The characterization of low pathogenic avian influenza viruses isolated from wild birds in northern Vietnam from 2006-2009

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1

ABSTRACT

Due to concerns that wild birds could possibly spread H5N1 viruses, surveillance was conducted to monitor the types of avian influenza viruses circulating among the wild birds migrating to or inhabiting in northern Vietnam from 2006-2009. An H5N2 virus isolated from a Eurasian woodcock had a close phylogenetic relationship to H5 viruses recently isolated in South Korea and Japan, suggesting that H5N2 has been shared between Vietnam, South Korea, and Japan. An H9N2 virus isolated from a Chinese Hwamei was closely related to two H9N2 viruses that were isolated from humans in Hong Kong in 2009, suggesting that an H9N2 strain relevant to the human isolates had been transmitted to and maintained among the wild bird population in Vietnam and South China. The results support the idea that wild bird species play a significant role in the spread and maintenance of avian influenza and that this also occurs in Vietnam.

Keywords: wild bird / avian influenza virus / Vietnam

1. Introduction

Since late 2003, highly pathogenic H5N1 influenza viruses have spread among poultry and wild aquatic birds in southern China and southeastern Asian countries, including Vietnam [1]. Multiple sublineages of highly pathogenic avian influenza (HPAI) virus (H5N1) have been detected in poultry in Vietnam since 2001 [2]. However, the introduction of the subtype H5N1 genotype Z virus in 2003 resulted in an unprecedented increase in outbreaks of the genotype in poultry in northern Vietnam [3, 4, 5, 6]. These viruses became endemic in poultry in the area, causing repeated outbreaks, and have been transmitted to other Southeast Asian countries, where these viruses have also caused outbreaks in poultry [3, 4]. In Vietnam, a series of human cases of H5N1 virus infections have occurred sporadically in areas where the virus is endemically circulating [5], and as of March 2012, 123 confirmed cases with 61 deaths have been reported since 2003 [7].

The surveillance of wild birds for avian influenza viruses (AIV) has increased substantially worldwide in recent years due to the spread of the H5N1 HPAI viruses among domestic and wild birds. Wild bird sampling for surveillance of AIV also intensified in South-East Asia, and H5N1 HPAI viruses have been detected in migrating wild birds since 2005 [8, 9]. The ecological success of this virus in diverse species of both poultry and wild birds with frequent introduction to humans suggests that this virus is a likely source of the next human pandemic. Despite the significance of these events, which pose a serious threat to animal and public health in Vietnam, very little is known about AIV circulating in the wild birds in Vietnam. Therefore, we conducted a surveillance of AIV in wild birds in northern Vietnam.

In this study, throat and cloacal swab samples were obtained from wild birds in

northern Vietnam from 2006-2009 and were subjected to virus isolation and nucleotide sequencing to characterize the isolated viruses. The implications for the role of wild birds in the global spread of AI viruses were investigated.

2. Materials and Methods

2. 1. Study sites and sample collection

A total of 651 live wild birds were collected by mist-netting in Hanoi city in November and December, 2006; Ha Tay province in November, 2006 and 2009; Nam Dinh province in November and December, 2006; November, 2007 and October, 2008; Vinh Phuc province in November and December, 2006; and Bac Ninh province in November, 2009 in northern Vietnam (Fig. 1 and Table 1). The latest outbreaks during study period occurred at the end of October 2005, the beginning of December 2005, and the end of December 2005, in Hanoi, Vinh Phuc and Nam Dinh provinces, respectively. The fourth endemic wave had started before initiating the second survey. During the fourth epidemic wave in 2007, the number of outbreaks reported in Nam Dinh province was the highest among the three provinces; therefore, we concentrated our surveillance in Nam Dinh in 2007 and 2008. Ha Tay and Bac Ninh provinces, where outbreaks were reported in February and June in 2007, were chosen as study sites in November 2009. Throat and cloacal secretion specimens were taken from each wild bird and were suspended in 2 ml of phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin, 10,000 units/ml of penicillin, 10 mg/ml of streptomycin sulfate, and 0.3 mg/ml of gentamicin sulfate. All of the specimens were kept at 4 °C for 4 to 6 hrs during transportation to the laboratory and were kept frozen at -80 °C until inoculation into embryonated eggs for virus isolation.

2. 2. Virus isolation and hemagglutinin (HA) and neuraminidase (NA) subtyping

A hundred (100) µl portion of each specimen was inoculated into the allantoic cavity of two 10-day-old specific-pathogen free embryonated hen's eggs and incubated at 35°C for 72 hrs unless death of the embryo was detected. After a 72 hr-incubation or upon embryo death, the allantoic fluids were tested for hemagglutination as previously described [10]. All of the allantoic fluids containing hemagglutinating agents were further tested to determine the subtype of HA and NA by HA-inhibition (HI) [11] and NA-inhibition (NI) [12] tests using specific antisera prepared with A/PR/8/34 (H1N1), A/swine/Iowa/15/30 (H1N1), A/Singapore/1/57 (H2N2), A/duck/Ukraine/1/63 (H3N8), A/duck/Czech/56 (H4N6), A/whistling swan/Shimane/499/83 (H5N3), A/turkey/Massachusetts/65 (H6N2), A/seal/Massachusetts/1/80 (H7N7), A/turkey/Ontario/6118/68 (H8N4), A/turkey/Wisconsin/66 (H9N2), A/chicken/Germany/"N"/49 (H10N7), A/duck/England/56 (H11N6), A/duck/Alberta/60/76 (H12N5), A/gull/Maryland/704/77 (H13N6), and A/duck/Memphis/564/74 (H11N9), as described in previous studies [10]. An antiserum specific to the Newcastle Disease virus (NDV) strain Miyadera was also used to exclude hemagglutinating agents induced by NDV in the test.

2. 3. Nucleotide sequencing

To characterize isolated viruses, nucleotide sequences of eight fragments of the influenza A virus (polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and non-structural (NS)) were determined as described elsewhere [13].

Briefly, viral RNA was extracted from all allantoic fluids containing the influenza A virus using TRIzol LS Reagent (Invitrogen) and was reverse-transcribed with the Uni12 primer [13] and M-MLV Reverse Transcriptase (Invitrogen). The full-length genome of each gene segment was amplified by polymerase chain reaction with gene-specific primer sets [13]. A series of PCR-amplified fragments corresponding to each of the eight segments were purified with the QIAquick Gel Extraction Kit, and direct sequencing was performed on a CEQ 8000 DNA sequencer (Beckman Coulter, Inc.) using the CEQ DTCS-Quick Start Kit. Nucleotide sequence alignment was performed on ClustalW methods. Phylogenetic trees were generated by the maximum likelihood method using the general time-reversible model [14] of nucleotide substitution and gamma distributed rates among sites as parameters. We performed 1,000 bootstrap replicates for each tree. All nucleotide sequence alignments and phylogenetic tree generations were conducted using MEGA version 5 [15]. H5 segments representing each of the clades 1 to 2.5 of the HA, and representative segments PB2, PB1, PA, HA, NP, NA, M, and NS of Influenza A virus, in GenBank were used for references and compared to the strains isolated in this study by phylogenetic analysis.

Amino acid sequences translated from nucleotide sequences of each gene segment were examined for the human-associated amino acids that have been described in several reports [16-18], as reported previously [19].

3. Results

3. 1. Isolation of avian influenza A viruses in northern Vietnam from 2006 to 2009

In the period between 2006 and 2009, 651 wild birds (26 species) were collected in Hanoi city and four different provinces in northern Vietnam, and throat and cloacal secretions

were taken for virus isolation (Table 1). A total of 18 influenza A virus strains, consisting of 17 H9N2 strains and one H5N2 strain, were isolated in the study. H5N2 was isolated from a Eurasian woodcock (*Scolopax rusticola)* collected in 2007; and H9N2 strains were isolated from one Chinese Hwamei (*Garrulax canorus*) collected in 2006, and from one rock pigeon (*Columba livia*), one African stonechat (*Saxicola torquatus*), and 14 Japanese quail (*Coturnix japonica*) collected in 2009 (Table 2). Sequence analysis was performed on the eight segments of each strain isolated in this study. The 18 strains were divided into six different groups, and six representative strains from each group were chosen for the analyses as follows: A/Chinese Hwamei/Vietnam/38/2006 (H9N2), A/Eurasian Woodcock/Vietnam/8/2007 (H5N2), A/Rock Pigeon/Vietnam/6/2009 (H9N2), A/African Stonechat/Vietnam/8/2009 (H9N2), A/Japanese quail/Vietnam/4/2009 (H9N2), and A/Japanese quail/Vietnam/7/2009 (H9N2) (Table 2).

3.2. Phylogenetic analysis of the H5N2 virus

A phylogenetic tree rooted to highly pathogenic historical H5 isolates (A/chicken/Scotland/59 and A/Tern/South Africa/61) was constructed. Phylogenetic analysis indicated that the surface genes of A/Eurasian Woodcock/Vietnam/8/07 were related to those of the low pathogenic H5N2 viruses isolated from wild birds in South Korea [20] and Japan [21] in recent years, and not to those of recent H5N1 HPAI isolates (Fig. 2). This H5N2 virus also has close phylogenetic relationships in six other segments to H5 viruses that have been recently isolated in Korea and Japan, but not with most other H5 viruses (data not shown). These results indicate that some populations of wild birds, such as Eurasian Woodcock, that migrate between Far East Asia and Southeast Asia carry A/Eurasian

Woodcock/Vietnam/8/2007 (H5N2)-like viruses (Fig. 4).

3.3. Amino acid sequence analysis of the H5N2 virus

Amino acid sequence analysis of A/Eurasian Woodcock/Vietnam/8/2007 (H5N2) identified a low cleavable sequence (KETK), which is typically found in the HA molecule of LPAI strains. Recently, it was reported that the amino acid composition at 20 different positions in the receptor-binding site of the H5 HA region are important in conferring the preference to a receptor, the avian- or the human-type. Of these, amino acid positions, 157, 224, 226 and 318 are critical [22]. For the A/Eurasian Woodcock/Vietnam/8/2007 (H5N2) strain, none of the amino acid residues at the aforementioned four positions were identical with those typically found in strains of human origin (Table 3), indicating that the HA molecule of A/Eurasian Woodcock/Vietnam/8/2007 (H5N2) is of avian origin. Contrary to the HA gene, analyses on the translated amino acid sequences of all the internal genes of the A/Eurasian Woodcock/Vietnam/8/2007 (H5N2) identified M, K, and S at amino acid position 64 of PB2, at 293 of NP, and at 82 of PB1-F2, respectively, which are typically found in strains of human origin [16-18] (Table 3).

3. 4. Phylogenetic analysis of the H9N2 viruses

Phylogenetic analyses were performed on all of the eight segments (HA, NA, PB2, PB1, PA, NP, M and NS) of all of the five representative H9N2 strains (A/Chinese Hwamei/Vietnam/38/2006, A/Japanese quail/Vietnam/4/2009, A/Rock Pigeon/Vietnam/6/2009, A/Japanese quail/Vietnam/7/2009 and A/African Stonechat/Vietnam/8/2009) isolated from the wild birds from northern Vietnam in this study

and were compared with those of the H9N2 strains deposited in GenBank (Fig. 3).

Phylogenetic analysis on all eight gene segments revealed that each of the five representative H9N2 strains carried genes closely related to those of viruses isolated from Chinese poultry (Fig. 3). The analysis of the HA gene revealed that four H9N2 strains collected in 2009 (A/Japanese quail/Vietnam/4/2009, A/Rock Pigeon/Vietnam/6/2009, A/Japanese quail/Vietnam/7/2009, and A/African Stonechat/Vietnam/8/2009) belonged to the Ck/Bei/94-like lineage; however, one strain from 2006 (A/Chinese Hwamei/Vietnam/38/2006) belonged to the G1-like lineage (Fig. 3). When the phylogenetic relationship of all eight genes was considered (genotyping), the closest strain to the four representative 2009 H9N2 isolates (A/Japanese quail/Vietnam/4/2009, A/Rock Pigeon/Vietnam/6/2009, A/Japanese quail/Vietnam/7/2009 and A/African Stonechat/Vietnam/8/2009) was A/quail/Shantou/6794/2004. The genetic relatedness of each of the four 2009 isolates to the closest counterpart (A/quail/Shantou/6794/2004) was different; that is, A/Japanese quail/Vietnam/7/2009 possessed the PA gene relatively isolated from that of A/quail/Shantou/6794/2004 and three other 2009 strains, whereas the opposite relationship was observed in the PB2 gene (Fig. 3 and Table 4). Surprisingly, all of the eight genes of A/Chinese Hwamei/Vietnam/38/2006, isolated from a wild bird in Vietnam, categorized into a close lineage with those of two H9N2 strains (A/Hong Kong/33982/2009 and A/Hong Kong/35820/2009) isolated from humans in Hong Kong in 2009 [23] (Fig. 3 and Table 4).

3.5. Amino acid sequence analysis of the H9N2 viruses

The alignments of the critical amino acid residues of the HA and NA proteins of the

five H9N2 viruses isolated in this study and two human H9N2 isolates collected in Hong Kong in 2009 (A/Hong Kong/33982/2009 and A/Hong Kong/35820/2009) are shown in Table 5. Translated amino acid sequences on the receptor-binding site of A/Chinese Hwamei/Vietnam/38/2006 and the human H9N2 isolates were identical, with H, D, Q, Q and G at the position of 191, 198, 234, 235 and 236, respectively. This composition is typically found in strains of avian origin that preferentially bind to sialic acid linked to galactose by α2, 3-linkages. The translated amino acid sequences of the cleavage site of HA of A/Chinese Hwamei/Vietnam/38/2006 and the human H9N2 isolates were RSSR and RSNR, respectively, both of which are typically found in low pathogenic avian influenza strains. Moreover, the deduced amino acid sequences of the hemadsorbing sites of NA were identical in the A/Chinese Hwamei/Vietnam/38/2006 and the human H9N2 isolates from Hong Kong in 2009. However, these similarities were not observed in the other four representative strains.

Because A/Chinese Hwamei/Vietnam/38/2006 was categorized in the same genotype with that of the human H9N2 isolates (Table 4), we further examined the translated amino acid sequences of all the internal proteins (PB2, NP, PB1-F2, and PA) of the five representative H9N2 isolates from northern Vietnam to find the human-associated amino acids. We identified that the same amino acids were present at position 661 in PB2, at 136 in NP, at 73 in PB1-F2, and at 57 in PA among A/Chinese Hwamei/Vietnam/38/2006 and the two human H9N2 isolates, except for K at 73 in PB1-F2 in A/Hong Kong/33982/2009 (Table 6) [16-18]. However, the amino acids of other representative Vietnamese H9N2 isolates were typically found in strains of avian origin (Table 6).

4. Discussion

H9N2 influenza A viruses have been reported to cause infection in the poultry population around the globe since the mid-1990s [24]. Three distinct sublineages of H9N2 AIV were circulating in poultry in several countries in the Eurasia region: Ck/Bei/94-like, represented by A/chicken/Beijing/1/94; G1-like, represented by A/Quail/Hong Kong/G1/97; and Korean-like, represented by A/chicken/Korea/38349-p96323/96. The G1-like viruses provided an internal gene for the human H5N1 influenza virus in 1997 [25]. In southern China, the G1-like and Ck/Bei/94-like lineages of the H9N2 viruses have been co-circulating [26]. In 1998, it was observed that domestic pigs in Hong Kong were infected with H9N2 influenza viruses [27]. Several human cases of H9N2 infection have been recorded since 1997 in Hong Kong and China in children and adults exhibiting influenza-like symptoms and mild upper respiratory tract infections [27-31]. Genetic analysis of the H9N2 virus isolated from a child in Hong Kong in 2003 showed that the human H9N2 virus was of purely avian origin and was closely related to some H9N2 viruses isolated in Hong Kong live bird markets [28]. Furthermore, those from Hong Kong live bird markets showed the preferential binding to sialic acid linked to galactose by α2,6-linkages (Sia α2,6Gal; human receptors) [32]. Taken together, these findings indicated the possibility of the inter-species transmission of H9N2 viruses, which could pose a persistent threat to the human population. It is noteworthy that A/Chinese Hwamei/Vietnam/38/2006 (H9N2) isolated from wild birds in Vietnam in this study was closely related to the two H9N2 strains (A/Hong Kong/33982/2009 and A/Hong Kong/35820/2009) isolated from humans in Hong Kong in 2009 [23] at all eight gene segments (Fig. 3 and Table 5).

Analyses of the translated amino acids revealed that the surface proteins of A/Chinese Hwamei/Vietnam/38/2006 (H9N2) maintained the typical features of

non-pathogenic viruses (Table 5), although the virus was closely related to the two H9N2 human isolates from Hong Kong in 2009 (Tables 5 and 6, Fig. 3) [23]. Analysis of the HA cleavage site showed that the human H9N2 strains isolated in 2009 had a different cleavage site than A/Chinese Hwamei/Vietnam/38/2006. Almost all H9N2 viruses, including A/Chinese Hwamei/Vietnam/38/2006 isolated from 1997 to 2008 worldwide, retain a conserved amino acid pattern at the cleavage site; RSSR [23]. Presence of the R-S-S-R motif is suggestive of H9N2 viruses with low pathogenicity that have adapted to the chicken host [33-35]. However, the human H9N2 viruses isolated in 2009 have a different pattern (RSNR), due to the substitution mutation S to N at position 337. This is a major difference between A/Chinese Hwamei/Vietnam/38/2006 and the human N9N2 isolates (Table 5). These findings suggest that A/Chinese Hwamei/Vietnam/38/2006 could evoke and acquire this significant mutation for increasing pathogenicity in humans. The significance of this mutation on viral stability or increased pathogenicity is not fully understood and requires further study, as the cleavage site is an indicator of pathogenicity.

The translated amino acid residues at positions 402 and 403 of the hemadsorbing sites of NA of A/Chinese Hwamei/Vietnam/38/2006 and the H9N2 human isolates from Hong Kong in 2009 were N and S, respectively (Table 5). The most prominent residues that are typical in human pandemic H2N2 and H3N2 viruses are N and S at positions 402 and 403, respectively. The typical mutations of the hemadsorbing sites of NA in human pandemic H2N2 and H3N2 viruses are at position 402 from I to N/ S and at position 403 from R to W/S [32, 36]. Although the biological significance of any of these substitutions in the hemadsorbing site is not yet known, A/Chinese Hwamei/Vietnam/38/2006 could already have an NA protein with a better replication rate in humans.

Phylogenic analyses revealed interesting evidence of the circulation of the virus between Southeast Asia and the Far East. The H5N2 virus isolated from a Eurasian woodcock in northern Vietnam in this study (A/Eurasian Woodcock/Vietnam/8/2007) had a close phylogenetic relationship with H5 viruses recently isolated in the Far East countries of South Korea and Japan, but not with most of other H5 viruses. The Eurasian Woodcock has an extensive Palaearctic distribution and winters in Europe, North Africa, the Middle East, India and Southeast Asia to Japan. These findings suggest that the H5N2 viruses are circulating and are being maintained in the area including Vietnam, South Korea and Japan where the Asian population of the Eurasian Woodcock winters (Fig. 4). Moreover, it is known that the species is also susceptible to the H5N1 virus and could be threatened by future outbreaks of the virus [37], indicating that the Eurasian Woodcock could play a significant role in maintaining and transmitting viruses that can cause disease outbreaks in northern Vietnam.

Wildlife trade and emerging infectious diseases pose significant threats to human and animal health and global biodiversity. Legal and illegal trade of domestic and wild birds has played a significant role in the global spread of the highly pathogenic avian influenza H5N1. The Chinese Hwamei, or the melodious laughing thrush (*Leucodioptron canorum*, formerly *Garrulax canorus*), is a passerine bird of eastern Asia. The species is a popular cage bird because of its attractive song. The Chinese Hwamei was a major species that was observed during the survey of the bird markets in Hanoi in 2007 [38]. Although the trade of wild birds in the Hanoi markets during the survey in 2007 had declined compared with surveys conducted before the H5N1 outbreak, the consensus obtained from the study was that most people in Hanoi were buying birds for decoration and for their song. These facts indicate

that the Chinese Hwamei may have a pivotal role in maintaining and transmitting the virus to humans in northern Vietnam.

In this study, we discuss and demonstrate the possibility that some wild bird species may play a significant role in the global spread of new epidemics. Therefore, it is important to monitor the prevalence of influenza viruses among wild birds to understand and prevent the emergence of new epidemics.

Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Figure legends

Fig 1. Location of Hanoi, Vinh Phuc, Nam Dihn, Ha Tay and Bac Ninh provinces (Shadow areas) in northern Vietnam where the sampling were performed between 2006 and 2009.

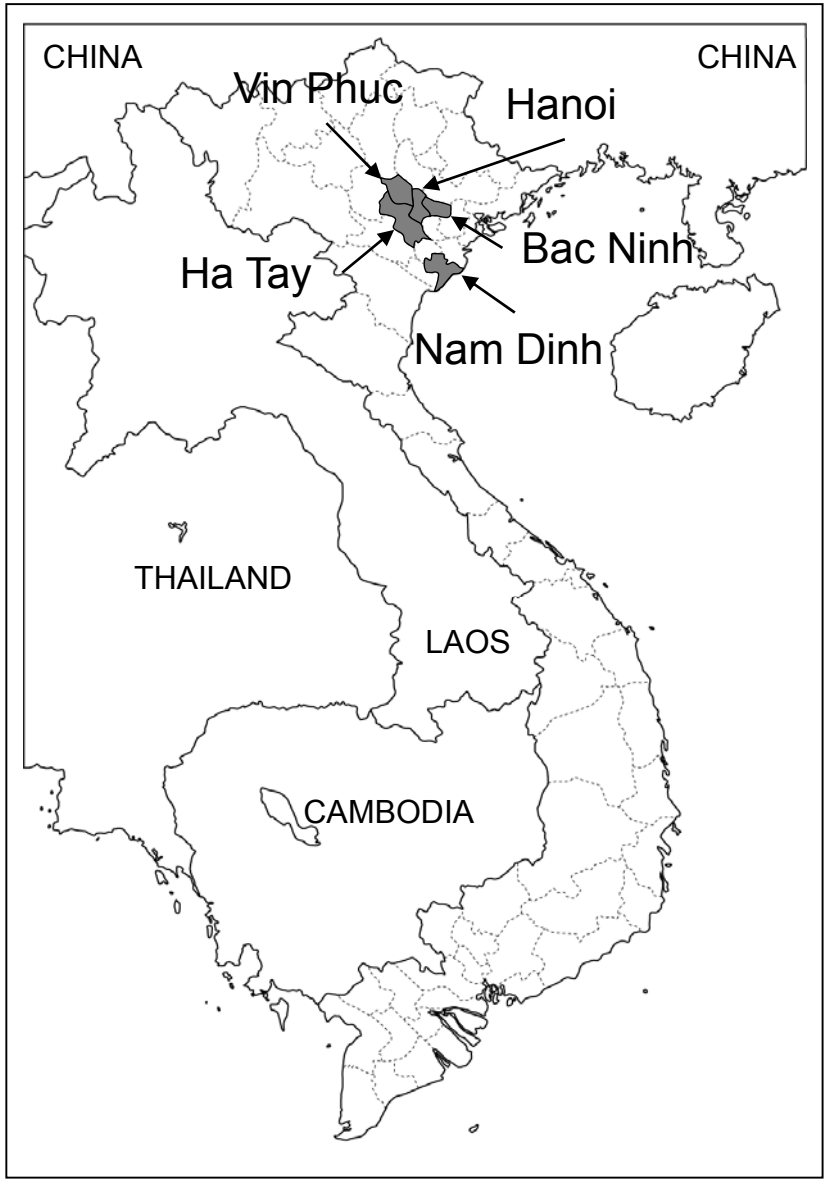
Fig. 2. Phylogenetic relationship of the surface glycoprotein genes of A/Eurasian Woodcock/Vietnam/8/2007 (H5N2) and selected reference isolates. Trees are shown for the HA and NA genes of the strain. Phylogenetic trees were rooted to A/Tern/South Africa/61 (H5N3) for H5, A/Yurkey/MO/24093/99 (H1N2) for N2, respectively. The coding sequences of the full genomes of all viruses were sequenced and analyzed phylogenetically. The viruses isolated from East Asia are indicated with a red bar. The highly pathogenic avian influenza (HPAI) viruses are indicated with a blue bar. Numbers above and below branch nodes indicate represent bootstrap values. Scale bar, 0.01 substitutions per site.

Fig. 3. Phylogenetic relationship of the eight gene segments of the H9N2 viruses isolated in northern Vietnam. Phylogenetic trees include all the five representative strains isolated from wild birds in northern Vietnam from 2006-2009 and selected reference isolates. Trees are shown for all 6 gene segments of each isolate as follows: HA, NA, PB2, PB1, PA, NP, M and NS. All phylogenetic trees are rooted to A/Turkey/Wisconsin/1/1966 (H9N2). The viruses isolated during this study are indicated with red underlining, except for A/Chinese Hwamei/Vietnam/38/2006 which is enclosed in a red rectangle. The coding sequences of the full genomes of all viruses were sequenced and analyzed phylogenetically. Blue lines are shown for each lineage as follows. Unknown: unknown avian host; Ck/Bei/94: the lineage of H9N2 viruses established in chickens since 1994; G1: the lineage of H9N2 viruses established in quail and the putative donor of the internal genes for HK156/97-like H5N1

viruses; H5N1/01: the new gene segments detected only in H5N1 viruses from Hong Kong in 2001. Numbers above and below branch nodes indicate represent bootstrap values. Scale bar, 0.01 substitutions per site.

Fig. 4. Map of Southeast and East Asia showing the predicted transmission pathways of the H5N2 and H9N2 viruses circulating between Vietnam and East Asia. The distribution area of the Eurasian Woodcock in Asia is shown in green(*"Eurasian Woodcock Scolopax rusticola*". *Datazone*. BirdLife International. http://www.birdlife.org/datazone/speciesfactsheet.php?id=2978).

Figure 1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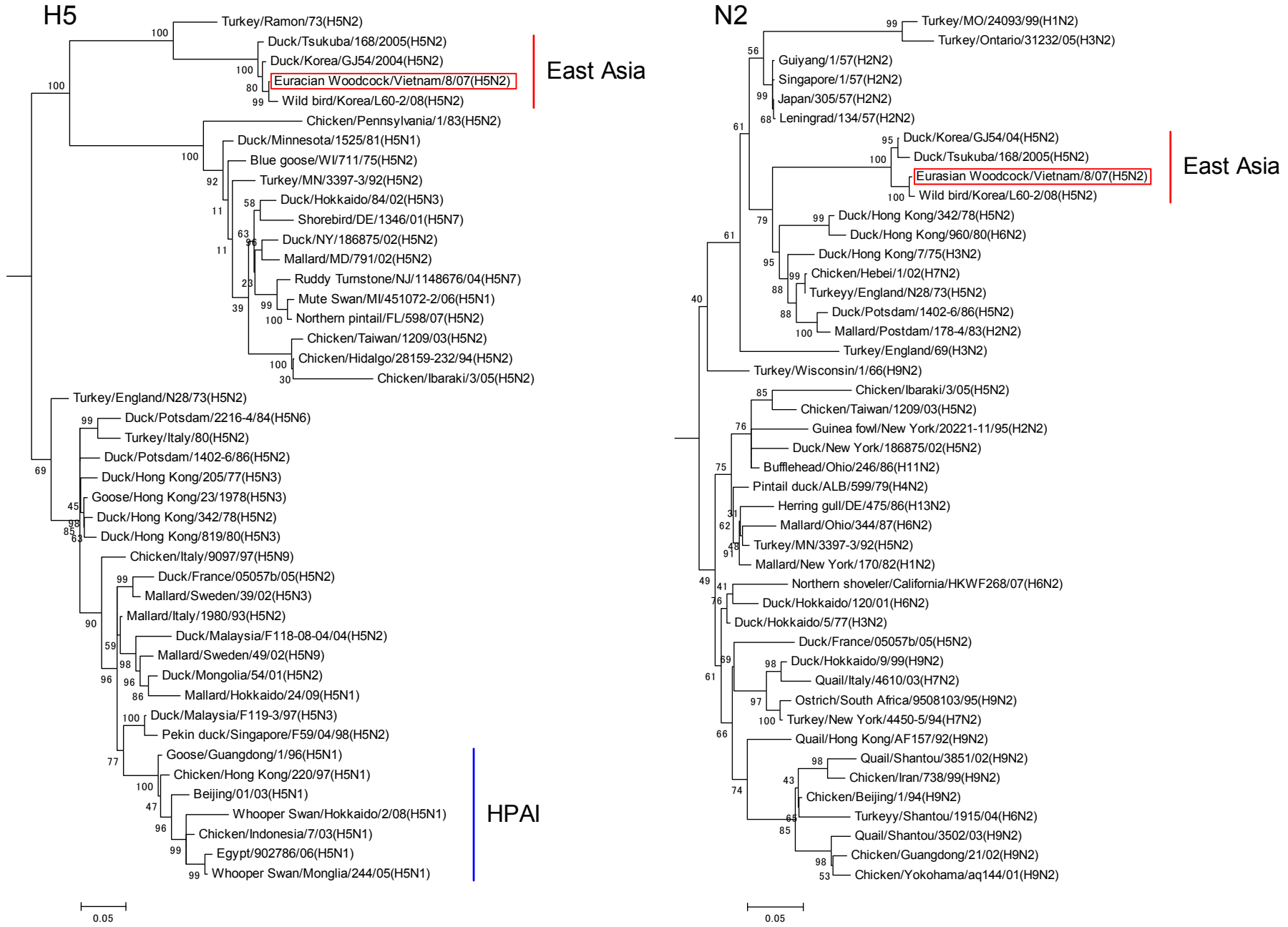
Hanoi
Bac Ninh
Ha Tay
Nam Dinh
THAILAND
LAOS
CAMBODIA

Figure 2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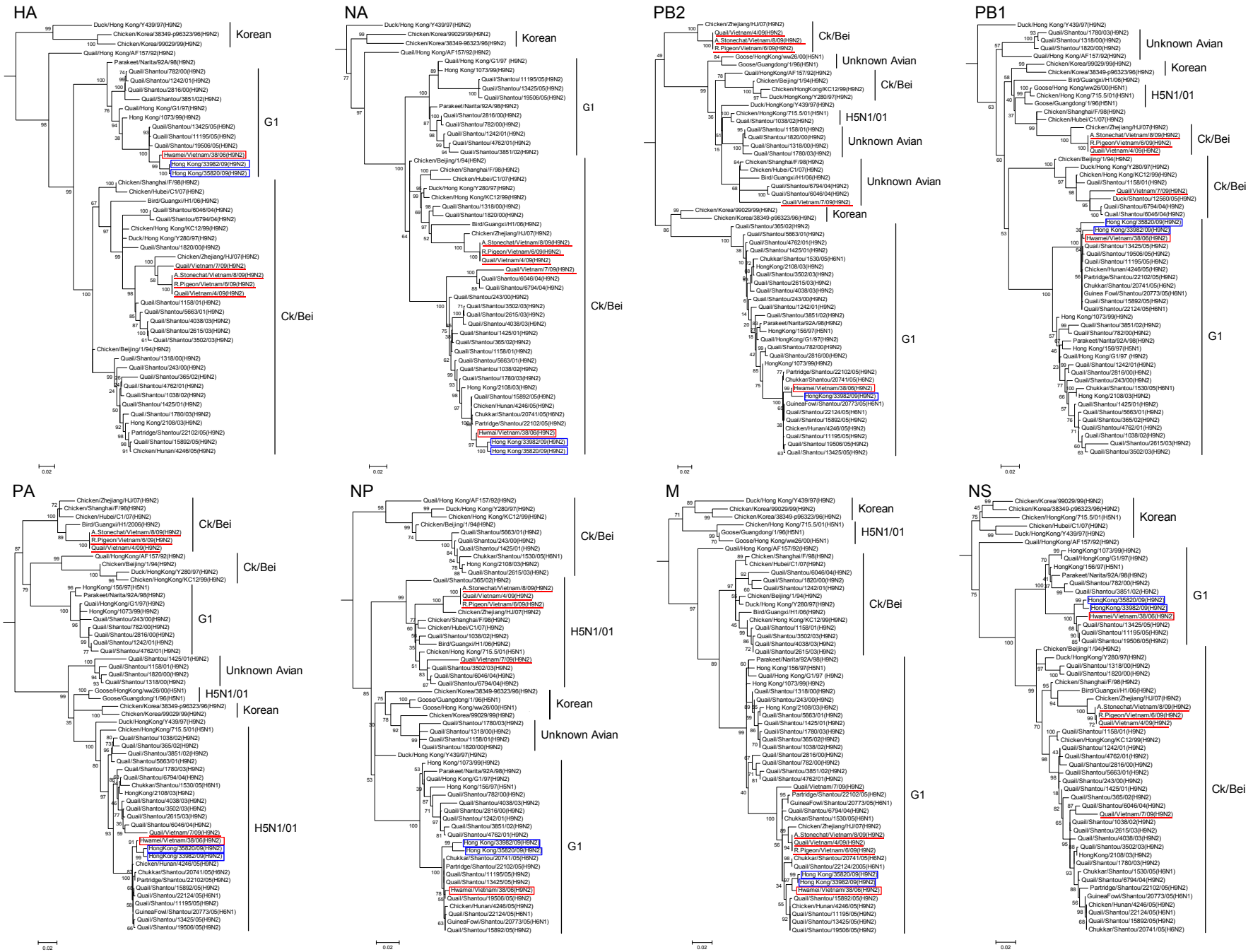
Duck/Tsukuba/168/2005(H5N2)
Duck/Korea/GJ54/2004(H5N2)
Euracian Woodcock/Vietnam/8/07(H5N2)
Wild bird/Korea/L60-2/08(H5N2)
East Asia
Chicken/Pennsylvania/1/83(H5N2)
Duck/Minnesota/1525/81(H5N1)
Blue goose/WI/711/75(H5N2)
Turkey/MN/3397-3/92(H5N2)
Duck/Hokkaido/84/02(H5N3)
Shorebird/DE/1346/01(H5N7)
Duck/NY/186875/02(H5N2)
Mallard/MD/791/02(H5N2)
Ruddy Turnstone/NJ/1148676/04(H5N7)
Mute Swan/MI/451072-2/06(H5N1)
Northern pintail/FL/598/07(H5N2)
Chicken/Taiwan/1209/03(H5N2)
Chicken/Hidalgo/28159-232/94(H5N2)
Chicken/Ibaraki/3/05(H5N2)
Turkey/England/N28/73(H5N2)
Duck/Potsdam/2216-4/84(H5N6)
Turkey/Italy/80(H5N2)
Duck/Potsdam/1402-6/86(H5N2)
Duck/Hong Kong/205/77(H5N3)
Goose/Hong Kong/23/1978(H5N3)
Duck/Hong Kong/342/78(H5N2)
Duck/Hong Kong/819/80(H5N3)
Chicken/Italy/9097/97(H5N9)
Duck/France/05057b/05(H5N2)
Mallard/Sweden/39/02(H5N3)
Mallard/Italy/1980/93(H5N2)
Duck/Malaysia/F118-08-04/04(H5N2)
Mallard/Sweden/49/02(H5N9)
Duck/Mongolia/54/01(H5N2)
Mallard/Hokkaido/24/09(H5N1)
Duck/Malaysia/F119-3/97(H5N3)
Pekin duck/Singapore/F59/04/98(H5N2)
Goose/Guangdong/1/96(H5N1)
Chicken/Hong Kong/220/97(H5N1)
Beijing/01/03(H5N1)
Whooper Swan/Hokkaido/2/08(H5N1)
Chicken/Indonesia/7/03(H5N1)
Egypt/902786/06(H5N1)
Whooper Swan/Monglia/244/05(H5N1)
HPAI
0.05
N2
Turkey/MO/24093/99(H1N2)
Turkey/Ontario/31232/05(H3N2)
Guiyang/1/57(H2N2)
Singapore/1/57(H2N2)
Japan/305/57(H2N2)
Leningrad/134/57(H2N2)
Duck/Korea/GJ54/04(H5N2)
Duck/Tsukuba/168/2005(H5N2)
Eurasian Woodcock/Vietnam/8/07(H5N2)
Wild bird/Korea/L60-2/08(H5N2)
East Asia
Duck/Hong Kong/342/78(H5N2)
Duck/Hong Kong/960/80(H6N2)
Duck/Hong Kong/7/75(H3N2)
Chicken/Hebei/1/02(H7N2)
Turkeyy/England/N28/73(H5N2)
Duck/Potsdam/1402-6/86(H5N2)
Mallard/Postdam/178-4/83(H2N2)
Turkey/England/69(H3N2)
Turkey/Wisconsin/1/66(H9N2)
Chicken/Ibaraki/3/05(H5N2)
Chicken/Taiwan/1209/03(H5N2)
Guinea fowl/New York/20221-11/95(H2N2)
Duck/New York/186875/02(H5N2)
Bufflehead/Ohio/246/86(H11N2)
Pintail duck/ALB/599/79(H4N2)
Herring gull/DE/475/86(H13N2)
Mallard/Ohio/344/87(H6N2)
Turkey/MN/3397-3/92(H5N2)
Mallard/New York/170/82(H1N1)
Northern shoveler/California/HKWF268/07(H6N2)
Duck/Hokkaido/120/01(H6N2)
Duck/Hokkaido/5/77(H3N2)
Duck/France/05057b/05(H5N2)
Duck/Hokkaido/9/99(H9N2)
Quail/Italy/4610/03(H7N2)
Ostrich/South Africa/9508103/95(H9N2)
Turkey/New York/4450-5/94(H7N2)
Quail/Hong Kong/AF157/92(H9N2)
Quail/Shantou/3851/02(H9N2)
Chicken/Iran/738/99(H9N2)
Chicken/Beijing/1/94(H9N2)
Turkeyy/Shantou/1915/04(H6N2)
Quail/Shantou/3502/03(H9N2)
Chicken/Guangdong/21/02(H9N2)
Chicken/Yokohama/aq144/01(H9N2)
0.05

Figure 3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G1
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NA
Korean
G1
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PB2
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Unknown Avian
Unknown Avian
Korean
G1
PB1
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Korean
H5N1/01
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G1
PA
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G1
Ck/Bei

Figure 4

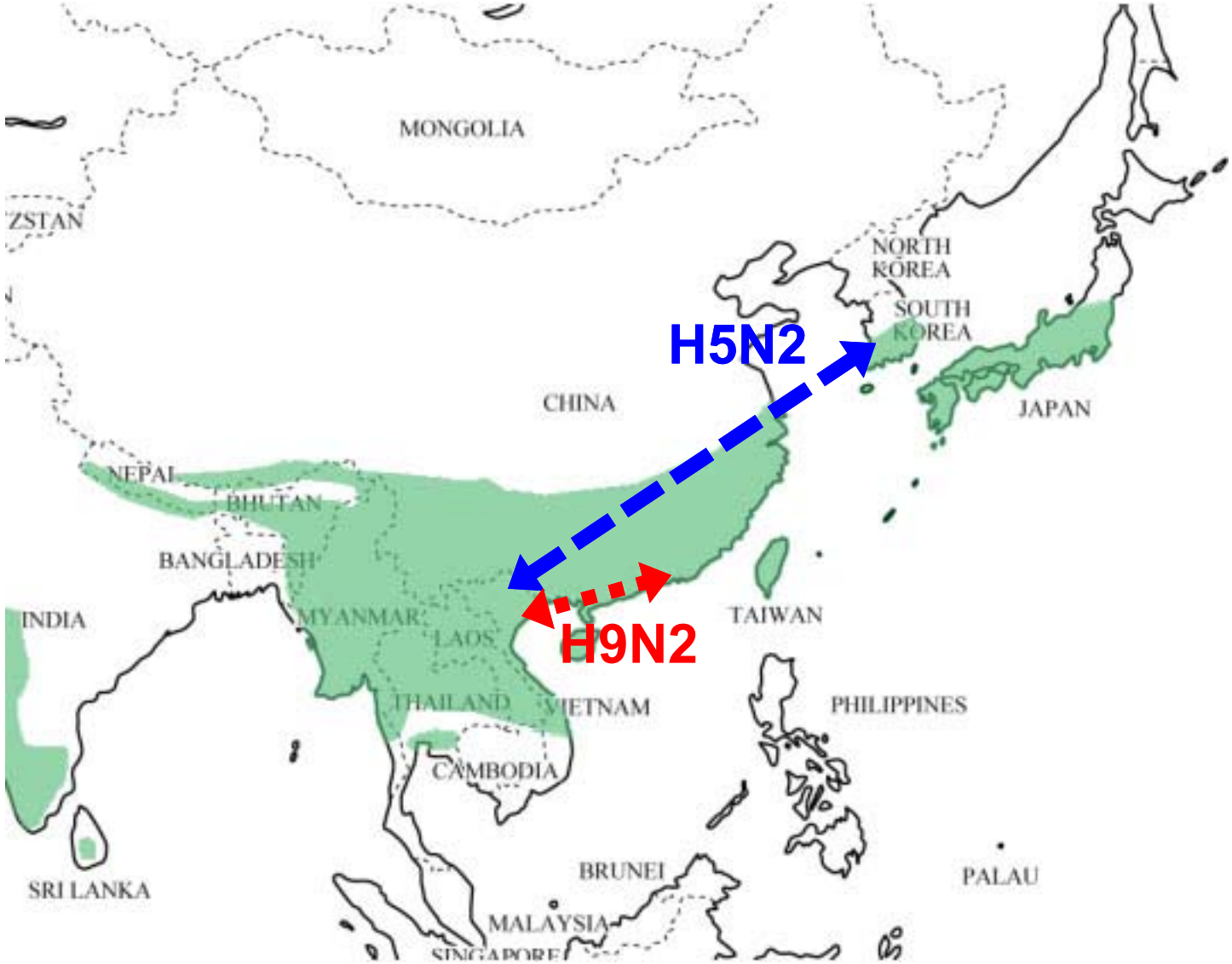
MONGOLIA
ZSTAN
NORTH KOREA
SOUTH KOREA
H5N2
CHINA
JAPAN
NEPAL
BHUTAN
BANGLADESH
INDIA
MYANMAR
LAOS
H9N2
TAIWAN
THAILAND
VIETNAM
PHILIPPINES
CAMBODIA
BRUNEI
PALAU
SRI LANKA
MALAYSIA
SINGAPORE

Table 1. Species of wild birds collected for virus isolation in this study

			Period	2006 November-December				2007 November	2008 October	2009 November		Total
			Site of wild bird collection	Hanoi	Ha Tay	Nam Dinh	Vin Phuc	Nam Dinh	Nam Dinh	Ha Tay	Bac Ninh	
Order	Family	*Genus*	Species									
Columbiformes	Columbidae	*Streptopelia*	Red Turtle Dove	23	4		20	35	25	19	8	134
			Oriental Turtle-dove	10			39	31	4			84
			Spotted Dove	40		27						67
		Columba	Rock Pigeon	8		10			10	10		38
Passeriformes	Passeridae	*Passer*	Eurasian Tree Sparrow	5			1	10	25			41
			Red-whiskered Bulbul	5		21	3					29
			Red-vented Bulbul		7							7
	Estrildidae	*Lonchura*	Scaly-breasted Munia		4	10	10					24
			White-rumped Munia	5								5
	Muscicapidae	*Saxicola*	African Stonechat							15		15
		Copsychus	White-rumped Shama				4					4
	Zosteropidae	*Zosterops*	Japanese White-eye		2				5			7
	Timaliidae	*Garrulax*	Chinese Hwamei	5								5
			Black-throated Laughingthrush		3							3
		Leiothrix	Red-billed Leiothrix	5								5
Gruiformes	Rallidae	*Gallinula*	Common Moorhen	5		10	5	12	25	14	5	76
		Amaurornis	White-breasted Waterhen	5								5
Galliformes	Phasianidae	*Coturnix*	Japanese Quail							35		35
	Numididae		Guineafowl				1		2			3
Charadriiformes	Scolopacidae	*Scolopax*	Eurasian Woodcock	2				25				27
		Tringa	Tringa				8					8
Ciconiiformes	Ardeidae	*Egretta*	Little Egret			5	12					17
		Bubulcus	Cattle Egret				1		4			5
Anseriformes	Anatidae	*Anas*	Garganey				3	1				4
			Common Teal								2	2
Podicipediformes	Podicipedidae	*Tachybaptus*	Little Grebe							1		1
			Total	118	20	83	107	114	100	94	15	651

Number of wild birds collected in the study was shown in the table. Throat and cloacal secretion specimens were taken from each of wild birds and inoculated into embryonated eggs for virus isolation.

Table 2. Avian influenza viruses isolated from wild birds in this study

Year	Place	Subtype	Number of isolates	Representative strain	Accession number
2006	Hanoi	H9N2	1	A/Chinese Hwamei/Vietnam/38/2006	AB753174- AB753181
2007	Nam Dinh	H5N2	1	A/Eurasian Woodcock/Vietnam/8/2007	AB753182- AB753189
2009	Ha Tay	H9N2	8*[1]	A/Japanese quail/Vietnam/4/2009	AB753190- AB753197
2009	Ha Tay	H9N2	1	A/Rock Pigeon/Vietnam/6/2009	AB753198- AB753205
2009	Ha Tay	H9N2	6*[2]	A/Japanese quail/Vietnam/7/2009	AB753206- AB753213
2009	Ha Tay	H9N2	1	A/African Stonechat/Vietnam/8/2009	AB753214- AB753220

*[1]:The HA gene of 8 H9N2 isolates was almost identical with strains A/Rock Pigeon/Vietnam/6/2009 (H9N2) and A/African Stonechat/Vietnam/8/2009 (H9N2)

*[2]:The HA genes of these 6 strains were almost (98%) identical to each other.

Table 3. Alignment of amino acids compositions associated with host preference

Protein		HA				PB2	NP	PB1-F2
Amino acid position		157	224	226	318	64	293	82
Host*[1]	Avian	K	N	Q	T	T	R	L
	Human	E	K	L	I	M	K	S
A/Eurasian Woodcock/Vietnam/8/2007		-*[2]	-	-	-	M	K	S

*[1]: Host-associated amino acids in predicted virus internal gene products (Finkelstein et al., 2007; Shaw et al., 2002; Chen et al., 2006).

*[2]: - indicates the same amino acid residue used in the corresponding positions of strains of Avian origin.

Table 4. Genotyping of H9N2 influenza virus

Strains	Lineage of gene segment							
	PB2	PB1	PA	HA	NP	NA	M	NS
A/Japanese quail/Vietnam/7/2009	**Unknown**	Ck/Bei/94	Ck/Bei/94	Ck/Bei/94	H5N1/01	Ck/Bei/94	G1	Ck/Bei/94
A/quail/Shantou/6794/2004	**Unknown**	Ck/Bei/94	**H5N1/01**	Ck/Bei/94	H5N1/01	Ck/Bei/94	G1	Ck/Bei/94
A/Japanese quail/Vietnam/4/2009	Ck/Bei/94	Ck/Bei/94	**H5N1/01**	Ck/Bei/94	H5N1/01	Ck/Bei/94	G1	Ck/Bei/94
A/Rock Pigeon/Vietnam/6/2009	Ck/Bei/94	Ck/Bei/94	**H5N1/01**	Ck/Bei/94	H5N1/01	Ck/Bei/94	G1	Ck/Bei/94
A/African Stonechat/Vietnam/8/2009	Ck/Bei/94	Ck/Bei/94	**H5N1/01**	Ck/Bei/94	H5N1/01	Ck/Bei/94	G1	Ck/Bei/94
A/Chinese Hwamei/Vietnam/38/2006	G1	G1	H5N1/01	G1	G1	**Ck/Bei/94**	G1	G1
A/Hong Kong/33982/2009	G1	G1	H5N1/01	G1	G1	**Ck/Bei/94**	G1	G1

Genotypes were determined by phylogenetic relationships. The upper part shows the genotype comparison between four H9N2 isolates and the closest avian isolate from the Influenza Virus Resource of GenBank (Accession number: A/quail/Shantou/6794/2004, EF154892, EF154965, EF155038, EF155111, EF155184, EF155257, EF155330, EF155403). The lower part of the table shows the genotype comparison between A/Chinese Hwamei/Vietnam/38/2006 and the closest strain from the GenBank (Accession number: A/Hong Kong/33982/2009, CY055137-CY055144). Strains in bold type indicate viruses characterized in this study. Bold type shows important lineages for comparison. Unknown: unknown avian host; Ck/Bei/94: the lineage of H9N2 viruses established in chickens since 1994; G1: the lineage of H9N2 viruses established in quail and the putative donor of the internal genes for HK156/97-like H5N1 viruses; H5N1/01: the new gene segments detected only in H5N1 viruses from Hong Kong in 2001.

Table 5. Comparison of critical amino acid residues in HA and NA proteins among the H9N2 viruses

Virus	HA						NA		
	Receptor Binding Site					Cleavage Site	Hemadsorbing Site		
	191	198	234	235	236	335-338	366-373	399-404	431-433
A/Hong Kong/33928/2009	H	D	Q	Q	G	RSNR	IEKDSRSG	DRDNSS	PKE
A/Hong Kong/35820/2009	H	D	Q	Q	G	RSNR	IEKDSRSG	DRDNSS	PKE
A/Chinese Hawmai/Vietnam/38/2006	H	D	Q	Q	G	RS**S**R	IEKDSRSG	DRDNSS	PKE
A/Japanese quail/Vietnam/4/2009	**N**	**V**	**L**	Q	G	RS**S**R	IEKDSRSG	D**S**DNSS	PKE
A/Japanese quail/Vietnam/7/2009	**N**	**V**	**L**	Q	G	RS**S**R	IEKDSRSG	D**S**DNSS	PKE
A/Rock Pigeon/Vietnam/6/2009	**N**	**V**	**L**	Q	G	RS**S**R	I**KSG**SRSG	D**S**D**SG**S	P**Q**E
A/African Stonechat/Vietnam/8/2009	**N**	**V**	**L**	Q	G	RS**S**R	I**KSG**SRSG	D**S**D**SG**S	P**Q**E

Bold letters indicate different amino acid residues from those of the human H9N2 isolates.

Table 6. Human-associated amino acids identified in viral proteins of A/Chinese Hwamei/Vietnam/38/2006 (H9N2)

Protein		PB2	NP	PB1-F2	PA
Amino acid position		661	136	73	57
Host*[1]	Avian	A	L	K	R
	Human	T	M	R	Q
A/Hong Kong/33982/2009		T	M	K	Q
A/Hong Kong/35820/2009		nd*[2]	M	R	Q
A/Chinese Hwamei/Vietnam/38/2006		T	M	R	Q
A/Japanese quail/Vietnam/4/2009		-*[3]	-	-	-
A/Rock Pigeon/Vietnam/6/2009		-	-	-	-
A/Japanese quail/Vietnam/7/2009		-	-	-	-
A/African Stonechat/Vietnam/8/2009		-	-	-	-

*[1]: Host-associated amino acids in predicted virus gene products (Finkelstein et al., 2007; Shaw et al., 2002; Chen et al., 2006).

*[2]: Sequence data is not available.

*[3]: - indicates the same amino acid residue with the avian-associated amino acid of each protein.