



Low Pathogenic Avian Influenza A (H5N7) Virus Isolated from a Domestic Duck in Dongting Lake Wetland of China, 2016

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Dear Editor,

The avian influenza virus (AIV) H5N7 was first isolated from wild birds in North America in 2001 (Spackman *et al.* 2007), and information on only 25 strains of this virus has been deposited in the Global Initiative on Sharing All Influenza Data (GISAID-<http://platform.gisaid.org/epi3/frontend#493de3>) database until October 30, 2018. Twenty viruses were identified in the United States from 2001 to 2017, three viruses in Denmark in 2003, and two viruses in Mongolia in 2014 (GISAID-<http://platform.gisaid.org/epi3/frontend#493de3>). All these H5N7 viruses were low pathogenicity and were discovered in wild migratory birds.

Wild migratory birds serve as natural reservoir of AIVs and disseminate the viruses during long-distance migration. Wetlands are important for wild migratory birds as stop-over for resting and breeding. In China, millions of domestic ducks are also raised near Dongting Lake Wetland Nature Reserve (Hunan Province Rural Social and

Economic Investigation Team 2008), and thus, these sites play key roles in the transmission of AIVs from wild birds to domestic birds (Ma *et al.* 2018). Moreover, domestic ducks are usually asymptomatic after AIV infection (Kuchipudi *et al.* 2014), and these healthy appearing ducks may serve as a hidden infection source. For instance, the low-pathogenic AIVs (LPAIVs) H7N9 and H10N8, which cause human infections, were very likely transmitted from wild birds to poultry through domestic ducks (Liu *et al.* 2013; Qi *et al.* 2014). In particular, research has shown that the LPAIV H7N9 became highly pathogenic AIV (HPAIV) after consecutive passages within poultry. If this happened, it will not only cause economic losses to the poultry industry but also pose a great threat to human health (Zhang *et al.* 2017). Herein, we report a novel H5N7 LPAIV that was isolated in China in 2016 from a healthy domestic duck in the Dongting Lake wetland, a key stop-over site for migratory waterfowls in the East Asian-Australasian flyway.

From November 2016 to March 2017, we performed routine surveillances in Central China and collected approximately 4,000 samples from wild birds and ~ 2,400 samples from live poultry markets (LPMs). From a fecal sample collected from a healthy duck near Dongting Lake wetland, we isolated a strain of influenza virus H5N7 (A/Duck/Dongting/76-1/2016, DT-H5N7) (Detailed methods were described in the Supplementary Materials). We sequenced the entire viral genome and deposited the sequences in GenBank (accession numbers: MF362100–MF362107). A BLAST search (Detailed phylogenetic analysis was shown in the Supplementary Materials) showed that the *HA* gene shared 97.2%–97.3% identities with those of H5N2 LPAIVs obtained from wild waterfowls in Xianghai (China) in 2011, and the *NA* gene was similar to those of H10N7 and H6N7 from wild birds and H7N7 from chicken in Jiangxi Province of China (identities 97.1%–98.9%). The internal genes shared 98.2%–99.5% identities with those of the wild waterfowl viruses circulating in Eurasia during 2008–2016 (Table 1).

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Table 1 The highest nucleotide similarity analysis of the internal genes of DT-H5N7 isolate in this study.

Gene	Viruses with the highest nucleotide identity	Accession number	Identity (%)
<i>PB2</i>	A/Environment/Chongqing/45208/2015(H5N6)	EPI749574	98.2
	A/common coot/Republic of Georgia/1/2010 (H6N2)	EPI618102	98.2
	A/common goldeneye/Mongolia/1216/2010 (H1N1)	EPI527564	98.2
<i>PBI</i>	A/duck/Mongolia/66/2015 (H10N2)	EPI693712	98.6
	A/duck/Mongolia/154/2015 (H0N2)	EPI704305	98.5
	A/teal/Chany/135/2016 (H3N8)	EPI774481	98.0
<i>PA</i>	A/duck/Mongolia/520/2015 (H1N1)	EPI704397	99.2
	A/duck/Mongolia/154/2015 (H10N2)	EPI704306	99.0
	A/duck/Mongolia/66/2015 (H10N2)	EPI693713	99.0
<i>HA</i>	A/baikal teal/Xianghai/426/2011 (H5N2)	EPI401775	97.3
	A/spot-billed duck/Xianghai/427/2011 (H5N2)	EPI401776	97.2
	A/little grebe/Xianghai/429/2011 (H5N2)	EPI401800	97.2
<i>NP</i>	A/wild bird/Korea/SK14/2014 (H1N1)	EPI969336	99.5
	A/Anseriformes/Anhui/S107/2014 (H1N1)	EPI1006621	99.3
	A/chicken/Wuhan/WHJF/2014 (H5N2)	EPI683062	99.3
<i>NA</i>	A/duck/Mongolia/742/2015 (H10N7)	EPI704546	98.9
	A/waterfowl/Bangladesh/12301/2013 (H6N7)	EPI540406	97.4
	A/chicken/Jiangxi/10784/2014 (H7N7)	EPI594131	97.1
<i>M</i>	A/duck/Mongolia/543/2015 (H4N6)	EPI704417	99.5
	A/pintail/Chany/198/2016 (H3N8)	EPI925975	99.2
	A/mallard/Chany/185/2016 (H3N8)	EPI925967	99.2
<i>NS</i>	A/mallard duck/Netherlands/3/2008 (H6N2)	EPI890842	99.0
	A/mallard/Republic of Georgia/1/2012 (H1N1)	EPI617896	99.0
	A/Eurasian teal/Netherlands/1/2008 (H6N1)	EPI890921	98.8

The H5N7 isolate possesses a pattern of PQRETRGLF at the cleavage site of HA, which is an indication of low pathogenicity (Senne *et al.* 1996) (Table 2). At the receptor-binding sites, HA contains Q226 and G228 (H3 numbering), implying a high binding affinity to avian receptors (Matrosovich *et al.* 2000). Of note, no amino acid mutations associated with viral replication in mammalian hosts (Q591, E627, and D701 in PB2) or drug resistance (H274 in NA (N2 numbering) and S31 in M2) were observed (Table 2).

Table 2 Characterization of the key molecular markers of the DT-H5N7 virus in this study.

Protein	Sites	Amino acid(s)
HA (H3 numbering)	Cleavage site	PQRETRGLF
	226	Q
	228	G
NA (N2 numbering)	274	H
	Stalk deletion	NO
PB2	591	Q
	627	E
	701	D
M2	31	S

The phylogenetic analysis showed that all eight genes derived from viral genes in wild migratory waterfowls (Fig. 1, Supplementary Figure S1). The HA belonged to the Eurasian low-pathogenic H5 lineage. The most closely related strains were from wild birds along the East Asian-Australian flyway (Fig. 1A, Supplementary Figure S1D). The phylogeny of NA showed that the NA gene also belonged to the Eurasian lineage and fell within the clade of viruses from the Central Asian flyway and East Asian-Australian flyway (Fig. 1B, Supplementary Figure S1F).

All the internal genes, except NS gene, were closely related to those of the H5N8 viruses circulating in Eurasia in 2016–2017. This was based on the finding that the PB2, PBI, and M genes clustered with strains from wild birds in the Central Asian flyway (Fig. 1C, Supplementary Figure S1A, S1B and S1G) and the PA and NP genes clustered with strains from wild birds in the East Asian-Australian flyway (Supplementary Figure S1C and S1E). The NS gene fell into the clade containing a mixture of genes from the East Asian-Australian, Central Asian, and Mediterranean-Black sea flyways (Fig. 1D, Supplementary Figure S1H).

We then explored the presence of molecular clock in the datasets using TempEst (Rambaut *et al.* 2016) v1.5.1 with the Best-fitting root and estimated the potential

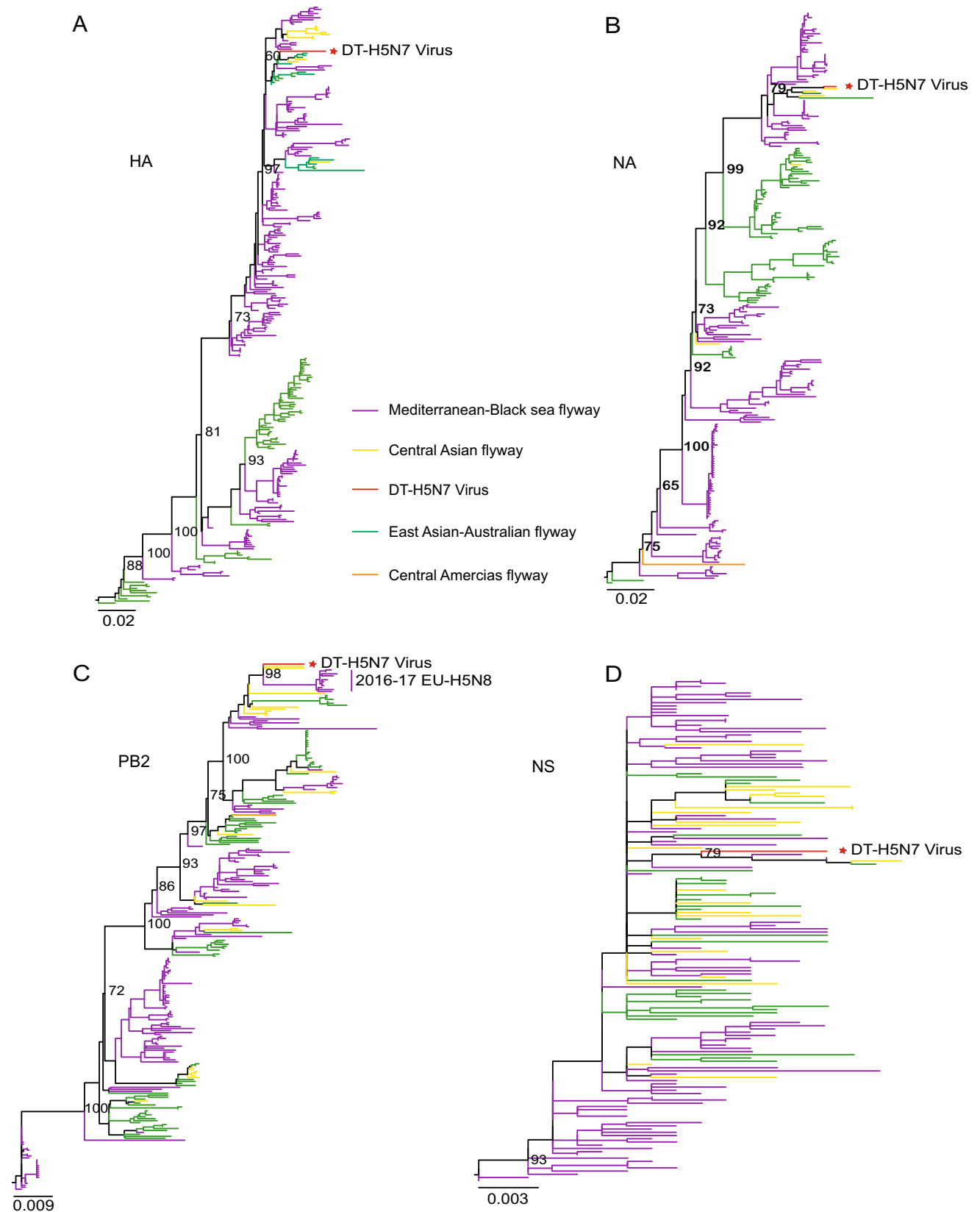


Fig. 1 Phylogenetic analysis of the **A** HA (1695 nucleotides), **B** NA (1416 nucleotides), **C** PB2 (2280 nucleotides), and **D** NS (858 nucleotides) genes of the DT-H5N7 isolate. The DT-H5N7 virus branch is colored in red and marked with a red five-pointed star, the

isolates in the Mediterranean-Black sea flyway are marked in purple, those in the Central Asian flyway are colored in yellow, those in the Central Americas flyway are colored in orange, and those in the East Asian-Australian flyway are labeled in green.

reassortment process by molecular dating. The results showed that the most recent common ancestor (tMRCA) of *HA* possibly emerged in September 2007 [95% highest posterior density (August 2006, August 2008)] and that the tMRCA of *NA* possibly emerged in July 2014 (November 2013, February 2015) (Fig. 2, Supplementary Figure S2D and S2F). The internal genes probably reassorted through three independent events, as the tMRCA of the *NS* gene was estimated to have emerged in December 2009 (July 2008, February 2011), the tMRCA of the *PB2* and *PB1* genes probably emerged in August 2012 (December 2011, June 2013) and January 2013 (January 2012, January 2014), respectively, and those of the *PA*, *NP*, and *M* genes emerged in June 2015 (March 2015, September 2015), September 2013 (October 2012, August 2014), and March 2014 (September 2012, June 2015), respectively (Fig. 2, Supplementary Figure S2). It is very likely that the H5N7 virus has experienced sequential reassortments within wild waterfowls and was then finally transmitted to domestic ducks.

This is the first report of the isolation of an LPAI H5N7 virus in China. The most recent LPAI H5N7 virus isolates were identified in samples from ruddy turnstones in New Jersey of the United States in May 2017 (GISAID-<http://platform.gisaid.org/epi3/frontend#493de3>). Moreover, this is the first identification of H5N7 LPAIV in the domestic duck.

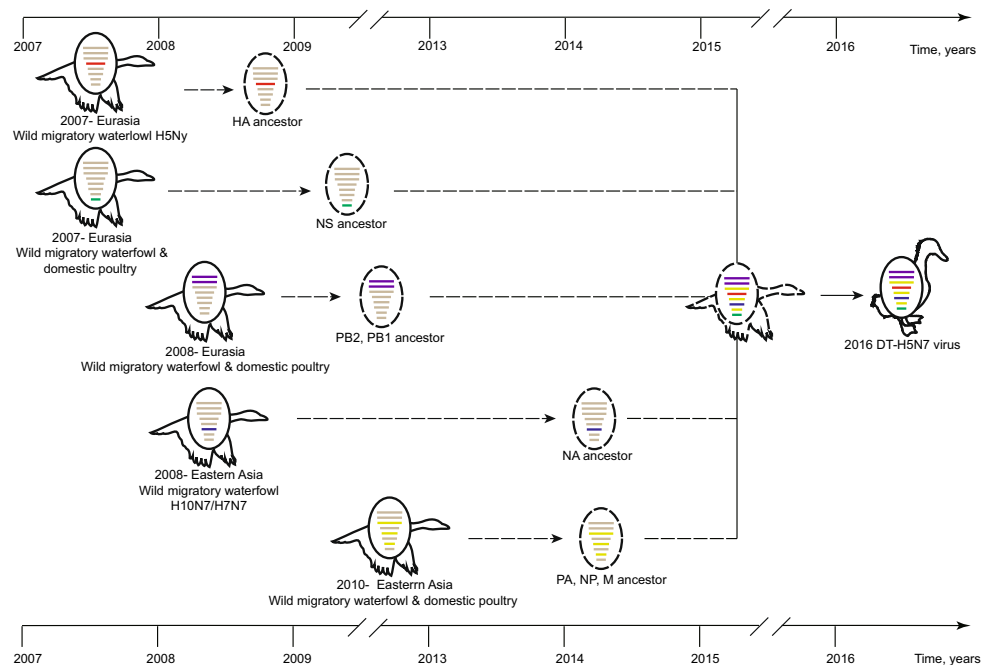
Phylogenetic analysis revealed that the genome of the H5N7 isolate belonged to the Eurasian lineage and originated from wild migratory waterfowls. Notably, the H5N7

virus shared high similarity of internal genes with the Eurasian-H5N8 HPAIV in 2016–2017, which formed the prevalent AIVs in wild birds (Adlhoch *et al.* 2016; Li *et al.* 2017). The process of internal genes reassortment showed extremely high complexity, possibly caused by unavailability of the surveillance data of LPAIV. Whether this combination of internal genes is adaptive to wild birds requires further investigation.

The lack of deletion in the NA stalk suggested that the H5N7 virus still possesses the adaptive feature to wild birds (Munier *et al.* 2010). Moreover, the absence of any signs of drug resistance suggested that the transmission from wild birds to the domestic duck occurred recently. The ancestor of the circulating H5N1 HPAIV has experienced the deletion of NA stalk and became adaptive to domestic ducks (Li *et al.* 2004). Since its emergence in 1996, 10 phylogenetic clades of H5 viruses have emerged, and two of them have crossed the barriers to infect humans (Abdel-Ghaffar *et al.* 2008). The circulating H5N1 HPAIV in ducks has become resistant to amantadine and zanamivir (Abdel-Ghaffar *et al.* 2008; Li *et al.* 2004). Moreover, the H7N9 strain has also experienced the deletion of the NA stalk and acquisition of drug resistance in the process of “genetic tuning” to adapt to domestic hosts and humans (Wang *et al.* 2013).

It has been proved that LPAIVs may also serve as precursors of HPAIVs (Monne *et al.* 2014). For instance, the A/Goose/Guangdong/1/96 (H5N1) virus, the ancestor of the current H5 HPAIV, was considered to have been derived from the H5 LPAIV circulating in migratory birds

Fig. 2 Hypothetical reassortment pathway of the DT-H5N7 virus. The gene segments are colored according to their ancestors.



(Duan *et al.* 2007). It would follow that this H5N7 LPAIV adapted to the domestic duck after it began circulating. Our research showed that routine surveillance for influenza virus at wetlands where wild birds interact with domestic birds should not be neglected. Furthermore, the traditional breeding mode of ducks in an uncontrolled manner in wetlands should be regulated to reduce the risk of AIVs spreading from wild to domestic birds.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Animal and Human Rights Statement This article does not contain any studies with human subjects performed by any of the authors. All studies involving animals were conducted according to the animal welfare guidelines of the World Organisation for Animal Health.

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