Assessment of virus movement across continents: using Northern Pintails (*Anas acuta*) as a test

A collaborative study between U.S. and Japanese researchers

Progress Report
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Summary

Personnel from the USGS Alaska Science Center, National Wildlife Health Center, and Western Ecological Research Center collaborated with colleagues from the U.S. Fish and Wildlife Service, University of Tokyo, Miyagi Prefectural Izunuma-Uchinuma Foundation, and the Yamashina Institute of Ornithology to study whether migratory birds might carry the highly pathogenic H5N1 avian influenza virus from Asia to North America. We are using Northern Pintails (*Anas acuta*) as a model species to study intercontinental virus transmission because the species frequently migrates between Asia and North America, is abundant, and often carries avian influenza viruses. The various aspects of this project include: (1) an evaluation of spatial and temporal overlap of North American and Asian pintail populations through analysis of band recoveries, and marking of pintails with satellite transmitters on their Japanese wintering areas, (2) comparison of genetic differentiation of Asian and North American pintails to determine the degree to which populations are reproductively isolated, and (3) contrasting of strains of non-H5N1 viruses found in Northern Pintails on Japanese wintering areas and those on North American breeding and wintering areas to assess the recent and historic transcontinental transmission of avian viruses. Here we report on progress in these three areas of the study in 2007.

We marked 27 pintails with satellite transmitters at one of their main wintering areas in Japan in February 2007. Fourteen pintails survived with operable transmitters to depart Japan for breeding areas in eastern Russia, and nine successfully completed migration to likely nesting and molting areas. Preliminary results suggest that after departing the northern Japanese island of Hokkaido, pintails either migrate to Sakhalin Island before going to the Russian mainland, or make a non-stop migration of >1600 km over the Sea of Okhotsk to the Kamchatka Peninsula or eastern Chukotka. Though results of the satellite telemetry are preliminary, there was considerable overlap between satellite telemetry locations of Japanese pintails on Russian nesting and molting areas, and areas that had a 95% likelihood of use by North American pintails, as delineated from band recovery data.

In 2007, we collected DNA samples from 77 wintering pintails in Japan and 147 pintails throughout wintering areas in California. We used mitochondrial and nuclear DNA markers to assess the degree of genetic contact (i.e., gene flow) between groups of Northern Pintail ducks that winter in Japan and California. There was no evidence for genetic differentiation between the two groups using either type of genetic marker. Thus, we conclude there is strong evidence for gene flow between pintails that winter in Japan and California.

We examined genetic characteristics of low pathogenic avian influenza viruses collected via cloacal swabs from wild Northern Pintails throughout the state of Alaska in 2006. All samples were analyzed at the USGS National Wildlife Health Center. To examine relationships between virus sequences from Alaskan pintail samples to those in Asia, we obtained a representative sample of sequences from GenBank for each of the eight viral RNA segments. A total of 299 viral RNA segments from 38 Alaska Northern Pintails were sequenced. All segments except the matrix (M) segment showed that at least one sample was more closely related to Asian than North American lineages. Across all segments, we observed 8.6% to exhibit a closer association to Asian than North American lineages. Thus, there is evidence for viral reassortment among Eurasian and North American lineages within wild Northern Pintails sampled in Alaska.

Preliminary satellite telemetry and band recovery data indicate a large area of sympatry between Asian and North American pintails in eastern Russia. Genetic data indicate no evidence of restricted gene flow between continents, and suggests that interbreeding of Asian and North American pintails frequently occurs. Preliminary genetic analysis of low pathogenic viruses carried by Northern Pintails in Alaska provides evidence of historical transmission of Eurasian viruses to North America. Our combined satellite telemetry, banding, genetic, and virus data suggest that Northern Pintails can be used as an effective model for estimating rates of viral exchange. This study will continue in 2008 and 2009.

I. Spatial Overlap of North American and Asian Pintails

Evaluating exchange of avian influenza subtypes between Asia and North America by Northern Pintails requires a clear understanding of where birds from each continent are likely to come into contact. In 2007 we marked pintails with satellite transmitters on one of their main wintering areas in Japan in order to evaluate their distribution on Russian nesting and molting areas. Satellite telemetry, combined with band recovery data will enable us to model the spatial and temporal distribution of Asian pintails during migration and breeding, and to more clearly define geographic overlap with North American pintails.

Methods

We collaborated with colleagues from the University of Tokyo, Laboratory of Biodiversity Science to capture 158 pintails at Lake Izunuma-Uchinuma in the Miyagi Prefecture of northern Honshu on 13 February, 2007 (Figure 1). Capture was made via a traditional Japanese "clap-trap". Captured pintails were transported to the Izunuma-Uchinuma Environmental Foundation where we weighed each bird, and classified sex and age based on wing plumage. We also collected contour feathers from a sample of birds for genetic analysis.

We attached satellite transmitters to 16 adult males and 11 adult females that were above-average body mass. We used 18 g solar-powered platform transmitting terminals (PTTs) manufactured by Microwave Telemetry Inc. Japanese colleagues modified the PTTs prior to our arrival by attaching a wire frame to the base of transmitters (Figure 2). The frame held the PTT approximately 1 cm above the back of a bird to prevent feathers from covering the solar panel. Japanese biologists attached PTTs to the backs of birds via Teflon ribbon that was secured using a modified figure eight harness. Pintails were released *en masse* near the capture site after marking.

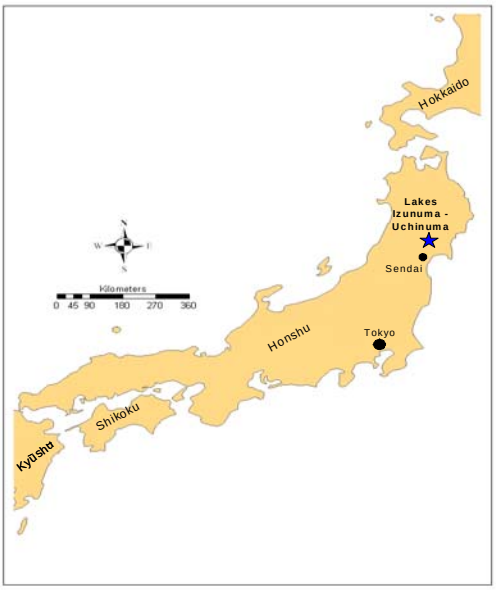

Figure 1. Location in Japan where Northern Pintail ducks were captured and radiomarked.

Figure2. Attachment of a solar-powered PTT to a male Northern Pintail.

PTTs were programmed to transmit 10 hours within each 34 hour period. We received data through the Argos Data Collection and Location System (Largo, Maryland). Location class (LC) was assigned as described by Argos (2008). We filtered PTT data via a computer program that enabled us to remove unlikely locations based on the rate of movement, distance, and angle between locations (Douglas 2006). A location was retained if it was $\leq$30 km from the previous or subsequent positions, or if the rate of movement between adjacent locations was $\leq$100 km/hour. We selected the location that had the highest quality within a 10-hour transmission cycle to represent the bird's location on that day. We collected data until no further signals were obtained from a PTT, or until no further movement was apparent, indicating the bird had died or the PTT had fallen off the bird. If we suspected mortality or transmitter loss, we deleted data past the last date on which movement was observed. Data presented here are for movements of pintails through 5 October, although PTTs were monitored until 26 November to ensure no further movements were observed.

We examined records from the USGS Bird Banding Laboratory to identify recovery locations in Asia of Northern Pintails banded in North America. We restricted the analysis to birds banded on North American wintering areas and recovered during the breeding season in Asia. That was so the recovery distribution represented that for the component of the population that is most likely to come into contact with Asian pintails, and to potentially carry viruses back to wintering areas in North America. We used a kernel home range analysis to draw 95% and 50% utilization distributions around recoveries in Asia to define the region where North American pintails were most likely to occur.

Results

Mean body mass of marked females was 851 g (SD = 42) whereas that of all captured females was 759 g (SD = 78). Average mass of marked males (960 g, SD = 51) was higher than for all captured males (919 g, SD = 84). Mean duration that PTTs were monitored was 130 days (range 18-234 days), with an average of 98 (range 10-187) 10-hour transmissions from each PTT. On average we received 6 locations during each 10 hour transmission for a PTT. We observed a higher proportion of invalid locations (LC Z) or locations with unknown error (LC A, B) when pintails were in Japan, although the proportion of poor quality locations diminished as pintails migrated north (Figure 3).

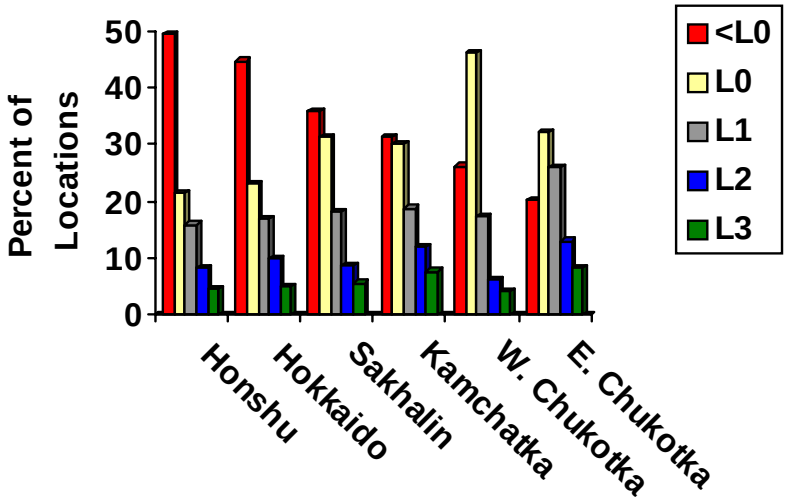

Figure 3. Quality of Northern Pintail satellite telemetry locations when birds were in different regions of Asia. Location Class <LO includes class A, B, and Z. Based on 18,215 locations.

Median departure (movement of >20 km) date of pintails from Lake Izunuma-Uchinuma was 28 February (13 February – 12 March). Median departure dates of males (27 February) was similar to that of females (28 February). After departure, pintails mainly moved to agricultural regions of Honshu that were north and west of the capture site (Figure 4). Twenty pintails (13 males, 7 females) survived to depart Honshu. Median departure date from Honshu was 6 April. Pintails spent a median of 44 days on Honshu after their departure from Lake Izunuma-Uchinuma.

All birds that departed Honshu stopped on Hokkaido where they mainly used agricultural habitats in the western region of the island (Figure 4). For 14 pintails that survived to depart Hokkaido, median duration of spring staging on Hokkaido was 28 days, and median departure date was 16 May (range 27 April – 23 July). Median departure of five females was slightly earlier (14 May) than that of 9 males (21 May). Three pintails died in transit over the Sea of Okhotsk after migrating 680-1140 km from

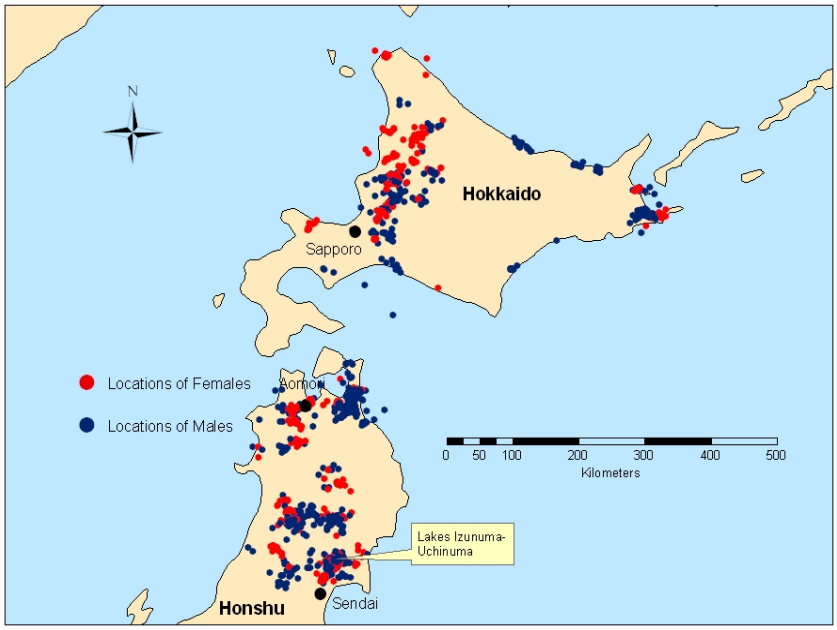

Figure 4. Satellite telemetry locations in Japan of 27 Northern Pintails marked with PTTs at Lake Izunuma-Uchinuma, February – June, 2007.

Hokkaido. One female returned to Hokkaido after migrating 280 km over the Sea of Okhotsk and made no further effort to migrate.

Ten pintails (5 males, 5 females) completed migration to Russia after departing Hokkaido (Figure 5). Five pintails that migrated to the Kamchatka Peninsula made long nonstop flights (1370-1680 km) over the Sea of Okhotsk. Two of those pintails continued approximately 1000 km to the Anadyr Lowlands where they arrived between 25 May and 1 June after stopping for 8-23 days on the Kamchatka Peninsula (Figure 5). Five pintails that migrated to Sakhalin Island made relatively short initial flights (230-640 km) from Hokkaido, and then migrated to the northern end of the Sakhalin Island. After spending 15-30 days on Sakhalin Island, three of those individuals made flights of 630-990 km across the Sea of Okhotsk to the vicinity of Magadan or the Kolyma River, where they arrived between 4—8 June. A male pintail migrated 1270 km from Sakhalin Island to the Kamchatka Peninsula on 27 May. Only one PTT continued to transmit as a pintail made a return migration from its northward terminus. That individual migrated from the Anadyr region on 23 September to the northern Kamchatka Peninsula, but made no further movements between 5 October and 26 November when monitoring terminated.

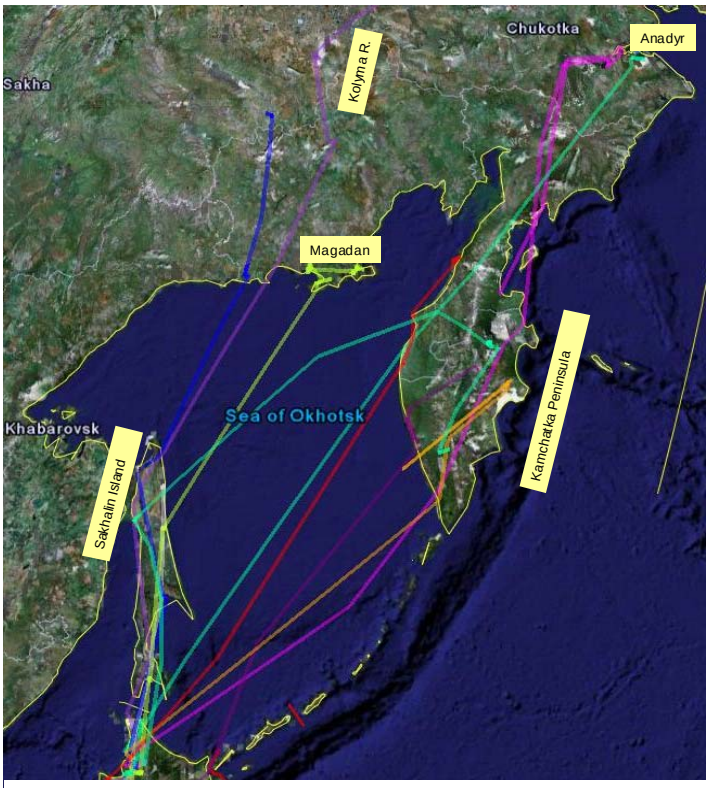

Figure 5. Migration routes of 10 Northern Pintail ducks from Japan to Russia, April-July, 2007.

There were 130 breeding season recoveries in Asia of pintails banded in winter in North America. The majority of recoveries were in eastern Chukotka and the Kamchatka Peninsula (Figure 6). There were a smaller number of recoveries near Magadan and the Kolyma River. Though results from satellite telemetry studies are preliminary, these are largely the same regions used by pintails marked with PTTs in Japan (Figure 7).

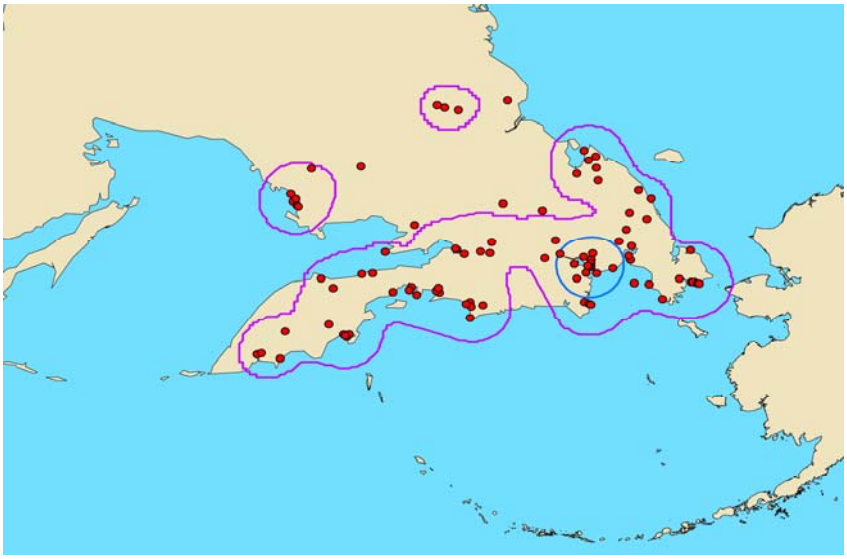

Figure 6. Recovery distribution in Russia of Northern Pintails banded during winter in North America. The outer purple contour line represents a 95% utilization distribution as determined from a kernel home range analysis. The inner blue contour is a 50% utilization distribution.

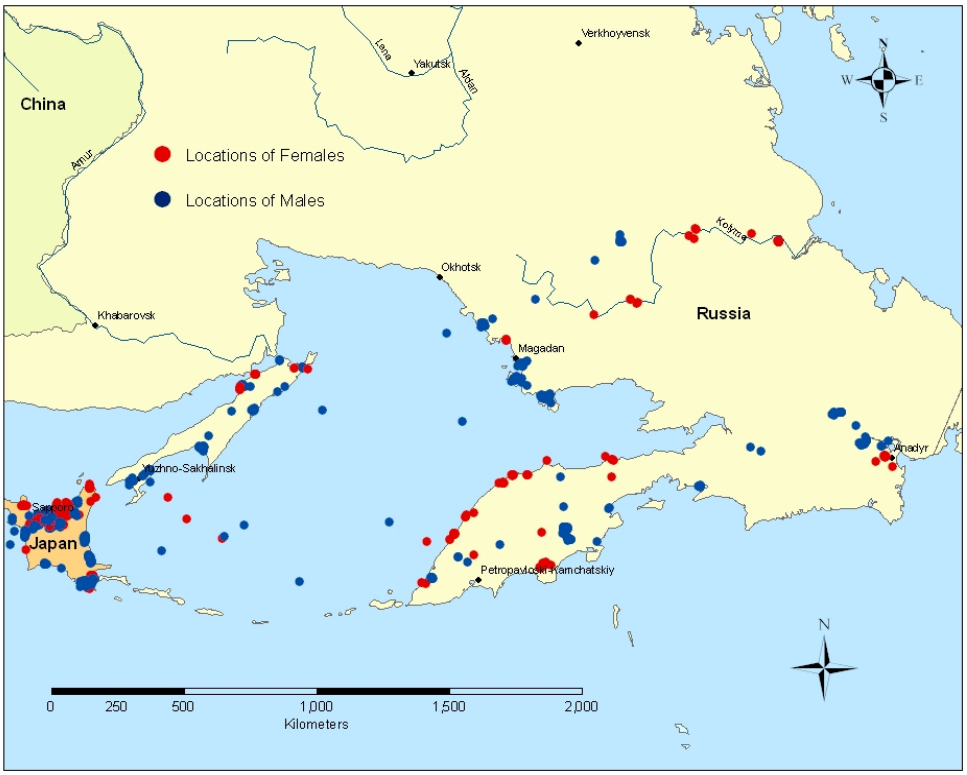

Figure 7. Locations in Russia of 10 Northern Pintails marked with satellite transmitters in Japan, May-October 2007.

Discussion

Location frequency of PTTs was high and there were relatively few transmission periods in which signals were not received by satellites. As a result, we received a large number of locations (18,566), and we were able to select high quality locations (LC 1, 2, or 3) within most transmission periods for analysis, even though the proportion of such locations was relatively low when birds were in Japan. The higher proportion of poor quality locations in Japan may have resulted from electronic interference that exists in Eastern Asia (Microwave Telemetry, Inc. 2007). Location quality likely improved as pintails migrated because birds moved into regions with less interference and more favorable satellite configuration.

Although location frequency was high, PTTs or harnesses failed, or birds died at a higher rate than observed by Miller et al. (2005) for 26-g battery powered PTTs attached to pintails in California via external harness (Figure 8). Adult female pintails that we marked in Japan weighed slightly less than adult female pintails marked by Miller et al (2005; 900-950 g). A higher mortality rate is plausible if Japanese pintails were in poorer physiological condition. However PTT configuration and harness attachment also differed from Miller et al.'s study.

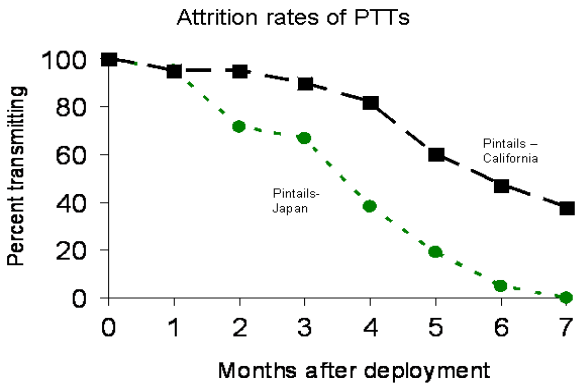

Figure 8. Comparison of the rates at which PTTs failed or marked birds died for Northern Pintails marked in Japan versus California

After departing their winter site at Lake Izunuma-Uchinuma, pintails spent a prolonged premigratory period ($\bar{x}$ = 83 days) on Honshu and Hokkaido before migrating to Russia. This migration behavior is comparable to that of many pintails that winter in the Central Valley of California, and which have a prolonged (up to 2 months) staging interval in southern Oregon and northeastern California before continuing

migration to Alaska and Canada (Miller et al. 2005). In each, pintails use migratory habitats a relatively short distance north of their winter area before embarking on longer migrations to nesting areas. Pintails which migrated to the Kamchatka Peninsula did so via non-stop migrations over the Sea of Okhotsk. A prolonged staging period before departure from Japan is likely necessary for pintails to acquire endogenous reserves for long-distance flights. Birds that migrated to Sakhalin Island made shorter initial migrations, but also made long non-stop flights over the Sea of Okhotsk. Overall, some pintails migrated up to 3,700 km from their wintering area at Lake Izunuma-Uchinuma to breeding areas in Russia. That distance is comparable to the migration that North American pintails make between California wintering areas and breeding areas in western Alaska.

The number of pintails that reached Russia in 2007 was too small for analysis of distribution on the breeding area. However, preliminary results suggest there was considerable overlap with areas where band recoveries of pintails marked in North America have been reported. Preliminary results also suggest two migration routes may exist; (1) migration through Sakhalin Island to western Chukotka or (2) migration to the Kamchatka Peninsula with some birds continuing to the eastern Chukotka Peninsula. Seven of the 27 pintails that we marked reached the breeding range of Japanese pintails in Russia outlined by Bianki and Dobrynina (1997). Additional marking of pintails with satellite transmitters is needed to define spatial and temporal distribution in Russia of pintails that winter in Japan.

Future Plans

We plan to again collaborate with Japanese colleagues to mark 52 Northern Pintails with PTTs in Japan in 2008, and to mark a comparable number in 2009. The sample size of marked birds will be increased to compensate for attrition of PTTs observed in 2007. In an attempt to reduce failure of PTTs and mortality of marked pintails, we will deploy 26-g battery powered PTTs in 2008 and use the same harness design employed by Miller et al. (2005). In 2008, we will deploy 30-40 PTTs at Lake Izunuma-Uchinuma, and the remaining PTTs in the Iwate Prefecture, approximately 150 km to the north. We will obtain band recovery for pintails from the Yamashina Institute of Ornithology in order to contrast recovery distributions in Russia of pintails banded in Japan versus those banded in North America.

Project personnel

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The Yamashina Institute of Ornithology: *Kiyoaki Ozaki*

II. Population genetic characteristics of Northern Pintails wintering in Japan and California: evidence for genetic exchange

Contact among groups of organisms can be assessed in a variety of ways. In 2007, we collected DNA samples from wintering pintails in Japan and throughout the state of California. We used mitochondrial (mt) and nuclear DNA markers to assess the degree of genetic contact (i.e., gene flow) between groups of Northern Pintail ducks that winter in Japan and California. Portions of these wintering populations appear to breed in sympatry in northeastern Russia and Alaska, based on banding and radio telemetry studies.

Methods

During the winter of 2005-2006, we collected 147 tissue samples from wings of Northern Pintails that were submitted to the U.S. Fish and Wildlife Service parts collection survey. Samples were obtained from throughout the Central Valley of California where the bulk of Northern Pintails winter and samples came from both males and females and a variety of age classes. We collected feather samples from 77 Northern Pintails in Japan during February of 2007. DNA was extracted from tissue and feather samples following the protocols used by Pearce et

al. (2005). The protocols described by Pearce et al. (2005) for mtDNA sequencing and microsatellite DNA genotyping were identical to those used in this study, except that the microsatellite primers differed. For Northern Pintails, we used primers for the following loci: Aph01, Aph07 and Aph09 (Maak et al. 2003), Bca10, Bca11 and Hhi5 (Buccholz et al. 1998), Sfi4, Sfi5, Sfi7and Sfi8 (K. Scribner, unpubl. data).

For each microsatellite locus, we calculated allele frequencies, allelic richness, and observed (H_o) and expected (H_e) heterozygosity using Program ARLEQUIN version 3.01 (Schneider et al. 1997). GENPOP was used to conduct exact probability tests for deviations from Hardy-Weinberg equilibrium in each sampling area. We used the program GENEPOP (Raymond and Rousset 1995) to test genotypic linkage disequilibrium for each pair of loci in each sampling area. Deviations from Hardy-Weinberg were also assessed by estimating Wright's inbreeding coefficient, F_{IS}, across all loci for each sampling area using Program FSTAT version 2.9.3 (Goudet 1995). Positive values of F_{IS} indicate heterozygote deficiency, a signal of inbreeding or population admixture (i.e., Wahlund effect), whereas negative values indicate heterozygote excess. For mtDNA sequence data, we graphically displayed the relationship of all mtDNA haplotypes using a network diagram constructed with the program Network version 4.2 (Bandelt et al. 1999).

To examine patterns of genetic differentiation between Japan and California samples, we used an analysis of molecular variance (AMOVA) in ARLEQUIN to generate estimates of inter-population variance in nuclear allele (F_{ST}) and mtDNA haplotype (Φ_{ST}) frequency. For mtDNA sequence data, *F*-statistic analogs were generated using the Tamura and Nei model of nucleotide evolution as identified by the Program MODELTEST (Posada and Crandall 1998).

Results

We obtained microsatellite genotypes for 138 California samples and 77 Japan samples. There was no difference between the two groups for standard indices of genetic diversity, such as observed heterozygosity, allelic richness, or levels of inbreeding. There was no evidence for genetic differentiation between the two groups (F_{ST} = 0.002, P = 0.96), suggesting gene flow. We obtained mtDNA sequence data for 425 bases of the control region (domain I) for 53 Japan samples and 60 California samples. We observed 26 haplotypes in Japan and 23 in California. Seven haplotypes were shared between the two groups (Fig. 9). The remainder were single haplotypes in both areas, which is typical for species that have a large population size and that have experienced population growth. With mtDNA, there was also no evidence for genetic differentiation between the Japan and California samples (Φ_{ST} = 0.002, P = 0.44). A haplotype network (Fig. 9) shows that there is no clear sorting of mtDNA lineages by sampling locale, which is suggestive of either gene flow or recent isolation and no gene flow. However, the latter scenario is unlikely given the movement of pintails that we have documented between continents using band recovery data. Thus, we conclude that both nuclear and mtDNA are suggestive of gene flow between winter sampling areas in Japan and California.

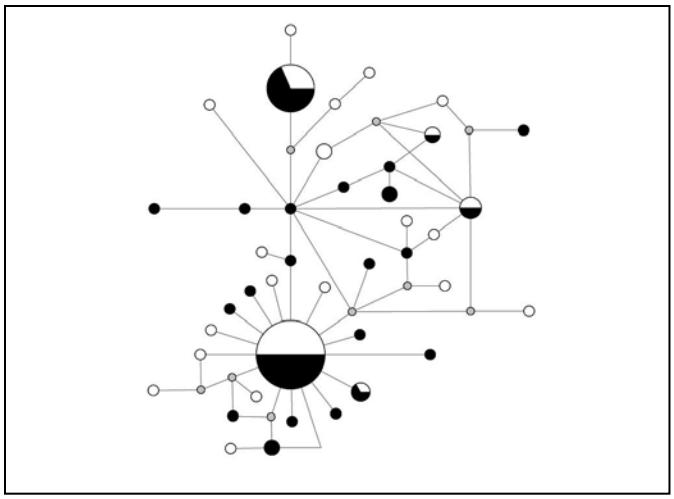

Figure 9. MtDNA haplotype network for all haplotypes observed among wintering pintails sampled in Japan (black circles) and California (white circles). Grey circles are inferred haplotypes and lines are single mutations that connect haplotypes. Circles size represents the number of haplotypes observed in each area, the smallest circles equaling a single sample.

Discussion

We observed evidence from putatively neutral genetic markers that gene flow occurs between pintails that winter in both Japan and California, a result that is expected given the overlap of band recoveries and satellite telemetry locations of birds marked in the two locations. This result is similar to the findings of Cronin et al. (1996) who observed that Northern Pintails sampled at different locales across North America had similar mtDNA types. The banding and telemetry data also suggest that gene flow is on-going and that our genetic data is not the result of recent divergence and little gene flow.

Future directions

This segment of the project is completed as we do not plan to sample additional pintails for DNA. Initial results presented here will be formally summarized in a manuscript that will be submitted to a peer-reviewed journal by summer of 2008.

Project personnel

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III. Exchange of influenza A viral segments between continents: initial evidence via comparative data

With this portion of the project, our main objective is to determine whether Northern Pintails that breed in sympatry in northeastern Russia – arriving there from wintering areas in both Asia and North America – also share viruses. If so, then we predict that over the long term, Eurasian segments of viral RNA should be present in Northern Pintail influenza A viruses isolated in both Alaska and the lower-48 United States. Similarly, we also predict that North American strains of these same low pathogenic influenza viruses should also be present in wintering Northern Pintails sampled in Japan.

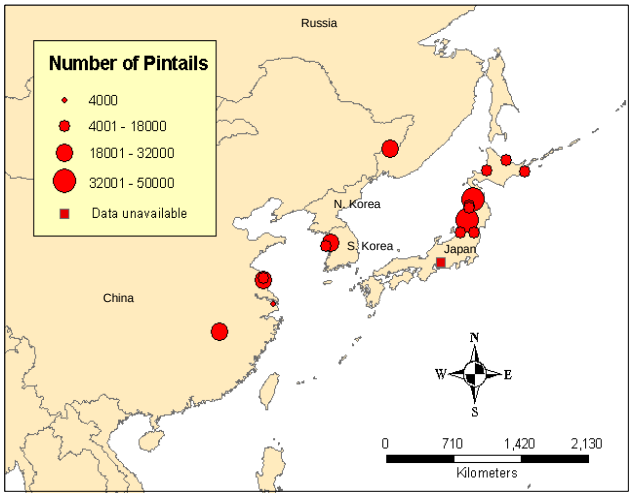

Figure 10. Locations of major wintering and staging areas for Northern Pintails in eastern Asia . Figure adapted from Miyabayashi and Mundkur (1999).

Methods

For this portion of the project, we used cloacal swab samples from wild Northern Pintails that were collected throughout the state of Alaska in 2006. All samples were analyzed at the USGS National Wildlife Health Center. Samples for which live virus culture was isolated were then subjected to RNA extraction and direct sequencing. We used a variety of PCR primers to amplify the full length of all eight RNA gene segments present in influenza A viruses. At this stage, 38 samples from Alaska have been isolated and sequenced. To examine relationships between virus sequences from these Alaskan pintail samples to those in Asia, we obtained a representative sample of sequences from GenBank for each of the eight viral RNA segments. Geographic sampling from GenBank for Asian sequences was restricted to areas where large concentrations of Northern Pintails winter (Fig. 10). We

then used MEGA (Kumar et al. 2004) to depict these relationships graphically as phylogenetic trees.

Results

A total of 299 viral RNA segments from 38Alaska Northern Pintails were sequenced (Table 1). All segments except the matrix (M) segment showed that at least one sample was more closely related to Asian than North American lineages. Across all segments, we observed 8.6% to exhibit a closer association to Asian than North American lineages (Table 1). Evidence for these reassortment events is also visible in phylogenetic trees for these segments For example, the tree for the NP gene segment shows a clear separation between Asian and North American virus lineages, which has been previously reported (Widjaja et al. 2004), and also the location of five Alaska Northern Pintails that possess more Asian-line lineages for the gene segment (Fig. 11).

Table 1. Summary of Alaska Northern Pintail viral RNA sequencing effort (by gene segment) and frequency of reassortment events between North American and Asian lineages based on comparisons to GenBank samples.

	RNA gene segment								Total
	PB2	PB1	PA	HA	NP	NA	M	NS	
Total segments	36	37	37	38	37	38	38	38	299
Asian lineage	1	1	5	11	4	3	0	1	26
N. America lineage	35	36	32	27	32	35	38	37	272
Asian to N. America reassortment (%)	2.7%	2.7%	13.5%	28.9%	10.8%	7.8%	0.0%	2.63%	8.6%

Discussion

We observed some evidence for viral reassortment, among Eurasian and North American lineages, within wild Northern Pintails sampled in Alaska. Similar findings were recently reported for waterfowl (Widjaja et al. 2004). However, those authors argued that since only a few of the eight gene segments were observed to have Eurasian affinities and no "wholly" Eurasian viruses were found, that incidence of intercontinental transfer is a rare event. Our combined banding, genetic, and virus data prompts a different conclusion. While the mechanism of virus reassortment remains unknown at this time, Eurasian lineages present in Alaskan Northern Pintails suggest that this species can be used as an effective model for estimating rates of viral exchange.

Future directions

A formal summary of our virus sequencing effort to date is being prepared for the Alaska samples. Additional samples from wintering Northern Pintails in California are also being processed and will ultimately be compared to virus samples collected from Northern Pintails wintering in Japan. Samples from Japan will be analyzed in collaboration with Japanese virologists.

Project Personnel

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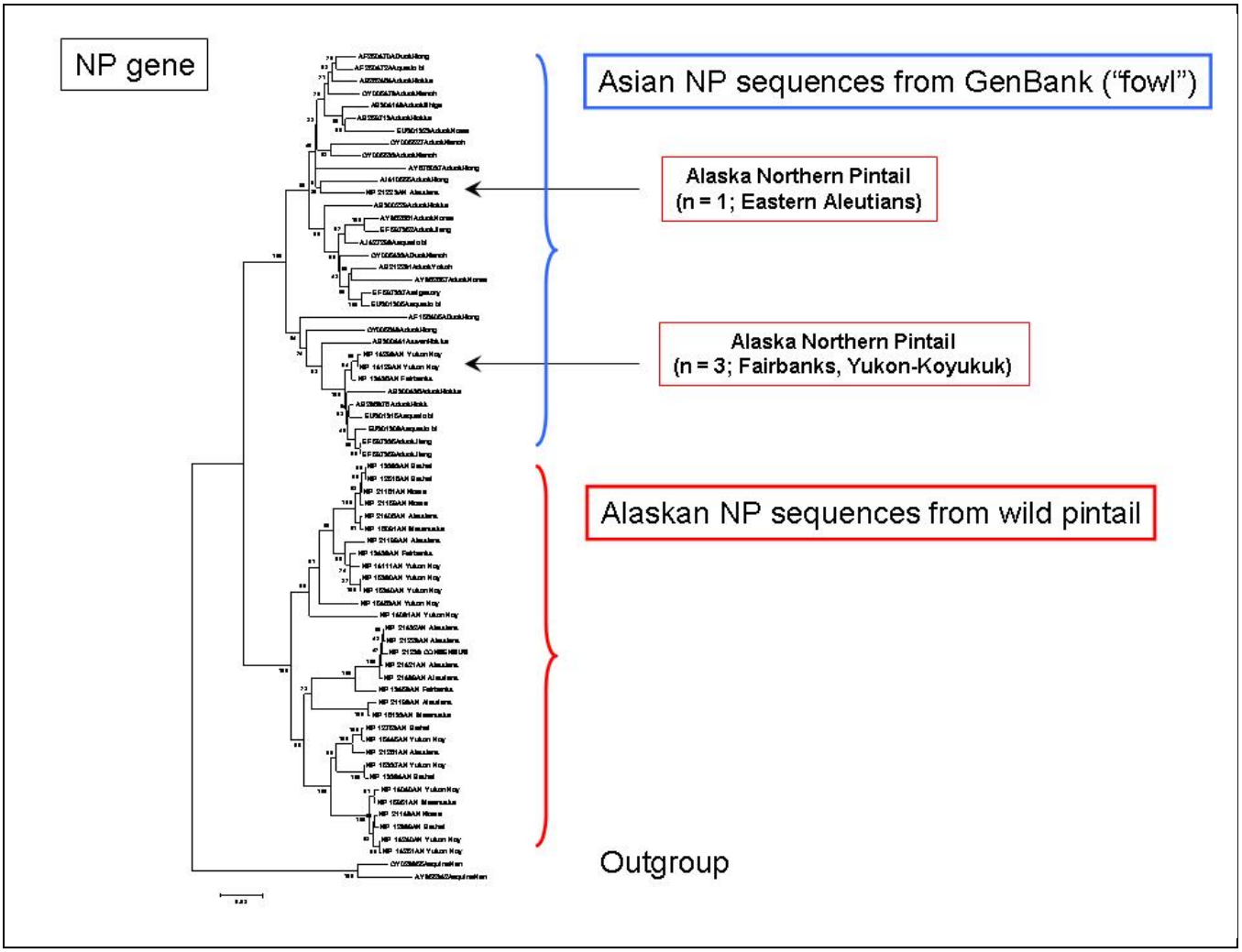

Figure 11. Phylogenetic tree of the NP RNA gene segment of influenza A for Northern Pintail from Alaska in comparison to a sample of available Asian sequences on GenBank for this same segment. Blue and red lines show the deep split between viral lineages and the location of four Northern Pintail samples from Alaska that exhibit Asian lineages.

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