

Genetic characteristics of highly pathogenic H5N8 avian influenza viruses isolated from migratory wild birds in South Korea during 2014–2015

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Abstract The continuous worldwide spread of highly pathogenic avian influenza (HPAI) H5N8 viruses among wild birds and poultry is a potential threat to public health. In the present study, we investigated the genetic characteristics of recent H5N8 viruses continuously isolated from migratory birds over two winters (2013–2014 and 2014–2015) in South Korea. Genetic and phylogenetic analysis demonstrated that the 2014–2015 HPAI H5N8 viruses are closely related to the 2013–2014 viruses, including virulence markers; however, all eight gene segments of 2014–2015 H5N8 viruses clustered in different phylogenetic branches from 2013–2014 H5N8 viruses, except the A/Em/Korea/W492/2015 virus. The H5N8 viruses of Europe and North America belong to sublineages of the 2013–2014 Korean H5N8 viruses but differ from the 2014–2015 Korean H5N8 viruses. Further hemagglutination inhibition (HI) assay results showed that there were 2-to-4 fold differences in HI titer between 2013–2014 and 2014–2015 H5N8 viruses. Taken together, our results suggested that the 2014–2015 Korean H5N8 viruses were genetically and serologically different from those of 2013–2014 winter season H5N8 viruses, including those from Europe and North America.

Introduction

Highly pathogenic avian influenza (HPAI) H5 viruses have been continuously isolated from wild birds and domestic poultry since 1996 [1, 2]. These viruses cause high mortality, resulting in serious economic losses in the poultry industry [3]. After first being identified in Southeast Asia, the H5N1 virus spread across broad regions of Eurasia and Africa [4, 5]. The spread of H5N1 virus was not only confined to animals, as it also infected humans with a case fatality rate of approximately 59 % [6]. While the human fatality rate of this virus is quite high, human-to-human transmission of the HPAI A/H5N1 virus is not yet efficient and will require the virus to mutate or exchanges genes with a circulating human influenza virus [7]. Eradication of HPAI-virus-infected poultry is a temporary measure to prevent virus exposure to humans, but migratory ducks and geese are asymptotically infected with these avian influenza viruses, making it impossible to completely avoid contact with the virus [8, 9]. Thus, active surveillance and monitoring of HPAI viruses with subsequent development of HPAI vaccines is considered to be the optimal strategy for the prevention and control of an influenza pandemic.

In addition to HPAI A/H5N1 outbreaks, a newly-emerged highly pathogenic avian influenza (HPAI) A/H5N8 virus caused poultry outbreaks in the Republic of Korea (Korea) in mid-January 2014 [9]. Despite control measures on A/H5N8-infected farms, the virus still managed to cause sporadic outbreaks in South Korea, resulting in the culling of more than 16 million poultry within a short period of time. Furthermore, the HPAI A/H5N8 viruses spread to Europe and North America, where they were detected in domestic and wild birds [8]. Although no cases of human infection with A/H5N8 have

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been detected, the wide spread of novel A/H5N8 viruses by wild migratory birds is a growing concern for public health.

Since the huge HPAI A/H5N8 outbreak in domestic poultry in South Korea during 2013-2014, additional HPAI A/H5N8 virus outbreaks have mainly occurred in wild birds in the 2004-2015 winter seasons. In this study, we analyzed the genetic characteristics of HPAI A/H5N8 viruses isolated from wild birds during the 2014-2015 winter seasons and compared them with those of 2013-2014 HPAI A/H5N8 viruses. Further, we carried out a hemagglutination inhibition (HI) assay to investigate the serologic relationships between the H5N8 viruses from two winters (2013-2014 and 2014-2015).

Materials and methods

Viruses

The HPAI A/H5N8 viruses were isolated from wild bird fecal samples taken during the winter seasons of 2013-2014 ($n = 5$) and 2014-2015 ($n = 21$) and grown in specific-pathogen-free (SPF) 10-day-old embryonated chicken eggs. Supernatants (allantoic fluids and cell culture) were harvested at 48 hours postinfection (hpi), aliquoted into cryo vials (1 mL each), and stored at -80°C until use. The HPAI H5N8 virus, A/Mallard Duck/Korea/W452/2014 (MDk/W452/2014 (2013-2014 isolate), was included in this study as a standard strain [10].

Genomic sequencing and phylogenetic analysis

Sequences were prepared and analyzed as described previously [10]. Briefly, gene sequences of H5N8 viruses were obtained by Cosmo Genetech (Seoul, Korea) using an ABI 3730XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed and compiled with DNASTar 5.0 (DNASTAR, Madison, WI, USA); closely related viruses were identified using Basic Local Alignment SearchTool (BLAST). Phylogenetic trees were built by aligning published reference avian influenza virus sequences obtained from wild birds, domestic poultry and humans that are available in GenBank together with the closely related avian virus sequences obtained from the BLAST results. Complete genome sequences were aligned in Clustal X [11, 12], and phylogenetic trees were generated using Bayesian Markov chain Monte Carlo coalescent analysis to investigate the recent evolutionary history of HPAI A/H5N8 in South Korea [13]. Using the HKY model and 40 million steps of MCMC, different models of population dynamics were tested (constant population size, exponential population growth, expansion population

growth, logistic population growth, and Bayesian skyline). The uncorrelated exponential molecular clock was selected according to comparison of Bayes factors with estimates obtained for strict and uncorrelated lognormal local clocks. Statistical uncertainty in the data was reflected by the 95 % highest probability density (HPD) values. Results were examined using the TRACER v1.6 program from the BEAST package [13]. Convergence was assessed with ESS (effective sample size) values after a burn-in of 4 million steps. Models were compared by calculating the Bayes factor (BF) from the posterior output of each of the models using the TRACER v1.6 program as explained on the BEAST website http://beast.bio.ed.ac.uk/Model_comparison. A log BF (natural log units) >2.3 indicates strong evidence against the null model [13, 14]. Maximum-clade-credibility trees were generated using Tree Annotator from the BEAST package, and FigTree v1.4.3 (available at <http://tree.bio.ed.ac.uk>) was used for visualization of the annotated trees. Complete sequences of the eight viral gene segments of all viruses collected in this study were submitted to GenBank. Accession numbers assigned to the sequences determined in this study are KX297862-KX298061.

Antigenic analysis

Hyperimmune sera were obtained from 5-week-old BALB/c mice that had been immunized with representative A/H5N8 viruses (Table 3). To investigate the cross-reactivity between H5N8 viruses from two winters (2013-2014 and 2014-2015), we performed a hemagglutination inhibition (HI) assay as described previously [15] using antibodies against two variants of 2013-2014 isolates (A/MDk/Korea/W452/2014 and A/MDk/Korea/W456/2014) and four variants of 2014-2015 viruses (A/Em/Korea/W468/2014, A/Em/Korea/W483/2015, A/Em/Korea/W488/2015, and A/Em/Korea/W492/2015).

Ethics statement

All animal experiments including serologic testing were carried out in an enhanced biosafety level 3 (BSL-3+) facility at Chungbuk National University (approval no.: BSL-ABSL-15-003).

Procedures for the use of mice were carried out according to relevant policies regarding animal handling as mandated under the Guidelines for Animal Use and Care of the Korea Center for Disease Control (K-CDC), which was approved by the Medical Research Institute and by the Laboratory Animal Research Center (approval no.: CBNUA-767-14-11), which is a member of the Institutional Animal Care and Use Committee of Chungbuk National University.

Results

Isolation and sequencing of HPAI H5N8 viruses from wild birds

During this study, a total 26 (5 in 2013-2014 and 21 in 2014-2015) H5N8 viruses were isolated over two winters from migratory birds in South Korea (Table 1). During the 2013-2014 winter season, two viruses were isolated from deceased migratory birds that were found in common migratory bird habitats. In contrast, all 2014-2015 H5N8 viruses were isolated from fecal samples from migratory birds. The first 2013-2014 virus was isolated January 17, 2014, just after large outbreaks of H5N8 virus reportedly occurred in domestic poultry [10, 16], and during the portion of the season when most migratory birds had just started to move back to Mongolia and Siberia. However, the 2014-2015 H5N8 viruses were mainly isolated in mid-season (December 2014 to January of 2015), except A/Em/Korea/W492/2015, which was isolated on February 27,

2015. To determine the genetic origin of these two different seasonal H5N8 viruses, whole-genome sequencing was performed on all isolates. The sequencing results revealed that the genome segments of H5N8 viruses were quite homologous within the same winter (98.9 to 100 %), while relatively low homologies (97.6 to 99.0 %) were observed between 2013-2014 and 2014-2015 H5N8 viruses.

Molecular analysis

Genetic analysis revealed that all H5N8 viruses were HPAI viruses that contained the RERRRKRGLF cleavage site within their HA protein (Table 2). Protein sequence analysis of 2014-2015 H5N8 viruses revealed relatively high homology to the first 2013-2014 season H5N8 viruses (97.6 to 99.0 %); however, in the 2014-2015 viruses, the specific amino acid substitutions N100K, P148Y, K165E, and R499G were observed in HA, in addition to PA_{V379M}, NP_{S492I}, and NS1_{V180I}, events that have not been

Table 1 List of H5N8 viruses isolated from migratory birds over two winters (2013-2014 and 2014-2015) in South Korea

Isolation season	Virus name	HPAI subtype	Isolation date	Isolation region	Sample type	Genetic group
2013-2014	A/MDk/Korea/W452/2014	H5N8	2.5	CB	Feces	A
	A/Em/Korea/W454/2014	H5N8	2.6	CB	Feces	A
	A/MDk/Korea/W456/2014	H5N8	2.14	CB	Cloacal swabs	A
	A/MDk/Korea/W457/2014	H5N8	2.14	CB	Cloacal swabs	A
	A/Em/Korea/W458/2014	H5N8	2.18	CN	Carcass	A
2014-2015	A/Em/Korea/W464/2014	H5N8	12.21	CN	Feces	B
	A/Em/Korea/W465/2014	H5N8	12.21	CN	Feces	B
	A/Em/Korea/W466/2014	H5N8	12.21	CB	Feces	B
	A/Em/Korea/W467/2014	H5N8	12.21	CB	Feces	B
	A/Em/Korea/W468/2014	H5N8	12.21	CN	Feces	B
	A/Em/Korea/W469/2014	H5N8	12.21	CN	Feces	B
	A/Em/Korea/W470/2014	H5N8	12.21	CN	Feces	B
	A/Em/Korea/W471/2014	H5N8	12.21	CN	Feces	B
	A/Em/Korea/W472/2014	H5N8	12.21	CB	Feces	B
	A/Em/Korea/W473/2014	H5N8	12.21	CB	Feces	B
	A/Em/Korea/W474/2014	H5N8	12.21	CN	Feces	B
	A/Em/Korea/W475/2014	H5N8	12.21	CN	Feces	B
	A/Em/Korea/W476/2014	H5N8	12.21	CN	Feces	B
	A/Em/Korea/W477/2014	H5N8	12.21	CN	Feces	B
	A/Em/Korea/W482/2015	H5N8	1.14	GG	Feces	B
	A/Em/Korea/W483/2015	H5N8	1.14	CB	Feces	B
	A/Em/Korea/W486/2015	H5N8	1.21	GG	Feces	B
	A/Em/Korea/W487/2015	H5N8	1.21	CB	Feces	B
	A/Em/Korea/W488/2015	H5N8	1.21	GG	Feces	B
	A/Em/Korea/W490/2015	H5N8	1.21	GG	Feces	B
	A/Em/Korea/W492/2015	H5N8	2.27	CB	Feces	A

JB Jeonbuk province, *CB* Chungbuk province, *CB* Chungnam province, *GG* Gyeonggi province, *Em* environment, *MDk* mallard duck

Table 2 Molecular characterization of 2014 to 2015 avian H5N8 influenza viruses

Virus name	HA ^a										NA ^b										M2	NS1
	Cleavage site					Receptor-binding site (highly conserved residues)					NP					PB2 Virulence marker						
	327-333	100	148	165	222	224	226	499	40	202	274	292	492	379	63	66	627	701	31	180	C-terminus	
A/BTI/Donglim3/2014	REKRR_KR/GLF	N	F	K	Q	N	Q	R	S	V	H	R	S	V	S	S	E	D	N	V	ESEVRGNGKMAD	
A/MDK/Korea/W452/2014(5) ^c	REKRR-KR/GLF	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	
A/Ck/Kumamoto/1-7/2014	RERRR_KR/GLF	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	
A/EWn/Netherlands/1/2014	RERRR_KR/GLF	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	
A/GFI/Oregon/41613-1/2014	RERRR_KR/GLF	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	
A/Em/Korea/W492/2015	RERRR_KR/GLF	•	•	•	•	•	•	G	•	•	•	•	•	•	F	•	•	•	•	•	•	
A/Em/Korea/W468/2014(14)	REKRR-KR/GLF	K	Y	E	•	•	•	G	G	I	•	•	I	M	F	•	•	•	•	I	•	
A/Em/Korea/W483/2015(2)	REKRR-KR/GLF	K	Y	E	•	•	•	G	G	I	•	•	I	M	F	•	•	•	•	I	•	
A/Em/Korea/W488/2015(4)	RERRR_KR/GLF	K	Y	E	•	•	•	G	G	I	•	•	I	M	F	•	•	•	•	I	•	

Deduced amino sequences were used to compare and examine known virulence markers and pathogenicity determinants

B71 Baikal teal, *Ck* chicken, *Em* environment, *EWn* Eurasian wigeon, *GFI* guinea fowl, *MDK* mallard duck

^a H3 numbering

^b N2 numbering

^c The number of viruses showing the same molecular characteristics

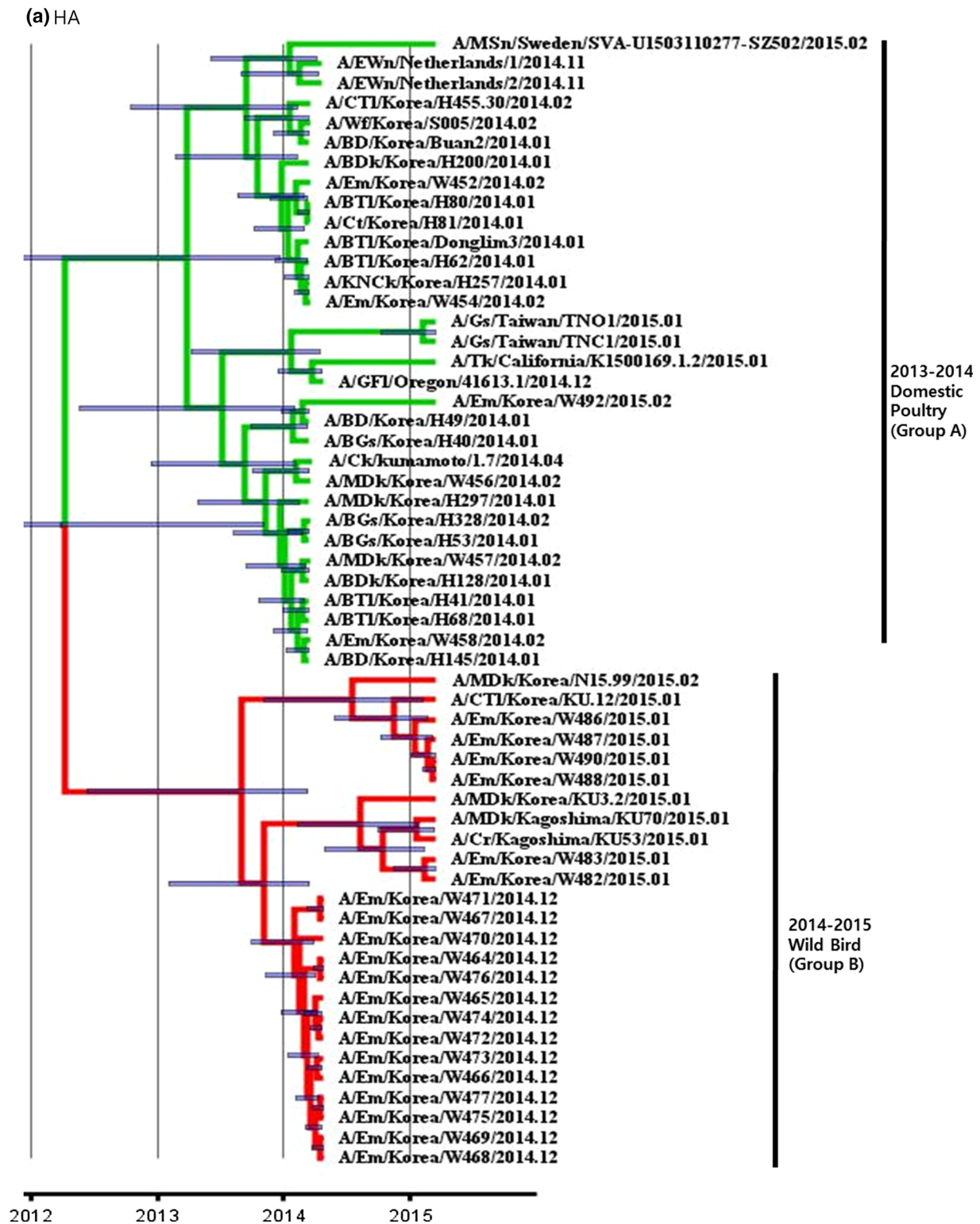


Fig. 1 Maximum-clade-credibility trees of HA (a) and NA (b) for HPAI H5N8 viruses isolated from migratory birds in the two winter seasons of 2013 to 2015 and compared with nucleotide sequences of H5N8 strains available in GenBank. Time-scaled phylogenies (dates shown on the horizontal axis) were inferred using strict-clock Bayesian Markov chain Monte Carlo analysis. Times of most recent common ancestors with 95 % highest posterior density intervals are indicated by horizontal bars at each

node. The two distinct 2013-2014 and 2014-2015 Korean H5N8 lineages are indicated in group A (green lines) and group B (red lines). The dates indicate the month of sampling. Abbreviations: BTI, Baikal teal; BGs, bean goose; BDk, breeder duck; BD, broiler duck; Ck, chicken; CTI, common teal; Ct, coot; Cr, crane; Em, environment; EWn, Eurasian wigeon; Gs, goose; GFI, guinea fowl; Gf, gyrfalcon; KNCK, Korean native chicken; MDK, mallard duck; MSn, mute swan; Tk, turkey; Wf, waterfowl

(b) NA

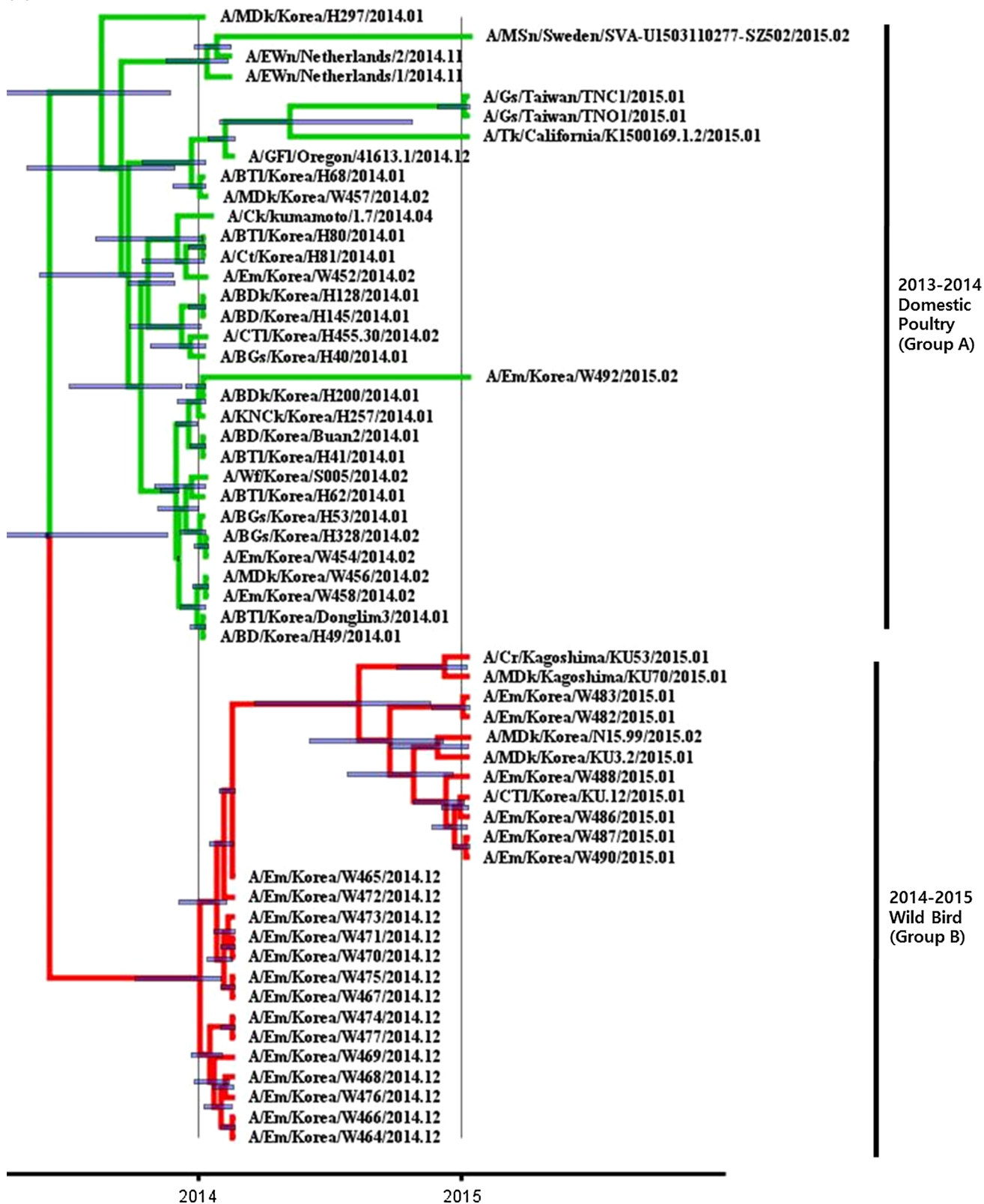


Fig. 1 continued

previously reported in other H5N8 viruses (Table 2). All Korean HPAI H5N8 isolates maintained a glutamine residue at position 222 (226 H3 numbering) and a glycine residue at position 224 (228 H3 numbering), suggestive of preferential binding to α -2,3-linkage sialic acid receptors for influenza viruses of avian species [17].

In addition, while all H5N8 viruses exhibited characteristics of low-pathogenic viruses in their PB2 genes (glutamic acid [E] and aspartic acid [D] at position 627 and 701, respectively), they had functional PB1-F2 proteins (PB1-F2_{66S}), which have been shown to affect host defense mechanisms and, in turn, enhance pathogenicity in mammalian hosts [18]. Further, the non-structural 1 (NS1) protein in these viruses harbored an NS 42S mutation associated with increased virulence in mice [19], and seven additional amino acid residues (ESEVRGNKMAD) were observed in the C-terminal region. Regarding antiviral resistance, all of the H5N8 viruses were predicted to be resistant to amantadine-derived compounds due to a matrix 2 ion-channel protein 31_{Asn} mutation, but to have retained their sensitivity to NA inhibitors (Table 2) [20, 21]. No other mammalian-adaptive molecular determinants were observed in the viral genome [22, 23].

Phylogenetic analysis

Phylogenetic trees were constructed for each gene segment to investigate the evolutionary relationships between the 2013-2014 (group A) and 2014-2015 (group B) H5N8 viruses, including the recent spread of HPAI H5N8 viruses in the wild (Figs. 1, 2). Phylogenetic analysis of the HA genes showed that all 2014-2015 H5N8 viruses belonged to clade 2.3.4.4 of HPAI H5 viruses but were on separate branches from the 2013-2014 H5N8 viruses, with the exception of the A/Em/Korea/W492/2015(H5N8) isolate (Fig. 1a). Furthermore, the NA genes of 2014-2015 H5N8 viruses, except the A/Em/Korea/W492/2015(H5N8) isolate, clustered together but formed a different branch from the 2013-2014 H5N8 viruses (Fig. 1b). However, all HA and NA genes of 2013-2014 H5N8 viruses were closely related to A/MDk/Korea/W452/2014-like virus and clustered together with Korean domestic-poultry isolates, which continued to spread widely to Europe and North America (Fig. 1). It is noteworthy that all gene segments of A/Em/Korea/W492/2015 were more closely related to 2013-2014 winter-season H5N8 viruses than to the 2014-2015 H5N8 viruses.

Similar to the surface genes, phylogenetic analysis of the other internal genes revealed separation between 2014-2015 viruses and 2013-2014 HPAI H5N8 viruses (Fig. 2a-f). Of the 2014-2015 viruses, A/Em/Korea/W486/2015, A/Em/Korea/W487/2015, A/Em/Korea/W488/2015, and A/Em/Korea/W490/2015 clustered together throughout

the 2014-2015 gene segments (Fig. 2a-f). In addition, the M genes of three 2014-2015 isolates (A/Em/Korea/W467/2014, A/Em/Korea/W470/2014, and A/Em/Korea/W471/2014) clustered separately from the other 2014-2015 H5N8 viruses (Fig. 2e). Thus, these results clearly demonstrate that all 2015 HPAI H5N8 viruses, except the A/Em/Korea/W492/2015 isolate, originated from genetic gene pools of HPAI H5N8 viruses that differed from the 2013-2014 Korean, European, and North American HPAI H5N8 viruses. However, the A/Em/Korea/W492/2015 isolate was phylogenically closely related to 2013-2014 winter-season H5N8 viruses.

Antigenic cross-reactivity

Based on phylogenic and molecular analysis of the HA gene, we could distinguish at least seven different variants: two variants of 2013-2014 isolates (A/MDk/Korea/W452/2014 and A/MDk/Korea/W456/2014) and four variants of 2014-2015 viruses (A/Em/Korea/W468/2014, A/Em/Korea/W483/2015, A/Em/Korea/W488/2015, and A/Em/Korea/W492/2015) (Fig. 1a; Table 2). To investigate whether these differences lead to serological differences between the variants, we performed HI assays using sera from mice infected with each virus. Each virus showed the highest HI titer against the homologous strain, but there were 2- to 4-fold decreases in cross-reactivity between the 2013-2014 and 2014-2015 H5N8 viruses (Table 3). Specifically, the antisera of A/Em/Korea/W468/2014 and A/Em/Korea/W483/2015 viruses exhibited approximately 4-fold differences against 2013-2015 viruses, including the A/Em/Korea/W492/2015 virus. Although the A/Em/Korea/W492/2015 virus was isolated in the 2014-2015 winter season, the antiserum showed a 4-fold difference in HI titers against 2014-2015 H5N8 viruses.

Discussion

Since the first report of HPAI A/H5N8 virus outbreaks in South Korea in early 2014, outbreaks of the same H5N8 subtype were reported in poultry and wild birds in Europe, Asia and North America during the winter of 2014-2015 [16, 24-26]. Furthermore, South Korea also experienced another H5N8 outbreak in wild birds and domestic poultry during the winter of 2014-2015 [27]. In this study, we compared the genetic characteristics of HPAI A/H5N8 viruses isolated from these seasons (2013-2014 and 2014-2015 winter seasons) and compared them with other HPAI A/H5N8 reported outside of South Korea. Molecular and phylogenetic studies demonstrated that all of the 2013-2014 and 2014-2015 H5N8 viruses clustered with A/Mallard Duck/Korea/W452/2014-like virus (clade

(a) PB2

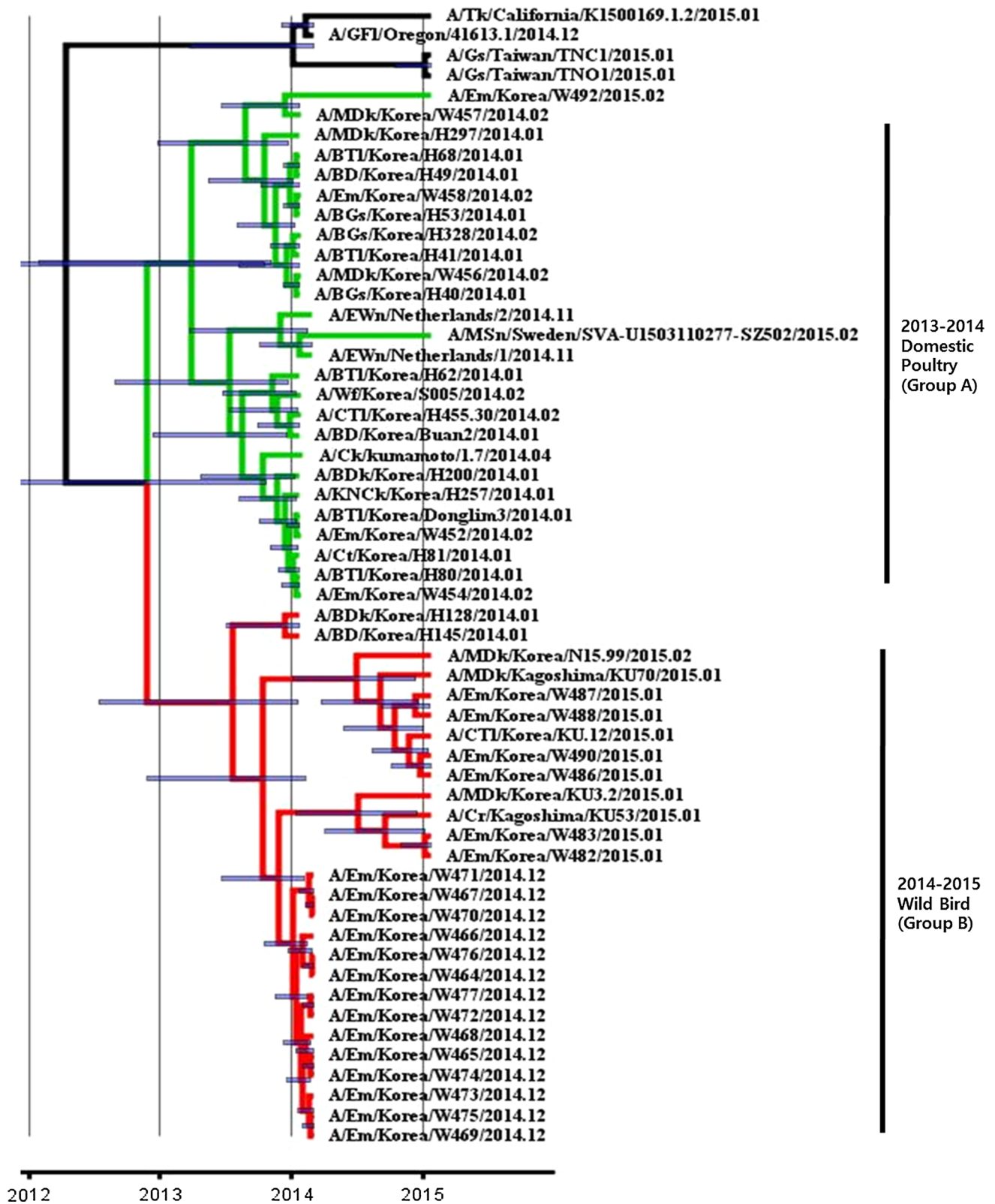


Fig. 2 Maximum-clade-credibility trees of the PB2 (a), PB1 (b), PA (c), NP (d), M (e), and, NS (f) genes of HPAI H5N8 viruses isolated from migratory birds in the two winter seasons of 2013 to 2015 and compared with nucleotide sequences from H5N8 strains available in GenBank. Time-scaled phylogenies (dates shown on the horizontal axis) were inferred using strict-clock Bayesian Markov chain Monte Carlo analysis. Times of most recent common ancestors with 95 % highest posterior density intervals are indicated by horizontal bars at each node. The two distinct 2013-2014 and 2014-2015 Korean H5N8 lineages are indicated in group A (green lines) and group B (red lines). The dates indicate the month of sampling. Abbreviations: BTI, Baikal teal; BGs, bean goose; BDK, breeder duck; BD, broiler duck; Ck, chicken; CTL, common teal; Ct, coot; Cr, crane; Em, environment; EWn, Eurasian wigeon; Gs, goose; GFL, guinea fowl; Gf, gyrfalcon; KNCK, Korean native chicken; MDK, mallard duck; MSN, mute swan; Tk, turkey; Wf, waterfowl

2.3.4.4) and Korean domestic poultry H5N8 viruses. Furthermore, with the exception of A/Em/Korea/W492/2015, the 2014-2015 viruses clustered into a novel branch separated from the Korean 2013-2014 H5N8 viruses and the European/North American viruses (Figs. 1, 2). In addition, sequence analysis showed that specific amino acid substitutions were observed throughout all eight gene segments of the 2014-2015 viruses (Table 2). Interestingly, only A/Em/Korea/W492/2015 shared the same molecular characteristics with 2013-2014 viruses and was phylogenetically more closely related to 2013-2014 winter-season H5N8 viruses than to the other Korean 2014-2015 H5N8 viruses (Figs. 1, 2; Table 2), suggesting that this particular virus could be a descendent of 2013-2014 H5N8 viruses, or that a wild bird might have been infected with virus from domestic poultry harboring H5N8 viruses. Since sequence information on the H5N8 viruses from 2014-2015 domestic poultry is unavailable in GenBank, detailed analysis of the relationship between A/Em/Korea/W492/2015 and domestic poultry H5N8 viruses is still pending. Furthermore, we cannot define the specific origins of the 2014-2015 H5N8 viruses at this point. When we conducted

BLAST analysis with each gene segment of 2014-2015 viruses, the most closely related viruses were the 2013-2014 Korean viruses. However, based on the results of time-scaled phylogenetic trees, they were differently clustered as group A and group B (Figs. 1, 2), and these different phylogenetic groups might have diverged around late 2012 to early 2013, which was at least one year earlier than the first report of HPAI/H5N8 viruses in South Korea [10, 27]. Nevertheless, our results demonstrate that the surface genes of the 2014-2015 H5N8 viruses in Asia, Europe and North America were sublineages of Korean 2013-2014 HPAI H5N8 viruses but different from the Korean 2014-2015 H5N8 viruses. In addition, some of the spread viruses evolved further by genetic reassortment with pre-existing avian influenza viruses and generated more diverse subtypes and genotypes [14, 24, 27, 28]. Taken together, our results suggest that the 2014-2015 Korean HPAI H5N8 viruses were newly introduced into South Korea sometime early in the winter of 2014-2015 by migratory birds, and were not simply a reintroduction of older 2013-2014 Korean HPAI H5N8-like viruses (Table 2; Figs. 1, 2).

In addition, serological analysis revealed 2- to 4-fold differences in HI titers between 2013-2014 and 2014-2015 viruses (Table 3). Although at least a 4-fold difference would be expected between different serotypes according to the common definition of significant HI titer differences, all antisera showed relatively high cross-reactive HI titers (160-640 HI units), suggesting that the H5N8 viruses tested in this study were still the same serogroup.

This study highlights the role of migratory birds in the perpetuation and spread of HPAI A/H5N8 viruses in South Korea. With the changing pathobiology among wild and poultry birds caused by H5 viruses, continued surveillance of influenza viruses among migratory bird species remains crucial for effective monitoring of high-pathogenicity and pandemic influenza viruses.

(b) PB1

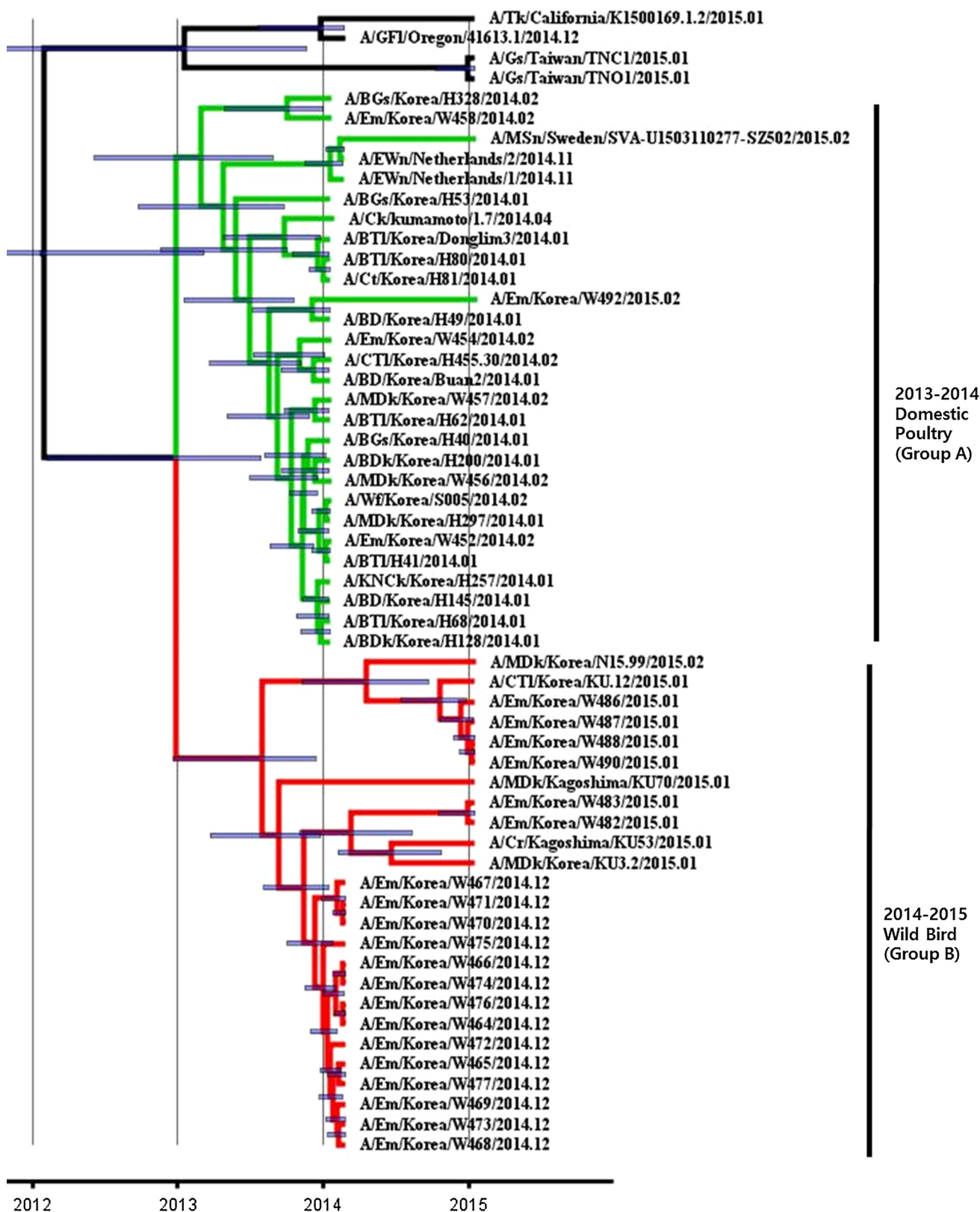


Fig. 2 continued

(c) PA

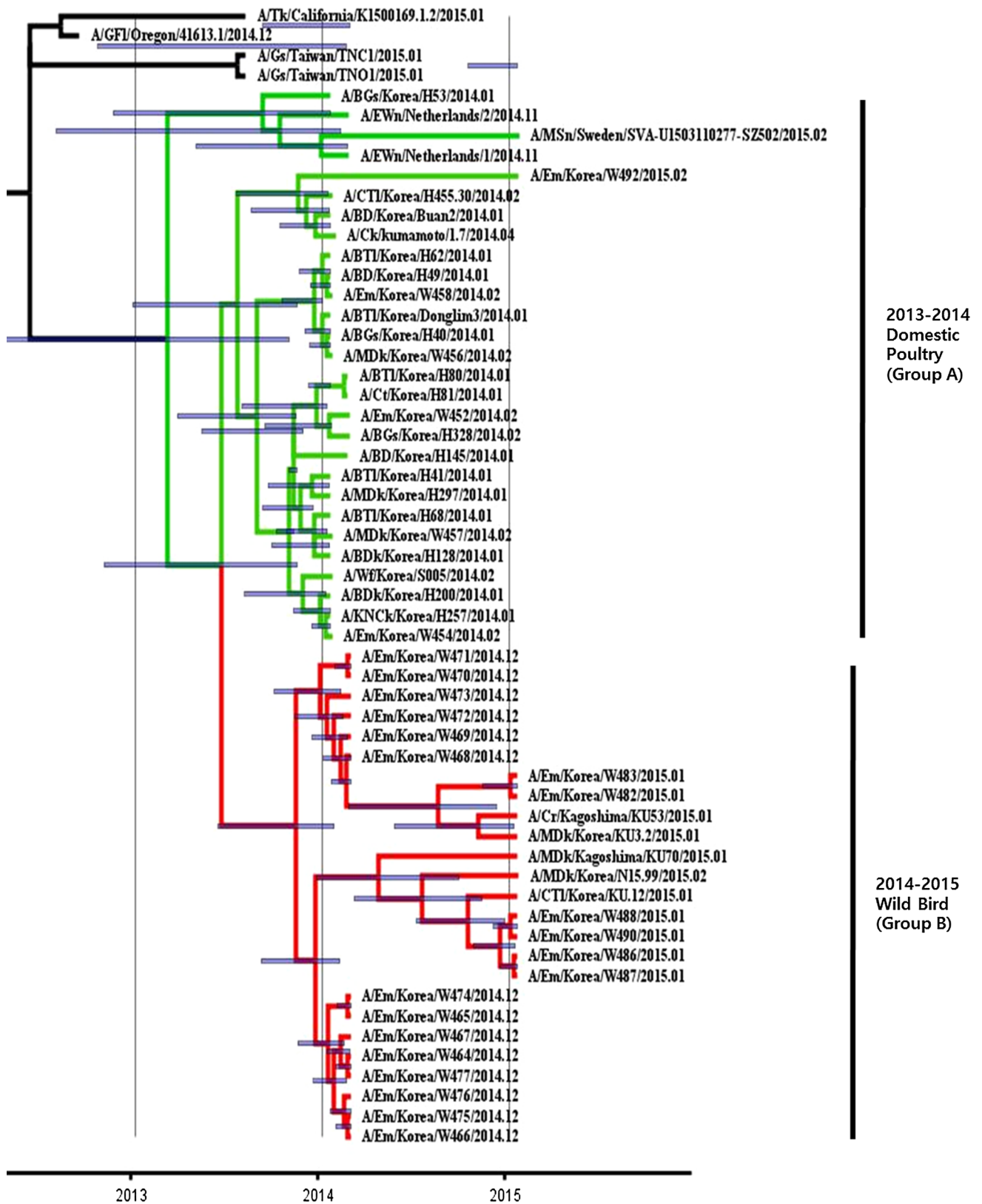


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(d) NP

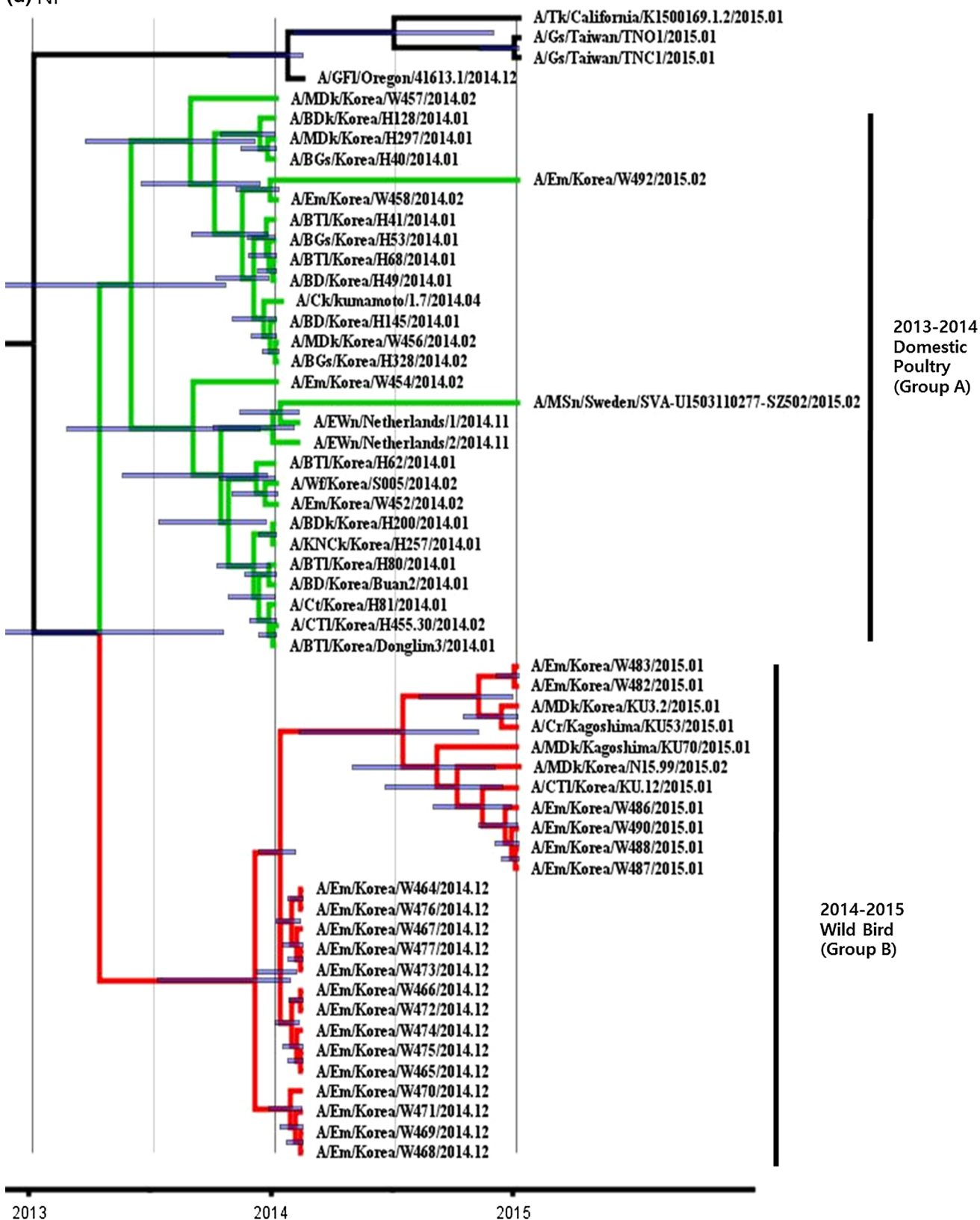


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(e)M

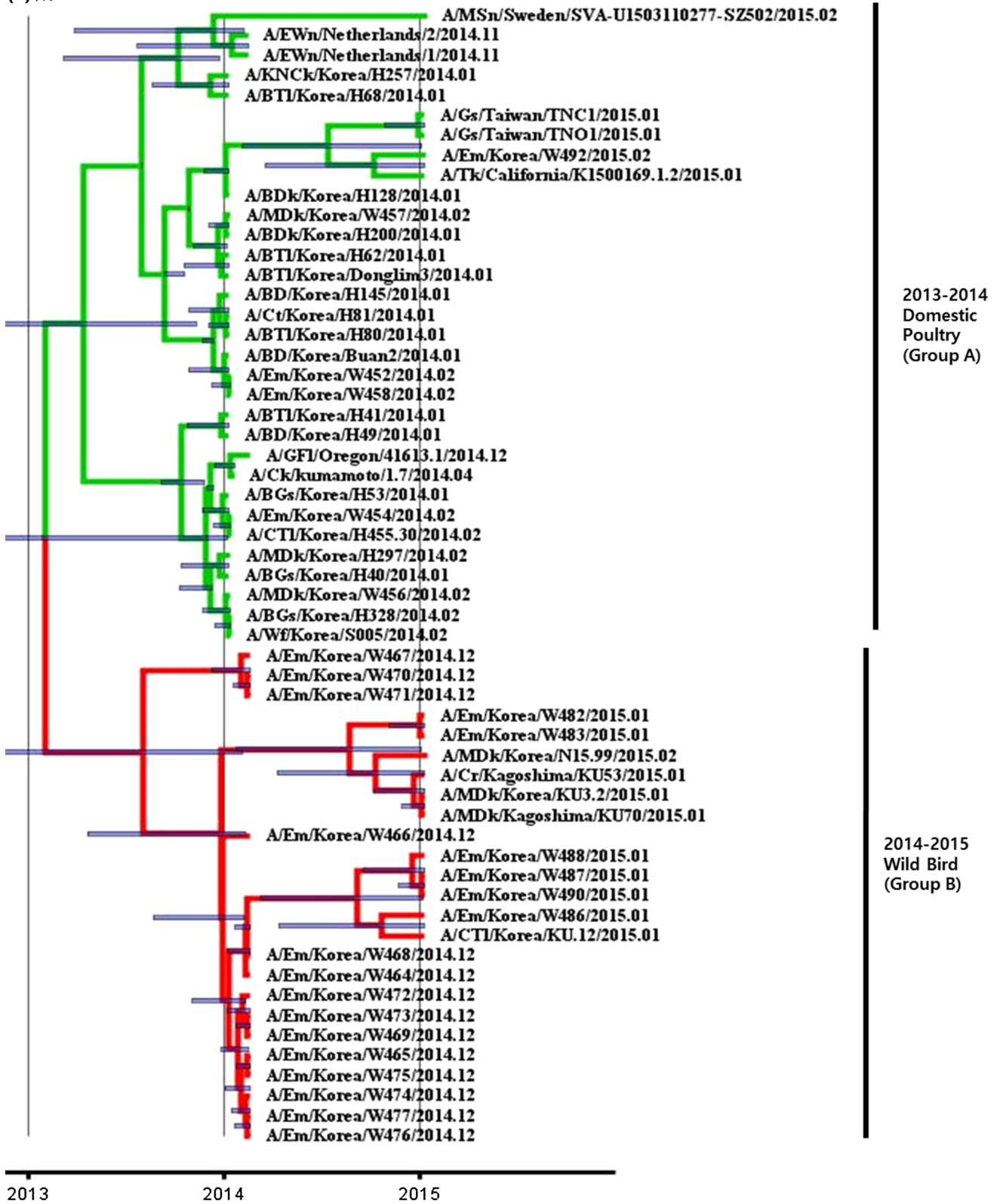


Fig. 2 continued

(f) NS

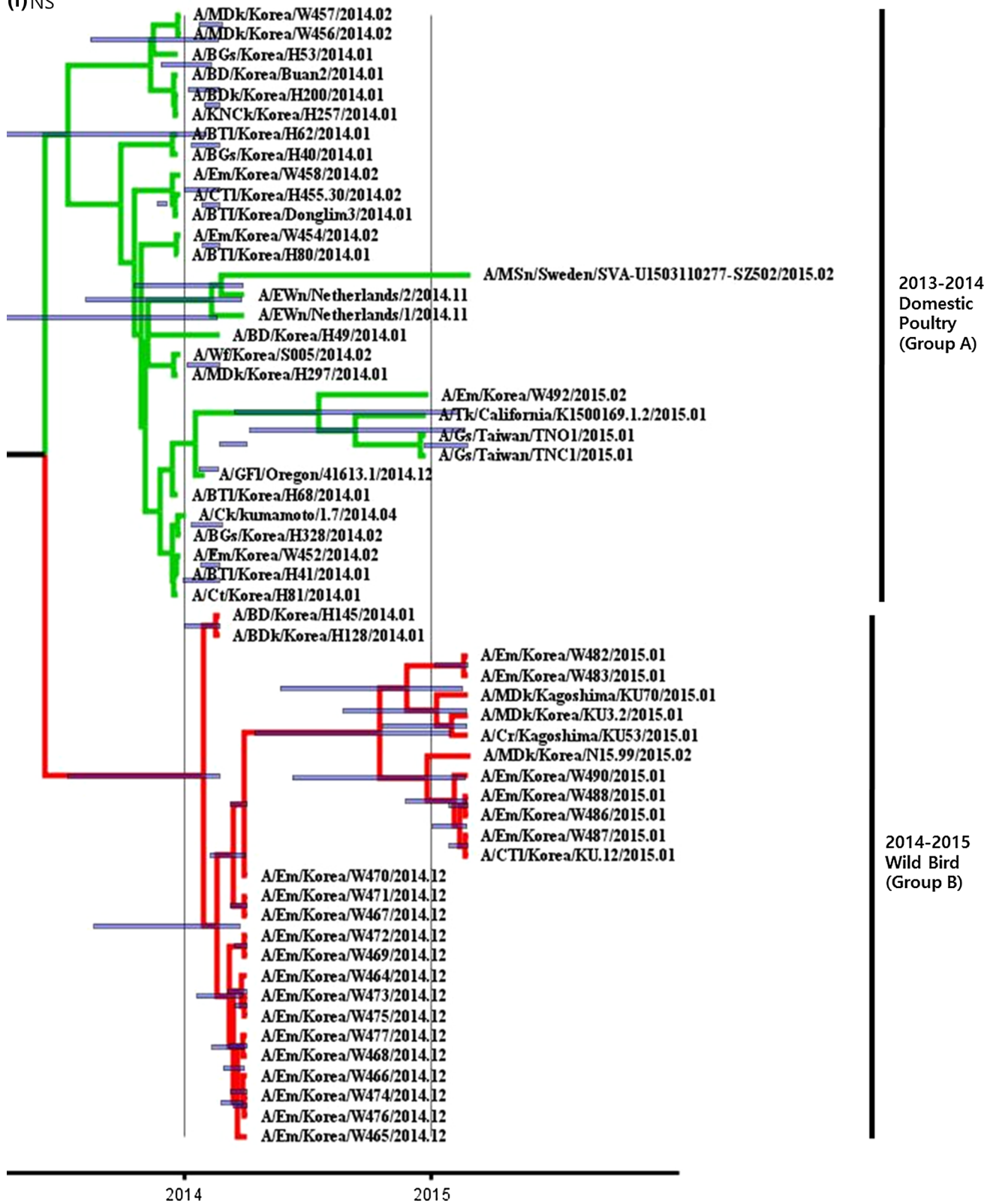


Fig. 2 continued

Table 3 Antigenic analysis of HPAI H5N8 viruses isolated from the two winter seasons of 2013 and 2015

Virus	Genetic group	Antiserum (HI GMT ^a)					
		W452	W456	W468	W483	W488	W492
A/MDk/Korea/W452/2014	A	557	320	320	320	320	367
A/MDk/Korea/W456/2014	A	320	640	160	211	160	367
A/Em/Korea/W468/2014	B	320	320	640	640	320	160
A/Em/Korea/W483/2015	B	320	160	367	1114	320	211
A/Em/Korea/W488/2015	B	320	211	320	640	640	160
A/Em/Korea/W492/2015	A	367	320	183	320	160	640

Em environment, *MDk* mallard duck

^a Geometric mean titers (GMT) of five independent tests

Titers in boldface represent homologous virus reactivity

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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