Influenza A(H5N6) Virus Reassortant, Southern China, 2014

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To the Editor: Avian influenza A viruses generally do not cause disease in aquatic birds, the natural reservoir of these viruses (*1*). Influenza A(H5N6) was first isolated from mallards by García et al. in 1975 (*2*). Influenza viruses continue to evolve and reassort to generate novel, highly pathogenic viruses. Novel H5 highly pathogenic avian influenza virus subtypes, such as H5N2, H5N5, and H5N8, have been reported (*3*,*4*). Highly pathogenic influenza A viruses are endemic to many countries (http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2015/), cause tremendous economic losses to the poultry industry, and represent a serious threat to public health.

In March 2014, an influenza A(H5N6) outbreak caused the death of 457 birds in Laos (http://www.oie.int/wahis_2/public%5C..%5Ctemp%5Creports/en_imm_0000015052_20140507_182757.pdf). During the same month, a flock of ducks in Guangdong Province in southern China exhibited typical respiratory signs of influenza A virus infection. This flock also had 70% decreased egg production and a slightly increased mortality rate. Throat swab specimens were taken from the symptomatic and dead ducks, and the samples were used to inoculate chicken embryos for virus isolation. Hemagglutination (HA) and neuraminidase (NA) inhibition assays were performed to identify the subtype of the isolated virus, which was designated A/duck/Guangdong/GD01/2014 (H5N6) (GD01/2014). The complete RNA genome was amplified by reverse transcription PCR and cloned into the pMD-19T vector for sequencing (*5*). The complete genome sequence of the GD01/2014 virus was submitted to GenBank (accession nos. KJ754142–KJ754149).

Multiple-sequence alignments showed that the HA gene of GD01/2014 shared 97.5% nt identity with A/wild duck/Shandong/628/2011 (H5N1) and NA genes shared 96.6% and 98.3% nt identity with A/swine/Guangdong/K6/2010 (H6N6) and A/duck/Shantou/1984/2007 (H6N6), respectively. All internal genes shared high levels of nucleotide identity (97.6%–98.5%) with A/wild duck/Fujian/2/2011(H5N1). The whole genes of GD01/2014 and the H5N6 viruses in Laos (LAO/2014) shared 98.2%–99.7% nt identity, indicating the same genotype. Phylogenetic analysis of the HA gene revealed that the isolated virus belonged to clade 2.3.4.6 (online Technical Appendix Figure, panel A, http://wwwnc.cdc.gov/EID/article/21/7/14-0838-Techapp.pdf) (*6*). The NA gene of GD01/2014 was clustered with some H6N6 viruses circulating in China (online Technical Appendix Figure, panel B). The 6 internal genes of GD01/2014 were closely related with A/wild duck/Fujian/2/2011(H5N1) and A/wild duck/Fujian/1/2011(H5N1) (online Technical Appendix Figure, panels C–H). Phylogenetic analysis showed that all 8 genes of GD01/2014 and LAO/2014 were closely related although genetically distant from the earlier isolated H5N6 viruses (online Technical Appendix Figure). These findings suggest that GD01/2014 and LAO/2014 are reassortants of wild duck H5N1 and H6N6 viruses, both of which have pathogenic and potential pandemic capacity in southern China. A previous report that H5N1 and H6N6 co-infected a duck suggests that GD01/2014 might be generated from the co-infection of H5N1 and H6N6 in the same host (*7*).

The intravenous pathogenicity index of GD01/2014 was 3.0, which indicates that the isolate is highly pathogenic for chickens. GD01/2014 had multiple basic amino acids (LRERRRKR/GLF) at the cleavage site between HA1 and HA2; this characteristic is typical of highly virulent influenza viruses (*8*). The HA protein contained E190, R220, G225, Q226, and G228 (H3 numbering) residues at the receptor-binding pockets, indicating that the virus preferentially binds to the sialic acid-2,3-NeuAcGal of the avian-like receptor (*9*). The HA protein has 7 potential N-glycosylation sites (PGSs); the HA1 protein has 5 PGSs; the HA2 protein has 2 PGSs. The NA protein of GD01/2014 and LAO/2014 had a deletion of 11 aa residues at positions 59–69 (N6 numbering) in the NA stalk region. Moreover, a deletion of 5 aa residues from positions 80–84 in the nonstructural 1 protein was found in GD01/2014 and LAO/2014. The position 627 and 701 of the polymerase basic 2 protein were E and D, respectively, characteristics of the avian influenza virus (*10*)

In summary, in 2014, outbreaks of H5N6 virus occurred in China, Laos, and Vietnam and caused the deaths of infected humans in Sichuan province, China (http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2014/; http://www.wpro.who.int/china/mediacentre/releases/2014/20140507/en/). We characterized the novel reassortant H5N6 virus in China and found that it

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was the same genome type as and was highly homologous with the H5N6 virus in Laos. The findings in this study are also supported by the previous genetic characterization of these viruses by Wong et al. (*11*). However, the adaptation, host range, and virulence of this reassortant H5N6 virus are still unclear and should be further investigated. Furthermore, the potential for infection, outbreaks, and pandemic in other poultry and mammals should be carefully monitored.

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Characterization of 3 Megabase-Sized Circular Replicons from *Vibrio cholerae*

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To the Editor: Prokaryotes typically have a single circular chromosome. However, some bacteria have >1 chromosome. *Vibrio* bacteria, for example, have 2 circular chromosomes: 1 (Ch1) and 2 (Ch2) (*1–3*). Most recognizable genes responsible for essential cell functions and pathogenicity are located on Ch1. Ch2 is also thought to encode some genes essential for normal cell function and those associated with virulence. Both chromosomes are controlled coordinately in their replication and segregation (*4*). Evidence suggests that Ch2 was originally a mega-plasmid captured by an ancestral *Vibrio* species (*2,5*). We report the characterization of recent isolates of *V. cholerae* O1 from Thailand that carry a novel gigantic replicon (Rep.3) in addition to Ch1 and Ch2.

Cholera outbreaks occurred in Tak Province, Thailand, during March–December 2010. We obtained 118 isolates of *V. cholerae* O1 and subjected their *Not*I digests to pulsed-field gel electrophoresis (PFGE), which differentiated the isolates into 8 different patterns (*6*). The profile of PFGE type A6 was identical to that of PFGE type A4, except that a large DNA band existed in type A6. The PFGE profile of the intact (undigested) DNA of the type A6 isolates exhibited a unique genome structure consisting of 3 large replicons (Figure, http://wwwnc.cdc.gov/EID/article/21/7/14-1055-F1.htm).

Three isolates of PFGE type A6 (TSY216, TSY241, and TSY421) were obtained during June 3–July 5, 2010, from 3 unrelated residents of a village near the Thailand–Myanmar border. The isolates were classified as multilocus variable-number tandem-repeat analysis type 16, suggesting that they are of clonal origin (*6*). Next, we performed whole-genome sequencing of TSY216, as a representative of PFGE type A6 isolates, by using the GS FLX Titanium

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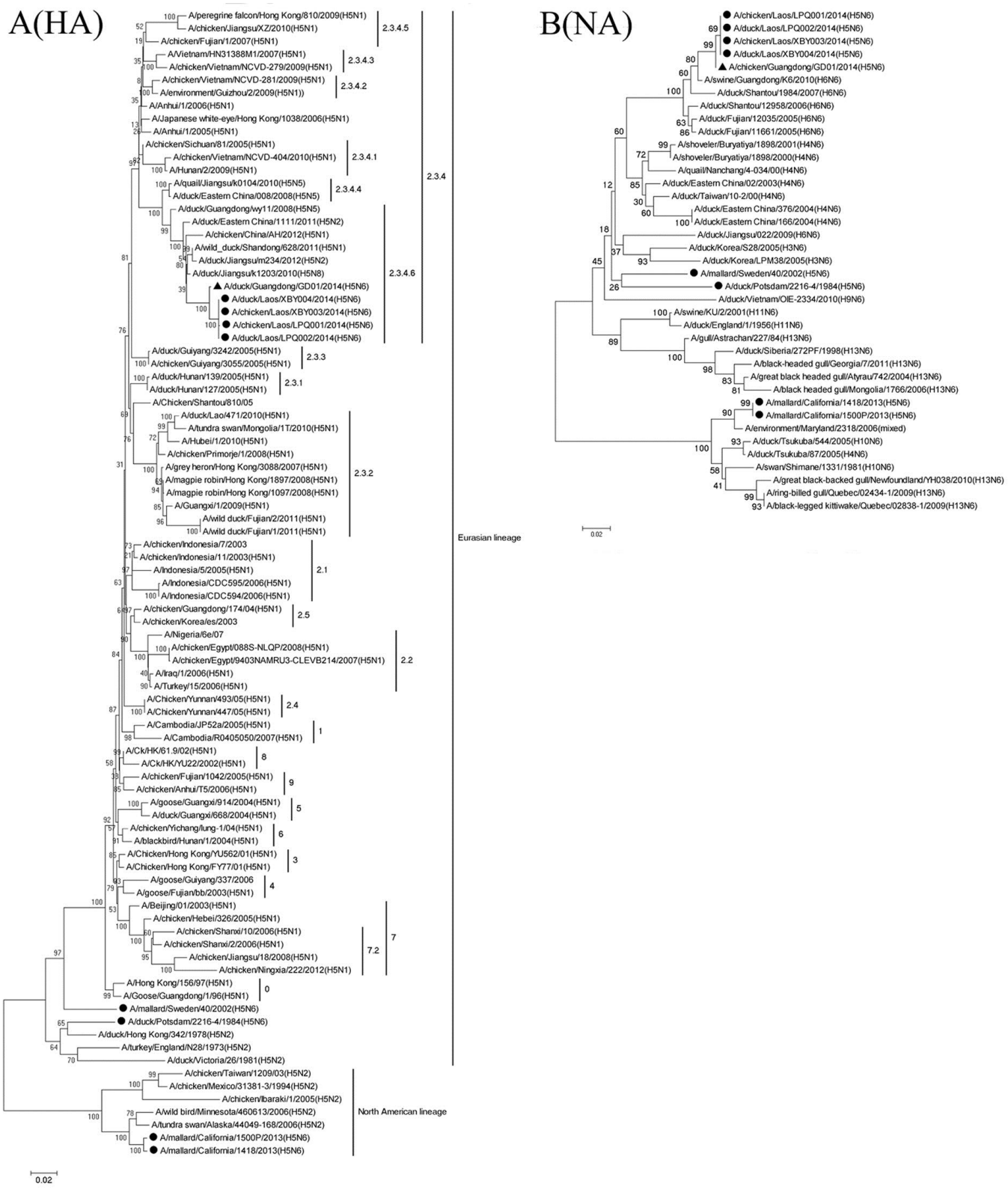
Technical Appendix

Technical Appendix Figure (following pages). Phylogenetic relationship of genes of A/duck/Guangdong/GD01/2014 (H5N6), a novel virus isolated from ducks in Guangdong Province in southern China during March 2014, compared with reference strains from GenBank. Phylogenetic relationship of specific genes are shown by panel: A) HA, hemagglutinin; B) NA, neuraminidase; C) PB2, polybasic protein 2; D) PB1, polybasic protein 1; E) PA, polymerase acidic protein; F) NP, nucleocapsid protein; G) M, matrix protein; H) NS, nonstructural protein. Triangles indicate the virus characterized in this study. Circles indicate other H5N6 viruses available in GenBank. The tree was constructed by using the neighbor-joining method of MEGA5.05 (www.megasoftware.net/) with 1,000 bootstrap replicates.


A(HA)
A/peregrine falcon/Hong Kong/810/2009(H5N1)
A/chicken/Jiangsu/XZ/2010(H5N1)
A/chicken/Fujian/1/2007(H5N1)
2.3.4.5
A/Vietnam/HN31388M1/2007(H5N1)
A/chicken/Vietnam/NCVD-279/2009(H5N1)
2.3.4.3
A/chicken/Vietnam/NCVD-281/2009(H5N1)
A/environment/Guizhou/2/2009(H5N1))
2.3.4.2
A/Anhui/1/2006(H5N1)
A/Japanese white-eye/Hong Kong/1038/2006(H5N1)
A/Anhui/1/2005(H5N1)
A/chicken/Sichuan/81/2005(H5N1)
A/chicken/Vietnam/NCVD-404/2010(H5N1)
A/Hunan/2/2009(H5N1)
2.3.4.1
A/quail/Jiangsu/k0104/2010(H5N5)
A/duck/Eastern China/008/2008(H5N5)
2.3.4.4
2.3.4
A/duck/Guangdong/wy11/2008(H5N5)
A/duck/Eastern China/1111/2011(H5N2)
A/chicken/China/AH/2012(H5N1)
A/wild_duck/Shandong/628/2011(H5N1)
A/duck/Jiangsu/m234/2012(H5N2)
A/duck/Jiangsu/k1203/2010(H5N8)
A/duck/Guangdong/GD01/2014(H5N6)
A/duck/Laos/XBY004/2014(H5N6)
A/chicken/Laos/XBY003/2014(H5N6)
A/chicken/Laos/LPQ001/2014(H5N6)
A/duck/Laos/LPQ002/2014(H5N6)
2.3.4.6
A/duck/Guiyang/3242/2005(H5N1)
A/chicken/Guiyang/3055/2005(H5N1)
2.3.3
A/duck/Hunan/139/2005(H5N1)
A/duck/Hunan/127/2005(H5N1)
2.3.1
A/Chicken/Shantou/810/05
A/duck/Lao/471/2010(H5N1)
A/tundra swan/Mongolia/1T/2010(H5N1)
A/Hubei/1/2010(H5N1)
A/chicken/Primorje/1/2008(H5N1)
A/grey heron/Hong Kong/3088/2007(H5N1)
A/magpie robin/Hong Kong/1897/2008(H5N1)
A/magpie robin/Hong Kong/1097/2008(H5N1)
A/Guangxi/1/2009(H5N1)
A/wild duck/Fujian/2/2011(H5N1)
A/wild duck/Fujian/1/2011(H5N1)
2.3.2
A/chicken/Indonesia/7/2003
A/chicken/Indonesia/11/2003(H5N1)
A/Indonesia/5/2005(H5N1)
A/Indonesia/CDC595/2006(H5N1)
A/Indonesia/CDC594/2006(H5N1)
2.1
A/chicken/Guangdong/174/04(H5N1)
A/chicken/Korea/es/2003
2.5
A/Nigeria/6e/07
A/chicken/Egypt/088S-NLQP/2008(H5N1)
A/chicken/Egypt/9403NAMRU3-CLEVB214/2007(H5N1)
A/Iraq/1/2006(H5N1)
A/Turkey/15/2006(H5N1)
2.2
A/Chicken/Yunnan/493/05(H5N1)
A/Chicken/Yunnan/447/05(H5N1)
2.4
A/Cambodia/JP52a/2005(H5N1)
A/Cambodia/R0405050/2007(H5N1)
1
A/Ck/HK/61.9/02(H5N1)
A/Ck/HK/YU22/2002(H5N1)
8
A/chicken/Fujian/1042/2005(H5N1)
A/chicken/Anhui/T5/2006(H5N1)
9
A/goose/Guangxi/914/2004(H5N1)
A/duck/Guangxi/668/2004(H5N1)
5
A/chicken/Yichang/lung-1/04(H5N1)
A/blackbird/Hunan/1/2004(H5N1)
6
A/Chicken/Hong Kong/YU562/01(H5N1)
A/Chicken/Hong Kong/FY77/01(H5N1)
3
A/goose/Guiyang/337/2006
A/goose/Fujian/bb/2003(H5N1)
4
A/Beijing/01/2003(H5N1)
A/chicken/Hebei/326/2005(H5N1)
A/chicken/Shanxi/10/2006(H5N1)
A/chicken/Shanxi/2/2006(H5N1)
A/chicken/Jiangsu/18/2008(H5N1)
A/chicken/Ningxia/222/2012(H5N1)
7.2
7
A/Hong Kong/156/97(H5N1)
A/Goose/Guangdong/1/96(H5N1)
0
A/mallard/Sweden/40/2002(H5N6)
A/duck/Potsdam/2216-4/1984(H5N6)
A/duck/Hong Kong/342/1978(H5N2)
A/turkey/England/N28/1973(H5N2)
A/duck/Victoria/26/1981(H5N2)
Eurasian lineage
A/chicken/Taiwan/1209/03(H5N2)
A/chicken/Mexico/31381-3/1994(H5N2)
A/chicken/Ibaraki/1/2005(H5N2)
A/wild bird/Minnesota/460613/2006(H5N2)
A/tundra swan/Alaska/44049-168/2006(H5N2)
A/mallard/California/1500P/2013(H5N6)
A/mallard/California/1418/2013(H5N6)
North American lineage
0.02
B(NA)
A/chicken/Laos/LPQ001/2014(H5N6)
A/duck/Laos/LPQ002/2014(H5N6)
A/chicken/Laos/XBY003/2014(H5N6)
A/duck/Laos/XBY004/2014(H5N6)
A/chicken/Guangdong/GD01/2014(H5N6)
A/swine/Guangdong/K6/2010(H6N6)
A/duck/Shantou/1984/2007(H6N6)
A/duck/Shantou/12958/2006(H6N6)
A/duck/Fujian/12035/2005(H6N6)
A/duck/Fujian/11661/2005(H6N6)
A/shoveler/Buryatiya/1898/2001(H4N6)
A/shoveler/Buryatiya/1898/2000(H4N6)
A/quail/Nanchang/4-034/00(H4N6)
A/duck/Eastern China/02/2003(H4N6)
A/duck/Taiwan/10-2/00(H4N6)
A/duck/Eastern China/376/2004(H4N6)
A/duck/Eastern China/166/2004(H4N6)
A/duck/Jiangsu/022/2009(H6N6)
A/duck/Korea/S28/2005(H3N6)
A/duck/Korea/LPM38/2005(H3N6)
A/mallard/Sweden/40/2002(H5N6)
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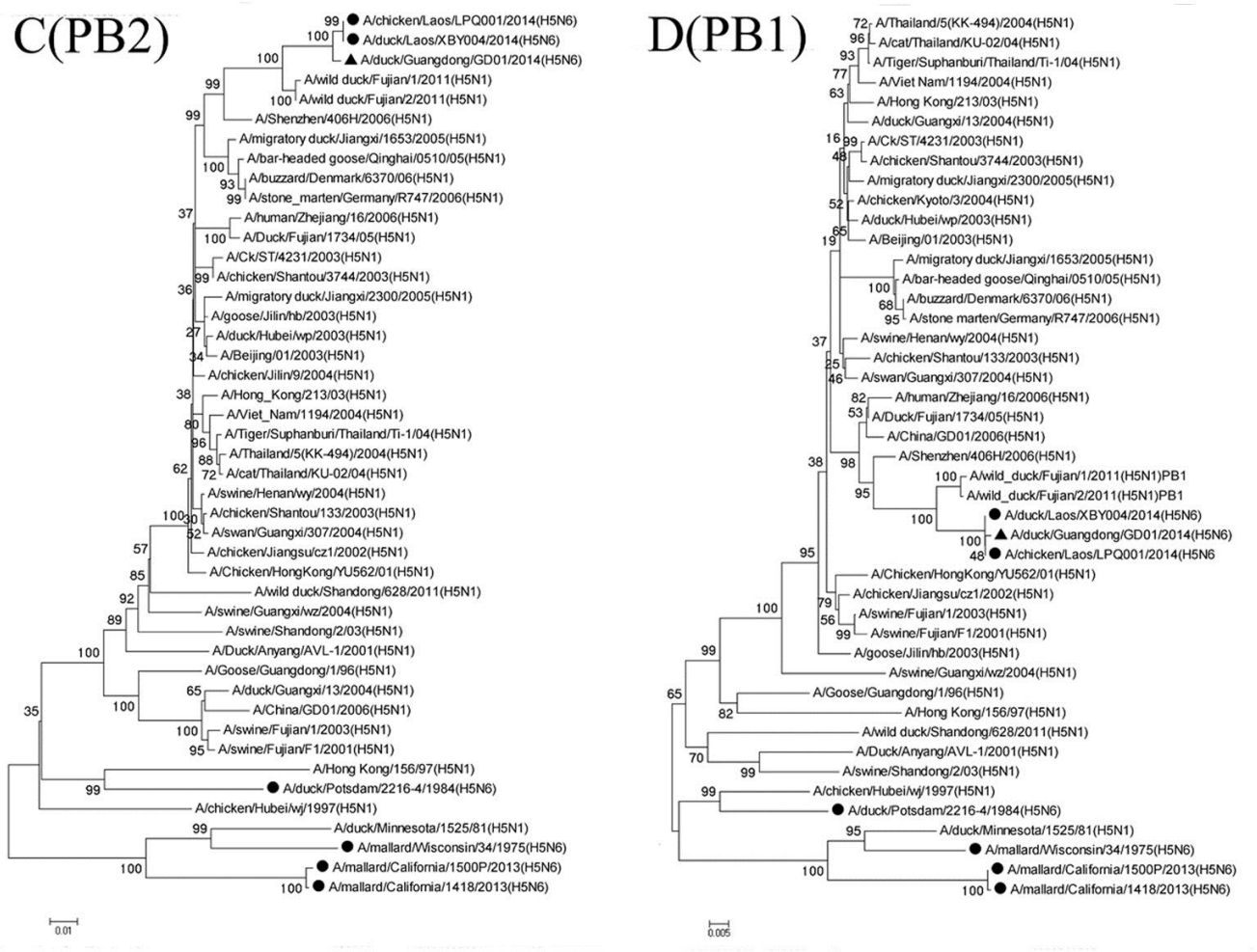
A/duck/Vietnam/OIE-2334/2010(H9N6)
A/swine/KU/2/2001(H11N6)
A/duck/England/1/1956(H11N6)
A/gull/Astrachan/227/84(H13N6)
A/duck/Siberia/272PF/1998(H13N6)
A/black-headed gull/Georgia/7/2011(H13N6)
A/great black headed gull/Atyrau/742/2004(H13N6)
A/black headed gull/Mongolia/1766/2006(H13N6)
A/mallard/California/1418/2013(H5N6)
A/mallard/California/1500P/2013(H5N6)
A/environment/Maryland/2318/2006(mixed)
A/duck/Tsukuba/544/2005(H10N6)
A/duck/Tsukuba/87/2005(H4N6)
A/swan/Shimane/1331/1981(H10N6)
A/great black-backed gull/Newfoundland/YH038/2010(H13N6)
A/ring-billed gull/Quebec/02434-1/2009(H13N6)
A/black-legged kittiwake/Quebec/02838-1/2009(H13N6)
0.02


C(PB2)
A/chicken/Laos/LPQ001/2014(H5N6)
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A/wild duck/Fujian/1/2011(H5N1)
A/wild duck/Fujian/2/2011(H5N1)
A/Shenzhen/406H/2006(H5N1)
A/migratory duck/Jiangxi/1653/2005(H5N1)
A/bar-headed goose/Qinghai/0510/05(H5N1)
A/buzzard/Denmark/6370/06(H5N1)
A/stone_marten/Germany/R747/2006(H5N1)
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A/Beijing/01/2003(H5N1)
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A/Viet_Nam/1194/2004(H5N1)
A/Tiger/Suphanburi/Thailand/Ti-1/04(H5N1)
A/Thailand/5(KK-494)/2004(H5N1)
A/cat/Thailand/KU-02/04(H5N1)
A/swine/Henan/wy/2004(H5N1)
A/chicken/Shantou/133/2003(H5N1)
A/swan/Guangxi/307/2004(H5N1)
A/chicken/Jiangsu/cz1/2002(H5N1)
A/Chicken/HongKong/YU562/01(H5N1)
A/wild duck/Shandong/628/2011(H5N1)
A/swine/Guangxi/wz/2004(H5N1)
A/swine/Shandong/2/03(H5N1)
A/Duck/Anyang/AVL-1/2001(H5N1)
A/Goose/Guangdong/1/96(H5N1)
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A/bar-headed goose/Qinghai/0510/05(H5N1)
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A/swan/Guangxi/307/2004(H5N1)
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A/Shenzhen/406H/2006(H5N1)
A/wild_duck/Fujian/1/2011(H5N1)PB1
A/wild_duck/Fujian/2/2011(H5N1)PB1
A/duck/Laos/XBY004/2014(H5N6)
A/duck/Guangdong/GD01/2014(H5N6)
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A/wild duck/Shandong/628/2011(H5N1)
A/Duck/Anyang/AVL-1/2001(H5N1)
A/swine/Shandong/2/03(H5N1)
A/chicken/Hubei/wj/1997(H5N1)
A/duck/Potsdam/2216-4/1984(H5N6)
A/duck/Minnesota/1525/81(H5N1)
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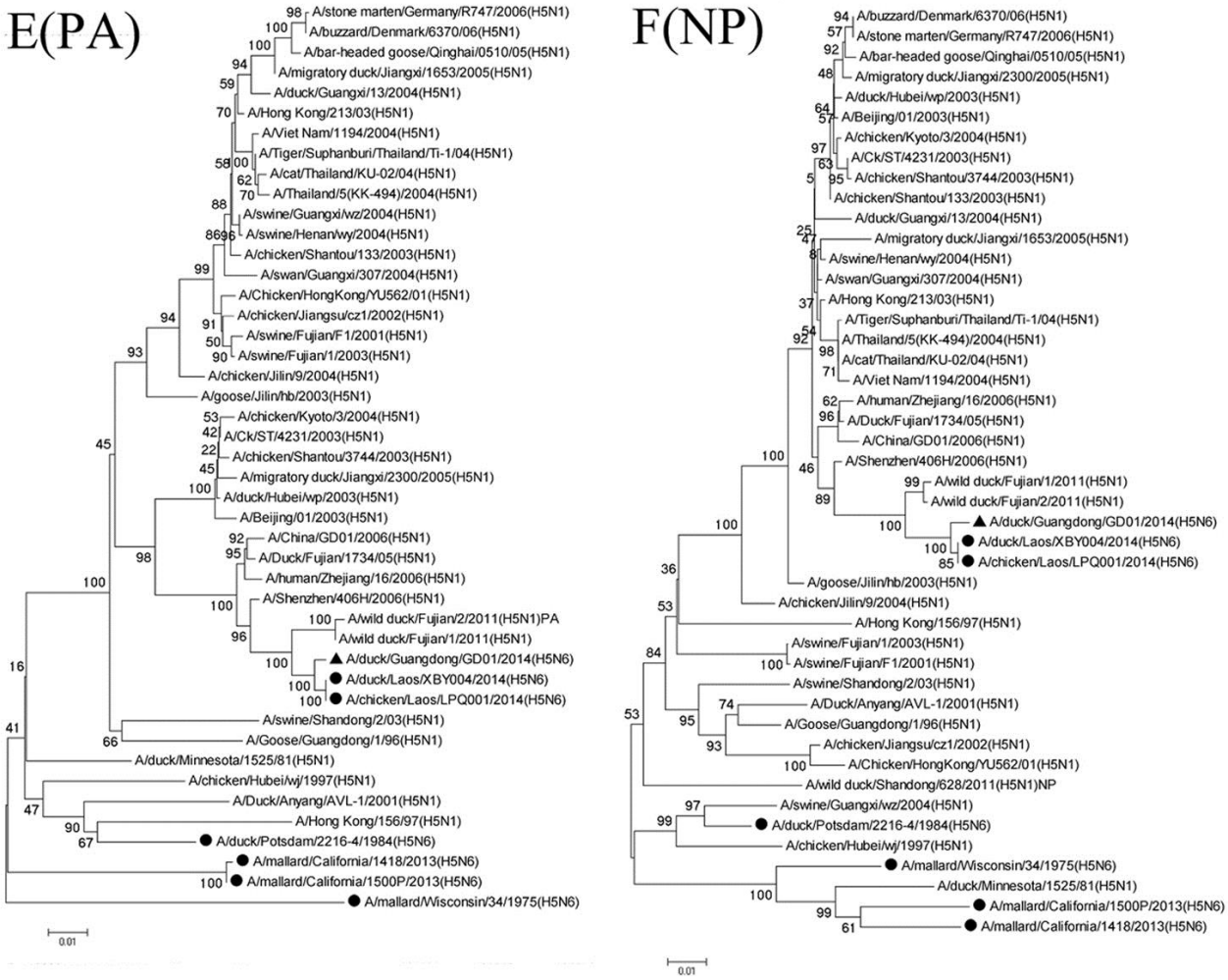
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A/duck/Guangxi/13/2004(H5N1)
A/Hong Kong/213/03(H5N1)
A/Viet Nam/1194/2004(H5N1)
A/Tiger/Suphanburi/Thailand/Ti-1/04(H5N1)
A/cat/Thailand/KU-02/04(H5N1)
A/Thailand/5(KK-494)/2004(H5N1)
A/swine/Guangxi/wz/2004(H5N1)
A/swine/Henan/wy/2004(H5N1)
A/chicken/Shantou/133/2003(H5N1)
A/swan/Guangxi/307/2004(H5N1)
A/Chicken/HongKong/YU562/01(H5N1)
A/chicken/Jiangsu/cz1/2002(H5N1)
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A/chicken/Jilin/9/2004(H5N1)
A/goose/Jilin/hb/2003(H5N1)
A/chicken/Kyoto/3/2004(H5N1)
A/Ck/ST/4231/2003(H5N1)
A/chicken/Shantou/3744/2003(H5N1)
A/migratory duck/Jiangxi/2300/2005(H5N1)
A/duck/Hubei/wp/2003(H5N1)
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A/China/GD01/2006(H5N1)
A/Duck/Fujian/1734/05(H5N1)
A/human/Zhejiang/16/2006(H5N1)
A/Shenzhen/406H/2006(H5N1)
A/wild duck/Fujian/2/2011(H5N1)PA
A/wild duck/Fujian/1/2011(H5N1)
A/duck/Guangdong/GD01/2014(H5N6)
A/duck/Laos/XBY004/2014(H5N6)
A/chicken/Laos/LPQ001/2014(H5N6)
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A/Goose/Guangdong/1/96(H5N1)
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A/duck/Hubei/wp/2003(H5N1)
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A/China/GD01/2006(H5N1)
A/Shenzhen/406H/2006(H5N1)
A/wild duck/Fujian/1/2011(H5N1)
A/wild duck/Fujian/2/2011(H5N1)
A/duck/Guangdong/GD01/2014(H5N6)
A/duck/Laos/XBY004/2014(H5N6)
A/chicken/Laos/LPQ001/2014(H5N6)
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A/chicken/Jilin/9/2004(H5N1)
A/Hong Kong/156/97(H5N1)
A/swine/Fujian/1/2003(H5N1)
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A/swine/Shandong/2/03(H5N1)
A/Duck/Anyang/AVL-1/2001(H5N1)
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A/Chicken/HongKong/YU562/01(H5N1)
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A/duck/Potsdam/2216-4/1984(H5N6)
A/chicken/Hubei/wj/1997(H5N1)
A/mallard/Wisconsin/34/1975(H5N6)
A/duck/Minnesota/1525/81(H5N1)
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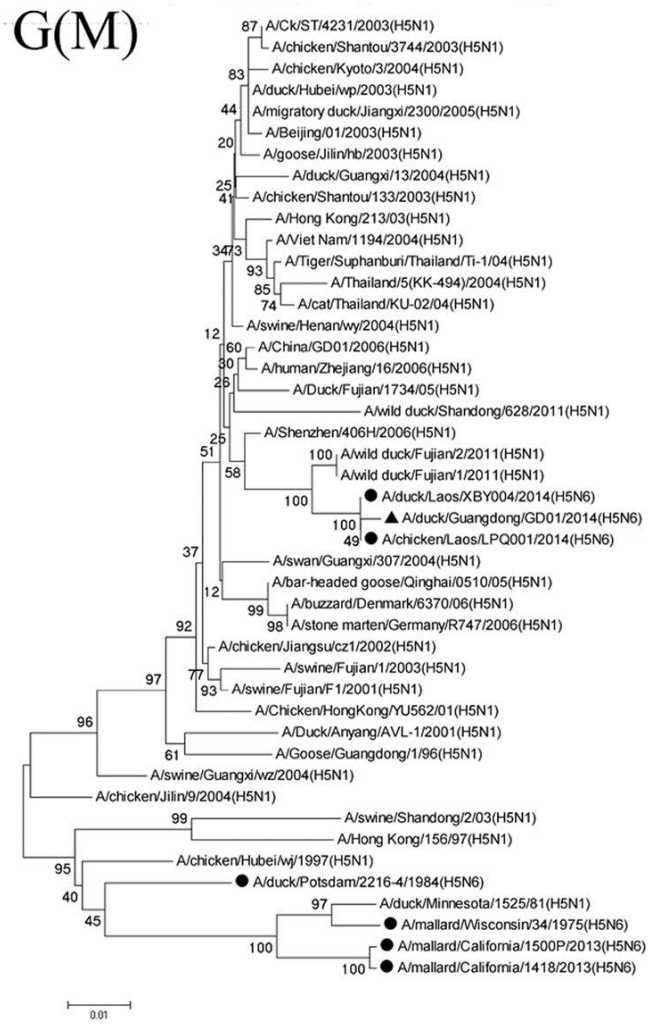
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A/Beijing/01/2003(H5N1)
A/goose/Jilin/hb/2003(H5N1)
A/duck/Guangxi/13/2004(H5N1)
A/chicken/Shantou/133/2003(H5N1)
A/Hong Kong/213/03(H5N1)
A/Viet Nam/1194/2004(H5N1)
A/Tiger/Suphanburi/Thailand/Ti-1/04(H5N1)
A/Thailand/5(KK-494)/2004(H5N1)
A/cat/Thailand/KU-02/04(H5N1)
A/swine/Henan/wy/2004(H5N1)
A/China/GD01/2006(H5N1)
A/human/Zhejiang/16/2006(H5N1)
A/Duck/Fujian/1734/05(H5N1)
A/wild duck/Shandong/628/2011(H5N1)
A/Shenzhen/406H/2006(H5N1)
A/wild duck/Fujian/2/2011(H5N1)
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A/duck/Laos/XBY004/2014(H5N6)
A/duck/Guangdong/GD01/2014(H5N6)
A/chicken/Laos/LPQ001/2014(H5N6)
A/swan/Guangxi/307/2004(H5N1)
A/bar-headed goose/Qinghai/0510/05(H5N1)
A/buzzard/Denmark/6370/06(H5N1)
A/stone marten/Germany/R747/2006(H5N1)
A/chicken/Jiangsu/cz1/2002(H5N1)
A/swine/Fujian/1/2003(H5N1)
A/swine/Fujian/F1/2001(H5N1)
A/Chicken/HongKong/YU562/01(H5N1)
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A/Goose/Guangdong/1/96(H5N1)
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A/mallard/Wisconsin/34/1975(H5N6)
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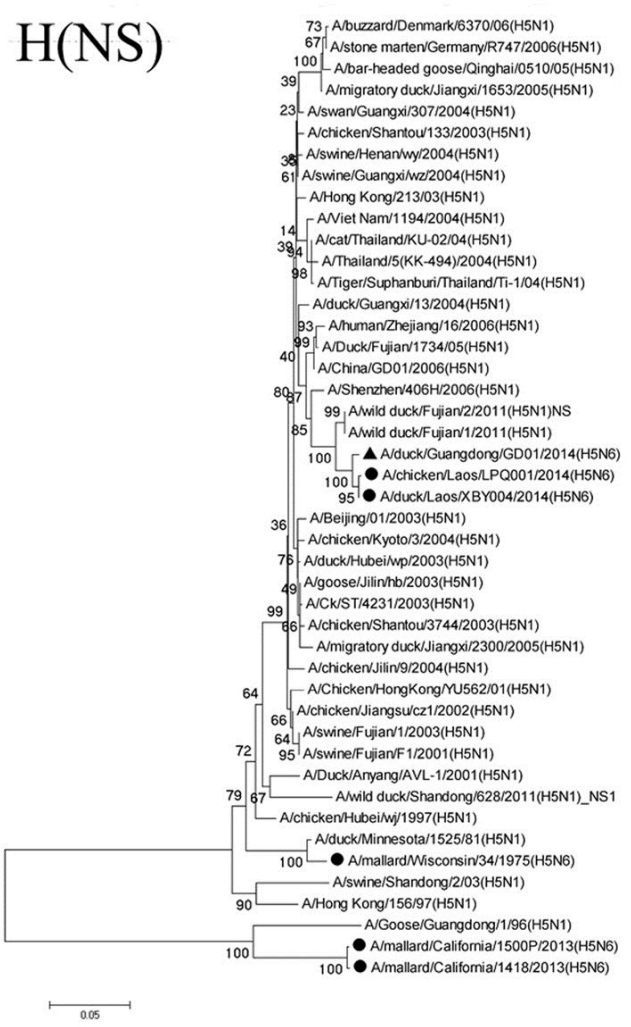
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A/chicken/Shantou/133/2003(H5N1)
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A/Hong Kong/213/03(H5N1)
A/Viet Nam/1194/2004(H5N1)
A/cat/Thailand/KU-02/04(H5N1)
A/Thailand/5(KK-494)/2004(H5N1)
A/Tiger/Suphanburi/Thailand/Ti-1/04(H5N1)
A/duck/Guangxi/13/2004(H5N1)
A/human/Zhejiang/16/2006(H5N1)
A/Duck/Fujian/1734/05(H5N1)
A/China/GD01/2006(H5N1)
A/Shenzhen/406H/2006(H5N1)
A/wild duck/Fujian/2/2011(H5N1)NS
A/wild duck/Fujian/1/2011(H5N1)
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A/chicken/Laos/LPQ001/2014(H5N6)
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A/Chicken/HongKong/YU562/01(H5N1)
A/chicken/Jiangsu/cz1/2002(H5N1)
A/swine/Fujian/1/2003(H5N1)
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A/mallard/Wisconsin/34/1975(H5N6)
A/swine/Shandong/2/03(H5N1)
A/Hong Kong/156/97(H5N1)
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A/mallard/California/1500P/2013(H5N6)
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