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Research Article

Global spatiotemporal transmission patterns of human enterovirus 71 from 1963 to 2019

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Abstract

Enterovirus 71 (EV71) can cause large outbreaks of hand, foot, and mouth disease (HFMD) and severe neurological diseases, which is regarded as a major threat to public health, especially in Asia-Pacific regions. However, the global spatiotemporal spread of this virus has not been identified. In this study, we used large sequence datasets and a Bayesian phylogenetic approach to compare the molecular epidemiology and geographical spread patterns of different EV71 subgroups globally. The study found that subgroups of HFMD presented global spatiotemporal variation, subgroups B0, B1, and B2 have caused early infections in Europe and America, and then subgroups C1, C2, C3, and C4 replaced B0-B2 as the predominant genotypes, especially in Asia-Pacific countries. The dispersal patterns of genotype B and subgroup C4 showed the complicated routes in Asia and the source might in some Asian countries, while subgroups C1 and C2 displayed more strongly supported pathways globally, especially in Europe. This study found the predominant subgroup of EV71 and its global spatiotemporal transmission patterns, which may be beneficial to reveal the long-term global spatiotemporal transmission patterns of human EV71 and carry out the HFMD vaccine development.

Key words: enterovirus 71; hand; foot; mouth disease; phylogeographic analysis; spatial transmission

1. Introduction

Enterovirus 71 (EV71) is known to be one of the main causative agents of hand, foot, and mouth disease (HFMD) and some severe neurological complications, including brainstem encephalitis, acute flaccid paralysis, and poliomyelitis-like disease (Cardosa et al., 2003; Solomon et al., 2010). By 2018, almost 20.5 million HFMD cases and 3,667 deaths have been reported by China Center for Disease Control and Prevention (CDC) (Fu et al., 2019).

Since first isolated from neurological disease in California in 1969 (Schmidt, Lennette, and Ho 1974), EV71 has been identified sporadically around the world in the 1970s, with severe outbreaks in Bulgaria (Shindarov et al., 1979) in 1975 and Hungary (Nagy et al., 1982) in 1978 and sporadic reports in other European countries, Japan (Gobara et al., 1977; Ishimaru et al., 1980) (1973 and 1978) and Australia (Kennett et al., 1973) (1973). In the 1980s, small EV71 outbreaks associated with a severe neurological disease with low mortality were reported in Australia (Gilbert et al., 1988), Asia (Samuda et al., 1987), and the USA (Alexander et al., 1997). The late 1990s witnessed several large EV71 outbreaks in the Asia-Pacific region, especially in Malaysia (AbuBakar et al., 1999) in 1997 with at least 34 deaths and Taiwan (Yan et al., 2000) in 1998 and 2000 with at least 78 and 41 fatal cases, respectively. Since then, EV71 became endemic in the Asian-Pacific region and typically caused severe outbreaks every 3–4 years. In 2007 and 2008, two Chinese cities—Lin Yi (Zhang et al., 2009) and Fu Yang (Ding et al., 2009)—experienced large-scale HFMD outbreaks caused by EV71 with severe neurological complications and deaths, which spread quickly over other provinces in China (Zhang et al., 2009; Mao et al., 2010; Yu et al., 2010; De et al., 2011; Tan et al., 2011; Yang et al., 2011; Liu et al., 2011b), including Anhui, Guangdong, Zhejiang, Beijing, and Hong Kong, with serious public health events leading to the EV71 infections in China being continuously high until now.

As a member of the genus *Enterovirus* A belonging to the family *Picornaviridae*, EV71 is a small positive-sense, single-stranded, and non-enveloped RNA virus of ∼7,400 nucleotides (Solomon et al., 2010). The genome consists of some essential cis-acting elements for viral RNA replication and translation, including 5′ and 3′ untranslated regions (UTRs) and a polyadenylated tail of variable length in the flank, and a single open reading frame (ORF) encoding a large polyprotein between the two UTRs. The ORF is proteolytically cleaved by the viral protease into structural protein

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P1 (VP1–VP4), and non-structural proteins P2 (2A–2C) and P3 (3A–3D). Due to a high degree of diversity and lack of involvement in recombination, capsid protein VP1 is likely to be the most suitable region for sequence analysis to determine genetic diversity (Brown and Pallansch 1995; Lee et al., 2012). At present, the genetic evolution of EV71 based on this region can be classified into eight genotypes, A–H. Genotype A was first isolated in the USA in 1969, including the prototype BrCr strain (Brown et al., 1999) and re-emerged in China in 2008 (Yu et al., 2010). Genotypes B and C can be further divided into genotypes B0–B5 and C1–C5, which are globally circulating over the past decades. Genotype B0 was only isolated based on a retrospective analysis of the Netherlands in the 1960s (van der Sanden et al., 2010), and B1and B2 circulated in Europe (Brown et al., 1999; Yoke-Fun and AbuBakar 2006; van der Sanden et al., 2010), Australia (Brown et al., 1999), and the USA (Brown and Pallansch 1995; Brown et al., 1999) from 1971 to 1986. Since 1997, B3–B5 have been circulating in Southeast Asia, particularly in Malaysia (McMinn et al., 2001; Herrero et al., 2003; Chan et al., 2012) and Singapore (McMinn et al., 2001). Genotype B5 was also predominant with the outbreaks in Vietnam (Nguyen et al., 2016), Thailand (Linsuwanon et al., 2014; Puenpa et al., 2018), Brunei (AbuBakar et al., 2009), Taiwan (Huang et al., 2008, 2009; Luo et al., 2015), and Japan (Mizuta et al., 2014) after 2000. Then genotype C1–C5 was first isolated in Australia after 1986 (Brown et al., 1999). C1 emerged in 1986 and C2 emerged in 1995 and has been frequently reported in countries in Europe (Witso et al., 2007; Diedrich, Weinbrecht, and Schreier 2009; Vallet et al., 2009; van der Sanden et al., 2010; Hassel et al., 2015) and Australia (Sanders et al., 2006). Genotype C3 was mainly circulating in Japan (Cardosa et al., 2003; Iwai et al., 2009) and Korea (Jee et al., 2003). C4 was first isolated in Japan in 1997 (Iwai et al., 2009), and then was classified into subgroups C4a and C4b, circulating predominantly in Asia-Pacific countries, especially in China since 2000 (Zhang et al., 2009; Thoa le et al., 2013; Lin et al., 2015; Chen et al., 2016; Duong et al., 2016; Puenpa et al., 2019). Genotype C5 was mainly circulating in Vietnam (Van Tu et al., 2007; Thoa le et al., 2013; Duy et al., 2017), Thailand (Chatproedprai et al., 2010), and Taiwan (Huang et al., 2008). Genotypes D and G were only found in India (Rao, Yergolkar, and Shankarappa 2012) and Bangladesh (Oberste et al., 2013). Although genotypes E and F were recently discovered only in Africa like Nigeria, Niger, Central African Republic, Madagascar, and Cameroon, the epidemiology of EV71 has been largely unexplored here (Bessaud et al., 2014; Fernandez-Garcia et al., 2016). New genotype H has only been described in Pakistan (Majumdar et al., 2018).

The past few decades have seen the prevalence and several large outbreaks of HFMD caused by EV71. Interestingly, phylogenetic analyses and molecular clock dating suggested that these large EV71 outbreaks were associated with (sub)genotype switches, sometimes accompanied by recombination events (Huang et al., 2015). Many previous research studies have explored the epidemiological and overall spatiotemporal distribution pattern of some genotypes, some even focused on their virulence, antigenicity, and genetic evolution (Solomon et al., 2010; Huang, Cheng, and Wang 2019). However, the evolutionary dynamics of this pathogen, particularly its global spatiotemporal spread, have not yet been identified. In particular, how virus transmission occurs over time, across space, and among genotypes has not been extensively studied in geographical areas.

In this study, we used a Bayesian phylogenetic approach to investigate the spatiotemporal evolutionary heterogeneous dynamics and migration of EV71 globally. To this aim, we collected almost all sequence datasets of virus isolates sampled worldwide from Genbank and Virus Pathogen Resource (ViPR) and used some representative sequence data of each EV71 (sub)genotype circulating in Europe, America, and Asia-Pacific regions to complete phylogenetic analyses. To our knowledge, this is the first global research based on such a large scale and scope.

2. Material and methods

2.1 Data collection and preprocessing of nucleotide sequence datasets

At first, we collected almost all available EV71 sequences from GenBank of the National Center for Biotechnology Information (NCBI) and ViPR by 31 October 2019, 14,343 from NCBI and 13,491 from ViPR were obtained, their accession numbers were also provided. Meanwhile, the basic sequence information like year, location, subtype, nucleotide length, and whether the sequence belongs to VP1 was also collected. Considering some missing information from these two databases, we obtained the source literature of these sequences by their PUBMED or PMID number. By extracting their evolution tree, table, text content, or supplementary document from more than 250 articles (Supplementary File 1), most sequences with missing information were retrieved. After discarding erroneously annotated sequences that share less than 60 per cent identity with reference sequences and short sequences that overlap less than 270 nucleotides (nt) with the partial VP1 fragment, a total of 11,752 strains between 1963 and 2019 were obtained here. All the sequences were corresponding to virtually EV71 VP1 genes reported in previous studies (Brown et al., 1999; McMinn et al., 2001; Cardosa et al., 2003; Bessaud et al., 2014; Hassel et al., 2015; Puenpa et al., 2019). To minimize the calculation during the evolution tree construction, only one representative subtype sequence was kept if there were two or more sequences in the same country in the same year. Eventually, 635 EV71 VP1 sequences (Supplementary File 2) were used in this article. In order to ameliorate potential sampling biases, we subsequently randomly subsampled these datasets by each year and each country to create a more equitable spatiotemporal distribution of EV71 sequences (Supplementary File 3). The VP1 sequence of the CA16 prototype strain, G-10, was included in the phylogenetic analysis as an outgroup.

2.2 Phylogenetic analysis of EV71

In this study, multiple sequence alignment of the entire VP1 nucleotide sequences of the EV71 was performed using ClustalX2.1 and later manually edited with MEGA7 (Kumar et al., 2018). The optimal nucleotide substitution model was selected by the Akaike Information Criterion and a hierarchical likelihood ratio test using model generator (Goss et al., 2014). Eventually, HKY + I + G model was the best-fit substitution model for subgroup B and GTR + G model for subgroup C. Phylogenetic relationships of the representative sequences of each (sub)genotype were constructed by Bayesian phylogenetic analysis using the Markov Chain Monte Carlo (MCMC) framework implemented in BEAST 1.10.4 (Suchard et al., 2018). Bayesian phylogenetic methods use virus isolation times as an additional parameter in inferring a phylogenetic tree that better reflects the actual virus evolution (Nascimento, Reis, and Yang 2017). The MCMC was run for $2*10^7$ to 10^8 generations, and trees were sampled every 10,000 generations. In addition, an effective sample size of parameters was checked to ensure that they were above 200 at the end of running using Tracer 1.7.1 (available at http://tree.bio.ed.ac.uk/software/tracer/) for convergence and mixing, with the first 10 per cent of sampled

trees discarded as burn-in. Maximum clade credibility (MCC) trees were annotated using TreeAnnotator v.1.10.8 (available at http://evolve.zoo.ox.ac.uk/Evolve/Software.html). Branch substitution rates were also extracted from the MCC tree file. At last, MCC trees were visualized with FigTree v.1.4.4 (available at http://tree.bio.ed.ac.uk/software/tracer/).

2.3 Phylogeographic reconstruction of EV71 subgroups

To gain insight into the circulation of EV71 subgroups through time and different regions, we reconstructed spatial transmission patterns using a phylogeographic analysis by an asymmetric continuous-time Markov chain model, coupled with model averaging using Bayesian stochastic search variable selection (BSSVS) (Lemey et al., 2009) in BEAST. We used an uncorrelated lognormal relaxed molecular clock model (Drummond et al., 2006), the HKY + I + G nucleotide substitution model (Shapiro, Rambaut, and Drummond 2006) for genotype B and GTR + F + G nucleotide substitution model for genotype C (Shapiro, Rambaut, and Drummond 2006), and the Coalescent-based Bayesian Skyline plots (Minin, Bloomquist, and Suchard 2008). The locations where the virus strains were collected were used as discrete character states to estimate changes in geographical locations here. At first, we counted the expected number of transitions among each pair of locations using the robust counting approach and plotted the total number of state counts for migration into and out of each location. We then used those inferred transitions to identify the early estimated EV71 introductions into new regions. The Bayes factor (BF) test was used to determine which diffusion links were statistically significant based on the standard BSSVS protocol (Suchard, Weiss, and Sinsheimer 2001). Here, we considered that the pairwise diffusion pathways were significantly supported when $BF \geq 3$ and the node posterior probability ≥ 0.5. The description of the globally geographic spread of main EV71 subgroups was obtained by MCMC sampling from the plausible set of trees using the procedures outlined above.

We also performed the phylogenetic temporal structure by obtaining root-to-tip regressions in TempEst v1.5 (Rambaut et al., 2016), which showed a strong temporal signal (Fig. 5). To estimate the population dynamics of different EV71 genotypes, we constructed the past population dynamics of genotype B, subgroups C1, C2, and C4 using the coalescent-based Bayesian Skyline plots, which revealed the changes in genetic diversity of EV71 viruses.

2.4 Visualizing phylogeographic diffusion

To summarize the posterior distribution of ancestral location states, the nodes in the MCC trees were annotated with their modal location states using TreeAnnotator, simultaneously, the MCC trees were visualized by TreeAnnotator and FigTree. The phylogeographic analysis results were summarized with SPREAD3 (Bielejec et al., 2011). To provide a spatial projection, we converted the diffusion process into a keyhole markup language (KML) file suitable for viewing with Google Earth (http://earth.google.com). The EV71 subgroups B4, B5, C1, C2, and C4 diffusion process KML files are included as supplementary files. In addition, we also used ggplot2 package in R software for embellishing the global evolutionary history.

3. Results

3.1 Geographical distributions of EV71 subgroups

As of 31 October 2019, 14,343 from NCBI and 13,491 from ViPR EV71 sequences were obtained in this study, after discarding erroneously annotated sequences, short sequences with less than 270 nucleotides, and sequences with missing information, we screened out 11,752 strains. The number of different EV71 genotypes from 1963 to 2019 is shown in Supplementary File 4. In total, 2,338 strains belong to genotype B, 9,321 strains belong to genotype C, only 25 strains belong to genotype A, 29 strains belong to D, 10 strains belong to E, 6 strains belong to F, 19 strains belong to G, and 4 strains belong to H. At the early stage (1963–1988), subgroup B0 was first isolated in 1963, 1964–1967, followed by predominant subgroups B1 and B2. The appearance of subgroup C1 in the mid-1980s replaced the predominance from 1989 to 1996. Since 1997, the number of EV71 isolates grew exponentially, along with a number of novel subgroups, has been sequentially isolated, including subgroups B3, B4, and B5 and subgroups C2, C3, C4, and C5. In 1997, the predominant subgroup was B3, followed by C2 in 1998 and 1999, B4 in 2000–2002, C4 in 2003–2005, B5 in 2006 and C2, C4 in 2007. In the recent stage (2008–2019), the number of EV71 isolates was propelled a step further, and the predominant subgroups were B5, C1, C2, and C4.

Figure 1 shows the geographical distributions of different EV71 genotypes in three periods (1963–1996, 1997–2007, and 2007–2019), more detailed geographical distributions of EV71 B and C subgroups in three periods are displayed in Supplementary Figs S5 and S6, respectively. Genotype A was first emerged in 1970 in California and America and re-emerged in China (2008–2012), Thailand (2014), and Kenya (2009). Genotypes D, G, and H were only found in some South Asian countries (India, Bangladesh, and Pakistan), while genotypes E and F were only circulating in some Africa countries (Table 1). B0 was only isolated in the 1960s in the Netherlands, B1 and B2 were the predominant subgroups in Europe and North America in the 1970s and 1980s, while some sporadic cases in East Asia, Austria, and Africa were also caused by B1 or B2. In contrast, after 1987, genotype B was replaced by genotype C, especially C1 and C2. Since 1997, B3-B5 were mainly isolated in Asia-Pacific region and circulated for more than 10 years. In recent years, B3 and B4 have already disappeared, while B5 was one of the predominant subgroups in some Asia-Pacific countries (Table 2, Supplementary Fig. S5). To date, C1 and C2 were still cocirculating in Europe and North America since 1997. C4, particularly the C4a lineage, was the main subgroup in East Asia, especially in China since 2004. C3 and C5 were only detected in some East and Southeast Asian countries (Table 2, Supplementary Fig. S6).

3.2 Phylogenetic analysis of EV71 subgroups

Phylogenetic trees are shown in Figs 2 and 3. The dendrogram revealed seven distinct genotypes, including A, B, C, D, E, F, and G. The strains belonging to genotype H had less than 270 nucleotides, so the result did not include the genotype H. Detailed phylogenetic relationships of genotypes B and C are displayed in Fig. 3A and B. We inferred that genotype B emerged earlier than A although prototype strain BrCr, which belonged to genotype A, was the first isolated strain. The genotypes F and G appeared around 2000. Figure 3 shows that there existed six distinct subgroups B0–B5 of genotype B and there were five distinct subgroups C1–C5 of genotype C. Phylogeographic analysis revealed that all these subgroups except B0, C3, and C5 displayed a global distribution, whereas B0, C3, and C5 lineages were restricted to the Netherlands, Korea, and Vietnam, respectively. Cocirculation of multiple subgroups was observed in many countries especially in Asia-Pacific region.

Since mid-1960, the genotype of B appeared in succession. Early subgroups (B1 and B2) mainly circulated in North America,

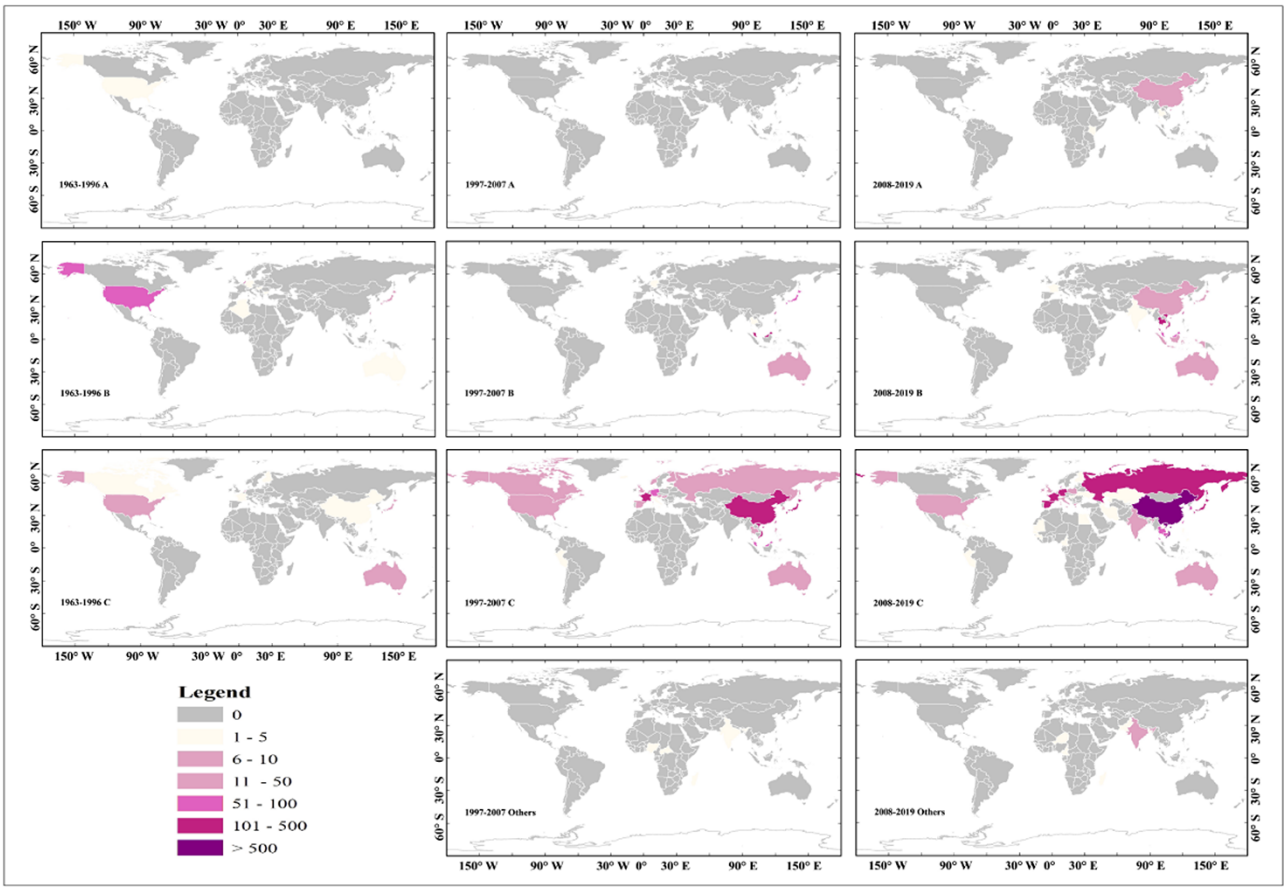

Figure 1. Geographical distribution of EV71 genogroups. Different colors represented the number of isolates.

Table 1. A summary of EV71 subgenogroups circulating in different regions, 1960–2019.

	Years					
Regions	1960–1969	1970–1979	1980–1989	1990–1996	1997–2007	2008–2019
EU	**B0**	**B1**, B2	**B2**, C1	**C1**	B2, **C1, C2**, C4	B5, **C1, C2**, C4, C5
NAm	–	A, **B1, B2**	B1, **B2, C1**, C2	B1, **C1**	C1, **C2**, C4	**C1, C2**
SAm	–	–	–	–	**C1**	C1
AUS	–	B1	C1	C1, C2	**B3, B4**, B5, C1, **C2**	B3, **B5, C2**, C4
ME	–	–	–	–	C1, C2, C4	C1, C2, C4
EA	–	B1	**B1**, B2, B4, **C1**	B1, B2, **C1, C2**, C3	**B1, B2**, B3, **B4, B5**, C1, C2-C5	**A**, B1-B4, **B5**, C1, **C2**, C3, C4, C5
SEA	–	–	B2, C2	–	**B3, B4, B5**, C1, C2, C4, C5	A, **B5**, C1, C2, **C4, C5**
SA	–	–	–	–	D	B5, **C1, D, G,** H
AF	–	–	B2	–	E, F	A, C1, C2, C4, E, F

Note: Bold indicates predominant genotype; AF, Africa; AUS, Australia; EA, East Asia; EU, Europe; NAm, North America; SEA, Southeast Asia; SAm, South America; SA, South Asia.

Europe, and Asia in the 1970s and 1980s. Subgroups B3, B4, and B5 then replaced them and became predominant and circulated endemically in the Asia-Pacific region. In contrast, subgroup C1 has continuously been identified in various countries since its initial detection in Australia and the USA in the mid-1980s. Later in the 1990s, a number of novel subgroups (C2, C4, and C5) emerged in the Asia-Pacific region, including several fatal HFMD cases in Taiwan (1998) (Ho et al., 1999), China (2008) (Yang et al., 2009; Tee et al., 2010), and Vietnam (2005) (Van Tu et al., 2007), respectively. Subgroups C3 circulated endemically in Korea and China Taiwan. Of note, subgroup C4 was mainly detected in China and some other Southeast Asian countries after the outbreak of HFMD in 2008 in Fuyang, China.

Our Bayesian analysis showed that the Netherlands might be the root of genotype B, with a posterior probability of 0.47, followed by America (0.34). We have dated the genotype B to 1955.6 (95 per cent HPD interval (1948.6, 1963.7)) and genotype C to 1980.2 (95 per cent HPD interval (1976.5 and 1983.3)). Figure 4 shows the root state posterior probability of mainly subgroups C1, C2, C4. America, the Netherlands, and China were the origin of subgroups C1, C2, and C4, with the posterior probability of 0.35, 0.25, and 0.96, respectively. The mean substitution rate of genotype B was $3.29*10^{-4}$ substitutions/site/year (95 per cent credibility interval $2.48*10^{-4}$–$4.17*10^{-4}$), and the mean substitution rates of subgroups C1, C2, and C4 were $3.38*10^{-4}$ substitutions/site/year (95 per cent credibility interval $2.28*10^{-4}$–$3.98*10^{-4}$), $2.65*10^{-4}$

Table 2. A summary of EV71 subgenogroups circulating in Asia-Pacific countries, 1960–2019.

	Years					
Countries	1960–1969	1970–1979	1980–1989	1990–1996	1997–2007	2008–2019
Singapore	–	–	B2		**B3, B4**, C1, C5	C1, C2, C4
Malaysia	–	–	–		**B3, B4, B5**, C1, C2	**B5**
Australia	–	–	**C1**	**C1, C2**	**B3, B4**, B5, C1, C2	B3, **B5, C2**, C4
Japan	–	B1	**B1, C1**	B1, B2, C1, C2, C3	B1, B3, **B4, B5**, C1, C2, C4	**B5**, C1, **C2**, C4
Korea	–	–	–		**C3, C4**	**C4**, C5
China Taiwan	–	–	B1, B2, B4		B1, B2, **B4, B5**, C1, C2, C3, C4, C5	B4, **B5**, C2, **C4**, C5
China Mainland	–	–	–	C2	C2, C3, **C4**	A, B1-B5, C1, C2, C3, **C4**
Vietnam					C1, C4, **C5**	**B5, C4, C5**
Thailand					B4, B5, C1, C2, C4, **C5**	A, **B5**, C1, C2, **C4**, C5
Brunei					B4, **B5**	
Cambodia						B5, **C4**
Indonesia						B5
Philippines					C2	C2
Laos						C4

Note: Bold indicates predominant genotype.

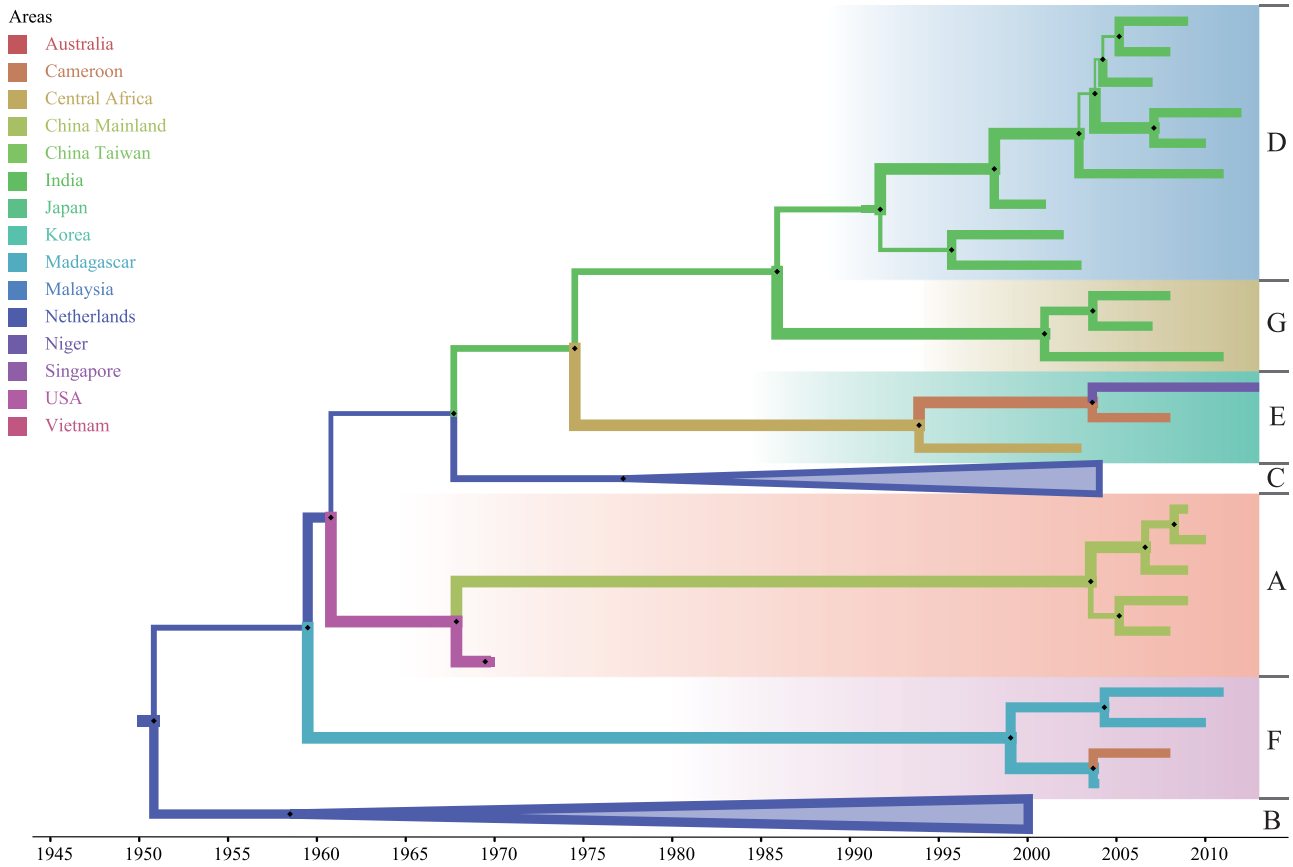

Figure 2. Phylogenetic analysis of EV71 VP1 gene sequences. The tree topology has been chosen to maximize the product of node posterior probabilities. Note: The branch colors meant the location of these strains. Branch lengths are scaled in units of time, as indicated by the time axis, with the branches colored by different countries. The thickness of the line represents the posterior probability. Thicker line represented higher posterior probability.

substitutions/site/year (95 per cent credibility interval $2.01*10^{-4}$ –$3.33*10^{-4}$), and $3.56*10^{-4}$ substitutions/site/year (95 per cent credibility interval $2.98*10^{-4}$–$4.15*10^{-4}$). Additionally, a strong temporal signal tested in TempEst of genotypes B and C is shown in Fig. 5.

In Fig. 6, the Bayesian Skyline plots depict the changes in genetic diversity of different EV71 (sub)genotypes via time, which reflect the predominant (sub)genotype in different periods. The population of genotype B encountered a slow upward trend until it suffered a sharp decline in the late 1990s and soon returned

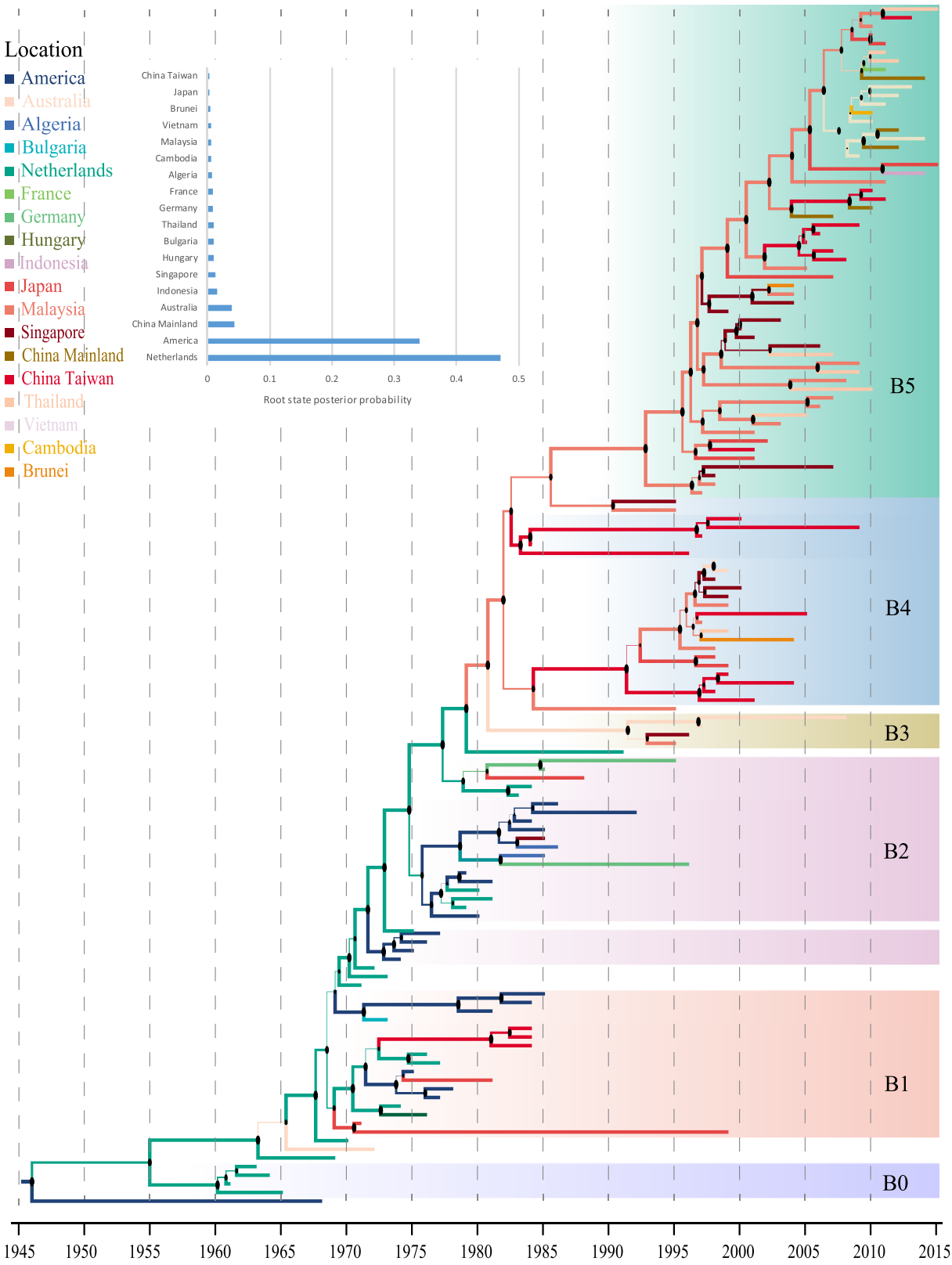

Figure 3A. (A) Phylogenetic analysis of EV71 genotype B VP1 gene sequences. The tree topology has been chosen to maximize the product of node posterior probabilities. The upper left was the root location state posterior probability distributions. Note: The branch colors meant the location of these strains. Branch lengths are scaled in units of time, as indicated by the time axis with the branches colored by different countries. The thickness of the line represents the posterior probability. Thicker line represented higher posterior probability. (B) Phylogenetic analysis of EV71 genotype C VP1 gene sequences. The tree topology has been chosen to maximize the product of node posterior probabilities.

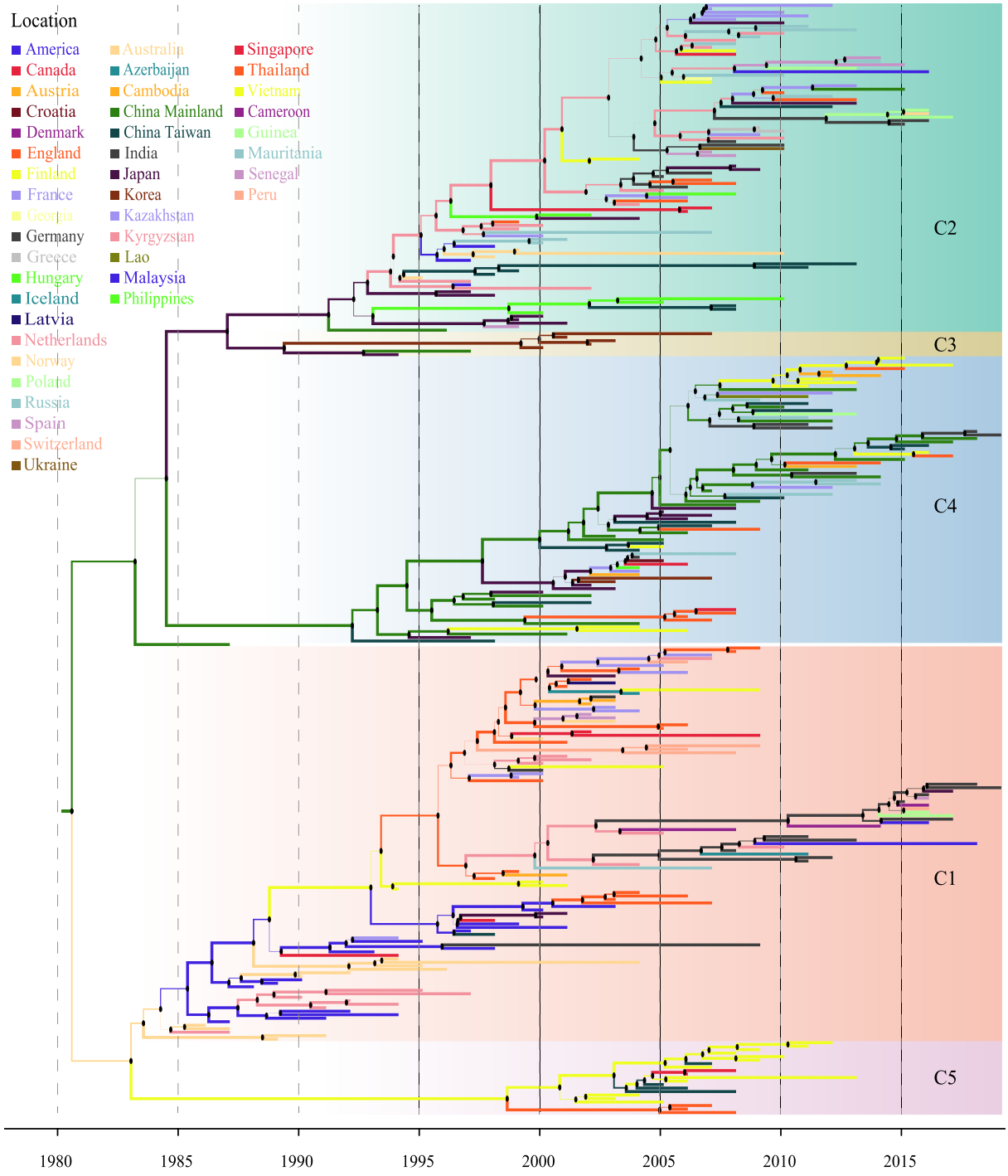

Figure 3B. (A) Phylogenetic analysis of EV71 genotype B VP1 gene sequences. The tree topology has been chosen to maximize the product of node posterior probabilities. The upper left was the root location state posterior probability distributions. Note: The branch colors meant the location of these strains. Branch lengths are scaled in units of time, as indicated by the time axis with the branches colored by different countries. The thickness of the line represents the posterior probability. Thicker line represented higher posterior probability. (B) Phylogenetic analysis of EV71 genotype C VP1 gene sequences. The tree topology has been chosen to maximize the product of node posterior probabilities.

to the previous peak, after that, it gradually decreased and leveled off in 2010. The rise time of C1 was about 1998, which corresponded to the time of the decline of genotype B. It gradually decreased in about 2007 and leveled off in 2015. In about 2002, C2 went through an obvious expansion prior to a period of stability in 2005. The trend of C4 was similar to C1, with a peak in 2007–2013.

3.3 Global Spatial dynamics of EV71 subgroups

To understand the global circulation of EV71, we reconstructed the past spatial transmission patterns for two main genotypes (B and C), inferred from estimates of genetic diversity for different countries. We found that subgroups B0, B1, and B2 have caused early infections in Europe and America and then subgroups C1, C2, and C4 replaced B0–B2 as the predominant genotypes.

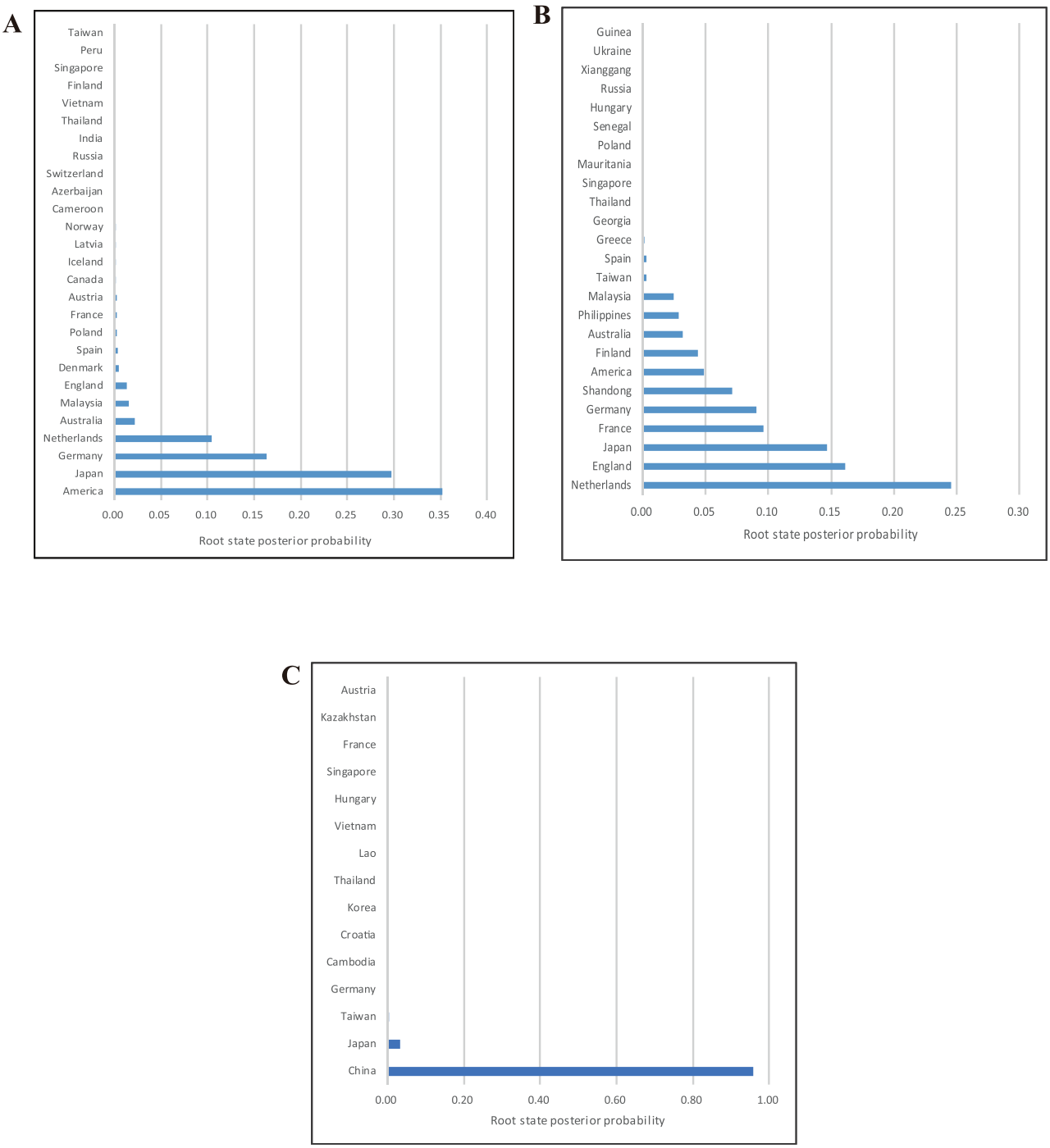

Figure 4. Root state posterior probability of EV71 genotype C.
A: subgroup C1, B: subgroup C2, C: subgroup C4. (Note: China means China Mainland).

3.3.1 *Spread of EV71 B genotype between distinct countries*

Figure 7 shows the significant epidemiological unidirectional pathways of EV71 B genotype globally. In total, 17 migration pathways were supported by our Bayesian phylogeographic analysis, two originated from the Netherlands to Hungary and Bulgaria within Europe, 10 within Asia and five diffused globally. The migrations from Thailand to France and Singapore to Australia, and migration from the USA to Algeria were decisively supported with BF $\geq$ 100. In Asia, the diffusion pathways were more complicated than Europe. Four decisive routes originated from Vietnam to China Mainland and Cambodia, from Malaysia to Japan, and from China Taiwan to China Mainland. Four very strongly supported diffusion rates originated from Malaysia to other Southeast Asian countries and one from Japan to Indonesia.

3.3.2 *Spread of EV71 C genotype between distinct countries*

In Figs 8–10 and Supplementary File 7–8, the significant global migration routes of EV71 C subgroups are displayed here. Globally, six significant migration routes (decisive support with BF $\geq$ 100) and 14 very strongly (100>BF $\geq$ 10) supported diffusion routes

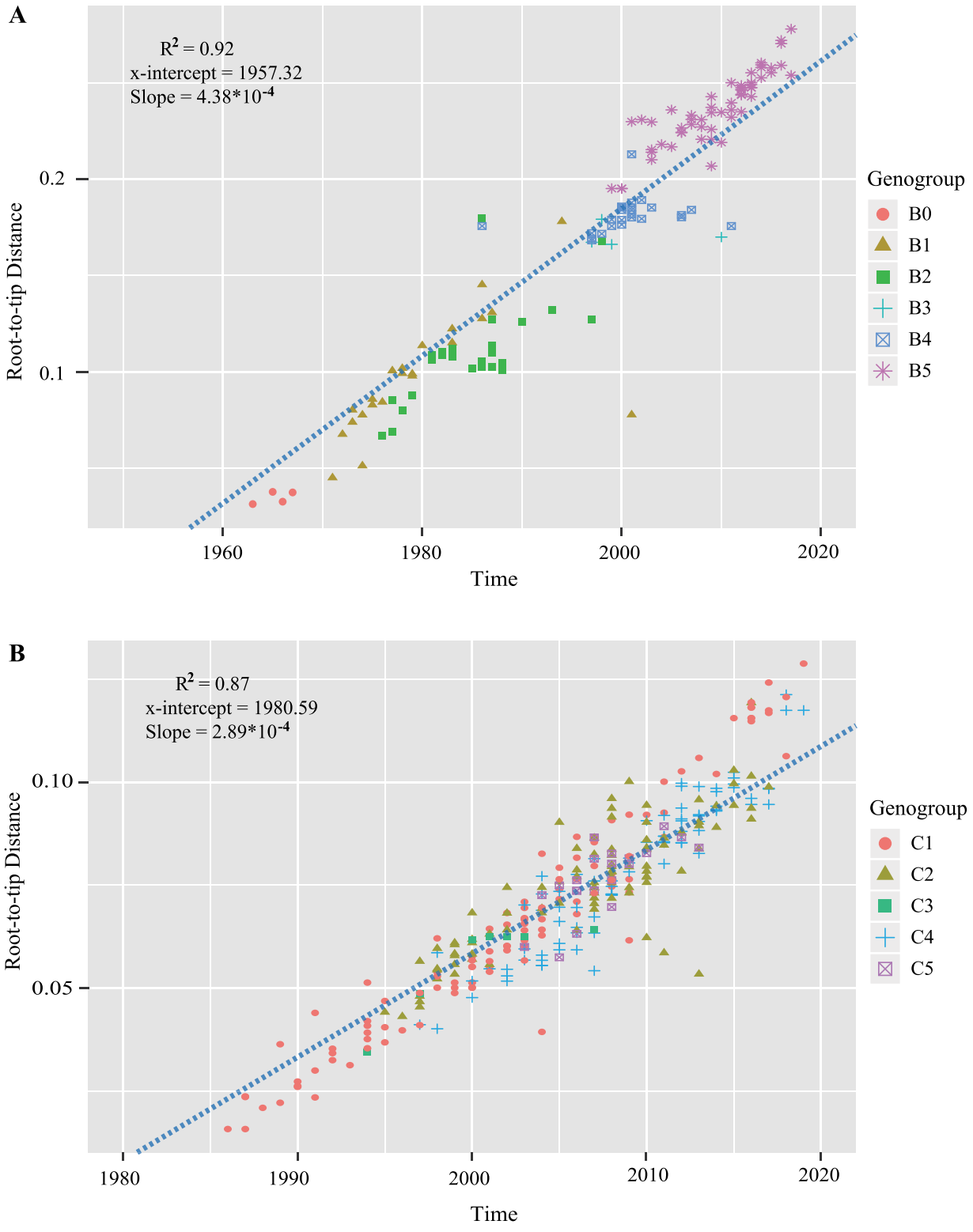

Figure 5. Strong temporal signal tested in TempEst of EV71 genotype B (A) and genotype C (B).

We used the subsampled dataset of VP1 gene sequences of each genotype of EV71. The y-axis corresponds to the root-to-tip distance of phylogenetic trees with branch lengths in units of substitutions per site. The x-axis represents calendar time. Each point corresponds to a tip in the tree. The top left values represent the R-squared, the slope (substitution rate) and time of origin, respectively.

existed in C1 (Fig. 8). Most of the EV71 C1 migration routes existed in Europe, only one decisive support pathway from Malaysia to India (posterior probability of 0.91), and a very strong support pathway from Malaysia to China Taiwan existed in Asia. In addition, the spread in Asia was introduced from Germany to Malaysia, Finland to Thailand (decisive support). Although numerous spread events occurred in European countries, the migrations from Germany to France and Austria were much

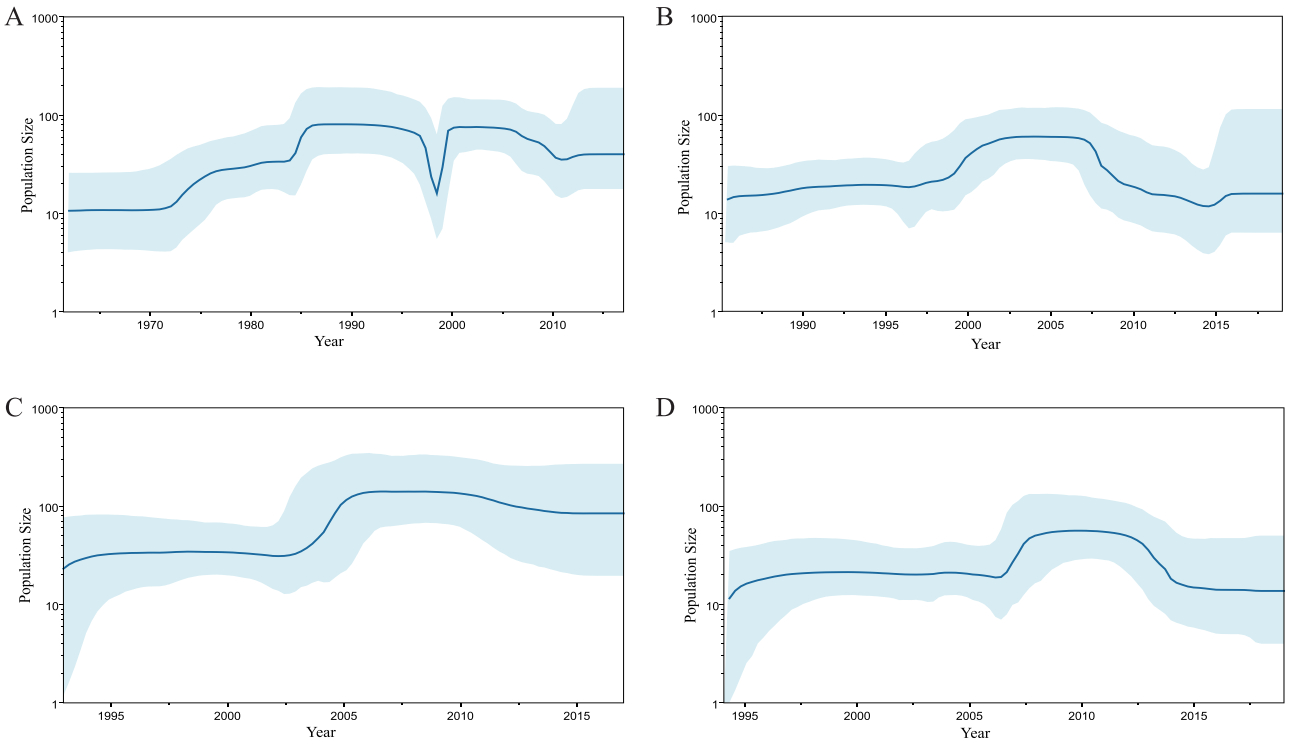

Figure 6. Bayesian skyline plots of genotype EV71 B and C, showing population size (y-axis) through time (x-axis).
A: subgroup B, B: subgroup C1, C: subgroup C2, D: subgroup C4. Solid lines represent the median estimates of population size and the shaded areas indicate the corresponding 95 per cent credibility intervals.

greater than other pathways. The transmissions from England to Azerbaijan (posterior probability of 0.94) were the decisive routes for C1.

In Fig. 9, three significant migration routes (decisive support with BF $\geq$ 100) existing for C2 are shown. Subgroup C2 was diffusing globally, especially between North America, Europe, Asia, West Africa, and Australia, with the decisive transmission routes from West Africa (Senegal) to Hong Kong and from Canada to Greece. As described in the previous section, subgroup C4 was detected mainly in China and some other Asian countries. The global migration routes displayed the prominent role of some Asian countries, considering that almost all decisive pathways were from Asia to other countries. In Fig. 10, one decisive support pathway from China to Germany in the global vision and five decisive support pathways in Asia are shown. Of these, China was likely the country of origin in four significant pathways. No strong supported pathway was found in Europe.

Supplementary Files 7 and 8 show the epidemiological migration routes of EV71 C3 and C5 subgroups in some regions, where C3 originated from China to Korea and Japan and C5 originated from Vietnam to Finland and some Southeast Asian countries.

4. Discussion

EV71 can cause large outbreaks of HFMD and severe neurological diseases, which is regarded as a major threat to public health, especially in Asia-Pacific regions. Although a number of recent studies have focused on the molecular epidemiology, pathogenesis, and vaccine development of EV71 (Solomon et al., 2010; Lukashev et al., 2014; Chang, Chen, and Chen 2018; Huang, Cheng, and Wang 2019; Puenpa et al., 2019), to our knowledge, the evolutionary origins and spatiotemporal spread of EV71 in geographical areas have not yet been analyzed explicitly with recently developed phylogeographical models (Lemey et al., 2009). In this study, we first displayed the global geographical distributions of EV71 subgroups over the past six decades and then applied phylogeographical analyses to display the dispersal patterns of EV71 subgroups globally. This study concluded the predominant subgroup and its global spatiotemporal transmission patterns.

Given the widespread geographical distribution of EV71 subgroups over the past 60 years, we systematically elaborated the epidemiologic features of EV71 infections all over the world. The first genotype A was identified in 1970, and it disappeared until its reintroduction in China in 2008 (Yang et al., 2013; Vakulenko, Deviatkin, and Lukashev 2019). A new subgroup B0 found by a retrospective report in the Netherlands (van der Sanden et al., 2009, 2010), together with B1and B2, have caused early infections in Europe and America successively. The 1990s witnessed a shift in predominance from B0, B1, and B2 to subgroups C1, C2, C3, and C4, later subdivided into C4a and C4b, generating major EV71-associated HFMD outbreaks. Simultaneously, the main pandemic regions have also moved from Europe and the USA to the Asia-Pacific region, which have been associated with fatal HFMD cases in Taiwan (Lin et al., 2006; Kung et al., 2007) (1998) and China (Yang et al., 2009; Zhang et al., 2010) (2008). Subgroup B5 has been reported to be antigenically distinct from B1, B4, C2, and C4 and could therefore pose a potential risk for epidemic spread outside the Asia region (Chang, Chen, and Chen 2018). Subgroups C3 and C5 were only circulating in a small scale in Asia. Genotype D was initially identified in India, genotypes E and F were initially identified in Africa, and genotypes G and H were only identified in Bangladesh and Pakistan. Currently, the global geographical distribution pattern of EV71 subgroups was that C1 and C2 circulated endemically in Europe and North America, and subgroups

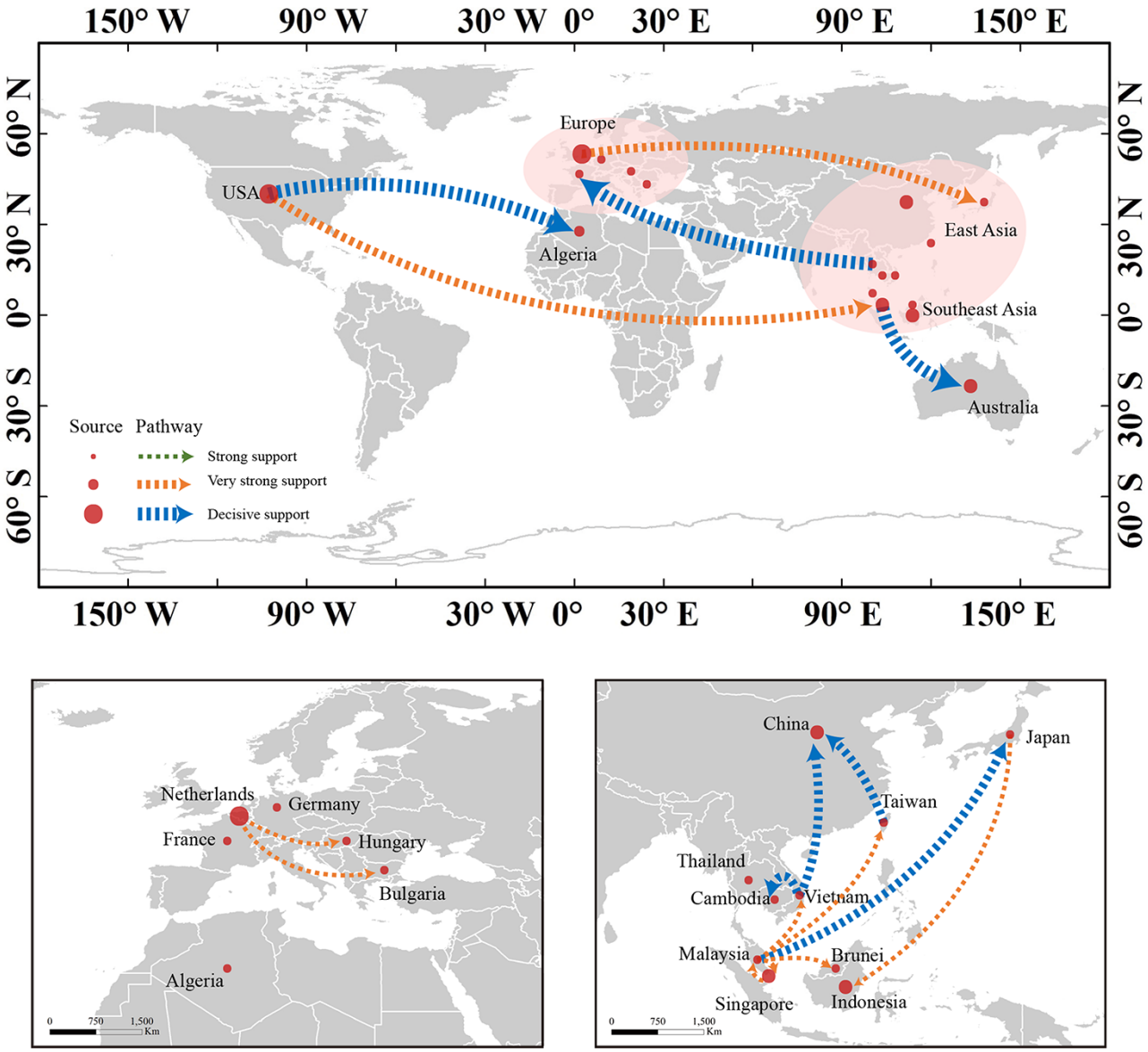

Figure 7. Spatial diffusion pathways of EV71 genotype B.

B5 and C4 were the predominant genotypes in most Asia-Pacific countries. A molecular epidemiological study suggested that the evolution of the EV71 virus has global characteristics. Global herd immunity against C1 and C2 viruses could possibly explain why epidemics caused by subgroups B4 and C4 are restricted to the Asia-Pacific region.

We found that the evolution rates of subgroups C1 and C4 were higher than that of C2. Our phylogeographic analysis revealed that the diffusion patterns of C1 mainly occurred in Europe and C4 in Asia, while the diffusion patterns of C2 mainly occurred globally. We can infer that the diffusion process on a small regional scale may accelerate the evolution of the virus. Our Bayesian analysis also showed that the Netherlands might be the root of genotype B, and the America, the Netherlands, and China were the origin of subgroups C1, C2, and C4, respectively. Our estimated dates of the tMRCA of genotype B and subgroups C1, C2, and C4 were consistent with previous studies (Hassel et al., 2015).

Moreover, the transmission patterns of genotypes B and C reflected different characteristics globally. The diffusion of genotype B mainly occurred before the 2000s, while genotype C mainly after 2000. We used the BF of each subgroup to compare their transmission rates. The transmission rates of genotype C were higher than genotype B. The BF of subgroup C1 was highest, followed by C4 and C2. The transmission routes of genotype B in Asia-Pacific region were more complicated than Europe countries. This was in accordance with the previous outbreaks since 1997 in Malaysia (Herrero et al., 2003), China Taiwan (Chu et al., 2001), and Singapore (McMinn et al., 2001; Puenpa et al., 2019), when subgroups B3, B4, and B5 have replaced B1 and B2 and become predominant and circulated endemically in the Asia-Pacific region. The source of China Taiwan was originated from Japan, Malaysia, and Singapore (Fig. 3A).

On the contrary, the transmission history of subgroup C1 displayed more strongly support pathways globally, especially in Europe. Some European countries, for example, Germany

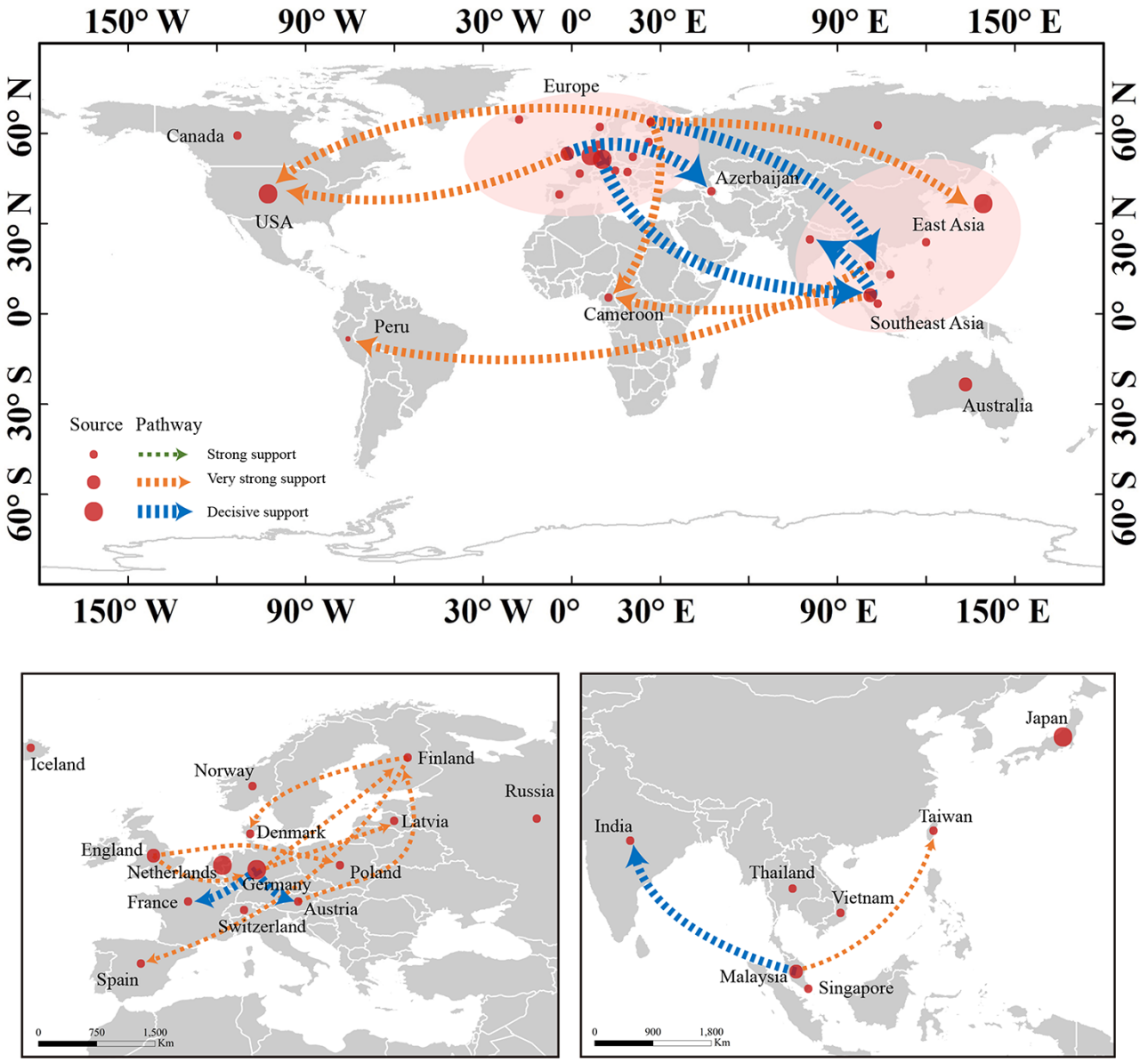

Figure 8. Spatial diffusion pathways of EV71 subgroup C1.

and Finland, were the source of multiple migration routes to Asia, North America, and Africa. The phylogenetic patterns also showed multiple virus introduction events occurring in Europe (Hassel et al., 2015). It was particularly noteworthy that the transmission of EV71 strains C1 and C2 in Europe was mainly dependent on the frequency of virus spread events between neighboring countries, which has been previously described (Mizuta et al., 2005). Three obvious waves of infections in 2007, 2010, and 2013 were found in many European countries, especially the Netherlands (van der Sanden et al., 2010), France, and Germany (Mirand et al., 2010), which was the common dissemination mode in Europe for both subgroups C1 and C2. Although the epidemiological and biological factors involved were still unknown, the occurrence of these infections indicated that the immunity elicited by the C1 and C2 infections is cross-protective, as suggested by earlier studies (Liu et al., 2011a).

The consistent transmission chain caused by a C4 virus strain also suggested that the virus persisted between 2003 and 2005, which was consistent with the HFMD outbreaks in China (Yang et al., 2009; Zhang et al., 2009; Wang et al., 2015; Fu et al., 2019). Only sporadic introductions from some Asian Countries occurred in Europe. More decisive dispersal routes were endemic in Asia, especially in China. Therefore, it is not surprising that China was inferred as the potential origin of four decisive spread events, respectively, to Lao, China Taiwan, Korea, and Singapore, and numerous strongly support routes to neighboring countries. For example, the phylogeny pattern indicated that between 2001 and 2002 a C4 virus strain spread from China to Japan and from there to Europe in early 2003 (Fig. 10).

This study established the early origin, spatiotemporal dispersal, and epidemiological dynamics of EV71 throughout the world, providing new insights into spatial transmission patterns of main EV71 subgroups. Here, we implemented ancestral reconstruction of discrete states in a Bayesian statistical framework for evolutionary hypothesis testing that is geared towards

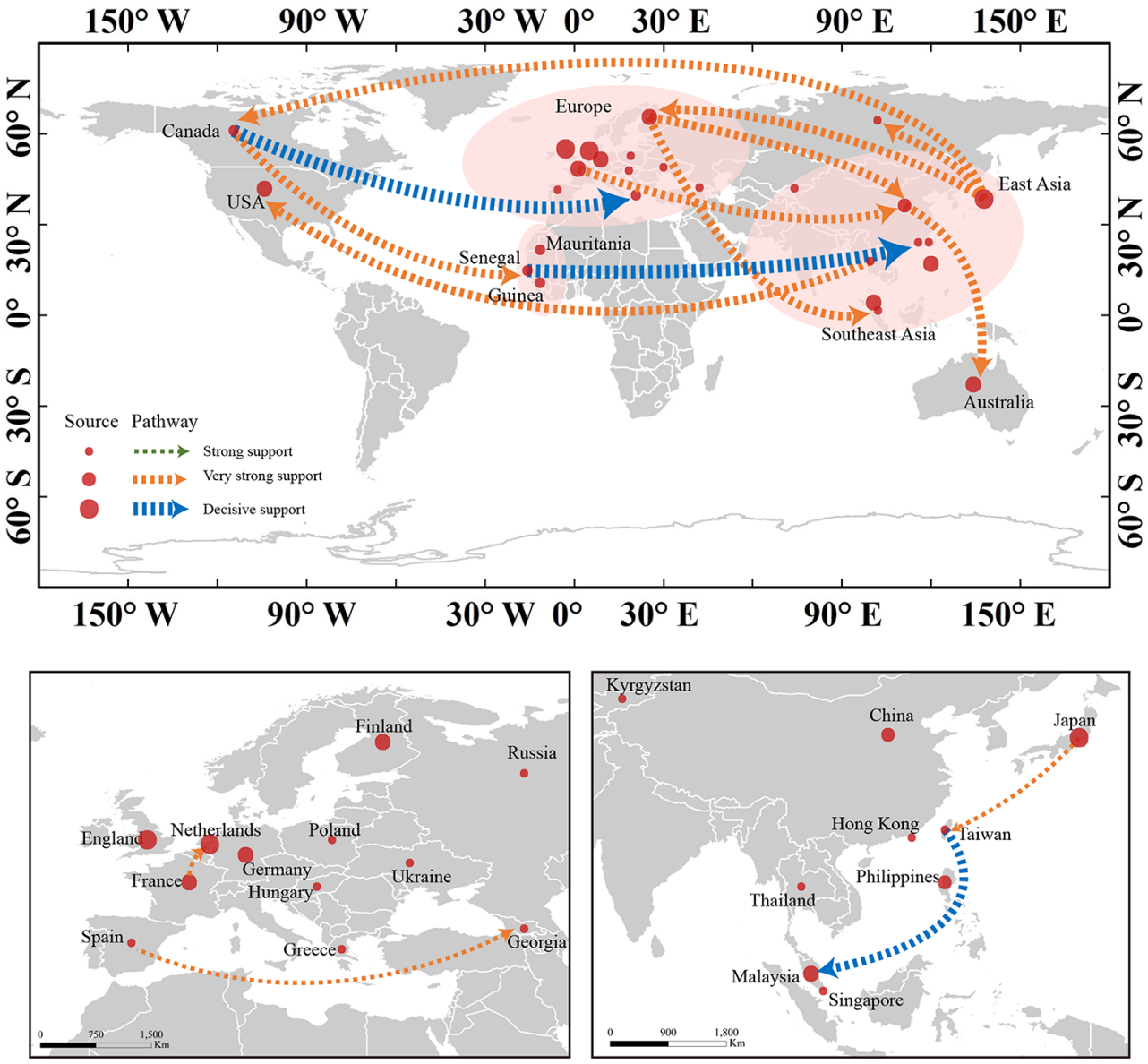

Figure 9. Spatial diffusion pathways of EV71 subgroup C2.

rooted, time-measured phylogenies. We used this full probabilistic approach to study viral phylogeography and extended the Bayesian implementation to a mixture model in which exchange rates in the Markov model are allowed to be zero with some probability. This BSSVS enabled us to construct a BF test that identifies the most parsimonious description of the phylogeographic diffusion process. We also demonstrated how the geographical distribution of the sampling locations can be incorporated as prior specifications. Through feature-rich visual summaries of the space-time process, we offered insights into the spatial dispersal patterns of EV71.

Additionally, our phylogeographic analysis could help us to find the predominant subgroup that contributed to the main HFMD epidemic in a given geographic location, which is beneficial to the HFMD vaccine development. Critically, it is unclear whether viral evolution is the driver of the emergence and large outbreaks of HFMD in Southeast Asia or a consequence of the greater number of infected hosts. Our results would have profound implications for vaccine development, necessitating active surveillance and periodic vaccine updates. Taken together, the available data highlight the importance of understanding EV71 evolution and spatial dispersal patterns within and between endemic countries, which may be essential for understanding and controlling this emerging infection, including vaccine development.

Nevertheless, the results of this paper also have some limitations. First, we have studied spatiotemporal transmission patterns of predominant EV71 subgroups from a global perspective at broad spatial scales. The predominant subgroups and the dispersal patterns within some countries were unable to capture. Our results only represent the data that we can collect. The actual spatial diffusion pathways might be more complicated. Second, considering full-length genomic sequences (including UTRs) are mostly not available, we only studied VP1 sequences. However, full-length genomic sequences often can offer more comprehensive information on the genetic and evolution of enteroviruses. Third, this study displayed the discrete trait just by BSSVS methodology, which may get some bias, especially under uneven sampling conditions. The next study should improve the accuracy. Besides, we

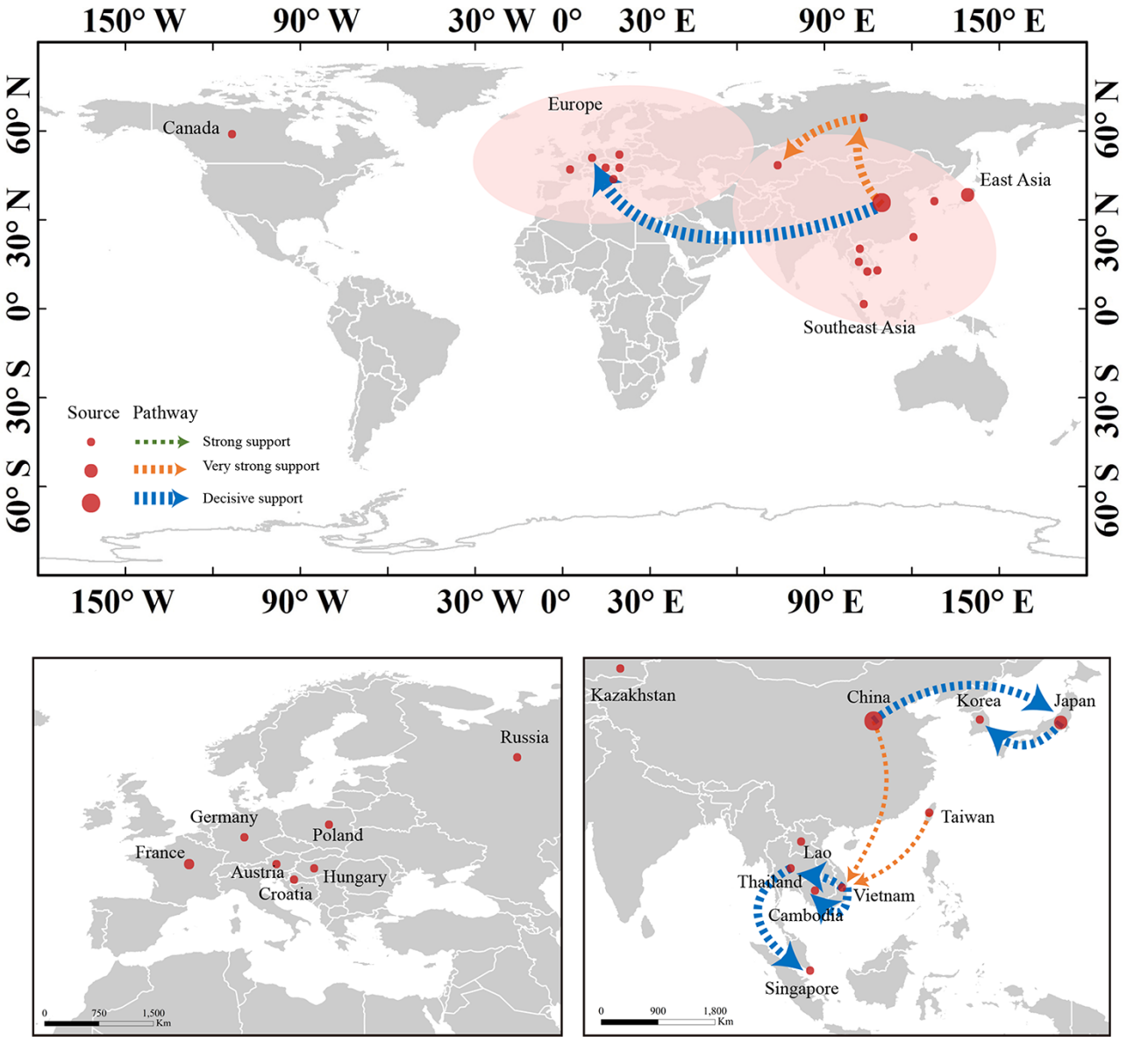

Figure 10. Spatial diffusion pathways of EV71 subgroup C4.

will focus further research on the small-scale dispersal patterns in Southeast Asia or in China. Furthermore, except EV71, many other viruses can also lead to HFMD, especially coxsackievirus A16 (CVA16). This study only displayed the results of EV71, and future research should also focus on the dispersal patterns of CVA16 and other enteroviruses.

5. Conclusions

Reconstruction of EV71 virus time to coalesce from temporal phylogenies showed that different geographical regions generated local epidemics circulating different predominant subgroups. In Europe and America, C1 and C2 replaced B1 and B2 as the predominant genotypes and C4 in Asia-Pacific countries. Our results highlighted that subgroups B1 and B2 played a great role in the early stage, while C1, C2, and C4 have played distinct roles since the late 1990s. This could help us to find the predominant subgroup in a given geographic location at a certain time, which may be beneficial to the HFMD vaccine development.

Data availability

Accession numbers of the sequences analyzed in this study have been provided in the Supplementary Data. The sequence data produced in this study are available from NCBI GenBank database.

Supplementary data

Supplementary data is available at *Virus Evolution* online.

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