Characterization of H3N6 avian influenza virus isolated from a wild white pelican in Zambia.

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Abstract

We characterized an influenza virus isolated from the Great White Pelican in Zambia. Phylogenetic analyses showed that all gene segments belonged to the Eurasian lineage and that they appear to have evolved in distinct geographical regions in Europe, Asia, and Africa, suggesting reassortment of virus genes maintained in wild aquatic birds whose flyways overlap across these continents. It was notable that this virus might possess some genes of the same origin as those of highly pathogenic H7 and H5 viruses isolated in Eurasia. The present study underscores the need for continued monitoring of avian influenza viruses in Eurasia and Africa.

Aquatic birds of the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and shorebirds) are thought to constitute the major natural reservoir for avian influenza (AI) A virus [20,24]. All known influenza A virus subtypes with respect to two surface glycoproteins, hemagglutinin (HA) (H1-H16) and neuraminidase (NA) (N1- N9) and most HA/NA combinations have been identified in wild birds and poultry [11,24]. Influenza A viruses of avian origin have been implicated in outbreaks of influenza in other hosts [13,20,24], indicating that a vast influenza virus gene pool for future epidemics in other animal species including human pandemics exists in avian sources.

Highly pathogenic (HP) H5N1 AI virus has spread from Asia to other regions, including Europe, the middle East, and Africa causing outbreaks in domestic poultry and wild birds [5,10,15]. As of 17 June 2009, Egypt had recorded the highest number of H5N1 human infections in Africa, 78 confirmed cases with 27 fatalities [25]. The origins and transmission routes of HP H5N1 virus initially from Asia to Africa remain unclear. The potential spread of HP H5N1 virus by wild birds over large geographical regions and the direct zoonotic threat posed by several AI viruses of the Eurasian lineage underscore the need for more information on ecology and evolution of AI A viruses circulating in the wild bird reservoir globally.

In attempting to narrow the knowledge gap that exists in the ecology of AI viruses circulating in wild birds in Africa, virologic surveillance studies were initiated in Zambian wetlands frequented by migratory birds. We report the characterization of the first influenza virus isolate from an avian host in Zambia.

In August 2006, fifty one fresh fecal samples were collected from apparently healthy pelicans in

Lochinvar national park (15^{o} 40' S; 27^{o} 15' E), in Southern province of Zambia. Virus isolation was attempted in 10- to 11-day- old embryonated chicken's eggs. One influenza virus isolate was obtained and designated A/pelican/Zambia/01/06 (H3N6) (Zb06) following subtyping by standard hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests using specific antisera to the reference strains of influenza viruses. We then prepared chicken antisera against Zb06. Briefly, purified virus was inactivated with 0.1% formalin at 4^{o} C for one week. Three-month-old specific-pathogen-free chickens were immunized intramuscularly and subcutaneously with 100µl of 300µg inactivated virus with Complete Freund's Adjuvant (DIFCO). The chickens were re-immunized two weeks later similarly but with Incomplete Freund's Adjuvant. The chickens were given a third intravenous booster injection without adjuvant three weeks after the second immunization. One week after the final immunization, the chickens were sacrificed to obtain serum. We used A/Puerto Rico/8/34 (H1N1) (PR8), A/duck/Hong Kong/836/80 (H3N1) (DHK836), A/Aichi/2/68 (H3N2) (Aichi), A/Memphis/1/96 (H3N2) (Mem96), A/Czeckoslovakia/56 (H4N6) (Czec56), A/duck/England/1/56 (H11N6) (Eng56), A/gull /Maryland/704/77 (H13N6) (MD77), and Zb06 for antigenic characterization by HI and NI assays. Chicken antiserum were raised against these viruses except DHK836. Chicken erythrocytes (0.5%) and Fetuin, Fetal Bovine Serum (CALBIOCHEM), were used in the HI and NI assays, respectively.

In antigenic analyses, chicken antiserum raised against Zb06 showed high HI and NI titers roughly equally to all the H3 and N6 influenza viruses tested, including the relatively recent human strain, Mem96 (Table 1), indicating that chicken antiserum raised against Zb06 has high cross reactivity. The reason for the high cross reactivity is unclear, but one possibility is that antibodies raised against Zb06 predominantly recognize the conserved epitopes of the surface

glycoproteins of the viruses tested. Chicken antiserum raised against Zb06 could therefore be useful in diagnosis of H3 and N6 influenza viruses. On the other hand, Zb06 did not react with chicken antisera raised against Mem96 but reacted with antiserum against Aichi (<40 and 320 HI titers, respectively), confirming antigenic drift which has been observed since this virus was first introduced into the human population [2].

For genetic analyses, viral RNA was extracted and amplified by RT-PCR as described previously [12]. PCR products were purified from agarose gels and then sequenced directly using BigDye terminator cycle sequencing ready reaction kit and analyzed on a 3130 Genetic analyzer (Applied Biosystems). The nucleotide sequences obtained in this study will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers **AB470293** to **AB470300**. Phylogenetic trees were constructed using the neighbor-joining bootstrap method (1,000 replicates) in MEGA4.

The entire genome of Zb06 was completely sequenced and analyzed with the basic local alignment search tool (BLAST) available from Genbank (Table 2). We found that the HA, PB2, and NS genes were highly similar (97-99%) to duck/South Africa/1108/04 (H3N8) (SAH3). The NP and PB1 genes showed 97% nucleotide similarity with H7N1 and H7N3 influenza viruses isolated from Italian poultry, respectively. The closest relative of Zb06 M gene was duck/Mongolia/54/2001 (H5N2) (98% nucleotide similarity). The NA segment was close to mallard/Germany/Wv1806-09k/03 (H4N6) with 96% nucleotide identity. The PA gene showed close sequence identity (98%) to H5N3 virus, teal/Italy/3812/05.

The HA, NA, NP, and PA gene phylograms are shown in Fig. 1. Phylogenetic analysis of the HA

gene of Zb06 showed the separation of the viruses into the Eurasian, American, and human-swine lineages (Fig. 1a). Sublineages 1-3 are distinguishable within the Eurasian lineage. The HA gene of Zb06 was closely related to that of SAH3, and belonged to the first sublineage, comprising viruses isolated mainly from the Far East and Europe. The much older virus, duck/Ukraine/1/63 (H3N8) and a swine isolate from Mongolia constituted the second Eurasian sublineage. The third sublineage is composed of H3N2 viruses isolated from fecal specimens collected from live poultry markets in Korea [21]. The NA gene tree of Zb06 revealed the assortment of viruses into the Eurasian, Eurasian-American, Oceania, and American lineages (Fig. 1b). Two sublineages were apparent within the Eurasian lineage, "contemporary" and 1970s and 1980s viruses (designated 1 and 2, respectively). The NA gene of Zb06 fell in the "contemporary" sublineage and was closely related to H4N6 viruses isolated from Germany and Norway. Aside from Zb06 and the two H4N6 European strains, all viruses of the "contemporary" sublineage were of Asian origin. Except for three viruses, Eng56, Czec56, and duck/Siberia/272/98 (H13N6) the Eurasian-American lineage was composed exclusively of shorebird and gull viruses isolated in America and Eurasia.

Phylograms of the internal protein genes (NP and PA) (Fig. 1c, and d) of Zb06 showed the clustering of strains of the Eurasian lineage into sublineages as previously described [9]. In the NP phylogeny, four groups or sublineages are recognized. The first group consists of recent isolates from Europe, Asia and South Africa, including HP H5N1 viruses isolated from ducks and chickens in China, and from whooper swans in Japan. The NP gene of Zb06 belonged to the second group, consisting of "early" European strains represented by Dk/Potsdam/2216-4/84 (H5N6) and some recent isolates, including H7N1 Italian poultry viruses [1]. The third sublineage was composed of a single isolate, Dk/Hokkaido/120/01 (H6N2). The fourth group of

the Eurasian lineage comprises 3 strains isolated from 1956 to 1961. The PA phylogram was topologically similar to that of the NP gene tree. In contrast to the NP gene that clustered with H7N1 Italian poultry viruses, the PA gene of Zb06 was closely related to that of teal/Italy/3812/05 (H5N3) and grouped together with those of the Asian HP H5N1 viruses, suggesting a common source of the PA gene of these viruses.

The close relation of the HA, and internal (NS and PB2) protein genes (Supplementary Fig. S1) of Zb06 to those of wild bird isolates from South Africa suggests that some reassortment may have occurred within sub-Saharan Africa due to the interaction of wild birds through the intra-African flyways. Ring recoveries of water birds in Southern Africa have shown that some waterbirds are migratory within southern Africa, while others show dispersal as far as central Africa [23]. Phylogenies of the NP, and other internal (NS, PB1, and M) protein genes (Supplementary Fig. S1) of Zb06 showed that they were closely related to H7 influenza viruses isolated from Italian poultry in 1999, suggesting that viruses of the same origin as Zb06 may have contributed some internal protein genes to viruses that caused epidemics of AI of H7 viruses that have been observed in Europe since 1997 [1,3,4,8]. Phylogenetic analyses of AI viruses isolated from wild ducks and domestic poultry in Italy revealed that the precursor H7 virus for AI in domestic poultry was directly introduced from migratory birds [4]. The close similarity of these genes of Zb06 to those of the viruses isolated in Italy leads us to speculate that these viruses may have infected their avian hosts on the Black Sea/Mediterranean flyway which, together with the East Africa/West Asia flyway pass through Zambia. We acknowledge the need for caution in interpreting our data because only very limited sequence data from African wild birds are available in Genbank.

Until now, there was no report of influenza virus isolation from the Great White Pelican (*Pelecanus onocrotalus*). Influenza virus (H6N1) isolation from Great Cormorant, a member of the order Pelecaniformes, has been reported [22]. Other studies did not yield positive results of influenza virus isolation from this order [17,19,20]. The Great White pelican is endemic in Southern Africa. Limited breeding sites exist in the region including two in South Africa, and one in Namibia [6,7]. Large colonies of white pelicans congregate in Lochinvar national park sharing same habitat with other bird species in which AI viruses have been frequently isolated worldwide. The role of "minor" bird reservoirs in influenza virus ecology is unclear. It remains to be determined in which of these species influenza viruses are endemic and in which the virus is a temporary pathogen [19,20].

Available evidence suggests that the rapid spread of HP H5N1 virus from Qinghai lake, China, to Europe and Africa may have involved migratory birds and possibly the poultry trade [14]. The close relation of the PA gene of Zb06 to those of the Asian HP H5N1 viruses implies that wild birds could carry and spread, at least in part, genes of the same origin as those of HP AI viruses over large geographical regions. The overlap of multiple migratory flyways within Eurasia and Africa permits virus-infected birds of different bird populations to transmit pathogens to new hosts that may carry them to new areas [20].

While AI A viruses have evolved into two genetically distinct lineages, Eurasian and American, possibly due to long-term confinement of birds to distinct flyways [20,24], transcontinental introduction of AI virus genes has been described between the two lineages [16,18]. For instance, PB2 and PA genes of the American lineage were detected in H2 viruses isolated in Japan, and the H2 HA genes of Eurasian lineage was present in American birds. Our findings

highlight that the gene segments of Zb06 appear to have been derived from multiple virus sources in Eurasia and Africa. Furthermore, our results indicate that wild waterfowl could play a role in the dissemination of genes of common origin as those of HP AI viruses over large geographical regions, thus underscoring the need for continued AI virus surveillance in Zambian wetlands as part of a global program.

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TABLE 1. Antigenic characterization of Zb06 by HI and NI assays

		HI titer of chicken antisera						NI titer of chicken antisera				
Virus	Subtype	Zb06	Aichi	Mem96	PR8	Virus	Subtype	Zb06	Eng56	MD77	Czec56	PR8
Zb06	H3N6	**5,120**[a]	320	<40	<40	Zb06	H3N6	**2,560**[a]	2,560	640	320	<10
Aichi	H3N2	5,120	**10,240**	160	<40	Eng56	H11N6	1,280	**2,560**	320	320	<10
Mem96	H3N2	2,560	<40	**10,240**	<40	MD77	H13N6	5,120	2,560	**1,280**	640	<10
DHK836	H3N1	2,560	640	<40	<40	Czec56	H4N6	2,560	2,560	1,280	**1,280**	<10
PR8	H1N1	<40	<40	<40	**5,120**	PR8	H1N1	<10	<10	<10	<10	**1,280**

[a] Homologous HI and NI titers are in boldface type and are underlined

TABLE 2. Influenza viruses with highest nucleotide sequence similarity to Zb06

Gene (nucleotide positions of Zb06 compared)	Virus with highest degree of sequence identity	Subtype	Identity (%)	GeneBank accession no.
HA (77-1063)	A/duck/South Africa/1108/2004	H3N8	97	EF041487
NS (57-711)	A/duck/South Africa/1108/2004	H3N8	98	EF041491
PB2 (1468-2193)	A/duck/South Africa/1108/2004	H3N8	99	EF041493
NP (751-1,483)	A/turkey/Italy/3560/1999	H7N1	97	CY025168
PB1 (1429-2178)	A/turkey/Italy/9739/2002	H7N3	97	CY031617
M (197-868)	A/duck/Mongolia/54/2001	H5N2	98	AB301916
NA (38-1,264)	A/mallard/Germany/Wv1 806-09k/2003	H4N6	96	AM933235
PA (1,456-2,149)	A/teal/Italy/3812/2005	H5N3	98	CY022650

Fig. 1 Phylogenetic relationships of the HA (a), NA (b), NP (c), and PA (d) genes of Zb06. Numbers next to the branches indicate neighbor-joining bootstrap values of ≥ 50%. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The virus strain sequenced in this study is in bold and underlined. Analysis was based on the following nucleotides: HA (77-1,063), NA (38-1,264), NP (751-1,483), and PA (1,456-2,149). AQB, aquatic bird; BHG, black-headed gull; Bsn, Bewick's swan; Ck, chicken; Dk, duck; Eq, equine; Gl, gull; Gt, gray teal; HG, herring gull; MDk, migratory duck; Mal, mallard; Pel, pelican; Pin, pintail; Qa, quail; RTs, ruddy turnstone; Sb, shorebird; Sl, seal; Sn, swan; Sw, swine; Te, tern; Ts, turnstone, Ty, turkey; ; Wsn, whooper swan.

Figure

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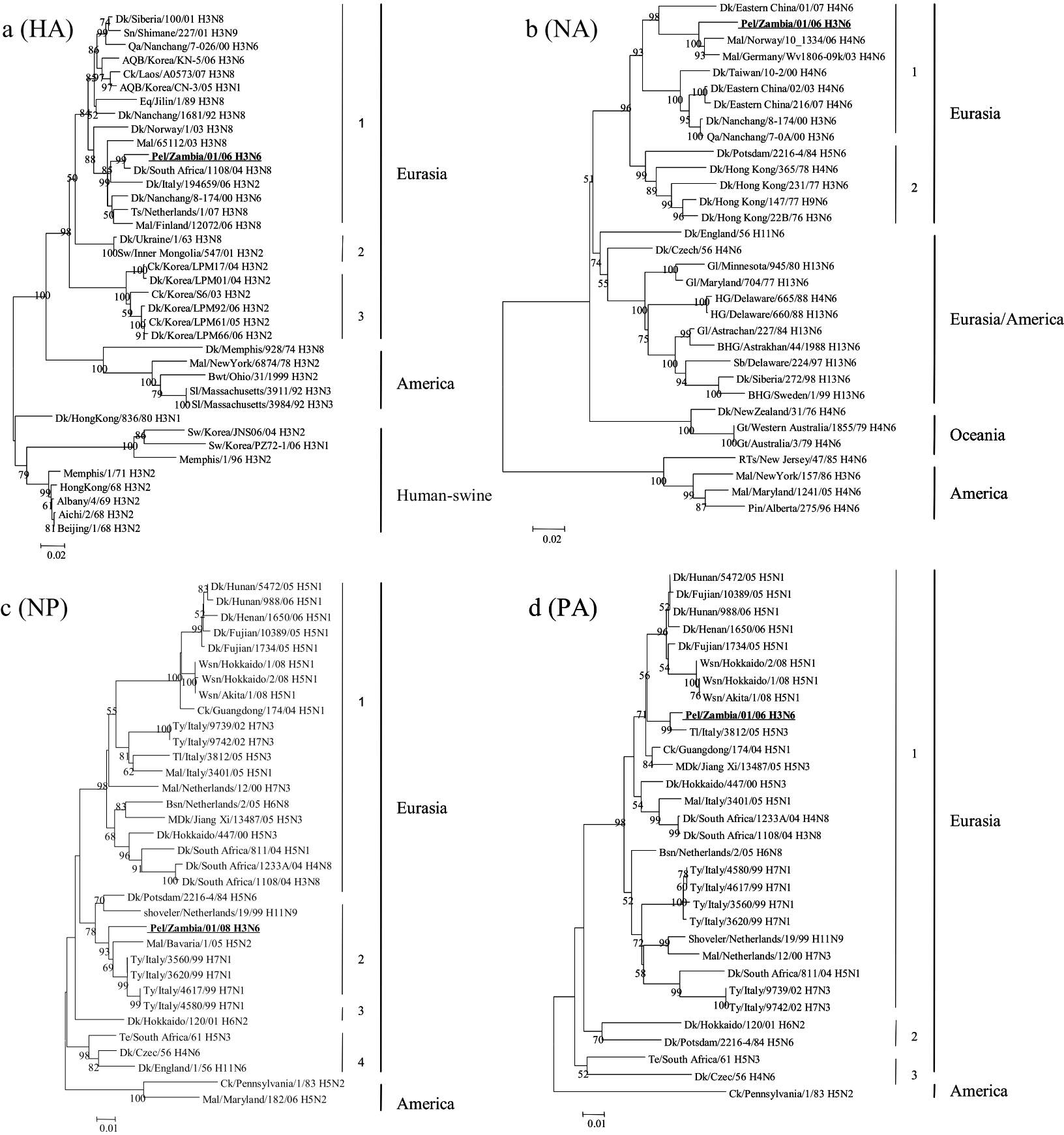

Figure

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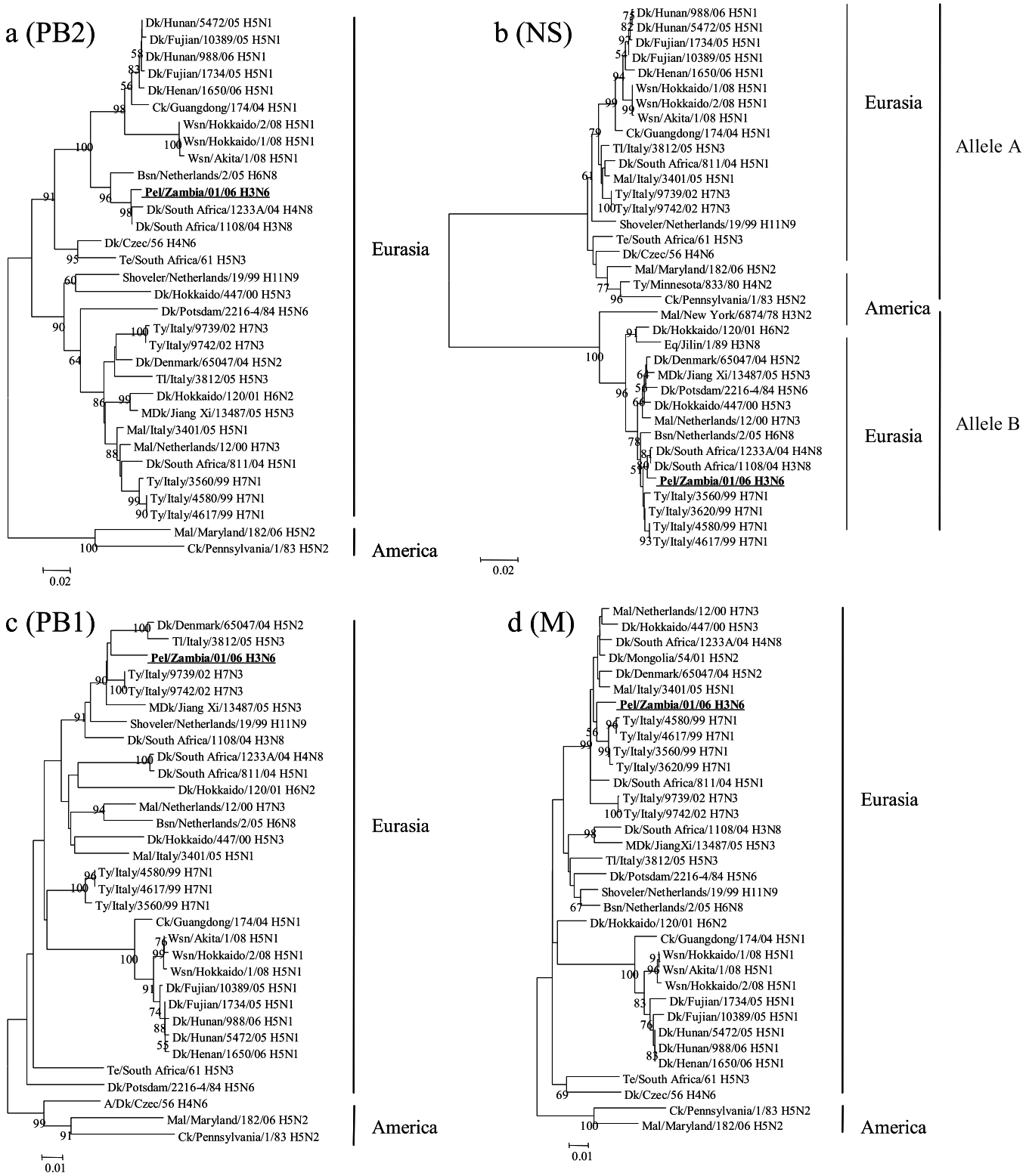

Supplementary Fig. S1 Phylogenetic relationships of the PB2 (a), NS (b), PB1 (c), and M (d) genes of Zb06. Numbers next to the branches indicate neighbor-joining bootstrap values of ≥ 50%. The virus strain sequenced in the present study is in bold and underlined. Analysis was based on the following nucleotides: PB2 (1,468-2,193), NS (57-711), PB1 (1,429-2,178), and M (197-868).