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## Low-Pathogenic Influenza A Viruses in North American Diving Ducks Contribute to the Emergence of a Novel Highly Pathogenic Influenza A(H7N8) Virus

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# Low-Pathogenic Influenza A Viruses in North American Diving Ducks Contribute to the Emergence of a Novel Highly Pathogenic Influenza A(H7N8) Virus

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**ABSTRACT** Introductions of low-pathogenic avian influenza (LPAI) viruses of subtypes H5 and H7 into poultry from wild birds have the potential to mutate to highly pathogenic avian influenza (HPAI) viruses, but such viruses' origins are often unclear. In January 2016, a novel H7N8 HPAI virus caused an outbreak in turkeys in Indiana, USA. To determine the virus's origin, we sequenced the genomes of 441 wild-bird origin influenza A viruses (IAVs) from North America and subjected them to evolutionary analyses. The results showed that the H7N8 LPAI virus most likely circulated among diving ducks in the Mississippi flyway during autumn 2015 and was subsequently introduced to Indiana turkeys, in which it evolved high pathogenicity. Preceding the outbreak, an isolate with six gene segments (PB2, PB1, PA, HA, NA, and NS) sharing >99% sequence identity with those of H7N8 turkey isolates was recovered from a diving duck sampled in Kentucky, USA. H4N8 IAVs from other diving ducks possessed five H7N8-like gene segments (PB2, PB1, NA, MP, and NS; >98% sequence identity). Our findings suggest that viral gene constellations circulating among diving ducks can contribute to the emergence of IAVs that affect poultry. Therefore, diving ducks may serve an important and understudied role in the maintenance, diversification, and transmission of IAVs in the wild-bird reservoir.

**IMPORTANCE** In January 2016, a novel H7N8 HPAI virus caused a disease outbreak in turkeys in Indiana, USA. To determine the origin of this virus, we sequenced and analyzed 441 wild-bird origin influenza virus strains isolated from wild birds inhabiting North America. We found that the H7N8 LPAI virus most likely circulated among diving ducks in the Mississippi flyway during autumn 2015 and was subsequently introduced to Indiana turkeys, in which it evolved high pathogenicity. Our results suggest that viral gene constellations circulating among diving ducks can contribute to the emergence of IAVs that affect poultry. Therefore, diving ducks may play an important and understudied role in the maintenance, diversification, and transmission of IAVs in the wild-bird reservoir. Our study also highlights the importance of a coordinated, systematic, and collaborative surveillance for IAVs in both poultry and wild-bird populations.

**KEYWORDS** highly pathogenic avian influenza, subtype H7N8, low-pathogenic avian influenza, wild birds, diving duck, genetic reassortment, evolutionary network

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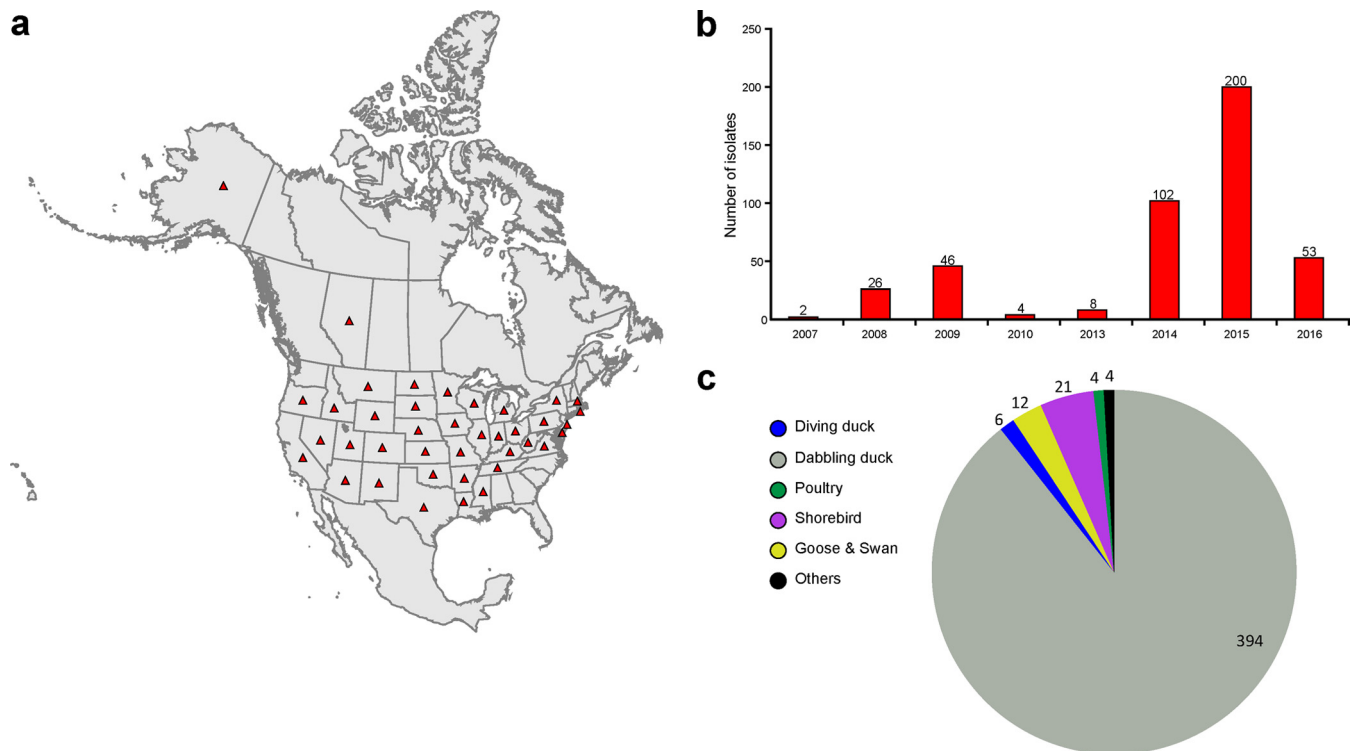
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Influenza A viruses (IAVs) exist in a complex ecosystem that involves various hosts, including humans, swine, horses, dogs, sea mammals, and numerous wild and domestic bird species. Among these hosts, wild aquatic birds are considered the natural reservoir for IAVs (H1 to H16), and bird migration plays an important role in the dispersal of viruses. Interactions of migratory birds at congregation sites enable transmission of IAVs and facilitate genetic diversity through reassortment. Periodic introduction of IAVs from wild birds to domestic poultry occasionally causes outbreaks among domestic birds. Following such introductions into domestic poultry, low-pathogenic avian influenza (LPAI) H5 and H7 viruses have the potential to mutate into highly pathogenic avian influenza (HPAI) viruses through mechanisms such as (i) acquisition of basic amino acids in the cleavage region of the hemagglutinin (HA) protein by insertion or substitution (1) and (ii) recombination with another gene segment(s) or host genome (2–4). HPAI viruses are of concern due to the direct and indirect losses that may be incurred by the poultry industry through mortality of affected birds, culling of flocks to prevent spread, trade restrictions, and increased costs associated with outbreak response. Non-H5 and -H7 strains can also have significant impacts, such as the H9N2 viruses that have become endemic in poultry in Asia and the Middle East (5); such endemicity can also contribute to the emergence of novel strains posing public health challenges, such as H7N9 viruses (6, 7).

The consequences of IAV introduction from wild birds to domestic poultry can be dramatic, as evidenced by the emergence of goose-Guangdong lineage H5N1 HPAI viruses (8) in Asia. This virus lineage spilled back into wild birds and has caused the longest HPAI outbreak to date, affecting many countries. The impacts of wild-bird lineage LPAI viruses, such as H7N1 (9) and H7N7 viruses (10), mutating into HPAI viruses after introduction and replication in poultry has been repeatedly demonstrated. In the Americas, H5 HPAI viruses caused outbreaks among domestic poultry in 1983 (11), 1994 (12), and 2014 and 2015 (13). Subtype H7 HPAI viruses have also periodically been reported in poultry in the Americas. There were four reported H7N3 HPAI outbreaks between 2002 and 2016: one outbreak in Chile (2002) (2), two distinct outbreaks in Canada (2004 and 2007) (14, 15), and one outbreak in Mexico (2012) (4). In addition to the impacts in domestic poultry, highly pathogenic H5N1, H7N3 (Canada in 2004 and Mexico in 2012), and H7N7 viruses have also caused human infections (8, 16–18).

Retrospective studies have previously been conducted to identify putative precursors of viruses that cause outbreaks among domestic poultry. Wild birds have repeatedly been recognized as the most probable source for outbreak strains, or at least a few of the gene segments that were incorporated into the genomes of the outbreak strains (19, 20). However, in many instances, evidence is lacking regarding the time of emergence, location of the reassortment, and bird species associated with the genesis of a particular IAV outbreak strain. Limited availability of contemporary wild-bird origin IAV isolates and corresponding genetic sequences may impede any attempt to investigate potential mechanisms underlying emergence.

On 15 January 2016, the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (APHIS) announced the detection of a novel H7N8 HPAI virus in a commercial turkey flock experiencing significant mortality in Dubois County, IN. The detection of this virus represented the first report of an H7N8 HPAI virus in any domestic species. Subsequently, APHIS reported the identification of H7N8 LPAI virus in eight turkey flocks during control zone surveillance. The epidemiologic investigation and phylogenetic analysis of the outbreak suggested a point source introduction followed by secondary spread (21, 22). The H7N8 virus was eradicated from Indiana after quarantine and depollution of 414,223 commercial birds, including 258,045 turkeys and 156,178 chickens, on 10 commercial turkey farms. No human infection associated with H7N8 HPAI virus was reported. A pathogenesis study conducted subsequent to the outbreak suggested that, in mice, the H7N8 HPAI strain produced greater weight loss and displayed greater replicative ability in the respiratory tract than the H7N8 LPAI strain, and in ferrets, the H7N8 HPAI and LPAI strains both replicated efficiently in the respiratory tract, but only the HPAI strain led to moderate morbidity



**FIG 1** Geographic, temporal, and taxon distributions of 441 influenza A virus strains subjected to genomic sequencing in this study. (a) The triangles indicate the state/province where the samples were collected. (b) The number above each bar indicates the number of influenza A virus isolates from that year. (c) Distribution of strains among distinct host groups. Map of North America with U.S. states, Canadian provinces, and Mexico by FreeVectorMaps.com (<https://freevectormaps.com/world-maps/north-america/WRLD-NA-02-0003>).

and mortality. The transmission study in ferrets showed that only the H7N8 LPAI strain caused transmission among one out of three cohoused ferrets through direct contact (23).

The objective of this study was to understand the molecular mechanisms leading to the emergence of this novel H7N8 virus in wild birds. We hypothesized that the H7N8 virus identified in turkeys in Indiana was initially introduced from wild birds and acquired high pathogenicity during replication in turkeys. In a collaborative effort, our team sequenced 441 IAVs obtained through wild-bird influenza surveillance from 2007 to 2016 across North America (Fig. 1). We used sequence data generated for this study, together with the IAV genomes recovered from the H7N8 outbreak in Indiana turkeys and publicly available sequence data, to infer the possible origin(s), evolutionary pathway(s), and transmission route(s) of this novel H7N8 HPAI virus.

**RESULTS**

**H7N8 virus originated from IAVs in North American wild birds.** To infer the ancestry of each gene segment of the H7N8 viruses detected in Indiana turkeys, we first performed preliminary phylogenetic analyses with a collection of representative genomic sequences to identify the major genetic lineage to which the H7N8 turkey strains were most closely related. Phylogenetic trees support two geographically dependent lineages of IAV (North American and Eurasian) for eight gene segments (a full set of results is available at <http://sysbio.cvm.msstate.edu/files/H7N8HPAI/>). The H7N8 turkey strains were found to share genetic ancestry with IAVs from North America for all eight gene segments.

We then performed phylogenetic analyses, focusing on genomic sequences of IAVs isolated from wild and domestic birds in the Americas. The phylogenetic tree for the H7 gene showed that IAV strains from the Americas could be divided into two genetic sublineages: those from North America and those from South America. The South

American sublineage included H7N3 HPAI viruses isolated during an outbreak in Chile in 2002. Three major genetic clusters were identified in the North American sublineage: cluster I was comprised of viruses isolated from wild and domestic birds in North America from the 1970s to the early 1990s, cluster II contained H7N2 IAVs isolated from live-poultry markets in the northeastern United States from 1994 to 2006, and cluster III represents contemporary H7 IAVs circulating in North America, including viruses isolated from wild and domestic birds. The novel H7N8 turkey strains grouped with H7 viruses in cluster III and were most closely related to IAV strains isolated from wild birds (see Fig. S1 in the supplemental material). The H7N3 HPAI viruses isolated during three previous outbreaks in North America also grouped with contemporary H7 IAVs in cluster III; however, these viruses were associated with monophyletic clades divergent from the clade containing H7N8 turkey strains. Phylogenetic analyses of the other seven gene segments also showed that the H7N8 turkey strains clustered with North American wild-bird origin IAV strains and in monophyletic clades different from those containing sequences for H7N3 HPAI viruses (see Fig. S1 in the supplemental material). Moreover, in the phylogenetic trees for eight gene segments, no poultry origin IAV gene segments were found to be closely related to the H7N8 turkey strains.

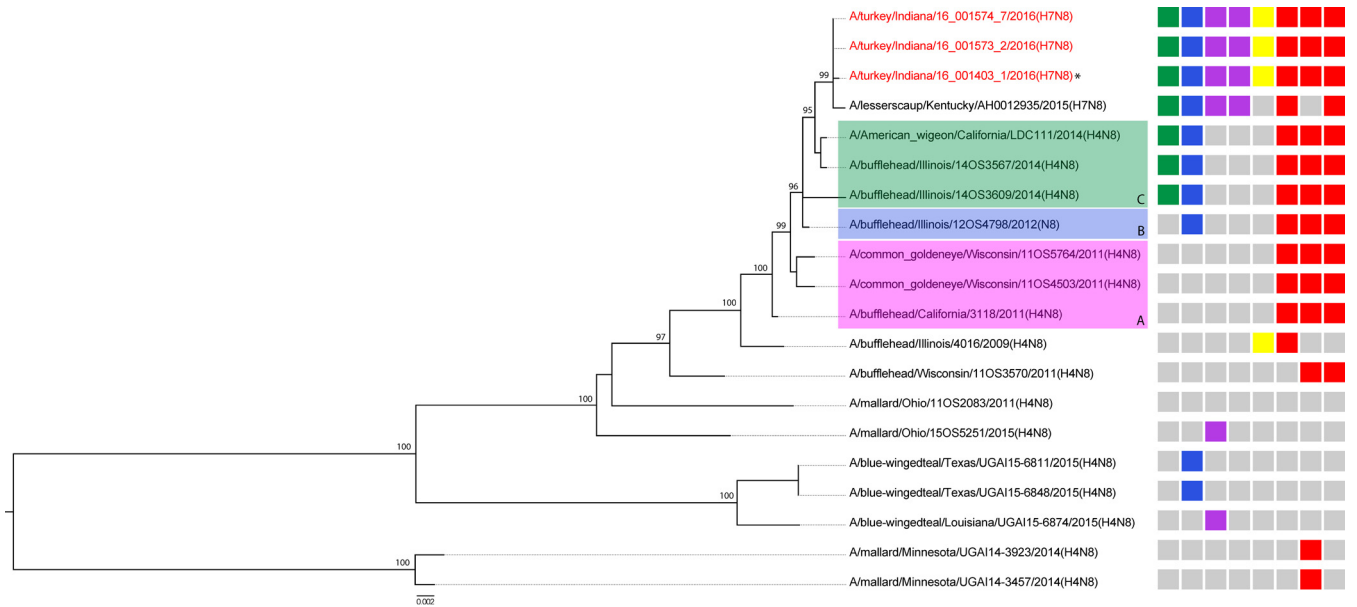
To infer the more detailed evolutionary history of the novel H7N8 viruses, we conducted molecular clock analyses for each gene segment using sequences in the clades containing the H7N8 turkey strains and the most closely related strains. The resulting time-scaled maximum clade credibility phylogenetic trees were used to identify viruses most closely related to H7N8 turkey strains for each gene segment (see Fig. S1 in the supplemental material). The gene segments most closely related to H7N8 turkey strains originated from a single isolate, A/Lesser scaup/Kentucky/AH0012935/2015(H7N8) (Kentucky/AH0012935), for six gene segments (PB2, PB1, PA, HA, NA, and NS). This isolate was recovered from a sample collected in Kentucky on 28 November 2015, approximately 7 weeks before the outbreak and approximately 200 kilometers from the Indiana turkey farm where the outbreak was detected. The close relatedness between Kentucky/AH0012935 and the novel H7N8 HPAI strain in six gene segments was supported by high nucleotide sequence identities, ranging from 99.00% (HA gene) to 99.95% (PA gene). The NP gene segments of two isolates recovered from samples collected in Ohio on 18 October 2014, A/Northern pintail/Ohio/14OS2209/2014(H5N9) (Ohio/14OS2209) and A/Northern pintail/Ohio/14OS2210/2014(H5N9) (Ohio/14OS2210), were most closely related (98.40% nucleotide sequence identity) to the NP gene of the novel H7N8 HPAI strain. The MP gene segment of A/Bufflehead/Illinois/14OS3567/2014(H4N8), collected on 15 November 2014, was most closely related (99.19% nucleotide sequence identity) to that of the H7N8 HPAI strain.

Three H7N8 turkey strains were estimated to share an ancestor among eight gene segments between March 2015 (MP gene) and December 2015 (HA gene). The mean estimated times to most recent common ancestor (TMRCA) for seven of eight gene segments (excluding the MP gene) was summer and fall (30 June to 4 December) 2015, which overlaps the late breeding and autumn migration periods of many wild birds inhabiting North America. The HA gene was estimated to have the latest TMRCA (mean, 2 December 2015; 95% highest posterior density [HPD], 27 October 2014 to 1 January 2016), which is close to the time when the sample from which Kentucky/AH0012935 was isolated was collected. The mean evolutionary rate for the H7 HA gene was estimated to be  $5.74 \times 10^{-3}$  substitutions per site per year (95% HPD,  $5.23 \times 10^{-3}$  to  $6.29 \times 10^{-3}$ ), which is significantly higher than that for the other seven gene segments.

The sampling time of the most closely related strain for the NP gene segment does not overlap the estimated TMRCA (between 28 February 2015 and 7 August 2015), suggesting a gap in surveillance and that neither Ohio/14OS2209 nor Ohio/14OS2210 is the direct predecessor of H7N8 turkey strains. This observation is consistent with the genetic distance separating H7N8 turkey strains from other viruses in the NP phylogenetic tree (see Fig. S1 in the supplemental material).

**The gene constellation of H4N8 virus in diving ducks contributed to the emergence of the novel H7N8 virus.** We investigated possible genetic events that



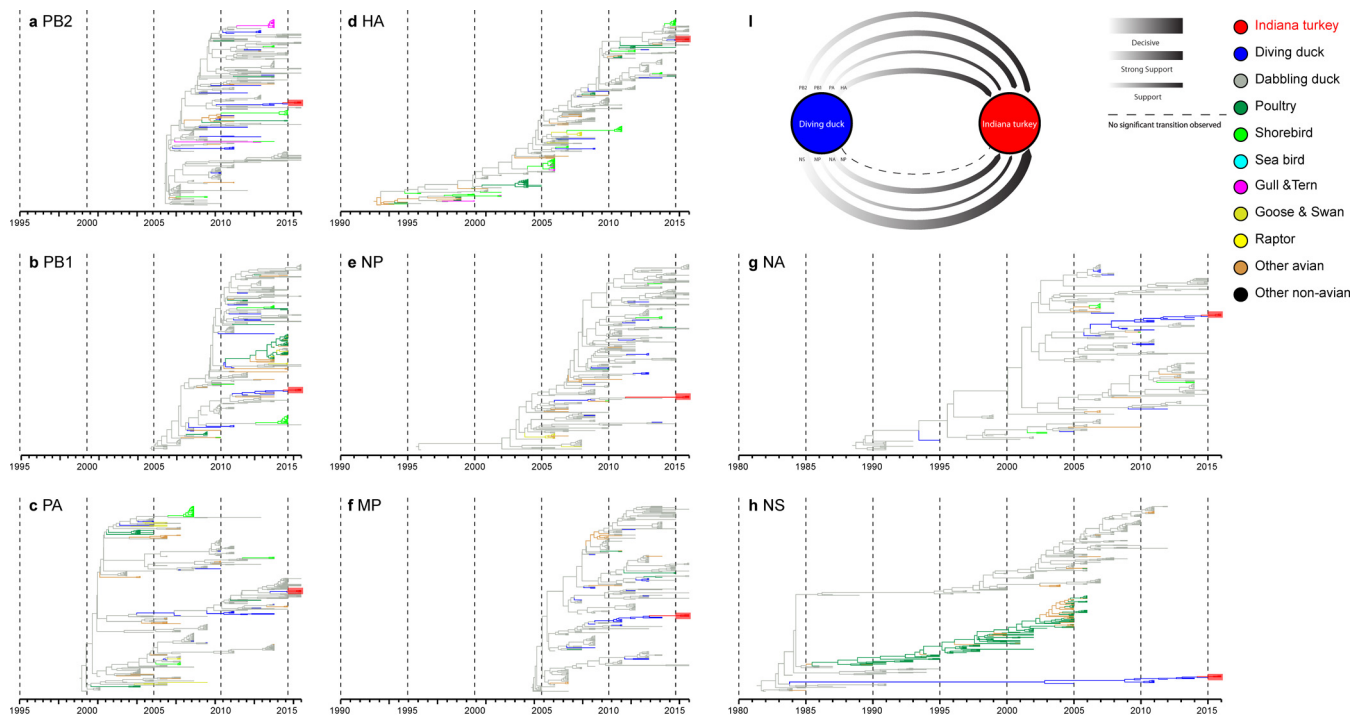


**FIG 2** Maximum-likelihood phylogenetic tree for the N8 gene segment of the H7N8 viruses detected in Indiana turkeys and H4N8 influenza A viruses isolated from wild birds. H7N8 strains associated with the outbreak of disease in Indiana turkeys are indicated in red, and the highly pathogenic strain is marked with an asterisk. The genotype of each strain is shown on the right of the tree, with eight columns of blocks representing eight gene segments (from left to right, PB2, PB1, PA, HA, NP, NA, MP, and NS). Distinct colors denote the cluster assignment, and color codes among different columns are independent. Gray indicates gene segments that are not the same as those of the H7N8 turkey strains in genotyping analysis. Three gene constellations, A, B, and C are indicated by purple, blue, and green shading, respectively.

contributed to the generation of the H7N8 precursor virus in wild birds. Examination of the eight gene segment-specific phylogenetic trees indicated that a group of H4N8 IAVs isolated from diving ducks (referred to as H4N8-DD) from 2011 to 2014 had five gene segments (PB2, PB1, NA, MP, and NS) closely clustered with those of the H7N8 turkey strains (see Fig. S1 in the supplemental material).

To better understand the contribution of H4N8-DD viruses to the generation of the H7N8 precursor virus, we assigned genotypes to H4N8 IAVs based on the results of phylogenetic analyses. We analyzed the temporal dynamics of gene constellations that possessed at least one gene segment that was assigned to the same cluster as a gene segment in the H7N8 turkey strains. Gene constellation A possessed three gene segments (NA, MP, and NS) that were of the same genotype as those in the H7N8 turkey strains; this constellation was first detected at the end of 2011 and persisted for the next 3 years (Fig. 2). Gene constellation B possessed the same three H7N8-like gene segments as constellation A plus an H7N8-like PB1 gene segment; this constellation was first detected at the end of 2012 and continued to exist in H4N8-DD viruses isolated in 2014. Gene constellation C possessed five H7N8-like gene segments (PB2, PB1, NA, MP, and NS) and was first detected in late 2014. The close genetic relationship between H4N8-DD viruses and the H7N8 HPAI turkey strain at these five gene segments was supported by high nucleotide sequence identities (>99.00%).

Using comparative phylogenetic analyses on host groups, we sought to further understand if diving ducks were the most likely source of H7N8 turkey strains. The results showed significant diffusion pathways from diving ducks to domestic turkey flocks in Indiana for seven gene segments: transition with decisive support was observed for the NS gene segment; strongly supported transition was observed for the PB2, PB1, HA, and NA gene segments; and supported transition was observed for the PA and MP gene segments (Fig. 3; see Fig. S2 in the supplemental material). No significant transition for the NP gene segment was observed between any wild-bird species and Indiana turkeys (Fig. 3; see Fig. S2 in the supplemental material). In total, the resulting transition patterns support IAV gene flow from diving ducks to Indiana turkeys and provide evidence that IAVs from diving ducks were the most likely genetic source of the H7N8 virus that was detected in turkeys in Indiana.



**FIG 3** Diffusion of influenza A virus between different host groups of wild birds and Indiana turkeys. (a to h) Maximum clade credibility phylogenetic trees for eight gene segments. The trees are constructed on the basis of the maximum clade credibility phylogenetic trees shown in Fig. S2 in the supplemental material. The branches are colored according to the estimated ancestral state of the host group from discrete trait reconstruction. The H7N8 viruses detected in Indiana turkeys are indicated by red boxes. (i) Summary of the diffusion pathway of influenza A virus between diving ducks and Indiana turkeys.

Taken together, these results provide evidence that gene constellations of H4N8 IAVs isolated primarily from diving ducks contributed to the generation of an H7N8 precursor virus in wild birds that was ultimately introduced into domestic turkeys. Furthermore, a relatively persistent gene constellation possessing H7N8-like gene segments emerged in or before 2011 and apparently acquired additional gene segments closely related to the H7N8 outbreak strain from 2011 to 2014.

**H7N8 LPAI virus evolved into an HPAI strain in turkeys.** We also investigated how the H7N8 virus evolved high pathogenicity after its introduction into domestic turkeys. The cleavability of the HA protein is considered a major determinant of pathogenicity, although the pathogenicity of IAVs is polygenic. We investigated the sequence in the cleavage region of the HA protein of H7N8 turkey strains. Indiana/16-001403-1 has an insert of three basic amino acids (KRK) at the cleavage site that results in a protein sequence motif: PENPKRKRTRGLF (Fig. 4). The other two H7N8 turkey strains, Indiana/16-001573-2 and Indiana/16-001574-7, have identical and typical LPAI cleavage sites with no insert. Analyses of H7 HPAI strains from previous outbreaks in the Americas identified multiple insertion patterns and insertions of 6 to 10 amino acids (Fig. 4). Comparison showed that the novel H7N8 HPAI strain has a unique cleavage site different from those of earlier H7 HPAI strains in the Americas.

Comparison of the genome of the H7N8 HPAI strain and those of the two H7N8 LPAI turkey strains identified five amino acid substitutions in five distinct proteins. Moreover, five amino acid substitutions in three different proteins were detected between the genomes of three H7N8 turkey strains and that of Kentucky/AH0012935. The profiles of three amino acid positions (E105K, F260L, and E278K [H3 numbering]; 95, 251, and 269 [H7 numbering]) in the HA1 protein were analyzed for all H7 IAVs isolated from wild and domestic birds in the influenza virus database. The amino acid K at position 278 (H3 numbering) in the HA1 protein was observed in one poultry origin isolate, A/chicken/Guanajuato/07437-15/2015(H7N3), which presents multiple basic amino acids in the HA protein cleavage region. Amino acid K was also observed in six H7N3 HPAI strains



	Cleavage region																↓				
A/CITE/Bolivia/4537/01(H7N3)	P	E	K	P	K	-	-	-	-	-	-	-	-	-	T	R	G	L	F		
A/CK/Chile/4957/02(H7N3)	P	E	K	P	K	T	C	S	P	L	S	R	C	R	K	T	R	G	L	F	
A/CK/Chile/184240-1/02(H7N3)	P	E	K	P	K	T	C	S	P	L	S	R	C	R	E	T	R	G	L	F	
A/BWTE/TX/578585/02(H7N3)	P	E	N	P	K	-	-	-	-	-	-	-	-	-	-	T	R	G	L	F	
A/CK/BC/NS-1319-2/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	Y	H	K	R	M	T	R	G	L	F	
A/CK/BC/NS-2035-12/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	C	Q	K	R	M	T	R	G	L	F	
A/CK/BC/NS-1479-1/04(H7N3)	P	E	N	P	R	-	-	-	Q	A	Y	R	K	R	M	T	R	G	L	F	
A/Canada/rv504/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	Y	Q	K	R	M	T	R	G	L	F	
A/CK/BC/CN-7/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	Y	R	K	R	M	T	R	G	L	F	
A/CK/BC/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	Y	Q	K	Q	M	T	R	G	L	F	
A/CK/BC/NS1337-1/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	Y	K	K	R	M	T	R	G	L	F	
A/CK/BC/CN12/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	H	Q	K	R	M	T	R	G	L	F	
A/GADW/AZ/A00663934/09(H7N7)	P	E	N	P	K	-	-	-	-	-	-	-	-	-	-	T	R	G	L	F	
A/CK/SK/HR-00011/07(H7N3)	P	E	N	P	K	-	-	-	-	T	T	K	P	R	P	R	R	G	L	F	
A/REDH/OH/13OS0406/13(H7N3)	P	E	N	P	K	-	-	-	-	-	-	-	-	-	-	T	R	G	L	F	
A/CK/Jalisco/12283/12(H7N3)	P	E	N	P	K	-	-	D	R	K	S	R	H	R	R	T	R	G	L	F	
A/Mexico/InDRE7218/12(H7N3)	P	E	N	P	K	-	-	D	R	K	S	R	H	R	R	T	R	G	L	F	
A/LESC/KY/AH0012935/15(H7N8)	P	E	N	P	K	-	-	-	-	-	-	-	-	-	-	T	R	G	L	F	
A/TK/IN/16-001403-1/16(H7N8)	P	E	N	P	K	-	-	-	-	-	-	-	K	R	K	T	R	G	L	F	
A/TK/IN/16-001573-2/16(H7N8)	P	E	N	P	K	-	-	-	-	-	-	-	-	-	-	T	R	G	L	F	
A/TK/IN/16-001574-7/16(H7N8)	P	E	N	P	K	-	-	-	-	-	-	-	-	-	-	T	R	G	L	F	

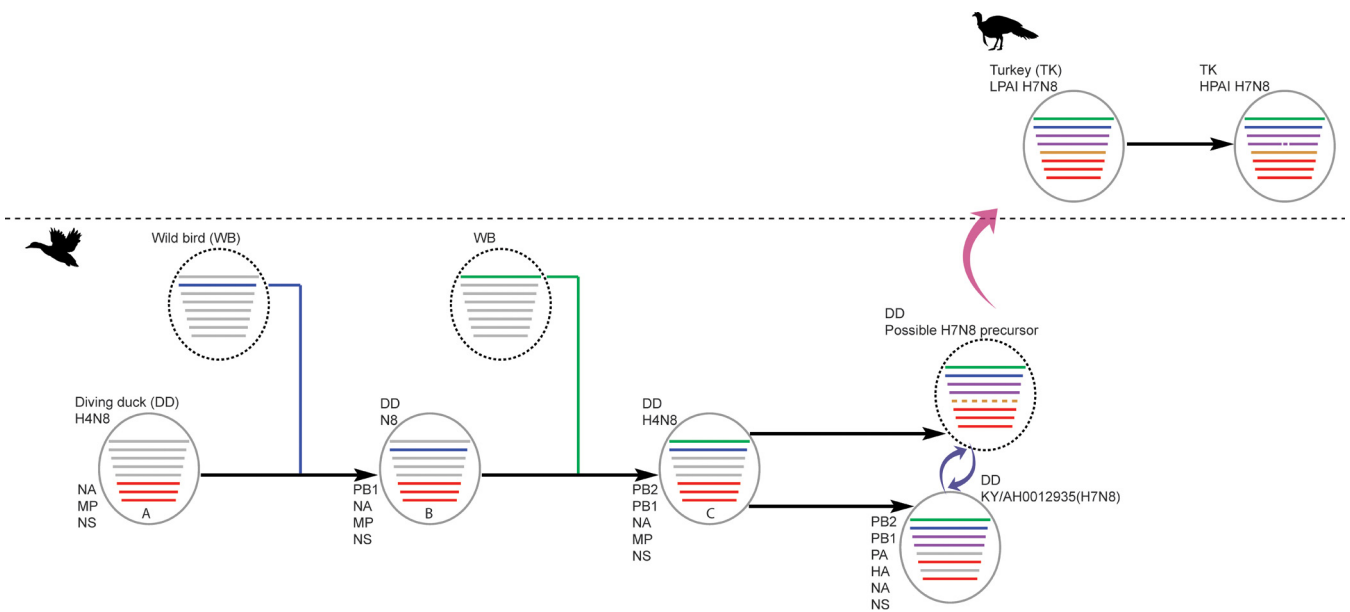
**FIG 4** Insertion patterns in the HA cleavage region of the H7 viruses detected in Indiana turkeys and those from four previous outbreaks in the Americas. The H7N8 viruses detected in Indiana turkeys are in red, and the highly pathogenic avian influenza viruses isolated from previous outbreaks are in green. Wild-bird origin influenza A viruses containing the HA gene segment that is most closely related to those of outbreak strains are in black. Inserts of basic amino acids are color coded accordingly. Host species: BWTE, blue-winged teal; CITE, cinnamon teal; CK, chicken; GADW, gadwall; LESC, lesser scaup; REDH, redhead; and TK, turkey. Geographic locations: AZ, Arizona; BC, British Columbia; IN, Indiana; KY, Kentucky; OH, Ohio; SK, Saskatchewan; and TX, Texas.

isolated during an outbreak in Pakistan in 2004. These amino acid substitutions were potentially linked to the host adaptation of H7N8 viruses from wild waterfowl to turkey and evolution from low-pathogenic to highly pathogenic forms.

To understand whether the H7N8 isolates evolved from low-pathogenic to highly pathogenic forms in Indiana turkeys, we analyzed the next-generation sequencing data for three H7N8 turkey isolates to determine the presence of viral quasispecies, especially at the positions within the HA cleavage site. The reads from each sample were aligned with the sequences of the H7N8 HPAI turkey isolate. The results showed that two H7N8 LPAI isolates do not possess low-frequency variations of polybasic amino acids at the HA cleavage site. Variations were observed in five positions across the HA (residues 302 and 374), PA (218 and 379), and NP (128) segments. The lack of low-frequency variations of polybasic amino acids within the HA cleavage site in the H7N8 LPAI isolates could be due to the adaptations in viral isolation. Further studies are needed, for example, sequencing a large number of clinical samples from the Indiana turkeys, especially those collected before the H7N8 outbreak.

**DISCUSSION**

Introduction of IAVs from wild birds to domestic poultry presents a continuous threat to livestock health. In this study, we provided evidence that the H7N8 isolates associated with the outbreak of disease in Indiana turkeys was generated through a



**FIG 5** Proposed evolutionary pathway leading to the generation of the highly pathogenic avian influenza (H7N8) virus that caused the outbreak in Indiana turkeys. Influenza A viruses are represented by ovals containing horizontal bars that represent eight gene segments (from top to bottom, PB2, PB1, PA, HA, NP, NA, MP, and NS). Solid ovals represent virus strains isolated from wild and domestic birds, and dotted ovals represent hypothetical virus strains. The dashed line in segment five (NP) indicates the unknown source of the gene segment, and the broken bar in segment four (HA) indicates an insert of basic amino acids at the cleavage site. A, B, and C indicate the three gene constellations shown in Fig. 2. Genetic events occurring in wild and domestic birds are separated by the dashed line.

series of genetic events likely occurring in wild birds and subsequently introduced into an Indiana turkey flock, where the virus evolved into high pathogenicity.

We propose one possible evolutionary model leading to the generation of H7N8 HPAI virus in Indiana turkeys on the basis of available evidence. The H7N8 precursor virus may have been generated in wild birds through two phases of sequential reassortment events (Fig. 5). The first phase of reassortment may have occurred between H4N8-DD viruses and other IAVs circulating in North American wild birds from 2011 to 2014, generating A/Bufflehead/Illinois/14OS3609/2014-like virus, possessing five H7N8-like gene segments (PB2, PB1, NA, MP, and NS), in diving ducks. In the second phase, further reassortment between A/Bufflehead/Illinois/14OS3609/2014-like virus and locally circulating IAVs in wild birds that had H7N8-like PA, HA, and NP gene segments led to the generation of H7N8 precursor virus. IAVs that are most closely related to the H7N8 turkey strains, including Kentucky/AH0012935 and H4N8-DD isolates, were mainly recovered from samples collected in Kentucky, Wisconsin, Illinois, Ohio, and Louisiana, all of which are included within the Mississippi flyway. In addition, sample collection sites for IAVs isolated from diving ducks included locations within all four major flyways of North America. Thus, we predict that the reassortment events leading to the generation of H7N8 precursor viruses likely occurred within the Mississippi flyway. Regarding the time of introduction of H7N8 virus to Indiana turkeys, we observed that the TMRCA for three H7N8 turkey strains among seven of eight gene segments were summer and fall 2015. In North America, waterfowl generally begin staging for autumn migration in July and August. Southern migration begins as early as August for blue-winged teal and in late September, October, and early November for most waterfowl. It was likely that waterfowl, including diving ducks, may have carried the H7N8 precursor virus during their southern migration and introduced the virus to Indiana turkeys through an unidentified interface between wild waterfowl and domestic poultry. After introduction to Indiana turkeys, H7N8 LPAI virus evolved into a highly pathogenic form through acquisition of three basic amino acids (KRR) in the cleavage region of the HA protein. Although we identified one IAV isolate, Kentucky/AH0012935, that possesses six gene segments most closely related to those of the H7N8 HPAI

isolate, with nucleotide sequence identity of  $\geq 99.00\%$ , the approximately 7-week and 200-kilometer gap in epidemiology could allow the existence of unknown intermediate host species in the evolutionary process. In addition, the sampling times of the most closely related strains for the NP and MP gene segments were in 2014 instead of 2015 or 2016. Thus, we emphasize that other models cannot be excluded.

Worldwide, LPAI viruses have been recovered from at least 105 wild-bird species representing 26 different taxonomic families (24). Most of the 26 families are in the order *Anseriformes* (including ducks, geese, and swans), followed by the order *Charadriiformes* (including shorebirds and gulls); both are considered major natural reservoirs for IAVs (25). Among these bird species, shorebirds and gulls were previously suggested to be the source of the precursor of H5 and H7 HPAI viruses in North America (26). A more recent study indicated that the precursors of H7N3 HPAI viruses in the Americas most likely originated from wild waterfowl, particularly dabbling ducks (19). In Eurasia, precursors of H7 HPAI viruses were generally found in dabbling ducks (20). Our findings add to the knowledge supporting the idea that introduction of IAVs from wild birds plays an important role in the emergence of IAVs in domestic poultry. This study differs from previous work, though, in providing evidence that diving ducks may also contribute to the emergence of an HPAI strain, like dabbling ducks, shorebirds and gulls, and other bird species. Diving ducks appeared to have contributed five gene segments (PB2, PB1, NA, MP, and NS) to the novel H7N8 virus and may have harbored the H7N8 LPAI precursor virus; however, the mechanism of exposure to Indiana turkeys remains unclear. The behavior of diving ducks may contribute to the long-term perpetuation of specific IAV genes or strains. Compared with dabbling ducks and many other birds, diving ducks utilize open-water habitats that include deeper water. It has been shown that IAVs may remain infectious in water for several months (27), but infectivity is adversely affected by repeated freeze-thaw cycles that would be more likely to occur in the shallower water utilized by dabbling ducks (28). In aquatic habitats highly contaminated by waterfowl feces, deeper-water habitats that undergo limited or no freeze-thaw cycles could also increase the risk for IAV coinfection, virus reassortment, and the generation of novel IAV strains, as occurred with the H7N8 precursor viruses.

Persistence of an IAV gene constellation has been previously shown to be associated with the emergence of novel IAV strains that infect mammals. The existence of triple-reassortant internal gene constellations has been well established in multiple subtypes of IAVs that infect swine, and such constellations contributed to the genesis of the pandemic influenza A(H1N1) pdm09 virus (29). This internal gene constellation was suggested to confer a selective advantage over other strains (30). In contrast, IAVs that infect wild birds generally form diverse and transient genotypes through reassortment of functionally equivalent gene segments (31). Specific studies devoted to H7 IAVs in wild birds inhabiting North America and Eurasia found similar patterns (32, 33). In addition, extensive gene flow between IAVs in North American wild birds could further facilitate genetic diversity, although the migratory flyway and geographic distance may impose short-term restrictions on gene flow (34, 35). We observed that as many as five H7N8-like gene segments (PB2, PB1, NA, MP, and NS) formed a relatively persistent gene constellation in H4N8 IAVs isolated from diving ducks. This finding suggests that reassortment between IAVs in wild birds may not be random: some gene segments may be more likely to form specific linkages within the context of diverse and transient genotypes. Alternatively, reassortment may be restricted in some groups of birds, like diving ducks, if prevalence and the probability of infection with two or more viruses are low. The significance, in the context of transient genotypes of IAVs in wild birds, of the relatively persistent gene constellation observed in this study is not known.

Surveillance for IAVs in wild birds in North America likely has considerable bias toward bird species that are present in large numbers in nature, are most frequently harvested, and are easily caught (24). Diving ducks are underrepresented in both historic and current IAV surveillance. We investigated the host origins of genomic sequences deposited in the Influenza Research Database (36) and found that diving

duck origin strains comprise around 1.50% of the total number of IAV strains isolated from wild and domestic birds in the Americas. Among the 441 IAV strains that we sequenced in this study, only 6 originated from diving ducks (Fig. 1). In addition, data from a large-scale surveillance program in wild birds throughout the United States showed that, among the 197,885 samples collected from over 200 wild-bird species (2007 to 2011), diving ducks accounted for only 7.72% of all samples collected and 3.58% of the IAV-positive samples determined by M gene-based quantitative RT-PCR; dabbling ducks accounted for 62.73% of the samples collected and 86.44% of the IAV-positive samples (37). Although data from the same study showed that the IAV-positive rate for diving ducks was 5.30% compared to 15.80% for dabbling ducks, our study highlights the contribution of diving ducks to the emergence of the novel H7N8 virus and suggests that diving ducks may contribute to the maintenance, diversification, and transmission of IAVs in wild birds. Thus, we recommend additional sampling of diving ducks in future surveillance efforts. Moreover, the genomes of 441 wild-bird origin IAVs were sequenced in a collaborative effort in this study. This large number of IAV isolates and sequences enabled us to conduct detailed evolutionary analyses and revealed a few key aspects regarding the evolutionary pathway leading to the novel H7N8 virus. The experience from this study demonstrates the need for a coordinated, systematic, and collaborative approach to active surveillance in wild birds.

In summary, our investigation provides information on the putative viral ancestors, possible evolutionary pathways, and probable host species involved in the emergence of a novel H7N8 virus that caused outbreaks among turkey flocks in Indiana during 2016. Our findings indicate that diving ducks contributed to the emergence of the novel H7N8 virus and may contribute to the maintenance, diversification, and transmission of IAVs in wild birds. The repeated introduction of IAVs into domestic poultry from wild birds highlights important gaps in existing biosecurity systems and provides evidence that surveillance in wild birds can be useful for understanding possible evolutionary pathways of emergence.

## MATERIALS AND METHODS

**Viruses and sequences.** To determine the genetic ancestry of the H7N8 IAV strains associated with the outbreak of disease in Indiana turkeys, we sequenced a collection of 441 archived wild-bird origin IAVs obtained throughout North America from influenza surveillance from 2007 to 2016 (Fig. 1; see Table S1 in the supplemental material). The wild-bird origin isolates represent strains recovered from Alberta, Canada, and from 38 states within the United States of America. IAVs from migratory birds from the Atlantic, Central, Mississippi, and Pacific flyways were included in our data set. Complete genomes of H7N8 IAVs detected in Indiana turkeys were downloaded from GenBank (accession no. [KU558903](#) to [KU558910](#) and [KU585905](#) to [KU585920](#)). One of the strains was an HPAI virus, A/turkey/Indiana/16-001403-1/2016(H7N8) (Indiana/16-001403-1), and the other two strains were LPAI viruses, A/turkey/Indiana/16-001573-2/2016(H7N8) (Indiana/16-001573-2) and A/turkey/Indiana/16-001574-7/2016(H7N8) (16-001574-7).

**Phylogenetic analyses.** Preliminary phylogenetic analyses were performed with the complete genomes of three H7N8 strains originating from turkeys in Indiana and a genomic sequence data set (32) that represents the two major geographically dependent genetic lineages, North American and Eurasian, for eight gene segments. The next round of phylogenetic analyses was conducted with an integrated data set that comprised the complete genomes of three H7N8 turkey strains, sequence data for 441 IAVs generated in this study, and sequence data downloaded in May 2016 from the Influenza Research Database (36) for IAVs isolated from wild and domestic birds in the Americas. In order to perform more detailed analysis on the timing of the emergence of the H7N8 viruses, sequences closely related to the H7N8 turkey strains (referred to here as the H7N8 outbreak lineage) were selected for each gene segment from the phylogenetic trees.

Gene segment-specific phylogenetic trees were generated using the maximum-likelihood method implemented in RAxML v8.1.17 (38). A general time-reversible model of nucleotide substitution and a gamma-distributed rate variation among sites were applied throughout the analyses. Sequence alignments were performed by using MUSCLE v3.8 (39). We manually examined alignments to ensure accuracy and retained only the coding region for phylogenetic analyses.

**Molecular clock.** We estimated the time-scaled phylogenies, nucleotide substitution rates, and TMRCA using the Bayesian Markov chain Monte Carlo method implemented in BEAST v1.8.0 (40). Genomic sequences with complete sampling dates (exact month, day, and year) were selected for each gene segment from the H7N8 outbreak lineage on the basis of phylogenetic trees. Before the analysis, the temporal signal of each data set was investigated using a root-to-tip regression of genetic distances against sampling time in the program TempEst v1.5 (41), and the clock-like behavior was measured by the  $R^2$  values. We used the SRD06 partitioned substitution model, strict and uncorrelated lognormal

relaxed clock models, and Bayesian skyline coalescent tree prior in the analyses. Estimates from two molecular clock models were compared by analyzing the values of the coefficient of variation in the uncorrelated lognormal relaxed clock model. Rate estimates from the strict clock model were reported if the lower 95% limit of the coefficient of variation was close to zero, which indicated clock-like evolutionary behavior. For each gene segment, we performed two independent runs with a chain length of 100 to 300 million steps sampled every 10,000 steps (which results in >10,000 samples per run). The results were analyzed in Tracer v1.6 (<http://tree.bio.ed.ac.uk/software/tracer/>). Adequate burn-in was determined from the trace of each run, and 2% to 10% of the initial steps, representing poor configuration, were removed from further analysis. The convergence of each run and consistency between two runs were assessed, and the results from two runs were combined for analyses to ensure an adequate effective sample size (>200) was reached for relevant parameters. The maximum clade credibility trees were summarized with TreeAnnotator v1.8.0 (<http://beast.bio.ed.ac.uk/TreeAnnotator/>) and edited in FigTree v1.4.0 (<http://tree.bio.ed.ac.uk/software/figtree/>).

**Comparative phylogenetic analyses on discrete traits.** The hosts of IAVs in our study were categorized into 11 different groups: dabbling duck, diving duck (including sea duck), goose and swan, gull and tern, passerine, poultry, raptor, seabird, shorebird, other avian, and other nonavian hosts. In an attempt to keep the number of sequences per host group as balanced as possible, we conducted subsampling for sequences within the H7N8 outbreak lineage for each gene segment. For each sampling year, 20 sequences were selected for each host group; if a host group had <20 sequences, all the sequences were retained. For the analysis, we used an asymmetric substitution model with the Bayesian stochastic search variable selection and a strict clock model. Two independent runs with chain lengths of 150 million steps with sampling every 10,000 steps (which results in 15,000 samples per run) were performed for each gene segment. Similar approaches for analyzing the resulting log files of molecular clock data were applied to analyze the burn-in and convergence of each run and to generate maximum clade credibility phylogenetic trees. Specifically, >10% of the initial steps representing poor configuration were removed as burn-in. The mean transition rate and the corresponding indicator were calculated from the resulting log files. Bayes factor (BF) support was calculated with the indicator to assess statistical support. Significant transition between host groups was determined based on the combination of a BF of >3 and a mean indicator of >0.5. Therefore, support levels were defined as support ( $3 \leq \text{BF} < 10$ ), strong support ( $10 \leq \text{BF} < 100$ ), very strong support ( $100 \leq \text{BF} < 1,000$ ), and decisive support ( $\text{BF} \geq 1,000$ ).

**Genotyping.** Genotypes were defined on the basis of the gene segment-specific phylogenetic trees for H7N8 turkey strains and 135 H4N8 IAV strains. A monophyletic clade was identified by two criteria: (i) it was supported by a bootstrap value above 70 and (ii) all sequences in the clade had nucleotide sequence identities of >98%, as determined by using the hierarchical clustering method implemented in R (<https://www.r-project.org/>). We implemented a stringent cutoff of 98% to identify IAV strains closely related to the H7N8 turkey strains. The genotype of a genome is the combination of the cluster assignment of eight gene segments.

**Ethics statement.** Viral samples were collected within the United States with U.S. Fish and Wildlife Service permits MB66162B-0, MB-124922-0, and MB779238-3; Louisiana Department of Natural Resources permit LNHP 15-086; and Texas Parks and Wildlife permit SPR-0807-1407. Banding of ducks in Alberta, Canada, was conducted in accordance with the Environment Canada scientific permit 10810 to capture and band migratory birds. Samples collected from hunter-harvested birds and the environment did not require permits.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.02208-16>.

**SUPPLEMENTAL FILE 1**, XLSX file, 0.1 MB.

**SUPPLEMENTAL FILE 2**, PDF file, 2.2 MB.

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