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BRIEF REPORT

Two novel reassortant H11N8 avian influenza viruses occur in wild birds found in East Dongting Lake, China

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Abstract

During the surveillance of avian influenza viruses in East Dongting Lake, China (2014–2015), two H11N8 avian influenza viruses were detected in the bean goose (*Anser fabalis*) and the falcated teal (*Anas falcata*). Phylogenetic analysis showed that these two novel reassortant H11N8 avian influenza viruses contain genes from poultry and wild birds. This is the first report detecting the H11N8 subtype influenza virus from wild birds in Asia. These findings indicate a dissemination of avian influenza virus along the East Asian-Australian flyway. In addition, the interaction between poultry and wild birds was addressed suggesting the need for intensive surveillance of wild bird populations.

H11 avian influenza viruses (AIVs) have been widely detected in domestic poultry and in wild birds [2, 15, 33]. H11N1 virus was identified in India among wild aquatic birds in 2007 [23]. By 2010, the H11N2 and H11N8 viruses were detected among wild bird populations in the Ukraine [21]. Subsequently, in 2011 and 2013, the H11N3 virus was isolated from domestic ducks in China [3, 29]. Since 1997, the H11N9 virus has been identified from geographic locations ranging from Eastern Asia to North America [13, 15, 18].

The East Dongting Lake National Nature Reserve (29.32°N, 112.98°E) in the Hunan Province, China, is a large natural area forming part of the more extensive Dongting Lake wetlands. This environment is listed as an important bird area for water birds in large numbers and serves as a significant overwintering area for migratory birds along the East Asia-Australia flyway [31]. Typically, there is a peak in the number of wild birds between December and February [19]. In this geographic location, domestic ducks share the same water area with migratory birds. This juxtaposition of populations provides an opportunity for AIVs to spread between migratory birds and domestic poultry via the direct or indirect fecal-oral-route. This dissemination appears to be the cause of viral reassortants [8, 27]. The cocirculating AIVs of different origins and subtypes may serve as donors of diverse gene segments for each other and generate new genetic combinations (reassortants).

Two H11N8 AIVs were identified from fecal samples of waterfowl in the East Dongting Lake between December 2014 and February 2015. Fresh feces were collected in 2 ml Eppendorf tubes with 1 ml of viral transport media. Within 6 h, the samples were transported to the laboratory at 4 °C and stored at − 80 °C until further use. The viral RNAs present in the feces were extracted by the MagMAX™ Pathogen RNA/DNA kit (Applied Biosystems, Foster City, CA, USA) on the Magmax-96 Express (Applied Biosystems) instrument following the manufacturer's instructions. Influenza A viral RNA was detected by Real time reverse transcription-PCR on ABI 7500 (Applied Biosystems) targeting the matrix gene [12]. Eight gene segments of each virus were amplified with PrimeScript™ One-step RT-PCR kit Ver.2 kit (TaKaRa, Biotechnology [Dalian]

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Co., Ltd, Dalian, China) and the genome amplification was performed with MightyAmp® DNA Polymerase Ver.3 kit (TaKaRa, Biotechnology [Dalian] Co., Ltd, Dalian, China). PCR products were purified with MinElute® Gel Extraction Kit (QIAGEN, Valencia, CA, USA) and sent to the sequencing company for next-generation sequencing. The bird species was determined by using primers for the mitochondrial cytochrome oxidase I gene [5]. The strains were designated as A/Anser fabalis/Dongting/664/2014(H11N8) (DT-664) and A/Anas falcata/Dongting/D257/2015(H11N8) (DT-D257). The complete genome sequences of AIVs in this study have been deposited in GenBank under accession numbers MH547047-MH547054 (DT-D257) and MH547055-MH547062 (DT-664).

Sequence alignment was carried out using BioEdit 7.2.6.1. Phylogenetic trees were constructed using the maximum-likelihood method with bootstrap analysis (1,000 replicates) using MEGA 7.0.26. Homology analyses of nucleic acids were performed on the NCBI website with BLAST. All the reference sequences used for phylogenetic analysis were obtained from GenBank (https://www.ncbi.nlm.nih.gov) and the GISAID EpiFlu database (http://platform.gisaid.org/epi3/frontend#43209d).

Sequence comparison of DT-664 and DT-D257 viruses showed that these viruses shared 99.1%–100% nucleotide sequence identity among the six gene segments tested, including: polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acidic (PA), hemagglutinin (HA), neuraminidase (NA), and matrix (M). Homology of the nucleoprotein (NP) and nonstructural (NS) genes in these viruses was 94.3% and 95.8% respectively. The HA gene of DT-664 and DT-D257 showed the same highest identity (99.41%) with the Eurasian A/duck/Jiangxi/22537/2012(H11N9) virus, whereas the NA gene sequence showed 99.51% identity with the Eurasian A/great white-fronted goose/Netherlands/4/2008(H6N8) virus. The NA gene was 87.9% identical to A/duck/Jiangsu/k1203/2010(H5N8) and was 78.9% identical to A/Jiangxi-Donghu/346/2013(H10N8). The highly pathogenic avian influenza H5N8 virus, first detected in poultry in eastern China in 2010, caused outbreaks in South Korea and Japan in 2014 [34]. In 2013, a reassortant avian influenza H10N8 virus was found to cause the first human infection in China [4]. The nucleotide sequences showed high similarity with other AIVs from wild birds or poultry. The viruses in GenBank that had the highest nucleotide sequence homology to our newly identified viruses are shown in Table 1.

Phylogenetic analysis showed that all eight segments of two H11N8 viruses in this study clustered in the Eurasian lineage (Fig. 1 and Figure S1, Supplementary Material). The HA genes were very closely related to A/duck/Jiangxi/22537/2012(H11N9) and H11 viruses circulating in eastern Asia from 2011 to 2015. It was also shown that the HA genes shared a higher sequence similarity with H11 AIVs from poultry than the others from wild birds. However, the NA genes were in a separate subgroup from H5N8 and H10N8 AIVs (Fig. 1). The NA gene phylogeny of these two strains indicated that the H5N8 and H10N8 AIVs were not the closest common ancestor for this gene.

The PB2 gene of DT-664 and DT-D257 clustered with A/chicken/Wuhan/WHJF/2014(H5N2). The PB1 gene and M gene clustered with A/duck/Hokkaido/W26/2012(H12N1) and A/duck/Mongolia/709/2015(H10N7), respectively. The PA gene clustered with A/duck/Mongolia/372/2010(H4N6) and A/mallard/Mongolia/1551/2010(H3N1), respectively. The NP gene clustered with A/wild bird feces/Anhui/L258/2014(H9N2) and A/goose/Zhejiang/1120085/2014(H1N2), respectively. The NS gene clustered with A/goose/Zhejiang/1120078/2014(H1N2) and A/duck/Jiangxi/15846/2013(H10N3), respectively (Fig. 1 and Figure S1, Supplementary Material).

Based on the deduced amino acid sequence of the HA gene, the HA cleavage sites of the 2 H11N8 viruses were AIASR↓GLF, displaying the properties of low-pathogenic avian influenza viruses. The Q226 and G228 residue of HA indicated that the receptor binding sites were similar to all

Table 1 Nucleotide identities of the highest homologs in GenBank database with the novel H11N8 viruses detected in the East Dongting Lake, China

Gene	GenBank ID	Virus	DT-664(%)	GenBank ID	Virus	DT-D257 (%)
PB2	KU143562.1	A/chicken/Wuhan/WHJF/2014(H5N2)	99.43(2266/2279)	KU143562.1	A/chicken/Wuhan/WHJF/2014(H5N2)	99.47(2267/2279)
PB1	LC339665.1	A/duck/Hokkaido/W26/2012(H12N1)	99.03(2255/2277)	LC339665.1	A/duck/Hokkaido/W26/2012(H12N1)	98.99(2254/2277)
PA	LC349406.1	A/duck/Mongolia/372/2010(H4N6)	98.42(2117/2151)	KF454809.1	A/mallard/Mongolia/1551/2010(H3N1)	98.88(2127/2151)
HA	KF259202.1	A/duck/Jiangxi/22537/2012(H11N9)	99.41(1688/1698)	KF259202.1	A/duck/Jiangxi/22537/2012(H11N9)	99.41(1688/1698)
NP	KY347761.1	A/wild bird feces/Anhui/L258/2014(H9N2)	99.13(1484/1497)	KY971153.1	A/goose/Zhejiang/1120085/2014(H1N2)	99.60(1491/1497)
NA	KX979084.1	A/great white-fronted goose/Netherlands/4/2008(H6N8)	99.51(1420/1427)	KX979084.1	A/great white-fronted goose/Netherlands/4/2008(H6N8)	99.51(1417/1424)
M	LC121439.1	A/duck/Mongolia/709/2015(H10N7)	99.60(1006/1010)	LC121439.1	A/duck/Mongolia/709/2015(H10N7)	99.49(977/982)
NS	KY971234.1	A/goose/Zhejiang/1120078/2014(H1N2)	98.81(828/838)	KP285481.1	A/duck/Jiangxi/15846/2013(H10N3)	98.45(825/838)

DT-664, A/Anser fabalis/Dongting/664/2014; DT-D257, A/Anas falcata/Dongting/D257/2015

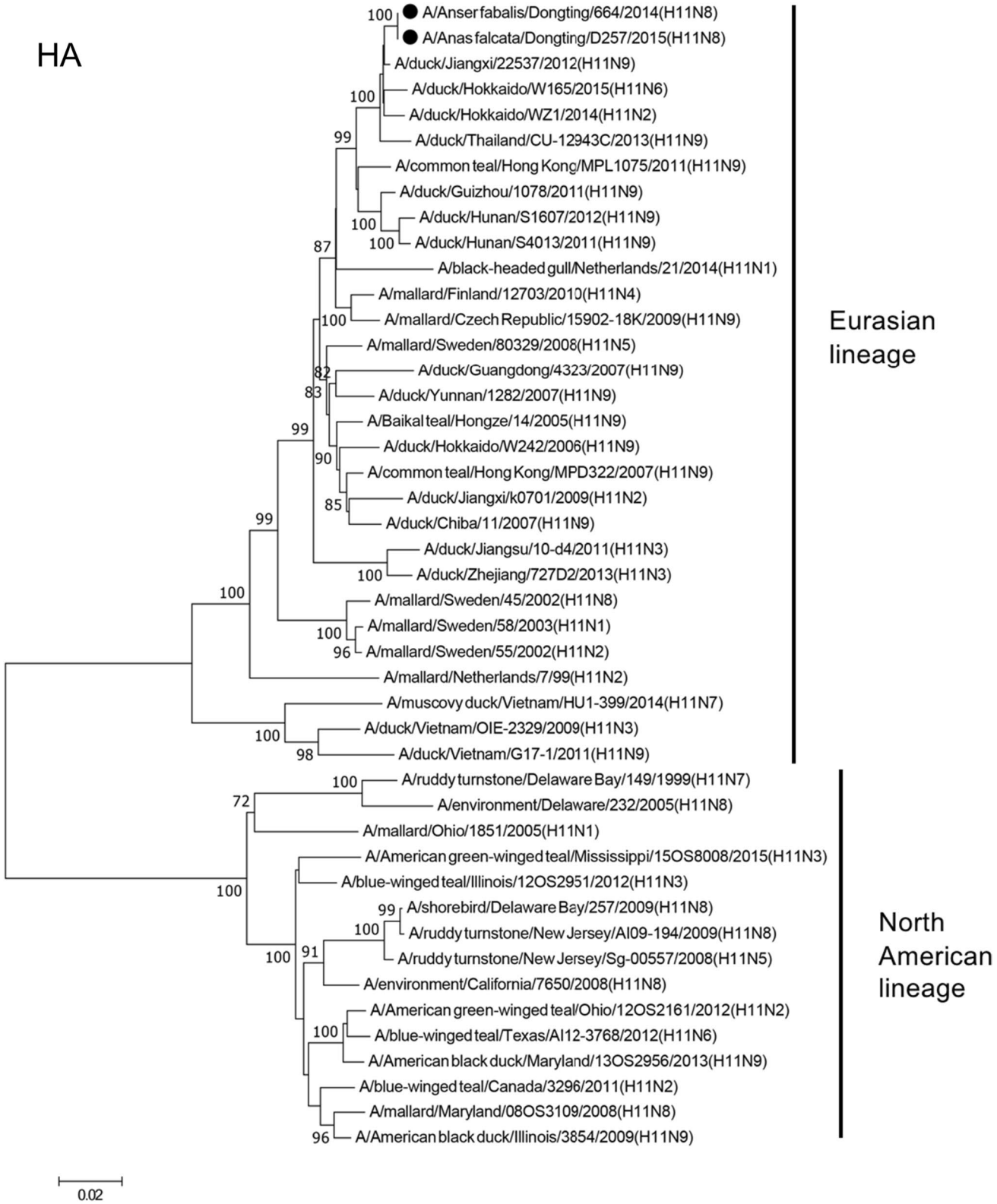

Fig. 1 Phylogenetic tree of the HA and NA genes of the H11N8 AIVs. The trees were generated using MEGA 7.0.26 software, and the bootstrap value was tested by 1000 replications. The H11N8 virus characterized is highlighted by a dot. The novel HPAI H5N8 virus originated in China highlighted by a triangle and the novel 2013 H10N8 influenza virus which caused human infection highlighted by a diamond. The scale bar shows nucleotide substitutions per site

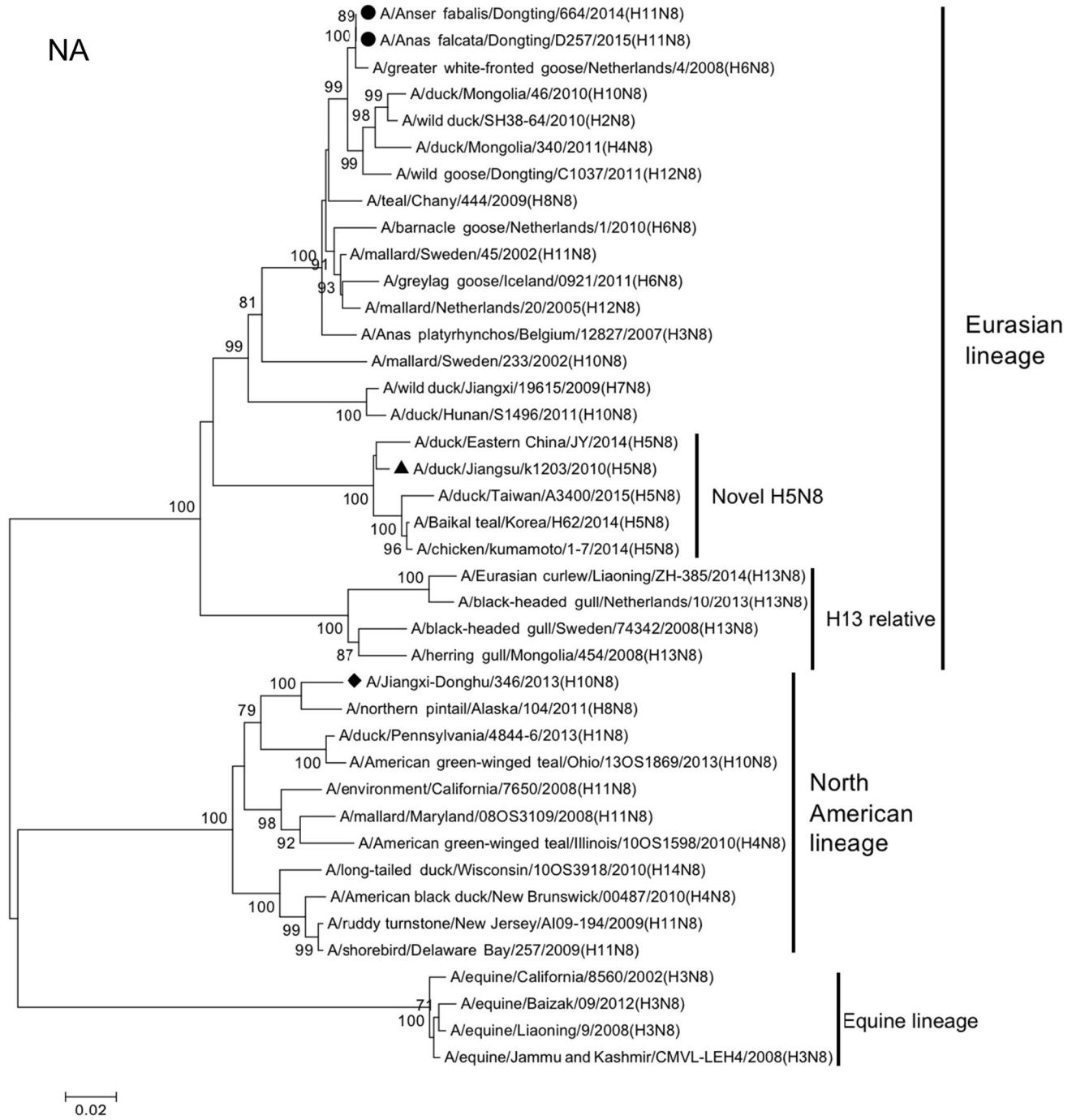

Fig. 1 (continued)

H11 AIVs clustered in Eurasian or North American lineages showing likely binding to α-2,3-linked sialic acid receptors (in H3 numbering), which are predominant in avian species [20]. Six potential N-glycosylation sites (Asn at 26 or 27, 39, 181, 304, 496 and 556) were detected in the HA protein.

E119, R152, H274, and R292 (in N2 numbering) of the NA protein suggested the susceptibility to the NA inhibitors oseltamivir and zanamivir [1]. The NA protein contained six potential glycosylation sites at amino acid positions 46, 54, 84 144, 293, 398. The mammalian adaptation mutations of PB2 such as E627 and D701 indicated an avian origin [6, 24]. The M1 mutations D30 and A215, known to increase the pathogenesis in mice, were detected [9]. No substitutions associated with resistance to adamantine were found in the M2 protein [11]. NS1 had S42 which is related to increased pathogenesis in mammals

Table 2 Specific amino acid residues analysis of two H11N8 AIVs

Protein	Mutation Site (aa)	Viruses		Function
		DT-664	DT-D257	
HA (H3 numbering)	Q226L	Q	Q	increased binding to human-type influenza receptor
	G228S	G	G	
		AIASR↓GLF	AIASR↓GLF	HA cleavage site
NA (N2 numbering)	E119V	E	E	resistance to oseltamivir and zanamivir
	R152K	R	R	
	H274Y	H	H	
	R292K	R	R	
PB2	E627K	E	E	Mammalian adaption mutations
	D701N	D	D	
M1	N30D	D	D	increased pathogenesis in mice
	T215A	A	A	
M2	S31N	S	S	resistance to amantadine and remantadine
NS1	P42S	S	S	increased pathogenesis in mammal

[14]. The primary amino acid mutations are shown in Table 2.

Up to date, although H11 AIVs have been isolated in both domestic poultry and wild birds, they are not detected as frequently as other subtypes [28]. However, H11N8 AIVs in duck have been reported in Hong Kong [13], in wild birds in the Ukraine, in Europe and in North America from the GenBank and GISAID EpiFlu databases. To our knowledge, this is the first report of the genomic sequence and phylogenetic analysis of H11N8 AIVs detected from wild birds in Asia.

In general, wild waterfowl have been deemed as the natural reservoir for AIVs. Wild waterfowl function for viral propagation and transmission, usually the low pathogenic avian influenza virus (LPAIV) [22]. Migrating birds travel between overwintering and breeding sites annually, which harbor the AIVs along the migration flyway. In this study, although H11N8 viruses have not been reported in other regions in Asia, viral infection may have been distributed to other places by migratory birds along the East Asia-Australia flyway.

Genomic analysis of DT-664 showed that the PB1, PA, HA, and M genes were similar to those from duck-origin AIVs. The PB2 and NS genes were closely related to chicken and goose- AIVs, respectively. The NP and NA genes were similar to wild bird AIVs. DT-D257 was similar to DT-664, which contained genes from poultry and wild bird AIVs. In Asia, most domestic ducks and geese are reared in free-range farms and live in a shared environment with wild birds [7]. Therefore, the origination of reassorted H11N8 AIVs might arise when wild birds have close contact with domestic poultry.

In the Dongting Lake wetlands, over 10 million birds congregate each winter. This is an area highly populated with approximately 15 million people [30]. Previous studies have shown that H1, H5, H6, H7, H9, H10, and H12 AIVs have been isolated from wild birds in this region [17, 25, 26, 31, 32]. However, H11N8 AIVs were detected from wild birds in this study, indicating increased diversity of LPAIV circulating locally. Several studies have reported that H11 virus could also infect people [10, 16]. Therefore, intensive surveillance and continual monitoring, especially on reassortant viruses, should be carried out. These data suggest that H11 virus might be a potential threat to public health.

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Compliance with ethical standards

Conflict of interest The authors declare there are no conflicts of interest.

Research involving human participants and/or animals The research did not involve human participants or animals.

Informed consent The research did not involve human participants or animals.

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