

**Table.** Summary of diagnostic and laboratory findings, animal species, sample materials, and region where samples were collected in the study of Shuni virus infection in ruminants, Israel, 2014–15\*

Animal no.	Laboratory no.	Species	Clinical manifestation	Region	PCR-positive sample	Vero isolation	Virus isolation in mice
1	2417/1/14	Sheep	Malformed, aborted fetus	Northern valley	Brain, placenta	Negative	Negative
2	2417/2/14	Sheep	Malformed, aborted fetus	Northern valley	Brain	Negative	Negative
3	267/2/14	Sheep	Malformed, aborted fetus	Northern valley	Brain	Not done	Not done
4	267/3/14	Sheep	Malformed, aborted fetus	Northern valley	Brain	Not done	Not done
5	267/4/14	Sheep	Malformed, aborted fetus	Northern valley	Brain	Not done	Not done
6	2498/1/14	Sheep	Weak lamb syndrome	Northern valley	Brain, EDTA-blood	Negative	Negative
7	2504/1/14	Sheep	Malformed aborted fetus	Northern valley	Brain	Not done	Not done
8	2504/2/14	Sheep	Malformed, aborted fetus	Northern valley	Brain, placenta	Negative	Negative
9	273/14	Sheep†	Malformed, aborted fetus	Negev	Brain	Not done	Not done
10	274/14	Sheep	Aborted fetus	Northern valley	Brain, placenta	Not done	Not done
11	2504/3/14	Sheep†	Malformed, aborted fetus	Northern valley	Brain, placenta	Positive	Positive
12	275/1/14	Sheep	Malformed, aborted fetus	Northern valley	Brain, placenta	Negative	Negative
13	275/2/14	Sheep	Malformed aborted fetus	Northern valley	Brain, placenta	Not done	Not done
14	263/14	Goat	Malformed, aborted fetus	Northern valley	Brain, placenta	Not done	Not done
15	215/14	Cattle	Aborted fetus	Upper Galilee	Brain	Negative	Negative

\*Not done, not performed if insufficient brain material was available for cerebral inoculation or if the infected brain failed to propagate in the cell line. For some animals, >1 sample was collected.

†Sequences used to build the phylogenetic trees in online Technical Appendix Figure 2 (<http://wwwnc.cdc.gov/EID/21/12/15-0804-Techapp1.pdf>).

activity. Thus, isolation of SHUV from malformed brains may indicate strong neurotropism of this putative pathogen. The possibility of its replication in the fetal nervous system should also be considered because an affected fetus that is born alive is likely a reservoir. Indeed, AKAV was identified in the hippocampus (only) of adult lactating cows (data not shown), and similar epidemiologic evidence might result from other Simbu virus infections.

A serologic survey conducted in Israel during the 2001–2003 outbreaks of AHS showed reactivity of AINV to serum samples of ruminants in Israel's southern regions (3). Because AINV and SHUV are known to have a strong serologic cross-reaction, SHUV has likely previously infiltrated Israel. However, whether the seroreactivity results from AINV or SHUV remains unresolved.

The emergence and reemergence of arboviruses should interest medical practitioners, particularly epidemiologists. The appearance of exotic viruses in unexpected locations might result in more severe pathology in newly invaded regions than in the original arbovirus-endemic areas. Furthermore, SHUV has been detected in a child with febrile illness (2), a finding that suggests a potential zoonotic problem.

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## Genetic Characterization of Highly Pathogenic Avian Influenza A(H5N6) Virus, Guangdong, China

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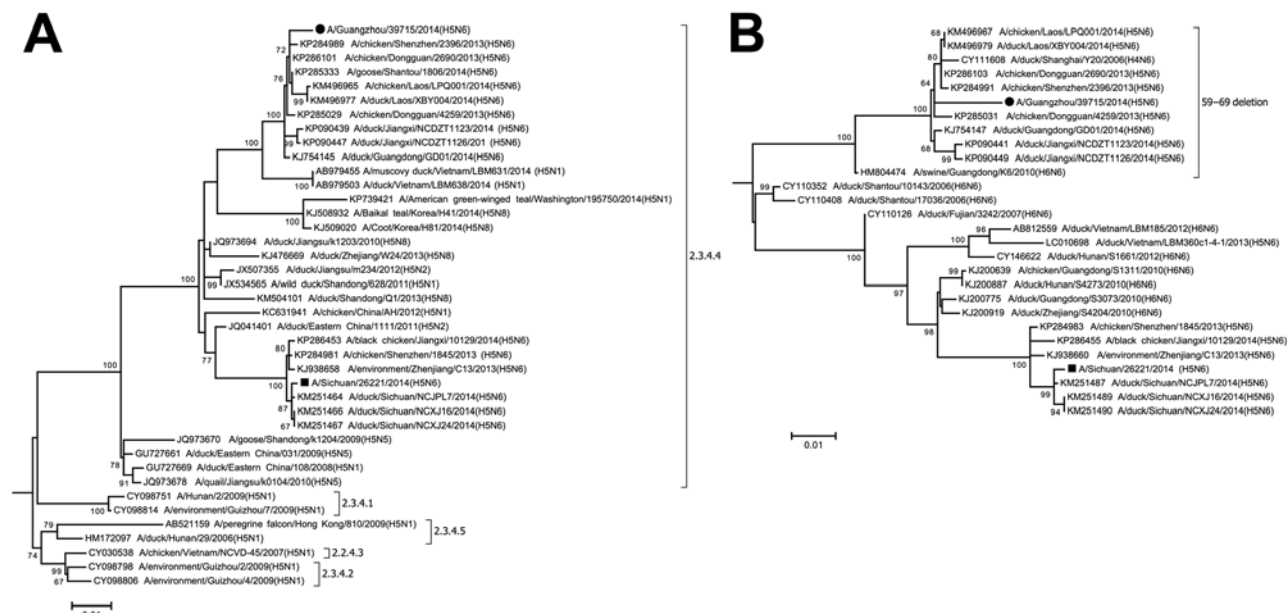
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**To the Editor:** Since the first detection of the influenza A(H5N1) virus in geese in China during 1996 (<http://www.cdc.gov/flu/avianflu/h5n1-virus.htm>), H5 subtype viruses have continued to reassort and evolve, giving rise to multiple virus clades and gene constellations. Recently, clade 2.3.4.4 viruses have shown a predilection for genetic reassortment, giving rise to H5N2, H5N5, H5N6, and H5N8 virus subtypes, and have become globally widespread, causing infections in wild birds or poultry elsewhere in Asia, and in Europe and North America (1–3). The H5N6 subtype viruses have circulated in China since 2013 and have been mainly identified in ducks or chickens in the southern (Jiangxi, Guangdong) or western (Sichuan) areas (4,5). Two lineages of H5N6 viruses with distant genetic background were found among the H5N6 viruses isolated in China (5).

In China, there have been 3 cases of H5N6 virus infection among humans, causing 2 deaths. We recently reported the clinical characteristics and progression of a patient infected by the H5N6 virus in Guangzhou City, China, who was the second reported case-patient

infected with this subtype (6). After having contact with poultry, he began to manifest an influenza-like illness on December 3, 2014, and progressed to a primary viral pneumonia. The H5N6 virus A/Guangzhou/39715/2014 (GenBank accession nos. KP765785–KP765792) was isolated from a throat swab specimen collected on day 8 of his illness by inoculation into 9–11-day-old, specific pathogen-free embryonated chicken eggs. He recovered from his infection and was discharged from the hospital on day 58.

Multiple sequence alignments showed that the hemagglutinin (HA) and neuraminidase (NA) genes of A/Guangzhou/39715/2014 shared the highest nucleotide identity with A/chicken/Dongguan/2690/2013 (H5N6) (99.4% and 98.3%, respectively) (online Technical Appendix 1, <http://wwwnc.cdc.gov/EID/article/21/12/15-0809-Techapp1.pdf>). All internal genes were also closely related to A/chicken/Dongguan/2690/2013 (H5N6), ranging from 98.5% nucleotide identity for the polymerase acidic (PA) gene and 100.0% for the matrix (M) gene. The genome segments were also 98.2%–99.7% identical to A/duck/Guangdong/GD012014 and 98.3%–99.4% identical to A/chicken/Laos/LPQ001/2014, which caused outbreaks in domestic ducks and poultry, respectively, indicating that these viruses have the same genotype.



**Figure.** Phylogenetic trees of influenza A(H5N6) virus isolate A/Guangzhou/39715/2014 compared with other influenza viruses based on the A) hemagglutinin (HA) and B) neuraminidase (NA) genes, China. Maximum-likelihood trees were constructed by using the general time reversible plus gamma distribution plus invariant sites (GTR+G+I) model in MEGA 6.06 (<http://www.megasoftware.net>). Bootstrap values were calculated on 1,000 replicates; only values >60% are shown. A/Guangzhou/39715/2014 and A/Sichuan/26221/2014 are indicated by a circle and a square, respectively. Brackets denote H5 subtype virus clades. Scale bars indicate nucleotide substitutions per site. Full HA and NA trees are provided in online Technical Appendix 1 (<http://wwwnc.cdc.gov/EID/article/21/12/15-0809-Techapp1.pdf>).

HA gene phylogeny confirmed that this virus belonged to clade 2.3.4.4 (online Technical Appendix 1). Notably, the HA genes of the H5N1, H5N2, and H5N8 viruses that were recently detected in wild birds in North America also belong to this clade, indicating that viruses from this clade are becoming globally widespread. More specifically, this isolate clustered within a sublineage that includes H5N6 isolates from poultry from Guangdong and Jiangxi provinces, China, and from Laos (5, 7). The A/Sichuan/26221/2014 (H5N6) virus that recently caused a fatal human infection in Sichuan Province, China is also within clade 2.3.4.4, but clusters in a distinct sub-lineage (Figure, panel A).

The HA cleavage site of both human isolates contained multiple basic amino acids, suggesting that they are highly pathogenic avian influenza viruses. Amino acid substitutions E190D, Q226L, or G228S (H3 numbering) in the HA gene that are known to enhance binding to mammalian receptors were not found. The NA gene phylogeny showed that A/Guangzhou/39715/2014 is likely originated from group II lineage influenza A(H6N6) viruses that circulate among domestic ducks in China (8) (Figure, panel B). An 11-aa deletion at the residue 59–69 position of the NA protein was identified in the isolate of this study, in the other H5N6 viruses of the same cluster, and in an H4N6 virus isolate from a duck in Shanghai, China. This deletion was monophyletic and likely originated from A/swine/Guangdong/K6/2010 (H6N6)-like viruses (Figure, panel B). However, it was not observed in other 2.3.4.4 viruses, such as A/Sichuan/26221/2014.

No mutations associated with oseltamivir or amantadine resistance was found in NA or M2 genes. The internal genes of the current H5N6 isolate were similar to 2.3.2.1b H5N1 subtype viruses found in domestic ducks from south-central and eastern China (5, 7–10; online Technical Appendix 1). The 6 internal genes are 97%–99% homologous to another isolate from a human, A/Sichuan/26221/2014, suggesting that the internal genes of the viruses may reassort from a common origin.

The phylogenetic clustering observed for the HA gene was also conserved for the internal genes. In contrast with all avian viruses within this clade, the current human isolate contains the mammalian adaptation mutation PB2-E627K, and A/Sichuan/26221/2014 has acquired PB2-D701N, suggesting a rapid acquisition of mammalian adaptation changes that likely arose after human infection.

There is still limited information on human disease caused by the emerging H5 lineage. Our genetic analysis suggests that the H5N6 virus isolated from the patient is originated from the avian host. Although the genetic background of H5N6 virus isolated from the third case in Yunnan Province, China, on January 2015 is still not known, the isolates from the human cases of H5N6 infection reported to date show distant genetic diversity, indicating

that viruses from both clusters may pose a threat to humans. This rapidly evolving and globally spreading virus lineage thus provides a threat to global public health.

### Acknowledgments

We thank the authors and originating and submitting laboratories of the sequences in the EpiFlu Database from Global Initiative on Sharing Avian Influenza Data, on which this research is based (online Technical Appendix 2, <http://wwwnc.cdc.gov/EID/article/21/12/15-0809-Techapp2.xlsx>).

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## Surveillance for Ebola Virus in Wildlife, Thailand

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**To the Editor:** Active surveillance for zoonotic pathogens in wildlife is particularly critical when the pathogen has the potential to cause a large-scale outbreak. The recent outbreak of Ebola virus (EBOV) disease in West Africa in 2014 was initiated by a single spillover event, followed by human-to-human transmission (1). Projection of filovirus ecologic niches suggests possible areas of distribution in Southeast Asia (2). Reston virus was discovered in macaques exported from the Philippines to the United States in 1989 and in sick domestic pigs in the Philippines in 2008 (with asymptomatic infection in humans) (3). Dead insectivorous bats in Europe were found to be infected by a filovirus, similar to other members of the genus *Ebolavirus* (4).

Although EBOV has historically been viewed as a virus from Africa, recent studies found that bat populations in Bangladesh and China contain antibodies against EBOV and Reston virus recombinant proteins, which suggests that EBOVs are widely distributed throughout Asia (5,6). Thus, an outbreak in Asian countries free of EBOV diseases may not only be caused by importation of infected humans and/or wildlife from Africa but may arise from in-country

filovirus-infected wildlife. Serologic and molecular evidence for filoviruses suggests that members of the order Chiroptera (bats) may be their natural reservoir (7).

As part of a proactive biosurveillance program, we conducted a cross-sectional study for EBOV infection in bats and macaques in Thailand. We screened 500 *Pteropus lylei* bats collected from 10 roosting sites during March–June 2014 (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/20/12/15-0860-Techapp1.pdf>) for antibodies against EBOV antigen by using an ELISA validated by the Centers for Disease Control and Prevention (Atlanta, GA, USA) (8).

Bats and macaques were captured with permission from the Department of National Parks, Wildlife and Plant Conservation. The Institutional Animal Care and Use Committee at the University of California, Davis (protocol #16048) approved the capture and sample collection protocols.

To further screen a wide range of wildlife species in Thailand for active EBOV infection, we sampled and tested 699 healthy bats, representing 26 species, and 50 long-tailed macaques (*Macaca fascicularis*). Additional bat species were randomly captured ( $\geq 50$ /site) in 6 provinces in Thailand during 2011–2013 and identified by morphologic traits. Macaques were captured and sampled in March 2013 from 1 site at Khao Chakan, Sa Kaeo Province, and released at the same site. Blood, saliva, urine, and feces were collected from anesthetized macaques or nonanesthetized bats. All animals were released after sample collection. Details on species screened, sample sizes, and trapping localities are provided in the Table.

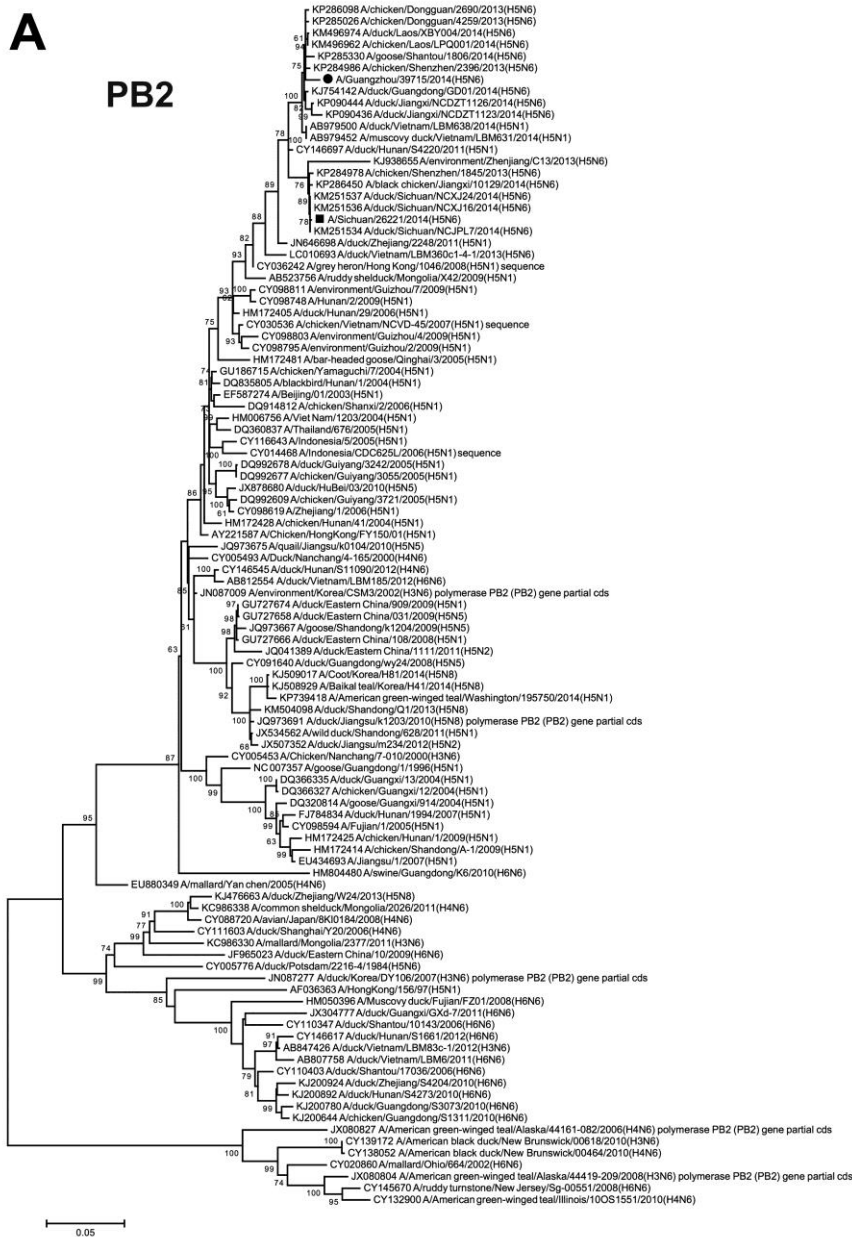
All nonblood specimens were collected in nucleic acid extraction buffer (lysis buffer) and transported on ice to the World Health Organization Collaborating Centre for Research and Training on Viral Zoonoses laboratory (Bangkok, Thailand) for storage and testing. Three types of specimen (saliva, urine, and serum) were collected from individual animals and pooled.

Nucleic acid was then extracted with NucliSENS easy-MAG (bioMérieux, Boxtel, the Netherlands) and analyzed by reverse transcription PCR (RT-PCR). A consensus RT-PCR was used to screen for all known species of Ebola virus and Marburg virus, including EBOV (9). In total, 5 RT-PCRs were performed on each specimen, a regimen that included 4 sets of primers specific to known filoviruses and 1 degenerate primer set to detect novel viruses in this family. The sensitivity of RT-PCR on synthetic standard was 50–500 copies/reaction (9). We ran 3,745 PCRs, covering a range of assays, to increase detection sensitivity. All specimens examined were negative for filoviruses by EBOV ELISA and PCR (Table). For *P. lylei* ELISA screening, optical density values for all 500 bats ranged from 0.000 to 0.095, well below the potential positive cutoff value of 0.2.

Assuming a population size of  $\approx 5,000$  bats/roost and a sample size of 50 bats/site, we have 95% confidence that

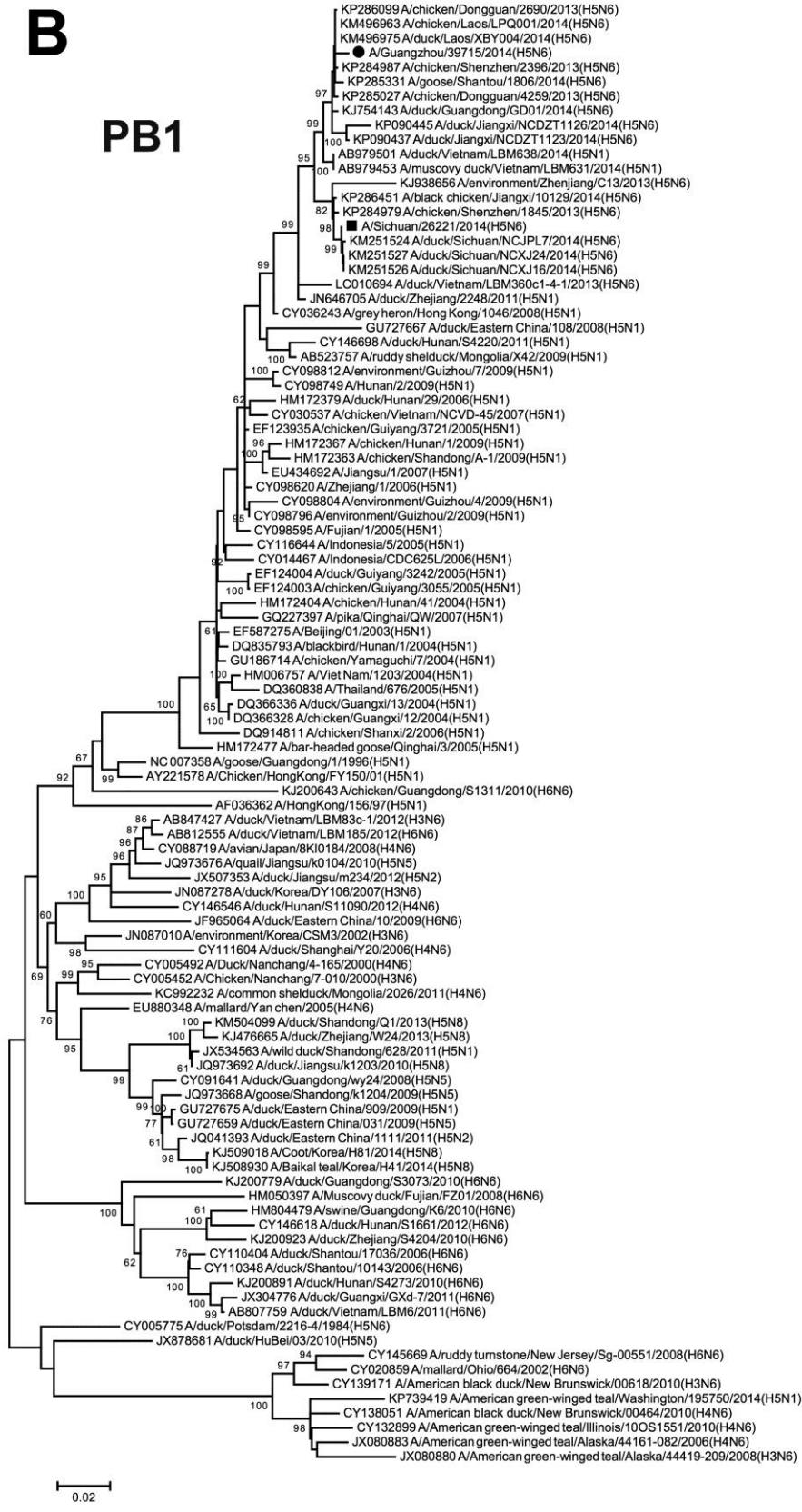
# Genetic Characterization of Highly Pathogenic Avian Influenza A(H5N6) Virus, Guangdong, China

## Online Technical Appendix 1



**B**

**PB1**



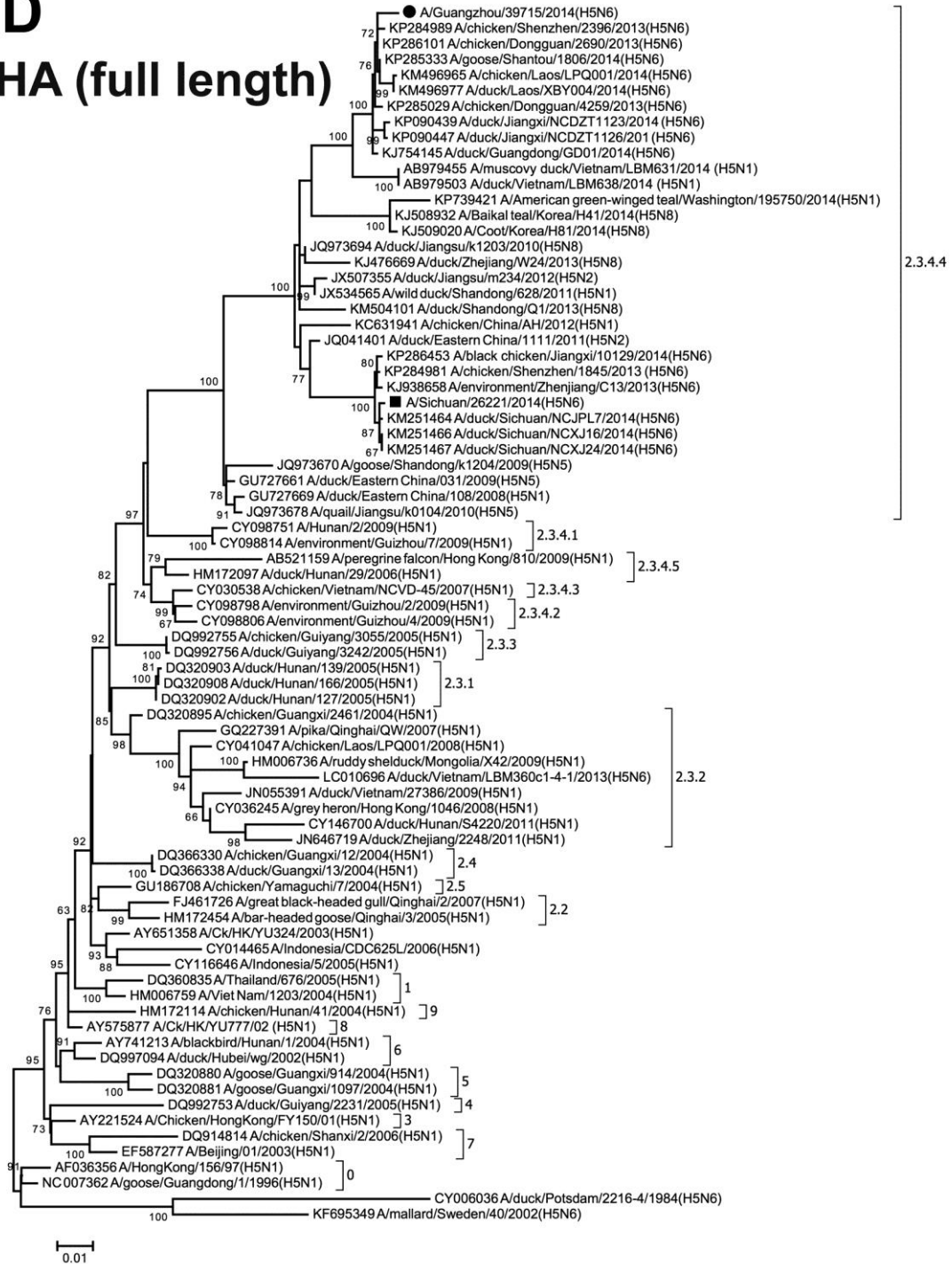
C

PA



# D

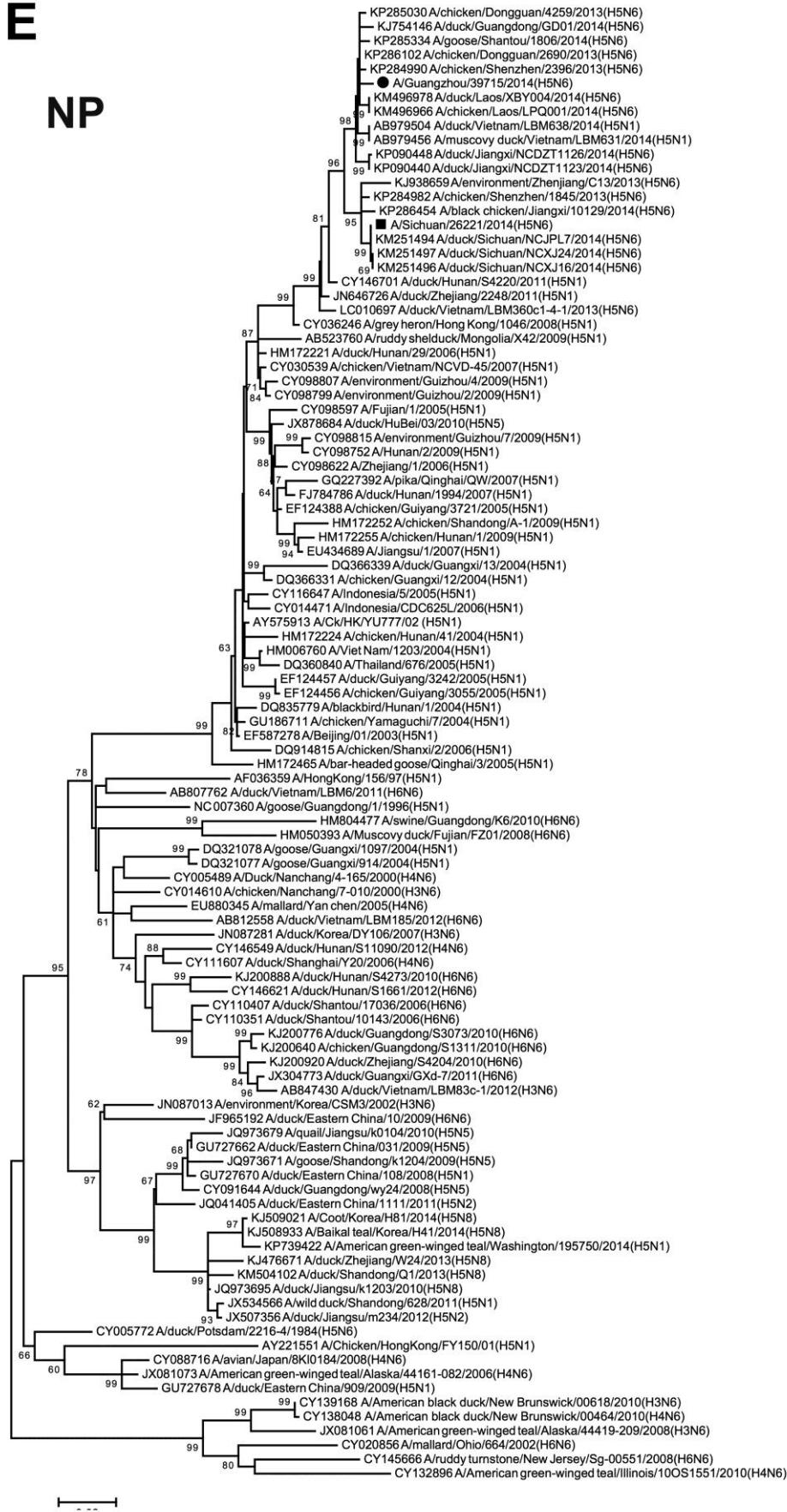
## HA (full length)





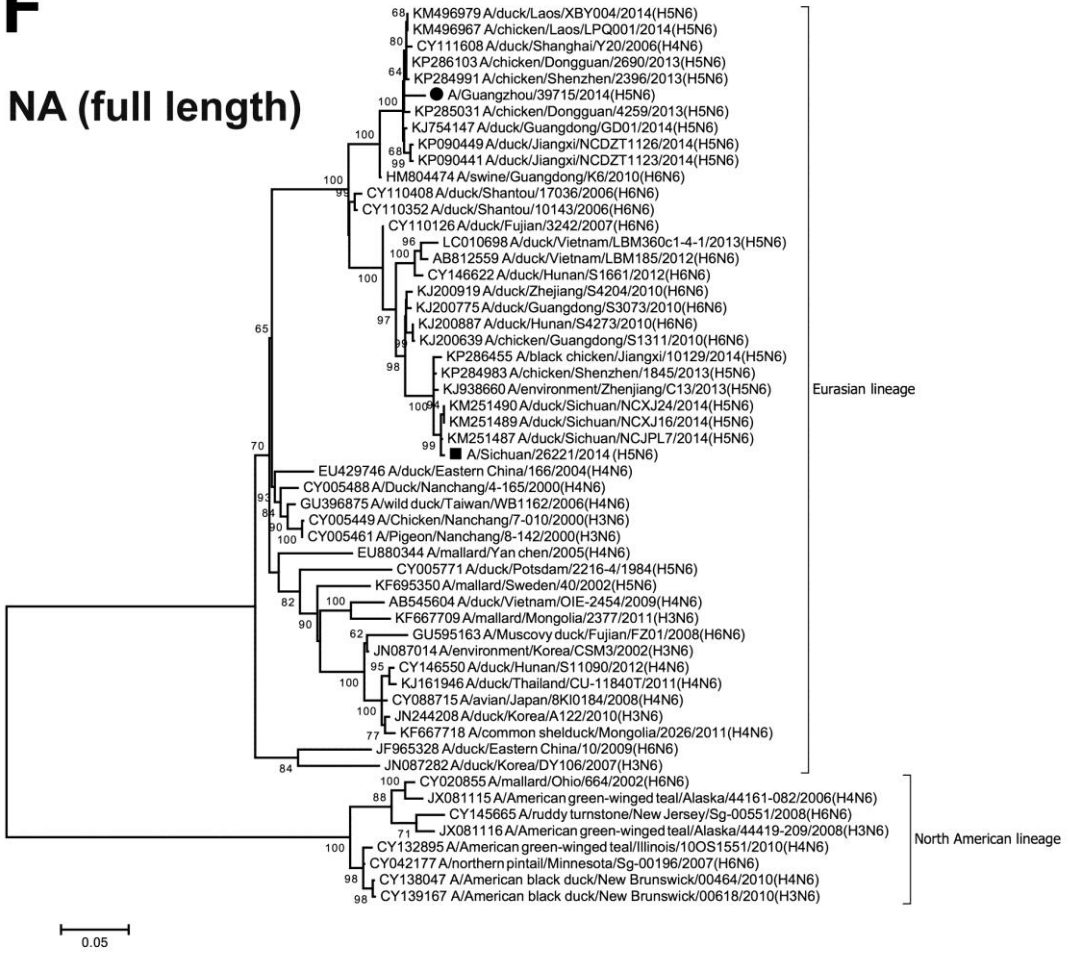
E

NP



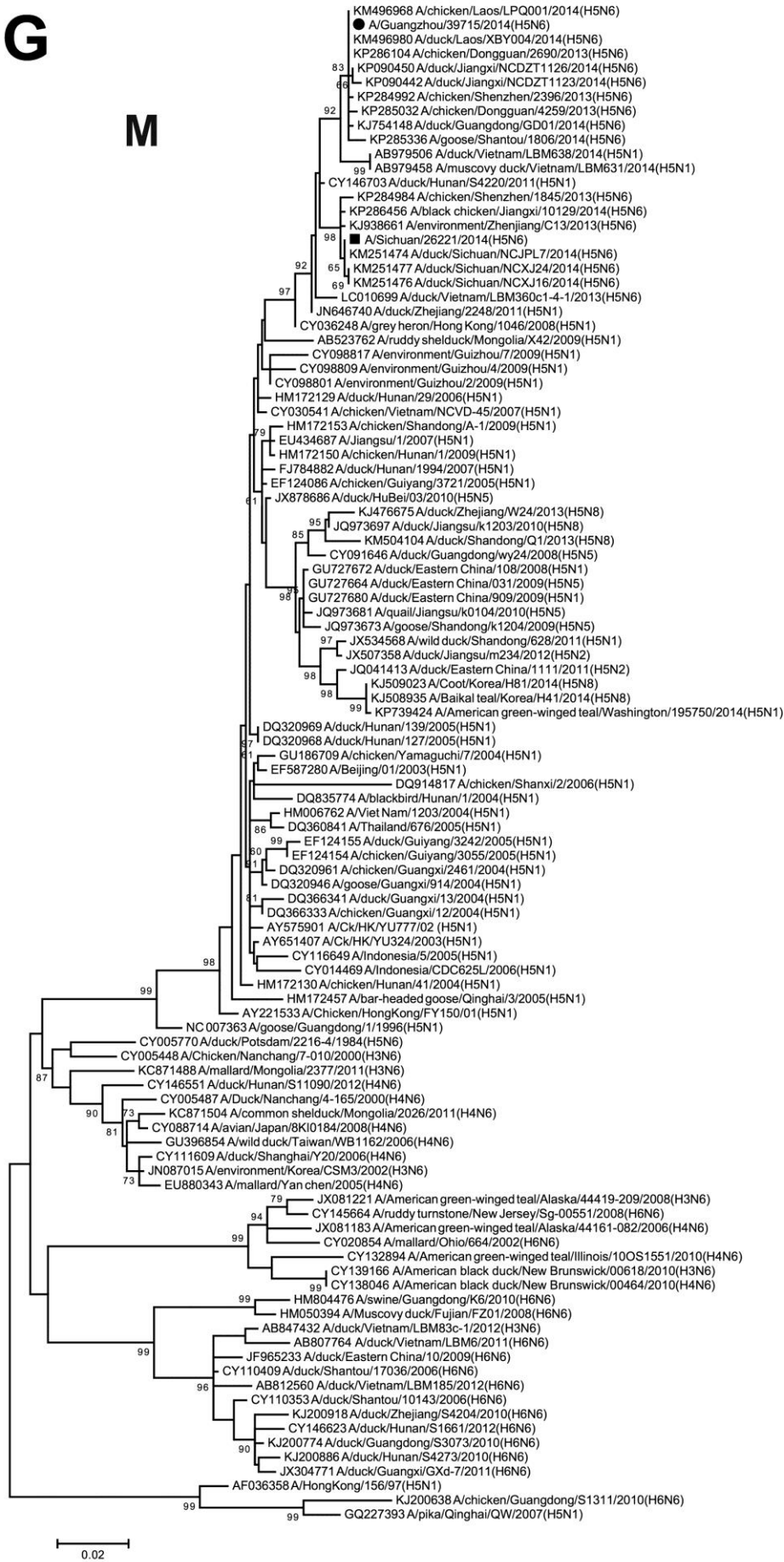
**F**

**NA (full length)**



G

M



H

NS



Full phylogenetic trees of polymerase basic (PB2) (Technical Appendix Figure 1A), PB1 (Technical Appendix Figure 1B), polymerase acidic (PA) (Technical Appendix

Figure 1C), hemagglutinin (HA) (Technical Appendix Figure 1D), nucleoprotein (NP) (Technical Appendix Figure 1E), neuraminidase (NA) (Technical Appendix Figure 1F), M (Technical Appendix Figure 1G), and nonstructural (NS) (Technical Appendix Figure 1H) genes. Maximum-likelihood trees were constructed by using the general time reversible + gamma distribution + invariant sites (GTR +  $\Gamma$  + I) model in MEGA 6.06 (<http://www.megasoftware.net>). Bootstrap values were calculated on 1,000 replicates; only values >60% are shown. A/Guangzhou/39715/2014 and A/Sichuan/26221/2014 are marked by a circle and a square, respectively. Scale bars indicate nucleotide substitutions per site.