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GENETIC DIVERSITY AND EVOLUTION

Influenza A Viruses of Swine (IAV-S) in Vietnam from 2010 to 2015: Multiple Introductions of A(H1N1)pdm09 Viruses into the Pig Population and Diversifying Genetic Constellations of Enzootic IAV-S

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ABSTRACT Active surveillance of influenza A viruses of swine (IAV-S) involving 262 farms and 10 slaughterhouses in seven provinces in northern and southern Vietnam from 2010 to 2015 yielded 388 isolates from 32 farms; these viruses were classified into H1N1, H1N2, and H3N2 subtypes. Whole-genome sequencing followed by phylogenetic analysis revealed that the isolates represented 15 genotypes, according to the genetic constellation of the eight segments. All of the H1N1 viruses were entirely A(H1N1)pdm09 viruses, whereas all of the H1N2 and H3N2 viruses were reassortants among 5 distinct ancestral viruses: H1 and H3 triple-reassortant (TR) IAV-S that originated from North American pre-2009 human seasonal H1, human seasonal H3N2, and A(H1N1)pdm09 viruses. Notably, 93% of the reassortant IAV-S retained M genes that were derived from A(H1N1)pdm09, suggesting some advantage in terms of their host adaptation. Bayesian Markov chain Monte Carlo analysis revealed that multiple introductions of A(H1N1)pdm09 and TR IAV-S into the Vietnamese pig population have driven the genetic diversity of currently circulating Vietnamese IAV-S. In addition, our results indicate that a reassortant IAV-S with human-like H3 and N2 genes and an A(H1N1)pdm09 origin M gene likely caused a human case in Ho Chi Minh City in 2010. Our current findings indicate that human-to-pig transmission as well as cocirculation of different IAV-S have contributed to diversifying the gene constellations of IAV-S in Vietnam.

IMPORTANCE This comprehensive genetic characterization of 388 influenza A viruses of swine (IAV-S) isolated through active surveillance of Vietnamese pig farms from 2010 through 2015 provides molecular epidemiological insight into the genetic diversification of IAV-S in Vietnam after the emergence of A(H1N1)pdm09 viruses. Multiple reassortments among A(H1N1)pdm09 viruses and enzootic IAV-S yielded 14 genotypes, 9 of which carried novel gene combinations. The reassortants that carried M genes derived from A(H1N1)pdm09 viruses became predominant, replacing those of the IAV-S that had been endemic in Vietnam since 2011. Notably, one of the novel reassortants likely caused a human case in Vietnam. Given that Vietnam is the second-largest pig-producing country in Asia, continued monitoring of IAV-S is highly important from the viewpoints of both the swine industry and human public health.

KEYWORDS Vietnam, influenza, phylogeny, pig, reassortment

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Influenza A viruses (IAV) are respiratory pathogens of vertebrates, including birds, humans, pigs, and other mammals (1). Host glycans with terminal sialic acid are the receptors for hemagglutinin (HA) proteins of influenza A virus. Whereas avian viruses prefer α2-3-linked sialic acid, which is found mainly on the duck intestine, human viruses prefer α2-6-linked sialic acid, which is expressed predominately on the human tracheal epithelial cells (2–4). In contrast, the epithelial cells of the upper and lower trachea of pigs carry both α2-3- and α2-6-linked sialic acid receptors for influenza virus (3, 5). Domestic pigs are therefore considered to be an intermediate host for influenza and a "mixing vessel" in which viruses with novel genetic constellations are generated. Influenza A viruses of swine (IAV-S) do not usually infect humans, but a novel reassortant virus generated from pigs would pose a public health concern because it could be capable of infecting and causing a pandemic in the human population. Therefore, monitoring swine influenza is important for public health reasons as well as to avoid economic loss.

IAV-S can be divided genetically into several distinct lineages. Each lineage of IAV-S has become enzootic in a particular region or country, such as North America, Europe, and Japan, because the movements of pigs are limited. Historically, IAV-S were first recognized in 1918 in the United States and were thought to be related to the human pandemic Spanish flu (6). Designated the classical swine (CS) H1N1 influenza virus, the descendant of the 1918 pandemic virus in pigs has spread worldwide from the United States to Asia since the 1970s (7) and to Europe since the 1950s (8). In 1979, IAV-S that originated from an avian influenza virus emerged in Belgium and has established a distinct lineage as European avian-like (EA-like) IAV-S in European countries. EA-like IAV-S and its reassortant with a human seasonal influenza virus have been endemic in Europe since the 1980s (9). Thereafter, EA-like IAV-S have spread to Thailand (10) and China (11, 12).

In 1998, a novel H3N2 IAV-S, which later was designated a triple reassortant (TR), emerged in the United States. The NP, M, and NS genes of the TR viruses were those of CS influenza virus; their PB1, hemagglutinin (HA), and neuraminidase (NA) genes derived from a human seasonal influenza virus; and the PB2 and polymerase acidic (PA) genes originated from an avian influenza virus (13). The H3N2 TR virus and its H1N2 reassortant with CS or human-seasonal H1 influenza virus have since been predominant in the United States (14, 15). As these viruses have continued to evolve, four clusters (α, β, γ, and δ) of the H1 TR IAV-S and four clusters (I to IV) of the H3 TR IAV-S have emerged (16, 17). The H1 genes of the α, β, and γ clusters evolved from the CS virus and that of the δ cluster derived from the human seasonal influenza viruses circulating during 2003 (18). TR IAV-S have spread to China (19) and Korea (20) but not to Europe thus far (21).

In 2009 in California, USA, a boy with asthma developed fever, cough, and vomiting; the novel H1N1 influenza virus isolated was a reassortant between a North American TR virus and EA-like IAV-S. This reassortant was designated "pandemic A(H1N1)2009 virus" [A(H1N1)pdm09v] and spread rapidly among humans to ultimately affect 214 countries and regions. Nearly 19,000 laboratory-confirmed deaths occurred in the first 14 months since its recognition in 2009 (WHO [http://www.who.int/csr/don/2010_08_06/en/]). Since its emergence in humans, unlike pre-2009 human seasonal H1 and H3 viruses, A(H1N1)pdm09v strains have frequently been transmitted back to pigs worldwide (22, 23).

Genetic studies since 2009 have revealed the particular characteristics of IAV-S in many countries (12, 24–28) but not Vietnam. Our previous study demonstrated that an IAV-S isolated in southern Vietnam, A/swine/Binh Duong/03-06/2010 (H3N2), was a reassortant between a human H3N2 virus circulating from 2004 to 2006 and a TR IAV-S (29). Specifically, the HA and NA genes of the Vietnamese reassortant originated from the human virus and its internal genes derived from the TR IAV-S, thus suggesting human-to-pig transmission and incursion of North American TR IAV-S into Vietnam. In addition, a novel reassortant between the TR IAV-S and A(H1N1)pdm09v was reported in northern Vietnam in 2013 (30).

To reveal the genetic characteristics of Vietnamese IAV-S, we performed active virological surveillance in northern and southern Vietnam between 2010 and 2015. The surveillance yielded a total of 388 IAV-S isolates. Phylogenetic analysis of these isolates revealed the diverse genetic constellations in Vietnamese IAV-S, which resulted from multiple introductions of A(H1N1)pdm09v, human seasonal viruses, and TR IAV-S from other countries. Our findings contribute to a fundamental understanding of IAV-S and fill information gaps regarding Southeast Asian IAV-S. In addition, these data will facilitate the early detection of IAV-S infection in humans.

RESULTS

Genotypic characterization of Vietnamese IAV-S. During active surveillance from 2010 to 2015 in Vietnam, we isolated 388 IAV-S from 32 pig farms in 7 provinces (Table 1; see also Table S1 in the supplemental material). Three subtypes—H1N1, H1N2, and H3N2—were endemic in both northern and southern Vietnam. After whole-genome sequencing followed by phylogenetic analysis, these 388 Vietnamese IAV-S were grouped into 15 genotypes (designated 1 through 15) according to their gene combinations (Fig. 1; Table S1). The genetic origins of each segment represented a maximum of 6 lineages: A(H1N1)pdm09, H1-TR, pre-2009 human-like H1, human-like H3, and H3-TR. In addition, the H3-TR internal genes formed two distinct phylogenetic clusters: the NV cluster of viruses, circulating in northern Vietnam, and the SV cluster, in southern Vietnam (Fig. 1). All of the gene segments in all 92 H1N1 IAV-S were entirely derived from A(H1N1)pdm09v (genotype 1), whereas all of the genotypes among the 97 H1N2 viruses (genotypes 2 through 10) and the 199 H3N2 IAV-S (genotypes 11 through 15) were reassortants (Fig. 1). Genotypes 1 and 8 were isolated in both northern and southern Vietnam, whereas the remaining genotypes were prevalent in either northern or southern Vietnam (Fig. 1 and Table 1).

Analyzing the genetic origin of each segment retained among the 296 reassortant H1N2 and H3N2 IAV-S revealed several preferred gene combinations (Fig. 2a). For example, 93% and 80% of the reassortants retained M and NP genes, respectively, derived from A(H1N1)pdm09 (pdm) (Fig. 2a). Acquisition of pdm-M and pdm-NP genes sharply increased in 2011 and 2012, respectively, whereas all of the reassortants isolated in 2010 possessed TR-M and TR-NP genes (Fig. 2b and c). Consequently, more than 98% and 88% of the reassortants isolated in 2015 contained pdm-M and pdm-NP genes, respectively. In contrast, only 2.0% of reassortants had pdm-H1 genes, and no viruses with pdm-N1 were isolated. In addition, reassortants with TR-PB1 genes were isolated more frequently than were those with pdm-PB1, about 30% of the reassortants retained HA and NA genes from human seasonal H3N2 viruses, and 5.1% had pre-2009 human-like H1 genes (Fig. 2a). However, neither reassortants with pre-2009 N1 nor internal genes from human pre-2009 H1 or H3 viruses were ever isolated during our surveillance.

Phylogenetic analyses. (i) pdm-H1 viruses (genotypes 1 and 2). In the phylogenetic tree for the pdm-H1 genes of the isolates composed entirely of A(H1N1)pdm09 segments (genotype 1), 7 virus groups—designated BV01/13, TG02-17-1/12, BV02/11, ND-V/11, TG02-25-1/11, ND11/10, and BD01/10—formed independent clusters with greater than 99% posterior support, except for TG02-25-1/11 (Fig. 3a). Whereas each of the 7 virus groups was detected once during our surveillance, another four virus groups—BN1/14, BN111/14, BN13-2/15, and BN11-3/15—formed a single cluster, that is, the Bac Ninh A(H1N1)pdm09 cluster (Fig. 3a), and were detected at 4 different sampling dates. When we estimated the divergence times from the most recent human A(H1N1)pdm09v for all eight gene segments of Vietnamese swine A(H1N1)pdm09v (genotype 1), all of the segments in each group, except for the NP genes of TG02-07-1/12 and the Bac Ninh A(H1N1)pdm09 cluster and the M genes of BN111/14, diverged from one ancestral human A(H1N1)pdm09v around the same time (Fig. 3b). For example, all of the segments of the ND-V/11 group were estimated to be diverged from a human ancestral virus circulating around 2010 (Fig. 3b; Table 2). These findings indicate that multiple independent introductions of A(H1N1)pdm09v had occurred in

TABLE 1 Summary of information regarding viruses isolated at each pig farm

Farm ID	Virus group abbreviation	Subtype	Genotype No.	No. of viruses isolated during each surveillance round and year[a]													Number of specimens collected at each round
				1	2	3	4	5	6	7	8	9	10	11	12	13	
				2010		2011			2012		2013		2014	2015			
Farms in Nam Dinh province																	
ND11	ND11/10	H1N1	1	5													17
ND31	ND-V/11	H1N1	1			3											17
ND37	ND-V/11	H1N1	1			1											17
ND38	ND-V/11	H1N1	1			5											17
Farms in Bac Ninh province																	
BN11	BN1/14	H1N1	1						-				1	-	-	5	30
	BN11-3/15																
	BN102/12	H3N2	14						3				-	-	-	-	
	BN243/15		15						-				-	12	-	-	
BN12	BN11-3/15	H1N1	1						-				-	-	-	20	30
	BN102/12	H3N2	14						3				-	-	-	-	
BN13	BN13-2/15	H1N1	1									-	-	-	3	-	30
	BN354/13	H1N2	9									1	-	-	-	-	
	BN102/12	H3N2	14						24			-	-	-	-	-	
	BN346/13		15									5	-	-	-	-	
BN25	BN11-3/15	H1N1	1							-			-	-	-	5	30
	BN344/12	H1N2	8							9			-	-	-	-	
BN43	BN111/14	H1N1	1									-	1	-	-	-	30
	BN388/13	H1N2	8									1	-	-	-	-	
	BN243/15 BN43-2/15 BN43-3/15	H3N2	15									-	-	15	1	23	
BN26	BN11-3/15	H1N1	1							-		-	-	-	-	1	30
	BN386/12	H3N2	14							2		-	-	-	-	-	
BN14	BN242/14	H3N2	15										13	-	-	-	30
BN40	BN11-3/15	H1N1	1										-	-	-	4	30
	BN43-3/15	H3N2	15										-	-	-	8	
BN41	BN11-3/15	H1N1	1										-	-	-	7	30
BN42	BN11-3/15	H1N1	1										-	-	-	3	30
BN44	BN242/14	H3N2	15										10	-	-	-	30
Farms in Binh Duong province																	
BD01	BD01/10	H1N1	1	7		-											25
BD02	BD02/10	H1N2	10	1		-											25
BD03[b]	BD03/10	H3N2	11	6		-											25
Farms in Ba Ria Vung Tau province																	
BV01	BV01/13	H1N1	1				-	-	-	-	2	-	-	-	-	-	30
BV02	BV02/11	H1N1	1				-	14	-	-	-	-	-	-	-	-	30
	BV02/15	H1N2	6				-	-	-	-	-	-	-	7	-	-	
Farm in Ho Chi Minh City																	
HCM01	HCM01/11	H3N2	12				-	14	-	-	-	-					30
Farms in Dong Nai province																	
DN03	DN03/11	H1N2	3				-	6									30
DN07	DN07-1/12 DN07-2/12 DN07-01-2/13	H3N2	13						4	6	-	5	-	-	-	-	30
	DN07-10-2/13	H1N2	4						-	-	-	2	-	-	-	-	
	DN07/14		8						-	-	-	-	1	-	-	-	
	DN07-1/15 DN07-3/15		7						-	-	-	-	-	-	3	22	
DN10	DN10/13	H3N2	13						-	-	3	-					13
DN08	DN08-1/15	H3N2	13										-	8	-	-	30
	DN08-2/15	H1N2	7										-	-	5	-	30
DN09	DN09/14	H3N2	13										10	-	-	-	30
	DN09-06-1/15		12										-	3	-	-	
	DN09-16-1/15	H1N2	8										-	2	-	-	
DN12	DN12/15	H3N2	13										-	-	-	15	30
Farms in Tien Giang province																	
TG01	TG01/11	H1N2	5				-	10	-	-							30
	TG01/12		4				-	-	13	-							
TG02	TG02-25-1/11 TG02-17-1/12	H1N1	1				1	-	4	-							30
	TG02-2/11	H3N2	12				-	6	-	-							
	TG02-1/12 TG02-2/12	H1N2	2				-	-	4	1							
TG03	TG03/13	H1N2	2						-	-	1						13
TG07	TG07/15	H1N2	6										-	2	-	-	30
TG08	TG08/14	H1N2	4										6	-	-	-	30

[a]Shading indicates that no specimens were collected; —, no isolates obtained. Surveillance rounds by date: 1, March 2010; 2, August or December 2010; 3, February or March 2011; 4, August 2011; 5, December 2011; 6, July or September 2012; 7, December 2012; 8, July 2013; 9, December 2013; 10, July 2014; 11, February 2015; 12, July 2015; 13, December 2015.

[b]Reported by Ngo et al. (29).

Genotype No.	Subtype	HA	NA	PB2	PB1	PA	NP	M	NS	Number of isolates	Virus groups	Region
1	H1N1									92	BD01/10, ND11/10, ND-V/11, TG02-25-1/11, BV02/11 , TG02-17-1/12, BV01/13, BN1/14, BN111/14,BN13-2/15, BN11-3/15	North and South
2	H1N2									6	TG02-1/12, TG02-2/12, TG03/13	South
3	H1N2									6	DN03/11	South
4	H1N2									21	TG01/12, DN07-10-2/13, TG08/14	South
5	H1N2									10	TG01/11	South
6	H1N2									9	TG07/15, BV02/15	South
7	H1N2									30	DN07-1/15, DN07-3/15, DN08-2/15	South
8	H1N2									13	BN344/12, BN388/13, DN07/14, DN09-16-1/15	North and South
9	H1N2									1	BN354/13	North
10	H1N2									1	BD02/10	South
11	H3N2									6	BD03/10	South
12	H3N2									23	TG02-2/11, HCM01/11, DN09-06-1/15	South
13	H3N2									51	DN07-1/12, DN07-2/12, DN07-01-2/13, DN10/13, DN08-1/15, DN09/14, DN12/15	South
14	H3N2									32	BN102/12, BN386/12	North
15	H3N2									87	BN346/13,BN242/14, BN243/15, BN43-2/15, BN43-3/15	North

FIG 1 The 15 genotypes identified among Vietnamese IAV-S from 2010 to 2015. The color of the block denotes the virus lineage from which the gene segment originated. Green, A(H1N1)pdm09v; pink, H1 triple reassortant (TR) (C-TR cluster); orange, H3N2 TR circulating in northern Vietnam (NV cluster); blue, TR circulating in southern Vietnam (SV cluster); red, seasonal H3N2 human-like virus; purple, H1 pre-2009 human-like virus. The geographic region (northern or southern Vietnam) from which each genotype was isolated is shown in the rightmost column.

the Vietnamese pig population. Of these introduced viruses, only Bac Ninh A(H1N1)pdm09 cluster viruses had been maintained in the pig population for more than 2 years (Fig. 3a and b).

Notably, the NP genes of TG02-17-1/12 and the Bac Ninh A(H1N1)pdm09 cluster shared common ancestors with other genotypes (Fig. 4). TG02-17-1/12 belonged to a large cluster containing genotypes 4, 7, and 13 and Chinese reassortant IAV-S (Fig. 4). Bac Ninh A(H1N1)pdm09 cluster viruses acquired their NP genes from genotype 15 (Fig. 4), and BN111/14 viruses acquired M genes from Bac Ninh reassortant IAV-S, which have the same genotype (no. 15) as the source of the pdm-NP genes (Fig. 4; see also Fig. S1 in the supplemental material). These results suggest that TG02-07-1/12 and the Bac Ninh A(H1N1)pdm09 cluster viruses might be reassortants between Vietnamese reassortant IAV-S and A(H1N1)pdm09v, although their genomes overall are from A(H1N1)pdm09v.

Genotype 2 IAV-S—represented by TG02-1/12, TG02-2/12, and TG03/13—contained pdm-H1 PB2, PB1, PA, M, and NS, human-like N2, and TR-NP genes (Fig. 1). In particular, the H1 genes of genotype 2 formed a distinct cluster from other Vietnamese pdm-H1 genes (Fig. 3) and occurred only in southern Vietnam. TG02-1/12 and TG02-2/12 were isolated at the same farm in September and December 2012, respectively. In addition, the farm at which TG03/13 was isolated in July 2013 was only 4 km from the farm that gave rise to the TG02 viruses. Therefore, genotype 2 viruses had circulated for at least 10 months without further reassortment, but whether viral transmission from farm TG02 to farm TG03 occurred or whether the same viruses had been prevalent in the two areas is unclear.

(ii) TR-H1 viruses (genotypes 3 through 7). Viruses of genotypes 3 through 7 had TR-H1 genes and clustered with the IAV-S isolated in Guangxi, China: A/swine/Guangxi/

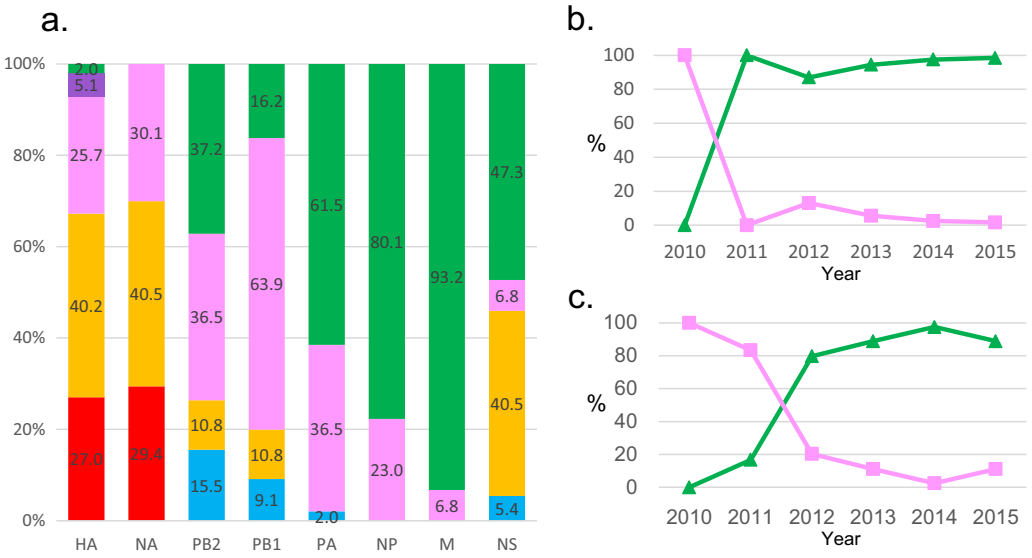

FIG 2 (a) Contributions (%) of various genetic lineages to gene segments retained in the 296 reassortant IAV-S isolated in Vietnam during 2010 through 2015; the color code is that described for Fig. 1. (b) Frequencies of A(H1N1)pdm09-origin (green) and TR-origin (pink) M genes among the 296 reassortant IAV-S isolated in Vietnam during 2010 through 2015. (c) Frequencies of A(H1N1)pdm09 origin (green) and TR origin (pink) NP genes among the 296 reassortant IAV-S isolated from 2010 through 2015 in Vietnam.

3075/2011 (H1N2) and A/swine/Guangxi/2887/2011 (H1N2) (that is, the C-TR cluster) (Fig. 5). The C-TR cluster diverged from southern Chinese H1N2 IAV-S, which originated from H1-TR viruses circulating in North America. A putative common ancestor of the C-TR cluster was estimated to have diverged between April 1999 and December 2000 (95% highest posterior density interval [HPD] calculated from H1 gene data; Table 2). In addition, the N2 genes of genotypes 3 through 7 similarly derived from the Chinese H1N2-TR group, with phylogenetic topology similar to that of the HA phylogeny (see Fig. S2 in the supplemental material). Viruses with a gene highly similar to the Vietnamese H1 TR origin HA gene have not yet been isolated except in China, suggesting that circulation of this sublineage was restricted to this region or to an area with incomplete virological surveillance.

The PB2 genes of genotypes 3 through 6 formed a cluster that had been circulating in southern Vietnam (the SV-TR cluster), but the origin of the PB2 segment likely differed from the previously described southern Chinese H1N2 IAV-S group that gave rise to the HA and NA genes (Fig. 6). The SV-TR cluster is closely related to North American cluster IV H3N2 TR, such as A/swine/Oklahoma/SG1473/2005 (H3N2). As seen from the PB2 phylogeny, all of the SV-TR genes (that is, PB1 genes of genotypes 3 and 4, PA genes of genotype 3, and NS genes of genotypes 3 and 5) were very closely related to North American TR viruses (see Fig. S3 for TR-PB1 phylogeny, Fig. S4 for TR-PA phylogeny, and Fig. S5 for TR-NS phylogeny in the supplemental material). The NP genes of genotypes 5 and 6 and the PB1 segment of genotype 7 were derived from the Chinese H1N2 TR group (that is, the C-TR cluster; see Fig. S6 and S3 in the supplemental material), as seen in the HA and NA phylogenies. The estimated time of divergence from a common ancestor differed markedly between the SV-TR and C-TR clusters: the C-TR cluster diverged between May 1998 and February 2001, and the SV-TR diverged between October 2004 and August 2005 (Table 2).

The remaining segments of genotypes 3 through 7 were derived from A(H1N1) pdm09v (Fig. 1). The pdm-PB1 genes of genotype 5 were closely related to those of genotypes 2 and 12 (described below), whose cluster diverged from human A(H1N1)pdm09v in 2009 (see Fig. S7 in the supplemental material). The pdm-PA genes of genotype 5 clustered with the human A(H1N1)pdm09v that circulated in early 2010 (95% HPD, October 2009 to January 2010; Table 2), whereas those of genotypes 4 and

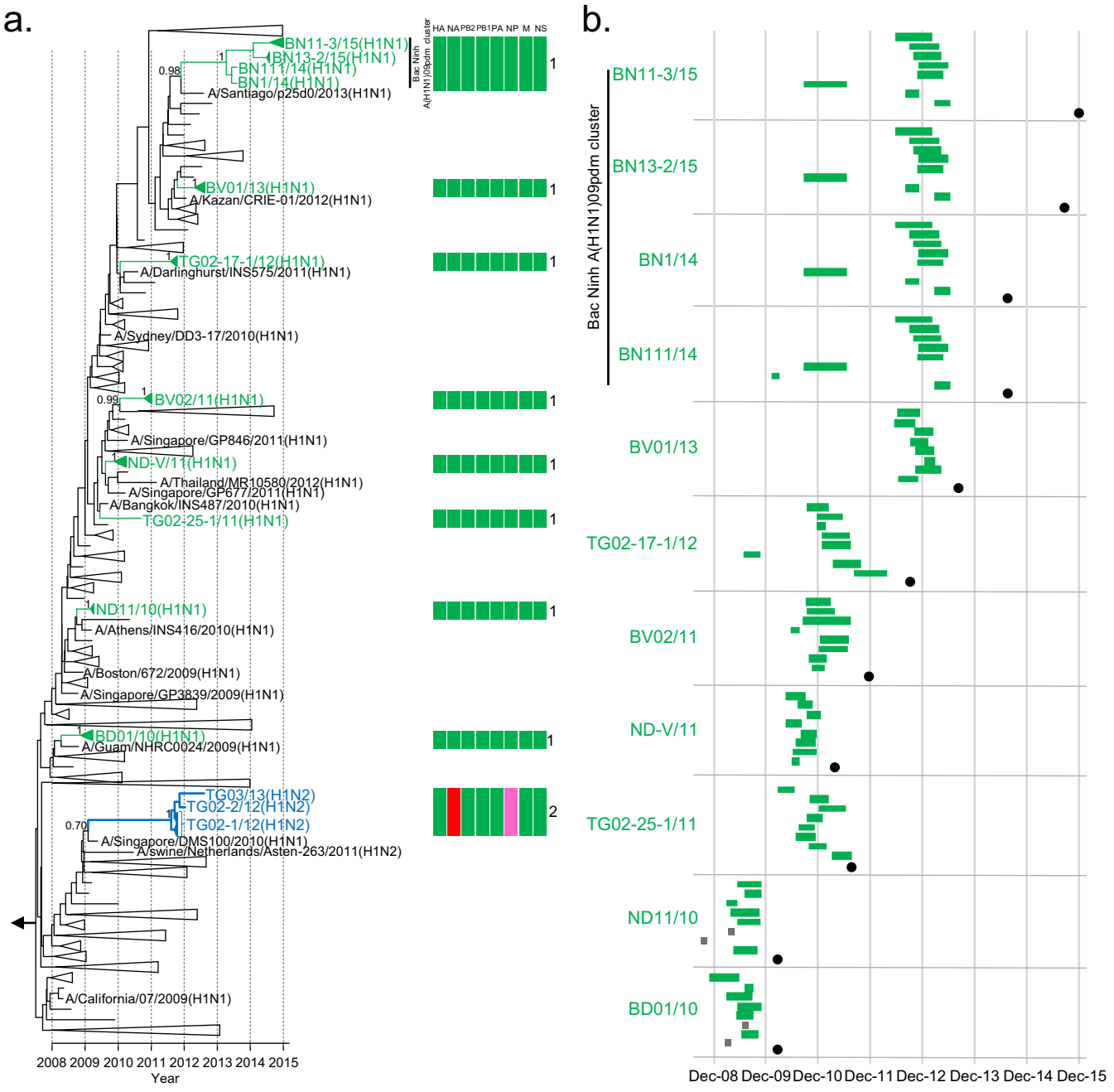

FIG 3 (a) Maximum clade credibility phylogenetic tree of A(H1N1)pdm09 origin HA genes. The names of the Vietnamese IAV-S (virus group) isolated in this study are colored according to the isolate's genotype. At the right of the tree, the phylogenetic origin of each segment (depicted as a square) is indicated according to the color code described for Fig. 1, with the isolate's genotype number beside the segment origin map. Posterior probabilities of >0.7 are provided for key nodes. (b) Divergence time from the most recent human A(H1N1)pdm09v in each segment of Vietnamese swine A(H1N1)pdm09v (genotype 1). Each column shows the 95% highest posterior density interval (HPD) for time of divergence from the most recent ancestral human A(H1N1)pdm09 virus; in order from the top, HA, NA, PB2, PB1, PA, NP, M, NS. When the 95% HPD could not be calculated because of a low posterior value at the node from the ancestral viruses, the time of divergence was estimated from the distance to the node and indicated in gray bars. The sampling date in each group is indicated by a black dot.

7 formed a large cluster with Chinese reassortant IAV-S (see Fig. S8 in the supplemental material). This large cluster seemed to originate from human A(H1N1)pdm09v circulating in 2009 (95% HPD, July to August 2009 [Table 2]). In addition, the pdm-PA genes of genotype 6 formed a Vietnamese IAV-S cluster with TG02-17-1/12 (genotype 1), and DN12/15 viruses (genotype 13, described below [Fig. S8]) were estimated to arise from human A(H1N1)pdm09v circulating in 2009 (95% HPD, January to August 2011 [Table 2]). This result suggested that the PA genes of genotypes 4 and 7, 5, and 6 originated

TABLE 2 Estimated divergence time from the common ancestor of each virus group[a]

Genotype	Virus group	Subtype	Divergence period for each segment							
			HA	NA	PB2	PB1	PA	NP	M	NS
1	BD01/10	H1N1	2008/11/25 ~2009/6/23	2009/7/24 ~9/25	2009/3/21 ~9/24	2009/6/6 ~11/21	2009/5/29 ~9/25	2009/7/6[b]	2009/7/6 ~11/4	2009/2/25
	ND11/10		2009/6/6 ~11/26	2009/7/24 ~11/26	2009/3/25 ~6/5	2009/4/22 ~11/9	2009/6/10 ~11/15	2009/3/17	2008/8/25	2009/5/9 ~10/26
	ND-V/11		2010/5/13 ~10/1	2010/8/6 ~11/20	2010/10/9 ~2011/1/12	2010/5/14 ~8/29	2010/8/23 ~12/13	2010/7/20 ~12/11	2010/6/28 ~12/16	2010/6/24 ~8/16
	TG02-25-1/11		2010/3/16 ~7/14	2010/10/24 ~2011/3/12	2010/12/27 ~2011/7/5	2010/10/5 ~2011/1/26	2010/8/9 ~12/2	2010/7/20 ~12/11	2010/10/20 ~2011/2/23	2011/3/29 ~8/15
	BV02/11		2010/9/30 ~2011/3/23	2010/10/9 ~2011/4/23	2010/9/5 ~2011/8/8	2010/6/12 ~8/17	2011/1/6 ~7/27	2010/12/28 ~2011/7/23	2010/10/20 ~2011/2/23	2010/11/13 ~2011/2/6
	TG02-17-1/12		2010/10/4 ~2011/3/7	2010/12/13 ~2011/6/19	2010/12/14 ~2011/2/17	2011/1/19 ~8/7	2011/1/21 ~8/10	2009/7/23 ~11/13	2011/4/4 ~10/21	2011/9/2 ~2012/4/23
	BV01/13		2012/7/3 ~12/6	2012/6/5 ~11/4	2012/10/27 ~2013/3/8	2012/9/26 ~2013/1/31	2012/11/5 ~2013/3/17	2013/1/7 ~3/25	2012/11/6 ~2013/5/5	2012/7/10 ~11/21
	BN111/14		2012/6/15 ~2013/3/2	2012/9/23 ~2013/4/21	2012/10/17 ~2013/5/5	2012/11/23 ~2013/6/26	2012/11/14 ~2013/5/18	2010/9/17 ~2011/7/16	2010/2/3 ~4/2	2013/3/19 ~7/7
	BN1/14								2012/8/28 ~12/3	
	BN13-2/15									
	BN11-3/15									
2	TG02-1/12, TG02-2/12, TG03/13	H1N2	2009/10/1 ~2010/3/25	2003/7/29 ~2004/3/6	2009/8/22 ~11/24	2009/10/23	2009/7/3 ~10/13	1998/4/18 ~2000/6/16	2009/4/19 ~9/10	2009/12/3 ~2010/4/24
3	DN03/11		1999/4/18 ~2000/12/30	1998/7/23 ~2000/7/17	2004/10/11 ~2005/8/5	2004/8/24 ~2005/10/13	2004/1/22 ~2005/7/16	2009/7/16 ~2010/1/15		2003/12/18 ~2005/6/9
4	TG01/12, DN07-10-2/13, TG08/14						2009/7/8~8/24	2009/7/23 ~11/13		2009/5/28 ~9/9
5	TG01/11					2009/10/23	2009/10/18 ~2010/1/6	1998/4/18 ~2000/6/16		2003/12/18 ~2005/6/9
6	TG07/15, BV02/15					2011/1/19 ~8/7	2011/1/21 ~8/10		2011/4/4 ~10/21	2011/9/2 ~2012/4/23
7	DN07-1/15, DN08-2/15				2009/8/22 ~11/24	1999/6/3 ~2000/9/1	2009/7/8 ~8/24	2009/7/23 ~11/13	2009/4/19 ~9/10	2009/8/10 ~10/27
	DN07-3/15				2010/12/14 ~2011/2/17					
8	BN344/12, BN388/13, DN07/14, DN09-16-1/15		2004/11/12 ~2005/8/15	2010/12/22 ~2011/11/3	1999/3/11 ~2000/11/24		1998/6/6 ~2000/1/29	1998/4/18 ~2000/6/16	1998/7/18 ~2001/2/26	1996/11/5 ~2000/6/8
9	BN354/13							2010/9/17 ~2011/7/16	2010/2/3 ~4/2	2006/10/10 ~2007/9/28
10	BD02/10							1998/4/18 ~2000/6/16	1998/7/18 ~2001/2/26	1996/11/5 ~2000/6/8
11	BD03/10									
12	TG02-2/11, HCM01/11		2004/4/26 ~2005/1/16	2003/7/29 ~2004/3/6	2009/8/22 ~11/24	2009/10/23	2009/7/3 ~10/13		2009/4/19 ~9/10	2009/12/3 ~2010/3/24
	DN09-06-1/15								2010/2/3 ~4/2	
13	DN07-1/12, DN07-2/12, DN07-01-2/13, DN10/13,DN08-1/15, DN09/14	H3N2				1999/6/3 ~2000/9/1	2009/7/8 ~8/24	2009/7/23 ~11/13	2009/4/19 ~9/10	2009/8/10 ~10/27
	DN12/15				2010/12/14 ~2011/2/17		2011/1/21 ~8/10		2011/4/4 ~10/21	2011/9/2 ~2012/4/23
14	BN102/12, BN386/12		2010/11/16 ~2011/9/19	2010/12/22 ~2011/11/3	2006/1/7 ~2007/2/9	2005/4/10 ~2006/12/15	2009/1/14 ~9/7	2010/9/17 ~2011/7/16	2010/2/3 ~4/2	2006/10/10 ~2007/9/28
15	BN346/13,BN242/14, BN243/15, BN43-2/15, BN43-3/15				1999/3/11 ~2000/11/24	1999/6/3 ~2000/9/1	1998/6/6 ~2000/1/29			

[a]The divergence time from the most recent human isolate was calculated for A(H1N1)pdm09 and human origin segments, and that from the Chinese or North American triple reassortant clusters was calculated for TR origin segments.

[b]When the 95% highest posterior density interval could not be calculated because of a low posterior value at the node from the ancestral viruses, the time was estimated from the distance to the node.

from different introductions of human A(H1N1)pdm09v. As seen for the pdm-PA genes, the pdm-NP genes of genotypes 4 and 7 formed a large cluster with Chinese reassortant IAV-S, whereas genotype 3 formed a distinct cluster with swine A(H1N1)pdm09v in Japan and North America (Fig. 4).

Finally, the pdm-M genes of genotypes 3 through 5 and 7 formed a single large cluster with other southern Vietnamese IAV-S (genotypes 2, 12, and 13) and swine

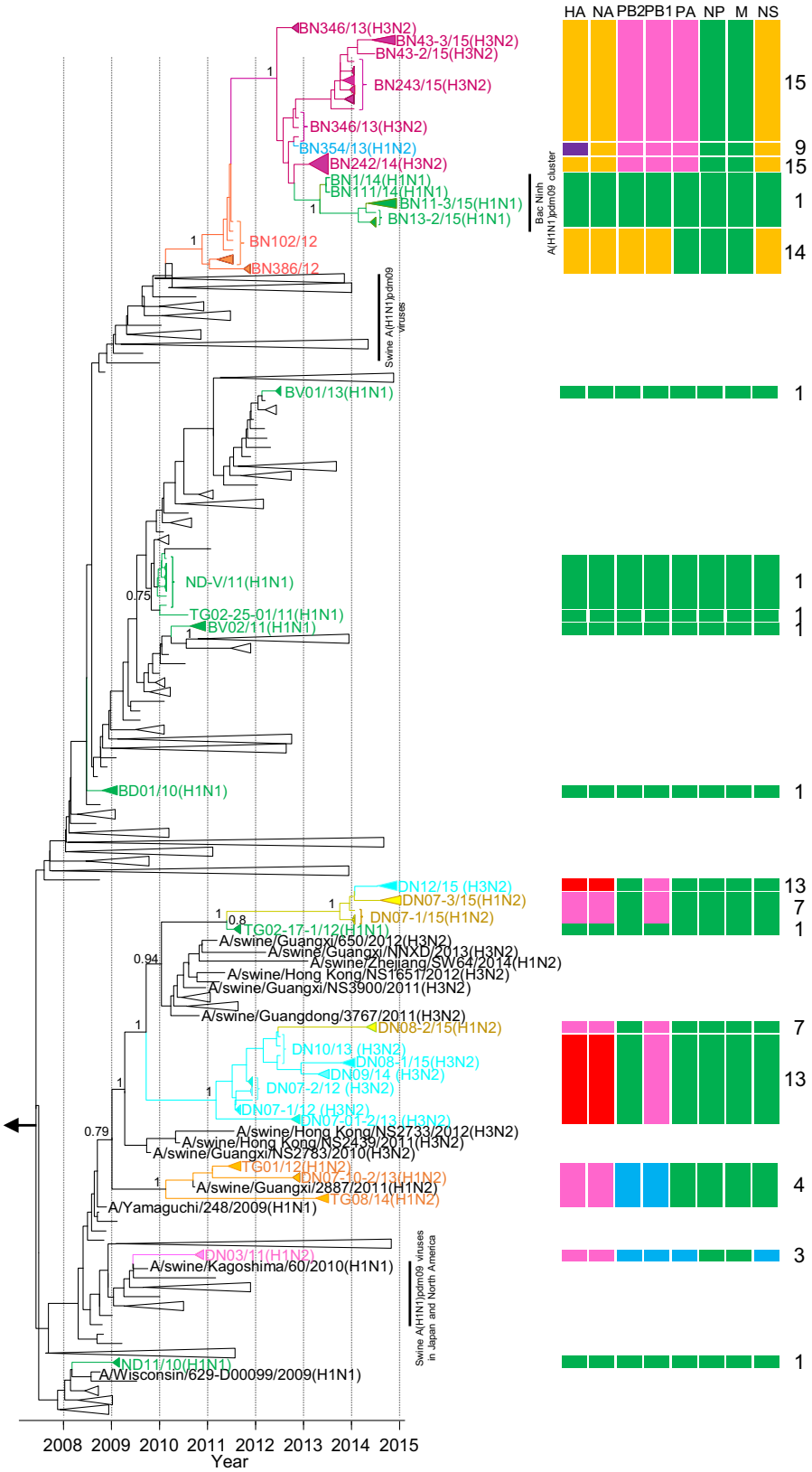

FIG 4 Maximum clade credibility phylogenetic tree of NP genes of A(H1N1)pdm09v. The names of the Vietnamese IAV-S (virus group) isolated in this study are colored according to the isolate's genotype. At the right of the tree, the phylogenetic origin of each segment (depicted as a square) is indicated according to the color code described for Fig. 1. The isolate's genotype number is given next to the segment origin map. Posterior probabilities of >0.7 are provided for key nodes.

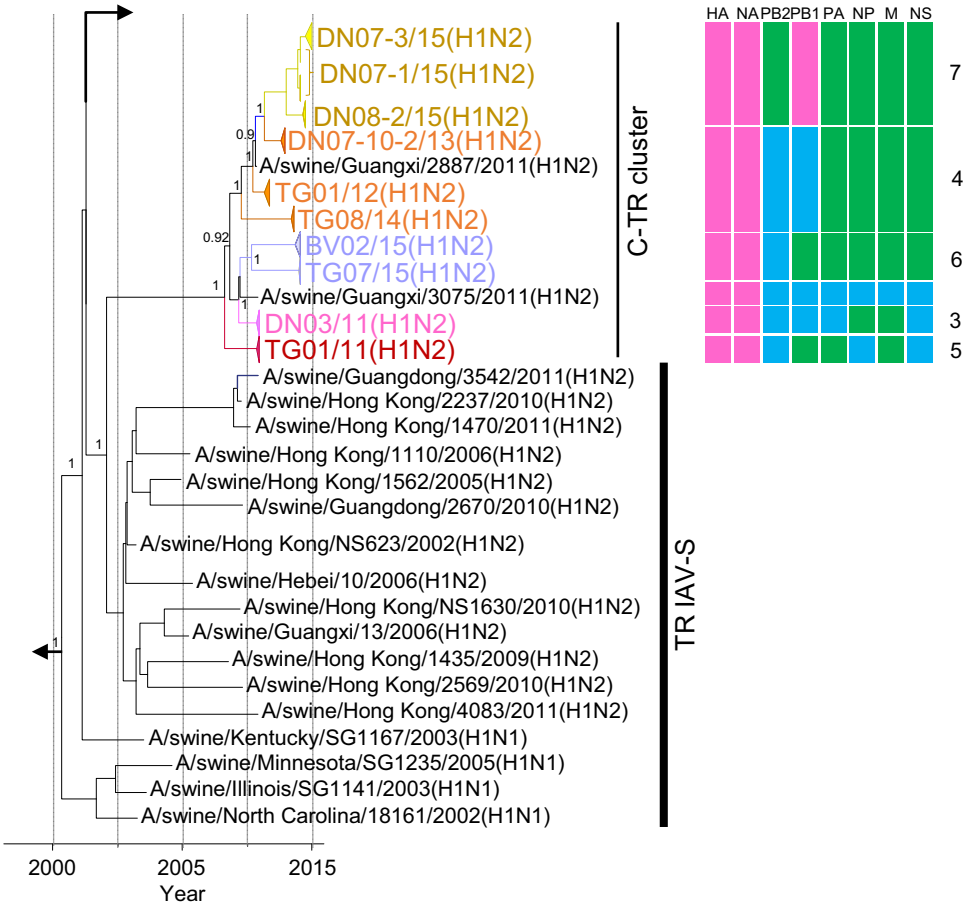

FIG 5 Maximum clade credibility phylogenetic tree of HA genes of H1 triple-reassortant (TR) IAV-S isolated in Southern Vietnam. The names of the Vietnamese IAV-S (virus group) isolated in this study are colored according to the isolate's genotype. At the right of the tree, the phylogenetic origin of each segment (depicted as rectangles) is indicated according to the color code described for Fig. 1, with the isolate's genotype number beside the segment origin map. Posterior probabilities of >0.7 are provided for key nodes.

A(H1N1)pdm09v from China and North America (Fig. S1). The ancestor of the large M-gene cluster was estimated to have diverged from human A(H1N1)pdm09v circulating between April and September 2009 (95% HPD [Table 2]).

(iii) Pre-2009 human-like H1 viruses (genotypes 8 through 10). The HA genes of genotypes 8, 9, and 10 were derived from pre-2009 human seasonal H1 viruses and formed a single cluster in the human lineage (Fig. 7). This cluster consists exclusively of Vietnamese IAV-S so far. Calculation of the time of divergence from the ancestral human seasonal H1N1 virus suggested that human-to-pig transmission occurred between September 2004 and August 2005 (95% HPD [Table 2]). A pre-2009 H1 virus (genotype 10) was first isolated in March 2010 in Binh Duong, southern Vietnam, after which various genotype 8 viruses were isolated in 2012 and 2013 at different farms in Bac Ninh, northern Vietnam (Table 1). Genotype 8 is the only reassortant found in both north and south Vietnam that has not reassorted with A(H1N1)pdm09v after 2010.

The N2 genes of genotypes 8 through 10 had different origins. Those of genotypes 8 and 9 arose from TR-N2 but belonged to two distinct clusters, with genotype 8 belonging to the C-TR cluster but genotype 9 belonging to the cluster IV TR viruses circulating in the North American pig population, such as A/swine/Manitoba/SG1431/2008 (H3N2) (NV-TR cluster [Fig. S2]). The NV-TR cluster of N2 genes was formed by other northern Vietnamese IAV-S (genotypes 14 and 15; described below). In comparison, genotype 10 had a human-like N2 gene and formed a single cluster with other southern Vietnamese IAV-S (genotypes 2 and 11 through 13, described below) and

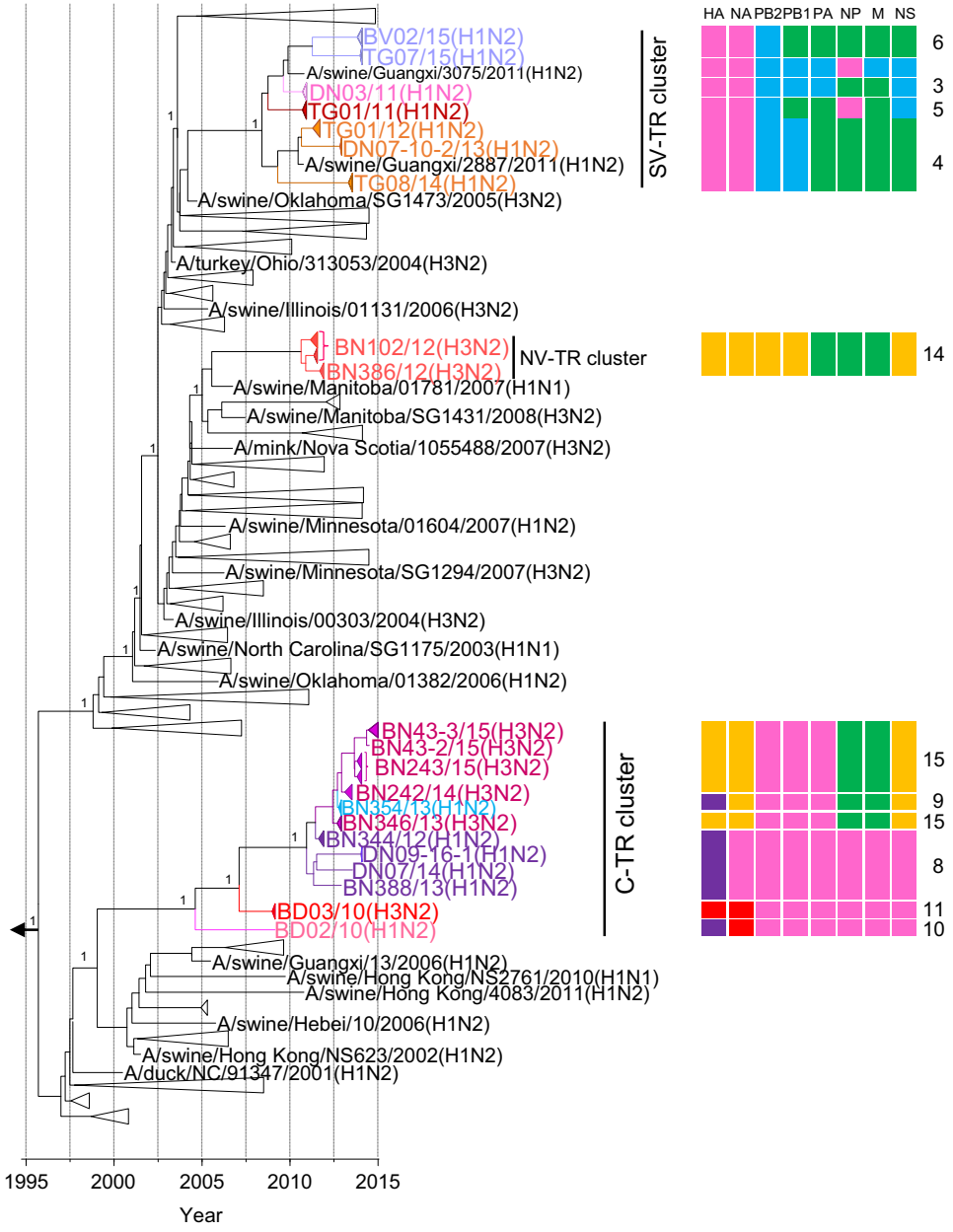

FIG 6 Maximum clade credibility phylogenetic tree of PB2 genes of triple-reassortant (TR) IAV-S. The names of the Vietnamese IAV-S (virus group) isolated in this study are colored according to the isolate's genotype. At the right of the tree, the phylogenetic origin of each segment (depicted as rectangles) is indicated according to the color code described for Fig. 1, with the isolate's genotype number beside the segment origin map. Posterior probabilities of >0.7 are provided for key nodes.

Chinese IAV-S in Hong Kong and Guangxi (see Fig. S9 in the supplemental material). All of the internal genes of genotypes 8 and 10 and the polymerase (PB2, PB1, and PA) genes of genotype 9 belonged to the C-TR cluster (Fig. 1 and 6; see also Fig. S2 through S6 and S10 in the supplemental material).

The NS genes of genotype 9 belonged to the NV-TR cluster, as did the N2 genes (Fig. S2). The NP and M genes of genotype 9 were derived from A(H1N1)pdm09v, and those clustered with genotypes 14 and 15 (described later), respectively, which were isolated in Bac Ninh province in northern Vietnam (Fig. 4 and S1).

(iv) Human-like H3 viruses (genotypes 11 through 13). Genotype 11 viruses, which were reassortants between a human H3N2 seasonal virus and TR IAV-S, were first isolated in March 2010 in southern Vietnam (29). Afterward, genotype 11 viruses

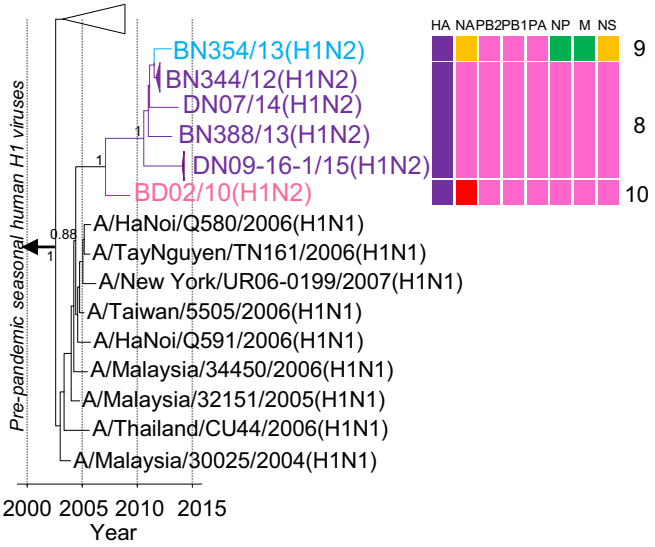

FIG 7 Maximum clade credibility phylogenetic tree of HA genes of pre-2009 human H1 lineage. The names of the Vietnamese IAV-S (virus group) isolated in this study are colored according to the isolate's genotype. At the right of the tree, the phylogenetic origin of each segment (depicted as rectangles) is indicated according to the color code described for Fig. 1, with the isolate's genotype number beside the segment origin map. Posterior probabilities of >0.7 are provided for key nodes.

appeared to yield genotypes 12 and 13 through reassortment with A(H1N1)pdm09v, because all of the TR origin internal genes of genotypes 12 and 13 belonged to the C-TR cluster, as did those of genotype 11 (Fig. S3 and S6). The H3 genes of genotypes 11, 12, and 13 formed a single cluster with Chinese IAV-S that were isolated in Hong Kong and Guangxi (Fig. 8). The human-like H3 genes in Vietnam had diverged from the ancestral H3N2 human-seasonal influenza viruses between April 2004 and January 2005 (95% HPD [Table 2]), whereas human-like N2 genes diverged between July 2003 and March 2004 (95% HPD [Table 2]), thus suggesting that a contemporary H3N2 virus had been introduced into the pig population.

The remaining internal genes of genotypes 12 and 13 originated from A(H1N1) pdm09v (Fig. 1). Interestingly, all of the pdm origin genes of DN12/15 (genotype 13) were clearly distinct from those of viruses belonging to the other 13 genotypes (Fig. 4; see also Fig. S1, S8, S11, and S12 in the supplemental material). In addition, pdmNP phylogeny showed that DN12/15 clustered with DN07-3/15 and DN07-1/15 (genotype 7 [Fig. 4]), suggesting that genetic reassortment between genotypes 7 and 13 had occurred. The PB2 genes of genotype 12 and 13 viruses except DN12/15 were derived from common ancestral viruses (Fig. S11). In addition, the pdm-M genes of genotypes 12 and 13 except DN12/15 and DN09-06-1/15 clustered with southern Vietnamese IAV-S, as described earlier (Fig. S1). However, the pdm-PA and pdm-NS genes of these genotypes seem to have arisen due to different introductions of A(H1N1)pdm09v (Fig. S8 and S12).

It is noteworthy that the H3 gene of A/Ho Chi Minh/459.6/2010 (H3N2), which was from a human isolate, was closely related to the genotype 12 virus HCM01/11 (Fig. 8). Information regarding the N2 and M genes of A/Ho Chi Minh/459.6/2010 (H3N2) was available in GenBank (31) and indicated that the N2 genes of A/Ho Chi Minh/459.6/2010 (H3N2) and HCM01/11 arose from the same ancestor identified through HA phylogeny (Fig. S9). In addition, the M gene of the human isolate was located at the root of the large M-gene cluster (described earlier) containing southern Vietnamese IAV-S, including genotypes 12 and 13, North American IAV-S, and Chinese IAV-S (Fig. S1). These phylogenetic relationships between pig and human isolates suggest that pig-to-human transmission of influenza viruses with (at least) common surface and M genes had occurred in or near Ho Chi Minh City.

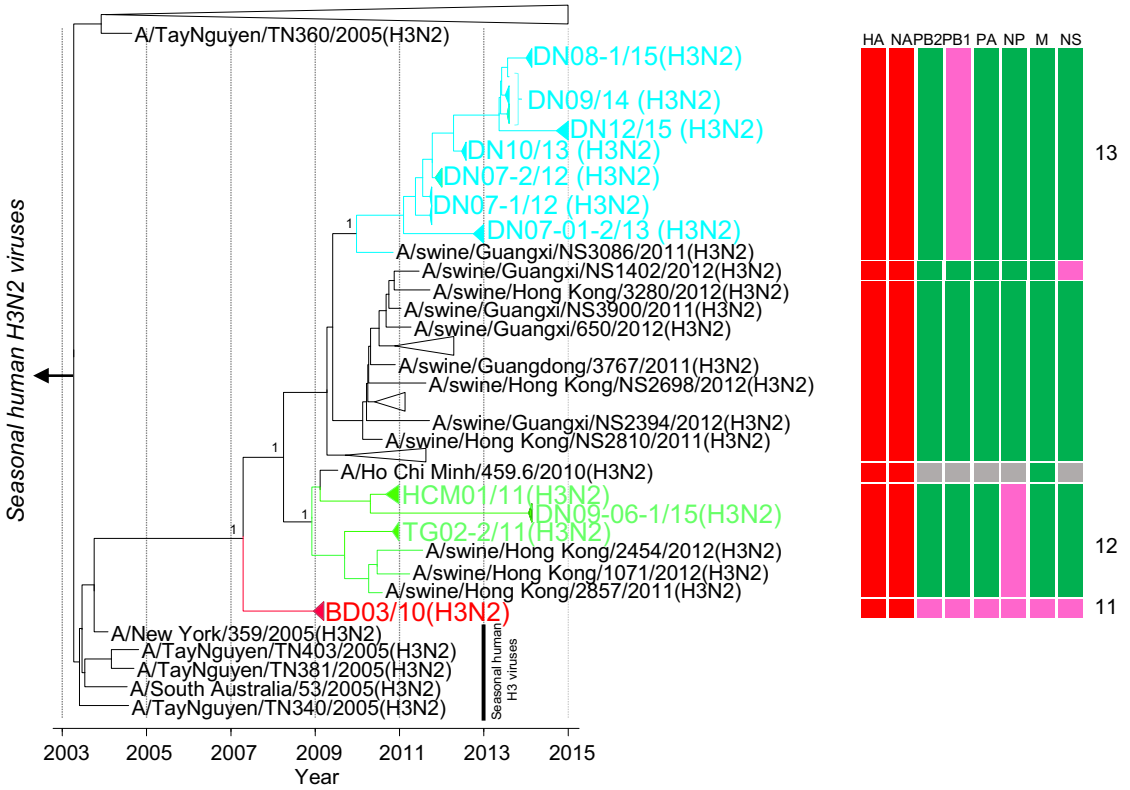

FIG 8 Maximum clade credibility phylogenetic tree of HA genes of human-lineage H3 viruses. The names of the Vietnamese IAV-S (virus group) isolated in this study are colored according to the isolate's genotype. At the right of the tree, the phylogenetic origin of each segment (depicted as rectangles) is indicated according to the color code described for Fig. 1; gray, unknown origin. The isolate's genotype number is beside the segment origin map. Posterior probabilities of >0.7 are provided for key nodes.

(v) H3TR viruses (genotypes 14 and 15). The H3 HA genes of two genotypes, 14 and 15, formed a single cluster (NV-TR cluster) with the H3N2 TR cluster IV IAV-S in North America (Fig. 9). A/swine/Hanoi/422/2013 (H3N2) and A/swine/Hanoi/415/2013 (H3N2), isolated in another IAV-S surveillance (30), were included in this cluster. Bac Ninh province, where genotypes 14 and 15 were isolated, is next to Hanoi City, suggesting that the NV-TR cluster viruses have been prevalent in northern Vietnam. The ancestral virus of the NV-TR cluster was estimated to enter the Vietnamese pig population between November 2010 and September 2011 (95% HPD calculated from H3 gene data [Table 2]). The NA and NS genes of genotypes 14 and 15 similarly belonged to the NV-TR cluster, as did genotype 9 (Fig. S2 and S5). In addition, the PB2 (Fig. 6) and PB1 (Fig. S3) genes of genotypes 14 and 15 and the PA (Fig. S4) genes of genotype 15 all were derived from TR origin viruses, but those of genotype 14 belonged to the NV-TR cluster, whereas those of genotype 15 belonged to the C-TR cluster. Phylogenetic analysis showed that the PA genes of genotype 14 were closely related to the human A(H1N1)pdm09 viruses circulating around 2009 (Fig. S8).

(vi) Vietnamese and Chinese IAV-S. Three Chinese genotypes were identical to Vietnamese IAV-S. All gene segments of Vietnamese genotype 13 viruses and A/swine/Guangxi/3086/2011 (H3N2), genotype 12 and A/swine/Hong Kong/2857/2011 (H3N2), and genotype 4 and A/swine/Guangxi/2887/2011 (H1N2) showed high similarity, suggesting that these pairs of viruses had diverged from a common ancestor despite their geographic isolation. Among genotype 12 viruses, TG02-2/11 was more similar to A/swine/Hong Kong/2857/2011 (H3N2) than to HCM01/11. Each gene segment of A/swine/Guangxi/2887/2011 (H1N2) was highly similar (more than 99.1% identical) to DN07-10-2/13 (genotype 4). The gene constellations of A/swine/Guangxi/3075/2011 (H1N2) and genotype 3 viruses were identical, except for the M and NP genes, whose similarities exceeded 99%. Interestingly, 13 genotypes of the reassortant viruses (that is,

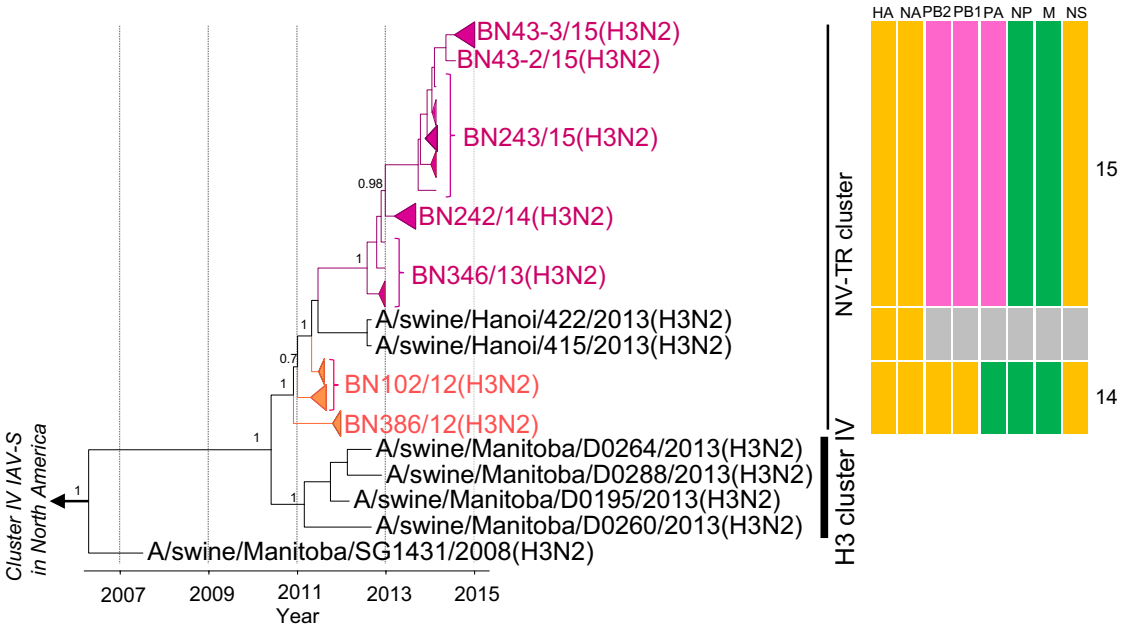

FIG 9 Maximum clade credibility phylogenetic trees of HA genes of H3 triple-reassortant (TR) IAV-S. The names of the Vietnamese IAV-S (virus group) isolated in this study are colored according to the isolate's genotype. At the right of the tree, the phylogenetic origin of each segment (depicted as rectangles) is indicated according to the color code described for Fig. 1; gray, unknown origin. The isolate's genotype number is beside the segment origin map. Posterior probabilities of >0.7 are provided for key nodes.

all except genotype 14) retained at least one gene segment of H1-TR (C-TR) origin that had been circulating in North America since 1999.

DISCUSSION

Our active IAV-S surveillance from 2010 to 2015 in southern and northern Vietnam yielded 388 IAV-S isolates. Of these, 92 isolates were A(H1N1)pdm09v and the remaining 296 isolates were reassortants. The human A(H1N1)pdm09v was introduced in early June 2009, and 11,208 laboratory-confirmed cases and 58 deaths had been reported by March 2010 in Vietnam (32). The introduction of A(H1N1)pdm09v from humans into the pig population was estimated to start during this period. Our present study shows that current IAV-S lineages circulating in Vietnam are highly influenced by human A(H1N1)pdm09v. Once A(H1N1)pdm09 origin gene segments were introduced as components of reassortants, they were more likely to be sustained in the pig population than was the entire A(H1N1)pdm09v. Therefore, controlling the risk of transmission of human A(H1N1)pdm09v at pig farms is important to prevent the expansion of novel reassortants containing A(H1N1)pdm09 origin genes.

Except for viruses of the Bac Ninh A(H1N1)pdm09 cluster, the the A(H1N1)pdm09 origin viruses whose genomes overall are from A(H1N1)pdm09v tended to not be sustained in the pig population. This trend agrees with the pattern seen globally. For example, weekly or fortnightly sampling in southern China and Hong Kong showed that the entire A(H1N1)pdm09v failed to persist in the pig population after each introduction (12). In addition, although an estimated 133 human-to-swine transmissions have occurred worldwide between 2009 and 2014 (23, 33), the A(H1N1)pdm09v does not appear to be retained in the U.S. pig population (33). Interestingly, Ban Ninh pdm cluster viruses that were A(H1N1)pdm09v but that acquired their pdm-NP genes from reassortant Vietnamese IAV-S (genotype 15) have been circulating for more than 2 years in Vietnam. This finding suggests that whereas most A(H1N1)pdm09v are difficult to sustain through pig-to-pig transmission, reassortment may allow them to be maintained even though the entire genome is derived from A(H1N1)pdm09v.

The gene combinations of the reassortant IAV-S in this study showed various patterns. In particular, 93% of the 296 Vietnamese reassortants retained pdm-M gene

segments. Experimentally, the A(H1N1)pdm09 origin M-gene segment is a critical factor for efficient transmission in the guinea pig model (34), because it increases neuraminidase activity and contributes to contact transmission (35). Therefore, during reassortment between enzootic IAV-S and A(H1N1)pdm09v, a pdm-M gene might be advantageous for propagation in and transmission to pigs, although the amino acid residues of pdm-M that are responsible for this function are unknown. It is noteworthy that A/Ho Chi Minh/459.6/2010 (H3N2), a human isolate transmitted from pigs in Vietnam, had pdm-M gene as did the "H3N2v" that were North American human isolates of H3N2-TR viruses possessing pdm-M genes. H3N2v caused 386 human cases in North America from 2011 to 2015 (http://www.cdc.gov/flu/swineflu/h3n2v-case-count.htm), suggesting that a pdm-M gene might also increase the risk of pig-to-human transmission. In addition, we have never isolated a reassortant IAV-S in which both the HA and NA genes were from A(H1N1)pdm09v. In particular, all of the reassortants in the current study lacked pdm-N1 genes, consistent with previous reports (12, 27, 33). Only 1 of 444 U.S. IAV-S collected from 2009 to 2014 contained a pdm-NA gene (33), suggesting that the pdm-N1 segment was rarely retained during reassortment events between A(H1N1)pdm09v and TR IAV-S. Coinfection of swine testis cells with swine A(H1N1)pdm09v and H1N2-TR viruses generated two kinds of reassortants, both of which possessed TR origin surface antigens and pdm-M genes (36). Further studies are required to elucidate the significance of these IAV-S gene combinations in pigs.

To our knowledge, 9 of the 14 genotypes of reassortant IAV-S that we collected during the current study have not been reported in other countries (12, 25, 27, 37–39), suggesting that such reassortants had been generated in the Vietnamese pig population. In contrast, 3 of the remaining 5 genotypes (that is, genotypes 4, 12, and 13) are also found in China, but they are unlikely to have been introduced into Vietnam from China. The EA-like IAV-S lineage has been constantly isolated in the Chinese pig population since 2002 (11, 12), and long-term serological surveillance suggests that the EA-like IAV-S lineage had been predominant in China since 2003 (40). Nonetheless, neither EA-like IAV-S nor IAV-S containing EA-like genes have been found in Vietnam to date. The United States exported 110 to 5,155 live pigs to Vietnam, China, and Hong Kong annually from 2000 to 2002 (http://www.ers.usda.gov/data-products/livestock-meat-international-trade-data.aspx). One possible scenario for the presence of the Chinese genotypes in Vietnam is that the ancestors of these three genotypes initially arose in North America and expanded first to Vietnam and then to China either concurrently or sequentially.

In conclusion, Bayesian Markov chain Monte Carlo phylogenetic analysis of 388 Vietnamese IAV-S isolated from 2010 through 2015 revealed that repeated introduction of A(H1N1)pdm09v followed by reassortments with enzootic IAV-S increased the genetic diversity of Vietnamese IAV-S. In addition to local transmission, the movement of pigs between North American countries, China, and Vietnam promotes the expansion of these IAV-S genotypes. Therefore, to prevent further introduction of different IAV-S, quarantine systems between both countries and farms need to be strengthened and an IAV-S diagnosis system must be developed. Active surveillance of IAV-S in Vietnam continues to deepen our understanding of IAV-S ecology and supports the development of an early detection system for pig-to-human transmission of IAV-S.

MATERIALS AND METHODS

Sample collection and virus isolation. Nasal swab samples were collected 26 times in total during our active IAV-S surveillance of pig farms and slaughterhouses in Vietnam from February 2010 to December 2015. Nasal swabs were collected one to three times a year during the period in both northern and southern regions (Table 1). The specimens were collected from 209 farms in 3 provinces (Bac Ninh, Hanoi, and Nam Dinh) in the northern region and from 53 farms and 10 slaughterhouses in 7 provinces (Ba Ria Vung Tau, Binh Duong, Dong Nai, Long An, Ho Chi Minh, Soc Trang, and Tien Giang) in the southern region. Except for sample collection at the 10 slaughter houses in the southern region in December 2010, all of the swabs were collected from pig farms. We collected a total of 10,424 nasal-swab samples from clinically healthy pigs, comprising 290 suckling pigs, 5,489 weanling pigs, 4,012 fattening pigs, 375 finishing pigs, 240 sows, and 18 boars. Pooled specimens (2 to 5 nasal swabs per pool) were screened for the influenza A virus M gene in real-time PCR analyses using SYBR Premix *Ex Taq* (TaKaRa

Bio, Shiga, Japan), as previously described (29). Component specimens of positive pooled samples were filtered individually (pore size, 0.45 μm; Millipore, Billerica, MA, USA); and 160 μl of filtrate was inoculated into floating MDCK cells (41) and incubated for 4 days at 37°C in 5% CO_2. When viruses could not be recovered after a second round of floating MDCK cells, the original materials were inoculated into 9- to 11-day embryonated chicken eggs or primary cultures of porcine alveolar epithelial cells as previously described (42). Hemagglutination activity (HA) was tested by using 0.55% red blood cells from guinea pigs and chickens (41).

Sequencing. The complete genomes of the 388 IAV-S isolates were obtained through next-generation sequencing (Miseq; Illumina, San Diego, CA, USA) or Sanger sequencing (ABI model 3130; Applied Biosystems, Foster City, CA, USA). RNA was extracted from isolates by using an RNeasy minikit (Qiagen, Hilden, Germany). cDNA libraries for next-generation sequencing were prepared by using a NEBNext Ultra RNA Library Prep kit for Illumina (NEB, Ipswich, MA, USA) and sequenced by using a Miseq Reagent kit v2 (Illumina, San Diego, CA, USA). Consensus sequences of the IAV-S isolated before 2013 were obtained by mapping to the reference sequences of influenza A viruses (CLC Genomics Workbench, Qiagen, Hilden, Germany). To identify the most appropriate reference sequences, we initially downloaded representative sequences of each lineage (i.e., human seasonal influenza viruses, CS influenza viruses, EA-like IAV-S, and TR IAV-S) from the NCBI influenza database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). Using the mapped partial sequences as queries in a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), we obtained the full-length influenza virus sequences and used them as reference sequences. In addition, the resulting full-length genomic sequences of Vietnamese IAV-S could subsequently be used as reference sequences when appropriate. The consensus sequences of the IAV-S isolated in 2014 and 2015 were obtained by using FLUGAS (beta version; World Fusion, Tokyo, Japan) and Genomics Workbench software. The nucleotide sequences and isolation information of the viruses analyzed in the present study were deposited in the GISAID Epiful database (http://www.gisaid.org). Isolate ID numbers and isolation information are listed in Table S1 in the supplemental material.

Phylogenetic analysis. In April 2016, we downloaded 19,550 complete genomic sequences of H1 and H3 influenza viruses from various species from the NCBI influenza database (http://www.ncbi.nlm-.nih.gov/genomes/FLU/FLU.html) and GISAID database (http://platform.gisaid.org/epi3/frontend). CD-HIT clustering software (43) was used to reduce the number of sequences by clustering together all genomic sequences in which the HA sequences were at least 99.5% identical. After clustering, 1615 H1 and 1440 H3 strains were extracted for further analysis. NA and internal genes were selected from the same strains as the HA gene sets by using BioEdit version 7.2.5 (44). Each gene set was assigned according to its phylogenetically distinct lineage, such as A(H1N1)pdm09 origin, pre-2009 human seasonal H1 origin, and CS lineages. For example, the 1615 H1 genes were phylogenetically classified into 416 avian viruses, including avian-like IAV-S, 447 human and swine A(H1N1)pdm09v, 372 pre-2009 human seasonal H1 viruses, and 380 CS viruses. The 1,440 H3 genes were classified into 463 seasonal human viruses and human-like IAV-S since 2000, 244 TR IAV-S, and other groups. Then, Vietnamese IAV-S sequences obtained during our current surveillance and previous studies (30, 31) were added to each data set. Ultimately, we used 545 A(H1N1)pdm09 H1 genes consisting of 322 human, 222 swine, and 1 avian strains (length, 1,694 bp), 385 pre-2009 human seasonal H1 genes consisting of 184 human and 201 swine strains (1,694 bp), and 457 classical H1 swine genes consisting of 5 human, 448 swine, 3 avian, and 1 other strains (1,694 bp) for H1 phylogenetic analysis. We also used 365 TR origin (cluster IV) H3 genes consisting of 2 human, 358 swine, 4 avian, and 1 other strains (1,701 bp) and 544 H3 genes of human seasonal and human-like IAV-S since 2000 consisting of 435 human and 109 swine strains (1,701 bp) for H3 phylogeny; 465 A(H1N1)pdm09 genes consisting of 293 human, 171 swine, and 1 avian strains (1,410 bp) for N1 phylogeny; and 619 TR origin (cluster IV) genes consisting of 4 human, 609 swine, 5 avian, and 1 other strains (1,410 bp) and 547 human seasonal and human-like IAV-S genes consisting of 436 human and 111 swine strains (1,410 bp) for N2 phylogeny. Internal gene phylogenetic analysis involved A(H1N1)pdm09 origin and TR origin genes: 715 A(H1N1)pdm09 genes consisting of 319 human, 392 swine, 2 avian, and 2 other strains and 772 TR origin genes consisting of 7 human, 759 swine, 4 avian, and 2 other strains (2,280 bp) for PB2; 637 A(H1N1)pdm09 genes consisting of 317 human, 317 swine, 1 avian, and 2 other strains and 846 TR origin genes consisting of 7 human, 832 swine, 5 avian, and 2 other strains (2,274 bp) for PB1; 880 A(H1N1)pdm09 genes consisting of 317 human, 558 swine, 2 avian, and 3 other strains and 600 TR origin genes consisting of 6 human, 589 swine, 4 avian, and 1 other strains (2,151 bp) for PA; 923 A(H1N1)pdm09 genes consisting of 319 human, 599 swine, 2 avian, and 3 other strains and 561 TR origin genes consisting of 6 human, 550 swine, 4 avian, and 1 other strains (1,497 bp) for NP; 1,103 A(H1N1)pdm09 genes consisting of 323 human, 775 swine, 2 avian, and 3 other strains and 370 TR origin genes consisting of 3 human, 362 swine, 4 avian, and 1 other strains (982 bp) for M; and 748 A(H1N1)pdm09 genes consisting of 320 human, 425 swine, 1 avian, and 2 other strains and 736 TR origin genes consisting of 7 human, 722 swine, 5 avian, and 2 other strains (838 bp) for NS.

The final nucleotide sequences of each segment were aligned by using MAFFT software (45). Phylogenetic trees of each lineage were constructed according to the Bayesian Markov chain Monte Carlo method in Beast software package version 1.8.2 (46). The SRD06 nucleotide substitution model (47) with uniform clock rate, strict-clock model, and random starting tree model was applied. Each chain was 1×10^8 to 1×10^9 steps in length, in which the steps were determined to obtain an effective sample size of more than 200 (as assessed by Tracer version 1.6 [http://beast.bio.ed.ac.uk/Tracer]), and then was sampled every 10,000 to 60,000 steps. The first 10% of the states was discarded as burn-in when the maximum clade credibility (MCC) trees were constructed. The exact sampling dates of the isolates used in the phylogenetic tree were downloaded with the nucleotide sequences to estimate the time to the common ancestor of Vietnamese IAV-S; when only the month and year of sampling were known, the day

was assumed to be the 15th of the month, and when the month and day were unknown, the sampling date was set to be 1 July. The time to the common ancestor was estimated from the node height of the 95% highest posterior density interval (HPD); when the 95% HPD could not be calculated because of a low posterior value at the node from the ancestral virus, the time was estimated from the distance from the node. Analyses through the Beast software package were conducted by using the SGI Rackable Standard Depth Server C2108-RP2 of AFFRIT, MAFF, Japan.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.01490-16.

DATASET S1, XLSX file, 0.04 MB.

TEXT S2, PDF file, 1.6 MB.

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