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Evidence of Influenza A Virus RNA in Siberian Lake Ice▽

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Influenza A virus infects a large proportion of the human population annually, sometimes leading to the deaths of millions. The biotic cycles of infection are well characterized in the literature, including in studies of populations of humans, poultry, swine, and migratory waterfowl. However, there are few studies of abiotic reservoirs for this virus. Here, we report the preservation of influenza A virus genes in ice and water from high-latitude lakes that are visited by large numbers of migratory birds. The lakes are along the migratory flight paths of birds flying into Asia, North America, Europe, and Africa. The data suggest that influenza A virus, deposited as the birds begin their autumn migration, can be preserved in lake ice. As birds return in the spring, the ice melts, releasing the viruses. Therefore, temporal gene flow is facilitated between the viruses shed during the previous year and the viruses newly acquired by birds during winter months spent in the south. Above the Arctic Circle, the cycles of entrapment in the ice and release by melting can be variable in length, because some ice persists for several years, decades, or longer. This type of temporal gene flow might be a feature common to viruses that can survive entrapment in environmental ice and snow.

Influenza A virus is infamous for its ability to cause seasonal human epidemics; it affects approximately 10 to 20% of the world's population every year (31). Occasionally, it exhibits extreme virulence in poultry as well, bringing about unparalleled economic losses. Pandemics of influenza A virus in 1918 (subtype H1N1), 1957 (subtype H2N2), and 1968 (subtype H3N2) led to over half a million human deaths in the United States alone. The World Health Organization and the Centers for Disease Control and Prevention continually plan for the next worldwide pandemic and have stressed the importance of both disease and virus surveillance. Therefore, it is important to identify all of the biotic as well as abiotic reservoirs for this virus. Ice potentially constitutes an abiotic reservoir of prime importance for influenza virus over short and long periods of time, particularly in the Siberian region, which encompasses several migration routes of a variety of waterfowl.

Aquatic birds are the primary biotic reservoir for all influenza viruses (8). All influenza A virus subtypes (H1 to H16 and N1 to N9) have been isolated from birds and are considered to have descended from a primordial avian source (7, 30). Infection in domestic and wild species is usually asymptomatic, with occasional epizootic activity (29). Subclinically infected ducks have been shown to excrete enormous quantities of viral particles (up to 10^8 particles/gram feces). Although the primary hosts are birds, a variety of influenza A virus subtypes have been isolated from mammals, including swine, horses, seals, whales, felines, canines, and humans. It is of considerable note that H1N1, the subtype that resulted in over 40 million human deaths during the pandemic of 1918, continues to circulate in avian species (15). Other subtypes currently circulating (e.g., H5N1) have the potential to cause similar degrees of human mortality.

Influenza A virus is primarily an apathogenic enteric virus of birds and secondarily a pathogenic respiratory virus of mammals. Moreover, the spectrum of its avian hosts immeasurably exceeds that of its mammalian ones; the virus is thought to be fundamentally an archaic parasite of ancestral waterfowl. It is a prominent waterborne virus, persisting chiefly through fecal-oral circulation, and a common virus of holarctic migratory aquatic birds.

The Chinese-Siberian axis populations of migratory waterfowl play a role of paramount importance in the evolutionary course and dynamics of influenza A virus, due to the high percentage of infected individuals, the wide exchangeability of viral RNAs, and the broad range of enduring variant viruses (both at a given point of time and throughout long eras). Those parameters are interrelated and yet independent of each other. One outcome of their coincidence is the periodical emergence of pandemic and epizootic influenza A virus strains in the Chinese-Siberian domain.

Most studies of influenza A viruses have focused on biotic modes of transmission. Abiotic sources tested have almost exclusively been samples of water. In previous studies, we documented the preservation of viruses, bacteria, and fungi in glacial ice up to 140,000 years old (2–4, 10–12, 24). In the present study, we report the results from testing for the presence of influenza A virus genes in ice and water samples from three lakes in northeastern Siberia. The lakes are covered by

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FIG. 1. Locations of lakes assayed. All of the lakes are in northeastern Siberia, near the Kolyma River (indicated by large arrow on upper map). The lower satellite view shows the locations for each lake (indicated by arrows). Hundreds of lakes cover the Kolyma River delta region. Most, including Lake Park, are in the flood plains of the rivers and rivulets. Some of the lakes, including Lake Edoma and Lake Shchychie, are on an elevated area above the flood plain.

ice for more than 6 months annually and are frequented by large populations of migratory waterfowl, some of which travel to North America and others that travel as far as southern Asia, Europe, and Africa.

MATERIALS AND METHODS

We sampled ice or water from three northeastern Siberian lakes in the Kolyma River region (Fig. 1) and tested the samples for the presence of influenza A virus using reverse transcription-PCR (RT-PCR) specific for the H1 version of the hemagglutinin gene. The lakes are approximately 100 km from the Arctic Ocean, in an area covered by hundreds of lakes. Ice forms on the lakes early in October and thaws early in June. The lakes sampled in our study do not contain permanent or semipermanent ice cover, as might probably be the case for Siberian lakes located north of 70°N. The significance of the lakes we sampled is for annual virus preservation and for demonstrating the feasibility of viral endurance in environmental ice. Maximum ice thickness ranged from 0.65 to 1.40 m. Lake water temperatures varied from 2.0 to 8.0°C at the bottom to 22.0 to 27°C at the surface during the summer, and from 0.3 to 2.5°C at the bottom to 0.0°C immediately below the ice during winter. Lake Edoma (also called Yedoma) and Lake Shchychie (also called Shuchi) are thermokarst lakes on a Late Pleistocene fluvial plain isolated from the Kolyma River, the primary river in the area. Lake Park also is a thermokarst lake but is within the floodplain of the Kolyma River between two residual outcrops of Late Pleistocene fluvial plain. Periodically, Lake Park is flooded by the Kolyma River. Due to its distance from human settlements, Lake Park is often visited by birds and has large avian nesting areas. Each of the lakes is frequented by migratory birds (although the visitation and nesting frequencies vary [Table 1]). The lakes are along the flight paths of migratory waterfowl, which fly into temperate and tropical Asia, North America, Europe, and Africa for wintering.

Samples (300 to 500 ml each) were collected in September 2001 (water), from Lake Edoma, and March 2002 (ice), from Lake Park and Lake Shchychie. Water samples were collected in autumn during the beginning of mass migration of birds. The water was collected in sterile bottles at stations that were 1.5 to 2.0 m from the lake edge, very close to areas frequented by migratory waterfowl. Samples were kept at temperatures between 1 and 5°C during the transportation to the lab and then were frozen at −80°C. Ice was collected during the winter at 10 to 15 m toward the lake center from the edge (very close to areas frequented by waterfowl) to avoid the deep near-shore snow cover. Ice was removed with sterilized instruments. The ice samples were placed into a double plastic pack and melted in the lab without contact with air. Then, the meltwater was placed into sterilized bottles and frozen at −80°C.

In the lab, rigorous attention to avoidance of contamination was maintained throughout the procedures (10, 11, 12, 17, 19). The sterile culture room and sterile biosafety laminar flow hood were bathed with germicidal UV radiation for at least 30 min prior to each work session (as well as 30 min after each session). All laboratory benches were cleaned with undiluted Clorox (5.25% sodium hypochlorite) and 70% ethanol prior to, and following, a work session. The ice and frozen water samples were melted at room temperature in the sterile laminar

TABLE 1. List of lakes assayed or to be assayed for the presence of influenza A virus

Lake	Elevation (m)	Width × length; depth range (m)	Waterfowl observed	Influenza A virus H1 gene (isolation date, source)
Edoma	25	200 × 610; 1.0–14.0	Ducks (*Anas* spp. and *Aythya* spp.), gulls (*Larus* spp.), loons (*Gavia arctica* and *G. stellata*, some of which nested near the lake), sandpipers (*Calidris* spp., *Limosa* spp., and *Tringa* spp.), and terns (*Sterna* spp.); all fewer than for Lake Park and similar to Lake Shchychie	1 sequence (September 2001, water)
Park	5	750 × 1250; 3.0–3.5	Cranes (*Grus canadensis*), ducks (broad range of species, including *Anas acuta*, *Anas clypeata*, *Anas formosa*, *Anas penelope*, *Aythya fuligula*, *Aythya marila*, *Clangula hyemalis*, *Melanitta fusca*, and *M. nigra*), geese (*Anser* spp.; *A. fabalis* is the dominant species, with some *A. erythropus*), gulls (*Larus* spp.; *L. argentatus* and *L. canus* are the dominant species, with some *L. ridibundus* and rarely *Rhodostethia rosea*), loons (*Gavia arctica* and *G. stellata*, some of which nested near the lake), sandpipers (*Actitis* spp., *Alidris* spp., *Calidris* spp., Limosa spp., *Phalaropus* spp., *Philomachus pugnax*, and *Tringa* spp.), swans (*Cygnus bewickii* and *C. cygnus*), and terns (usually *Sterna paradisaea* and *S. hirundo*)	83 unique sequences (March 2002, ice)
Shchychie	25	220 × 450; 11.0–14.0	Ducks (*Anas* spp.), gulls (*Larus* spp.), sandpipers (*Tringa* spp., *Calidris* spp., and *Xenus* spp.), and terns (*Sterna* spp.); all fewer than for Lake Park and similar to Lake Edoma	None detected (March 2002, ice)

flow hood. The meltwater was distributed into sterile 1.5-ml microfuge tubes. Meltwater from five tubes for each sample was assayed immediately (as described below) while the other samples were frozen and stored at −80°C until needed.

Aliquots of 10 μl per sample were subjected to RT-PCR amplification. Primers (forward, H1-1f [ATGCSAACAACTCAACCGACAC]) and reverse (an equimolar mixture of H1-5ra [GGGTTCCAGCAGAGTCCAGTAGTA] and H1-5rb [GGG TTCTAGCAAGGTCCAGTAATA]) were used at 25 pmol each, in 25-μl reaction mixtures. Reverse transcription was performed with a GeneAmp EZ r*Tth* RNA PCR kit (Applied Biosystems, Inc., Foster City, CA) using the reaction mix provided (10 μM Tris-HCl [pH 8.3]; 300 μM [each] dATP, dCTP, dGTP, and dTTP; 2.5 mM manganese acetate; 50 mM bicine; 115 mM potassium acetate; 8% [wt/vol] glycerol; and 2.5 U *rTth* DNA polymerase) at 60°C for 30 min. This was followed by PCR (with the same reaction mix and enzyme) using the following temperature regime: 94°C for 4 min and 35 cycles of 94°C for 1 min and 60°C for 90 s, followed by a final extension at 60°C for 8 min. Next, nested PCR was performed using 0.5 μl of the RT-PCR mixtures described above. The nested primers consisted of a forward primer (an equimolar mixture of H1-2fa [TCAA CCTACTTGAGGACAGTCACA] and H1-2fb [TTAACCTGCTCGAAGACA GCCACA]) and a reverse primer (H1-4r [CGGGTGATGAACACCCCATAG TA]) specific for the influenza A virus hemagglutinin H1 gene (25 pmol each). The following temperature regime was used: 94°C for 5 min; then 45 cycles of 94°C for 1 min, 54°C for 1 min, a 0.3°C-per-s increase to 72°C, and 72°C for 1 min; followed by 72°C for 8 min.

The PCR products were subjected to electrophoresis on 1.5% standard agarose (Bio-Rad Laboratories, Hercules, CA) gels in TBE (89 mM Tris base, 89 mM borate, 2 mM EDTA, pH 8.0, with 0.5 μg/ml ethidium bromide). Following electrophoresis at 5 V/cm, the gels were viewed and photographed under 300 nm UV light. In total, 373 amplification attempts were performed using meltwater from Lake Park ice. An additional 40 amplification attempts were made using meltwater from Lake Shchychie ice. A total of 161 amplification attempts were performed with water from Lake Edoma. Multiple positive and negative controls were included for every set of RT-PCR experiments. When amplification bands of the expected molecular weight were observed, another aliquot of the reaction mixture was subjected to electrophoresis on a 1.0% low-melting-point agarose gel (NuSieve GTG; FMC, Rockville, ME) in TBE. The bands were then eluted from the gels (26, 27) and rehydrated in water. The eluted amplicons were then ligated into plasmid vectors (pCR2.1-TOPO; Invitrogen Corp., Carlsbad, CA). Following transformation of host bacteria, the recombinant clones were identified by growth on selective medium. The recombinant plasmids were extracted from the bacterial host cells and purified, and the inserts were amplified using M13 primers (forward primer, CAGGAAACAGCTATGAC; reverse primer, GTAAAACGACGGCCAG). The amplicons were gel purified (as described above) and then used in DNA sequencing reactions. For sequencing, approximately 30 ng of DNA was added to a reaction with the Terminator Ready Reaction kit, version 3.0 (ABI, Foster City, CA). The cycling program was as follows: 1 min at 94°C and then 30 cycles of 94°C for 10 s, 50°C for 30 s, and 60°C for 4 min. The amplified DNA was precipitated by adding ethanol to a final concentration of 65% and incubating at room temperature for 15 min. Then the DNA was subjected to centrifugation in a microfuge (13,200 rpm) for 20 min. The pellets were washed with 80% ethanol and then dried under a vacuum. The dried pellets were rehydrated with template suppression reagent (ABI, Foster City, CA), denatured at 94°C for 4 min, and chilled on ice. Then the solutions were loaded into an ABI (Foster City, CA) 310 automated DNA sequencer for determination of the influenza A virus H1 gene sequences.

The sequences were compared to those in NCBI databases by using BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences exhibiting the highest similarity to influenza A virus H1 sequences (a total of 84) were aligned using CLUSTALW (http://searchlauncher.bcm.tcm.edu/multi-align/multi-align.html). A broad selection of H1 sequences from the NCBI database also were included in the alignment. Following alignment, the sequences were examined, and small manual adjustments were made as needed. Phylogenetic analyses were performed with PAUP (phylogenetic analysis using parsimony [25]) using neighbor-joining and maximum parsimony. Consistency, homoplasy, retention, and rescaled consistency indices were calculated in the parsimony analysis.

RESULTS

The highest frequencies of influenza A virus H1 genes were found in ice from Lake Park. Twenty of the 373 RT-PCRs using Lake Park ice meltwater yielded amplification bands of the expected size. After cloning, a total of 83 unique sequences resulted from these 20 positive reactions. Lake Park has the highest concentration of birds, including cranes, ducks, geese, gulls, loons, sandpipers, swans, and terns (Table 1). In 40 attempts to amplify (by RT-PCR) H1 genes from Lake Shchychie ice, no amplification was evident. This lake had the lowest bird visitation rate (including no observed visits by geese) of the three lakes (Table 1). Only 1 of the 161 attempts to amplify the H1 gene from Lake Edoma water yielded an amplicon of the expected molecular weight. A single sequence resulted. This lake is only occasionally visited by geese and other birds (Table 1).

In BLAST searches of NCBI databases, the H1 gene amplicons were most similar to those previously isolated with neuraminidase gene subtypes N1, N2, and N5. Phylogenetic analyses indicated that while the viruses exhibit genetic diversity (Fig. 2), they form a monophyletic cluster (Fig. 3) in contrast to other H1 sequences. Comparison with a wide variety of H1 gene sequences indicates that the population in the Lake Park ice is most closely related to subtypes that were isolated from both avian and porcine hosts in the 1930s and 1960s (Fig. 3). They are distantly related to the H1 subtype from the 1918 pandemic.

Our results indicate the following: (i) the highest frequencies of detection of influenza A virus RNAs are in the lakes with the highest concentrations of migratory waterfowl; (ii) influenza A virus RNA is preserved in higher concentrations in lake ice than in lake water (also of note is that the fragment we consistently were able to amplify was 610 nucleotides in length, indicating good preservation of the RNA, which implies good preservation of the virus); (iii) the H1 gene population in the lakes is genetically heterogeneous; (iv) the single H1 gene found in Lake Edoma is similar to the H1 genes in Lake Park, indicating that this gene likely is from the same population of virions; (v) the H1 sequences in this study are closest to those found in Europe during the 1930s and in Asia during the 1960s; and (vi) the H1 sequence from an H1N1 specimen (Brevig Mission, Alaska, 1918) (15) is distantly related to all of the H1 genes from Lake Park and Lake Edoma that were characterized in this study.

DISCUSSION

This is the first report of the persistence of influenza A virus in lake ice, as reflected by enduring genes. It indicates a potential long-term survival mechanism for the virus. Ice may act as a reservoir for influenza A viruses, preserving them for later release and infection of animals, including migratory waterfowl and humans. Surveillance of Arctic and subarctic lakes for influenza virus may aid health professionals to improve prediction of influenza virus subtypes that are circulating at particular points in time, thus facilitating long-term vaccination strategies. Furthermore, surveillance may shed some light on a fundamental apparatus allowing for abiotic long-term perpetuation of multiform influenza A virus strains.

Cold temperatures and freezing preserve most types of viruses, including influenza virus (14, 17, 18, 22). Experimentally, the feasibility of influenza A virus endurance in the frozen state has been demonstrated, implying its survivability in frozen lakes. Inactivation of 99% of a virus population occurs in approximately 1 week when water temperatures are between

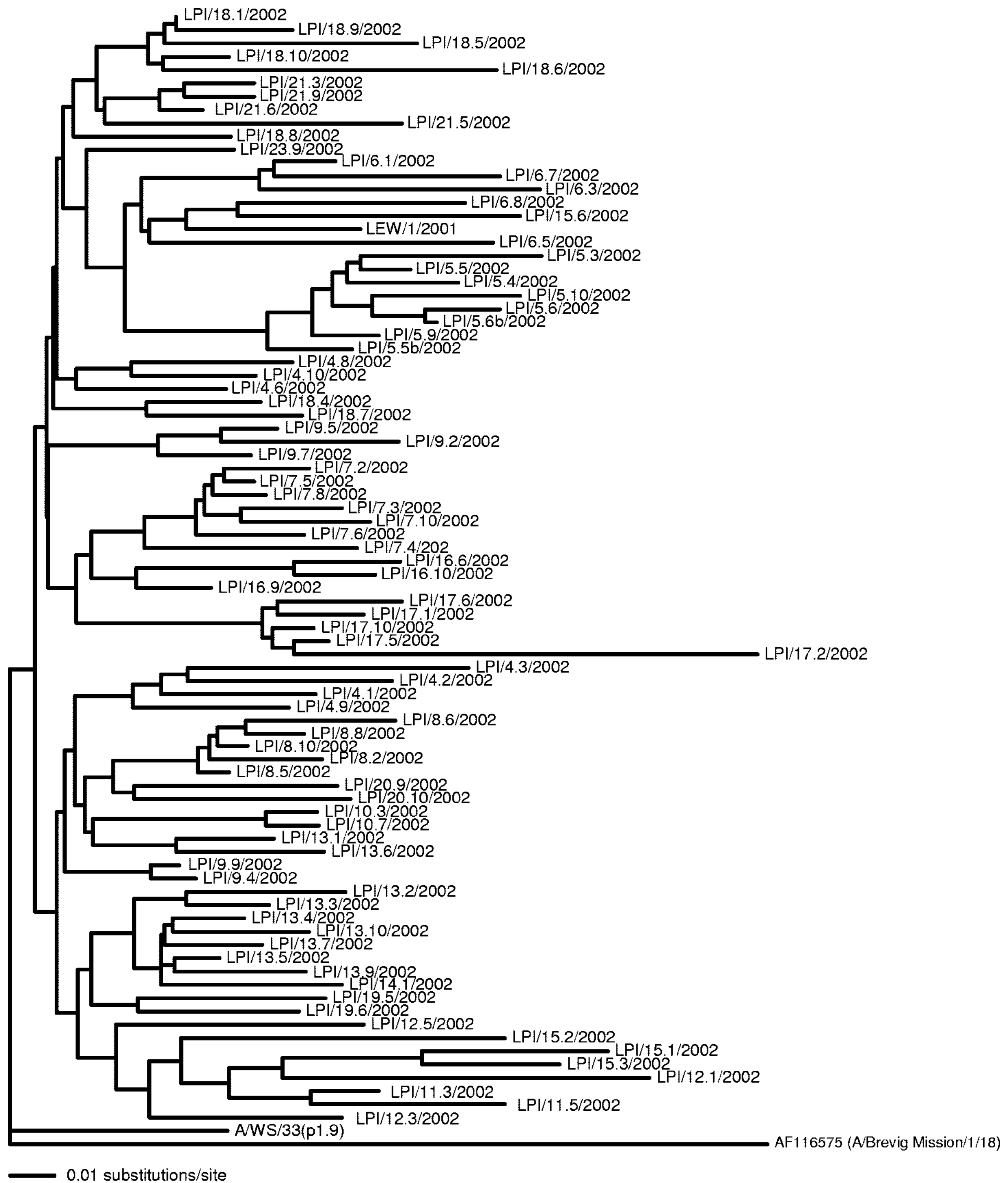

FIG. 2. Neighbor-joining phylogram of the influenza virus hemagglutinin H1 gene sequences isolated from Lake Park ice (collected in March 2002) and Lake Edoma water (collected in September 2001). Numbers indicate sequences from clones derived from nested RT-PCR mixtures. The number before the decimal point indicates the RT-PCR number, while the number after the decimal point indicates each unique clone from the reaction. Sequences from the control virus (A/WS/33, clone p1.9) and from the Brevig Mission, Alaska, subtype H1 (accession number AF116575), are also shown. The Brevig Mission sequence was used as the outgroup. LPI indicates cloned sequences from Lake Park ice, while LEW indicates the one clone from Lake Edoma water.

22 and 25°C. However, 10 weeks is required to inactivate the same proportion of the virus population if the temperature is between 3 and 5°C. The rate of virus degradation slows down to an even greater extent at and below freezing, and it continues to decrease as the temperature is lowered. This trend continues to below −80°C. We have found that viruses and bacteriophage (as prophage) frozen in glaciers can be preserved for well over 100,000 years (3, 4). We previously

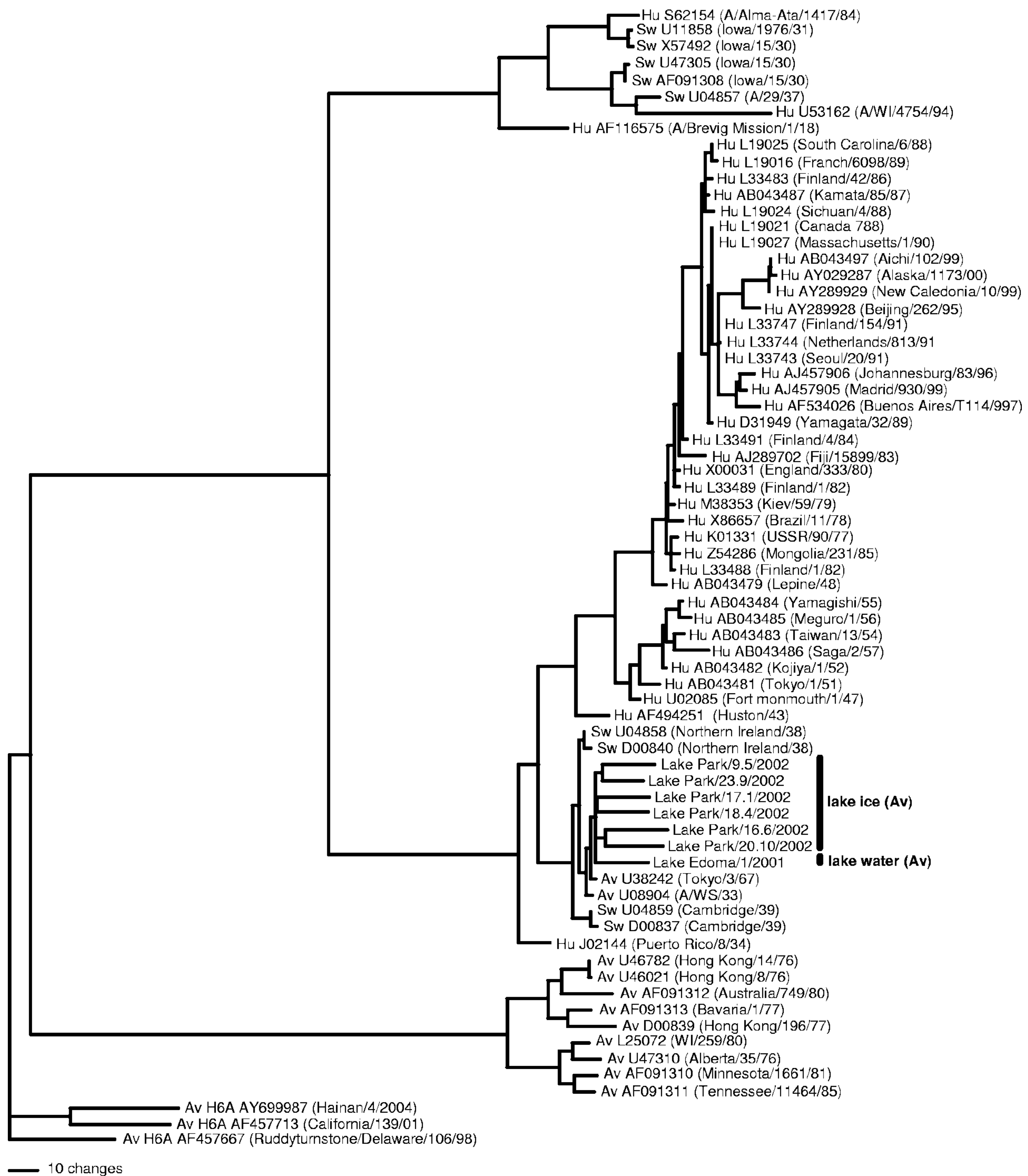

FIG. 3. Maximum parsimony phylogram of a wide selection of hemagglutinin H1 gene sequences, including selected sequences from this study. Gaps were scored as a fifth base. There were more than 2,000 most-parsimonious trees, with placement of several of the sequences varying. Primarily, very closely related sequences shifted relative to one another. However, the relationships of the influenza A virus H1 sequences from this study with the other sequences were consistent in all of the trees. The tree shown has 1,128 steps, with consistency, homoplasy, retention, and rescaled consistency indices equal to 0.5417, 0.4583, 0.8562, and 0.4638, respectively. The H1 genes from Lake Park ice and Lake Edoma water are closest to those from avian strains isolated in Asia in 1933 and 1967. Also, they were related to strains from 1938 and 1939 isolated from swine in the United Kingdom. These are embedded within a large clade that includes a wide range of H1 sequences isolated from humans from the 1930s to the present. There is a more distant relationship with the H1 gene from the 1918 H1 influenza A virus (upper clade) and avian strains from 1976 through 1985 (lower ingroup clade). Sequences from H6 influenza virus strains were used as representatives of the outgroup. Abbreviations for sources: Av, avian; Hu, human; Sw, swine.

reported on viral RNA preserved in ice that was approximately 140,000 years old (4) and have additional unpublished data supporting preservation to many times this age. Therefore, glaciers and ice-covered lakes may be an unrecognized major reservoir of microbes. For pathogens, this is advantageous, since upon reemergence specific genotypes may interact with host populations that may lack resistance or immunity.

Some influenza virus strains have appeared, disappeared, and then reemerged decades later virtually unchanged (13, 20). This may indicate the presence of an abiotic mode of preservation. For example, the Russian influenza virus subtype (H1N1) that caused an epidemic in 1977 was nearly identical to the subtype (H1N1) that caused an epidemic in 1950. Other strains, most notably specific genotypes of H2N2 and H3N2 and several H1 variants, have made similar returns. Since influenza virus is an RNA virus, the rates of mutation should have been rapid if the viruses had been reproducing in biotic hosts during those years. A possible explanation for the slow rates of mutation is that the strains may have been preserved in some way during the decades between the epidemics. Preservation in ice is a possible explanation (2, 14, 18, 22, 23). Ice and ice-covered lakes (as well as glaciers) may act as huge reservoirs of preserved viruses. Therefore, annually and perennially frozen lakes (some are frozen continuously for decades or longer) along the paths of waterfowl migration routes have the potential for being major sources of viruses that cause pandemics and epizootics in birds and other animals. Virological surveillance of these lakes is needed in order to assess the relationships between the prevalence of current as well as earlier influenza A virus subtypes (including sequence characteristics) and endemic and epidemic occurrence of disease. This probably relates to other diseases as well, but this awaits thorough examination. One expectation in relation to this phenomenon would be an increased rate of release of these microbes during times of global (or local) warming events and a decrease during cooler periods.

Bird populations maintain extensive, long-term contact with the most northerly bodies of water, particularly in remote Siberian lakes, which represent perennial freezing-thawing periodicities of high variability, reaching, at the maximum, intervals of decades or longer. This means that influenza A viruses may be preserved in those lakes for years or perhaps much longer (e.g., the viability of microbes encased in ice for hundreds of thousands of years has been demonstrated in many studies [1–6, 9–12, 16, 17, 24, 28]). Thawing releases entrapped viruses of various age and thus seeds the water with concurrent strains regularly harbored by nearby sojourning birds. Until refreezing takes place, viruses of both present and past strains may be contracted by the waterfowl, whereas the remaining viruses would again be encapsulated by the subsequent formation of ice. Conceivably, such ongoing perpetual mechanisms have been operating cyclically throughout the virus's evolution, enabling recurrent emergence of past genes and genomes.

Most of the birds that carry influenza A virus are migratory, such that the disease readily moves within the bird population from one locale to another. During the spring they move northward as the frozen lakes thaw. Starting in fall they move southward as the lakes freeze. The Kolyma River lowland birds travel along major migration paths to Southeast Asia, North America, or the northwestern Pacific Ocean, while some travel to Europe and North Africa. As the birds visit lakes along their paths they shed viruses into the lakes and onto the ice (when present) and drink water containing viruses discharged by other birds or released from the ice by thawing. Therefore, these lakes become abiotic mixing pools for the viruses, while the birds are the biotic vessels where mixing occurs (including replication and recombination). Since there are susceptible hosts along their migration path, they may pass the viruses to other birds as well as to swine, humans, or other animals. Our results support the hypothesis that ice acts as a long-term abiotic storage matrix for influenza virus and other microbes, including pathogens (18, 21–23). Furthermore, the cold lake water is also capable of preserving the virus, although presumably for shorter time periods.

Although the findings of this study are limited to the testing of three lakes, they point to a principal mechanism that may underlie a wider natural apparatus of abiotic long-term preservation of avian influenza viruses. The prevalence and extent of such a mechanism, which may bear a wealth of implications, should be further demonstrated through additional studies, including the exploration of more geographical sites, assays for related genes, and examination of viral endurance. At present, we describe the feasibility of the mechanism and supportive evidence at the level of gene recovery and analysis.

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ERRATUM

Evidence of Influenza A Virus RNA in Siberian Lake Ice

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Volume 80, no. 24, p. 12229–12235, 2006. Page 12231, column 2, line 20: “avian” should read “human.”

Page 12233, Fig. 3. Sequences U38242 (Tokyo/3/67) and U08904 (A/WS/33) were isolated from humans; therefore, they should have the Hu prefix rather than the Av prefix.

Page 12233, legend to Fig. 3, line 6. “avian strains isolated in Asia in 1933 and 1967” should read “human and swine strains isolated in the UK during the 1930s and from a human in Asia in 1967.”