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Evidence for common ancestry among viruses isolated from wild birds in Beringia and highly pathogenic intercontinental reassortant H5N1 and H5N2 influenza A viruses

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ABSTRACT

Highly pathogenic clade 2.3.4.4 H5N8, H5N2, and H5N1 influenza A viruses were first detected in wild, captive, and domestic birds in North America in November–December 2014. In this study, we used wild waterbird samples collected in Alaska prior to the initial detection of clade 2.3.4.4 H5 influenza A viruses in North America to assess the evidence for: (1) dispersal of highly pathogenic influenza A viruses from East Asia to North America by migratory birds via Alaska and (2) ancestral origins of clade 2.3.4.4 H5 reassortant viruses in Beringia. Although we did not detect highly pathogenic influenza A viruses in our sample collection from western Alaska, we did identify viruses that contained gene segments sharing recent common ancestry with intercontinental reassortant H5N2 and H5N1 viruses. Results of phylogenetic analyses and estimates for times of most recent common ancestry support migratory birds sampled in Beringia as maintaining viral diversity closely related to novel highly pathogenic influenza A virus genotypes detected in North America. Although our results do not elucidate the route by which highly pathogenic influenza A viruses were introduced into North America, genetic evidence is consistent with the hypothesized trans-Beringian route of introduction via migratory birds.

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1. Introduction

Highly pathogenic (HP) H5N1 subtype influenza A viruses (IAVs) of the A/goose/Guandong/1/1996 (Gs/Gd) lineage have circulated among domestic poultry in eastern Asia since 1996, resulting in outbreaks of disease in wild birds, domestic poultry, and humans (Li et al., 2004; Chen et al., 2005) and ultimately leading to the emergence of numerous HP reassortants including H5N2, H5N5, H5N6, and H5N8 (Zhao et al., 2012, 2013; Qi et al., 2014). In 2014, Gs/Gd lineage H5N8 IAVs were detected in South Korea and associated with widespread outbreaks of clinical disease in domestic poultry and wild birds (Jeong et al., 2014; Lee et al., 2014). Despite extensive efforts to control and eradicate HP H5N8 IAVs in South Korea, these viruses were purportedly maintained in domestic and wild waterfowl in the region (Hill et al., 2015), dispersed through migration to breeding grounds in northern Russia, and eventually detected in wild, captive, and domestic birds in Europe and North America beginning in November–December 2014 (Ip et al., 2015; Lee et al., 2015; Verhagen et al., 2015). Although evidence for the intercontinental spread of HP H5N8 IAVs by wild birds is circumstantial, previous research conducted in western Alaska provides evidence for dispersal of viruses by migratory birds between East Asia and North America via a trans-Beringian pathway (Ramey et al., 2015).

Concurrent with the initial detection of Gs/Gd lineage H5N8 IAVs in North America, a reassortant HP H5N2 subtype virus was associated with an outbreak of disease in domestic poultry in British Columbia, Canada (Pasick et al., 2015) and, shortly thereafter, a highly similar virus was identified from a sample collected from a wild northern pintail (*Anas acuta*) in Washington, USA (Ip et al., 2015). These viruses were characterized as having five gene segments (PB2, PA, HA, M, and NS) closely related to Gs/Gd lineage H5N8 IAVs, including an H5 subtype hemagglutinin gene segment designated as belonging to clade 2.3.4.4, as well as three North American lineage gene segments (PB1, NP, and NA). Viruses of the H5N2 subtype were subsequently associated with numerous outbreaks of disease in domestic poultry in the Midwestern USA (OIE, 2015). Additionally, HP clade 2.3.4.4 (henceforth HP) reassortant IAVs of the H5N1 subtype were detected from two samples collected from wild ducks in Washington, USA during December 2014–January 2015 (OIE, 2015; Torchetti et al., 2015). Similar to the intercontinental reassortant HP H5N2 viruses, these H5N1 viruses were comprised of gene segments closely related to Gs/Gd lineage H5N8 IAVs (PB2, HA, NP, and MA) and others of North American wild bird lineages (PB1, PA, NA, and NS).

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In the autumn of 2014, prior to the detection of clade HP H5 IAVs in North America, we collected samples from wild waterbirds in western Alaska as part of ongoing research to understand the intercontinental dispersal of viruses. In this study, our objectives were to analyze these samples to assess the evidence for: (1) the dispersal of HP H5 IAVs from East Asia to North America by migratory birds via Alaska and (2) possible ancestral origins of HP reassortant viruses in Beringia. Specifically, we screened samples from western Alaska collected temporally proximate to the first detection of HP H5 viruses in North America for HP IAVs, viruses with highly similar gene segments to HP H5N8 IAVs associated with outbreaks in East Asia, and possible progenitor strains to intercontinental reassortant HP H5N2 and H5N1 viruses detected in North America.

2. Materials and methods

During September and October of 2014, cloacal and environmental samples were collected from wild birds at Izembek National Wildlife Refuge (NWR) located on the Alaska Peninsula (Fig. 1). Cloacal samples were collected from hunter-harvested birds and environmental samples were obtained by swabbing freshly deposited feces from locations where monospecific flocks were congregated. Swab samples were submersed in viral transport media and frozen in dry shippers charged with liquid nitrogen prior to laboratory analysis. A total of 993 samples were collected from 19 species (Table 1). Samples were screened for the influenza A virus MA gene using real-time RT-PCR (Spackman et al., 2002) and those yielding cycle threshold values ≤45 were inoculated into embryonated chicken eggs (n = 99) for virus isolation (Woolcock, 2008) resulting in the recovery of 41 isolates (Table 1).

Viral RNA for the 41 isolates was extracted using MagMax AI/NDV RNA extraction kit (Ambion Inc.). Virus genomes were amplified in a multiplex RT-PCR using Super ScriptTM III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen), and primers and protocols described in Zhou et al. (2009). Excess dNTPs and primers were removed using ExoSAP-IT ® (USB Corporation). PCR products were quantified using a Quant-iT dsDNA HS Assay Kit (Invitrogen) and prepared for sequencing using a Nextera XT DNA library preparation kit (Illumina, Inc.). Indexed libraries were pooled and sequenced on the Illumina MiSeq using a 500 cycle reagent kit with paired-end reads. Sequence reads were assembled with Bowtie 2 version 2.2.3 (Langmead and Salzberg, 2012) using reference data for IAVs obtained from GenBank (Bao et al., 2008) to map reads. Consensus sequences were generated with SAMtools version 1.1 (Li et al., 2009). An average of 627,843 reads were aligned per isolate (range 133,986–1,096,362). For sequences with gaps and poor coverage resulting in low alignment quality scores, Sanger sequencing data was generated using specific primers to complete consensus sequences. Consensus sequences were verified using FLuANotation (FLAN). GenBank accession numbers for viruses isolated as part of this study are: KT338310–KT338613.

Nucleotide sequences for the complete coding regions of isolates originating from wild birds sampled at Izembek NWR were compared to those reported for the initial isolates of HP H5N8, H5N2, and H5N1 recovered from wild birds (or a captive bird that had recently fed on wild birds in the case of H5N8) in North America as available on the GenBank public database (Ip et al., 2015; Torchetti et al., 2015). The phylogenetic relationships of gene segment sequences of isolates from Izembek NWR that shared ≥99% nucleotide identity with reference HP H5N8, H5N2, and H5N1 isolates from North America were further explored using BEAST version 1.8.2 (Drummond et al., 2012). Phylogenies were reconstructed using sequence data for gene segments from Izembek NWR isolates and HP H5 IAV isolates from North America identified as sharing 99% nucleotide identity, as well as the top 100 BLAST (Zhang et al., 2000) hits per gene segment for each HP H5 reference isolate (BLAST searches performed on 15 July 2015; incomplete sequences for the full coding region of each gene were excluded from analyses; n = 99–115 sequences per analysis;). Nucleotide sequences were aligned using Sequencher version 5.1 (Gene Codes Corp.) and trimmed to the complete open reading frame per gene segment. Maximum clade credibility (MCC) Bayesian phylogenetic trees were estimated for each gene segment with an uncorrelated lognormal relaxed molecular clock (Drummond et al., 2006) that allows for rate variation across lineages. The SRD06 codon model with two partitions (codon position 1 + 2 and codon position 3), incorporating a HKY85 model for each partition, was used with a Bayesian skyline coalescent tree prior (10 groups). A

Table 1
Number of wild bird samples collected at Izembek National Wildlife Refuge, Alaska during September–October 2014 by species and results of molecular screening (MA+) and virus isolation (VI+)[†].

Species	n =	MA+	VI+
American green-winged teal (*Anas crecca*)	69	17	8
American wigeon (*Anas americana*)	11	1	0
Black brant (*Branta bernicla*)	21	0	0
Black scoter (*Melanitta americana*)	1	0	0
Bufflehead (*Bucephala albeola*)	10	2	2
Canvasback (*Aythya valisineria*)	1	0	0
Common eider (*Somateria mollissima*)	1	0	0
Common goldeneye (*Bucephala clangula*)	2	0	0
Emperor goose (*Chen canagica*)[*]	294	14	3
Eurasian wigeon (*Anas penelope*)	1	0	0
Greater scaup (*Aythya marila*)	21	0	0
Glaucous-winged gull (*Larus glaucescens*)[*]	348	6	3
Gyrfalcon (*Falco rusticolus*)	1	0	0
Harlequin duck (*Histrionicus histrionicus*)	2	0	0
Mallard (*Anas platyrhynchos*)	49	18	8
Northern pintail (*Anser acuta*)	147	40	17
Northern shoveler (*Anas clypeata*)	5	1	0
Red-breasted merganser (*Mergus serrator*)	6	0	0
White-winged scoter (*Melanitta deglandi*)	3	0	0
Totals	993	99	41

[*] Unpooled fecal samples; all other samples were cloacal swabs.
[†] MA+, cycle threshold value ≤45 using real-time reverse transcriptase PCR specific for influenza A virus matrix gene; VI+, virus isolated in specific pathogen free eggs.

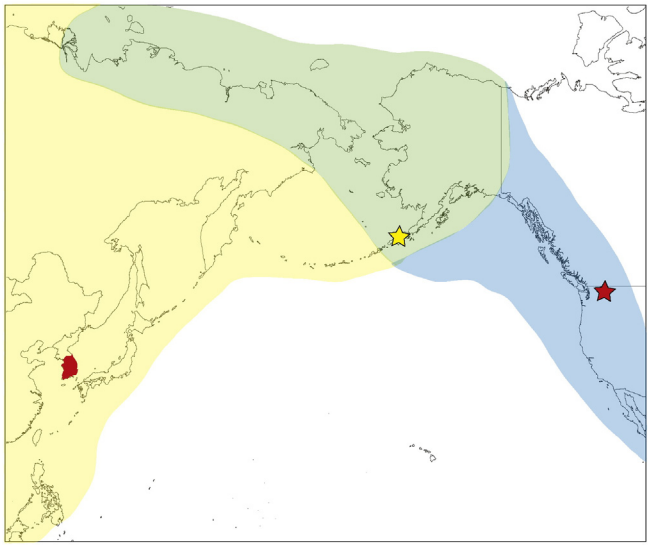

Fig. 1. Map depicting the location of Izembek National Wildlife Refuge (yellow star) within overlapping (green polygon) northern regions of the East Asian-Australasian (yellow polygon) and Pacific Americas (blue polygon) flyways. South Korea, which experienced repeated outbreaks of clade 2.3.4.4 H5N8 influenza A in wild and domestic birds in early 2014, prior to the breeding season of migratory birds, is indicated in red. The approximate location of Whatcom County, Washington (USA) at which clade 2.3.4.4 H5N1, H5N2, and H5N8 influenza A viruses were first detected in wild birds in North America in December 2014 (or a captive bird that had recently fed on wild birds in the case of H5N8), subsequent to the breeding season for migratory birds, is depicted with a red star.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

minimum of three independent chains of 50 million generations were combined after removal of burn-in (~10–25% of the samples) to achieve an Effective Sample Size of ≥200 as diagnosed in Tracer v1.6.

3. Results

We did not detect HP H5 IAVs through genomic characterization of 41 isolates from samples collected at Izembek NWR in autumn of 2014, although we did isolate two low pathogenic H5 subtype viruses (A/American green-winged teal/Alaska/472/2014 (H5N2) and A/mallard/Alaska/103/2014 (H5N3)) as inferred through deduced amino acid motifs of the HA gene at the fusion cleavage site. Additionally, we did not identify any isolates that shared ≥99% nucleotide identity with any gene segment of A/gyrfalcon/WA/41009-6/2014(H5N8), the initial HP H5N8 IAV isolate recovered in North America (Supplemental Tables S1–S8). We did, however, identify isolates recovered from wild birds in western Alaska that shared ≥99% identity with all three North American lineage gene segments (PB1, NP, and NA) of A/northern pintail/WA/40,964/2014 (H5N2), the initial HP H5N2 IAV reassortant isolate recovered from a wild bird in North America (Table 2). Additionally, we identified isolates recovered from our sampling efforts that shared ≥99% identity with gene segments (PA and NS) of A/American green-winged teal/WA/19570/2014 (H5N1), the only HP H5N1 IAV isolate recovered to date in North America for which nucleotide sequence information was publically available at the time of manuscript preparation (Table 2).

MCC phylogenetic analyses provided support for the formation of clades comprised of sequences for the PB1, NP, and NA genes of isolates derived from wild birds at Izembek NWR and wild and domestic birds infected with HP H5N2 IAVs (Figs. 2–4). For the PB1 gene, 15 isolates from Izembek NWR formed a clade with 24 HP H5N2 IAV isolates from North America (Fig. 2). The mean time of most recent common ancestry (TMRCA) between clades comprised of 24 HP H5N2 IAV isolates from North America and the five most closely related isolates derived from wild birds in western Alaska was estimated to be late 2013 (mean TMRCA; 95% Bayesian credible interval [BCI]: 2013.8; 2013.3–2014.3) for the PB1 gene segment (Fig. 2). For the NP and NA genes, nucleotide sequence data for the low pathogenic isolate A/American green-winged teal/Alaska/472/2014 (H5N2), derived from a sample collected at Izembek NWR on 10 October 2014, formed a clade with sequence data for HP H5N2 IAV isolates derived from wild and domestic birds in Canada and the USA. The TMRCA was estimated to be early 2014 in both cases (2014.3; 2013.8–2014.6 and 2014.0; 2013.4–2014.6, respectively; Figs. 3, 4).

Relatively close genetic relationships were also identified between nucleotide sequences for PA and NS gene segments of isolates derived from samples collected at Izembek NWR and the HP H5N1 IAV detected in Washington, USA through MCC analyses (Figs. 5, 6). Sequence data for the PA gene of Izembek NWR isolate A/mallard/Alaska/138/2014(H1N1) and A/American green-winged teal/Washington/195750/2014 (H5N1) were nested within the same clade and had an estimated TMRCA of early 2011 (2011.1; 2009.6–2013.0; Fig. 5). Similarly, sequence data for the NS gene of Izembek NWR low pathogenic isolate A/American green-winged teal/Alaska/472/2014 (H5N2) formed a clade with HP H5 isolate A/American green-winged teal/Washington/195750/2014(H5N1) and the TMRCA was estimated to be late 2013 (2013.8; 2012.7–2014.7; Fig. 6).

Table 2
Nucleotide identity among gene segments of influenza A virus isolates derived from migratory birds at Izembek National Wildlife Refuge, Alaska and highly pathogenic clade 2.3.4.4 H5 influenza A virus isolates detected in North America, 2014. Only information for isolates from Alaska sharing ≥99% identity with one or more highly pathogenic clade 2.3.4.4 H5 influenza A virus is presented here. For complete nucleotide identity comparisons refer to Supplemental Tables S1–S8. Values >99% are shaded gray.

	H5N2[†]			H5N1[*]	
	PB1	NP	NA	PA	NS
A/emperor goose/Alaska/50/2014 (H11N2)	99.2%	97.4%	97.6%	91.1%	96.4%
A/mallard/Alaska/103/2014 (H5N3)	99.3%	93.9%	< 85.0%[§]	90.9%	96.0%
A/mallard/Alaska/138/2014 (H1N1)	94.3%	94.1%	< 85.0%[§]	99.2%	96.3%
A/American green-winged teal/Alaska/306/2014 (H3N3)	99.1%	97.3%	< 85.0%[§]	90.9%	96.1%
A/mallard/Alaska/417/2014 (H4N1)	99.0%	93.7%	< 85.0%[§]	90.8%	95.8%
A/mallard/Alaska/441/2014 (mixed)	99.1%	NC[¥]	< 85.0%[§]	NC[¥]	96.0%
A/emperor goose/Alaska/462/2014 (mixed)	99.2%	97.4%	97.4%	NC[¥]	96.2%
A/mallard/Alaska/468/2014 (H4N6)	99.2%	93.7%	< 85.0%[§]	90.9%	96.0%
A/American green-winged teal/Alaska/472/2014 (H5N2)	96.9%	99.8%	99.6%	88.7%	99.8%
A/American green-winged teal/Alaska/478/2014 (H3N1)	99.1%	93.8%	< 85.0%[§]	90.6%	96.0%
A/northern pintail/Alaska/576/2014 (H1N1)	99.3%	94.0%	< 85.0%[§]	91.0%	96.3%
A/glaucous-winged gull/Alaska/670/2014 (H11N2)	99.2%	97.3%	97.8%	91.0%	96.2%
A/emperor goose/Alaska/676/2014 (H4N6)	99.1%	93.7%	< 85.0%[§]	90.8%	96.0%
A/American green-winged teal/Alaska/816/2014 (H4N6)	99.1%	93.7%	< 85.0%[§]	90.8%	96.0%
A/northern pintail/Alaska/819/2014 (H4N6)	99.0%	93.7%	< 85.0%[§]	90.6%	95.8%
A/northern pintail/Alaska/861/2014 (H3N8)	99.1%	93.7%	< 85.0%[§]	90.9%	95.7%
A/mallard/Alaska/898/2014 (H4N6)	99.1%	93.7%	< 85.0%[§]	90.8%	< 85.0%[§]

4. Discussion

The lack of detection of HP H5 IAVs at Izembek NWR in autumn of 2014 does not provide any direct evidence that such viruses were present in western Alaska during September–October; although, the geographic coverage of sampling was limited and not all taxa potentially involved in IAV ecology in this region were adequately sampled so as to provide rigorous statistical support for absence of HP IAVs. The detection of viruses at Izembek NWR comprised of gene segments sharing high nucleotide similarity to intercontinental reassortant HP H5N2 and H5N1 viruses is, however, consistent with the hypothesis of dispersal of HP H5N8 IAVs to Beringia by migratory birds and subsequent reassortment. Furthermore, inferred phylogenetic relationships and estimates of the TMRCA provide support for common ancestry between viruses detected in western Alaska during autumn 2014 and intercontinental reassortant HP H5 IAVs detected at lower latitudes of North America in late autumn/winter of 2014–2015.

Although HP H5 IAVs were not identified at Izembek NWR during autumn of 2014, it is possible that the extent or timing of sample collection precluded detection despite our surveillance strategy targeting: (1) species well-established as contributing to the natural reservoir of IAVs, (2) taxa in which Gs/Gd lineage IAVs have previously been detected, and (3) locally abundant species with intercontinental migratory tendencies, such as northern pintail ducks and emperor geese (*Chen canagica*; Miller et al., 2005; Hupp et al., 2007, 2011). The probability of detection for HP H5 IAVs in wild birds is unknown and sampling of locally uncommon species with intercontinental migratory tendencies was limited at Izembek NWR during 2014 (e.g., Eurasian wigeon [*Anas penelope*]; Table 1). Thus, the possibility that HP H5 viruses were present yet undetected at Izembek NWR during September–October 2014 cannot be excluded.

Another plausible scenario is that HP H5 viruses were dispersed to North America via Alaska after the September–October sampling period at Izembek NWR. HP H5 IAVs were not initially detected in North America until late November 2014 (Pasick et al., 2015). During approximately 7–9 November 2014, western Alaska was affected by the strongest storm on record for the Bering Sea (as measured by barometric pressure) as Typhoon Nuri dissipated and the associated weather system moved across the Pacific Ocean from East Asia to North America. It is well established that meteorological factors such as wind speed and direction, precipitation, and changes in temperature may affect bird migration (Richardson, 1978). Thus, it is plausible that HP H5 viruses were dispersed from East Asia to North America via wild bird movements associated with this storm event. Anecdotally, waterfowl hunters at Izembek NWR observed unusually large numbers of Eurasian teal (*Anas crecca crecca*) coincident with this storm (J. Wasley pers. comm.).

A third possibility is that HP H5 IAVs were introduced into western Alaska at a location other than Izembek NWR, such as the Yukon-Kuskokwim Delta, where evidence for interhemispheric dispersal of

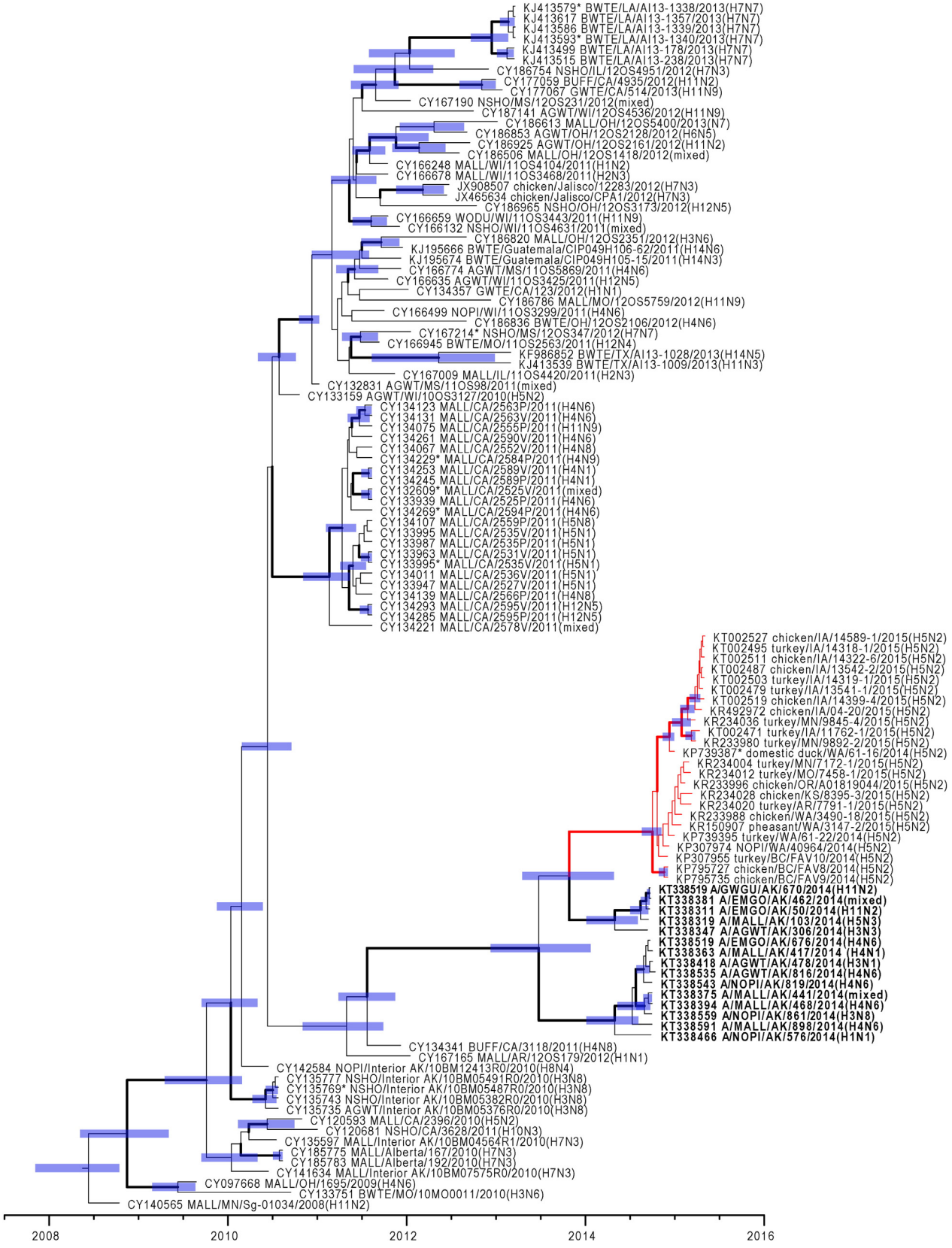

Fig. 2. Maximum clade credibility phylogeny for influenza A virus gene segments depicting inferred genetic relationship among nucleotide sequences for genetically similar influenza A virus PB1 gene segments (see Materials and methods). Nucleotide sequences for PB1 gene segments of highly pathogenic clade 2.3.4.4 H5N2 subtype influenza A viruses (red branch tips) and strains derived from samples collected at Izembek National Wildlife Refuge (bold) are indicated. Nucleotide sequences for viruses that share 100% identity with other strains reported on the GenBank public database are identified with an asterisk. Complete strain names (year/month/day) appear on tip labels with American Ornithologists' Union four letter codes used for wild bird taxa and standard two letter abbreviations for United States. Bayesian credible intervals for estimates of common ancestry are depicted with horizontal purple bars for clades with Bayesian posterior probability values >0.5. Clades with Bayesian posterior probability values >0.95 are indicated with thickened branches. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

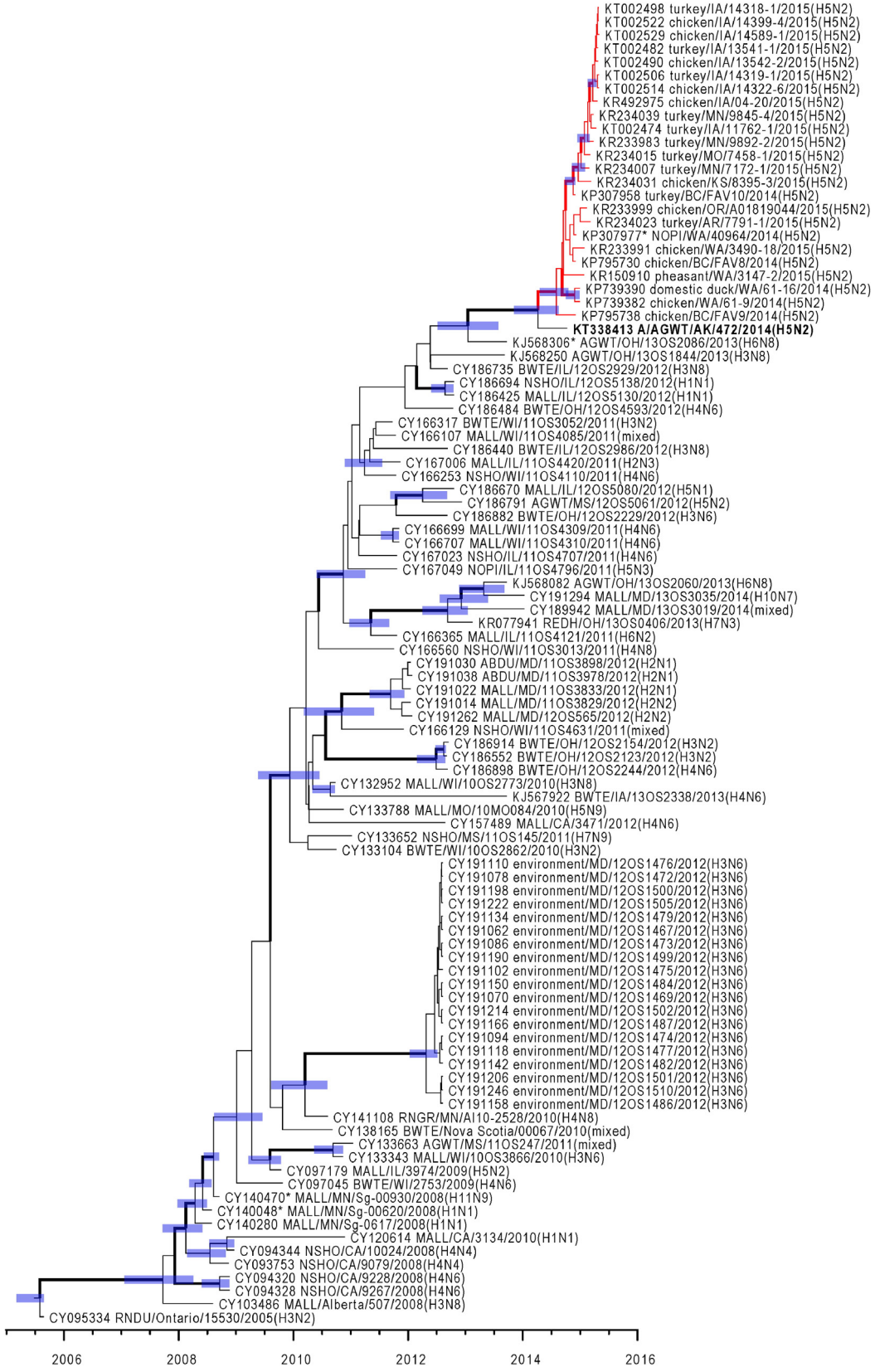

Fig. 3. Maximum clade credibility phylogeny for influenza A virus gene segments depicting inferred genetic relationship among nucleotide sequences for genetically similar influenza A virus NP gene segments (see Materials and methods). Nucleotide sequences for NP gene segments of highly pathogenic clade 2.3.4.4 H5N2 subtype influenza A viruses (red branch tips) and strains derived from samples collected at Izembek National Wildlife Refuge (bold) are indicated. Nucleotide sequences for viruses that share 100% identity with other strains reported on the GenBank public database are identified with an asterisk. Complete strain names (year/month/day) appear on tip labels with American Ornithologists' Union four letter codes used for wild bird taxa and standard two letter abbreviations for United States. Bayesian credible intervals for estimates of common ancestry are depicted with horizontal purple bars for clades with Bayesian posterior probability values >0.5. Clades with Bayesian posterior probability values >0.95 are indicated with thickened branches. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

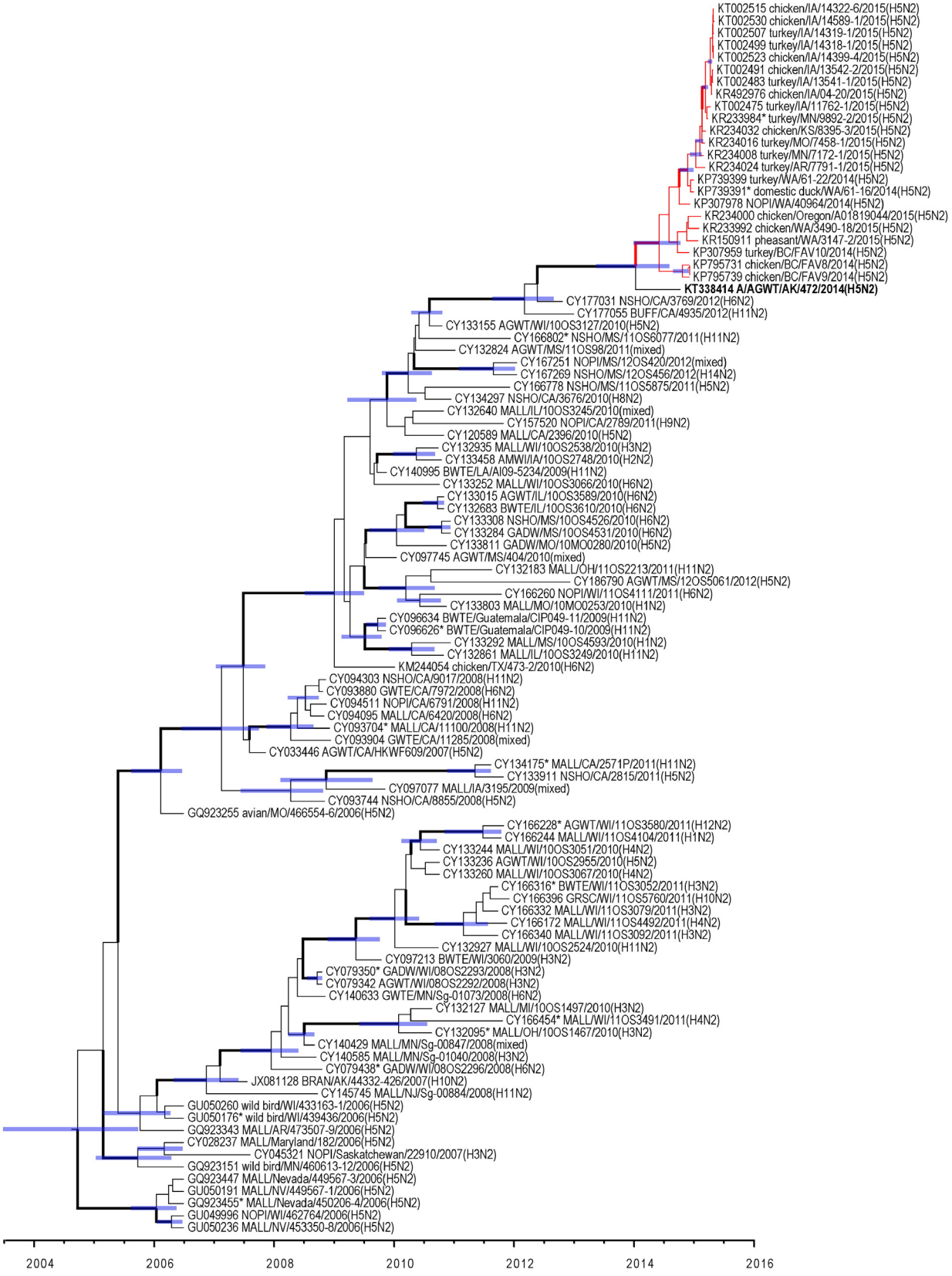

Fig. 4. Maximum clade credibility phylogeny for influenza A virus gene segments depicting inferred genetic relationship among nucleotide sequences for genetically similar influenza A virus NA gene segments (see Materials and methods). Nucleotide sequences for NA gene segments of highly pathogenic clade 2.3.4.4 H5N2 subtype influenza A viruses (red branch tips) and strains derived from samples collected at Izembek National Wildlife Refuge (bold) are indicated. Nucleotide sequences for viruses that share 100% identity with other strains reported on the GenBank public database are identified with an asterisk. Complete strain names (year/month/day) appear on tip labels with American Ornithologists' Union four letter codes used for wild bird taxa and standard two letter abbreviations for United States. Bayesian credible intervals for estimates of common ancestry are depicted with horizontal purple bars for clades with Bayesian posterior probability values >0.5. Clades with Bayesian posterior probability values >0.95 are indicated with thickened branches. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

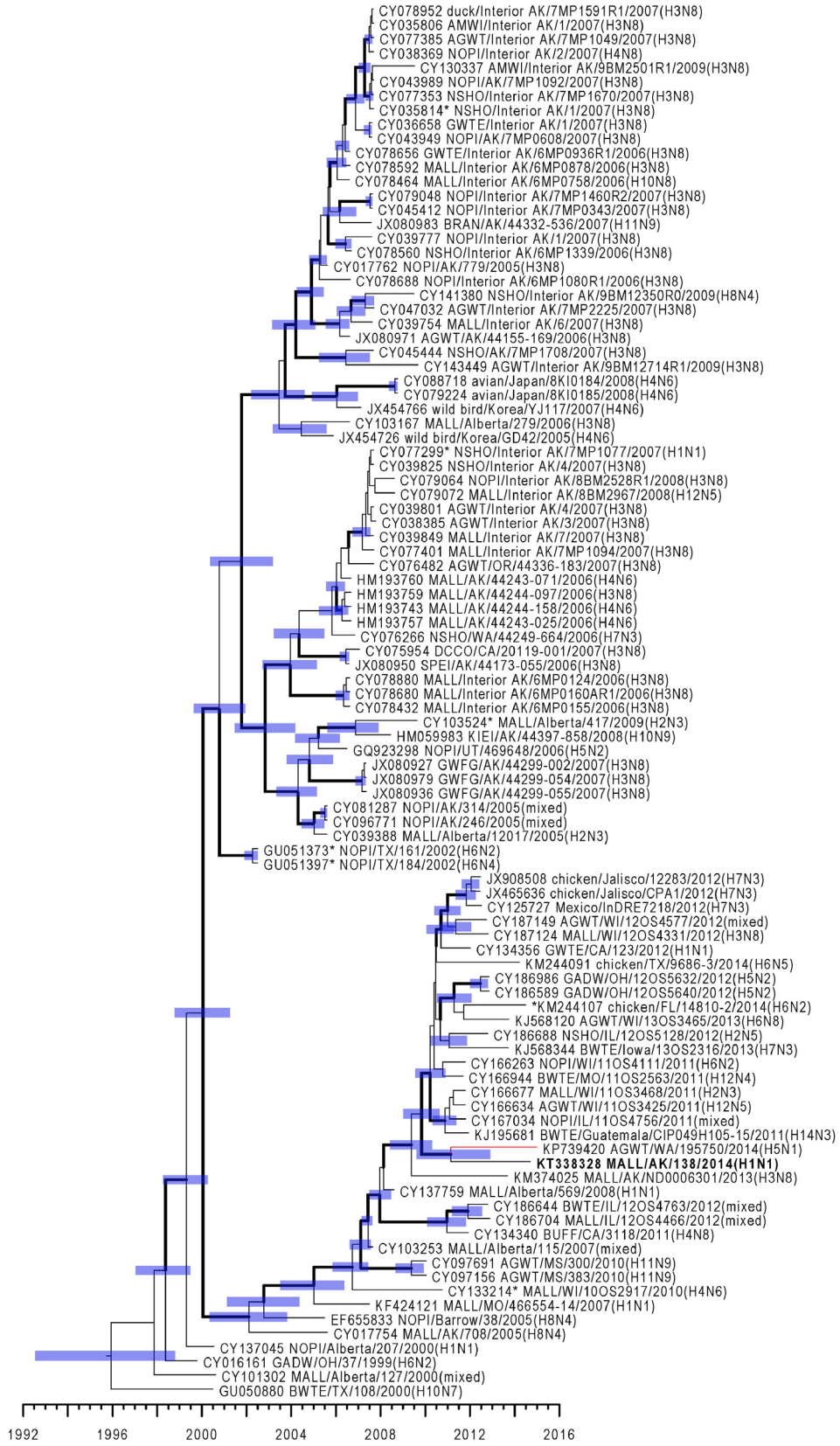

Fig. 5. Maximum clade credibility phylogeny for influenza A virus gene segments depicting inferred genetic relationship among nucleotide sequences for genetically similar influenza A virus PA gene segments (see Materials and methods). Nucleotide sequences for PA gene segments of highly pathogenic clade 2.3.4.4 H5N1 subtype influenza A viruses (red branch tips) and strains derived from samples collected at Izembek National Wildlife Refuge (bold) are indicated. Nucleotide sequences for viruses that share 100% identity with other strains reported on the GenBank public database are identified with an asterisk. Complete strain names (year/month/day) appear on tip labels with American Ornithologists' Union four letter codes used for wild bird taxa and standard two letter abbreviations for United States. Bayesian credible intervals for estimates of common ancestry are depicted with horizontal purple bars for clades with Bayesian posterior probability values >0.5. Clades with Bayesian posterior probability values >0.95 are indicated with thickened branches. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

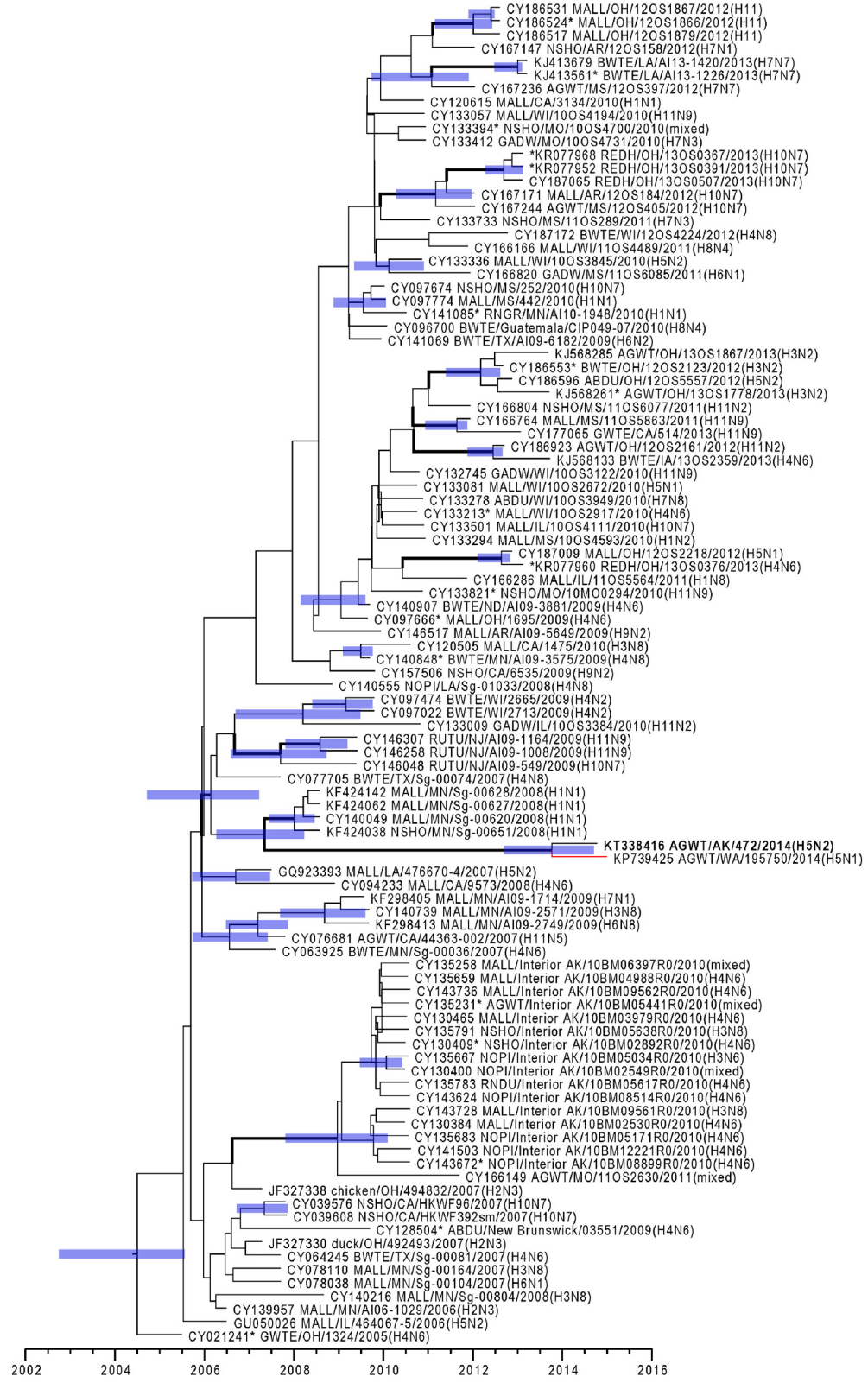

Fig. 6. Maximum clade credibility phylogeny for influenza A virus gene segments depicting inferred genetic relationship among nucleotide sequences for genetically similar influenza A virus NS gene segments (see Materials and methods). Nucleotide sequences for NS gene segments of highly pathogenic clade 2.3.4.4 H5N1 subtype influenza A viruses (red branch tips) and strains derived from samples collected at Izembek National Wildlife Refuge (bold) are indicated. Nucleotide sequences for viruses that share 100% identity with other strains reported on the GenBank public database are identified with an asterisk. Complete strain names (year/month/day) appear on tip labels with American Ornithologists' Union four letter codes used for wild bird taxa and standard two letter abbreviations for United States. Bayesian credible intervals for estimates of common ancestry are depicted with horizontal purple bars for clades with Bayesian posterior probability values >0.5. Clades with Bayesian posterior probability values >0.95 are indicated with thickened branches. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

viruses is also relatively common (Reeves et al., 2013). Thus, the geographic coverage of our autumn sampling effort may not have been sufficient for the early detection of HP H5 IAVs into North America. Finally, a fourth possibility is that HP IAVs were not introduced into North America via migratory birds and that common ancestry of gene segments between HP viruses and those isolated from samples collected at Izembek NWR is a function of broad geographic distribution of these lineages in avian hosts during 2014, including areas spatially and temporally proximate to the initial point of introduction.

MCC phylogenetic analyses provide support for recent common ancestry for PB1, NP, and NA gene segments of influenza A virus isolates from Izembek NWR and HP H5N2 IAVs detected in North America. Given estimates of TMRCA and overlap of associated BCIs, it is plausible that the intercontinental dispersal of viruses leading to the reassortment of a HP H5N8 virus with IAVs comprised of North American genes occurred during the spring of 2014 in Beringia with the arrival of birds on breeding grounds. However, given this scenario, it remains unclear from our analysis if the subsequent reassortment event or events between HP H5N8 and other IAVs with North American lineage gene segments occurred in northeastern Russia, Alaska, or areas further south. Although limited data exist regarding IAVs infecting wild birds inhabiting northeastern Russia, intercontinental reassortant IAVs are relatively common in western Alaska (Ramey et al., 2010; Reeves et al., 2013). It is therefore reasonable to infer that interhemispheric reassortment events may commonly occur in western Beringia (i.e., northeastern Russia).

Alternatively, TMRCA estimates also provide support for the possibility that the reassortment event(s) leading to HP H5N2 IAVs detected in North America occurred during late autumn/winter of 2013–2014. However, under this scenario, progenitor IAVs to the HP H5N2 viruses would have circulated undetected for approximately one year despite surveillance efforts for IAVs in East Asia and the USA. Furthermore, the reassortment event(s) leading to HP H5N2 IAVs would have presumably coincided with more southerly staging/wintering distributions of migratory birds when and where the detection of intercontinental reassortants has been less common (i.e., as compared to Alaska; Pearce et al., 2009). The identification of additional nucleotide sequences for PB1, NP, and NA gene segments that are closely related to North American HP H5N2 IAVs may help to refine inference of the genetic ancestry of these emergent pathogenic viruses.

Our analyses also provide support for common genetic ancestry between IAV isolates originating from Izembek NWR and a HP H5N1 IAV detected in an American green-winged teal (*Anas crecca carolinensis*) in Washington, USA. However, we found closely related genetic sequences for only two of the four North American lineage gene segments of A/American green-winged teal/Washington/195750/2014(H5N1) in isolates derived from samples collected at Izembek NWR in September–October 2014. Additionally, mean TMRCA estimates had relatively large confidence intervals for both the PA and NS genes (i.e., >2 years). Thus, although there is evidence that viral diversity maintained in migratory birds in Beringia may have contributed towards the emergence of this HP H5N1 IAV in North America; additional nucleotide sequence information is needed to make robust conclusions about the genetic ancestry of this reassortant virus.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.02.035.

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