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1 Original article

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3 **H13 influenza viruses in wild birds have undergone genetic and antigenic diversification in nature**

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24

25 **Abstract**

26 Among 16 haemagglutinin (HA) subtypes of avian influenza viruses (AIVs), H13 AIVs have rarely been isolated
27 in wild waterfowl. H13 AIVs cause asymptomatic infection and are maintained mainly in gull and tern populations;
28 however, recorded antigenic information relating to the viruses has been limited. In this study, 2 H13 AIVs,
29 A/duck/Hokkaido/W345/2012 (H13N2) and A/duck/Hokkaido/WZ68/2012 (H13N2), isolated from the same area in the
30 same year in our surveillance, were genetically and antigenically analyzed with 10 representative H13 strains including
31 a prototype strain, A/gull/Maryland/704/1977 (H13N6). The HA genes of H13 AIVs were phylogenetically divided into
32 3 groups (I, II, and III). A/duck/Hokkaido/W345/2012 (H13N2) was genetically classified into Group III. This virus
33 was distinct from a prototype strain, A/gull/Maryland/704/1977 (H13N6), and the virus, A/duck/Hokkaido/WZ68/2012
34 (H13N2), both belonging to Group I. Antigenic analysis indicated that the viruses of Group I were antigenically closely
35 related to those of Group II, but distinct from those of Group III, including A/duck/Hokkaido/W345/2012 (H13N2). In
36 summary, our study indicates that H13 AIVs have undergone antigenic diversification in nature.

37

38 **Keywords:** Avian influenza, H13 subtype, Antigenicity, Genetics

39

40 **Introduction**

41 Avian influenza viruses (AIVs) belong to the genus *influenzavirus A* of the family *Orthomyxoviridae*. The genomes
42 of these viruses consist of 8 negative-stranded RNA segments. AIVs have been serologically divided into different
43 subtypes based on the antigenicity of their viral surface glycoproteins: haemagglutinin (HA: H1–H16 subtypes) and
44 neuraminidase (NA: N1–N9 subtypes) [1]. AIVs of all subtypes are naturally isolated from wild waterfowl, primarily
45 Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, shorebirds, and terns) [1–5]. Wild birds infected
46 with AIVs usually do not display any clinical signs of disease, but shed the virus during their migration [6]. Surveillance
47 of AIVs is important to monitor virus prevalence and transmission between birds. In our study, fecal samples were
48 collected from the ground at each habitat during the annual waterfowl migration season [7, 8].

49 H13 low-pathogenic AIV (LPAIV) was first isolated from gull in 1977 [9] and is rarely detected in avian species
50 other than Charadriiformes, suggesting that the viruses were maintained in gull and tern populations [10–12]. H13 AIVs
51 have rarely been isolated anywhere in the world, and so genetic and antigenic information on the viruses has been
52 limited [10]. In this study, 2 H13 AIVs, A/duck/Hokkaido/W345/2012 (H13N2) and A/duck/Hokkaido/WZ68/2012
53 (H13N2), isolated from the same area in the same year in our surveillance, were genetically and antigenically analyzed
54 with 10 representative H13 strains including a prototype strain, A/gull/Maryland/704/1977 (H13N6).

55
56 **Materials and methods**

57 **Viruses**

58 A total of 12 H13 AIVs were used in this study (Table 1). A/duck/Hokkaido/W186/2006 (H13N6),
59 A/duck/Hokkaido/W189/2006 (H13N6), A/duck/Hokkaido/W345/2012 (H13N2), A/duck/Hokkaido/WZ68/2012
60 (H13N2), and A/duck/Siberia/272PF/1998 (H13N6) were derived from the feces of migratory ducks in our surveillance
61 in Japan and Siberia [7, 8, 13]. A/gull/Maryland/704/1977 (H13N6), A/laughing gull/Delaware Bay/2838/1987
62 (H13N2), A/sanderling/Delaware Bay/221/2006 (H13N9), A/sanderling/Delaware Bay/224/2006 (H13N9), and A/red
63 knot/Delaware Bay/424/2007 (H13N9) were from St. Jude Children's Research Hospital, TN, USA [14].
64 A/mallard/Korea/SH38-45/2010 (H13N2) was from the Avian Disease Division, Animal and Plant Quarantine Agency,

65 South Korea [11]. A/whistling swan/Shimane/1343/1981 (H13N6) was kindly provided by Dr. Koichi Otsuki, Tottori
66 University, Japan.

67 Viruses were inoculated into 10-day-old embryonated chicken eggs and incubated for 48 h at 37°C. After
68 incubation, the infectious allantoic fluid was harvested and a hemagglutination titer was determined using 0.5% chicken
69 red blood cells. Aliquots of each virus were stored at –80°C until use[15].

70

71 **Sequencing and phylogenetic analysis**

72 Viral RNA extraction and amplification of full-length cDNAs from the 12 viruses was performed as described
73 previously [16]. Direct sequencing of HA gene segments for these 12 viruses was performed using the ABI 3500
74 Genetic Analyzer (Life Technologies, USA). The genome sequences identified in this study have been registered in
75 GenBank/EMBL/DDBJ (Table 1).

76 For phylogenetic analysis, nucleotide sequences of the 12 viruses, together with those from a public database
77 (<https://www.fludb.org>), were aligned using the Clustal W algorithm [17]. A phylogenetic tree was constructed using the
78 maximum likelihood method with 1000 bootstrap replicates using MEGA 6.0 software [18]. In addition, deduced amino
79 acid sequences of these 12 H13 AIVs were aligned to identify amino acid differences among the viruses. HA numbering
80 and antigenic sites were based on the H3 HA [19]. The positions of amino acid differences in the HA molecule were
81 analyzed on a 3-dimensional model of the H13 trimer HA of A/gull/Maryland/704/1977 (H13N6), obtained from the
82 Protein Databank (PDB accession number, 4KPQ) [20], by PyMOL presentation (DeLano Scientific, San Carlos, CA,
83 USA).

84

85 **Antigenic analysis**

86 Hyperimmunized antisera were prepared from chickens immunized with representative H13 AIVs inactivated with
87 formalin according to a previously described method [21]. Antigenic analysis of H13 viruses was performed using
88 antisera in the hemagglutination inhibition (HI) test as previously described [16]. Antisera raised against
89 A/gull/Maryland/704/1977 (H13N6), A/duck/Hokkaido/WZ68/2012 (H13N2), A/laughing gull/Delaware

90 Bay/2838/1987 (H13N2), A/red knot/Delaware Bay/424/2007 (H13N9), A/duck/Hokkaido/W345/2012 (H13N2), and
91 A/sanderling/Delaware Bay/224/2006 (H13N9) were named Gull/Maryland, Duck/WZ68, Gull/2838, Red knot/424,
92 Duck/W345, and Sanderling/224, respectively.

93

94 **Ethics statements**

95 Animal experiments for preparation of antisera were authorized by the Institutional Animal Care and Use
96 Committee of Hokkaido University (approval number: 13-0108), and all experiments were performed according to the
97 guidelines of the committee. All applicable international, national, and/or institutional guidelines for the care and use of
98 animals were followed. The Faculty of Veterinary Medicine, Hokkaido University has had accreditation from the
99 Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) since
100 2007.

101

102 **Results**

103 **Phylogenetic analysis of H13 HA genes**

104 To analyze the genetic relation of representative H13 AIVs, HA genes of 12 H13 AIVs were used for phylogenetic
105 tree analysis together with those of H13 virus strains from a public database. Phylogenetic analysis showed that H13
106 HA genes were clearly divided into 3 groups (Group I, II, and III) (Fig. 1). Group I consists of viruses isolated in
107 Eurasia and North America in the 20th century and isolated in Eurasia in recent years. Group II comprises only viruses
108 isolated in North America. Group III comprises viruses isolated in Eurasian and American continents.
109 A/gull/Maryland/704/1977 (H13N6), belonging Group I, was previously classified in North American lineage, however,
110 the virus was newly clustered with the viruses isolated in Eurasian lineage. Furthermore, phylogenetic analysis showed
111 that Group III is genetically more closely related to Group II than Group I. Interestingly, the HA genes of 2 viruses
112 isolated in the same year in Hokkaido were classified in the different genetic groups: the HA gene of
113 A/duck/Hokkaido/WZ68/2012 (H13N2) belongs to group I, and the HA gene of A/duck/Hokkaido/W345/2012 (H13N2)
114 belongs to Group III together with A/mallard/Korea/SH38-45/2010 (H13N2) and H13N8 viruses isolated in Qinghai

115 Lake, China.

116

117 **Antigenic analysis of H13 viruses**

118 To compare the antigenicity of H13 AIVs, 12 representative strains were antigenically analyzed by HI test (Table
119 2). Six Group I viruses showed similar reactivity patterns against all antisera tested. Group I and Group II viruses
120 reacted with antisera prepared from Group I and II viruses with high HI titers similarly to the homologous viruses;
121 however they reacted with antisera of Group III viruses with low HI titers. In contrast, Group III viruses showed
122 different patterns of reactivity against antisera from Group I and Group II viruses. Among Group III viruses, the
123 reactivity pattern of A/duck/Hokkaido/W345/2012 (H13N2) against these antisera was similar to
124 A/mallard/Korea/SH38-45/2010 (H13N2). HI titers of A/duck/Hokkaido/W345/2012 (H13N2) and
125 A/mallard/Korea/SH38-45/2010 (H13N2) viruses were relatively high compared with A/sanderling/Delaware
126 Bay/221/2006 (H13N9) and A/sanderling/Delaware Bay/224/2006 (H13N9). The reactivity patterns of antigenicity
127 showed that the Group I viruses are closely related to Group II viruses, but more distantly related to Group III viruses.
128 These antigenic virus relationships were also revealed using antigenic cartography methods based on the results of HI
129 tests (Supplementary Fig. 1).

130

131 **Positions of substitutions in H13 HA**

132 To estimate antigenic variation in H13 viruses, deduced amino acid sequences of the HAs from 12 H13 AIVs were
133 aligned, and the positions of substitutions in the H13 HAs were identified (Fig. 2). These positions were also mapped
134 on the H13 HA structure to determine whether these amino acids are exposed on the surface of the molecule as shown
135 in Supplementary Fig. 2. Based on the information of antigenic sites in H3 HA numbering [19], the HA of Group III
136 viruses had many amino acid differences from those of Group I and II viruses both within and outside of these
137 supposed antigenic sites, suggesting that these differences are likely associated with antigenic variation in Group III
138 viruses.

139

140 Discussion

141 The HA genes of H13 AIVs were phylogenetically divided into 3 groups (I, II, and III) in the present study. While
142 previous studies have demonstrated that H13 AIVs can be divided into 2 groups: North American lineage (Containing
143 the viruses belong to Group I and II in this study) and Eurasian lineage (Containing the viruses belong to Group III in
144 this study) [11, 22]. In this study, Group I, which includes A/gull/Maryland/704/1997 (H13N6), consisted not only of
145 viruses isolated in North America, but also viruses isolated in Eurasia. Also, it was revealed that Group III contains both
146 North American and Eurasian viruses. A/duck/Hokkaido/W345/2012 (H13N2) and A/duck/Hokkaido/WZ68/2012
147 (H13N2) isolated in the same year and the same place were antigenically and genetically different characters,
148 suggesting that these viruses have circulated together between North America and Eurasia in wild birds (Fig. 1 and
149 Supplementary Fig. 1). Furthermore, Group III was genetically more closely related to Group II than to Group I (Fig. 1).
150 All subtypes AIVs can be divided into two lineages, Eurasian and American, as a result of long-term ecological and
151 geographical separation of host [1]. The geographical distribution of the H13 AIVs was not well described but clearly
152 different from that of other subtype AIVs in the present study, possibly due to differences in the migration routes of gull
153 and tern species. Some ducks (e.g. Northern pintail, *Anas acuta*) and shorebird species cross the Bering Strait and could
154 provide an intercontinental bridge for AIVs, but the overlap in distribution of ducks is not profound as that of
155 Charadriiformes, such as shorebirds [1, 23, 24]. Also, the host species of fecal samples, from which viruses were
156 isolated, were not identified genetically; however, morphology of the feces were clearly that of ducks. So, we concluded
157 that H13 viruses were isolated from ducks in the present study. Previous studies have indicated that H13 subtype is
158 strongly adapted to gull host, but infections in anomalous hosts (*i.e.*, turkeys and ducks) could possibly occur [10].
159 Actually, black-headed gull (*Larus ridibundus*), black-tailed gull (*Larus crassirostris*), and herring gull (*Larus*
160 *argentatus*) were observed at the lake on the day when the samples were collected. Thus, we assumed that the H13
161 viruses were transmitted from Charadriiformes to the ducks.

162 Antigenicity of H13 viruses was previously reported by Chamberes *et al.* in 1989 [25]. They concluded that H13
163 AIVs were antigenically distinct between the Eurasian virus, A/gull/Astrakhan/176/1986 (H13N2), and the North
164 American viruses, A/gull/Maryland/704/1977 (H13N6) and A/pilot whale/Maine/328 HN/1984 (H13N2); however,

165 there has been no information relating to antigenicity in more recent years. In the present study, the antigenicities of
166 H13 AIVs tested were clearly distinct even in viruses isolated in the same area and the same year. Furthermore,
167 antigenic analysis showed that Group III was antigenically different from the other 2 groups and several amino acid
168 differences with one amino acid deletion. These differences seemed to be related to the antigenic differences among the
169 groups. In LPAIVs of other subtypes, there was no clear antigenic variation in viruses isolated from wild ducks [7]. Our
170 result indicates that serological diagnosis of H13 viruses should be performed with consideration of this antigenic
171 variation.

172 In conclusion, H13 AIVs that have rarely been isolated from natural hosts are genetically and antigenically diverse.
173 This contrasts AIVs of other subtypes which are mainly isolated from Anseriformes. To reveal more about the nature of
174 this diversity, further studies on topics such as virus–host interactions and the ecology of Charadriiformes are required.

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183 Agency for Medical Research and Development (AMED).

185 **Statement of author contributions**

186 Z-J.W. wrote this study and performed genetic and antigenic analysis. Y.K., L.T.N., T.H., S.K., R.W., and Y-J.L.
187 performed genetic and antigenic analysis. K.M., M.O., and H.K. provided laboratory management support and
188 manuscript editing. Y.S. managed this research project. All authors read and approved the final manuscript.

189

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195 **Compliance with Ethical Standards**

196 **Conflict of Interest** The authors declare no conflicts of interest.

197 **Ethical approval** Animal experiments in this study were authorized by the Institutional Animal Care and Use
198 Committee of Hokkaido University (approval number: 13-0108), and all experiments were performed according to the
199 guidelines of the committee. All applicable international, national, and/or institutional guidelines for the care and use of
200 animals were followed. The Faculty of Veterinary Medicine, Hokkaido University has had accreditation from the
201 Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) since
202 2007. This article does not contain any studies with human participants performed by any of the authors.

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Figure Legends

Fig. 1 Phylogenetic tree for the H13 HA AIVs. Full-length HA genes of 12 H13 subtype viruses, and reference strains, were analyzed using the maximum likelihood method with 1000 bootstrap replicates using MEGA 6.0 software

(<http://www.megasoftware.net/>), and A/glaucous-winged gull/South Central Alaska/16MB03160/2016 (H16N3) was used to root the tree. The viruses used in our study are underlined. The isolates from the same area in the same year in our surveillance, A/duck/Hokkaido/W345/2012 (H13N2) and A/duck/Hokkaido/WZ68/2012 (H13N2), are highlighted in grey.

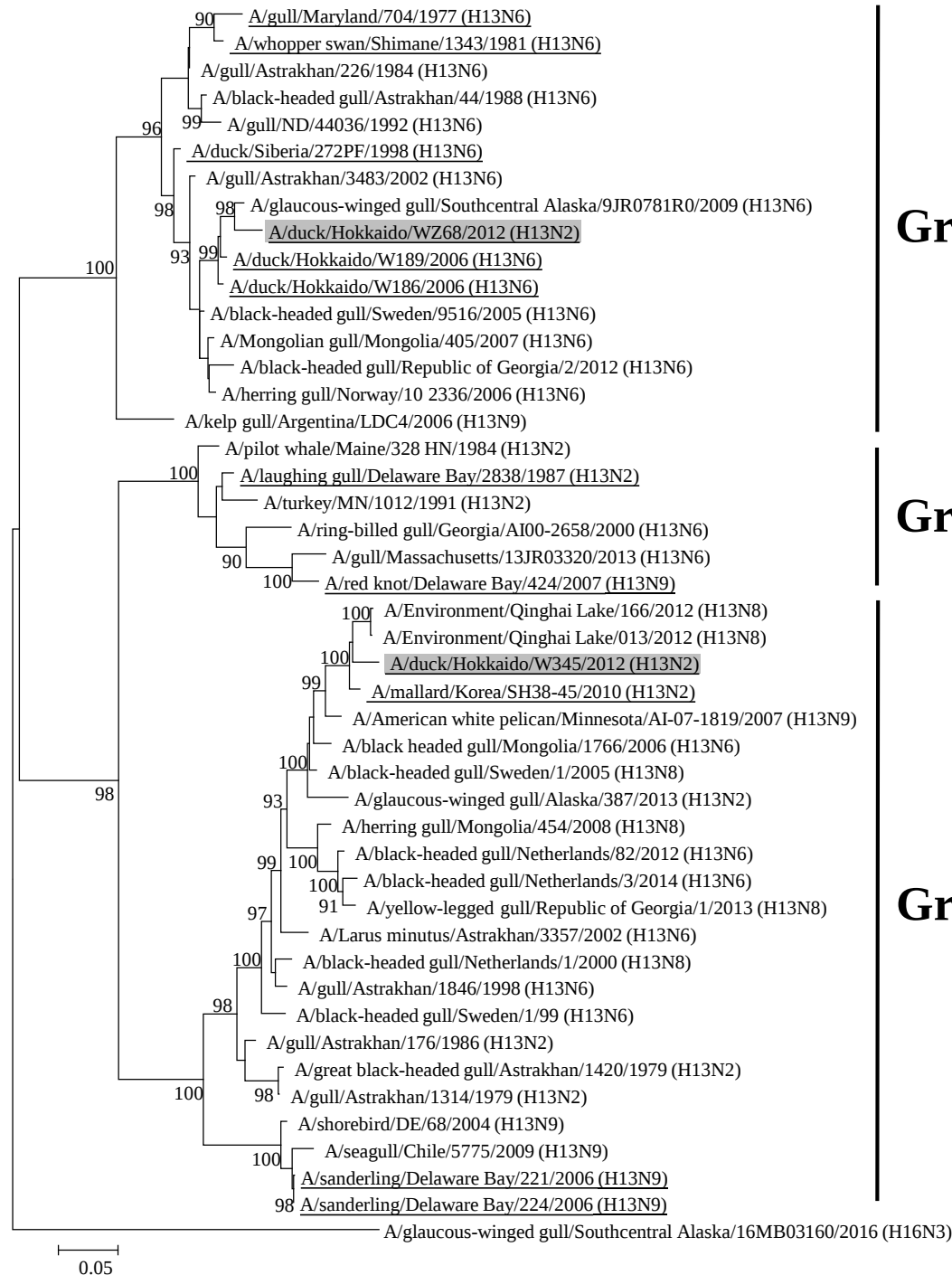
Fig. 2 Aligned amino acid sequences of H13 HA genes. Alignment and comparison of complete HA coding sequences for the H13 AIVs. The sequence of A/gull/Maryland/704/1977 (H13N6) is shown in its entirety on the top line. Underlined amino acids are in antigenic sites proposed by H3 HA [16]. Asterisks show supposed key amino acid differences in Group III viruses. Abbreviations of strains are A/gull/Maryland/704/1977 (Gull/MD/704/77), A/whistling swan/Shimane/1343/1981 (Ws/Shimane/1343/81), A/laughing gull/Delaware Bay/2838/1987 (Lg/DE Bay/2838/87), A/duck/Siberia/272PF/1998 (Duck/Siberia/272PF/98), A/sanderling/Delaware Bay/221/2006 (S/DE Bay/221/06), A/sanderling/Delaware Bay/224/2006 (S/DE Bay/224/06), A/duck/Hokkaido/W186/2006 (Duck/Hokkaido/W186/06), A/duck/Hokkaido/W189/2006 (Duck/Hokkaido/W189/06), A/red knot/Delaware Bay/424/2007 (Rk/DE Bay/424/07), A/mallard/Korea/SH38-45/2010 (Mallard/Korea/SH38-45/10), A/duck/Hokkaido/W345/2012 (Duck/Hokkaido/W345/12), and A/duck/Hokkaido/WZ68/2012 (Duck/Hokkaido/WZ68/12).

Supplementary Fig. 1 Antigenic cartography of a panel of immune sera against corresponding H13 AIVs. The antigenic cartography was constructed to better understand the antigenic data from the HI test, shown in Table 2, using AntigenMap [26]. The HI test data were used to construct two-dimensional (2D) antigenic map in which the distance between points represents the antigenic distance as measured by a HI test. One unit of antigenic distance on the antigenic map corresponds to a two-fold difference in the serological assay. The web-based software for Antigenic Cartography is available at <http://www.antigenic-cartography.org/>.

Supplementary Fig. 2 Amino acid substitutions on a 3-dimensional model of the Group III H13 HA based on H3 numbering. The crystallographic structure of the H13 trimer HA of A/gull/Maryland/704/1977 (H13N6), Protein

291 Databank accession number: 4KPQ [20], is represented. Amino acid substitutions at the antigenic site (based on H3
292 antigenic sites) are shown in red, and those outside of the antigenic site are shown in blue.

Fig. 1.
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I Gull/MD/704/77	1	DRICVGYLSTNSSERVDTLLENGVPVTSSIDLIETNHTGTGCSLNGVSPVHLGDCSFEQWIVGNPACTSNFGIREWSYLIEDPAAPHGLCYPGELNNNGELRHLFSGIRS	110
Ws/Shimane/1343/81		T.....	
Duck/Siberia/272PF/98		V.....T.....G.....	
Duck/Hokkaido/W186/06		V.....G.....	
Duck/Hokkaido/W189/06		V.....G.....	
Duck/Hokkaido/WZ68/12		I.....G.....	
II Lg/DE Bay/2838/87		T.K.....D.....V.....D.....S.....D.....K.....	
Rk/DE Bay/424/07		T.K.....D.....V.....S.....DE.....K.....	
III Duck/Hokkaido/W345/12		K.....N.....V.V.....A.G.I.....A..L.....S.....D.....	
S/DE Bay/224/06		K.....D.....V.V.....I.....S.A..L.....S.....D.....K.....	
S/DE Bay/221/06		K.....D.....V.V.....I.....S.A..L.....S.....D.....K.....	
Mallard/Korea/SH38-45/10		K.....N.....V.V.....A.G.I.....A..L.....S.....D.....	
I Gull/MD/704/77	111	FSRTELIPPTSWGEVLDTTACRDNTGTNSFYRNLVFIKKNNRYPVISKTYNNTTGRDVLVLWGIHHPVSVDETCTLYVNSDPYTLVSTKSWSEKYKLETGVRPGYNG	220
Ws/Shimane/1343/81		A.....V...K...R.....E.....I.....	
Duck/Siberia/272PF/98		A.....DK.....V.....E.....	
Duck/Hokkaido/W186/06		A.....DK.....V.....E...A.....	
Duck/Hokkaido/W189/06		V.....A.....E.....V.....E...A.....	
Duck/Hokkaido/WZ68/12		A.....A.....DK.....V.....E.....	
II Lg/DE Bay/2838/87		A.....N.A.....S.....V.RG.K...R.....I.....T...S.....S...K.....	
Rk/DE Bay/424/07		A.....A.....S.....V.....R.....TG.IQ...A.....RL...K.....	
III Duck/Hokkaido/W345/12		A.....A.N..VSA..T.-R.AS.....VNRG.N...RGA.....I.....T..VRQ..AKDN.....R...R...N.....	219
S/DE Bay/224/06		A.....A.N..VS...Q.-K.AS.....VERGKK...RG.....M.....E..ARK..I..N.....G...K...N...I.....	
S/DE Bay/221/06		A.....A.N..VS...Q.-K.AS.....VERGKK...RG.....M.....E..ARK..I..N.....G...K...N...I.....	
Mallard/Korea/SH38-45/10		A.....A.N..VS...T.-K.AS.....VERGKN...RGA.....I.....T...ARK..AKDN.....R...R...N.....	
I Gull/MD/704/77	221	QRSWMKIYWLSIHPGEMITFESNGGFLAPRYGYIIIEYGGKGRIFQSRIRMSRCNTKCQTSVGGINTNRTFQNIIDKNALGDCPKYIKSGQLKLATGLRNPVPAISNRG	326
Ws/Shimane/1343/81		L.....I.....	
Duck/Siberia/272PF/98		L.....K.....	
Duck/Hokkaido/W186/06		L.....L.....L.K.....	
Duck/Hokkaido/W189/06		L.....L.....L.K.....	
Duck/Hokkaido/WZ68/12		L.....L.....L.K.....	
II Lg/DE Bay/2838/87		V.M...S.....L.....P..VA.....K.....ER...N.....ST...	
Rk/DE Bay/424/07		D.M...S.....L.....P..VA.....K.....ER...N.....K.....	
III Duck/Hokkaido/W345/12	220	.K.....Y.L...S.S.....L..K.....IAK..A.....K.....ER.....	325
S/DE Bay/224/06		.K.....M...S.S.....L.....H..AGK.....K.....ER.....T.AS..	
S/DE Bay/221/06		.K.....M...S.S.....L.....H..AGK.....K.....ER.....T.AS..	
Mallard/Korea/SH38-45/10		Y.L...S.S.....L..K.....F.KA.H.IAK.....K.....ER.....R.....	

Fig. 2.
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Table 1. Representative H13 AIVs using in this study

Viruses	Subtypes	Accession No. of HA gene	Reference
A/gull/Maryland/704/1977	H13N6	CY130086	[25]
A/whistling swan/Shimane/1343/1981	H13N6	LC336770	This study
A/laughing gull/Delaware Bay/2838/1987	H13N2	CY005979	[25]
A/duck/Siberia/272PF/1998	H13N6	AB285094	[13]
A/sanderling/Delaware Bay/221/2006	H13N9	CY043888	This study
A/sanderling/Delaware Bay/224/2006	H13N9	CY043896	This study
A/duck/Hokkaido/W186/2006	H13N6	LC336771	This study
A/duck/Hokkaido/W189/2006	H13N6	LC336772	This study
A/red knot/Delaware Bay/424/2007	H13N9	CY127799	This study
A/mallard/Korea/SH38-45/2010	H13N2	JX030406	[11]
A/duck/Hokkaido/W345/2012	H13N2	LC336769	This study
A/duck/Hokkaido/WZ68/2012	H13N2	AB812744	[6]

Table 2. Antigenic characterization of H13 AIVs belonged to 3 genetic groups using HI test

Groups	Viruses	Subtypes	HI titers of antisera ^a					
			I		II		III	
			Gull/Maryland	Duck/WZ68	Gull/2838	Red knot/424	Duck/W345	Sanderling/224
I	Gull/MD/704/77	H13N6	<u>8,192</u>	16,384	16,384	16,384	128	256
	Ws/Shimane/1343/81	H13N6	2,048	8,192	16,384	16,384	64	512
	Duck/Siberia/272PF/98	H13N6	4,096	16,384	8,192	16,384	256	128
	Duck/Hokkaido/W186/06	H13N6	4,096	16,384	4,096	8,192	256	128
	Duck/Hokkaido/W189/06	H13N6	4,096	16,384	4,096	8,192	256	128
	Duck/Hokkaido/WZ68/12	H13N2	4,096	<u>16,384</u>	2,048	8,192	128	32
II	Lg/DE Bay/2838/87	H13N2	1,024	4,096	<u>4,096</u>	512	256	128
	Rk/DE Bay/424/07	H13N9	1,024	4,096	4,096	<u>4,096</u>	32	128
III	Duck/Hokkaido/W345/12	H13N2	128	512	8,192	512	<u>8,192</u>	16,384
	S/DE Bay/224/06	H13N9	64	128	256	128	1,024	<u>4,096</u>
	S/DE Bay/221/06	H13N9	32	128	1,024	1,024	512	8,192
	Mallard/Korea/SH38-45/10	H13N2	256	1,024	8,192	128	16,384	8,192

^aThe homologous titers were underlined.