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Sequence and phylogenetic analyses of five low pathogenic avian influenza H5N2 viruses isolated in China

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Summary. – Five H5N2 avian influenza viruses (AIVs) were isolated in Xianghai National Nature Reserve, Jilin Province, China in October 2011. They were all identified as low pathogenic AIVs (LPAIVs) based on their deduced amino acid sequences at the cleavage site of HA protein. Phylogenetic analysis revealed that all gene segments clustered in the Eurasian lineage and that the nucleotide homology of the five isolates was greater for HA and NA genes than for the genes for internal proteins PB2, PB1, PA, M, NP and NS.

Keywords: avian influenza virus; H5N2; low pathogenicity; sequence analysis; phylogenetic analysis

Introduction

In recent years, AIV of H5 subtype has exerted a major influence worldwide. Studies have shown that H5N1 highly pathogenic avian influenza viruses (HPAIVs) were the cause of repeated outbreaks in poultry and wild birds and also the cause fatal diseases among humans and other mammals, and that H5N2 HPAIVs cause sporadic outbreaks in poultry and have brought economic losses to the local industry (Horimoto, *et al*., 1995). H5N2 low pathogenic avian influenza viruses (LPAIVs) can cause outbreaks with low mortality and declines in egg production, or real economic losses (Halvorson, 2009). Both HPAI and LPAI H5N2 viruses infect in nature not only wild birds but also mammals, including humans (Lee J. H. *et al*., 2009; Yamazaki *et al*., 2009; Jiao *et al*., 2012).

Some H5N2 LPAIVs can mutate to HPAIVs, and several mechanisms have been suggested for the emergence of HPAIVs from LPAIV precursors (Kawaoka *et al*., 1984; Garcia *et al*., 1996; Suarez *et al*., 2004; Pasick *et al*., 2005). It has been demonstrated that H5N2 LPAIVs are widely present in wild birds, which play an important role in the spread of AIVs. AIV surveillance in wild birds may reflect the status of virus carriage and provide valuable information about genetic variation.

In October 2011, 300 wild waterfowl samples were collected from Xianghai Nature Reserve of China and five H5N2-positive isolates were obtained. In this study, five H5N2 LPAIVs were cloned and subjected to sequence and phylogenetic analyses. The purpose of this study was to understand the molecular and genetic variation of the five H5N2 isolates, explore their recombination, and provide a scientific basis for in-depth study of AIV molecular epidemiology.

Materials and Methods

Swab samples colleted in Xianghai National Nature Reserve were inoculated in SPF chicken embryos for three generations. Five H5N2

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Abbreviations: AIV(s) = avian influenza virus(es); HPAIVs = highly pathogenic avian influenza viruses; LPAIVs = low pathogenic avian influenza viruses

isolates were chosen according to their different hosts: A/baikal teal/Xianghai/426/2011(H5N2), A/spot-billed duck/Xianghai/427 /2011 (H5N2), A/canvasback/Xianghai/428/2011(H5N2), A/little grebe/Xianghai/429/2011(H5N2), and A/green-winged teal/ Xianghai/430/2011(H5N2). Virus names are shortened to 426, 427, 428, 429 and 430 for convenience.

Each gene was amplified with one or two pairs of specific primers. HA, PA, PB1 and PB2 genes were divided into two segments for amplification, and NA, M, NS and NP genes were amplified as a whole. All eight genes of the isolates were cloned and sequenced.

Homology and phylogenetic trees were calculated using DNASTAR (version 5.06) and Mega (version 4.0). The resultant sequences were spliced with Seqman in DNASTAR. MegAlign was used for determination of nucleotide and amino acid homology. Kimura distances were calculated with MEGA 4.0 using the Kimura 2 parameters model. Phylogenetic trees were calculated using Mega 4.0 with the neighbor-joining method by using the Kimura 2 parameters model and 1000 replicates.

Results

Five AIV H5N2 subtype strains were isolated, cloned and sequenced (GenBank Acc. Nos. JX570831–JX570870).

HA and NA genes

HA ORFs of all five isolates consist of 1695 bp, and these ORFs encode proteins of 564 aa. The deduced amino acid sequences at the cleavage site of all HA proteins were PQRETR↓GLFGAI, which corresponds to the characteristics of LPAIVs.

The phylogenetic tree of HA gene (Fig. 1) contained five clusters. Clusters A–D were grouped in the Eurasian lineage, and the cluster E was in the North American lineage. The HPAIVs (H5N1 and H5N2) were included in clusters B and C. Our isolates belonged to the cluster A and were far from clusters B and C; they were most closely related to A/mallard/Bavaria/1/2005(H5N2). The HA genes of the five isolates were 98.9–99.8% homologous.

NA ORFs of all five isolates consist of 1410 bp, ORFs encode a proteins of 469 aa. The phylogenetic tree of the NA gene (Fig. 2) contained three clusters. Clusters A and B were N2 strains isolated in Asia from 2005 to 2011 and were grouped in the Eurasian lineage. The cluster C was of North American lineage. The five isolates were in the cluster A, and closely related to A/duck/Mongolia/ OIE-7799/2011(H3N2). The NA genes of the five isolates were 99.6–99.9% homologous.

Genes of internal proteins

The ORF length and nucleotide homologies of PB2, PB1, PA, NP and M genes of the five isolates were 2280, 2274, 2151, 1497 and 759 bp, and 91.4–99.4%, 92.2–99.8%, 94.7–99.5%, 93.1–99.8% and 92.6–100.0%, respectively. Phylogenetic trees (Figs. 3–7) revealed that they all belonged to the Eurasian lineage and originated from different H and N subtypes.

The NS genes of the isolates 426, 427, 428 and 430 contained two complete ORFs of 693 and 363 bp, which encoded two proteins of 230 aa and 120 aa, respectively. It is worth noting that the two complete ORFs of 429 NS were 654 and 363 bp, which encoded two proteins of 217 and 120 aa, respectively. The phylogenetic tree of the NS gene (Fig. 8) consisted of four clusters. The first three clusters belonged to the Eurasian lineage and the last cluster was in the North American lineage. The phylogenetic tree indicated that 426NS and 428NS had a common ancestor A/aquatic bird/Korea/ CN9/2009(H6N8) and shared 99.8% homology; 427NS, 429NS and 430NS were derived from A/wild duck/Korea/ SH5-26/2008(H4N6), A/duck/Jiangxi/7348/2007(H6N2), and A/migratory duck/Hong Kong/MP206/2004(H5N2), respectively. The homology of the five NS genes of our isolates was 94.4–97.7%, and 429NS had the lowest homology with the other four isolates.

Discussion

Full genetic characterization of five H5N2 viruses analyzed in this study indicated that they all belonged to the Eurasian AIV lineage. Nucleotide sequence comparison between the five isolates and reference strains showed different genetic relations and different levels of homology in the HA, NA genes and the genes of internal proteins. For example, the HA, NA genes were most closely related to A/mallard/Bavaria/1/2005 (H5N2) (A) or A/duck/Mongolia/ OIE-7799/2011(H3N2) (B) and shared very high homology among the five isolates. However, in each of the genes of internal proteins, there was a certain level of difference in homology between the five isolates, as shown by the fact that their reference strains (strains with the highest homology) were different, and a high level of variation of these reference strains was also found between the genes of internal proteins of each of the five isolates. Overall, the results suggest that these five isolates probably resulted from genetic reassortment between circulating viruses.

Reassortment of the viral genome is a universal phenomenon in wild birds and produces a variety of transient constellations that are continually reshuffled by reassortment (Dugan *et al.*, 2008). The direct result of gene reassortment is the production of new viruses, resulting in a wide variety of AIV variants or genotypes, and possible influence on viral replication, stability and virulence (Kaverin *et al.*, 1998; Li *et al.*, 2006). Therefore, exploring the dynamic change in the constellations of AIV in wild birds is an important means for understanding the regular patterns of genome reassortment.

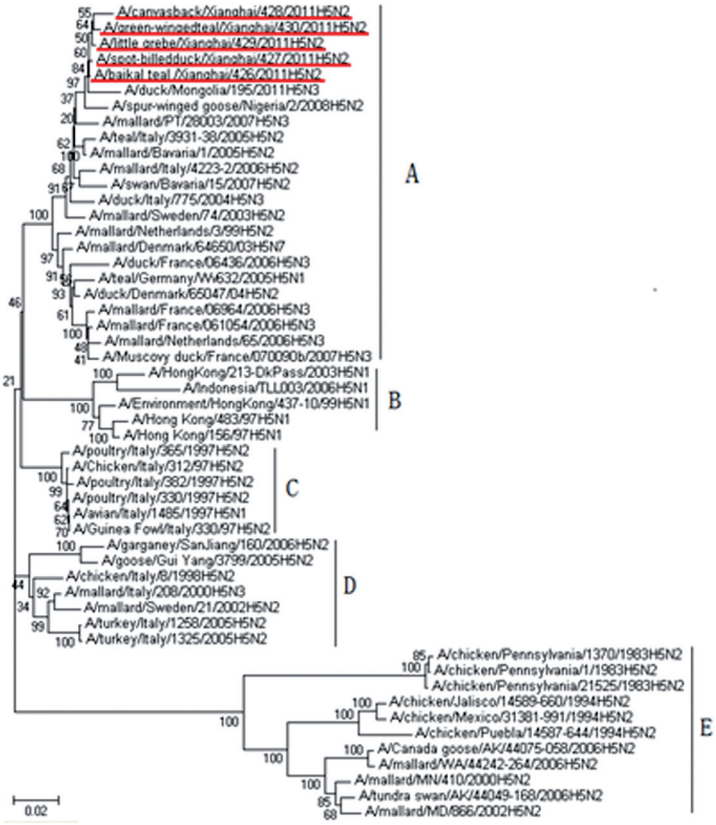

Fig. 1
Phylogenetic tree of HA genes

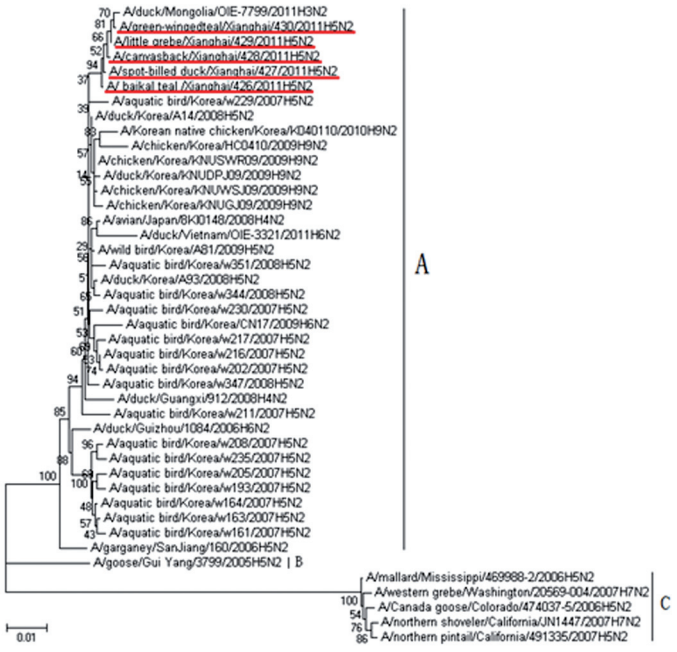

Fig. 2
Phylogenetic tree of HA genes

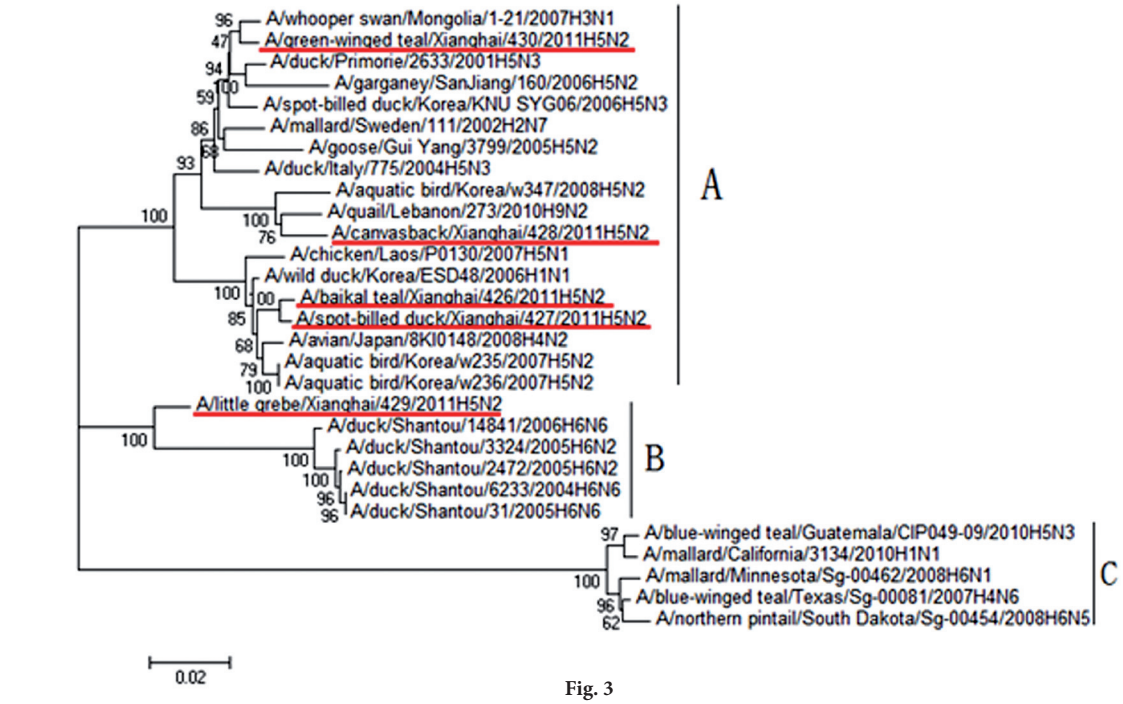

Fig. 3
Phylogenetic tree of PB2 genes

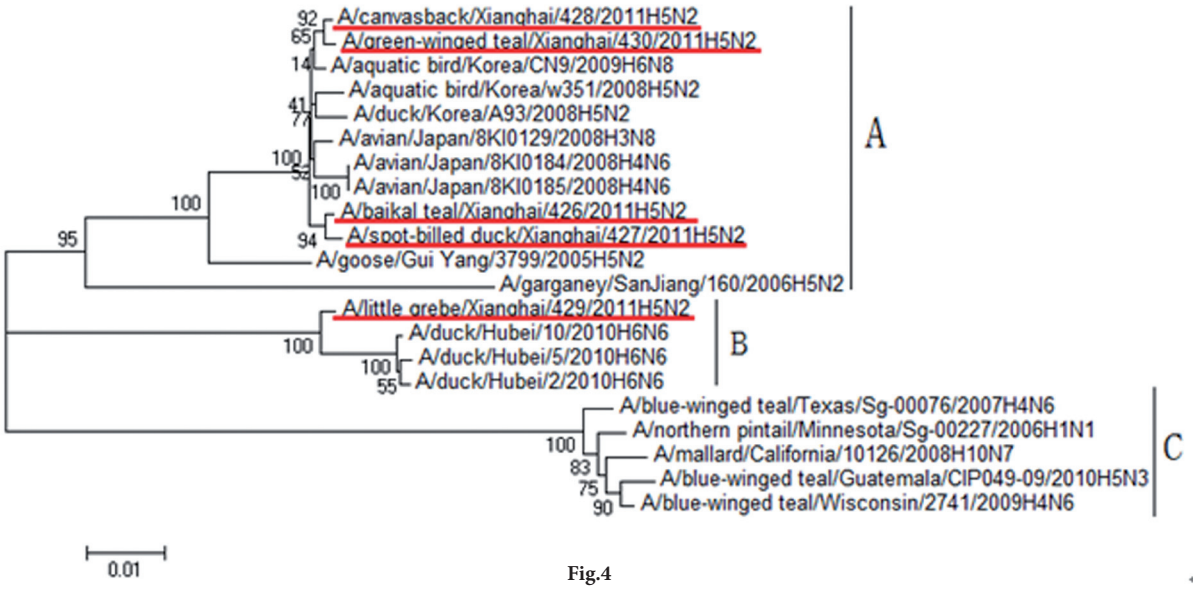

Fig.4
Phylogenetic tree of PB1 genes

Nucleotide sequences with homologies ≥95% were sorted as one constellation, otherwise they have been sorted to different constellations. All eight genes of these five isolates fell into three different genome constellations as marked with red, blue and yellow colors. For 426 and 427, 428 and 430, each pair shared the same constellation. 429 possessed a single constellation that differed considerably from the other two constellations, in which most of the segments of its genes of internal proteins seemed to have originated from different strains. In these constellations, the origin of differences of the genes of internal proteins revealed that the gene reassortment may occur frequently between different subtypes of AIVs, even though the HA and NA genes are highly homologous.

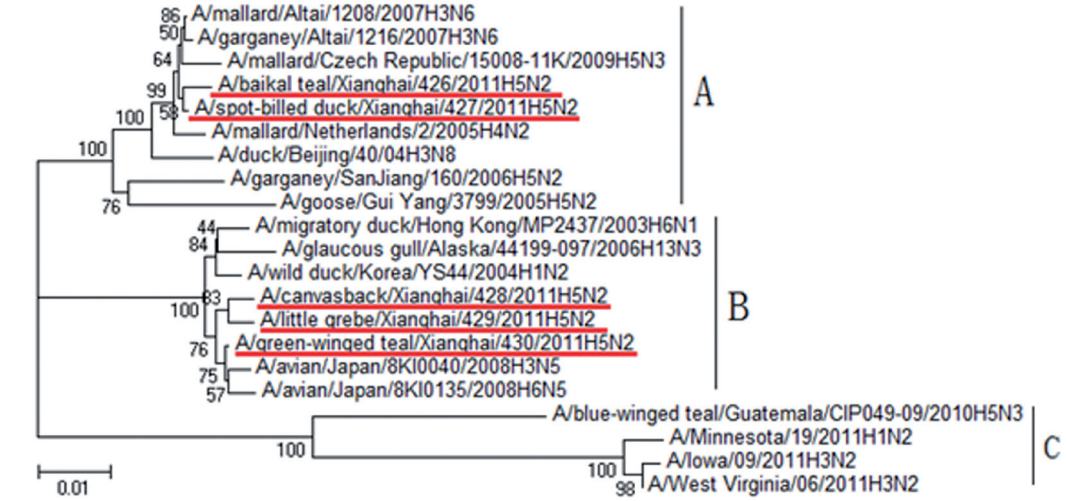

Fig. 5
Phylogenetic tree of PA genes

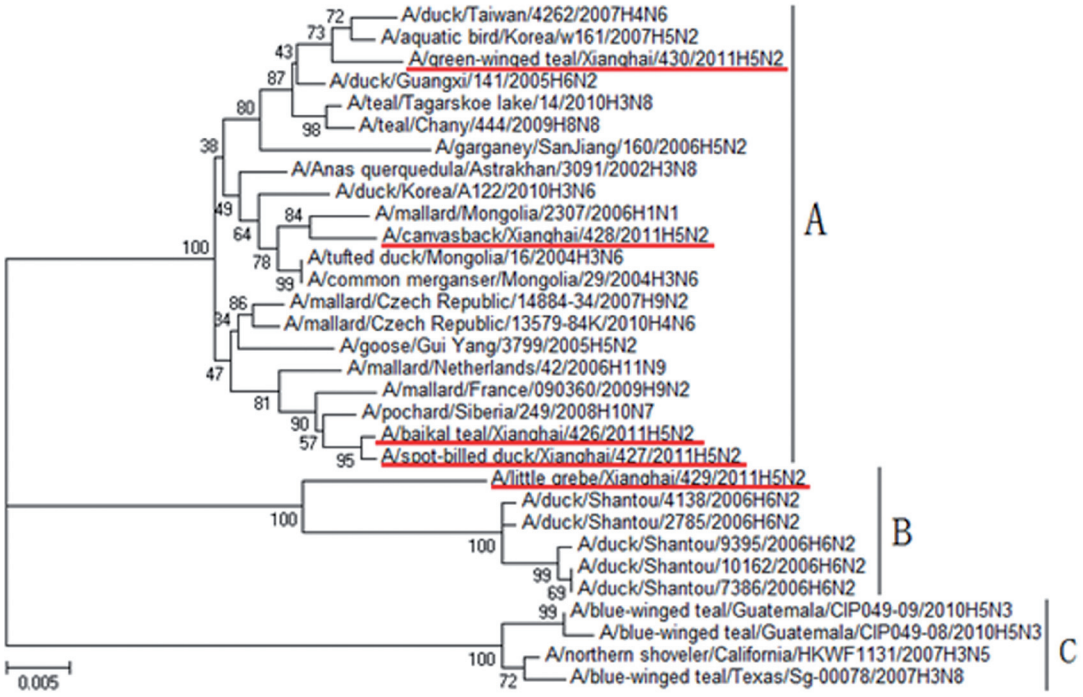

Fig. 6
Phylogenetic ttree of M genes

The high homology of the HA and NA genes indicates that these five isolates arose from a common original H5N2 virus. When the virus co-infected with other AIVs in different bird species, reassortment of genes of internal proteins may have been influenced by the host species. Hosts of 426 (baikal teal), 427 (spot-billed duck), 428 (canvasback) and 430 (green-winged teal) all belong to the duck family, and perhaps because of the similar hosts, there were fewer differences in the genes of internal proteins between the four isolates. However, the host of 429 (little grebe) belongs to the grebe family, and species differences may have led to significant differences in the genes of internal proteins between 429 and the other four isolates. Apparently, the proximity of the host genetic relationship is able to affect the homology in genes of internal proteins.

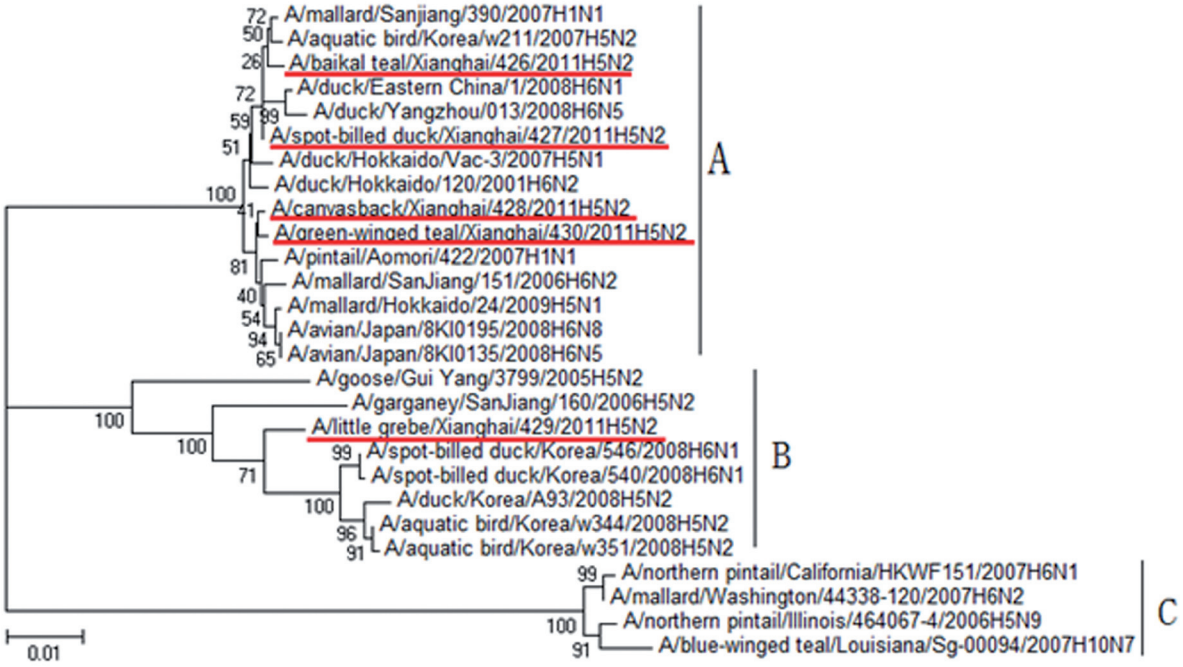

Fig. 7
Phylogenetic tree of NP genes

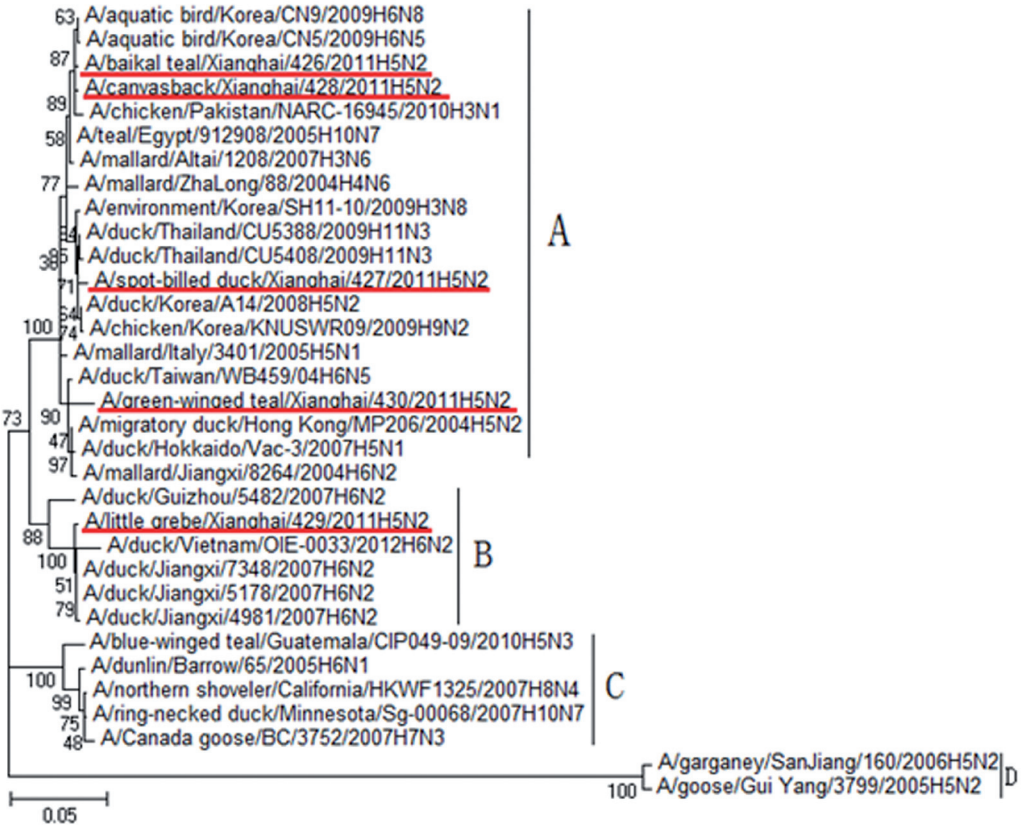

Fig. 8
Phylogenetic tree of NS genes

All segments of the five isolates were clustered with different viruses of the Eurasian lineage, and reassortment occurred with the viruses from this lineage among wild birds and poultry. Comparison of the homology of the genes of internal proteins revealed that five of six reference strains that had the highest homology with genes of internal proteins of 429 differed from the corresponding reference strains of the other four isolates, except the reference strain of PA gene. From the phylogenetic trees, these five genes of internal proteins, except 429 PA, were clustered in an independent B sublineage.

The five isolates were obtained from different bird species. However, they were collected at the same time and place, probably sharing a common migration route or stopover place. The HA and NA genes of the five isolates were highly homologous, revealing that their hosts had close contact in time and space, and with a high possibility could be infected by a group of AIVs with the same HA and NA genes, or by viruses from the same H5N2 subtype quasispecies. The different levels of gene reassortment in the genes of internal proteins resulted in genome constellations that varied to different degrees, and which were closely related to the proximity of the genetic relationship between host species. These cases indicated that during infection, the viral quasispecies, including a group of complex and dynamic distributions of variants, existed widely in the migratory wild bird populations (Ramakrishnan *et al.*, 2009).

Previous studies have shown that PA and PB1 genes of H5N1 HPAIVs are associated with lethality in mallards (Hulse-Post *et al.*, 2007). The amino acids 515T of PA protein and 207 K and 436 Y of PB1 proteins in the five isolates, revealed low or no pathogenicity in mallards. One single-amino-acid substitution of serine for proline at the position 42 (P42S) in the NS1 protein dramatically increases virulence in mice (Jiao *et al.*, 2008). Multiple sequence alignment showed that there was 42S in the five NS1 proteins, indicating possible higher virulence in mice. Seo *et al.* (2002) have found that swine infected with recombinant human H1N1 virus that carried the H5N1 NS gene experienced significantly greater and more prolonged viremia, fever and weight loss than swine infected with wild-type human H1N1 virus. These effects required the presence of glutamic acid at the position 92 of the NS1 molecule. 92E inhibited the antiviral activity of the host cells and reduced virus eradication, thus contributing to virus infection. Multiple sequence alignment indicated that there was 92E present in the five NS1 genes of our isolates, thus these possibly come from NS genes of HPAIVs.

The analysis of these virulence-associated loci in the deduced amino acid sequences of the five isolates was not sufficient to confirm whether they were pathogenic in corresponding animals. However, accumulation of new information about the occurrence of virulence-associated amino acids in LPAIVs should be helpful for understanding of how these viruses mutate into HPAIVs. It is generally believed that wild birds, especially waterfowl, appear to serve as the reservoir for most influenza A viruses and play an important role in the spread of AIVs. Even though most influenza virus strains found in wild birds have been defined as low pathogenic, studies have demonstrated that wild birds could carry both LPAIVs and HPAIVs (Wallensten, 2007). When reaching the same habitat with different segments of AIVs in them, migratory birds can turn into virus mixers, which increases the risk of gene reassortment and gene flow between the viruses in wild birds and domestic birds that share the same habitat, and make it possible that LPAIVs change into HPAIVs. Evidence has shown that each outbreak of HPAIVs originated from low or non-pathogenic AIVs (Rohm *et al.*, 1995; Alexander, 2000). Which LPAIV could mutate into HPAIV is unpredictable, but it is possible that when H5 and H7 LPAIVs are endemic for some time in birds, certain isolates can mutate into HPAIVs (Kawaoka *et*

Table 1. Nucleotide sequence homology (%) and genome constellations

	HA	NA	PB2	PB1	PA	M	NP	NS
426	A:98.8%	B:99.6%	C:98.8%	G:99.5%	I:99.5%	K:99.3%	O:99.7%	G:99.8%
427	A:98.6%	B:99.6%	C:98.8%	G:99.4%	I:99.8%	M:99.9%	O:99.8%	U:99.4%
428	A:97.9%	B:99.6%	D:98.0%	G:99.6%	J:99.2%	L:99.0%	P:99.7%	G:99.8%
429	A:98.6%	B:99.8%	E:95.2%	H:98.7%	J:98.9%	N:96.9%	R:98.5%	S:99.5%
430	A:98.4%	B:99.8%	F:98.6%	G:99.6%	J:99.7%	Q:98.9%	P:99.6%	T:98.6%

A to U - the reference strains with the highest nucleotide sequence homology. A: A/mallard/Bavaria/1/2005(H5N2); B: A/duck/Mongolia/OIE-7799/2011(H3N2); C: A/wild duck/Korea/ESD48/2006(H1N1); D: A/quail/Lebanon/273/2010(H9N2); E: A/duck/Shantou/14841/2006(H6N6); F: A/spot-billed duck/Korea/KNU SYG06/06(H5N2); G: A/aquatic bird/Korea/CN9/2009(H6N8); H: A/duck/Hubei/2/2010(H6N6); I: A/mallard/Altai/1208/2007(H3N6); J: A/avian/Japan/8KI0040/2008(H3N5); K: A/pochard/Siberia/249/2008(H10N7); L: A/tufted duck/ Mongolia/16/2004(H3N6); M: A/duck/Thailand/Cu-10507T/2011(H7N4); N: A/wild duck/Shantou/311/2011(H6N9); O: A/duck/Sanjiang/390/2007(H1N1); P: A/pintail/Aomori/422/2007(H1N1); Q: A/duck/Mongolia/54/2001(H5N2); R: A/aquatic bird/Korea/w344/2008(H5N2); S: A/duck/Jiangxi/7348/2007(H6N2); T: A/migratory duck/Hong Kong/MP206/2004(H5N2); U: A/wild duck/Korea/SH5-26/2008(H4N6). Red, blue and yellow: the nucleotide sequence homologies of gene segments highlighted with the same color were ≥95% among the five virus isolates, and nucleotide sequence homology <95% are shown in different colors.

al., 1984; Garcia *et al.*, 1996; Suarez *et al.*, 2004; Pasick *et al.*, 2005; Okamatsu *et al.*, 2007).

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