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Genomic phylogenetic analyses of four major hand, foot and mouth disease-related enteroviruses

Yajuan Dong[1,2], Zhenzhou Wan[3], Shenwei Li[4], Jian-Hua Wang[5], Xia Jin[2], Guoying Yu[1*], Chiyu Zhang[2*]

[1]College of Life Sciences, Henan Normal University, Xinxiang 453007, P. R. China; [2]Shanghai Public Health Clinical Center, Fudan University, Shanghai 201508, P. R. China; [3]Medical Laboratory of Taizhou Fourth People's Hospital, Taizhou 225300, P. R. China; [4]Shanghai International Travel Healthcare Center, Shanghai 200335, P. R. China; [5]Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, P. R. China

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Summary. – Enteroviruses had diverged into many types, some of which cause hand, foot and mouth disease (HFMD) in children. The predominant enterovirus types associated with HFMD are EVA71, CVA16, CVA6 and CVA10. Four enterovirus types were classified into subtypes based on VP1 sequences. However, the phylogenetics of these enteroviruses is rarely concerned at the genomic level. In this study, we performed the phylogenetic analyses of the EVA71, CVA16, CVA6 and CVA10 using available full-length genomic sequences. We found that the topologies of phylogenetic trees of full-length genomic sequences and VP1 sequences were almost consistent, except few subtypes of EVA71 and CVA10. The mean genetic divergence was 15.8–27% between subtypes and less than 12% within subtypes/sub-subtypes at genomic level. Comparison of phylogenetic topologies between genomic and VP1 sequences helped us to identify two new EVA71 inter-subtype recombinants RF01_CC4 and RF02_CC4. Furthermore, EVA71 subtypes C1 and C2 and CVA10 subtype D were found to originate through inter-subtype recombination. The genomic reference sequences of these enteroviruses are provided here for subtyping. The results provide important insights into the understanding of the evolution and epidemiology of the four enteroviruses.

Keywords: enterovirus; hand; foot and mouth disease; classification; genetic distance; recombination

Introduction

Hand, foot and mouth disease (HFMD) is a common contagious disease of childhood. It is caused by infection with various non-polio and non-rhinovirus enteroviruses and characterized by fever and skin eruptions on the hands and feet, and vesicles in the mouth (Zaoutis and Klein 1998; Lei *et al.,* 2015). HFMD has been a major public health burden across the Asia-Pacific region with an estimated number of cases with over 1.38 million per year worldwide (Lei *et al.,* 2015).

Enterovirus belongs to the *Picornaviridae*, a family of small, non-enveloped viruses with a positive-stranded RNA genome of approximately 7.4 kilobase in size (Hyypia *et al.,* 1997), and it is highly divergent and hierarchically classified into 15 species including enterovirus A-L and rhinovirus A-C based on sequence identity and genome organization (Hyypia *et al.,* 1997; Muehlenbachs *et al.,* 2015; Lukashev *et al.,* 2018). Enterovirus types are further classified into a large number of genotypes based on a genetic distance of over 25% at nucleotide level (Lukashev and Vakulenko, 2017; Lukashev *et al.,* 2018). Distinct enterovirus types can exhibit various biological properties related to virulence, transmissibility and pathogenesis, and they cause different diseases (Zaoutis and Klein, 1998; Muehlenbachs *et al.,* 2015; Wang C *et al.,* 2018; Fu *et al.,* 2020).

*Corresponding authors. E-mail: zhangcy1999@hotmail.com; phone: +86-158-0078-1776 (C.Z.); 201508. E-mail: 2018043@htu.edu.cn; phone: +86-0373-3326341 (G.Y.); 453007.

Abbreviations: HFMD = hand, foot and mouth disease; ICTV = International Committee on Taxonomy of Viruses; ML = maximum likelihood NJ = Neighbor-Joining; RF = recombinant form

HFMD is mainly attributed to some enterovirus types within enterovirus A and B species (Zaoutis and Klein, 1998; Lei *et al.,* 2015; Muehlenbachs *et al.,* 2015).

Enterovirus A71 (EVA71) and coxsackievirus A16 (CVA16) are the two most commonly detected enteroviruses A among HFMD cases. However, the molecular epidemiology of HFMD-related enteroviruses was changing during the past decade, with a progressive increase of CVA6, CVA10 and other enterovirus types (Fu *et al.,* 2020). Currently, the four dominant enterovirus types were divided into various numbers of subtypes mainly based on their phylogenetic relationships of partial VP1 gene sequences (Oberste *et al.,* 1999a,b; Perera *et al.,* 2007; He *et al.,* 2013; Saxena *et al.,* 2015; Xu *et al.,* 2015; Zhang *et al.,* 2015; Song *et al.,* 2017; Weng *et al.,* 2017; Ji *et al.,* 2018). For example, previous studies classified EVA71 into A-G (Bessaud *et al.,* 2014; Saxena *et al.,* 2015; Fernandez-Garcia *et al.,* 2018), CVA16 into A, B1, B2, D (Hassel *et al.,* 2017; Wang J *et al.,* 2018), CVA6 into A-D (Song *et al.,* 2017) and CVA10 into A-G (Tian *et al.,* 2017; Ji *et al.,* 2018) subtypes.

Enteroviruses contain four structural viral proteins VP1, VP2, VP3 and VP4, of which VP1, VP2, and VP3 are external, whereas VP4 is completely internalized. VP1 is the most important capsid protein on the surface of enteroviruses and serves as the main neutralizing antigen determinant of enteroviruses. Previously VP1 capsid protein was used in neutralization assay to determine virus type, and more recently this classification was correlated to VP1 sequences. However, the phylogenetics of enteroviruses at genomic level is rarely concerned, missing thus some inter-subtype recombinants. The increasing publicly available number of genomic sequences allows us to further analyze phylogeny of HFMD-related enteroviruses (Bessaud *et al.,* 2014; Saxena *et al.,* 2015; Hassel *et al.,* 2017; Song *et al.,* 2017; Tian *et al.,* 2017; Fernandez-Garcia *et al.,* 2018; Ji *et al.,* 2018; Wang *et al.,* 2018).

Materials and Methods

Sequence collection. All available full-length genomic sequences of EVA71, CVA16, CVA10 and CVA6 of enteroviruses A were downloaded from the GenBank on November 5, 2019. According to the prototype strains of EVA71 (BrCr: U22521), CVA16 (G-10: U05876), CVA6 (Gdula: AY421764), and CVA10 (Kowalik: AY421767) in the International Committee on Taxonomy of Viruses (ICTV), the sequences with a length of <90% of full-length genomic sequences were removed. The selected sequences were trimmed to a same length according to prototype EVA71 strain, BrCr; CVA16 G-10; CVA6 Gdula; CVA10 Kowalik (7149 nt for EVA71, 7250 nt for CVA16, 7293 nt for CVA6, 6874 nt for CVA10). The selected full-length genomic sequences and their complete VP1 sequences were subjected to the phylogenetic analyses. All available near complete VP1 gene sequences (about 880–915 nt) of the four enteroviruses in GenBank were also downloaded on November 5, 2019.

Phylogenetic analyses. All sequence alignments were performed using MUSCLE implemented in MEGA-X. To investigate the phylogenies of the four predominant HFMD-related enteroviruses EVA71, CVA16, CVA6, and CVA10, maximum likelihood (ML) trees were constructed based on the full-length genomic sequences (7149 nt for EVA71, 7250 nt for CVA16, 7293 nt for CVA6, 6874 nt for CVA10) using MEGA-X with 1000 bootstrap replications. The ML trees were also constructed using the complete VP1 sequences from the full-length genomic sequences, together with additional near-complete VP1 sequences from GenBank, including those without full-length genomic sequences. The model used for the ML tree construction were General Time Reversible model (GTR) with Gamma Distributed With Invarient Sites (G+I), and partial deletion of sequence gaps with site coverage cut off 50%. To minimize the calculation time in ML tree construction, about 100 full-length genomic sequences of each enterovirus were selected for the phylogenetic analyses. To achieve the goal, only one representative sequence was included if there were two or more sequences sharing sequence similarity of more than 97% for EVA71, 98% for CVA6, and 99% for both CVA10 and CVA16 (the number of sequences with high similarity is shown at the end of the name of strain in the phylogenetic tree). The use of 97% similarity criterion for EVA71 is due to too many (913) available full-length genomic sequences. To confirm the results by ML method, Neighbor-Joining (NJ) trees were further constructed.

Recombination analysis. To detect potential recombination occurring in enteroviruses, bootscan and similarity plot analyses were performed using SimPlot v.3. 5. 1 (Lole *et al.,* 1999). The default parameter with Kimura two-parameter substitution model with a transition/transversion ratio of 2 was used in the analysis. The plots show the percentages of permuted trees and similarity of the query sequence to a panel of reference sequences in a sliding window along the sequence alignment.

Results

Genomic phylogenetic analysis of EVA71

All 922 full-length genomic sequences of EVA71 available in GenBank were downloaded (on November, 2019). Of them, 913 with a length of more than 7000 nt were subject to sequence alignment. After removing highly homologous sequences with more than 97% sequence similarity, 131 representative genomic sequences were used to construct maximum likelihood (ML) tree. The ML tree of the full-length genomic sequences showed that the vast majority of EVA71 strains were clustered within three well-supported large clades (with 100% bootstrap value),

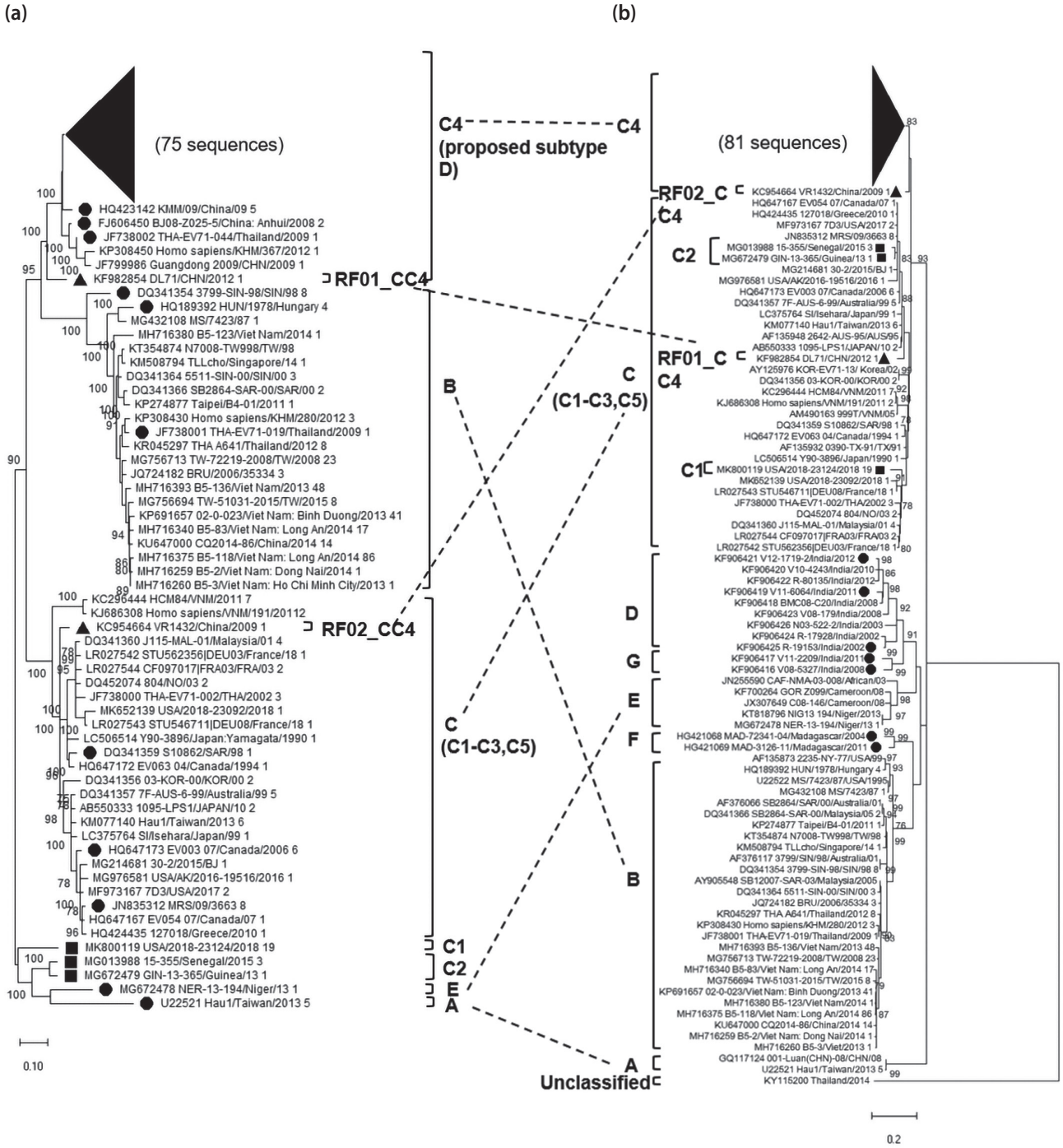

Fig. 1

Phylogenetic analysis of EV-A71 using the ML method based on near full-length genomic (a) and complete VP1 (b) sequences
The stability of the nodes was assessed by bootstrap analysis with 1000 replications, and only the bootstrap values of >75% are shown at the nodes. According to the phylogeny of full-length genomic sequences, C4 was proposed as subtype D by previous studies (Chan *et al.*, 2010; Yip *et al.*, 2010). Two subtypes experiencing early recombination events are highlighted in black solid boxes. Two recombinants are highlighted in black solid triangles. The reference sequences recommended are highlighted in black solid circles. The last number in the name of each strain indicates the number of completely identical genomic sequences. The last number "1" indicates that there was only one unique genomic sequence.

Table 1. Mean genetic distance and standard error among subtypes of EVA71

Subtype	A	B	C	C4	D	E	F	G
A	NA/0.01±0.00	0.215±0.014	0.208±0.012	0.207±0.013	0.222±0.012	0.234±0.014	0.218±0.013	0.231±0.014
B	0.235±0.006	0.09±0.00/ 0.09±0.00	0.206±0.010	0.185±0.010	0.215±0.011	0.221±0.011	0.194±0.011	0.207±0.012
C	0.242±0.005	0.235±0.005	0.11±0.00/ 0.10±0.01	0.135±0.007	0.208±0.010	0.200±0.010	0.202±0.011	0.192±0.010
C4	0.239±0.006	0.215±0.005	0.205±0.004	0.07±0.00/ 0.06±0.00	0.204±0.011	0.197±0.011	0.200±0.011	0.197±0.012
D	NA	NA	NA	NA	NA/0.11±0.01	0.188±0.010	0.205±0.011	0.172±0.009
E	0.225±0.006	0.222±0.005	0.235±0.005	0.238±0.005	NA	NA/0.09±0.01	0.208±0.011	0.195±0.011
F	NA	NA	NA	NA	NA	NA	NA/0.09±0.01	0.200±0.011
G	NA	NA	NA	NA	NA	NA	NA	NA/0.11±0.01

The data obtained from full-length (7149 nt) and complete VP1 sequences (891 nt) are shown in lower left and top right quarters, respectively. NA: not available. It means that there is only one, no sequence, or more than two completely identical sequences.

and the others formed small clusters or independent phylogenetic branches (Fig. 1a).

To compare the phylogeny of EVA71 between genomic and VP1 sequences, complete VP1 sequences from 131 full-length genomic sequences were subjected to further phylogenetic analysis, together with 31 additional near-complete VP1 sequences retrieved from GenBank (Fig. 1b). The phylogeny of EVA71 was almost consistent between the ML phylogenetic trees of genomic and VP1 sequences except few strains. The strains from the same subtypes (A-G) determined by VP1 sequences often clustered together to form independent clusters, supporting the previous classification (Fig. 1) (Saxena *et al.,* 2015; Fernandez-Garcia *et al.,* 2018), which was validated by NJ trees (Supplementary Fig. S1A and B). Different subtypes appeared to diverge from each other (Table 1). The mean inter-subtype genetic distances ranged from 20.5% to 24.2% at the genome level and 17.2% to 23.4% at VP1 gene level. The mean within-subtype distance ranged from 7% to 11% at the genome level and 1% to 11% at VP1 gene level (Table 1).

Some EVA71 strains (earlier called C4) were found to form an independent clade and diverge from the most related clade of subtype C with a genetic distance of 20.5% in the tree of full-length genomic sequences (Table 1), and they had been confirmed as a new recombinant subtype D based on full-length genomic sequences in previous with studies (Chan *et al.,* 2010; Yip *et al.,* 2010). Because of the lack of available full-length genomic sequences, some strains at the root of the VP1 tree were unable to be analyzed.

Interestingly, we found that three representative strains (C1_2018-23124, C2_15-355, C2_GIN-13-365) had inconsistent topological location between the trees of full-length genomic and near-complete VP1 sequences, and formed two independent clusters in the genomic phylogenetic tree (Fig. 1a), which suggests that recombination event might have occurred as previously reported (Fernandez-Garcia *et al.,* 2018). Bootscan analyses confirmed that the strain (15-355) was involved in recombination between subtypes C and E, and another strain (2018-23124) was involved in recombination among subtypes C4, E and C (Supplementary Fig. S2A and B).

Besides above three strains, there were two additional strains (DL71/CHN/2012 and VR1432/CHN/2009) that also showed inconsistent topological location in both the full-length genomic and near-complete VP1 trees, and were highly suspected to be inter-subtype recombinants. DL71/CHN/2012 clustered between subtypes C4 (proposed subtype D) and B in the full-length genomic sequence tree, but within the clade of subtype C (C1-C3, C5) in the VP1 tree. Bootscan analyses demonstrated that DL71/CHN/2012 originated recombination between subtypes C and C4, and had a mosaic genome structure of C-C4-C-C4-C-C4-C (Fig. 2a). The recombination pattern was further confirmed by separate phylogenetic analyses (Supplementary Fig. S3). Another strain VR1432/China/2009 in the full-length genomic sequence tree was found to cluster between subtypes C4 and C in the VP1 tree, suggesting the presence of recombination at least in VP1 region (Fig. 1). Bootscan and separate phylogenetic analyses confirmed that VR1432/China/2009 was a recombinant between subtypes C4 and C with a mosaic genome structure of C4-C-C4-C-C4-C (Fig. 2b and Supplementary Fig. S4). The recombinant strains DL71/CHN/2012 and VR1432/China/2009 were named as EVA71 recombinant form RF01_CC4 and RF02_CC4, respectively.

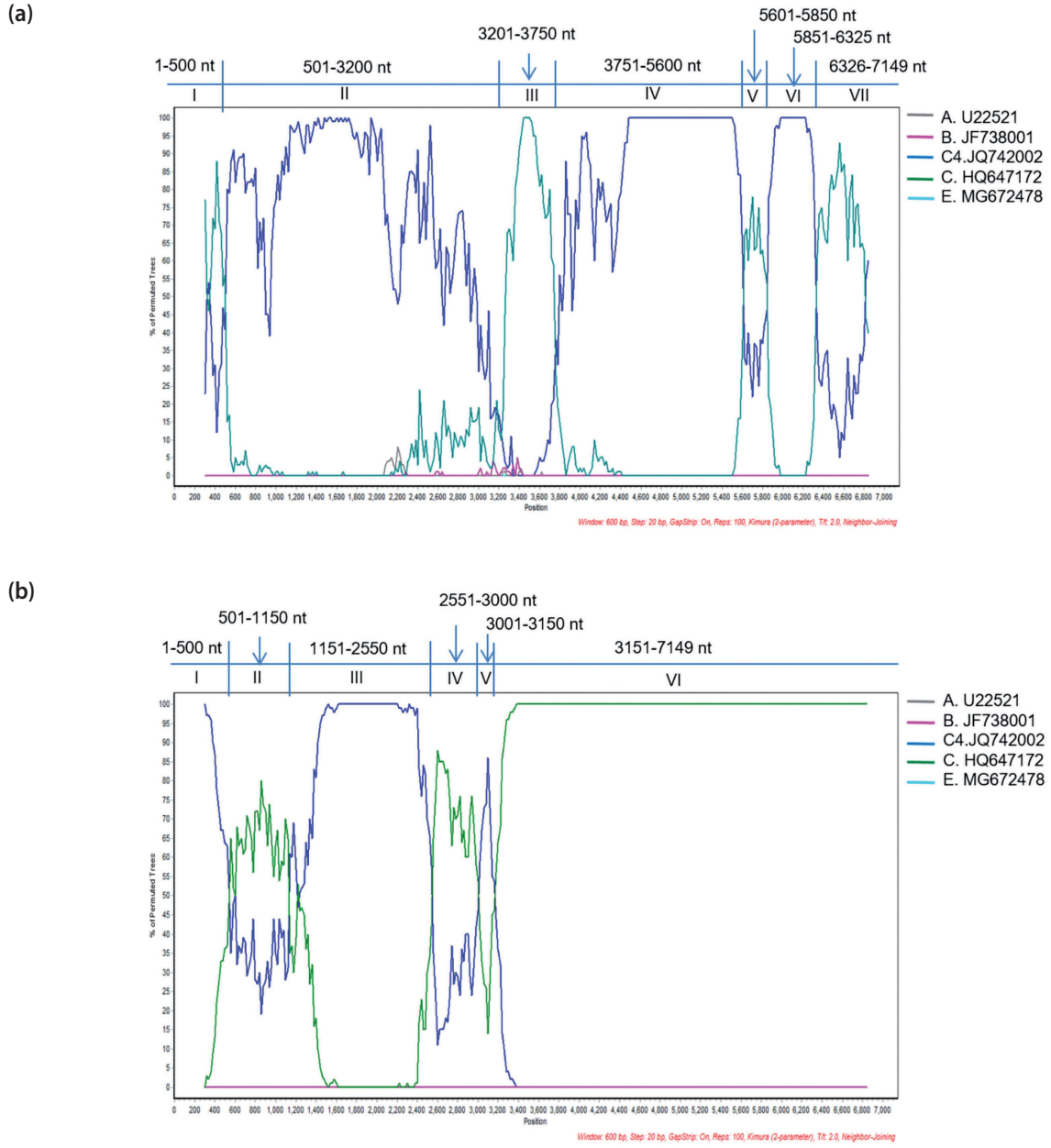

Fig. 2

Bootscan analysis of two inter-subtype recombinants of EV-A71

(a) RF01_CC4; **(b)** RF02_CC4. The reference strains used in the analyses were subtypes A (U22521), B (JF738001), C4 (JQ742002), C (HQ647172) and E (MG672478). The analyses were performed using a sliding 600 bp window with 20 bp steps.

Genomic phylogenetic analysis of CVA16

All 142 available near full-length genomic sequences of CVA16 were retrieved from GenBank (on November, 2019). Of them, 138 had a genomic sequence of more than 7000 nt, and were used in sequence alignment. After the removal of 36 similar sequences, 102 representative genomic sequences were used in the phylogenetic analysis. Furthermore, a phylogenetic analysis using 102 VP1 sequences of the representative CVA16 strains and 19 additional VP1 sequences was performed. The topologies of both the full-length genomic and near-complete VP1 sequences were well consistent in both ML (Fig. 3) and NJ trees (Supplementary Fig. S5A and B). The subtype B1 had mean genetic distances of 29.4% and 16.3% to subtypes A and D at VP1 gene level and the mean genetic distances

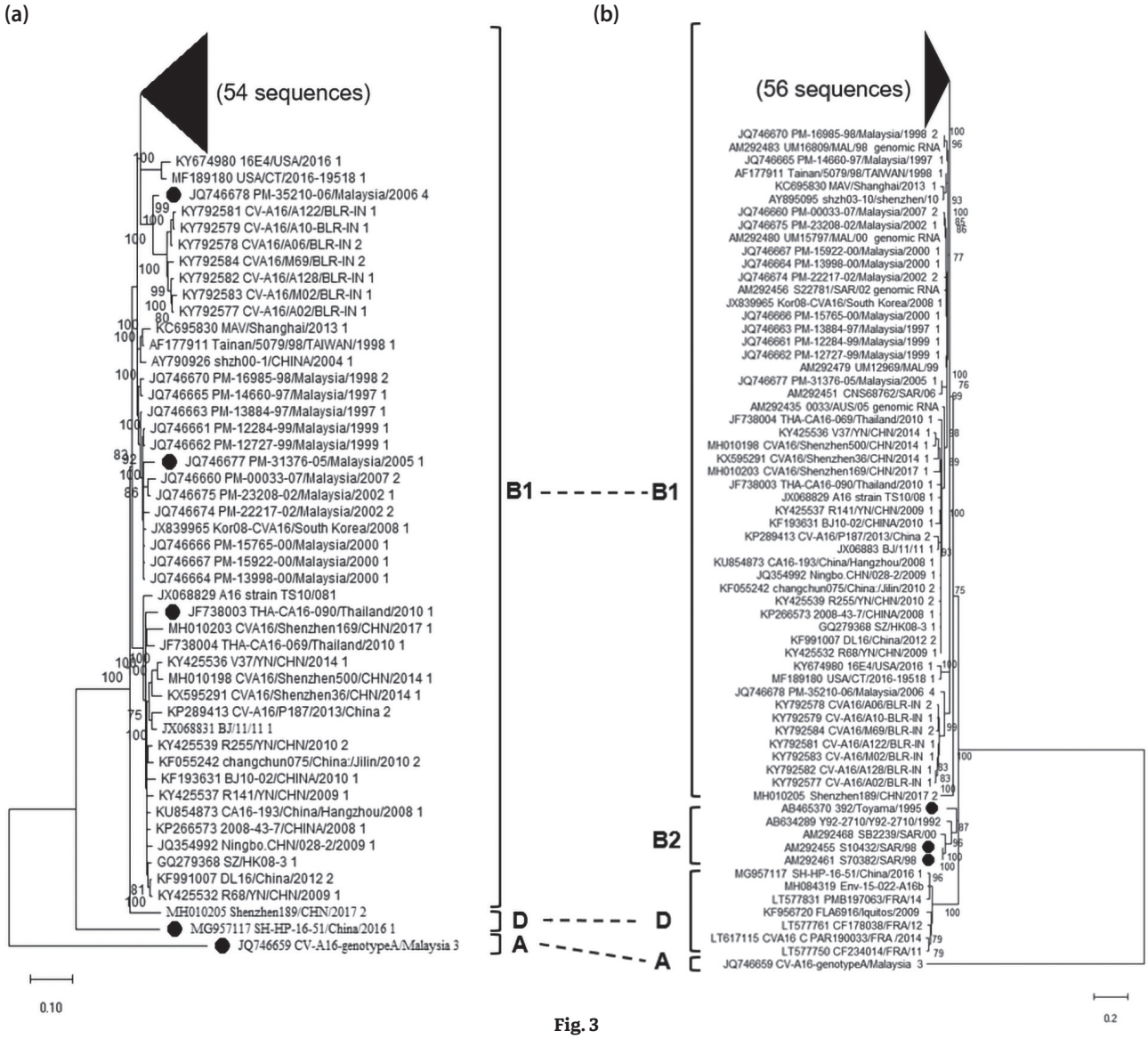

Fig. 3

Phylogenetic analysis of CV-A16 using the ML method based on near full-length genomic (a) and complete VP1 (b) sequences
The stability of the nodes was assessed by bootstrap analysis with 1000 replications, and only the bootstrap values of >75% are shown at the nodes. The reference sequences recommended are highlighted in black solid circles. The last number in the name of each strain indicates the number of completely identical genomic sequences. The last number "1" indicates that there was only one unique genomic sequence.

of 25.7% and 20.8% at the genome gene level, and subtype B2 had mean genetic distances of 29.8%, 12.2% and 16.4% to subtypes A, B1 and D at VP1 gene level, respectively (Supplementary Table S1). The vast majority of the circulating CVA16 strains belonged to subtype B1.

Genomic phylogenetic analysis of CVA6

A total of 230 near full-length genomic sequences of CVA6 were available in GenBank (on November, 2019), and 229 of them had a genomic sequence of more than 7000 nt. After the removal of 160 similar sequences, 69 full-length genomic sequences were used in the phylogenetic analyses based on ML (Fig. 4a) and NJ methods (Supplementary Fig. S6A). Simultaneously, the VP1 sequences of the 69 representative strains were also subjected to the phylogenetic analysis together with 17 additional VP1 sequences (Fig. 4b and Supplementary Fig. S6B). CVA6 was previously classified into four subtypes A, B, C, and D at VP1 gene sequence level (Feng *et al.*, 2015; Tan *et al.*, 2015; Song *et al.*, 2017). Two additional independent branches represented by two strains (40428/TKM/2011, NIV43883/India/2004)

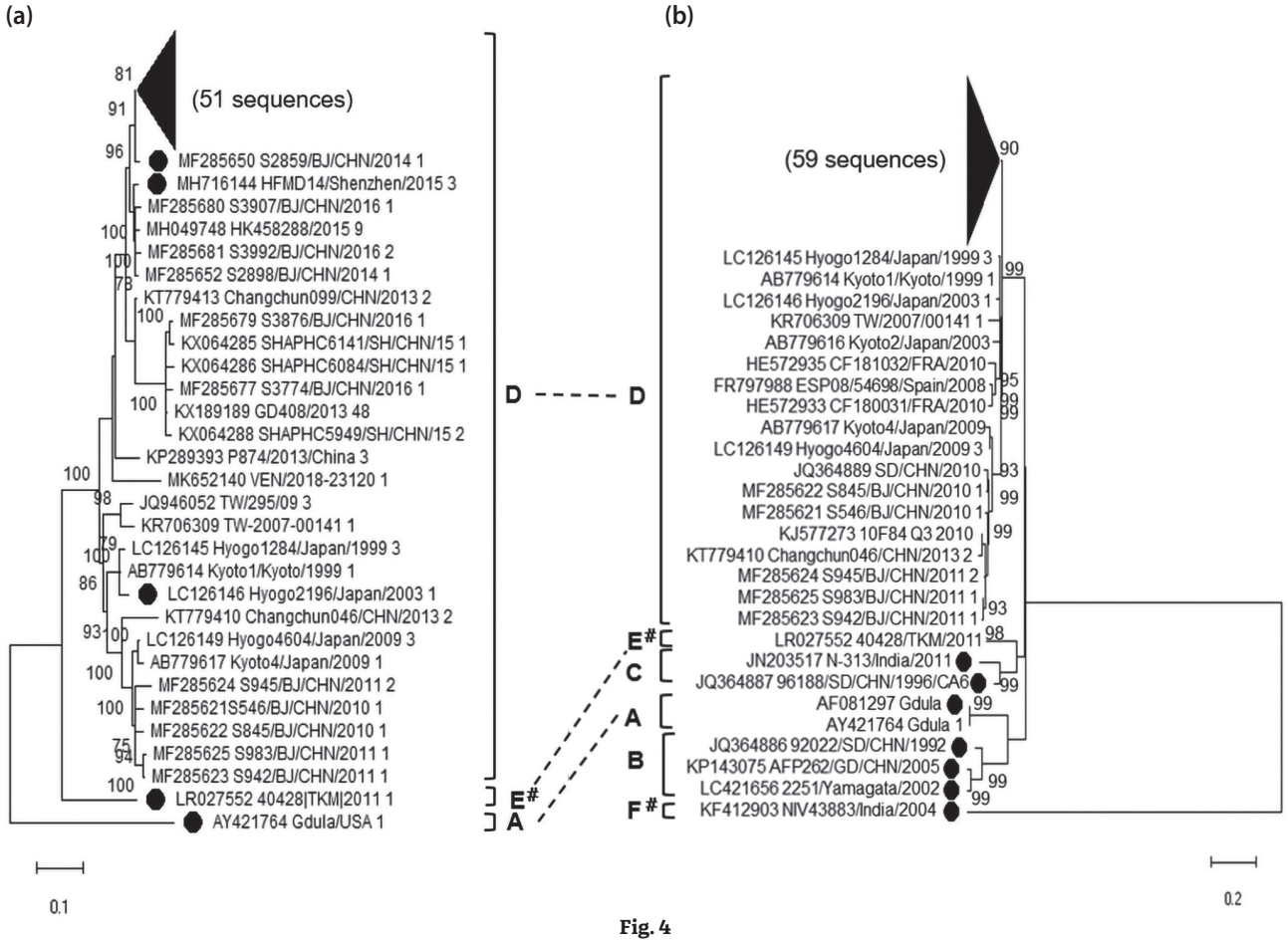

Fig. 4

Phylogenetic analysis of CV-A6 using the ML method based on near full-length genomic (a) and complete VP1 (b) sequences
The stability of the nodes was assessed by bootstrap analysis with 1000 replications, and only the bootstrap values of >75% are shown at the nodes. The letters labeled with well number suggested new subtypes. The reference sequences recommended are highlighted in black solid circles. The last number in the name of each strain indicates the number of completely identical genomic sequences. The last number "1" indicates that there was only one unique genomic sequence.

were identified in the VP1 tree (Fig. 4b). Because the two strains met the phylogenetic relationship and genetic distance criterion, they were suggested to be defined as new subtypes E and F (Fig. 4b and Supplementary Table S2). New subtype E was supported by the phylogenetic analysis of full-length genomic sequences (Fig. 4a). The mean inter-subtype genetic distances were all over 15% regardless of whether being analyzed at genomic or VP1 sequence levels (Supplementary Table S2).

Genomic phylogenetic analysis of CVA10

A total of 127 near full-length genomic sequences of CVA10 were downloaded from GenBank (on November, 2019), and 124 that had a genomic sequence of more than 6800 nt were used. After the removal of 22 similar sequences, 102 representative genomic sequences were used in the phylogenetic analyses based on ML (Fig. 5a) and NJ methods (Supplementary Fig. S7A). Phylogenetic analyses of VP1 sequences were also performed using those from the representative strains and 19 additional VP1 sequences retrieved from GenBank (Fig. 5b and Supplementary Fig. S7B). Seven CVA10 subtypes were previously identified (Tian *et al.*, 2017; Ji *et al.*, 2018). The phylogeny of CVA10 was consistent between the trees of full-length genomic and near complete VP1 sequences (Fig. 5). A new subtype H was suggested and previous subtype F could be sub-divided into two sub-subtypes F1 and F2 (Fig. 5a and Supplementary Table S3). The vast majority of the circulating CVA10 strains belonged to subtypes C and F (F1 and F2).

Interestingly, we found an inconsistent location of the clade of subtype D between the ML trees of genomic sequence and VP1 sequence (Fig. 5). The subtype D clade was

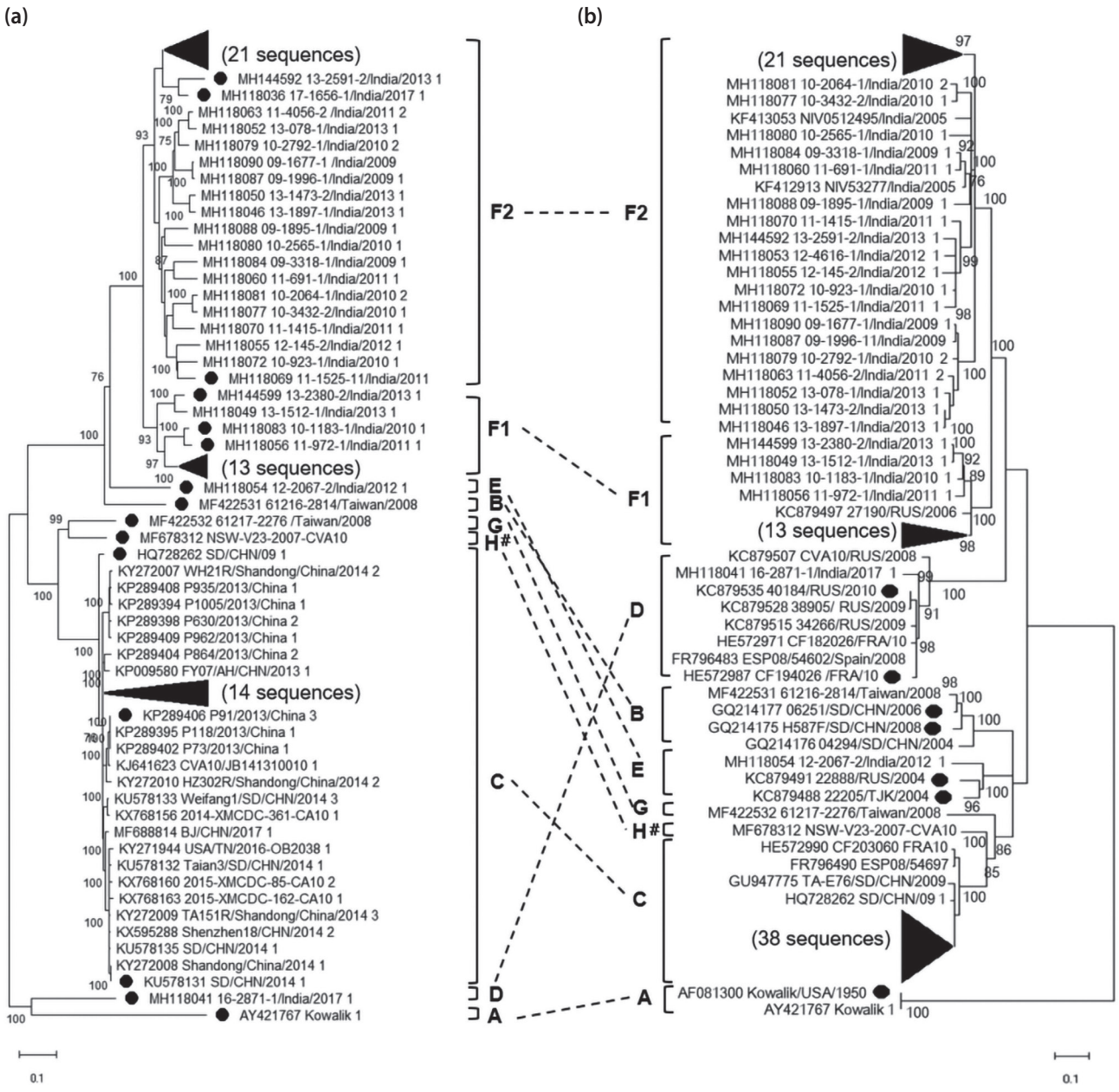

Fig. 5

Phylogenetic analysis of CV-A10 using the ML method based on near full-length genomic (a) and complete VP1 (b) sequences
The stability of the nodes was assessed by bootstrap analysis with 1000 replications, and only the bootstrap values of >75% are shown at the nodes. One suggested new subtype is labeled with well number. The reference sequences recommended are highlighted in black solid circles. The last number in the name of each strain indicates the number of completely identical genomic sequences. The last number "1" indicates that there was only one unique genomic sequence.

located at the root of the ML tree of genomic sequences, but it was located at middle, and closely clustered with the clade of subtype F (including F1 and F2) in the VP1 sequence tree. This result implies that recombination events between subtype D and other subtypes have occurred during the evolution of subtype D strains. To confirm this hypothesis, we performed bootscan and phylogenetic analyses. The bootscan analysis showed that several genomic segments from subtypes F and B split the backbone of subtype A into seven segments (Supplementary Fig. S2C), which was further confirmed by separate phylogenetic analyses (data not shown). Be-

Table 2. Suggested genomic reference sequences of enteroviruses

Enteroviruses	Subtypes		Reference strains
EVA71	A		U22521, GU434678, AB204853
	B		DQ341354, HQ189392, JF738001
	C	C (C1-C3,C5)	HQ647173, JN835312, DQ341359
		C4 (proposed subtype D)	HQ423142, FJ606450, JF738002
	D		KF906421*, KF906419*, KF906425*
	E		MG672478
	F		HG421068*, HG421069*
	G		KF906417*, KF906416*
CVA16	A		U05876, JQ746659, EU812514
	B1		JQ746677, JF738003, JQ746678
	B2		AB465370*, AM292455*, AM292461*
	D		MG957117, LT577761*, LT617115*
CVA6	A		AY421764, AF081297*
	B		JQ364886*, KP143075*, LC421656*
	C		JN203517*, JQ364887*
	D (D1-3)		MH716144, MF285650, LC126146
	E#		LR027552
	F#		KF412903*
CVA10	A		AY421767, AF081300*
	B		MF422531, GQ214177*, GQ214175*
	C		HQ728262, KP289406, KU578131
	D		MH118041, KC879535*, HE572987*
	E		MH118054, KC879491*, KC879488*
	F	F1	MH144599, MH118083, MH118056
		F2	MH118069, MH118036, MH144592
	G		MF422532
	H#		MF678312

*Complete VP1 sequence. Letters with well number (#) indicate the suggested subtypes or sub-subtypes by this study.

cause subtype D was genetically closely related to subtype A, the subtype A strain used in the bootscan analysis can reflect the early strain of subtype D. Therefore, the bootscan analysis indicated that several subtypes F, B and C-related genomic segments were inserted into the genomic backbone of subtype D by recombination during the evolution of subtype D.

Genomic reference sequences of four dominant enteroviruses

According to previous classification and genomic phylogenetic analyses, currently, EVA71, CVA16, CVA6 and CVA10 contain seven, three, four and seven subtypes, respectively (Table 2). New CVA6 subtypes E-F, and CVA10 subtypes F1 and H were suggested in this study. Because of good correlation in the phylogenetic analyses based on whole genome and VP1 sequences, and VP1 relatively easy acquisition, VP1 was recommended for use for typing of enteroviruses. However, genomic sequences are also encouraged when they are available. For molecular epidemiological investigation, GenBank Acc. Nos. of genomic reference sequences and the sequence alignments are provided in Table 2 and Supplementary file S1, respectively.

Discussion

Genetic variation affects virus transmission, pathogenicity and epidemics (Zaoutis and Klein, 1998; Muehlenbachs *et al.*, 2015; Wang *et al.*, 2018; Fu *et al.*, 2020). HFMD is caused by various enteroviruses that belong to RNA

family with high genetic diversity (Hyypia *et al.,* 1997; Muehlenbachs *et al.,* 2015; Lukashev and Vakulenko, 2017; Lukashev *et al.,* 2018). A large number of molecular epidemiological investigations have suggested that EVA71, CVA16, CVA6 and CVA10 were the predominant enteroviruses responsible for HFMD in China and other countries (Fu *et al.,* 2020). The classification of the four enteroviruses had been proposed previously according to the phylogeny of VP1 sequences. In this study, we performed systematical phylogenetic analyses of these enteroviruses (EVA71, CVA16, CVA6 and CVA10) using all available genomic sequences.

Genomic phylogenetic analyses showed almost consistent phylogenies of the four dominant enteroviruses to those of VP1 sequences. The subtypes identified based on VP1 sequences can be well confirmed by the phylogenetic analyses of genomic sequences except several recombinants (e.g. RH01_CC4, RH02_CC4) and potential new subtypes (CVA6 subtype E, F and CVA10 subtype H). A cut-off of 25% genetic divergence in the complete VP1 region was previously suggested to distinguish or define novel types of enteroviruses (Lukashev and Vakulenko 2017; Lukashev *et al.,* 2018). We found that the mean genetic distance of genomic sequences was 15.8–27% between subtypes and less than 12% within subtype/sub-subtypes. Inter-subtype genetic distance higher than 25% were commonly observed between subtype A and other subtypes in CVA16 and CVA10. High genetic distance between subtypes and the findings of new potential subtypes indicate that enteroviruses are more divergent than previously thought.

In most scenarios, full-length genomic sequences and near-complete VP1 sequences generate consistent phylogeny of enteroviruses. In some cases, however, inconsistent topologies of certain strains were observed between the phylogenetic trees of full-length genomic sequences and near-complete VP1 sequences, which might be indicative of the presence of inter-subtype recombination events, which frequently occur during the evolution of enteroviruses (Oberste *et al.,* 2004; Simmonds and Welch, 2006; Zhang *et al.,* 2015). Most observed inter-subtype recombination events in enteroviruses occurred in the distant past, and the recombinants had evolved into well-defined subtypes during a long evolutionary history. This was especially true for EVA71 sub-subtypes C1 (2018-23124/USA/2018) and C2 (15-335/Senegal/2015 and GIN-13-365/Guinea/2013), which were demonstrated to experience recombination during evolution (Fernandez-Garcia *et al.,* 2018) and CVA10 subtype D (16-2871-1/India/2017). However, more recent recombination events were rarely observed in enteroviruses, albeit they can generate some strains having distinct phylogenetic location and being difficult to be classified as certain subtype in the analyses of genomic and/or complete VP1 sequences. In this study, we identified two EVA71 recombinants (RF01_CC4 and RF02_CC4), which originated from recombination between subtypes C and C4.

Currently, the molecular epidemiology of four dominant enteroviruses is already changing in China and surrounding countries. The genomic and VP1 phylogenetic analyses revealed the on-going evolution. One major limitation of this study is that we did not investigate the adaptation evolution of these enteroviruses and the selective pressure influencing them. Furthermore, phylodynamics of these enteroviruses, including epidemiology and phylogeographic history, also deserves further investigation.

In summary, we performed genomic phylogenetic analyses of four major HFMD-related enteroviruses (EVA71, CVA16, CVA6 and CVA10). The phylogenies of these enteroviruses were consistent between the genomic and VP1 sequences. Comparison of phylogenetic topologies between genomic and VP1 sequence helped us to identify two new EVA71 inter-subtype recombinants (i.e. RF01_CC4 and RF02_CC4) and several potential subtypes (e.g. CVA6 subtypes E-F, and CVA10 subtype H). Furthermore, we provide GenBank Acc. Nos. of genomic reference sequences and the sequence alignments for molecular epidemiological investigation. These results provide important insights into the understanding of the evolution, epidemiology and transmission of the four enteroviruses.

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Supplementary information is available in the online version of paper.

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