Genetic and antigenic characterization of H5, H6 and H9 avian influenza viruses circulating in live bird markets with intervention in the center part of Vietnam

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Abstract

A total of 3,045 environmental samples and oropharyngeal and cloacal swabs from apparently healthy poultry have been collected at three live bird markets (LBMs) at which practices were applied to reduce avian influenza (AI) virus transmission (intervention LBMs) and six conventional LBMs (non-intervention LBMs) in Thua Thien Hue province in 2014 to evaluate the efficacy of the intervention LBMs. The 178 AI viruses, including H3 (19 viruses), H4 (2), H5 (8), H6 (30), H9 (114), and H11 (5), were isolated from domestic ducks, muscovy ducks, chickens, and the environment. The prevalence of AI viruses in intervention LBMs (6.1%; 95% CI: 5.0 to 7.5) was similar to that in non-intervention LBMs (5.6%; 95% CI: 4.5 to 6.8; $\chi^2 = 0.532$; $df = 1$; $P = 0.53$) in the study area. Eight H5N6 highly pathogenic avian influenza (HPAI) viruses were isolated from apparently healthy ducks, muscovy ducks, and an environmental sample in an intervention LBM. The hemagglutinin genes of the H5N6 HPAI viruses belonged to the genetic clade 2.3.4.4, and the antigenicity of the H5N6 HPAI viruses differed from the H5N1 HPAI viruses previously circulating in Vietnam. Phylogenetic and antigenic analyses of the H6 and H9 viruses isolated in both types of LBMs revealed that they were closely related to the viruses isolated from domestic birds in China, Group II of H6 viruses and Y280 lineage of H9 viruses. These results indicate that the interventions currently applied in LBMs are insufficient to control AI. A risk analysis should be conducted to identify the key factors contributing to AI virus prevalence in intervention LBMs.

Key words: avian influenza; antigenic analysis; phylogenetic analysis; live bird market; surveillance

1. Introduction

Transmission of avian influenza (AI) viruses among wild and domestic birds is an important target of control measures aiming to minimize the risk to both human and animal health worldwide (Kilpatrick et al., 2006; Nomura et al., 2012; Okamatsu et al., 2013). Vietnam has a large population of poultry (approximately 308 million), and a majority of these animals (approximately 80%) are raised under backyard conditions at households in rural areas without biosecurity application (General Statistics Office of Vietnam. Results of the 2012 Rural, Agricultural and Fishery Census. https://www.gso.gov.vn/default_en.aspx?tabid=778; Hanh et al., 2007). In Vietnam, H5N1 highly pathogenic avian influenza (HPAI) viruses have caused a large number of outbreaks in poultry since 2003 (Minh et al., 2009). In our previous study, we conducted surveillance of AI viruses in live bird markets (LBMs) and households that raise poultry in a large number of Vietnamese provinces in 2009. We found that the prevalence of AI viruses was substantially higher in LBMs than in backyard farms, and H5N1 viruses were isolated from apparently healthy domestic ducks and chickens in LBMs (Okamatsu et al., 2013; Nomura et al., 2012). The findings suggest that in Vietnam, LBMs are more responsible for the amplification, maintenance, circulation, and transmission of AI viruses than backyard farms. Therefore, continuous surveillance of AI viruses in LBMs in Vietnam is essential to understand the distribution of AI viruses and to minimize the risk to public and animal health.

LBMs are ubiquitous and integral parts of the poultry industry in Vietnam and other developing countries in Asia (Wan et al., 2011; Indriani et al., 2010). In China, LBMs have become

a major source of human infection with H5 HPAI viruses and H7 low pathogenic AI viruses (He et al., 2014; Yu et al., 2014; Wan et al., 2011). When keeping live birds overnight in LBMs was banned, the rate of virus isolation declined (Leung et al., 2012). In addition, it was reported that closing LBMs effectively reduced the risk of virus transmission to the public (Fournié et al., 2011; He et al., 2014; Yu et al., 2014). However, banning LBMs in developing countries remains challenging because changing the traditional market style should take a long time. Therefore, government intervention to improve the biosecurity measures employed at LBMs has been thought to represent a promising strategy to minimize the transmission of viruses in Asian countries including Vietnam. To support for the evaluation of local authority on the current intervention, we surveyed the prevalence of AI viruses at nine LBMs with or without the intervention of Vietnam Avian and Human Influenza Control and Preparedness Project (VAHIP) in Thua Thien Hue province, located in the central region of Vietnam. The VAHIP was a project funded by the World Bank aiming to reduce the risk of AI virus transmission to humans (The World Bank, Projects and Operations. Vietnam - Avian and Human Influenza Control and Preparedness Project. http://documents.worldbank.org/curated/en/2011/03/14026433/vietnam-additional-financing-vietnam-avian-human-influenza-control-preparedness-project-environmental-impact-assessment.). The project developed the new model of LBM in selected provinces in Vietnam by deploying infrastructure for the poultry markets and operating periodic disinfection in these intervention LBMs. In this study, various subtypes of AI viruses were isolated during surveillance of LBMs with or without intervention in August and December, 2014. The representative isolates were

103 phylogenetically and antigenically analyzed to characterize the genetic and antigenic variation of
104 the AI viruses currently circulating in LBMs in Vietnam.
105
106 **2. Materials and methods**
107 *2.1. Sample collection*
108 The surveillance was conducted in August and December, 2014. Oropharyngeal, cloacal swabs
109 and fecal samples from domestic birds and water troughs (environmental samples) were collected at
110 nine LBMs (Supplemental Fig. 1). At three of the nine LBMs, a biosecurity infrastructure had been
111 established by the VAHIP program in Thua Thien Hue province, Vietnam (intervention LBMs;
112 Table 1; Supplemental Fig. 1). The other six LBMs were conventional markets at which no
113 particular biosecurity infrastructure was established and at which poultry and other animals were
114 usually mixed together in low biosecurity conditions (non-intervention LBMs; Table 1;
115 Supplemental Fig. 1).
116 All collected samples were stored in sterile tubes with transport medium (minimum essential
117 medium, Nissui, Japan) containing 10,000 U/ml penicillin G (Meiji Seika, Japan), 10 mg/ml
118 streptomycin (Meiji Seika, Japan), 0.3 mg/ml gentamicin (Schering Plough, USA), 250 U/ml
119 nystatin (Sigma, USA), and 0.5% bovine serum albumin fraction V (Roche, Switzerland) at −80°C
120 until use.
121
122 *2.2. Isolation and identification of AI viruses*

123 Samples were resuspended in virus transport medium and centrifuged at 2,000 rpm for 5 min.
124 The supernatant was inoculated into the allantoic cavity of a 10-day-old chicken embryo. After
125 incubation at 35°C for 30–48 h, allantoic fluids exhibiting hemagglutination activity were collected
126 for subtyping of influenza viruses by hemagglutination-inhibition (HI) and neuraminidase-
127 inhibition tests with antisera to the reference influenza virus strains (Kida et al., 1979).
128
129 *2.3. Sequencing and phylogenetic analysis*
130 Viral RNA was extracted from the 250 μl of allantoic fluids by TRIzol LS Reagent (Life
131 Technologies, USA) following the manufacturer's protocol and reverse transcribed with the Uni12
132 primer (Hoffmann et al., 2001) and M-MLV Reverse Transcriptase (Life Technologies, USA). Full-
133 length cDNAs of the eight gene segments were amplified by polymerase chain reaction with Ex-
134 Taq (TaKaRa, Shiga, Japan) and gene-specific primer sets (Hoffmann et al., 2001). Direct
135 sequencing of each gene segment was performed using 3500 Genetic Analyzer (Life Technologies,
136 USA).
137 For phylogenetic analysis, nucleotide sequences of the isolates, together with those from a
138 public database, were aligned using Clustal W. Phylogenetic trees were constructed using the
139 maximum-likelihood (ML) method with 1,000 bootstrap replicates using MEGA 5.0 software
140 (Tamura et al., 2011). The genome sequences identified in this study have been registered in
141 GenBank/EMBL/DDBJ (Table 2).
142

2.4. Antigenic analysis

Polyclonal antisera were prepared from chickens immunized with reference AI virus strains that had been inactivated with formalin (Kida et al., 1979). Antigenic analysis of H5, H6 and H9 viruses was performed using polyclonal antisera by HI test.

2.5. Pathogenicity of an H5N6 AI virus in chickens

To assess the pathogenicity of the representative H5N6 virus in chickens, 0.2 ml of the 1:10-diluted fresh allantoic fluid of chicken embryos infected with A/duck/Vietnam/HU1-1151/2014 (H5N6) was inoculated intravenously into four 7-week-old chickens (*Gallus gallus*). Each chicken was housed in a self-contained isolator unit (Tokiwa Kagaku, Japan) in a BSL3 biosafety facility in the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

2.6. Knowledge, attitude, and practices survey

The KAP survey was designed to investigate the knowledge, attitude, and practice of individual poultry sellers that associated with AI in LBMs using questionnaires written in Vietnamese. A self-designed questionnaire set comprised 47 questions about the general background of the sellers, the source of poultry, and application of any biosecurity measures for the identification of possible risk factors contributing to AI virus circulation in LBMs.

2.7. Ethics statements

Animal experiments were authorized by the Institutional Animal Care and Use Committee of Hokkaido University (approval number: 13-0108), and all experiments were performed according to the guidelines of the committee.

3. Results

3.1. Identification of AI viruses circulating in intervention and non-intervention LBMs

A total of 178 viruses were identified from 3,045 cloacal and oropharyngeal samples of domestic birds and environmental samples (Table 1). In total, 19 H3, 2 H4, 8 H5, 30 H6, 114 H9, and 5 H11 AI viruses were isolated from samples collected at nine LBMs. At the individual market level, the prevalence of AI viruses in No market was the highest (12.2%; 95% CI: 9.6 to 15.4) in the group of intervention LBMs as well as that of Phu Bai market (13.3%; 95% CI: 9.9 to 17.6) in the group of non-intervention LBMs. At the type of LBMs level, the prevalence of AI viruses in intervention LBMs (6.1%; 95% CI: 5.0 to 7.5) was similar to that in non-intervention LBMs (5.6%; 95% CI: 4.5 to 6.8; $\chi^2 = 0.532$; $df = 1$; $P = 0.53$). The subtypes of AI viruses isolated in LBMs with intervention were H3N2 (7), H3N6 (1), H4N6 (2), H6N2 (10), H6N6 (9), H9N2 (49), H9N6 (2), H11N6 (1), and H11N7 (3). The subtypes of AI viruses isolated in non-intervention LBMs were H3N2 (11), H6N2 (4), H6N6 (7), H9N2 (60), H9N6 (3), and H11N7 (1). Eight H5N6 viruses were isolated from apparently healthy ducks (5), muscovy ducks (2), and an environmental sample at the "No" market with intervention. To assess the pathogenicity of the H5N6 viruses, a representative

182 H5N6 virus, A/duck/Vietnam/HU1-1151/2014 (H5N6) was inoculated intravenously into four 7-
183 week-old chickens. All chickens died within 1 day of infection.
184
185 *3.2. Genetic and antigenic analysis of H5 viruses*
186 The full-length nucleotide sequences of the HA genes of H5 representative isolates were
187 phylogenetically analyzed and divided into two lineages: Eurasian and North American. The H5
188 viruses in the Eurasian lineage were identified on the basis of the nomenclature defined by
189 WHO/OIE/FAO H5N1 Evolution Working Group in 2014 (Smith et al., 2015). The HA genes of
190 six representative H5N6 Vietnam isolates were classified into the clade 2.3.4.4, which was newly
191 established in 2014 (Smith et al., 2015) and is closely related to the
192 A/environment/Zhenjiang/C13/2013 (H5N6) virus isolated in China (Qi et al., 2014) (Fig. 1). All
193 the H5N6 isolates contain multiple basic amino acids, R and K, at the proteolytic cleavage site of
194 the hemagglutinin protein, PLREKRRKR/GLF, identical to the typical cleavage site motif of H5
195 HPAI viruses. The residues at position 190, 225, 226, and 228 (H3 numbering) are associated with
196 influenza virus receptor specificity. In these viruses, those residues were E, G, Q, and G,
197 respectively; all of them are of avian-type motif (Paulson et al., 2013).
198 Four representative strains of the H5 isolate were antigenically analyzed by cross HI test (Table
199 3). Chicken antiserum against the reference virus of the clade 2.3.4.4, A/chicken/Kumamoto/1-
200 7/2014 (H5N8) (Kanehira et al., 2015), effectively inhibited hemagglutination of A/muscovy
201 duck/Vietnam/HU1-1144/2014 (H5N6), A/duck/Vietnam/HU1-1151/2014 (H5N6), and A/muscovy

202 duck/Vietnam/HU2-26/2014 (H5N6) and partly inhibited hemagglutination of
203 A/environment/Vietnam/HU1-1434/2014 (H5N6) causing a 4-fold reduction in HI titer. In contrast,
204 these viruses exhibited low HI titers in comparison with homologous titers in reactions with the
205 antisera of different clades (HI titer from 20 to 80).
206
207 *3.3. Genetic and antigenic analysis of H6 viruses*
208 The H6 HA genes were phylogenetically divided into two lineages: Eurasian and North
209 American. The H6 viruses in the Eurasian lineage were clustered into five different sublineages:
210 Group II, W312, Group III, Early and Group I, as described in our previous study (Okamatsu et al.,
211 2013). All H6 viruses isolated in Vietnam in 2014 were found to belong to the Group II sublineage
212 (Fig. 2), as were the viruses previously isolated in Vietnam between 2010 and 2012 and those
213 isolated in China between 2003 and 2011. However, the H6 viruses isolated in this study and in our
214 previous study occupied different clusters.
215 Four strains representative of the H6 isolates were antigenically analyzed by cross HI test
216 (Table 4) using a panel of chicken antisera against four viruses of different sublineages. These H6
217 viruses weakly reacted with A/duck/Vietnam/OIE-4429/2010 (H6N2) antiserum, a virus of the
218 same genetic sublineage, Group II. In reaction with viruses of other sublineages, these H6 viruses
219 exhibited HI titers 4-fold lower than the homologous groups. In addition, these H6 viruses share
220 equivalent HI titers with homologous hyperimmune serum against A/duck/Vietnam/HU1-637/2014
221 (H6N6) virus, while A/duck/Vietnam/OIE-4429/2010 (H6N2) virus reacted weakly with the

antiserum with 16-fold lower HI titers. These results suggest that the antigenicity of the H6 viruses we isolated differed from viruses isolated in Vietnam between 2010 and 2012 (Okamatsu et al., 2013).

3.4. Genetic and antigenic analysis of H9 viruses

The HA genes of H9 isolates were phylogenetically divided into two lineages: Eurasian and North American lineages. All H9 viruses isolated at LBMs in Thua Thien Hue province in 2014 were classified as Y280 sublineage of the Eurasian lineage (Okamatsu et al., 2013; Nomura et al., 2012). These viruses were genetically related to the A/chicken/Vietnam/OIE-1611/2012 (H9N2) virus isolated in the North Vietnam in 2012 and other viruses isolated from poultry in China in 1997 and 2012, which were also classified into the Y280 sublineage (Fig. 3).

Representative strains of the H9 isolates were antigenically analyzed by cross HI test (Table 5). All viruses from Vietnam reacted with the antiserum of A/duck/Hong Kong/Y280/1997 (H9N2) virus, which belonged to the Y280 sublineage. These Vietnamese H9N2 viruses reacted weakly with antisera of other H9N2 viruses classified into different sublineages, such as the G1 or North American lineages, and reacted moderately with antisera to Y349 sublineage virus. This suggests that the antigenicities of the H9N2 viruses isolated in Vietnam have been stable in the poultry population.

3.5. The neuraminidase genes of AI viruses isolated in LBMs

For neuraminidase (NA) gene segments, the names of groups were defined based on previous studies (Kim et al., 2013; Okamatsu et al., 2013). The N2 NA genes of H6 and H9 AI viruses were phylogenetically categorized into two groups, Group II and Y280 (Supplemental Fig. 2a). All N6 NA genes of the H5 viruses belonged to Group II and of H6 viruses belonged to Group I (Supplemental Fig. 2b).

3.6. Genetic diversity of AI viruses isolated in LBMs

The six internal gene segments of the AI viruses were then phylogenetically analyzed to investigate the genetic diversity of AI viruses circulating in Vietnamese LBMs (Supplemental Fig. 3). For each internal gene segment, the names of groups were defined as previously described (Kim et al., 2013; Okamatsu et al., 2013). In this study, the six internal gene segments were also classified into H6 Group I, H6 Group II, H6 Group III, JX8264 like, and Gs/Gd like, indicating that these viruses were closely related to the viruses isolated in China. For instance, the PB2 and PA genes of the A/muscovy duck/Vietnam/HU1-1144/2014 (H5N6) were classified as JX8264 like. The NP, M, and NS genes of this H5N6 virus were grouped into Gs/Gd like and H6 Group I where Chinese viruses were also grouped into. Phylogenetic analysis also indicated that the PB2, PB1, PA, and NS internal genes of H9N2 viruses, A/chicken/Vietnam/HU1-1050/2014 and A/chicken/Vietnam/HU1-976/2014, were classified into the same groups: Vietnam II, Vietnam, and JX8286 like. However, other gene segments, NP and M genes, were classified into different groups (Supplemental Fig. 3).

3.7. Knowledge, attitude, and practices

A total of 52 sellers working at LBMs were interviewed during the first sampling collection, 29 sellers working at intervention LBMs and 23 working at non-intervention LBMs. All respondents working at intervention LBMs declared that their shops were cleaned by chemical disinfection twice per day before the LBM was opened and after closing their business. In contrast, only 17.4% of respondents working at non-intervention LBMs made this claim. In intervention LBMs, 52.0% of sellers acquired their poultry from within Thua Thien Hue province, within a 5 km radius. The other 48.0% of sellers imported their poultry from neighboring provinces, from within a radius of 50 km. In contrast, 100% of the sellers working at non-intervention LBMs sell poultry that originated from the local areas surrounding the LBMs.

4. Discussion

LBMs provide an ideal environment for genetic reassortment events and interspecies transmission of AI viruses (He et al., 2014; Yu et al., 2014; Okamatsu et al., 2013; Wan et al., 2011). In this study, 178 AI viruses were isolated from poultry in both intervention and non-intervention LBMs. The H5N6 viruses were isolated from apparently healthy domestic ducks, muscovy ducks, and an environmental sample at an intervention LBM, indicating that subclinical HPAI virus infections are endemic in domestic birds at LBMs. These results remind us that LBMs play an important role as a hotspot for the transmission of AI viruses, including HPAI viruses, in Vietnam. Although closure of LBMs or banning the storage of poultry overnight at the markets represents a

282 highly effective method to reduce amplification and persistence of viruses in LBMs (He et al.,
283 2014; Yu et al., 2014; Leung et al., 2012; Fournié et al., 2011), it is hard to change the LBMs
284 system in Vietnam due to well-established traditional cultures and the behavior of customers.
285 Therefore, biosecurity measures were developed with the intention of reducing AI transmission. In
286 this study, we conducted surveillance of AI viruses in both intervention and non-intervention LBMs
287 in a central province of Vietnam. We sampled viruses on two occasions and found the AI virus
288 prevalence at intervention LBMs to be 6.1%. The prevalence of AI viruses at non-intervention
289 LBMs did not differ significantly from that at non-intervention LBMs, which was 5.6%. All the
290 viruses identified in this study were isolated from apparent healthy LBM poultry. In addition,
291 various subtypes of AI viruses were identified at intervention LBMs. The H6 and H9 viruses
292 isolated in intervention LBMs were genetically similar to H6 and H9 viruses isolated at non-
293 intervention LBMs. Although disinfection was reported to be performed twice per day at
294 intervention LBMs, AI viruses still contaminated the environment. These results indicate that the
295 current intervention LBMs remain several limitations for the control of AI contamination such as
296 problems of periodic disinfection procedure and concentration of chemical disinfection. The
297 intervention LBMs are generally larger capability to hold poultry than these of non-intervention
298 LBMs meaning that intervention LBMs can contain poultry originating from many different sources,
299 including neighboring provinces. In addition, KAP surveys indicated that the sources of poultry in
300 intervention LBMs in Thua Thien Hue province are widely distributed from local area to other
301 provinces. Therefore, the source of poultry may play an important role in AI transmission at LBMs,

302 and this factor should be further studied. As a next step, a risk analysis will be conducted to identify
303 the risk factors contributing to the appearance of various subtypes of AI viruses at intervention
304 LBMs.

305 The H5N1 HPAI viruses were first detected in Vietnam in 2001, and outbreaks have been
306 regularly reported since the end of 2003 (Minh et al., 2009). The H5 viruses circulating in Vietnam
307 were classified into seven major genetic clades: 1.1, 2.3.2.1, 2.3.4.1, 2.3.4.2, 2.3.4.3, 7.1, and 7.2
308 (Nguyen et al., 2014; Okamatsu et al., 2013; Nguyen et al., 2012). In this study, we report for the
309 first time the characterization of H5N6 viruses isolated in an intervention LBM in a center province
310 of Vietnam. The HA genes of the H5N6 viruses isolated from poultry and an environmental sample
311 at an intervention LBM in Thua Thien Hue province were classified as clade 2.3.4.4. Genetic
312 analysis indicated that these H5N6 viruses were closely related to an H5N6 virus isolated in
313 Zenjiang, China and differed slightly from the H5N6 viruses isolated in Laos, indicating an existing
314 genetic diversity within this new clade 2.3.4.4 (Food and Agriculture Organization of the United
315 Nations. Avian influenza A (H5N6): the latest addition to emerging zoonotic avian influenza threats
316 in East and Southeast Asia, 2014; www.fao.org/ag/empres.html). The H5 viruses found in northern
317 Vietnam may have been introduced from China. In April, 2014, the first outbreak of H5N6 was
318 found in a flock of chickens in Lang Son province, located near the border with China. Then
319 another outbreak was reported in a flock of ducks in Ha Tinh province, located in central part of
320 Vietnam and China (World Animal Health Information Database Interface, Disease Information,
321 2015; http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid=15802). In

322 Vietnam and China, AI vaccines are used to control H5 HPAI virus infections in poultry (Hu et al.,
323 2015; Le et al., 2014). However, immunological selection pressure has driven development of
324 antigenic variants of H5 HPAI viruses (Grund et al., 2011; Lee et al., 2004). The H5N6 HPAI
325 viruses we identified were also antigenically distinct from the clade 2.3.4 virus used in the Re-5
326 vaccine, which has been applied in China and Vietnam (Hu et al., 2015; Le et al., 2014). Recently,
327 several human H5N6 AI virus infections were reported, and avian-originated H5N6 viruses were
328 also isolated from healthy pigs in China, although the H5N6 AI virus has not adapted to the swine
329 population yet (Pan et al., 2016; Li et al., 2015). In Thua Thien Hue province, Vietnam, piglets, and
330 poultry are often housed together in non-intervention LBMs. This LBM system may facilitate
331 influenza virus reassortment and transmission of influenza viruses between poultry and from
332 poultry to pigs or humans. Therefore, it will be important to monitor swine influenza, and strict
333 controls should be applied to limit interspecies transmission of influenza viruses.

334 H6 and H9 AI viruses are widely distributed among poultry and wild birds in Asia (Huang et
335 al., 2010; Moon et al., 2010). H9N2 viruses have become the most prevalent subtype detected in
336 poultry populations in China since 2004 (Choi et al., 2004) and in Vietnam since 2009 (Okamatsu
337 et al., 2013; Nomura et al., 2012). In this study, H6 and H9 viruses isolated from domestic ducks,
338 chickens, and environment samples were genetically identical in both types of LBMs in Thua Thien
339 Hue province, Vietnam. These viruses were phylogenetically closely related to viruses previously
340 isolated in poultry in China. The HA genes of H9 viruses did not differ from those previously
341 isolated in 2011 and 2012, indicating that these viruses were maintained in poultry in Vietnam.

342 Antigenic analysis of H6 viruses indicated that all the Vietnamese H6 viruses we isolated in 2014
343 exhibited low cross reactivity with antiserum of A/duck/Vietnam/OIE-4429/2010 (H6N2), another
344 Group II virus. In addition, genetic analysis also indicated that the H6 viruses isolated in this study
345 differed from other Vietnamese H6 viruses isolated in our previous study (Okamatsu et al., 2013).
346 These results suggest that the antigenicity of these Vietnamese H6 viruses isolated in 2014 differed
347 from those isolated in Vietnam between 2010 and 2012. Perhaps the H6 viruses circulating in
348 Vietnam in 2014 exhibited altered antigenicity as a result of repetitive infections of the poultry
349 population. Further studies should be conducted to characterize the antigenic variation of H6 viruses
350 circulating in Vietnam.

351 In Vietnam, domestic birds are mainly raised in households in a free-range manner and are
352 transported to LBMs by their owners or poultry sellers. AI viruses are transmitted and spread within
353 the poultry population. Although domestic birds are vaccinated against H5N1 in Vietnam (Le et al.,
354 2014; Soares et al., 2010; Minh et al., 2009), HPAI viruses have silently spread in the poultry
355 population. Our study provides initial evidences for the improvement of intervention strategies in
356 study LBMs. The intervention with supporting a good infrastructure as “a good hardware” need to
357 comprise well with “a good software” like increasing of education level of people involved in
358 poultry trade and improvement of hygiene procedures by local authority at LBMs. Active
359 surveillance program of AI and hygiene practice performance monitoring in LBMs will be essential
360 to eradicate HPAI from Vietnam as well as Asian countries.

361

Conflict of interest

None.

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502

503 **Figure captions**

504 **Fig. 1.** Phylogenetic tree for the influenza virus H5 HA genes. Full-length HA genes of six H5
505 subtype viruses, and reference strains were analyzed using the maximum-likelihood method with
506 MEGA 5.0 software and divided into two lineages: Eurasian and North American. The Eurasian H5
507 viruses were identified on the basis of the nomenclature defined by WHO/OIE/FAO H5N1
508 Evolution Working Group, 2014 (Smith et al., 2015). Horizontal distances are proportional to the
509 minimum number of nucleotide differences required to join nodes and sequences. Digits at the
510 nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications.
511 The viruses isolated in this study are highlighted in gray. The viruses isolated in our previous study
512 are underlined. The black circle indicates the viruses isolated in an intervention LBM.

513

514 **Fig. 2.** Phylogenetic tree for the influenza virus H6 HA genes. Nucleotides 51–943 (893 bp) of the
515 HA genes of four viruses of H6 subtype and reference strains were analyzed using the maximum-
516 likelihood method with MEGA 5.0 software and divided into two lineages: Eurasian and North
517 American. The Eurasian H6 viruses were clustered into five different sublineages: Group II, W312,
518 Group III, Early, and Group I (Okamatsu et al., 2013). Horizontal distances are proportional to the
519 minimum number of nucleotide differences required to join nodes and sequences. Digits at the
520 nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications.
521 The viruses isolated in this study are highlighted in gray, and the representative of each sublineage

522 is indicated in bold. The virus isolated in a previous study is underlined. The viruses isolated at
523 intervention LBMs are indicated by black circles, and the virus isolated at a non-intervention LBM
524 is indicated by a white circle.
525
526 **Fig. 3.** Phylogenetic tree for the influenza virus H9 HA genes. Full-length HA genes of eight
527 viruses of H9N2 subtype, and reference strains were analyzed using the maximum-likelihood
528 method with MEGA 5.0 software and divided into two lineages: Eurasian and North American
529 lineages. The Eurasian H9 viruses were clustered into three different sublineages: Y280, G1, and
530 Y439 (Nomura et al., 2012; Okamatsu et al., 2013). Horizontal distances are proportional to the
531 minimum number of nucleotide differences required to join nodes and sequences. Digits at the
532 nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications.
533 The viruses isolated in this study are highlighted in gray, and the representative of each sublineage
534 is indicated in bold. The virus isolated in a previous study is underlined. The viruses isolated in
535 intervention LBMs are indicated by black circles, and the viruses isolated in non-intervention LBMs
536 are indicated by white circles. The H9N2 subtype of each virus strain is omitted.

Table 1 Viruses isolated from LBMs in Vietnam in 2014

Type of LBMs	Name of LBMs	Latitude/Longitude	Number of samples	Number of isolates	% Positive (95% CI)	Subtype of isolates (number of isolates)
Intervention	An Lo	16.545962/107.452388	500	18	3.6 (2.3–5.6)	H3N2 (1) H4N6 (1) H6N6 (1) H9N2 (10) H9N6 (2) H11N7 (3)
	No	16.511793/107.600336	500	61	12.2 (9.6–15.4)	H3N2 (4) H3N6 (1) H5N6 (8) H6N2 (9) H6N6 (3) H9N2 (36)
	Thuy Phuong	16.433257/107.635256	496	13	2.6 (1.5–4.4)	H3N2 (2) H4N6 (1) H6N2 (1) H6N6 (5) H9N2 (3) H11N6 (1)
Total			1,496	92	6.1 (5.0–7.5)	
Non-intervention	Phu Bai	16.408326/107.677989	300	40	13.3 (9.9–17.6)	H6N2 (3) H6N6 (1) H9N2 (35) H11N7 (1)
	Phu Da	16.434797/107.710014	200	1	0.5 (0.1–2.8)	H3N2 (1)
	Than Phu	16.424635/107.656268	199	12	6.0 (3.5–10.2)	H3N2 (1) H6N6 (5) H9N2 (3) H9N6 (3)
	Quang Phuoc	16.57431/107.51984	212	6	2.8 (1.3–6.0)	H9N2 (6)
	Tay Ba	16.537018/107.560679	338	25	7.4 (5.1–10.7)	H3N2 (9) H6N2 (1) H9N2 (15)
	Vinh Thanh	16.431796/107.783475	300	2	0.7 (0.2–2.4)	H6N6 (1) H9N2 (1)
Total			1,549	86	5.6 (4.5–6.8)	

CI, confidence interval

Table 2 Representative H5, H6, and H9 viruses isolated in live bird markets in Vietnam in 2014

HA subtypes	Type of LBMs	Name of LBMs	Isolates	Accession numbers							
				PB2	PB1	PA	HA	NP	NA	M	NS
H5	Intervention	No	A/muscovy duck/Vietnam/HU1-1144/2014 (H5N6)	LC069835	LC069867	LC069898	LC041323	LC069953	LC041324	LC070009	LC070040
			A/duck/Vietnam/HU1-1151/2014 (H5N6)	LC041310	LC041311	LC041312	LC041313	LC086330	LC041314	LC041315	LC041316
			A/environment/Vietnam/HU1-1434/2014 (H5N6)	LC069834	LC069866	LC069897	LC041321	LC069952	LC041322	LC070008	LC070039
			A/duck/Vietnam/HU1-1507/2014 (H5N6)	LC069832	LC069864	LC069895	LC041317	LC069950	LC041318	LC070006	LC070037
			A/duck/Vietnam/HU1-1511/2014 (H5N6)	LC069833	LC069865	LC069896	LC041319	LC069951	LC041320	LC070007	LC070038
H6	Intervention	An Lo	A/duck/Vietnam/HU1-1245/2014 (H6N2)	LC069813	LC069846	LC069877	LC069908	LC069932	LC069963	LC069988	LC070019
		No	A/environment/Vietnam/HU1-1423/2014 (H6N2)	LC069836	LC041334	LC041335	LC041336	LC041337	LC041338	LC041339	LC041340
			A/environment/Vietnam/HU1-1426/2014 (H6N2)	LC069837	LC069868	LC069899	LC041341	LC069954	LC041342	LC070010	LC070041
	Non-intervention	Than Phu	A/duck/Vietnam/HU1-637/2014 (H6N6)	LC069817	LC069850	LC069881	LC069912	LC069936	LC069967	LC069992	LC070023
H9	Intervention	An Lo	A/chicken/Vietnam/HU1-3/2014 (H9N2)	LC069838	LC069869	LC069900	LC041350	LC069955	LC069980	LC070011	LC070042
			A/duck/Vietnam/HU1-225/2014 (H9N2)	LC069842	LC069873	LC069904	LC069928	LC069959	LC069984	LC070015	LC070046
		No	A/chicken/Vietnam/HU1-1286/2014 (H9N2)	LC069840	LC069871	LC069902	LC069926	LC069957	LC069982	LC070013	LC070044
			A/environment/Vietnam/HU1-1424/2014 (H9N2)	LC069841	LC069872	LC069903	LC069927	LC069958	LC069983	LC070014	LC070045
			A/duck/Vietnam/HU1-1512/2014 (H9N2)	LC041343	LC041344	LC041345	LC041346	LC086331	LC041347	LC041348	LC041349
	Non-intervention	Phu Bai	A/chicken/Vietnam/HU1-381/2014 (H9N2)	LC069839	LC069870	LC069901	LC069925	LC069956	LC069981	LC070012	LC070043
		Than Phu	A/duck/Vietnam/HU1-675/2014 (H9N2)	LC069820	LC069853	LC069884	LC069915	LC069939	LC069970	LC069995	LC070026
		Quang Phuoc	A/chicken/Vietnam/HU1-786/2014 (H9N2)	LC069843	LC069874	LC069905	LC069929	LC069960	LC069985	LC070016	LC070047

Table 3 Cross-reactivity of H5 influenza viruses with antisera by HI test

Lineage	Viruses	Clade	Antiserum to						
			Vac-3	Mon/05	Hok/08	HK/09	Km/14	Yama/04	Ibr/05
Eurasia	A/duck/Hokkaido/Vac-3/2007 (H5N1)	−	10,240	10,240	640	1,280	1,280	5,120	2,560
	A/whooper swan/Mongolia/3/2005 (H5N1)	2.2	80	640	80	40	20	1,280	80
	A/whooper swan/Hokkaido/1/2008 (H5N1)	2.3.2.1	80	1,280	1,280	80	80	1,280	40
	A/peregrine falcon/Hong Kong/810/2009 (H5N1)	2.3.4	40	20	40	2,560	40	160	20
	A/muscovy duck/Vietnam/HU1-1144/2014 (H5N6)	2.3.4.4	80	40	20	640	640	80	<20
	A/duck/Vietnam/HU1-1151/2014 (H5N6)	2.3.4.4	40	20	20	640	640	40	<20
	A/environment/Vietnam/HU1-1434/2014 (H5N6)	2.3.4.4	20	20	20	160	160	20	<20
	A/muscovy duck/Vietnam/HU2-26/2014 (H5N6)	2.3.4.4	40	40	20	640	640	80	<20
	A/chicken/Kumamoto/1-7/2014 (H5N8)	2.3.4.4	20	20	20	320	640	80	<20
	A/chicken/Yamaguchi/7/2004 (H5N1)	2.5	640	10,240	640	160	160	10,240	320
North America	A/chicken/Ibaraki/1/2005 (H5N2)	−	160	640	20	20	<20	2,560	10,240

The H5 isolates identified in the present study are shown in bold.

Vac-3, A/duck/Hokkaido/Vac-3/2007; Mon/05, A/whooper swan/Mongolia/3/2005; Hok/08, A/whooper swan/Hokkaido/1/2008; HK/09, A/peregrine falcon/Hong Kong/810/2009; Km/14, A/chicken/Kumamoto/1-7/2014; Yama/04, A/chicken/Yamaguchi/7/2004; Ibr/05, A/chicken/Ibaraki/1/2005

Homologous titers are underlined.

"−" indicates that the virus does not belong to clade 0–9

Table 4 Cross-reactivity of H6 influenza viruses with antisera by HI test

Lineage	Sublinage	Viruses	Antiserum to				
			HK/960/80	VN/OIE-4429/10	VN/HU1-637/14	Aus/1/72	Mas/3740/65
Eurasia	Early	A/duck/Hong Kong/960/1980 (H6N2)	5,120	640	640	640	640
	Group II	A/duck/Vietnam/OIE-4429/2010 (H6N2)	640	5,120	640	160	2,560
		A/duck/Vietnam/HU1-637/2014 (H6N6)	640	1,280	10,240	80	640
		A/duck/Vietnam/HU1-1245/2014 (H6N2)	160	160	2,560	20	160
		A/environment/Vietnam/HU1-1423/2014 (H6N2)	320	640	2,560	40	320
		A/environment/Vietnam/HU1-1426/2014 (H6N2)	320	640	2,560	80	320
–		A/shearwater/South Australia/1/72 (H6N5)	2,560	40	80	1,280	640
North American		A/turkey/Masachusetts/3740/65 (H6N2)	2,560	1,280	40	1,280	5,120

The H6 isolates identified in the present study are shown in bold.

HK/960/80 , A/duck/Hong Kong/960/1980 (H6N2); VN/OIE-4429/10, A/duck/Vietnam/OIE-4429/2010 (H6N2); VN/HU1-637/14, A/duck/Vietnam/HU1-637/2014 (H6N6); Aus/1/72, A/shearwater/South Australia/1/72 (H6N5); Mas/3740/65 , A/turkey/Masachusetts/3740/65 (H6N2)

Homologous titers are underlined.

"–" indicates that the virus does not belong to any lineage.

Table 5 Cross-reactivity of H9N2 influenza viruses with antisera by HI test

Lineage	Sublinage	Viruses	Antiserum to			
			HK/Y280/97	HK/G1/97	Hok/49	Wis/66
Eurasia	Y280	A/duck/Hong Kong/Y280/1997	10,240	1,280	2,560	80
		A/chicken/Vietnam/HU1-3/2014	10,240	1,280	1,280	80
		A/duck/Vietnam/HU1-225/2014	2,560	1,280	640	80
		A/chicken/Vietnam/HU1-381/2014	10,240	640	1,280	40
		A/chicken/Vietnam/HU1-786/2014	20,480	1,280	2,560	80
		A/chicken/Vietnam/HU1-1286/2014	10,240	640	640	80
		A/environment/Vietnam/HU1-1424/2014	10,240	320	1,280	40
		A/duck/Vietnam/HU1-1512/2014	10,240	640	2,560	40
	G1	A/quail/Hong Kong/G1/1997	1,280	5,120	640	80
	Y439	A/duck/Hokkaido/49/1998	640	80	2,560	320
North America	−	A/turkey/Wisconsin/1/1966	80	40	320	320

The H9 isolates identified in the present study are shown in bold.

HK/Y280/97, A/duck/Hong Kong/Y280/1997; HK/G1/97, A/quail/Hong Kong/G1/1997; Hok/49, A/duck/Hokkaido/49/1998; Wis/66, A/turkey/Wisconsin/1/1966

Homologous titers are underlined.

"−" indicates that the virus does not belong to any sublinage.

Fig. 1. Chu *et al.*

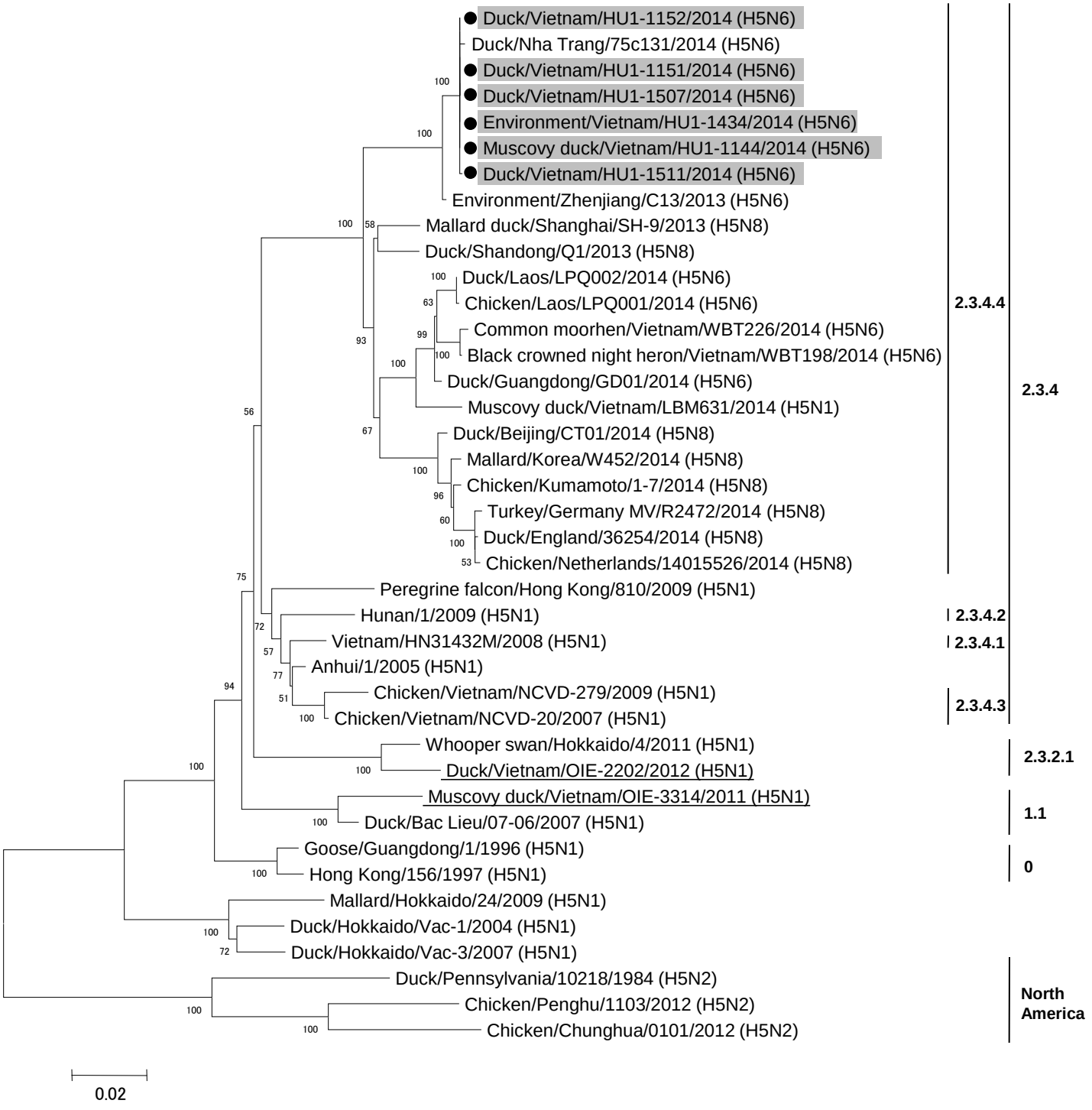

Fig. 2. Chu *et al.*

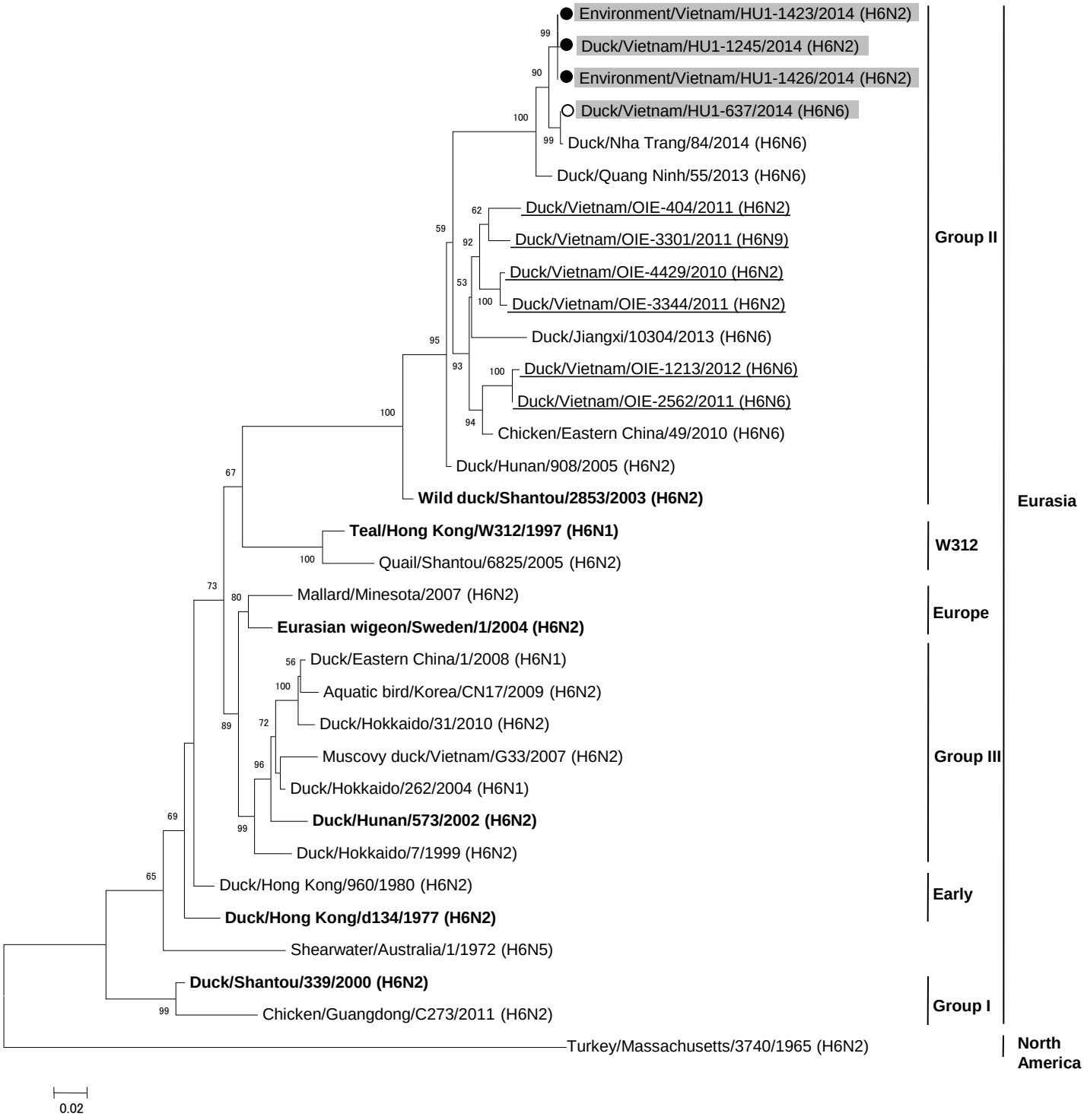

Fig. 3. Chu *et al.*