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INFLUENZA

Role for migratory wild birds in the global spread of avian influenza H5N8

The Global Consortium for H5N8 and Related Influenza Viruses*†

Avian influenza viruses affect both poultry production and public health. A subtype H5N8 (clade 2.3.4.4) virus, following an outbreak in poultry in South Korea in January 2014, rapidly spread worldwide in 2014–2015. Our analysis of H5N8 viral sequences, epidemiological investigations, waterfowl migration, and poultry trade showed that long-distance migratory birds can play a major role in the global spread of avian influenza viruses. Further, we found that the hemagglutinin of clade 2.3.4.4 virus was remarkably promiscuous, creating reassortants with multiple neuraminidase subtypes. Improving our understanding of the circumpolar circulation of avian influenza viruses in migratory waterfowl will help to provide early warning of threats from avian influenza to poultry, and potentially human, health.

In 2014, highly pathogenic avian influenza (HPAI) virus of the subtype H5N8 caused disease outbreaks in poultry in Asia, Europe, and North America (1–3). Avian influenza viruses are a threat both to global poultry production and to public health; they have the potential to cause severe disease in people and to adapt to transmit efficiently in human populations (4). This was the first time since 2005 that a single subtype of HPAI virus had spread over such a large geographical area and the first time that a Eurasian HPAI virus had spread to

North America. The rapid global spread of HPAI H5N8 virus outbreaks raised the question of the routes by which the virus had been transmitted.

The segment encoding for the hemagglutinin (HA) surface protein of the HPAI H5N8 viruses is a descendant of the HPAI H5N1 virus (A/Goose/Guangdong/1/1996), first detected in China in 1996 (5). Since then, HPAI H5N1 viruses have become endemic in poultry populations in several countries. The H5 viruses have developed new characteristics by mutation and by reassortment with other avian influenza (AI) viruses, both in poultry and in wild birds. In 2005–2006, HPAI H5N1 spread from Asia to Europe, the Middle East, and Africa during the course of a few months. Although virus spread traditionally had been

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attributed to transport of infected poultry, infected poultry products, or HPAI-virus-contaminated materials, several observations in the 2005–2006 epidemic suggested that wild birds also might have carried the virus to previously unaffected areas (6).

A HPAI H5N8 virus with genes from viruses of the influenza A (H5N1) A/Goose/Guangdong/1/1996 lineage was first detected in birds at live bird markets in China in 2010 (7). This HPAI H5N8 virus was a reassortant virus with the HA gene segment from HPAI H5N1 virus and other gene segments from multiple other AI viruses circulating in eastern China (7) and is now categorized as HPAI H5 virus clade 2.3.4.4 (7). This clade is unusually promiscuous and has been found in combination with six different neuraminidase (NA) segments, and multiple H5Nx viruses may be circulating at the same time and in the same region (8, 9). The propensity of HPAI H5 virus clade 2.3.4.4 to form novel subtypes capable of rapid, global spread is a major concern.

HPAI H5N8 virus caused a large avian influenza outbreak in poultry in South Korea in the winter of 2013–2014 and subsequently spread to Japan, North America, and Europe, causing outbreaks there between autumn 2014 and spring 2015 (table S1). However, it is not clear by which routes HPAI H5N8 virus spread so rapidly around the world. Although there have been reports on parts of these outbreaks (1, 2, 10) and speculation on possible routes of transmission (3), no comprehensive global analysis has yet been performed.

The goal of this study was to analyze the available genetic, epidemiological, and ornithological data for evidence of the relative contributions from poultry trade and from wild bird movements (3, 6) for the global spread of clade 2.3.4.4 during 2014–2015. For this purpose, we performed phylogeographic analysis of HPAI H5N8 viruses detected in wild birds and poultry from this global outbreak. In addition, we analyzed migration patterns of wild birds found infected with HPAI H5N8 virus, epidemiological investigations of HPAI H5N8 virus outbreaks, and poultry-trade records from countries where HPAI H5N8 virus was reported (11).

Initial phylogenetic analysis was performed using HA sequences from HPAI H5 clade 2.3.4.4 viruses of poultry and wild birds from around the world between 2004 and 2015, including subtypes H5N1, H5N2, H5N3, H5N5, H5N6, and H5N8. From 2004 to 2012, clade 2.3.4.4 viruses were circulating predominantly in Eastern Asia (China), with some transmission to Southeastern Asia (Fig. 1 and fig. S1). During this period, transmission involving domestic anseriforms (ducks and geese) appears to dominate, although some contribution from domestic galliforms (chickens and turkeys) and short-distance migratory wild birds (e.g., mallard ducks) is also evident (Fig. 1). Unlike H5 segments from other clades, which are mostly found as H5N1, the HPAI H5 segment of the clade 2.3.4.4 viruses reassorts frequently, acquiring NA segments from cocirculating low pathogenic avian influenza (LPAI) subtypes, including N5 (from 2006 to 2010), N2

(from 2008 to 2012), N8 (from 2010), and, more recently, N6 (from 2013) (8). To indicate the host species and regions in which the reassortments are thought to have occurred, a reassortment measure was calculated using the number of branches in the posterior set of phylogenetic trees for which the NA subtype changed while the host species and region traits remained the same (normalized by branch lengths). This measure suggests that most of the observed reassortants were generated in domestic anseriforms (fig. S2), and particularly domestic anseriforms in Eastern Asia (China) within the time period 2004 to 2012 (fig. S3).

The time to the most recent common ancestor (TMRCA) for the HA segment of all clade 2.3.4.4 HPAI H5N8 sequences was estimated as June 2010 [95% highest posterior density (HPD), January to October 2010]; the TMRCA for the corresponding NA segments was similar (September 2010; 95% HPD, April to December 2010). Clade 2.3.4.4 HA H5N8 sequences were found in two subclades (Fig. 1). The smaller and earlier subclade (a in Fig. 1) contained the first sequenced 2.3.4.4 HPAI H5N8 virus [A/Duck/Jiangsu/ki203/2010 (H5N8)]. The larger and more recent subclade (b in Fig. 1) contained sequences from outbreaks in South Korea and other countries included in this study and caused multiple HPAI outbreaks in 2014 and 2015 globally. The TMRCA of subclade b was September 2013 for both HA (95% HPD, July to November 2013) and NA (95% HPD, May to November 2013). Consistent with earlier findings (1, 10), the phylogeny indicates that HPAI H5N8 was introduced into South Korea by long-distance migrant wild birds that acquired it from the pool of HPAI H5 viruses circulating in domestic anseriforms in Eastern Asia (China), although we

formally cannot exclude the possibility that HPAI H5 viruses were circulating in unsampled locations (Fig. 1).

Distinct, well-supported clades were identified in South Korea, likely originating in the transmission of HPAI H5N8 viruses from long-distance migrants to other wild and domestic birds (10). One clade (c in Fig. 1) was ancestral to the European outbreak and another (d in Fig. 1) was ancestral to the North American outbreak. Again, we cannot exclude the possibility that viruses from these subclades were present in unsampled locations.

More detailed phylogenetic analyses, using only clade 2.3.4.4 H5N8 HA sequences with location coordinates (11), showed that the virus spread along two main long-distance migration routes: one from the east Asia coast/Korean peninsula, north to the Arctic coast of the Eurasian continent, then west to Europe; and the other north from the Korean peninsula, then east across the Bering Strait, and south along the northwest coast of the North American continent to Canada and the United States (Fig. 2 and movie S1). The reconstruction did not indicate any spread between Europe and North America. The TMRCA for European HA segments was August 2014 (95% HPD, July to October 2014), and September to October 2014 (95% HPD, August to November 2014) for the North American HA segments (table S2, a and b). Similar results were found from analysis of the NA segments (table S2, c and d). There were also four separate introductions into Japan, the first estimated around February 2014 (ancestral date of single virus A/Chicken/Kumamoto/1-7/2014), and then three more, all with TMRCA in October and November 2014. The sequences from one Japanese introduction were most closely related to sequences from Taiwan and those from

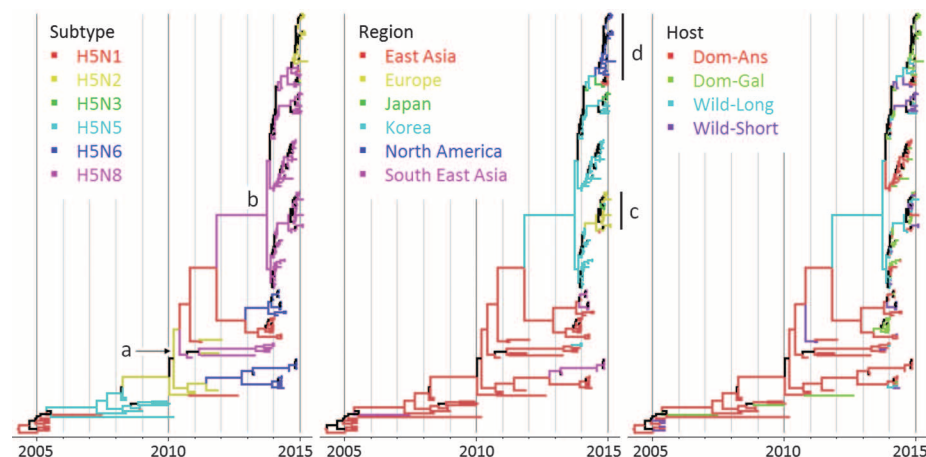


Fig. 1. Maximum clade credibility (MCC) time-scaled phylogenetic tree of multisubtype HA sequences colored by subtype, region, and host-type traits. The clades marked a and b contain H5N8 sequences, and c and d contain sequences from Europe and North America, respectively. The displayed MCC tree was obtained from a posterior set of trees inferred using the Bayesian Evolutionary Analysis Sampling Trees (BEAST) program (13) with the SRD06 nucleotide substitution model, uncorrelated relaxed clock model, and constant population size tree prior. The branches are colored according to the most probable ancestral trait, and ancestral traits were inferred by a symmetric (subtype and region) or asymmetric discrete trait model (host-type) upon the posterior tree set (14). Host types are Dom-Ans (red), domestic anseriform birds; Dom-Gal (green), domestic galliform birds; Wild-Long (blue), long-distance migratory wild birds; Wild-Short (purple), short-distance migratory wild birds.

another introduction to the Russian (A/Wigeon/Sakha/1/2014) and European sequences.

The phylogenetic data were also used to infer the ancestral host categories of the most recent common ancestor of the European and North American outbreak sequences, thus providing evidence for which host type had introduced the viruses into those areas (Fig. 3, figs. S4 and S5, and table S2). The most likely ancestral host category for the North American outbreak for both HA and NA segments was long-distance migrants (HA, 66%; NA, 84%). A similar result was obtained for Europe (HA, 66%; NA, 70%).

Several wild bird species with known HPAI H5N8 sequences were long-distance migrants at different stages of their migratory cycle, depending on time and place found (table S3): Five of the nine species found in South Korea in winter 2013–2014 were long-distance migrants at their wintering sites or on spring migration. Both in North America and Europe, two of the four species found in winter 2014–2015 were long-distance migrants at their wintering sites or on autumn migration (17) (tables S4 and S5 and fig. S6).

The April 2014 HPAI H5N8 virus outbreak in Japan had different characteristics from the later

outbreaks in North America and Europe. The Japan outbreak was the only one that was contemporaneous with the outbreak in South Korea, and no wild birds were found positive for HPAI H5N8 virus in Japan during that outbreak.

Qualitative analysis of data from outbreak investigations on affected poultry farms in North America, Europe, and Japan (17) (table S6) showed that the likelihood of virus introduction via contaminated water, feed, and poultry was negligible (Germany). Furthermore, no links between the outbreaks in one country and those in other countries could be attributed to personnel contacts or

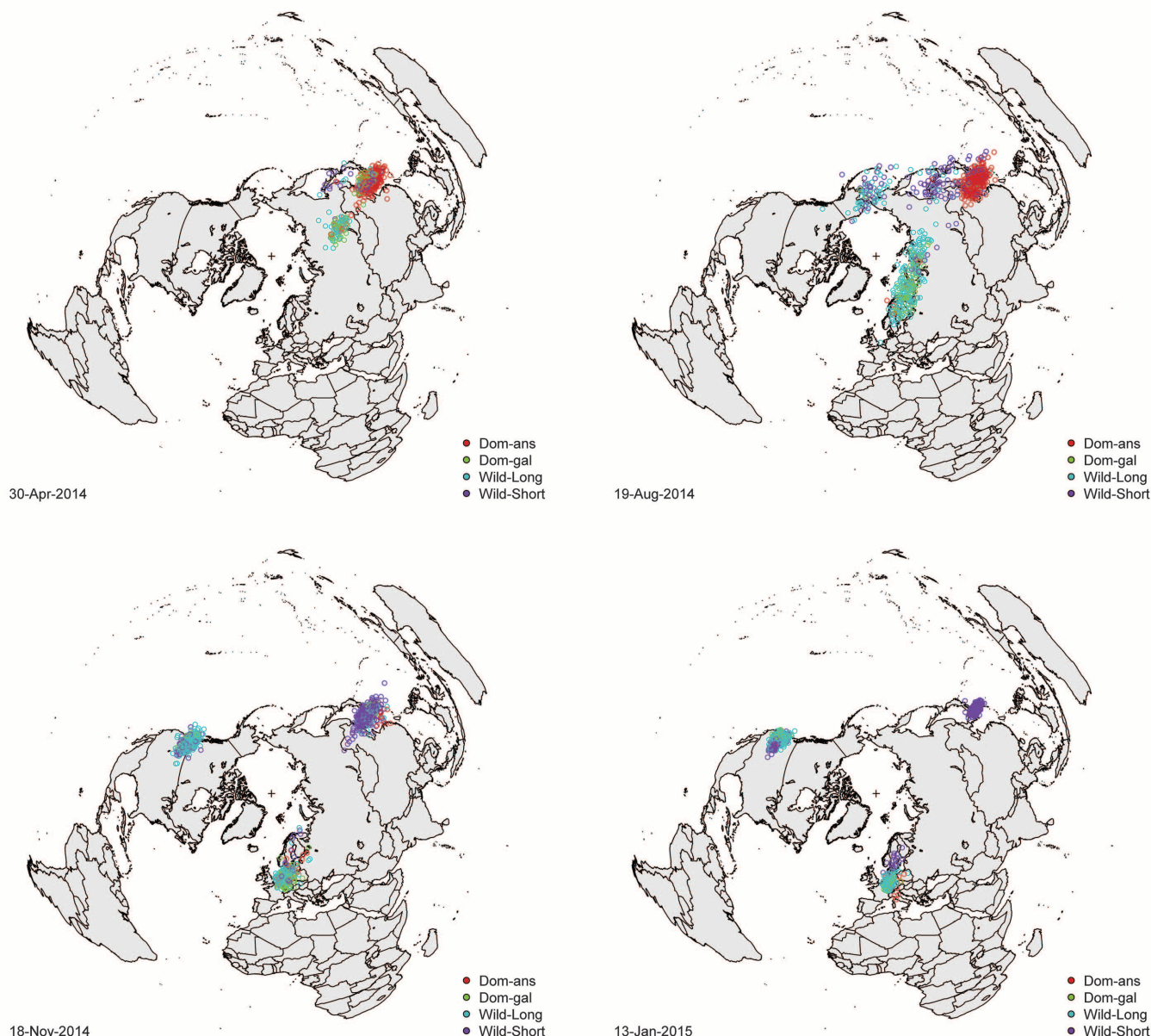


Fig. 2. Reconstruction of the transmission routes using phylogenetic data only from H5N8 HA sequences. At each time slice, the host-type and location coordinates on the branches of the posterior set of phylogenetic trees are inferred and plotted as a cloud of points. The host type was inferred by discrete trait model (as Fig. 1) (14), and the continuous location coordinates were inferred using a homogeneous Brownian motion diffusion model (15). The map projection used is the azimuthal equal areas projection, centered on the North Pole, which is marked with a + sign. Color key as for Fig. 1; see also movie S1.

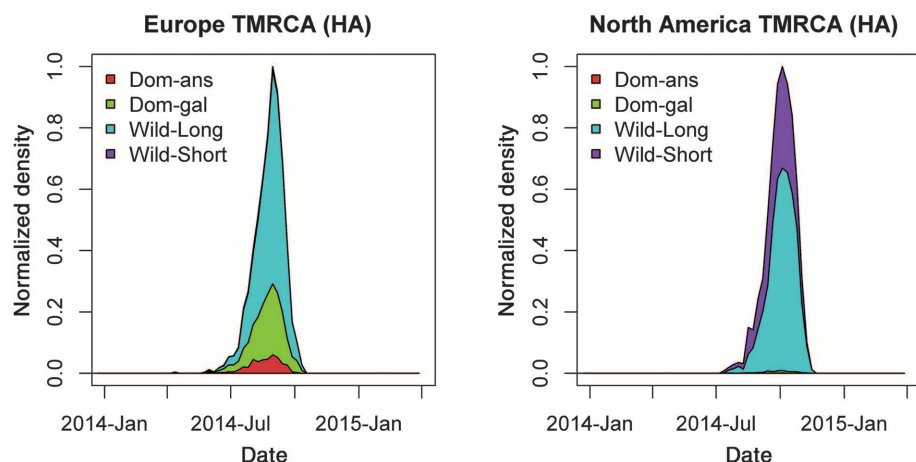


Fig. 3. Posterior distributions of TMRCA of HA sequences from Europe and North America with H5N8 subtype only, including host-type reconstructions, based upon a posterior set of phylogenetic trees generated as in Fig. 1. Color key as for Fig. 1.

trade of live animals, feed, or products of animal origin (Germany, Netherlands, United Kingdom, and Canada). Many affected poultry farms were located in areas where wild waterfowl are abundant (Germany, Netherlands, United Kingdom, Italy, and Canada). Direct contact with infected wild birds (United States) or indirect contact with materials (e.g., bedding material, boots, and wheels of vehicles) contaminated with wild-bird feces was considered the most likely route of introduction into poultry holdings (United States, Germany, Netherlands, United Kingdom, and Italy). In some outbreaks, the source of infection was unknown or inconclusive (Japan and Hungary).

We reviewed data from the Food and Agriculture Organization of the United Nations (FAO) (12) for 2011 to 2013 on export and import of live domestic ducks and chickens of affected countries to estimate the risk of spread of HPAI virus from South Korea to other countries via the international poultry trade (table S7). Data on the export of live poultry from North Korea and Mongolia, also in East Asia, were not available from FAO. Although all countries (Japan, Canada, United States, Germany, Netherlands, United Kingdom, Italy, and Hungary) where HPAI H5N8 virus emerged between November 2014 and February 2015 imported live chickens and live domestic ducks in 2013, South Korea reported the export of a low number of live chickens and no export of live domestic ducks, although unreported cross-border trade cannot be excluded. Nevertheless, based on these data, it seems unlikely that international trade in live poultry played a major role in the long-distance spread of South Korean clade HPAI H5N8 virus in 2014–2015.

Our analysis, using four different sources of data, indicates that the main routes of large-scale geographical spread of HPAI H5N8 virus were most probably via long-distance flights of infected migratory wild birds, first in spring 2014 from South Korea or other unsampled locations in the

region to northern breeding grounds and then in autumn 2014 from these breeding grounds along migration routes to wintering sites in North America and Europe.

Recognition of a likely role of wild birds in the spread of HPAI reinforces the need to improve biosecurity on poultry farms and to exclude wild birds from the immediate vicinity of poultry farms. Culling wild birds and draining or disinfecting wetlands would not be effective because these viruses disseminate on rapid time scales over very large distances, making reactive interventions of this kind impractical and ineffective, as well as contravening commitments made by signatory countries to the Convention on Migratory Species and the Ramsar Convention on Wetlands.

The potential role of wild birds in the circumpolar circulation of influenza viruses does point to the need to increase our knowledge about the connectedness at the vast circumpolar (sub)arctic breeding areas between migratory waterfowl populations originating from different wintering areas. Surveillance of waterfowl at the crossroads of migratory flyways to wintering areas in Europe, Asia, and North America would inform epidemiological risk analysis and provide early warning of specific HPAI threats to poultry, and potentially human, health.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/354/6309/213/suppl/DC1
Materials and Methods

Figs. S1 to S6

Tables S1 to S10

Movie S1

References (16–59)

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Supplementary Materials for

Role for migratory wild birds in the global spread of avian influenza H5N8

The Global Consortium for H5N8 and Related Influenza Viruses*[†]

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Materials and Methods
Figs. S2 to S6
Tables S1 and S3 to S8
References

Other Supplementary Material for this manuscript includes the following:
(available at www.sciencemag.org/cgi/content/full/354/6309/213/DC1)

Fig. S1
Tables S2, S9, and S10 as Excel files
Movie S1

Materials and Methods

Sequence data

Sequence data obtained from field isolates collected from domestic and wild birds were contributed by the partners from 16 member countries in the Global Consortium for H5N8 Avian Influenza Viruses. The data were curated into a Global Initiative on Sharing All Influenza Data (GISAID) workset to facilitate sharing between the partners. GISAID promotes the sharing of all influenza type virus sequences, related clinical and epidemiological data associated with human isolates, and geographic and species-specific data associated with avian and other animal isolates. The GISAID workset used here included the sequence data themselves as well as information on host species, date of isolation and location of isolation. Publically available background sequences from GISAID were also used, including other clade 2.3.4.4 sequences. The final data consisted of (i) 219 HA multiple subtype sequences from 2005-2015 (ii) 130 HA H5N8 sequences (2010-2015) and (iii) 84 NA H5N8 sequences (2010-2015); and each contained sequences from several global regions, as well as from wild and domestic avian hosts (**Tables S8 and S9**).

Phylogenetic analysis

Bayesian time-resolved phylogenetic trees were created using BEAST 1.8 (*13*). Several nucleotide, molecular clock and tree prior / effective population size models were evaluated on HA data using AICM in Tracer 1.6. The selected models were: the SRD06 nucleotide substitution model (one HKY model for codon positions 1 and 2, and another HKY for codon position 3, both with site-site rate variation assuming 4 categories of rate variation taken from a gamma distribution, which allows site to site rate variation ranging from highly conserved to very variable sites), an uncorrelated relaxed clock with a log-normal distribution, and a constant population size tree prior (for the large multiple subtype HA dataset), or skygrid flexible effective population size tree prior (for the H5N8 only datasets) (*16*). Three independent MCMC chains for the HA and NA H5N8 only datasets were run, each chain consisted of 50,000,000 steps, was sampled every 5,000 steps, and the first 10% of samples discarded as burn-in. For the multiple subtype data, four independent chains were run, the chain length was increased to 100,000,000 steps (and sampled every 10,000) and the first 20% of samples discarded as burn-in. For each dataset, the post-burn-in independent runs were combined, and then down sampled to create posterior tree sets containing 1000 trees. The above MCMC settings were chosen to achieve a post burn-in effective sample size of at least 200 in all parameters, as recommended on the BEAST website (*17*), and at least two independent runs were combined to mitigate non-convergence between runs, but in some cases we combined four independent runs in order to achieve the best effective sample size.

Phylogenetic discrete and continuous traits models were inferred for each data set using the posterior tree sets as input and MCMC chain lengths of 11,000 steps with sampling every 1,000 steps. An asymmetric model was used for the Host discrete trait, and symmetric models were used for Subtype and Region discrete traits (the choices were made by comparing symmetric and asymmetric models using AICM in Tracer 1.6). To perform phylogeographic diffusion with continuous spatial coordinates using

homogeneous Brownian motion, the latitude and longitude of the sampling locations were first transformed into a north-pole map projection using equal azimuthal areas. The traits models were run independently over the posterior tree sets. Results from Host and continuous spatial coordinates models (H5N8 data sets), and Host, Subtype and Region (H5NX data set) were amalgamated (after discarding burn-in samples) for posterior tree sets containing 1000 trees, to obtain approximately 10 multiple trait mappings on each tree of the posterior set. In order to compare original to randomized trait reconstructions, Host and Region discrete trait labels were permuted (100 times for host only, and 10 times for Host + Region) and run over the posterior tree sets containing 1000 trees.

Using the posterior tree sets with Host, Subtype and Region (H5NX data set) discrete trait mappings, we identified branches where the (i) Host species and (ii) Host species and Region, were the same at the ancestral and child nodes. To get a measure of where reassortment was occurring, for the identified branches, we calculated the normalized subtype changes per host (or per Host-Region) as the sum of the branch lengths for branches where the subtype changed divided by the sum of the branch lengths (of the identified branches). Additionally, to examine the reassortments per Host-Region, we also calculated the normalized subtype changes for branches occurring before and after 2012 separately.

Hosts were divided into the following categories: poultry were divided into domestic anseriform birds and domestic galliform birds; wild birds were divided into long-distance (listed in **Table S4**) and short-distance migratory birds (listed in **Table S5**). To test the effect of wild bird categorisation on phylogenetic analysis, wild birds were also examined as one group (Wild) or divided into two different groups, anseriforms (Wild-anseriform) and other species (Wild-other) (**Table S2**). Subtypes were HPAI viruses with the HA subtype 5 and NA subtypes 1, 2, 3, 5, 6, or 8. Regions were either individual countries (Japan or South Korea) or the following regions: Eastern Asia (China, East Russia, and Taiwan), Europe (Germany, Hungary, Italy, Netherlands, Sweden, and UK), North America (Canada and USA), or South Eastern Asia (Laos, Malaysia and Vietnam). South Korea and Japan were categorized individually because there were enough sequenced viruses to do so (>80 from South Korea, 12 from Japan) and because of our specific interest to evaluate the assumed sources of the viruses causing later outbreaks. Regional categorization was done after phylogenetic analysis, which was therefore not affected by the regional categories chosen.

Epidemiological outbreak investigations

After the HPAI H5N8 outbreak in South Korea, which started in January 2014, the virus was first detected in a chicken farm in Japan in April 2014. The origin of the infection was unknown or inconclusive, and Japan declared freedom from HPAI from July 2014 (18). Starting in November 2014, HPAI H5N8 virus again was detected in Japan, in faecal samples of tundra swans (*Cygnus columbianus*) and of unspecified wild ducks (Anatidae), as well as in tissues of individual sick or dead wild birds (white-naped crane [*Grus vipio*], hooded crane [*Grus monacha*], Mandarin duck [*Aix galericulata*], and mallard [*Anas platyrhynchos*]). In December 2014 and January 2015, several chicken farms (broiler, broiler breeder, layer) also were infected with HPAI H5N8 virus. Again,

the origin of the infection was unknown or inconclusive, and Japan declared freedom from HPAI from April 2015 (18).

Between November 2014 and February 2015, HPAI H5N8 was detected in a total of 11 poultry farms (turkey, duck, chicken) and other holdings, as well as in a small number of wild waterfowl, in five European countries, as well as in two mute swans (*Cygnus olor*) in Sweden. In Germany, epidemiological outbreak investigations were performed to identify potential routes of entry via water, feed, animals, people, bedding material, other fomites (e.g., equipment and vehicles) and the presence of wild birds near affected holdings (12, 19–22).. Virus introduction via contaminated water, feed and poultry introduced into the farms was considered negligible in all cases, based on the retrospective, qualitative assessment of risk factors for the introduction of HPAIV H5N8 by use of a template of the World Organisation for Animal Health for risk analysis . Furthermore, no links between the outbreaks in Germany and those in other countries were detected regarding person contacts or trade of live animals, feed, or products of animal origin. In contrast, the presence of wild birds near the affected holdings was the factor with the highest mean risk score. All outbreaks in Germany occurred in the north of the country, and all were in areas with an abundant presence of wild waterbirds. A few days before the outbreak in one of the three commercial poultry farms, large aggregations of bean geese (*Anser fabalis*) and greylag geese (*Anser anser*) were observed in surrounding pastures, and the ground around the poultry houses was notably contaminated with bird faeces. Considerable numbers of wild waterbirds also were observed in the vicinity of the other two commercial poultry farms. The highest risk of virus introduction into poultry farms was considered to be indirect contact with materials (e.g., bedding material, boots, wheels of vehicles) contaminated with wild bird faeces. Introduction via contaminated boots or bedding material on the two turkey farms is supported by the fact that turkeys in the poultry houses next to the entrances of the farms were the first to be affected, and by the identification of gaps in biosecurity measures. In the two small free-range holdings and the zoo, risk of introduction by direct contact with wild birds was considered high.

In the Netherlands, all five affected poultry farms kept their animals indoors and were located in areas where wild waterfowl were abundant. Epidemiological outbreak investigations did not reveal possible dangerous contacts (e.g., professional visitors and transport of contaminated feed or bedding material) between the affected Dutch farms and affected farms in Germany, the U.K., or Asia, or between the affected Dutch farms themselves, except one possible farm-to-farm transmission. The most likely route of virus introduction was suggested to be persons wearing contaminated clothes or boots, carrying contaminated materials or feed, or by contaminated vermin or flies (23).

The single affected poultry farm in the U.K. was located about 30 km north of the Humber Estuary, one of the most important wetland sites in the U.K., with up to 130,000 wildfowl and waders making use of saltmarshes and mudflats during migration and overwintering. Epidemiological investigation showed no direct connections between affected holdings in the U.K., Germany and the Netherlands, or to the Far East. There was no evidence of direct introduction of HPAI H5N8 virus by purchased poultry, by

indirect contact with contaminated people or fomites, or by importation of infected products (e.g., feed) (24, 25).

The single affected poultry farm in Italy was located in close proximity to wetlands and marshlands of the Po River Delta, which is one of the most populated wintering sites for migratory birds and wild waterfowl in Italy. Results of epidemiological investigation suggested that HPAI H5N8 virus may have been introduced into the farm by use of litter contaminated by infected wild birds where it was kept outside (26).

The single affected farm in Hungary was located 30 km south of the 32,000 hectare Hortobágyi National Park, where important numbers of many species of migratory waterbirds feed and rest (27). Results of epidemiological investigation indicated that there was no transport to the affected holding from outside Hungary, and the source of infection was unknown or inconclusive (28).

Two out of ten mute swans found dead in central Stockholm, Sweden, were autopsied as part of general wildlife health surveillance and found positive for HPAI H5N8 virus. There are no commercial poultry farms in the near vicinity, and HPAI H5N8 virus was not reported in any poultry farms in Sweden (29).

Between November 2014 and July 2015, HPAI H5N8 and related HPAI H5N2 and HPAI H5N1 outbreaks were detected in poultry farms and wild birds in western and central North America. In Canada, outbreaks occurred in poultry farms in British Columbia and Ontario, affecting a total of 324,504 birds (18). Field epidemiological data from the outbreaks in Canada were not available, but it was noted for one outbreak in Ontario that wild waterfowl was present around the barns beforehand, and that HPAI H5N8 virus was detected in a single American wigeon (18). In the USA, outbreaks occurred in poultry farms of 15 western and central states, affecting over 48 million birds (30), as well as a wide range of wild bird species, mainly ducks, geese, and raptors (30, 31). Based on both epidemiological and virological analyses, direct or indirect contact with infected wild birds was considered to be responsible for initial introduction of HPAI H5N8 and related viruses into commercial poultry in the USA. However, insufficient application of biosecurity measures likely allowed the virus to subsequently spread between farms in other ways, such as sharing of equipment between infected and non-infected farms, employees moving between infected and non-infected farms, lack of cleaning and disinfection of vehicles moving between farms, and the presence of rodents or small wild birds inside poultry houses. There also was evidence that virus was transmitted through air from infected to non-infected farms (30).

Annual migration patterns of wild birds involved in HPAI H5N8 outbreaks

The phylogenetic analyses indicate that migrating wild birds carried HPAI H5N8 virus to North America and Europe in 2014/2015. Therefore, we reviewed the migratory patterns of wild birds found positive for HPAI H5N8 virus (**Table S3, S4, S6 and Fig. S6**).

Times and routes of migration are consistent with virus spread by migratory birds via their breeding grounds. The best supported scenario is as follows: in winter 2013/2014, wild birds wintering in South Korea or possibly neighbouring unsampled regions became infected; during spring migration, they transported the virus to northern breeding grounds; in summer 2014, virus was maintained at northern breeding grounds by serial infection of wild birds and/or virus persistence in water, and spread to wild birds originating from other wintering areas; in autumn 2014, infected wild birds transported the virus to wintering areas in North America and Europe. This scenario fits with the synchronous detection of HPAI H5N8 or related viruses in these geographical areas in November 2014.

Fig. S1 (separate PDF file)

Time scaled Bayesian phylogenetic tree of the 219 multiple subtype HA sequences, with sequence names and 95% highest posterior density confidence intervals for the internal node heights (time to most recent common ancestors).

Normalised Subtype Changes Per Host Type

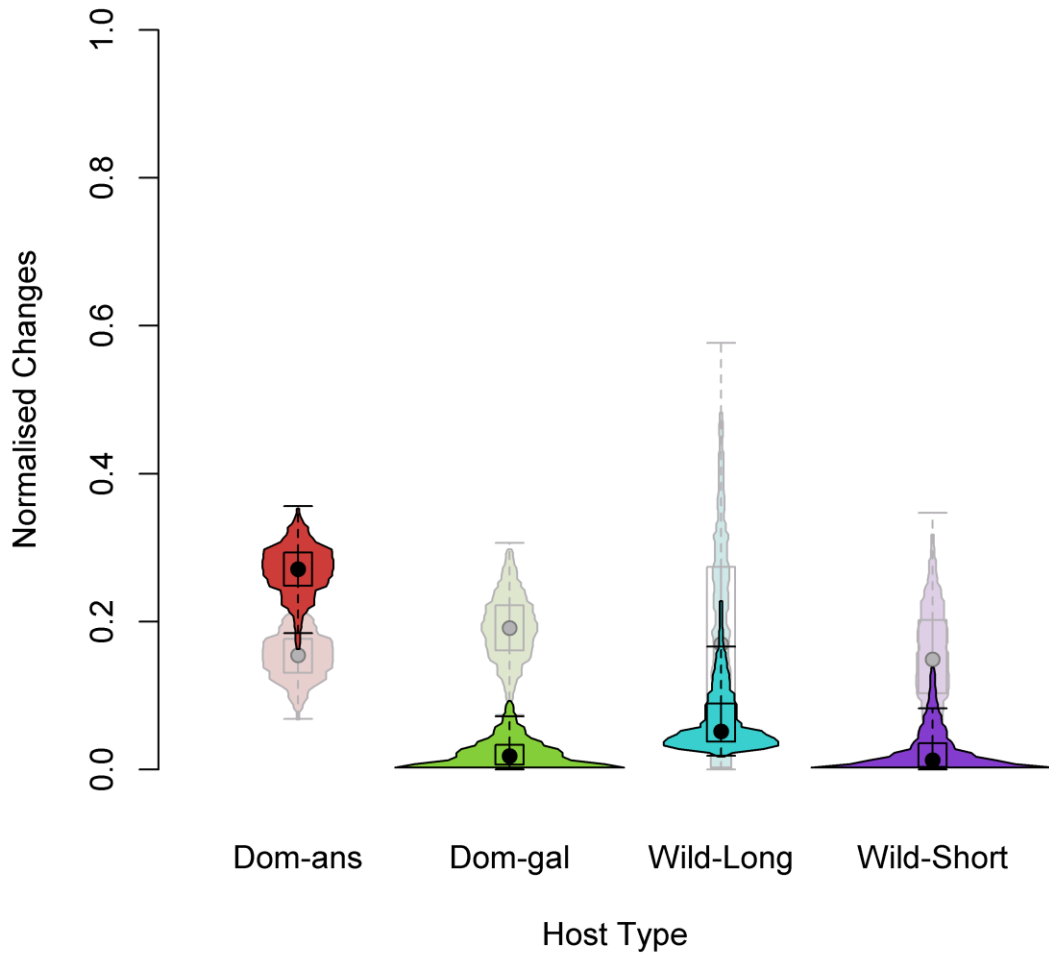


Fig. S2

Number of NA-subtype changes with respect to HA trees per host type, normalised by branch lengths. The dark coloured densities are for the original data (10 reconstructions per tree, over 1000 trees), the pale colours are for trees with permuted host labels (10 per tree, over 1000 trees). The dots represent the median values and the boxes show the interquartile range. Host types are: Dom-ans (red): domestic anseriform birds, Dom-gal (green): domestic galliform birds, Wild-Long (blue): long-distance migratory wild birds, Wild-Short (purple): short-distance migratory wild birds. Numerical values of the descriptive statistics are provided in Table S10.

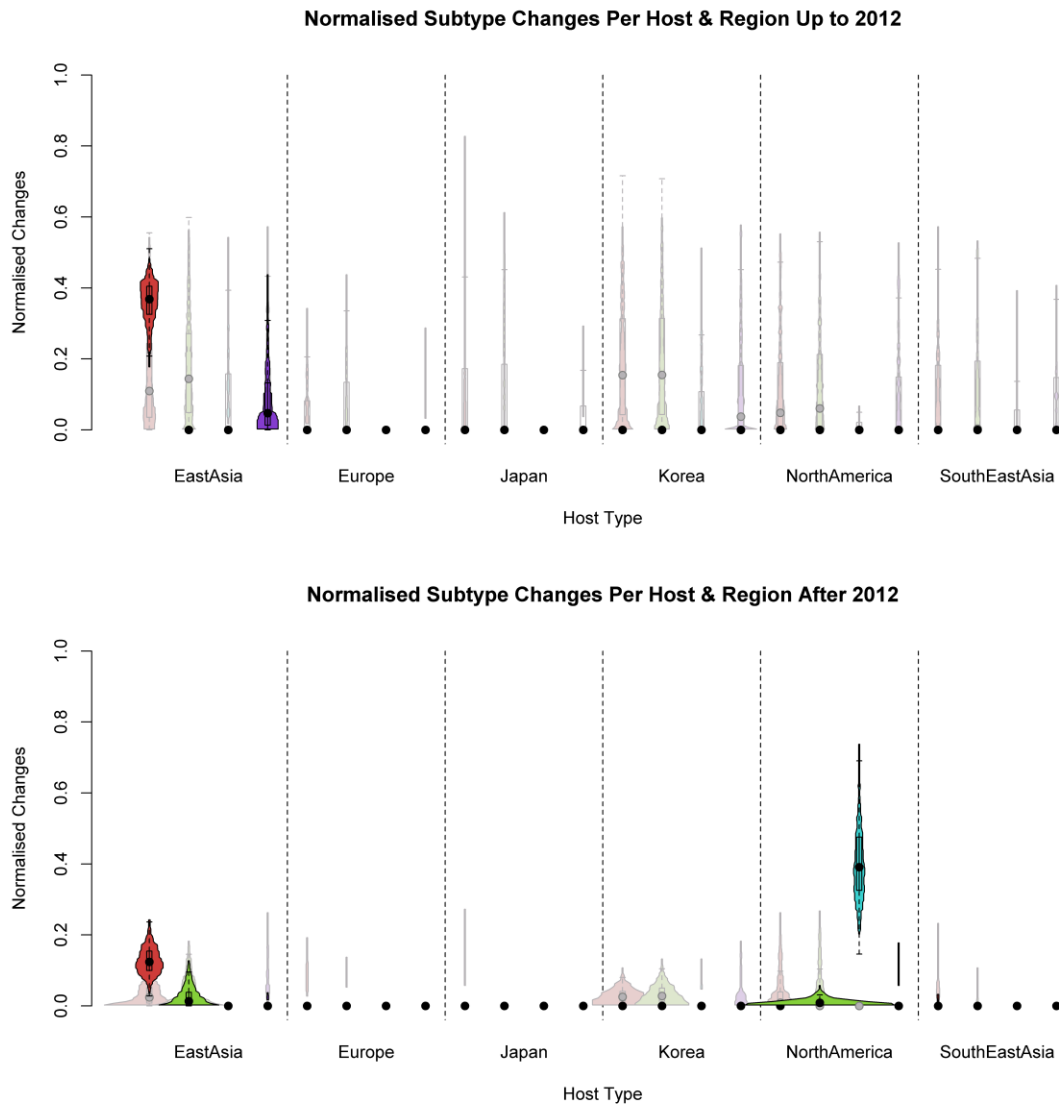


Fig. S3

Number of NA-subtype changes with respect to HA trees per combined host type and region traits, normalised by branch lengths. The dark coloured densities are for the original data (10 reconstructions per tree, over 1000 trees), the pale colours are for trees with permuted host labels (10 per tree, over 1000 trees). The dots represent the median values and the boxes show the interquartile range. Color key as for Fig. S2. Numerical values of the descriptive statistics are provided in Table S10.

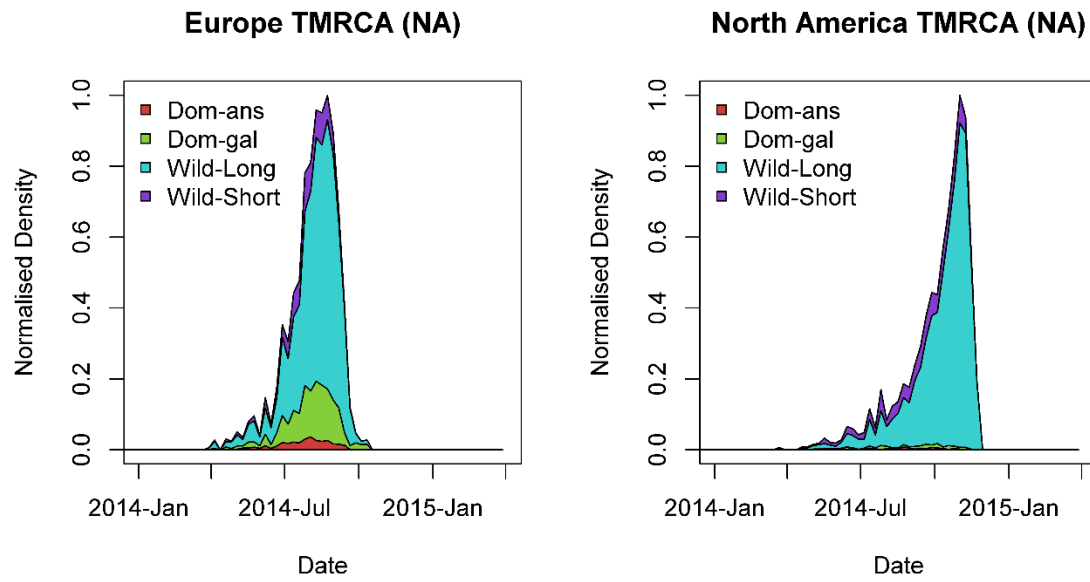


Fig . S4

Posterior distributions of time to most recent common ancestor (TMRCA) of NA sequences from Europe and North America with H5N8 subtype only, including Host type reconstructions, based upon a posterior set of phylogenetic trees generated as in Fig 1. Color key as for Fig. S2.

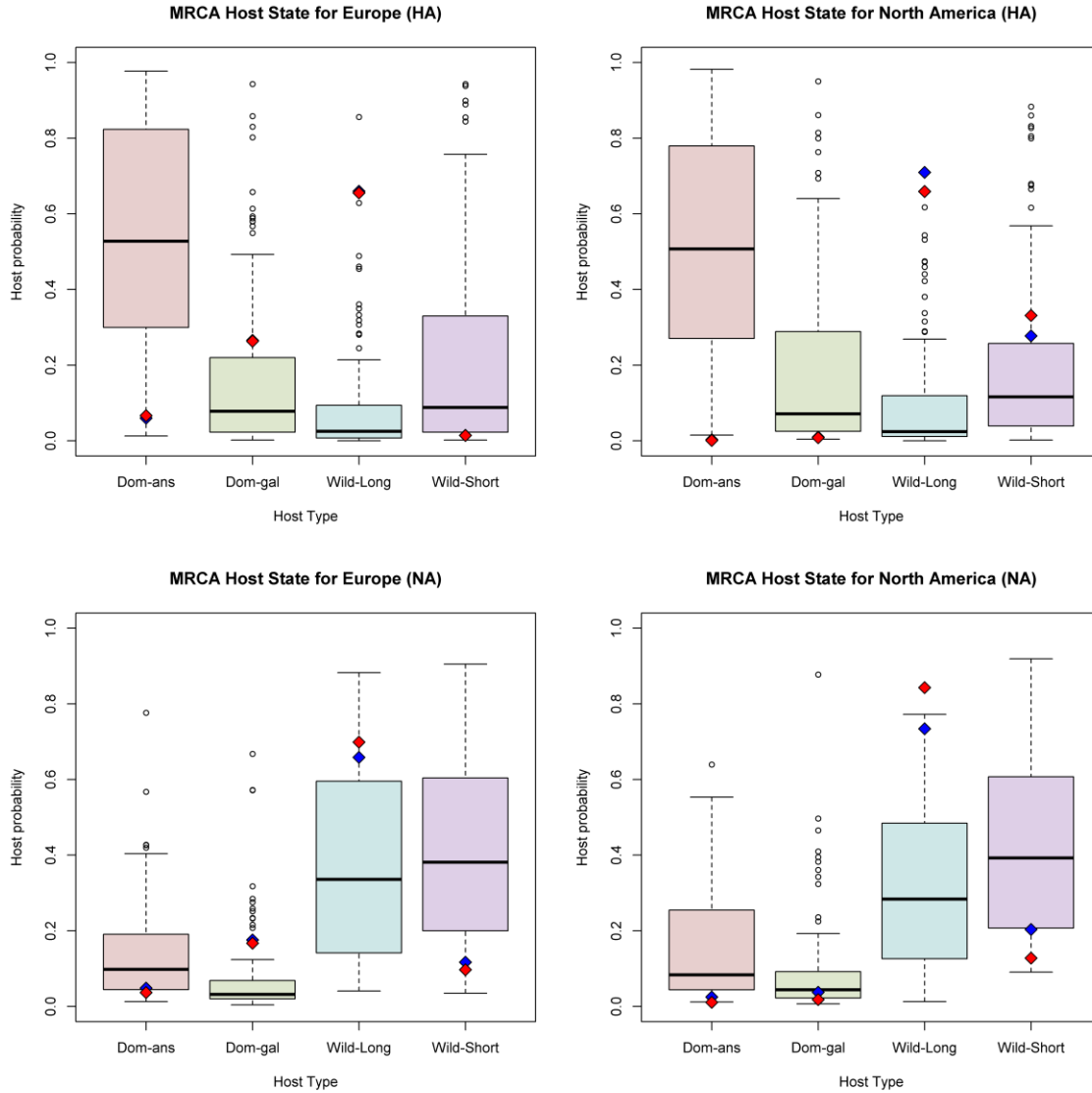


Fig. S5

Host reconstructions at the TMRCA for H5N8 European and H5N8 North American sequences (HA upper, NA lower), averaged over 1000 trees, showing original data (red diamonds, from the constant population size trees; blue diamonds, from the skygrid flexible population trees) and distribution of values from reconstructions using permuted host labels (100 per tree over 1000 skygrid flexible population trees, pale colors). The bars represent median values and the boxes show the interquartile range for the permuted data. Color key as for Fig. S2.

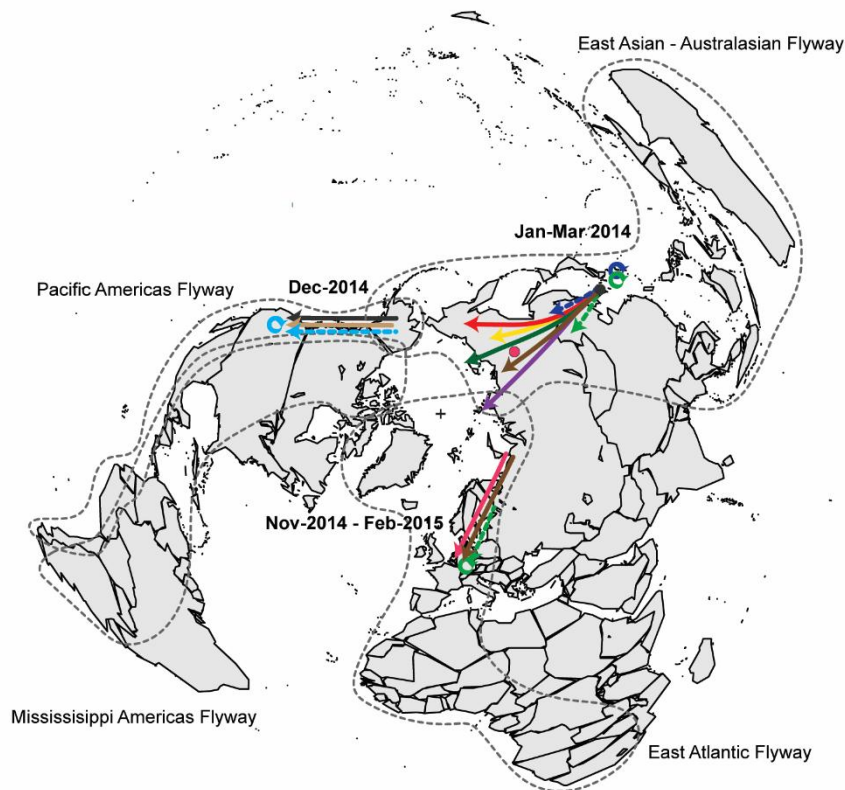


Fig. S6

Overview of possible migration routes of long-distance migratory wild water birds with clade 2.3.4.4. HPAIV H5Nx in 2014 and early 2015. The map projection used is the azimuthal equidistant projection, centered on the North Pole, which is marked with a + sign. Arrows indicate the main known migration routes of each bird species after (South Korea) or before (Europe and North-America) a HPAIV clade 2.3.4.4. H5Nx positive sample was collected in 2014. Dashed colored lines indicate the migration patterns of bird species of which certain populations of these birds are also sedentary (small circles). (Eurasian coot: blue, Baik al teal: red, bean goose: yellow, greater white-fronted goose: dark green, common teal: dark brown, tundra swan: purple, mallard: light green, Eurasian wigeon: dark pink, American green-winged teal: light blue, northern pintail: black, American wigeon: beige). The dark pink dot indicates the territory of Belaya Gora settlement in Sakha Republic (Russia), where clade 2.3.4.4 HPAIV was detected in a sample collected from a Eurasian wigeon. Relevant flyways of waders/shorebirds are indicated with dashed light-grey lines.

Table S1.

Global spread of clade 2.3.4.4 HPAIV A(H5N8) and A(H5N2) viruses.

Date of first report	Geographical location	Environment	Host species	Virus	Reference
May 2010	China	Live bird market	Domestic duck	HPAIV H5N8	(32)
January 2014	South Korea	Poultry farms and wetlands	Multiple domestic and wild birds	HPAIV H5N8	(33)
April 2014	Japan	Poultry farm	Chicken	HPAIV H5N8	(34)
September 2014	Sakha Republic Russia	Wild bird	Eurasian wigeon	HPAIV H5N8	(35)
September 2014	China	Slaughterhouse and wetland of river	Domestic duck and environmental sample	HPAIV H5N8	(18)
November 2014	Germany, Netherlands, U.K.	Poultry farms and wild birds	Chicken, duck, turkey	HPAIV H5N8	[(20, 24, 36–38)
November 2014	Canada	Poultry farm	Chicken, turkey	HPAIV H5N2	(18, 39)
November 2014	Japan	Poultry farms and wild birds	Chicken and wild birds	HPAIV H5N8	(18)
December 2014	Italy	Poultry farm	Turkey	HPAIV H5N8	(38)
December 2014	USA	Backyard flock and wild birds	Various	HPAIV H5N8 and H5N2	(18)
February 2015	Hungary	Poultry farm	Domestic duck	HPAIV H5N8	(18)
February 2015	Sweden	Wild birds; central Stockholm	Mute swan	HPAIV H5N8	(29)

Table S2 (separate excel spread sheet file).

Time to Most Recent Common Ancestors (TMRCA) with probability of inferred ancestral host-type (as percentage of trees with reconstructed host-type), overall transition rate between host-types (host clock rate), branch length between the most recent common ancestor and its ancestor, and number of expected host-type transitions along this branch. Each sub-table is repeated for HA and NA segments, constant population size and skygrid trees and for the 4-host-state model (domestic anseriform, domestic galliform, wild long-range migrants, wild short-range migrants), a 3-host-state model (domestic anseriform, domestic galliform, wild) and an alternative 4-host-state model (domestic anseriform, domestic galliform, wild anseriform, wild other). In addition, the corresponding relative transition rates between host-types for each model are provided.

Table S3.

Overview of wild bird species in which clade 2.3.4.4 HPAIV H5N8 with known sequence information were detected in 2014-early 2015.

Branch	Country	Wild bird species	Date of sample collection	Stage	IUCN status(40)
European branch	South Korea	Eurasian coot (<i>Fulica atra</i>)	Jan-2014	wintering	Least concern
	South Korea	Baikal teal (<i>Anas formosa</i>)	Jan-2014	wintering	Least concern
	South Korea	Mallard (<i>Anas platyrhynchos</i>)	Feb-2014	sedentary/wintering	Least concern
	South Korea	Goose (unknown species)	--	unknown	-
	South Korea	Common teal (<i>Anas crecca</i>)	Mar-2014	wintering	Least concern
	South Korea	Tundra swan (<i>Cygnus columbianus</i>)	Feb-2014	wintering	Least concern
	South Korea	Spot-billed duck (<i>Anas poecilorhyncha</i>)	Feb-2014	wintering	Least concern
	Russia	Eurasian wigeon (<i>Anas penelope</i>)	Sep-2014	breeding/autumn migration	Least concern
	Netherlands	Eurasian wigeon (<i>Anas penelope</i>)	Nov-2014	wintering	Least concern
	Germany	Common teal (<i>Anas crecca</i>)	Nov-2014	autumn migration/wintering	Least concern
	Sweden	Mute swan (<i>Cygnus olor</i>)	Feb-2015	sedentary	Least concern
	Germany	Mallard (<i>Anas platyrhynchos</i>)	Jan-2015	wintering/sedentary	Least concern
North American branch	South Korea	Greater white-fronted goose (<i>Anser albifrons</i>)	Jan-2014	wintering	Least concern
	South Korea	Mallard (<i>Anas platyrhynchos</i>)	Jan-2014	wintering/sedentary	Least concern
	South Korea	Bean goose (<i>Anser fabalis</i>)	Feb-2014	wintering	Least concern
	South Korea	Baikal teal (<i>Anas formosa</i>)	Jan-2014	wintering	Least concern
	South Korea	Common teal (<i>Anas crecca</i>)	Mar-2014	spring migration	Least concern
	South Korea	Spot-billed duck (<i>Anas poecilorhyncha</i>)	Dec-2014	sedentary	Least concern
	South Korea	Gadwall (<i>Anas strepera</i>)	May-2014	sedentary or spring migration	Least concern
	Japan	Hooded crane (<i>Grus monacha</i>) and White-naped crane (<i>Grus vipio</i>)	Nov-2014 - Jan-2015	wintering	Vulnerable, vulnerable
	USA (Washington)	Gyr Falcon (<i>Falco rusticolus</i>)	Dec-2014	wintering	Least concern
	USA (Washington)	American wigeon (<i>Anas americana</i>)	Dec-2014	wintering	Least concern
	USA (Washington)	Northern pintail (<i>Anas acuta</i>)	Dec-2014	wintering/autumn migration	Least concern
	USA (Washington)	Canada goose (<i>Branta canadensis</i>)	Jan-2015	sedentary/wintering	Least concern

Table S4.

Annual migration patterns of long-distance migratory wild bird species in which clade 2.3.4.4 HPAIV H5N8 was detected.

Region	Species	Analyzed data (1)	Wintering area	Period of migration to breeding area	Breeding area	Period of migration to wintering area	Reference
East Asia	Baikal teal (<i>Anas formosa</i>)	Population	Japan, South Korea and China	Mid-March- early April	Eastern Siberia	September to November	(41)
East Asia	Bean goose (<i>Anser fabalis</i>)	<i>Anser fabalis middendorfi</i> and <i>Anser fabalis serrirostris</i>	China, Korea, Japan	Start nesting in early-May to early-June	Taiga zone located in eastern Siberia to Russian Far East	September to October	(42)
East Asia	Common teal (<i>Anas crecca</i>)	Particular (sub)population, known to change between flyways (2)	South Korea	Late-February (peaking in March-April)	Eastern Russia	October to November	(43)
East Asia	Greater white fronted goose (<i>Anser albifrons</i>)	Subspecies	Eastern part of China, North and South Korea, Japan	Start nesting in late May	Arctic (subspecies A.a. frontalis: Arctic tundra from Lena delta east to Russian Far east)	Late August to late autumn	(44)
East Asia	Tundra swan (<i>Cygnus columbianus</i>)	Whole species	East-Asia: east part of China, North and South Korea, Japan	March-June	Arctic	September to October	(45)
Northwestern Europe	Common teal (<i>Anas crecca</i>)	Population	Northwestern Europe	Late February (peaking in March-April)	Fennoscandia, the Baltic states, northwest Russia, northern Poland, Germany and Denmark	October to November	(43, 46)
Northwestern Europe	Eurasian wigeon (<i>Anas penelope</i>)	Population	Northwestern Europe	March-May	Fennoscandia and European Russia	September to November	(46, 47)
North America	American wigeon (<i>Anas americana</i>)	Whole species	British Columbia in Canada, the near complete United States, Central America and the West Indies	February-May	Northern part of the USA, Alaska and Canada	Late July- December	(48, 49)
North America	Northern pintail (<i>Anas acuta</i>)	Population	British Columbia in Canada, the near complete United States, Central America and the West Indies	Late February- May	Northern part of the USA, Alaska and Canada	Mid-August onwards	(50, 51)

(1) When data about a particular population in an area were available, these data were analyzed. This is indicated with ‘population’.

(2) Population has a very large range, different populations have different breeding and wintering locations, species can also change between flyways.

(3) Populations in more temperate regions are sedentary.

Table S5.

Overview of annual migration patterns of wild bird species in which clade 2.3.4.4. HPAIV H5N8 was detected that migrate only over relatively short distances.

Region	Species	Analyzed data (1)	Comments	References
East Asia	Eurasian coot (<i>Fulica atra</i>)	Population	Spring migration route and exact site of location in summer are unknown. They most probably stay during spring and summer in the mainland of east or northern Asia.	(52)
East Asia	Gadwall (<i>Anas strepera</i>)	Species	Partly sedentary. Northern breeding populations fly to their breeding grounds in March-April and to their wintering grounds in July-August.	(53)
East Asia	Mallard (<i>Anas platyrhynchos</i>)	Population	Widely distributed. Northern breeding populations stay much further south in the winter. Populations of temperate regions are more sedentary.	(46, 54)
East Asia	White-naped crane (<i>Antigone vipio</i>)	Species	Breeds in Dauria on the border of Russia, Mongolia and China, the Amur and Ussuri basins on the Sino-Russian border and the Songnen and Sanjiang plains, China. Migrates to its wintering grounds in the Yangtze basin, the Demilitarised Zone in North Korea/South Korea, and to southern Kyushu in Japan.	(55)
East Asia	Hooded crane (<i>Grus monacha</i>)	Species	Breeds in south-central and south-eastern Siberia, Russia. Breeding is suspected in Mongolia and two breeding sites have recently been found in the region of Heilongjiang, China. Majority of the population winters in Japan, with smaller numbers in China and South Korea.	(56)
Northwestern Europe	Mallard (<i>Anas platyrhynchos</i>)	Population	Partially migratory. Birds that breed in the northern part of Europe in general migratory, birds that breed in temperate regions are more sedentary.	(46)
Northwestern Europe	Mute swan (<i>Cygnus olor</i>)	Species	Essentially sedentary, or only locally migratory. Exchange between the NW/Central European population and the Black Sea/Sea of Azov population.	(57)
North America	Mallard (<i>Anas platyrhynchos</i>)	Population	Partially migratory. Birds that breed in the northern part are in general migratory, birds that breed in temperate regions are more sedentary	(46)
North America	Gyrfalcon (<i>Falco rusticolus</i>)	Population	Breeds in the far north part of Canada and Alaska and comes south in the winter to Canada and the northern parts of the USA	(58)
North America	Canada goose (<i>Branta canadensis</i>)	Population	Large range, breeding in Canada, Alaska and northern parts of the USA, wintering in North America and Mexico. Also resident populations present in the USA, which live south of the normal breeding range.	(59)

(1) When data about a particular population in an area were available, these data were analyzed. This is indicated with ‘population’.

Table S6.

Overview of field epidemiological and sequence analysis data of outbreaks of HPAIV clade 2.3.4.4 H5N8 among commercial poultry farms.

Country	Species and farm	City	Animals on farm	Housing	Field epidemiological data	Sequence analysis data	Reference
Germany	fattening turkeys	Heinrichswalde, Vorpommern-Greifswald	31,000	indoor	Farm surrounded by fields and forest and with restricted access. Area with low poultry density, about 1.3 km east of a lake frequently visited by wild birds. All surrounding poultry holdings tested AIV negative.	No data related other German strains	(19–21)
Germany	fattening turkeys	Barssel, district Cloppenburg	19,200	indoor	All poultry in surveillance zone tested AIV negative. Contact tracing: all negative.	No data related other German strains	(19, 22)
Germany	fattening ducks and few hens	Neuborger, district Emsland	about 11,000	indoor	All poultry in surveillance zone tested AIV negative. Contact tracing: all negative.	No data related other German strains	(19)
Hungary	fattening ducks	Füzesgyarmat	22,000	indoor	No poultry have been moved from the affected or contact holdings to EU member states or Third Countries. In the last 21 days the only transport was to a slaughterhouse in Bekes country. No transport to the affected holding from outside Hungary	Only outbreak in this country	(28)
Italy	fattening turkeys	Porto Viro	35,000	indoor	Farm located in close proximity to wetlands and marshlands, wintering site for migratory birds and wild waterfowl.	Only outbreak in this country	(26)
Japan (April 2014)	broiler chicken	Kuma-gun, Kumamoto	113,100	indoor	No data.		(18)
Japan (November 2014-January 2015)	broiler breeders	Nobeoka-shi, Miyazaki	4,031	indoor	No data.		(18)
Japan (November 2014-January 2015)	chicken broilers	Miyazaki-shi, Miyazaki	42,030	indoor	No data.		(18)
Japan (November 2014-January 2015)	chicken broiler breeders	Natato-shi, Yamaguchi	33,017	indoor	No data.		(18)
Japan (November 2014-January 2015)	chicken broilers	Arita-cho, Kumamoto	72,908	indoor	No data.		(18)
Netherlands	laying hens	Hekendorp	150,000	indoor	Farm situated next to a river and in the middle of peat land, with abundant presence of wild waterfowl. All samples collected in frame of the official control system tested AIV negative.	Separate introduction	(23)
Netherlands	laying hens	Ter Aar	43,000	indoor	Farm situated in an area with abundance presence of waterfowl.	Separate introduction	(23)
Netherlands	pullet-rearing	Kamperveen	11,100	indoor	Farm located on a stretch of farmland boarded by a lake and the IJssel river. Large numbers of wild waterfowl were present.	Separate introduction	(23)
Netherlands	meat ducks	Kamperveen	15,000	indoor	Farm located 550 m from the other farm of Kamperveen.	Transmission between farms outbreak 3 > outbreak 4)	(23)
Netherlands	layer hens	Zoeterwoude	29,000	indoor	No data available.	Separate introduction	(23)
United Kingdom	breeding ducks	Nafferton, East Yorkshire	6,000	indoor	Contact tracing: all samples tested AIV negative.	Only outbreak in this country	(24, 25)
USA	turkey	Stanislaus County, California	145,000	indoor	No data available.	No data	(18)
USA	chickens and ducks	Kings County, California	114,000	indoor (chickens)	No data available.	No data	(18)

Table S7.

Export and import of live domestic ducks and chickens from 2011 to 2013, in countries affected by HPAI H5N8 virus outbreaks in 2014 and 2015 using data available from the FAO (12).

Region	Country	Year	Domestic ducks		Chickens	
			Export	Import	Export	Import
North America	Canada	2013	1470 ¹	121	5874	60652
		2012	1397	78	6066	53397
		2011	972	134	6345	57099
	USA	2013	500	822	87015	5350
		2012	593	620	84803	5551
		2011	901	82	80605	6020
Europe	Netherlands	2013	1177	3680	330181	274401
		2012	998	1892	297854	290786
		2011	1	386	335475	296137
	Hungary	2013	1781	232	70031	18116
		2012	1070	272	41174	18648
		2011	70	30	32194	12086
	UK	2013	163	51	18652	11549
		2012	316	235	27739	9235
		2011	0	0	23922	13375
	Italy	2013	6	147	12435	9241
		2012	13	136	13728	8283
		2011	0	51	15169	6054
	Germany	2013	992	816	270858	166356
		2012	979	1496	290584	184023
		2011	476	218	254984	177549
Asia	China	2013	0	68	7168	9867
		2012	0	76	7360	9786
		2011	0	77	7235	10173
	Taiwan	2013	0	9	0	298
		2012	0	0	0	236
		2011	0	3	0	352
	Japan	2013	no data	5	0	557
		2012	no data	6	0	784
		2011	no data	1000	0	772
	South Korea	2013	0	139	3	608
		2012	0	319	0	624
		2011	0	1063	2	1036

¹ x 1000 heads

Table S8.

Host-type, subtype, and region distribution of sequence data sets.

Trait	Data Set	H5NX HA 219	H5N8 HA 130	H5N8 NA 84
	Number of sequences	219	130	84
Host-type	Domestic anseriform	87	44	19
	Domestic galliform	71	30	14
	Wild long-range migrant	22	25	24
	Wild sedentary or short-range migrant	39	31	27
Subtype	H5N1	25	0	0
	H5N2	33	0	0
	H5N3	1	0	0
	H5N5	9	0	0
	H5N6	30	0	0
	H5N8	121	130	84
Region	East Asia	58	8	8
	Europe	17	16	16
	Japan	12	11	11
	Korea	79	80	34
	North America	33	15	15
	South East Asia	20	0	0

Table S9 (separate excel spread sheet file).

Tables of HA and NA sequences used for phylogenetic analysis. These sequences consist of 219 HA multiple subtype sequences from 2005-2015, 130 HA H5N8 sequences from 2010 to 2015 and 84 NA H5N8 sequences from 2010 to 2015. The tables contain the subtype, host-type, phylogeographic region trait, assumed latitude and longitude, and also the accession numbers and submitter details from GISAID. We gratefully acknowledge all authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu™ Database on which this research is based, and those who have also submitted their sequences to Genbank.

Table S10 (separate excel spread sheet file).

Numerical values of the descriptive statistics for the number of NA-subtype changes with respect to HA trees per host type, normalised by branch lengths (Figure S2) and for the number of NA-subtype changes with respect to HA trees per combined host type and region traits, normalised by branch lengths (Figure S3).

Movie S1 (separate mp4 file)

Animation of Figure 2. Continuous trait phylogeographic reconstruction using 130 H5N8 HA sequences coloured by host-type reconstruction.

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