



Title	Reintroduction of H5N1 highly pathogenic avian influenza virus by migratory water birds, causing poultry outbreaks in the 2010-2011 winter season in Japan
Author(s)	Sakoda, Yoshihiro; Ito, Hiroshi; Uchida, Yuko; Okamatsu, Masatoshi; Yamamoto, Naoki; Soda, Kosuke; Nomura, Naoki; Kuribayashi, Saya; Shichinohe, Shintaro; Sunden, Yuji; Umemura, Takashi; Usui, Tatsufumi; Ozaki, Hiroichi; Yamaguchi, Tsuyoshi; Murase, Toshiyuki; Ito, Toshihiro; Saito, Takehiko; Takada, Ayato; Kida, Hiroshi
Citation	Journal of General Virology, 93(3), 541-550 <a href="https://doi.org/10.1099/vir.0.037572-0">https://doi.org/10.1099/vir.0.037572-0</a>
Issue Date	2012-03
Doc URL	<a href="http://hdl.handle.net/2115/52103">http://hdl.handle.net/2115/52103</a>
Rights	This is an author manuscript that has been accepted for publication in Journal of General Virology, copyright Society for General Microbiology, but has not been copy-edited, formatted or proofed. J Gen Virol March 2012 vol. 93 no. 3 541-550. This version of the manuscript may not be duplicated or reproduced, other than for personal use or within the rule of 'Fair Use of Copyrighted Materials' (section 17, Title 17, US Code), without permission from the copyright owner, Society for General Microbiology. The Society for General Microbiology disclaims any responsibility or liability for errors or omissions in this version of the manuscript or in any version derived from it by any other parties. The final copy-edited, published article, which is the version of record, can be found at <a href="http://vir.sgmjournals.org">http://vir.sgmjournals.org</a> , and is freely available without a subscription 12 months after publication.
Type	article (author version)
File Information	JGV93-3_541-550.pdf



[Instructions for use](#)

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

3

4 Yoshihiro Sakoda<sup>1†</sup>, Hiroshi Ito<sup>2, 3†</sup>, Yuko Uchida<sup>4†</sup>, Masatoshi Okamatsu<sup>1</sup>, Naoki Yamamoto<sup>1</sup>,  
5 Kosuke Soda<sup>1, 3</sup>, Naoki Nomura<sup>1</sup>, Saya Kuribayashi<sup>1</sup>, Shintaro Shichinohe<sup>1</sup>, Yuji Sunden<sup>5</sup>, Takashi  
6 Umemura<sup>5</sup>, Tatsufumi Usui<sup>3, 6</sup>, Hiroichi Ozaki<sup>3, 7</sup>, Tsuyoshi Yamaguchi<sup>3, 6</sup>, Toshiyuki Murase<sup>3, 7</sup>,  
7 Toshihiro Ito<sup>2, 3</sup>, Takehiko Saito<sup>4</sup>, Ayato Takada<sup>8</sup>, Hiroshi Kida<sup>1, 8, 9\*</sup>

8

9 <sup>1</sup> Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary  
10 Medicine, Hokkaido University, Sapporo 060-0818, Japan

11 <sup>2</sup> Laboratory of Veterinary Public Health, Faculty of Agriculture, Tottori University, Tottori 680-8553,  
12 Japan

13 <sup>3</sup> Avian Zoonosis Research Center, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan

14 <sup>4</sup> Research Team for Zoonotic Diseases, National Institute of Animal Health, Tsukuba 305-0856,  
15 Japan

16 <sup>5</sup> Laboratory of Comparative Pathology, Department of Veterinary Clinical Sciences, Graduate School  
17 of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

18 <sup>6</sup> Laboratory of Veterinary Hygiene, Faculty of Agriculture, Tottori University, Tottori 680-8553,  
19 Japan

20 <sup>7</sup> Laboratory of Veterinary Microbiology, Faculty of Agriculture, Tottori University, Tottori 680-8553,  
21 Japan

22 <sup>8</sup> Research Center for Zoonosis Control, Hokkaido University, Sapporo 001-0020, Japan

23 <sup>9</sup> Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi 332-0012, Japan

24

25 † These authors contributed equally to this work.

26 \*Corresponding author: Laboratory of Microbiology, Department of Disease Control, Graduate  
27 School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

28 Tel.: +81-11-706-5207; Fax: +81-11-706-5273

29 E-mail: kida@vetmed.hokudai.ac.jp

30 Key words: avian influenza, H5N1, surveillance, migratory water birds

31 Running head: Characterization of H5N1 isolates in Japan

32

33 **Abstract**

34 H5N1 highly pathogenic avian influenza virus (HPAIV) was reintroduced and caused outbreaks  
35 in chickens in 2010-2011 winter season in Japan, that had been free from highly pathogenic avian  
36 influenza (HPAI) since 2007 when HPAI outbreaks occurred and were controlled. On October 14,  
37 2010 at Lake Ohnuma, Wakkanai, the northernmost part of Hokkaido, Japan, H5N1 HPAIVs were  
38 isolated from fecal samples of ducks flying from their nesting lakes in Siberia. Since then, in Japan,  
39 H5N1 HPAIVs have been isolated from 63 wild birds in 17 prefectures and caused HPAI outbreaks in  
40 24 chicken farms in 9 prefectures by the end of March in 2011. Each of these isolates was  
41 genetically closely related to the HPAIV isolates at Lake Ohnuma, and those in China, Mongolia,  
42 Russia, and Korea, belonging to genetic clade 2.3.2.1. In addition, these isolates were genetically  
43 classified into 3 groups, suggesting that the viruses were transmitted by migratory water birds  
44 through at least 3 different routes from their northern territory to Japan. These isolates were  
45 antigenic variants, which is consistent with the selection in poultry under the immunological  
46 pressure induced by vaccination. To prevent the perpetuation of viruses in the lakes where water  
47 birds nest in summer in Siberia, prompt eradication of HPAIVs in poultry is urgently needed in  
48 Asian countries where the HPAI has not been controlled.

49 <219 words>

50

51

52 **INTRODUCTION**

53 Avian influenza caused by infection with H5N1 highly pathogenic avian influenza virus (HPAIV)  
54 has spread in poultry in more than 60 countries in Eurasia and Africa since 1996, when the first  
55 outbreak occurred at a goose farm in Guangdong province in China (Smith *et al.*, 2006; Xu *et al.*,  
56 1999). H5N1 HPAIV infections have become endemic in several countries and cause accidental  
57 transmissions to humans. H5N1 viruses are thus now recognized as one of the most likely  
58 candidates for the next pandemic (Li *et al.*, 2004; Peiris *et al.*, 2007). The widespread presence of  
59 H5N1 HPAIVs in poultry, especially in domestic ducks reared in free range, has inevitably resulted  
60 in the water-borne transmission of viruses to wild bird populations since domestic ducks and geese  
61 infected with HPAIV shed progeny viruses with feces into ponds at farms, where migratory water  
62 birds visit. In the past, such infections had been restricted to wild birds found dead in the vicinity  
63 of infected poultry farms, but it is now a concern that infections in wild birds in which HPAIV has  
64 caused mild clinical signs (e.g., ducks) could result in the spread of viruses to large areas (Kim *et al.*,  
65 2009; Smith *et al.*, 2009). Infection with HPAIVs in many wild bird species at 2 water bird parks in  
66 Hong Kong was reported in 2002 (Ellis *et al.*, 2004) and further, more significant outbreaks in wild  
67 water birds occurred at Lake Qinghai in Western China, and Khunt and Erkhel Lakes in Mongolia in  
68 2005 (Chen *et al.*, 2005; Sakoda *et al.*, 2010). H5N1 HPAIV infections in poultry and wild birds  
69 have now spread in Asia, Europe, and Africa, and it has been suggested that the H5N1 virus could  
70 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; Robertson *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

109 from 63 dead wild birds (63 isolates) and chickens of 24 farms (24 isolates) in Japan (Fig. 1b and  
110 Table 1). The multiple basic amino acids (RERRRKR/G), which is a marker of HPAIVs (OIE, 2011),  
111 was found at the cleavage site of the deduced amino acid sequence of the hemagglutinin (HA) of all  
112 87 isolates. The pathogenicity of the representative 4 isolates, A/duck/Fukushima/2/2011 (H5N1),  
113 A/whooper swan/Hokkaido/4/2011 (H5N1), A/peregrine falcon/Tochigi/15/2011 (H5N1), and  
114 A/peregrine falcon/Aomori/7/2011 (H5N1) to chickens was evaluated with intravenous pathogenicity  
115 index (IVPI) test. All chickens inoculated with each virus died within 3 days post-inoculation and  
116 IVPI scores were from 2.80 to 2.98, being categorized as HPAIV in chickens. The nucleotide  
117 sequences of the representative H5N1 isolates obtained in the present study have been registered in  
118 GenBank/EMBL/DDBJ (Supplementary Table S1).

119

#### 120 **Phylogenetic analysis of the H5N1 isolates**

121 For the phylogenetic analysis of HA genes, 30 isolates were selected from 63 isolates of wild  
122 birds and 3 isolates were also selected from 24 isolates of chickens. The HA genes of the  
123 representative 33 H5N1 isolates were analyzed by the neighbor-joining method along with those of  
124 other HPAIVs recently isolated in Asia (Fig. 2a and 2b). The HA genes of the isolates in the  
125 2010-2011 winter season in Japan were closely related to the isolates from poultry or wild birds in  
126 China, Mongolia, Russia and Korea in 2009-2011, and were classified into clade 2.3.2.1. These  
127 isolates in Japan were divided into 3 groups (A, B, and C) based on the results of phylogenetic

128 analysis (Fig. 2b and Table 1). This classification by neighbor-joining method was supported by the  
129 analyses using maximum likelihood and most parsimony methods with 1,000 bootstrap replicates  
130 (data not shown). In particular, A/duck/Hokkaido/WZ83/2010 (H5N1), the first isolate at Lake  
131 Ohnuma, Wakkanai, Hokkaido, in October 2010, indicated with asterisk in Fig. 2b, was classified  
132 into group C, not group A containing subsequent isolates in Hokkaido (A/pintail/Hokkaido/1/2011,  
133 A/greater scaup/Hokkaido/2/2011, A/whooper swan/Hokkaido/3/2011, A/whooper  
134 swan/Hokkaido/4/2011, A/whooper swan/Hokkaido/6/2011, A/whooper swan/Hokkaido/13-21/2011,  
135 A/whooper swan/Hokkaido/13-27/2011, A/greater scaup/Hokkaido/28/2011, A/whooper  
136 swan/Hokkaido/A13/2011) and Fukushima (A/tufted duck/Fukushima/2/2011, A/tufted  
137 duck/Fukushima/4/2011, A/tufted duck/Fukushima/5/2011, A/tufted duck/Fukushima/7/2011,  
138 A/tufted duck/Fukushima/16/2011, A/tundra swan/Fukushima/207/2011). All occurrences in  
139 Hokkaido after January 2011 were only in the eastern Kushiro area, 350 km southeast from Lake  
140 Ohnuma, Wakkanai (Fig. 1b). The cases in the Kushiro area in Hokkaido started in mid-January  
141 2011, and ended in mid-February 2011 (Table 1). The isolates from wild birds in this area were  
142 genetically closely related to each other and classified into group A (Fig. 2b). In the group B, all  
143 viruses were isolated only from western areas (Aichi, Kyoto, Hyogo, Tokushima, and Shimane). In  
144 the group C, viruses were isolated from whole of country (Hokkaido, Aomori, Tochigi, Aichi, Mie,  
145 Tottori, Yamaguchi, Kochi, Oita, Nagsaki, Miyazaki, and Kagoshima). In addition, A/mandarin  
146 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

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

206 Concerning the origin of these H5N1 viruses, the HA genes of isolates from chickens and wild  
207 birds in China (Jiang *et al.*, 2010; Li *et al.*, 2011) and from wild birds in Mongolia and Russia in 2009  
208 and 2010 (Sakoda *et al.*, 2010; Sharshov *et al.*, 2010) were closely related to those of the present  
209 isolates in Japan. The isolates in Laos in 2010 were recently released in the public database  
210 (accession No. CY098351), although epidemiological information is not available. The season of  
211 isolation of these viruses from wild birds in China, Mongolia, and Russia in 2009 was May to July,  
212 the period when migratory water birds return to their nesting lakes in Siberia. Since Japan and  
213 Mongolia are located on the flyways of migratory water birds that flew from their nesting lakes in  
214 Siberia to the south in autumn, intensive surveillance of avian influenza has been performed in  
215 Hokkaido, Japan, and Mongolia every year since 1996. No HPAIV was found in a total of 634 virus  
216 isolates from 13,740 fecal samples of migratory water birds until 2009 (Sakoda *et al.*, 2010;  
217 Yamamoto *et al.*, 2011). These results suggest that the origin of the viruses isolated from wild birds  
218 in China, Mongolia, and Russia in 2009 was poultry in China, and these viruses did not perpetuate  
219 at their nesting areas in Siberia until 2009. The isolation of H5N1 HPAIVs in 2010 spring in  
220 Mongolia and Russia demonstrates that virus spread from poultry to wild birds occurred again in  
221 China and H5N1 HPAIVs circulated in wild water birds since last summer at their nesting lakes in  
222 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.

242 In the 2010-2011 winter season in Japan, outbreaks of H5N1 HPAIV infection in chicken farms  
243 were sporadic, except in Miyazaki Prefecture (13 cases), although a large number of infections in  
244 wild birds occurred and the natural environment was contaminated with H5N1 HPAIVs all over the  
245 country. In Japan, each of the outbreaks in poultry was controlled by culling, intensive surveillance,  
246 improved biosecurity measures, and compensation, without the use of vaccine, and ended in March  
247 2011. H5N1 HPAIV strains have persisted throughout the world for more than 15 years, and  
248 antigenic variants have been selected because some countries use vaccines for the control of HPAIV  
249 infection. In the chickens vaccinated against HPAIV, it is hardly to find infected ones because they  
250 do not show clinical signs, in spite of shedding of viruses. As a result, HPAIV returned to migratory  
251 water birds from domestic poultry, and many feral water birds died on the way back to their northern  
252 territory in Siberia in spring. Some migratory water birds infected with the virus must have  
253 returned to their nesting lakes in Siberia, then disseminate the virus to other birds though  
254 water-born transmission at their nesting lakes. To prevent the perpetuation of HPAIVs among  
255 migratory water birds at their nesting lakes in Siberia, HPAIVs should be contained within poultry  
256 in Asia. We, thus, strongly propose that a stamping-out strategy is the only way to achieve prompt  
257 eradication of H5N1 HPAIV and that vaccination may be an optional tool for the control of HPAI in  
258 addition to the stamping-out policy. Otherwise, disasters will occur every year throughout Asian  
259 countries.  
260

261 **METHODS**

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

267

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

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

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

287

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

295

296 **Sequencing and phylogenetic analysis.** For the genetic analysis, 30 isolates were selected from 63  
297 isolates of wild birds and 3 isolates were also selected from 24 isolates of chickens. Viral RNA was  
298 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/>).

318

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

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

331

### 332 **ACKNOWLEDGEMENTS**

333 We deeply appreciate the kind cooperation of the Ministry of Environment and Ministry of  
334 Agriculture, Forestry and Fisheries, Government of Japan. We also thank Ms. M. Jizou, Ms. M.  
335 Endo, and Ms. Y. Sato of Hokkaido University for their technical support. The present work was  
336 supported in part by the J-GRID; the Japan Initiative for Global Research Network on Infectious

337 Diseases and Japan Science and Technology Agency Basic Research Programs.

338

339 REFERENCES

- 340 Cattoli, G., Milani, A., Temperton, N., Zecchin, B., Buratin, A., Molesti, E., Aly, M. M., Arafa, A. & Capua,  
341 I. (2011). Antigenic Drift in H5N1 Avian Influenza Virus in Poultry Is Driven by Mutations in  
342 Major Antigenic Sites of the Hemagglutinin Molecule Analogous to Those for Human Influenza  
343 Virus. *J Virol* **85**, 8718-8724.
- 344 Chen, H. (2009). Avian influenza vaccination: the experience in China. *Rev Sci Tech* **28**, 267-274.
- 345 Chen, H., Smith, G. J., Li, K. S., Wang, J., Fan, X. H., Rayner, J. M., Vijaykrishna, D., Zhang, J. X., Zhang,  
346 L. J., Guo, C. T., Cheung, C. L., Xu, K. M., Duan, L., Huang, K., Qin, K., Leung, Y. H., Wu, W. L.,  
347 Lu, H. R., Chen, Y., Xia, N. S., Naipospos, T. S., Yuen, K. Y., Hassan, S. S., Bahri, S., Nguyen, T. D.,  
348 Webster, R. G., Peiris, J. S. & Guan, Y. (2006). Establishment of multiple sublineages of H5N1  
349 influenza virus in Asia: implications for pandemic control. *Proc Natl Acad Sci U S A* **103**,  
350 2845-2850.
- 351 Chen, H., Smith, G. J., Zhang, S. Y., Qin, K., Wang, J., Li, K. S., Webster, R. G., Peiris, J. S. & Guan, Y.  
352 (2005). Avian flu: H5N1 virus outbreak in migratory waterfowl. *Nature* **436**, 191-192.
- 353 Duan, L., Bahl, J., Smith, G. J., Wang, J., Vijaykrishna, D., Zhang, L. J., Zhang, J. X., Li, K. S., Fan, X. H.,  
354 Cheung, C. L., Huang, K., Poon, L. L., Shortridge, K. F., Webster, R. G., Peiris, J. S., Chen, H. &  
355 Guan, Y. (2008). The development and genetic diversity of H5N1 influenza virus in China,  
356 1996-2006. *Virology* **380**, 243-254.
- 357 Ellis, T. M., Bousfield, R. B., Bissett, L. A., Dyrting, K. C., Luk, G. S., Tsim, S. T., Sturm-Ramirez, K.,  
358 Webster, R. G., Guan, Y. & Malik Peiris, J. S. (2004). Investigation of outbreaks of highly  
359 pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. *Avian*  
360 *Pathol* **33**, 492-505.
- 361 Ellis, T. M., Dyrting, K. C., Wong, C. W., Chadwick, B., Chan, C., Chiang, M., Li, C., Li, P., Smith, G. J.,  
362 Guan, Y. & Malik Peiris, J. S. (2009). Analysis of H5N1 avian influenza infections from wild bird  
363 surveillance in Hong Kong from January 2006 to October 2007. *Avian Pathol* **38**, 107-119.
- 364 Grund, C., Abdelwhab, E. S., Arafa, A. S., Ziller, M., Hassan, M. K., Aly, M. M., Hafez, H. M., Harder, T. C.  
365 & Beer, M. (2011). Highly pathogenic avian influenza virus H5N1 from Egypt escapes  
366 vaccine-induced immunity but confers clinical protection against a heterologous clade 2.2.1  
367 Egyptian isolate. *Vaccine*.
- 368 Hoffmann, E., Stech, J., Guan, Y., Webster, R. G. & Perez, D. R. (2001). Universal primer set for the  
369 full-length amplification of all influenza A viruses. *Arch Virol* **146**, 2275-2289.
- 370 Jiang, W. M., Liu, S., Chen, J., Hou, G. Y., Li, J. P., Cao, Y. F., Zhuang, Q. Y., Li, Y., Huang, B. X. & Chen, J.  
371 M. (2010). Molecular epidemiological surveys of H5 subtype highly pathogenic avian influenza

372 viruses in poultry in China during 2007-2009. *J Gen Virol* **91**, 2491-2496.

373 **Kajihara, M., Matsuno, K., Simulundu, E., Muramatsu, M., Noyori, O., Manzoor, R., Nakayama, E.,**  
374 **Igarashi, M., Tomabechi, D., Yoshida, R., Okamatsu, M., Sakoda, Y., Ito, K., Kida, H. & Takada, A.**  
375 **(2011).** An H5N1 highly pathogenic avian influenza virus that invaded Japan through waterfowl  
376 migration. *Jap J Vet Res* **59**, 89-100.

377 **Kim, J. K., Negovetich, N. J., Forrest, H. L. & Webster, R. G. (2009).** Ducks: the "Trojan horses" of H5N1  
378 influenza. *Influenza Other Respi Viruses* **3**, 121-128.

379 **Kou, Z., Li, Y., Yin, Z., Guo, S., Wang, M., Gao, X., Li, P., Tang, L., Jiang, P., Luo, Z., Xin, Z., Ding, C., He,**  
380 **Y., Ren, Z., Cui, P., Zhao, H., Zhang, Z., Tang, S., Yan, B., Lei, F. & Li, T. (2009).** The survey of  
381 H5N1 flu virus in wild birds in 14 Provinces of China from 2004 to 2007. *PLoS One* **4**, e6926.

382 **Kwon, H. I., Song, M. S., Pascua, P. N., Baek, Y. H., Lee, J. H., Hong, S. P., Rho, J. B., Kim, J. K., Poo, H.,**  
383 **Kim, C. J. & Choi, Y. K. (2011).** Genetic characterization and pathogenicity assessment of highly  
384 pathogenic H5N1 avian influenza viruses isolated from migratory wild birds in 2011, South Korea.  
385 *Virus Res* **160**, 305-315.

386 **Lee, C. W., Senne, D. A. & Suarez, D. L. (2004).** Effect of vaccine use in the evolution of Mexican lineage  
387 H5N2 avian influenza virus. *J Virol* **78**, 8372-8381.

388 **Li, K. S., Guan, Y., Wang, J., Smith, G. J., Xu, K. M., Duan, L., Rahardjo, A. P., Puthavathana, P.,**  
389 **Buranathai, C., Nguyen, T. D., Estoepangestie, A. T., Chaisingh, A., Auewarakul, P., Long, H. T.,**  
390 **Hanh, N. T., Webby, R. J., Poon, L. L., Chen, H., Shortridge, K. F., Yuen, K. Y., Webster, R. G. &**  
391 **Peiris, J. S. (2004).** Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus  
392 in eastern Asia. *Nature* **430**, 209-213.

393 **Li, Y., Liu, L., Zhang, Y., Duan, Z., Tian, G., Zeng, X., Shi, J., Zhang, L. & Chen, H. (2011).** New avian  
394 influenza virus (H5N1) in wild birds, Qinghai, China. *Emerg Infect Dis* **17**, 265-267.

395 **Mase, M., Tsukamoto, K., Imada, T., Imai, K., Tanimura, N., Nakamura, K., Yamamoto, Y., Hitomi, T.,**  
396 **Kira, T., Nakai, T., Kiso, M., Horimoto, T., Kawaoka, Y. & Yamaguchi, S. (2005).** Characterization  
397 of H5N1 influenza A viruses isolated during the 2003-2004 influenza outbreaks in Japan. *Virology*  
398 **332**, 167-176.

399 **Monne, I., Fusaro, A., Al-Blawi, M. H., Ismail, M. M., Khan, O. A., Dauphin, G., Tripodi, A., Salviato, A.,**  
400 **Marangon, S., Capua, I. & Cattoli, G. (2008).** Co-circulation of two sublineages of HPAI H5N1  
401 virus in the Kingdom of Saudi Arabia with unique molecular signatures suggesting separate  
402 introductions into the commercial poultry and falconry sectors. *J Gen Virol* **89**, 2691-2697.

403 **Muramoto, Y., Le, T. Q., Phuong, L. S., Nguyen, T., Nguyen, T. H., Sakai-Tagawa, Y., Iwatsuki-Horimoto,**  
404 **K., Horimoto, T., Kida, H. & Kawaoka, Y. (2006).** Molecular characterization of the hemagglutinin  
405 and neuraminidase genes of H5N1 influenza A viruses isolated from poultry in Vietnam from 2004  
406 to 2005. *J Vet Med Sci* **68**, 527-531.

407 **OIE (2011).** Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011 "Avian Influenza".  
408 Paris: The World Organization for Animal Health,  
409 [http://www.oie.int/eng/normes/mmanual/A\\_summry.htm](http://www.oie.int/eng/normes/mmanual/A_summry.htm)

410 Okamatsu, M., Tanaka, T., Yamamoto, N., Sakoda, Y., Sasaki, T., Tsuda, Y., Isoda, N., Kokumai, N.,  
411 Takada, A., Umemura, T. & Kida, H. (2010). Antigenic, genetic, and pathogenic characterization of  
412 H5N1 highly pathogenic avian influenza viruses isolated from dead whooper swans (*Cygnus*  
413 *cygnus*) found in northern Japan in 2008. *Virus Genes* **41**, 351-357.

414 Peiris, J. S., de Jong, M. D. & Guan, Y. (2007). Avian influenza virus (H5N1): a threat to human health.  
415 *Clin Microbiol Rev* **20**, 243-267.

416 Reid, S. M., Shell, W. M., Barboi, G., Onita, I., Turcitu, M., Cioranu, R., Marinova-Petkova, A.,  
417 Goujgoulova, G., Webby, R. J., Webster, R. G., Russell, C., Slomka, M. J., Hanna, A., Banks, J.,  
418 Alton, B., Barrass, L., Irvine, R. M. & Brown, I. H. (2011). First reported incursion of highly  
419 pathogenic notifiable avian influenza A H5N1 viruses from clade 2.3.2 into European poultry.  
420 *Transbound Emerg Dis* **58**, 76-78.

421 Robertson, S. I., Bell, D. J., Smith, G. J., Nicholls, J. M., Chan, K. H., Nguyen, D. T., Tran, P. Q., Streicher,  
422 U., Poon, L. L., Chen, H., Horby, P., Guardo, M., Guan, Y. & Peiris, J. S. (2006). Avian influenza  
423 H5N1 in viverrids: implications for wildlife health and conservation. *Proc Biol Sci* **273**, 1729-1732.

424 Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic  
425 trees. *Mol Biol Evol* **4**, 406-425.

426 Sakoda, Y., Sugar, S., Batchluun, D., Erdene-Ochir, T. O., Okamatsu, M., Isoda, N., Soda, K., Takakuwa,  
427 H., Tsuda, Y., Yamamoto, N., Kishida, N., Matsuno, K., Nakayama, E., Kajihara, M., Yokoyama, A.,  
428 Takada, A., Sodnomdarjaa, R. & Kida, H. (2010). Characterization of H5N1 highly pathogenic  
429 avian influenza virus strains isolated from migratory waterfowl in Mongolia on the way back from  
430 the southern Asia to their northern territory. *Virology* **406**, 88-94.

431 Salzberg, S. L., Kingsford, C., Cattoli, G., Spiro, D. J., Janies, D. A., Aly, M. M., Brown, I. H.,  
432 Couacy-Hymann, E., De Mia, G. M., Dung do, H., Guercio, A., Joannis, T., Maken Ali, A. S.,  
433 Osmani, A., Padalino, I., Saad, M. D., Savic, V., Sengamalay, N. A., Yingst, S., Zaborsky, J.,  
434 Zorman-Rojs, O., Ghedin, E. & Capua, I. (2007). Genome analysis linking recent European and  
435 African influenza (H5N1) viruses. *Emerg Infect Dis* **13**, 713-718.

436 Sharshov, K., Silko, N., Sousloparov, I., Zaykovskaya, A., Shestopalov, A. & Drozdov, I. (2010). Avian  
437 influenza (H5N1) outbreak among wild birds, Russia, 2009. *Emerging Infect Dis* **16**, 349-351.

438 Shivakoti, S., Ito, H., Otsuki, K. & Ito, T. (2010). Characterization of H5N1 highly pathogenic avian  
439 influenza virus isolated from a mountain hawk eagle in Japan. *J Vet Med Sci* **72**, 459-463.

440 Smith, G. J., Fan, X. H., Wang, J., Li, K. S., Qin, K., Zhang, J. X., Vijaykrishna, D., Cheung, C. L., Huang,  
441 K., Rayner, J. M., Peiris, J. S., Chen, H., Webster, R. G. & Guan, Y. (2006). Emergence and  
442 predominance of an H5N1 influenza variant in China. *Proc Natl Acad Sci U S A* **103**, 16936-16941.

443 Smith, G. J., Vijaykrishna, D., Ellis, T. M., Dyrting, K. C., Leung, Y. H., Bahl, J., Wong, C. W., Kai, H.,  
444 Chow, M. K., Duan, L., Chan, A. S., Zhang, L. J., Chen, H., Luk, G. S., Peiris, J. S. & Guan, Y.  
445 (2009). Characterization of avian influenza viruses A (H5N1) from wild birds, Hong Kong,  
446 2004-2008. *Emerg Infect Dis* **15**, 402-407.

447 Soda, K., Ozaki, H., Sakoda, Y., Isoda, N., Haraguchi, Y., Sakabe, S., Kuboki, N., Kishida, N., Takada, A.

448           **& Kida, H. (2008).** Antigenic and genetic analysis of H5 influenza viruses isolated from water  
449           birds for the purpose of vaccine use. *Arch Virol* **153**, 2041-2048.

450   **Starick, E., Beer, M., Hoffmann, B., Staubach, C., Werner, O., Globig, A., Strebelow, G., Grund, C.,**  
451           **Durban, M., Conraths, F. J., Mettenleiter, T. & Harder, T. (2008).** Phylogenetic analyses of highly  
452           pathogenic avian influenza virus isolates from Germany in 2006 and 2007 suggest at least three  
453           separate introductions of H5N1 virus. *Vet Microbiol* **128**, 243-252.

454   **Suarez, D. L., Perdue, M. L., Cox, N., Rowe, T., Bender, C., Huang, J. & Swayne, D. E. (1998).**  
455           Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens  
456           from Hong Kong. *J Virol* **72**, 6678-6688.

457   **Tanimura, N., Tsukamoto, K., Okamatsu, M., Mase, M., Imada, T., Nakamura, K., Kubo, M., Yamaguchi,**  
458           **S., Irishio, W., Hayashi, M., Nakai, T., Yamauchi, A., Nishimura, M. & Imai, K. (2006).** Pathology  
459           of fatal highly pathogenic H5N1 avian influenza virus infection in large-billed crows (*Corvus*  
460           *macrorhynchos*) during the 2004 outbreak in Japan. *Vet Pathol* **43**, 500-509.

461   **Uchida, Y., Mase, M., Yoneda, K., Kimura, A., Obara, T., Kumagai, S., Saito, T., Yamamoto, Y., Nakamura,**  
462           **K., Tsukamoto, K. & Yamaguchi, S. (2008).** Highly pathogenic avian influenza virus (H5N1)  
463           isolated from whooper swans, Japan. *Emerg Infect Dis* **14**, 1427-1429.

464   **WHO/OIE/FAO H5N1 Evolution Working Group (2008).** Toward a unified nomenclature system for highly  
465           pathogenic avian influenza virus (H5N1). *Emerging Infect Dis* **14**, e1.

466   **WHO/OIE/FAO H5N1 Evolution Working Group (2009).** Continuing progress towards a unified  
467           nomenclature for the highly pathogenic H5N1 avian influenza viruses: divergence of clade 2.2  
468           viruses. *Influenza Other Respi Viruses* **3**, 59-62.

469   **WHO/OIE/FAO H5N1 Evolution Working Group (2011).** Continued evolution of highly pathogenic avian  
470           influenza A (H5N1): updated nomenclature. *Influenza Other Respi Viruses*, in press.

471   **Xu, X., Subbarao, K., Cox, N. J. & Guo, Y. (1999).** Genetic characterization of the pathogenic influenza  
472           A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1  
473           viruses from the 1997 outbreaks in Hong Kong. *Virology* **261**, 15-19.

474   **Yamamoto, N., Sakoda, Y., Motoshima, M., Yoshino, F., Soda, K., Okamatsu, M. & Kida, H. (2011).**  
475           Characterization of a non-pathogenic H5N1 influenza virus isolated from a migratory duck flying  
476           from Siberia in Hokkaido, Japan, in October 2009. *Virol J* **8**, 65.

477  
478  
479  
480

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 distinct 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*

502 *al.*, 2011). HA genes of A/chicken/Pennsylvania/1/1983 (H5N2) and A/chicken/Ibaraki/1/2005  
503 (H5N2) belong to the North American lineage. (b) Phylogenetic trees of HA genes of H5N1 HPAIVs  
504 including the isolates in the 2010-2011 winter season in Japan. To assess genetic relationships  
505 among H5 avian influenza virus isolates, nucleotide sequences of HA genes of each isolate in the  
506 present study were compared with those of recent isolates in Asia in 2007-2011, belonging to genetic  
507 clade 2.3.2.1. Phylogenetic trees were constructed by the neighbor-joining method and bootstrap  
508 testing (n = 1000). Phylogenetic trees were rooted to A/whooper swan /Hokkaido/1/2008 (H5N1).  
509 The HA genes of the recent isolates in this study (highlighted) was divided into 3 genetic groups (A, B,  
510 and C). A/duck/Hokkaido/WZ83/2010, H5N1 HPAIV isolated from fecal samples on October 14,  
511 2010 at Lake Ohnuma, Hokkaido, Japan (Kajihara *et al.*, 2011) was indicated with an asterisk. The  
512 isolates in Korea, Russia, Mongolia, China, Laos, and Vietnam in 2007-2011 were underlined.  
513 Horizontal distances are proportional to the minimum number of nucleotide differences required to  
514 join nodes and sequences. HA and NA subtypes were left out for the names of H5N1 viruses.  
515 Abbreviation: Ck (chicken).  
516



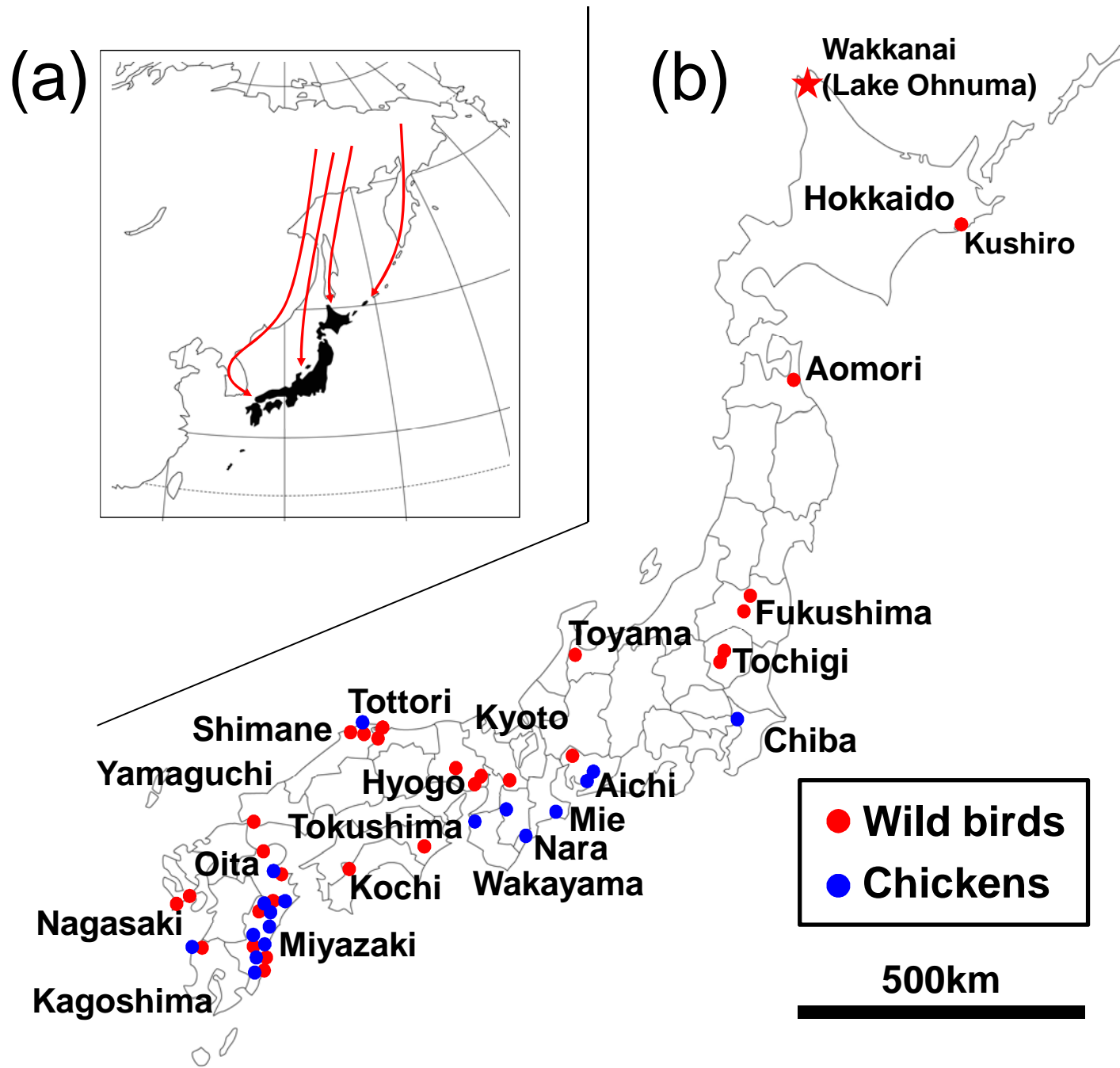


Fig. 1 Sakoda et al.

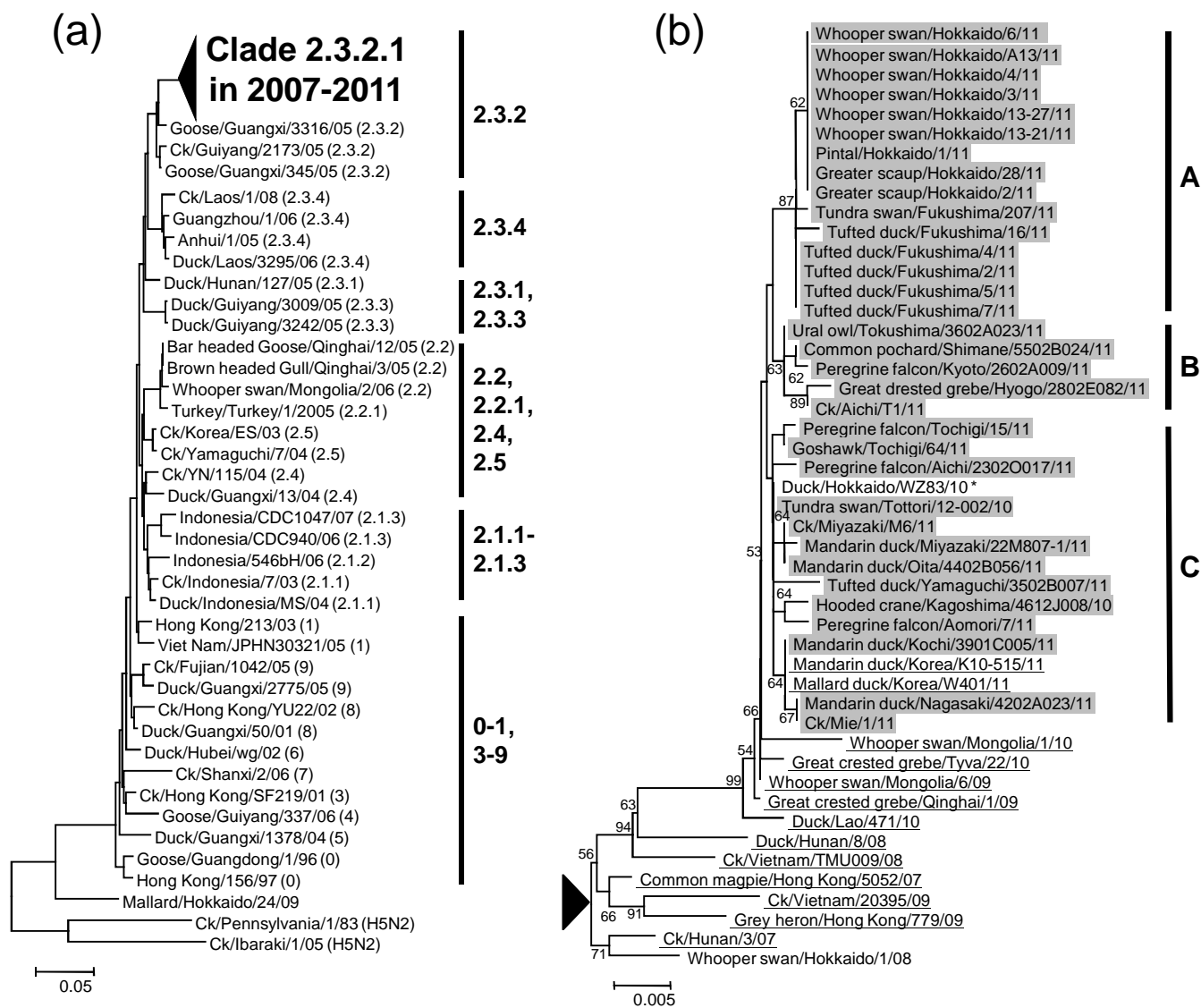


Fig. 2 Sakoda et al.

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	<u>Mar. 12, 16 (2011)</u>	<u>Chicken (2)</u>	- ¶	
	Aichi	Feb. 17 (2011) <u>Jan. 27, Feb. 14 (2011)</u>	Peregrine falcon (1) <u>Chicken (2)</u>	B, C	
	Toyama	Dec. 16 (2010)	Mute swan (1)	-	
	Mie	<u>Feb. 15, 26 (2011)</u>	<u>Chicken (2)</u>	C	
	Wakayama	<u>Feb. 15 (2011)</u>	<u>Chicken (1)</u>	-	
	Kyoto	Feb. 16 (2011)	Peregrine falcon (1)	B	
	Nara	<u>Feb. 28 (2011)</u>	<u>Chicken (1)</u>	-	
	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) <u>Nov. 29 (2010)</u>	Tufted duck (4), Common pochard (1) <u>Chicken (1)</u>	B	
	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) <u>Feb. 2 (2011)</u>	Mandarin duck (4), Grey heron (1) <u>Chicken (1)</u>	C	
	Miyazaki	Feb. 1, 2, 8, 11, 14, 15, 18 (2011) <u>Jan. 22, 24, 27, 28, 29, 30, Feb. 1, 4, 5, 6, 7, 17, Mar. 5 (2011)</u>	Mandarin duck (3), Peregrine falcon (3), Little grebe (1) <u>Chicken (13)</u>	C	
	Kagoshima	Dec. 19, 20, 21, 24 (2010), Feb. 13 (2011) <u>Jan. 26 (2011)</u>	Hooded crane (7) <u>Chicken (1)</u>	C	

\* Information about the case in chicken farm is underlined.

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

‡ Based on the phylogenetic tree 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)	Mal/Hok/09 (H5N1)	Ws/Hok/08 (H5N1)	
		D101/1	A310/39	64/1	B9/5	B220/1	B59/5	25/2			
<b>NPAIV</b>											
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)	—	+	+	+	+	+	+	+	+	<u>1280</u>	160
Dk/Hokkaido/101/2010 (H5N2)	—	+	+	+	+	+	+	+	+	640	80
<b>HPAIV</b>											
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	<u>1280</u>
Ws/Mongolia/6/2009 (H5N1)	2.3.2.1	+	—	—	—	—	—	—	—	80	1280
Ws/Mongolia/1/2010 (H5N1)	2.3.2.1	+	—	—	—	—	—	—	—	80	640
<b>Dk/Hokkaido/WZ83/2010 (H5N1)</b>	<b>2.3.2.1</b>	+	—	—	—	—	—	—	—	<b>40</b>	<b>320</b>
<b>Ws/Hokkaido/4/2011 (H5N1)</b>	<b>2.3.2.1</b>	+	—	—	—	—	—	—	—	<b>40</b>	<b>320</b>
<b>Pf/Aomori/7/2011 (H5N1)</b>	<b>2.3.2.1</b>	+	—	—	—	—	—	—	—	<b>40</b>	<b>320</b>

\* 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.