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Molecular characterization of highly pathogenic avian influenza H5N8 viruses isolated from Baikal teals found dead during a 2014 outbreak in Korea

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Nineteen highly pathogenic avian influenza (HPAI) H5N8 viruses were isolated from wild birds in the Donglim reservoir in Gochang, Jeonbuk province, Korea, which was first reported to be an outbreak site on January 17, 2014. Most genes from the nineteen viruses shared high nucleotide sequence identities (*i.e.*, 99.7% to 100%). Phylogenetic analysis showed that these viruses were reassortants of the HPAI H5 subtype and the H4N2 strain and that their hemagglutinin clade was 2.3.4.4, which originated from Eastern China. The hemagglutinin protein contained Q222 and G224 at the receptor-binding site. Although the neuraminidase protein contained I314V and the matrix 2 protein contained an S31N substitution, other mutations resulting in oseltamivir and amantadine resistance were not detected. No substitutions associated with increased virulence and enhanced transmission in mammals were detected in the polymerase basic protein 2 (627E and 701D). Non-structural-1 was 237 amino acids long and had an ESEV motif with additional RGNKMAD amino acids in the C terminal region. These viruses caused deaths in the Baikal teal, which was unusual, and outbreaks occurred at the same time in both poultry and wild birds. These data are helpful for epidemiological understanding of HPAI and the design of prevention strategies.

Keywords: H5N8, avian influenza, phylogenetic analysis, surveillance, wild bird

Introduction

Highly pathogenic avian influenza (HPAI) viruses cause economic losses and pose a threat to public health [15]. Avian influenza viruses (AIV) have segmented genomes, and antigenic drift and shift are important mechanisms for producing rapid diversity [16]. H5N1 is the representative subtype of HPAI in Asia and has evolved into over 32 clades distinguished by their hemagglutinin (HA) genes [13].

In Korea, there have been four outbreaks of H5N1 HPAI [6]. The A/chicken/Korea/Es/2003 (H5N1) caused the first outbreak in 2003 and 2004. This virus clustered with the A/Duck/China/E319-2/03 (H5N1), which is designated as clade 2.5. The virus involved in the second outbreak, A/chicken/Korea/Is/2006 (H5N1), belongs to the Qinghai-like H5N1 HPAI viruses [7,9]. In the third outbreak, the causative virus was A/chicken/Korea/Gimje/2008 (H5N1) from clade 2.3.2, which was similar to A/Muscovy duck/Vietnam/1455/06. The fourth outbreak, caused by A/duck/Korea/Cheonan/2010 (H5N1), occurred in 2010–2011. In this outbreak the strain was clustered into clade 2.3.2.1 and was similar to the A/Whooper swan/Mongolia/21/10 (H5N1) [5]. This virus has been found in various wild bird species such as the mallard, Baikal teal, mandarin duck, whooper swan, and Eurasian eagle owl [5].

On January 16, 2014, the fifth outbreak was caused by a strain of H5N8 in Korea. The first reported case was in a breeder duck farm in Gochang in Jeonbuk province. On January 17, another infection was reported in broiler ducks in Buan (near Gochang), and flocks of Baikal teal carcasses were found in the Donglim reservoir in Gochang [10].

Here, we obtained nineteen H5N8 viral isolates from wild Baikal teal in the Donglim reservoir in Gochang, Korea, where

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the 2014 outbreak was first reported [7]. To obtain further genetic information regarding these viruses, all eight gene segments were sequenced and characterized by molecular and phylogenetic analysis.

Materials and Methods

Sampling and virus isolation

Nineteen viruses were isolated from cloacal and tracheal swab samples of 27 wild Baikal teals that were found dead at Donglim reservoir in Gochang, Jeonbuk province, Korea on January 20, 2014. Virus isolation was performed in 9- to 11-day-old specific-pathogen-free embryonated chicken eggs. After 72 h of incubation at 37°C, the eggs were chilled, and allantoic fluids were harvested and tested for hemagglutinin activity according to the WHO manual [17]. Necropsy and virus isolation were performed under biosafety level 3 conditions at Konkuk University in Korea.

Genome sequencing

RNA was extracted using a RNeasy kit (Qiagen, USA) according to the manufacturer's instructions. AIV-positive specimens were further characterized by complete genome sequencing. The eight genes of AIV were amplified by multiplex RT-PCR using previously described primers [1]. Next, 2 μg of the RT-PCR amplicons of all eight gene segments was used for preparation of the Ion Fragment sequencing library (Life Technologies, USA) according to the manufacturer's instructions. Briefly, amplicons were loaded onto beads, and emPCR or emulsion PCR was conducted prior to sequencing with the Ion 318 Chip on the Ion Torrent Personal Genome Machine. *De novo* and directed assembly of genome sequences was performed using the Geneious R7 program [4]. Nucleotide sequences for gene segments have been deposited in GenBank under accession No. KJ756562 to KJ756713.

Phylogenetic analysis

The basic local alignment search tool, BLAST (National Center for Biotechnology Information, USA) was used to search for homologous genes. Phylogenetic analysis was conducted using the MEGA 6 program and inferences were made using the neighbor-joining method from 1,000 bootstrap values. For phylogenetic analysis, the nucleotide sequences used in this study were deposited in GISAID (Friedrich-Loeffler-Institut Germany's Federal Research Institute for Animal Health, Germany) and in GenBank (National Center for Biotechnology Information, USA). The viral isolates were named according to their sample numbers: for example, A/Baikal teal/Korea/D2402/2014 (H5N8) was 2402.

Results

Nineteen viruses were isolated from cloacal and tracheal swab samples of a flock of Baikal teal from Donglim reservoir. The sampling area was mainly in the north-east area of Donglim reservoir (panel C in Fig. 1), where 89 Baikal teal, seven bean goose, one common coot, and one whooper swan were found dead.

Subsequently, all eight gene segments of the 19 different viruses were sequenced by high-throughput sequencing using

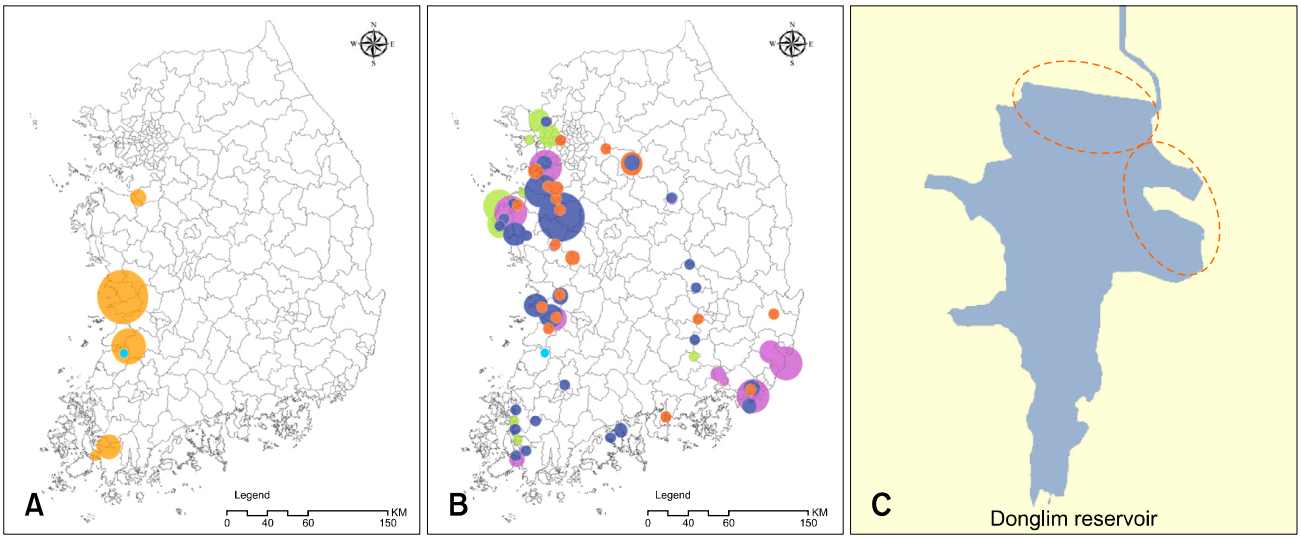

Fig. 1. Waterfowl distribution during the HPAI H5N8 outbreak in Korea based on a survey conducted by the National Institute of Biological Resources from January 21–23, 2014. Light blue circles indicate Donglim reservoir. (A) Distribution of Baikal teal (yellow). (B) Distribution of spot-billed duck (orange), mallard (blue), bean goose (light green) and common coot (purple). (C) Sampling region of Donglim reservoir (dashed circle).

the Ion Torrent PGM platform. The number of obtained sequencing reads from each sample was between 1,207 and 48,200 (average of 15,736 reads). Furthermore, mapping of the reads to the reference genome (A/baikal teal/Korea/Donglim3/2014 (H5N8); GenBank accession No. KJ413847-KJ413854) was performed at a depth of 124.43−11177.50 (Supplementary Fig. 1). The reads covered 99.88−100% of the total genome, which suggests that all eight genes were completely sequenced. Homology analysis of the nineteen viral genome sequences showed that most of the genes shared high nucleotide sequence identity of 99.7% to 100% (Supplementary Fig. 2). All 19 viral isolates were homologous to viral isolates from Eastern China (Table 1), with 97% to 99% similarity at the nucleotide level. These viruses were also homologous to previous Korean isolates of H5N8 from Buan (GenBank accession No. KJ413839-KJ413846) and Donglim (KJ413847-KJ413854), with 99.6% similarity. Deduced amino acid sequence analysis of the complete genome sequence compared to sequences of Buan and Donglim strains, revealed that viruses sequenced in this study had 1 to 8 variations (Table 2).

Compared to the sequences of the nineteen viral isolates, the PB2 and HA genes in A/wild duck/Shandong/628/2011 (H5N1) showed 98.8% and 97.2% to 97.3% similarity, and NP had 98.9% to 99.0% similarity with the respective sequence in the A/wild duck/Shandong/1/2011 (H5N1). The PB1, PA, NS, and M genes of the nineteen viruses showed 98.6%, 98.2% to 98.4%, 98.7% to 98.8%, and 98.7% to 98.9% similarity to the respective nucleotide sequences of A/duck/Jiangsu/1-15/2011 (H4N2), whereas the NA genes were closely related to those of A/duck/Jiangsu/k1203/2010 (H5N8) with 97.9% to 98.1% similarity.

Table 1. H5N8 virus genes and their closest relatives based on nucleotide sequence

Gene	Relative nucleotide	Similarity (%)*
PB2	A/wild duck/Shandong/628/2011 (H5N1)	98.8
PB1	A/duck/Jiangsu/1-15/2011 (H4N2)	98.6
PA	A/duck/Jiangsu/1-15/2011 (H4N2)	98.2
HA	A/wild duck/Shandong/628/2011 (H5N1)	97.2−97.3
NP	A/wild duck/Shandong/1/2011 (H5N1)	98.9−99.0
NA	A/duck/Jiangsu/k1203/2010 (H5N8)	97.9−98.1
M	A/duck/Jiangsu/1-15/2011 (H4N2)	98.7
NS	A/duck/Jiangsu/1-15/2011 (H4N2)	98.4

*Similarity was expressed relative to 19 Korean H5N8 isolates from this study.

Table 2. Amino acid substitutions of 19 H5N8 viruses

Name*	PB2	PB1	PA	HA	NP	NA	M	NS
1437	−	K54Q	−	D473G	−	E72K	−	−
1441	R497	−	M261V	A9S	−	−	−	T197I
1445	−	−	L163I	−	−	−	−	−
1446	R497	−	−	−	−	−	−	−
1447	−	−	−	N184K	−	−	−	−
1448	I57L	−	−	T247A	−	−	−	−
1449	−	−	K158R	−	−	V106I	−	−
1452	−	−	K104Q	−	−	−	−	−
1454	R497	K578N	−	A156E	−	−	−	A60V
1456	I57L	−	−	−	−	−	−	R88L, V237I
1457	T471A	−	−	−	−	−	−	−
1458	−	−	−	D171N	−	−	−	−
2399	I57L	−	−	R343K	−	S337N	−	V237I
2402	A59G, R70I, G682R, V690L, G693E, F694I	−	D419N	D59N	−	−	−	−
2403	−	−	−	−	S84N	−	−	−
2406	I57L	−	−	T247A	−	−	−	V237I
2414	T471A	−	−	-	−	−	−	−
2416	−	−	−	H311N	−	−	−	−
2417	−	−	−	−	−	−	A206V	−
Buan2	S497	−	−	−	−	−	−	−
Donglim3	R497	−	−	−	−	−	−	−

*Viruses were named according to the analysis number; e.g., 1437 indicates A/baikal teal/Korea/D1437/2014 (H5N8).

Phylogenetic analysis of the HA genes indicated these viruses belonged to H5 clade 2.3.4.4. (Fig. 2). The HA genes of the viral isolates in this study were located in the same cluster as the H5 Eastern China isolates, such as A/wild duck/Shandong/628/2011 (H5N1). These HA genes also formed a branch with other Korean H5N8 isolates available in GenBank: A/breeder duck/Korea/Gochang1/2014 (Gochang1), A/broiler duck/Korea/Buan2/2014 (Buan2), and A/baikal teal/Korea/Donglim3/2014 (Donglim3). The HA genes had 99.6% similarity to those in Buan2 and Donglim3 and 96.4% to 96.6% similarity with

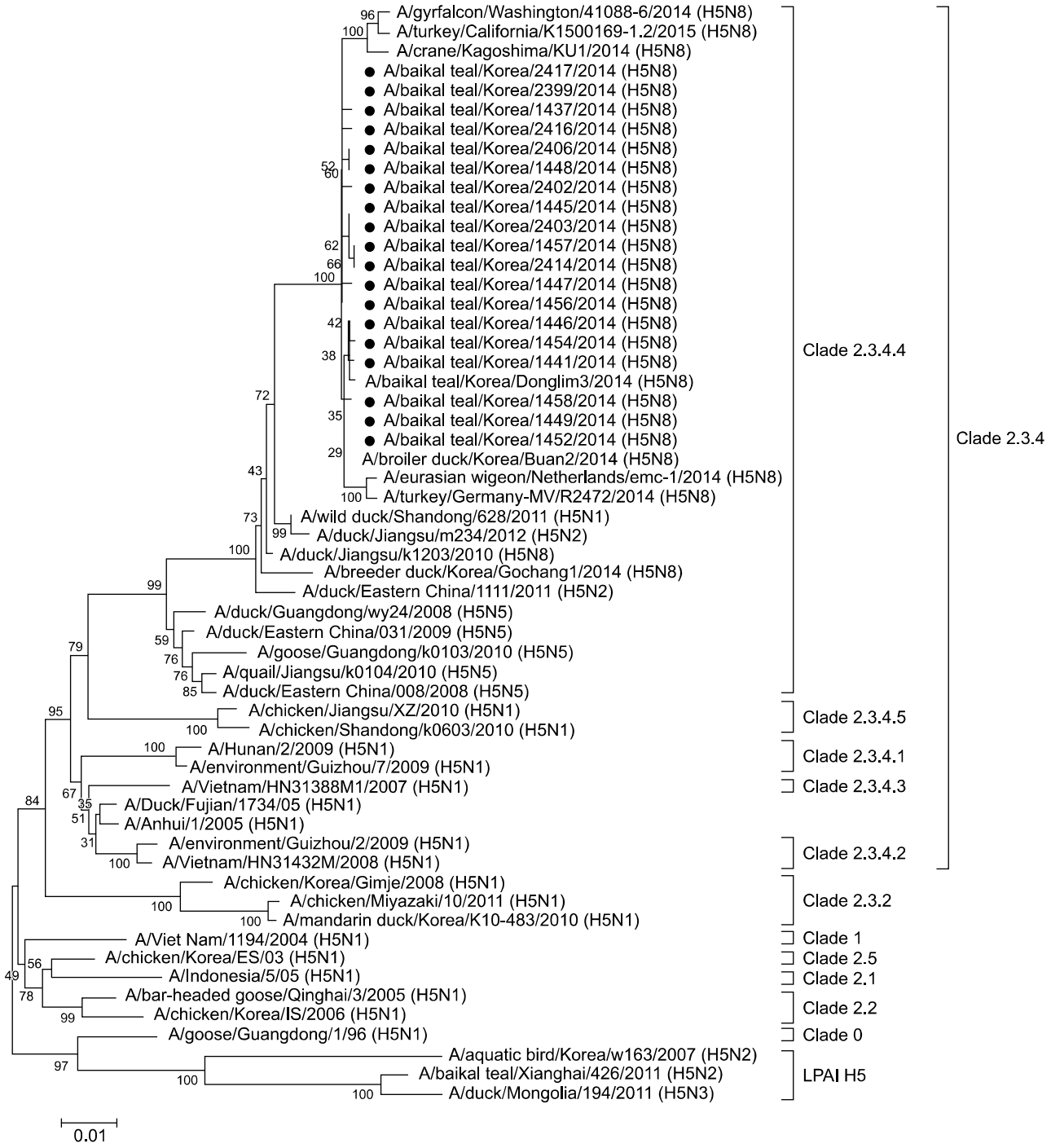

Fig. 2. Neighbor-joining phylogenetic tree for the H5 gene (nucleotide positions: 49–1649). The black circle (●) indicates the genes of isolates from this study. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

Gochang1 (GenBank accession No. KJ413831–413838). The NA genes were 99.8% similar to those in Buan2 and Donglim3 and had 97.8% to 97.9% similarity with those in the Gochang1 isolates. Phylogenetic analysis of the NA genes indicated that these viruses belonged to the N8 subtype of the Eurasian lineage, and they clustered with the H3N8 isolates (Fig. 3). Phylogenetic analysis of the six internal genes indicated that these 19 strains were reassortant viruses with genes derived from H5N2, H4N2, H5N5 and H5N8 viruses from eastern China.

All of the viruses in this study have a highly pathogenic motif with multiple basic amino acids, such as PLRERRRKRG at the HA cleavage site (Table 3), and they have Q222 and G224 at the receptor-binding sites of the HA gene, similar to H5N8 viruses. The other amino acid sequences were similar to those of clade 2.3.4.4 viruses, with the exception of K156A in antigenic site A and S94T in antigenic site D. Although the neuraminidase protein contained I314V and the matrix 2 protein contained an S31N substitution, other mutations resulting in oseltamivir and amantadine resistance were not evident (Table 4). Other amino acid residue changes known to be relevant for virulence or transmission included 627E and 701D in the PB2 protein (Table 5). The NS1 proteins had a length of 237 amino acids and the ESEV PDZ-binding motif (PBM) with additional RGNKMAD

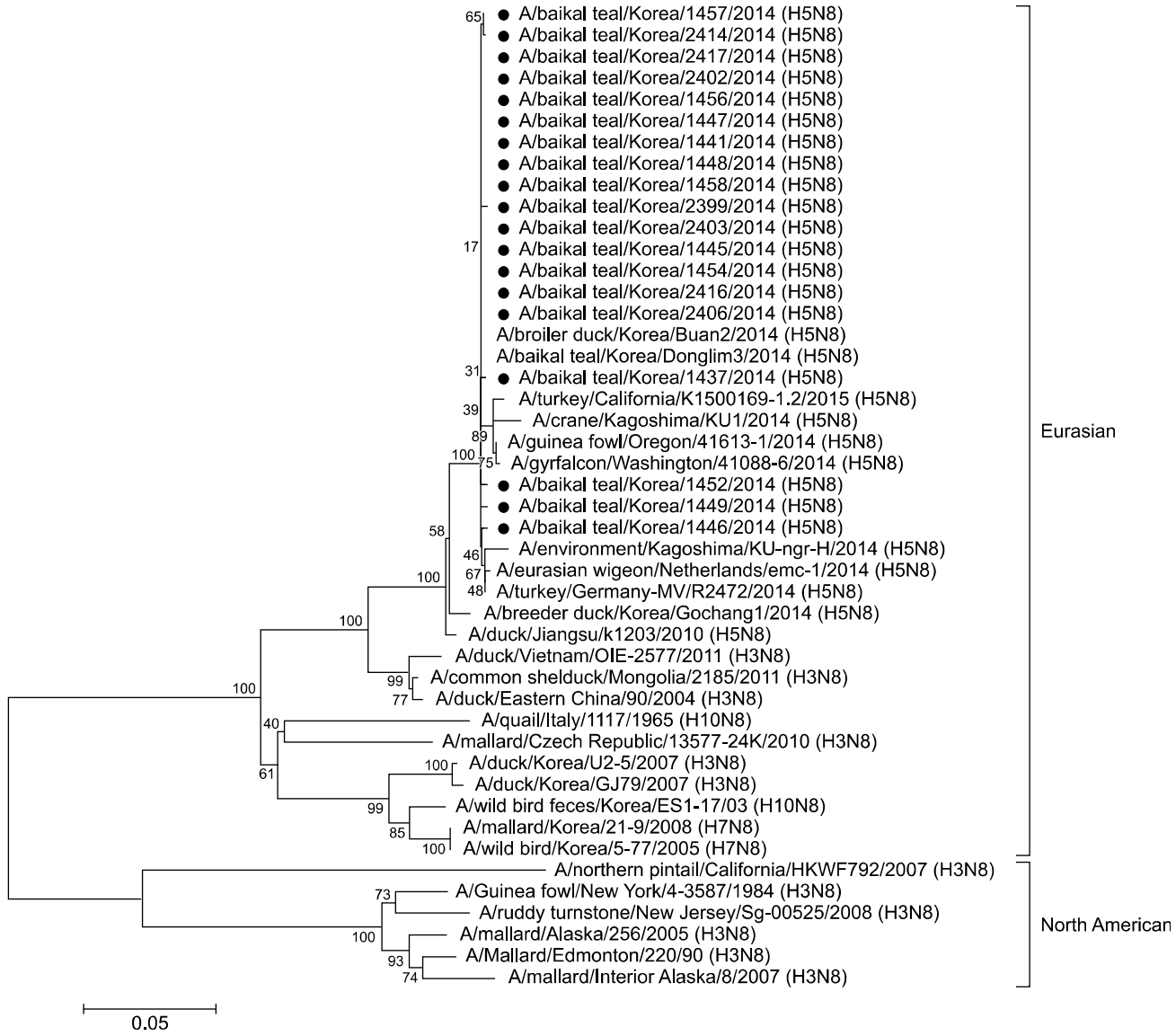

Fig. 3. Neighbor-joining phylogenetic tree of N8 gene (nucleotide positions: 31–1374). The black circle (●) identifies the genes of isolates used in this study. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

Table 3. Amino acid characteristics of the HA protein of H5N8 isolates

Amino acids*	k1203†	Sd628‡	H5N8§	Comments
222	Q	Q	Q	Receptor binding site
224	G	G	G	
140	T	A	A	Antigenic site A
141	P	P	S	
94	S	N	T	Antigenic site D
226	M	M	M	
227	D	D	D	
124	D	D	N	Virulence determinant
156	A	A	A	
212	K	K	K	
263	T	T	T	
154	NDA	NDA	NDA	N-glycosylation
321	PLREKRR-KR	PLREKRR-KR	PLRERRR-KR	Cleavage site

*Amino acids were named according to H3 numbering. †A/duck/Jiangsu/k1203/2010 (H5N8). ‡A/wild duck/Shandong/628/2011 (H5N1). §19 Korean H5N8 isolates from this study.

Table 4. Amino acid characteristics in the NA and M proteins associated with resistance against antivirals

Gene	Antivirals	Substitution*	k1203	H5N8
Neuraminidase gene	Oseltamivir resistance	I117V	I	I
		E119V	E	E
		D198N	D	D
		H274Y	H	H
		R292K	R	R
		N294S	N	N
		I314V	V	V
	Zanamivir resistance	V116A	V	V
		R118K	R	R
		Q136K	Q	Q
		D151E	D	D
		R152K	R	R
		R224K	R	R
		E276D	E	E
		R371K	R	R
Matrix gene	Amantadine resistance	L26F	L	L
		V27A	V	V
		A30T/S	A	A
		S31N	N	N
		G34E	G	G

*Amino acid substitutions of neuraminidase were named according to N2 numbering.

Table 5. Amino acid characteristics of internal proteins associated with transmission and virulence

Function	Substitution		k1203	Sd628	JS1-15*	H5N8
Enhanced transmission	PB2	A199S	A	A	A	A
		A661T	A	A	A	A
		K702R	R	K	K	R
		V667I	V	V	V	V
	PA	S409N	S	S	S	S
	NP	L136M	L	L	L	L
	M2	A16G	E	E	E	E
		C55F	L	L	L	L
Virulence determinant	PB2	E627K	E	E	E	E
		D701N	D	D	D	D
	PB1-F2	N66S	S	S	S	S
	NS1	T92E	D	D	D	D
		PBM	EPEV	ESEV	ESEV	ESEV

*A/duck/Jiangsu/1-15/2011 (H4N2).

amino acids in the C terminal region [11].

Discussion

On January 17, 2014, an outbreak of HPAI H5N8 was reported in the Donglim reservoir in Gochang, Korea. AIV infections in wild waterfowl are usually mild or asymptomatic [2]; however, this virus caused deaths in the Baikal teal (*Anas Formosa*) [10]. In addition, during the outbreak period, H5N8 infection was identified in carcasses, feces, and cloacal and tracheal swabs of wild birds such as the bean goose (*Anser fabalis*), common coot (*Fulica atra*), and mallard (*Anas platyrhyncos*). In this study, we sequenced and analyzed the complete genome sequences of 19 H5N8 viral isolates using high-throughput sequencing with an Ion Torrent PGM platform and provided detailed information regarding their molecular characteristics.

Two opinions exist regarding the origin of the H5N8 viruses, Gochang1 and Buan2. In March 2014, Lee *et al*. [10] suggested that the H5N8 viruses were reassorted in Eastern China and introduced by migratory birds. In May 2014, Wu *et al*. [18] described a novel reassortant H5N8 virus isolated in 2013 that is the precursor of Gochang1. However, Ku *et al*. [6] suggested that Buan2 may have reassorted in Korea because the HA gene of A/waterfowl/Korea/S005/2014 (H5N8) has 97% homology to the closest matching gene in GenBank. Phylogenetic analysis of our H5N8 viruses showed that they are genetically similar to viruses isolated from Eastern China since 2010 [3]. The PB2, HA, NP, and NA genes were from H5 subtype viruses, and four other genes were from the H4N2 strain A/duck/Jiangsu/1-15/2011 (KC282877-78, KC282882-83). According to Zhao et al., this H4N2 virus was a natural reassortant with regard to

the HA gene from A/wild duck/Korea/CSM4-28/2010 (H4N6) and seven other genes from HPAI H5N2 strains, including A/duck/Eastern china/1111/2011 and A/goose/Eastern China/1112/2011 [19]. Therefore, the 19 viral isolates in this study were likely derived from HPAI H5 reassorted viruses, and their origin appears to be in China. Additionally, H5N8 viruses identified in Korea were genetically similar to H5N8 viruses in clade 2.3.4.4 reported in Europe, Japan, and North America since late autumn 2014 [8].

The HA of the H5N8 viruses contains Q226 and G228, which indicates that these viruses preferentially bind to avian-like α-2,3 sialic acid linkages on host receptors. The NA of the H5N8 viruses has the I314V substitution, which is a molecular marker for oseltamivir resistance [10]. However, other molecular markers of oseltamivir resistance (I117V, E119V, D198N, H274Y, R292K and N294S) and zanamivir resistance (V116A, R118K, E119G/A/D, Q136K, D151E, R152K, R224K, E276D, R292K and R371K) [12] were not observed (N2 numbering). The PB2 protein contained the mutations 627E and 701D, and the NS1 protein contained the ESEV motif at the C-terminus. In particular, when the 19 H5N8 viruses were compared with Buan2, some point mutations became evident and were presumed to be host adaptations resulting from neutral evolution of the Baikal teal. PB2 of 2402 (KJ756575) showed the most changes, with variations in 11 nucleotides corresponding to six amino acid changes: A59G, R70I, G682R, V690L, G693E, and F694I. G693E may alter the nuclear localization signal (NLS) [14], whereas the potential function of the other substitutions is unknown.

In summary, 19 H5N8 viruses were isolated from Baikal teal in Donglim reservoir in Gochang, Korea at a time when an H5N8 outbreak was occurring in both poultry and wild birds. Phylogenetic analysis showed that the H5N8 outbreak strains were related to highly pathogenic H5 and H4N2 reassortant viruses that originated from Eastern China. Although the route of virus spread still needs to be clarified, our results revealed a close relationship between HPAI isolates identified in 2014 from Korea and Eastern China. Therefore, enhanced surveillance of AIV from poultry farms and wild bird populations could increase the epidemiological understanding of HPAI and facilitate the design of prevention strategies.

Acknowledgments

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Conflict of Interest

There is no conflict of interest.

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