ABSTRACT

Title of Document: **MOLECULAR EPIDEMIOLOGY AND SURVEILLANCE OF AVIAN INFLUENZA IN WILD AND DOMESTIC BIRDS**

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Surveillance of the existence of avian influenza virus in birds is essential in understanding its epidemiology and potential zoonosis. Point surveillance was made on December 2004 and May to August 2005 in wild birds, domestic poultry and environment. Seven out of 67 samples were positive for avian influenza infection resulting to a 10.4 % isolation rate during the winter. Partial sequencing revealed that all isolates were of H11N3 subtype. In the summer, a total of 584 tracheal, cloacal and environmental swabs were tested in the laboratory through virus isolation, real-time PCR and RT-PCR. All samples were negative. To understand the evolution and ecology of the isolated virus, further sequencing was done for all eight genes of H11N3 and each gene sequence was phylogenetically analyzed with available sequences in the Influenza Sequence Database. Replication and transmission of H11N3 were also investigated through experimental infection of chicken and quail.

MOLECULAR EPIDEMIOLOGY AND SURVEILLANCE OF AVIAN INFLUENZA IN WILD AND DOMESTIC BIRDS

By

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Thesis submitted to the Faculty of the Graduate School of the University of Maryland, College Park, in partial fulfillment of the requirements for the degree of Master of Science
2006

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Dedication

To all the birds that remain to be unwilling hosts of avian influenza virus ---

may their beautiful character shine more than ever.

Acknowledgements

I am indebted to Dr. Nathaniel Tablante, my very kind adviser. I appreciate all the help and support that he has given me during the course of my study. Being one of the best poultry veterinarian I have ever known, he imparted a great deal of knowledge on poultry diseases, industry practices and avian influenza to me. He exposed me to the real drama of the field. Being an adviser, he always had a listening ear in spite of his busy schedule. Doc Nats will always be a role model for me and will never be forgotten.

I have a special case in the department wherein I have a co-adviser, Dr. Daniel Perez, an expert on influenza. First of all, I want to thank him for sharing his knowledge on flu and giving me opportunities to learn laboratory techniques and develop critical thinking. He has pushed me to learn skills which I never thought I would be able to do on my own.

Dr. Bill Pierson has been an integral part of my committee. His intelligence and life-long experience in the veterinary field have inspired me to do better in the future. I thank him for being so honest and patient with me. I am very pleased to know him and I hope to see him again.

My former professor, Dr. Daniel Bautista, has been very helpful in my research. I thank him for his kindness and willingness to help. Most of my isolations were with him and the H11N3 were studied because of him. I admire his dedication to his work. I would also like to mention that the Maryland Department of Agriculture in Salisbury has been very accommodating to me. I thank them for that.

I would also like to express my sincere gratefulness to all who collaborated with our study: Mr. Jason Miller of the US Fish and Wildlife Services, US Army Corps of Engineers in Poplar Island, Dr. Sue Trock and Miss Lisa Weise of the New York Department of Agriculture as well as the owners of the two live bird markets.

The members of the Perez Laboratory have trained me in different aspects of research on avian influenza and have welcomed me sincerely. I would like to express my gratitude to Dr. Haichen Song, Dr. Hongquan Wan, Dr. Md Jaber Hossain, Miss Erin Sorrell, Mrs.

Sharon Azogue-Zimmer, Dr. Gloria Ramirez-Nieto, Dr. Ivan Gomez-Osorio, Mrs. Andrea Ferrero-Perez, Miss Beth Barnes, Miss Daniel Hickman and Miss Erin Graf.

Dr. Siba Samal and all of the VA-MD Regional College of Veterinary Medicine have given our department an atmosphere of continuous learning. You made me proud to graduate from this department and from University of Maryland.

My roommates in St. Andrews Place, Jolie, Masa and Jim, were wonderful pseudo-big sister and big brothers. My close friends in Virginia, Cris and Armie Escano, Van Villar – thank you for the unending support. The VKV-VLV fraternity/sorority---thank you for continuing to be a family.

To Mommy, Daddy and Lola---I thank you all for making me grow in your love. I would choose you guys to be my parents and grandma if I get to live again. I love you so much. You inspired me to live. My siblings, Chris Anne, Michael Angelo and Angelica---you continue to be such delights to me. For that, I love you. I miss our happy times at home in the Philippines. Albert, your kindness, laughter and patience make me a strong and independent person. You are a wonderful part of my life. My pets and past pets---you inspire me to further my studies in veterinary medicine. You make me go places.

Thank you to my Creator…everything is possible with You.

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Chapter I: Introduction

1.1 Overview

Continuous surveillance of the presence of subtypes of avian influenza virus (AIV) in a wide range of birds is critical in its epidemiology and potential zoonosis. It may be called avian influenza virus in general, yet, it appears to cause asymptomatic infections and recurrent epidemics of mild-to-severe disease not only in wild and domestic birds but also in pigs, horses, ferrets, cats, dogs, seals, whales and humans. Online influenza database (www.flu.lanl.gov) displayed that most of the surveillance are done in birds. Detection of AIV in wild birds, particularly in birds from the orders Anseriformes (ducks, geese, swans) and Charadriiformes (terns, sandpipers, gulls), is essential to trace the movement of the virus between these bird populations (Lamb and Choppin 1983; Stallknecht and Shane 1988) and other avian and mammalian species. Wild aquatic birds are considered the natural reservoir of the virus and many of their species migrate in great distances. Therefore, the dissemination and transmission of the virus become efficient because of their migration. During their movement, they commingle with other migrating and resident birds while their droppings can contaminate areas of heightened human and animal traffic.

Avian influenza is widely monitored in domestic poultry including chickens, turkeys, quails, game birds, domestic ducks, ratites and commercially-raised birds. The disease can result to severe economic loss if not detected immediately. If

detected, depopulation of the affected and exposed flock and neighboring farms is carried out to contain the virus and prevent the spread of disease. Depopulating or culling is the most economical and straightforward method of controlling the virus but this kind of measure is not possible in a wildlife setting. For this reason, surveillance of avian influenza in wild birds is increasingly becoming intricate, applying molecular techniques for quicker diagnosis and enhanced understanding of the ecology of the disease. The knowledge that will be gained from surveillance can succor in protecting veterinary industry and public health. The potential of a low pathogenic avian influenza (LPAI) virus from a wild bird of becoming highly pathogenic when transmitted to susceptible poultry population is beyond human control but surveillance can render authorities prepared for potential disease outbreaks. It is also requisite in epidemiological investigations involving the determination of the cause of such an outbreak: (a) whether it is a result of direct transmission of the virus from wild birds to poultry; (b) how long and what subtypes of AIV has been circulating in these birds and; (c) whether there is virulence shift occurring in AIV co-infections.

Eradication of AIV is implausible at present because of the convergence of factors that come into play. These are bird migration and species interactions, highly concentrated poultry and swine farming, dense human and animal populations in cities and presence of traditional live animal markets. There is a relentless concern on AIV circulating in the live-bird markets (LBMs) because the subtypes that have been isolated in some of these markets are associated with the highly pathogenic

phenotypes, the H5 and H7 subtypes (Senne et al. 2003). This is because LBMs, where strict biosecurity measures are hardly implemented, are common places where different species of live birds including wild waterfowl are sold for public consumption. Birds from a myriad of sources are transported in a common vehicle of middle persons and delivered to markets that may or may not have other birds from other suppliers. These circumstances promote circulation of AIV within the LBM. For more than ten years, the number of LPAI-positive markets persisted and increased until three years ago in the Northeast (Mullaney 2003; Trock et al. 2003). These markets are now closely monitored by regular inspection and surveillance by the US Department of Agriculture.

Avian Influenza Type A virus can cause both Highly Pathogenic Avian Influenza (HPAI) and Low Pathogenic Avian Influenza (LPAI). The disease can be highly pathogenic to one species but can cause low pathogenicity to another. HPAI can cause severe, systemic disease with high mortality in chickens, turkeys, and other gallinaceous birds. It is not normally pathogenic to wild birds until recently when a HPAI cause mortalities in wild birds in Asia and Europe. Clinical signs or gross lesions may be absent in peracute cases. However, in acute cases, observable lesions are cyanosis and edema of the head, comb, and wattle; edema and discoloration of the shanks and feet due to subcutaneous ecchymotic hemorrhages; petecchial hemorrhages on visceral organs and in muscles; and blood-tinged oral and nasal discharges. Neurological signs can include torticollis, opisthotonos, or incoordination (Kahn 2005).

The Office International des Epizooties (OIE), otherwise known as the World Organization for Animal Health listed HPAI in the List A disease. They classified AIV as HPAI if it conforms to the following criteria: (a) any influenza virus that is lethal for six, seven or eight of eight 4 to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free, infective allantoic fluid; (b) for subtypes other than H5 and H7, there should be growth of the virus in cell culture with cytopathic effect or plaque formation in the absence of trypsin. If no growth is observed, the isolate is not considered to be a HPAI isolate; (c) for all H5 and H7 viruses of low pathogenicity and for other influenza viruses, if growth is observed in cell culture without trypsin, the amino acid sequence of the cleavage site of the hemagglutinin gene must be determined. If the sequence is similar to that observed for other HPAI isolates, the isolate being tested will be considered to be HPAI (Pearson 2003).

LPAI, on the other hand, causes mild disease in poultry and occasionally causes subclinical infections. AIV from wild birds, once introduced into a poultry population, can become endemic among the flock, manifesting itself as an inapparent infection to a mucosal respiratory infection. Symptoms include reduced egg production, reduced activity and reduced feed consumption. Other signs may include increased oculonasal discharge, skin lesions, nervous disorders, and diarrhea (Swayne and Suarez 2000). The ultimate impact on the poultry industry and farmers is enormous because affected farms have to be depopulated, disinfected and left without birds for an indefinite period of time. On a global perspective, a ban on poultry

products from an AI-infected country may be imposed by an importing country, resulting in significant economic losses to the poultry industry of the exporting country. When avian influenza, also known as bird flu, has cropped up in birds in Delaware in early 2004, Japan , South Korea and Russia imposed a ban against US poultry products.

The need for a large-scale surveillance of AIV is also based on the etiologic agent's characteristics. For an RNA virus like AIV, mutation, selection and reassortment occur making the mechanisms of their evolution and epidemiologic history complex and should be incessantly analyzed (Moya et al. 2004). The RNA-dependent RNA polymerase does not have an associated exonuclease proofreading activity, thus, increasing the chance of errors during replication (Steinhauer et al. 1992). This infidelity of the polymerase makes the RNA virus prone to mutations. Combined with the predisposing factors in the environment and the state of the host, the characteristics of the virus are generally the basis for the viral antigenic drift and shift. The mutations are accidental in nature but could have an adverse effect on the ability of the virus to cause disease and widen its host range. This is the reason why an outbreak occurs every now and then.

To keep pace with the evolving virus, investigators involved in disease surveillance around the globe have inferred to phylogenetic relationships of the genes of emerging and re-emerging subtypes of AIV by analyzing large amount of data with the use of bioinformatics and statistical algorithms. Results can be correlated to

describe the origin, occurrence and biological relationships of AIVs, to determine genes responsible for virulence and to assess what kind of gene reassortments have been taking place aside from recombinations of the 16 hemagglutinin and nine neuraminidase subtypes.

1.2 Research Objectives

The main goal of this research is to contribute to the existing knowledge on the nucleotide changes and reassortment taking place in AIV genome in wild birds necessary to become adapted to terrestrial birds and mammals thereby causing disease. This study is designed to fulfill two objectives at hand. First is to carry out point surveillance of AIV in wild and domestic birds. This activity will bring about data on the prevalence of avian influenza in an area and will entail active collaborations to aid in the procurement of samples. Collaborating to different agencies is an integral part of the surveillance as expertise on different aspects is put into one goal. The second objective is to perform genetic and biological characterization of AIV in order to aid in understanding phylogenetic relationships among isolates and representative subtypes. The gene sequence will be determined, aligned with other sequences and subjected to statistical algorithms for the generation of phylogenetic trees. A tree will be built for every gene segment.

In a larger perspective, surveillance of AIV will lead to understanding of its ecology, socioeconomic impact and temporal and spatial patterns, thereby, effectively

aiding in the design of control programs for poultry production, defining risks to public health and monitoring of circulating viruses that can help in development of vaccines.

Chapter II: Review of Literature

2.1 Genus Influenzavirus A

Avian influenza viruses refer to the avian strains of influenza A virus that comprise the Genus *Influenzavirus A* of the family *Orthomyxoviridae*. Although it has an avian description, the virus can infect a wide variety of vertebrate hosts during its entire life cycle (Buechen-Osmond and Dallwitz 1996). It is an enveloped, spherical or pleiomorphic to filamentous structure of 80 to 120 nm in diameter (Figure 1). The envelope is a lipid bilayer derived from the plasma membrane of the infected host cell. It contains two types of surface glycoproteins called the hemagglutinin (HA) protein and, in lesser abundance, the neuraminidase (NA). Another integral protein contained in the envelope is the matrix 2 (M2) protein. Underlying the lipid bilayer is the viral matrix (M1) protein that brings togetherwith the ribonucleoprotein core (RNP) and the envelope. The coiled RNPs (Heggeness et al. 1982) consist of the nucleocapsid (NP) encapsidating the RNA segments of the viral genome and the heterotrimeric RNA-dependent RNA polymerase complex (Polymerase basic protein 1, PB1; Polymerase basic 2, PB2; Polymerase acid protein, PA). The viral genome is segmented into eight single-stranded negative sense RNA of 890 to 2,341 nucleotides (Table 1) (Fields et al. 2001) (Lamb and Choppin 1983).

The shortest RNA segment of 890 nucleotides encodes for the nonstructural protein, NS1 and NS2. NS1 has antagonistic effects on interferon (IFN) α/β (Weber

et al. 2004), cytokines that have antiviral activity and immunoregulatory function (Johnson and Baron 1976). Late in the viral infection, RNPs are transported out of the nucleus to the plasma membrane mediated by a protein adaptor molecule NS2, otherwise known as the nuclear export protein (NEP) (O'Neill et al. 1998).

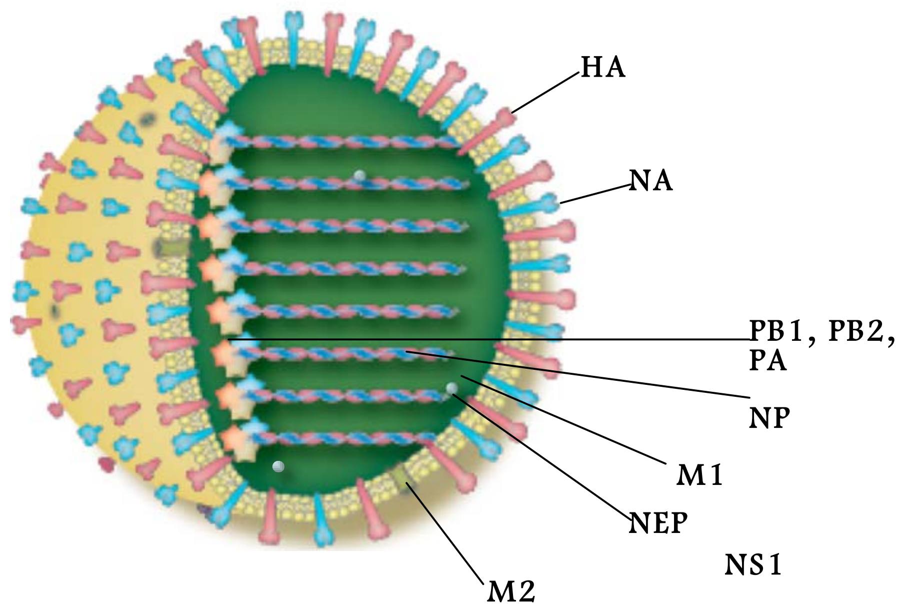

Figure 1. A schematic diagram of the structure of influenza A virus. This diagram is taken from www.agnr.umd.edu/avianflu. Integral proteins HA, NA and M2 are found in the envelope. Underneath the envelope is the viral genome consisting of eight segmented, minus-sense RNA. Each RNA is associated with NP and RNA-dependent RNA polymerase complex (PB2, PB1 and PA) forming the ribonucleoprotein (RNP). RNPs are closely associated with the M1 which underlies the envelope. NS1 proteins are produced in AIV-infected cells. NEP has a role in exporting of RNPs out of the nucleus during the late stage of viral infection.

Table 1. Influenza A virus* genome RNA segments

Segment	Nucleotide Length	Encoded polypeptide	
1	2,341	Polymerase Basic 2	PB2
2	2,341	Polymerase Basic 1	PB1
3	2,233	Polymerase Acid	PA
4	1,778	Hemagglutinin	HA
5	1,565	Nucleoprotein	NP
6	1,413	Neuraminidase	NA
7	1,027	Matrix	M1, M2
8	890	Nonstructural Protein	NS1, NS2

* based on A/PR/8/34 strain (In: Fields Virology)

Efficient virion formation is preceded by the presence of all eight segments of the viral RNA (vRNA) and selective incorporation of these segments is aided by the signal from the coding region of the NA viral RNA (Fujii et al. 2003). The incorporated segments are arranged in a distinct pattern wherein seven segments of varying length surround a central segment (Noda et al. 2006). M1 and all eight RNPs are brought to the apical plasma membrane containing lipids and transmembrane proteins in polarized epithelial cells. The RNPs are situated perpendicular to the plasma membrane prior to budding. Viral and host factors pushes the membrane outward until buds are formed at the assembly site and virus particles are released in the extracellular environment following closure of buds (Nayak et al. 2004). While

RNP's are made in the host nucleus and transported with the help of NEP, M1 is synthesized by the cytosolic ribosomes. M1 has an L motif (Nayak et al. 2004) which is central to budding mechanism. M1 molecules bind vRNP's as well as plasma membrane, possibly via the cytoplasmic tails of the two surface glycoproteins (Ruigrok et al. 2000).

The entry of enveloped viruses like the influenza virus into cells is carried out through fusion of the viral membrane with the cell membrane. HA protrudes on the viral surface and binds to a sialic acid-containing receptor on the cell surface for attachment. Epizootics are associated with changes in antigenic structure of HA because it is the major antigen against which the neutralizing antibodies of the host are made (Cross et al. 2001). This pressure exerted by the immune system is believed to be a reason for the genetic mutations. HA is also responsible for viral entry into the cytoplasm through facilitated fusion of the membrane of the endocytosed virus particle with the endosomal membrane. The other surface glycoprotein, NA, is important in the removal of sialic acid residues from virion components in order to prevent the aggregation of virus particles (Kaverin et al. 1998) as well as sialic acid cleavage from the host cell to promote virus release and spread (Stray et al. 2000).

In other words, influenza virus particles carry both a sialic acid–specific lectin (HA) for entry into cell and a sialidase (NA) for release of virions (Stray et al. 2000). Efficient infection requires fusion of HA to the respective cell receptors containing either a sialic acid linked to galactose by an alpha-2,3 linkage (SAalpha2,3Gal) or by

an alpha-2,6 linkage (SAalpha2,6Gal). All subtypes of HA found in avian species prefer binding to sialic acid in an 2,3-linkage to galactose. Important to note is that the HAs of human viruses recognize sialic acid in 2,6-linkage. Some species of birds like quail has both receptors in the respiratory and intestinal tract, therefore, can act as an intermediate host between the avian-like viruses and human-like viruses (Wan and Perez 2006). Aside from the receptor-specificity, the cross-species transfer of avian viruses into humans requires a change in binding specificity (Gamblin et al. 2004).

2.2 History of Avian Influenza

The virus was not classified as influenza virus until 1955, yet, outbreaks before the classification of the virus were already described. In 1878, fowl plague or a highly pathogenic form of avian influenza was reported by Perroncito in Italy. It was confused with an acute septicemic form of fowl cholera until 1880 when Rivolto and Delprato classified the two diseases based on clinical and pathological signs. After 12 years, another outbreak in chickens occurred in Northern Italy and spread to Austria, Germany, Belgium and France. It became endemic in many parts of Europe since then. In the US, however, an outbreak of highly pathogenic avian influenza began in 1924 in the live poultry markets of New York, followed by New Jersey and Pennsylvania. By middle of the twentieth century, highly pathogenic avian influenza was diagnosed in Europe, Middle East, Russia, Asia, North Africa, North and South America. During this time also, a milder form of the disease was recognized in chickens, domestic ducks and turkeys causing respiratory distress and drops in egg

production (Saif 2003). There were no reports of AIV outbreaks in wild aquatic birds until 1961 when common terns (*Sterna hirundo*) died in South Africa. This was caused by a HP form of avian influenza (Swayne and Suarez 2000). In 1972, surveillance of Newcastle disease in migratory birds led to coincidental isolation of AIV (Slemons et al. 1974). Surveys revealed that many wild birds were positive for AIV infection based on isolation and serology but did not show any clinical sings of the disease. It was established, since then, that healthy wild birds are primordial reservoirs of AIV. They harbor the virus without causing disease to them. These birds act as silent reservoirs that can shed viruses in huge amounts which can further infect other poultry and mammals as well (Figure 2). This was a very important finding because it led to an active surveillance of AIV in wild birds.

2.3 Role of Migratory Birds

Avian influenza virus have been found in many wild bird species, most commonly in wild waterfowls with the highest frequency of isolation from mallard ducks, *Anas platyrhynchos* (Friend et al. 1999). Other families of wild birds that shed avian influenza virus are geese (e.g. *Branta canadensis*), swans (*Cygnus olor*), gulls (e.g. *Larus argentatus)*, terns, waders (e.g. *Calidris canutus*), rails (e.g. *Fulica americana*), petrels (e.g *Pterodroma lessonii*), cormorants (e.g *Phalacrocorax auritus*), quail (*Coturnix cotunix*), pheasants (*Phasianus colchicus*) and ratites (e.g. *Dromaius novaehollandiae*) (Friend et al. 1999) (Olsen et al. 2006). All 16 HA and

nine NA subtypes are found in these birds (Fouchier et al. 2005) (Hinshaw et al. 1982) (Kawaoka et al. 1990) (Krauss et al. 2004) (Rohm et al. 1996). AIVs infect cells lining the intestinal tract of birds and are excreted in high concentrations in their feces. The mode of transmission is through feco-oral route (Fouchier et al. 2005).

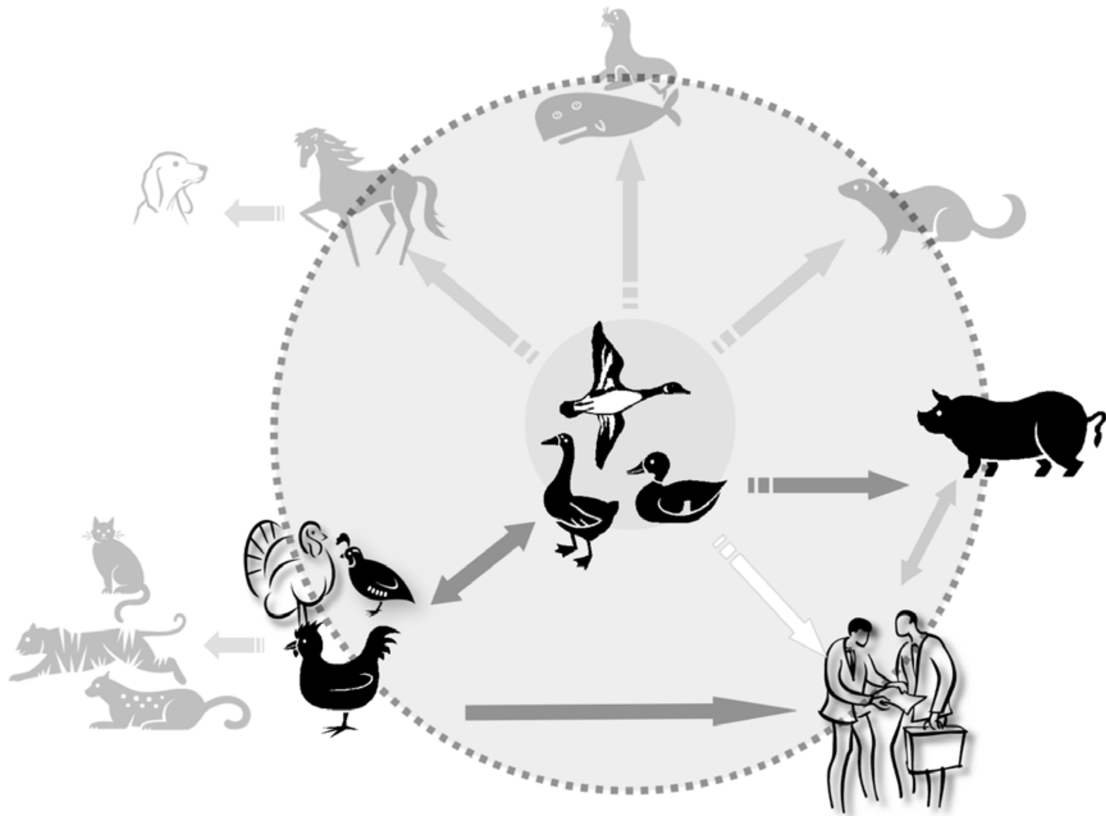

Figure 2. Wild waterfowls and shorebirds are natural reservoirs of avian influenza virus. These birds can shed AIV subtypes that can infect a variety of species of animals including human. While AIVs are generally nonpathogenic in wild birds, they sometimes cause morbidity and mortality upon transmission to domestic birds and mammals.

During migration of these birds, they can infect other waterfowls. For instance, in 1998, five hemagglutinin subtypes (H2, H3, H6, H9, and H12), six neuraminidase subtypes (N1, N2, N4, N5, N6, and N8), resulting to nine HA-NA combinations were isolated from the resident ducks in the eastern shore of Maryland. These viruses were introduced by migrating birds during the late summer as surveillance in these birds in the start of summer did not result in any positive isolations (Slemons et al. 2003). This finding makes resident ducks serve as sentinels for AIV brought about by migratory fowls and at the same time, they can be amplifiers of infection to other birds in the area.

The intermixing of bird population is previously described. Matrix genes of viruses isolated from Canadian ducks and those of shorebird and gull viruses in the Delaware Bay have common ancestors as that of the M genes of most North American poultry viruses. Some North American AIVs contained M genes closely related to those of Eurasian lineage suggesting an interregional mixing of the two clades (Widjaja et al. 2004). Intermixing of bird species as well as interregional mixing greatly contribute to the reassortment of the virus which can result to a strain that can adapt to domestic birds and other mammals.

The role of migratory birds in the biological evolution of a low pathogenic to a high pathogenic form of the virus is evident in the current situation of the circulation of the subtype H5N1 in Asia, Europe, Middle East and some parts of

Africa. In a timeline published by the World Health organization on 8th May 2006, the H5N1 can be traced from 1996 when a high pathogenic (HP) form of this subtype was isolated from a farmed goose in Guangdong Province, China. In 1997, outbreaks of the HP H5N1 occurred in farms and wet markets in Hong Kong. Most of the birds in the wet markets of Hong Kong come from the Guangdong Province. The transport of the birds itself is a method of spreading the infecton. Eighteen cases of human infections were also documented in Hong Kong. This was the first evidence of H5N1 causing fatal disease in man. Interestingly, outbreaks of H5N1 in waterfowls in recreational parks as well as in migratory birds in Hong Kong in late 2002 displayed disruption of the stable agent-host relationship between AIV and its natural reservoirs (Sturm-Ramirez et al. 2005). Inoculation of this 2002 isolate to experimental mallard ducks gave surprising results. The virus replicated in multiple organs and ducks develop acute disease including neurologic dysfunction followed by death (Sturm-Ramirez et al. 2004). There were only two influenza viruses that caused deaths in aquatic birds: A/tern/South Africa/61 (H5N3), mentioned previously, and the HP H7N1 that caused outbreaks in Muscovy ducks (*Cairina moschata*) in Italy during 1999-2000 (Capua et al. 2002). Late 2003, there was a postmortem isolation of H5N1 from tissue samples of two tigers and two leopards fed with chicken carcasses in a Thailand zoo. This was the first time an H5N1 was isolated from these animals. This was followed by a report of H5N1 infection in poultry by the Republic of Korea, Vietnam, Japan, Thailand, Cambodia, Laos, Indonesia and China. Another group of H5N1 fatalities occurred in a Thailand zoo in 2004 wherein one-third of the tiger population in the zoo either died or were euthanized.

Three years after the reported isolation of AIV from waterfowls in Hong Kong, thousands of migratory birds in Qinghai Lake died of the virus. Many people frequently visit the lake. In the same year, 2005, Russia and Kazakhstan reported H5N1 in poultry and dead migratory birds were seen in the area of outbreaks. This year, Azerbaijan confirmed H5N1 in migratory birds. Bulgaria, Greece, Slovenia and Italy had isolates from swans. Iran, Austria, UK and Germany also reported isolations from swans. The current situation bothered the public and the authorities alike because of the deaths of wild birds from AIV. And it seemed that the virus was spreading westward across the globe. Although a HP H5N1 had been killing its natural hosts, experimental infection of a 2004 isolate to ducks showed low pathogenicity in the experimental animals. This result showed that H5N1 can revert to a nonpathogenic form in order to maintain itself in its natural host. The experimental animals shed viruses in large amounts which suggest that the virus can possibly be maintained within the wild bird population and continue to circulate (Hulse-Post et al. 2005).

Chapter III: Materials and Methods

3.1 Sampling sites

Sample collections were done in the winter of 2004 and summer of 2005. There were 67 samples processed in the laboratory during the winter and 584 samples processed in summer. The 67 samples were a mixture of cloacal and tracheal swabs as well as fecal samples. The Maryland Department of Agriculture (MDA) in Salisbury submitted these samples. It was December of 2004 when duck die-offs in Chesapeake Bay were reported to MDA. The birds were brought to MDA for necropsy and further investigation.

During the summer, a total of 584 samples including tracheal and cloacal swabs, fecal and environmental samples were tested in the laboratory. Most of the samples were pooled into five. A pool of samples consists of either five tracheal swabs or five cloacal swabs from different birds of the same species during the same day of collection. These samples were collected in the months May to August 2005 from migratory and non-migratory waterfowls, other wild birds, domestic poultry and environment. Birds included Canada geese, snow geese (*Chen caerulescens*), mallard ducks, muscovy ducks, wood ducks (*Aix sponsa*), mute swans, herring gulls, glaucous gulls (*Larus hyperboreus*), sandpipers (*Actitis macularia*), pheasants, and various strains of chickens (*Gallus domesticus*). There was a mixture of adult and juvenile

birds. Places of collections were Chesapeake Bay particularly on Poplar and Kent Islands, along the Wicomico River in Salisbury, Maryland, Salisbury Zoo and two live bird markets in Queens, New York. All samples collected were inoculated in embryonated chicken eggs (B and E eggs, PA) and passaged two more times performing hemagglutination assay after each passage. To verify some results, 105 cloacal swabs were subjected to real-time Polymerase Chain Reaction and 37 samples in Reverse Transcriptase-PCR (RT-PCR).

3.2 Sample collection in wild birds

The December 2004 isolates were from dead ducks submitted to the Maryland Department of Agriculture Laboratory in Salisbury, MD. The species of the ducks were not specified, but the ducks were residents of the area. Cloacal and tracheal swabs were obtained from dead ducks by Dr. Daniel Bautista. The swabs were placed in 3.7% Brain Heart Infusion (BD, Franklin Lakes, NJ) medium with Penicillin-Strpetomycin, Gentamicin and Amphotercin B and transported on ice to Avrum Gudelsky Veterinary Center, University of Maryland College Park, for AI testing.

There were four field collections from wild birds made from May to August of 2005. One of the sites was Poplar Island, a five-acre island located in the upper middle Chesapeake Bay, southeast of Annapolis and northwest of Tilghman Island in Talbot County, Maryland. It was once a large island covering around 1,100 acres but

through the years, the land slowly eroded. The US Army Corps of Engineers and other local government entities started rebuilding it in 1998 to save it from further erosion. They are working to improve it as a nesting area for migrating birds.

According to the 2002-2003 Bird Surveys on Poplar Island and Vicinity (www.nab.usace.army.mil/projects/Maryland/PoplarIsland/birds.html), many birds from the orders Anseriformes, Charadriiformes, Ciconiiformes, Columbiformes, Coraciiformes, Falconiformes, Gaviiformes, Gruiformes, Passeriformes, Podicipediformes, Pelicaniformes, and Strigiformes were sighted on the island. Overpopulation of some species of birds is inevitable and control measures are more often implemented. On June 2005, the Animal and Plant Health Inspection Service (APHIS) began Canada goose and gull control on the island. Two visits to the island were made with Jason Miller of the US Fish and Wildlife Service. Cloacal swabs were taken from dead birds that were rounded up. The swabs were placed in 3.7% BHI broth with antibiotics (Penicillin-Streptomycin-Gentamycin; Amphotercin B) and immediately brought to the laboratory.

Similarly, mute swan control was also implemented in other sites of the Chesapeake Bay and the birds were submitted to MDA-Salisbury for necropsy. The cloacal swabs taken were sent to Avrum Gudelsky Veterinary Center, UMCP, as previously described.

Water and fresh fecal samples were collected from the Wicomico River in Salisbury, MD. Different species of birds were sighted in this area which were previously mentioned. Cotton swabs were dipped into the water and feces separately and placed separately in 3.7% BHI broth containing antibiotics and transported on ice to Avrum Gudelsky Veterinary Center, UMCP.

Water and fresh fecal samples were also obtained as previously described from different parts of the Salisbury Zoo including cages housing North and South American ducks, an open pen with llamas in which mallard ducks and swan were found, parking lots, and picnic area which attracted brant geese. Swabs were immersed in 3.7% BHI broth with antibiotics and immediately placed in ice.

3.3 Sample collection in live bird markets

Two live bird markets were visited in Queens, New York together with Lisa Weiss, a trained technician of the New York Department of Agriculture. Apart from the tracheal and cloacal swab samples, floors, cages, walls and drains were also swabbed. The NYDA regularly monitors most of the live bird markets in the area. The samples taken were placed in BHI transport medium prepared by National Veterinary Services Laboratories (NVSL), placed in ice and immediately hand carried to Avrum Gudelsky Veterinary Center, UMCP.

3.4 Collection procedures used in sites

The transport medium, brain-heart infusion broth, were kept in ice at all times during the collection. Gloves were used when taking swabs. Most of the swabs from the live bird markets were pooled by type of swab ,whether it was tracheal, cloacal or environmental, and by species of bird. Cloacal swabs were visibly coated with fecal material. If the bird was too small and could be harmed by swabbing, the collection of fresh feces served as an adequate alternative. The glass vials containing the transport medium were labeled and placed on ice. As soon as the vials with samples reached the laboratory, they were either inoculated immediately to embryonated chicken eggs or stored in an ultra-low freezer (-70ºC). Samples were thawed once and immediately processed after thawing.

New disposable boots and coveralls, caps and gloves were used for every market visited. Used swabs and protective clothing were left at the visited market for disposal. Live bird markets naturally sell a variety of species of birds. For land-based birds such as chicken and pheasants, both tracheal and cloacal swabs were collected. On the other hand, only cloacal swabs were collected from waterfowls such as ducks and geese. Freshly voided fecal samples were collected from the environment.

There was no handling or trapping of live wild birds involved as far as sample collection is concerned. Samples taken in the field were either from dead birds or from fecal samples voided by wild birds. Cloacal swabs were immediately done upon

rounding up of dead birds as a result of the geese and swan control program. Swabbing of the cloaca involved stroking the cotton tip against the wall of lower intestinal tract and engorging it with feces.

3.5 Processing of samples

The easiest way of propagating avian influenza virus is by inoculating it into the allantoic cavity filled with allantoic fluid of an embryonated chicken egg. The virus replicates in the cells lining the cavity and progeny viruses are released into the allantoic fluid as the cells are disrupted. Before the actual inoculation, the eggs were incubated for 9-10 days at 37ºC with 55-60% humidity. At 10th day, the viability of the embryo was checked by candling the eggs. The blood vessels should be well-defined and the embryo should show movement. A line was drawn on the shell marking the edge of the air sac.

The samples in glass vials were vortexed for 3 seconds, transferred into 15 ml polypropylene tubes (Fisherbrand, Hampton, NH) and spun down at 1500 rpm (252 g) for 15 minutes in a refrigerated centrifuge (4°C). Meanwhile, 10-day old embryonated eggs were prepared by spraying the surfaces with 70% ethyl alcohol. A hole was gently drilled in the inoculation site (2-5 mm above the air cell line). Then, using a 1 ml syringe with gauge 23 needle, 0.6 ml of the supernatant fluid was drawn out from each tube. Exactly 0.2 ml was introduced into each of the three eggs. The holes were covered with melted paraffin. Eggs were then incubated at 37ºC on a still platform in a humidified incubator with 5% CO_2 for 48 hours.

3.6 Hemagglutination Assay

After 48-hour incubation, the allantoic fluid was harvested from the eggs. As soon as the eggs were taken out of the incubator, they were placed in a refrigerator (4ºC) overnight or in a freezer (-20ºC) for not more than 45 minutes. During this span, a 96-well round bottom plate was labeled. There were eight rows and 12 columns in a plate and a line was drawn at the middle of the eight rows. Lines were drawn every other three columns resulting to eight groups of wells consisting of four rows and three columns for each group. Each well group was allocated for each sample given that each sample was inoculated into three eggs. Included in the allocation of well groups were positive and negative controls. Several metal scoopula, a metal round-bottom scapula and a pair of forceps were boiled in distilled water. The eggs were then taken out of the refrigerator or freezer, placed under the hood and sprayed with ethyl alcohol. The blunt end was slowly tapped using a spatula. When the shell cracked, the broken pieces were slowly taken out with the use of forceps exposing the air cell cavity. The air cell membrane was punctured using a sterilized scoopula placing it deep down into the allantoic cavity until allantoic fluid flowed out. One hundred µl of the fluid was pipetted out of each infected egg and placed into a first-row well of the plate.

Fifty µl of 1X Phosphate Buffered Saline was added into the second, third and fourth rows of wells using a multichannel pipettor. Then, 50 µl of allantoic fluid from the first well was removed, placed into the second well, and gently mixed by pipetting. Fifty µl of the mixture was then discarded. This was repeated, taking 50 ul

from the second row of wells and placing it in the third row, thus producing two-fold serial dilutions. Fifty μl of 0.5% chicken red blood cells was added to all the wells. One edge of the plate was tapped against to distribute the red blood cells in the wells. The plate was incubated at room temperature for 45 minutes to 1 hour. The presence of virus in an uncontaminated sample produced agglutination of the red blood cells i.e., the mixture in the well appeared diffusely pink in color. Negative results were indicated by the presence of red "button" on the bottom of the well due to settling of the red blood cells.

3.7 RNA Extraction and RT-PCR

For each sample, Beta-mercaptoethanol and RLT buffer (guanidine isothiocyanate-containing buffer) solution was prepared at a ratio of 10:1000. In 1.5ml tube, 350 μl of the solution was added to 200 μl of allantoic fluid or BHI to lyse and homogenize the sample inactivating the RNases. Five hundred fifty ul of 70% ethanol was then added to adjust binding conditions. The mix was transferred to an RNeasy (Qiagen, Valencia, CA) spin column for adsorption of RNA to membrane. The column was spun at 15,000 rpm in a microfuge. The contaminants were washed off three times using wash buffers RW1 and RPE. The RNA was eluted using 40 ul millipore water.

Complementary DNA (cDNA) was prepared by incubating 4.0 ul RNA, 0.5 ul Uni12 primer AGCAAAAGCAGG (1.0 ug/ul) (Widjaja et al. 2004) and 5.5 ul

millipore water in a tube in 70°C bath for five minutes, then, immediately placed in ice. Into the mixture, 4.0 ul of 2.5 mM dNTP's, 4.0 ul of 5x Reverse Transcriptase buffer (Promega, Madison, WI), 1.0 ul RNaseout (Invitrogen, Carlsbad, CA) and 1.0 ul Reverse Transcriptase AMV (Promega, Madison, WI) were added and immediately incubated in a 42°C water bath for 1 hour. The sample was heat inactivated at 70°C for 10 minutes.

To amplify a gene segment or fragment as in the case of large polymerase genes, 1.0 μl of cDNA was mixed with 1.5 μl each of forward and reverse primers (Invitrogen, Carlsbad, CA) of 100 ng/μl concentration, 1.0 μl 50X dNTP's (Invitrogen), 5.0 μl 10x Taq buffer (Invitrogen) 0.75 μl Taq Polymerase (Invitrogen) and 39.0 μl millipore water in a 200 μl tube. The tube was then placed in ice until the thermal cycler reached a temperature of 90°C. The tube with the sample was then transferred to the PTC-200 Peltier thermal cycler (Bio-Rad, Hercules, CA). The sample was incubated at 94°C for 4 minutes and then subjected to 30 cycles of 94°C for 20 seconds for denaturation, 55°C for 30 seconds for primer annealing and 72°C for 5 minutes for polymerization. After 30 cycles, the sample was then further subjected to 72°C for 10 minutes before putting it to 4°C.

The PCR product was then ran on an agarose gel to determine if the desired gene was amplified. The gel was cut and extracted using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

The sequence of template DNA was determined using synthetic oligonucleotides, 40 ng/ul concentration of either reverse or forward primer and rhodamine or dRhodamine Dye-Terminator Cycle Sequencing Ready Reaction Kits with AmpliTaq DNA polymerase FS (Applied Biosystems, Inc. Foster City, CA). The amplicons were sequenced on an automated Applied Biosystems 373 AI 3100 system using cycle sequencing dye terminator chemistry.

3.8 Real-Time PCR

A two-step method was used in the performance of real-time PCR. The hydrolysis probe used has the following sequence of the matrix gene nucleotide position 64-83: 5'FAM-TCA GGC CCC CTC AAA GCC GA-3'TAMRA with a final concentration of 300 nM. The reporter dye was 6-carboxyfluorescein (FAM), which has its emission spectra quenched due to the spatial proximity of a second fluorescent dye, 6-carboxy-tetramethyl-rhodamine (TAMRA). As for the forward primer, it has the following sequence: 5'AGA TGA GTC TTC TAA CCG AGG TCG 3'. The sequence was based on the M gene nucleotide position 23-46. On the other hand, the reverse primer sequence was based on nucleotide position 123-100: 5'TGC AAA AAC ATC TTC AAG TCT CTG3'. Both primers had final concentrations of 300nM.

Brilliant QPCR Master Mix (Stratagene, La Jolla, CA) protocol for mixture preparation was used. A three-step cycling protocol was followed according to the manufacturer's recommendations. The PTC-200 DNA Engine Thermal Cycler with

Chromo-4 detector (Bio-Rad, Hercules, CA) with the corresponding Opticon Monitor software were used to process and analyze the samples.

3.9 Phylogenetic Analysis

The gene fragment sequences were compiled with the Seqman II program (DNASTAR, Madison, WI), and nucleotide sequences were aligned with close sequences from the Influenza Sequence Database. Primers were designed based on the initial sequencing done on the virus. When full-length sequence of the gene was done, consensus sequences were exported to EditSeq (DNASTAR, Madison, WI). Using MegAlign (DNASTAR, Madison, WI), the sequence of the isolate was entered and best-local-homology rapid search procedure (BLAST) was carried out to look for similar sequences in the database. The sequences chosen from the database were used for multiple alignment with the virus sequenced using the slow-accurate mode of ClustalW. Aligned sequences were exported as NEXUS files. This was done to facilitate opening of the file in MacClade 4 (Sinauer Associates, Inc, Sunderland, MA) and PAUP 4.0b10 Altivec (Sinauer Associates, Inc, Sunderland, MA) for alignment. All phylogenetic relationships of the aligned sequence for each gene segment were generated with the use of a statistical algorithm called maximum parsimony method in conjunction with boostrapping in 100 replicates and stepwise-addition method of full heuristic search (Hall 2001). Trees were saved in either .tre or PICT format and opened with web-downloadable TreeviewX and Adobe Illustrator 10.0 (Adobe, San Jose, CA).

Chapter IV: Results and Discussion

4.1 Viral isolation

There were a total of seven samples positive for AIV out of the 67 samples processed in December 2004. This resulted to a 10.4% isolation rate in the winter. A total of 584 samples were collected in the months of May to August 2005. After testing these samples, none turned out positive. Serum samples could have been helpful in monitoring and surveillance as antibodies against AIV can be detected in much longer time than the duration of detection of the virus itself in birds.

The reported die-offs of ducks in the Chesapeake Bay cannot be attributed to the apparent isolation of AIV alone because partial sequencing of the virus after the detection revealed an H11N3 subtype which is an apathogenic form of avian influenza. The isolation, though, was further investigated through phylogenetic analysis to know if the virus was related to other isolates in the area, especially in the Delmarva region which has more than 2,000 poultry farms. In the early part of 2004, 300,000 birds were destroyed due to an outbreak of avian influenza in the region.

All seven isolates were of H11N3 subtype as revealed by partial sequences of the eight genes of the different isolates. One bird was positive in both tracheal and cloacal swabs, another bird was positive in tracheal swab, three birds were positive in cloacal swabs and one fecal sample was positive for AIV.

4.2 Phylogenetic trees

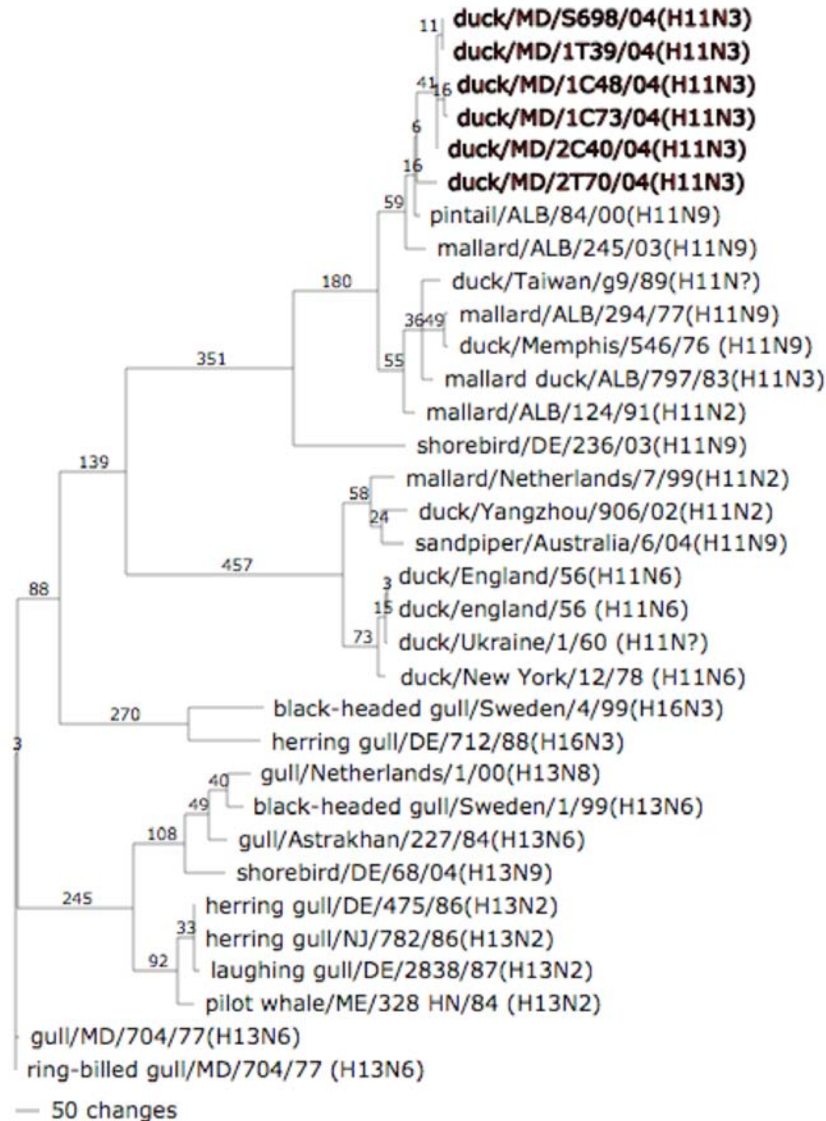

Figure 3. Phylogenetic relationships of Hemagglutinin (HA) genes of A/duck/MD/S698/04 (H11N3), A/duck/MD/1T39/04 (H11N3), A/duck/MD/1C48/04 (H11N3), A/duck/MD/1C73/04 (H11N3), A/duck/MD/2C40/04 (H11N3), A/duck/MD/2T70/04 (H11N3) in bold text and representative avian influenza virus isolates rooted to A/ring-billed gull/MD/704/77. The tree was generated by using maximum parsimony in the PAUP 4.0b10. Bootstrap values from 100 replicates with full heuristic search method are indicated adjacent to the branches. Analysis was based on nucleotides 1-1765 (1765 bp) of the HA gene. Scale bar = 50 nucleotide changes.

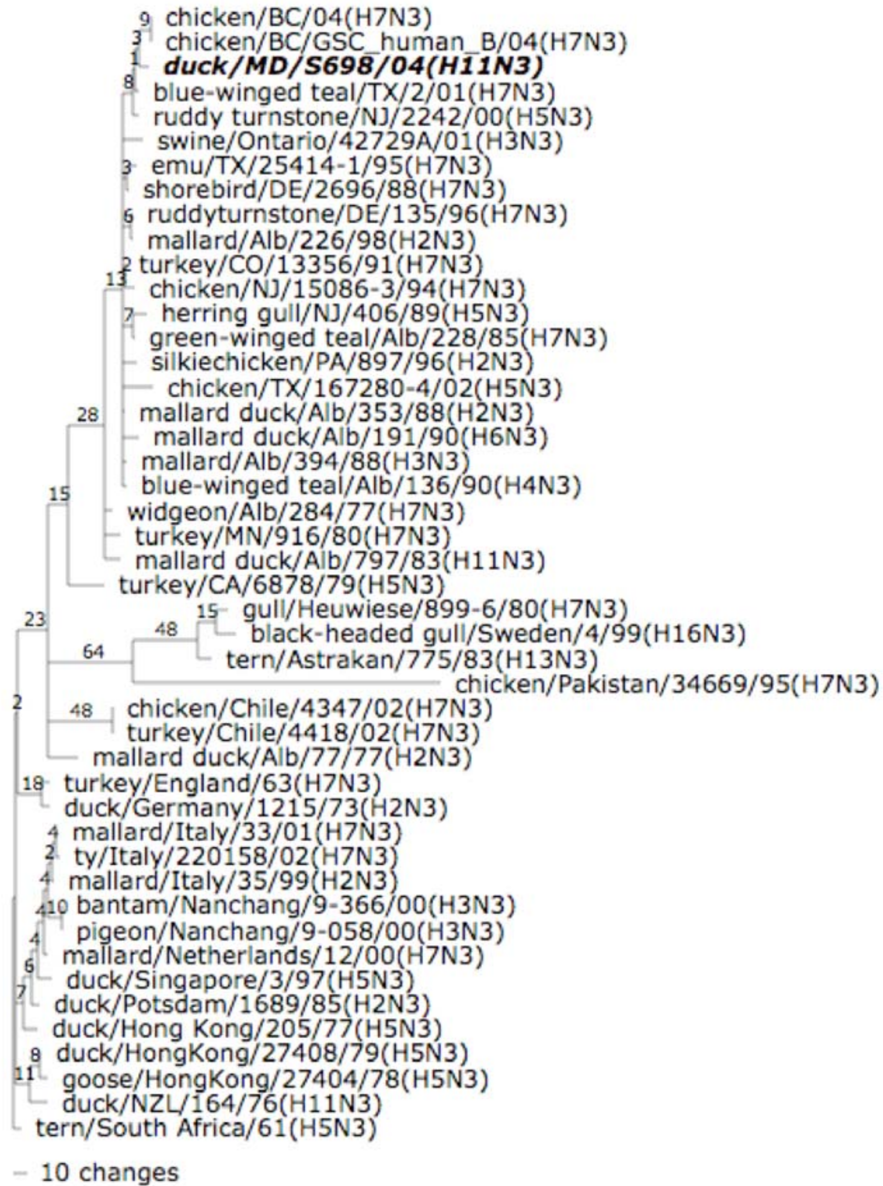

Figure 4. Phylogenetic relationships of Neuraminidase (NA) genes of A/duck/MD/S698/04 (H11N3) in bold text and representative avian influenza virus isolates rooted to A/tern/South Africa/61 (H5N3). The tree was generated by using maximum parsimony in the PAUP 4.0b10. Bootstrap values from 100 replicates with full heuristic search method are indicated adjacent to the branches. Analysis was based on nucleotides 970-1369 (400 bp) of the NA gene. Scale bar = 10 nucleotide changes.

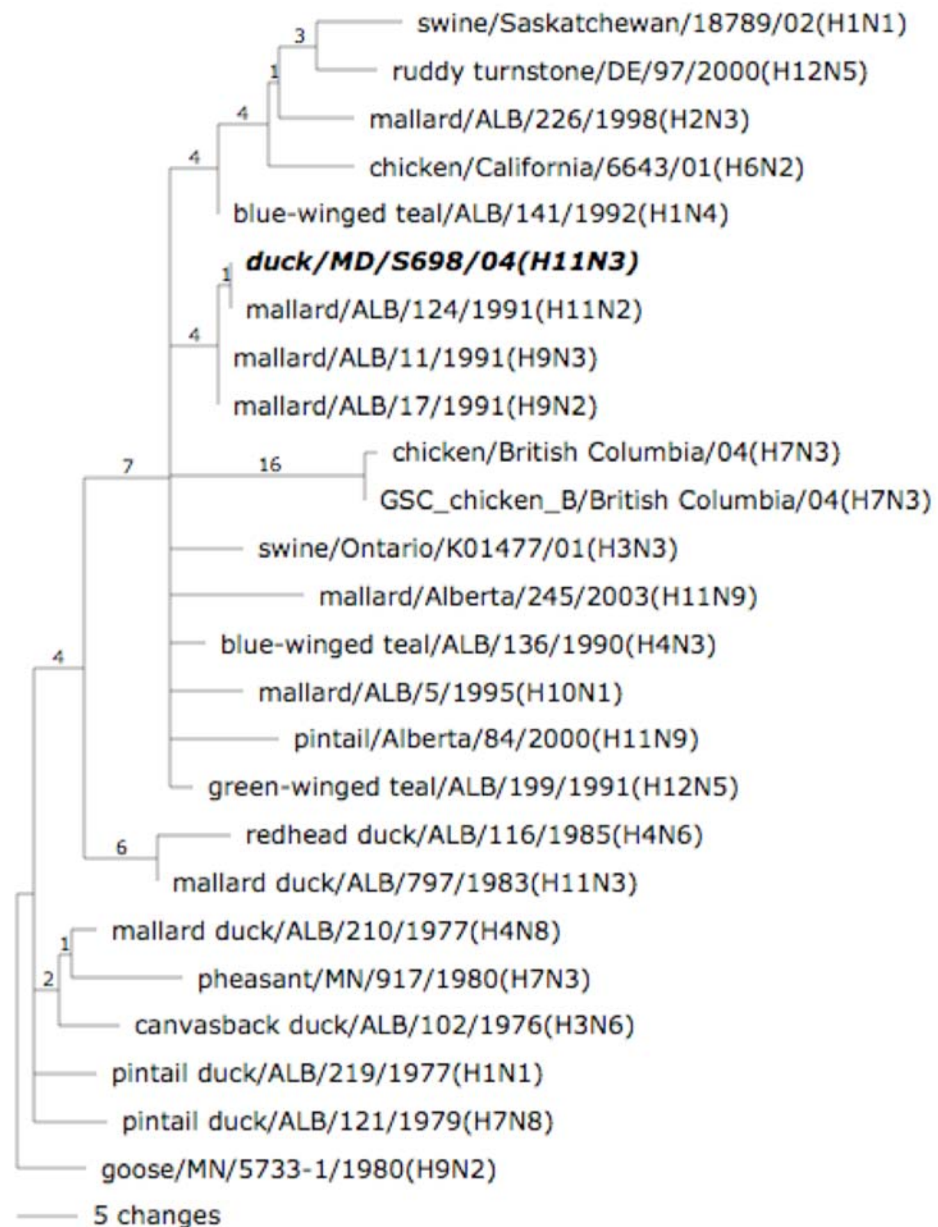

Figure 5. Phylogenetic relationships of polymerase basic protein 2 (PB2) genes of A/duck/MD/S698/04 (H11N3) in bold text and representative avian influenza virus isolates rooted to A/goose/MN/5733-1/80 (H9N2). The tree was generated by using maximum parsimony in the PAUP 4.0b10. Bootstrap values from 100 replicates with full heuristic search method are indicated adjacent to the branches. Analysis was based on nucleotides 1300-1699 (400 bp) of the PB2 gene. Scale bar = 10 nucleotide changes.

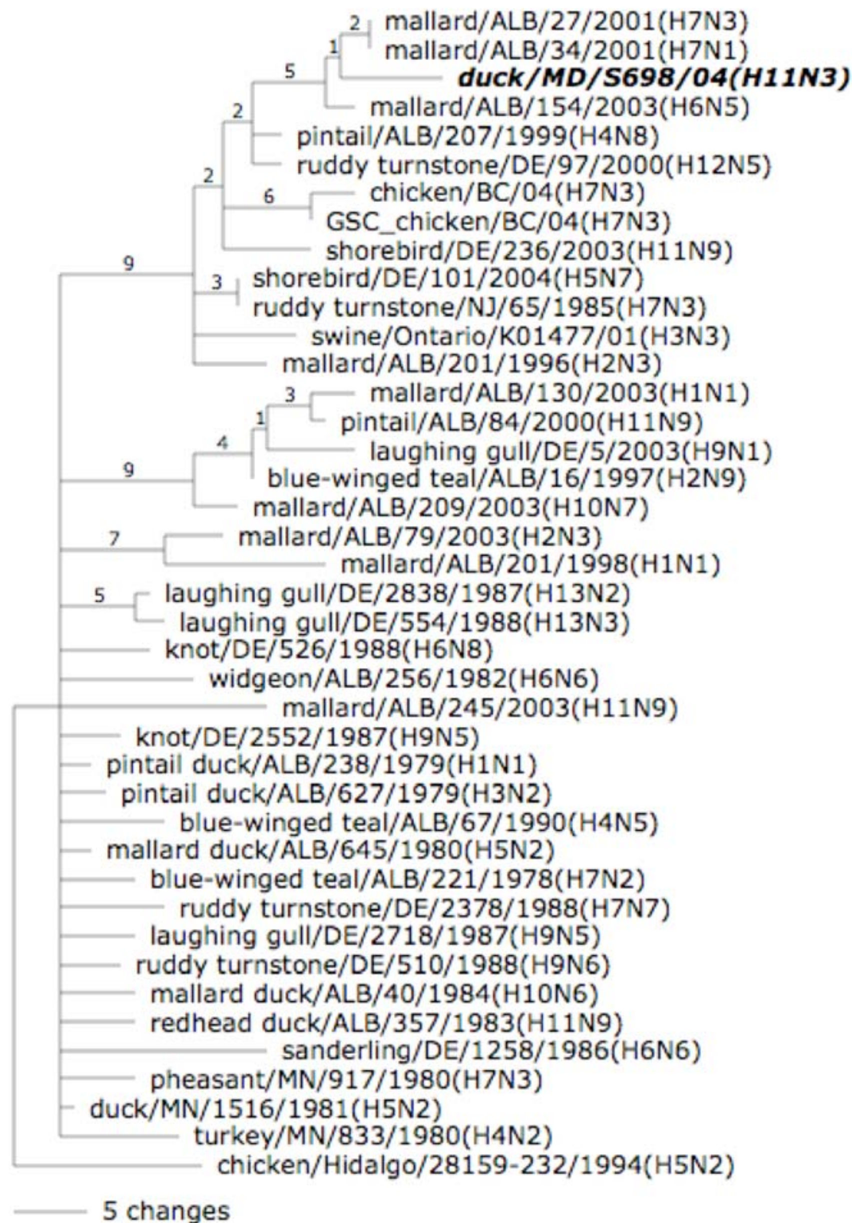

Figure 6. Phylogenetic relationships of polymerase basic protein 1 (PB1) genes of A/duck/MD/S698/04 (H11N3) in bold text and representative avian influenza virus isolates rooted to A/chicken/Hidalgo/28159-232/94 (H5N2). The tree was generated by using maximum parsimony in the PAUP 4.0b10. Bootstrap values from 100 replicates with full heuristic search method are indicated adjacent to the branches. Analysis was based on nucleotides 1740-2139 (400 bp) of the PB1 gene. Scale bar = 5 nucleotide changes.

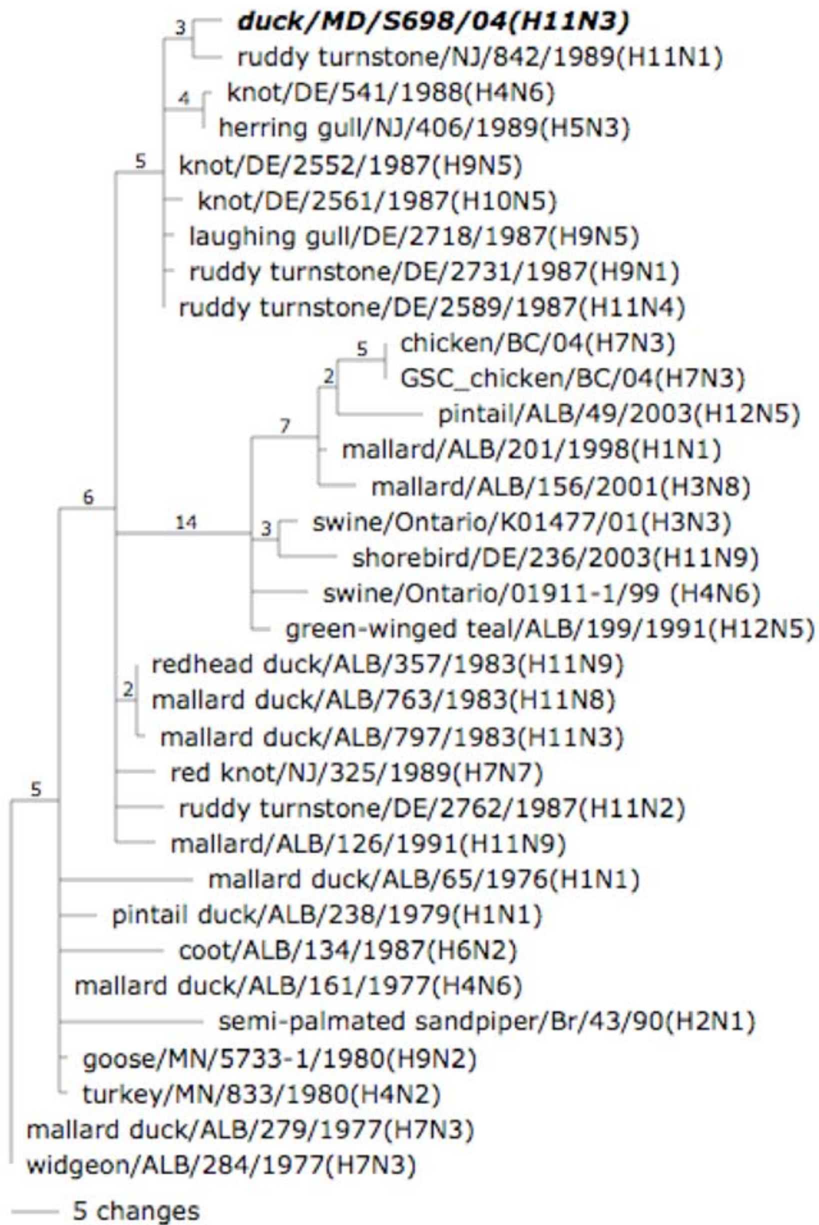

Figure 7. Phylogenetic relationships of polymerase acidic protein (PA) genes of A/duck/MD/S698/04 (H11N3) in bold text and representative avian influenza virus isolates rooted to A/widgeon/Alberta/284/77 (H7N3). The tree was generated by using maximum parsimony in the PAUP 4.0b10. Bootstrap values from 100 replicates with full heuristic search method are indicated adjacent to the branches. Analysis was based on nucleotides 1250-1649 (400 bp) of the PA gene. Scale bar = 5 nucleotide changes.

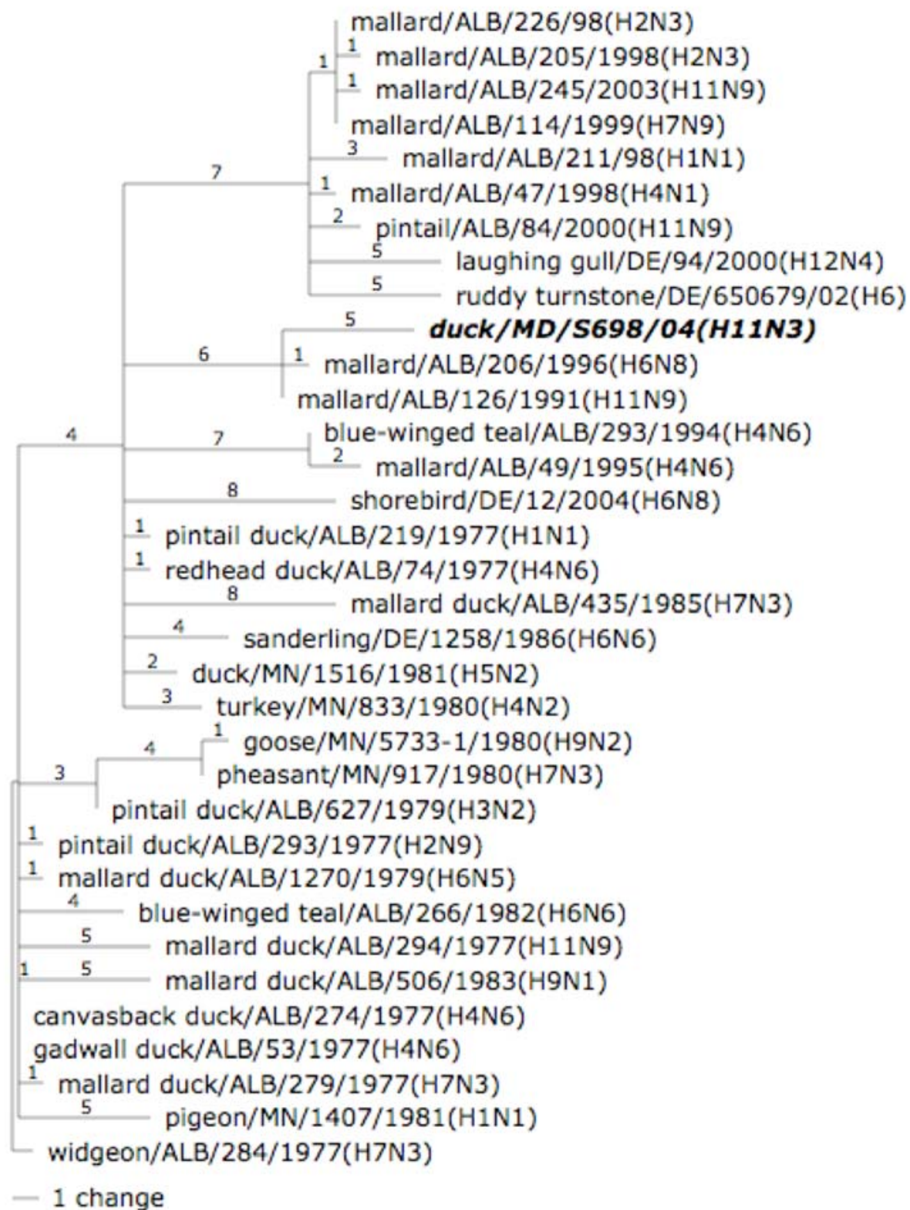

Figure 8. Phylogenetic relationships of Nucleoprotein (NP) genes of A/duck/MD/S698/04 (H11N3) in bold text and representative avian influenza virus isolates rooted to A/widgeon/Alberta/284/77 (H7N3). The tree was generated by using maximum parsimony in the PAUP 4.0b10. Bootstrap values from 100 replicates with full heuristic search method are indicated adjacent to the branches. Analysis was based on nucleotides 137-536 (400 bp) of the NP gene. Scale bar = 1 nucleotide change.

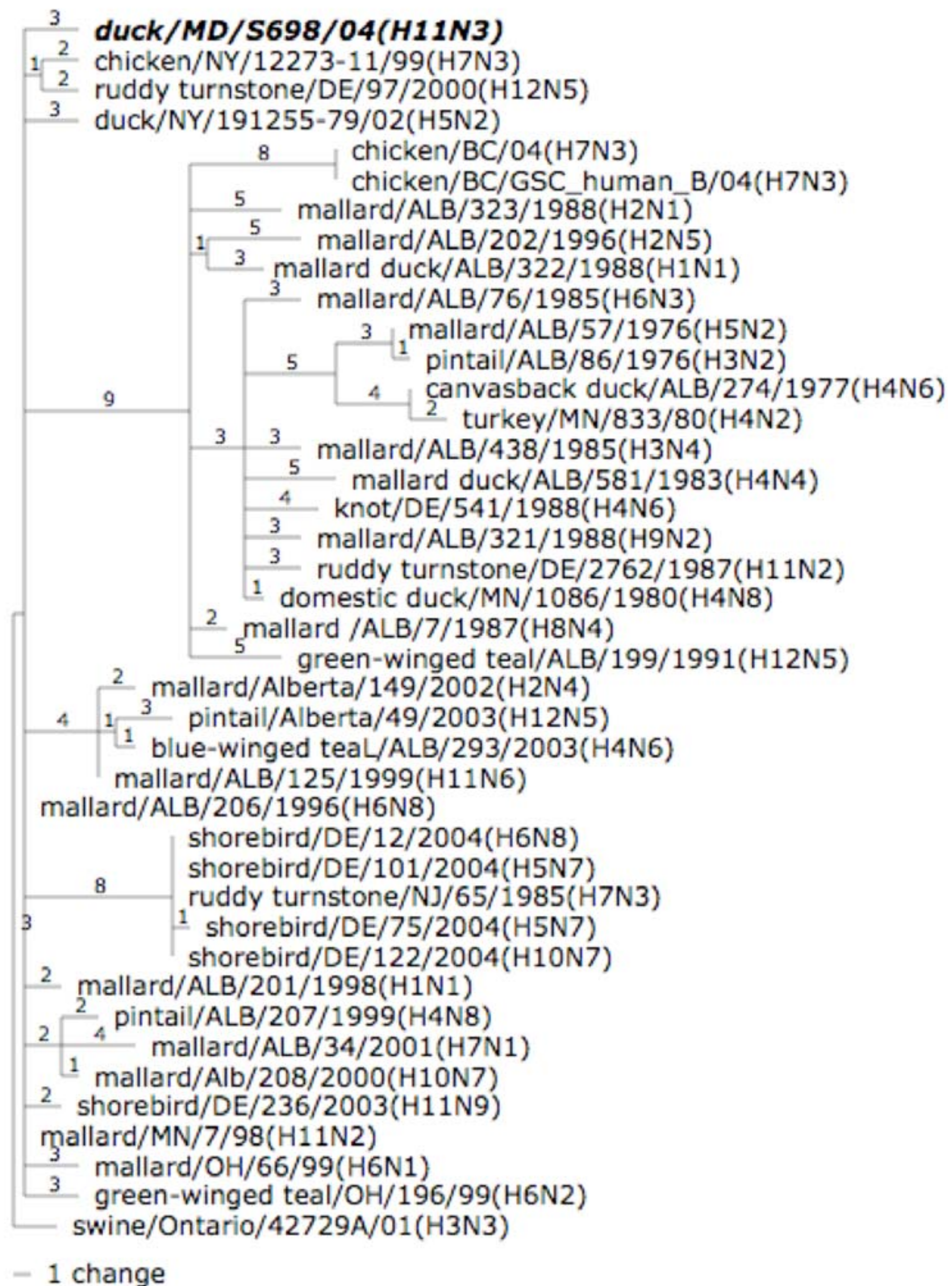

Figure 9. Phylogenetic relationships of Matrix protein (M) genes of A/duck/MD/S698/04 (H11N3) in bold text and representative avian influenza virus isolates rooted to A/swine/Ontario/42729A/01 (H3N3). The tree was generated by using maximum parsimony in the PAUP 4.0b10. Bootstrap values from 100 replicates with full heuristic search method are indicated adjacent to the branches. Analysis was based on nucleotides 315-714 (400 bp) of the M gene. Scale bar = 1 nucleotide change.

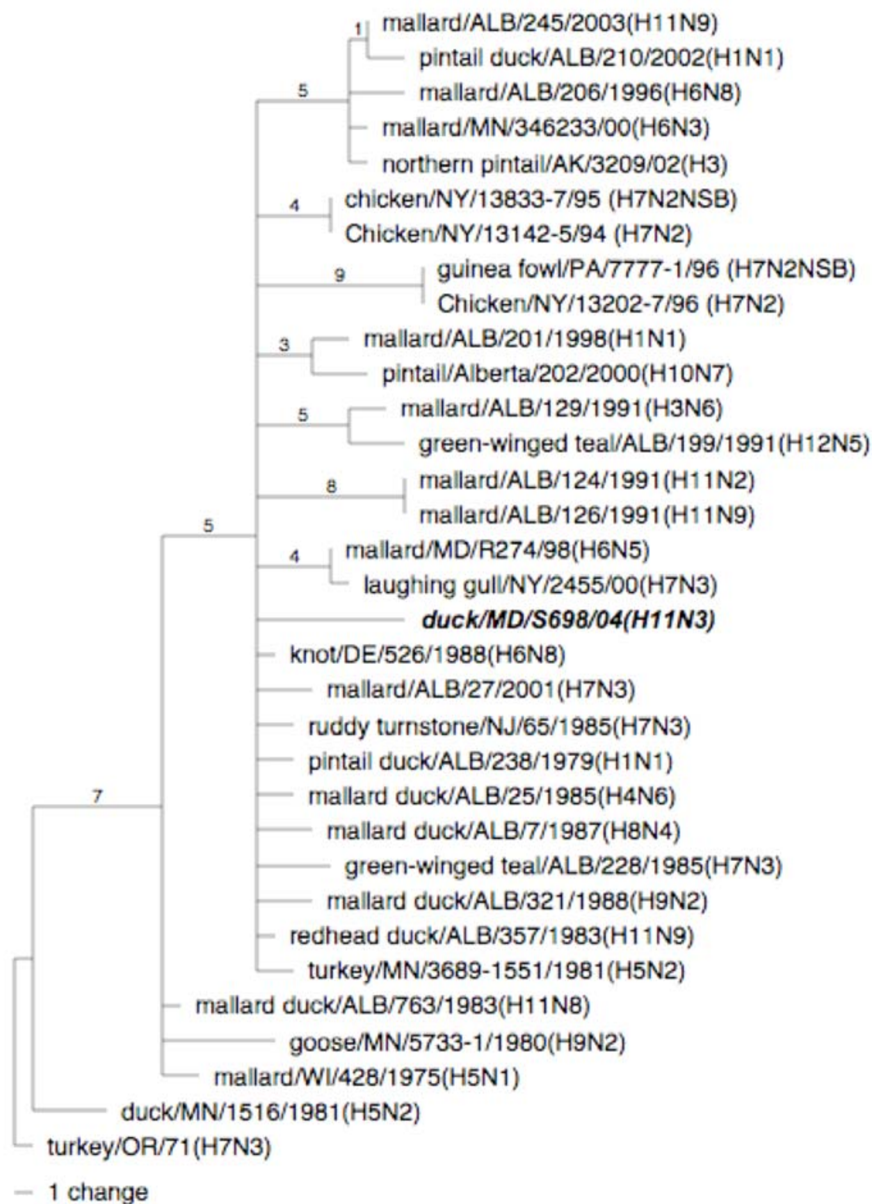

Figure 10. Phylogenetic relationships of Nonstructural protein (NS) genes of A/duck/MD/S698/04 (H11N3) in bold text and representative avian influenza virus isolates rooted to A/swine/Ontario/42729A/01 (H3N3). The tree was generated by using maximum parsimony in the PAUP 4.0b10. Bootstrap values from 100 replicates with full heuristic search method are indicated adjacent to the branches. Analysis was based on nucleotides 410-809 (400 bp) of the M gene. Scale bar = 1 nucleotide change.

4.3 Discussion

Some terms to describe the phylogenetic relationships are explained as follows. It is said that two genes are to be orthologous if they diverged after a speciation event while two genes are to be paralogous if they diverged after a duplication event (Fitch 1970). In other words, two genes belonging to different species are orthologous (ortho = exact) to each other when they diverged from exactly the same root. When a gene is duplicated descending from a root, it is paralogous (para = parallel) to the copy.

The result of the phylogenetic analysis of the Hemagglutinin gene of A/Duck/Maryland/S698/04 (H11N3) is shown in Figure 3. Five other isolates form a cluster of homologous sequences. The tree was rooted to A/ring-billed gull/Maryland/1977 (H13N6) which generated distinct groups of H13, H16 and H11. Throughout the H11 sequences, two distinct clusters were observed, one cluster where the Maryland/04 belonged, is of North American lineage and another cluster is of Eurasian lineage. All the H11 in both clusters were isolated either from ducks, pintail, shorebird and sandpiper. Among the Eurasian lineage was A/duck/England/1956 (H11N6) which is the first H11 subtype isolated. This suggests that H11 has been circulating within the wild bird population and positive selection occurring within the gene has only been for further adaptation in these birds. If the tree was rooted to England/56, this would still form outgroups of H11, H13 and H16. Speciation from an H11 to H13 and H16 would still generate viruses adapted to wild

birds which are not pathogenic to them. An exception is one isolate A/pilot whale/Maine/328 HN/84 (H13N6) which is isolated from stranded pilot whales. The hemagglutinin sequence of the whale isolate is similar to isolates of H13 from gulls. Although H13 is an avian virus, it can enter marine mammal population but cannot be maintained as it caused mortality in the population (Chambers et al. 1989). The similarity index of Maryland/04 to Maine/84 whale isolate is very low at 62.8%, suggestive of a minimal potential of an H11 to speciate to an H13 which could cause outbreaks in pilot whales.

Multiple alignment of the H11 and H13 in the tree revealed that a nine-nucleotide (nt 30-38) deletion resulting in three-amino-acid-deletion from residues 7-9 of the consensus sequence was present in the non-coding regions of H11 isolates. There was an insertion of six nucleotides at positions 479-484 of the consensus which corresponds to insertion of two amino acid residues serine (S) and glycine (G) at positions 154 and 155, respectively, of Maryland/04. Like most other H11s, Maryland/04 posesses the sequence PAIATR⇓G at the cleavage site (indicated by arrow). Sequence combination of multiple basic amino acids (R, K and H) flanking the cleavage site of HA0 (Hemagglutinin precursor) are found in HPAI viruses. However, LPAI viruses contain two basic amino acids at positions -1 and -4 from the cleavage site for H5 and at positions -1 and -3 for the H7. Basic residues at the cleavage site allows HA precursor, HA0 be cleaved by host proteases present in many parts of the body allowing replication of AIV in different tissues. For instance, the recent die-offs of swans in Italy (Terregino et al. 2006) is caused by H5N1 having

basic amino acids in -1,-2,-3,-4 from the cleavage site plus an insertion of four nucleotides flanking the multibasic residues (Steinhauer 1999). AIV does not normally cause mortalities in wild birds but a sequence change in the cleavage site seems to be a determinant of its virulence even in its natural host.

Figure 11. A/duck/MD/S698/04 (H11N3) sequence is compared to sequence in the cleavage site of other HA

Phylogenetically, the Neuraminidase (NA) protein gene of Maryland/04 assorted into paralogous clusters of exclusively of N3 subtype. The tree (Figure 4) is rooted to A/tern/South Africa/61 (H5N3) with a similarity index of 86.8% and minimally diverged into a North American, South American and Eurasian lineages. In the North American lineage, Maryland/04 descended similarly with A/chicken/British Columbia/04 (H7N3) and A/Chicken/British Columbia/GSC human B/04 (H7N3). It is not surprising, however, that the NA sequences share a close common ancestor because if the pathways to the wintering ground of migratory birds are considered, then, there is an enormous possibility that these migratory birds that carried the AIV for the emergence of Maryland/04 to the Chesapeake Bay may have come from Canada.

The NA genes of other AIV among this clade have been isolated not only from wild birds but also from pigs and domestic poultry. Many of the subtypes are of H7N3. In the tree, a distinct South American clade is the A/chicken/Chile/4347/02 (H7N3) was formed. This suggested that viruses from the North America were different from isolates in South America. Maryland/04 is 83.5% similar to this virus. This LPAI Chilean virus was among the first AIV isolated from a broiler breeder flock in Chile which was followed by isolation of the same subtype of AIV a month later but is highly pathogenic. The pathogenicity of the virus was characterized also by basic amino acids in the HA1 cleavage site.

NA is the other surface glycoprotein of AIV that determines the antigenicity of the virus. It functions mainly in the cleavage of sialic acid residues in which HA's are bound. It also promotes penetration of the mucin layer of the respiratory tract to infect the epithelial cells. The function may not be exhibited by the NA of wild birds in particular because they mainly infect the lower digestive tract. But since the Maryland/04 NA is close to NA of AIV that caused morbidities in Chilean chickens then it can be a potential donor of the NA gene in the event of reassortment in suitable or intermediate host. It can even affect its natural host in the event of acquiring an HA with polybasic amino acids.

The topology of the phylogenetic tree of Polymerase Basic protein 2, PB2 gene (Figure 5) completely showed viruses that were of North American lineage isolated from ducks and other aquatic birds, pigs and domestic poultry. It was rooted

to A/goose/MN/5733-1/1980 (H9N2). The Maryland/04 PB2 nucleotide sequence was compared with AIV from chicken and pig. It is most paralogous to viruses isolated from ducks including an H9N2 from Alberta, Canada and orthologous to clusters containing mixture of avian-like and swine-like AIV. Like the NA, the PB2 gene of Maryland/04 has close relationship to A/chicken/British Columbia/04 (H7N3) with a similarity index of 95.5% .

The taxa included in the analysis of Polymerase Basic protein 1, PB1 (Figure 6) are combinations of various HA and NA subtypes. Most are of aquatic bird origin but a distinct sublineage that includes the Maryland/04 is diversified to isolates from chicken and pig. A/chicken/British Columbia/04 (H7N3) and A/swine/Ontario/K01477/01 (H3N3) PB1's close association with Maryland/04 is also evident in analysis of the NA and PB2 genes.

Interestingly, the root used in this phylogeny, A/chicken/Hidalgo 28159-232/1994 (H5N2) which has similarity index of 95.2% to the PB1 sequence of Maryland/04. Other H5N2's in the consensus tree are of aquatic bird in origin isolated in the 1980's. From the data given, it is convincing that Hidalgo/94 may have originally been circulating in wild bird species before its introduction into a flock of chickens causing high morbidity and mortality in Mexico (Swayne 1997).

The sequence of Polymerase Acidic (PA) gene of Maryland/04 is closely related to PA of AIV in shorebirds isolated in the 1980's (Figure 7). They emerged

from a common node that diverged into two more outgroups. One of the outgroups shows that few nucleotide changes in PA and other internal genes apparently increase the host range of relative viruses. This outgroup includes contemporary isolates (1991-2004) from the A/chicken/British Columbia/04 (H7N3) and A/swine/Ontario/01911-1/99 (H4N6). The ancestral relationship between Maryland/04 PA gene and A/chicken/British Columbia/04 (H7N3) has been the same for all the above-mentioned gene segments except for HA. The tree formed only included isolates of North American lineage.

Nucleoprotein (NP) sequences related to the Maryland/04 are conserved in the avian population, in particular with birds from the order Anseriformes. There were few isolates from shorebirds indicating intermixing of bird species. From the topology of the tree (Figure 8), one can infer that most of the isolates are from birds in Alberta, Canada. This supports the earlier statement that Maryland/04 may have originated from a bird migrating from Canada. Another inference that can be made from the tree topology is that the lineage closest to the root A/widgeon/ALB/284/1977 (H7N3) in the 1970's to early 80's. The cluster of AIV that has the most nucleotide changes contains more contemporary isolates. This is a clear example of a virus undergoing positive selection to maintain itself in the population. NP is not exposed to the immune system so the evolution may be the result of nonsynonymous substitutions which are continuously occurring for further host-specific adaptation (Gorman et al. 1991). There were no insertions or deletions in the NP sequence of Maryland/04.

The Matrix (M) gene encodes for overlapping proteins, M1 and M2. M1 has conserved amino acid sequence of 252 while M2 has 97 amino acids. Like the NP gene, the evolution of M gene relies on host-specific adaptation (Widjaja et al. 2004) but the evolutionary steps are very minimal which involve nucleotide substitutions. The taxa included in the trees have more than 90% similarity with each other although some nucleotide changes within the taxa created clusters. Based on the topology (Figure 9) Maryland/04 directly evolved from a swine virus, A/swine/Ontario/427/729A/01 (H3N3) and had duplicated sequences of A/duck/New York/191255-79-/02 (H5N2) and A/chicken/NY 12273-11/99 (H7N3). The majority of the viruses are of duck and shorebird origin which do not create a distinct speciation from A/swine/Ontario/427/729A/01 (H5N2). There is only one distinct lineage observed suggestive of a relatively longer branch length. One of the terminal branches belong to A/chicken/British Columbia/04 (H7N3) which is a frequent member taxon with more than 90% similarity with the polymerase and NA genes.

The nonstructural (NS) protein gene of AIV has the least length of nucleotides which is 890 long. Phylogenetic analysis (Figure 10) revealed synomymous branches of predominantly ducks and shorebirds from Canada. There were four out of 33 taxa that were of terrestrial bird origin.

Maryland/04 may be nonpathogenic to the ducks from which it was isolated because of the genetic character of its hemagglutinin gene but the NA and polymerase

genes have attributes from LPAI and HPAI based on their evolution history. To assess the replication and transmission of the virus, experiments involving inoculation of Maryland/04 were performed. Separate experiments were carried out in two-week-old SPF white leghorn chickens (Charles River Laboratories, Wilmington, MA) and three-week-old quail (UMCP, MD). Summary of the experiments is tabulated (Table 1).

No replication was detected from the three contact birds, therefore, there was no transmission of H11N3 in chickens. The three chickens infected with 1.0 ml of A/duck/MD/S698/04 inoculum with 5 x 10^6 eID_{50}/ml showed no clinical signs. Intratracheal, intranasal, oral and cloacal inoculation were done. There was an apparent replication in the lower intestinal tract of infected birds as suggested by viral shedding detected through HA Assay and real time PCR. All three chickens were positive for cloacal swabs at three day post-inoculation with a high virus titer of $10^{4.6}$ eID_{50}/ml. At five day post-inoculation, the titer of viral shedding of two out of three birds was $10^{3.4}$ eID_{50}/ml. Oropharyngeal and cloacal swabs were collected at day seven and nine. No shedding were observed on these days. Necropsy of the birds was performed 14 days postinoculation. There were no significant findings except for some edematous lesions in the lungs of birds given 500 ul of the viral inoculum intratracheally. One infected bird had pinpoint hemorrhages on the inner surface of the cecal tonsils.

The three quail were infected with 0.5 ml of A/duck/MD/S698/04 inoculum with 5 x 10^6 eID_{50}/ml. Both the infected and contact quail were positive for viral shedding at day 3 and day 5 post-inoculation. The replication in quail respiratory tract generated higher virus titers than chickens. This may contribute to a more efficient transmission of the virus but this needs further study. Serous exudates were observed in the oral cavity of the birds throughout the experiment though no decrease in activity was observed. Fourteen days post-inoculation, the birds were necropsied. Gross lesions in the lungs of both infected and contact quail included edema and ventral pneumonia. Petecchial hemorrhages were found in the upper respiratory tract.

The experiments suggest that Maryland/04, though a duck virus, can replicate in chicken and quail. Important to note is that there were differences in the tissue tropism of the same H11N3 virus as suggested by the results of the experiments. Maryland/04 replicated in the lower intestinal tract of chickens while in quail, it replicated in the respiratory tract. The chicken may be a dead end host because it cannot transmit the virus to other chicken but shedding in the lower intestinal tract suggests replication of the virus. In contrast Maryland/04 can replicate and transmit in quail causing subclinical infection making these birds potential amplifiers of the virus.

Table 2. Replication and transmission of A/duck/MD/S698/04 (H11N3) virus in chicken and quail.

	Virus shedding on day 3 postinoculation				Virus shedding on day 5 postinoculation			
	Oropharyngeal		Cloacal		Oropharyngeal		Cloacal	
	No. of birds shedding/total	Virus titer, log10eID50/ml	No. of birds shedding/total	Virus titer, log10eID50/ml	No. of birds shedding/total	Virus titer, log10eID50/ml	No. of birds shedding/total	Virus titer, log10eID50/ml
Chicken								
Infected	0/3	0	3/3	4.6	0/3	0	2/3	3.4
Contact	0/3	0	0/3	0	0/3	0	0/3	0
Quail								
Infected	3/3	5.2	0/3	0	1/3	2.7	0/3	0
Contact	3/3	4.9	0/3	0	2/3	4.1	0/3	0

Two-week-old specific pathogen free (SPF) White Leghorn chicks were used in the replication and transmission studies of H11N3. Three chicks were infected and three chicks served as sentinel birds.

Three-week-old quail were used in the replication and transmission studies of H11N3. Three quail were infected and three quail remained as sentinel birds.

Chapter V: Summary and Conclusion

5.1 Meeting the objectives

Surveillance of wild and domestic birds have been useful to many investigators to understand the ecology and molecular epidemiology of AIV, to determine the multifaceted nature of its virulence and assess the evolutionary relationships between the existing and emerging strains. The first objective of carrying out a point surveillance of avian influenza in wild and domestic birds were met. This resulted in the isolation of an H11N3 in the Chesapeake Bay and based on the study, it could be an introduction of the virus in the area from migrating birds from Canada. Seven AIVs isolated were identical. The sequence of the representative virus, A/duck/MD/S698/04 (H11N3) was genetically characterized and its evolution was traced through phylogenetic analysis, which was the second objective of the study. The hemagglutinin gene did not contain the polybasic cleavage site that is a characteristic of highly pathogenic avian influenza. MD/04 HA clustered with most isolates from Alberta, Canada associated with LPAI. But the other integral membrane protein, neuraminidase, was closely related to N3's associated with LPAI and HPAI in historical outbreaks in domestic poultry, wild birds and other mammals. The internal viral genes, particularly, the polymerase genes have homologous sequences with an HPAI H7N3. Moreover, the topology of the internal genes of H11N3 is suggestive that it had undergone reassortment. It also insinuated mixing or interactions of waterfowls and shorebirds can actually increase host range with minimal or absence of mutations. A year-round surveillance should be planned in

order to get intensive with tracking of the viruses introduced by migratory birds as well as to investigate the possible evolution of circulating viruses.

Infection of experimental birds with Maryland/04 did not show any significant clinical signs. Lower intestinal tract replication was apparent in chicken while oropharyngeal and lung replication were observed in quail. This virus can be classified as a nonpathogenic to a low pathogenic avian influenza.

5.2 Significance of the study

During the course of the study further knowledge was gained on the role of wild birds in the dissemination of the virus to different species. It proved that surveillance is useful in understanding the ecology of existing and emerging avian influenza viruses. In the future, we look forward to potential isolation of a virus that can be a prototype for vaccine production.

Appendices

Appendix A. Two-Step Real-Time PCR Protocol

1. Perform the experiments aseptically.

2. Prepare the experimental reaction in amber-colored tube by adding the following components in order:

Reagent Mixture

6.5 ul	RNase-free water
12.5 ul	2X Brilliant QPCR Master Mix (Stratagene Cat. No. 600549)
1.5 ul	300 nM probe M 64-83F
1.5 ul	300 nM forward primer M 23-46F
1.5 ul	300 nM reverse primer M123-100R

3. Gently mix the reactions by pipetting up and down without creating bubbles. 23.5 ul reagent mixture is placed into each well of 96-well white hard-shell PCR plate.

4. Add 1.5 ul cDNA, gDNA or plasmid DNA

5. Cover the plate and it is ready to load into the DNA Engine Thermal Cycler PTC-200 Peltier Cycler with Chromo4 Detector.

6. Run the PCR program below:

Three-step cycling protocol

Cycles	Duration of Cycle	Temperature
1	10 minutes	95°C
40	30 seconds	95°C
	1.0 minute	55-60°C
	30 seconds	72°C

*Place the plate into the instrument when the temperature of the block reaches 50-60°C.

Appendix B. 3.7% Brain Heart Infusion Broth

Materials:

Double-distilled water
Brain Heart Infusion (BHI) broth powder (BBL Cat no. 211060)
Gentamicin
Penicillin-Streptomycin
Amphotercin B
Two 1 liter volumetric flasks
Ten sterile 125 ml glass bottles
Weigh boats, spatula, stir bars, foil

Procedures:

1. Place 1000 ml ddH20 in the 1L flask.
2. Add 37 g of BHI powder and dissolve by stirring over low heat.
3. Put half of the solution into another 1L flask. Cover the two flasks with aluminum foil. Autoclave.
4. After the two flasks cool down, add 2.5 ml Gentamicin and 5 ml Pen-Strep-AmpB into each flask. This should be done under the hood.
5. Distribute the BHI broth into10 glass botltes. Label each bottle, put paraffin tape around the cap and bottle and store in the freezer until needed. The solution should be stable for 5-6 months in the freezer.
6. The broth can also be poured into glass vials. Under the hood, pipette 1.0 ml of BHIB into each vial and freeze until needed for swab collection.

Appendix C. Pair distances of HA genes using ClustalW slow-accurate method

Percent Identity (upper triangle) / Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32		
1		99.4	99.3	99.2	99.8	99.2	92.2	62.2	61.6	61.7	83.1	62.3	92.1	95.2	62.6	62.3	61.4	62.0	93.7	95.5	96.5	91.3	80.7	80.8	79.9	80.1	93.2	79.4	80.2	61.8	61.6	62.8	1	duck/MD/S698/04(H11N3)
2	0.6		99.4	98.8	99.3	98.8	91.9	62.1	61.4	61.5	82.5	62.2	92.0	95.1	62.6	62.2	61.1	61.9	93.5	95.4	96.4	91.1	80.4	80.4	79.5	78.4	92.1	77.7	78.5	61.7	61.6	62.6	2	duck/MD/1C48/04(H11N3)
3	0.7	0.6		98.7	99.2	98.7	91.8	62.0	61.3	61.5	82.6	62.1	92.1	95.2	62.4	62.1	61.1	61.8	93.6	95.5	96.5	91.1	80.4	80.4	79.6	78.7	91.5	78.5	79.1	61.6	61.4	62.6	3	duck/MD/1C73/04(H11N3)
4	0.8	1.3	1.3		99.1	99.8	92.2	62.0	61.4	61.7	82.4	62.2	92.7	95.8	62.5	62.2	61.2	61.9	94.2	96.1	97.2	91.7	80.3	80.3	79.6	80.1	93.2	79.4	80.2	61.5	61.7	62.7	4	duck/MD/2C40/04(H11N3)
5	0.0	0.6	0.7	0.7		99.0	92.2	62.1	61.4	61.6	82.9	62.2	92.0	95.1	62.5	62.2	61.2	61.9	93.6	95.4	96.4	91.1	80.5	80.7	79.7	80.1	93.2	79.4	80.2	61.7	61.5	62.6	5	duck/MD/1T39/04(H11N3)
6	0.8	1.1	1.3	0.1	0.7		92.4	62.1	61.5	61.8	82.3	62.3	92.8	96.0	62.6	62.3	61.2	62.0	94.4	96.2	97.4	91.9	80.3	80.3	79.6	80.4	93.8	79.4	80.2	61.6	61.7	62.8	6	duck/MD/2T70/04(H11N3)
7	8.5	8.8	8.9	8.5	8.5	8.4		58.6	57.9	57.2	80.4	58.7	93.5	92.5	57.8	58.8	57.3	58.5	94.9	94.4	93.2	89.4	77.8	78.0	76.7	82.1	92.9	79.7	80.2	58.0	57.2	58.5	7	duck/Taiwan/g9/89(H11N?)
8	51.8	51.9	52.3	52.3	51.9	52.2	58.8		95.4	72.4	61.9	83.6	61.8	62.1	89.0	83.6	73.3	83.2	61.7	61.7	61.7	62.1	60.9	60.8	62.2	62.5	59.0	56.8	58.8	93.0	78.2	83.8	8	gull/Netherlands/1/00(H13N8)
9	53.0	53.2	53.6	53.3	53.0	53.2	60.4	4.8		72.4	61.4	83.3	61.0	61.4	89.3	83.2	73.5	82.8	61.1	61.1	61.1	61.5	60.0	59.9	61.4	62.5	59.0	57.6	59.6	93.7	77.8	83.3	9	black-headed gull/Sweden/1/99(H13N6)
10	52.9	53.5	53.4	53.0	53.1	52.9	62.9	34.0	34.0		62.9	73.6	61.2	61.5	72.2	73.5	82.2	73.4	61.5	61.1	61.3	61.0	60.5	61.1	61.1	62.5	62.4	59.9	60.2	73.1	71.9	73.2	10	black-headed gull/Sweden/4/99(H16N3)
11	19.6	20.4	20.3	20.6	19.7	20.7	23.4	52.6	53.3	50.6		62.0	81.8	81.3	61.7	62.0	61.6	61.6	80.9	81.3	81.5	80.0	90.9	91.6	87.5	88.7	81.1	88.1	88.4	62.3	61.4	62.3	11	mallard/Netherlands/7/99(H11N2)
12	51.2	51.4	51.5	51.4	51.2	51.3	58.2	18.9	19.3	32.0	52.2		61.8	62.2	83.0	99.8	73.5	99.0	61.7	62.0	62.4	61.6	60.8	60.3	60.5	64.1	59.9	59.3	61.9	84.7	80.4	96.0	12	herring gull/DE/475/86(H13N2)
13	8.6	8.6	8.5	7.9	8.5	7.8	7.0	52.8	54.3	54.1	21.4	52.4		93.0	62.2	61.9	60.9	61.6	95.4	94.5	94.1	90.7	79.6	79.9	79.6	80.7	95.8	80.8	80.8	61.2	61.3	62.1	13	mallard/ALB/294/77(H11N9)
14	5.0	5.1	5.0	4.4	4.9	4.3	8.2	52.1	53.4	53.7	22.2	51.6	7.6		62.4	62.2	61.1	61.9	94.5	97.1	97.9	92.3	79.3	79.3	79.4	81.4	92.4	80.2	79.9	61.4	61.9	62.6	14	mallard/ALB/245/03(H11N9)
15	50.7	50.8	51.1	51.0	50.7	50.9	60.7	12.3	11.8	34.3	52.9	19.6	51.8	51.3		82.9	72.8	82.6	61.9	62.1	62.0	62.1	60.1	60.5	60.6	62.1	60.5	57.9	59.6	90.4	78.6	82.6	15	shorebird/DE/68/04(H13N9)
16	51.2	51.4	51.5	51.4	51.2	51.3	58.0	18.9	19.4	32.1	52.1	0.2	52.2	51.6	19.8		73.5	99.2	61.9	62.0	62.4	61.7	60.8	60.3	60.5	64.1	59.9	59.3	61.9	84.7	80.5	96.1	16	herring gull/NJ/782/86(H13N2)
17	53.1	53.5	53.6	53.4	53.1	53.4	61.5	32.7	32.4	20.7	52.6	32.2	54.2	53.7	33.5	32.2		73.8	60.9	60.9	61.2	60.7	59.3	59.9	60.5	63.1	61.9	62.1	60.5	73.1	72.5	73.1	17	herring gull/DE/712/88(H16N3)
18	52.0	52.2	52.3	52.2	52.0	52.1	58.7	19.4	20.0	32.3	52.9	0.9	52.9	52.3	20.3	0.8	31.7		61.5	61.6	62.0	61.4	60.6	60.0	60.5	63.8	60.2	59.0	61.6	84.4	79.9	95.4	18	laughing gull/DE/2838/87(H13N2)
19	6.7	6.9	6.8	6.1	6.7	6.0	5.4	53.1	54.2	53.7	22.7	52.6	4.9	5.9	52.6	52.3	54.3	53.1		96.2	95.5	91.6	79.3	79.9	79.2	81.4	96.6	80.8	80.2	61.0	61.7	62.1	19	mallard duck/ALB/797/83(H11N3)
20	4.7	4.8	4.7	4.1	4.6	3.9	6.0	52.9	54.1	54.5	22.2	52.0	5.9	3.0	51.9	52.0	54.3	52.8	4.0		98.0	93.7	79.7	79.9	79.7	82.7	92.9	81.4	81.6	60.9	61.8	62.3	20	mallard/ALB/124/91(H11N2)
21	3.6	3.7	3.6	2.9	3.5	2.7	7.4	53.1	54.3	54.1	21.8	51.2	6.4	2.1	52.2	51.2	53.4	52.0	4.8	2.0		93.0	79.7	79.9	79.6	81.7	93.8	81.1	81.4	61.0	61.7	62.8	21	pintail/ALB/84/00(H11N9)
22	9.5	9.6	9.7	9.0	9.5	8.9	11.9	52.2	53.3	54.8	24.0	53.0	10.3	8.4	52.0	52.7	54.7	53.5	9.1	6.7	7.5		78.6	78.9	78.4	79.7	89.8	78.8	79.7	61.6	61.5	62.6	22	shorebird/DE/236/03(H11N9)
23	20.9	21.3	21.3	21.5	20.9	21.5	24.1	52.4	54.5	54.6	8.0	52.5	22.6	23.1	54.1	52.5	56.2	53.0	23.1	22.5	22.4	24.1		93.9	90.0	79.4	73.2	76.8	80.2	60.5	59.7	60.4	23	duck/Yangzhou/906/02(H11N2)
24	21.1	21.8	21.7	21.9	21.2	21.9	24.5	53.1	54.9	53.1	7.6	54.0	22.6	23.5	53.4	54.0	55.1	54.7	22.6	22.6	22.6	24.1	6.3		89.8	82.4	75.7	81.9	83.6	60.5	59.9	59.9	24	sandpiper/Australia/6/04(H11N9)
25	22.1	22.6	22.4	22.4	22.2	22.6	25.8	49.4	51.0	53.0	12.2	53.2	22.5	22.9	52.9	53.2	53.3	53.2	23.1	22.4	22.5	24.3	11.5	11.2		88.0	73.7	81.4	87.6	61.6	58.4	60.7	25	duck/England/56(H11N6)
26	25.0	27.4	27.0	25.0	25.0	25.0	22.5	46.3	46.4	50.0	13.6	44.5	24.5	23.5	47.2	44.5	46.1	45.2	23.5	21.5	22.9	25.9	13.4	11.6	1.6		81.7	91.0	97.3	62.8	60.5	62.8	26	duck/England/56 (H11N6)
27	6.7	8.0	8.7	6.7	6.7	6.4	7.3	54.2	54.3	49.6	23.0	53.6	4.0	8.0	50.9	53.6	48.7	52.9	3.1	7.3	6.3	11.2	24.1	22.2	23.2	23.0		80.5	80.5	60.7	59.0	59.0	27	duck/Memphis/546/76 (H11N9)
28	25.1	27.6	26.3	25.1	25.1	25.6	25.2	59.2	57.1	55.1	13.4	54.6	23.3	24.2	56.7	54.6	48.0	55.3	23.4	22.5	22.9	26.3	18.6	13.1	11.7	10.5	23.1		89.5	59.3	60.2	57.9	28	duck/New York/12/78 (H11N6)
29	23.9	26.4	25.6	23.9	23.9	24.4	24.4	54.4	52.5	54.6	13.1	49.0	23.5	24.8	52.6	49.0	51.6	49.6	24.3	22.2	22.6	25.1	13.4	10.7	3.4	2.9	23.3	12.3		61.0	56.2	60.7	29	duck/Ukraine/1/60 (H11N?)
30	52.6	52.7	53.1	53.2	52.7	53.1	60.5	7.5	6.7	33.0	51.7	17.4	54.0	53.6	10.5	17.4	33.2	17.9	54.6	54.8	54.7	53.1	53.3	53.5	50.8	46.4	51.1	53.9	50.1		78.3	85.1	30	gull/Astrakhan/227/84(H13N6)
31	52.4	52.5	52.9	52.3	52.5	52.3	61.7	26.5	27.1	34.8	53.2	23.6	53.2	51.9	25.8	23.3	34.0	24.2	52.4	52.1	52.3	52.8	54.7	54.5	57.5	51.3	54.8	51.8	61.3	26.5		80.9	31	ring-billed gull/MD/704/77 (H13N6)
32	50.1	50.2	50.4	50.2	50.1	50.0	58.6	18.7	19.3	32.4	51.3	4.1	51.5	50.5	20.2	4.0	32.9	4.7	51.6	51.1	50.1	50.5	53.2	54.7	52.5	46.5	54.8	57.2	50.7	17.0	22.8		32	pilot whale/ME/328 HN/84 (H13N2)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32		

Appendix D. Pair distances of NA genes using ClustalW slow-accurate method

Percent Identity (upper triangle); Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29		
1		85.5	92.0	85.3	93.0	95.0	94.3	93.0	86.8	90.1	95.7	86.7	89.1	87.4	97.3	83.5	83.4	85.4	95.6	98.8	88.7	96.1	96.2	94.2	95.6	95.8	94.0	94.4	91.9	1	duck/MD/S698/04(H11N3)
2	15.2		86.9	93.7	85.8	86.2	86.4	86.2	91.5	86.7	86.2	81.2	82.3	81.4	86.6	83.8	83.7	96.5	86.2	84.8	84.8	85.5	84.8	86.1	85.3	85.1	85.9	86.0	85.7	2	bantam/Nanchang/9-366/00(H3N3)
3	7.4	15.1		86.2	94.8	95.0	96.2	93.8	87.6	95.5	93.9	87.4	90.7	88.5	94.0	86.4	86.4	87.2	94.1	91.8	90.7	95.0	93.8	94.9	95.0	94.3	94.3	95.7	95.7	3	widgeon/Alb/284/77(H7N3)
4	15.5	6.8	16.1		85.7	86.1	86.0	85.1	91.8	86.0	85.8	80.3	81.9	81.2	86.2	83.2	83.0	94.5	86.2	84.4	84.9	85.0	84.8	84.7	84.8	84.7	84.9	85.0	85.0	4	duck/Singapore/3/97(H5N3)
5	6.2	16.5	5.6	16.6		95.9	97.8	95.0	87.3	92.4	95.0	87.7	92.2	90.0	95.0	84.8	84.8	86.4	95.1	92.8	88.7	96.4	94.7	94.1	96.0	95.7	93.9	94.8	92.4	5	emu/TX/25414-1/95(H7N3)
6	3.9	16.1	5.3	16.1	4.3		97.4	95.7	87.6	92.8	96.9	89.2	92.3	90.4	96.9	85.3	85.3	87.0	96.8	94.7	88.5	96.8	97.6	94.3	96.3	96.4	94.3	95.0	92.7	6	ruddyturnstone/DE/135/96(H7N3)
7	4.7	15.7	4.0	16.2	2.2	2.7		96.5	87.5	93.3	96.5	89.0	93.4	91.1	96.4	84.9	84.8	86.8	96.2	94.0	89.0	98.0	96.0	95.7	97.8	97.3	95.1	96.2	93.6	7	shorebird/DE/2696/88(H7N3)
8	6.1	16.0	6.7	17.4	5.3	4.5	3.7		86.8	91.4	94.7	88.0	91.6	89.7	95.0	83.9	83.9	86.1	94.8	92.7	87.3	95.9	94.5	93.4	95.1	96.3	93.3	93.8	91.1	8	silkiechicken/PA/897/96(H2N3)
9	13.6	9.4	14.3	9.1	14.6	14.3	14.3	15.2		87.2	87.6	80.9	82.7	81.8	88.1	84.4	84.3	91.7	87.6	86.2	87.1	86.4	86.3	85.9	86.5	86.0	86.2	86.1	86.2	9	tern/South Africa/61(H5N3)
10	9.3	15.1	4.5	16.0	8.1	7.6	7.1	9.3	14.5		91.9	85.3	88.4	86.9	91.8	86.3	86.2	87.0	91.9	89.8	90.5	92.2	91.4	92.1	91.9	91.7	92.0	92.6	93.3	10	turkey/CA/6878/79(H5N3)
11	3.1	16.0	6.6	16.5	5.3	3.2	3.7	5.7	14.1	8.7		87.8	91.3	89.4	97.8	84.6	84.5	86.7	97.5	95.4	87.8	95.6	95.6	93.4	95.1	95.0	93.3	93.8	91.0	11	ruddy turnstone/NJ/2242/00(H5N3)
12	6.8	15.6	7.6	16.9	7.1	5.3	5.6	6.8	16.1	10.0	7.0		88.8	88.0	88.3	79.5	79.3	81.5	87.6	86.2	81.6	88.3	87.7	86.5	87.6	87.9	86.2	86.8	85.3	12	chicken/TX/167280-4/02(H5N3)
13	4.7	15.0	4.3	15.4	2.6	2.5	1.3	3.2	14.4	7.3	3.7	5.3		96.3	91.1	80.8	80.7	82.7	91.0	88.9	84.1	92.7	91.0	90.3	92.1	92.2	89.6	90.6	87.9	13	turkey/CO/13356/91(H7N3)
14	7.2	16.6	7.3	16.9	5.6	5.1	4.3	5.9	16.2	9.6	6.2	6.7	3.6		89.6	80.5	80.4	81.7	89.0	87.5	83.1	90.4	89.1	88.0	89.8	90.0	87.6	88.5	86.2	14	chicken/NJ/15086-3/94(H7N3)
15	1.5	15.5	6.5	16.0	5.3	3.2	3.8	5.4	13.5	8.9	2.3	6.5	3.8	6.1		84.8	84.8	86.8	97.5	96.8	88.0	95.6	95.6	93.6	95.1	95.2	93.3	93.9	91.1	15	blue-winged teal/TX/2/01(H7N3)
16	18.1	19.2	15.7	20.0	17.9	17.2	17.8	19.1	18.4	15.6	18.3	18.1	17.1	18.0	17.9		99.8	83.3	85.2	83.6	86.2	84.3	84.2	83.9	84.2	83.8	84.0	84.1	84.0	16	chicken/Chile/4347/02(H7N3)
17	18.1	19.3	15.8	20.3	18.0	17.3	17.9	19.2	18.5	15.7	18.4	18.3	17.2	18.1	18.0	0.2		83.3	85.1	83.5	86.1	84.2	84.1	83.8	84.1	83.7	83.9	84.0	83.9	17	turkey/Chile/4418/02(H7N3)
18	15.3	3.7	14.7	5.9	15.7	15.0	15.2	16.0	9.1	14.6	15.3	15.1	14.3	16.1	15.2	19.9	20.0		86.6	84.9	85.3	85.9	85.6	86.1	85.6	85.7	86.2	86.2	85.9	18	mallard/Italy/35/99(H2N3)
19	3.3	16.0	6.3	16.0	5.2	3.4	4.0	5.6	14.1	8.7	2.6	7.3	4.0	6.7	2.6	17.4	17.5	15.4		95.4	88.1	95.3	95.5	93.4	95.0	94.8	93.3	93.5	91.3	19	swine/Ontario/42729A/01(H3N3)
20	1.2	16.1	7.6	16.7	6.4	4.3	5.1	6.5	14.3	9.7	3.5	7.4	5.0	7.1	2.0	17.8	17.9	16.0	3.5		88.8	95.7	96.0	93.8	95.3	95.5	93.7	94.1	91.6	20	chicken/BC/GSC_human_B/04(H7N3)
21	12.6	16.2	8.8	16.1	11.2	11.5	10.9	13.0	13.2	8.8	12.3	13.5	10.9	12.7	12.2	14.4	14.5	15.6	12.0	12.5		90.7	89.7	90.1	90.5	90.3	90.5	90.9	90.9	21	mallard duck/Alb/77/77(H2N3)
22	4.1	15.3	3.9	15.9	2.5	2.0	0.8	3.0	14.2	6.8	3.3	5.0	0.6	3.6	3.3	16.9	17.0	14.7	3.6	4.4	10.3		97.9	97.1	99.1	99.2	96.6	97.5	94.8	22	mallard duck/Alb/353/88(H2N3)
23	3.9	16.1	5.3	16.1	4.3	1.2	2.8	4.5	14.2	7.8	3.3	5.6	2.5	5.1	3.3	17.1	17.2	15.1	3.4	4.1	11.4	2.1		95.5	97.5	97.5	95.3	96.2	93.8	23	mallard/Alb/226/98(H2N3)
24	6.2	14.5	4.0	16.3	4.9	4.8	3.1	5.8	14.8	7.0	5.8	7.1	3.3	6.5	5.5	17.5	17.6	14.4	5.8	6.6	10.9	3.0	4.8		96.9	96.6	95.9	97.3	94.7	24	mallard duck/Alb/191/90(H6N3)
25	4.6	15.5	4.0	16.2	2.8	2.5	1.0	3.8	14.0	7.2	3.8	5.7	1.3	4.3	3.8	17.1	17.2	15.1	4.0	4.9	10.5	0.9	2.5	3.2		98.4	96.2	97.3	94.6	25	mallard/Alb/394/88(H3N3)
26	4.4	15.8	4.7	16.3	3.1	2.5	1.5	2.5	14.6	7.5	3.9	5.4	1.2	4.1	3.7	17.7	17.7	15.0	4.2	4.7	10.7	0.8	2.5	3.6	1.6		95.9	96.8	94.2	26	blue-winged teal/Alb/136/90(H4N3)
27	6.4	14.8	4.7	16.0	5.2	4.7	3.8	5.8	14.4	7.1	5.8	7.5	4.1	6.9	5.8	17.4	17.4	14.3	5.8	6.7	10.4	3.6	4.9	4.3	3.9	4.3		97.7	94.2	27	herring gull/NJ/406/89(H5N3)
28	5.9	14.6	3.2	15.9	4.1	4.0	2.7	5.3	14.5	6.4	5.3	6.7	3.0	5.9	5.1	17.2	17.3	14.3	5.6	6.3	10.0	2.5	4.0	2.8	2.8	3.3	2.4		95.2	28	green-winged teal/Alb/228/85(H7N3)
29	8.8	15.0	3.2	16.0	6.9	6.5	5.5	8.3	14.3	5.6	8.5	8.6	6.1	8.7	8.4	17.4	17.4	14.8	8.1	9.2	9.9	5.4	6.6	5.6	5.7	6.1	6.2	5.0		29	mallard duck/Alb/797/83(H11N3)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29		

Appendix E. Pair distances of PB2 genes using ClustalW slow-accurate method

Percent Identity (upper triangle); Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25		
1		95.1	95.5	93.8	94.9	95.6	96.4	96.0	96.2	97.9	95.3	95.8	95.7	97.2	95.8	98.1	98.1	97.6	95.9	98.0	96.3	97.1	96.2	96.4	94.5	1	duck/MD/S698/04(H11N3)
2	4.5		93.3	94.5	94.1	93.5	95.9	93.6	94.1	96.7	93.5	93.7	93.6	94.7	93.8	95.8	95.8	95.3	93.8	95.6	94.3	95.0	95.2	94.4	92.5	2	chicken/California/6643/01(H6N2)
3	4.7	6.5		92.3	94.8	99.8	94.6	94.6	95.3	96.0	94.5	94.7	94.8	95.8	94.7	96.5	96.5	96.1	95.1	96.4	96.2	97.1	94.1	95.2	93.6	3	chicken/British Columbia/04(H7N3)
4	5.3	5.3	7.1		94.0	92.4	94.9	92.6	92.9	95.2	92.3	92.4	92.6	93.7	92.8	94.4	94.4	94.1	92.7	94.2	93.2	94.1	95.4	93.1	91.4	4	swine/Saskatchewan/18789/02(H1N1)
5	4.1	5.7	4.2	6.4		95.0	94.6	94.1	95.1	95.7	93.9	94.4	94.4	95.5	94.5	96.1	96.1	95.7	94.8	95.9	95.5	96.9	93.7	95.1	93.3	5	swine/Ontario/K01477/01(H3N3)
6	4.5	6.3	0.2	7.0	4.1		94.8	94.8	95.5	96.2	94.6	94.8	94.9	96.0	94.9	96.7	96.7	96.2	95.2	96.6	96.4	97.3	94.3	95.3	93.7	6	GSC_chicken_B/British Columbia/04(H7N3)
7	3.8	3.6	5.7	4.1	4.4	5.5		95.0	95.6	97.9	94.7	95.1	94.9	96.3	95.2	97.1	97.1	96.6	95.0	97.0	95.6	96.7	96.6	95.7	93.9	7	mallard/ALB/226/1998(H2N3)
8	4.1	6.2	5.7	6.7	5.1	5.5	5.3		95.2	96.4	94.5	94.8	94.9	96.1	94.8	97.0	97.0	96.8	94.9	96.9	95.4	96.3	95.1	95.4	93.9	8	mallard/Alberta/245/2003(H11N9)
9	3.9	5.6	4.9	6.4	4.0	4.7	4.6	5.1		97.0	97.6	98.0	97.1	97.0	98.1	97.4	97.4	96.8	97.1	97.2	96.0	96.9	95.4	98.5	96.6	9	pintail duck/ALB/219/1977(H1N1)
10	2.1	2.8	4.1	3.8	3.3	3.9	2.1	3.7	3.1		96.2	96.5	96.4	97.8	96.6	98.7	98.7	98.2	96.6	98.6	97.0	98.1	97.4	97.2	95.2	10	blue-winged teal/ALB/141/1992(H1N4)
11	4.9	6.3	5.8	7.1	5.2	5.7	5.6	5.8	2.5	4.0		98.1	96.0	96.2	97.1	96.7	96.7	96.2	96.1	96.5	95.2	96.0	94.6	97.5	96.5	11	canvasback duck/ALB/102/1976(H3N6)
12	4.4	6.0	5.5	7.0	4.7	5.4	5.2	5.4	2.0	3.7	2.0		96.7	96.5	97.8	96.8	96.8	96.4	96.7	96.7	95.4	96.3	94.9	98.1	97.2	12	mallard duck/ALB/210/1977(H4N8)
13	4.5	6.2	5.4	6.7	4.7	5.3	5.4	5.4	3.0	3.7	4.1	3.4		96.5	96.6	96.9	96.9	96.5	98.2	96.8	95.3	96.2	94.7	97.0	95.4	13	redhead duck/ALB/116/1985(H4N6)
14	2.8	4.9	4.4	5.5	3.5	4.2	3.8	4.0	3.1	2.2	3.9	3.6	3.7		96.7	98.5	98.5	97.9	96.7	98.3	96.8	97.7	95.7	97.1	95.3	14	blue-winged teal/ALB/136/1990(H4N3)
15	4.4	6.0	5.5	6.5	4.6	5.3	5.0	5.5	1.9	3.5	3.0	2.3	3.5	3.4		96.8	96.8	96.5	96.7	96.7	95.5	96.3	95.2	98.0	96.3	15	pintail duck/ALB/121/1979(H7N8)
16	2.0	3.8	3.6	4.7	2.9	3.4	2.9	3.1	2.7	1.3	3.4	3.3	3.2	1.6	3.2		100.0	98.7	97.0	99.9	97.5	98.4	96.6	97.4	95.5	16	mallard/ALB/11/1991(H9N3)
17	2.0	3.8	3.6	4.7	2.9	3.4	2.9	3.1	2.7	1.3	3.4	3.3	3.2	1.6	3.2	0.0		98.7	97.0	99.9	97.5	98.4	96.6	97.4	95.5	17	mallard/ALB/17/1991(H9N2)
18	2.4	4.3	4.1	5.0	3.3	3.9	3.5	3.3	3.3	1.8	3.9	3.7	3.7	2.2	3.7	1.3	1.3		96.6	98.5	97.0	97.9	96.2	96.9	95.2	18	mallard/ALB/5/1995(H10N1)
19	4.2	6.0	5.1	6.6	4.2	5.0	5.3	5.4	3.0	3.5	4.0	3.4	1.8	3.4	3.4	3.1	3.1	3.5		96.8	95.8	96.5	94.9	97.2	95.5	19	mallard duck/ALB/797/1983(H11N3)
20	2.0	3.9	3.7	4.9	3.0	3.5	3.1	3.2	2.8	1.4	3.6	3.4	3.3	1.7	3.4	0.1	0.1	1.5	3.3		97.4	98.2	96.5	97.3	95.4	20	mallard/ALB/124/1991(H11N2)
21	3.8	5.4	3.9	6.0	3.5	3.8	4.5	4.8	4.2	3.1	5.1	4.8	4.9	3.3	4.7	2.6	2.6	3.1	4.4	2.7		97.8	95.0	96.1	94.1	21	pintail/Alberta/84/2000(H11N9)
22	2.9	4.6	2.9	5.0	2.0	2.8	3.4	3.8	3.2	1.9	4.2	3.9	3.9	2.3	3.8	1.6	1.6	2.1	3.6	1.8	2.3		96.0	97.0	95.1	22	green-winged teal/ALB/199/1991(H12N5)
23	3.9	4.4	6.2	3.5	5.5	6.1	3.5	5.2	4.8	2.7	5.7	5.3	5.6	4.5	5.0	3.5	3.5	4.0	5.4	3.7	5.2	4.2		95.5	94.1	23	ruddy turnstone/DE/97/2000(H12N5)
24	3.8	5.3	5.1	6.2	3.9	4.9	4.4	4.8	1.5	2.8	2.6	1.9	3.1	3.0	2.0	2.6	2.6	3.2	2.9	2.8	4.0	3.1	4.7		96.6	24	goose/MN/5733-1/1980(H9N2)
25	5.8	7.4	6.8	8.1	5.9	6.7	6.5	6.5	3.5	5.1	3.6	2.9	4.8	5.0	3.8	4.7	4.7	5.1	4.7	4.8	6.3	5.2	6.3	3.5		25	pheasant/MN/917/1980(H7N3)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25		

Appendix F. Pair distances of PB1 genes using ClustalW slow-accurate method

Percent Identity (upper right); Divergence (lower left)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41		
1		97.0	93.9	97.1	97.6	95.8	94.5	94.4	94.6	98.1	94.9	98.3	94.8	94.6	96.8	96.8	94.5	94.7	95.3	94.6	95.5	96.3	98.1	95.4	94.4	94.5	94.4	98.4	94.6	94.4	94.7	94.7	95.3	96.8	97.5	94.4	94.4	95.2	94.7	95.2	95.1	1	duck/MD/S698/04(H11N3)
2	3.1		94.1	99.5	97.7	96.1	94.6	94.2	94.8	97.4	95.2	97.6	95.3	95.0	97.2	97.2	94.4	94.9	95.3	94.7	95.6	96.7	98.0	95.4	94.4	94.3	94.4	97.7	94.5	94.2	94.5	94.7	95.4	97.4	97.6	94.1	94.2	95.4	94.6	95.2	95.1	2	chicken/BC/04(H7N3)
3	3.5	3.2		97.2	98.5	96.7	95.4	94.6	95.6	97.4	96.1	97.4	95.7	95.3	98.3	98.3	95.0	95.7	96.0	95.2	96.4	97.4	98.0	96.2	95.1	94.9	94.9	97.5	95.0	94.9	95.3	95.5	96.2	96.7	97.4	94.8	94.6	96.2	95.2	95.9	95.8	3	swine/Ontario/K01477/01(H3N3)
4	2.9	0.5	2.9		97.9	96.4	94.7	94.4	95.0	97.6	95.4	97.8	95.4	95.4	97.5	97.5	94.6	95.2	95.6	94.9	95.8	96.8	98.2	95.6	94.7	94.6	94.7	97.9	94.7	94.5	94.7	95.0	95.7	97.6	97.7	94.4	94.5	95.6	94.9	95.4	95.4	4	GSC_chicken/BC/04(H7N3)
5	2.5	2.4	1.5	2.1		97.5	96.0	95.4	96.2	98.2	96.6	98.3	96.4	96.2	98.4	98.4	95.7	96.2	96.8	96.0	97.1	98.2	98.8	96.9	95.9	95.7	95.5	98.4	95.8	95.6	96.0	96.1	96.8	97.7	98.3	95.6	95.5	96.8	95.9	96.5	96.6	5	mallard/ALB/201/1996(H2N3)
6	4.4	4.0	3.4	3.8	2.6		95.9	95.1	95.6	96.6	98.8	96.6	98.5	96.2	97.0	97.0	95.3	98.5	96.3	95.6	96.5	98.5	97.2	96.4	95.6	95.3	95.1	96.7	95.3	95.1	95.5	95.6	99.1	95.9	96.7	95.2	95.0	97.3	95.6	96.1	96.0	6	blue-winged teal/ALB/16/1997(H2N9)
7	5.8	5.7	4.8	5.5	4.2	4.3		95.8	96.6	95.2	95.1	95.0	95.3	94.5	95.3	95.3	96.1	94.8	97.1	97.4	97.5	96.4	95.5	97.1	96.2	95.9	95.8	95.1	96.2	96.0	96.3	96.5	95.3	94.5	95.2	95.8	95.5	95.1	96.3	96.9	96.8	7	mallard/ALB/79/2003(H2N3)
8	5.9	6.1	5.6	5.8	4.8	5.1	4.4		96.5	95.4	94.4	95.0	94.3	93.4	95.0	95.0	99.1	94.2	97.4	95.1	97.2	95.5	95.1	97.5	96.3	97.7	98.1	95.0	99.2	98.9	96.7	96.7	94.7	94.3	94.7	97.5	95.8	94.3	96.7	98.2	97.1	8	knot/DE/526/1988(H6N8)
9	5.7	5.5	4.6	5.3	3.9	4.6	3.5	3.6		95.3	95.0	95.3	95.0	94.2	95.7	95.7	97.0	94.5	98.0	95.9	97.9	96.3	95.7	98.2	97.2	96.7	96.7	95.4	97.0	96.7	97.2	97.6	95.2	94.7	95.3	96.6	96.3	95.3	97.3	98.0	98.1	9	widgeon/ALB/256/1982(H6N6)
10	2.0	2.7	2.7	2.4	1.8	3.5	5.0	4.8	4.9		95.8	98.8	95.4	95.3	97.6	97.6	95.3	95.5	96.3	95.5	96.3	97.1	98.6	96.1	95.1	95.1	95.2	98.9	95.4	95.2	95.6	95.3	95.9	97.3	98.0	95.1	95.1	95.9	95.5	96.1	95.9	10	mallard/ALB/154/2003(H6N5)
11	5.3	5.1	4.1	4.8	3.5	1.2	5.2	5.9	5.2	4.4		95.7	97.6	95.2	96.4	96.4	94.5	97.7	95.6	94.8	95.8	97.6	96.3	95.6	94.7	94.7	94.5	95.8	94.5	94.4	94.8	94.9	99.1	95.3	95.8	94.6	94.3	96.6	94.9	95.4	95.3	11	mallard/ALB/130/2003(H1N1)
12	1.7	2.4	2.6	2.3	1.7	3.5	5.2	5.3	4.9	1.2	4.5		95.4	95.3	97.6	97.6	95.0	95.6	95.9	95.3	96.2	97.1	98.9	96.0	95.0	95.0	94.9	99.9	95.2	95.0	95.6	95.3	95.9	97.4	98.4	94.9	95.0	95.9	95.3	95.9	95.9	12	mallard/ALB/27/2001(H7N3)
13	5.4	4.9	4.4	4.8	3.7	1.5	4.9	6.0	5.3	4.8	2.5	4.8		95.2	96.0	96.0	94.5	97.3	95.6	94.8	95.9	97.5	96.0	95.6	94.8	94.5	94.5	95.5	94.6	94.4	94.6	94.9	97.9	95.2	95.8	94.5	94.3	96.2	95.0	95.3	95.2	13	mallard/ALB/209/2003(H10N7)
14	5.7	5.2	4.9	4.8	3.9	3.9	5.8	7.0	6.1	4.9	5.0	5.0	5.0		95.6	95.6	93.8	94.9	94.8	94.4	95.2	97.0	95.6	94.8	94.2	94.1	93.7	95.3	93.8	93.7	94.2	94.6	95.6	94.8	95.3	93.9	93.9	95.6	94.2	94.6	94.7	14	mallard/ALB/245/2003(H11N9)
15	3.3	2.8	1.7	2.6	1.6	3.1	4.9	5.2	4.5	2.5	3.8	2.5	4.2	4.6		100.0	95.3	95.7	96.3	95.3	96.5	97.4	98.0	96.4	95.3	95.3	95.2	97.7	95.3	95.2	95.4	95.7	96.4	97.1	97.5	95.2	95.3	96.2	95.6	96.1	96.1	15	shorebird/DE/101/2004(H5N7)
16	3.3	2.8	1.7	2.6	1.6	3.1	4.9	5.2	4.5	2.5	3.8	2.5	4.2	4.6	0.0		95.3	95.7	96.3	95.3	96.5	97.4	98.0	96.4	95.3	95.3	95.2	97.7	95.3	95.2	95.4	95.7	96.4	97.1	97.5	95.2	95.3	96.2	95.6	96.1	96.1	16	ruddy turnstone/NJ/65/1985(H7N3)
17	5.8	5.9	5.3	5.7	4.4	5.0	4.0	0.9	3.1	4.9	5.8	5.2	5.8	6.5	4.9	4.9		94.3	97.8	95.4	97.8	95.9	95.2	98.0	96.9	98.2	98.7	95.1	99.8	99.5	97.0	97.3	94.7	94.5	94.9	98.1	96.1	94.7	97.1	98.6	97.6	17	knot/DE/2552/1987(H9N5)
18	5.6	5.3	4.5	5.1	3.9	1.6	5.4	6.1	5.8	4.7	2.4	4.5	2.8	5.3	4.5	4.5	6.0		95.3	94.5	95.6	97.1	96.0	95.3	94.4	94.2	94.2	95.7	94.3	94.1	94.5	94.6	97.9	95.2	95.6	94.2	94.2	96.2	94.6	95.0	94.9	18	laughing gull/DE/5/2003(H9N1)
19	4.9	4.9	4.2	4.6	3.4	3.8	3.0	2.7	2.0	3.9	4.6	4.2	4.6	5.4	3.9	3.9	2.2	4.9		96.4	98.8	97.1	96.3	99.0	97.9	97.4	97.6	96.0	97.9	97.6	97.9	98.6	95.7	95.6	95.8	97.4	97.7	95.9	98.2	98.8	98.8	19	pintail duck/ALB/238/1979(H1N1)
20	5.7	5.5	5.0	5.3	4.2	4.6	2.7	5.1	4.3	4.7	5.4	4.9	5.4	5.9	5.0	5.0	4.8	5.8	3.7		96.7	96.2	95.6	96.5	95.7	95.3	95.1	95.4	95.6	95.3	95.7	95.9	95.2	94.6	95.3	95.3	95.2	94.9	95.7	96.4	96.5	20	mallard/ALB/201/1998(H1N1)
21	4.7	4.6	3.7	4.4	2.9	3.6	2.6	2.9	2.1	3.9	4.4	3.9	4.3	5.0	3.6	3.6	2.3	4.6	1.3	3.4		97.4	96.5	98.8	97.9	97.4	97.4	96.3	97.7	97.5	97.9	98.2	95.9	95.6	96.1	97.4	97.0	96.1	98.0	98.8	98.6	21	pintail duck/ALB/627/1979(H3N2)
22	3.8	3.4	2.7	3.3	1.8	1.5	3.8	4.7	3.8	3.0	2.4	3.0	2.6	3.1	2.7	2.7	4.3	2.9	2.9	3.9	2.7		97.6	97.1	96.2	96.0	95.7	97.2	95.9	95.8	96.2	96.5	97.8	96.5	97.3	96.0	95.6	98.1	96.2	96.8	96.8	22	blue-winged teal/ALB/67/1990(H4N5)
23	2.0	2.0	2.1	1.8	1.3	2.9	4.7	5.1	4.5	1.4	3.9	1.1	4.1	4.6	2.0	2.0	5.0	4.2	3.8	4.6	3.6	2.5		96.4	95.4	95.1	95.0	99.0	95.2	95.0	95.6	95.6	96.5	97.9	98.9	95.0	95.1	96.3	95.6	96.1	96.2	23	pintail/ALB/207/1999(H4N8)
24	4.8	4.8	4.0	4.5	3.2	3.8	3.0	2.6	1.8	4.0	4.6	4.1	4.6	5.4	3.8	3.8	2.0	4.9	1.0	3.6	1.2	2.9	3.7		97.8	97.7	97.7	96.1	98.0	97.8	97.9	98.5	95.8	95.6	96.0	97.7	97.1	95.9	98.1	98.9	98.9	24	mallard duck/ALB/645/1980(H5N2)
25	5.9	5.9	5.1	5.6	4.3	4.6	3.9	3.8	2.9	5.1	5.6	5.2	5.5	6.1	4.9	4.9	3.2	5.9	2.2	4.5	2.1	3.9	4.8	2.3		96.5	96.5	95.1	96.8	96.6	96.9	97.4	95.0	94.4	94.8	96.5	95.9	95.0	97.1	97.8	97.7	25	blue-winged teal/ALB/221/1978(H7N2)
26	5.8	6.0	5.4	5.7	4.5	4.9	4.2	2.4	3.4	5.1	5.5	5.2	5.8	6.3	5.0	5.0	1.8	6.1	2.7	5.0	2.7	4.1	5.1	2.4	3.6		98.1	95.1	98.3	98.0	96.8	97.1	95.0	94.5	94.8	98.9	95.7	94.9	96.7	98.4	97.3	26	laughing gull/DE/2838/1987(H13N2)
27	5.8	5.9	5.4	5.6	4.7	5.1	4.4	1.9	3.4	5.0	5.8	5.3	5.8	6.6	5.0	5.0	1.3	6.1	2.4	5.1	2.7	4.5	5.2	2.4	3.7	2.0		95.0	98.8	98.5	96.8	97.1	94.7	94.4	94.7	97.9	95.8	94.6	96.8	98.4	97.4	27	ruddy turnstone/DE/2378/1988(H7N7)
28	1.6	2.4	2.5	2.2	1.6	3.4	5.1	5.2	4.8	1.1	4.4	0.1	4.7	4.9	2.4	2.4	5.1	4.5	4.1	4.8	3.9	2.9	1.0	4.0	5.2	5.1	5.2		95.3	95.0	95.7	95.3	96.0	97.5	98.5	95.0	95.0	96.0	95.3	96.0	95.9	28	mallard/ALB/34/2001(H7N1)
29	5.7	5.8	5.2	5.5	4.4	4.9	4.0	0.8	3.1	4.8	5.8	5.1	5.7	6.6	4.9	4.9	0.2	6.0	2.2	4.6	2.3	4.2	5.0	2.0	3.3	1.7	1.3	5.0		99.6	97.0	97.3	94.8	94.5	95.0	98.2	96.2	94.8	97.1	98.7	97.6	29	laughing gull/DE/2718/1987(H9N5)
30	5.9	6.1	5.4	5.8	4.5	5.1	4.2	1.1	3.4	5.0	5.9	5.2	5.9	6.7	5.0	5.0	0.5	6.2	2.4	4.9	2.6	4.4	5.2	2.3	3.5	2.0	1.5	5.2	0.4		96.8	97.1	94.6	94.4	94.8	97.9	95.9	94.6	96.8	98.4	97.4	30	ruddy turnstone/DE/510/1988(H9N6)
31	5.5	5.8	4.9	5.6	4.2	4.7	3.8	3.4	2.9	4.6	5.5	4.6	5.7	6.1	4.8	4.8	3.1	5.8	2.1	4.5	2.1	3.9	4.6	2.1	3.2	3.3	3.3	4.5	3.1	3.3		97.6	95.0	94.8	95.3	96.8	96.1	95.0	97.2	97.9	97.9	31	mallard duck/ALB/40/1984(H10N6)
32	5.6	5.6	4.7	5.3	4.1	4.5	3.6	3.4	2.5	4.9	5.3	5.0	5.4	5.7	4.5	4.5	2.8	5.7	1.4	4.2	1.9	3.7	4.6	1.5	2.7	3.0	3.0	4.9	2.8	3.0	2.5		95.3	95.1	95.1	97.1	97.0	95.3	97.9	98.3	98.4	32	redhead duck/ALB/357/1983(H11N9)
33	4.9	4.8	3.9	4.5	3.3	0.9	4.9	5.6	5.1	4.2	0.9	4.3	2.1	4.6	3.7	3.7	5.5	2.1	4.5	5.1	4.2	2.2	3.6	4.4	5.3	5.2	5.6	4.2	5.5	5.7	5.3	4.9		95.5	96.0	94.9	94.7	96.7	95.0	95.6	95.5	33	pintail/ALB/84/2000(H11N9)
34	3.3	2.6	3.4	2.4	2.4	4.3	5.8	6.0	5.6	2.8	5.0	2.6	5.1	5.4	2.9	2.9	5.8	5.0	4.6	5.7	4.5	3.6	2.1	4.6	5.9	5.8	5.9	2.5	5.8	5.9	5.4	5.2	4.7		97.5	94.4	94.6	95.3	94.8	95.2	95.6	34	shorebird/DE/236/2003(H11N9)
35	2.6	2.5	2.7	2.3	1.7	3.4	5.1	5.5	5.0	2.0	4.4	1.6	4.4	4.9	2.5	2.5	5.3	4.5	4.4	5.0	4.1	2.8	1.1	4.2	5.5	5.5	5.6	1.6	5.3	5.5	4.9	5.1	4.2	2.6		94.8	94.7	96.0	95.0	95.6	95.7	35	ruddy turnstone/DE/97/2000(H12N5)
36	5.9	6.2	5.5	5.9	4.6	5.0	4.4	2.5	3.5	5.1	5.7	5.3	5.8	6.5	5.0	5.0	1.9	6.1	2.6	5.0	2.7	4.2	5.2	2.4	3.7	1.1	2.1	5.2	1.8	2.1	3.3	3.0	5.4	6.0	5.4		95.8	94.8	96.8	98.4	97.4	36	laughing gull/DE/554/1988(H13N3)
37	5.9	6.1	5.6	5.8	4.7	5.2	4.7	4.4	3.8	5.1	6.0	5.3	6.0	6.5	4.9	4.9	4.0	6.1	2.4	5.0	3.1	4.6	5.1	2.9	4.2	4.5	4.3	5.2	3.9	4.3	4.0	3.1	5.6	5.7	5.6	4.4		94.6	96.5	97.0	97.1	37	sanderling/DE/1258/1986(H6N6)
38	5.0	4.8	3.9	4.5	3.2	2.7	5.1	5.9	4.8	4.3	3.5	4.2	3.8	4.5	3.8	3.8	5.5	3.9	4.2	5.3	4.0	1.9	3.8	4.2	5.1	5.3	5.6	4.1	5.4	5.6	5.1	4.9	3.3	4.9	4.1	5.4	5.7		95.2	95.7	95.6	38	chicken/Hidalgo/28159-232/1994(H5N2)
39	5.6	5.7	5.0	5.4	4.3	4.6	3.9	3.4	2.8	4.7	5.3	5.0	5.3	6.1	4.6	4.6	3.0	5.7	1.8	4.5	2.0	3.9	4.5	1.9	3.0	3.4	3.3	4.9	3.0	3.3	2.9	2.2	5.2	5.4	5.2	3.3	3.6	5.0		98.1	98.2	39	pheasant/MN/917/1980(H7N3)
40	5.1	5.1	4.3	4.8	3.6	4.0	3.2	1.8	2.0	4.1	4.8	4.3	4.9	5.7	4.1	4.1	1.4	5.2	1.3	3.8	1.3	3.3	4.0	1.1	2.2	1.6	1.6	4.2	1.3	1.6	2.1	1.7	4.6	5.0	4.6	1.6	3.1	4.4	2.0		98.7	40	duck/MN/1516/1981(H5N2)
41	5.1	5.1	4.4	4.8	3.5	4.2	3.3	3.0	2.0	4.2	4.9	4.3	5.0	5.6	4.1	4.1	2.5	5.3	1.2	3.7	1.4	3.3	4.0	1.1	2.4	2.8	2.7	4.2	2.4	2.7	2.2	1.6	4.7	4.6	4.4	2.7	3.0	4.6	1.8	1.3		41	turkey/MN/833/1980(H4N2)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41		

Appendix G. Pair distances of PA genes using ClustalW slow-accurate method

Percent Identity (upper triangle); Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33		
1		94.6	97.1	91.4	96.8	93.9	96.5	96.1	96.0	95.3	97.1	93.6	96.5	95.2	96.3	96.2	95.0	95.0	95.2	96.2	96.0	95.7	95.7	95.6	96.2	95.3	96.6	94.8	94.7	96.0	93.6	94.7	94.9	1	duck/MD/S698/04(H11N3)
2	5.6		95.2	92.5	94.9	93.9	95.1	95.7	95.4	95.4	96.0	94.0	95.6	95.4	95.3	95.2	95.0	95.0	94.8	95.7	95.5	95.8	95.8	95.7	95.7	95.2	95.3	94.9	95.7	97.2	93.9	94.8	95.1	2	swine/Ontario/01911-1/99 (H4N6)
3	2.9	5.0		91.9	99.7	93.3	97.8	95.2	95.0	94.7	98.4	93.4	97.8	94.6	94.8	94.8	94.4	94.4	94.3	95.3	95.2	95.0	95.0	94.9	95.2	94.4	94.8	94.1	95.3	96.5	93.3	94.0	94.4	3	chicken/BC/04(H7N3)
4	5.3	4.1	4.8		95.2	94.1	95.1	96.1	96.1	95.4	96.3	93.6	96.1	95.5	95.9	95.7	95.1	95.1	94.7	96.2	96.2	96.0	96.0	95.9	96.1	95.3	95.8	95.3	98.7	97.7	94.2	95.1	95.4	4	swine/Ontario/K01477/01(H3N3)
5	3.1	5.2	0.2	4.9		93.2	97.6	95.0	94.8	94.5	98.3	93.2	97.6	94.4	94.7	94.7	94.3	94.3	94.1	95.1	95.0	94.9	94.9	94.8	95.1	94.3	94.7	93.9	95.2	96.3	93.3	93.9	94.2	5	GSC_chicken/BC/04(H7N3)
6	6.4	6.4	7.1	6.2	7.3		93.5	96.2	96.0	97.1	94.1	95.6	93.6	97.4	96.1	96.1	97.2	97.2	95.9	96.3	96.0	96.8	96.8	96.7	96.2	95.6	96.1	95.9	94.1	95.3	95.3	96.8	97.0	6	mallard duck/ALB/65/1976(H1N1)
7	3.6	5.1	2.2	5.2	2.4	7.0		95.0	94.8	94.5	98.1	93.5	97.5	94.5	94.8	94.8	94.4	94.4	94.0	95.1	94.9	95.0	95.0	94.9	95.1	94.3	94.7	94.1	95.1	96.4	93.2	93.9	94.3	7	pintail/ALB/49/2003(H12N5)
8	3.9	4.5	5.1	4.0	5.2	3.9	5.2		99.6	98.2	96.0	96.0	95.7	98.0	99.2	99.0	97.4	97.4	97.5	99.6	99.6	98.6	98.6	98.5	99.8	97.5	99.1	97.6	95.8	97.4	96.2	97.4	97.5	8	knot/DE/2552/1987(H9N5)
9	4.1	4.8	5.3	4.0	5.4	4.1	5.4	0.4		98.1	95.8	95.9	95.5	97.9	99.1	98.9	97.4	97.4	97.4	99.6	99.8	98.5	98.5	98.4	99.7	97.4	99.1	97.6	95.8	97.3	96.0	97.3	97.4	9	knot/DE/2561/1987(H10N5)
10	4.9	4.8	5.6	4.8	5.8	2.9	5.8	1.9	2.0		95.5	97.1	95.2	99.4	98.0	97.8	98.6	98.6	97.4	98.3	98.1	98.8	98.8	98.7	98.3	97.5	98.0	97.7	95.4	96.9	97.2	98.6	98.7	10	pintail duck/ALB/238/1979(H1N1)
11	2.9	4.1	1.6	3.8	1.8	6.3	1.9	4.1	4.3	4.7		94.3	99.1	95.5	95.8	95.7	95.3	95.3	94.9	96.1	95.9	96.0	96.0	96.0	96.1	95.4	95.7	95.2	96.2	97.5	93.9	95.1	95.3	11	mallard/ALB/201/1998(H1N1)
12	6.8	6.3	7.1	6.8	7.2	4.6	6.9	4.1	4.2	3.0	6.1		93.9	97.3	96.0	95.8	96.7	96.7	95.7	96.2	95.9	96.8	96.8	96.7	96.1	95.8	96.0	95.9	93.7	95.3	95.0	96.7	97.0	12	coot/ALB/134/1987(H6N2)
13	3.6	4.6	2.3	4.0	2.5	6.8	2.5	4.5	4.7	5.0	0.9	6.5		95.2	95.5	95.3	94.9	94.9	94.7	95.8	95.6	95.7	95.7	95.7	95.7	95.2	95.4	94.8	96.0	97.2	93.9	94.8	94.9	13	mallard/ALB/156/2001(H3N8)
14	5.0	4.8	5.7	4.7	5.9	2.6	5.8	2.0	2.1	0.6	4.7	2.8	5.0		98.0	97.9	98.8	98.8	97.5	98.2	97.9	98.7	98.7	98.7	98.2	97.4	98.0	97.7	95.5	96.9	97.1	98.8	99.0	14	mallard duck/ALB/161/1977(H4N6)
15	3.8	4.9	5.4	4.3	5.6	4.0	5.5	0.8	0.9	2.0	4.4	4.2	4.7	2.1		99.3	97.5	97.5	97.4	99.4	99.1	98.4	98.4	98.3	99.4	97.4	99.5	97.4	95.6	97.2	96.0	97.3	97.4	15	knot/DE/541/1988(H4N6)
16	3.8	5.0	5.5	4.4	5.6	4.0	5.5	1.0	1.1	2.2	4.4	4.3	4.9	2.2	0.7		97.3	97.3	97.3	99.1	98.9	98.2	98.2	98.1	99.1	97.2	99.1	97.2	95.4	97.1	95.7	97.2	97.3	16	herring gull/NJ/406/1989(H5N3)
17	5.2	5.2	5.9	5.1	6.1	2.9	5.9	2.6	2.7	1.5	4.9	3.4	5.3	1.2	2.6	2.8		100.0	96.9	97.6	97.4	98.0	98.0	97.9	97.6	96.9	97.4	97.1	95.2	96.3	96.6	98.2	98.4	17	widgeon/ALB/284/1977(H7N3)
18	5.2	5.2	5.9	5.1	6.1	2.9	5.9	2.6	2.7	1.5	4.9	3.4	5.3	1.2	2.6	2.8	0.0		96.9	97.6	97.4	98.0	98.0	97.9	97.6	96.9	97.4	97.1	95.2	96.3	96.6	98.2	98.4	18	mallard duck/ALB/279/1977(H7N3)
19	5.0	5.4	6.1	5.5	6.2	4.3	6.3	2.5	2.6	2.7	5.3	4.4	5.6	2.5	2.7	2.8	3.2	3.2		97.6	97.4	98.0	98.0	97.9	97.6	98.3	97.4	96.8	94.7	96.1	95.3	96.8	97.0	19	red knot/NJ/325/1989(H7N7)
20	3.8	4.5	5.0	3.9	5.1	3.9	5.1	0.4	0.4	1.7	4.0	3.9	4.4	1.8	0.6	0.9	2.4	2.4	2.4		99.6	98.7	98.7	98.7	99.8	97.7	99.3	97.8	95.9	97.5	96.3	97.5	97.7	20	laughing gull/DE/2718/1987(H9N5)
21	4.1	4.7	5.1	3.9	5.2	4.1	5.3	0.4	0.2	2.0	4.2	4.2	4.6	2.1	0.9	1.1	2.7	2.7	2.6	0.4		98.4	98.4	98.3	99.6	97.5	99.0	97.5	95.8	97.3	96.0	97.3	97.4	21	ruddy turnstone/DE/2731/1987(H9N1)
22	4.5	4.4	5.2	4.2	5.4	3.3	5.3	1.5	1.5	1.2	4.2	3.3	4.5	1.3	1.6	1.8	2.1	2.1	2.1	1.3	1.5		100.0	99.9	98.7	98.1	98.4	98.2	95.9	97.4	96.7	98.1	98.2	22	redhead duck/ALB/357/1983(H11N9)
23	4.5	4.4	5.2	4.2	5.4	3.3	5.3	1.5	1.5	1.2	4.2	3.3	4.5	1.3	1.6	1.8	2.1	2.1	2.1	1.3	1.5	0.0		99.9	98.7	98.1	98.4	98.2	95.9	97.4	96.7	98.1	98.2	23	mallard duck/ALB/763/1983(H11N8)
24	4.6	4.5	5.3	4.3	5.5	3.4	5.4	1.5	1.6	1.3	4.2	3.4	4.5	1.4	1.7	1.9	2.1	2.1	2.1	1.4	1.6	0.1	0.1		98.6	98.0	98.3	98.2	95.8	97.3	96.6	98.0	98.1	24	mallard duck/ALB/797/1983(H11N3)
25	3.9	4.5	5.0	4.0	5.2	3.9	5.2	0.2	0.3	1.7	4.1	4.0	4.4	1.9	0.6	0.9	2.5	2.5	2.5	0.2	0.3	1.3	1.3	1.4		97.7	99.3	97.7	95.8	97.5	96.3	97.5	97.6	25	ruddy turnstone/DE/2589/1987(H11N4)
26	4.9	5.0	5.9	4.9	6.0	4.6	6.0	2.5	2.6	2.6	4.8	4.3	5.1	2.6	2.7	2.9	3.2	3.2	1.7	2.3	2.5	2.0	2.0	2.1	2.4		97.3	97.1	94.9	96.4	95.5	96.9	97.0	26	ruddy turnstone/DE/2762/1987(H11N2)
27	3.5	5.0	5.4	4.4	5.6	4.1	5.6	0.9	0.9	2.0	4.5	4.2	4.8	2.1	0.5	0.9	2.7	2.7	2.7	0.7	0.9	1.6	1.6	1.7	0.7	2.8		97.4	95.5	97.1	96.0	97.3	97.4	27	ruddy turnstone/NJ/842/1989(H11N1)
28	5.5	5.3	6.3	5.0	6.4	4.2	6.3	2.5	2.5	2.3	5.1	4.3	5.5	2.4	2.6	2.8	3.0	3.0	3.3	2.3	2.5	1.8	1.8	1.8	2.3	2.9	2.7		95.2	96.6	95.9	97.3	97.3	28	mallard/ALB/126/1991(H11N9)
29	5.5	4.5	4.9	1.4	5.0	6.2	5.1	4.4	4.4	4.8	3.9	6.7	4.2	4.7	4.6	4.8	5.1	5.1	5.6	4.3	4.3	4.3	4.3	4.4	4.3	5.3	4.7	5.0		97.4	94.1	95.0	95.4	29	shorebird/DE/236/2003(H11N9)
30	4.1	2.9	3.7	2.4	3.8	5.0	3.7	2.6	2.8	3.2	2.6	5.0	2.9	3.2	2.9	3.0	3.8	3.8	4.0	2.5	2.7	2.7	2.7	2.8	2.6	3.8	2.9	3.6	2.6		95.3	96.1	96.5	30	green-winged teal/ALB/199/1991(H12N5)
31	6.8	6.5	7.1	6.1	7.2	4.9	7.2	3.9	4.1	2.9	6.5	5.3	6.5	3.0	4.2	4.4	3.5	3.5	4.9	3.9	4.1	3.4	3.4	3.5	3.8	4.7	4.2	4.2	6.2	5.0		96.5	96.5	31	semi-palmated sandpiper/Br/43/90(H2N1)
32	5.5	5.4	6.3	5.2	6.5	3.3	6.5	2.7	2.8	1.4	5.2	3.4	5.5	1.2	2.8	2.9	1.8	1.8	3.3	2.6	2.8	1.9	1.9	2.0	2.5	3.2	2.8	2.8	5.2	4.0	3.7		98.8	32	goose/MN/5733-1/1980(H9N2)
33	5.3	5.1	6.0	4.8	6.1	3.1	6.0	2.6	2.7	1.3	4.9	3.1	5.3	1.0	2.6	2.8	1.6	1.6	3.0	2.4	2.7	1.8	1.8	1.9	2.4	3.0	2.6	2.8	4.8	3.7	3.7	1.2		33	turkey/MN/833/1980(H4N2)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33		

Appendix H. Pair distances of NP genes using ClustalW slow-accurate method

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33		
1		94.3	94.2	98.1	98.5	97.5	98.8	97.9	98.1	97.4	98.5	97.9	98.3	97.9	98.1	97.8	97.4	97.9	97.8	97.1	97.1	97.1	97.6	97.2	92.8	93.8	93.8	92.6	94.4	94.2	94.9	94.8	94.3	1	duck/MD/S698/04(H11N3)
2	3.0		96.2	98.7	97.1	97.9	97.0	98.5	98.5	97.8	96.9	97.1	98.3	98.5	98.0	98.3	97.6	98.5	97.7	97.0	97.0	96.9	97.2	96.4	96.0	94.9	94.9	95.7	99.7	96.2	97.5	98.0	96.5	2	chicken/NY/13833-7/95 (H7N2NSB)
3	3.1	4.0		97.2	96.8	96.9	96.9	97.0	97.2	96.5	96.8	97.5	97.7	97.0	97.0	97.0	96.5	97.0	96.9	96.6	96.6	96.6	96.6	96.1	94.5	94.8	94.8	94.2	96.3	100.0	96.9	96.8	95.7	3	guinea fowl/PA/7777-1/96 (H7N2NSB)
4	1.8	1.3	2.8		98.2	99.2	98.1	99.8	99.3	99.1	98.0	98.2	99.3	99.6	99.1	99.3	98.9	99.6	98.5	97.9	97.9	97.6	98.3	97.3	94.3	93.3	93.3	94.0	96.1	94.5	95.7	96.3	94.6	4	knot/DE/526/1988(H6N8)
5	1.4	3.0	3.3	1.8		97.4	99.2	98.0	98.0	97.3	99.1	98.0	98.4	98.0	98.0	97.9	97.3	98.0	97.6	97.2	97.2	97.4	97.4	96.9	92.9	94.4	94.4	92.7	94.5	94.0	94.8	94.9	93.9	5	mallard/ALB/206/1996(H6N8)
6	2.4	2.1	3.2	0.8	2.7		97.5	99.0	98.5	98.3	97.4	97.9	98.5	98.8	98.5	98.5	98.3	98.8	98.0	97.3	97.3	97.1	97.8	97.0	93.6	92.7	92.7	93.4	95.5	94.2	94.9	95.5	94.0	6	mallard/ALB/27/2001(H7N3)
7	1.1	3.1	3.2	1.9	0.8	2.5		97.9	97.9	97.2	99.4	97.9	98.3	97.9	98.1	97.8	97.2	97.9	97.5	97.1	97.1	97.1	97.3	97.2	92.8	94.5	94.5	92.6	94.4	94.2	94.7	94.8	94.3	7	mallard/ALB/245/2003(H11N9)
8	2.1	1.5	3.1	0.2	2.1	1.0	2.2		99.1	98.9	97.8	98.0	99.1	99.3	98.9	99.1	98.7	99.3	98.3	97.6	97.6	97.4	98.1	97.1	94.3	93.0	93.0	94.0	95.8	94.3	95.5	96.1	94.4	8	ruddy turnstone/NJ/65/1985(H7N3)
9	1.8	1.5	2.8	0.7	2.1	1.5	2.2	0.9		98.5	97.8	98.2	99.1	99.1	99.3	98.9	98.2	99.1	98.8	97.9	97.9	97.6	98.3	97.5	94.0	93.0	93.0	93.8	95.8	94.5	96.0	96.1	94.8	9	pintail duck/ALB/238/1979(H1N1)
10	2.5	2.2	3.6	0.9	2.8	1.7	2.9	1.1	1.5		97.1	97.3	98.4	98.7	98.2	98.4	98.9	98.7	97.6	97.4	97.4	96.7	97.5	96.4	93.4	92.4	92.4	93.1	95.2	93.8	94.8	95.4	93.7	10	mallard/ALB/201/1998(H1N1)
11	1.4	3.2	3.3	2.1	0.9	2.7	0.6	2.3	2.3	3.0		97.8	98.2	97.8	98.0	97.6	97.1	97.8	97.4	97.0	97.0	97.0	97.2	97.1	92.7	94.4	94.4	92.5	94.3	94.0	94.8	94.7	94.2	11	pintail duck/ALB/210/2002(H1N1)
12	2.1	3.0	2.6	1.8	2.1	2.2	2.2	2.1	1.8	2.8	2.3		98.7	98.0	98.0	97.8	97.3	98.0	97.9	97.6	97.6	98.5	97.6	97.3	92.7	93.0	93.0	92.5	94.5	94.7	95.3	94.9	94.2	12	mallard/ALB/129/1991(H3N6)
13	1.6	1.8	2.4	0.7	1.6	1.5	1.7	0.9	0.9	1.6	1.8	1.4		99.1	98.9	98.9	98.4	99.1	98.8	98.5	98.5	98.1	98.5	97.5	93.8	93.5	93.5	93.6	95.6	94.9	96.0	95.8	94.6	13	mallard duck/ALB/25/1985(H4N6)
14	2.1	1.5	3.1	0.5	2.1	1.3	2.2	0.7	0.9	1.4	2.3	2.1	0.9		98.9	99.1	98.4	99.3	98.3	97.6	97.6	97.4	98.1	97.1	94.0	93.0	93.0	93.8	95.8	94.3	95.6	96.1	94.4	14	mallard duck/ALB/7/1987(H8N4)
15	1.8	2.0	3.1	0.9	2.1	1.5	1.9	1.1	0.7	1.8	2.1	2.1	1.1	1.1		98.8	98.0	98.9	98.5	97.6	97.6	97.4	98.3	97.5	93.8	93.0	93.0	93.6	95.4	94.3	95.7	95.8	94.8	15	green-winged teal/ALB/228/1985(H7N3)
16	2.2	1.8	3.1	0.7	2.2	1.5	2.3	0.9	1.1	1.6	2.4	2.3	1.1	0.9	1.2		98.2	99.1	98.1	97.4	97.4	97.2	97.9	96.9	93.8	92.9	92.9	93.6	95.6	94.3	95.3	95.8	94.2	16	mallard duck/ALB/321/1988(H9N2)
17	2.5	2.5	3.6	1.1	2.8	1.7	2.9	1.4	1.8	1.1	3.0	2.8	1.6	1.6	2.1	1.8		98.4	97.6	97.6	97.6	96.7	97.5	96.6	93.3	92.4	92.4	93.0	95.2	93.8	94.8	95.2	93.7	17	pintail/Alberta/202/2000(H10N7)
18	2.1	1.5	3.1	0.5	2.1	1.3	2.2	0.7	0.9	1.4	2.3	2.1	0.9	0.7	1.1	0.9	1.6		98.3	97.6	97.6	97.4	98.1	97.1	94.0	93.0	93.0	93.8	95.8	94.3	95.5	96.1	94.4	18	redhead duck/ALB/357/1983(H11N9)
19	2.2	2.4	3.2	1.5	2.4	2.1	2.5	1.7	1.2	2.4	2.6	2.2	1.2	1.7	1.5	1.9	2.4	1.7		97.5	97.5	97.4	98.7	97.9	93.5	92.7	92.7	93.3	95.1	94.2	96.1	95.3	94.9	19	mallard duck/ALB/763/1983(H11N8)
20	2.9	3.1	3.5	2.2	2.9	2.8	3.0	2.4	2.2	2.6	3.1	2.4	1.5	2.4	2.4	2.6	2.4	2.4	2.5		100.0	97.3	97.3	96.7	92.5	92.4	92.4	92.2	94.4	93.9	94.7	94.6	93.6	20	mallard/ALB/124/1991(H11N2)
21	2.9	3.1	3.5	2.2	2.9	2.8	3.0	2.4	2.2	2.6	3.1	2.4	1.5	2.4	2.4	2.6	2.4	2.4	2.5	0.0		97.3	97.3	96.7	92.5	92.4	92.4	92.2	94.4	93.9	94.7	94.6	93.6	21	mallard/ALB/126/1991(H11N9)
22	2.9	3.2	3.5	2.4	2.7	3.0	3.0	2.6	2.4	3.4	3.1	1.5	1.9	2.7	2.7	2.9	3.4	2.7	2.6	2.8	2.8		97.1	96.3	92.1	92.2	92.2	91.9	94.3	93.9	94.5	94.4	93.1	22	green-winged teal/ALB/199/1991(H12N5)
23	2.3	2.8	3.5	1.7	2.7	2.3	2.8	1.9	1.7	2.5	2.9	2.4	1.5	1.9	1.7	2.2	2.5	1.9	1.4	2.8	2.8	3.0		97.4	93.3	92.7	92.5	93.0	94.6	93.9	95.6	94.8	94.3	23	goose/MN/5733-1/1980(H9N2)
24	2.8	3.7	4.1	2.8	3.2	3.1	2.9	3.0	2.5	3.7	3.0	2.8	2.5	3.0	2.5	3.2	3.5	3.0	2.2	3.4	3.4	3.8	2.6		92.2	92.1	92.1	92.0	93.8	93.4	95.3	94.0	96.2	24	duck/MN/1516/1981(H5N2)
25	2.3	1.9	3.5	0.8	2.3	1.6	2.4	0.8	1.1	1.8	2.5	2.5	1.3	1.1	1.3	1.3	1.9	1.1	1.7	2.8	2.8	3.2	1.9	3.0		97.8	97.8	99.8	98.3	96.6	98.3	98.5	97.2	25	mallard/MD/R274/98(H6N5)
26	1.2	3.0	3.2	1.9	0.7	2.5	0.6	2.2	2.2	2.9	0.7	2.2	1.7	2.2	2.2	2.3	2.9	2.2	2.5	2.9	2.9	3.0	2.5	3.2	2.3		99.5	97.5	97.2	96.9	97.5	97.6	96.8	26	mallard/MN/346233/00(H6N3)
27	1.2	3.0	3.2	1.9	0.7	2.5	0.6	2.2	2.2	2.9	0.7	2.2	1.7	2.2	2.2	2.3	2.9	2.2	2.5	2.9	2.9	3.0	2.8	3.2	2.3	0.5		97.5	97.2	96.9	97.5	97.6	96.8	27	northern pintail/AK/3209/02(H3)
28	2.5	2.2	3.8	1.1	2.5	1.8	2.7	1.1	1.3	2.0	2.8	2.8	1.6	1.3	1.6	1.6	2.2	1.3	1.9	3.0	3.0	3.4	2.2	3.3	0.2	2.5	2.5		98.1	96.3	98.1	98.2	96.9	28	laughing gull/NY/2455/00(H7N3)
29	2.9	0.3	3.8	1.2	2.9	1.8	3.0	1.4	1.4	2.1	3.1	2.9	1.6	1.4	1.9	1.6	2.1	1.4	2.2	3.0	3.0	3.1	2.7	3.6	1.7	2.9	2.9	1.9		96.3	97.6	98.2	96.6	29	Chicken/NY/13142-5/94 (H7N2)
30	3.1	4.0	0.0	2.8	3.3	3.2	3.2	3.1	2.8	3.6	3.3	2.6	2.4	3.1	3.1	3.1	3.6	3.1	3.2	3.5	3.5	3.5	3.5	4.1	3.5	3.2	3.2	3.8	3.8		96.9	96.8	95.7	30	Chicken/NY/13202-7/96 (H7N2)
31	2.2	2.6	3.2	1.5	2.5	2.4	2.6	1.8	1.3	2.5	2.5	2.0	1.3	1.6	1.5	2.0	2.5	1.8	1.2	2.6	2.6	2.8	1.6	2.0	1.7	2.5	2.5	1.9	2.5	3.2		98.0	97.9	31	mallard/WI/428/1975(H5N1)
32	2.4	2.0	3.3	0.9	2.4	1.8	2.5	1.2	1.2	1.9	2.6	2.4	1.4	1.2	1.4	1.4	2.1	1.2	2.0	2.7	2.7	3.0	2.5	3.3	1.6	2.4	2.4	1.8	1.9	3.3	2.0		96.9	32	turkey/MN/3689-1551/1981(H5N2)
33	3.0	3.6	4.5	2.7	3.5	3.3	3.1	3.0	2.5	3.7	3.2	3.2	2.7	3.0	2.5	3.2	3.7	3.0	2.4	3.8	3.8	4.3	3.1	1.1	2.9	3.3	3.3	3.2	3.5	4.5	2.1	3.2		33	turkey/OR/71(H7N3)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33		

Appendix I. Pair distances of M genes using ClustalW slow-accurate method

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41		
2	1.0		99.0	95.1	99.1	95.1	95.1	95.3	95.8	95.2	96.8	95.5	94.9	95.8	95.7	95.7	96.6	95.4	94.8	95.2	94.8	95.4	94.9	96.2	96.0	95.6	95.5	95.9	95.3	96.6	95.7	95.1	96.3	96.5	95.4	96.5	95.3	99.3	99.0	98.9	94.8	2	chicken/NY/12273-11/99(H7N3)
3	0.8	1.0		95.4	99.5	95.4	95.2	95.2	96.0	95.3	97.0	96.2	95.0	96.2	96.5	96.5	96.8	95.5	95.0	95.3	95.0	95.5	95.0	96.4	96.2	96.4	95.6	96.1	95.4	96.8	96.5	95.2	96.5	96.9	95.5	96.5	95.4	99.5	99.4	99.3	95.0	3	duck/NY/191255-79/02(H5N2)
4	1.8	2.6	2.2		95.7	100.0	98.2	98.2	97.7	98.2	98.1	97.3	98.1	97.5	97.6	97.6	97.9	98.5	98.1	98.3	97.9	98.5	98.1	97.8	97.9	97.5	98.8	97.5	98.4	98.1	97.6	98.2	98.0	98.0	98.3	97.7	98.4	95.5	95.4	95.5	97.9	4	chicken/BC/04(H7N3)
5	0.5	0.9	0.5	1.9		95.7	95.7	95.7	96.1	95.8	97.1	95.9	95.5	96.3	96.2	96.2	96.9	96.0	95.4	95.8	95.4	96.0	95.5	96.7	96.3	96.1	96.1	96.4	95.7	97.1	96.2	95.7	96.6	97.2	96.0	96.6	95.9	99.6	99.7	99.4	95.4	5	swine/Ontario/42729A/01(H3N3)
6	1.8	2.6	2.2	0.0	1.9		98.2	98.2	97.7	98.2	98.1	97.3	98.1	97.5	97.6	97.6	97.9	98.5	98.1	98.3	97.9	98.5	98.1	97.8	97.9	97.5	98.8	97.5	98.4	98.1	97.6	98.2	98.0	98.0	98.3	97.7	98.4	95.5	95.4	95.5	97.9	6	chicken/BC/GSC_human_B/04(H7N3)
7	1.9	2.6	2.4	1.8	1.9	1.8		98.6	97.5	98.2	98.1	97.1	98.3	97.5	97.4	97.4	98.1	98.7	98.2	98.5	97.9	98.5	98.4	97.8	97.5	97.3	99.0	97.5	98.6	98.1	97.4	98.6	97.8	98.0	98.7	97.8	98.8	95.5	95.4	95.5	97.9	7	mallard/ALB/323/1988(H2N1)
8	1.9	2.4	2.4	1.8	1.9	1.8	1.4		97.5	98.2	98.1	97.1	98.1	97.3	97.4	97.4	98.1	98.9	98.1	98.5	97.9	98.5	98.4	97.8	97.5	97.3	98.8	97.5	98.6	98.1	97.4	98.6	97.8	98.0	98.5	97.6	98.6	95.5	95.4	95.5	97.9	8	mallard/ALB/202/1996(H2N5)
9	1.1	1.8	1.5	2.4	1.4	2.4	2.6	2.6		97.6	98.8	97.9	97.3	98.6	98.1	98.1	98.6	97.8	97.2	97.6	97.2	97.8	97.5	98.2	98.8	98.1	97.9	98.2	97.7	98.6	98.1	97.5	99.1	98.5	97.8	98.3	97.7	96.3	96.0	95.9	97.4	9	mallard/Alberta/149/2002(H2N4)
10	1.8	2.5	2.3	1.8	1.8	1.8	1.8	1.8	2.5		98.1	97.4	98.5	97.4	97.7	97.7	98.0	98.5	98.4	99.3	98.2	98.9	98.6	97.9	97.6	97.6	98.6	97.8	99.0	98.4	97.7	99.2	97.9	98.2	98.3	97.7	99.2	95.6	95.5	95.6	98.2	10	mallard/ALB/76/1985(H6N3)
11	0.5	0.7	0.5	2.0	0.4	2.0	2.0	2.0	1.2	1.9		98.4	97.9	98.8	98.7	98.7	99.6	98.3	97.8	98.1	97.8	98.3	97.9	99.2	99.0	98.6	98.4	98.9	98.2	99.6	98.7	98.1	99.3	99.5	98.3	99.3	98.2	97.3	97.0	96.9	97.8	11	mallard/ALB/206/1996(H6N8)
12	1.3	2.2	1.3	2.8	1.7	2.8	3.0	3.0	2.2	2.7	1.6		96.9	98.1	99.5	99.5	98.4	97.4	96.8	97.4	96.6	97.4	96.9	97.9	98.1	99.4	97.5	97.9	97.5	98.2	99.5	97.3	98.3	98.3	97.4	98.1	97.3	95.9	95.8	95.9	96.8	12	shorebird/DE/12/2004(H6N8)
13	2.1	2.8	2.6	1.9	2.1	1.9	1.7	1.9	2.8	1.5	2.2	3.2		97.1	97.2	97.2	97.7	98.4	99.9	98.6	98.9	98.6	98.3	97.6	97.3	97.1	98.7	97.3	98.5	98.1	97.2	98.7	97.6	97.8	98.4	97.6	98.9	95.3	95.2	95.3	98.7	13	mallard/ALB/57/1976(H5N2)
14	1.1	1.8	1.3	2.6	1.2	2.6	2.6	2.8	1.4	2.7	1.2	2.0	3.0		98.3	98.3	98.6	97.6	97.0	97.4	97.4	97.6	97.1	98.2	99.0	98.2	97.7	98.0	97.5	98.6	98.3	97.3	99.1	98.7	97.6	98.3	97.5	96.3	96.2	96.1	97.6	14	pintail/Alberta/49/2003(H12N5)
15	1.0	1.9	1.0	2.5	1.3	2.5	2.7	2.7	1.9	2.4	1.3	0.5	2.9	1.7		100.0	98.7	97.7	97.1	97.7	96.9	97.7	97.2	98.1	98.3	99.9	97.8	98.1	97.8	98.5	100.0	97.6	98.6	98.6	97.7	98.2	97.6	96.2	96.1	96.2	97.1	15	shorebird/DE/101/2004(H5N7)
16	1.0	1.9	1.0	2.5	1.3	2.5	2.7	2.7	1.9	2.4	1.3	0.5	2.9	1.7	0.0		98.7	97.7	97.1	97.7	96.9	97.7	97.2	98.1	98.3	99.9	97.8	98.1	97.8	98.5	100.0	97.6	98.6	98.6	97.7	98.2	97.6	96.2	96.1	96.2	97.1	16	ruddy turnstone/NJ/65/1985(H7N3)
17	0.7	0.9	0.7	2.2	0.6	2.2	2.0	2.0	1.4	2.1	0.4	1.6	2.4	1.4	1.3	1.3		98.1	97.6	98.1	97.6	98.1	97.9	99.0	98.8	98.6	98.2	98.7	98.2	99.4	98.7	98.1	99.1	99.3	98.1	99.1	98.1	97.1	96.8	96.7	97.6	17	mallard/ALB/201/1998(H1N1)
18	1.6	2.3	2.1	1.5	1.5	1.5	1.3	1.1	2.3	1.5	1.7	2.7	1.6	2.5	2.4	2.4	1.9		98.3	98.6	98.1	98.8	98.5	98.1	97.8	97.6	99.1	98.0	98.7	98.4	97.7	98.7	98.1	98.2	98.8	97.9	98.9	95.8	95.7	95.8	98.1	18	mallard duck/ALB/322/1988(H1N1)
19	2.2	2.9	2.7	2.0	2.2	2.0	1.8	2.0	2.9	1.6	2.3	3.3	0.1	3.1	3.0	3.0	2.5	1.7		98.5	98.8	98.5	98.2	97.5	97.2	97.0	98.6	97.2	98.4	98.1	97.1	98.6	97.5	97.7	98.3	97.5	98.8	95.2	95.1	95.2	98.6	19	pintail/ALB/86/1976(H3N2)
20	1.8	2.5	2.3	1.7	1.8	1.7	1.5	1.5	2.5	0.7	1.9	2.7	1.4	2.7	2.4	2.4	1.9	1.4	1.5		98.3	99.2	98.9	97.9	97.6	97.6	98.7	97.8	99.3	98.4	97.7	99.5	97.9	98.1	98.6	97.7	99.3	95.6	95.5	95.6	98.3	20	mallard/ALB/438/1985(H3N4)
21	2.2	2.9	2.7	2.2	2.2	2.2	2.2	2.2	2.9	1.8	2.3	3.5	1.1	2.7	3.2	3.2	2.5	1.9	1.2	1.7		98.5	98.1	97.5	97.2	96.8	98.2	97.2	98.2	98.1	96.9	98.4	97.5	97.7	98.0	97.3	98.6	95.2	95.1	95.0	99.8	21	canvasback duck/ALB/274/1977(H4N6)
22	1.6	2.3	2.1	1.5	1.5	1.5	1.5	1.5	2.3	1.1	1.7	2.7	1.4	2.5	2.4	2.4	1.9	1.2	1.5	0.8	1.5		98.7	98.1	97.8	97.6	98.9	97.8	98.9	98.6	97.7	98.9	98.1	98.2	98.6	97.9	99.1	95.8	95.7	95.8	98.5	22	mallard duck/ALB/581/1983(H4N4)
23	2.1	2.8	2.6	2.0	2.1	2.0	1.6	1.6	2.6	1.4	2.2	3.2	1.7	3.0	2.9	2.9	2.2	1.5	1.8	1.1	2.0	1.3		97.6	97.3	97.1	98.6	97.3	99.0	98.1	97.2	99.0	97.6	97.8	98.3	97.4	99.0	95.3	95.2	95.3	98.1	23	knot/DE/541/1988(H4N6)
24	0.9	1.3	1.1	2.3	0.8	2.3	2.3	2.3	1.8	2.2	0.8	2.2	2.5	1.8	1.9	1.9	1.0	2.0	2.6	2.2	2.6	2.0	2.5		98.4	98.1	98.1	98.7	98.0	99.4	98.1	97.8	98.9	98.9	98.1	98.7	98.0	96.7	96.4	96.3	97.5	24	pintail/ALB/207/1999(H4N8)
25	0.9	1.5	1.3	2.2	1.2	2.2	2.6	2.6	1.2	2.5	1.0	2.0	2.8	1.0	1.7	1.7	1.2	2.3	2.9	2.5	2.9	2.3	2.8	1.6		98.2	98.1	98.1	97.7	98.8	98.3	97.5	99.3	98.7	97.8	98.5	97.7	96.5	96.2	96.1	97.4	25	blue-winged teaL/ALB/293/2003(H4N6)
26	1.1	2.0	1.1	2.6	1.4	2.6	2.8	2.8	2.0	2.5	1.4	0.6	3.0	1.8	0.1	0.1	1.4	2.5	3.1	2.5	3.3	2.5	3.0	2.0	1.8		97.7	98.1	97.7	98.4	99.9	97.5	98.5	98.5	97.6	98.1	97.5	96.1	96.0	96.1	97.0	26	shorebird/DE/75/2004(H5N7)
27	1.5	2.2	2.0	1.2	1.4	1.2	1.0	1.2	2.2	1.4	1.6	2.6	1.3	2.4	2.3	2.3	1.8	0.9	1.4	1.3	1.8	1.1	1.4	1.9	2.0	2.4		97.9	98.8	98.5	97.8	98.8	98.1	98.3	99.1	98.1	99.0	95.9	95.8	95.9	98.2	27	mallard /ALB/7/1987(H8N4)
28	1.2	1.6	1.4	2.6	1.1	2.6	2.6	2.6	1.8	2.3	1.1	2.2	2.8	2.1	1.9	1.9	1.3	2.1	2.9	2.3	2.9	2.3	2.8	1.3	1.9	2.0	2.2		97.9	99.1	98.1	97.7	98.4	98.6	97.8	98.6	97.7	96.4	96.1	96.0	97.2	28	mallard/ALB/34/2001(H7N1)
29	1.7	2.4	2.2	1.6	1.9	1.6	1.4	1.4	2.4	1.0	1.8	2.6	1.5	2.6	2.3	2.3	1.8	1.3	1.6	0.7	1.8	1.1	1.0	2.1	2.4	2.4	1.2	2.2		98.5	97.8	99.4	98.0	98.0	98.5	97.8	99.2	95.7	95.4	95.7	98.2	29	mallard/ALB/321/1988(H9N2)
30	0.5	0.9	0.7	1.9	0.4	1.9	1.9	1.9	1.4	1.6	0.4	1.8	1.9	1.4	1.5	1.5	0.6	1.6	2.0	1.6	2.0	1.4	1.9	0.6	1.2	1.6	1.5	0.9	1.5		98.5	98.3	99.1	99.3	98.4	99.1	98.5	97.1	96.8	96.7	98.1	30	mallard/Alb/208/2000(H10N7)
31	1.0	1.9	1.0	2.5	1.3	2.5	2.7	2.7	1.9	2.4	1.3	0.5	2.9	1.7	0.0	0.0	1.3	2.4	3.0	2.4	3.2	2.4	2.9	1.9	1.7	0.1	2.3	1.9	2.3	1.5		97.6	98.6	98.6	97.7	98.2	97.6	96.2	96.1	96.2	97.1	31	shorebird/DE/122/2004(H10N7)
32	1.9	2.6	2.4	1.8	1.9	1.8	1.4	1.4	2.6	0.8	2.0	2.8	1.3	2.8	2.5	2.5	2.0	1.3	1.4	0.5	1.6	1.1	1.0	2.3	2.6	2.6	1.2	2.4	0.6	1.7	2.5		97.8	98.0	98.5	97.6	99.4	95.5	95.4	95.5	98.4	32	ruddy turnstone/DE/2762/1987(H11N2)
33	0.6	1.2	1.0	2.1	0.9	2.1	2.3	2.3	0.9	2.2	0.7	1.7	2.5	0.9	1.4	1.4	0.9	2.0	2.6	2.2	2.6	2.0	2.5	1.1	0.7	1.5	1.9	1.6	2.1	0.9	1.4	2.3		99.0	98.1	98.8	98.0	96.8	96.5	96.4	97.7	33	mallard/ALB/125/1999(H11N6)
34	0.8	1.0	0.6	2.1	0.3	2.1	2.1	2.1	1.5	1.8	0.5	1.7	2.3	1.3	1.4	1.4	0.7	1.8	2.4	2.0	2.4	1.8	2.3	1.1	1.3	1.5	1.7	1.4	2.1	0.7	1.4	2.1	1.0		98.2	99.0	98.1	97.0	97.1	96.8	97.7	34	shorebird/DE/236/2003(H11N9)
35	1.6	2.3	2.1	1.7	1.5	1.7	1.3	1.5	2.3	1.7	1.7	2.7	1.6	2.5	2.4	2.4	1.9	1.2	1.7	1.4	2.1	1.4	1.7	2.0	2.3	2.5	0.9	2.3	1.5	1.6	2.4	1.5	2.0	1.8		98.1	98.7	95.8	95.7	95.8	98.0	35	green-winged teal/ALB/199/1991(H12N5)
36	1.0	1.0	1.0	2.4	0.9	2.4	2.3	2.5	1.7	2.4	0.7	1.9	2.5	1.7	1.8	1.8	0.9	2.2	2.6	2.4	2.8	2.2	2.7	1.3	1.5	1.9	1.9	1.4	2.3	0.9	1.8	2.5	1.2	1.0	2.0		97.8	96.8	96.5	96.4	97.3	36	ruddy turnstone/DE/97/2000(H12N5)
37	1.6	2.3	2.1	1.5	1.6	1.5	1.1	1.3	2.3	0.7	1.7	2.7	1.0	2.5	2.4	2.4	1.9	1.0	1.1	0.6	1.3	0.8	0.9	2.0	2.3	2.5	0.9	2.3	0.7	1.4	2.4	0.5	2.0	1.8	1.2	2.2		95.7	95.6	95.7	98.6	37	domestic duck/MN/1086/1980(H4N8)
38	0.5	0.7	0.5	2.1	0.4	2.1	2.1	2.1	1.2	2.0	0.2	1.7	2.3	1.2	1.3	1.3	0.4	1.8	2.4	2.0	2.4	1.8	2.3	0.8	1.0	1.4	1.6	1.1	1.9	0.4	1.3	2.1	0.7	0.5	1.8	0.7	1.8		99.5	99.4	95.2	38	mallard/MN/7/98(H11N2)
39	0.8	1.0	0.6	2.2	0.3	2.2	2.2	2.2	1.5	2.1	0.5	1.8	2.4	1.3	1.4	1.4	0.7	1.9	2.5	2.1	2.5	1.9	2.4	1.1	1.3	1.5	1.8	1.4	2.2	0.7	1.4	2.2	1.0	0.4	1.9	1.0	1.9	0.5		99.3	95.1	39	mallard/OH/66/99(H6N1)
40	0.9	1.1	0.7	2.1	0.6	2.1	2.1	2.1	1.7	2.0	0.6	1.6	2.3	1.4	1.3	1.3	0.8	1.8	2.4	2.0	2.6	1.8	2.3	1.2	1.4	1.4	1.6	1.5	1.9	0.8	1.3	2.1	1.1	0.7	1.8	1.1	1.8	0.6	0.7		95.0	40	green-winged teal/OH/196/99(H6N2)
41	2.0	2.9	2.7	2.2	2.2	2.2	2.2	2.2	2.7	1.8	2.3	3.3	1.3	2.5	3.0	3.0	2.5	1.9	1.4	1.7	0.2	1.5	2.0	2.6	2.7	3.1	1.8	2.9	1.8	2.0	3.0	1.6	2.4	2.4	2.1	2.8	1.3	2.4	2.5	2.6		41	turkey/MN/833/80(H4N2)

Appendix J. Pair distances of NS genes using ClustalW slow-accurate method

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33		
1		94.3	94.2	98.1	98.5	97.5	98.8	97.9	98.1	97.4	98.5	97.9	98.3	97.9	98.1	97.8	97.4	97.9	97.8	97.1	97.1	97.1	97.6	97.2	92.8	93.8	93.8	92.6	94.4	94.2	94.9	94.8	94.3	1	duck/MD/S698/04(H11N3)
2	3.0		96.2	98.7	97.1	97.9	97.0	98.5	98.5	97.8	96.9	97.1	98.3	98.5	98.0	98.3	97.6	98.5	97.7	97.0	97.0	96.9	97.2	96.4	96.0	94.9	94.9	95.7	99.7	96.2	97.5	98.0	96.5	2	chicken/NY/13833-7/95 (H7N2NSB)
3	3.1	4.0		97.2	96.8	96.9	96.9	97.0	97.2	96.5	96.8	97.5	97.7	97.0	97.0	97.0	96.5	97.0	96.9	96.6	96.6	96.6	96.6	96.1	94.5	94.8	94.8	94.2	96.3	100.0	96.9	96.8	95.7	3	guinea fowl/PA/7777-1/96 (H7N2NSB)
4	1.8	1.3	2.8		98.2	99.2	98.1	99.8	99.3	99.1	98.0	98.2	99.3	99.6	99.1	99.3	98.9	99.6	98.5	97.9	97.9	97.6	98.3	97.3	94.3	93.3	93.3	94.0	96.1	94.5	95.7	96.3	94.6	4	knot/DE/526/1988(H6N8)
5	1.4	3.0	3.3	1.8		97.4	99.2	98.0	98.0	97.3	99.1	98.0	98.4	98.0	98.0	97.9	97.3	98.0	97.6	97.2	97.2	97.4	97.4	96.9	92.9	94.4	94.4	92.7	94.5	94.0	94.8	94.9	93.9	5	mallard/ALB/206/1996(H6N8)
6	2.4	2.1	3.2	0.8	2.7		97.5	99.0	98.5	98.3	97.4	97.9	98.5	98.8	98.5	98.5	98.3	98.8	98.0	97.3	97.3	97.1	97.8	97.0	93.6	92.7	92.7	93.4	95.5	94.2	94.9	95.5	94.0	6	mallard/ALB/27/2001(H7N3)
7	1.1	3.1	3.2	1.9	0.8	2.5		97.9	97.9	97.2	99.4	97.9	98.3	97.9	98.1	97.8	97.2	97.9	97.5	97.1	97.1	97.1	97.3	97.2	92.8	94.5	94.5	92.6	94.4	94.2	94.7	94.8	94.3	7	mallard/ALB/245/2003(H11N9)
8	2.1	1.5	3.1	0.2	2.1	1.0	2.2		99.1	98.9	97.8	98.0	99.1	99.3	98.9	99.1	98.7	99.3	98.3	97.6	97.6	97.4	98.1	97.1	94.3	93.0	93.0	94.0	95.8	94.3	95.5	96.1	94.4	8	ruddy turnstone/NJ/65/1985(H7N3)
9	1.8	1.5	2.8	0.7	2.1	1.5	2.2	0.9		98.5	97.8	98.2	99.1	99.1	99.3	98.9	98.2	99.1	98.8	97.9	97.9	97.6	98.3	97.5	94.0	93.0	93.0	93.8	95.8	94.5	96.0	96.1	94.8	9	pintail duck/ALB/238/1979(H1N1)
10	2.5	2.2	3.6	0.9	2.8	1.7	2.9	1.1	1.5		97.1	97.3	98.4	98.7	98.2	98.4	98.9	98.7	97.6	97.4	97.4	96.7	97.5	96.4	93.4	92.4	92.4	93.1	95.2	93.8	94.8	95.4	93.7	10	mallard/ALB/201/1998(H1N1)
11	1.4	3.2	3.3	2.1	0.9	2.7	0.6	2.3	2.3	3.0		97.8	98.2	97.8	98.0	97.6	97.1	97.8	97.4	97.0	97.0	97.0	97.2	97.1	92.7	94.4	94.4	92.5	94.3	94.0	94.8	94.7	94.2	11	pintail duck/ALB/210/2002(H1N1)
12	2.1	3.0	2.6	1.8	2.1	2.2	2.2	2.1	1.8	2.8	2.3		98.7	98.0	98.0	97.8	97.3	98.0	97.9	97.6	97.6	98.5	97.6	97.3	92.7	93.0	93.0	92.5	94.5	94.7	95.3	94.9	94.2	12	mallard/ALB/129/1991(H3N6)
13	1.6	1.8	2.4	0.7	1.6	1.5	1.7	0.9	0.9	1.6	1.8	1.4		99.1	98.9	98.9	98.4	99.1	98.8	98.5	98.5	98.1	98.5	97.5	93.8	93.5	93.5	93.6	95.6	94.9	96.0	95.8	94.6	13	mallard duck/ALB/25/1985(H4N6)
14	2.1	1.5	3.1	0.5	2.1	1.3	2.2	0.7	0.9	1.4	2.3	2.1	0.9		98.9	99.1	98.4	99.3	98.3	97.6	97.6	97.4	98.1	97.1	94.0	93.0	93.0	93.8	95.8	94.3	95.6	96.1	94.4	14	mallard duck/ALB/7/1987(H8N4)
15	1.8	2.0	3.1	0.9	2.1	1.5	1.9	1.1	0.7	1.8	2.1	2.1	1.1	1.1		98.8	98.0	98.9	98.5	97.6	97.6	97.4	98.3	97.5	93.8	93.0	93.0	93.6	95.4	94.3	95.7	95.8	94.8	15	green-winged teal/ALB/228/1985(H7N3)
16	2.2	1.8	3.1	0.7	2.2	1.5	2.3	0.9	1.1	1.6	2.4	2.3	1.1	0.9	1.2		98.2	99.1	98.1	97.4	97.4	97.2	97.9	96.9	93.8	92.9	92.9	93.6	95.6	94.3	95.3	95.8	94.2	16	mallard duck/ALB/321/1988(H9N2)
17	2.5	2.5	3.6	1.1	2.8	1.7	2.9	1.4	1.8	1.1	3.0	2.8	1.6	1.6	2.1	1.8		98.4	97.6	97.6	97.6	96.7	97.5	96.6	93.3	92.4	92.4	93.0	95.2	93.8	94.8	95.2	93.7	17	pintail/Alberta/202/2000(H10N7)
18	2.1	1.5	3.1	0.5	2.1	1.3	2.2	0.7	0.9	1.4	2.3	2.1	0.9	0.7	1.1	0.9	1.6		98.3	97.6	97.6	97.4	98.1	97.1	94.0	93.0	93.0	93.8	95.8	94.3	95.5	96.1	94.4	18	redhead duck/ALB/357/1983(H11N9)
19	2.2	2.4	3.2	1.5	2.4	2.1	2.5	1.7	1.2	2.4	2.6	2.2	1.2	1.7	1.5	1.9	2.4	1.7		97.5	97.5	97.4	98.7	97.9	93.5	92.7	92.7	93.3	95.1	94.2	96.1	95.3	94.9	19	mallard duck/ALB/763/1983(H11N8)
20	2.9	3.1	3.5	2.2	2.9	2.8	3.0	2.4	2.2	2.6	3.1	2.4	1.5	2.4	2.4	2.6	2.4	2.4	2.5		100.0	97.3	97.3	96.7	92.5	92.4	92.4	92.2	94.4	93.9	94.7	94.6	93.6	20	mallard/ALB/124/1991(H11N2)
21	2.9	3.1	3.5	2.2	2.9	2.8	3.0	2.4	2.2	2.6	3.1	2.4	1.5	2.4	2.4	2.6	2.4	2.4	2.5	0.0		97.3	97.3	96.7	92.5	92.4	92.4	92.2	94.4	93.9	94.7	94.6	93.6	21	mallard/ALB/126/1991(H11N9)
22	2.9	3.2	3.5	2.4	2.7	3.0	3.0	2.6	2.4	3.4	3.1	1.5	1.9	2.7	2.7	2.9	3.4	2.7	2.6	2.8	2.8		97.1	96.3	92.1	92.2	92.2	91.9	94.3	93.9	94.5	94.4	93.1	22	green-winged teal/ALB/199/1991(H12N5)
23	2.3	2.8	3.5	1.7	2.7	2.3	2.8	1.9	1.7	2.5	2.9	2.4	1.5	1.9	1.7	2.2	2.5	1.9	1.4	2.8	2.8	3.0		97.4	93.3	92.7	92.5	93.0	94.6	93.9	95.6	94.8	94.3	23	goose/MN/5733-1/1980(H9N2)
24	2.8	3.7	4.1	2.8	3.2	3.1	2.9	3.0	2.5	3.7	3.0	2.8	2.5	3.0	2.5	3.2	3.5	3.0	2.2	3.4	3.4	3.8	2.6		92.2	92.1	92.1	92.0	93.8	93.4	95.3	94.0	96.2	24	duck/MN/1516/1981(H5N2)
25	2.3	1.9	3.5	0.8	2.3	1.6	2.4	0.8	1.1	1.8	2.5	2.5	1.3	1.1	1.3	1.3	1.9	1.1	1.7	2.8	2.8	3.2	1.9	3.0		97.8	97.8	99.8	98.3	96.6	98.3	98.5	97.2	25	mallard/MD/R274/98(H6N5)
26	1.2	3.0	3.2	1.9	0.7	2.5	0.6	2.2	2.2	2.9	0.7	2.2	1.7	2.2	2.2	2.3	2.9	2.2	2.5	2.9	2.9	3.0	2.5	3.2	2.3		99.5	97.5	97.2	96.9	97.5	97.6	96.8	26	mallard/MN/346233/00(H6N3)
27	1.2	3.0	3.2	1.9	0.7	2.5	0.6	2.2	2.2	2.9	0.7	2.2	1.7	2.2	2.2	2.3	2.9	2.2	2.5	2.9	2.9	3.0	2.8	3.2	2.3	0.5		97.5	97.2	96.9	97.5	97.6	96.8	27	northern pintail/AK/3209/02(H3)
28	2.5	2.2	3.8	1.1	2.5	1.8	2.7	1.1	1.3	2.0	2.8	2.8	1.6	1.3	1.6	1.6	2.2	1.3	1.9	3.0	3.0	3.4	2.2	3.3	0.2	2.5	2.5		98.1	96.3	98.1	98.2	96.9	28	laughing gull/NY/2455/00(H7N3)
29	2.9	0.3	3.8	1.2	2.9	1.8	3.0	1.4	1.4	2.1	3.1	2.9	1.6	1.4	1.9	1.6	2.1	1.4	2.2	3.0	3.0	3.1	2.7	3.6	1.7	2.9	2.9	1.9		96.3	97.6	98.2	96.6	29	Chicken/NY/13142-5/94 (H7N2)
30	3.1	4.0	0.0	2.8	3.3	3.2	3.2	3.1	2.8	3.6	3.3	2.6	2.4	3.1	3.1	3.1	3.6	3.1	3.2	3.5	3.5	3.5	3.5	4.1	3.5	3.2	3.2	3.8	3.8		96.9	96.8	95.7	30	Chicken/NY/13202-7/96 (H7N2)
31	2.2	2.6	3.2	1.5	2.5	2.4	2.6	1.8	1.3	2.5	2.5	2.0	1.3	1.6	1.5	2.0	2.5	1.8	1.2	2.6	2.6	2.8	1.6	2.0	1.7	2.5	2.5	1.9	2.5	3.2		98.0	97.9	31	mallard/WI/428/1975(H5N1)
32	2.4	2.0	3.3	0.9	2.4	1.8	2.5	1.2	1.2	1.9	2.6	2.4	1.4	1.2	1.4	1.4	2.1	1.2	2.0	2.7	2.7	3.0	2.5	3.3	1.6	2.4	2.4	1.8	1.9	3.3	2.0		96.9	32	turkey/MN/3689-1551/1981(H5N2)
33	3.0	3.6	4.5	2.7	3.5	3.3	3.1	3.0	2.5	3.7	3.2	3.2	2.7	3.0	2.5	3.2	3.7	3.0	2.4	3.8	3.8	4.3	3.1	1.1	2.9	3.3	3.3	3.2	3.5	4.5	2.1	3.2		33	turkey/OR/71(H7N3)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33		

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