Tropical Biomedicine 40(2): 220-235 (2023)
https://doi.org/10.47665/tb.40.2.014
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RESEARCH ARTICLE

Molecular characterization and phylogenetic analysis of avian influenza H3N8 virus isolated from imported waterfowl in Malaysia

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ARTICLE HISTORY

Received: 3 February 2023
Revised: 26 April 2023
Accepted: 26 April 2023
Published: 30 June 2023

ABSTRACT

Wild aquatic birds are natural reservoirs of influenza A viruses and H3 subtype is one of the most prevalent subtypes in waterfowl. Two H3N8 viruses of low pathogenic avian influenza (LPAI) were isolated via egg inoculation technique from the fecal swab specimens from imported barnacle goose and paradise shelduck in Veterinary Research Institute Ipoh, Malaysia. The full length of eight gene segments of the two viruses were amplified and sequenced with specific primers. The sequences were molecularly characterized, and the sequence identity were assessed with other published sequences. The two viruses are identical and they possess the same amino acid sequences for all the eight gene segments. The viruses were highly similar to the H3 virus from Netherlands and N8 virus from Belgium respectively. Phylogenetic analysis revealed that all the eight gene segments were grouped in the Eurasian lineage, and genetic reassortment may occur between the internal genes of the H3 viruses and other AI subtypes. Though four amino acid substitutions were identified in the hemagglutinin gene, the viruses retained most of the avian-type receptor binding preference. Few amino acid substitutions were observed in all internal genes. Most of the neuraminidase inhibitors and adamantine resistance related mutation were not seen in the viruses. The replicative capacity, cross species transmissibility, and potential zoonotic risk of the viruses are worth further investigation. As H3 virus poses potential threats to both human and animals, and with the increase in the international trade of birds; strict quarantine practice at the entry point and good laboratory diagnostic capabilities is crucial to prevent the introduction of new AI virus into our country.

Keywords: avian influenza; imported waterfowl; H3N8 virus; molecular characterization; Malaysia.

INTRODUCTION

Avian influenza virus (AIV) belongs to the genus *influenza A virus* in the *Orthomyxoviridae* family. The viral genome consists of eight segments of negative sense and single-stranded RNA which code for at least twelve viral proteins including the hemagglutinin (HA), neuraminidase (NA), polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), PB1-F2 protein, polymerase protein (PA), PA-X protein, nucleoprotein (NP), non-structural proteins 1 and 2 (NS1, NS2), and matrix proteins 1 and 2 (M1, M2) (Gora *et al*., 2014; Suttie *et al*., 2019). Newly discovered proteins such as N40, PA-N182, PA-N155, and M42 were also reported (Gora *et al*., 2014).

Waterfowls (order Anseriformes especially ducks, swans, and geese) and shorebird (order Charadriiformes especially waders, gulls, and terns) are the natural reservoirs for influenza A virus (Gora *et al*., 2014; Bailey *et al*., 2016; Suttie *et al*., 2019). Novel reassortants resulting from co-infection with different AIVs in waterfowl serve them as a gene pool providing genetic diversity to animal and human influenza A virus (Webster *et al*., 1992; Li *et al*., 2016; Yang *et al*., 2021). The infected migratory wild bird particularly waterfowl can promote the spread of AIVs among wild birds and different geographical area, and plays a role in viral transmission to domestic poultry, pig, and human (Bailey *et al*., 2016). These waterfowl AIVs circulate in domestic flocks as low pathogenic strain (Suttie *et al*., 2019), evolve silently in nature (Yao *et al*., 2022), and lead to the establishment of stable AIV lineage in poultry (Suttie *et al*., 2019). This low pathogenic avian influenza (LPAI) virus can contribute its gene segments to high pathogenic avian influenza (HPAI) viruses through genetic reassortment and recombination leads to the emergence of novel strains (Yao *et al*., 2022).

AIVs can be divided into subtypes based on the antigenicity of the surface glycoproteins, the HA and NA (OIE, 2021). To date, 16 HA (H1-H16) and 9 NA (N1-N9) subtypes have been identified from waterfowl except for the H17, H18, N10, and N11 which have been only detected from bats (Li *et al*., 2016; Suttie *et al*, 2019; Yang *et al*, 2021). Among all the AI subtypes, the H3 subtype is one of the most prevalent AIVs detected in waterfowl with the highest detection rate in ducks (Pu *et al*., 2009; Yang *et al*., 2021). The H3N8 virus has been isolated from a broad range of wild bird species including Anseriformes and Charadriiformes (Zhang *et al*., 2021). Apart from the avian, the H3N8 virus has also been detected in mammals such as pigs, dogs, horses, seals, and donkeys (Zhang *et al*., 2021). Studies revealed that avian origin H3N8 virus can replicate in the respiratory tract of mice (Driskell *et al*., 2010; Baz *et al*., 2013), as

well as transmitted efficiently to guinea pigs (Zhang *et al.*, 2021). Experiment demonstrated that the avian seal H3N8 virus can be transmitted via respiratory droplet in ferret model (Karlsson *et al.*, 2014) and infect swine without prior adaptation (Solórzano *et al*., 2015). These findings indicate the ability of the avian-origin H3N8 virus to cause cross-species transmission.

In the present study, two H3N8 viruses were isolated from the imported shelduck and goose. In order to better understand the genetic characteristic of the viruses, all eight gene segments were genetically and phylogenetically characterized.

MATERIALS AND METHODS

Viruses

In August 2018, two referral cases were received from the regional veterinary laboratory for AI screening for import purposes. The fecal swab sample was collected from barnacle goose (*Branta leucopsis*) and paradise shelduck (*Tadorna variegata*) respectively at the Kuala Lumpur International Airport Animal Quarantine Station. Virus isolation was carried out in 9 to 11 days old Specific Pathogen Free (SPF) embryonated chicken eggs via the intra-allantoic route. The eggs were incubated at 37°C for three days and the infected allantoic fluid was harvested for hemagglutination activity test (OIE, 2021). The hemagglutination inhibition (HI) test was used to identify the HA subtype (OIE, 2021).

Reverse transcription - polymerase chain reaction (RT-PCR) and gene sequencing

The viral ribonucleic acid (RNA) was extracted from the infected allantoic fluid using the IndiSpin Pathogen Kit (Indical Bioscience, Germany). For further virus subtyping, RT-PCR was conducted with subtype-specific HA and NA primers. With the aim of amplification of all the eight gene segments of AIV, the RNA was transcribed into full-length complementary deoxyribonucleic acid (cDNA) by AMV reverse transcriptase (Promega, US) using the Uni12 primer as described by Hoffmann *et al.* (2001). The RT reaction was performed at 45°C for 45 min. The cDNA was then amplified by GoTaq® Green Master Mix (Promega, USA) for the five segments (HA, NA, NP, NS, and M) using the universal primers for the influenza A virus (Hoffmann *et al.*, 2001). In the amplification, the initial denaturation was set at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 7 min with a final extension for 10 min at 72°C.

For the viral polymerase subunits, the PB2, PB1, and PA genes, primers designed by Li *et al.* (2007) were used for the full-length amplification from the extracted RNA using the SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen, USA). The amplification was performed in T100 Thermal Cycler (Bio-Rad, USA). In the amplification, the RT was carried out at 48°C for 30 min. The reaction mixture was then subjected to 94°C for 5 min for initial denaturation, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 68°C for 2 min with a final extension for 10 min at 68°C.

The amplicons were loaded on 1.0% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen). Gel electrophoresis is performed to separate the amplicons. The results were viewed under UV transilluminator. The amplified PCR products were cut from the gel and sent for Sanger sequencing (Apical Scientifics (M) Sdn Bhd). Primers used in the Sanger sequencing are the same as those used in the PCR amplification.

Phylogenetic analysis and molecular characterization

The assembly of the nucleotide sequences was performed using the SeqMan Pro software (DNA Star Lasergene, USA). BioEdit Sequence Alignment Editor version 7.1.9 (Hall, 1999) was used in the alignment and comparison of all the eight gene segments of AIV of the two viruses with other published sequences. Basic Local Alignment Search Tool (BLAST) searches the regions of local similarity between the sequences with the other published sequences in the GenBank National Center for Biotechnology Information (NCBI) for the eight gene segments was also performed (NCBI Resource Coordinators, 2016). The phylogenetic tree was constructed by Neighbor-Joining statistical method based on the Kimura 2-parameter model and bootstrapping 1,000 replicates using Molecular Evolutionary Genetic Analysis (MEGA) version 11 (Tamura *et al.*, 2021). The phylogram is used to analyze the genetic relationship of the eight gene segments of the two viruses and other respective strains in the world. In the genetic analysis of the HA and NA gene, the NetNGlyc 1.0 Server program (Chen *et al.*, 2012) was used to predict the potential glycosites.

RESULTS

Virus isolation and gene sequencing

The viruses were successfully isolated in the SPF embryonated chicken eggs from the fecal swab sample for the two referral cases. The infected allantoic fluids were tested positive for the HA test. In the HI assay, the two viruses were neutralized by antiserum specific to AI subtype H3. The two isolates were further confirmed as H3N8 viruses by RT-PCR using the HA and NA primers targeting the H3 and N8 gene, respectively. The two viruses were designated as A/barnacle goose/Malaysia/6728/2018 (H3N8) and A/paradise shelduck/Malaysia/6731/2018 (H3N8) respectively. The full length of the eight gene segments for the two viruses were sequenced. Both the H3N8 viruses are identical and they possessed the same amino acid sequences for all eight gene segments. BLAST analysis showed that the two viruses were highly similar to the H3 virus from Netherlands and N8 virus from Belgium with 99% and 100% nucleotide identity respectively (Table 1). The nucleotide identities for the other six inner gene segments of the two H3N8 viruses with the closest homologs in the GenBank database obtained via BLAST search were shown in Table 1. The nucleotide sequences of the eight gene segments of the two viruses were deposited to the GenBank and their accession numbers are shown in Table 1.

Phylogenetic analysis

Phylogenetic analysis of all eight gene segments was performed to determine the genetic relationship of the two H3N8 viruses with other respective strains including human and swine viruses. The HA genes of the two H3 viruses were phylogenetically grouped into the Eurasian lineage (Figure 1A). The H3 viruses were clustered in the group 2 viruses and showed the highest sequence homologies with the H3 viruses from the Netherlands, Belgium, and Mongolia. The phylogram showed that the H3 viruses were highly similar to those in European countries. However, surprisingly, the H3 viruses that were isolated from the aquatic birds in the study were also closely related to the H3 viruses isolated from jungle fowl (Gallus gallus) (A/gallus gallus/Belgium/609/2019) in Belgium and chickens (A/chicken/Pakistan/NARC-16945/2010) from Pakistan, South Asia. The N8 gene of the two H3 viruses was located in the Eurasian lineage along with the N8 viruses detected from waterfowls in Belgium, Iceland, and Netherlands (Figure 1B).

Likewise, the phylogenetic analysis also indicated that the six internal genes (PB2, PB1, PA, NS, M, and NP) of the two H3N8 viruses were belonged to the Eurasian lineage (Figure1C to 1H). All the internal genes were clustered with the AIVs detected in waterfowl or shorebirds from European countries. Among these genes, the NS can be phylogenetically divided into A-allele and B-allele lineage. Both the H3N8 viruses were belonged to the B-allele lineage together with AIVs including H3, H4, H5, and H6 viruses from the Netherlands, Moscow, and Belgium (Figure 1F). It is noteworthy that reassortment in the internal genes of the two

Table 1. The GenBank accession numbers of the eight gene segments for the A/barnacle goose/Malaysia/6728/2018 (H3N8) and A/paradise shelduck/Malaysia/6731/2018 (H3N8) viruses and the nucleotide sequence identities between the two H3N8 viruses and the closest homologs in the GenBank database via BLAST search

Gene	GenBank accession number		Nucleotide sequence identities between the A/barnacle goose/Malaysia/6728/2018 and A/paradise shelduck/Malaysia/6731/2018 to the closest homologs in the GenBank database			
	A/barnacle goose/Malaysia/6728/2018	A/paradise shelduck/Malaysia/6731/2018	Virus	Subtype	GenBank accession number	Nucleotide sequence identity (%)
HA	OQ359378	OQ359379	A/Mallard/Netherlands/37/2015	H3N8	MK414733	98.66
NA	OQ359380	OQ359381	A/Arenaria interpres/Belgium/11446/2016	H6N8	MT407139	98.87
PB2	OQ359383	OQ359384	A/Anas platyrhynchos/Belgium/10409/2018	H2N3	MT406840	99.26
PB1	OQ359385	OQ359386	A/Anas platyrhynchos/Belgium/9074/2016	H9N2	MT407095	99.00
PA	OQ363219	OQ363220	A/mallard duck/Netherlands/16/2014	H2N9	MF146627	98.32
NS	OQ363287	OQ363288	A/greater white-fronted goose/Netherlands/5/2010	H5N3	KX977601	99.10
M	OQ363284	OQ363285	A/avian/Israel/824/2005	H10N2	JN641305	98.36
NP	OQ359412	OQ359413	A/sanderling/Iceland/6743/2017	H2N5	MZ717596	98.33

H3N8 viruses and other AIV subtypes may occur. In particular, the PB1 gene of the H3N8 viruses formed a distinct branch with the H9N2 virus from Belgium (Figure 1D). Whereas the PB2 (Figure 1C) and NP (Figure 1H) genes formed a separate cluster with H2N3 and H2N5 viruses respectively. Likewise, the M gene also constructed a specific group with the H10N7 virus (Figure 1G). In the same way, the NS gene formed a unique cluster with the H6N8 virus (Figure 1F). On the other hand, the PA gene grouped with H2N9 and H4N6 virus from the Netherlands and Iceland respectively (Figure 1E).

Molecular characterization of surface genes

The A/barnacle goose/Malaysia/6728/2018 (H3N8) and A/paradise shelduck/Malaysia/6731/2018 (H3N8) exhibited mono-basic cleavage sites, the PEKQTR/GLF in the HA gene. Both H3 viruses possessed the conserved glutamine (Q) at position 226 and glycine (G) at position 228 respectively (H3 numbering). Four amino acid substitutions, the S126N, I155T, T160A, and V214I were recognized in the HA gene (Table 2). Both the H3 viruses remain the avian receptor binding preference sites including the histidine (H) and glutamic acid

Table 2. Amino acid substitutions detected in the eight gene segments of A/barnacle goose/Malaysia/6728/2018 (H3N8) and A/paradise shelduck/Malaysia/6731/2018 (H3N8) viruses

Gene	Amino acid substitutions /motif	Phenotype	References
HA	S126N, I155T, T160A, and V214I	increased preferentially binding of the virus to mammalian α-2,6 receptor	Burke & Smith, 2014; Suttie *et al.*, 2019
NA	"TEI" deletion at position 63-65	enhanced fatality in mice	Yao *et al.*, 2022
	I314V	reduced susceptibility to oseltamivir	Suttie *et al.*, 2019
PB2	251R	virus growth adaptation in mammals	Yao *et al.*, 2022
	L89V	increased polymerase activity in mammalian cells and increased virulence in mice	Yao *et al.*, 2022
	I292V, K389R, V598T, G309D, T399K, I495V, A676T, and R477G	increased polymerase activity in mammalian cells and increased virulence in mice	Suttie *et al.*, 2019
	E358V	decreased virulence in mice	Desrochers *et al.,* 2016
	S715N	decreased virulence in mice	Sun *et al.,* 2015
PB1	D622G	increased polymerase activity and virulence in mice	Suttie *et al.*, 2019; Yao *et al.*, 2022
	D3V	increased polymerase activity and viral replication in avian and mammalian cell lines	Suttie *et al.*, 2019
	P598L	decreased polymerase activity and viral replication in mammalian cell lines	Suttie *et al.*, 2019
PB1-F2	T51M, and V56A	decreased polymerase activity, replication and virulence in duck	Suttie *et al.*, 2019
PA	S37A, N383D, N409S, H266R, and S515T	increased polymerase activity in mammalian cell lines	Suttie *et al.*, 2019
	P190S	decreased virulence in mice	Suttie *et al.*, 2019
NS1	I106M, C138F, V149A, P3S, R41K, and K66E	enhanced replication in mammalian cells, increased virulence, and decreased interferon response in chickens	Suttie *et al.*, 2019
	^{227}ESEV^{230}	decreased viral replication in human and duck cell line	Suttie *et al.*, 2019
M1	N30D, and T215A	increased virulence of H5 subtypes in mice	Suttie *et al.*, 2019
	I43M	increased virulence of H5 subtypes in mice and increased virulence in ducks and chickens	Suttie *et al.*, 2019
NP	M105V and A184K	enhanced viral replication and increased virulence in chickens	Suttie *et al.*, 2019

(E) at position 183 and 190 respectively. Seven N-linked glycosylation sites were predicted in the HA gene. They are 24NST, 38NGT, 54NAT, 142NWT, 181NVT, 301NGS, and 499NGT (Table 3).

Genetic analysis of the NA gene of the two H3N8 viruses showed that there is no amino acid deletion at residue 54-72 (N2 numbering) in the NA stalk region when compared to A/goose/Guangdong/1/1996 (H5N1) virus. Deletion of three amino acids "TEI" at position 63-65 at the NA stalk region was observed. An amino acid substitution I314V associated with reduced susceptibility to oseltamivir was noticed in both H3 viruses. Six potential glycosites were identified, the 46NGT, 54NET, 67NTS, 84NNT, 144NGT, 293NWT, and 398NWS (Table 4).

Molecular characterization of inner genes

The amino acid substitutions detected in the inner genes of the two H3N8 viruses were shown in the Table 2. In the PB2 gene, several mutations were observed in the two H3N8 viruses. They were L89V, I292V, K389R, V598T, G309D, T399K, I495V, A676T, R477G, E358V, S715N, and 251R substitution. Molecular characterization showed that three mutations, the D622G, D3V, and P598L were identified in the PB1 gene for both the H3N8 viruses. Whereas, the T51M and V56A substitutions were noticed in the PB1-F2 gene. Substitutions S37A, N383D, N409S, H266R, S515T, and P190S in the PA gene were found. The two H3N8 viruses possessed a full-length PA-X gene and no truncation was seen. The NS1 gene of the two H3N8 viruses possessed the I106M, C138F, V149A, P3S, R41K, K66E substitutions. No elongation was observed in position 230 to 237 in the NS1 protein and the PDZ domain in the NS1 protein was the ^{227}ESEV^{230} motif. No mutation was seen in the NS2 protein. The M1 gene of the H3N8 viruses consisted of three substitutions, the N30D, I43M, and T215A. Mutation related to adamantane resistance in the M2 protein was not noticed. On the other hand, the M105V and A184K mutations were recognized in the NP gene for the two viruses in the study.

Table 3. N-linked glycosylation analysis of HA protein for A/barnacle goose/Malaysia/6728/2018 (H3N8) (OQ359378) and A/paradise shelduck/Malaysia/6731/2018 (H3N8) (OQ359379) virus. (Threshold=0.5)

Sequence Name	Position	Potential	Jury agreement	*N-Glyc result
OQ359378, OQ359379	24 NSTA	0.7721	(9/9)	+++
OQ359378, OQ359379	38 NGTI	0.6873	(9/9)	++
OQ359378, OQ359379	54 NATE	0.5083	(6/9)	+
OQ359378, OQ359379	142 NWTG	0.5657	(6/9)	+
OQ359378, OQ359379	181 NVTM	0.7512	(9/9)	+++
OQ359378, OQ359379	301 NGSI	0.6790	(9/9)	++
OQ359378, OQ359379	499 NGTY	0.5628	(8/9)	+

The number in red indicates the highest score of the jury agreement.
*–: non-glycosylated sites; +, ++, +++: glycosylated sites.

Table 4. N-linked glycosylation analysis of NA protein for A/barnacle goose/Malaysia/6728/2018 (H3N8) (OQ359380) and A/paradise shelduck/Malaysia/6731/2018 (H3N8) (OQ359381) virus. (Threshold=0.5)

Sequence Name	Position	Potential	Jury agreement	*N-Glyc result
OQ359380, OQ359381	46 NGTI	0.7724	(9/9)	+++
OQ359380, OQ359381	54 NETV	0.6382	(8/9)	+
OQ359380, OQ359381	67 NTSV	0.7506	(9/9)	+++
OQ359380, OQ359381	84 NNTE	0.5230	(5/9)	+
OQ359380, OQ359381	144 NGTV	0.6830	(9/9)	++
OQ359380, OQ359381	293 NWTG	0.5584	(6/9)	+
OQ359380, OQ359381	398 NWSG	0.3928	(8/9)	–

The number in red indicates the highest score of the jury agreement.
*–: non-glycosylated sites; +, ++, +++: glycosylated sites.

Figure 1A.

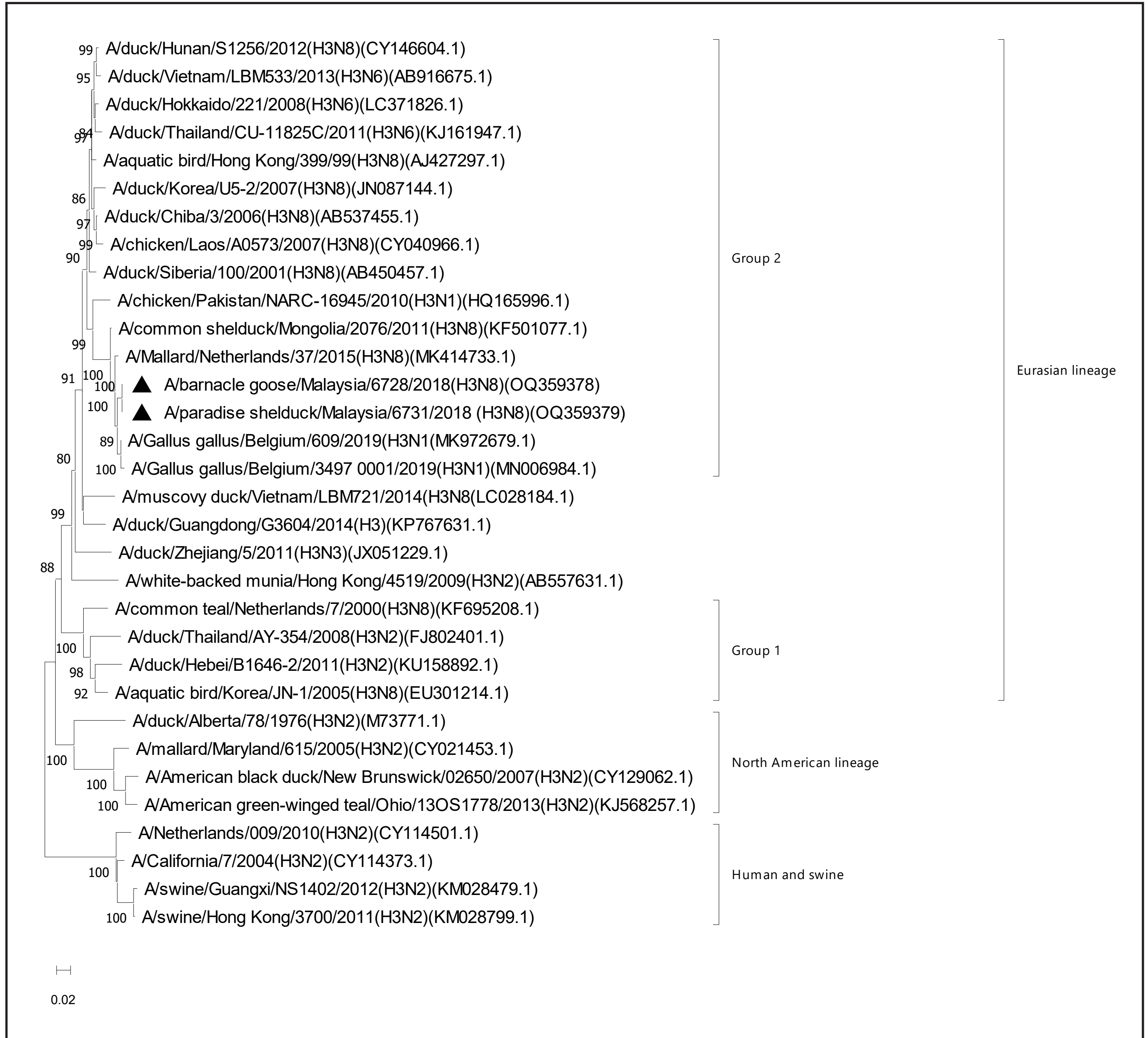

Figure 1B.

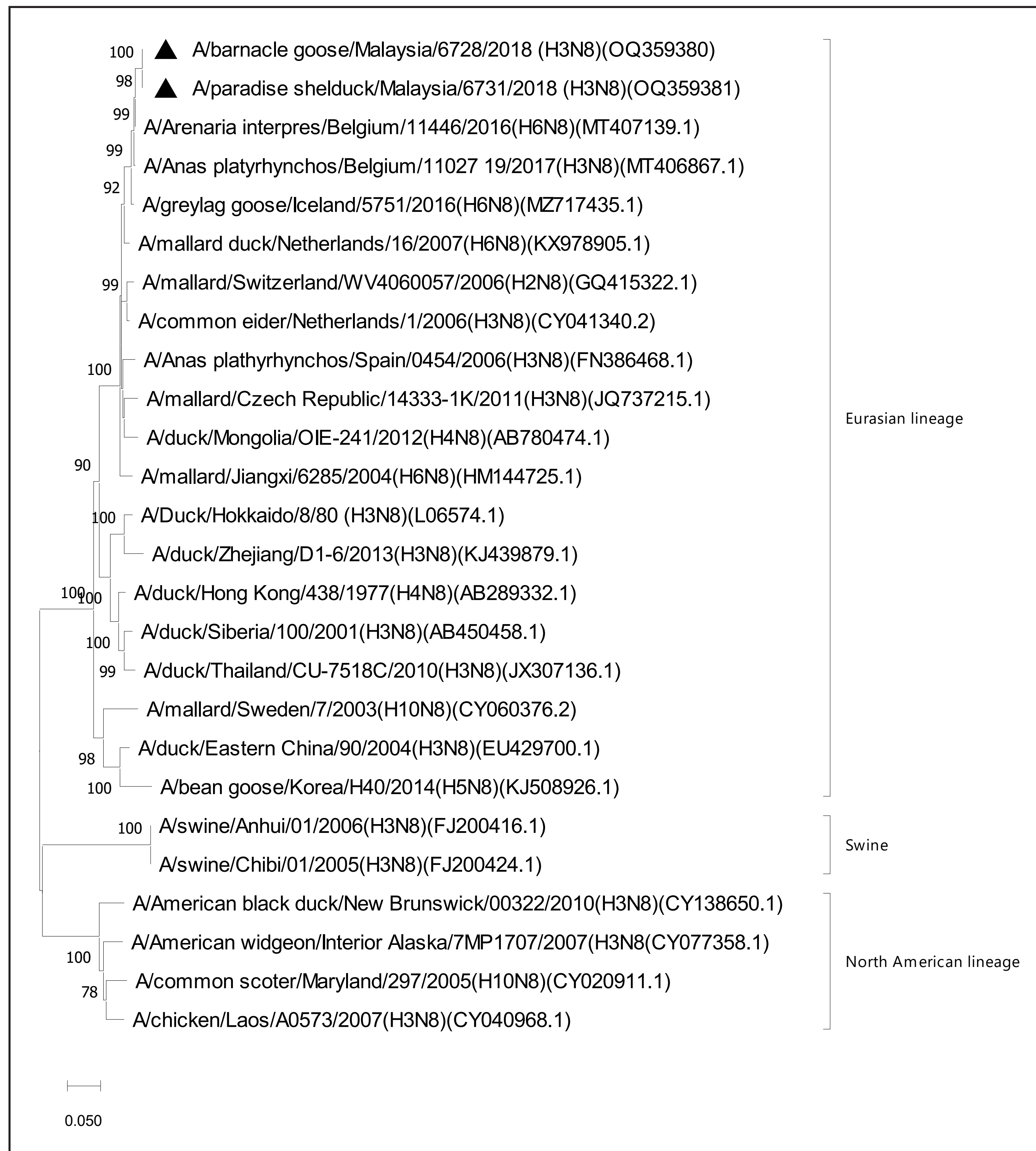

Figure 1C.

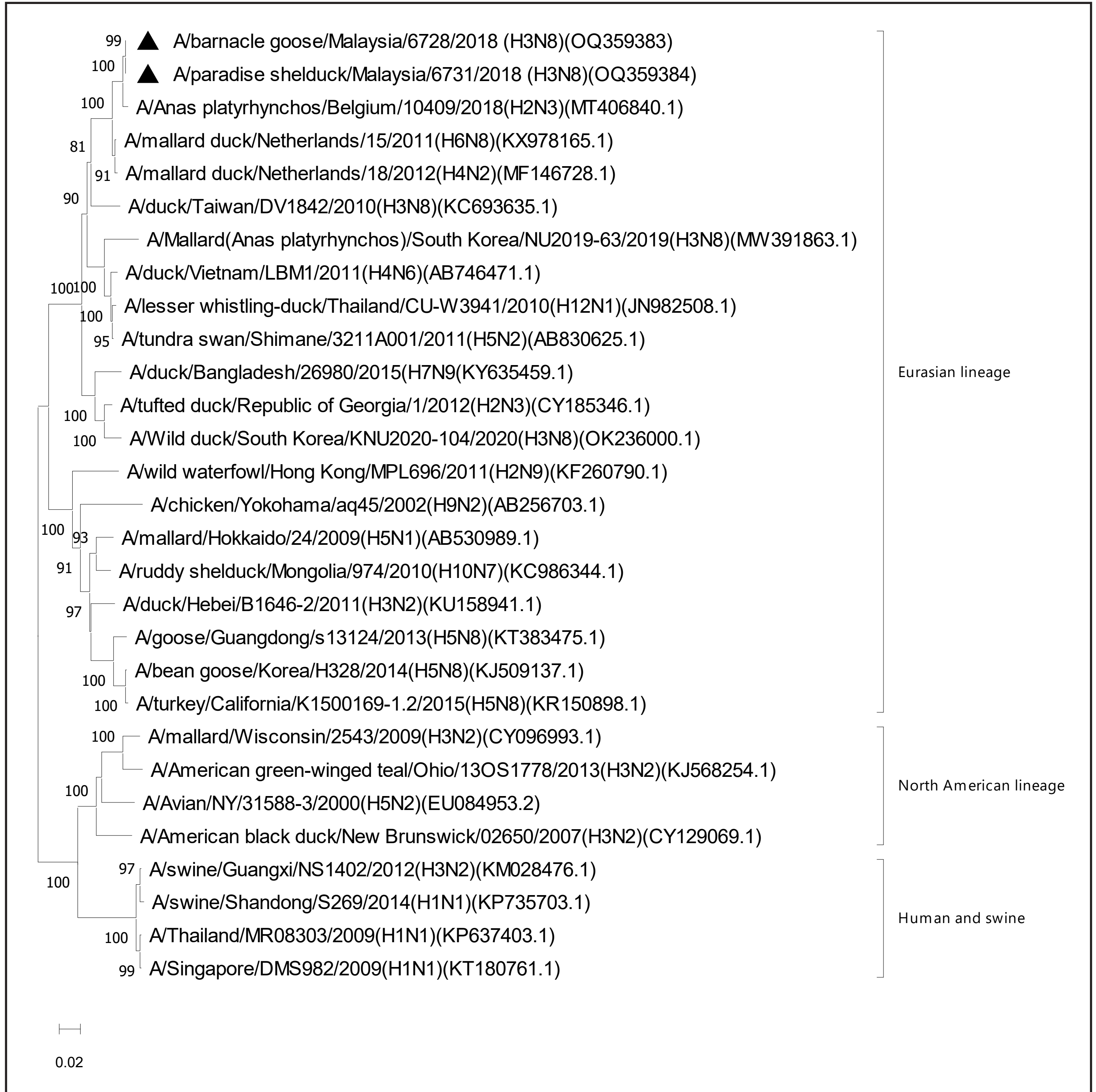

Figure 1D.

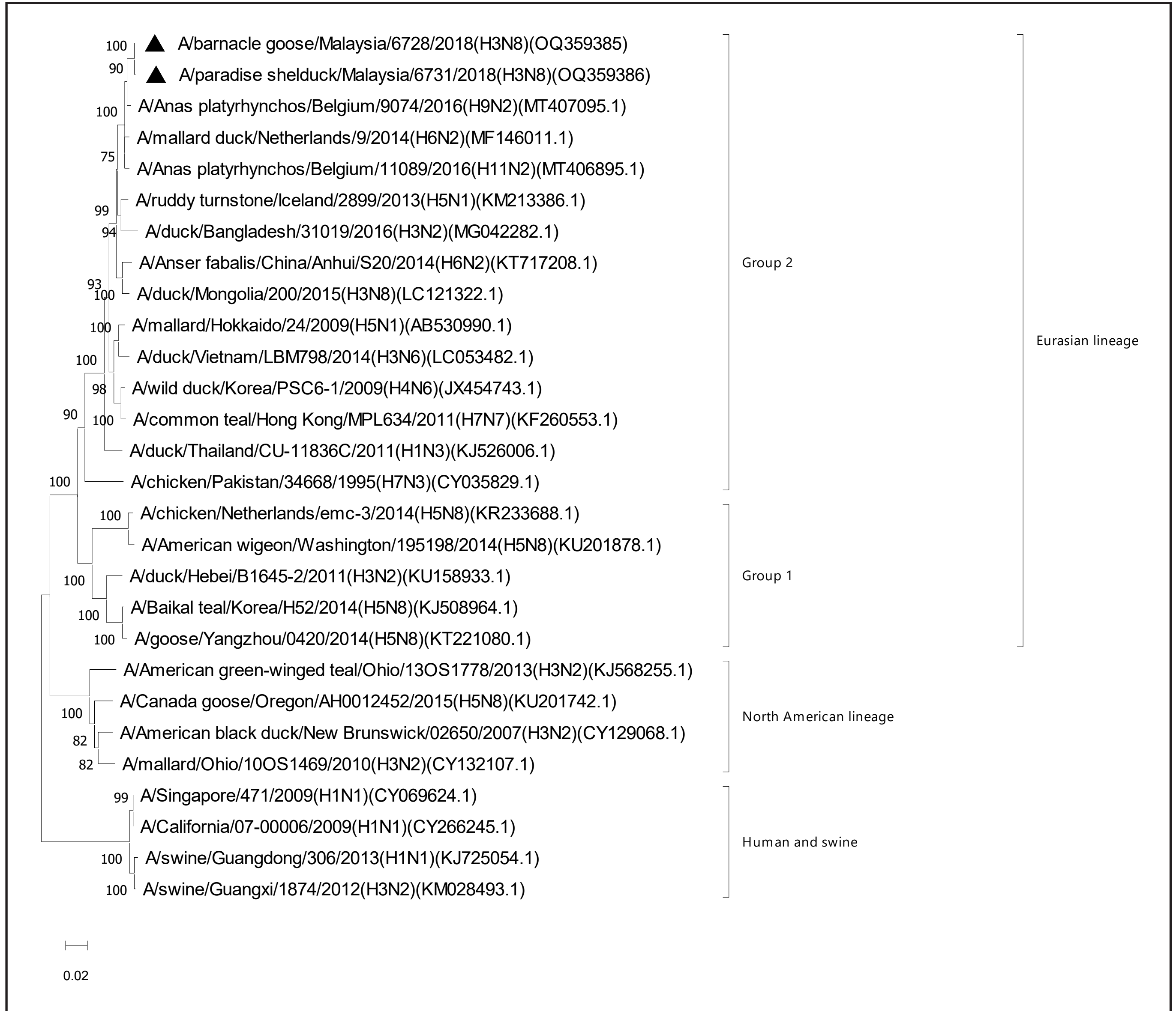

Figure 1E.

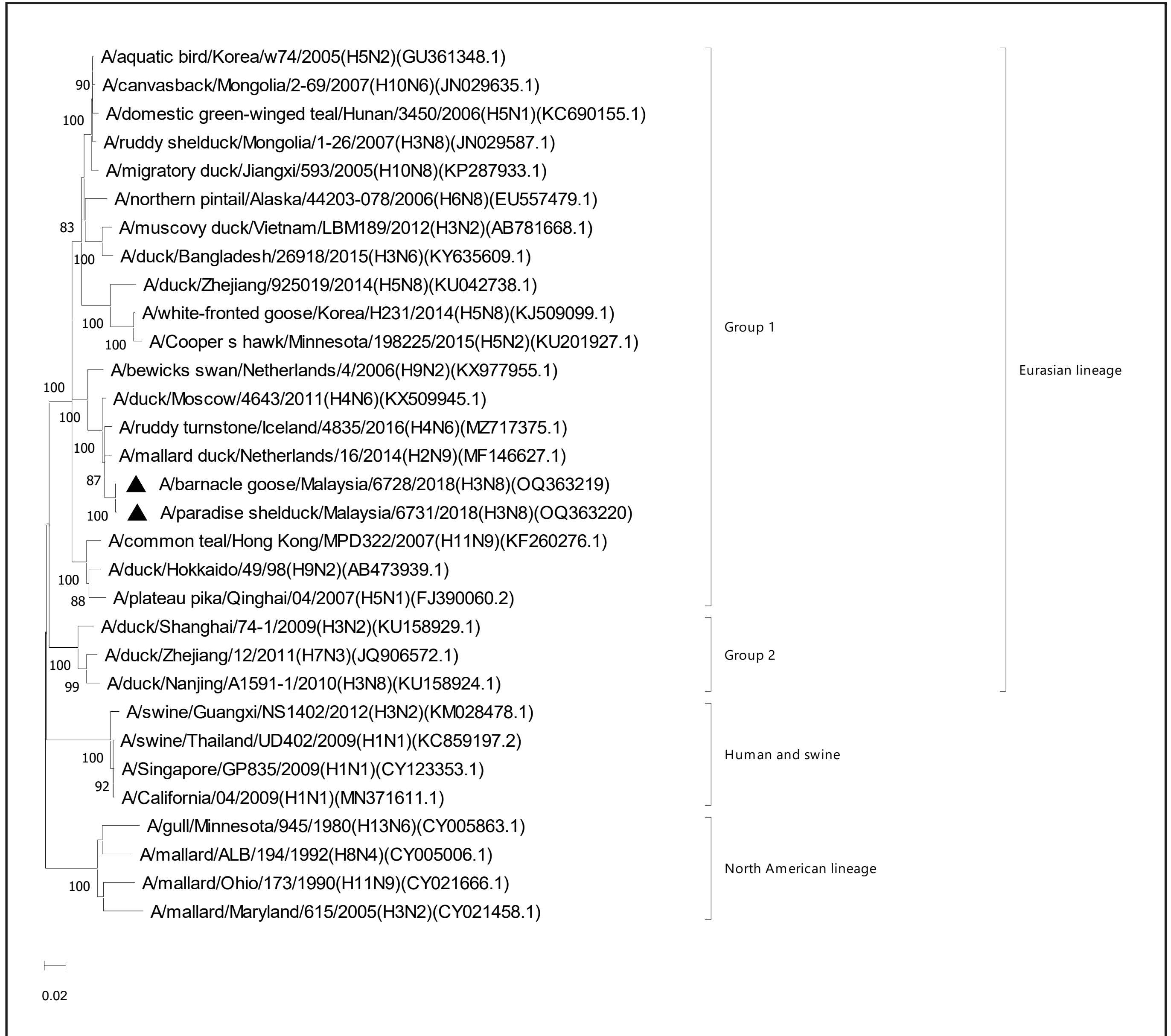

Figure 1F.

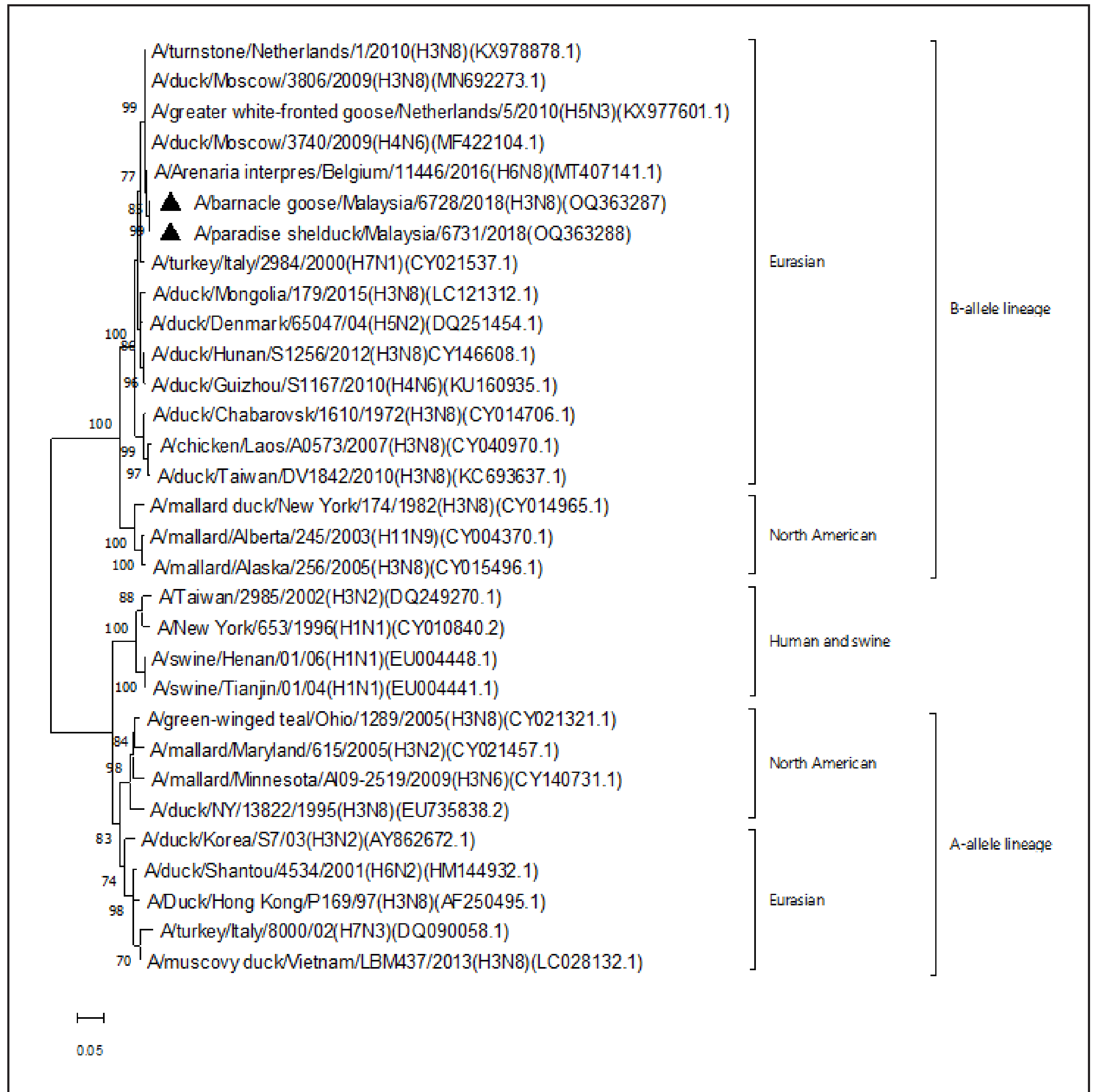

Figure 1G.

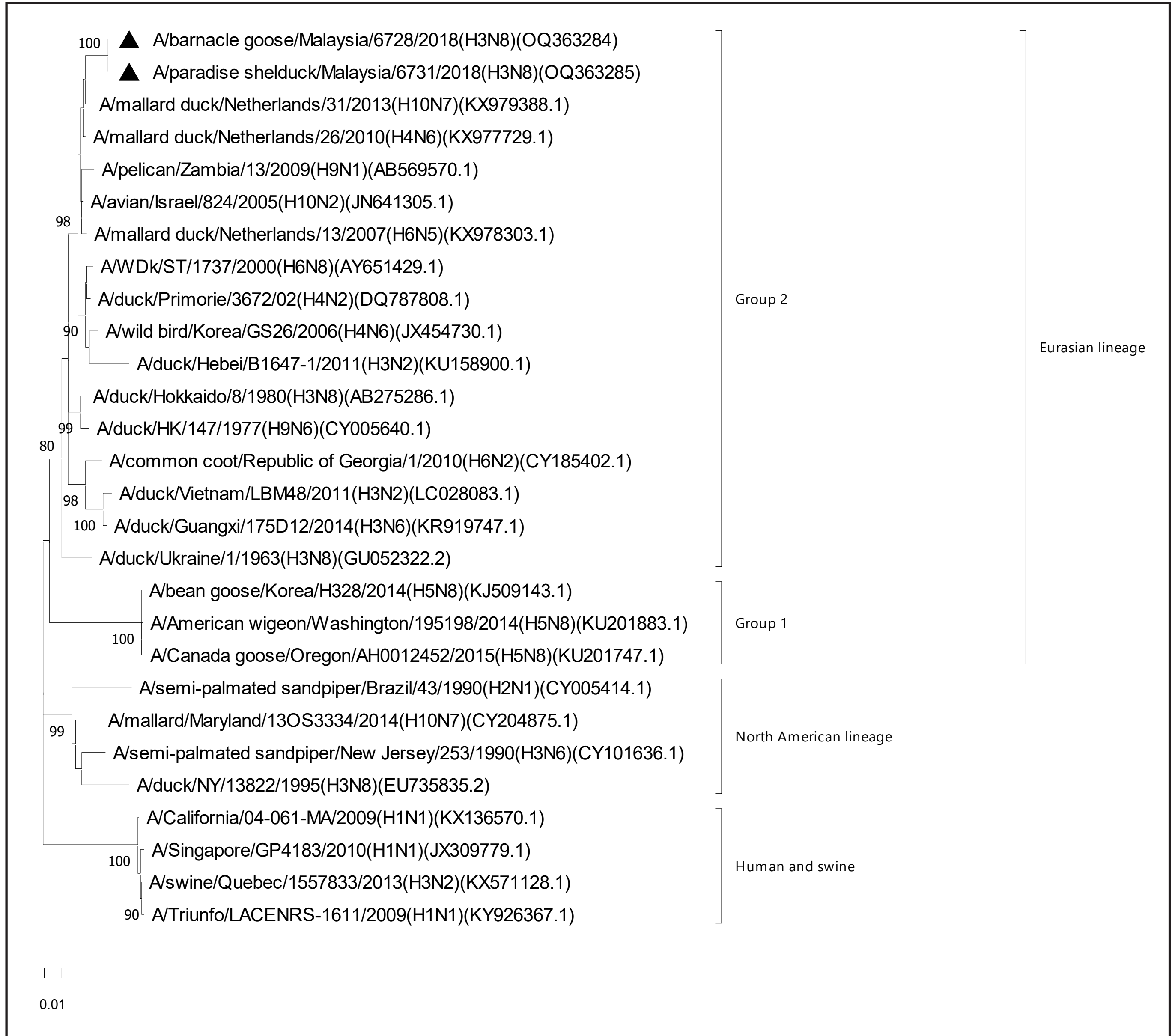

Figure 1H.

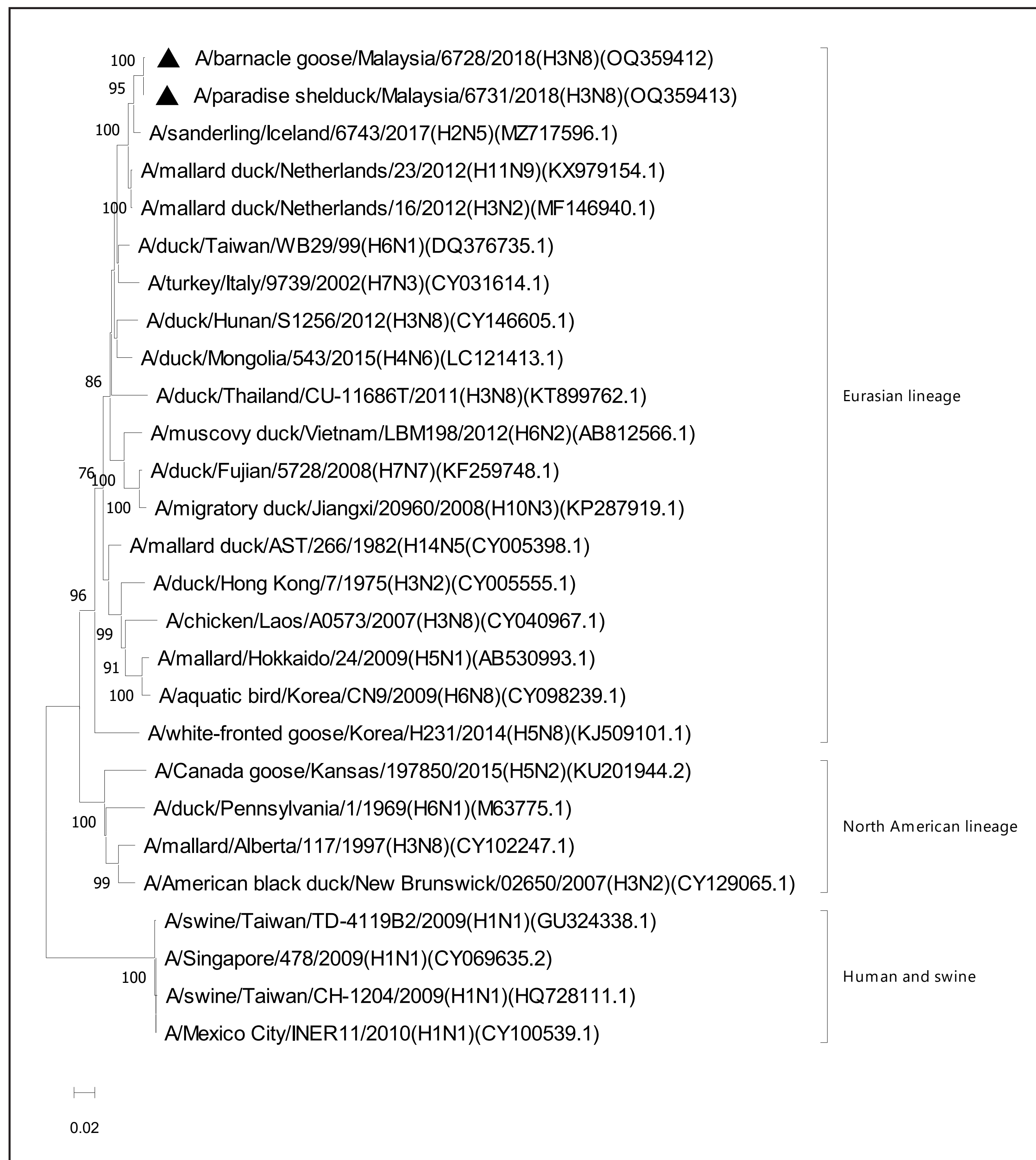

Figure 1. Phylogenetic trees of all eight gene segments of A/barnacle goose/Malaysia/6728/2018 (H3N8) and A/paradise shelduck/Malaysia/6731/2018 (H3N8) with other published sequences. Phylogeny of the HA (Fig. 1A), NA (Fig. 1B), PB2 (Fig. 1C), PB1 (Fig. 1D), PA (Fig. 1E), NS (Fig. 1F), M (Fig. 1G), and NP (Fig. 1H) genes was constructed by Neighbor-Joining statistical method based on the Kimura 2-parameter model and setting bootstrap 1,000 replicates using MEGA version 11. Nodes with bootstrap values of more than 70% are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The black triangles (▲) represent the two H3N8 viruses. The local strain name is suffixed by VRI disease investigation number / species / year detected / country / GenBank accession number.

DISCUSSION

Detection of the H3N8 virus of LPAI strain in the imported goose and shelduck is corresponding to Verhagen *et al.* (2014) that birds from aquatic environments are prone to LPAI especially order Anseriformes. All eight gene segments of A/banarcle goose/Malaysia/6728/2018 (H3N8) and A/paradise shelduck/Malaysia/6731/2018 (H3N8) were phylogenetically grouped into the Eurasian lineage that clustered with viruses detected in water birds from European countries. Interestingly, the phylogenetic analysis of the HA gene indicated that the two H3 viruses isolated from aquatic birds in the study also revealed a close relationship with H3 viruses isolated from chickens in Pakistan. This is not an uncommon finding as AIV subtypes such as H3N2, H3N6 and H3N8 have been isolated from domestic poultry worldwide (Li *et al.*, 2016).

In the study, the phylogenetic analysis of all the internal genes of the two H3 viruses were grouped together with other AIV subtypes indicating that the reassortment event may take place between the two H3 viruses with other AIV strains of different subtypes. Genetic reassortment among the influenza A viruses can promote the virus variation, evolution, and emergence of novel strains giving rise to zoonotic and pandemic influenza strains (Pu *et al.*, 2009; Yao *et al.*, 2022). This can be seen in 1968 Hong Kong H3N2 pandemic caused by the reassortment of avian origin H3N8 virus and human H2N2 virus (Bailey *et al.*, 2016) where the H3 virus was believed to donate the HA and PB1 genes to the pandemic strain (Pu *et al.*, 2009). This H3N2 strain has become endemic, leading to yearly seasonal flu in humans (Bailey *et al.*, 2016, Soda *et al.*, 2020) and the antigenicity continues to change (Soda *et al.*, 2020). Concerning the reassortment event that may occur in the study, the possibility of the virus mutating, evolving, and having capability to transmit to domestic poultry and mammals including humans cannot be neglected. As such, the zoonotic risk of the viruses worth a thorough study.

A crucial characteristic of AIVs is that they can transmit interspecies generally from avian species to mammals (Webster *et al.*, 1992). Both the H3 viruses possessed conserved glutamine (Q) at position 226 and glycine (G) at position 228 (H3 numbering) revealing the preferential binding of the virus to the avian α-2,3 receptor. However, four amino acid substitutions, the S126N, I155T, T160A, and V214I which are associated with increased preferentially binding of the virus to mammalian α-2,6 receptor (Burke & Smith, 2014; Suttie *et al.*, 2019) were recognized in the HA gene. Despite substitutions related to mammalian receptor binding preference, the H3 viruses maintained the avian receptor binding preference sites such as 183 (H) and 190 (E) (Pu *et al.*, 2009). Q226L and G228S in the HA gene are the key substitutions that recognize the mammalian receptor binding sites. Though four amino acid mutations related to the increased preferentially binding to mammalian receptors were detected, the H3 viruses still maintained most of the avian receptor binding preference sites indicating the risk of the virus transmission to mammalian species is relatively low.

The presence of a typical low pathogenic motif (PEKQTR/GLF) in the HA gene of the two H3 viruses indicating that the viruses are of LPAI strain and it was identical to those previously reported avian H3 viruses (Pu *et al.*, 2009; Wu *et al.*, 2014; Cui *et al.*, 2016; Li *et al.*, 2016). The A138S mutation that can increase the infectivity in SIAT cells (Burke & Smith, 2014; Suttie *et al.*, 2019) which contributes to the virus virulence in mammalian species (Yao *et al.*, 2022) is not seen in both H3 viruses.

N-Glycosylation is essential in the protein biological function, folding, maturation, transport, and degradation (Chen *et al*., 2012; Suttie *et al*., 2019). The potential glycosites of the HA and NA protein of AIVs can escape host immune detection by providing similar glycans to the host's glycans to shield the antigenic site (Chen *et al*., 2012; Suttie *et al*., 2019). The potential glycosites identified in the HA protein in the study were similar to the previous report by Anthony *et al.* (2012) and Wu *et al.* (2014) except for the 142NWT. The finding of this different glycosites is not a rare observation as AIV evolves rapidly, there is an accumulation of adaptive mutations and glycosylation sites due to long-term cross-species infection (Chen *et al.*, 2012). So, there is a possibility that more or new glycosites will be discovered.

A 19 amino acid deletion in the NA stalk region associated with enhanced replication in mice is generally seen in highly pathogenic H5N1 viruses (Suttie *et al.*, 2019). Shortened NA stalk is crucial as it is related to viral adaptation from wild birds to domestic poultry (Suttie *et al.*, 2019; Zou *et al.*, 2020). This deletion is not noticed in the H3 viruses in the study and is in agreement with the research on other avian H3 viruses by Li *et al.* (2016) and Zou *et al.* (2020). The "TEI" deletion in the NA stalk in the study which linked with enhanced fatality in mice (Yao *et al*., 2022) is not an uncommon finding as Wu *et al.* (2014) have published similar results. The deletion together with some amino acid mutation in the HA gene may increase the pathogenicity of AIVs in multiple hosts and provide selective advantage (Wu *et al.*, 2014). Substitution I314V associated with reduced susceptibility to oseltamivir (Suttie *et al.*, 2019) was noticed in both H3 viruses. Nonetheless, substitutions such as V116A, E119G, Q136L, R152K, H274Y, R292K, and I222M/K/R which are related to antiviral resistance to neuraminidase inhibitors (NAI) (Suttie *et al.*, 2019; Yao *et al.*, 2022) were not found. The NAI resistance is rising as a result of broadly used antidrug (Suttie *et al.*, 2019). In this context, environmental contamination by NAI plays a role in the increased antiviral resistance as well (Suttie *et al.*, 2019). Despite the I314V substitution, H274Y and other antiviral resistance markers were not identified in the study. Hence, the possibility of the H3N8 viruses resistant to NAI is relatively low.

In the PB2 gene, the 251R and 590S substitutions are associated with virus growth adaptation in mammals (Yao *et al.*, 2022). The H3N8 viruses acquired the 251R substitution, but not the 590S in the PB2 gene. Substitutions that have been proven to increase virulence and polymerase activity in mammalian cell lines such as E627K, T271A, A588I/V, Q591K, and D701N (Gora *et al.*, 2014; Suttie *et al.*, 2019; Yao *et al.*, 2022) were not noticed in the two viruses. Two well-known mutations in PB2 protein, the E627K and D701N are crucial in the virulence and transmission of AIVs in mammals (Zou *et al.*, 2020) where these mutations can enhance the adaptability of the virus in mammalian cells, allowing them to replicate well in mammals (Yang *et al.*, 2021). These two mammalian adaptation-associated residues were not present in the study indicating reduced efficient adaptation of the H3 viruses to mammals. Several substitutions that were associated with increased polymerase activity in mammalian cells and increased virulence in mice such as L89V (Yao *et al.*, 2022), I292V, K389R, V598T, G309D, T399K, I495V, A676T and R477G (Suttie *et al.*, 2019) were seen in the H3 viruses. Interestingly, two mutations linked to decreased virulence in mice, namely E358V and S715N were also observed. These two mutations has been experimentally demonstrated to decrease the virulence of virulent H7N3 and H5N1 virus respectively in mice (Sun *et al.,* 2015; Desrochers *et al*., 2016).

The K577E and D622G mutations in the PB1 gene are known to increase polymerase activity and virulence in mice (Suttie *et al.*, 2019; Yao *et al.*, 2022). The D622G was identified in the two H3N8 viruses, and yet the K577E was not detected. D3V mutation that can increase polymerase activity and viral replication in avian and mammalian cell lines (Suttie *et al.*, 2019) was observed in the PB1 gene in the study. Conversely, the P598L that was linked with decreased polymerase activity and replication in mammalian cell lines (Suttie *et al.*, 2019) was also recognized in the two H3 viruses. S524G substitution that enhances virulence and fitness for transmission in mammals reported by Zhang *et al.* (2021) was not seen in the PB1 protein. In the PB1-F2 protein, the N66S substitution is related to enhance replication, virulence, and antiviral response in mice (Gora *et al.*, 2014; Suttie *et al.*, 2019; Yao *et al.*, 2022). The two

H3 viruses in the study encoded the full-length PB1-F2 protein, but the N66S was not seen in the viruses. Though the N66S mutation was not found, the T51M and V56A substitutions in the PB1-F2 were noticed. These two mutations were linked to decreased polymerase activity, replication, and virulence in ducks (Suttie *et al.*, 2019).

In the PA gene, there are several single amino acid substitutions that can increase AIV virulence in mice including the V63I, T97I, K142N/E, K356R, S421I, R443K, and K615N (Suttie *et al.*, 2019). None of these mutations were detected in the viruses in the study. However, substitutions S37A, N383D, N409S, H266R, and S515T which can increase polymerase activity in mammalian cell lines (Suttie *et al.*, 2019) were found. N383D mutation in PA only increases polymerase activity in mammalian and avian cell line (Suttie *et al.*, 2019). If this mutation together with S224P substitution, both mutation will increase AIV H5N1 virus virulence in mice and ducks (Suttie *et al.*, 2019). The combination of these mutations in affecting the virulence is different from the previously mentioned mutation where a single mutation in the polymerase protein typically influences the virulence in mammals with minimal changes in the polymerase activity, replication, or virulence in avian (Suttie *et al.*, 2019). Fortunately, only the N383D mutation is identified in the study. Thus, the risk of the increased virulence of the viruses in the host such as duck is relatively low. On the other hand, the P190S that was associated with decreased virulence in mice (Suttie *et al.*, 2019) was also observed in the two H3N8 viruses.

The influenza virus genomic segment 8 encodes the NS1, NS2 (also known as nuclear export protein, NEP), and NS3 (Suttie *et al.*, 2019). An important mutation in the NS1 protein, the P42S which increases virulence and decreases antiviral response in mice (Gora *et al.*, 2014; Suttie *et al.*, 2019; Yao *et al.*, 2022) was not observed in the two H3N8 viruses. In contrast to this finding, mutations that related to enhanced replication in mammalian cells, increase virulence, and decrease interferon response in chickens including I106M, C138F, V149A, P3S, R41K, K66E (Suttie *et al.*, 2019) were identified in both H3 viruses. In the NS1 protein, the effect of the PDZ motif on the viral phenotype depends on the infected host and virus strain (Suttie *et al.*, 2019). The avian viruses generally contain the ESEV or EPEV motif (Suttie *et al.*, 2019) which supports the finding of the ESEV motif in the H3N8 viruses in the study. In general, the PDZ domain is not a major factor in AIV pathogenicity as the ESEV motif does not affect the virulence in chickens (Suttie *et al.*, 2019) though the ESEV motif is usually associated with decreased viral replication in human and duck cell line (Suttie *et al.*, 2019). No elongation was observed in position 230 to 237 in the NS1 protein where the elongation can increase replication and inflammatory cytokine production in chickens (Suttie *et al.*, 2019). In comparison, there is no mutation seen in the NS2 protein including the M16I, Y41C, and E75G that is thought to be linked with increased polymerase activity in mammalian cells (Gora *et al.*, 2014; Suttie *et al.*, 2019). NS3 protein was just recognized recently and the functions remain unclear. Therefore, there is no information regarding the amino acid mutation yet (Suttie *et al.*, 2019).

Segment 7 of the influenza virus encodes the matrix 1 and 2 protein. There are four amino acid substitutions in M1 that can increase the virulence of H5 subtypes in mice, namely N30D, I43M, T139A, and T215A (Suttie *et al.*, 2019; Yao *et al.*, 2022). Among these mutations, the I43M also increases virulence in ducks and chickens (Suttie *et al.*, 2019). Both the H3N8 viruses consisted the above four mentioned substitutions except the T139A. In the M2 protein, mutations L26F, V27A, A30V/T/S, S31N/G, and G34E that corresponding to adamantane resistance (Suttie *et al.*, 2019; Yao *et al.*, 2022) was not noticed in the two H3 viruses. Besides NA, M2 is one of the targets for antidrug usage. Adamantane which is used in seasonal human influenza is no longer suggested as adamantane resistance is rising (Suttie *et al.*, 2019). In this study, no mutation associated with adamantane resistance was observed.

N319K substitution in the NP gene is famous for increased polymerase activity and replication in mammalian cell lines (Suttie *et al.*, 2019; Yao *et al.*, 2022). This mutation was not detected in the two H3N8 viruses. Nevertheless, the M105V and A184K that involve in enhanced viral replication and increased AIV virulence in chickens (Suttie *et al.*, 2019) were recognized. The M105V substitution noticed in the NP gene in the two H3N8 viruses can influence virus replication in chicken but not in duck embryo fibroblast (Suttie *et al.*, 2019). Hence, this mutation may play a role in the spillover adaptation from ducks to chickens (Suttie *et al.*, 2019).

Migratory birds may enhance the globalization of the transmission of influenza viruses (Yang *et al.*, 2022) and H3 virus is generally of LPAI (Yang *et al*., 2021) with most of the infected animals are asymptomatic (Soda *et al.*, 2020). Thus, disease screening for imported animals and/or animal products at the entry point is important to prevent the introduction of new pathogens into our country. Fortunately, in this case, we managed to identify the presence of AIV in the imported birds before they are released into the country. It is not unusual for the virus to be isolated from imported birds as Anseriformes are able to carry the virus over long distances (Kayed *et al.*, 2019). Furthermore, sharing cages and drinking water during transportation may cause viral transmission to occur as well (Kayed *et al.*, 2019). Also, the LPAI is prone to replicate in the digestive tract in water fowl and the virus can be shed in the feces for more than 20 days (Spackman, 2020). This is corresponding to the recovery of the H3 viruses in the fecal swab in the study.

There is currently no evidence that the avian-origin influenza viruses can transmit efficiently from human to human through aerosol (Suttie *et al.*, 2019). Rarely, human is infected by AIVs and other zoonotic influenzas (WHO, 2022). In April and May 2022, first two human infections with avian origin H3N8 viruses in China were reported (Yang *et al.*, 2022). The novel virus was resulted from triple reassortment with the Eurasian avian H3 gene, North American avian N8 gene, and internal genes of the H9N2 viruses (Yang *et al.*, 2022). Notably, the NA gene of the virus might have originated in Alaska and been transmitted by migratory birds through the East Asian flyway. This human infection indicates that avian H3N8 virus is capable to reassort and adapt in mammalian host (Yang *et al.*, 2022). More importantly, with the first human death from AI H3N8 virus reported in China recently (March 2023) where the deceased has exposed to live poultry and wild birds before she become ill; and the environmental specimens from the wet market that she had visited was positive for influenza H3 virus (CDC, 2023); the potential risk of the avian H3N8 virus in causing future influenza pandemic should not be underestimated.

In our case, although four substitutions related to mammalian receptor binding preferences were recognized, the H3N8 viruses maintain most of the avian receptor binding sites including the Q226 and G228. Also, the two mammalian adaptation associated residue in the PB2 gene, the E627K and D701N were not observed in the viruses. Moreover, the S524G in the PB1 gene that can enhance the fitness for transmission in mammals was not seen. Taken together, the likelihood that the viruses can spread into mammals is relatively low. Nevertheless, the study on the replicative capacity of the H3 viruses in the mammalian cells and study on the cross species transmissibility in the animal model worth an in-depth investigation.

CONCLUSION

Two LPAI H3N8 viruses were isolated from imported waterfowl and genetic reassortment may occur in the internal genes of the AIVs. Though the genetic characteristics of all the eight gene segments, amino acid substitutions, receptor binding properties, and antiviral resistance have been discussed; the replicative capacity, transmissibility, and the potential risk of the virus spreading into mammals are worth in-depth investigation. Lastly, with the increase

in the international trade of birds; strict quarantine practices at the entry point and good, efficient laboratory diagnostic capabilities are crucial to prevent the introduction of new virus strains into our country.

ACKNOWLEDGEMENTS

Thanks to the Director General of Veterinary Services Malaysia (DVS) for her permission to publish this scientific paper. A great appreciation to Sabah Department of Veterinary Services and Kota Kinabalu Veterinary Diagnostic Laboratory for providing the samples. Not forget to thank the Director of Veterinary Research Division, DVS for her fully support in this study. This finding of the study was supported by VRI Ipoh.

Conflict of interest

The author declares that they have no conflict of interest.

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