Potential role of Whooper Swans (*Cygnus cygnus*) in reassortment and dissemination of avian influenza A (H5N2) in Eastern Asia

Running title: The role of whooper swans in spread of H5N2

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Abstracts: Surveillance of whooper swan migration is important for monitoring avian influenza transmission risk potential from east to west China between the East Asian–Australasian and Central Asian flyways. Here, we characterised the evolutionary and reasssortment history of H5N2 viruses isolated from 1866 fresh faeces samples of wintering whooper swans collected in the Sanmenxia Reservoir area, China. This was combined with information on the migration routes of whooper swans in Eastern Asia to elucidate the role of whooper swans in spreading the virus. All segments of the new H5N2 isolates belong to the Eurasian avian-like lineage and are closely related to wild-bird viruses from China, Korea and Mongolia covering the wintering, stopover and breeding grounds in migration routes of whooper swans. We further found that the temporal-spatial migration process of whooper swans was identical with the virus transmission and reassortment pathway in Eastern Asia particularly.

Keywords: Eastern Asia; H5N2; Migration routes; Reassortment; Whooper swans

1 Introduction

Avian influenza virus (AIV) spreads through migratory wild birds and poultry (Fusaro et al., 2019, Meng et al., 2019, Newman et al., 2009, Yang et al., 2020). Wild waterfowl serve as the natural reservoir of AIV, and information on their migration pathways is crucial to understanding the evolution and genesis of new lineages (Tian et al., 2015). An HPAI H5N1 outbreak in whooper swans (*Cygnus cygnus*) in the Sanmenxia Reservoir area, middle China in 2015, sparked concerns about AIV transmission by migratory swans (Li et al., 2018), which are known to be highly

susceptible to H5 AIV (Newman et al., 2009). Whooper swans are the most abundant wild waterfowl in Eastern Asia, and through migration they spread HPAI infections in the East Asian countries, including China, Korea, Japan, Mongolia, and Far East Russia. In recent years, H5N2 viruses have often been isolated from wild waterfowl in Eastern Asia including Japan, Korea and Mongolia (Baek et al., 2010, Hiono et al., 2015). For these reasons we speculated that whooper swans may be involved in the geographic dissemination and reassortment history of H5N2 AIVs.

To address this, we have collected and tested fresh faeces samples from whooper swans from December to January every year in the Sanmenxia Reservoir area since 2015, where more than 90% of waterfowl are wintering whooper swans. In addition, we also collected faeces samples of wild waterfowl along the migration route of whooper swans in spring (March–May) and autumn (July–September or October–November) of 2018 and 2019. Among the samples collected, we only isolated 14 H5N2 from whooper swans wintering in the Sanmenxia Reservoir area in December of 2018–January of 2019, and sequenced the full genome of 14 H5N2 AIV isolates (except only 10 complete NS gene fragments for analysis), and other faeces samples collected were negative. These isolates' evolutionary and reassortment history was characterized in light of the migration route of whooper swans to explore their potential role in reassortment of H5N2 viruses in Eastern Asia and in the spread of H5N2.

2 Methods

2.1 Virus Isolation and Identification

A total of 1866 fresh faeces samples were collected from wintering whooper swans in the Sanmenxia Reservoir area of China on 10, 20 and 25 December 2018 and 15 January 2019. To further explore the role of whooper swans migration in H5N2 virus spread, we collected shorebirds, wild ducks and geese fresh faeces on the whooper swans migration routes; whooper swan feaces could not be collected on their migration route as difficult to reach estuaries and lakes serve as stopover sites of whooper swans. A total of 3949 fresh faeces samples of shorebirds and wild ducks were collected on Cangzhou, Tangshan and Langfang of Hebei and Bohai Bay of Tianjin where on whooper swans migration routes from Mongolia to the eastern wintering sites from March to May in spring and from July to September in autumn of 2018, and 1069 fresh faeces samples were collected from March to May in spring of 2019; 1252 and 1150 fresh faeces samples of wild geese and ducks on Lingwu and Zhongning of Ningxia and Wuliangsu Lake of Inner Mongolia where on of whooper swans migration route from Mongolia to Sanmenxia Reservoir area from October to November in autumn of 2018and 2019. It was very easy to make a distinction on fresh faeces between shorebirds, wild geese, ducks and whooper swans because of the obvious difference on shape and size.

Fresh faeces samples in sterile PBS were centrifuged and supernatants were taken. Virus RNA were extracted from supernatants by using T014 Virus DNA/RNA Extraction Kit (magnetic beads method; Xi'an Tianlong Technology, China) and GeneRotex96 Nucleic Acid Extraction System (Xi'an Tianlong Technology, China)

which based on the magnetic beads method and the rotating nucleic acid extraction technology following the manufacturer's protocol, then further analysis by real-time quantitative polymerase chain reaction ((RT-)qPCR). Virus RNA was subjected to real-time reverse transcription and gel electrophoresis. The reverse transcription products of viral RNA were then purified and used to construct a gene library and the avian influenza virus genome was sequenced using next-generation sequencing with an Illumina MiSeq system (Illumina Inc., Shanghai, China) according to the manufacturer's instructions.

2.2 Phylogenetic Analysis

Sequences of AIVs from avian hosts collected globally were downloaded from the GISAID database on 1–2 May 2020. The final numbers of reference sequences for each gene segment were as follows: PB2, n = 111; PB1, n = 112; PA, n = 99; H5, n = 101; NP, n = 95; N2, n = 108; M, n = 86; and NS, n = 122. Coding regions were extracted and aligned using MAFFT v.7.450 (Katoh et al., 2002), and the alignment was manually edited in MEGA 7.0 (Kumar et al., 2016). FastTree v.2.1.11 (Price et al., 2010) was used to estimate the evolutionary history for each segment and branch support was evaluated by 1000 bootstrap replicates. Sequence homology analysis was performed using DNASTAR's MegAlign (Burland, 1999).

2.3 Migration Routes and Virus Transmission

Fourteen whooper swans were captured in the Sanmenxia Reservoir area in 2018. The birds were caught with the approval of National Forestry and Grassland Administration of China. Each swan was tagged with a global positioning system transmitter using a backpack method, to obtain detailed information about the bird's migration route. Banding and recovery data for whooper swans wintering in Rongcheng, China, in 2007–2019 were also downloaded from the database of the National Bird Banding Centre of China to investigate their migration routes. Information on the migration routes of whooper swans wintering in Korea was also obtained from the database of the National Bird Banding Centre of China.

To investigate the relation between virus migration and H5N2 evolution, the whooper swan migration data were analysed in light of the phylogenetic reconstructions. Satellite tracking data of other birds including tundra swans (*Cygnus columbianus*) and bar-headed geese (*Anser indicus*) were downloaded from the database of the National Bird Banding Centre of China as a supplement.

3 Results

3.1 Virus Isolation and Phylogenetic Analysis

Fourteen H5N2 AIVs were isolated. Analyses of the H5 gene showed that the cleavage site in all 14 viruses was PQRETR↓GLF, corresponding to a low-pathogenic pathotype. The homology of the 14 H5N2 AIVs eight genes were more than 99.8%, this showed that the 14 viruses isolated were almost the same.

14 H5N2 AIVs all belonged to the Eurasian lineage (Figure 1; Figure S1). A set of H5N3 virus circulating in Korea was the most closely related to the newly obtained

H5 segments. The N2 gene segment had the closest relatedness to the H5N2 virus also isolated in Korea. The PB2 gene segments was most closely related to viruses from the eastern coastal region of China, and the PB1 and M genes to viruses from Anhui and Yunnan of China, the PA gene to viruses from Korea. The NP and NS gene segments all had close relationships with AIVs isolated from wild ducks in Mongolia (Table 1). Overall, all the gene segments from the H5N2 isolates were derived from strains resident in Eurasian migratory wild birds, and not from domestic poultry viruses (Table 1).

3.2 Whooper Swan Migration and Virus Transmission

From eastern wintering sites to Mongolia, H5, N2, PB2 and PA Genes

The H5, N2 and PA gene segments from the H5N2 isolate were most closely related to viruses isolated from spot-billed ducks (*Anas poecilorhyncha*) wintering in Korea, and the PB2 gene segment was most closely related to the virus isolated from eastern coastal region of China which include many wintering sites for whooper swans, such as Rongcheng and Yellow River delta wetland (Table 1). These sites are located in the eastern migration route of the whooper swans. According to the test results of samples collected in Cangzhou, Tangshan and Langfang of Hebei and Bohai Bay of Tianjin along the eastern wintering whooper swans migration route in spring and autumn of 2018 and spring of 2019 (Figure 2), shorebirds and wild ducks (not whooper swans) on the migration route were not infected with this virus. These segments' pattern of reletedness with other isolates renders it plausible that they originate from spot-billed ducks and other wild birds, and eventually recombined with viruses carried by whooper swans wintering in the same areas in Korea and Rongcheng. Whooper swans most likely carried these segments to the breeding sites in Mongolia; during this migration they got transmitted to other swans, via which these segments eventually reached the Sanmenxia Reservoir area (Figure 2; Figure 3).

Anhui and Yunnan of China to Sanmenxia Reservoir area, PB1 and M Genes

The strain most closely related to the PB1 and M genes of the H5N2 isolate were from bean geese (*Anser fabalis*) of Anhui and black-necked cranes (*Grus nigricollis*) of Yunnan, China (Table 1). Because bean geese and black-necked cranes have no shared geographical distribution in winter, we believed the geese and cranes first transmit the virus to other wild birds in the same area, for example tundra swans and bar-headed geese, which in turn spread the PB1 and M genes to whooper swans at some sites such as upper and middle reaches of the Yellow River and Mongolia during the spring migration (Figure 2; Figure 3).

Mongolia to Sanmenxia Reservoir area, NP and NS Genes

The NP and NS genes from the H5N2 isolate were all most closely related to viruses isolated from geese and mallards (*Anas platyrhynchos*) breeding in Mongolia (Table 1). Many wild geese, ducks, and other waterbirds breed in north-central Mongolia, and the satellite tracking results confirmed that this was also a breeding site for whooper swans wintering in the Sanmenxia Reservoir area (Figure 2; Figure 3). These

two gene fragments could thus have been obtained by reassortment of viruses in wild geese, ducks, and Sanmenxia Reservoir whooper swans during the breeding periods. In autumn of 2018 and 2019, the results of wild geese and ducks samples collected from Wuliangsu Lake of Inner Mongolia and Lingwu and Zhongning of Ningxia on the way back from Mongolia to Sanmenxia Reservoir area were all negative, which indicated that the virus did not spread between wild geese and ducks during these periods, and that whooper swans were more likely to be the host of AIV. However, no faeces samples of whooper swans were collected on the migration route from Mongolia to Sanmenxia Reservoir area, it was not confirmed whether swans had been infected with H5N2 in Mongolia, and it was possible that Mongolia was not the final site of H5N2 virus formation.

Based on the result above, whooper swans returned from their breeding sites to overwinter in the Sanmenxia Reservoir area in winter 2018, and the eight gene fragments from different countries and regions underwent reassortment to generate the H5N2 isolate.

4. Discussion

The main finding of this study was that the 14 H5N2 isolates with small sequence divergence from whooper swans in the Sanmenxia Reservoir area were likely generated by reassortment of AIVs isolated from a diverse set of wild birds at multiple distant locations, including the wintering, stopover and breeding sites of whooper swans who carry the viruses over long distances during their migration. The H5N2 isolates were characterized as LPAVs. Overall, our results emphasize the important role of whooper swans in the transmission and reassortment history of the H5N2.

Whooper swans breed in northern Eurasia and winter in Europe and Eastern Asia, including China, Korea, and Japan (Shimada et al., 2014). Wintering whooper swans in South Korea often mix with spot-billed ducks. Spot-billed ducks are generally considered to be carriers of AIVs, and many subtypes of AIVs have been detected in spot-billed ducks from South Korea (Jeong et al., 2014, Lee et al., 2013). Whooper swans wintering in South Korea may thus have become infected with AIVs by mixing with spot-billed ducks and then carried the viruses to their breeding sites during spring migration and transmitted them to whooper swans wintering in the Sanmenxia Reservoir area, coincident with the swans' migration on a temporal-spatial scale. During the three consecutive migration periods from 2018 to spring of 2019, the faeces samples of shorebirds and wild ducks on the migration route were all negative, which also indicated that shorebirds and wild ducks were not the key species of the H5N2 transmission, and the whooper swans, which was highly susceptible to avian influenza, should be focused on. Furthermore, whooper swans are usually faithful to their wintering grounds (Newman et al., 2009, Shimada et al., 2014), and swans wintering in South Korea could thus transmit the closely related strains collected in different years with different gene fragments of H5N2 isolates to swans wintering in the Sanmenxia Reservoir area in different years, which is compatible with the idea that AIVs are spread among China, Mongolia, and Korea by swan migration.

Rongcheng is located on the eastern coast of the Shandong Peninsula, which is an important wintering site for whooper swans in eastern China. Whooper swans wintering in Rongcheng and the Sanmenxia Reservoir area have shared breeding sites in Mongolia, increasing opportunities for virus exchange and reassortment between H5N2 viruses at the breeding sites in Mongolia among whooper swans frequenting eastern and central China. Besides, whooper swans from South Korea and Rongcheng share the same migration routes and breeding area in Mongolia, indicating a high risk of virus transmission between South Korea and Rongcheng. Eastern China is the most important region for waterbirds, especially ducks, geese, and swans, on the East Asian–Australasian Flyway in Eastern Asia (Cao et al., 2008). We therefore suggest that avian influenza surveillance should be strengthened in eastern China to prevent the emergence of influenza viruses with pandemic potential.

Anhui Province, south of China, is an important wintering site for bean geese; and Zhaotong of Yunnan Province, southwest of China, is an important wintering ground for black-necked cranes, and also for bar-headed geese. In winter 2018, the migration routes of one tundra swans at middle and lower Yangtze River at the boundary between Hubei and Anhui Provinces and one bar-headed goose nearby the Zhaotong, were studied by satellite tracking. The results shows that the Yellow River stretch in Inner Mongolia is an important stopover site for tundra swan and north-central Mongolia is a breeding site of bar-headed goose (Figure 2), which are the same as satellite tracking results of Huang et al. (Huang et al., 2018) and Prosser et al. (Prosser et al., 2009) to tundra swans and bean geese wintering in south of China, and the areas also same as the whooper swans wintering in Sanmenxia Reservoir area. Therefore, we believe that bean geese indirectly transmit the virus to the whooper swans at Yellow River stretch in Inner Mongolia via tundra swans, and black-necked cranes indirectly passed the virus to whooper swans at north-central Mongolia through bar-headed geese.

Mongolia is the breeding ground for swans wintering in Korea and in central and eastern China. Call et al. (2019) surveyed 96 wetlands in north-central Mongolia and recorded more than 12,000 waterbirds in these areas every year, indicating that north-central Mongolia included important breeding, moulting, and stopover sites for waterbirds from the central and east Asian flyways. Reassortment of virus genes is commonly detected in AIVs originating from areas where migratory routes overlap (Hurtado et al., 2015). This further indicates that whooper swans may serve as a common vector in north-central Mongolia during the breeding period (Call et al., 2019), and through the migration of swans facilitating the dissemination of AIVs and the formation of reassortant AIVs from lineages carried by waterfowl from different regions and countries, then promote AIVs including H5N2 spread on their migration routes. The negative results of wild geese and ducks faeces samples from the Yellow River stretch in Ningxia and Inner Mongolia from October to November in autumn of 2018 and 2019 also suggested that it is whooper swans, not the other wild waterbirds, that were important host for the H5N2 formation.

In this study, we collected and tested fresh faeces samples from whooper swans wintering in Sanmenxia Reservoir area for six consecutive years, and fresh faeces

samples of other waterbirds in the same area on the migration route of the whooper swans in spring and autumn of 2018 and 2019 were also collected and tested (because of the geographical factors, we could not get close to the stopover sites of whooper swans during the migration, so no faeces samples of the whooper swans were collected). Despite this large effort, only 14 H5N2 strains were isolated from the samples in one winter season, which limits reconstructing the reassortment history that underlies the H5N2 segments constellation. Nonetheless, a parsimonious view on the phylogenetic analyses and whooper swans migration route provides clues on the gensis of H5N2. Moreover, the avian influenza surveillance results of wild waterbirds in multiple stopover sites along the migration route of whooper swans were negative for two consecutive years, point to an important role of whooper swans in the transmission and formation of H5N2.

The viruses most closely related to the newly obtained H5N2 isolates were collected along the East Asian–Australasian flyway. The Sanmenxia Reservoir area is located at the intersection of the Central Asian and East Asian–Australasian flyways (Li et al., 2018), and AIV was confirmed to spread from east to west along the migration routes of whooper swans along these flyways (Meng et al., 2019). We therefore speculate that swan migration increases the potential of AIVs spreading to western China.

Acknowledgments

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Compliance with ethical standards

The data collected comply with the current laws of China in which they were performed. Approval for Whooper Swans capture was granted by Forestry and Grassland Department of Henna Province of China (No. 260, Yulin [2019])

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378
379**Table 1.** AIVs with closest relationships to H5N2 viruses isolated in Sanmenxia Reservoir
380area.

Gene	Country	Isolate	Host
H5	Korea	A/spot-billed duck/Korea/H422-7/2016(H5N3)	Spot-billed duck
N2	Korea	A/spot-billed duck/ Korea /H51/2017(H5N2)	Spot-billed duck
PB2	China	A/wild bird/Eastern China/1758/2017(H5N3)	Wild bird
PB1	China	A/Anser fabalis/China/Anhui/L221/2014(H6N1)	Bean goose
PA	Korea	A/spot-billed duck/Korea/A09-1-2/2017(H5N3)	Spot-billed duck
NP	Mongolia	A/duck/Mongolia/734/2018(H3N8)	Geese
M	China	A/black-necked crane/Zhaotong/ZT-12/2013(H1N2)	Black-necked crane
NS	Mongolia	A/duck/Mongolia/543/2015(H4N6)	Mallard

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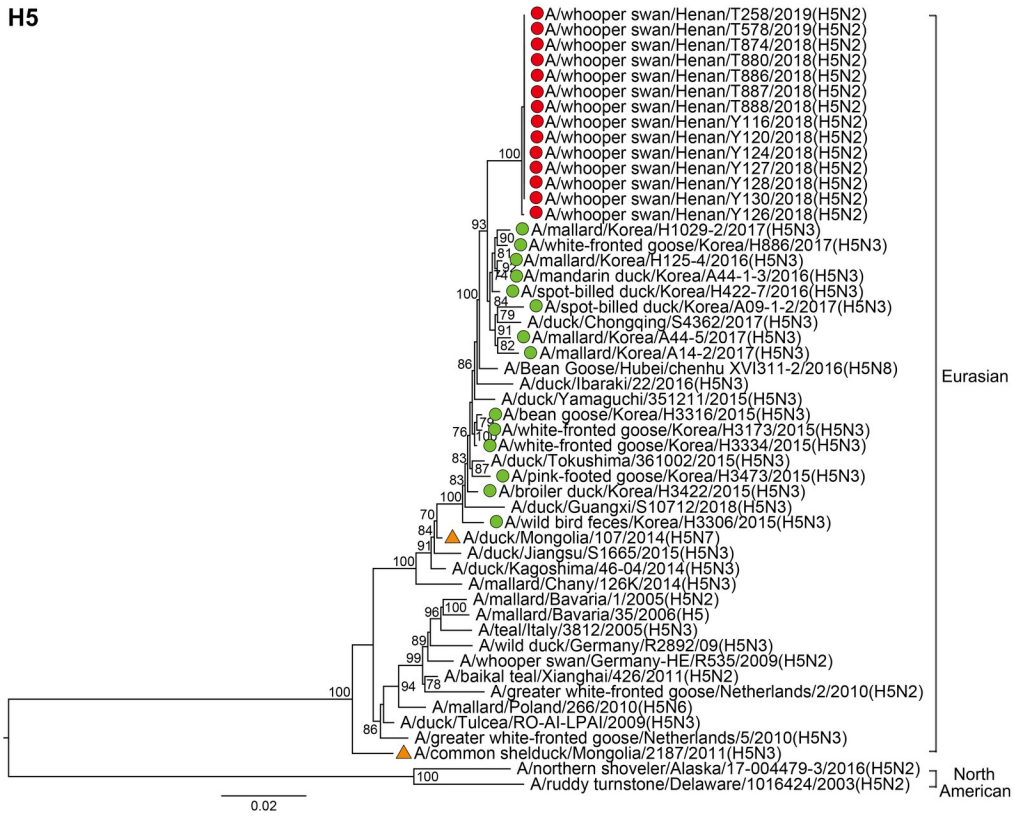
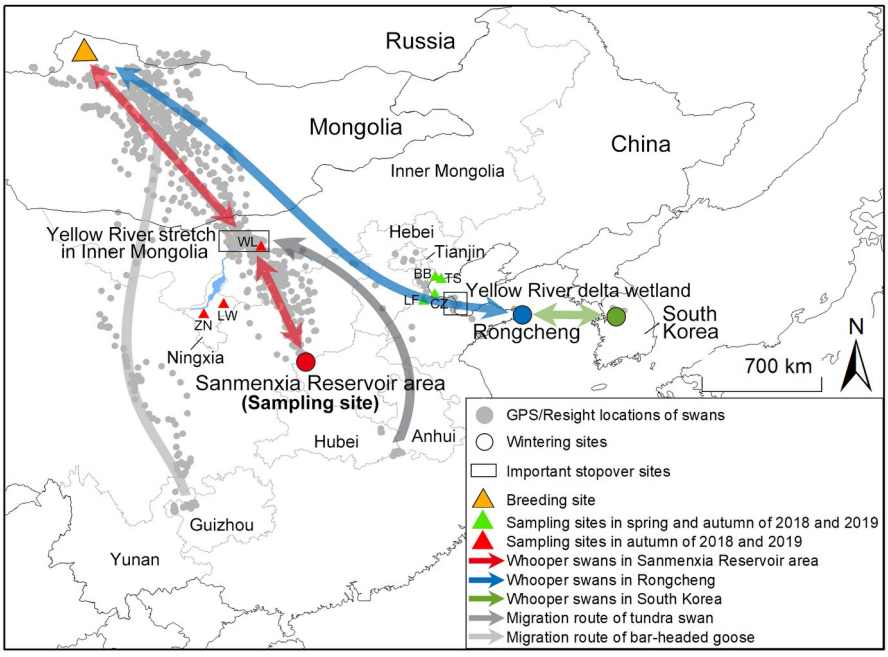


382

383**Figure 1.** Maximum likelihood phylogeny of the H5 AIV HA gene. Numbers at each
384node indicate bootstrap values ≥70 %. Scale bar indicates nucleotide substitutions per
385site. Circle represented the AIV strains isolated from wintering ground of whooper
386swans, and H5N2 strains with red circles isolated in this study; Triangle represented
387the AIV strains isolated from breeding ground of whooper swans.

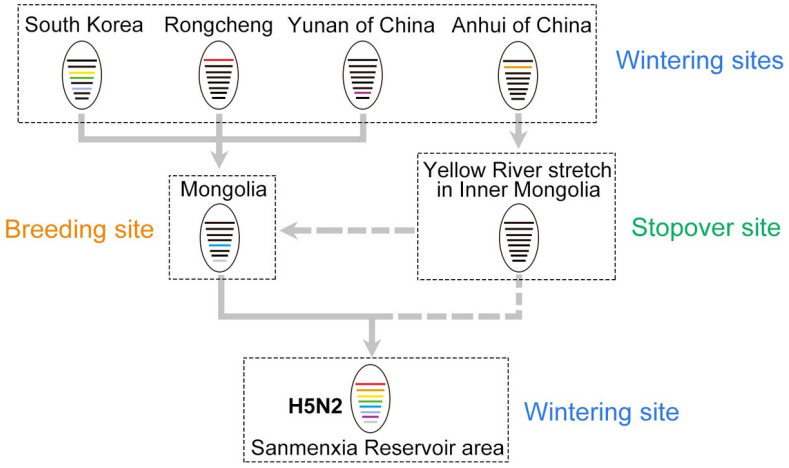
388



389

390**Figure 2.** Migration routes of whooper swans wintering in Sanmenxia Reservoir area,
391Rongcheng and South Korea, and tundra swan and bean goose wintering in south of

392China. (BB: Bohai Bay of Tianjin; CZ: Cangzhou of Hebei; LF: Langfang of Hebei;
393LW: Lingwu of Ningxia; TS: Tangshan of Hebei; ZN: Zhongning of Ningxia; WL:
394Wuliangsu Lake of Inner Mongolia. Migration routes of whooper swans wintering in
395Sanmenxia Reservoir area based on satellite tracking data; migration routes of
396whooper swans wintering in Rongcheng and South Korea based on banding and
397recovery data; migration routes of tundra swan and bean goose based on satellite
398tracking data.)
399



400

401**Figure 3.** Most likely geographic origin of the H5N2 AIV segments based on
402phylogenetic analysis of virus segments and migration routes of whooper swans. (The
403eight gene segments are (horizontal bars starting from top to bottom of the virion)
404PB2, PB1, PA, HA, NP, NA, M, and NS, different colors represent different gene
405segments. Gray solid arrow represents the transmission path of the virus, and the
406dotted arrow represents the possible transmission path of the virus.)