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Evolutional dynamics of highly pathogenic avian influenza H5N8 genotypes in wintering bird habitats: Insights from South Korea's 2020–2021 season

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ABSTRACT

The winter of 2020–2021 in South Korea witnessed severe outbreaks of Highly Pathogenic Avian Influenza (HPAI) viruses, specifically multiple genotypes of the H5N8 subtype. These outbreaks prompted an extensive investigation into the genetic characteristics and evolutionary dynamics of these viruses. Under the auspices of the National Institute of Wildlife Disease Control and Prevention (NIWDC), we conducted a nationwide surveillance program, collecting 7588 specimens from diverse wild bird habitats. Influenza A viruses were isolated at a rate of 5.0%, with HPAI H5N8 viruses accounting for 38.5% of isolates, predominantly found in wild bird carcasses (97.3%). Genetic analysis revealed the emergence of novel HPAI genotypes due to genetic reassortment events. G1 and G2 viruses were separately introduced into Korea, with G1 viruses displaying dynamic behavior, resulting in diverse sub-genotypes (G1–1 to G1–5) and mainly isolated from clinical specimens. Conversely, the G2 virus, introduced later, became the dominant strain consistently isolated mainly from bird carcasses (88.9%). These findings underscore the emergence of numerous novel HPAI genotypes shaped by multiple reassortment events in high-density wintering grounds of migratory birds. These sites act as hotspots for genetic exchanges, significantly influencing avian ecology, including resident bird species, and contributing to HPAI H5N8 evolution. The genetic diversity and ongoing evolution of these viruses highlight the need for vigilant surveillance and adaptive control measures. Recognizing the potential spillover to human populations, a One Health approach is essential to mitigate the evolving threats posed by avian influenza.

1. Introduction

Migratory birds, especially wild waterfowl, are widely recognized as natural reservoirs of avian influenza viruses (AIV), playing a pivotal role in the maintenance and transmission of these viruses along migratory flyways [1]. The emergence of the highly pathogenic avian influenza (HPAI) H5 Gs/Gd lineage (A/Goose/Guangdong/1/1996, H5N1) in the mid-1990s marked the beginning of continuous identification of multiple HPAI H5 subtypes worldwide. These outbreaks have not only resulted in significant economic losses in the poultry industry but have also raised serious public health concerns due to human infections [2–5].

South Korea experienced its first HPAIV H5N1 outbreak in 2003, with subsequent detections of various HPAI H5Nx subtypes, including H5N1, H5N6, and H5N8, in both wild birds and domestic poultry at intervals of two to three years [6–10]. Of these, the clade 2.3.4.4 H5N8 virus, initially identified in poultry in South Korea in 2014 [9], has since led to numerous outbreaks in domestic and wild birds globally, spanning Asia, Europe, and North America [11,12]. Notably, the clade 2.3.4.4 H5N8 virus has evolved into novel reassortant H5Nx viruses with multiple subtypes [1,13].

Annually, South Korea witnesses the introduction of over 1.6 million

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migratory birds [14], creating high-density environments, particularly during the winter season, due to limited wintering sites. These conditions foster heightened ecological interactions among diverse bird species, increasing the likelihood of generating novel genotypes of avian influenza viruses. To comprehensively understand the dynamics of evolutionary reassortment events in migratory bird habitats during the winter season, a detailed genome analysis of viruses isolated from various sources and a study of their pathogenicity is imperative. During the 2020–2021 winter season, South Korea experienced multiple outbreaks of novel genotypes of HPAI H5N8 viruses, leading to significant mortality among various species of wild birds [15]. In this context, our study aims to investigate the genetic diversity of HPAI H5 viruses, identify their respective wild bird host species, and analyze reassortment events in the wild bird habitats of South Korea during the specified winter season. Furthermore, through genotyping of the 2020–2021 HPAI H5N8 isolates according to the timelines of virus isolation, we tried to evaluate the dominant virus strain among the various genotypic viruses.

2. Materials and methods

2.1. Virus isolation

In this study, Highly Pathogenic Avian Influenza (HPAI) H5N8 viruses were isolated from wild bird habitats and major streams in South Korea between September 2020 and February 2021 as part of avian influenza (AI) surveillance conducted by the Ministry of Environment. During active surveillance, fecal specimens and oropharyngeal or cloacal swabs were collected from wild birds. Carcasses discovered through passive surveillance were also examined to determine the cause of death in wild birds. All samples were resuspended in antibiotic solution and thoroughly mixed by vortexing, followed by centrifugation (3000 rpm for 15 min at 4 °C). Centrifuged supernatants were inoculated into the allantoic cavity of 9–11-day-old specific pathogen-free (SPF) embryonated eggs according to previously established protocols [16] and maintained at 37 °C for 48 h.

2.2. Avian host identification

Identification of avian hosts for AI-positive fecal samples was conducted using cytochrome C oxidase I (COI) barcoding, as described [17].

2.3. Genomic sequencing

Viral RNA was extracted from allantoic fluids of SPF chicken eggs using the Maxwell® RSC simply RNA Tissue Kit (Promega, Madison, WI, USA). Complementary DNA (cDNA) was synthesized using M-MLV Reverse Transcriptase (Enzynomics, Daejeon, Korea). PCR fragments were synthesized and amplified using nTaq-Tenuto (Mg^{2+} Plus) DNA Polymerase (Enzynomics, Daejeon, Korea), following the protocol outlined by Zhou et al. [18]. Whole influenza virus genome cDNA libraries were meticulously prepared using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) for next-generation sequencing (NGS). The sequencing process was conducted on a MiniSeq system (Illumina, San Diego, CA, USA) employing a MiniSeq mid-output kit. Nucleotide sequences derived from this study were assembled using the CLC Workbench program (version 10.1.1; CLC bio). Complete genome sequences were deposited in the GISAID's EpiFlu Database (http://www.gisaid.org) (Supplementary Table 1) for broader accessibility and reference.

2.4. Phylogenetic analysis

To construct phylogenetic trees for all segments, available sequence information was retrieved for HPAI H5Nx isolates collected in Eurasia and North America throughout this study. The National Center for Biotechnology Information (NCBI) Influenza Virus Database (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi) and the GISAID's EpiFlu Database were utilized for data collection. Multiple-sequence alignments for each dataset were conducted using the MAFFT program [19]. Phylogenetic relationships of each segment were inferred using a maximum-likelihood method by running RAxML v8.2.10. An ML tree of a subset of H5 and N8 genes was generated using MEGA (version 6). A general time-reversible model of nucleotide substitution with a γ-distributed rate variation among sites was applied throughout the analysis.

2.5. Genotyping

Genotypes were determined based on each segment-specific phylogenetic tree using the following criteria: a minimum bootstrap value of 70 for tree topology and a nucleotide sequence having ≥97% identity. Genotypes were assigned based on the unique combination of gene clustering for each segment of the virus.

3. Results

3.1. Isolation of HPAI H5N8 viruses from wild birds

A comprehensive active surveillance program for avian influenza viruses (AIVs) in South Korea, conducted by the National Institute of Wildlife Disease Control and Prevention (NIWDC), collected a total of 7588 wild bird specimens from September 2020 to February 2021 (Table 1). Throughout this national AIV surveillance of wild birds, clinical fecal specimens ($n = 4741$) were obtained from 87 distinct wild bird habitat sites, while oropharyngeal or cloacal swabs ($n = 2400$) were collected by capturing wild birds using cannon netting. Additionally, environmental divisions affiliated with local governments retrieved a total of 447 wild bird carcasses discovered during the monitoring of wild bird habitats. The supernatants of processed specimens were inoculated into 10-day embryonated chicken eggs for virus isolation, resulting in the isolation of a total of 385 AIVs (with a virus isolation rate of 5.0% from the total tested specimens) (Table 1). Among the clinical specimens, wild bird carcasses exhibited the highest AIV-positive rate (25%), followed by bird feces (5.5%) and oropharyngeal (OP) and cloacal (CL) swab specimens (0.3%). Notably, HPAI H5N8 viruses ($n = 148$) accounted for 38.5% of the isolated AIVs, while the remaining 61.5% were categorized as Low Pathogenic Avian Influenza (LPAI) viruses ($n = 236$) (Table 1). Specifically, H5N8 HPAIs were predominantly isolated from wild bird carcasses ($n = 109$, 97.3%), followed by fecal samples ($n = 32$, 12.1%), and OP and CL swab specimens ($n = 7$, 77.7%).

Mitochondrial DNA sequence analysis of the fecal specimens revealed that HPAI H5N8 viruses were primarily isolated from Mandarin Ducks ($n = 15$, 10.1%), followed by Wild Ducks ($n = 9$, 6.0%), Wild Birds (n = 3, 2.0%), Spot-billed Ducks (n = 3, 2.0%), and Northern Pintails ($n = 2$, 1.3%) (Table 1). Additionally, the H5N8 virus was predominantly isolated from the carcasses of Whooper Swans ($n = 36/109$, 24.3%), followed by White-fronted Geese ($n = 24/109$, 16.2%), Egrets ($n = 16/109$, 10.8%), and other minor avian species (Table 1).

3.2. Phylogenetic analysis of HPAI H5N8 viruses

In pursuit of comprehensive insights into the genetic evolution of Korean H5 HPAIs, we conducted an extensive phylogenetic analysis, with a primary focus on the clade 2.3.4.4b HPAI H5N8 virus isolates (Fig. 1, Supplementary Table 1). Although all 2020–2021 Korea H5N8 viruses belonged to the clade 2.3.4.4b yet they were separately clustered into two sub-groups: Group A and Group B (Fig. 1A). Group A displayed a close genetic affinity with H5N8 viruses predominantly identified in Eastern European regions, including Slovakia, Poland, the Czech Republic, and Hungary, during the 2019–2020 winter season. In contrast, Group B viruses exhibited closer genetic associations with H5 viruses

Table 1
Number of highly pathogenic avian influenza viruses (HPAIVs) isolated from wild birds in the South Korea between September 2020 and February 2021.

Sample		Feces	OP, CL swab	Carcass	Subtotal
No. of total samples		4741	2400	447	7588
No. of AIV-positive samples (prevalence[a])		**263 (5.5%)**	**9 (0.3%)**	**112 (25.0%)**	**384 (5.0%)**
No. of positive samples for HPAI H5N8	Mallard		1		1 (0.7%)
	Spot-billed Duck	3	3	7	13 (8.7%)
	Mandarin Duck	15			15 (10.1%)
	Green-winged Teal			1	1 (0.7%)
	Gadwall			1	1 (0.7%)
	Eurasian Wigeon		1		1 (0.7%)
	Northern Pintail	2	1		3 (2.0%)
	Tufted Duck		1		1 (0.7%)
	Wild Duck	9			9 (6.0%)
	Egret			16	16 (10.8%)
	White-fronted Goose			24	24 (16.2%)
	Bean Goose			8	8 (5.4%)
	Whooper Swan			36	36 (24.3%)
	Mute Swan			1	1 (0.7%)
	Grey Heron			9	9 (6.0%)
	Eurasian Curlew			1	1 (0.7%)
	Eurasian Spoonbill			1	1 (0.7%)
	Hooded Crane			1	1 (0.7%)
	Black-tailed Gull			1	1 (0.7%)
	Eurasian Eagle Owl			2	2 (1.3%)
	Wild Birds	3			3 (2.0%)
	Subtotal (proportion[b])	**32 (12.1%)**	**7 (77.7%)**	**109 (97.3%)**	**148 (38.5%)**
No. of positive samples for LPAIVs	Mallard		1		1 (0.4%)
	Spot-billed Duck		1		1 (0.4%)
	Mandarin Duck	2			2 (0.8%)
	Common Teal	5			5 (2.1%)
	Eurasian Wigeon	1			1 (0.4%)
	Northern Pintail	3			3 (1.2%)
	Wild Duck	49			49 (20.7%)
	White-fronted Goose	8			8 (3.3%)
	Bean Goose	12		1	13 (5.5%)
	Whooper Swan			2	2 (0.8%)
	Swan Goose	1			1 (0.4%)
	Greylag goose	2			2 (0.8%)
	Coot	1			1 (0.4%)
	Northern Shoveler	2			2 (0.8%)
	Falcated Duck	1			1 (0.4%)
	Wild Bird	144			144 (61.0%)
	Subtotal (proportion[c])	**231 (87.8%)**	**2 (22.2%)**	**3 (2.7%)**	**236 (61.5%)**

[a] [(Number of AIV-positive samples)/(number of total samples)] × 100 (%).
[b] [(Number of HPAI H5N8 viruses)/(number of AIV-positive samples)] × 100 (%).
[c] [(Number of LPAI viruses)/(number of AIV-positive samples)] × 100 (%).

detected in Western European regions, encompassing England, Scotland, Wales, France, Italy, and the Netherlands, between 2020 and 2021 (Fig. 1B). Notably, a similar pattern was observed in the neuraminidase (NA) genes, which could be categorized into two sub-groups corresponding to the HA gene groups, Group A and Group B. Specifically, N8 Group A originated from H5N8 viruses that emerged in Korea in 2016, while Group B was linked to the H5N8 viruses that initially emerged in Uvs-Nuur Lake (Tyva Republic) in 2016 [20] (Fig. 1C).

Regarding the internal genes, although all H5N8 internal genes belonged to the Eurasian lineage, they exhibited multiple genetic backbones for each gene segment, with particular diversity observed in PB2 ($n = 4$), PB1 ($n = 3$), PA (n = 3), NP (n = 3), M ($n = 2$), and NS (n = 3) (Supplementary Fig. 1). Consequently, this diversity led to distinct subgroups among the viruses (Table 2). Collectively, our analysis of the 148 H5N8 2020–2021 isolates revealed genetic diversity, primarily characterized by two groups based on surface proteins and five subgroups based on internal genes (Table 2, Supplementary Fig. 1). This genetic diversity underscores the dynamic nature of HPAI H5N8 viruses and their capacity for genetic adaptation and evolution.

3.3. Investigation of genetic reassortment events 2021–2022 HPAI H5N8 viruses

To explore the emergence of novel genotypes of HPAI H5N8 viruses in winter migratory bird habitats, we conducted extensive genetic analyses, incorporating sequencing data from co-circulating LPAI viruses during the 2021–2022 winter season. Aligned with the timeline of virus isolation, we initially designated the first H5N8 isolate during the 2020–2021 winter season as the G1 genotype. These G1 viruses exhibited significant genetic similarity, sharing approximately 98.45–99.80% homology with H5N8 viruses previously identified in Eastern Europe between 2019 and 2020 (Supplementary Table 2) [21]. Following the introduction of the G1 viruses, the G1 genotype underwent rapid reassortment with co-circulating LPAI viruses within winter habitats, resulting in the emergence of distinct sub-genotypes denoted as G1–1 to G1–5, contingent upon the timing of virus isolation during the 2020–2021 winter season. The G1–1-like viruses contained the NP segment from an A/Fd/Korea/JB42–30/2020-like H9N2 virus within the G1 backbone (Fig. 2A). Three unique genotypes emerged through genetic reassortment between November and December 2020, differing from G1–1-like viruses due to the incorporation of PB2 and PB1 in G1–2, PB2, PB1, and NS in G1–3, or PB2 and PA in G1–4, derived from co-circulating LPAIs.

On the other hand, G2 viruses shared a close genetic relationship with H5N8 viruses detected in Western Europe in late 2020/early 2021, exhibiting approximately 96.9–99.9% homology (Fig. 2B, Supplementary Table 3) [22]. Interestingly, the G1–5 virus isolated from Egret at the end of December 2020 carried HA and NA genes from G1-like viruses but had acquired PB1, M, and NS genes from G2-like viruses. Remarkably, the PB2 gene of G1–5 viruses exhibited the highest nucleotide sequence homology (95.0%) with that of the A/Md/Korea/JB22–3/2019(H5N3) strain. This observation suggested that the G1–5 genotype virus had undergone multiple reassortment events involving at least four different subtypes (H3N2, H5N3, H5N8, and H9N2) co-circulating among migratory birds during the 2020–2021 winter season (Fig. 2A). Collectively, these findings provide valuable insights into the intricate genetic dynamics of HPAI H5viruses in South Korea, driven by migratory bird populations.

3.4. Prevalence of H5N8 viruses by genotype in migratory birds

To elucidate the infection patterns associated with the two distinct highly pathogenic avian influenza (HPAI) H5N8 viruses in Korea, we analyzed waterfowl species and their respective HPAI genotypes. The G1 and G1 sub-genotypes were predominantly isolated from wild ducks, accounting for 64.7% of the cases after their initial identification in

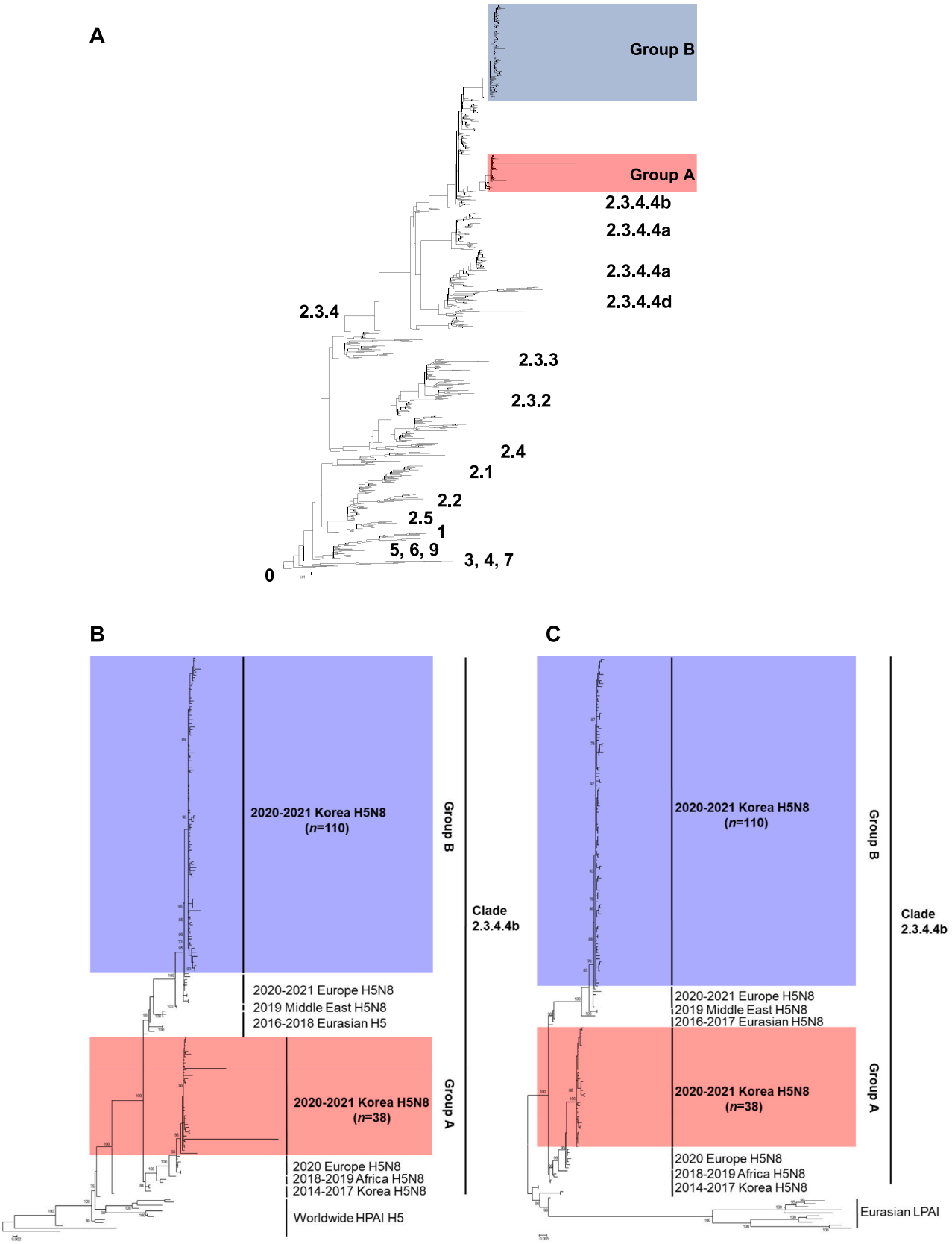

Fig. 1. Genetic phylogenetic analysis of H5N8 highly pathogenic avian influenza isolated in Korea in 2020–2021. Maximum likelihood phylogenetic tree of the complete genome sequence concatenated with the H5Nx virus HA gene (A). Bold text indicates the clade of H5Nx, and the viruses analyzed in this study were labeled as subgroups A and B of the 2.3.4.4 clade. Phylogenetic tree of the hemagglutinin gene of the H5 subtype (B). Phylogenetic tree of the neuraminidase gene of the N8 subtype (C). The phylogenetic tree sequenced 148 H5N8 HPAI viruses isolated from 7588 samples collected from wild birds between October 2020 to February 2021.

Table 2
The names of the reassortants, and the phylogenetic groups within each gene segment.

Reassortant name	Subgroup	Phylogenetic group within each gene segment							
		PB2	PB1	PA	H5	NP	N8	M	NS
AAAAAAAA[a]	G1	A	A	A	A	A	A	A	A
AAAACAAA	G1–1	A	A	A	A	C[c]	A	A	A
CCAACAAA	G1–2	C	C	A	A	C	A	A	A
DCAACAAC	G1–3	D[c]	C	A	A	C	A	A	C
DACACAAA	G1–4	D	A	C	A	C	A	A	A
CBCACABB	G1–5	C	B	C	A	C	A	B	B
BBBBBBBB[b]	G2	B	B	B	B	B	B	B	B

[a] According to phylogenetic analysis (Supplementary fig. 1), the genotype belonging to the G1 group was designated as A.
[b] According to phylogenetic analysis (Supplementary fig. 1), the genotype belonging to the G2 group was designated as B.
[c] New genotypes were reassortments, and genotypes that did not belong to groups A and B were designated as C or D.

mandarin ducks on October 24, 2020. Subsequently, these G1-like viruses were also detected mainly in migratory bird species, including white-fronted geese, whooper swans, grey herons, and Eurasian spoonbills. Specimens for G1 and G1-like cluster viruses were primarily obtained from clinical swabs and feces specimens of live birds, representing 56.4% of the cases, while 43.6% were isolated from bird carcasses (Table 1, Fig. 3).

Although initially identified in spot-billed duck carcass on December 3, 2020, subsequent isolation of G2 viruses were predominantly from bird carcasses, constituting a total of 88.9% of cases ($n = 97/109$). These isolations included swans (32.9%), geese (31.9%), egrets (12.3%), herons (8.2%), and ducks (7.2%), encompassing various regions across South Korea until February 15, 2021. Intriguingly, G2-like viruses were also recovered from carcasses of distinct avian hosts such as hooded cranes, Eurasian spoonbills, as well as resident black-tailed gulls and Eurasian eagle owl species in Korea. This observation suggests the potential spread of the virus not only among migratory birds but also among resident wild birds, highlighting the complex dynamics of H5N8 transmission among avian populations (Fig. 3).

3.5. Temporal dynamics of two clusters of H5N8 viruses

An investigation into the temporal dynamics of H5N8 viruses unveiled distinct phases in their occurrence and shifts in dominant viral strains. The initial detection of the H5N8 virus, belonging to the G1 cluster, transpired in samples (feces and swabs) obtained from wild migratory birds in October 2020, persisting until the third week of November (Fig. 4). Subsequently, G2 cluster viruses were first identified in early December 2020, originating from carcasses of wild birds and continued to be isolated until the conclusion of the winter season, which extended until the third week of February 2021 (Fig. 4). Following the emergence of the G1 genotype virus, five recombinant genotypes emerged over time in collected samples. Notably, G1 and G1 subgenotypic variants were primarily identified in clinical specimens obtained from captured live wild birds, although some were also isolated from certain species of bird carcasses (Fig. 4). However, G2 and G2-like gene-containing viruses (G1–5) were predominantly detected in the carcasses of most avian species examined in this study, displaying an association with higher mortality rates among various wild bird species compared to those infected with the G1 virus (Fig. 4).

4. Discussion

Highly pathogenic avian influenza (HPAI) viruses, particularly the H5N8 subtype, continue to pose a formidable threat to global public health and the poultry industry. The significant outbreaks observed globally, including in South Korea, underscore the urgency of understanding the evolutionary dynamics and potential impacts of these viruses [11,12]. In the late 2020–2021 winter season, South Korea experienced a large-scale HPAI H5N8 outbreak, prompting extensive surveillance efforts by the Ministry of Environment across diverse wild bird habitats, yielding 7588 clinical specimens for avian influenza virus (AIV) surveillance.

The phylogenetic analysis of the HA gene of the 2020–2021 Korea H5N8 viruses all belonged to the H5 clade 2.3.4.4b, forming two discernible genetic clusters (Group 1 and Group 2), which may indicate introductions of two genetically distinct H5N8 clade 2.3.4.4 viruses at a juncture in the 2020–2021 winter season. Moreover, these viruses further underwent multiple reassortment events, generating the emergence of different subgroups of HPAI H5N8 viruses. The Group 1 Korean H5N8 isolates clustered closely to the H5N8 viruses mainly identified from Eastern Europe including Slovakia, Poland, Czech Republic, and Hungary during the 2019–2020 winter season, whereas, Group 2 Korean H5N8 isolates were closely associated with H5 viruses detected in Western Europe including England, Scotland, Wales, France, Italy, and the Netherlands between 2020 and 2021, which also include the H5 gene of the HPAI H5N8 that was recently reported in Astrakhan in Russia which causes human infections. Furthermore, The NA gene clusters also formed two distinct groups corresponding to the HA gene groups, emphasizing the intricate genetic diversity within the H5N8 viruses.

The observed genetic reassortment events within the HPAI H5N8 viruses during the 2021–2022 winter seasons underscore the dynamic nature of these viruses within the winter migratory bird habitats in South Korea. The introduction of the G1 genotype, characterized by its genetic similarity to H5N8 viruses from Eastern Europe, marked the initiation of a series of rapid reassortment events with co-circulating LPAI viruses. The emergence of distinct sub-genotypes (G1–1 to G1–5) suggests a complex interplay between HPAI and LPAI viruses within the high-density wintering habitats of migratory birds. Notably, the G1–5 genotype, isolated from an Egret carcass, demonstrated multiple reassortment events involving at least four different subtypes (H3N2, H5N3, H5N8, and H9N2) co-circulating among migratory birds, including the G2 genotype. This complexity in the genetic makeup of the G1–5 virus highlights the potential for diverse viral strains to coexist and exchange genetic material within the concentrated populations of migratory birds during the winter season. Similarly, the G2 viruses, initially identified in a Spot-billed Duck and the consistent identification among various waterfowl species across South Korea and the generation of G1–5 viruses (containing PB1, M, and NS segments of G2 genotype) further support the idea of ongoing genetic evolution and reassortment within the winter migratory bird habitats, contributing to the ongoing evolution of HPAI H5N8.

The high-density setting of wintering places for migratory birds in South Korea; exemplified by the presence of 500,000 ducks in the Geum estuary alone [23], provides an ideal environment for the intricate genetic dynamics observed in the HPAI H5Nx viruses. These wintering sites, acting as hubs for migratory birds, enable the viruses to adapt to new hosts and reassort with co-circulating strains, as demonstrated by the G2 viruses. The broader spectrum of avian hosts for the G2 cluster viruses highlights the potential for interspecies transmission and the maintenance of viral diversity compared to the G1 viruses, which were

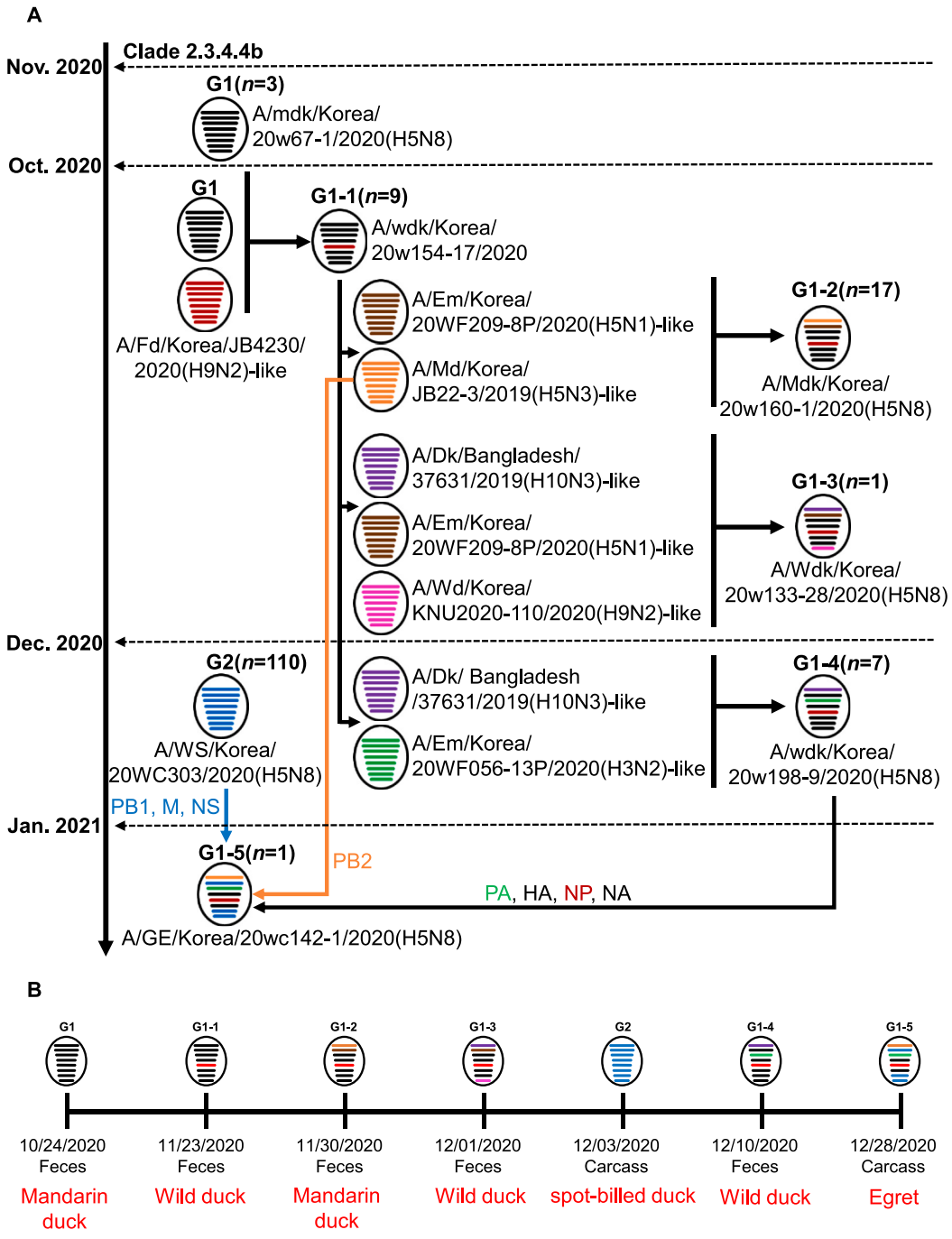

Fig. 2. Schematic representation depicting various H5N8 HPAI reassortment events in Korea. Formation of H5N8 reassortment viruses (A). Virus particles are illustrated as colored ovals with horizontal bars denoting eight gene segments (from top to bottom: PB2, PB1, PA, HA, NP, NA, M, and NS). Descendant virus segments are colour-coded to indicate their genetic ancestry, with the donor virus adjacent to the arrow tail. Arrowheads indicate reassortants resulting from genetic reorganization. The timeline on the left represents when the virus emergence or reassortment event occurred. Genotype-based isolates information of reassortant viruses (B). The isolation date and source sample of the virus, organized by genotype, are presented. The red text indicates the wild bird species from which the virus was isolated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

predominantly isolated among wild ducks. Furthermore, the varied isolation patterns and the detection of G2-like viruses in both migratory and residential wild bird species suggest that these high-density settings serve as hotspots for viral transmission, influencing the ecology and evolution of HPAI H5N8. Noteworthy is the contrast between G1 and G2 viruses, where the latter exhibited a higher isolation rate mainly from wild bird carcasses. Accordingly, the evolution of G1 into a more diverse subcluster virus may have been facilitated by its low pathogenicity, which reduced the mortality of infected wild birds, thereby creating conditions conducive to the generation of recombinant viruses.

In summary, the H5N8 viruses identified in Korea during the 2020–2021 season underwent multiple reassortment events, actively evolving through genetic exchanges with co-circulating avian influenza strains (Fig. 2). The Korean Peninsula serves as an important stopover, breeding, and wintering site for many migratory bird species, such as those birds from Australia and New Zealand en route to Siberia [24]. These bird species include the Hooded Crane, Baikal Teal, Whooper Swan, Tundra Swan, Eurasian Spoonbill, and various species of geese

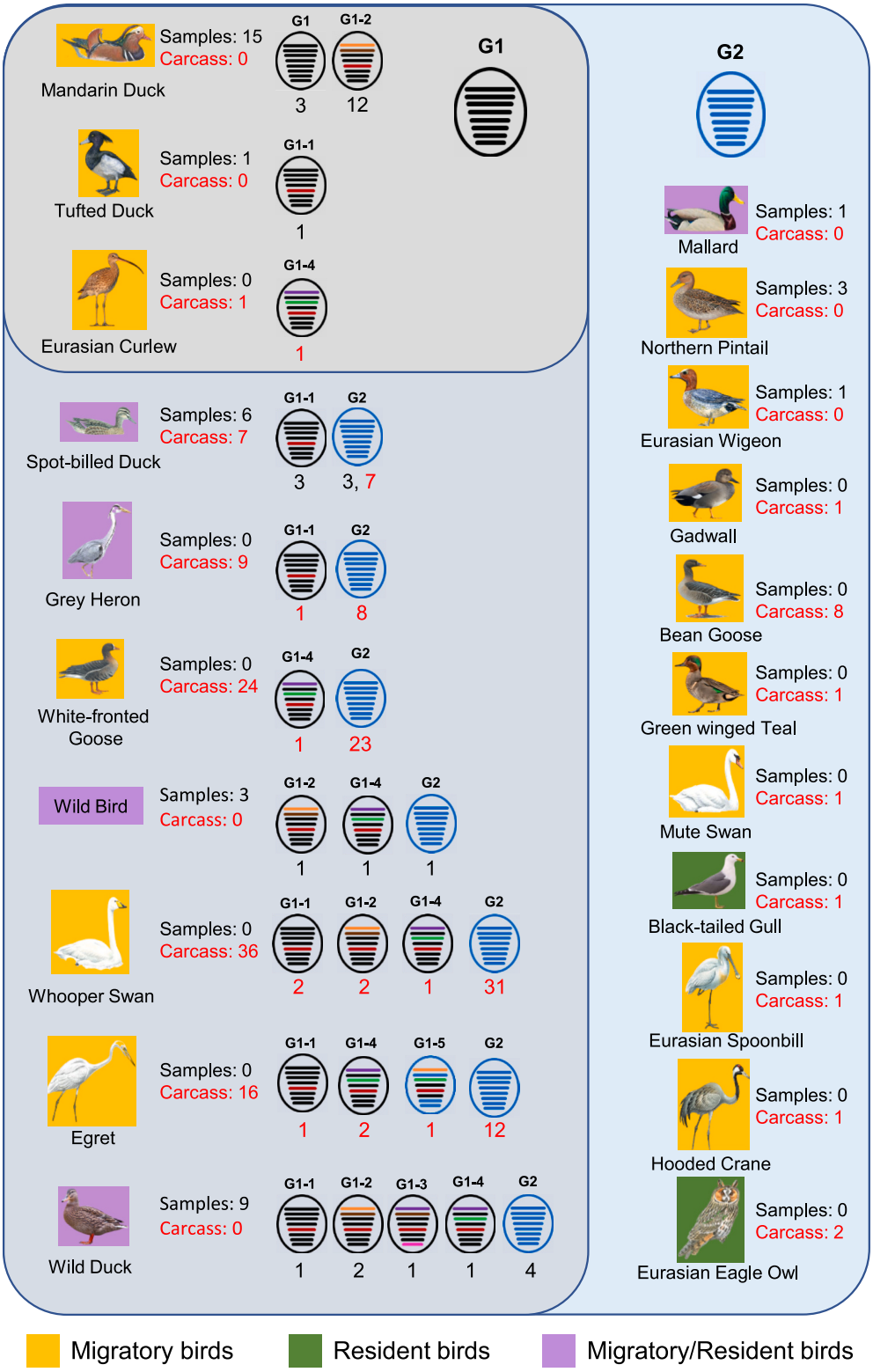

Fig. 3. The genotype distribution of H5N8 viruses in various wild bird species. Presents the genotype and quantity of H5N8 viruses obtained from diverse wild birds. The black numbers below each genotype signify the number of isolates from each sample, while the red numbers denote the number of isolates from the carcasses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and ducks that migrate from Northern Europe and Mongolia [24]. As these migratory birds cross contents and diverse habitats with other migratory birds, they are potential carriers of diverse strains of Influenza viruses across vast geographic regions. Understanding the significance of these wintering sites in shaping the genetic landscape of avian influenza viruses is crucial for developing targeted surveillance strategies and interventions to mitigate the risk of emerging strains with pandemic potential. These findings underscore the need for ongoing monitoring and research to unravel the complexities of virus ecology in these critical habitats and inform effective control measures. While

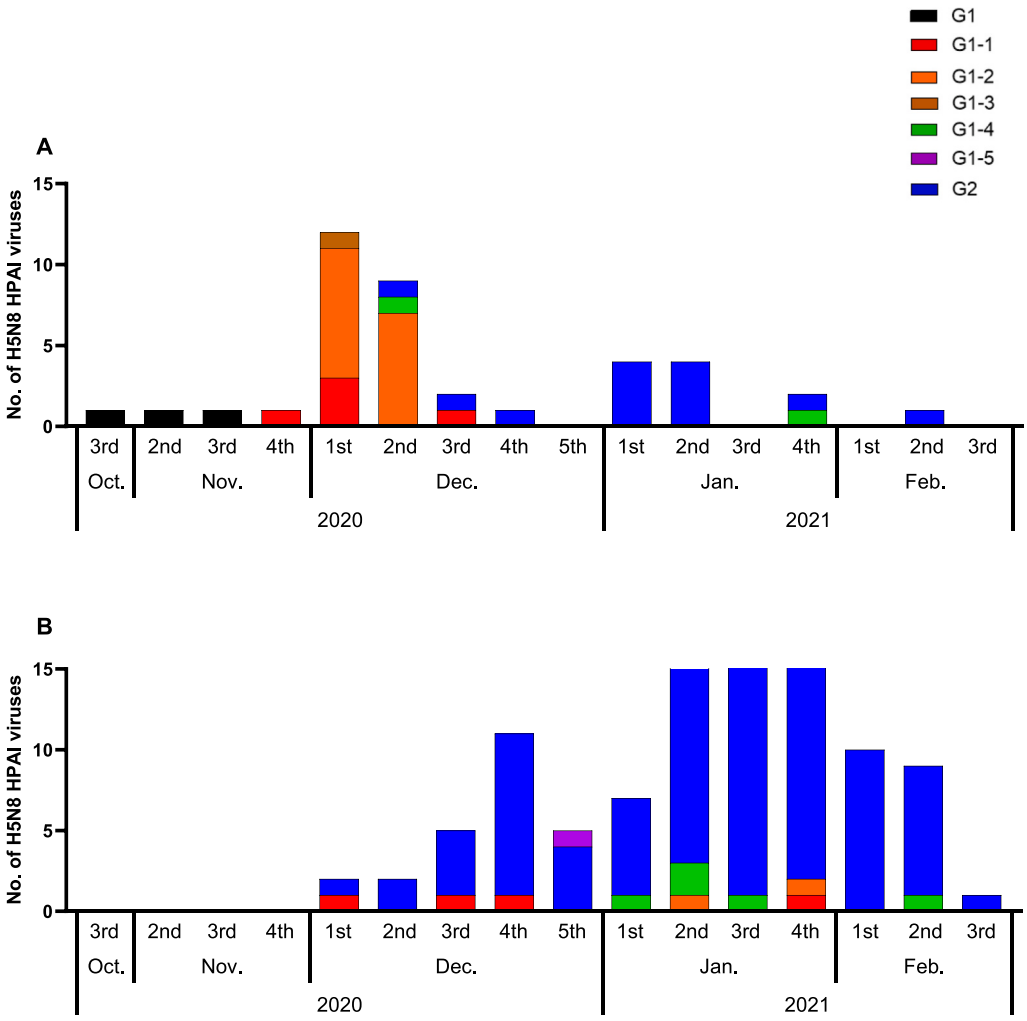

Fig. 4. Prevalence trends of H5N8 virus across different genotypes in Korea during 2020–21. From each isolation time point, the stacked bar graphs showing the total number of viruses from each genotype and were displayed based on the specific sample type (feces, swab, and carcass) from which the virus was isolated.

human infections with HPAI H5N8 are rare, they remain a matter of concern and require a comprehensive One Health approach, acknowledging the interconnectedness of human, animal, and environmental health. The potential for zoonotic transmissions, as highlighted by our study and reports from Russia, underscores the significance of maintaining vigilance and conducting further investigations into the ongoing evolution of HPAI H5N8 viruses. These efforts are essential to both safeguarding avian populations and minimizing any potential risks to human health.

CRediT authorship contribution statement

Young Jae Si: Conceptualization, Formal analysis. **Seung-gyu Jang:** Formal analysis, Investigation. **Mark Anthony B. Casel:** Writing – review & editing. **Dong-ju Kim:** Investigation. **Ho Young Ji:** Investigation. **Jeong Ho Choi:** Investigation. **Ju Ryeon Gil:** Investigation. **Rare Rollon:** Investigation. **Hyunwoo Jang:** Investigation. **So Youn Cheun:** Investigation. **Eun-Ha Kim:** Validation. **Hyesung Jeong:** Resources, Writing – review & editing. **Young Ki Choi:** Conceptualization, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2024.100719.

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