
Highly Pathogenic Avian Influenza A(H5N8) Virus, Democratic Republic of the Congo, 2017

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In 2017, highly pathogenic avian influenza A(H5N8) virus was detected in poultry in the Democratic Republic of the Congo. Whole-genome phylogeny showed the virus clustered with H5N8 clade 2.3.4.4B strains from birds in central and southern Asia. Emergence of this virus in central Africa represents a threat for animal health and food security.

The detection of highly pathogenic avian influenza (HPAI) infections in poultry has greatly increased in the past decades, in particular as a consequence of the spread of the HPAI virus subtype H5, descendent of the H5N1 virus A/goose/Guangdong/1/1996 (Gs/GD), which was detected in China in 1996 (1). The evolution of the Gs/GD H5 lineage has resulted in the emergence of multiple clades characterized by distinct antigenic properties and zoonotic potential (2). Among them, the HPAI H5 clade 2.3.4.4 has stood out for its concerning ability to reassort and combine with different neuraminidase (NA) subtypes and to spread rapidly to and within multiple continents (3).

In late 2016, a reassortant HPAI H5N8 virus (clade 2.3.4.4 group B) began to spread from China (4) and the Russian Federation (5) to Asia, the Middle East, Europe, and western Africa and for the first time reached central, eastern, and southern Africa. Egypt, Tunisia, and Nigeria reported HPAI H5N8 virus in late autumn 2016, and virus detection continued to occur across Africa in the winter, spring, and summer of 2017 (6). This study provides

insights from the epidemiologic and viral genome analysis on the outbreaks in the Democratic Republic of the Congo (DRC).

The Study

In late April 2017, high death rates in domestic chickens and ducks were reported in 4 localities of the Ituri province (Bunia territory) of DRC, which is situated at the edge of Albert Lake between the Rwenzori Mountains and the Republic of Uganda (Figure 1). Because this outbreak followed an HPAI H5N8 outbreak in Uganda in January 2017 (7,8), this alert led to a strong suspicion of HPAI.

Clinical signs in the affected poultry included prostration, dyspnea, yellowish-colored diarrhea, generalized weakness, torticollis, and, in some cases, recumbency before death. Necropsies on carcasses revealed petechiae, hemorrhage, or both in all organs; hemorrhagic liver with soft consistency; and an empty gizzard with epithelial hemorrhage.

We sampled 22 birds (9 duck carcasses, 12 live ducks, and 1 live chicken) in the 4 infected villages. We collected tracheal and cloacal swabs from living birds showing clinical signs and collected organs including lung, intestine, trachea, and heart from dead birds.

We performed a rapid test for avian influenza virus (AIV) type A detection in the field using the AIV Ag Test Kit (BioNote, Hwaseong-si, South Korea). Of the 22 birds sampled, 6 ducks tested positive with the rapid test; real-time reverse transcription PCR analysis confirmed 11 H5-positive ducks. The Central Veterinary Laboratory of Kinshasa (Kinshasa, DRC) submitted the samples to the World Organisation for Animal Health (OIE) Reference Laboratory and the Food and Agriculture Organization of the United Nations (UN-FAO) Reference Center for Animal Influenza at the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Italy) for confirmatory diagnosis and genetic analysis.

Using an Illumina MiSeq platform (Illumina, San Diego, CA, USA), we obtained whole-genome sequences for 4 viruses selected as being representative of the 4 affected areas in Ituri province (Table 1; online Technical Appendix Table 1, <https://wwwnc.cdc.gov/EID/article/24/7/17-2123-Techapp1.pdf>). We submitted the full genomes to GenBank (accession nos. MG607401–32) (Table 1; online Technical Appendix 1 Table 1) and used the

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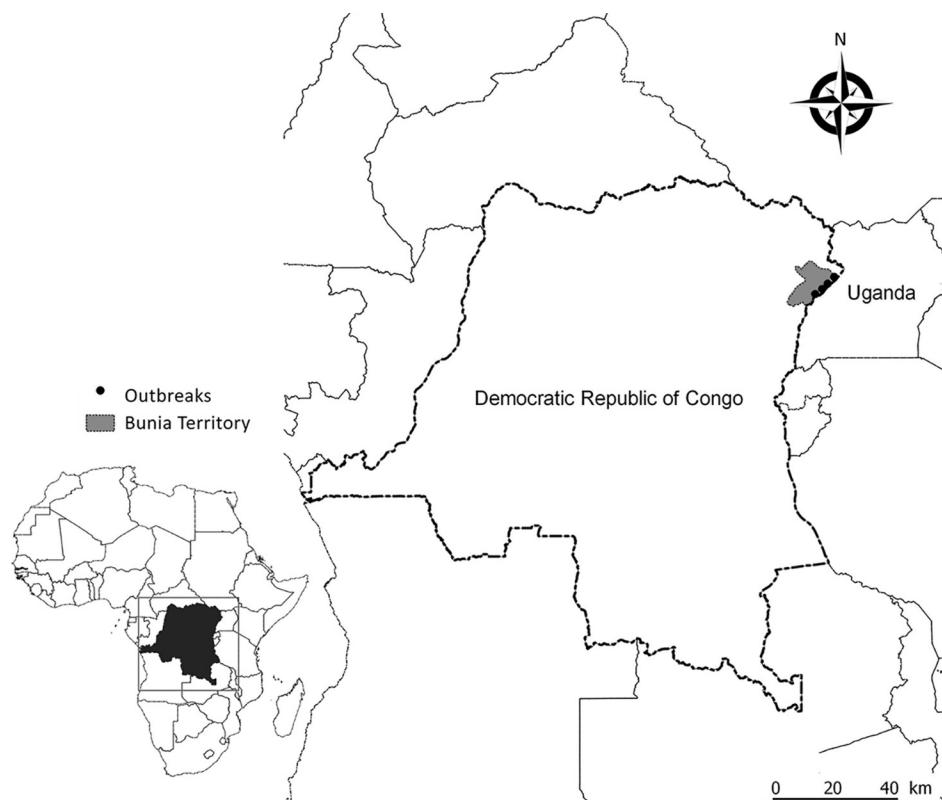


Figure 1. Location of confirmed highly pathogenic avian influenza virus A (H5N8) infection in Bunia territory, on the border with Uganda, Democratic Republic of the Congo, 2017. Inset shows location of Democratic Republic of the Congo in Africa.

maximum-likelihood method to generate phylogenetic trees for each gene segment using PhyML 3.1 (<http://www.atgc-montpellier.fr/phyml/versions.php>).

Among the 4 H5N8 viruses sequenced, A/duck/Democratic_Republic_of_the_Congo/17RS882-5/2017 and A/duck/Democratic_Republic_of_the_Congo/17RS882-40/2017 had identical hemagglutinin (HA) genes; these 2 sequences displayed a similarity of 99.9% with the HA sequences of A/duck/Democratic_Republic_of_the_Congo/17RS882-33/2017 and 99.6% similarity with the HA sequences of A/duck/Democratic_Republic_of_the_Congo/17RS882-29/2017. The topology of the phylogenetic tree based on the HA gene segment showed that the H5N8 viruses from DRC belonged to clade 2.3.4.4 group B (9) and grouped together with viruses collected in Qinghai, China; southern Russia; and India in 2016. The highest similarity (99.2%) was with an Indian virus (A/duck/India/10CA01/2016) (Figure 1; online Technical Appendix 1 Figure 1). For the NA gene, the sequences of the viruses A/duck/Democratic_Republic_of_the_Congo/17RS882-5/2017,

A/duck/Democratic_Republic_of_the_Congo/17RS882-33/2017, and A/duck/Democratic_Republic_of_the_Congo/17RS882-40/2017 were identical (100% similarity); these 3 sequences displayed 99.6% similarity with the NA sequence of A/duck/Democratic_Republic_of_the_Congo/17RS882-29/2017(H5N8) (online Technical Appendix 1 Figure 2). The phylogenetic trees based on the NA and the internal gene segments (online Technical Appendix 1 Figures 1–8), except for the nucleoprotein (NP) gene segment, reflected the same topology of the HA tree, indicating that the H5N8 viruses from DRC were closely related to the virus A/duck/India/10CA01/2016. The topology of the phylogenetic tree based on the NP gene segment (online Technical Appendix 1 Figure 4) revealed a different clustering, with the viruses grouped with H5N8 viruses collected from wild birds in Qinghai and southern Russia in 2016. As discussed by Nagarajan et al. (10), it is possible that the Indian virus has been involved in a reassortment event that resulted in NP gene distinct from that described in the Qinghai and southern Russian viruses.

Table. Details of highly pathogenic avian influenza A(H5N8) viruses isolated from birds, Democratic Republic of the Congo, 2017

Date of sample collection	Sampling site	Isolate	GenBank accession no. for hemagglutinin gene
May 14	Tchomia	A/duck/Democratic_Republic_of_the_Congo/17RS882-5/2017	MG607416
May 15	Joo	A/duck/Democratic_Republic_of_the_Congo/17RS882-29/2017	MG607413
May 14	Mahagi	A/duck/Democratic_Republic_of_the_Congo/17RS882-33/2017	MG607414
May 13	Kafe	A/duck/Democratic_Republic_of_the_Congo/17RS882-40/2017	MG607415

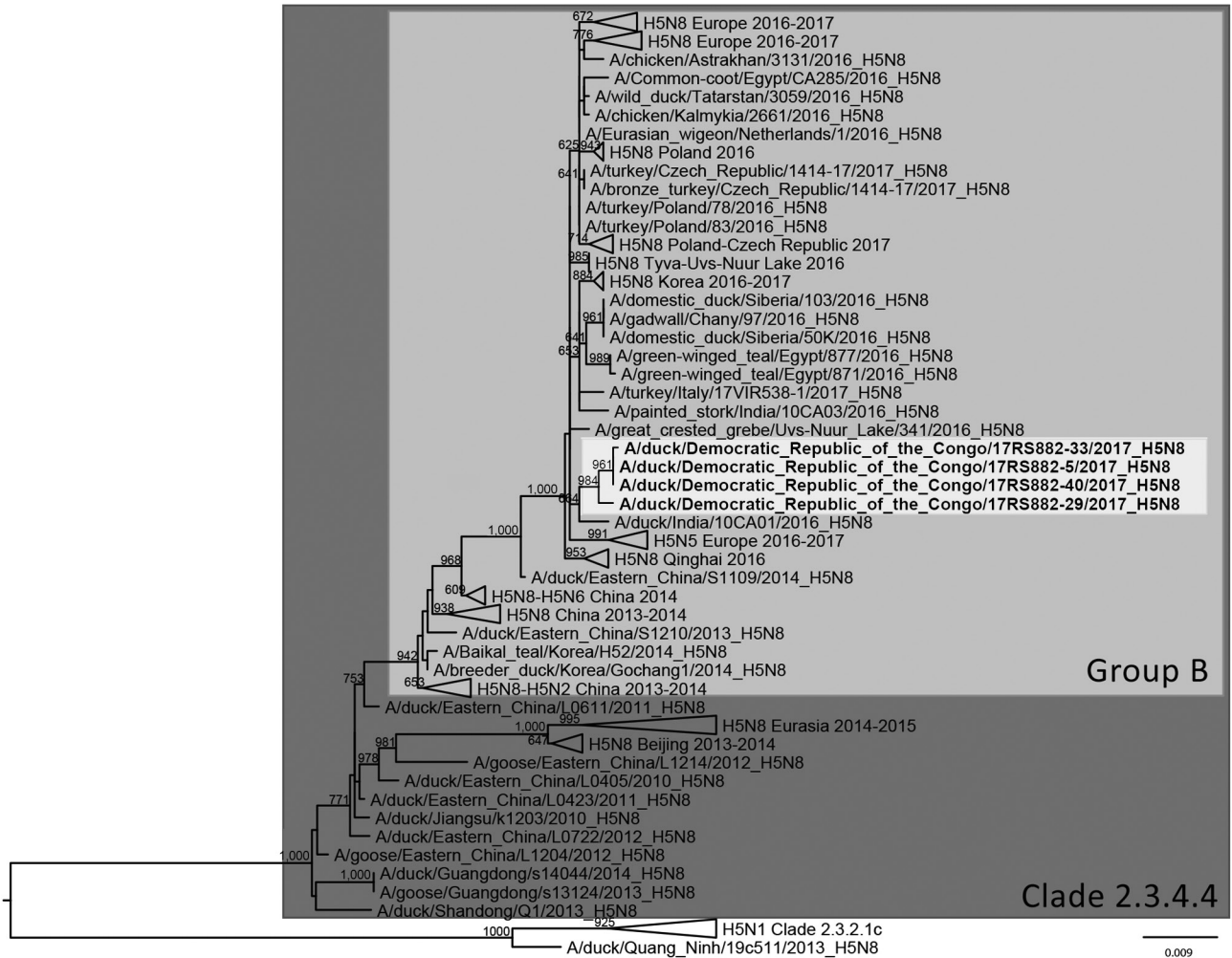


Figure 2. Phylogenetic tree constructed by the maximum-likelihood method of the hemagglutinin gene segment of 4 isolates of highly pathogenic avian influenza A(H5N8) viruses from the Democratic Republic of the Congo (light gray shading) and reference viruses. Bootstrap supports >600/1,000 are indicated above the nodes. Scale bar indicates number of nucleotide substitutions per site.

The time to the most recent common ancestor estimated for the HA gene suggested that the H5N8 virus may have reached DRC during March 2016–February 2017 (online Technical Appendix 1 Figure 9). However, the paucity of data in the public databases reduces the accuracy of the evolutionary analyses and limits the possibility to reconstruct the early transmission dynamics of the H5N8 virus in DRC.

Conclusions

Before 2017, no HPAI H5 goose/Guangdong lineage viruses had been reported in DRC. We have attempted to retrace the origin of the H5N8 outbreaks identified in the Ituri province through the evolutionary analysis of viral gene sequences. Considering the close phylogenetic relationship identified between the DRC viruses and those detected in wild and domestic birds in Asia and the over-

lap of the West Asian–East African flyway with the zones affected by the H5N8 infection, it is reasonable to assume that migratory birds may have been involved in the introduction of the virus in the eastern and central parts of Africa. The inter-African movements of wild birds and the commercial trade between countries could also have favored the spread of AIVs across the region. The outbreaks in Uganda in January 2017 and in DRC in April 2017 could exemplify this scenario because of the close contact between the 2 countries, even though no public information about the genetic characteristics of the Ugandan viruses is available for comparison. According to reports from the DRC veterinary service (www.au-ibar.org/2012-10-01-13.../348-newcastle-disease), different regions of the country have previously reported mortalities in wild and domestic birds; however, these were considered Newcastle disease cases because of the endemic status of this

disease in the country and therefore were not investigated further. For the HPAI H5N8 outbreak, the awareness of DRC veterinary services, as well as of the population, was raised following the Uganda HPAI outbreak notification, highlighting the crucial role of sharing information in the control of this transboundary disease.

Because DRC hosts many sites for residential and migratory wild birds and is considered a stopover point along the West Asian–East African flyway, surveillance in wild and domestic birds should be implemented for early detection of the virus and efficient control of its spread. However, the challenges for the sustainable development of strategies for the effective prevention and control of this disease are vast and deeply ingrained. Investments to overcome infrastructure obstacles hindering the implementation of a true early-warning system are urgently needed to reduce the risk of onward spread of the virus in the region.

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Technical Appendix

Materials and Methods

Genome Amplification and Sequencing

We purified total RNA from 4 HPAI H5N8-positive clinical samples, collected in the Democratic Republic of the Congo (DRC), using the Nucleospin RNA kit (Macherey-Nagel, Düren, Germany). We amplified whole influenza A virus genomes using the SuperScript III One-Step RT-PCR system with Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA) (1). Following the manufacturer's instructions, we obtained sequencing libraries using the Nextera DNA XT sample preparation kit (Illumina, San Diego, CA, USA) and quantified them using the Qubit dsDNA High Sensitivity kit (Invitrogen). We determined the average fragment length using the Agilent High Sensitivity Bioanalyzer Kit (Agilent Technologies, Santa Clara, CA, USA). We sequenced the indexed libraries in multiplex for 250 bp paired-end on Illumina MiSeq.

High-Throughput Sequencing Data Analysis

We assessed read quality using FastQC v0.11.2 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc>). We filtered the raw data by removing reads with more than 10% of undetermined (N) bases, reads with more than 100 bases with Q score below 7, and duplicated paired-end reads. We clipped the remaining reads from Illumina Nextera XT adaptors with scythe v0.991 (<https://github.com/vsbuffalo/scythe>) and trimmed them with sickle v1.33 (<https://github.com/najoshi/sickle>). We aligned high-quality reads against a reference genome using BWA v0.7.12 (2). We processed alignments with Picard-tools v2.1.0 (<http://picard.sourceforge.net>) and GATK v3.5 (3–5). We called single nucleotide

polymorphisms were called using LoFreq v2.1.2 (6) and used the outputs to generate the consensus sequences.

Phylogenetic Analyses

We aligned consensus sequences of the complete genome of the 4 samples from DRC (Technical Appendix 1 Table 1) using MAFFT v7 (7) and compared them with the most related sequences available in the Global Initiative on Sharing All Influenza Data (GISAID) database (<https://www.gisaid.org>) (Technical Appendix 2, <https://wwwnc.cdc.gov/EID/article/24/7/17-2123-Techapp2.xlsx>). In detail, for each gene segment, we aligned the sequences of the H5N8 viruses from DRC with all the H5 sequences belonging to clade 2.3.4.4 group B available in GISAID at the time of writing and with the 50 most related sequences resulted from a BLAST search (<https://blast.ncbi.nlm.nih.gov/Blast>). We obtained maximum-likelihood phylogenetic trees using the best-fit general time-reversible model of nucleotide substitution with gamma-distributed rate variation among sites (with 4 rate categories, Γ_4) and a heuristic SPR branch-swapping search (8) available in PhyML v3.1 (<http://www.atgc-montpellier.fr/phyml/versions.php>). We performed 1,000 bootstrap replicates. Phylogenetic trees were visualized using FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>) (Technical Appendix 1 Figures 1–9).

Estimation of Time to the Most Recent Common Ancestor

Using BEAST v.1.8.4 software (9), we estimated the tMRCA of the HPAI H5N8 identified in the DRC for the HA gene segment. We selected a HKY85 + Γ_4 nucleotide substitution model with 2 data partitions reflecting codon positions (1st + 2nd positions, 3rd position) with base frequency unlinked across all codon position (SRD06 substitution model). We adopted a relaxed uncorrelated lognormal molecular clock and constant population size as the tree prior. We used Markov chain Monte Carlo (MCMC) and chain lengths of 100 million iterations to achieve convergence. We used TreeAnnotator v1.8.4 to summarize the maximum clade credibility (MCC) phylogenetic tree from the posterior distribution of trees, after the removal of a burn-in of 10% of the samples. We used FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>) to visualize the MCC tree (Technical Appendix 1 Figure 9).

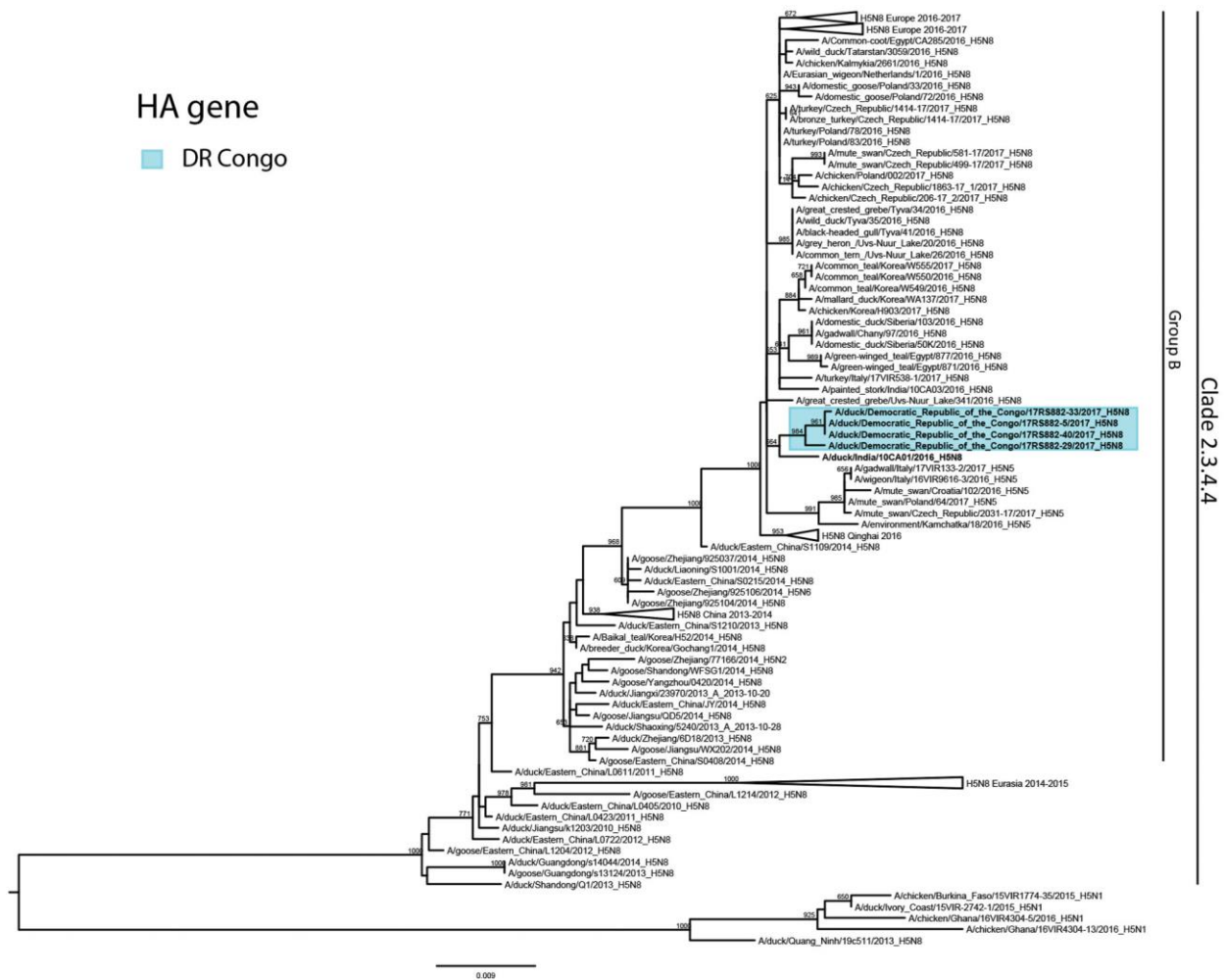
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Technical Appendix Table. Epidemiologic information and gene segments accession numbers of the H5N8 viruses characterized in this study, Democratic Republic of the Congo, May 2017

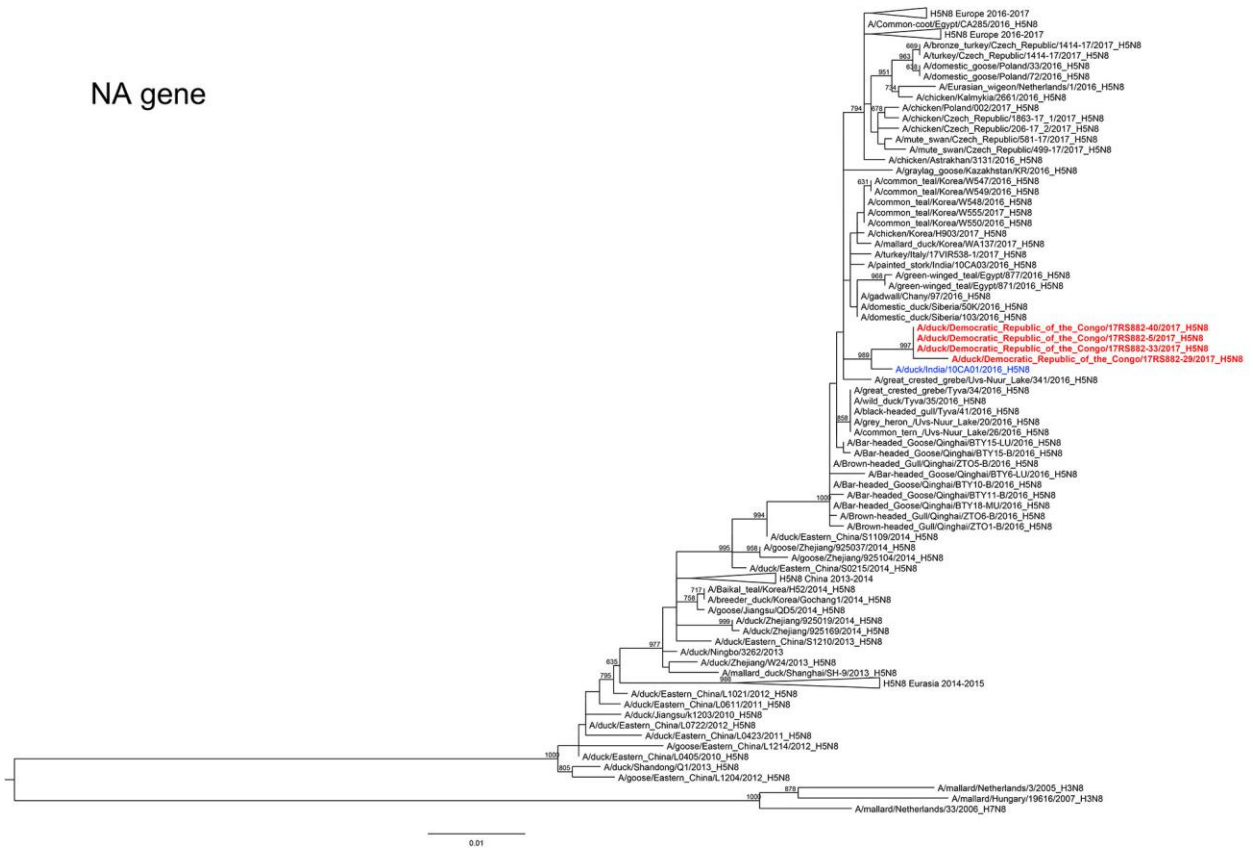
Date of sample collection	Sampling site	Isolate	Accession number*
May 14	TCHOMIA	A/duck/Democratic Republic of the Congo/17RS882-5/2017 (H5N8)	MG607404 for PB2 gene; MG607408 for PB1 gene; MG607412 for PA gene; MG607420 for NP gene; MG607424 for NA gene; MG607428 for MP gene; MG607432 for NS gene
May 15	JOO	A/duck/ Democratic Republic of the Congo/17RS882-29/2017 (H5N8)	MG607401 for PB2 gene; MG607404 for PB1 gene; MG607409 for PA gene; MG607417 for NP gene; MG607421 for NP gene; MG607425 for MP gene; MG607429 for NS gene
May 14	MAHAGI	A/duck/ Democratic Republic of the Congo/17RS882-33/2017 (H5N8)	MG607402 for PB2 gene; MG607406 for PB1 gene; MG607410 for PA gene; MG607418 for NP gene; MG607422 for NA gene; MG607426 for MP gene; MG607430 for NS gene
May 13	KAFE	A/duck/ Democratic Republic of the Congo/17RS882-40/2017 (H5N8)	MG607403 for PB2 gene; MG607407 for PB1 gene; MG607411 for PA gene; MG607419 for NP gene; MG607423 for NA gene; MG607427 for MP gene; MG607431 for NS gene

*HA, hemagglutinin; MP, matrix protein; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural protein; PA, polymerase acidic protein; PB1, polymerase basic protein 1; PB2, polymerase basic protein 2



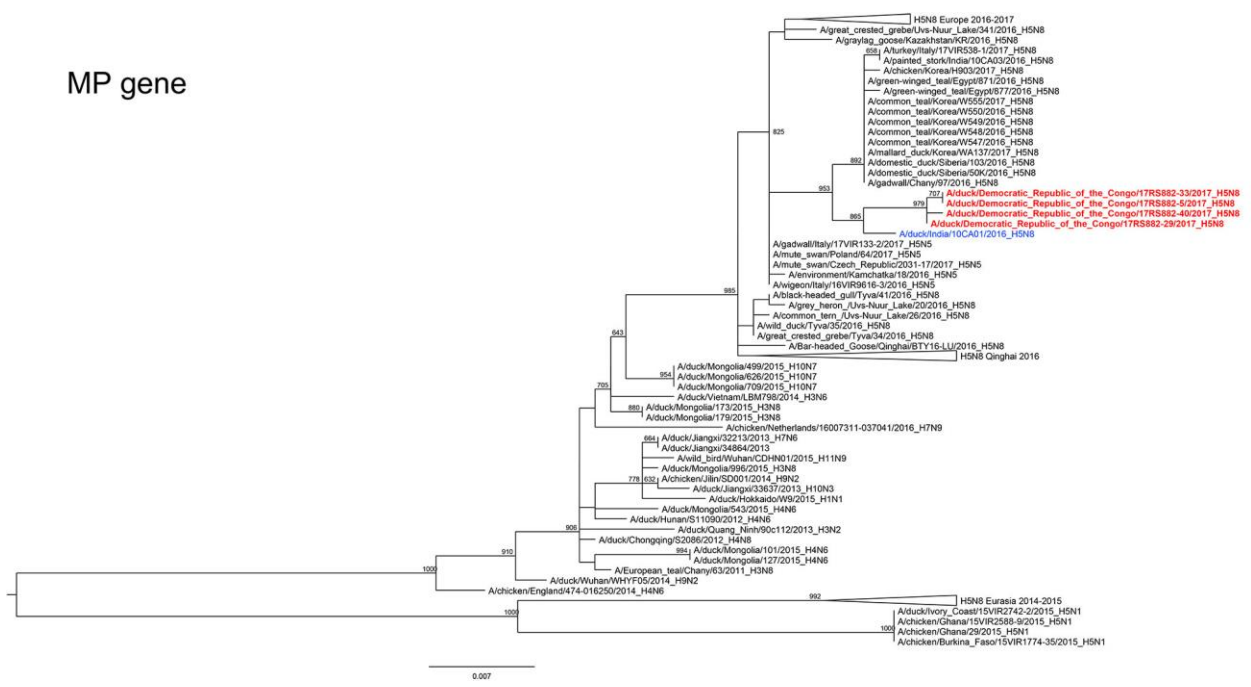
Technical Appendix Figure 1. Phylogenetic trees constructed by the maximum-likelihood method of the hemagglutinin (HA) gene segment of the 4 isolates of highly pathogenic avian influenza virus A(H5N8) from the Democratic Republic of the Congo in May 2017. Viruses analyzed in this study are highlighted in red. Bootstrap supports higher than 600/1000 are indicated above the nodes. Scale bar indicates number of nucleotide substitutions per site.

NA gene



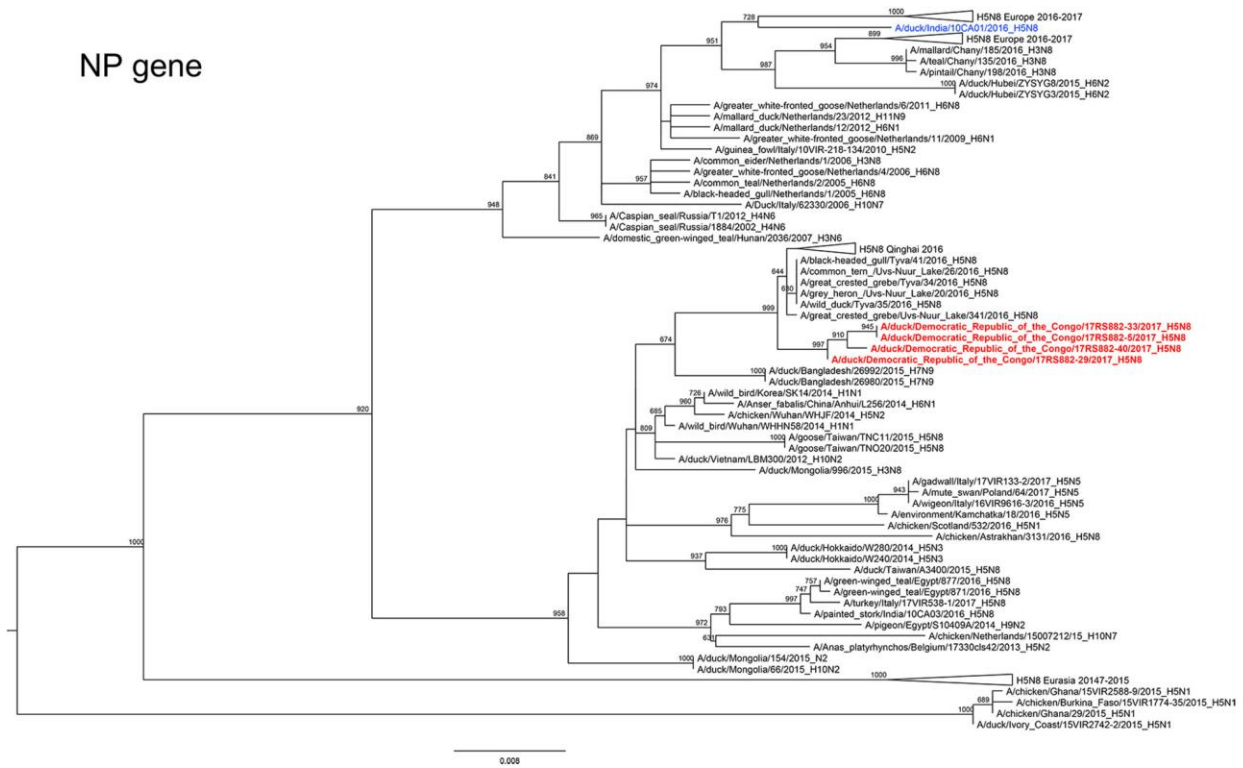
Technical Appendix Figure 2. Maximum-likelihood phylogenetic tree of the neuraminidase (NA) gene. The H5N8 viruses from the Democratic Republic of the Congo are marked in red. Bootstrap supports higher than 600/1000 are indicated next to the nodes. Scale bar indicates number of nucleotide substitutions per site.

MP gene



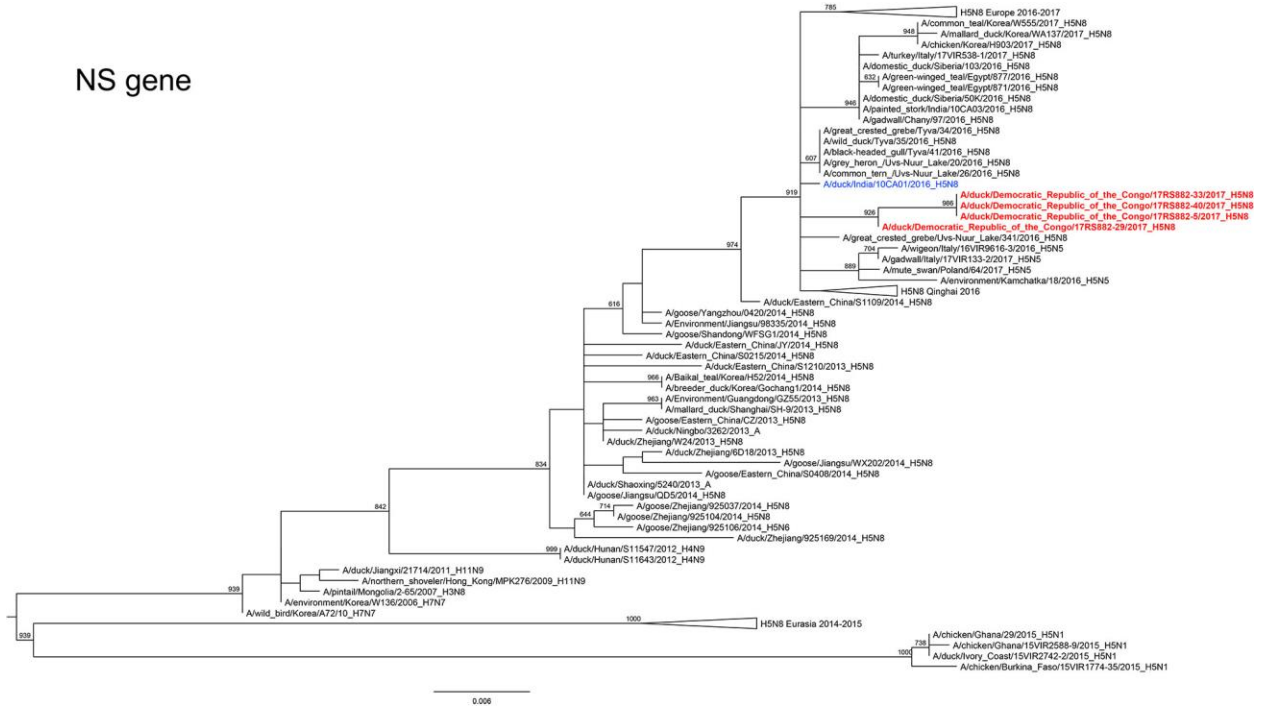
Technical Appendix Figure 3. Maximum-likelihood phylogenetic tree of the matrix protein (MP) gene. The H5N8 viruses from the Democratic Republic of the Congo are marked in red. Bootstrap supports higher than 600/1000 are indicated next to the nodes. Scale bar indicates number of nucleotide substitutions per site.

NP gene



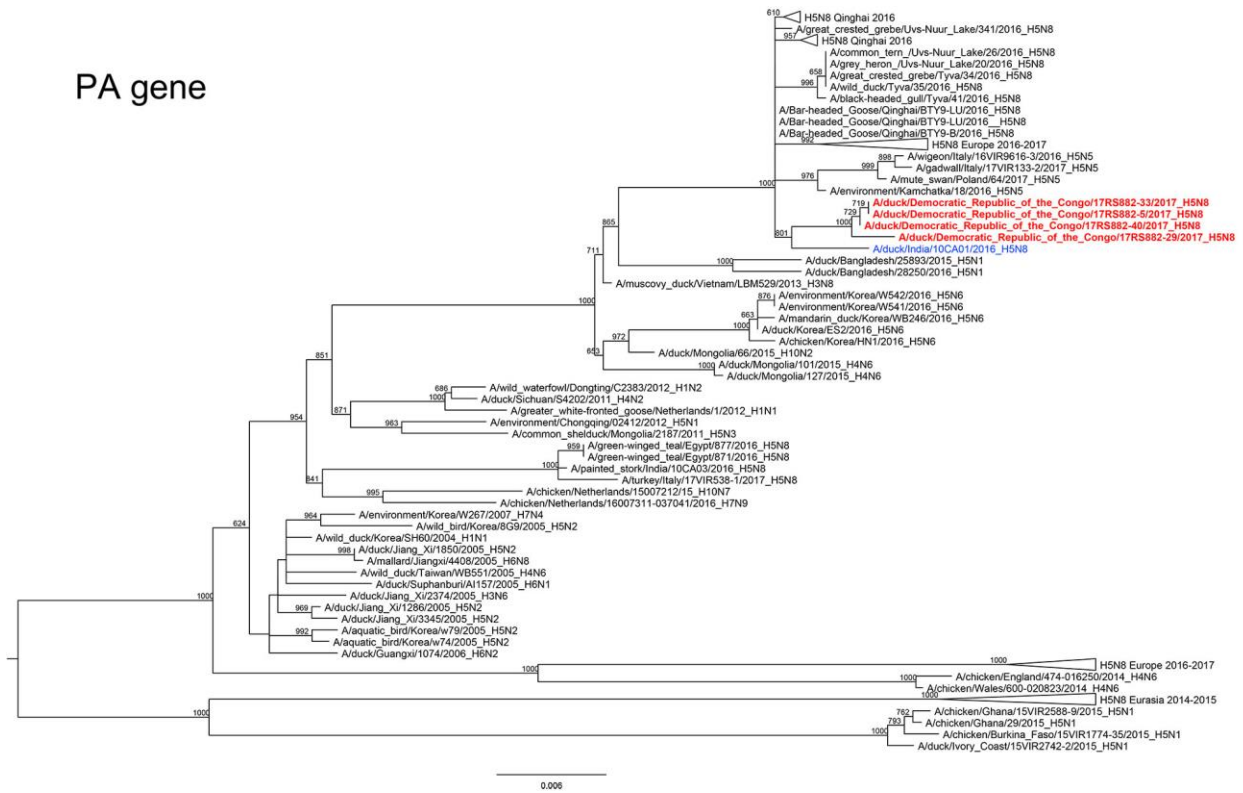
Technical Appendix Figure 4. Maximum-likelihood phylogenetic tree of the nucleoprotein (NP) gene. The H5N8 viruses from the Democratic Republic of the Congo are marked in red. Bootstrap supports higher than 600/1000 are indicated next to the nodes. Scale bar indicates number of nucleotide substitutions per site.

NS gene



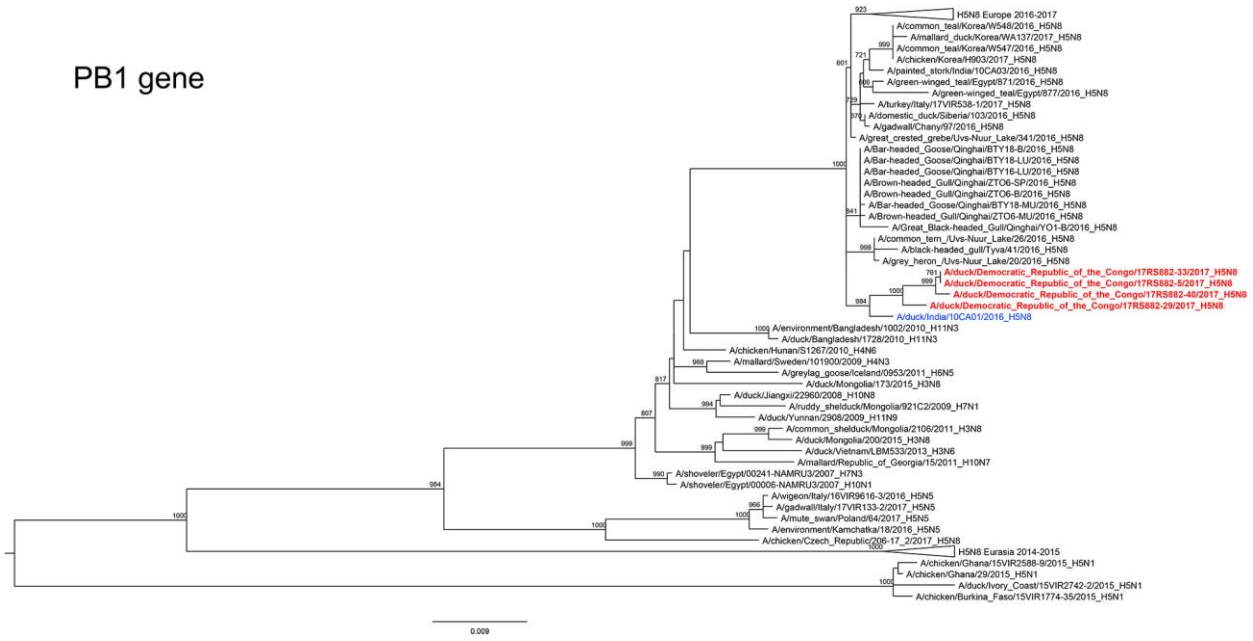
Technical Appendix Figure 5. Maximum-likelihood phylogenetic tree of the nonstructural protein (NS) gene. The H5N8 viruses from the Democratic Republic of the Congo are marked in red. Bootstrap supports higher than 600/1000 are indicated next to the nodes. Scale bar indicates number of nucleotide substitutions per site.

PA gene



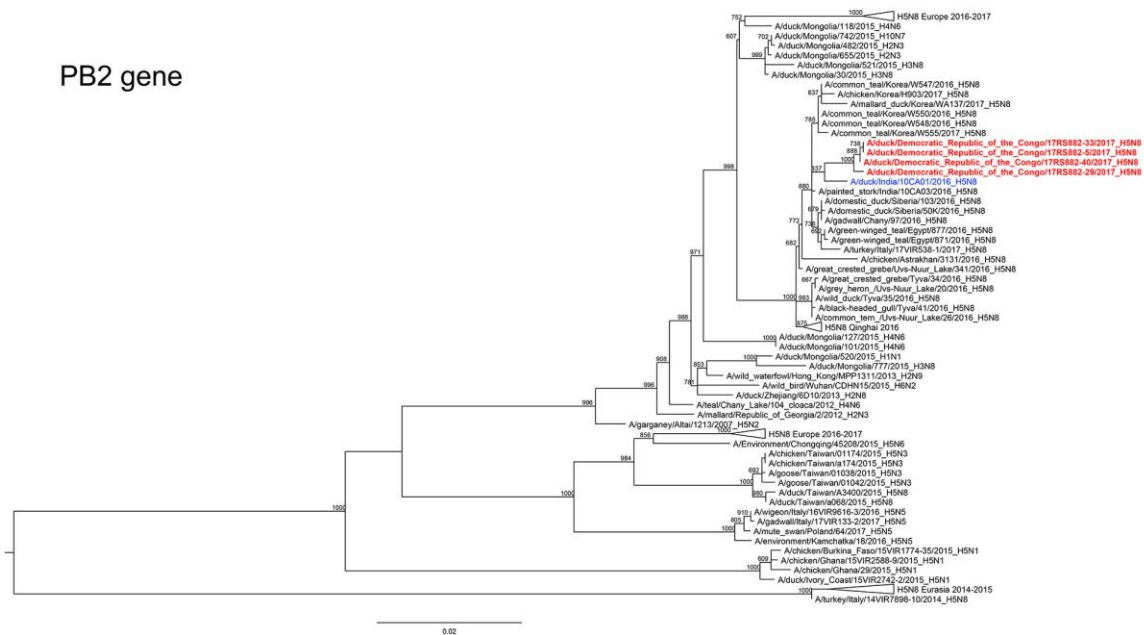
Technical Appendix Figure 6. Maximum-likelihood phylogenetic tree of the polymerase acidic protein (PA) gene. The H5N8 viruses from the Democratic Republic of the Congo are marked in red. Bootstrap supports higher than 600/1000 are indicated next to the nodes. Scale bar indicates number of nucleotide substitutions per site.

PB1 gene

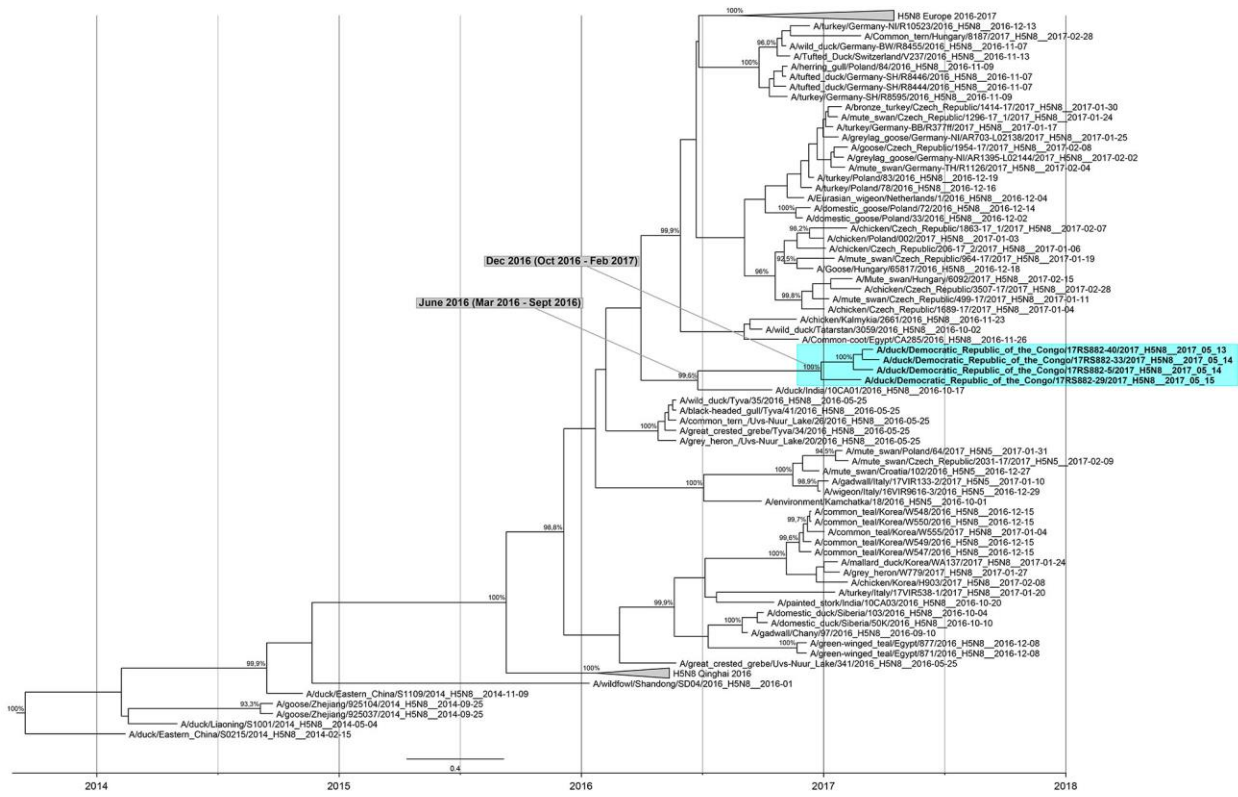


Technical Appendix Figure 7. Maximum-likelihood phylogenetic tree of the polymerase basic protein 1 (PB1) gene. The H5N8 viruses from the Democratic Republic of the Congo are marked in red. Bootstrap supports higher than 600/1000 are indicated next to the nodes. Scale bar indicates number of nucleotide substitutions per site.

PB2 gene



Technical Appendix Figure 8. Maximum-likelihood phylogenetic tree of the polymerase basic protein 2 (PB2) gene. The H5N8 viruses from the Democratic Republic of the Congo are marked in red. Bootstrap supports higher than 600/1000 are indicated next to the nodes. Scale bar indicates number of nucleotide substitutions per site.



Technical Appendix Figure 9. Maximum clade credibility phylogenetic tree of the hemagglutinin (HA) gene. The H5N8 viruses from the Democratic Republic of the Congo are highlighted in the light blue box. Posterior probability values higher than 90% are indicated next to the nodes. The mean time to the most recent common ancestor (tMRCA) and the 95% highest posterior density intervals of the relevant nodes are indicated in the gray boxes.