. Studies on antigenic variation of enterovirus A71 (EV-A71) have shown that amino acid changes in residues on antigenic sites might lead to viral structure changes, resulting in binding alteration[22,26,27]. Regarding to EV-D68, the previous study showed that the antibody level against EV-D68 collected from pregnant women serum in Finland in 1983, 1993, and 2002 had decreased by times, suggesting for a possible antigenic drift among the circulating EV-D68 strains[28]. An accumulation of amino substitutions on predicted antigenic sites has been noted between the Fermon virus and circulating strains from 1989 to 2015[29-36]. A study[37] in 2015 showed a difference in neutralization titres of Fermon antiserum against different EV-D68 strains (14-18949, 14-18952, and 14-18953 of lineage 2), which were isolated from outbreaks in the United States in 2014. This evidence suggests that antigenic epitopes of Fermon strain differed from the EV-D68 strains that have been co-circulating in the epidemic areas.

Global distribution of EV-D68

EV-D68 was first reported in 1962 in the United States[5]. Since then, the virus was found in a few numbers of clinical cases. Only 26 EV-D68 cases were detected from enterovirus surveillance in the United States during 1970–2005[17]. Until late 2000s, evidences of respiratory infection associated with EV-D68 have been more frequently reported from different part of the world, including China[38,39], England[8], France[40], Gambia[21], Italy[41-43],

Japan[19,20,44], Netherlands[33,45], New Zealand[46], Philippines[2,3], Senegal[21], South Africa[21], Thailand[31], and United States[21,47], as summarized by Imamura and Oshitani[1] (Table 2). However, an increase of EV-D68 might be affected by reporting bias or improvement of molecular detection method. Retrospective studies had been conducted by testing EV-D68 in stored respiratory samples collected in the Netherlands during 1994–2010[33] and in Japan during 2005–2009[19]. The report from the Netherlands revealed that EV-D68 positive samples were less than 10 cases per year between 1994 and 2009 while a total of 24 EV-D68 positive samples were detected solely in 2010[33]. An increasing trend of EV-D68 was also reported in Japan that EV-D68 was found in 10 samples in 2005, 1 sample in 2006, 2 samples in 2007, 2 samples in 2009, and 40 samples in 2010[19]. Therefore, the findings from both studies confirmed the actual increase of EV-D68 in recent years.

During 2013–2014 in the North America, the outbreaks of EV-D68 associated with respiratory illness in the United States and Canada was reported with more than 1,000 confirmed cases[48]. Like polioviruses (PVs) and EV-A71, EV-D68 also occasionally infects central nervous system (CNS), resulting in acute flaccid-like symptom which can lead to death. Neurological diseases-associated with EV-D68 infection such as AFP and Guillain-Barre syndrome (GBS) have been occasionally reported from England[49], France[50], Norway[51] and United States, in which such severe cases were found especially among children ages under 5 years old[52]. The findings emphasized that the virus is not only an emerging respiratory pathogen but also a major concern due to EV-D68-associated acute flaccid myelitis during the post eradication of poliovirus.

Recent epidemiological surveillance demonstrated that EV-D68 lineages 2 and 3 were responsible for the global EV-D68 outbreak during 2013–2014[4,10,14,30,31,34,36,53]. The European surveillance showed a co-detection of lineages 2 and 3 viruses across 12 countries during

July–December in 2014. But majority viruses belonged toEV-D68 lineage 2, which was genetically related to the North America strains detected between 2013 and 2014[34]. Co-circulation of EV-D68 lineages 2 and 3 has also been documented recently in various countries in Asia[4,10,30,31]. The genetic changes of these recently circulating viruses compared to the prototype Fermon strain have been noted[27,29,34,35,52], indicating that EV-D68 has been constantly evolving. The recent large outbreaks with more severe complications including acute flaccid myelitis that occurred in the North America, Europe, and Asia have raised the question of whether viral mutations are responsible for the increased in incidence and severity. However, it is still unknown if any virological changes such as antigenicity, viral fitness and pathogenesis had recently occurred.

In the Philippines, studies on respiratory viruses have been conducted among patients with severe acute respiratory infection (sARI) and influenza-like illness (ILI). Rhinovirus and respiratory syncytial virus were commonly detected viruses in these studies[15]. EV-D68 was detected unexpectedly by PCR targeting for 5'UTR of rhinoviruses and it was then confirmed by sequence identity to previously reported EV-D68. From the study, EV-D68 was revealed to be a possible causative agent for 21 cases with severe respiratory illnesses during mid May 2008–mid May 2009 with two deaths[2]. Continuous monitoring of the virus identified subsequent outbreaks of EV-D68 especially among severe cases[3,4]. These findings confirm the public health impact of EV-D68 in the Philippines. Therefore I did further molecular characterization of EV-D68 in the Philippines. In total, four outbreaks of EV-D68 had been identified in the Philippines during 2008–2015 (Figure 6). EV-D68 lineages 2 and 3 viruses had been co-circulating in 2008 and 2011. Thereafter, the re-emergence of individual EV-D68 lineage was documented; lineages 3 virus was reported during November 2012–May 2013 and a large

number lineage 2 virus was reported during October 2013–March 2014. Moreover, sporadic cases of lineages 2 and 3 viruses were found during late 2015. In previous studies, the distribution of all three major lineages (lineages 1, 2, and 3) had been documented in Japan (2005–2010)[19], Italy (2008–2012)[41-43] and the Netherlands (2011–2014)[33,35], indicating continuous co-circulation of EV-D68 in countries. Also, evolutionary analyses revealed sequences diversity among the emerged viruses[21,31,54], leading to the identification of novel clades in recent years[14,36]. Therefore, genetic analysis of EV-D68 collected in different periods of time would provide better understanding on the molecular evolution of the virus.

3. **OBJECTIVES**

In order to draw the overall picture of EV-D68 in this Philippines, this study aimed to gain insight into molecular characteristics of EV-D68 in the Philippines and to infer its genetic relationship with viruses from other countries in Asia and other regions. This study also aimed to reveal evolutionary dynamics of EV-D68 in Asia over the past 10 years.

4. METHODOLOGIES

4.1 Sample collection

A total of 1,854 nasopharyngeal swabs (NPS) were collected from patients hospitalized with severe acute respiratory infection (sARI) from 3 hospitals in the Philippines; Eastern Visayas Regional Medical Center (EVRMC; Tacloban City, Leyte), Biliran Provincial Hospital (BPH; Naval, Biliran), and Ospital ng Palawan (ONP; Puerto Princesa City, Palawan) between September 2012 and February 2014.

This study was approved by the Ethics Committee of Tohoku University Graduate School of Medicine, Japan and the Institutional Review Board of the Research Institute for Tropical Medicine, Philippines. The written informed consent was obtained from parents or guardians of all children involved in the study.

4.2 Molecular detection and DNA sequencing

Viral genome extraction was conducted using the QIAamp Viral Mini Kit (Qiagen) and complementary DNA (cDNA) was then synthesized by RT reaction using M-MLV and random primers (Invitrogen) according to the manufacturer's instruction (Invitrogen). All samples were tested for respiratory viruses, including EV-D68, adenovirus, cytomegalovirus, human metapneumovirus, influenza virus, parainfluenza virus, respiratory syncytial virus, rhinovirus, and other enteroviruses (including coxsackievirus, echovirus, and poliovirus), by PCR using previously described methods[15]. EV-D68 was detected by a primer pair DK001[12] and DK004[11] targeting 5'UTR of rhinoviruses and enteroviruses genome. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and sequenced using Big Dye Terminator version 3.1 cycle sequencing kit and Genetic Analyzer 3730 (Applied

Biosystems, Foster City, the United States). EV-D68 from clinical samples in the Philippines was confirmed based on a high sequence identity shared with those of EV-D68 reference strains available in GenBank database using NCBI BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM). All EV-D68 samples were further characterized to determine genetic lineages by PCR and sequence analysis targeting partial VP1 using two primer pairs of EV68-VP1F and EV68-VP1R[2], and VP1-RCF and 485[13]. Also, all EV-D68 samples were chosen for a whole genome sequencing using genes-specific primers as shown in Table 3.

4.3 Phylogenetic analyses of EV-D68

4.3.1 Molecular characterization of EV-D68 in the Philippines from 2008 to 2015

In order to determine the relationship of EV-D68 sequences in the Philippines, I re-analyzed the Filipino sequences collected during 2008–2011[2,3] with the additional sequences obtained from EV-D68 samples during 2013–2015.

A phylogenetic tree based on the genetic sequences of partial VP1 regions (546 nucleotides) was inferred using the maximum-likelihood method based on the Tamura 3-parameter (T92) model with gamma (G) distribution implemented in Molecular Evolutionary Genetics Analysis version 6 (MEGA6) software[55].

4.3.2 Genetic relationship between EV-D68 detected in the Philippines and worldwide strains from 1962 to 2015

This analysis included a total of 442 partial VP1 sequences (546 nucleotides), which consisted of 52 sequences from Philippines during 2008–2015 and 390 sequences (selected based on sampling dates and locations, as shown in Table 4) from other countries during

1962–2015, as of July 2016. Maximum-likelihood phylogenetic trees were constructed under T92 + G model implemented in MEGA6 software[55]. Statistical support was evaluated by bootstrap analysis with 1,000 replicates and bootstrap values of over 80% are shown at nodes.

To investigate if there is clustering of EV-D68 sequences by place or time, I further analysed the spatial (continent and country) and temporal (year) distribution of EV-D68 circulating worldwide which were calculated by using the association index (AI)[56] and parsimony score (PS)[57] available in Bayesian Tip-Significance Testing (BaTS) software[58]. The posterior sets of trees (PSTs) were constructed by using the previous VP1 dataset. This analysis was run for 50 million (of all lineages), 30 million (of lineage 2), and 15 million (of lineage 3) generations with subsampling every 50000, 30000, and 15000 iterations, respectively. The average standard deviation of split frequencies value was less than 0.1, indicating convergence and the first 10% of tree states were then removed as burn-in. The strength of phylogenetic clustering by traits was indicated by the index ratio of estimated the observed to expected (null) values.

4.4 Evolutionary analyses of EV-D68 in Asia

To determine the genetic relationship of EV-D68 circulating in Asia, a total of 266 partial VP1 sequences (546 nucleotides) from 7 countries and regions were collected from GenBank as of July 2016. The dataset consisted of sequences from Japan (n = 77, 2005–2015), Taiwan (n = 35, 2007–2014), Philippines (n = 52, 2008–2015), China (n = 37, 2008–2015), Thailand (n = 6, 2009–2011), Hong Kong (n = 54, 2010–2014), and Malaysia (n = 5, 2012). A phylogenetic tree was constructed using the maximum-likelihood method based on T92 + G model implemented in MEGA6[55].

To further investigate the phylodynamics of EV-D68 circulating in Asia, the temporal change in genetic diversity of the virus was estimated using the strict molecular clock with Gaussian Markov random-fields (GMRF) Bayesian skyride model[59] as a tree prior as implemented in the BEAST package[60]. The most appropriate model was selected based on the lowest value of the Akaike's information criterion through Markov chain Monte Carlo (AICM), which provides a better fit to the data. Bayesian Markov chain Monte Carlo (MCMC) chains were run for 100 million generations with subsampling every 10000 iterations under the General Time Reversible (GTR) + G substitution model. A 10% burn-in was removed. All effective sample size (ESS) values were more than 300, which indicated sufficient mixing to achieve convergence. The uncertainty of the estimates was shown by 95% highest-posterior density (HPD) intervals by using Tracer ver1.6. The outputs were then generated to describe the population dynamics of EV-D68 in Asia over time.

4.5 EV-D68 whole genome sequence analysis

The phylogenetic relationships of EV-D68 genome were constructed from partial 5' UTR and complete sequence of individual gene, including VP1, VP2, VP3, VP4, 2A, 2B, 2C, 3A, 3B, 3C, and 3D. A total of 67 randomly selected sequences (registered sequences in GenBank as of July 2016) were used in this analysis. To evaluate sequence variability of whole EV-D68 genome, 11 datasets of protein-coding genes (VP1-VP4, 2A-2C, and 3A-3D) were used and consensus sequences were generated by using Consensus Maker (http://www.hiv.lanl.gov/content/sequence/CONSENSUS/consensus.html). Deduced amino acid sequences were analysed further in MEGA6 software. Sequence variability scores per coding protein were calculated relative to individual protein consensus sequence obtained from each dataset. In addition, complete VP1 amino acid sequence was used to evaluate the

amino acid variability within VP1 capsid protein by Shannon entropy (Hx) analysis. This analysis was implemented in BioEdit software[61]. The score of zero (0) indicated a complete conserved amino acid at individual site in VP1 capsid while a value of greater than 0.2 was considered variable.

4.6 Selection analysis

The previous datasets of whole sequences were used to define the selective pressure on specific codons across the whole genome of EV-D68 circulating worldwide. The ratio of non-synonymous to synonymous (dN/dS) substitution per site (ω value) was determined using the single likelihood ancestor counting (SLAC) method implemented in the Hypothesis testing using the Phylogenies (HYPHY) package[62]. The amino acid position, in which ω value is higher than 1, is considered to be a positively selected site. The significant level for selections was accepted at p-value <0.05. For each codon site, the difference (dN-dS) is indicated instead of dN/dS value because there is a possibility of dS value of zero (0), which results an undetermined ratio[62]. Codons with normalized dN-dS value higher than 0 were considered as positive selected.

4.7 Mapping of amino acid substitutions on the VP1 protein model.

The structure of full-length VP1 protein was inferred based on the Fermon strain (4WM8)[63] as a modelling template in this study. The individual amino acid sequences were subjected into SWISS-MODEL homology modelling sever (http://swissmodel.expasyorg/ workspace/index.php?func=modelling_simple1&userid=USERID&token=TOKEN) in order to generate the Protein Data Bank (PDB) file. PyMOL software (http://www.pymol.org) was

used to visualize and map the amino acid mutations on the antigenic BC and DE loops of VP1 protein.

5. RESULTS

5.1 EV-D68 detection

A total of 1,854 NPS collected from hospitalized patients with severe acute respiratory infection (sARI) between September 2012 and February 2014 were tested for the presence of respiratory viruses. In this study, EV-D68 was detected at 1.0% (20 of 1,854). The most common viral pathogen identified among sARI cases was respiratory syncytial virus (29.3%), followed by rhinovirus (14.0%) and influenza virus (5.1%). Among three study sites (Leyte, Billiran, and Palawan islands), interestingly, thirteen EV-D68 positive cases were found after the typhoon hit (Figure 7 and Table 5). The ages of EV-D68-positive patients ranged from 1 month to 4 years (median, 14 months).

In terms of coinfection, this study found 3 cases positive for both EV-D68 and respiratory syncytial virus. One patient (2 months of age) coinfected with the viruses died during the study period. All the other patients who were infected with EV-D68 recovered. During this study period, 35% (7 of 20) of EV-D68-positive sARI patients exhibited wheezing.

5.2 Molecular characterization of EV-D68 in the Philippines from 2008 to 2015

To understand the genetic relationship of EV-D68 in the Philippines, VP1 sequences identified in the Philippines during 2008–2015 were analyzed. Phylogenetic analysis showed that EV-D68 sequences in the Philippines were classified into two lineages, lineages 2 and 3. None of EV-D68 sequence belonging to lineage 1 was detected in this study (Figure 8). The Filipino sequences obtained in 2008 and 2011 formed a distinct cluster in lineage 2 while the recent EV-D68 sequences obtained from 2012 to 2015 were divided into two distinct

sublineages, including A11 (in lineage 3) and PL13 (in lineage 2). All EV-D68-positive samples collected before May 2013 were classif.ied into the A11 sublineage, which also consisted of strains from Thailand and China collected in 2011 and 2012. However, EV-D68-positive samples after October 2013 formed a distinct sublineage, PL13. Although the strains in the PL13 sublineage were closely related to the strains collected in Thailand in 2011, this sublineage was clearly distinct. Notably, I found amino acid substitutions in the predicted antigenic sites of the VP1 gene in the PL13 sublineage: threonine (T) to alanine (A) at position 98 (T98A) in the BC loop and methionine (M) to valine (V) at position 148 (M148V) in the DE loop. In contrast, any of these amino acid substitutions were not observed among lineage 2 sequences identified in 2008 and 2011 in the Philippines. Sporadic cases of lineages 3 viruses were detected recently in 2015. These lineage 3 sequences were more closely related to sequences identified in France and Spain in 2014, and Philippines in 2011 than those sequences in A-11 sublineage.

5.3 Genetic relationship between EV-D68 detected in the Philippines and worldwide strains from 1962 to 2015

A total of 52 EV-D68 sequences of partial VP1 gene from the Philippines between 2008 and 2015 were analyzed along with 390 sequences from 23 countries and regions in Africa, Asia, Europe, North America, and Oceania from 1962 to 2015. Phylogenetic analysis of these sequences revealed that recent EV-D68 strains were classified into three major genetic groups; lineages 1, 2, and 3 (Figure 9A).

Most of recent large outbreaks occurred from 2012 to 2015 in North America, Europe, and Asia were caused by viruses in lineage 2. Distribution of multiple EV-D68 lineages was observed in many countries, for instance lineages 1, 2 and 3 in Italy, Japan, and

the United States; lineages 2 and 3 in China, France, Netherlands, Philippines, Spain, Taiwan, and Thailand. In this analysis, each of lineages 2, and 3 was further divided in to various sublineages which were named after continents, including Asia (AS), North America (NA), Europe (EU), and worldwide (W). Sequences from the Philippines were divided into 4 sublineages; AS07 and AS13 of lineage 2 and W07 and W08 of lineage 3 (Figure 9A).

Within lineage 2 (Figure 9B), EV-D68 identified in the Philippines in 2008 and 2011 were classified into the AS07 sublineage, which also includes sequences from Taiwan detected between 2007 and 2008. Sequences of lineage 2 identified during 2013–2015 in the Philippines were classified as AS13, which includes sequences from other countries mainly in Asian and some sequences from Canada. Most of Asian viruses in lineage 2 were clustered separately from North American and European viruses, which formed distinct clusters as NA14 and EU09 sublineages, respectively.

In addition, the lineage 3 viruses in the Philippines collected in 2008, 2012, and 2013 were classified into W08 sublineage while the viruses collected in 2011 and 2015 were clustered in W07 sublineage (Figure 9C). Two lineage 3 viruses (TTa-11-Ph224 and TTa-11-Ph272) detected in 2011 clustered with viruses from Europe and Oceania (New Zealand), while other two strains, TB8-15-Ph508 and TB9-15-Ph380 (in the yellow box), detected in 2015 formed a distinct genetic group, which shared only 93% identity with the viruses detected in 2011 (Figure 9C). The W07 and W08 sublineages of lineage 3 included sequences from different countries in different regions during 2007–2015. Furthermore, analysis of spatial and temporal structures of EV-D68 showed a significant clustering by continent in lineage 2 which was more strongly observed than in lineage 3 (AI, $p<0.01$; PS, $p<0.01$). However, a strong temporal clustering was not observed in this study (Table 6).

5.4 Evolution of EV-D68 in Asia

In this study, I analysed 266 VP1 sequences (546 nucleotides) of EV-D68 collected from China, Hong Kong, Japan, Malaysia, Philippines, Taiwan, and Thailand during 2005–2015 (Table 7). EV-D68 sequences consisted of lineage 1 (n = 51), lineage 2 (n = 145), and lineage 3 (n = 70). As shown in Figure 10, phylogenetic tree showed the obvious clustering by countries. To further investigate the evolution dynamic of EV-D68 lineages circulating in Asia, the strict clock model with GMRF Bayesian skyride prior was performed to determine the changes in genetic diversity or population size of virus through times. The evolutionary tree (Figure 11E) and the GMRF skyride plots show the continuous expansion of lineage 1 since 2005, which was detected in Japan for the first time in Asia. However, it was not detected after 2010 (Figure 11A and 11B). The trend of lineage 2 showed fluctuation in genetic diversity over time with the highest peak in 2014 (Figure 11C), when the significant increase of EV-D68 cases in North America and Europe was also observed. On the other hand, the high genetic diversity of lineage 3 was relatively constant over the same time period (Figure 11D).

5.5 EV-D68 whole genome sequence analysis

Phylogenetic trees of EV-D68 that were constructed from a total of 67 sequences of all genes showed a similar pattern of lineage clustering with VP1 (Figure 12). Analysis of deduced amino acid sequence of EV-D68 genome showed that VP1 protein is the most variable protein (amino acid positions 553–861) (Figure 13A) with the highest substitution density of 27.8% compared to other proteins, which ranged from 1.2 to 22.7% (Table 8). Two amino acid sites, 98 in BC loop (T98A) and 148 in DE loop (M148V), had highest frequency of amino acid substitution, which were mainly observed among lineage 2 viruses detected in

recent years (Figure 14). In addition, Shannon entropy analysis of VP1 amino acid residues further supported the high genetic variability accumulating on antigenic BC and DE loops (Figure 13B).

5.6 Evolutionary selection

Using the previous dataset of 67 whole genome sequences, the ratio of dN/dS substitution (ω) was calculated. Using the SLAC method, result showed that the evolution of EV-D68 is driven by strong purifying selection ($\omega < 1$), with mean dN/dS values ranged from 0.03 to 0.10 in all lineages (Table 9). Codon with normalized dN-dS values higher than 0 was considered as positive selected. In antigenic BC and DE loops of VP1 region, residues 97 (BC loop), 103 (BC loop), 141 (DE loop) and 148 (DE loop) were found under positive selection without statistical significance ($p < 0.05$) (Figure 15).

5.7 Amino acid diversity among recently circulating strains compared to the prototype Fermon virus

The VP1 protein structure of EV-D68 showed amino acid variability in antigenic BC and DE loops that are located on the external surface of the capsid complex when compared to the Fermon virus (Figure 16). At least 5 amino acid substitutions were found on each antigenic loop (indicated in yellow). Notably, the amino acid substitutions at positions 98 of BC and 148 of DE loops (indicated in red) were found among viruses in lineages 2 in recent years.

5.8 Nucleotide sequence accession numbers

Nucleotide sequences of EV-D68 identified in the Philippines (2012–2015) and Sendai, Japan (2015) reported in this study were deposited in the GenBank database under accession numbers AB992413–AB992443 and KX789218–KX789267.

6. DISCUSSION

The recent increase of EV-D68 cases worldwide including severe complications emphasizes the importance of deeper understanding of the molecular evolution of the virus. This study described the molecular characteristics of EV-D68 detected in the Philippines and their relationship with viruses in other countries especially in Asia.

In this study, the detection rate of EV-D68 was found at 1%, which is slightly different from previous reports at 2.6% in 2008[2] and 0.23% in 2011[3]. During the study period, Typhoon Haiyan (Yolanda), which hit the Philippines on 8 November 2013[64], caused extensive damage to large areas of the country, including some of study sites. The study sites included EVRMC in Tacloban City, which is located on Leyte Island, one of the municipalities most severely damaged by the typhoon. It should be noted that research projects were temporarily discontinued after the typhoon; thus, samples were unable to be collected continuously in this region. Among twenty EV-D68-positive cases during 2013–2014, thirteen cases were observed after the typhoon hit (Table 5). It is, however, unclear whether the typhoon had any impact on EV-D68 circulation. Infectious disease outbreaks after major disasters have been reported. For example, a huge outbreak of cholera was documented after the earthquake in Haiti in 2010, which is believed to have been introduced to the country by the relief teams[65]. Factors including population displacement, inadequate basic needs and sanitations facilities after natural disasters are likely to favor for disease transmission[66].

Phylogenetic analysis of EV-D68 identified 3 major lineages 1, 2, and 3 in different countries and regions. This study found the strong geographic clustering in lineage 2 viruses but not in lineage 3 viruses. Within lineage 2 (Figure 9B), phylogenetic analysis showed that

viruses in AS07 sublineage include strains detected in Taiwan between 2007 and 2008[67] and the Philippines in 2008 and 2011[3]. These AS07 viruses were the first lineage 2 viruses that were identified during 2007–2008. It is possible that these viruses may be the origin of the recent expansion of lineage 2. However, it is difficult to draw any conclusions regarding the location of the emergence of lineage 2 viruses due to the small number of registered sequences before 2010. Sequences in lineage 2 showed strong geographic clustering with most sequences in AS13 were detected in Asia, most of NA14 in North America and most of EU09 in Europe. This suggests that a strong geographic clustering of lineage 2 viruses might reflect the recent expansion of these viruses in Asia, Europe, and North America. However, this analysis found small clusters of sequences from Japan and North America which grouped together with European (EU09), sequences from France grouped with North American (NA14), and sequences from Canada[68] grouped with Asian (AS13) sublineages, suggesting an inter-regional transmission. In addition, phylogenetic analysis showed the presence of two sublineages in lineage 3; W07 and W08 (Figure 9C). Most of viruses in the W07 sublineage were detected before 2012 while many viruses in the W08 sublineage were detected during 2012–2014. The W08 sublineage was recently proposed as the novel genetic group by another classification system[36], known as clade D. In this study, Filipino sequences identified in 2008, 2012, and 2013 of W08 sublineage were also classified into the clade D, which had unique residues, 92T in BC loop and 143N and 148V in DE loop, when compared to clade A[36] or W07 sublineage of lineage 3. This result indicates a distinct in genetic diversity of EV-D68 lineage 3 circulating worldwide.

Selective pressure analysis of whole genome sequences showed that the viral evolution was under strong purifying selection ($p < 0.05$). A high degree of amino acid conservation implies that EV-D68 is likely to maintain its proteins structure and function. In

general, genes encoding structural proteins that contain important domains such as neutralizing epitopes and antigenic sites are more considered across the viral genome. In this study, VP1 protein showed the highest frequency of mutations among protein-coding genes (Figure 13) and four positions in antigenic sites of VP1 region, including residues 97, 103, 141, and 148 were under positive selection (without statistical significance) (Figure 15). The result is consistent with one previous study in Hong Kong in which residues 97 of BC loop showed the similar finding[30]. In other studies, selection analyses of each lineage showed that substitutions under selective pressure were found frequently among viruses in lineage 2[32] or clade B[36] and could differentiate this lineage/clade from others. These observations clearly indicate the evolution by increased mutations among the outbreak EV-D68 strains. In addition, amino acid substitutions, T98A and M148V, in VP1 are localized in antigenic BC and DE loops (Figure 14), which are considered for receptor interaction and neutralizing immunogenicity[32,63]. These two amino acid mutations were detected among the recently expanding lineage 2 viruses which were clustered in AS13, NA14, and EU09 sublineages (Figure 9B). The M148V substitution was observed commonly in AS13, NA14, and EU09 sublineages, which is consistent with other reports[30,34,36]. It is of note that amino acid substitution in antigenic BC and DE loops of VP1 had the effect on neutralization activity among enteroviruses. A single amino acid mutation of VP1 protein at position 84 (BC loop) of coxsackie B4 virus[24] and at positions 98 (BC loop), 145 (DE loop) and 165 of EV-A71[22] led to neutralization reduction against patients's antisera. Amino acid changes in these residues possibly caused the antigenic changes of viruses which might explain for the impaired reactivity with the specific antibodies. Regarding to EV-D68, previous studies showed the differences in neutralization titres of Fermon antiserum against EV-D68 lineages 1, 2, 3[32] and the United States outbreak strains[37]. In addition, another report showed that

there is a limited cross reactivity against antisera generated for EV-D68 lineages 1, 2, 3 collected from Yamagata prefecture in Japan in 2010 and Philippines in 2011[32]. These evidences indicate that the recent strains are antigenically different from the prototype Fermon virus and also are antigenically diversified. Therefore, antigenic variations might be one of the possible reasons for its expansion in recent years.

Several studies have investigated whether genetic changes among recent EV-D68 could possibly be associated with more severe clinical manifestations and complications[14,27,52,53]. Neurological diseases-associated with EV-D68 infection such as AFP and Guillain-Barre syndrome (GBS) have been reported from France[50], Norway[51], United Kingdom[49], and United States[52]. AFP was mainly associated with clade B1 (lineage 2) viruses and some amino acid substitutions that have been found as neurovirulence mutations of polioviruses were identified[52]. In addition, some predicted mutations were suggested to alter the protease cleavage efficiency that might be a potential factor for an increasing rate of EV-D68 replication[14]. However, these possible associations have not been confirmed to date. It is possible that other factors such as antigenic change and different virus fitness might also play a significant role.

In this study, phylodynamic analysis showed the changing trends of virus population size of each lineage in Asia from 2005 to 2015. In other studies, the overall trend of population size of EV-D68 appeared to increase as three lineages had emerged and spread rapidly in the Netherlands[33] and worldwide[21] during 2010 which are concordant with result in this study. It suggests that the evolutionary pattern of EV-D68 in Asia represents the global trend. All findings clearly imply large outbreaks that had occurred in recent years. In Asia, phylodynamics analysis identified 4 peaks of viral population in lineage 2, which is consistent with increased detection of EV-D68 in Philippines[2] and Taiwan[67] in 2007–2010,

China[39], and Hong Kong[30] in 2011–2012, Philippines in 2013[4], and China[10], Hong Kong[30], Taiwan[53], and Japan (unpublished) in 2014–2015 (Figure 11A and 11C). However, this phenomenon was not observed in lineage 3. Previously, the trend of worldwide lineage 3 was found to increase in genetic diversity[21]. The discrepancy might be caused by the analyzed dataset. This study analyzed only strains from Asia while the previous study analyzed strains from over the world which contained several sequenced strains identified from other countries outside Asia, including Gambia, Senegal, South Africa, and United States. A large proportion of these strains might reflect the increasing trend in worldwide dataset. The lineage 3 viruses in Asia may have been continuously circulating in the population and have maintained their high genetic diversity over time. In some countries, EV-D68 lineage 1 was the most predominant virus, for instance in Japan during 2005–2010[19,20]. This coincides with the sharp increase in genetic diversity in 2010 (Figure 11A and 11B). EV-D68 lineage 1 was also detected in Italy in 2008[43]. Thereafter, detection of EV-D68 lineage 1 had been rarely reported worldwide. There is only one lineage 1 virus detected after 2010: (CA/RESP/09-871) from the United States in 2013. Phylogenetic tree showed that lineage 1 strains detected in Japan, Italy and the United States shared high homology based on VP1 sequences (Figure 9A). To summarize, the temporal structure of EV-D68 at the early period might be represented by the spread of lineage 1 virus. The fluctuated trends, which were mainly triggered by large scale outbreaks of lineage 2 and sustained circulation of lineage 3 viruses, were then observed during 2010–2015. These finding indicated that EV-D68 from Asian countries exhibited dynamics changes in genetic diversity of multiple EV-D68 lineages over time.

Limitations of this study include the discrepancy of sampling time period and surveillance system among countries. In this study, the lineage replacement is not clearly

observed. It is possible that lineage 1 viruses circulate at subclinical level, which may explain its non-detection in recent outbreaks. However, this possibility remains to be elucidated. Long-term observation of EV-D68 in different geographic regions with improved laboratory diagnostic may contribute a clearer picture of genetic distribution and evolutionary dynamics of EV-D68 worldwide.

In conclusion, this study highlights on the molecular characteristics of worldwide EV-D68 and provides the better understanding on the evolutionary mechanisms associated with the global spread of multiple lineages in recent years. High frequency of genetic mutations within predicted antigenic sites may play an important role among epidemic strains circulating in communities. This study also revealed the dynamic signature among EV-D68 lineages in Asia over time.

7. CONCLUSION

This study reported the increasing number of EV-D68 cases, in particular, after the typhoon Haiyan hit the country during 2013–2014. Furthermore, phylogenetic analysis showed an expansion of lineage 2 and 3 viruses across countries in recent years. EV-D68 lineage 2 identified in the Philippines in 2008 and 2011 formed a unique cluster while others identified during 2013–2015 were genetically related to strains from other countries mainly in Asia. A strong spatial clustering was found among lineage 2 viruses by continent. But such a pattern was not observed for viruses in lineage 3. This study described the evolutionary dynamics of EV-D68 circulating in Asia over a decade. GMRF skyride coalescent analysis revealed an early expansion of lineage 1. Genetic diversity of lineage 2 viruses was fluctuating, while that of lineage 3 viruses was stable. These finding indicated that EV-D68 from Asian countries exhibited dynamics changes in genetic diversity of multiple EV-D68 lineages over time. Whole genome analyses of selected viruses identified frequent amino acid substitutions in BC and DE loops of VP1 which are considered for receptor interaction and neutralizing immunogenicity. This study provides a better understanding on EV-D68 evolution in the recent years. Further research on the interplay between genetic diversity and virus antigenic variation and evolutionary dynamics of EV-D68 in different countries will advance current knowledge of potential mechanism that might be responsible for changing epidemiology and pathogenesis of the virus so that better control strategies can be established.

8. REFERENCES

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9. FIGURES

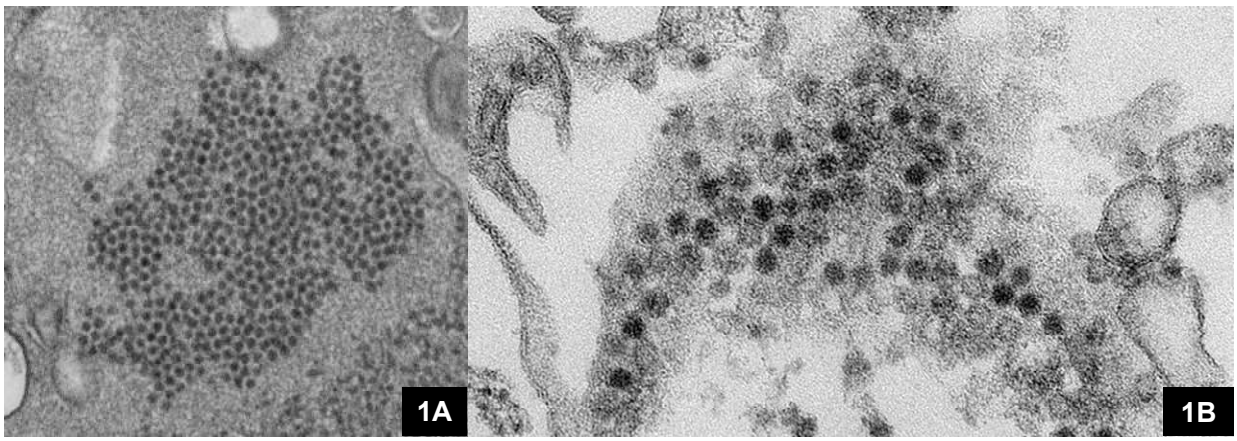


Figure 1 Electron micrograph of EV-D68 particles. A thin section of EV-D68 **(Fig 1A and 1B)** showing the numerous, spherical viral particles. The images are kindly contributed by Cynthia S. Goldsmith and Yiting Zhang, CDC (available at http://www.cdc.gov/non-polio-enterovirus/resources-ev68-photos.html).

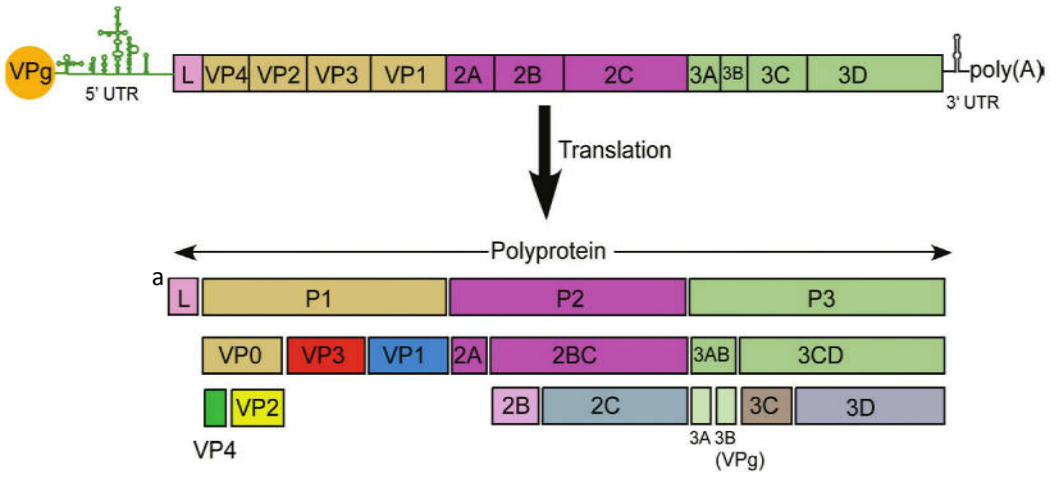


Figure 2 Organization of picornavirus[b] genome. Viral genomic RNA has a viral protein (VPg) at the 5' untranslated region (UTR) containing the internal ribosomal entry site (IRES), the protein coding regions, the 3' UTR containing a pseudoknot, and the poly(A) tail. Coding regions for the viral proteins are indicated. The P1 region encodes the structural polypeptides. The P2 and P3 regions encode the nonstructural proteins associated with replication[6].

Notes:

[a]L (Leader protein) is found in other genus e.g. erboviruses, cardioviruses, and aphthoviruses which also belong to *Picornaviridae* family.

[b]Picornavirus is a virus belonging to the *Picornaviridae* family, including enterovirus D68 (EV-D68).

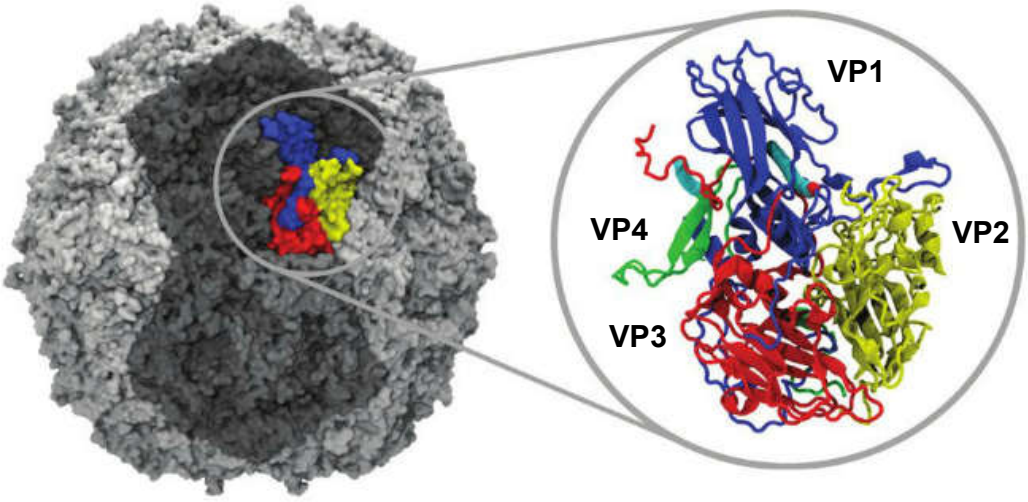


Figure 3 Schematic of enterovirus capsid showing the packing of VP1 (blue), VP2 (yellow), VP3 (red) on the surface. VP4 (green) is on the inner capsid (adapted from[6]).

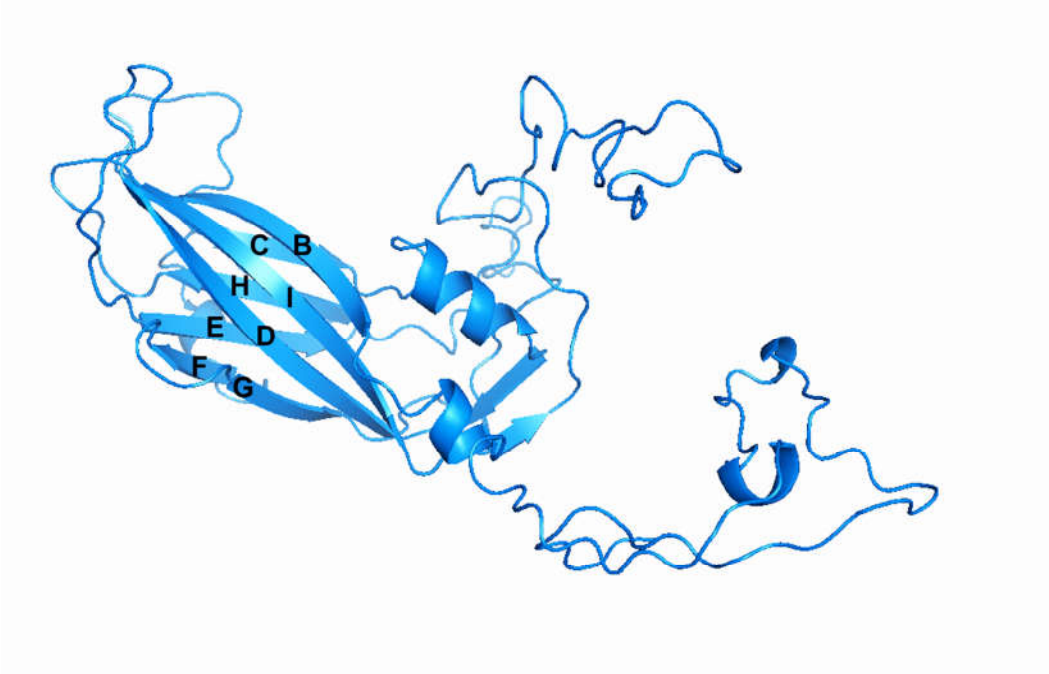
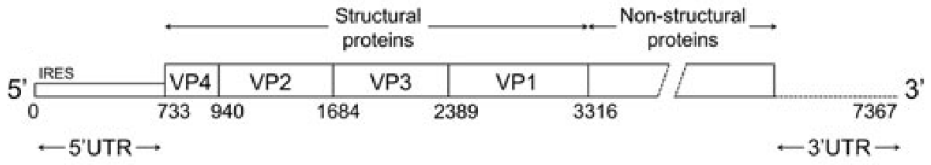
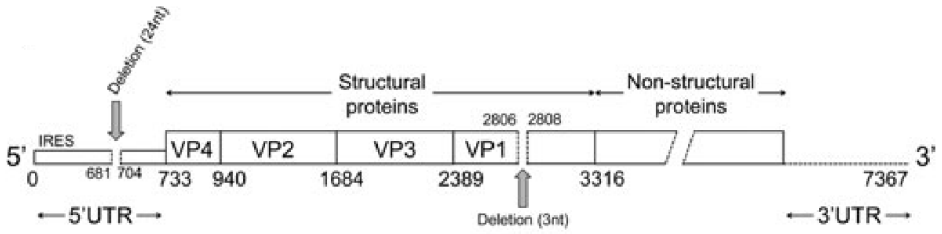


Figure 4 The ribbon diagram of VP1 β-barrel jelly roll in EV-D68. One of the sheets is composed of the antiparallel strands BIDG and the other by the antiparallel strands CHEF (adapted from[63] reconstructed by using PyMOL software).

A) Fermon virus



B) Lineage 3 virus



C) Lineages 1 and 2 viruses

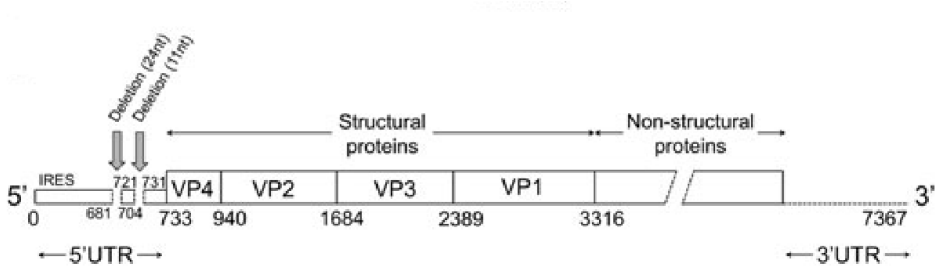


Figure 5 Genome structure of EV-D68 lineages 1, 2, and 3. The genome structures of the Fermon strain **(Fig 5A)**, lineage 3 **(Fig 5B)**, and lineages 1 and 2 **(Fig 5C)** of EV-D68 are demonstrated. Each genome region is indicated with a bar, and nucleotide positions of these regions are annotated with numbers below the bar (adapted from[1]).

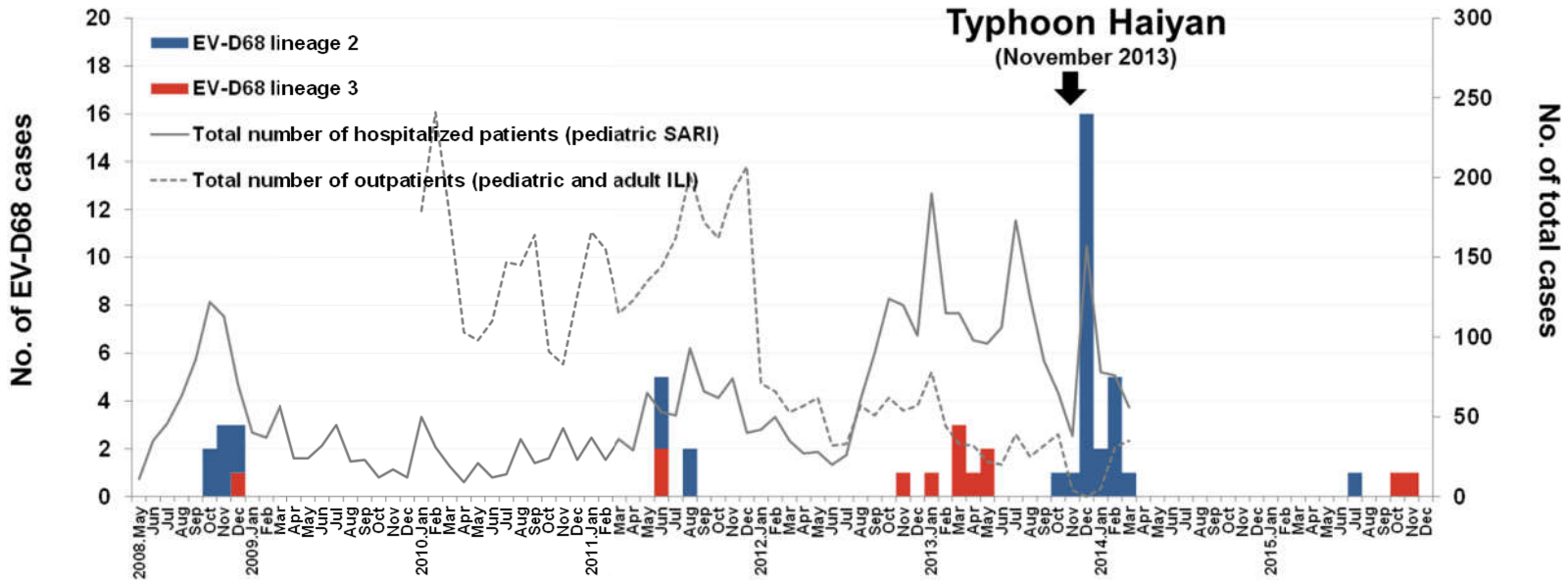


Figure 6 Temporal distribution of EV-D68 lineages in the Philippines between 2008 and 2015. The bar graph showed the number of EV-D68 from respiratory cases in each month during 7 years study period, combined with previous reports[2-4]. The total number of tested samples is shown in line graph. The genetic lineages were differentiated by colors (lineage 2=blue and lineage 3=red).

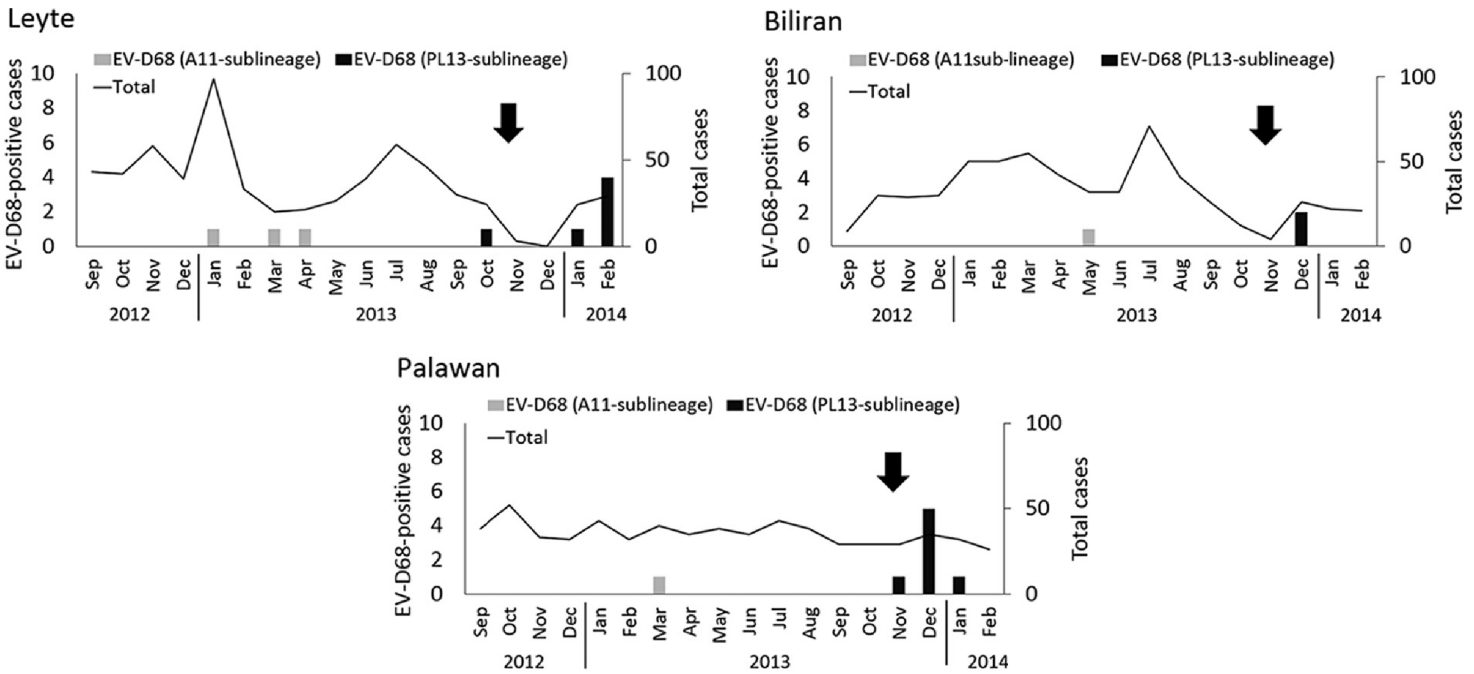


Figure 7 Monthly distribution of EV-D68-positive cases at each study site from 2012 to 2014. The total number and number of EV-D68-positive cases are shown. Arrows indicate the occurrence of Typhoon Haiyan.

Figure 8 A phylogenetic tree of EV-D68 strains in Philippines from 2008 to 2015 and reference strains. Significant mutations (T98A and M148V) mentioned in the body are shown in red letters at nodes. Filled circles with different colors indicate strains collected from our study sites, including Leyte; TTa and TEv, Biliran; TBp, and Palawan; TOp, in 2008 (blue), 2011 (green), 2012 (wheat), 2013 (purple), 2014 (red), and 2015 (olive).

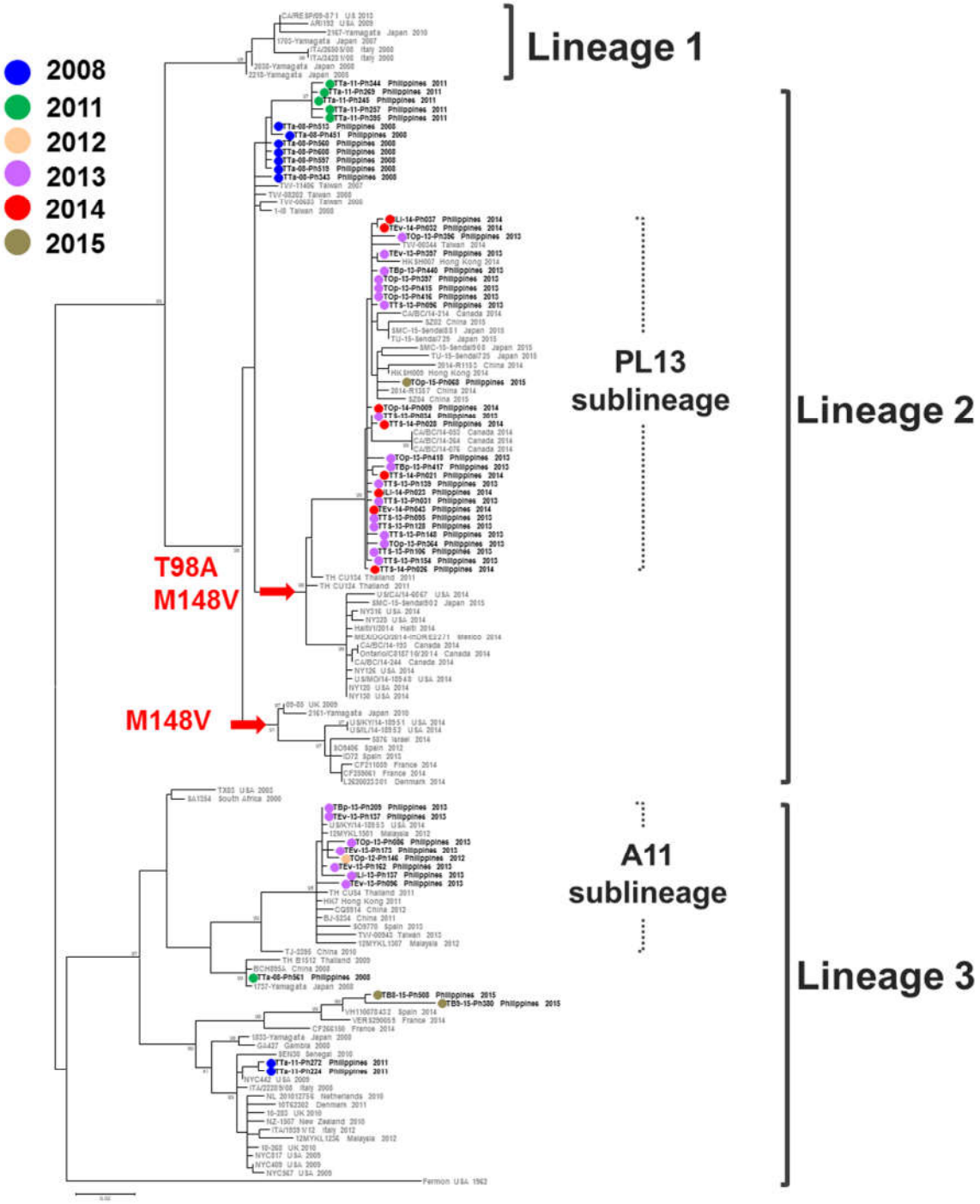
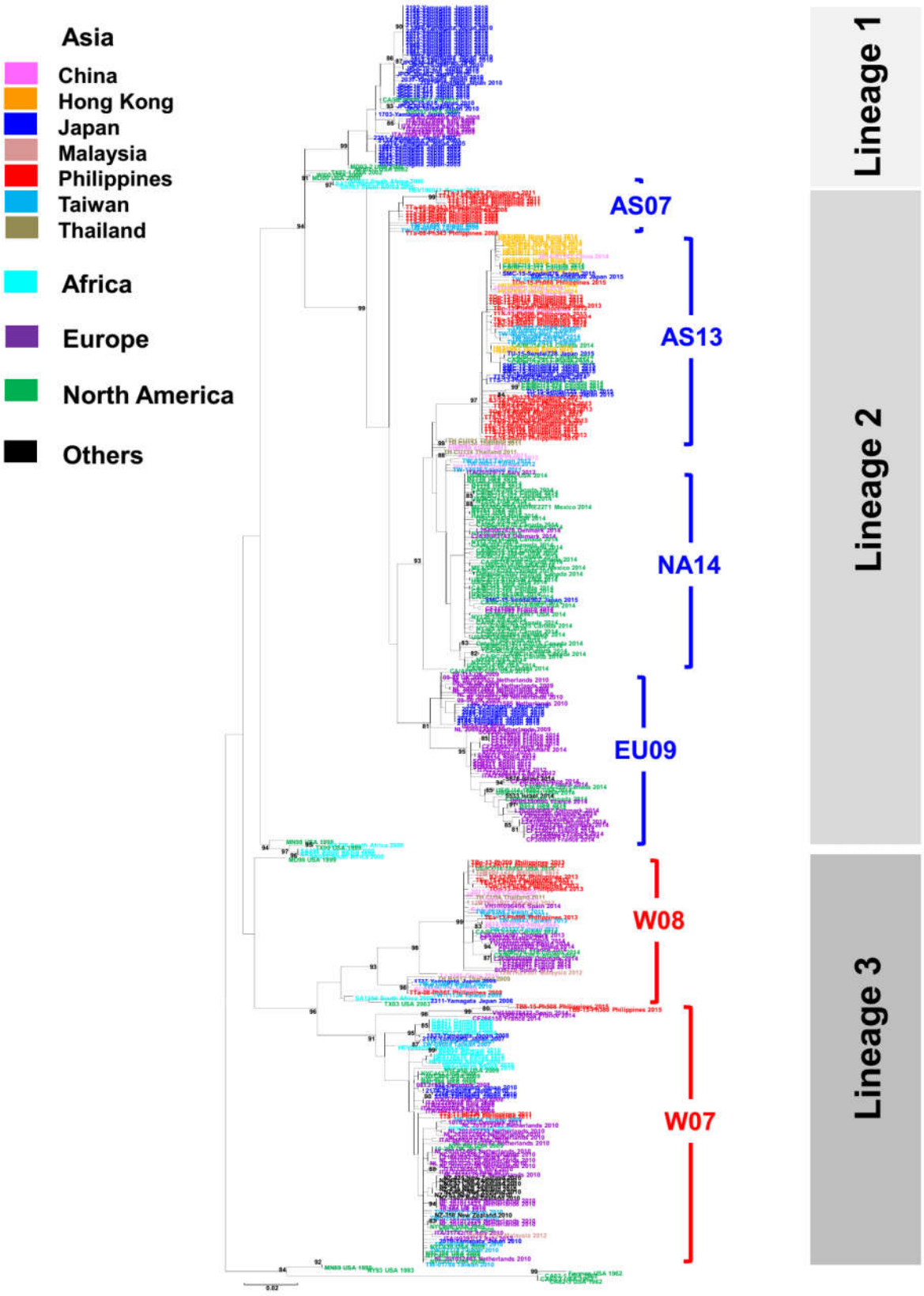

2008
2011
2012
2013
2014
2015
Lineage 1
Lineage 2
PL13
sublineage
Lineage 3
A11
sublineage
T98A
M148V
M148V

Figure 9A The phylogenetic relationship of worldwide EV-D68. Three major lineages 1, 2, 3 and sublineages are indicated at a vertical blanket at the right side with names. Significant mutations (T98A and M148V) are shown in red letters at nodes. Bootstrap values of > 80% (in 1000 tests) are shown. The continents/countries/regions (AS; Asia, EU; Europe, NA; North America, W; worldwide) included in this analysis are differentiated by colors.

Lineages 1, 2, and 3



Lineage 2 (enlarged)

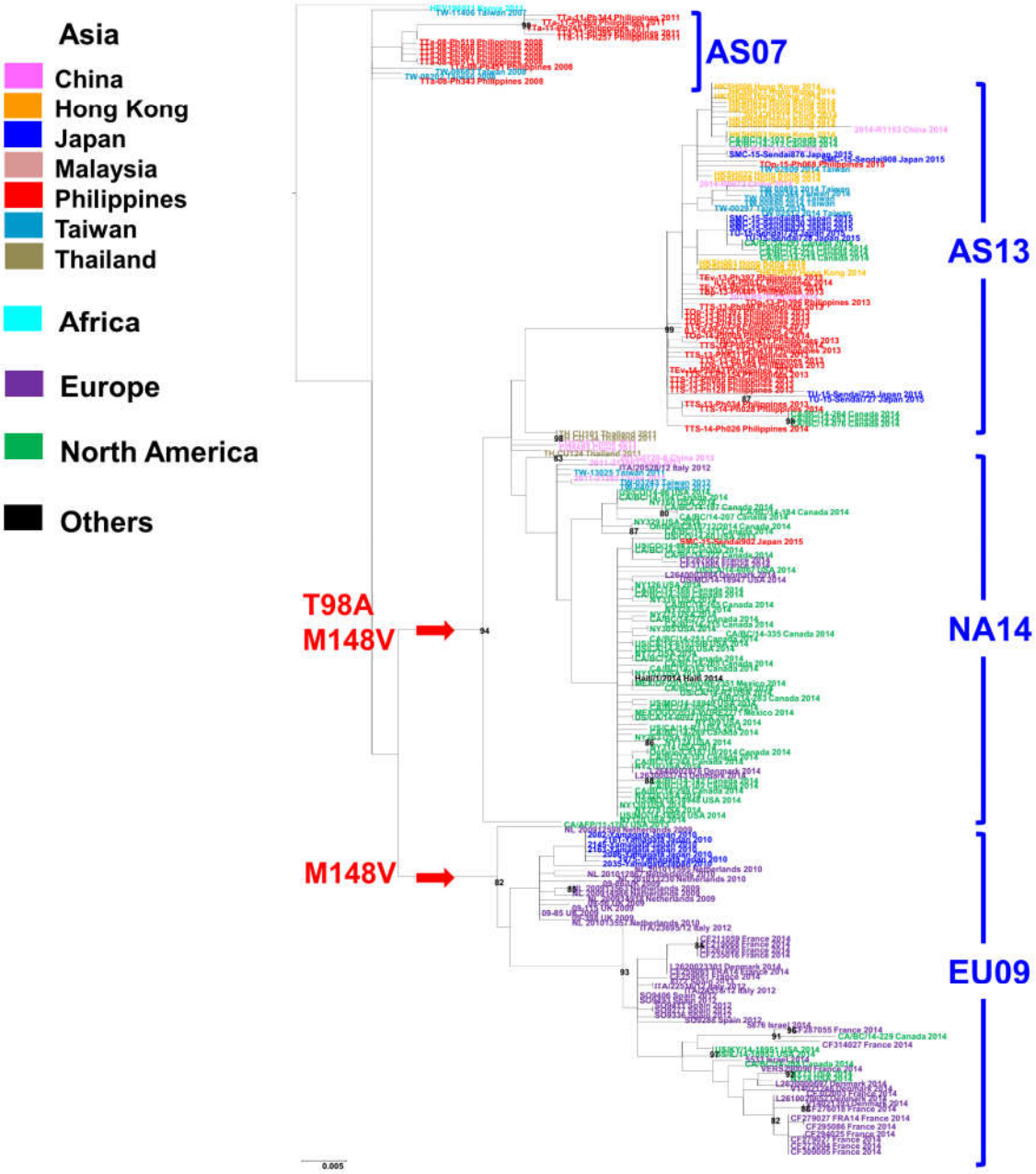


Figure 9B The phylogenetic relationship of worldwide lineage 2 viruses. Four sublineages, including AS07, AS13, NA14, and EU09 of lineage 2 are indicated.

Lineage 3 (enlarged)

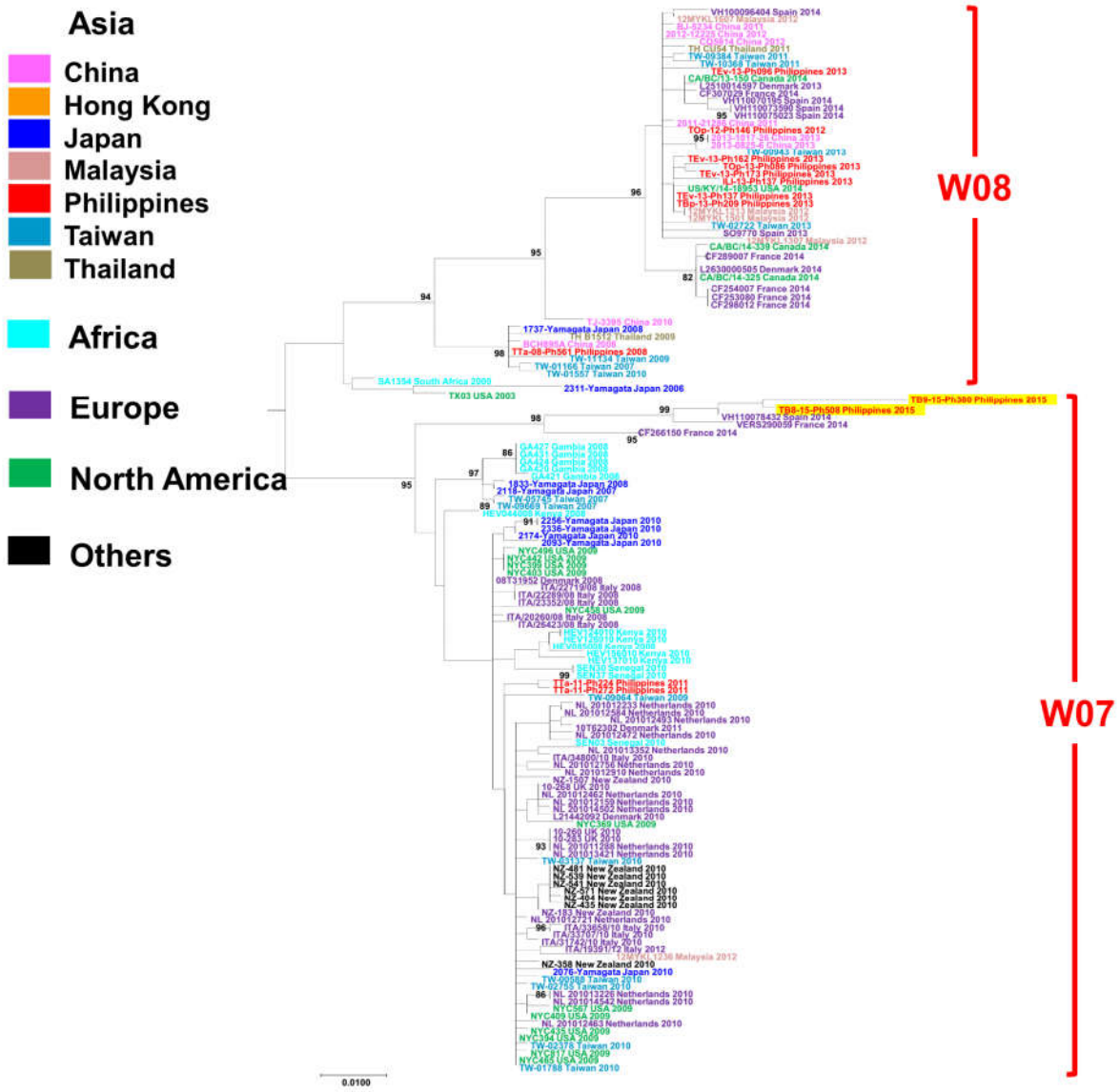


Figure 9C The phylogenetic relationship of worldwide lineage 3 viruses. Two sublineages (W07 and W08) of lineage 3 are shown. Within W07 sublineage, sequences of TB8-15-Ph508 and TB9-15-Ph380 are shown in yellow box.

Figure 10 The phylogenetic relationship of EV-D68 in Asia. EV-D68 lineages 1, 2, 3 are indicated at a vertical blanket at the right side with names. Bootstrap values of > 80% (in 1000 tests) are shown. Asian countries/regions included in this analysis are differentiated by colors.

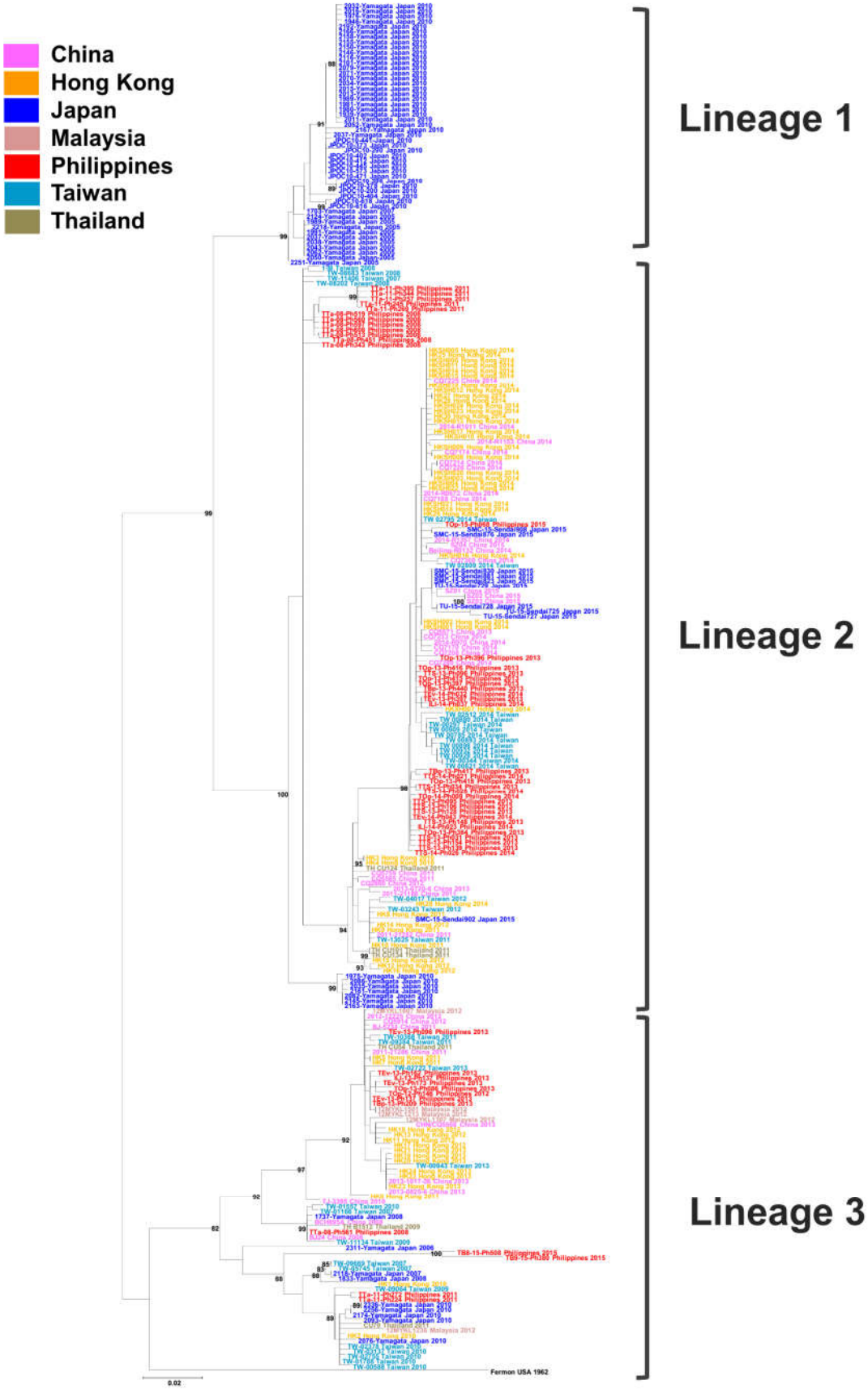
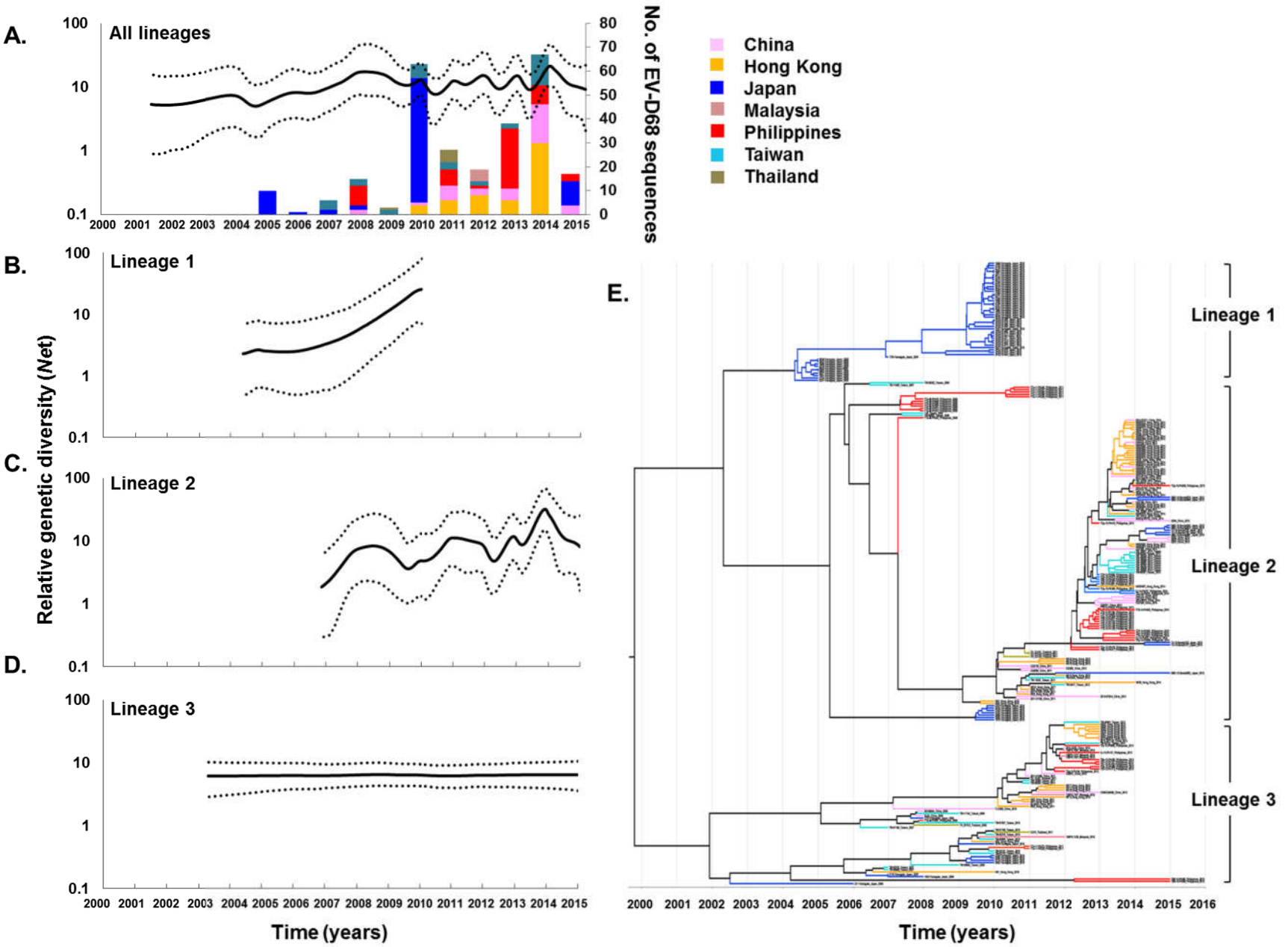

China
Hong Kong
Japan
Malaysia
Philippines
Taiwan
Thailand
Lineage 1
Lineage 2
Lineage 3
Fermon USA 1962
0.02

Figure 11 Phylodynamics of EV-D68 in Asia. Phylodynamics plots **(Fig 11A to 11D)** and Bayesian tree **(Fig 11E)** of EV-D68 lineages 1, 2, and 3 circulating in Asia from 2005 to 2015 are indicated. The dash and solid lines indicate mean values and the upper and lower 95% HPD values, respectively. The temporal distribution of EV-D68 in Asia is also indicated **(Fig 11A)**. The countries and regions included in this study are differentiated by colors.


A.
All lineages
No. of EV-D68 sequences
China
Hong Kong
Japan
Malaysia
Philippines
Taiwan
Thailand
B.
Lineage 1
C.
Lineage 2
D.
Lineage 3
Relative genetic diversity (Net)
Time (years)
E.
Lineage 1
Lineage 2
Lineage 3
Time (years)

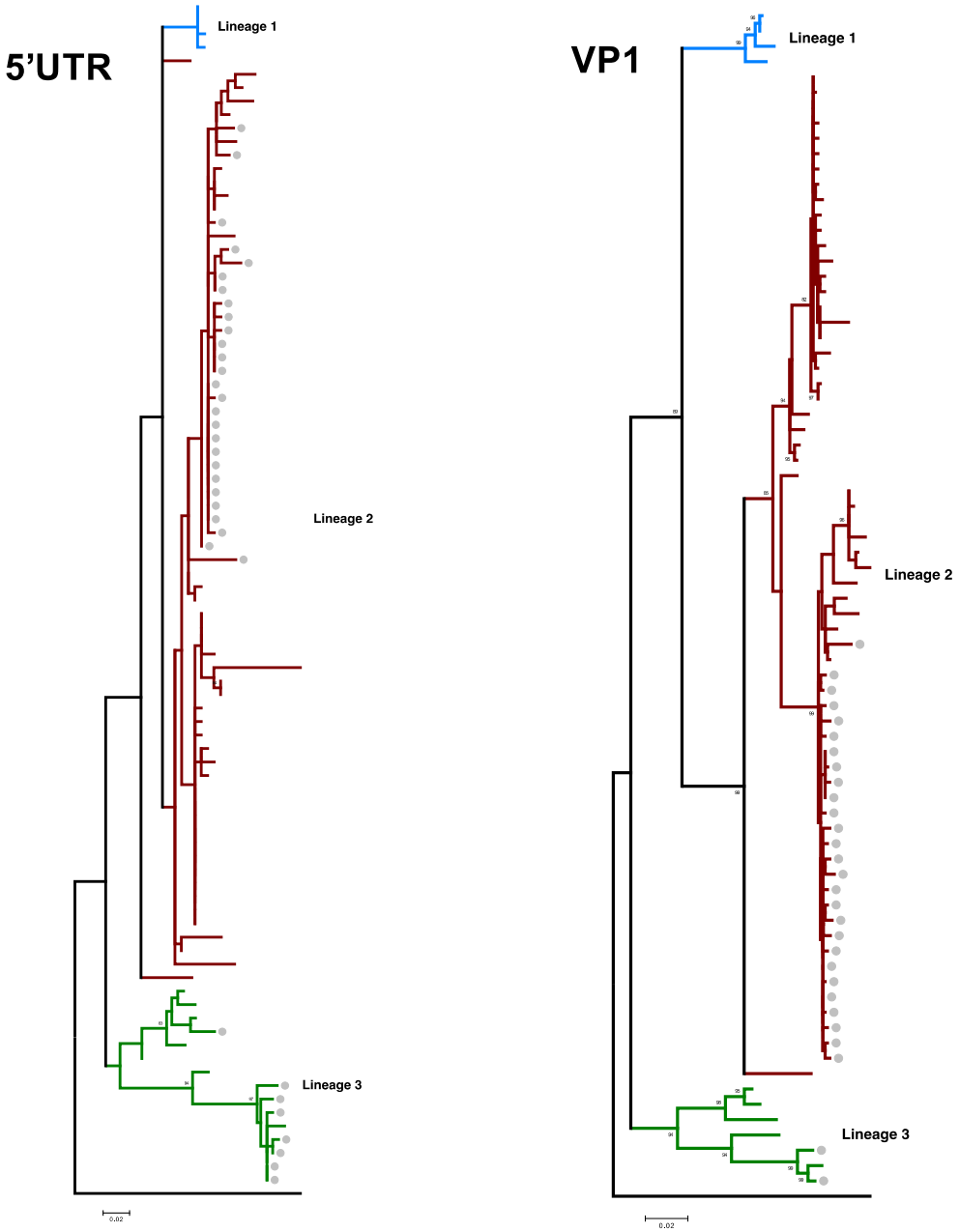


Figure 12 Phylogenetic trees of individual genes of EV-D68 genome using Maximum likelihood method. Each cluster of lineages 1, 2, and 3 was indicated in blue, brown, and green, respectively. The Philippines strains were marked with gray dots.

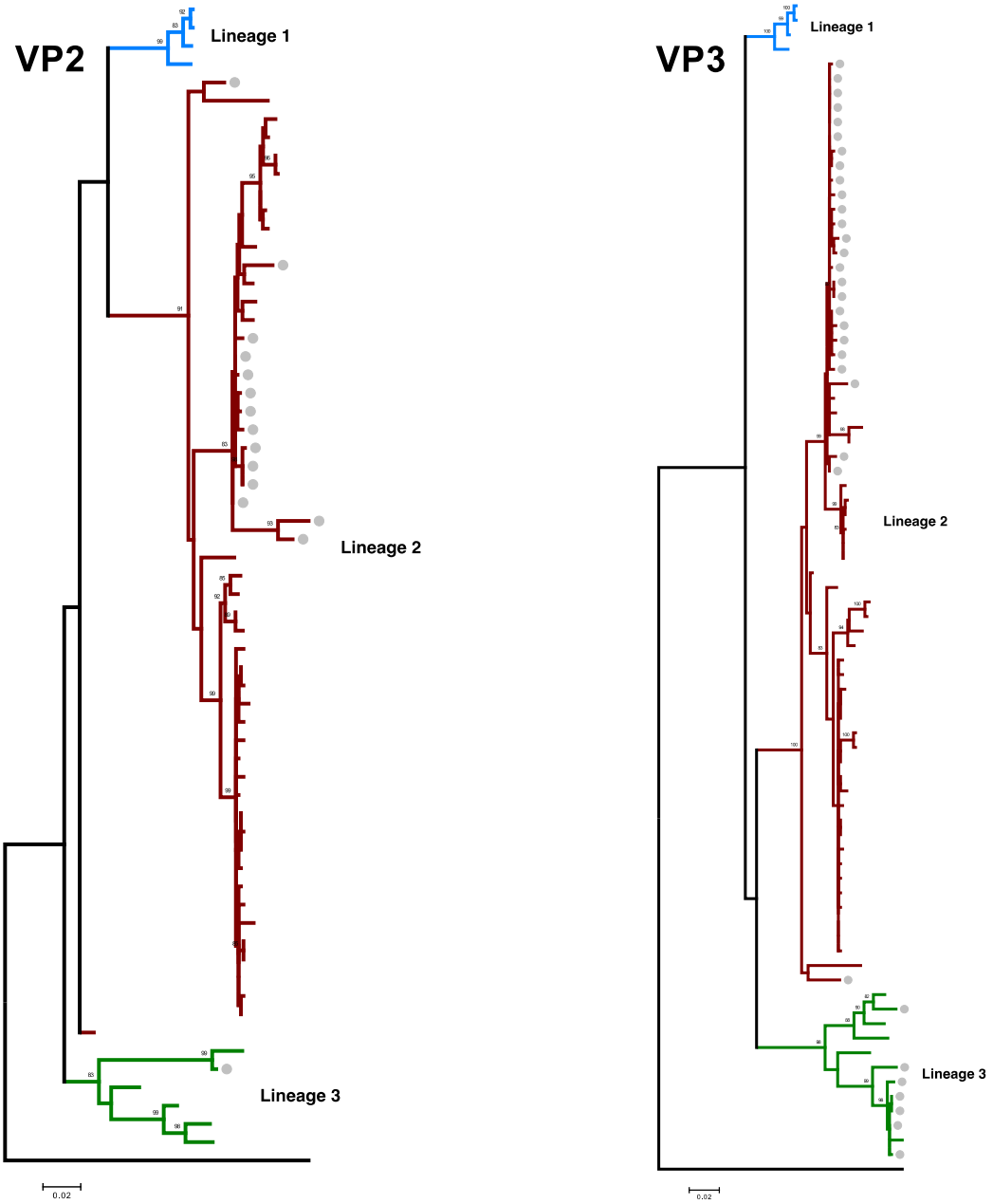


Figure 12 (continued)

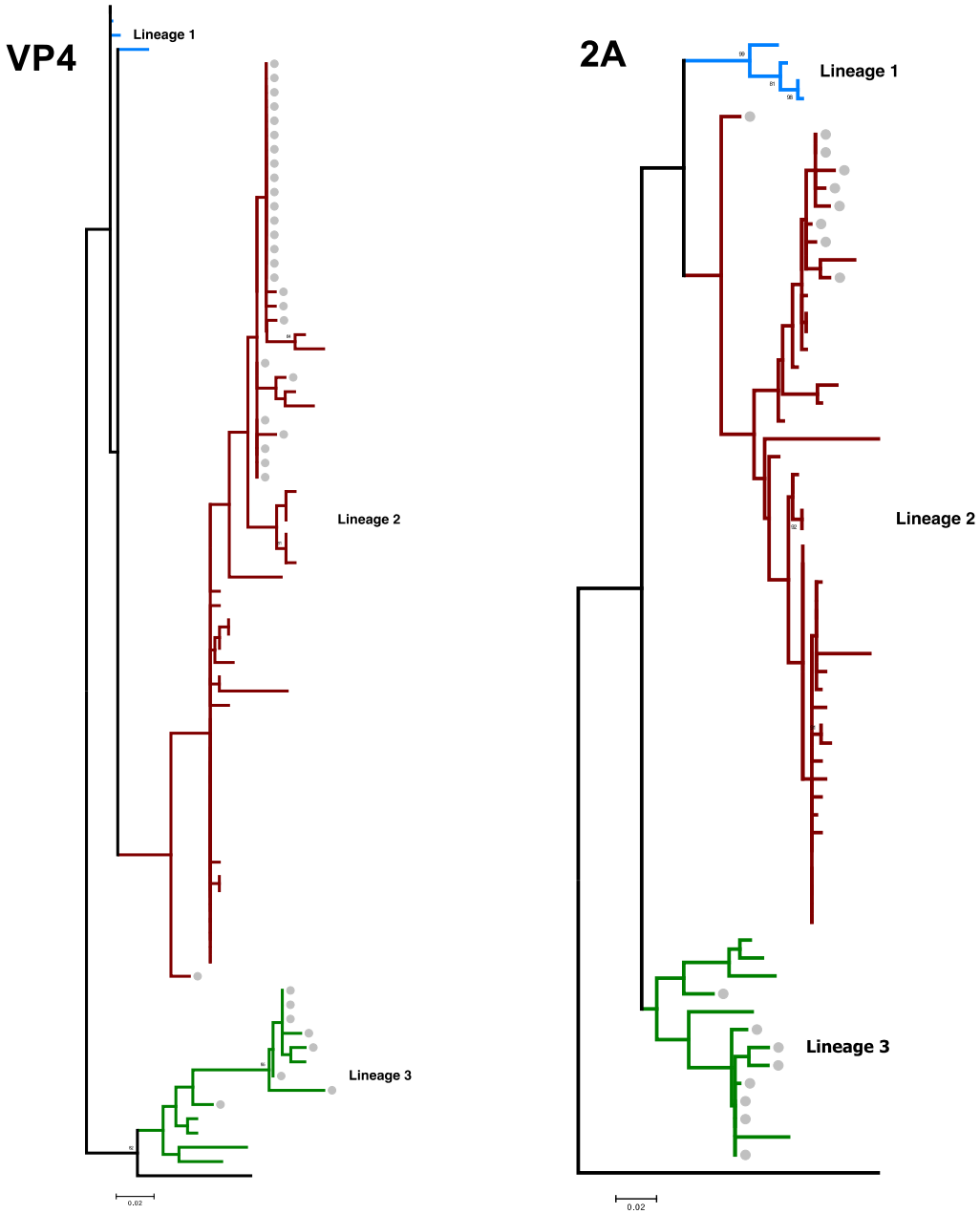


Figure 12 (continued)

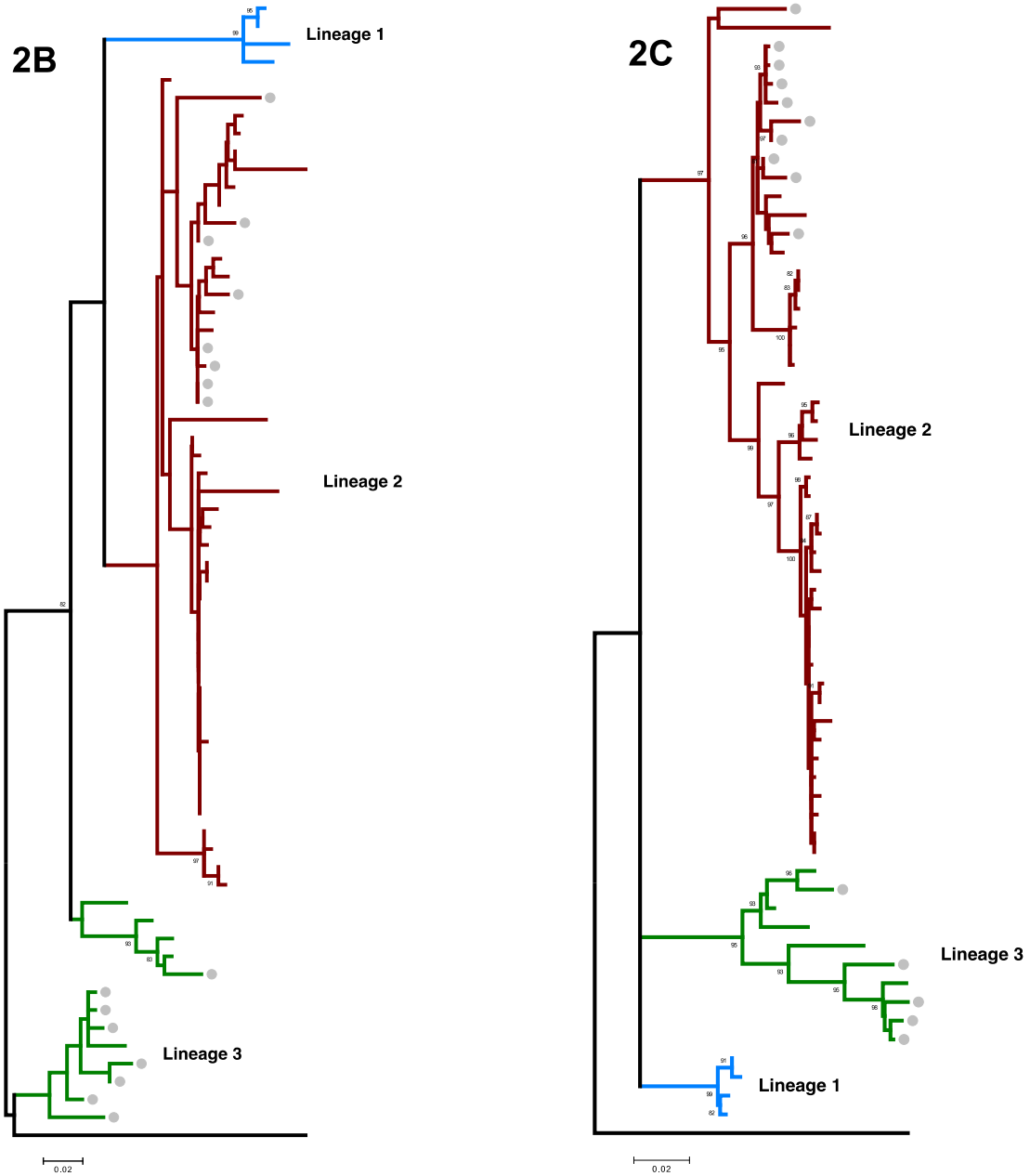


Figure 12 (continued)

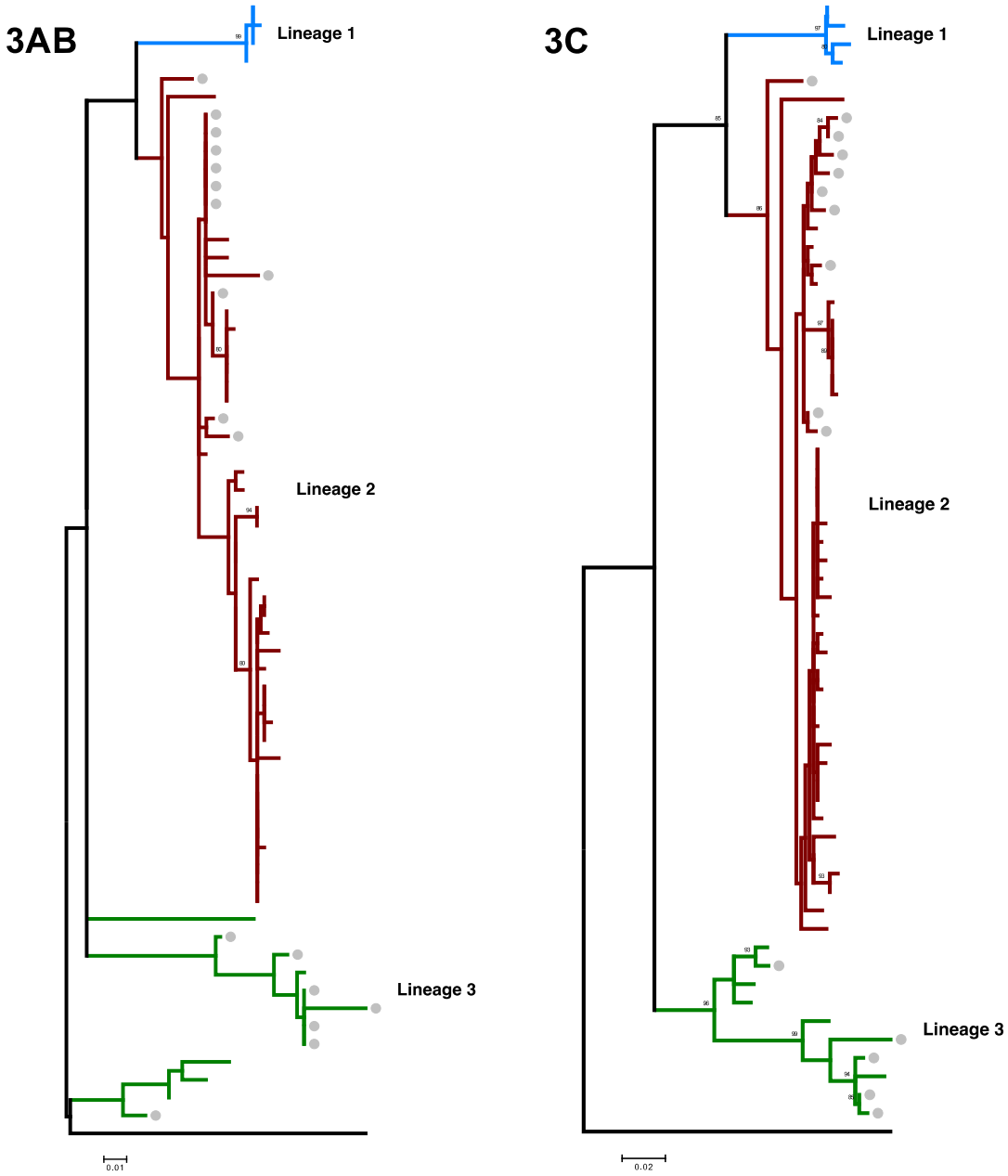


Figure 12 (continued)

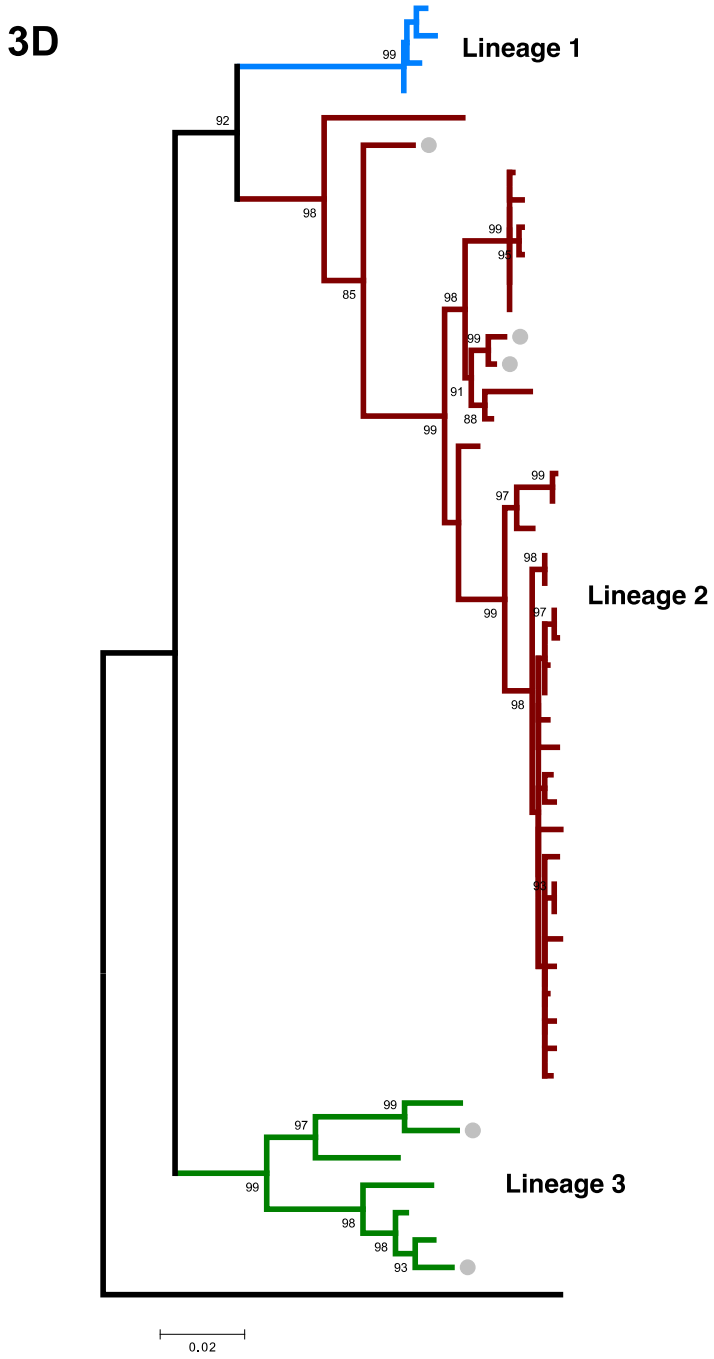
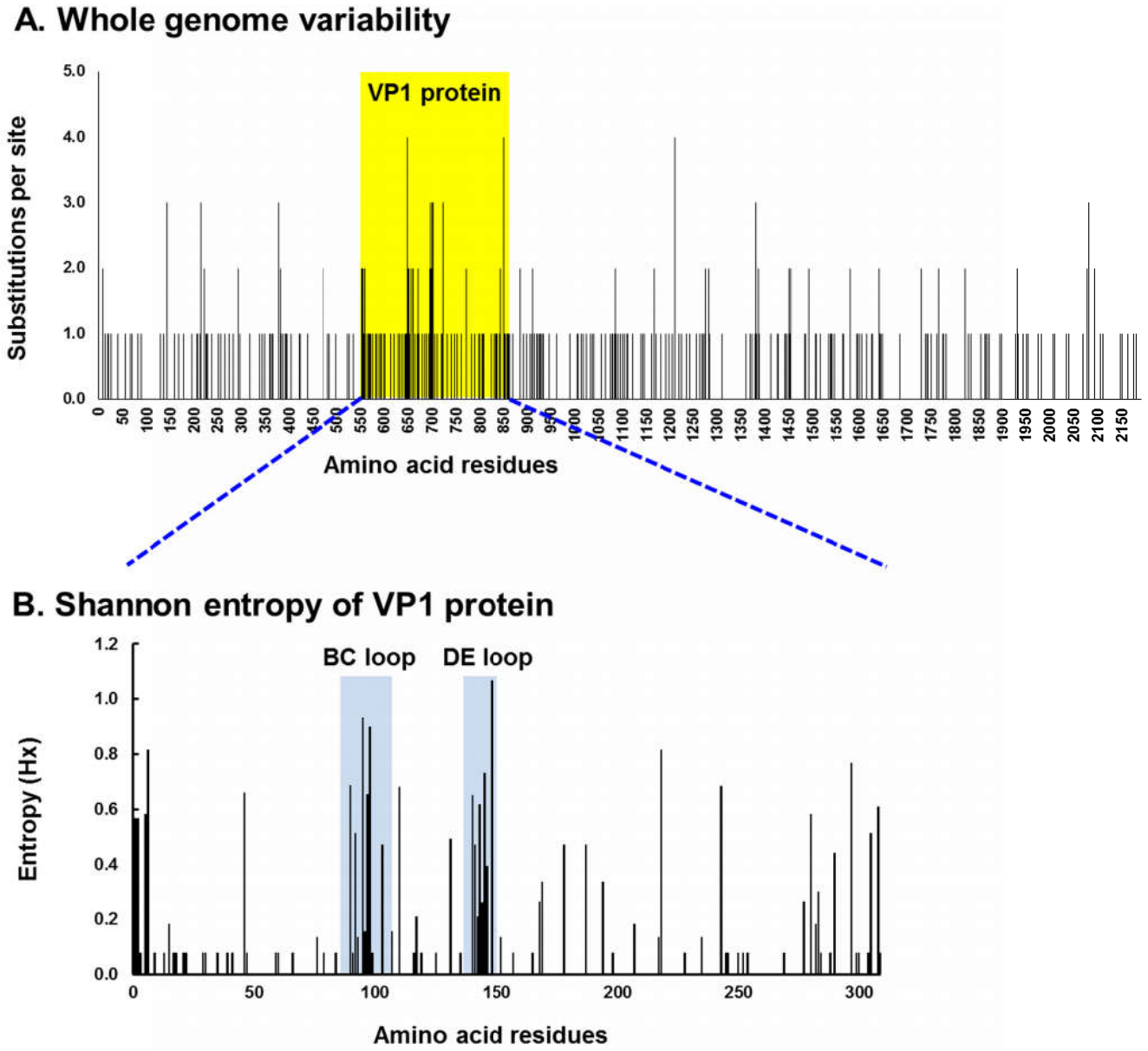

3D
Lineage 1
Lineage 2
Lineage 3
99
92
98
85
99
98
99
91
88
99
99
97
98
99
97
98
99
97
99
98
98
93
0.02

Figure 12

Figure 13 EV-D68 amino acid sequence variability in whole genome and Shannon entropy of VP1 protein. A number of substitution per site was calculated per strains relative to the consensus sequences generated from individual gene of worldwide strains. VP1 coding protein was indicated in the yellow box **(Fig 13A)**. The VP1 dataset was further analysed to point out the genetic variability located on antigenicity BC and DE loops of EV-D68 as shown by Shannon entropy of VP1 protein **(Fig 13B)**.


A. Whole genome variability
VP1 protein
Substitutions per site
5.0
4.0
3.0
2.0
1.0
0.0
0 50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000 1050 1100 1150 1200 1250 1300 1350 1400 1450 1500 1550 1600 1650 1700 1750 1800 1850 1900 1950 2000 2050 2100 2150
Amino acid residues
B. Shannon entropy of VP1 protein
BC loop
DE loop
Entropy (Hx)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 50 100 150 200 250 300
Amino acid residues

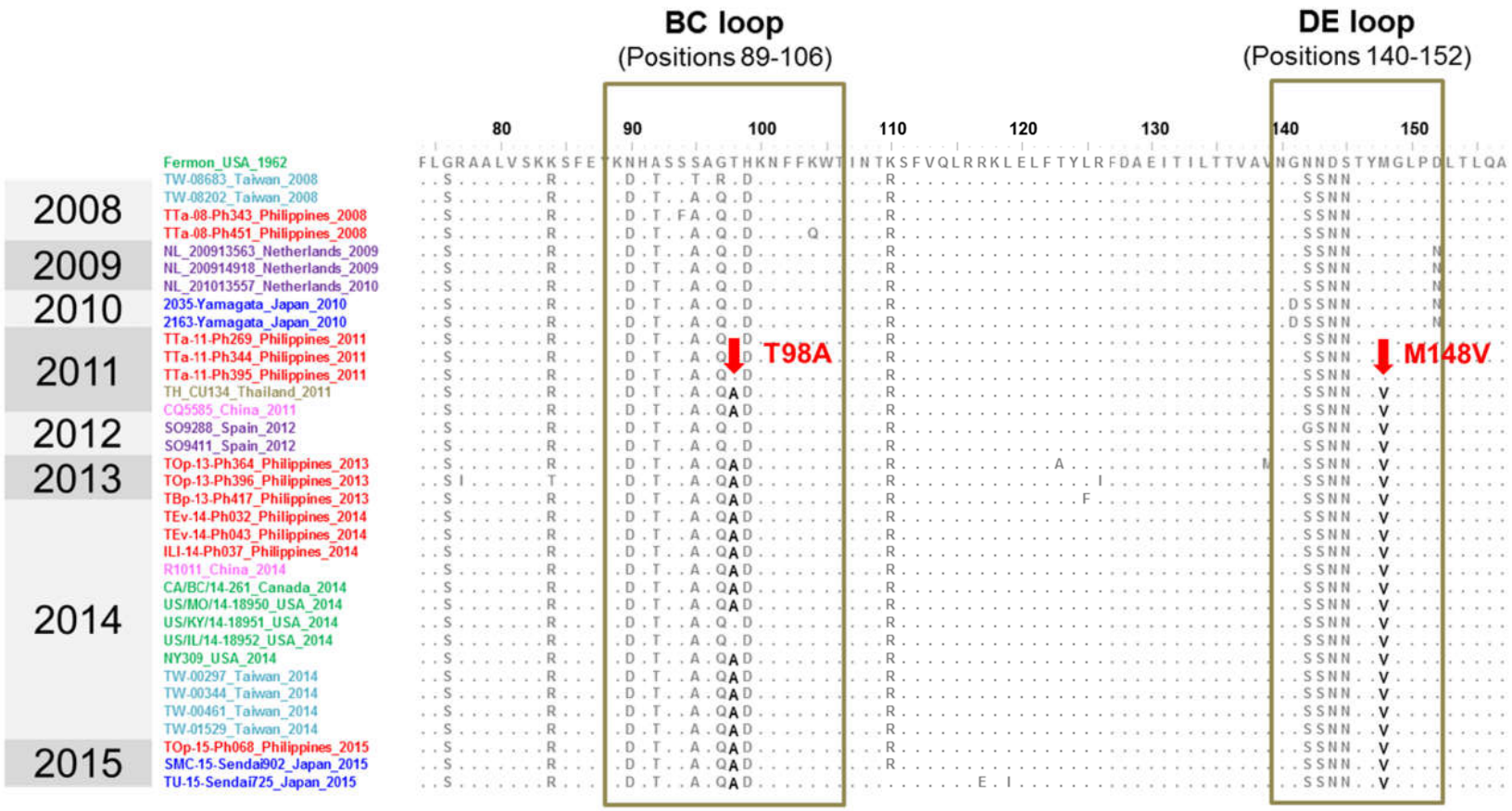


Figure 14 Multiple alignment of partial VP1 amino acid sequences. The amino acid sequences of representative EV-D68 lineage 2 collected in different years were aligned and compared to the prototype Fermon. Identical sequences were indicated with dots. The antigenic BC and DE loops were shown in boxes.

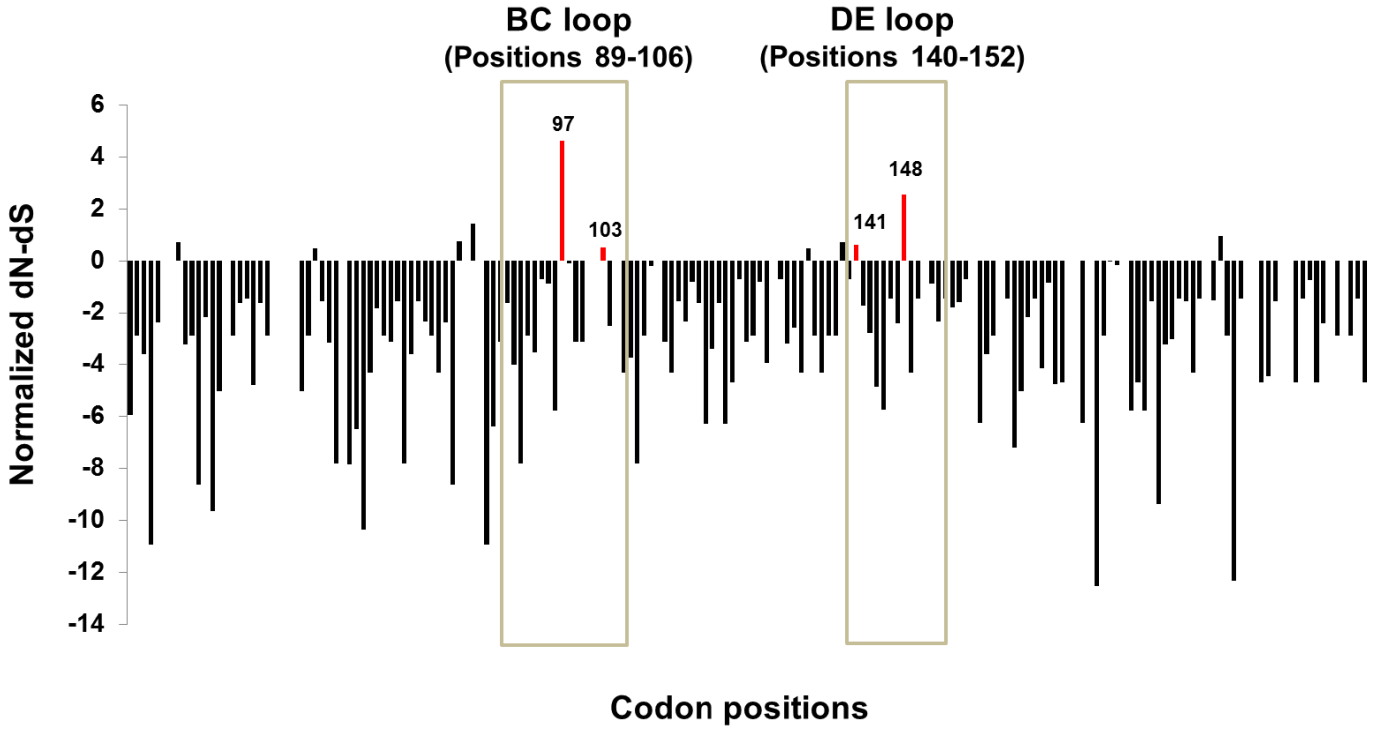


Figure 15 Codons under selective pressure in VP1 region by the SLAC method. (shown in red bars). Normalized dN-dS values > 0 indicated positive selection while values < 0 indicate negative selection. The amino acid positions in antigenic BC and DE loops were indicated in boxes.

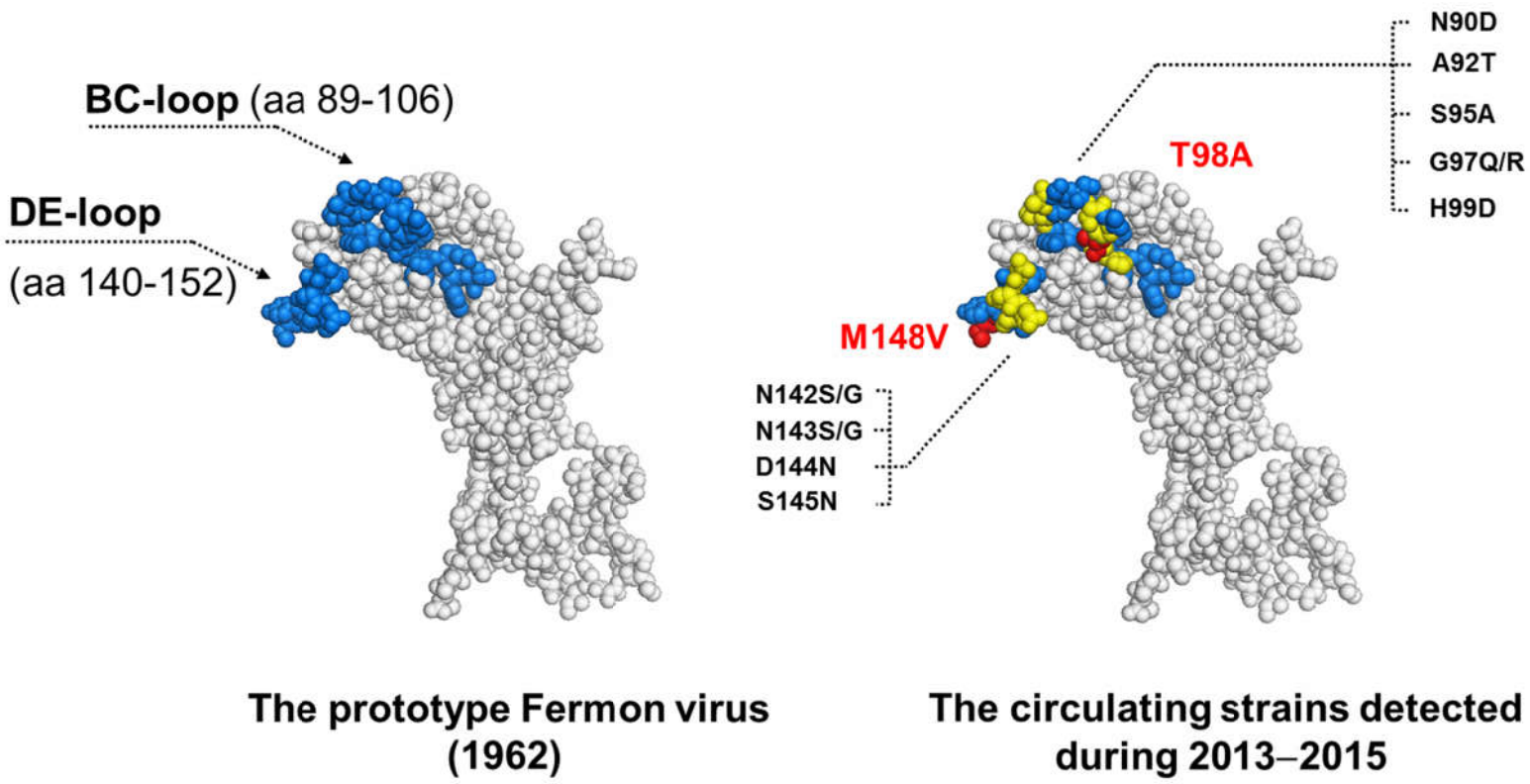


Figure 16 The structure models of VP1 protein of EV-D68. The accumulation of amino acid substitutions on BC and DE loops was obtained from EV-D68 circulating in Asia, Europe, and North America during 2013–2015.

10. TABLES

Table 1. Enterovirus species D (EV-D).

Types	Prototype strains	Geographical region	Illnesses in person with prototype	Accession number
EV-D68	Fermon (human)	California	Lower respiratory illness	AY426531[13]
EV-D70	J670/71 (human)	Japan and Singapore	Acute hemorrhagic conjunctivitis	D00820[69]
EV-D94	E210 (human)	Egypt	Detected in sewage	DQ916376[70]
EV-D111	KK2640 (chimpanzee)	Cameroon	none	JF416935[71]
	17-04 (human)	Democratic Republic of Congo	Acute flaccid paralysis	EF127249[72]
EV-D120	MB6201 (gorilla)	Cameroon	none	KF040080[73]
	MB6128 (gorilla)	Cameroon	none	KF040081[73]

Table 2. Incidence of worldwide EV-D68 infection in recent years[1].

Location [references]	Period of EV-D68 detection	Study period	Study population	Number of EV-D68-positive cases (overall detection rate, %)	Number of fatal cases	Age of EV-D68-positive cases	Diagnosis
The Netherlands [8]	1996, 1997, 1998, 1999, 2000, 2001, 2003, 2006, 2007, 2008, 2009, 2010	1994–2010	ILI surveillance, children cohorts for wheezing illness, cystic fibrosis, and amniotic fluid study	70 (70/12,743, 0.55%)	0	0.8 months–80s	ILI, ARI
South Africa [7]	May 2000–May 2001	ND	Hospitalized children	8	0	5 months–1 year 11 months	Respiratory illness (hospitalized)
Japan (Yamagata) [11]	Sep–Oct 2005, Sep 2006, Aug–Oct 2007, Sep 2009, Aug–Oct 2010	2005–2010	Pediatric outpatients with ARI	55 (55/6307, 0.87%)	0	5 months–15 years (mean: 5.2 years)	URTI, LRTI, others (e.g., asthma)
China (Beijing) [14]	Aug–Oct 2006, Aug 2008	Aug 2006–Apr 2010	Adult patients admitted to outpatient clinics and hospitals	13 (13/6942, 0.18%)	0	18–67 years (median: 34 years)	Mild ARTI
The Gambia [7]	Jun 2008	ND	Hospitalized children	5	0	<2 years	Respiratory illness (hospitalized)
Philippines (Leyte) [6,10]	Oct 2008–Feb 2009, Jun–Aug 2011	May 2008–Dec 2011	Hospitalized pneumonia patients, outpatients with ILI	33 (33/6056, 0.54%)	4	1 month–76 years (median: 1 year 8 months)	Pneumonia, ILI
Italy (Pavia) [16,17]	Oct 2008–Apr 2009	Oct 2008–Sep 2009	Pediatric and adult patients hospitalized with ARI	12 (12/1500, 0.80%)	0	1 month–57 years	URTI, LRTI

USA (New Hampshire) [37]	Autumn 2008	CR	CR	1	1	5 years	Meningomyeloencephalitis
USA (Arizona) [7]	Oct 2009	ND	ND	1	0	1 year 11 months	Fever, cough, and rhinitis
The Gambia [7]	Jun 2008	ND	Hospitalized children	5	0	<2 years	Respiratory illness (hospitalized)
Thailand [13]	Jun–Sep 2009, Feb–Oct 2010, Jun–Sep 2011	Feb 2006–Nov 2011	Children with ARTI	25 (25/1810, 1.38%)	0	7 months–15 years (mean: 7.6 years)	Pneumonia, ILI
China (Chongqing, Beijing, Tianjin) [15]	Jun 2009–Jun 2012	Sep 2010–Aug 2011	Hospitalized children and adult outpatients with ARTI	9 (9/2150, 0.42%)	0	7 children, 2 adults	ARTI (e.g., pneumonia, URTI, and asthma)
USA (New York) [7]	Aug–Oct 2009	ND	Outpatients with respiratory illnesses	16	0	14–47 years	Respiratory illness (outpatients)
France (Champagne-Ardenne) [19]	Sep–Nov 2009	Sep 2009–Jun 2010	Pediatric patients admitted to a hospital with acute airway diseases	10 (10/651, 1.53%)	0	6 months–10 years (median: 3.8 years)	Asthma, bronchiolitis
USA (Arizona) [7]	Oct 2009	ND	ND	1	0	1 year 11 months	Fever, cough, and rhinitis
England (London) [21]	Nov–Dec 2009, Sep–Dec 2010	Nov 2009–Dec 2010	Patients with respiratory symptoms attending primary care and hospitals	17	1	7 weeks–45 years (mean: 12.2 years, median: 5.8 years)	Respiratory symptoms
Italy (Pavia) [18]	Jan 2010–Dec 2012	2010–2012	Patients who stayed or visited a hospital with respiratory tract infections	9 (9/3736, 0.24%)	0	7 pediatrics (4 months–6 years, median 15 months), 2 adults	URTI, LRTI
Senegal [7]	Feb–Mar 2010	ND	Hospitalized children	3	0	2, 23, and 32 years	Respiratory illness (hospitalized)
New Zealand [22]	Mar–Aug 2010	ND	ND	15	0	1 months–48 years (median: 11 years)	Bronchiolitis, asthma, cough,

(Continues)

Location [references]	Period of EV-D68 detection	Study period	Study population	Number of EV-D68-positive cases (overall detection rate, %)	Number of fatal cases	Age of EV-D68-positive cases	Diagnosis
							coryza, wheeze, strider, pertussis, sepsis, heart failure, burns
Japan (Osaka) [12]	Jul–Sep 2010	Oct 2009–Oct 2010	Children with RTI	15 (15/448, 3.35%)	0	3 months–5 years (mean: 2 years 10 months)	Pneumonia, bronchopneumonia, bronchitis, LRTI, asthma, pharyngitis, febrile convulsion
Japan (Yamaguchi) [32]	Jul–Sep 2010	Jul–Sep 2010	Hospitalized children with history of asthma	26 (26/35, 74.3%)	0	Mean: 4 years	Asthma attack
Netherlands [20]	Aug–Nov 2010	2009–Jan 2011	Children hospitalized with respiratory infections	24 (24/252, 9.52%)	0	1 months–72 years (median: 14 years)	Respiratory infections (hospitalized)

Summary information for EV-D68 incidence. The data include the study sites, year of detection, case numbers, age distribution, and diagnosis. The numbers of reported cases of EV-D68-positive respiratory illnesses in the USA in 2014 have not been included in this table because of the limited information at present.

ILI, influenza-like illness; RTI, respiratory tract infections; URTI, upper respiratory tract infections; LRTI, lower respiratory infections; ARTI, acute respiratory tract infections; ARI, acute respiratory infections; ND, unknown; CR, case report.

Table 3 Primers used for whole EV-D68 genome amplification and DNA sequencing.

Genes/ Regions	Primer name	Sequence (5'-3')	Position	References
5'UTR	DK001	CAAGCACTTCTGTTTCCC	164-181	[12]
	DK004	CACTACTTTGGGTGTCCGTG	540-559	[11]
	5UTRF	CCATGGAGCAAGTGCTCAC	475-493	This study
	VP4R	GACCCATCAAAATTRACT	877-894	
VP4	VP4F	GGACCCATCAAAATTCACTG	876-895	[2]
	VP2R	CAATATTTCCACATCAATGG	1451-1470	
VP2	VP2F	CCAGGGTTCGATGATATCATG	1360-1380	
	VP3R	GACATACAGTTAGACGGGCC	1942-1961	
VP3	VP3F	GCACATTCCAGGGCAGGTCC	1785-1804	
	VP1RFH	GATTAACGCCCGAACTTGGTG	2469-2489	
VP1	VP1F	ACCATTTACATGCAGCAGAGG	2393-2413	
	485	GTNGAYTGGCANTCAGATGT	3406-3425	[13]
	VP1R	TTGTCCATTTGAAAAAGTTCTTGTC	2683-2707	
	VP1RCF	GACAAGAACTTTTTCAAATGGAC	2683-2705	This study
2A	2AF	ATGTRGAYTGGCAATCAGAYGT	3404-3425	
	2BR	GGYATTCCYTATGTACCTAGACAG	4030-4053	
2B	2BF	ACACTAGCATTGYTGGGATGC	3961-3981	
	2CR	GGCTCAATACATGCACCAACA	4726-4746	
2C	2CF	ATCCAGATGGGAATGACAT	4595-4613	
	3AR	ACTCTACAAGCCATTGCCA	5233-5251	
3AB	3AF	AGTTAGGGATTATTGCCAA	5142-5160	
	3CR	CCAGTTGGACAAGTCACTAAC	5719-5739	
3C	3CF	TATGAGGATGATTACAATGACGC	5653-5675	
	3DR1	TATGGAGTTGACYTACCTT	6355-6373	
3D	3DF1	GATTCCCTTACTTRCTACAAG	6263-6283	
	3DR2	TGGATTAGTAATGACACCAGC	6963-6983	
3'UTR	3DF2	ATGGTGGAATGCCCTCTGGT	6761-6780	
	3UTR	AATTTTGGTCACTTGGGGGC	7347-7367	

Nucleotide positions correspond to the prototype Fermon virus (AY426531).

Table 4 Accession numbers of VP1 sequence used in this study.

Strains	Accession numbers
09-115 UK 2009	JQ586224
09-398 UK 2009	JQ586226
09-56 UK 2009	JQ586221
09-85 UK 2009	JQ586222
09-86 UK 2009	JQ586223
10-260 UK 2010	JQ586230
10-268 UK 2010	JQ586231
10-283 UK 2010	JQ586232
1703-Yamagata Japan 2007	AB667895
1737-Yamagata Japan 2008	AB667899
1833-Yamagata Japan 2008	AB667898
1939-Yamagata Japan 2010	AB614406
1946-Yamagata Japan 2010	AB614408
1975-Yamagata Japan 2010	AB614409
1976-Yamagata Japan 2010	AB614410
1980-Yamagata Japan 2010	AB614411
1981-Yamagata Japan 2010	AB614412
1989-Yamagata Japan 2005	AB667885
1989-Yamagata Japan 2010	AB614413
1991-Yamagata Japan 2005	AB667886
2034-Yamagata Japan 2010	AB614414
2035-Yamagata Japan 2010	AB614427
2037-Yamagata Japan 2005	AB667887
2037-Yamagata Japan 2010	AB614428
2038-Yamagata Japan 2005	AB667888
2043-Yamagata Japan 2005	AB667889
2050-Yamagata Japan-2005	AB667890
2052-Yamagata Japan 2010	AB614429

Table 4 (continued)

Strains	Accession numbers
2062-Yamagata Japan 2005	AB667891
2070-Yamagata Japan 2010	AB614416
2071-Yamagata Japan 2010	AB614417
2076-Yamagata Japan 2010	AB614440
2079-Yamagata Japan 2010	AB614418
2082-Yamagata Japan 2010	AB614430
2086-Yamagata Japan 2010	AB614433
2093-Yamagata Japan 2010	AB614441
2101-Yamagata Japan 2010	AB614434
2116-Yamagata Japan 2010	AB614435
2118-Yamagata Japan 2007	AB667897
2124-Yamagata Japan 2005	AB667892
2145-Yamagata Japan 2010	AB614431
2146-Yamagata Japan 2010	AB614436
2150-Yamagata Japan 2010	AB614419
2155-Yamagata Japan 2010	AB614420
2158-Yamagata Japan 2010	AB614421
2161-Yamagata Japan 2010	AB614437
2163-Yamagata Japan 2010	AB614432
2166-Yamagata Japan 2010	AB614438
2167-Yamagata Japan 2010	AB614439
2174-Yamagata Japan 2010	AB614442
2192-Yamagata Japan 2010	AB614422
2218-Yamagata Japan 2005	AB667893
2251-Yamagata Japan 2005	AB667894
2256-Yamagata Japan 2010	AB614443
2311-Yamagata Japan 2006	AB667896
2336-Yamagata Japan 2010	AB614444
ARI192 USA 2009	JX101786

Table 4 (continued)

Strains	Accession numbers
TX02-1 USA 2002	AY426495
BCH895A China 2008	KF726085
BJ-5234 China 2011	JQ924864
Fermon USA 1962	AY426531
CA62-1 USA 1962	AY426486
CA62-2 USA 1962	AY426487
CA62-3 USA 1962	AY426488
CQ5585 China 2011	JX898785
CQ5759 China 2011	JQ924867
CQ5914 China 2012	JX898786
GA420 Gambia 2008	JX101790
GA421 Gambia 2008	JX101791
GA424 Gambia 2008	JX101792
GA427 Gambia 2008	JX101793
GA431 Gambia 2008	JX101794
ID72 Spain 2013	KF254913
ITA/19962/08 Italy 2008	KC763160
ITA/20528/12 Italy 2012	KC763162
ITA/24281/08 Italy 2008	KC763168
ITA/26505/08 Italy 2008	KC763171
JPOC10-200 Japan 2010	AB601872
JPOC10-290 Japan 2010	AB601882
JPOC10-373 Japan 2010	AB601873
JPOC10-378 Japan 2010	AB601883
JPOC10-396 Japan 2010	AB601884
JPOC10-402 Japan 2010	AB601874
JPOC10-404 Japan 2010	AB601885
JPOC10-412 Japan 2010	AB601875
JPOC10-441 Japan 2010	AB601876

Table 4 (continued)

Strains	Accession numbers
JPOC10-445 Japan 2010	AB601877
JPOC10-471 Japan 2010	AB601878
JPOC10-573 Japan 2010	AB601879
JPOC10-616 Japan 2010	AB601880
JPOC10-618 Japan 2010	AB601881
MD02-1 USA 2002	AY426491
MD02-2 USA 2002	AY426492
MD99 USA 1999	AY426499
MN89 USA 1989	AY426489
MN98 USA 1998	AY426497
MO00 USA 2000	AY426493
NL 200912598 Netherlands 2009	JF896311
NL 200913563 Netherlands 2009	JF896308
NL 200914918 Netherlands 2009	JF896310
NL 200914986 Netherlands 2009	JF896309
NL 201011288 Netherlands 2010	JF896311
NL 201011595 Netherlands 2010	JF896304
NL 201012159 Netherlands 2010	JF896293
NL 201012233 Netherlands 2010	JF896298
NL 201012462 Netherlands 2010	JF896291
NL 201012463 Netherlands 2010	JF896296
NL 201012472 Netherlands 2010	JF896300
NL 201012493 Netherlands 2010	JF896301
NL 201012584 Netherlands 2010	JF896299
NL 201012721 Netherlands 2010	JF896294
NL 201012756 Netherlands 2010	JF896295
NL 201012867 Netherlands 2010	JF896307
NL 201012910 Netherlands 2010	JF896290
NL 201013226 Netherlands 2010	JF896287

Table 4 (continued)

Strains	Accession numbers
NL 201013230 Netherlands 2010	JF896306
NL 201013352 Netherlands 2010	JF896289
NL 201013421 Netherlands 2010	JF896312
NL 201013557 Netherlands 2010	JF896302
NL 201014502 Netherlands 2010	JF896292
NL 201014542 Netherlands 2010	JF896288
NY93 USA 1993	AY426490
NYC369 USA 2009	JX101803
NYC394 USA 2009	JX101804
NYC399 USA 2009	JX101805
NYC403 USA 2009	JX101806
NYC409 USA 2009	JX101808
NYC435 USA 2009	JX101809
NYC442 USA 2009	JX101807
NYC458 USA 2009	JX101810
NYC465 USA 2009	JX101811
NYC496 USA 2009	JX101812
NYC567 USA 2009	JX101813
NYC817 USA 2009	JX101814
NZ-1507 New Zealand 2010	JQ713904
NZ-183 New Zealand 2010	JQ713905
NZ-358 New Zealand 2010	JQ713906
NZ-404 New Zealand 2010	JQ713907
NZ-435 New Zealand 2010	JQ713909
NZ-481 New Zealand 2010	JQ713910
NZ-539 New Zealand 2010	JQ713911
NZ-541 New Zealand 2010	JQ713912
NZ-571 New Zealand 2010	JQ713913
SA1354 South Africa 2000	JX101802

Table 4 (continued)

Strains	Accession numbers
SA402 South Africa 2000	JX101795
SA498 South Africa 2000	JX101796
SA551 South Africa 2000	JX101797
SA553 South Africa 2000	JX101798
SA563 South Africa 2000	JX101799
SA726 South Africa 2000	JX101801
SA792 South Africa 2000	JX101800
SEN03 Senegal 2010	JX101787
SEN30 Senegal 2010	JX101788
SEN37 Senegal 2010	JX101789
SO9277 Spain 2012	KF254920
SO9288 Spain 2012	KF254922
SO9336 Spain 2012	KF254917
SO9406 Spain 2012	KF254924
SO9411 Spain 2012	KF254921
SO9493 Spain 2012	KF254918
SO9770 Spain 2013	KF254914
TH B1512 Thailand 2009	JQ411802
TH CU101 Thailand 2011	JQ411804
TH CU124 Thailand 2011	JQ411807
TH CU134 Thailand 2011	JQ411803
TH CU54 Thailand 2011	JQ411805
TJ-3395 China 2010	JQ924862
TOp-13-Ph086 Philippines 2013	AB992427
TOp-13-Ph364 Philippines 2013	AB992432
TOp-13-Ph396 Philippines 2013	AB992433
TOp-13-Ph397 Philippines 2013	AB992434
TOp-13-Ph415 Philippines 2013	AB992435
TOp-13-Ph416 Philippines 2013	AB992436

Table 4 (continued)

Strains	Accession numbers
TOp-14-Ph009 Philippines 2014	AB992440
TTa-08-Ph343 Philippines 2008	AB817702
TTa-08-Ph451 Philippines 2008	AB817703
TTa-08-Ph513 Philippines 2008	AB817704
TTa-08-Ph519 Philippines 2008	AB817705
TTa-08-Ph561 Philippines 2008	AB817707
TTa-08-Ph560 Philippines 2008	AB817706
TTa-08-Ph597 Philippines 2008	AB817708
TTa-08-Ph608 Philippines 2008	AB817709
TTa-11-Ph224 Philippines 2011	AB861413
TTa-11-Ph272 Philippines 2011	AB817714
TTa-11-Ph245 Philippines 2011	AB817711
TTa-11-Ph257 Philippines 2011	AB817712
TTa-11-Ph269 Philippines 2011	AB817713
TTa-11-Ph344 Philippines 2011	AB861414
TTa-11-Ph395 Philippines 2011	AB817716
TEv-13-Ph397 Philippines 2013	AB992429
TBp-13-Ph417 Philippines 2013	AB992430
TTa-13-Ph137 Philippines 2013	AB992425
TTa-13-Ph096 Philippines 2013	AB992424
TBp-13-Ph209 Philippines 2013	AB992428
ILI-13-Ph137 Philippines 2013	AB992413
TTa-13-Ph162 Philippines 2013	AB992426
TBp-13-Ph440 Philippines 2013	AB992431
TTS-13-Ph031 Philippines 2013	AB992414
TTS-13-Ph034 Philippines 2013	AB992415
TTS-13-Ph095 Philippines 2013	AB992416
TTS-13-Ph096 Philippines 2013	AB992417
TTS-13-Ph106 Philippines 2013	AB992418

Table 4 (continued)

Strains	Accession numbers
TTS-13-Ph128 Philippines 2013	AB992419
TTS-13-Ph139 Philippines 2013	AB992420
TTS-13-Ph148 Philippines 2013	AB992421
TTS-13-Ph154 Philippines 2013	AB992422
TOp-13-Ph418 Philippines 2013	AB992441
TTS-14-Ph021 Philippines 2014	AB992437
TTS-14-Ph026 Philippines 2014	AB992438
TTS-14-Ph028 Philippines 2014	AB992439
TEv-14-Ph032 Philippines 2014	AB992442
TEv-14-Ph043 Philippines 2014	AB992443
ILI-14-Ph023 Philippines 2014	AB992423
ILI-14-Ph037 Philippines 2014	KX789243
TOp-15-Ph068 Philippines 2015	KX789231
TOp-12-Ph146 Philippines 2012	KX789232
TTa-13-Ph173 Philippines 2013	KX789246
TB9-15-Ph380 Philippines 2015	KX789238
TB8-15-Ph508 Philippines 2015	KX789239
TU-15-Sendai725 Japan 2015	KX789221
TU-15-Sendai727 Japan 2015	KX789224
TU-15-Sendai728 Japan 2015	KX789223
TU-15-Sendai729 Japan 2015	KX789222
SMC-15-Sendai902 Japan 2015	KX789226
SMC-15-Sendai908 Japan 2015	KX789225
SMC-15-Sendai823 Japan 2015	KX789264
SMC-15-Sendai830 Japan 2015	KX789265
SMC-15-Sendai876 Japan 2015	KX789266
SMC-15-Sendai881 Japan 2015	KX789267
US/MO/14-18947 USA 2014	KM851225
US/MO/14-18948 USA 2014	KM851226

Table 4 (continued)

Strains	Accession numbers
US/MO/14-18949 USA 2014	KM851227
US/MO/14-18950 USA 2014	KM851228
US/KY/14-18951 USA 2014	KM851229
US/IL/14-18952 USA 2014	KM851230
US/KY/14-18953 USA 2014	KM851231
US/CO/14-94 USA 2014	KP100793
US/CO/13-60 USA 2013	KP100794
US/CO/14-86 USA 2014	KP126912
US/CA/14-6092 USA 2014	KP100792
US/CA/14-6103SIB USA 2014	KP100795
US/CA/14-R1 USA 2014	KP126909
US/CA/14-R2 USA 2014	KP126908
US/CA/14-6100 USA 2014	KP100796
US/CA/14-6067 USA 2014	KP126910
WI00 USA 2000	AY426494
TX99 USA 1999	AY426498
TX03 USA 2003	AY426500
VH110070195 Spain 2014	KP122208
VH110073590 Spain 2014	KP090456
VH100096404 Spain 2014	KP090457
VH110075023 Spain 2014	KP090458
VH110078432 Spain 2014	KP090459
NY120 USA 2014	KP745751
NY124 USA 2014	KP745752
NY126 USA 2014	KP745753
NY130 USA 2014	KP745754
NY153 USA 2014	KP745755
NY160 USA 2014	KP745756
NY210 USA 2014	KP745757

Table 4 (continued)

Strains	Accession numbers
NY263 USA 2014	KP745758
NY275 USA 2014	KP745759
NY278 USA 2014	KP745760
NY305 USA 2014	KP745761
NY309 USA 2014	KP745762
NY314 USA 2014	KP745763
NY316 USA 2014	KP745764
NY326 USA 2014	KP745765
NY328 USA 2014	KP745766
NY329 USA 2014	KP745767
NY73 USA 2014	KP745768
NY74 USA 2014	KP745769
NY77 USA 2014	KP745770
CF253080 France 2014	LN681316
CF254007 France 2014	LN681317
CF211059 France 2014	LN681318
CF219068 France 2014	LN681319
CF259061 France 2014	LN681320
CF235016 France 2014	LN681321
CF266150 France 2014	LN681322
CF272004 France 2014	LN681323
CF276018 France 2014	LN681324
CF279027 France 2014	LN681325
CF287055 France 2014	LN681326
CF287062 France 2014	LN681327
CF289007 France 2014	LN681328
CF294025 France 2014	LN681329
CF295086 France 2014	LN681330
CF298012 France 2014	LN681331

Table 4 (continued)

Strains	Accession numbers
CF300005 France 2014	LN681334
CF302003 France 2014	LN681335
CF307029 France 2014	LN681336
CF311065 France 2014	LN681337
CF314027 France 2014	LN681338
VERS290059 France 2014	LN681339
VERS290090 France 2014	LN681340
ITA/18641/08 Italy 2008	KC763157
ITA/19179/08 Italy 2008	KC763158
ITA/26868/08 Italy 2008	KC763172
ITA/22516/12 Italy 2012	KC763164
ITA/24518/12 Italy 2012	KC763169
ITA/23695/12 Italy 2012	KC763167
ITA/27708/08 Italy 2008	KC763173
ITA/31742/10 Italy 2010	KC763174
ITA/33658/10 Italy 2010	KC763175
ITA/33707/10 Italy 2010	KC763176
ITA/34800/10 Italy 2010	KC763177
ITA/19391/12 Italy 2012	KC763159
ITA/20260/08 Italy 2008	KC763161
ITA/22289/08 Italy 2008	KC763163
ITA/22719/08 Italy 2008	KC763165
ITA/23352/08 Italy 2008	KC763166
ITA/26423/08 Italy 2008	KC763170
L2610020652 Denmark 2014	KP729103
L2620000697 Denmark 2014	KP729104.
L2620023301 Denmark 2014	KP729105
L2630000505 Denmark 2014	KP729106
L2630003743 Denmark 2014	KP729107

Table 4 (continued)

Strains	Accession numbers
V14021246 Denmark 2014	KP729108
V14021393 Denmark 2014	KP729109
08T31952 Denmark 2008	KR108018
L2510014597 Denmark 2013	KR108021
10T62302 Denmark 2011	KR108019
L21442092 Denmark 2010	KR108020
L2640002876 Denmark 2014	KR108022
L2640003884 Denmark 2014	KR108023
EV-D68/Haiti/1/2014 Haiti 2014	KT266905
2011-21282 China 2011	KT285320
2014-R1357 China 2014	KT280496
2014-R1153 China 2014	KT280497
2013-0720-6 China 2013	KT280504
2011-21186 China 2011	KT280503
2014-R1011 China 2014	KT280498
2014-R970 China 2014	KT280499
2014-R0672 China 2014	KT280500
2011-21286 China 2011	KT306743
2012-12225 China 2012	KT285319
2013-1017-26 China 2013	KT280501
2013-0825-6 China 2013	KT280502
CA/AFP/11-1767 USA 2013	KM892501
TW-01166 Taiwan 2007	KP657701
TW-05745 Taiwan 2007	KP657702
TW-09669 Taiwan 2007	KP657703
TW-11406 Taiwan 2007	KP657704
TW-08683 Taiwan 2008	KP657705
TW-09064 Taiwan 2009	KP657707
TW-08202 Taiwan 2008	KP657706

Table 4 (continued)

Strains	Accession numbers
TW-11134 Taiwan 2009	KP657708
TW-01557 Taiwan 2010	KP657709
TW-00588 Taiwan 2010	KP657710
TW-03137 Taiwan 2010	KP657711
TW-01788 Taiwan 2010	KP657712
TW-02378 Taiwan 2010	KP657713
TW-02755 Taiwan 2010	KP657714
TW-09384 Taiwan 2011	KP657715
TW-10368 Taiwan 2011	KP657716
TW-13025 Taiwan 2011	KP657717
TW-03243 Taiwan 2012	KP657718
TW-04017 Taiwan 2012	KP657719
TW-02722 Taiwan 2013	KP657720
TW-00943 Taiwan 2013	KP657721
TW-00297 Taiwan 2014	KP657722
TW-00344 Taiwan 2014	KP657723
5533 Israel 2014	KP317481
5876 Israel 2014	KP317480
HEV044008 Kenya 2008	KJ472878
HEV085008 Kenya 2008	KJ472880
HEV156010 Kenya 2010	KJ472882
HEV124010 Kenya 2010	KJ472883
HEV126010 Kenya 2010	KJ472884
HEV137010 Kenya 2010	KJ472885
HEV196011 Kenya 2011	KJ472886
HKSH012 Hong Kong 2014	KT762415
HKSH013 Hong Kong 2014	KT762416
HKSH024 Hong Kong 2014	KT762427
HKSH023 Hong Kong 2014	KT762426

Table 4 (continued)

Strains	Accession numbers
HKSH022 Hong Kong 2014	KT762425
HKSH009 Hong Kong 2014	KT762412
HKSH008 Hong Kong 2014	KT762411
HKSH007 Hong Kong 2014	KT762410
HKSH006 Hong Kong 2014	KT762409
HKSH005 Hong Kong 2014	KT762408
HKSH004 Hong Kong 2014	KT762407
HKSH003 Hong Kong 2014	KT762406
HKSH002 Hong Kong 2014	KT762405
HKSH001 Hong Kong 2014	KT762404
MEX/DF/2014-InDRE2351 Mexico 2014	KT825142
MEX/DGO/2014-InDRE2271 Mexico 2014	KT803995
Ontario/C818712/2014 Canada 2014	KT835408
Ontario/C818710/2014 Canada 2014	KT835407
CA/BC/14-334 Canada 2014	KT873664
CA/BC/14-166 Canada 2014	KT873670
CA/BC/14-200 Canada 2014	KT873671
CA/BC/14-275 Canada 2014	KT873652
CA/BC/14-306 Canada 2014	KT873650
CA/BC/14-244 Canada 2014	KT873649
CA/BC/14-265 Canada 2014	KT873648
CA/BC/14-193 Canada 2014	KT873638
CA/BC/14-165 Canada 2014	KT873630
CA/BC/14-251 Canada 2014	KT873631
CA/BC/14-289 Canada 2014	KT873632
CA/BC/14-215 Canada 2014	KT873633
CA/BC/14-259 Canada 2014	KT873634
CA/BC/14-239 Canada 2014	KT873554
CA/BC/14-291 Canada 2014	KT873551

Table 4 (continued)

Strains	Accession numbers
CA/BC/14-053 Canada 2014	KT873550
CA/BC/14-264 Canada 2014	KT873549
CA/BC/14-076 Canada 2014	KT873548
CA/BC/14-229 Canada 2014	KT873540
CA/BC/14-288 Canada 2014	KT873539
CA/BC/14-321 Canada 2014	KT873555
CA/BC/14-214 Canada 2014	KT873556
CA/BC/14-212 Canada 2014	KT873562
CA/BC/14-103 Canada 2014	KT873563
CA/BC/14-184 Canada 2014	KT873565
CA/BC/14-197 Canada 2014	KT873570
CA/BC/14-207 Canada 2014	KT873574
CA/BC/14-142 Canada 2014	KT873595
CA/BC/14-162 Canada 2014	KT873599
CA/BC/14-322 Canada 2014	KT873604
CA/BC/14-299 Canada 2014	KT873605
CA/BC/14-309 Canada 2014	KT873613
CA/BC/14-331 Canada 2014	KT873577
CA/BC/14-104 Canada 2014	KT873583
CA/BC/14-283 Canada 2014	KT873590
CA/BC/14-335 Canada 2014	KT873592
CA/BC/14-102 Canada 2014	KT873594
CA/BC/14-325 Canada 2014	KT873536
CA/BC/13-150 Canada 2014	KT873535
CA/BC/14-339 Canada 2014	KT873537
12MYKL1213 Malaysia 2012	KR018811
12MYKL1236 Malaysia 2012	KR018812
12MYKL1307 Malaysia 2012	KR018813
12MYKL1501 Malaysia 2012	KR018814

Table 4

Strains	Accession numbers
12MYKL1607 Malaysia 2012	KR018815
TW 02809 Taiwan 2014	KT711087
TW 00880 Taiwan 2014	KT711078
TW 00898 Taiwan 2014	KT711082
TW 02512 Taiwan 2014	KT711086
TW 00893 Taiwan 2014	KT711079
TW 02809 Taiwan 2014	KT711087
TW 00880 Taiwan 2014	KT711078
TW 00898 Taiwan 2014	KT711082
TW 02512 Taiwan 2014	KT711086
TW 00893 Taiwan 2014	KT711079
CF267090 France 2014	LN626610
CA/RESP/09-871 USA 2013	KM892497

Table 5 Distribution of EV-D68-positive cases at each study site in the Philippines from August 2012 to February 2014.

Patients	Regions	Before the typhoon Haiyan (Sep 2012-Nov 8, 2013)		After the typhoon Haiyan (After Nov 8, 2013- Feb 2014)	
		Total no.	EV-D68 cases (%)	Total no.	EV-D68 cases (%)
Hospitalized patients (pediatric sARI)	Leyte	580	4 (0.7)	53	5 (9.4)
	Biliran	512	1 (0.2)	70	2 (2.9)
	Palawan	525	2 (0.4)	114	6 (5.3)
	Total no.	**1617**	**7 (0.4)**	**237**	**13 (5.5)**

Table 6 Association index (AI) and Parsimony (PS) values of EV-D68 clustering by spatial and temporal traits

Traits		EV-D68 dataset	Association index (AI) values		Ratio Observed/Null	p-values	Parsimony (PS) values		Ratio Observed/Null	p-values
			Observed	Null			Observed	Null		
Spatial	continent	All lineages	4.96	29.24	0.17	<0.01	65.25	212.36	0.31	<0.01
		Lineage 2	1.69	19.20	0.09	<0.01	23.99	135.18	0.18	<0.01
		Lineage 3	2.31	9.58	0.24	<0.01	34.01	70.27	0.48	<0.01
	country	All lineages	11.96	40.09	0.30	<0.01	148.90	325.70	0.46	<0.01
		Lineage 2	6.14	19.60	0.31	<0.01	74.33	158.60	0.47	<0.01
		Lineage 3	5.12	13.13	0.39	<0.01	64.47	108.29	0.60	<0.01
Temporal	year	All lineages	7.15	34.99	0.20	<0.01	92.92	260.91	0.36	<0.01
		Lineage 2	2.82	12.91	0.22	<0.01	36.65	85.34	0.43	<0.01
		Lineage 3	3.36	11.16	0.30	<0.01	40.74	81.78	0.50	<0.01

Note: The sequences of lineage 1 did not analyze separately due to a limitation of temporal and spatial information.

Table 7 Number of VP1sequences (546 nucleotides) of EV-D68 in Asia from 2005 to 2015 obtained from GenBank as of July 2016.

Countries or regions	Years											
	2005	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**Total**
China				2		1	6	3	5	16	4	37
Hong Kong						4	6	8	6	30		54
Japan	10	1	2	2		52					10	77
Malaysia								5				5
Philippines				8			7	1	25	8	3	52
Taiwan			4	3	2	6	3	2	2	13		35
Thailand					1		5					6
Total	**10**	**1**	**6**	**15**	**3**	**63**	**27**	**19**	**38**	**67**	**17**	**266**

Table 8 Substitution density of a whole EV-D68 genome.

Genes	VP4	VP2	VP3	VP1	2A	2B	2C	3A	3B	3C	3D
Substitution density	14.5%	11.3%	12.8%	27.8%	15.0%	20.2%	12.7%	13.5%	22.7%	1.2%	2.6%

Table 9 Mean dN/dS values of whole EV-D68 genome using the SLAC method**.**

Genes	Mean dN/dS values
VP1	0.10
VP2	0.04
VP3	0.05
VP4	0.07
2A	0.06
2B	0.07
2C	0.04
3A	0.05
3B	0.04
3C	0.03
3D	0.04

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