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Reassortant low-pathogenic avian influenza H5N2 viruses in African wild birds

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To investigate the presence and persistence of avian influenza virus in African birds, we monitored avian influenza in wild and domestic birds in two different regions in Nigeria. We found low-pathogenic avian influenza (LPAI) H5N2 viruses in three spur-winged geese (*Plectropterus gambensis*) in the Hadejia–Nguru wetlands. Phylogenetic analyses revealed that all of the genes, except the non-structural (NS) genes, of the LPAI H5N2 viruses were more closely related to genes recently found in wild and domestic birds in Europe. The NS genes formed a sister group to South African and Zambian NS genes. This suggested that the Nigerian LPAI H5N2 viruses found in wild birds were reassortants exhibiting an NS gene that circulated for at least 7 years in African birds and is part of the African influenza gene pool, and genes that were more recently introduced into Africa from Eurasia, most probably by intercontinental migratory birds. Interestingly the haemagglutinin and neuraminidase genes formed a sister branch to highly pathogenic avian influenza (HPAI) H5N2 strains found in the same wild bird species in the same wetland only 1 year earlier. However, they were not the closest known relatives of each other, suggesting that their presence in the wetland resulted from two separate introductions. The presence of LPAI H5N2 in wild birds in the Hadejia–Nguru wetlands, where wild birds and poultry occasionally mix, provides ample opportunity for infection across species boundaries, with the potential risk of generating HPAI viruses after extensive circulation in poultry.

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INTRODUCTION

Wild birds, in particular of the orders Charadriiformes and Anseriformes, are considered natural asymptomatic reservoirs of low pathogenic avian influenza (LPAI) virus (Suarez, 2000; Webster *et al.*, 1992) and the source of influenza viruses in other species including poultry (Webster *et al.*, 1992). All subtypes (H1–16 and N1–9) of avian influenza have been found in wild birds (Alexander & Brown, 2000; Fouchier *et al.*, 2005). Migration after the breeding season along distinct flyways contributes to long-distance dissemination of influenza viruses (Garamszegi & Møller, 2007), and the aquatic environment of waterfowl supports efficient short-range virus transmission of LPAI viruses by the faecal–oral route (Ito *et al.*, 1995; Webster *et al.*, 1992). High densities of mixed bird populations at stopovers and non-breeding sites also promote intra- and interspecies virus transmission (Olsen *et al.*, 2006).

Wetlands in Africa are preferred non-breeding sites for many Eurasian migratory waterbirds. Although Eurasian species can mix with resident African birds or intra-African migrants in many important bird areas (Ezealor, 2001; Scott & Rose, 1996), only a few avian influenza viruses (AIV) have been reported from Africa before 2006 (Abolnik, 2007a; Abolnik *et al.*, 2006, 2007, 2009; Allwright *et al.*, 1993; Banks *et al.*, 2000; Becker, 1966; Pfitzer *et al.*, 2000; Röhm *et al.*, 1995; Saad *et al.*, 2007). The first seroprevalence study in sub-Saharan Africa, conducted in commercial poultry in Nigeria between 1999 and 2004, did not detect antibodies to influenza viruses (Owoade *et al.*, 2006). In a first wild-bird surveillance effort, a wide variety of LPAI viruses were identified in migratory waterbirds (Gaidet *et al.*, 2007). Interest in AIV in wild birds in Africa further increased in

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Colour versions of Figs 1 and 2, showing phylogenies of the H5 and NS genes, and six supplementary figures showing phylogenies of the NA, PA, PB1, PB2, matrix and NP genes are available with the online version of this paper.

2006 when highly pathogenic avian influenza (HPAI) H5N1 virus was first identified in Africa in Nigerian poultry farms (ProMED, 2006). After the independent introduction of three sublineages of clade 2.2 viruses into poultry, HPAI H5N1 viruses have undergone multiple reassortments and reassorted viruses have largely replaced the initial sublineages (Owoade *et al.*, 2008). The route of introduction of H5N1 is unknown and both migratory birds and poultry trade might be suspected (Ducatez *et al.*, 2006, 2007a; Fusaro *et al.*, 2009). Although efforts to isolate influenza viruses from wild birds in Africa intensified, few met with success (Ducatez *et al.*, 2007b; Saad *et al.*, 2007). Vultures have probably been infected by scavenging H5N1-infected poultry (Ducatez *et al.*, 2007b) but the infection route of the common teal (*Anas crecca*) from Egypt is not known (Saad *et al.*, 2007).

In this study, we investigated the presence of avian influenza in wild and domestic birds in two different locations in Nigeria in 2008. The Dagona wildlife sanctuary is a protected area within the Hadejia–Nguru wetlands, located in north-eastern Nigeria, and is part of the Chad Basin national park. Large numbers of migratory birds mix with local wild birds in the Hadejia–Nguru wetlands. The Hadejia–Nguru wetlands are also the most important bird area in the region, from which the first HPAI H5N1 outbreak in Africa was reported. The Amurum Forest reserve is also a protected area located 15 km north-east of

Table 1. List of sampled domestic and wild bird species (order and family) in Dagona wildlife sanctuary and in the Amurum forest reserve and villages around Jos, Nigeria in 2008

For each family, the number of samples from domestic birds is given in parentheses.

Order	Family	Dagona wildlife sanctuary		Amurum Forest reserve and villages around Jos	
		No. species per family	No. samples per family	No. species per family	No. samples per family
Accipitiformes	Accipitridae	2	4		
Anseriformes	Anatidae	4	262 (133)	1	5 (5)
Charadriiformes	Jacanidae	2	32		
	Scolopacidae	2	18		
Ciconiiformes	Ardeidae	1	3		
Coliiformes	Coliidae			1	9
Columbiformes	Columbidae	6	162	2	4
Coraciformes	Alcedinidae	2	20	1	1
	Coraciidae	1	1		
Cuculiformes	Cuculidae	1	1	1	1
Galliformes	Meleagrididae			1	7 (7)
	Phasianidae	1	240 (240)	1	362 (362)
Gruiformes	Rallidae	1	1		
Piciformes	Indicatoridae	1	1	1	2
	Lybiidae			4	16
Passeriformes	Alaudidae			1	1
	Dicruridae	1	1		
	Emberizidae			1	1
	Estrildidae	5	14	6	38
	Fringillidae	1	37		
	Hirundinidae	3	9		
	Malaconotidae			3	7
	Monarchidae			1	2
	Motacillidae	2	14	1	1
	Muscicapidae			1	3
	Nectariniidae			3	8
	Passeridae	3	56	2	2
	Platysteiridae			1	1
	Ploceidae	5	123	4	12
	Pycnonotidae			1	3
	Sylviidae	1	4	13	32
	Turdidae			2	5
	Viduidae	2	21	1	1
	Zosteropidae			1	4
Total		**47**	**1024**	**55**	**528**

Fig. 1. Dated phylogeny of H5 genes. The horizontal axis represents calendar years and nodes correspond to mean TMRCAs. Posterior probability values >0.75 are shown. Representative strains from Africa, Europe and Asia are included. Virus strains characterized in this study are shown in boldface type. *, HPAI strains. Node I represents the mean TMRCAs of the LPAI H5N2 cluster. Node II corresponds to the most recent common ancestor of the Nigerian LPAI and HPAI supported by a high posterior probability value.

Jos (Plateau State), very close to the urban community (Ezealor, 2001). We did not find any HPAI H5N1 virus, but did find LPAI H5N2 strains that contained genes from the Eurasian gene pool and a non-structural (NS) gene that was most closely related to other African viruses. Interestingly the haemagglutinin (HA) genes were part of a cluster that also contained HA genes from HPAI H5N2 viruses found in the same bird species and in the same region 1 year earlier.

RESULTS

Avian influenza prevalence is low

A total of 1024 samples were collected in the Dagona wildlife sanctuary from 44 wild bird species, including waterfowl, waders and passerines, and from 373 domestic poultry such as chickens, ducks and geese (Table 1). Around Jos, cloacal samples were collected from 374 domestic birds (362 chickens, 5 ducks and 7 turkeys). In addition 154 wild birds corresponding to 52 species, mainly passerines, were sampled in the Amurum Forest reserve (Table 1). Only three fresh faecal samples collected from spur-winged geese (*Plectropterus gambensis*) in a single location in the Dagona wildlife sanctuary (12°44.676 N, 10°40.001 E) were influenza A positive. They were collected on 31 March 2008 (A/spur-winged goose/Nigeria/2/2008) and on 3 April 2008 (A/spur-winged goose/Nigeria/210/2008 and A/spur-winged goose/Nigeria/226/2008). Thus, in our study, the prevalence of AIV in the Dagona wildlife sanctuary was 0.3 % (3/1024). All samples collected in Plateau State were influenza A negative.

Molecular analyses show no marker of virulence

Genotype-specific PCRs were positive for H5 and N2 for the three viruses and all of the genes were sequenced using previously published or newly designed primers. Analyses of the HA gene showed that the predicted amino acid sequence of the cleavage site of the three viruses corresponded to a low pathogenic pathotype (PQRETR*GLF). They had a glutamine at position 226 and a glycine at position 228 (numbering for the H3 subtype) indicating a higher binding affinity for sialic acid α2,3, which is characteristic of avian cell-surface receptors (Matrosovich *et al.*, 1997; Rogers *et al.*, 1983). No stalk deletion in the neuraminidase (NA) gene, nor additional predicted glycosylation sites, both proposed to be associated with poultry adaptation, were detected (Baigent & McCauley, 2003; Matrosovich *et al.*, 1999). No genetic marker associated with increased virulence in mammals [PB2 627E, 701D, 714S, PB1 678S, PA 615K and NP 319N (Gabriel *et al.*, 2005)] or drug resistance [M2 26L, 27V, 30A, 31S and 34G (Hay *et al.*, 2008); NA 119E (Kiso *et al.*, 2004); 274H (Gubareva *et al.*, 2001); 292R (Kiso *et al.*, 2004); 294N (Kiso *et al.*, 2004; Le *et al.*, 2005)] was found.

Phylogenetic analyses of the eight genes reveal a separate origin for the NS gene

To explore the evolutionary origin and the time of introduction of LPAI H5N2 to Nigeria, tree topologies were assessed and times to the most recent common ancestor (TMRCAs) were estimated using BEAST version 1.5.3. For each gene, the three LPAI H5N2 viruses were genetically highly similar (Kimura distance from 0 to 0.9 %) and always clustered together (100 % posterior probabilities for all genes), suggesting that the three strains recently evolved from a common ancestor and resulted from a single introduction event in the Nigerian wetlands. All of the genes belonged to the Eurasian and not the American lineage, but none of them were related to HPAI H5N1 strains from Nigeria, from other parts of Africa or from Eurasia.

Phylogenetic analyses of the HA gene revealed that the LPAI H5N2 viruses were most closely related to a European H5N3 isolate (A/duck/Tulcea/RO-AI-LPAI/2009) recently found in a sentinel duck in Romania (Fig. 1). The NA genes of the LPAI H5N2 viruses were most closely related to an H2N2 strain from the Netherlands (A/mallard/Netherlands/14/2007) (Supplementary Fig. S1, available in JGV Online). The LPAI H5N2 viruses formed a sister clade to strains recently isolated from wild birds in Europe (HA and NA genes) and Asia (NA gene). Interestingly they also formed a sister branch with highly pathogenic H5N2 strains that had been isolated one year earlier in a similar location (10 km away) from the same wild bird species (HA and NA genes). In the same HA cluster, closely related HPAI and LPAI H5N2 viruses, both from South African ostriches (A/ostrich/South Africa/AI1160/2006, LPAI; and A/ostrich/South Africa/AI1091/2006, HPAI), formed another sister branch to the Nigerian LPAI H5N2 viruses. However, all African isolates did not share a direct common ancestor, suggesting that they did not directly evolve from each other. The TMRCAs of the LPAI H5N2 HA and NA genes ranged from February 2007 to March 2008 (95 % highest posterior density interval, HPD) and October 2006 to March 2008 (95 % HPD), respectively.

The influenza virus PA polymerase subunit (PA) gene of the LPAI H5N2 viruses formed a sister clade with PA genes from a goose and wild and captive ducks from France, the Netherlands and Sweden (Supplementary Fig. S2, available

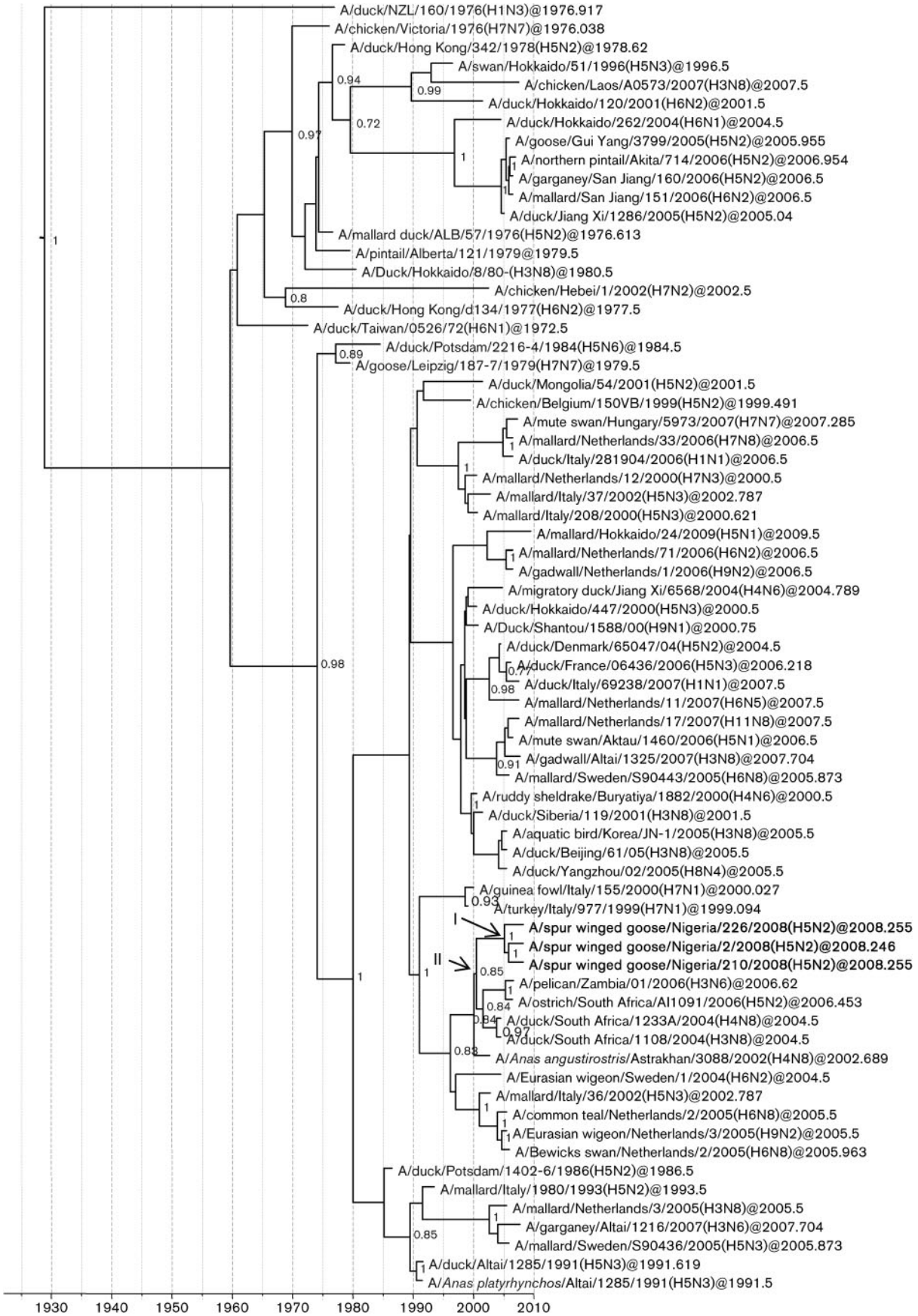

Fig. 2. Dated phylogeny of NS genes clustering in allele B. The horizontal axis represents calendar years and nodes correspond to mean TMRCAs. Posterior probability values >0.75 are shown. Representative strains from Africa, Europe and Asia are included. Virus strains characterized in this study are presented in boldface type. Node I represents the mean TMRCAs of the LPAI H5N2 cluster. Node II corresponds to the most recent common ancestor of the African NS genes, including the Nigerian LPAI H5N2.

in JGV Online). The TMRCA of the LPAI H5N2 cluster was estimated as being between July 2005 and February 2008 (95 % HPD). The polymerase basic protein 1 (PB1) gene showed that the LPAI H5N2 viruses formed a sister clade with two wild-bird viruses from the Netherlands (A/turnstone/Netherlands/1/2007, H3N8 and A/mallard/Netherlands/17/2007, H11N8) and a virus from one domestic goose from the Czech Republic (A/goose/Czech Republic/1848/2009, H7N9; Supplementary Fig. S3, available in JGV Online). The TMRCA of the LPAI H5N2 cluster was estimated as being between June 2006 and March 2008 (95 % HPD). The PB2 genes of the three H5N2 strains were most closely related to A/quail/Italy/4610/2003 (H7N2) and the TMRCA of the LPAI H5N2 cluster ranged from June 2005 to March 2008 (95 % HPD; Supplementary Fig. S4, available in JGV Online).

The matrix gene sequences were more closely related to an H9N2 matrix gene from the Netherlands (A/Bewick's swan/Netherlands/5/2007) and the TMRCA ranged from June 2005 to January 2008 (95 % HPD; Supplementary Fig. S5, available in JGV Online). The three nucleoprotein (NP) genes clustered with viral genes isolated from four ducks, one swan and one turnstone from Italy, the Netherlands, Hungary, Germany and Sweden; the TMRCA of the cluster formed by these three genes ranged from June 2005 to February 2008 (95 % HPD; Supplementary Fig. S6, available in JGV Online).

The NS gene sequences clustered in allele B and formed a sister group to three South African NS sequences (A/ostrich/South Africa/AI1091/2006, H5N2; A/duck/South Africa/1108/2004, H3N8; and A/duck/South Africa/1233A/2004, H4N8) and to an H3N6 gene from Zambia (A/pelican/Zambia/01/2006) (Fig. 2). Values larger than 95 % HPD were observed for the TMRCA of the Nigerian LPAI H5N2 cluster (January 2002 to February 2007), which may indicate an older origin for that gene, although we cannot exclude that our dataset for the NS segment does not contain sufficient phylogenetic signal to provide precise estimates. Also, the exclusion of the distantly related NS gene of A/duck/NZL/160/1976 (H1N3) from the phylogenetic analysis had little influence on the TMRCA. The close relationship suggests that all seven viruses shared a recent common ancestor that was introduced to Africa (TMRCA of the African NS cluster, node II in Fig. 2, November 1997 to May 2003).

DISCUSSION

Although avian influenza has been extensively studied for decades in wild birds from the northern hemisphere, with few exceptions (Abolnik, 2007a; Abolnik *et al.*, 2006, 2007, 2009; Allwright *et al.*, 1993; Banks *et al.*, 2000; Becker, 1966; Pfitzer *et al.*, 2000; Röhm *et al.*, 1995; Saad *et al.*, 2007) AIV has received attention in Africa only after the introduction of HPAI H5N1 viruses. In our survey, three of 1024 (0.3 %) samples collected in the Dagona wildlife sanctuary, a part of the Hadejia–Nguru wetlands, in north-eastern Nigeria were positive for influenza A. This corresponds to a prevalence of 1 % (3/312) in birds from the orders Charadriiformes and Anseriformes that are considered the natural reservoir of AIV (Webster *et al.*, 1992). In spur-winged geese we found an infection rate of 2.4 % (3/123) compared with 8.2 % (8/97) in the earlier study (Gaidet *et al.*, 2008). The three viruses found were low-pathogenic H5N2 viruses most closely related to each other over their full genomes. A tenfold higher prevalence (3.9 %) was found in waterbirds in the Hadejia–Nguru wetlands in the previous year (Gaidet *et al.*, 2008). This is similar to the overall prevalence (3.5 %) found in a previous study in wetlands throughout 12 African countries, including several neighbouring countries of Nigeria (Gaidet *et al.*, 2007). Many factors including the year, season, location, species and age of birds influence the prevalence of AIV, as is known from wild birds in Europe and America (Ip *et al.*, 2008; Munster *et al.*, 2007; Munster & Fouchier, 2009; Stallknecht *et al.*, 1990; Wallensten *et al.*, 2007).

Phylogenetic analyses revealed that all genes of our LPAI H5N2 viruses were most closely related to genes circulating in the Eurasian wild-bird influenza gene pool. It has been shown that migratory birds can carry LPAI viruses from one continent to another (Fouchier *et al.*, 2007; Olsen *et al.*, 2006), introducing new genes and mixing gene pools (Dugan *et al.*, 2008; Koehler *et al.*, 2008; Spackman *et al.*, 2005). Three main migratory flyways link Africa to Eurasia (the East Atlantic flyway, the Black Sea–Mediterranean flyway and the East Africa–West Asia flyway; Olsen *et al.*, 2006) and Nigeria is located where they intersect. Thus, low pathogenic avian influenza genes may have been introduced from Eurasia to Africa by migratory birds. However, spur-winged geese are in principle a sedentary sub-Saharan species, normally making only short daily flights, and only rarely longer flights, depending on the availability of water. The species is widespread throughout sub-Saharan Africa, but does not leave the continent (Brown *et al.*, 1982; Scott & Rose, 1996). During the dry season, spur-winged geese are highly gregarious around permanent waterbodies (Brown *et al.*, 1982) such as in the Hadejia–Nguru wetlands where they mix with Afro-tropical birds and Eurasian migratory birds such as garganey (*Anas querquedula*), northern pintail (*Anas acuta*) or ferruginous duck (*Aythya nyroca*) (Brown *et al.*, 1982; Ezealor, 2001; Scott &

Rose, 1996). The mingling of Eurasian migratory and African bird species in the Hadejia–Nguru wetlands seems to be reflected also in the AIV gene pools.

Interestingly, the NS gene sequences of the three LPAI H5N2 viruses from Nigeria shared a common ancestor with South African and Zambian influenza isolates from 2004 and 2006, suggesting another origin for the NS gene, in contrast to all of the other genes. Also, the TMRCA of the Nigerian LPAI H5N2 NS genes suggested that the cluster emerged in approximately October 2004 (January 2002–February 2007; 95 % HPD; node I in Fig. 2), whereas the mean TMRCAs for the other genes were found to be during late 2006 or 2007 (Table 2). The TMRCA of the African monophyletic cluster, which included the Nigerian, South African and Zambian strains, suggested that a virus was introduced to Africa between 1997 and 2003. This ancestral virus evolved and probably reassorted with other viruses present in the African wild bird population, as has already been suggested (Abolnik, 2007a; Abolnik *et al.*, 2006; Simulundu *et al.*, 2009). The NS gene was acquired by viruses that later spread throughout Africa. Ring recoveries have shown that some intra-African migratory birds from South Africa [e.g. comb duck (*Sarkidiornis melanotos*)] sometimes migrate as far north as West Africa (Hockey *et al.*, 2005) and thus would be able to transmit AIV over long distances within Africa. The presence of similar genes over a four year period (2004–2008; Fig. 3) and its probable introduction at least 7 years ago suggests that AIV can persist in the African bird population.

The Eurasian–African AIV interface resembles the situation in the Bering Strait where interregional transmission of influenza viruses occurs between North American and Eurasian birds. Genes from the Asian lineage have been found in Alaska (Koehler *et al.*, 2008; Ramey *et al.*, 2010) and genes from the American lineages have been found in Japan (Liu *et al.*, 2004), producing a variety of reassortants. Also, similarly to the persistence of Eurasian genes in Africa, now constituting the African gene pool, H6 genes from the Eurasian lineages have been introduced into North America and gradually replaced the American H6 strains (zu Dohna *et al.*, 2009).

The presence of LPAI H5N2 viruses in African wild birds represents a certain risk also for poultry. Infected wild birds, such as spur-winged geese, may introduce LPAI viruses into free-ranging domestic ducks reared in the wetlands in north-eastern Nigeria with whom they occasionally mix (Cecchi *et al.*, 2008). Similar situations have already been observed in South Africa where wild birds were suspected to have infected domestic birds with whom they shared similar genes (Fig. 3). Farmed ostriches were infected with H6N8 in 1998 (Abolnik, 2007b) and 2007 (Abolnik *et al.*, 2010), with H5N2 viruses in 2004 and 2006 (Abolnik, 2007a; Abolnik *et al.*, 2009), and with H9N2 in 2008. H10N7 was also found in domestic ducks (Abolnik *et al.*, 2010) (Fig. 3). In addition, HPAI viruses are thought to emerge after extensive circulation of H5 (and H7) LPAI subtypes and adaptation in poultry (Alexander, 2007; Lupiani & Reddy, 2009; Munster *et al.*, 2005; Röhm *et al.*, 1995). This is of particular concern since the Nigerian LPAI H5N2 strains belong to a genetic cluster that seems to have an increased propensity to develop the highly pathogenic phenotype. Indeed, from a common node (node II, Fig. 1) that emerged between June 2002 and May 2004 (95 % HPD), highly pathogenic H5N2 strains emerged twice in South Africa in 2004 (not shown in Fig. 1 because of its shorter sequence; Abolnik, 2007a, b) and in 2006 (Abolnik, 2007a, b), and in Nigeria in 2007 (Gaidet *et al.*, 2008).

Interestingly, the latter Nigerian HPAI H5N2 viruses were found only 1 year earlier, about 10 km away in the same wetlands and in the same wild bird species as the LPAI H5N2 described in this study. For both the HA and NA genes, the HPAI H5N2 formed a sister branch to the LPAI H5N2 but were not the closest known relatives of each other, suggesting that their presence in the wetlands resulted from two separate introductions. The question

Table 2. Estimated TMRCAs of the Nigerian LPAI H5N2 cluster, nucleotide substitution model and sequence length used in the Bayesian analyses

General time-reversible (GTR) substitution model with a gamma (Γ) and invariant (I) site heterogeneity model.

	Mean TMRCA (95 % HPD)	Nucleotide substitution model	Sequence length (nt)
PB2	March 2007 (Jun 05 – Mar 08)	GTR+I+Γ	1593
PB1	June 2007 (Jun 06 – Mar 08)	GTR+I+Γ	1692
PA	March 2007 (Jul 05 – Feb 08)	GTR+I+Γ	2151
HA	October 2007 (Feb 07 – Mar 08)	GTR+I+Γ	1710
NP	January 2007 (Jun 05 – Feb 08)	GTR+I+Γ with simplifications: CP3.cg=CP3.gt, CP1+2.at=CP1+2.gt=CP1+2.cg	855
NA	August 2007 (Oct 06 – Mar 08)	GTR+I+Γ	1377
M	December 2006 (Jun 05 – Jan 08)	GTR+I+Γ with simplifications: CP3.cg=CP3.at, CP1+2.at=CP1+2.cg	759
NS	October 2004 (Jan 02 – Feb 07)	GTR+Γ with simplifications: CP3.cg=CP3.gt	690

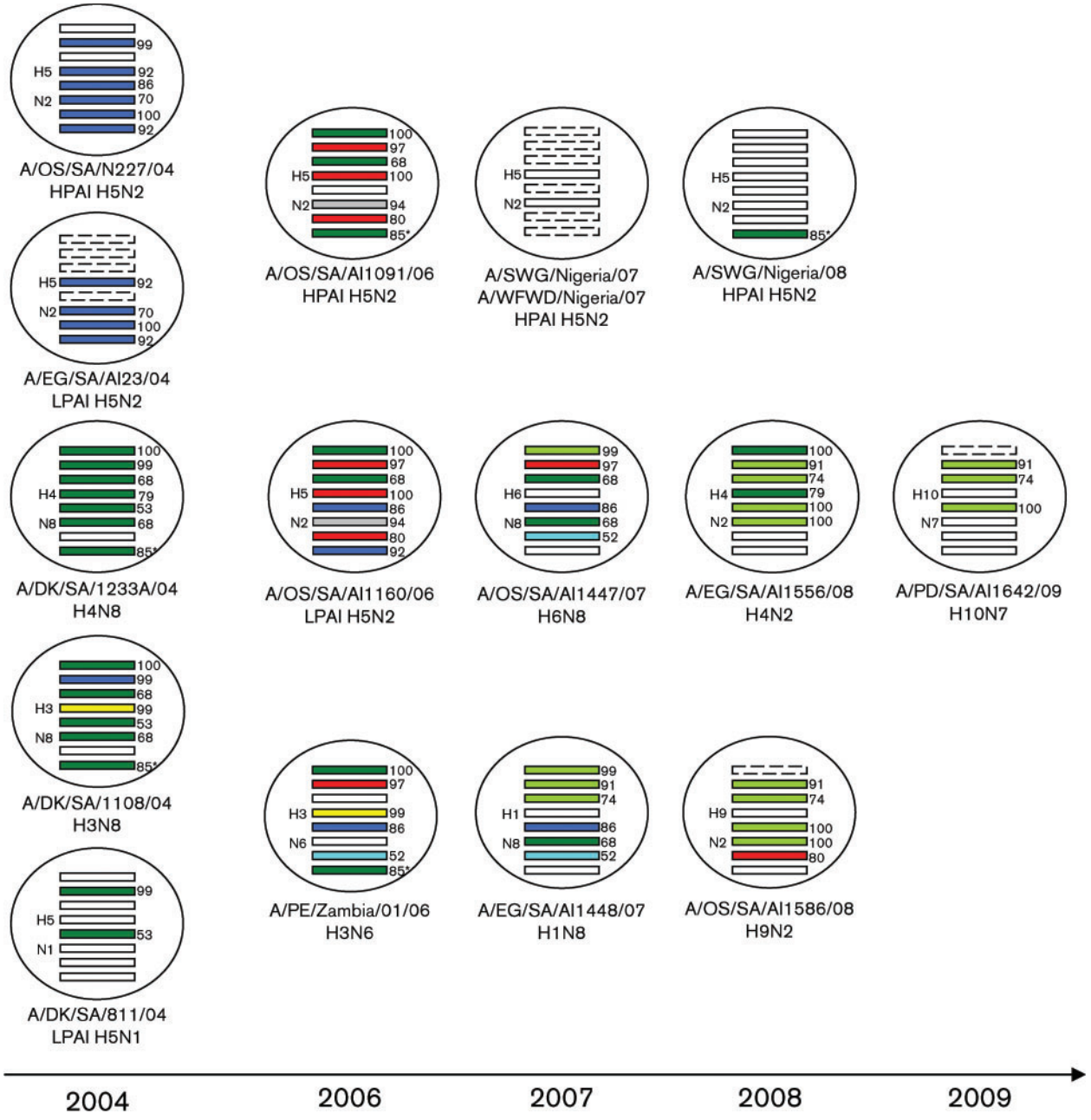

Fig. 3. Genetic relationship of African AIV viruses, other than HPAI H5N1 viruses, sequenced since 2004, by year, in wild and domestic birds. Genes have the same colour code if they share a direct common ancestor. Unsequenced genes are indicated as dotted bars and genes that have no African sister gene are shown as white bars. Phylogenetic relationships were first assessed by comparing the African strains with all avian influenza strains downloaded on to the NCBI Influenza Virus Resource database, but based on shorter fragments, depending on the shortest sequences available for the African strains. Representative strains were selected for each gene based on these preliminary analyses and trees were calculated using MEGA4 (Tamura *et al.*, 2007) by using the neighbour-joining method and by using the Kimura two parameters model and 1000 bootstrap replicates. *, Values corresponding to the probability values from Fig. 2. The figure suggests, based on the available data (from South Africa, Nigeria and Zambia), that some avian influenza viruses may be maintained in the African wild-bird population where reassortment events can occur, and that these viruses can be transmitted from wild to domestic birds. Only A/DK/SA/811/04 (LPAI H5N1) does not share any gene with a domestic bird strain. OS, Ostrich; EG, Egyptian goose; SWG, spur-winged goose; WFWD, white-faced whistling duck; PD, pekin duck; PE, pelican; SA, South Africa.

also remains as to where the Nigerian HPAI H5N2 virus acquired its HPAI phenotype. The wetlands in north-eastern Nigeria provide ample opportunity for cross-species infection and perhaps even the generation of HPAI viruses, which normally only occur after circulation and adaptation in poultry.

In conclusion, we report the presence of LPAI H5N2 viruses in wild birds in an African wetland, which were reassortants with genes from the Eurasian and African gene pools, as strong evidence of the introduction of low-pathogenic avian influenza into Africa by Eurasian migratory birds. Furthermore, the circulation of LPAI and HPAI H5N2 strains in wild birds in African wetlands that emerged from a cluster that had an increased propensity to develop the highly pathogenic phenotype represents a high risk for poultry, especially in areas with low biosecurity that provide opportunities for cross-species infections.

METHODS

Wild bird surveillance. Wild birds were captured with mist nets in the Dagona wildlife sanctuary in north-eastern Nigeria between 28 March and 22 April 2008. Oropharyngeal and cloacal swabs as well as fresh faecal samples were collected after ensuring the species of origin. Domestic poultry in the villages around the wetlands were also sampled. All samples were collected in triplicate with cotton swabs, stored in virus transport medium [PBS pH 7.0 with 2000 U penicillin ml^{-1}, 200 mg streptomycin ml^{-1}, 2000 U polymyxin B ml^{-1}, 250 μg gentamicin ml^{-1}, 60 μg ofloxacin ml^{-1}, 200 μg sulfamethoxazole ml^{-1} and 2.5 μg amphotericin B ml^{-1}] and placed directly into liquid nitrogen in the field. In addition, between 4 December 2007 and 5 March 2008, swabs were collected from wild birds in the Amurum forest reserve (Plateau State) and from backyard poultry in five villages around Jos.

RNA Extraction, RT-PCR and sequencing. RNA was extracted from 50 μl of virus transport medium by using a MagMAX-96 AI/ND Viral RNA Isolation kit (Ambion) and a KingFisher 96 (Thermo Fisher). Influenza A-positive specimens were detected by using a real-time RT-PCR assay targeting the matrix gene and previously published gene-specific primers (Ward *et al.*, 2004) and probe (Ducatez *et al.*, 2006). RT-PCRs were carried out using the following cycling conditions: reverse transcription for 30 min at 50 °C, denaturation at 95 °C for 15 min followed by 40 cycles of amplification at 95 °C for 10 s, 60 °C for 20 s. Amplifications were performed with a Qiagen OneStep RT-PCR kit using 2 μl of RNA in a final volume of 25 μl. Matrix-positive samples were tested for H5 (Ducatez *et al.*, 2006), H7 (http://www.defra.gov.uk/vla/science/docs/sci_ai_vi536.pdf) and N1 (Payungporn *et al.*, 2006) genotypes. The eight genes were then amplified by using several PCRs targeting overlapping fragments (primer sequences and details available upon request). PCR products were purified using a JetQuick PCR Purification Spin kit (Genomed). Sequencing was performed as previously described (Snoeck *et al.*, 2009) using PCR primers as sequencing primers.

Molecular and phylogenetic analyses. Sequence assembly and analyses were performed using SeqScape version 2.5 (Applied Biosystems) and BioEdit (Hall, 1999). The nucleotide sequences are available in the GenBank/EMBL/DDBJ databases under the accession numbers FR771823–FR771846. Potential *N*-linked glycosylation sites were predicted for HA and NA by using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). Kimura distances were calculated with MEGA4 (Tamura *et al.*, 2007) using the Kimura two parameters model. For each gene, phylogenetic relationships were inferred by comparing the LPAI H5N2 strains with all avian influenza strains downloaded to the NCBI Influenza Virus Resource database (up until 15 December 2009; www.ncbi.nlm.nih.gov./genomes/FLU/) after removing short sequences and sequences with insertions or deletions resulting in frame shifts. Datasets were aligned using CLUSTAL W (Thompson *et al.*, 1994). Coding regions were used for phylogenetic analyses and only the first ORFs were used for the matrix and NS genes. Trees were calculated using MEGA4 (Tamura *et al.*, 2007) with the neighbour-joining method by using the Kimura two parameters model and 1000 bootstrap replicates. Representative strains were selected for each gene based on these preliminary analyses.

Tree topologies, substitution rates and TMRCAs were estimated by a Bayesian Markov-chain Monte Carlo (MCMC) method (Drummond *et al.*, 2002) implemented in BEAST version 1.5.3 (Drummond & Rambaut, 2007). Depending on the available details on isolation dates, the exact isolation dates, the mid-month dates (15th), the mid-interval dates or the mid-year dates were used as calibration points. For each dataset, different substitution models with two codon partitions to allow independent estimates for the third codon position, two uncorrelated relaxed-clock models (log-normal and exponential distributions; Drummond *et al.*, 2006) and two coalescent models (constant population size and Bayesian skyline; Drummond *et al.*, 2005) were compared visually in TRACER version 1.5.3 (Rambaut & Drummond, 2009) and statistically using a Bayes factor test (the ratio of the marginal likelihoods of two models) (Kass & Raftery, 1995; Suchard *et al.*, 2001), as implemented in TRACER version 1.5.3, in order to identify the model that fitted the data best. Evidence against the null model, which is the model with the lowest marginal likelihood, was assessed by the method proposed by Kass & Raftery (1995). When there was no evidence against the null model or when this evidence was weak, the simplest model was kept to avoid unnecessary overparameterization. Analyses revealed that the GTR substitution model, assuming an uncorrelated exponential relaxed clock and a constant population size, was the model that best fitted the data for all genes. For matrix, NP and NS genes, the GTR model was further simplified to avoid overparameterization (Table 2). Two to three runs of $50–100 \times 10^6$ generations of the MCMC method were performed and sampled to produce 10 000 trees each. Convergence of the runs was confirmed in TRACER version 1.5.3. The results of multiple runs were combined using LogCombiner version 1.5.3 (Drummond & Rambaut, 2007) with a burn-in of 10–25 %, summarized into the maximum clade credibility tree using TreeAnnotator version 1.5.3 (Drummond & Rambaut, 2007) and visualized in FigTree version 1.3.1 (Rambaut, 2009).

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