The United Graduate School of Veterinary Science,

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Insights into the evolution of influenza A viruses circulating in pigs and birds

豚及び鳥類間で循環するＡ型インフルエンザウイルスの進化に関する見識

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Contents

Preface

Influenza viruses belong to the family *Orthomyxoviridae,* whose genomes are single-stranded RNA, and include four types of viruses: A, B, C, and D influenza viruses. Influenza A viruses (IAVs) are of wide concern and infect different species, including humans, pigs, horses, marine mammals, and wild and domestic birds [104]. IAV was not determined as the etiological agent of human influenza disease until 1933, shortly after the advent of electron microscopy [91]. Nevertheless, the influenza virus was implicated in the Russian pandemic in 1889 and the Spanish flu pandemic in 1918 [26, 43]; the etiological agent for the two pandemics was determined to be the influenza virus, based on serological studies and molecular clock analysis involving people who lived during those times. The results revealed that influenza viruses of H3N8 and H1N1 subtypes were the etiological agents for the Russian and Spanish flu pandemics, respectively [96, 110]. Following the Spanish flu pandemic, influenza viruses caused four additional pandemics: Asian flu (caused by influenza virus H2N2 subtype in 1957), Hong Kong flu (caused by influenza virus H3N2 subtype in 1968), Russian flu (caused by influenza virus H1N1 subtype in 1977), and the swine-origin influenza virus H1N1 subtype in 2009 [43, 86].

IAVs are single-stranded, negative-sense, segmented RNA viruses that encode at least 11 proteins. Based on two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), IAVs are classified into 16 HA and nine NA subtypes [104]. Moreover, two novel influenza viruses, H17N10 and H18N11, were detected in bats [97, 98]. The high mutation rate, attributed to the negative-sense RNA segmented genome and low replicative fidelity, providing IAVs with rapid evolution that hinders the control strategies for influenza virus-associated diseases [52]. Two evolutionary mechanisms are involved in the evasion of the host immune system by

the influenza virus: antigenic drift and antigenic shift. Antigenic drift is the introduction of minor changes/mutations during viral replication in the antigenic gene segments, hemagglutinin (HA) and neuraminidase (NA). These minor changes are mainly attributed to low fidelity and lack of proofreading abilities of the viral polymerases, allowing the influenza virus to evade the host immune response [93, 99]. The antigenic shift involves a sudden major change in the HA and NA gene segments of the influenza virus, resulting in a virus to which humans have limited or no previous immunity, and thereby, in the occurrence of pandemics [103].

Swine are considered a mixing vessel for IAVs due to the susceptibility to IAVs from different species, resulting in genetic reassortment by swapping the gene segments between two viruses that infect the same individual pig and the subsequent emergence of pandemic-potential strains [83]. The influenza A (H1N1) pandemic in 2009 is an example of a swine-origin pandemic influenza virus. Triple reassortant swine influenza viruses (swIAVs) and Eurasian swIAVs generated the influenza A (H1N1) pandemic 2009 virus (hereafter called A(H1N1)pdm09 virus), which emerged in North America, causing the first pandemic of the 21st century [88]. The A(H1N1)pdm09 virus has been circulating in humans as a seasonal influenza virus since 2009, and A(H1N1)pdm09-related swIAVs have been frequently isolated from swine populations worldwide [29, 44]. However, the evolution of A(H1N1)pdm09-related swIAVs, particularly in Asian countries, remains largely uncharacterized. In Chapter I, a variation in HA antigenicity of A(H1N1)pdm09-related swIAVs was elucidated, and the key viral determinants for the antigenic differences in swIAV Has were identified.

Aside from human and swine circulating IAVs, viruses circulating in birds, known as avian influenza viruses (AIVs), constitute a major concern for poultry production and public health. Wild aquatic birds of the order *Anseriformes* are considered the primary natural reservoir for the 16 HA and nine NA subtypes. Based on their pathogenicity in chickens, AIVs

are classified as highly pathogenic AIVs (HPAIVs) and low pathogenic AIVs (LPAIVs) [104]. In addition to the severe economic losses in poultry production induced by AIVs, particularly the HPAIVs, several spillover events of different AIV subtypes, such as H5N1, H5N6, H5N8, H7N9, and H9N2, have been reported in humans, which indicate the possible emergence of pandemic-potential strains from birds [50]. Thus, the investigation of the circulation and epidemiology of AIVs in wild birds is needed to understand the ecology of AIVs and intervene in the occurrence of occasional pandemics.

The Izumi plain in Kagoshima Prefecture at the southern tip of Kyushu Island in Japan is a well-known overwintering site for tens of thousands of migratory birds, including cranes and wild aquatic birds of the order *Anseriformes,* the natural reservoirs for AIVs [104]. These cranes are classified as vulnerable species according to the International Union for Conservation of Nature Red List, and the plain is famous for chicken farming; therefore, we surveyed AIVs on the Izumi plain every winter season since 2012 and isolated AIVs of various subtypes. During the AIV surveillance on the Izumi plain in the 2017/18 winter season, three AIVs of two subtypes were isolated: two AIVs of the H4N6 subtype and one AIV of the H3N8 subtype. In Chapter II, three AIV isolates were genetically characterized to compare the genetic constellations of the three isolates with their counterparts of the same subtype isolated from the Izumi plain during previous seasons.

During the 2020/21 winter season, multiple outbreaks of H5N8 HPAIVs have been reported in several European and Asian countries, including Japan. Additionally, seven human cases of H5N8 HPAIVs in the Russian Federation were reported on February 18, 2021, which was the first report of H5N8 HPAIVs in humans [106]. Two H5N8 HPAIVs were isolated from the feces of Falcated ducks and environmental water samples collected from the Izumi plain during the 2020/21 winter season. The potential role of falcated ducks was revealed in the dissemination of H5N8 HPAIVs first reported in Japan (Chapter III).

Chapter I

Variation in the HA antigenicity of A(H1N1)pdm09-related swine influenza viruses

Abstract

Since the influenza pandemic in 2009, the causative agent "A(H1N1)pdm09 virus", has been circulating in both human and swine populations. Although phylogenetic analyses of the hemagglutinin (HA) gene segment have revealed broader genetic diversity of A(H1N1)pdm09-related swine influenza A viruses (swIAVs) compared with human A(H1N1)pdm09 viruses, it remains unclear whether the genetic diversity reflects the antigenic differences in HA. To assess the impact of the diversity of the HA gene of A(H1N1)pdm09-related swIAVs on HA antigenicity, we characterized 12 swIAVs isolated in Japan from 2013–2018. We used a ferret antiserum and a panel of anti-HA mouse monoclonal antibodies (mAbs) raised against an early A(H1N1)pdm09 isolate. The neutralization assay with the ferret antiserum revealed that five of the 12 swIAVs were significantly different in their HA antigenicity from the early A(H1N1)pdm09 isolate. The mAbs also showed differential neutralization patterns depending on the swIAV strains. In addition, the single amino acid substitution at position 190 of HA, which was found in one of the five antigenically different swIAVs but not in human isolates, was shown to be one of the critical determinants for the antigenic difference of swIAV HAs. Two potential N-glycosylation sites at amino acid positions 185 and 276 of the HA molecule were identified in two antigenically different swIAVs. These results indicated that the genetic diversity of HA in the A(H1N1)pdm09-related swIAVs is associated with their HA antigenic variation. Our findings highlighted the need for surveillance to monitor the emergence of swIAV antigenic variants with public health importance.

Introduction

In April 2009, a novel swine-origin influenza A virus of the H1N1 subtype emerged in humans in North America and rapidly spread around the world, resulting in the first influenza pandemic of the 21st century [60, 88]. Since then, the influenza A virus that caused the pandemic in 2009, the so-called A(H1N1)pdm09 virus, has been circulating among humans as one of the seasonal influenza viruses [2, 10]. Conversely, the A(H1N1)pdm09-related swine influenza A virus (swIAV) has been frequently isolated from pigs worldwide since 2009 [29, 44], indicating its re-introduction to swine populations and sustained circulation of the viruses in pigs. We previously isolated swIAVs in Japan from 2013-2016 and found that their hemagglutinin (HA) gene segments are phylogenetically classified into the A(H1N1)pdm09 virus lineage [66, 73].

The HA glycoprotein, an influenza viral envelope protein, is responsible for receptor binding and membrane fusion [108]. HA is the primary target of neutralizing antibodies and is thus exposed to selective immune pressure as the main viral antigenic determinant [67, 111]. Therefore, genetic and antigenic analyses of HA provide critical insights into the diversity and evolution of influenza A viruses. In fact, one of the main purposes of global influenza surveillance coordinated by the World Health Organization (WHO) is monitoring the HA antigenicity of human influenza viruses to update recommendations for the selection of vaccine strains [17, 22]. In contrast, global swine influenza surveillance has not yet been established. Whereas the HA antigenicity of swIAVs isolated from North America and Europe has been characterized in several studies [5, 47], that of swIAVs isolated in Asia remains largely uncharacterized.

In general, the genetic evolution rate of swIAVs is considered to be lower than that of human influenza viruses [18, 94]. However, in our previous study, the phylogenetic analysis

of the HA gene segments from A(H1N1)pdm09-related swIAVs isolated in Japan revealed that the genetic diversity of the swIAVs is broader than that of the human viruses [66]. Here, to assess the impact of the genetic diversity of the swIAV HA genes on HA antigenicity, we characterized the HA of 12 swIAVs isolated in Japan from 2013–2018. In addition, we investigated the key amino acid residue(s) responsible for substantial differences in HA antigenicity.

Materials and methods

Cells

AX4 cells, which are Madin-Darby canine kidney (MDCK) cells that overexpress human α2,6-sialyltransferase I and allow replication of human influenza viruses more efficiently than wild-type MDCK cells [23], were maintained in minimum essential medium (MEM) (Thermo Fisher Scientific, Waltham, MA) supplemented with 5% newborn calf serum and puromycin (2 μg/mL). AX4 cells inoculated with viruses were cultured in MEM containing 0.3% bovine serum albumin (BSA) and 1 μg/mL tolysulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin. Human embryonic kidney-derived 293T cells were maintained in Dulbecco's modified Eagle medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal calf serum. 293T cells transfected with plasmids were cultured in Opti-MEM (Thermo Fisher Scientific). All cell cultures were maintained in a humidified incubator at 37°C in 5% CO_2.

Viruses

Twelve swIAVs (Table 1) were subjected to genetic and antigenic characterization. A/swine/Aichi/KU-OE7/2017 (H1N2) was isolated in this study (see below in detail), while A/swine/Aichi/101/2018 (H1N1) was isolated in the Kyodoken Institute for Animal Science Research & Development (Kyoto, Japan). The remaining ten viruses were previously isolated during 2013–2016 [66, 73]. In addition, A/California/04/2009 (H1N1) (CA04 strain), which is a representative early human isolate of the A(H1N1)pdm09 viruses propagated in MDCK cells [34], and its HA mutants were generated by plasmid-based reverse genetics [57] (see below in detail).

Titers of the stock viruses were determined using 50% tissue culture infective dose ($TCID_{50}$) assays in AX4 cells. Briefly, AX4 cells were inoculated with 10-fold serial dilutions

of the viruses in MEM/0.3% BSA containing 1 μg/mL TPCK-treated trypsin and cultured at 37°C for three days. The virus titers were calculated using the Reed-Muench method [78] based on the cytopathic effects (CPE) in the cells observed under the microscope.

Isolation and subtyping of swIAV

Ten nasal swab specimens collected from growing pigs aged 120–150 days from a pig farm in Aichi prefecture, Japan, in October 2017, were subjected to swIAV isolation. These specimens were filtered with a 0.22 μm pore-size membrane (Merck, Darmstadt, Germany) and inoculated into AX4 cells, followed by culture at 37°C for 2-3 days. swIAV isolation was confirmed by observation of CPE in the inoculated AX4 cells, followed by real-time reverse transcription PCR-based detection of the partial influenza A viral M gene segment as previously described (41). The HA and neuraminidase (NA) subtypes of the isolated swIAVs were determined as described previously [40] with a panel of subtype-specific primer sets [100].

Sequence analysis of swIAV genes

Supernatants collected from AX4 cells inoculated with A/swine/Aichi/KU-OE7/2017 (H1N2) and A/swine/Aichi/101/2018 (H1N1) were subjected to viral RNA extraction, complementary DNA synthesis, and amplification of the eight-segmented influenza virus genes by PCR as previously described [40]. Nucleotide sequences of open reading frames of all viral gene segments were determined by Sanger sequencing at FASMAC Co., Ltd. (Atsugi, Japan). The determined sequences were deposited in the Global Initiative on Sharing Avian Influenza Data (GISAID) database (http://platf orm.gisaid.org/) (Table 2). The primer sequences used for these sequence analyses are available upon request.

Phylogenetic analysis

The sequenced HA viral gene segments from the 12 swIAVs were phylogenetically analyzed with representative counterparts, retrieved from the GISAID database on January 10, 2021, that reflected the temporal and spatial distribution of human and swine viruses belonging to the A(H1N1)pdm09 virus. The nucleotide sequences were aligned using the MAFFT software version 7.397 (https://mafft.cbrc.jp/alignment/software/). The phylogenetic tree for the HA genes was constructed using the maximum likelihood (ML) method and the general time-reversible model in the MEGA X software with a bootstrapping set of 1,000 replicates [45].

Antibodies

Mouse monoclonal antibodies (mAbs) raised against A/Narita/1/2009 (H1N1) (Narita strain), which is the first A(H1N1)pdm09 virus isolate in Japan, were generated as described previously [42, 116]. Briefly, two 6-week-old female BALB/c mice were immunized subcutaneously using Freund's complete adjuvant or intranasally using the cholera toxin B subunit as an adjuvant (Wako Pure Chemical Industries, Osaka, Japan) twice at 2-week intervals with 100 μg of formalin (0.2%)-inactivated Narita strain. One to three months after the second immunization, the mice were intraperitoneally boosted with inactivated purified virus alone. Three days later, spleen cells from the mice and mouse myeloma p3u1 cells were fused and maintained as previously described [42]. The resultant hybridomas were screened for secretion of HA-specific mAbs by enzyme-linked immunosorbent assay (ELISA) as previously described [42]. ELISA-positive mAbs were tested for hemagglutination inhibition (HI) activity using 0.5% chicken erythrocytes. The neutralization activity of the mAbs was measured by plaque reduction assays in MDCK cells. Escape mutants of the Narita strain were isolated under virus propagation in the presence of mAbs and characterized genetically and antigenically as previously described [42].

Ferret antiserum raised against A/California/07/2009 (H1N1) (CA07 strain), which is an initial vaccine strain for the pandemic in 2009 [69], was kindly provided by Dr. Shinji Watanabe at the Influenza Virus Research Center, National Institute of Infectious Diseases [55].

Microneutralization (MN) assay

The HA antigenicity of the 12 swIAVs was characterized by microneutralization (MN) assays as described previously [71] with the ferret antiserum raised against the CA07 strain and 20 mouse mAbs established in this study. Briefly, two-fold serial dilutions of each mAb ascites fluid or receptor-destroyed enzyme (RDE II, Denka Seiken Co., Ltd., Tokyo, Japan)-treated ferret antiserum were mixed with each virus tested, followed by incubation at 37°C for 1 h. AX4 cells were inoculated with the virus-antibody mixtures containing 100 $TCID_{50}$ of each virus tested in duplicate and cultured at 37°C for three days. The neutralization titer was determined based on the CPE observed under a light microscope.

Reverse genetics

The wild-type CA04 strain was generated by reverse genetics as described previously [57]. Briefly, eight plasmids for the expression of CA04 strain-derived gene segments [48] and four plasmids for the expression of A/WSN/1933 (H1N1)-derived viral proteins (i.e., PB2, PB1, PA, and NP) that are required for the transcription and replication of influenza viral RNA genes [57] were transfected into 293T cells maintained in Opti-MEM (Thermo Fisher Scientific) with transfection reagent TransIT-293 (Mirus, Madison, WI) according to the manufacturer's instructions. At 48 h post-transfection, the supernatant containing the wild-type CA04 virus was harvested and inoculated into AX4 cells for virus propagation.

To generate CA04 strain-based HA mutants, a single desired nucleotide substitution(s) was introduced into the plasmid for the expression of the CA04 strain-derived HA gene segment by site-directed mutagenesis. One of the resultant plasmids was then used for reverse genetics instead of the counterpart for the wild-type HA gene segment.

Prediction of glycosylation sites

The web-based NetNglyc server was used to predict N-glycosylation sequon (Asn-Xaa-Ser/Thr) on the HA protein (http://www.cbs.dtu.dk) [21] using default settings. Only scores crossing the default threshold of 0.5 were considered positive for potential glycosylation sites, as previously described [20]. In addition, the prevalence of any amino acid substitution that could affect the N-glycosylation of the HA protein of A(H1N1)pdm09 viruses was checked using WebLogo 3 (http://weblogo.threeplusone.com) [14].

Results

Isolation of swIAVs from nasal swab specimens

We irregularly receive pig specimens, including nasal swabs, tracheal swabs, and lung homogenate specimens from pig farmers and swine veterinarians in Japan for diagnostic purposes. In 2017, ten nasal swab specimens collected from growing pigs were subjected to swIAV isolation. The CPE observation followed by swIAV-specific gene detection indicated that five out of the ten specimens contained infectious swIAVs. HA and NA subtyping showed that all five swIAVs were classified as the same subtype, H1N2.

Sequence and phylogenetic analyses of swIAVs

To genetically characterize the isolated swIAVs, one of the five isolated swIAVs, A/swine/Aichi/KU-OE7/2017 (H1N2), was selected for further analyses. In addition, A/swine/Aichi/101/2018 (H1N1), which was isolated from the Kyodoken Institute for Animal Science Research & Development, was subjected to genetic characterization. The nucleotide sequences of the open reading frames of all eight gene segments from both isolates were determined, followed by the Basic Local Alignment Search Tool (BLAST) search in the GISAID database (Table 2). The results indicated that the HA gene segments from both isolates belong to the A(H1N1)pdm09 virus lineage.

We further investigated the phylogenetic relationship of the HA genes from two A(H1N1)pdm09-related swIAVs characterized in this study together with those from ten previously characterized swIAVs isolated during 2013–2016 [66, 73], and representative human influenza viruses reflecting the temporal and spatial distribution of human A(H1N1)pdm09 viruses (Fig. 1). Phylogenetic analysis revealed that the HA genes of A/swine/Aichi/KU–MI1-1/2016 (H1N1) and A/swine/Aichi/KU–MI2-3/2016 (H1N1) formed a cluster with their counterparts from the human A(H1N1)pdm09 viruses, suggesting that the

ancestors of these two swIAVs could be linked to recent human transmission, as described previously [66]. In contrast, the HA genes of the remaining ten swIAVs, including A/swine/Aichi/KU-OE7/2017 (H1N2) and A/swine/Aichi/101/2018 (H1N1), were phylogenetically different from the main evolutionary pathway of the human A(H1N1)pdm09 viruses and exhibited broader genetic diversity.

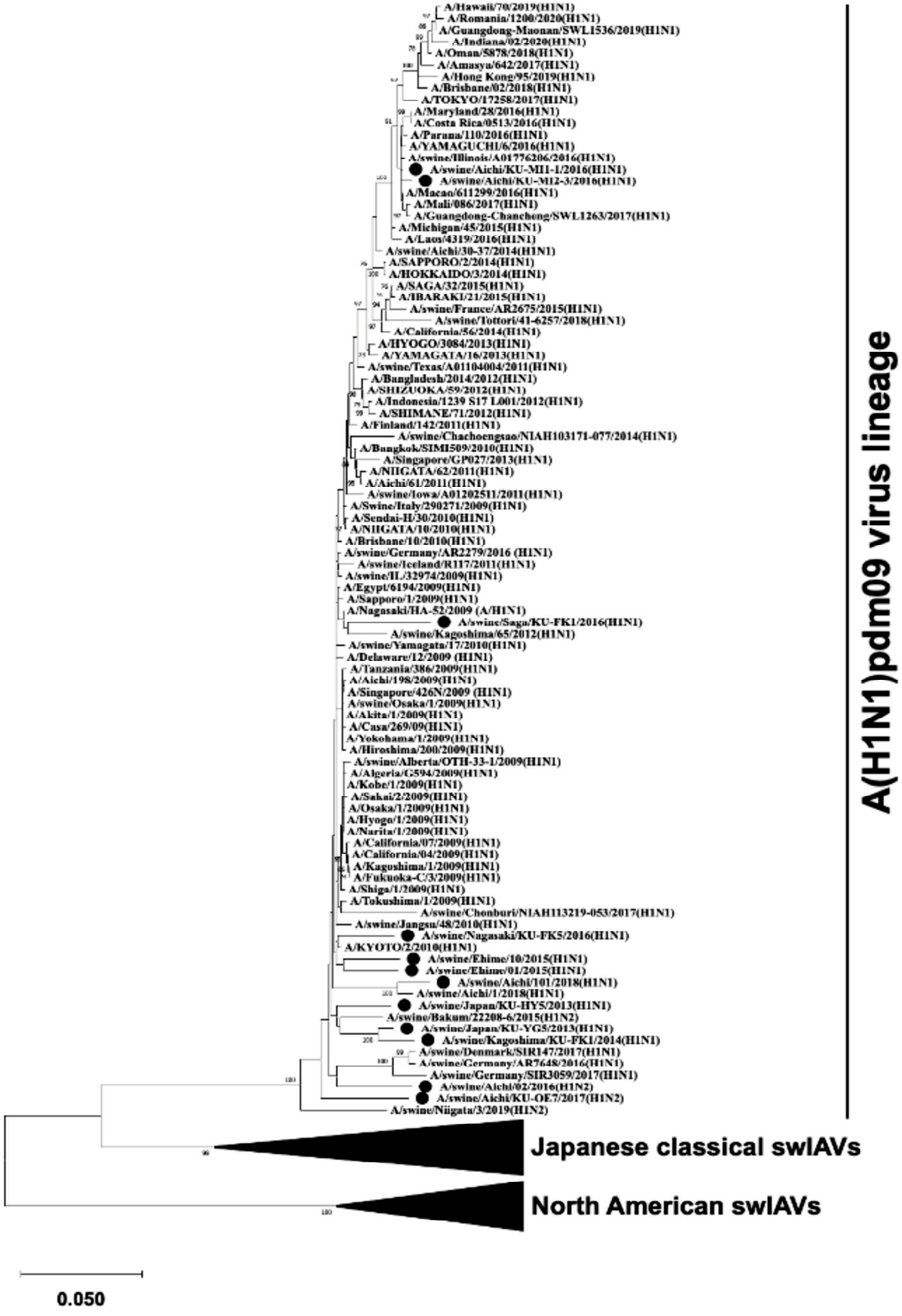

A/Hawaii/70/2019(H1N1)
A/Romania/1200/2020(H1N1)
A/Guangdong-Maonan/SWL1536/2019(H1N1)
A/Indiana/02/2020(H1N1)
A/Oman/5878/2018(H1N1)
A/Amasya/642/2017(H1N1)
A/Hong Kong/95/2019(H1N1)
A/Brisbane/02/2018(H1N1)
A/TOKYO/17258/2017(H1N1)
A/Maryland/28/2016(H1N1)
A/Costa Rica/0513/2016(H1N1)
A/Paruna/110/2016(H1N1)
A/YAMAGUCHI/6/2016(H1N1)
A/swine/Illinois/A01776206/2016(H1N1)
A/swine/Aichi/KU-MI1-1/2016(H1N1)
A/swine/Aichi/KU-MI2-3/2016(H1N1)
A/Macao/611299/2016(H1N1)
A/Muli/086/2017(H1N1)
A/Guangdong-Chancheng/SWL1263/2017(H1N1)
A/Michigan/45/2015(H1N1)
A/Laos/4319/2016(H1N1)
A/swine/Aichi/30-37/2014(H1N1)
A/SAPPORO/2/2014(H1N1)
A/HOKKAIDO/3/2014(H1N1)
A/SAGA/32/2015(H1N1)
A/IBARAKI/21/2015(H1N1)
A/swine/France/AR2675/2015(H1N1)
A/swine/Tottori/41-6257/2018(H1N1)
A/California/56/2014(H1N1)
A/HYOGO/3084/2013(H1N1)
A/YAMAGATA/16/2013(H1N1)
A/swine/Texas/A01104004/2011(H1N1)
A/Bangladesh/2014/2012(H1N1)
A/SHIZUOKA/59/2012(H1N1)
A/Indonesia/1239 S17 L001/2012(H1N1)
A/SHIMANE/71/2012(H1N1)
A/Finland/142/2011(H1N1)
A/swine/Chachoengsao/NIAH103171-077/2014(H1N1)
A/Bangkok/SIM1509/2010(H1N1)
A/Singapore/GP027/2013(H1N1)
A/NIIGATA/62/2011(H1N1)
A/Aichi/61/2011(H1N1)
A/swine/Iowa/A01202511/2011(H1N1)
A/Swine/Italy/290271/2009(H1N1)
A/Sendai-H/30/2010(H1N1)
A/NIIGATA/10/2010(H1N1)
A/Brisbane/10/2010(H1N1)
A/swine/Germany/AR2279/2016 (H1N1)
A/swine/Iceland/R117/2011(H1N1)
A/swine/IL/32974/2009(H1N1)
A/Egypt/6194/2009(H1N1)
A/Sapporo/1/2009(H1N1)
A/Nagasaki/HA-52/2009 (A/H1N1)
A/swine/Saga/KU-FK1/2016(H1N1)
A/swine/Kagoshima/65/2012(H1N1)
A/swine/Yamagata/17/2010(H1N1)
A/Delaware/12/2009 (H1N1)
A/Tanzania/386/2009(H1N1)
A/Aichi/198/2009(H1N1)
A/Singapore/426N/2009 (H1N1)
A/swine/Osaka/1/2009(H1N1)
A/Akita/1/2009(H1N1)
A/Cusa/269/09(H1N1)
A/Yokohama/1/2009(H1N1)
A/Hiroshima/200/2009(H1N1)
A/swine/Alberta/OTH-33-1/2009(H1N1)
A/Algeria/G594/2009(H1N1)
A/Kobe/1/2009(H1N1)
A/Saka/2/2009(H1N1)
A/Osaka/1/2009(H1N1)
A/Hyogo/1/2009(H1N1)
A/Narita/1/2009(H1N1)
A/California/07/2009(H1N1)
A/California/04/2009(H1N1)
A/Kagoshima/1/2009(H1N1)
A/Fukuoka-C/3/2009(H1N1)
A/Shiga/1/2009(H1N1)
A/Tokushima/1/2009(H1N1)
A/swine/Chonburi/NIAH113219-053/2017(H1N1)
A/swine/Jangsu/48/2010(H1N1)
A/swine/Nagasaki/KU-FK5/2016(H1N1)
A/KYOTO/2/2010(H1N1)
A/swine/Ehime/10/2015(H1N1)
A/swine/Ehime/01/2015(H1N1)
A/swine/Aichi/101/2018(H1N1)
A/swine/Aichi/1/2018(H1N1)
A/swine/Japan/KU-HY5/2013(H1N1)
A/swine/Bakum/22208-6/2015(H1N2)
A/swine/Japan/KU-YG5/2013(H1N1)
A/swine/Kagoshima/KU-FK1/2014(H1N1)
A/swine/Denmark/SIR147/2017(H1N1)
A/swine/Germany/AR7648/2016(H1N1)
A/swine/Germany/SIR3059/2017(H1N1)
A/swine/Aichi/02/2016(H1N2)
A/swine/Aichi/KU-OE7/2017(H1N2)
A/swine/Niigata/3/2019(H1N2)
A(H1N1)pdm09 virus lineage
Japanese classical swIAVs
North American swIAVs
0.050

Fig. 1. Phylogenetic tree of HA gene segments of H1 subtype

Nucleotide sequences of the HA gene segment from our swIAVs (indicated by circles) were phylogenetically analyzed with their counterparts from representative human and swine A(H1N1)pdm09 viruses and classical Japanese swIAVs.

Characterization of HA antigenicity of swIAVs with ferret antiserum

We then antigenically characterized the 12 swIAV isolates and an early human isolate of the A(H1N1)pdm09 viruses, CA04 strain, by MN assays using ferret antiserum raised against the CA07 strain. Notably, ferret antiserum is a gold standard material for characterizing the HA antigenicity of influenza viruses [75, 90]. In addition, it was previously demonstrated that there is no antigenic difference between CA04 and CA07 HAs, as shown by the limited cross-reactivity of the corresponding ferret antisera [51]. In general, a tested virus is considered antigenically different when showing a more than eight-fold reduction in the MN titer relative to a reference strain [48]. The MN assays with the ferret antiserum (Table 3) revealed that five swIAVs, A/swine/Japan/KU-YG5/2013 (H1N1), A/swine/Japan/KU-HY5/2013 (H1N1), A/swine/Kagoshima/KU-FK1/2014 (H1N1), A/swine/Aichi/101/2018 (H1N1), and A/swine/Aichi/KU-OE7/2017 (H1N2), were substantially antigenically different from the CA04 strain (i.e., showed an eight-fold reduction in the MN titer). In contrast, the MN titers of the remaining seven swIAVs, A/swine/Ehime/01/2015 (H1N1), A/swine/Ehime/10/2015 (H1N1), A/swine/Nagasaki/KU-FK5/2016 (H1N1), A/swine/Aichi/KU–MI1-1/2016 (H1N1), A/swine/Aichi/KU–MI2-3/2016 (H1N1), A/swine/Aichi/02/2016 (H1N2), and A/swine/Saga/KU-FK1/2016 (H1N1) were comparable to and/or higher than those of the CA04 strain.

Characterization of HA antigenicity of swIAVs with mouse monoclonal antibodies

To further characterize the HA antigenicity of the 12 swIAVs, we attempted to generate mouse mAbs that neutralize A(H1N1)pdm09 Narita strain, whose HA antigenicity was indistinguishable from that of the CA04 strain [51], and obtained 20 mAbs with both HI and neutralization activities against Narita strain. By selecting escape mutants of Narita strain followed by their genetic and antigenic characterization, we identified antigenic epitopes of the 20 mAbs (Table 4). Out of the five previously reported HA antigenic sites (i.e., antigenic sites

Sa, Sb, Ca1, Ca2, and Cb) [7], three antigenic sites were covered by our 20 mAbs: Sa (recognized by 13 mAbs), Sb (4 mAbs), and Ca2 (3 mAbs).

We then determined MN titers against the 12 swIAVs and CA04 strains using the panel of the 20 mAbs (Table 3). We found that mAbs N345 and 3N28 showed no detectable neutralizing activity against any of the five antigenically different swIAVs. Likewise, neutralizing activities of mAbs N230, N408, and 3N74 were not detectable against all five swIAVs, except for one isolate, A/swine/Aichi/101/2018 (H1N1). More importantly, none of the 12 swIAVs and CA04 strains yielded consistent MN titer patterns with the 20 mAbs. These results suggested that the 12 swIAVs repeatedly replicated in different swine herds for a long time, rather than genetically and antigenically similar strains were shared among the swine populations in Japan.

Identification of the key amino acid residues for the substantially different HA antigenicity of the selected swIAVs

To determine the amino acid residues responsible for altering the HA antigenicity of the selected swIAVs, we compared the deduced amino acid sequences of the HAs from the five swIAVs whose HA antigenicity was substantially different from that of the CA04 strain (Table 5). Notably, our panel of 20 mouse mAbs targeted nine amino acid positions at three HA antigenic sites (Table 4). Among these nine amino acid positions, six substitutions at three positions were identified in at least one of the five swIAVs, compared to the human isolates; an alanine-to-glutamic acid substitution at position 141 (H1 numbering was applied throughout the manuscript) (A141E), S183P, S183T, S190N, S190T, and S190R (Table 5 and Fig. 2).

To assess the effect of the six identified substitutions on HA antigenicity, we generated six CA04 strain-based mutants possessing one of the six substitutions in the HA and determined their MN titers with the ferret antiserum. Among the CA04 HA mutants, the S190R mutant showed an eight-fold reduction in the MN titer relative to the wild-type CA04 strain (Table 6).

To further confirm the impact of the S190R substitution, we determined the MN titers against the S190R mutant using two mouse mAbs [that is, 3N1 and 3N51, which likely interact with a serine at position 190 of the HA (Table 4)]. The S190R mutant showed an eight-fold reduction in the MN titer of both mAbs relative to the wild-type CA04 strain (Table 6). These results indicated the critical impact of the S190R substitution at the antigenic site Sb on the antigenicity of the A(H1N1)pdm09-related swIAVs.

Potential N-glycosylation sites in HA of the antigenically different swIAVs

Glycans, particularly the N-glycans, close to the antigenic sites of influenza virus HAs, have the potential to mask epitopes from binding of the antibodies [84] and to contribute to evading the viruses from the host immune response [38]. To further investigate the possible determinants of the differential HA antigenicity of the five swIAVs, we predicted the N-glycosylation sequon (Asn-Xaa Ser/Thr) in the HAs by using the web-based NetNglyc server. Besides the N-glycosylation sites previously reported in the HA molecule of human A(H1N1)pdm09 viruses (i.e., amino acid positions 11, 23, 87, 162, 287, 481, and 540) [3], we identified two new potential N-glycosylation sites at amino acid positions 185 and 276 in the HAs of A/swine/Kagoshima/KU-FK1/2014 (H1N1) and A/swine/Japan/KU-YG5/2013 (H1N1), respectively (Fig. 2). The amino acid at position 185 is located in the antigenic site Sb of the HA head domain [12, 75], while that at position 276 is located ahead of the stem region of the HA. These findings suggest that the two N-glycosylation sites, particularly at position 185, might be one of the determinants of HA antigenicity.

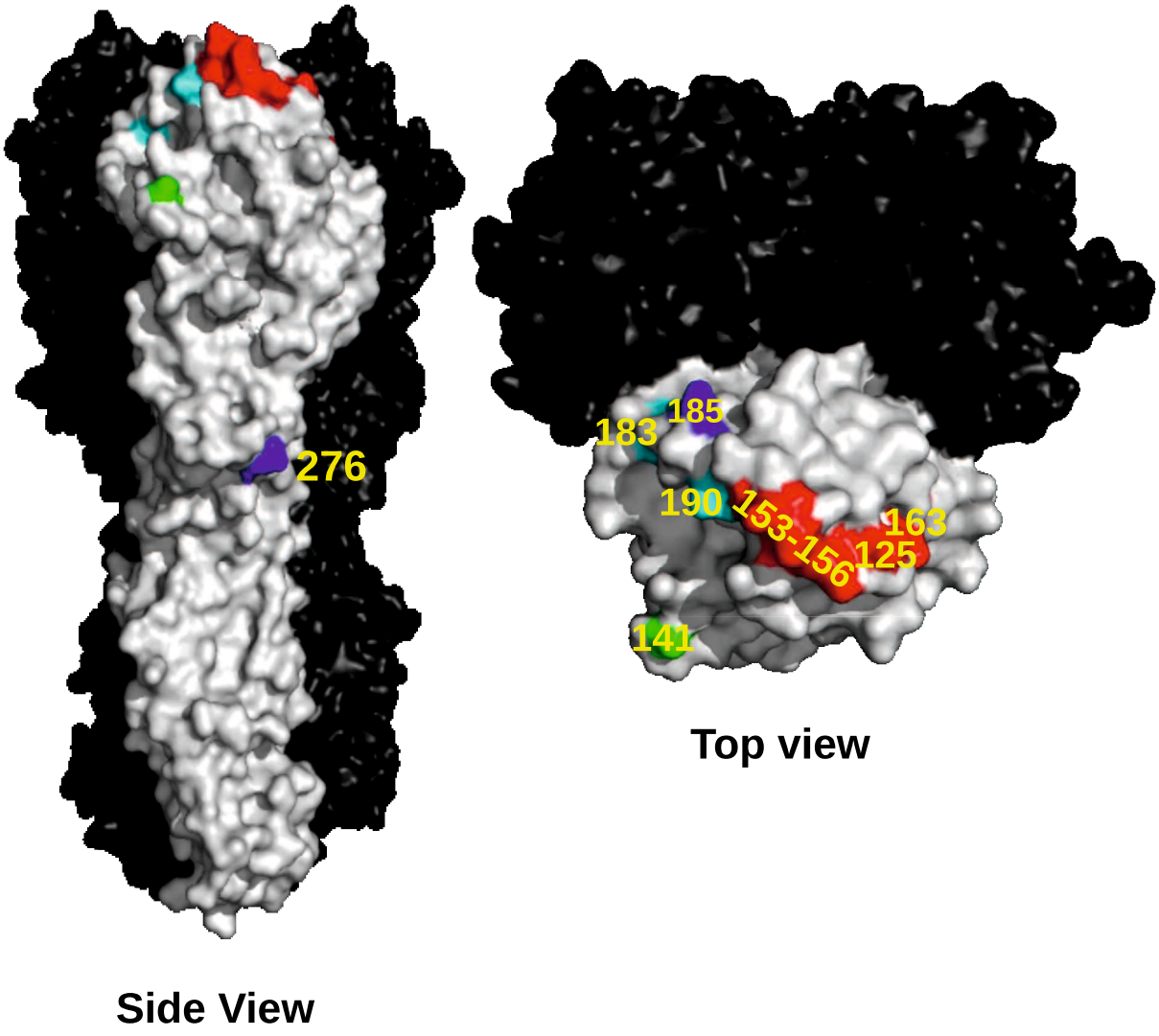


Fig. 2. Molecular structure of the HA showing the epitopes targeted by the panel of mAbs and potential N-glycosylation sites

The molecular structure of HA of A/California/04/2009 (H1N1) (PDB 3AL4) was visualized using the software program PYMOL (https://pymol.org/2/). The molecular surface of HA trimers viewed on its side (left) and top (right) are shown. One monomer (center) is colored gray, and the others are colored dark gray. Amino acid positions recognized by the panel of 20 mAbs tested were labeled; red (125, 153-156, and 163 located at the antigenic site Sa), cyan (183 and 190 at the antigenic site Sb), and green (141 at the antigenic site Ca2). Amino acid

positions of the potential N-glycosylation sites identified in this study (185 and 276) are shown in blue.

Discussion

The genetic diversity among the HAs of A(H1N1)pdm09-related swIAVs isolated in Japan during 2013–2018 has been reported to be broader than that of their human counterparts [66]. Here, we demonstrated that genetic diversity is associated with the antigenic difference of HA (Fig. 1 and Table 3). We also identified the amino acid substitution S190R in the antigenic site Sb of HA as a key determinant of the different antigenicity of A(H1N1)pdm09-related swIAVs (Fig. 2 and Table 6). In addition, two potential N-glycosylation sites that had not been previously reported in the HA of human A(H1N1)pdm09 viruses were identified in two swIAVs whose HA antigenic characteristics are substantially different from that of the CA04 strain (Fig. 2).

Of the 12 A(H1N1)pdm09-related swIAVs antigenically characterized using the ferret antiserum in this study, five swIAVs phylogenetically distinct from human isolates, A/swine/Japan/KU-YG5/2013 (H1N1), A/swine/Japan/KU-HY5/2013 (H1N1), A/swine/Kagoshima/KU-FK1/2014 (H1N1), A/swine/Aichi/101/2018 (H1N1), and A/swine/Aichi/KU-OE7/2017 (H1N2) (Fig. 1), were substantially different from the putative ancestor CA04 strain in the HA antigenicity (Table 3). More importantly, not only the five swIAVs, but also the remaining seven swIAVs, showed variable neutralization patterns in the assays with the panel of 20 mouse mAbs, indicating remarkable variations in HA antigenicity (Table 3). These results suggested that the five swIAVs evolved individually in each pig population, although we did not attest to the antigenic relationships between the swIAVs. In fact, we [66] and others [46, 47, 56, 102] previously described that swIAVs are genetically and antigenically variable by region even among strains of the same subtype, most likely due to the limited international and/or intercontinental movement of pigs. In Japan, because no swine influenza vaccine against the A(H1N1)pdm09 virus lineage is available, the immune-selective

pressure in pig populations may be more heterogeneous, and thus affect the genetic and antigenic diversity of circulating swIAVs.

Using CA04 strain-based HA mutants generated by reverse genetics, we identified the S190R substitution in the antigenic site Sb of HA as one of the critical determinants for the differential HA antigenicity of A(H1N1)pdm09-related swIAVs (Table 6). It should be noted that the S190R substitution in HA was previously reported as an egg-adapted mutation of human A(H1N1)pdm09 viruses [11], although A/swine/Aichi/KU-OE7/2017 (H1N2), which is the only isolate possessing the S190R substitution in HA among all the A(H1N1)pdm09-related human and swine viruses available in the GISAID database (Table 5), was isolated in canine-origin AX4 cells. Importantly, we confirmed by nucleotide sequencing that the HA gene segment detected in the original specimen for A/swine/Aichi/KU-OE7/2017 (H1N2) indeed encoded arginine at amino acid position 190 (data not shown). These results indicated that the S190R substitution in the HA of A/swine/Aichi/KU-OE7/2017 (H1N2) swIAV was introduced during replication in swine herds, but not in cultured cells.

The N-glycosylation site prediction allowed us to identify two amino acid substitutions creating potential N-glycosylation sites at positions 185 and 276 in HA, which are unique to A/swine/Kagoshima/KU-FK1/2014 (H1N1) and A/swine/Japan/KU-YG5/2013 (H1N1) swIAVs, respectively (Fig. 2). GISAID database search revealed that among all the other A(H1N1)pdm09-related human and swine viruses, only one swine isolate—A/swine/Kagoshima/SVB-167/2017 (H1N1)—encoded a potential N-glycosylation site at position 276. Although further studies are needed to confirm the N-glycosylations and their effect on the HA antigenicity, these amino acid substitutions may contribute to the substantially different HA antigenicity of the two swIAVs from the ancestor CA04 strain likely by interfering with the binding of neutralizing antibodies.

In addition to the nine amino acid positions that were recognized by our panel of mAbs, we found that the five antigenically different swIAVs possessed several amino acids that have

not been reported in human isolates: P124S and K160E in Sa; D187S, Q189R, and A195V in Sb; I166V and D168N in Ca1; K142R/S and D222N in Ca2; and S71Y in Cb. To elucidate the impact of these amino acid substitutions on HA antigenicity and to assess the public health risk posed by swIAVs, further experiments using antisera raised against recent human isolates are warranted.

In conclusion, our results revealed the various evolution patterns of the HA genes among A(H1N1)pdm09-related swIAVs. In addition, our findings highlight the potential risk of re-introduction of A(H1N1)pdm09 variants from pigs to humans, thus underlining the importance of swIAV surveillance not only for swine production but also for public health.

Table 1. List of tested swIAVs in MN assays

Virus	Accession No*.
A/swine/Japan/KU-YG5/2013 (H1N1)	KM596715
A/swine/Japan/KU-HY5/2013 (H1N1)	KM596713
A/swine/Kagoshima/KU-FK1/2014 (H1N1)	EPI1057378
A/swine/Ehime/01/2015 (H1N1)	EPI1057410
A/swine/Ehime/10/2015 (H1N1)	EPI1057419
A/swine/Nagasaki/KU-FK5/2016 (H1N1)	EPI1057488
A/swine/Aichi/KU-MI1-1/2016 (H1N1)	EPI1057436
A/swine/Aichi/KU-MI2-3/2016 (H1N1)	EPI1057462
A/swine/Aichi/02/2016 (H1N2)	EPI1057427
A/swine/Saga/KU-FK1/2016 (H1N1)	EPI1057496
A/swine/Aichi/KU-OE7/2017 (H1N2)	EPIISL576565
A/swine/Aichi/101/2018 (H1N1)	EPIISL576566

*Accession numbers in the NCBI (A/swine/Japan/KU-YG5/2013 (H1N1) and A/swine/Japan/KU-HY5/2013 (H1N1) and GISAID database (for the remaining virus strains) are listed.

Table 2. Closest relatives of each gene segment of the two characterized swIAVs

Virus	Gene	Accession No.*	Closest relative**	Identity (%)
A/swine/Aichi/101/2018 (H1N1)	PB2	EPI1255829	A/swine/Aichi/1/2018 (H1N1)	98.9
	PB1	EPI1255830	A/swine/Aichi/1/2018 (H1N1)	99.9
	PA	EPI1255828	A/swine/Aichi/1/2018 (H1N1)	99.1
	HA	EPI1255832	A/swine/Aichi/1/2018 (H1N1)	98.1
	NP	EPI1255825	A/swine/Aichi/1/2018 (H1N1)	100
	NA	EPI1057498	A/swine/Saga/KU-FK1/2016 (H1N1)	92.5
	M	EPI1255827	A/swine/Aichi/1/2018 (H1N1)	99.9
	NS	EPI585132	A/swine/Minnesota/A01134221/2011 (H1N2)	97.9
A/swine/Aichi/KU-OE7/2017 (H1N2)	PB2	EPI1057383	A/swine/Aichi/10/2015 (H1N1)	98.9
	PB1	EPI1057425	A/swine/Aichi/02/2016 (H1N2)	97.8
	PA	EPI1057385	A/swine/Aichi/10/2015 (H1N1)	98.3
	HA	EPI334810	A/Aichi/1250/2009 (H1N1)	96.1
	NP	EPI1057428	A/swine/Aichi/02/2016 (H1N2)	98.1
	NA	EPI1057429	A/swine/Aichi/02/2016(H1N2)	95.9
	M	EPI1057389	A/swine/Aichi/10/2015 (H1N1)	98.5
	NS	EPI1057431	A/swine/Aichi/02/2016 (H1N2)	98.4

*Accession numbers in the GISAID database are listed.

** Representative viruses with the highest nucleotide identity retrieved from the GISAID database on January 10, 2021.

Table 3. MN titers of 12 swIAVs and CA04 strain with the ferret antiserum and mouse mAbs

Virus	MN titer* with:												
	Ferret antiserum	antigenic site Sa-targeting mAb**											
		N73	N230	N327	N329	N334	N343	N345	N347	N408	3N37	3N38	3N64	3N74
A/California/04/2009 (H1N1)	640	16	32	16	256	16	64	8	64	64	8	32	4	128
A/swine/Japan/KU-YG5/2013 (H1N1)	80	16	<***	512	16	8	<	<	4	<	4	<	<	<
A/swine/Japan/KU-HY5/2013 (H1N1)	80	1	<	2	16	4	<	<	8	<	<	<	<	<
A/swine/Kagoshima/KU-FK1/2014 (H1N1)	80	1	<	512	8	16	1	<	4	<	2	32	32	<
A/swine/Ehime/01/2015 (H1N1)	320	2	<	8	4	4	<	<	2	<	1	<	<	<
A/swine/Ehime/10/2015 (H1N1)	320	32	<	32	16	32	512	128	8	4	64	128	16	256
A/swine/Nagasaki/KU-FK5/2016 (H1N1)	2560	1	1	64	16	128	512	256	256	512	128	128	16	256
A/swine/Aichi/KU-MI1-1/2016 (H1N1)	2560	1	1	256	4	16	4	<	16	2	16	4	2	8
A/swine/Aichi/KU-MI2-3/2016 (H1N1)	2560	8	<	256	4	16	2	<	8	2	16	4	2	16
A/swine/Aichi/02/2016 (H1N2)	160	1	<	1	8	16	16	2	8	2	4	1	1	<
A/swine/Saga/KU-FK1/2016 (H1N1)	640	16	1	512	512	512	512	1	32	2	32	16	8	32
A/swine/Aichi/101/2018 (H1N1)	80	32	8	4	4	16	2	<	16	32	16	64	2	256
A/swine/Aichi/KU-OE7/2017 (H1N2)	80	2	<	32	32	16	4	<	16	<	<	<	2	<

*The MN titer is expressed as the reciprocal of the highest antibody dilution to retain a confluent cell monolayer.

**Two-fold serial dilutions of mAbs N73-N327, N343, N345, N408-3N38, 3N74, 3N28, 3N22-3N47 starting at 1:1000 dilution and mAbs N329, N334, N347, 3N64, 3N1, 3N44, and 3N51 mAbs starting at 1:100 dilution were individually mixed with each virus tested, followed by incubation at 37°C for 1 h.

***<: Neutralization was not detected.

Table 3 (continued).

Virus	MN titer* with:						
	antigenic site Sb-targeting mAb				antigenic site Ca2-targeting mAb		
	3N1	3N28	3N44	3N51	3N22	3N35	3N47
A/California/04/2009 (H1N1)	64	1	4	8	8	8	16
A/swine/Japan/KU-YG5/2013 (H1N1)	<	<	<	8	64	256	256
A/swine/Japan/KU-HY5/2013 (H1N1)	2	<	8	1	<	<	<
A/swine/Kagoshima/KU-FK1/2014 (H1N1)	8	<	2	8	64	512	512
A/swine/Ehime/01/2015 (H1N1)	4	<	2	2	64	512	128
A/swine/Ehime/10/2015 (H1N1)	128	32	128	32	128	128	128
A/swine/Nagasaki/KU-FK5/2016 (H1N1)	128	4	32	16	128	512	512
A/swine/Aichi/KU-MI1-1/2016 (H1N1)	512	64	256	128	2456	512	512
A/swine/Aichi/KU-MI2-3/2016 (H1N1)	512	64	512	128	256	512	512
A/swine/Aichi/02/2016 (H1N2)	8	<	16	8	32	4	16
A/swine/Saga/KU-FK1/2016 (H1N1)	512	512	512	256	512	4	16
A/swine/Aichi/101/2018 (H1N1)	16	<	2	4	2	<	<
A/swine/Aichi/KU-OE7/2017 (H1N2)	16	<	4	1	<	<	<

Table 4. Comparative biological assays of the mouse mAbs produced against the A/Narita/1/2009 (H1N1) strain

mAb clone	Isotype	Neutralization titer*	HI titer**	Amino acid substitution in escape mutant***	Antigenic site
N73	IgA	640,000	10,240	N125D, G155E	Sa
N230	IgG1	1,280,000	5,120	G155E	Sa
N327	IgG1	800,000	>20,480	N125T, N125S, G155E, K163N	Sa
N329	IgG1	5,120	1,280	K153E, G155E	Sa
N334	IgG1	32,000	2,560	G155E	Sa
N343	IgG1	3,200,000	>20,480	N125D, G155E	Sa
N345	IgG1	6,400,000	>20,480	N125D, K153E, G155E	Sa
N347	IgG2b	160,000	1,280	N156D	Sa
N408	IgG2b	6,400,000	>20,480	N125T, N125D, G155E, K163N	Sa
3N37	IgA	1,280,000	20,480	K154E	Sa
3N38	IgG1	1,600,000	>20,480	E153K & K154T, G155E	Sa
3N64	IgA	5,120	1,280	K154E, G155E	Sa
3N74	IgG2a	640,000	>20,480	K154E, N156D	Sa
3N1	IgA	40,000	10,240	K153E & S190R, N156D & S190R	Sb
3N28	IgG2a	409,600	>20,480	K153E & S190R, N156D & S190R	Sb
3N44	IgG2a	204,800	>20,480	S183P	Sb
3N51	IgA	51,200	10,240	K153E & S190R, N156D & S190R	Sb
3N22	IgA	10,240,000	>20,480	A141E	Ca2
3N35	IgG2b	1,280,000	>20,480	A141E	Ca2
3N47	IgG1	5,120,000	>20,480	A141E	Ca2

*Neutralization titer was determined by plaque reduction assays against the Narita strain. Neutralization titer was expressed as the reciprocal of the highest antibody dilution inducing a >50% reduction in plaque number.

**HI titer to Narita strain was determined by the standard method using 0.5% chicken erythrocytes. The HI titer was expressed as the reciprocal of the highest antibody dilution that completely inhibited hemagglutination. ***Amino acid positions are shown by H1 numbering.

Table 5. Amino acid residues at the selected positions of the HA of human A(H1N1)pdm09 viruses and the antigenically different swIAVs

Virus	Amino acid residue								
	Sa**						Sb		Ca2
	125***	153	154	155	156	163	183	190	141
Human pdm09 viruses*	N	K/E/N	K	G/E	N/D/K	K/Q/I	S/P	S	A
A/swine/Japan/KU-YG5/2013 (H1N1)	N	K	K	E	N	K	S	T	A
A/swine/Japan/KU-HY5/2013 (H1N1)	N	K	K	G	N	Q	P	N	E
A/swine/Kagoshima/KU-FK1/2014 (H1N1)	N	K	K	E	N	K	T	T	A
A/swine/Aichi/101/2018 (H1N1)	N	K	K	G	D	K	P	S	A
A/swine/Aichi/KU-OE7/2017 (H1N2)	N	N	K	E	N	I	P	R	E

* All human isolates of A(H1N1)pdm09 virus lineage isolated during 2009–2020. Genetic data were retrieved from the GISAID on January 10, 2021 and analyzed using the WebLogo 3 (51).

**The HA antigenic sites previously defined (55) are shown.

***Amino acid positions are shown by H1 numbering.

Table 6. MN titers of the ferret antiserum and selected mAbs against wildtype CA04 strain and its HA mutants

Virus	MN titer with:		
	Ferret antiserum	mAb 3N1*	mAb 3N51*
Wild-type CA04 strain	640	64	8
CA04-based HA mutants with A141E**	640	ND***	ND
CA04-based HA mutants with S183P	640	ND	ND
CA04-based HA mutants with S183T	640	ND	ND
CA04-based HA mutants with S190T	640	ND	ND
CA04-based HA mutants with S190N	320	ND	ND
CA04-based HA mutants with S190R	80	8	1

*Two-fold serial dilutions of both mAbs starting at 1:100 dilution were mixed with each virus tested, followed by incubation at 37°C for 1 h.

**CA04-based mutants possessing the indicated single HA substitution were generated by reverse genetics.

*** ND: not done.

Chapter II

Transition in genetic constellations of H3N8 and H4N6 low-pathogenic avian influenza viruses isolated from an overwintering site in Japan throughout different winter seasons

Abstract

The Izumi plain in Kagoshima Prefecture, Japan, is an overwintering site for migratory ducks and endangered cranes. We have surveyed avian influenza viruses (AIVs) in this area since 2012 and isolated low-pathogenic AIVs (LPAIVs) of various subtypes every winter season. H3N8 LPAIVs were isolated during the 2012/13 and 2016/17 seasons, and H4N6 LPAIVs were isolated during the 2012/13 and 2013/14 seasons. In the 2017/18 season, one H3N8 and two H4N6 LPAIV strains were isolated from environmental water samples. Genetic and phylogenetic analysis for each gene segment from these H3N8 and H4N6 LPAIVs suggested that our isolates were genetic reassortants generated by intermixing between AIVs circulating not only in Eurasia but also in Africa and/or North America. Comparison of the genetic constellations of our three isolates with their counterparts isolated during previous seasons from the Izumi plain revealed a drastic transition in the genetic constellations of both subtypes. These findings emphasize the importance of continuous surveillance of AIVs on the Izumi plain.

Introduction

Influenza A virus, a member of the family Orthomyxoviridae, is a single-stranded negative-sense segmented RNA virus. The viral genome consists of eight negative-sense, single-stranded RNAs containing the polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and non-structural protein (NS) genes, which encode at least 10 proteins [104]. Among the influenza A viruses, isolates from avian species—the avian influenza viruses (AIVs)— are of great concern because of their economic impact on the poultry industry and their zoonotic potential [58]. AIVs are classified into 16 HA and nine NA subtypes based on the antigenicity of two surface glycoproteins, HA and NA [15, 104].

The Izumi plain in Kagoshima Prefecture at the southern tip of Kyushu Island in Japan is a well-known overwintering site for over 17,000 migratory birds. Approximately 90% of the global population of the hooded crane (Grus monacha)—which breeds in south-central and southeastern Siberia—and 50% of the white-naped crane (Grus vipio)—which breeds in the northeastern regions of Mongolia and China—share the Izumi plain as their overwintering site. Because these cranes are classified as vulnerable species according to the International Union for Conservation of Nature Red List, several conservation measures (e.g., feeding, monitoring, and creating artificial wet paddies as roosts for cranes) have been undertaken every winter season. In addition to cranes, wild ducks of the order Anseriformes—the primary natural reservoir of AIVs [67, 104]—also share this overwintering site [54, 64]. Thus, there is a potential risk of AIV infection involving the endangered crane populations in this area. HPAIVs of H5N1, H5N8, and H5N6 subtypes were isolated from dead or debilitated cranes from the Izumi plain during the 2010/11, 2014/15, and 2016/17 winter season, respectively [63, 72, 74, 81]. In addition, because the Izumi plain is famous for chicken farming, the local population is concerned with the risk of AIV infection in their chicken populations. In fact, one of the chicken farms on the Izumi plain

was affected by H5N1 HPAIVs in 2010 [81]. Therefore, AIV surveillance on the Izumi plain, especially bird roosts, is imperative for risk assessment of HPAIV infections in both endangered cranes and local chickens.

Since 2012, we have surveyed AIVs on the Izumi plain every winter season by collecting samples from cranes and ducks (feces and carcasses) and from roosts. We isolated both HPAIVs [63, 72, 74] and LPAIVs of various subtypes [54, 64, 65, 74]. In particular, LPAIVs of subtypes H3N8 and H4N6 have been isolated repeatedly throughout different winter seasons; H3N8 LPAIVs were isolated during the 2012/13 [64] and 2016/17 [74] seasons, and H4N6 LPAIVs were isolated during the 2012/13 [64] and 2013/14 [65] seasons.

Here, we report the isolation of one H3N8 LPAIV and two H4N6 LPAIVs from the Izumi plain during the 2017/18 winter season. Genetic characterization of these isolates, as well as previous isolates from the same overwintering site, revealed the diversity and transition of genetic constellations involving H3N8 and H4N6 LPAIVs.

Materials and methods

Sample collection

In the Izumi plain, two rectangular wet paddies (approximately 2,000 m2 each) are transiently converted into roosts for cranes every winter. A total of 256 environmental water samples (50 mL/sample, 10–14 samples/week, 6-8 samples from each wet paddy) were collected from the crane roosts during the 2017/18 winter season (from November 2017 to March 2018). The collected samples were kept on ice until processing for virus isolation.

Influenza virus isolation

The water samples were processed for virus isolation as described previously [64]. Briefly, the potential viruses in the water samples were concentrated by adsorption to chicken red blood cells and inoculated into embryonated chicken eggs (two eggs/sample). The allantoic fluids harvested from the inoculated eggs were subjected to hemagglutination assays for confirmation of virus isolation as described previously [54].

Detection of influenza A virus genes and HA and NA subtyping

RNA was extracted from allantoic fluids that were positive for hemagglutination activity using an innuPREP Virus DNA/RNA Kit (Analytik Jena AG, Jena, Germany) and subjected to real-time reverse transcription polymerase chain reaction (RT-PCR)-based influenza virus gene detection using Brilliant III Ultra-Fast SYBR Green QRT-PCR Master Mix (Agilent Technologies, Santa Clara, CA) and primers specific for a conserved region of the influenza A virus M gene as described previously [64]. The extracted RNAs that were positive for the influenza A virus M gene were reverse transcribed using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA) and subjected to standard PCR-based HA and NA

subtyping using Ex Taq DNA Polymerase (TaKaRa Bio Inc., Otsu, Japan) with a panel of subtype-specific primer sets [100].

Sequence analysis of influenza virus genes

The complementary DNAs (cDNAs) for the eight-segmented influenza virus genes were amplified by standard PCR using TaKaRa Tks Gflex DNA Polymerase (TaKaRa Bio Inc.) with gene segment-specific primer sets [28]. Nucleotide sequences of open reading frames of all viral gene segments were determined by Sanger sequencing at FASMAC Co., Ltd. (Atsugi, Japan), and the sequences were deposited in the GISAID database (Table 1).

Genetic and phylogenetic analysis of influenza virus genes

The closest relatives of the sequenced viral gene segments were identified using the Basic Local Alignment Search Tool (BLAST) and the Global Initiative on Sharing All Influenza Data (GISAID) database (http://platf orm.gisaid.org/). The sequenced viral gene segments were phylogenetically analyzed with their representative counterparts reflecting the temporal and spatial distribution of AIVs, and with viral gene segments from LPAIVs previously isolated from the Izumi plain; nucleotide sequences were retrieved from the GISAID and National Center for Biotechnology Information (NCBI) (https ://www.ncbi.nlm.nih.gov) databases. We then defined the geographical clusters in the phylogenic trees based on the countries where the majority of viral strains in each cluster were isolated. The nucleotide sequences for each viral gene segment were aligned using MAFFT software version 7.397 (https ://mafft.cbrc.jp/alignment/software/). Phylogenetic trees for each viral gene segment were constructed using the neighbor-joining method based on the maximum composite likelihood model in the MEGA software version 6.0 (https ://www.megasoftware.net) with a bootstrapping set of 1,000 replicates. Notably, the conformations of each phylogenetic tree constructed using the maximum-likelihood method (data not shown) were essentially the same as those shown in Fig. 1.

Results and discussion

Isolation of AIVs from crane roosts on the Izumi plain

AIVs have been reported to survive in water for only up to 32 days at 4 °C and 4 days at 22 °C, [105]. In addition, the wet paddies from which samples were collected are used for cultivating rice from spring through fall, and the water in these areas is kept running throughout the season. Therefore, AIV surveillance activity on the Izumi plain is imperative every winter. As part of our AIV surveillance activity on the Izumi plain, a total of 256 environmental water samples were collected from crane roosts during the 2017/18 winter season for AIV isolation. By inoculating embryonated chicken eggs with the water samples, followed by hemagglutination assays, we isolated three viruses exhibiting hemagglutination activity. Each of the three isolates was positive for the M gene of influenza A virus. PCR-based HA and NA subtyping revealed that these three isolates included one H3N8 AIV, A/ environment/Kagoshima/KU-ngr-G/2018 (H3N8), and two H4N6 AIVs, A/environment/Kagoshima/KU-ngr-C/2018 (H4N6) and A/environment/Kagoshima/KU-ngr-E/2018 (H4N6) (Table 1).

Genetic analysis of the AIV isolates

To genetically characterize our three AIV isolates, the nucleotide sequences of all eight gene segments of each isolate were determined (Table 1). The nucleotide sequences revealed that the two H4N6 AIV isolates were genetically almost identical. While the samples for these two isolates were collected from the same wet paddy, there was a considerable time lag (approximately 1 month) in sampling. These results suggest that AIVs are genetically stable in their primary natural reservoirs. The deduced amino acid sequence at the HA cleavage site of the H3N8 and H4N6 AIVs was SEKQPR/GLF and PEKASR/GLF, respectively. As a monobasic amino acid residue at this site is characteristic of LPAIVs that cause local infection in respiratory and digestive organs, all three isolates were defined as LPAIVs.

To determine the closest relatives of all of the viral gene segments of each isolate, their nucleotide sequences were subjected to BLAST searches (Table 1). While most of the closest relatives of our three isolates were from East Asian countries, the NA gene of our H3N8 LPAIV isolate was genetically close to that of a North American isolate, A/northern pintail/Alaska/993/2011 (H3N8). These findings suggested that our H3N8 AIV isolate was a genetic reassortant between AIVs circulating in Asian and the North American countries and that our H4N6 AIV isolates were genetic reassortants between AIVs circulating in Asian countries. Intriguingly, the PB2 gene segments from our H4N6 LPAIV isolates were genetically similar to that of an AIV isolate from a hooded crane in Korea, A/hooded crane/Korea/1176/2016 (H1N1). However, the scenario in which hooded cranes would be potential carriers of LPAIVs remains inconclusive, because only a few LPAIVs have been isolated from their feces [49, 54]. The lower sequence identity in the HA (95.8%) and NA (96.5%) gene segments from the H3N8 isolate and NA gene segments from H4N6 isolates (96.9 and 93.4%) compared with other gene segments could be attributed to the fact that the encoded viral proteins, HA and NA, are expressed on the virion surface and thus evolve more rapidly under immune selective pressure in the hosts [79].

Phylogenetic analysis of AIV isolates

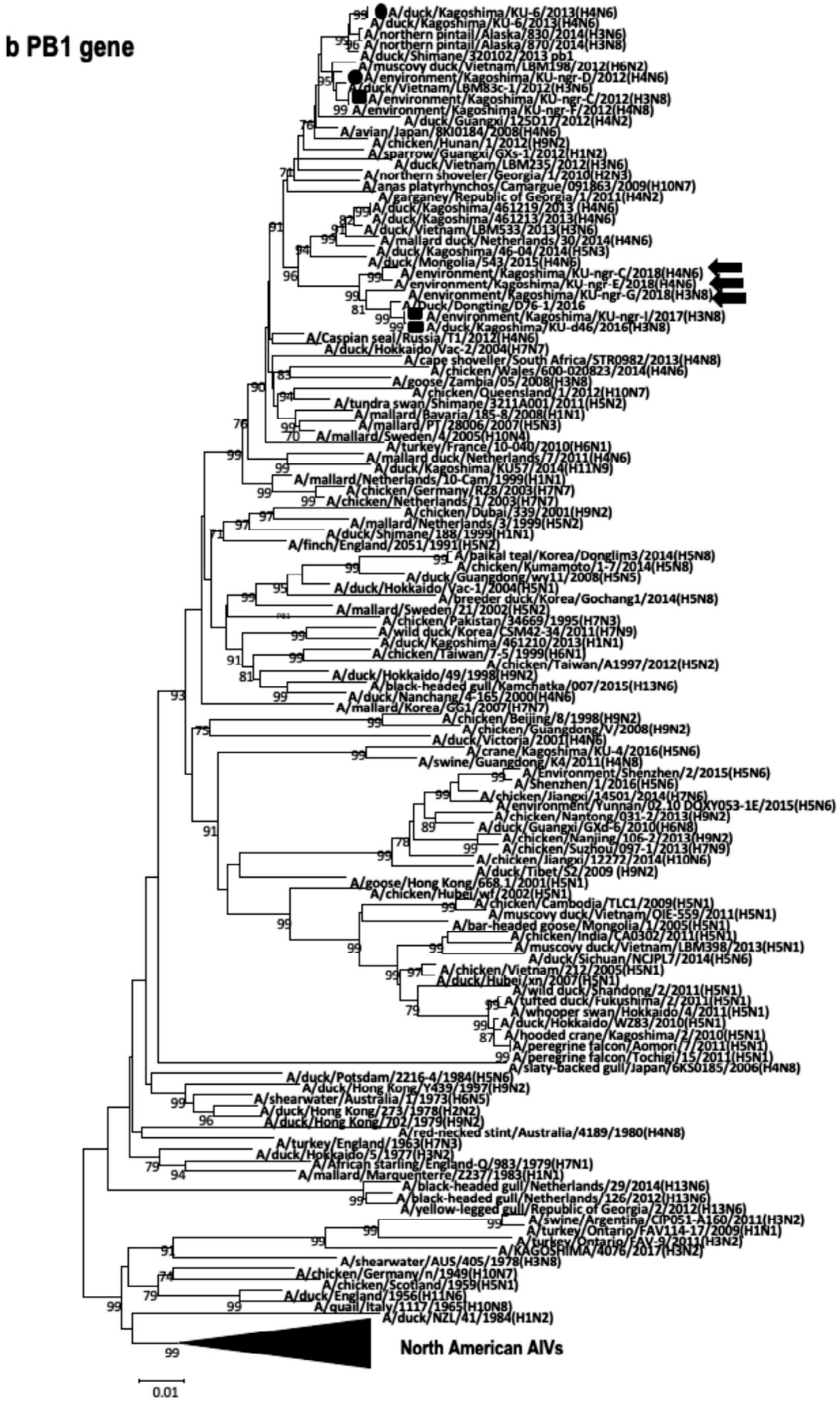
To further investigate the genetic relationships involving our three isolates and global isolates, the nucleotide sequence of each viral gene segment was phylogenetically analyzed together with their counterparts from isolates that reflect the temporal and spatial distribution of AIVs. In addition, the nucleotide sequence of each viral gene segment from AIVs previously isolated from the Izumi plain [64, 65, 74] was included in the analysis.

Phylogenetic analysis revealed that five of the eight viral gene segments—specifically the PB2, PB1, PA, NP, and M gene segments—from our H3N8 LPAIV isolate were phylogenetically close to their counterparts from AIVs isolated in Asian and European countries (Fig. 1a, b, c, d, and e). By contrast, the NA gene segment—whose closest relative was from the North American

isolate (Table 1)—formed a small cluster along with another North American isolate, A/ northern pintail/Alaska/870/2014 (H3N8), and a relatively old Japanese isolate, A/duck/Hokkaido/W2/2004 (H6N8) (Fig. 1f). In addition, previous studies have also identified several genetic reassortments that have occurred between AIVs circulating in North American and Asian countries [77, 109]. Furthermore, the NS and HA gene segments formed clusters with their counterparts from various Eurasian and African isolates (Fig. 1g and h), although the closest relatives of these gene segments were detected in China and Japan, respectively (Table 1).

The PB2, NS, HA, and NA gene segments from our two H4N6 LPAIV isolates were closely related to those of Eurasian and African isolates (Fig. 1a, g, i and j), although their closest relatives were detected in Asian countries (Table 1). The other four gene segments (PB1, PA, NP and M) were phylogenetically close to their counterparts from AIVs isolated in Asian and European countries (Fig. 1b-e). In particular, the PB1 gene segments of our H3N8 and H4N6 LPAIV isolates share a recent common ancestor (Fig. 1b), while the origins of the remaining five internal gene segments of our H3N8 and H4N6 LPAIV isolates were phylogenetically distinguishable (Fig. 1a, c, d, e, and g). These findings suggest that our H3N8 and H4N6 LPAIV isolates are genetic reassortants that were generated by intermixing between AIVs circulating in Eurasian, African, and/or North American countries.


a PB2 gene
A/chicken/Jiangxi/14501/2014(H7N6)
A/chicken/Nanjing/106-2/2013(H9N2)
A/chicken/Suzhou/097-1/2013(H7N9)
A/Environment/Shenzhen/2/2015(H5N6)
A/chicken/Jiangxi/12272/2014(H10N6)
A/duck/Guangxi/GXd-6/2010(H6N8)
A/environment/Yunnan/02.10 DQXY053-1E/2015(H5N6)
A/chicken/Hunan/1/2012(H9N2)
A/duck/Tibet/52/2009(H9N2)
A/swine/Guangdong/K4/2011(H4N8)
A/mallard/Korea/GG3/2007(H7N7)
A/northern pintail/Alaska/870/2014(H3N8)
A/common teal/Hong Kong/MPM1645/2011(H7N1)
A/environment/Kagoshima/KU-ngr-C/2012(H3N8)
A/environment/Kagoshima/KU-ngr-F/2012(H4N8)
A/mallard/Netherlands/3/1999(H5N2)
A/chicken/Iran/S54/2009(H9N2)
A/chicken/Iran/S57/2011(H9N2)
A/mallard duck/Netherlands/30/2014(H4N6)
A/environment/Kagoshima/KU-ngr-G/2018(H3N8)
A/duck/Aichi/231001/2016(H8N4)
A/environment/Kagoshima/KU-ngrI/2014(H6N2)
A/duck/Hokkaido/Vac-2/2004(H7N7)
A/tundra swan/Shimane/3211A001/2011(H5N2)
A/wild duck/Korea/CSM42-34/2011(H7N9)
A/chicken/Germany/R28/2003(H7N7)
A/chicken/Netherlands/1/2003(H7N7)
A/mallard/Sweden/21/2002(H5N2)
A/mallard/Bavaria/185-8/2008(H1N1)
A/duck/Hokkaido/Vac-3/2007(H5N1)
A/avian/Japan/8KI0184/2008(H4N6)
A/breeder duck/Korea/Gochang1/2014(H5N8)
A/Caspian seal/Russia/T1/2012(H4N6)
A/chicken/Wales/600-020823/2014(H4N6)
A/mallard/Sweden/4/2005(H10N4)
A/environment/Kagoshima/KU-ngr-C/2018(H4N6)
A/environment/Kagoshima/KU-ngr-E/2018(H4N6)
A/hooded crane/Korea/1176/2016(H1N1)
A/duck/Mongolia/543/2015(H4N6)
A/avian/Egypt/920431/2006(H9N2)
A/cape shoveller/South Africa/STR0982/2013(H4N8)
A/mallard duck/Netherlands/7/2011(H4N6)
A/duck/Thailand/CU-LM728BC/2010(H7N6)
A/sparrow/Guangxi/GXs-1/2012(H1N2)
A/duck/Vietnam/LBM235/2012(H3N6)
A/duck/Kagoshima/KU-d26/2016(H1N1)
A/environment/Kagoshima/KU-ngr-I/2017(H3N8)
A/duck/Kagoshima/KU-d46/2016(H3N8)
A/garganey/Republic of Georgia/1/2011(H4N2)
A/northern shoveler/Georgia/1/2010(H2N3)
A/bean goose/Korea/SH20-17/2008(H7N3)
A/crane/Kagoshima/KU-T40/2015(H11N9)
A/duck/Kagoshima/KUS7/2014(H11N9)
A/duck/Kagoshima/461210/2013(H1N1)
A/duck/Kagoshima/46-04/2014(H5N3)
A/duck/Vietnam/LBM533/2013(H3N6)
A/duck/Kagoshima/461219/2013(H4N6)
A/duck/Kagoshima/461213/2013(H4N6)
A/mallard/Marquenterre/2237/1983(H1N1)
A/budgerigar/Hokkaido/1/1977(H4N6)
A/mallard/Netherlands/10-Cam/1999(H1N1)
A/chicken/Dubai/339/2001(H9N2)
A/chicken/Pakistan/34669/1995(H7N3)
A/duck/Potsdam/ZZ16-4/1984(H5N6)
A/crane/Kagoshima/KU-4/2016(H5N6)
A/African starling/England-Q/983/1979(H7N1)
A/duck/Shimane/188/1999 (H1N1)
A/finch/England/2051/1991(H5N2)
A/quail/Italy/1117/1965(H10N8)
A/turkey/England/1963(H7N3)
A/duck/England/1956(H11N6)
A/chicken/Scotland/1959(H5N1)
A/chicken/Germany/n/1949(H10N7)
A/shearwater/Australia/1/1973(H6N5)
A/duck/Hokkaido/5/1977(H3N2)
A/duck/Hong Kong/273/1978(H2N2)
A/duck/Guangxi/125D17/2012(H4N2)
A/slaty-backed gull/Japan/6K50185/2006(H4N8)
A/chicken/Queensland/1/2012(H10N7)
A/duck/Victoria/2001(H4N6)
A/chicken/Beijing/8/1998(H9N2)
A/chicken/Hubei/01-MA01/1999(H9N2)
A/chicken/Guangdong/V/2008(H9N2)
A/anas platyrhynchos/Camargue/091863/2009(H10N7)
A/turkey/France/10-040/2010(H6N1)
A/mallard/PT/28006/2007(H5N3)
A/duck/Hong Kong/Y439/1997(H9N2)
A/chicken/Taiwan/7-5/1999(H6N1)
A/duck/Hokkaido/49/1998(H9N2)
A/duck /Zambia/03/2008(H6N2)
A/ostrich/South Africa/AI2887/2011(H1N2)
A/duck/Zambia/12/2009(H11N9)
A/goose/Zambia/07/2008(H4N6)
A/chicken/Taiwan/A1997/2012(H5N2)
A/duck/Hong Kong/702/1979(H9N2)
A/shearwater/AUS/405/1978(H3N8)
A/baikal teal/Korea/Donglim3/2014(H5N8)
A/chicken/Kumamto/1-7/2014(H5N8)
A/duck/Guangdong/wy11/2008(H5N5)
A/environment/Kagoshima/KU-ngr-D/2012(H4N6)
A/duck/Miyazaki/451115/2012(H11N3)
A/muscovy duck/Vietnam/LBM198/2012(H6N2)
A/duck/Kagoshima/KU-6/2013(H4N6)
A/northern pintail/Alaska/830/2014(H3N6)
A/duck/Hunan/S11090/2012(H4N6)
A/muscovy duck/Vietnam/LBM455/2013(H6N2)
A/duck/Hokkaido/Vac-1/2004(H5N1)
A/duck/Nanchang/4-165/2000(H4N6)
A/duck/Hubei/xn/2007(H5N1)
A/goose/Hong Kong/668.1/2001(H5N1)
A/chicken/Hubei/wf/2002(H5N1)
A/chicken/Cambodia/TLC1/2009(H5N1)
A/muscovy duck/Vietnam/OIE-559/2011(H5N1)
A/chicken/Menofia/CAI39/2010(H5N1)
A/chicken/Sharkia/CAI38/2009(H5N1)
A/chicken/Egypt/Q2247B/2010(H5N1)
A/bar-headed goose/Mongolia/1/2005(H5N1)
A/chicken/Bangladesh/BL-470/2010(H5N1)
A/turkey/Egypt/S6405A/2012(H5N1)
A/duck/Sichuan/NCJPL7/2014(H5N6)
A/muscovy duck/Vietnam/LBM398/2013(H5N1)
A/chicken/Vietnam/212/2005(H5N1)
A/chicken/India/CA0302/2011(H5N1)
A/wild duck/Shandong/2/2011(H5N1)
A/hooded crane/Kagoshima/2/2010(H5N1)
A/tufted duck/Fukushima/2/2011(H5N1)
A/whooper swan/Hokkaido/4/2011(H5N1)
A/peregrine falcon/Tochigi/15/2011(H5N1)
A/duck/Hokkaido/WZ83/2010(H5N1)
A/peregrine falcon/Aomori/7/2011(H5N1)
A/seagull/Chile/5775/2009(H13N9)
A/black-headed gull/Netherlands/29/2014(H13N6)
A/yellow-legged gull/Republic of Georgia/2/2012(H13N6)
A/duck/NZL/41/1984(H1N2)
A/red-necked stint/Australia/4189/1980(H4N8)
A/mallard/Chile/C948/2015(H4N2)
A/yellow-billed pintail/Chile/6/2013(H4N6)
A/KAGOSHIMA/4076/2017(H3N2)
North American AIVs
Eurasian/African AIVs
0.02


b PB1 gene
A/duck/Kagoshima/KU-6/2013(H4N6)
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A/northern pintail/Alaska/870/2014(H3N8)
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A/muscovy duck/Vietnam/LBM198/2012(H6N2)
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A/duck/Guangxi/125D17/2012(H4N2)
A/avian/Japan/8KI0184/2008(H4N6)
A/chicken/Hunan/1/2012(H9N2)
A/sparrow/Guangxi/GXs-1/2012(H1N2)
A/duck/Vietnam/LBM235/2012(H3N6)
A/northern shoveler/Georgia/1/2010(H2N3)
A/anas platyrhynchos/Camargue/091863/2009(H10N7)
A/garganey/Republic of Georgia/1/2011(H4N2)
A/duck/Kagoshima/461219/2013 (H4N6)
A/duck/Kagoshima/461213/2013(H4N6)
A/duck/Vietnam/LBM533/2013(H3N6)
A/mallard duck/Netherlands/30/2014(H4N6)
A/duck/Kagoshima/46-04/2014(H5N3)
A/duck/Mongolia/543/2015(H4N6)
A/environment/Kagoshima/KU-ngr-C/2018(H4N6)
A/environment/Kagoshima/KU-ngr-E/2018(H4N6)
A/environment/Kagoshima/KU-ngr-G/2018(H3N8)
A/Duck/Dongting/D76-1/2016
A/environment/Kagoshima/KU-ngr-I/2017(H3N8)
A/duck/Kagoshima/KU-d46/2016(H3N8)
A/Caspian seal/Russia/T1/2012(H4N6)
A/duck/Hokkaido/Vac-2/2004(H7N7)
A/cape shoveller/South Africa/STR0982/2013(H4N8)
A/chicken/Wales/600-020823/2014(H4N6)
A/goose/Zambia/05/2008(H3N8)
A/chicken/Queensland/1/2012(H10N7)
A/tundra swan/Shimane/3211A001/2011(H5N2)
A/mallard/Bavaria/185-8/2008(H1N1)
A/mallard/PT/28006/2007(H5N3)
A/mallard/Sweden/4/2005(H10N4)
A/turkey/France/10-040/2010(H6N1)
A/mallard duck/Netherlands/7/2011(H4N6)
A/duck/Kagoshima/KU57/2014(H11N9)
A/mallard/Netherlands/10-Cam/1999(H1N1)
A/chicken/Germany/R28/2003(H7N7)
A/chicken/Netherlands/1/2003(H7N7)
A/chicken/Dubai/339/2001(H9N2)
A/mallard/Netherlands/3/1999(H5N2)
A/duck/Shimane/188/1999(H1N1)
A/finch/England/2051/1991(H5N2)
A/baikal teal/Korea/Donglim3/2014(H5N8)
A/chicken/Kumamoto/1-7/2014(H5N8)
A/duck/Guangdong/wy11/2008(H5N5)
A/duck/Hokkaido/Vac-1/2004(H5N1)
A/breeder duck/Korea/Gochang1/2014(H5N8)
A/mallard/Sweden/21/2002(H5N2)
A/chicken/Pakistan/34669/1995(H7N3)
A/wild duck/Korea/CSM42-34/2011(H7N9)
A/duck/Kagoshima/461210/2013(H1N1)
A/chicken/Taiwan/7-5/1999(H6N1)
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A/black-headed gull/Kamchatka/007/2015(H13N6)
A/duck/Nanchang/4-165/2000(H4N6)
A/mallard/Korea/GG1/2007(H7N7)
A/chicken/Beijing/8/1998(H9N2)
A/chicken/Guangdong/V/2008(H9N2)
A/duck/Victoria/2001(H4N6)
A/crane/Kagoshima/KU-4/2016(H5N6)
A/swine/Guangdong/K4/2011(H4N8)
A/Environment/Shenzhen/2/2015(H5N6)
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A/chicken/Jiangxi/14501/2014(H7N6)
A/environment/Yunnan/02.10 DQXY053-1E/2015(H5N6)
A/chicken/Nantong/031-2/2013(H9N2)
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A/chicken/Nanjing/106-2/2013(H9N2)
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A/chicken/Jiangxi/12272/2014(H10N6)
A/duck/Tibet/S2/2009 (H9N2)
A/goose/Hong Kong/668.1/2001(H5N1)
A/chicken/Hubei/wf/2002(H5N1)
A/chicken/Cambodja/TLC1/2009(H5N1)
A/muscovy duck/Vietnam/OIE-559/2011(H5N1)
A/bar-headed goose/Mongolia/1/2005(H5N1)
A/chicken/India/CA0302/2011(H5N1)
A/muscovy duck/Vietnam/LBM398/2013(H5N1)
A/duck/Sichuan/NCJPL7/2014(H5N6)
A/chicken/Vietnam/212/2005(H5N1)
A/duck/Hubei/xn/2007(H5N1)
A/wild duck/Shandong/2/2011(H5N1)
A/tufted duck/Fukushima/2/2011(H5N1)
A/whooper swan/Hokkaido/4/2011(H5N1)
A/duck/Hokkaido/WZ83/2010(H5N1)
A/hooded crane/Kagoshima/2/2010(H5N1)
A/peregrine falcon/Aomori/7/2011(H5N1)
A/peregrine falcon/Tochigi/15/2011(H5N1)
A/slaty-backed gull/Japan/6KS0185/2006(H4N8)
A/duck/Potsdam/2216-4/1984(H5N6)
A/duck/Hong Kong/Y439/1997(H9N2)
A/shearwater/Australia/1/1973(H6N5)
A/duck/Hong Kong/273/1978(H2N2)
A/duck/Hong Kong/702/1979(H9N2)
A/red-necked stint/Australia/4189/1980(H4N8)
A/turkey/England/1963(H7N3)
A/duck/Hokkaido/5/1977(H3N2)
A/African starling/England-Q/983/1979(H7N1)
A/mallard/Marquenterre/2237/1983(H1N1)
A/black-headed gull/Netherlands/29/2014(H13N6)
A/black-headed gull/Netherlands/126/2012(H13N6)
A/yellow-legged gull/Republic of Georgia/2/2012(H13N6)
A/swine/Argentina/CIP051-A160/2011(H3N2)
A/turkey/Ontario/FAV114-17/2009(H1N1)
A/turkey/Ontario/FAV-9/2011(H3N2)
A/KAGOSHIMA/4076/2017(H3N2)
A/shearwater/AUS/405/1978(H3N8)
A/chicken/Germany/n/1949(H10N7)
A/chicken/Scotland/1959(H5N1)
A/duck/England/1956(H11N6)
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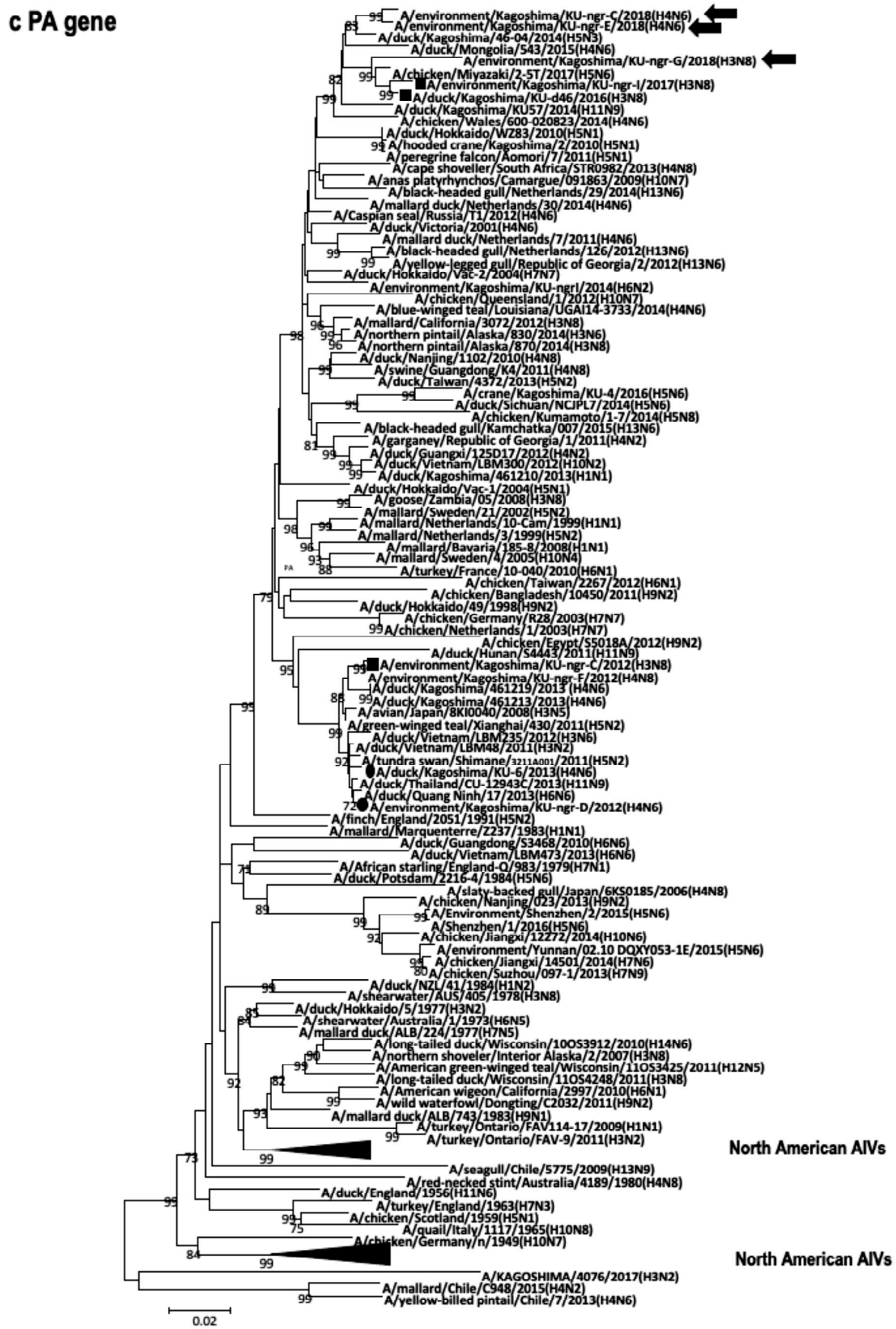
A/duck/NZL/41/1984(H1N2)
North American AIVs
0.01


c PA gene
A/environment/Kagoshima/KU-ngr-C/2018(H4N6)
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A/duck/Mongolia/S43/2015(H4N6)
A/environment/Kagoshima/KU-ngr-G/2018(H3N8)
A/chicken/Miyazaki/2-5T/2017(H5N6)
A/environment/Kagoshima/KU-ngr-I/2017(H3N8)
A/duck/Kagoshima/KU-d46/2016(H3N8)
A/duck/Kagoshima/KU57/2014(H11N9)
A/chicken/Wales/600-020823/2014(H4N6)
A/duck/Hokkaido/WZ83/2010(H5N1)
A/hooded crane/Kagoshima/2/2010(H5N1)
A/peregrine falcon/Aomori/7/2011(H5N1)
A/cape shoveller/South Africa/STR0982/2013(H4N8)
A/anas platyrhynchos/Camargue/091863/2009(H10N7)
A/black-headed gull/Netherlands/29/2014(H13N6)
A/mallard duck/Netherlands/30/2014(H4N6)
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A/duck/Victoria/2001(H4N6)
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A/northern pintail/Alaska/870/2014(H3N8)
A/duck/Nanjing/1102/2010(H4N8)
A/swine/Guangdong/K4/2011(H4N8)
A/duck/Taiwan/4372/2013(H5N2)
A/crane/Kagoshima/KU-4/2016(H5N6)
A/duck/Sichuan/NCJPL7/2014(H5N6)
A/chicken/Kumamoto/1-7/2014(H5N8)
A/black-headed gull/Kamchatka/007/2015(H13N6)
A/garganey/Republic of Georgia/1/2011(H4N2)
A/duck/Guangxi/125D17/2012(H4N2)
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A/duck/Potsdam/2216-4/1984(H5N6)
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A/duck/Hokkaido/5/1977(H3N2)
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A/chicken/Scotland/1959(H5N1)
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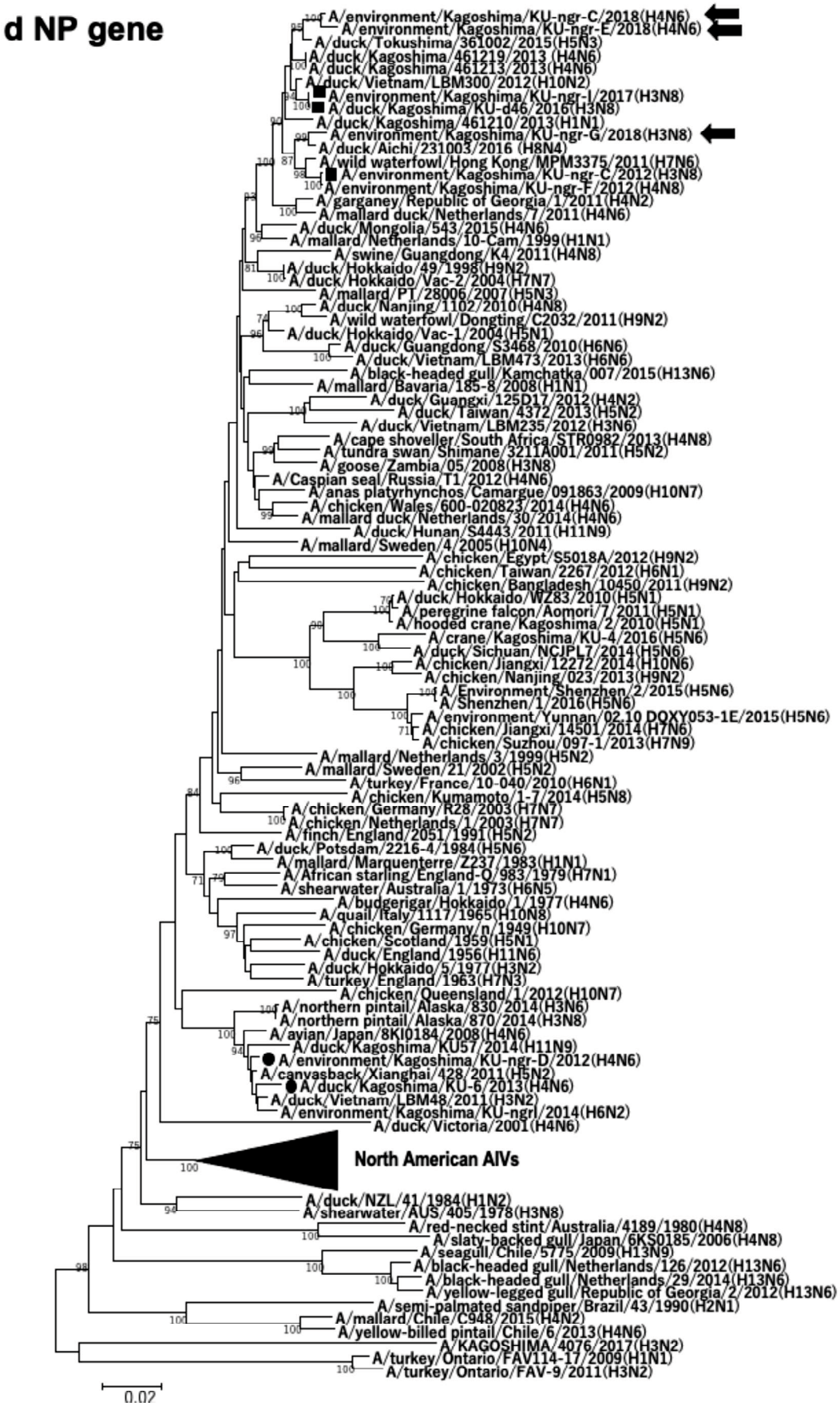
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A/duck/Mongolia/543/2015(H4N6)
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A/swine/Guangdong/K4/2011(H4N8)
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A/chicken/Taiwan/2267/2012(H6N1)
A/chicken/Bangladesh/10450/2011(H9N2)
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A/peregrine falcon/Aomori/7/2011(H5N1)
A/hooded crane/Kagoshima/2/2010(H5N1)
A/crane/Kagoshima/KU-4/2016(H5N6)
A/duck/Sichuan/NCJPL7/2014(H5N6)
A/chicken/Jiangxi/12272/2014(H10N6)
A/chicken/Nanjing/023/2013(H9N2)
A/Environment/Shenzhen/2/2015(H5N6)
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A/shearwater/Australia/1/1973(H6N5)
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A/duck/Hokkaido/5/1977(H3N2)
A/turkey/England/1963(H7N3)
A/chicken/Queensland/1/2012(H10N7)
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North American AIVs
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A/yellow-legged gull/Republic of Georgia/2/2012(H13N6)
A/semi-palmated sandpiper/Brazil/43/1990(H2N1)
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A/yellow-billed pintail/Chile/6/2013(H4N6)
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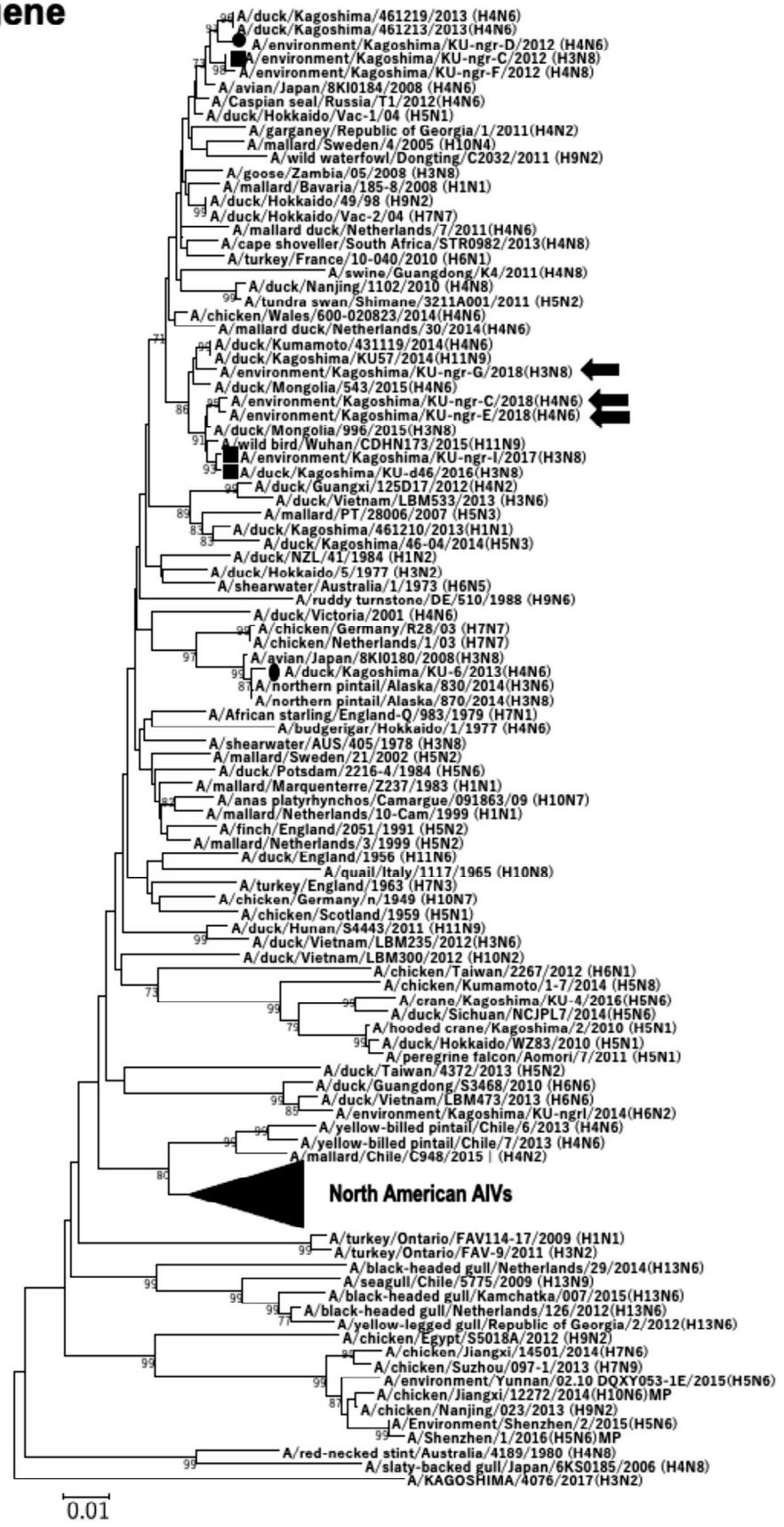
e M gene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A/budgerigar/Hokkaido/1/1977 (H4N6)
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A/hooded crane/Kagoshima/2/2010 (H5N1)
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A/peregrine falcon/Aomori/7/2011 (H5N1)
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North American AIVs
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A/Shenzhen/1/2016(H5N6)MP
A/red-necked stint/Australia/4189/1980 (H4N8)
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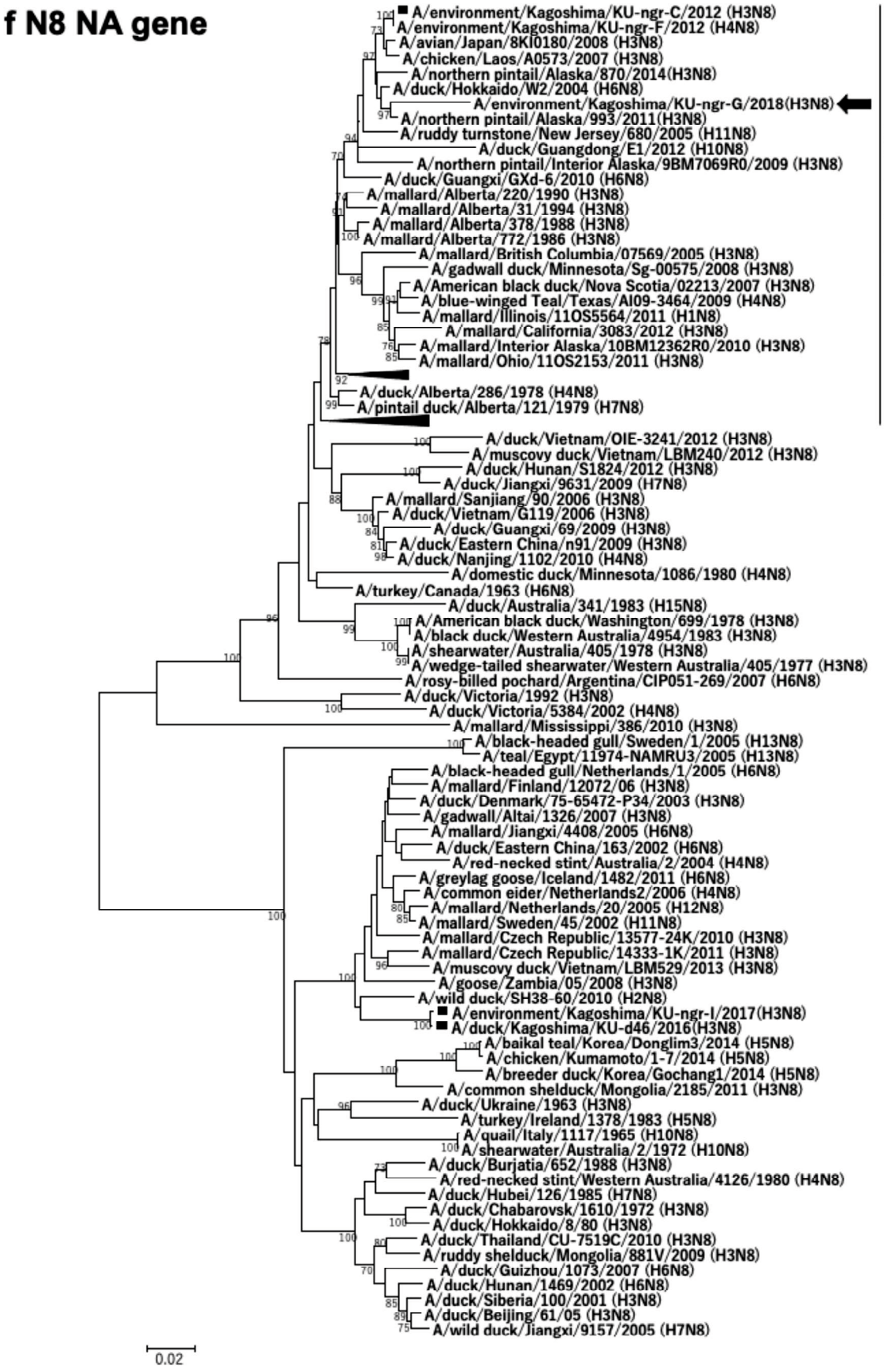
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A/shearwater/Australia/405/1978 (H3N8)
A/wedge-tailed shearwater/Western Australia/405/1977 (H3N8)
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A/wild duck/SH38-60/2010 (H2N8)
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A/baikal teal/Korea/Donglim3/2014 (H5N8)
A/chicken/Kumamoto/1-7/2014 (H5N8)
A/breeder duck/Korea/Gochang1/2014 (H5N8)
A/common shelduck/Mongolia/2185/2011 (H3N8)
A/duck/Ukraine/1963 (H3N8)
A/turkey/Ireland/1378/1983 (H5N8)
A/quail/Italy/1117/1965 (H10N8)
A/shearwater/Australia/2/1972 (H10N8)
A/duck/Burjatia/652/1988 (H3N8)
A/red-necked stint/Western Australia/4126/1980 (H4N8)
A/duck/Hubei/126/1985 (H7N8)
A/duck/Chabarovsk/1610/1972 (H3N8)
A/duck/Hokkaido/8/80 (H3N8)
A/duck/Thailand/CU-7519C/2010 (H3N8)
A/ruddy shelduck/Mongolia/881V/2009 (H3N8)
A/duck/Guizhou/1073/2007 (H6N8)
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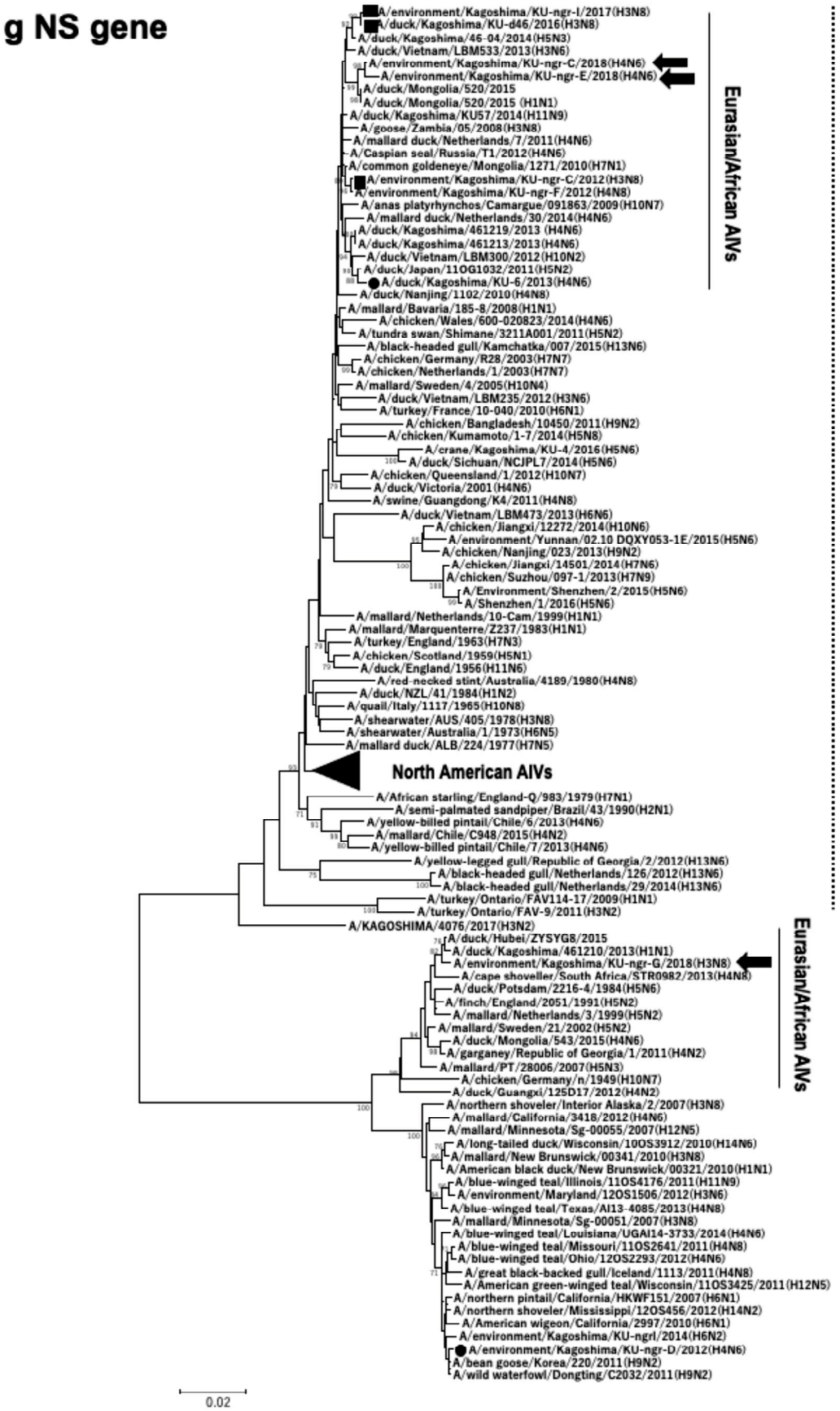
North American AIVs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A/duck/Vietnam/LBM300/2012(H10N2)
A/duck/Japan/11OG1032/2011(H5N2)
A/duck/Kagoshima/KU-6/2013(H4N6)
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A/mallard/Bavaria/185-8/2008(H1N1)
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A/black-headed gull/Kamchatka/007/2015(H13N6)
A/chicken/Germany/R28/2003(H7N7)
A/chicken/Netherlands/1/2003(H7N7)
A/mallard/Sweden/4/2005(H10N4)
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A/turkey/France/10-040/2010(H6N1)
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North American AIVs
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A/semi-palmated sandpiper/Brazil/43/1990(H2N1)
A/yellow-billed pintail/Chile/6/2013(H4N6)
A/mallard/Chile/C948/2015(H4N2)
A/yellow-billed pintail/Chile/7/2013(H4N6)
A/yellow-legged gull/Republic of Georgia/2/2012(H13N6)
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A/turkey/Ontario/FAV-9/2011(H3N2)
A/KAGOSHIMA/4076/2017(H3N2)
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A/cape shoveller/South Africa/STR0982/2013(H4N8)
A/duck/Potsdam/2216-4/1984(H5N6)
A/finch/England/2051/1991(H5N2)
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A/duck/Mongolia/543/2015(H4N6)
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A/American wigeon/California/2997/2010(H6N1)
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A/environment/Kagoshima/KU-ngr-D/2012(H4N6)
A/bean goose/Korea/220/2011(H9N2)
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Eurasian/African AIVs
Eurasian/African AIVs
NS allele A
NS allele B
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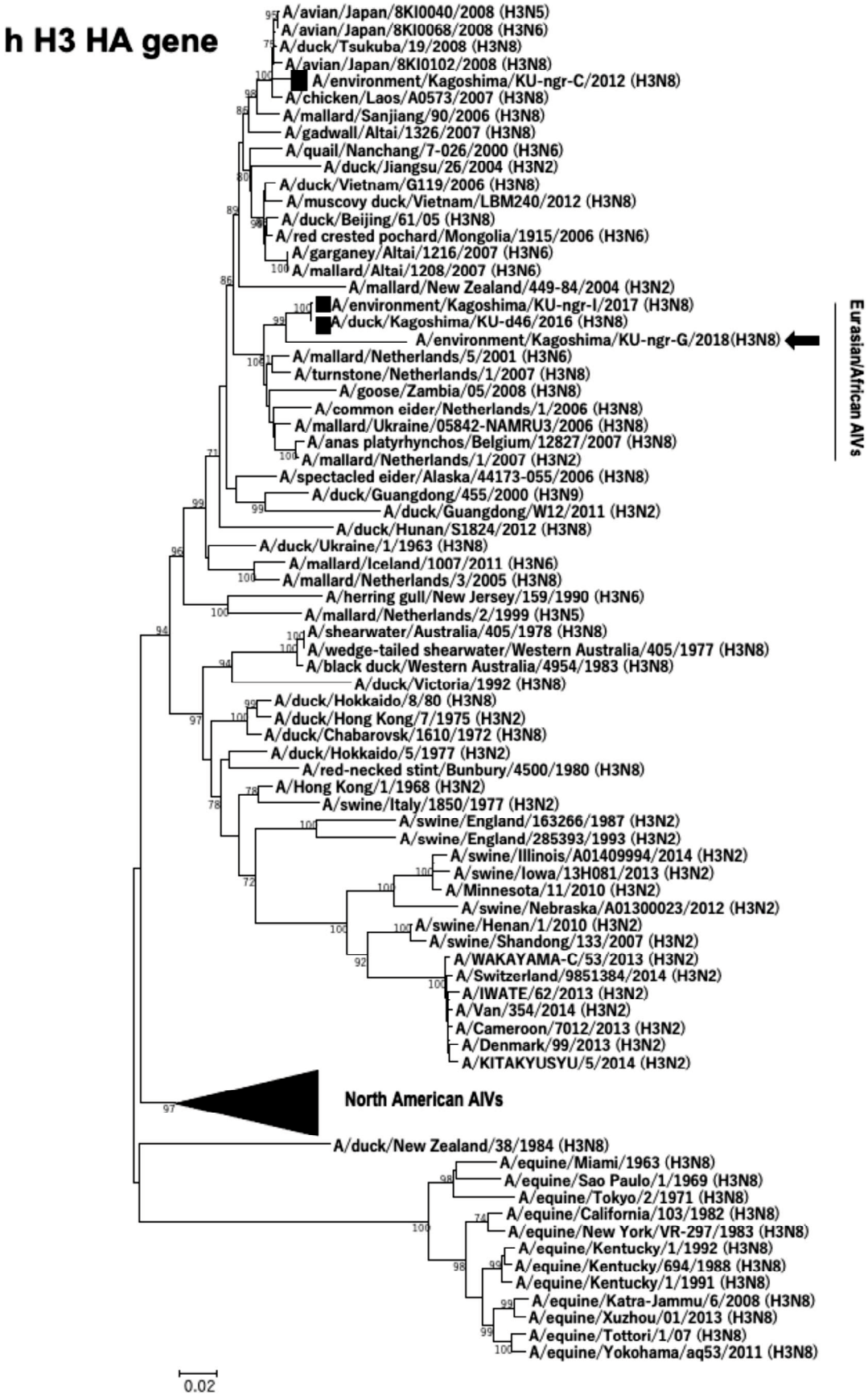
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A/duck/Jiangsu/26/2004 (H3N2)
A/duck/Vietnam/G119/2006 (H3N8)
A/muscovy duck/Vietnam/LBM240/2012 (H3N8)
A/duck/Beijing/61/05 (H3N8)
A/red crested pochard/Mongolia/1915/2006 (H3N6)
A/garganey/Altai/1216/2007 (H3N6)
A/mallard/Altai/1208/2007 (H3N6)
A/mallard/New Zealand/449-84/2004 (H3N2)
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A/turnstone/Netherlands/1/2007 (H3N8)
A/goose/Zambia/05/2008 (H3N8)
A/common eider/Netherlands/1/2006 (H3N8)
A/mallard/Ukraine/05842-NAMRU3/2006 (H3N8)
A/anas platyrhynchos/Belgium/12827/2007 (H3N8)
A/mallard/Netherlands/1/2007 (H3N2)
A/spectacled eider/Alaska/44173-055/2006 (H3N8)
A/duck/Guangdong/455/2000 (H3N9)
A/duck/Guangdong/W12/2011 (H3N2)
A/duck/Hunan/S1824/2012 (H3N8)
A/duck/Ukraine/1/1963 (H3N8)
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A/black duck/Western Australia/4954/1983 (H3N8)
A/duck/Victoria/1992 (H3N8)
A/duck/Hokkaido/8/80 (H3N8)
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A/duck/Chabarovsk/1610/1972 (H3N8)
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A/WAKAYAMA-C/53/2013 (H3N2)
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A/IWATE/62/2013 (H3N2)
A/Van/354/2014 (H3N2)
A/Cameroon/7012/2013 (H3N2)
A/Denmark/99/2013 (H3N2)
A/KITAKYUSYU/5/2014 (H3N2)
North American AIVs
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A/equine/Miami/1963 (H3N8)
A/equine/Sao Paulo/1/1969 (H3N8)
A/equine/Tokyo/2/1971 (H3N8)
A/equine/California/103/1982 (H3N8)
A/equine/New York/VR-297/1983 (H3N8)
A/equine/Kentucky/1/1992 (H3N8)
A/equine/Kentucky/694/1988 (H3N8)
A/equine/Kentucky/1/1991 (H3N8)
A/equine/Katra-Jammu/6/2008 (H3N8)
A/equine/Xuzhou/01/2013 (H3N8)
A/equine/Tottori/1/07 (H3N8)
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Eurasian/African AIVs
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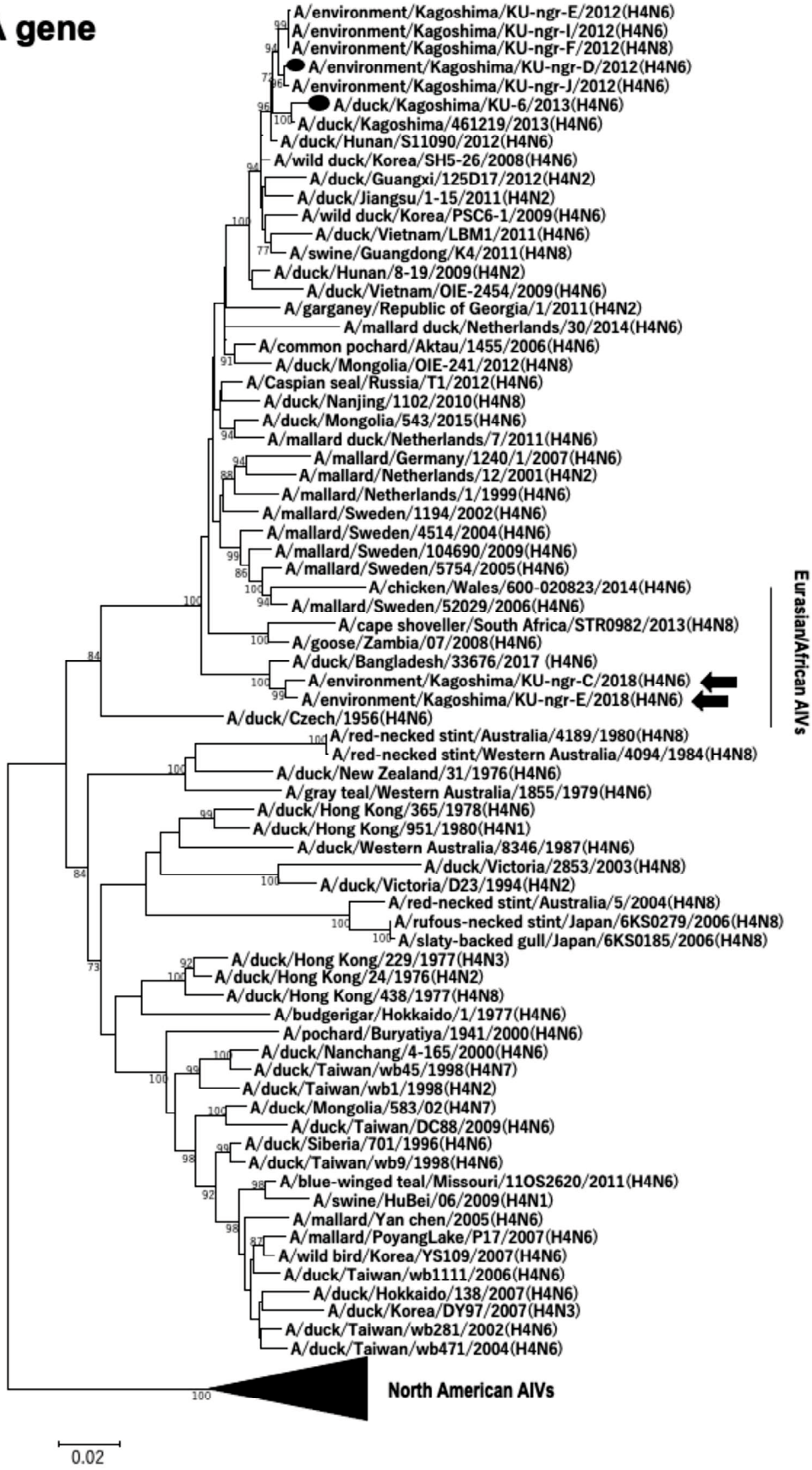
i H4 HA gene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A/duck/Vietnam/OIE-2454/2009(H4N6)
A/garganey/Republic of Georgia/1/2011(H4N2)
A/mallard duck/Netherlands/30/2014(H4N6)
A/common pochard/Aktau/1455/2006(H4N6)
A/duck/Mongolia/OIE-241/2012(H4N8)
A/Caspian seal/Russia/T1/2012(H4N6)
A/duck/Nanjing/1102/2010(H4N8)
A/duck/Mongolia/543/2015(H4N6)
A/mallard duck/Netherlands/7/2011(H4N6)
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A/goose/Zambia/07/2008(H4N6)
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A/duck/Victoria/2853/2003(H4N8)
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A/rufous-necked stint/Japan/6KS0279/2006(H4N8)
A/slaty-backed gull/Japan/6KS0185/2006(H4N8)
A/duck/Hong Kong/229/1977(H4N3)
A/duck/Hong Kong/24/1976(H4N2)
A/duck/Hong Kong/438/1977(H4N8)
A/budgerigar/Hokkaido/1/1977(H4N6)
A/pochard/Buryatiya/1941/2000(H4N6)
A/duck/Nanchang/4-165/2000(H4N6)
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A/duck/Taiwan/wb1/1998(H4N2)
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A/mallard/PoyangLake/P17/2007(H4N6)
A/wild bird/Korea/YS109/2007(H4N6)
A/duck/Taiwan/wb1111/2006(H4N6)
A/duck/Hokkaido/138/2007(H4N6)
A/duck/Korea/DY97/2007(H4N3)
A/duck/Taiwan/wb281/2002(H4N6)
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North American AIVs
Eurasian/African AIVs
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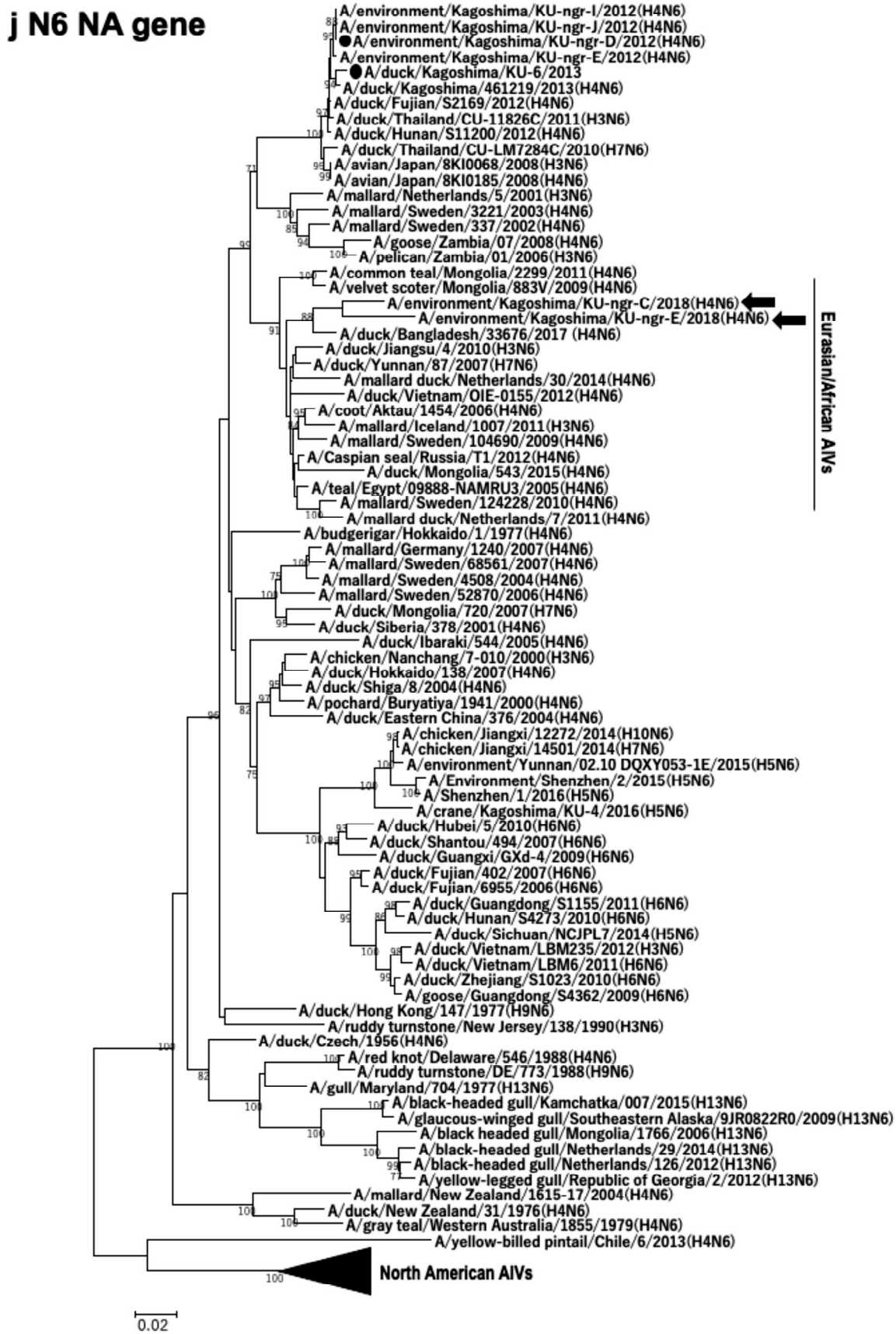
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A/duck/Fujian/S2169/2012(H4N6)
A/duck/Thailand/CU-11826C/2011(H3N6)
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A/mallard/Sweden/3221/2003(H4N6)
A/mallard/Sweden/337/2002(H4N6)
A/goose/Zambia/07/2008(H4N6)
A/pelican/Zambia/01/2006(H3N6)
A/common teal/Mongolia/2299/2011(H4N6)
A/velvet scoter/Mongolia/883V/2009(H4N6)
A/environment/Kagoshima/KU-ngr-C/2018(H4N6)
A/environment/Kagoshima/KU-ngr-E/2018(H4N6)
A/duck/Bangladesh/33676/2017 (H4N6)
A/duck/Jiangsu/4/2010(H3N6)
A/duck/Yunnan/87/2007(H7N6)
A/mallard duck/Netherlands/30/2014(H4N6)
A/duck/Vietnam/OIE-0155/2012(H4N6)
A/coot/Aktau/1454/2006(H4N6)
A/mallard/Iceland/1007/2011(H3N6)
A/mallard/Sweden/104690/2009(H4N6)
A/Caspian seal/Russia/T1/2012(H4N6)
A/duck/Mongolia/543/2015(H4N6)
A/teal/Egypt/09888-NAMRU3/2005(H4N6)
A/mallard/Sweden/124228/2010(H4N6)
A/mallard duck/Netherlands/7/2011(H4N6)
Eurasian/African AIVs
A/budgerigar/Hokkaido/1/1977(H4N6)
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A/mallard/Sweden/4508/2004(H4N6)
A/mallard/Sweden/52870/2006(H4N6)
A/duck/Mongolia/720/2007(H7N6)
A/duck/Siberia/378/2001(H4N6)
A/duck/Ibaraki/544/2005(H4N6)
A/chicken/Nanchang/7-010/2000(H3N6)
A/duck/Hokkaido/138/2007(H4N6)
A/duck/Shiga/8/2004(H4N6)
A/pochard/Buryatiya/1941/2000(H4N6)
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A/chicken/Jiangxi/14501/2014(H7N6)
A/environment/Yunnan/02.10 DQXY053-1E/2015(H5N6)
A/Environment/Shenzhen/2/2015(H5N6)
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A/crane/Kagoshima/KU-4/2016(H5N6)
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A/ruddy turnstone/DE/773/1988(H9N6)
A/gull/Maryland/704/1977(H13N6)
A/black-headed gull/Kamchatka/007/2015(H13N6)
A/glaucous-winged gull/Southeastern Alaska/9JR0822R0/2009(H13N6)
A/black headed gull/Mongolia/1766/2006(H13N6)
A/black-headed gull/Netherlands/29/2014(H13N6)
A/black-headed gull/Netherlands/126/2012(H13N6)
A/yellow-legged gull/Republic of Georgia/2/2012(H13N6)
A/mallard/New Zealand/1615-17/2004(H4N6)
A/duck/New Zealand/31/1976(H4N6)
A/gray teal/Western Australia/1855/1979(H4N6)
A/yellow-billed pintail/Chile/6/2013(H4N6)
North American AIVs
0.02

Fig. 1. Phylogenetic trees of all gene segments from our three isolates. All of the internal gene segments—PB2 (a), PB1 (b), PA (c), NP (d), M (e), and NS (g)—from our three isolates [A/environment/Kagoshima/KU-ngr-G/2018 (H3N8), A/environment/Kagoshima/KU-ngr-C/2018 (H4N6), and A/environment/Kagoshima/KU-ngr-E/2018 (H4N6)] and the glycoprotein gene segments from both subtypes: H3 HA (h) and N8 NA (f), from the H3N8 isolate and H4 HA (i) and N6 NA (j) from H4N6 isolates, are indicated by arrows in the trees. All gene segments (a-j) were phylogenetically analyzed with their representative counterparts, including those of previous H3N8 and H4N6 isolates from the Izumi plain (indicated by squares and circles for the H3N8 and H4N6 isolates, respectively).

Estimation of pathogenicity, mammalian adaptability, and antiviral sensitivity of AIV isolates

To estimate the pathogenicity, mammalian adaptation, and antiviral sensitivity of our three LPAIV isolates, the deduced amino acid sequences were analyzed to identify residues that are known to affect these properties. The PB1-F2 protein, which is a small polypeptide (87-90 amino acids) encoded in a +1 reading frame of the PB1 gene segment, is known to induce apoptosis and modulate interferon responses in virus infected cells [9, 82]. Moreover, the serine residue at position 66 in the PB1-F2 protein is thought to be responsible for its high pathogenicity [13]; however, our three LPAIV isolates had an asparagine encoded at this position.

The influenza virus NS1 protein is an interferon antagonist [16]. In addition, the four C-terminal residues of the NS1 protein from most human and several animal isolates comprise an X-S/T-X-V-type PDZ ligand motif [61]—which typically serves as an interaction domain in modular proteins [85]—and have been shown to influence pathogenicity in mice [35]. Sequence analysis of the NS gene segment revealed that our three LPAIV isolates have the sequence ESEV at the C-terminus of the NS1 protein. Although this PDZ ligand motif was previously reported to enhance pathogenicity in mice [35], it is also encoded by the NS gene segments of most avian influenza viruses.

The HA protein is a major determinant of the host tropism of influenza A viruses. Several mutations involving the HA protein of AIVs have been shown to enhance the binding specificity for mammalian-type cellular receptors, including a threonine-to-alanine substitution at position 160 (H3 numbering) (T160A), N186K, and Q226L [30, 59, 114]. Likewise, T271A, G590S, Q591R, E627K, D701N, and S714R mutations in the PB2 protein—which is one of the subunits of influenza virus RNA polymerase—are known to enhance mammalian adaptation of AIVs [6, 24, 53, 92, 113]. Sequence analysis revealed that none of our isolates harbored any of these mammalian adaptive mutations in the HA or PB2 proteins.

The M2 protein—which forms ion channels on the virion surface and plays an important role in the release of the viral ribonucleoprotein complex into infected cells—and the NA protein—which is critical for the efficient release of progeny viruses from infected cells—are targets for two types of approved anti-influenza drugs: M2 ion-channel blockers (e.g., amantadine) and NA inhibitors (e.g., oseltamivir). L26P, V27A, A30T, and S31N mutations in the M2 protein [25, 76] and H274Y and N294S (N2 numbering) mutations in the NA protein [1, 4] are known to be associated with resistance to these antiviral agents. Sequence analysis revealed that none of our isolates encoded any of these antiviral resistance mutations.

Transition in the genetic constellations of H3N8 and H4N6 LPAIVs isolated from the Izumi plain throughout different seasons

In our previous studies, three H3N8 LPAIVs were isolated from the Izumi plain during the 2012/13 season, i.e., A/environment/Kagoshima/KU-ngr-C/2012 (H3N8) [64], and the 2016/17 season, i.e., A/duck/Kagoshima/KU-d46/2016 (H3N8) and A/environment/Kagoshima/KU-ngr-I/2017 (H3N8) [74]. Likewise, five H4N6 LPAIVs were isolated from the same overwintering site during the 2012/13 season, i.e., A/environment/Kagoshima/KU-ngr-D/2012 (H4N6), A/environment/Kagoshima/KU-ngr-E/2012 (H4N6), A/environment/Kagoshima/KU-ngr-I/2012 (H4N6), and A/environment/Kagoshima/KU-ngr-J/2012 (H4N6) [64], and the 2013/14 season, i.e., A/duck/Kagoshima/KU-6/2013 (H4N6) [65]. To clarify the transition in the genetic constellations of LPAIVs of these two representative subtypes on the Izumi plain, the genetic relationships of individual gene segments between our three isolates and the previous isolates were investigated.

Phylogenetic analysis revealed that the genetic constellation of H3N8 LPAIVs isolated from the Izumi plain changed drastically over the three seasons (Fig. 1a-h and Fig. 2a). For example, the PB2 gene segments from the H3N8 LPAIVs isolated during the 2012/13, 2016/17,

and 2017/18 seasons were individually derived from three different gene pools (Fig. 1a). The PB1, PA, M, and HA gene segments were divided into two genetic groups; the gene segments from the 2012/13 isolates were phylogenetically distant from those from the 2016/17 and 2017/18 isolates (Fig. 1b, c, e, and h). Accordingly, the HA gene segment from the H3N8 LPAIV from the 2017/18 season showed more genetic similarity to those isolated in the 2016/17 season (96.0%) than to those from the 2012/13 season (91.0%). Similarly, NA gene segments were divided into two genetic groups; the NA gene segment from the 2017/18 season showed more genetic similarity to that from the 2012/13 season (95.2%) than to those from the 2016/17 season (77.9%) (Fig. 1f). In contrast, the NP gene segments from the H3N8 LPAIVs during the three seasons formed a single cluster in the phylogenetic tree (Fig. 1d), indicating that the NP gene segments were derived from the same gene pool.

Likewise, the genetic constellation of the H4N6 LPAIVs isolated from the Izumi plain also demonstrated drastic changes over the three seasons (Fig. 1a-e, g, i, and j and Fig. 2b). The M gene segments from H4N6 LPAIVs isolated during the 2012/13, 2013/14, and 2017/18 seasons were individually derived from three different gene pools (Fig. 1e). In contrast, the remaining seven gene segments were phylogenetically divided into two genetic groups, although the grouping combination for the NS gene segment (where the 2012/13 isolates were phylogenetically distinct from the 2013/14 and 2017/18 isolates) (Fig. 1g) was different from those of the other gene segments (where the 2012/13 and 2013/14 isolates were phylogenetically distinct from the 2017/18 isolates) (Fig. 1a-d, i and j). The HA and NA gene segments of the H4N6 LPAIVs isolated in the 2017/18 season were 93-94 and 90-91% identical, respectively, to their counterparts isolated during the previous seasons (i.e., the 2012/13 and 2013/14 seasons). It is noteworthy that all internal gene segments of both H4N6 isolates from the 2017/18 season were closely related to those of the H3N8 isolates from the 2016/17 season (Fig. 1a-e and g and Fig. 2). Because both H3N8 and H4N6 LPAIVs were isolated only intermittently from the Izumi plain, the changing genetic constellation revealed in this study is unlikely to reflect the precise dynamics

of the AIVs of these subtypes. Nevertheless, these findings indicate an active transition in the genetic constellations of both H3N8 and H4N6 LPAIVs isolated from the same overwintering site throughout different seasons, which implies that the genetic constellation of AIVs circulating on the Izumi plain each season is not conserved, even for the same subtype. Additionally, this highlights the role of wild migratory birds in the genesis and spread of reassortant influenza viruses.

In conclusion, we isolated three LPAIVs from the Izumi plain that showed evidence of global genetic reassortment between AIVs circulating not only in East Asian countries but also in European, African, and/or North American countries. Furthermore, we revealed a transition in the genetic constellations of both H3N8 and H4N6 LPAIVs isolated from the Izumi plain throughout different seasons. Such diversity highlights the role of migratory birds in dispersing AIVs across the globe and emphasizes the importance of continuous surveillance of AIVs on the Izumi plain.

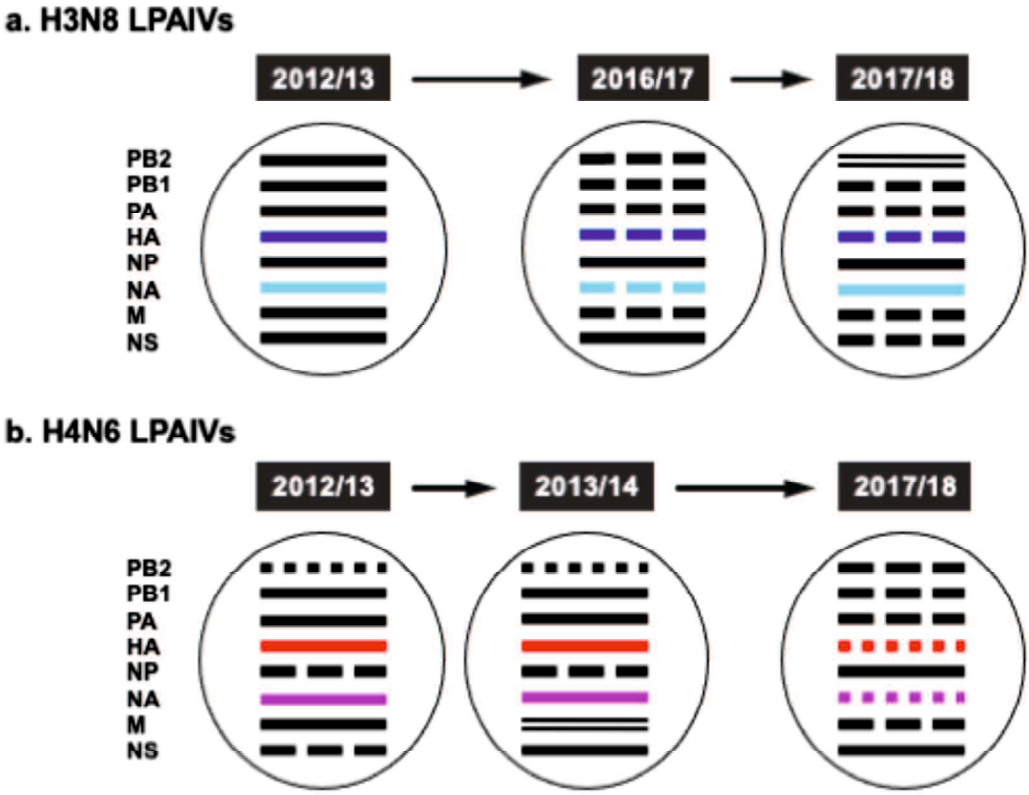

Fig. 2. Schematic diagram of transition in the genetic constellations of H3N8 and H4N6 LPAIVs isolated from the Izumi plain. The eight gene segments of (a) H3N8 and (b) H4N6 LPAIVs isolated from the Izumi plain throughout different seasons are represented by horizontal lines. To clarify transition in the genetic constellations of the viruses, the different genetic origins of each gene segment are represented by different line styles (i.e., solid lines, dashed lines, dotted lines, and double lines). H3 HA, N8 NA, H4 HA, N6 NA, and the internal gene segments are shown in blue, light blue, red, pink, and black, respectively.

Table 1. Closest relatives of the viral genes of the isolated viruses

Isolated virus	Collection date	Gene	Accession No.*	Closest relative**	Identity (%)
A/environment/Kagoshima/ KU-ngr-G/2018 (H3N8)	January 22, 2018	PB2	EPI1478542	A/duck/Aichi/231001/2016 (H8N4)	98.8
		PB1	EPI1478543	A/duck/Dongting/D76-1/2016 (H5N7)	98.4
		PA	EPI1478544	A/chicken/Miyazaki/2-5T/2017 (H5N6)	97.2
		HA	EPI1478545	A/duck/Kagoshima/KU-d46/2016 (H3N8)	95.8
		NP	EPI1478546	A/duck/Aichi/231003/2016 (H8N4)	99.1
		NA	EPI1478547	A/northern pintail/Alaska/993/2011 (H3N8)	96.5
		M	EPI1478548	A/duck/Kumamoto/431119/2014 (H4N6)	99.3
		NS	EPI1478549	A/duck/Hubei/ZYSYG8/2015 (H6N2)	99.2
A/environment/Kagoshima/ KU-ngr-C/2018 (H4N6)	January 22, 2018	PB2	EPI1478550	A/hooded crane/Korea/1176/2016 (H1N1)	98.4
		PB1	EPI1478551	A/duck/Dongting/D76-1/2016 (H5N7)	98.4
		PA	EPI1478552	A/duck/Kagoshima/46-04/2014 (H5N3)	98.1
		HA	EPI1478553	A/duck/Bangladesh/33676/2017 (H4N6)	98.3
		NP	EPI1478554	A/duck/Tokushima/361002/2015 (H5N3)	99.1
		NA	EPI1478555	A/duck/Bangladesh/33676/2017 (H4N6)	96.9
		M	EPI1478557	A/duck/Mongolia/996/2015 (H3N8)	99.5
		NS	EPI1478558	A/duck/Mongolia/520/2015 (H1N1)	99.3
A/environment/Kagoshima/ KU-ngr-E/2018 (H4N6)	February19, 2018	PB2	EPI1478559	A/hooded crane/Korea/1176/2016 (H1N1)	97.1
		PB1	EPI1478560	A/duck/Dongting/D76-1/2016 (H5N7)	98.4
		PA	EPI1478561	A/duck/Kagoshima/46-04/2014 (H5N3)	98.1
		HA	EPI1478562	A/duck/Bangladesh/33676/2017 (H4N6)	98.3
		NP	EPI1478563	A/duck/Tokushima/361002/2015 (H5N3)	98.5
		NA	EPI1478564	A/duck/Bangladesh/33676/2017 (H4N6)	93.4
		M	EPI1478565	A/duck/Mongolia/996/2015 (H3N8)	99.6
		NS	EPI1478566	A/duck/Mongolia/520/2015 (H1N1)	98.9

* Accession numbers in the GISAID database are listed.

**Representative viruses with the highest nucleotide identity retrieved from the GISAID database, except A/hooded crane/Korea/1176/2016 (H1N1) found in the NCBI GenBank data base, on October 11, 2018.

Chapter III

Genetic Characterization of H5N8 Highly Pathogenic Avian Influenza Viruses Isolated from Falcated Ducks and Environmental Water in Japan in November 2020

Abstract

We isolated two highly pathogenic avian influenza viruses (HPAIVs) of subtype H5N8 clade 2.3.4.4b from falcated duck (Anas falcata) feces and environmental water collected at an overwintering site in Japan. Our isolates were almost genetically identical to each other and showed high genetic similarity with H5N8 HPAIVs recently isolated in South Korea, a distant part of Japan, and European countries. These results suggest the potential role of falcated ducks in the dissemination of HPAIVs.

Since the detection of A/Goose/Guangdong/1/1996 (H5N1) (Gs/GD96) from domestic poultry in China in 1996, highly pathogenic avian influenza viruses (HPAIVs) of the H5Nx subtype have been circulating in wild and domestic birds [89]. In addition, the hemagglutinin (HA) of the Gs/GD96 strain has evolved into multiple distinct phylogenetic clades, subclades, and lineages worldwide [19]. Since 2014, H5Nx HPAIVs of clade 2.3.4.4 have been circulating in wild and domestic birds in several countries [36, 72, 115, 117], resulting in further classification into four subclades, namely clades 2.3.4.4a–2.3.4.4d [107]. In the winter of 2019/2020, H5N8 HPAIVs belonging to clade 2.3.4.4b caused outbreaks in wild and domestic birds in Europe . Similarly, in the winter of 2020/2021, genetically similar H5N8 HPAIVs from clade 2.3.4.4b were disseminated not only in European countries, but also in South Korea and Japan [33, 37], which is most likely due to the migration of wild aquatic birds that are considered natural reservoirs of avian influenza viruses (AIVs) [104], as suggested in previous studies on the dissemination of H5N8 HPAIVs [36, 72, 115, 117]. Here, we describe the isolation of two H5N8 HPAIVs clade 2.3.4.4b from a fecal sample of falcated ducks (Anas falcata) and an environmental water sample collected in Japan in November 2020.

The Izumi plain, which is in the Kagoshima Prefecture at the southern tip of Kyushu Island in Japan, is an overwintering site for several tens of thousands of wild migratory birds, including approximately 90% of the global population of the hooded crane (Grus monachal) and 50% of the white-naped crane (Grus vipio) [112]. Since these two cranes are classified as vulnerable species [31, 32], various conservation measures, such as creating artificial wet paddies as roosts, have been conducted every winter. In addition to cranes, wild ducks, including mallards, northern pintails, and Eurasian wigeon, which are considered the primary natural reservoir of AIVs [104], also share this overwintering site [65]. In fact, both HPAIVs and low pathogenic AIVs were previously isolated from dead or debilitated cranes, duck feces, and water samples from the Izumi plain [40, 64, 72].

On 5 November 2020, duck fecal samples were collected by the local government authority at the Komenotsu River mouth, approximately 6 km away from a crane roost in the Arasaki area, during a public AIV surveillance. Influenza viral RNA was detected in one of the five pooled fecal samples using reverse transcription loop-mediated isothermal amplification (RT-LAMP) at the National Institute for Environmental Studies, as described previously [68]. We then inoculated the AIV gene-positive fecal specimen into embryonated chicken eggs for virus isolation, as described previously [65]. Using the rapid diagnostic test ESPLINE A Influenza (Fujirebio Inc., Tokyo, Japan), the allantoic fluids harvested from the inoculated eggs were found to test positive for influenza A viral antigen. To identify the duck species of the fecal sample, the cytochrome c oxidase I (COI) gene of the mitochondrial DNA was sequenced as described previously [27]. A Basic Local Alignment Search Tool (BLAST) search on the National Center for Biotechnology Information (NCBI) database revealed that the fecal sample was derived from falcated ducks. Subsequent genetic analyses of the allantoic fluid revealed that we isolated an AIV of the H5N8 subtype, named A/falcated duck/KU-d3/2020 (H5N8). Furthermore, on 9 November 2020, we collected environmental water samples from the crane roost in the Arasaki area during a private AIV surveillance and the water samples were subjected to AIV isolation in embryonated chicken eggs, as described previously [64]. We then isolated another H5N8 AIV, named A/environment/Kagoshima/KU-ngr-J2/2020 (H5N8).

To genetically characterize the two H5N8 isolates, the nucleotide sequences of all eight gene segments from both isolates were determined (Table 1). The partial sequence of the HA genes showed a cleavage site motif of REKRRKR#GLF, indicating the high pathogenicity in chickens. All eight gene segments from both isolates were almost identical to each other, with the nucleotide sequences of the HA and M genes from both isolates sharing 100% identity. These results suggest that feces from falcated ducks are a source of water contamination of crane roosts on the Izumi plain (Table 2).

To identify the closest relatives of all the viral gene segments from A/falcated duck/KUd3/2020 (H5N8) isolate, the sequences were subjected to BLAST search against the Global Initiative on Sharing Avian Influenza Data (GISAID) and NCBI databases (Table 2). All eight gene segments from A/falcated duck/KU-d3/2020 (H5N8) showed high similarities (99.34–99.90%) to their counterparts from two H5N8 HPAIVs of clade 2.3.4.4b isolated in East Asia in October 2020, namely A/Mandarin duck/Korea/H242/2020 (H5N8) [37] and A/northern pintail/Hokkaido/M13/2020 (H5N8) [33]. Although the gene segments from A/falcated duck/KU-d3/2020 (H5N8) also showed high similarity to European poultry isolates from the winter of 2019/2020 [87], they were less similar than those against the Asian isolates. These findings indicated that H5N8 HPAIVs recently isolated in East Asia and Europe share a recent common ancestor without genetic reassortment. These results also confirm the critical role of migratory birds, including the falcated duck, in the dissemination of recent H5N8 HPAIVs.

Falcated ducks, also known as falcated teals, are dabbling ducks that have a wide breeding range spanning eastern Siberia and Mongolia to northeastern China and northern Japan, with wintering grounds in southeast Asia to eastern India [80]. Hence, falcated ducks are considered one of the migratory ducks that may facilitate the dissemination of AIVs through their migratory flyways. In fact, falcated ducks, along with other wild birds, were implicated in the dissemination of H5N8 HPAIVs in East Asia during the outbreak in South Korea in January 2014 [117]. However, falcated ducks are a minor duck species that host AIVs: among 18,502 AIVs isolated from duck species deposited in the GISAID database as of 9 December 2020, only 0.037% were associated with falcated duck, whereas more AIVs were isolated from mallards (30.82%), northern pintails (4.79%), and Eurasian wigeons (0.28%). These three duck species can be also observed at the Izumi plain every winter as stated above. More importantly, A/falcated duck/KU-d3/2020 (H5N8) is the first falcated duck isolate in Japan. Our findings imply the potential of falcated ducks as one of the migratory ducks facilitating the dissemination of AIVs to Japan.

In conclusion, we isolated two H5N8 HPAIVs clade 2.3.4.4b from falcated duck feces and environmental water collected at an overwintering site for wild migratory birds in Japan in November 2020. Genetic analyses revealed that our isolates were almost identical, suggesting the potential of feces from falcated ducks as a source of environmental water contamination. In addition, the genomes of our isolates shared high similarity with those from H5N8 HPAIVs recently isolated in South Korea and the northern part of Japan, Hokkaido, implying the potential role of falcated ducks in the dissemination of HPAIVs. Our results highlight the importance of global surveillance of AIVs.

Table 1. Accession number of each gene segment of the two characterized AIVs.

Virus	Gene	Accession No.
A/falcated duck/KU-d3/2020 (H5N8)	PB2	MW342697*
	PB1	MW342698
	PA	MW342699
	HA	MW342700
	NP	MW342701
	NA	MW342702
	M	MW342703
	NS	MW342704
A/environment/Kagoshima/KU-ngr-J2/2020(H5N8)	PB2	EPI1815131**
	PB1	EPI1815132
	PA	EPI1815133
	HA	EPI1815134
	NP	EPI1815135
	NA	EPI1815136
	M	EPI1815137
	NS	EPI1815138

*Accession numbers in the GenBank database are listed.

**Accession numbers in the GISAID database are listed.

Table 2. Percent nucleotide identity of A/falcated duck/KU-d3/2020 (H5N8) with its closest relatives.

Gene	Accession No.*	Closest relative**	Identity (%)
PB2	MW342697	A/environment/Kagoshima/KU-ngr-J2/2020 (H5N8)	99.52
		A/Mandarin duck/Korea/H242/2020 (H5N8)	99.42
		A/northern pintail/Hokkaido/M13/2020 (H5N8)	99.34
		A/domestic duck/Poland/285/2020 (H5N8)	99.16
PB1	MW342698	A/environment/Kagoshima/KU-ngr-J2/2020 (H5N8)	99.87
		A/Mandarin duck/Korea/H242/2020 (H5N8)	99.86
		A/northern pintail/Hokkaido/M13/2020 (H5N8)	99.78
		A/duck/Hungary/1565_20VIR749-2/2020 (H5N8)	99.42
PA	MW342699	A/environment/Kagoshima/KU-ngr-J2/2020 (H5N8)	99.91
		A/Mandarin duck/Korea/H242/2020 (H5N8)	99.81
		A/northern pintail/Hokkaido/M13/2020 (H5N8)	99.81
		A/domestic goose/Poland/028/2020 (H5N8)	99.39
HA	MW342700	A/environment/Kagoshima/KU-ngr-J2/2020 (H5N8)	100.0
		A/Mandarin duck/Korea/H242/2020 (H5N8)	99.76
		A/northern pintail/Hokkaido/M13/2020 (H5N8)	99.71
		A/chicken/Germany-BW/AI00049/2020 (H5N8)	99.53
NP	MW342701	A/environment/Kagoshima/KU-ngr-J2/2020 (H5N8)	99.87
		A/Mandarin duck/Korea/H242/2020 (H5N8)	99.93
		A/northern pintail/Hokkaido/M13/2020 (H5N8)	99.80
		A/chicken/Poland/003/2020 (H5N8)	99.53
NA	MW342702	A/environment/Kagoshima/KU-ngr-J2/2020 (H5N8)	99.93
		A/Mandarin duck/Korea/H242/2020 (H5N8)	99.57
		A/northern pintail/Hokkaido/M13/2020 (H5N8)	99.79
		A/turkey/Poland/096/2020 (H5N8)	99.07
M	MW342703	A/environment/Kagoshima/KU-ngr-J2/2020 (H5N8)	100.0
		A/Mandarin duck/Korea/H242/2020 (H5N8)	99.89
		A/northern pintail/Hokkaido/M13/2020 (H5N8)	99.90
		A/domestic duck/Poland/271/2020 (H5N8)	99.79
NS	MW342704	A/environment/Kagoshima/KU-ngr-J2/2020 (H5N8)	99.88
		A/Mandarin duck/Korea/H242/2020 (H5N8)	99.52
		A/northern pintail/Hokkaido/M13/2020 (H5N8)	99.52
		A/turkey/Czech Republic/3071/2020 (H5N8)	98.92

*Accession numbers in the GenBank database are listed.

**Top four viruses with the highest nucleotide identity found in the GISAID and/or GenBank databases on December 2, 2020 are listed. Note that the top three viruses for all gene segments are our isolate from the environmental water sample, followed by the recent South Korean isolate or the recent isolate from the northern part of Japan.

Concluding Remarks

Human influenza viruses result in 250,000–500,000 deaths annually [70]. In addition, the introduction of swIAVs and AIVs in human populations can result in insidious pandemics. For instance, the avian-origin H1N1 Spanish flu pandemic in 1918 and the swine-origin H1N1 pandemic in 2009 reported an estimated number of 50 million and 284,000 deaths, respectively [8, 39]. Therefore, investigating the ecology and evolution of swIAVs and AIVs in their primary hosts can help intervene in the occurrence of occasional pandemics.

Although the genetic evolution rate of swIAVs is considered lower than that of human influenza viruses [18, 94], genetic diversity among HAs of A(H1N1)pdm09-related swIAVs isolated in Japan during 2013–2018 has been reported to be broader than that of their human counterparts [66]. In Chapter I, a variation in the HA antigenicity of A(H1N1)pdm09-related swIAVs was revealed, and the genetic diversity associated with the antigenic difference of HA was demonstrated, which indicates various evolution patterns of A(H1N1)pdm09-related swIAVs in swine populations. Additionally, the antigenically different swIAVs evolved individually in each pig population, which is consistent with the fact that swIAVs are genetically and antigenically variable by region, even among strains of the same subtype [46, 47, 56, 66, 102]. Overall, insights were provided into the variable evolution patterns of swIAVs, and the critical importance of continuous surveillance of swIAVs were highlighted as public health threats.

As part of our AIV surveillance activity on the Izumi plain, an overwintering site in Kagoshima prefecture, Japan, three AIVs of two subtypes were isolated: two AIVs of the H4N6 subtype and one AIV of the H3N8 subtype during the 2017/18 winter season (Chapter II). The three isolates were revealed to be genetic reassortants. The genetic constellation of these isolates, compared with their counterparts isolated during previous seasons in the Izumi plain, are not conserved throughout different seasons. The results imply the role of wild birds in the genesis and spread of AIVs, and the importance of continuous AIV surveillance on the Izumi plain.

In the 2020/21 winter season, several outbreaks in wild and domestic birds were induced by H5N8 HPAIVs belonging to clade 2.3.4.4b in European [62] and Asian countries [33, 37]. In addition, unprecedented spillover events of H5N8 HPAIVs were reported in humans in the Russian Federation during February 2021 [106]. In Chapter III, two H5N8 HPAIVs were isolated from the feces of a falcated duck, Anas falcata, and environmental water samples were collected from the Izumi plain during the 2020/21 winter season. The falcated duck was also identified as a potential source of water contamination in the Izumi plain by genetic characterization of both isolates. This is the first report of H5N8 HPAIV isolation from a falcated duck in Japan, particularly because they are minor host species for AIVs compared with mallards, northern pintails, and Eurasian wigeons [41]. These results imply the role of wild birds in the dissemination of H5N8 HPAIVs during the 2020/21 winter season in Japan.

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