


## ORIGINAL ARTICLE

# Genetic characterization of influenza A virus subtypes H11N6, H11N7, and H11N9 isolated from free-grazing ducks, Thailand

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## Funding information

PMU-B (NXPO), Grant/Award Number: B17F640011; Agricultural Research Development Agency, Grant/Award Number: PRP6405031220; TSRI, Grant/Award Numbers: CU\_FRB640001\_01\_31\_1, CUFRB65\_Food\_182\_31\_01; Thailand Research Fund, Grant/Award Number: PHD/0181/2553; One Health Research Cluster; Chulalongkorn University, Grant/Award Number: GCUGR1125614076D

## Abstract

Influenza A viruses (IAVs) infect avian species and several mammalian species including humans. Anseriformes water birds are an important reservoir of IAVs. In this study, we identified and characterized IAV subtypes H11N6 (n = 5), H11N7 (n = 3), and H11N9 (n = 3) isolated during the influenza surveillance program in free-grazing ducks from 2012 to 2015 in Thailand. Eleven IAV-H11 viruses were characterized by either whole genome sequencing (n = 5) or HA and NA gene sequencing (n = 6) for phylogenetic and amino acid analyses. Phylogenetic analysis showed that Thai IAV-H11 were grouped into Avian Eurasian lineage. Amino acid analysis showed that all Thai IAV-H11 viruses have low pathogenic avian influenza (LPAI) characteristics and sensitive to Oseltamivir and Amantadine. Novel reassortant viruses (IAV-H11N7 and IAV-H11N9) have been observed. The reassortant viruses contained NP, M, and NS gene segments which originate from intercontinental sources which never been reported in Thai IAVs. In summary, this study demonstrated high genetic diversity of IAV-H11 circulating in free-grazing ducks. Free-grazing ducks infected with IAVs generated novel reassortant IAV-H11. Thus, surveillance of IAVs in free-grazing ducks should be routinely conducted to monitor novel reassortant viruses and subsequently potential virulence viruses.

## KEYWORDS

characterization, ducks, H11N6, H11N7, H11N9, influenza

## 1 | INTRODUCTION

Influenza A virus (IAV) or Alphainfluenzavirus is an enveloped, segmented, single-stranded RNA virus of the family *Orthomyxoviridae*. The virus can infect avian species and several mammal species including humans. IAVs can be classified into subtypes based on hemagglutinin and neuraminidase proteins (18HA and 11NA).<sup>1–4</sup> Several IAV subtypes, H1–H16 and N1–N9, circulate in avian species especially in

order *Anseriformes*. Waterfowls are susceptible to infection by IAVs and serve as natural reservoirs and can spread the viruses to other avian and mammal species. It has been documented that IAV reservoir hosts are important for generating novel reassortant viruses.<sup>5–7</sup>

IAV subtype H11 (IAV-H11) have been reported in avian species worldwide. Most of the IAV-H11 were found in avian species of order *Anseriformes* and a few were reported in order *Charadriiformes*, and rarely were found in order *Galliformes*.<sup>8</sup> It has also been reported that

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IAV-H11 rarely infect humans. For example, IAV-H11 had been reported to infect humans who were exposed to wild birds.<sup>8</sup> The previous study showed that receptor binding site of IAV-H11 has preferential binding to both avian (SA  $\alpha$  2,3-Gal) and mammalian (SA  $\alpha$  2,6-Gal) receptors.<sup>9</sup> In addition, IAV-H11N6 had been reported to infect pigs in South Korea.<sup>10</sup> The IAV-H11 viruses (H11N2, H11N3, and H11N9) have been reported in domestic ducks from live bird markets in China.<sup>11–13</sup>

In Thailand, IAV-H11 were isolated from free-grazing ducks (FGDs) during the influenza surveillance program in 2012–2015. The FGDs (mainly *Anas platyrhynchos*) are semidomestic ducks raised in open rice field in flocks of >1000 birds for egg and meat production in Southeast Asia. The ducks are raised to free graze for food in rice fields after harvesting and frequently moved among rice fields for new food sources. FGDs potentially interface with wild birds and domestic birds such as backyard ducks, chickens, and quails. Because FGDs are reservoirs of the IAVs, therefore, interspecies transmission of IAVs commonly occurred.<sup>14–19</sup> It is also noted that FGDs could be infected with HPAI-H5N1 without clinical signs and can carry virulent viruses to other animals. Thus, novel reassortant viruses with virulence genes can arise.<sup>20,21</sup> In this study, we selected IAV-H11 (H11N6, H11N7, and H11N9) isolated from FGDs in Thailand and performed genetic characterization of IAV-H11 by whole genome sequencing to determine genetic diversity of the viruses and subsequently monitor potential virulence viruses.

## 2 | MATERIAL AND METHODS

### 2.1 | Viruses

The IAVs were isolated from oropharyngeal swabs (OP) and cloacal swabs (CS) collected from FGDs during 2012–2015 influenza surveillance program in Thailand. The FGD flocks were selected based on owner collaboration, and approximately 50 ducks from each flock were randomly selected and sampled. The IAVs were isolated by egg inoculation into 9- to 11-day-old embryonated chicken eggs.<sup>22</sup> The allantoic fluid was tested for influenza virus by hemagglutination (HA) test. The allantoic fluid with the HA titer  $\geq 2$  HA unit were interpreted as positive influenza virus. The HA positive samples were confirmed for the presence of IAV by using real-time Reverse Transcriptase-PCR (rRT-PCR) specific to the Matrix (M) gene.<sup>23</sup> In this study, IAVs ( $n = 11$ ) isolated from FGDs were included for genetic characterization. This study was conducted under the approval of the Chulalongkorn University Animal Care and Use Protocol (IACUC# 2031050 and 2031051).

### 2.2 | IAV detection

RNA extraction from the allantoic fluid was carried out by Nucleospin<sup>®</sup> RNA virus (Macherey-Nagel, Germany) according to the manufacturer's instruction. The viral RNA was subjected to IAV

detection by rRT-PCR specific to Matrix (M) gene.<sup>23</sup> In brief, the rRT-PCR was performed by using the SuperScript<sup>®</sup> III Platinum<sup>®</sup> One-Step Quantitative RT-PCR System (Invitrogen<sup>®</sup>). The 30  $\mu$ l of reagent mixture contained 4  $\mu$ l of RNA template, 1 $\times$  master mix buffer, 0.8  $\mu$ M of M gene specific primers, 0.2  $\mu$ M of probe, 0.6 mM MgSO<sub>4</sub>, 1 unit of Superscript III reverse transcriptase, and RNase-free water. Amplification was done by rRT-PCR which contained three steps: (1) reverse transcription at 50°C for 30 min, (2) predenaturation at 95°C for 15 min, and (3) denaturation for 50 cycles of 95°C for 15 s and annealing–extension at 60°C for 30 s. The rRT-PCR results were evaluated by cycle threshold (Ct), and values less than 36 were considered positive, greater than 40 were negative, and between 36 and 40 were interpreted as inconclusive.

### 2.3 | IAV subtype identification

The positive RNA samples were synthesized for cDNA by using Improm-II Reverse Transcription System (Promega, Madison, WI, USA) with universal primer for IAVs. The cDNA samples were subjected to IAV subtype identification. The specific primers of each influenza subtypes, H1–H15 and N1–N9, were used for influenza subtyping by PCR using primers previously described.<sup>24–26</sup> In detail, 30  $\mu$ l of PCR mixture contained 1  $\mu$ l of cDNA, 1 $\times$  master mix buffer (TopTaq<sup>™</sup>), 0.8  $\mu$ M of primers for each subtype, and distilled water. The PCR conditions were 94°C for 3 min and 35 cycles of 94°C for 30 s, 50°C (for H1–H15) or 45°C (for N1–N9) for 30 s, and 72°C for 30 s. The PCR product was run in 1.2% of agarose gel with Red safe in 0.5 $\times$  Tris borate EDTA (TBE).

### 2.4 | IAV characterization

Eight segments of the IAVs were amplified using TopTaq master mix (Qiagen, Hilden, Germany) with specific primer sets and newly designed primers for sequencing.<sup>27</sup> In brief, 30  $\mu$ l of PCR mixture contained 1.5  $\mu$ l of cDNA, 1.2  $\mu$ M of each forward and reverse primers, 1 $\times$  Top Taq Master Mix (QIAGEN), 1 $\times$  loading dye, and distilled water. PCR condition was set as initial denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing temperature depend on primers for 45 s, and extension at 72°C for 1 min; and final extension at 72°C for 7 min. PCR products were then visualized by gel electrophoresis with 1.2% of agarose gel in 0.5 $\times$  Tris borate EDTA. The amplicons were purified using Nucleospin<sup>®</sup> PCR clean up kit. The purified PCR products were then sequenced using BigDye<sup>®</sup> Terminator v3.1 cycle sequencing kit (1st Base Laboratories, Kembangan, Malaysia). The nucleotide sequences of each gene segment were validated and assembled using SeqMan software v.5.03 (DNASTAR Inc., Madison, WI, USA).

To determine nucleotide identity, the nucleotide sequences of each gene segment were compared with those of the reference IAVs from the GenBank database by using nucleotide BLAST tool. For pairwise comparison, nucleotide sequences and deduced amino acids

of the IAVs in this study were aligned with those of reference IAV sequences from different subtypes and geographic locations (Eurasian and North America lineages) by using Muscle version 3.6 and MegAlign version 5.03 (DNASTAR Inc., Madison, WI, USA) software. Phylogenetic trees of eight gene segments were generated by using MEGA v10.0 applying neighbor-joining method with 1000 replications for bootstrap analysis. The reference data set for phylogenetic analysis was selected to represent geographic locations (Eurasian and North America lineages) and available sequences of Thai IAV-H11Nx, HxN6, HxN7, and HxN9.

### 3 | RESULTS

In this study, we tested oropharyngeal swabs ( $n = 780$ ) and cloacal swabs ( $n = 780$ ) collected from 18 FGD flocks during 2012–2015 (Table S1). We detected and isolated IAVs ( $n = 11$ ) from FGD flocks sampled from three provinces of central and northern Thailand (Ang Thong, Sukhothai, and Kamphaengphet) (Table 1 and Figure 1). All viral RNA samples were confirmed as IAV by rRT-PCR assay and had cycle threshold value  $\leq 20$ . Then, the IAVs were subtyped as IAV-H11N6 ( $n = 5$ ), IAV-H11N7 ( $n = 3$ ), and IAV-H11N9 ( $n = 3$ ). The representative viruses IAV-H11N6 ( $n = 2$ ), IAV-H11N7 ( $n = 1$ ), and IAV-H11N9 ( $n = 2$ ) were selected for whole genome sequencing, and the other IAV-H11 ( $n = 6$ ) were subjected to HA and NA gene sequencing. The nucleotide sequences of IAV-H11 were submitted to the GenBank database under the accession# MW857483-857534 (Table 1).

#### 3.1 | Genetic characteristics of HA gene of Thai IAV-H11

For H11 gene, Thai IAV-H11 viruses possessed high nucleotide identities to IAVs of Eurasian lineage (Tables S2 and S3). Phylogenetic tree of the H11 gene showed that the viruses can be divided into North American lineage and Eurasian lineage. The Thai IAV-H11 were clustered with the IAVs of the Eurasian lineage. It is noted that Thai IAV-H11 could be further grouped into two different subgroups. The subgroup 1 (Ang Thong subgroup) included IAV-H11N6 ( $n = 5$ ) and IAV-H11N9 ( $n = 2$ ), which were recovered from the same province (Ang Thong). These viruses were closely related to the Thai IAV-H11N3 isolated in 2009 (95.44–95.68%). The subgroup 2 (Sukhothai and Kamphaeng Phet subgroup) included IAV-H11N9 ( $n = 1$ ) and H11N7 ( $n = 3$ ), which were closely related to the Chinese H11N9 isolated in 2012 (98.78–98.88%) (Figure 2).

#### 3.2 | Genetic characteristics of NA gene of Thai IAV-H11

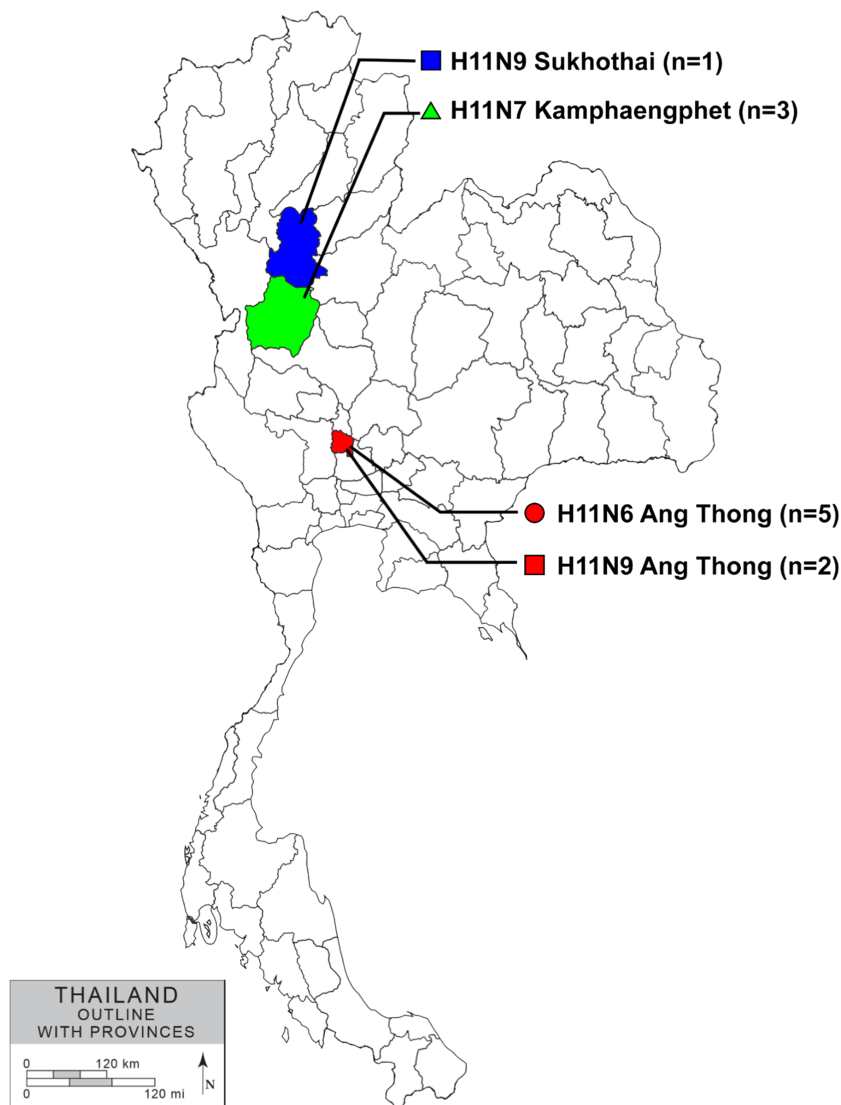
The phylogenetic tree of N6 gene can be divided into North American lineage and Eurasian lineage. The N6 gene of Thai IAV-H11N6 ( $n = 5$ ) were clustered in avian Eurasian lineage and were closely related to

TABLE 1 Description of Thai IAV-H11 from free-grazing ducks characterized in this study

Virus	Strain name	Subtype	Year	Location	Flock	Source	Date	Sequencing	GenBank #
CU-12657 (H11N6)	A/duck/Thailand/CU-12657T/2012	H11N6	2012	Ang Thong	1	OP	Oct 2012	WGS	MW857483-90
CU-12658 (H11N6)	A/duck/Thailand/CU-12658T/2012	H11N6	2012	Ang Thong	1	OP	Oct 2012	HA, NA	MW857491-92
CU-12661 (H11N6)	A/duck/Thailand/CU-12661T/2012	H11N6	2012	Ang Thong	1	OP	Oct 2012	HA, NA	MW857493-94
CU-12677 (H11N6)	A/duck/Thailand/CU-12677T/2012	H11N6	2012	Ang Thong	2	OP	Oct 2012	WGS	MW857495-02
CU-12678 (H11N6)	A/duck/Thailand/CU-12678C/2012	H11N6	2012	Ang Thong	2	CS	Oct 2012	HA, NA	MW857503-04
CU-12660 (H11N9)	A/duck/Thailand/CU-12660T/2012	H11N9	2012	Ang Thong	1	OP	Oct 2012	WGS	MW857505-12
CU-12662 (H11N9)	A/duck/Thailand/CU-12662T/2012	H11N9	2012	Ang Thong	1	OP	Oct 2012	HA, NA	MW857513-14
CU-14442 (H11N9)	A/duck/Thailand/CU-14442C/2014	H11N9	2014	Sukhothai	3	CS	Feb 2014	WGS	MW857515-22
CU-16340 (H11N7)	A/duck/Thailand/CU-16340C/2015	H11N7	2015	Kamphaengphet	4	CS	Feb 2015	WGS	MW857523-30
CU-16345 (H11N7)	A/duck/Thailand/CU-16345C/2015	H11N7	2015	Kamphaengphet	4	CS	Feb 2015	HA, NA	MW857531-32
CU-16347 (H11N7)	A/duck/Thailand/CU-16347C/2015	H11N7	2015	Kamphaengphet	4	CS	Feb 2015	HA, NA	MW857533-34

Abbreviations: CS, cloacal swabs; HA, hemagglutination; NA, neuraminidase; OP, oropharyngeal swabs; WGS, characterization by whole genome sequencing.

**FIGURE 1** Map of Thailand and provinces where the Thai IAV-H11 were isolated in this study



Thai IAV-H4N6. For N7 gene, the phylogenetic tree of N7 gene possessed two major groups, the North American and Eurasian lineages. The Thai IAV-H11N7 ( $n = 3$ ) were clustered in avian Eurasian lineage and were closely related with IAV-H10N7 from Mongolia. For N9 gene, the phylogenetic tree of N9 gene can be divided into North American lineage and Eurasian lineage. The Thai IAV-H11N9 ( $n = 3$ ) were clustered in avian Eurasian lineage. It is noted that the Thai IAV-H11N9 could be grouped into two subgroups: subgroup 1 (IAV-H11N9 from Ang Thong) and subgroup 2 (IAV-H11N9 from Sukhothai). Both subgroups were in a separated group to IAV-H7N9 in China (Figure 3).

### 3.3 | Genetic characteristics of internal gene segments of Thai IAV-H11

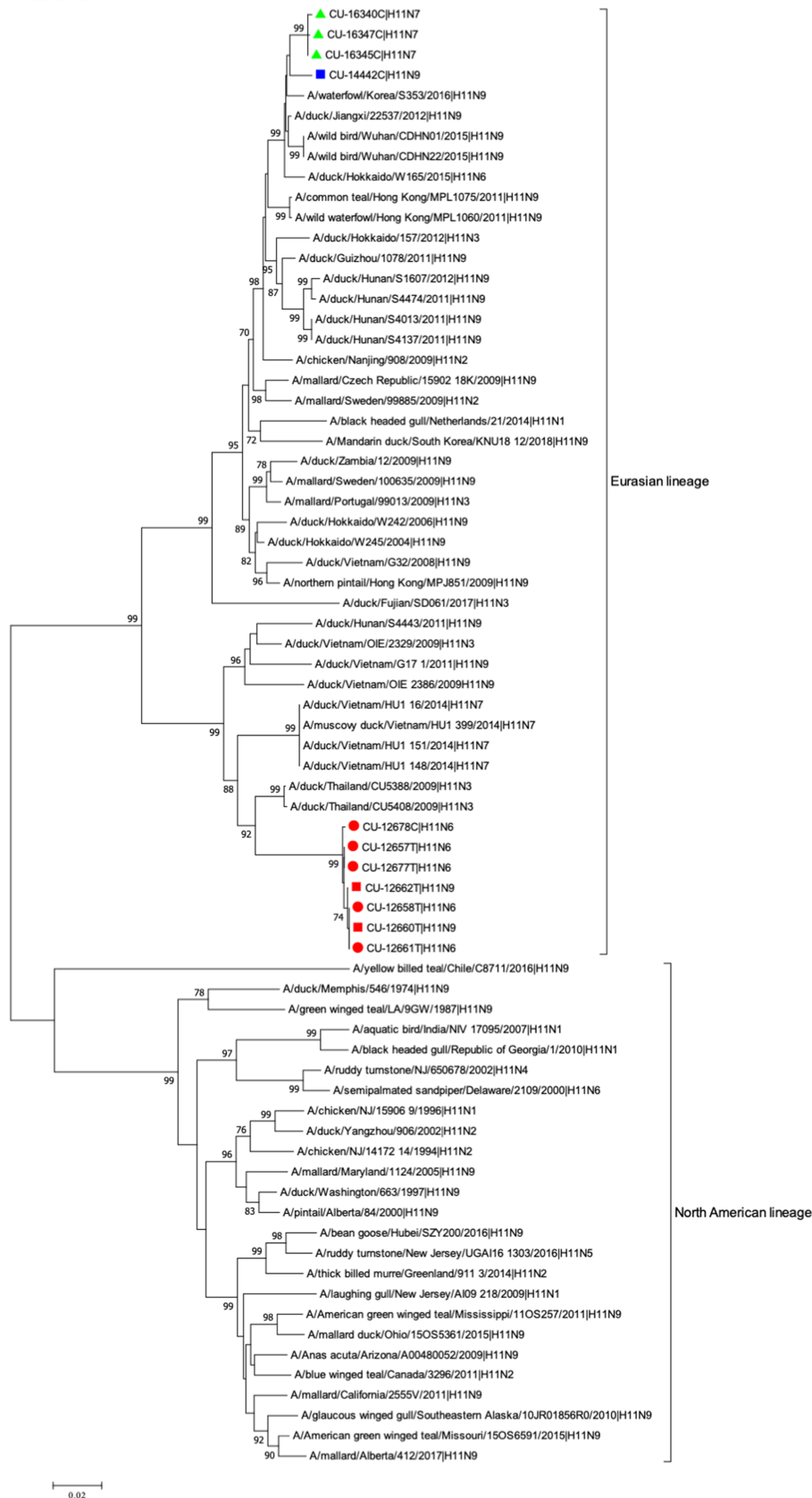
Phylogenetic trees of internal gene segments of IAV-H11 revealed possible reassortment of Thai IAV-H11 (Table 2). The common origin of each gene lineages of Thai IAVs is Eurasian lineage (EA). However,

in this study, some internal gene segments of IAV-H11N9 (CU-12660) and IAV-H11N7 (CU-16340) were originated from uncommon Eurasian and North American lineages. Phylogenetically, the NS gene of IAV-H11N9 (CU-12660) belongs to the avian Eurasian lineage (allele B) (EA-B) but not common avian Eurasian lineage (allele A) (EA) (CU-12657, CU-12677, CU-14442, and CU-16340). The M gene of IAV-H11N7 (CU16340) belongs to the avian North American lineage (NA) which closely related to A/mallard/Hokkaido/24/2009 (H5N1). Phylogenetic tree of NP gene can be divided into four lineages: (1) North American lineage (NA), (2) Eurasian lineage (EA), (3) Asian group 1 (A1), and (4) Asian group 2 (A2). The NP genes of IAV-H11N9 (CU-12660) and H11N7 (CU-16340) belong to Asian group 1 (A1) and Asian group 2 (A2), respectively (Figure 4).

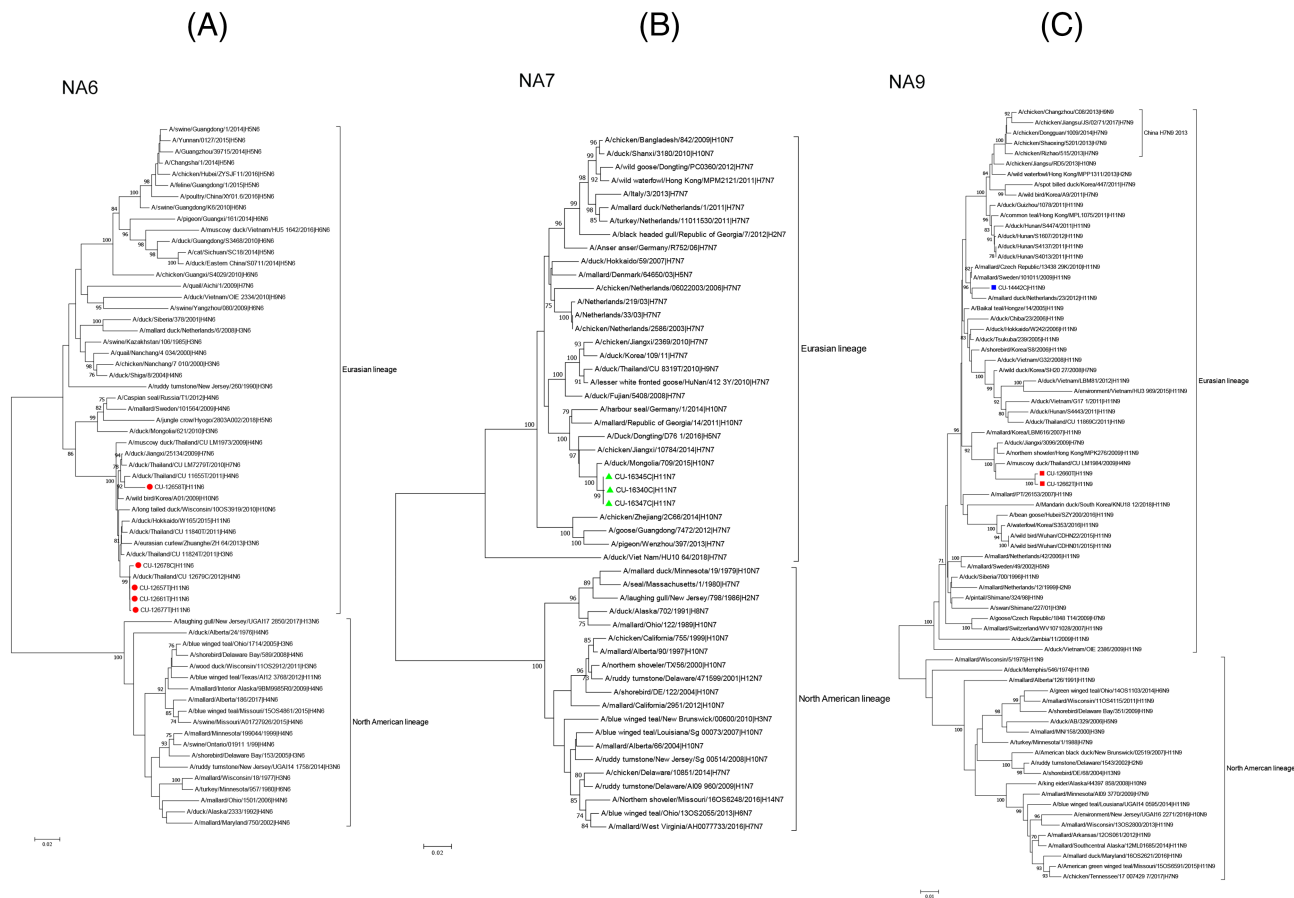
### 3.4 | Amino acid analysis of Thai IAV-H11

In this study, amino acid analysis for the translated genetic sequences of the Thai IAV-H11 was conducted. At the HA cleavage site, the

## HA11



**FIGURE 2** Phylogenetic tree of H11 gene of Thai IAV-H11. The phylogenetic tree was generated by the neighbor-joining algorithm with 1000 replications of bootstrap analysis by using the MEGA7.0 program. Red circle represents IAV-H11N6, red square represents IAV-H11N9 (subgroup 1), blue square represents IAV-H11N9 (subgroup 2), and green triangles represent IAV-H11N7



**FIGURE 3** (A) Phylogenetic tree of N6 gene of Thai IAV-H11. (B) Phylogenetic tree of N7 gene of Thai IAV-H11. (C) Phylogenetic tree of N9 gene of Thai IAV-H11. The phylogenetic tree was generated by the neighbor-joining algorithm with 1000 replications of bootstrap analysis by using the MEGA7.0 program. Red circle represents IAV-H11N6, red square represents IAV-H11N9 (subgroup 1), blue square represents IAV-H11N9 (subgroup 2), and green triangles represent IAV-H11N7

**TABLE 2** Genetic constellation of Thai IAV-H11 representing the lineages of all eight gene segments

Virus	Subtype	Year	Location	PB2	PB1	PA	HA	NP	NA	M	NS
CU-12657 (H11N6)	H11N6	2012	Ang Thong	EA	EA	EA	EA	EA	EA	EA	EA
CU-12677 (H11N6)	H11N6	2012	Ang Thong	EA	EA	EA	EA	EA	EA	EA	EA
CU-12660 (H11N9)	H11N9	2012	Ang Thong	EA	EA	EA	EA	A1	EA	EA	EA-B
CU-14442 (H11N9)	H11N9	2014	Sukhothai	EA	EA	EA	EA	EA	EA	EA	EA
CU-16340 (H11N7)	H11N7	2015	Kamphaengphet	EA	EA	EA	EA	A2	EA	NA	EA
CU-12658 (H11N6)	H11N6	2012	Ang Thong				EA		EA		
CU-12661 (H11N6)	H11N6	2012	Ang Thong				EA		EA		
CU-12678 (H11N6)	H11N6	2012	Ang Thong				EA		EA		
CU-12658 (H11N6)	H11N6	2012	Ang Thong				EA		EA		
CU-12662 (H11N9)	H11N9	2012	Ang Thong				EA		EA		
CU-16345 (H11N7)	H11N7	2015	Kamphaengphet				EA		EA		
CU-16347 (H11N7)	H11N7	2015	Kamphaengphet				EA		EA		

Abbreviations: A1, avian group 1; A2, avian group 2; EA, avian Eurasian lineage (allele A); EA-B; avian Eurasian lineage (allele B); NA, avian North American lineage.



**FIGURE 4** Phylogenetic tree of internal gene segments of Thai IAV-H11. The phylogenetic tree was generated by the neighbor-joining algorithm with 1000 replications of bootstrap analysis by using the MEGA7.0 program. Red circle represents IAV-H11N6, red square represents IAV-H11N9 (subgroup 1), blue square represents IAV-H11N9 (subgroup 2), and green triangles represent IAV-H11N7

**TABLE 3** Amino acid analysis of Thai IAV-H11 at the HA, NA, and internal gene segments

Virus	Subtype	Location	Host	Year	HA cleavage site 320-329	Receptor-binding site (RBS)										Right edge of RBS 134-138
						98	153	155	183	190	194	195	224-229	274		
North American consensus	H11Nx		Avian	—	PAIATR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
Eurasian consensus	H11Nx		Avian	—	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
Hokkaido/W165/15	H11N6	Japan	Duck	2015	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
Vietnam/HU1-399/14	H11N7	Vietnam	Duck	2014	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
Vietnam/HU1-151/14	H11N7	Vietnam	Duck	2014	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
Hongkong/MPL1075/11	H11N9	Hong Kong	Teal	2011	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
Hunan/S4443/11	H11N9	China	Duck	2011	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
Jiangxi/22537/12	H11N9	China	Duck	2012	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
Korea/KNU18-12/18	H11N9	Korea	Duck	2018	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
Rizhao/515/13	H7N9	China	Chicken	2013	PEIPKGR	Y	W	L	H	E	L	Y	NGLSGR	GVTSA		
Jiangsu/JS-02/17	H7N9	China	Chicken	2017	PEIPKGR	Y	W	L	H	E	L	Y	NGLSGR	GVTGA		
This study																
CU-12657 (H11N6)	H11N6	Ang Thong	Duck	2012	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
CU-12658 (H11N6)	H11N6	Ang Thong	Duck	2012	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
CU-12661 (H11N6)	H11N6	Ang Thong	Duck	2012	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
CU-12677 (H11N6)	H11N6	Ang Thong	Duck	2012	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
CU-12678 (H11N6)	H11N6	Ang Thong	Duck	2012	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
CU-12660 (H11N9)	H11N9	Ang Thong	Duck	2012	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
CU-12662 (H11N9)	H11N9	Ang Thong	Duck	2012	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAA		
CU-14442 (H11N9)	H11N9	Sukhothai	Duck	2014	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAS		
CU-16340 (H11N7)	H11N7	Kamphaengphet	Duck	2015	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAS		
CU-16345 (H11N7)	H11N7	Kamphaengphet	Duck	2015	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAS		
CU-16347 (H11N7)	H11N7	Kamphaengphet	Duck	2015	PAIASR	Y	W	I	H	E	L	Y	NGQAGR	GVTAS		
Virus	Subtype	Location	Host	Year	Stalk region 49-68			Oseltamivir resistance <sup>a</sup>								
North American consensus	H11Nx		Avian	—	No del			119	222	274	292	R				
Eurasian consensus	H11Nx		Avian	—	No del			E	I	H	R	R				
Hokkaido/W165/15	H11N6	Japan	Duck	2015	No del			E	I	H	R	R				
Vietnam/HU1-399/14	H11N7	Vietnam	Duck	2014	No del			E	I	H	R	R				
Vietnam/HU1-151/14	H11N7	Vietnam	Duck	2014	No del			E	I	H	R	R				
Hongkong/MPL1075/11	H11N9	Hong Kong	Teal	2011	No del			E	I	H	R	R				
Hunan/S4443/11	H11N9	China	Duck	2011	No del			E	I	H	R	R				
Jiangxi/22537/12	H11N9	China	Duck	2012	No del			E	I	H	R	R				

(Continues)



TABLE 3 (Continued)

Virus	Subtype	Location	Host	Year	Stalk region 49–68	Oseltamivir resistance <sup>a</sup>							
						119	222	274	292				
Korea/KNUI8-12/18	H11N9	Korea	Duck	2018	No del	E	I	H	R				
Rizhao/515/13	H7N9	China	Chicken	2013	No del	E	I	H	R				
Jiangsu/JS-02/17	H7N9	China	Chicken	2017	No del	E	I	H	R				
This study													
CU-12657 (H11N6)	H11N6	Ang Thong	Duck	2012	No del	E	I	H	R				
CU-12658 (H11N6)	H11N6	Ang Thong	Duck	2012	No del	E	I	H	R				
CU-12661 (H11N6)	H11N6	Ang Thong	Duck	2012	No del	E	I	H	R				
CU-12677 (H11N6)	H11N6	Ang Thong	Duck	2012	No del	E	I	H	R				
CU-12678 (H11N6)	H11N6	Ang Thong	Duck	2012	No del	E	I	H	R				
CU-12660 (H11N9)	H11N9	Ang Thong	Duck	2012	No del	E	I	H	R				
CU-12662 (H11N9)	H11N9	Ang Thong	Duck	2012	No del	E	I	H	R				
CU-14442 (H11N9)	H11N9	Sukhothai	Duck	2014	No del	E	I	H	R				
CU-16340 (H11N7)	H11N7	Kamphaengphet	Duck	2015	No del	E	I	H	R				
CU-16345 (H11N7)	H11N7	Kamphaengphet	Duck	2015	No del	E	I	H	R				
CU-16347 (H11N7)	H11N7	Kamphaengphet	Duck	2015	No del	E	I	H	R				
Virus	Subtype	Location	Host	Year	M gene	Amantadine resistance <sup>b</sup>		PB2		NS1			
						26	27	30	31	34	627	701	80–84
North American consensus	H11Nx		Avian	–	Q	R	D	V	G	E	D	No del	D
Eurasian consensus	H11Nx		Avian	–	Q	R	D	V	G	E	D	No del	D
Hokkaido/W165/15	H11N6	Japan	Duck	2015	Q	R	D	V	G	E	D	No del	D
Vietnam/HU1-399/14	H11N7	Vietnam	Duck	2014	Q	R	D	V	G	E	NA	No del	D
Vietnam/HU1-151/14	H11N7	Vietnam	Duck	2014	Q	R	D	V	G	E	D	No del	D
Hongkong/MPL1075/11	H11N9	Hong Kong	Teal	2011	Q	R	D	V	G	E	D	No del	D
Hunan/S4443/11	H11N9	China	Duck	2011	Q	R	D	V	G	E	D	No del	D
Jiangxi/22537/12	H11N9	China	Duck	2012	Q	R	D	V	G	E	D	No del	D
Korea/KNUI8-12/18	H11N9	Korea	Duck	2018	Q	R	D	V	G	E	D	No del	D
Rizhao/515/13	H7N9	China	Chicken	2013	Q	R	D	V	G	E	D	No del	D
Jiangsu/JS-02/17	H7N9	China	Chicken	2017	Q	R	D	V	G	E	D	No del	D
This study													
CU-12657 (H11N6)	H11N6	Ang Thong	Duck	2012	Q	R	D	V	G	E	D	No del	D
CU-12658 (H11N6)	H11N6	Ang Thong	Duck	2012	–	–	–	–	–	–	–	–	–
CU-12661 (H11N6)	H11N6	Ang Thong	Duck	2012	–	–	–	–	–	–	–	–	–

(Continues)

TABLE 3 (Continued)

Virus	Subtype	Location	Host	Year	M gene					PB2		NS1	
					Amantadine resistance <sup>b</sup>					Virulence determinant		Virulence determinant	
					26	27	30	31	34	627	701	80-84	92
CU-12677 (H11N6)	H11N6	Ang Thong	Duck	2012	Q	R	D	V	G	E	D	No del	D
CU-12678 (H11N6)	H11N6	Ang Thong	Duck	2012	-	-	-	-	-	-	-	-	-
CU-12660 (H11N9)	H11N9	Ang Thong	Duck	2012	Q	R	D	V	G	E	D	No del	D
CU-12662 (H11N9)	H11N9	Ang Thong	Duck	2012	-	-	-	-	-	-	-	-	-
CU-14442 (H11N9)	H11N9	Sukhothai	Duck	2014	Q	R	D	V	G	E	D	No del	D
CU-16340 (H11N7)	H11N7	Kamphaengphet	Duck	2015	Q	R	D	V	G	E	D	No del	D
CU-16345 (H11N7)	H11N7	Kamphaengphet	Duck	2015	-	-	-	-	-	-	-	-	-
CU-16347 (H11N7)	H11N7	Kamphaengphet	Duck	2015	-	-	-	-	-	-	-	-	-

<sup>a</sup>Osetamivir resistant amino acids: E119V, I222L, H274Y, and R292K.

<sup>b</sup>Amantadine resistant amino acids: Q26F, R27A, D30T/V, V31N, and G34E.

IAV-H11 possessed “PAIASR/GLF” suggesting low pathogenic avian influenza (LPAI) characteristic. Analysis of receptor binding site showed that Thai IAV-H11 contained Q226 and G228 suggesting preferential binding for  $\alpha$  2-3-linked sialic acid receptor or avian receptor.<sup>28,29</sup> Moreover, Thai IAV-H11N7 had single amino acid difference near the receptor binding site (134–138; GVTAS) which is different from that of other IAV-H11 (134–138; GVTA) (Table 3). For NA gene analysis, the Thai IAV-H11 did not contain amino acid deletion in the NA stalk region and amino acid substitutions associated with neuraminidase resistance suggesting that the IAV-H11 were sensitive to Osetamivir.<sup>30,31</sup> For PB2 gene analysis, the PB2-627 of IAV-H11 contained glutamic acid (E), whereas one IAV-H11N9 (CU-12660) contained glycine (G).<sup>32,33</sup> Interestingly, glycine at PB2-627 position is very rare because most amino acid at PB2-627 is 627E in avian viruses and 627K in mammalian viruses (Table 3).

The analysis of genomic signatures related to host specificity of the internal gene segments of IAV-H11 showed that the IAV-H11N9 (CU14442) contained lysine (K) at position NP109, which is rare in avian viruses (Table 4). For IAV-H11N7 (CU16340), the virus contained four unusual amino acids at NP293 (K), NP305 (H), NP313 (L), and NP455 (E), which NP293 (K) and NP455 (E) were predominantly observed in human viruses. It is noted that NP305 (H) and NP313 (L) were rarely reported in any IAVs in the GenBank (Table 3).

## 4 | DISCUSSION

FGD is one of the important reservoirs for influenza virus. FGDs can be infected with IAVs without clinical signs. Thus, FGDs can receive and/or spread IAVs to and/or from wild birds, domestic birds, domestic animals, and humans.<sup>15,17</sup> In Thailand, during HPAI-H5N1 outbreaks, the FGD production system was considered to be an important potential risk pathway for HPAI-H5N1 outbreaks.<sup>14</sup> The HPAI-H5N1 infected ducks showed no clinical signs with low mortality and morbidity. As FGDs can carry IAVs without clinical appearance and their nature of frequent movement among rice fields, these factors contributed to increasing risk of IAVs to widely spread in the country.<sup>16</sup> Since 2008, the HPAI-H5N1 outbreak has not been reported in Thailand; however, surveillance of IAV in FGDs has been routinely conducted to monitor the status of HPAI and LPAI viruses circulating in the country.

FGDs shared the habitats (rice fields) with wild birds and other domestic animals. Sharing habitat between wild birds and FGDs increased the risk of influenza virus transmission between both populations. For example, there was evidence that identical LPAI viruses could be isolated from both domestic ducks and wild birds in China. In Thailand, IAV subtypes H4N6, H4N9, H7N6, and H10N3 have been reported in Muscovy ducks housing in live bird markets.<sup>34,35</sup> Additionally, other LPAI viruses, IAV-H12N1, were isolated from watercock and lesser-whistling ducks.<sup>35</sup> There are also several reports of IAVs circulating in ducks in Asia, for example, IAV-H5N6 in Vietnam, IAV-H7N4 in Cambodia, and IAV-H5N3 in waterfowl and domestic ducks in China.<sup>36,37</sup> From the surveillance of IAVs in FGDs

TABLE 4 Amino acid analysis of genomic signatures of avian IAV-H11

Virus	Subtype	Location	PB2																PB1			
			44	199	271	475	567	588	613	627	702	327	336	372	422	442	455					
Avian IAVs			A	A	T	L	D	A	V	E	K	R	V									
Human IAVs			S	S	A	M	N	I	T	K	R	K	I									
pdmH1N1 2009	H1N1	Ang Thong	A	A	A	L	D	T	V	E	K	R	I									
CU-12657 (H11N6)	H11N6	Ang Thong	A	A	T	L	D	A	V	E	K	R	V									
CU-12677 (H11N6)	H11N6	Ang Thong	A	A	T	L	D	A	V	E	K	R	V									
CU-12660 (H11N9)	H11N9	Ang Thong	A	A	T	L	D	A	V	G	K	R	V									
CU-14442 (H11N9)	H11N9	Sukhothai	A	A	T	L	D	A	V	E	K	R	V									
CU-16340 (H11N7)	H11N7	Kamphaengphet	A	A	T	L	D	A	V	E	K	R	V									
NP																						
Virus	Subtype	Location	16	61	100	109	214	283	293	305	313	357	372	422	442	455						
Avian IAVs			G	V	I	R	R	L	R	R	F	Q	E	R	T	D						
Human IAVs			D	I	L	V	K	P	K	K	Y	K	D	K	A	E						
pdmH1N1 2009	H1N1	Ang Thong	G	I	I	V	R	L	R	K	V	K	E	R	T	D						
CU-12657 (H11N6)	H11N6	Ang Thong	G	V	I	R	R	L	R	R	F	Q	E	R	T	D						
CU-12677 (H11N6)	H11N6	Ang Thong	G	V	I	R	R	L	R	R	F	Q	E	R	T	D						
CU-12660 (H11N9)	H11N9	Ang Thong	G	V	I	R	R	L	R	R	F	Q	E	R	T	D						
CU-14442 (H11N9)	H11N9	Sukhothai	G	V	I	K	R	L	R	R	F	Q	E	R	T	D						
CU-16340 (H11N7)	H11N7	Kamphaengphet	G	V	I	R	R	L	K	H	L	Q	E	R	T	E						
M1																			NS1			
Virus	Subtype	Location	115	121	137	11	20	57	86	93	81	227	107									
Avian IAVs			V	T	T	T	S	Y	V	N	I	E	L									
Human IAVs			I	A	A	I	N	H	A	S	M	R	F									
pdmH1N1 2009	H1N1	Ang Thong	V	T	T	T	S	Y	V	N	I	del	L									
CU-12657 (H11N6)	H11N6	Ang Thong	V	T	T	T	S	Y	V	N	I	E	L									
CU-12677 (H11N6)	H11N6	Ang Thong	V	T	T	T	S	Y	V	N	I	E	L									
CU-12660 (H11N9)	H11N9	Ang Thong	V	T	T	T	S	Y	V	N	I	E	L									
CU-14442 (H11N9)	H11N9	Sukhothai	V	T	T	T	S	Y	V	N	I	E	L									
CU-16340 (H11N7)	H11N7	Kamphaengphet	V	T	T	T	S	Y	V	N	I	E	L									

Abbreviation: IAV, influenza A virus.

in Thailand during 2012–2015, we able to identify IAV-H11 ( $n = 11$ ). In 2012, the IAV-H11N6 ( $n = 5$ ) and IAV-H11N9 ( $n = 2$ ) could be isolated from FGD flocks in Ang Thong province. Then, in 2014, the IAV-H11N9 ( $n = 1$ ) was detected in FGD flock in Sukhothai province. In 2015, we identified IAV-H11N7 ( $n = 3$ ) in FGD flocks in Kamphaengphet province. Our results suggested that IAV-H11 were constantly circulating in FGDs located in several provinces in Thailand during this period.

The phylogenetic analysis of Thai IAV-H11 revealed two novel reassortant IAV-H11 including rIAV-H11N9 (CU12660) and rIAV-H11N7 (CU16340). It has been known that the common lineages of eight gene segments of Thai IAVs is Eurasian lineage (EA); however, in this study, the novel reassortant IAV-H11 contained gene segments originating from uncommon lineages. For example, the NP gene of IAV-H11N9 (CU12660) and IAV-H11N7 (CU16340) clusters with Asian group 1 (A1) and Asian group 2 (A2), respectively. Both groups were diverged from avian lineage before the separation of the North American (NA) and Eurasian lineage (EA). The Thai IAV-H11N9 (CU12660) was clustered in Asian group 1 (A1) which includes IAV-H3N8 from Vietnam and IAV-H7N3 and IAV-H7N7 from China,<sup>38</sup> and Thai IAV-H11N7 (CU16340) was clustered in Asian group 2 (A2), which included IAV-H5N2 from China and Korea.<sup>39,40</sup> Interestingly, the M gene of IAV-H11N7 (CU16340) was grouped into North American lineage (NA). The NS gene of IAV-H11N9 (CU12660) was grouped into EA (allele B), whereas other Thai IAVs were grouped into EA (allele A) (EA). Because novel reassortment of Thai IAV-H11 have been observed in this study suggesting that IAV-H11 with internal gene segments originated from several sources are circulating in FGDs in Thailand, it should also be noted that novel reassortment of Thai IAV-H11 was identified based on whole genome sequencing of five IAV-H11. On the other hands, we could not conclude the reassortment of other IAV-H11 with select gene segments (HA and NA) characterization.

Because most reported IAV-H11 have been isolated from the Anseriformes order and few IAV-H11N9 was detected in Charadriiformes order and rarely in Galliformes order.<sup>41</sup> The FGDs are one of the reservoir species that are frequently infected with IAV-H11. It has been reported that IAV-H11 has zoonotic potential. There is evidence of IAV-H11 exposure in humans, for example, seropositivity of H11 antibody among chicken growers, duck hunters, and wildlife professionals.<sup>8</sup> In this study, Thai IAV-H11 did not contain some virulence determinants, for example, PB2-627 mutation (E627K), which relating to viral replication and more virulence of IAVs in mammals.<sup>42,43</sup> However, the Thai IAV-H11N9 (CU12660) contained glycine (G) at PB2-627, which is rarely reported in avian viruses and never been reported in mammalian viruses and need further investigation. For the analysis of genomic signatures, PA and NP gene segments of Thai IAV-H11 contained identical amino acids in both avian and human viruses.

In summary, this study provided genetic information of Thai IAV-H11 isolated from FGDs. The Thai IAV subtypes H11N6, H11N7, and H11N9 were characterized. Phylogenetic analysis showed that some IAV-H11N9 and IAV-H11N7 are novel reassortant viruses in

Thailand. From amino acid analysis, the HA cleavage site and receptor binding sites of IAV-H11 showed low pathogenic characteristics suggesting less potential to be zoonotic or virulence viruses. Novel reassortant IAV-H11N9 and IAV-H11N7 suggested that IAVs originated from several sources are circulating in FGDs in Thailand.

## ACKNOWLEDGEMENTS

We would like to thank Chulalongkorn University for its financial support to the Center of Excellence for Emerging and Re-emerging Infectious Diseases in Animals (CUEDIAs) and the One Health Research Cluster. We thank the 90th Anniversary of Chulalongkorn University Fund for scholarship to the first author (GCUGR1125614076D) and the Thailand Research Fund for the Royal Golden Jubilee (RGJ) Ph.D. scholarship to the first author (PHD/O181/2553). This study was financially supported by the Chulalongkorn University for the research fund under the TSRI fund (CU\_FRB640001\_01\_31\_1 and CUFRB65\_Food\_182\_31\_01), the Agricultural Research Development Agency (ARDA) fund (PRP6405031220), and the PMU-B (NXPO) fund (B17F640011).

## AUTHOR CONTRIBUTIONS

**Supassama Chaiyawong:** Data curation; formal analysis; investigation; methodology. **Kamonpan Charoenkul:** Investigation; methodology; visualization. **Kitikhun Udom:** Investigation; methodology; visualization. **Ekkapat Chamsai:** Investigation; methodology; software. **Waleemas Jairak:** Formal analysis; investigation; methodology; validation; visualization. **Supanat Boonyapisitsopa:** Investigation; methodology. **Napawan Bunpapong:** Investigation; methodology. **Alongkorn Amonsin:** Conceptualization; formal analysis; supervision; validation.

## PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/irv.12960>.

## DATA AVAILABILITY STATEMENT

The nucleotide sequence data that support the findings of this study are openly available in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>), under accession numbers MW857483-857534.

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## SUPPORTING INFORMATION

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**How to cite this article:** Chaiyawong S, Charoenkul K, Udom K, et al. Genetic characterization of influenza A virus subtypes H11N6, H11N7, and H11N9 isolated from free-grazing ducks, Thailand. *Influenza Other Respi Viruses.* 2022; 16(4):726-739. doi:[10.1111/irv.12960](https://doi.org/10.1111/irv.12960)