Infection, Genetics and Evolution 81 (2020) 104264

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research Paper

Evolutionary dynamics of influenza B strains detected from paediatric acute respiratory infections in central Vietnam

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ARTICLE INFO

Keywords:
Influenza B
Phylogenetic
Molecular evolution
ARI
Vietnam
Paediatric infectious disease

ABSTRACT

Influenza virus B belongs to the family *Orthomyxoviridae* with segmented negative-sense RNA genomes. Since 1970s, influenza B has diverged intoVictoria and Yamagata, which differs in antigenic and evolutionary characteristics. Yet, molecular-epidemiological information of influenza B from developing nations is limited. In central Vietnam, influenza A subtype-specific circulation pattern and clinical characteristics were previously described. However, molecular evolutionary characteristics of influenza B has not been discussed to date.

We utilized the influenza B positives obtained from paediatric ARI surveillance during 2007–2013. Influenza B *HA* and *NA* genes were amplified, sequenced, and phylogenetic/molecular evolutionary analysis was performed using Maximum Likelihood and Bayesian MCMC. Phylodynamics analysis was performed with Bayesian Skyline Plot (BSP). Furthermore, we performed selection pressure analysis and estimated N-glycosylation sites. In the current study, overall positive rate for influenza B was 3.0%, and Victoria lineage immediately became predominant in post-A/H1N1pdm09 period. The noticeable shift in Victoria lineage WHO Group occurred. With respect to the evolutionary rate (substitutions/site/year), Victoria lineage *HA* gene was evolving faster than Yamagata lineage (2.43×10^{-3} vs 2.00×10^{-3}). Furthermore, the evolutionary rate of Victoria Group 5 was greater than Group 1. BSP presented the rapid growth in Effective Population Size (EPS) of Victoria lineage occurred soon after the 1st A/H1N1pdm09 case was detected whereas the EPS of Yamagata lineage was stable for both genes. N-glycosylation pattern between lineages and among WHO Groups were slightly different, and *HA* gene had a total of 6 amino acid substitutions under positive section pressure (4 for Victoria and 2 for Yamagata).

The current results highlight the importance of Victoria lineage in post-A/H1N1pdm09 period. Difference in evolutionary characteristics and phylodynamics may indicate lineage and WHO Group-specific evolutionary dynamics. It is necessary to further continue the molecular-epidemiological surveillance in local setting to gain a better understanding of local evolutionary characteristics of influenza B strains.

1. Introduction

Influenza virus belongs to the family *Orthomyxoviridae* with segmented negative-sense RNA genomes (Lamb and Choppin, 1983; Nerome et al., 1998). Among three influenza types, A, B and C, type A and B circulate among human population and impart a great proportion of morbidity and mortality among children and elder population (Chan et al., 2013; Paul Glezen et al., 2013; Roy et al., 2011). Seasonal peaks of influenza-related acute respiratory infections (ARI) may differ geographically where influenza-related ARI incidence increase during early

Abbreviations: MCMC, Markov Chain Monte Carlo; AICM, Akaike's Information Criterion through MCMC; ESS, Effective Sample Size; MCC, Maximum Clade Credibility; HPD, Highest Probability Density; tMRCA, time to the most recent common ancestor; BSP, Bayesian Skyline Plot; EPS, Effective Population Size; ML, Maximum Likelihood; SLAC, Conservative Single Likelihood Ancestor Counting; FEL, Fixed Effects Likelihood; IFEL, Internal Fixed Effects Likelihood; MEME, Mixed Effects Model for Episodic Diversifying Selection

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https://doi.org/10.1016/j.meegid.2020.104264
Received 30 September 2019; Received in revised form 7 February 2020; Accepted 22 February 2020
Available online 24 February 2020

autumn through winter season in temperate region (Shek and Lee, 2003) whereas no clear influenza peaks are seen in tropics (Beckett et al., 2004; Chittaganpitch et al., 2012; Nguyen et al., 2007; Western Pacific Region Global Influenza and Response, 2012).

Historically, the molecular-epidemiological and evolutionary characteristics of influenza A have been deeply investigated at the global scale up to date (Bahl et al., 2011; Bedford et al., 2011; Rambaut et al., 2008; Russell et al., 2008), yet the information of influenza B is comparatively limited worldwide. Since the initial discovery of influenza B in 1940s (Francis Jr., 1940), influenza B has been circulating alongside with influenza A (Klimov et al., 2012). Influenza B infection is mainly limited to human population, and few sporadic infections were previously reported in animal species (Osterhaus et al., 2000; Racaniello and Palese, 1979). Therefore, reassortment of genetic component within influenza B is considered to be relatively rare event, and influenza B does not possess multiple subtypes as influenza A. In fact, genetic drift, including nucleotide substitutions, insertions and deletions impart a major role on molecular evolution of influenza B in order to form its genetic variability as well as the evolutionary mechanism on host immune evasion, which may explain the slower molecular evolution rate and the smaller capacity of causing large ARI outbreak among human population (Barr et al., 2010; McCullers et al., 1999).

In 1970s, influenza B has diverged and categorized into two genetically and antigenically distinct lineages, Victoria and Yamagata lineages, represented by B/Yamagata/16/88 and B/Victoria/2/87 respectively (Kanegae et al., 1990; Rota et al., 1990). Since its lineage diversion event, two lineages have been co-circulating in many countries (Members of the Western Pacific Region Global Influenza Surveillance Response et al., 2013). Regardless of their similarity in terms of genetic organization and protein structure, influenza B Victoria and Yamagata lineages may differ in their preference on receptor binding, antigenicity and evolutionary characteristics. Furthermore, the difference in transmission dynamics between Victoria and Yamagata lineages has also been reported (Vijaykrishna et al., 2015). However, there still exists a gap in knowledge with respect to the molecular evolutionary characteristics of locally circulating influenza B lineages up to date particularly in developing nations including Vietnam.

In our study site in central Vietnam, the first A/H1N1pdm09-related paediatric ARI hospitalization case was detected in July 2009, and the seasonal circulation pattern of influenza A subtypes was previously documented by utilizing ARI cases obtained from the population-based paediatric ARI surveillance during January 2007–March 2011 (Le et al., 2014). Since then, A/H1N1pdm09 has been co-circulating along with A/H3N2. In addition, we recently presented the clinical aspect of influenza B infections and that there was an approximately four times greater risk of influenza B-related ARI hospitalization in post-A/H1N1pdm09 era (Yoshihara et al., 2018).

On the other hand, the detailed phylogenetic and molecular evolutionary characteristics of influenza B strains circulating in the current study site have not been previously revealed. Therefore in this study, we aim to gain a better understanding of the molecular evolutionary characteristics of influenza B strains circulating among paediatric ARI hospitalization cases during the period of February 2007 through June 2013 in central Vietnam.

2. Materials and methods

2.1. Study site and case enrollment

A population-based prospective paediatric ARI surveillance was initiated at Khanh Hoa General Hospital (KHGH), Nha Trang, Khanh Hoa Province, Vietnam since February 2007 (Yoshida et al., 2010). The study area covered approximately 198,729 populations living in 42,770 households from 16 communities with 13,631 children under the age of five: detailed demographic characteristics of the targeted population was previously described (Yoshida et al., 2010). All children less than 15 years from the catchment area admitted to the paediatric ward of KHGH presenting ARI symptom (cough and/or difficulty breathing) were enrolled in the current study. Prior to the enrollment, written informed consents were obtained from the parents or guardians of all the enrolled ARI children.

This study was approved by the institutional ethical review board of both National Institute of Hygiene and Epidemiology (NIHE), Vietnam, and Institute of Tropical Medicine (NEKKEN), Nagasaki University, Japan. The study was conducted in accordance with the approved guidelines.

2.2. Period of sample collection

For the current study, paediatric ARI hospitalization cases enrolled in the paediatric ward of KHGH during the period of February 2007 through June 2013 were selected and utilized for the data analysis.

2.3. Viral screening and nucleotide sequencing of influenza B HA and NA genes

Viral nucleic acids were extracted from the nasopharyngeal (NP) swabs collected from the enrolled paediatric ARI hospitalization cases using QIA Viral RNA Minikit (QIAGEN Inc., Valencia, CA, USA). Four multiplex PCR assays were performed for screening 13 respiratory viruses including influenza B as previously described (Yoshida et al., 2010). For influenza B confirmed ARI samples, the genetic regions encoding for Haemagglutinin (HA) (1.7 kb) and Neuraminidase (NA) (1.4 kb) were amplified using Reverse Transcription (RT)-PCR with specific primer pairs: (*HA* gene: BHA1-N (forward) AATATCCACAAA ATGAAGGC and HA2B-1867-1887R (reverse) AGTAGTAACAAGAGCA TTTTT) and (*NA* gene: BNA-F5v2 (forward) TCAAAACTGAAGCAAAT AGGCCA) and BNA-R1498–1472 (reverse): AATAGGAACAAAGGGTTT AGAACAGA). After the purification of amplified PCR products with ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA), BigDye Terminator ver.1.1 (Applied Biosystem, Foster City, CA, USA) was used for the sequencing reaction with the sequencing primers available in National Institute of Infectious Diseases (Japan)(https://www.niid.go.jp/niid/en/). Nucleotide sequence analysis was performed with 3730 DNA Analyzer (Applied Biosystem, Foster City, CA, USA).

2.4. Phylogenetic and molecular evolutionary analysis with Bayesian Markov Chain Monte Carlo (MCMC) and Maximum Likelihood (ML) methods

Sequenced fragments of influenza B *HA* and *NA* genes for each sample were assembled using GeneStudio ver.2.2. Influenza B genomic reference strains including WHO recommended vaccine strains were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and EpiFlu database within Global Initiative on Sharing All Influenza Data (GISAID) (http://platform.gisaid.org). Nucleotide sequences were aligned and edited using ClustalW algorithm implemented within MEGA ver.6.0 (Tamura et al., 2013). KAKUSAN4 (http://www.fifthdimension.jp/products/kakusan/) was utilized for the selection of best-fit nucleotide substitution model based on Bayesian Information Criterion (BIC) (Tanabe, 2011). The information of reference strains used in the current data analysis was summarized in Supplementary Table 1.

We constructed the phylogenetic trees of both influenza B *HA* and *NA* genes using Maximum Likelihood (ML) method under Hasegawa, Kishino and Yano (HKY) 1985-gamma nucleotide substitution model with 1000 bootstrap replications in order to estimate the evolutionary distances with MEGA ver.6.0 (Tamura et al., 2013). The best-fit nucleotide substitution model for each ML tree constructed was selected based on the Bayesian Information Criterion (BIC) using KAKUSAN4 (Tanabe, 2011). All the genetic classification within Victoria and Yamagata lineage used WHO groups in the analysis in order to make the

Table 1
Demographic and clinical information of paediatric ARI hospitalizations and yearly prevalence of influenza B ARIs.

	Year of sample collection (January 2007–June 2013)						
	(Jan. - Dec. 2007)	(Jan. - Dec. 2008)	(Jan. - Dec. 2009)	(Jan. - Dec. 2010)	(Jan. - Dec. 2011)	(Jan. - Dec. 2012)	(Jan. - Jun. 2013)
Paediatric ARI cases (n)	(*n* = 788)	(*n* = 600)	(*n* = 726)	(*n* = 542)	(*n* = 513)	(*n* = 801)	(*n* = 459)
Influenza B ARI cases (%)	2 (0.3%)	11 (1.8%)	11 (1.5%)	33 (6.1%)	9 (1.8%)	57 (7.1%)	10 (2.2%)
Victoria lineage (%)	1 (50.0%)	2 (18.2%)	4 (36.4%)	23 (69.7%)	8 (88.9%)	29 (50.9%)	5 (50.0%)
Yamagata lineage (%)	0	6 (54.5%)	1 (9.1%)	0	0	10 (17.5%)	2 (20.0%)
Demographic information							
Sex: Male (%)	449 (57.0%)	378 (63.0%)	445 (61.3%)	310 (57.2%)	293 (57.1%)	449 (56.1%)	278 (60.6%)
Female (%)	339 (43.0%)	222 (37.0%)	281 (38.7%)	232 (42.8%)	220 (42.9%)	352 (43.9%)	181 (39.4%)
Age (in month)[a]	17 (IQR: 9–25)	18 (IQR: 9–30)	16 (IQR: 9–28)	17 (IQR: 9–26)	18 (IQR: 9–31)	15 (IQR: 7–26)	16 (IQR: 8–25)
Clinical symptom(s)							
URTI (%)[b]	566 (71.8%)	501 (83.5%)	583 (80.3%)	436 (80.4%)	449 (87.5%)	533 (66.5%)	320 (69.7%)
LRTI (%)[c]	222 (28.2%)	99 (16.5%)	143 (19.7%)	106 (19.6%)	64 (12.5%)	268 (33.5%)	139 (30.3%)

[a] Data are presented as median (IQR (Interquartile Range): 1st - 3rd).
[b] URTI: upper respiratory tract infection (URTI condition refers to the enrolled ARI children without LRTI condition.)
[c] LRTI: lower respiratory tract infection (The definition of LRTI is based on the modified WHO Integrated Management of Children Illnesses algorithms.)

naming consistent with our previous study from the current study site (Yoshihara et al., 2018).

Phylogenetic and molecular evolutionary analyses were performed with Bayesian Markov Chain Monte Carlo (MCMC) method using BEAST ver.1.8.0 (Drummond and Rambaut, 2007). For the selection of best-fit demographic and clock model in each analysis, four demographic models (Constant Size, Exponential Growth, Logistic Growth and Expansion Growth) and four clock models (Strict clock, Uncorrelated lognormal relaxed clock, Uncorrelated exponential clock and Random local clock) were compared based on Akaike's Information Criterion through MCMC (AICM) (Kimura et al., 2015; Nagasawa et al., 2015; Suchard et al., 2001) using Tracer ver.1.6 (http://tree.bio.ed.ac.uk/software/tracer/) (Supplementary Table 2 and 3). The model with the lowest AICM was selected as the best-fit model and used for each analysis. Detailed condition for each analysis is summarized in Supplementary Table 4. The MCMC chains were run for 200,000,000 steps for all the analyses to achieve convergence with sampling every 2000 steps. The convergence was assessed using Tracer ver.1.6, and the parameters with Effective Sample Sizes (ESS) of 200 or greater after 10% burn-in were accepted. The time-scaled Maximum Clade Credibility (MCC) trees were generated by Tree Annotator ver.1.8.0 after removing the first 10% of trees as burn-in. Time-scaled MCC trees were viewed and edited with FigTree ver.1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). Furthermore, molecular evolutionary rates were estimated using BEAST ver.1.8.0 under the models summarized in Supplementary Table 4 (Drummond and Rambaut, 2007). Also, the best-fit model for each analysis was confirmed with log marginal likelihood value using path sampling and stepping-stone sampling (Baele et al., 2012; Baele et al., 2013) (Supplementary Table 5 and 6).

2.5. Pairwise distance (p-distance) frequency distribution

Pairwise distances (*p*-distances) were estimated in order to investigate the genetic variability of influenza B *HA* and *NA* genes circulating in the current study site using MEGA ver.6.0 (Tamura et al., 2013). ARI strains with 100% nucleotide sequence identity were intentionally excluded from the analysis.

2.6. Phylodynamic analysis using Bayesian Skyline Plot (BSP)

In order to assess the change in time-course trend of Effective Population Size (EPS) of both influenza B Victoria and Yamagata lineages circulating in central Vietnam during the current investigation period, Bayesian Skyline Plots (BSP) were constructed using BEAST ver.1.8.0 (Drummond and Rambaut, 2007). Selection of the best-fit nucleotide substitution models were performed with KAKUSAN4 based on the comparison of BIC value (Tanabe, 2011). Four clock models were compared based on AICM using Tracer ver.1.6 (Kimura et al., 2015; Nagasawa et al., 2015), and the model with the lowest AICM were selected as the best-fit model (Supplementary Table 7). Also, the model selection was confirmed with path sampling and stepping-stone methods (Supplementary Table 8).

The MCMC chains were run for 200,000,000 steps with sampling every 2000 steps under the uncorrelated exponential relaxed clock model with HKY85-gamma substitution model (Supplementary Table 4).

2.7. Prediction of potential N-glycosylation sites

Prediction of the potential N-glycosylated sites within influenza B *HA* and *NA* genes were performed using NetNGlyc 1.0 Server (www.cbs.dtu.dk/services/NetNGlyc/). Amino acid sequence stretches containing Asn-Xaa-Ser/Thr (where Xaa was not Proline) were considered to be the potential N-glycosylation sites.

2.8. Positive and negative selection pressure analysis

Amino acid substitutions within *HA* and *NA* genes of central Vietnam influenza B strains under positive and negative selection pressure were estimated by calculating synonymous (*dS*) and non-synonymous (*dN*) substitution rates at every codon using Datamonkey (http://www.datamonkey.org/) (Pond and Frost, 2005). In the current analysis, four selective pressure models, Conservative Single Likelihood Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL), Internal Fixed Effects Likelihood (IFEL) and Mixed Effects Model for Episodic Diversifying Selection (MEME) methods were performed for the accurate estimation (Kimura et al., 2016; Kobayashi et al., 2015). For all the selection pressure analyses, the cut-off values for *p*-value were set at 0.05.

2.9. Statistical analysis

In order to compare the molecular evolutionary rates between two independent groups, Welch's test was performed. All the statistical analyses in the current study were performed using STATA ver.12.1 (StataCorp LP, College Station, TX, USA). *P*-values less than 0.05 were considered to be statistically significant.

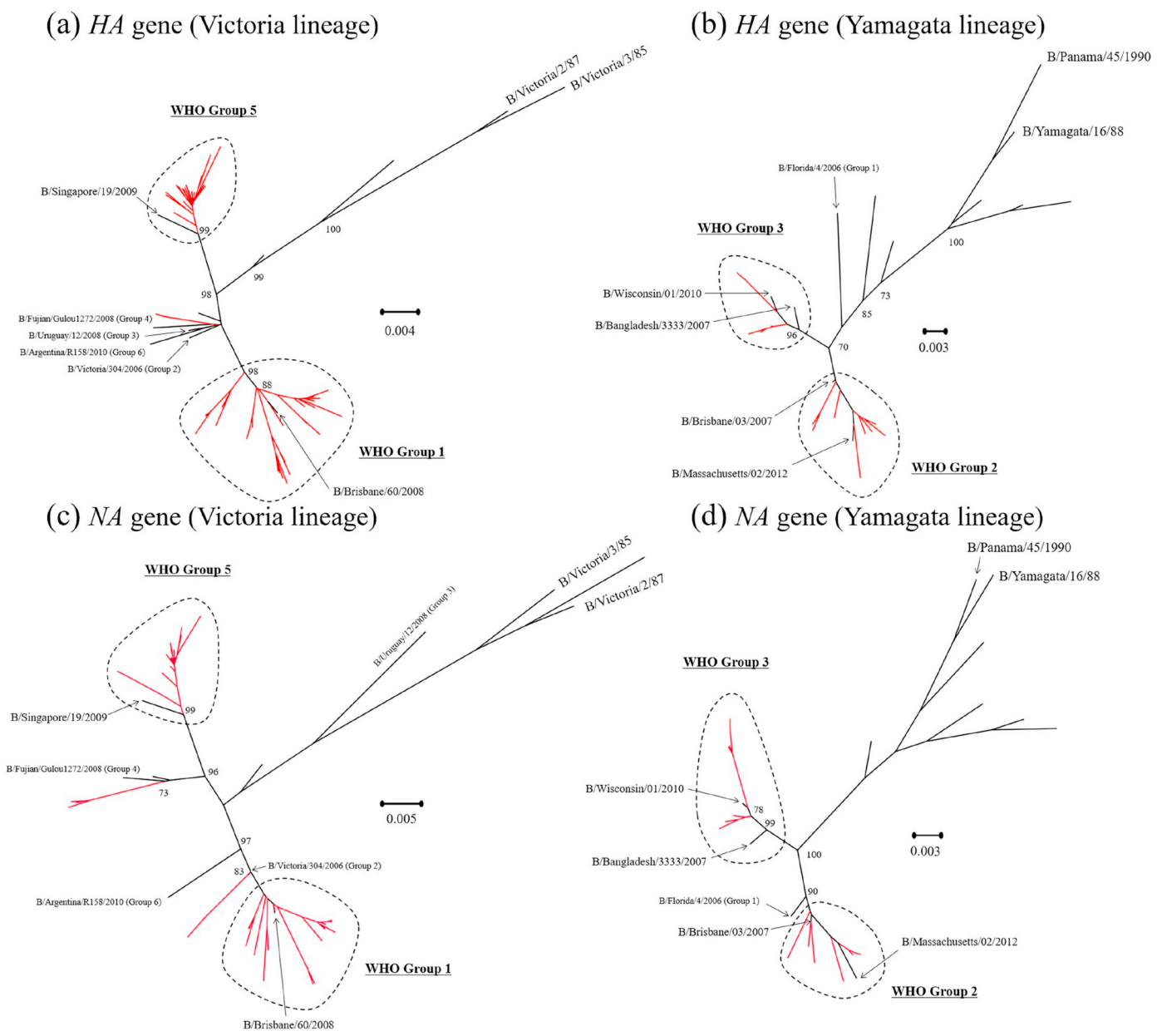

Fig. 1. Phylogenetic trees of influenza B *HA* and *NA* genes of Victoria and Yamagata lineages using Maximum Likelihood (ML) method.
Phylogenetic trees with Maximum Likelihood (ML) method were constructed for influenza B *HA* gene Victoria (a), Yamagata (b), *NA* gene Victoria (c) and Yamagata (d) lineages respectively. The Vietnamese influenza B strains obtained from the current study site were indicated with the RED-solid lines. Scale bar indicates the nucleotide substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Phylogenetic analyses of influenza B HA and NA genes using Maximum Likelihood (ML) and Bayesian Markov Chain Monte Carlo (MCMC)

During the investigation period of January 2007 through June 2013, a total of 4429 paediatric ARI hospitalization cases were enrolled in the study. Demographic and clinical characteristics of the paediatric ARI cases were summarized in Table 1. Among the enrolled ARI cases, 133 were confirmed to be influenza B positive, and yearly prevalence of influenza B varied among seasons (Table 1). Furthermore, the monthly cumulative influenza B positive cases as well as age-category specific hospitalization incidence due to influenza B infection in the current study was summarized in our previous manuscript (Yoshihara et al., 2018), in which the clinical epidemiological characteristics were also discussed.

Among influenza B positives, 106 *HA* and 100 *NA* genes were amplified, and 91 (85.9%) of *HA* and 84 (84.0%) of *NA* genes were successfully sequenced and proceeded to the phylogenetic and molecular evolutionary analysis.

Regarding the phylogenetic analysis of *HA* gene, Maximum Likelihood (ML) tree presented the genetic clustering in Group 1 (B/Brisbane/60/2008) and Group 5 (B/Singapore/19/2009) of Victoria lineage (Fig. 1 (a)). In addition, a few Vietnamese ARI strains were detected in Group 4 (B/Fujian/Gulou1272/2008). The time-scaled Maximum Clade Credibility (MCC) tree presented the time to most recent common ancestor (tMRCA) was around 1931 (95%HPD: 1917–1941), and the major lineage diversion event occurred around

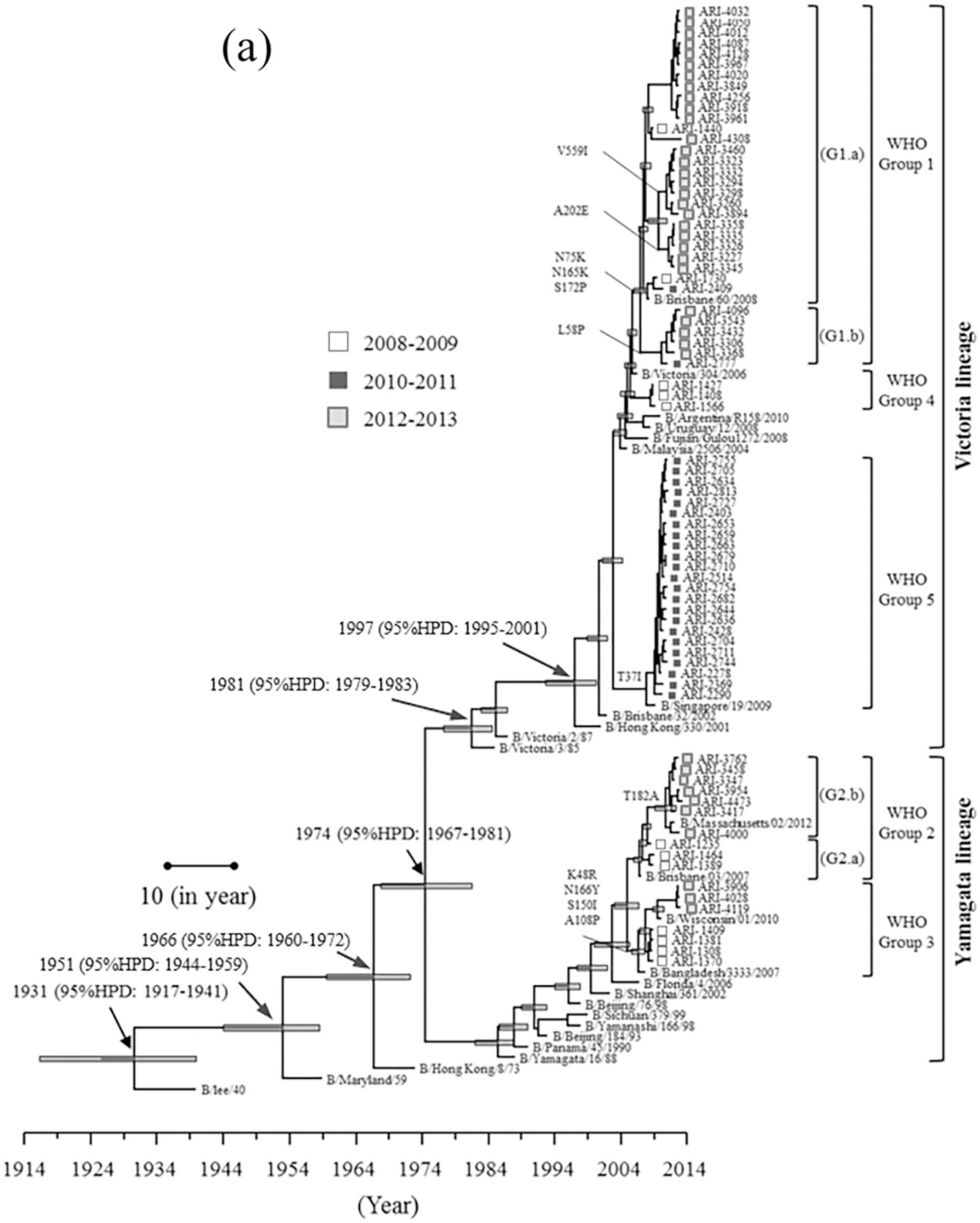

Fig. 2. Time-scaled phylogenetic trees of influenza B *HA* (a) and *NA* (b) genes using Bayesian Markov Chain Monte Carlo (MCMC) method.
The Vietnamese strains included in the current study were indicated in the boxes with distinct colors: BLANK for 2008–2009, GREY for 2010–2011 and BLACK for 2012–2013. Uncertainties for the year of divergence were indicated as 95%HPD (Highest Probability Density) in the horizontal light-blue boxes at each branch node. The scale bar represents the unit of time (in year). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1974 (95%HPD: 1967–1981) (Fig. 2 (a)). Victoria lineage Group 5, dominant in 2010–2011 season, possessed an additional substitution at T37I. Victoria lineage Group 1, the dominant group in 2012–2013 season, formed genetic clusters represented by the amino acid substitutions, V559I, A202E, and L58P.

With respect to Yamagata lineage of *HA* gene, Vietnamese strains

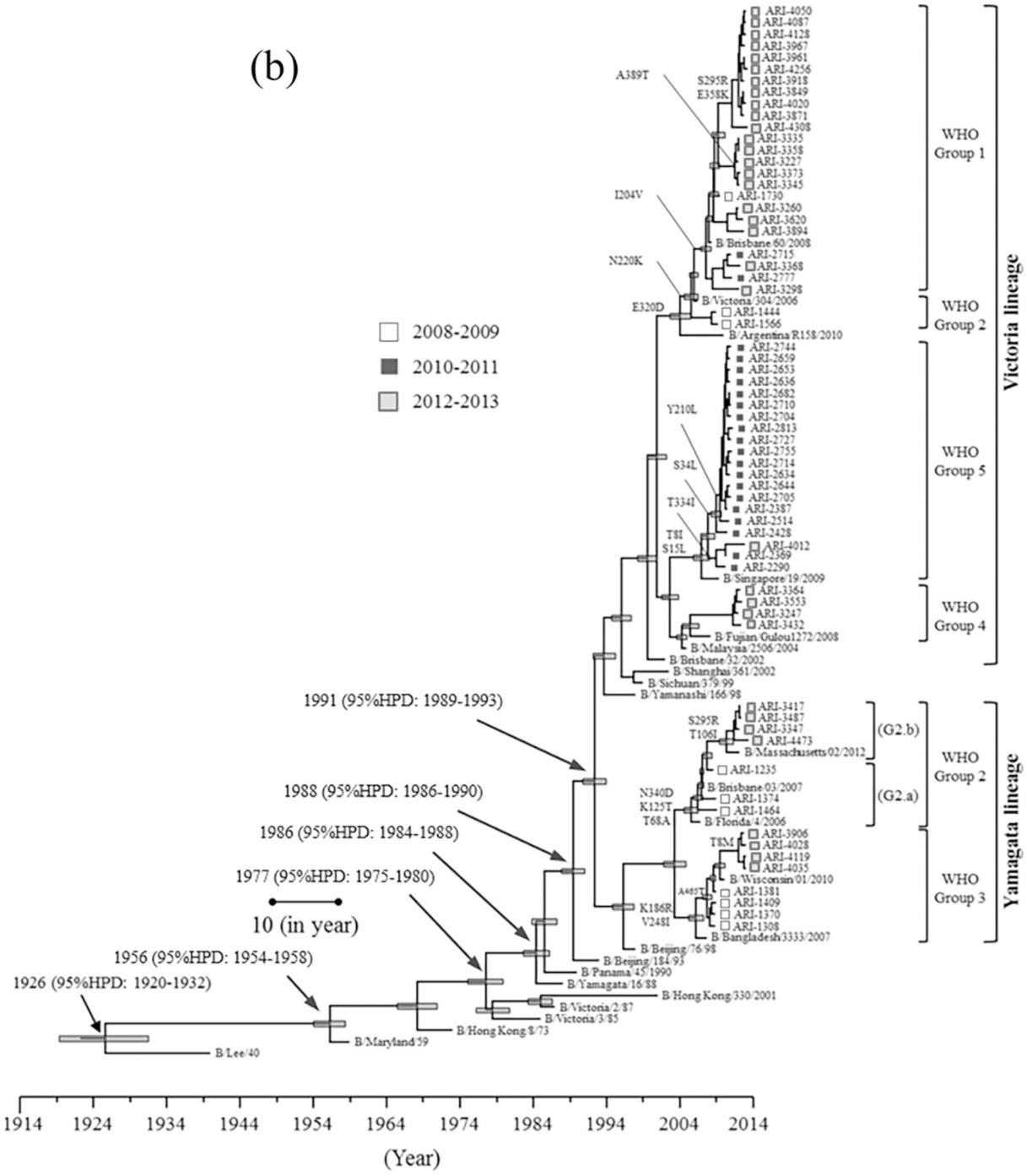

Fig. 2. (*continued*)

clustered in Group 2 (B/Brisbane/03/2007) and Group 3 (B/Bangladesh/3333/2007) (Fig. 1 (b)). Regarding Group 2, ARI strains from 2012 to 2013 season possessed an additional substitution at T182A, which clustered with B/Massachusetts/02/2012 (Fig. 2 (a)). For Group 3, ARI strains from 2012 to 2013 seasons formed the cluster with B/Wisconsin/01/2010.

Next, the overall genetic clustering patterns in both lineages of *NA* gene were similar to *HA* gene (Fig. 1 (c) and (d)). The time-scaled MCC tree presented that tMRCA was around 1926 (95%HPD: 1920–1932) (Fig. 2 (b)). With respect to Victoria lineage Group 1 in the current study, there were genetic clusters with unique substitutions, including S295R, E358K, and A389T. Furthermore, Group 5 possessed additional substitutions, T334I, S34L and Y210L. In addition to Group 1 and 5, a few cases of Group 2 in 2008–2009 season and Group 4 in 2012–2013 season were detected. For Yamagata lineage, both Group 2 and 3 were co-circulating during the investigation period. Group 2 strains from 2012 to 2013 season possessed additional substitutions at S295R and T106I, which genetically resembled to B/Massachusetts/02/2012. Furthermore, Group 3 strains from 2012 to 2013 season were with T8M substitution, which clustered with B/Massachusetts/02/2012.

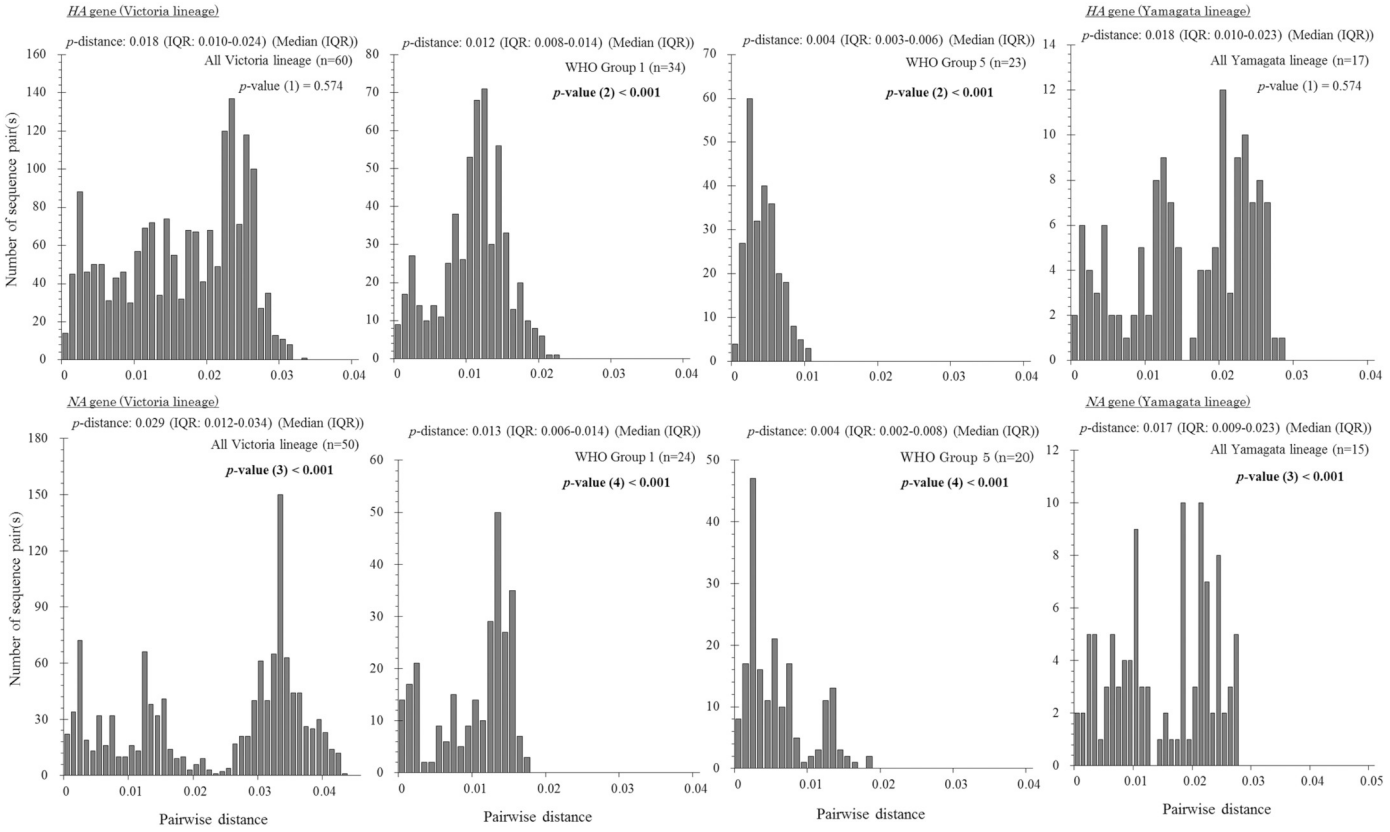

Fig. 3. Pairwise distance (*p*-distance) frequency distributions of influenza B *HA* (top) and *NA* (bottom) genes from central Vietnam.

3.2. Pairwise distance frequency distributions of influenza B HA and NA genes

In order to investigate the genetic variability of influenza B *HA* and *NA* genes circulating in the current study site, we calculated the pairwise distances (*p*-distance) of both lineages and WHO Groups (Fig. 3). Regarding the lineage-specific *p*-distance comparison in *HA* gene, no significant difference was detected ($p = .574$). In the comparison between Group 1 and 5 of Victoria lineage, *p*-distance of Group 1 was greater than Group 5: (0.012 (IQR: 0.008–0.014), Group 1 vs. 0.004 (IQR: 0.003–0.006), Group 5, $p < .001$).

With respect to *NA* gene, *p*-distance of Victoria lineage was greater in comparison to Yamagata lineage: (0.029 (IQR: 0.012–0.034), Victoria vs. 0.017 (IQR: 0.009–0.023), Yamagata, $p < .001$). Furthermore, within the Victoria lineage, *p*-distance of Group 1 was greater than Group 5: (0.013 (IQR: 0.006–0.014), Group 1 vs. 0.004 (IQR: 0.002–0.008), Group 5, $p < .001$).

3.3. Molecular evolutionary rates of influenza B lineages and WHO groups

Molecular evolutionary rates of lineages and WHO Groups in both *HA* and *NA* genes were estimated through Bayesian MCMC (Table 2). Firstly, the nucleotide substitution rate of overall *HA* gene was faster in comparison to *NA* gene: (3.11×10^{-3} (95%HPD: 1.97×10^{-3} -

Table 2
Molecular evolutionary rate comparison between lineages and WHO Groups of central Vietnam influenza B strains.

	No. of ARI strains (n)	Mean molecular evolution rate (substitutions/site/year) (95%HPD[a])	*p*-value
Overall *HA* gene	($n = 91$)	**3.11×10^{-3} (1.97×10^{-3} - 4.24×10^{-3})**	**< 0.001[b,***]**
Yamagata lineage	($n = 19$)	2.00×10^{-3} (7.57×10^{-4} - 3.34×10^{-3})	**0.015[c,*]**
Victoria lineage	($n = 72$)	**2.43×10^{-3} (1.60×10^{-3} - 3.30×10^{-3})**	
WHO Group 1	($n = 42$)	1.67×10^{-3} (8.13×10^{-4} - 2.52×10^{-3})	**< 0.001[d,***]**
WHO Group 5	($n = 26$)	**4.77×10^{-3} (1.09×10^{-3} - 9.27×10^{-3})**	
Overall *NA* gene	($n = 84$)	1.69×10^{-3} (1.06×10^{-3} - 2.32×10^{-3})	
Yamagata lineage	($n = 16$)	1.76×10^{-3} (9.68×10^{-4} - 2.58×10^{-3})	0.125[e]
Victoria lineage	($n = 68$)	1.95×10^{-3} (1.08×10^{-3} - 2.93×10^{-3})	
WHO Group 1	($n = 31$)	1.64×10^{-3} (5.18×10^{-4} - 2.98×10^{-3})	0.920[f]
WHO Group 5	($n = 27$)	1.65×10^{-3} (5.93×10^{-4} - 2.82×10^{-3})	

Statistically significant *p*-values were indicated in bold style. As the index of statistically significant values: * were used for *p*-values < .05, ** for *p*-values < .01 and *** for *p*-values≦0.001.

[a] 95%HPD stands for 95% Highest Probability Density.
[b] Molecular evolution rates were compared between overall *HA* and *NA* genes.
[c] Molecular evolution rates were compared between Victoria and Yamagata lineages of *HA* gene.
[d] Molecular evolution rates were compared between Victoria lineage WHO Group 1 and 5 of *HA* gene.
[e] Molecular evolution rates were compared between Victoria and Yamagata lineages of *NA* gene.
[f] Molecular evolution rates were compared between Victoria lineage WHO Group 1 and 5 of *NA* gene.

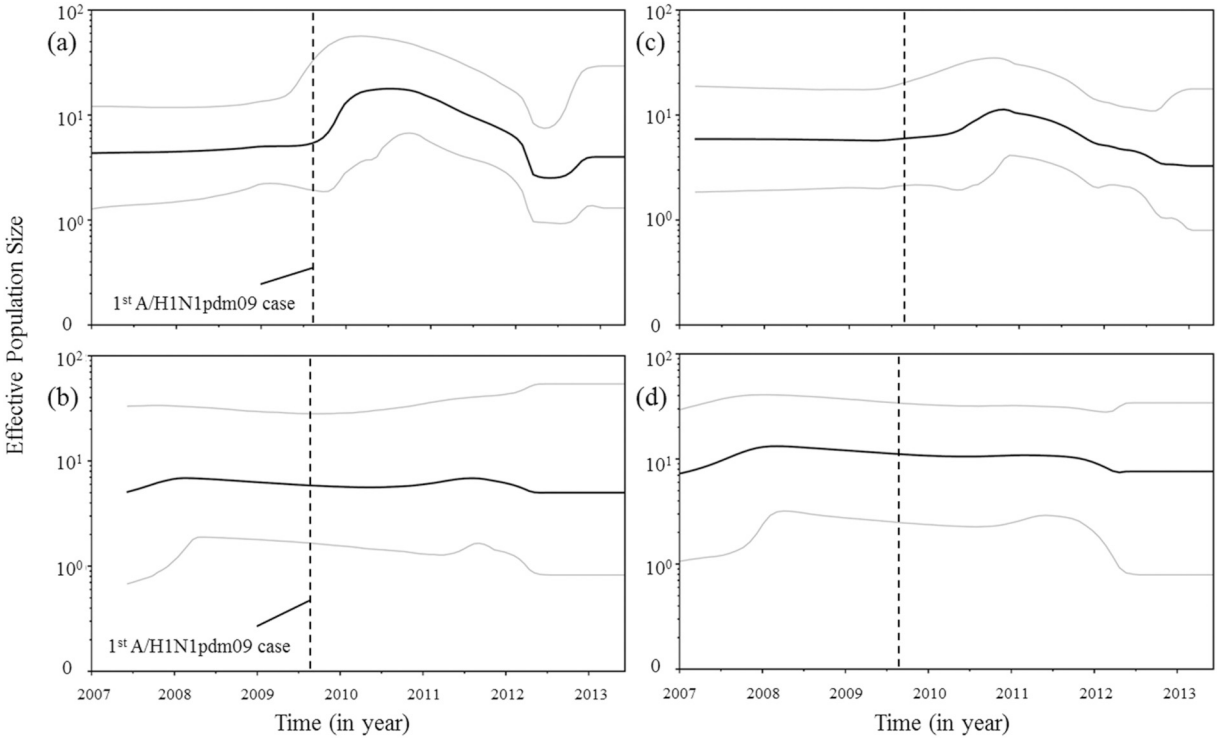

Fig. 4. Bayesian Skyline Plot (BSP) of influenza B *HA* gene Victoria (a), Yamagata (b), *NA* gene Victoria (c) and Yamagata (d) lineages from central Vietnam. The Y-axes represent the effective population size, and the X-axes represent the generation time (in year). The BLACK-solid lines indicate the median effective population size, and the GREY lines present the range for 95%HPD (Highest Probability Density). The first A/H1N1pdm09 strain-related paediatric ARI case detected in the current study site (July 2009) was indicated as the vertical dotted-lines.

4.24×10^{-3}), *HA* vs. 1.69×10^{-3} (95%HPD: 1.06×10^{-3} - 2.32×10^{-3}), *NA*, $p < .001$). With respect to the comparison between lineages of *HA* gene, Victoria lineage presented the greater substitution rate: (2.43×10^{-3} (95%HPD: 1.60×10^{-3} - 3.30×10^{-3}), Victoria vs. 2.00×10^{-3} (95%HPD: 7.57×10^{-4} - 3.34×10^{-3}), Yamagata, $p = .015$). Furthermore, within Victoria lineage, evolutionary rate of Group 5 was greater than Group 1: (4.77×10^{-3} (95%HPD: 1.09×10^{-3} - 9.27×10^{-3}), Group 5 vs. 1.67×10^{-3} (95%HPD: 8.13×10^{-4} - 2.52×10^{-3}), Group 1, $p < .001$). On the contrary, there was no significant difference in evolutionary rate comparison within *NA* gene (Table 2).

3.4. Bayesian skyline plot of influenza B Victoria and Yamagata lineages

We estimated the time-course trend of effective population size (EPS) in both Victoria and Yamagata lineages of *HA* and *NA* genes using Bayesian Skyline Plot (BSP) (Fig. 4). Regarding the *HA* gene, Victoria lineage experienced rapid increase in the size of EPS immediately after the detection of 1st A/H1N1pdm09-associated ARI case in July 2009 in the current study site (Fig. 4 (a)), the peak of which reached the highest around the middle of 2010. Since then, EPS gradually decreased towards 2011/2012 and 2012/2013 seasons. The lowest EPS was detected in the middle of 2012, and the EPS slightly increased towards the end of 2012, which was followed by the steady phase. With respect to Yamagata lineage, the size of EPS had stayed flat throughout the current investigation period (Fig. 4 (b)).

Furthermore, we examined the time-course trend of EPS in *NA* gene. Overall, similar time-course trend of EPS were observed in both Victoria and Yamagata lineages. However, the size of increase in EPS observed after the detection of 1st A/H1N1pdm09 ARI case was relatively small in comparison to *HA* gene (Fig. 4 (c)). Regarding the Yamagata lineage, BSP presented that EPS of Yamagata lineage stayed flat throughout the current investigation period (Fig. 4 (d)).

3.5. N-glycosylation sites comparison between influenza B lineages and WHO groups

We estimated the positions of N-glycosylated site within both *HA* and *NA* genes of influenza B strains obtained from central Vietnam. With respect to *HA* gene, both Victoria and Yamagata lineages possessed seven conserved N-glycosylation at 25–28, 59–62, 145–148, 166–169, 197–200, 304–307 and 333–336 (Supplementary Table 10). In addition, Victoria lineage possessed one additional N-glycosylation at 233–236 (NQTE). Within each lineage, no WHO Group specific N-glycosylation pattern was detected.

Regarding the N-glycosylation analysis of *NA* gene, four conserved sites were found at 56–59, 64–67, 144–147 and 284–287 in both lineages (Supplementary Table 10). Within Victoria lineage, Group 4 possessed an additional N-glycosylation at 463–466 (NMTL). Furthermore, Group 3 of Yamagata lineage also had additional N-glycosylation site at 463–466 (NMTL).

3.6. Positive and negative selection pressure analysis of influenza B HA and NA genes

We investigated the amino acid substitutions under positive and negative selection pressure using four different selection models. With respect to the positive selection pressure in *HA* gene, none of the amino acid substitution was agreed by all four selection algorithms (Table 3). Among central Vietnam Victoria lineage strains, substitution at V146I/A/D was agreed by FEL and MEME. Other substitutions at A202E/K, K203S and I331V of Victoria lineage and G230S/D and G151P of Yamagata lineage were under the positive selection pressure either by IFEL or MEME model.

Table 3
Positive selection sites with amino acid substitutions in *HA* and *NA* genes.

Influenza B gene and lineage	Selection method(s)	Positive selection sites with amino acid substitutions	Mean *dN* / *dS*
HA gene Victoria lineage	SLAC[a]	· · ·	0.150
	FEL[b]	V146I, V146A, V146D	
	IFEL[c]	· · ·	
	MEME[d]	V146I, V146A, V146D, A202E, A202K, K203S, L331V	
HA gene Yamagata lineage	SLAC	· · ·	0.201
	FEL	· · ·	
	IFEL	G230S, G230D	
	MEME	G151P	
NA gene Victoria lineage	SLAC	· · ·	0.259
	FEL	A395T, A395V, A395N	
	IFEL	L34S	
	MEME	· · ·	
NA gene Yamagata lineage	SLAC	· · ·	0.232
	FEL	· · ·	
	IFEL	· · ·	
	MEME	· · ·	

Values of *p*-value less than 0.05 were considered to be statistically significant.
[a] SLAC: Single Likelihood Ancestor Counting
[b] FEL: Fixed Effects Likelihood
[c] IFEL: Internal Fixed Effects Likelihood
[d] MEME: Mixed Effects Model for Episodic Diversifying Selection

In regard to the positive selection pressure on *NA* gene, two sites (A395T/V/N with FEL and L34S with IFEL) were detected in Victoria lineage (Table 3). On the other hand, no positive selection site was found in Yamagata lineage.

Furthermore, we analyzed the amino acid substitution sites under negative selection pressure with SLAC, FEL and IFEL models. Both lineages of *HA* gene possessed several negative selection sites agreed by all three selection models (Table 4). With respect to *NA* gene, two negative selection sites were agreed by all three models in Victoria lineage whereas no negative selection site in Yamagata lineage was agreed by all three selection models.

4. Discussion

We investigated the evolutionary characteristics of influenza B circulating between 2007 and 2013 among hospitalized paediatric ARI cases in central Vietnam. Victoria and Yamagata lineages were co-circulating with Victoria lineage as the dominant type in all seasons, yet these two lineages differed in terms of evolutionary characteristics. Furthermore, the seasonal dominance of genetically distinct WHO Groups within Victoria lineage shifted from season to season whereas two Yamagata lineage WHO Groups co-circulated. In majority of developing countries in southeast Asia, including Vietnam, influenza vaccination is not readily available in public hospital. The current study together with our previous study (Yoshihara et al., 2018).

will emphasize the increased risk of hospitalization due to influenza B infection among child population as well as the unique evolutionary dynamics of influenza B strains in south-central region of Vietnam, which would be useful for future vaccination program.

Victoria lineage predominated over Yamagata lineage in post-A/H1N1pdm09 period in the current study. Similar trends have been reported from southern China, Taiwan, and Cambodia (Horm et al., 2014; Tan et al., 2013; Yang et al., 2012). The trend of influenza B lineage dominance would be attributed by the population-based herd-immunity against each lineage (Chen and Holmes, 2008). Also, the difference in age of infection between lineages may contribute to the trend as previous study suggested a higher basic reproductive number (R*e*) in younger population (Lunelli et al., 2013). The lower sero-positivity against Victoria lineage among younger group might have resulted in the rapid increase of Victoria lineage ARIs in the current study. Furthermore, the difference in viral infectious capacity may play a role for Victoria lineage being dominant after the emergence of A/H1N1pdm09 strain. The reports from China, Australia, and New Zealand stated that Victoria lineage may possess the greater viral infectivity (Tan et al., 2013; Vijaykrishna et al., 2015), based on the higher basic reproductive number (R*e*) compared to Yamagata lineage. Furthermore, a recent mathematical modeling study, which took into account the cross-reactivity between lineages and waning immunity, also estimated a greater R*e* in Victoria lineage (Nyirenda et al., 2016).

Genetic drift plays an important role in seasonal circulation of influenza B virus, and some studies suggested the difference in

Table 4
Negative selection sites with amino acid substitutions in *HA* and *NA* genes.

Influenza B gene and lineage	Selection method(s)	Negative selection sites with amino acid substitutions[d]
HA gene Victoria lineage	SLAC[a]	I3, **V15**, **K71**, **R112**, **R118**, **F153**, P210, G312, K316, P328, A380, **E460**, A464, T513, **G528**, **S544**
	FEL[b]	I3, G6, **V15**, H40, R50, V65, **K71**, P108, **R112**, **R118**, H122, **F153**, N197, E198, P210, Y224, P232, K272, K281, I290, H297, G312, K316, P328, T372, A380, L405, D428, **E460**, A464, T513, **G528**, Y537, **S544**, D562
	IFEL[c]	**V15**, **K71**, **R112**, **R118**, **F153**, D196, **E460**, **G528**, **S544**
HA gene Yamagata lineage	SLAC	**H85**, **A382**
	FEL	G28, G74, **H85**, K129, S195, V381, **A382**, N400, N409, R412, S440, E508, N531
	IFEL	**H85**, **A382**, N409, R412, N531
NA gene Victoria lineage	SLAC	**A47**, **V430**
	FEL	I45, **A47**, N64, F103, F172, A181, K186, A282, V353, T368, D392, Y409, G412, C420, **V430**, A441, G461
	IFEL	I45, **A47**, N64, G89, A181, V353, D392, C420, **V430**, A441, G461
NA gene Yamagata lineage	SLAC	E308
	FEL	V18, P83, G162, I191, P196, E308, G434
	IFEL	· · ·

Values of *p*-value less than 0.05 were considered to be statistically significant.
[a] SLAC: Single Likelihood Ancestor Counting.
[b] FEL: Fixed Effects Likelihood.
[c] IFEL: Internal Fixed Effects Likelihood
[d] Negative selection sites agreed by all three selection models were indicated in bold style.

evolutionary rate between influenza B lineages (Langat et al., 2017). In the current study, Victoria lineage of *HA* gene presented the faster evolutionary rate (substitutions/site/year) than Yamagata lineage (2.43×10^{-3}, Victoria vs 2.00×10^{-3}, Yamagata, $p = .015$), Other studies from Cameroon, Australia, and New Zealand also presented the faster genetic evolutionary rates in Victoria lineage (Monamele et al., 2018; Vijaykrishna et al., 2015). In contrast, the recent analysis with Bayesian evolutionary model, using global dataset presented no difference between lineages (Langat et al., 2017), which was in line with a previous study (Bedford et al., 2014). The difference in evolutionary rate among studies could be due to the difference in the investigation year covered in each study, during which the genetic component of circulating strains may differ. In addition, geographic diversity within the dataset may result in the difference as the circulation dynamics of Victoria lineage may differ between temperate and tropical climate regions (Langat et al., 2017).

In the current study, WHO Groups of Victoria lineage shifted from season to season in post-A/H1N1pdm09 period. Group 5 predominated in 2010–2011, then it was replaced by Group 1 in 2012–2013. Circulation of Group 5 in 2010 in Cambodia, Lao PDR, and Thailand implied the consistent influenza B trend with neighboring Asian countries (Horm et al., 2014; Tewawong et al., 2017). The lack of herd-immunity against Group 5 prior to 2010–2011 may also have driven the local endemic by Group 5. Interestingly, the study from Australia and New Zealand presented the viral infectivity of Victoria lineage varied among epidemics (Vijaykrishna et al., 2015). Therefore, two dominant Victoria lineage WHO Groups in this study may differ in viral infectivity, which may have resulted in a favorable transmission by Group 5 in 2010–2011.

Victoria lineage WHO Group 1 and 5 differed in *HA* gene amino acid composition (Group 1: N75K, N165K, and S172P, Group 5: T37I), which may link with host-receptor binding affinity or viral infectivity as these are located within or close proximity to the previously described antigenic sites (Wang et al., 2008) (Supplementary Table 11). Interestingly, Victoria lineage Group 1 of both *HA* and *NA* genes in our study formed distinct genetic clusters for the 1st-peak (December 2011–June 2012) and 2nd-peak (October 2012–April 2013) respectively. Majority of Group 1 strains from the first peak clustered with either A202E or V559I for *HA* and A389T for *NA* genes, neither of which was detected in the second peak. A202E is located within 190-helix and Receptor Binding Pocket (Wang et al., 2008). Therefore, this substitution may be linked with alternation of influenza B antigenicity. Another unique genetic cluster seen in 2012–2013 from our study site, with L58P (Group 1B), was also reported from Beijing, China in the same time period (Lei et al., 2019). This may imply the similar circulation trend of Victoria lineage strains with other Asian countries. The different genetic component of influenza B strains in different peaks within same season implied the importance of antigenic drift as a viral evolutionary mechanism for host-immune evasion as described (Hay et al., 2001). Alternative explanation for multiple Group 1 peaks might have been due to the introduction of Group 1 genetic variants from more than one geographical location. The previous studies from China have described the introduction of multiple dominantly circulating strains from other area within China and international source within a single year (Chen et al., 2016; Tan et al., 2013).

On the other hand, Victoria lineage Group 5, the dominant type in 2010–2011, was genetically conserved compared to Group 1, which implied the introduction of 1st Group 5 case within the community might have triggered the rapid transmission among naïve population. Within *HA* gene, Victoria lineage Group 5 presented the faster substitution rate in comparison with Group 1. Possible factors may be due to different herd-immunity against these two WHO Groups in the community and/or the distinct transmission dynamics of the two genetically different Groups. The viral infectivity represented by (R*e*) of Victoria lineage varied among seasons (Vijaykrishna et al., 2015), which implied the role of Victoria lineage genetic diversity on viral transmission capability.

The phylodynamics of influenza B lineages in the current study were assessed through Bayesian Skyline Plots (BSP). BSPs of Victoria and Yamagata lineages in both *HA* and *NA* genes presented distinct trend over time. The Effective Population Size (EPS) of Victoria lineage presented seasonal fluctuation whereas the EPS of Yamagata lineage was relatively invariant throughout the investigation period, which resembled to the study in East Australia and New Zealand (Vijaykrishna et al., 2015). Victoria lineage tends to go through selection bottleneck event from season to season, pattern of which resembles to influenza A/H3N2 (Bedford et al., 2011; Zinder et al., 2013). On the other hand, the relatively invariant EPS of Yamagata lineage implied the co-circulation of multiple genetic variants within same season, which was in line with the co-circulation of Group 2 and 3 in the current study (Yoshihara et al., 2018). Yamagata lineage may less likely be going through selection bottleneck event compared to Victoria lineage. Furthermore, the magnitude of EPS oscillation in Victoria lineage differed between seasons. For example, the EPS of Group 5, the dominant group in 2010–2011, was noticeably greater in comparison with Group 1 in 2012–2013. The viral evolution rate of Victoria lineage Groups may coincide with the EPS trend.

As limitation of the current study, the investigation period of post-A/H1N1pdm09 period was till 2013, which may have been short. In addition, we were not able to investigate the biological importance of different amino acid composition as well as positively selected amino acid substitutions on receptor binding preference, antigenicity, and evolutionary mechanism. Therefore future in vivo experiments are warranted. Furthermore, the biological role of HA glycoprotein N-glycosylation on influenza B strains needs to be investigated as its' critical role on host innate immune response and viral virulence of influenza A/H1N1pdm09 strain has been reported (Sun et al., 2013; York et al., 2019). Although, inter-lineage reassortment was seen only in one case, its biological role needs to be addressed in the future study as the previous study suggested its evolutionary importance on influenza B strains (Monamele et al., 2018). Therefore, it is important to further continue the influenza surveillance in the current study setting to investigate the lineage-specific circulation trend as well as the evolutionary dynamics of circulating influenza strains in Vietnam.

5. Conclusion

The faster evolutionary rate of influenza B *HA* gene implied the stronger immunological pressure on *HA* compared to *NA* gene. Our study highlights the difference in evolutionary dynamics between Victoria and Yamagata lineages as well as WHO Groups in Victoria lineage. Victoria lineage is likely to go through evolutionary bottleneck event from season to season whereas Yamagata lineage WHO Groups may co-circulate. Further strengthening of influenza surveillance system at community-level would be beneficial for the future vaccination program as well as gaining a better understanding of the molecular evolutionary aspects of circulating influenza strains in Vietnam.

Financial disclosure

The authors have no financial relationships relevant to this article to disclose.

Funding

This study was supported by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID)(10008012), Japan Agency for Medical Research and Development (AMED) (JP19fm0108001), and Grants-in-Aid for Scientific Research (Japan Society for the Promotion of Science). This study was supported in part by the Joint Usage / Research Center on Tropical Disease, Institute of Tropical Medicine, Nagasaki University, Japan.

The values of median *p*-distances between two independent groups were compared using Mann-Whitney *U* test: *p*-value (1) for *HA* gene Victoria and Yamagata lineages, *p*-value (2) for *HA* gene Victoria lineage WHO Group 1 and 5, *p*-value (3) for *NA* gene Victoria and Yamagata lineages and *p*-value (4) for *NA* gene Victoria lineage WHO Group 1 and 5.

Declaration of Competing Interest

The authors have declared no competing interests.

Acknowledgment

We would like to thank for medical doctors, nurses and laboratory technician in Khanh Hoa General Hospital for supportive technical assistance for patient's clinical data and samples collection and management. Also, we acknowledge staffs in Khanh Hoa Health Service for data entry procedure and management utilized in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2020.104264.

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