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Full Length Research Paper

Evolutionary characteristics of swine-origin H1N1 influenza virus that infected humans from sporadic to pandemic

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Evolutionary process of swine-origin H1N1 influenza A viruses that infected humans from sporadic to pandemic is of high epidemiological significance but still remains obscure. To understand this process, we performed phylogenetic, bootscan, and adaptive evolution analyses using the sequences of the 8 gene segments from swine-origin H1N1 influenza A viruses that infected humans and the reference viruses. Classic swine H1N1 viruses occasionally infected humans before 1998. Sporadic human infection with the triple-reassortant swine-origin H1N1 viruses was firstly identified in 1998 and has become increasingly frequent since 2005. Except genes encoding the neuraminidase and matrix protein of swine influenza viruses of Eurasian lineage, other 6 genes of A/H1N1/2009 pandemic strain were most closely linked to those of A/Iowa/CEID23/2005(H1N1), a representative swine-origin triple-reassortant virus that infected humans sporadically. Potential positive selections acting on the haemagglutinin gene evolved from classic swine H1N1 viruses to the triple-reassortant H1N1 viruses and on the neuraminidase gene evolved from Eurasian swine viruses to A/H1N1/2009 pandemic viruses might play a role in cross-species transmission and human infection. Surveillance of genetic evolution of influenza A viruses in swine workers might provide useful clues of influenza pandemic.

Key words: Swine, H1N1 influenza virus, evolution, sporadic, pandemic.

INTRODUCTION

Classic swine influenza A viruses cause sporadic human infection via animals to human transmission, especially in swine workers (Myers et al., 2007; Olsen et al., 2002). A swine influenza virus was firstly isolated from autopsy lung tissue of human in 1974 (Smith et al., 1976). Like the infections with avian influenza A viruses, sporadic cases of humans infected with classical swine influenza A viruses in recent decades have rarely resulted in human-to-human spread. Pigs have receptors to swine, avian, and human influenza virus strains and act as a “mixing vessel” in which genetic material of the viruses can be exchanged (Ito et al., 1998). The reassortment of swine, avian, and human influenza viruses in swine possibly results in interspecies transmission of influenza and generation of novel progeny viruses to which humans are immunologically naive and highly susceptible (Scholtissek et al., 1985; Webster et al., 1992). The emergence of pandemic H1N1/2009 influenza demonstrated that pandemic viruses could be generated in pigs.

The novel H1N1/2009, triple-reassortant swine-origin influenza A virus, shows a strong ability to transmit from human to human and has caused influenza A pandemic worldwide since its first emergence in Mexico in March 2009 (MMWR Morb Mortal Weekly Report, 2009). This novel virus contains 8 gene segments encoding haemagglutinin (*HA*), nucleoprotein (*NP*), and nonstructural protein (*NS*) from classic swine influenza A virus of North American lineage, the polymerase basic 2 (*PB2*) and the polymerase acidic (*PA*) from avian influenza of North American lineage, the polymerase basic 1 (*PB1*) from human seasonal influenza A H3N2, and neuraminidase

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(*NA*) and matrix protein (*MP*) from swine influenza A of Eurasian lineage (Babakir-Mina et al., 2009; Dawood et al., 2009; Garten et al., 2009; Lu et al., 2009). The reassortment of swine lineages may have occurred years before emergence in humans. However, the nature and location of the genetically closest swine viruses reveal little about the immediate origin of the epidemic (Smith et al., 2009).

Between the 1930s and the 1990s, classic swine H1N1 influenza A underwent little change. However, by the late 1990s, multiple strains and subtypes (H1N1, H3N2, and H1N2) of triple-reassortant swine influenza A (H1) viruses whose genomes included combinations of avian, swine, and human influenza virus gene segments had emerged in pigs (Olsen, 2002). Since 2005, the triple-reassortant swine-origin H1N1 influenza viruses have been frequently reported to be able to infect humans sporadically (Gray et al., 2007; Newman et al., 2008; Shinde et al., 2009). In this study, we retrieved the representative DNA sequences of swine-origin H1N1 influenza A viruses that infected humans and reference viruses from GenBank and analyzed evolutionary relationships using phylogenetic analysis, bootscan analysis, and adaptive evolution analysis. The reassortment of swine-origin H1N1 with avian influenza virus of North America lineage and human seasonal influenza virus before 2005 in North America was found to be an important step during evolutionary process of A/H1N1/2009 pandemic virus.

EXPERIMENTAL

Searching for the sequences of influenza A viruses

The genome sequences of the novel A/H1N1/2009 pandemic viruses were downloaded from the NCBI Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). We searched the PubMed database up to March 2009, the time point before the outbreak of novel influenza A viruses, using the searching terms “swine”, “influenza”, and “human”, to identify articles describing sporadic cases of human infection thought to be caused by swine influenza virus strains. Reference lists from the articles selected by electronic searching were searched to identify further relevant articles. The 8 gene segments of the corresponding viruses were retrieved from GenBank. The sequences of reference influenza A viruses sampled from human, avian and swine in North America, Europe and Asia during the period 1918 to 2009 were also retrieved from GenBank, respectively.

Phylogenetic analysis

Sequences of the 8 gene segments of the reference viruses, those of all available swine-origin H1N1 influenza A viruses that infected humans sporadically in North America, and those of selected pandemic A/H1N1/2009 viruses were used for phylogenetic analysis, respectively. Sequence alignments for the gene segments of *PB2* (full-length 2,281 bp; partial 1,573 bp), *PB1* (2,274 bp), *PA* (full-length 2,151 bp; partial 1,470 bp), *HA* (1,701 bp), *NP* (1,497 bp), *NA* (1,410 bp), *MP* (982 bp), and *NS* (842 bp) and phylogenetic analysis were performed using MEGA 4.0 software package (Tamura et al., 2007). We deleted some of the phylogenetically closely related sequences from reference influenza viruses isolated in the same years and locations and then construct phylogenetic tree. A bootstrap resampling process (1000 replicates) using the neighbor-joining (NJ) method was used to assess the robustness of individual nodes on the phylogeny.

Bootscan analysis

Full-length genomic sequences containing the *PB2*, *PB1*, *PA*, *HA*, *NP*, *NA*, *MP*, and *NS* gene segments in order of a given influenza isolate were generated using MEGA 4.0 software. Bootscan analyses over full-length sequences were carried out by using SimPlot software (version 3.5) (200 bp window size, 20 bp step size, 100 bootstrap replicates, using gap-stripped alignments and neighbor-joining analysis) (Cavinta et al., 2009). Intact sequences of the test viruses, a pandemic A/H1N1/2009 strain (A/Wisconsin/629-D01773/2009) and a representative triple-reassortant swine-origin strain (A/Iowa/CEID23/2005), were compared with those of the previously isolated reference viruses whose gene segments were the closest genetic neighbors of the test viruses.

Adaptive evolution analysis

Positive selection drives viral evolution during cross-species transmission and human infection. To investigate whether A/Iowa/CEID23/2005 (H1N1) and A/H1N1/2009 viruses were driven by positive selections, the standard McDonald–Kreitman test (http://mkt.uab.es/mkt/MKT.asp) was applied to detect the natural selection (Egea et al., 2008). For the analyses of the *NA* and *MP* gene segments, the clades of the novel A/H1N1/2009 viruses were added into “species 1” and their nearest genetic clades (Eurasian swine influenza strains) were added into “species 2”, respectively. For the analysis of the *HA*, two steps of the selections were performed. Firstly, the clade of the novel A/H1N1/2009 viruses was added into “species 1” and the clade of the triple-reassortant swine H1N1 that sporadically infected humans in or after 2005 was added into “species 2”; Secondly, the clade of the triple-reassortant swine H1N1 that sporadically infected humans in or after 2005 was added into “species 1” and the clade of the classic swine H1N1 that sporadically infected humans before 1998 was added into “species 2”. The Neutrality Index (NI) was used to indicate the extent to which the levels of amino acid polymorphism depart from the expected in the neutral model. A NI value < 1 indicated an excess of fixation of non-neutral replacements due to positive selection, 1 means under neutral selection, and > 1 reflected negative selection that prevented the fixation of harmful mutations (Egea et al., 2008; Ding et al., 2009).

RESULTS

Genetic characteristics of swine-origin influenza viruses that sporadically infected humans before 2009

A total of 49 sporadic influenza patients caused by swine-origin influenza viruses were reported in North America. Four infections occurred in 1980s and 5 in 1990s. However, 15 infections occurred between December 2005 and February 2009 and 10 of which occurred in or after 2007 (Table 1). Some nucleotide sequences of the 8 gene segments of swine-origin influenza viruses sporadically infected humans in North America were available in GenBank. Of the 49 cases, 46 were infected with H1N1

Table 1. Cases of human infections with swine-origin H1N1 influenza viruses in North America and their corresponding viral gene segments.

Patient	Reference	Year	Residence	Subtype	Access no. of gene segments in GenBank							
					PB2	PB1	PA	HA	NP	NA	MP	NS
1	Myers et al. (2007)	1974	Minnesota	H1N1	-	-	-	-	-	-	-	-
2	Myers et al. (2007)	1975	Wisconsin	H1N1	-	-	-	-	-	-	-	-
3	Myers et al. (2007)	1975	Virginia	H1N1	-	-	-	-	-	-	-	-
4	Myers et al. (2007)	1975	Virginia/NY	H1N1	-	-	-	-	-	-	-	-
5	Myers et al. (2007)	1975	Tennessee	H1N1	-	-	-	-	-	-	-	-
6	Myers et al. (2007)	1976	Missouri	H1N1	-	-	-	-	-	-	-	-
7	Myers et al. (2007)	1976	Wisconsin	H1N1	-	-	-	-	-	-	-	-
8	Myers et al. (2007)	1976	Wisconsin	H1N1	-	-	-	-	-	-	-	-
9	Myers et al. (2007)	1976	NA	H1N1	-	-	-	-	-	-	-	-
10-23	Myers et al. (2007)	1976	New Jersey	H1N1	-	-	-	-	-	-	-	-
24	Shinde et al. (2009)	1976	Wisconsin	H1N1	CY026146	CY026145	CY026144	CY026139	CY026142	CY026141	CY026140	CY026143
25	Myers et al. (2007)	1979	Texas	H1N1	-	-	-	-	-	-	-	-
26	Myers et al. (2007)	1980	Texas	H1N1	-	-	-	-	-	-	-	-
27	Myers et al. (2007)	1982	Nevada	H1N1	-	-	-	-	-	-	-	-
28	Shinde et al. (2009)	1988	Ohio	H1N1	CY024932	CY024931	CY024930	CY024925	CY024928	CY024927	CY024926	CY024929
29	Myers et al. (2007)	1988	Wisconsin	H1N1	-	-	-	-	-	-	-	-
30	Myers et al. (2007)	1991	Maryland	H1N1	CY039916	CY039915	CY039914	CY039909	CY039912	CY039911	CY039910	CY039913
31	Myers et al. (2007)	1994	Wisconsin	H1N1	U53159*	U53157*	U53161*	U53163	U53165*	U53167*	U53169	U53171
32	Myers et al. (2007)	1994	Wisconsin	H1N1	U53158*	U53156*	U53160*	U53162	U53164*	U53166	U53168	U53170
33	Myers et al. (2007)	1995	Minnesota	H1N1	-	-	-	-	-	-	-	-
34	Myers et al. (2007)	1998	Wisconsin	H1N1	AF342824*	AF342823	AF342822*	AF342821*	AF342819	AF342820	AF342818	AF342817
35	Gray et al. (2007)	2005	Iowa	H1N1	DQ889682	DQ889683	DQ889684	DQ889689	DQ889686	DQ889687	DQ889688	DQ889685
36	Newman et al.(2008)	2005	Wisconsin	H1N1	-	-	-	FJ986619	-	-	-	-
37	Olsen et al.(2006)	2005	Canada	H3N2	DQ469955	DQ469956	DQ469957	DQ469962	DQ469959	DQ469960	DQ469961	DQ469958
38	Shinde et al. (2009)	2006	Missouri	H1N1	-	-	-	-	-	-	-	-
39	Shinde et al. (2009)	2006	Iowa	H1N1	-	-	-	FJ986618	-	-	-	-
40	Shinde et al. (2009)	2007	Ohio	H1N1	-	-	-	FJ986620	-	-	-	-
41	Shinde et al. (2009)	2007	Ohio	H1N1	-	-	-	FJ986621	-	-	-	-
42	Shinde et al. (2009)	2007	Michigan	H1N2	-	-	-	FJ986622	-	-	-	-
43	Shinde et al. (2009)	2007	Illinois	H1N1	-	-	-	-	-	-	-	-
44	Shinde et al. (2009)	2007	Iowa	H1N1	-	-	-	-	-	-	-	-
45	Vincent et al. (2009)	2007	Ohio	HIN1	EU604691	EU604692	EU604693	EU604689	EU604694	EU604690	EU604695	EU604696
46	Bastien et al. (20098)	2007	Canada	H3N2	EU399758	EU399757	EU399756	EU399751	EU399754	EU399753	EU399752	EU399755
47	Shinde et al. (2009)	2008	Minnesota	H1N1	-	-	-	-	-	-	-	-
48	Shinde et al. (2009)	2008	Texas	H1N1	-	-	-	-	-	-	-	-
49	Shinde et al. (2009)	2009	Iowa	H1N1	-	-	-	-	-	-	-	-

"-", not available in GenBank; "*", partial sequences.

influenza A. Phylogenetic analysis with 30, 27, 27, 35, 28, 28, 30, and 30 full-length sequences of *PB2*, *PB1*, *PA*, *HA*, *NP*, *NA*, *MP*, and *NS* genes of the influenza viruses are performed, respectively (Figure 1). All of the 8 gene segments of swine H1N1 viruses that sporadically infected humans in 1970s to 1990s clustered with those of classic swine influenza viruses of North American lineage, except A/Wisconsin/10/98, an unreported isolate from a human case in Wisconsin in 1998. Partial *PB2* (1,573 bp) and *PA* (1,470 bp) segments and other 6 full-length gene segments of A/Wisconsin/10/98 were retrieved from GenBank. Phylogenetic analyses with the partial sequences indicated that the *PB2* and *PA* segments of A/Wisconsin/10/98 were closely related to that of A/Iowa/CEID23/2005(H1N1) (Figure 2). The full-length *PB1* gene of A/Wisconsin/10/98 was closely related to that of A/Iowa/CEID23/2005(H1N1) (Figure 1). Bootscan analysis indicated that A/Iowa/CEID23/2005 (H1N1), a representative swine-origin triple-reassortant influenza that sporadically infected humans in North America, shared 92.3 and 93.0% identities in the *PB2* and *PA* genes with avian influenza virus of North American lineage, 94.6% identity in the *PB1* gene with human H3N2 virus, and 92.8 to 96.5% identities in another 5 genes with classic swine influenza H1N1 virus of North American lineage, respectively, as shown in Table 2. The 8 fragments of a strain isolated in 2007, A/swine/OH/511445/2007(H1N1), were phylogenetically linked to those of A/Iowa/CEID23/2005(H1N1) (Figure 1). The *PB2* and *PA* segments, *PB1* segment, and other 5 gene segments of swine-origin H1N1 viruses that infected humans sporadically between 2005 and 2007 (or 2009) were closely related to those of avian influenza viruses of North American lineage, human seasonal H3N2 influenza viruses, and classic swine influenza viruses of North American lineage, respectively. Since human infections with the triple-reassortant swine-origin H1N1 viruses had been increasingly frequent several years before the outbreak of the novel H1N1 influenza in 2009, we hypothesize that introduction of the *PB2* and *PA* genes of avian influenza viruses and the *PB1* gene from human seasonal H3N2 viruses facilitate the sporadic infection of swine-origin H1N1 influenza A virus in humans since 2005.

Evolutionary relationship of swine-origin H1N1 influenza viruses that infected humans from sporadic to pandemic

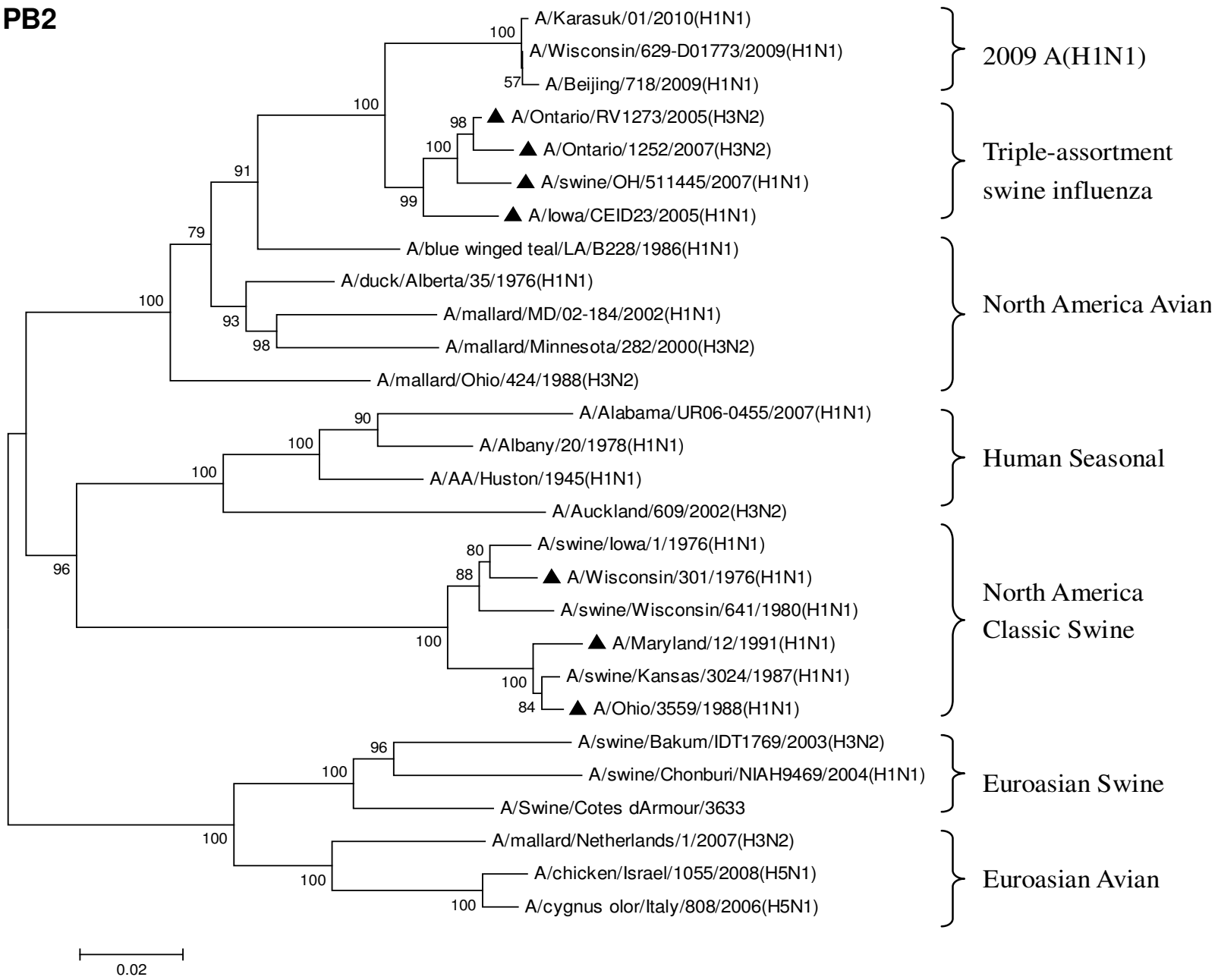
As shown in Figure 1, the A/H1N1/2009 pandemic strains clustered in a unique clade for each of the 8 gene segments, respectively. Within the clades of the nearest genetic neighbors, the *PB2, PB1*, *PA*, *HA*, *NP*, and *NS* gene segments of the novel H1N1/2009 strains were most closely linked to those of A/Iowa/CEID23/2005 (H1N1) and those of other triple-reassortant swine-origin viruses that sporadically infected humans since 2005 (Figures 1 and 2). The *PB1*, *NP*, and *NS* were also closely linked to those of a triple-reassortant swine-origin virus A/Wisconsin/10/98 (H1N1) that sporadically infected humans. The *HA* was also closely linked to the gene of triple-reassortant swine viruses A/Ohio/01/2007 (H1N1), A/Ohio/02/2007 (H1N1), A/Wisconsin/87/2005 (H1N1), and A/Iowa/01/2006 (H1N1) that sporadically infected humans in North America. The *NA* and *MP* were closely related to those of influenza A H1N1 viruses circulating in swine populations in Eurasia. Bootscan analysis showed that the A/H1N1/2009 pandemic strain shared great homology with A/Iowa/CEID23/2005 (H1N1) in the *PB2* (95.3%), *PB1* (95.1%), *PA* (95.2%), *HA* (90.3%), *NP* (95.2%), and *NS* (94.7%) genes. The *NA* and *MP* genes of the A/H1N1/2009 pandemic strain were most closely related to those of A/Swine/Spain/50047/2003 (H1N1), a swine virus of Eurasian lineage, sharing 90.2 and 94.4% identities, respectively, as shown in Figure 3. The genetic components of the swine-origin H1N1 viruses that infected humans from sporadic to pandemic at different phases were shown in Figure 4.

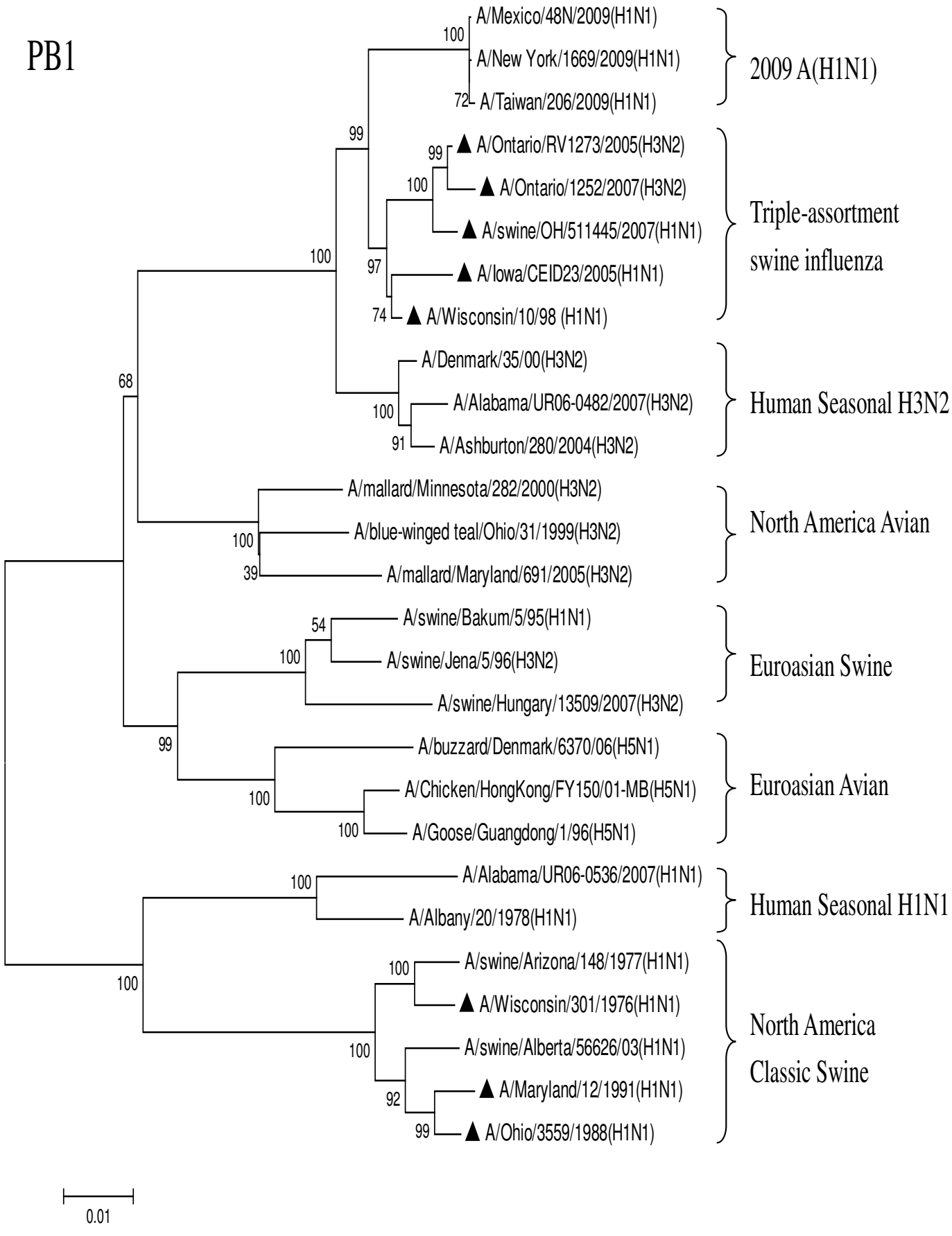
Positive selection drives the evolution of swine-origin H1N1 viruses that infected humans from sporadic to pandemic

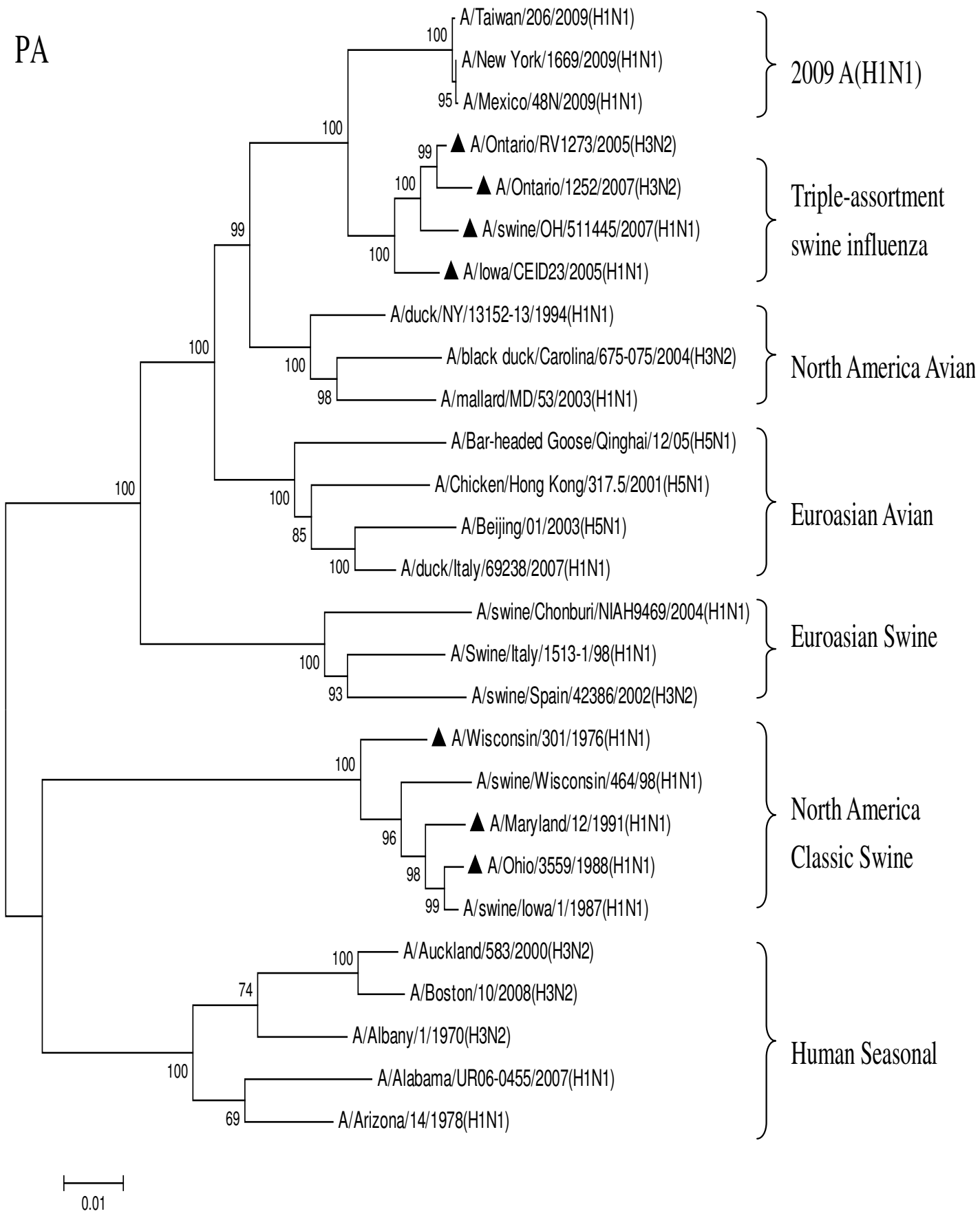
Table 3 shows the results of the McDonald– Kreitman tests. A HI value of 0.817 was observed in the *HA* segment between the classic swine H1N1 viruses that occasionally infected humans before 1998 and the triple-reassortant H1N1 viruses that sporadically infected humans in or after 2005, implying potential positive selection acting on this gene evolved from the classic swine H1N1 viruses to the triple-reassortant H1N1 viruses. However, positive selection was not observed on this gene between the triple-reassortant H1N1 viruses and A/H1N1/2009 pandemic viruses. Moreover, a HI value of 0.773 was observed acting on the *NA* gene between A/H1N1/2009 viruses and their nearest genetic neighbors (Euroasian swine H1N1 viruses), implying a positive selection was acting on this gene before or during the reassortment of this gene segment into A/H1N1/2009 pandemic viruses.

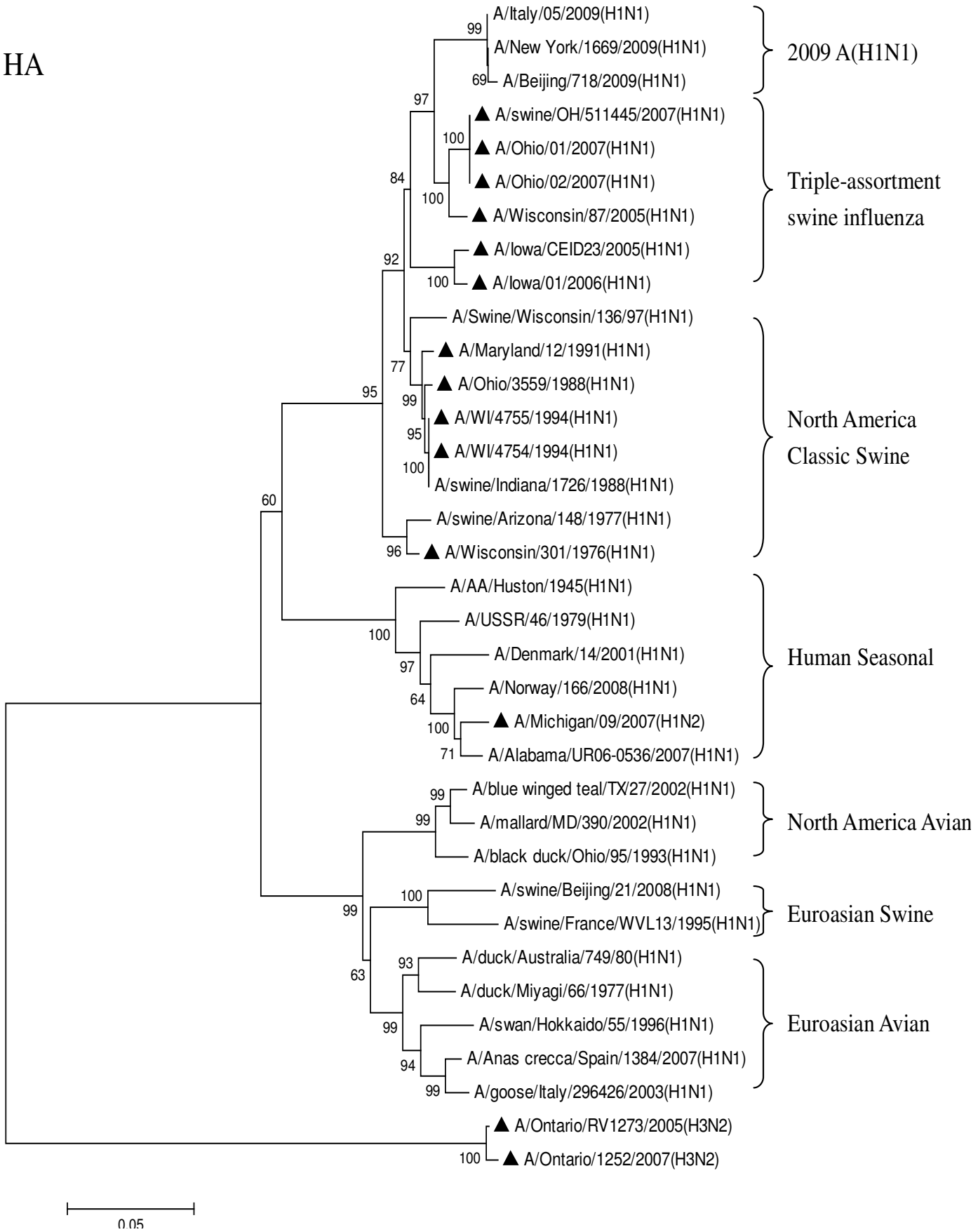
DISCUSSION

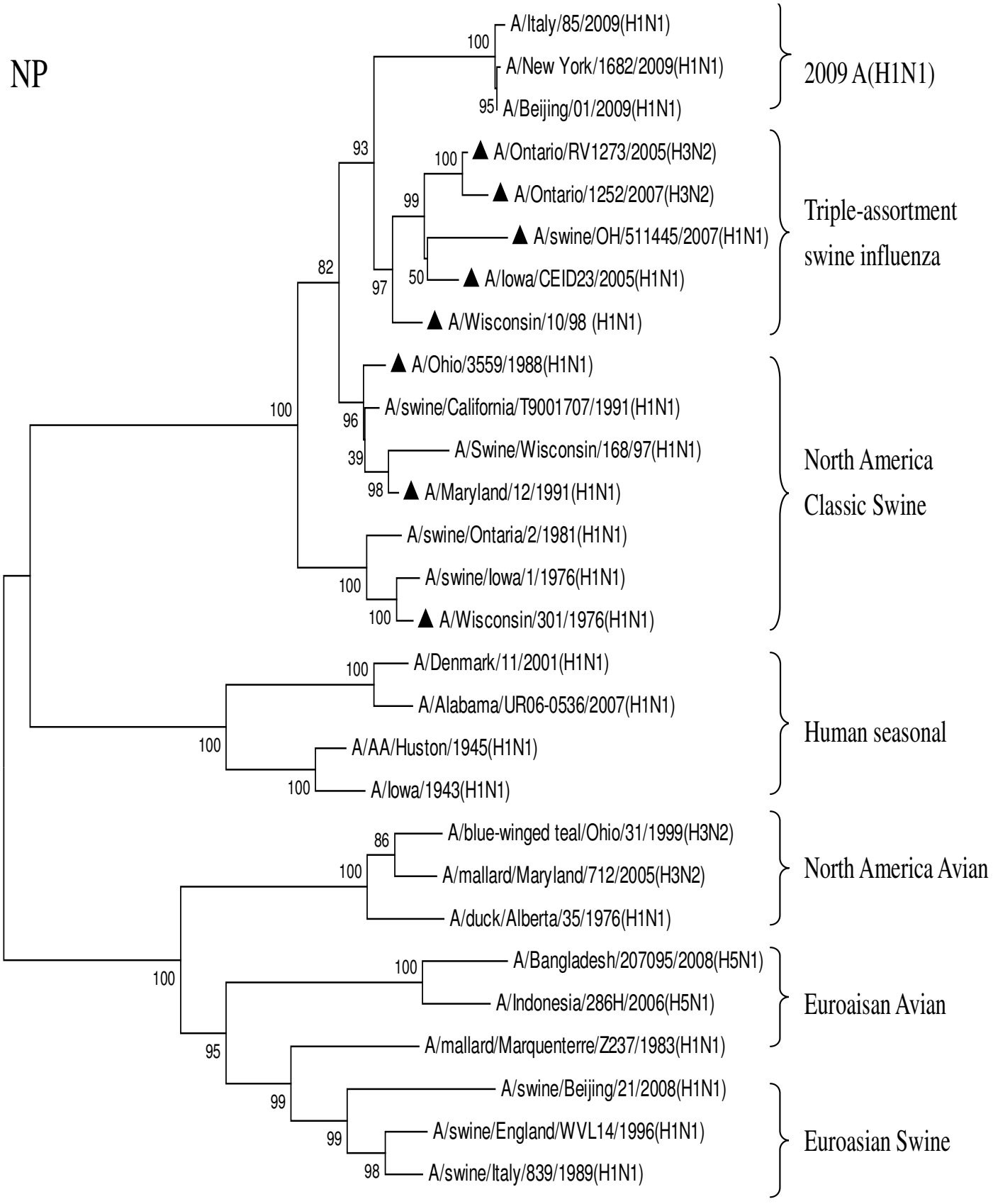
The reassortments between gene segments are essential steps for the evolution of influenza viruses. This study indicated that swine-origin H1N1 influenza viruses that infected humans from sporadic to pandemic have experienced two critical steps of the reassortments. The first critical step is the introduction of the PB2 and *PA* genes of avian influenza viruses and the *PB1* gene from human seasonal H3N2 viruses which might facilitate the


PB2
A/Karasuk/01/2010(H1N1)
A/Wisconsin/629-D01773/2009(H1N1)
A/Beijing/718/2009(H1N1)
A/Ontario/RV1273/2005(H3N2)
A/Ontario/1252/2007(H3N2)
A/swine/OH/511445/2007(H1N1)
A/Iowa/CEID23/2005(H1N1)
A/blue winged teal/LA/B228/1986(H1N1)
A/duck/Alberta/35/1976(H1N1)
A/mallard/MD/02-184/2002(H1N1)
A/mallard/Minnesota/282/2000(H3N2)
A/mallard/Ohio/424/1988(H3N2)
A/Alabama/UR06-0455/2007(H1N1)
A/Albany/20/1978(H1N1)
A/AA/Huston/1945(H1N1)
A/Auckland/609/2002(H3N2)
A/swine/Iowa/1/1976(H1N1)
A/Wisconsin/301/1976(H1N1)
A/swine/Wisconsin/641/1980(H1N1)
A/Maryland/12/1991(H1N1)
A/swine/Kansas/3024/1987(H1N1)
A/Ohio/3559/1988(H1N1)
A/swine/Bakum/IDT1769/2003(H3N2)
A/swine/Chonburi/NIAH9469/2004(H1N1)
A/Swine/Cotes dArmour/3633
A/mallard/Netherlands/1/2007(H3N2)
A/chicken/Israel/1055/2008(H5N1)
A/cygnus olor/Italy/808/2006(H5N1)
2009 A(H1N1)
Triple-assortment swine influenza
North America Avian
Human Seasonal
North America Classic Swine
Euroasian Swine
Euroasian Avian
0.02


PB1
A/Mexico/48N/2009(H1N1)
A/New York/1669/2009(H1N1)
A/Taiwan/206/2009(H1N1)
2009 A(H1N1)
A/Ontario/RV1273/2005(H3N2)
A/Ontario/1252/2007(H3N2)
A/swine/OH/511445/2007(H1N1)
A/Iowa/CEID23/2005(H1N1)
A/Wisconsin/10/98 (H1N1)
Triple-assortment swine influenza
A/Denmark/35/00(H3N2)
A/Alabama/UR06-0482/2007(H3N2)
A/Ashburton/280/2004(H3N2)
Human Seasonal H3N2
A/mallard/Minnesota/282/2000(H3N2)
A/blue-winged teal/Ohio/31/1999(H3N2)
A/mallard/Maryland/691/2005(H3N2)
North America Avian
A/swine/Bakum/5/95(H1N1)
A/swine/Jena/5/96(H3N2)
A/swine/Hungary/13509/2007(H3N2)
Euroasian Swine
A/buzzard/Denmark/6370/06(H5N1)
A/Chicken/HongKong/FY150/01-MB(H5N1)
A/Goose/Guangdong/1/96(H5N1)
Euroasian Avian
A/Alabama/UR06-0536/2007(H1N1)
A/Albany/20/1978(H1N1)
Human Seasonal H1N1
A/swine/Arizona/148/1977(H1N1)
A/Wisconsin/301/1976(H1N1)
A/swine/Alberta/56626/03(H1N1)
A/Maryland/12/1991(H1N1)
A/Ohio/3559/1988(H1N1)
North America Classic Swine
0.01


PA
A/Taiwan/206/2009(H1N1)
A/New York/1669/2009(H1N1)
A/Mexico/48N/2009(H1N1)
2009 A(H1N1)
A/Ontario/RV1273/2005(H3N2)
A/Ontario/1252/2007(H3N2)
A/swine/OH/511445/2007(H1N1)
A/Iowa/CEID23/2005(H1N1)
Triple-assortment swine influenza
A/duck/NY/13152-13/1994(H1N1)
A/black duck/Carolina/675-075/2004(H3N2)
A/mallard/MD/53/2003(H1N1)
North America Avian
A/Bar-headed Goose/Qinghai/12/05(H5N1)
A/Chicken/Hong Kong/317.5/2001(H5N1)
A/Beijing/01/2003(H5N1)
A/duck/Italy/69238/2007(H1N1)
Euroasian Avian
A/swine/Chonburi/NIAH9469/2004(H1N1)
A/Swine/Italy/1513-1/98(H1N1)
A/swine/Spain/42386/2002(H3N2)
Euroasian Swine
A/Wisconsin/301/1976(H1N1)
A/swine/Wisconsin/464/98(H1N1)
A/Maryland/12/1991(H1N1)
A/Ohio/3559/1988(H1N1)
A/swine/Iowa/1/1987(H1N1)
North America Classic Swine
A/Auckland/583/2000(H3N2)
A/Boston/10/2008(H3N2)
A/Albany/1/1970(H3N2)
A/Alabama/UR06-0455/2007(H1N1)
A/Arizona/14/1978(H1N1)
Human Seasonal
0.01


HA
A/Italy/05/2009(H1N1)
A/New York/1669/2009(H1N1)
A/Beijing/718/2009(H1N1)
2009 A(H1N1)
A/swine/OH/511445/2007(H1N1)
A/Ohio/01/2007(H1N1)
A/Ohio/02/2007(H1N1)
A/Wisconsin/87/2005(H1N1)
A/Iowa/CEID23/2005(H1N1)
A/Iowa/01/2006(H1N1)
Triple-assortment swine influenza
A/Swine/Wisconsin/136/97(H1N1)
A/Maryland/12/1991(H1N1)
A/Ohio/3559/1988(H1N1)
A/WI/4755/1994(H1N1)
A/WI/4754/1994(H1N1)
A/swine/Indiana/1726/1988(H1N1)
A/swine/Arizona/148/1977(H1N1)
A/Wisconsin/301/1976(H1N1)
North America Classic Swine
A/AA/Huston/1945(H1N1)
A/USSR/46/1979(H1N1)
A/Denmark/14/2001(H1N1)
A/Norway/166/2008(H1N1)
A/Michigan/09/2007(H1N2)
A/Alabama/UR06-0536/2007(H1N1)
Human Seasonal
A/blue winged teal/TX/27/2002(H1N1)
A/mallard/MD/390/2002(H1N1)
A/black duck/Ohio/95/1993(H1N1)
North America Avian
A/swine/Beijing/21/2008(H1N1)
A/swine/France/WVL13/1995(H1N1)
Euroasian Swine
A/duck/Australia/749/80(H1N1)
A/duck/Miyagi/66/1977(H1N1)
A/swan/Hokkaido/55/1996(H1N1)
A/Anas crecca/Spain/1384/2007(H1N1)
A/goose/Italy/296426/2003(H1N1)
Euroasian Avian
A/Ontario/RV1273/2005(H3N2)
A/Ontario/1252/2007(H3N2)
0.05


NP
A/Italy/85/2009(H1N1)
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A/Beijing/01/2009(H1N1)
2009 A(H1N1)
A/Ontario/RV1273/2005(H3N2)
A/Ontario/1252/2007(H3N2)
A/swine/OH/511445/2007(H1N1)
A/Iowa/CEID23/2005(H1N1)
A/Wisconsin/10/98 (H1N1)
Triple-assortment swine influenza
A/Ohio/3559/1988(H1N1)
A/swine/California/T9001707/1991(H1N1)
A/Swine/Wisconsin/168/97(H1N1)
A/Maryland/12/1991(H1N1)
A/swine/Ontaria/2/1981(H1N1)
A/swine/Iowa/1/1976(H1N1)
A/Wisconsin/301/1976(H1N1)
North America Classic Swine
A/Denmark/11/2001(H1N1)
A/Alabama/UR06-0536/2007(H1N1)
A/AA/Huston/1945(H1N1)
A/Iowa/1943(H1N1)
Human seasonal
A/blue-winged teal/Ohio/31/1999(H3N2)
A/mallard/Maryland/712/2005(H3N2)
A/duck/Alberta/35/1976(H1N1)
North America Avian
A/Bangladesh/207095/2008(H5N1)
A/Indonesia/286H/2006(H5N1)
A/mallard/Marquenterre/Z237/1983(H1N1)
Euroaisan Avian
A/swine/Beijing/21/2008(H1N1)
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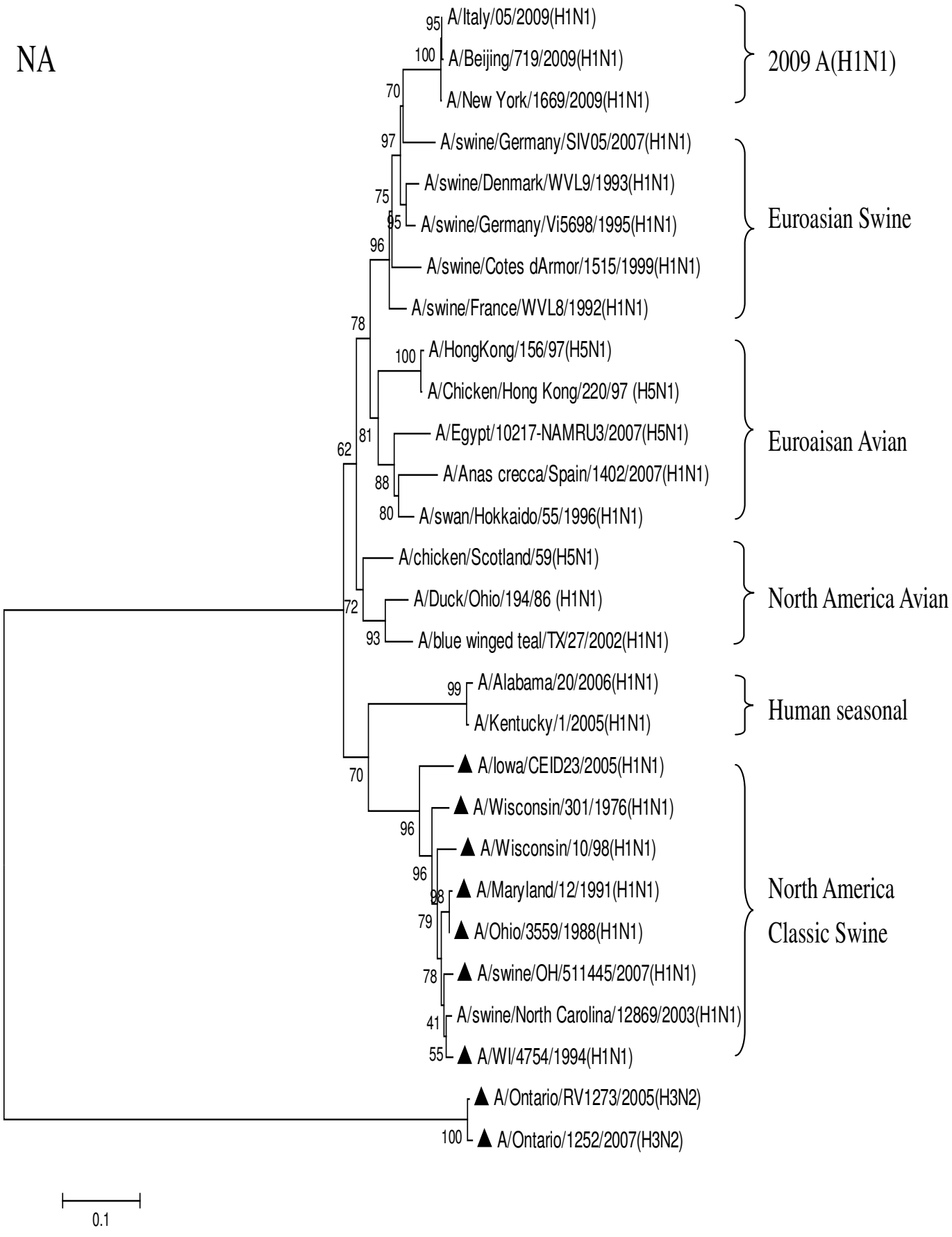
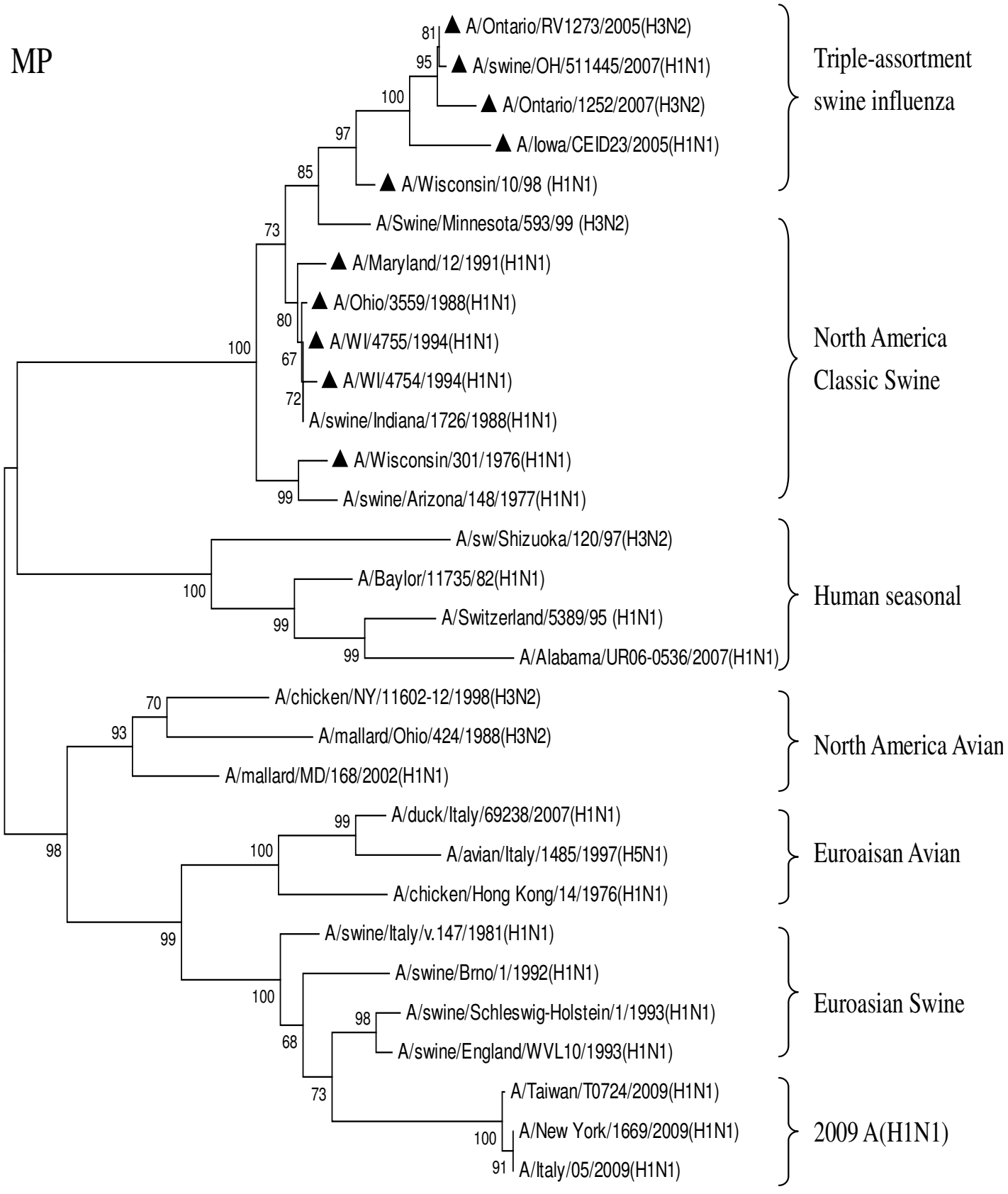
A/swine/Italy/839/1989(H1N1)
Euroasian Swine
0.01


NA
A/Italy/05/2009(H1N1)
A/Beijing/719/2009(H1N1)
A/New York/1669/2009(H1N1)
2009 A(H1N1)
A/swine/Germany/SIV05/2007(H1N1)
A/swine/Denmark/WVL9/1993(H1N1)
A/swine/Germany/Vi5698/1995(H1N1)
A/swine/Cotes dArmor/1515/1999(H1N1)
A/swine/France/WVL8/1992(H1N1)
Euroasian Swine
A/HongKong/156/97(H5N1)
A/Chicken/Hong Kong/220/97 (H5N1)
A/Egypt/10217-NAMRU3/2007(H5N1)
A/Anas crecca/Spain/1402/2007(H1N1)
A/swan/Hokkaido/55/1996(H1N1)
Euroaisan Avian
A/chicken/Scotland/59(H5N1)
A/Duck/Ohio/194/86 (H1N1)
A/blue winged teal/TX/27/2002(H1N1)
North America Avian
A/Alabama/20/2006(H1N1)
A/Kentucky/1/2005(H1N1)
Human seasonal
A/Iowa/CEID23/2005(H1N1)
A/Wisconsin/301/1976(H1N1)
A/Wisconsin/10/98(H1N1)
A/Maryland/12/1991(H1N1)
A/Ohio/3559/1988(H1N1)
A/swine/OH/511445/2007(H1N1)
A/swine/North Carolina/12869/2003(H1N1)
A/WI/4754/1994(H1N1)
North America
Classic Swine
A/Ontario/RV1273/2005(H3N2)
A/Ontario/1252/2007(H3N2)
0.1


MP
A/Ontario/RV1273/2005(H3N2)
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A/Ontario/1252/2007(H3N2)
A/Iowa/CEID23/2005(H1N1)
A/Wisconsin/10/98 (H1N1)
A/Swine/Minnesota/593/99 (H3N2)
A/Maryland/12/1991(H1N1)
A/Ohio/3559/1988(H1N1)
A/WI/4755/1994(H1N1)
A/WI/4754/1994(H1N1)
A/swine/Indiana/1726/1988(H1N1)
A/Wisconsin/301/1976(H1N1)
A/swine/Arizona/148/1977(H1N1)
A/sw/Shizuoka/120/97(H3N2)
A/Baylor/11735/82(H1N1)
A/Switzerland/5389/95 (H1N1)
A/Alabama/UR06-0536/2007(H1N1)
A/chicken/NY/11602-12/1998(H3N2)
A/mallard/Ohio/424/1988(H3N2)
A/mallard/MD/168/2002(H1N1)
A/duck/Italy/69238/2007(H1N1)
A/avian/Italy/1485/1997(H5N1)
A/chicken/Hong Kong/14/1976(H1N1)
A/swine/Italy/v.147/1981(H1N1)
A/swine/Brno/1/1992(H1N1)
A/swine/Schleswig-Holstein/1/1993(H1N1)
A/swine/England/WVL10/1993(H1N1)
A/Taiwan/T0724/2009(H1N1)
A/New York/1669/2009(H1N1)
A/Italy/05/2009(H1N1)
Triple-assortment swine influenza
North America Classic Swine
Human seasonal
North America Avian
Euroaisan Avian
Euroasian Swine
2009 A(H1N1)
0.01

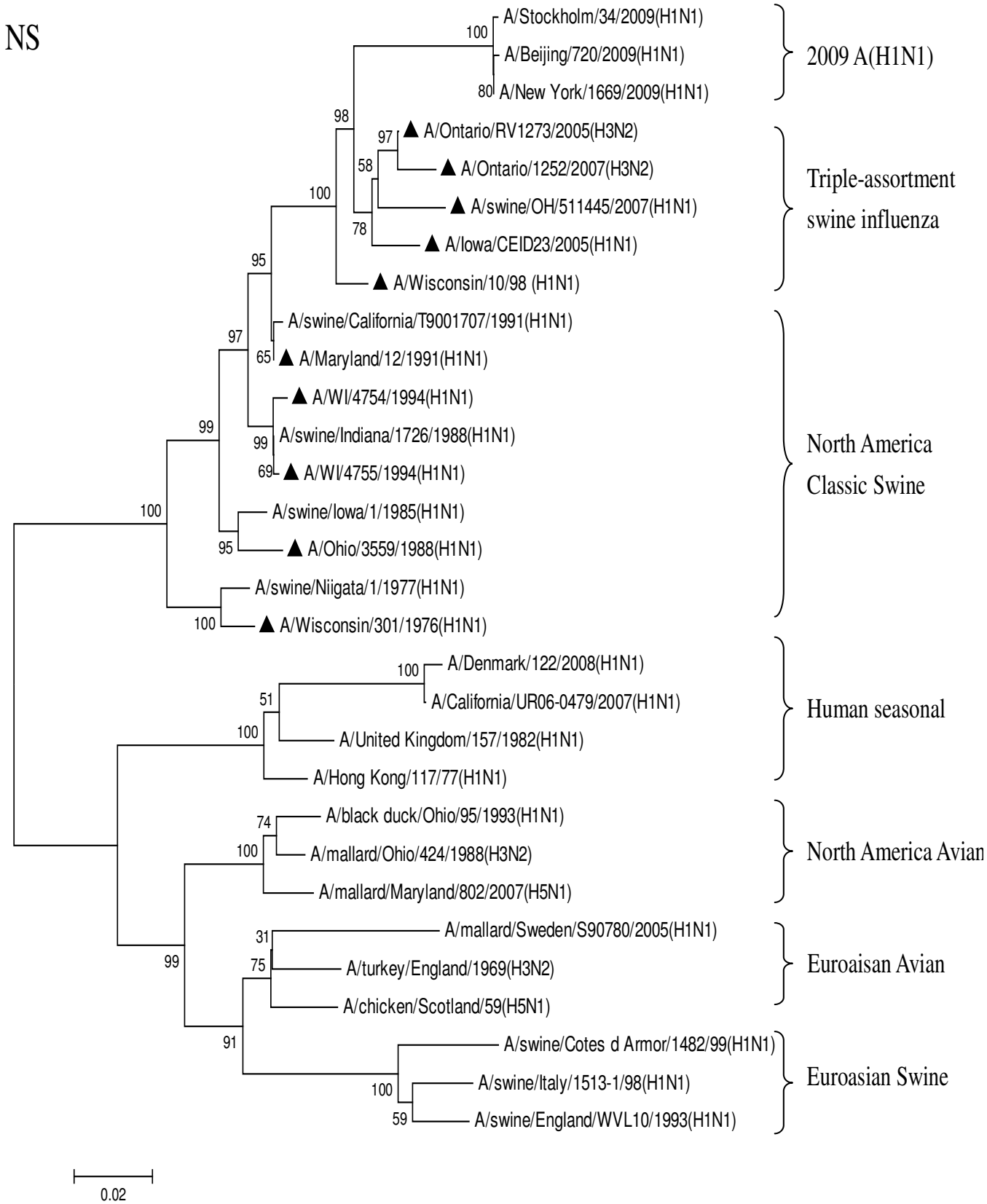
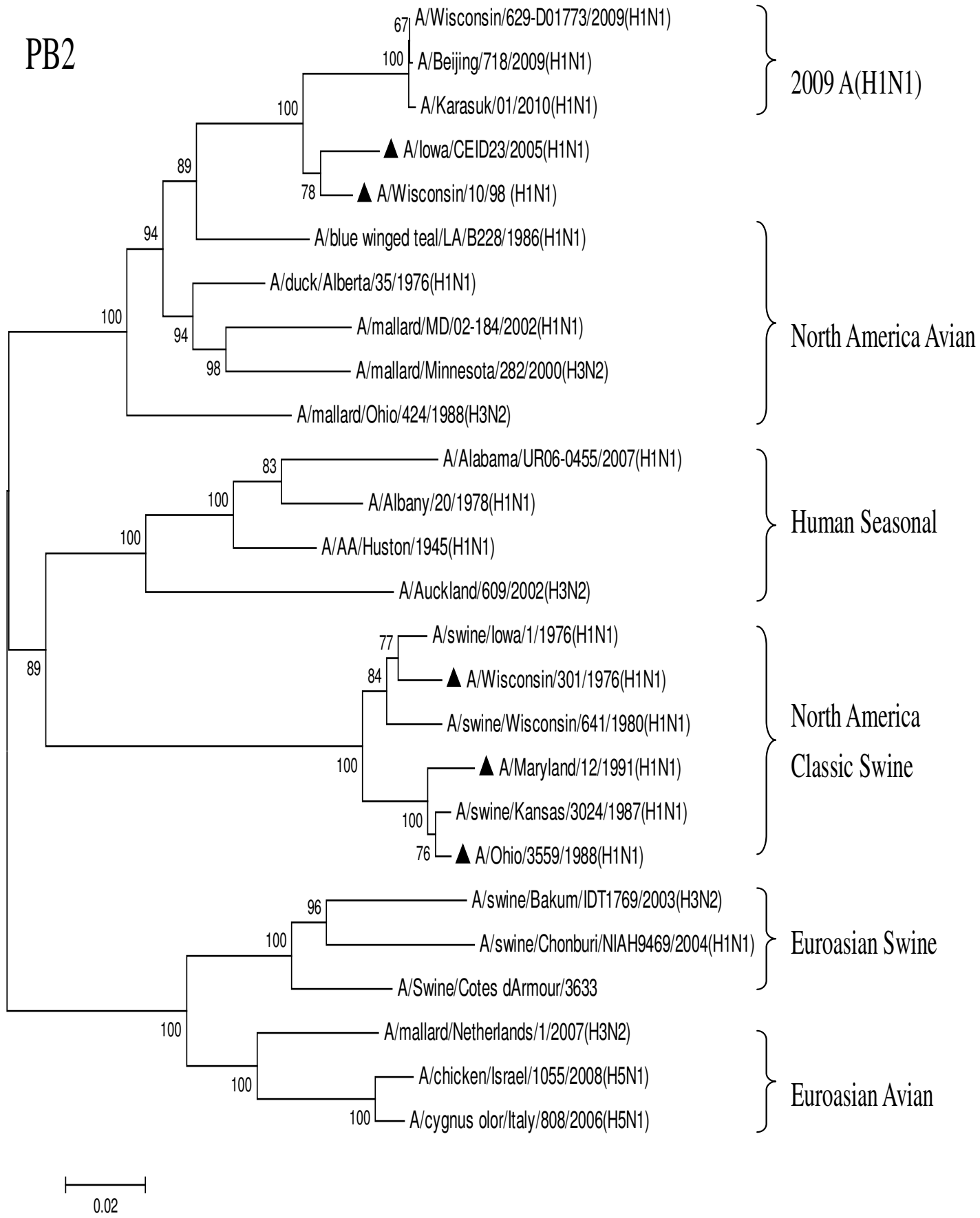


Figure 1. Phylogentic analyses of the 8 full-length gene segments (*PB2*, *PB1*, *PA*, *HA*, *NP*, *NA*, *MP*, and *NS*) of the selected pandemic A/H1N1/2009 strains, all available swine-origin influenza viruses that infected humans sporadically, and the selected reference influenza A viruses. ▲ indicates the strains infected humans sporadically.


PB2
A/Wisconsin/629-D01773/2009(H1N1)
A/Beijing/718/2009(H1N1)
A/Karasuk/01/2010(H1N1)
A/Iowa/CEID23/2005(H1N1)
A/Wisconsin/10/98 (H1N1)
A/blue winged teal/LA/B228/1986(H1N1)
A/duck/Alberta/35/1976(H1N1)
A/mallard/MD/02-184/2002(H1N1)
A/mallard/Minnesota/282/2000(H3N2)
A/mallard/Ohio/424/1988(H3N2)
A/Alabama/UR06-0455/2007(H1N1)
A/Albany/20/1978(H1N1)
A/AA/Huston/1945(H1N1)
A/Auckland/609/2002(H3N2)
A/swine/Iowa/1/1976(H1N1)
A/Wisconsin/301/1976(H1N1)
A/swine/Wisconsin/641/1980(H1N1)
A/Maryland/12/1991(H1N1)
A/swine/Kansas/3024/1987(H1N1)
A/Ohio/3559/1988(H1N1)
A/swine/Bakum/IDT1769/2003(H3N2)
A/swine/Chonburi/NIAH9469/2004(H1N1)
A/Swine/Cotes dArmour/3633
A/mallard/Netherlands/1/2007(H3N2)
A/chicken/Israel/1055/2008(H5N1)
A/cygnus olor/Italy/808/2006(H5N1)
2009 A(H1N1)
North America Avian
Human Seasonal
North America
Classic Swine
Euroasian Swine
Euroasian Avian
0.02

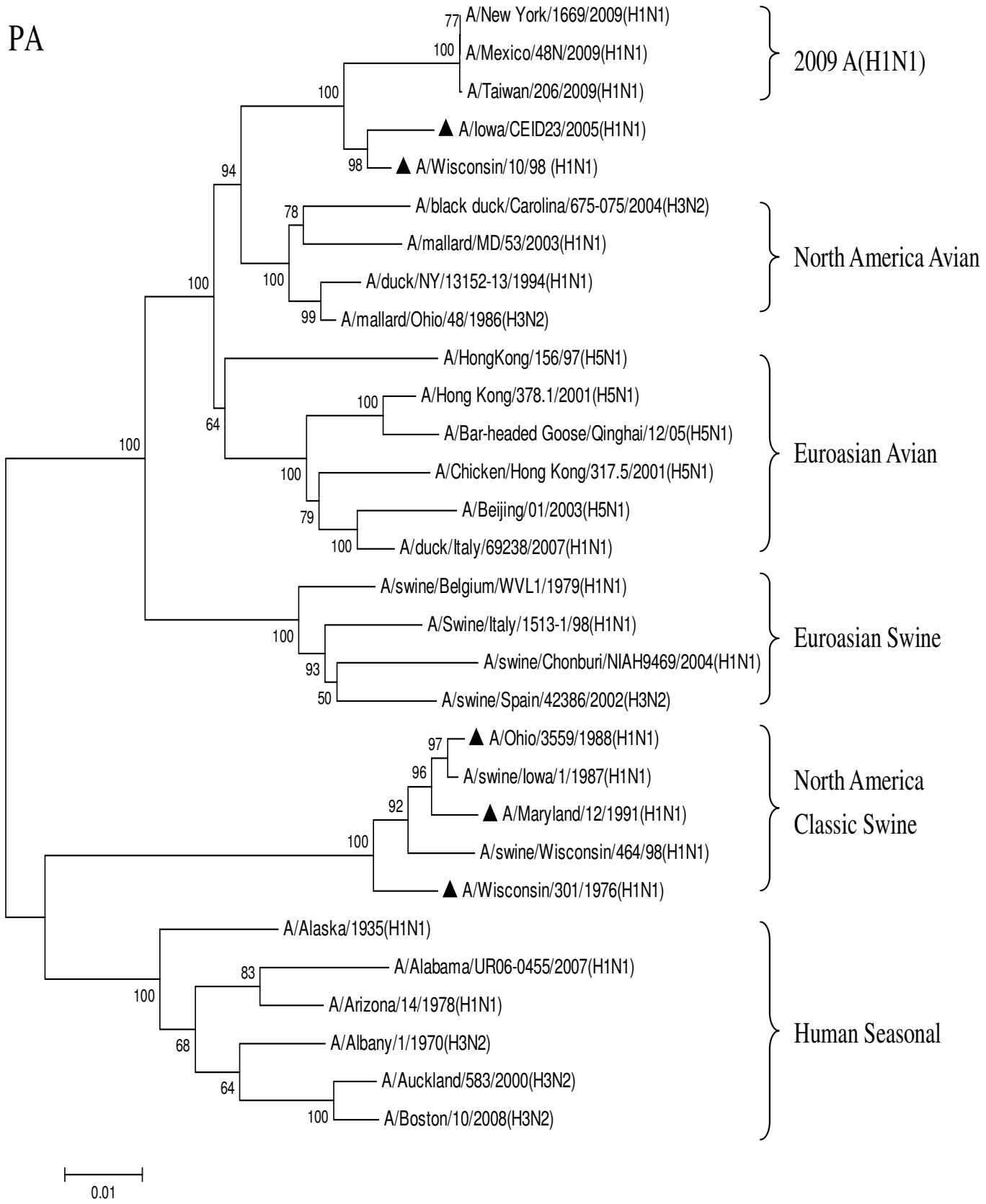


Figure 2. Phylogentic analyses of the partial *PB2* (1,573 bp) and *PA* (1,470 bp) sequences of the selected pandemic A/H1N1/2009 strains, swine-origin H1N1 influenza viruses that infected humans sporadically, and the selected reference influenza A viruses. ▲ indicates the strains infected humans sporadically.

Table 2. Sequence identities of the 8 gene segments of a representative swine-origin triple reassortant virus that infected humans in 2005 with those of selected reference strains using Bootscan analysis.

Reference influenza virus strains	Identities (%) with A/Iowa/CEID23/2005 (H1N1)								
	Full	PB2	PB1	PA	HA	NP	NA	MP	NS
A/Swine/Spain/50047/2003 (H1N1)	80.6	81.0	83.4	84.3	64.5	80.9	77.2	85.5	78.9
A/Swine/Wisconsin/1915/1988 (H1N1)	86. 8	81.1	75.5	78.9	92.9	94.2	93.1	96.2	94.9
A/Ohio/3559/1988 (H1N1)*	86.9	81.4	75.6	78.5	92.8	96.1	92.8	96.5	93.6
A/mallard/Ohio/48/1986 (H3N2)	83.9	92.3	86.8	92.7	29.9	80.8	29.3	89.7	62.7
A/Turkey/Ontario/84/1983 (H5N1)	83.4	90.0	86.6	93.0	41.4	80.5	78.0	89.4	83.3
A/Yucatan/ME6057/2003 (H3N2)*	73.5	81.4	94.6	79.5	30.0	79.9	2.9	85.9	79.5

* Infected humans sporadically.

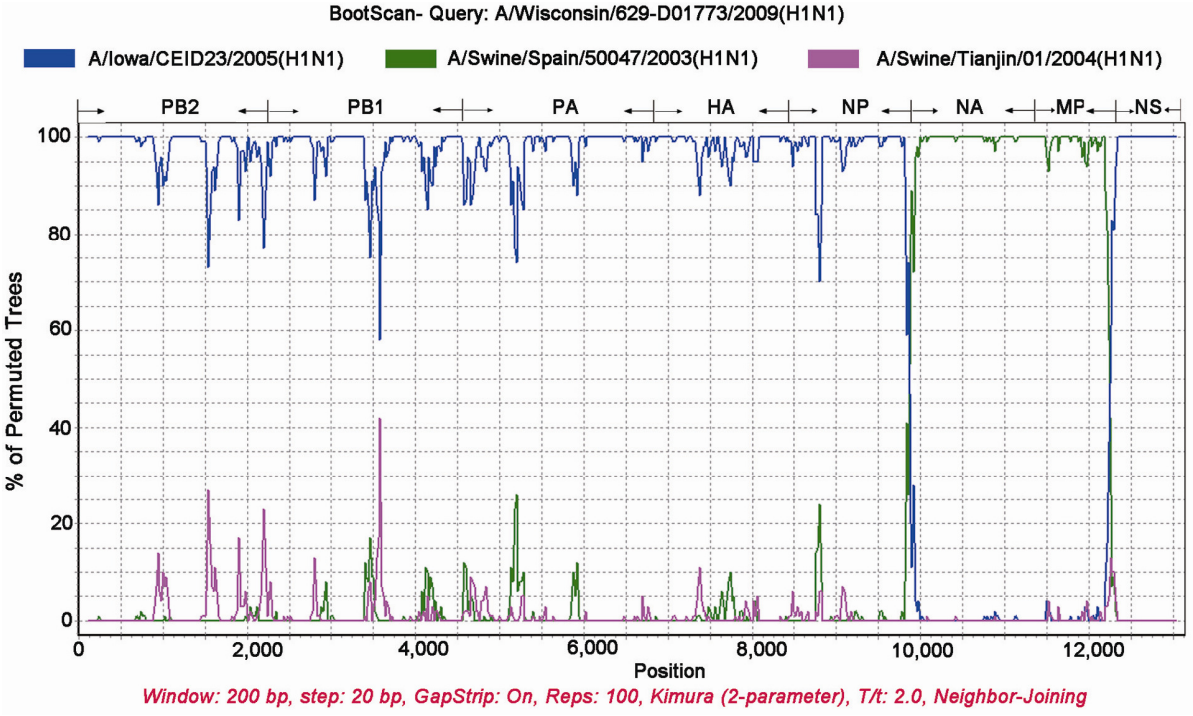


Figure 3. Bootscan analysis for full-length of A/H1N1/2009 pandemic strain with those of 3 representative reference strains.

sporadic infection of swine-origin H1N1 influenza A virus in humans since 2005. The other step is the reassortment with the *NA* and *MP* gene segments of swine H1N1 viruses of Eurasian lineage which might enable the triple-reassortant swine-origin H1N1 viruses to cause the pandemic in 2009.

Stochastic mutation in nucleotides and directional immune selection may play an important role in determining the direction of the evolution of influenza viruses. The reassortments between gene segments are essential steps for the evolution of influenza viruses. This study shows that swine-origin H1N1 influenza viruses that infected humans from sporadic to pandemic have experienced two critical steps of the reassortments. The gene segments of the swine H1N1 viruses that occasionally infected persons who ever exposed to pigs in North America before 1998 were phylogenetically linked to those of classic swine H1N1 influenza viruses. Phylogenetic analyses with the partial *PB2* and *PA* gene segments and the full-length *PB1* gene segment of A/Wisconsin/10/98 indicate that the triple-reassortant H1N1 virus started to infect humans in late 1990s. The swine-origin H1N1 viruses that sporadically infected humans between 2005 and 2009 were of the triple-reassortant viruses (Figure 4). The triple-reassortant virus may be considered as “backbone frame” of the A/H1N1/2009 pandemic strain or “intermediate” in evolutionary process of the A/H1N1/2009 pandemic

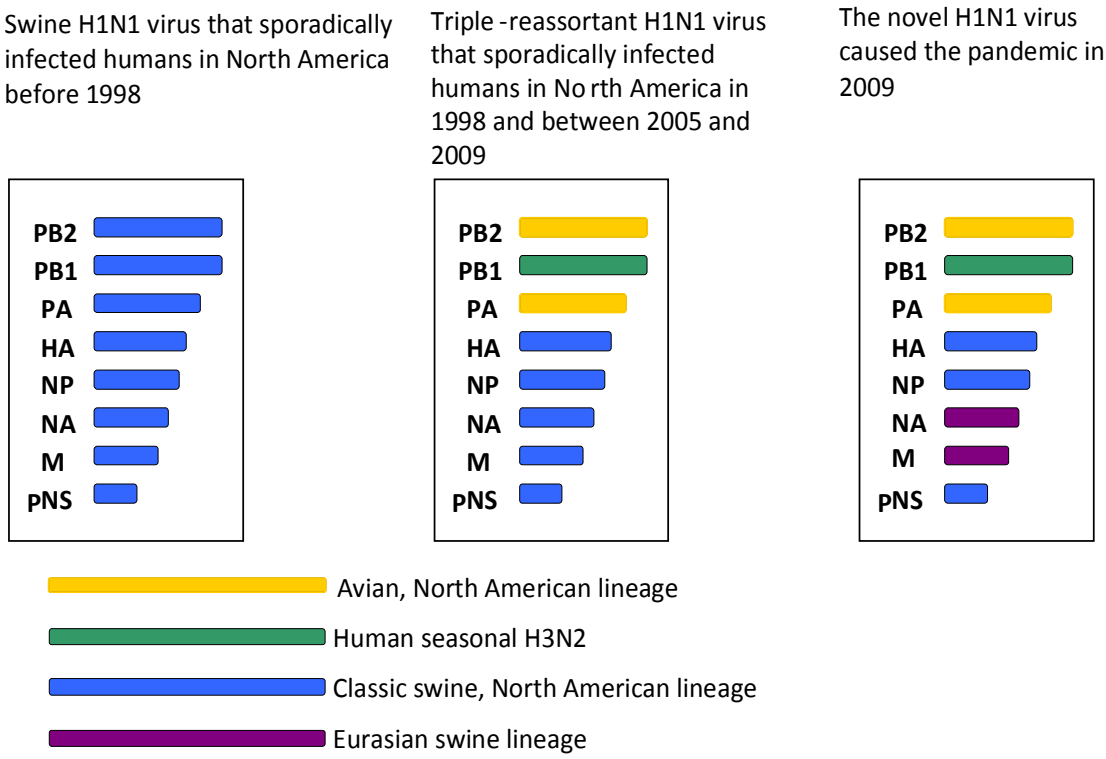


Figure 4. The genetic components of the swine-origin H1N1 viruses that infected humans from sporadic to pandemic at different phases.

Table 3. McDonald-Kreitman test for the natural selection of 3 gene segments of novel influenza A/H1N1/2009 viruses.

Gene	Neutral		Non-neutral		Neutrality Index (NI)	Proportion of adaptive substitutions	p
	Polymorphism	Divergence	Polymorphism	Divergence			
*HA**	206	13.30	89	7.02	0.817	0.182	0.677
*HA***	126	32.79	71	17.15	1.077	-0.077	0.822
NA	797	25.37	97	16.16	0.773	0.226	0.450
MP	50	14.60	15	4.01	1.091	-0.091	0.890

*The clade of the triple-reassortant swine H1N1 that sporadically infected humans in or after 2005 was added into “species 1” and the clade of the classic swine H1N1 that sporadically infected humans before 1998 was added into “species 2”; ** The clade of the novel A/H1N1/2009 pandemic strains was added into “species 1” and the clade of the triple-reassortant swine H1N1 that sporadically infected humans in or after 2005 was added into “species 2”.

strain. As shown in Figure 3, the H1N1/2009 pandemic strain shared great homology with a representative triple-reassortant strain, A/Iowa/CEID23/2005 (H1N1), in the *PB2*, *PB1*, *PA*, *HA*, *NP*, and *NS* gene segments. The reassortment with the *NA* and *MP* gene segments of swine H1N1 viruses of Eurasian lineage might enable the triple-reassortant swine-origin H1N1 viruses to cause the pandemic.

Since the HA protein is critical for binding to cellular receptors and fusion of the viral and endosomal membranes (Neumann et al., 2009), we investigated the natural selection acting on this gene of the swine-origin viruses that infected humans from sporadic to pandemic. A positive selection might exist during the evolution of the *HA* gene from the classic swine H1N1 viruses that sporadically infected humans before 1998 to the triple-reassortant H1N1 viruses that sporadically infected humans from 2005 to 2009, possibly driving the adaptation of the classic swine H1N1 viruses to human at this stage. A positive selection might also act on the *NA* gene segment from the swine H1N1 virus of Eurasian lineage to the A/H1N1/2009 pandemic strain. The *NA* protein facilitates virus release from infected cells by removing sialic acids from cellular and viral *HA* and *NA* proteins (Neumann et al., 2009). Alterations of the *NA* are needed for the emergence of pandemic influenza viruses (Uhlendorff et al., 2009). The positive selection of the *NA* gene might drive the adaptation of this gene segment to

human. Thus, in addition to the reassortment between gene segments of influenza A viruses of different lineages, nucleotide variations within a given gene segment and subsequent selection might play a role in the evolutionary process of swine-origin H1N1 viruses infected humans from sporadic to pandemic. However, there are population bottlenecks that might limit evidential power of the McDonald-Kreitman tests.

This study has a number of limitations. Firstly, the viral sequences of the swine-origin viruses used in this study were mostly from the United States because sporadic infections with classic swine H1N1 before 1998 and with triple-reassortant swine H1N1 influenza A viruses since 2005 in persons with exposure to pigs were well-documented in this country, not in other countries.

Secondly, the patients with slight symptom and syndrome might not be reported, possibly resulting in underestimation of human infections with swine-origin H1N1 viruses before the outbreak. Thirdly, not all sequences from the reported cases were available in GenBank, which might have resulted in bias of the analyses based on the incomplete data.

In conclusion, this study clearly characterized the evolutionary process of swine-origin H1N1 influenza viruses that infected humans from sporadic to pandemic. In addition to the present knowledge, this study indicates that a representative triple-reassortant strain with intact sequence data of the 8 gene segments, A/Iowa/CEID23/2005 (H1N1), may be a suitable “intermediate” in the evolutionary process of A/H1N1/2009 pandemic viruses. Monitoring the evolutionary steps of animal-origin influenza A viruses that occasionally infect humans from swine workers is of epidemiological significance for the prediction of novel influenza pandemic.

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