Original Article

Phylogenetic and Molecular Analysis of an H7N7 Avian Influenza Virus Isolated in East Dongting Lake in 2012

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Abstract

Objective In March 2012, an H7N7 subtype avian influenza virus (AIV) named A/wild goose/Dongting/PC0360/2012 (H7N7) (DT/PC0360) was recovered from a wild goose in East Dongting Lake. We performed whole-genome sequencing of the isolate, and analyzed the phylogenetic and molecular characterization.

Methods RNA was extracted from environment samples (including fecal samples from wild bird or domestic ducks, and water samples) for detecting the presence of Influenza A Virus targeting Matrix gene, using realtime RT-PCR assay. The positive samples were performed virus isolation with embryonated eggs. The subtype of the isolates were identified by RT-PCR assay with the H1-H16 and N1-N9 primer set. The whole-genome sequencing of isolates were performed. Phylogenetic and molecular characterizations of the eight genes of the isolates were analyzed.

Results Our results suggested that all the eight gene segments of DT/PC0360 belonged to the Eurasian gene pool, and the HA gene were belonged to distinct sublineage with H7N9 AIV which caused outbreaks in Mainland China in 2013. The hemagglutinin cleavage site of HA of DT/PC0360 showed characterization of low pathogenic avian influenza virus.

Conclusion Strengthening the surveillance of AIVs of wild waterfowl and poultry in this region is vital for our knowledge of the ecology and mechanism of transmission to prevent an influenza pandemic.

Key words: Avian influenza viruses; Wild geese; East Dongting Lake wetland; H7N7 subtype

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INTRODUCTION

Influenza A virus belong to the *Orthomyxoviridae* family, and its genome consists of eight segmented, negative single-stranded RNA, and codes 12-14 proteins[1-3]. They can infect a wide variety of animals, including birds, pigs, sea mammals, and humans, and cause a range of disease from asymptomatic to mild and severely symptomatic, and even death[4]. It is

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generally accepted that aquatic birds, especially Anseriformes (ducks, geese, and swans) and Charadriiformes (terns, gulls, and waders), are the major natural reservoirs of AIVs, and all the H1-H16 and N1-N9 subtypes of AIVs can be recovered from them[5-7]. Moreover, it is recognized that wild birds, especially migratory birds, play an important role in AIV transmission and dissemination[8]. Long-term surveillance of AIVs of wild birds in North America and Europe has been conducted since 1976 and 1998[8-9], respectively. However, little information about the ecology of AIVs in wild birds is available in Asia. Before 2013, no outbreak of H7 subtype AIVs was recorded in Mainland China, although there were sporadic reports of H7 viruses isolated mainly from ducks, including H7N1, H7N2, H7N3, H7N7, H7N6, and H7N8 subtypes, and all were of low pathogenicity[10-11].

H7 subtype influenza A viruses have been circulating in both wild birds and terrestrial poultry, and have caused numerous outbreaks in commercial poultry in the American and Eurasian continents since the last century[12-13]. Occasionally, they can be transmitted to mammals including equines, seals, and even humans. Thus, they are considered to pose a potential pandemic threat to human health. Before 2013, the H7 subtype avian influenza viruses (AIVs) had led to >100 cases of human infection worldwide, and one person died because of acute respiratory distress syndrome[14]. In February 2013, a novel reassortant H7N9 subtype AIV suddenly emerged in Eastern China[15]. As of August 20, 2013, 134 H7N9 human cases with 45 fatalities have been reported[16]. Several biological feature analyses revealed that the pandemic potential of the novel H7N9 viruses cannot be excluded[17], which raises concern about the threat of H7 subtype AIVs to animal and human health worldwide.

During November 2011 and April 2012, we collected 6621 environmental samples (fresh fecal and water samples) from wild birds and domestic ducks in Eastern Dongting lake, China. All the samples were detected the presence of influenza A viruses by realtime RT-PCR assay. The positive samples were performed isolation using embryonated eggs. An H7N7 subtype AIV named A/wild goose/Dongting/PC0360/2012 (H7N7) was recovered from a wild goose in March 2012. We performed whole-genome sequencing of the isolate, and analyzed the phylogenetic and molecular characterization. Our findings suggested that all the eight gene segments of the dt/PC0360 belonged to the Eurasian gene pool. Moreover, the hemagglutinin (HA) gene was distinct from the novel H7N9 virus.

MATERIALS AND METHODS

Sample Collection

Fresh Fecal samples from wild birds were taken by cotton swabs, and put in 15-mL tubes containing 4 mL virus transport medium [Medium 199 (Thermo Scientific Hyclone, Logan, UT, USA) containing 0.5% BSA (Roche, USA), 10% glycerol, 2×10^6 U/L penicillin G, 200 mg/L streptomycin, 2×10^6 U/L polymyxin B sulfate, 250 mg/L gentamicin, 60 mg/L ofloxacin HCl, 0.2 g/L sulfamethoxazole, and 5×10^5 U/L nystatin (Sigma, St Louis, MO, USA)]. Samples were shipped immediately to the laboratory at 4 °C, and then stored at -80 °C until use.

Virus Isolation

RNA was extracted and purified from 200-μL samples using the QIAamp One-For-All Nucleic Acid Kit (Qiagen, Hilden, Germany) with the BioRobot Universal system, following the kit handbook. Influenza A virus RNA was detected by real-time RT-PCR (AgPath; Applied Biosystems, Foster City, CA, USA) targeted Matrix gene on a Stratagene Mx3005P thermocycler machine. The positive samples were inoculated into the allantoic cavity of 9-day-old embryonated chicken eggs (ECEs). The ECEs were incubated at 37 °C for 48 h, and chilled at 4 °C overnight. The allantoic fluid was harvested and a hemagglutination assay was performed with 0.5% turkey red blood cells for virus identification.

Isolates Subtyping and Sequencing

The viral RNA from allantoic fluid was extracted using the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. RNA was reverse transcribed with the SuperRT cDNA Kit (CWBIO, Beijing, China), using the Uni12 primer (5'-AGCAAAAGCAGG-3'). Isolate subtyping was performed by PCR using 16 sets of HA (H1-H16) primers and nine sets of neuraminidase (NA) (N1-N9) primers designed by the Chinese National Influenza Center. The complete genome amplification was performed using specific primers (primer sequences available on request), with 2×Es Taq MasterMix Kit (CWBIO). The PCR products of the expected sizes were purified with a QIAquick PCR purification kit (Qiagen). Sequencing was performed using the

BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM 3700xl DNA Analyzer (Applied Biosystems), following the manufacturer's instructions.

Phylogenetic Analysis

Sequences were assembled and edited with Lasergene 8.1 (DNASTAR); mafft 6 was used for alignment. Neighbor-joining (NJ) trees were constructed using MEGA 4.0. Estimates of the phylogenies were calculated by performing 1000 NJ bootstrap replicates. The full-length genome sequence of the isolates was deposited in the GenBank database under accession numbers KC876680-KC876687. All the reference sequences used in the phylogenetic comparison were obtained from the GenBank influenza database and GISIAD.

RESULTS

Virus Isolation and Sequence Analysis

During November 2011 and April 2012, we conducted an investigation of prevalence of avian influenza viruses circulated in wild birds and domestic ducks in East Dongting lake area. 6621 environmental samples (fresh fecal and environmental water) were collected from wild birds and domestic ducks. The positive rate of influenza A virus was approximately 5.21%, and the various subtypes of influenza A viruses were identified. More details were reported in our previous published paper[18]. An H7N7 AIV, termed A/wild goose/Dongting/PC0360/2012 (H7N7, DT/PC0360), was isolated from a wild goose in East Dongting Lake Nature Reserves in March 2012. The full genome of DT/PC0360 was sequenced. The whole genome of the isolate consists of eight segments, including PB2, PB1, PA, HA, NP, NA, M, NS. The full lengths of each segment were 2341, 2341, 2233, 1732, 1565, 1442, 1027, and 890 bp, respectively. Each segment was used in Blast search to identify similar virus sequences with 98%-99% homology (Table 1). The HA gene was most closely related to that of A/duck/Fukui/160104/2012(H7N7). NA gene showed 99% nucleic acid similarity with that of A/environment/Hunan/S4484/2011(H12N7). The nucleic acid sequences of PB2, PB1, PA, M, NS gene of DT/PC0360 had 98%-99% homology with those of strains isolated birds in East Asia. The nucleic acid sequence of NP gene showed 99% identity with that of H6N2 subtype influenza A virus isolated from wild birds in Europe (Table 1).

Phylogenetic Analysis

We downloaded all the sequences of H7N7 subtype AIVs from GenBank and the highest identity virus sequences in GenBank, and performed phylogenetic analysis of all eight gene segments. As shown in Figure 1, the phylogenetic tree of HA gene was clustered into four lineages, including American, Eurasian, Australian, and Equine lineages. The Eurasian lineage could be divided into three sublineages. Four H7 subtype AIVs isolated in Mainland China at an earlier time were clustered into sublineage I. H7 viruses isolated in recent years fell within sublineage II and III. The DT/PC0360 fell within sublineage II, and had high identity with the strain isolated from ducks in Japan, which had the same migratory route (East Asia-Australian flyway) with our sampling sites[9]. H7N6, H7N7, and H7N1 viruses isolated in Yunnan, Fujian, and Hunan Province during 2007-2010 were also clustered into sublineage II. However, most H7 subtype AIVs isolated in Mainland China (including the novel H7N9

Table 1. Nucleotide Identity (%) of A/Wild Goose/Dongting/PC0360/2012 H7N7 Virus with the Most Closely Related Isolates in GenBank Database

Segement	Viruses in the Genebank Showing Highest Similarity	Nucleotide Identity (%)	Accession Number
PB2	A/green-winged teal/Xianghai/430/2011(H5N2)	99	JX570867
PB1	A/wild duck/Korea/SH5-26/2008(H4N6)	99	JX454751
PA	A/wild duck/Korea/CSM20-5/2009(H4N6)	99	JX454703
HA	A/duck/Fukui/160104/2012(H7N7)	99	AB813068
NP	A/mallard/Czech Republic/15902-17K/2009(H6N2)	99	HQ244431
NA	A/environment/Hunan/S4484/2011(H12N7)	99	CY146766
M	A/duck/Guangxi/GXd-1/2011(H1N2)	99	KF013919
NS	A/environment/Mongolia/1-33/2007(H4N6)	98	JN029606

viruses) belonged to sublineage III. This indicated that there may be a variety of origin of H7 AIVs that have been transmitted by migratory waterfowl along the East Asian flyway and introduced into poultry during recent decades. The phylogenetic tree of NA also showed four lineages, including Equine American, Australian and Eurasian lineages. The Eurasian lineage could be divided into two sublineages further. The NA gene of DT/PC0360 fell within the sublineage I, and was clustered with other earlier isolates from European wild birds before 2009. Without exception, the six internal genes of DT/PC0360 all belonged to the Eurasian gene pool, and had a close relation with the strains circulating in East Asia.

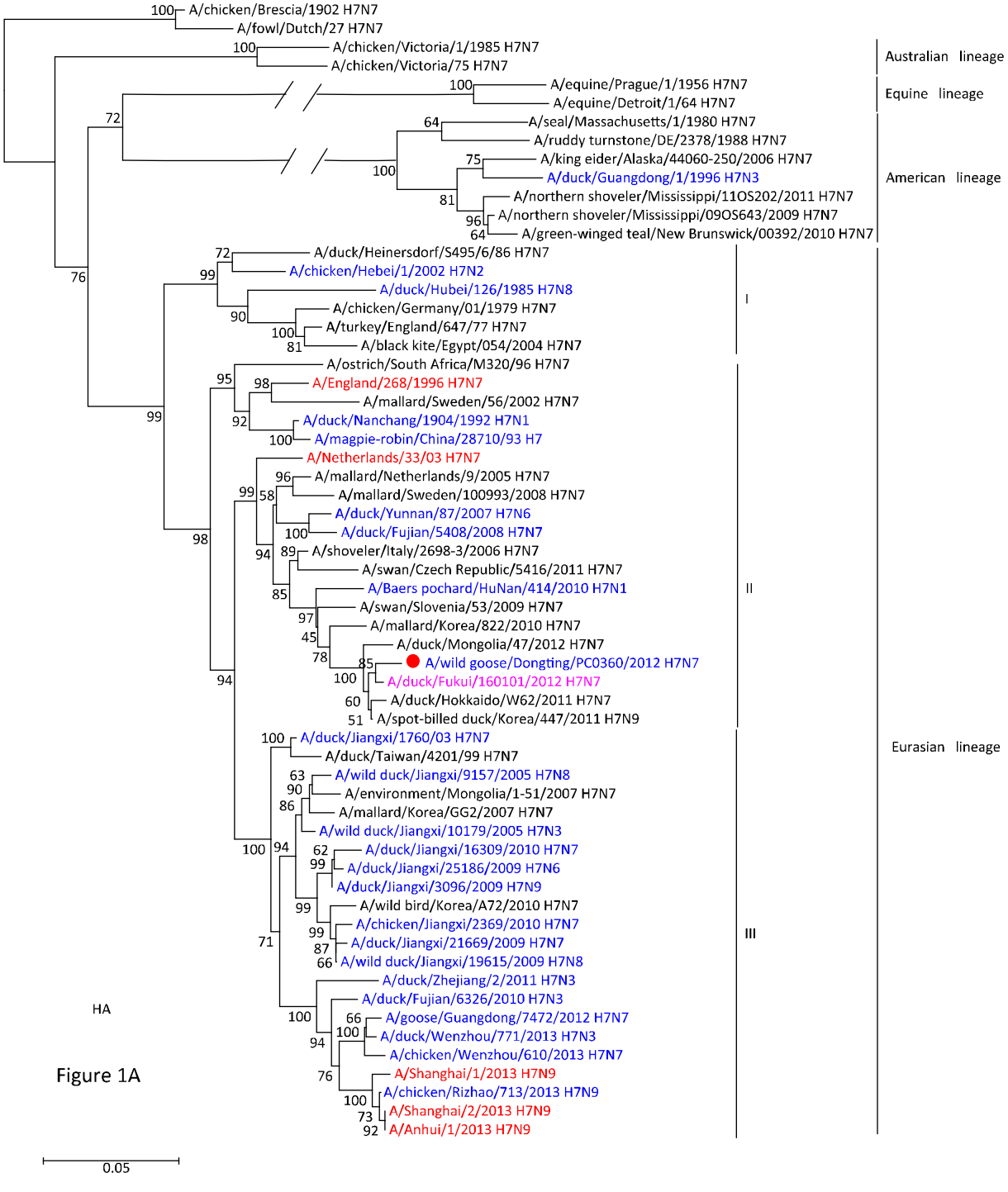

Figure 1A

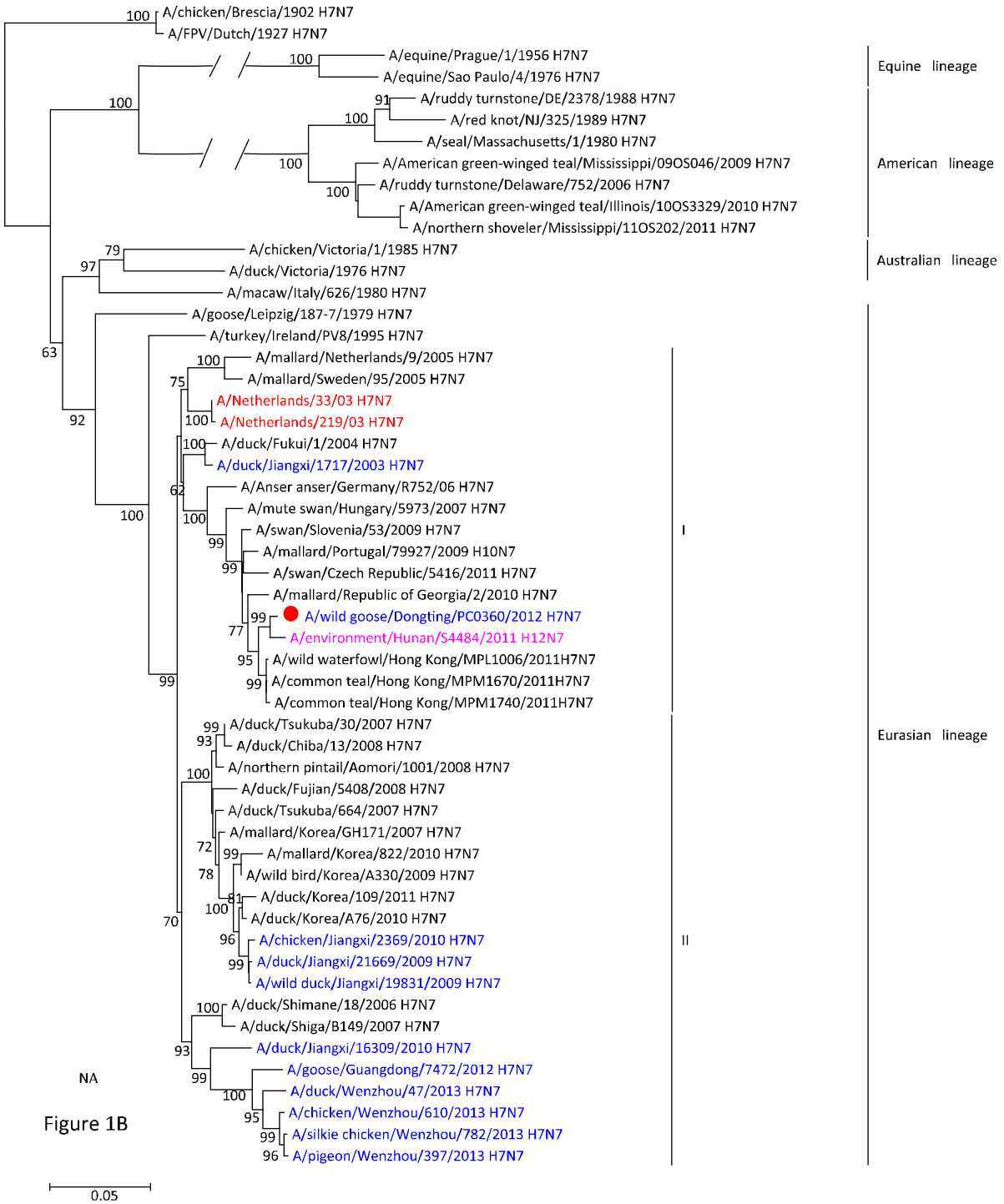

Figure 1B

Figure 1. NJ phylogenetic tree of the HA (A), NA (B), PB2 (C), PB1 (D), PA (E), NP (F), M (G), and NS(H) genes of A/wild goose/Dongting/PC0360/2012 H7N7 isolated from the fecal samples of wild geese in East Dongting Lake Wetland. NJ trees were constructed using MEGA 4.0. Estimates of the phylogenies were calculated by performing 1000 NJ bootstrap replicates; all rooted to A/chicken/Brescia/1902 H7N7. The human H7 subtype viruses are highlighted in red; DT/PC0360 and all the other H7 subtype AIVs isolated in Mainland China are highlighted in blue; and DT/PC0360 is marked with the red dot. The highest identity of each gene segment with DT/PC0360 is highlighted in purple. (Additional detail of PB2 (C), PB1 (D), PA (E), NP (F), M (G), and NS (H) could be found on website of www. besjournal.com)

Molecular Analysis

To compare further the genetic difference between DT/PC0360 and the other H7 subtype virus, we downloaded the nucleotide sequences of H7 subtype AIVs isolated in Mainland China (including the sequence of the novel H7N9 AIVs) and some other H7 subtype isolates from Eurasia. DT/PC0360 had a VPELPKGR↓GLF in the connecting peptide that is a typical characteristic of low pathogenicity AIVs. The HA receptor-binding pocket of HA protein maintained the avian-like motif, Q226 and G228 (H3 numbering, Table 2), suggesting that its binding preference was for sialic acid α-2,3 receptors. Analysis of potential glycosylation site motifs N-X-S/T of HA protein revealed six sites at positions 30, 46, 141, 249, 421, and 493 (Table 3). Sequence analysis suggested that the HA gene of DT/PC0360 was similar to that of the strain isolated from Japan, and distinct from that of the novel H7N9 subtype viruses isolated from Mainland China in 2013.

The NA gene of DT/PC0360 was full length, and no deletion was observed in the NA stalk region. DT/PC0360 possessed amino acids E and D at positions 627 and 701 of PB2 protein, respectively, which are characteristic of AIV. We did not observe H274Y or N294S mutations in the NA protein, which confer resistance to oseltamivir, which indicated that it was sensitive to oseltamivir. No amino acid mutations associated with drug resistance were observed in the M2-ion channel protein of DT/PC0360, which indicated that the isolate was sensitive to amantadine inhibition. No substitution associated with increasing virulence in mammals was observed in PB2, PB1 and NS proteins[19] (data not shown).

Table 2. Amino Acid Sequences of Specific Sites in HA of A/Wild Goose/Dongting/PC0360/2012 H7N7 AIV

Strains	Accession Numbers	RBS					HA Cleavage Site
		160	186	190	226	228	
A/wild goose/Dongting/PC0360/2012 H7N7	KC876685	A	G	E	Q	G	PELPKGR----------GLF
A/duck/Jiangxi/1760/03 H7N7	EU158101	*	*	*	*	*	PEI PKGR----------GLF
A/Baer's pochard/HuNan/414/2010 H7N1	JQ973643	*	*	*	*	*	PELPKGR----------GLF
A/duck/Zhejiang/2/2011_H7N3	KC876683	*	*	*	*	*	PETPKGR----------GLF
A/wild duck/Jiangxi/9157/2005 H7N8	KF258999	*	*	*	*	*	PEI PKGR----------GLF
A/duck/Yunnan/87/2007 H7N6	KF258991	*	*	*	*	*	PELPKGR----------GLF
A/duck/Fujian/5408/2008 H7N7	KF258959	*	*	*	*	*	PETPKGR----------GLF
A/duck/Jiangxi/3096/2009 H7N9	KF258951	*	*	*	*	*	PELPKGR----------GLF
A/wild duck/Jiangxi/19615/2009 H7N8	KF258972	*	*	*	*	*	PEI PKGR----------GLF
A/duck/Jiangxi/25186/2009 H7N6	KF258978	*	*	*	*	*	PEI PKGR----------GLF
A/chicken/Rizhao/713/2013 H7N9	KF259058	*	V	*	*	*	PEI PKGR----------GLF
A/chicken/Wenzhou/662/2013 H7N7	KF259029	*	V	*	*	*	PEI PKGR----------GLF
A/duck/Zhejiang/DK10/2013 H7N3	KC961628	*	*	*	*	*	PEI PKGR----------GLF
A/Shanghai/1/2013 H7N9	EPI439486	*	*	*	*	*	PEI PKGR----------GLF
A/Shanghai/2/2013 H7N9	EPI439502	*	V	*	L	*	PEI PKGR----------GLF
A/Anhui/1/2013 H7N9	EPI439507	*	V	*	L	*	PEI PKGR----------GLF
A/duck/Fukui/160101/2012 H7N7	AB813068	*	*	*	*	*	PELPKGR----------GLF
A/Netherlands/216/03 H7N7	AY338459	*	*	*	*	*	PEI PKRRRR----------GLF

Note. *Standed for the identical glycosylation sites between the DT/PC0360 and the other reference strains.

DISCUSSION

High-and low-pathogenicity AIVs of the H7N1, H7N2, H7N3, H7N4, and H7N7 subtypes have caused outbreaks in poultry that have resulted in the culling of 10 million birds. Notably, the geographic diversity of countries affected by the H7 subtype in poultry, which includes countries in the Eurasian and American continents[20-24], readily demonstrates the global scale of animal production and the public health risk posed by viruses of H7 subtype. Both H7 subtype AIVs of Eurasian and American lineages are associated with human infections[21,24-25]. In addition, limited human-to-human transmission has been recorded, based on viral and/or serological evidence[25]. In 2013, a novel H7N9 AIV suddenly emerged in humans in Eastern China, and has caused >30% mortality, suggesting that H7 subtype AIVs are potential pandemic candidates once again.

Table 3. The Comparison of Potential Glycosylation Sites in HA of A/Wild Goose/Dongting/ PC0360/2012 H7N7 AIV with Strains in China and Two Isolates in Eurasian

Isolates	Potential Glycosylation Sites						
	30-32	46-48	141-143	249-251	421-423	493-595	516-518
A/wild goose/Dongting/PC0360/2012 H7N7	NGT	NAT	NGT	NDT	NWT	NNT	NLS
A/duck/Hubei/126/1985 H7N8	*	*	-	*	*	*	-
A/duck/Nanchang/1904/1992 H7N1	*	*	-	*	*	*	-
A/duck/Guangdong/1/1996 H7N3	*	*	*	*	*	*	*
A/chicken/Hebei/1/2002 H7N2	*	*	*	*	*	*	-
A/duck/Jiangxi/1760/03 H7N7	*	*	-	*	*	*	-
A/duck/Jiangxi/1760/03 H7N7	*	*	-	*	*	*	-
A/wild duck/Jiangxi/9157/2005 H7N8	*	*	-	*	*	*	-
A/duck/Yunnan/87/2007 H7N6	*	*	-	*	*	*	-
A/duck/Fujian/5408/2008 H7N7	*	*	-	*	*	*	-
A/duck/Jiangxi/3096/2009 H7N9	*	*	-	*	*	*	-
A/duck/Jiangxi/16309/2010 H7N7	*	*	-	*	*	*	-
A/duck/Fujian/6326/2010 H7N3	*	*	-	*	*	*	-
A/goose/Guangdong/7472/2012 H7N7	*	*	-	*	*	*	-
A/chicken/Rizhao/715/2013 H7N9	*	*	-	*	*	*	-
A/chicken/Wenzhou/662/2013 H7N7	*	*	-	*	*	*	-
A/duck/Zhejiang/DK10/2013 H7N3	*	*	-	*	*	*	-
A/Baer's pochard/HuNan/414/2010 H7N1	*	*	-	*	*	*	-
A/duck/Zhejiang/2/2011_H7N3	*	*	-	*	*	*	-
A/duck/Zhejiang/DK10/2013 H7N3	*	*	-	*	*	*	-
A/Shanghai/1/2013 H7N9	*	*	-	*	*	*	-
A/Shanghai/2/2013 H7N9	*	*	-	*	*	*	-
A/Anhui/1/2013 H7N9	*	*	-	*	*	*	-
A/duck/Fukui/160101/2012 H7N7	*	*	-	*	*	*	-
A/Netherlands/33/03 H7N7	*	*	-	*	*	*	-

Note. *Standed for the identical glycosylation sites between the DT/PC0360 and the other reference strains. - Standed for the absence of the glycosylation sites in the HA protein of the other reference strains.

In our study, an H7N7 subtype AIV was recovered from the fecal sample of wild geese in East Dongting Lake Nature Reserve, which was the first report of H7N7 subtype AIV isolated in this region. The results of genetic and phylogenetic analysis suggested all the gene segments of DT/PC0360 were belonged to the Eurasian gene pool. Its HA gene was clustered with the strains isolated in Eurasia in recent years, but not with the novel H7N9 subtype AIVs within the same sublineage. This indicated that DT/PC0360 had a distinct origin from that of the novel H7N9 AIVs causing human infection. Evolutionary analysis of H7 viruses circulating in China (conducted by Guan et al. using the data of active surveillance since 2000 in Southern China and virus archives) suggested that H7 viruses from East Asian migratory birds were introduced into domestic ducks in China on several occasions during recent decades. The H7 viruses then reassorted with various subtypes of AIVs, which generated the novel H7N9 viruses and a related, previously unrecognized H7N7 lineage in China[11]. Therefore, it is of concern whether the DT/PC0360 could be introduced into domestic ducks, subsequently reassort with other AIV subtypes, and generate a novel pandemic H7 subtype AIV.

Since the first H7 virus was isolated in 1985 in Mainland China, the H7 viruses have sporadically been isolated in poultry (mostly in domestic ducks). We compared the nucleotide identity of the HA gene of DT/PC0360 with all the other H7 subtype AIVs isolated in Mainland China (including H7N1, H7N2, H7N3, H7N7, H7N7, H7N8, and H7, and the novel H7N9 subtype AIVs). Sequence analysis revealed that DT//PC0360 shared 76.2%-95.3% nucleotide homology with the other H7 subtypes, indicating that DT/PC0360 is largely distinct from the previous H7 subtype viruses circulating in Mainland China. In addition, the majority of H7 viruses isolated in Mainland China, including the novel H7N9 viruses, formed a Eurasian sublineage of (sublineage III). However, DT/PC0360 clustered with the H7 viruses isolated from Europe and East Asia within sublineage II, indicating the existence of an additional H7 sublineage in Mainland China. N-link glycosylation sites of HA and NA proteins can affect the host specificity, infectivity or virulence of influenza A virus, when they are in receptor binding regions, antigenic regions or cleavage sites[27-28]. DT/PC0360 showed the same glycosylation sites in HA proteins with the strain isolated in Guangdong province in 1996 (A/duck/Guangdong/1/1996 H7N3).

Some evidence shows that the outbreaks of H7 viruses in poultry usually originated in wild birds[10], which were commonly recognized as the natural reservoir of the influenza A viruses. Three of the eight migrating flyways of the world cross and overlap in Mainland China. Hunan East Dongting Lake Nature Reserve, which is one of the largest wetland nature reserves in China, is located in the East Asia/Australia migrating flyway. It is a major overwintering and staging site for migratory birds. Every winter, wild birds gather here, which provides an opportunity to come into contact with poultry, especially domestic ducks, and humans[18]. Therefore, a variety of subtypes of AIVs circulating in this region were prone to reassort to generate some novel AIVs, and then the novel reassortant viruses can be disseminated to other areas by the migrating birds or transmitted to poultry or humans. More novel reassortant AIVs were isolated and identified from wild waterfowl, lake water, or domestic ducks in Dongting lake wetland during 2007-2012, such as H9N2, H10N8, and H4N2 viruses[29-32]. Our results highlighted the necessity of continuous surveillance of AIVs in Dongting lake wetland to be helpful for our knowledge of the ecology and evolution of the AIVs in wild birds.

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