Genetic diversity of highly pathogenic H5N8 avian influenza viruses at a single overwintering site of migratory birds in Japan, 2014/15

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We isolated eight highly pathogenic H5N8 avian influenza viruses (H5N8 HPAIVs) in the 2014/15 winter season at an overwintering site of migratory birds in Japan. Genetic analyses revealed that these isolates were divided into three groups, indicating the co-circulation of three genetic groups of H5N8 HPAIV among these migratory birds. These results also imply the possibility of global redistribution of the H5N8 HPAIVs via the migration of these birds next winter.

In January 2014, newly discovered highly pathogenic H5N8 avian influenza viruses (H5N8 HPAIVs) caused outbreaks in poultry and wild birds in South Korea [1], although their ancestor had been isolated in China in 2013 [2]. Thereafter, these viruses have been circulating in both avian populations in South Korea [3,4] and sporadically in neighbouring countries, including China and Japan. Since November 2014, H5N8 HPAIVs have also appeared in poultry and wild birds in Europe [5,6]. Genetic analyses revealed that these isolates were closely related to the H5N8 viruses circulating in Korean birds. More recently, genetically similar HPAIVs also caused outbreaks in various avian species in North America [7]. These findings suggest that the H5N8 viruses have circulated and evolved in migratory birds.

Characteristics of the study area

The Izumi plain, which is located at the southern tip of Japan's mainland, is a major overwintering site of the white-naped crane (*Grus vipio*) and hooded crane (*Grus monacha*), both of which are categorised as vulnerable species on the International Union for Conservation of Nature Red List (Figure 1).

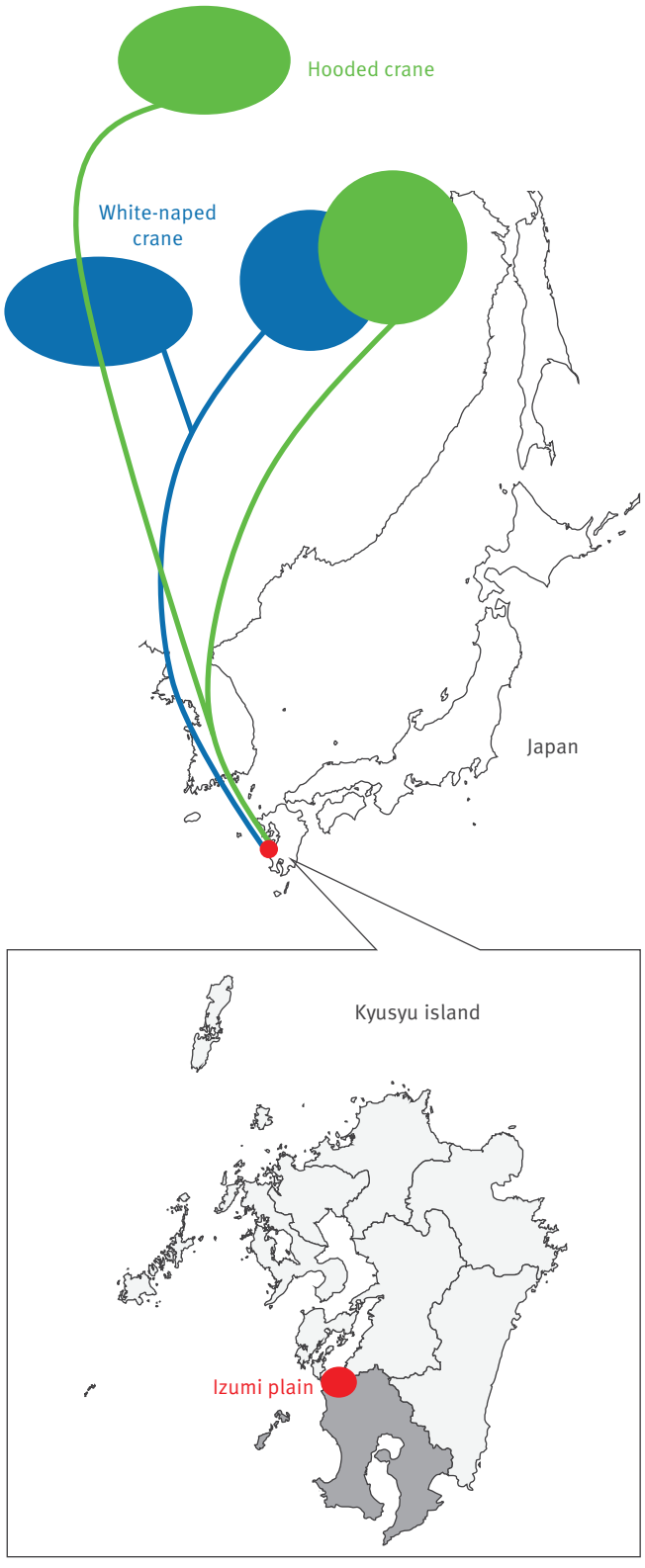
Over 10,000 cranes visit this plain in the winter season (arriving around November to December and leaving around February to March). For the purpose of protecting these endangered bird species, the local government creates artificial wet paddy areas for roosting cranes every winter. In addition to the cranes, many other migratory birds including wild ducks, a natural reservoir of influenza A viruses [8], also overwinter at this plain and share the wet paddies. Avian influenza viruses are therefore likely to be transmitted among the migratory birds, including the endangered cranes, at the Izumi plain. In fact, H5N1 HPAIVs were isolated from seven dead cranes in the 2010/11 winter season [9]. We have also isolated low pathogenic avian influenza viruses from duck faeces and the cranes' roost water collected at this area over the last two winter seasons (data not shown).

Influenza isolates from birds in the study area

On 23 November 2014, a debilitated white-naped crane was captured at the Izumi plain. Tracheal and cloacal swabs were collected and subjected to RNA extraction for the detection of influenza A viral genes and inoculation into embryonated chicken eggs for virus isolation. Influenza A viral M gene was detected in the RNA from the tracheal swab by conventional reverse transcription PCR. The allantoic fluids of the inoculated eggs showed haemagglutination activity. Further genetic analyses of the allantoic fluid revealed that the isolate was the H5N8 subtype influenza A virus. The infected white-naped crane died on 29 November 2014; investigations are under way into the cause of death. The partial sequence of the haemagglutinin (HA) gene revealed that the isolate encoded RERRRKR↓G at the

Figure 1

Map of crane flyways around the Izumi plain, Japan, 2014/15



The location of the Izumi plain is indicated in red circles. Breeding grounds (circles) and flyways (lines) of the white-naped crane (blue) and hooded crane (green) are also shown

HA cleavage sites, suggesting their potential to cause systemic infection, subsequently leading to high pathogenicity.

Since 23 November 2014 when the infected crane was found, wild birds within a 10 km radius of the point where the infected crane was found have been placed under active surveillance for HPAIVs. Local government staff searched for sick and dead wild birds in the area, and sent us the swab specimens and/or dead bodies to test for avian influenza viruses. As of 21 March 2015, eight H5N8 HPAIVs have been isolated from six debilitated or dead cranes, two dead mallard ducks (*Anas platyrhynchos*) and a water sample collected from the cranes' roost at the Izumi plain (Table 1). These virus isolations were reported in a timely manner to the World Organisation for Animal Health via the Ministry of Agriculture, Forestry and Fisheries of Japan [10].

To genetically characterise these H5N8 HPAIV isolates, we determined the complete genome sequences of the eight H5N8 HPAIV isolates, and deposited the sequences in the Global Initiative on Sharing Avian Influenza Data (GISAID) database (Table 2). Overall sequence data show that each gene segment of these isolates was genetically similar to the counterpart H5N8 HPAIVs recently isolated elsewhere in the world, suggesting that these isolates had not experience gene reassortment since their parental viruses caused outbreaks in South Korea in early 2014.

Phylogenetic analysis

To understand the genetic relationship between our isolates and related viruses, the HA and neuraminidase (NA) genes were phylogenetically analysed with counterparts from the representative avian influenza H5 (Figure 2A) and N8 (Figure 2B) subtypes, respectively. We found that the H5 genes from our eight isolates belonged to clade 2.3.4.4 and were genetically divided into three groups. The water isolate, A/environment/Kagoshima/KU-ngr-H/2014(H5N8), fell into a phylogenetic cluster together with the European isolates and was closely related to two wild duck isolates in Japan (Group A, indicated in green in the Figures). The first and second crane isolates, A/crane/Kagoshima/KU1/2014(H5N8) and A/crane/Kagoshima/KU13/2014(H5N8), were genetically similar to the North American isolates (Group B, blue in the Figures). The HA genes of the rest of our isolates (Group C, red in the Figures), as well as a poultry isolate from Japan were clearly distinct from those of the other recent H5N8 isolates. These findings suggest that three genetically distinct groups of H5N8 HPAIVs were independently circulating among the migratory birds at the Izumi plain. Intriguingly, the genetic grouping of our isolates matched broadly the dates of sampling; the forth to eighth isolates were categorised into Group C, while earlier isolates were categorised into Group A or B. To determine whether this virus group has genetic characteristics that become predominant among the migratory birds over the remaining virus groups, further investigation would be needed.

To further characterise the three genetic groups of H5N8 HPAIVs, the nucleotide sequences of the remaining six

genes were phylogenetically analysed with their counterparts from the representative avian viruses of various subtypes (Figure 2).

The bootstrap values between the isolates in Groups A and C and among the isolates in Group B in the phylogenetic trees of the PB2 (Figure 3A) and PB1 (Figure 3B) genes were 100%. Similarly, the bootstrap value between the isolates in Group A and the isolates in Groups B and C in the phylogenetic tree of the NS genes (Figure 3F) were 99%. These results support our findings in the phylogenetic trees of the HA and NA genes.

No mutations were found that are known to confer the ability to infect mammalian hosts or to provide resistance against anti-influenza drugs to avian influenza viruses, with the exception of an asparagine at position 31 in the M2 protein, which confers resistance to the M2 ion channel blocker amantadine [11].

Conclusion

We isolated eight H5N8 HPAIVs from migratory birds and the water in their environment at the Izumi plain in southern Japan. Based on their genome sequences, these isolates were genetically divided into three groups. These results indicate the co-circulation of at least three genetic groups of H5N8 HPAIVs among the migratory birds overwintering at a single site in Japan. These H5N8 HPAIVs are most likely to be derived from wild ducks [12], rather than from cranes whose flyways were restricted to East Asian countries (Figure 1A). These findings also imply the possibility of global redistribution of the H5N8 HPAIVs via migration of these ducks next winter.

Table 1

H5N8 influenza A viruses isolated in this study, Izumi plain, Japan, 2014/15 (n = 8)

Isolate	Collection date	Host	Specimen source
A/crane/Kagoshima/KU0.5014(H5N8)	23 November 2014	Sick white-naped crane	Tracheal and cloacal swabs
A/environment/Kagoshima/KU-ngr-H/2014(H5N8)	1 December 2014	NA[a]	Water sample
A/crane/Kagoshima/KU13/2014(H5N8)	7 December 2014	Dead hooded crane	Tracheal and cloacal swabs
A/crane/Kagoshima/KU21/2014(H5N8)	17 December 2014	Dead hooded crane	Tracheal and cloacal swabs
A/crane/Kagoshima/KU41/2014(H5N8)	24 December 2014	Dead hooded crane	Tracheal and cloacal swabs
A/crane/Kagoshima/KU53/2015(H5N8)	3 January 2015	Dead hooded crane	Tracheal and cloacal swabs
A/mallard duck/Kagoshima/KU70/2015(H5N8)	14 January 2015	Dead mallard duck	Conjunctival swab
A/mallard duck/Kagoshima/KU116/2015(H5N8)	13 February 2015	Dead mallard duck	Conjunctival swab

[a] NA, not applicable.

Table 2

Nucleotide identity of the H5N8 influenza A isolates and their closest relatives, Izumi plain, Japan, 2014/15 (n = 8)

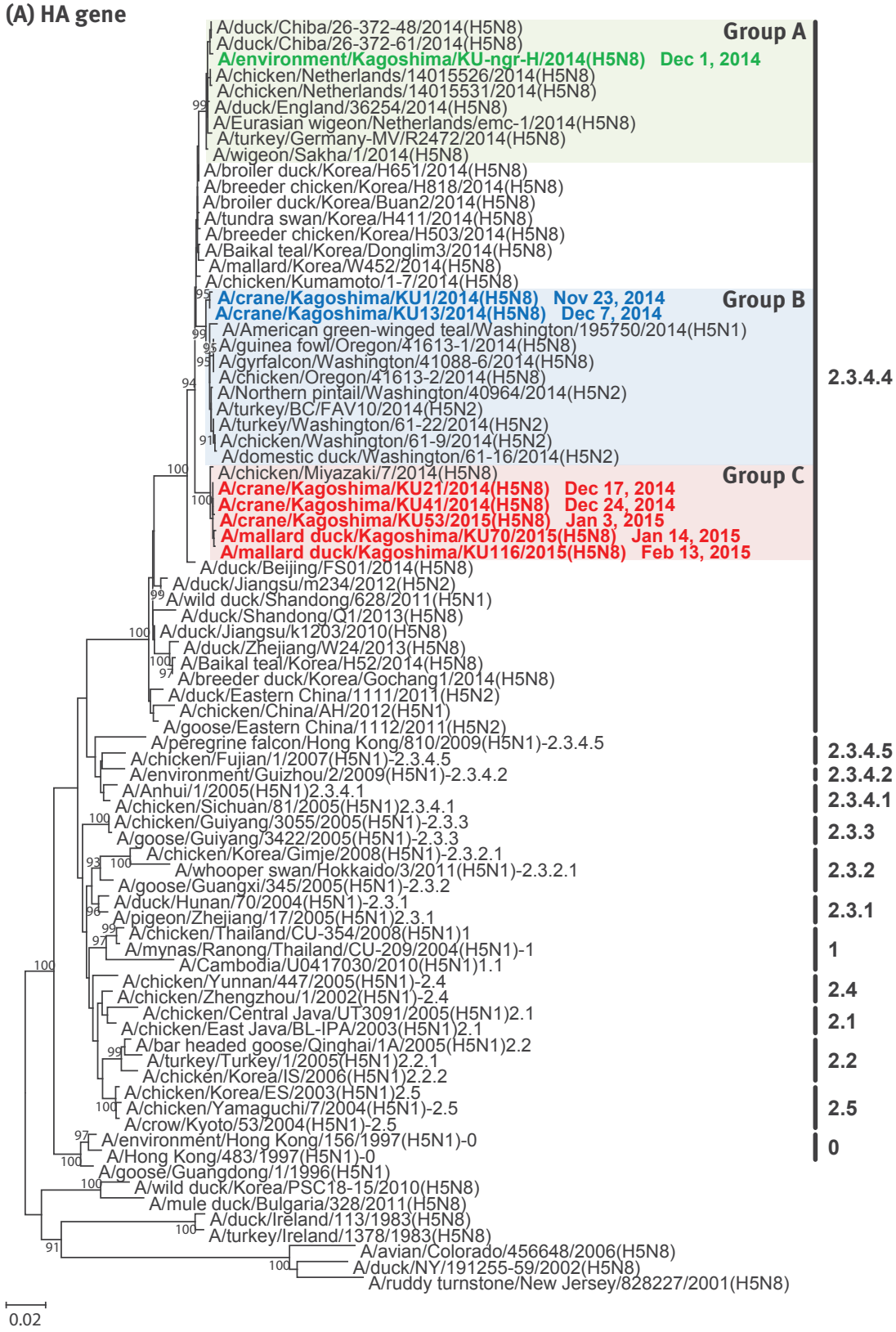
Isolate	Gene	Accession number[a]	Closest relative[b]	Identity (%)
A/crane/Kagoshima/KU0.5014(H5N8)	PB2	EPI553205	A/gyrfalcon/Washington/41088–6/2014(H5N8)	99.65
	PB1	EPI553206	A/gyrfalcon/Washington/41088–6/2014(H5N8)	99.43
	PA	EPI553207	A/Northern pintail/Washington/40964/2014(H5N2)	99.87
	HA	EPI553208	A/Northern pintail/Washington/40964/2014(H5N2)	99.29
	NP	EPI553209	A/Northern pintail/Washington/40964/2014(H5N2)	99.53
	NA	EPI553210	A/guinea fowl/Oregon/41613–1/2014(H5N8)	98.94
	M	EPI553211	A/Baikal teal/Korea/Donglim3/2014(H5N8)	100.00
	NS	EPI553212	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.76
A/environment/Kagoshima/KU-ngr-H/2014(H5N8)	PB2	EPI553359	A/duck/Chiba/26–372–61/2014(H5N8)	99.78
	PB1	EPI553360	A/duck/Chiba/26–372–61/2014(H5N8)	98.86
	PA	EPI553361	A/duck/Chiba/26–372–61/2014(H5N8)	99.81
	HA	EPI553362	A/duck/Chiba/26–372–61/2014(H5N8)	99.76
	NP	EPI553363	A/duck/Chiba/26–372–61/2014(H5N8)	99.87
	NA	EPI553364	A/turkey/Germany-MV/R2472/2014(H5N8)	98.86
	M	EPI553365	A/duck/Chiba/26–372–61/2014(H5N8)	100.00
	NS	EPI553366	A/duck/Chiba/26–372–61/2014(H5N8)	99.76
A/crane/Kagoshima/KU13/2014(H5N8)	PB2	EPI573635	A/gyrfalcon/Washington/41088–6/2014(H5N8)	99.52
	PB1	EPI573636	A/gyrfalcon/Washington/41088–6/2014(H5N8)	99.60
	PA	EPI573637	A/Northern pintail/Washington/40964/2014(H5N2)	99.44
	HA	EPI573638	A/Northern pintail/Washington/40964/2014(H5N2)	99.53
	NP	EPI573639	A/gyrfalcon/Washington/41088–6/2014(H5N8)	99.67
	NA	EPI573640	A/guinea fowl/Oregon/41613–1/2014(H5N8)	98.65
	M	EPI573641	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.80
	NS	EPI573642	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.76
A/crane/Kagoshima/KU21/2014(H5N8)	PB2	EPI573643	A/chicken/Miyazaki/7/2014(H5N8)	99.82
	PB1	EPI573644	A/chicken/Miyazaki/7/2014(H5N8)	99.69
	PA	EPI573645	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.86
	HA	EPI573646	A/chicken/Miyazaki/7/2014(H5N8)	99.71
	NP	EPI573647	A/breeder duck/Korea/H158/2014(H5N8)	99.73
	NA	EPI573648	A/chicken/Miyazaki/7/2014(H5N8)	99.79
	M	EPI573649	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NS	EPI573650	A/chicken/Miyazaki/7/2014(H5N8)	99.76
A/crane/Kagoshima/KU41/2014(H5N8)	PB2	EPI573651	A/chicken/Miyazaki/7/2014(H5N8)	99.82
	PB1	EPI573652	A/chicken/Miyazaki/7/2014(H5N8)	99.64
	PA	EPI573653	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.81
	HA	EPI573654	A/chicken/Miyazaki/7/2014(H5N8)	99.71
	NP	EPI573655	A/breeder chicken/Korea/H250/2014(H5N8)	99.73
	NA	EPI573656	A/chicken/Miyazaki/7/2014(H5N8)	99.72
	M	EPI573657	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NS	EPI573658	A/chicken/Miyazaki/7/2014(H5N8)	99.88
A/crane/Kagoshima/KU53/2015(H5N8)	PB2	EPI573661	A/chicken/Miyazaki/7/2014(H5N8)	99.78
	PB1	EPI573662	A/chicken/Miyazaki/7/2014(H5N8)	99.60
	PA	EPI573663	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.81
	HA	EPI573664	A/chicken/Miyazaki/7/2014(H5N8)	99.65
	NP	EPI573665	A/chicken/Miyazaki/7/2014(H5N8)	99.73
	NA	EPI573666	A/chicken/Miyazaki/7/2014(H5N8)	99.65
	M	EPI573667	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NS	EPI573668	A/chicken/Miyazaki/7/2014(H5N8)	99.88
A/mallard duck/Kagoshima/KU70/2015(H5N8)	PB2	EPI573669	A/chicken/Miyazaki/7/2014(H5N8)	99.60
	PB1	EPI573670	A/mallard/Korea/H297/2014(H5N8)	99.60
	PA	EPI573671	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.53
	HA	EPI573672	A/chicken/Miyazaki/7/2014(H5N8)	99.59
	NP	EPI573673	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NA	EPI573674	A/chicken/Miyazaki/7/2014(H5N8)	99.72
	M	EPI573675	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NS	EPI573676	A/chicken/Miyazaki/7/2014(H5N8)	99.76
A/mallard duck /Kagoshima/KU116/2015(H5N8)	PB2	EPI573677	A/chicken/Miyazaki/7/2014(H5N8)	99.56
	PB1	EPI573678	A/mallard/Korea/H297/2014(H5N8)	99.52
	PA	EPI573679	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.39
	HA	EPI573680	A/chicken/Miyazaki/7/2014(H5N8)	99.59
	NP	EPI573681	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NA	EPI573682	A/chicken/Miyazaki/7/2014(H5N8)	99.72
	M	EPI573683	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NS	EPI573684	A/chicken/Miyazaki/7/2014(H5N8)	99.88

[a] Accession numbers in the GISAID (http://platform.gisaid.org/) database are listed.

[b] Representative viruses with the highest nucleotide identity found in the GISAID and/or GenBank (http://www.ncbi.nlm.nih.gov/genbank/) databases on 23 March 2015 are listed. We thank the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database on which this research is based.

Figure 2A

Phylogenetic trees of the HA and NA genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)



HA: haemagglutinin; HPAIV: highly pathogenic avian influenza viruses; NA: neuraminidase.

The nucleotide sequences of the HA (A) and NA (B) genes from our H5N8 isolates were phylogenetically analysed with counterparts from other H5 and N8 subtype viruses, respectively, using the neighbour-joining method with a bootstrapping set of 1,000 replicates. Our isolates in Groups A, B and C are indicated in green, blue and red, respectively (see main text for details), with the dates of sampling. Bootstrap values of>90% are shown at the nodes. The scale bar indicates the number of nucleotide substitutions per site.

Figure 2B

Phylogenetic trees of the HA and NA genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)

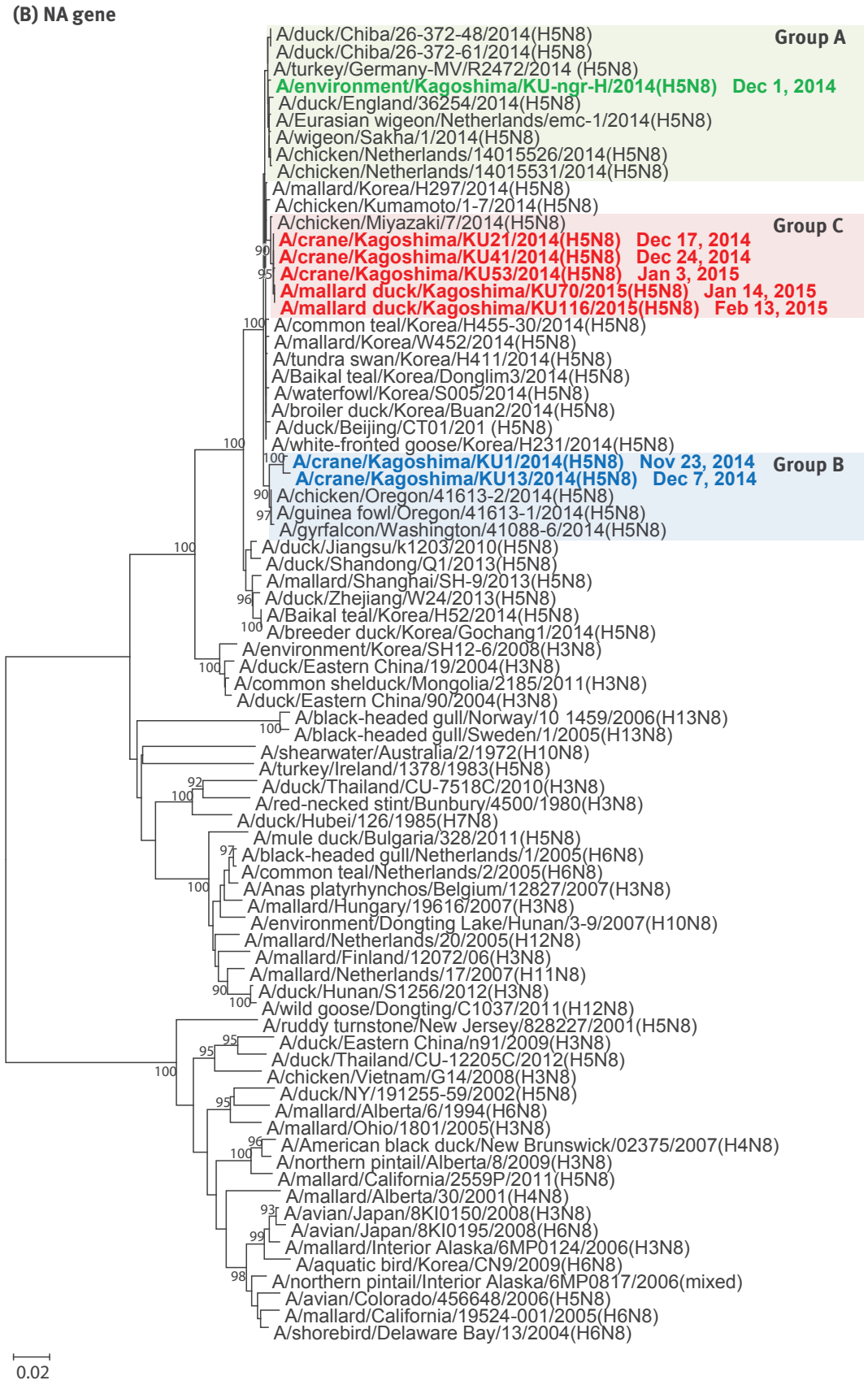


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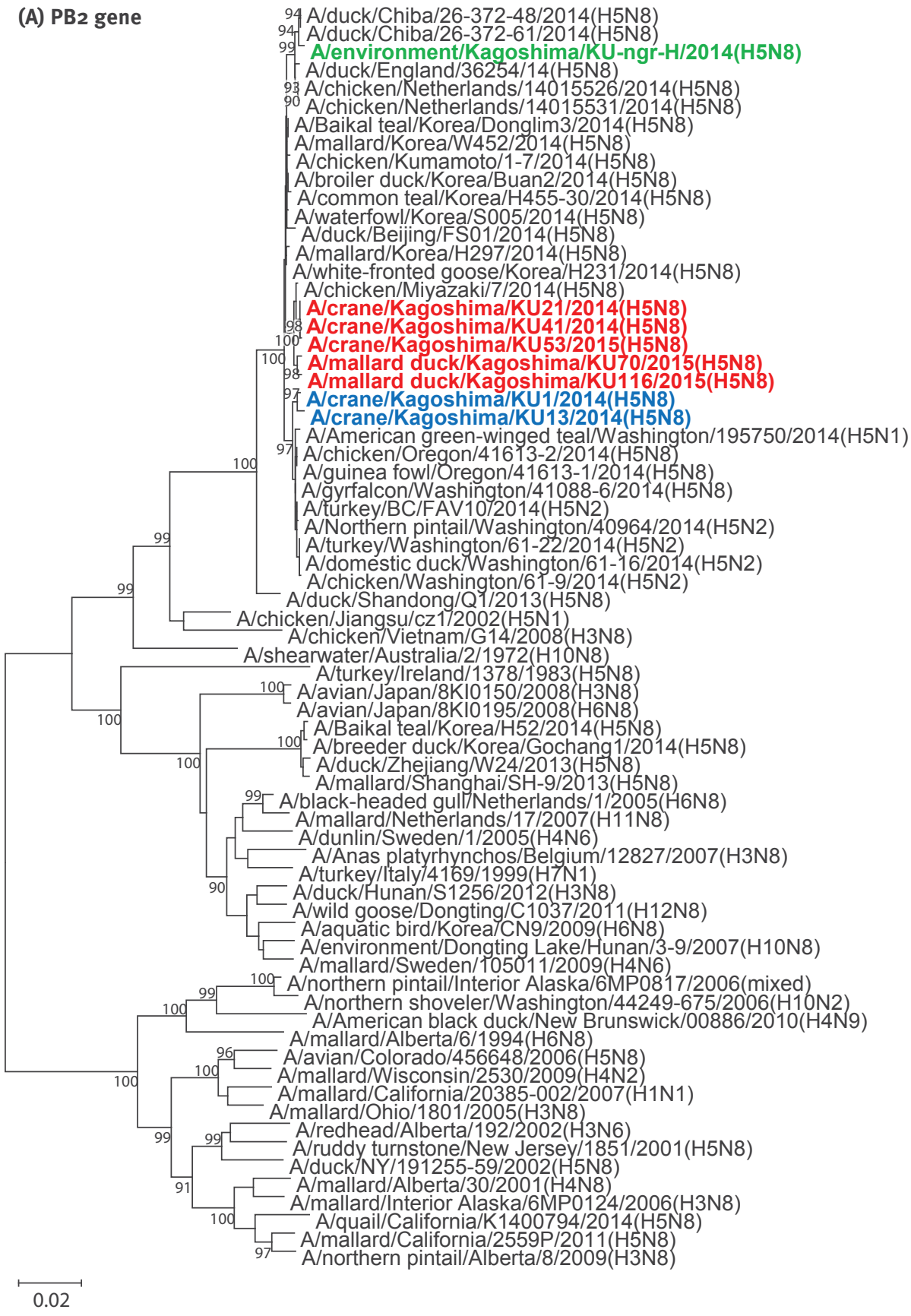
Figure 3A

Phylogenetic trees of six non-envelope genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)



HPAIV: highly pathogenic avian influenza viruses.

The nucleotide sequences of the PB2 (A), PB1 (B), PA (C), NP (D), M (E) and NS (F) genes from our H5N8 isolates were phylogenetically analysed with counterparts from the representative avian viruses of various subtypes by using the neighbour-joining method with a bootstrapping set of 1,000 replicates. Our isolates in Groups A, B and C are indicated in green, blue and red, respectively (see main text for details). Bootstrap values of >90% are shown at the nodes. The scale bar indicates the number of nucleotide substitutions per site.

Figure 3B

Phylogenetic trees of six non-envelope genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)

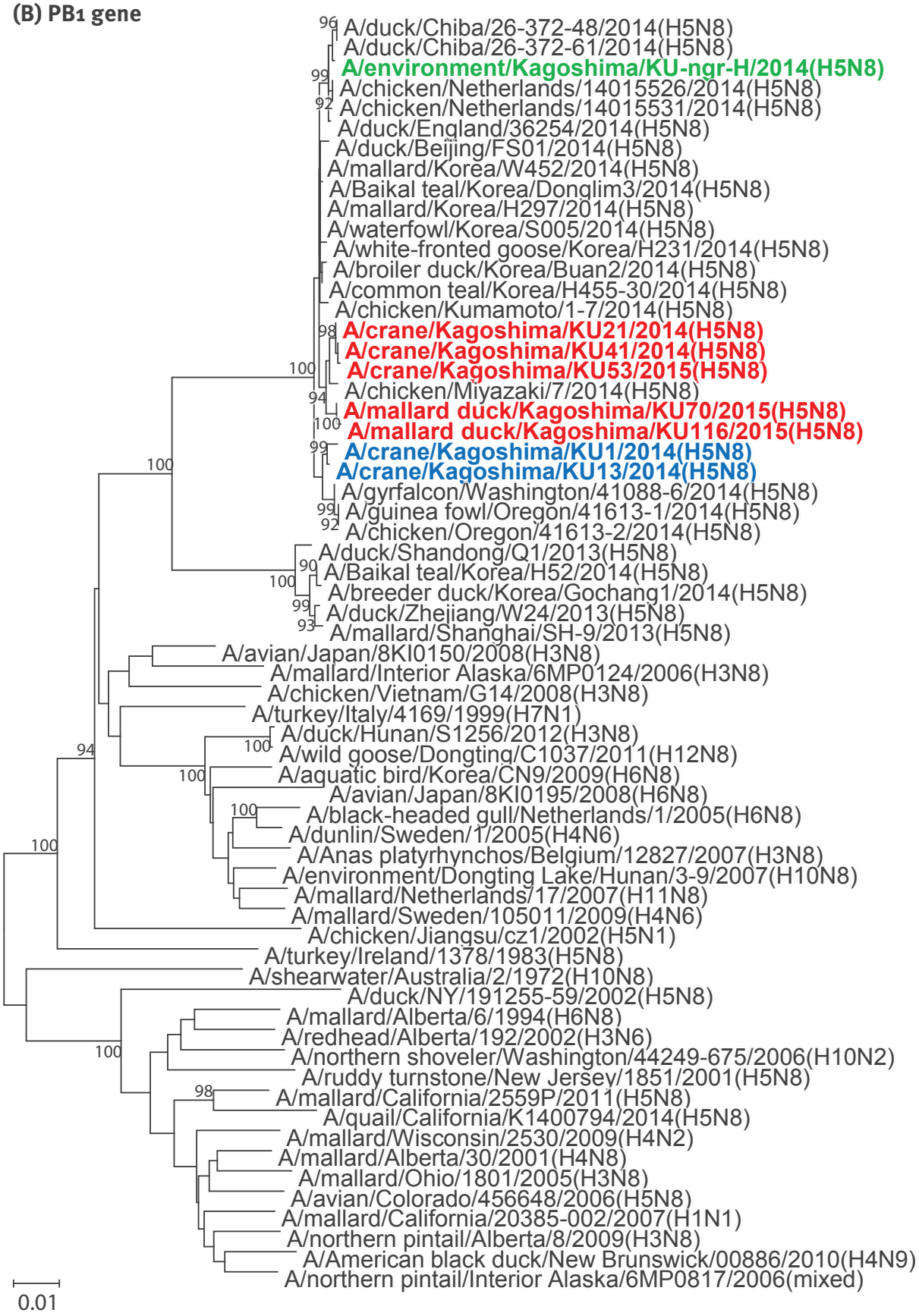


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Figure 3C

Phylogenetic trees of six non-envelope genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)

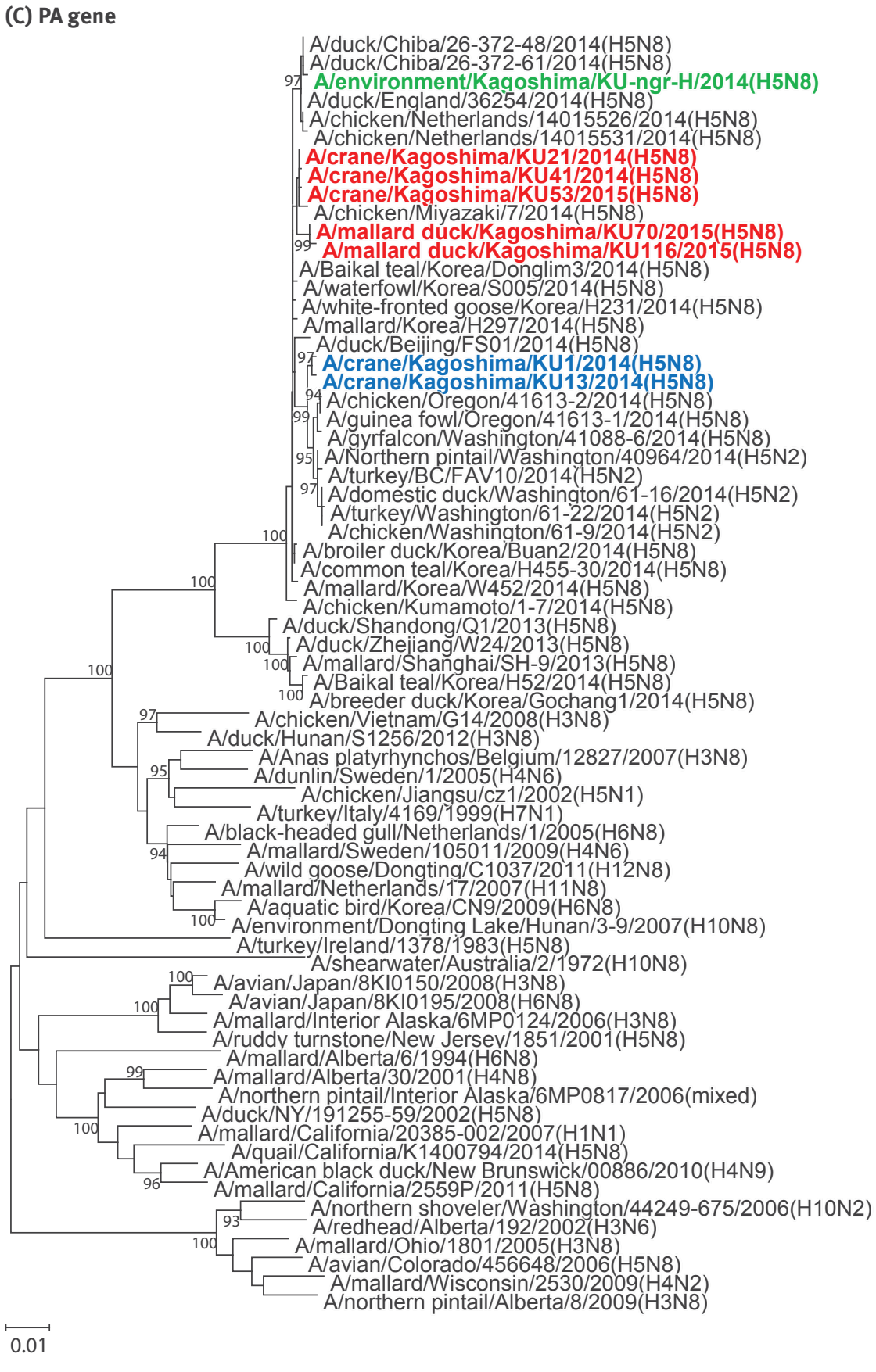

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Figure 3d

Phylogenetic trees of six non-envelope genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)

(D) NP gene

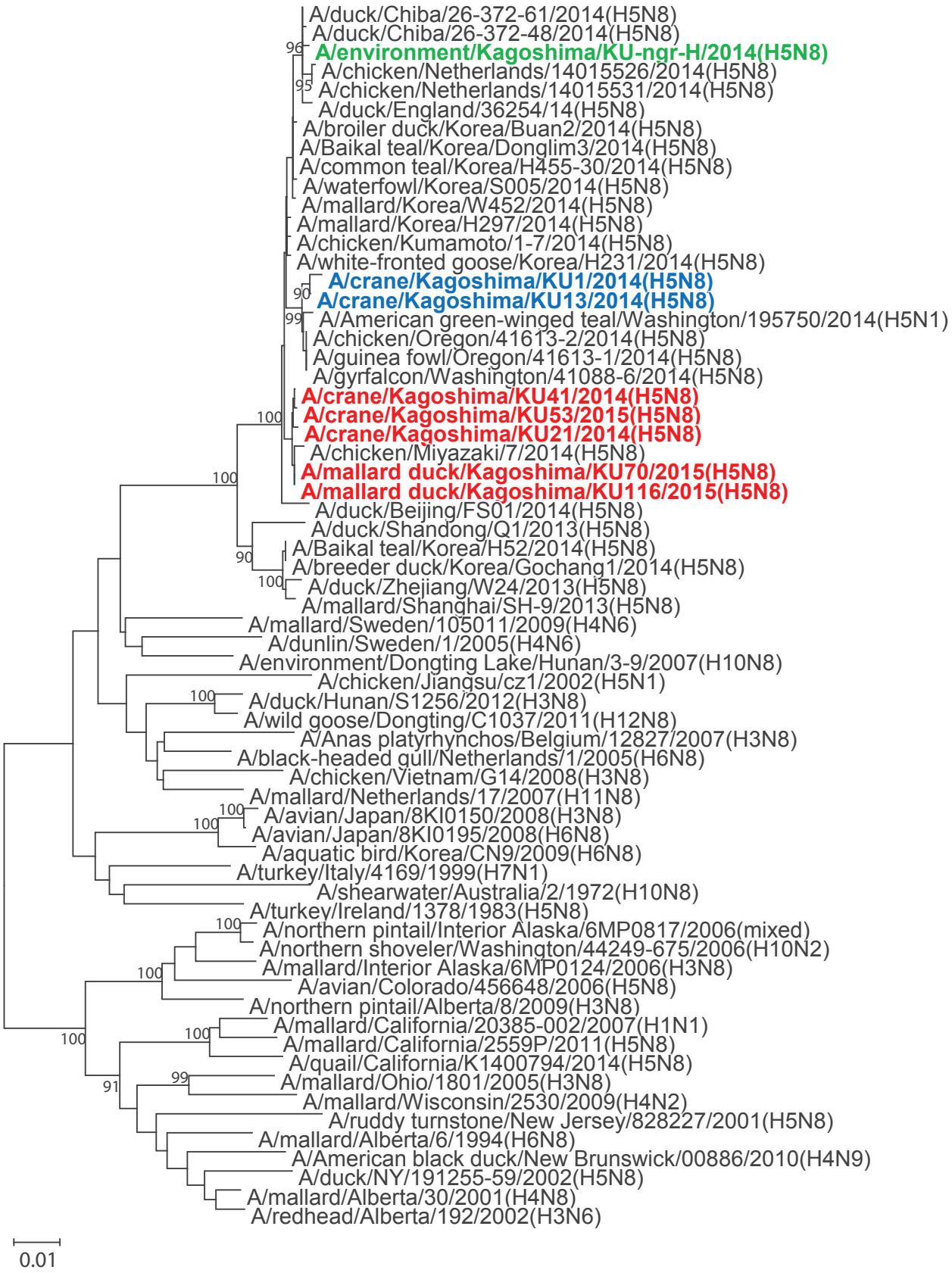

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Figure 3E

Phylogenetic trees of six non-envelope genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)

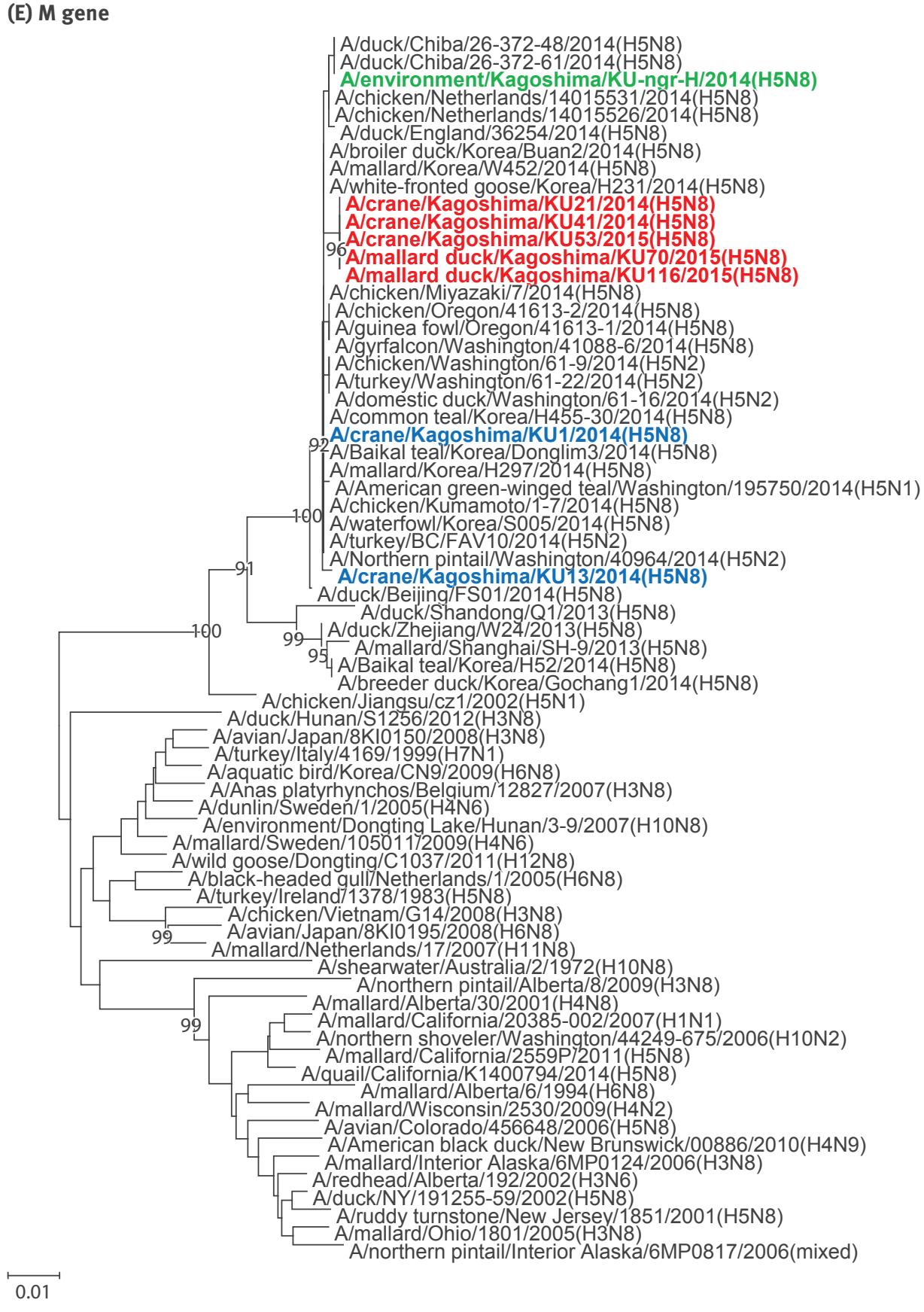

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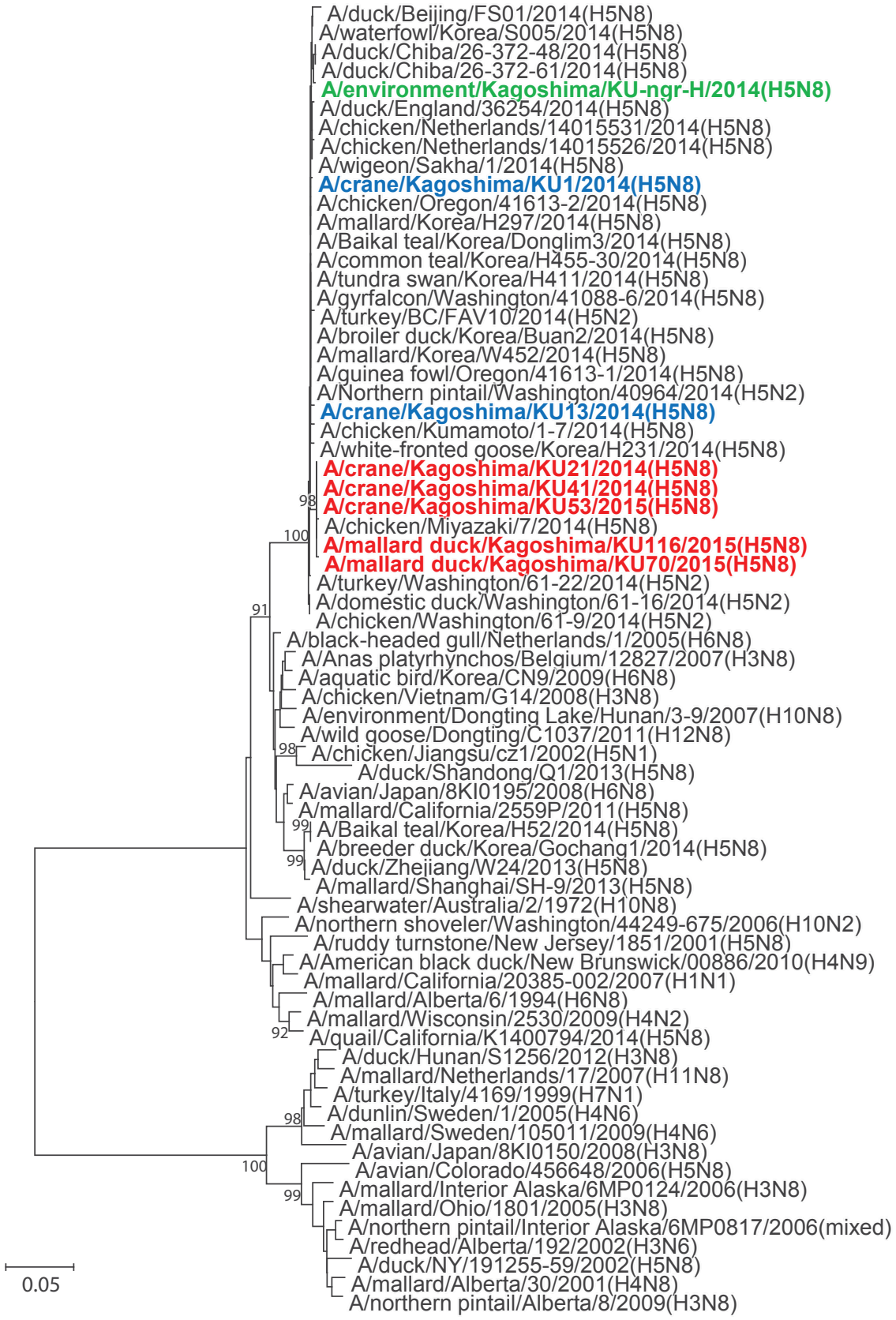
Figure 3F

Phylogenetic trees of six non-envelope genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)

(F) NS gene



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The nucleotide sequences of the PB2 (A), PB1 (B), PA (C), NP (D), M (E) and NS (F) genes from our H5N8 isolates were phylogenetically analysed with counterparts from the representative avian viruses of various subtypes by using the neighbour-joining method with a bootstrapping set of 1,000 replicates. Our isolates in Groups A, B and C are indicated in green, blue and red, respectively (see main text for details). Bootstrap values of >90% are shown at the nodes. The scale bar indicates the number of nucleotide substitutions per site.

Acknowledgments

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Conflict of interest

None declared.

Authors' contributions

Makoto Ozawa and Aya Matsuu designed the study; Makoto Ozawa, Aya Matsuu, Kaori Tokorozaki, Masayuki Horie, Tatsunori Masatani, Hiroko Nakagawa, Kosuke Okuya, Toshiko Kawabata, and Shigehisa Toda performed the experiments; Makoto Ozawa drafted the manuscript; Makoto Ozawa, Aya Matsuu, Kaori Tokorozaki, Masayuki Horie, and Tatsunori Masatani reviewed the manuscript.

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