Genetic Diversity of Highly Pathogenic Avian Influenza A(H5N8/H5N5) Viruses in Italy, 2016–17

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In winter 2016–17, highly pathogenic avian influenza A(H5N8) and A(H5N5) viruses of clade 2.3.4.4 were identified in wild and domestic birds in Italy. We report the occurrence of multiple introductions and describe the identification in Europe of 2 novel genotypes, generated through multiple reassortment events.

In spring 2016, highly pathogenic avian influenza (HPAI) outbreaks caused by the H5N8 subtype of clade 2.3.4.4 (group B) were reported in migratory wild birds in Qinghai Lake, China (*1*), and in the salt lake system of Uvs Nuur on the Russian Federation–Mongolia border (*2*). Since then, HPAI A(H5N8) viruses have been detected in several countries in Asia, Europe, and Africa. In Europe, the virus was detected for the first time in October 2016 in Hungary (*3*). Here, we describe the occurrence of multiple introductions of reassortant HPAI A(H5N8) and A(H5N5) viruses in Italy, in both wild and domestic birds.

The Study

During December 2016–January 2017, a Eurasian wigeon (*Anas penelope*) and a gadwall (*Anas strepera*) found dead at Grado Lagoon in northeastern Italy tested positive for HPAI A(H5N5). A second wigeon tested positive for HPAI A(H5N8). Since then, additional HPAI A(H5N8) cases were observed in a common shelduck (*Tadorna tadorna*) and in a mute swan (*Cygnus olor*) and in birds on 6 commercial turkey farms, 1 layer farm, and 3 backyard flocks (Table 1; Figure 1). All of the cases in domestic poultry farms occurred in areas in close proximity to wetlands that are listed as important resting sites for migratory waterfowl. The onset of clinical signs in all the affected poultry species was generally associated with depression, reluctance to move, and a drop in feed consumption. The clinical condition often evolved into a more severe respiratory and nervous syndrome associated with an increased mortality rate (average mortality rate is 1.62% [95% CI 1.10%–2.14%]). Depopulation measures on the infected farms and 7 neighboring poultry premises considered at risk involved ≈510,000 birds.

The genomes of 10 positive samples collected from wild (n = 4) and domestic (n = 6) birds were fully sequenced (online Technical Appendix 1, https://wwwnc.cdc.gov/EID/article/23/9/17-0539-Techapp1.pdf). Phylogenetic analysis of the hemagglutinin (HA) gene showed that the HPAI A(H5N5) and A(H5N8) viruses clustered within the 2.3.4.4 clade, group B (Figure 1). However, the characterization of the complete genome (online Technical Appendix 1 Figures 1–8) revealed that these viruses belong to 4 distinct genotypes, which had very likely originated from multiple reassortment events.

Phylogenetic analyses indicated that the HPAI H5N5 viruses had been generated through intersubtype reassortment events between the H5N8 viruses from Asia (H5N8-Gs/Qinghai/2016-like) and the low pathogenicity avian influenza (LPAI) viruses of the Eurasian lineage (Figure 2). The A(H5N8) viruses from Asia were the source of the HA, polymerase acidic, matrix, and nonstructural protein genes. HPAI A(H5N5) viruses with similar HA and neuraminidase genes were identified in Croatia and Czech Republic in 2016–17. The time to the most recent common ancestor (tMRCA) estimated by pooling the information across all the gene segments in a hierarchical model (online Technical Appendix 1) suggested that a virus with this gene constellation emerged during October–December 2016 (Table 2; online Technical Appendix 1 Table 1).

Among the 8 HPAI A(H5N8) viruses in Italy investigated during this study, 5 were collected from wild and domestic birds in the Veneto region. In all the phylogenetic trees, these viruses clustered within the main European A(H5N8) group (A/wild duck/Poland/82A/

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Table 1. Epidemiologic information for highly pathogenic avian influenza A(H5N5) and A(H5N8) viruses isolated from birds in Italy, 2016–17

Isolate	Type	Collection date	Region	Location	Site type	EpiFlu accession no.*
A/wigeon/Italy/16VIR9616-3/2016	H5N5	2016 Dec 29	Friuli Venezia Giulia	Grado (Gorizia)	Natural park	EPI888600-01, EPI954800-05
A/wigeon/Italy/17VIR57-3/2017	H5N8	2017 Jan 03	Friuli Venezia Giulia	Grado (Gorizia)	Natural park	EPI888085-92
A/gadwall/Italy/17VIR133-2/2017	H5N5	2017 Jan 10	Friuli Venezia Giulia	Grado (Gorizia)	Natural park	EPI954616-23
A/swan/Italy/17VIR537-2/2017	H5N8	2017 Jan 19	Friuli Venezia Giulia	Aquileia (Udine)	Natural park	EPI954552-59
A/turkey/Italy/17VIR538-1/2017	H5N8	2017 Jan 20	Veneto	Mira (Venice)	Fattening turkeys farm	EPI954560-67
A/turkey/Italy/17VIR576-11/2017	H5N8	2017 Jan 23	Veneto	Piove di Sacco (Padua)	Fattening turkeys farm	EPI954568-75
A/chicken/Italy/17VIR653-12/2017	H5N8	2017 Jan 25	Veneto	Porto Viro (Rovigo)	Laying hens farm	EPI954576-83
A/turkey/Italy/17VIR973-2/2017	H5N8	2017 Feb 01	Emilia Romagna	Sorbolo (Parma)	Fattening turkeys farm	EPI954584-91
A/turkey/Italy/17VIR1338-3/2017	H5N8	2017 Feb 14	Lombardy	Monzambano (Mantova)	Fattening turkeys farm	EPI954592-99
A/turkey/Italy/17VIR1452-22/2017	H5N8	2017 Feb 16	Veneto	Gazzo Veronese (Verona)	Fattening turkeys farm	EPI954600-07

*GISAID EpiFlu database (http://platform.gisaid.org).

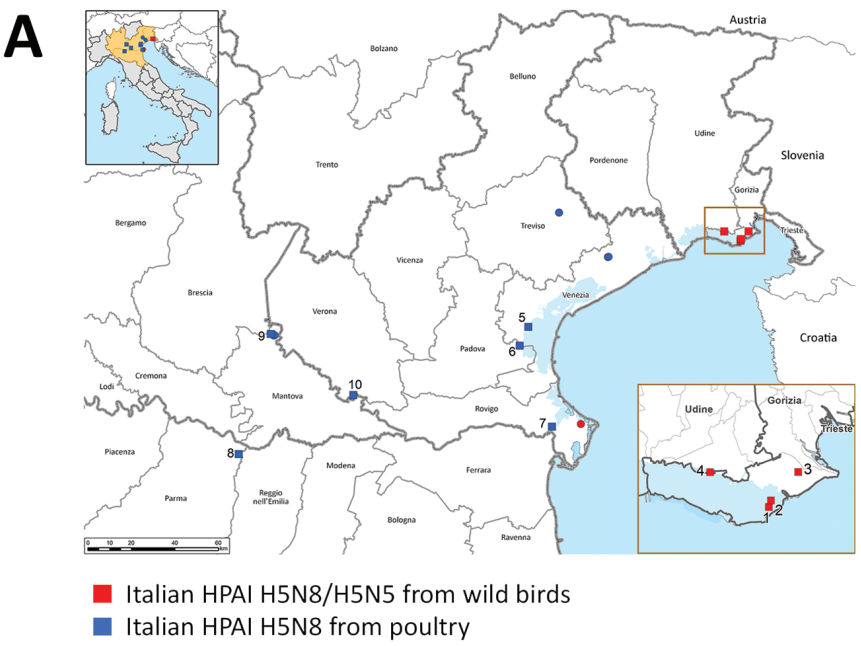
2016-like) (Figure 2), previously described by Pohlmann et al. (*4*). The tMRCA for this group was May–June 2016 in the hierarchical gene segment model (Table 2; online Technical Appendix 1 Table 1). The first HPAI A(H5N8) virus detected in a turkey farm in the Veneto region displayed the gene composition of a virus isolated in October 2016 from a painted stork in an Indian zoo (*5*), which had not previously been reported in Europe (Figure 2). The tMRCA of this Indian–Italian group is July–October 2016, according to the hierarchical gene segment model (Table 2; online Technical Appendix 1 Table 1). The 2 outbreaks reported in 2 commercial turkey farms in the Emilia-Romagna and Lombardy regions were caused by HPAI A(H5N8) reassortant viruses containing the polymerase basic protein 2 and nucleoprotein genes of LPAI viruses of the Eurasian lineage and the remaining genes from the H5N8-Gs/Qinghai/2016-like genotype (Figure 2). Viruses with a similar gene pool were identified in Croatia and France. Estimation of the tMRCA by the hierarchical gene segment model indicated that this genotype might have emerged during June–August 2016 (Table 2; online Technical Appendix 1 Table 1).

Analyses of the phylogenetic topologies revealed that most of the sequences found in Italy were dispersed throughout the trees, indicating the occurrence of several independent introductions of the A(H5N8) virus into poultry farms from wild birds (online Technical Appendix 1 Figures 1–8). These results were confirmed by our median-joining network analyses for the HA gene (online Technical Appendix 1 Figure 9), which showed that the ancestral sequences of the samples from Italy represent viruses collected in other countries. In most cases ≥1 median vector, representing the lost ancestral sequences, separated these viruses from the hypothetical progenitor. The only exception was for A/turkey/Italy/17VIR576-11/2017 and A/turkey/Italy/17VIR1452-22/2017, which proved to be almost identical for all the genes (similarity of 99.9%–100%), although they were collected 24 days apart in 2 turkey flocks located at a distance of ≈90 km from one another and no evident contacts were observed between them. However, because the 2 outbreaks had occurred in 2 farms operated by the same company, an exchange of virus cannot be ruled out.

Intravenous pathogenicity indexes obtained for 8 representative A(H5N8) and A(H5N5) isolates ranged from 2.85–3, comparable to an index of 2.93 for 2016 A(H5N8) viruses from Germany and 2.75–2.84 for 2016 A(H5N8) viruses from Russia (*2,4*). These data confirm that both of the A(H5N8) and A(H5N5) viruses from Italy, which shared the same HA cleavage site (PLREKRRKR), are highly pathogenic for poultry.

Conclusions

Since its emergence in China in 2013, the HPAI H5 of clade 2.3.4.4 has evolved in different genetic groups, namely A to D (*6*). Here, we describe the introductions of 4 different H5 viral genotypes of clade 2.3.4.4 group B in northern Italy. As previously observed for the 2014–15 A(H5N8) epidemic wave (*7*), our results confirm that these strains have a high propensity to reassort with co-circulating LPAI and HPAI viruses, causing the generation of several subtypes and genotypes with unique gene constellations. Unfortunately, the lack of sequences of the potential progenitors, exemplified by the



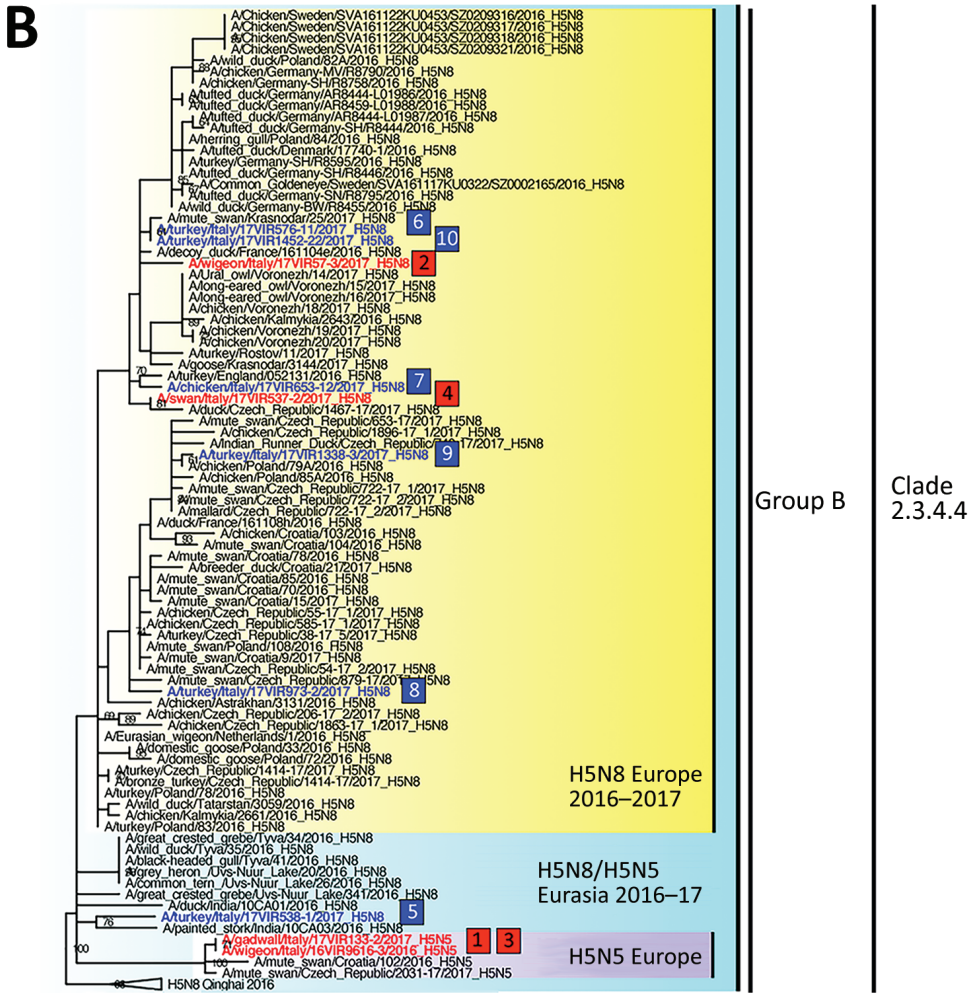


Figure 1. Highly pathogenic avian influenza A(H5N8) and A(H5N5) in birds, Italy, 2016–17). A) Geographic distribution of cases in wild (red) and domestic (blue) birds in northern Italy. Squares indicate the samples sequenced in this study; circles indicate positive samples for which no genetic information was available at the time of writing. B) Maximum-likelihood phylogenetic tree of the hemagglutinin gene of clade 2.3.4.4 viruses. Viruses analyzed in this study are indicated with red (wild birds) and blue (domestic birds) squares, numbered according to the collection date. Bootstrap supports >60% are indicated above the nodes. Scale bar indicates nucleotide substitutions per site.

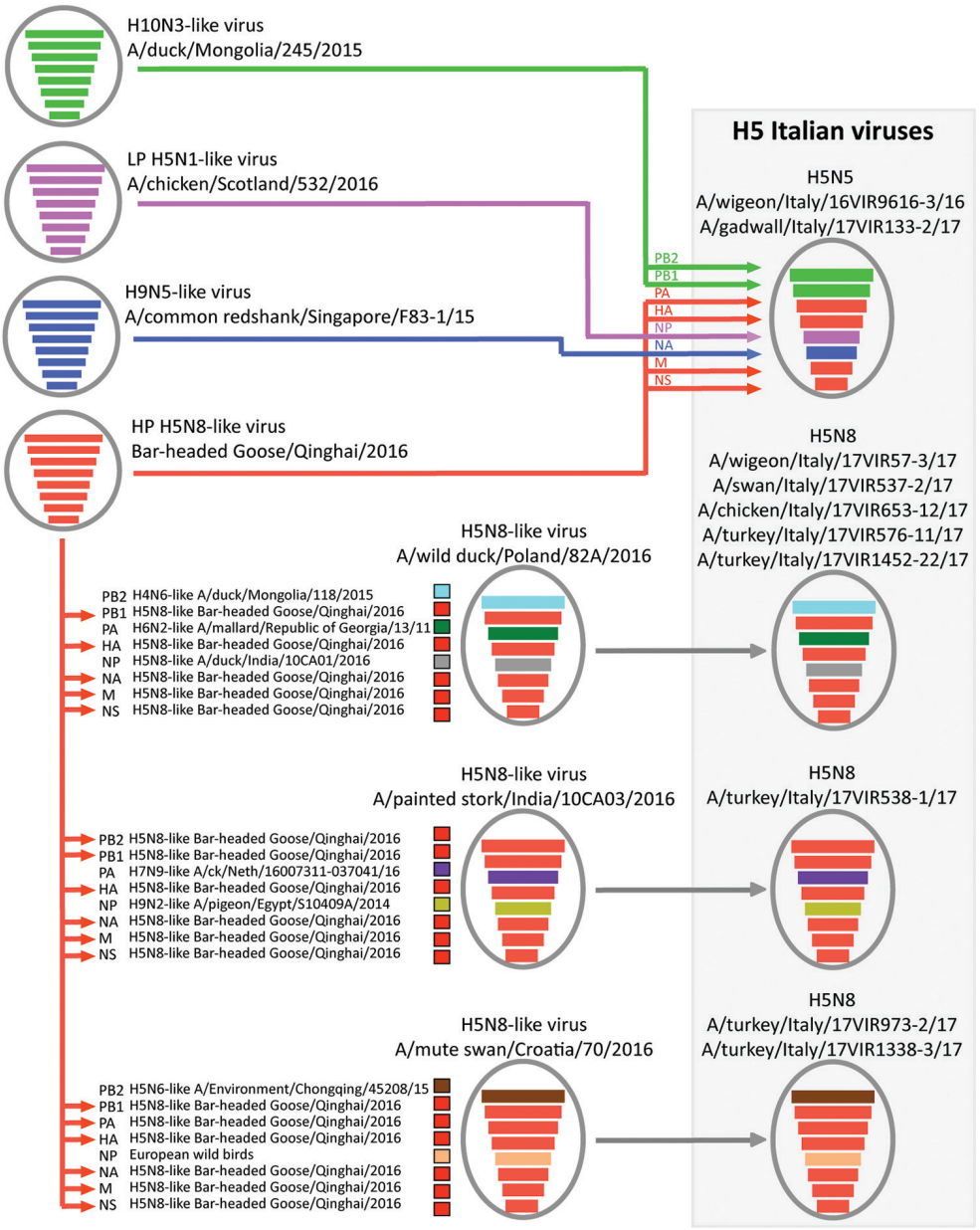


Figure 2. Probable genesis of highly pathogenic avian influenza A(H5N8) and A(H5N5) reassortant viruses identified in Italy, 2016–17 (gray box). Virus particles are represented by ovals containing horizontal bars that represent the 8 gene segments, colored according to their origin.

long branches observed in particular in the polymerase basic protein 2, polymerase acidic, and nucleoprotein phylogenies, makes it difficult to determine when and where these genotypes emerged. The genetic variability observed in the viruses identified in domestic birds, the similarity to viruses circulating in Europe and India, and the close proximity of the infected poultry farms to wetlands all suggest that wild birds did play a major role in the multiple and independent introductions of the virus into poultry holdings.

Table 2. tMRCA for the 4 avian influenza A(H5N5) and A(H5N8) virus genotypes identified in Italy, 2016–17*

Genotype	tMRCA	
	Mean	95% HPD
H5N5	November 2016	October–December 2016
H5N8 A/wild duck/Poland/82A/2016-like	May 2016	May–June 2016
H5N8 A/painted stork/India/10CA03/2016-like	August 2016	July–October 2016
H5N8 A/mute swan/Croatia/70/2016-like	July 2016	June–August 2016

*tMRCAs estimated for each gene segments are reported in online Technical Appendix 1 Table 1 (https://wwwnc.cdc.gov/EID/article/23/9/17-0539-Techapp1.pdf). HPD, highest posterior density; tMRCA, time to most recent common ancestor.

Our study highlights the importance of generating complete viral genome sequences in a timely fashion, which may help to monitor the viral spread and define appropriate disease control strategies. This, coupled with intensified wild bird surveillance on wetlands of ecologic importance for avian influenza viruses, can improve our understanding of the virus dissemination routes and support early detection of viruses highly pathogenic to poultry or believed to be of immediate concern to human health.

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EID Podcast: Novel Eurasian Highly Pathogenic Avian Influenza A H5 Viruses in Wild Birds, Washington, USA, 2014

The novel Eurasian lineage clade 2.3.4.4 highly pathogenic avian influenza (HPAI) A(H5N8) virus spread rapidly and globally during 2014, substantially affecting poultry populations. The first outbreaks were reported during January 2014 in chickens and domestic ducks in South Korea and subsequently in China and Japan, reaching Germany, the Netherlands, and the United Kingdom by November 2014 and Italy in early December 2014. Also in November 2014, a novel HPAI H5N2 virus was reported in outbreaks on chicken and turkey farms in Fraser Valley, British Columbia, Canada. This H5N2 influenza virus is a reassortant that contains the Eurasian clade 2.3.4.4 H5 plus 4 other Eurasian genes and 3 North American wild bird lineage genes. Taiwan has recently reported novel reassortants of the H5 clade 2.3.4.4 with other Eurasian viruses (H5N2, H5N3).

The appearance of highly similar Eurasian H5N8 viruses in Asia, Europe, and now the United States suggests that this novel reassortant may be well adapted to certain waterfowl species, enabling it to survive long migrations. These appearances also represent a major change in Eurasian H5 virus circulation. After the reported spread of HPAI H5N1 virus in Asia, a large, interagency avian influenza virus (AIV) surveillance effort was implemented throughout the United States during April 2006–March 2011. Of nearly 500,000 wild bird samples tested, none harbored Eurasian subtype H5 AIV. The overall prevalence of AIV was ≈11%, and most viruses (86%) were detected in dabbling ducks (family *Anatidae*). Although H5N8 subtype viruses have been detected previously in the United States, all have been low pathogenicity AIV of North American wild bird lineage.

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Genetic Diversity of Highly Pathogenic Avian Influenza A(H5N8/H5N5) Viruses in Italy, 2016–17

Technical Appendix 1

Materials and Methods

Genome sequencing

Total RNA was purified from 8 HPAI H5N8 and 2 HPAI H5N5 positive clinical samples using the Nucleospin RNA kit (Macherey–Nagel, Duren, Germany). Complete influenza A virus genomes were amplified with the SuperScript III One-Step RT-PCR system with Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA) using one pair of primers complementary to the conserved elements of the influenza A virus promoter as described in (*1*). Sequencing libraries were obtained using Nextera DNA XT Sample preparation kit (Illumina) following the manufacturer's instructions and quantified using the Qubit dsDNA High Sensitivity kit (Invitrogen, USA). The average fragment length was determined using the Agilent High Sensitivity Bioanalyzer Kit. The indexed libraries were pooled in equimolar concentrations and sequenced in multiplex for 250 bp paired-end on Illumina MiSeq, according to the manufacturer's instructions.

High-throughput sequencing data analysis

Illumina reads quality was assessed using FastQC v0.11.2. Raw data were filtered by removing: i) reads with more than 10% of undetermined ("N") bases; ii) reads with more than 100 bases with Q score below 7; iii) duplicated paired-end reads. Remaining reads were clipped from Illumina Nextera XT adaptors with scythe v0.991 (https://github.com/vsbuffalo/scythe) and trimmed with sickle v1.33 (https://github.com/najoshi/sickle). Reads shorter than 80 bases or unpaired after previous filters were discarded. High-quality reads were aligned against a reference genome using BWA v0.7.12 (*2*). Alignments were processed with Picard-tools v2.1.0 (http://picard.sourceforge.net) and GATK v3.5 (*3*–*5*) to correct potential errors, realign reads

around indels, and recalibrate base quality. Single Nucleotide Polymorphisms (SNPs) were called using LoFreq v2.1.2 (*6*) and the outputs were used to generate the consensus sequences.

Phylogenetic analyses

Consensus sequences of the complete genome of the 10 samples were aligned using MAFFT v. Seven (*7*) and compared with the most related sequences available in GISAID (accessed February 28, 2017). Maximum likelihood (ML) phylogenetic trees were obtained for each gene segment using the best-fit general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites (with 4 rate categories, Γ4) and a heuristic SPR branch-swapping search (*8*) available in the PhyML program version 3.1. To assess the robustness of individual nodes of the phylogeny, 100 bootstrap replicates were performed. Phylogenetic trees were visualized with the program FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

The HA gene segment of the HPAI H5N8 and H5N5 influenza viruses collected in Eurasia in 2016–2017 was aligned and used to construct a phylogenetic network using the Median Joining method implemented in the program NETWORK 4.5 (http://www.fluxus-engineering.com) (*9*). This method uses a parsimony approach to reconstruct the relationships between highly similar sequences, and allows the creation of "median vectors," which represent unsampled sequences that are used to connect the existing genotypes in the most parsimonious way. The parameter *epsilon* was set to 0.

Estimation of the Time to the Most Recent Common Ancestor (tMRCA)

We estimated the tMRCAs of the HPAI H5N8 and H5N5 genotypes identified in Italy by applying a Bayesian hierarchical model to all 8 gene segments using the BEAST software (*10*). To model the substitution process in each gene segment, we employed an HKY85 + Γ_4 model with two partitions (1st + 2nd positions versus 3rd position), base frequencies and Γ-rate heterogeneity unlinked across all codon positions (the SRD06 substitution model). We specified an independent uncorrelated lognormal relaxed clock and Bayesian skyride tree prior for each segment. To allow pooling of information across segments in estimating the tMRCAs of the HPAI H5N8 and H5N5 genotypes, we specified a hierarchical prior distribution over each of the corresponding tMRCAs of the genotypes (*11*). Specifically, we assume that the log of the tMRCAs are drawn from a normal distribution with a mean and a variance that is also unknown

and simultaneously estimated along with all the sequence evolution parameters. We used Markov chain Monte Carlo (MCMC) to draw inference under this model and used chain lengths of 50 million iterations to achieve convergence as assessed using Tracer v1.6 (http://beast.bio.ed.ac.uk/Tracer). Maximum Clade Credibility (MCC) phylogenetic trees were summarized from the posterior distribution of trees using TreeAnnotator v1.6.1 (*10*) after the removal of an appropriate burn-in (10% of the samples). The MCC trees were visualized using the program FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

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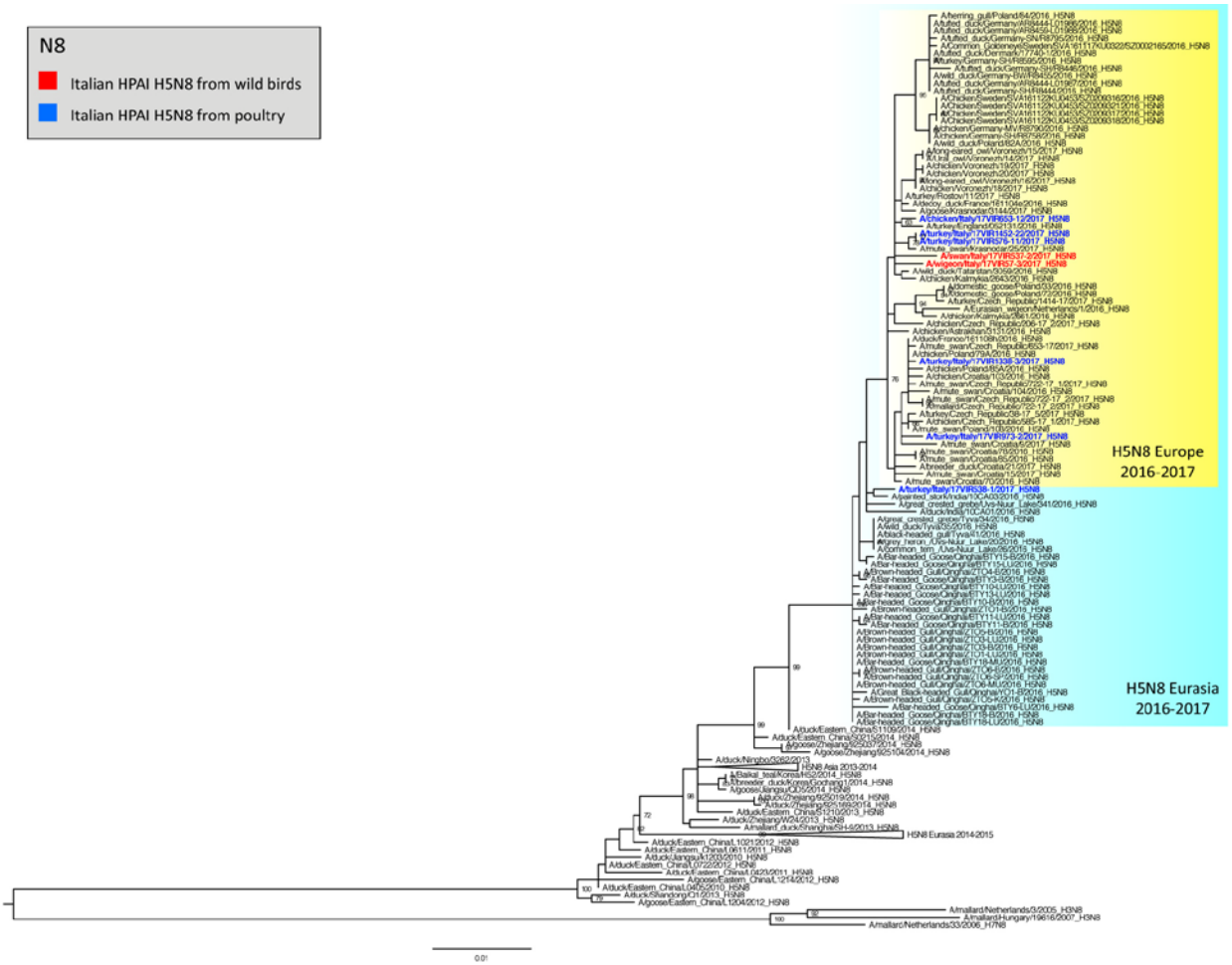
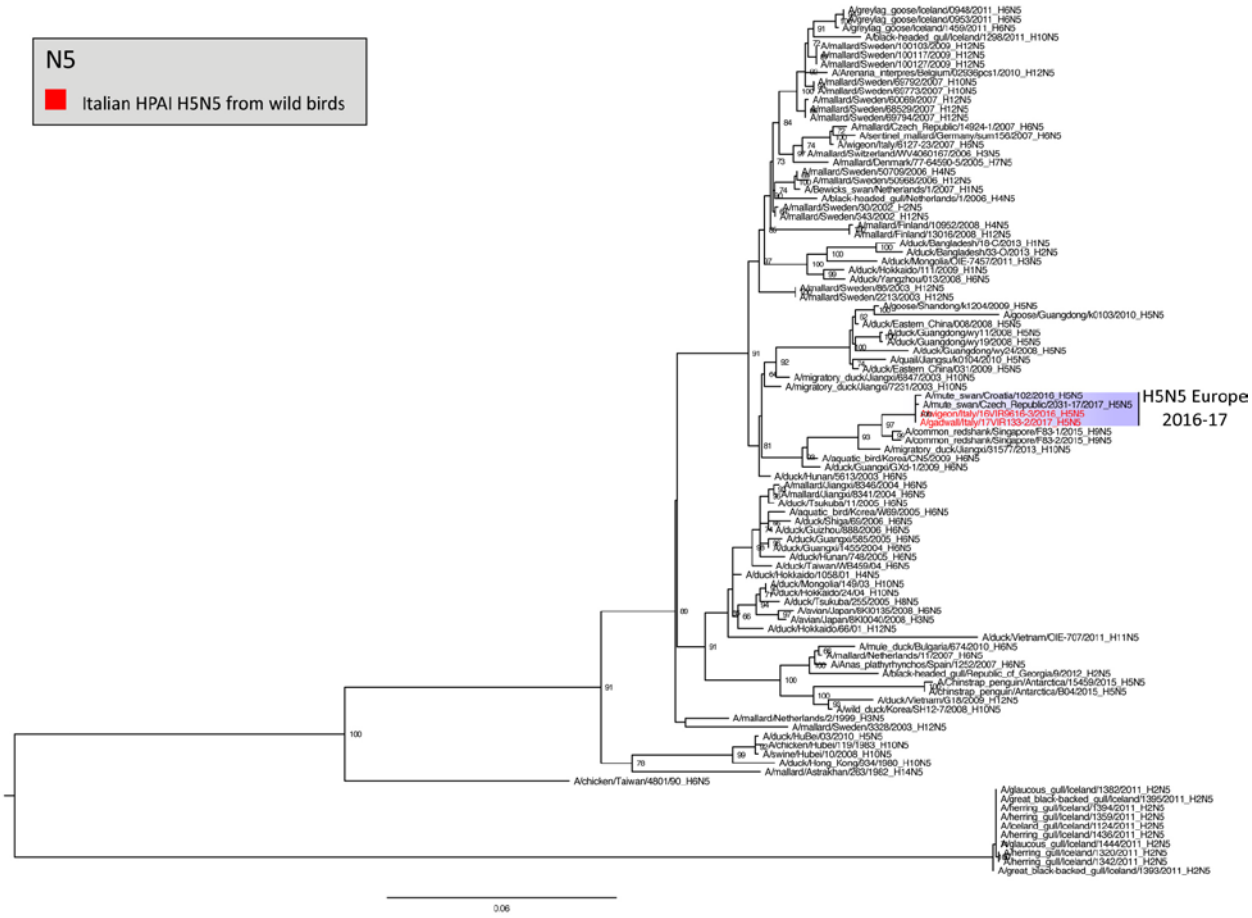
Technical Appendix Table 1. Time to the most recent ancestor (tMRCA) for each gene segment of the 4 genotypes identified in Italy

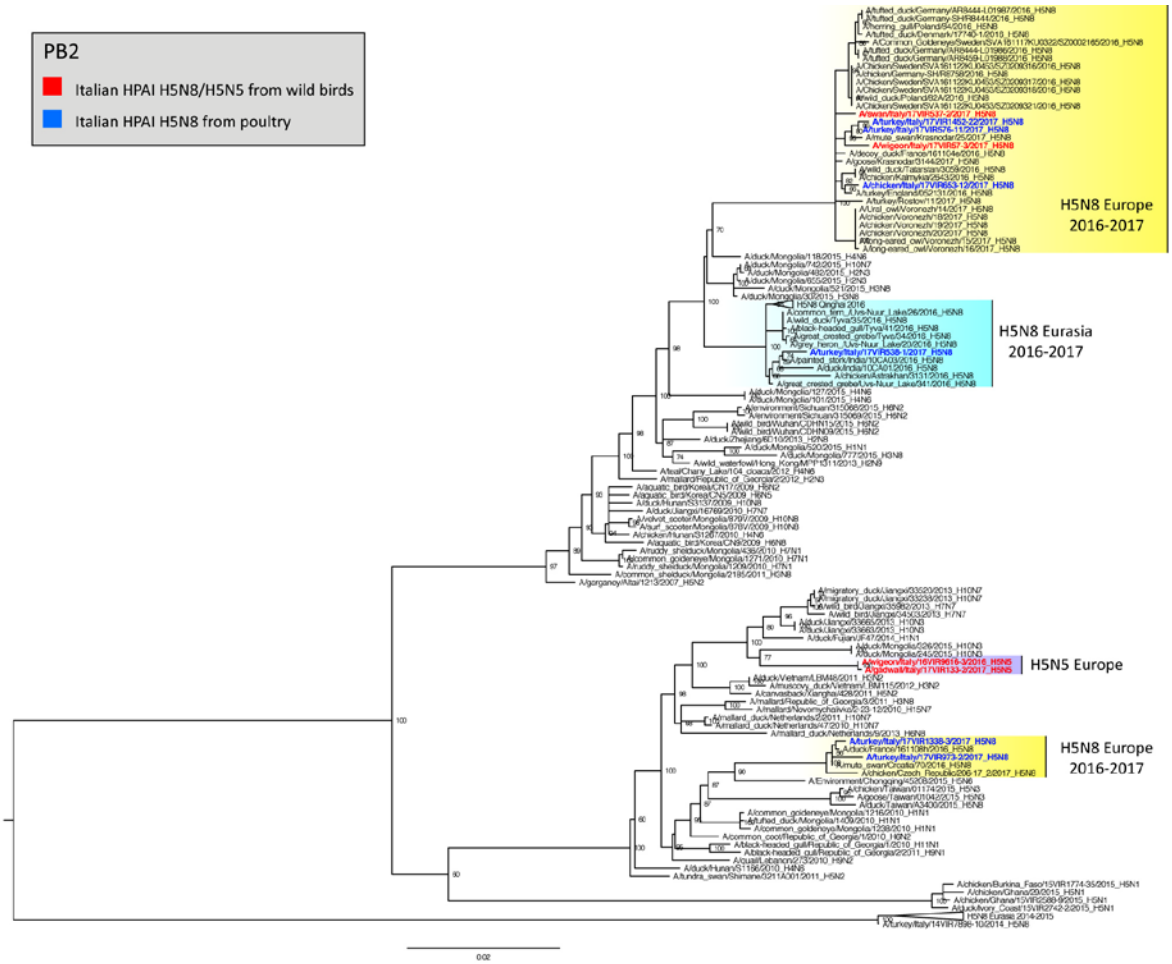
	Genotype							
	H5n5		H5n8 A/Wild Duck/Poland/82a/16-Like		H5n8 A/Painted Stork/India/10ca03/16-Like		H5n8 A/Mute Swan/Croatia/70/16-Like	
Gene	Mean	95% Hpd	Mean	95% Hpd	Mean	95% Hpd	Mean	95% Hpd
PB2	Nov 2016	Oct–Dec 2016	May 2016	May–Jun 2016	Sep 2016	Jul–Oct 2016	Jul 2016	Jun–Sep 2016
PB1	Nov 2016	Oct–Dec 2016	May 2016	May–Jun 2016	Aug 2016	Jul–Oct 2016	Jul 2016	Jun–Aug 2016
PA	Nov 2016	Sep–Dec 2016	May 2016	May–Jun 2016	Aug 2016	Jul–Oct 2016	Jul 2016	Jun–Aug 2016
HA	Nov 2016	Oct–Dec 2016	Jun 2016	May–Jun 2016	Aug 2016	Jul–Sep 2016	Jul 2016	Jun–Sep 2016
NP	Nov 2016	Oct–Dec 2016	May 2016	May–Jun 2016	Aug 2016	Jul–Oct 2016	Jul 2016	Jun–Aug 2016
NA	Nov 2016	Sep–Dec 2016	Jun 2016	May–Jun 2016	Aug 2016	Jul–Oct 2016	Jul 2016	Jun–Aug 2016
M	Nov 2016	Oct–Dec 2016	Jun 2016	May–Jun 2016	Sep 2016	Aug–Oct 2016	Jul 2016	Jun–Aug 2016
NS	Nov 2016	Oct–Dec 2016	Jun 2016	May–Jun 2016	Sep 2016	Aug–Oct 2016	Jul 2016	Jun–Aug 2016



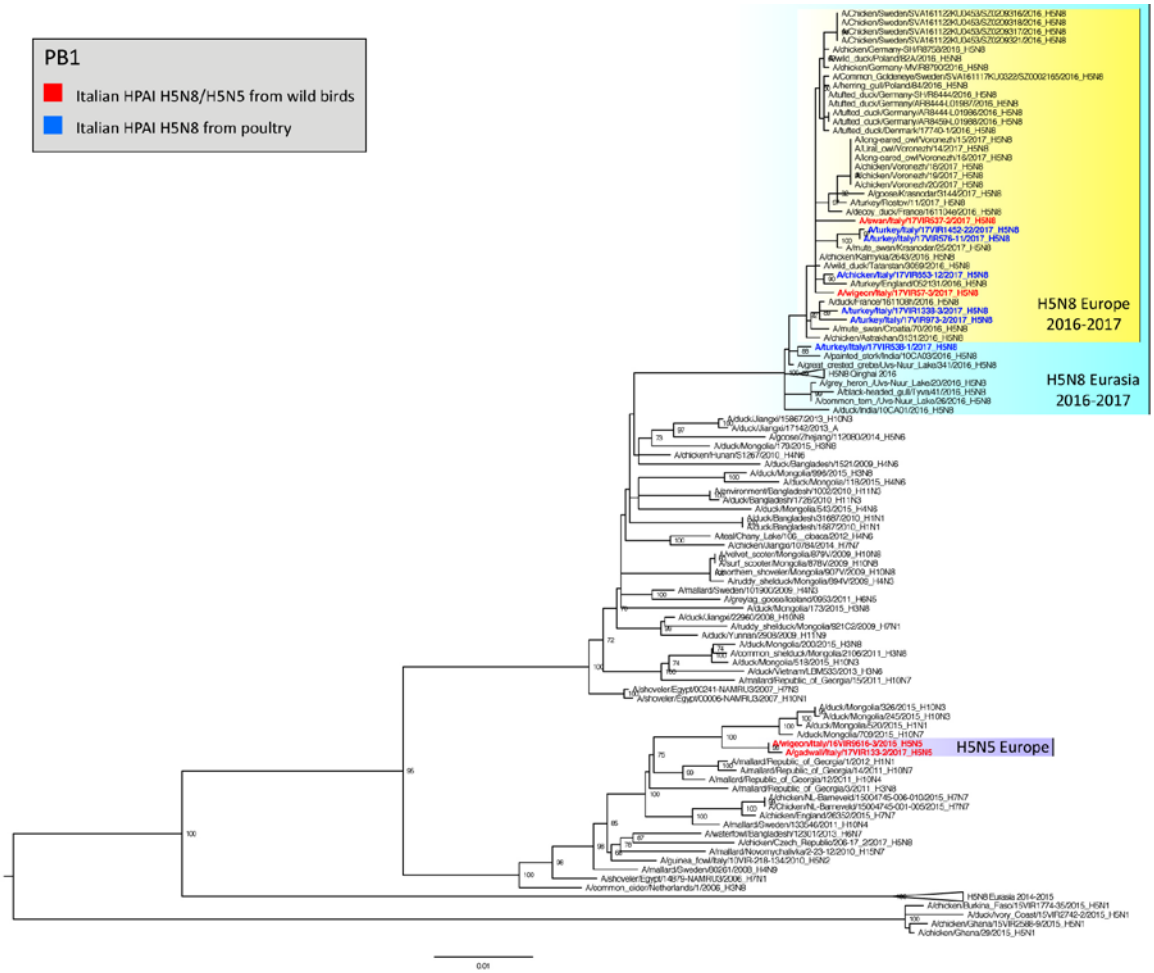
Technical Appendix Figure 1. Maximum Likelihood phylogenetic tree of the NA gene of the N8 subtype. HPAI H5N8 viruses were collected in Italy from wild (red) and domestic (blue) birds. Bootstrap supports higher than 60% are indicated next to the nodes, while branch lengths are scaled according to the number of nucleotide substitutions per site. The tree is midpoint rooted for clarity only.



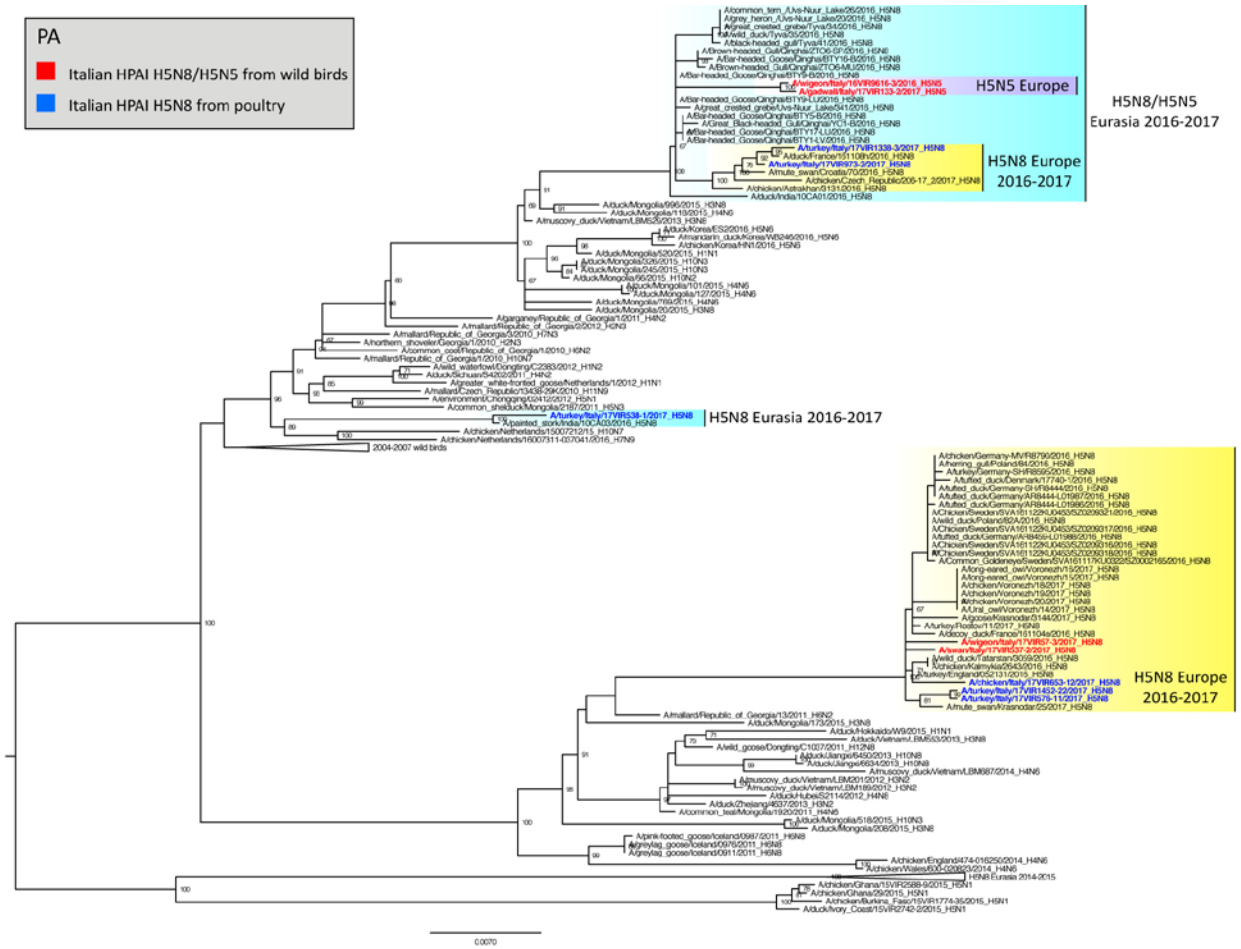
Technical Appendix Figure 2. Maximum Likelihood phylogenetic tree of the NA gene of the N5 subtype. HPAI H5N5 viruses collected in Italy from wild birds are marked in red. Bootstrap supports higher than 60% are indicated next to the nodes, while branch lengths are scaled according to the number of nucleotide substitutions per site. The tree is midpoint rooted for clarity only.



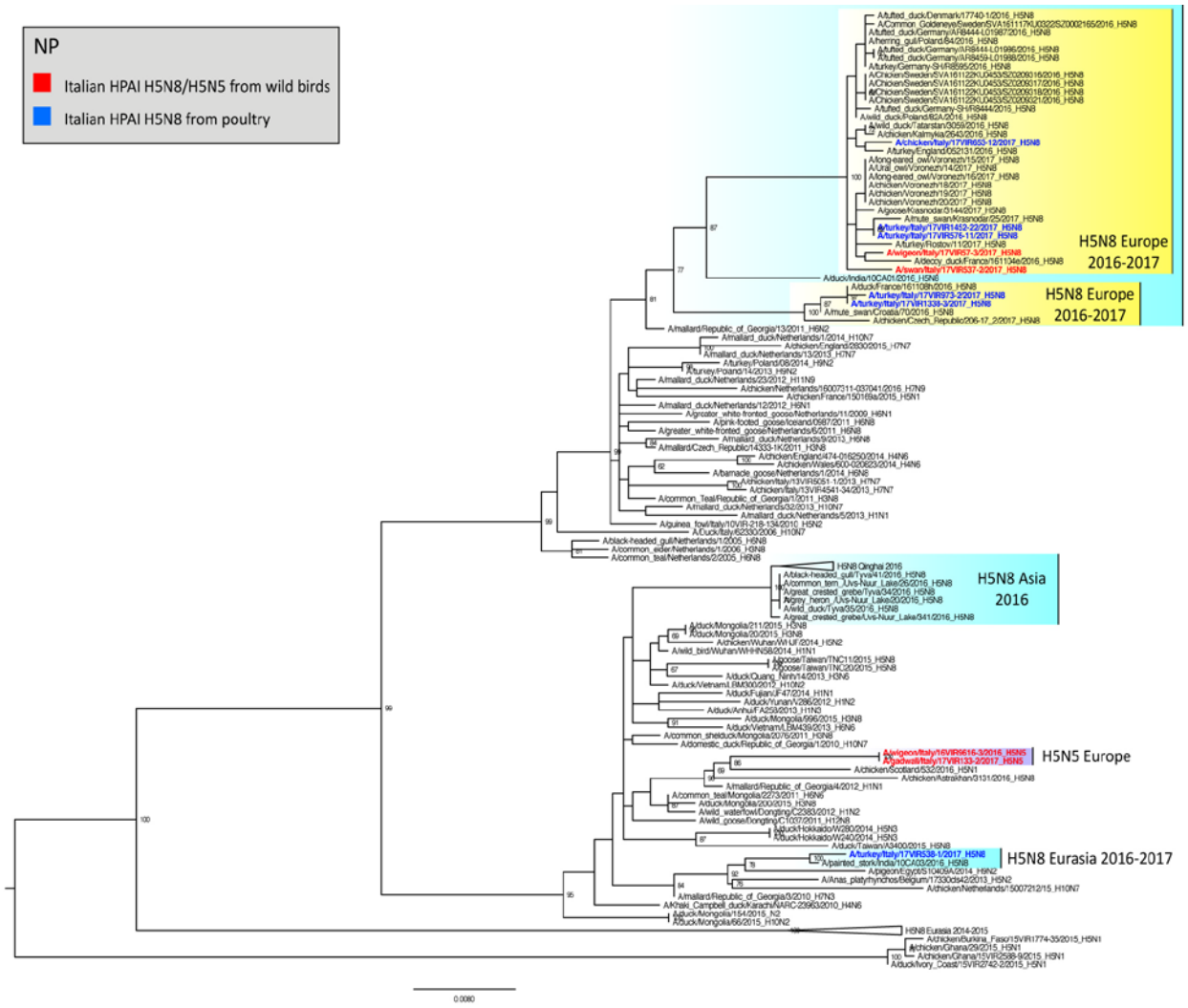
Technical Appendix Figure 3. Maximum Likelihood phylogenetic tree of the PB2 gene. HPAI H5N8/H5N5 viruses were collected in Italy from wild (red) and domestic (blue) birds. Bootstrap supports higher than 60% are indicated next to the nodes, while branch lengths are scaled according to the number of nucleotide substitutions per site. The tree is midpoint rooted for clarity only.



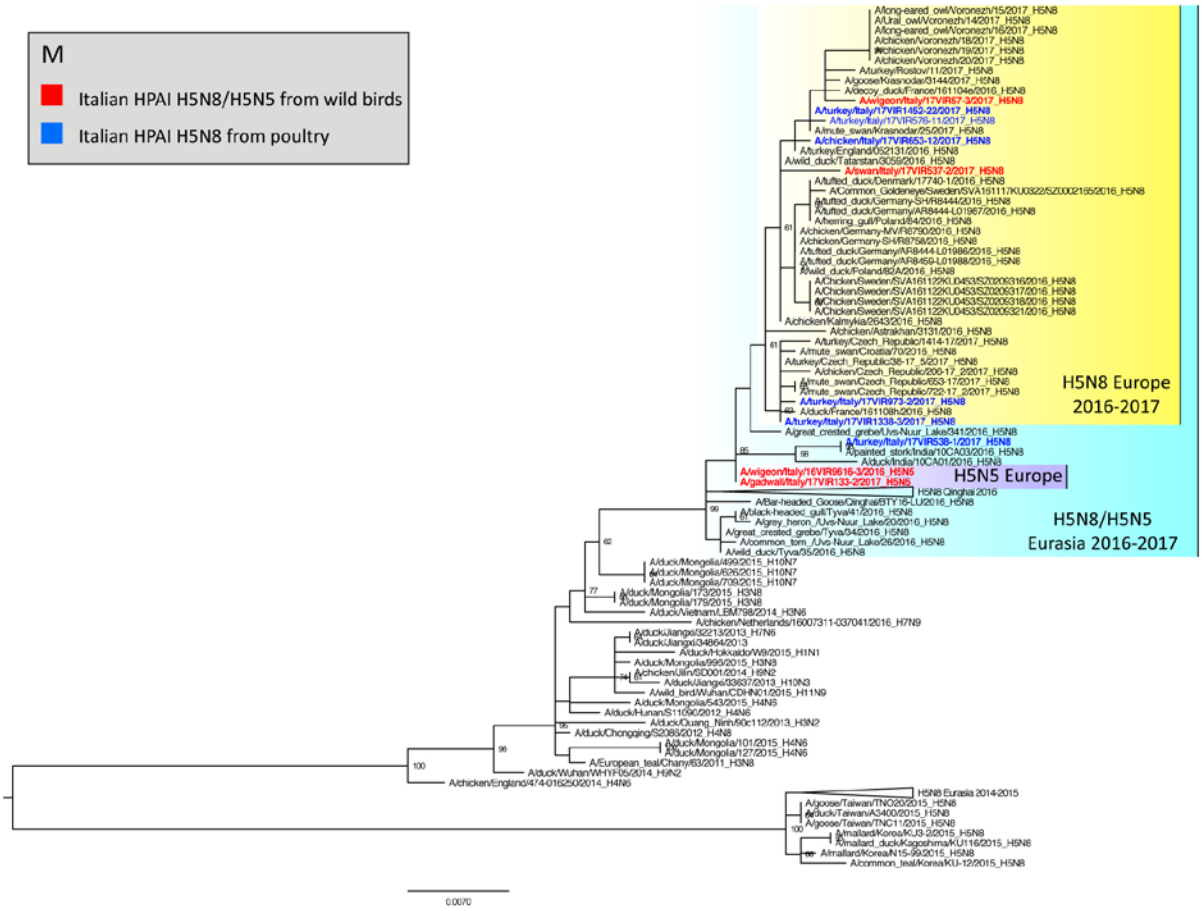
Technical Appendix Figure 4. Maximum Likelihood phylogenetic tree of the PB1 gene. HPAI H5N8/H5N5 viruses were collected in Italy from wild (red) and domestic (blue) birds. Bootstrap supports higher than 60% are indicated next to the nodes, while branch lengths are scaled according to the number of nucleotide substitutions per site. The tree is midpoint rooted for clarity only.



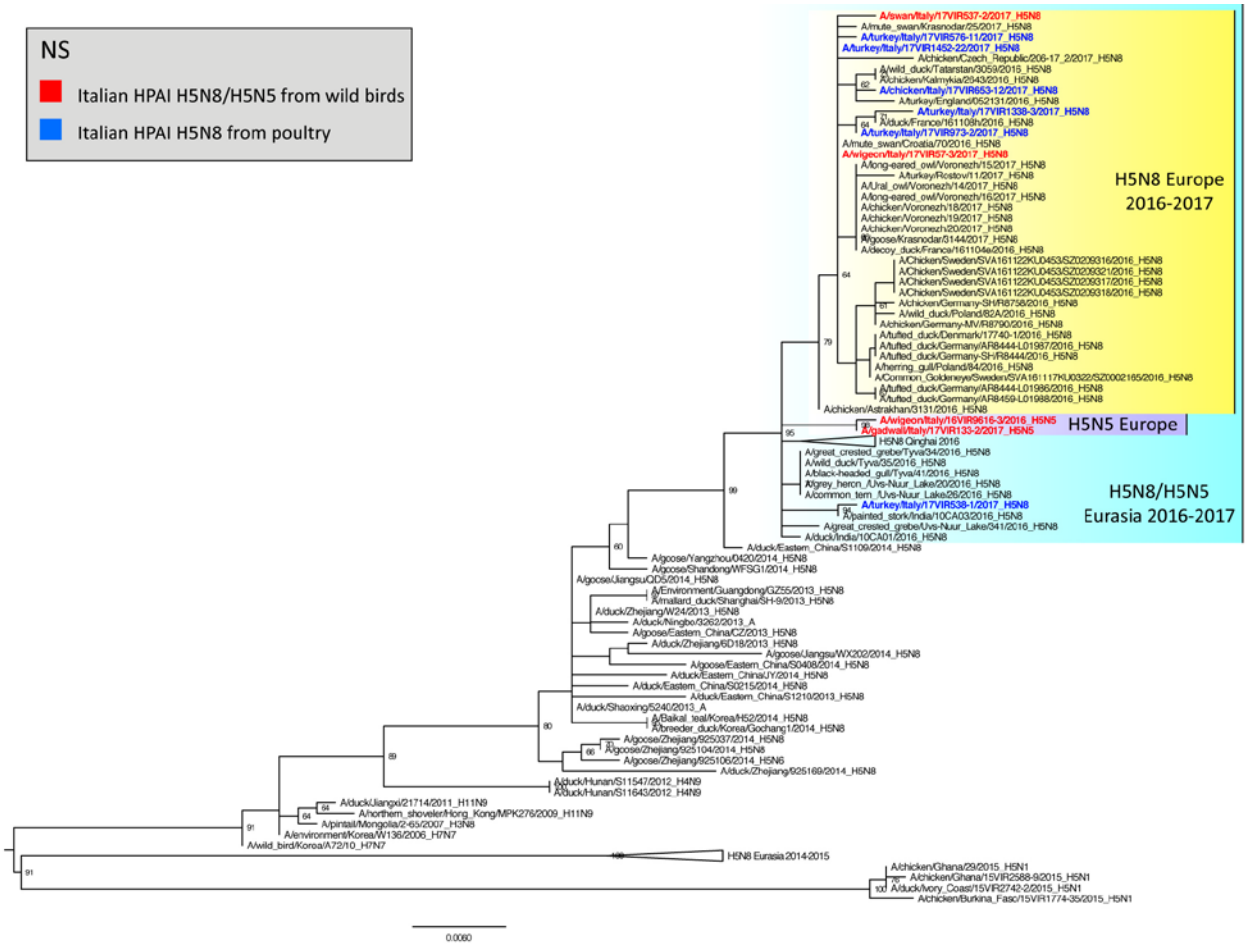
Technical Appendix Figure 5. Maximum Likelihood phylogenetic tree of the PA gene. HPAI H5N8/H5N5 viruses were collected in Italy from wild (red) and domestic (blue) birds. Bootstrap supports higher than 60% are indicated next to the nodes, while branch lengths are scaled according to the number of nucleotide substitutions per site. The tree is midpoint rooted for clarity only.



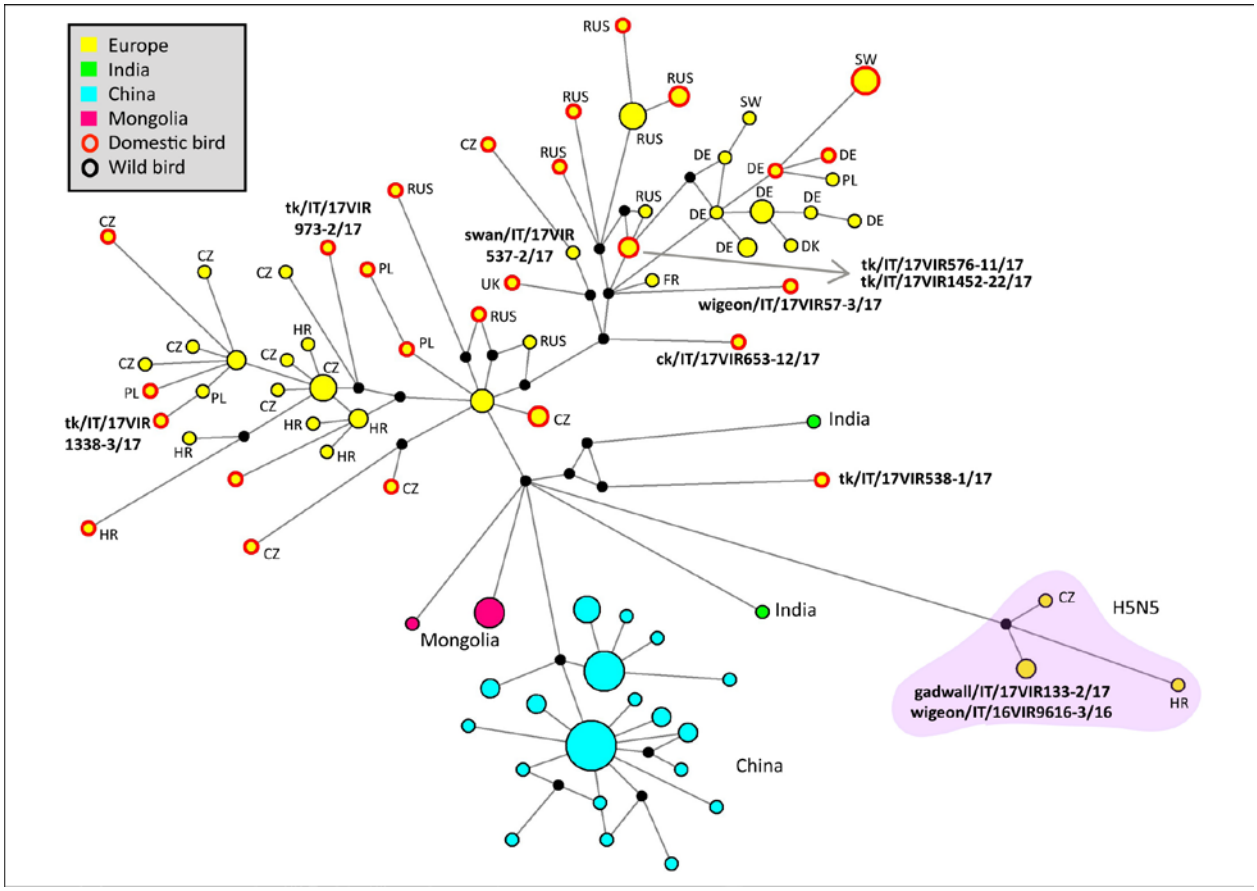
Technical Appendix Figure 6. Maximum Likelihood phylogenetic tree of the NP gene. HPAI H5N8/H5N5 viruses were collected in Italy from wild (red) and domestic (blue) birds. Bootstrap supports higher than 60% are indicated next to the nodes, while branch lengths are scaled according to the number of nucleotide substitutions per site. The tree is midpoint rooted for clarity only.



Technical Appendix Figure 7. Maximum Likelihood phylogenetic tree of the M gene. HPAI H5N8/H5N5 viruses were collected in Italy from wild (red) and domestic (blue) birds. Bootstrap supports higher than 60% are indicated next to the nodes, while branch lengths are scaled according to the number of nucleotide substitutions per site. The tree is midpoint rooted for clarity only.



Technical Appendix Figure 8. Maximum Likelihood phylogenetic tree of the NS gene. HPAI H5N8/H5N5 viruses were collected in Italy from wild (red) and domestic (blue) birds. Bootstrap supports higher than 60% are indicated next to the nodes, while branch lengths are scaled according to the number of nucleotide substitutions per site. The tree is midpoint rooted for clarity only.



Technical Appendix Figure 9. Median-joining phylogenetic network of the HA gene sequences of the 2016 HPAI H5N8/H5N5 viruses from Eurasia. Each unique sequence genotype is represented by a circle sized relatively to its frequency in the dataset. Branches represent the shortest trees and are proportional to the number of nucleotide mutations that separate each node. Median vectors are indicated as black circles. The pink shading shows the H5N5 viruses.