depend on the presense of P3-like viruses in the inoculum.

The intranasal pathogenicity of the mutants of H9N2 virus was different from those of H5N1 mutants while these viruses replicated in MDCK cells in the absence of trypsin and killed chickens when inoculated via intravenous route (Tables 1 and 4). The viruses were recovered from the brain and the blood of some chickens infected with rgVac1 mutants (H5N1), and morbidity was closely associated with viral titers in the brain (Table 5). No viruses were recovered from the brain and the blood of chickens infected with rgY55 mutants (H9N2), indicating the reason why rgY55sub-P10 (H9N2) did not show intranasal pathogenicity. All the viruses passaged in the air sacs of chicks killed chicken embryos by 48 hours post allantoic inoculation (data not shown). rgVac1sub-P3 (H5N1) and rgVac1ins-P3 (H5N1) were more pathogenic to chicken embryos than rgY55sub-P10 (H9N2); the allantoic fluids obtained from the embryonated eggs inoculated with the H5N1 viruses passaged in the air sacs were turbid. It was reported that infection of a highly pathogenic virus was strictly confined to endothelial cells in chicken embryos or chickens [34,35]. Therefore, it is suggested that the difference of endotheliotropism between the H9N2 and H5N1 viruses passaged in the air sacs affected their intranasal pathogenicity. rgY55sub-P10 (H9N2) was not recovered from the brain and the blood of chickens although it caused systemic infection (Table 5), indicating that high levels of viremia followed by replication in the vascular endothelial cells was prerequisite for the virus to cross the blood-brain barrier and consequently replicated in the brain. This hypothesis is supported by the result that rgY55sub-P10 (H9N2) showed intravenous pathogenicity in chickens; direct injection of the virus to the blood vessels readily caused viremia, leading to invasion of the virus to the brain. (Table 1).

H9N2 viruses which have the PARSKR or PARSRR motifs at their HA cleavage site have been isolated from turkeys, ostriches, and chickens in Israel and quails in China [14] although PARSSR motif has been found in most H9N2 isolates, indicating that such substitutions with basic amino acid residues occur in nature. If serine at the c-terminus of the HA1 of the H9 virus was substituted with lysine, the amino acid motif would be consistent with that of rgY55sub (H9N2) which acquired intravenous pathogenicity on consecutive passages in the air sacs of chicks. LPAI caused by H9N2 strains in poultry is now causing serious economic losses [11-19], and its eradication is still difficult because of its low pathogenicity, frequently causing inapparent infections. The present study demonstrated that H9N2 viruses circulating in chicken flocks can acquire intravenous pathogenicity. It is predicted that co-infections of rgY55-sub-P10 (H9N2) with bacteria exacerbate not only intravenous pathogenicity but intranasal pathogenicity in chickens as shown in a previous study [21]. Therefore, continuous monitoring in poultry is important to prevent the emergence of pathogenic H9 viruses.

Materials and methods

Viruses

A/chicken/Yokohama/aq-55/2001 (Y55) (H9N2) isolated from chicken meat imported from China upon quarantine was kindly provided by Dr. M. Eto, Animal Quarantine Service (Yokohama, Kanagawa, Japan) [36]. A/duck/Hokkaido/Vac-1/2004 (Vac1) (H5N1) was generated by the standard genetic reassortment procedure from non-pathogenic viruses, A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1) [37-39]. Viruses were propagated in ten-day-old embryonated chicken eggs for 48 hours at 35°C.

The complete nucleotide sequences of Y55 (H9N2) and Vac1 (H5N1) have been registered in GenBank/EMBL/DDBJ (Accession numbers: AB256671-AB256678 [36] and AB259709-AB259716 [37], respectively).

Reverse genetics

Viral RNA was extracted from the allantoic fluid of embryonated chicken eggs infected with the Y55 and Vac1 strains using a commercial kit (TRIzol LS Reagent, Sigma-Aldrich, St. Louis, MO, U.S.A) and reverse-transcribed with the Uni12 primer [40] and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A). PCR-based amplification of the full genomes of the eight gene segments was performed with universal primer sets [41]. The PCR products were cloned into the vector pCR2.1-TOPO (Invitrogen) or pGEM-T Easy Vector (Promega, Mannheim, Germany). After confirmatory sequencing, T-vector clones were digested with *BsmBI* and inserted into the vector pHW2000 [42]. MDCK cells and 293T cells were maintained in Minimum Essential Medium (MEM, Nissui Pharmaceutical, Tokyo, Japan) containing 10% calf serum and D-MEM (Invitrogen) containing 10% FBS, respectively. Before transfection, confluent 293T and MDCK cells in 75 cm^2 flasks were trypsinized, and 10% of each cell line was mixed in 12 ml of Opti-MEM I (Invitrogen); 2 ml of the suspension was seeded into each well of six-well tissue culture plates (Nunc Inc., Naperville, IL). The cocultured 293T and MDCK cells were used for the transfection. TransIT-293 (Panvera, Madison, WI) was used to transfect cells according to the manufacturer's directions. Briefly, two microliters of TransIT-293 per microgram of DNA was mixed, incubated at room temperature for 45 minutes, and added to the cells. The transfection mixture was replaced with Opti-MEM I after six hours of incubation at 37°C. Thirty hours later, Opti-MEM I containing one microgram per microliter

of trypsin was added. At 48 to 72 hours post-transfection, the culture supernatant was collected and propagated in ten-day-old embryonated chicken eggs.

Site-directed-mutagenesis

To generate H9 and H5 mutant viruses with basic amino acid residue substitutions (sub) or insertions (ins) at the HA cleavage site, mutations were introduced into the HA genes of the Y55 and Vac1 strains using a QuikChange II site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions. The mutant viruses, rgY55sub (H9N2), rgVac1sub (H5N1), and rgVac1ins (H5N1), were rescued by reverse genetics as described above, and the entire genomes of the eight gene segments were sequenced to confirm the existence of the introduced mutations and the absence of undesired mutations.

Plaque assay

Ten-fold dilutions of viruses were inoculated onto confluent monolayers of MDCK cells and incubated at 35°C for one hour. Unbound viruses were removed by washing the cells with MEM. Cells were then overlaid with MEM containing 0.7% Bacto-agar (Difco, Sparks, MD) in the presence or absence of trypsin (5 μg/ml). After 48 hours of incubation at 35°C, cells were stained with 0.005% neutral red.

Consecutive passage in the air sacs of chicks

The caudal thoracic air sacs of three 3-day-old chicks were inoculated with 200 μl of each of the mutant Y55 and Vac1 viruses. The chicks were sacrificed, and their lungs and brains were collected at three days post-inoculation. Serial passages in the air sacs of three to six 3-day-old chicks were performed with 200 μl of a pooled 10% tissue suspension of infected organs. Brain samples were used as the inoculum when both samples (lungs and brains) tested positive for the virus. Isolates were identified by their parental strain's name, mutation (substitution or insertion), and number of passages. For example, the designation rgY55sub-P10 (H9N2) indicates that the amino acids at the HA cleavage site of the Y55 virus were substituted with basic amino acids as shown in Figure 1A, then passaged ten times in the air sacs. Passaged viruses were propagated in the allantoic cavities of ten-day-old embryonated chicken eggs for 48 hours at 35°C. The allantoic fluid was harvested and stored at -80°C.

Experimental infection of chickens with mutant virus strains

Four-week-old Boris Brown chickens were used to test the pathogenicity of the passaged viruses. Eight chickens were intravenously inoculated with 200 μl of each virus (1:10 diluted allantoic fluid), and examined for clinical signs at intervals of 24 hours over a period of ten days. Similarly, three chickens were infected intranasally with 100 μl of allantoic fluid containing each virus at a EID_{50} of $10^{6.5}$ and observed for 14 days. Specific antibodies against homologous viruses after 14 days of infection were detected in serum using the hemagglutination inhibition (HI) test and/or enzyme-linked immunosorbent assay (ELISA) as described previously [43]. To study viral replication, each virus was inoculated into three chickens at an EID_{50} of $10^{6.5}$. The birds were euthenized three days post-challenge, and tissue and blood were collected aseptically. To make a 10% suspension with MEM, the tissue samples were homogenized using a Muti-Beads Shocker (Yasui Kikai, Osaka, Japan). These suspensions were serially diluted ten-fold with PBS and inoculated into ten-day-old embryonated eggs and incubated at 35°C for 48 hours. Viral titers were calculated by the method of Reed and Muench [44] and expressed as EID_{50} per gram and milliliter of tissue and blood, respectively.

All experiments were carried out in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) at a BSL3 biosafety facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. The experiments were performed according to the guidelines of the institutional animal care and use committee of the Graduate School of Veterinary Medicine.

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Authors' contributions
HK is the leader of the study group. KS carried out the experiments and wrote the manuscript. SA helped in passaging study. KS, MO, YS, and HK designed the experiments and analyzed the data. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Characterization of influenza A viruses isolated from wild waterfowl in Zambia

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Although the quest to clarify the role of wild birds in the spread of the highly pathogenic H5N1 avian influenza virus (AIV) has yielded considerable data on AIVs in wild birds worldwide, information regarding the ecology and epidemiology of AIVs in African wild birds is still very limited. During AIV surveillance in Zambia (2008–2009), 12 viruses of distinct subtypes (H3N8, H4N6, H6N2, H9N1 and H11N9) were isolated from wild waterfowl. Phylogenetic analyses demonstrated that all the isolates were of the Eurasian lineage. Whilst some genes were closely related to those of AIVs isolated from wild and domestic birds in South Africa, intimating possible AIV exchange between wild birds and poultry in southern Africa, some gene segments were closely related to those of AIVs isolated in Europe and Asia, thus confirming the inter-regional AIV gene flow among these continents. Analysis of the deduced amino acid sequences of internal proteins revealed that several isolates harboured particular residues predominantly observed in human influenza viruses. Interestingly, the isolates with human-associated residues exhibited higher levels of virus replication in the lungs of infected mice and caused more morbidity as measured by weight loss than an isolate lacking such residues. This study stresses the need for continued monitoring of AIVs in wild and domestic birds in southern Africa to gain a better understanding of the emergence of strains with the potential to infect mammals.

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INTRODUCTION

Avian influenza viruses (AIVs) are zoonotic pathogens maintained in nature mainly in wild aquatic birds (Olsen *et al.*, 2006; Webster *et al.*, 1992). Viruses of 16 different haemagglutinin (HA) (H1–H16) and nine neuraminidase (NA) (N1–N9) subtypes have been identified in waterfowl reservoirs. These viruses are usually non-pathogenic for their natural hosts. It is generally accepted that highly pathogenic AIVs (HPAIVs), particularly of the H5 and H7 subtypes, emerge from low-pathogenic AIV (LPAIV) precursors once introduced into poultry and that they may not be harboured by wild birds (Capua & Alexander, 2006; Röhm *et al.*, 1995). After emerging in China in 1996, H5N1 HPAIV spread rapidly throughout Asia, Europe, the

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this study are AB569476–AB569571.

Supplementary data on the phylogenetic relationships of AIVs from wild birds in Zambia are available with the online version of this paper.

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Middle East and Africa, causing unprecedented outbreaks in wild birds, poultry and occasional human infections that have risen to pose a significant pandemic threat (Ducatez *et al.*, 2006; Li *et al.*, 2004; Smith *et al.*, 2006; Wang *et al.*, 2008). The rapid spread of the H5N1 HPAIV and the detection of H5N2 AIVs with an HP viral genotype in healthy wild waterfowl in Africa (Gaidet *et al.*, 2008) have heightened the possibility of the existence of a wild-bird reservoir for HPAIVs and underscore the need to improve our current understanding of the eco-epidemiological dynamics of AIVs in nature.

As early as 1961, Africa recorded the first outbreak of HPAIV in wild birds, which caused the death of approximately 1300 common terns (Capua & Alexander, 2006). Until 2004 when H5N2 HPAIV caused an outbreak in South African ostriches, there had been no reported cases of HP avian influenza in Africa. The continent's first experience with the Asian-origin H5N1 HPAIV was in 2006 in Nigeria (Ducatez *et al.*, 2006). The virus has since spread to several African countries, affecting a range of avian species with sporadic spillover into humans. Egypt is the African country that has recorded the highest number of human infections with the H5N1 HPAIV to date, with 115 confirmed cases, of which 38 were fatal (World Health Organization, 2010). Despite the significance of these events, which pose a serious threat to animal and public health, as well as to food security in Africa, very little is known about AIVs circulating in wild birds in Africa. Presently, there is very limited GenBank coverage (no more than three complete genomes) of non-pathogenic/LP viral genes of AIVs isolated from African wild birds.

Repeated direct transmissions of AIVs from poultry to humans and other mammals have stimulated investigations into the pathogenicity and transmission mechanisms of AIVs in mammals. Prior to the H5N1 'bird flu' incident in Hong Kong in 1997, which marked the first recorded instance of a purely AIV infecting and causing death in humans (Peiris *et al.*, 2007), investigations on the potential of AIVs from waterfowl to infect mammals, including humans, monkeys, pigs, ferrets and cats, have revealed a spectrum of replication, mostly with no significant disease signs (Beare & Webster, 1991; Hinshaw *et al.*, 1981; Kida *et al.*, 1994; Murphy *et al.*, 1982). In recent years, considerable advances have been made in elucidating the determinants of pathogenicity and adaptation of AIVs in mammals, especially for HP isolates involved in human infections (de Wit *et al.*, 2008). However, the mechanisms of pathogenicity and replicative capacity of LPAIVs isolated from wild birds in mammals are still poorly understood.

Sub-Saharan Africa where Zambia is located supports large populations of indigenous waterfowl and is an over-wintering area for some Eurasian birds (Olsen *et al.*, 2006). Hitherto, no cases of H5N1 HPAIV have been recorded in southern Africa. Thus, AIV surveillance in wild birds and poultry in this region could provide timely information on the possible introduction of H5N1 HPAIV for mitigation purposes. Additionally, data obtained on LPAIVs in wild birds would expand our current understanding of the ecology and epidemiology of AIVs in this region.

During AIV surveillance conducted between 2008 and 2009 in Zambia, 12 viruses were isolated from wild waterfowl in Lochinvar National Park. Whole-genome sequencing was performed on each isolate, and bioinformatics approaches were employed to characterize the viruses genetically. Furthermore, based on genetic characterization results, we evaluated the replication and pathogenicity of some of the isolates in a mouse model.

RESULTS

Surveillance and virus isolation

AIV surveillance has been ongoing in Zambia since 2006 (Simulundu *et al.*, 2009). Between April 2008 and November 2009, a total of 3094 wild waterfowl faecal specimens were collected in Lochinvar National Park. On average, about 200 faecal specimens were collected every month except during the rainy season (December to March) when the wetland becomes inaccessible due to extreme flooding. Twelve AIVs were isolated (Table 1). Of the 12 isolates, seven were from ducks, four from geese and one from a great white pelican (*Pelecanus onocrotalus*). We identified five different HA (H3, H4, H6, H9 and H11) and NA (N1, N2, N6, N8 and N9) subtypes (Table 1). Among these subtypes, the H11N9 subtype is relatively uncommon, whilst H9N1 is a rare HA/NA combination. Currently, only ten H9N1 isolates are available in GenBank and none has been reported from Africa or Europe.

Phylogenetic analysis of the HA and NA genes

To understand the evolutionary relationships of AIVs isolated from wild birds in Zambia in detail, we sequenced the entire genome of each isolate and conducted phylogenetic analyses. To include, as much as possible, some AIV sequences of isolates from African birds in our analyses, some partial sequences were used. The HA and NA genes of all the viruses characterized in this study belonged to the Eurasian avian lineage (Figs 1 and 2 and Supplementary Figs S1 and S2, available in JGV Online). They clustered mostly with those of AIVs isolated in southern Africa. It was noted that the HA and NA genes of H11N9 viruses reported here formed a distinct sublineage within the Eurasian lineage (see Supplementary Figs S1c and S2c). In this report, only the H6 and H9 HA and the N2 and N8 NA gene trees are described in more detail, because these subtypes have been involved in avian influenza outbreaks in southern Africa.

The topology of the H6 HA phylogenetic tree conformed to that described previously by Bahl *et al.* (2009), particularly in the classification of isolates into the American and

Table 1. AIVs isolated from wild waterfowl in Zambia (2006–2009)

Host	Strain name	Abbreviation	Sampling date
Wild duck	A/duck/Zambia/02/08 (H6N2)	Zb02 (H6N2)	June 2008
	A/duck/Zambia/03/08 (H6N2)	Zb03 (H6N2)	June 2008
	A/duck/Zambia/04/08 (H3N8)	Zb04 (H3N8)	June 2008
	A/duck/Zambia/08/09 (H6N2)	Zb08 (H6N2)	August 2009
	A/duck/Zambia/10/09 (H6N2)	Zb10 (H6N2)	September 2009
	A/duck/Zambia/11/09 (H11N9)	Zb11 (H11N9)	September 2009
	A/duck/Zambia/12/09 (H11N9)	Zb12 (H11N9)	September 2009
Wild goose	A/goose/Zambia/05/08 (H3N8)	Zb05 (H3N8)	July 2008
	A/goose/Zambia/06/08 (H3N8)	Zb06 (H3N8)	July 2008
	A/goose/Zambia/07/08 (H4N6)	Zb07 (H4N6)	September 2008
	A/goose/Zambia/09/09 (H11N9)	Zb09 (H11N9)	September 2009
Wild pelican	A/pelican/Zambia/01/06 (H3N6)*	Zb01 (H3N6)	August 2006
	A/pelican/Zambia/13/09 (H9N1)	Zb13 (H9N1)	November 2009

*The first influenza virus isolate from an avian host in Zambia (Simulundu *et al.*, 2009).

Eurasian/American lineages (Fig. 1a). The H6 HA genes reported in this study belonged to a group of viruses of the Eurasian/American lineage that consisted of contemporary H6 strains isolated from wild aquatic birds in Africa, Asia and America, including those viruses that were introduced into terrestrial poultry in Taiwan and South Africa (Fig. 1a). They shared a common ancestor with an H6N8 virus that caused avian influenza in South African ostriches in 2007 (Abolnik *et al.*, 2010) (Fig. 1a). The H6 HA genes of the isolates obtained in Zambia were distinct from those that caused an avian influenza outbreak in chickens in South Africa in 2002.

Genetic and antigenic analyses of the HA genes of H9N2 AIVs have shown that these viruses separate into three main Eurasian lineages (Xu *et al.*, 2007). These lineages are represented by chicken/Beijing/1/94, quail/Hong Kong/G1/97 and duck/Hong Kong/Y439/97 (Fig. 1b). The HA gene of Zb13 (H9N1) belonged to the duck/Hong Kong/Y439/97-like lineage (also called the Korean lineage) and was most closely related to that of ostrich/South Africa/AI1586/08 (H9N2).

In the N2 NA gene tree, AIVs isolated in this study fell in a Eurasian sublineage composed of viruses isolated mainly from wild aquatic birds in Asia, Europe and Africa (Fig. 2a). They clustered together with strains isolated from an ostrich and from a wild goose in South Africa in 2008, as well as two other strains isolated in China and Japan. The H5N2 AIVs with a genotype characteristic of HPAIVs detected from wild ducks in Nigeria also belonged to this sublineage. The N2 NA phylogeny further revealed that the N2 genes of viruses that caused outbreaks of avian influenza in South Africa in 2002 were distinct from those characterized in this study, a finding that is in concordance with their HA phylogenetic comparisons (Figs 1a and 2a).

Phylogenetic analysis of the N8 NA genes showed several sublineages within the Eurasian avian lineage, namely, early 1 and 2, contemporary 1 and 2 and European gull isolates (Fig. 2b). The NA genes of Zb04 (H3N8), Zb05 (H3N8) and Zb06 (H3N8) belonged to the contemporary 1 sublineage, which consisted of AIVs isolated mostly from wild birds in Europe and southern Africa.

Phylogenetic analysis of the internal protein genes

Broadly, the topologies of the internal protein gene trees showed assortment of the AIVs into the American and Eurasian avian lineages, with early and contemporary sublineages being identifiable in the latter lineage, as described previously by Duan *et al.* (2007). The Eurasian contemporary sublineage was further divided into two to three groups.

Phylogenetic analysis of the PB2 polymerase subunit gene showed that, except for Zb04 (H3N8) which fell in group 2 of the Eurasian contemporary sublineage, all the viruses isolated in Zambia from 2006 to 2009 clustered together in group 1 (Fig. 3a). H5N1 HPAIVs also joined this group, but they were not closely related to the viruses reported here. The PB2 gene of Zb04 (H3N8) showed a close relationship to those of two H5N2 viruses isolated from domestic ducks in China.

In the PB1 polymerase subunit gene tree, three groups were observed in the Eurasian contemporary sublineage (Fig. 3b). All isolates from waterfowl in Zambia belonged to group 3. Whilst the majority of the strains isolated between 2006 and 2009 in Zambia grouped together as an independent branch, the PB1 genes of Zb04 (H3N8), Zb08 (H6N2), Zb10 (H6N2) and Zb13 (H9N1) belonged to a cluster of viruses that included two recent wild-bird isolates from South Africa, two H5N2 influenza viruses isolated from pigs in South Korea and two H5N1 HPAIVs isolated in Laos (Fig. 3b).

In the PA polymerase subunit phylogeny, all the viruses reported here belonged to group 1 and the majority of the

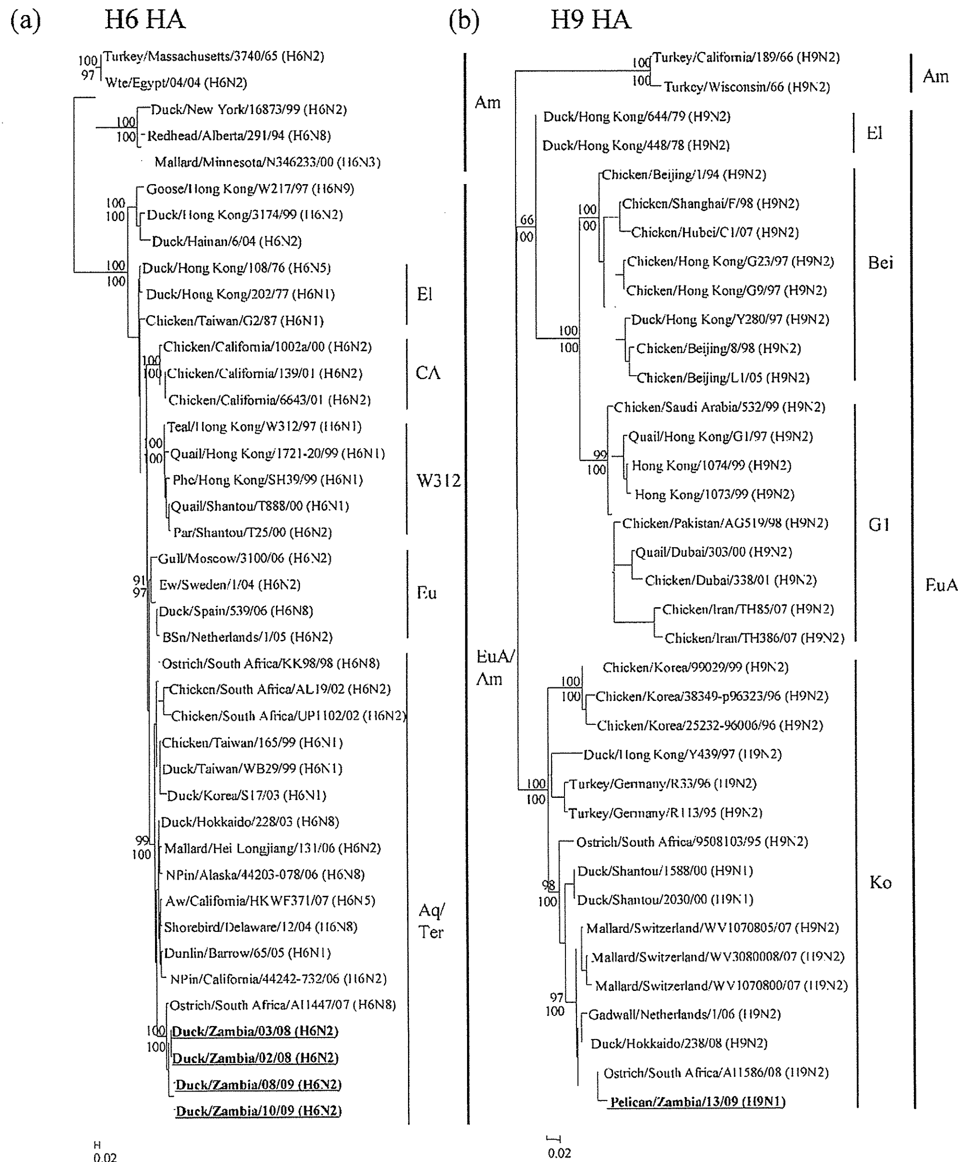

Fig. 1. Phylogenetic relationships of the H6 HA (a) and H9 HA (b) genes of AIVs isolated from wild birds in Zambia. Analysis was based on nt 44–1066 (1023 bp) of H6 HA and 97–1228 (1132 bp) of H9 HA. Numbers above and below branch nodes indicate neighbour-joining bootstrap values of ⩾50 % and Bayesian posterior probabilities of >95 %, respectively. Owing to space limitations, not all supports are indicated. Virus strains characterized in the present study are bold and underlined. Bars, number of substitutions per site. Lineages: Am, American; Aq/Ter, aquatic/terrestrial; Bei, chicken/Beijing/1/94-like; CA, California; El, early; Eu, Europe; EuA, Eurasian; G1, quail/Hong Kong/G1/97-like; Ko, Korean-like; W312, teal/Hong Kong/W312-like. Strain names: Aw, American wigeon; BSn, Bewick's swan; Ew, Eurasian wigeon; NPin, northern pintail; Par, partridge; Phe, pheasant; Wte, whiskered tern.

viruses grouped with those of AIVs isolated from wild birds and ostriches in South Africa (Fig. 3c). The PA gene of Zb04 (H3N8) showed a close relationship to viruses isolated from wild birds in the Netherlands. The PA gene of Zb01 (H3N6) belonged to group 2 and clustered with those of the Asian H5N1 HPAIVs, as we reported previously (Simulundu *et al.*, 2009).

The nucleoprotein (NP) gene tree showed the division of the Eurasian contemporary sublineage into three groups (Fig. 4a). All the viruses characterized in this study assorted to group 3. Eight of these strains, along with those isolated recently from wild and domestic birds in South Africa, formed a distinct cluster within this sublineage. The other four isolates clustered with viruses isolated mostly from

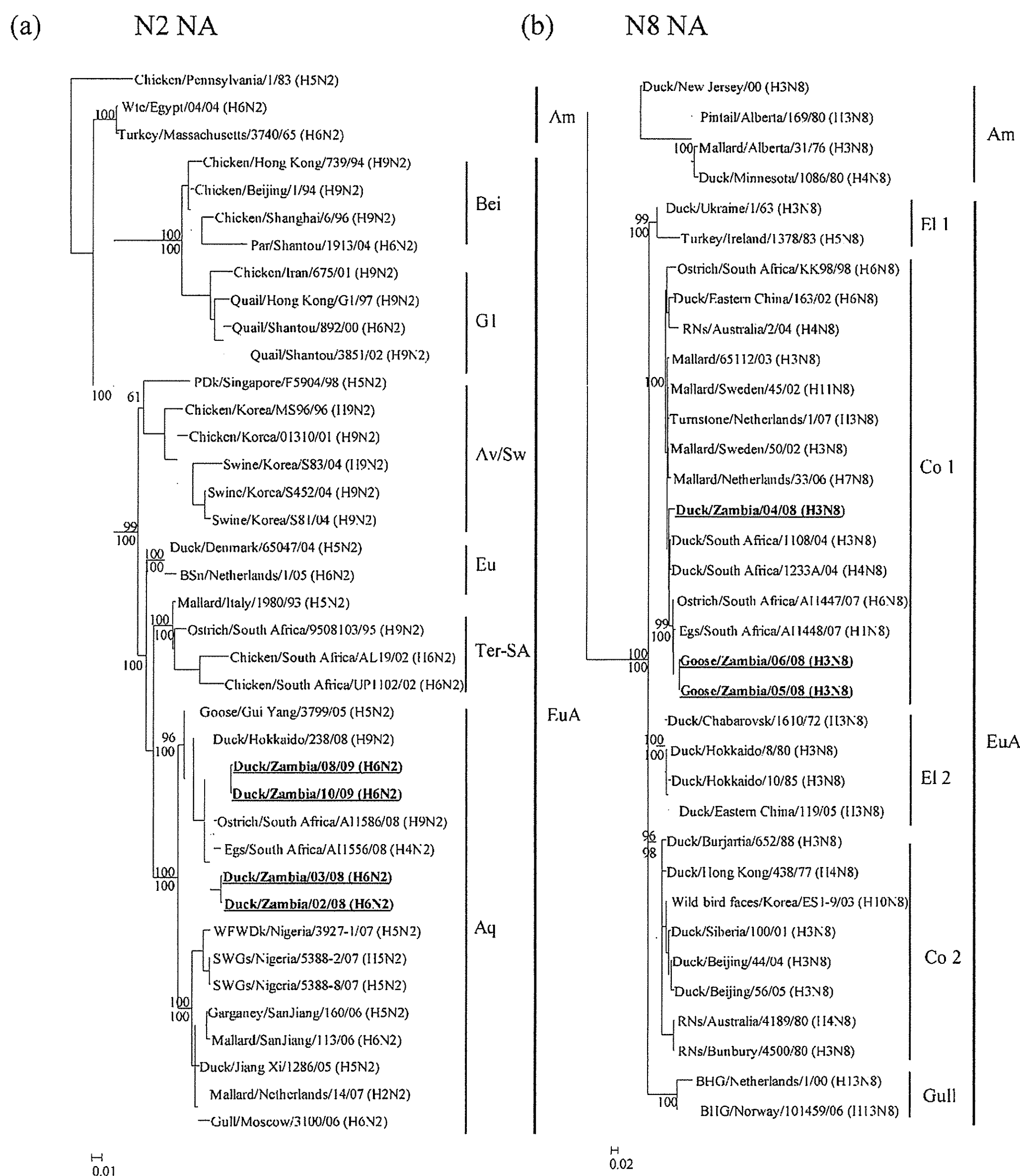

Fig. 2. Phylogenetic relationships of the N2 NA (a) and N8 NA (b) genes of AIVs isolated from wild birds in Zambia. Analysis was based on nt 78–1396 (1319 bp) of N2 NA and 54–1343 (1290 bp) of N8 NA. Numbers above and below branch nodes indicate neighbour-joining bootstrap values of ⩾50 % and Bayesian posterior probabilities of >95 %, respectively. Owing to space limitations, not all supports are indicated. Virus strains characterized in the present study are in bold and underlined. Bars, number of substitutions per site. Lineages: Aq, aquatic; Av/Sw, avian/swine; Co, contemporary; Ter-SA, terrestrial, South Africa. Strain names: BHG, black-headed gull; Egs, Egyptian goose; PDk, pekin duck; RNs, red-necked stint; SWGs, spur-winged goose; WFWDk, white-faced whistling duck. Other abbreviations are listed in the legend of Fig. 1.

Europe. The NP gene of Zb01 (H3N6) was closely related to that of ostrich/South Africa/AI1447/07 (H6N8) and both these strains belonged to a group of viruses comprising early and contemporary strains.

The matrix (M) gene tree showed that all the viruses isolated from wild birds in Zambia were in group 3, but they did not all cluster together (Fig. 4b). The majority of the viruses reported in this study grouped with isolates obtained from wild and domestic birds in South Africa and appear to have been derived from A/mallard/Netherlands/1/06 (H8N4)-like viruses. The M genes of Zb01 (H3N6) and Zb04 (H3N8) were closely related to that of turkey/Italy/3620/99 (H7N1), whilst those of Zb02 (H6N2), Zb03 (H6N2), Zb05 (H3N8) and Zb06 (H3N8) grouped with that of an H9N2 virus isolated from an ostrich in South Africa.

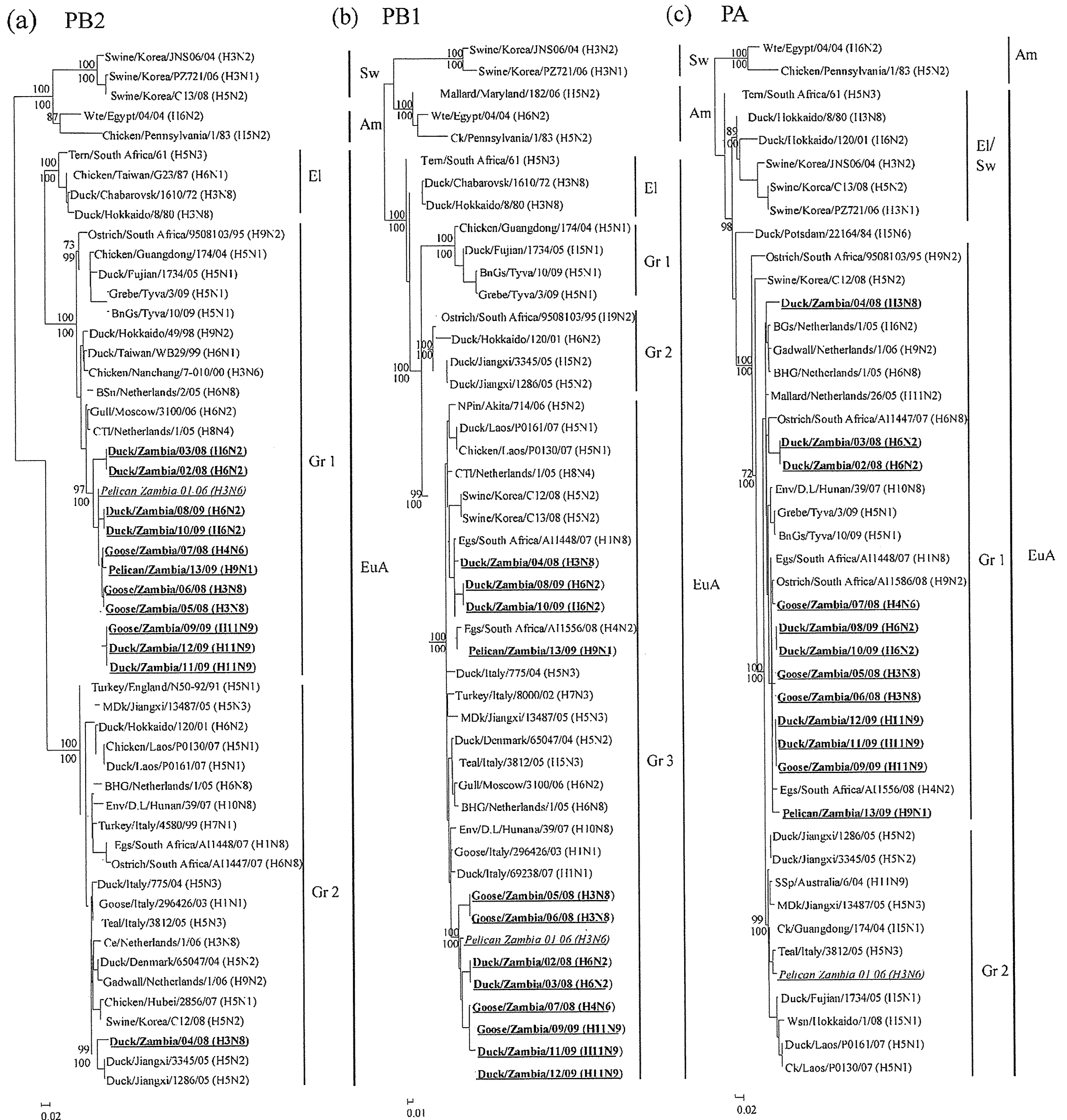

Fig. 3. Phylogenetic relationships of the PB2 (a), PB1 (b) and PA (c) genes of AIVs isolated from wild birds in Zambia. Analysis was based on nt 56–2285 (2230 bp) of PB2, 64–2281 (2218 bp) of PB1 and 30–2098 (2069 bp) of PA. Numbers above and below branch nodes indicate neighbour-joining bootstrap values of ⩾50 % and Bayesian posterior probabilities of >95 %, respectively. Due to space limitations, not all supports are indicated. Virus strains characterized in the present study are in bold and underlined, whilst the first AIV isolate in Zambia is italicized and underlined. Bars, number of substitutions per site. Lineages: El/Sw, early/swine; Sw, swine. Strain names: BGs, barnacle goose; BnGs, bean goose; Ce, common eider; Ck, chicken; CTl, common teal; D.L, Dongting Lake; Env, environment; MDk, migratory duck; SSp, sharp-tailed sandpiper; Wsn, whooper swan. Other abbreviations are listed in the legends of Figs 1 and 2.

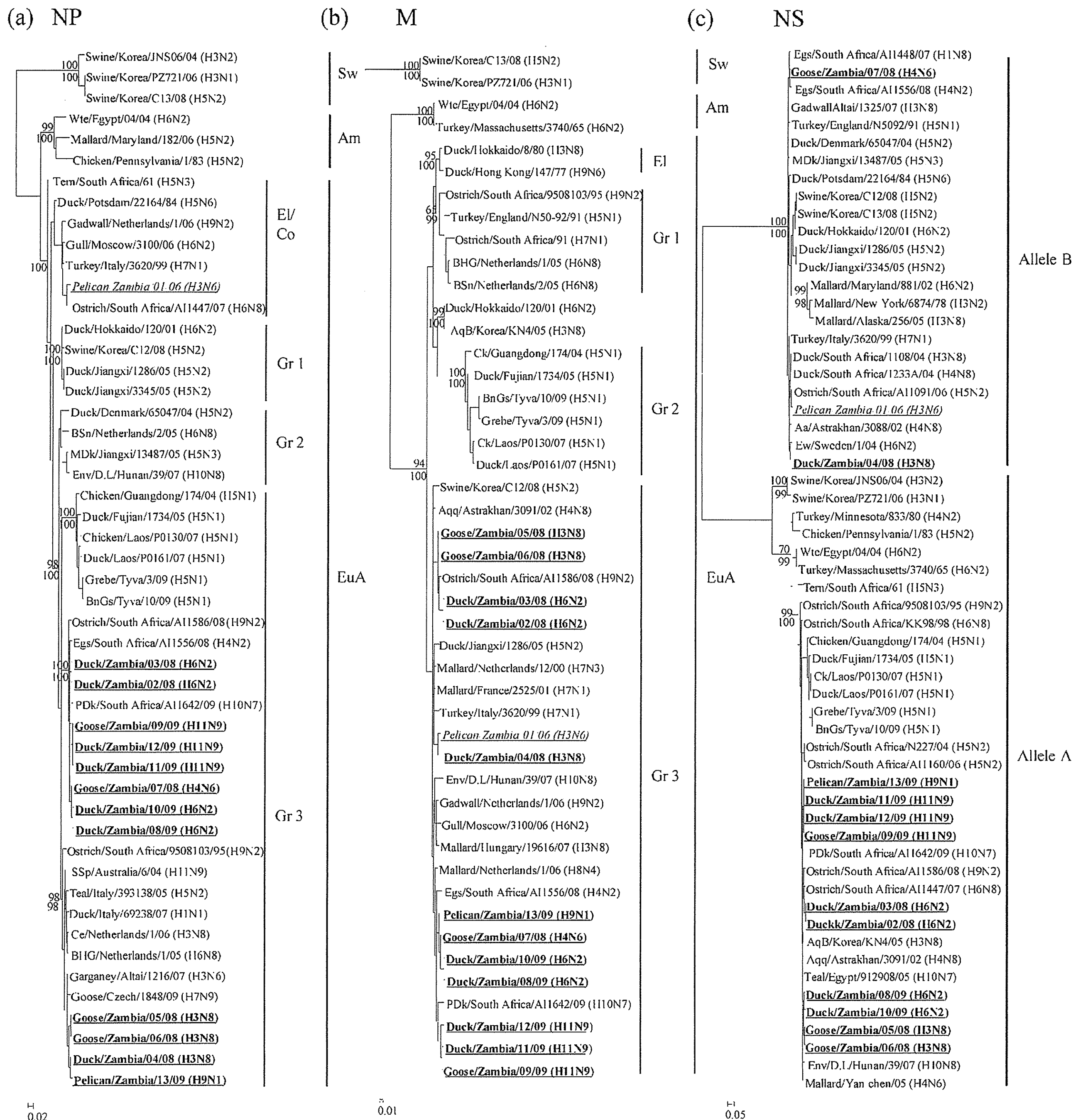

Fig. 4. Phylogenetic relationships of the NP (a), M (b) and NS (c) genes of AIVs isolated from wild birds in Zambia. Analysis was based on nt 46–1489 (1444 bp) of NP, 32–753 (722 bp) of M and 57–705 (649 bp) of NS. Numbers above and below the branch nodes indicate neighbour-joining bootstrap values of ≥50 % and Bayesian posterior probabilities of >95, respectively. Owing to space limitations, not all supports are indicated. Virus strains characterized in the present study are in bold and underlined, whilst the first influenza virus isolate from an avian host in Zambia is italicized. Bars, number of substitutions per site. Lineages: El/Co, early/contemporary. Strain names: Aa, *Anas angustirostris*; AqB, aquatic bird; Aqq, *Anas querquedula*. Other abbreviations are listed in the legends of Figs 1–3.

Phylogenetic analysis of the non-structural (NS) gene indicated that the NS genes of ten of the viruses from wild birds in Zambia comprised the A allele, whilst the other three were of the B allele (Fig. 4c). Some of the NS genes were closely related to viruses isolated mainly in Asia and Africa, particularly those isolated in South Africa (Fig. 4c).

The NS gene tree clearly demonstrated that, among the viruses examined, the A allele was predominant and that two genetically distinct gene pools, corresponding to NS alleles A and B, were co-circulating in wild birds in this region during the surveillance period.

Amino acid sequence analysis

Although it is difficult to ascertain the capacity of a non-pathogenic AIV/LPAIV from wild waterfowl to cause interspecies transmission into other animals, close monitoring of host-associated signatures in viral proteins may provide some clues regarding an isolate's zoonotic potential. Several amino acids that are preferentially associated with human influenza viruses have been described (Chen *et al.*, 2006; Finkelstein *et al.*, 2007; Shaw *et al.*, 2002). We examined the deduced amino acid sequences of all the internal proteins of all the wild-bird isolates from Zambia and identified some human-associated amino acids in the genome of some strains (Table 2). Zb08 (H6N2) and Zb10 (H6N2) possessed the human-associated amino acid methionine at position 475 of the PB2 protein, which has been described to be 100 % conserved in the influenza viruses that caused the 1918, 1957 and 1968 human pandemics (Finkelstein *et al.*, 2007). Zb13 (H9N1) had a serine at position 66 of the PB1-F2 polypeptide, which was shown previously to contribute to increased virulence in mice (Conenello *et al.*, 2007). Zb13 (H9N1) also possessed the human-associated amino acid alanine at position 76 of the PB1-F2 protein. Six isolates were found to have the human-associated amino acid serine at position 82 of the PB1-F2 protein, whilst only Zb08 (H6N2) and Zb10 (H6N2) had the human-associated amino acid glycine at position 87 of this polypeptide. In the M2 protein, Zb04 (H3N8) possessed the human-associated amino acid valine at position 28. At position 55 of the M2 protein, Zb07 (H4N6), Zb08 (H6N2), Zb10 (H6N2) and Zb12 (H11N9) were found to possess the human-associated amino acid phenylalanine. It is noteworthy that, although the human-associated amino acids found in some of the virus isolates analysed in this report are not unique to these isolates, these residues are rarely found among AIVs isolated from members of the orders Anseriformes and Charadriiformes (our unpublished data).

Table 2. Human-associated amino acids identified in viral proteins of AIVs isolated in Zambia

Protein	Aa position*	Host		Isolate†
		Avian	Human	
PB2	475	L	M	Zb08 (H6N2)
				Zb10 (H6N2)
PB1-F2	66	N	S‡	Zb13 (H9N1)
	76	V	A	Zb13 (H9N1)
	82	L	S	Zb04 (H3N8)
				Zb05 (H3N8)
				Zb06 (H3N8)
				Zb08 (H6N2)
				Zb10 (H6N2)
				Zb13 (H9N1)
	87	E	G	Zb08 (H6N2)
				Zb10 (H6N2)
M2	28	I	V	Zb04 (H3N8)
	55	L	F	Zb07 (H4N6)
				Zb08 (H6N2)
				Zb10 (H6N2)
				Zb12 (H11N9)

*For references of human-associated residues at these specific positions, see Chen *et al.* (2006), Finkelstein *et al.* (2007) and Shaw *et al.* (2002).
†Names of isolates possessing human-associated amino acid residues.
‡The amino acid serine at position 66 of the PB1-F2 protein is not a human-associated residue but was shown previously to increase virulence in mice (Conenello *et al.*, 2007).

Replication and pathogenicity of selected viruses in mice

Amino acid sequence analysis revealed that several isolates from wild birds in Zambia had human-associated residues in their genome (Table 2). Therefore, we sought to investigate whether there could be a difference in virus replication and/or pathogenicity in a mammalian host between viruses either possessing or lacking human-associated residues. For this purpose, we compared the replication ability and pathogenicity of two isolates, Zb03 (H6N2) and Zb10 (H6N2), in mice. Four human-associated residues were identified in some viral proteins of Zb10 (H6N2), whilst none was observed in the genome of Zb03 (H6N2) (Table 2). We also tested the replication capacity and pathogenicity of Zb04 (H3N8) in mice, because it had two human-associated residues in its genome and was of a subtype distinct from that of Zb10 (H6N2).

All the tested viruses replicated in the lungs of mice without prior adaptation, with virus titres ranging from $10^{3.3}$ to $10^{4.8}$ EID_{50} g^{-1} (Table 3). None of the viruses was detected in the brain. It was noted that mice inoculated with Zb10 (H6N2) showed higher virus titres that were statistically significantly different from those of Zb03 (H6N2)-infected mice (Table 3). Virus was detected in the lungs of all five mice inoculated with Zb04 (H3N8) and Zb10 (H6N2), whilst, in Zb03 (H6N2)-inoculated mice, virus was detected in three of the five mice.

Mice infected with Zb10 (H6N2) exhibited more weight loss and delayed weight gain [weight returned to baseline after day 7 post-inoculation (p.i.)] than those inoculated with Zb03 (H6N2) (Fig. 5). Zb04 (H3N8)-inoculated mice showed significant weight loss early on p.i. when compared with Zb03 (H6N2)- or mock-inoculated control mice (Fig. 5). Mild to considerable ruffled fur was noted between days 1 and 3 p.i. in mice infected with Zb10 (H6N2) and Zb04 (H3N8) but not in Zb03 (H6N2)-inoculated mice.

Table 3. Replication of selected AIVs isolated from wild waterfowl in Zambia in BALB/c mice

Virus	No. positive/ total	Mean virus titre of positive samples ($\log_{10}$ EID_{50} g^{-1})		*P* value
		Lung	Brain	
Zb03 (H6N2)*	3/5	3.3	$<10^{1.5}$	–
Zb04 (H3N8)†	5/5	3.6	$<10^{1.5}$	0.099
Zb10 (H6N2)†	5/5	4.8	$<10^{1.5}$	0.001‡

*Virus with no apparent human/mammalian-associated residues in its genome.
†Viruses with human-associated residues in their genome.
‡Virus titre in the lungs of mice inoculated with Zb10 (H6N2) was significantly higher than that of Zb03 (H6N2)-inoculated mice (Student's *t*-test, $P<0.05$).

All the mice survived the infection for the 14-day observation period.

DISCUSSION

In this study, we genetically and biologically characterized AIVs isolated from wild birds in Zambia. During the surveillance period, AIVs were isolated mainly between June and November, a time frame encompassing the period when palearctic migrants are absent or rare, as well as when they are present. Palearctic birds usually start to arrive in Zambia between September and December and leave between January and May. Our isolation of AIVs between June and August of 2008 and 2009 when palearctic migrants were scarce raises the possibility of yearly persistence of AIVs in indigenous waterfowl in southern Africa. This idea is further supported by our phylogenetic analyses, which showed the separate clustering of southern African isolates, with the glycoprotein genes of H11N9 viruses characterized in this report forming a distinct sublineage within the Eurasian lineage (Fig. 3a, b and Supplementary Figs S1c and S2c). In neighbouring Zimbabwe, AIVs were also detected in Afro-tropical waterfowl in periods when palearctic birds were rare (Caron *et al.*, 2010). Moreover, AIVs were detected from Afro-tropical bird species in several major wetlands in Africa (Gaidet *et al.*, 2007). These data not only support the notion of a possible endemicity of AIVs in Afro-tropical ecosystems where high temperatures experienced in these regions may restrict the persistence and transmissibility of AIVs (Brown *et al.*, 2009), but also raise the possibility that palearctic migrants may also carry AIVs from Africa into Eurasia. However, the extent to which Afro-tropical ecosystems depend on introductions of AIVs by Eurasian migrants to sustain the possible endemic state remains to be clarified.

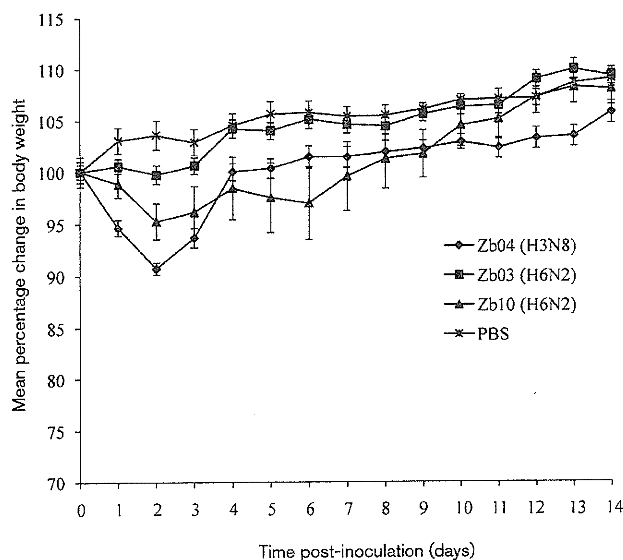

Fig. 5. Weight loss in mice inoculated with selected AIVs from wild waterfowl in Zambia. Data are presented as mean body weight change per group ±SD. Statistically significant weight loss (Student's *t*-test, $P<0.05$) was observed in Zb04 (H3N8)-inoculated mice at days 1 ($P=0.03$), 2 ($P=0.01$) and 3 ($P=0.03$) p.i. compared with mock-inoculated control mice.

The detection of five distinct HA and NA subtypes suggested that a variety of subtypes could be circulating in wild birds in this region. Whilst 11 of the isolates were detected in wild ducks and geese, confirming the major role of these birds in the perpetuation of AIVs (Olsen *et al.*, 2006; Webster *et al.*, 1992), Zb13 (H9N1) was isolated from an atypical avian host, a great white pelican. Despite several AIV surveillance studies that involved sampling from the Pelecaniformes worldwide (Gaidet *et al.*, 2007; Munster *et al.*, 2007; Olsen *et al.*, 2006), the number of AIVs detected from this order has remained low. Thus, we consider the two instances in which we isolated AIVs from these birds as incidental findings, but we do not exclude the possibility that white pelicans, which are native to southern Africa, may also play a major role in influenza virus ecology in this region.

Phylogenetic analyses demonstrated that all the gene segments of the viruses reported in this study clustered with contemporary viruses of the Eurasian avian lineage. Most genes were closely related to those of AIVs isolated from wild and domestic birds in South Africa. AIVs originating in wild birds have been implicated in avian influenza outbreaks in farmed birds in South Africa with serious economic consequences (Abolnik, 2007; Abolnik *et al.*, 2007, 2010; Alexander, 2007; Brown, 2010). These data highlight the need for continued monitoring of AIVs in wild and domestic birds in southern Africa for avian influenza control. It is also important to clarify the extent of influenza virus exchange between wild birds and domesticated birds (including ostriches) in the region, as

results from a study in China demonstrated that a two-way transmission of influenza viruses between terrestrial and aquatic birds may increase opportunities for the generation of reassortant viruses with pandemic potential (Li *et al.*, 2003). Furthermore, the potential role of human related activities (e.g. the poultry trade) in AIV dissemination should not be ignored.

A number of human-associated amino acids were observed in some viral proteins of some viruses tested. The two possible means by which AIVs may acquire 'novel' amino acids are either through genetic reassortment or through point mutations. Genetic analyses of AIVs isolated from wild and terrestrial birds in southern Africa have demonstrated the involvement of ostriches in the evolution and epidemiology of AIVs in this region (Abolnik, 2007; Abolnik *et al.*, 2007, 2010; this study). Recently, Shinya *et al.* (2009) demonstrated that ostriches may be involved in the emergence of viruses possessing mammalian-associated amino acids lysine and asparagine at positions 627 and 701 of the PB2 protein, respectively. Indeed, an examination of PB2 gene sequences of viruses isolated from ostriches in South Africa between 1995 and 2008 showed that four viruses had lysine and one virus possessed asparagine at positions 627 and 701 of the PB2 protein, respectively (data not shown). Therefore, if a two-way transmission of AIVs between ostriches and wild aquatic birds in southern Africa exists, these data indicate that the human-associated amino acids observed in some internal proteins of some of the isolates examined here may have been acquired through genetic reassortment with viruses from ostriches. Unfortunately, the lack of complete internal protein gene sequences of isolates from ostriches in South Africa for a comprehensive study makes it difficult to reach this conclusion. Moreover, genetic analyses of the deduced amino acids revealed that the surface proteins of the viruses listed in Table 2 maintained typical features of non-pathogenic wild waterfowl isolates, including conservation of putative glycosylation sites and no NA stalk deletions, and did not exhibit evidence for accelerated or increased amino acid substitutions, suggesting that these viruses may not have circulated extensively in land-based avian species. These observations leave open the possibility that the human-associated amino acids in the viral proteins of some isolates from Zambia may have been acquired in wild waterfowl or other non-gallinaceous birds. Whether some African waterfowl may provide an environment that may lead to the selection of AIVs with human/mammalian-associated amino acids is a question deserving further exploration.

In a mouse model, we demonstrated that all the tested viruses replicated in mouse lung without prior adaptation and that mice infected with isolates having human-associated residues displayed increased virus titres and caused increased morbidity, as measured by weight loss, than those inoculated with Zb03 (H6N2). Although it is tempting to conclude that possession of human-associated residues may have impacted on virus replication and pathogenicity in mice, there is need for caution, because the influence of other residues was not ruled out in the current study. In fact, there were 68 amino acid differences in viral proteins between Zb03 (H6N2) and Zb10 (H6N2). Therefore, investigations employing reverse genetics and site-directed mutagenesis may be needed to explain more fully the observed differences. To our knowledge, the present study is the first to demonstrate the ability of non-HPAIVs from wild birds in Africa to replicate without adaptation and cause illness in a mammalian host. Elsewhere, although few in number, AIVs from wild birds of considerable numbers of HA subtypes have been shown to replicate in mice and ferrets without adaptation, causing varied degrees of morbidity (Driskell *et al.*, 2010; Gillim-Ross *et al.*, 2008; Joseph *et al.*, 2007; Kim *et al.*, 2010; Wan *et al.*, 2008). These studies have highlighted the potential risk of direct transmission of non-HPAIVs from wild birds to mammalian species. Whilst direct transmission of AIVs from wild birds to humans has not been reported, serological evidence of AIV infection in three persons with substantial exposure to wild waterfowl and game birds argues for a possible direct transmission of AIVs from wild birds to humans (Gill *et al.*, 2006). Moreover, both natural and experimental infections of humans with AIVs, together with serological data, have emphasized the susceptibility of humans to several AIV subtypes (Myers *et al.*, 2007; Peiris *et al.*, 2007; Shortridge, 1992). Thus, the potential threat posed to both animal and public health by some of the viruses characterized currently cannot be overemphasized.

Here, we demonstrated that the 12 influenza viruses isolated from wild waterfowl in Zambia belonged to the contemporary Eurasian avian lineage. We have shown the possibility that AIVs could persist in wild waterfowl in a Zambian ecosystem, with transmission of viruses involving wild and domestic avian species in southern Africa, Europe and Asia. This study further established that some AIVs from wild waterfowl in Zambia may have the potential to infect mice directly without adaptation. Overall, the present study raises concerns for continued monitoring of AIVs in wild and domestic birds in southern Africa and suggests that complete characterization of isolates may help in the identification of strains that may have potential for future incursions into humans and other animals.

METHODS

Viruses and sequencing. The viruses characterized in the present study were isolated from wild waterfowl faecal specimens collected in Lochinvar National Park between April 2008 and November 2009 (Table 1). All virus isolation was performed using 10–11-day-old embryonated chicken's eggs. The isolates were subtyped by standard HA inhibition and NA inhibition tests, as well as by sequencing of the HA and NA genes. The viruses were passaged once in eggs before being used in this study. Viral RNA extraction, cDNA synthesis, PCR and sequencing were carried out as described previously (Simulundu *et al.*, 2009).

Phylogenetic analyses. Phylogenetic trees were constructed by the neighbour-joining bootstrap method with 1000 replicates applied

using MEGA4 (Tamura *et al.*, 2007). The gene tree topologies obtained in MEGA4 were then confirmed using Bayesian methods implemented in MRBAYES version 3.1.2 (Huelsenbeck & Ronquist, 2001). Specifically, we used the program ModelTest version 3.7 (Posada & Crandall, 2001), applied in PAUP* version 4.0 (Swofford, 2001), to determine the appropriate evolutionary model that best fitted the data. The HA, NA, PB2, PB1, PA and NP nucleotide sequence data were best fitted by the general time reversible plus invariant sites plus gamma-distributed (GTR+I+G) model, whilst the Hasegawa–Kishino–Yano (plus invariant sites) plus gamma-distributed models (HKY+G and HKY+I+G) were preferred for the NS and M sequence data, respectively. In Bayesian analysis, we used one to four replicates of 1 million generations, with four chains sampled every 100 generations. All replicates converged with less than 0.01 SD of split frequencies.

Experimental infection of mice. Groups of 6-week-old BALB/c mice (ten mice per group) were lightly anaesthetized with isoflurane and inoculated intranasally with 0.05 ml virus-infected chorioallantoic fluid containing Zb03 (H6N2), Zb04 (H3N8) or Zb10 (H6N2) ($10^{7.5}$ EID_{50} ml^{-1}). To serve as a control, a group of five mice was mock infected with sterile PBS. Mice were observed daily for morbidity (weight loss, ruffled fur and hunching) and mortality for 14 days. On day 3 p.i., half of the virus-inoculated mice were euthanized, and the titres of virus in the lung and brain were determined using eggs. Briefly, a 10 % lung and brain tissue homogenate was prepared using minimal essential medium (Gibco) containing antibiotics. The tissue homogenates were clarified by centrifugation and titrated in 10–11-day-old embryonated chicken's eggs. The virus titre was calculated as the $\log_{10}$ EID_{50} (g tissue)$^{-1}$ by the method of Reed & Muench (1938).

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FULL PAPER

Virological surveillance and phylogenetic analysis of the PB2 genes of influenza viruses isolated from wild water birds flying from their nesting lakes in Siberia to Hokkaido, Japan in autumn

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Abstract

Recent introduction of H5N1 highly pathogenic avian influenza virus (HPAIV) in wild birds from poultry in Eurasia signaled the possibility that this virus may perpetuate in nature. Surveillance of avian influenza especially in migratory birds, therefore, has been conducted to provide information on the viruses brought by them to Hokkaido, Japan, from their nesting lakes in Siberia in autumn. During 2008-2009, 62 influenza viruses of 21 different combinations of hemagglutinin (HA) and neuraminidase (NA) subtypes were isolated. Up to September 2010, no HPAIV has been found, indicating that H5N1 HPAIV has not perpetuated at least dominantly in the lakes where ducks nest in summer in Siberia. The PB2 genes of 54 influenza viruses out of 283 influenza viruses isolated in Hokkaido in 2000-2009 were phylogenetically analysed. None of the genes showed close relation to those of H5N1 HPAIVs that were detected in wild birds found dead in Eurasia on the way back to their northern territory in spring.

Keywords: *Avian influenza, migratory ducks, PB2 gene, surveillance*

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Introduction

Ecological studies have revealed that a vast influenza virus gene pool for avian and mamalian influenza exists in migratory ducks[10]. Each of the sixteen hemagglutinin (HA) and nine neuramidase (NA) subtypes of influenza A viruses are perpetuated among migratory ducks and their nesting lake water in nature[4,7,15,23]. Transmission of H5 or H7 influenza viruses to domestic birds and especially in chickens may result in the emergence of highly pathogenic avian influenza viruses (HPAIV)[14].

Since 2003, HPAIVs H5N1 have spread to 62 countries in Eurasia and Africa and seriously affected poultry in Asia. Over 400 million birds have died from the infection or been killed for control purposes. A HPAIV is generated when a non-pathogenic virus brought in by migratory birds from nesting lakes in the north is transmitted to chickens via domestic ducks, geese, quails, turkeys and acquires pathogenicity for chickens. During over-wintering, some migratory birds were conversely infected with HPAIV H5N1 from poultry and have been found dead at lakes in northern China, Mongolia, Japan, Russia, Europe and Africa in April to May on the way back to their nesting lakes in northern territories. It was found that each of the viruses isolated from these birds were genetically closely related to those of the isolates from poultry in China[2,10,16,17]. Thus HPAIV strains that are currently circulating in poultry have returned to migratory water birds and spread world wide[10].

Since it is of concern that this H5N1 virus may perpetuate in the lakes in Siberia where migratory ducks nest in summer, virological surveillance and phylogenetic analysis of influenza viruses have been carried out in autumn when these birds flew to Hokkaido, Japan in 2008-2009.

It is known that the PB2 protein is a component of the viral polymerase complex that plays an important role in virus replication[5,11,19], and is a determinant of host range and pathogenicity of influenza viruses[18,20]. Therefore, PB2 genes of influenza viruses isolated from migratory ducks have been phylogenetically analyzed in the present study.

Materials and Methods

Sample collection and virus isolation: A total of 1,626 fecal samples of wild water birds were collected in autumn in 2008-2009 from Lake Ohnuma, Wakkanai, and Ohno pond, Hokkaido University, Sapporo, Japan. The fecal samples collected were kept in chilled containers and transported to our laboratory. Virus isolation and subtyping were performed as previously described[9]. One virus of each of the HA and NA combinations was selected randomly by year of isolation for genetic analyses (Table 1).

RNA extraction, RT-PCR, and nucleotide sequencing: RNA extraction and RT-PCR were conducted as previously described[13]. Partial-length PB2 genes were amplified using PB2 gene-specific primer set PB2-625F (5'-CAT GTA TGC TAC CAT CAA GGG-3'), and the universal primer Ba-PB2-2341R[6]. The PCR products were separated by 0.8% agarose gel electrophoresis and purified using the MiniElute™ Gel Extraction Kit (Qiagen, USA) as recommended by the manufacturer. The purified products were used as templates in sequencing reactions using a BigDye terminator cycle sequencing ready reaction kit and analyzed on a 3130 Genetic Analyzer (Applied Biosystems). DNA sequences were assembled and edited using the program Genetyx ATGC (2008 Genetyx Corp.). The accession numbers of PB2 genes sequenced in this study are available from DDBJ/EMBL/GenBank under accession numbers given in Table 2.

Phylogenetic analysis of the PB2 genes: Phylogenetic analysis was conducted using PB2 gene sequences of 36 representative strains from a total of 54 that were sequenced. Published sequences used in this study for phylogenetic comparison were obtained using BLAST homology searches from the influenza sequence database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). The PB2 gene tree was generated using the Neighbor Joining (NJ) bootstrap method (1,000 replicates) implemented in the Molecular Evolutionary Genetics Analysis program (MEGA, version 4.0)[22]. The evolutionary distances were calculated by the Maximum Composite Likelihood method[21].

Results

Influenza A viruses isolated from fecal samples of wild water birds flying from their nesting lakes in Siberia to Hokkaido, Japan in autumn

In the surveillance of avian influenza conducted in Hokkaido in autumn 2008-2009, 62 influenza viruses have been isolated from a total of 1,626 fecal samples. The HA (H1, H3-H7, H9-H12) and NA (N1-N3, N5-N9) subtypes of the isolates were identified. Twenty one HA and NA combinations were detected in the present study (Table 1). In the surveillance studies in 2000-2009 performed by our laboratory, no H5N1 HPAIV was isolated from wild water birds that flew from their nesting lakes in Siberia to Hokkaido, Japan in autumn[13].

Table 1. Influenza viruses isolated from fecal samples of free-flying water birds 2008-2009

Subtypes of influenza viruses isolated in following years	
2008	2009
H3N2 (1)[a]	H1N3 (1)
H3N6 (3)	H1N5 (1)
H4N6 (11)	H4N6 (5)
H5N2 (1)	H5N1 (1)
H6N1 (4)	H5N2 (1)
H6N2 (1)	H6N1 (4)
H6N5 (1)	H6N8 (2)
H6N8 (1)	H11N9 (3)
H6N9 (1)	H12N5 (1)
H7N7 (1)	
H9N5 (1)	
H9N9 (1)	
H10N9 (2)	
H10N7 (11)	
H11N9 (2)	
H12N2 (1)	

[a]Number of isolates of subtypes were shown in parenthesis.

Sequencing and phylogenetic analysis of the PB2 genes of influenza virus isolates from migratory birds

Randomly selected 54 isolates out of 283 avian influenza viruses isolated in the surveillance studies in 2000-2009 were sequenced of which 36 were phylogenetically analyzed. A phylogenetic tree was constructed on the basis of the partial nucleotide sequences of the PB2 genes (positions 1425-2192) of viruses isolated from wild water birds in Hokkaido in 2000 to 2009 (Fig. 1).

Phylogenetic tree of the PB2 genes was divided into American and Eurasian lineages. Duan *et al.*[3] showed that Eurasian lineage could be further divided into early and contemporary sublineages. Phylogenetic analysis of the PB2 genes of the isolates in the present study, belonged to the Eurasian lineage and were grouped (bootstrap values more than 85) into contemporary sublineages I and II. All the viruses analyzed in the present study belonged to either contemporary sublineage I or sublineage II. The majority of the PB2 genes under study clustered in different groups of sublineage I. They either clustered together or showed close relation to the PB2 genes of influenza viruses isolated from domestic and wild birds in China, Russia, Australia and Korea. It was noted that some of the strains, A/duck/Hokkaido/WZ76/2008