Antigenic and genetic characterization of a European avian-like H1N1 swine influenza virus from a boy in China in 2011

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Abstract Cross-species transmission of influenza A viruses from swine to human occurs occasionally. In 2011, an influenza A H1N1 virus, A/Jiangsu/ALS1/2011 (JS/ALS1/2011), was isolated from a boy who suffered from severe pneumonia in China. The virus is closely related antigenically and genetically to avian-like swine H1N1 viruses that have recently been circulating in pigs in China and that were initially detected in European pig populations in 1979. The isolation of JS/ALS1/2011 provides additional evidence that swine influenza viruses can occasionally infect humans and emphasizes the importance of reinforcing influenza virus surveillance in both pigs and humans.

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Introduction

Influenza A viruses belong to the family *Orthomyxoviridae* and contain an eight-segmented, single-stranded, negative-sense RNA genome. They are classified into different subtypes based on structure and antigenic properties of the two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA) [47]. Wild aquatic birds are the primary natural reservoir of influenza A viruses and harbor all 16 of the currently known HA and all nine of the NA subtypes [11]. Although influenza A viruses can infect a variety of animals, each subtype has a limited number of hosts, i.e., humans (H1N1, H3N2, H2N2), pigs (H1N1, H3N2, and H1N2), horses (H3N8 and H7N7), dogs (H3N8), and terrestrial poultry (H5N1, H5N2, H7N7, H6N6 and H9N2) [32, 47]. In general, because of independent evolution of each virus subtype with its animal host, it is difficult for one subtype to cross interspecies barriers and infect another species. The molecular basis of host-range restriction and adaptation to a new host species is not completely understood, but the receptor-binding preference of the HA protein is believed to be an important factor in determining host-species tropism [32, 47]. Human influenza viruses prefer sialic acid (SA)-2,6-Gal-terminated saccharides that are abundant in human upper respiratory epithelium, whereas avian influenza viruses prefer those terminating with SA-a-2,3-Gal, abundant in duck intestinal epithelium. Therefore, avian influenza viruses usually cannot efficiently infect humans, despite sporadic human infection with avian H5N1 viruses in recent years. However, pigs are susceptible to both avian and human viruses because their tracheal epithelium possesses both types of receptors [19], and pigs have therefore been postulated to serve as mixing vessels for the generation of human, avian, and/or swine reassortant viruses with the potential to cause

human pandemics, as exemplified by the A (H1N1) pandemic 2009 (pdm09) virus that arose through reassortment of swine-, avian-, and human-origin viruses [39].

Influenza virus infection is an important febrile respiratory disease in pigs. Swine influenza viruses (SIV) are enzootic in pigs worldwide. Currently, three predominant subtypes of influenza virus are prevalent in pig populations worldwide: H1N1, H3N2, and H1N2 [5, 21, 45, 46]. To date, the antigenic and genetic diversity of swine H1N1 viruses have been documented in different regions of the world. The classical swine virus (H1N1) evolved from the 1918 pandemic flu virus and was first isolated in 1930. Since then, its progeny viruses have continued to circulate in swine populations in Asia and North America [5, 46]. Since 1979, the previously dominant classical H1N1 swine viruses have been replaced by the avian-like H1N1 viruses, which are closely related to Eurasia avian H1N1 viruses antigenically and genetically [5, 23]. These two distinct lineages of swine H1N1 viruses (classical and avian-like) showed different evolutionary trajectories during adaptation in swine [9]. Since 1984, reassortant H3N2 and H1N2 viruses containing human HA, NA, and avian-like internal genes (PB2, PB1, PA, NP, M, and NS) have circulated in pigs in Europe [5]. In North America, classical swine H1N1 viruses predominantly circulated in pigs before 1998, and triple-reassortant H1N1, H1N2, and H3N2 viruses containing genes of human, swine, and avian viruses have become established in pigs since then [5, 31, 46, 51].

The occurrence of cross-species transmission of influenza viruses from swine to humans is infrequent. Over 70 cases of human infections with swine influenza virus have been documented since the 1950s in North America, Europe, and Asia, and most cases were due to exposure to pigs [3, 25, 29, 38, 43]. In this study, we isolated and characterized a swine influenza virus from a child in China with severe pneumonia in January 2011. All genomic segments of this virus were closely related to those of European avian-like swine H1N1 viruses, which were concurrently circulating in pigs in China.

Materials and methods

Virus isolation and identification

Madin–Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco) with TPCK-trypsin (2 µg/ml), and the cell cultures were used for virus isolation. A bronchoalveolar aspirate sample was taken from the patient, stored in saline, and inoculated in MDCK cells. The cultures were incubated at 37 °C for 7 days and examined daily for cytopathic effects. The isolates were genotyped with a set of specific primers

in real-time reverse transcription-PCR (RT-PCR) assays for identifying influenza A and B [28].

Antigenic analysis

Hemagglutinin inhibition (HI) assays were used to determine the antigenic properties of influenza A virus isolates with hyperimmune rabbit sera to recent human seasonal H1N1, A(H1N1) pdm09, classical swine H1N1, and European avian-like H1N1. All antiserum samples were treated overnight with receptor-destroying enzymes and subsequently incubated at 56 °C for 1 h with 20 % chicken red blood cells. Twofold serial dilutions of each antiserum, starting from a 1:10 dilution, were tested for their ability to inhibit agglutination of chicken erythrocytes by four hemagglutinating units of influenza A virus. All HI assays were performed in duplicate.

Genomic sequencing and phylogenetic analysis

Viral RNAs were extracted from 140 µl of bronchoalveolar aspirate sample with an RNeasy Mini Kit (QIAGEN) as directed by the manufacturer. The primer Uni12 (5'-AG CGAAAGCAGG-3') was used for reverse transcription. PCR was performed with a set of primers that were specific for each gene segment of influenza A virus [18]. PCR products were purified by using a QIAamp Gel Extraction Kit (QIAGEN) and sequenced using an ABI 3730 DNA Analyzer (Applied Biosystems). All primer sequences are available upon request. Sequences were compiled with the Lasergene sequence analysis software package (DNAStar, Madison, WI, USA). Nucleotide BLASTn analysis (http://www.ncbi.nlm.nih.gov/BLAST) was used to identify related reference viruses, and reference sequences were obtained from GenBank. Pairwise sequence alignments were also performed with the MegAlign program (DNASTAR) to determine nucleotide and amino acid sequence similarities. To understand the evolutionary characterization of viruses isolated in this study, phylogenetic analysis of the aligned sequences for eight genomic segments was performed by the maximum composite likelihood method using MEGA 4.1 software [24]. The reliability of the unrooted neighbor-joining tree was assessed by bootstrap analysis with 1,000 replications; only bootstrap values >70 % are shown. Horizontal distances are proportional to genetic distance. Alignments of each influenza virus sequence were created using the program ClustalX 1.83.

Results

The patient

On December 31, 2010, a 3-year-old boy with onset of fever, cough, and fatigue was admitted to a hospital.

Shortly after administration procedures, the patient stopped breathing and was supported with mechanical ventilation. The patient became comatose and died 40 days later. Bilateral patchy infiltrates were observed by chest radiograph. The patient lived in a country house with cows, swine, chickens, geese, and ducks. The patient and his family had direct contact with the pigs, some of which showed signs of illness before he became ill. No other family members or persons who had close contact with the patient reported flu-like symptoms before or after his illness. From January to April 2011, we obtained 60 lung samples from slaughtered pigs collected from neighboring villages near where the patient had lived. Six viruses were isolated, and HI and NI assay showed that they belonged to European avian-like swine H1N1 subtype. Genomic sequencing and BLASTn analysis further confirmed these findings. Two of the isolates, A/swine/Jiangsu/s15/11/2011 (JS/s15/11) and A/swine/Jiangsu/s15/11/2011 (JS/s16/11), were selected as representing all six viruses. The complete genomes of s15/11 and s16/11 were sequenced, and their nucleotide sequences were deposited in the GenBank database under accession numbers JF820274-JF820289.

Isolation of virus from the patient

A bronchoalveolar aspirate sample was taken from the patient after his admission to the hospital on January 2, 2011. The specimen was sent to the influenza surveillance network laboratory of Jiangsu Provincial Center for Disease Prevention and Control, a member of the China influenza surveillance system. The sample tested positive for influenza A virus by real-time RT-PCR, but negative for human subtypes H1, A (H1N1) pdm09, H3, and avian H5, H7, and H9 influenza viruses.

A virus with hemagglutination activity was isolated from a bronchoalveolar aspirate sample using both 10-day-old embryonating eggs and MDCK cultures, and was designated A/Jiangsu/ALS1/2011. The isolate was determined to be of the European avian-like swine H1N1 subtype by genomic sequencing and nucleotide BLASTn analysis.

Antigenic characterization of the human isolate

HI tests revealed that JS/ALS1/2011 is antigenically related to swine H1N1 and A(H1N1) pdm09 viruses, such as A/Zhejiang/1/2007 (Sw/ZJ/1/2007), A/Jiangsu/1/2009 (JS/1/2009), and A/swine/Shanghai/1/2005 (Sw/SH/1/05), and is clearly distinguishable from seasonal human H1N1, represented by A/Nanjing/xu1/2009 (NJ/xu1/2009) (Table 1). The results also showed that HI cross-reactivity was observed among A (H1N1) pdm09, avian-like swine H1N1, and classical swine H1N1 viruses.

Phylogenetic analysis

The complete genome of JS/ALS1/2011 was sequenced, and the nucleotide sequences were deposited in the GenBank database under accession numbers HQ908437-HQ908444. The genotype and genetic origin of JS/ALS1/2011 were initially determined by a BLAST search of the GenBank database and pairwise comparisons of each gene segment to the corresponding sequences of reference viruses. The eight genomic segments of JS/ALS1/2011 shared the highest nucleotide acid sequence similarity with those of European avian-like H1N1 isolates circulating in China since 2006 (97.5 %-99.5 %) (Table 2), and the amino acid sequence similarity ranged from 97.5 % (Nuclear export protein, NEP) to 100 % (Matrix1, M1 and Matrix2, M2) (data not shown). This is clearly distinct from the corresponding genes of recent classical swine H1N1, human seasonal H1N1, and avian H1N1 virus isolates, represented by A/swine/Shanghai/1/2005 (Sw/SH/1/05), A/Brisbane/59/2007 (Brisbane/59/07), and A/duck/Italy/69238/2007 (Dk/Italy/69238/07), respectively (Table 2). NA and M genes of JS/ALS1/2011 were less closely related to those of reference A/Jiangsu/1/2009 (JS/1/2009; 89.9 % and 95.6 % similarity, respectively), a 2009 pandemic influenza A (H1N1) virus with the NA and M genomes derived from European avian-like swine H1N1 viruses. In contrast, relatively low sequence similarities (<84.4 %) were found with the other six genomic segments between JS/ALS1/2011 and A/JS/1/2009, including HA, nucleoprotein

Table 1 Haemagglutination-inhibition (HI) tests of A/Jiangsu/ALS1/1011 and recent human and swine H1N1 viruses

	Hyperimmune rabbit sera, titer*					Lineage
	JS/ALS1/2011	JS/1/2009	Sw/SH/1/2005	Sw/ZJ/1/2007	NJ/xu1/2009	
JS/ALS1/2011	**640**	320	320	640	<10	European avian-like swine H1N1
JS/1/2009	320	**1280**	320	320	<10	Pandemic 2009 A/H1N1
Sw/SH/1/2005	160	640	**1280**	640	<10	Classical swine H1N1
Sw/ZJ/1/2007	640	320	640	**640**	<10	European avian-like swine H1N1
NJ/xu1/2009	<10	<10	<10	<10	**1280**	Seasonal human H1N1

* Homologous hemagglutination-inhibition titers are in bold

(NP), nonstructural (NS), polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), and polymerase acidic (PA) gene. To characterize the genetic origin of the genome segments of JS/ALS1/2011 more precisely, we constructed phylogenic trees with reference viruses consisting of H1N1 viruses isolated from poultry, humans, and swine (Fig. 1). In the HA1 tree, there were seven distinct lineages, including seasonal human H1N1, classical swine H1N1, A (H1N1) pdm09, northern American triple reassortment H1, Eurasian avian H1N1, American avian H1N1, and European avian-like swine H1N1 viruses. JS/ALS1/2011 was clustered with European avian-like swine H1N1 viruses and located within a sub-lineage comprising swine viruses isolated in China from 2006 to 2010. The other seven lineages, revealed by the phylogenic trees of the other seven genes (NA, NP, M, NS, PB2, PB1 and PA), grouped JS/ALS1/2011 with European avian-like swine lineage in each of the seven trees. These results further confirmed that the eight genomic segments of JS/ALS1/2011 were closely related to those of the avian-like H1N1 viruses circulating in pig populations, especially those isolated in China.

Molecular characterization

Based on the deduced amino acid sequence, the human isolate contained an amino acid motif PSIQSR↓G at its HA cleavage sites, which is characteristic of low-pathogenic influenza viruses. Compared to reference H1N1 viruses, four potential glycosylation sites (Asn-X-Ser/Thr) were observed at positions 13, 26, 198, and 277 (according to H3 numbering) in the HA1 protein of A/Jiangsu/ALS1/2011. The receptor-binding specificity of HA has been proposed as a major determinant of the host range of a given influenza virus [27, 48]. Some studies have revealed that two amino acid mutations of H1 HA, E190D and G225D/E could cause a shift in receptor-binding specificity from the

Fig. 1 Phylogenetic trees for the HA1, NA, NP, M, NS, PB2, PB1, ▶ and PA gene segments of A/Jiangsu/ALS1/2011 and related reference viruses. The unrooted neighbor-joining phylogenetic trees were generated by the maximum composite likelihood model in MEGA 4.1 software (http://www.megasoftware.net). The reliability of the tree was assessed by bootstrap analysis with 1,000 replications. Bootstrap values are shown for selected nodes (only for those with a frequency greater than 70 %). Horizontal distances are proportional to genetic distance. The sequence length compared is as follows: HA (444 bp), NA (787 bp), NP (817 bp), M (729 bp), NS (786 bp), PB2 (2274 bp), PB1 (817 bp) and PA (833 bp). A/Jiangsu/ALS1/2011 is indicated with a black circle (•)

avian SA–a-2,3-Gal to the human SA-a-2,6-Gal [12, 42]. In this study, the two isolates possessed 190D and 225E, which might imply that the viruses had the same receptor-binding preference as human viruses (Table 3).
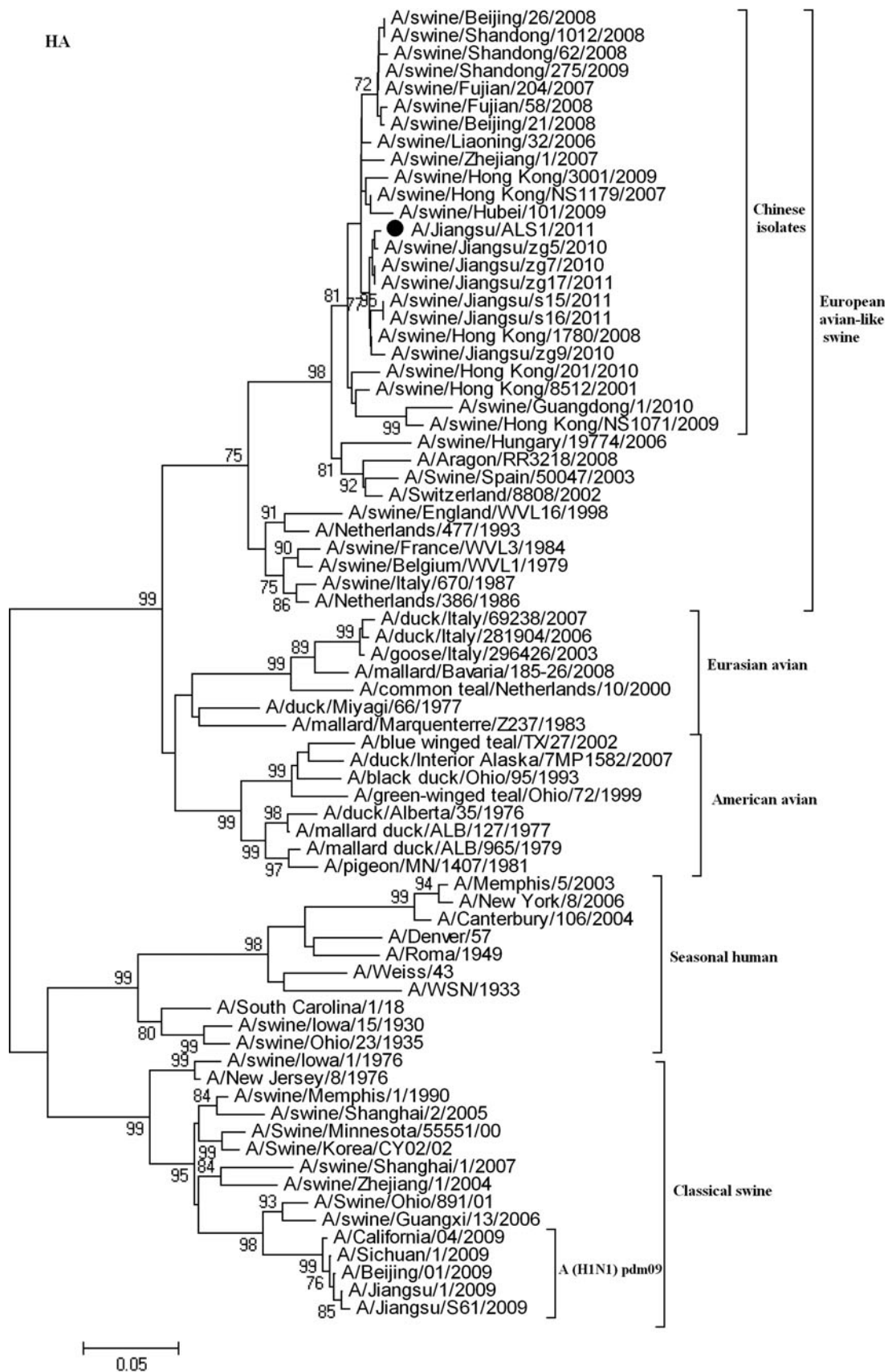
The isolates had a full-length stalk of NA, which is conserved in all classical swine H1N1 viruses. The amino acid substitutions (H274Y and N294S) were not observed in the NA proteins of A/Jiangsu/ALS1/2011, which suggests that it is sensitive to oseltamivir and zanamivir. The M2 proteins of the isolate have N rather than S at residue 31, which confers resistance to amantadine and rimantadine antivirals, a characteristic marker of the European swine viruses (H1N1, H3N2 and H1N2) since about 1987 [15, 23].

Based on studies of the 1918 pandemic H1N1 and highly pathogenic H5N1 viruses, many virulence determinants have been implicated in PB1-F2, a non-structural protein encoded by an alternative reading frame of the PB1 segment [2, 22]. PB1-F2 has been shown to localize to the mitochondria and induce apoptosis, which is an important pathogenic mechanism in influenza A virus infection and, in turn, to enhance viral virulence in a mouse model [6, 49]. The truncated PB1-F2 (with fewer than 87 amino acid residues) does not contain the mitochondrial translocation signal at the C-terminal end and may therefore lose its PB1-F2 function. With stop codons at amino acid positions

Table 2 Nucleotide sequence identity between A/Jiangsu/ALS1/2011 and reference strains available in GenBank

Gene[b]	Similarity (%)				
	Greatest similarity (%)	Sw/SH/1/2005	JS/1/2009	Brisbane/59/2007	Dk/Italy/69238/2007
PB2	Sw/HK/1780/2008 (99.0)	81.4	83.7	82.0	86.6
PB1	Sw/HK/1780/2008 (98.6)	80.8	84.4	80.8	88.4
PA	Sw/HK/1780/2008 (99.1)	80.7	84.2	82.8	85.6
HA	Sw/HK/1780/2008 (99.1)	75.5	74.0	74.3	82.8
NP	Sw/HK/1780/2008 (99.3)	82.2	82.4	83.3	89.6
NA	Sw/JS/zg5/2010 (98.6)	78.9	89.6	77.0	85.0
M	Sw/JS/s16/2011 (99.7)	87.3	95.6	86.2	92.2
NS	Sw/JS/s16/2011 (98.6)	83.1	81.1	83.3	71.0

[b] Nucleotide sequence identities were based on comparisons of the nucleotide sequences of PB2 (28-2307), PB1 (25-2298), PA (25-2175), HA (33-1733), NP (46-1542), NA (21-1430), M (26-1007) and NS (27-864)
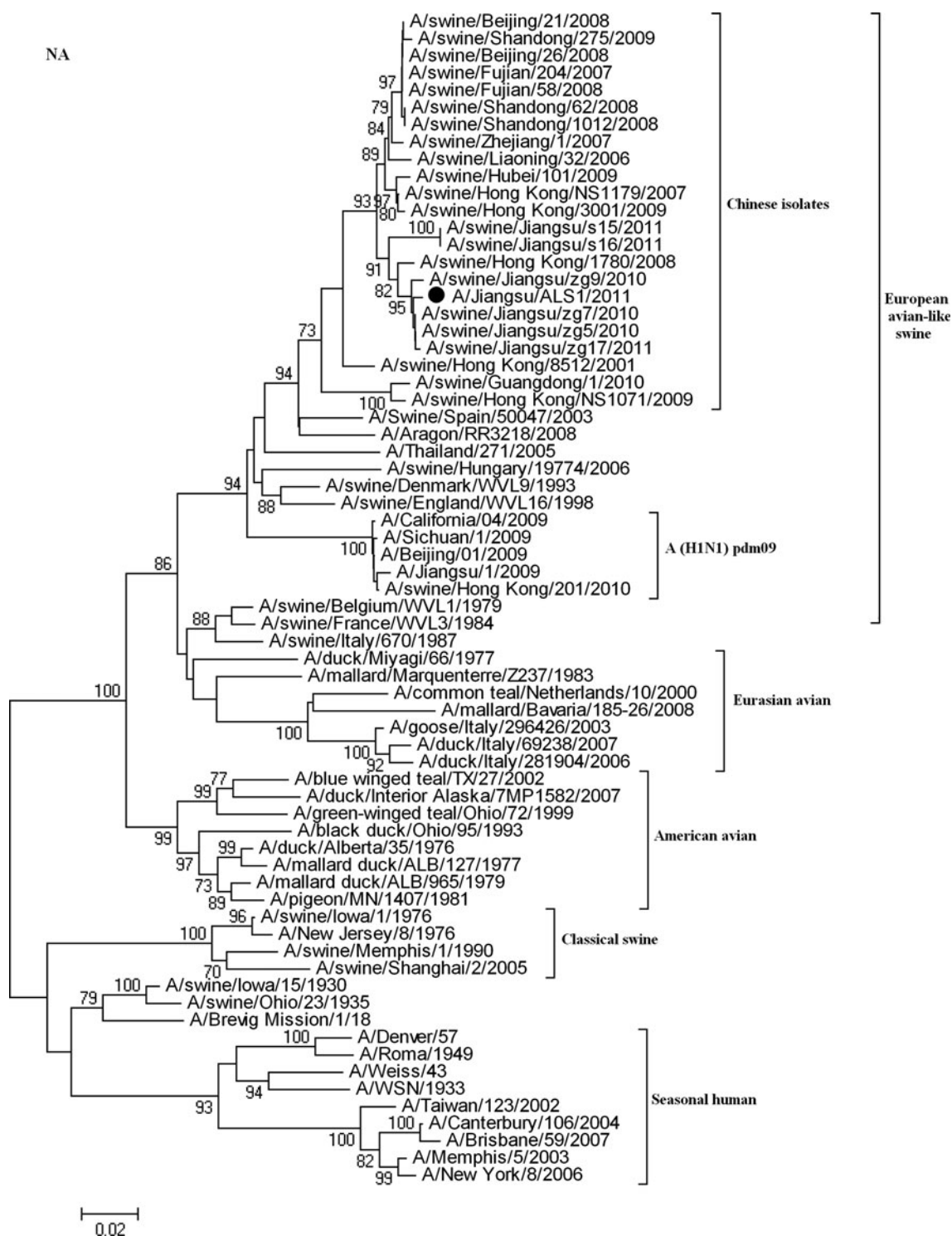
HA

A/swine/Beijing/26/2008
A/swine/Shandong/1012/2008
A/swine/Shandong/62/2008
A/swine/Shandong/275/2009
72 A/swine/Fujian/204/2007
A/swine/Fujian/58/2008
A/swine/Beijing/21/2008
A/swine/Liaoning/32/2006
A/swine/Zhejiang/1/2007
A/swine/Hong Kong/3001/2009
A/swine/Hong Kong/NS1179/2007
A/swine/Hubei/101/2009
● A/Jiangsu/ALS1/2011
81 A/swine/Jiangsu/zg5/2010
A/swine/Jiangsu/zg7/2010
A/swine/Jiangsu/zg17/2011
A/swine/Jiangsu/s15/2011
A/swine/Jiangsu/s16/2011
A/swine/Hong Kong/1780/2008
A/swine/Jiangsu/zg9/2010
98 A/swine/Hong Kong/201/2010
A/swine/Hong Kong/8512/2001
A/swine/Guangdong/1/2010
99 A/swine/Hong Kong/NS1071/2009
A/swine/Hungary/19774/2006
A/Aragon/RR3218/2008
81 A/Swine/Spain/50047/2003
92 A/Switzerland/8808/2002
91 A/swine/England/WVL16/1998
A/Netherlands/477/1993
90 A/swine/France/WVL3/1984
A/swine/Belgium/WVL1/1979
75 A/swine/Italy/670/1987
86 A/Netherlands/386/1986

Chinese
isolates

European
avian-like
swine

75
99

A/duck/Italy/69238/2007
99 A/duck/Italy/281904/2006
89 A/goose/Italy/296426/2003
99 A/mallard/Bavaria/185-26/2008
A/common teal/Netherlands/10/2000
A/duck/Miyagi/66/1977
A/mallard/Marquenterre/Z237/1983

Eurasian avian

A/blue winged teal/TX/27/2002
A/duck/Interior Alaska/7MP1582/2007
99 A/black duck/Ohio/95/1993
A/green-winged teal/Ohio/72/1999
98 A/duck/Alberta/35/1976
99 A/mallard duck/ALB/127/1977
A/mallard duck/ALB/965/1979
97 A/pigeon/MN/1407/1981

American avian

99

94 A/Memphis/5/2003
99 A/New York/8/2006
A/Canterbury/106/2004
98 A/Denver/57
A/Roma/1949
A/Weiss/43
A/WSN/1933

Seasonal human

99
A/South Carolina/1/18
80 A/swine/Iowa/15/1930
99 A/swine/Ohio/23/1935
99 A/swine/Iowa/1/1976
A/New Jersey/8/1976
84 A/swine/Memphis/1/1990
A/swine/Shanghai/2/2005
99 A/Swine/Minnesota/55551/00
84 A/Swine/Korea/CY02/02
95 A/swine/Shanghai/1/2007
A/swine/Zhejiang/1/2004
93 A/Swine/Ohio/891/01
A/swine/Guangxi/13/2006
98 A/California/04/2009
A/Sichuan/1/2009
99 A/Beijing/01/2009
76 A/Jiangsu/1/2009
85 A/Jiangsu/S61/2009

Classical swine

A (H1N1) pdm09

0.05

Fig. 1 continued

11 and 77, our isolates contained truncated PB1-F2 proteins. The amino acid at 627 of PB2 has previously been shown to be a determinant of host specificity, and the majority of avian influenza viruses have E at this position, whereas all human influenza viruses (H1N1, H2N2, and H3N2) have K [17, 41]. In this study, the isolate possessed an E at position 627 of PB2, which is characteristic of avian influenza viruses. A previous study demonstrated that
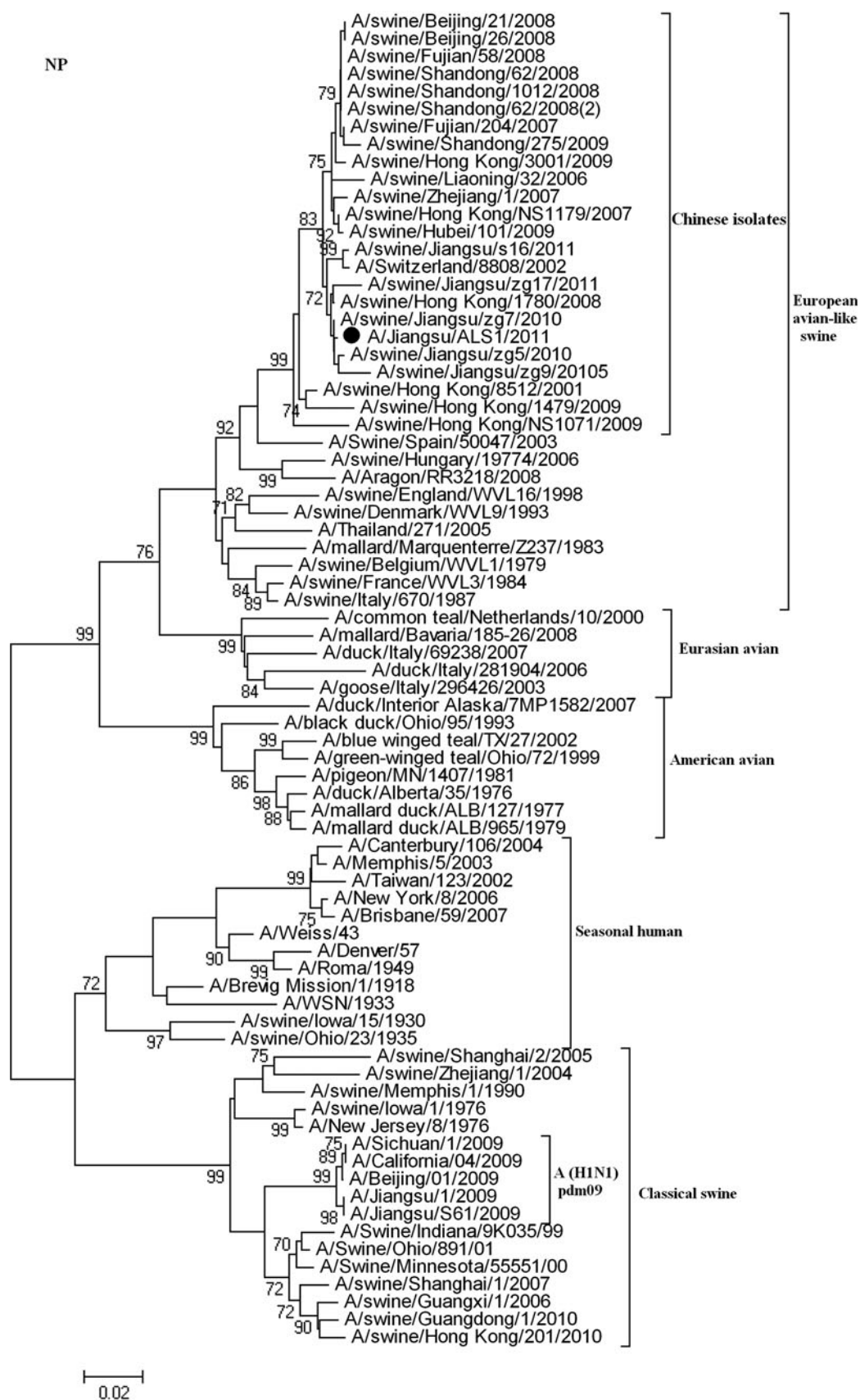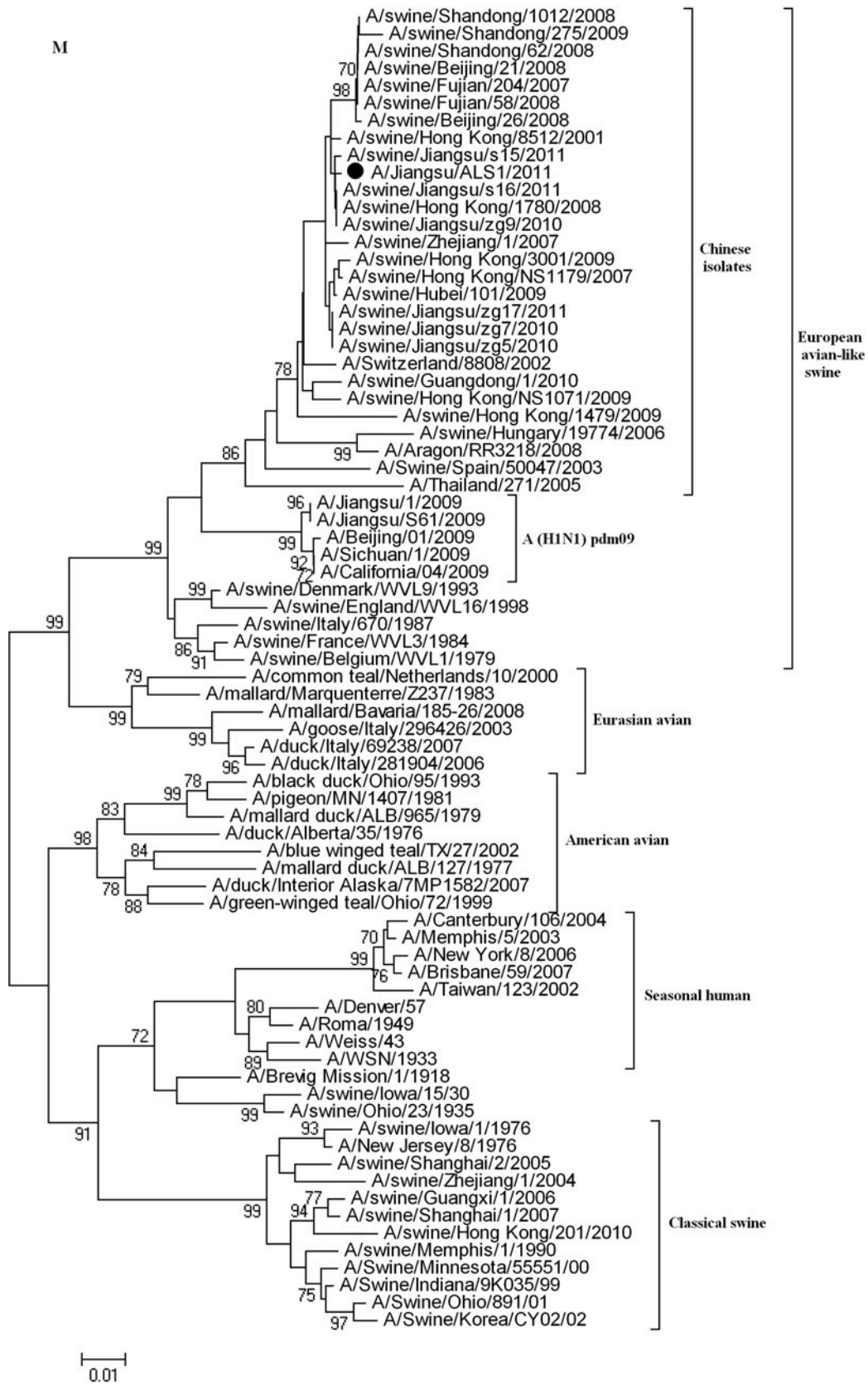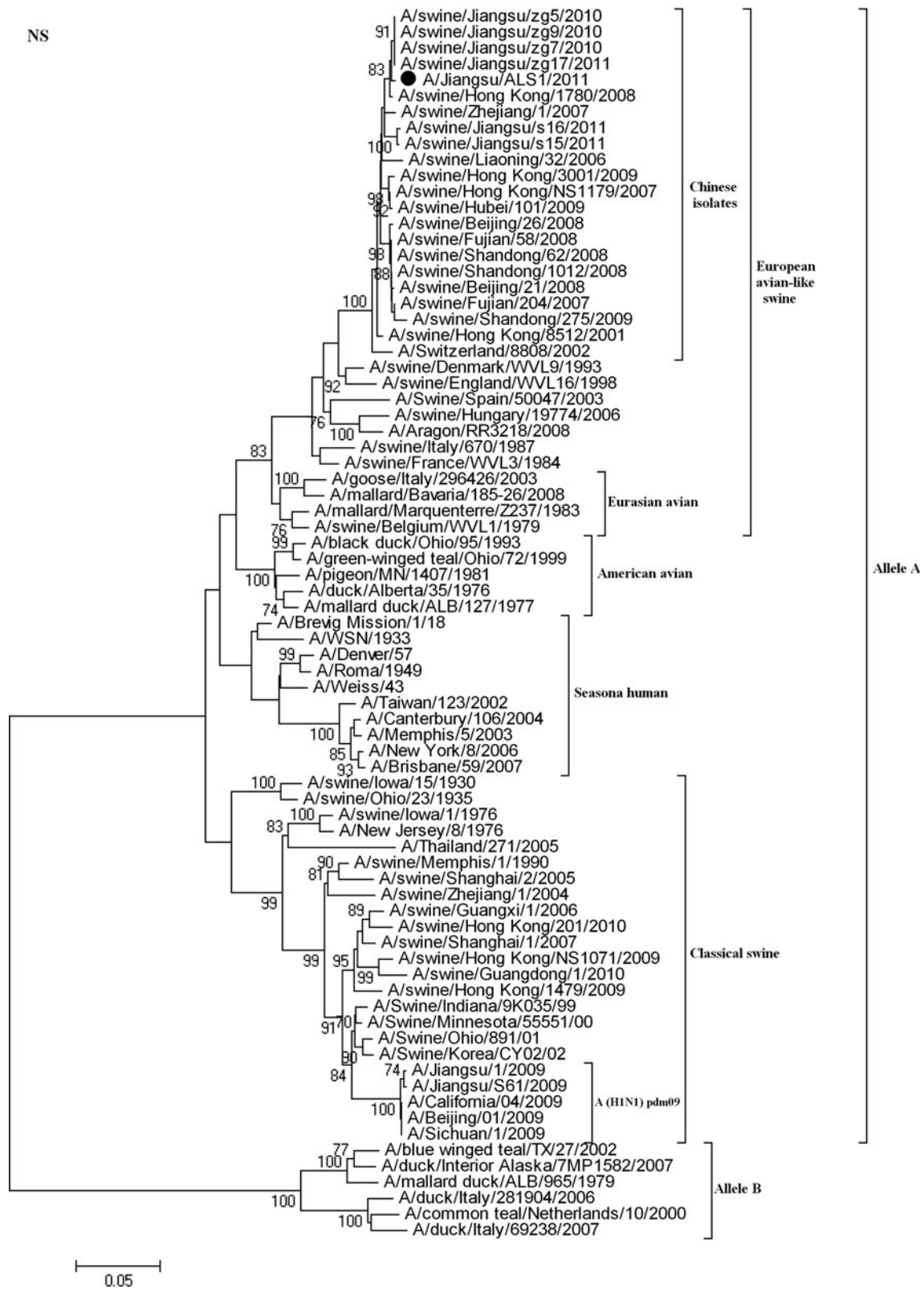
NP

A/swine/Beijing/21/2008
A/swine/Beijing/26/2008
A/swine/Fujian/58/2008
A/swine/Shandong/62/2008
A/swine/Shandong/1012/2008
A/swine/Shandong/62/2008(2)
A/swine/Fujian/204/2007
A/swine/Shandong/275/2009
A/swine/Hong Kong/3001/2009
A/swine/Liaoning/32/2006
A/swine/Zhejiang/1/2007
A/swine/Hong Kong/NS1179/2007
A/swine/Hubei/101/2009
A/swine/Jiangsu/s16/2011
A/Switzerland/8808/2002
A/swine/Jiangsu/zg17/2011
A/swine/Hong Kong/1780/2008
A/swine/Jiangsu/zg7/2010
A/Jiangsu/ALS1/2011
A/swine/Jiangsu/zg5/2010
A/swine/Jiangsu/zg9/20105
A/swine/Hong Kong/8512/2001
A/swine/Hong Kong/1479/2009
A/swine/Hong Kong/NS1071/2009
A/Swine/Spain/50047/2003
A/swine/Hungary/19774/2006
A/Aragon/RR3218/2008
A/swine/England/WVL16/1998
A/swine/Denmark/WVL9/1993
A/Thailand/271/2005
A/mallard/Marquenterre/Z237/1983
A/swine/Belgium/WVL1/1979
A/swine/France/WVL3/1984
A/swine/Italy/670/1987

Chinese isolates

European avian-like swine

A/common teal/Netherlands/10/2000
A/mallard/Bavaria/185-26/2008
A/duck/Italy/69238/2007
A/duck/Italy/281904/2006
A/goose/Italy/296426/2003

Eurasian avian

A/duck/Interior Alaska/7MP1582/2007
A/black duck/Ohio/95/1993
A/blue winged teal/TX/27/2002
A/green-winged teal/Ohio/72/1999
A/pigeon/MN/1407/1981
A/duck/Alberta/35/1976
A/mallard duck/ALB/127/1977
A/mallard duck/ALB/965/1979

American avian

A/Canterbury/106/2004
A/Memphis/5/2003
A/Taiwan/123/2002
A/New York/8/2006
A/Brisbane/59/2007
A/Weiss/43
A/Denver/57
A/Roma/1949
A/Brevig Mission/1/1918
A/WSN/1933
A/swine/Iowa/15/1930
A/swine/Ohio/23/1935

Seasonal human

A/swine/Shanghai/2/2005
A/swine/Zhejiang/1/2004
A/swine/Memphis/1/1990
A/swine/Iowa/1/1976
A/New Jersey/8/1976
A/Sichuan/1/2009
A/California/04/2009
A/Beijing/01/2009
A/Jiangsu/1/2009
A/Jiangsu/S61/2009
A/Swine/Indiana/9K035/99
A/Swine/Ohio/891/01
A/Swine/Minnesota/55551/00
A/swine/Shanghai/1/2007
A/swine/Guangxi/1/2006
A/swine/Guangdong/1/2010
A/swine/Hong Kong/201/2010

A (H1N1) pdm09

Classical swine

0.02

Fig. 1 continued

Fig. 1 continued

NS

Fig. 1 continued

Fig. 1 continued

PB1

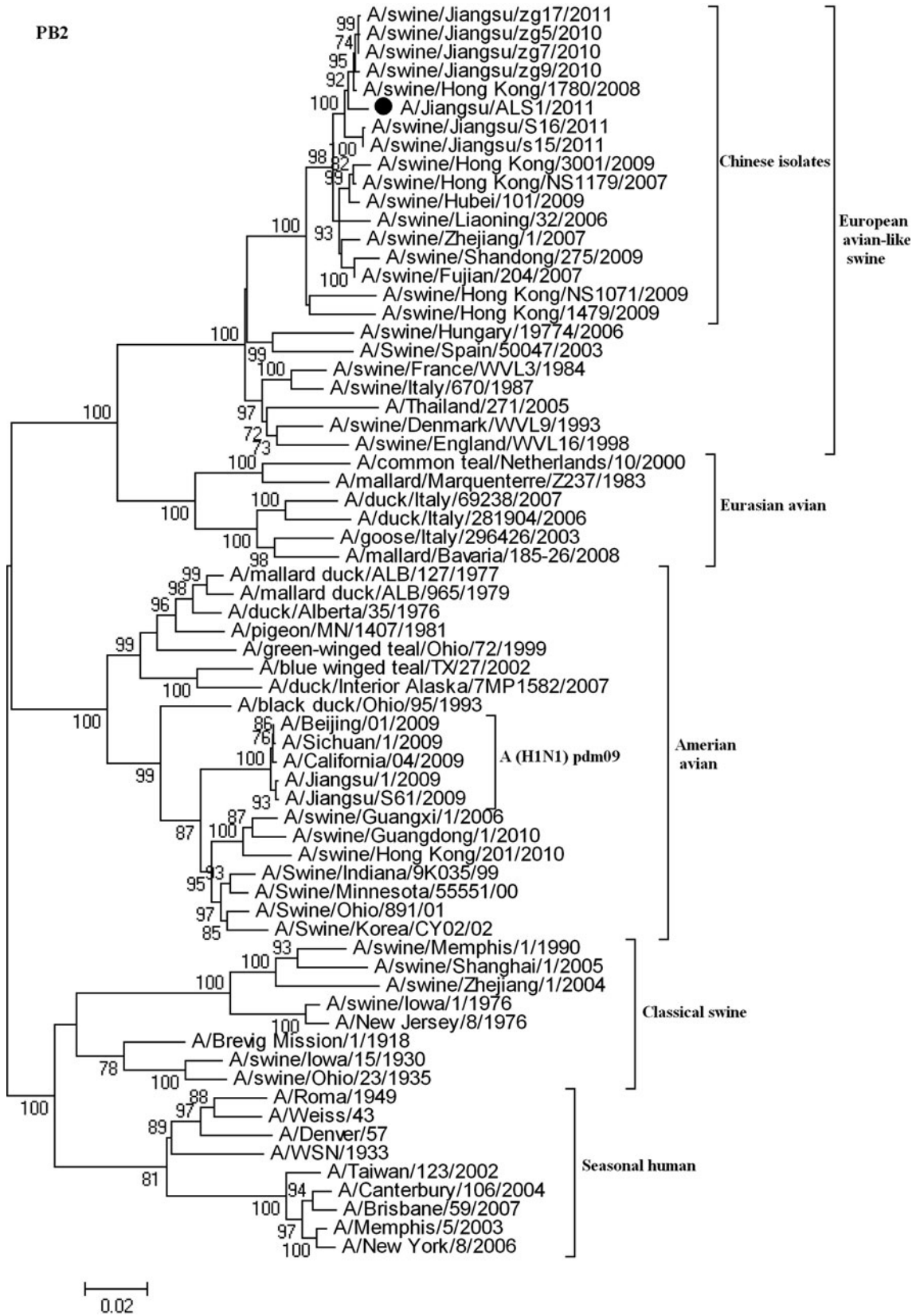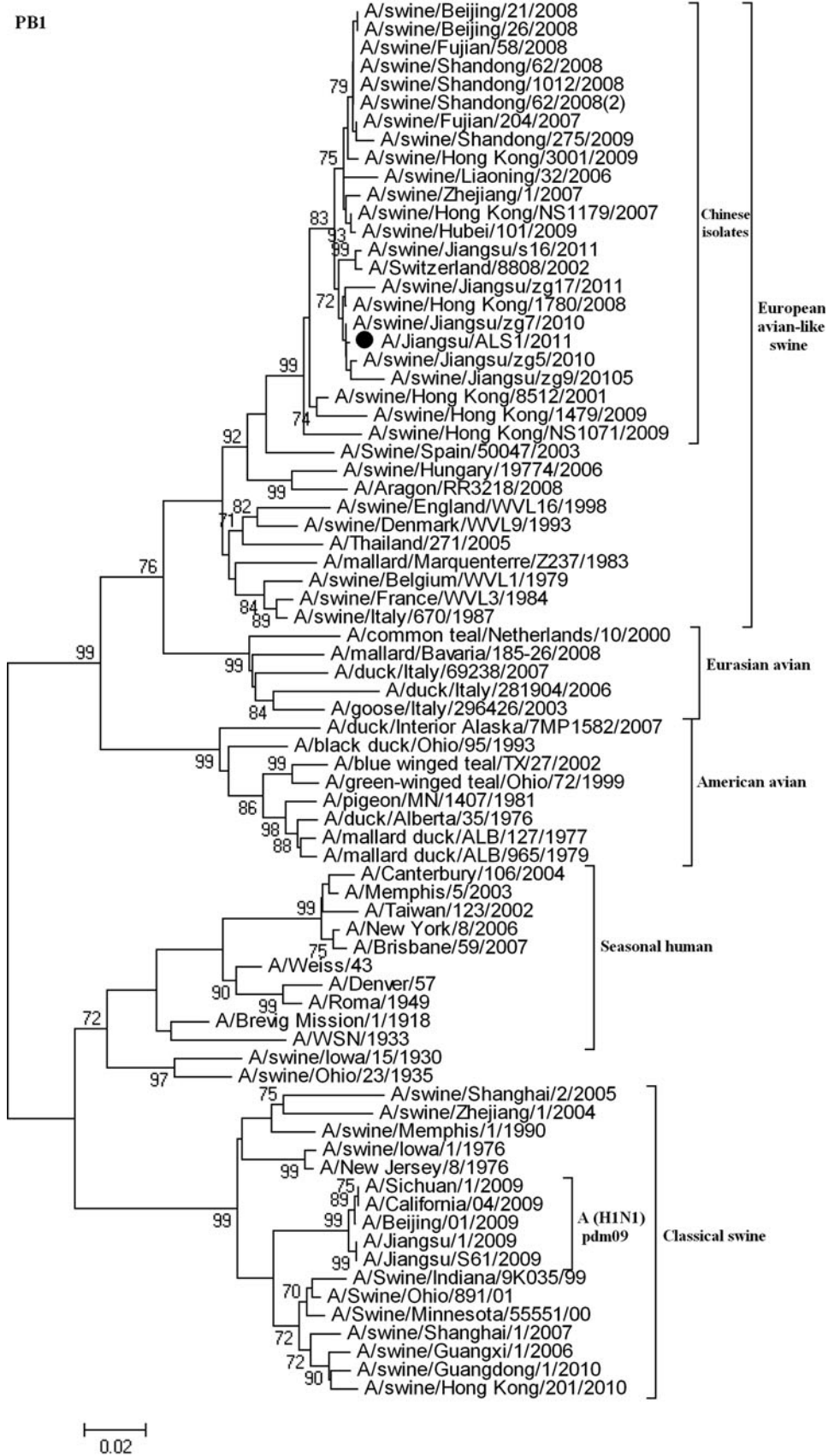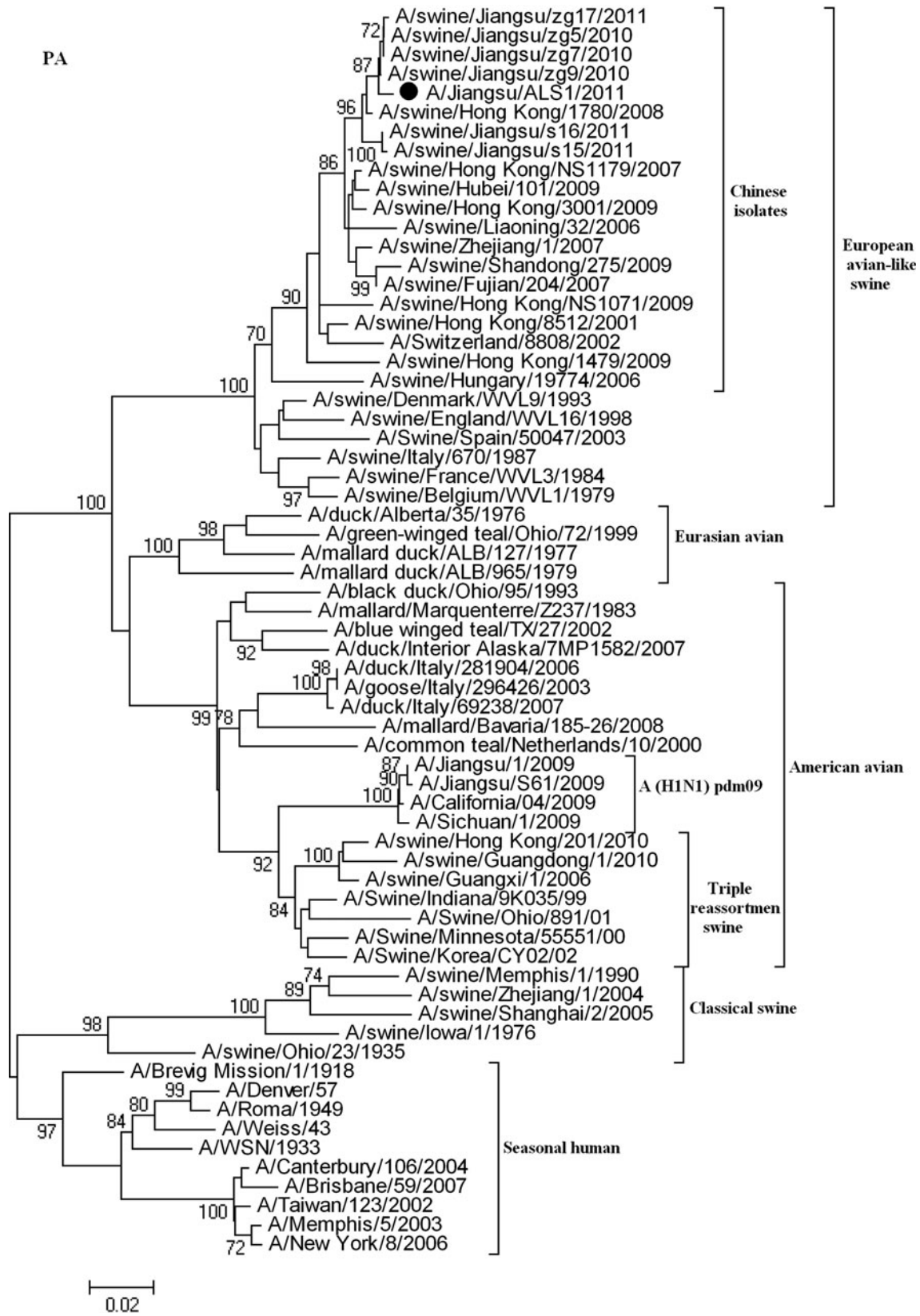
Fig. 1 continued

PA

Fig. 1 continued

Table 3 Amino acid residues at receptor-binding sites of HA proteins of A/Jiangsu/ALS1/2011 and the reference viruses

Virus	Residues at receptor-binding sites of HA proteins*								Lineage
	71	138	155	159	186	190	194	225	
A/California /04/2009	E	A	V	N	S	D	L	D	Pandemic 2009, A/H1N1
A/Jiangsu/ALS1/2011	D	A	V	N	S	D	L	E	Avian-like swine, Europe
A/swine/Shanghai/1/2005	E	A	V[#]	N	P	D	L	G	Classical swine
A/Brisbane/59/2007	E	S	T[#]	G[#]	P	D	L	D	Seasonal human
A/Duck/Italy/69238/07	D[#]	A[#]	T[#]	T[#]	P[#]	E[#]	L[#]	G[#]	Avian
A/swine/BJ/226/2008	D	A	V	N	P	D	L	G	Avian-like swine, Europe
A/South Carolina/1/181)	D	A	T[#]	S[#]	P	D[#]	L	D	Spain pandemic, 1918

* Residues numbering is aligned to the H3 virus HA

[#] The amino acid is conserved in all sequences in this host

a mutation at position D92E of the NS1 gene may increase the virulence of H5N1 viruses in pigs [37]. In this study, the NS1 protein of the isolates possessed D rather than E at position 92. As a PDZ ligand domain involved in cellular signaling pathways, the four C-terminal residues of the NS1 protein, ESEV or EPEV, were found from avian influenza as well as the 1918 H1N1 viruses, which might be a new virulence factor for influenza A viruses [20]. The NS1 protein of our isolates contained a GPKV motif in the PDZ ligand domain, which is distinct from those of avian, human, and classical swine viruses.

Discussion

Our study showed that JS/ALS1/2011 was closely related, antigenically and genetically, to avian-like swine H1N1 viruses initially detected in European pig populations in 1979 and recently found circulating in pigs in China. Phylogenetic analysis indicated that all eight genomic segments of JS/ALS1/2011 grouped with the European avian-like swine lineage, and reassortment with human or avian viruses was not observed. Recently, Zhao et al. isolated 32 avian-like swine H1N1 influenza viruses from 1,030 pig nasal swab sample collected from May 2010 to May 2011, and the eight gene segments of some of those isolates had 99.1-99.8 % sequence identity with JS/ALS1/2011 [50]. Their results indicate that avian-like H1N1 viruses remain endemic in swine in China, which is in agreement with our findings in this study. The isolation of JS/ALS1/2011 provides additional evidence that swine influenza viruses can occasionally infect humans, even though they are antigenically and genetically distinct from human influenza viruses.

To our knowledge, this is the first human infection with a swine influenza virus in mainland China. Since January 1986, at least nine human infections with European avian-like swine viruses have taken place: six caused by avian-like H1N1 and three by reassortant avian-like H3N2 virus [1, 7, 8, 14, 15, 29, 36]. Among these events, a case of infection with swine H3N2 virus likely derived from pigs in southern China was reported in Hong Kong in 1999, and this was the first case of infection with similar viruses reported outside Europe [15]. Few data pertaining to swine influenza virus infections in humans have been available in China since then.

Swine husbandry practices provide close contact between pigs and humans and present the opportunity for interspecies transmission of influenza viruses in China, where over 60 % of the world's pig population is raised. Serologic studies in the United States indicate that humans exposed to swine, especially those working with them, are at increased risk for zoonotic influenza virus infection [13, 30]. In contrast, actual incidence rates of infections with swine influenza viruses in the general population in China are unknown, which stresses the need for surveillance in humans exposed to pigs, especially those in close, regular contact with them.

Influenza virus infection in pigs was first described in 1918 in China, which coincided with the so-called Spanish pandemic in humans [5, 13]. Studies on influenza viruses from pigs in China revealed that the classical swine H1N1 viruses, co-circulating with human-like H3N2 viruses, circulated in the pig population until 2006 [13, 33, 35]. In 1993-1994, avian-like H1N1 viruses that were antigenically and genetically distinguishable from those in Europe were detected in pigs and co-circulated with classical H1N1 viruses in southern China [16]. European avian-like swine H1N1 began to appear in 2006, and may have been prevailing in pigs in China since then [4, 26, 45]. A previous study revealed that European reassortant swine H3N2 entered into pigs in southern China during the 1990s [15]. North American triple-reassortant swine H1N2 and H3N2 viruses were also isolated in recent years [45]. In 2004, we detected three reassortant H1N2 viruses that were genetically distinguishable from other H1N2 viruses found in

pigs worldwide [34]. These findings demonstrate that genetic evolution of influenza viruses is complex and diverse in pigs in China. Genetic reassortment may be a primary mechanism for generating new pandemic strains, such as the pandemic influenza viruses of 1957 (H2N2), 1968 (H3N2), and A (H1N1) pdm09 derived from reassortment of avian, human and/or swine viruses. Because of susceptibility to both avian and human viruses, pigs may serve as ideal intermediate hosts for the generation of potential pandemic influenza viruses by reassortment. Following the generation of the A (H1N1) pdm09 viruses, pdm09-like and reassortant viruses containing genes of pdm09-like and other influenza viruses have often been detected in pigs from different countries, including China [40, 44, 45, 52]. Most recently, new H3N2 reassortant viruses with the pdm09 internal genes have prevailed in pigs in southern China [10]. From August to December 2011, 11 human cases were reported to be infections with novel triple reassortant H3N2 viruses of swine origin that have an M gene from A(H1N1) pdm09 [25]. All of these findings further emphasize the importance of surveillance for genetic diversity of influenza A viruses in pigs and raise more concerns about the occurrence of cross-species transmission events.

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