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Genetic evidence of intercontinental movement of avian influenza in a migratory bird: the northern pintail (*Anas acuta*)

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Abstract

The role of migratory birds in the movement of the highly pathogenic (HP) avian influenza H5N1 remains a subject of debate. Testing hypotheses regarding intercontinental movement of low pathogenic avian influenza (LPAI) viruses will help evaluate the potential that wild birds could carry Asian-origin strains of HP avian influenza to North America during migration. Previous North American assessments of LPAI genetic variation have found few Asian reassortment events. Here, we present results from whole-genome analyses of LPAI isolates collected in Alaska from the northern pintail (*Anas acuta*), a species that migrates between North America and Asia. Phylogenetic analyses confirmed the genetic divergence between Asian and North American strains of LPAI, but also suggested inter-continental virus exchange and at a higher frequency than previously documented. In 38 isolates from Alaska, nearly half (44.7%) had at least one gene segment more closely related to Asian than to North American strains of LPAI. Additionally, sequences of several Asian LPAI isolates from GenBank clustered more closely with North American northern pintail isolates than with other Asian origin viruses. Our data support the role of wild birds in the intercontinental transfer of influenza viruses, and reveal a higher degree of transfer in Alaska than elsewhere in North America.

Keywords: low pathogenic, migration, reassortment, virus sequencing, waterfowl

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Introduction

A critical question surrounding emergence of the highly pathogenic (HP) Asian H5N1 avian influenza is the role of wild migratory birds in the geographic redistribution of this virus. While virus translocation via migratory birds is suspected in outbreaks of HP H5N1 in Africa and Europe, other mechanisms of transmission, such as illegal trafficking of wild birds and international trade of poultry cannot be discounted (Kilpatrick *et al*. 2006; Salzberg *et al*. 2007). As a result, the potential dissemination of HP H5N1 within Eurasia and to Russia and North America via migratory movements of birds remains the subject of considerable discussion (Feare 2007; Gauthier-Clerc *et al*. 2007) and has two independent components. First, can wild birds be

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inapparent carriers, becoming infected with and shedding the virus, yet healthy enough to migrate? To date, there are no data to definitively answer this question (Feare 2007; Flint 2007), and while recent studies have demonstrated that infection with HP H5N1 is not fatal to some waterfowl species (Brown *et al*. 2006; Kalthoff *et al*. 2008; Keawcharoen *et al*. 2008), experimental studies may not reflect natural conditions. Second, is there direct intercontinental movement of infected individuals, or contact between Asian and North American migrants which results in transfer of viruses between continents? In the case of several HP H5N1 outbreaks, wild birds have been dismissed as potential sources of the virus as there were no known migratory movements from HP avian influenza endemic regions to the area of the new outbreak (Kilpatrick *et al*. 2006). However, direct movement may not be necessary to facilitate virus movement. Viruses may also spread via sequential contact among wild birds along a wide range of migratory

pathways and through environmental reservoirs (Webster *et al*. 1992; Lang *et al*. 2008; Uchida *et al*. 2008), although the exact conditions necessary for host-to-host transfer, such as species assemblages, animal densities, and environmental characteristics remain largely unknown.

Phylogenetic analysis of whole low pathogenic avian influenza (LPAI) genomes has the potential to answer questions about wild bird contact and associated viral exchange, provided appropriate species and geographic locales are sampled. Wild migratory birds, primarily in the orders Charadriiformes (gulls and shorebirds) and Anseriformes (ducks, geese, and swans), are the natural reservoirs of a large diversity of LPAI subtypes (Webster *et al*. 1992; Clark & Hall 2006). Several lines of evidence from recent surveys of LPAI in wild birds have led to speculation that intercontinental transfer of avian influenza viruses from Asia to North America via wild birds is rare (Kilpatrick *et al*. 2006; Krauss *et al*. 2007; Winker *et al*. 2007). These include lack of detection of HP H5N1 in North America, phylogenetic divergence between Asian and North American lineages of LPAI (Ito *et al*. 1995; Widjaja *et al*. 2004), and low levels of reassortment between Asian and North American lineages of LPAI (Marakova *et al*. 1999; Wallensten *et al*. 2005; Krauss *et al*. 2007; Dugan *et al*. 2008; Kim *et al*. 2008). However, most of these studies examined viruses obtained from species that are not transcontinental migrants or from mid-latitude locales of North America, which are far removed from sources of Asian lineages of avian influenza. Genetic characterization of LPAI viruses obtained from a large sample of known intercontinental migrants and from an area close to the Asian continent would provide a better test of whether migratory birds can transfer Asian lineages of LPAI into North America.

In this study, we focus on the northern pintail (*Anas acuta*), which is a wide-ranging migratory bird with a Holarctic breeding and wintering distribution (Kear 2005) and a model species to test hypotheses about the movement of avian influenza viruses. The northern pintail is one of the most abundant duck species at high latitudes during summer and is sympatric with a diverse array of other waterbirds on wintering areas in Asia and North America. Thus, intra- and interspecific transfer of LPAI viruses are likely on both breeding and wintering areas, facilitating intercontinental viral exchange indirectly. In addition, information from banding and radio-transmitter studies has revealed occasional direct migratory movements of this species within and between Asia and North America (Miyabayashi & Mundkur 1999; Miller *et al*. 2005; Nicolai *et al*. 2005). Lastly, northern pintails regularly carry numerous strains of LPAI at some of the highest prevalence among species of water birds (Hinshaw *et al*. 1980; Ip *et al*. 2008; Parmley *et al*. 2008).

We therefore conducted whole-genome analysis of LPAI viruses isolated from wild northern pintails in Alaska, an area at the crossroads of Eurasian and North American migratory flyways. We hypothesized that a phylogenetic examination of LPAI strains isolated from northern pintails in Alaska would demonstrate higher frequencies of intercontinental transfer of avian influenza viruses than lower latitude locations in North America that can be considered farther from the source of Asian origin strains. We compared northern pintail sequences from Alaska to selected reference samples in the National Center for Biotechnology Information (NCBI) Influenza Virus Resource database (Bao *et al*. 2008) from areas in Eastern Asia (China, South Korea, and Japan) where northern pintails are known to winter. We interpret these results in the context of using LPAI phylogenetic analysis to infer the degree to which wild birds disperse Asian lineages of viruses across intercontinental boundaries.

Materials and methods

Sampling and virus isolation

Samples were collected from 1426 live and hunter-harvested birds in Alaska in 2006. The live bird samples came primarily from five National Wildlife Refuges (Koyukuk, Yukon Flats, Innoko, Yukon-Kuskokwim Delta, and Izembek) and one State Game Refuge (Minto Flats) located in central and western Alaska, whereas hunter-harvested samples were collected near the communities of Nome and Palmer, Alaska, and from two National Wildlife Refuges (Yukon-Kuskokwim Delta, and Izembek). Detailed maps of these sampling locations can be viewed in Ip *et al*. (2008) and at http://alaska.usgs.gov/science/biology/avian_influenza/monitoring.html. From the total 1426 samples, 793 were analysed using molecular detection of avian influenza viruses via virus isolation in embryonated eggs (Purchase *et al*. 1989). Following this analysis, allantoic fluids from each egg were tested for the presence of hemagglutinating virus using chicken and turkey red blood cells. This resulted in a total of 57 LPAI positive samples that were then subtyped (see Ip *et al*. 2008). From these 57 positive samples, we selected 38 for whole-genome sequencing. This reduction in number of samples sequenced is based on our selection of one to three samples of each virus subtype per location. Additional isolates were excluded if they duplicated the subtype combination of a previously selected sample collected from the same location and date.

RNA extraction, PCR and sequencing

Viral RNA was extracted from allantoic fluid with the MagMAX AI/NDV RNA extraction kit (Ambion Inc.). All eight segments were amplified with the QIAGEN one-step RT PCR kit using a combination of previously published primers (Zou 1997; Hoffmann *et al*. 2001; Phipps *et al*. 2004;
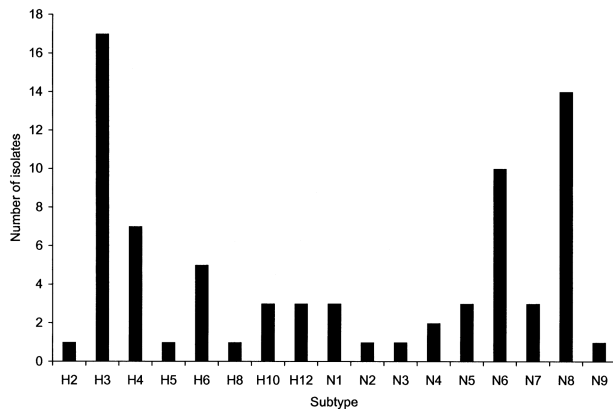
Fig. 1 Distribution of HA and NA subtypes observed among 38 northern pintail avian influenza isolates from Alaska.

Bragstad *et al.* 2005; Chan *et al.* 2006; Obenauer *et al.* 2006; Li *et al.* 2007). A number of additional primers were also specifically designed for this study (see Appendix SI, Supporting Information). Amplified products were gel purified and extracted using the QIAquick gel extraction kit (QIAGEN, Inc.) or treated with ExoSap-IT (USB) without additional purification before sequencing. Cycle sequencing was performed with identical primers used in the PCR along with BigDye Terminator version 3.1 mix (Applied Biosystems). Samples were analysed on an Applied Biosystems 3730xl automated DNA sequencer (Applied Biosystems).

Sequence analysis

We sequenced 294 (out of 304) segments from eight genes for this study. The lengths (in parentheses) of the nucleotides sequenced for each gene segment are as follows: PB2 (2240), PB1 (2257), PA (2142), HA (1665–1701), NP (1457), NA (1397–1422), M1 protein (737), NS1 protein (671). Final sequences were assembled and edited with Sequencher version 4.7 (Gene Codes Corp.).

Our main objective was to examine the placement of all segments from each isolate into either Asian or North American clades as defined by our reference samples (below). Therefore, we used phylogenetic analyses (distance and Bayesian) to determine the most likely continental affinity of each gene segment, as Asian and North American clades of LPAI are well-differentiated (Ito *et al.* 1995; Widjaja *et al.* 2004). To test the clade affinities of each segment, we first selected two groups of reference sequences from the NCBI database (Bao *et al.* 2008). For each of the eight gene segments, we selected 12–31 sequences of LPAI strains from the database. Where possible, we selected sequences of Asian influenza viruses isolated in Japan, South Korea, and China, since these are areas where northern pintails are known to overwinter in large numbers (Perennou *et al.*

1994; Miyabayashi & Mundkur 1999). We also selected five to seven North American LPAI waterfowl sequences for each segment. No LPAI sequences of northern pintail are available from Asian sampling locales on the NCBI database and many sequences are not identified to host species. Therefore, the Asian reference samples we selected included those classified as 'aquatic bird', 'migratory duck', 'duck', and 'wild bird faeces'. Additionally, four samples classified as 'swan', three as 'egret', and one as 'gull' were also included. The majority of these reference sequences were from viruses isolated within the past 15 years. Sequences for each gene segment were then aligned using ClustalW version 1.4 in BioEdit 7.0.9 (Hall 1999).

We used PAUP* version 4.0b (Swofford 2003) to generate neighbour-joining trees using the branch-and-bound method with 10 000 bootstrap replicates. The best-approximating model of nucleotide evolution (GTR), as determined by MODELTEST version 3.06 (Posada & Crandall 1998), was incorporated into the analysis. For Bayesian analysis, we used the program MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003) to construct posterior probabilities of support for clade differences. Each analysis was run for 5×10^6 generations using four heated chains following a burn-in of 5000 generations. Average posterior probabilities of the 50% majority rule consensus tree topologies were estimated using a sampling of likelihood parameters every 100 generations. Trees were visualized with TreeView (Page 1996).

Following the construction of phylograms, we determined that a viral reassortment event had occurred between Asian and North American viruses when a gene segment isolated from a northern pintail in North America was most closely related to Asian reference genes. In summarizing these events, we first tabulated all events per gene segment. Next, to make direct comparisons to the reassortment events as reported in Krauss *et al.* (2007), we determined if several closely related northern pintail strains formed a clade within the Asian strains. In such cases, only a single event was considered to have occurred. Nucleotide sequences for all gene segments in this article have been submitted to GenBank under Accession nos EU557376–EU557669.

Results

We generated complete sequence data for 294 segments and obtained virus subtype information for all 38 isolates. Sampling locales of these 38 isolates came from a large geographic area, including four National Wildlife Refuges (Yukon-Kuskokwim Delta, $n = 5$; Yukon Flats and Koyukuk, $n = 12$; and Izembek, $n = 11$), one State Game Refuge (Minto Flats, $n = 4$), as well as locations near Palmer ($n = 3$) and Nome, Alaska ($n = 3$). We observed eight HA subtypes, with H3 (44.7%) as the most common (Fig. 1). All
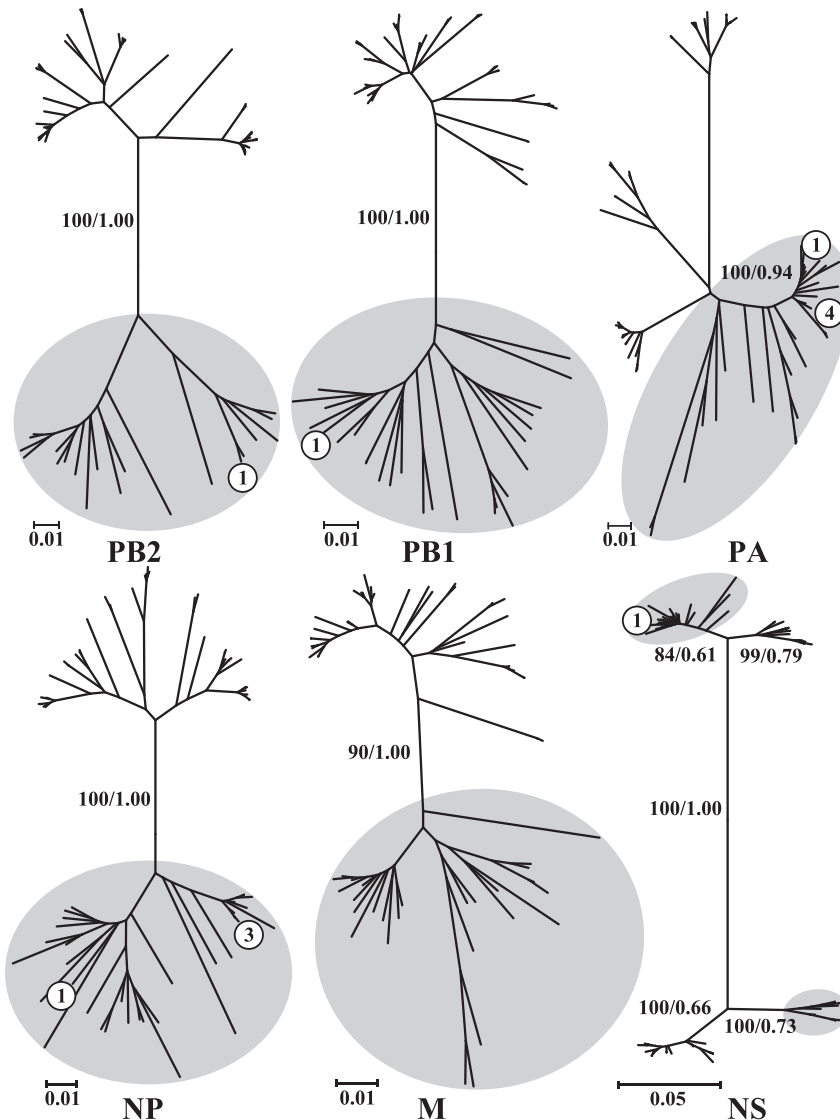
Fig. 2 Unrooted neighbour-joining phylograms of Asian and North American lineages of PB2, PB1, PA, NP, M, and NS genes. Shaded areas indicate Asian lineages. Circled numbers indicate the number of reassortment events for Alaskan northern pintails at each gene segment. The level of neighbour-joining bootstrap support and Bayesian posterior probabilities between major groups is shown along branches (separated by a slash). Scale bar indicates substitutions per site.

NA subtypes were represented (Fig. 1) with the most frequent being N8 (36.8%). The most common subtype combination among northern pintails was H3N8 (34.2% of all samples), followed by H4N6 (18.4%), H10N7, H12N5, H3N6 (all 7.8%), plus eight additional subtype combinations (H2N3, H3N1, H5N9, H6N1, H6N2, H6N4, H6N8, H8N4) that occurred at low frequency (< 6%). No H5N1 subtype combination was observed.

The phylogenetic separation of Asian and North American lineages of LPAI was well supported for all gene segments by both distance and Bayesian methods (Figs 2–5). Seventeen of the 38 Alaskan isolates (45%) had one or more gene segments that were more closely related to Asian isolates than those found in North America and 12 of these occurred within the PB2, PB1, PA, NP, and NS segments (Fig. 2). Thirteen of these 17 isolates had a single reassortment event, whereas three had two events and one had

three. Across all 294 segments, we observed 22 (7.5%) that were more closely related to Asian reference samples (Table 1), with the largest number occurring among the HA genes (27%). When closely related sequences were removed, following the methodology of Krauss *et al.* (2007), a total of nine (3.1%) Asian reassortment events were determined to have occurred (Table 1, see Discussion).

Phylogenetic analysis of the most common LPAI subtypes (H3 and N8) revealed contrasting patterns of lineage ancestry. The phylogram of H3 sequences (Fig. 3) shows six Alaskan samples nested within the Asian group of reference sequences. Three of these isolates were identical across all nucleotides of the H3 gene. The H6 phylogram (Fig. 4) is similar to that of the H3 gene, with four Alaskan isolates nested within the Asian group. Phylograms of the remaining HA subtypes contained no reassortment events (not shown). The N8 phylogram (Fig. 5) revealed five
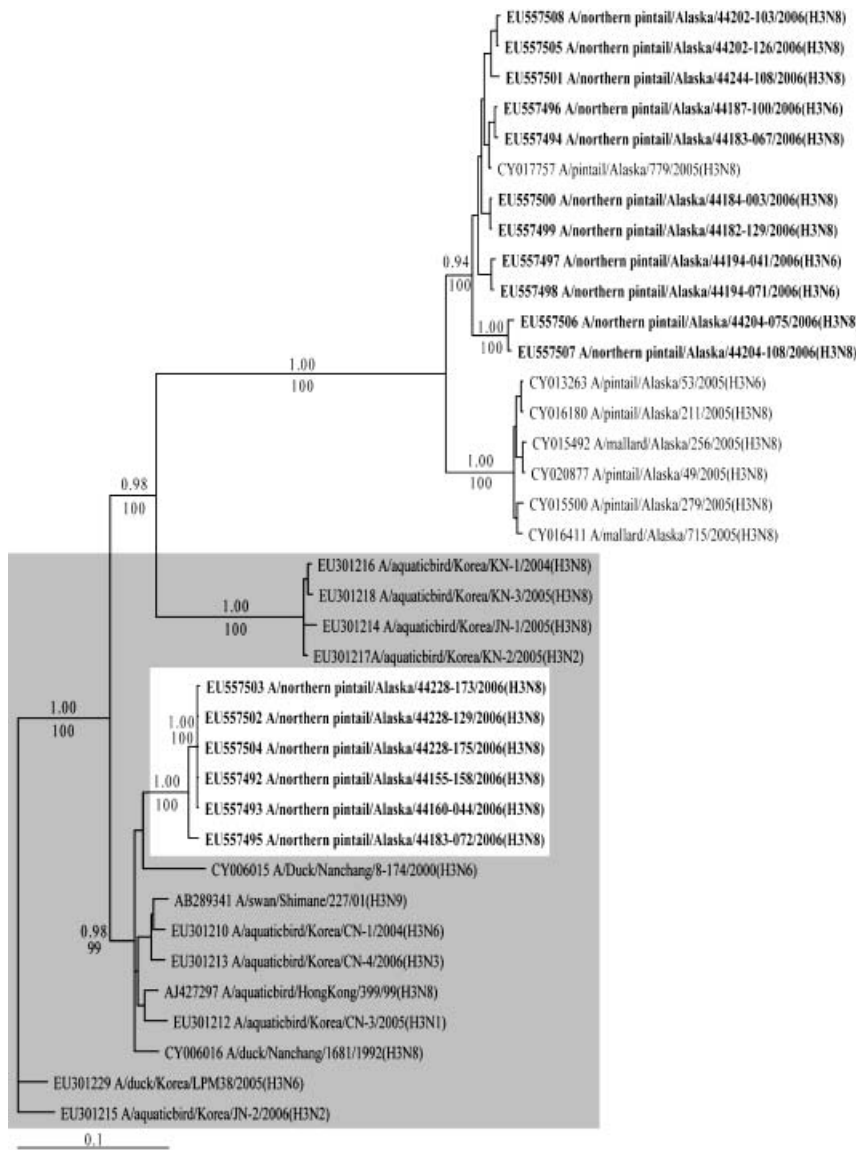
Fig. 3 Phylogram of the most common HA subtype (H3) for 17 Alaskan northern pintail samples (shown in bold). Shaded areas indicate Asian lineages. Comparative Asian and North American sequences are shown in non-bold font. Bayesian posterior probabilities and the level of neighbour-joining bootstrap support between major groups are shown above and below branches, respectively. Scale bar indicates substitutions per site.

Table 1 Frequency of Asian-origin avian influenza lineages among viral sequences of northern pintails sampled in Alaska. Numbers in brackets are values after removing closely related sequences (see Materials and methods)

	PB2	PB1	PA	HA	NP	NA	M	NS	Total
Segments analysed	36	37	37	37	36	35	38	38	294
Asian segments in North American clades	1 (1)	1 (1)	5 (2)	10 (2)	4 (2)	0 (0)	0 (0)	1 (1)	22 (9)
Per cent of Asian events/segment	2.8 (2.8)	2.7 (2.7)	13.5 (5.4)	27.0 (5.4)	11.1 (5.6)	0.0 (0.0)	0.0 (0.0)	2.6 (2.6)	7.5 (3.1)

Asian reference sequences nested within the group of northern pintail isolates and North American reference samples. A similar pattern was observed for the phylogram of the N1 subtypes, but the number of Asian lineages within the North American group was limited to a single sequence (not shown).

Discussion

The observed frequency of reassortment events (45%) of Asian and North American virus lineages in our study is considerably higher (7.5×) than what was found in a recent global study (Dugan *et al.* 2008) that reported 6%
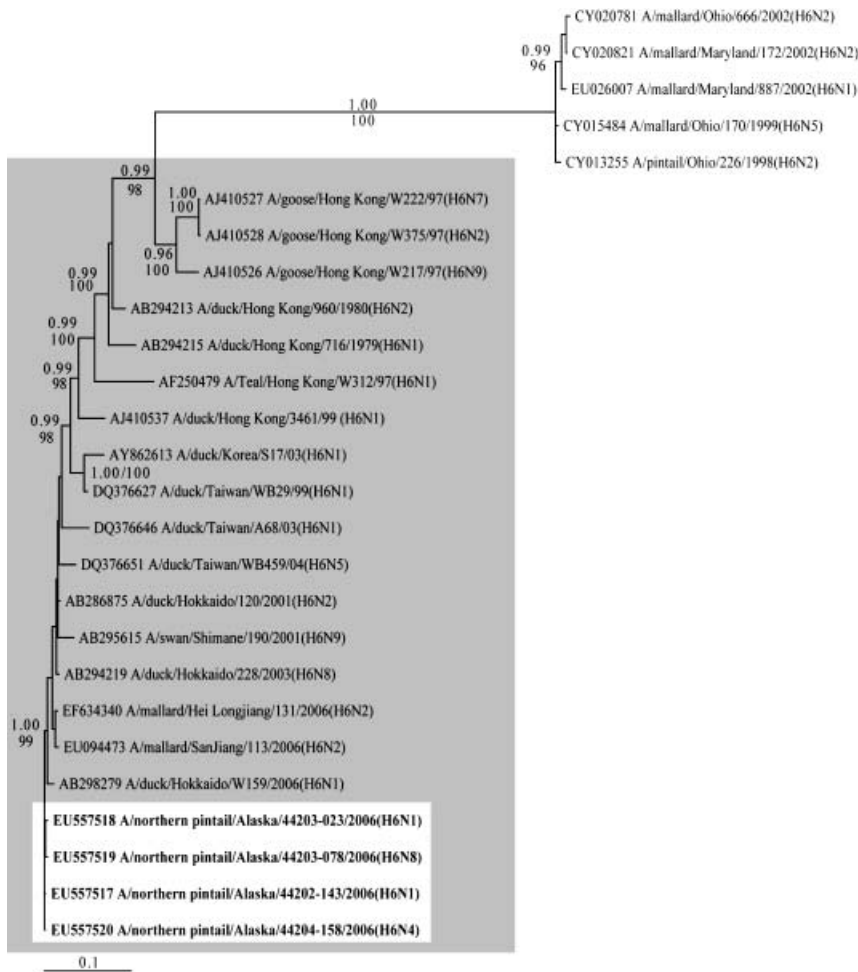
Fig. 4 Phylogram of the H6 HA subtype for four Alaskan northern pintail samples (shown in bold). Shaded areas indicate Asian lineages. Comparative Asian and North American sequences are shown in non-bold font. Bayesian posterior probabilities and the level of neighbour-joining bootstrap support between major groups is shown above and below branches, respectively. Scale bar indicates substitutions per site.

hemispheric reassortment. Krauss *et al.* (2007) excluded closely related LPAI replicates, arguing that they are not necessarily representative of independent events, and reported the frequency of intercontinental exchange at the gene segment level as 0.64%. Following this methodology, the number of Asian reassortment events in our study is reduced to 3.1% (Table 1) and that of Dugan *et al.* (2008) is reduced to < 1%. Thus, regardless of the exclusion of closely related lineages, we found a considerably higher frequency of reassortment events that contain Asian lineages than previously reported. Because the likelihood of detecting intercontinental reassortment events is directly related to the degree of contact among host populations, we found a higher frequency of intercontinental reassortment because Alaska northern pintails are closer to the source of Asian lineages. The lower frequency of LPAI strains found with Asian lineages by Krauss *et al.* (2007) and Dugan *et al.* (2008) is likely caused by dilution (i.e. further reassortment) related to the increased distance from areas where Asian lineages commonly circulate.

Although our data support intercontinental transfer of LPAI segments, we suspect that our assessment of the level of transfer is still biased low. To date, all studies of hemispheric reassortment among LPAI viruses in North American birds, including ours, have made comparisons to Asian gene sequences available on the NCBI database. Because many strains in the database are not classified to species, we are likely comparing northern pintail LPAI strains to species that do not exhibit similar migratory tendencies or patterns of breeding and wintering distribution as have been documented for the northern pintail (Kear 2005; Miller *et al.* 2005). Accordingly, we likely underestimated the degree to which viruses are exchanged between wild migratory birds in Alaska and Asia. We suspect that a whole-genome comparison of LPAI viruses sampled from northern pintails in Asia and Alaska would show greater evidence of exchange than we report here. Furthermore, we predict that such a comparison would reveal individuals with a mixture of both Asian and North American lineages, as suggested by our data for the N8 and N1 lineages (Fig. 4). Similar observations were made in a single green-winged teal (*Anas crecca*) wintering in Japan (Kida *et al.* 1987; Bean *et al.* 1992).

We also question whether LPAI viruses in which all eight gene segments are of Asian descent (i.e. completely
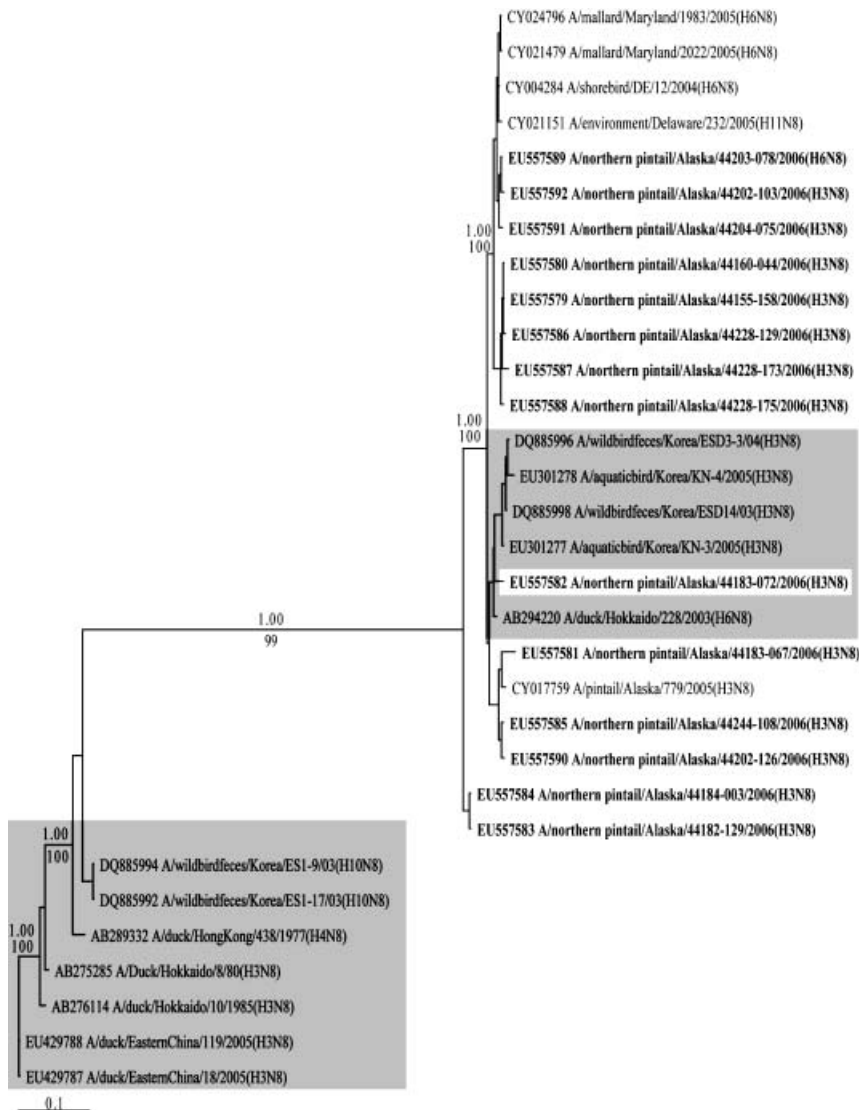
Fig. 5 Phylogram of the most common NA subtype (N8) for 14 northern pintail samples (shown in bold). Shaded areas indicate Asian lineages. Comparative Asian and North American sequences are shown in non-bold font. Bayesian posterior probabilities and the level of neighbour-joining bootstrap support between major groups is shown above and below branches, respectively. Scale bar indicates substitutions per site.

Asian-origin viruses) persist in substantial frequency in northern pintails. Our study, along with others (Krauss *et al*. 2007; Dugan *et al*. 2008), observed no completely Asian viruses, but this may be related to the fact that northern pintails tend to show high rates of LPAI virus exposure (Ito *et al*. 1995; Runstadler *et al*. 2007; Ip *et al*. 2008) combined with 'extremely frequent' reassortment (Dugan *et al*. 2008). Reassortment can only occur when individuals are co-infected by multiple LPAI strains (Sharp *et al*. 1997) and the probability of co-infection appears positively related to overall virus prevalence. Wang *et al*. (2008) found co-infection in 16% of samples where the overall prevalence of influenza viruses was 26% (i.e. 61% of the positive samples were co-infections). Furthermore, novel virus types may be more likely to result in co-infections (Sharp *et al*. 1997).

Thus, dispersal of northern pintails within and between continental wintering populations, as demonstrated with satellite telemetry, banding data, and putatively neutral genetic markers (Cronin *et al*. 1996; Miyabayashi & Mundkur 1999; Miller *et al*. 2005), should over time facilitate contact with novel virus lineages, but perhaps only at northern latitudes during the breeding period when different populations come into contact. Northern pintails that migrate along the Pacific coast of both Asia and North America spend winters within, what we suspect, are isolated LPAI gene pools as demonstrated by deep phylogenetic divergence (Ito *et al*. 1995; Krauss *et al*. 2007; this study) and remarkably different virus subtype combinations. Influenza subtypes H3N8 and H4N6 were the most common among northern pintails sampled in Alaska (Fig. 1), similar to previous surveys of LPAI in Alaskan wild waterfowl (Ito *et al*. 1995; Runstadler *et al*. 2007), but different from subtype combinations found among northern pintails in Japan during winter (Jahangir *et al*. 2008). Additional research is

needed on whole LPAI genome surveys of wintering populations of northern pintails in both Asia and North America, but initial indications are that the number of Asian lineages per isolate is lower on wintering areas (Dugan *et al.* 2008), likely a result of continuous reassortment and distance from the source of Asian lineages.

In summary, while our data support previous conclusions of large genetic differences between hemispheric populations of LPAI, we find greater evidence that wild migratory birds in Alaska are a mechanism for the movement of LPAI viruses across this phylogenetic boundary. Alaska is a major crossroad of Eurasian and North American migratory flyways, and thus, may harbour migratory birds with greater frequencies of co-circulating LPAI lineages than other areas of North America, especially among those species that exhibit high virus prevalence, such as the northern pintail. We suggest that species such as northern pintails that maintain relatively high prevalence of influenza viruses (Runstadler *et al.* 2007; Ip *et al.* 2008) and migrate long-distances among divergent virus gene pools (Miller *et al.* 2005), may have a greater chance for co-infection, and thus reassortment, with novel viruses that they come into contact with. When viewed collectively with previous surveys of LPAI genetic variation in North America (e.g. Krauss *et al.* 2007; Dugan *et al.* 2008), our data demonstrate that substantial geographic and species variation likely exist in levels of intercontinental gene exchange in LPAI viruses. Such variation should serve as a valuable tool for directing future avian influenza surveillance programmes in wild birds. For example, full-genome LPAI phylogenetic analyses could be used to optimize and prioritize surveillance sampling by targeting habitats used by species with the highest frequencies of virus prevalence, as well as the greatest likelihood of contact with areas where HP H5N1 occurs. Geographic patterns of LPAI genome variation should also inform our understanding of mechanisms of circulation and persistence of avian influenza viruses in wild birds.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Previously and newly designed polymerase chain reaction primers used in the amplification of Alaska northern pintail avian influenza gene segments

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