Original Article

Genetic characterization of LPAI-H4N2 and H4N8 viruses recovered from free-grazing ducks in Thailand

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Abstract

Influenza A viruses (IAVs) can infect birds and various mammals. In this study, we retrospectively isolated and characterized IAVs (n=8) from oropharyngeal and cloacal swab samples of free-grazing ducks collected during December 2018 to December 2021, by using egg inoculation and whole-genome sequencing. The IAVs could be subtyped as IAV-H4N2 (n = 3) and IAV-H4N8 (n = 5) using real-time RT-PCR assays. Genetic and phylogenetic analyses revealed that all eight genes of Thai IAV-H4 belong to the Avian Eurasian lineage. Both Thai IAV-H4N2 and IAV-H4N8 exhibited low pathogenic avian influenza (LPAI) characteristics and were susceptible to oseltamivir and amantadine. This study did not detect any reassortant viruses among IAV-H4N2 and IAV-H4N8. Overall, our findings suggest that IAV-H4N2 and IAV-H4N8 are endemic and circulating among free-grazing ducks in Thailand. Therefore, routine surveillance of IAVs in free-grazing ducks is necessary to monitor the emergence of new reassortant and potential virulent viruses.

Keywords: Characterization, Free-grazing ducks, Genetic, H4N2, H4N8

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Introduction

Influenza A virus (IAV) can infect various animal species, including avian and mammalian species, as well as humans. IAVs are categorized into subtypes based on the hemagglutinin and neuraminidase proteins (Fouchier *et al.*, 2005). These subtypes, designated H1-H16 and N1-N9, are commonly found in avian species, particularly wild and aquatic birds. Waterfowl, especially free-grazing ducks (FGDs), serve as natural reservoirs for IAVs and can transmit the viruses to other species of birds and mammals. As FGDs serve as reservoirs and sources of gene pools for IAVs, novel reassortant IAVs could emerge within FGD populations (Su *et al.*, 2012; Hagag *et al.*, 2019; Yang *et al.*, 2019).

Influenza A virus subtype H4 (IAV-H4) has been detected in avian species worldwide, such as chickens, ducks, turkeys, shorebirds, and various wild birds. IAV-H4 is one of the most prevalent subtypes identified in wild birds in North America, Europe, and Asia (Okazaki *et al.*, 2000; Olsen *et al.*, 2006; Kang *et al.*, 2013). For instance, IAV-H4, which exhibits high genetic variation, has been reported in wild aquatic birds and migratory birds in the southwestern United States (Spackman *et al.*, 2005; Scotch *et al.*, 2014). In South America, IAV-H4N2 and IAV-H4N8 have been reported in wild birds in Argentina. The viruses had a genetic composition associated with those from the South American lineage (Rimondi *et al.*, 2018). In Europe, IAV-H4 was reported in the black-legged kittiwake in Norway (Toennessen *et al.*, 2011). In Asia, IAV-H4N6 was reported in domestic ducks in Vietnam. The viruses belonged to a single genetic group related to domestic ducks and wild birds across Asian countries (Kim *et al.*, 2013). IAV-H4 has also been reported in domestic ducks and chickens in live-bird markets and slaughter sites in Vietnam (Okamatsu *et al.*, 2013). Similarly, IAV-H4 has also been found in ducks, geese, and chickens in live bird markets in Southern China (Peng *et al.*, 2013; Luo *et al.*, 2017). In Korea, IAV-H4N2, H4N6, and H4N3 were identified in both domestic ducks and wild birds (Kang *et al.*, 2013). In Thailand, IAV-H4N6 and IAV-H4N9 were first reported in domestic ducks from live bird markets (Wisedchanwet *et al.*, 2011a; Wisedchanwet *et al.*, 2011b).

Interspecies transmission of IAV-H4 from avian species to other mammals has also been reported. For example, the avian-like IAV-H4N8 was detected in pigs in southern China (Su *et al.*, 2012). Another study reported a novel IAV-H4N1 isolated from pigs in central China, which caused severe respiratory syndrome in pigs. This IAV-H4N1 was closely related to the influenza virus found in migratory birds outside mainland China, indicating the transmission of IAVs from migratory birds to pigs (Hu *et al.*, 2012). IAV-H4N6 was reported in pigs with pneumonia in Canada. The viruses possessed genetic similarities to IAVs of the North American lineage (Karasin *et al.*, 2000). Similarly, IAV-H4N6 with avian lineage was identified in pigs with respiratory symptoms on farms in the Midwestern United States (Abente *et al.*, 2017). However, there is currently limited information about IAV-H4 infection in humans. For instance, a previous serological study demonstrated the presence of antibodies against H4 and H11 avian influenza viruses in chicken growers in the Middle East (Kayali *et al.*, 2011). In Italy, a serological study showed that IAV-H4N6 and antibodies against H4 can be detected in birds but not in humans with at-risk occupations (De Marco *et al.*, 2021). Another serological study in the US found antibodies against low-pathogenic avian H5, H6, and H7 viruses in bird hunters and poultry workers but did not detect antibodies against H4 viruses (Gray *et al.*, 2008). In this study, we retrospectively isolated and characterized IAV-H4N2 and IAV-H4N8 recovered from free-grazing ducks using whole-genome sequencing to assess the genetic diversity of the viruses.

Materials and Methods

Influenza A virus isolation and detection: Sample collection was conducted under the approval of the Chulalongkorn University Animal Care and Use Protocol, IACUC number 1831105. In this study, we retrospectively selected oropharyngeal swabs (OP) and cloacal swabs (CS) from 27 free-grazing duck (FGD) flocks (20 FGDs/flock) in 6 provinces of Thailand (Chai Nat, Suphan Buri, Ayutthaya, Nakhon Sawan, Sukhothai, and Phitsanulok) between December 2018 and December 2021. The inclusion criteria for sample selection for viral isolation are based on 1) history of Influenza A positive with high RNA quality (Low Ct value), 2) geographical locations (provinces), 3) year and date of sample collection. To isolate the influenza A virus (IAV), oropharyngeal swabs (OP) and cloacal swabs (CS) were subjected to virus isolation using egg inoculation (WHO, 2005). In brief, the sample supernatant was inoculated into three 9 to 11-day-old embryonated chicken eggs per sample. After incubation for 3 days, the allantoic fluid was tested for influenza virus using the Hemagglutination (HA) test. The HA-positive sample was determined by observing a hemagglutination titer of ≥2 HA units in the allantoic fluid. The presence of the influenza A virus was confirmed in HA-positive samples using real-time RT-PCR (rRT-PCR) specific to the Matrix (M) gene (Spackman *et al.*, 2002).

To confirm influenza A virus (IAVs), the positive allantoic samples were subjected to RNA extraction using a commercial DNA/RNA extraction kit (GENTi™ viral DNA/RNA, GeneAll®, South Korea), according to the manufacturer's instructions. The detection of IAV was performed using real-time RT-PCR (rRT-PCR) specific to the Matrix (M) gene with the SuperScript® III Platinum® One-Step Quantitative RT-PCR System (Invitrogen®) (Spackman *et al.*, 2002). In brief, the reagent mixture included 5 µl of RNA template, 1x master mix buffer, 0.8 µM of M gene-specific primers, 0.2 µM of probe, 0.6 mM $MgSO_4$, 1 unit of Superscript III reverse transcriptase, and RNase-free water, with a total volume of 50 µl. The rRT-PCR was conducted, which included reverse transcription at 50°C for 30 minutes, pre-denaturation at 95°C for 15 minutes, denaturation for 50 cycles at 95°C for 15 seconds, and annealing-extension at 60°C for 30 seconds. The result was interpreted based on the cycle threshold (Ct) value. Samples with a Ct value of

less than 36 were considered positive, those between 36 and 40 were considered suspected, and > 40 were considered negative.

Influenza A virus subtype identification and characterization: To subtype the influenza A virus (IAV), RNA samples were synthesized into cDNA using the Improm-II Reverse Transcription System (Promega, Madison, WI, USA). To identify the subtype of the influenza A virus, cDNA samples were subjected to PCR using specific primers for each subtype (H1-H15 and N1-N9) (Tsukamoto *et al*., 2008; VanDalen *et al*., 2008; Tsukamoto *et al*., 2009). In brief, a mixture of 30 µl was prepared, containing 1 µl of cDNA, 1x master mix buffer (Toptaq ™), 0.8 µM of primers for each subtype, and distilled water. The PCR was carried out under the following conditions: 94ºC for 3 minutes, followed by 35 cycles of 94ºC for 30 seconds, 50ºC (for H1-H15) or 45ºC (for N1-N9) for 30 seconds, and 72ºC for 30 seconds. The PCR product was then analyzed by gel electrophoresis.

To select the influenza A virus (IAV) for characterization, the criteria for selecting the viruses are based on 1) history of influenza A positive with high RNA quality (Low Ct value), and time of sample collection. To characterize the influenza A virus (IAV), eight genes of the IAVs were amplified using PCR with Toptaq master mix (Qiagen, Hilden, Germany) with previously reported and newly designed primer sets for influenza virus whole genome sequencing (Hoffmann *et al*., 2001). To perform the PCR, a mixture of 50 µl was prepared, containing 2 µl of cDNA, 1.2 µM of each forward and reverse primers, 1× Top Taq Master Mix (QIAGEN), 1x loading dye, and distilled water. The PCR was carried out under the following conditions: initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at a temperature dependent on the primers for 45 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. After PCR, the amplicons were visualized by gel electrophoresis. The amplicons were then purified using the Nucleospin® PCR clean-up kit and sequenced using the Oxford Nanopore sequencing with Rapid Sequencing Kit (Cat#SQK-RAD004) (Oxford Nanopore Technologies, Oxford, UK). The original FASTQ file was then mapped to the IAV reference sequences using CLC Genomic Workbench software version 20.0 (Qiagen, Hilden, Germany). Finally, the consensus sequence of each virus gene segment was exported in FASTA file format for analysis. To identify the nucleotide identities, the nucleotide BLAST tool was used to compare the nucleotide sequences of each gene segment against those of the reference IAVs. The deduced amino acid sequences of Thai IAV-H4 were aligned with those of the reference IAVs of different subtypes from Eurasian and North American lineages using MEGA X software (Kumar *et al*., 2018). The phylogenetic tree of each gene of IAVs characterized in the study was generated using the neighbor-joining algorithm with 1,000 bootstrap replications in MEGA X software (Kumar *et al*., 2018).

Results

In this study, eight IAV-H4 viruses were retrospectively isolated and subjected to whole genome sequencing (n=6) and HA, NA gene sequencing (n=2). In detail, the viruses IAV-H4N2 (n = 3) and IAV-H4N8 (n = 3) underwent whole-genome sequencing, and the other IAV-H4N8 (n = 2) were subjected to HA and NA gene sequencing due to the low quality of PCR product. The nucleotide sequences of IAV-H4N2 and IAV-H4N8 were submitted to the GenBank database under the accession ##OR512565-OR512616 (Table 1).

Nucleotide and phylogenetic analysis showed that the H4 gene of all Thai IAV-H4 viruses (n=8) possessed high nucleotide identities to IAVs of the Avian Eurasian lineage (A/mallard/Korea/CL45/2017 (H4N6) and A/mallard/South Korea/JB17-85/2019 (H4N6)) at 98.58%-98.70% (Table 2). The phylogenetic tree of the H4 gene showed that the viruses can be divided into the North American lineage and the Eurasian lineage. The Thai IAV-H4 (n=8) was clustered with the IAVs of the Avian Eurasian lineage. It is noted that Thai IAV-H4 could be further grouped into separate clusters, Clusters 1 and 2. Cluster 1, IAV-H4N2 from Chai Nat, the viruses were closely related to the Korean IAV-H4N6 (CL45) isolated in 2017, with a similarity of 98.58%-98.70%. Cluster 2, IAV-H4N8 from Suphan Buri, the viruses were closely related to the Korean IAV-H4N6 (JB17-85) isolated in 2019 (98.64%-98.70%). Notably, the H4 gene from IAV-H4N2 and IAV-H4N8 from this study were found to be in separate clusters from IAV-H4N6, which was previously isolated in Thailand (Figure 1 and Table 2).

Nucleotide and phylogenetic analysis of the N2 gene showed that the Thai IAV-H4N2 (n=3) were clustered in the Avian Eurasian lineage and were closely related to IAV-H4N2 (820) from Mongolia in 2019, with 99.08% to 99.15%. For the N8 gene, the phylogenetic tree revealed two major groups, North American and Eurasian lineages. The Thai IAV-H4N8 (n=5) clustered in the Avian Eurasian lineage and were closely related to IAV-H3N8 (KNU2019-52) from Korea in 2019, with a similarity of 98.51% to 98.66% (Figures 2 and 3, and Table 2).

Table 1 Description of Thai IAV-H4 from free-grazing ducks characterized in this study

Virus	Strain name	Date	Location	Source	GenBank#
CU-22668 (H4N2)*	A/duck/Thailand/CU-22668/2018	Nov 2018	Chai Nat	CS	OR512577- OR512584*
CU-22671 (H4N2)*	A/duck/Thailand/CU-22671/2018	Nov 2018	Chai Nat	CS	OR512585- OR512592*
CU-22673 (H4N2)*	A/duck/Thailand/CU-22673/2018	Nov 2018	Chai Nat	OP	OR512565- OR512572*
CU-25525 (H4N8)*	A/duck/Thailand/CU-25525/2020	Nov 2020	Suphan Buri	OP	OR512593- OR512600*
CU-25536 (H4N8)*	A/duck/Thailand/CU-25536/2020	Nov 2020	Suphan Buri	CS	OR512601- OR512608*
CU-25541 (H4N8)*	A/duck/Thailand/CU-25541/2020	Nov 2020	Suphan Buri	CS	OR512609- OR512616*
CU-25533 (H4N8)	A/duck/Thailand/CU-25533/2020	Nov 2020	Suphan Buri	CS	OR512573- OR512574
CU-25534 (H4N8)	A/duck/Thailand/CU-25534/2020	Nov 2020	Suphan Buri	OP	OR512575- OR512576

*WGS; characterization by whole genome sequencing

Phylogenetic analyses of the remaining six internal genes of IAV-H4N2 and IAV-H4N8 revealed that all internal genes clustered within the Avian Eurasian lineage, consistent with both HA and NA genes. Since the origin of each gene lineage of Thai IAV-H4 belongs to the Avian Eurasian lineage, there is no evidence of possible reassortment of the viruses characterized in this study. In detail, the NS gene of IAV-H4N2 and IAV-H4N8 belongs to the Avian Eurasian lineage (allele A) (EA-A). In contrast, the NS gene of IAV-H11N9, previously characterized in Thailand, clustered into the Avian North American lineage (allele B) (EA-B). The M gene of IAV-H4N2 and IAV-H4N8 belongs to the Avian Eurasian lineage, but in a separate cluster from the North American lineage. Similarly, the NP gene of IAV-H4N2 and IAV-H4N8 belongs to the Avian Eurasian lineage but in a separate subgroup of IAV-H5N1. PB2, PB1, and PA genes from Thai IAV-H4N2 and IAV-H4N8 belong to the Avian Eurasian lineage but are separated from the IAV-H5N1, IAV-H7N9, and IAV-H9N2 clusters (Figure 4).

Genetic analysis of the deduced amino acids of the Thai IAV-H4 showed that the viruses contained "PAKASR/GLF" at the HA cleavage site, similar to the IAV-H4 of the Eurasian consensus and differing from the IAV-H4 of the North American consensus (PEKATR/GLF). This HA cleavage site suggested Low Pathogenic Avian Influenza (LPAI) characteristics of Thai IAV-H4N2 and IAV-H4N8. The left and right edges of the receptor binding site of IAV-H4N2 and IAV-H4N8 contained RGQSGR (position 224-229) and GKSGA (position 134-138), which are observed in both IAV-H4 of Eurasian and North American viruses. It is noted that the Thai IAV-H4 contained Q226 and G228, suggesting preferential binding to the α 2-3-linked sialic acid receptor or avian receptor (Table 3). For NA gene analysis, the Thai IAV-H4 did not contain amino acid deletions in the NA stalk region compared to IAV-H9N2 (deletion at positions NA 63-65). No amino acid mutations at positions E119V, I222L, H274Y, and R292K are associated with oseltamivir resistance. For M gene analysis, there are no amino acid substitutions at positions Q26F, R27A, D30T/V, V31N, and G34E of the M2 protein, suggesting that the Thai IAV-H4N2 and IAV-H4N8 are not resistant to amantadine. For PB2 gene analysis, IAV-H4 contained E627 and D701, suggesting low pathogenic characteristics (Table 3). The genetic analysis of Thai IAV-H4 revealed that IAV-H4N2 and IAV-H4N8 contained signature amino acids associated with host specificity at PB2, PB1, NP, M1, and M2, similar to those found in avian IAVs consensus (Table 4).

Table 2 Nucleotide identities of the genome of Thai IAV-H4 (H4N2 and H4N8)

Virus	Gene	Closest reference virus	% nucleotide identity
CU-22668 (H4N2)	PB2	A/wild bird/Eastern China/1758/2017(H5N3)	99.25%
	PB1	A/duck/Yunnan/YN-E2/2011(H4N6)	98.20%
	PA	A/wild goose/dongting lake/121/2018(H6N2)	99.12%
	HA4	A/mallard/Korea/CL45/2017(H4N6)	98.58%
	NP	A/duck/Mongolia/200/2015(H3N8)	98.40%
	NA2	A/duck/Mongolia/820/2019(H4N2)	99.15%
	M	A/Spot-billed_duck/China/51(2)/2016(H3N8)	98.88%
	NS	A/duck/Cambodia/C70W14M/2018(H7N5)	99.40%
CU-22671 (H4N2)	PB2	A/wild bird/Eastern China/1758/2017(H5N3)	99.25%
	PB1	A/duck/Yunnan/YN-E2/2011(H4N6)	98.20%
	PA	A/wild goose/dongting lake/121/2018(H6N2)	99.02%
	HA4	A/mallard/Korea/CL45/2017(H4N6)	98.70%
	NP	A/duck/Mongolia/200/2015(H3N8)	98.40%
	NA2	A/duck/Mongolia/820/2019(H4N2)	99.08%
	M	A/Spot-billed_duck/China/51(2)/2016(H3N8)	98.88%
	NS	A/duck/Cambodia/C70W14M/2018(H7N5)	99.40%
CU-22673 (H4N2)	PB2	A/wild bird/Eastern China/1758/2017(H5N3)	99.21%
	PB1	A/duck/Yunnan/YN-E2/2011(H4N6)	98.17%
	PA	A/wild goose/dongting lake/121/2018(H6N2)	99.12%
	HA4	A/mallard/Korea/CL45/2017(H4N6)	98.70%
	NP	A/duck/Mongolia/200/2015(H3N8)	98.40%
	NA2	A/duck/Mongolia/820/2019(H4N2)	99.15%
	M	A/Spot-billed_duck/China/51(2)/2016(H3N8)	98.88%
	NS	A/duck/Cambodia/C70W14M/2018(H7N5)	99.40%
CU-25525 (H4N8)	PB2	A/Green-winged teal(Anas crecca)/South Korea/KNU 2019-69/2019(H6N2)	98.55%
	PB1	A/Wild Duck/South Korea/KNU2020-11/2020(H4N6)	99.21%
	PA	A/spot-billed duck/South Korea/JB32-105/2019(H4N2)	99.26%
	HA4	A/mallard/South Korea/JB17-85/2019(H4N6)	98.64%
	NP	A/Mallard(Anas platyrhynchos)/South Korea/KNU2019-51/2019(H5N3)	99.13%
	NA8	A/Mallard(Anas platyrhynchos)/South Korea/KNU2019-52/2019(H3N8)	98.66%
	M	A/mallard/Tottori/31C/2019(H7N7)	99.49%
	NS	A/Mallard(Anas platyrhynchos)/South Korea/KNU2021-48/2021(H7N7)	99.28%
CU-25536 (H4N8)	PB2	A/Green-winged teal(Anas crecca)/South Korea/KNU 2019-69/2019(H6N2)	98.64%
	PB1	A/Wild Duck/South Korea/KNU2020-11/2020(H4N6)	99.25%
	PA	A/spot-billed duck/South Korea/JB32-105/2019(H4N2)	99.30%
	HA4	A/mallard/South Korea/JB17-85/2019(H4N6)	98.70%
	NP	A/Mallard(Anas platyrhynchos)/South Korea/KNU2019-51/2019(H5N3)	99.06%
	NA8	A/Mallard(Anas platyrhynchos)/South Korea/KNU2019-52/2019(H3N8)	98.58%
	M	A/Anas poecilorhyncha/South Korea/JB32-81/2019(H11N2)	99.59%
	NS	A/Mallard(Anas platyrhynchos)/South Korea/KNU2021-48/2021(H7N7)	99.28%
CU-25541 (H4N8)	PB2	A/Green-winged teal(Anas crecca)/South Korea/KNU 2019-69/2019(H6N2)	98.53%
	PB1	A/Wild Duck/South Korea/KNU2020-11/2020(H4N6)	99.15%
	PA	A/spot-billed duck/South Korea/JB32-105/2019(H4N2)	99.21%
	HA4	A/mallard/South Korea/JB17-85/2019(H4N6)	98.70%
	NP	A/Mallard(Anas platyrhynchos)/South Korea/KNU2019-51/2019(H5N3)	99.13%
	NA8	A/Mallard(Anas platyrhynchos)/South Korea/KNU2019-52/2019(H3N8)	98.51%
	M	A/mallard/Tottori/31C/2019(H7N7)	99.49%
	NS	A/Mallard(Anas platyrhynchos)/South Korea/KNU2021-48/2021(H7N7)	99.28%
CU-25533 (H4N8)	HA4	A/mallard/South Korea/JB17-85/2019(H4N6)	98.69%
	NA8	A/Mallard(Anas platyrhynchos)/South Korea/KNU2019-52/2019(H3N8)	98.60%
CU-25534 (H4N8)	HA4	A/mallard/South Korea/JB17-85/2019(H4N6)	98.69%
	NA8	A/Mallard(Anas platyrhynchos)/South Korea/KNU2019-52/2019(H3N8)	98.60%

Figure 1 Phylogenetic tree of H4 gene of Thai IAV-H4. The phylogenetic tree of H4 gene was generated using the neighbor-joining algorithm with 1,000 bootstrap replications in the MEGA X program. IAV-H4N2 and IAV-H4N8 are represented by red circles (cluster 1) and blue squares (cluster 2), respectively.

Figure 2 Phylogenetic tree of N2 gene of Thai IAV-H4. The phylogenetic tree of N2 gene was generated using the neighbor-joining algorithm with 1,000 bootstrap replications in the MEGA X program. IAV-H4N2 are represented by red circles.

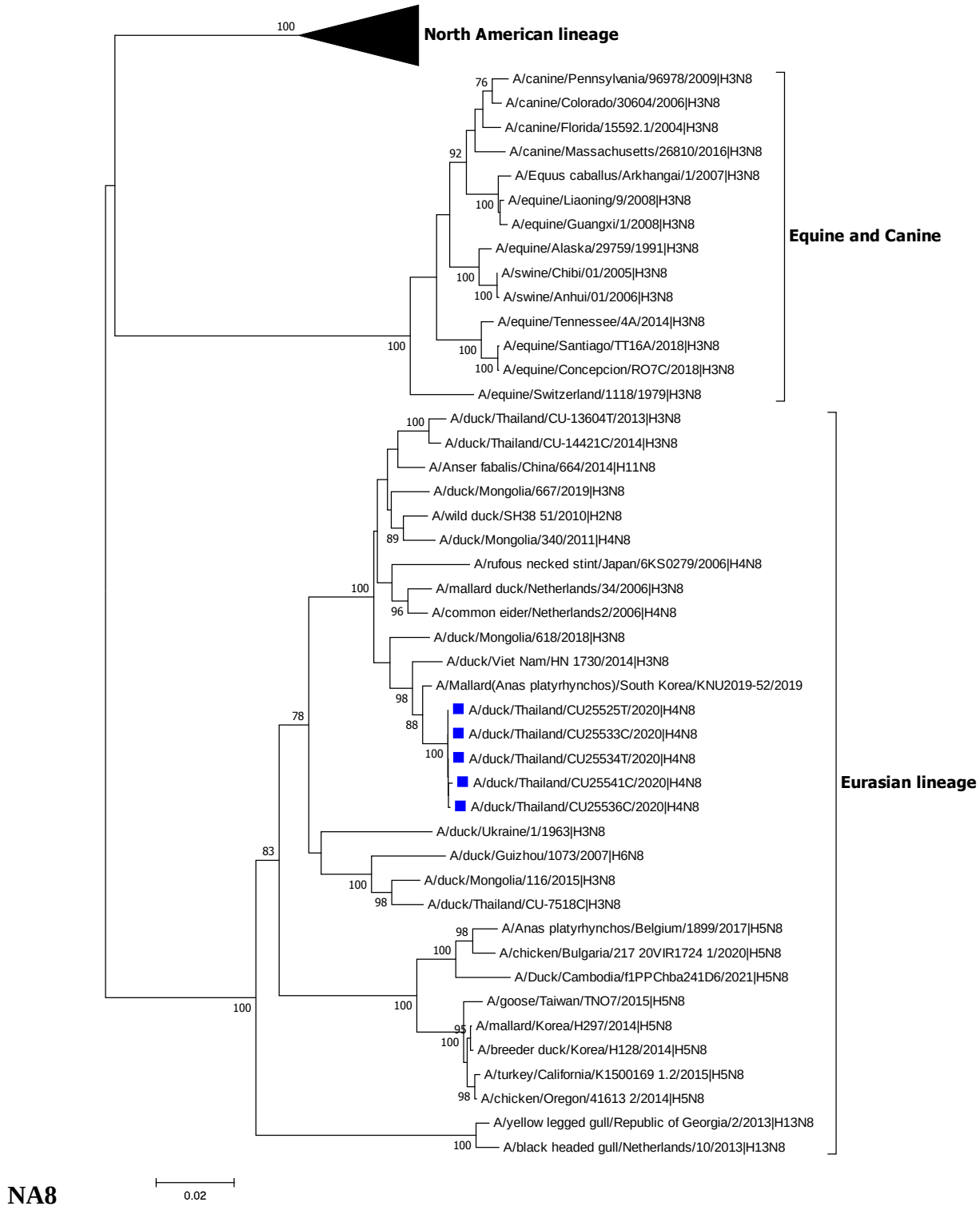


Figure 3 Phylogenetic tree of N8 gene of Thai IAV-H4. The phylogenetic tree of N8 gene was generated using the neighbor-joining algorithm with 1,000 bootstrap replications in the MEGA X program. IAV-H4N8 are represented by blue squares.

Table 3 Genetic analysis of Thai IAV-H4 at the HA, NA, and internal genes

Virus	Subtype	Location	Host	Year	HA cleavage site	Receptor -binding site (RBS)						Left edge of RBS	Right edge of RBS
H3 numbering system					**320-329**	**98**	**153**	**155**	**183**	**190**	**195**	**224-229**	**134-138**
H4 position					**338-343**	**110**	**165**	**167**	**196**	**203**	**208**	**237-242**	**146-150**
North American consensus	H4Nx		Avian	-	PEKATR	Y	W	T	H	E	Y	RGQSGR	GKSGA
Eurasian consensus	H4Nx		Avian	-	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA
A/duck/Zhejiang/77136/2014	H4N2		Duck	2014	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA
A/duck/Hokkaido/491003/2014	H4N2		Duck	2014	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA
A/duck/Mongolia/340/2011	H4N8		Duck	2011	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA
A/greylag goose/Changsha/CS-1983/2013	H4N8		Goose	2013	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA
A/wild waterfowl/Korea/F7-18/2018	H4N8		Waterfowl	2018	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA
A/duck/China/D4/2018	H9N2		Duck	2018	PSRSSR	Y	W	T	N	T	Y	NGQQGR	GTSTA
A/chicken/Dongguan/2701/2013	H9N2		Chicken	2013	PSRSSR	Y	W	T	N	A	Y	NGLMGR	GTSKA
A/chicken/Fujian/SD056/2017	H9N2		Chicken	2017	PSRSSR	Y	W	T	N	T	Y	NGLMGR	GTSAA
This study													
CU-22668 (H4N2)	H4N2	Chai Nat	Duck	2018	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA
CU-22671 (H4N2)	H4N2	Chai Nat	Duck	2018	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA
CU-22673 (H4N2)	H4N2	Chai Nat	Duck	2018	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA
CU-25525 (H4N8)	H4N8	Suphan Buri	Duck	2020	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA
CU-25536 (H4N8)	H4N8	Suphan Buri	Duck	2020	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA
CU-25541 (H4N8)	H4N8	Suphan Buri	Duck	2020	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA
CU-25533 (H4N8)	H4N8	Suphan Buri	Duck	2020	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA
CU-25534 (H4N8)	H4N8	Suphan Buri	Duck	2020	PEKASR	Y	W	V	H	E	Y	RGQSGR	GKSGA

Virus	Subtype	Location	Host	Year	Stalk region	Oseltamivir resistance*			
					63-65 (N2)	E119V (N2)	I222L (N2)	H274Y (N2, N8)	R292K (N2)
North American consensus	H4Nx		Avian	-	No del	E	I	H	R
Eurasian consensus	H4Nx		Avian	-	No del	E	I	H	R
A/duck/Zhejiang/77136/2014	H4N2		Duck	2014	No del	E	I	H	R
A/duck/Hokkaido/491003/2014	H4N2		Duck	2014	No del	E	I	H	R
A/duck/Mongolia/340/2011	H4N8		Duck	2011	No del	E	I	H	R
A/greylag goose/Changsha/CS-1983/2013	H4N8		Goose	2013	No del	E	I	H	R
A/wild waterfowl/Korea/F7-18/2018	H4N8		Waterfowl	2018	No del	E	I	H	R
A/duck/China/D4/2018	H9N2		Duck	2018	Del	E	I	H	R
A/chicken/Dongguan/2701/2013	H9N2		Chicken	2013	Del	E	I	H	R
A/chicken/Fujian/SD056/2017	H9N2		Chicken	2017	Del	E	I	H	R
This study									
CU-22668 (H4N2)	H4N2	Chai Nat	Duck	2018	No del	E	I	H	R
CU-22671 (H4N2)	H4N2	Chai Nat	Duck	2018	No del	E	I	H	R
CU-22673 (H4N2)	H4N2	Chai Nat	Duck	2018	No del	E	I	H	R
CU-25525 (H4N8)	H4N8	Suphan Buri	Duck	2020	No del	E	I	H	R
CU-25536 (H4N8)	H4N8	Suphan Buri	Duck	2020	No del	E	I	H	R
CU-25541 (H4N8)	H4N8	Suphan Buri	Duck	2020	No del	E	I	H	R
CU-25533 (H4N8)	H4N8	Suphan Buri	Duck	2020	No del	E	I	H	R
CU-25534 (H4N8)	H4N8	Suphan Buri	Duck	2020	No del	E	I	H	R

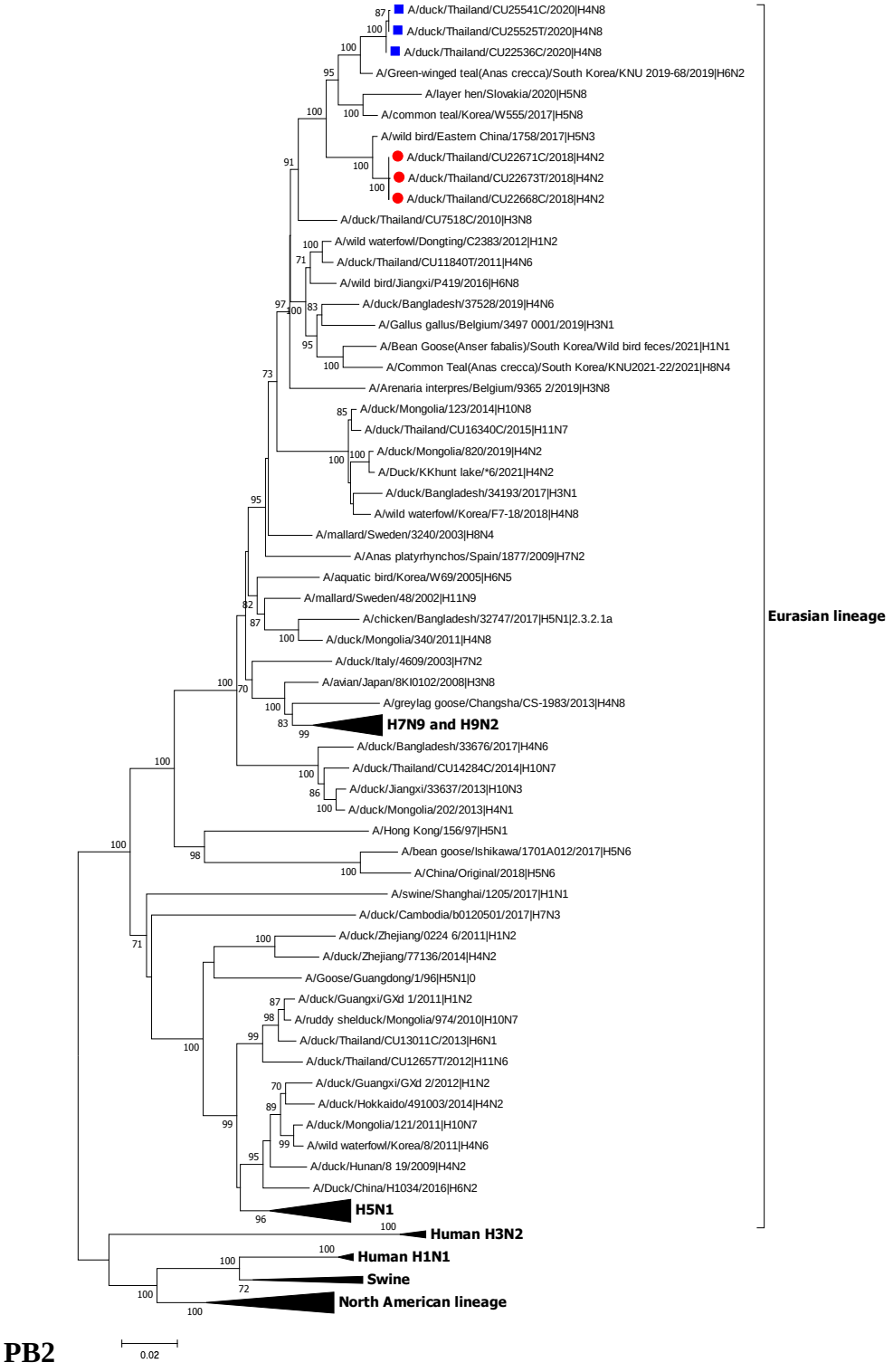
Virus	Subtype	Location	Host	Year	M gene					PB2		NS1	
					Amantadine resistance**					Virulence determinant		Virulence determinant	
					26	27	30	31	34	627	701	80-84	92
North American consensus	H4Nx		Avian	-	Q	R	D	V	G	E	D	No del	D
Eurasian consensus	H4Nx		Avian	-	Q	R	D	V	G	E	D	No del	D
A/duck/Zhejiang/77136/2014	H4N2		Duck	2014	Q	R	D	V	G	E	D	No del	D
A/duck/Hokkaido/491003/2014	H4N2		Duck	2014	Q	R	D	V	G	E	D	No del	D
A/duck/Mongolia/340/2011	H4N8		Duck	2011	Q	R	D	V	G	E	D	No del	D
A/greylag goose/Changsha/CS-1983/2013	H4N8		Goose	2013	Q	R	D	V	G	E	D	No del	D
A/wild waterfowl/Korea/F7-18/2018	H4N8		Waterfowl	2018	Q	R	D	V	G	E	D	No del	D
A/duck/China/D4/2018	H9N2		Duck	2018	Q	R	D	V	G	E	D	No del	D
A/chicken/Dongguan/2701/2013	H9N2		Chicken	2013	Q	R	D	V	G	E	D	No del	D
A/chicken/Fujian/SD056/2017	H9N2		Chicken	2017	Q	R	D	V	G	E	D	No del	D
This study													
CU-22668 (H4N2)	H4N2	Chai Nat	Duck	2018	Q	R	D	V	G	E	D	No del	D
CU-22671 (H4N2)	H4N2	Chai Nat	Duck	2018	Q	R	D	V	G	E	D	No del	D
CU-22673 (H4N2)	H4N2	Chai Nat	Duck	2018	Q	R	D	V	G	E	D	No del	D
CU-25525 (H4N8)	H4N8	Suphan Buri	Duck	2020	Q	R	D	V	G	E	D	No del	D
CU-25536 (H4N8)	H4N8	Suphan Buri	Duck	2020	Q	R	D	V	G	E	D	No del	D
CU-25541 (H4N8)	H4N8	Suphan Buri	Duck	2020	Q	R	D	V	G	E	D	No del	D
CU-25533 (H4N8)	H4N8	Suphan Buri	Duck	2020	-	-	-	-	-	-	-	-	-
CU-25534 (H4N8)	H4N8	Suphan Buri	Duck	2020	-	-	-	-	-	-	-	-	-

Table 4 Genetic analysis of Thai IAV-H4 at amino acids related to species preferences

Virus	Subtype	Location	PB2									PB1	
			44	199	271	475	567	588	613	627	702	327	336
Human IAVs			S	S	A	M	N	I	T	K	R	K	I
pdmH1N1 2009	H1N1		A	A	A	L	D	T	V	E	K	R	I
Avian IAVs			A	A	T	L	D	A	V	E	K	R	V
CU-22668 (H4N2)	H4N2	Chai Nat	A	A	T	L	D	A	V	E	K	R	V
CU-22671 (H4N2)	H4N2	Chai Nat	A	A	T	L	D	A	V	E	K	R	V
CU-22673 (H4N2)	H4N2	Chai Nat	A	A	T	L	D	A	V	E	K	R	V
CU-25525 (H4N8)	H4N8	Suphan Buri	A	A	T	L	D	A	V	E	K	R	V
CU-25536 (H4N8)	H4N8	Suphan Buri	A	A	T	L	D	A	V	E	K	R	V
CU-25541 (H4N8)	H4N8	Suphan Buri	A	A	T	L	D	A	V	E	K	R	V

Virus	Subtype	Location	NP													
			16	33	61	100	109	214	283	293	305	313	357	372	422	442	455
Human IAVs			D	I	L	V	V	K	P	K	K	Y	K	D	K	A	E
pdmH1N1 2009	H1N1		G	I	I	V	I	R	L	R	K	V	K	E	R	T	D
Avian IAVs			G	V	I	R	I	R	L	R	R	F	Q	E	R	T	D
CU-22668 (H4N2)	H4N2	Chai Nat	G	V	I	R	I	R	L	R	R	F	Q	E	R	T	D
CU-22671 (H4N2)	H4N2	Chai Nat	G	V	I	R	I	R	L	R	R	F	Q	E	R	T	D
CU-22673 (H4N2)	H4N2	Chai Nat	G	V	I	R	I	R	L	R	R	F	Q	E	R	T	D
CU-25525 (H4N8)	H4N8	Suphan Buri	G	V	I	R	I	R	L	R	R	F	Q	E	R	T	D
CU-25536 (H4N8)	H4N8	Suphan Buri	G	V	I	R	I	R	L	R	R	F	Q	E	R	T	D
CU-25541 (H4N8)	H4N8	Suphan Buri	G	V	I	R	I	R	L	R	R	F	Q	E	R	T	D

Virus	Subtype	Location	M1			M2					NS1		NS2
			115	121	137	11	20	57	86	93	81	227	107
Human IAVs			I	A	A	I	N	H	A	S	M	R	F
pdmH1N1 2009	H1N1		V	T	T	T	S	Y	V	N	I	del	L
Avian IAVs			V	T	T	T	S	Y	V	N	I	E	L
CU-22668 (H4N2)	H4N2	Chai Nat	V	T	T	T	S	Y	V	N	I	E	L
CU-22671 (H4N2)	H4N2	Chai Nat	V	T	T	T	S	Y	V	N	I	E	L
CU-22673 (H4N2)	H4N2	Chai Nat	V	T	T	T	S	Y	V	N	I	E	L
CU-25525 (H4N8)	H4N8	Suphan Buri	V	T	T	T	S	Y	V	N	I	E	L
CU-25536 (H4N8)	H4N8	Suphan Buri	V	T	T	T	S	Y	V	N	I	E	L
CU-25541 (H4N8)	H4N8	Suphan Buri	V	T	T	T	S	Y	V	N	I	E	L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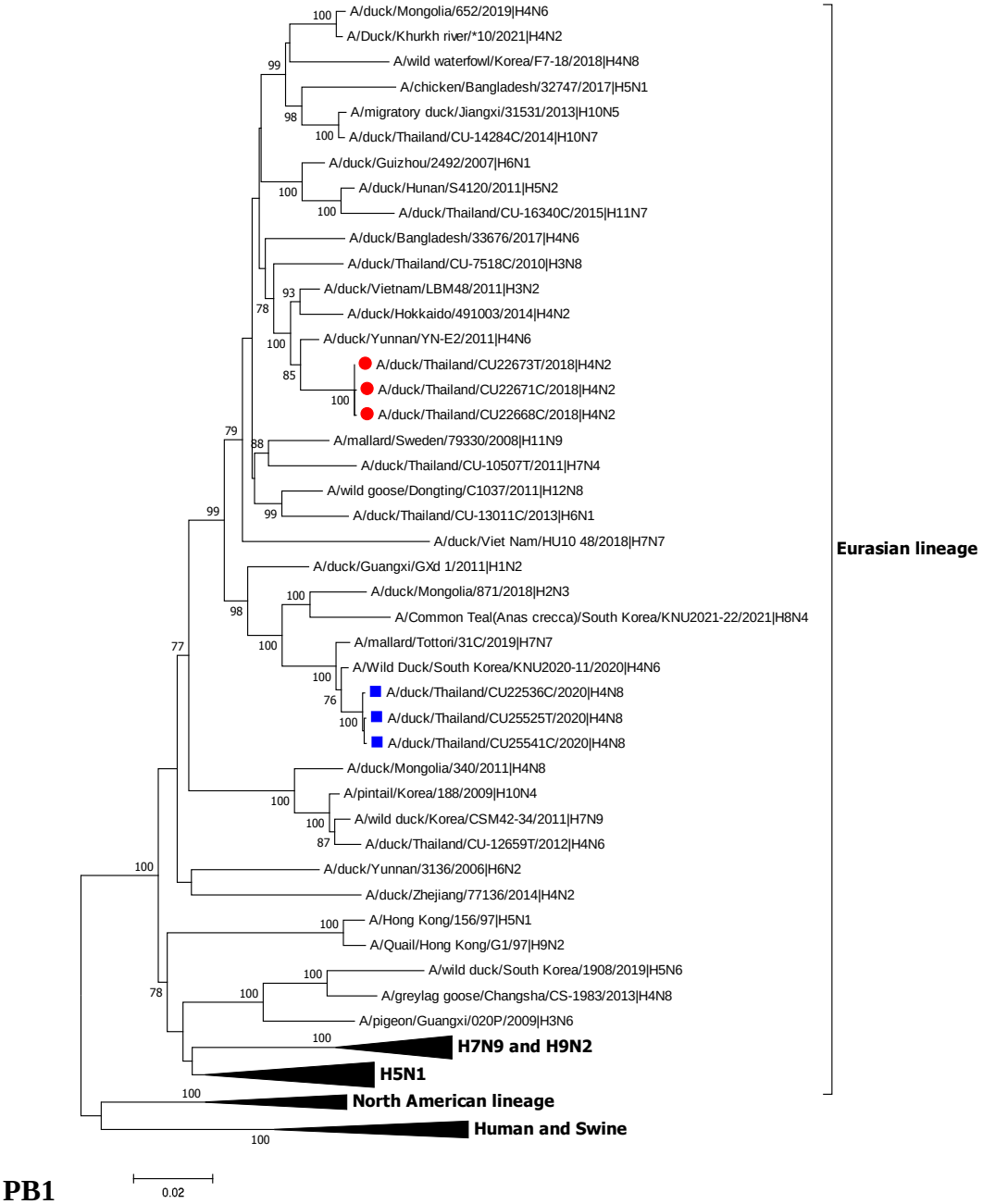
A/Green-winged teal(Anas crecca)/South Korea/KNU 2019-68/2019|H6N2
A/layer hen/Slovakia/2020|H5N8
A/common teal/Korea/W555/2017|H5N8
A/wild bird/Eastern China/1758/2017|H5N3
A/duck/Thailand/CU22671C/2018|H4N2
A/duck/Thailand/CU22673T/2018|H4N2
A/duck/Thailand/CU22668C/2018|H4N2
A/duck/Thailand/CU7518C/2010|H3N8
A/wild waterfowl/Dongting/C2383/2012|H1N2
A/duck/Thailand/CU11840T/2011|H4N6
A/wild bird/Jiangxi/P419/2016|H6N8
A/duck/Bangladesh/37528/2019|H4N6
A/Gallus gallus/Belgium/3497 0001/2019|H3N1
A/Bean Goose(Anser fabalis)/South Korea/Wild bird feces/2021|H1N1
A/Common Teal(Anas crecca)/South Korea/KNU2021-22/2021|H8N4
A/Arenaria interpres/Belgium/9365 2/2019|H3N8
A/duck/Mongolia/123/2014|H10N8
A/duck/Thailand/CU16340C/2015|H11N7
A/duck/Mongolia/820/2019|H4N2
A/Duck/KKhunt lake/*6/2021|H4N2
A/duck/Bangladesh/34193/2017|H3N1
A/wild waterfowl/Korea/F7-18/2018|H4N8
A/mallard/Sweden/3240/2003|H8N4
A/Anas platyrhynchos/Spain/1877/2009|H7N2
A/aquatic bird/Korea/W69/2005|H6N5
A/mallard/Sweden/48/2002|H11N9
A/chicken/Bangladesh/32747/2017|H5N1|2.3.2.1a
A/duck/Mongolia/340/2011|H4N8
A/duck/Italy/4609/2003|H7N2
A/avian/Japan/8KI0102/2008|H3N8
A/greylag goose/Changsha/CS-1983/2013|H4N8
H7N9 and H9N2
A/duck/Bangladesh/33676/2017|H4N6
A/duck/Thailand/CU14284C/2014|H10N7
A/duck/Jiangxi/33637/2013|H10N3
A/duck/Mongolia/202/2013|H4N1
A/Hong Kong/156/97|H5N1
A/bean goose/Ishikawa/1701A012/2017|H5N6
A/China/Original/2018|H5N6
A/swine/Shanghai/1205/2017|H1N1
A/duck/Cambodia/b0120501/2017|H7N3
A/duck/Zhejiang/0224 6/2011|H1N2
A/duck/Zhejiang/77136/2014|H4N2
A/Goose/Guangdong/1/96|H5N1|0
A/duck/Guangxi/GXd 1/2011|H1N2
A/ruddy shelduck/Mongolia/974/2010|H10N7
A/duck/Thailand/CU13011C/2013|H6N1
A/duck/Thailand/CU12657T/2012|H11N6
A/duck/Guangxi/GXd 2/2012|H1N2
A/duck/Hokkaido/491003/2014|H4N2
A/duck/Mongolia/121/2011|H10N7
A/wild waterfowl/Korea/8/2011|H4N6
A/duck/Hunan/8 19/2009|H4N2
A/Duck/China/H1034/2016|H6N2
H5N1
Human H3N2
Human H1N1
Swine
North American lineage
Eurasian lineage
0.02

PB2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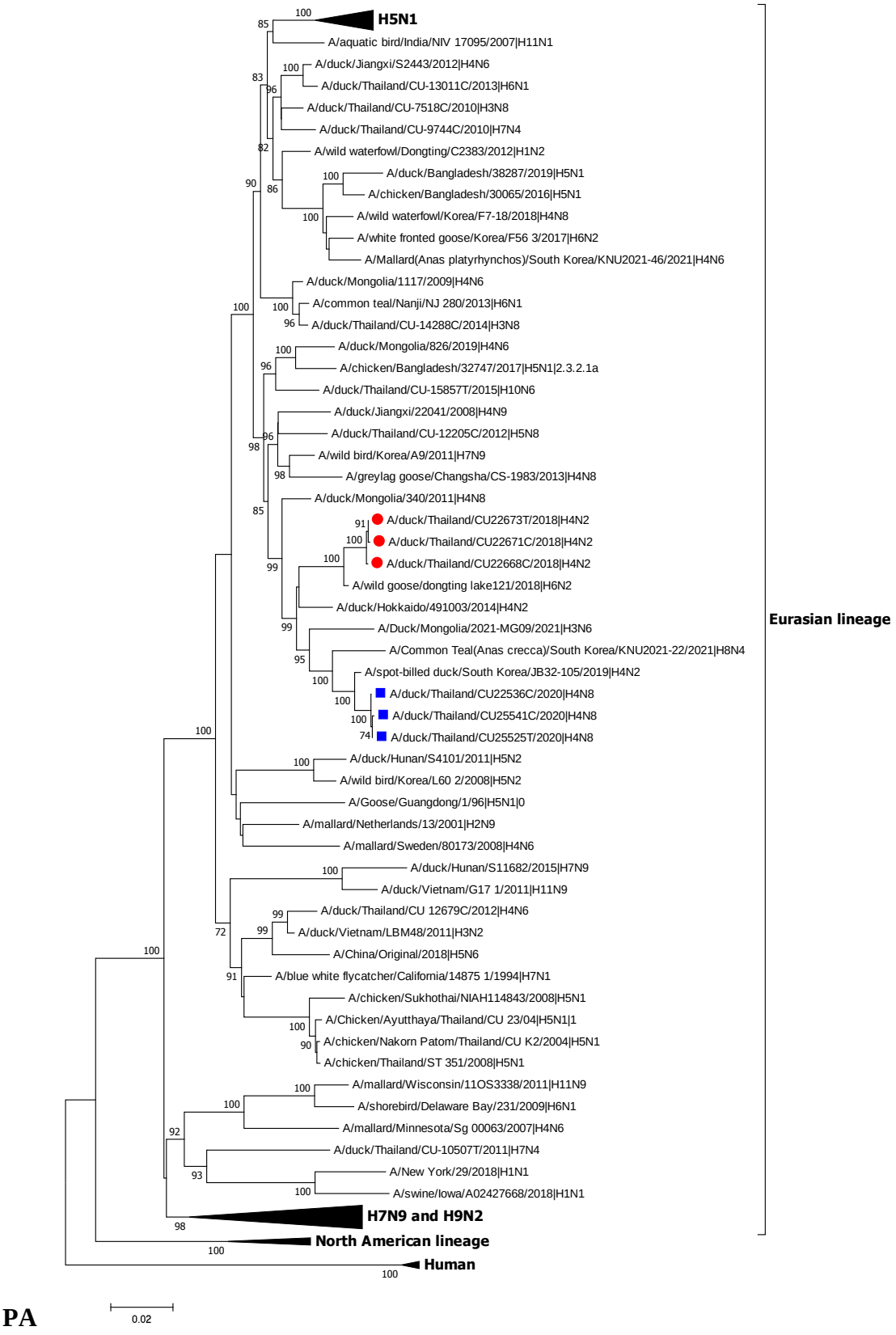
A/chicken/Bangladesh/32747/2017|H5N1
A/migratory duck/Jiangxi/31531/2013|H10N5
A/duck/Thailand/CU-14284C/2014|H10N7
A/duck/Guizhou/2492/2007|H6N1
A/duck/Hunan/S4120/2011|H5N2
A/duck/Thailand/CU-16340C/2015|H11N7
A/duck/Bangladesh/33676/2017|H4N6
A/duck/Thailand/CU-7518C/2010|H3N8
A/duck/Vietnam/LBM48/2011|H3N2
A/duck/Hokkaido/491003/2014|H4N2
A/duck/Yunnan/YN-E2/2011|H4N6
A/duck/Thailand/CU22673T/2018|H4N2
A/duck/Thailand/CU22671C/2018|H4N2
A/duck/Thailand/CU22668C/2018|H4N2
A/mallard/Sweden/79330/2008|H11N9
A/duck/Thailand/CU-10507T/2011|H7N4
A/wild goose/Dongting/C1037/2011|H12N8
A/duck/Thailand/CU-13011C/2013|H6N1
A/duck/Viet Nam/HU10 48/2018|H7N7
A/duck/Guangxi/GXd 1/2011|H1N2
A/duck/Mongolia/871/2018|H2N3
A/Common Teal(Anas crecca)/South Korea/KNU2021-22/2021|H8N4
A/mallard/Tottori/31C/2019|H7N7
A/Wild Duck/South Korea/KNU2020-11/2020|H4N6
A/duck/Thailand/CU22536C/2020|H4N8
A/duck/Thailand/CU25525T/2020|H4N8
A/duck/Thailand/CU25541C/2020|H4N8
A/duck/Mongolia/340/2011|H4N8
A/pintail/Korea/188/2009|H10N4
A/wild duck/Korea/CSM42-34/2011|H7N9
A/duck/Thailand/CU-12659T/2012|H4N6
A/duck/Yunnan/3136/2006|H6N2
A/duck/Zhejiang/77136/2014|H4N2
A/Hong Kong/156/97|H5N1
A/Quail/Hong Kong/G1/97|H9N2
A/wild duck/South Korea/1908/2019|H5N6
A/greylag goose/Changsha/CS-1983/2013|H4N8
A/pigeon/Guangxi/020P/2009|H3N6
H7N9 and H9N2
H5N1
North American lineage
Human and Swine
Eurasian lineage
0.02

PB1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A/duck/Thailand/CU-7518C/2010|H3N8
A/duck/Thailand/CU-9744C/2010|H7N4
A/wild waterfowl/Dongting/C2383/2012|H1N2
A/duck/Bangladesh/38287/2019|H5N1
A/chicken/Bangladesh/30065/2016|H5N1
A/wild waterfowl/Korea/F7-18/2018|H4N8
A/white fronted goose/Korea/F56 3/2017|H6N2
A/Mallard(Anas platyrhynchos)/South Korea/KNU2021-46/2021|H4N6
A/duck/Mongolia/1117/2009|H4N6
A/common teal/Nanji/NJ 280/2013|H6N1
A/duck/Thailand/CU-14288C/2014|H3N8
A/duck/Mongolia/826/2019|H4N6
A/chicken/Bangladesh/32747/2017|H5N1|2.3.2.1a
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A/duck/Jiangxi/22041/2008|H4N9
A/duck/Thailand/CU-12205C/2012|H5N8
A/wild bird/Korea/A9/2011|H7N9
A/greylag goose/Changsha/CS-1983/2013|H4N8
A/duck/Mongolia/340/2011|H4N8
A/duck/Thailand/CU22673T/2018|H4N2
A/duck/Thailand/CU22671C/2018|H4N2
A/duck/Thailand/CU22668C/2018|H4N2
A/wild goose/dongting lake121/2018|H6N2
A/duck/Hokkaido/491003/2014|H4N2
A/Duck/Mongolia/2021-MG09/2021|H3N6
A/Common Teal(Anas crecca)/South Korea/KNU2021-22/2021|H8N4
A/spot-billed duck/South Korea/JB32-105/2019|H4N2
A/duck/Thailand/CU22536C/2020|H4N8
A/duck/Thailand/CU25541C/2020|H4N8
A/duck/Thailand/CU25525T/2020|H4N8
A/duck/Hunan/S4101/2011|H5N2
A/wild bird/Korea/L60 2/2008|H5N2
A/Goose/Guangdong/1/96|H5N1|0
A/mallard/Netherlands/13/2001|H2N9
A/mallard/Sweden/80173/2008|H4N6
A/duck/Hunan/S11682/2015|H7N9
A/duck/Vietnam/G17 1/2011|H11N9
A/duck/Thailand/CU 12679C/2012|H4N6
A/duck/Vietnam/LBM48/2011|H3N2
A/China/Original/2018|H5N6
A/blue white flycatcher/California/14875 1/1994|H7N1
A/chicken/Sukhothai/NIAH114843/2008|H5N1
A/Chicken/Ayutthaya/Thailand/CU 23/04|H5N1|1
A/chicken/Nakorn Patom/Thailand/CU K2/2004|H5N1
A/chicken/Thailand/ST 351/2008|H5N1
A/mallard/Wisconsin/11OS3338/2011|H11N9
A/shorebird/Delaware Bay/231/2009|H6N1
A/mallard/Minnesota/Sg 00063/2007|H4N6
A/duck/Thailand/CU-10507T/2011|H7N4
A/New York/29/2018|H1N1
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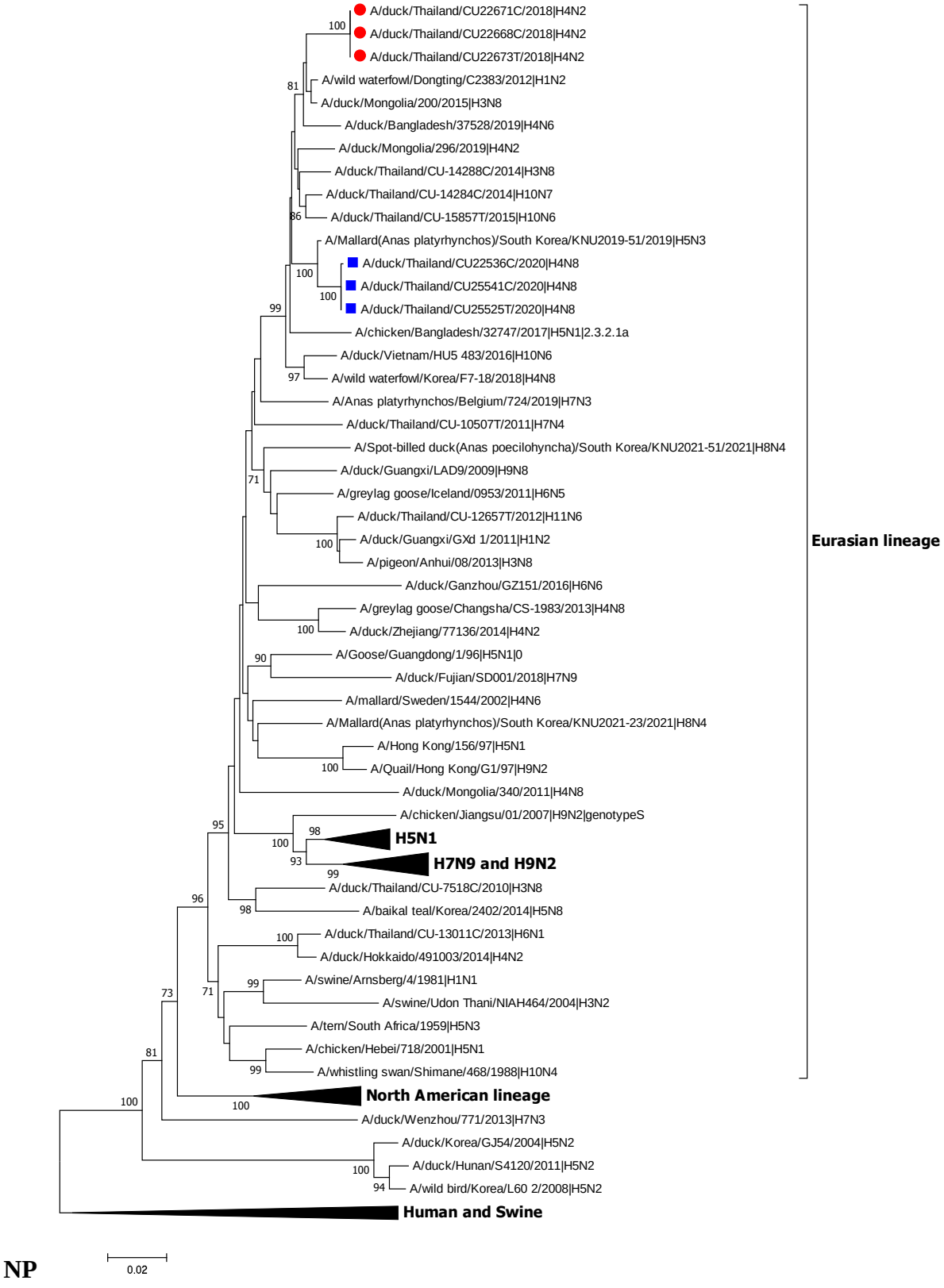
H7N9 and H9N2
North American lineage
Human
Eurasian lineage
0.02

PA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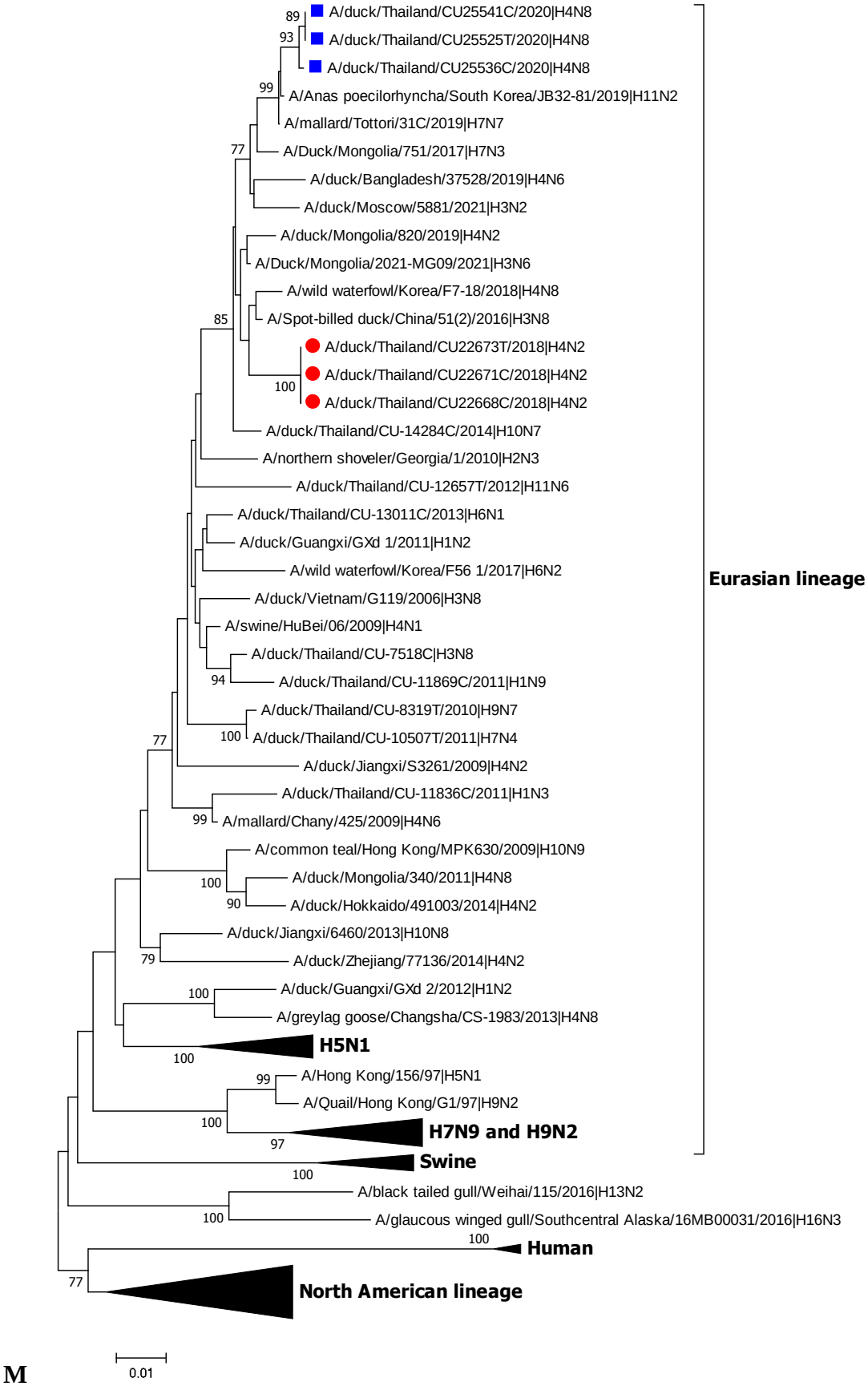
A/wild waterfowl/Dongting/C2383/2012|H1N2
A/duck/Mongolia/200/2015|H3N8
A/duck/Bangladesh/37528/2019|H4N6
A/duck/Mongolia/296/2019|H4N2
A/duck/Thailand/CU-14288C/2014|H3N8
A/duck/Thailand/CU-14284C/2014|H10N7
A/duck/Thailand/CU-15857T/2015|H10N6
A/Mallard(Anas platyrhynchos)/South Korea/KNU2019-51/2019|H5N3
A/duck/Thailand/CU22536C/2020|H4N8
A/duck/Thailand/CU25541C/2020|H4N8
A/duck/Thailand/CU25525T/2020|H4N8
A/chicken/Bangladesh/32747/2017|H5N1|2.3.2.1a
A/duck/Vietnam/HU5 483/2016|H10N6
A/wild waterfowl/Korea/F7-18/2018|H4N8
A/Anas platyrhynchos/Belgium/724/2019|H7N3
A/duck/Thailand/CU-10507T/2011|H7N4
A/Spot-billed duck(Anas poecilohyncha)/South Korea/KNU2021-51/2021|H8N4
A/duck/Guangxi/LAD9/2009|H9N8
A/greylag goose/Iceland/0953/2011|H6N5
A/duck/Thailand/CU-12657T/2012|H11N6
A/duck/Guangxi/GXd 1/2011|H1N2
A/pigeon/Anhui/08/2013|H3N8
A/duck/Ganzhou/GZ151/2016|H6N6
A/greylag goose/Changsha/CS-1983/2013|H4N8
A/duck/Zhejiang/77136/2014|H4N2
A/Goose/Guangdong/1/96|H5N1|0
A/duck/Fujian/SD001/2018|H7N9
A/mallard/Sweden/1544/2002|H4N6
A/Mallard(Anas platyrhynchos)/South Korea/KNU2021-23/2021|H8N4
A/Hong Kong/156/97|H5N1
A/Quail/Hong Kong/G1/97|H9N2
A/duck/Mongolia/340/2011|H4N8
A/chicken/Jiangsu/01/2007|H9N2|genotypeS
H5N1
H7N9 and H9N2
A/duck/Thailand/CU-7518C/2010|H3N8
A/baikal teal/Korea/2402/2014|H5N8
A/duck/Thailand/CU-13011C/2013|H6N1
A/duck/Hokkaido/491003/2014|H4N2
A/swine/Arnsberg/4/1981|H1N1
A/swine/Udon Thani/NIAH464/2004|H3N2
A/tern/South Africa/1959|H5N3
A/chicken/Hebei/718/2001|H5N1
A/whistling swan/Shimane/468/1988|H10N4
North American lineage
A/duck/Wenzhou/771/2013|H7N3
A/duck/Korea/GJ54/2004|H5N2
A/duck/Hunan/S4120/2011|H5N2
A/wild bird/Korea/L60 2/2008|H5N2
Human and Swine
Eurasian lineage
0.02

NP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A/Anas poecilorhyncha/South Korea/JB32-81/2019|H11N2
A/mallard/Tottori/31C/2019|H7N7
A/Duck/Mongolia/751/2017|H7N3
A/duck/Bangladesh/37528/2019|H4N6
A/duck/Moscow/5881/2021|H3N2
A/duck/Mongolia/820/2019|H4N2
A/Duck/Mongolia/2021-MG09/2021|H3N6
A/wild waterfowl/Korea/F7-18/2018|H4N8
A/Spot-billed duck/China/51(2)/2016|H3N8
A/duck/Thailand/CU22673T/2018|H4N2
A/duck/Thailand/CU22671C/2018|H4N2
A/duck/Thailand/CU22668C/2018|H4N2
A/duck/Thailand/CU-14284C/2014|H10N7
A/northern shoveler/Georgia/1/2010|H2N3
A/duck/Thailand/CU-12657T/2012|H11N6
A/duck/Thailand/CU-13011C/2013|H6N1
A/duck/Guangxi/GXd 1/2011|H1N2
A/wild waterfowl/Korea/F56 1/2017|H6N2
A/duck/Vietnam/G119/2006|H3N8
A/swine/HuBei/06/2009|H4N1
A/duck/Thailand/CU-7518C|H3N8
A/duck/Thailand/CU-11869C/2011|H1N9
A/duck/Thailand/CU-8319T/2010|H9N7
A/duck/Thailand/CU-10507T/2011|H7N4
A/duck/Jiangxi/S3261/2009|H4N2
A/duck/Thailand/CU-11836C/2011|H1N3
A/mallard/Chany/425/2009|H4N6
A/common teal/Hong Kong/MPK630/2009|H10N9
A/duck/Mongolia/340/2011|H4N8
A/duck/Hokkaido/491003/2014|H4N2
A/duck/Jiangxi/6460/2013|H10N8
A/duck/Zhejiang/77136/2014|H4N2
A/duck/Guangxi/GXd 2/2012|H1N2
A/greylag goose/Changsha/CS-1983/2013|H4N8
H5N1
A/Hong Kong/156/97|H5N1
A/Quail/Hong Kong/G1/97|H9N2
H7N9 and H9N2
Swine
A/black tailed gull/Weihai/115/2016|H13N2
A/glaucous winged gull/Southcentral Alaska/16MB00031/2016|H16N3
Human
North American lineage
Eurasian lineage
89
93
99
77
85
100
94
100
77
99
100
90
79
100
100
97
100
100
100
77
0.01

M

Figure 4 (A-F) Phylogenetic tree of internal genes (PB2, PB1, PA, M, NP, and NS) of Thai IAV-H4. The phylogenetic trees of internal genes were generated using the neighbor-joining algorithm with 1,000 bootstrap replications in the MEGA X program. IAV-H4N2 and IAV-H4N8 are represented by red circles and blue squares, respectively

Discussion

Free-grazing ducks (FGDs) are ducks that are allowed to roam freely and forage for food in natural or semi-natural environments, such as open rice fields. FGDs feed on natural foods found in the open rice fields, such as insects, snails, small aquatic animals, and rice seeds. However, since FGDs are exposed to various pathogens present in the environment, including influenza, they can pose a risk of spreading the viruses. FGDs can serve as a reservoir for the influenza virus and facilitate viral transmission, as they can carry the virus without showing clinical symptoms, allowing the virus to spread to other birds or even humans (Gilbert *et al*., 2006). Therefore, it is essential to monitor FGD settings to minimize the risk of viral contamination and transmission, including regular influenza surveillance in FGDs, implementing biosecurity measures on FGD premises, and controlling animal movement. The influenza A virus subtypes H4N2 and H4N8 found in FGDs can be a potential risk to birds, domestic animals, and humans. The viruses can be shed in feces and contaminate the environment in grazing areas such as rice fields. This setting provides a suitable environment for the transmission of viruses among birds, wild birds, and domestic animals in the same areas. Consequently, a small outbreak of IAV-H4 in FGDs may occur. For example, IAV-H4N8 causes small avian influenza outbreaks in chicken farms in the US and China (Johnson and Maxfield, 1976; Liu *et al*., 2003). Fortunately, the IAV-H4N2 and H4N8 circulating in these FGD flocks have low virulence and limited reports of infecting humans. However, if HPAI or newly reassorted viruses with high virulence circulate in the areas, this could contribute to a new gene pool and generate novel reassortant viruses.

In this study, phylogenetic analysis of Thai IAV-H4N2 and IAV-H4N8 revealed that the common lineages of the eight gene segments of Thai IAVs were from the Avian Eurasian lineage (EA). No reassortment of Thai IAV-H4 was observed in this study, unlike IAV-H11, which has recently shown reassortments in Thailand, as reported by our group. The M gene of IAV-H11N7 belonged to the North American lineage (NA), indicating the circulation of IAV-H11 with internal genes from various sources in FGDs (Chaiyawong *et al*., 2022). Phylogenetically, the HA gene of Thai IAV-H4 characterized in this study can be grouped into two separate clusters - Cluster 1 (IAV-H4N2) and Cluster 2 (IAV-H4N8). Notably, the H4 gene of Thai IAV-H4 belonged to a different cluster from IAV-H4N6, which was previously isolated in Thailand. This suggested that the unique gene pool of Thai IAV-H4 was present in the IAV-H4 in Thailand (Wisedchanwet *et al*., 2011b). In previous studies, the HA gene of IAV-H4 could be assigned to at least nine genotypes. Most genotypes belonged to the Avian Eurasian lineage, while two genotypes were from the North American lineage (Kang *et al*., 2013; Song *et al*., 2017). These viruses were commonly found in wild birds and can subsequently be transmitted to domestic poultry. Both endemic and intercontinental transmission have been observed (Song *et al*., 2017). For the NA gene, the Thai IAV-H4N2 and IAV-H4N8 clustered within the Avian Eurasian lineage. Similarly, the six internal genes of Thai IAV-H4N2 and IAV-H4N8 also clustered in the Avian Eurasian lineage, indicating that there was no reassortment or intercontinental gene pool contribution.

In this study, genetic analysis showed that Thai IAV-H4N2 and IAV-H4N8 exhibited low pathogenic avian influenza characteristics. The HA cleavage site and receptor binding sites of Thai IAV-H4 were conserved and resembled LPAI viruses. A similar pattern of HA cleavage site and receptor binding sites was also observed in IAV-H4N6 and IAV-H4N9, which were previously isolated from ducks in live bird markets in Thailand (Wisedchanwet *et al*., 2011a; Wisedchanwet *et al*., 2011b). The amino acids at the HA cleavage site of Thai IAV-H4N2 and IAV-H4N8 were "PEKASR/G", which is identical to that of IAV-H4N6 and IAV-H4N9 previously isolated in Thailand (Wisedchanwet *et al*., 2011b), and similar to most avian viruses of the Avian Eurasian lineage (Table 3). On the other hand, the HA cleavage site of the North American lineage was "PEKATR/G". It has been known that multiple basic amino acids at the cleavage site, such as Arginine (R) and Lysine (K), can convert LPAI virus into HPAI virus (Horimoto *et al*., 1995). There was also a report of IAV-H4N2 from quails (CA12) with multiple basic amino acids at HA cleavage sites (PEKRRTR/G). However, the virus (CA12) lacked virulence and did not cause clinical signs in chickens, suggesting that the IAV-H4N2 virus can acquire multiple basic amino acids similar to those found in H5 and H7 viruses (Wong *et al*., 2014). The amino acids of IAV-H4N2 and IAV-H4N8 at positions 224-229 (left edge of RBS) and 134-138 (right edge of RBS) were "RGQSGR" and "GKSGA", respectively. It is noted that the receptor binding sites (Q226 and G228) of Thai IAV-H4 are similar to those of IAV-H4 from the Avian Eurasian lineages, suggesting preferential binding to α 2,3-linked sialic acid receptors, which are dominant in avian species (Bateman *et al*., 2008). In contrast, IAV-H4 (H4N6) found in pigs contained amino acid substitutions at 226L and 228S, suggesting preferential binding to α 2,6-linked sialic acid receptors, which are dominant in mammalian species (Karasin *et al*., 2000). In this study, none of the IAV-H4 had deletions in NA stalk regions or D92E substitutions in the NS protein. It has been documented that an amino acid substitution at 92E of the NS1 protein can lead to severe pathology in mammals (Seo *et al*., 2002). All Thai IAV-H4 retained the E627 in the PB2 protein. The amino acid change at position 627K was associated with increased replication of IAVs in mammalian cells (Hatta *et al*., 2001; Shinya *et al*., 2004). The M2 protein of all Thai IAV-H4 contained the amino acids at positions Q26, R27, D30, V31, and G34 and were known as the amantadine-sensitive markers. IAV-H4 resistance to amantadine had been reported in IAV-H4 from wild birds in Korea (Song *et al*., 2017).

There were some reports of interspecies transmission of IAV-H4 viruses. For example, interspecies transmission of IAV-H4N8 from avian to domestic pigs was reported in China. The viruses contained all eight gene segments of the Eurasian lineage, and the NP gene was closely related to that of IAV-H5N1. This implied that IAV-H5 contributed

genes through reassortment and generated the avian-like IAV-H4N8, which caused severe respiratory problems in pigs (Su *et al.*, 2012). Some studies showed serological evidence of IAV-H4 infection in occupations at risk, such as farmers, hunters, and poultry workers in the Middle East and North America (Gray *et al.*, 2008; Kayali *et al.*, 2011; De Marco *et al.*, 2021). Even though there is limited information on IAV-H4 infection in humans, a previous experimental study of avian-to-human receptor-binding adaptation of IAV-H4N6 indicated that the shift in receptor-binding at positions Q226L and G228S of avian influenza viruses played a critical role in transmission from avian to human hosts. Thus, the IAV-H4 virus has the potential to cause pandemics in humans (Song *et al.*, 2017).

In summary, this study characterized the Thai IAV subtypes H4N2 and H4N8 isolated from free-grazing ducks in Thailand. Phylogenetic analysis revealed that IAV-H4N2 and IAV-H4N8 were closely related to the Avian Eurasian viruses and showed no reassortment from North American viruses. According to genetic analysis, the HA cleavage site and receptor binding sites of IAV-H4 displayed low pathogenic characteristics, suggesting a diminished potential to be zoonotic or virulent viruses.

Ethics approval and consent to participate: This study was conducted under the approval of the Institute for Animal Care and Use Protocol of CU-VET, Chulalongkorn University (IACUC no. 1831105).

Consent for publication: Not applicable

Data Availability Statement: The authors declare that the data supporting the findings of this study are available upon request from the first author. The nucleotide sequence data that support the findings of this study are openly available in the GenBank database at https://www.ncbi.nlm.nih.gov/genbank , under accession numbers #OR512565-OR512616.

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Author's contributions : SB, SC, KU, EC, and NB performed virus isolation, molecular detection, whole genome characterization, and phylogenetic analysis. SC, KC, KU, and EC participated in whole genome sequencing and phylogenetic analysis. SB drafted the manuscript. SP and AA designed the study, performed data analysis, drafted, revised, and approved the manuscript. All authors reviewed the manuscript.

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