Acta virologica **65**: 104 – 110, 2020 doi: 10.4149/av_2020_113

Genomic characteristics and phylogenetic analysis of the first H12N2 influenza A virus identified from wild birds, China

J. LIU[1], L. ZHOU[2], Z. LUO[3], Y. ZOU[4], J. LV[1], T. WANG[1,5*]

[1]School of Life Sciences, East China Normal University, Shanghai 200062, P. R. China; [2]Shanghai Zoo, Shanghai 200335, P. R. China; [3]School of Environment and Life Sciences, Kaili University, Kaili 556011, Guizhou, P. R. China; [4]Key Laboratory of Agro-ecological Processes in Subtropical Regions, Chinese Academy of Sciences, Changsha 410125, Hunan, P. R. China; [5]Institute of Eco-Chongming, East China Normal University, Shanghai 200062, P. R. China

Received May 28, 2019, revised July 7, 2019; accepted February 6, 2020

Summary. – In this report, an H12N2 influenza A virus was identified from a fecal sample in the falcated teal, *Anas falcata*, located in the Dongting Lake wetland. This is the first report of H12N2 IAV detected from wild birds in China. Phylogenetic analysis showed that all eight segments of this H12N2 virus clustered in the Eurasian lineage. This strain was also shown to be closely related to those from duck-origin, goose origin and wild bird origin. This suggested a transmission of influenza A virus along the East Asian-Australian flyway. In addition, these results implied an interaction between wild birds and domestic poultry and established the need of a long-term and systematic surveillance of wild bird populations.

Keywords: influenza A virus; H12N2 subtype; Dongting Lake wetland; wild birds; phylogenetic analysis

Introduction

Influenza A viruses (IAVs) are divided into various subtypes on the basis of two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). These strains cause influenza in birds and other mammals worldwide (Webster *et al*., 1992). With the exception of H17N10 and H18N11 discovered in bats, all 16 HA subtypes (H1-H16) and nine NA subtypes (N1-N9) have been identified in wild aquatic birds (Fouchier *et al*., 2005; Tong *et al*., 2013; Zhu *et al*., 2013). Aquatic birds act as a natural reservoir by carrying the gene pool for IAVs, especially low pathogenic avian influenza virus (LPAIV) (Olsen *et al*., 2006). Because many aquatic birds can harbor the IAVs without any clinical signs, they are capable of disseminating the viruses along their migratory routes (Alexander, 2007).

The Dongting Lake wetland in the Hunan Province, China, is one of the most important overwintering spots for migratory birds in China. Large populations of aquatic birds, especially from the taxonomic the order *Anseriformes*, stop in this location each winter (Zhang *et al*., 2011). Moreover, numerous poultry farms are found in the same area, which offer more chances for virus reassortment between poultry and wild birds (Deng *et al*., 2013).

In 1976, H12 viruses were first detected in Canada (Hinshaw and Webster, 1979). H12 subtype viruses are relatively rare (Wille *et al*., 2018). The first H12N2 subtype was isolated from mallards found in Wisconsin in 1977 according to the GenBank database. Recently, only 11 viruses of H12N2 subtypes have been deposited in GenBank and the GISAID EpiFlu database. In 2015, the first H12N2 subtype virus in China was isolated from a duck farm (Zhang *et al*., 2017), however this study provides the first report of an H12N2 virus detected from wild birds in China.

*Corresponding author. E-mail: thwang@bio.ecnu.edu.cn; phone: +86-21-62233012.

Abbreviations: HA = hemagglutinin; IAV(s) = influenza A virus(es); NA = neuraminidase

Materials and Methods

A total of 921 fresh fecal samples were collected during an active surveillance in Dongting Lake wetland in February 2015. The samples were placed into 2 ml tubes containing 1 ml viral transport medium. Samples were maintained at 4°C during transport then stored at -80°C until further use.

The viral RNA was extracted using the MagMAX™ Pathogen RNA/DNA kit (Applied Biosystems, Foster City, CA, USA) on the Magmax-96 Express (Applied Biosystems) according to the manufacturer's instructions. The viruses were confirmed by real-time reverse transcription-PCR on an ABI 7500 (Applied Biosystems) targeting the matrix gene (Hindiyeh *et al*., 2005). Eight gene segments of each virus were amplified with PrimeScript™ One-step RT-PCR kit Ver.2 kit (TaKaRa, Biotechnology [Dalian] Co., Ltd, Dalian, China). The genome amplification was performed with MightyAmp® DNA Polymerase Ver.3 kit (TaKaRa, Biotechnology [Dalian] Co., Ltd, Dalian, China). PCR products were purified with MinElute® Gel Extraction Kit (Qiagen, Valencia, CA, USA) and sent to the sequencing company for next-generation sequencing. Host species were identified by using primers for the mitochondrial cytochrome-oxidase I gene (Cheung *et al*., 2009).

The MEGA 7.0.26. was used to conduct sequence alignment and phylogenetic analysis. The neighbor joining method with the Kimura 2 parameter distance model was used with 1000 bootstrap replicates. Homology analyses of nucleic acids were performed by BLAST. All of the reference sequences used in the phylogenetic analysis were obtained from GenBank and the GISAID EpiFlu database. Glycosylation sites and transmembrane segments of the HA protein were identified using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

Results

Thirty-eight IAVs from 921 fecal samples were detected in this study. The H6N2, H9N2, H11N8 and H12N2 subtypes were identified and an H12N2 IAV identified from the falcated teal, *Anas falcata*, was named A/Anas falcata/Dongting/D29/2015(H12N2) (Dongting-D29). The nucleotide sequences obtained in this study have been deposited in GenBank Acc. Nos. MK301256-MK301263.

The complete viral genome consists of eight gene segments including *PB2* (2280 bp), *PB1* (2277 bp), *PA* (2151 bp), *HA* (1705 bp), *NP* (1497 bp), *NA* (1467 bp), *M* (1027 bp) and *NS* (838 bp). The genetic analysis showed that multiple gene segments of Dongting-D29 shared high homology with viruses isolated from wild bird and poultry in the Eurasian region. The nucleic acid sequence of the *PB2* gene showed 99.6% identity with A/Anseriformes/Anhui/L259/2014(H1N1). The nucleic acid sequence of the *PB1* gene shared 99.3% identity with A/goose/Hunan/S2466/2011(H4N8). The *PA* gene showed 99.2% nucleotide similarity with A/duck/Jiangxi/5461/2014(H7N3). The closest relative of the *HA* gene was A/duck/Hokkaido/W26/2012(H12N1) (99.1% nucleotide similarity). The nucleic acid sequences of the *NP* and *NA* genes of the H12N2 virus showed identity of 99.9 and 99.6%, respectively, with the virus A/goose/Zhejiang/1120085/2014(H1N2). The *M* segment was closely related to A/duck/Mongolia/258/2011(H8N4), with 99.2% nucleotide identity. The nucleic acid sequence of the *NS* gene showed 98.8% identity with the virus A/duck/Jiangxi/15846/2013(H10N3) (Table 1).

Phylogenetic analysis showed that all eight segments of Dongting-D29 clustered in the Eurasian lineage (Fig. 1 and Fig. S1). As seen from the phylogenetic tree, the *HA* genes of H12 subtype viruses were divided into Eurasian and North American lineages and the *HA* of Dongting-D29 belonged to Eurasian lineage. The *HA* gene was very closely related to A/Environment/Jiangxi/08488/2015(H12N2) (JX-H12N2) which was reported the first H12N2 subtype virus isolated from the Jiangxi province, China and to H12 viruses that were circulating in eastern Asia from 2009 to 2015. When compared with H12N2 subtype viruses isolated from Norway and Switzerland, the *HA* gene of Dongting-D29 was more closely related to H12N7 and H12N8 in the Dongting Lake region, Hunan province. However, the phylogenetic tree of the *NA* gene was clustered

Table 1. Homology analyses of Dongting-D29 with isolates in GenBank

Gene	GenBank ID	Virus	Homology (%)
PB2	KU881717.1	A/Anseriformes/Anhui/L259/2014(H1N1)	99.6
PB1	KU161073.1	A/goose/Hunan/S2466/2011(H4N8)	99.3
PA	KP417016.1	A/duck/Jiangxi/5461/2014(H7N3)	99.2
HA	LC339667.1	A/duck/Hokkaido/W26/2012(H12N1)	99.1
NP	KY971153.1	A/goose/Zhejiang/1120085/2014(H1N2)	99.9
NA	KY971182.1	A/goose/Zhejiang/1120085/2014(H1N2)	99.6
M	LC349338.1	A/duck/Mongolia/258/2011(H8N4)	99.2
NS	KP285481.1	A/duck/Jiangxi/15846/2013(H10N3)	98.8

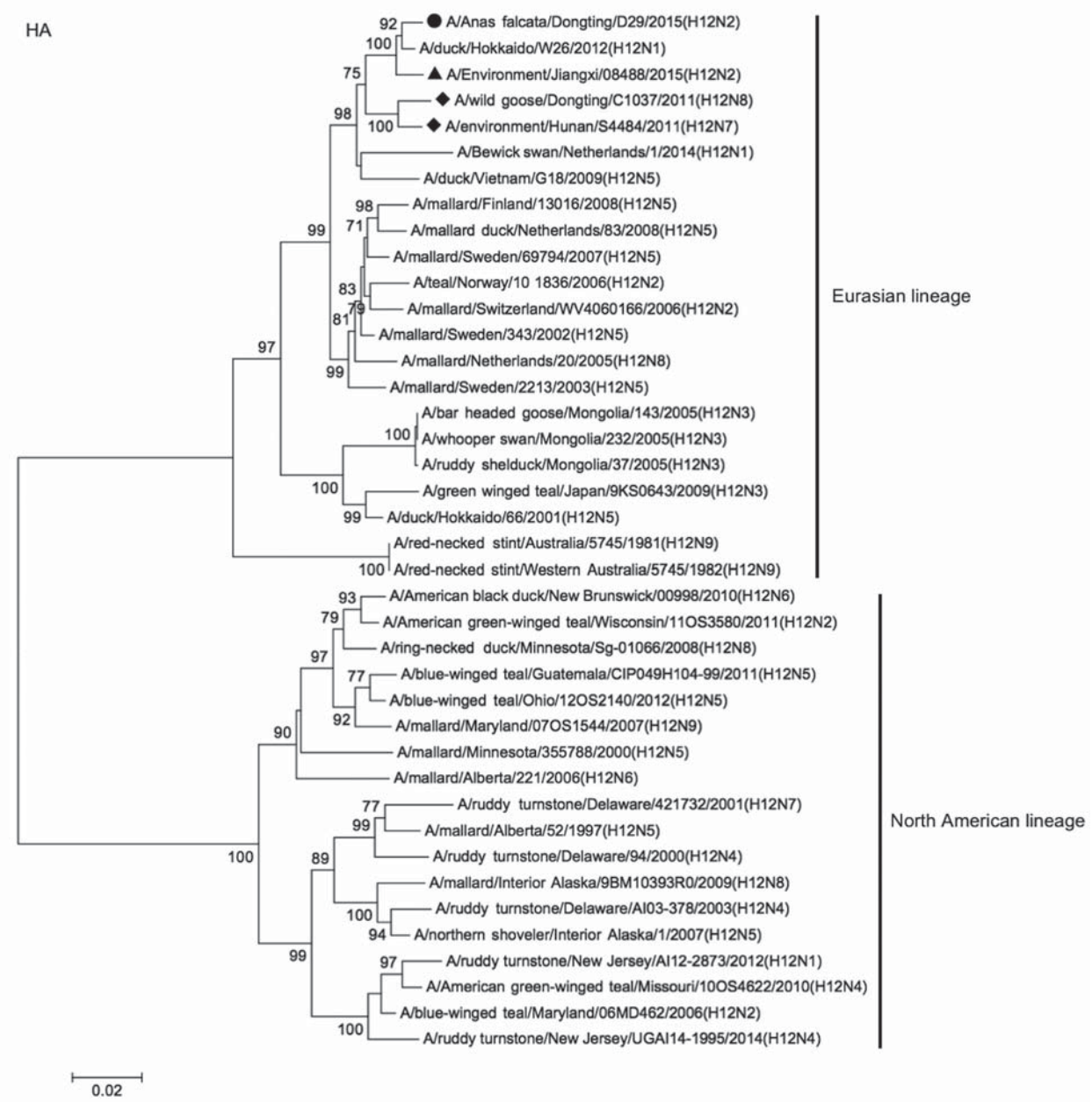

Fig. 1 Continued

into five lineages, including Eurasian, North American I, North American II, human and swine lineages. The *NA* gene of Dongting-D29 fell into the Eurasian lineage. The *NA* gene phylogeny indicated that Dongting-D29 was more closely related to H6N2, H9N2 subtype viruses circulating in the Anhui province than to JX-H12N2. In addition, the *NA* gene of Dongting-D29 was in a separate subgroup from the H5N2 virus, which caused the epidemic outbreak of 1997–1998 in Italy (Fig. 1) (Capua *et al*., 1999). This result indicated that Dongting-D29 had a different ancestor from the H5N2 virus.

Based on the deduced amino acid sequence of the *HA* gene, the *HA* cleavage sites of Dongting-D29 with a single basic amino acid (R) was PQAQDR↓GLF. This observation suggested that this was a low-pathogenic avian influenza virus (Alexander, 2000). The Q226 and G228 (*H3* numbering) residue of the *HA* gene indicated that this strain would preferentially bind to α-2,3-linked sialic acid receptors, which are dominant in avian species (Matrosovich *et al*., 2000). Six potential N-glycosylation sites in the *HA* gene (28, 140, 151, 152, 302 and 309) were detected in this study according to the Asn-X-Ser/Thr glycosylation site motifs.

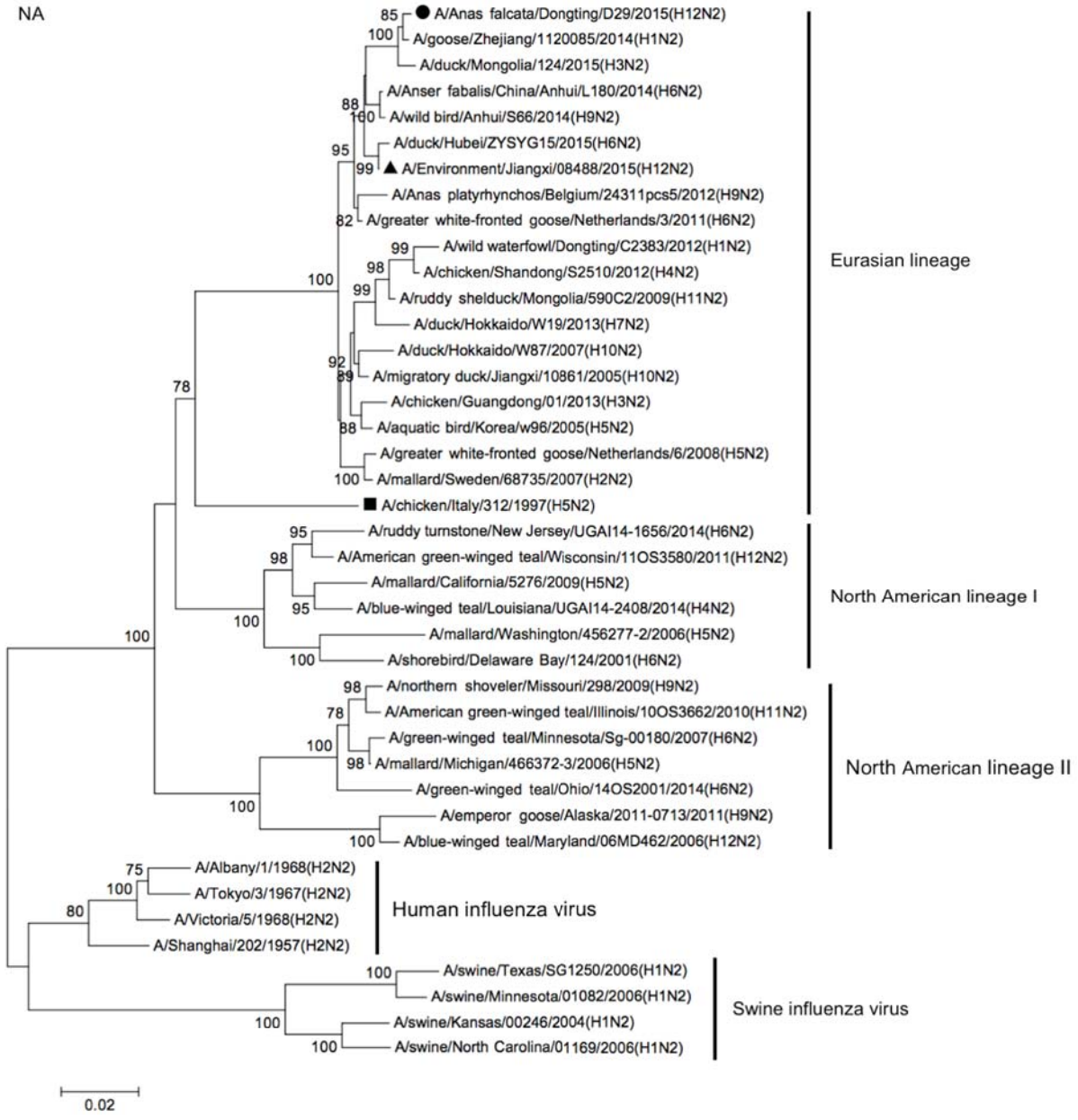

Fig. 1

Phylogenetic tree of the *HA* and *NA* genes of the H12N2 IAV

The phylogenetic trees were generated using MEGA 7.0.26 software, and the bootstrap value was tested by 1000 replications. The H12N2 virus characterized is highlighted by a dot. The H12N2 virus isolated in the Jiangxi province is highlighted by a triangle. The H12 virus originating in the Dongting Lake wetland isolated in 2011 is highlighted by a diamond. The HPAI H5N2 virus, which caused the epidemic outbreak of 1997–1998 in Italy, is highligted by a square. The Scale bar shows nucleotide substitutions per site.

Five potential N-glycosylation sites in the *NA* gene (61, 69, 70, 146 and 234) were detected in this study. There were no deletions in the *NA* gene stalk region, and no mutations (E119A, H274Y, N294S, *N2* numbering) were found in the NA protein which suggested the susceptibility to the NA inhibitors oseltamivir and zanamivir (Aoki *et al*., 2007). The amino acids E and D at positions 627 and 701 of the PB2 protein indicated an avian origin (Ping *et al*., 2010). The mutation P13 and V473 in the PB1 protein suggested the increased polymerase activity, replication efficiency and virulence in mammals (Gabriel *et al*., 2005; Xu *et al*., 2012). In the PA protein, L672 was found to increase airborne

Table 2. Specific amino acid residues analysis of Dongting-D29 with JX-H12N2

Protein	Mutation site	Dongting-D29	JX-H12N2	Possible function
		PQAQDR↓GLF	PQAQDR↓GLF	Low pathogenic influenza virus.
HA[α]	Q226L	Q	Q	Binding α-2,3-linked sialic acid receptor.
	G228S	G	G	
	E119A	E	E	
NA[β]	H274Y	H	H	Resistance to oseltamivir and zanamivir.
	N294S	N	–	
PB2	E627K	E	E	Molecular marker of IAVs replicated in avian cell.
	D701N	D	D	
PB1	L13P	P	P	Increased polymerase activity and virulence in mammals.
	L473V	V	V	Increased polymerase activity and replication efficiency.
PA	F672L	L	L	Increased airborne transmissibility between chickens.
M1	N30D	D	D	Increased pathogenesis in mice.
	T215A	A	A	
M2	S31N	S	–	Resistance to amantadine and rimantadine.
NS1	P42S	S	–	Increased pathogenesis in mammal.

[α]H3 numbering; [β]N2 numbering; (–) Not mentioned in the article (Zhang *et al*., 2017).

transmissibility between chickens (Zhong *et al*., 2014). The M1 mutations D30 and A215 were detected which can increase pathogenesis in mice but no substitutions associated with resistance to adamantine were found in the M2 protein (Fan *et al*., 2009; Hay *et al*., 1985). NS1 had a mutation S42 related to increased pathogenesis in mammals (Jiao *et al*., 2008). The primary amino acid mutations of Dongting-D29 with JX-H12N2 are shown in Table 2.

Discussion

The Dongting Lake wetland is located along the East Asian-Australasian Flyway which harbors a great abundance of migratory birds. Many of these birds carry the IAVs as they travel between overwintering and breeding sites along the migration flyway (Zhang *et al*., 2011). In the same area, a large population of domestic ducks normally breeds around the lake (Zhang *et al*., 2012). This combination of features makes the Dongting Lake wetland an ideal location for cocirculation of IAVs of different subtypes and origins. Not surprisingly, the genomic analysis of Dongting-D29 showed that the *PA, HA, M* and *NS* genes were similar to those from duck-origin. The *PB1, NP* and *NA* genes were closely related to goose-origin. The *PB2* gene was similar to wild bird IAV.

Previous studies have shown that H3, H4, H5, H6, H9, H10, H11 and H12 subtype viruses have been isolated from domestic duck (Deng *et al*., 2013), while H1, H5, H6, H7, H9, H12 subtype viruses originate from wild birds in this region (Shi *et al*., 2014; Wang *et al*., 2014). The phylogenetic tree data shown that the *HA* gene of Dongting-D29 was very closely related to H12N7 and H12N8 isolated in the Dongting Lake region. However, the first H12N2 IAV was detected from wild birds in this study, indicating increased diversity of LPAIV circulating locally. Additionally, the similarity between Dongting-D29 and JX-H12N2 in gene sequences and amino acid mutations maybe because the sampling sites along the same flyway were not far from each other which increased the chances for virus transmission between poultry and wild birds.

Long-term surveillance of IAVs in wild birds in Europe and North America has occurred since 1998 and 1976, respectively (Munster *et al*., 2006; Olsen *et al*., 2006). However, little systematic surveillance has been conducted in wild birds in China. Based on the sequence record in GenBank and in the GISAID EpiFlu database, all 10 of the eleven subtype H12N2 viruses have been reported in the past 15 years. These data may suggest the result of increased surveillance globally. Therefore, increased and continual surveillance should be implemented in order to understand the ecology and evolution of IAV in this region, and to promote the study of correlation between wild bird migration and virus transmission.

Acknowledgments. We thank Dongting Lake Station for Wetland Ecosystem Research for field sampling assistance. This work was supported by Shanghai Wildlife Epidemic Disease Monitoring Program (G061255), Shanghai Science and Technology Committee Project (2013QLG001) and Shanghai forestry bureau (GZ180409).

Supplementary information is available in the online version of the paper.

References

Alexander DJ (2000): A review of avian influenza in different bird species. Vet. Microbiol. 74, 3–13. https://doi.org/10.1016/S0378-1135(00)00160-7

Alexander DJ (2007): An overview of the epidemiology of avian influenza. Vaccine 25, 5637–5644. https://doi.org/10.1016/j.vaccine.2006.10.051

Aok FY, Boivin G, Roberts N (2007): Influenza virus susceptibility and resistance to oseltamivir. Antivir. Ther. 12, 603–616.

Capua I, Marangon S, Selli L, Alexander DJ, Swayne DE, Dalla Pozza M, Parenti E, Cancellotti FM (1999): Outbreaks of highly pathogenic avian influenza (H5N2) in Italy during October 1997 to January 1998. Avian Pathol. 28, 455–460. https://doi.org/10.1080/03079459994470

Cheung PP, Leung YHC, Chow CK, Ng CF, Tsang CL, Wu YO, Ma SK, Sia SF, Guan Y, Peiris JSM (2009): Identifying the species-origin of faecal droppings used for avian influenza virus surveillance in wild-birds. J. Clin. Virol. 46, 90–93. https://doi.org/10.1016/j.jcv.2009.06.016

Deng GH, Tan D, Shi JZ, Cui PF, Jiang YP, Liu LL, Tian GB, Kawaoka Y, Li CJ, Chen HL (2013): Complex Reassortment of Multiple Subtypes of Avian Influenza Viruses in Domestic Ducks at the Dongting Lake Region of China. J. Virol. 87, 9452–9462. https://doi.org/10.1128/JVI.00776-13

Fan S, Deng G, Song J, Tian G, Suo Y, Jiang Y, Guan Y, Bu Z, Kawaoka Y, Chen H (2009): Two amino acid residues in the matrix protein M1 contribute to the virulence difference of H5N1 avian influenza viruses in mice. Virology 384, 28–32. https://doi.org/10.1016/j.virol.2008.11.044

Fouchier RAM, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus ADME (2005): Characterization of a novel influenza a virus hemagglutinin subtype (H16) obtained from black-headed gulls. J. Virol. 79, 2814–2822. https://doi.org/10.1128/JVI.79.5.2814-2822.2005

Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, Stech J (2005): The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc. Natl. Acad. Sci. USA. 102, 18590–18595. https://doi.org/10.1073/pnas.0507415102

Hay AJ, Wolstenholme AJ, Skehel JJ, Smith MH (1985): The Molecular-Basis of the Specific Anti-Influenza Action of Amantadine. EMBO. J. 4, 3021–3024. https://doi.org/10.1002/j.1460-2075.1985.tb04038.x

Hindiyeh M, Levy V, Azar R, Varsano N, Regev L, Shalev Y, Grossman Z, Mendelson E (2005): Evaluation of a multiplex real-time reverse transcriptase PCR assay for detection and differentiation of influenza viruses A and B during the 2001–2002 influenza season in Israel. J. Clin. Microbiol. 43, 589–595. https://doi.org/10.1128/JCM.43.2.589-595.2005

Hinshaw VS, Webster RG (1979): Characterization of a new avian influenza virus subtype and proposed designation of this haemagglutinin as Hav10. J. Gen. Virol. 45, 751–754. https://doi.org/10.1099/0022-1317-45-3-751

Jiao P, Tian G, Li Y, Deng G, Jiang Y, Liu C, Liu W, Bu Z, Kawaoka Y, Chen H (2008): A single-amino-acid substitution in the NS1 protein changes the pathogenicity of H5N1 avian influenza viruses in mice. J. Virol. 82, 1146–1154. https://doi.org/10.1128/JVI.01698-07

Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, Donatelli I, Kawaoka Y (2000): Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J. Virol. 74, 8502–8512. https://doi.org/10.1128/JVI.74.18.8502-8512.2000

Munster VJ, Veen J, Olsen B, Vogel R, Osterhaus AD, Fouchier RA (2006): Towards improved influenza A virus surveillance in migrating birds. Vaccine 24, 6729–6733. https://doi.org/10.1016/j.vaccine.2006.05.060

Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA (2006): Global patterns of influenza a virus in wild birds. Science 312, 384–388. https://doi.org/10.1126/science.1122438

Ping JH, Dankar SK, Forbes NE, Keleta L, Zhou Y, Tyler S, Brown EG (2010): PB2 and Hemagglutinin Mutations Are Major Determinants of Host Range and Virulence in Mouse-Adapted Influenza A Virus. J. Virol. 84, 10606–10618. https://doi.org/10.1128/JVI.01187-10

Shi J, Gao L, Zhu Y, Chen T, Liu Y, Dong L, Liu F, Yang H, Cai Y, Yu M, Yao Y, Xu C, Xiao X, Shu Y (2014): Investigation of avian influenza infections in wild birds, poultry and humans in Eastern Dongting Lake, China. Plos One 9, e95685. https://doi.org/10.1371/journal.pone.0095685

Tong SX, Zhu XY, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen XF, Recuenco S, Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu WL, McBride R, Carney PJ, Gilbert AT, Chang J, Guo Z, Davis CT, Paulson JC, Stevens J, Rupprecht CE, Holmes EC, Wilson IA, Donis RO (2013): New World Bats Harbor Diverse Influenza A Viruses. Plos Pathog. 9. https://doi.org/10.1371/journal.ppat.1003657

Wang B, Liu Z, Chen Q, Gao Z, Fang F, Chang H, Chen J, Xu B, Chen Z (2014): Genotype diversity of H9N2 viruses isolated from wild birds and chickens in Hunan Province, China. Plos One 9, e101287. https://doi.org/10.1371/journal.pone.0101287

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992): Evolution and ecology of influenza A viruses. Microbiol. Rev. 56, 152–179. https://doi.org/10.1128/MMBR.56.1.152-179.1992

Wille M, Latorre-Margalef N, Tolf C, Halpin R, Wentworth D, Fouchier RAM, Raghwani J, Pybus OG, Olsen B, Waldenström J (2018): Where do all the subtypes go? Temporal dynamics of H8-H12 influenza A viruses in waterfowl. Virus evolution 4, vey025–vey025. https://doi.org/10.1093/ve/vey025

Xu C, Hu WB, Xu K, He YX, Wang TY, Chen Z, Li TX, Liu JH, Buchy P, Sun B (2012): Amino acids 473V and 598P of PB1 from an avian-origin influenza A virus contribute to polymerase activity, especially in mammalian cells. J. Gen. Virol. 93, 531–540. https://doi.org/10.1099/vir.0.036434-0

Zhang H, Chen Q, Chen Z (2012): Characterization of an H4N2 avian influenza virus isolated from domestic duck in Dongting Lake wetland in 2009. Virus Genes 44, 24–31. https://doi.org/10.1007/s11262-011-0658-9

Zhang H, Xu B, Chen Q, Chen J, Chen Z (2011): Characterization of an H10N8 influenza virus isolated from Dongting lake wetland. Virol. J. 8, 42. https://doi.org/10.1186/1743-422X-8-42

Zhang H, Yuan H, Chen T, Zeng XX, Zhao X, Fu WJ, Xie Y, Zhu F, Jiao M, Liu XQ, Wang DY, Shu YL (2017): Genomic characteristics and phylogenetic analyses of the first avian influenza H12N2 subtype virus identified in China. Chin. J. Virol. 33, 129–135.

Zhong L, Wang XQ, Li QH, Liu D, Chen HZ, Zhao MJ, Gu XB, He L, Liu XW, Gu M, Peng DX, Liu XF (2014): Molecular Mechanism of the Airborne Transmissibility of H9N2 Avian Influenza A Viruses in Chickens. J. Virol. 88, 9568–9578. https://doi.org/10.1128/JVI.00943-14

Zhu XY, Yu WL, McBride R, Li Y, Chen LM, Donis RO, Tong SX, Paulson JC, Wilson IA (2013): Hemagglutinin homologue from H17N10 bat influenza virus exhibits divergent receptor-binding and pH-dependent fusion activities. Proc. Natl. Acad. Sci. USA 110, 1458–1463. https://doi.org/10.1073/pnas.1218509110

Acta virologica **65**: 104 – 110, 2020 doi: 10.4149/av_2020_113

SUPPLEMENTARY INFORMATION

Genomic characteristics and phylogenetic analysis of the first H12N2 influenza A virus identified from wild birds, China

J. LIU[1], L. ZHOU[2], Z. LUO[3], Y. ZOU[4], J. LV[1], T. WANG[1,5*]

[1]School of Life Sciences, East China Normal University, Shanghai 200062, P. R. China; [2]Shanghai Zoo, Shanghai 200335, P. R. China; [3]School of Environment and Life Sciences, Kaili University, Kaili 556011, Guizhou, P. R. China; [4]Key Laboratory of Agro-ecological Processes in Subtropical Regions, Chinese Academy of Sciences, Changsha 410125, Hunan, P. R. China; [5]Institute of Eco-Chongming, East China Normal University, Shanghai 200062, P. R. China

Received May 28, 2019; revised July 7, 2019; accepted February 6, 2020

[*]Corresponding author. E-mail: thwang@bio.ecnu.edu.cn; phone: +86-21-62233012.

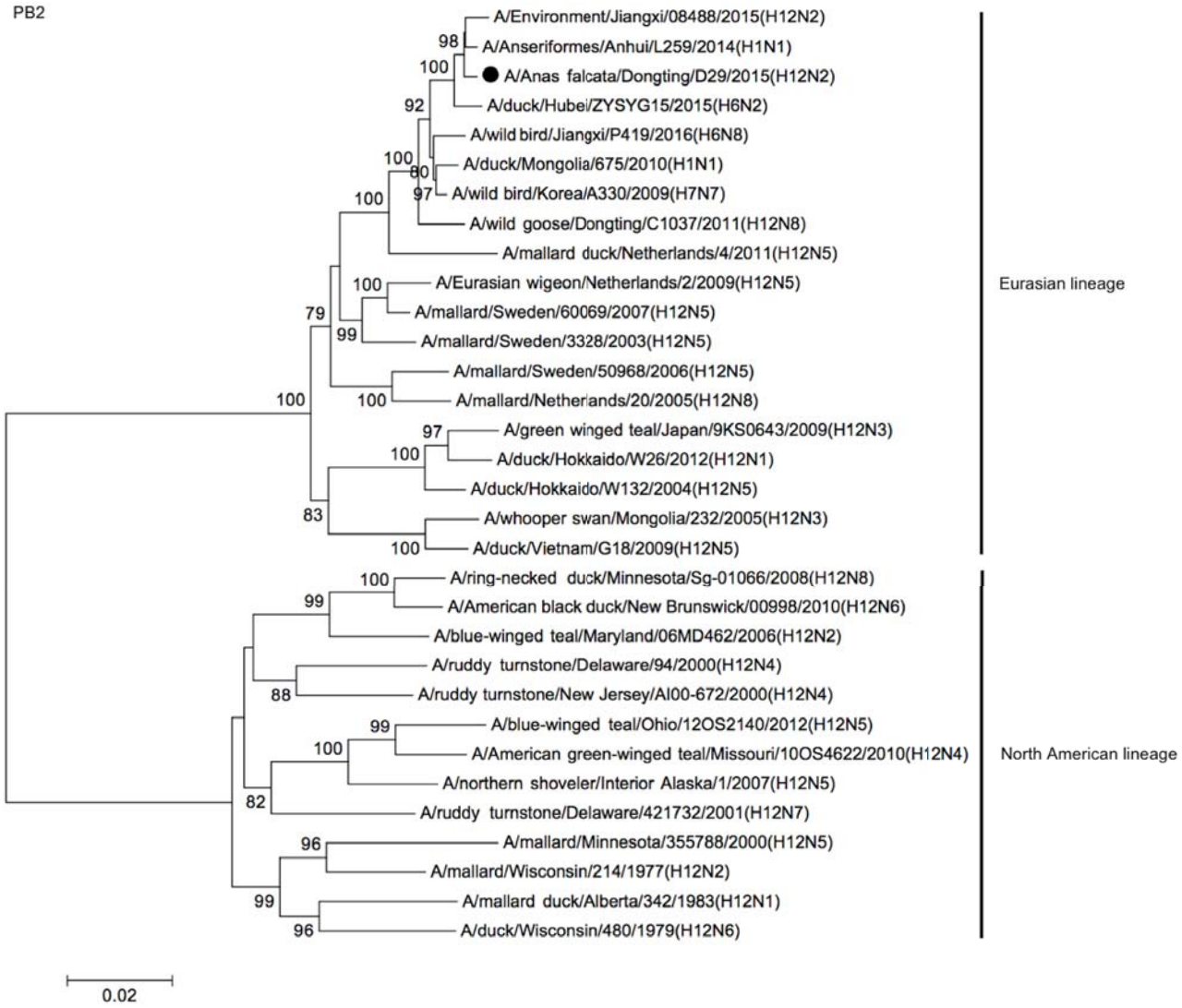

Fig. S1 Continued

Phylogenetic tree of the *PB2* gene of the H12N2 AIV

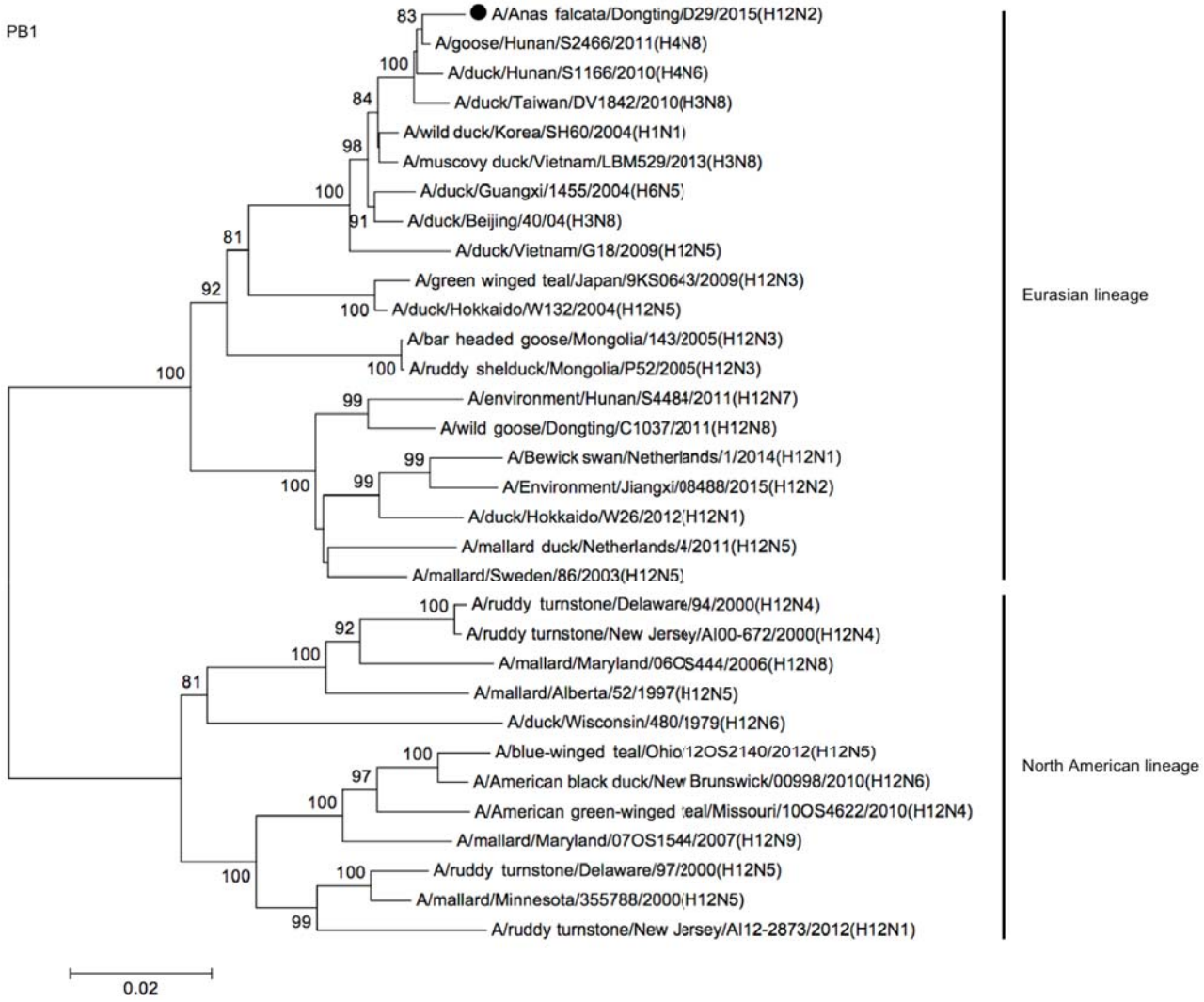

Fig. S1 Continued

Phylogenetic tree of the *PB1* gene of the H12N2 AIV

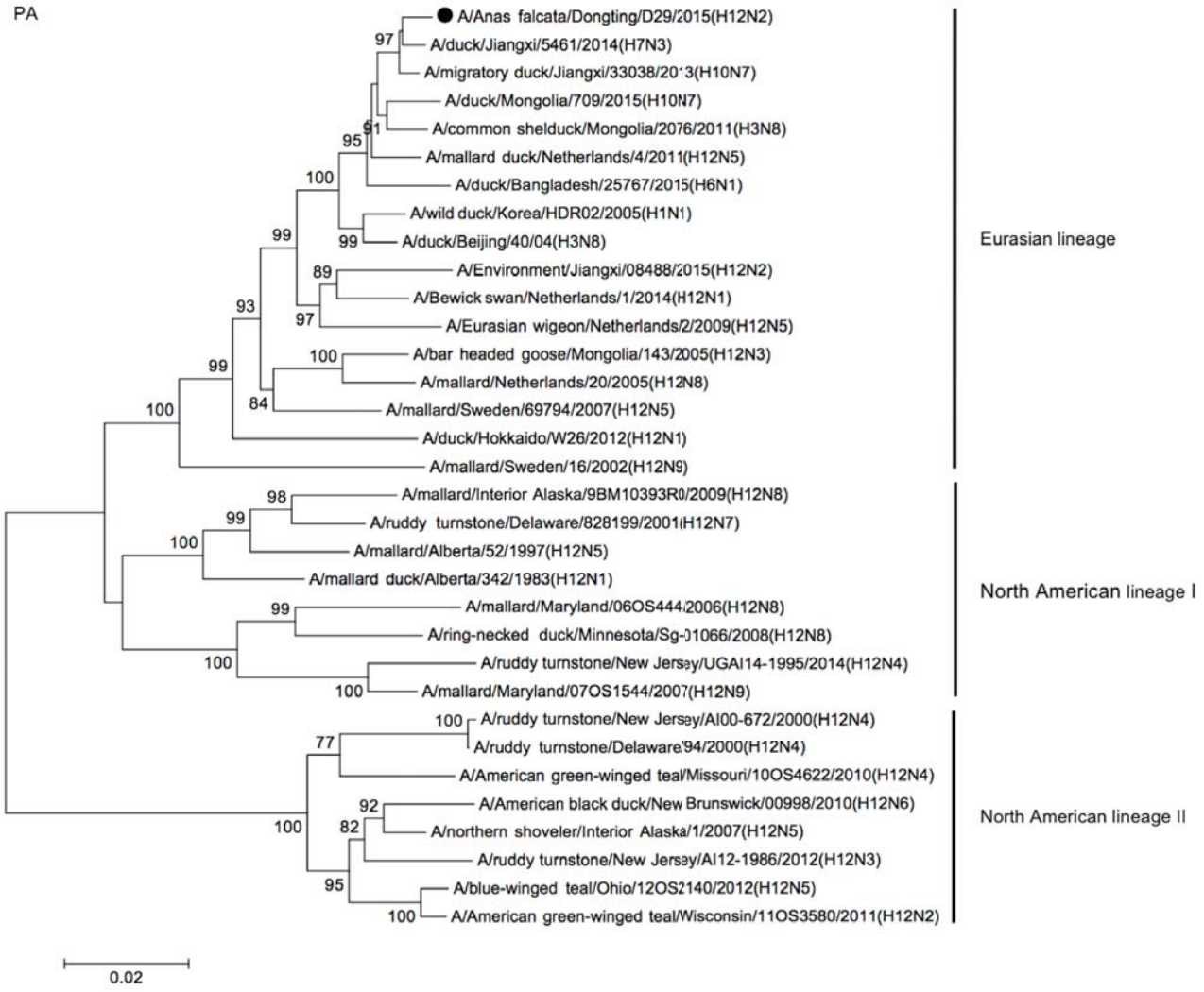

Fig. S1 Continued

Phylogenetic tree of the *PA* gene of the H12N2 AIV

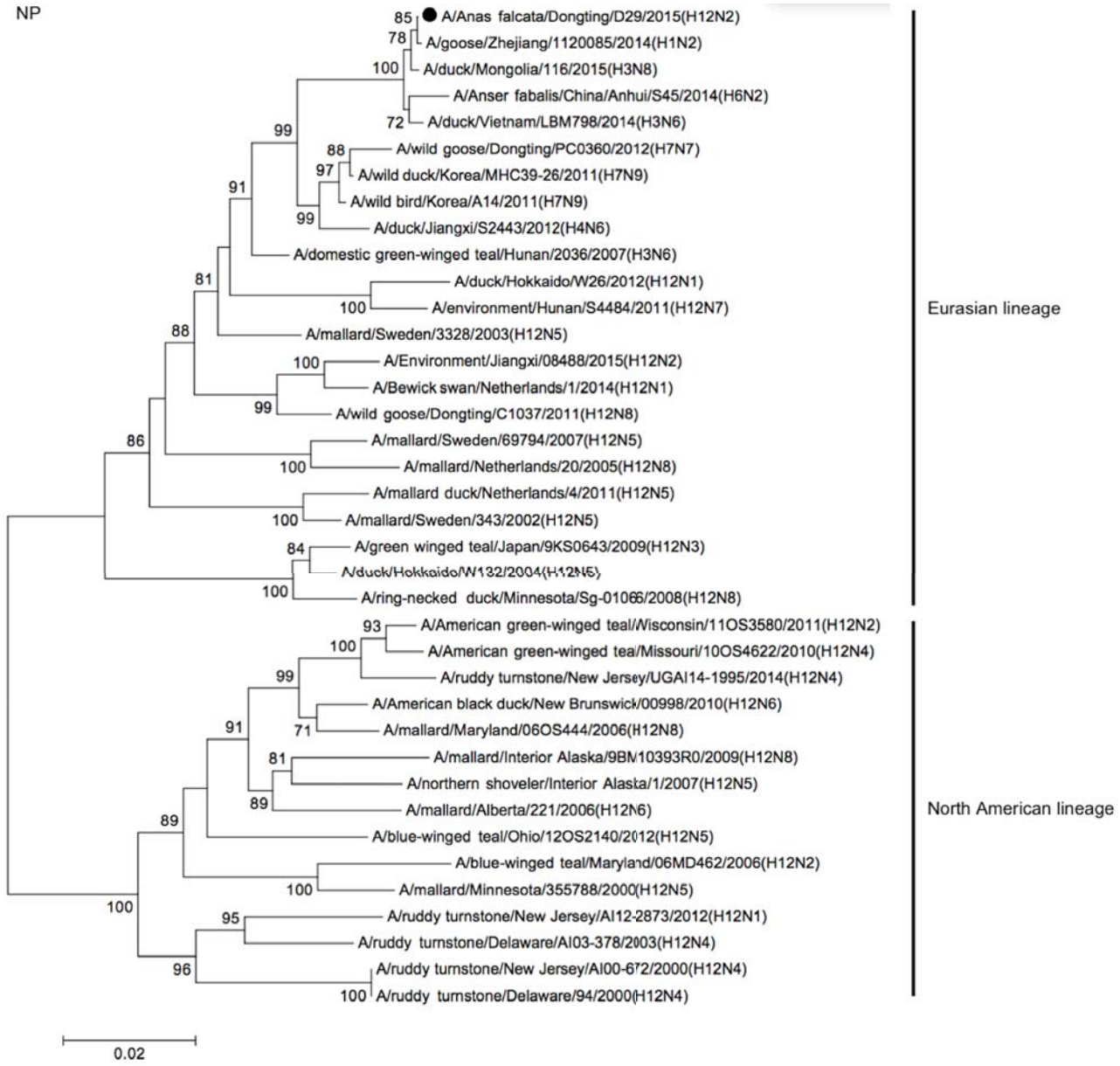

Fig. S1 Continued

Phylogenetic tree of the *NP* gene of the H12N2 AIV

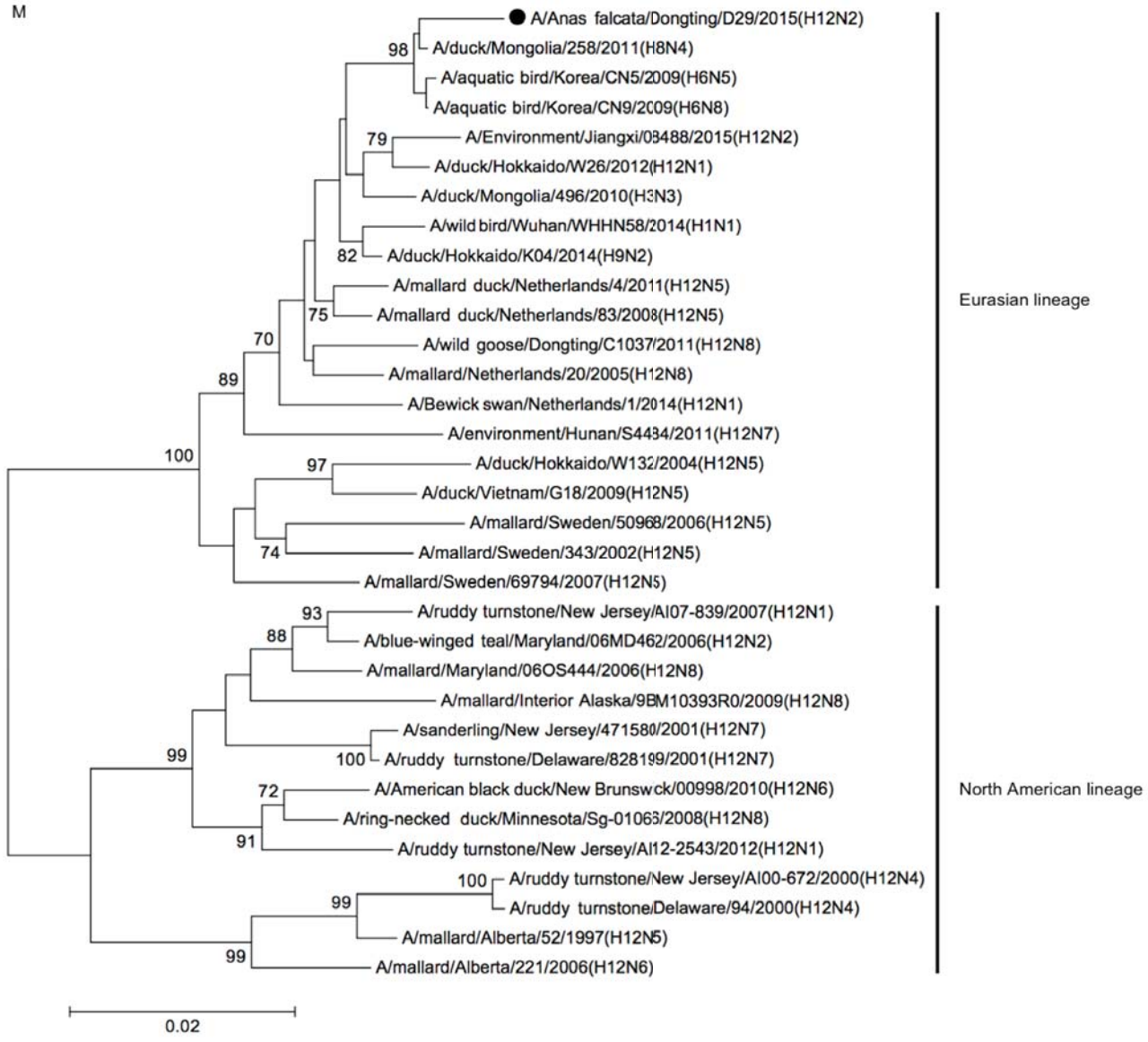

Fig. S1 Continued

Phylogenetic tree of the *M* gene of the H12N2 AIV

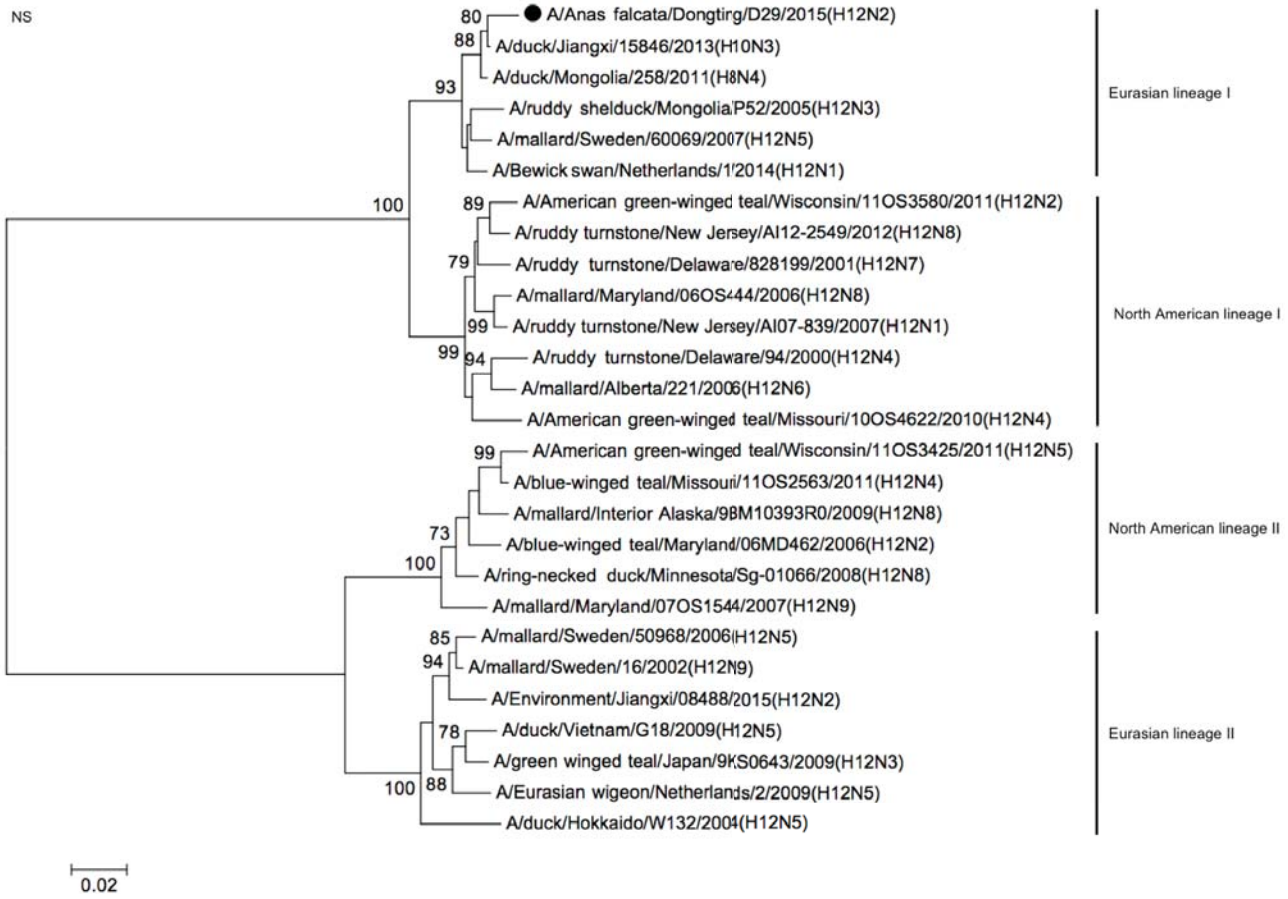

Fig. S1

Phylogenetic tree of the *NS* gene of the H12N2 AIV

The phylogenetic trees were generated using MEGA 7.0.26 software, and the bootstrap value was tested by 1000 replications. The H12N2 virus characterized is highlighted by a dot. The Scale bar shows nucleotide substitutions per site.