Origins of the new influenza A(H1N1) virus: time to take action

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To gain insight into the possible origins of the 2009 outbreak of new influenza A(H1N1), we performed two independent analyses of genetic evolution of the new influenza A(H1N1) virus. Firstly, protein homology analyses of more than 400 sequences revealed that this virus most likely evolved from recent swine viruses. Secondly, phylogenetic analyses of 5,214 protein sequences of influenza A(H1N1) viruses (avian, swine and human) circulating in North America for the last two decades (from 1989 to 2009) indicated that the new influenza A(H1N1) virus possesses a distinctive evolutionary trait (genetic distinctness). This appears to be a particular characteristic in pig-human interspecies transmission of influenza A. Thus these analyses contribute to the evidence of the role of pig populations as "mixing vessels" for influenza A(H1N1) viruses.

Introduction

On 24 April, the World Health Organization (WHO) released the first alert indicating the occurrence of confirmed human cases of swine influenza A(H1N1) in North America [1]. A few days later, the Centers for Disease Control and Prevention in the United States confirmed that these human influenza cases were caused by the same new influenza A(H1N1) virus [2]. Soon after, it was proposed that the current flu outbreak is caused by a new influenza A(H1N1) virus generated from a triple reassortment of human, swine and avian viruses [2-8]. Other publications, including our study presented here, demonstrate that this new influenza A(H1N1) virus most likely evolved from recent swine viruses [9-11].

Methods and results

Protein homology analysis

We used more than 400 protein sequences to analyse the genetic evolution of the new influenza A(H1N1) virus. This set of protein sequences included polymerases PB2, PB1 and PA, hemagglutinin (HA), nucleocapsid (NP), neuraminidase (NA), matrix 1 (MP1), nonstructural 1 (NS1) encoded by the new influenza A(H1N1) virus as well as other homologous proteins from influenza viruses from past flu seasons. Phylogenetic tree topologies revealed that the closest homologies for the new influenza A(H1N1) virus are swine influenza viruses that have been circulating in the United States and Asia for the last decade (Figure 1, Supplementary materials: Figure 1 and Table 1).

Figure 1. Possible origins of the influenza 2009 A(H1N1) virus: a) hemagglutinin and b) neuraminidase proteins

(See Below)

These findings indicate that domestic pigs in North America may have a central role in the generation and maintenance of this virus. This idea is also supported by the observation that protein sequences of the new influenza A(H1N1) virus have close homology to proteins of swine influenza viruses that infected humans in the recent past (Supplementary materials: Figure 1, Figure 2 and Table 2). In fact, a common element of these swine influenza zoonotic transmissions was that humans (mostly swine farm workers) were in direct contact with infected pigs [12-15].

Phylogenetic analysis

To further examine the possible genetic origins of the new influenza A(H1N1) virus, we compared all the available sequences of influenza A(H1N1) viruses circulating in North America for the last two decades (from 1989 to 2009). Protein sequences from avian, swine and human influenza viruses were obtained from the Influenza Virus Resource [16], a database that integrates information gathered from the Influenza Genome Sequencing Project of the National Institute of Allergy and Infectious Diseases (NIAID) and the GenBank of the National Center for Biotechnology Information (NCBI). A total of 5,214 protein sequences were found in this database. After removing identical sequences, a set of 1,699 influenza A proteins including PB2, PB1, PA, HA, NP, NA, MP1, and NS1 proteins were used for analyses of the genetic evolution of influenza A(H1N1) viruses. These analyses provide additional evidence of the role of pig populations as "mixing vessels" for influenza A(H1N1) viruses (Figure 2).

Figure 2. Genetic distinctness of the influenza 2009 A(H1N1) virus: a) hemagglutinin (HA) and b) neuraminidase (NA) proteins; c) phylogenetic trees for PB2, PB1, PA, NP, MP1, and NS1 proteins

(See Below)

Secondly, our analyses also revealed that the new influenza A(H1N1) virus possesses a distinctive evolutionary trait (genetic distinctness), that seems to be characteristic in pig-human interspecies transmission of influenza A (reported cases occurred in Iowa, Maryland and Wisconsin, United States between 1991 and 2006) (Figure 2, Supplementary materials: Figure 2 and Table 3).

Discussion and conclusion

Although limited in sample size, our analyses substantiate the value of molecular screening and phylogenetic assessment

for understanding the evolution of influenza viruses and, most importantly, for the early detection of emerging novel viruses that could lead to influenza pandemics. Notably, our analyses revealed that the new influenza A(H1N1) virus is genetically distinct from other influenza A(H1N1) viruses that have been circulating for the last twenty flu seasons (Figure 2 and Supplementary materials: Figure 2). Influenza viruses with novel antigens (genetic drift) can escape from immune responses induced by prior infection or vaccination and can lead to a pandemic [17].

These observations also reiterate the potential risk of pig populations as the source of the next influenza virus pandemic. Although the role of swine as "mixing vessels" for influenza A(H1N1) viruses was established more than a decade ago [18,19], it appears that the policy makers and scientific community have underestimated it. In fact, in 1998 influenza experts proposed the establishment of surveillance in swine populations as a major part of an integrated early warning system to detect pandemic threats for humans [18,19] but, to some extent, this task was overlooked. For example, a search of influenza sequences in the Influenza Virus Resource [16] revealed that the total number of swine influenza A sequences (as of 19 May 2009) is ten-times smaller than the corresponding number of human and avian influenza A sequences (4,648 compared to 46,911 and 41,142 sequences, respectively). More significantly, in some countries, such as the United States, the national strategy for pandemic influenza [20] assigned the entire preparedness budget (3.8 billion US dollars) for the prevention and control of avian A(H5N1) influenza, overlooking the swine threat [20-22]. In our (the authors') opinion, in this plan, a substantial effort was dedicated to prevent and contain the foreign threat of Asian avian flu, neglecting the influenza threat that the North American swine population presents [23]. Specifically, we believe that the aforementioned strategy ignores the swine farm and industry workers which constitute the population at higher risk of contracting and spreading the hypothetical pandemic influenza virus [24-26].

The current new influenza A(H1N1) outbreak caused by a virus of swine origin represents a new challenge for animal and human health experts. Our institution, the College of Veterinary Medicine at the National Autonomous University of Mexico (Universidad Nacional Autonoma de Mexico, UNAM) is placing a strong emphasis on the establishment of influenza surveillance in swine and avian species to identify novel genetic assortment of the new influenza A(H1N1) and other influenza viruses circulating in Mexico. For example, since 2002, we have been monitoring the genetic evolution of influenza A viruses circulating in Mexican poultry farms [27]. Now, a similar surveillance system will be applied to swine farms. This effort prioritises the use of genetic distinctness as a marker for the detection of novel viruses that could lead to influenza pandemics.

The recent influenza pandemic threat in North America reveals that it is time to take action towards the development of a systemic surveillance system which integrates phylogenetic information of influenza viruses circulating in humans and livestock.

Supplementary materials: Figure 1, Figure 2, Table 1, Table 2, Table 3:
(See Below)

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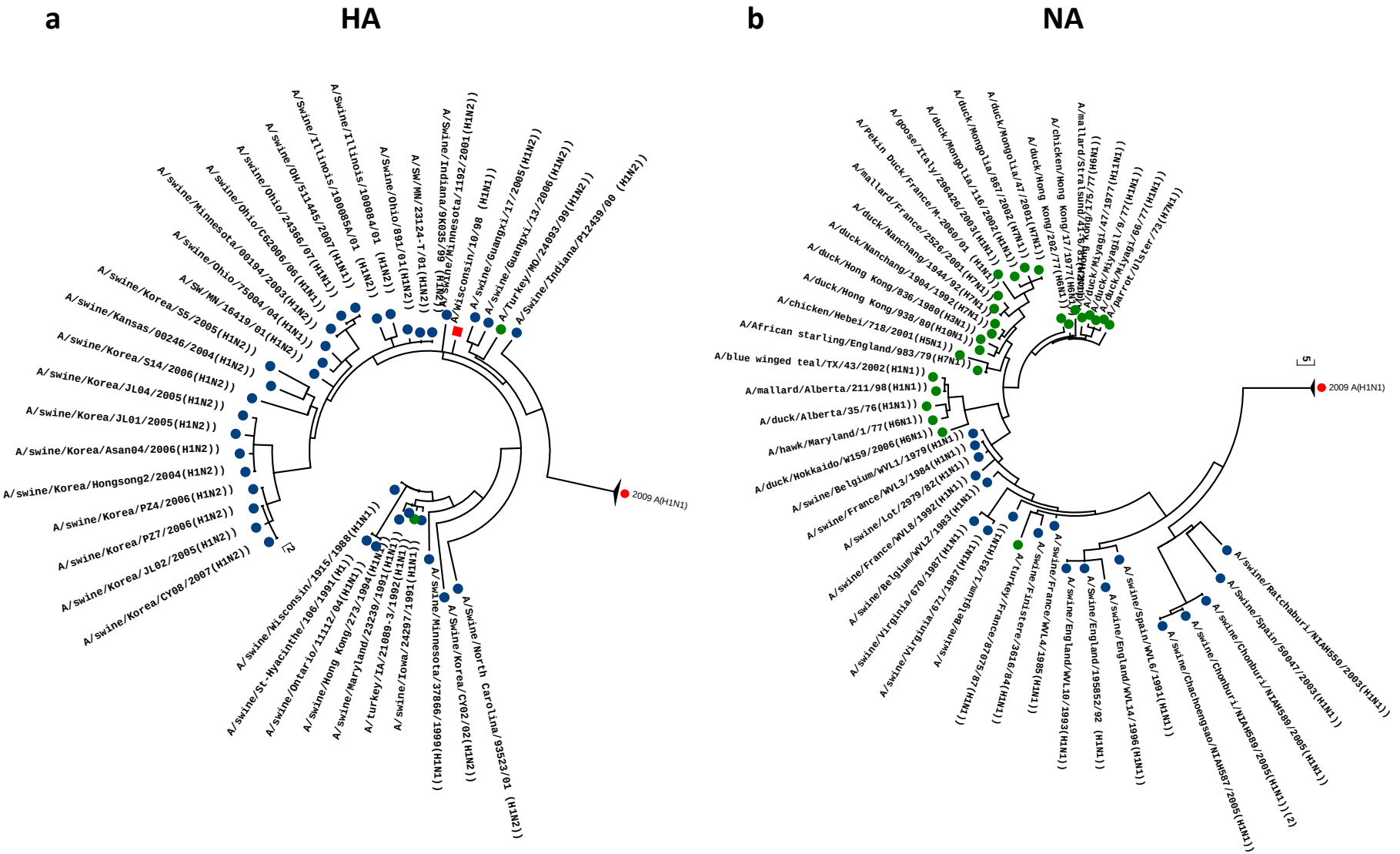
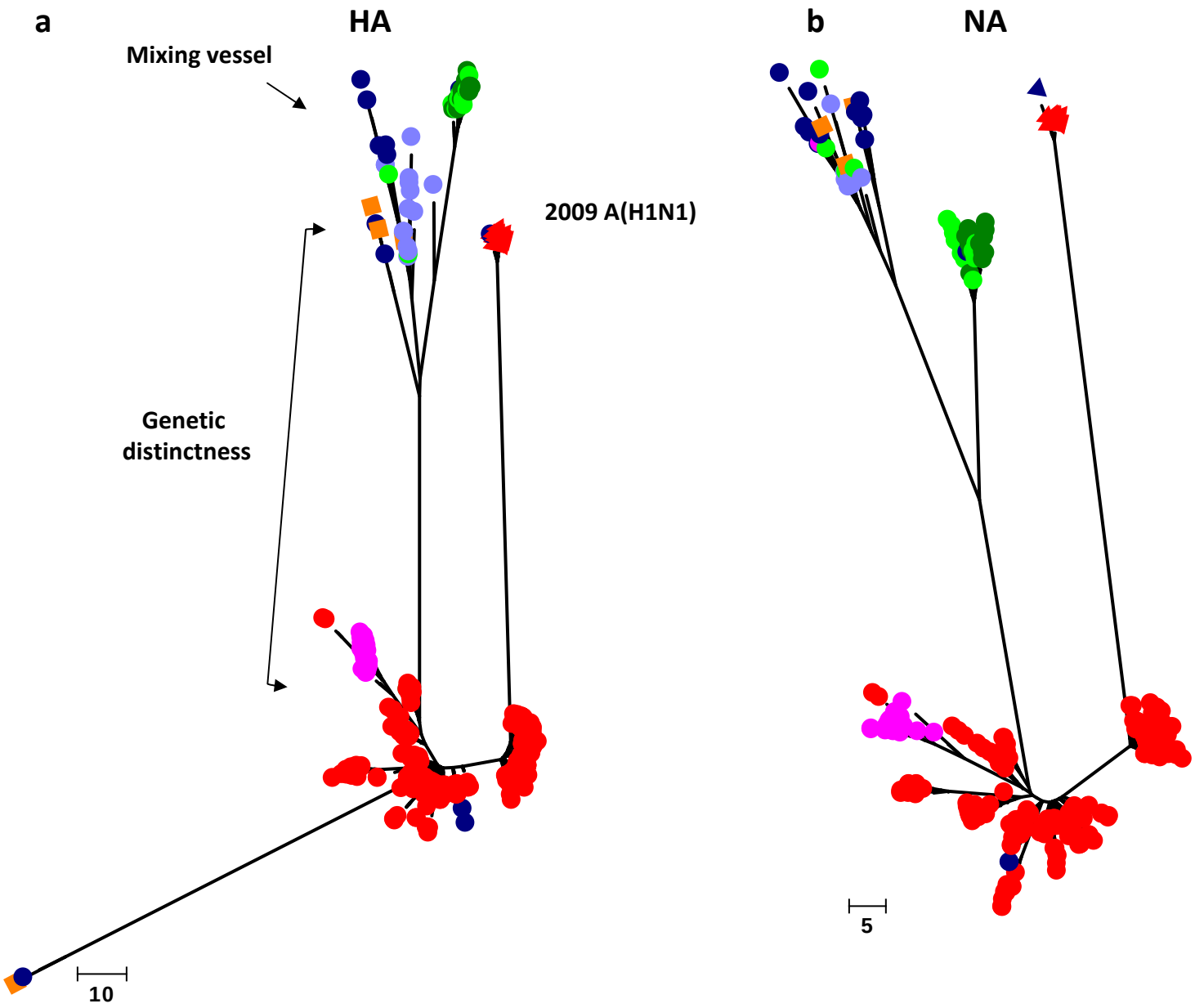
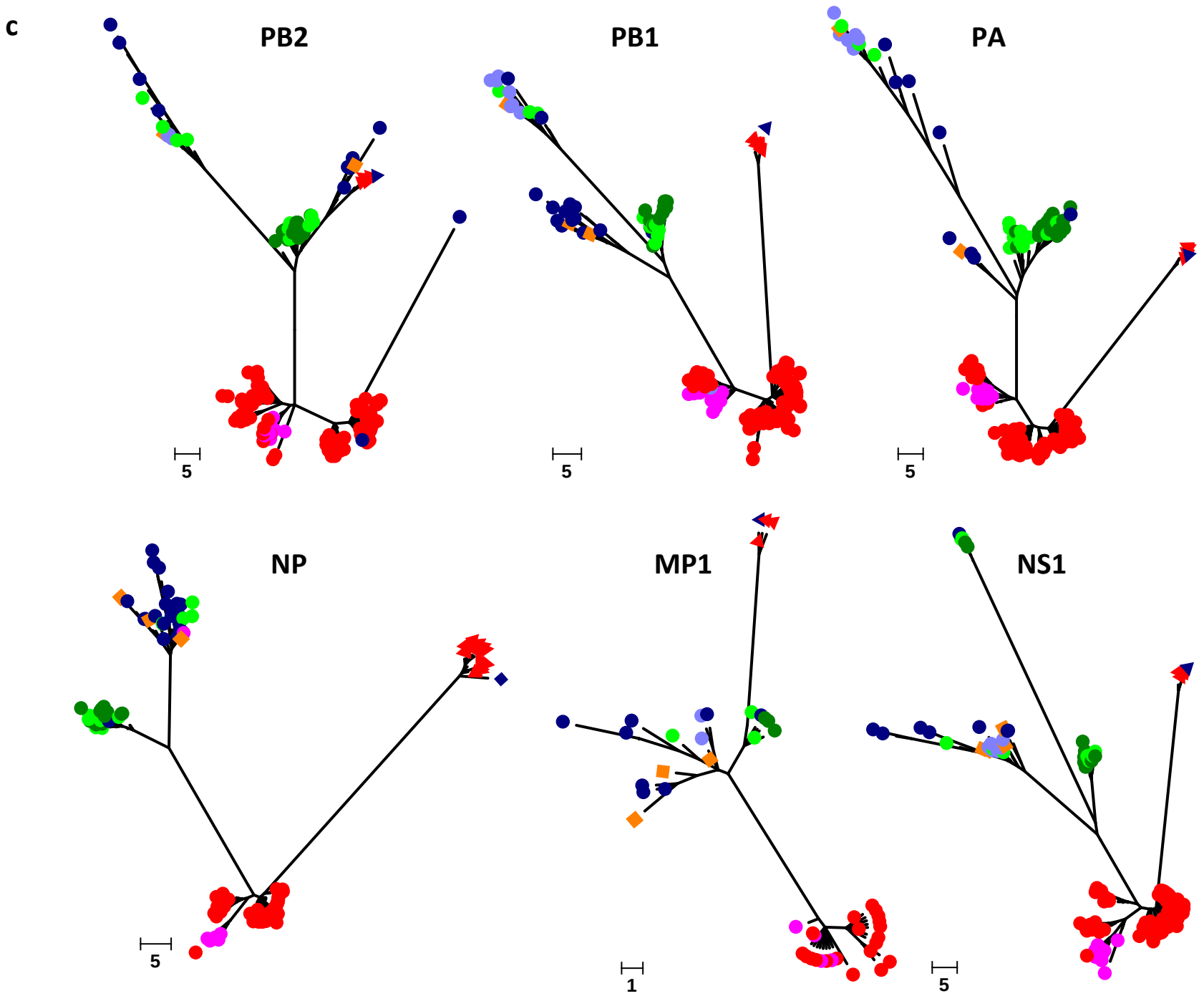
Figure 1. Possible origins of the influenza 2009 A(H1N1) virus: a) hemagglutinin and b) neuraminidase proteins



Protein sequences from the 2009 A(H1N1) virus were retrieved and used for BLAST searches versus the all-species NCBInr protein database. Top-fifty best hits were retrieved from GenBank and used for phylogenetic tree reconstruction using the maximum parsimony method. Phylogenetic trees were rooted using the earliest influenza virus found with the analysis. Proteins from the 2009 A(H1N1) virus (red circles) showed close homology to proteins from swine influenza viruses circulating in Asia, Europe and US (blue circles) and swine influenza viruses that have infected humans in recent past (red squares). Protein relationships with avian influenza virus (green circles) were more distant. Scale bar indicates the number of changes over the whole sequence. Phylogenetic trees for PB2, PB1, PA, NP, MP1, and NS1 proteins, and details of statistical significance of branch order are provided in Supplementary Materials - Figure 1.

Figure 2. Genetic distinctness of the influenza 2009 A(H1N1) virus: a) hemagglutinin (HA) and b) neuraminidase (NA) proteins; c) phylogenetic trees for PB2, PB1, PA, NP, MP1, and NS1 proteins




c
PB2
PB1
PA
NP
MP1
NS1
5
5
5
5
1
5

Legend:

a) and b)

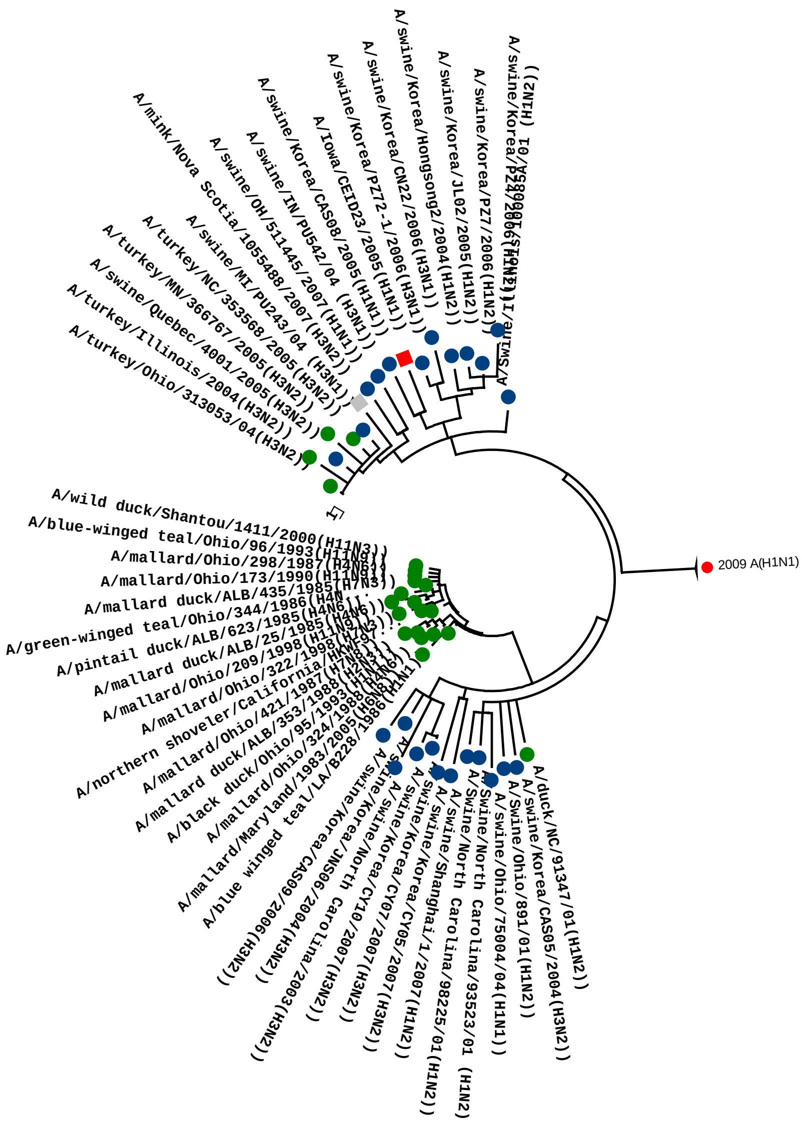
Protein sequences from avian, swine and human influenza A(H1N1) viruses circulating in North America from 1989 to 2009 were retrieved from the Influenza Virus Resource. Sequences were used for the reconstruction of unrooted phylogenetic trees with the maximum parsimony method. Proteins from the influenza 2009 A(H1N1) virus (red triangles), earlier human (red and pink circles) swine (navy blue and purple circles) and avian (green circles) viruses are shown. Light colors (pink, purple and green) correspond to viruses found between 1989 and 1999 and dark (red, navy blue and green) colors to viruses found between 2000 and 2009. Arrow indicates the influenza swine cluster containing an assortment of avian and human viruses. Genetic distinctness between pig-human interspecies transmission of influenza A viruses (orange squares; cases occurred in Iowa, Maryland and Wisconsin, US between 1991 and 2006) and the main cluster of human influenza viruses are indicated with the left bracket. Similar noticeable phylogenetic differences are observed between the influenza 2009 A(H1N1) virus and the main human influenza virus cluster. Scale bar indicates the number of changes over the whole sequence.

c)

Detailed phylogenetic tree topology, protein accession numbers, virus strains and serotypes, and statistical significance of branch order are presented in Supplementary Materials - Figure 1.

Supplementary Fig. 1. Possible origins of influenza 2009 A(H1N1) virus. **a**, PB2; **b**, PB1 and **c**, PA polymerases; **d**, hemagglutinin; **e**, nucleocapsid protein; **f**, neuraminidase; **g**, matrix protein 1; **h**, nonstructural protein 1. Protein sequences from the 2009 A(H1N1) virus were used for BLAST searches versus the all-species NCBInr protein database. Top fifty best hits were retrieved from GenBank and used for phylogenetic tree reconstruction using the maximum parsimony method. Phylogenetic trees were constructed with the maximum parsimony method using the MEGA software version 4.0 and rooted using the earliest influenza virus isolates obtained with the analyses. The statistical significance of branch order was estimated by the generation of 100 replications of bootstrap resampling of the originally-aligned amino acid sequences. Scale bar indicates the number of changes over the whole sequence.

a **PB2**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A/wild duck/Shantou/1411/2000(H11N3)
A/blue-winged teal/Ohio/96/1993(H11N9)
A/mallard/Ohio/298/1987(H4N6)
2009 A(H1N1)
A/blue winged teal/LA/B228/1986(H1N1)
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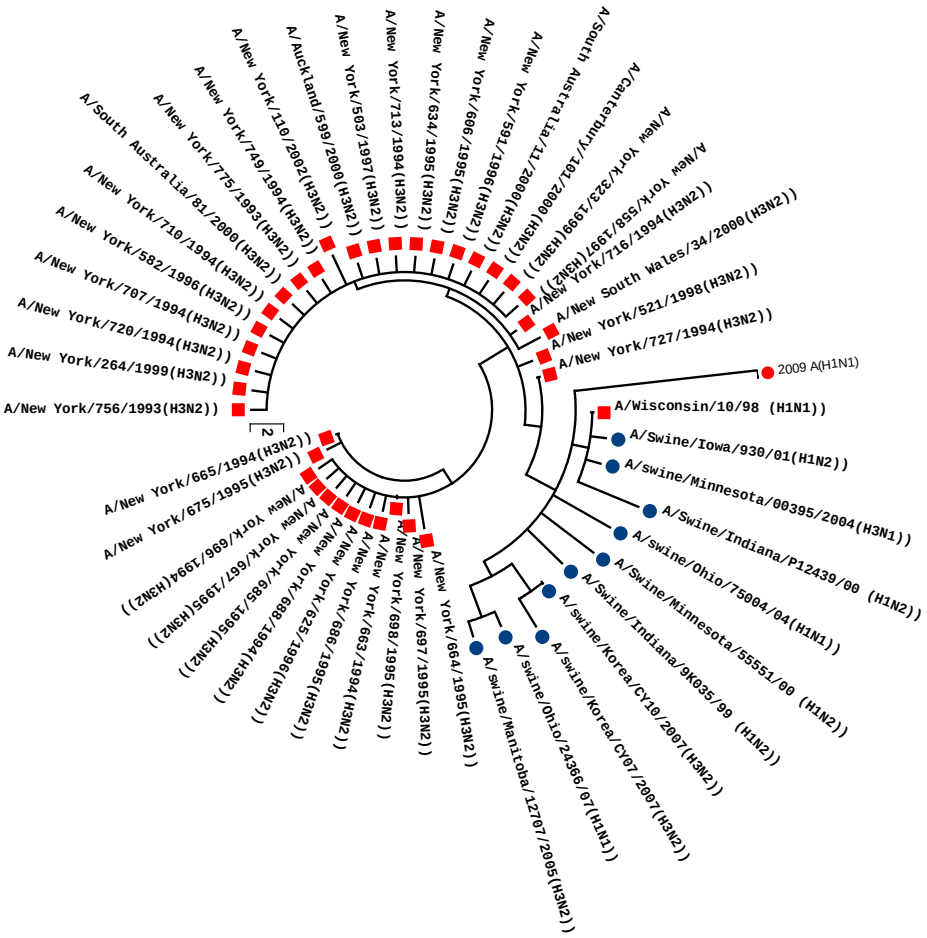
b **PB1**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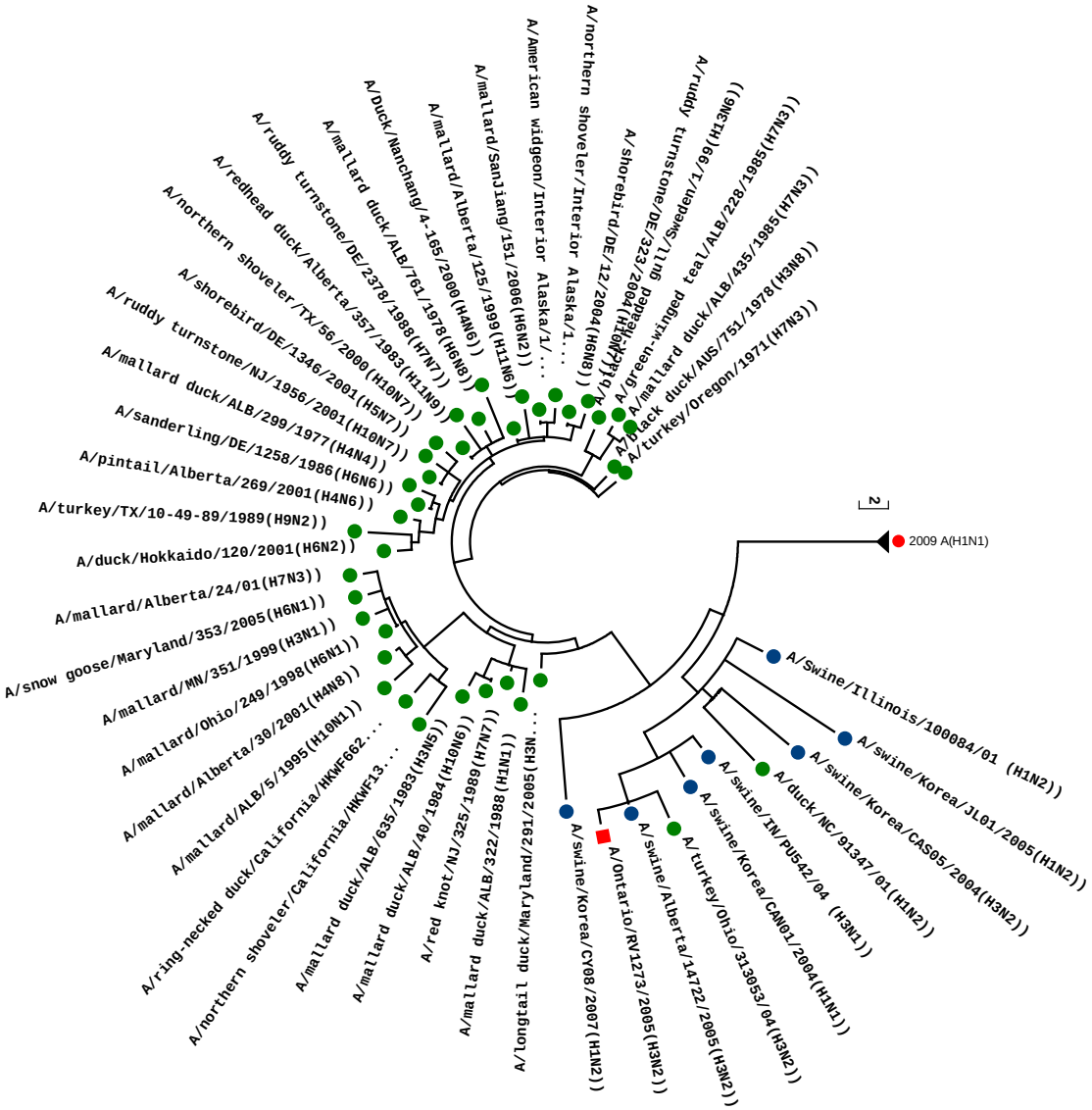
c **PA**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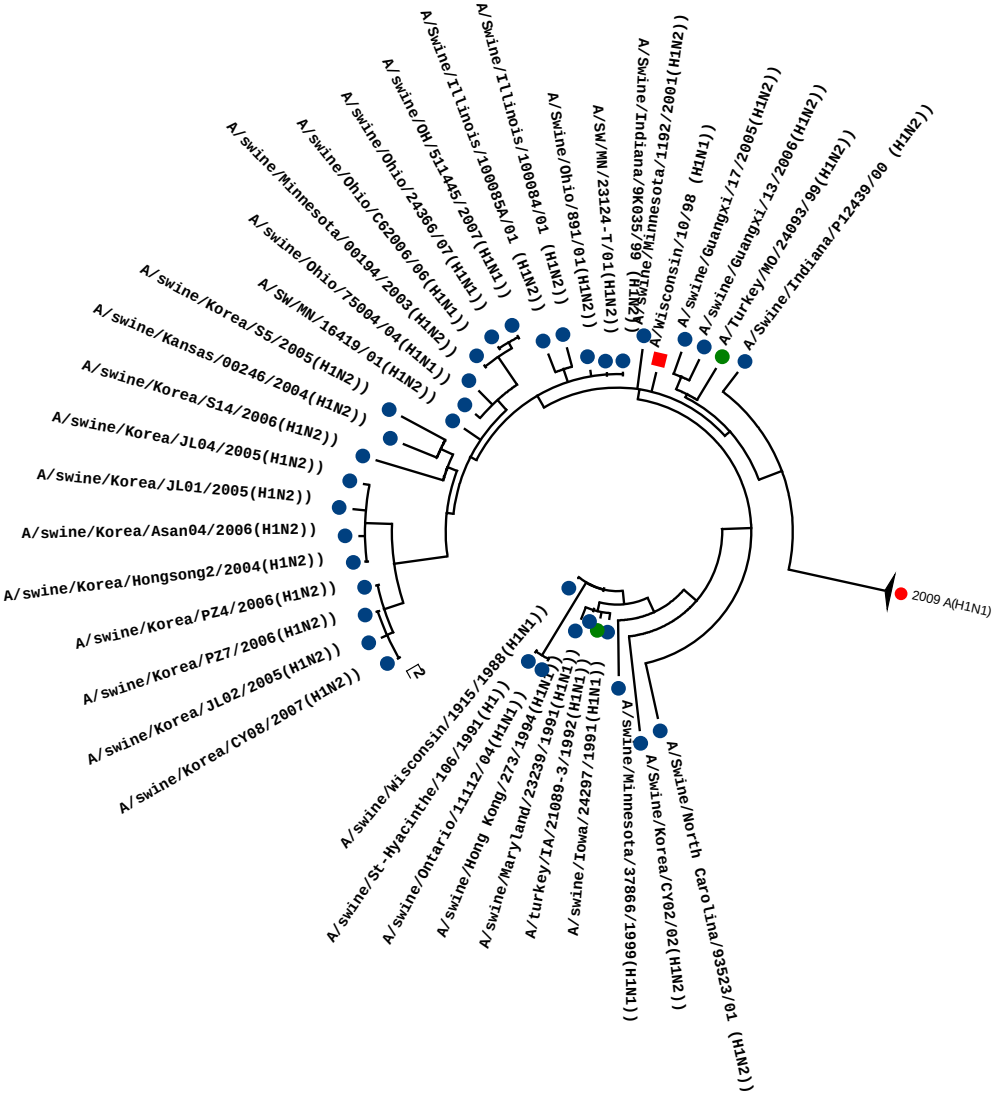
A/northern shoveler/Interior Alaska/1/...
2

d **HA**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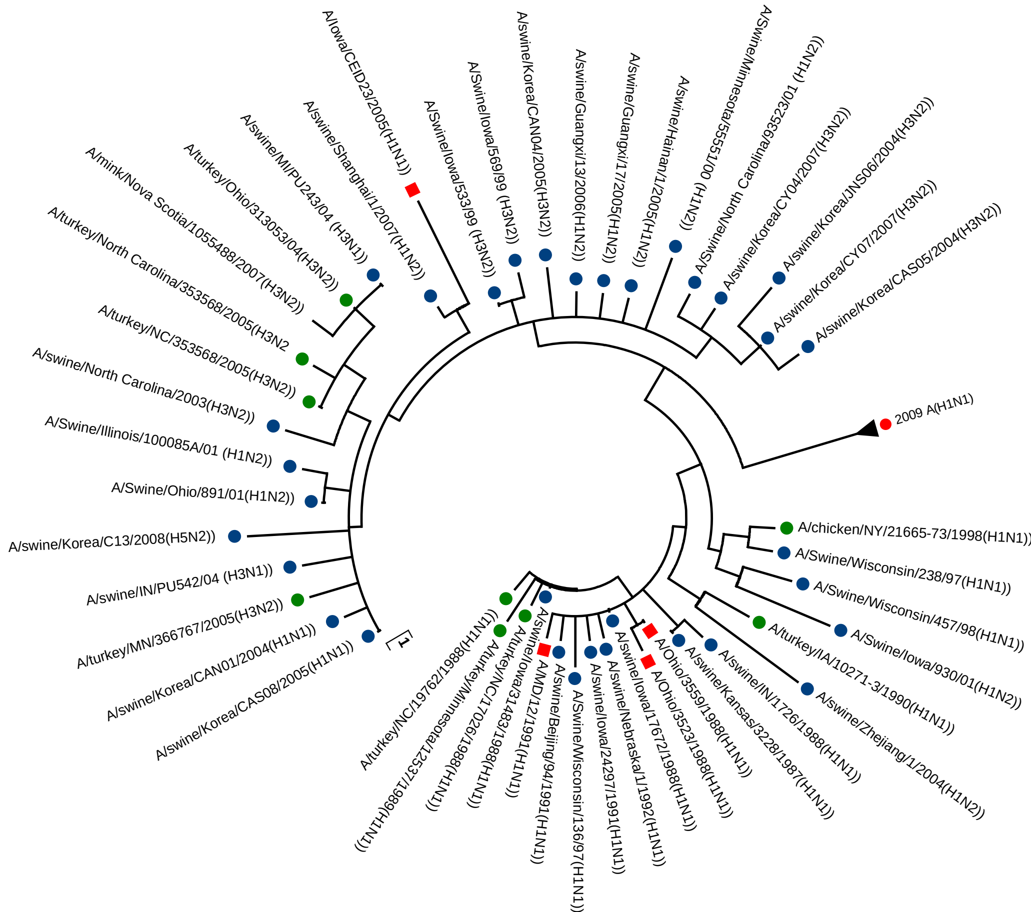
e **NP**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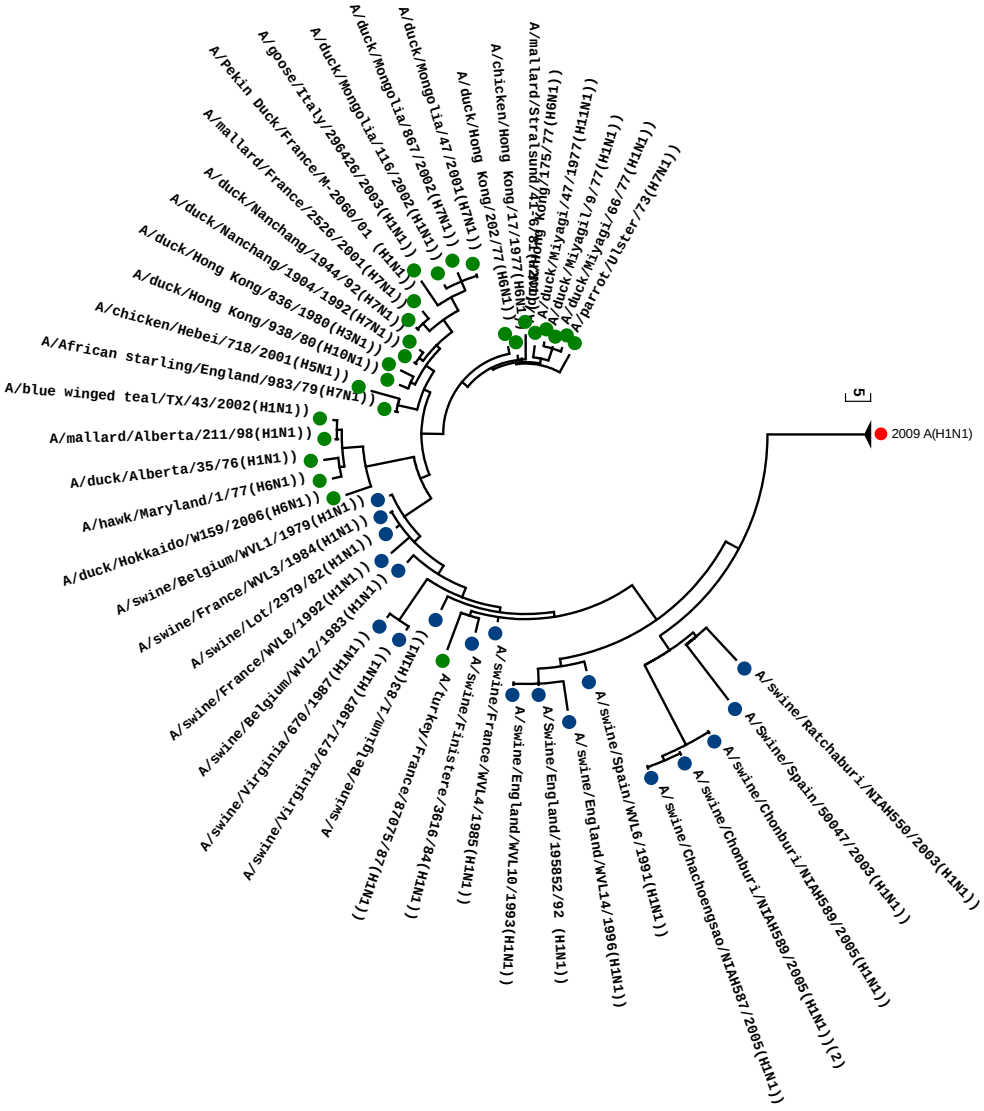
f **NA**


5
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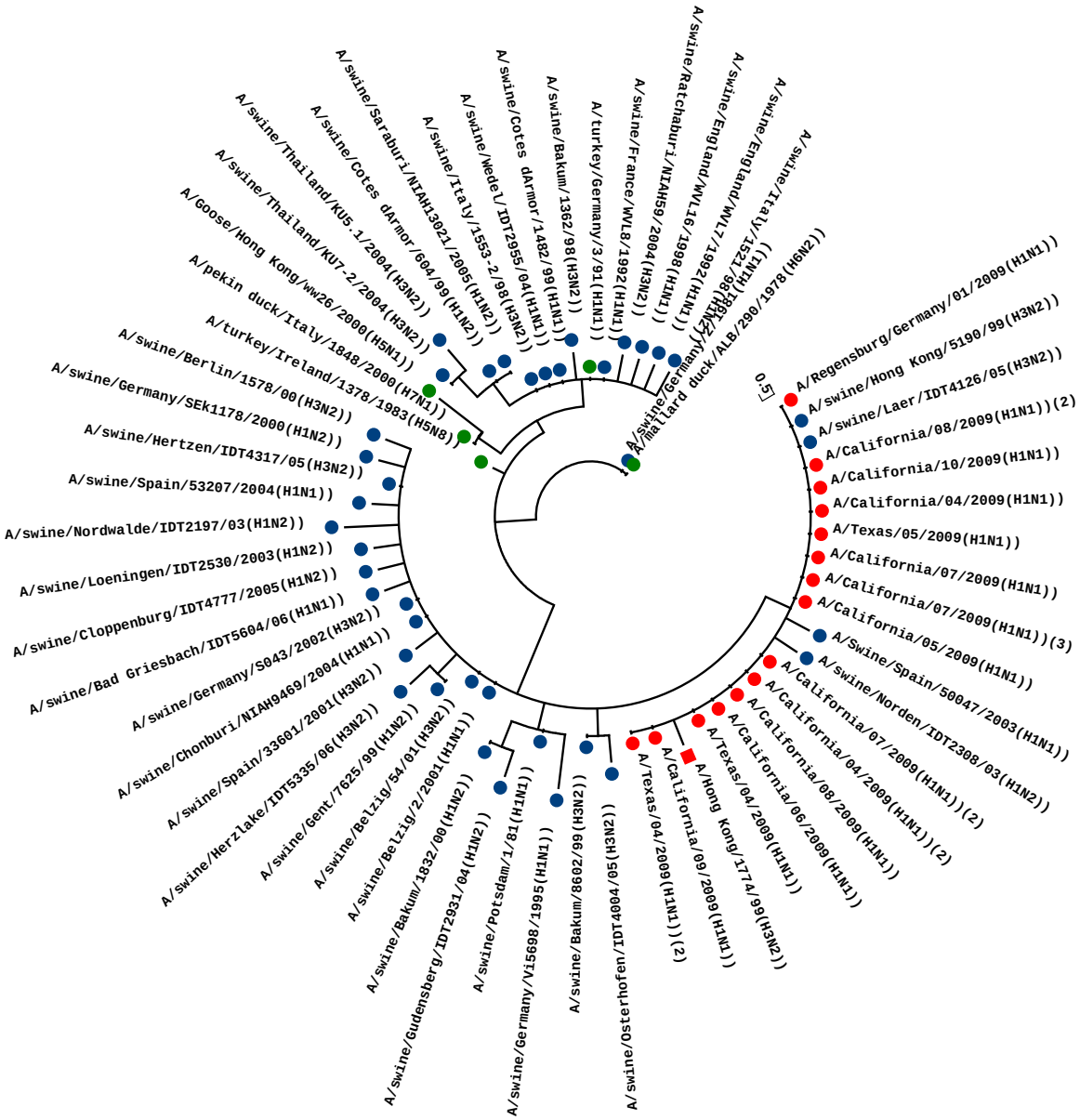
g **MP1**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A/Texas/04/2009(H1N1))
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A/Goose/Hong Kong/ww26/2000(H5N1))
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A/swine/France/WA18/1992(H1N1))
A/swine/Ratchaburi/NIAH5/2004(H3N2))
A/swine/England/WL16/1998(H1N1))
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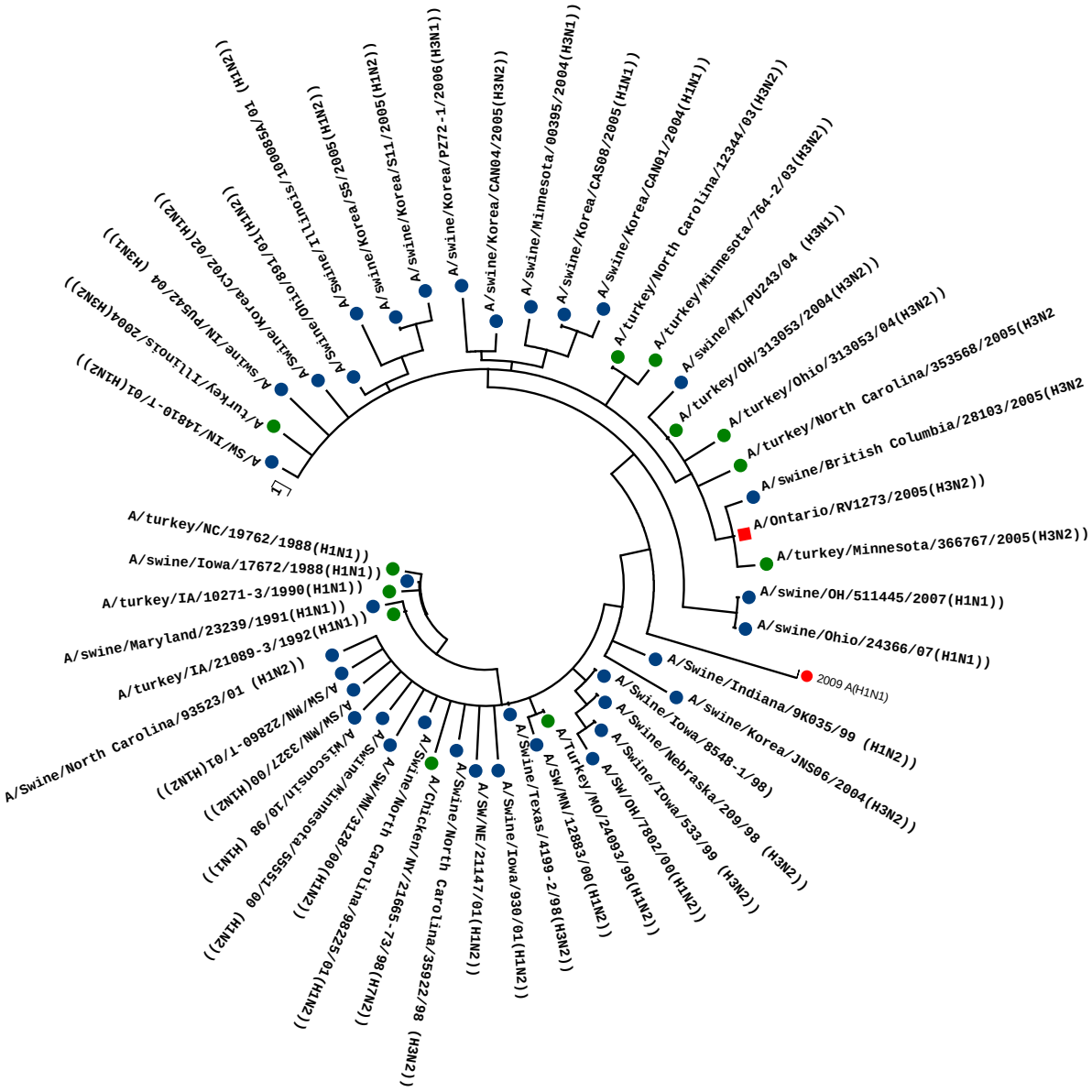
0.5

h **NS1**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A/Swine/Nebraska/209/98 (H3N2))
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A/SW/NE/21147/01(H1N2))
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A/turkey/NC/19762/1988(H1N1))
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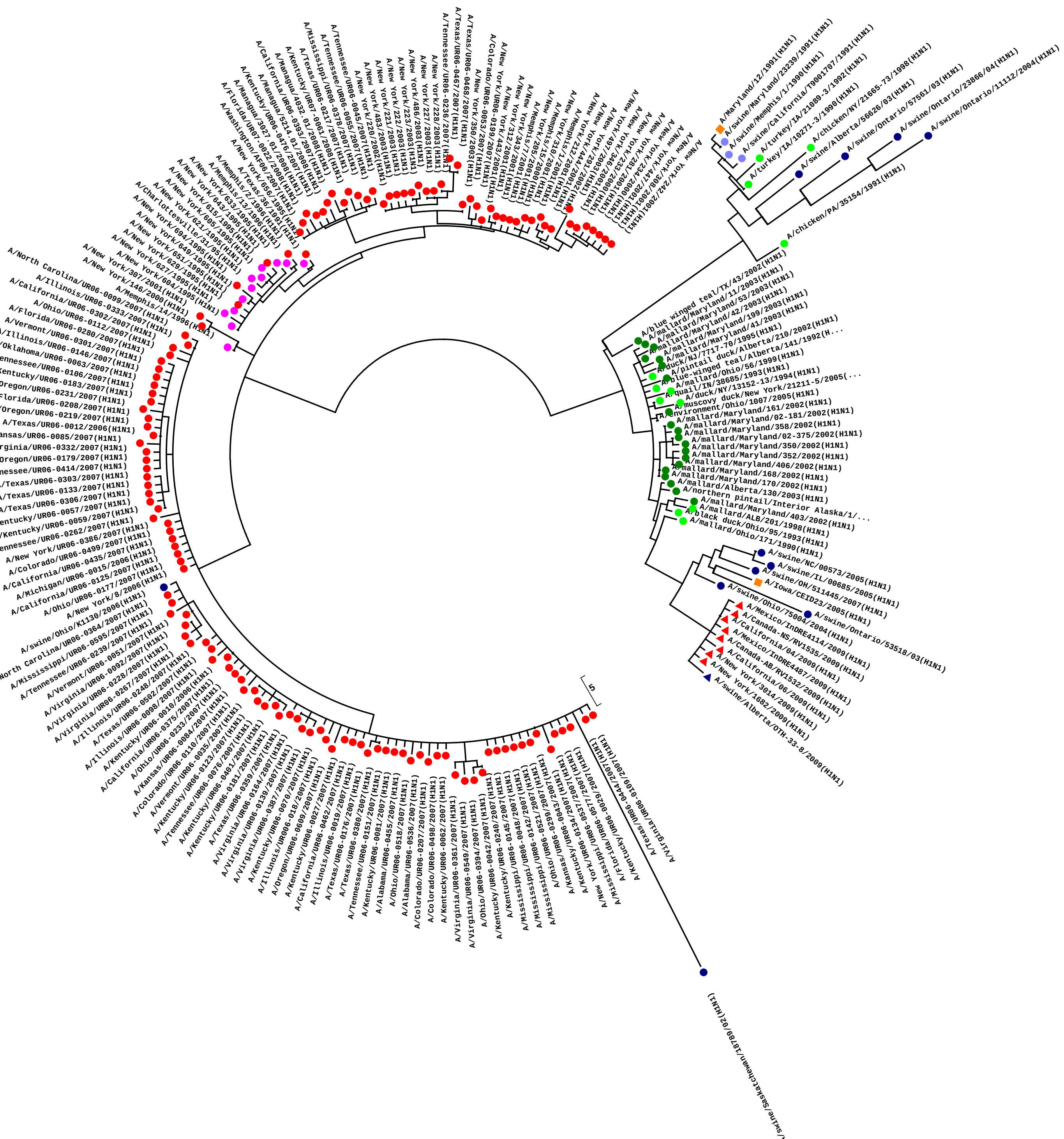
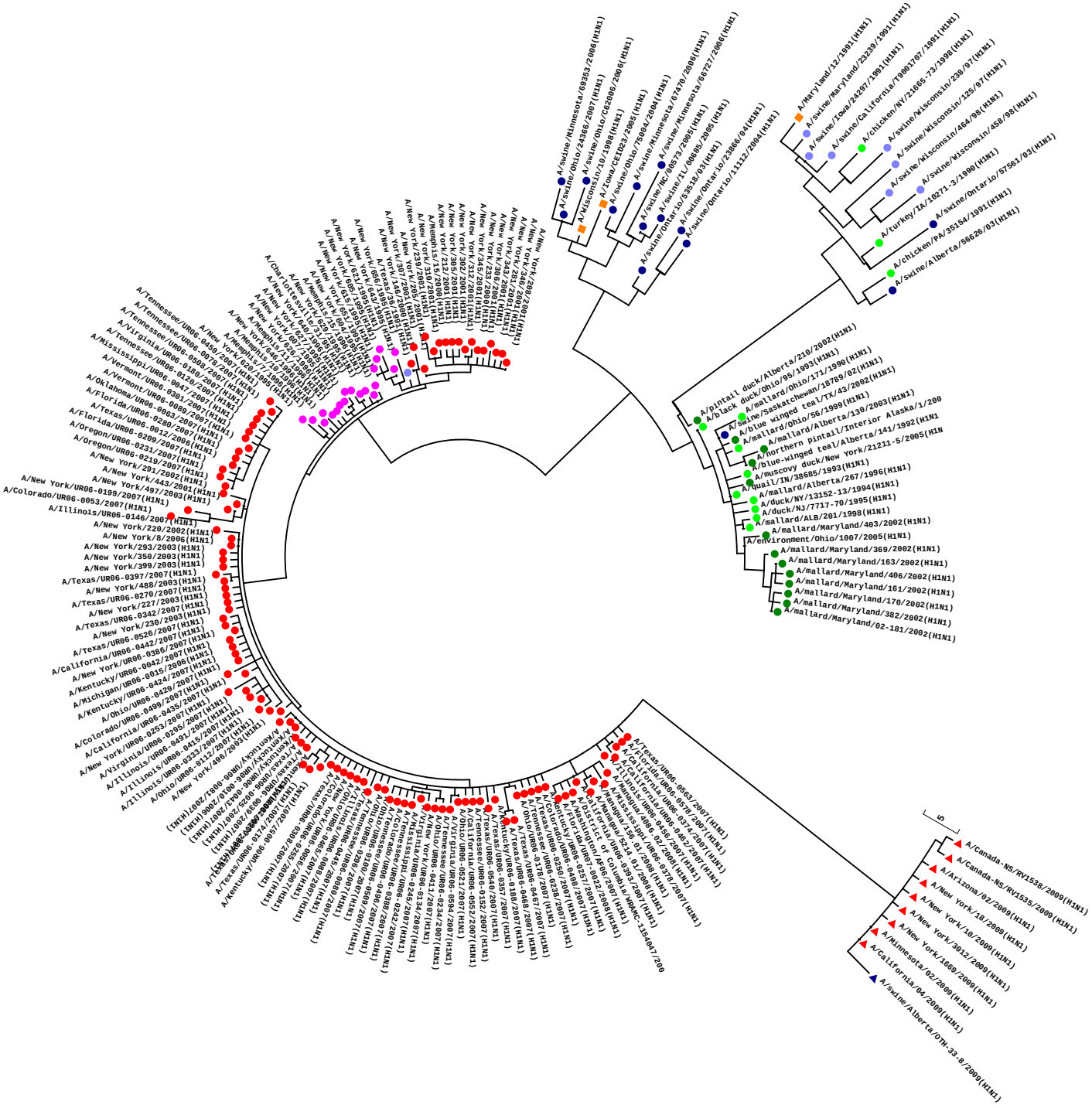
A/Swine/Korea/CY02/02(H1N2))
A/Swine/Ohio/891/01(H1N2))
A/Swine/Illinois/J0088A/01 (H1N2))
A/Swine/Korea/S1/2005(H1N2))
A/swine/Korea/S1/2005(H1N1))

Supplementary Fig. 2. Genetic distinctness of the influenza 2009 A(H1N1) virus. **a,** PB2; **b,** PB1 and **c,** PA polymerases; **d,** hemagglutinin (HA); **e**, nucleocapsid protein (NP); **f,** neuraminidase (NA); **g,** matrix protein 1 (MP1); **h,** nonstructural protein 1 (NS1). Protein sequences from avian, swine and human influenza A (H1N1) viruses circulating in North-America from 1989 to 2009 were retrieved from the Influenza Virus Resource. Sequences were used for unrooted phylogenetic tree construction with the maximum parsimony method. Proteins from the influenza 2009 A(H1N1) virus (red triangles), earlier human (red and pink circles) swine (navy blue and purple circles) and avian (green circles) viruses are shown. Light colors (pink, purple and green) correspond to viruses found between 1989 and 1999 and dark colors (red, navy blue and green) to viruses found between 2000 and 2009. Orange squares represent pig-human interspecies transmission of influenza A cases occurred in Iowa, Maryland and Wisconsin, USA between 1991 and 2006. Scale bar indicates the number of changes over the whole sequence. Phylogenetic trees were constructed with the MEGA software version 4.0. The statistical significance of branch order was estimated by the generation of 100 replications of bootstrap resampling of the originally-aligned amino acid sequences. Scale bar indicates the number of changes over the whole sequence.

a **PB2**



b **PB1**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A/chicken/NY/21665-73/1998(H1N1)
A/swine/Wisconsin/238/97(H1N1)
A/swine/Wisconsin/125/97(H1N1)
A/swine/Wisconsin/464/98(H1N1)
A/swine/Wisconsin/458/98(H1N1)
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A/turkey/IA/10271-3/1990(H1N1)
A/swine/Ontario/57561/03(H1N1)
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A/swine/Minnesota/67470/2006(H1N1)
A/swine/NC/08573/2005(H1N1)
A/swine/Minnesota/68727/2006(H1N1)
A/swine/IL/08635/2008(H1N1)
A/swine/Ontario/53518/03(H1N1)
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A/pintail duck/Alberta/210/2002(H1N1)
A/black duck/Ohio/95/1993(H1N1)
A/mallard/Ohio/171/1990(H1N1)
A/swine/Saskatchewan/18789/92(H1N1)
A/blue winged teal/TX/43/2002(H1N1)
A/mallard/Ohio/56/1999(H1N1)
A/mallard/Alberta/130/2003(H1N1)
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A/blue-winged teal/Alberta/1/1992(H1N1)
A/muscovy duck/New York/Z1211-5/2005(H1N
A/quail/IN/38685/1993(H1N1)
A/mallard/Alberta/267/1996(H1N1)
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A/duck/NJ/7717-70/1995(H1N1)
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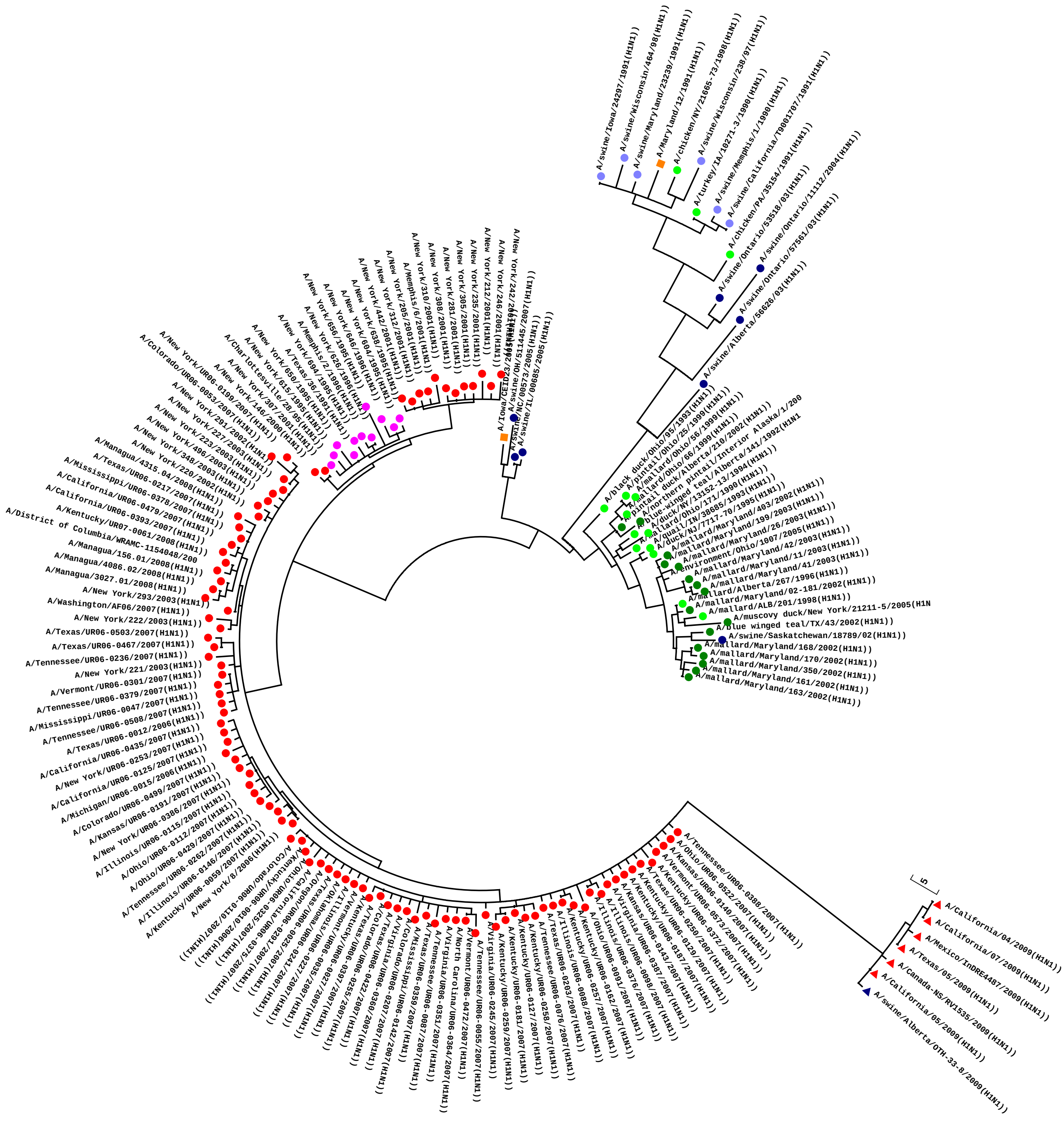
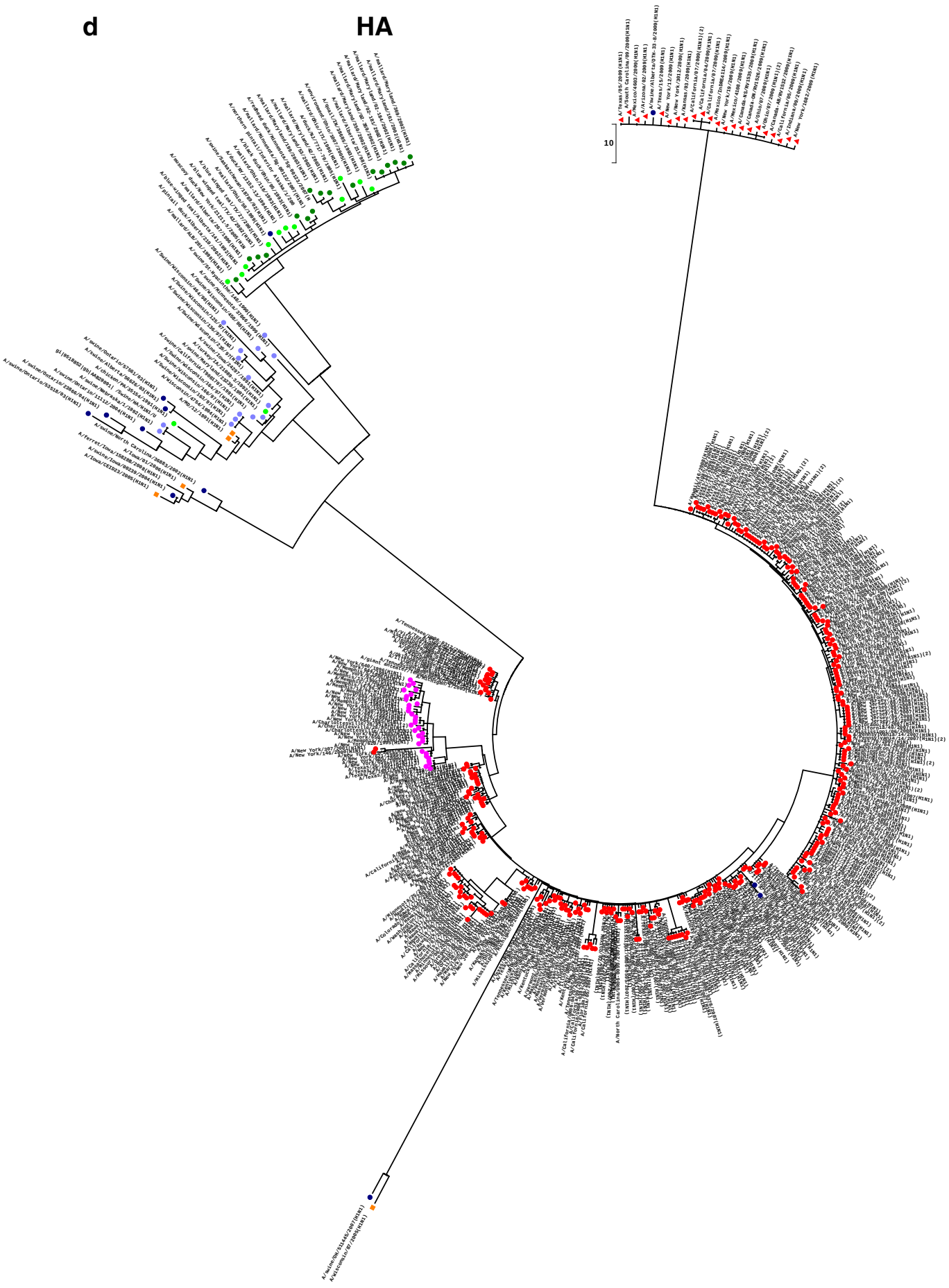
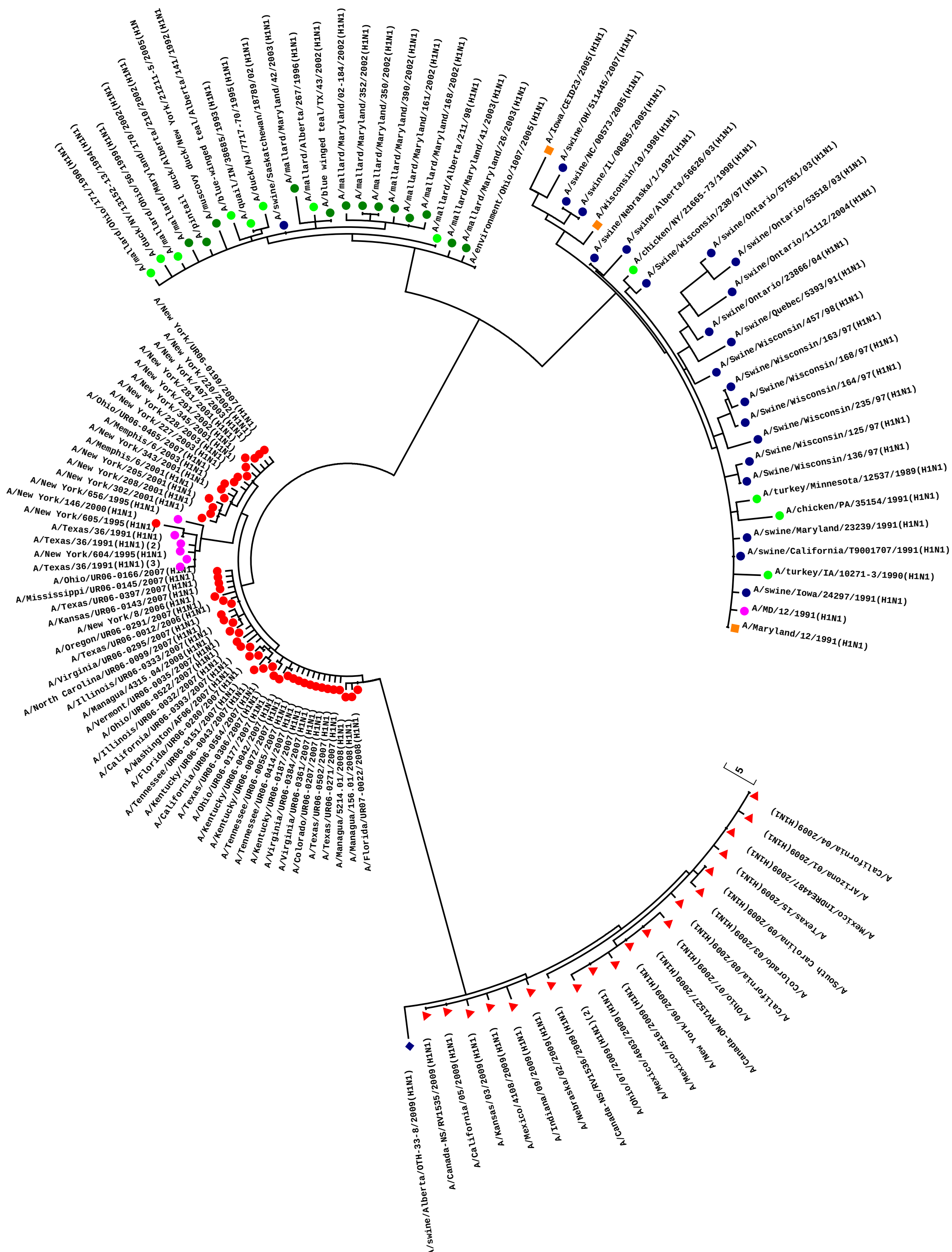
A/swine/Alberta/OTH-33-8/2009(H1N1)

c **PA**

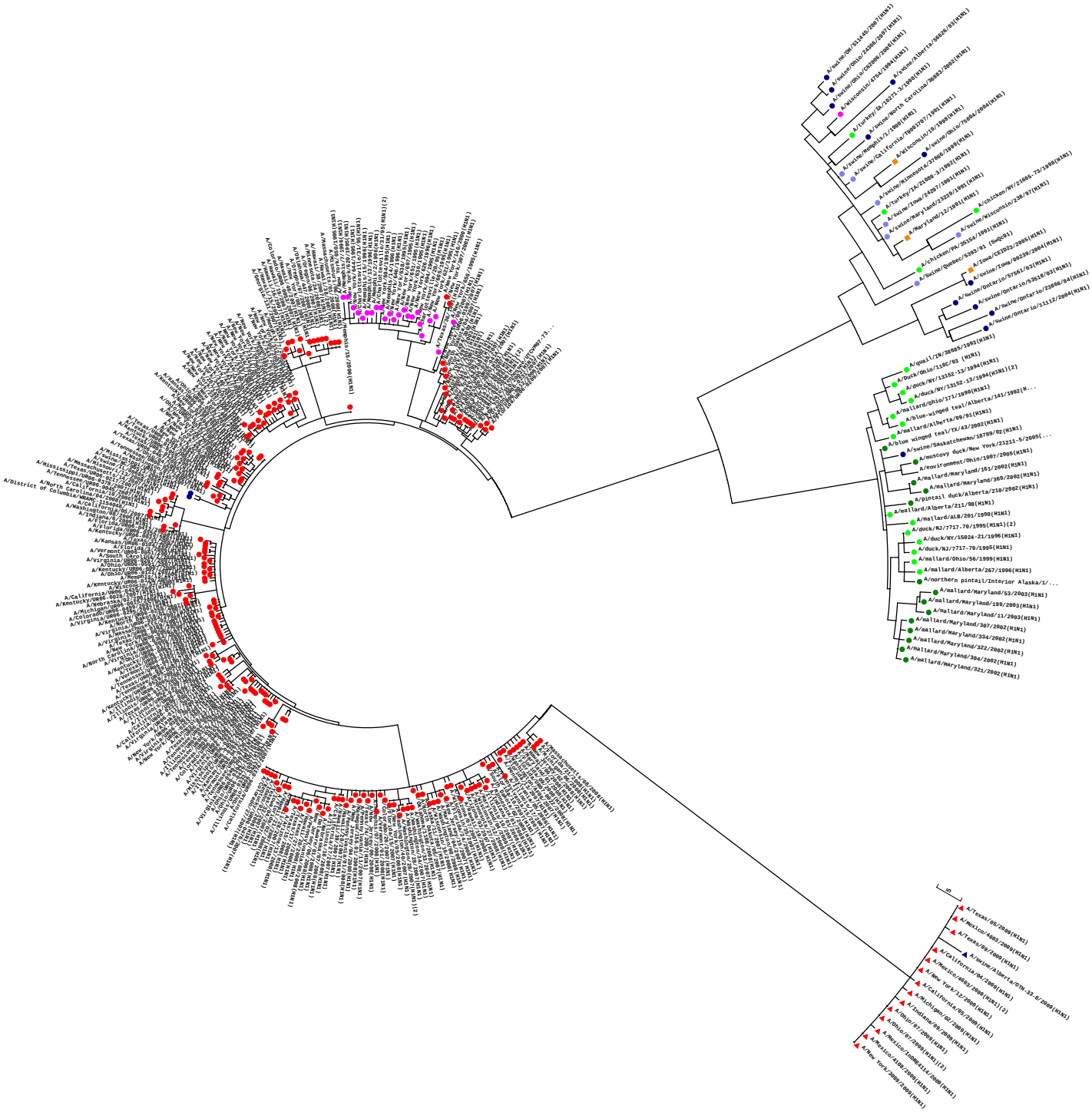



d
HA
10

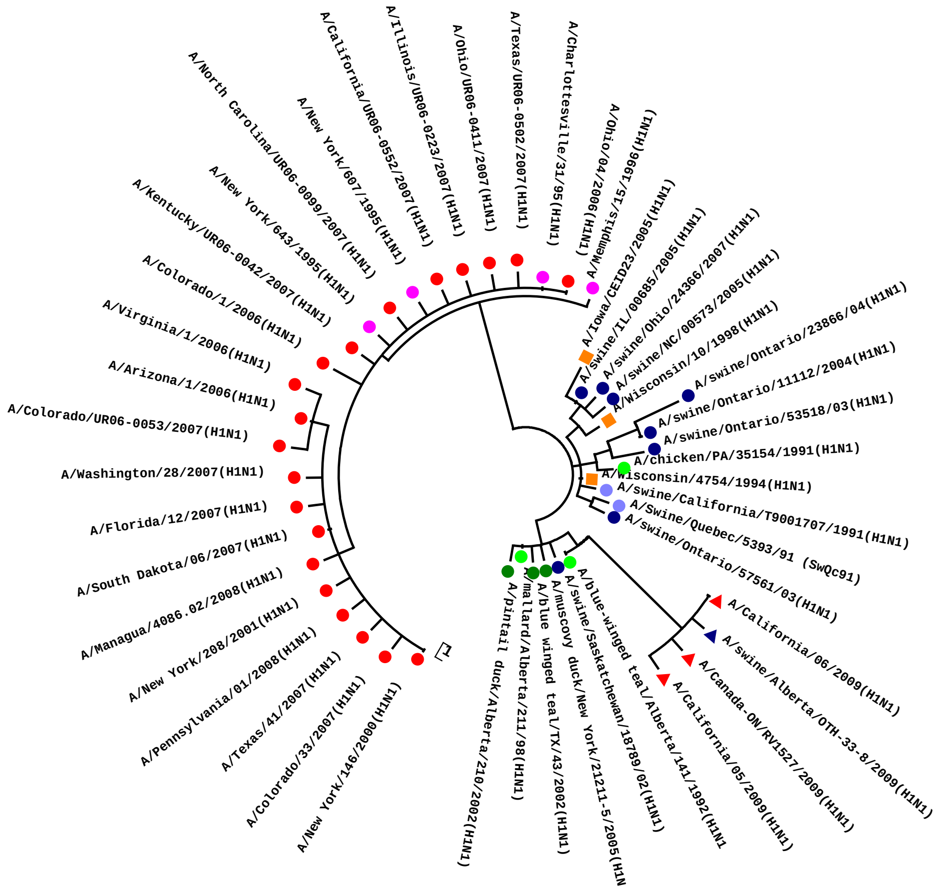
e **NP**



f **NA**



g **MP1**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A/California/UR06-0552/2007(H1N1)
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A/North Carolina/UR06-0099/2007(H1N1)
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A/Colorado/1/2006(H1N1)
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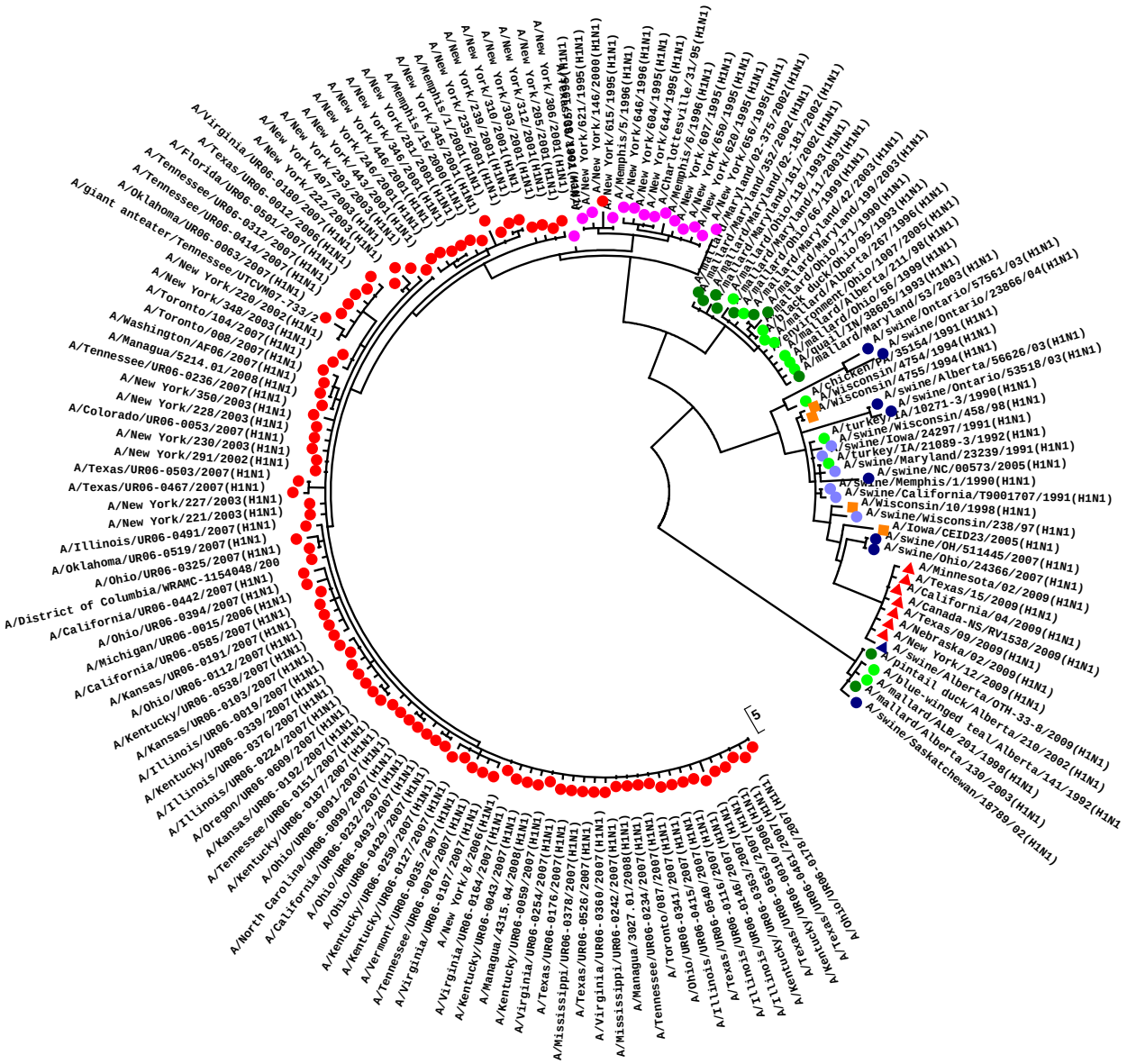
h **NS1**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A/New York/658/1995(H1N1)
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A/Maryland/Maryland/352/2002(H1N1)
A/Maryland/Maryland/02-181/2002(H1N1)
A/Maryland/Ohio/118/1993(H1N1)
A/Maryland/Ohio/66/1999(H1N1)
A/Maryland/Maryland/11/2003(H1N1)
A/Maryland/Maryland/42/2003(H1N1)
A/Maryland/Ohio/171/1990(H1N1)
A/Maryland/Ohio/95/1993(H1N1)
A/Maryland/Alberta/267/1996(H1N1)
A/Maryland/Ohio/1007/2006(H1N1)
A/Maryland/Alberta/211/98(H1N1)
A/Maryland/Ohio/66/1999(H1N1)
A/Maryland/38685/1993(H1N1)
A/Maryland/53/2003(H1N1)
A/swine/Ontario/57561/03(H1N1)
A/swine/Ontario/23866/04(H1N1)
A/swine/3515A/1991(H1N1)
A/Wisconsin/4754/1994(H1N1)
A/Wisconsin/4755/1994(H1N1)
A/swine/Alberta/56626/03(H1N1)
A/swine/Ontario/53518/03(H1N1)
A/swine/IA/10271-3/1990(H1N1)
A/turkey/IA/21089-3/1992(H1N1)
A/swine/Iowa/24297/1991(H1N1)
A/turkey/Wisconsin/458/98(H1N1)
A/swine/Maryland/23239/1991(H1N1)
A/swine/NC/00573/2005(H1N1)
A/Swine/Memphis/1/1990(H1N1)
A/Swine/California/T9001707/1991(H1N1)
A/Wisconsin/10/1998(H1N1)
A/swine/Wisconsin/238/97(H1N1)
A/Iowa/CEID23/2005(H1N1)
A/swine/OH/511445/2007(H1N1)
A/swine/Ohio/24366/2007(H1N1)
A/Minnesota/02/2009(H1N1)
A/Texas/15/2009(H1N1)
A/California/04/2009(H1N1)
A/Canada-NS/RV1538/2009(H1N1)
A/Texas/09/2009(H1N1)
A/Nebraska/02/2009(H1N1)
A/New York/12/2009(H1N1)
A/Swine/Alberta/OTH-33-8/2009(H1N1)
A/pintail duck/Alberta/210/2002(H1N1)
A/blue-winged teal/Alberta/141/1992(H1N1)
A/mallard/ALB/201/1998(H1N1)
A/swine/Saskatchewan/18789/02(H1N1)
A/Ohio/UR06-0178/2007(H1N1)
A/Texas/UR06-0463/2007(H1N1)
A/Kentucky/UR06-0610/2007(H1N1)
A/Illinois/UR06-0563/2007(H1N1)
A/Texas/UR06-0415/2007(H1N1)
A/Illinois/UR06-0516/2007(H1N1)
A/Ohio/UR06-0341/2007(H1N1)
A/Tennessee/UR06-0234/2007(H1N1)
A/Toronto/087/2007(H1N1)
A/Managua/3027.01/2008(H1N1)
A/Virginia/UR06-0360/2007(H1N1)
A/Mississippi/UR06-0242/2007(H1N1)
A/Texas/UR06-0526/2007(H1N1)
A/Mississippi/UR06-0378/2007(H1N1)
A/Texas/UR06-0176/2007(H1N1)
A/Kentucky/UR06-0059/2007(H1N1)
A/Virginia/UR06-0254/2007(H1N1)
A/Kentucky/UR06-0043/2007(H1N1)
A/Managua/A315.04/2008(H1N1)
A/Kentucky/UR06-0164/2007(H1N1)
A/Virginia/UR06-0107/2007(H1N1)
A/New York/8/2006(H1N1)
A/Kentucky/UR06-0232/2007(H1N1)
A/Kentucky/UR06-0429/2007(H1N1)
A/Vermont/UR06-0127/2007(H1N1)
A/Tennessee/UR06-0035/2007(H1N1)
A/Virginia/UR06-0078/2007(H1N1)
A/North Carolina/UR06-0099/2007(H1N1)
A/California/UR06-0187/2007(H1N1)
A/Ohio/UR06-0151/2007(H1N1)
A/Kentucky/UR06-0891/2007(H1N1)
A/Kansas/UR06-0192/2007(H1N1)
A/Oregon/UR06-0609/2007(H1N1)
A/Illinois/UR06-0224/2007(H1N1)
A/Kentucky/UR06-0376/2007(H1N1)
A/Illinois/UR06-0339/2007(H1N1)
A/Kansas/UR06-0019/2007(H1N1)
A/Kentucky/UR06-0103/2007(H1N1)
A/Ohio/UR06-0538/2007(H1N1)
A/Kansas/UR06-0112/2007(H1N1)
A/California/UR06-0191/2007(H1N1)
A/Michigan/UR06-0585/2007(H1N1)
A/Ohio/UR06-0915/2006(H1N1)
A/California/UR06-0394/2007(H1N1)
A/California/UR06-0442/2007(H1N1)
A/District of Columbia/WRAMC-1154048/200
A/Ohio/UR06-0325/2007(H1N1)
A/Oklahoma/UR06-0519/2007(H1N1)
A/Illinois/UR06-0491/2007(H1N1)
A/New York/221/2003(H1N1)
A/New York/227/2003(H1N1)
A/Texas/UR06-0467/2007(H1N1)
A/Texas/UR06-0503/2007(H1N1)
A/New York/291/2002(H1N1)
A/New York/230/2003(H1N1)
A/Colorado/UR06-0053/2007(H1N1)
A/New York/228/2003(H1N1)
A/New York/350/2003(H1N1)
A/Tennessee/UR06-0236/2007(H1N1)
A/Managua/5214.01/2008(H1N1)
A/Washington/AF06/2007(H1N1)
A/Toronto/008/2007(H1N1)
A/Toronto/104/2007(H1N1)
A/New York/348/2003(H1N1)
A/New York/220/2002(H1N1)
A/giant anteater/Tennessee/UTCVM07-733/2
A/Tennessee/UR06-0063/2007(H1N1)
A/Oklahoma/UR06-0414/2007(H1N1)
A/Tennessee/UR06-0312/2006(H1N1)
A/Florida/UR06-0501/2007(H1N1)
A/Texas/UR06-0180/2007(H1N1)
A/Virginia/UR06-0012/2006(H1N1)
A/New York/497/2003(H1N1)
A/New York/222/2003(H1N1)
A/New York/293/2003(H1N1)
A/New York/443/2001(H1N1)
A/New York/246/2001(H1N1)
A/New York/346/2001(H1N1)
A/Memphis/282/2001(H1N1)
A/Memphis/15/2001(H1N1)
A/New York/345/2001(H1N1)
A/New York/235/2001(H1N1)
A/New York/239/2001(H1N1)
A/New York/310/2001(H1N1)
A/New York/303/2001(H1N1)
A/New York/312/2001(H1N1)
A/New York/295/2001(H1N1)
A/New York/306/2001(H1N1)
A/New York/146/2000(H1N1)
A/New York/621/1995(H1N1)

Supplementary table 1. Closest protein homology of influenza 2009 A(H1N1) viruses

Gene	Best Hit	Identity	Lineage
PB2	A/Swine/Illinois/100085A/01 (H1N2)	98%	Swine
PB1	A/Wisconsin/10/98 (H1N1)	98%	Human
PA	A/Swine/Illinois/100084/01 (H1N2)	98%	Swine
HA	A/Swine/Indiana/P12439/00 (H1N2)	95%	Swine
NP	A/swine/Guangxi/13/2006 (H1N2)	98%	Swine
NA	A/swine/Spain/WVL6/1991 (H1N1)	94%	Swine
MP1	A/swine/Laer/IDT4126/05 (H3N2)	99%	Swine
NS1	A/SW/IN/14810-T/01 (H1N2)	94%	Swine

Supplementary table 2. Closest protein homology of influenza 2009 A(H1N1) viruses with swine influenza viruses that have infected humans

Protein	Cases	Identity	Lineage	Reference
PB2	A/Iowa/CEID23/2005(H1N1)	98%	Human	[1]
PB1	A/New York/727/1994(H3N2)	98%	Human	ABG48024
PA	A/Ontario/RV1273/2005(H3N2)	97%	Human	[2]
HA	A/Wisconsin/10/98 (H1N1)	93%	Human	AAO88265
NP	A/Iowa/CEID23/2005(H1N1)	97%	Human	[1]
	A/MD/12/1991(H1N1)	98%	Human	AAA51491
	A/Ohio/3559/1988(H1N1)	98%	Human	ABU80404
	A/Ohio/3523/1988(H1N1)	97%	Human	AAA73104
NA	Novel protein	Nf	Nf	Nf
MP1	A/Hong Kong/1774/99(H3N2)	99%	Human	[3]
NS1	/Wisconsin/10/1998(H1N1)	93%	Human	AAO88260
	A/Ontario/RV1273/2005(H3N2)	94%	Human	[2]

Nf = Not found

Supplementary table 3. Reported cases of pig-human interspecies transmission of influenza A (H1N1) occurred in Iowa, Maryland and Wisconsin, USA between 1991 and 2006. These influenza virus subtypes possess genetic distinctness compared to main cluster of human influenza A (H1N1) viruses

Influenza A virus subtype	Evidence linking pig-human infection	Reference
A/Iowa/CEID23/2005 (H1N1)	Yes	[1]
A/Wisconsin/10/1998	Insufficient data	[4]
A/Wisconsin/4754/1994	Yes	[5]
A/Maryland/12/1991	Yes	[5]
A/MD/12/1991	Yes	[5]
A/Wisconsin/4755/1994	Yes	[5]
A/Wisconsin/87/2005	Yes	[6]
A/Iowa/01/2006	Yes	[6]

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