Avian Pathology

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Genetic characterization of the whole genome of four avian influenza H7N7 viruses isolated in three successive winter seasons (2007 to 2009) from wild mallards in three cities in Poland was performed. All of the tested strains were of low pathogenicity and no molecular marker associated with an increased adaptation to poultry, mammals or resistance to antiviral drugs was found. The major outcome of the phylogenetic studies was that the isolate A/mallard/Poland/446/09 (detected in December 2009) shared a recent common ancestor with A/mallard/Poland/41/09 (isolated in February 2009) in relation to HA and PB1 genes, with A/mallard/Poland/16/09 (found in January 2009) regarding NA and NS genes, and with A/mallard/Poland/01/08 (recovered in December 2007) as regards the NS gene. Interestingly, A/mallard/Poland/16/09 and A/mallard/Poland/446/09 were isolated at the same sampling site almost exactly 1 year apart, which points to resident population of mallards (and other resident waterfowl) as responsible for the perpetuation of avian influenza virus (AIV) in the given area between successive winters. On the other hand, the ornithological data discussed in detail in the paper strongly suggest that the virus transmission between close sites but located in different urban areas is most probably achieved by migratory birds, a fact additionally supported by a close relatedness between different gene segments of Polish H7N7 and AIV detected in wild birds in Europe. A high heterogeneity of the gene pool found in the study is indicative of frequent reassortment events. Additionally, two H7N7 isolates were shown to possess selected genes closely related to AIV detected in domestic poultry in Italy and the Czech Republic. The present study corroborates the importance of active surveillance in wild birds as a valuable tool for early warning of avian influenza in poultry.

Introduction

Avian influenza A virus (AIV) is an orthomyxovirus with a genome consisting of eight single-stranded RNA segments encoding for at least 10 different viral proteins: PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, and NS2 (Suarez, 2008). The current classification into subtypes is based on the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA), and so far 16 HA subtypes and nine NA subtypes have been described for which wild aquatic birds are considered the natural reservoir (Webster *et al.*, 1992; Swayne & Suarez, 2000; Fouchier *et al.*, 2005). In wild birds, AIVs usually persist in the low-pathogenic form but some of the H5 and H7 subtypes have the capacity to become highly pathogenic (HP). The emergence of HP AIV from low-pathogenic AIV is believed to take place in poultry through a number of processes, of which acquisition of multiple amino acids at the HA2/HA1 cleavage site plays a crucial role (Swayne & Suarez, 2000)

In recent years, HP outbreaks caused by viruses of H7 subtypes, even though overshadowed by the H5N1 epidemic (Sims & Brown, 2008), have also had devastating effects on the poultry industry; for example, resulting in 13 million birds dead or culled in Italy during 1999 and 2000 (HPAI H7N1) and 25.6 million birds stamped out in the Netherlands in 2003 (HPAI H7N7) (Capua *et al.*, 2000; Alexander *et al.*, 2008). In 2008 and 2009, outbreaks of HPAI H7N7 were recorded in the UK and Spain but they were contained rapidly and did not spread (OIE Online Reports, 2010).

Despite the great importance, there are few reports describing phylogenetic analysis of H7N7 strains isolated from their natural reservoir (Campitelli *et al.*, 2008; Jahangir *et al.*, 2010; Metreveli *et al.*, 2010). Furthermore, there is still little information dealing with virus-host ecology.

In the present study we have analysed the molecular features and phylogenetic relationship of AIV H7N7 isolated from wild mallards in Poland during 2007 to 2009 based on partial or full-length sequencing of all eight segments of the viral genome and compared the results with AIV sequences available in the public domain.

Materials and Methods

Viruses. Four avian influenza H7N7 viruses isolated from cloacal swabs collected from wild mallards (*Anas platyrhynchos*) were used (Table 1). The viruses were isolated in three successive winters: 2007/08 (isolate A/mallard/Poland/01/08), 2008/09 (isolates A/mallard/Poland/16/09 and A/mallard/Poland/41/09), and 2009/10 (isolate A/mallard/Poland/446/09). The geographical location of sampled mallards is presented in Figure 1. Noteworthy is the fact that the isolates A/mallard/Poland/16/09 and A/mallard/Poland/446/09 were detected on the same pond (~ 3500 m^2) almost exactly 1 year apart (January 2009 to December 2009). All of the viruses were isolated in specific pathogen free embryonated chicken eggs followed by identification in a haemagglutination inhibition test according to standard procedures (OIE, 2008). Allantoic fluids from inoculated eggs of the first passage exhibiting haemagglutinating activity were used directly for RNA isolation or stored at –80°C until use.

RNA extraction, reverse transcriptase-polymerase chain reaction and gene sequencing. The RNA was extracted from infected allantoic fluid using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions. Reverse transcription (RT) and polymerase chain reaction (PCR) were performed in one tube using the Qiagen RT-PCR kit using specific primers (sequences and cycling conditions kindly provided by Dr W. Dundon, IZSVe, Legnaro, Italy; available upon request). The complete coding sequences or large portions of all eight genes were generated using BigDye Terminator v3.1 (Applied Biosystems). The products of the sequencing reactions were sequenced in the 3730xl DNA Analyzer (Applied Biosystems) in Genomed, Warsaw.

Phylogenetic analyses. The nucleotide regions investigated in the phylogenetic analyses were as follows: PB2, 27 to 1203; PB1, 1 to 1920; PA, 983 to 2135; HA, 26 to 1637; NA, 20 to 1264; NP, 46 to 1442; M, 1 to 937; NS, 1 to 812. For sequence editing and analysis, modules SeqMan (assembly of the consensus sequence from electrophoregrams) and EditSeq (translation from nucleotide into amino acid sequences) of DNAStar LaserGene (version 8.1.3) were used. Sequences were compared with published sequences of AIV of Eurasian and North American lineages using the BLAST local alignment search tool available from the National Center for Biotechnology Information, Bethesda, Maryland, USA (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were established and phylogenies were reconstructed with MEGA 4 software (Tamura *et al.,* 2007) using Clustal W and the neighbour-joining tree inference method with the Kimura two-parameter substitution model with 1000 bootstrap replicates. Potential *N*-glycosylation sites were predicted with the use of the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). Gene sequences were submitted to the EpiFLU nucleotide sequence database (platform.gisaid.org) with the following isolate identifications: EPI_ISL_26339, EPI_ISL_60707, EPI_ISL_60708, EPI_ISL_74018.

Results

Molecular characterization of AIV H7N7. All of the investigated isolates had the monobasic amino-acid

cleavage site motif at the HA cleavage site: PEIPKGR-GLF (three isolates) and PELPKGR-GLF (one isolate) indicating low pathogenicity (Table 1). The amino-acid residues 226Q and 228G (H3 amino acid numbering) at the receptor binding sites, associated with preferential binding to α(2,3)-linked sialic acid (Vines *et al.*, 1998), were also found in all of the isolates. The H7 HA amino acid sequence revealed five possible glycosylation sequons but not at positions 123 or 149 (H7 numbering), a feature shared by H7 strains isolated from poultry (Banks *et al.*, 2000, 2001). The PB2 E627K mutation, related to an increased adaptation to mammals (Subbarao *et al.*, 1993; Shinya *et al.*, 2004), was not observed. The S31N amino acid substitution in the M2 protein, responsible for conferring resistance to amantadines (Scholtissek *et al.*, 1998), was not detected in any of the tested H7N7 viruses. The NS1 protein did not show any deletion and the D92E substitution, associated with viral resistance to cytokines in pigs (Seo *et al.*, 2002), was not observed.

Phylogenetic analysis. All of the gene segments of the viruses analysed in the present study were of avian origin and clearly belonged to Eurasian lineage (Figures 2 to 5; see also Supplementary Figures S1 to S4). With regard to the HA gene, all of the Polish H7 isolates fell into the same major clade of phylogenetic tree (Figure 2) with the sequence homology 96.2 to 99.3%, and the highest similarity was observed between A/mallard/Poland/41/09 and A/mallard/Poland/446/09 (99.3%). The A/mallard/Poland/16/09 was most closely related to A/goose/Czech Republic/1848/2009(H7N9) (99.8%), isolated from poultry.

The NA genes of the tested strains were 90.3 to 98.7% homologous to each other, and A/mallard/Poland/16/09 and A/mallard/Poland/446/09 (isolated at the same sampling site almost exactly 1 year apart) were the most closely related (Figure 3). Both isolates were also related to A/mute swan/Hungary/5973/2007 (H7N7) with sequence identity up to 97.9%. Interestingly, in contrast to HA gene analysis, the nucleotide homology percentage of the NA gene of A/mallard/Poland/41/09 and A/mallard/Poland/446/09 was very low (90.3%) and these isolates were located on two distant branches of the phylogenetic tree.

With reference to the PB2 gene, two Polish isolates A/mallard/Poland/01/08 and A/mallard/Poland/41/09 clustered in the same subgroup (sequence homology 97.3%), A/mallard/Poland/16/09 was located within a sister subgroup of the same group with up to 96.0% of sequence similarity. The A/mallard/Poland/446/09 showed no significant similarity in relation to the other

Table 1. *Molecular characteristics of the H7N7 Polish isolates in the present study.*

Isolate	Location	Sample collection date (ring number of bird)	HA cleavage site (pathogenicity)	PB2 amino acid at 627	NS1 amino acid at 92	M2 amino acid at 31
A/mallard/Poland/01/08	Bialystok	28.12.2007 (SN 8884)	PEIPKGR-GLF (low pathogenic)	E	D	S
A/mallard/Poland/16/09	Gdańsk	12.01.2009 (SN 10243)	PELPKGR-GLF (low pathogenic)	E	D	S
A/mallard/Poland/41/09	Słupsk	16.02.2009 (SN 10266)	PEIPKGR-GLF (low pathogenic)	E	D	S
A/mallard/Poland/446/09	Gdańsk	27.12.2009 (SN 11463)	PEIPKGR-GLF (low pathogenic)	E	D	S

Polish H7N7 (93.5 to 94.2%) or sequences available at GenBank (Figure 4).

Regarding the PB1 gene, H7N7 viruses isolated in Poland clustered in three different subgroups of the phylogenetic tree. The phylogenic position of A/mallard/Poland/41/09 and A/mallard/Poland/446/09 on the same branch (sequence homology 99.1%) resembled their location on the phylogenetic tree constructed for the HA gene. Both isolates were similar to A/mute swan/Hungary/5973/2007 (H7N7) (nucleotide sequence similarity up to 98.0%; Supplementary Figure S1). The A/mallard/Poland/01/08 shared a very similar PB1 gene with A/turnstone/Netherlands/1/2007 (nucleotide identity 99.1%).

As for the PA gene, a high diversity was found among Polish isolates, which clustered within three different clades with sequence similarity ranging from 94.0 to 96.6% (Supplementary Figure S2). Some of them, however, showed a very high degree of relationship with PA gene detected in wild waterfowl in Europe; for example, A/mallard/Poland/01/08 to A/mallard/Netherlands/22/2007 (H7N1) (99.2%), A/mallard/Italy/299/05 (H7N7) (99.0%) or A/common teal/Netherlands/1/2005 (H8N4) (99.0%).

A high heterogeneity was also found in relation to the NP gene because all of the H7N7 detected in Poland clustered into four different subgroups (homology of nucleotide sequences ranged from 93.9 to 96.0%; Supplementary Figure S3). Similarly to the HA analysis, the highest sequence similarity (99.4%) was found between A/mallard/Poland/16/09 and A/goose/Czech Republic/1848/2009 (H7N9). The A/mallard/Poland/01/08 grouped closely to AIV H7 detected in poultry in Italy: A/turkey/Italy/2685/2003 (H7N3) and A/turkey/Italy/220185/2003 (H7N3), with 98.5% similarity of sequences.

In the case of the M gene, 97.0 to 98.8% of nucleotide sequence identity was found but none of the Polish isolates were directly related to one another. On the other hand, a high degree of relationship was observed between A/mallard/Poland/01/08 and A/turnstone/Netherlands/1/2007 (99.5% nucleotide sequence similarity; Figure 5).

The analysis of NS gene demonstrated that the Polish H7N7 viruses belonged to allele A (Ludwig et al., 1991;

Zohari et al., 2008). The isolates A/mallard/Poland/01/08, A/mallard/Poland/16/09, and A/mallard/Poland/446/09 showed a high percentage of nucleotide homology (99.0 to 99.1%), and their position on the phylogenetic tree clearly suggests their common origin (Supplementary Figure S4). The highest nucleotide homology with reference to sequences available in GenBank was observed between A/mallard//Poland/01/08, A/mallard/Netherlands/1/2006 (99.8%) and A/mallard/Altai/1208/2007 (99.6%).

Discussion

Mallards constitute an important host for AIV. According to Munster et al. (2007), an average prevalence of AIV infections in this species established in a large-scale surveillance study was 7.3%. However, the frequency of AIV isolations in mallards is dependent on season and geographical location. At the sampling sites in Northern Europe, AIV isolation was about three-fold higher as compared with wintering sites in the West, with peak in the early fall migration (Munster et al., 2007). Among mallards breeding in Poland, like in other European countries and in North America, two groups can be distinguished (Cramp & Simmons, 1977; Heusmann, 1981). Mallards resident in urban areas do not migrate while most of the birds breeding outside urban regions show regular movements between breeding and wintering sites. However, in mild climate areas of Western Europe, breeding populations of this species are sedentary making only local movements during severe weather conditions (Scott & Rose, 1996). Mallards ringed in Poland during the breeding season were recovered in Western Europe and South-Western Europe. On the other hand, Poland is the wintering site for mallards breeding in Northern Europe and North-Eastern Europe (e.g. Scandinavia, Russia, Baltic states) (Fransson & Petterson, 2001; unpublished data of the Polish Ringing Center). According to Stallknecht & Brown (2008), the AIV maintenance cycle is based on virus transmission on the breeding and wintering grounds between migratory and resident birds but the mechanism is still poorly understood. It is known that in urban areas in Poland

Figure 1. *Geographical location of the LPAIV H7N7 cases in wild mallards: A, A/mallard/Poland/01/08; B, A/mallard/Poland/16/09; C, A/mallard/Poland/41/09; D, A/mallard/Poland/446/09. Isolates B and D detected at the same sampling location. Map reproduced by kind permission of www.mapykonturowe.pl.*
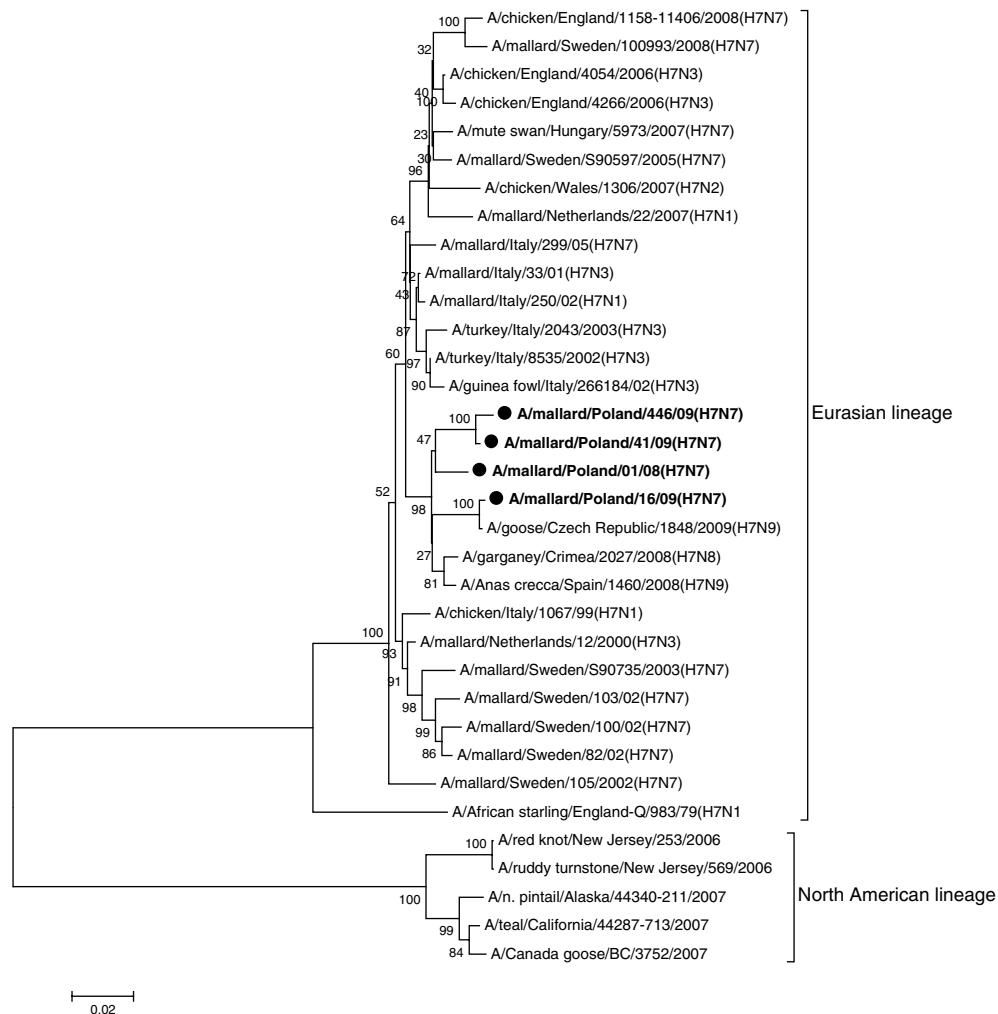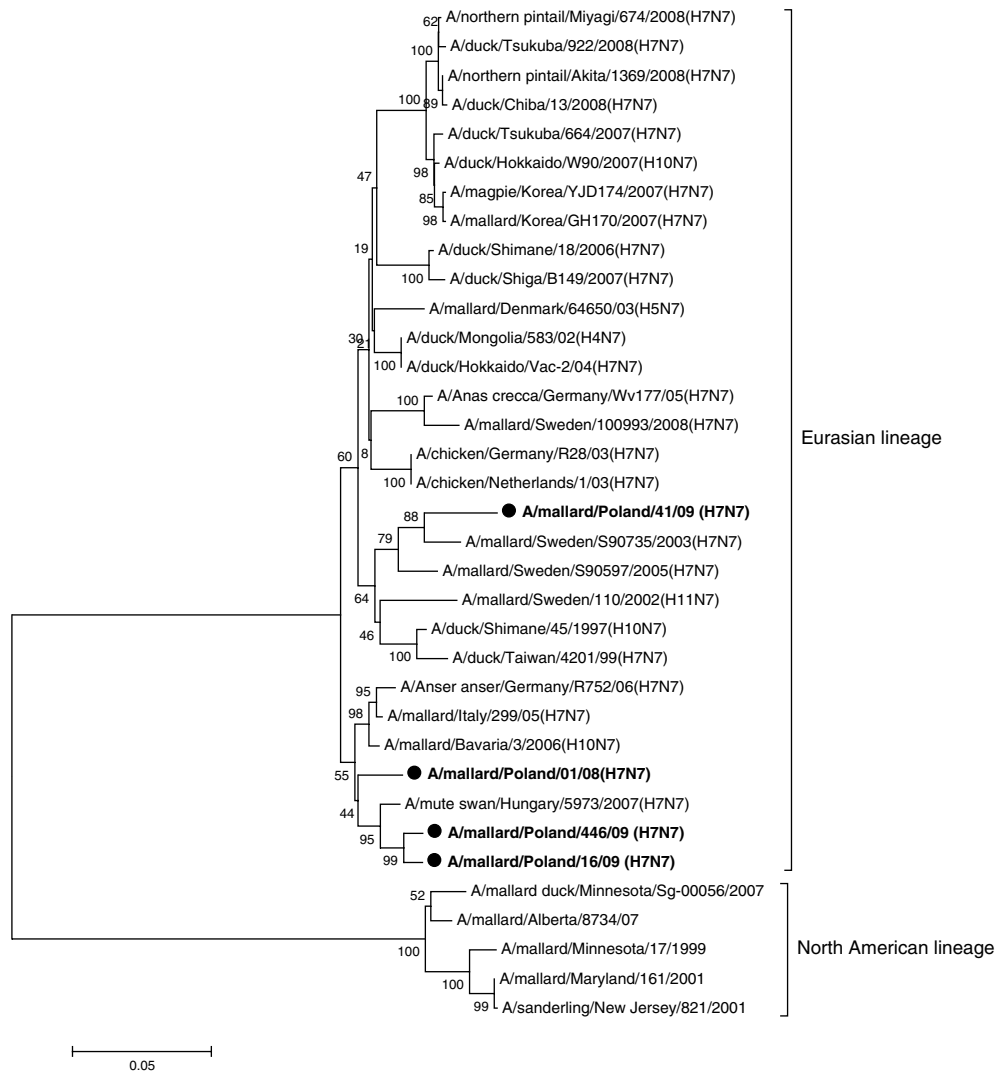
Figure 2. *Phylogenetic tree for the HA gene (nucleotides 26 to 1637) constructed by the neighbour-joining method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.*

both sedentary and migratory mallards form mixed populations in winter. Pair formation in mallards takes place in autumn and winter (Cramp & Simmons, 1977). Females reveal very high philopatry to the breeding grounds, higher than males, and in spring males follow females to the breeding sites (Cramp & Simmons, 1977; Rohwer & Anderson, 1988; Evrard, 1999). Therefore some males of the sedentary populations may join migratory populations whereas some migratory males can stay put on the wintering ground. Such behavioural features create a perfect environment for the transmission of AIV between birds from different populations. Due to the fact that mallards are the most numerous waterfowl in Europe (population estimated at ∼7.5 million; Wetlands International, 2006), this species may play a crucial role in the long-lasting perpetuation of AIV, as suggested by Munster & Fouchier (2009).

In our study we analysed molecular and phylogenetic characteristics of four H7N7 strains isolated from wild mallards in northern and north-eastern Poland in three successive winters (2007/08, 2008/09, and 2009/10). Even though the molecular markers revealed a profile typical of AIV of wild bird origin (low pathogenicity, no markers related to increased adaptation to poultry and mammals or resistance to some antiviral drugs), these

features needed to be analysed even in AIV isolated from wild birds. There have been sporadic cases of HPAI H7 in wild birds (Swayne, 2008). Moreover, some amino acid substitutions in the binding receptor site of HA and mutations associated with resistance to amantadine in a mallard-derived H7 in Italy have also been found (Campitelli *et al.*, 2008).

The phylogenetic study demonstrated a high degree of inter-relationship between Polish H7N7 but only in relation to selected genes. The major conclusion is that the isolate A/mallard/Poland/446/09 is a reassortant virus that shares a very recent common ancestor with A/mallard/Poland/41/09 (in relation to HA and PB1 genes), A/mallard/Poland/16/09 (with respect to NA and NS genes) and A/mallard/Poland/01/08 (as regards the NS gene). The spatial and temporal distribution of the cases was significant: the sampling site of birds carrying A/mallard/Poland/16/09 and A/mallard/Poland/446/09 isolates was located on the same pond in Gdansk (January to December 2009), approximately 100 km away from the city of Slupsk, in which A/mallard/Poland/41/09 had been detected in March 2009. Both sites were located 330 to 430 km from the sampling location of the mallard from which A/mallard/Poland/01/08 had been recovered at the end of 2007. The
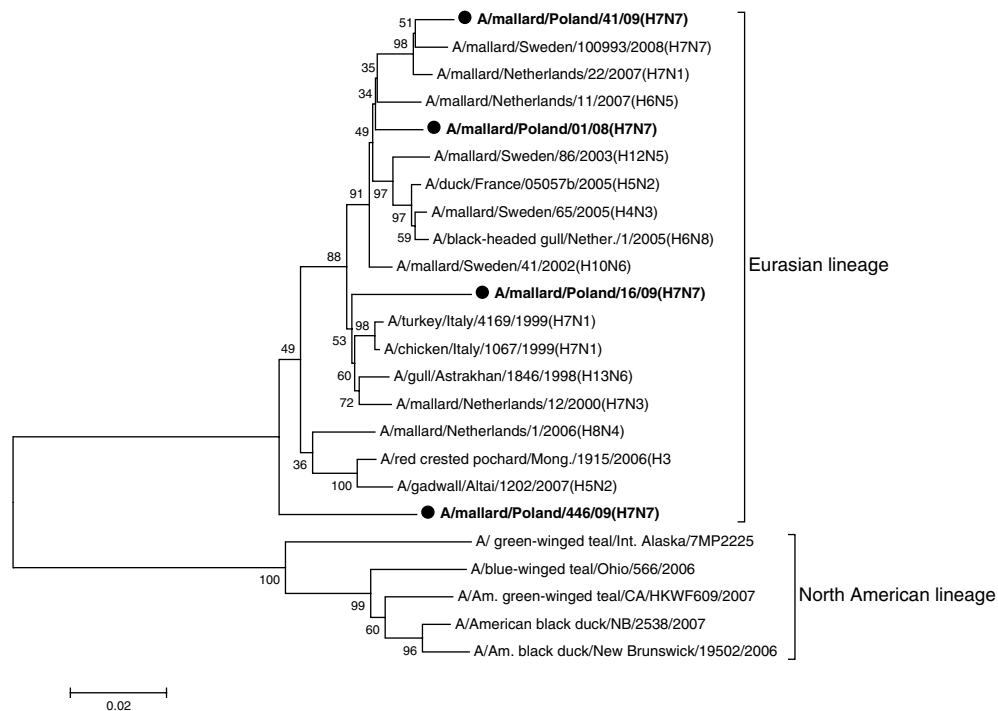
Figure 3. *Phylogenetic tree for the NA gene (nucleotides 20 to 1264) constructed by the neighbour-joining method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.*

ornithological observations resulted in several resightings of the male (ring number SN 11463), the host of A/mallard/Poland/446/09 isolate. The bird was ringed and sampled on a pond in Gdansk on 27 December 2009 and it was recovered at the same site on 23 February, 1 May and early June 2010. Additionally, it was seen on 7 January and 7 March in the area within a radius of 2 km from the sampling site (observation by W. Meissner). These findings confirm that an individual carrying A/mallard/Poland/446/09 strain was a resident bird and it reinforces our hypothesis that resident mallards contribute to the long-term circulation of AIV in the given area between successive winters. We suggest that the NA and NS segments of A/mallard/Poland/16/09 and A/mallard/Poland/446/09 detected in mallards at the same sampling site 1 year apart were derived from AIV precursor strain(s) that persisted in the Gdansk area for a long period of time and emerged in January 2009 and then in December 2009. Interestingly, Jahangir *et al.* (2009) found a high isolation rate of AIV H7N7 in northern pintails (*Anas acuta*) in wintering season 2007/08 in Japan, and similarly to our studies, on one occasion the virus was detected twice at one site, 2 months apart. A phylogenetic study of H7 and N7 genes revealed that these isolates were very similar or virtually identical (Jahangir *et al.*, 2010).However, the high frequency of H7N7 isolation in that region was noted exclusively in one wintering season as opposed to our study in which we demonstrated the presence of AIV with genetically related genes between two consecutive winter seasons in the same area.

On the other hand, the introduction of new AIVs and their efflux from the area is caused by migratory waterfowl, a fact supported by ornithological observations and by our phylogenetic studies in which we found a high degree of relationship between some of the genome segments of the Polish H7N7 and AIV detected in wild birds in Europe. Despite a very close relationship between HA and PB1 of AIV H7N7 isolated from mallards at close locations in Gdansk (A/mallard/Poland/446/09) and Slupsk (A/mallard/Poland/41/09)—that is, 102 km away from each other—it is the most probable that the precursor viruses bearing these gene segments were transmitted between these sites via migratory birds since urban mallards show a very high fidelity to their whereabouts and usually move within very restricted areas of the city in which they stay (Heusmann, 1981;

Figure 4.   *Phylogenetic tree for the PB2 gene (nucleotides 27 to 1203) constructed by the neighbour-joining method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.*
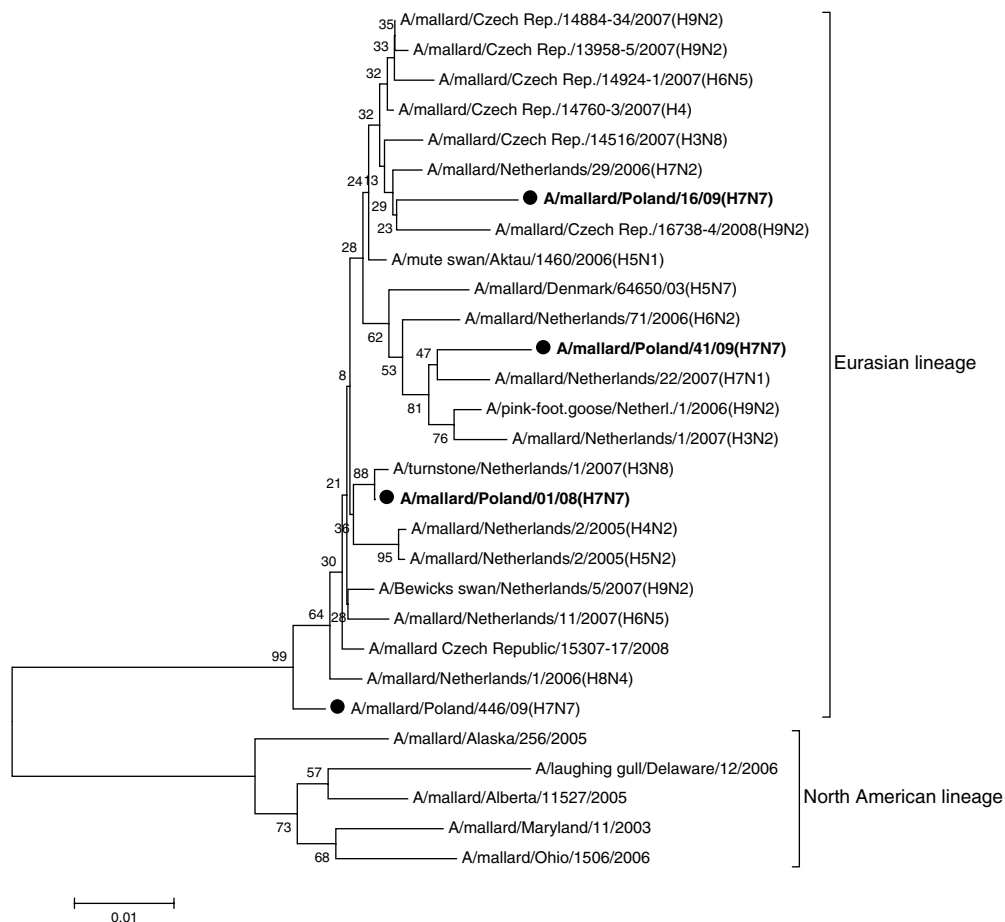
Figure 5.   *Phylogenetic tree for the M gene (nucleotides 1 to 937) constructed by the neighbour-joining method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.*

W. Meissner, unpublished data from studies conducted in three Polish cities).

The AIV undergoes frequent reassortment events: the H7N7 isolates detected in Poland never shared the same constellation of all genome segments, and the close relatedness ($>98.5\%$) was only found in relation to single genes—indicating in most cases different evolutionary pathways. Of special significance is the fact that despite A/mallard/Poland/41/09 and A/mallard/Poland/446/09 sharing a very closely related HA gene ($>99\%$ nucleotide identity), their NA genes were completely different ($\sim90\%$ nucleotide similarity). Other studies have also proved that reassortment events between AIV of various origins are frequent (Hatchette et al., 2004; Macken et al., 2006; Campitelli et al., 2008; Briand et al., 2010).

Interestingly, unlike other genes, the H7 gene seems to have established a relatively conserved sublineage for wild mallards in Poland. However, the assumption that this is a result of an adaptive process to this species of bird requires more evidence due to a limited number of available H7 isolates. The fragmentary data of virological surveillance in Poland are on one hand caused by the number and species of birds tested (approximately 3000 a year, mostly mallards), and on the other hand possible insufficient sampling and storage conditions of swabs and faeces, which may have also contributed to the reduction of an overall AIV detection rate ($<2\%$ a year). Consequently, it cannot be excluded that there may have been other undetected AIV H7 strains of the same and different sublineages circulating in mallards and other species of waterfowl. This issue will be raised in future after more intensive and targeted surveillance in places with previous records of AIV H7.

Another noteworthy aspect of the phylogenetic studies is a close relationship between two genes (HA and NP) of A/mallard/Poland/16/09 and AIV H7N9 isolated from a flock of domestic geese in the Czech Republic. On the other hand, as mentioned above, a high relatedness of its NA and NS genes to A/mallard/Poland/446/09 was shown. The isolate A/mallard/Poland/01/08 was also found to have an NP gene most closely related to H7N3 viruses detected in poultry in Italy in 2003. There is also strong evidence from other studies that low-pathogenic AIVs spill over to poultry, as phylogenetic studies have shown a direct relationship between AIV genes from wild and domestic birds, even from HP AIV outbreaks (Campitelli et al., 2004, 2008; Munster et al., 2005; Terregino et al., 2007; Briand et al., 2010; Metreveli et al., 2010). The present study corroborates the importance of active surveillance in free-living birds as a valuable tool of an early warning system of low-pathogenic AIV and HP AIV outbreaks. It is very important in relation to H7N7 viruses, for which the number of available sequences is still very limited. We suggest that the survey programmes for AIV should not only be targeted at migratory population at major flyways but that resident waterfowl should also be considered.

Acknowledgements

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## Appendix A: Supplementary material available online

Supplementary Figures S1 to S4 showing phylogenetic trees for the PB1, PA, NP and NS genes.

*Supplementary material for* K. Smietanka *et al.* Evidence of persistence and multiple genetic modifications of H7N7 low-pathogenic avian influenza virus in wild mallards in Poland provided by phylogenetic studies. *Avian Pathology*, 2011.
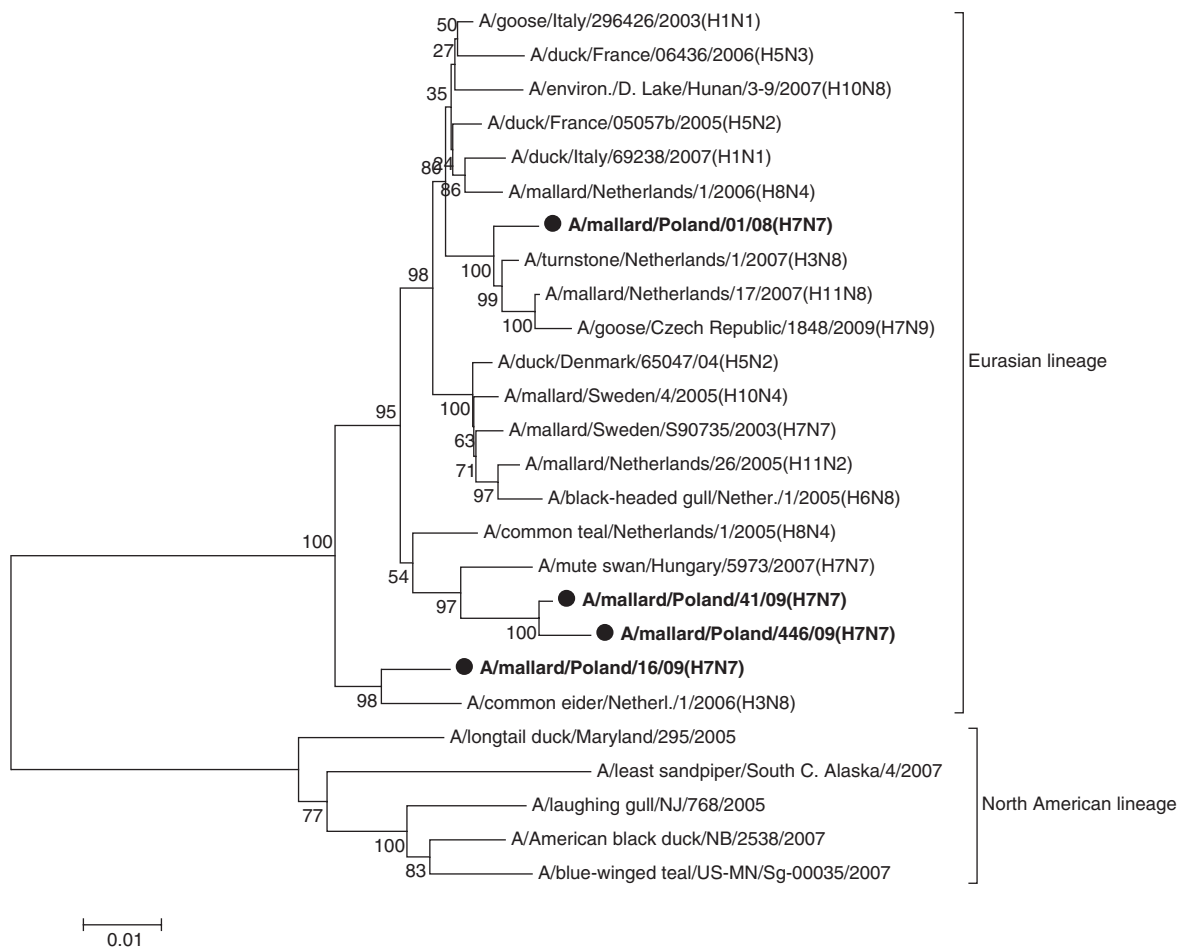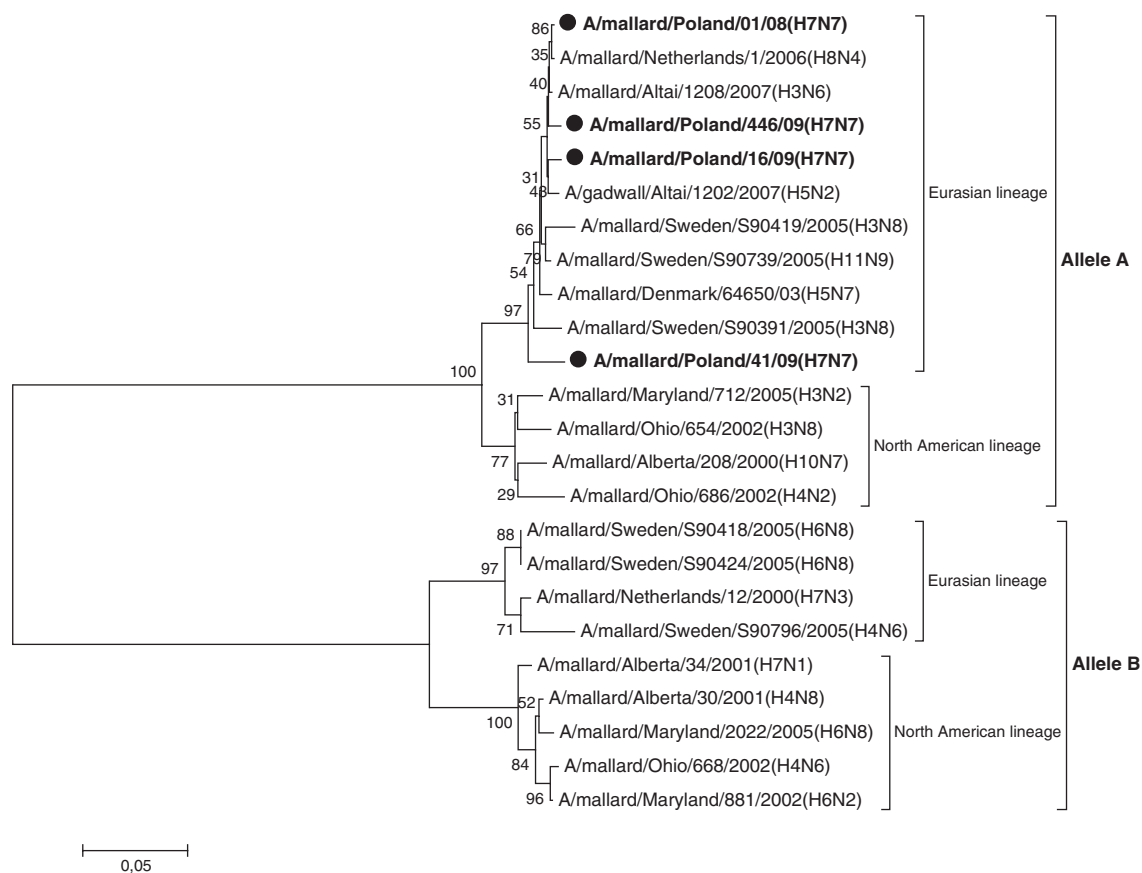

Supplementary Figure S1. *Phylogenetic tree for the PB1 gene (nucleotides 1 to 1920) constructed by the neighbour-joining method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.*
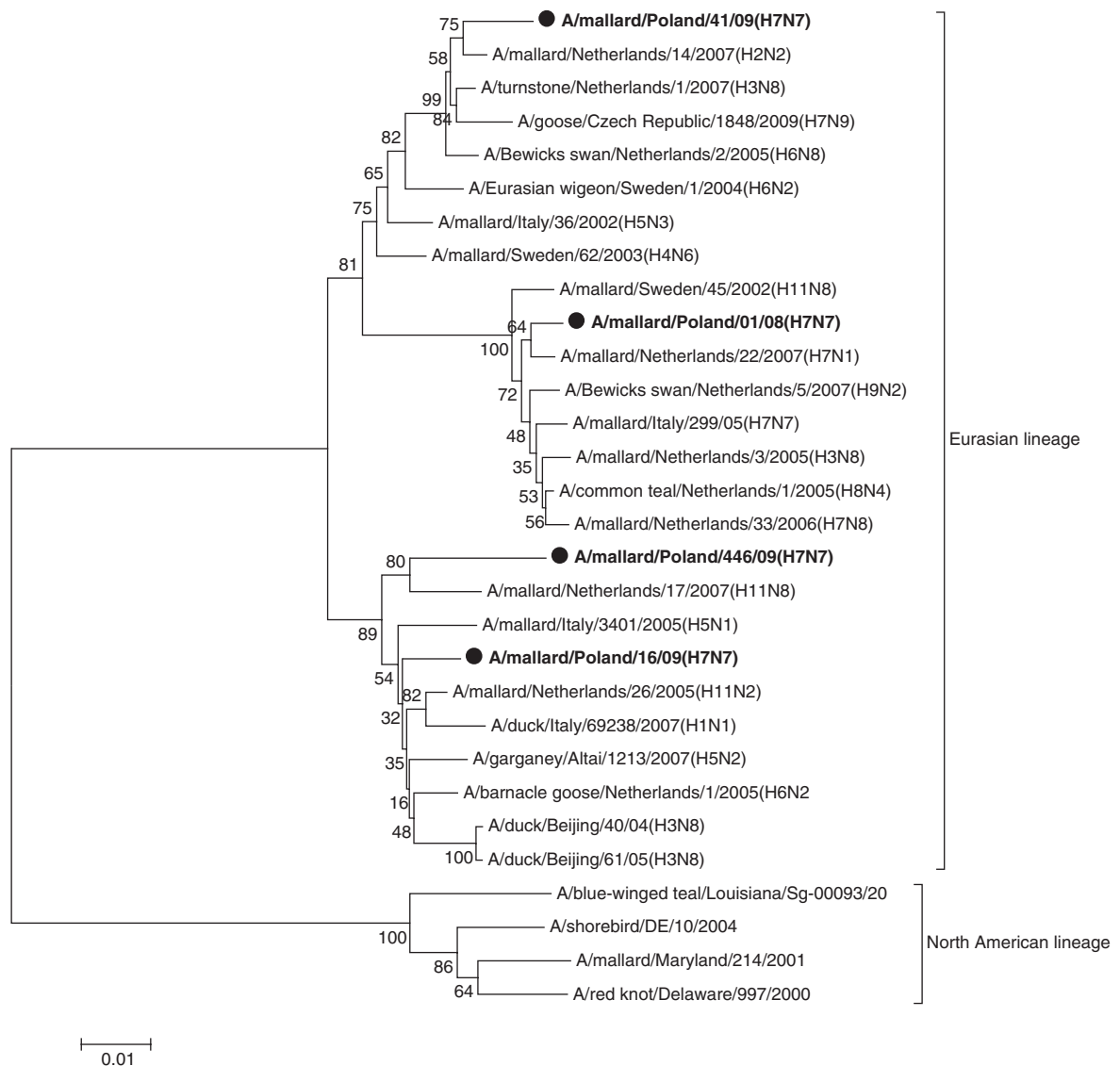
Supplementary Figure S2. *Phylogenetic tree for the PA gene (nucleotides 983 to 2135) constructed by the neighbour-joining method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.*
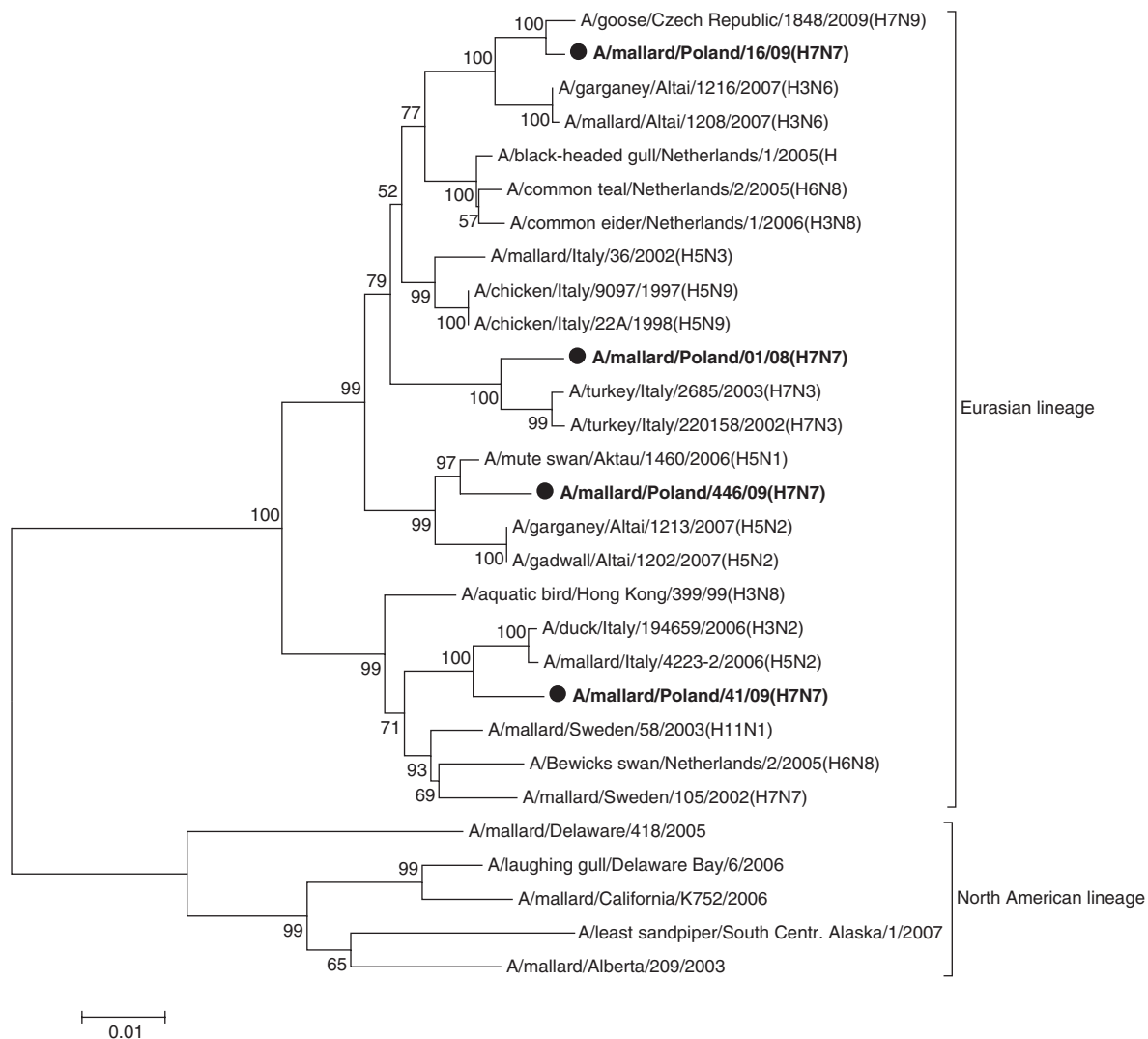
Supplementary Figure S3. *Phylogenetic tree for the NP gene (nucleotides 46 to 1442) constructed by the neighbour-joining method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.*

Supplementary Figure S4. *Phylogenetic tree for the NS gene (nucleotides 1 to 812) constructed by the neighbour-joining method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.*