

Genetic analysis of novel avian A(H7N9) influenza viruses isolated from patients in China, February to April 2013

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Citation style for this article:

Kageyama T, Fujisaki S, Takashita E, Xu H, Yamada S, Uchida Y, Neumann G, Saito T, Kawaoka Y, Tashiro M. Genetic analysis of novel avian A(H7N9) influenza viruses isolated from patients in China, February to April 2013. *Euro Surveill.* 2013;18(15):pii=20453. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20453>

Article submitted on 08 April 2013 / published on 12 April 2013

Novel influenza viruses of the H7N9 subtype have infected 33 and killed nine people in China as of 10 April 2013. Their haemagglutinin (HA) and neuraminidase genes probably originated from Eurasian avian influenza viruses; the remaining genes are closely related to avian H9N2 influenza viruses. Several characteristic amino acid changes in HA and the PB2 RNA polymerase subunit probably facilitate binding to human-type receptors and efficient replication in mammals, respectively, highlighting the pandemic potential of the novel viruses.

Humans are rarely infected with avian influenza viruses, with the exception of highly pathogenic avian influenza A(H5N1) viruses, which have caused 634 infections and 371 deaths as of 12 March 2013 [1]. A few isolated cases of human infection with viruses of the H7N2, H7N3, and H7N5 subtypes have been reported, but none were fatal [2-11]. In 2003, in the Netherlands, 89 people were infected with an influenza virus of the H7N7 subtype that caused conjunctivitis and one fatality [5,7].

On 19 February 2013, an 87 year-old man in Shanghai developed a respiratory infection and died on 4 March, and on 27 February 2013, a 27 year-old pork seller in a Shanghai market became ill and died on 10 March. A 35 year-old woman in Chuzhou City in Anhui province (west of Shanghai), who had contact with poultry, became ill on 15 March 2013, and remains hospitalised in critical condition. There is no known epidemiological relationship among these three cases. A 38 year-old man in Hangzhou (Zhejiang province, south of

Shanghai) became ill on 7 March 2013 and died on 27 March. All four cases presented with respiratory infections that progressed to severe pneumonia and breathing difficulties.

On 31 March 2013, the Chinese Centre for Disease Control and Prevention announced the isolation in embryonated eggs of avian influenza viruses of the H7N9 subtype (designated A/Shanghai/1/2013, A/Shanghai/2/2013, and A/Anhui/1/2013) from the first three cases. The sequences of the coding regions of all eight viral genes were deposited in the influenza sequence database of the Global Initiative on Sharing All Influenza Data (GISAID) on 31 March (Table 1). On 5 April 2013, the Hangzhou Center for Disease Control and Prevention deposited the haemagglutinin (HA), neuraminidase (NA), and matrix (M) gene sequences of A/Hongzhou/1/2013 virus (Table 1), which was isolated in cell culture from samples obtained from the 38 year-old man.

All four human influenza A(H7N9) viruses are similar at the nucleotide and amino acid levels, suggesting a common ancestor. The HA gene of the novel viruses belongs to the Eurasian lineage of avian influenza viruses and shares ca. 95% identity with the HA genes of low pathogenic avian influenza A(H7N3) viruses isolated in 2011 in Zhejiang province (south of Shanghai) (Figure 1, Table 2). The NA gene of the novel viruses is ca. 96% identical to the low pathogenic avian influenza A(H11N9) viruses isolated in 2010 in the Czech Republic (Figure 1, Table 2).

TABLE 1

Origin of influenza A(H7N9) isolates included in the phylogenetic analysis, China, February–April 2013 (n=7)

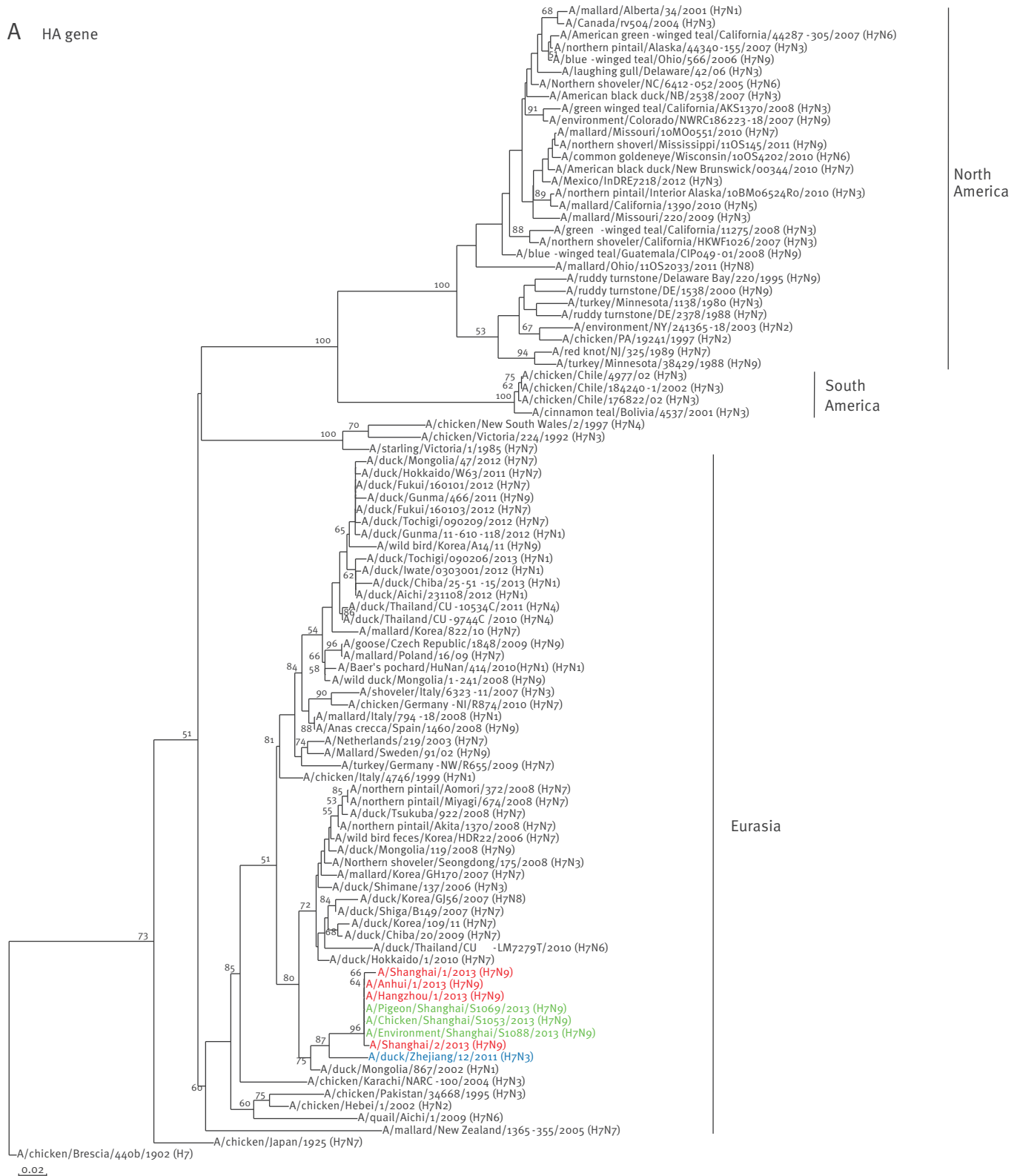
| Segment ID | Segment | Isolate name | Collection date | Originating Laboratory | Submitting Laboratory | Submitter/ Authors |
|------------|---------|-------------------------------------|-----------------|--|--|--|
| EPI439488 | PB2 | A/Shanghai/1/2013 | 2013 | - | WHO Chinese National Influenza Center | Lei Yang |
| EPI439489 | PB1 | | | | | |
| EPI439490 | PA | | | | | |
| EPI439486 | HA | | | | | |
| EPI439491 | NP | | | | | |
| EPI439487 | NA | | | | | |
| EPI439493 | M | | | | | |
| EPI439494 | NS | | | | | |
| EPI439495 | PB2 | A/Shanghai/2/2013 | 2013 | - | WHO Chinese National Influenza Center | Lei Yang |
| EPI439501 | PB1 | | | | | |
| EPI439498 | PA | | | | | |
| EPI439502 | HA | | | | | |
| EPI439496 | NP | | | | | |
| EPI439500 | NA | | | | | |
| EPI439497 | M | | | | | |
| EPI439499 | NS | | | | | |
| EPI439504 | PB2 | A/Anhui/1/2013 | 2013 | - | WHO Chinese National Influenza Center | Lei Yang |
| EPI439508 | PB1 | | | | | |
| EPI439503 | PA | | | | | |
| EPI439507 | HA | | | | | |
| EPI439505 | NP | | | | | |
| EPI439509 | NA | | | | | |
| EPI439506 | M | | | | | |
| EPI439510 | NS | | | | | |
| EPI440095 | HA | A/Hangzhou/1/2013 | 2013-03-24 | Hangzhou Center for Disease Control and Prevention | Hangzhou Center for Disease Control and Prevention | Li,J; Pan,JC; Pu,XY; Yu,XF; Kou,Y; Zhou,YY |
| EPI440096 | NA | | | | | |
| EPI440097 | M | | | | | |
| EPI440682 | PB2 | A/Chicken/Shanghai /S1053/2013 | 2013-04-03 | Harbin Veterinary Research Institute | Harbin Veterinary Research Institute | Huihui Kong |
| EPI440683 | PB1 | | | | | |
| EPI440681 | PA | | | | | |
| EPI440685 | HA | | | | | |
| EPI440678 | NP | | | | | |
| EPI440684 | NA | | | | | |
| EPI440680 | M | | | | | |
| EPI440679 | NS | | | | | |
| EPI440690 | PB2 | A/Environment/ Shanghai /S1088/2013 | 2013-04-03 | Harbin Veterinary Research Institute | Harbin Veterinary Research Institute | Huihui Kong |
| EPI440691 | PB1 | | | | | |
| EPI440689 | PA | | | | | |
| EPI440693 | HA | | | | | |
| EPI440686 | NP | | | | | |
| EPI440692 | NA | | | | | |
| EPI440688 | M | | | | | |
| EPI440687 | NS | | | | | |
| EPI440698 | PB2 | A/Pigeon/Shanghai /S1069/2013 | 2013-04-02 | Harbin Veterinary Research Institute | Harbin Veterinary Research Institute | Huihui Kong |
| EPI440699 | PB1 | | | | | |
| EPI440697 | PA | | | | | |
| EPI440701 | HA | | | | | |
| EPI440694 | NP | | | | | |
| EPI440700 | NA | | | | | |
| EPI440696 | M | | | | | |
| EPI440695 | NS | | | | | |

We gratefully acknowledge the authors and laboratories for originating and submitting these sequences to the EpiFlu database of the Global Initiative on Sharing All Influenza Data (GISAID); these sequences were the basis for the research presented here.

All submitters of data may be contacted directly via the GISAID website www.gisaid.org

FIGURE 1

Phylogenetic analysis of the haemagglutinin (A) and neuraminidase (B) genes of the novel influenza A(H7N9) viruses, China, February–April 2013 (n=7)



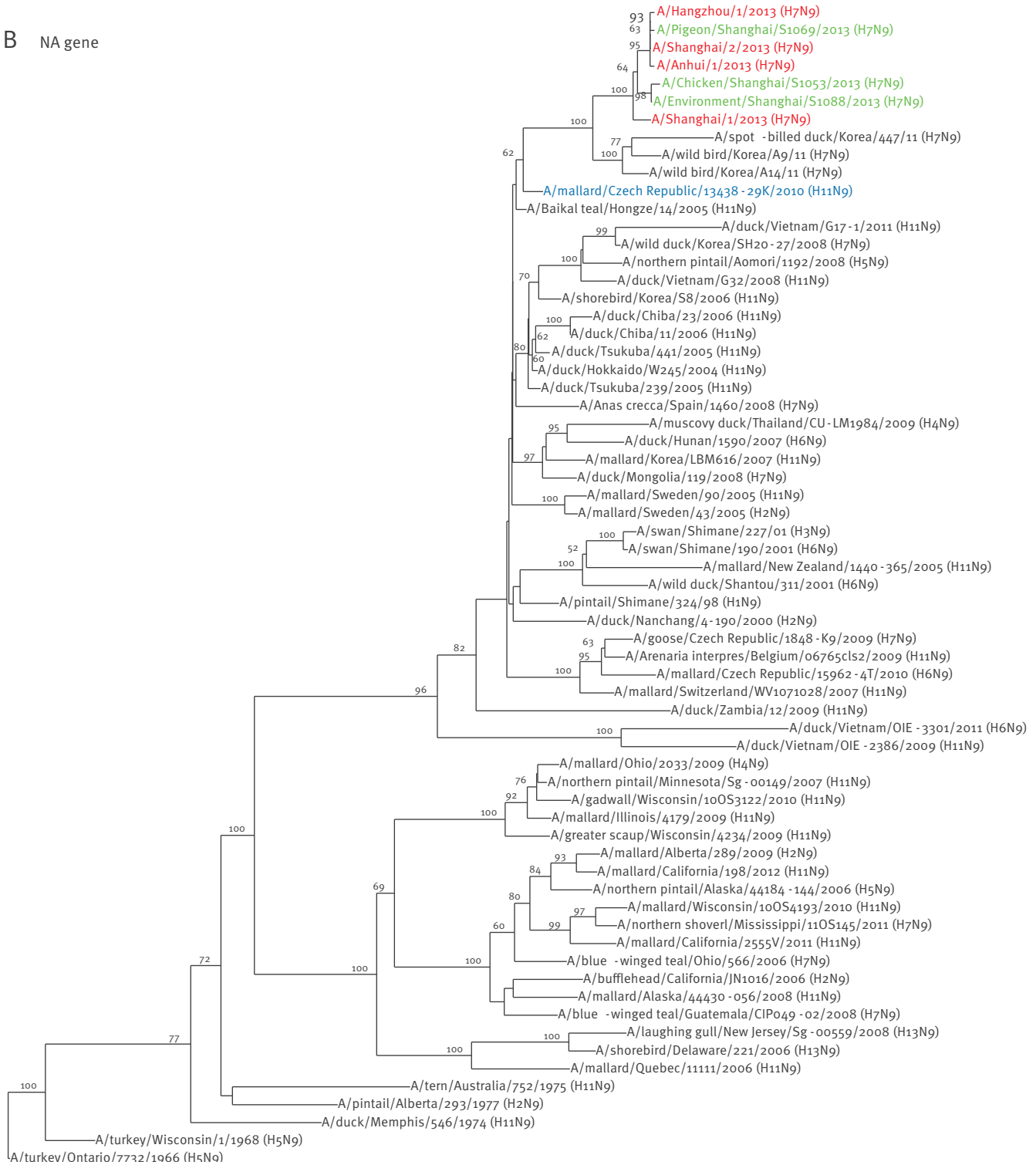
Multiple alignments were constructed by using the CLUSTAL W algorithm. Genetic distances were calculated by using the Kimura's 2-parameter method [26], and phylogenetic trees were constructed by using the neighbour-joining method with bootstrap analyses of 1,000 replicates in CLUSTAL W. Numbers next to nodes indicate bootstrap value percentages (>50%).

Novel human H7N9 viruses are shown in red; novel H7N9 viruses from birds and the environment are shown in green; viruses with the highest similarities to the novel viruses are shown in blue. The HA clade names, North America, South America, and Eurasia, are based on epidemiological studies of H7 viruses [27,28].

FIGURE 1

Phylogenetic analysis of the haemagglutinin (A) and neuraminidase (B) genes of the novel influenza A(H7N9) viruses, China, February–April 2013 (n=7)

B NA gene



HA: haemagglutinin; NA: neuraminidase.

Multiple alignments were constructed by using the CLUSTAL W algorithm. Genetic distances were calculated by using the Kimura's 2-parameter method [26], and phylogenetic trees were constructed by using the neighbour-joining method with bootstrap analyses of 1,000 replicates in CLUSTAL W. Numbers next to nodes indicate bootstrap value percentages (>50%).

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TABLE 2

Nucleotide identity of novel influenza A(H7N9) virus genes and their closest relative, China, February–April 2013

| Viral gene | Closest influenza virus relative | Nucleotide identity (%) |
|------------|--|-------------------------|
| PB2 | A/brambling/Beijing/16/2012(H9N2) | 99 |
| PB1 | A/chicken/Jiangsu/Q3/2010(H9N2) | 98 |
| PA | A/brambling/Beijing/16/2012(H9N2) | 99 |
| HA | A/duck/Zhejiang/12/2011(H7N3) | 95 |
| NP | A/chicken/Zhejiang/611/2011(H9N2) | 98 |
| NA | A/mallard/Czech Republic/13438-29K/2010(H11N9) | 96 |
| M | A/chicken/Zhejiang/607/2011(H9N2) | 98 |
| NS | A/chicken/Dawang/1/2011(H9N2) | 99 |

HA: haemagglutinin; M: matrix gene; NA: neuraminidase; NP: nucleoprotein; NS: non-structural gene; PA: RNA polymerase acidic subunit; PB1: RNA polymerase basic subunit 1; PB2: RNA polymerase basic subunit 2.

The sequences of the remaining viral genes are closely related (>97% identity) to avian influenza A(H9N2) viruses, which recently circulated in poultry in Shanghai, Zhejiang, Jiangsu, and neighbouring provinces of Shanghai (Table 2, Figure 2). These findings strongly suggest that the novel influenza A(H7N9) viruses are reassortants that acquired their H7 HA and N9 NA genes from avian influenza viruses, and their remaining genes from recent influenza A(H9N2) poultry viruses (Figure 1, Figure 3, Table 2).

At the nucleotide level, A/Shanghai/2/2013, A/Anhui/1/2013, and A/Hangzhou/1/2013 share more than 99% identity and differ by no more than three nucleotides per gene, even though they were isolated in different cities several hundred kilometres apart. On 7 April 2013, the Harbin Veterinary Research Institute deposited the full genome sequences of isolates from a pigeon (A/pigeon/Shanghai/S1069/2013), a chicken (A/chicken/Shanghai/S1053/2013), and an environmental sample (A/environment/Shanghai/S1088/2013) that were collected on 2 and 3 April from a Shanghai market (Table 1). All eight genes of these three isolates are similar to those of A/Shanghai/2/2013 and A/Anhui/1/2013 at the nucleotide level, except for the PB1 gene of A/pigeon/Shanghai/S1069/2013, which belongs to a different lineage than the PB1 of the other H7N9 isolates (Figures 1 and 2).

Interestingly, A/Shanghai/1/2013 and A/Shanghai/2/2013 differ by 52 nucleotides (for example, there are 13 nucleotide and nine amino acid differences in their HA sequences) even though these two cases were identified in the same city and at around the same time. These findings suggest that A/Shanghai/2/2013, A/Anhui/1/2013, A/Hangzhou/1/2013, as well as the viruses from the chicken and the environment, share a closely related source of infection, whereas A/Shanghai/1/2013 and A/pigeon/Shanghai/S1069/2013 are likely to have originated from other sources.

Highly pathogenic avian influenza viruses are characterised by a series of basic amino acids at the HA cleavage site that enable systemic virus spread. The HA cleavage sequence of the novel influenza A(H7N9) viruses possesses a single basic amino acid (EIPKGR*GL; *indicates the cleavage site), suggesting that these viruses are of low pathogenicity in avian species.

The amino acid sequence of the receptor-binding site (RBS) of HA determines preference for human- or avian-type receptors. At this site, A/Shanghai/1/2013 encodes an A138S* mutation (H3 numbering; Figure 4, Table 3), whereas A/Shanghai/2/2013, A/Anhui/1/2013, the two avian isolates, and the virus from the environmental sample encode G186V and Q226L mutations; any of these three mutations could increase the binding of avian H5 and H7 viruses to human-type receptors [12–14]. The finding of mammalian-adapting mutations in the RBS of these novel viruses is cause for concern. The A/Hangzhou/1/2013 isolate encodes isoleucine at position 226, which is found in seasonal influenza A(H3N2) viruses.

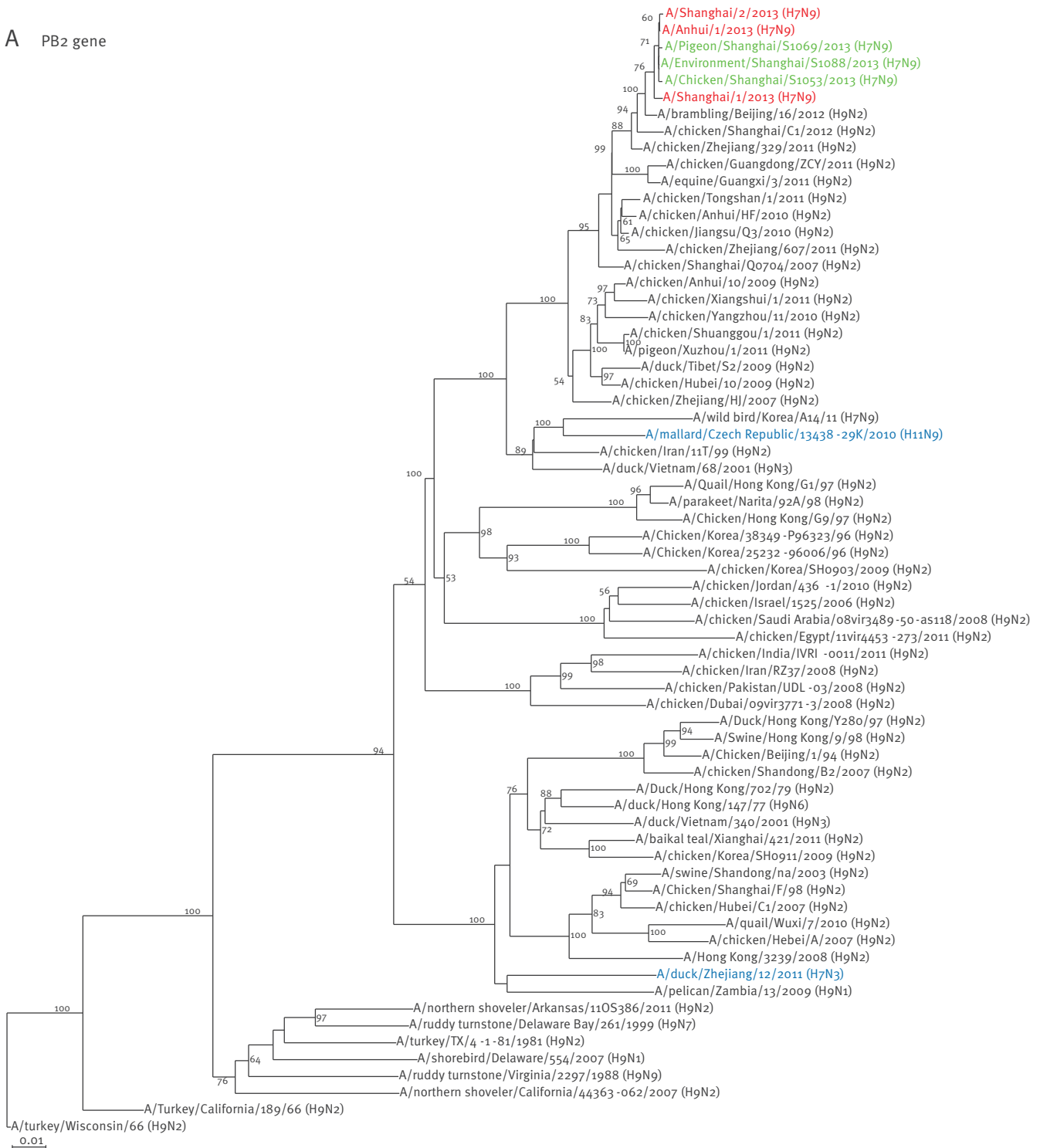
In addition, all seven influenza A(H7N9) viruses possess a T160A substitution (H3 numbering; Table 3) in HA, which is found in recently circulating H7 viruses; this mutation leads to the loss of an *N*-glycosylation site at position 158 (H3 numbering; position 149 in H7 numbering), which results in increased virus binding to human-type receptors [15].

Lysine at position 627 of the polymerase PB2 protein is essential for the efficient replication of avian influenza viruses in mammals [16] and has been detected in highly pathogenic avian influenza A(H5N1) viruses and in the influenza A(H7N7) virus isolated from the fatal case in the Netherlands in 2003 [17]. PB2-627K is rare among avian H9N2 PB2 proteins (i.e. it has been found in only five of 827 isolates). In keeping with this finding, the avian and environmental influenza A(H7N9)

FIGURE 2

Phylogenetic analysis of the six remaining genes of the novel influenza A(H7N9) viruses, China, February–April 2013 (n=7)

A PB2 gene



PB2: RNA polymerase basic subunit 2.

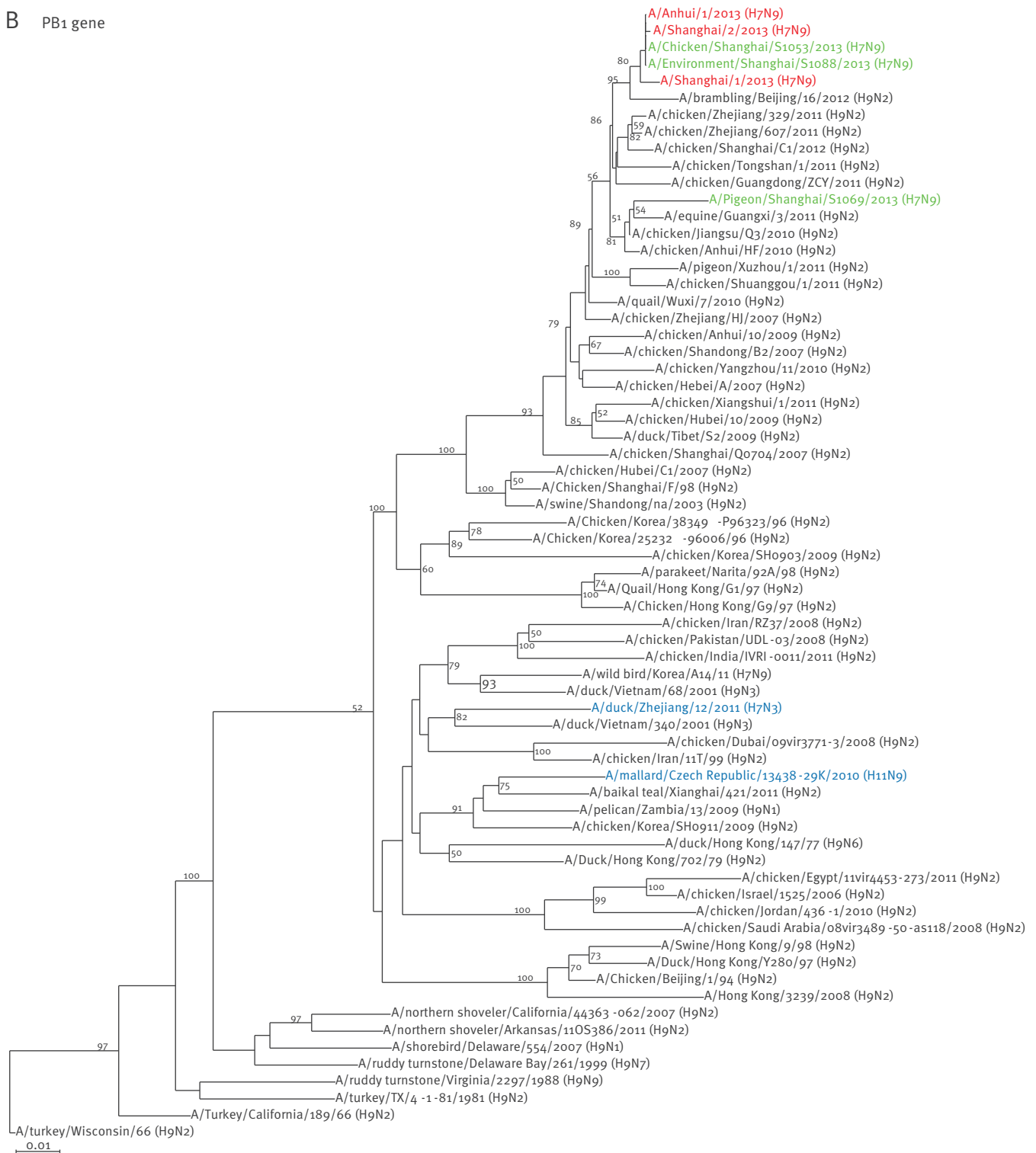
Multiple alignments were constructed by using the CLUSTAL W algorithm. Genetic distances were calculated by using the Kimura's 2-parameter method [26], and phylogenetic trees were constructed by using the neighbour-joining method with bootstrap analyses of 1,000 replicates in CLUSTAL W. Numbers next to nodes indicate bootstrap value percentages (>50%).

The novel human H7N9 viruses are shown in red; novel H7N9 viruses from birds and the environment are shown in green. Influenza viruses whose HA and NA genes are most closely related to the novel human H7N9 viruses are shown in blue.

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B PB1 gene



PB1: RNA polymerase basic subunit 1.

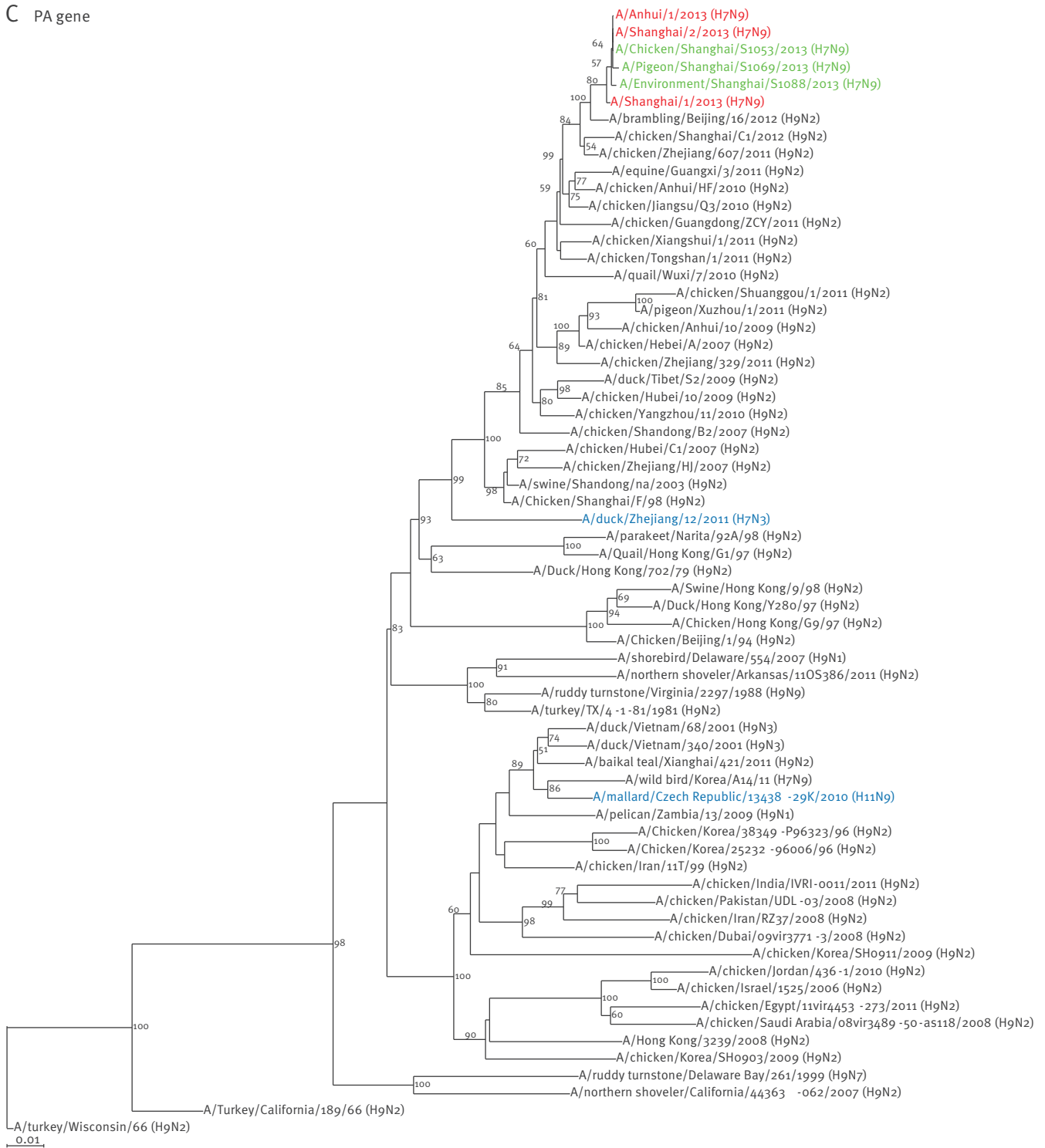
Multiple alignments were constructed by using the CLUSTAL W algorithm. Genetic distances were calculated by using the Kimura's 2-parameter method [26], and phylogenetic trees were constructed by using the neighbour-joining method with bootstrap analyses of 1,000 replicates in CLUSTAL W. Numbers next to nodes indicate bootstrap value percentages (>50%).

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C PA gene



PA: RNA polymerase acidic subunit.

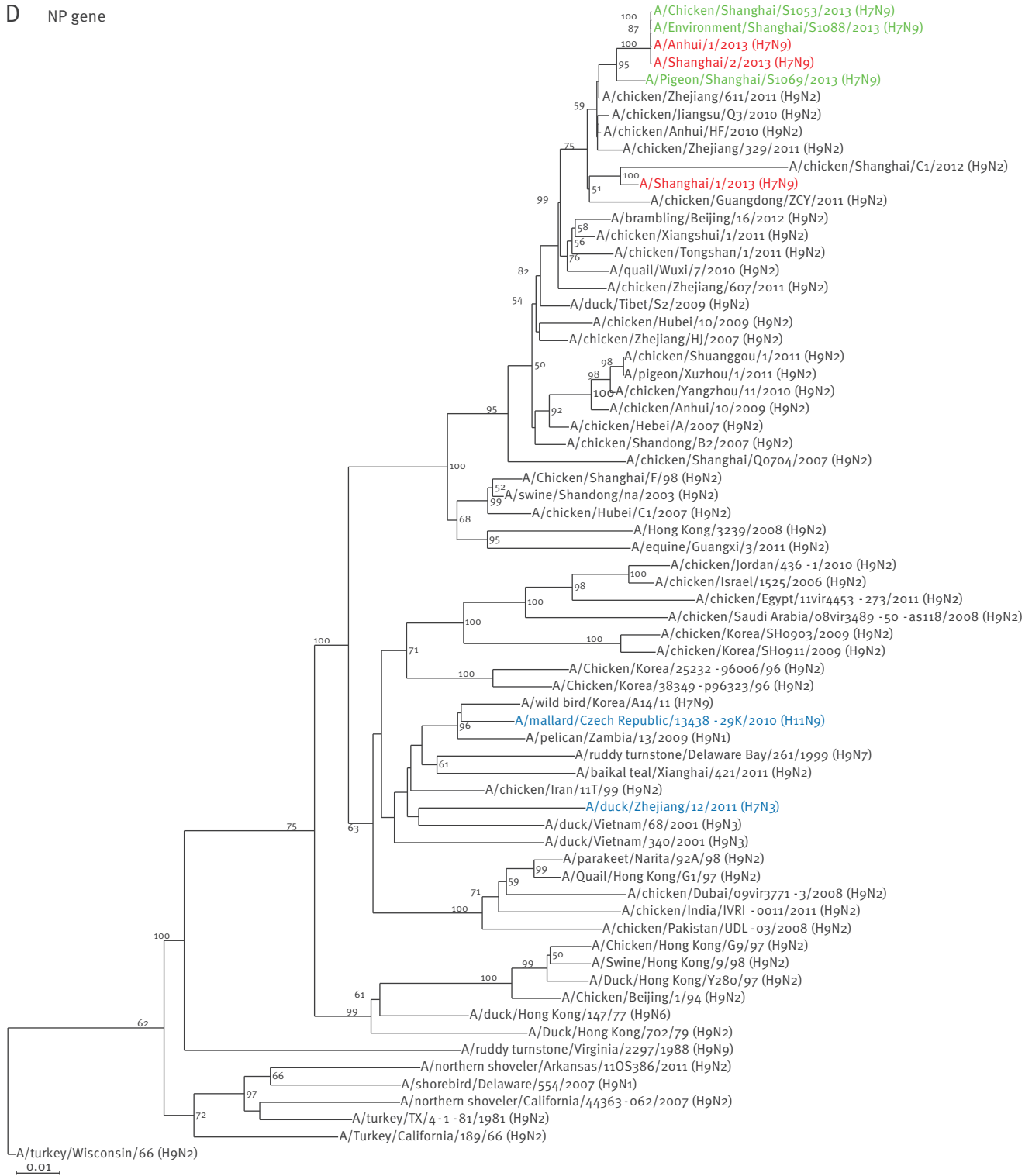
Multiple alignments were constructed by using the CLUSTAL W algorithm. Genetic distances were calculated by using the Kimura's 2-parameter method [26], and phylogenetic trees were constructed by using the neighbour-joining method with bootstrap analyses of 1,000 replicates in CLUSTAL W. Numbers next to nodes indicate bootstrap value percentages (>50%).

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D NP gene



NP: nucleoprotein.

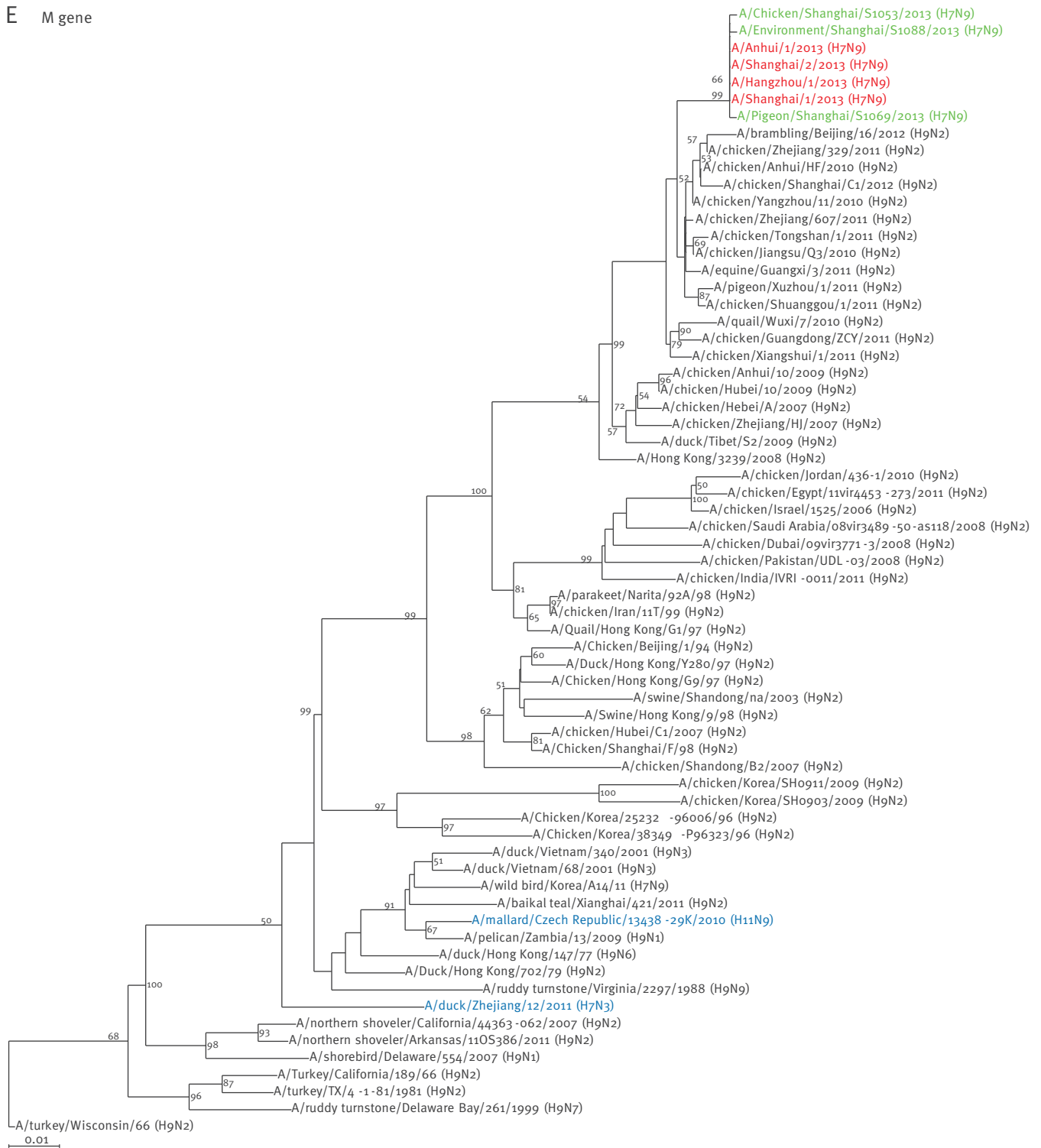
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E M gene



M: matrix gene.

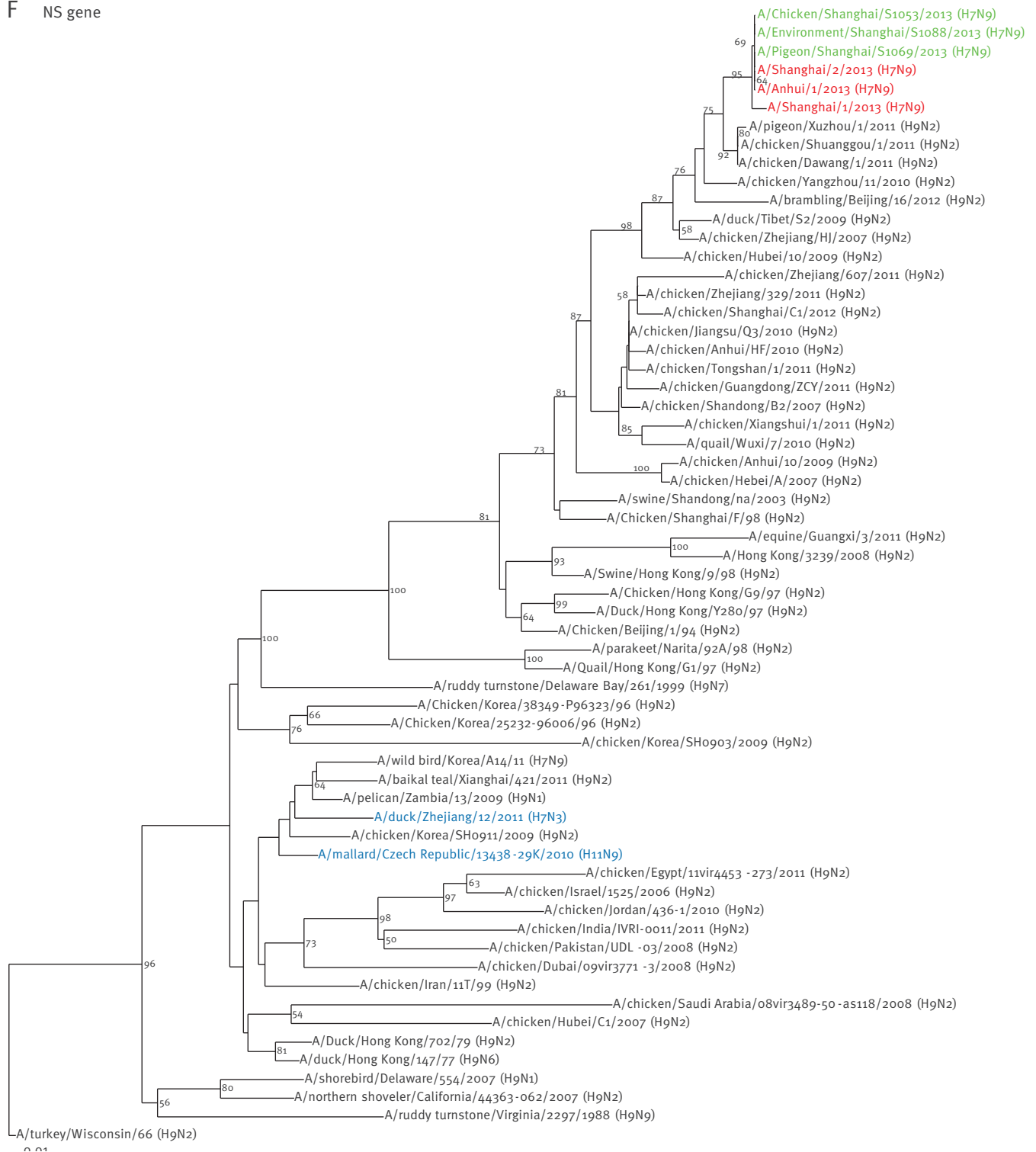
Multiple alignments were constructed by using the CLUSTAL W algorithm. Genetic distances were calculated by using the Kimura's 2-parameter method [26], and phylogenetic trees were constructed by using the neighbour-joining method with bootstrap analyses of 1,000 replicates in CLUSTAL W. Numbers next to nodes indicate bootstrap value percentages (>50%).

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F NS gene



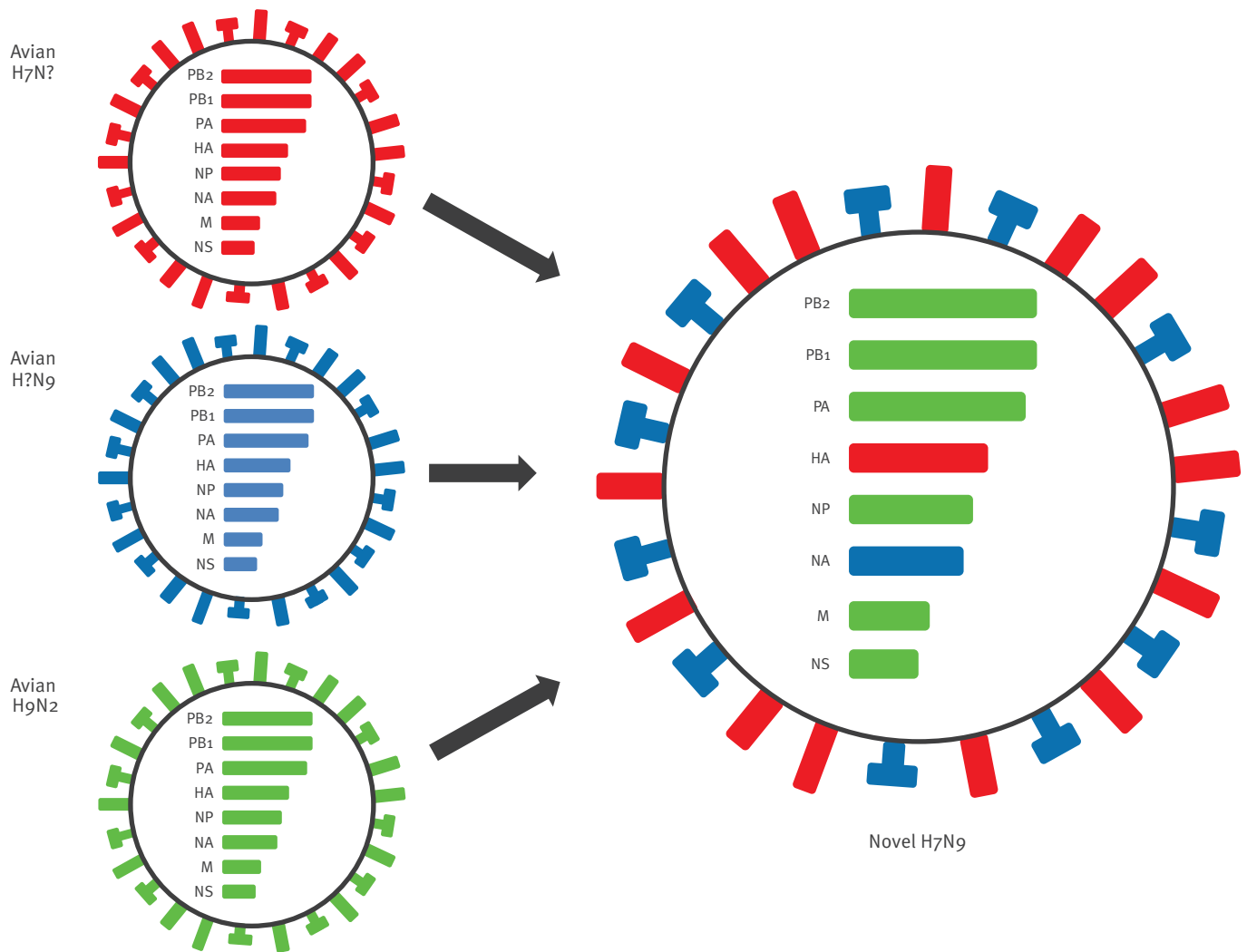
NS: non-structural gene.

Multiple alignments were constructed by using the CLUSTAL W algorithm. Genetic distances were calculated by using the Kimura's 2-parameter method [26], and phylogenetic trees were constructed by using the neighbour-joining method with bootstrap analyses of 1,000 replicates in CLUSTAL W. Numbers next to nodes indicate bootstrap value percentages (>50%).

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FIGURE 3

Schematic diagram of novel influenza A(H7N9) virus generation



HA: haemagglutinin; NA: neuraminidase.

The novel influenza A(H7N9) viruses are likely to have acquired their HA gene from an avian H7 virus of unknown NA subtype, their NA gene from an avian N9 virus of unknown HA subtype, and their remaining six viral segments from avian H9N2 viruses circulating in poultry.

viruses analysed here encode PB2-627E. By contrast, all four human H7N9 viruses analysed here encode PB2-627K (Table 3).

Antiviral compounds are the first line of defense against novel influenza viruses until vaccines become available. All seven novel influenza A(H7N9) viruses sequenced to date encode the S31N substitution in the viral ion channel M2 (encoded by the M segment) (Table 3), which confers resistance to ion channel inhibitors [18,19]. Based on the sequences of their NA proteins, all H7N9 viruses analysed here, with the exception of A/Shanghai/1/2013, should be sensitive to neuraminidase inhibitors (Table 3). However, the R294K mutation in the NA protein of A/Shanghai/1/2013 is known to

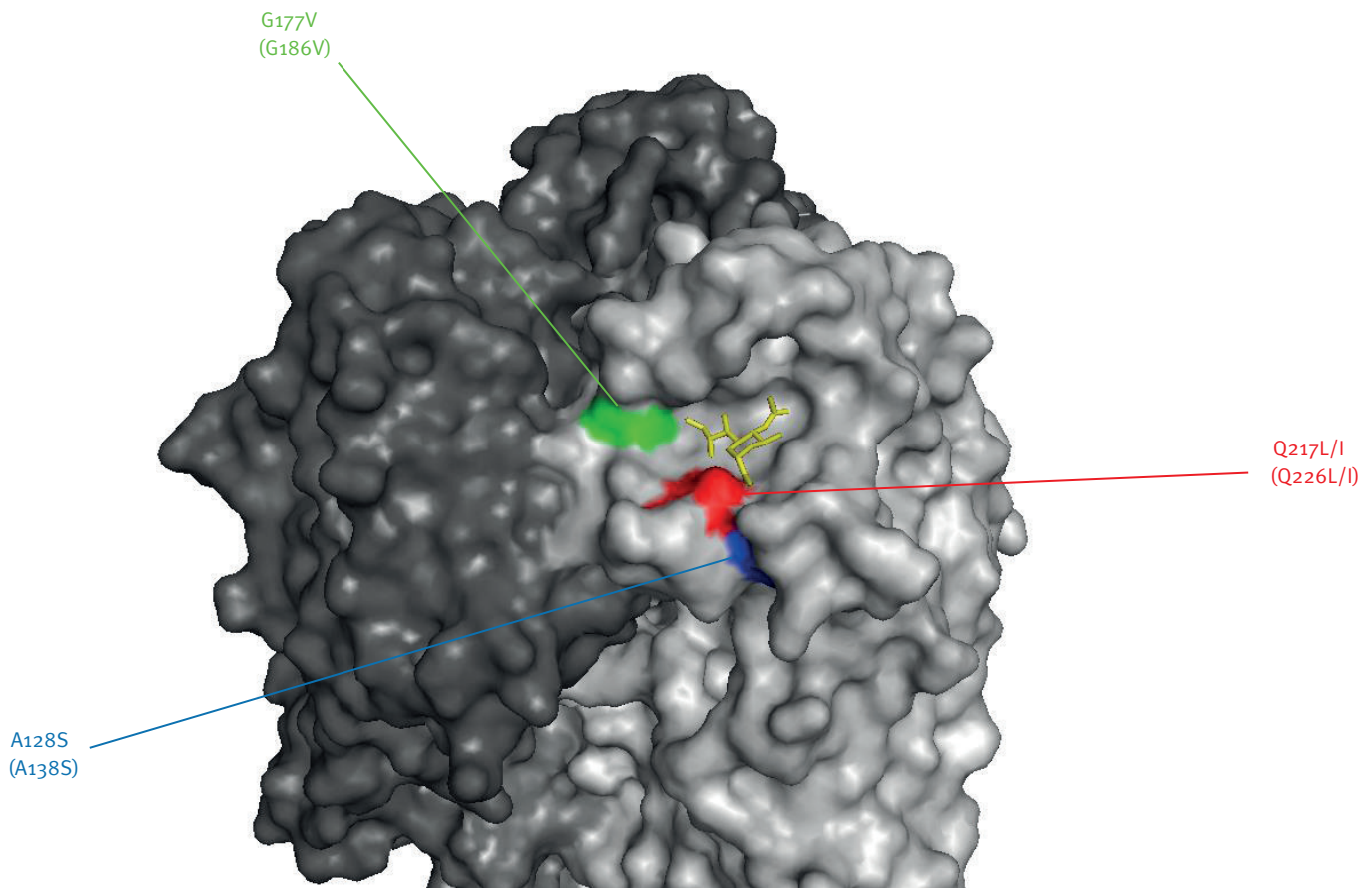
confer resistance to NA inhibitors in N2 and N9 subtype viruses [20], and is therefore of great concern.

All H7N9 viruses encode a deletion at positions 69–73 of the NA stalk region (Table 3), which is reported to occur upon virus adaptation to terrestrial birds. This finding suggests that the novel H7N9 viruses (or their ancestor) may have circulated in terrestrial birds before infecting humans. Moreover, this deletion is associated with increased virulence in mammals [21].

The influenza A virus PB1-F2 protein (encoded by the PB1 segment) is also associated with virulence. The available sequences indicate that the H7N9 PB1 genes of all of the human viruses encode a full-length PB1-F2 of 90 amino acids, but lack the N66S mutation that is

FIGURE 4

Amino acid changes in the three novel influenza A(H7N9) viruses that may affect their receptor-binding properties, China, February–April 2013 (n=7)



H7 numbering
(H3 numbering)

Shown is the three-dimensional structure of three monomers (light and dark gray) of the influenza A(H7N7) virus (A/Netherlands/219/2003) haemagglutinin (accession code 4DJ8). Also shown is the part of 6'-sialyl-N-acetylglucosamine (a sialyloligosaccharide) to which human viruses bind preferentially (yellow). Indicated are amino acid changes in the H7N9 virus haemagglutinin protein at positions known to increase binding to human-type receptors.

associated with the increased pathogenicity of the 1918 pandemic virus and the highly pathogenic avian influenza A(H5N1) viruses [22]. Interestingly, the pigeon isolate encodes a truncated PB1-F2 of only 25 amino acids; the significance of this truncation is unknown.

The NS1 protein (encoded by the NS segment) is an interferon antagonist with several functions in the viral life cycle. All available H7N9 NS1 sequences lack the C-terminal PDZ domain-binding motif; the lack of the PDZ domain-binding motif may attenuate these viruses in mammals [23].

Other amino acids in the NS1 and matrix (M1; encoded by the M segment) proteins of the novel viruses are also associated with increased virulence (Table 3) [24,25]. However, these amino acids are found in many

avian influenza viruses, and therefore, their significance for the biological properties of the novel influenza A(H7N9) viruses is currently unclear.

In conclusion, we here present a biological evaluation of the sequences of the avian influenza A(H7N9) viruses that caused fatal human infections in China. These viruses possess several characteristic features of mammalian influenza viruses, which are likely to contribute to their ability to infect humans and raise concerns regarding their pandemic potential.

*Authors' correction:

The mutation A138S was erroneously written as S138A in the original publication. This mistake was corrected on 13 April 2013

TABLE 3

Selected characteristic amino acids of the three novel influenza A(H7N9) viruses, China, February–April 2013 (n=7)

| Viral protein | Amino acid position | Shanghai/1/2013 | Shanghai/2/2013 | Anhui/1/2013 | Hangzhou/1/2013 | Chicken/Shanghai/S1053/2013 | Environment/Shanghai/S1088/2013 | Pigeon/Shanghai/S1069/2013 | Human influenza viruses | Avian influenza viruses | Comments | Reference(s) |
|---------------|--------------------------|-----------------|-----------------|-----------------|-----------------|-----------------------------|---------------------------------|----------------------------|--------------------------|-------------------------|--|--------------|
| PB2 | 627 | K | K | K | Nd | E | E | E | K | E | E627K: Mammalian host adaptation | 16 |
| | 128/138 ^a | S | A | A | A | A | A | A | A | A ^b | S138A: Increased virus binding to human-type receptors | 13 |
| HA | 151/160 ^b | A | A | A | A | A | A | A | K | A^b | T160A: Loss of N-glycosylation and increased virus binding to human-type receptors | 15 |
| | 177/186 ^a | G | V | V | V | V | V | V | G | G ^b | G186V: Increased virus binding to human-type receptors | 14 |
| | 217/226 ^a | Q | L | L | I | L | L | L | I | Q ^b | Q226L: Increased virus binding to human-type receptors | 12 |
| NA | 69–73 ^c | Deletion | Deletion | Deletion | Deletion | Deletion | Deletion | Deletion | No deletion | No deletion | Deletion of amino acids 69–73: Increased virulence in mice | 21 |
| | 289/294/292 ^d | K | R | R | R | R | R | R | R | R | R294K: Reduced susceptibility to oseltamivir and zanamivir | 20 |
| M1 | 30 | D | D | D | D | D | D | D | D(S) | D | N30D: Increased virulence in mice (most influenza A viruses encode 30D) | 24 |
| | 215 | A | A | A | A | A | A | A | A | A | T215A: Increased virulence in mice (most avian influenza A viruses encode 215A) | 24 |
| M2 | 31 | N | N | N | N | N | N | N | S/N | S/(N) | S31N: Reduced susceptibility to amantadine and rimantadine | 18,19 |
| | 42 | S | S | S | Nd | S | S | S | S | S/A | P42S: Increased virulence in mice (most avian influenza A viruses encode 42S) | 25 |
| NS1 | 218–230 | Deletion | Deletion | Deletion | Nd | Deletion | Deletion | Deletion | No deletion ^e | No deletion/Deletion | Lack of PDZ domain binding motif: Decreased virulence in mice | 23 |

Substitutions of particular concern are shown in bold.

Nd: not determined.

^a H7/H3 numbering.^b H7 virus.^c N9 numbering.^d H7N9/avian N9/N2 numbering.^e Influenza A(H1N1)pdm09 viruses from the 2009 influenza pandemic have the deletion.

Acknowledgements

We are grateful to Dr. Shu Yuelong, Chinese National Influenza Center, Chinese Center for Disease Control and Prevention, Beijing, China, for his rapid publication of the entire gene sequence data of A(H7N9) viruses isolated from human cases in China, and also for his information sharing and advice to this study. We also thank Susan Watson for scientific editing. This work was supported by Grants-in-Aid for Pandemic Influenza Research (TK, SF, HX, and MT) and Grant-in-Aid for Specially Promoted Research (MT) from the Ministry of Health, Labour and Welfare, Japan, by the NIAID-funded Center for Research on Influenza Pathogenesis (CRIP, HHSN266200700010C)(YK), by a Grant-in-Aid for Specially Promoted Research, by the Japan Initiative for Global Research Network on Infectious Diseases from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (YK), and by ERATO, Japan (YK).

Authors contributions

Designed the analyses: TK, SF, ET, SY, GN, YK, MT. Analysed and interpreted data: TK, SF, ET, HX, SY, YU, GN, YK, MT. Drafted the article: TK, SF. Revised the article: ET, GN, TS, YK, MT.

Conflict of interest

None declared.

This manuscript was accepted on 11 April 2013
and published online on 12 April 2013

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