Highly Pathogenic Avian Influenza A (H5N8) Virus in Swans, China, 2020

Appendix

Materials and Methods

Samples

Two sick swans, whooper swan (*Cygnus cygnus*) and mute swan (*Cygnus olor*) were found almost at the same site in Wuliangsuhai Lake in Bayannur city, Inner Mongolia, China (41.826234°N, 107.54972°E) on 17 October 2020. The swans died soon and virus was detected and collected from organs at the same day. We collected multiple organs (brains, larynx, liver, lung, pancreas, kidney, spleen and rectum) from two dead swans. We sequenced the H5N8 genomes directly from organs, and we used them in whole genetic analysis. The egg passages were used to confirm the genome and to get the long-term preserved viral strains. We inoculated 10-day-old specific pathogen-free chicken embryos (National Poultry Laboratory Animal Resource Center, Harbin Veterinary Research Institute, Chinese Academy of Agriculture Sciences, Harbin 150069, China) with the homogenates of mixed organs, respectively. All chicken embryos dead within 48 hours, the allantoic fluid was harvested, and the hemagglutinin (HA) activity was assayed. Subtypes of influenza viruses were identified initially by using the hemagglutination inhibition (HI) test. Viral RNA was extracted from organs or HA positive samples from incubated allantoic fluid using a QIAamp Viral RNA Mini Kit (Qiagen, Germany), reverse transcribed using the primer Un12 and subjected to RT-PCR using the method described in the WHO manual (World Health Organization [WHO], 2002) to further confirm AIV positive. The PCR products of eight fragments of the isolates were sequenced using a set of specific sequencing primers listed in a previous dissertation (*1*). The sequence data were compiled using the SeqMan program (DNASTAR, Madison, WI, United States). Two H5N8 influenza viruses, A/whooper swan/Inner Mongolia/W1–1/2020(H5N8) and A/mute swan/Inner Mongolia/W2–1/2020(H5N8), were isolated.

Genetic Analysis

A BLASTn search was performed against sequences in the GISAID database to identify the closest relatives of the two Inner Mongolia H5N8 isolates (QH-H5N8) in early January 2021, and eight datasets were derived from the top 100 BLASTn hits. The genome of clade 2.3.4.4 H5N8 viruses isolated in 2020 were also downloaded. Sequences were aligned using MAFFT (*2*) implemented in PhyloSuite 1.21 (*3*). The alignment lengths for each dataset were: PB2 2,277 nt (nt), PB1 2,271 nt, PA 2,148 nt, HA 1,681 nt, NP 1,494 nt, NA 1,407 nt, M 979 nt, NS 835 nt. For all eight datasets, sequences without full alignment length were removed. We reconstructed the phylogenetic trees using selected representative sequences of 2.3.4.4 and sequences derived from GISAID. Maximum likelihood phylogenies were inferred using IQ-TREE (*4*) under the best-fit substitution model for 10000 ultrafast bootstraps (*5*). Best-fit substitution model was selected using the Bayesian information criterion by ModelFinder (*6*) implemented in PhyloSuite 1.21 (*3*).

References

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Appendix Table 1. Virus isolates sharing the highest nucleotide similarity (top 2) with the two Inner Mongolia H5N8 isolates as identified on global initiative on sharing all influenza data (GISAID) in early January 2021

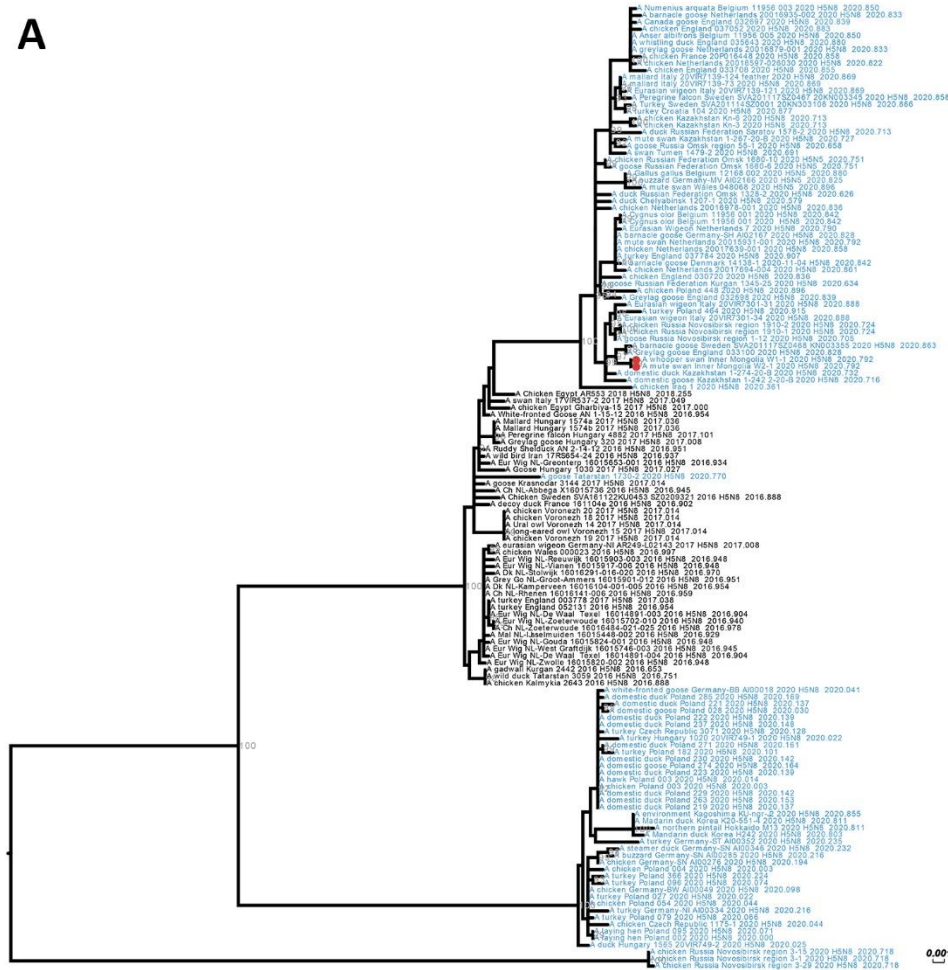| Segment	Virus name	Accession number*	similarity
A/whooper swan/Inner Mongolia/W1–1/2020(H5N8) [(A/mute swan/Inner Mongolia/W2–1/2020(H5N8)]			
PB2	A/Greylag goose/England/033100/2020(H5N8)	EPI1837929	99%
	A/barnacle goose/Sweden/SVA201117SZ0468/KN003355/2020(H5N8)	EPI1814739	99%
PB1	A/goose/Russia Novosibirsk region/1–12/2020(H5N8)	EPI1839239	99%
	A/domestic duck/Kazakhstan/1–274–20-B/2020(H5N8)	EPI1811615	99%
PA	A/domestic duck/Kazakhstan/1–274–20-B/2020(H5N8)	EPI1811615	99%
	A/goose/Russia Novosibirsk region/1–12/2020(H5N8)	EPI1839239	99%
HA	A/goose/Russia Novosibirsk region/1–12/2020(H5N8)	EPI1839239	99%
	A/domestic duck/Kazakhstan/1–274–20-B/2020(H5N8)	EPI1811615	99%
NP	A/duck/Russian Federation Omsk/1328–2/2020(H5N8)	EPI1811690	99%
	A/domestic duck/Kazakhstan/1–274–20-B/2020(H5N8)	EPI1811615	99%
NA	A/turkey/England/038115/2020(H5N8)	EPI1837953	99%
	A/turkey/England/037784/2020(H5N8)	EPI1837937	99%
M	A/barnacle goose/Sweden/SVA201117SZ0468/KN003355/2020(H5N8)	EPI1814739	99%
	A/Greylag_goose/England/033100/2020(H5N8)	EPI1837929	99%
NS	A/chicken/England/037052/2020(H5N8)	EPI1837911	99%
	A/Eurasian wigeon/Italy/20VIR7301–34/2020(H5N8)	EPI1815377	99%

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

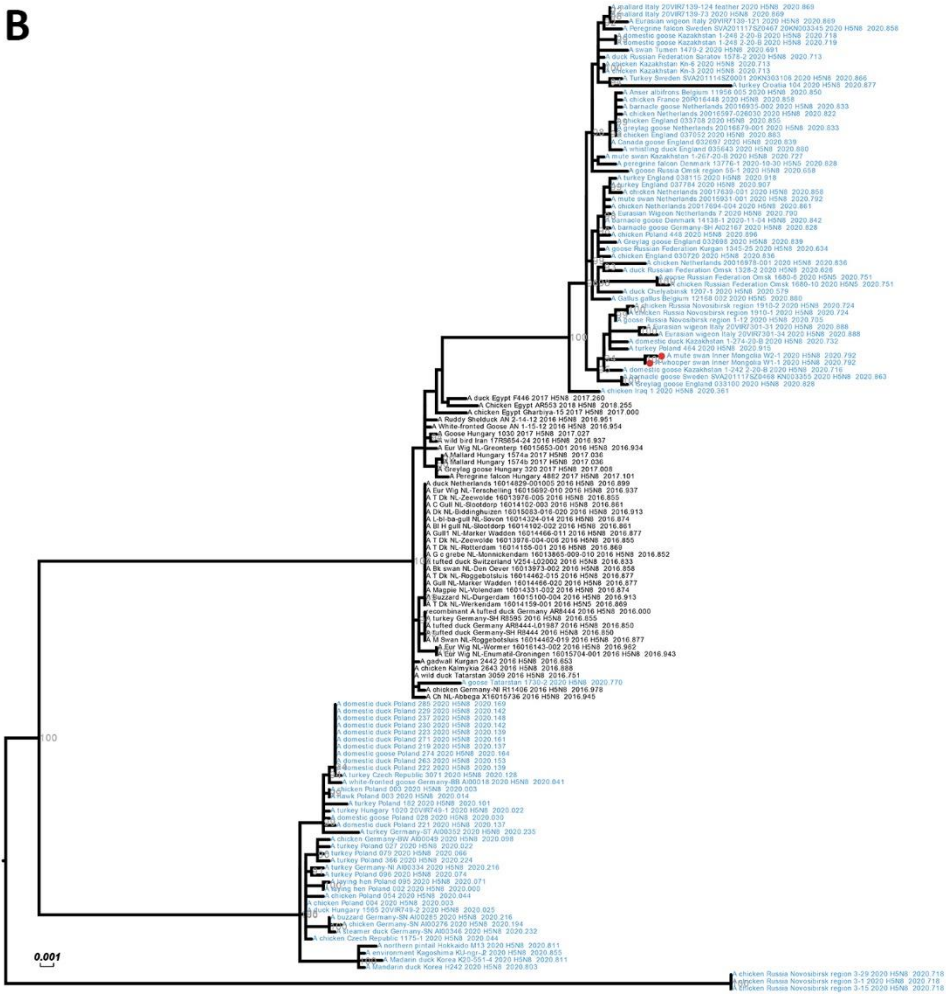**Appendix Table 2.** Information of viruses related the two Inner Mongolia isolates according to the HA genes

Date	Location	Isolate name	Isolate ID
2020-May-12	Iraq	A/chicken/Iraq/1/2020(H5N8)	EPI_ISL_623074
2020-Sep-18	Kazakhstan	A/chicken/Kazakhstan/Kn-3/2020(H5N8)	EPI_ISL_739686
2020-Sep-18	Kazakhstan	A/chicken/Kazakhstan/Kn-6/2020(H5N8)	EPI_ISL_739687
2020-Sep-19	Kazakhstan	A/domestic goose/Kazakhstan/1–242_2–20-B/2020(H5N8)	EPI_ISL_615073
2020-Sep-20	Kazakhstan	A/domestic goose/Kazakhstan/1–248_2–20-B/2020(H5N8)	EPI_ISL_615068
2020-Sep-22	Kazakhstan	A/domestic chicken/Kazakhstan/1–261_1–20-B/2020(H5N8)	EPI_ISL_615070
2020-Sep-23	Kazakhstan	A/mute swan/Kazakhstan/1–267–20-B/2020(H5N8)	EPI_ISL_614401
2020-Sep-25	Kazakhstan	A/domestic duck/Kazakhstan/1–274–20-B/2020(H5N8)	EPI_ISL_615072
2020-Jul-31	Russia	A/duck/Chelyabinsk/1207–1/2020(H5N8)	EPI_ISL_637098
2020-Aug-17	Russia	A/duck/Russian Federation Omsk/1328–2/2020(H5N8)	EPI_ISL_626650
2020-Sep-10	Russia	A/swan/Tumen/1479–2/2020(H5N8)	EPI_ISL_661178
2020-Sep-15	Russia	A/goose/Russia Novosibirsk region/1–12/2020(H5N8)	EPI_ISL_739684
2020-Sep-18	Russia	A/duck/Russian Federation/Saratov/1578–2/2020 (H5N8)	EPI_ISL_626649
2020-Sep-20	Russia	A/chicken/Russia Novosibirsk region/3–1/2020(H5N8)	EPI_ISL_739690
2020-Sep-20	Russia	A/chicken/Russia Novosibirsk region/3–15/2020(H5N8)	EPI_ISL_739691
2020-Sep-20	Russia	A/chicken/Russia Novosibirsk region/3–29/2020(H5N8)	EPI_ISL_739692
2020-Sep-22	Russia	A/chicken/Russia Novosibirsk region/1910–1/2020(H5N8)	EPI_ISL_739688
2020-Sep-22	Russia	A/chicken/Russia Novosibirsk region/1910–2/2020(H5N8)	EPI_ISL_739689
2020-Oct-02	Russia	A/chicken/Russian Federation/Omsk/1680–10/2020(H5N5)	EPI_ISL_626647
2020-Oct-02	Russia	A/goose/Russian Federation/Omsk/1680–6/2020(H5N5)	EPI_ISL_626648
2020-Oct-16	Netherlands	A/Eurasian Wigeon/Netherlands/1/2020(H5N1)	EPI_ISL_603133
2020-Oct-16	Netherlands	A/Eurasian Wigeon/Netherlands/4/2020(H5N1)	EPI_ISL_603134
2020-Oct-16	Netherlands	A/Eurasian Wigeon/Netherlands/5/2020(H5N1)	EPI_ISL_603135
2020-Oct-16	Netherlands	A/Eurasian Wigeon/Netherlands/7/2020(H5N8)	EPI_ISL_603136
2020-Oct-17	Netherlands	A/mute swan/Netherlands/20015931–001/2020(H5N8)	EPI_ISL_591075
2020-Oct-28	Netherlands	A/chicken/Netherlands/20016597–026030/2020(H5N8)	EPI_ISL_603132
2020-Oct-28	Netherlands	A/greylag goose/Netherlands/20016582–004/2020(H5N1)	EPI_ISL_632314
2020-Nov-01	Netherlands	A/barnacle goose/Netherlands/20016935–002/2020(H5N8)	EPI_ISL_632317
2020-Nov-01	Netherlands	A/greylag goose/Netherlands/20016879–001/2020(H5N8)	EPI_ISL_632318
2020-Nov-02	Netherlands	A/eurasian teal/Netherlands/20016896–013/2020(H5N1)	EPI_ISL_632315
2020-Nov-02	Netherlands	A/chicken/Netherlands/20016978–001/2020(H5N8)	EPI_ISL_641377
2020-Nov-10	Netherlands	A/chicken/Netherlands/20017639–001/2020(H5N8)	EPI_ISL_641394
2020-Nov-11	Netherlands	A/chicken/Netherlands/20017694–004/2020(H5N8)	EPI_ISL_641395
2020-Dec-14	Netherlands	A/chicken/Netherlands/20019879–001005/2020(H5N1)	EPI_ISL_711055
2020-Oct-29	Germany	A/buzzard/Germany-MV/AI02166/2020(H5N5)	EPI_ISL_614399
2020-Oct-30	Germany	A/barnacle goose/Germany-SH/AI02167/2020(H5N8)	EPI_ISL_614400
2020-Oct-30	Denmark	A/peregrine falcon/Denmark/13776–1/2020–10–30(H5N5)	EPI_ISL_644737
2020-Nov-04	Denmark	A/barnacle goose/Denmark/14138–1/2020–11–04(H5N8)	EPI_ISL_644824
2020-Oct-30	England	A/Greylag goose/England/033100/2020(H5N8)	EPI_ISL_710508
2020-Nov-02	England	A/chicken/England/030720/2020(H5N8)	EPI_ISL_626652
2020-Nov-03	England	A/Canada goose/England/032697/2020(H5N8)	EPI_ISL_710506
2020-Nov-03	England	A/Greylag goose/England/032698/2020(H5N8)	EPI_ISL_710507
2020-Nov-09	England	A/chicken/England/033708/2020(H5N8)	EPI_ISL_710509
2020-Nov-18	England	A/whistling duck/England/035643/2020(H5N8)	EPI_ISL_710512
2020-Nov-19	England	A/chicken/England/037052/2020(H5N8)	EPI_ISL_710511
2020-Nov-24	England	A/mute swan/Wales/048068/2020(H5N5)	EPI_ISL_683999
2020-Nov-28	England	A/turkey/England/037784/2020(H5N8)	EPI_ISL_710504
2020-Dec-02	England	A/turkey/England/038115/2020(H5N8)	EPI_ISL_710505
2020-Nov-04	Belgium	A/Cygnus olor/Belgium/11956_001/2020(H5N8)	EPI_ISL_644735
2020-Nov-07	Belgium	A/Numenius arquata/Belgium/11956_003/2020(H5N8)	EPI_ISL_664102
2020-Nov-07	Belgium	A/Anser albifrons/Belgium/11956_005/2020(H5N8)	EPI_ISL_661313
2020-Nov-18	Belgium	A/Gallus gallus/Belgium/12168_002/2020(H5N5)	EPI_ISL_660264
2020-Nov-10	Sweden	A/Peregrine falcon/Sweden/SVA201117SZ0467/20KN003345/2020(H5N8)	EPI_ISL_668456
2020-Nov-12	Sweden	A/barnacle goose/Sweden/SVA201117SZ0468/KN003355/2020(H5N8)	EPI_ISL_668457
2020-Nov-13	Sweden	A/Turkey/Sweden/SVA201114SZ0001/20KN303106/2020(H5N8)	EPI_ISL_647969
2020-Nov-10	France	A/chicken/France/20P016448/2020(H5N8)	EPI_ISL_667810
2020-Nov-14	Italy	A/mallard/Italy/20VIR7139–124_feather/2020(H5N8)	EPI_ISL_683594
2020-Nov-14	Italy	A/mallard/Italy/20VIR7139–73/2020(H5N8)	EPI_ISL_654958
2020-Nov-14	Italy	A/Eurasian wigeon/Italy/20VIR7139–121/2020(H5N8)	EPI_ISL_683593
2020-Nov-21	Italy	A/Eurasian wigeon/Italy/20VIR7301–206/2020(H5N1)	EPI_ISL_683592
2020-Nov-21	Italy	A/Eurasian wigeon/Italy/20VIR7301–31/2020(H5N8)	EPI_ISL_683751
2020-Nov-21	Italy	A/Eurasian wigeon/Italy/20VIR7301–34/2020(H5N8)	EPI_ISL_683752
2020-Nov-24	Poland	A/chicken/Poland/448/2020(H5N8)	EPI_ISL_661177
2020-Dec-01	Poland	A/turkey/Poland/464/2020(H5N8)	EPI_ISL_779129

Appendix Figure. Maximum-likelihood phylogenetic trees. Our Inner Mongolia H5N8 isolates were marked with red circle and viruses isolated in 2020 are shown in blue. A UFBoot support values ≥90 was shown. Segments shown: A) polymerase basic; B) polymerase basic; C) polymerase; D) hemagglutinin; E) nucleoprotein; F) neuraminidase; G) matrix protein; H) nonstructural protein.
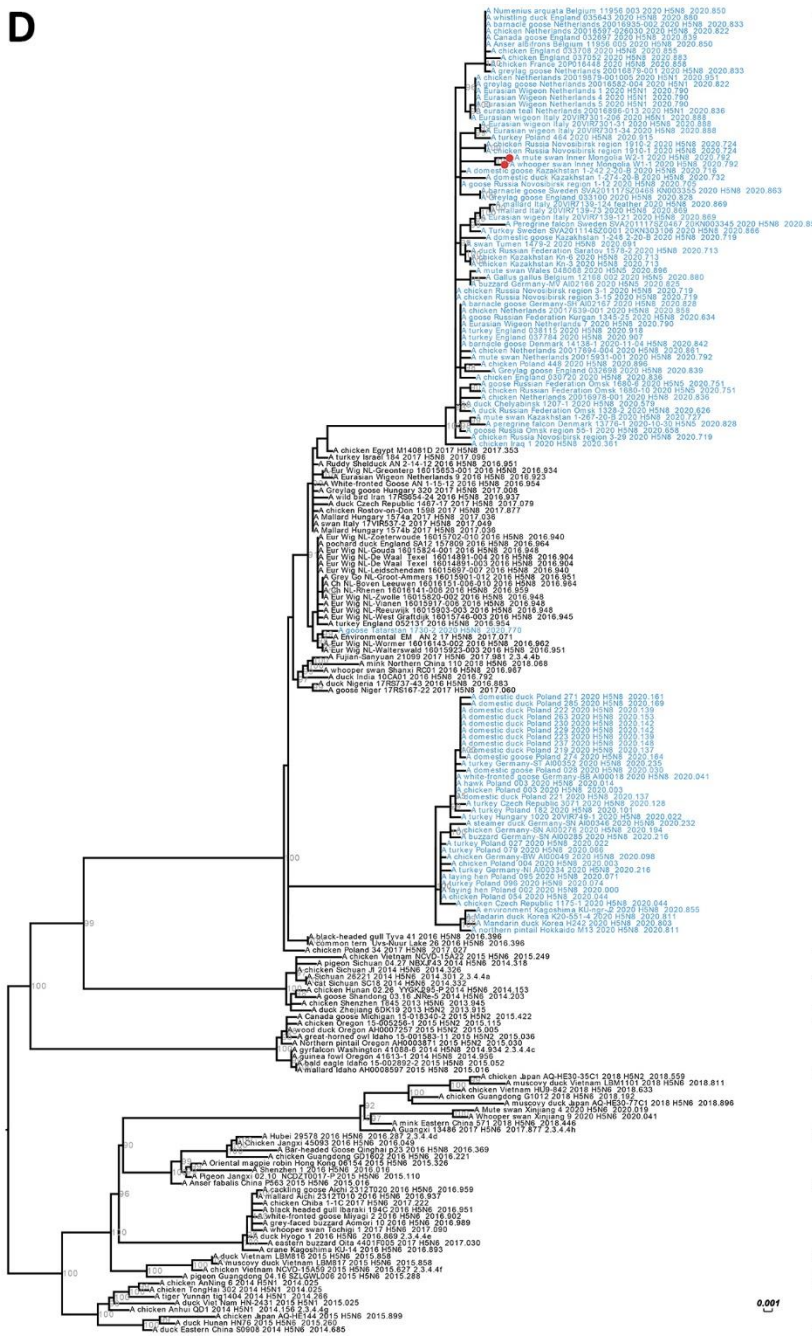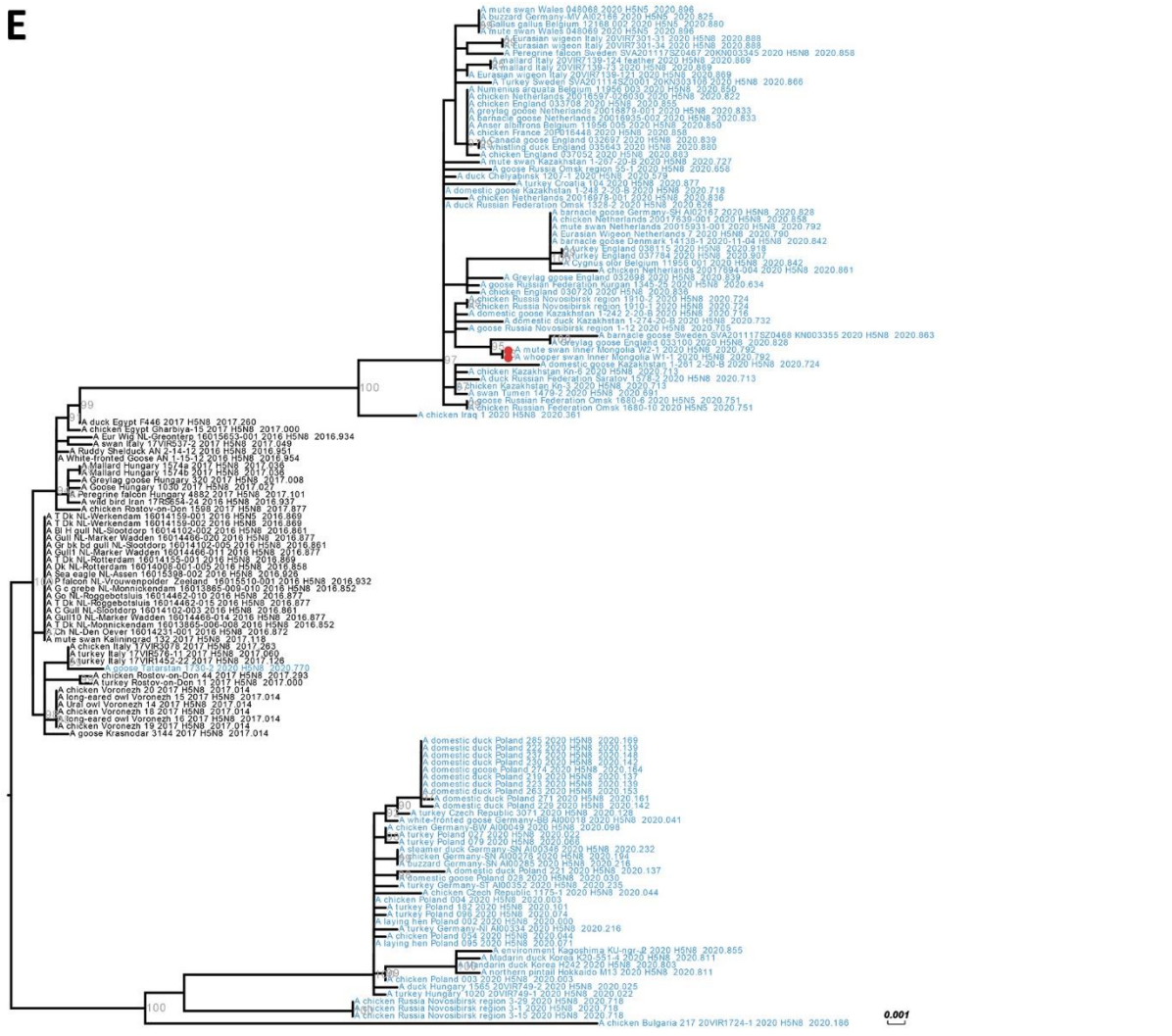
B

C

D

2.3.4.4b

2.3.4.4a

2.3.4.4c

2.3.4.4h

2.3.4.4d

2.3.4.4e

2.3.4.4f

2.3.4.4g

0.001

E

A mute swan Wales 048068 2020 H5N5_2020.896
A buzzard Germany-MV AI02188 2020 H5N5_2020.825
A Gallus gallus Belgium 12188 002 2020 H5N8_2020.880
A mute swan Wales 048069 2020 H5N5_2020.896
A Eurasian wigeon Italy 20VIR7301-31 2020 H5N8_2020.888
A Eurasian wigeon Italy 20VIR7301-34 2020 H5N8_2020.888
A Peregrine falcon Sweden SVA20111175Z0467 20KN003345 2020 H5N8_2020.858
A mallard Italy 20VIR7139-124 feather 2020 H5N8_2020.869
A mallard Italy 20VIR7139-124 2020 H5N8_2020.869
A Eurasian wigeon Italy 20VIR7139-121 2020 H5N8_2020.869
A Turkey Sweden SVA20111145Z0001 20KN003108 2020 H5N8_2020.866
A Numenius arquata Belgium 11956 005 2020 H5N8_2020.850
A chicken Netherlands 20016567 026020 2020 H5N8_2020.622
A greylag goose Netherlands 20016679-001 2020 H5N5_2020.833
A barnacle goose Netherlands 20016935-002 2020 H5N8_2020.833
A Anser albifrons Belgium 11956 006 2020 H5N8_2020.850
A chicken England 033708 2020 H5N8_2020.858
A Canada goose England 032697 2020 H5N8_2020.839
A Whistling duck England 032643 2020 H5N8_2020.880
A chicken England 037052 2020 H5N8_2020.803
A mute swan Kazakhstan 1-267-20-B 2020 H5N8_2020.727
A goose Russia Omsk region 55-1 2020 H5N8_2020.656
A duck Chelyabinsk 1207-1 2020 H5N8_2020.579
A turkey Croatia 184 2020 H5N8_2020.877
A domestic goose Kazakhstan 1-248 2-20-B 2020 H5N8_2020.718
A chicken Netherlands 20016678-001 2020 H5N5_2020.836
A duck Russian Federation Omsk 1328-2 2020 H5N8_2020.828
A barnacle goose Germany-SH AI02167 2020 H5N8_2020.828
A chicken Netherlands 20017839-001 2020 H5N5_2020.858
A mute swan Netherlands 20015931-001 2020 H5N8_2020.792
A Eurasian Wigeon Netherlands 7 2020 H5N8_2020.790
A barnacle goose Denmark 14138-1 2020-11-04 H5N8_2020.842
A turkey England 038115 2020 H5N8_2020.918
A turkey England 037784 2020 H5N8_2020.907
A chicken Netherlands 20017894-004 2020 H5N8_2020.861
A Cygnus olor Belgium 11956 001 2020 H5N8_2020.850
A Greylag goose England 032898 2020 H5N8_2020.828
A goose Russian Federation Kurgan 1345-25 2020 H5N8_2020.634
A chicken England 030720 2020 H5N8_2020.836
A chicken Russia Novosibirsk region 1910-2 2020 H5N8_2020.724
A chicken Russia Novosibirsk region 1910-1 2020 H5N8_2020.724
A domestic goose Kazakhstan 1-242 2-20-B 2020 H5N8_2020.718
A domestic duck Kazakhstan 1-274-20-B 2020 H5N8_2020.732
A goose Russia Novosibirsk region 1-12 2020 H5N8_2020.705
A barnacle goose Sweden SVA20111175Z0468 KN003355 2020 H5N8_2020.863
A Greylag goose England 033100 2020 H5N8_2020.828
A mute swan Inner Mongolia W2-1 2020 H5N8_2020.792
A whopper swan Inner Mongolia W1-1 2020 H5N8_2020.792
A chicken Kazakhstan Kri-6 2020 H5N8_2020.724
A domestic goose Kazakhstan 1-261 2-20-B 2020 H5N8_2020.724
A duck Russian Federation Saratov 1578-2 2020 H5N8_2020.713
A chicken Kazakhstan Kri-3 2020 H5N8_2020.713
A swan Tumen 1479-2 2020 H5N8_2020.691
A goose Russian Federation Omsk 1680-6 2020 H5N5_2020.751
A chicken Russian Federation Omsk 1680-10 2020 H5N8_2020.751
A chicken Iraq 1 2020 H5N8_2020.361

A duck Egypt F446 2017 H5N8_2017.260
A chicken Egypt Gharbiya-15 2017 H5N8_2017.000
A Eur Wid NL-Greonterp 16015653-001 2016 H5N8_2016.934
A swan Italy 17VIR537-2 2017 H5N8_2017.049
A Ruddy Shelduck AN 2-14-12 2016 H5N8_2016.951
A White-fronted Goose AN_1-15-12 2016 H5N8_2016.954
A Mallard Hungary 15748 2017 H5N8_2017.038
A Mallard Hungary 15746 2017 H5N8_2017.038
A Greylag goose Hungary 320 2017 H5N8_2017.008
A Goose Hungary 1030 2017 H5N8_2017.022
A Peregrine falcon Hungary 4882 2017 H5N8_2017.101
A wild bird Iran 17R8654-24 2016 H5N8_2016.937
A chicken Rostov-on-Don 1598 2017 H5N8_2017.877
A T Dk NL-Werkendam 16014159-001 2016 H5N5_2016.869
A T Dk NL-Werkendam 16014159-002 2016 H5N8_2016.869
A Bl H gull NL-Slootdorp 16014102-002 2016 H5N8_2016.861
A Gr bk bd gull NL-Slootdorp 16014446-020 2016 H5N8_2016.877
A Gull NL-Marker Wadden 16014102-005 2016 H5N8_2016.877
A T Dk NL-Rotterdam 16014155-001 2016 H5N8_2016.869
A Dk NL-Rotterdam 16014008-001-005 2016 H5N8_2016.628
A Sea eagle NL-Assen 16015398-002 2016 H5N8_2016.932
A P falcon NL-Vrouwenpolder Zeeland 16015510-001 2016 H5N8_2016.932
A G e grebe NL-Nbonnickendam 18013865-009-010 2016 H5N8_2016.852
A Go NL-Roggebotsluis 16014462-010 2016 H5N8_2016.877
A T Dk NL-Roggebotsluis 16014462-015 2016 H5N8_2016.877
A C Gull NL-Slootdorp 16014102-003 2016 H5N8_2016.861
A Gull10 NL-Marker Wadden 16014466-014 2016 H5N8_2016.877
A T Dk NL-Monnickendam 16013865-006-008 2016 H5N8_2016.852
A Cn NL-Den Oever 16014231-001 2016 H5N8_2016.872
A mute swan Kaliningrad 132 2017 H5N8_2017.118
A chicken Italy 17VIR3078 2017 H5N8_2017.263
A turkey Italy 17VIR576-11 2017 H5N8_2017.060
A turkey Italy 17VIR1452-22 2017 H5N8_2017.126
A goose Tatarstan 1730-2 2020 H5N8_2020.770
A chicken Rostov-on-Don 44 2017 H5N8_2017.000
A chicken Rostov-on-Don 11 2017 H5N8_2017.293
A chicken Voronezh 20 2017 H5N8_2017.014
A long-eared owl Voronezh 15 2017 H5N8_2017.014
A Ural owl Voronezh 14 2017 H5N8_2017.839
A chicken Voronezh 18 2017 H5N8_2017.014
A long-eared owl Voronezh 16 2017 H5N8_2017.014
A chicken Voronezh 19 2017 H5N8_2017.014
A goose Krasnodar 3144 2017 H5N8_2017.014

A domestic duck Poland 285 2020 H5N8_2020.169
A domestic duck Poland 335 2020 H5N8_2020.139
A domestic duck Poland 333 2020 H5N8_2020.148
A domestic goose Poland 274 2020 H5N8_2020.164
A domestic duck Poland 219 2020 H5N8_2020.137
A domestic duck Poland 323 2020 H5N8_2020.139
A domestic duck Poland 263 2020 H5N8_2020.153
A domestic duck Poland 271 2020 H5N8_2020.161
A domestic duck Poland 229 2020 H5N8_2020.142
A turkey Czech Republic 3071 2020 H5N8_2020.128
A white-fronted goose Germany-BB AI00018 2020 H5N8_2020.041
A chicken Germany-BW AI00049 2020 H5N8_2020.098
A turkey Poland 077 2020 H5N8_2020.083
A turkey Poland 079 2020 H5N8_2020.088
A shoveler duck Germany-SN AI00348 2020 H5N8_2020.232
A chicken Germany-SN AI00285 2020 H5N8_2020.216
A buzzard Germany-SN AI00285 2020 H5N8_2020.137
A domestic goose Poland 221 2020 H5N8_2020.030
A turkey Germany-ST AI00352 2020 H5N8_2020.235
A chicken Czech Republic 1175-1 2020 H5N8_2020.044
A chicken Poland 004 2020 H5N8_2020.003
A turkey Poland 182 2020 H5N8_2020.101
A turkey Poland 096 2020 H5N8_2020.074
A laying hen Poland 002 2020 H5N8_2020.216
A turkey Germany-NI AI00334 2020 H5N8_2020.003
A chicken Poland 054 2020 H5N8_2020.044
A laying hen Poland 095 2020 H5N8_2020.071
A environment Kagoshima KU-ngr-2 2020 H5N8_2020.855
A Madarin duck Korea K20-551-4 2020 H5N8_2020.811
A chicken Poland 003 2020 H5N8_2020.003
A Mandarin duck Korea H242 2020 H5N8_2020.803
A northern pintail Hokkaido M13 2020 H5N8_2020.811
A duck Hungary 1565 20VIR749-2 2020 H5N8_2020.025
A turkey Hungary 1020 20VIR749-1 2020 H5N8_2020.025
A chicken Russia Novosibirsk region 3-29 2020 H5N8_2020.718
A chicken Russia Novosibirsk region 3-1 2020 H5N8_2020.718
A chicken Russia Novosibirsk region 3-15 2020 H5N8_2020.718
A chicken Bulgaria 217 20VIR1724-1 2020 H5N8_2020.186

0.001

F

0.01

G

H

0.001