Reintroduction of H5N1 highly pathogenic avian influenza virus by migratory water birds, causing poultry outbreaks in 2010-2011 winter season in Japan

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Running head: Characterization of H5N1 isolates in Japan

Abstract

H5N1 highly pathogenic avian influenza virus (HPAIV) was reintroduced and caused outbreaks in chickens in 2010-2011 winter season in Japan, that had been free from highly pathogenic avian influenza (HPAI) since 2007 when HPAI outbreaks occurred and were controlled. On October 14, 2010 at Lake Ohnuma, Wakkanai, the northernmost part of Hokkaido, Japan, H5N1 HPAIVs were isolated from fecal samples of ducks flying from their nesting lakes in Siberia. Since then, in Japan, H5N1 HPAIVs have been isolated from 63 wild birds in 17 prefectures and caused HPAI outbreaks in 24 chicken farms in 9 prefectures by the end of March in 2011. Each of these isolates was genetically closely related to the HPAIV isolates at Lake Ohnuma, and those in China, Mongolia, Russia, and Korea, belonging to genetic clade 2.3.2.1. In addition, these isolates were genetically classified into 3 groups, suggesting that the viruses were transmitted by migratory water birds through at least 3 different routes from their northern territory to Japan. These isolates were antigenic variants, which is consistent with the selection in poultry under the immunological pressure induced by vaccination. To prevent the perpetuation of viruses in the lakes where water birds nest in summer in Siberia, prompt eradication of HPAIVs in poultry is urgently needed in Asian countries where the HPAI has not been controlled.

<219 words>

INTRODUCTION

Avian influenza caused by infection with H5N1 highly pathogenic avian influenza virus (HPAIV) has spread in poultry in more than 60 countries in Eurasia and Africa since 1996, when the first outbreak occurred at a goose farm in Guangdong province in China (Smith *et al.*, 2006; Xu *et al.*, 1999). H5N1 HPAIV infections have become endemic in several countries and cause accidental transmissions to humans. H5N1 viruses are thus now recognized as one of the most likely candidates for the next pandemic (Li *et al.*, 2004; Peiris *et al.*, 2007). The widespread presence of H5N1 HPAIVs in poultry, especially in domestic ducks reared in free range, has inevitably resulted in the water-borne transmission of viruses to wild bird populations since domestic ducks and geese infected with HPAIV shed progeny viruses with feces into ponds at farms, where migratory water birds visit. In the past, such infections had been restricted to wild birds found dead in the vicinity of infected poultry farms, but it is now a concern that infections in wild birds in which HPAIV has caused mild clinical signs (e.g., ducks) could result in the spread of viruses to large areas (Kim *et al.*, 2009; Smith *et al.*, 2009). Infection with HPAIVs in many wild bird species at 2 water bird parks in Hong Kong was reported in 2002 (Ellis *et al.*, 2004) and further, more significant outbreaks in wild water birds occurred at Lake Qinghai in Western China ,and Khunt and Erkhel Lakes in Mongolia in 2005 (Chen *et al.*, 2005; Sakoda *et al.*, 2010). H5N1 HPAIV infections in poultry and wild birds have now spread in Asia, Europe, and Africa, and it has been suggested that the H5N1 virus could spread by migratory water birds to the west and south, since genetically closely related H5N1

71 viruses (clade 2.2) have been isolated in several countries since 2005 (Monne *et al.*, 2008; Salzberg *et*
72 *al.*, 2007; Starick *et al.*, 2008).

73 In Japan, the outbreaks caused by H5N1 HPAIVs occurred in chicken farms in 2004 (Mase *et al.*,
74 2005) and 2007. The H5N1 HPAIV isolates in 2004 and 2007 were genetically classified into clade
75 2.5 and 2.2, respectively. Both outbreaks were controlled by the culling of chickens of the farms
76 where the outbreaks occurred (4 farms in each year), intensive surveillance, and improved
77 biosecurity measures. In addition, the H5N1 HPAIVs were isolated from the jungle crows,
78 mountain hawk eagle, and whooper swans in 2004, 2007, and 2008, respectively (Shivakoti *et al.*,
79 2010; Tanimura *et al.*, 2006; Uchida *et al.*, 2008). Since then, it was confirmed that Japan was free
80 from HPAIV infection in poultry and wild birds by intensive surveillance.

81 H5N1 viruses of clade 2.3.2 were first isolated from ducks, geese and other mammals in China
82 and Vietnam in 2005 (Chen *et al.*, 2006; Roberton *et al.*, 2006). In intensive surveillance studies in
83 China, viruses belonging to clade 2.3.2, have been characterized as the dominant isolates in poultry
84 and wild birds (Ellis *et al.*, 2009; Jiang *et al.*, 2010; Kou *et al.*, 2009; Smith *et al.*, 2009). In the
85 updated unified nomenclature of H5 HPAIVs, recent H5N1 isolates belonging to the clade 2.3.2 were
86 defined as clade 2.3.2.1 (WHO/OIE/FAO H5N1 Evolution Working Group, 2011). H5N1 HPAIVs of
87 clade 2.3.2.1 were isolated from migratory water birds in Japan in 2008, in China in 2009, in
88 Mongolia in 2009 and 2010, in Russia in 2009 and 2010, and in Korea in 2010 and 2011 (Kwon *et al.*,
89 2011; Li *et al.*, 2011; Sakoda *et al.*, 2010; Sharshov *et al.*, 2010; Uchida *et al.*, 2008). In addition, the

90 infections of chickens and wild birds with HPAIVs belonging to clade 2.3.2.1 have now spread to
91 Europe (Reid *et al.*, 2011). These H5N1 HPAIVs were isolated from migratory water birds only on
92 the way back to their northern territory, and not from those flying to the south from their nesting
93 lakes in Siberia in autumn, suggesting that H5N1 HPAIVs had not dominantly perpetuated at their
94 nesting lakes in Siberia until 2009 (Sakoda *et al.*, 2010; Yamamoto *et al.*, 2011).
95 On October 14, 2010 at Lake Ohnuma, Wakkanai, the northernmost part of Hokkaido, Japan,
96 H5N1 HPAIVs were isolated from fecal samples from ducks flying from their nesting lakes in Siberia
97 (Kajihara *et al.*, 2011). Since then, in Japan, H5N1 HPAIVs have been isolated from 63 wild birds
98 and caused HPAI outbreaks in 24 chicken farms by the end of March. The aim of the present study
99 is to characterize genetically and antigenically H5N1 viruses isolated from wild birds and chickens
100 in Japan.
101
102 **RESULTS**
103 **Isolation and identification of H5N1 HPAIVs from wild birds and chickens**
104 In the intensive surveillance of HPAIV infection in poultry and wild birds, H5N1 HPAIV had not
105 been isolated from migratory water birds that flew from their nesting lakes in Siberia to Japan until
106 the 2009-2010 winter season (data not shown). In the 2010-2011 winter season, 5,591 dead wild
107 birds of about 100 species were found in Japan. After the isolation of H5N1 HPAIVs from fecal
108 samples of ducks at Lake Ohnuma, Hokkaido (Kajihara *et al.*, 2011), H5N1 viruses were isolated

from 63 dead wild birds (63 isolates) and chickens of 24 farms (24 isolates) in Japan (Fig. 1b and Table 1). The multiple basic amino acids (RERRRKR/G), which is a marker of HPAIVs (OIE, 2011), was found at the cleavage site of the deduced amino acid sequence of the hemagglutinin (HA) of all 87 isolates. The pathogenicity of the representative 4 isolates, A/duck/Fukushima/2/2011 (H5N1), A/whooper swan/Hokkaido/4/2011 (H5N1), A/peregrine falcon/Tochigi/15/2011 (H5N1), and A/peregrine falcon/Aomori/7/2011 (H5N1) to chickens was evaluated with intravenous pathogenicity index (IVPI) test. All chickens inoculated with each virus died within 3 days post-inoculation and IVPI scores were from 2.80 to 2.98, being categorized as HPAIV in chickens. The nucleotide sequences of the representative H5N1 isolates obtained in the present study have been registered in GenBank/EMBL/DDBJ (Supplementary Table S1).

Phylogenetic analysis of the H5N1 isolates

For the phylogenetic analysis of HA genes, 30 isolates were selected from 63 isolates of wild birds and 3 isolates were also selected from 24 isolates of chickens. The HA genes of the representative 33 H5N1 isolates were analyzed by the neighbor-joining method along with those of other HPAIVs recently isolated in Asia (Fig. 2a and 2b). The HA genes of the isolates in the 2010-2011 winter season in Japan were closely related to the isolates from poultry or wild birds in China, Mongolia, Russia and Korea in 2009-2011, and were classified into clade 2.3.2.1. These isolates in Japan were divided into 3 groups (A, B, and C) based on the results of phylogenetic

analysis (Fig. 2b and Table 1). This classification by neighbor-joining method was supported by the analyses using maximum likelihood and most parsimony methods with 1,000 bootstrap replicates (data not shown). In particular, A/duck/Hokkaido/WZ83/2010 (H5N1), the first isolate at Lake Ohnuma, Wakkanai, Hokkaido, in October 2010, indicated with asterisk in Fig. 2b, was classified into group C, not group A containing subsequent isolates in Hokkaido (A/pintail/Hokkaido/1/2011, A/greater scaup/Hokkaido/2/2011, A/whooper swan/Hokkaido/3/2011, A/whooper swan/Hokkaido/4/2011, A/whooper swan/Hokkaido/6/2011, A/whooper swan/Hokkaido/13-21/2011, A/whooper swan/Hokkaido/13-27/2011, A/greater scaup/Hokkaido/28/2011, A/whooper swan/Hokkaido/A13/2011) and Fukushima (A/tufted duck/Fukushima/2/2011, A/tufted duck/Fukushima/4/2011, A/tufted duck/Fukushima/5/2011, A/tufted duck/Fukushima/7/2011, A/tufted duck/Fukushima/16/2011, A/tundra swan/Fukushima/207/2011). All occurrences in Hokkaido after January 2011 were only in the eastern Kushiro area, 350 km southeast from Lake Ohnuma, Wakkanai (Fig. 1b). The cases in the Kushiro area in Hokkaido started in mid-January 2011, and ended in mid-February 2011 (Table 1). The isolates from wild birds in this area were genetically closely related to each other and classified into group A (Fig. 2b). In the group B, all viruses were isolated only from western areas (Aichi, Kyoto, Hyogo, Tokushima, and Shimane). In the group C, viruses were isolated from whole of country (Hokkaido, Aomori, Tochigi, Aichi, Mie, Tottori, Yamaguchi, Kochi, Oita, Nagsaki, Miyazaki, and Kagoshima). In addition, A/mandarin duck/Kochi/3901C005/2011 (H5N1) isolated in Kochi Prefecture, in southwestern Japan, belonging to

147 group C, had the highest nucleotide identity of the HA gene with A/mallard duck/Korea/W401/2011
148 (H5N1) and A/mandarin duck/Korea/K10-515/2011 (H5N1) isolated in Korea in the 2010-2011 winter
149 season (Kwon *et al.*, 2011).

150 To assess the genetic relationship of the HPAIVs in gene segments other than the HA, the
151 nucleotide sequences of the representative 30 H5N1 isolates were analyzed and compared with those
152 of other H5N1 HPAIVs (Supplementary Fig. S1 - S7). These viruses are the isolates from wild birds
153 and were used for the phylogenetic tree analysis of HA gene. Genes of these isolates were closely
154 related to each other, and no genetic reassortment with other previous HPAIVs has been identified.
155 Each of the PB2, PB1, NP, NA, and M genes of the isolates was divided into 3 genetic groups,
156 corresponding to the classification of the HA genes (group A, B, and C), although a few isolates were
157 not divided into these groups (Supplementary Fig. S1 - S5). Because the sequence identities of PA
158 and NS genes were so high that the genes of these isolates were not classified completely into groups
159 A, B, and C (Supplementary Fig. S6 - S7).

160

161 *Antigenic analysis of the HA of the H5N1 HPAIV isolates*

162 The HAs of H5N1 isolates were antigenically analyzed using a panel of monoclonal antibodies
163 (MAbs) recognizing six different epitopes on the HA of A/duck/Pennsylvania/10218/84 (H5N2)
164 (Okamatsu *et al.*, 2010; Soda *et al.*, 2008; Yamamoto *et al.*, 2011) (Table 2). Each of the
165 non-pathogenic avian influenza viruses (NPAIVs) isolated from migratory ducks in Mongolia and

166 Hokkaido in 2000-2010 bound to all MAbs used in the present study. Each of the H5N1 HPAIVs
167 isolates before 2005, A/Hong Kong/483/1997 (H5N1), A/Vietnam/1194/2004 (H5N1),
168 A/chicken/Yamaguchi/7/2004 (H5N1), and A/whooper swan/Mongolia/3/2005 (H5N1) bound to most
169 MAbs; however, each of the H5N1 viruses belonging to genetic clade 2.3.2.1, including 2 strains
170 isolated in the present study and A/duck/Hokkaido/WZ83/2010 (H5N1) isolated at lake Ohnuma,
171 Wakkanai, bound only to MAb D101/1.
172 These H5N1 isolates were also antigenically analyzed using hyperimmunized chicken antisera
173 to A/mallard/Hokkaido/24/2009 (H5N1) and A/whooper swan/Hokkaido/1/2008 (H5N1) (Table 2).
174 A/mallard/Hokkaido/24/2009 (H5N1) was isolated from fecal sample and the antigenicity and
175 pathogenicity of this isolate in chickens were similar to those of other H5 NPAIVs isolated from
176 migratory ducks (Yamamoto *et al.*, 2011). The reactivity of the present H5N1 isolates in Japan with
177 the antiserum to A/mallard/Hokkaido/24/2009 (H5N1) was quite low. In contrast, the reactivity of
178 these H5N1 isolates with antiserum to A/whooper swan/Hokkaido/1/2008 (H5N1) was comparatively
179 high. These results indicate that the HAs of H5N1 isolates in the 2010-2011 winter season in Japan
180 are antigenically distinct from H5 NPAIVs and HPAIVs isolated before 2005.
181
182 **DISCUSSION**
183 In October 2010, H5N1 viruses were isolated from fecal samples of ducks at Lake Ohnuma,
184 Wakkanai, Hokkaido on their way to the south from their nesting lakes in Siberia (Kajihara *et al.*,

185 2011). Since then, nationwide H5N1 HPAIV infections in wild birds and chickens have occurred in
186 Japan, and 63 and 24 isolates were identified from wild birds and chickens, respectively. The
187 present results indicate that the viruses isolated from wild birds and chickens from November 2010
188 onward were genetically related to the isolates from migratory ducks at Lake Ohnuma, Wakkanai in
189 October 2010. In Hokkaido, H5N1 viruses were isolated in two areas, Wakkanai and Kushiro (Fig.
190 1b). A/duck/Hokkaido/WZ83/2010 (H5N1), the first isolate at Lake Ohnuma, Wakkanai, was
191 identified as a member of genetic group C, not group A containing subsequent isolates in Kushiro in
192 January and February 2011. Based on the genetic analysis, A/duck/Hokkaido/WZ83/2010 (H5N1)
193 was closely related to A/tundra swan/Tottori/12-002/2010 (H5N1) belonging to the group C. The
194 isolates of group C were detected in the whole of country and some isolates of group C had the
195 highest nucleotide identity to that from wild ducks in Korea (Kwon *et al.*, 2011). By contrast, the
196 isolates of group B were detected only in the western area. Wild water birds start migration from
197 their nesting lakes in the northern territory to the south in the middle of August. The migratory
198 routes of water birds are from Siberia to northern Japan via the Kamchatka Peninsula or Sakhalin
199 Island, and to southern Japan via the Korean Peninsula or the coast of northeastern China (Fig 1a).
200 Our results indicate that the viruses circulating in different populations of wild migratory birds at
201 their nesting lakes in Siberia in summer were transmitted through at least 3 different routes via
202 China, Korea or Russia to Japan in the 2010-2011 winter season. Then, further virus spread
203 occurred in wild birds at the resting lakes of birds in Japan by water-borne transmission or

predation of carcass. Taken together, our results raise the possibility that hat H5N1 HPAIVs perpetuated at the nesting lakes in Siberia before the migration of water birds to Japan.

Concerning the origin of these H5N1 viruses, the HA genes of isolates from chickens and wild birds in China (Jiang *et al.*, 2010; Li *et al.*, 2011) and from wild birds in Mongolia and Russia in 2009 and 2010 (Sakoda *et al.*, 2010; Sharshov *et al.*, 2010) were closely related to those of the present isolates in Japan. The isolates in Laos in 2010 were recently released in the public database (accession No. CY098351), although epidemiological information is not available. The season of isolation of these viruses from wild birds in China, Mongolia, and Russia in 2009 was May to July, the period when migratory water birds return to their nesting lakes in Siberia. Since Japan and Mongolia are located on the flyways of migratory water birds that flew from their nesting lakes in Siberia to the south in autumn, intensive surveillance of avian influenza has been performed in Hokkaido, Japan, and Mongolia every year since 1996. No HPAIV was found in a total of 634 virus isolates from 13,740 fecal samples of migratory water birds until 2009 (Sakoda *et al.*, 2010; Yamamoto *et al.*, 2011). These results suggest that the origin of the viruses isolated from wild birds in China, Mongolia, and Russia in 2009 was poultry in China, and these viruses did not perpetuate at their nesting areas in Siberia until 2009. The isolation of H5N1 HPAIVs in 2010 spring in Mongolia and Russia demonstrates that virus spread from poultry to wild birds occurred again in China and H5N1 HPAIVs circulated in wild water birds since last summer at their nesting lakes in Siberia. These viruses have been maintained in wild migratory bird populations and were brought

223 to Japan in the 2010-2011 winter season. To clarify whether H5N1 HPAIV has dominantly
224 perpetuated at their nesting lakes in Siberia and viruses are brought by migratory birds from
225 Siberia to the south in autumn, intensive surveillance of avian influenza in migratory birds should
226 be strengthened.

227 HPAIVs are not under immunological selection pressure in the non-vaccinated chicken
228 population since HPAIV causes acute infection and death in chickens. The generation of escape
229 mutants against H5 HPAIV was first observed in the follow-up phase of H5N2 HPAIV outbreaks in
230 Mexico in the 1990s (Lee *et al.*, 2004). Since vaccine use for poultry has increased in several
231 counties, antigenic variants have been selected in H5N1 HPAIVs under immunological selection
232 pressure (Cattoli *et al.*, 2011; Chen, 2009; Grund *et al.*, 2011). The present results support the
233 findings that H5N1 viruses belonging to clade 2.3.2.1 were antigenically distinct from other HPAIVs
234 and NPAIVs of H5 subtype (Okamatsu *et al.*, 2010; Smith *et al.*, 2009). The vaccination was applied
235 based on the optimistic expectation to prevent H5N1 influenza virus infection in poultry and
236 humans; however, several countries using vaccines against H5 HPAIV could not eliminate viruses
237 yet in poultry because the efficacy of vaccine against HPAI is limited to suppress virus replication,
238 and does not confer the immunity to prevent infection with the virus. It is reasonable to argue that
239 vaccination of poultry results in the selection of antigenic variants and the vaccine does not confer
240 immunity against antigenic variants for humans and animals. To stop the infection with H5 HPAIV
241 in poultry, thorough culling of infected birds must be carried out in the world.

In the 2010-2011 winter season in Japan, outbreaks of H5N1 HPAIV infection in chicken farms were sporadic, except in Miyazaki Prefecture (13 cases), although a large number of infections in wild birds occurred and the natural environment was contaminated with H5N1 HPAIVs all over the country. In Japan, each of the outbreaks in poultry was controlled by culling, intensive surveillance, improved biosecurity measures, and compensation, without the use of vaccine, and ended in March 2011. H5N1 HPAIV strains have persisted throughout the world for more than 15 years, and antigenic variants have been selected because some countries use vaccines for the control of HPAIV infection. In the chickens vaccinated against HPAIV, it is hardly to find infected ones because they do not show clinical signs, in spite of shedding of viruses. As a result, HPAIV returned to migratory water birds from domestic poultry, and many feral water birds died on the way back to their northern territory in Siberia in spring. Some migratory water birds infected with the virus must have returned to their nesting lakes in Siberia, then disseminate the virus to other birds though water-born transmission at their nesting lakes. To prevent the perpetuation of HPAIVs among migratory water birds at their nesting lakes in Siberia, HPAIVs should be contained within poultry in Asia. We, thus, strongly propose that a stamping-out strategy is the only way to achieve prompt eradication of H5N1 HPAIV and that vaccination may be an optional tool for the control of HPAI in addition to the stamping-out policy. Otherwise, disasters will occur every year throughout Asian countries.

METHODS

Viruses. The H5N1 viruses isolated in the present study and reference H5 viruses shown in Table 2 were propagated in 10-day-old embryonated chicken eggs. As reference strains, H5 NPAIVs isolated from fecal material of migratory ducks (Yamamoto *et al.*, 2011) and H5N1 HPAIVs shown in Table 2 (Kajihara *et al.*, 2011; Mase *et al.*, 2005; Muramoto *et al.*, 2006; Okamatsu *et al.*, 2010; Sakoda *et al.*, 2010; Suarez *et al.*, 1998) were used for antigenic analyses.

Isolation and identification of viruses. Virus isolation has been carried out from fecal samples, tracheal and cloacal swabs, or homogenates of the tissues of wild birds and chickens throughout a year. Fecal samples were mixed with the transport medium containing minimum essential medium (Nissui, Japan), 10,000 U/ml penicillin G (Meiji Seika, Japan), 10 mg/ml streptomycin (Meiji Seika), 0.3 mg/ml gentamicin (Merck, USA), 250 U/ml nystatin (Sigma, USA), and 0.5% bovine serum albumin fraction V (Roche, Switzerland) to yield a 10–20% suspension. Tracheal and cloacal swabs were mixed with 2ml of transport medium. Organ tissue was homogenized with transport medium to yield 10% suspension. Samples from wild birds and chickens were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs and subtypes of the HA and NA of influenza virus isolates were identified by hemagglutination-inhibition (HI) and neuraminidase-inhibition tests, respectively, according to the standard protocol (OIE, 2011).

H5N1 HPAIVs were isolated from 17 species of dead or diseased wild birds, whooper swans

(*Cygnus cygnus*), greater scaups (*Aythya marila*), pintail (*Anas acuta*), peregrine falcons (*Falco peregrinus*), tufted ducks (*Aythya fuligula*), mute swans (*Cygnus olor*), common pochards (*Aythya ferina*), little grebes (*Tachybaptus ruficollis*), great crested grebes (*Podiceps cristatus*), tundra swans (*Cygnus columbianus*), black-headed gull (*Larus ridibundus*), black swan (*Cygnus atratus*), ural owl (*Strix uralensis*), mandarin ducks (*Aix galericulata*), grey heron (*Ardea cinerea*), hooded cranes (*Grus monacha*), and goshawk (*Accipiter gentilis*), found at the waterside of their resting areas and the gardens of private houses in November 2010 - March 2011 (Table 1).

Experimental infection of chickens with H5N1 isolates. To assess the pathogenicity of the representative H5N1 virus isolates, A/duck/Fukushima/2/2011 (H5N1), A/whooper swan/Hokkaido/4/2011 (H5N1), A/peregrine falcon/Tochigi/15/2011 (H5N1), and A/peregrine falcon/Aomori/7/2011 (H5N1), were inoculated intravenously into 4- to 6-week-old chickens (*Gallus gallus*) for the IVPI test according to the standard protocol (OIE, 2011). Each bird was housed in a self-contained isolator unit (Tokiwa Kagaku, Japan) at a BSL-3 facility at Hokkaido University, Japan.

Sequencing and phylogenetic analysis. For the genetic analysis, 30 isolates were selected from 63 isolates of wild birds and 3 isolates were also selected from 24 isolates of chickens. Viral RNA was extracted from the allantoic fluid of embryonated chicken eggs by TRIzol LS Reagent (Invitrogen,

299 USA) and reverse-transcribed with the Uni12 primer (Hoffmann *et al.*, 2001) and M-MLV Reverse
300 Transcriptase (Invitrogen). The full-length or partial sequence of each gene segment was amplified
301 by polymerase chain reaction with gene-specific primer sets reported previously (Hoffmann *et al.*,
302 2001) or designed exclusively in the present study. The sequences of primers designed in the
303 present study are as follows: PB2-826F: GTTAGGAGAGCAACAGTATCAG, PB2-2135R:
304 TCATTGATGCTCAATGCCGG, PB1-547F: ACACATTTCCAGAGAAAGAG, PB1-2128R:
305 TCCACCATGCTAGAAATCCC, PA-38F: GTGCGACAATGCTTCAATCC, PA-1372R:
306 CCTGCAATGGGATACTTCCGC, NP-57F: TGGAAACTGGTGGAGAACGC, NP-1456R:
307 TTGTCTCCGAAGAAATAAGA, M-19F: GTCGAAACGTACGTTCTCTC, M-853R:
308 GAATCCACAATATCAAGTGCAAG, and NS-848R: TCATTAAATAAGCTGGAACG. Direct
309 sequencing of each gene segment was performed using an auto sequencer, 3130 and 3500 Genetic
310 Analyzer (Applied Biosystems, USA). To assess the genetic relationship among influenza virus
311 isolates, the nucleotides 34-1,019 (986 bp) of HA, 197-1,206 (1,010 bp) of NA, 1,017-1,929 (913 bp) of
312 PB2, 1,064-1,657 (594 bp) of PB1, 269-1,218 (950 bp) of PA, 760-1,329 (570 bp) of NP, 97-771 (675 bp)
313 of M, and 73-750 (678 bp) of NS of isolates in the present study were compared with those of other
314 recent H5N1 isolates in Asia. For the NA and internal genes, reference strains of each genotype
315 according to the previous report (Duan *et al.*, 2008) were included. Phylogenetic trees were
316 constructed by the neighbor-joining method (Saitou & Nei, 1987) by MEGA 5 software
317 (http://www.megasoftware.net/).

Antigenic analysis. The antigenic properties of the representative H5 viruses, A/duck/Hokkaido/WZ83/2010 (H5N1), A/whooper swan/Hokkaido/4/2011 (H5N1), A/peregrine falcon/Aomori/7/2011 (H5N1), were compared with those of the reference H5 viruses by the fluorescent antibody method using MAbs against H5 HA (Soda *et al.*, 2008). MDCK cells infected with H5 influenza viruses were fixed with cold 100 % acetone at 8 hours post-inoculation. The reactivity patterns of the H5 viruses with MAbs were investigated with a FITC-conjugated goat IgG to mouse IgG (MP Biomedicals, USA) by a fluorescence microscope, Axiovert 200 (Carl Zeiss, Germany).

The antigenic properties of the representative H5 viruses were also assessed using hyperimmunized chicken antisera against A/mallard/Hokkaido/24/2009 (H5N1) and A/whooper swan/Hokkaido/1/2008 (H5N1) by HI test according to the standard protocol (OIE, 2011). HI titers were expressed as the reciprocals of the highest serum dilutions that showed complete HI.

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338

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481

482 **Figure legends**

483

484 **Fig. 1 H5N1 HPAIV infections in wild birds and chickens in the 2010-2011 winter season in Japan.**

485 (a) Geographical location of Japan in Asia and migration routes of wild water birds from Siberia in

486 autumn. (b) On October 14, 2010 at Lake Ohnuma, Wakkanai, Hokkaido, Japan (denoted by red

487 star), H5N1 HPAIVs were isolated from fecal samples from ducks that had flown from their nesting

488 lakes in Siberia (Kajihara *et al.*, 2011). H5N1 HPAIVs had been isolated from 63 wild birds in 17

489 prefectures (denoted by red circles) and chickens of 24 farms in 9 prefectures (denoted by blue circles)

490 by the end of March 2011. The occurrences at different geographical location were indicated by star

491 or circles, and the subsequent cases at the same place were omitted.

492

493 **Fig. 2 Phylogenetic trees of HA genes of the isolates in the 2010-2011 winter season in Japan.** (a)

494 Phylogenetic tree of H5 avian influenza viruses. The unified nomenclature of the

495 A/goose/Guangdong/1/1996 lineage of Eurasian HPAIVs was based on the homology of HA gene and

496 classified into 10 district clades (clade 0-9) containing second (or third) order clades proposed by the

497 WHO/OIE/FAO H5N1 Evolution Working Group (2008; 2009). Recently, new classification was

498 proposed by the same group (2011) and 2.3.2.1 is one of the new nomenclature system. The H5N1

499 HPAIVs isolated in this study were classified into clade 2.3.2.1 with other recent isolates in Asia

500 from 2007 onward. A/mallard/Hokkaido/24/09 (H5N1) is indicated as representative strain of

501 NPAIV isolated from water birds and its HA gene was classified into Eurasian lineage (Yamamoto *et*

al., 2011). HA genes of A/chicken/Pennsylvania/1/1983 (H5N2) and A/chicken/Ibaraki/1/2005 (H5N2) belong to the North American lineage. (b) Phylogenetic trees of HA genes of H5N1 HPAIVs including the isolates in the 2010-2011 winter season in Japan. To assess genetic relationships among H5 avian influenza virus isolates, nucleotide sequences of HA genes of each isolate in the present study were compared with those of recent isolates in Asia in 2007-2011, belonging to genetic clade 2.3.2.1. Phylogenetic trees were constructed by the neighbor-joining method and bootstrap testing (n = 1000). Phylogenetic trees were rooted to A/whooper swan /Hokkaido/1/2008 (H5N1). The HA genes of the recent isolates in this study (highlighted) was divided into 3 genetic groups (A, B, and C). A/duck/Hokkaido/WZ83/2010, H5N1 HPAIV isolated from fecal samples on October 14, 2010 at Lake Ohnuma, Hokkaido, Japan (Kajihara *et al.*, 2011) was indicated with an asterisk. The isolates in Korea, Russia, Mongolia, China, Laos, and Vietnam in 2007-2011 were underlined. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. HA and NA subtypes were left out for the names of H5N1 viruses. Abbreviation: Ck (chicken).

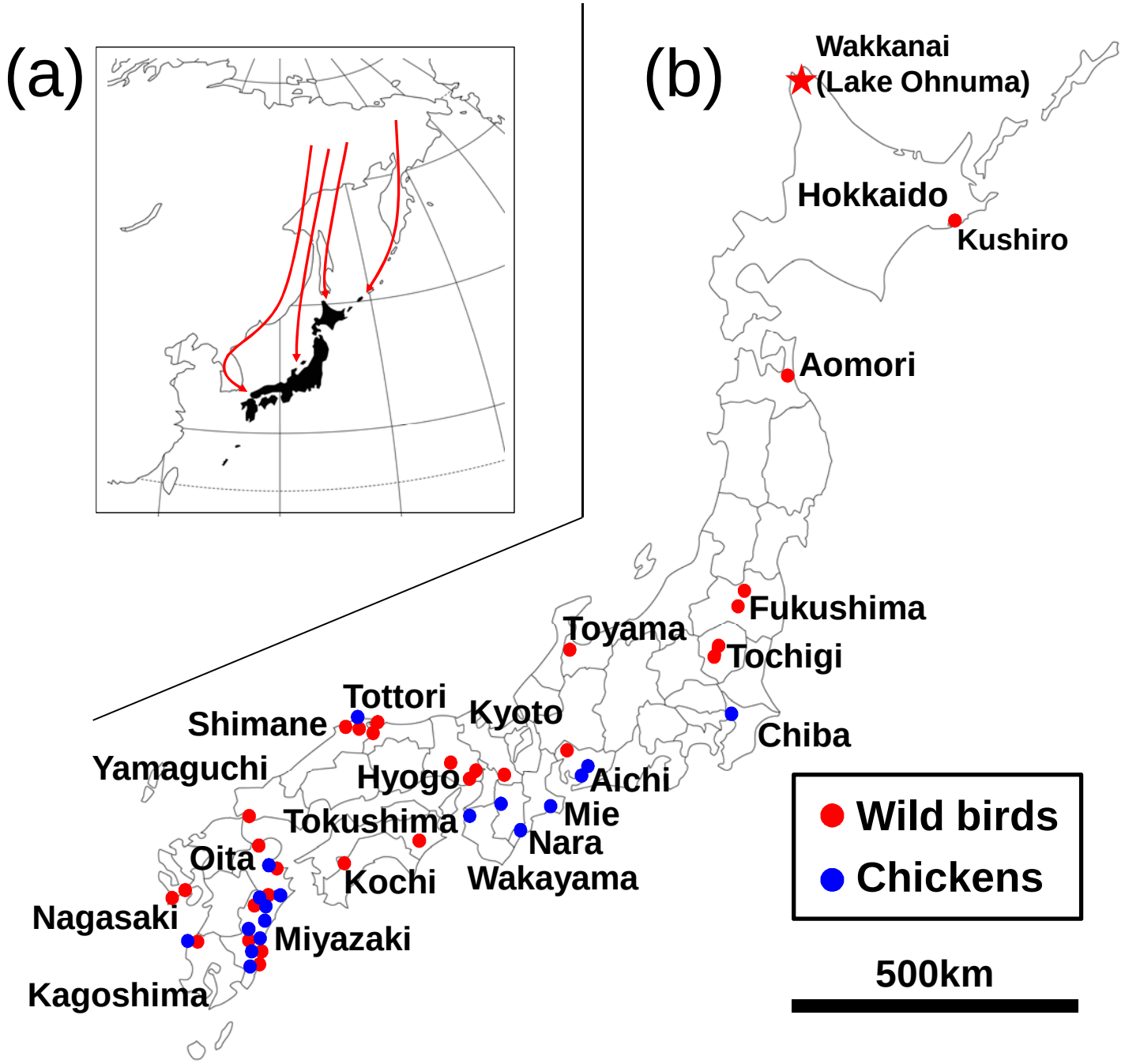

Fig. 1 Sakoda et al.

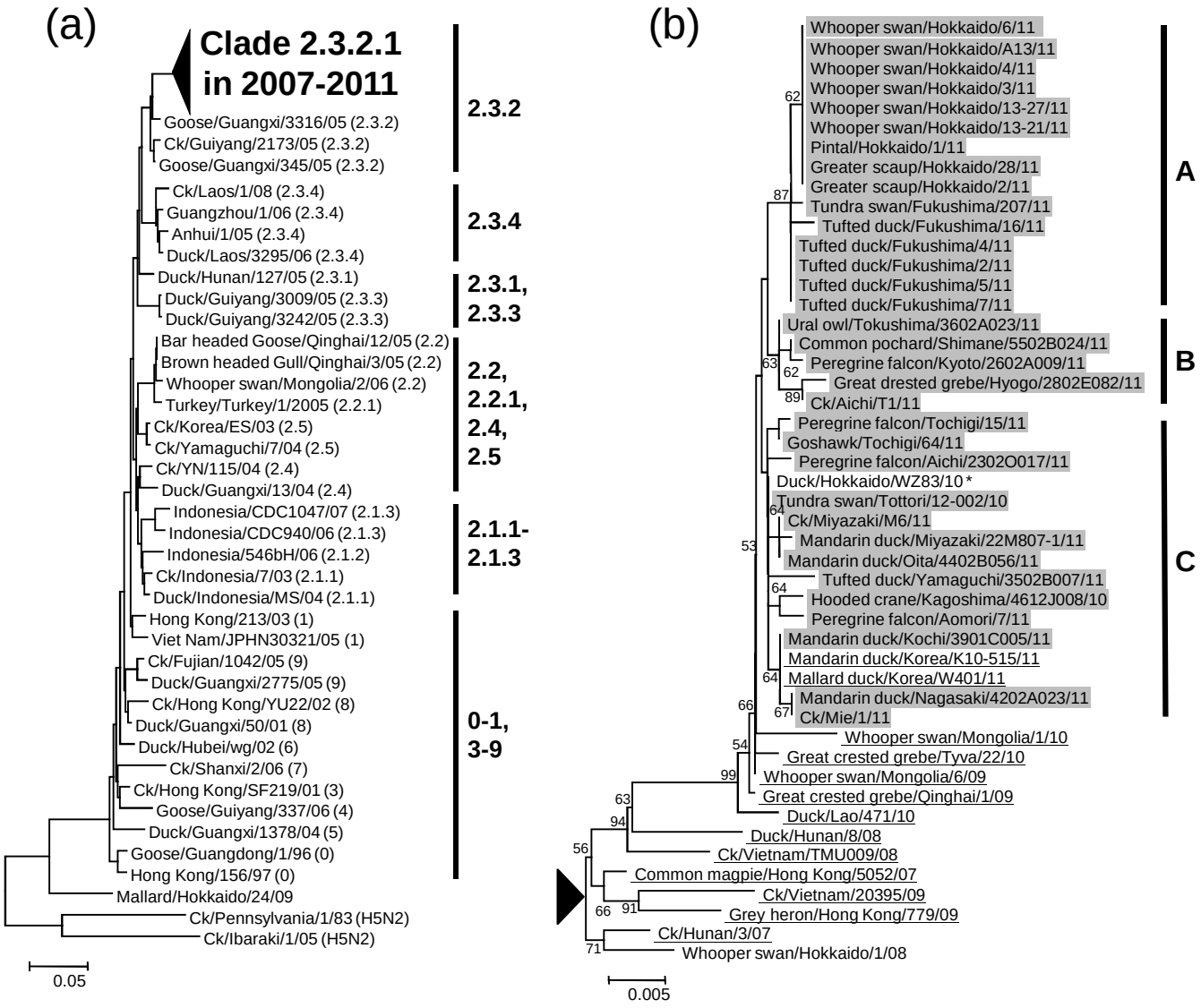

Fig. 2 Sakoda et al.

Table 1. Cases of infection with H5N1 HPAIVs in Japan in 2010-2011 winter season *

Areas	Prefectures	Date of reports	Species of birds †	Genetic subgroup of representative isolates ‡
Hokkaido	Hokkaido	Oct. 14 (2010) §, Jan. 12, 17, 18, 19, 28, Feb. 3, 7, 17 (2011)	Duck (2) §, Whooper swan (6), Greater scaup (2), Pintail (1)	A, C §
Honshu	Aomori	Mar. 10 (2011)	Peregrine falcon (1)	C
	Fukushima	Jan. 4, 5, 7, 10, 23, Feb. 10 (2011)	Tufted duck (5), Tundra swan (1)	A
	Tochigi	Feb. 14, March 25 (2011)	Peregrine falcon (1), Goshawk (1)	C
	Chiba	Mar. 12, 16 (2011)	Chicken (2)	-¶
	Aichi	Feb. 17 (2011)	Peregrine falcon (1)	B, C
		Jan. 27, Feb. 14 (2011)	Chicken (2)	
	Toyama	Dec. 16 (2010)	Mute swan (1)	-
	Mie	Feb. 15, 26 (2011)	Chicken (2)	C
	Wakayama	Feb. 15 (2011)	Chicken (1)	-
	Kyoto	Feb. 16 (2011)	Peregrine falcon (1)	B
	Nara	Feb. 28 (2011)	Chicken (1)	-
	Hyogo	Jan. 12, 25, Feb. 11, 22 (2011)	Common pochard (1), Little grebe (1), Mute swan (1), Great crested grebe (1)	B
	Tottori	Dec. 4 (2010), Jan. 19, 24, Feb. 1, 3, 6 (2011)	Tundra swan (1), Black-headed gull (1), Tufted duck (2), Common pochard (1), Peregrine falcon (1)	C
	Shimane	Jan. 14, Feb. 1, 8 (2011)	Tufted duck (4), Common pochard (1)	B
		Nov. 29 (2010)	Chicken (1)	
	Yamaguchi	Feb. 6, 9 (2011)	Tufted duck (1), Black swan (1)	C
Shikoku	Tokushima	Feb. 8 (2011)	Ural owl (1)	B
	Kochi	Jan. 26 (2011)	Mandarin duck (1)	C
Kyushu	Nagasaki	Jan. 31, Feb. 4, 12 (2011)	Mandarin duck (3), Peregrine falcon (1)	C
	Oita	Feb. 7, 8, 9, 15 (2011)	Mandarin duck (4), Grey heron (1)	C
		Feb. 2 (2011)	Chicken (1)	
	Miyazaki	Feb. 1, 2, 8, 11, 14, 15, 18 (2011)	Mandarin duck (3), Peregrine falcon (3), Little grebe (1)	C
		Jan. 22, 24, 27, 28, 29, 30, Feb. 1, 4, 5, 6, 7, 17, Mar. 5 (2011)	Chicken (13)	
	Kagoshima	Dec. 19, 20, 21, 24 (2010), Feb. 13 (2011)	Hooded crane (7)	C
		Jan. 26 (2011)	Chicken (1)	

* Information about the case in chicken farm is underlined.

† Number of dead wild birds or outbreaks in chciken farm is in parentheses.

‡ Based on the phylogenetic treee of HA gene shown in Fig. 1.

§ Viruses were isolated from fecal sample (Kajihara *et al.*, 2011).

¶ Not tested.

Table 2 Antigenic analyses of H5 influenza viruses

Viruses *	Clades	Monoclonal antibodies †							Polyclonal antibodies ‡	
		I (88)	II (145)	III (157)	IV (168)		V (169)	VI (205)		
		D101/1	A310/39	64/1	B9/5	B220/1	B59/5	25/2	Mal/Hok/09 (H5N1)	Ws/Hok/08 (H5N1)
NPAIV										
Dk/Pennsylvania/10218/1984 (H5N2)	−	+	+	+	+	+	+	+	1280	80
Dk/Mongolia/54/2001 (H5N2)	−	+	+	+	+	+	+	+	640	80
Dk/Hokkaido/167/2007 (H5N3)	−	+	+	+	+	+	+	+	1280	160
Dk/Hokkaido/WZ21/2008 (H5N2)	−	+	+	+	+	+	+	+	2560	80
Mal/Hokkaido/24/2009 (H5N1)	−	+	+	+	+	+	+	+	1280	160
Dk/Hokkaido/101/2010 (H5N2)	−	+	+	+	+	+	+	+	640	80
HPAIV										
Hong Kong/483/1997 (H5N1)	0	−	+	+	+	+	+	+	1280	320
Vietnam/1194/2004 (H5N1)	1	+	+	+	+	+	−	+	640	640
Ck/Yamaguchi/7/2004 (H5N1)	2.5	−	+	+	+	+	−	+	1280	1280
Ws/Mongolia/3/2005 (H5N1)	2.2	+	−	+	+	+	−	+	320	640
Ws/Hokkaido/1/2008 (H5N1)	2.3.2.1	+	−	−	−	−	−	−	40	1280
Ws/Mongolia/6/2009 (H5N1)	2.3.2.1	+	−	−	−	−	−	−	80	1280
Ws/Mongolia/1/2010 (H5N1)	2.3.2.1	+	−	−	−	−	−	−	80	640
Dk/Hokkaido/WZ83/2010 (H5N1)	**2.3.2.1**	**+**	**−**	**−**	**−**	**−**	**−**	**−**	**40**	**320**
Ws/Hokkaido/4/2011 (H5N1)	**2.3.2.1**	**+**	**−**	**−**	**−**	**−**	**−**	**−**	**40**	**320**
Pf/Aomori/7/2011 (H5N1)	**2.3.2.1**	**+**	**−**	**−**	**−**	**−**	**−**	**−**	**40**	**320**

* Viruses indicated in bold were the isolates in 2010-2011 winter season in Japan. Abbreviations: Dk (duck), Mal (mallard), Ck (chicken), Ws (whooper swan), Pf (peregrine falcon).

† Reactivity of monoclonal antibodies against the HA of A/duck/Pennsylvania/10218/1984 (H5N2) to the representative H5 viruses were compared in fluorescent antibody methods. Location of amino acid substitutions in antigenic variants selected in the presence of respective monoclonal antibodies (Soda et al., 2008) is indicated in parentheses.

‡ HI titers of hyperimmunized polyclonal antibodies against representative H5 viruses were measured.