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# Phylogenic analysis of reassorted avian influenza viruses isolated from Korean domestic ducks from 2005 to 2007

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**Abstract** Ducks have been regarded as animals that can perpetuate most avian influenza viruses since they generally do not show the clear clinical signs such as death and reduced body weight when they are infected. Here, we characterized two H3N2 and one H3N6 avian influenza viruses isolated from ducks on the local farms in Korea from 2005 to 2007. Genetic analysis of these viruses showed that most segments of isolates except NP genes belonged to Eurasian lineage. NP genes of two H3N2 isolates, A/Duck/Korea/S71/07, and A/Duck/Korea/S72/07 belonged to North American lineage. Our results suggest that the genetic reassortment among avian influenza viruses can occur in domestic ducks.

**Keywords** Avian influenza virus · Ducks · H3N2 · H3N6

Aquatic birds harbor all the known influenza A viruses including 16 hemagglutinin (HA) and nine neuraminidase (NA) subtypes [1, 2]. The highly pathogenic H5 and H7 subtypes of influenza viruses contain additional basic amino acids at the connecting peptide of HA and are highly pathogenic to chickens, quail, and pheasants [3, 4]. Generally, most avian influenza viruses do not cause diseases in ducks [5, 6]. However, recent events suggest that the equilibrium between influenza viruses and aquatic birds may have been disrupted. During H7N1 avian influenza outbreak in Italy from 1999 to 2000, two domestic geese and two Muscovy ducks died of infections [7]. In two Hong Kong parks, H5N1 outbreaks in 2002 resulted in the deaths of many resident avian species including waterfowl [8]. In Thailand, it appears that free-gazing ducks in rice puddies played a critical role in the persistence and spread of highly pathogenic H5N1 influenza viruses during the outbreak from July 2004 to May 2005 [9].

In Korea, the previous surveillance in live poultry market in 2003 reported that H9N2, H3N2, and H6N1 avian influenza viruses were isolated in ducks [10]. In this study, we genetically characterized one H3N6 and two H3N2 avian influenza viruses isolated from ducks on the farm from 2005 to 2007.

About 200 fecal samples from 20 farms were collected from ducks on the farms in the Province of Chungcheong namdo (Fig. 1) in Korea from 2005 to 2007. They were collected in 1 ml of isolation media (PBS, pH 7.4) supplemented with glycerol (50%), Potassium penicillin, Streptomycin sulfate, Gentamicin sulfate, Polymixin B, and Nystatin), and were inoculated into 10-day-old SPF embryonated eggs by allantoic route. The eggs were incubated at 35°C for 72 h and then chilled at 4°C overnight. The presence of viruses in the allantoic fluids was confirmed by hemagglutination (HA) assay. HA assay was performed in V-bottomed microtiter plates with 0.5% Turkey red blood cells, and determined 30 min after incubation at room temperature.

To perform the sequencing of isolates, viral RNAs were directly extracted from infected allantoic fluid using the

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**Fig. 1** Map of Korea showing the location where samples were collected. Samples were collected at the province of Chungcheong namdo

Rneasy Protect Mini Kit (Qiagen, Valencia, CA, USA). The RNAs were transcribed into cDNA using the ImProm-II TM Reverse Transcription System (Promega, Madison, WI, USA) and the Uni12 primer (AGCAAAAGCAGG) set (10). Eight genes in each virus were amplified by PCR with GoTaq DNA Polymerase (Promega, Madison, WI, USA) and a segment-specific primer set (10). These PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and were sequenced by Solgent corporation (Daejeon, Korea). The sequenced genes were compiled and edited using the Lasergene sequence analysis software package (DNA Star software version 4.0, Madison, WI, USA). Phylogenic analysis was performed by analyzing the nucleotide sequences of our isolates with those of influenza viruses obtained from the GenBank database by neighbor-joining trees and MEGA4: molecular evolutionary genetics analysis(MEGA) software version 4.0. The nucleotide regions used in the phylogenic analysis were PB2: 1-1150, PB1: 1-993, PA: 1-1138, HA: 1-1038, NP: 1-1494, NA: 1-766, M: 1-895, and NS: 1-838. The nucleotide sequences were deposited into GenBank under the accession numbers: EU912501-EU912524.

We identified three isolates, one H3N6 and two H3N2 avian influenza viruses, from domestic ducks in Korea from 2005 to 2007. A/duck/Korea/S71/07 (H3N2) and A/duck/Korea/S72/07 (H3N2) viruses were isolated on the same farm, and A/duck/Korea/S28/05 (H3N6) was isolated on the farm which is about 10 km away from the farm where the other two viruses were isolated.

We first analyzed the HA and NA lineage by means of phylogenic analysis. The analysis revealed that the HA genes and NA genes of A/duck/Korea/S28/05 (H3N6), A/duck/Korea/S71/07 (H3N2), and A/duck/Korea/S72/07 (H3N2) belonged to the Eurasian lineage, not to North American lineage (Fig. 2a–c), and NA genes of the two H3N2 and one H3N6 avian influenza viruses belonged to Eurasian lineage (Fig. 2b and c). When we analyzed the NP lineage, we found that NP genes of A/duck/Korea/S71/07 (H3N2) and A/duck/Korea/S72/07 (H3N2) were closely clustered with North American lineage (Fig. 2d).

When we genetically analyzed the genes in internal proteins of the three isolates, most genes except NP were clustered with the Eurasian lineage (Table 1). NP genes of A/duck/Korea/S28/05 (H3N6) belonged to the Eurasian lineage, while those of A/duck/Korea/S71/07 (H3N2) and A/duck/Korea/S72/07 (H3N2) belonged to the North American lineage.

It seems that H3N2 avian influenza viruses are continuously circulating in ducks on Korean farms. A previous study showed that 6 H3N2 avian influenza viruses were isolated from ducks in the live bird markets in Korea in 2003 [11]. When we serologically surveyed H3N2 infections in domestic ducks, about 5% of sera were positive (data not shown).

We isolated one H3N6 avian influenza virus in Korean domestic ducks. All genes except NA are similar to H3N2 viruses isolated in Korean ducks in 2003. The N6 gene of A/duck/Korea/S28/05 (H3N6) may be introduced by reassortment in ducks between 2003 H3N2 viruses and avian influenza viruses containing N6 from wild birds. This suggests that ducks may play a role in creating new subtypes of influenza viruses.

NP genes of H3N2 isolates belong to North American lineage. NP genes of H3N2 isolates may be introduced by migratory birds shedding avian influenza viruses with NP genes originated from North American lineage. It was reported that influenza A viruses have been diversified into two separate avian lineages, North American and Eurasian [12]. This suggests that migratory birds sometimes cross Atlantic and Pacific flyway, resulting in the creation of reassorted avian influenza viruses. Alternatively, the imported chickens to Korea from nations located in North America might have harbored avian influenza viruses of which NP genes were the source for the reassorted H3N2 viruses.

Considering that the reassorted H3N2 influenza viruses were created in ducks, it would be possible that these ducks

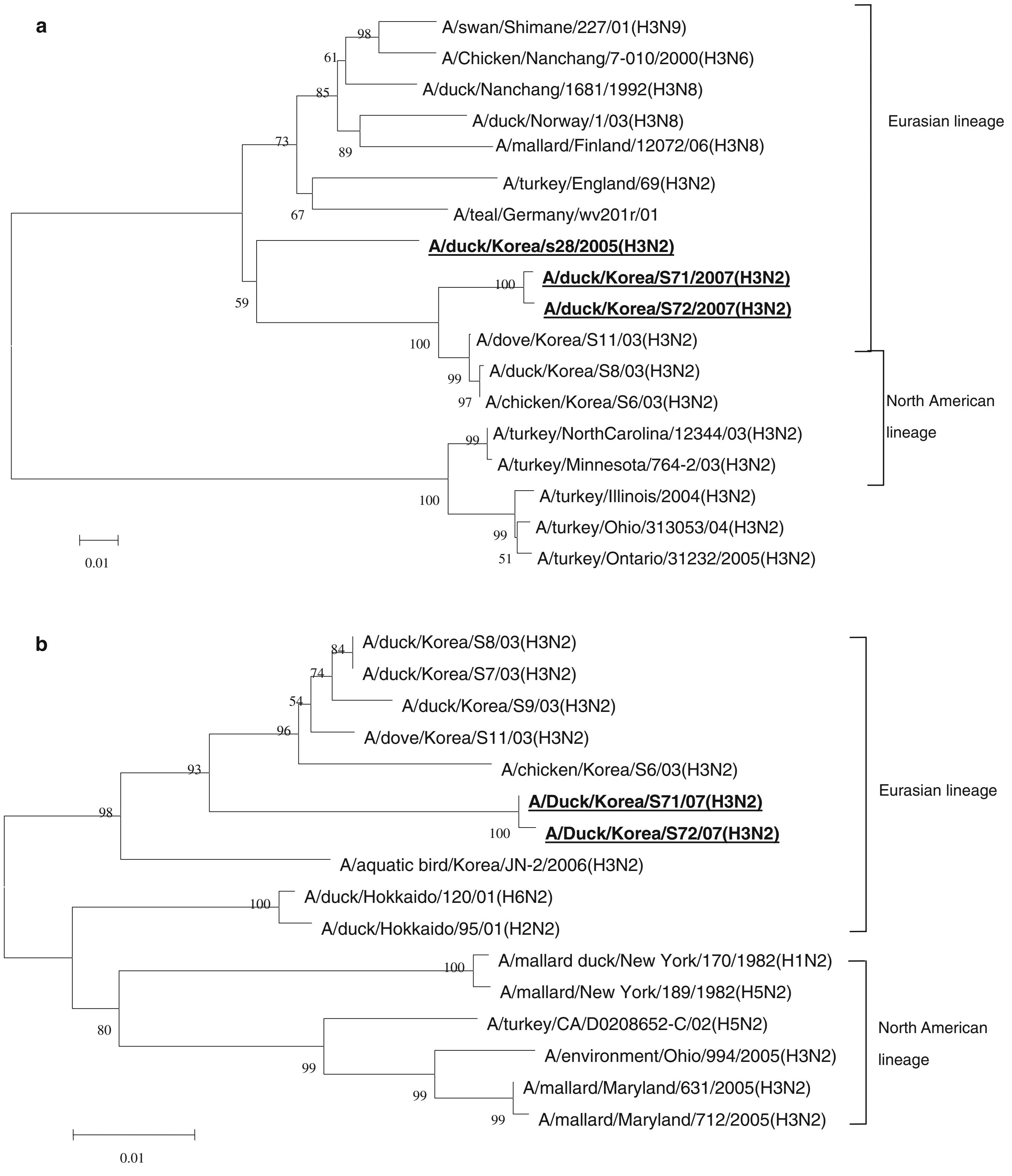

**Fig. 2** Phylogenic analysis of HA and genes of isolates. Phylogenic analysis was performed by analyzing the data of our isolates with those of sequences of influenza viruses obtained from the GenBank database by 1,000 neighbor-joining bootstrap replicates using MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0 (http://megasoftware.net/). The numbers beside branch points represent the neighbor-joining bootstrap values and the scale of bar is 0.01. **a** HA, **b** N2, **c** N6, **d** NP

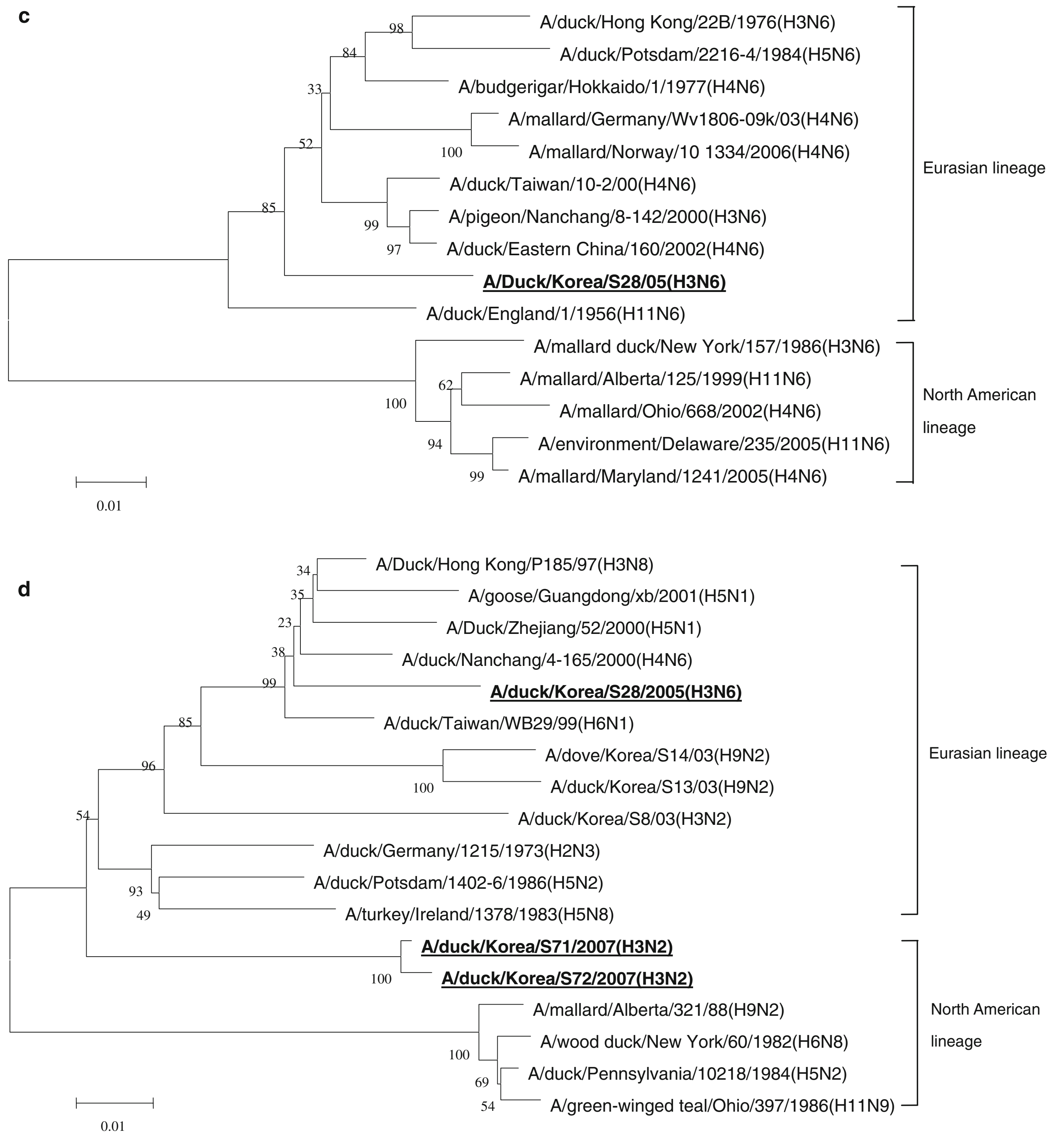

**Fig. 2** continued

could act as reassortant vessels for creating H5N1 influenza viruses. In China, novel H9N2 influenza viruses that were double or even triple reassortant were isolated in ducks from 2000 to 2001 [13]. Some of them contained gene segments that are closely related to those in A/Hong Kong/156/97 (H9N2) or A/Quail/Hong Kong/G1/97 (Gl-like, H9N2). Continuous surveillance of avian influenza viruses in the domestic poultry including ducks should be performed to prevent the emergence of next pandemic influenza viruses in humans.

**Table 1** Lineage analysis of internal genes of isolates

| Isolates | Genes | | | | | |
|---|---|---|---|---|---|---|
| | PB2 | PB1 | PA | NP | M | NS |
| A/Duck/Korea/S28/05(H3N6) | * | * | * | * | * | * |
| A/Duck/Korea/S71/07(H3N2) | * | * | * | ** | * | * |
| A/Duck/Korea/S72/07(H3N2) | * | * | * | ** | * | * |

* Eurasian lineage

** North American lineage

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