

Transmission Dynamics of Highly Pathogenic Avian Influenza Virus A(H5Nx) Clade 2.3.4.4, North America, 2014–2015

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Eurasia highly pathogenic avian influenza virus (HPAIV) H5N1 clade 2.3.4.4 emerged in North America at the end of 2014 and caused outbreaks affecting >50 million poultry in the United States before eradication in June 2015. We investigated the underlying ecologic and epidemiologic processes associated with this viral spread by performing a comparative genomic study using 268 full-length genome sequences and data from outbreak investigations. Reassortant HPAIV H5N2 circulated in wild birds along the Pacific flyway before several spillover events transmitting the virus to poultry farms. Our analysis suggests that ≥ 3 separate introductions of HPAIV H5N2 into Midwest states occurred during March–June 2015; transmission to Midwest poultry farms from Pacific wild birds occurred ≈ 1.7 –2.4 months before detection. Once established in poultry, the virus rapidly spread between turkey and chicken farms in neighboring states. Enhanced biosecurity is required to prevent the introduction and dissemination of HPAIV across the poultry industry.

Highly pathogenic avian influenza virus (HPAIV) HA(H5N1) emerged in 1996 in Guangdong, China (A/goose/Guangdong/1/1996 [Gs/GD]), and has since caused outbreaks in poultry, infections in wild birds, and often fatal clinical cases in humans in >80 countries (1). In late autumn 2014, Gs/GD H5N8 clade 2.3.4.4 was reported in East Asia, Europe, and the west coast of North America (2,3). The timing and direction of this virus's dissemination, which coincided with waterfowl migration patterns, together with phylogenetic and geospatial data (3,4) support the hypothesis that Gs/GD H5N8 clade 2.3.4.4 was introduced into North America through the Beringian Crucible via intercontinental movement of migratory waterfowl (5,6).

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DOI: <https://doi.org/10.3201/eid2410.171891>

In November and December 2014, novel reassortant H5N1, H5N2, and H5N8 viruses were detected in wild waterfowl along the Pacific flyway (2,7). Reassortant H5N2 virus was identified as the causative agent of influenza outbreaks in poultry farms in British Columbia, Canada, and among wild waterfowl in the northwestern United States (Video, <https://wwwnc.cdc.gov/EID/article/24/10/17-1891-V1.htm>) (8). This virus subsequently was the predominant strain during HPAIV outbreaks among poultry in the United States in 2015, particularly during March–June (9,10), and was detected in wild birds migrating along the Pacific, Central, and Mississippi flyways (11,12). The last reported virus detection related to the outbreak in the United States was in June 2015 from a Canada goose in Michigan. Despite increased surveillance efforts in the United States, HPAIV was rarely detected in wild bird populations during the latter half of 2015 and 2016 (13). In 2015, the virus was detected just 2 times by real-time reverse transcription PCR in mallards, once in July (from bird banding efforts in Utah) and once in November (from a hunter harvest in Oregon) (14). In 2016, H5N2 virus clade 2.3.4.4 was detected in 2 mallards sampled in Alaska and Montana, providing evidence for low-level maintenance of this virus in wild birds in northwestern North America (15,16). Despite disagreement about the role of flyways in limiting viral spread among wild birds (17,18), the rapid spatial diffusion and transmission of HPAIV in wild and domestic birds highlight the need to further investigate the processes involved in viral emergence and spread.

We sequenced and analyzed the full-length genome sequences of 249 H5N2 and 19 H5N8 HPAIVs collected during the 2014–2015 outbreaks in the United States. Viruses came from 32 wild birds, 7 raptors, 14 backyard poultry farms, and 196 commercial poultry sites. We used a molecular epidemiologic approach involving genome sequences and outbreak information to determine the evolution and spread patterns of these viruses.

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Materials and Methods

Genome Sequencing

The National Animal Health Laboratory Network conducts PCR testing for avian influenza in the United States, and per title 9, Code of Federal Regulations, nonnegative samples are forwarded to the National Veterinary Services Laboratories (Ames, Iowa, USA) for confirmation testing and genome sequencing. In December 2014, interagency wild bird surveillance started including testing for the HPAIV Gs/GD lineage in wild birds in the Pacific flyway, and by summer 2015, surveillance was further expanded to include testing for this virus in all flyways (19). In our study, we included all available wild bird ($n = 32$) and raptor ($n = 7$) viruses from these surveillance efforts and, for poultry, just 1 virus from each affected site with available samples ($n = 210$; 14/21 affected backyard sites, 196/211 affected commercial flocks) (20).

We extracted viral RNA from 249 H5N2-positive and 19 H5N8-positive oral or cloacal swab samples using MagMAX Viral RNA Isolation Kit (Ambion, Foster City, CA, USA). We synthesized complementary DNA and amplified all 8 segments by reverse transcription PCR using SuperScript III (Invitrogen, Carlsbad, CA, USA) (21). We conducted genome sequencing using the Ion Torrent PGM (Life Technologies, Waltham, MA, USA) or MiSeq (Illumina, San Diego, CA, USA) platforms as previously described (7,16). We assembled genome sequences using SeqMan NGen version 4 (<http://www.dnastar.com/t-next-gen-seqman-ngen.aspx>). We deposited nucleotide sequences in GenBank (online Technical Appendix Table 5, <https://wwwnc.cdc.gov/EID/article/24/10/17-1891-Techapp1.pdf>).

Maximum-Likelihood Phylogenetic Analysis of US HPAIV H5N2

Of the 265 H5N2 sequences analyzed, we retrieved the 16 viruses from Canada from the GISAID EpiFlu database (<http://platform.gisaid.org>) and sequenced the 249 viruses from the United States. In all, 32 viruses were from wild waterfowl in 10 states (Alaska, Idaho, Kansas, Kentucky, Michigan, Missouri, Montana, Oregon, Washington, Wyoming); 7 viruses were from captive and wild raptors in 5 states (Idaho, Minnesota, Missouri, Washington, Wisconsin); and 210 viruses were from backyard and commercial poultry farms in 13 states (Arkansas, Idaho, Iowa, Kansas, Minnesota, Missouri, Montana, Nebraska, North Dakota, Oregon, South Dakota, Washington, Wisconsin). Data from Minnesota (1 backyard farm, 99 commercial poultry farms, 1 wild bird) and Iowa (69 commercial poultry farms), where most commercial poultry were affected, predominated in this sample.

To demonstrate the phylogenetic organization of the HPAIV outbreak, we built a maximum-likelihood

phylogenetic tree of the concatenated genome (all 8 genome segments) with RAxML version 8.0.0 (22) using default parameters and a general time-reversible model with gamma-distributed rate variation among sites. We divided the 265 H5N2 isolates into subgroups of related clusters by their bootstrap value ($>70\%$) on maximum-likelihood phylogenetic analysis (23). To assess relatedness support, we performed rapid bootstrapping, with bootstrap convergence criterion yielding 1,000 bootstrap iterations. We illustrated the time series of outbreaks by subgroup and state using Tableau (<https://www.tableau.com/>).

Median-Joining Network Analysis

We concatenated all 8 genome segments of each virus isolate to generate a single alignment. We used these alignments to construct a phylogenetic network using the median-joining method implemented in program NETWORK version 5.0 (http://www.fluxus-engineering.com/sharenet_rn.htm) with epsilon set to 1 (24).

Ancestral State Reconstruction of Geographic Location and Host Type

To investigate virus transmission between host types over large spatial scales, we reconstructed the virus transmission history between states geographically using an ancestral state reconstruction approach with a Bayesian stochastic search variable selection to determine the most probable spatial and ecologic transmission history. For all phylogeographic analyses, we used an uncorrelated log-normal distribution relaxed-clock method with a Hasegawa, Kishino, and Yano nucleotide substitution model and Bayesian sky-ride coalescent prior.

Using a globally derived dataset ($n = 127$ taxa) of HPAIV hemagglutinin (HA) sequences from Asia, Europe, and North America, we tested whether the HPAIV H5 outbreak in North America resulted from a single virus introduction or multiple virus introductions into the bird population of North America. We then developed a more refined model to estimate viral diffusion and transmission between wild and domestic populations and assess the most likely route of spread. We incorporated HPAIV HA sequences from the US and Canada 2014–2015 outbreaks (11) into our discrete phylogenetic model. In this model, we defined geographic region and host type as discrete nominal categories. We categorized geographic region of virus collection by migratory flyway (Pacific, Central, Mississippi) and host type as chicken, turkey, wild bird, or backyard poultry. We estimated ancestral state transition rate and model parameters from a set of 5,000 empirical trees simulated from HPAIV H5 HA gene data collected throughout the outbreaks. For the discrete ancestral state model, we used a nonreversible continuous-time Markov chain model to estimate geographic and host transitions among wild and

domestic birds (25). We used Bayesian stochastic search variable selection to identify important transitions using a binary indicator (*I*) (18,26,27), enabling the calculation of Bayes factor with SPREAD version 1.0.6 (28). A transition was considered important when *I* was >0.5 and Bayes factor was >4.0 (18,26). We used the last 500 trees from each posterior distribution to construct heat maps representing the average number of transitions per month (online Technical Appendix).

Results

The highly pathogenic phenotype of viruses sequenced in this study were confirmed by presence of the polybasic amino acid motif at the HA cleavage site and through experimental infection of chickens by intravenous inoculation (online Technical Appendix). Analysis of globally circulating Gs/GD-lineage H5Nx clade 2.3.4.4 viruses showed a widespread distribution and confirmed that the lineage causing the outbreak in North America probably resulted from a single introduction (online Technical Appendix Table 2, Figure 1). During December 2014–March 2015, H5N2 was detected in poultry farms of British Columbia and in wild waterfowl and backyard poultry of states along the Pacific flyway (Figure 1). Subsequently, during March–June 2015, H5N2 predominated in poultry outbreaks in the Midwest.

We generated a maximum-likelihood phylogenetic tree and median-joining phylogenetic network of HPAIV H5N2 using 265 concatenated full-genome sequences (249 from the United States, 16 from Canada) to investigate the relatedness of isolates (Figure 2; online Technical Appendix

Figure 2). The H5N2 viruses formed 2 major genetic groups, group 1 and group 2 (includes subgroups 2a, 2b, 2c, 2d, 2e, and 2 nonclustered [2nc]), with maximum-likelihood phylogeny bootstrap proportions >75%. Group 1 contains 73 viruses from 11 US states and 2 Canada provinces across the Pacific (Alaska, Idaho, Montana, Oregon, and Washington, USA; British Columbia), Central (Kansas, South Dakota, and Wyoming, USA), and Mississippi (Arkansas, Minnesota, and Missouri, USA; Ontario, Canada) flyways (online Technical Appendix Figures 3, 4). Group 2 predominantly contained viruses isolated from poultry from states along the Mississippi flyway: 2a (n = 39; Iowa, Minnesota, Missouri, Wisconsin); 2b (n = 35; Minnesota); 2c (n = 11; Iowa, Minnesota); 2d (n = 4; Wisconsin); and 2e (n = 81; Iowa, Minnesota; Nebraska, South Dakota in Central flyway). Twenty-two 2nc viruses were detected in 6 states along the Central (North Dakota, South Dakota) and Mississippi (Kentucky, Michigan, Minnesota, Wisconsin) flyways.

Both group 1 and 2 viruses were found in wild birds and gallinaceous poultry (Figure 2; online Technical Appendix Figure 3). Wild birds infected with group 2 viruses were only infected with subgroup 2nc viruses. Group 1 viruses were recovered from all host types represented (28 wild birds, 5 raptors, 11 backyard poultry, 14 commercial poultry [1 chicken, 13 turkeys]). Subgroups 2c and 2d were found only in turkeys and had limited geographic spread. The remaining subgroups affected several hosts. Subgroup 2nc viruses were isolated from 6 wild birds, 13 commercial turkey sites, and 3 commercial chicken sites. Subgroup 2a was found in 2 backyard sites, 36 commercial turkey sites,

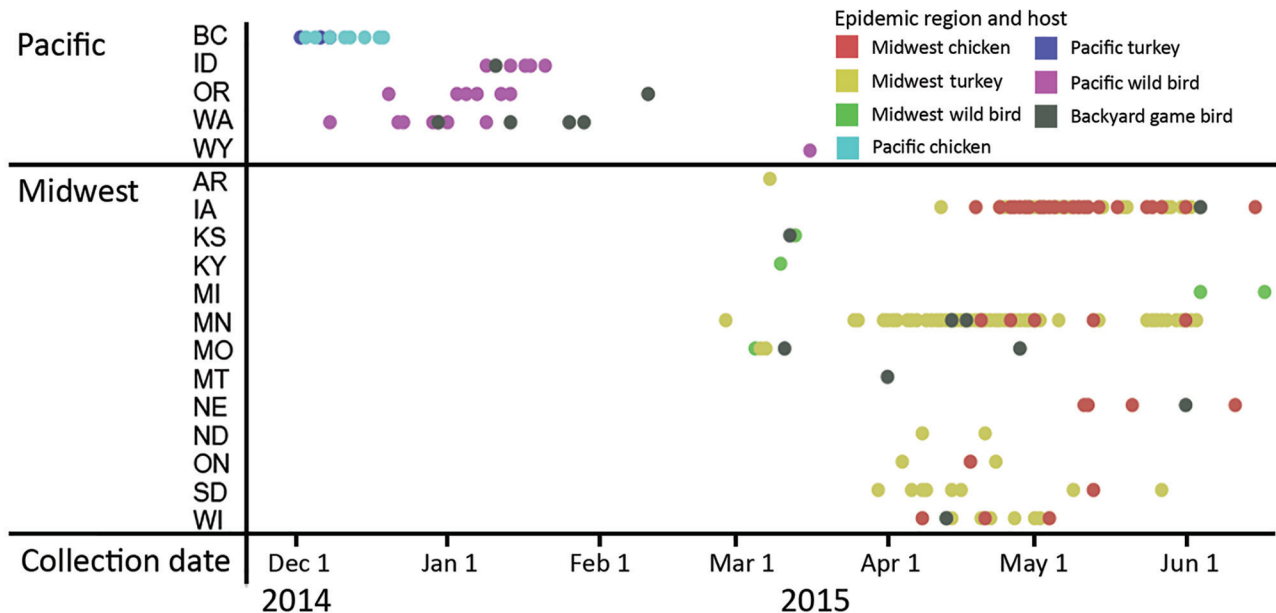


Figure 1. Time series of reassortant highly pathogenic avian influenza virus A(H5N2) distribution, by US state and Canada province, December 2014–June 2015. Virus region and host are indicated. BC, British Columbia, Canada; ON, Ontario, Canada.

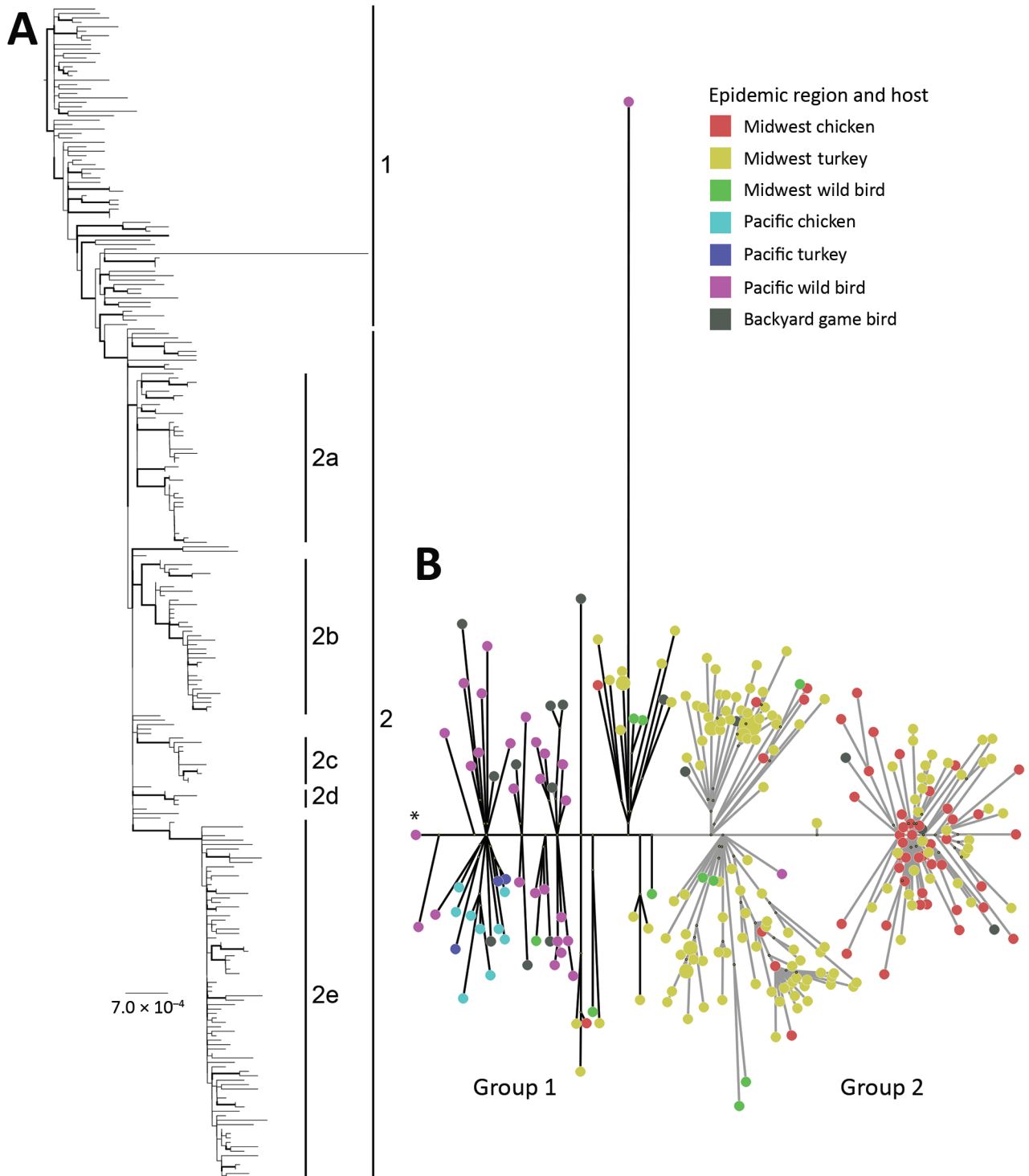


Figure 2. Genetic characterization of reassortant highly pathogenic avian influenza virus A(H5N2) clade 2.3.4.4, North America, 2014–2015. A) Maximum-likelihood phylogeny of concatenated complete genome sequences. Labels indicate genetic subgroups. Scale bar indicates nucleotide substitutions per site. The full version of the phylogenetic tree is available in online Technical Appendix Figure 2 (<https://wwwnc.cdc.gov/EID/article/24/10/17-1891-Techapp1.pdf>). B) Median-joining phylogenetic network constructed by using concatenated complete genome sequences. This network includes the most parsimonious trees linking the sequences. Each unique sequence is represented by a circle sized relative to its frequency in the dataset. Virus region and host are indicated. *US index H5N2 virus (A/Northern_pintail/Washington/40964/2014).

and 1 commercial chicken site. Subgroup 2b predominantly affected commercial turkey sites (n = 32) and 3 commercial chicken flocks; subgroup 2e was recovered from 2 backyard, 36 commercial turkey, and 42 commercial chicken sites and 1 game bird flock. Genetic clustering of

subgroups 2a, 2b, and 2e in maximum-likelihood analysis and the close genetic relatedness of Midwest poultry isolates in network analysis provide strong evidence for extensive interfarm transmission (Figure 2; online Technical Appendix Figure 2).

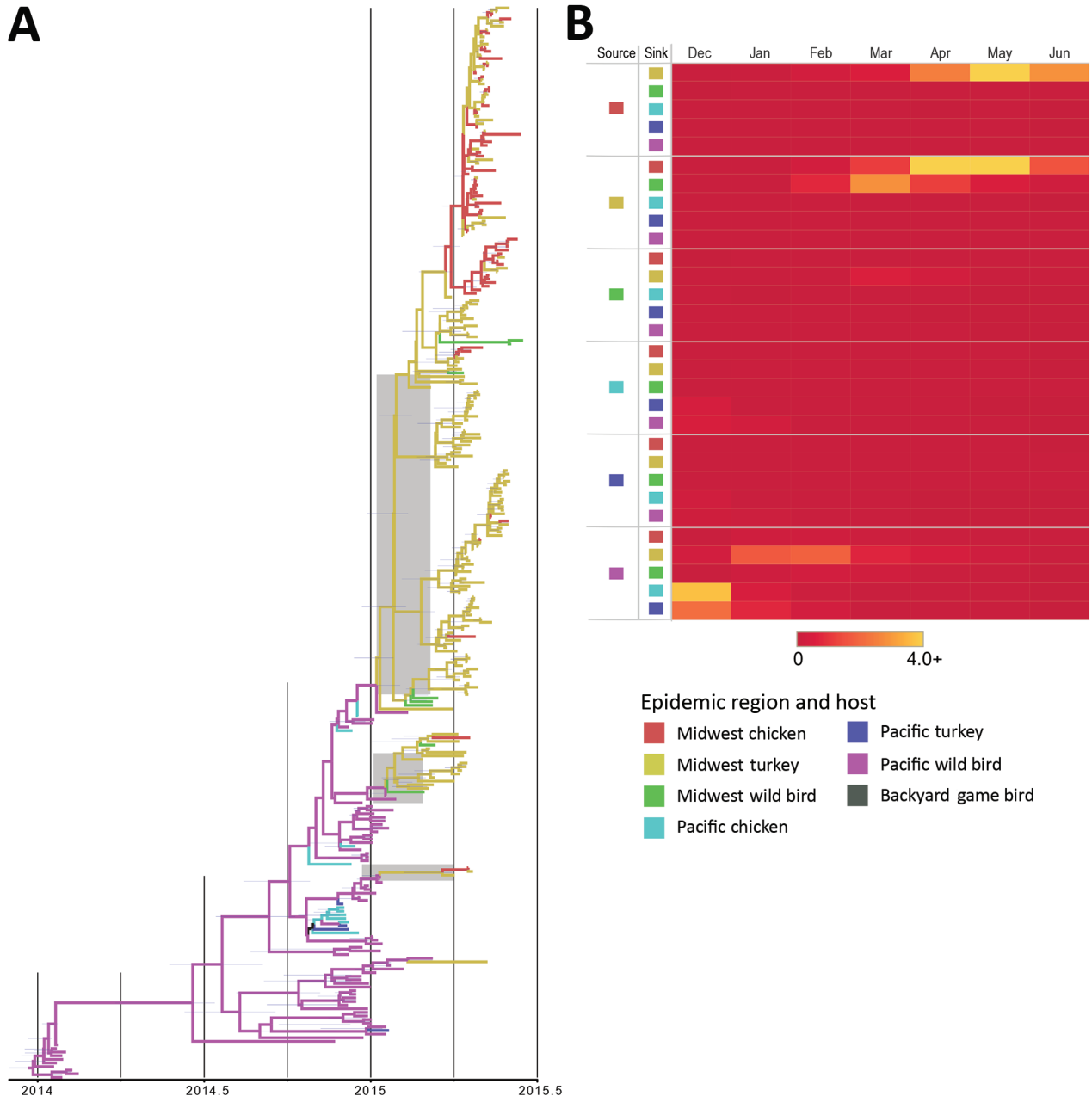


Figure 3. Phylogeographic reconstruction of source–sink dynamics of highly pathogenic avian influenza virus A(H5N2) outbreak, United States, 2014–2015. A) Phylogenetic tree of hemagglutinin gene of H5N2 isolates. The geographic region and host type were defined in the model as discrete nominal character states, and the number of state transitions at tree nodes was counted. The character states included in the phylogenetic model (Midwest chicken, Midwest turkey, Midwest wild bird, Pacific chicken, Pacific turkey, and Pacific wild bird) are indicated. Black nodes and branches represent an ancestral reconstruction that is highly uncertain, sharing equal probability between Pacific wild bird and Pacific turkey. The shaded boxes represent the time between the introduction of the virus into Midwest poultry populations and the first detection of the virus within the population. B) Heat map showing source–sink dynamics and average number of transitions per location per year (0–4.0).

Our phylogenetic analysis suggests that the outbreak in the Midwest was initiated through multiple independent HPAIV H5N2 introductions with group 1 viruses of wild bird origin (Figures 2, 3). To formally test the role of various host populations and elucidate the most likely route for viral spread in the United States, we integrated host type (wild birds, domestic chickens and turkeys, backyard poultry) into our geographic model. Our source–sink and phylogenetic network analyses suggest that the virus detected in the Midwest shares a common ancestor with the viruses detected in the Pacific flyway 1.7–2.4 months before the first detection in Midwest poultry facilities (Figure 3; online Technical Appendix Figure 5). Undetected infected hosts also could have migrated from the Pacific Coast through the Midwest, depositing infectious material on the US landscape, leading to the outbreaks in Midwest poultry populations. The virus subsequently rapidly transmitted between commercial turkey and chicken farms. The rapid rate of transmission suggests that underlying epidemiologic factors were driving the spread within and between farm systems.

Bayesian simulation results showed that turkey farms and chicken farms were equally supported as the source of infection of domestic flocks after establishment of the virus in Midwest US farms (Table). Although the discrete state transition rate from chicken farms to turkey farms was higher, this difference is likely an artifact of the substantially larger flocks found in chicken farms, which predominantly use layer houses. Our analysis showed high rates of transmission between turkey and chicken farms in the Midwest, probably confounding control efforts and contributing to the overall extent of the outbreak (Figure 3, panel B). No support exists for viral spread from Midwest wild birds to Midwest commercial farms, suggesting that this epidemiologic pathway likely did not play a role in the spread of the outbreak after the initial introduction into the Midwest (Table), which has further been supported by epidemiologic investigations (29). In addition, a transition from Midwest turkey to wild birds is supported by a high Bayes factor (4798.91) and posterior probability (0.999), suggesting HPAIV spillback from domestic poultry to wild birds at a low transition rate (Table).

We identified 14 common substitutions across the entire genome of HPAIV H5N2 when comparing with US index virus A/northern pintail/WA/40964/2014 (H5N2). These substitutions include 10 nonsynonymous substitutions in group 1 and 2 viruses (L386V and V649I in polymerase basic 2; L8F, N130T, and S157P in HA; R253K, E368K, and V412A in neuraminidase; Q78R in matrix 2; I176T in nonstructural protein 1) and 4 nonsynonymous substitutions in group 2 viruses (R215K in polymerase basic 1, A337V in polymerase acidic, N60H in nuclear export protein, K217T in nonstructural protein 1) (online Technical Appendix Table 3).

Discussion

Phylogenetic reconstruction of source–sink dynamics supports multiple independent introductions of HPAIV H5N8 into the United States during late 2014 from wild birds. Despite the frequent detection of HPAIV H5 in wild birds of the Pacific flyway up to February 2015 (30), only 2 commercial poultry and 1 backyard farm flocks became infected with HPAIV H5N8 and 7 backyard poultry flocks with HPAIV H5N2 in states along the Pacific flyway. No HPAIV H5N2 infections were found at commercial poultry sites in states along the Pacific flyway (31). In contrast with infrequent events in states along the Pacific flyway, 213 H5N2 events in commercial and backyard poultry were reported during March–June 2015 in the Midwest. Our analysis demonstrates extensive interfarm transmission of virus subgroups 2a, 2b, and 2c but not of group 1 viruses, which could also infect wild birds, suggesting a change in the epidemiologic processes driving viral spread. Although a national level–structured surveillance effort was not initiated until July 2015 (19), H5N2 detections were far less frequent in the Midwest than in the Pacific flyway, suggesting few H5N2-infected wild waterfowl were in the Central and Mississippi flyways during the outbreak period (32,33). Nonetheless, detection of H5N2 viruses from wild birds in the Midwest in early 2015 and our phylogenetic analysis suggest that the HPAIV H5N2 moved from the Pacific flyway to the Mississippi and Central flyways as early as January or February 2015, with subsequent interfarm transmission in and between states of the Midwest.

When performing the source–sink phylogenetic analysis, we made assumptions regarding sampling and population structure, which should be considered when interpreting results. For instance, sampling in this analysis is most likely biased toward commercial poultry because of the nature of HPAIV reporting. Oversampling of a specific population might lead to overestimation of that group as a sink to viral migration (34); however, a secondary simulation in which viral host characteristics were continuously randomized to observe the influence of sampling heterogeneity (35) revealed that sampling does not appear to bias the ancestral reconstruction in this data. Also, in the model, we included the complete random population mixing assumption, even though a structured coalescent approach might have helped limit bias introduced from ecologic barriers that structure wild and domestic bird populations; many of the HPAIV sequences from poultry hosts used in our study represented a single farm. No data were available regarding the size of farms, number of animals infected on those farms, or number of farms affected, all of which might further bias spatial diffusion parameter estimates in a structured coalescent analysis.

Table. Well-supported discrete trait viral transitions among chicken, turkey, and wild bird populations during highly pathogenic avian influenza virus H5 outbreak, Midwest and Pacific regions, USA, 2014–2015*

Transition from	Transition to	Bayes factor	Posterior probability	Mean transition rate
Midwest chicken	Midwest turkey	38,421.1688	1.0	5.0917
Midwest turkey	Midwest chicken	38,421.1688	1.0	1.8376
Midwest turkey	Midwest wild bird	4,798.9103	0.9991	0.8344
Pacific wild bird	Pacific chicken	464.3334	0.9909	0.8478
Pacific wild bird	Midwest turkey	227.2091	0.9816	0.8557
Pacific wild bird	Pacific turkey	201.2141	0.9792	0.5687

*Only the migrations supported by a Bayes factor >4 and posterior probability >0.5 are indicated.

The hypothesis that HPAIV H5N2 was introduced into the Midwest through wild birds rather than poultry production channels is supported by epidemiologic evidence; during February 27–April 20, 2015, H5N2 virus was detected 17 times across 5 states and 16 counties in different hosts (wild bird, raptor, backyard flocks, turkey flocks), and epidemiologic links could not be found between Pacific Coast and Midwest farms for any virus subgroup. The widespread detection of the HPAIV H5 lineage in healthy wild birds (30), subclinical infection with high virus shedding, and transmission among mallard ducks experimentally infected with HPAIV H5N2 (36) support the hypothesis of HPAIV H5 dissemination to multiple states through wild waterfowl over a short period during wild aquatic bird migrations. For the outbreak in the Midwest (February–June 2015), events (infections with viruses of groups 1 and 2nc) detected early during the outbreak (February–April 2015) probably represent independent introductions, followed by limited secondary spread, and events (infections with viruses of subgroups 2a–e) detected later during the outbreak (April–June 2015) were largely caused by extensive interfarm transmission. Previous estimates of basic reproductive number and viral migration among host types in domestic and wild birds support our findings that HPAIV H5N2 rapidly spread between poultry facilities in the Midwest after the initial introduction of a virus of wild bird origin (37). Grear et al. showed that the H5N2 and H5N8 HPAIV outbreak did not encounter sufficient transmission barriers to prevent persistence when introduced to wild or domestic birds (37). Consistent with our data, Grear et al. concluded that once the HPAIV H5N2 entered the poultry production system in the Midwest, transmission was driven through poultry production–related mechanisms (37). That conclusion was based on evidence of a close phylogenetic distance among sequences from poultry facilities, relatively infrequent cross-species transmission, and a high estimated proportion of virus diversity in addition to the lack of detection of this lineage in reservoir hosts when using other surveillance data. Our Bayesian simulation also supports the high probability of 2-way transmission between poultry and wild bird populations.

The epidemiology of the HPAIV H5 detections and pathobiologic features suggest that the early H5N8 and H5N2 group 1 viruses detected in the United States were

highly adapted to waterfowl and poorly adapted to chickens and turkeys (36,38–40). Index wild bird viruses [A/gyrfalcon/Washington/40188–6/2014 (H5N8), A/northern pintail/Washington/40964/2014 (H5N2)] required a high mean bird infectious dose of 50% (BD₅₀; 10^{5.7} EID₅₀) to infect chickens; directly inoculated and in-contact exposed survivors did not seroconvert (38). Increased virus adaptation to chickens and turkeys, specifically poultry, was observed among the H5N2 group 2 viruses and not the H5N2 group 1 viruses (39,40). In chickens, the BD₅₀ of H5N2 group 2 viruses for poultry (10^{3.2–3.6} EID₅₀) was lower than that of group 1 viruses for poultry (10^{5.1} EID₅₀) and index wild birds (10^{5.7} EID₅₀).

Examination of mutational frequencies and patterns showed common mutations among H5N2 subgroup 2a–e viruses fixed in the population during circulation in poultry. In particular, substitutions in the nucleoprotein amino acid 105 (M105V, M105I) were identified among H5N2 group 2 viruses from the United States and a virus isolate from a chicken in British Columbia (11) and has been suggested as a determinant for virus adaptation from ducks to chickens (41). Consistent with our previous study (39), we identified common substitutions (R215K polymerase basic 1, A337V polymerase acidic, N60H nuclear export protein, K217T nonstructural protein 1) in H5N2 group 2 viruses that increased infectivity and pathogenicity in chickens in comparison with group 1 viruses. In addition, we found substitution S157P at an antigenic site of the HA protein, which might affect the immunogenicity profile of the virus (42).

In summary, we analyzed the transmission dynamics of Asia-origin HPAIV H5 in the United States. The H5N8 virus came from East Asia, entered North America during the fall 2014 migration season (7), and spread rapidly along the wild bird flyways in the United States starting in December 2014. In the Pacific flyway, Eurasia H5N8 virus circulated in wild birds and disseminated to backyard and commercial poultry farms largely by point-source introductions; a single detection occurred in a backyard flock in Indiana outside the Pacific states. Furthermore, influenza A genome segments of the Eurasia H5N8 virus mixed with segments of the North America low pathogenicity avian influenza viruses, creating new reassortant HPAIVs H5N1, H5N2, and H5N8. For the reassortant H5N2 virus in the Midwest, our results strongly support introduction from the

Pacific flyway early in 2015, with ≥ 3 initial independent introductions and late events involving extensive secondary spread between poultry farms. These H5N8 and H5N2 viruses cause substantial illness and death in poultry but have, thus far, not been implicated in human infection. Mammalian studies in mice and ferrets suggest low public health risk (43,44).

During 2016–2017, HPAIV H5N8 clade 2.3.4.4 group B (Gochang-like) reemerged and caused outbreaks in wild birds and domestic poultry across Europe, Asia, and Africa (45,46). Enhanced biosecurity is required to prevent the introduction and dissemination of HPAIVs across the poultry industry; the role that human activity can play in viral spread via fomites should not be underestimated. Continued surveillance, virus characterization, and infectivity studies remain invaluable to monitoring the spread and evolution of these H5 viruses; such efforts could further the design of improved prevention strategies. Additional research is needed to decipher the potential mechanisms of virus introduction into poultry systems.

Acknowledgments

We thank the Veterinary Services and Center of Epidemiology and Animal Health staffs (especially Philip D. Riggs) of the Animal and Plant Health Inspection Service, US Department of Agriculture, for their contribution to data visualization, and we thank the Diagnostic Virology Laboratory avian diagnostic team of the National Veterinary Services Laboratories. We thank the authors of the research we cited and staff of laboratories contributing sequences to the GISAID's EpiFlu database, on which this research is based. A detailed list of acknowledgments is provided in online Technical Appendix Table 4.

J.B. and J.H. are partially supported by the National Institutes of Health Centers for Excellence in Influenza Research and Surveillance (contract no. HHSN272201400006C).

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Transmission Dynamics of Highly Pathogenic Avian Influenza Virus A(H5Nx) Clade 2.3.4.4, North America, 2014–2015

Technical Appendix

Ancestral State Reconstruction of the Geographic Location and Host Type

To investigate the viral transmission between host types over large spatial scales, we reconstructed the viral transmission history between geographic states using an ancestral state reconstruction approach with a Bayesian stochastic search variable selection to determine the most probable spatial and ecologic transmission history. For all phylogeographic analyses, we used an uncorrelated lognormal distribution relaxed-clock method with a Hasegawa, Kishino, and Yano nucleotide substitution model and the Bayesian skyride coalescent prior. A Markov chain Monte Carlo method to sample trees and evolutionary parameters was run for 7.0×10^7 generations. At least 3 independent Markov chains were performed to achieve output that converged well, on the basis of the criteria of an effective sampling size of >200 as calculated by Tracer version 1.5 with a 10% burn-in (<http://tree.bio.ed.ac.uk/software/tracer/>). A maximum clade credibility tree was generated for each data set by using TreeAnnotator (<http://www.phylo.org/index.php/tools/treeannotator.html>) in BEAST (<http://beast.community/>). FigTree 1.3.1 (<http://tree.bio.ed.ac.uk/>) was used for visualization of trees.

Using a globally derived data set ($n_{tax} = 127$), we tested whether the highly pathogenic avian influenza virus (HPAIV) H5 that caused the North America outbreak was the result of a single introduction or multiple introductions into the North America wild bird population using hemagglutinin (HA) sequences of HPAIVs from Asia, Europe, and North America. We then developed a more refined model to estimate viral diffusion and transmission between wild and domestic populations and assess the most likely route for spread. Sequences of the HPAIV HAs from the United States and Canada (*I*) during the 2014–15 outbreak and the 12 most closely related H5N8 sequences from Asia (determined from our global scale analysis), which were

collected in 2014, were incorporated into our analysis discrete phylogenetic model. The geographic region and host type were defined in the model as discrete nominal categories. The geographic region of collection was categorized by migration flyway (Pacific, Central, Mississippi). No H5Nx viruses were detected in US poultry from the Atlantic flyway region, and there was no US Department of Agriculture Wildlife Services wild bird surveillance conducted in the Atlantic flyway during the period of interest. Sequences of viruses from the Central and Mississippi flyways were combined into 1 category due to the sparsity of sequences from the Central flyway. Host type was categorized into chicken, turkey, wild bird, and backyard poultry. We assumed that backyard poultry operations were small-scale operations with lower biosecurity than large-scale commercial poultry production. We, therefore, treated the backyard poultry category as ambiguous state, which is a general class accounting for various-sized backyard operations. We estimated the ancestral state transition rate and model parameters from a set of 5,000 empirical trees simulated using HPAIV H5 HA gene data collected throughout the outbreak. We assumed a constant coalescent tree before produce the empirical phylogenetic tree set. For the discrete ancestral state model, we used a nonreversible continuous-time Markov chain model with a strict clock assumption to estimate geographic and host transitions among wild and domestic birds in the US outbreak during December 2014–June 2015 (2). Bayesian stochastic search variable selection was used to identify important transitions by using a binary indicator (I) (3–5), enabling the calculation of Bayes factors with SPREAD version 1.0.6 (6). A transition was considered important when $I > 0.5$ and Bayes factors > 4.0 (3,4). At least 3 independent runs of Markov chains were performed to achieve output that converged well. The last 500 trees from each posterior distribution of trees were used to construct heat maps representing the average number of transitions per month. Convergence of tree topologies was assessed by using RWTY, an R implementation of Are We There Yet (7).

Intravenous Pathogenicity Index in Chickens

The intravenous pathogenicity index (IVPI) test of 20 H5 isolates, representing at least the index cases for each state and domestic species, was conducted according to the World Organisation for Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (<http://www.oie.int/en/international-standard-setting/terrestrial-manual>). In brief, 0.1 mL of infectious allantoic fluid was inoculated intravenously into ten 6-week-old specific–

pathogen-free chickens and chickens monitored for 10 days for morbidity and mortality. Isolates with IVPI >1.2 were characterized as HPAIV. The challenge studies and all experiments with live viruses were conducted in a biosafety level 3 facility at the National Veterinary Services Laboratories, Animal and Plant Health Inspection Service, US Department of Agriculture in Ames, Iowa, USA, and in accordance with approved institutional animal care and use protocols. Twenty representative isolates were highly pathogenic in chickens on intravenous inoculation and IVPIs of each isolate ranged 2.57–2.99 (Technical Appendix Table 1).

Single-Nucleotide Polymorphism (SNP) Analysis for HPAIV H5N2

To identify genetic changes, full-genome sequence analysis of the US HPAIV H5N2 isolates was conducted. For phylogenetic analysis, complete coding regions of the US HPAIV H5 isolates were aligned by using MAFFT (<https://mafft.cbrc.jp/alignment/software/>) and were used for subsequent SNP testing. The coding sequences discriminating SNPs were classified as either nonsynonymous or synonymous depending on whether or not they corresponded to differences in encoded amino acid sequences. Nonsynonymous substitutions found in the alignment of 264 H5N2 complete genome sequences from the United States and Canada were compared with A/Northern pintail/Washington/40964/2014 (H5N2) virus, previously identified as the oldest US H5 2.3.4.4 representative at 5% of minimum variant frequency.

A total of 31 nonsynonymous substitutions across the entire genome were identified in HPAIV H5N2 when compared with the US index virus A/Northern Pintail/WA/ 2014 (Technical Appendix Table 3). A total of 10 nonsynonymous substitutions were identified in group 1 viruses and maintained in group 2 H5N2 viruses: L386V and V649I in polymerase basic 2; L8F, N130T, and S157P in HA; R253K, E368K, and V412A in neuraminidase; Q78R in matrix 2; and I176T in nonstructural protein 1. The nonsynonymous substitutions R215K in polymerase basic 1, A337V in polymerase acidic, P152S in HA, N60H in nuclear export protein, and K217T in nonstructural protein 1 were common substitutions of H5N2 group 2 viruses. The other 16 nonsynonymous substitutions were unique changes identified from each subgroup.

Transmission Pattern of H5N8 HPAIV in the United States

The 8 gene segments were concatenated to generate a single alignment that was used to construct a phylogenetic network by using the median-joining method implemented in the program NETWORK version 5.0 with epsilon set to 0 (8).

Phylogenetic network analysis of the HPAIV H5N8 suggests largely independent introductions with limited lateral spread (Technical Appendix Figure 6). There was a single detection of H5N8 outside the Pacific flyway in a backyard flock in Indiana. The phylogenetic reconstruction of source–sink dynamics supports the potential for wild bird introduction in Indiana (main text Figure 3; Technical Appendix Figure 5); however, there were no H5N8 detections in wild birds outside the Pacific flyway.

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Technical Appendix Table 1. Pathogenicity of highly pathogenic avian influenza viruses H5Nx in chickens during outbreaks, United States, 2014–15

Strain name	Date collected	IVPI index
A/gyrfalcon/Washington/41088–6/2014(H5N8)	2014 Dec 7	2.65
A/Northern_pintail/Washington/40964/2014(H5N2)	2014 Dec 8	2.57
A/guinea_fowl/Washington/41613–1/2014(H5N8)	2014 Dec 16	2.69
A/Coopers_hawk/Washington/197127/2014(H5N2)	2014 Dec 29	2.77
A/American_green-winged_teal/Washington/195750/2014(H5N1)	2014 Dec 29	2.99
A/chicken/Washington/61–9/2014(H5N2)	2014 Dec 30	2.87
A/chicken/Idaho/1711–7/2015(H5N2)	2015 Jan 11	2.70
A/turkey/California/K1500169–1.2/2015(H5N8)	2015 Jan 21	2.75
A/chicken/California/4912/2015(H5N8)	2015 Feb 6	2.96
A/chicken/Oregon/5256/2015(H5N2)	2015 Feb 11	2.67
A/turkey/Minnesota/7172–1/2015(H5N2)	2015 Feb 27	2.80
A/turkey/Missouri/15–007478–1/2015(H5N2)	2015 Mar 6	2.91
A/turkey/Arkansas/7791–1/2015(H5N2)	2015 Mar 8	2.81
A/chicken/Kansas/8395–3/2015(H5N2)	2015 Mar 12	2.66
A/turkey/South_Dakota/10371/2015(H5N2)	2015 Mar 30	2.86
A/chicken/Montana/10559/2015(H5N2)	2015 Apr 1	2.74
A/chicken/Wisconsin/11595–1/2015(H5N2)	2015 Apr 8	2.94
A/turkey/North_Dakota/15–011419–1/2015(H5N2)	2015 Apr 8	2.96
A/turkey/Iowa/11762–1/2015(H5N2)	2015 Apr 12	2.92
A/chicken/Indiana/15–014961–4/2015(H5N8)	2015 May 9	2.80

IVPI, intravenous pathogenicity index.

Technical Appendix Table 2. Migration of viruses from Asia well-supported by Bayes factors*

Migrations	Bayes factor	Posterior probability	Mean migration rate
China→Vietnam	73.343	0.9096	0.584
Japan→Taiwan	56.543	0.8859	1.352
China→Korea	42.831	0.8546	0.806
China→Laos	40.668	0.8481	0.589
Korea→Japan	35.5599	0.8300	2.563
Japan→North America	11.6459	0.6152	1.268
Japan→Europe	11.4887	0.6120	1.166

*Migrations supported by a Bayes factor >6 and posterior probability >0.5.

Technical Appendix Table 3. Nonsynonymous substitutions found in highly pathogenic avian influenza virus H5N2 isolates from United States outbreaks when compared with A/northern pintail/Washington/40964/2014 (H5N2), 2014–15*

Protein	Substitution	Codon change	Group or subgroup
PB2	K54R	AAA→AGA	2e
PB2	L386V	TTA→GTA	1, 2a, 2b, 2c, 2d, 2e
PB2	V649I	GTA→ATA	1, 2a, 2b, 2c, 2d, 2e
PB1	E180D	GAA→GAC	2e
PB1	R215K	AGG→AAG	2a, 2b, 2c, 2d, 2e
PB1	M317V	ATG→GTG	2e
PB1	R723L	CGA→CTA	2a
PA	A337V	GCT→GTT	2a, 2b, 2c, 2d, 2e
PA	A475T	GCA→ACA	2e
HA	L6I	CTT→ATT	2c
HA	L7P	CTT→CCT	2e
HA	L8F	CTT→TTT	1, 2a, 2b, 2c, 2d, 2e
HA	M82I	ATG→ATA	2e
HA	N130T	AAT→ACT	1, 2a, 2b, 2c, 2d, 2e
HA	P152S	CCA→TCA	2a, 2b
HA	S157P	TCC→CCC	1, 2a, 2b, 2c, 2d, 2e
HA	K250R	AAA→AGA	2a
HA	L338Q	CTA→CAA	2e
NP	M105V	ATG→GTG	2b

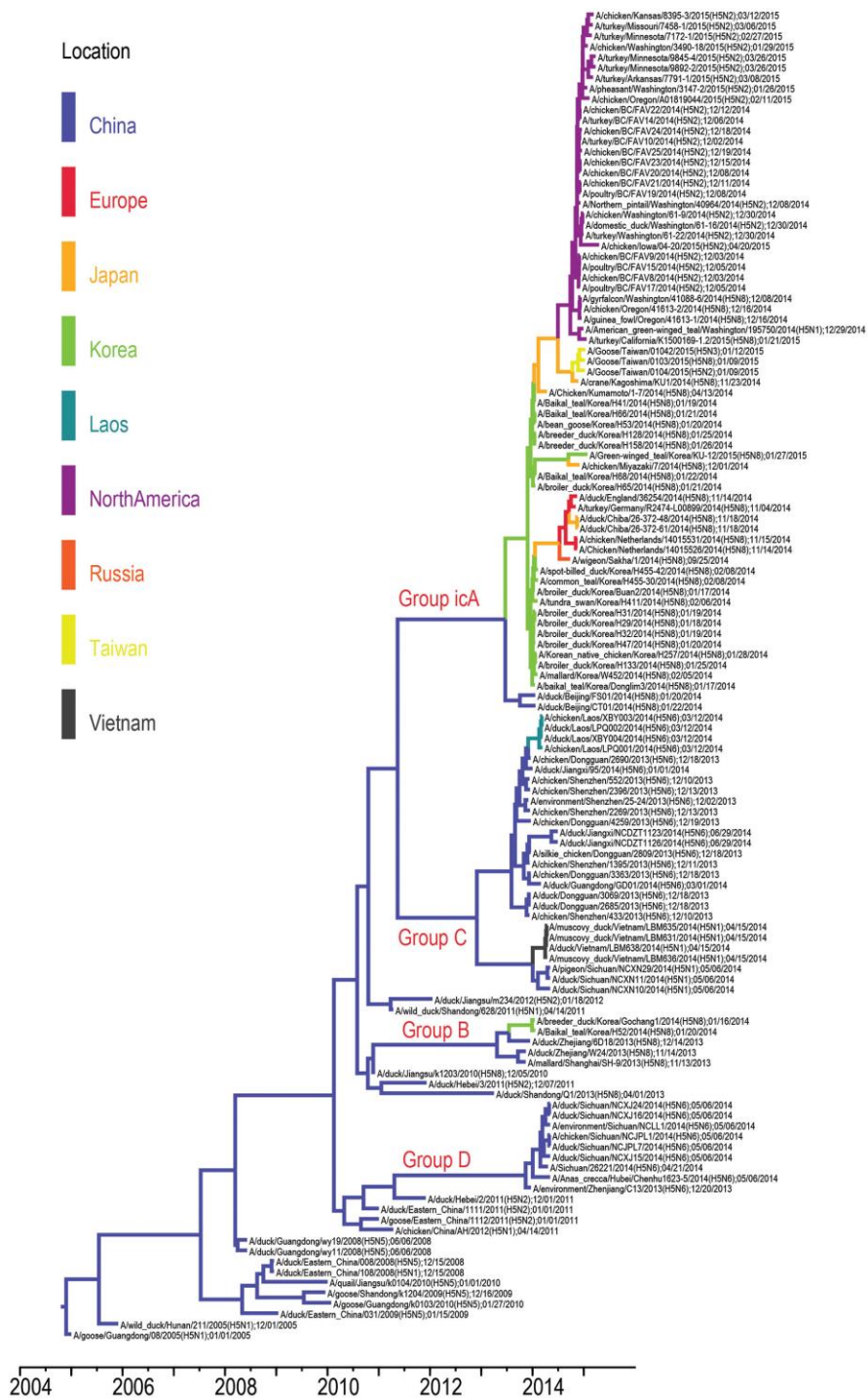
Protein	Substitution	Codon change	Group or subgroup
NP	M105I	ATG→ATA	2a
NP	I109T	ATC→ACC	2e
NA	R253K	AGA→AAA	1, 2a, 2b, 2c, 2d, 2e
NA	E368K	GAA→AAA	1, 2a, 2b, 2c, 2d, 2e
NA	T380I	ACC→ATC	2b
NA	V412A	GTT→GCT	1, 2a, 2b, 2c, 2d
NA	S416G	AGC→GGC	2e
NA	R430K	AGG→AAG	2b
M2	Q78R	CAG→CGG	1, 2a, 2b, 2c, 2d, 2e
NEP	N60H	AAC→CAC	2a, 2b, 2c, 2d, 2e
NS1	I176T	ATT→ACT	1, 2a, 2b, 2c, 2d, 2e
NS1	K217T	AAA→ACA	2a, 2b, 2c, 2d, 2e

*HA, hemagglutinin; M2, matrix 2; NA, neuraminidase; NEP, nuclear export protein; NP, nucleoprotein; NS1, nonstructural protein 1; PA, polymerase acidic; PB1, polymerase basic 1; PB2, polymerase basic 2.

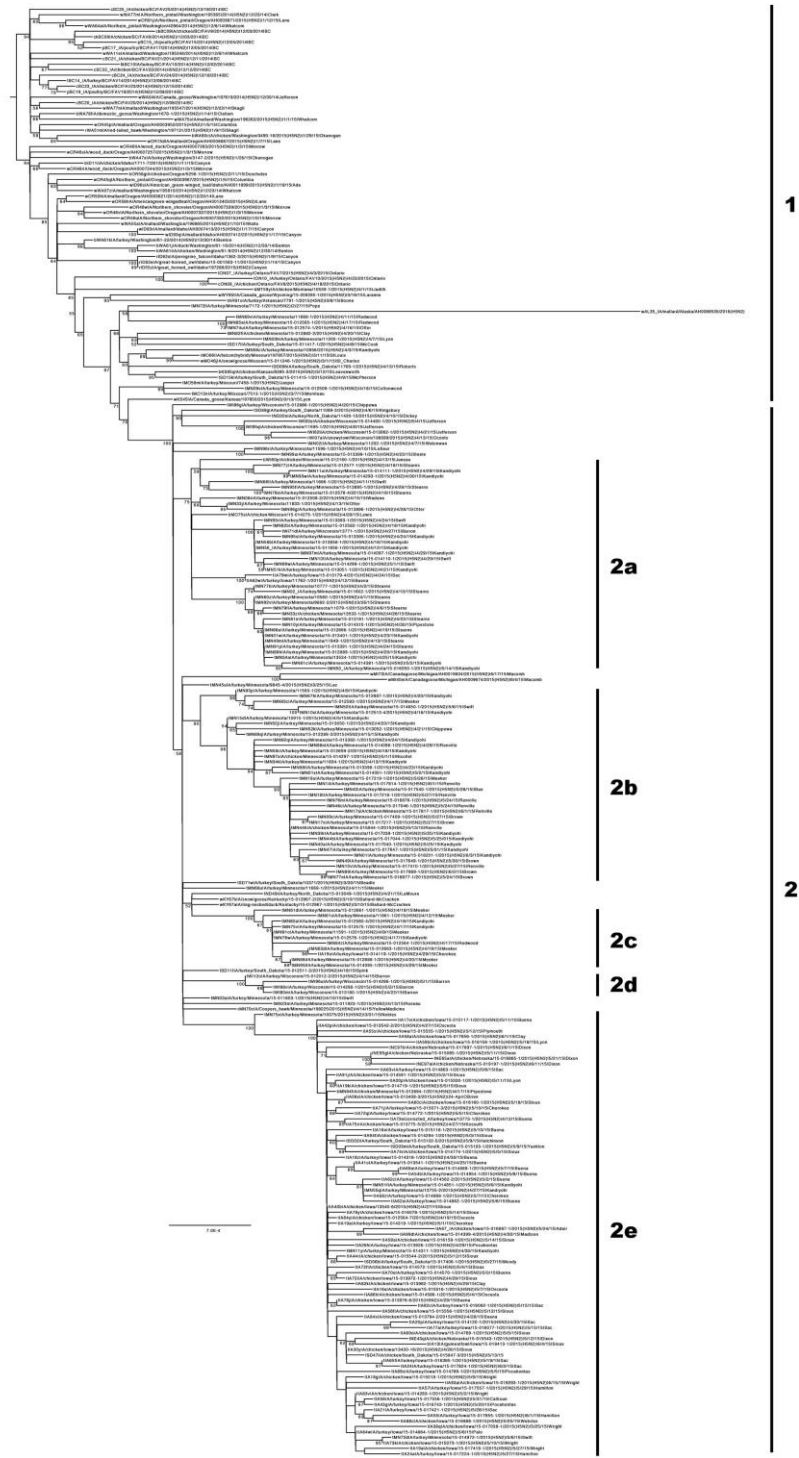
Technical Appendix Table 4. Acknowledgment of GISAID EpiFlu submitters*

Segment ID	Isolate name	Collection date	Submitter	Originating laboratory
EPI169427	A/wigeon/Sakha/1/2014	2014 Sep 25	Ivan Susloparov	State Research Center of Virology and Biotechnology Vector
EPI161973	A/environment/Shenzhen/25–24/2013	2013 Dec 2	Guang Liu	Beijing Genomics Institute Shenzhen
EPI137255	A/duck/Hebei/3/2011	2011 Dec 7	Di Liu	Institute of Microbiology, Chinese Academy of Sciences
EPI137254	A/duck/Hebei/2/2011	2011 Dec 1	Di Liu	Institute of Microbiology, Chinese Academy of Sciences
EPI163493	A/Sichuan/26221/2014	2014 Apr 21	Lei Yang	WHO Chinese National Influenza Center
EPI161972	A/duck/Jiangxi/95/2014	2014 Jan 10	Guang Liu	Beijing Genomics Institute Shenzhen
EPI169424	A/chicken/Miyazaki/7/2014	2014 Dec 16	Takehiko Saito	National Institute of Animal Health
EPI169390	A/crane/Kagoshima/KU1/2014	2014 Nov 23	Makoto Ozawa	Kagoshima University
EPI168075	A/chicken/Netherlands/14015531/2014	2014 Nov 15	Guus Koch	Central Veterinary Institute
EPI168025	A/duck/Chiba/26–372–61/2014	2014 Nov 18	Takehiko Saito	National Institute of Animal Health
EPI168024	A/duck/Chiba/26–372–48/2014	2014 Nov 18	Takehiko Saito	National Institute of Animal Health
EPI167905	A/Chicken/Netherlands/14015526/2014	2014 Nov 14	Guus Koch	Central Veterinary Institute
EPI167904	A/duck/England/36254/14	2014 Nov 14	Amanda Hanna	Animal and Plant Health Agency
EPI166694	A/duck/Beijing/CT01/2014	2014 Jan 22	Di Liu	Institute of Microbiology, Chinese Academy of Sciences
EPI166693	A/duck/Beijing/FS01/2014	2014 Jan 20	Di Liu	Institute of Microbiology, Chinese Academy of Sciences
EPI159719	A/Chicken/Kumamoto/1–7/2014	2014 Apr 13	Michiyo Yoshizawa Harada	National Institute of Animal Health
EPI173568	A/green-winged teal/Korea/KU-12/2015	2015 Jan 22	Dong-Hun Lee	Konkuk University
EPI169273	A/turkey/Germany/R2474-L00899/2014	2014 Nov 4	Anne Pohlmann	Friedrich-Loeffler-Institut
EPI586509	A/poultry/BC/FAV19/2014	2014 Dec 8	CFIA	Animal Health Centre, Ministry of Agriculture
EPI584079	A/turkey/BC/FAV14/2014	2014 Dec 6	CFIA	Animal Health Centre, Ministry of Agriculture
EPI586501	A/poultry/BC/FAV17/2014	2014 Dec 5	CFIA	Animal Health Centre, Ministry of Agriculture
EPI586493	A/poultry/BC/FAV15/2014	2014 Dec 5	CFIA	Animal Health Centre, Ministry of Agriculture
EPI586550	A/chicken/BC/FAV24/2014	2014 Dec 18	CFIA	Animal Health Centre, Ministry of Agriculture
EPI586542	A/chicken/BC/FAV23/2014	2014 Dec 15	CFIA	Animal Health Centre, Ministry of Agriculture
EPI586534	A/chicken/BC/FAV22/2014	2014 Dec 12	CFIA	Animal Health Centre, Ministry of Agriculture
EPI586526	A/chicken/BC/FAV21/2014	2014 Dec 11	CFIA	Animal Health Centre, Ministry of Agriculture
EPI586518	A/chicken/BC/FAV20/2014	2014 Dec 8	CFIA	Animal Health Centre, Ministry of Agriculture
EPI586558	A/chicken/BC/FAV25/2014	2014 Dec 19	CFIA	Animal Health Centre, Ministry of Agriculture

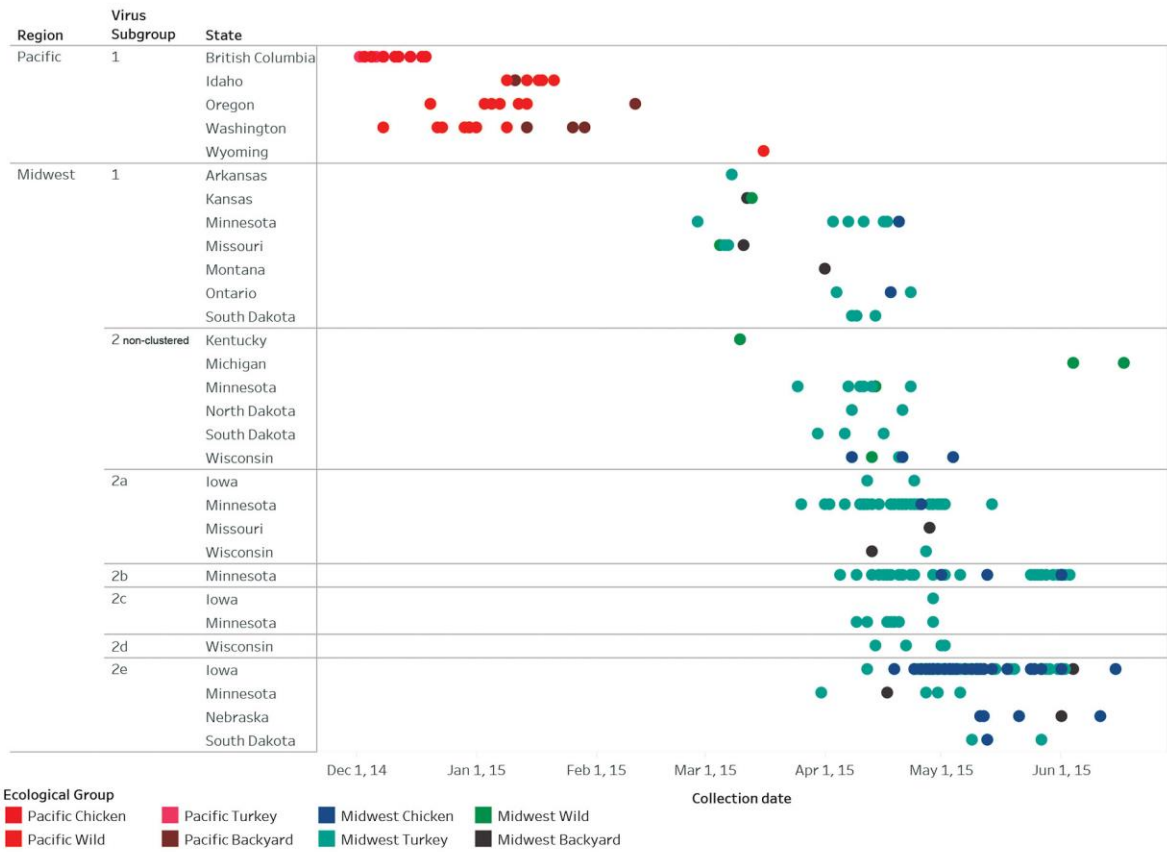
*CFIA, Canadian Food Inspection Agency; GISAID, global initiative on sharing all influenza data; ID, identification; WHO, World Health Organization.



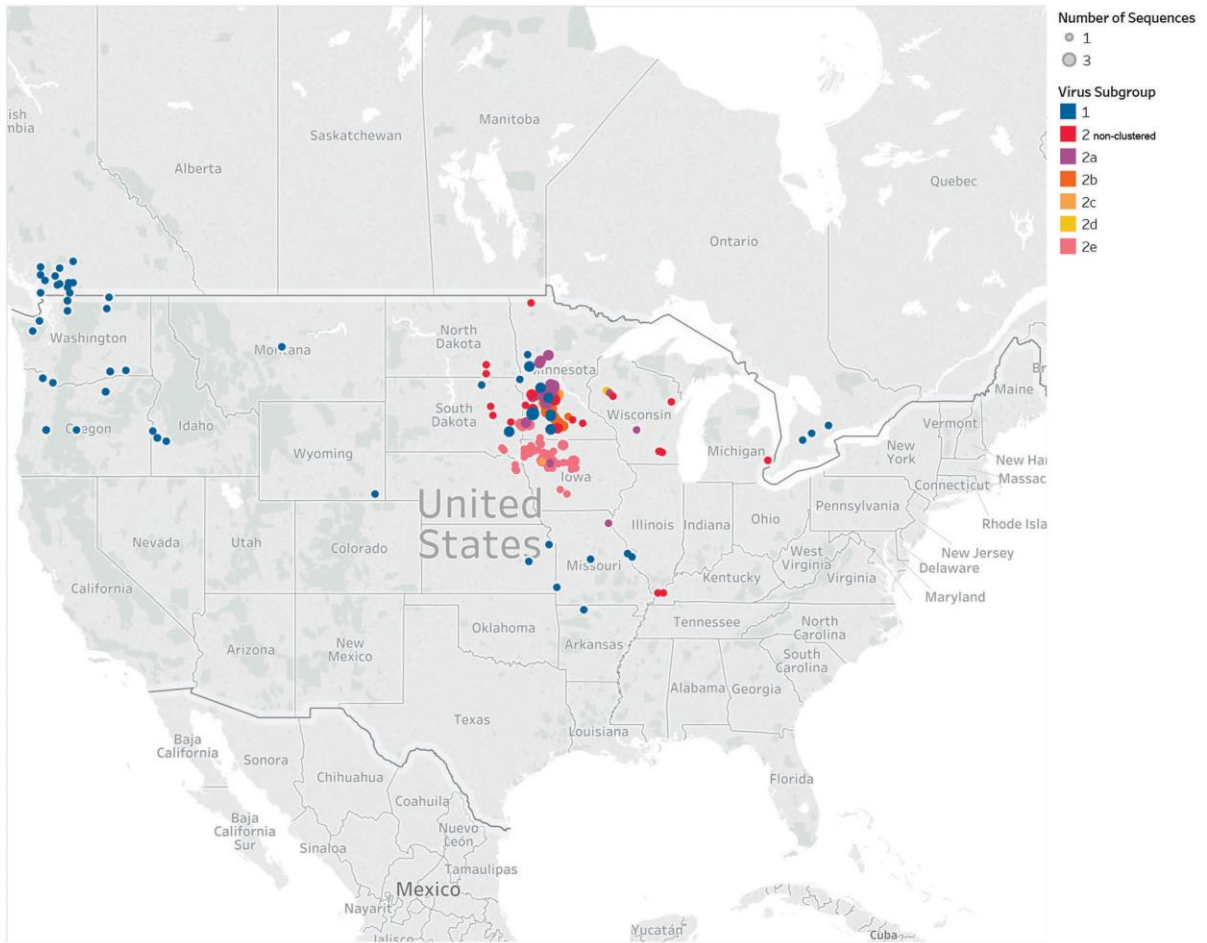
Technical Appendix Figure 1. Phylogeography of hemagglutinin of highly pathogenic avian influenza virus H5 clade 2.3.4.4 isolates from outbreaks, North America, 2014–2015. The phylogenetic relationships and temporal evolutionary history were estimated by molecular clock analysis. Group designations are indicated. Scale bar indicates year of isolation.



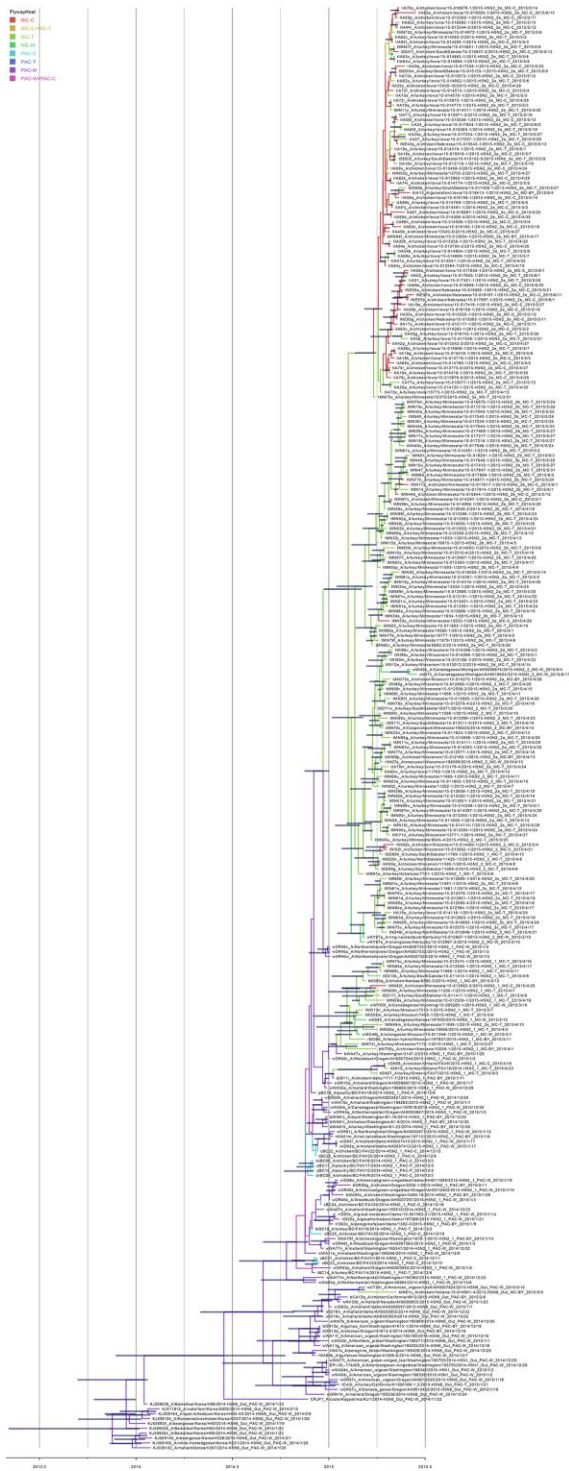
Technical Appendix Figure 2. Maximum-likelihood phylogeny of concatenated complete genome sequences of highly pathogenic avian influenza virus H5N2 clade 2.3.4.4, North America, 2014–2015. Strain names are displayed. Numbers along branches indicate bootstrap values >70%. Brackets indicate the genetic subgroups. Scale bar indicates nucleotide substitutions per site.



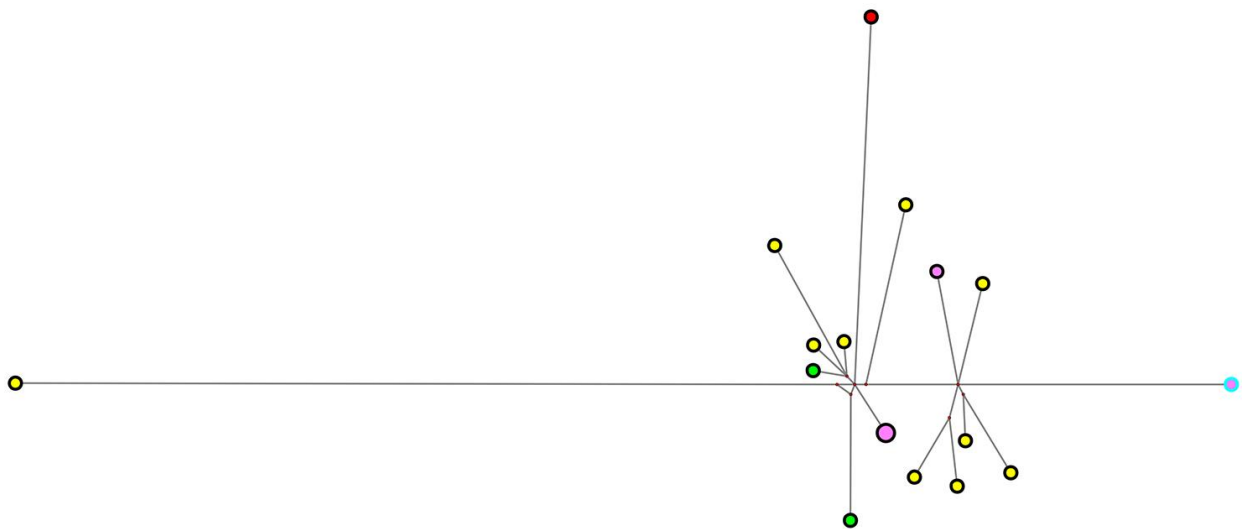
Technical Appendix Figure 3. Time series of highly pathogenic avian influenza virus H5N2 distribution during outbreaks by subgroup and US state or Canada province of North America, 2014–2015. Ecologic group is indicated.



Technical Appendix Figure 4. Distribution of highly pathogenic avian influenza virus H5N2 subgroup during outbreak, North America, 2014–2015. Virus subgroup is indicated.



Technical Appendix Figure 5. Bayesian phylogenetic analysis and ancestral state reconstruction of highly pathogenic avian influenza virus H5 hemagglutinin gene, North America. The outgroup contains avian influenza viruses from Asia. Color changes at nodes represent transition between discrete character states (a combination of geographic region and host type). Bars represent the node height 95% highest posterior density. C, chicken; MC, Mississippi-Central; PAC, Pacific; T, turkey; W, wild bird.



Technical Appendix Figure 6. Median-joining phylogenetic network of highly pathogenic avian influenza H5N8 viruses, North America, 2014–2015. The network was constructed from the concatenated entire genome of H5N8 viruses and includes all the most parsimonious trees linking the sequences. Each unique sequence is represented by a circle sized relative to its frequency in the data set. Circles were colored according to sample origin: red is commercial poultry, purple backyard poultry, yellow wild birds, and green raptors. Outer circle color indicates flyway: black Pacific and sky-blue Mississippi-Central.