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**Genetic and antigenic characterization of the first H7N7 low pathogenic avian influenza
viruses isolated in Vietnam**

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Abstract

During the annual surveillance of avian influenza viruses (AIVs) in Vietnam in 2018, three H7N7 AIV isolates were identified in domestic ducks in a single flock in Vinh Long province. The present study is the first documented report of H7N7 virus isolates in Vietnam and aimed to characterize these viruses, both genetically and antigenically. Deduced amino acid sequences for the hemagglutinins (HAs) indicated a low pathogenicity of these viruses in chickens. Phylogenetic analysis revealed that the H7 HA genes of these isolates were closely related to each other and belonged to the European–Asian sublineage, together with those of H7N3 viruses isolated from ducks in Cambodia during 2017. They were not genetically related to those of Chinese H7N9 or H7N1 viruses that were previously detected in Vietnam during 2012. Interestingly, the M genes of the two H7N7 virus isolates were phylogenetically classified into distinct groups, suggesting an ongoing reassortment event in domestic ducks because they were isolated from the same flock. These H7N7 viruses exhibited somewhat different antigenic characteristics compared with other representative H7 low pathogenic AIVs. Surprisingly, the antigenicity of Vietnamese H7N7 viruses is similar to Chinese H7N9 highly pathogenic AIV. The findings of this study suggest that H7N7 viruses may be undergoing reassortment and antigenic diversification in poultry flocks in Vietnam. The silent spread of Vietnamese H7N7 viruses in chickens may lead to acquire high pathogenicity in chickens although the zoonotic potential of the viruses seems to be low since these viruses retain typical avian-specific motifs in the receptor-binding site in the HA and there is no mutation related to mammalian adaptation in

49 PB2 gene. Thus, these results highlight the need for continuous and intensive surveillance of
50 avian influenza in Vietnam, targeting not only highly pathogenic AIVs but also low pathogenic
51 viruses.

52 **Keywords:** H7 avian influenza virus; Vietnam; genetics; antigenicity

1. Introduction

Avian influenza viruses (AIVs) are grouped in the genus *Alphainfluenzavirus* of the *Orthomyxoviridae* family and carry eight negative-stranded RNA segments as their genome (Smith et al., 2018). AIVs are categorized based on the antigenic differences of their surface glycoproteins; these glycoproteins comprise 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes. AIVs are also categorized into highly pathogenic AIVs (HPAIVs) and low pathogenic AIVs (LPAIVs), based on their pathogenicity in chickens. Wild aquatic birds are the natural reservoir of AIVs, being capable of harboring all subtypes of AIVs while not usually showing any clinical signs of AIV infection (Alexander, 2007). In this way, AIVs can be maintained and spread globally without being noticed or recognized. Because of their zoonotic potential, AIVs pose a major threat, not only to the global poultry industry but also for human public health (Koh et al., 2008).

Since the first isolation of an H7 influenza virus from a chicken during a 1902 outbreak (Alexander and Brown, 2009), infections caused by H7 viruses in both wild birds and domestic poultry have been reported globally (Campitelli et al., 2008; Hiono et al., 2015; Lee et al., 2017; Śmietanka et al., 2011). In particular, H7 HPAIVs, which are characterized by the insertion of a multibasic cleavage motif on the HA, have been under extensive monitoring based on the disease occurrence (OIE, 2015). There have been sporadic reports worldwide of outbreaks of H7 HPAIV infection, including in Asian countries [e.g., Pakistan in 1994 (Abbas et al., 2010) and North Korea in 2005 (FAO, 2005)]. Recently, an H7N9 LPAIV, capable of causing fatal disease in

humans, emerged in China (2013–present) and rapidly evolved into HPAIVs (Ke et al., 2017; Quan et al., 2018). Both H7N9 HPAIV and H7Nx AIVs can be continuously introduced into neighboring countries via the migration of wild birds, human movement, live-bird transportation, and the transportation of poultry products (Kim et al., 2012; Shibata et al., 2019; Shibata et al., 2018). Because of the potential impact of H7 AIVs, the occurrence of any viruses belonging to subtype H7 is notifiable, regardless of their actual pathogenicity; this process aims to reduce the spread of the disease and eventually to achieve its eradication (OIE, 2015).

While no outbreaks of H7 HPAIV infection have been reported in Southeast Asian countries, the detection of H7 LPAIVs has been reported in domestic poultry and wild birds, e.g., in Singapore [H7N1 in 1994–1995 (Banks et al., 2000)]; Thailand [H7N1 in 2009–2010, H7N6 in 2010, and H7N4 in 2010–2011 (FAO, 2019; Wongphatcharachai et al., 2014)]; Cambodia [H7N3, H7N4, H7N7, and H7N9 2017–2019 (FAO, 2019; Suttie et al., 2018)]; and Vietnam [H7N1 in 2012 (Okamatsu et al., 2013)]. Because of the potential for H5/H7 LPAIVs to evolve into HPAIVs through the insertion of polybasic amino acids into the HA cleavage site during the circulation of these viruses in poultry (Silvano et al., 1997) and the emergence of antigenic variants under the pressure of vaccine, which might lead to the failure of vaccination campaign (Eladl et al., 2011; Ibrahim et al., 2013; Bai et al., 2019; Jia et al., 2019), the circulation of H5/H7 LPAIVs in the poultry population should be minimized to enhance disease control efforts.

Our active surveillance program to control and prevent AIV infections in poultry in

93 Vietnam was begun in 2009 (Nomura et al., 2012; Okamatsu et al., 2013; Chu et al., 2016). In
94 2018, this surveillance program detected three H7N7 LPAIVs, which were isolated from the
95 same flock of domestic ducks. The present study reports on the genetic and antigenic
96 characteristics of these first H7N7 isolates to be identified in Vietnam, with the aim of
97 providing a better understanding of the H7 LPAIVs circulating in Asia.

2. Materials and Methods

2.1. Sample collection

In August 2018, surveillance for avian influenza was conducted in Vinh Long province, in the south of Vietnam (Fig. 1). Oropharyngeal and cloacal swabs were obtained from domestic birds housed at biosecurity or backyard farms, live-bird markets (LBMs), and poultry delivery stations (PDSs). Transport medium was used for the preservation of field samples; this medium comprised Eagle's minimum essential medium (Nissui, Japan) containing 10,000 U/ml penicillin G (Meiji Seika, Japan), 10 mg/ml streptomycin (Meiji Seika, Japan), 0.3 mg/ml gentamicin (Schering Plough, USA), 250 U/ml nystatin (Sigma, USA), and 0.5% bovine serum albumin fraction V (Roche, Switzerland). All samples were stored at -80°C until they could be tested.

2.2. Isolation and identification of AIVs

Each sample along with transport medium was inoculated into the allantoic cavity of a 10-day-old chicken embryo from conventional chicken flock tested free of avian influenza virus antibody. After incubation at 35°C for 30–48 h, allantoic fluid exhibiting hemagglutination activity was collected. The influenza virus subtypes were identified by hemagglutination inhibition (HI) and neuraminidase inhibition tests with antisera to the reference influenza virus strains (Kida and Yanagawa, 1979).

2.3. Sequencing and phylogenetic analysis

Viral RNA was extracted from 250 μl of allantoic fluid using TRIzol LS Reagent (Life Technologies, USA) according to the manufacturer's protocol, followed by reverse transcription

with the Uni12 primer (Hoffmann et al., 2001) and M-MLV Reverse Transcriptase (Life Technologies). Full-length cDNAs of the HA RNA gene segments were amplified by polymerase chain reaction with Ex-Taq (Takara Bio, Japan) and gene-specific primer sets (Hoffmann et al., 2001). Direct sequencing of the HA gene segment was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit and a 3500 Genetic Analyzer (Life Technologies). Next generation sequencing was applied to determine the whole genome sequences of the other seven gene segments as follows. MiSeq libraries were prepared using an NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, USA) and sequenced using a MiSeq system and MiSeq Reagent Kit v3 (600 cycles) (Illumina, USA). Sequence reads were assembled using CLC Genomics Workbench, version 12 (CLC Bio, Denmark; now Qiagen). The deduced amino acid sequence of the HA was interpreted from cDNA sequence information using GENETYX version 12 (Genetyx Corporation, Japan). The genome sequences identified in this study have been registered in GenBank/EMBL/DDBJ: A/duck/Vietnam/HU10-48/2017 (H7N7) (abbreviation: HU10-48; accession nos. MK629009–MK629016), A/duck/Vietnam/HU10-64/2017 (H7N7) (abbreviation: HU10-64; accession nos. MK629225–MK629232).

For phylogenetic analysis, nucleotide sequences of the isolates and those from a public database were aligned using Clustal W version 2.0 (Larkin et al., 2007). Phylogenetic trees were constructed using the maximum likelihood (ML) method, with 1,000 bootstrap replicates, using MEGA 7.0 software (Stecher et al., 2016).

2. 4. *Estimating the major antigenic regions of H7 hemagglutinin*

The amino acid sequence of an H7 virus HA, from A/Netherlands/219/2003 (H7N7), was aligned with that of the H3 HA from A/Aichi/2/1968 (H3N2). The antigenic sites in the H7 HA of viruses in the present study (HU10-48, HU10-64, and A/duck/Vietnam/OIE-0178/2012 (H7N1)) and other viruses from public database were inferred based on the corresponding location of epitopes (from A to E) of the H7 HA from A/Netherlands/219/2003 (H7N7) (Liu et al., 2015).

2.5. *HI assay and antigenic cartography*

Polyclonal antisera were prepared as previously described during our research (Hiono et al., 2015; Sakabe et al., 2008; Shibata et al., 2018), by hyperimmunizing chickens against various viruses [i.e., A/turkey/Italy/4580/1999 (H7N1), A/duck/Hokkaido/W19/2013 (H7N2), A/chicken/New South Wales/327/1997 (H7N4), A/duck/Hokkaido/Vac-2/2004 (H7N7), A/chicken/North Korea/7916/2005 (H7N7), A/duck/Taiwan/Ya103/1993 (H7N7), A/seal/Massachusetts/1/1980 (H7N7), A/Anhui/1/2013 (H7N9), and A/duck/Japan/AQ-HE29-22/2017 (H7N9)]. Freund's Complete Adjuvant and Freund's Incomplete Adjuvant (Becton, Dickinson and Company, USA) were mixed with 500 µg formalin-inactivated whole virus particles for the first and second immunization, respectively. Five days after a final immunization with a mixture of 500 µg formalin-inactivated whole virus particles and PBS, sera were collected and stored at -20°C until use. Then, the antigenic properties of the newly isolated H7 viruses were assessed using polyclonal antisera by performing a cross-HI test as follows.

158 Antibodies were two-fold serially diluted with PBS in 96-well U-bottom microtiter plates and
159 mixed with an amount of virus equivalent to four HA units, followed by incubation at room
160 temperature (approximately 25°C) for 60 min. After adding 50 µl 0.5% chicken red blood cells,
161 the mixtures were gently mixed and incubated at room temperature for a further 45 min. HI titers
162 were expressed as reciprocals of the highest serum dilutions that showed complete HI.

163 Antigenic cartography was estimated using the web-based software,
164 <http://www.antigenic-cartography.org/> (Smith et al., 2004). The resulting data containing cross-
165 HI titers were loaded to obtain x/y coordinates of each antiserum and antigen.

3. Results

3.1. Identification of AIVs circulating in PDSs, LBMs, and farms.

A total of 139 viruses were isolated from 1,846 cloacal and oropharyngeal samples of domestic birds and environmental samples. In total, 1 H3, 2 H4, 28 H5, 52 H6, 3 H7, 48 H9, 4 H11, and 1 H13 AIVs were identified from samples collected at 8 PDSs, 8 LBMs, and 75 farms (Table 1). The prevalence of AIVs in PDSs was the highest (20.3%; 95% confidence interval - CI: 16 to 25.3), followed by LBMs (18.3%; 95% CI: 14.1 to 23.2), next was biosecurity farm (2.5%; 95% CI: 1.5 to 3.9), and the last was backyard farm (0.8%; 95% CI: 0.2 to 2). A total of 17 H5N1 HPAIVs clade 2.3.2.1 were isolated from apparently healthy ducks (7), Muscovy ducks (4), geese (2), and chicken (3) in PDSs, LBMs, and farms. Eleven H5N6 HPAIVs were isolated from apparently healthy ducks (10) and Muscovy duck (1) and this is the first report of the detection of H5N6 HPAIVs clade 2.3.4.4 in Vinh Long province.

3.2. Isolation and identification of H7N7 LPAIVs from domestic ducks in Vietnam

Three H7N7 viruses were detected from apparently healthy ducks on the same farm in the southern region (Fig. 1). Two H7N7 viruses were selected in this study and virus strain names were assigned as A/duck/Vietnam/HU10-48/2018 (H7N7) and A/duck/Vietnam/HU10-64/2018 (H7N7). Deduced amino acid sequences of the HA gene segments of these H7N7 isolates were examined to estimate viral pathotypes and showed that these H7N7 viruses had amino acid motifs of PEPPKG/GLF at the HA cleavage site (Table 2), indicating that these isolates were likely to be LPAIVs.

3.3. *Phylogenetic analysis of the H7N7 LPAIVs*

To investigate the phylogenetic relationships between the Vietnamese H7N7 LPAIVs and other homologous virus subtypes, the full-length nucleotide sequences of their eight gene segments were analyzed using the ML method. In a phylogenetic tree based on the HA gene, H7 HA genes were phylogenetically divided into five lineages (i.e., Eurasian, Historical European, Australian, North American, and Equine; Fig. 2), as previously reported (Hiono et al., 2015). The Eurasian lineage comprised three sublineages: Old Eurasian, Far Eastern, and European–Asian. The present H7N7 viruses both belonged to the European–Asian sublineage and showed a close relationship with an HA gene segment of H7N3 viruses that were recently isolated in Cambodia during 2017. It should be noted that the H7 HA genes of the recent H7N7 viruses are genetically distinct from those of Chinese H7N9 viruses and two H7 viruses previously detected in Vietnam, in 2009 and 2012. In the NA phylogenetic tree, similarly to the HA-based phylogenetic trees, both HU10-48 and HU10-64 were grouped within the European–Asian sublineage of the Eurasian lineage, although they branched from the ancestor of the European–Asian sublineage together with an H10N7 virus previously isolated in Vietnam in 2012 (Supplementary Fig. 1a). In the previous study, the sequence of internal genes was phylogenetically divided into 10 genetic groups (Okamatsu et al., 2013), in this study the H7N9 China genetic group was newly proposed to make 11 genetic groups. Among the internal genes, the M-gene-based phylogenetic tree showed a different topology compared with the other phylogenetic trees (Fig. 2). The M gene segment of HU10-48 clustered separately from the HU10-64 virus, suggesting different

origins for these two M gene segment RNAs. All of the other internal genes were seen to be closely related to those of viruses isolated from domestic poultry in the same area as well as from poultry and wild birds in China and other East and Southeast Asian countries (Supplementary Fig. 1). However, the relationships with Cambodian H7N3 viruses differed among segments; the M gene of the HU10-64 virus and PB1 gene segments of both viruses was closely related to the Cambodian viruses, as the HA gene, while the M gene of HU10-48 virus, PB2, PA, NP, and NS gene segments of both viruses were distinct from them.

3.4. Antigenic analysis of HA of the H7N7 LPAIV isolates

The antigenicity of the H7N7 viruses isolated during the present study was analyzed by an HI test using a chicken hyper-antisera panel (Table 3). The results of the cross-HI test were used to produce the antigenic cartography necessary for projecting the dataset into 2D cartography (Fig. 3). The HI titers of HU10-48 and HU10-64 to each of the antisera against viruses in the Eurasian lineage were different by approximately 1 to 2 antigenic units, respectively. In addition, the two H7N7 viruses did not react with most of the antisera at lower concentrations compared with the homologous strain, indicating that these two strains are antigenically different from the majority of strains that belong to the Eurasian lineage. Based on the antigenic cartography, the H7Nx viruses examined in the present study were likely to form three distinct antigenic groups. The major antigenic group (green) comprised the majority of viruses, which belonged to the Eurasian, Australian, and North American lineages. The Historical European lineage was likely to form a single antigenic group (blue). The present

H7N7 viruses formed another antigenic group (red), together with A/turkey/Italy/4580/1999 (H7N1) and A/duck/Japan/AQ-HE29-22/2017 (H7N9). Furthermore, the antigenic drift of the A/duck/Japan/AQ-HE29-22/2017 (H7N9) from that of A/Anhui/1/2013 (H7N9) and A/duck/Hokkaido/W19/2013 (H7N7) was reported in our previous study (Shibata et al., 2018). The mass vaccination for H7 subtype was applied in China might generate the immune escape viral mutants, which was identified as the main cause of antigenic drift of AIVs (Nguyen et al., 2017). HU10-48 and HU10-64 were the most antigenically distant from the viruses of the major antigenic group [more than approximately 1 and 2 antigenic units from A/duck/Hokkaido/W19/2013 (H7N7) to HU10-48 and HU10-64, respectively]. This result suggested that there were important antigenic differences between the newly isolated H7N7 viruses in Vietnam and the majority of H7 LPAIVs isolated thus far elsewhere in the world.

3.5. Molecular characterization of viral amino acid sequences

The deduced viral amino acid sequences of the two H7N7 AIVs were examined to estimate the viral phenotypes. The amino acid residues at the receptor binding site in the HA protein are Q226 and G228 (Table 2) , same as H7 avian progenitor virus, which indicates an avian-like receptor binding preference (Xiong et al., 2013). Additionally, these strains have several differences in the antigenic region from A to E compared with the reference strain [A/duck/Hokkaido/W19/2013 (H7N2)], which is shown in Supplementary Table 1. In particular, the HU10-64 strain had one amino acid mutation at A138T in the 130-loop (Yang et al., 2012) compared with HU10-48.

246 The newly detected viruses have a full-length NA protein, with no amino acid deletions
247 in the stalk region (Table 2), meaning that these viruses have not yet adapted to terrestrial birds
248 (Cauldwell et al., 2014). No single mutations in the PB2 protein (i.e., Q591K, E627K, or
249 D701N), which are markers of high pathogenicity of influenza viruses in mammals (Subbarao et
250 al., 1993), were detected in the present H7N7 viruses. Furthermore, the retention of H274 in the
251 NA and L26, V/I27, A30, and S31 in the M2 amino acid sequences indicates that both newly
252 detected H7N7 viruses will still be sensitive to NA and M2 inhibitors (Pinto et al., 1992; Song et
253 al., 2015).

4. Discussion

H7N7 LPAIVs have been detected in several countries on the East Asian–Australasian Flyway [including Mongolia, Japan (Hiono et al., 2015), China (Liu et al., 2018), and South Korea (Kim et al., 2012)], indicating the circulation of these viruses in migratory waterfowl. In the present study, we identified two H7N7 isolates from domestic ducks in Vietnam; this is the first report of H7N7 LPAIV detection in the country. As previously reported (Kim et al., 2012), H7N7 LPAIVs circulate in wild birds and are highly likely to have been introduced into domestic ducks. Here, we characterized these two new isolates and revealed possible ongoing reassortment of H7 LPAIVs and antigenic diversification.

In the present phylogenetic analyses, possible transmission routes of the H7N7 viruses have been identified; the viruses carrying the H7 HA gene belonging to the European–Asian sublineage circulate in East Asia and were introduced first into Cambodia as an H7N3 virus and then into Vietnam. The virus carrying the N7 NA gene of the European–Asian sublineage has circulated in Vietnam since at least 2012 as an H10N7 virus, and likely shares this N7 gene with the present newly isolated H7N7 viruses. The internal genes, including two M genes, of viruses reported for the first time in this study, may be shared with other AIVs circulating in Vietnam and other viruses that have been isolated in East Asian–Australasian Flyway countries; HA, PB1, and M of HU10-64 seem to have been introduced into Vietnam from Cambodia. The M gene of HU10-48, as well as its PB2, PA, NP, NA, and NS genes are likely to be shared with other viruses in the same area and have been a result of spillover from wild birds and are now

maintained in the poultry population in Vietnam. The distinct M gene segments identified on the single farm in this study suggest an ongoing reassortment event, although reassortment during virus isolation is also a possibility. In addition, a previous field epidemiological study indicated that poultry movement across areas that border Cambodia might lead to proliferation of the H7 LPAIVs circulating in both Vietnam and Cambodia (Meyer et al., 2018). Taken together, these results suggest that Vietnam and Cambodia have a close relationship in terms of AIV ecology and further studies should focus on this relationship.

Interestingly, the two H7 AIVs in our study showed similar antigenicity to HPAIVs of two distinct sublineages: European–Asian [A/turkey/Italy/4580/1999 (H7N1)] and Far Eastern [A/duck/Japan/AQ-HE29-22/2017 (H7N9)], despite differences in their amino acid sequences (94% and 91% homology, respectively). The single mutation at A138T in the 130-loop may play an important role in the antigenic differences between HU10-64 and HU10-48, which are indicated in Fig. 3. In general, non-pathogenic AIVs circulate among wild ducks under the relatively low selective pressure of antibodies; thus, they are antigenically stable (Kida et al., 1987). However, the long-term circulation of H7 viruses in the poultry population may lead to selective pressure from antibodies induced by natural infections in poultry, accelerating antigenic variation. The H7 vaccine has not been used in Vietnam thus far, so the antigenic variance of the two H7 AIVs in this study suggests antigenic diversification occurred in domestic ducks following natural infections. Furthermore, the similarities in antigenic properties of the present H7N7 LPAIVs with Chinese H7N9 HPAIVs suggest that the multi-direction of antigenic

diversity of AIVs in poultry population and these newly isolated H7N7 viruses would be considered as potential candidates for vaccine strain. The similar of antigenicity despite the differences in amino acid sequence suggests the synonymous mutation on Vietnamese H7N7 viruses.

H7N9 AIVs of the Chinese group have not been detected during our influenza surveillance of poultry. However, domestic birds in Vietnam are mainly raised in households in a free-range manner, and poultry can come into direct contact with wild animals. Therefore, we should pay more attention to LPAIVs, because some H7 LPAIV strains are capable of undergoing systemic replication and efficient transmission in chickens (Lee et al., 2018), and the circulation of H7 LPAIVs in a poultry population can increase their pathogenicity (Silvano et al., 1997). The detection of H7 LPAIVs in the south of Vietnam at different time periods, as well as in Cambodia in recent years (FAO, 2019; Suttie et al., 2018), has acted as a warning of the silent circulation of AIVs in the southern border area. A previous study indicated that the transmission of AIVs occurs through a combination of local and long-distance spreading (Phan et al., 2009). Taking the above results together, the control of H7 LPAIVs by a number of measures, including stamping out LPAIVs completely, better hygiene practices, and improved biosecurity is key to controlling avian influenza in Vietnam. Of these, stamping out LPAIVs is highly recommended so as to remove them from the poultry population before they mutate into HPAIVs and become antigenically divergent viruses (Gonzales et al., 2014). Further studies would be necessary to monitor the circulation and analyze the epidemiology of H7 LPAIVs in Vietnam, giving a more

314 comprehensive data of the economic impact and human health risk of the viruses (Rushton et al.,
315 2005). Therefore, the implementation of effective control measures against H7 LPAIVs is
316 needed to prevent the economic losses and opportunity for a pandemic. Thus, active surveillance
317 should be conducted continuously in these areas to monitor the circulation of AIVs in Vietnam.

5. Conclusions

H7N7 low pathogenic AIVs were identified in domestic ducks in Vietnam in 2018. The findings of this study suggest that H7N7 viruses may be undergoing reassortment and antigenic diversification in poultry flocks in Southeast Asia. Thus, our results highlight the need for continuous and intensive surveillance of avian influenza in Vietnam, targeting not only HPAIVs but also LPAIVs.

Conflict of interest

The authors declare that no competing interests exist.

Acknowledgments

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528

529 **Fig. 3.** An antigenic map based on the cross hemagglutination inhibition (HI) tests on viruses and
530 sera of different lineages. In an antigenic map, both vertical and horizontal axes represent
531 antigenic distance. The spacing between grid lines represents a distance of 1 antigenic-unit
532 distance, corresponding to a 2-fold dilution in the HI assay (e.g. 2 units correspond to a 4-fold
533 dilution, 3 units correspond to an 8-fold dilution etc.). Different antigenic clusters are indicated
534 by different colors (green, blue, and red). Sera are indicated by a square symbol and antigens are
535 indicated by a round symbol. Dot line indicates homologous combination.

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537

538 **Supplementary Table 1.** Amino acid sequence of antigenic sites for HA of H7 viruses.

539

540 **Supplementary Fig. 1.** Phylogenetic analyses for the other genes of H7N7 influenza viruses
541 isolated in Vietnam. The full-lengths of the NA (A), PB2 (B), PB1 (C), PA (D), NP (E), and NS
542 (F) genes were used for ML phylogenetic analysis using MEGA 7.0 software. Digits at the nodes
543 indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications.
544 The H7 viruses isolated in this study are highlighted in gray, other viruses isolated in the same
545 area are indicated by black triangle, HPAIVs are indicated by black circles, Chinese H7N9
546 viruses are indicated in bold, and viruses previously isolated in Vietnam are underlined.

Table 1. Avian influenza viruses isolated in Vinh Long province in Vietnam in 2018

Sampling place	Sample type	No. of samples	No. of isolates	Isolation rate (%)	Subtype (No. of isolates)
PDS	Chicken	174	7	4.0	H5N1 (3) ; H9N2 (3); H13N9 (1)
	Duck	93	55	59.1	H5N1 (1) ; H5N6 (10) ; H6N6 (43); H9N6 (1)
	Geese	5	0	-	
	Muscovy duck	33	0	-	
LBM	Chicken	151	28	18.5	H9N2 (28)
	Duck	116	18	15.5	H4N6 (2); H5N1 (6) ; H6N6 (7); H9N2 (3)
	Geese	2	2	100.0	H5N1 (2)
	Muscovy duck	31	7	22.6	H5N1 (4) ; H5N6 (1) ; H9N2 (1); H11N1 (1)
Biosecurity farm	Chicken	360	9	2.5	H9N2 (9)
	Duck	368	9	2.5	H3N2 (1); H5N1 (1) ; H6N6 (2); <u>H7N7</u> (3); H9N2 (1); H11N9 (1)
	Geese	2	0	-	
Backyard farm	Chicken	246	2	0.8	H9N2 (2)
	Duck	225	2	0.9	H11N9 (2)
	Geese	1	0	-	
	Muscovy duck	39	0	-	
Total		1,846	139	7.5	

Highly pathogenic avian influenza viruses are highlighted in bold; H7 viruses analyzed in this study are underlined.

LBM: live bird market, PDS: poultry delivery station.

Table 2. Amino acid comparison of the newly detected H7N7 viruses

Virus	Subtype	HA				NA		PB2			M2			
		Cleavage site	138 [§]	225	226	274 [*]	Stalk deletion	591	627	701	26	27	30	31
A/duck/Vietnam/HU10-48/2018	H7N7	PEPPKGR/GLF	A	G	Q	H	No	Q	E	D	L	V	A	S
A/duck/Vietnam/HU10-64/2018	H7N7	PEPPKGR/GLF	T	•	•	•	•	•	•	•	•	I	•	•
A/duck/Cambodia/b0120501/2017	H7N3	PEPPKGR/GLF	•	•	•	•	•	•	•	•	•	I	•	•
A/duck/Vietnam/OIE-0178/2012	H7N1	PEGPKGR/GLF	•	•	•	•	•	•	•	•	•	I	•	•
A/turkey/Italy/4580/1999	H7N1	PEIPKGSRVRR/GLF	T	•	•	•	Yes	•	•	•	•	•	•	•
A/duck/Taiwan/Ya103/1993	H7N7	PEIPKKREKR/GLF	•	•	•	•	Yes	•	•	•	•	•	•	•

[§] H3 numbering

^{*} N2 numbering

“•” indicates the same amino acids as A/duck/Vietnam/HU10-48/2018 (H7N7)

Table 3. Antigenic analyses of H7 influenza viruses by cross HI test.

Lineage	Sub lineage	Virus	HI titers of the antiserum								Austra- lian	North American
			Eurasian									
			Far -Eastern				European - Asian		Historical Europe			
			Dk/Hok/ Vac- 2/04	Anhui/ 1/13	Ck/NK/ 7916/05	Dk/JP/AQ- 29-22/17	Dk/Hok/ W19/13	Ty/Italy/4 580/99	Dk/TW/ Ya103/9 3	Ck/NS W/327/9 7		
Eurasian	Far - Eastern	A/duck/Hokkaido/Vac-2/2004 (H7N7)	<u>20,480</u>	5,120	10,240	10,240	20,480	5,120	2,560	10,240	5,120	
		A/Anhui/1/2013 (H7N9)	10,240	<u>5,120</u>	5,120	20,480	10,240	5,120	1,280	5,120	1,280	
		A/chicken/North Korea/7916/2005 (H7N7)	2,560	2,560	<u>5,120</u>	10,240	5,120	256	640	5,120	320	
		A/duck/Japan/AQ-HE29-22/2017 (H7N9)	640	640	640	<u>5,120</u>	1,280	640	160	2,560	80	
	European - Asian	A/duck/Vietnam/HU10-48/2017 (H7N7)	1,280	640	1,280	5,120	2,560	640	640	2,560	640	
		A/duck/Vietnam/HU10-64/2017 (H7N7)	640	160	640	2,560	1,280	320	160	1,280	320	
		A/duck/Vietnam/OIE-0178/2012 (H7N1)	5,120	2,560	5,120	10,240	10,240	2,560	1,280	5,120	1,280	
		A/duck/Hokkaido/W19/2013 (H7N2)	5,120	2,560	2,560	10,240	<u>5,120</u>	2,560	640	5,120	1,280	
	Historical Europe	A/turkey/Italy/4580/1999 (H7N1)	320	160	640	2,560	640	<u>1,280</u>	320	1,280	160	
		A/duck/Taiwan/Ya103/1993 (H7N7)	160	320	640	1,280	640	160	<u>5,120</u>	320	80	
Australian	-	A/chicken/New South Wales/327/1997 (H7N2)	5,120	2,560	5,120	20,480	10,240	2,560	1,280	<u>10,240</u>	640	
North American	-	A/seal/Massachusetts/1/1980 (H7N7)	20,480	5,120	10,240	10,240	10,240	5,120	640	10,240	<u>5,120</u>	

Viruses isolated in this study are highlighted in italic.

HPAIVs are shown in bold.

Homologous titers are underlined.

Dk duck, Ck chicken, Ty Turkey, Sl Seal, Hok Hokkaido, JP Japan, NK North Korea, TW Taiwan, NSW New South Wales, Mass Massachusetts.



China

Laos

Vietnam

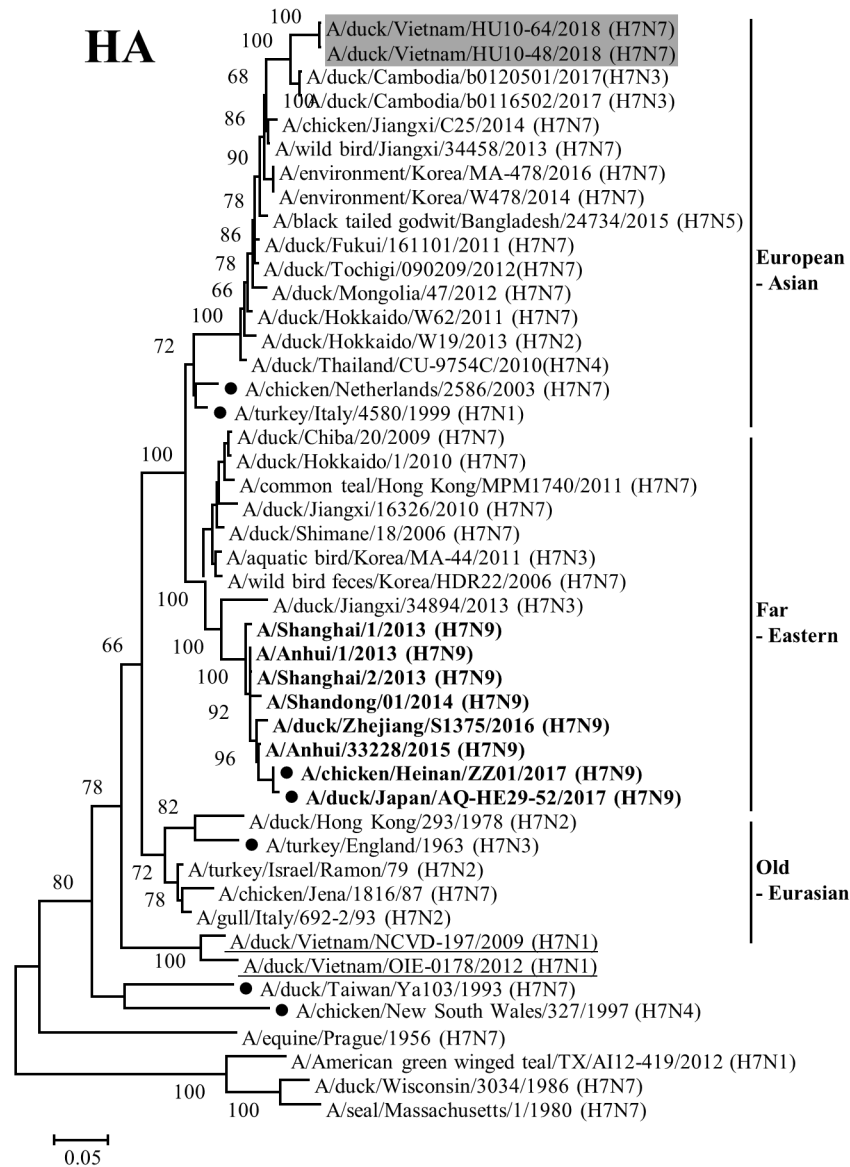
Thailand

Cambodia

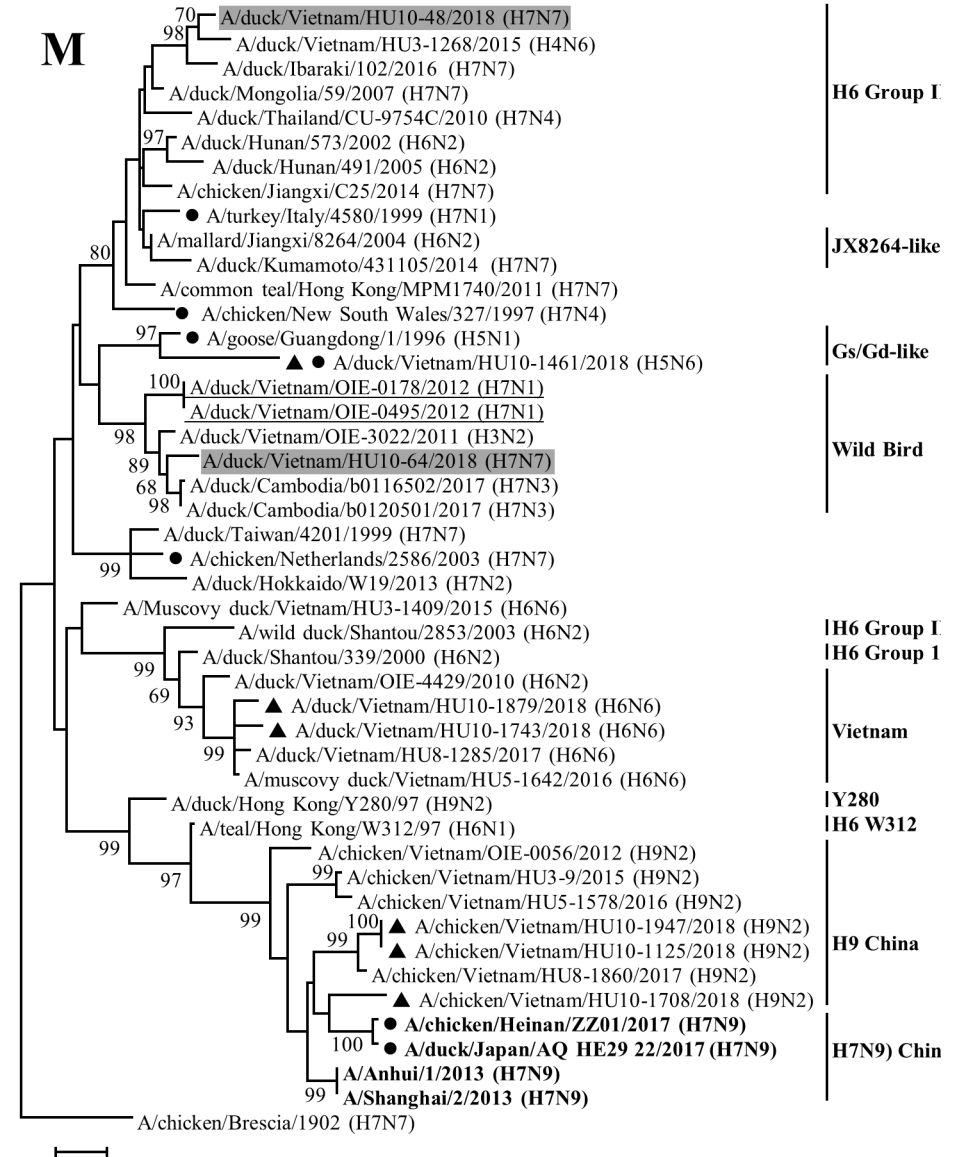
Vinh Long

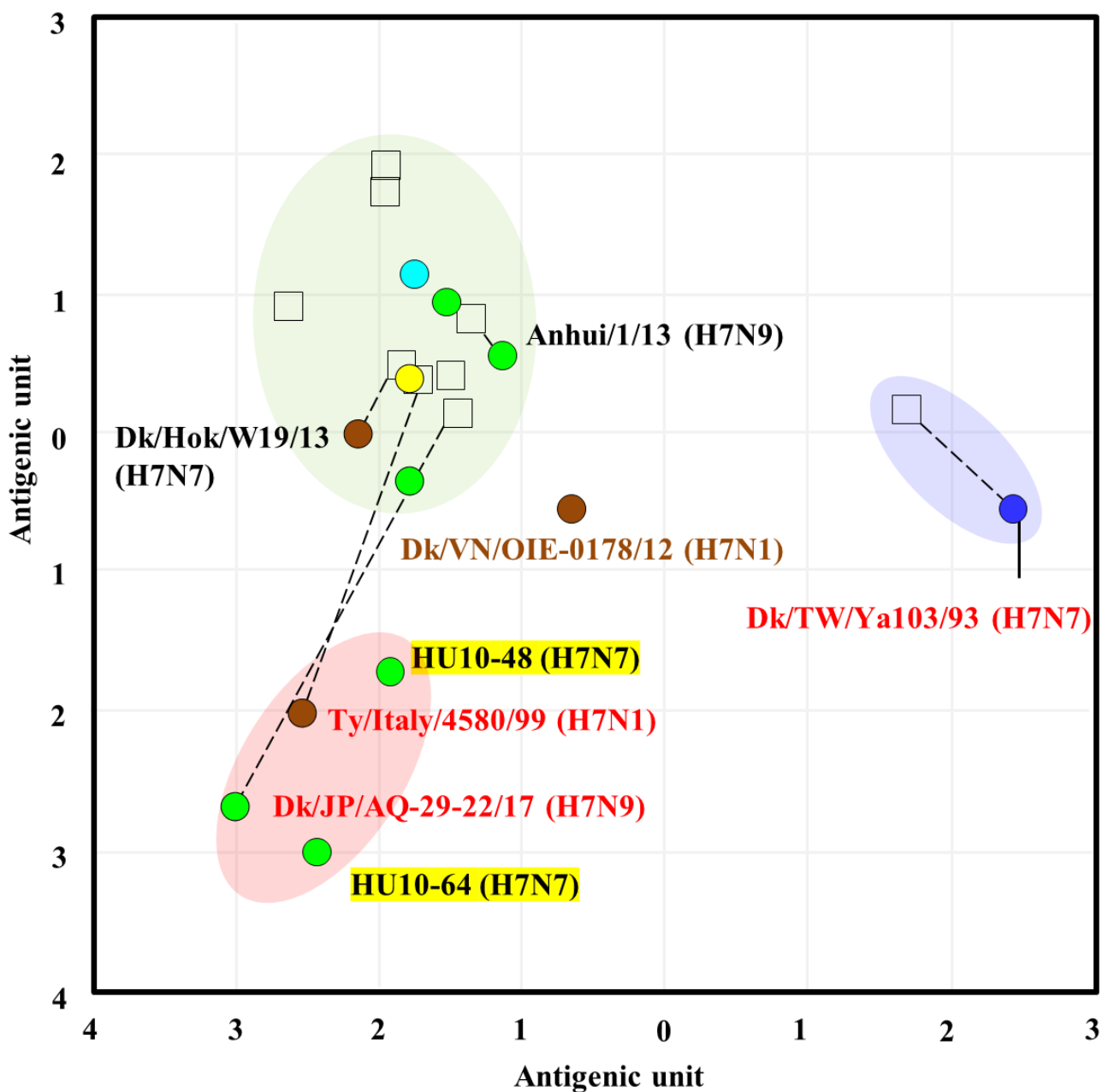
0 100 200 km

HA



M





Antigen

- : European-Asian
- : Far-Eastern
- : Historical Europe
- : Australian
- : North American

Antiserum

- Green highlight: Major antigenic group
- Blue highlight: Historical Europe antigenic group
- Red highlight: Antigenic variants group
- Viruses previously isolated in Vietnam
- H7 HPAIVs
- Viruses isolated in this study