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GENOMIC ANALYSIS OF AVIAN INFLUENZA VIRUSES FROM WATERFOWL IN WESTERN ALASKA, USA

Andrew B. Reeves,[1,4] John M. Pearce,[1] Andrew M. Ramey,[1] Craig R. Ely,[1] Joel A. Schmutz,[1] Paul L. Flint,[1] Dirk V. Derksen,[1] Hon S. Ip,[2] and Kimberly A. Trust[3]

[1] US Geological Survey, Alaska Science Center, 4210 University Drive, Anchorage, Alaska 99508, USA
[2] US Geological Survey, National Wildlife Health Center, 6006 Schroeder Road, Madison, Wisconsin 53711, USA
[3] US Fish and Wildlife Service, National Wildlife Refuge System, 4401 N. Fairfax Drive, Arlington, Virginia 22203, USA
[4] Corresponding author (email: areeves@usgs.gov)

ABSTRACT: The Yukon-Kuskokwim Delta (Y-K Delta) in western Alaska is an immense and important breeding ground for waterfowl. Migratory birds from the Pacific Americas, Central Pacific, and East Asian-Australasian flyways converge in this region, providing opportunities for intermixing of North American- and Eurasian-origin hosts and infectious agents, such as avian influenza virus (AIV). We characterized the genomes of 90 low pathogenic (LP) AIV isolates from 11 species of waterfowl sampled on the Y-K Delta between 2006 and 2009 as part of an interagency surveillance program for the detection of the H5N1 highly pathogenic (HP) strain of AIV. We found evidence for subtype and genetic differences between viruses from swans and geese, dabbling ducks, and sea ducks. At least one gene segment in 39% of all isolates was Eurasian in origin. Target species (those ranked as having a relatively high potential to introduce HP H5N1 AIV to North America) were no more likely than nontarget species to carry viruses with genes of Eurasian origin. These findings provide evidence that the frequency at which viral gene segments of Eurasian origin are detected does not result from a strong species effect, but rather we suspect it is linked to the geographic location of the Y-K Delta in western Alaska where flyways from different continents overlap. This study provides support for retaining the Y-K Delta as a high priority region for the surveillance of Asian avian pathogens such as HP H5N1 AIV.

Key words: Alaska, avian influenza virus, genome, migratory birds, surveillance, waterfowl.

INTRODUCTION

In response to potential introduction of highly pathogenic (HP) H5N1 avian influenza virus (AIV) from Eurasia to North America by migratory birds, a US Interagency Strategic Plan (hereafter the Plan) was developed to guide surveillance efforts for early detection of the virus (Interagency Working Group, 2006). Sampling birds in Alaska was a priority of the Plan due to its location along migratory flyways linking western and eastern hemispheres (Winker and Gibson, 2010). The Plan also identified and ranked wild bird species to be sampled based on criteria that assessed each species' potential for carrying HP H5N1 AIV to North America (Ip et al., 2008). The highest ranking species were identified as target species. Although HP H5N1 AIV has never been detected in North America, genomic analyses of low pathogenic (LP) AIVs isolated from surveillance sampling have enhanced monitoring efforts by identifying sampling locations and host species more likely to be associated with intercontinental movements of viruses or viral gene segments (Pearce et al., 2009; Ramey et al., 2010b). Entirely Eurasian-origin LP AIV genomes have not been documented in North America, although gene segments descended from Eurasian ancestors have (Krauss et al., 2007; Dugan et al., 2008; Koehler et al., 2008; Wille et al., 2011). These Eurasian-origin genes have been detected more frequently in western Alaska than in other locations in North America (Pearce et al., 2009; Ramey et al., 2010a). However, there has not been a study of viruses from a community of species that covers a large geographic area in this region.

The Yukon-Kuskokwim Delta (Y-K Delta) in western Alaska (Fig. 1) is a vast breeding ground of global significance for many species of migratory birds. The diversity and abundance of birds, many with migratory connectivity to Eurasia, combined with the feasibility of collecting samples, made the Y-K Delta one of the

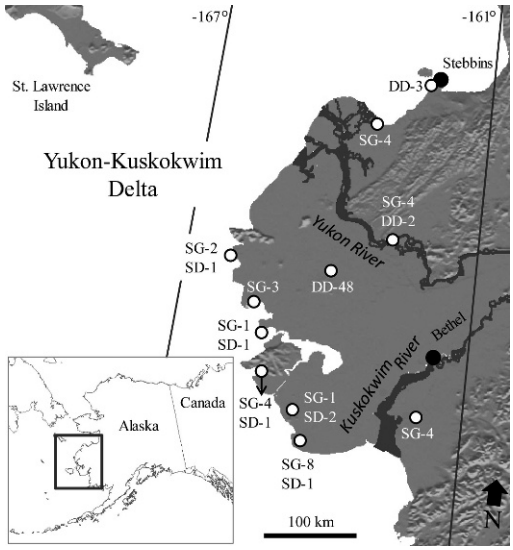

FIGURE 1. Map of the Yukon-Kuskokwim Delta in western Alaska, USA (inset), where waterfowl samples were collected 2006–2009 to characterize avian influenza virus (AIV) isolates. Species groups (swans and geese=SG, dabbling ducks=DD, and sea ducks=SD) and the number of avian influenza virus isolates characterized are provided for each of the approximated collection sites (open circles). Locations discussed in text and major geographic references are labeled.

most heavily sampled regions in the United States for HP H5N1 AIV surveillance (Ip et al., 2008). Therefore, molecular characterization of LP AIVs from birds across the Y-K Delta provide a unique opportunity to examine virus diversity among multiple hosts and investigate exchange of AIV lineages across a large community of migratory species. We examined subtype diversity from three taxonomic groups of waterfowl: swans and geese, dabbling ducks, and sea ducks. We also characterized LP AIV genomes from target and nontarget species sampled on the Y-K Delta to test whether target species were more likely to be infected with viruses with mixed continental lineages. We interpret these results in the context of past surveillance and future monitoring programs in North America, Europe, and Asia.

MATERIALS AND METHODS

Sampling, virus isolation, and sequencing

Cloacal swab samples were collected from wild birds between 2006 and 2009 during spring and fall subsistence harvests and live-captures (May–August). Samples were stored in viral transport media and then screened for AIVs at the US Geological Survey (USGS), National Wildlife Health Center (NWHC) using methods of Ip et al. (2008). We attempted to sequence AIVs isolated from cloacal swab samples collected on the Y-K Delta (boroughs of Wade Hampton and Bethel, and the geographically contiguous location of Stebbins within the Nome Borough; Fig. 1) if harvested allantoic fluid agglutinated chicken red blood cells and was positive for the influenza A matrix gene by rRT-PCR. Viral RNA was extracted from allantoic fluid with the MagMAX AI/NDV RNA extraction kits (Ambion Inc., Austin Texas, USA) and amplified with the One-Step RT PCR kit (QIAGEN Inc., Valencia, California, USA) using combinations of previously published primers (Zou, 1997; Hoffmann et al., 2001; Phipps et al., 2004; Bragstad et al., 2005; Chan et al., 2006; Obenauer et al., 2006; Li et al., 2007; Koehler et al., 2008; Pearce et al., 2011) and primers designed by researchers at the USGS Alaska Science Center (available upon request from the authors). PCR products were gel purified and extracted using the QIAquick Gel Extraction Kit (Qiagen) or treated with ExoSap-IT (USB Inc., Cleveland, Ohio, USA) without additional purification before sequencing. Cycle sequencing was performed with identical primers used for PCR along with BigDye Terminator version 3.1 mix (Applied Biosystems, Foster City, California, USA). Samples were analyzed on an Applied Biosystems 3730xl automated DNA sequencer. Sequences were aligned and edited using Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, Michigan, USA).

Isolates with evidence of coinfection (i.e., chromatograms with multiple peaks throughout a sequence) were excluded from further analyses ($n=3$) as were 16 isolates for which contamination concerns could not be ruled out. These included seven isolates collected from nonwaterfowl species (Pectoral Sandpiper, *Calidris melanotos*; and Sandhill Crane, *Grus canadensis*). As a result of their removal, our analyses focus exclusively on LP AIVs in waterfowl. Ninety LP AIV isolates were sequenced, including 24 from previous Alaska studies (Koehler et al., 2008; Ramey et al., 2010b; Pearce et al., 2011) and 66 obtained

specifically for this project. All eight gene segments were sequenced for 82 isolates; the remaining eight were missing data for one or two segments each. Thus, data from 709 of 720 potential gene segments were used in analyses. GenBank accession numbers for gene segments sequenced in this study are JX080722–JX081243. Strain names and accession numbers for sequences obtained in previous studies are available upon request from the authors. For phylogenetic analyses, sequences for each gene segment were cropped to the following number of nucleotides: PB2 (2189), PB1 (2216), PA (2146), HA (1470–1656), NP (1411), NA (1222–1388), M (732), and NS (666–669).

Subtype comparisons among waterfowl groups

Subtyping was determined using the NCBI BLAST tool (Altschul et al., 1990) for hemagglutinin (HA) and neuraminidase (NA) sequences and confirmed by phylogenetic comparisons to reference sequences. We compared distributions of HA and NA subtypes, and subtype combinations for viruses isolated from waterfowl groups using chi-square (χ^2) tests of homogeneity and calculated the exact P values using a randomized sampling distribution (1,000 replicates) of our data.

Analyses were based on the following organization structure of three waterfowl groups: swans and geese (Tundra Swan, *Cygnus columbianus*; Canada/Cackling goose, *Branta canadensis/B. hutchinsii*; Black Brant, *B. bernicla*; Greater White-fronted goose, *Anser albifrons*; Lesser Snow Goose, *Chen caerulescens*; and Emperor Goose, *C. canagica*), dabbling ducks (Mallard, *Anas platyrhynchos*; Green-winged Teal, *A. crecca*; and Northern Pintail, *A. acuta*), and sea ducks (King Eider, *Somateria spectabilis*; and Spectacled Eider, *S. fischeri*). We combined Tundra Swans and geese for analyses because both taxa are large-bodied grazers utilizing similar habitats. Isolates were grouped into two periods for each year; spring samples were collected in April and May, and fall samples from July–October.

Comparing genomic identities

To examine patterns of virus sharing, we quantified the similarity of AIV genomes by calculating nucleotide pairwise distances (PWD) for all gene segments, thus comparing entire genome constellations for all viruses. Virus genomes were considered highly similar when all eight gene segments between two isolates were >99% similar (PWD <0.01; Reeves et al., 2011). To evaluate persistence of highly similar genomes, we calculated the number of days between sample collections. The frequencies at which viruses were shared within and between species were calculated and observed differences between these were tested using a contingency table to compute χ^2.

Identifying intercontinental reassortment

Our phylogenetic methods for detecting Eurasian-origin gene segments were similar to those of Koehler et al. (2008). All gene segments in this study were compared to sequences of viruses isolated from wild waterfowl in Eurasia (internal segments, n=30–38; HA and NA segments, n=8–38) and North America (internal segments n=30–44; HA and NA segments, n=18–47) available from GenBank for each RNA segment to represent respective continental lineages (accession numbers for reference sequences are available upon request from the authors). Reference sequences were selected to cover wide geographic distributions of collection locations from both continents and restricted to samples collected from 2006–2009 with the exception of Eurasian references from 2000–2009 for H2, H8, H10, H11, H12, N2, N4, and N5 genes because few sequences of these genes were available from the more contemporary time period. Neighbor-joining trees were generated using MEGA Version 4.0.2 (Tamura et al., 2007) with the maximum composite likelihood model for nucleotide sequences and 10,000 bootstrap replicates. Sequences isolated from Y-K Delta samples phylogenetically embedded in continental clades of Eurasian reference sequences were considered evidence of intercontinental movement of influenza genes. NCBI BLAST results were used to assess independence of Eurasian-origin gene segments from our Y-K Delta viruses from lineages representing introduction events previously observed in North America. Sequences from the Y-K Delta where the highest identity matches in GenBank were to non–North American sequences were considered evidence of independent outsider events and subject to further phylogenetic analysis. We compared the frequencies at which isolates had at least one gene of Eurasian origin for target and nontarget species using a χ^2 test of homogeneity.

RESULTS

Isolates and subtypes

In total, 24,170 field swab samples were collected from 82 species of wild birds on

TABLE 1. Numbers of cloacal swab samples collected on the Yukon-Kuskokwim Delta, Alaska, USA, by species, 2006–2009, and numbers of avian influenza virus (AIV) genomes sequenced for each collection year. Numbers of species are given in parentheses for categories from which no isolates were sequenced. These data are not to be interpreted as prevalence of infection/isolate recovery rates, but rather are presented here to highlight extent of the surveillance effort on the Y-K Delta.

Species	Samples collected	AIV genomes sequenced				
		2006	2007	2008	2009	Total
Target Species	15,497	28	20	4	15	67
Tundra Swan	728	4	0	0	0	4
Lesser Snow Goose	1,571	1	1	0	0	2
Emperor Goose	1,930	8	4	0	0	12
Black Brant	3,388	1	4	1	0	6
Northern Pintail	1,317	9	11	3	14	37
King Eider	1,295	4	0	0	1	5
Spectacled Eider	576	1	0	0	0	1
Other target waterfowl species (n=3)	665	0	0	0	0	0
Other target nonwaterfowl species (n =12)	4,027	0	0	0	0	0
Non-Target Species	8,673	7	5	5	6	23
Greater White-fronted Goose	3,342	3	3	0	0	6
Canada Goose/Cackling Goose	3,183	0	1	0	0	1
Mallard	112	1	1	0	0	2
Green-winged Teal	611	3	0	5	6	14
Other nontarget waterfowl species (n=14)	583	0	0	0	0	0
Other nontarget nonwaterfowl species (n=43[a])	842	0	0	0	0	0
Total	24,170	35	25	9	21	90

[a] Three passerine species (Arctic Warbler, *Phylloscopus borealis*; Yellow Wagtail, *Motacilla tschutschensis*; and Gray-cheeked Thrush, *Catharus minimus*) were target species in 2006 but were removed from the priority list in subsequent years; thus sample sizes from these species are included here.

the Y-K Delta between 2006 and 2009 (Table 1). From these samples, we characterized the genomes of 90 LP AIVs from 11 waterfowl species (Table 1). Although we attempted to maximize the sample size of genomic sequence data from this region and time, the 90 LP AIVs in this study do not represent all of the possible AIV positive samples, and therefore the number of genomes sequenced should not be used to infer prevalence and isolate recovery rates. All viruses sequenced in this study were collected on the mainland of the Y-K Delta west of 161°0′0″W longitude (Fig. 1). Seven of the 11 species from which viruses were sequenced were target species (Interagency Working Group, 2006). There were significant differences in the frequency of HA types (χ^2=60.3, df=18, P<0.001), NA types (χ^2=77.01, df=16, P<0.001), and subtype combinations (χ^2=131.5, df=36, P<0.001) among waterfowl groups (Fig. 2). We detected 10 HA and nine NA subtypes paired in 19 combinations (Fig. 2) from 86 viruses for which both HA and NA were sequenced. H3N8 (n=19) and H4N6 (n=18), the most common subtype combinations, and H11N9 (n=3) were recovered from species of geese and dabbling ducks. Only one isolate from sea ducks shared a subtype combination (H3N8) with species from other waterfowl groups.

Highly similar genomes

Thirty-eight of 4,005 isolate-to-isolate genomic comparisons were highly similar with identities >99% (PWD<0.01) for all gene segments (Table 2). These highly similar genomes were observed more often within (68%) than between (32%) species (χ^2=50.0, df=1, P<0.001). Only one (3%) of the 38 comparisons with highly similar genomes occurred between viruses from species of different waterfowl groups: A/Northern Pintail/Alaska/44340-441/2007

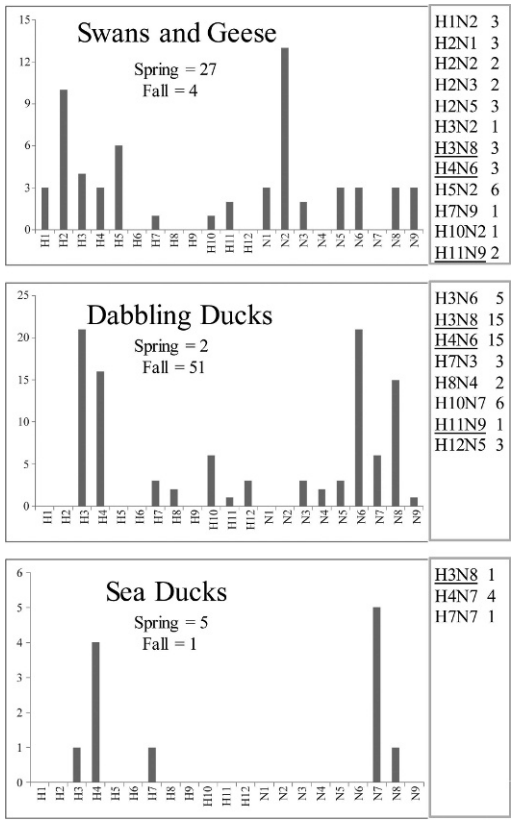

FIGURE 2. Subtype distributions for avian influenza viruses isolated from three waterfowl groups. Subtype combinations observed are given at right. Those combinations observed in more than one waterfowl group are underlined. The number of viruses sequenced for each group is provided by time of year samples were collected.

(H11N9) and A/Black Brant/Alaska/44391-190/2008 (H11N9). These strains also represent one of two comparisons where highly similar viruses were isolated from different sample collection years or seasons (Table 2); the other was between A/American Green-winged Teal/Alaska/44419-342/2008 (H3N8) and A/Northern Pintail/Alaska/44500-089/2009 (H3N8). All remaining comparisons of highly similar genomes ($n=36$, 95%) occurred between isolates collected within 15 days from one another.

Eurasian-origin gene segments in LP AIVs recovered from the Y-K Delta

Sequence clades representing major continental divisions were identified and supported by bootstrap values ≥99% (10,000 replicates) for all gene segments, except N2, for which a complex phylogeny similar to findings by Bahl et al. (2009) was observed. Two sequences from each contemporary North American N2 clade in Bahl et al. (2009) were included in our references for comparison. All 12 Y-K Delta N2 sequences from our study were similar to those North American sequences with Eurasian ancestry in Bahl et al. (2009). However, given the complexity of the N2 phylogeny and establishment of this lineage across all of North America for over a decade, N2 gene segments from the Y-K Delta were considered of North American origin for the purposes of this study.

Thirty-five of the 90 (39%) Y-K Delta isolates had at least one, and as many as four, Eurasian-origin gene segment (Fig. 3). Forty-eight of the 709 (6.8%) gene segments sequenced were of Eurasian origins. We obtained genomic sequence data for seven target species of the HP AIV surveillance program. Of those, five species had LP AIV genomes with ≥1 Eurasian-origin gene segment (Fig. 3). Green-winged Teal was the only nontarget species that had LP AIV genomes with Eurasian-origin gene segments (Fig. 3). Of all Eurasian-origin gene segments detected, H3 ($n=19$) was the most common, followed by NP ($n=8$), NS ($n=6$), PA ($n=4$), N7 ($n=4$), H2 ($n=3$), M ($n=2$), H4 ($n=1$), and N6 ($n=1$). BLAST results combined with phylogenetic analyses indicate 39 of the 48 Eurasian-origin sequences identified in this study were similar to Eurasian lineage sequences previously documented in Alaska /North America (i.e., the 39 are not unique outsider events). In contrast, all of the Eurasian-origin H4 ($n=1$), N6 ($n=1$), N7 ($n=4$), and M ($n=2$) gene segments in this study along with one of six NS segments with Eurasian ancestry (accession numbers: JX080775, JX081167, JX081151–JX081154, JX081239, JX081228, and JX081045) represent five outsider events where no closely related North American

TABLE 2. Highly similar avian influenza viruses detected by genetic pairwise distances among waterfowl species sampled on the Yukon-Kuskokwim Delta, Alaska, USA, 2006–2009. Sample details are provided for virus genomes where all eight gene segments between two isolates were >99%[a] similar. Target species are given in bold.

Species 1	Species 2	No. of events >99%[a]/total number of comparisons made	No. of days between collections of highly similar viruses[a]
Tundra Swan	**Tundra Swan**	3/6 (0.500)	0–15
Greater White-fronted Goose	Greater White-fronted Goose	4/15 (0.267)	0–9
King Eider	**King Eider**	1/10 (0.100)	0
Emperor Goose	**Emperor Goose**	6/66 (0.091)	0–11
Tundra Swan	Greater White-fronted Goose	2/24 (0.083)	2–11
Black Brant	**Black Brant**	1/15 (0.067)	0
Emperor Goose	**Black Brant**	2/72 (0.028)	0–2
Green-winged Teal	Green-winged Teal	2/91 (0.022)	4–6
Northern Pintail	**Northern Pintail**	9/666 (0.014)	0–2
Northern Pintail	Green-winged Teal	7/518 (0.014)	2–8, 361[b]
Northern Pintail	**Black Brant**	1/222 (0.005)	283[c]

[a] Nucleotides for all eight gene segments of influenza A viruses are >99% identical.

[b] Number of days between the collections of highly similar viruses: A/American Green-winged Teal/Alaska/44419-342/2008 (H3N8), 14 August 2008; and A/Northern Pintail/Alaska/44500-089/2009 (H3N8), 10 August 2009; hosts sampled <20 km apart.

[c] Number of days between collections of highly similar viruses: A/Northern Pintail/Alaska/44340-441/2007 (H11N9), 10 August 2007; and A/Black Brant/Alaska/44391-190/2008 (H11N9), 19 May 2008; hosts sampled ≈120 km apart.

acquired sequences have been previously reported to GenBank (data available upon request from the authors). These novel observations of outsider events were detected in isolates from King Eiders ($n=5$; N7 and NS) and Northern Pintails ($n=2$; H4, N6, and M).

The H3 segments in this study were more often of Eurasian lineage ($n=19$) than North American ($n=7$). Eurasian H3 segments isolated from the Y-K Delta formed two monophyletic clades within the greater Eurasian complex of reference sequences (data not shown). There was no significant difference in the frequency at which viruses from target (43%) and nontarget (26%) species contained at least one gene segment of Eurasian origin ($\chi^2=2.13$, df $=1$, $P=0.144$).

DISCUSSION

The Y-K Delta provides habitat for large concentrations of swans and geese, dabbling ducks, and sea ducks, yet our data provide limited evidence for the sharing of viruses among these waterfowl groups. This might be explained by seasonal patterns of prevalence in different groups of waterfowl sampled. AIV prevalence tends to be higher in spring for geese (Kleijn et al., 2010), whereas ducks show greater prevalence in fall (Krauss et al., 2004). Although our sample size data are not directly comparable to prevalence studies, swans, geese, and dabbling ducks were sampled in spring and fall, with most (87%) viruses sequenced from swans and geese collected in the spring and 96% of dabbling duck viruses sequenced from the fall (Fig. 2). Thus, temporal differences in our data fit this pattern and likely contribute to the minimal rates of viral exchange among species groups. Furthermore, our data show that sampling target species from the HP AIV surveillance program was a useful approach to detect Eurasian-origin AIV gene segments; however, this strategy might not be the most efficient. Whereas isolates from most target species showed evidence of Eurasian-origin gene segments, one nontarget species, the Green-winged Teal, also had a high frequency of Eurasian lineage genes; 43% of isolates had at least one Eurasian-origin gene segment. Despite the observed

Species	Proportion of viruses with at least one Eurasian gene segment	Eurasian gene segments detected
Target Species		
Tundra Swan *Cygnus columbianus*	0/4	
Lesser Snow Goose *Chen caerulescens*	0/2	
Emperor Goose *Chen canagica*	7/12 (58%)	H2(3) NS(4)
Black Brant *Branta bernicla*	1/6 (16%)	NS(1)
Northern Pintail *Anas acuta*	15/37 (41%)	H3(13) H4(1) NP(2) N6(1) M(2)
King Eider *Somateria spectabilis*	5/5 (100%)	PA(4) NP(3) N7(4) NS(1)
Spectacled Eider *Somateria fischeri*	1/1 (100%)	H3(1) NP(1)
Non-target Species		
Greater White-fronted Goose *Anser albifrons*	0/6	
Canada Goose/Cackling Goose *Branta candensis/ B. hutchinsii*	0/1	
Mallard *Anas platyrhynchos*	0/2	
Green-winged Teal *Anas crecca*	6/14 (43%)	H3(5) NP(2)

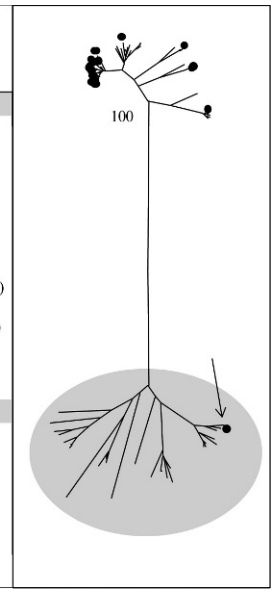

FIGURE 3. Summary of Eurasian and North American avian influenza virus (AIV) gene segment reassortment. Species sampled on the Yukon-Kuskokwim Delta (Y-K Delta), Alaska, USA from which viruses were sequenced are shown with the proportion of AIV isolates with at least one Eurasian-origin gene segment relative to the total number of isolates for each species. Gene segments of Eurasian origin are given with the number of isolates in parentheses. Neighbor-joining tree of the N6 gene exemplifies methods for identifying Eurasian lineages in our data. Dark circles indicate Y-K Delta sequences in relation to North American (nonshaded) and Eurasian (shaded oval) reference sequences. Bootstrap value from 10,000 replications is given for node of continental division. Arrow shows position of N6 gene for A/Northern Pintail/Alaska/44500-126/2009 (H4N6) embedded within the Eurasian clade.

genetic differences between LP AIVs among waterfowl groups in our data, we also demonstrate that AIVs circulate within such groups, similar to the findings of Munster et al. (2007) for species sampled in Europe. Thus, viruses present in a geographic area might be detected in any number of species and therefore, within a HP AIV surveillance framework, it might not be necessary to sample only target species. Sampling directed at sympatric species, based on abundance and ease of sample collection might be an alternative way to detect a virus introduced into a specific geographic area.

AIV diversity among waterfowl groups

Comparisons of genomes revealed only one instance of highly similar viruses between species from different waterfowl groups; a dabbling duck (Northern Pintail) and a goose (Black Brant) collected 283 days apart. The subtype differences detected, combined with the genetically dissimilar isolates, demonstrates that LP AIV populations might not be homogeneous among waterfowl groups. Additionally, of 17 subtype combinations identified in swans and geese, and dabbling ducks, 14 were unique to one group, whereas three (H3N8, H4N6, and H11N9) were observed in both groups. The sharing of these specific subtype combinations was not surprising because these are some of the most frequently reported in North American waterfowl (Sharp et al., 1997; Krauss et al., 2004; Wilcox et al., 2011). However, the sharing of subtype combinations between hosts is not synonymous with the sharing of virus strains (i.e., highly similar sequences), because there can be great genetic divergence even within the same HA and NA subtypes and in the other six gene segments. One H3N8 isolate was also detected in a sea

duck (A/Spectacled Eider/Alaska/44173-055/ [2006]) and was the only shared subtype combination between a sea duck isolate and viruses from other waterfowl groups. Although the H3 sequence from this isolate was highly similar (>99%) to nine other sequences in this study, the N8 sequence was <99% similar to all other N8 sequences (data not shown).

Although the total number of isolates from sea ducks ($n=6$) was fewer than swans and geese, and dabbling ducks in this study, phylogenetic analyses revealed some viral gene segments from a sea duck (King Eider) were differentiated from those in other host species and waterfowl groups examined. Sequences from the N7 gene observed in viruses from four King Eiders were of Eurasian origin and thus genetically divergent from North American N7 lineages found in dabbling ducks on the Y-K Delta (data not shown). To our knowledge, this is the first instance of Eurasian-origin N7 gene segments in Alaska. Additionally, whereas four H4 segments from King Eiders were more closely related to North American than Eurasian viruses, three (two in 2006 and one in 2009) were only 83.5% similar (PWD=0.165) to the closest sequence among our H4 data. BLAST results from these sequences indicate the highest identity (90–91%) was with a marine mammal virus (A/seal/Massachusetts/133/1982 [H4N5]) and the remaining top 10 results were samples collected from North American waterfowl from 1977–1983. This suggests the circulation of an H4 lineage that has not been identified or sequenced via contemporary surveillance efforts. Unlike Spectacled Eiders and Common Eiders (*Somateria mollissima*), King Eiders do not breed on the Y-K Delta, and sample collections of this species occurred during spring subsistence hunting along the coast as birds migrated northward. Different breeding distributions and life history attributes (e.g., their absence inland) for this species could explain the lack of genetic exchange of AIVs detected between sea ducks and other taxa evaluated in this study. For example, all isolates from sea ducks in this study were collected within 10 km of the ocean coast, whereas 96% and 26% of viruses from dabbling ducks, and swans and geese, respectively, were from samples further inland (>10 km; Fig. 1). Thus, differences in collection sites among waterfowl groups might have contributed to the restricted exchange of viruses we observed.

Highly similar genomes across species and time

The detection of highly similar genomes in our study indicates viruses are shared within, and to a lesser degree, among species groups on the Y-K Delta. We observed significant differences in the distribution of subtypes among waterfowl groups and a greater number of highly similar viruses occurred between hosts within a waterfowl group (37/38 comparisons), than between (1/38) them (Table 2). In most cases, closely related viruses were also temporally clustered. Spatiotemporal clustering of highly similar viruses can result from the epizootic nature of outbreaks or from common surveillance sample collection methods (e.g., sampling birds over a short time and within close proximity). Interestingly, we detected two events in which nearly identical viruses were collected across more than one breeding season (i.e., >283 days; Table 2). In spite of the transient nature of genomic constellations and high mutation rates of AIVs, it is possible genomic similarity across breeding seasons represents either overwinter maintenance of the virus in wild populations (Chen and Holmes, 2006) or in environmental reservoirs. Pearce et al. (2009) did not find evidence that viruses from Alaska were maintained in wintering populations of Northern Pintails in California, but controlled experiments have demonstrated that AIVs can survive in water for long periods and at temperatures similar to those on the Y-K Delta (Brown et al., 2009; Stallknecht et al., 2010). Furthermore, AIV RNA has been detected in winter sediment

samples from Alaska, although these viruses were not proven to be viable (Lang et al., 2008). Thus, we suspect that detections of identical viruses across breeding seasons are evidence of over-winter persistence in the environment.

Eurasian-origin gene segments in viruses from target and nontarget species

The frequency of isolates with Eurasian-origin genes (39%) in our data concurs with other studies that document numerous AIVs with mixed continental lineages in Alaska (Ramey et al., 2010a, b; Pearce et al., 2011). However, over half of viruses with Eurasian lineages in our study contained H3 gene segments of Eurasian origin. The two monophyletic clades with samples from subsequent years suggest either repeated entry of these Eurasian-origin H3 lineages across years or some mechanism of virus perpetuation within North America. Additionally, Eurasian-origin H3 viruses in this study were more common (73%) than those of North American origin (27%) and were found at a higher frequency than previously reported for Northern Pintails sampled in Alaska (44%; Ramey et al., 2010b). Overall, these data provide evidence that Eurasian-origin gene segments are regularly introduced to the Y-K Delta, supporting the contention that detection of nonindigenous viruses should focus on locations with the closest proximity to the source (Pearce et al., 2009; Ramey et al., 2010a).

All the novel outsider events (Eurasian-origin gene segment lineages detected for the first time from North American samples) in this study were linked to seven isolates from target species (King Eider and Northern Pintail). However, no difference was observed in our analyses between target and nontarget surveillance species sampled on the Y-K Delta with regard to the frequency of all Eurasian gene segments. This supports the tenet that geographic location is a good predictor of Eurasian-origin gene segment frequency (Pearce et al., 2009; Ramey et al., 2010a). Although a target species could be responsible for an initial introduction of gene segments from Eurasia, these lineages can spread upon arrival to other species and even persist across years. Our findings suggest that interspecies transmission occurs within waterfowl groups, and hence detecting Eurasian-origin lineages established in Alaska would be less dependent on the individual species sampled than it would be for identifying novel outsider events. Furthermore, because our data provide evidence that virus diversity is not evenly distributed among waterfowl groups (i.e, swans and geese, dabbling ducks, and sea ducks), restructuring species into target and nontarget categories might have confounded our ability to test for differences among species assemblages organized by surveillance priority. If HP H5N1 AIV was introduced via wild birds into Alaska and rapidly dispersed across species in a similar manner to some LP AIV genes, concentrating surveillance on target species could be advantageous to detection efforts at only the earliest stages of an introduction event. Thus, phylogenetic analyses of viruses from western Alaska continues to provide one of the best methods for characterizing intercontinental gene flow of AIVs into Alaska and North America from Asia.

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