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Molecular analysis of avian H7 influenza viruses circulating in Eurasia in 1999–2005: detection of multiple reassortant virus genotypes

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Avian influenza infections by high and low pathogenicity H7 influenza viruses have caused several outbreaks in European poultry in recent years, also resulting in human infections. Although in some cases the source of H7 strains from domestic poultry was shown to be the viruses circulating in the wild bird reservoir, a thorough characterization of the entire genome of H7 viruses from both wild and domestic Eurasian birds, and their evolutionary relationships, has not been conducted. In our study, we have analysed low pathogenicity H7 influenza strains isolated from wild and domestic ducks in Italy and southern China and compared them with those from reared terrestrial poultry such as chicken and turkey. Phylogenetic analysis demonstrated that the H7 haemagglutinin genes were all closely related to each other, whereas the remaining genes could be divided into two or more phylogenetic groups. Almost each year different H7 reassortant viruses were identified and in at least two different years more than one H7 genotype co-circulated. A recent precursor in wild waterfowl was identified for most of the gene segments of terrestrial poultry viruses. Our data suggest that reassortment allows avian influenza viruses, in their natural reservoir, to increase their genetic diversity. In turn this might help avian influenza viruses colonize a wider range of hosts, including domestic poultry.

INTRODUCTION

Influenza A viruses are negative-sense RNA viruses with a genome divided into eight segments coding for 11 proteins (Hay, 1998; Chen *et al*., 2001). They are further classified into subtypes according to the two surface glycoproteins: 16 different haemagglutinin (HA) and 9 neuraminidase (NA) subtypes have been identified so far (Fouchier *et al*., 2005). Influenza A virus host range includes wild waterfowl (their natural reservoir) and several other avian and mammalian species, including man (reviewed by Webster *et al*., 1992). Ecological studies have established that all influenza A viruses in nature are derived from strains circulating in wild aquatic birds, where they usually appear to be apathogenic (Webster *et al*., 1992). Transmission of avian influenza viruses (AIVs) from wild birds can cause disease in domestic poultry, and transfer of gene segments from avian to human viruses by reassortment has resulted in at least two human pandemics (Alexander, 2000;

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Scholtissek *et al.*, 1978). Of the 16 HA subtypes, two (H5 and H7) have the potential to become highly pathogenic (HP) for domestic poultry (Wood *et al.*, 1993; Senne *et al.*, 1996). Moreover, since the 1997 H5N1 influenza outbreak in Hong Kong, influenza A viruses belonging to several avian subtypes, including H5 and H7 HP viruses, have shown their ability to infect humans directly, suggesting that preliminary adaptation in an intermediate host through the generation of human–avian virus reassortants is not an absolute requirement to infect humans (Claas *et al.*, 1998; Fouchier *et al.*, 2004; Lin *et al.*, 2000; WHO, 2005). At present, the continued circulation of HP H5N1 viruses in domestic birds in Eurasian and northern African countries, accompanied by a growing number of human infections, mostly fatal, increases the likelihood that this virus may become transmissible from human to human and result in a pandemic (WHO, 2005). However, the type of aquatic bird influenza viruses capable of transmitting to domestic poultry is still unclear. In one instance, an H7N3 virus from wild waterfowl was shown to be the direct precursor of viruses of the same subtype that caused widespread and prolonged outbreaks of low pathogenicity (LP) avian influenza (AI) among commercial poultry farms in northern Italy in 2002–2003 (Capua *et al.*, 2002), during which serological evidence of H7 virus transmission to poultry workers was obtained (Puzelli *et al.*, 2005). Genetic analyses showed that only a few nucleotide and amino acid changes throughout the genome differentiated the wild from the domestic avian virus (Campitelli *et al.*, 2004). Given the important role of wild bird viruses for the emergence of viruses pathogenic for both domestic poultry and humans, and the fact that measures to contain these viruses are impossible to implement in the wild, improved knowledge about the ecology of influenza viruses and the properties of their gene pool in feral waterfowl is crucial to develop interventions aimed at limiting the risk of transmission to other species. Recent reports have highlighted the existence of multiple sublineages within the major lineage of AIVs isolated from their natural reservoir in North America, and suggested that reassortment events in the wild waterfowl reservoir occur more frequently than previously thought (Hatchette *et al.*, 2004; Widjaja *et al.*, 2004). However, few data are available on the genetic heterogeneity and extent of gene pool mixing among wild and domestic bird viruses isolated in the entire Eurasian region, despite their importance for animal and human health. Therefore, we have analysed and compared the genome of a group of H7 subtype AIVs isolated from Italy over a 6 year period with those co-circulating in Europe and China at the same time in order to characterize the genetic heterogeneity among H7 subtype viruses isolated from birds, including wild and domestic species, between 1999 and 2005. We have also evaluated the extent of reassortment in this gene pool and its role in the generation of viruses capable of infecting domestic avian species, and analysed the molecular determinants relative to receptor binding, host adaptation, virulence and antiviral susceptibility.

METHODS

Sample collection and virus isolation. Cloacal samples were obtained from wild mallard ducks (*Anas platyrhynchos*) in central and northern Italy, from turkeys and chickens raised in commercial poultry farms in northern Italy, and from domestic ducks raised in farms at Poyang Lake, China. Poyang Lake is located in the north Jiangxi Province, is the largest freshwater lake in China and is a major overwintering site for migratory birds in eastern Asia (Li & Mundkur, 2004). For influenza A virus detection and isolation from wild and domestic waterfowl from Italy, cloacal swabs were processed as follows: pools of five to six faecal specimens were prepared, viral RNA was extracted using the RNeasy kit (Qiagen), and RT-PCR with primers M52C and M253R, which are specific for a conserved region of the influenza matrix protein, was performed as described previously (Fouchier *et al.*, 2000). Samples from PCR-positive pools were inoculated into 10-day-old embryonated specific-pathogen-free hen's eggs, and influenza isolates were identified by both the haemagglutination test (according to standard procedures) and a double-antibody 'sandwich' ELISA for the detection of influenza A virus nucleoprotein (Siebinga & de Boer, 1988). For virus isolation from turkey and chicken, cloacal and/or tracheal samples were inoculated into embryonated hen's eggs, as described above. For those viruses isolated from China, virological samples were collected, isolated and characterized as described previously (Chen *et al.*, 2006).

Antigenic characterization. Subtype identification of influenza viruses was performed by using haemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) assays with a panel of reference antigens and antisera, as described previously (WHO, 2002). A more detailed analysis of HA antigenic reactivity was carried out by the HI test using a panel of hyperimmune rabbit and chicken sera to A/Ruddy turnstone/New Jersey/65/85 (H7N3), A/Turkey/Italy/2676/99 (H7N1) (the index case strain isolated during the 1999–2000 H7N1 Italian poultry outbreak) and A/Turkey/Italy/214845/02 (H7N3) virus. In addition, a panel of post-infection ferret sera against A/Chicken/Italy/13474/99 (H7N1) virus, also isolated during the 1999–2000 H7N1 outbreak, and a monoclonal antibody to A/Turkey/Italy/2676/99 were used. The HI test was performed using horse red blood cells (HRBCs) according to standard procedures with minor modifications (Stephenson *et al.*, 2003). Basically, a 1 % suspension of HRBCs in 0.5 % BSA/PBS was used throughout the procedure and HI titres were read after 60 min.

Gene sequencing. Viral RNA was extracted from infected allantoic fluids as described earlier and reverse-transcribed using a 12 bp oligonucleotide primer (5′-AGCAAAAGCAGG-3′) and Superscript II reverse transcriptase (Invitrogen) as described previously (Campitelli *et al.*, 2002). We PCR-amplified the coding region of the viral gene segments with gene-specific primers using the Expand High-Fidelity PCR system (Roche) according to the manufacturer's protocol. Amplified products of the expected size were purified with the QIAquick PCR purification kit (Qiagen), sequenced using the BigDye Terminator Cycle-Sequencing Ready Reaction (Applied Biosystems) and analysed on ABI Prism 310 DNA sequencer (Applied Biosystems).

Sequence and phylogenetic analysis. Sequence assembly was performed with the Lasergene package (version 4.0; DNASTAR). Sequences obtained in this study were aligned with selected sequences available from GenBank, using the CLUSTAL_X program (Thompson *et al.*, 1997), and edited with BioEdit 7.05 (Hall, 1999). Phylogenetic analysis was carried out in two steps: first, substitution models for each gene segment analysed were determined using MODELTEST 3.06 (Posada & Crandall, 1998); subsequently, maximum-likelihood (ML) phylogenetic trees were inferred using PAUP* 4.0 (Swofford, 2002), according to the selected models. Statistical analyses included bootstrap analyses (1000 bootstrap replicates) and the zero-branch-

length test (Swofford & Sullivan, 2003), also performed with PAUP*. Identification of potential glycosylation sites was done with the PPSearch program, available at www.ebi.ac.uk/ppsearch.

Virus genotyping. Genotypes were defined by gene phylogeny. Gene segments were assigned to a given phylogenetic grouping, or sublineage, according to their position on each segment-specific tree, when the identified cluster complied with the following criteria: (i) starting from the tip of a phylogeny, a monophyletic clade is identified when it is supported by a bootstrap support of >80 % and/or by *P* values $\leqslant 0.001$. Moreover, all taxa in the proposed clade must have 95 % or greater identity with each other; (ii) when the bootstrap score or the *P* value at the node supporting the proposed segment-specific clade is less than the arbitrary cut-off value, all the taxa in the clade must have at least 95 % or greater sequence identity with each other and less than 94.9 % identity with the gene segments of viruses belonging to other phylogenetic groups.

RESULTS

Epidemiological background and antigenic characterization

The viruses analysed in this study were collected from northern and central Italy and from China over a 5 year period, from wild and domestic birds (Table 1). H7 strains were isolated from wild migratory birds captured in the winter of 2001–2002 in the Orbetello Lagoon, a WWF protected wetland area in central Italy normally used as a stopover or wintering site by migratory as well as resident waterfowl, including sentinel birds, as described elsewhere (De Marco *et al.*, 2003). No serological nor virological evidence of H7 virus circulation in wild waterfowl had been detected during the previous 9 years in the same area (De Marco *et al.*, 2005).

In February 2005 an additional H7N7 strain was isolated from a mallard duck shot by a hunter in northern Italy. Between December 2002 and May 2003 several H7N3 viruses were obtained from a variety of domestic fowl species during an extensive epidemic of LPAI in northern Italy (Capua *et al.*, 2002; Campitelli *et al.*, 2004). Three of them were isolated from sentinel turkeys in commercial poultry farms that had been included in a vaccination plan against H7 viruses aimed at containing the spread of the epidemic (Capua *et al.*, 2004). In 2003, influenza surveillance in both wild and domestic waterfowl at Poyang Lake, Jiangxi Province, China led to the isolation of four H7N7 viruses from domestic ducks (Table 1).

Table 1. List of viruses analysed in this study

Virus	Subtype	Species	Origin	Isolation date	Place	Origin of sequence data	Genotype group
A/Turkey/Italy/2676/99*	H7N1	Turkey	Domestic	March 1999	Italy	Public database and this study†	I‡
A/Turkey/Italy/4169/99	H7N1	Turkey	Domestic	March 1999	Italy	Public database	I
A/Chicken/Italy/4746/99	H7N1	Chicken	Domestic	December 1999	Italy	Public database	I
A/Chicken/Italy/5093/99	H7N1	Chicken	Domestic	1999	Italy	Public database	I
A/Guinea fowl/Italy/155/00	H7N1	Guinea fowl	Domestic	January 2000	Italy	Public database	I
A/Mallard/Italy/33/01	H7N3	Mallard Duck	Wild	October 2001	Italy	Public database	II
A/Mallard/Italy/199/01	H7N3	Mallard Duck	Wild	December 2001	Italy	This study†	II‡
A/Mallard/Italy/250/02	H7N1	Mallard Duck	Wild	January 2002	Italy	This study	III
A/Turkey/Italy/214845/02	H7N3	Turkey	Domestic	October 2002	Italy	Public database	II
A/Guinea fowl/266184/02	H7N3	Guinea fowl	Domestic	December 2002	Italy	This study†	II‡
A/Chicken/Italy/270638/02	H7N3	Chicken	Domestic	December 2002	Italy	This study†	II‡
A/Turkey/Italy/68819/03	H7N3	Turkey	Domestic	March 2003	Italy	This study	II
A/Turkey/Italy/97500/03	H7N3	Turkey	Domestic	April 2003	Italy	This study	II
A/Turkey/Italy/121964/03	H7N3	Turkey	Domestic	May 2003	Italy	This study	II
A/Turkey/Italy/3620/03	H7N3	Turkey	Domestic	May 2003	Italy	Public database	II
A/Chicken/Netherlands/1/03	H7N7	Chicken	Domestic	February–May 2003	Netherlands	Public database	II
A/Netherlands/219/03	H7N7	Human	AV-like Domestic	February–May 2003	Netherlands	Public database	IV
A/Duck/Jiangxi/1742/03	H7N7	Duck	Domestic	August 2003	China	This study	V
A/Duck/Jiangxi/1786/03	H7N7	Duck	Domestic	August 2003	China	This study	V
A/Duck/Jiangxi/1814/03	H7N7	Duck	Domestic	August 2003	China	This study	V
A/Duck/Jiangxi/1760/03	H7N7	Duck	Domestic	August 2003	China	This study	V
A/Mallard/Italy/299/05	H7N7	Mallard Duck	Wild	February 2005	Italy	This study	VI

*Also known as A/Turkey/Italy/977/99 (Banks *et al.*, 2001).
†Partially sequenced.
‡Genotyping of internal genes based on similarity data; public databases: '*ISD: Influenza Sequence Database at LANL*' and '*Influenza virus resources NCBI*'.

Serological comparison of the H7 viruses circulating in Italy and China between 1999 and 2005 in different species revealed no significant differences in their antigenic pattern, except for the index case of the 1999 AI epidemic caused by H7N1 virus, A/Turkey/Italy/2676/99, that showed a fourfold higher titre against its homologous antiserum and monoclonal antibody compared with most of the other strains tested. Thus, only a limited antigenic drift was observed among these Eurasian viruses (Table 2).

Sequence and phylogenetic analysis

We sequenced the genome of 12 H7 viruses isolated in Italy and China from 2001 to 2005, and analysed them along with H7 viruses that have circulated in several European countries since 1999. The list of viruses used in this comparison is presented in Table 1. With regards to the HA gene, the comparable serological reactivity observed in the HI test was in agreement with a high degree of HA similarity at the amino acid level (ranging, for HA1, from 98.1 to 100 %), although, at nucleotide level, similarity percentages varied from 95.0 to 100 %. The highest degree of divergence was observed between the four Chinese strains and A/Guinea fowl/Italy/266184/02 (from 95.0 to 95.1 %) (data not shown). A higher range of variability was observed within the remaining genes: with regards to the NA gene, three different NA subtypes were associated with the H7 HAs (N1, N3 and N7), whereas similarity percentages for the internal protein genes varied from 97.6 to 100 % for the NS1 gene to 88.5 to 99.4 % for the PB2 gene at nucleotide level, although the corresponding proteins were much more conserved (ranging from 99.5 to 98.3 % similarity for PA to a maximum divergence of 100–97.1 % for NS1).

Phylogenetic relationships were inferred for each of the eight gene segments after selecting the best-fitting nucleotide substitution models (see Supplementary Table S1 available in JGV Online). All virus genes belonged to the main Eurasian avian lineage, as no evidence of reassortment with genes belonging to the North American avian gene pool was found. HA genes, including the Chinese strains, fell within the same subgroup with a high statistical support. They were clearly distinct from both an H7N3 virus isolated in Taiwan in 1998 and several H7 strains circulating in Europe from 1989 to 1996 (Fig. 1a). The monophyletic group observed for the HA gene contrasts with the situation observed in most other gene segments. Three different NA subtypes (N1, N3 or N7) were associated with the H7 viruses. In particular, A/Mallard/Italy/199/01 and A/Mallard/Italy/250/02, which were isolated 1 month apart in the same area, possessed an almost identical HA gene (99.8 % identity) but their NA genes belonged to the N3 and N1 subtypes, respectively. Noteworthy is the fact that A/Mallard/Italy/250/02 shares a very recent common ancestor with the N1 genes of the H7N1/99 Italian viruses responsible for the 1999–2000 epidemics in poultry, whose direct precursor in the wild bird reservoir had not been identified previously. Within each NA subtype, the viruses clustered in the same branches (Supplementary Fig. S1 available in JGV Online).

Phylogenies of the internal genes indicated a high level of heterogeneity and for most genes distinct subgroups, suggestive of different evolutionary pathways, could be identified (Figs 1b, 2, 3 and Supplementary Fig. S2). The highest number of sublineages among the H7 viruses under study was observed within the NP, PB1 and PA genes. With regards to the NP gene, the Italian H7N3 viruses formed a

Table 2. Antigenic analysis of the H7 influenza viruses by HI test

Abbreviations: RT/NJ, A/Ruddy turnstone/New Jersey/65/85; Ty/It/2676/99, A/Turkey/Italy/2676/99; Ty/It/214845/02, A/Turkey/Italy/214845/02; Ck/It/13474/99, A/Chicken/Italy/13474/99; Ty/It/2676/99, A/Turkey/Italy/2676/99. Homologous titres are in bold.

Virus	RT/NJ*	Ty/It/2676/99†	Ty/It/214845/02†	Ck/It/13474/99		Ty/It/2676/99
				F4/02‡	F5/02‡	Monoclonal antibody
A/Ruddy turnstone/New Jersey/65/85 H7N3	**5120**	320	320	40	10	160
A/Turkey/Italy/2676/99 H7N1	5120	**2560**	640	160	80	**1280**
A/Turkey/Italy/214845/02 H7N3	5120	640	**1280**	80	20	320
A/England/268/96 H7N7	2560	320	320	40	40	160
A/Mallard/Italy/33/01 H7N3	5120	640	640	80	20	320
A/Mallard/Italy/250/02 H7N1	5120	1280	640	160	80	320
A/Duck/Jiangxi/1742/03 H7N7	2560	640	640	80	40	160
A/Duck/Jiangxi/1760/03 H7N7	2560	640	640	80	40	160
A/Duck/Jiangxi/1786/03 H7N7	2560	640	640	80	40	160
A/Duck/Jiangxi/1814/03 H7N7	2560	640	640	80	40	160
A/Mallard/Italy/299/05 H7N7	2560	640	640	80	40	160

*Hyperimmune rabbit antisera.
†Hyperimmune chicken antisera.
‡Post-infection ferret antisera to antisera.

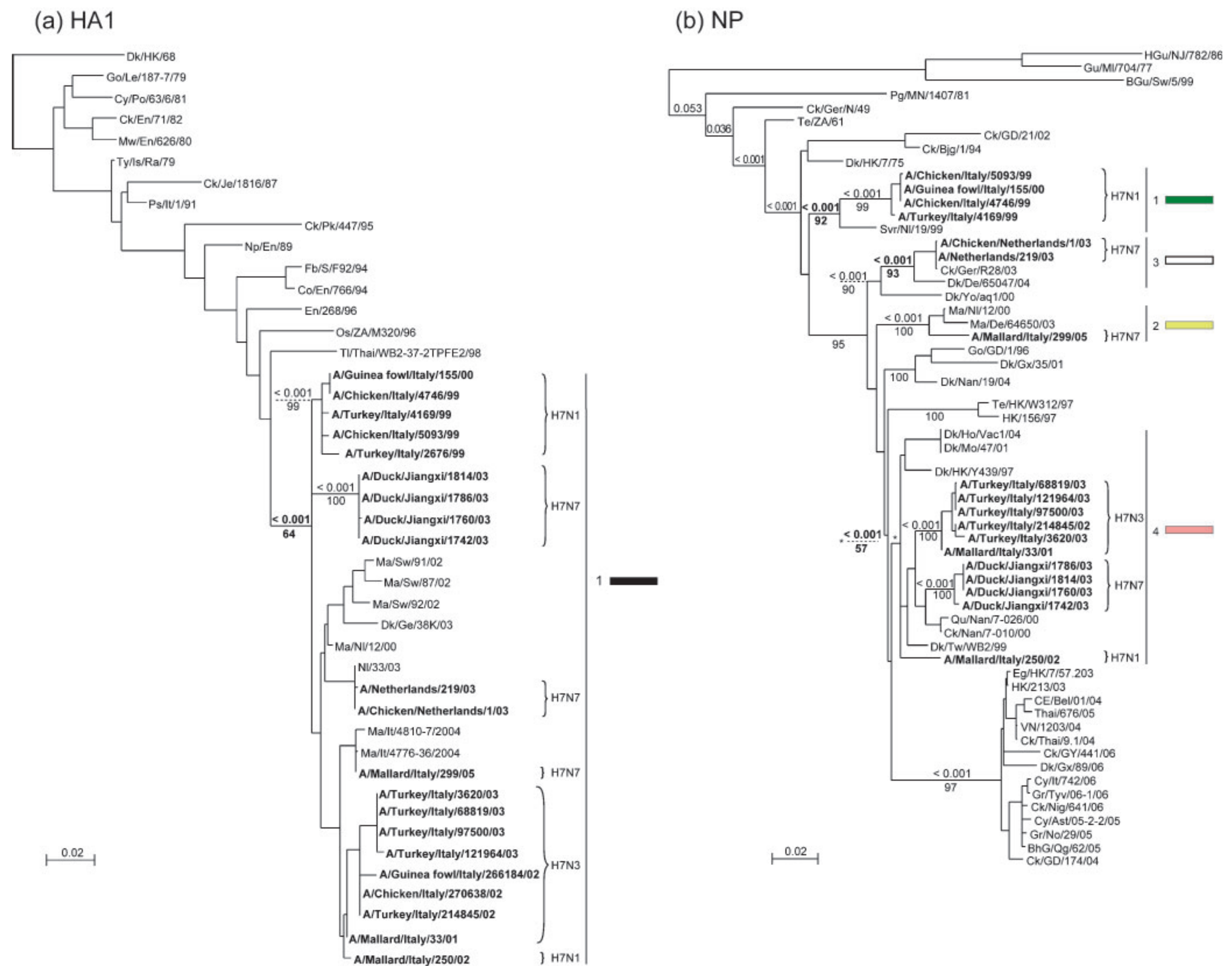

Fig. 1. Phylogenetic trees of the HA (subunit 1) and NP genes of H7 viruses (nucleotide positions: HA1, 55–1008; NP, 46–1524) analysed by maximum-likelihood using PAUP*. The distance bar indicates the number of nucleotide substitutions per site, numbers above and below nodes indicate *P* values, and bootstrap values $>50\,\%$, respectively. Not all supports are shown because of space constraints. Each of the identified phylogenetic groupings is indicated by supports in bold and is assigned different numbers and colours (to the right of each tree). Viruses compared in this study are in bold. (a) HA1 gene. The HA1 tree is rooted to A/Chicken/Germany/N/49 (H10N7). (b) NP gene. The tree is rooted to A/Equine/Prague/1/56. Species abbreviations used in this study: AfS, African Starling; BhG, Bar-headed Goose; BGu, Black-headed Gull; Bwt, Blue winged teal; Ck, Chicken; Co, Conjure; Ce, Crested Eagle; Cy, Cygnus Olor and/or Swan; Dk, Duck; Eg, Egret; E, Environment; Eq, Equine; Fb, Fairy blue bird; Fo, Fowl; Go, Goose; Gf, Guinea fowl; Gr, Grebe; Gu, Gull; HGu, Herring Gull; Ma, Mallard and/or mallard duck; Mw, Macaw; Np, Non-psittacine; Os, Oystercatcher; Pg, Pigeon; Pi, Pintail Duck; Ps, Psittacine; Qu, Quail; Rk, Red knot; Rh, Rhea; Rt, Ruddy turnstone; Svr, Shoveler; Sh, Shorebird; So, Softbill; Sw, Swine; Tl, Teal; Ter, Tern; Ty, Turkey; WS, Wilson-Smith (human). See Supplementary Table S2 for country abbreviations.

sister group with the Chinese H7N7 strains and with the A/Mallard/Italy/250/02 (H7N1) virus, whereas the H7N1/99 strains from domestic poultry, A/Mallard/Italy/299/05 (H7N7) strain and the Dutch H7N7 poultry strains were all clearly separated from each other and from the Italian H7N3 viruses (Fig. 1b). Significant variation was also observed among the PB1 genes: all the viruses analysed grouped on a sister branch to that of the H5N1 strains from Asia, and within this branch four subgroups were observed. A/Mallard/Italy/299/05 showed a separate evolution (Fig. 2a). In the case of PA gene, the Duck/Jiangxi viruses and the H7N1/99 poultry strains were on sister branches, A/Mallard/Italy/299/05 grouped with a Guangdong virus, and the Dutch and Italian H7N3 strains formed two additional clusters, respectively (Fig. 3a). The M1 genes of all H7 viruses in this study fell into three

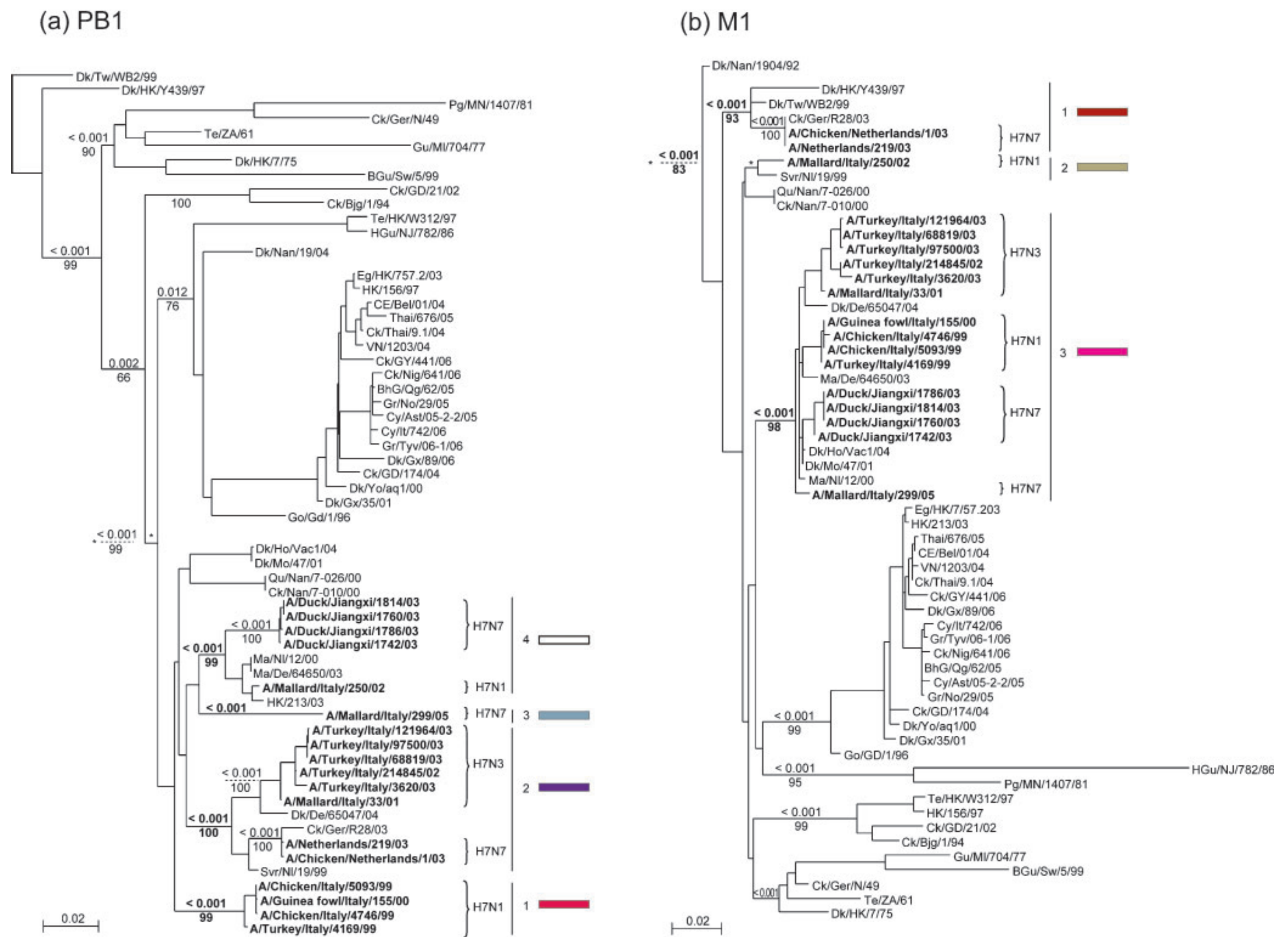

Fig. 2. Phylogenetic trees of PB1 and M1 genes (nucleotide positions: PB1, 31–2209; M1, 26–781) analysed with PAUP* using maximum-likelihood. The distance bar indicates the number of nucleotide substitutions per site, numbers above and below nodes indicate *P* values, and bootstrap values >50 %, respectively. Not all supports are shown because of space constraints. Viruses compared in this study are in bold. Each of the identified phylogenetic groupings is indicated by supports in bold and is assigned different numbers and colours (to the right of each tree). (a) PB1 gene. The tree is rooted to A/Equine/London/1416/73. (b) M1 gene. The tree is rooted to A/Equine/Prague/1/56. Abbreviations: see legend to Fig. 1.

clades, one of which comprised A/Mallard/Italy/299/05 and the Duck/Jiangxi strains, that appeared closely related to a duck strain isolated in Mongolia (Fig. 2b). The PB2 phylogeny indicated that most of the viruses shared a very recent common ancestor, except for A/Mallard/Italy/299/05, which was closely related to Goose/Guangdong/1/96-like viruses (Fig. 3b). Finally, the H7 NS genes were divided in two major groups: the H7N1/99 strains were all located within the allele B, the remaining viruses grouped together in the allele A (Supplementary Fig. S2).

Thus, in most instances viruses, clustered differently in different genes. Another notable feature is that, on the one hand, most genes from the Duck/Jiangxi strains were found to group more often with the Italian isolates than with geographically closer strains; on the other hand, PB2 and PA genes of A/Mallard/Italy/299/05 and A/Mallard/Italy/250/02 appeared more similar to Asian strains than to other Italian H7 viruses.

Virus genotyping

To illustrate better the diverse gene constellations within the H7 avian strains under study, each one of the phylogenetic lineages identified for every gene was assigned a different colour and a visual representation of the virus genotype of each representative virus isolate was obtained, as described by other authors (Hatchette *et al.*, 2004) (Fig. 4). These lineages are also represented on the phylogenetic trees (Figs 1–3). Throughout the period 1999–2005, six different genotype combinations were observed within the H7 subtype viruses identified in Eurasian countries. In each of the years of isolation a

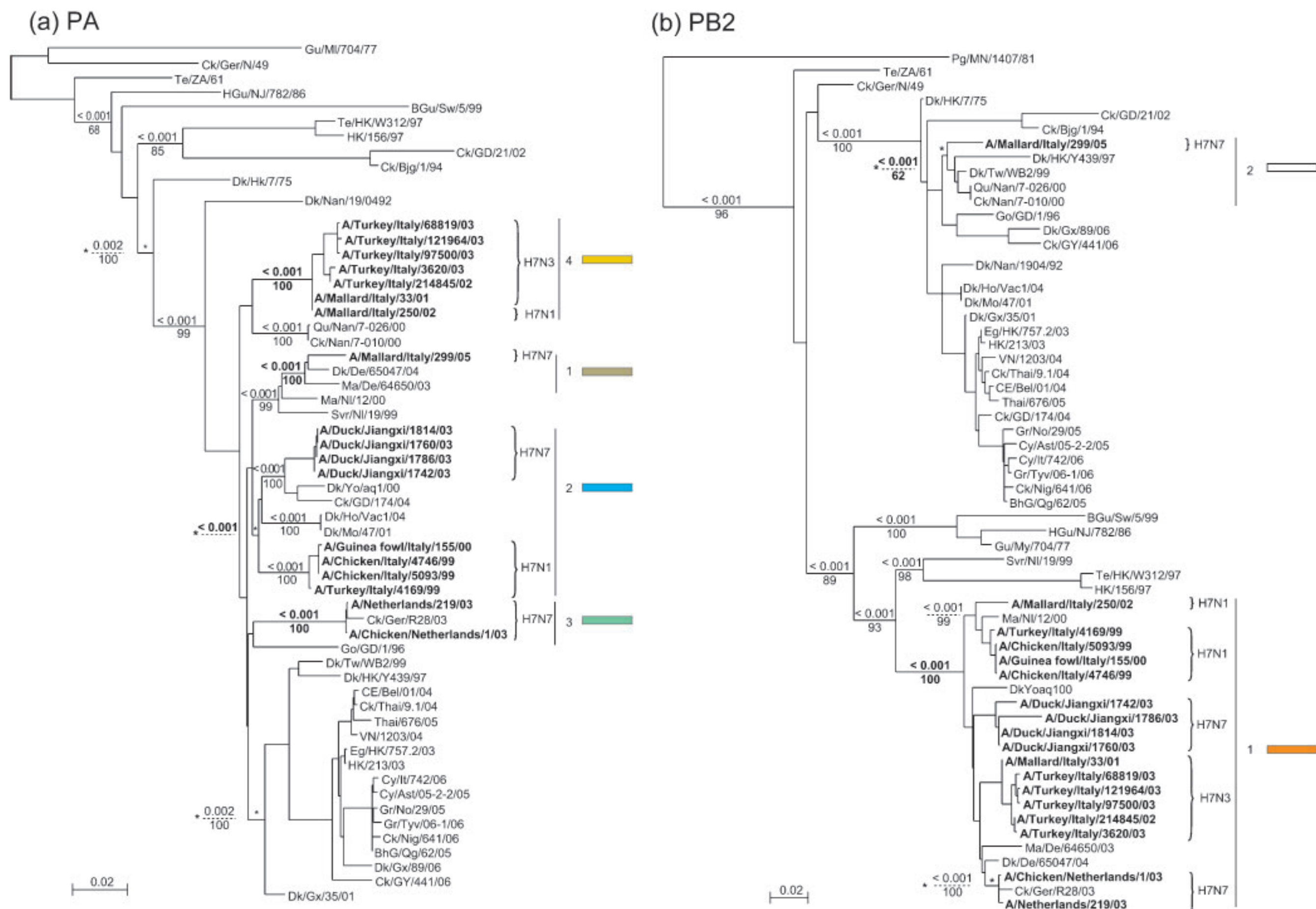

Fig. 3. Phylogenetic trees of PA and PB2 genes (nucleotide positions: PA, 81–1784; PB2, 58–2283) analysed with PAUP* using maximum-likelihood. The distance bar indicates the number of nucleotide substitutions per site, numbers above and below nodes indicate *P* values, and bootstrap values >50 %, respectively. Not all supports are shown because of space constraints. Viruses compared in this study are in bold. Each of the identified phylogenetic groupings is indicated by supports in bold and is assigned different numbers and colours (to the right of each tree). (a) PA gene. The tree is rooted to A/Equine/London/1416/73. (b) PB2 gene. The tree is rooted to A/Equine/Prague/1/56. Abbreviations: see legend to Fig. 1.

different genotype emerged, although in some years more than one genotype was detected. In 2002, H7N3 and H7N1 viruses were isolated in Italy; in 2003, two H7N7 subtype viruses with different genotypes circulated in the Netherlands and in China, and an H7N3 virus in Italy. The H7N3 genotype was identified in Italy for three consecutive years, in different avian species (wild waterfowl in 2001 and domestic poultry in 2002–2003), as a consequence of a likely interspecies transmission event from wild to domestic birds followed by a long-lasting epizootic in commercial poultry (Campitelli *et al.*, 2004).

Apart from the HA genes, which belonged to the same sublineage, all other influenza A segments appear to have reassorted in a random fashion, with the highest number of phylogenetic groupings observed in NP, PA and PB1 genes (four in each of the three genes).

Molecular characterization

HA gene. The H7 HAs of Eurasian viruses differed mainly because of the presence or absence of an insertion of multibasic amino acids at the cleavage site, known to confer an HP phenotype to H5 and H7 subtype viruses. This motif was found only in Italian H7N1 viruses isolated between December 1999 and March 2000 (represented by A/Guinea fowl/Italy/155/00 strain) and in all the H7N7 Dutch strains (Banks *et al.*, 2001; Fouchier *et al.*, 2004). We then looked at the amino acids that define the receptor-binding pocket, usually highly conserved throughout all avian subtypes (Nobusawa *et al.*, 1991). As expected, most of the H7 strains retained the avian consensus sequence of the receptor-binding site (RBS), known to confer the ability to bind preferentially to Neu5Acα2, 3-Gal receptor determinants. However, A/Mallard/Italy/250/02 (H7N1)

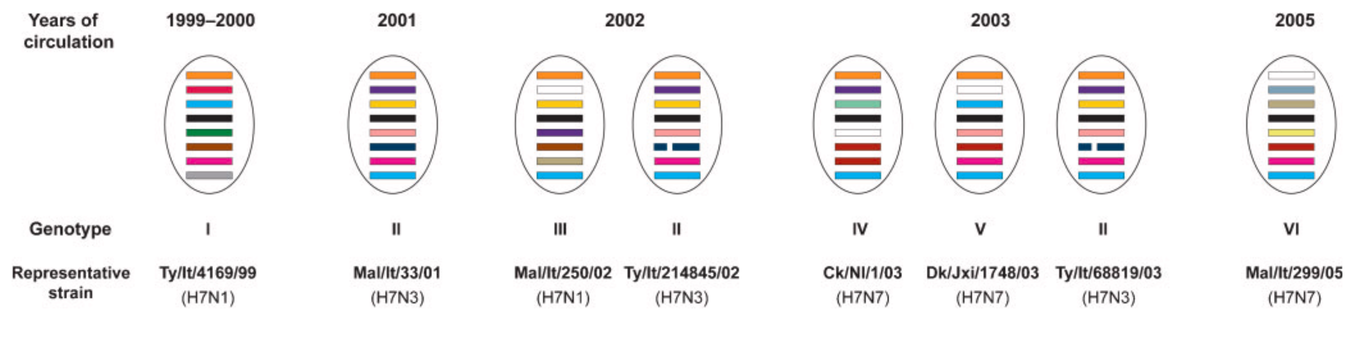

Fig. 4. Graphic representation of the genotypes identified by using the phylogenetic groupings derived from each phylogenetic tree. Different phylogenetic clusters are assigned different colours. For each genotype a representative virus has been indicated. Genes in each virus are (top to bottom): PB2, PB1, PA, HA, NP, NA, M and NS.

showed an amino acid substitution (A→S) at position 128 (138, according to H3 numbering), which is part of the right edge of the RBS.

The HA glycosylation pattern indicated that all wild and domestic duck viruses possessed five potential glycosylation (CHO) sites throughout the molecule (HA1 + HA2). This pattern was also retained by the H7N3 turkey and chicken strains, regardless of the species and time of isolation, and by the H7N7 Dutch strains, except for A/Netherlands/219/03, which displayed an additional CHO site at residue 123 (Fouchier *et al.*, 2003). On the other hand, almost all H7N1 viruses isolated in Italy in 1999–2000 had one of two extra CHO sites at positions 123 and 149 (Banks *et al.*, 2001).

NA gene. The sialidase active site (Colman *et al.*, 1993) was conserved in all the analysed viruses. The most remarkable feature, when comparing H7 virus NAs, was the presence or absence of a fairly long deletion in the NA stalk region. Both H7N1 and H7N3 subtype viruses from wild waterfowl had a full-length NA gene, whereas viruses of the same subtypes isolated from domestic poultry had a deletion of 22 and 23 aa, respectively, as already reported (Banks *et al.*, 2001; Campitelli *et al.*, 2004), even though they shared a high level of nucleotide similarity along the rest of the molecule (98.6–99.0 % among H7N3 viruses and 97.7 % between A/Mallard/Italy/250/02 and A/Turkey/Italy/4169/99). An NA deletion was not observed in any of the H7N7 viruses analysed, irrespective of their species of origin, of additional CHO sites in the HA, and of their pathogenic phenotype. For the N1 and N3 NA proteins, the number of glycosylation sites varied depending on the stalk deletion: wild bird viruses had six CHO sites, three of which, located within the stalk region, were lost in domestic poultry viruses. All N7 NA genes had nine glycosylation sites.

Internal protein genes. Several amino acid positions within the internal protein sequences have been associated with features such as virulence, host range and interspecies transmission, and resistance to antivirals. The NS1 genes of the H7 viruses coded for full-length proteins; thus, no deletions were observed either in the C-terminal region or within the protein. Amino acid changes at positions 92 (D92E) and 149 (V149A) have been associated with increased virulence of H5N1 influenza viruses in man and chickens, respectively (Seo *et al.*, 2002; Li *et al.*, 2006). All the H7 viruses in our study had 92D and 149A. The four C-terminal amino acids, recently shown to bind to protein interaction domains of intracellular scaffold proteins (Obenauer *et al.*, 2006) were represented by the motif ESEV, typical of AIVs.

Two changes in the virus polymerase complex that have been frequently associated with adaptation of an AIV to growth in a mammalian host are E627K and D701N of PB2 (Hatta *et al.*, 2001; Li *et al.*, 2005). The former had already been reported in the H7 Dutch virus that caused lethal viral pneumonia in man, A/Netherlands/219/03 (Fouchier *et al.*, 2004) but no other H7 virus in our study, either from poultry or from wild birds, possessed it. The latter change was not detected in any of the H7 strains.

Finally, we checked the M2 genes for the presence of mutations known to code for resistance to amantadine, an anti-influenza drug commonly used in swine and humans (Gubareva & Hayden, 2006). Interestingly, one of the wild waterfowl isolates, A/Mallard/Italy/250/02, showed the change S31N, considered sufficient to induce full resistance to amantadine.

DISCUSSION

We have analysed the antigenic and genetic characteristics of H7 influenza viruses isolated over 7 years in Eurasian countries from wild and domestic avian species. All the viruses in our study, whether circulating in different geographical areas or in the same regions, possess HA genes sharing a recent common ancestor, with limited sequence divergence. However, when considering the other gene segments, multiple sublineages exist at nucleotide level and six different reassortant genotypes were identified, based on phylogenetic and similarity data. The basis of influenza virus variability depends on two properties of the virus

genome: the lack of a proof-reading function in the virus RNA polymerase, that allows the emergence of virus variants by point mutation at a high rate (Domingo *et al.*, 2001), and the segmented nature of the virus genome, which permits the exchange of genetic material among different influenza A viruses during coinfection events, resulting in the emergence of novel 'reassortant' viruses. The importance of the latter phenomenon for influenza A virus evolution in avian species has become apparent since the appearance of the HP H5N1 virus in 1997 as a result of multiple reassortment events (Guan *et al.*, 2002), which have continued in the following years, leading to the emergence of several different genotypes, each circulating for no more than two years (Li *et al.*, 2004). A recent study has conservatively estimated an average three year time lapse between creation of a new genotype and its next segment reassortment, when considering the polymerase complex genes of viruses from domestic and terrestrial aquatic avian species (Macken *et al.*, 2006). Our data indicate that both point mutations and reassortment events account for the evolution observed within H7 subtype viruses, and the detection of one or more different H7 genotypes during each of the years considered, as well as their rapid replacement, supports the hypothesis that the generation of reassortants as a consequence of dual infection of avian species is not an infrequent event. This occurrence allows the virus to evolve at a faster rate than that achievable by point mutations alone (Macken *et al.*, 2006). In turn, this increase of genetic diversity may favour the emergence of virus variants capable of spreading more efficiently in avian species. Similar evidence has been obtained for viruses isolated from ducks in Canada (Hatchette *et al.*, 2004).

All the viruses analysed in our study are from Eurasia, an extremely wide geographical region within which multiple migratory flyways can be identified. Phylogenetic evidence that a number of genes of H7 viruses isolated in Italy are most closely related to viruses from southern China indicates that these major flyways overlap, allowing different bird populations to come in contact with each other (Olsen *et al.*, 2006), and further supports the hypothesis of a continuous evolution of the influenza gene pool through reassortment. Analogous patterns have also been identified for LP H5 viruses (Duan *et al.*, 2007).

Previous findings suggested that the number of potential reassortants arising from coinfection of ducks with two or more viruses is limited, possibly because of non-viability of defined gene constellations (Sharp *et al.*, 1997). In the present study, gene exchange appeared to occur randomly, without constraints among different gene sublineages of avian viruses: as an example, we found no correlation between the H7 HA genes and a specific sublineage of any of the remaining genome segments, in agreement with recent studies carried out on the feral Canadian duck gene pool and the AI polymerase gene complex (Widjaja *et al.*, 2004; Macken *et al.*, 2006).

Most genes of H7 poultry viruses (H7N1/IT/99 and H7N7/NL/03) share recent common ancestors with those of wild birds, and the Italian H7N3 poultry viruses have been shown to be the result of a direct introduction of a wild bird virus *in toto* in domestic poultry (Campitelli *et al.*, 2004). These data indicate that AIVs in poultry species do not constitute separate evolutionary cycles; rather, a continuous virus exchange between wild aquatic birds and domestic species seems likely, as also confirmed by studies on H6N2 viruses in chickens in California and by the ability shown by the HP H5N1 virus in Asia to move back from domestic to wild aquatic birds (Webby *et al.*, 2002; Guan *et al.*, 2002).

H7N1 and H7N3 strains isolated from poultry species possess a deleted NA protein in the stalk region, a feature considered a marker of adaptation of a virus from wild birds to poultry hosts, which is frequently accompanied by additional carbohydrate groups at the tip of the HA molecule (Matrosovich *et al.*, 1999). In this way, the virus is thought to compensate the impairment of sialidase activity by reducing binding affinity to sialylglycoconjugates, as evidenced by *in vitro* studies (Baigent & McCauley, 2001; Wagner *et al.*, 2002). However, only the H7N1 viruses have additional glycosylation groups on the HA globular head. On the other hand, the NA stalk of both chicken and human H7N7 viruses from The Netherlands is not deleted, although an additional CHO site was present on the HA protein of at least one strain, A/Netherlands/219/03 (Fouchier *et al.*, 2004). All of these data indicate that co-mutation of these two genes, aimed at balancing HA and NA activities, does not constitute an absolute requirement for an avian virus from aquatic birds to adapt to terrestrial species, under natural conditions.

Differences in receptor-binding specificity between human and avian viruses represent a major barrier to efficient transmission of an AIV among mammals (Matrosovich *et al.*, 2006). Moreover, as little as two amino acid substitutions may be sufficient to cause a change in receptor-binding preference from the avian α-2,3 to the human α-2,6 sialic acid receptor, as shown for the H5N1 and H1N1 subtype viruses (Yamada *et al.*, 2006; Tumpey *et al.*, 2007). Our study shows that a duck virus, A/Mallard/Italy/250/02, possesses an amino acid change (A138S, H3 numbering) on the right edge of the RBS, while not showing any additional glycosylation site on its globular head. As reported by other authors, A138 is a highly conserved position in avian viruses of all subtypes, whereas in several human H1 strains A138S substitution seems to increase their ability to bind to α-2,6 sialic acid receptors, specific for human viruses (Matrosovich *et al.*, 1997). Thus, virus variants circulating in wild waterfowl and bearing amino acid changes that might favour a partial or complete switch towards a human-type receptor specificity could facilitate transmission and adaptation to a mammalian host.

We also detected a mutation in the M2 gene of A/Mallard/Italy/250/02 virus (S31N) known to confer resistance to amantadine, an anti-influenza drug effective on both

human and animal influenza A viruses (Scholtissek *et al.*, 1998). Amantadine resistant viruses have been detected among avian strains of poultry origin, and have been associated, at least in South East Asia, with the use of amantadine against the H5N1 viruses in commercial poultry (Ilyushina *et al.*, 2005; Cheung *et al.*, 2006), whereas no data are available on wild waterfowl viruses. Due to the high frequency generation of random point mutations by the viral transcriptase, it is very likely that this substitution, as well as the one in the HA RBS, is the result of random selection of variants bearing these changes.

Our study highlights once again the close relationship between AIV in poultry and wild birds. Moreover, in order to understand the mechanisms and ecological interactions that favour the emergence of potentially dangerous influenza A viruses, it supports the idea that more extensive and long-term AI surveillance programmes, as well as a thorough genetic characterization of AIVs isolated all over the world, must be implemented. In this regard, attention should be placed not only on H5 and H7 subtype viruses that represent a more immediate threat for poultry industry but also on other subtypes of relevance for public health, such as H1, H2, H3 and H9.

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