Original article

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

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**Abstract**

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

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

## Introduction

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

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

## Materials and methods

### Viruses

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

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

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

**Sequencing and phylogenetic analysis**

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

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

**Antigenic analysis**

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

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

**Ethics statements**

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

**Results**

**Phylogenetic analysis of H13 HA genes**

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

Lake, China.

**Antigenic analysis of H13 viruses**

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

**Positions of substitutions in H13 HA**

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

## Discussion

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

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

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

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

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**Statement of author contributions**

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. performed genetic and antigenic analysis. K.M., M.O., and H.K. provided laboratory management support and manuscript editing. Y.S. managed this research project. All authors read and approved the final manuscript.

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**Compliance with Ethical Standards**

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

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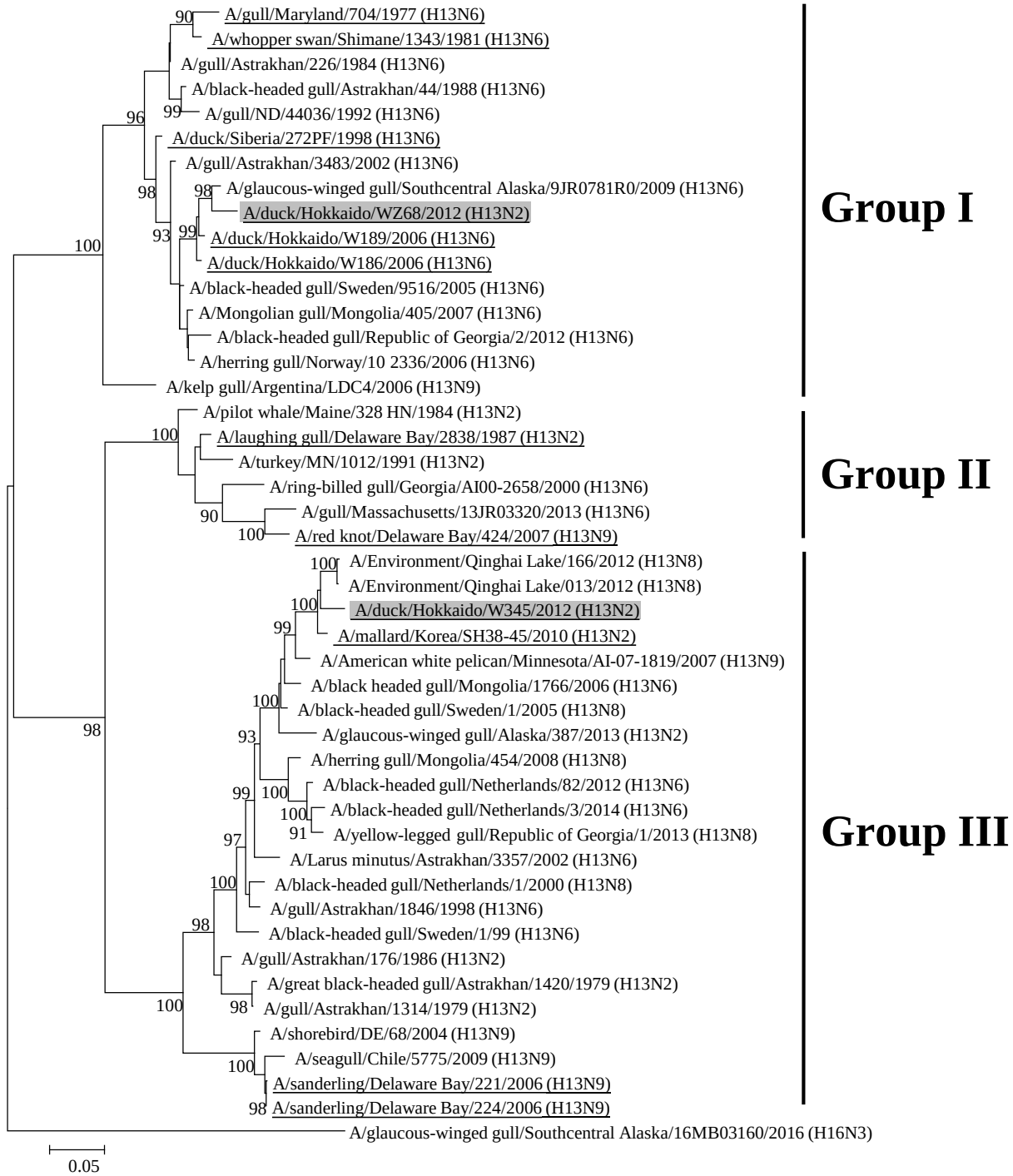

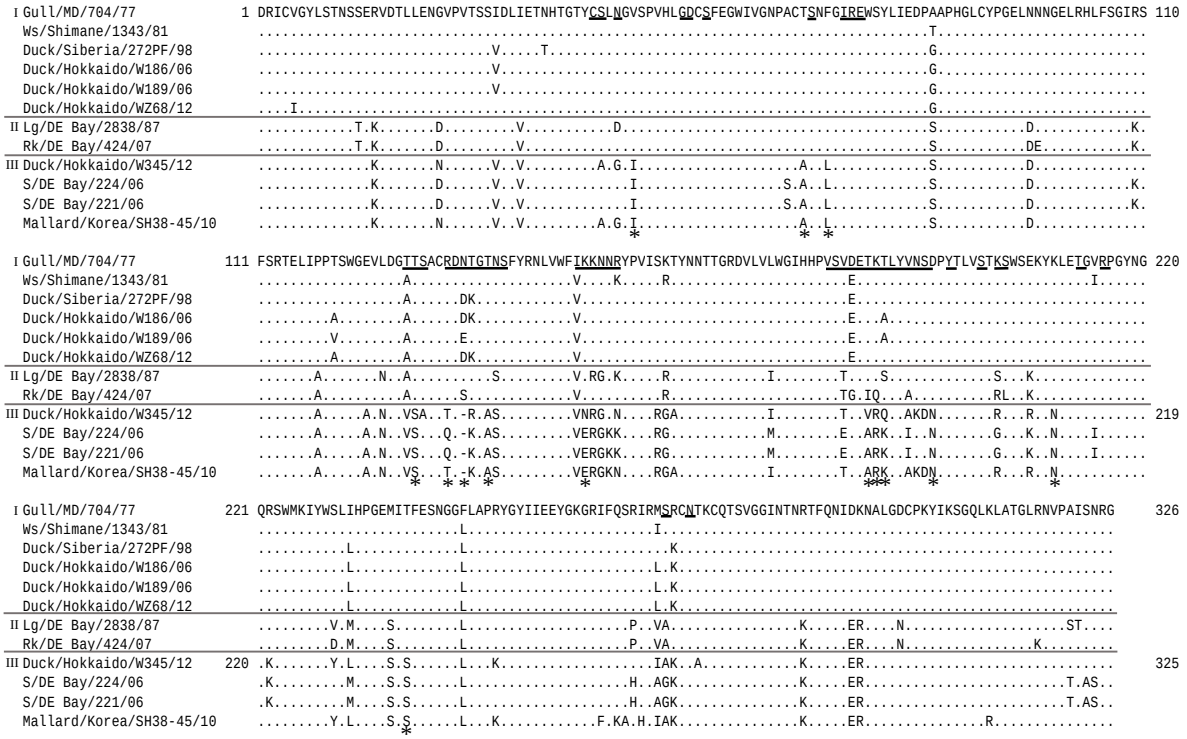


**Fig. 2.**
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**Table 1.** Representative H13 AIVs using in this study

| 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 | 8,192 | 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 | 16,384 | 2,048 | 8,192 | 128 | 32 |
| II | Lg/DE Bay/2838/87 | H13N2 | 1,024 | 4,096 | 4,096 | 512 | 256 | 128 |
| | Rk/DE Bay/424/07 | H13N9 | 1,024 | 4,096 | 4,096 | 4,096 | 32 | 128 |
| III | Duck/Hokkaido/W345/12 | H13N2 | 128 | 512 | 8,192 | 512 | 8,192 | 16,384 |
| | S/DE Bay/224/06 | H13N9 | 64 | 128 | 256 | 128 | 1,024 | 4,096 |
| | 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 |

[a]The homologous titers were underlined.