農研機構
NARO 国立研究開発法人 農業・食品産業技術総合研究機構

Five distinct reassortants of H5N6 highly pathogenic avian influenza A viruses affected Japan during the winter of 2016-2017

メタデータ	言語: eng 出版者: 公開日: 2019-05-14 キーワード (Ja): キーワード (En): 作成者: 竹前, 喜洋, 常國, 良太, 谷川, 太一朗, 内田, 裕子, 峯, 淳貴, 西藤, 岳彦 メールアドレス: 所属:
URL	https://repository.naro.go.jp/records/2565

This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 International License.

Virology 512 (2017) 8–20

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Five distinct reassortants of H5N6 highly pathogenic avian influenza A viruses affected Japan during the winter of 2016–2017

Nobuhiro Takemae[a,b], Ryota Tsunekuni[a,b], Kirill Sharshov[c], Taichiro Tanikawa[a,b], Yuko Uchida[a,b], Hiroshi Ito[d], Kosuke Soda[d], Tatsufumi Usui[d], Ivan Sobolev[c], Alexander Shestopalov[c], Tsuyoshi Yamaguchi[d], Junki Mine[a,b], Toshihiro Ito[d], Takehiko Saito[a,b,e,*]

[a] Division of Transboundary Animal Disease, National Institute of Animal Health, NARO, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan
[b] Thailand–Japan Zoonotic Diseases Collaboration Center, Kasetklang, Chatuchak, Bangkok 10900, Thailand
[c] Research Institute of Experimental and Clinical Medicine, 2, Timakova street, Novosibirsk 630117, Russia
[d] The Avian Zoonosis Research Center, Faculty of Agriculture, Tottori University, 4-101 Koyama-cho Minami, Tottori, Tottori 680-8550, Japan
[e] United Graduate School of Veterinary Sciences, Gifu University, 1-1, Yanagito, Gifu, Gifu 501-1112, Japan

ARTICLE INFO

Keywords:
Highly pathogenic avian influenza A viruses
H5N6
Clade 2.3.4.4
Poultry
Wild bird

ABSTRACT

To elucidate the evolutionary pathway, we sequenced the entire genomes of 89 H5N6 highly pathogenic avian influenza viruses (HPAIVs) isolated in Japan during winter 2016–2017 and 117 AIV/HPAIVs isolated in Japan and Russia. Phylogenetic analysis showed that at least 5 distinct genotypes of H5N6 HPAIVs affected poultry and wild birds during that period. Japanese H5N6 isolates shared a common genetic ancestor in 6 of 8 genomic segments, and the PA and NS genes demonstrated 4 and 2 genetic origins, respectively. Six gene segments originated from a putative ancestral clade 2.3.4.4 H5N6 virus that was a possible genetic reassortant among Chinese clade 2.3.4.4 H5N6 HPAIVs. In addition, 2 NS clusters and a PA cluster in Japanese H5N6 HPAIVs originated from Chinese HPAIVs, whereas 3 distinct AIV-derived PA clusters were evident. These results suggest that migratory birds were important in the spread and genetic diversification of clade 2.3.4.4 H5 HPAIVs.

1. Introduction

The Asian lineage of H5 highly pathogenic avian influenza viruses (HPAIVs), which was first reported in domestic geese in southern China in 1996, has continued to circulate and spread among poultry and wild birds in Asia, the Middle East, North America, and Africa (Cattoli et al., 2009; Harfoot and Webby, 2017; Lee et al., 2015; Saito et al., 2015; Su et al., 2015). Outbreaks of H5 HPAIVs have resulted in enormous economic losses to the poultry industry, and their sporadic transmission from poultry to humans in several countries highlights their potential threat to public health. The haemagglutinin (HA) genes of the Asian H5 HPAIVs have rapidly evolved into multiple clades as defined by WHO/OIE/FAO (Smith et al., 2015). To date, H5 genes of the Asian H5 HPAIV have diversified to generate 10 clades, clades 0–9, and some of the clades are further divided into as many as 4-digit subclades, such as 2.3.2.1, 2.3.4.4, and so on (Smith et al., 2015). All clades of the Asian H5 HPAIVs except one, clade 2.3.4.4, have been associated with the N1 neuraminidase (NA) since its first appearance in 1996.

The HA genes of clade 2.3.4.4 evolved from clade 2.3.4 as early as 2008 (Gu et al., 2011). This HA associated with N5 neuraminidase to form the H5N5 HPAIVs, such as A/duck/Guangdong/wy19/2008 (H5N5) (Liu et al., 2013). Since then, the clade 2.3.4.4 HA gene has been found in association with either N2, N5, N6, or N8 NA in poultry and wild birds. The H5N6 HPAIVs with the clade 2.3.4.4 HA have expanded in poultry in China since 2013 (Bi et al., 2016a, 2016b). In 2014, the first human infection with clade 2.3.4.4 H5N6 HPAIV was reported in China, followed by 16 human cases as of December 1, 2016 (Jiang et al., 2017). Starting in January 2014, clade 2.3.4.4 H5N8 HPAIVs caused massive outbreaks in poultry in South Korea through July 2014 (Lee et al., 2014; Yoon et al., 2015), as well as 1 poultry outbreak in April 2014 (Kanehira et al., 2015) and 5 between December 2014 and January 2015 in Japan (Saito et al., 2015). In addition, clade 2.3.4.4 H5N8 HPAIVs affected Canada and the United States in the winter of 2014–2015 (Bevins et al., 2016; Ip et al., 2015; Lee et al., 2016; Pasick et al., 2015). During the winter of 2016–2017, clade 2.3.4.4 H5N6 HPAIVs outbreaks occurred in South Korea (Jeong et al., 2017; Kwon et al., 2017; Lee et al., 2017b; Si et al., 2017), Taiwan, and Japan (Okamatsu et al., 2017), whereas clade 2.3.4.4 H5N8 viruses affected European countries (Pohlmann et al., 2017).

* Corresponding author at: Division of Transboundary Animal Disease, National Institute of Animal Health, NARO, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan.
E-mail address: taksaito@affrc.go.jp (T. Saito).

http://dx.doi.org/10.1016/j.virol.2017.08.035
Received 28 June 2017; Received in revised form 22 August 2017; Accepted 24 August 2017
Available online 09 September 2017
0042-6822/ © 2017 The Author(s). Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).

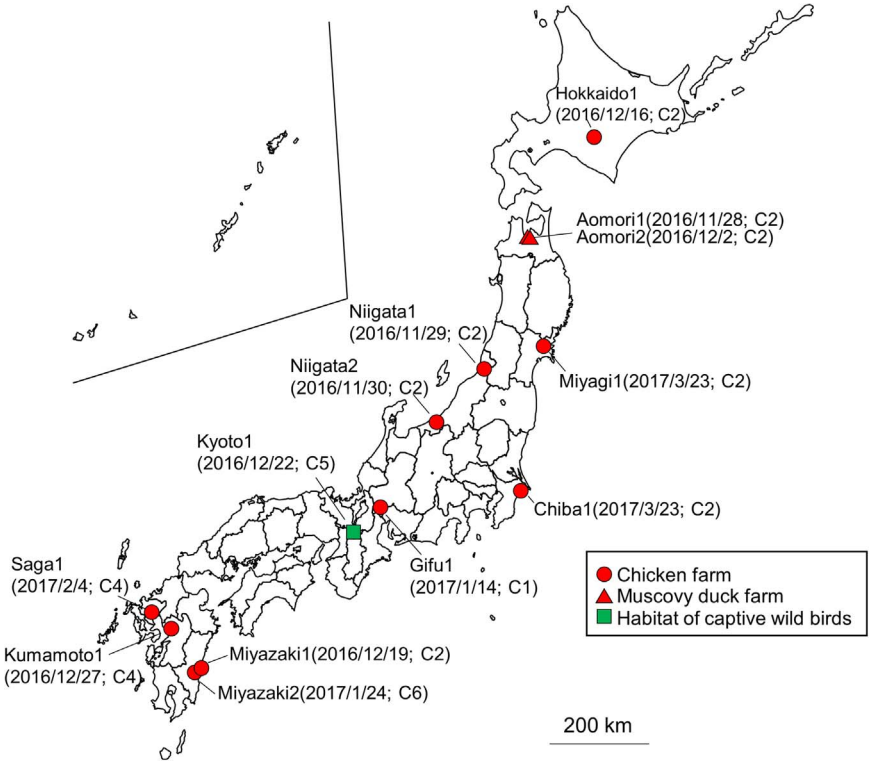

Fig. 1. Geographical location of the H5N6 HPAI outbreaks at twelve poultry farms and one habitat of captive wild birds in Japan analyzed in this study. The sample collection dates and genotypes isolated in each outbreak are shown in parenthesis.

During its evolution, clade 2.3.4.4 HA viruses have been classified into 4 groups. Group A (Buan2-like) and group B (Gochang1-like) viruses are represented by A/duck/Korea/Buan2/2014 and A/breeder duck/Korea/Gochang1/2014, respectively, which caused the Korean outbreaks in 2014–2015 (Lee et al., 2015, 2014). Group C and group D (Si et al., 2017) consist mainly of H5N6 HPAIVs and are designated as 'major group' and 'minor group', respectively, according to the number of viruses in each group (Bi et al., 2016a). Furthermore, according to the genetic constellation of 8 segments, the H5N6 HPAIVs isolated in China are classified at least into 34 distinct genotypes (Bi et al., 2016a). The genotype G1 derived from group C and G2 derived from group D, and their descendant genotypes, such as G1.1.x and G2.1.x, arose from multiple reassortments with other HPAIVs or AIVs (Bi et al., 2016a). The Korean H5N6 HPAIVs were divided into 5 distinct genotypes (designated as C1 through C5) according to the origins of their PA (polymerase acidic protein) and NS (non-structural protein) genes, which emerged via reassortment between Chinese H5N6 HPAIVs and Eurasian AIVs (Lee et al., 2017b).

The migration of wild birds has long been thought to contribute to both the expansion of HPAIVs (Olsen et al., 2016; Verhagen et al., 2015; Webster et al., 1992) as well as the genetic reassortment between HPAIVs and AIVs (Bevins et al., 2016; Lee et al., 2016; Uchida et al., 2012). Eight major intercontinental flyways of aquatic migratory birds are recognized (Boere and Stroud, 2006), and Siberia, which is the birds' breeding ground, sits at an intersection of several of those flyways (Alerstam et al., 2007; Boere and Stroud, 2006). The group A H5N8 HPAIVs in 2014–2015 were suspected to be carried by migratory birds from Siberia to various countries in Asia, Europe, and North America during the winter migration and from those regions back to Siberia during the spring migration (Bouwstra et al., 2015; Hanna et al., 2015; Ip et al., 2015; Lee et al., 2015; Ozawa et al., 2015). Group B H5N8 HPAIVs isolated from waterfowl and shorebirds at Uvs-Nuur Lake in western Siberia in Spring 2016 (Lee et al., 2017a; Marchenko et al., 2017) appeared in European countries by Autumn 2016 (Pohlmann et al., 2017), suggesting that those viruses were transported during the winter migration from Siberia to Europe.

From November 2016 through March 2017, H5N6 HPAIVs affected 12 poultry farms in Japan. Prior to these outbreaks, an H5N6 HPAIV belonging to clade 2.3.4.4 was first detected in feces of a northern pintail (*Anas acuta*) in the Tottori prefecture of Japan (Okamatsu et al., 2017). By March 2017, 218 cases among migratory and captive birds were reported in Japan. In the current study, we genetically and phylogenetically characterized a series of H5N6 viruses isolated from poultry and wild birds in Japan to elucidate the evolutionary pathway of the Japanese H5N6 viruses and to surmise how those viruses intruded into Japan.

2. Results

2.1. Outbreaks and virulence of Japanese H5N6 strains

Between November 28, 2016, and March 24, 2017, 12 poultry outbreaks occurred across 9 prefectures in Japan: 2 in Muscovy duck farms, 7 in layer chicken farms, and 3 in broiler chicken farms (Fig. 1 and Supplementary Table S1). The first outbreak in poultry was reported in a Muscovy duck farm (Aomori1) in Aomori prefecture and was the first occurrence of H5 HPAIV infection in a Japanese duck farm. The second and third HPAI outbreaks occurred at layer chicken farms (Niigata1, Niigata2) in Niigata prefecture on November 29 and 30, 2016, respectively. Although in the same prefecture, these farms were located 143 km away from each other, and were not epidemiologically related. Four days after the first HPAI outbreak, another outbreak occurred in a Muscovy duck farm (Aomori2) that was adjacent to but approximately 350 m away from Aomori1 and was managed by the same company. Subsequently, 3 outbreaks in chicken farms in 3 prefectures occurred in December 2016, 2 chicken farms in Miyazaki and Gifu prefectures were affected in January 2017, and 1 outbreak occurred in a chicken farm during February 2017. The last outbreaks during this season were on 2 layer chicken farms in Miyagi and Chiba prefectures on March 24, 2017. Hokkaido1 and Miyazaki2 were more than 1500 km apart, indicating that Japan was widely affected by H5N6 HPAIVs during the season. In sum, approximately 1.6 million chickens and 23,000 Muscovy ducks died or were euthanized during the outbreaks.

In the current study, 4 Japanese H5N6 strains (Aomori1-3T, Niigata1-1T, Kumamoto1-2C, and Miyazaki2-2C) among the 12 poultry outbreaks were tested for pathogenicity according to the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (World Organisation for Animal Health, 2016). All chickens inoculated intravenously with each strain died within 24 h of inoculation. In addition, sequence analysis revealed that all of the Japanese H5N6 AIVs we tested, except for the isolates from Saga1, have the multiple basic amino acid sequence RERRRKR in their HA cleavage sites, whereas the isolates from Saga1 have KERRRKR (Table 1). Both of these motifs are associated with high pathogenicity of AIV in chickens (World Organisation for Animal Health, 2016).

2.2. Molecular characteristics of Japanese H5N6 HPAIVs

We compared the molecular characteristics of the 8 gene segments among the representative Japanese H5N6 HPAIVs in each outbreak because the homology among the strains isolated at the same farm or habitat was more than 99.7% in each segment (Table 1 and Supplementary Table S1). The Saga strains had an additional potential N-glycosylation at position 240 of their HA1 protein, compared with other Japanese H5N6 HPAIVs. The amino acids at positions 138, 186, 224, 226, and 228 (H3 numbering) of the receptor-binding sites of the HA1 proteins indicated a preference for α2,3-linked sialic acid receptors (de Vries et al., 2014; Imai et al., 2012; Nidom et al., 2010; Srinivasan et al., 2013; Yang et al., 2010). The NA stalk of Japanese H5N6 viruses had a deletion of the 11 amino acid residues at positions 58–68 (N6 numbering). None of the viruses had the H274Y resistance mutation (N2 numbering) in the NA protein, indicating that these viruses likely are sensitive to drugs such as oseltamivir (Ilyushina et al., 2010). None of the Japanese H5N6 HPAIVs carried mutations associated with mammalian adaptation (Bussey et al., 2010; Hatta et al., 2001; Min et al., 2013; Steel et al., 2009; Yamada et al., 2010), such as T271A, Q591R, E627K, and D701N substitutions in the polymerase basic protein 2 (PB2). Similarly, neither the PB1–F2 truncation that causes delayed onset of clinical symptoms in ducks (McAuley et al., 2010) nor the N66S mutation that increases the efficiency of viral replication in the brains of mice (Schmolke et al., 2011) were found in these viruses. However the Japanese H5N6 HPAIVs carried the N30D and T215A mutations in the matrix protein 1 (M1) and the P42S mutation and deletion at 80–84 in the NS1 protein; these mutations increase viral virulence in mice and chickens (Fan et al., 2009; Jiao et al., 2008; Trapp et al., 2014). No Japanese H5N6 strains possessed the S31N mutation in their M2 protein that confers resistance to M2 channel inhibitors (Hay et al., 1985; Pinto et al., 1992), such as amantadine and rimantadine.

2.3. Origins of the Japanese H5N6 HPAIVs

Maximum-likelihood (ML) and maximum clade credibility (MCC) phylogenetic trees of each gene revealed the phylogenetic relationship of the Japanese H5N6 HPAIVs with other H5 HPAIVs and Eurasian AIVs. The surface protein genes of the Japanese H5N6 strains belonged to the group C within clade 2.3.4.4 HPAIVs and formed a single cluster with Korean H5N6 HPAIVs (Fig. 2a and b). Their ancestors diverged from the H5N6 HPAIVs isolated in January 2016 from a duck in China (designated as the G1.1.9 genotype; (Bi et al., 2016a)) (Supplementary Figs. S1 and S2). The PB2, PB1, nucleoprotein (NP), and M genes each likewise formed a Japanese–Korean cluster and diverged from the G1.1.9 genotype (Supplementary Figs. S3–S8). A putative common ancestor of the Japanese–Korean cluster was estimated to have diverged between October and November 2015 for HA, May and October 2015 for NA, July and December 2015 for PB2, and October 2015 and January 2016 for NP (95% highest posterior density interval (HPD); Fig. 3). The estimated tMRCA for the PB1 genes was March 2013 to May 2015, which is a wider window than for the other segments derived from the G1.1.9 genotype (95% HPD; Fig. 3). Gene segments (HA, NA, PB2, PB1, and NP genes) showed 98.7–100% sequence homology at the nucleotide and amino-acid levels among the representative strains of Japanese H5N6 HPAIVs listed in Supplementary Table S1.

The M genes of the Japanese H5N6 HPAIVs were derived from the G1.1-like genotype viruses, a progenitor genotype of G1.1.9 (Supplementary Figs. S9 and S10), and the tMRCA for the M genes derived from the G1.1 genotype was June to December 2015 (95% HPD; Fig. 3). The M genes showed more than 99.2% and 97.9% homology among Japanese H5N6 HPAIVs at the nucleotide and amino-acid levels, respectively. The NS genes, except the Gifu1 group, formed a single cluster, designated as NS-I in the current study, and diverged from the G1.1.9; in contrast, the Gifu isolates, designated as NS-II, diverged from G1.1-like genotype viruses (Supplementary Figs. S11 and S12). The tMRCAs were between July 2015 and January 2016 for the NS-I genes and November 2014 and October 2015 for NS-II genes (95% HPD; Fig. 3). The homology among the nucleotide sequences of the NS genes, the NS1 amino-acid sequences, and NS2 amino-acid sequences between the NS-I and NS-II clusters was 97.2–97.6%, 94.6–95.5%, and 96.5–98.2%, respectively.

The PA genes of the Japanese H5N6 HPAIVs formed 4 distinct clusters—designated as PA-I, PA-II, P-III, and PA-IV in this study (Fig. 4). There was 91.5–94.5% nucleotide homology and 96.3–98.8% amino-acid homology among the sequences of the representative strains for each distinct PA cluster (Supplementary Table S2); the greatest differences were between PA-I and PA-IV, whereas the highest similarities were between PA-II and PA-III. The PA-I cluster, containing the Miyazaki1, Niigata1 and 2, Hokkaido1, Aomori1 and 2, Gifu1, Chiba1, and Miyagi1 groups, diverged from G1.1.9 (Supplementary Fig. S13a). The tMRCA for PA-I was estimated as August to December 2015 (95% HPD; Fig. 3). The PA-II cluster, containing the Saga1, Kumamoto1 groups, and 9 Korean H5N6 strains, was in the Eurasian avian virus group isolated in Japan, Mongolia, Russia, China, and so on (Supplementary Fig. S13b). Viruses represented in the PA-II cluster were closely related to A/duck/Mongolia/520/2015 (H1N1), which was isolated in August 2015 from a migratory bird in Mongolia, and the AIVs circulating in the Primorsky region of Russia during April 2016 and in Japan during November and December 2016. The tMRCA for PA-II was estimated as October 2014 to July 2015 (95% HPD; Fig. 3). It is noteworthy that the PA-II cluster shared a putative common ancestor with the clade 2.3.4.4 H5N8 HPAIVs isolated from the wild birds and poultry at Qinghai Lake in China, Russia, France, and elsewhere in the winter of 2016–2017. The date of divergence (tMRCA2 in Supplementary Fig. S13b) from the putative common Eurasian AIVs was estimated as May 2012 to June 2013. Miyazaki2 group strains, designated as PA-III, were the most closely related to the Mongolian AIVs that circulated in August 2015. The tMRCA for the PA-III isolates was estimated to be between August 2012 and November 2014 (95% HPD; Fig. 3). The cluster including PA-III shared a common ancestor with European clade 2.3.4.4 H5N8 HPAIVs that were different from the European H5N8 HPAIVs in the PA-II phylogeny. The date of divergence (tMRCA2 in Supplementary Fig. S13c) from the putative common Eurasian AIVs was estimated as April 2009 to September 2010. The PA-IV cluster containing the Kyoto1 group, and the Japanese AIVs A/duck/Fukui/181006/2015 (H12N5) and A/duck/Fukui/181015/2015 (H12N5) shared a common ancestor (Supplementary Fig. S13d); the tMRCA for PA-IV was estimated to be between December 2010 and August 2014 (95% HPD; Fig. 3). The cluster including PA-IV and the Fukui strains shared a common ancestor with AIVs isolated in 2005–2007 from China, Hong Kong, and Korea and located as an outgroup (Supplementary Fig. S13d). In particular, a cluster consisting of those strains was a sister branch to the AIVs isolated in Australia. Because of the long branches from the nodes of the Japanese cluster to the tMRCA for PA-III and PA-IV (Supplementary Fig. S13c and d), the windows for the PA-III and PA-IV tMRCAs were much wider than those for PA-I and PA-II (Fig. 3).

Table 1
Comparison of the molecular characteristics of the clade 2.3.4.4 H5N6 viruses in Japan.

Protein	Phenotype/Function	Mutation[a]	Representative strains of each group												
			Aomori/1-1T	Aomori/2-1C	Niigata/1-1T	Niigata/2-5C	Hokkaido/1-3-7T	Miyazaki/1-4C	Miyagi/1-5T	Chiba/1-1T	Gifu/1-1T	Kumamoto/1-2C	Saga/1-1C	Kyoto/2T	Miyazaki/2-1C
	Connecting peptide		RERRRKR	RERRRKR	RERRRKR	RERRRKR	RERRRKR	RERRRKR	RERRRKR	RERRRKR	RERRRKR	RERRRKR	KERRRKR	RERRRKR	RERRRKR
HA	Receptor-binding specificity (Increased a2,6 SA preference)	129	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion
		A138S	A	A	A	A	A	A	A	A	A	A	A	A	A
		I151T	T	T	T	T	T	T	T	T	T	T	T	T	T
		T160A	A	A	A	A	A	A	A	A	A	A	A	A	A
		N186K	N	N	N	N	N	N	N	N	N	N	N	N	N
		N224K	N	N	N	N	N	N	N	N	N	N	N	N	N
		Q226L	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
		G228S	G	G	G	G	G	G	G	G	G	G	G	G	G
	Potential glycosylation sites	20–22	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
		33–35	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
		169–171	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
		197–199	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
		240–242	No	No	No	No	No	No	No	No	No	No	Yes	No	No
		289–291	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
NA	Stalk	58–68	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion
	Drug resistance	H274Y	H	H	H	H	H	H	H	H	H	H	H	H	H
PB2	Mammalian adaptation	T271A	T	T	T	T	T	T	T	T	T	T	T	T	T
		G590S	G	G	G	G	G	G	G	G	G	G	G	G	G
		Q591R	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
		E627K	E	E	E	E	E	E	E	E	E	E	E	E	E
		D701N	D	D	D	D	D	D	D	D	D	D	D	D	D
PB1-F2	Enhancement of virus-induced cell death	Length	90aa	90aa	90aa	90aa	90aa	90aa	90aa	90aa	90aa	90aa	90aa	90aa	90aa
		N66S	N	N	N	N	N	N	N	N	N	N	N	N	N
M1	Increased virulence in mice	N30D	D	D	D	D	D	D	D	D	D	D	D	D	D
		T215A	A	A	A	A	A	A	A	A	A	A	A	A	A
M2	Drug resistance	L26F	L	L	L	L	L	L	L	L	L	L	L	L	L
		V27IorA	V	V	V	V	V	V	V	V	V	V	V	V	V
		A30T	A	A	A	A	A	A	A	A	A	A	A	A	A
		S31AorN	S	S	S	S	S	S	S	S	S	S	S	S	S
		G34E	G	G	G	G	G	G	G	G	G	G	G	G	G
		F38L	L	L	L	L	L	L	L	L	L	L	L	L	L
		D44N	D	D	D	D	D	D	D	D	D	D	D	D	D
NS1	Increased virulence in mice	P42S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Increased virulence in chickens and mice	80–84	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion	deletion

[a] H3N2 numbering for HA and NA proteins.

Fig. 2. Part of the clade 2.3.4.4 H5 HA (a) and N6 NA (b) maximum-likelihood phylogenetic trees. The color of the branch denotes the virus subtype or country of isolation: red, Japanese and Korean H5N6 HPAIVs analyzed in this study; green, Chinese H5N6 viruses including Vietnamese and Laotian strains; blue, H5N8 HPAIVs; pink, other H5Nx HPAIVs; black, HxN6 AIVs.

Taken together, our analyses confirmed that 5 distinct genotypes among the Japanese H5N6 HPAIVs isolated during 2016–2017 were generated through the multiple reassortments among at least three potential progenitors (one G1.1.9- and two G1.1-like H5N6 HPAIVs) and three distinct Eurasian AIVs (Table 2 and Fig. 5). According to the previous study (Lee et al., 2017b), the genotypes in Japan were classified into groups C1, C2, C4, and C5. In the current study, we identified a novel genotype, C6, which included the Miyazaki2 strains. This C6 genotype carries PA-III and NS-I and has occurred only in chickens in Japan, whereas genotype C3, which contains PA-II and NS-II, has been obtained only in Korea so far (Lee et al., 2017b). Among 12 outbreaks in poultry farms, 7 poultry farms in northern Japan and a poultry farm (Miyazaki1) in southern Japan were affected by isolates of the C2 genotype (Fig. 1). Single introduction of C1 and C6 genotypes were found in the Gifu1 and Miyazaki2 farms, respectively. Both outbreaks in the Saga1 and Kumamoto1 farms in southern Japan were affected by the C4 genotype. According to the estimated tMRCAs, genotype C2, which was a reassortant between G1.1.9 and G1.1 genotypes, arose between late 2015 and early 2016. The long branches at the nodes of the NS-II, PA-II, PA-III, and PA-IV clusters preclude estimation of the divergence periods for C3, C4, C5, and C6.

2.4. Analysis of location-annotated PA trees

Because MCC phylogenetic analyses of the PA-II, -III, and -IV trees include the AIVs isolated from various countries or regions, we constructed location-annotated MCC trees for each PA cluster by using the 100 most genetically related strains to each Japanese PA cluster followed by visualization on a geographic map, to compare the spatial relationship between each PA cluster and its precursors (Fig. 6a–e). The precursors of the PA-I cluster had circulated in mainland China, Korea, and Japan (Fig. 6a). Although a direct connection between China and Japan–Korea was present, the Chinese strains of the G1.1.9 genotype that are the immediate precursors of Japanese–Korean strains were isolated 10 months before the outbreaks in Japan and Korea.

The PA-II map revealed many connections within countries or regions in Asia or Europe as well as across Europe and Asia (Fig. 6b). In particular, several connections between Mongolia and Japan indicated

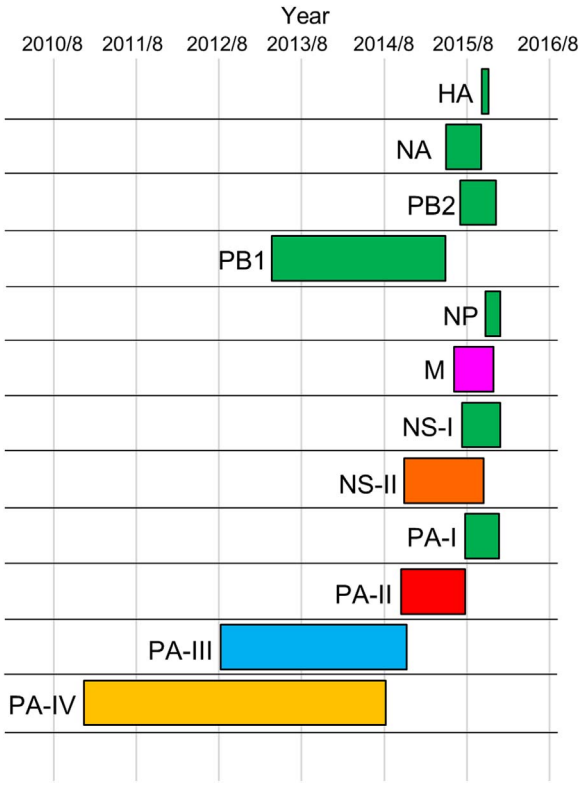

Fig. 3. The time to the most recent common ancestor (tMRCA) of each gene cluster or lineage found in the Japanese H5N6 HPAIVs. The tMRCA was estimated from the node height of the 95% highest posterior density interval. The colors of each segment denote the various origins: green, Chinese G1.1.9 genotype; pink and orange, Chinese G1.1-like genotypes; other colors, low-pathogenicity avian influenza lineages.

a close genetic relationship between Japanese and the Mongolian AIVs. In addition, the direct connection between Italian isolates and those from the Kamchatka peninsula of Russia arose due to the isolation of the group B H5N8 HPAIVs in both countries during 2016–2017 (Supplementary Fig. S13b).

The PA-III map showed 2 sets of branch clusters, that is, in Europe or the Near and Middle East regions and the Eastern Asian region (Fig. 6c). In addition, branch clusters in each region were connected, albeit with decreased genetic relatedness. The branches between Mongolia and Japan indicated the close genetic relationship between the PA-III Japanese HPAIVs and the Mongolian AIVs. The connection between Japanese isolates and isolates from the Kamchatka peninsula of Russia was established between Japanese AIVs isolated from migratory birds in 2015–2016, such as A/duck/Kyoto/261007/2015(H6N1) isolated in October 2015, and the Kamchaktkan AIVs isolated in May 2016, such as A/environment/Kamchatka/24/2016(H13N8) (Supplementary Fig. S13c). Nodes from Italy revealed the expansion of the clade 2.3.4.4 H5N8 HPAIVs in European countries in 2016–2017 (Supplementary Fig. S13c).

The map of the PA-IV tree revealed branches among China, Korea, and Japan as well as those between Japan and Mongolia or Russia (Fig. 6d). In addition, the cluster containing AIVs of wild birds from Egypt, Japan, and Sweden (Supplementary Fig. S13d) yielded connections between Egypt and Asia and between Egypt and Europe.

3. Discussion

Our results show that the clade 2.3.4.4 H5N6 HPAIVs that spread to Japan in 2016–2017 originated due to reassortment with co-circulating HPAIVs and AIVs (Fig. 5). It is noteworthy that the PA genes of the Japanese H5N6 HPAIVs arose from 3 distinct origins in AIVs circulating in wild birds. Reassortment events among Asian-lineage H5 HPAIVs with wild bird AIVs have been reported previously for other H5 HPAIV clades (Chen et al., 2009; Xiong et al., 2013). Our previous study showed that the PA genes of the clade 2.3.2.1 H5N1 HPAIVs that affected poultry and wild birds in Japan in 2010–2011 were closely related to Eurasian wild-bird isolates (Uchida et al., 2012). A recent analysis of 579 clade 2.3.4.4 H5N6 HPAIVs isolated from poultry, mammalian species such as pigs and human, and the environment in China revealed that 41%, 4.5%, and 2.6% of these viruses had likely acquired their PB2, PB1, and PA genes, respectively, from wild-bird AIVs (Bi et al., 2016a). In addition, novel-reassortant clade 2.3.4.4 H5Nx HPAIVs that have acquired one or more internal gene segments from AIVs in wild birds have been reported frequently worldwide since the emergence of Korean H5N8 HPAIVs in 2014 (Ip et al., 2015; Lee et al., 2016; Li et al., 2017; Nagarajan et al., 2017; Torchetti et al., 2015). How migratory birds contribute to the maintenance of Asian-lineage H5 HPAIVs is unknown, but although the habitats of wild birds and poultry are generally distinct, exchanges of AIV genome segments between wild birds and poultry populations undoubtedly have occurred (Chen et al., 2016; Uchida et al., 2012). Chen et al. (Chen et al., 2016) emphasize the importance of live poultry markets as focal points for the exchange of gene segments between wild birds and poultry in China. Thus, wild-bird populations have accelerated not only the genetic diversity of the recent Asian-lineage H5 HPAIVs but also may play an important role in their maintenance.

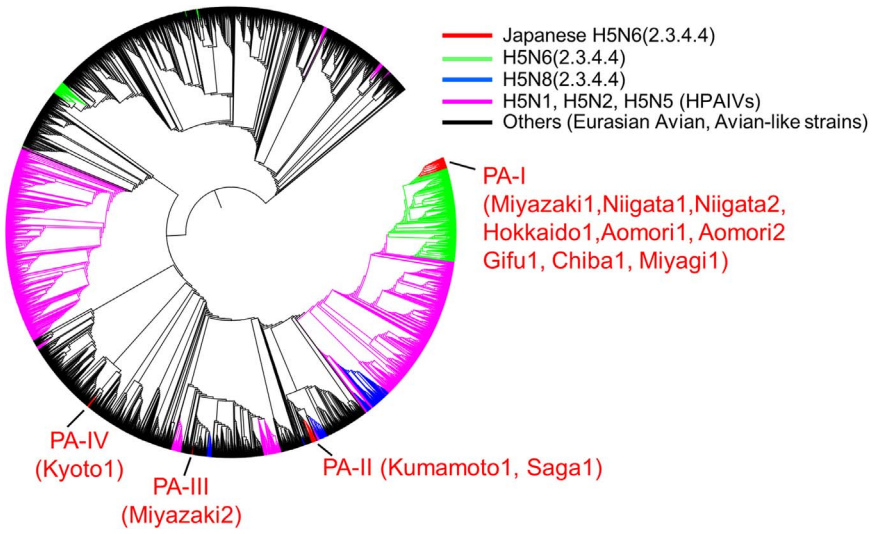

Fig. 4. Part of the maximum-likelihood phylogenetic tree of the PA genes that includes the Asian-lineage H5 HPAIVs and Eurasian avian and avian-like viruses. The color of the strain denotes the virus lineage: red, the 4 distinct PA clusters (PA-I to PA-IV) of the Japanese H5N6 HPAIVs analyzed in this study; green, H5N6 HPAIVs (clade 2.3.4.4); blue, H5N8 HPAIVs (clade 2.3.4.4); pink, other H5 HPAIVs; black, non-highly pathogenic Eurasian avian and avian-like viruses.

Table 2
Distinct genotypes of clade 2.3.4.4 H5N6 found in Japan and Korea in 2016–2017.

Genotype[a]	Genetic Origins of each gene segment								Group ID in Japan	Representative H5N6 strains isolated in South Korea
	HA	NA	PB2	PB1	PA	NP	M	NS		
C1	G1.1.9	G1.1.9	G1.1.9	G1.1.9	G1.1.9 (PA-I)	G1.1.9	G1.1	G1.1 (NS-II)	Gifu1	A/mandarin duck/Korea/T102-1/2016 (Lee et al.,2017b)
C2	G1.1.9	G1.1.9	G1.1.9	G1.1.9	G1.1.9 (PA-I)	G1.1.9	G1.1	G1.1.9 (NS-I)	Miyazaki1, Niigata1 and 2, Aomori1 and 2, Hokkaido1, Chiba1, Miyagi1	A/mandarin duck/Korea/K16-187-3/2016 (Kwon et al., 2017)
										A/duck/Korea/H15/2016 (Lee et al.,2017b)
C3	G1.1.9	G1.1.9	G1.1.9	G1.1.9	AIV (PA-II)	G1.1.9	G1.1	G1.1 (NS-II)	Not detected	A/chicken/Korea/HN1/2016 (Lee et al.,2017b)
C4	G1.1.9	G1.1.9	G1.1.9	G1.1.9	AIV (PA-II)	G1.1.9	G1.1	G1.1.9 (NS-I)	Kumamoto1, Saga1	A/duck/Korea/ES2/2016 (Lee et al.,2017b)
										A/environment/Korea/W544/2016 (Si et al., 2017)
										A/whooper swan/Korea/Gangjin_48/2016 (Jeong et al., 2017)
C5	G1.1.9	G1.1.9	G1.1.9	G1.1.9	AIV (PA-IV)	G1.1.9	G1.1	G1.1.9 (NS-I)	Kyoto1	A/whooper swan/Korea/Gangjin_49_1/2016 (Jeong et al., 2017)
										A/chicken/Korea/H23/2016 (Lee et al.,2017b)
C6	G1.1.9	G1.1.9	G1.1.9	G1.1.9	AIV (PA-III)	G1.1.9	G1.1	G1.1.9 (NS-I)	Miyazaki2	Not detected

[a] The categories of C1 through C5 are based on Lee et al. (2017b); C6 is newly designated in the current study.

The intracontinental connections apparent in the phylogenies of the AIV-derived PA genes of the Japanese H5N6 HPAIVs suggest the important contribution of the migration of wild birds to the movement of HPAIVs. The role of migratory birds in the dissemination of HPAIVs became clear in 2005, when clade 2.2 H5 HPAIVs spread from Qinghai Lake in China to European countries and the African continent (Chen et al., 2005; Liu et al., 2005). Qinghai Lake is a breeding site for migratory birds wintering in Southeast Asia and the wintering site for migratory birds that breed in Siberia; in other words, it is the intersection of different flyways (Lei et al., 2007). Moreover, recent evidence has shown that the intercontinental spread of clade 2.3.4.4 H5N8 HPAIVs from Asia to Europe and North America in 2014–2015 can be attributed to migratory birds (Global Consortium for H5N8 and Related Influenza Viruses, 2016; Lee et al., 2015). Japan is a wintering site for various species of migratory birds, including mallard ducks (*Anas platyrhynchos*) and hooded cranes (*Grus monacha*). Indeed, Eurasian lineage AIVs were frequently isolated from these species during the wintering season in Japan (Abao et al., 2013; Hiono et al., 2015; Okuya et al., 2015), suggesting the strong possibility that the H5N6 HPAIVs analyzed in the current study were carried into Japan by their hosts from breeding sites during their winter migration. The tMRCA we analyzed suggest that potential progenitors, such as genotype C2, which is derived from the G1.1.9 and G1.1 genotypes, had emerged before early 2016. In addition, each AIV-derived PA cluster was closely related to the Eurasian AIVs isolated in the summer or fall of 2015, whereas the H5N6 outbreaks in Japan began in Fall 2016. In light of the evolutional pathway of the Japanese–Korean H5N6 HPAIVs, the progenitor viruses might have been transported by migratory birds from China to their breeding sites in Spring 2016 and then travelled to both Korea and Japan in Fall 2016, when their hosts flew through the East Asian flyway for wintering. Although the live poultry trade is considered to be a risk factor for the wide geographic dissemination of Asian-lineage H5 HPAIVs (Claes et al., 2016), Japan bans the import of live poultry and poultry products from countries and areas that are affected by HPAI, including China and South Korea [http://www.maff.go.jp/aqs/english/news/pdf/EN_Poultry.pdf]. Therefore, it is less likely that a progenitor of Japanese H5N6 HPAIVs was introduced into Japan through the poultry trade.

Considering that the genetic flow of the PA gene might be related to the transport pathways of AIVs and HPAIVs by migratory wild birds, we overlaid the flyways that cover the Eurasian continent on the PA phylogenetic maps (Fig. 6e), revealing many phylogenetic connections across various migratory flyways. It is well documented that several flyways, such as the East Atlantic, East Asia–Australasian, West Asia-East Africa, Central Asia, and Black Sea-Mediterranean flyways, overlap in Siberia, where various migrating species breed during the summer months (Boere and Stroud, 2006; Dalby and Iqbal, 2015; Gaidet et al., 2010; Russell, 2016). For example, at least 228 migratory bird species breed in Sakha, in northeastern Siberia (Vladimirtseva and Germogenov, 2013). However, information about the AIVs circulating in Siberia and, in particular, the Russian Far East is limited compared with that for other countries or regions where HPAI outbreaks have been reported. Such limited information regarding AIVs in Siberia may explain why the genetic flow of the AIVs does not follow a flyway but rather moves directly across flyways. To evaluate this possibility, we analyzed the dispersal pattern of the H5 genes of clade 2.3.4.4 H5Nx viruses from 2014 by using representative strains in each country or region downloaded from GenBank together with the sequences we obtained in this study (Supplementary Fig. S14). The H5 gene map shows a similar pattern to that of the AIV-derived PA genes, except for a connection line from Sakha Republic of Russia to Europe. This line was established due to the isolation of a clade 2.3.4.4 HPAIV, A/wigeon/Sakha/1/2014 (H5N8), from a wild duck in September 2014 prior to the outbreaks of H5N8 HPAIVs in Europe, which started in November 2014. These results emphasize the importance of continuous surveillance in Siberia, and in particular, the Russian Far East.

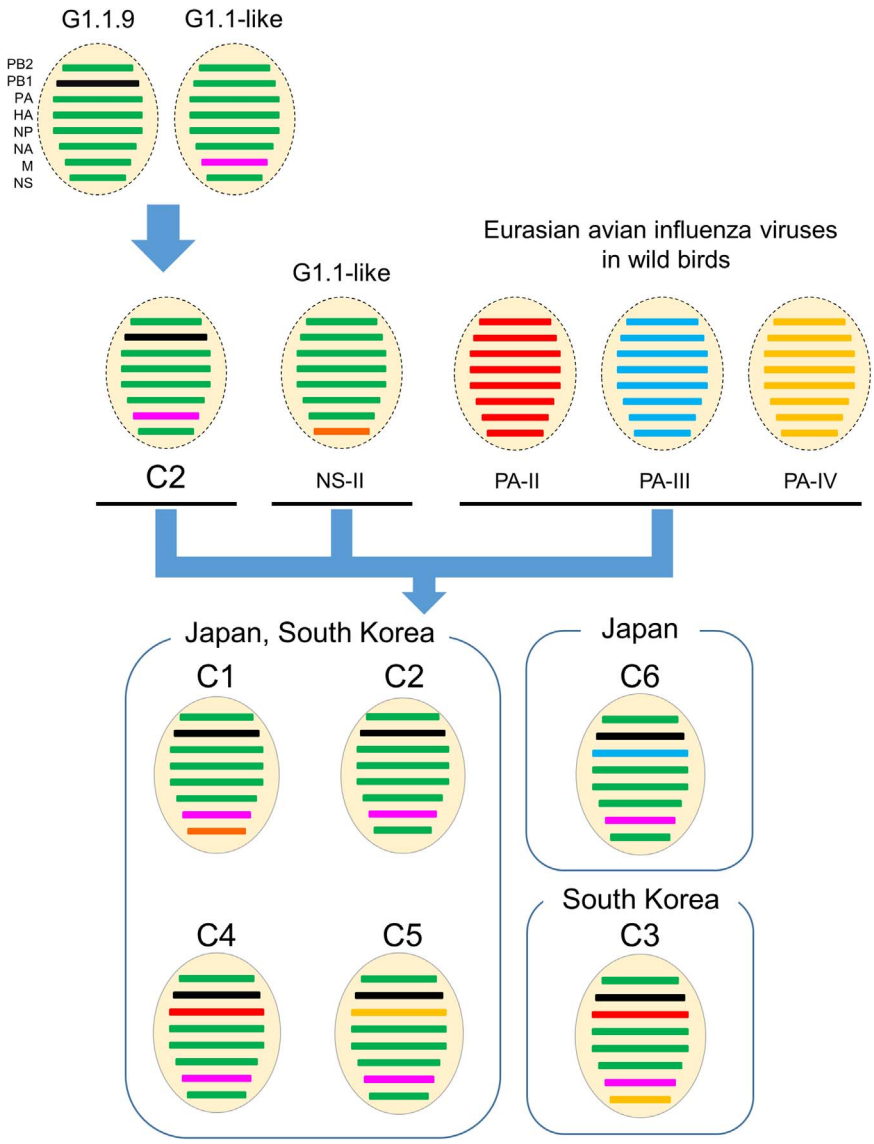

Fig. 5. Schematic gene flow of the Japanese H5N6 HPAIVs. The eight gene segments (PB2, PB1, PA, HA, NP, NA, M, and NS) are indicated by horizontal bars, from the top to the bottom of the virion. The colors of gene segments denote the genetic origins: green and black, Chinese G1.1.9 genotypes; pink and orange, Chinese G1.1-like genotypes; other colors, Eurasian avian influenza lineages.

From May 2014 through December 2016, 17 human cases of clade 2.3.4.4 H5N6 HPAIV infection were reported in China (Jiang et al., 2017). Our analysis indicated that amino acid substitutions responsible for adaptation to mammalian hosts (Bussey et al., 2010; Hatta et al., 2001; Min et al., 2013; Steel et al., 2009; Yamada et al., 2010) were missing from the Japanese H5N6 isolates. The T160A mutation in the HA protein is linked to the acquisition of binding specificity for α2,6-linked sialic acid receptors, which are predominant in the human upper respiratory tract (Gu et al., 2017; Wang et al., 2010); however, some clade 2.3.4.4 H5Nx HPAIVs that carry the T160A mutation show limited binding specificity for α2,6-linked receptors (Guo et al., 2017; Kaplan et al., 2016). Additional studies are needed to definitively understand the effect of T160A on the receptor specificity of clade 2.3.4.4 H5N6 HPAIVs. In addition, to our knowledge, no human cases resulting from a G1.1.9 H5N6 HPAIV that is a potential progenitor of Japanese H5N6 HPAIVs have been reported. Thus, the zoonotic potential of Japanese H5N6 HPAIVs is considered to be low. In contrast, H5N6 HPAIVs of genotypes G1, G1.1, G1.2, G2, and two novel G1.1-derived isolates have caused human infections in China so far (Bi et al., 2016a; Fang et al., 2016; Shen et al., 2016; Zhang et al., 2017). Apart from the G2 genotypes, the HA and NA genes of the human isolates belong to the major H5N6 lineage, as do Japanese H5N6 HPAIVs. However, several differences in genetic traits, such as the combination or origins of internal gene segments, emerged when the Japanese H5N6 HPAIVs and those that caused human infections in China were compared. For example, six internal genes of G1.2, the most frequently detected genotype among human cases (10 of 17) were derived from the poultry H7N9/H9N2 gene pool (Bi et al., 2016a). The two novel G1.1-derived isolates, A/Hunan/55555/2016 (H5N6) and A/Guangxi/55726/2016 (H5N6), were isolated during the same period as the H5N6 outbreaks in Japan; the former possessed the Eurasian AIV-derived PB1 gene and the G1.1-derived internal genes (PB2, PA, NP, M, and NS genes), and the latter carried the Eurasian AIV-derived PB1 and PA genes and the G1.1-derived internal genes (PB2, NP, M, and NS genes) (Zhang et al., 2017). Note that 11 of the 17 human H5N6 viruses carried mutations for adaptation to mammalian hosts, such as E627K and D701N in their PB2 gene (Bi et al., 2016a; Shen et al., 2016). The WHO currently considers the potential for international spread of these viruses to be low (http://www.who.int/). However, the ongoing increased prevalence and genetic diversity of H5N6 HPAIVs in China (Bi et al., 2016a; Du et al., 2017; Yang et al., 2016) may increase the opportunity for human infection with H5N6 isolates, as occurred with H5N1 HPAIVs.

In conclusion, we phylogenetically analyzed 206 AIVs, including clade 2.3.4.4 H5 HPAIVs and AIVs isolated in Japan and Russia. Our results show that at least 5 distinct genotypes of H5N6 HPAIVs simultaneously affected poultry and wild birds in Japan during the

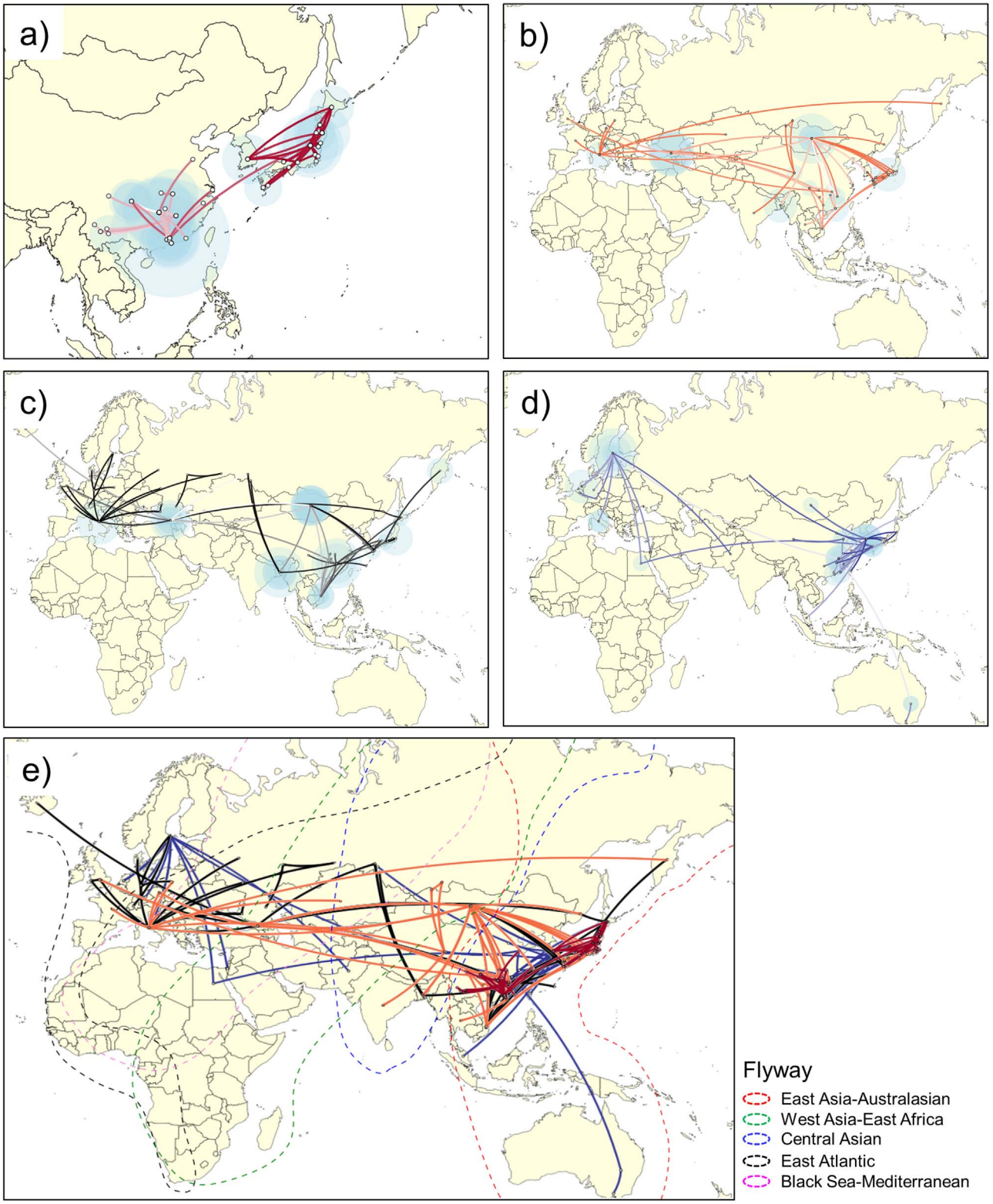

Fig. 6. Visualization of the location-annotated maximum clade credibility trees including the PA-I (a), PA-II (b), PA-III (c), and PA-IV (d) clusters on the world map, and their merged map (e). The lines between locations represent branches on the trees, and the color gradient of the lines indicates the height of the strains (i.e., dark red in Fig. 6a means a closer genetic relationship). The radius of the circle in blue is proportional to the number of tree lineages maintained in that location, and the color gradient of the circles indicates the transitions of the occurrences. Flyways are based on a previous report (Boere and Stroud, 2006).
The world map was generated using by SPreaD3 version 0.9.6 as licensed under the GNU Lesser GPL, and its source code is freely available from its repository: https://github.com/phylogeography/SPreaD3 (Bielejec et al., 2016).

winter of 2016–2017. Japanese H5N6 HPAIVs were generated from an ancestral H5N6 virus, presumably an H5N6 HPAIV that circulated in China, through several reassortment events with other H5N6 HPAIVs or 3 different lineages of AIVs (or both). The existence of 3 distinct AIV-derived PA genes in H5N6 HPAIVs highlights the importance of AIV and HPAIV surveillance to understand where and how novel HPAIVs that

pose a substantial economic burden to the poultry industry are generated and dispersed in wild birds and domestic poultry.

4. Materials and methods

4.1. Viruses and virus isolation

Tracheal or cloacal swabs (or both) collected in November 2016 through March 2017 from chickens and Muscovy ducks with suspected HPAIV infections were inoculated into embryonated chicken eggs for virus isolation at the diagnostic laboratories of the municipal livestock health centers that were managing each outbreak. Allantoic fluid that showed HA activity against chicken red blood cells was submitted to the National Institute of Animal Health of Japan for diagnosis. On December 22, 2016, the deaths of 8 mute swans raised at a horse racetrack in Kyoto Prefecture, Japan, were reported; tracheal and cloacal swabs collected from these 8 birds also were submitted to the National Institute of Animal Health. The specimens were diluted 10-fold with transport medium (MEM containing 1000 units/ML penicillin, 1000 µg/ML streptomycin, 25 µg/ML Fungizone [Thermo Fisher Scientific, Waltham, MA, USA], 0.01 M HEPES, and 0.5% bovine serum albumin), and then 200 µl of the diluted solution was inoculated into 9- to 11-day-old embryonated chicken eggs and incubated for 24–48 h at 37 °C. If viruses could not be recovered after one passage, the allantoic fluid collected was reinoculated into eggs. HA activity was tested by using 0.55% chicken red blood cells. A total of 82 H5N6 HPAIVs isolated from the 12 poultry outbreaks in Japan as well as 7 isolates from the racetrack incident were analyzed in this study (Supplementary Table S3).

In addition to the Japanese H5N6 HPAIVs, 15 Russian AIVs were included in this study. The Russian viruses (listed in Supplementary Table S3) were isolated in embryonated eggs from cloacal swabs or the internal organs of hunted–harvested birds or from dead birds at the Research Institute of Experimental and Clinical Medicine, Novosibirsk.

Furthermore, 79 Japanese AIVs were isolated from fecal samples from migratory ducks or wild birds that were collected as part of the periodic monitoring activities of the Ministry of the Environment of Japan during the winter migrations of 2012–2016. Virus isolation from the original specimens was conducted at the Animal Quarantine Service at the Chubu Airport Branch of the Ministry of Agriculture, Forestry, and Fisheries of Japan. The isolated viruses were then shipped to Tottori University or the National Institute of Animal Health for analysis. Another 8 Japanese AIV isolates from Ibaraki and 4 from Gunma Prefectures, obtained in 2016, were supplied to the National Institute of Animal Health by the Ibaraki Prefectural Kenpoku Livestock Hygiene Service Center and the Gunma Livestock Health Laboratory, respectively. The remaining 11 Japanese AIVs were isolated before 2012 and were obtained from the virus repository at the National Institute of Animal Health. Further information regarding the viruses analyzed in this study can be found in Supplementary Table S3.

4.2. Pathogenicity in chickens

Four Japanese H5N6 isolates—A/Muscovy duck/Aomori/1-3T/2016 (H5N6) (Aomori1-3T), A/chicken/Niigata/1-1T/2016 (H5N6) (Niigata1-1T), A/chicken/Kumamoto/1-2C/2016 (H5N6) (Kumamoto1-2C), and A/chicken/Miyazaki/2-2C/2017 (H5N6) (Miyazaki2-2C)—were used for experimental infections. According to the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2016* (World Organisation for Animal Health, 2016), we intravenously inoculated eight 4- to 8-week-old specific-pathogen-free White Leghorn chickens (Nissei-Bio, Yamanashi, Japan) per virus group with 200 ul of a 1:10 dilution of infectious allantoic fluid. All of the infected chickens were monitored daily for clinical signs of disease. Animal experiments were conducted in the Biosafety Level 3 facilities at the National Institute of Animal Health, Japan, and were performed in accordance with the Guidelines for Animal Experiments of the National Institute of Animal Health, NARO, Japan. In addition, the protocols for the animal experiments were approved by the Animal Care and Use Committee of the National Institute of Animal Health, NARO, Japan.

4.3. Whole-genome sequencing

The complete genomes of 191 Japanese strains were obtained through next-generation sequencing (Miseq, Illumina, San Diego, CA, USA). RNA was extracted from isolates by using an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA libraries for next-generation sequencing were prepared by using a NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) and sequenced by using a Miseq Reagent Kit v2 (Illumina). The consensus sequences were generated by using FLUGAS software (version 0.9.0, World Fusion, Tokyo, Japan) or Genomics Workbench software (version 9.5.3, Qiagen, Hilden, Germany) as previously described (Takemae et al., 2017). The nucleotide sequences and isolation information for the viruses analyzed were deposited in the GISAID EpiFlu database (http://www.gisaid.org); isolate ID numbers and isolation information are listed in Supplementary Table S3. The entire genomes of the Russian AIVs were sequenced at the SB RAS Genomics Core Facility (ICBFM SB RAS, Novosibirsk, Russia) and WHO National Influenza Center (Saint Petersburg, Russia) by using MiSeq (Illumina), and segment-specific sequences were obtained by using a Genomic Sequencer SOLiD 5500xl (Applied Biosystems).

4.4. Phylogenetic and phylogeographic analysis

On May 12, 2017, we downloaded all of the genomic sequences of the influenza A viruses publicly available in the GenBank and GISAID databases by using the GISAID EpiFlu database system (http://platform.gisaid.org/epi3/frontend). The numbers of sequences for each segment were: PB2 49,082; PB1 51,783; PA 51,521; H5 6296; NP 50,089; N6 2961; M 64,740; and NS 51,520. Sequences for each segment were aligned by using the online version of MAFFT (http://mafft.cbrc.jp/alignment/software/). Each gene segment except for H5 and N6 genes was then classified according to its phylogenetically distinct lineage, such as seasonal human and avian lineages; then all of the Eurasian avian viruses, including all of the Asian-lineage H5 HPAIVs, and avian-like viruses isolated from all of the hosts were collected. For the H5 and N6 genes, all of the data available from Genbank were used. The final nucleotide sequences for each segment, including the strains sequenced in this study (PB2 9659; PB1 11,313; PA 9849; H5 6017; NP 9206; N6 2590; M 10,334; and NS 11,174 sequences), then were obtained. Phylogenetic trees for each gene segment were constructed by using the ML method in RAxML software version 8.2.4 (Stamatakis, 2014) to reveal the detailed genetic relationships between Japanese H5N6 HPAIVs and other HPAIVs or AIVs.

Once the genetic relationship was clear according to each ML tree, the 200 strains that were the most genetically related to the Japanese H5N6 HPAIVs (Aomori1-1T for the H5, NA, PB2, PB1, NP, M, PA-I, and NS-I trees; Kumamoto/1-2C for the PA-II tree; Kyoto/2T for the PA-III tree; and Miyazaki2-1C for the PA-IV tree) were selected by using the online version of MAFFT (http://mafft.cbrc.jp/alignment/software/) for the construction of MCC trees for each segment. However, in the case of the NS genes, 2 distinct Japanese H5N6 clusters were revealed (see the Results section). Therefore, the number of NS gene sequences collected for the ML phylogenetic analysis was reduced by clustering together all of the NS sequences that were at least 99.6% identical according to CD-HIT software (Fu et al., 2012) before selecting the closest 200 strains to realize the NS gene analysis on one MCC phylogeny. The MCC trees for each segment were constructed according to the Bayesian Markov chain Monte Carlo method in BEAST software package version 1.8.2 (Drummond et al., 2012). JModeltest (Posada, 2008) was used to choose the most appropriate mutation models. Then the strict clock or lognormal relaxed clock model with the UPGMA or random starting

tree were applied to obtain a sufficient effective sample size (ESS). Each chain was 96 × 10^6 to 500 × 10^6 steps in length, where the number of steps was determined to obtain an ESS of more than 200 (as assessed by Tracer version 1.6 [http://beast.bio.ed.ac.uk/Tracer]), and was then sampled every 20,000 steps. The GMRF Bayesian Skyride coalescent was set as tree prior. The first 10% of the states was discarded as burn-in when the MCC trees were constructed. The time to the common ancestor was estimated from the node height of the 95% highest posterior density interval (HPD).

The location-annotated MCC trees for each PA cluster (see the Results section) were constructed according to a discrete phylogenetic model by using the BEAST software package version 1.8.2, as described earlier. The 100 strains that were the most genetically related to each Japanese PA cluster were selected from the sequences collected for the ML phylogenetic trees; but to construct the location-annotated MCC PA trees, the overlap between strains and collection sites and dates was reduced. Then each PA tree was visualized by means of spatial phylogenetic reconstruction of their evolutionary dynamics by using data-driven documents (SPreaD3) version 0.9.6 (Bielejec et al., 2016).

All of the analyses through RAxML, the BEAST software package, and SPreaD3 were conducted by using the SGI Rackable Standard Depth Server C2108-RP2 and the Large-scale Shared Memory System of AFFRIT, MAFF, Japan.

Acknowledgements

This research was supported in part by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) from the Ministry of Education, Culture, Sport, Science & Technology in Japan, and Japan Agency for Medical Research and Development (AMED); and by a research project for improving food safety and animal health of the Ministry of Agriculture, Forestry, and Fisheries of Japan.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2017.08.035.

References

Abao, L.N.B., Jamsransuren, D., Bui, V.N., Ngo, L.H., Trinh, D.Q., Yamaguchi, E., Vijaykrishna, D., Runstadler, J., Ogawa, H., Imai, K., 2013. Surveillance and characterization of avian influenza viruses from migratory water birds in eastern Hokkaido, the northern part of Japan, 2009–2010. Virus Genes 46, 323–329.

Alerstam, T., Bäckman, J., Gudmundsson, G.A., Hedenström, A., Henningsson, S.S., Karlsson, H., Rosén, M., Strandberg, R., 2007. A polar system of intercontinental bird migration. Proc. Biol. Sci. 274, 2523–2530.

Bevins, S.N., Dusek, R.J., White, C.L., Gidlewski, T., Bodenstein, B., Mansfield, K.G., DeBruyn, P., Kraege, D., Rowan, E., Gillin, C., Thomas, B., Chandler, S., Baroch, J., Schmit, B., Grady, M.J., Miller, R.S., Drew, M.L., Stopak, S., Zscheile, B., Bennett, J., Sengl, J., Brady, C., Ip, H.S., Spackman, E., Killian, M.L., Torchetti, M.K., Sleeman, J.M., Deliberto, T.J., 2016. Widespread detection of highly pathogenic H5 influenza viruses in wild birds from the Pacific Flyway of the United States. Sci. Rep. 6, 28980.

Bi, Y., Chen, Q., Wang, Q., Chen, J., Jin, T., Wong, G., Quan, C., Liu, J., Wu, J., Yin, R., Zhao, L., Li, M., Ding, Z., Zou, R., Xu, W., Li, H., Wang, H., Tian, K., Fu, G., Huang, Y., Shestopalov, A., Li, S., Xu, B., Yu, H., Luo, T., Lu, L., Xu, X., Luo, Y., Liu, Y., Shi, W., Liu, D., Gao, G.F., 2016a. Genesis, evolution and prevalence of H5N6 Avian Influenza viruses in China. Cell Host Microbe 20, 810–821.

Bi, Y., Liu, H., Xiong, C., Di, L., Shi, W., Li, M., Liu, S., Chen, J., Chen, G., Li, Y., Yang, G., Lei, Y., Xiong, Y., Lei, F., Wang, H., Chen, Q., Chen, J., Gao, G.F., 2016b. Novel avian influenza A (H5N6) viruses isolated in migratory waterfowl before the first human case reported in China, 2014. Sci. Rep. 6, 29888.

Bielejec, F., Baele, G., Vrancken, B., Suchard, M.A., Rambaut, A., Lemey, P., 2016. SpreaD3: interactive visualization of spatiotemporal history and trait evolutionary processes. Mol. Biol. Evol. 33, 2167–2169.

Boere, G.C., Stroud, D.A., 2006. The flyway concept: what it is and what it isn't. In: Boere, G.C., Galbraith, C.A., Stroud, D.A. (Eds.), Waterbirds Around the World. The Stationery Office, Edinburgh, UK, pp. 40–47.

Bouwstra, R.J., Koch, G., Heutink, R., Harders, F., van der Spek, A., Elbers, A.R., Bossers, A., 2015. Phylogenetic analysis of highly pathogenic avian influenza A(H5N8) virus outbreak strains provides evidence for four separate introductions and one between-poultry farm transmission in the Netherlands, November 2014. Eur. Surveill. 20 (pii: 21174).

Bussey, K.A., Bousse, T.L., Desmet, E.A., Kim, B., Takimoto, T., 2010. PB2 residue 271 plays a key role in enhanced polymerase activity of Influenza A Viruses in mammalian host cells. J. Virol. 84, 4395–4406.

Cattoli, G., Fusaro, A., Monne, I., Capua, I., 2009. H5N1 virus evolution in Europe—an updated overview. Viruses 1, 1351–1363.

Chen, H., Smith, G.J.D., Zhang, S.Y., Qin, K., Wang, J., Li, K.S., Webster, R.G., Peiris, J.S.M., Guan, Y., 2005. Avian flu H5N1 virus outbreak in migratory waterfowl. Nature 436, 191–192.

Chen, J., Yang, Z., Chen, Q., Liu, X., Fang, F., Chang, H., Li, D., Chen, Z., 2009. Characterization of H5N1 influenza A viruses isolated from domestic green-winged teal. Virus Genes 38, 66–73.

Chen, L.-J., Lin, X.-D., Guo, W.-P., Tian, J.-H., Wang, W., Ying, X.-H., Wang, M.-R., Yu, B., Yang, Z.-Q., Shi, M., Holmes, E.C., Zhang, Y.-Z., 2016. Diversity and evolution of avian influenza viruses in live poultry markets, free-range poultry and wild wetland birds in China. J. Gen. Virol. 97, 844–854.

Claes, F., Morzaria, S.P., Donis, R.O., 2016. Emergence and dissemination of clade 2.3.4.4 H5Nx influenza viruses-how is the Asian HPAI H5 lineage maintained. Curr. Opin. Virol. 16, 158–163.

Dalby, A.R., Iqbal, M., 2015. The European and Japanese outbreaks of H5N8 derive from a single source population providing evidence for the dispersal along the long distance bird migratory flyways. PeerJ 3, e934.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29, 1969–1973.

Du, Y., Chen, M., Yang, J., Jia, Y., Han, S., Holmes, E.C., Cui, J., 2017. Molecular evolution and emergence of H5N6 Avian Influenza Virus in Central China. J. Virol. 91 (pii: e00143-00117).

Fan, S., Deng, G., Song, J., Tian, G., Suo, Y., Jiang, Y., Guan, Y., Bu, Z., Kawaoka, Y., Chen, H., 2009. Two amino acid residues in the matrix protein M1 contribute to the virulence difference of H5N1 avian influenza viruses in mice. Virology 384, 28–32.

Fang, S., Bai, T., Yang, L., Wang, X., Peng, B., Liu, H., Geng, Y., Zhang, R., Ma, H., Zhu, W., Wang, D., Cheng, J., Shu, Y., 2016. Sustained live poultry market surveillance contributes to early warnings for human infection with avian influenza viruses. Emerg. Microbes Infect. 5, e79.

Fu, L., Niu, B., Zhu, Z., Wu, S., Li, W., 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics 28, 3150–3152.

Gaidet, N., Cappelle, J., Takekawa, J.Y., Prosser, D.J., Iverson, S.A., Douglas, D.C., Perry, W.M., Mundkur, T., Newman, S.H., 2010. Potential spread of highly pathogenic avian influenza H5N1 by wildfowl: dispersal ranges and rates determined from large-scale satellite telemetry. J. Appl. Ecol. 47, 1147–1157.

Global Consortium for H5N8 and Related Influenza Viruses, 2016. Role for migratory wild birds in the global spread of avian influenza H5N8. Science 354, 213–217.

Gu, M., Liu, W., Cao, Y., Peng, D., Wang, X., Wan, H., Zhao, G., Xu, Q., Zhang, W., Song, Q., Li, Y., Liu, X., 2011. Novel reassortant highly pathogenic Avian Influenza (H5N5) viruses in domestic ducks, China. Emerg. Infect. Dis. 17, 1060–1063.

Gu, M., Li, Q., Gao, R., He, D., Xu, Y., Xu, H., Xu, L., Wang, X., Hu, J., Liu, X., Hu, S., Peng, D., Jiao, X., Liu, X., 2017. The T160A hemagglutinin substitution affects not only receptor binding property but also transmissibility of H5N1 clade 2.3.4 avian influenza virus in guinea pigs. Vet. Res. 48, 7.

Guo, H., de Vries, E., McBride, R., Dekkers, J., Peng, W., Bouwman, K.M., Nycholat, C., Verheije, M.H., Paulson, J.C., van Kuppeveld, F.J., de Haan, C.A., 2017. Highly Pathogenic Influenza A(H5Nx) Viruses with Altered H5 Receptor-Binding Specificity. Emerg. Infect. Dis. 23, 220–231.

Hanna, A., Banks, J., Marston, D.A., Ellis, R.J., Brookes, S.M., Brown, I.H., 2015. Genetic characterization of highly pathogenic Avian Influenza (H5N8) virus from domestic ducks, England, November 2014. Emerg. Infect. Dis. 21, 879–882.

Harfoot, R., Webby, R.J., 2017. H5 influenza, a global update. J. Microbiol. 55, 196–203.

Hatta, M., Gao, P., Halfmann, P., Kawaoka, Y., 2001. Molecular basis for high virulence of Hong Kong H5N1 Influenza A viruses. Science 293, 1840–1842.

Hay, A.J., Wolstenholme, A.J., Skehel, J.J., Smith, M.H., 1985. The molecular basis of the specific anti-influenza action of amantadine. EMBO J. 4, 3021–3024.

Hiono, T., Ohkawara, A., Ogasawara, K., Okamatsu, M., Tamura, T., Chu, D.-H., Suzuki, M., Kuribayashi, S., Shichinohe, S., Takada, A., Ogawa, H., Yoshida, R., Miyamoto, H., Nao, N., Furuyama, W., Maruyama, J., Eguchi, N., Ulziibat, G., Enkhbold, B., Shatar, M., Jargalsaikhan, T., Byambadorj, S., Damdinjav, B., Sakoda, Y., Kida, H., 2015. Genetic and antigenic characterization of H5 and H7 Influenza viruses isolated from migratory water birds in Hokkaido, Japan and Mongolia from 2010 to 2014. Virus Genes 51, 57–68.

Ilyushina, N.A., Seiler, J.P., Rehg, J.E., Webster, R.G., Govorkova, E.A., 2010. Effect of neuraminidase inhibitor–resistant mutations on pathogenicity of Clade 2.2 A/Turkey/15/06 (H5N1) Influenza Virus in Ferrets. PLoS Pathog. 6, e1000933.

Imai, M., Watanabe, T., Hatta, M., Das, S.C., Ozawa, M., Shinya, K., Zhong, G., Hanson, A., Katsura, H., Watanabe, S., Li, C., Kawakami, E., Yamada, S., Kiso, M., Suzuki, Y., Maher, E.A., Neumann, G., Kawaoka, Y., 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature 486, 420–428.

Ip, H.S., Torchetti, M.K., Crespo, R., Kohrs, P., DeBruyn, P., Mansfield, K.G., Baszler, T., Badcoe, L., Bodenstein, B., Shearn-Bochsler, V., Killian, M.L., Pedersen, J.C., Hines, N., Gidlewski, T., DeLiberto, T., Sleeman, J.M., 2015. Novel Eurasian highly pathogenic Avian Influenza A H5 viruses in wild birds, Washington, USA, 2014. Emerg. Infect. Dis. 21, 886–890.

Jeong, J., Woo, C., Ip, H.S., An, I., Kim, Y., Lee, K., Jo, S.-D., Son, K., Lee, S., Oem, J.-K., Wang, S.-J., Kim, Y., Shin, J., Sleeman, J., Jheong, W., 2017. Identification of two novel reassortant Avian Influenza A (H5N6) viruses in whooper swans in Korea, 2016. Virol. J. 14, 60.

Jiang, H., Wu, P., Uyeki, T., He, J., Deng, Z., Xu, W., Lv, Q., Zhang, J., Wu, Y., Tsang, T., Kang, M., Zheng, J., Wang, L., Yang, B., Qin, Y., Feng, L., Fang, V., Gao, G., Leung, G.,

Yu, H., Cowling, B., 2017. Preliminary epidemiologic assessment of human infections with highly pathogenic avian influenza A(H5N6) virus, China. Clin. Infect. Dis. 65, 383–388.
Jiao, P., Tian, G., Li, Y., Deng, G., Jiang, Y., Liu, C., Liu, W., Bu, Z., Kawaoka, Y., Chen, H., 2008. A single-amino-acid substitution in the NS1 protein changes the pathogenicity of H5N1 Avian Influenza Viruses in mice. J. Virol. 82, 1146–1154.
Kanehira, K., Uchida, Y., Takemae, N., Hikono, H., Tsunekuni, R., Saito, T., 2015. Characterization of an H5N8 influenza A virus isolated from chickens during an outbreak of severe avian influenza in Japan in April 2014. Arch. Virol. 160, 1629–1643.
Kaplan, B.S., Russier, M., Jeevan, T., Marathe, B., Govorkova, E.A., Russell, C.J., Kim-Torchetti, M., Choi, Y.K., Brown, I., Saito, T., Stallknecht, D.E., Krauss, S., Webby, R. J., 2016. Novel Highly Pathogenic Avian A(H5N2) and A(H5N8) Influenza Viruses of Clade 2.3.4.4 from North America Have Limited Capacity for Replication and Transmission in Mammals. mSphere 1, pii: e00003-00016.
Kwon, J.H., Lee, D.H., Swayne, D.E., Noh, J.Y., Yuk, S.S., Erdene-Ochir, T.O., Hong, W.T., Jeong, J.H., Jeong, S., Gwon, G.B., Lee, S., CS, S., 2017. Reassortant clade 2.3.4.4 avian influenza A(H5N6) virus in a wild mandarin duck, South Korea, 2016. Emerg. Infect. Dis. 23, 822–826.
Lee, D.H., Torchetti, M.K., Winker, K., Ip, H.S., Song, C.S., Swayne, D.E., 2015. Intercontinental spread of Asian-Origin H5N8 to North America through Beringia by Migratory Birds. J. Virol. 89, 6521–6524.
Lee, D.H., Bahl, J., Torchetti, M.K., Killian, M.L., Ip, H.S., DeLiberto, T.J., Swayne, D.E., 2016. Highly pathogenic Avian Influenza viruses and generation of novel re-assortants, United States, 2014–2015. Emerg. Infect. Dis. 22, 1283–1285.
Lee, D.H., Sharshov, K., Swayne, D.E., Kurskaya, O., Sobolev, I., Kabilov, M., Alekseev, A., Irza, V., Shestopalov, A., 2017a. Novel reassortant Clade 2.3.4.4 Avian Influenza A(H5N8) virus in wild aquatic birds, Russia, 2016. Emerg. Infect. Dis. 23, 359–360.
Lee, E.K., Song, B.-M., Lee, Y.-N., Heo, G.-B., Bae, Y.-C., Joh, S.-J., Park, S.-C., Choi, K.-S., Lee, H.-J., Jang, I., Kang, M.-S., Jeong, O.-M., Choi, B.-K., Lee, S.-M., Jeong, S.C., Park, B.-K., Lee, H.-S., Lee, Y.-J., 2017b. Multiple novel H5N6 highly pathogenic avian influenza viruses, South Korea, 2016. Infect. Genet. Evol. 51, 21–23.
Lee, Y.J., Kang, H.M., Lee, E.K., Song, B.M., Jeong, J., Kwon, Y.K., Kim, H.R., Lee, K.J., Hong, M.S., Jang, I., Choi, K.S., Kim, J.Y., Lee, H.J., Kang, M.S., Jeong, O.M., Baek, J.H., Joo, Y.S., Park, Y.H., Lee, H.S., 2014. Novel reassortant Influenza A(H5N8) viruses, South Korea, 2014. Emerg. Infect. Dis. 20, 1087–1089.
Lei, F., Tang, S., Zhao, D., Zhang, X., Kou, Z., Li, Y., Zhang, Z., Yin, Z., Chen, S., Li, S., Zhang, D., Yan, B., Li, T., 2007. Characterization of H5N1 Influenza Viruses isolated from migratory birds in Qinghai Province of China in 2006. Avian Dis. 51, 568–572.
Li, M., Liu, H., Bi, Y., Sun, J., Wong, G., Liu, D., Li, L., Liu, J., Chen, Q., Wang, H., He, Y., Shi, W., Gao, G., Chen, J., 2017. Highly pathogenic Avian Influenza A(H5N8) virus in wild migratory birds, Qinghai Lake, China. Emerg. Infect. Dis. 23, 637–641.
Liu, C.-G., Liu, M., Liu, F., Lv, R., Liu, D.-F., Qu, L.-D., Zhang, Y., 2013. Emerging multiple reassortant H5N5 avian influenza viruses in ducks, China, 2008. Vet. Microbiol. 167, 296–306.
Liu, J., Xiao, H., Lei, F., Zhu, Q., Qin, K., Zhang, X.-w., Zhang, X.-l., Zhao, D., Wang, G., Feng, Y., Ma, J., Liu, W., Wang, J., Gao, G.F., 2005. Highly Pathogenic H5N1 Influenza Virus infection in migratory birds. Science 309, 1206.
Marchenko, V.Y., Susloparov, I.M., Komissarov, A.B., Fadeev, A., Goncharova, N.I., Shipovalov, A.V., Svyatchenko, S.V., Durymanov, A.G., Ilyicheva, T.N., Salchak, L.K., Svintitskaya, E.P., Mikheev, V.N., Ryzhikov, A.B., 2017. Reintroduction of highly pathogenic avian influenza A/H5N8 virus of clade 2.3.4.4. in Russia. Arch. Virol. 162, 1381–1385.
McAuley, J.L., Chipuk, J.E., Boyd, K.L., Van De Velde, N., Green, D.R., McCullers, J.A., 2010. PB1-F2 Proteins from H5N1 and 20th century Pandemic Influenza Viruses cause immunopathology. PLoS Pathog. 6, e1001014.
Min, J.Y., Santos, C., Fitch, A., Twaddle, A., Toyoda, Y., DePasse, J.V., Ghedin, E., Subbarao, K., 2013. Mammalian adaptation in the PB2 gene of Avian H5N1 Influenza Virus. J. Virol. 87, 10884–10888.
Nagarajan, S., Kumar, M., Murugkar, H., Tripathi, S., Shukla, S., Agarwal, S., Dubey, G., Nagi, R., Singh, V., Tosh, C., 2017. Novel reassortant highly pathogenic Avian Influenza (H5N8) virus in Zoos, India. Emerg. Infect. Dis. 23, 717–719.
Nidom, C.A., Takano, R., Yamada, S., Sakai-Tagawa, Y., Daulay, S., Aswadi, D., Suzuki, T., Suzuki, Y., Shinya, K., Iwatsuki-Horimoto, K., Muramoto, Y., Kawaoka, Y., 2010. Influenza A (H5N1) viruses from pigs, Indonesia. Emerg. Infect. Dis. 16, 1515–1523.
Okamatsu, M., Ozawa, M., Soda, K., Takakuwa, H., Haga, A., Hiono, T., Matsuu, A., Uchida, Y., Iwata, R., Matsuno, K., Kuwahara, M., Yabuta, T., Usui, T., Ito, H., Onuma, M., Sakoda, Y., Saito, T., Otsuki, K., Ito, T., Kida, H., 2017. Characterization of highly pathogenic Avian Influenza Virus A(H5N6), Japan, November 2016. Emerg. Infect. Dis. 23, 691–695.
Okuya, K., Kawabata, T., Nagano, K., Tsukiyama-Kohara, K., Kusumoto, I., Takase, K., Ozawa, M., 2015. Isolation and characterization of influenza A viruses from environmental water at an overwintering site of migratory birds in Japan. Arch. Virol. 160, 3037–3052.
Olsen, B., Munster, V., Wallensten, A., Waldenström, J., Osterhaus, A., Fouchier, R., 2016. Global patterns of influenza A virus in wild birds. Science 312, 384–388.
Ozawa, M., Matsuu, A., Tokorozaki, K., Horie, M., Masatani, T., Nakagawa, H., Okuya, K., Kawabata, T., Toda, S., 2015. Genetic diversity of highly pathogenic H5N8 avian influenza viruses at a single overwintering site of migratory birds in Japan, 2014/15. Eur. Surveill. 20 (pii: 21132).
Pasick, J., Berhane, Y., Joseph, T., Bowes, V., Hisanaga, T., Handel, K., Alexandersen, S., 2015. Reassortant highly pathogenic Influenza A H5N2 virus containing gene segments related to Eurasian H5N8 in British Columbia, Canada, 2014. Sci. Rep. 5, 9484.
Pinto, L.H., Holsinger, L.J., Lamb, R.A., 1992. Influenza virus M2 protein has ion channel activity. Cell 69, 517–528.
Pohlmann, A., Starick, E., Harder, T., Grund, C., Höper, D., Globig, A., Staubach, C., Dietze, K., Strebelow, G., Ulrich, R., Schinköthe, J., Teifke, J., Conraths, F., Mettenleiter, T., Beer, M., 2017. Outbreaks among wild birds and domestic poultry caused by reassorted Influenza A(H5N8) Clade 2.3.4.4 Viruses, Germany, 2016. Emerg. Infect. Dis. 23, 633.
Posada, D., 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256.
Russell, C., 2016. Sick birds don't fly…or do they? Science 354, 174–175.
Saito, T., Tanikawa, T., Uchida, Y., Takemae, N., Kanehira, K., Tsunekuni, R., 2015. Intracontinental and intercontinental dissemination of Asian H5 highly pathogenic avian influenza virus (clade 2.3.4.4) in the winter of 2014–2015. Rev. Med. Virol. 25, 388–405.
Schmolke, M., Manicassamy, B., Pena, L., Sutton, T., Hai, R., Varga, Z.T., Hale, B.G., Steel, J., Pérez, D.R., García-Sastre, A., 2011. Differential contribution of PB1-F2 to the virulence of highly pathogenic H5N1 Influenza A virus in mammalian and Avian Species. PLoS Pathog. 7, e1002186.
Shen, Y., Ke, C., Li, Q., Yuan, R., Xiang, D., Jia, W., Yu, Y., Liu, L., Huang, C., Qi, W., Sikkema, R., Wu, J., Koopmans, M., Liao, M., 2016. Novel Reassortant Avian Influenza A(H5N6) Viruses in Humans, Guangdong, China, 2015. Emerg. Infect. Dis. 22, 1507–1509.
Si, Y.J., Lee, I.W., Kim, E.H., Kim, Y.I., Kwon, H.I., Park, S.J., Nguyen, H.D., Kim, S.M., Kwon, J.J., Choi, W.S., Beak, Y.H., Song, M.S., Kim, C.J., Webby, R.J., Choi, Y.K., 2017. Genetic characterisation of novel, highly pathogenic avian influenza (HPAI) H5N6 viruses isolated in birds, South Korea, November 2016. Euro Surveill 22 (pii: 30434).
Smith, G., Donis, R., World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization (WHO/OIE/FAO) H5 Evolution Working Group, 2015. Nomenclature updates resulting from the evolution of avian influenza A(H5) virus clades 2.1.3.2a, 2.2.1, and 2.3.4 during 2013–2014. Influ. Other Respir. Viruses 9, 271–276.
Srinivasan, K., Raman, R., Jayaraman, A., Viswanathan, K., Sasisekharan, R., 2013. Quantitative description of Glycan-Receptor binding of Influenza A Virus H7 Hemagglutinin. PLoS One 8, e49597.
Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313.
Steel, J., Lowen, A.C., Mubareka, S., Palese, P., 2009. Transmission of Influenza Virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PLoS Pathog. 5, e1000252.
Su, S., Bi, Y., Wong, G., Gray, G.C., Gao, G.F., Li, S., 2015. Epidemiology, evolution, and recent outbreaks of Avian Influenza Virus in China. J. Virol. 89, 8671–8676.
Takemae, N., Harada, M., Nguyen, P.T., Nguyen, T., Nguyen, T.N., To, T.L., Nguyen, T.D., Pham, V.P., Le, V.T., Do, H.T., Vo, H.V., Le, Q.V.T., Tran, T.M., Nguyen, T.D., Thai, P.D., Nguyen, D.H., Le, A.Q.T., Nguyen, D.T., Uchida, Y., Saito, T., 2017. Influenza A viruses of swine (IAV-S) in Vietnam from 2010 to 2015: multiple Introductions of A(H1N1)pdm09 viruses into the pig population and diversifying genetic Constellations of Enzootic IAV-S. J. Virol. 91 (pii: e01490-01416).
Torchetti, M.K., Killian, M.L., Dusek, R.J., Pedersen, J.C., Hines, N., Bodenstein, B., White, C.L., Ip, H.S., 2015. Novel H5 Clade 2.3.4.4 Reassortant (H5N1) Virus from a Green-Winged Teal in Washington, USA. Genome Announc. 3 (pii: e00195-00115).
Trapp, S., Soubieux, D., Marty, H., Esnault, E., Hoffmann, T.W., Chandenier, M., Lion, A., Kut, E., Quéré, P., Larcher, T., Ledevin, M., Munier, S., Naffakh, N., Marc, D., 2014. Shortening the unstructured, interdomain region of the non-structural protein NS1 of an avian H1N1 influenza virus increases its replication and pathogenicity in chickens. J. Gen. Virol. 95, 1233–1243.
Uchida, Y., Suzuki, Y., Shirakura, M., Kawaguchi, A., Nobusawa, E., Tanikawa, T., Hikono, H., Takemae, N., Mase, M., Kanehira, K., Hayashi, T., Tagawa, Y., Tashiro, M., Saito, T., 2012. Genetics and infectivity of H5N1 highly pathogenic avian influenza viruses isolated from chickens and wild birds in Japan during 2010–11. Virus Res. 170, 109–117.
Verhagen, J.H., van der Jeugd, H.P., Nolet, B.A., Slaterus, R., Kharitonov, S.P., de Vries, P.P., Vuong, O., Majoor, F., Kuiken, T., Fouchier, R.A., 2015. Wild bird surveillance around outbreaks of highly pathogenic avian influenza A(H5N8) virus in the Netherlands, 2014, within the context of global flyways. Eur. Surveill. 20 (pii: 21069).
Vladimirtseva, M., Germogenov, N., 2013. Birds of Yakutia: fauna diversity, ecology, role in ecosystems and human life. J. Biodivers. Endanger. Species 1, 114.
de Vries, R.P., Zhu, X., McBride, R., Rigter, A., Hanson, A., Zhong, G., Hatta, M., Xu, R., Yu, W., Kawaoka, Y., de Haan, C.A.M., Wilson, I.A., Paulson, J.C., 2014. Hemagglutinin Receptor Specificity and Structural Analyses of Respiratory Droplet-Transmissible H5N1 Viruses. J. Virol. 88, 768–773.
Wang, W., Lu, B., Zhou, H., Suguitan, A.L., Cheng, X., Subbarao, K., Kemble, G., Jin, H., 2010. Glycosylation at 158N of the hemagglutinin protein and receptor binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated H5N1 A/Vietnam/1203/2004 vaccine virus in ferrets. J. Virol. 84, 6570–6577.
Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., Kawaoka, Y., 1992. Evolution and ecology of influenza A viruses. Microbiol. Rev. 56, 152–179.
World Organisation for Animal Health, 2016. Chapter 2.3.4 Avian influenza. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2016.
Xiong, C., Liu, Q., Chen, Q., Chen, J., 2013. Genome sequence of a reassortant H5N1 Avian Influenza virus isolated from domestic Green-Winged Teal. Genome Announc. 1 (e00639-00613).
Yamada, S., Hatta, M., Staker, B.L., Watanabe, S., Imai, M., Shinya, K., Sakai-Tagawa, Y., Ito, M., Ozawa, M., Watanabe, T., Sakabe, S., Li, C., Kim, J.H., Myler, P.J., Phan, I., Raymond, A., Smith, E., Stacy, R., Nidom, C.A., Lank, S.M., Wiseman, R.W., Bimber, B.N., O'Connor, D.H., Neumann, G., Stewart, L.J., Kawaoka, Y., 2010. Biological and structural characterization of a host-adapting amino acid in Influenza Virus. PLoS

Pathog. 6, e1001034.
Yang, H., Chen, L.-M., Carney, P.J., Donis, R.O., Stevens, J., 2010. Structures of receptor complexes of a North American H7N2 Influenza Hemagglutinin with a loop deletion in the receptor binding site. PLoS Pathog. 6, e1001081.
Yang, L., Zhu, W., Li, X., Bo, H., Zhang, Y., Zou, S., Gao, R., Dong, J., Zhao, X., Chen, W., Dong, L., Zou, X., Xing, Y., Wang, D., Shu, Y., 2016. Genesis and dissemination of highly pathogenic H5N6 avian influenza viruses. J. Virol. 91 (pii: e02199-02116).
Yoon, H., Moon, O.-K., Jeong, W., Choi, J., Kang, Y.-M., Ahn, H.-Y., Kim, J.-H., Yoo, D.-S., Kwon, Y.-J., Chang, W.-S., Kim, M.-S., Kim, D.-S., Kim, Y.-S., Joo, Y.-S., 2015. H5N8 Highly pathogenic avian influenza in the Republic of Korea: epidemiology during the first wave, from January through July 2014. Osong Public Health Res. Perspect. 6, 106–111.
Zhang, Y., Chen, M., Huang, Y., Zhu, W., Yang, L., Gao, L., Li, X., Bi, F., Huang, C., Kang, N., Zhang, H., Li, Z., Bo, H., Wang, D., Shu, Y., 2017. Human infections with novel reassortant H5N6 avian influenza viruses in China. Emerg. Microbes Infect. 6, e50.