

# Strengthening outbreak investigations using a next generation approach

Alice Fusaro, Bianca Zecchin, Gianpiero Zamperin, Alessia Schivo, Angela Salomoni, Annalisa Salviato, Silvia Ormelli, Sabrina Marciano, Lebara Bonfanti, Alessandra Azzolini, Giovanni Cunial, Paolo Mulatti, Stefano Marangon, Calogero Terregino, Isabella Monne

Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro (PD), Italy



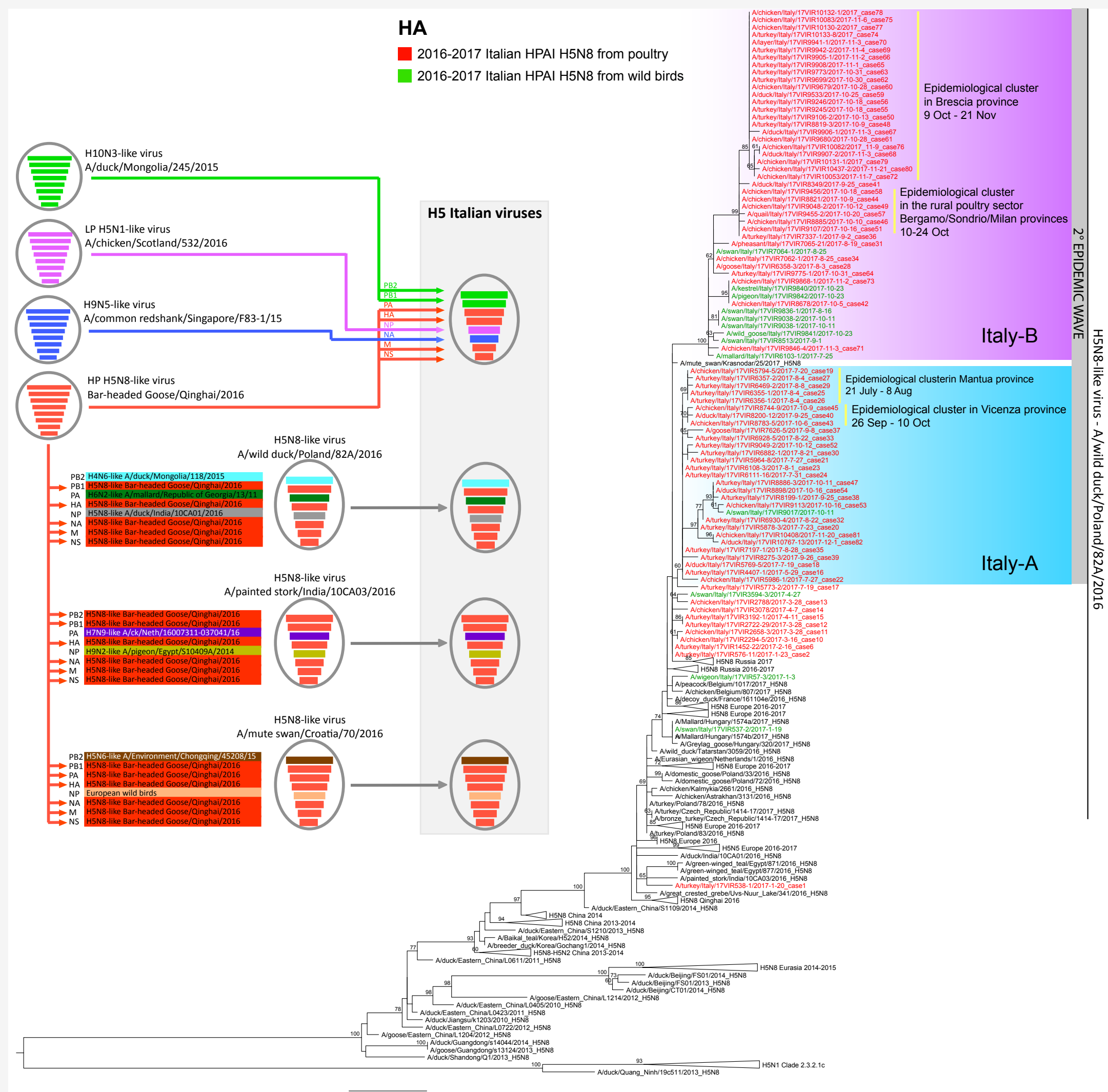
## The outbreak

Since the end of 2016, Italy has been experiencing circulation of Highly Pathogenic Avian Influenza (HPAI) H5N8 and H5N5 both in wild birds and domestic poultry. The first epidemic wave was observed between December 2016 – May 2017, with 16 outbreaks in poultry farms and 7 cases in migratory birds, while the second was registered between July-December 2017 when 67 HPAI H5N8 outbreaks in poultry farms and 10 in wild birds were confirmed.

## The aim

To shed light on the sources of the Italian H5N8 outbreaks reported since December 2016 and to investigate the inter- and intra-farm genetic diversity of the circulating viruses, we used an ultra-deep sequencing approach to characterize the complete genome of viruses from multiple hosts collected at each infected farm as well as from viruses identified in wild species, for a total of 115 samples and about 50 gigabases of data.

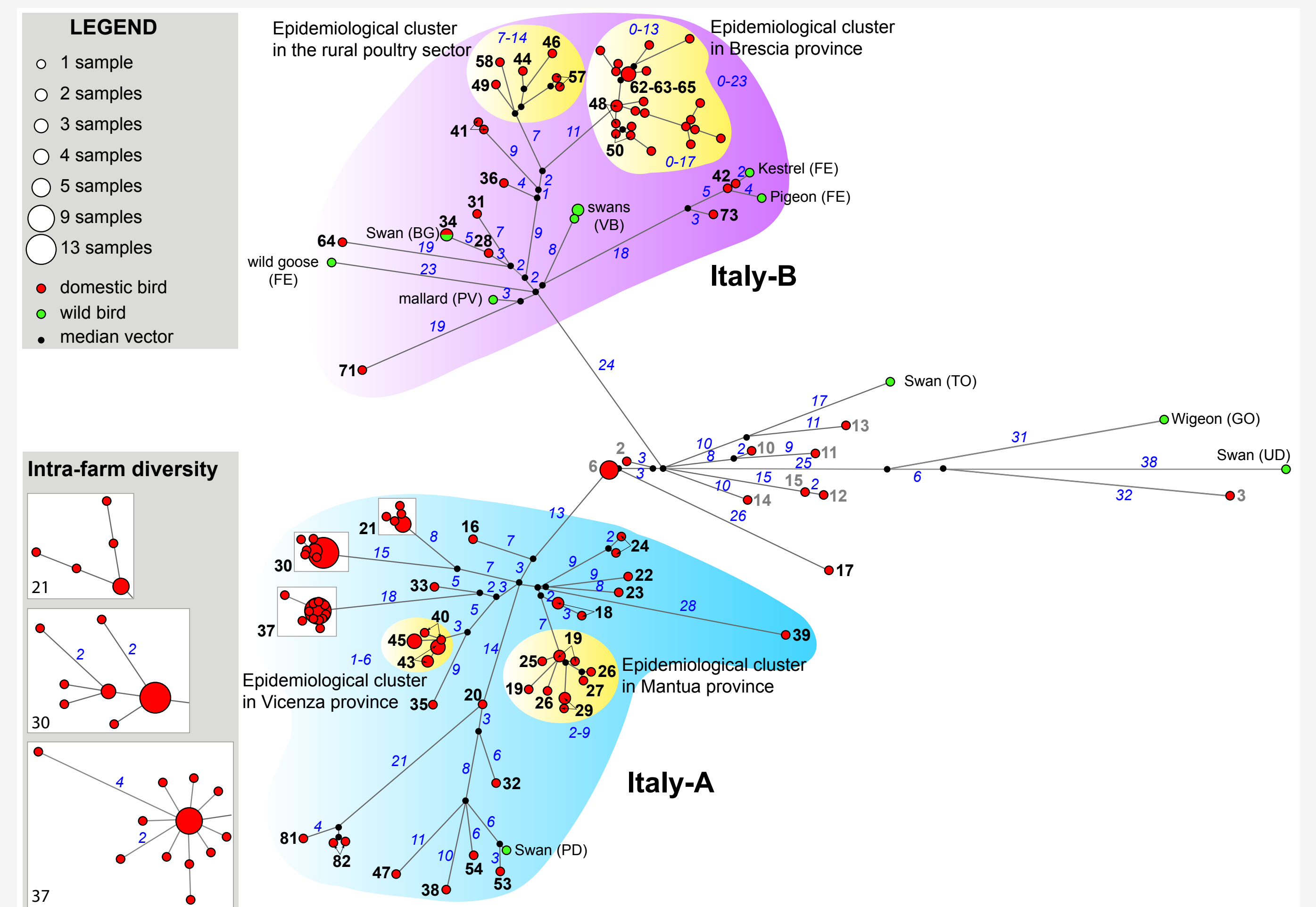
## Phylogenetic analyses identified four distinct genotypes



**Fig. 1.** A) 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, coloured according to their origin; B) Maximum-likelihood phylogenetic tree of the hemagglutinin (HA) gene of clade 2.3.4.4 viruses constructed using PhyML 3.1. H5N8 Italian viruses are highlighted in green (wild birds) and red (domestic birds). Bootstrap supports (100 replicates) >60% are indicated above the nodes.

- ❖ Maximum-likelihood phylogenetic trees of the eight gene segments identify the occurrence of multiple viral introductions of four distinct genotypes of HPAI H5N8 and H5N5 viruses in Italy at the beginning of the epidemic (December 2016-February 2017).
- ❖ Since March 2017 one single genotype, named H5N8-like virus A/wild duck/Poland/82A/2016 (Fusaro et al., EID, 2017), has been identified.
- ❖ During the second epidemic wave (July-December 2017) this genotype had further evolved into two main groups, namely Italy-A (light blue) and Italy-B (pink).

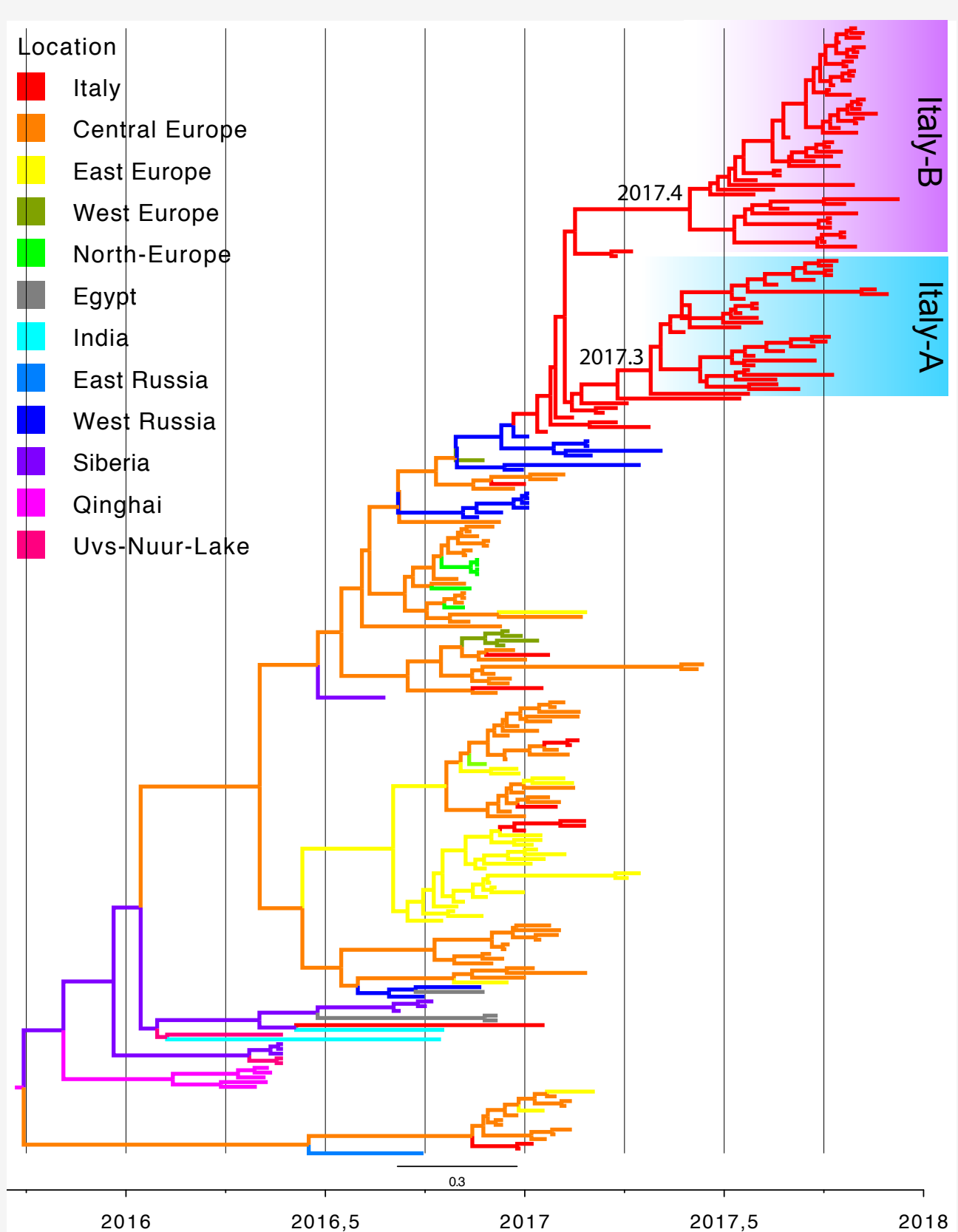
## Median-joining network analysis – 2° epidemic wave



**Fig. 2.** Median-joining phylogenetic network constructed from the consensus sequences of the eight concatenated gene segments of the Italian viruses of the H5N8-like A/wild duck/Poland/82A/2016 genotypes using the program NETWORK v.4.6 (epsilon=0). For each infected farm at least one virus was sequenced. Each unique sequence genotype is represented by a circle sized relatively to its frequency in the data set. Numbers next to the circles correspond to the case number. Branches represent the shortest trees, and blue numbers next to the branches represent the nucleotide mutations that separate each node. Median vectors (unsampled sequences) are indicated as black circles. Light blue and pink shadows identify respectively the groups Italy-A and Italy-B, which have been detected since July 2017. Yellow shadows represent the clusters of secondary outbreaks. The bottom left grey box shows a magnification of the intra-farm diversity of cases 21, 30 and 37.

- ❖ Median-joining network analysis identifies four clusters of secondary cases (in yellow), three in the industrial sector and one in the rural
- ❖ Network analyses of sequences of multiple samples within a farm (i.e., cases 21, 30 and 37) show a high intra-farm genetic diversity (0-9 nucleotide substitutions/genome), highlighting the importance of genetically characterizing viruses from multiple hosts, so as to correctly reconstruct the evolution and the transmission dynamics of an AI epidemic.

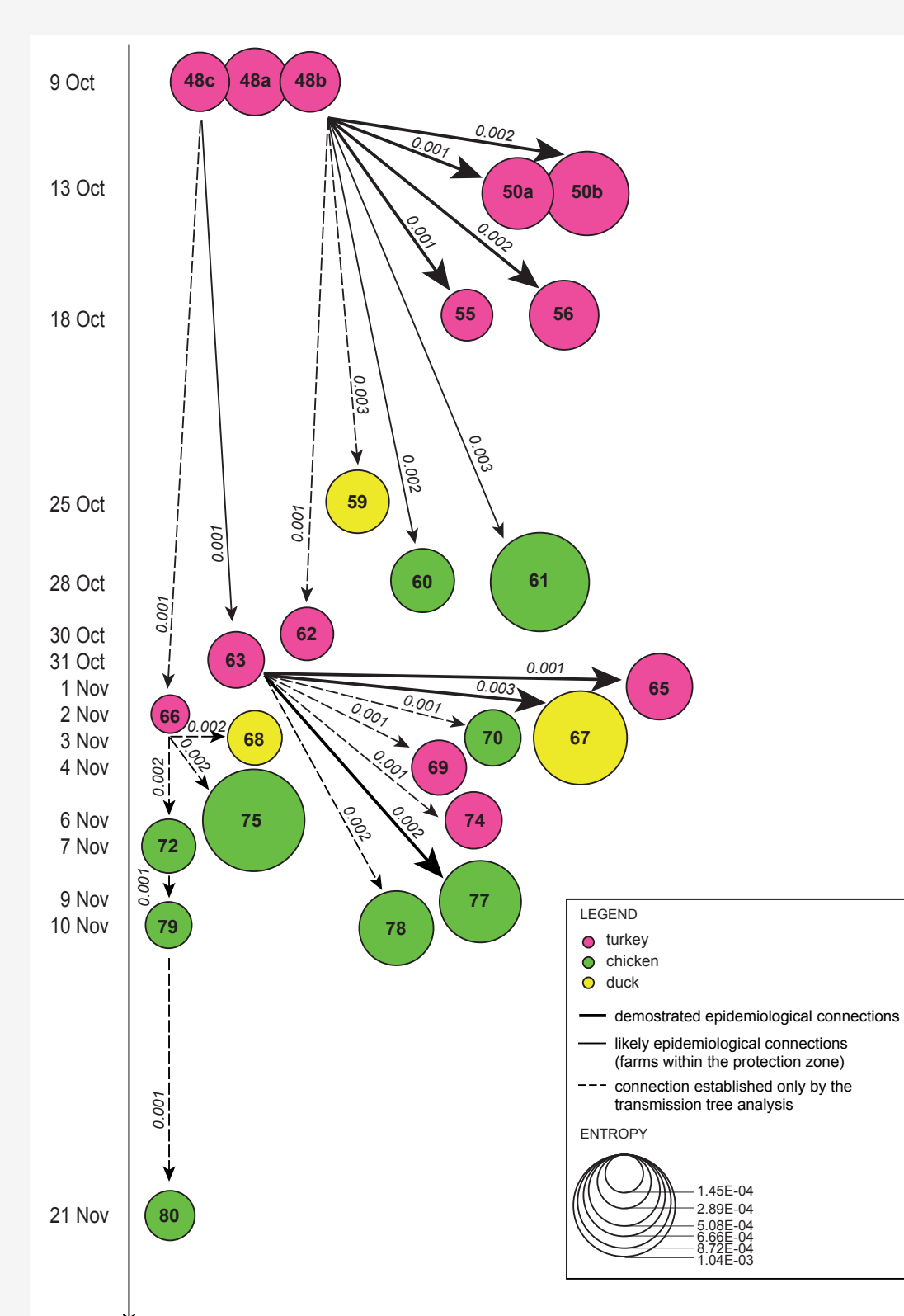
## Bayesian phylogeographic analysis identifies multiple introductions



- ❖ Discrete phylogeographic analyses performed using the BEAST v1.8.4 package (Lemey, et al., 2009) indicate that different HPAI H5N8 and H5N5 viruses were introduced in Italy probably through migratory birds from West Russia, Siberia, Central and East Europe.
- ❖ During the second epidemic wave Italy-A group probably emerged between February-April 2017 and has been circulating ever since in the north-east of Italy; differently, Italy-B group appeared between March-July 2017 and has mainly spread in the north-west of Italy.

**Fig. 3.** Maximum clade credibility (MCC) tree presenting the evolutionary relationships among the HPAI H5N8 and H5N5 viruses collected in Eurasia between 2016 and 2017. The color of each branch indicates the location where the analyzed viruses were collected.

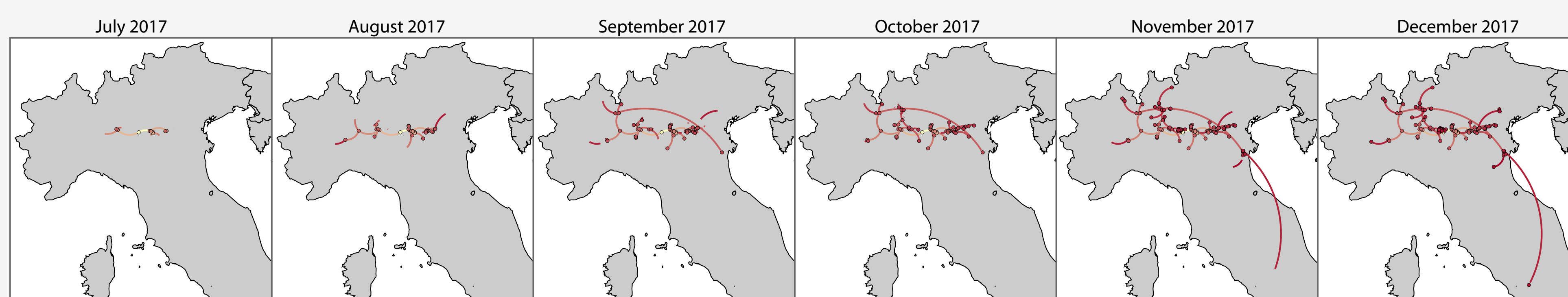
## Reconstruction of the transmission dynamics of the largest cluster of secondary outbreaks through ultra-deep data



Deep sequencing and epidemiological data were used to reconstruct the transmission pathway among the infected farms of the largest cluster of secondary outbreaks in the province of Brescia, which involved 22 farms between 9 October and 21 November 2017.

**Fig. 4.** Transmission tree obtained from deep-sequencing data (Illumina MiSeq) using SeqTrack (Poole et al., 2014). Each circle represents an individual sample, coloured according to the host species. The number within the circle corresponds to the number of the case. The size of the circles is proportional to the mean entropy value. The vertical axis represents the time of collection of each sample. Connecting arrows correspond to the results obtained from SeqTrack software. Specifically, thick black arrows represent proven epidemiological connections, thin black arrows are likely epidemiological links, whereas dashed arrows are transmission events identified only on the basis of genetic data. Numbers over the lines are the genetic distances between the complete genomes of the analysed samples calculated from the deep-sequencing data.

## Italy-A and Italy-B spread during the second epidemic wave



**Fig. 5.** Spread of the H5N8 viruses during the second Italian wave obtained using the continuous phylogeographic analysis (cauchy RRRW model) using the BEAST v1.8.4 software (Lemey et al., 2010). Visualization was obtained using SPREAD D3 (Bielejec et al., 2018). Connecting lines and dots (infected farms) are coloured according to the time (from yellow to dark red).

- ❖ Italy-A group seems to have emerged in Mantua province (Lombardy region) and then spread eastwards in the Veneto region.
- ❖ Italy-B group seems to have emerged in the central part of the Lombardy region and then spread westwards. This group was also responsible of the outbreaks in Emilia Romagna and Lazio regions.

## Conclusion

This study was instrumental to reconstruct the virus dissemination routes and indicated that wild and domestic birds from Lombardy most likely represented the key source for the re-emergence and spread of the HPAI virus during the second epidemic wave. This key spatial information will help to define appropriate disease control strategies on a local scale.

## REFERENCES:

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