ORIGINAL ARTICLE

Spatial transmission of H5N6 highly pathogenic avian influenza viruses among wild birds in Ibaraki Prefecture, Japan, 2016–2017

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Abstract

From 29 November 2016 to 24 January 2017, sixty-three cases of H5N6 highly pathogenic avian influenza virus (HPAIV) infections were detected in wild birds in Ibaraki Prefecture, Japan. Here, we analyzed the genetic, temporal, and geographic correlations of these 63 HPAIVs to elucidate their dissemination throughout the prefecture. Full-genome sequence analysis of the Ibaraki isolates showed that 7 segments (PB2, PB1, PA, HA, NP, NA, NS) were derived from G1.1.9 strains while the M segment was from G1.1 strains; both groups of strains circulated in south China. Pathological studies revealed severe systemic infection in dead swans (the majority of dead birds and the only species necropsied), thus indicating high susceptibility to H5N6 HPAIVs. Coalescent phylogenetic analysis using the 7 G1.1.9-derived segments enabled detailed analysis of the short-term evolution of these highly homologous HPAIVs. This analysis revealed that the H5N6 HPAIVs isolated from wild birds in Ibaraki Prefecture were divided into 7 groups. Spatial analysis demonstrated that most of the cases concentrated around Senba Lake originated from a single source, and progeny viruses were transmitted to other locations after the infection expanded in mute swans. In contrast, within just a 5-km radius of the area in which cases were concentrated, three different intrusions of H5N6 HPAIVs were evident. Multi-segment analysis of short-term evolution showed that not only was the invading virus spread throughout Ibaraki Prefecture but also that, despite the small size of this region, multiple invasions had occurred during winter 2016–2017.

Introduction

Highly pathogenic avian influenza viruses (HPAIVs) cause serious disease in chickens and other avian species. For example, the H5N1 HPAIV that caused an outbreak at a goose farm in Guangdong in China in 1996 [1] initiated a decades-long endemic throughout the world. In 1997, a descendant virus caused outbreaks on poultry farms as

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well as 18 human cases, including 6 deaths, in Hong Kong [2]. After a 5-year lull, progeny viruses caused devastating outbreaks in Asian countries in 2004 [3] and had spread to Europe, Africa, and the Middle East by 2006 [4, 5]. The numbers of countries and regions affected by H5N1 HPAIV peaked in 2006 [6]. Although local, regional, and international efforts had decreased the number of affected countries by 2013, these progeny viruses have since resurged, and the number of affected countries has rebounded. Recently, H5N2, H5N3, H5N6, and H5N8 HPAIVs that carry the H5 HA of clade 2.3.4.4 have emerged and have spread to Asia, Europe, and North America [7–10].

Wild birds play a key role in the transmission of influenza, because waterfowl are the natural reservoirs of avian influenza viruses. The first massive mortality due to Asian H5N1 HPAIVs that involved waterfowl and several other avian species occurred in two Hong Kong parks in 2002; before this date, waterfowl had been resistant to HPAIVs [11]. In 2005, numerous wild birds were found dead from H5N1 infection at Qinghai Lake in western China and Mongolia [12]. After the die-off at Qinghai Lake, the first outbreaks due to H5N1 HPAIVs in wild birds in Europe were reported in Romania and Croatia at the end of 2005 [13, 14]. During 2006–2007, several mute swans infected with H5N1 HPAIVs died on the island of Ruegen in Germany [15], and the infection of wild birds subsequently spread to the Baltic countries [16]. At the same time, African cases of wild-bird infection were reported in Burkina Faso, Côte d'Ivoire, and Nigeria [17]. Given the rapidity of the virus's spread, wild bird migration was considered to have contributed to the dissemination of the so-called "Qinghai lake strain," an H5 HPAIV of clade 2.2, throughout Africa, Europe, and the Middle East [18].

During winter 2010–2011, 63 cases of infection with H5N1 HPAIV, involving 18 species of wild birds, as well as 24 outbreaks at chicken farms occurred in Japan [19]. During the same season in South Korea, H5N1 HPAIV caused outbreaks at 53 poultry farms, including 32 domestic duck farms, and infected 20 wild birds [20]. In 2014, the first North American outbreaks due to Asian H5Nx HPAIVs occurred in wild birds and poultry [21]. H5N8 HPAIV also caused outbreaks in poultry and wild birds in Europe in 2016–2017.

South Korea experienced 2 waves of outbreaks due to H5N8 HPAIVs between 2014 and 2015 [22]. During these waves, 58 HAPIVs were isolated from wild birds through active and passive surveillance. In the same season, 12 cases of wild-bird infection and 5 outbreaks at poultry farms were reported in Japan [23]. Recently, H5N6 HPAIVs have emerged in wild birds and poultry [24].

The HPAI outbreak in black swans in Akita Prefecture on 15 November 2016 was the first case due to an H5N6 virus in Japan [25]. Subsequently, 212 cases in wild and captive birds as well as 12 cases on poultry farms occurred in Japan during winter 2016–2017 [26]. These cases were reported from Hokkaido in the north to Kyushu in the south. Among affected prefectures, Ibaraki had the most wild-bird cases—63 cases occurred from 29 November 2016 to 24 January 2017—although no poultry cases were reported. In the current study, we analyzed the genetic, temporal, and spatial relationships among the HPAIV strains isolated from wild birds in Ibaraki Prefecture to elucidate how the viruses had intruded into, and spread throughout, this region and its wild-bird populations.

Methods

Virus isolation

Tracheal and cloacal swabs were collected from 242 dead birds that were brought into Ibaraki Prefecture Kenpoku Livestock Hygiene Service Center and were transferred to PBS containing 1,000 U/mL penicillin, 1,000 μg/mL streptomycin and 25 μg/mL Fungizone (Table 1). The swabs were removed from the transport medium, and 0.2 mL of the supernatant was inoculated into 9- to 11-day-old embryonated eggs for virus isolation [27]. The inoculated eggs were incubated at 37 °C for 2 days after inoculation or until the embryos died. The eggs then were chilled at 4 °C, and the allantoic fluid was harvested and tested in hemagglutinin (HA) assays using chicken red blood cells to detect virus [27]. Allantoic fluid that was negative by HA assay was passaged once more in embryonated chicken eggs.

Pathology

Dead mute (*Cygnus olor*) and black (*Cygnus atratus*) swans, which comprised the majority of the dead birds at Senba Lake, were necropsied for the evaluation of gross lesions. Histopathology was performed on samples of brain, lung, heart, liver, pancreas, kidney, spleen, and intestinal tissue from birds that had gross lesions; the skin surrounding the rachis was evaluated in all dead birds. Tissue samples were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin. Paraffin blocks were sectioned at 3 μm and stained with hematoxylin and eosin. For AIV immunohistochemistry, paraffin sections were incubated with anti-influenza A virus matrix protein mouse monoclonal antibody (dilution, 1:1000; LSBio, Seattle, WA, USA). This was followed by incubation with universal immunoperoxidase polymer (Histofine Simple Stain MAX-PO [MULTI], Nichirei Biosciences, Tokyo, Japan) as the secondary antibody and 3,3′ diaminobenzidine tetrachloride as a chromogen.

Table 1 Cases of H5N6 HPAVI infection in wild birds in Ibaraki Prefecture, Japan, during winter 2016–2017

Area	Date	Species		Condition[a]	Location	Isolate name
Northern	2016/11/29	whooper swan	*Cygnus cygnus*	Dead	Otsukatyo	A/whooper swan/Ibaraki/188C/2016(H5N6)
	2016/12/6	black headed gull	*Larus ridibundus*	Dead	Senba lake	A/black-headed gull/Ibaraki/194T/2016(H5N6)
	2016/12/8	mute swan	*Cygnus olor*	Hyposthenic	Senba lake	A/mute swan/Ibaraki/202T/2016(H5N6)
	2016/12/10	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/208T/2016(H5N6)
	2016/12/11	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/210T/2016(H5N6)
	2016/12/12	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/211T/2016(H5N6)
	2016/12/13	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/214T/2016(H5N6)
	2016/12/13	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/217T/2016(H5N6)
	2016/12/14	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/218T/2016(H5N6)
	2016/12/14	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/219T/2016(H5N6)
	2016/12/14	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/220T/2016(H5N6)
	2016/12/14	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/221C/2016(H5N6)
	2016/12/14	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/222C/2016(H5N6)
	2016/12/14	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/223T/2016(H5N6)
	2016/12/14	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/232C/2016(H5N6)
	2016/12/15	black headed gull	*Larus ridibundus*	Dead	Izumityo	A/black-headed gull/Ibaraki/235T/2016(H5N6)
	2016/12/15	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/236T/2016(H5N6)
	2016/12/16	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/239C/2016(H5N6)
	2016/12/16	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/240C/2016(H5N6)
	2016/12/16	mute swan	*Cygnus olor*	Hyposthenic	Senba lake	A/mute swan/Ibaraki/242T/2016(H5N6)
	2016/12/16	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/245C/2016(H5N6)
	2016/12/16	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/247C/2016(H5N6)
	2016/12/17	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/248C/2016(H5N6)
	2016/12/17	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/249T/2016(H5N6)
	2016/12/17	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/250T/2016(H5N6)
	2016/12/18	black headed gull	*Larus ridibundus*	Dead	Senba lake	A/black-headed gull/Ibaraki/253T/2016(H5N6)
	2016/12/18	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/252T/2016(H5N6)
	2016/12/18	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/254C/2016(H5N6)
	2016/12/18	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/255C/2016(H5N6)
	2016/12/19	black swan	*Cygnus atratus*	Dead	Senba lake	A/black swan/Ibaraki/256C/2016(H5N6)
	2016/12/19	black swan	*Cygnus atratus*	Dead	Joto	A/black swan/Ibaraki/259T/2016(H5N6)
	2016/12/20	black headed gull	*Larus ridibundus*	Dead	Senba lake	A/black-headed gull/Ibaraki/265T/2016(H5N6)
	2016/12/20	black headed gull	*Larus ridibundus*	Dead	Senba lake	A/black-headed gull/Ibaraki/267T/2016(H5N6)
	2016/12/20	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/262C/2016(H5N6)
	2016/12/20	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/263T/2016(H5N6)
	2016/12/21	black swan	*Cygnus atratus*	Dead	Senba lake	A/black swan/Ibaraki/271C/2016(H5N6)
	2016/12/21	black swan	*Cygnus atratus*	Dead	Senba lake	A/black swan/Ibaraki/272C/2016(H5N6)
	2016/12/21	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/269T/2016(H5N6)
	2016/12/21	mute swan	*Cygnus olor*	Dead	Senba lake	A/mute swan/Ibaraki/270T/2016(H5N6)
	2016/12/21	pochard	*Aythya ferina*	Dead	Tanotyo	A/pochard/Ibaraki/268T/2016(H5N6)
	2016/12/22	black headed gull	*Larus ridibundus*	Dead	Senba lake	A/black headed gull/Ibaraki/282T/2016(H5N6)
	2016/12/22	mute swan	*Cygnus olor*	Dead	Senba lake	A/muteswan/Ibaraki/277T/2016(H5N6)
	2016/12/24	black headed gull	*Larus ridibundus*	Dead	Senba lake	A/black-headed gull/Ibaraki/284T/2016(H5N6)
	2016/12/25	great crested grebe	*Podiceps cristatus*	Dead	Senba lake	A/great crested grebe/Ibaraki/286T/2016(H5N6)
	2016/12/25	great crested grebe	*Podiceps cristatus*	Dead	Senba lake	A/great crested grebe/Ibaraki/287T/2016(H5N6)
	2016/12/26	black swan	*Cygnus atratus*	Dead	Senba lake	A/black swan/Ibaraki/290C/2016(H5N6)
	2016/12/26	black headed gull	*Larus ridibundus*	Dead	Tamiyahara	A/black-headed gull/Ibaraki/289T/2016(H5N6)
	2016/12/28	great crested grebe	*Podiceps cristatus*	Dead	Tanotyo	A/great crested grebe/Ibaraki/298C/2016(H5N6)
	2016/12/30	black swan	*Cygnus atratus*	Dead	Sakuragawa	A/black swan/Ibaraki/302C/2016(H5N6)
	2017/1/2	black swan	*Cygnus atratus*	Dead	Senba lake	A/black swan/Ibaraki/28-446T/2017(H5N6)

Table 1 (continued)

Area	Date	Species		Condition[a]	Location	Isolate name
	2017/1/4	black swan	*Cygnus atratus*	Dead	Tyuo	A/black swan/Ibaraki/310C/2017(H5N6)
	2017/1/16	black swan	*Cygnus atratus*	Dead	Otsukatyo	A/black swan/Ibaraki/341T/2017(H5N6)
	2017/1/17	black swan	*Cygnus atratus*	Hyposthenic	Otsukatyo	A/black swan/Ibaraki/345T/2017(H5N6)
	2017/1/18	black swan	*Cygnus atratus*	Dead	Otsukatyo	A/black swan/Ibaraki/348C/2017(H5N6)
	2017/1/19	black swan	*Cygnus atratus*	Hyposthenic	Otsukatyo	A/black swan/Ibaraki/350T/2017(H5N6)
	2017/1/21	black swan	*Cygnus atratus*	Dead	Otsukatyo	A/black swan/Ibaraki/356T/2017(H5N6)
	2017/1/22	black swan	*Cygnus atratus*	Dead	Otsukatyo	A/black swan/Ibaraki/357T/2017(H5N6)
	2017/1/24	black swan	*Cygnus atratus*	Hyposthenic	Otsukatyo	A/black swan/Ibaraki/365T/2017(H5N6)
Southern	2016/12/18	black headed gull	*Larus ridibundus*	Dead	Suga	A/black-headed gull/Ibaraki/258T/2016(H5N6)
	2016/12/26	black headed gull	*Larus ridibundus*	Dead	Tsumagi	A/black-headed gull/Ibaraki/291T/2016(H5N6)
	2016/12/28	whooper swan	*Cygnus cygnus*	Dead	Numao	A/whooper swan/Ibaraki/301T/2016(H5N6)
	2017/1/11	whooper swan	*Cygnus cygnus*	Dead	Maekawa	A/whooper swan/Ibaraki/331C/2017(H5N6)
	2017/1/19	whooper swan	*Cygnus cygnus*	Dead	Kashimashi	A/whooper swan/Ibaraki/351C/2017(H5N6)

[a]Condition of birds when being found. All birds had died before they were brought into Ibaraki Prefecture Kenpoku Livestock Hygiene Service Center

Full-genome sequencing of HPAIV isolates

Allantoic fluid was centrifuged for 5 min at 10,000 × g, and viral RNA was extracted from the supernatant by using an RNeasy Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Full-genome sequencing was performed by using a MiSeq sequencer (Illumina, Tokyo, Japan). The cDNA library was prepared by using the NEB-Next Ultra RNA Library Prep Kit (New England Biolabs, Tokyo, Japan) with NEBNext Multiplex Oligos for Illumina (New England Biolabs, Ipswich, MA, USA), and the concentration of the library was measured by using an NEB-Next Library Quant Kit for Illumina (New England Biolabs, Tokyo, Japan). We mixed 10 pM of each cDNA library with 10 pM of the PhiX control (Illumina), and the mixtures were loaded into the Miseq Reagent Kit version 2 (Illumina) for 2 rounds of 150 cycles. The output data were mapped to HPAIV genomes, and a consensus sequence for each isolate was generated by using FLUGAS software (World Fusion, Tokyo, Japan). All sequences were submitted to GISAID (http://platform.gisaid.org/epi3/start); accession numbers are listed in Online Resource 1.

Phylogenetic analysis

All AIV sequences registered to GISAID (http://platform.gisaid.org/epi3/start) as of 13 June 2017 were downloaded; the numbers of sequences downloaded for each gene were: PB2, 46,165; PB1, 45,393: PA, 44,838; H5, 9,776; NP, 46,082; N6, 3,168; M, 65,011; and NS, 47,497. To classify the HA clade of each isolate, 4,998 sequences were selected from the downloaded sequences by sorting for complete and unique sequences; these were then used to construct a maximum likelihood tree by using MEGA7 [28]. For detailed phylogenetic analysis, each set of downloaded sequences was aligned in its order of similarity to the sequence of A/whooper swan/Ibaraki/188C/2016 (H5N6), which was the first HPAIV isolated from a wild bird in Ibaraki Prefecture, by using MAFFT [29]; the top 200 most similar sequences were selected to generate the phylogenetic tree. Identical sequences from strains that were isolated on the same date, from the same species, and at the same place were removed from each sequence set. The alignment lengths of each segment are shown in Table 2. Maximum likelihood trees of each segment were generated by bootstrapping 1,000 times using MEGA7.

Table 2 Best-fit substitution model and nucleotide substitution rates

Segment	Length (nucleotides)	Substitution model[a]	Substitution rate (× 10^{-3})	
			Mean	95% HPD[b]
B2	2280	HKY	4.59	3.58–5.65
PB1	2274	HKY+I	6.01	4.43–7.60
PA	2151	TrN	4.72	3.73–5.83
HA	1701	TrN	7.62	5.88–9.48
NP	1497	HKY+I	4.10	2.92–5.40
NA	1380	HKY	5.47	4.17–6.88
M	948	[c]		
NS	823	HKY+G	4.23	2.80–5.73

[a]HKY: Hasegawa-Kishino-Yano model, +I: proportion of invariant sites, TrN: Tamura and Nei model

[b]HPD: highest posterior density

[c]M segment was not included in MCMC analysis

Bayesian phylogenetic trees were constructed by using BEAST (version 1.8.4) software [30]. From the 300 sequences with the highest level of identity using MAFFT [37], we selected sequence sets that belonged to 89 strains and that had 7 segments (PB2, PB1, PA, HA, NP, NA, NS) in common. M segments were removed from the sequence set because their origin differed from those of the others. The abovementioned 7 segments were used as partitions, and their sites and clock models were unlinked. Best-fit substitution models were selected by using the Bayesian Information Criterion implemented in jModeltest2 [31] and are shown in Table 2. The uncorrelated log-normal relaxed molecular clock was used to estimate the substitution rate of each segment. Two Markov chain Monte Carlo (MCMC) chains were run for 5×10^8 cycles and were thinned by sampling every 20,000 cycles. Two runs after 10% burn-in were combined by using LogCombiner in BEAST. Effective sample sizes were checked in Tracer version 1.6 (combined effective sample size >200), and maximum clade credibility (MCC) trees were generated from the MCMC samples by using TreeAnnotator (v1.8.4) software. Each MCC tree was viewed and edited by using FigTree v1.3.1 [32]. To elucidate how the H5N6 viruses were introduced into and expanded among wild-bird populations in Ibaraki Prefecture, we used SPREAD software [33] to perform discrete phylogeographical analysis, and the result was visualized in Google Earth (https://www.google.co.jp/intl/ja/earth/).

Results

Outbreaks of H5N6 influenza in Ibaraki Prefecture

From November 2016 through March 2017, a total of 212 cases of wild birds being affected by HPAIVs of the H5N6 subtype were identified through dead-bird surveillance conducted by Japan's Ministry of the Environment; 7 isolates were obtained from samples of feces or water samples through surveys by universities or the Ministry of Health, Labour, and Welfare of Japan (Fig. 1). These cases were reported from Hokkaido in the north to Kyushu in the south of Japan and included 16 infected captive birds in two zoos. In particular, 63 cases occurred in Ibaraki Prefecture: the first occurred on 29 November 2016 and the last on 24 January 2017 (Table 1). Whereas 58 cases occurred in the north of the prefecture (Izumityo, Joto, Otsukatyo, Sakuragawa, Senba Lake, Tamiyahara, Tanotyo, and Tyuo), the remaining 5 cases occurred in southern Ibaraki (Kashima, Tsumagi, Maekawa, Numao, and Suga) (Fig. 2). Otsukatyo, Senba Lake, and Tanotyo, which are in the northern area, are wintering or stopover sites of migratory waterfowl, and a total of 1,079 birds were observed in Senba Lake during the latter half of November 2016 [34]. In contrast, 5 locations in the southern region are near Kasumigaura, the second largest lake in Japan. Among the 63 cases in Ibaraki, most (that is, 49 cases) of the affected birds belonged to the genus *Cygnus*, including mute swans, black swans, and whopper swans; the remaining 14 birds were black-headed gulls, great crested grebes, and a pochard.

We pathologically analyzed the dead mute swans and black swans from Senba Lake to elucidate whether HPAIVs had contributed to their deaths, because the numbers of deaths in these species stood out (Table 3). On both gross and microscopic examination, all mute swans demonstrated hepatic and pancreatic necrosis, and half of these birds also showed intestinal hemorrhage (Fig. 3 a–d). Although not apparent grossly, pathologic lesions in the brain, kidney, and spleen were noted microscopically in some subjects (Online Resource 2 a and b). Immunostaining for AIV antigen revealed numerous positive cells in hepatic, pancreatic, and brain lesions (Fig. 3 e and f). No legions were present either grossly or in HE-stained samples from the lung, heart,
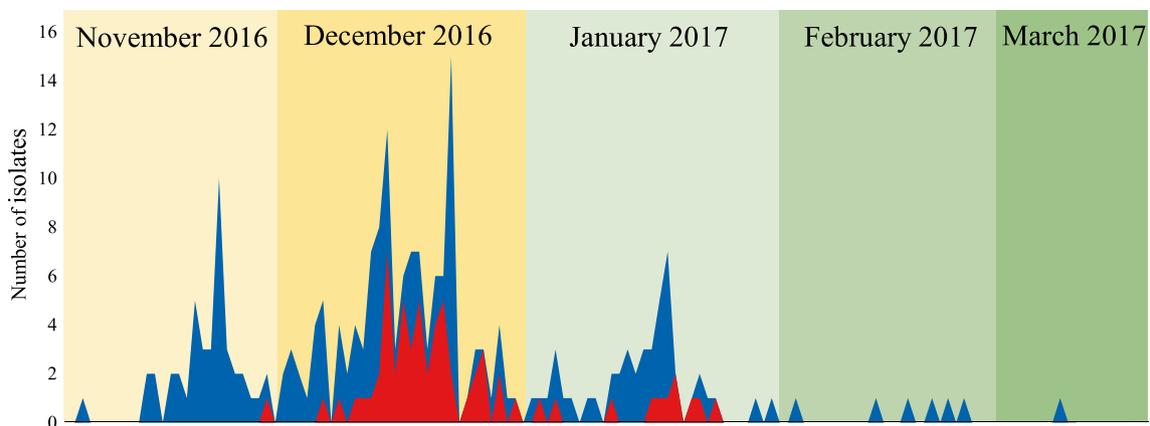

Fig. 1 Numbers of H5N6 HPAIVs isolated from wild birds in Japan, November 2016 to March 2017. Blue, total cases; red, Ibaraki cases

Fig. 2 Map of Ibaraki Prefecture. Arrowheads indicate sites of HPAI outbreaks. These places were grouped into two areas (circled in red): the northern area includes Izumityo, Joto, Otsukatyo, Sakuragawa, Senba Lake, Tamiyahara, Tanotyo, and Tyuo, and the southern area includes Kashima, Maekawa, Numao, Suga, and Tsumagi

or rachis, but numerous positive cells were revealed through immunostaining (Online Resource 2 c–f). In addition, AIV antigens were detected in the conjunctiva of a single mute swan (Online Resource 3). Similar hepatic, pancreatic, intestinal, brain, renal, and splenic lesions were present in dead black swans (Table 3).

Phylogenetic analysis of H5N6 isolates from wild birds in Ibaraki

To genetically characterize the viruses, we sequenced and then phylogenetically analyzed the entire genomes of 63 HPAIVs isolated from wild birds that had died in Ibaraki. In all isolates, the cleavage site of the HA protein had a series of basic amino acids (LRERRRKR/GLF), which is characteristic of HPAIVs. Maximum-likelihood phylogenetic analysis revealed that the HA genes of all 63 Ibaraki isolates belonged to clade 2.3.4.4, which includes the H5Nx

(H5N1, H5N2, H5N5, H5N6, and H5N8) HPAIVs responsible for the outbreaks in Asia, Europe, and North America since 2014 [35] (Online Resource 4). In addition, 7 segments (PB2, PB1, PA, HA, NP, NA, and NS) were derived from G1.1.9 strains, whereas the remaining segment (M) was derived from G1.1 strains; most of the ancestors of the Ibaraki viruses have been isolated in south China since 2014 [36] (Online Resources 5–12). The genetic constellation of the Ibaraki isolates was the same as that found predominantly among the H5N6 HPAIVs isolated throughout Japan during the same season. [27]. The nucleotide identity among the 63 Ibaraki isolates was 98.9% to 100%, and the identity at the amino acid level was 98.4% to 100%, depending on the gene (Table 4).

Next, to depict the temporal relationship among the isolates, we generated an MCC tree by using BEAST software. We performed a multi-locus coalescent analysis [30] using 7 segments (the M gene was omitted because of its different

Table 3 Distribution of pathologic lesions in mute swans and black swans infected with H5N6 HPAIVs

Species	Organ	Pathologic lesions (no. of organs with lesions/no. of organs analyzed)		
		Gross	HE	IHC
Mute swan	Brain	0/5	2/5	5/5
	Lung	0/6	0/6	6/6
	Heart	0/5	0/5	5/5
	Liver	6/6	6/6	6/6
	Pancreas	6/6	6/6	6/6
	Kidney	0/5	1/5	4/5
	Spleen	0/5	4/5	5/5
	Intestine	3/6	3/6	5/6
	Skin	0/30	0/30	30/30
Black swan	Brain	0/8	1/8	8/8
	Lung	0/10	0/10	9/10
	Heart	0/9	0/9	7/9
	Liver	11/12	11/12	12/12
	Pancreas	12/12	12/12	12/12
	Kidney	0/9	0/9	7/9
	Spleen	0/10	6/10	9/10
	Intestine	2/11	2/11	8/11
	Skin	0/13	0/13	12/13

HE hematoxylin and eosin stain, *IHC* immunohistochemistry for AIV M protein

genetic origin) to investigate the genetic relationships among the isolates, because using only a single segment might not have discriminated the isolates well owing to their high levels of identity (Table 4). The MCC tree displayed 7 distinguishable branches among the isolates, comprising two dominant groups (designated as groups 1 and 2) (Fig. 4). Group 1 consisted of 43 isolates from Senba Lake, 5 isolates from the vicinity in the northern area, as well as 1 additional isolate, A/black headed gull/Ibaraki/291T/2016, from Tsumagi, which is 40 km away from Senba Lake. Most (that is, 30) of the Senba Lake (that is, group 1) isolates were from mute swans, with 5 isolates from black swans, 6 from black-headed gulls, and 2 from great crested grebes. All of the viruses in group 2 were isolated from black swans in Otsukatyo. The first isolate in Otsukatyo, A/whooper swan/Ibaraki/188C/2006, was phylogenetically distinguishable from the other isolates in group 2, suggesting that this first isolate and the group 2 isolates were introduced independently.

Phylogeographic analysis of H5N6 isolates from Ibaraki Prefecture

Data from the MCC tree generated by multi-locus phylogenic analysis were entered on a map by using SPREAD software [33] to reveal the geographic and temporal relationships among HPAI outbreaks involving wild birds in Ibaraki Prefecture. Using this approach, we simulated the spatial spreading of the H5N6 HPAIVs in Ibaraki Prefecture during winter 2016–2017 (Fig. 5).

The first H5N6 virus in Ibaraki Prefecture was obtained from a whooper swan in Otsukatyo on 29 November 2017. On 6 December, an H5N6 virus was detected in a dead black-headed gull at Senba Lake, but it did not appear to be directly related to the previous isolate from Otsukatyo. Subsequently, the 6 December isolate spread among mute swans at Senba Lake from 8 through 22 December. Infections in and around Senba Lake continued until 2 January 2017, during which period two cases involving great crested grebes and another 5 cases in black-headed gulls were detected. The virus at Senba Lake appeared to spill over to infect black-headed gulls in Izumityo and Tamiyahara and black swans at Joto, Sakuragawa, and Tyuo (Fig. 5c). As indicated by the maximum likelihood tree, an isolate obtained from a black-headed gull in Tsumagi (in southern Ibaraki), A/black headed gull/Ibaraki/291T/2916, was derived from viruses at Senba Lake (Fig. 5c and 5f). Although Tanotyo is only 7 km from Senba Lake, the isolates detected in a pochard on 21 December and a great crested grebe on 26 December differed from the Senba Lake isolates (Fig. 5b).

The first H5N6 virus detected in southern Ibaraki, A/ black headed gull/Ibaraki/258T/2016, was obtained from a black-headed gull in Suga on 18 December. This virus did not represent spill-over from Senba Lake, even though the outbreak at Senba Lake had already reached 27 cases by then (Fig. 5e). In contrast, the virus isolated in Tsumagi on 26 December was introduced from Senba Lake, as mentioned earlier (Fig. 5e and 5g). On 28 December, a whooper swan in Numao was found dead from the H5N6 virus A/ whooper swan/Ibaraki/301T/2016, and a related virus, A/ whooper swan/Ibaraki/331C/2016, was obtained from a whooper swan in Maekawa on 11 January (Fig. 5f). Another whooper swan case occurred on 19 January in Kashima, but the virus isolated, A/whooper swan/Ibaraki/351C/2016, was not directly related to those in the cases at Numao and Maekawa.

Discussion

Here, we analyzed the evolutionary dynamics of several HPAIV outbreaks by using BEAST software and a Bayesian MCMC approach [37, 38]. This approach has been useful for analyzing virus transmission and spread among susceptible animals in different regions, because the MCC tree that is generated reflects not only the genetic relationship among viruses but also temporal and geographic information [39]. For example, MCMC modeling has been used to investigate

Fig. 3 Pathologic findings in dead mute swans. Liver (a, c, e) and pancreas (b, d, f) were collected from dead mute swans and investigated by gross pathological examination (a, b). Tissue sections were stained with hematoxylin and eosin (c, d). In immunohistochemical analysis for AIV M protein, the antigen stains red (e, f)
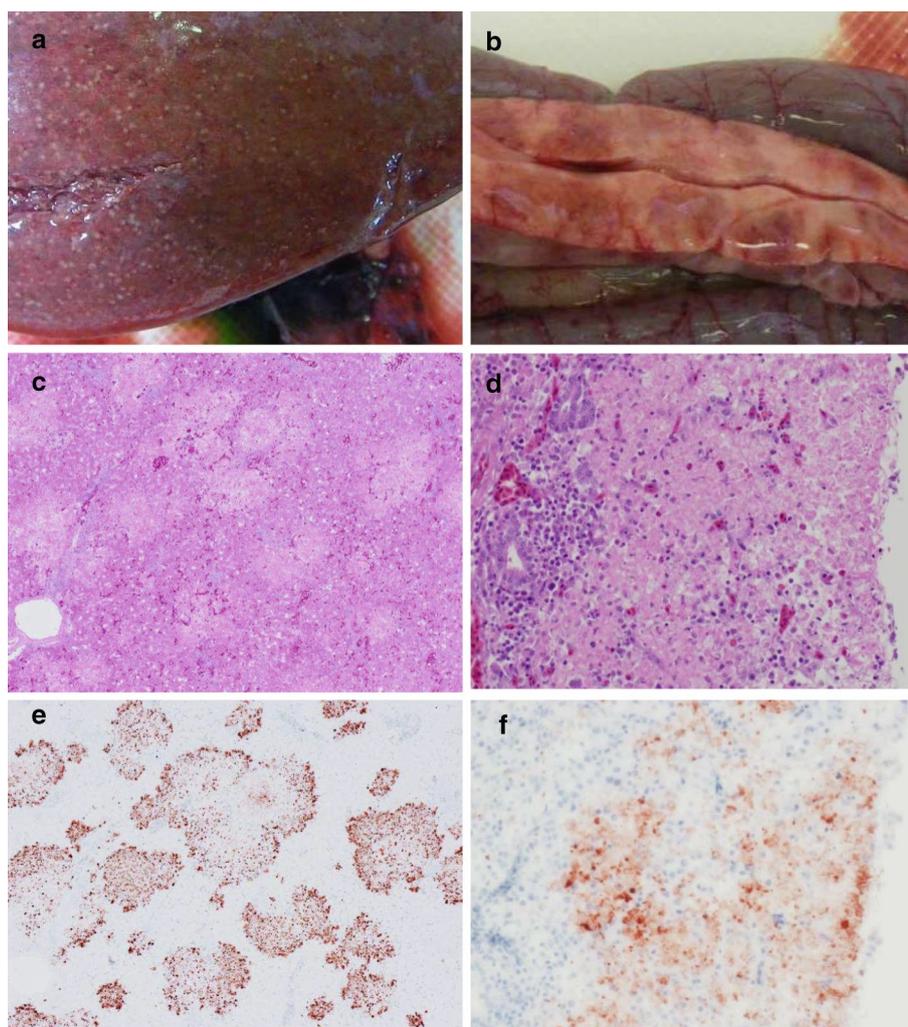

Table 4 Genetic diversity among Ibaraki isolates

| Gene | Identity (%) | | | | | |
| | Among Ibaraki isolates | | Among Group 1 isolates | | Among Group 2 isolates | |
	nucleotide	amino acid	nucleotide	amino acid	nucleotide	amino acid
HA	99.1–100	98.9–100	99.7–100	99.4–100	99.9–100	99.8–100
NA	98.9–100	98.4–100	99.6–100	99.3–100	99.8–100	99.7–100
PB2	99.2–100	99.4–100	99.7–100	99.7–100	99.9–100	100
PB1	99.3–100	99.4–100	99.6–100	99.6–100	99.8–100	99.6–100
PA	99.3–100	99.1–100	99.8–100	99.5–100	99.8–100	99.7–100
NP	99.2–100	99.1–100	99.8–100	99.7–100	99.7–100	99.7–100
M	99.5–100	99.6–100	99.7–100	99.6–100	100	100
NS	99.5–100	99.2–100	99.7–100	99.2–100	99.7–100	99.2–100

the relationship between the spread of H5N1 viruses during 2003–2012 and bird migration networks in Asia [40]. However, analyzing virus evolution within a small region or during a short period is difficult, because the genomic diversity among isolates in these contexts is generally quite low.

For example, the viruses analyzed in the present study were genetically similar (89.1% to 100%) to each other (Table 4). Using the concatenation or coalescent method with multilocus sequence sets can overcome such difficulty to allow detailed analysis of viral evolution. The concatenation
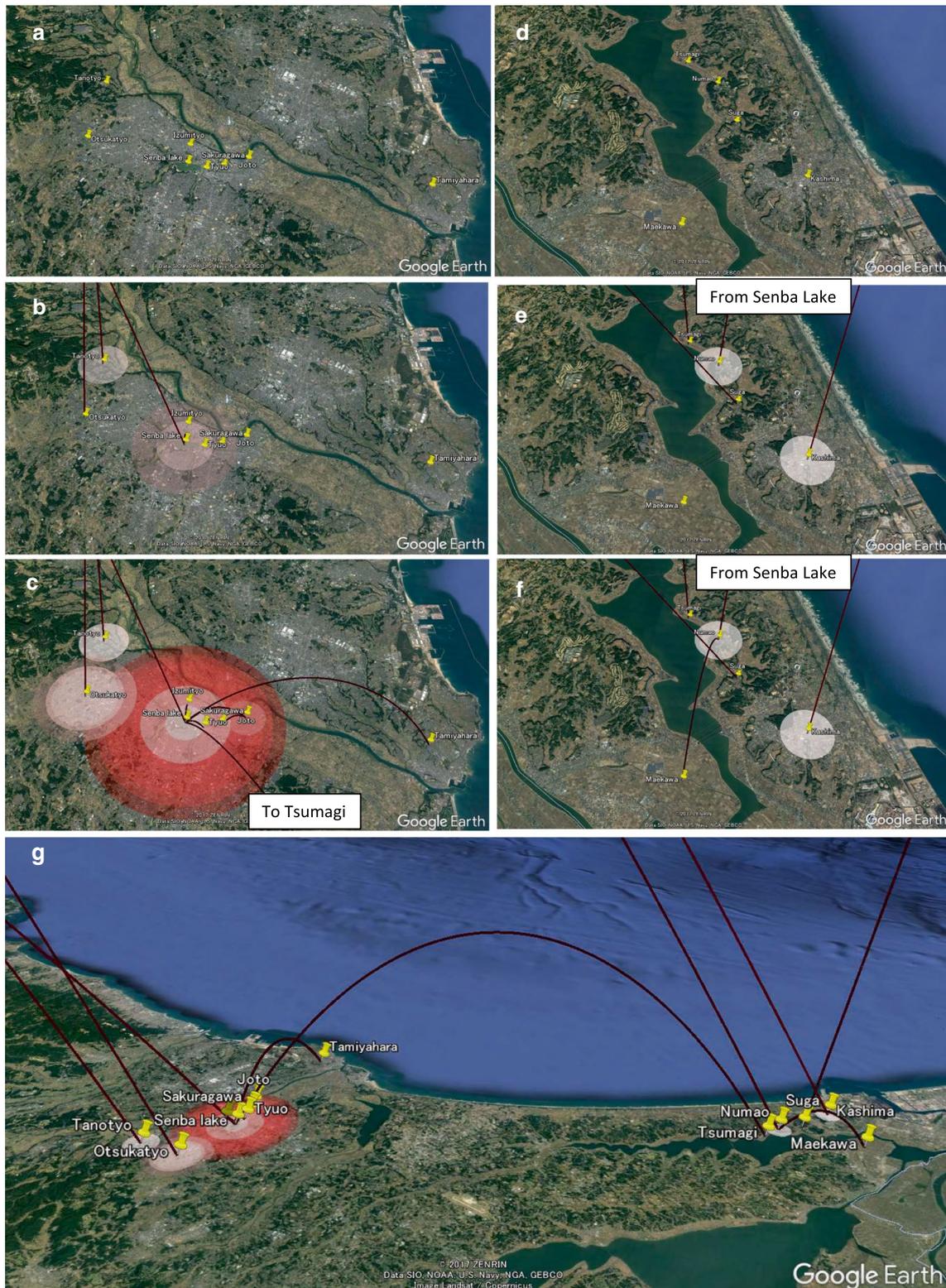
Fig. 4 Phylogenetic tree based on multiple segments of H5N6 HPAIV. The phylogenetic tree was generated from the PB2, PB1, PA, HA, NP, NA, and NS segments of the Ibaraki isolates and reference strains by using the MCMC method and BEAST v1.8.4 software. The color of the virus name indicates the site in Ibaraki at which the virus was isolated (red, northern region; blue, southern region). Two distinguishable groups emerged: group 1 (green highlighting) and group 2 (orange highlighting). Only posterior probability values above 0.80 are shown

Fig. 5 Spatial dynamics of H5N6 HPAIV in Ibaraki Prefecture. Panels a through c show sites in the northern region; panels d through f show sites in the southern region. Panel g shows an overview of virus spread

method involves a single supergene, which is produced by concatenating all gene variants; this method is generally used to construct a species tree because the estimation process is more rapid than with the coalescent method. However, the concatenation method can yield incorrect results when the evolution rate differs between gene sets [41].

In the coalescent method, estimation based on multi-locus sequence data is performed concurrently, and each sequence is treated as an independent neutral replicate; consequently, estimating the phylogenetic tree is computationally expensive [42]. In the present study, we used the coalescent approach to construct a phylogenetic tree from 7 segments (PB2, PB1, PA, HA, NP, NA, and NS), the substitution rates of which differed (Table 2). This 7-segment approach improved the posterior probability of monophyletic branches, compared with analysis that used a single segment (Online Resource 13), allowing us to perform a detailed investigation of virus evolution in a genetically closed population.

Phylogenic and phylogeographic analysis revealed that the HPAIV outbreaks among wild birds in Ibaraki Prefecture during winter 2016–2017 were not due to a single introduction of virus, followed by dissemination throughout the prefecture, but rather resulted from 7 independent introductions. Although Senba Lake, Otsukatyo, and Tanotyo are small areas within a 5-km radius (as are Tsumagi, Numao, Suga, and Kashima in southern Ibaraki), isolates from the respective areas did not share a Most Recent Common Ancestor with each other (Fig. 4). These multi-invasions may have resulted from the widespread prevalence of H5N6 HPAIVs in wild-bird populations throughout Japan, as evidenced by the dead-bird surveillance completed by the Ministry of the Environment of Japan [26]. In addition, susceptibility to HPAIVs varies among avian species, and in experimental infection studies infected birds have not always died [43–46]; furthermore, few reports have addressed the pathogenicity of H5N6 HPAIV against avian species except poultry. Furthermore, H5N6 viruses have been isolated from apparently healthy wild birds during AIV surveillance in China [47]. Therefore, the dead wild birds recovered through the dead-bird surveillance in Japan might have been only a small representation of the situation, and many more wild birds might have been infected asymptomatically and transmitted the virus among wild birds during the season.

Regarding the H5N6 HPAIVs in Ibaraki, 49 of the 63 cases occurred in swans: 30 cases involved mute swans, 15 black swans, and 4 whooper swans. Infections of swans with H5N1, H5N8, and H5N6 HPAIVs have been reported previously [48–50]. In 2006, 185 of the 347 cases of HPAI reported in Germany in wild birds and mammals involved swans [15]. Mute swans are highly susceptible to HPAIV and have been considered to act as virus spreaders because they shed large quantities of viruses from their respiratory

and digestive tracts before the onset of clinical signs [51]. Furthermore, 43 of the dead or hyposthenic birds in the current study (that is, 68% of those involved in the Ibaraki outbreaks) were found at Senba Lake during a single month (Table 1), and the Senba Lake viruses spilled over to sites as far as 10 km away (Fig. 5c). A possible explanation for the serious outbreaks at Senba Lake is that H5N6 HPAIV spread rapidly among the highly susceptible mute swans and that these viruses were subsequently transmitted to other species of wild birds in Senba Lake.

Most of the dead birds during the first half of the outbreaks at Senba Lake were mute swans; the infections in black swans, black-headed gulls, and great crested grebes followed in the second half. During these outbreaks, many wild birds flew into the Senba Lake site [34], and other species of wild birds—particularly mallard ducks, which have been subclinically infected with various H5N6 HPAIVs in an experimental study [52]—might have contributed to viral transmission. Some of these wild birds merely stopped over at Senba Lake before migrating elsewhere. Even birds wintering over or staying year-round did not remain continuously at that site but flew from place to place to forage [53].

In the present study, we demonstrated the short-term evolutionary dynamics of H5N6 viruses in Ibaraki Prefecture. Coalescent phylogenetic analysis of 7 segments of the HPAIV genome revealed multiple invasions of H5N6 HPAIVs during winter 2016–2017, and these viruses both spread among susceptible birds at the same site and expanded to other sites. Use of a multi-segment coalescent approach allowed detailed analysis of virus introduction and spread, even among small areas and during a short period; this depth of investigation would have been difficult to accomplish by using a single segment.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or live animals performed by any of the authors.

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