

Acknowledgments

The authors would like to express their appreciation to all of the study patients.

This study was supported by the Bill and Melinda Gates Foundation and the US Agency for International Development via the World Health Organization.

The authors declare that there is no conflict of interest regarding the publication of this paper. D.B., C.R., G.G., and P.R. are staff members of the World Health Organization. D.B., C.R., G.G., and P.R. are solely responsible for views expressed in this publication, and they do not necessarily represent decisions, policies, or views of the World Health Organization.

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Novel Reassortant Highly Pathogenic Avian Influenza (H5N8) Virus in Zoos, India

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DOI: <http://dx.doi.org/10.3201/eid2304.161886>

Highly pathogenic avian influenza (H5N8) viruses were detected in waterfowl at 2 zoos in India in October 2016. Both viruses were different 7:1 reassortants of H5N8 viruses isolated in May 2016 from wild birds in the Russian Federation and China, suggesting virus spread during southward winter migration of birds.

Since 1996, the hemagglutinin (HA) gene of subtype H5N1 highly pathogenic avian influenza (HPAI) viruses has evolved into multiple phylogenetic clades (1). During 2010, subtype H5N8 virus, bearing an H5N1 backbone and polymerase basic (PB) protein 1 (PB1), nucleoprotein (NP), and neuraminidase (NA) genes from non-H5N1 virus, emerged in China (2). In January 2014, a novel reassortant HPAI (H5N8) virus was detected in poultry and wild birds in South Korea (3) and subsequently spread to other counties in Asia and Europe before reaching North America by the end of 2014 (4). Because the H5N8-associated outbreaks coincided with bird migration routes, movement of wild waterfowl was suspected in intercontinental spread (5). Therefore, understanding the source and spread of the virus is a critical requirement for guidance of control measures. We report analysis of the genome of HPAI (H5N8) viruses isolated from waterfowl (domestic duck [*Anas platyrhynchos domesticus*] and painted stork [*Mycateria leucocephala*]) at 2 zoos in India in October 2016.

Twenty avian influenza viruses were isolated from 83 samples from National Zoological Park, Delhi, and Gandhi Zoological Park, Gwalior, Madhya Pradesh, India, in October 2016. The viruses were subtyped as H5N8 using reverse transcription PCR and real-time RT-PCR (online Technical Appendix 1, <https://wwwnc.cdc.gov/EID/article/23/4/16-1886-Techapp1.pdf>). One representative isolate each from Delhi (A/duck/India/10CA01/2016) and Madhya Pradesh (A/painted stork/India/10CA03/2016) were processed for pathogenic and molecular characterization. A detailed

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description of the methods for the intravenous pathogenicity index test and genetic analysis used are provided in online Technical Appendix 1. Nucleotide sequences were deposited in the GISAID EpiFlu database (<http://www.gisaid.org>) under accession nos. EP1858833–EP1858848.

Both isolates were highly pathogenic based on amino acid sequence at the HA cleavage region (PLREKRRKR/GLF), which was corroborated by using an intravenous pathogenicity index test of 3.00 (Delhi isolate) and 2.96 (Madhya Pradesh isolate). Amino acid markers in the neuraminidase protein and matrix protein 2 indicated sensitivity to neuraminidase inhibitors and amantadines. Markers for mammalian virulence and poultry adaptation, such as E627K and D701N in PB2 and amino acid deletion in nonstructural protein (NS) 1 (position 80–84), were absent in the H5N8 viruses. However, 42S and 13P mutations in NS and PB1 genes (6) associated with increased virulence of the virus to mice were present. The PB1-F2 protein was truncated because of nucleotide mutation C35A, leading to premature termination after 11 aa.

Except the polymerase acidic (PA) and NP genes, all other gene segments of both isolates shared high nucleotide identity, ranging from 99.2% to 99.5%. The nucleotide identity of the PA and NP gene was 95.8% and 94.8%, respectively, suggesting involvement of 2 gene pools of H5N8 virus in the waterfowl outbreaks at Delhi and Madhya Pradesh.

In the HA gene phylogeny, the India isolates clustered with H5N8 viruses from other countries in Asia and Europe within group B (intercontinental group B) (online Technical Appendix 1 Figures 1–8). A similar grouping pattern was observed in the neuraminidase and nonstructural (NS) gene phylogenies. Further, within intercontinental group B, the isolates shared >99% nucleotide sequence identity with H5N8 viruses isolated in Uvs-Nuur Lake (located at the Mongolia–Russia border) and Qinghai Lake, China, in May 2016 (online Technical Appendix 1 Table 2). However, PB1, PB2, and matrix protein genes grouped with low pathogenic avian influenza (LPAI) viruses isolated in Eurasia and H5N8 viruses isolated in Qinghai Lake, Uvs-Nuur Lake, and Tyva Republic (Russian Federation).

In the PA phylogeny, although the Delhi virus grouped with LPAI viruses isolated in Mongolia and Vietnam and viruses isolated in Qinghai Lake, Uvs-Nuur Lake, and Tyva Republic, the Madhya Pradesh virus shared close relationship with LPAI viruses from Eurasia. In the NP gene phylogeny, although the Delhi virus shared close relationship with the Eurasia group of LPAI viruses, whereas the Madhya Pradesh virus and H5N8 viruses from Qinghai Lake, Uvs-Nuur Lake, and Tyva Republic are closely related to the Eurasia 2 LPAI viruses. These results suggest that both isolates are 7:1 reassortant of the Tyva Republic and Uvs-Nuur Lake H5N8 viruses reported previously (7) with different gene constellations. A median-joining network analysis indicated that,

even though the contemporary H5N8 viruses isolated from wild birds in Qinghai Lake, Uvs-Nuur Lake, and Tyva Republic are not the direct ancestors, closely related precursor gene pools are source of the H5N8 viruses that caused outbreaks in waterfowls at the 2 zoos in India (online Technical Appendix 1 Figure 9).

The outbreak in waterfowls at both zoos coincided with winter migration of birds to India (September–March). The Uvs-Nuur Lake is an important habitat for 46 resident waterfowl species and 215 different species of birds migrating southward from Siberia (8). Therefore, different waves of migration of the wild birds might be the source of introduction of the H5N8 virus at the 2 zoos in India, as suggested by the observed spread of H5N1 clade 2.2 and 2.3.2.1c viruses (9,10).

Acknowledgments

We are thankful to the Indian Council of Agricultural Research, New Delhi, and the Indian Council of Agricultural Research–National Institute of High Security Animal Diseases, Bhopal, for providing necessary facilities to carry out this work. We are thankful to the Directors of Animal Husbandry Department of Delhi and Madhya Pradesh states in India for sharing the clinical samples used as part of this study. We gratefully acknowledge the authors and the originating and submitting laboratories for the sequences from the Global Initiative on Sharing Avian Influenza Data EpiFlu database (online Technical Appendix 2, <https://wwwnc.cdc.gov/EID/article/23/4/16-1886-Techapp2.xlsx>).

We acknowledge funding by the Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture and Farmers Welfare, Government of India under Central Disease Diagnostic Laboratory Grant.

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Acute Tetraplegia Caused by Rat Bite Fever in Snake Keeper and Transmission of *Streptobacillus moniliformis*

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DOI: <http://dx.doi.org/10.3201/eid2304.161987>

We report acute tetraplegia caused by rat bite fever in a 59-year old man (snake keeper) and transmission of *Streptobacillus moniliformis*. We found an identical characteristic

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bacterial pattern in rat and human samples, which validated genotyping-based evidence for infection with the same strain, and identified diagnostic difficulties concerning infection with this microorganism.

Human infections by *Streptobacillus moniliformis* are assumed to be caused by rats on the basis of epidemiologic information. We provide genotyping-based evidence for infection with the same bacterial strain in rat and human samples and highlight diagnostic difficulties concerning this microorganism and its potential for life-threatening consequences.

A 59-year-old man was admitted to Centre Hospitalier Universitaire de Tours (Tours, France) because he was unable to stand and had acute progressive onset of dyspnea and a 15-day history of fever and arthralgia (left knee, right wrist) but no signs of rash. He was sedated, mechanically ventilated, and admitted to the intensive care unit. The patient had a temperature of 39°C, a pulse rate of 63 beats/min, and a blood pressure of 126/68 mm Hg.

After discontinuation of sedation, physical examination showed cervical pain, flaccid tetraplegia, and sensitivity at the T4 level. His knees and left wrist were swollen and had joint effusions. There was little available information for the patient because he could not speak and had no known social contacts. Blood tests showed an increased leukocyte count (15×10^9 cells/L), predominantly neutrophils, and an increased C-reactive protein level (125 mg/L).

The patient was given antimicrobial drugs (amoxicillin and cloxacillin) after blood and synovia (knee) sampling. Cervical magnetic resonance imaging showed C5–T1 vertebral osteomyelitis and an epidural abscess with consecutive compression of the spinal cord (C5–T1) (Figure). Surgical spinal decompression and vertebral stabilization were not attempted because of extensiveness of injury and flaccid tetraplegia. Transthoracic and transesophageal echocardiograms showed no features of endocarditis. Blood cultures showed negative results. Joint effusions contained a culture-negative inflammatory liquid and uric acid crystals. The patient was given a tracheotomy and continuously ventilated.

A final diagnosis was obtained by sequencing the 16S rRNA gene obtained directly from synovia. An 897-nt partial 16S rRNA sequence showed 99.0% identity with sequences of *S. moniliformis* (GenBank accession nos. JQ087393 and CP001779).

The patient was a snake keeper who bred rats for snake food. He reported snake bites but not rat bites. We sampled his snakes (*Boa constrictor* and *Elaphe* sp.) and 1 of his feeder rats (*Rattus norvegicus*) by obtaining swab and biopsy specimens from oral cavities of all animals. All cultures were polymicrobial. We used desorption/ionization time-of-flight mass spectrometry (Bruker Daltonique,

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Technical Appendix 1

Materials and Methods

Outbreaks, Clinical Samples, Virus Isolation, and Confirmation

Mortality was observed in water fowls (ducks, goose, pelican and painted storks) at National Zoological Park, Delhi (28.6020° N, 77.2478° E) on 17th October, 2016 and in painted storks at Gandhi Zoological Park, Gwalior, Madhya Pradesh (26.2183° N, 78.1828° E) on 20th October, 2016.

A total of 83 samples from both the zoological parks were received during October 2016. Tissue samples from each dead bird were pooled and homogenized to make 10% suspension in PBS (pH 7.2). Tracheal and cloacal swabs, environmental and fecal samples were processed for virus isolation. Virus isolation was carried in 9–11-days old specific pathogen free embryonated chickens eggs (1).

Viral RNA was extracted from the clinical samples using QIAamp Viral RNA Mini Kit (Qiagen, Germany) as per the manufacturer's instructions. Type A and HA subtype identification of influenza viruses were carried out using one step RT-PCR and real time RT-PCR (2,3). The NA subtyping was carried out by RT-PCR (4). Avian influenza H5N8 virus positivity from the two zoological parks is given below (Technical Appendix 1 Table 1).

Genome sequencing

For molecular characterization of the H5N8 viruses, viral RNA was extracted from infected allantoic fluid using QIAamp Viral RNA Mini Kit (Qiagen, Germany) as per the manufacturer's instructions. All the 8 genes of the viruses were amplified by RT-PCR using Platinum *Taq* High Fidelity (Invitrogen, USA) as described previously (5). The PCR amplified products were purified using QIAquick gel extraction kit (Qiagen, Germany). Sequencing of the

gel purified products were carried out using BigDye cycle sequencing kit, version 3.1 (Applied Biosystems, USA) in an ABI 3130 Genetic analyzer (Applied Biosystems, USA).

Genetic Analysis

To know the evolutionary relationships of the H5N8 viruses isolated from waterfowls during October 2016 in India, homologous sequences were obtained by BLAST search at the GISAID (<http://www.gisaid.org>) and GenBank (<http://ncbi.nlm.nih.gov>) along with other reference sequences. Coding regions were aligned using BioEdit, ver. 7.2.5 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Tree reconstruction was carried out using MEGA 6 software (<http://www.megasoftware.net>). Maximum likelihood trees were reconstructed using GTR model of nucleotide substitution with γ -distributed rate variation among sites with 4 rate categories (4 discrete categories of γ) available in MEGA. Phylogenetic trees were statistically evaluated by bootstrap method using 1000 resampling datasets. A median-joining phylogenetic network was constructed by using NETWORK, version 5 (<http://www.fluxus-engineering.com/sharenet.htm>).

Intravenous pathogenicity index (IVPI)

Intravenous pathogenicity index (IVPI) of two H5N8 viruses was carried out following the method described earlier (1). The experiment was carried out in the BSL-3 containment animal wing of ICAR-NIHSAD, Bhopal as per the guidelines of CPCSEA, Government of India with approval from Institutional Animal Ethics Committee (IAEC approval No. 61/IAEC/HSADL/12). Briefly, eight 4-weeks old SPF chickens (white leghorn) were inoculated intravenously with 1:10 dilution of infected allantoic fluid. Birds were observed daily for any clinical signs/mortalities, and scored accordingly (0, healthy; 1, sick; 2, severely sick and 3, dead).

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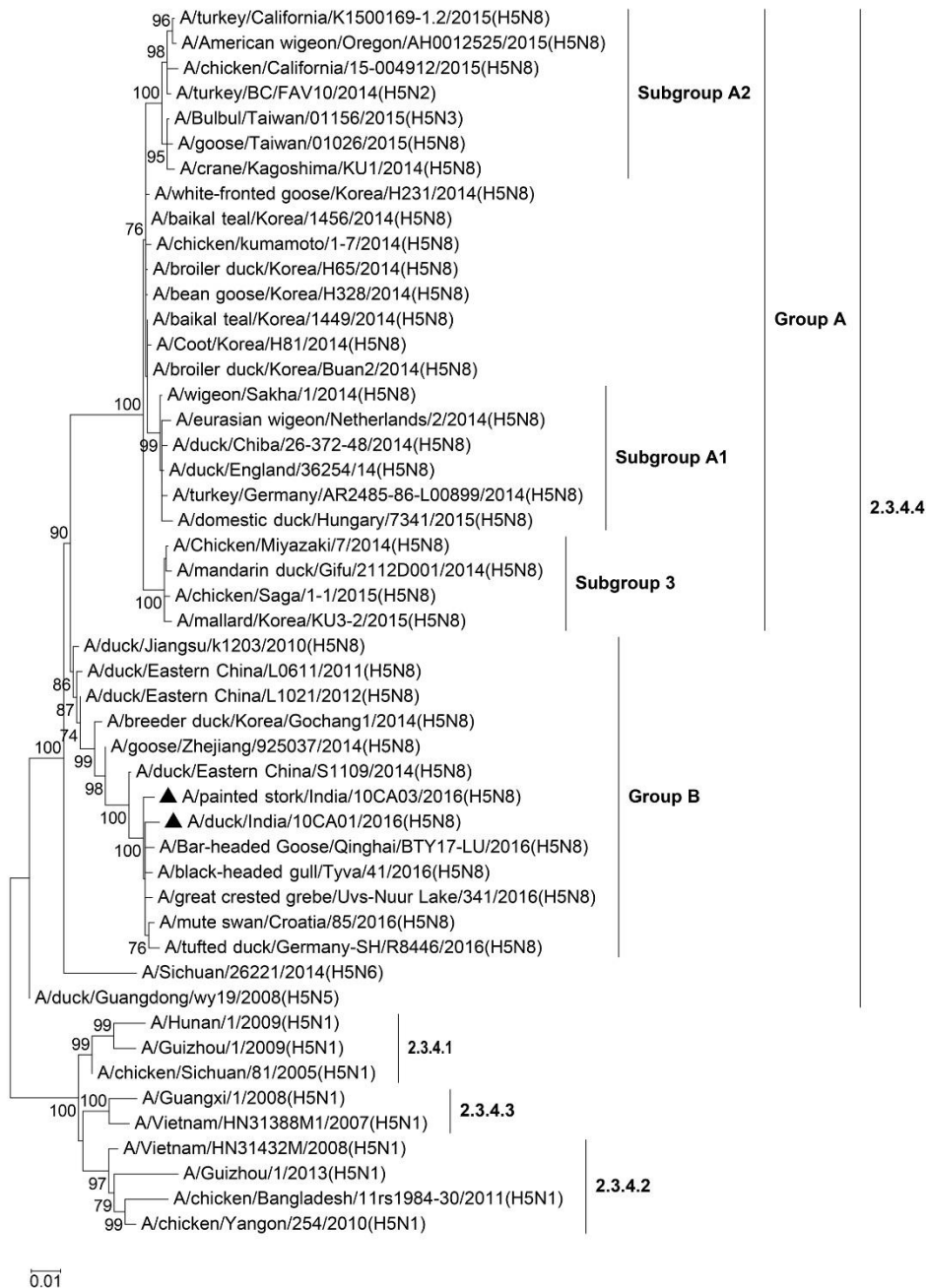
Technical Appendix 1 Table 1. Avian influenza H5N8 positive samples from zoological parks, India

Name of the zoological park	Species	Type of samples	No. positive samples
National Zoological Park, Delhi	Painted stork (<i>Mycteria leucocephala</i>)	Carcass	01
	Domestic duck (<i>Anas platyrhynchos domesticus</i>)		01
	Domestic goose (<i>Anser domesticus</i>)		01
	House crow (<i>Corvus splendens</i>)		01
	Great White Pelican (<i>Pelecanus onocrotalus</i>)		01
	Domestic duck (<i>Anas platyrhynchos domesticus</i>)	Cloacal swab	02
	Domestic duck (<i>Anas platyrhynchos domesticus</i>)	Tracheal swab	01
Gandhi Zoological Park, Gwalior, Madhya Pradesh	-	Environmental samples	02
	Painted stork (<i>Mycteria leucocephala</i>)	Carcass	02
	Painted stork (<i>Mycteria leucocephala</i>)	Tissue	06
Total	Painted stork (<i>Mycteria leucocephala</i>)	Fecal sample	02
			20

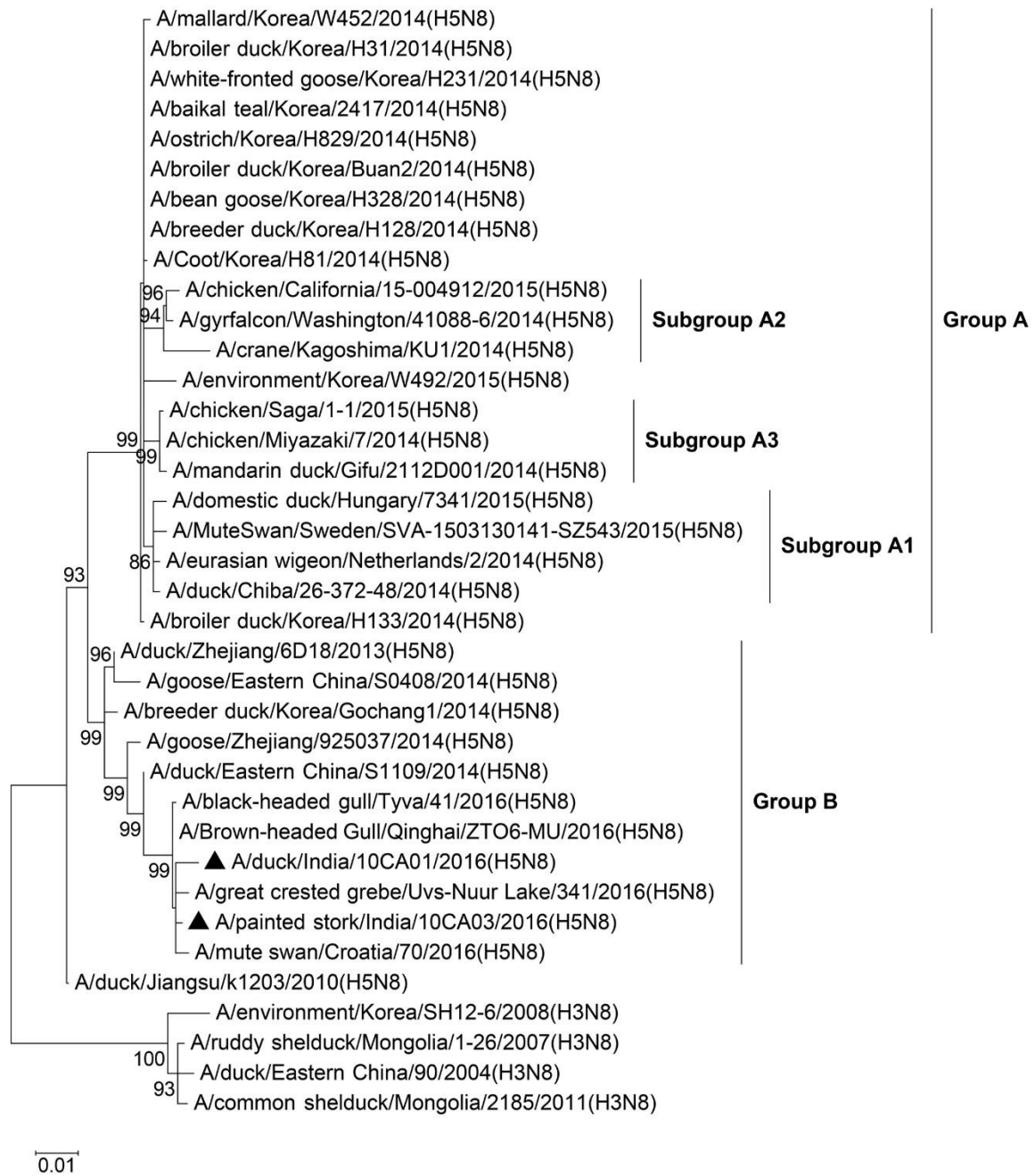
Technical Appendix 1 Table 2. Nucleotide sequence homology of H5N8 HPAI viruses isolates of India compared to available sequences in database

Segment	Position	Virus with the highest nucleotide identity	Homology (%)	<i>EpiFlu</i> / GenBank accession number*
Isolate 1: A/duck/India/10CA01/2016(H5N8)				
PB2	30–2309	A/great_crested_grebe/Uvs-Nuur_Lake/341/2016(H5N8)	99.4	EPI773754
PB1	25–2301	A/common_tern_/Uvs-Nuur_Lake/26/2016(H5N8)	99.4	EPI836612
		A/Brown-headed Gull/Qinghai/ZTO6-MU/2016(H5N8)		EPI774496
PA	25–2175	A/Bar-headed Goose/Qinghai/BTY9-LU/2016(H5N8)	99.5	EPI774258
HA	29–1735	A/great_crested_grebe/Uvs-Nuur_Lake/341/2016(H5N8)	99.3	EPI773757
		A/black-headed_gull/Tyva/41/2016(H5N8)		EPI823756
NP	46–1542	A/mallard/Republic of Georgia/13/2011(H6N2)	98.6	CY185580
NA	21–1433	A/Brown-headed_Gull/Qinghai/ZTO6-MU/2016(H5N8)	99.4	EPI774500
M	26–1004	A/great_crested_grebe/Uvs-Nuur_Lake/341/2016(H5N8)	98.9	EPI773760
NS	27–864	A/black-headed_gull/Tyva/41/2016(H5N8)	99.7	EPI823760
		A/Bar-headed Goose/Qinghai/BTY9-LU/2016(H5N8)		EPI774263
Isolate 2: A/painted stork/India/10CA03/2016(H5N8)				
PB2	30–2309	A/great_crested_grebe/Uvs-Nuur_Lake/341/2016(H5N8)	99.7	EPI773754
PB1	25–2301	A/Brown-headed Gull/Qinghai/ZTO6-MU/2016(H5N8)	99.5	EPI774496
PA	25–2175	A/northern shoveler/Georgia/1/2010(H2N3)	98.2	CY121988
HA	29–1735	A/great_crested_grebe/Uvs-Nuur_Lake/341/2016(H5N8)	99.3	EPI773757
		A/black-headed_gull/Tyva/41/2016(H5N8)		EPI823756
		A/Bar-headed_Goose/Qinghai/BTY17-LU/2016(H5N8)		EPI774394
		A/mute_swan/Croatia/85/2016(H5N8)		EPI864746
NP	46–1542	A/mallard/Republic of Georgia/3/2010(H7N3)	98.9	CY185436
NA	21–1433	A/Brown-headed_Gull/Qinghai/ZTO6-MU/2016(H5N8)	99.8	EPI774500
M	26–1004	A/great_crested_grebe/Uvs-Nuur_Lake/341/2016(H5N8)	99.0	EPI773760
NS	27–864	A/black-headed_gull/Tyva/41/2016(H5N8)	99.5	EPI823760
		A/Bar-headed Goose/Qinghai/BTY9-LU/2016(H5N8)		EPI774263

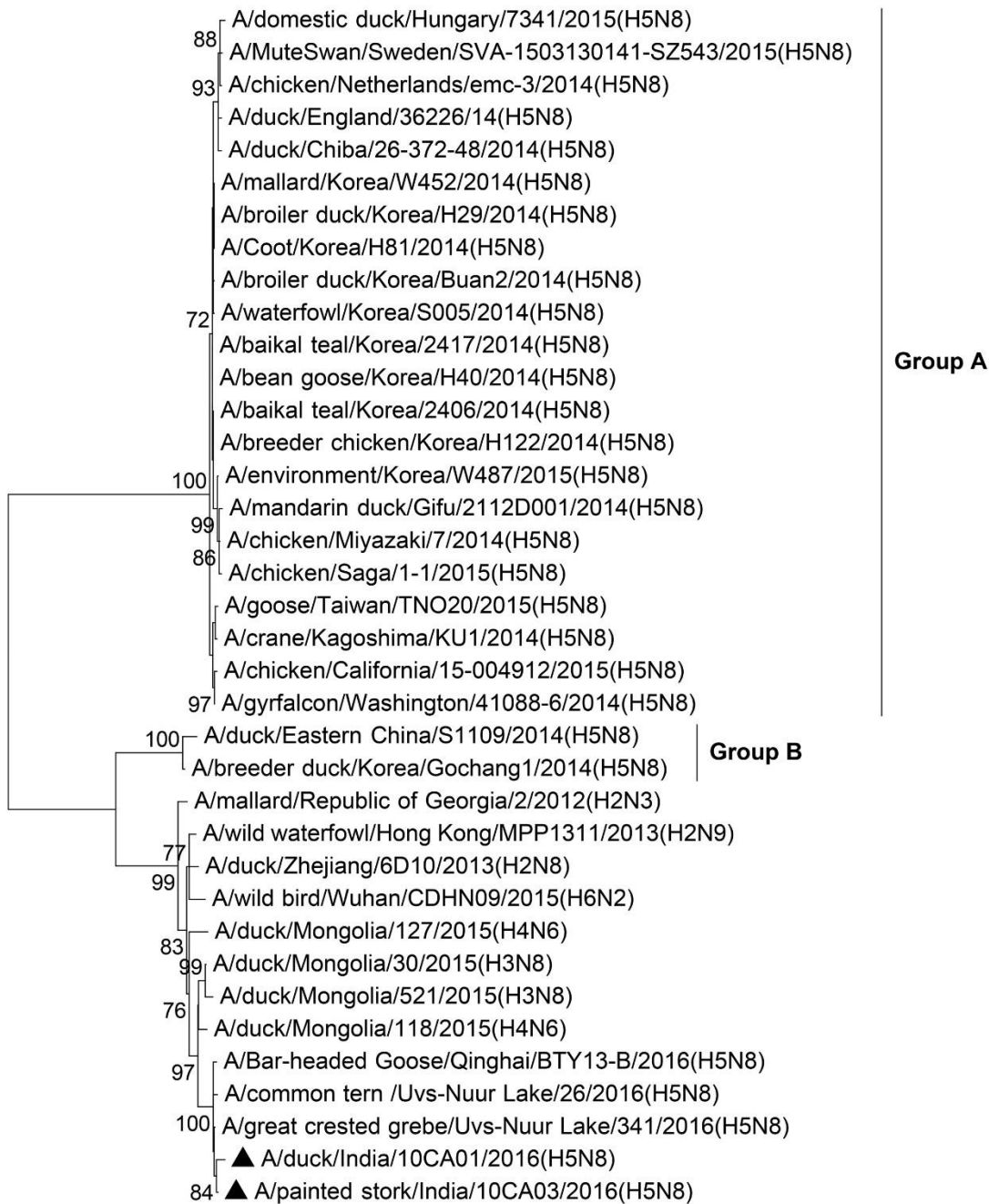
*EpiFlu Database of Global Initiative on Sharing All Influenza Data (GISAID).



Technical Appendix 1 Figure 1. Maximum-likelihood phylogenetic tree of HA gene (nucleotide positions 29–1735) of influenza A/H5 viruses. Clades and genetic groups are shown to the right. Bootstrap values ($\geq 70\%$) are shown near the nodes. Viruses sequenced in this study are highlighted with solid triangles. Scale bar indicates nucleotide substitution per site.

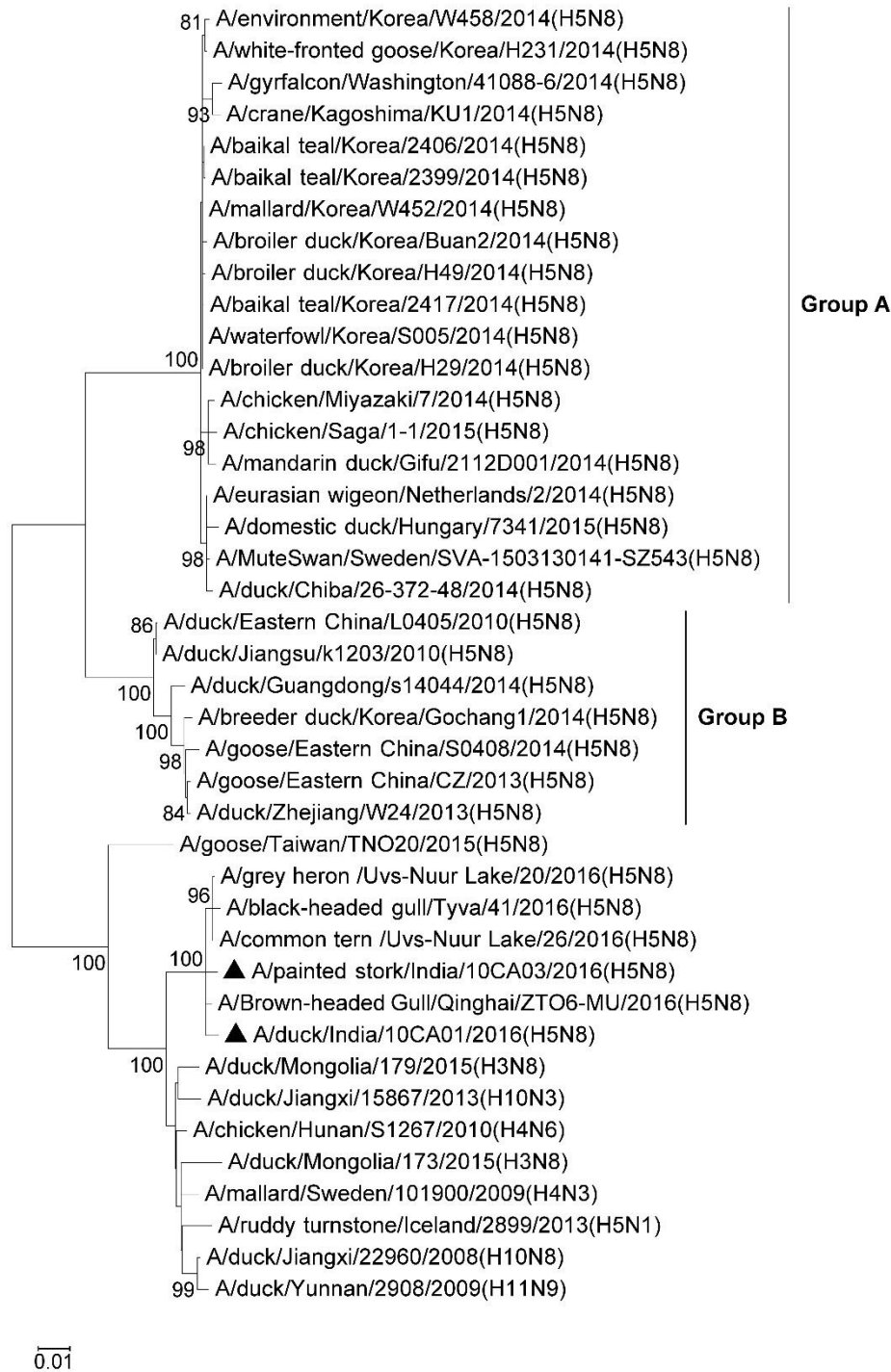


Technical Appendix 1 Figure 2. Maximum-likelihood phylogenetic tree of NA gene (nucleotide positions 21–1433) of influenza A/H5 viruses. Genetic groups are shown to the right. Bootstrap values ($\geq 70\%$) are shown near the nodes. Viruses sequenced in this study are highlighted with solid triangles. Scale bar indicates nucleotide substitution per site.

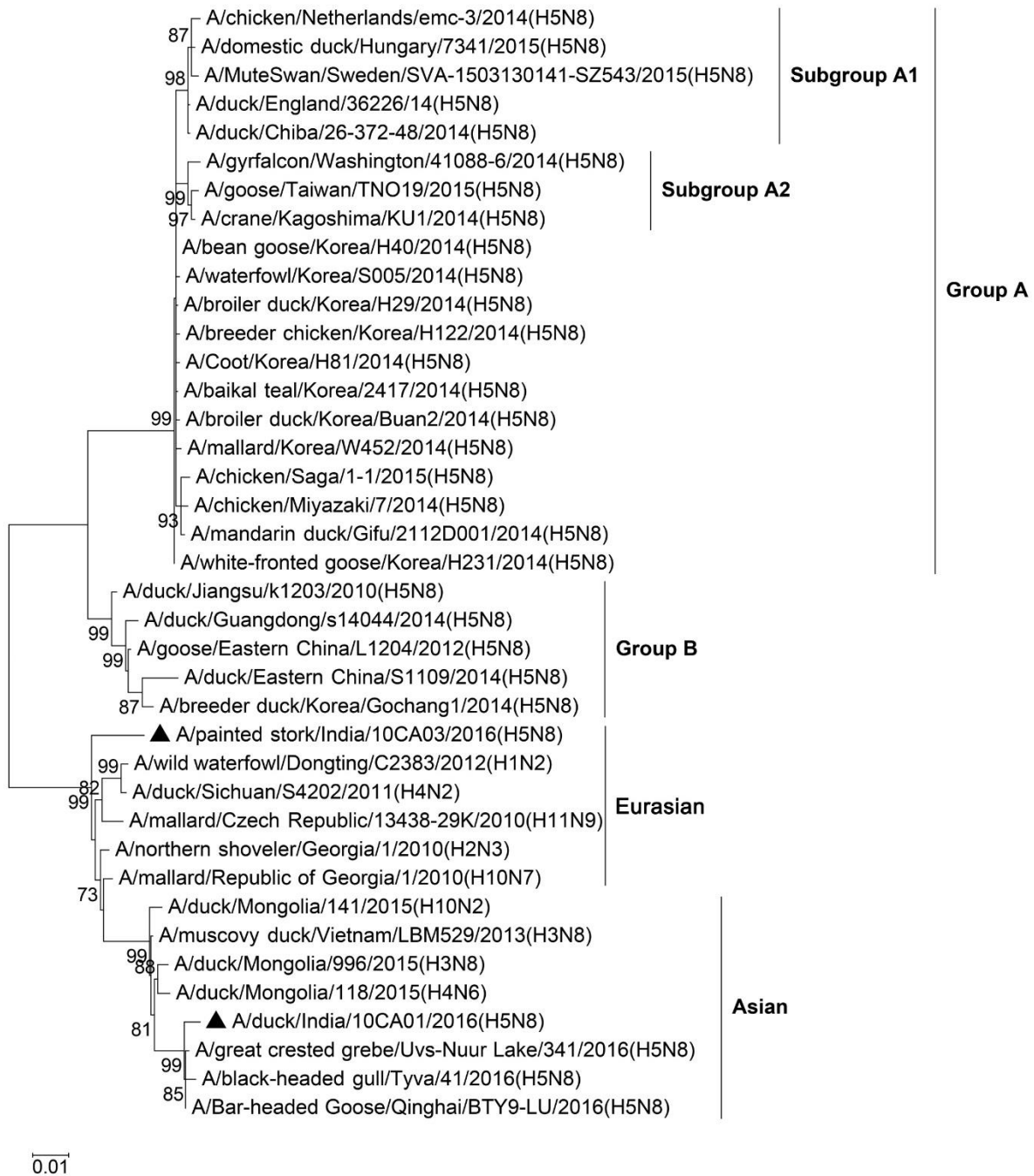


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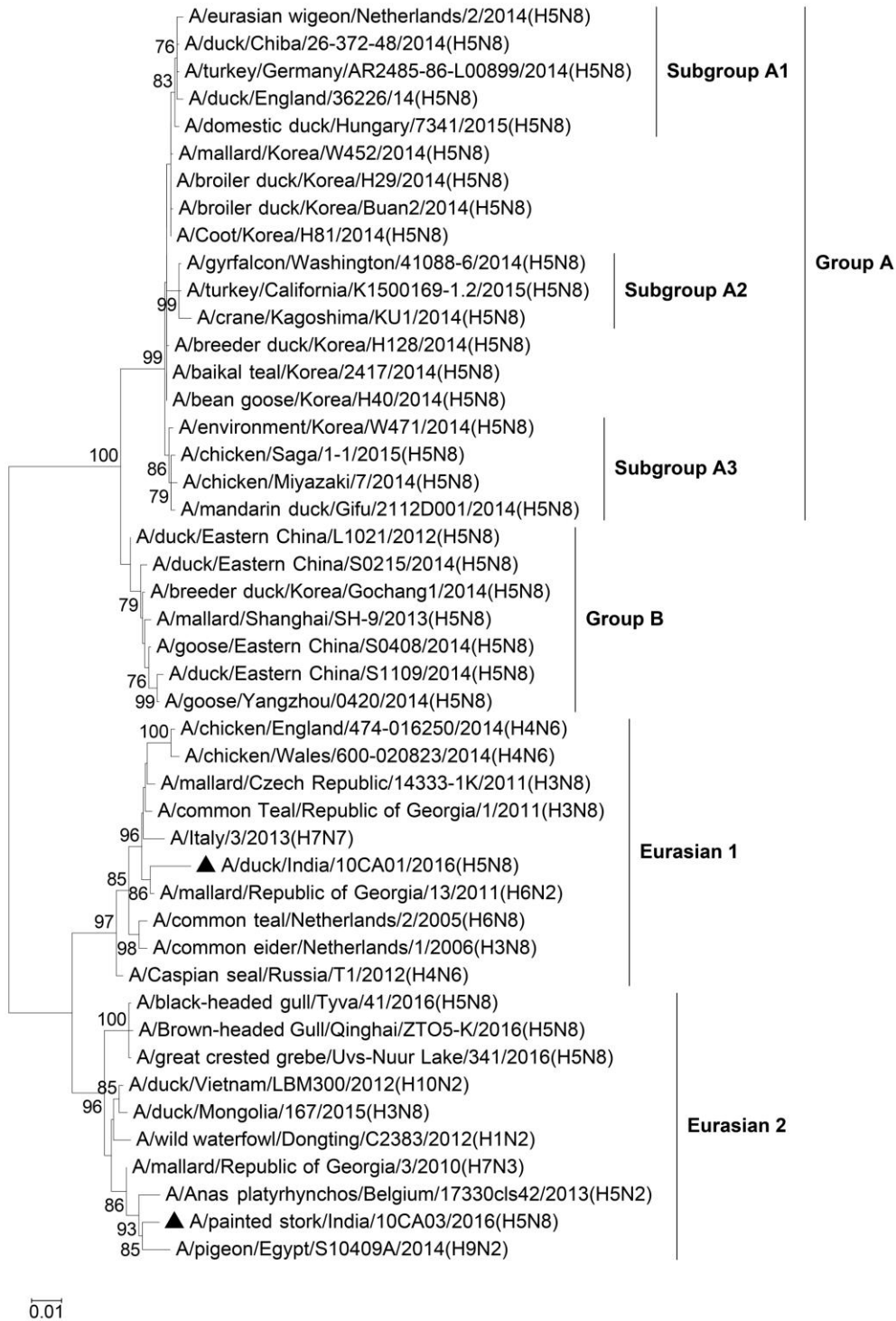
Technical Appendix 1 Figure 3. Maximum-likelihood phylogenetic tree of PB2 gene (nucleotide positions 30–2309) of influenza A/H5 viruses. Genetic groups are shown to the right. Bootstrap values ($\geq 70\%$) are shown near the nodes. Viruses sequenced in this study are highlighted with solid triangles. Scale bar indicates nucleotide substitution per site.



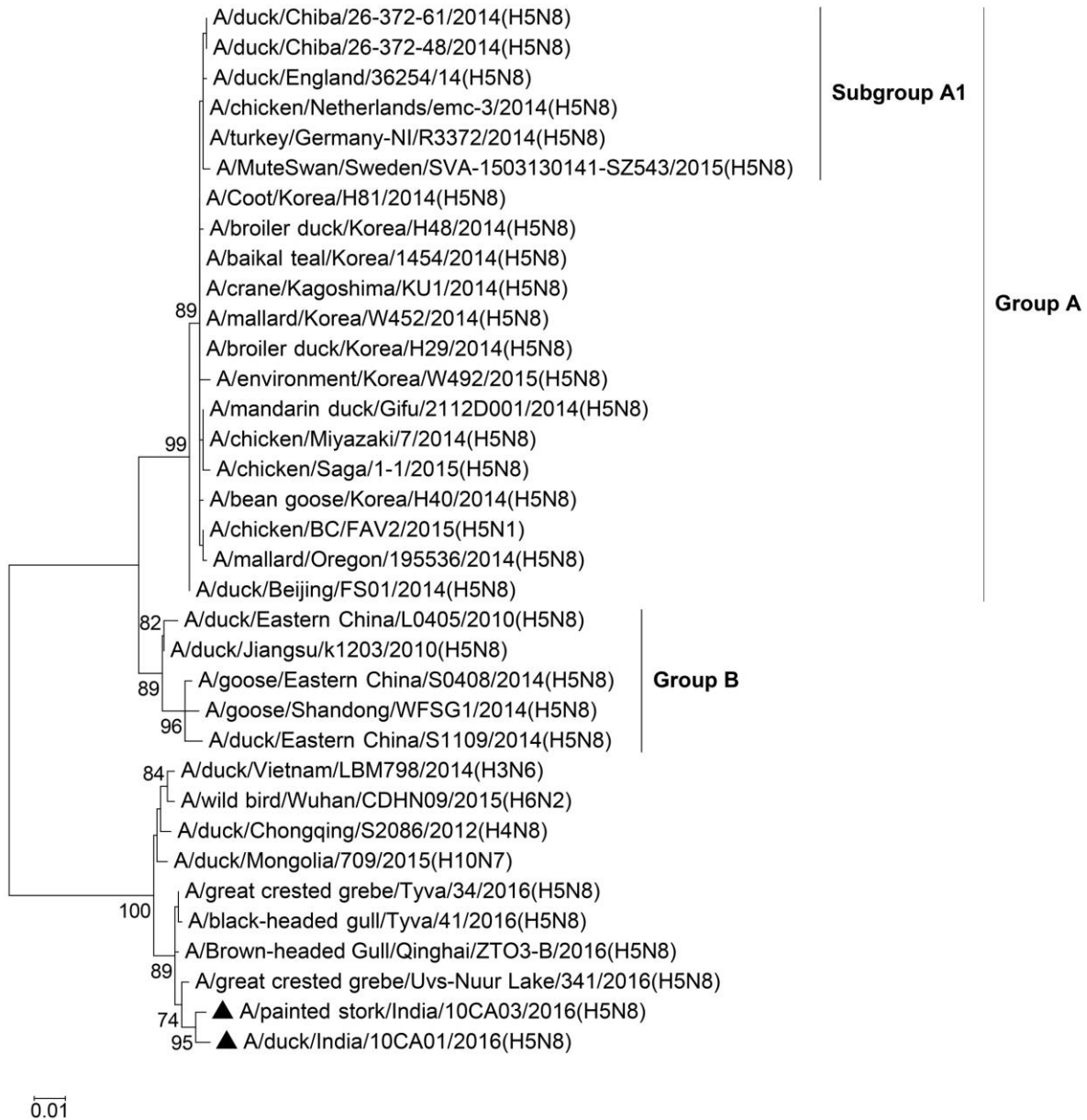
Technical Appendix 1 Figure 4. Maximum-likelihood phylogenetic tree of PB1 gene (nucleotide positions 25–2301) of influenza A/H5 viruses. Genetic groups are shown to the right. Bootstrap values ($\geq 70\%$) are shown near the nodes. Viruses sequenced in this study are highlighted with solid triangles. Scale bar indicates nucleotide substitution per site.



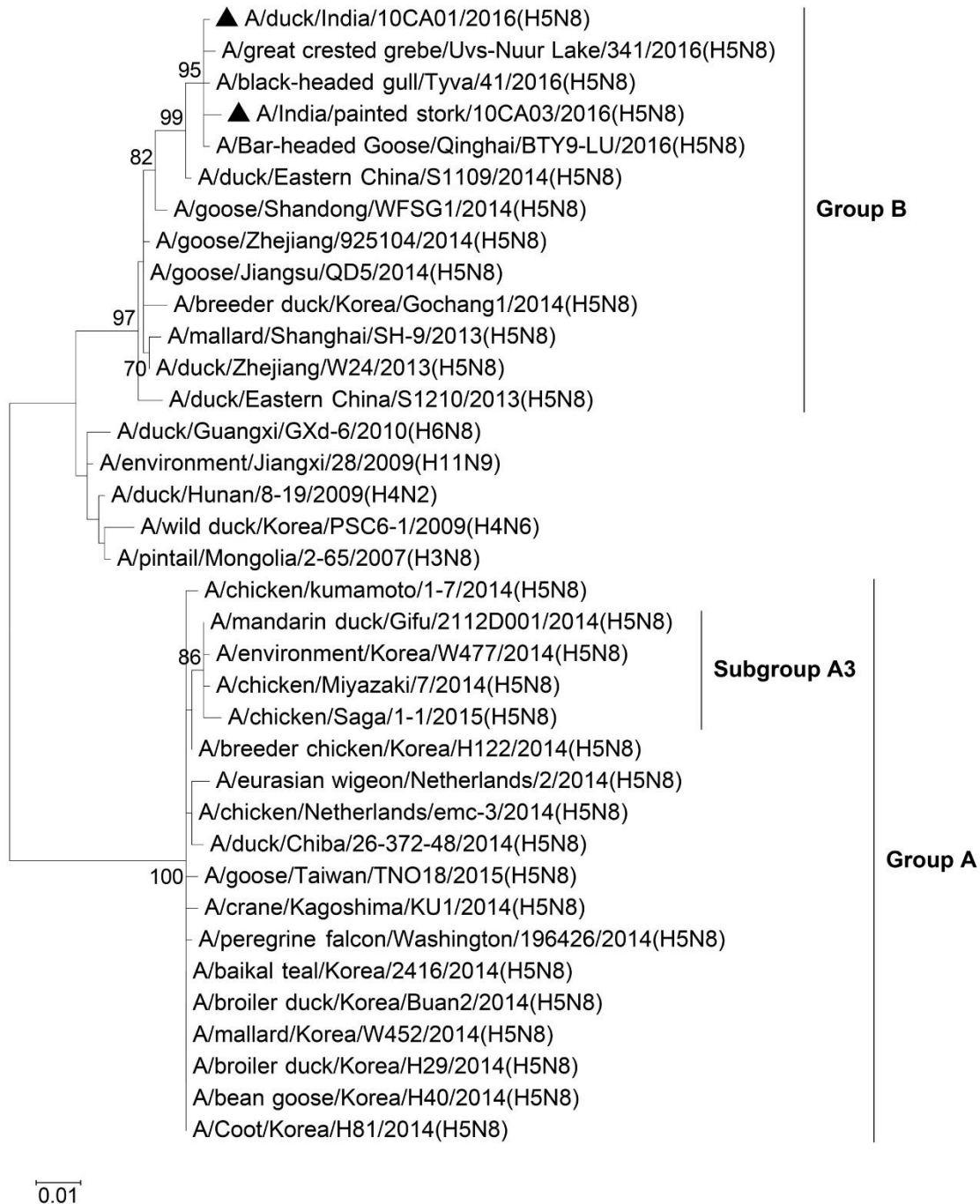
Technical Appendix 1 Figure 5. Maximum-likelihood phylogenetic tree of PA gene (nucleotide positions 25–2175) of influenza A/H5 viruses. Genetic groups are shown to the right. Bootstrap values ($\geq 70\%$) are shown near the nodes. Viruses sequenced in this study are highlighted with solid triangles. Scale bar indicates nucleotide substitution per site.



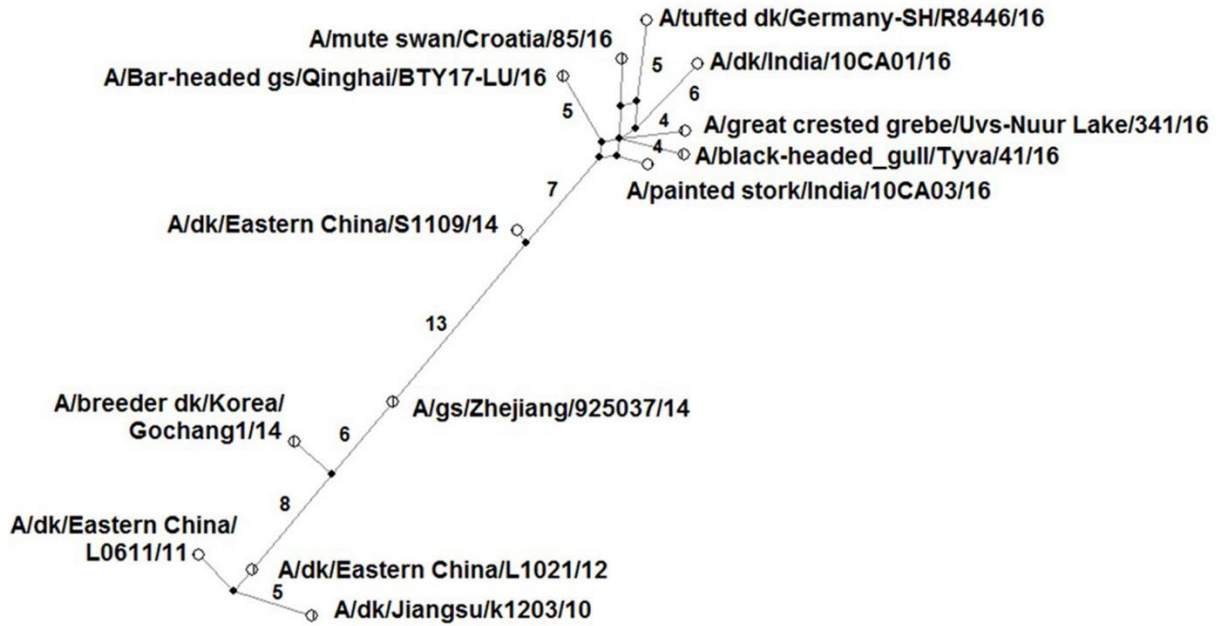
Technical Appendix 1 Figure 6. Maximum-likelihood phylogenetic tree of NP gene (nucleotide positions 46–1542) of influenza A/H5 viruses. Genetic groups are shown to the right. Bootstrap values ($\geq 70\%$) are shown near the nodes. Viruses sequenced in this study are highlighted with solid triangles. Scale bar indicates nucleotide substitution per site.



Technical Appendix 1 Figure 7. Maximum-likelihood phylogenetic tree of M gene (nucleotide positions 26–1004) of influenza A/H5 viruses. Genetic groups are shown to the right. Bootstrap values ($\geq 70\%$) are shown near the nodes. Viruses sequenced in this study are highlighted with solid triangles. Scale bar indicates nucleotide substitution per site.



Technical Appendix 1 Figure 8. Maximum-likelihood phylogenetic tree of NS gene (nucleotide positions 27–864) of influenza A/H5 viruses. Genetic groups are shown to the right. Bootstrap values ($\geq 70\%$) are shown near the nodes. Viruses sequenced in this study are highlighted with solid triangles. Scale bar indicates nucleotide substitution per site.



Technical Appendix 1 Figure 9. Median-joining phylogenetic network based on HA gene of H5N8 HPAI viruses of clade 2.3.4.4-B (as per HA gene phylogeny). Branch lengths are proportional to the number of mutations. Numbers along the branch represent the number of nucleotide substitutions distinguishing different nodes. Branches without number indicate 1–3 mutations. Dk, duck; Gs, goose.