Prevalence, Transmission and Evolution of Avian Influenza Viruses in Newfoundland and Labrador

by

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Abstract

The island of Newfoundland is an important location for the breeding and wintering of millions of subarctic birds. The adjacency of the island to Greenland and western Europe indicates the possibility of inter-continental viral transmission by pelagic birds. In this thesis, the prevalence and transmission of avian influenza viruses (AIVs) in 3 major bird groups (duck, gull and murre) were investigated to shed light on AIV ecology and evolution at this region.

The epidemiological study of AIVs in ducks (2008-2011) revealed an overall virus detection rate of 7.2%. The viral prevalence differed significantly by bird age and sampling season. Although the AIV detection rates were much lower in gulls (1.8%) and murres (3.9%) during 2009-2011, virus prevalence also displayed strong variability by bird age and season. In addition, serological study revealed a much higher frequency of AIV infection in ducks, gulls and murres compared to results by virus detection alone.

The gene sequences of 30 duck AIVs (2008-2011) in Newfoundland and 79 reference duck AIVs (2006-2010) from the Atlantic bird flyway of North America were analyzed to reveal their genetic structure and the extent of gene flow. The genetic structure differed amongst the 8 viral segments with the highest diversity being found in the HA and NA segments. These viruses showed rare inter-continental transmission, but frequent reassortment, and frequent interspecies and North American inter-flyway distribution.

The gull AIVs in Newfoundland (2009-2011) showed frequent inter-continental and cross-host group transmission. The study also revealed a larger than previously detected AIV gene reservoir in gulls in Atlantic Canada.

The 21 H1N2 AIVs identified from Common Murre in summer 2011 belonged to 4 genotypes. The major genotype had been circulating in the murre population for a while before detection, as indicated by its genetic heterogeneity. The murre viruses displayed a mainly waterfowl-related gene pool with considerable inter-continental and avian-gull gene reassortments.

The results of this thesis work provided a profile of AIV prevalence in Newfoundland, and increased our understanding of AIV ecology and evolution in wild birds of Atlantic Canada. Besides continuing the AIV surveillance in gulls, murres and dabbling ducks, future surveillance work should expand to include sea ducks and shorebirds, to better reveal the dynamics of AIV evolution and transmission in this region.

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List of Symbols, Nomenclature or Abbreviations

AIV	avian influenza A virus
ELISA	Enzyme-linked immunosorbent assay
HA	hemagglutinin
HPAI	highly pathogenic avian influenza
IAV	Influenza A virus
LRT	log-likelihood ratio tests
M1	matrix protein
M	matrix protein
MEGA	Molecular evolutionary genetic analysis
M2	ion channel protein
NA	neuraminidase
NEP	nucleotide export protein
NP	nucleocapsid protein
NS	non-structural protein
PA	polymerase acid protein
PB1	polymerase basic protein 1
PB2	polymerase basic protein 2
rRNA	ribosomal RNA
rRT-PCR	real-time RT-PCR
SA	sialic acid
tMRCA	the times of most common ancestor

vRNA	viral RNA
vRNP	viral ribonucleoprotein complex

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Appendix 5- Supplemental tables for Summary

Appendix 6 – Other scientific contributions

1. Wille M, Huang Y, Robertson GJ, Ryan P, Wilhelm SI, Fifield D, Bond AL, Granter A, Munro H, Buxton R, Jones IL, Fitzsimmons MG, Burke C, Tranquilla LM, Rector M, Takahashi L, Kouwenberg AL, Storey A, Walsh C, Hedd A, Montevecchi WA, Runstadler JA, Ojkic D, Whitney H and Lang AS. Evaluation of seabirds in Newfoundland and Labrador, Canada, as hosts of influenza A viruses. J Wildl Dis. 2014, 50(1):98-103.

2. Huang Y, Khan M and Măndoiu II. Neuraminidase Subtyping of Avian Influenza Viruses with PrimerHunter-Designed Primers and Quadruplicate Primer Pools. PLoS One. 2013, 8(11):e81842.

Chapter 1 - Introduction and Overview

1.1 The classification of avian influenza virus

Influenza viruses are a group of single-stranded, negative-sense and segmented RNA viruses belonging to the family *Orthomyxoviridae*. There are six viral genera in *Orthomyxoviridae*: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Isavirus*, *Thogotovirus* and the newly classified *Quaranjavirus* (Presti, Zhao et al. 2009, King, Adams et al. 2012). The three *Influenzavirus* genera are separated according to the antigenic differences in their matrix (M1) and nucleocapsid (NP) proteins (Palese 1977). Influenza viruses in different genera have different host ranges. Influenza A virus (IAV) has a broad spectrum of hosts including birds and mammals, influenza B virus is detected in human infections, while influenza C virus infects humans and pigs (Hinshaw, Naeve et al. 1983, Hinshaw, Bean et al. 1984, Hinshaw, Bean et al. 1986, Chambers, Hinshaw et al. 1991, Webster, Bean et al. 1992, Yoon, Cooper et al. 2005, Ducatez, Webster et al. 2008, Smith, Bahl et al. 2009).

According to the major host taxa, IAV is classified as swine influenza virus, human influenza virus, equine influenza virus, etc. Avian influenza viruses (AIVs) are IAVs that infect birds. Based on the genetic and antigenic differences of the two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), IAVs are classified into 18 HA and 11 NA subtypes, and all viral subtypes have been detected in birds except for the newly identified H17N10 and H18N11 viruses from bats (Hinshaw, Air et al. 1983, Webster, Bean et al. 1992, Krauss, Walker et al. 2004, Fouchier, Munster et al. 2005, Veits, Weber et al. 2012, Tong, Zhu et al. 2013).

1.2 The genome, replication and molecular features of avian influenza viruses

IAV virions are spherical, filamentous or pleomorphic in shape and 80-120 nm in size (Webster, Bean et al. 1992). The virus is enveloped by a host-derived membrane, with HA, NA and ion channel (M2) proteins embedded in the bi-layer membrane. Matrix proteins lie beneath the membrane and determine the structure of the IAV (Figure 1.1) (Webster, Bean et al. 1992, Horimoto and Kawaoka 2005).

The IAV genome has a total size of ~13.5kb and is composed of 8 viral RNA (vRNA) segments with lengths ranging from 0.89 to 2.3kb. These are segment 1 or PB2 gene (polymerase basic protein 2 gene, ~2.3kb), segment 2 or PB1 gene (polymerase basic protein 1 gene, ~2.3kb), segment 3 or PA gene (polymerase acid protein gene, ~2.2kb), segment 4 or HA gene (hemagglutinin gene, ~1.7kb), segment 5 or NP gene (nucleoprotein gene, ~1.6kb), segment 6 or NA gene (neuraminidase gene, ~1.4kb), segment 7 or M gene (matrix protein gene, ~1.0kb) and segment 8 or NS gene (non-structural protein gene, ~0.9kb) (Webster, Bean et al. 1992, Horimoto and Kawaoka 2005). For each of the 8 vRNA segments, there are 12 and 13 conserved nucleotides at their 3' and 5' ends, respectively, which are partially complementary and form closed panhandle structures (Robertson 1979, Desselberger, Racaniello et al. 1980). Each of the vRNA segments is associated with multiple copies of the NP monomer, and a single copy of the polymerase heterotrimer (PB2, PB1 and PA subunits), comprising the viral ribonucleoprotein (vRNP) complex in a helical supercoiled conformation, forming the core of the AIV virion (Murti, Webster et al. 1988, Horimoto and Kawaoka 2005).

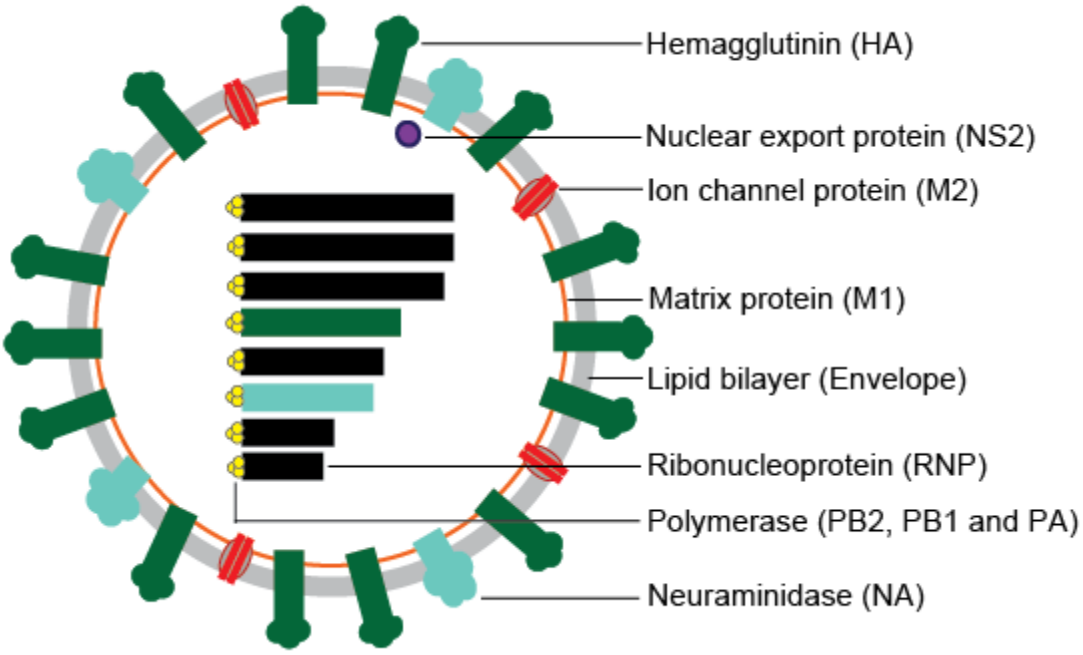

Figure 1.1. Structure of influenza A virus. The cartoon shows a spherical-shaped influenza A virus (IAV) particle. The virus is enveloped, with 3 proteins (HA, NA and M2) inserted in the lipid bilayer. The M1 protein lies beneath the envelope, and forms the shell of the virus. Each of the 8 gene segments is associated with multiple NP monomers (not shown) and a copy of RNA dependent RNA polymerase (PB2, PB1 and PA proteins) to form the core of the IAV (RNPs). One of the several non-structural proteins of IAV, NEP, is also illustrated in this picture.

The viral infection starts from the adsorption and entry of the viruses into target cells, a process accomplished by the HA protein (Skehel and Wiley 2000). HA is composed of the HA1 and HA2 subunits linked by a disulphide bond. HA binds to sialic acid (SA)-terminated cellular receptors in the form of a homotrimer (Wiley and Skehel 1987, Horimoto and Kawaoka 2005). The globular head of the HA trimer, composed of a

component of the HA1 subunit, possesses the receptor binding sites. The stem of the HA trimer, composed of the HA2 subunit and part of HA1, participates in the fusion of the virus envelope with the host cell membrane in a low pH condition (Wharton, Skehel et al. 1986, Daniels, Jeffries et al. 1987, Wiley and Skehel 1987, Patterson, Swainsbury et al. 1999, Skehel and Wiley 2000).

Once the viral membrane is fused with the endosomal membrane of the host cells, M2 proteins participate in the uncoating of IAVs by dissolving the M1 proteins and release of the viral core (vRNP polymerase complex) into the cell cytoplasm (Ito, Gorman et al. 1991, Horimoto and Kawaoka 2005). The vRNP polymerase complex is then transported to the nucleus and initiates the transcription and replication of the vRNAs (Coloma, Valpuesta et al. 2009, Resa-Infante, Recuero-Checa et al. 2010). The progeny vRNP is exported from the cell nucleus to the cytoplasm, a process mediated by the nuclear export protein (NEP, NS2) (Paterson and Fodor 2012). The NS1 protein also facilitates the above process through inhibiting the interferon function of the host cells, and restraining the nuclear export of host mRNA (Hale, Barclay et al. 2008, Hale, Randall et al. 2008, Paterson and Fodor 2012).

The translation of viral proteins takes place in the cell cytoplasm, while the assembly and budding of progeny virions take place on the membrane of the infected cells. The ion channel protein (M2) plays a crucial role in AIV budding by mediating the scission of the host cell membrane and facilitating the release of the virions (Rossman and Lamb 2011). NEP also participates in the viral assembly and budding process, as indicated by the interaction of NEP with the M1 protein, and with a cellular ATPase (Yasuda, Nakada et al. 1993, Gorai, Goto et al. 2012, Paterson and Fodor 2012). NA, the

homotetramized glycoprotein on the surface of the viral particle, also plays an important role in viral budding. It has sialidase enzymatic properties, which cleaves sialic acids from the viral receptor and releases the progeny virion from the cell surface (Meindl, Bodo et al. 1974, Palese, Schulman et al. 1974, Palese, Tobita et al. 1974).

Besides the 10 AIV proteins mentioned already (PB2, PB1, PA, HA, NP, NA, M1, M2, NS1 and NEP), additional proteins encoded by the IAV genome have been identified more recently (PB1-F2, PB1-N40, PA-X, PA-N155, PA-N182 and M42). Many IAVs express a functional PB1-F2 protein, which is encoded by the +1 open reading frame of the PB1 gene and contributes to viral pathogenicity (Chen, Calvo et al. 2001, Conenello, Tisoncik et al. 2011, Varga and Palese 2011). N40 is the third protein encoded by the PB1 gene. It lacks the 39 N-terminal amino acid (aa) of the PB1 protein and its function is yet unclear (Wise, Foeglein et al. 2009, Tauber, Ligertwood et al. 2012). Besides PA, the PA gene also encodes for the PA-X, PA-N155 and PA-N182 proteins. The PA-X protein (41 or 61 aa in length in most IAVs) decreased the pathogenicity of the virus during infection of mice (Jagger, Wise et al. 2012, Shi, Jagger et al. 2012). PA-N155 and PA-N182 are truncated PA proteins lacking the 11 and 13 N-terminal amino acids of PA, respectively. The presence of these two proteins facilitated viral replication and increased viral virulence in mouse infection models (Muramoto, Noda et al. 2013). Besides the M1 and M2 proteins, a third protein (M42), encoded by the M gene of IAVs, was recently discovered. It is a variant form of the M2 protein, but its function is not fully understood (Wise, Hutchinson et al. 2012).

1.3 The ecology of avian influenza virus

1.3.1 The natural reservoir of avian influenza virus

Previous research has shown that wild aquatic birds are the natural reservoir of influenza A virus with the viruses having been detected from at least 105 wild bird species globally (Webster, Bean et al. 1992, Olsen, Munster et al. 2006, Munster and Fouchier 2009). AIVs in wild birds transmit mainly through the fecal-oral mode and most often cause asymptomatic enteric infections (Webster, Bean et al. 1992, Fouchier and Munster 2009), but the first AIV isolate from wild birds, from the Common Tern (*Sterna hirundo*), was indeed highly pathogenic (Becker 1966). The distribution of AIVs in wild birds is greatly influenced by the birds' ecology, for example migratory pattern and population size (Munster and Fouchier 2009), but abiotic environments, such as water and lake sediment, also contribute greatly to the persistence of AIVs (Hinshaw, Webster et al. 1979, Lang, Kelly et al. 2008, Brown, Goekjian et al. 2009).

1.3.2 AIV transmission from wild birds to other host groups

The pathogenicity and transmissibility of AIVs may change during their transmission from wild birds to other host groups (e.g. poultry and mammals), which has caused great economic losses to the livestock and poultry industries and had negative outcomes for human health (Chen, Deng et al. 2004, Watanabe, Ibrahim et al. 2012). AIV infection has been frequently detected in domestic poultry (e.g. chickens and turkeys), less frequently in domestic mammals (e.g. swine, horses and dogs), sea mammals (e.g. seals and whales) and humans (Hinshaw, Naeve et al. 1983, Hinshaw, Bean et al. 1984, Hinshaw, Bean et al. 1986, Webster, Bean et al. 1992, Cardona, Xing et al. 2009). Ferrets and mice can also be infected by AIVs and are commonly used as experimental models

for AIV infection in humans (Matsuoka, Lamirande et al. 2009, Matsuoka, Lamirande et al. 2009).

1.3.3 AIV infection in poultry

Wild birds may transmit AIVs to domestic waterfowl via shared water use, and domestic waterfowl may then transmit the viruses to terrestrial poultry (e.g. chickens and turkeys) at farms, through commercial transportation or at live bird markets (Cardona, Xing et al. 2009). Direct transmission from wild birds to poultry can also occur. AIV infections in chickens and turkeys, of both lowly and highly pathogenic strains, are frequently reported (Alexander 2000). AIV infections in ducks and geese are more often asymptomatic or only mildly symptomatic, although the viral pathogenicity may change through mutation during long-time AIV circulation in bird populations (Webster, Peiris et al. 2006, Taubenberger and Kash 2010). Low pathogenic AIV infections in poultry may be asymptomatic or produce mild symptoms such as a decreases in activity, feed consumption and egg production, as well as respiratory symptoms (Alexander 2000). Highly pathogenic AIVs, on the other hand, may cause severe systemic hemorrhagic infections resulting in high mortality, which can be devastating for the poultry industry (Alexander 2000).

1.3.4 AIV infection in swine

Swine influenza is an important infectious disease that is a hazard to the swine industry (Van Reeth 2007). More importantly, swine can be infected by both human and avian influenza viruses, and may serve as the intermediate host in human infections of AIVs (Trifonov, Khiabanian et al. 2009). The viruses responsible for the last 4 influenza pandemics in human history, i.e. the H1N1/1918 IAV, H2N2/1957 IAV, H3N2/1968 IAV

and novel H1N1/2009 IAV, were all genetically related to contemporary swine influenza viruses (Garten, Davis et al. 2009, Smith, Bahl et al. 2009). The transmission of IAVs between swine and humans can be bi-directional, as was found in the 2009 pandemic H1N1 virus (H1N1/2009) (Nelson, Gramer et al. 2012). At the same time, swine influenza viruses with gene segments of avian-origin are commonly detected, and the above 4 pandemic influenza viruses all possessed avian-origin gene segments (Horimoto and Kawaoka 2005, Garten, Davis et al. 2009, Smith, Bahl et al. 2009). In fact, AIVs of subtypes H1, H3, H4, H5, H6 and H9 have been reported to cause infection in swine (He, Zhao et al. 2013); (Van Reeth 2007). Experimental study also demonstrated that low pathogenic AIVs with subtypes ranging from H4 to H13 could replicate in the respiratory tract of pigs and produce high antibody titres detected by the enzyme-linked immunosorbent assay (ELISA) and neutralization tests (Kida, Ito et al. 1994). Although AIVs are frequently detected in swine, only a few of them have become established in the swine population, indicating a host barrier that hinders viral establishment (Van Reeth 2007). For example, highly pathogenic H5N1 viruses only caused asymptomatic to mild infections in pigs with restricted viral replication, mainly in the lungs, and the viruses could not efficiently transmit through aerosol among pigs in experiments (Choi, Nguyen et al. 2005, Lipatov, Kwon et al. 2008). Epidemiological surveillance in Indonesia and China has also detected asymptomatic infection in pigs by highly pathogenic H5N1 AIVs (Nidom, Takano et al. 2010, He, Zhao et al. 2013).

1.3.5 AIV infection in humans

Human infections with influenza A viruses are zoonotic, directly or indirectly linked to viruses in birds or non-human mammals, especially swine. As introduced in

Section 1.3.4, swine may work as a "gene mixing vessel" between avian and human influenza viruses. In addition, humans can also be infected by AIVs directly from birds. Our understanding of this means of transmission started with human infection by the highly pathogenic H5N1 AIVs in 1997 in Hong Kong, China (Claas, Osterhaus et al. 1998, Webster, Peiris et al. 2006). The H5N1 viruses have been endemic since then in poultry, and pose great public concern due to their fast evolution and the potential for aerosol transmission among humans (Guan, Poon et al. 2004, Watanabe, Ibrahim et al. 2012).

AIVs of H5, H6, H7 and H9 subtypes have been reported to cause mild or fatal human infections (Subbarao, Klimov et al. 1998, Peiris, Yuen et al. 1999, Fouchier, Schneeberger et al. 2004, Tweed, Skowronski et al. 2004, Watanabe, Ibrahim et al. 2012, Yuan, Zhang et al. 2013, Yuan, Zhang et al. 2013). Fortunately, persistent human-to-human transmission of these AIVs has not occurred. However, recent studies showed that only several amino-acid changes were needed for a highly pathogenic H5N1 virus to acquire aerosol transmission in ferrets (Herfst, Schrauwen et al. 2012, Russell, Fonville et al. 2012), a preferred animal model of human IAV infection.

1.4 Evolution of avian influenza virus

AIVs undergo continuous evolution through time, space and host species (Taubenberger and Kash 2010). The mechanisms of AIV evolution include point mutation, deletion, insertion, recombination and reassortment. Point mutation and reassortment are the most commonly observed causes of AIV evolution.

1.4.1 Point mutation

The RNA-dependent RNA polymerase of IAVs lacks proof-reading activity, which causes errors in RNA synthesis and generates substitutions in the AIV genome at a rate of of 10^{-3} per site per year (Webster, Bean et al. 1992, Chen and Holmes 2006). Point mutation can help the virus respond to the selective pressure from changes in the cellular receptors and the activities of extracellular inhibitors (e.g. antibodies and drugs). It may also alter the pathogenicity and transmissibility of the viruses, especially during cross-host transmission.

1.4.1.1 Point mutations and antigenic drift

Antigenic drift may occur when mutation takes place in the two major immune-related proteins of IAVs, HA and NA. Such mutations help the virus to adapt to the selective pressure of host immunity generated by previous virus infections or vaccinations. Taking the highly pathogenic H5N1 AIV as an example, since its first detection in 1996 in China, multiple HA phylogenetic and antigenic clades have developed in poultry in Eurasia and Africa (Davis, Balish et al. 2010, WHO 2012). In countries that perform vaccination to control the H5N1 virus infection in poultry, the vaccine strains have been replaced and updated several times in the last decade to ensure their antigenic match to the circulating H5N1 viruses (Chen and Bu 2009). This situation poses a great challenge to future vaccine design and the formulation of virus control measures (Cattoli, Fusaro et al. 2011).

1.4.1.2 Point mutations and the switch of HA receptor-binding properties

The change of HA receptor binding affinity from avian α-2, 3 to mammalian α-2, 6 SA-linked cellular receptors is the prerequisite for AIVs to efficiently transmit among

mammals (Harvey, Martin et al. 2004, Taubenberger and Kash 2010). This mechanism has contributed to the origin of pandemic influenza strains (Matrosovich, Tuzikov et al. 2000). Experiments in the ferret model with two H2N2 viruses isolated during the 1957/1958 influenza pandemic, showed that the HA protein with amino acid combination of 226L/228S (H3 numbering) showed binding affinity to both α-2, 3 and α-2, 6 SA-linked cellular receptors and the IAV displayed efficient aerosol transmission among ferrets, while the other virus with the 226Q/228G HA protein preferred binding to α-2,3 SA-linked cellular receptors and showed inefficient viral transmission through droplets (Pappas, Viswanathan et al. 2010). The HA mutations at amino acid positions 190 and 225 (H3 numbering) of H1N1/1918 virus, changed both the receptor binding preference and the airborne transmissibility of the virus, but not the viral pathogenicity in ferrets (Pappas, Viswanathan et al. 2010).

The highly pathogenic H5N1 AIVs endemic in poultry of Eurasia and Africa possess HA proteins binding preferentially to α-2, 3 SA-linked cellular receptors (Matrosovich, Zhou et al. 1999), which probably explains their inefficient aerosol transmission in pig and ferret models (Yen, Lipatov et al. 2007). However, the experiment performed in 2012 with A/Indonesia/5/2005 A/H5N1influenza virus showed that only 5 amino acid mutations, 4 in the receptor binding sites of HA and 1 in the PB2 protein, were needed for the virus to gain efficient airborne transmissibility in the ferret model (Spekreijse, Bouma et al. 2011). The results reinforced again the pandemic potential of the highly pathogenic H5N1 viruses.

1.4.1.3 Point mutations and the change of viral pathogenicity

The HA protein is synthesized as a single polypeptide (HA0) that is then cleaved into two parts (HA1 and HA2). Mutation of the HA cleavage site from monobasic to polybasic amino acids will change its proteolytic properties and may increase AIV pathogenicity in birds, which has been observed in viruses of H5 and H7 subtypes in the field (Horimoto and Kawaoka 1994, Rohm, Suss et al. 1996, Selleck, Arzey et al. 2003, Duan, Campitelli et al. 2007, Berhane, Hisanaga et al. 2009, Stech, Veits et al. 2009), and also in H2, H4, H8, H9 and H14 viruses through experimentation (Gohrbandt, Veits et al. 2011, Veits, Weber et al. 2012). HA proteins containing monobasic amino acids at their cleavage sites can only be cleaved and activated by monobasic-specific trypsin-like proteases, which are mainly distributed in the respiratory and intestinal tracts of birds (Suarez and Schultz-Cherry 2000, Lebarbenchon and Stallknecht 2011). Consequently, these AIVs usually cause limited infection displaying mild or inapparent symptoms. On the other hand, if the amino acids at the HA cleavage site are mutated to a polybasic pattern, the protein can be cleaved by the ubiquitous protease furin, which will result in extensive viral replication and may cause multi-organ systemic infection in birds (Stieneke-Grober, Vey et al. 1992, Suarez and Schultz-Cherry 2000).

1.4.1.4 Point mutations and influenza drug resistance

Two types of influenza drugs are commercially available, the M2 ion channel inhibitors (amantadine and rimantadine) and the neuraminidase inhibitors (oseltamivir and zanamivir) (Beigel and Bray 2008). Unfortunately, single amino acid mutation of these viral proteins can produce drug-resistant viruses. Viruses containing the L26I, V27I, A30P, S31N or G34E mutation in their M2 protein are amantadine-resistant (Deyde, Nguyen et al. 2009). For example, the newly emerged novel H7N9 influenza A viruses

are resistant to amantadine because of the S31N mutation within the M2 proteins (Hay, Wolstenholme et al. 1985, Wang, Wu et al. 2013). In addition, the highly pathogenic H5N1 viruses circulating in poultry in Asian countries such as China, Vietnam, Malaysia, Indonesia and Cambodia are widely amantadine-resistant, coincident with the extensive use of amantadine in these countries (Hurt, Selleck et al. 2007, He, Qiao et al. 2008). Point mutation may also cause resistance to neuraminidase inhibitor treatment (Tisoncik-Go, Cordero et al. 2013). For example, the R292K mutation of the NA protein (N2 numbering) has been reported to confer resistance to NA inhibitors in N1, N2 and N9 subtype AIVs (McKimm-Breschkin, Sahasrabudhe et al. 1998, Kiso, Ozawa et al. 2011, Yen, McKimm-Breschkin et al. 2013).

1.4.1.5 Other point mutations during cross-host virus transmission

Besides the above-described AIV genes, point mutation in other AIV gene segments may also facilitate cross-host viral transmission or increase the pathogenicity of AIV infection, as reflected in the following examples. The E627K mutation in PB2 of the H5N1 AIVs could contribute to systemic infection and impaired T cell activation in mice (Hatta, Gao et al. 2001, Fornek, Gillim-Ross et al. 2009). The D701N mutation in PB2 was a prerequisite for the transmission of H5N1 viruses in a mammalian guinea pig model (Li, Chen et al. 2005). The D92E mutation in the NS1 protein is associated with increased pathogenicity of an H5N1 AIV in pigs (Seo, Hoffmann et al. 2002). Lastly, the N66S mutation in the PB1-F2 protein has been suggested to increase the pathogenesis of the H5N1/1997 and H1N1/1918 influenza viruses (Conenello, Zamarin et al. 2007).

1.4.2 Reassortment of AIV gene segments and antigenic shift

When two or more IAVs co-infect the same host cell, the 8 gene segments assembled in a progeny viral particle may possibly come from different parental viruses (Figure 1.2).

When this reassortment happens to the 2 surface glycoproteins (HA and NA), antigenic shift may occur. The reassortant virus may acquire novel antigenic features to facilitate its adaptation and establishment in new host groups (Webster, Bean et al. 1992, Greenbaum, Li et al. 2012). Reassortment is a key role in the generation of novel AIVs among birds (Dugan, Chen et al. 2008, Deng, Tan et al. 2013). For example, the highly pathogenic H5N1 AIVs and the novel H7N9 AIVs endemic in Asia both originated through reassortment, with their surface protein genes (HA and NA) emerging from wild aquatic birds, and the 6 internal protein genes originating from the co-circulating H9N2 AIVs in poultry (Guan, Shortridge et al. 1999, Watanabe, Ibrahim et al. 2012, Chen, Liang et al. 2013, Gao, Cao et al. 2013, Li, Yu et al. 2013).

Reassortment can also occur when the viruses transmit from birds to mammals (e.g. swine and human). For example, the HA and/or NA gene(s) of the pandemic influenza viruses in H2N2/1957, H3N2/1968 and the novel H1N1/2009 originated from swine or avian source, which reassorted and replaced the gene segment(s) of the circulating human influenza viruses during the epidemics (Webster, Bean et al. 1992, Kilbourne 2006, Furuse, Suzuki et al. 2010, Greenbaum, Li et al. 2012).

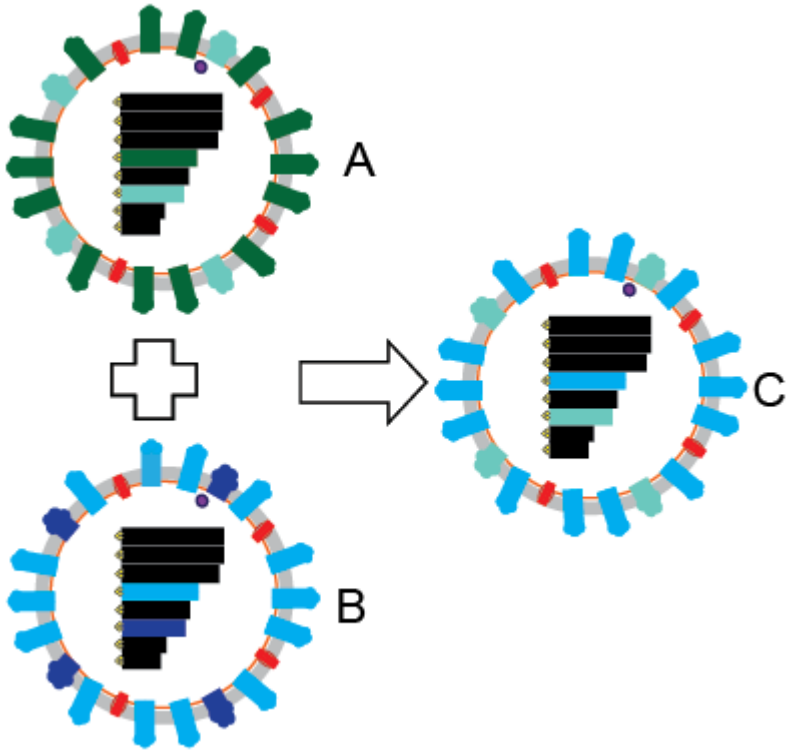

Figure 1.2. Generation of a novel influenza virus through reassortment. When two influenza A viruses (A and B) replicate in a single cell, novel viral progeny (C) can be generated through gene segment reassortment during viral assembly. The virus C contains genes originally from both viruses A and B.

IAV reassortment is restricted by segment mismatch of the different parental viruses during co-infection or lower fitness of the resulting reassortants (Li, Hatta et al. 2008, Greenbaum, Li et al. 2012). The efficiency of AIV reassortment also depends on the infection dose of the parental viruses and the timing of infection (Marshall, Priyamvada et al. 2013). The reassortment compatibility between different contemporary endemic/pandemic IAV strains has been studied in experiments. Octaviani and collegues

reported the high level of genetic compatibility between a highly pathogenic H5N1 virus and a 2009 pandemic H1N1 virus during co-infection in tissue culture experiments (Octaviani, Ozawa et al. 2010). Kimble and co-authors reported the compatibility of the surface genes of an H9N2 AIV with the internal genes of the 2009 pandemic H1N1 SIVs in reverse genetics experiments (Kimble, Sorrell et al. 2011). Sun and co-authors generated 127 H9 reassortants from an avian H9N2 and a pandemic H1N1 virus, and half of the hybrid viruses replicated well in tissue culture (Sun, Qin et al. 2011). Kimble's and Sun's studies also showed that AIV may gain novel properties through reassortment, such as higher pathogenicity and more efficient respiratory droplet transmission.

1.4.3 Other mechanisms of AIV evolution

Besides the major mutation forms of point mutation and reassortment, deletion and non-homologous recombination are occasionally detected during the process of cross-host AIV transmission.

1.4.3.1 Deletion

Different patterns of deletions at the stalk region of the NA gene in AIVs of multiple subtypes (N1–N3, N5–N7 and N9) have been detected from avian hosts such as quail, duck, chicken and turkey (Guo, Krauss et al. 2000, Spackman, Senne et al. 2003, Li, Zu Dohna et al. 2011). The NA stalk deletion is associated with the adaptation and transmission of AIVs in terrestrial birds (Baigent and McCauley 2001, Guan, Peiris et al. 2002, Sorrell, Song et al. 2010). For example, the H7N3 AIVs circulating in turkey in Italy during 2002-2003 possessed a 23-aa deletion in the NA stalk in comparison to the precursor viruses from wild ducks (Campitelli, Mogavero et al. 2004). The NA proteins of the novel H7N9 virus detected in both poultry and human infections in China contain a

4-aa deletion at the stalk region, compared to its precursor in wild birds (Gao, Cao et al. 2013). Research showed that such NA deletions might increase the AIV pathogenicity in chickens and mammals (Matsuoka, Swayne et al. 2009).

1.4.3.2 Non-homologous recombination

As described above, multi-basic cleavage sites in the HA protein are an important molecular signature of highly pathogenic AIVs (Lebarbenchon and Stallknecht 2011, Watanabe, Ibrahim et al. 2012). Besides the mechanism of point mutation, H7 subtype low pathogenic AIVs could acquire multi-basic cleavage sites through non-homologous recombination with either host ribosomal RNA (rRNA) or viral RNA, which is accompanied by the increased pathogenicity of the mutant strains (Maurer-Stroh, Lee et al. 2013). Viral mutants of this type have been observed during experimentation (Khatchikian, Orlich et al. 1989, Orlich, Gottwald et al. 1994) and also in natural infections (Suarez, Senne et al. 2004, Maurer-Stroh, Lee et al. 2013). For example, compared to its low pathogenic precursor, the highly pathogenic H7N3 AIV that caused poultry and human infections in Mexico in 2012, acquired an extended multi-basic cleavage site that was derived from a 24-nucleotide insert of chicken 28S rRNA (Lopez-Martinez, Balish et al. 2013). In addition, the highly pathogenic H7N3 AIVs responsible for the infections in chicken and humans in British Columbia, Canada, in 2004 originated from a low pathogenic AIV precursor with a 21-nucleotide insert of the NP gene at the HA cleavage site (Pasick, Handel et al. 2005).

1.5 Epidemiological surveillance of AIVs in wild birds and genetic structure of viral populations

Epidemiological studies of AIVs in wild birds have greatly increased our understanding of the infection, transmission and pathogenicity of AIVs in multiple host groups, including waterfowl, shorebirds and gulls (Hatchette, Walker et al. 2004, Obenauer, Denson et al. 2006, Dugan, Chen et al. 2008, Munster and Fouchier 2009). Surveillance of AIVs in wild birds started from the early 1970s through virus isolation and HA/NA antibody detection (Slepuskin, Pysina et al. 1972, Slemons, Johnson et al. 1974). Real-time RT-PCR and NP-antibody ELISA are now commonly used in large-scale AIV surveillance (Spackman, Senne et al. 2002, Brown, Stallknecht et al. 2009). AIVs are most frequently detected from birds in the order Anseriformes such as ducks, geese and swans, followed by birds in the order Charadriiformes such as gulls, shorebirds and terns (Becker 1966, Hinshaw, Air et al. 1983, Fouchier, Munster et al. 2005, Clark and Hall 2006, Maxted, Luttrell et al. 2012). However, seabirds are also hosts of AIVs (Wallensten, Munster et al. 2005, Ip, Flint et al. 2008, Granter, Wille et al. 2010, Ramey, Pearce et al. 2010). The employment of large-scale AIV genome sequencing and phylogenetic analysis has detected extensive reassortment and genetic diversity of AIVs in wild birds (Munster and Fouchier 2009). Due to the geographic separation of migratory flyways utilized by different bird species in different continents, AIV gene segments in wild birds are divided into Eurasian/Australian, North American and South American phylogenetic lineages in either waterfowl- or gull-related clades (Obenauer, Denson et al. 2006, Olsen, Munster et al. 2006, Dugan, Chen et al. 2008, Gonzalez-Reiche, Morales-Betoulle et al. 2012). However, AIV distribution does not always follow these constraints,

and inter-continental and interspecies AIV transmission has been detected from long-distance migratory bird species, such as shorebirds, gulls and waterfowl (Krauss, Obert et al. 2007, Dugan, Chen et al. 2008, Koehler, Pearce et al. 2008, Lee, Lee et al. 2011, Van Borm, Rosseel et al. 2012).

All 8 gene segments of AIVs in wild birds display high nucleotide substitution rates with magnitudes of 10^{-3} nucleotide/site/year (Chen and Holmes 2006). However, the genetic diversity of HA, NA and NS genes is much higher compared to the other 5 gene segments (Dugan, Chen et al. 2008). There are 16 HA and 9 NA subtypes detected in wild birds, and the NS gene was classified into 2 different alleles (A and B) (Munster and Fouchier 2009); Ludwig et al. 1991). In comparison, the other 5 internal genes showed much shallower evolutionary divergence (Obenauer, Denson et al. 2006). The deeper divergence in HA, NA and NS may be the outcome of long-term evolution under an increased host immunity they encounter (Dugan, Chen et al. 2008).

1.6 Research goal and outline of this thesis

Despite the extensive surveillance performed in some other areas of North America, the prevalence and diversity of AIVs in wild birds at the North Atlantic coast is understudied (Webster, Bean et al. 1992, Parmley, Lair et al. 2009). Newfoundland and Labrador (NL), Canada, lies at an important location with respect to wild bird connections between North America and Europe (Tuck 1971). Millions of subarctic birds utilize this province and the surrounding marine region yearly as breeding and/or wintering habitat. Previous studies in Atlantic Canada found evidence for inter-continental reassortant viruses, suggesting movement of viral gene segments by migratory

birds and thus a possible entry point for highly pathogenic (HP) Eurasian virus genes and potentially entire viruses (Krauss, Obert et al. 2007, Wille, Robertson et al. 2011, Hall, Teslaa et al. 2013). However, the prevalence of AIVs, the frequency of inter-continental and interspecies viral transmission, and the genetic structure of AIVs in wild birds in NL remain unclear. Since 2007, AIV epidemiological surveillance has been conducted in NL. From more than 5000 swab samples collected from waterfowl, gulls and seabirds, 167 samples were identified as AIV positive. The aim of my PhD thesis is to investigate the prevalence, transmission and evolution of AIVs in 3 major bird groups (ducks, gulls and murres) in NL through virological, serological, phylogenetic and genotypic approaches, to shed light on the AIV ecology of wild birds in NL, and on the Atlantic coast of North America. This will also provide data to allow evaluation of the potential impact of AIVs in wild birds on the local poultry industry and human health, and of the risk of the introduction of HP AIVs from Eurasia by migratory birds of different bird host groups in this area. In chapter 2, the prevalence of AIVs in ducks (2008-2011) was investigated to study the infection pattern, subtype distribution and perpetuation of duck AIVs in NL. In chapter 3, the genetic structure of contemporary duck AIVs (2006-2012) in the Atlantic bird migratory flyway of North America (including 30 viruses from NL) was analyzed to increase our understanding of the phylogeny, distribution and transmission of duck AIVs at the Atlantic coast of North America. In chapter 4, the prevalence, phylogeny and genotype of gull AIVs in NL (from 2009-2011) was studied to shed light on the epidemiological dynamics, population structure and transmission of AIVs in gulls in Atlantic Canada. In chapter 5, the epidemiology, phylogeny and genotype of murre AIVs in NL (from 2009-2011) were analyzed to increase our knowledge on AIV epidemiology

and transmission in these understudied seabirds. The overall results presented in chapters 2 to 5 are summarized in chapter 6.

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Co-authorship Statement

The epidemiological surveillance work performed in my thesis was the collaboration between Dr. Lang's lab in Memorial University of Newfoundland, the Newfoundland and Labrador Department of Natural Resources (Animal Health Division) and the Wildlife Research Division of Environment Canada (Mount Pearl). The epidemiological surveillance was designed and managed by Dr. Hugh Whitney, Dr. Andrew Lang, Dr. Gregory Robertson and myself. The virus screening was performed at the Animal Health Laboratory, University of Guelph (Dr. Davor Ojkic's lab).

Chapter 1 and 6 were drafted by me, with editorial advices from my supervisory committee members, Dr. Andrew Lang, Dr. Hugh Whitney, Dr. Gregory Robertson and Dr. Kensuke Hirasawa.

Chapter 2 is a version of a manuscript published in *Canadian Journal of Microbiology* (Huang Y, Wille M, Dobbin A, Robertson GJ, Ryan P, Ojkic D, Whitney H and Lang AS. A four-year study of avian influenza virus prevalence and subtype diversity in ducks of Newfoundland, Canada. *Canadian Journal of Microbiology.* 2013, 59(10):701-708.). Pierre Ryan collected all the swab samples with the assistance of Michelle Wille, Ashley Dobbin and David Fifield. I performed most of the lab work except the HA subtyping for 5 of the duck AIVs in 2008, which was done by Ashley Dobbin and Michelle Wille. The statistic analysis was conducted by Dr. Gregory Robertson. The manuscript was drafted by Dr. Lang and me with subsequent editorial input from the other co-authors.

Chapter 3 is a manuscript published in *PLoS One* (Huang Y, Wille M, Dobbin A, Walzthoni NM, Robertson GJ, Ojkic D, Whitney H and Lang AS. Genetic structure of avian influenza viruses from ducks of the Atlantic flyway of North America. *PLoS One*. 2014, 9(1):e86999.). I designed the experiment, carried out most of the data analysis and wrote the manuscript with subsequent editorial input from Dr. Lang and the other co-authors. For the gene sequences of the 30 duck AIVs in Newfoundland used in this chapter, Michelle Wille and Ashley Dobbin sequenced most of the 8 duck AIV genes in 2008, I sequenced the other genes, edited and submitted all the sequences to Genbank database. The statistical analysis was conducted by Dr. Gregory Robertson with advice from Dr. David Innes.

Chapter 4 is a manuscript recently published in *Virology* (Huang Y, Wille M, Benkaroun J, Munro H, Bond AL, Fifield DA, Robertson GJ, Ojkic D, Whitney H and Lang AS. Perpetuation and reassortment of gull influenza A viruses in Atlantic Canada. *Virology*. 2014, 456-457:353-363.). The research was designed by Dr. Lang and me. Dr. Gregory Robertson performed the statistical analysis. Dr. Gregory Robertson, Michelle Wille, Hannah Munro, Alexander L. Bond and David Fifield collected all the swab samples. I performed all the lab work, carried out the data analysis, submitted the gene sequences to the Genbank database and wrote the manuscript with editorial assistance from Dr. Lang, and subsequent editorial input from the other co-authors.

Chapter 5 is a manuscript published in *Infection, Genetics and Evolution* (Huang Y, Robertson GJ, Ojkic D, Whitney H and Lang AS. Diverse inter-continental and host lineage reassortant avian influenza A viruses in pelagic seabirds. *Infection, Genetics and*

Evolution. 2014, 22: 103–111.). The research was designed by Dr. Lang and myself. I performed all the lab work, carried out the data analysis, submitted the gene sequences to Genbank database and wrote the manuscript with editorial assistance from Dr. Lang, and subsequent editorial input from the other co-authors.

Chapter 2 A four-year study of avian influenza virus prevalence and subtype diversity in ducks of Newfoundland, Canada

Abstract

We report a 4-year avian influenza virus (AIV) epidemiological study in ducks in the St. John's region of Newfoundland, Canada. The overall prevalence of AIV detection in ducks during this study was 7.2%, with American Black Ducks contributing the vast majority of the collected samples and the AIV positives. The juvenile ducks showed a significantly higher AIV detection rate (10.6%) compared with adults (3.4%). Seasonally, AIV prevalence rates were higher in the autumn (8.4%), but positives were still detected in the winter (4.6%). Preliminary serology tests showed a high incidence of previous AIV infection (20/38, 52.6%). A total of 43 viruses were characterized for their HA-NA or HA subtypes, which revealed a large diversity of AIV subtypes and little recurrence of subtypes from year to year. Investigation of the movement patterns of ducks in this region showed that it is a largely non-migratory duck population, which may contribute to the observed pattern of high AIV subtype turnover. Phylogenetic analysis of 4 H1 and one H5 AIVs showed these viruses were highly similar to other low pathogenic AIV sequences from waterfowl in North America and assigned all gene segments into American-avian clades. Notably, the H1N1 viruses, which were identified in consecutive years, possessed homologous genomes. Such detection of homologous AIV genomes across years is rare, but indicates the role of the environmental reservoir in viral perpetuation.

2.1. Introduction

Wild birds such as waterfowl, shorebirds, and gulls are the natural reservoir of avian influenza A viruses (AIVs) within the family *Orthomyxoviridae* (Webster, Bean et al. 1992, Olsen, Munster et al. 2006). Most AIV strains show low pathogenicity and are generally assumed to cause asymptomatic infections in wild birds (Fouchier and Munster 2009, Munster and Fouchier 2009), although a recent review of the available evidence suggests there are effects on digestive tract function in infected birds (Kuiken 2013). The effects in poultry are generally more obvious and AIVs can cause mild to serious disease; therefore, these viruses pose a great threat to the poultry industry (Alexander 2007). Historically, outbreaks of highly pathogenic (HP) AIV in wild birds have been rare (Becker 1966), but in more recent years several H5N1 strains have been associated with fatal or subclinical infections in wild birds in Eurasia (Ellis, Bousfield et al. 2004, Chen, Smith et al. 2005, Liu, Xiao et al. 2005, Keawcharoen, van Riel et al. 2008). The HP H5N1 viruses have also been a significant source of problems in the poultry industry (Webster, Peiris et al. 2006) and in human health (Peiris, de Jong et al. 2007). This has resulted in concern about the potential risk of transmission of HP H5N1 to the Americas through wild bird migration (Krauss, Obert et al. 2007, Peterson, Benz et al. 2007, Koehler, Pearce et al. 2008, Ramey, Pearce et al. 2010). Additionally, AIV can be involved in the generation of novel strains that circulate in the human population, such as the H1N1 virus in the 2009 pandemic (Smith, Vijaykrishna et al. 2009). These wide-ranging effects make compelling arguments for the value of studying AIV in natural reservoir species.

Prior to 2006, wild bird AIV research in Canada was mainly conducted on waterfowl in Alberta (Hinshaw, Webster et al. 1978, Hinshaw, Webster et al. 1979, Hinshaw, Webster et al. 1980, Hinshaw, Wood et al. 1985, Sharp, Kawaoka et al. 1993, Hatchette, Walker et al. 2004, Krauss, Walker et al. 2004, Widjaja, Krauss et al. 2004) with fewer studies conducted in other provinces (Boudreault, Lecomte et al. 1980, Thorsen, Barker et al. 1980). Most of this work focused on wild waterfowl with a generally high prevalence of AIV infection found, especially in dabbling ducks. In 2005, Canada started a countrywide inter-agency wild bird influenza survey (Parmley, Bastien et al. 2008, Parmley, Lair et al. 2009), which has broadened the geographic scope of surveillance efforts although the overall coverage of different regions in Canada remains fairly limited. A summary of the survey for 2005-2007 showed that AIVs were detected from 30% of live ducks, and all viruses identified to date have been characterized as low pathogenic (Parmley, Bastien et al. 2008, Parmley, Lair et al. 2009, Pasick, Berhane et al. 2010).

To date, there has been limited published work on AIV on the island of Newfoundland, which represents the most easterly part of North America. The prevalence of AIVs in wild birds of different bird host groups in Newfoundland remains unclear. Newfoundland is geographically connected with the mainland Atlantic coast of North America as well as proximate to Greenland, Iceland and Europe. It is long known that migratory birds, from a wide variety of taxa, move between Newfoundland and these other locations (Tuck 1971, Bellrose and Kortright 1976) and Eurasian AIV genes have been detected on the island (Wille, Robertson et al. 2011). Therefore, performing AIV surveillance in Newfoundland is also valuable to monitor for the transmission of AIVs

into North America by trans-Atlantic migrants. We have performed an AIV epidemiological study from 2008 to 2011 in different duck species in the St. John's region of Newfoundland by approaches of both viral screening and serological tests in combination with bird band-recovery data, in order to provide insight into the AIV prevalence and subtype diversity in this location.

2.2. Materials and Methods

2.2.1. Ethics statement

This work was carried out under the guidelines specified by the Canadian Council on Animal Care with approved protocols 09-01-AL, 10-01-AL, and 11-01-AL from the Memorial University Institutional Animal Care Committee, and biosafety permit S-103-08 from the Memorial University Biosafety Committee.

2.2.2. Bird sampling, AIV screening, virus subtyping and sequencing

Ducks were caught by bait trapping in Quidi Vidi Lake (47 °34'42.18"N, 52 °41'59.18"W), Mundy Pond (47 °33'8.09"N, 52 °44'23.12"W), and Commonwealth Pond (47°30'2.54"N, 52°47'22.23"W) in or near to the city of St. John's, Newfoundland. without species priority In the St. John's region, the duck population is dispersed during the spring and summer months for breeding and then congregates to a smaller number of water bodies for the late summer, fall and winter months. This makes capture of birds most feasible beginning in August. Ducks were banded and evaluated for sex and age based on cloacal examination and plumage characters later in the season (Ashley, North et al. 2006). We use the terms juvenile to define birds in their first year of life and adult for birds older than 1 year. Oropharyngeal and cloacal swabs were collected from each

bird, with these paired swabs placed into a single tube of Multitrans viral transport media (Starplex Scientific, Etobicoke, Ontario, Canada) and representing a single sample. Samples were kept cool in the field for less than 24 hours and then stored at -80 ℃ until assayed. RNA was extracted from each sample using the MagMAX-96 viral RNA isolation kit (Ambion, Austin, Texas, USA) following the manufacturer's instructions. Screening of the samples for the presence of AIV was performed by real-time RT-PCR (rRT-PCR) targeting the M gene as described previously (Spackman, Senne et al. 2002, Granter, Wille et al. 2010) with C_t values <35 considered as positive. Hemagglutinin (HA) subtyping was performed by RT-PCR using the SuperScript III One-Step RT-PCR System with Platinum *Taq* (Life Technologies, Burlington, Ontario, Canada) and subsequent sequencing of the RT-PCR products (Chan, Lin et al. 2006). Neuraminidase (NA) subtyping was similarly performed with RT-PCR (Huang, Khan et al. 2010) followed by sequencing.

To address the public health concern raised by the previous human infections by the H1 and H5 influenza A viruses, four samples that were identified to contain H1N1 viruses and one sample that contained an H5N4 virus were selected for gene sequencing and phylogenetic analysis (section 2.2.5). RNA was extracted from the original swab samples with Trizol LS reagent (Life Technologies). One-step RT-PCR reactions were performed to amplify portions of the 8 AIV genes with segment-specific primers (Hoffmann, Stech et al. 2001, Chan, Lin et al. 2006) using the Superscript III One-Step RT-PCR System (Life Technologies). PCR products were purified with the QIAquick PCR purification kit (Qiagen, Toronto, Ontario, Canada) and sequenced. Sequencing was performed at the Centre for Applied Genomics (Hospital for Sick Children, Toronto,

Canada). The sequences have been submitted to the GenBank database under accession numbers: KC464555-KC464586, KC492275-KC492290 and KC492307-KC492330.

2.2.3. Serology

Between October 2011 and February 2012, blood samples were collected from captured individuals to test for previous AIV infection. Approximately 2 ml of blood was drawn from the brachial vein. Serum was separated by centrifugation at ~3000 *g* for 10 minutes to pellet the red blood cells. The AI MultiS-Screen Ab Test (IDEXX, Westbrook, ME) was used to test for anti-nucleoprotein (NP) antibodies, as recommended by the manufacturer, where a sample to negative control ratio (S/N) of <0.50 is considered positive.

2.2.4. Bird banding and recovery data

To study the influence of duck population structure on the prevalence and subtype pattern of AIVs, recovery data (reporting of a bird band) for hunter-killed ducks banded from December 2005 to January 2012 in St. John's region were obtained from the Bird Banding Office, Canadian Wildlife Service, Environment Canada.

2.2.5. Phylogenetic analysis

The H1N1 viruses and the H5N4 virus The nucleotide sequence data were compiled and analyzed using the Lasergene v7.1 sequence analysis software package (DNASTAR Inc., Madison, WI). The coding regions of each gene used for phylogenetic analyses are H1 (1147-1590 bp), H5 (1086-1686 bp), N1 (910-1344 bp), N4 (1004-1392 bp), PB2 (1804-2256 bp), PB1 (1624-2271 bp), PA (1-528 bp), NP (4-576 bp), M (40-939 bp) and NS (55-792 bp). Phylogenetic trees were reconstructed with the p-distance

based neighbor-joining method (with 1000 bootstrap replicates) in MEGA5 (Tamura, Peterson et al. 2011).

2.2.6. Statistical analysis

Prevalence rates by bird age, sex and season of sampling were compared using Fisher's Exact tests (Sokal and Rohlf 2012). P values <0.05 were taken to indicate a significant difference in the compared rates.

2.3. Results

2.3.1. AIV surveillance of ducks in the St. John's region of Newfoundland over four years

A total of 879 swab samples were collected from ducks between September 2008 and February 2012 and 7.2% (63 samples) were positive by real-time RT-PCR. Most of the samples (694/879, 79.0%) and positives (51/63, 81.0%) came from American Black Ducks (*Anas rubripes*), with an overall prevalence of 7.3% for this species. The other positive samples were from Mallard (*A. platyrhynchos*), Northern Pintail (*A. acuta*), American Black Duck-Mallard hybrids, 2 unidentified (pre-fledgling) ducks in the Mallard-American Black Duck complex, and 1 feral domestic duck (Table 2.1).

Most of the samples were collected during the late summer and fall months (596 from August to October), while 283 samples were collected during the winter (November to February) (Figure 2.1; Table S2.1). Within our sampling windows, the peaks of AIV-positive samples were in the fall months (Figure 2.1). Positive samples were also detected during November and December (13 positives) but not in January or February. The AIV

prevalence was higher (50/596, 8.4%) in the fall compared to winter (13/283, 4.6%) ($P = 0.049$).

In the 879 samples, 461 were from juvenile ducks, 417 were from adults, and one was from a bird of unknown age (Table S2.1). The overall AIV prevalence rate in juveniles (49/461, 10.6%) was significantly higher than in the adult ducks (14/417, 3.4%) over the period of our study ($P < 0.0001$). Within age classes, the AIV detection rate in adult males (9/282, 3.2%) was similar to that in adult females (4/131, 3.1%) ($P = 1.00$), whereas prevalence was higher in juvenile males (38/288, 13.2%) than in juvenile females (11/171, 6.4%) ($P = 0.028$). One juvenile Mallard was trapped 6 times over a 22-day period in 2010 (22 and 23 November; 1, 3, 6, and 15 December). This individual was AIV positive by rRT-PCR on 23 November but negative at the other samplings, providing a duration of detected virus shedding of $\leq$8 days.

Table 2. 1. Surveillance data for AIV infection by rRT-PCR by species, age, gender, and season in ducks in the St. John's region of Newfoundland

	Species						Age		Sex		Season*	
	American Black Duck	Mallard	Northern Pintail	Hybrid†	Mallard type‡	Domestic	Juvenile	Adult	Male	Female	Summer-Fall	Winter
Samples	694	109	43	27	4	2	461	417	571	302	596	283
Positives	51	3	4	2	2	1	49	14	47	15	50	13
%	7.3	2.8	9.3	7.4	50	50	10.6	3.4	8.2	5.0	8.4	4.6

* Summer-Fall, May-October; Winter, November-February

† American Black Duck x Mallard hybrid

‡ Pre-fledgling, exact identification not possible; either American Black Duck, Mallard or a hybrid

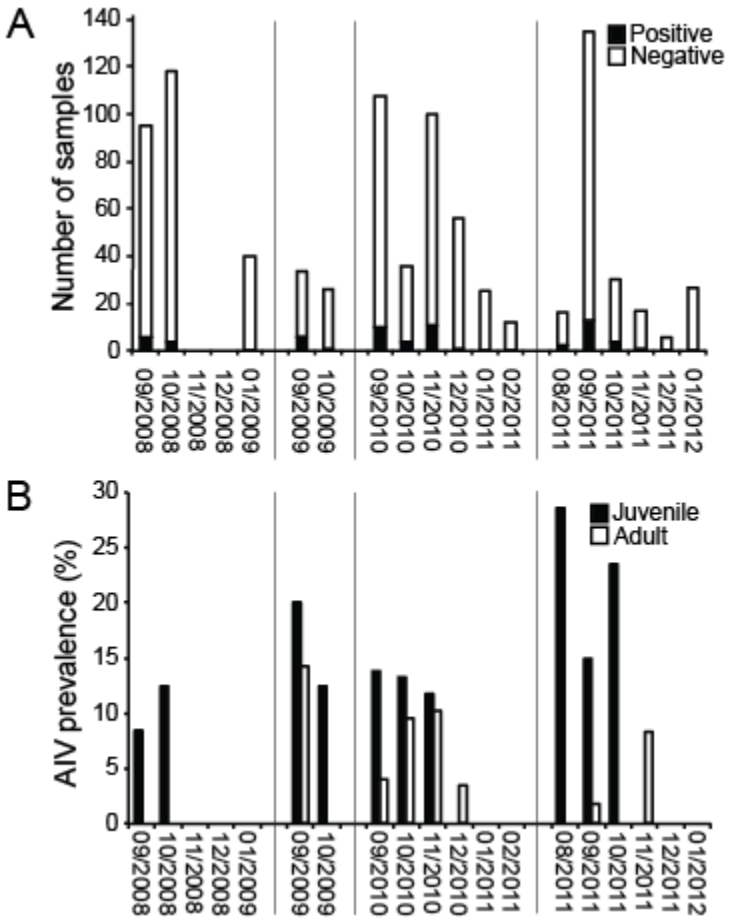

Figure 2. 1. Avian influenza virus (AIV) surveillance data for ducks in the St. John's region of Newfoundland, Canada. Total AIV sample numbers (A) and AIV prevalence according to bird age (B) by monthly sampling period. Detailed data are provided in Table S1. Dates are formatted as month/year.

2.3.2. Serological study of ducks during the 2011 sampling season

In the 2011 sampling season, serum samples were collected from 38 birds between October 2011 and February 2012. Of these, 20 samples (52.6%) were positive for anti-AIV antibodies (Table 2.2), which demonstrated a high frequency of previous AIV infection in these ducks. The results by species were 14/23 for American Black Duck, 5/8 for Mallard, 0/6 for hybrid American Black Duck-Mallard, and 1/1 for Northern Pintail. There do appear to be possible differences when comparing the data by bird age and gender, but the sample sizes are limited. One of the 38 birds tested, an adult American Black Duck, was positive both by serology and rRT-PCR on the day of sample collection.

Table 2. 2. Serological detection of AIV NP-antibodies in ducks in St. John's, NL

Month	Age		Gender		Total
	Juvenile	Adult	Male	Female	
October 2011	1/3	6/9	6/11	1/1	7/12
November 2011	0/3	5/9	4/10	1/2	5/12
December 2011	0/2	2/4	2/4	0/2	2/6
January 2012	NS*	5/7	4/6	1/1	5/7
February 2012	NS	1/1	1/1	0/0	1/1
Total	1/8	19/30	17/32	3/6	20/38

* None sampled.

2.3.3. Subtype distribution of the duck AIVs

The HA and NA subtypes were determined for 33 of the detected viruses, and HA subtypes were also determined for an additional 10 viruses for which we were unable to determine the NA subtype (Figure 2.2). There were 8 different HA subtypes (4 H1, 6 H2, 13 H3, 8 H4, 1 H5, 6 H6, 2 H11, and 3 H12) and 7 different NA subtypes (4 N1, 10 N2, 1 N3, 4 N4, 9 N6, 4 N8, and 1 N9) identified, resulting in 14 different HA/NA combinations. H4N6, H3N2, and H1N1 were the most numerous, and only 3 of the 14 subtypes, H1N1, H3N8, and H4N6, were detected in more than 1 year.

2.3.4. Genetic features of the H1 and H5 viruses

Phylogenetic trees for each segment of the H1N1 and H5N4 AIVs were constructed (Figure 2.3 and Figure S2.1). All gene segments of the viruses were assigned to American-avian clades, closely related to genes previously isolated from wild waterfowl in North America. Remarkably, each of the 8 gene segments of the 4 H1N1 viruses shared nucleotide identity of more than 99%, so these viruses could be considered homologous. Three of the H1N1 AIVs were isolated from American Black Ducks on 25 September, 2009 at Mundy Pond, and the other one (A/Domestic duck/Newfoundland/MW668/2010) was detected 1 year later (17 September, 2010) from a domestic duck at Commonwealth Pond. Comparison of the internal genes of the H1N1 isolates with those of the H5N4 AIV (A/American black duck/ Newfoundland/1181/2009) showed that the PB1 genes had nucleotide homology >99%, the PB2, NP1 and M genes had >95% nucleotide identity, while the PA and NS genes were more distantly related (<90% nucleotide homology), indicating distinct phylogenetic origin of some of the internal genes and suggesting complex past reassortment histories for these viruses.

	2008-09	2009-10*	2010-11	2011-12
H1N1		3	1	
H2N2				2
H2N4	2			
H2N6	1			
H2N?	1			
H3N2				8
H3N6			1	
H3N8	2			1
H3N?			1	
H4N4	1			
H4N6	2		3	
H4N?			2	
H5N4		1		
H6N6			2	
H6N8			1	
H6N?			3	
H11N3			1	
H11N9			1	
H12N?				3

*Samples only from September and October

Figure 2. 2. Avian influenza virus subtype detection for ducks in St. John's region of Newfoundland, Canada. Subtypes detected in each sampling year are shaded, with numbers of viruses detected for each of the different subtypes indicated. * means that the samples were only collected from September and October.

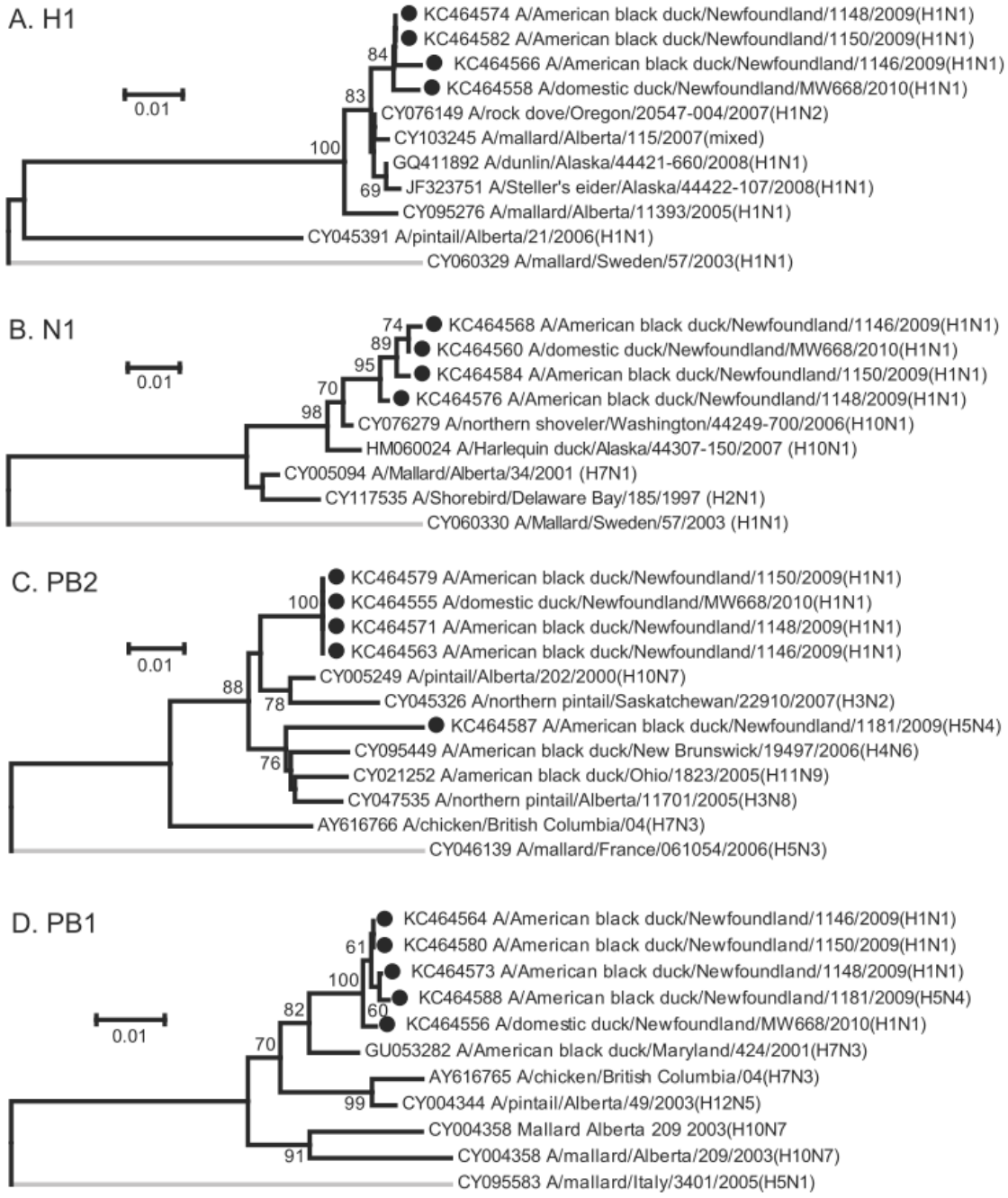

Figure 2. 3. Phylogenetic trees of selected genes from the H1N1 and H5N4 viruses identified in ducks in Newfoundland. The trees in panels A through D represent analyses of the H1, N1, PB2 and PB1 sequences, respectively. The avian influenza viruses in this

study (●) are assigned in American avian clades (black branches) with Eurasian-avian sequences as the outgroups (grey branches). The trees were constructed with MEGA 5 by the neighbour-joining method based on p-distance. Support for the branches is indicated as percentages based on 1000 bootstrap replicates, shown only for values ≥60%. The scale bars indicate substitutions per site. Analyses of the remaining gene segments are provided in Figure S2.1.

2.3.5. Movement patterns of the duck population

Over the period of this AIV screening work, 976 American Black Ducks, 126 Mallards, 99 Northern Pintail, and 32 American Black Duck-Mallard hybrids were banded in the St. John's region. There have been subsequent band recoveries from these for 90 American Black Ducks, 12 Mallards, 10 Northern Pintails and 1 American Black Duck-Mallard hybrid over this period. The recovered American Black Ducks, Mallard, and hybrid individuals have almost exclusively been recovered on the island of Newfoundland, and mostly near St. John's. A single American Black Duck was recovered off the island, in New Jersey. In contrast, 4 of 10 Northern Pintail recoveries have been off the island, with 1 in Quebec, 1 in Delaware and 2 in North Carolina.

2.4. Discussion

The location of the island of Newfoundland is such that it has migratory bird connections with both mainland North America and Europe (Tuck 1971, Bellrose and Kortright 1976), which makes it a compelling location to study AIV in wild birds. This is the first multiyear study on AIV epidemiology in ducks of Newfoundland, Canada, and

one of the few in which American Black Ducks are the prominent species. The screening of swab samples by rRT-PCR to test for active AIV shedding was also supplemented by a preliminary serological study to provide information about previous AIV infection rates. The duck population in this area appears to be composed of mostly non-migratory American Black Ducks and Mallards, along with a smaller component of more migratory Northern Pintails. The non-migratory population allowed sampling to continue into the winter, which has also provided valuable insights into the AIV infection dynamics in this location. The viruses identified with HA subtypes of high animal or public health concern, H1 and H5 in this study, were also analyzed for their genetic features. Further genetic characterization of all of the identified viruses is needed to determine how they compare with viruses from other regions of North America and from other bird groups.

We have found both shared and unique features with respect to studies in other locations regarding duck AIV prevalence and diversity. The AIV prevalence was higher in juvenile ducks compared with adult ducks, as frequently observed elsewhere (Hinshaw, Webster et al. 1980, Olsen, Munster et al. 2006, Munster, Baas et al. 2007, Runstadler, Happ et al. 2007, Parmley, Lair et al. 2009, Pasick, Berhane et al. 2010, Wilcox, Knutsen et al. 2011) (Figure 2.1; Table 2.1). The serology data also showed a higher prevalence of previous infection in older birds in comparison with young birds (Table 2.2), fitting this paradigm of increased susceptibility and infection rates in younger immunologically naïve birds. The detected AIV prevalence was higher in the later breeding season (autumn), as would be expected (Olsen, Munster et al. 2006), but continued sampling into the winter months in this study also resulted in positive samples during this period (Figure 2.1; Table 2.1). Because we do not have consistent sampling of the population throughout

the year, we cannot rule out that prevalence is actually higher at other times outside our sampling windows. Surveillance of waterfowl in the winter has received less attention than during the migratory periods, but several recent studies have also documented active winter infections in waterfowl (Munster, Baas et al. 2007, Kleijn, Munster et al. 2010, Hoye, Munster et al. 2011, Lewis, Javakhishvili et al. 2013). The number of samples from male ducks in this survey is almost double that from females (577 vs. 302), and males showed overall higher AIV detection rates compared with females. This likely is a reflection of male duck behavior where these individuals are very mobile in their search for food and possible mates, which is reflected by the fact that juvenile males are shot at higher rates than any other age-sex class (Krementz, Conroy et al. 1987). Increased movement would increase the interactions with different birds and the exposure to different possible environmental sources of AIV, thereby leading to higher infection rates. An increased likelihood of infection for males was also found in a large-scale surveillance study in Alaska (Ip, Flint et al. 2008) as well as in a modeling and meta-analysis based on widespread surveillance data from the U.S.A. (Farnsworth, Miller et al. 2012). On the other hand, the overall AIV detection rate in this study is noticeably lower compared with several recent reports from Canada (Parmley, Bastien et al. 2008, Parmley, Lair et al. 2009, Pasick, Berhane et al. 2010), which might reflect differences in sampling strategy where our samples were collected without duck-age bias and sampling continued into the winter months.

Viruses with H5 and H7 subtypes have caused previous influenza outbreaks in poultry and/or humans in the Americas and Eurasia (Claas, Osterhaus et al. 1998, Pasick, Handel et al. 2005, Chen, Smith et al. 2006, Spackman, McCracken et al. 2006, Gao, Cao

et al. 2013), and the 2009 pandemic H1N1 strain also contained genes of AIV origin (Trifonov, Khiabanian et al. 2009). We detected 4 H1N1 viruses, and 1 H5N4 virus but no H7 AIVs during this study. The gene segments of these H1N1 and H5N4 viruses all fall into low pathogenic American-avian AIV phylogenetic clades. Interestingly, the 4 H1N1 AIVs are very similar (Figure 2.3 and S2.1). Three of these were found on the same day in 2009 and the fourth was found in a domestic duck at another pond the following year. This high level of conservation across years is a rare but not unprecedented finding (Reeves, Pearce et al. 2011) and may reflect maintenance of the virus in the environment as opposed to continual perpetuation among host individuals over extended periods because of the high level of sequence conservation across this length of time.

We repeatedly captured and sampled one individual bird in which the infection status changed from negative to positive and then back to negative. This allowed estimation of the duration of detectable virus shedding for this individual at ≤8 days, which is similar to findings from an extensive field dataset (Latorre-Margalef, Gunnarsson et al. 2009) and experimental infection studies (Keawcharoen, van Riel et al. 2008, Costa, Brown et al. 2011, Hénaux and Samuel 2011).

One duck positive for AIV by rRT-PCR was also positive for previous infection by serology. This most likely resulted from the duck having been infected by an AIV in the past and then infected by a different virus subtype at the time it was swabbed (Latorre-Margalef, Grosbois et al. 2013). However, it is also possible that the duck exhibited a prolonged virus shedding period (Costa, Brown et al. 2010), while its immune system had begun to produce detectable anti-AIV antibodies. Immunologically naïve

Mallards were shown to seroconvert to anti-AIV positive within 14 days of release to a natural setting where they interacted with wild ducks, and individuals were repeatedly infected by different subtypes over the course of a year (Tolf, Latorre-Margalef et al. 2013). Although serological studies are more labor-intensive and challenging for large-scale epidemiological surveillance, they are becoming more common in wild bird surveillance work (Velarde, Calvin et al. 2010, Lewis, Javakhishvili et al. 2013, Tolf, Latorre-Margalef et al. 2013, Wille, Huang et al. 2013, Wilson, Hall et al. 2013). These results could help to bridge the gap in information about anti-AIV immune response in different host species and the frequency of infection, and could further elucidate the dynamics of perpetuation and evolution of different AIVs in these host species. Serological data are still likely underestimates of past exposures to influenza in the light of studies showing that antibodies can drop to undetectable levels in recently infected individuals (Kida, Yanagawa et al. 1980, Tolf, Latorre-Margalef et al. 2013).

The banding and recovery data show that the population of American Black Ducks in the St. John's region is largely non-migratory, which is unusual for a population in such a northern climate (Diefenbach, Nichols et al. 1988, Zimper and Conroy 2006). This is in contrast to Northern Pintails, for which we found a much higher proportion of movement off the island of Newfoundland. Considering the number of viruses that were characterized, we found a high level of subtype diversity (Figure 2.2). Additionally, there was a high subtype turnover over the period of our study, and most subtypes were detected in one of the 4 years. We interpret the high level of year-to-year subtype turnover in the identified viruses to be a result of the largely non-migratory population contributing the majority of our samples and identified viruses. A virus that circulates in

this fairly small, local, non-migratory population would have a limited host pool and would be removed from circulation because of increased herd immunity, which is frequently suggested as the reason for subtype turnover (Dugan, Chen et al. 2008, Fouchier and Munster 2009, Munster and Fouchier 2009, Chen and Holmes 2010). This also agrees with observations in a repeatedly sampled collection of captive Mallards, in which subtypes found the first year were not observed the second year (Tolf, Latorre-Margalef et al. 2013). The new viruses that appear in this population each year may come from local environmental reservoirs or may be introduced via the lower numbers of migratory individuals (i.e. Northern Pintails). This is a very different pattern than what has been found in multi-year studies conducted on populations that are dominated by migratory individuals in which subtype recurrence in consecutive years is more common (Hinshaw, Webster et al. 1980, Ramey, Pearce et al. 2010, Wilcox, Knutsen et al. 2011, Gunnarsson, Latorre-Margalef et al. 2012).Consistent AIV surveillance in ducks of Newfoundland without species bias will increase our understanding of this phenomenon.

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Chapter 3 Genetic structure of avian influenza viruses from ducks of the Atlantic flyway of North America

Abstract

Wild birds, including waterfowl such as ducks, are reservoir hosts of influenza A viruses. Despite the increased number of avian influenza virus (AIV) genome sequences available, our understanding of AIV genetic structure and transmission through space and time in waterfowl in North America is still limited. In particular, AIVs in ducks of the Atlantic flyway of North America have not been thoroughly investigated. To begin to address this gap, we analyzed 109 AIV genome sequences from ducks in the Atlantic flyway to determine their genetic structure and to document the extent of gene flow in the context of sequences from other locations and other avian and mammalian host groups. The analyses included 25 AIVs from ducks from Newfoundland, Canada, from 2008-2011 and 84 available reference duck AIVs from the Atlantic flyway from 2006-2011. A vast diversity of viral genes and genomes was identified in the 109 viruses. The genetic structure differed amongst the 8 viral segments with predominant single lineages found for the PB2, PB1 and M segments, increased diversity found for the PA, NP and NS segments (2, 3 and 3 lineages, respectively), and the highest diversity found for the HA and NA segments (12 and 9 lineages, respectively). Identification of inter-hemispheric transmissions was rare with only 2% of the genes of Eurasian origin. Virus transmission between ducks and other bird groups was investigated, with 57.3% of the genes having highly similar ($\geqslant$99% nucleotide identity) genes detected in birds other than ducks.

Transmission between North American flyways has been frequent and 75.8% of the genes were highly similar to genes found in other North American flyways. However, the duck AIV genes did display spatial distribution bias, which was demonstrated by the different population sizes of specific viral genes in one or two neighbouring flyways compared to more distant flyways.

3.1. Introduction

Epidemiological surveillance of avian influenza viruses (AIVs) has revealed a huge and dynamic virus reservoir in wild birds, especially in ducks. The genetic diversity of AIVs in wild birds is shaped by many factors such as the high error rate of the replicase, the segmented nature of the viral genome, broad host range properties of the viruses, and bird migration patterns (Webster, Bean et al. 1992, Olsen, Munster et al. 2006, Dugan, Chen et al. 2008). Decades of studies in wild birds have revealed seasonal patterns of viral prevalence (Olsen, Munster et al. 2006) and the segregation of AIV sequences into Eurasian and North American (Figure S3.1) (Olsen, Munster et al. 2006) and now also South American geographic phylogenetic lineages (Pereda, Uhart et al. 2008, Gonzalez-Reiche and Perez 2012).

The extensive set of AIV genomes sequences accumulated during recent decades has made it possible to study the patterns of intra-continental virus distribution in North America. Analysis of the duck AIV genomes available from 1976 to 2005 showed strong patterns of AIV distribution by sampling locations and time, but not by duck species (Chen and Holmes 2009). A large-scale statistical phylogeographic investigation of AIVs demonstrated a strong link between AIV phylogeny, spatial distance and migratory

flyway (Lam, Ip et al. 2012). Another recent large-scale analysis did not find that flyway separation affected the distribution of AIVs over long periods (Bahl, Krauss et al. 2013), supporting extensive movement of viruses among regions in North America.

Recent work with ducks in Alaska and California has provided important insights into AIV ecology at the western edge of North America. Comparison of AIVs from these two locations from 2006 to 2008 revealed the transmission of AIVs was strongly associated with duck species, locations and sampling times (Girard, Runstadler et al. 2012). Comparison of 161 Alaskan dabbling duck AIV genomes from 2005 to 2008 detected inter-species transmission of viruses between Northern Pintail (*Anas acuta*), Mallard (*A. platyrhynchos*), American Green-winged Teal (*A. carolinensis*) and Northern Shoveler (*A. clypeata*) during all sampling years (Reeves, Pearce et al. 2011). Northern Pintails in Alaska have shown a higher inter-continental AIV detection rate compared to other duck species in Alaska as well as ducks from other locations of North America (Krauss, Obert et al. 2007, Koehler, Pearce et al. 2008, Pearce, Ramey et al. 2009, Ramey, Pearce et al. 2010). Mallards in Alaska also carry inter-continental AIV reassortants but, unlike Northern Pintails that move between Alaska and Asia, these are due to secondary infection from other bird species (Pearce, Reeves et al. 2011).

In comparison to the large amount of work performed in Pacific North America, our knowledge of the ecology of AIVs in ducks in eastern North America and the Atlantic flyway, especially at the northern end in Atlantic Canada, is more limited. The province of Newfoundland and Labrador, Canada, is located at the northeastern margin of North America; yet, the role of ducks from this region, and the Atlantic flyway in general, in intra- and inter-continental AIV transmission is understudied. We have analyzed AIV

genome sequence data from ducks on the island of Newfoundland, Canada, along with available reference Atlantic flyway duck virus sequences from 2006-2011 to increase our knowledge of AIV dynamics in this region of North America. Along with available sequences from other North American bird flyways. we have used these data to examine the genetic structure of these viruses and to determine the amount of gene flow amongst different bird groups and North American migratory flyways.

3.2. Materials and methods

3.2.1. Ethics statement

The samples used in this study came from ducks (*Anas* spp.) that were caught by bait trapping and banded under banding permit 10559 from Environment Canada at public locations requiring no access permits. This study did not involve endangered or protected species. Swabs of the duck oropharyngeal cavity and cloaca were collected. This work was carried out under the guidelines specified by the Canadian Council on Animal Care with approved protocols 09-01-AL, 10-01-AL, and 11-01-AL from the Memorial University Institutional Animal Care Committee, and biosafety permit S-103-08 from the Memorial University Biosafety Committee.

3.2.2. Determination of AIV genome sequences from ducks in Newfoundland, Canada

Genome sequence data were successfully acquired for twenty-five AIVs from ducks in the St. John's region of Newfoundland, Canada, that were identified during AIV epidemiological surveillance over 2008-2011 (Huang, Wille et al.) (Table S3.1). RNA was extracted from the swabs, which were kept cool in the field after collection and then

stored at -80 ℃ until use, with Trizol LS (Life Technologies, Burlington, Canada) and the AIV sequences were amplified by RT-PCR using the SuperScript® III One-Step RT-PCR System with Platinum *Taq* (Life Technologies) and previously published primers (Hoffmann, Stech et al. 2001, Phipps, Essen et al. 2004, Chan, Lin et al. 2006, Obenauer, Denson et al. 2006, Huang, Khan et al. 2013). The resulting PCR products were purified with the QIAquick PCR purification kit (Qiagen, Toronto, Canada), and sequenced in both directions by Sanger sequencing at the Centre for Applied Genomics (Hospital for Sick Children, Toronto, Canada). Samples that showed evidence of mixed sequences were excluded from further analysis. The nucleotide sequence data were compiled and analyzed using the Lasergene v7.1 sequence analysis software package (DNASTAR Inc., Madison, WI). The GenBank accession numbers for the sequences are KC492244-KC492440.

3.2.3. Reference AIV genome sequences from ducks in the Atlantic flyway

Eighty-four reference AIV genomes, representing all available sequences (as of August 2012) from ducks in the Atlantic flyway over the period of 2006 to 2011, were downloaded from the NCBI influenza database (Bao, Bolotov et al. 2008). The numbers of viruses from different locations were: Newfoundland, 7; Quebec, 20; New Brunswick, 32; Prince Edward Island, 9; Ontario, 1; Nova Scotia, 2; New York, 4; Pennsylvania, 1; Delaware, 1; Maryland, 6; and Florida, 1 (Table S2).

3.2.4. Reference AIV genome sequences from other North American flyways

The genotypes of duck AIVs from 2006 to 2011 from the Pacific, Central and Mississippi flyways were analyzed in the Flugenome database (http://www.flugenome.org/), which employs a nucleotide difference of 10% (pairwise

distance analysis) as the cut-off value for an individual gene lineage, with the genotype of a certain AIV determined by the combination of gene lineages for segments 1 to 8 (Lu, Rowley et al. 2007). Viral genome sequences from different flyways representing all available genotype varieties were downloaded (1 genome per genotype in each bird flyway) from the NCBI influenza database for use in comparisons with the Atlantic flyway viruses. This provided 141 reference duck AIV genomes, with 102 genotypes from the Pacific flyway (35 from Alaska and 67 from California), 13 genotypes from the Central flyway, 21 genotypes from the Mississippi flyway, and 5 genotypes from Guatemala (a location of overlapping flyways). In addition, AIV gene segments from host species other than ducks that were highly related (≥99% nucleotide identity) to those in the Atlantic flyway duck AIVs were also downloaded from the NCBI database to study the distribution of the viral genes in different host groups.

3.2.5. Phylogenetic analysis of the Atlantic flyway duck AIVs

Two sets of phylogenetic trees were constructed for each segment. The first set included 250 duck AIV genome sequences, 109 from the Atlantic flyway and 141 from other bird flyways in North America and Guatemala, to compare the phylogenetic features of the Atlantic flyway sequences to those from the other flyways. The second set was performed to determine the occurrence of the Atlantic flyway genes in non-duck (avian and mammal) host species. The nucleotide sequence alignments and pairwise distance analyses were performed with the Jotun Hein method in Lasergene v7.1 (DNAStar, Inc., Madison, WI). Phylogenetic trees were constructed with the neighbour-joining method with bootstrap value of 1000 in MEGA5 (Tamura, Peterson et al. 2011). The nucleotide ranges of the coding regions used for each gene were: PB2, 1804-2256 bp;

PB1, 1624-2271 bp; PA, 1-528 bp; NP, 4-576 bp; M, 40-939 bp; NS, 55-792 bp; H1, 1147-1590 bp; H2, 1180-1689 bp; H3, 1102-1680 bp; H4, 1120-1659 bp; H5, 1086-1686 bp; H6, 1018-1665 bp; H7, 691-1659 bp; H11, 1087-1656 bp; H12, 1123-1662 bp; H13, 493-1695 bp; H16, 13-1668 bp; N1, 910-1344 bp; N2, 1102-1410 bp; N3, 760-1347 bp; N4, 1004-1392 bp; N6, 904-1383 bp; N8, 856-1404 bp; N9, 844-1395 bp.

3.2.6. Gene lineage and genotype assignments of the Atlantic flyway duck AIVs

On the basis of the phylogenetic trees, the 109 Atlantic flyway duck AIVs were analyzed to categorize their gene lineages and genotypes following the nomenclature of the Flugenome database (Lu, Rowley et al. 2007). The sequences were also classified by geographic and host group lineage (i.e. North American avian, Eurasian avian, North American gull or Eurasian gull) according to the origins of the reference sequences in the same phylogenetic clades. To study the genetic structure of the Atlantic flyway duck AIV population at an even finer level, gene sub-lineages were further determined with the criterion of ≥95% nucleotide identity (pairwisedistance) for a sub-lineage. Sub-genotypes of the viruses were then determined accordingly. We employed the Flugenome gene lineage and genotype determination approach in this study because it works well as a universal phylogenetic classification method for AIV genes and virus genotyping (Lu, Rowley et al. 2007, Ramey, Pearce et al. 2010). This was chosen to allow representation of the genetic structure of the viruses in a standardized way that also makes it easier to directly compare between different studies.

3.2.7. Identification of highly similar genes and genomes among the Atlantic flyway duck AIVs

To study the patterns of distribution and maintenance of the Atlantic flyway duck AIVs, highly related genes and genomes were identified within the identified gene sub-lineages and virus sub-genotypes. Sequences with nucleotide identity of ≥99% (pairwisedistance) were considered as highly similar genes or the same gene type. Viruses with nucleotide identities ≥99% for all 8 segments were considered as homologous genomes (Reeves, Pearce et al. 2011). The identified highly similar genes and genomes were analyzed for their spatial and temporal patterns of detection.

3.2.8. Investigation of the distribution of Atlantic flyway duck AIVs through space, time and host species

Each of the 248 gene types identified in the Atlantic flyway duck AIVs was used to search the NCBI database for highly related reference genes by BLAST (nucleotide identity ≥99% and coverage ≥95%) (Altschul, Madden et al. 1997). The longest gene sequence of the same gene type was chosen as the query sequence when applicable. The sequences highly related to the Atlantic flyway sequences were categorized for detection date, location and host species.

Differences among proportions of gene types detected for >5 and >10 years for the eight segments were analysed using standard χ^2 contingency table tests. Individual contributions of each segment to the overall χ^2 test were examined, and deemed to be significantly different from the overall proportion when the χ^2 was greater than the critical χ^2 value for α, where α was set to 0.05 and adjusted downward for multiple comparisons

(0.05/8 segments = 0.0063). All tests were 2-tailed and statistics were calculated using R 3.0.1 (www.R-project.org/).

3.3. Results

3.3.1. Phylogenetic analysis, gene typing and genotyping of the Atlantic flyway duck AIVs

Phylogenetic trees of the 250duck AIV genome sequences were constructed (Figure S3.2). This allowed delineation of the Atlantic flyway sequences into 34 gene lineages, 76 sub-lineages and 248 different gene types (summarized in Table 3.1; full details provided in Table S3.2). Of the 248 gene types, 235 (95.5%) were of North American avian origin. The other 13 gene types, from 6 different viruses, represented 11 gull-related genes and 2 Eurasian avian genes (Table 3.2). At the genomic level, 43 different genotypes and 70 sub-genotypes were identified amongst the 109 viruses (Table S3.2). These high levels of diversity were not artifacts of the large geographical representation over the flyway because diverse gene types (Table S3.3) and genotypes (Table S3.4) were also observed in the individual locations that contributed ≥20 AIVs (Newfoundland, Quebec and New Brunswick; Table S3.4).

3.3.2. Genetic features of each gene segment of the Atlantic flyway duck viruses

All 8 segments of the 109 Atlantic flyway viruses had diverse gene types, but their genetic structures had pronounced differences (Table 3.1; Figure 3.1). Predominantly single lineages and sub-lineages were found in the PB2, PB1 and M genes. The NP segments were represented by a single dominant lineage, with a number of different sub-lineages within this lineage. The PA segments were from 2 main lineages, each with very

different sub-lineage structures. The NS segments were from 2 main lineages, with a major sub-lineage for each. The HA and NA segments had the highest diversity, with multiple lineages and sub-lineages identified. Investigation of the phylogenetic and epidemiological features of the 109 Atlantic flyway duck AIVs revealed the following information for each gene segment.

PB2. The PB2 sequences belonged to 2 gene lineages, J and C, with a single J sub-lineage and 5 C sub-lineages (Figures 3.1 and S3.2; Table S3.2). The 2 genes in the J-1 lineage were of Eurasian gull origin, and this lineage has also been detected in shorebirds and gulls of the Atlantic flyway (Figure 3.2). The remaining 106 genes were classified in C lineages, with the predominant C-2 sub-lineage detected in 79 viruses with 17 different gene types. In general, the C lineage of the PB2 segment is abundant in birds (waterfowl, gulls and poultry) of North America, but it has also been found in Eurasian ducks and seabirds, and in the 2009 H1N1 pandemic virus (Flugenome database).

Table 3.1. Summary of gene lineage and gene type diversity by segment for the 109 Atlantic flyway duck AIVs from 2006-2011.

Category[a]	HA	NA	PB2	PB1	PA	NP	M	NS	Total
Gene lineages	12	9	2	1	2	3	2	3	34
Gene sub-lineages	19	20	6	8	7	9	3	4	76
Gene types	38	38	29	31	34	32	23	23	248
Gene types identified in species other than ducks	14	14	14	18	22	19	22	18	141

[a] Gene lineages, sub-lineages and types are defined as sharing ≥90%, ≥95% and ≥99% nucleotide identity, respectively.

Table 3.2. Inter-continental reassortants identified in the 109 Atlantic flyway duck AIVs

Virus	PB2	PB1	PA	HA	NP	NA	M	NS
A/mallard/Maryland/802/2007(H5N1)	C-2.7	F-7.1	H-1.13	5C-1.2	F-1.1[a]	1E- 2.1	E-1.5	1D-1.7
A/hooded merganser/New Brunswick/03750/2009(H13N6)	J-1.1[b]	F-8.1[c]	E-6.1[c]	13A-1.1[c]	D-1.1[c]	6A-1.2	J-1.1[b]	1C-1.1[c]
A/mallard/Quebec/02916-1/2009(H16N3)	J-1.2[b]	F-8.1[c]	E-6.2[c]	16D-1.1[b]	D-1.1[c]	3D-1.1[b]	J-1.1[b]	1C-1.1[c]
A/American black duck/Newfoundland/MW733/2010(H6N6)	C-2.3	F-3.1	H-1.4	6B-1.1[a]	H-1.3	6A-3.2	E-1.1	1D-1.4
A/American black duck/Newfoundland/PR007/2010(H6N6)	C-2.1	F-3.1	H-1.5	6B-1.1[a]	H-1.1	6A-2.1	E-1.6	1D-1.5
A/duck/Newfoundland/MW721/2010(H6N8)	C-2.1	F-3.1	H-1.4	6B-1.1[a]	H-1.3	8A-1.3	E-1.1	1D-1.4

[a] Eurasian avian origin

[b] Eurasian gull origin

[c] North American gull origin

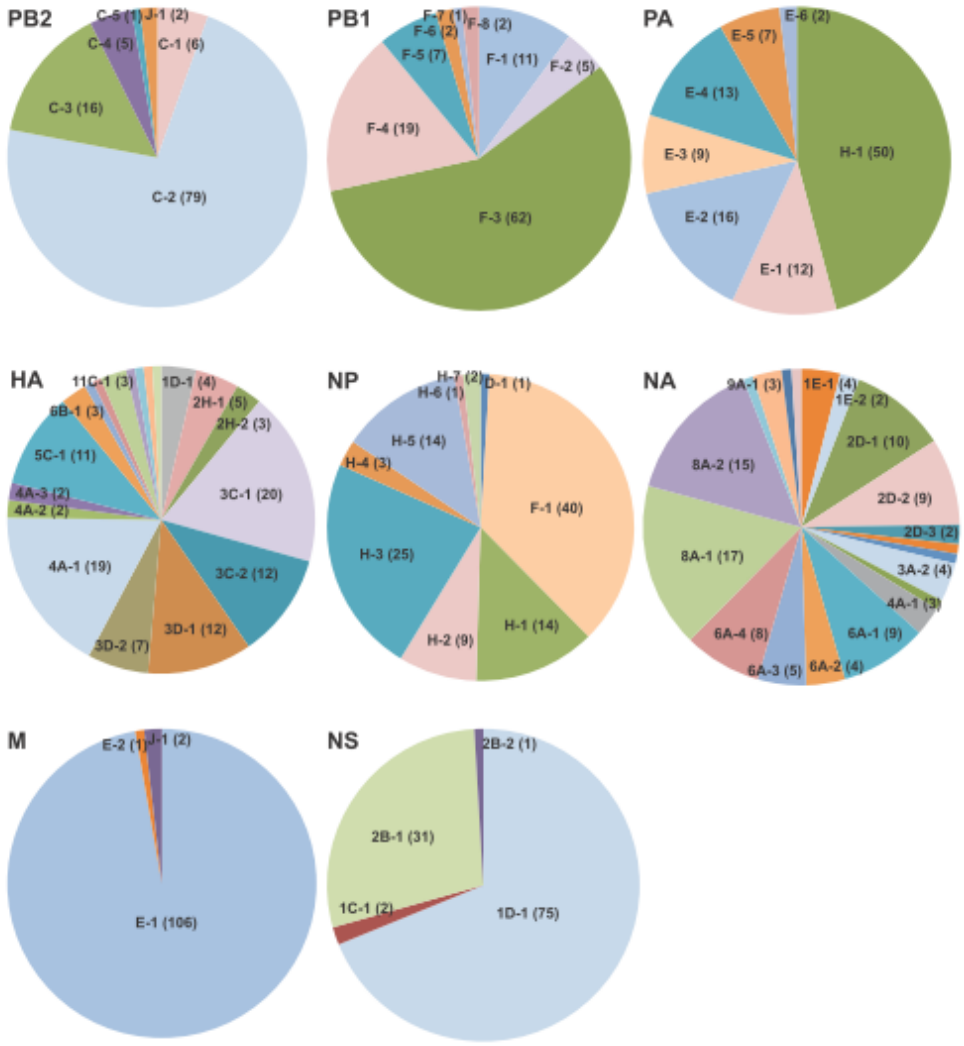

Figure 3.1. Genetic structure of the gene lineages and sub-lineages of the Atlantic flyway duck AIVs. The numbers of genes in each detected sub-lineage for each of the segments of the 109 Atlantic flyway duck AIVs are shown in the pie charts. AIV genes with ≥95% nucleotide identity by pairwise distance analysis were assigned in a sub-lineage. For the HA genes, sub-lineages 7F-1, 7F-2, 11C-2, 12A-1, 13A-1 and 16D-1 were each detected once and are not labelled. Similarly for the NA genes, sub-lineages 2G-1, 3A-1, 3D-1, 8A-3, 9A-2 and 9A-3 were each detected once and are not labelled.

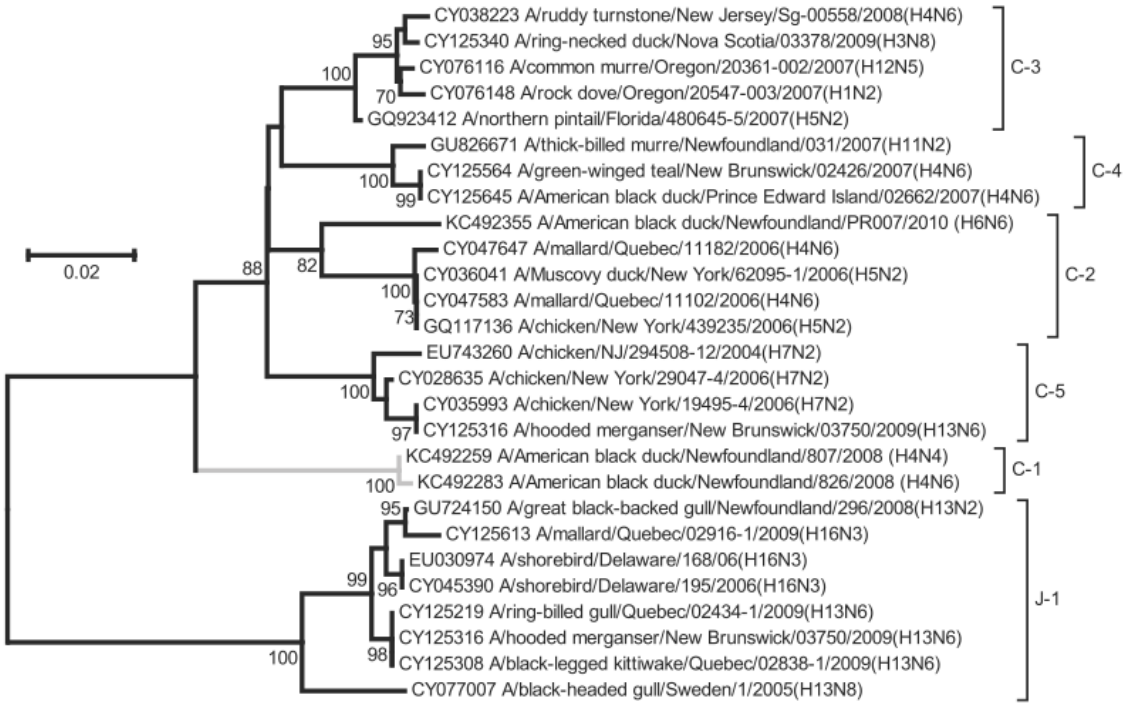

Figure 3.2. Phylogenetic analysis demonstrating the presence in Atlantic flyway ducks of non-duck AIV lineages. The phylogenetic tree contains representative sequences from the Atlantic flyway duck AIVs for each of the 6 PB2 sub-lineages, with sub-lineages indicated on the right. The 5 sub-lineages (C-2 through C-5 and J-1) that contained genes detected from non-duck host species are displayed with black branches. The sub-lineage C1, which did not contain genes from non-duck host species, is displayed with grey branches. The neighbour-joining tree was constructed with MEGA 5 and support values based on 1000 bootstrap replicates are shown as percentages where ≥70%. The scale bar indicates nucleotide substitutions per site.

PB1. All of the PB1 sequences belonged to the F lineage, with 8 sub-lineages (Figure 3.1). The F-3 sub-lineage was predominant and comprised of 12 gene types from 62 viruses (Figure S3.2; Table S3.2). In general, the F lineage of the PB1 gene is common in birds of North America, and has spilled over to swine (1999-2002) and humans (2003-2004). Several F-lineage genes have also been detected in Eurasian ducks and seabirds (Flugenome database).

PA. The PA sequences fell within 2 lineages (E and H; Figure 3.1) with 34 different gene types (Table S3.2). Browsing through the Flugenome database, the H lineage is common in birds of North America, but several genes of this lineage have been reported in Eurasia. The PA gene of the 2009 H1N1 pandemic virus is also assigned to the H lineage. E-lineage PA genes are common in birds from both North America and Eurasia.

HA. There were 11 subtypes found in the HA genes, with 4 H1, 8 H2, 51 H3, 23 H4, 11 H5, 3 H6, 2 H7, 4 H11, 1 H12, 1 H13 and 1 H16 categorized into 12 gene lineages and 19 sub-lineages (Figures 3.1 and S3.2; Table S3.2). The 4 H1 genes belonged to the 1D lineage, which is abundant in waterfowl in North America and distinct from the 1A lineage often found in poultry in North America and found in the 2009 H1N1 pandemic strain (Flugenome database). The H2 genes all belonged to the 2H lineage, which is distinct from the 2D lineage that invaded North America from Eurasia (Makarova, Kaverin et al. 1999, Lu, Rowley et al. 2007). All of the H3 and H4 genes of the Atlantic flyway duck AIVs were from lineages typically identified in waterfowl in North America (Figure S3.2). The 11 H5 genes all belonged to the 5C lineage, which is abundant in waterfowl and poultry of North America and distinct from the H5 genes of the highly pathogenic H5N1 viruses in Eurasia, which belong to the 5J lineage (Flugenome

database). The 3 H6 genes belonged to the 6B lineage of Eurasian-avian origin, which is known to have become the predominant H6 lineage in North America in the last decade (zu Dohna, Li et al. 2009). The 2 H7 genes were classified in the 7F lineage (Figure S3.3; Table S3.2), which is abundant in waterfowl and poultry of North America with several genes detected from European poultry, while most H7 genes from waterfowl and poultry in Eurasia belonged to 7A lineage (Flugenome database). The 4 H11 genes belong to the 11C lineage (Table S3.2), which is abundant in waterfowl of North America and occasionally detected from shorebirds and poultry (Flugenome database). Several 11C-lineage genes were also detected from storks and ducks in Eurasia, although most H11 genes detected in Eurasia belonged to 11A lineage (Flugenome database). The H12 gene in this study belongs to the 12A lineage (Table S3.2) and the most similar sequences in the NCBI Influenza database (~98% nucleotide identity) were from shorebirds of New Jersey and Delaware in 2008 (Figure S3.3). HA genes in the 12A lineage were detected from waterfowl, shorebirds, gulls and murres in North America, while those from Eurasia are mainly in the 12B lineage (Flugenome database). The one H13 gene belonged to the 13A lineage and may have recently been transmitted from gulls to ducks because the same gene type was detected in gulls from Quebec in the same year (2009) and these genes are closely related to an H13 gene found in a Newfoundland gull in 2008 (Figure S3.3). The one H16 gene was of Eurasian-gull origin and related to genes detected in shorebirds and gulls in Delaware and gulls in Europe (Figure S3.3). There are 3 lineages of H16 genes in the Flugenome database (16A, 16B and 16C) but the H16 gene from the Atlantic flyway duck in this study was divergent from these (≤90% nucleotide identity) and we therefore assigned it as the 16D lineage (Figure S2).

NP. The 109 NP sequences belonged to 3 lineages (F, H and D) but almost all (106/109) were from the H lineage (Figures 3.1 and S3.2; Table S3.2), which is the predominant lineage in birds of North America (Flugenome database). NP genes of this lineage have also been detected in humans and swine, and 8 Eurasian H-lineage genes from Asia were found in the Flugenome database. The 2 NP genes in the D lineage were of American-gull origin (Table S3.2). Several D-lineage NP genes were also detected from gulls of Eurasia (Flugenome database). The single NP gene we found in the F lineage was of Eurasian-avian origin, and this lineage is common in Eurasian waterfowl and poultry (Flugenome database). Phylogenetic analyses indicated that these genes might have been brought to North America by ducks in the Pacific flyway (Figure S3.2), which is also supported by the epidemiological data because 49 of the 53 F-lineage NP genes in the Flugenome database from North America are from Pacific flyway waterfowl. The NP genes from some human AIV infections (H5N1 and H9N2 subtypes) in Asia also belong to the F lineage.

NA. The 108 NA sequences available from the 109 Atlantic flyway viruses grouped into 7 NA subtypes with 9 gene lineages and 20 sub-lineages (Figures 3.1 and S3.1; Table S3.2). The 6 N1 genes belonged to 2 sub-lineages of the 1E lineage, which is common in waterfowl and shorebirds of North America (Flugenome database). Most of the N2 genes (26/27) belonged to the 2D lineage, which is mainly found in waterfowl of North America and occasionally in other bird species such as chickens, seabirds and gulls. One N2 gene belonged to the 2G lineage, which is common in birds of North America but which has also been found in human AIVs in North America (Flugenome database). The 6 N3 genes were from 2 lineages, 3A and 3D. The 3A lineage is common in birds of

North America and sometimes also detected in swine and human AIV viruses. The gene from the 3D lineage was of Eurasian-gull origin (Table S3.2). The 4 N4 genes belonged to the 4A lineage, which is mainly found in waterfowl of North America. The 26 N6 genes are from 4 sub-lineages of the 6A lineage, which is abundant in waterfowl and shorebirds in North America and occasionally detected in birds in Eurasia (Flugenome database). The gene type 6A-1.2 was also detected in gulls from Quebec in 2009 (Figure S3.3). Considering the predominance of the 6A-lineage NA genes in ducks of North America, this likely represents a recent cross-species transmission of this gene from ducks to gulls. There were 34 N8 genes and all belonged to the 8A lineage (Figure S2). In general, the 8A lineage is abundant in waterfowl, shorebirds and gulls of North America but also occasionally detected in aquatic birds in Asia (Flugenome database). The 5 N9 genes belonged to 3 sub-lineages of the 9A lineage, which has mainly been detected in waterfowl, shorebirds and gulls of North America but there were also 33 NA genes of 9A lineage from Eurasia in the Flugenome database.

M. The M sequences belonged to the E and F lineages. The E-1 sub-lineage was predominant and included 106 of the viruses (Figure S3.2; Table S3.2). The E-lineage is common in birds of North America with several genes detected in human and swine cases of AIV infection. Thirteen E-lineage genes from birds and swine in Asia were also found in the Flugenome database. The M gene of the F-1 sub-lineage was of Eurasian gull origin, which has previously been detected in American gulls and shorebirds, and has now also been detected in Atlantic flyway ducks (Figure S3.3).

NS. Both alleles 1 and 2 (allele A and B by conventional nomenclature) were detected in the Atlantic flyway duck AIVs. There were 77 genes from lineage 1, with 75 genes from

lineage 1D and 2 from lineage 1C (Figures 3.1 and S3.2; Table S3.2). The 1D lineage is abundant in waterfowl and shorebirds of North America, and has also been detected in poultry, seabirds, gulls, swine and humans in North America (Flugenome database). The 2 genes from the 1C lineage were of American gull origin (Figure S3.2). The remaining 32 NS genes belonged to lineage 2B, which is common in waterfowl and poultry of North America and also has been detected in other hosts (Flugenome database).

3.3.3. Spatial and temporal detection of AIV genes and genomes within Atlantic flyway ducks

The 248 Atlantic flyway gene types were sorted according to year and location of detection (Figures S3.4 and S3.5). Circulation of the genes in the Atlantic flyway through space and time was common, with 81 of the 248 gene types (32%) detected in more than one year or at different locations over 2006-2011, and 22 gene types (9%) were maintained in Atlantic flyway ducks for 3 to 6 years during 2006-2011 (Table S3.5). For example, the NA gene type 2D-1.1 was found in several locations of the Atlantic flyway over the 6 years and the HA gene type 3D-1.1 was perpetuated in Newfoundland over 4 years (2007-2011) (Table S3.5). Different locations possessed both unique and shared gene types. For example, amongst the 78 total gene types from Newfoundland, 18 were also detected elsewhere in the Atlantic flyway and 60 were restricted to Newfoundland. This was different than what was observed for the viruses from Quebec, where 30 of the 51 identified gene types were also detected at other locations in the Atlantic flyway during 2006-2011 and 21 genes were exclusively found in that province. However, the limited number of AIV reference sequences in Atlantic flyway ducks could cause under-estimation of virus transmission within Atlantic flyway, because searching of the NCBI

database showed that 69 of the 78 gene types from Newfoundland have also been found in other bird flyways (Table S3.6 and S3.7).

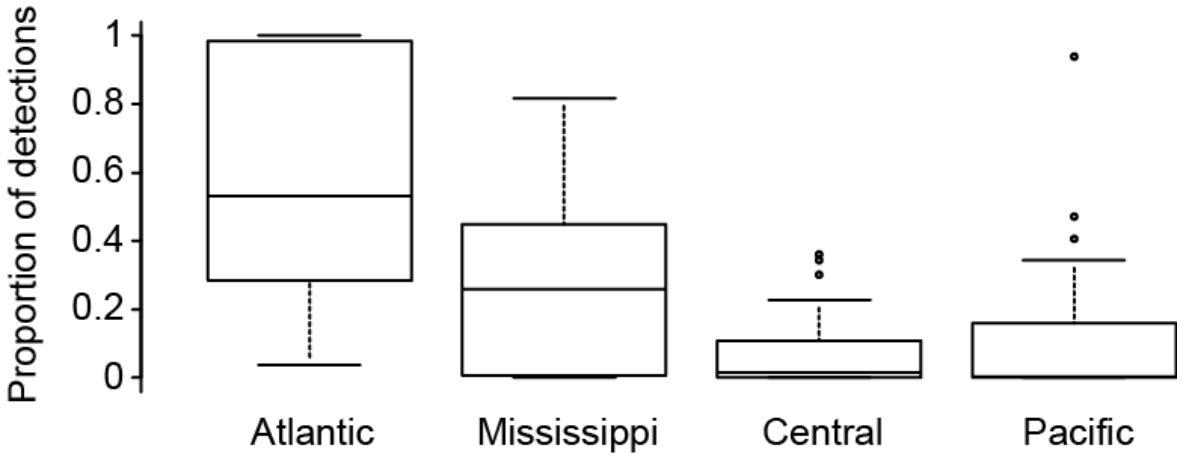

Figure 3.3. Spatial distribution of detections of the Atlantic flyway duck AIV sub-lineages in North American flyways. The box plots show the proportions of detections in each of the flyways for the gene sub-lineages that were identified in the 2006-2011 Atlantic flyway duck AIVs. The solid line in the box is the median. The top and bottom of the box are the first and third quartiles, respectively. The upper and lower horizontal lines represent 1.5X the difference between the first and third quartiles and any values falling outside of those are plotted as points. The detailed results for the each segment are provided in Figure S3.6.

At the genomic level, 12 homologous genomes were detected more than once (Table S3.2). Distinct from the long perpetuation of individual gene segments, most (9/12)

of the homologous genomes were detected during the same year and from the same location. The exceptions to this were H1N1 viruses detected in consecutive years (both 2009 and 2010 in Newfoundland), H3N8 viruses detected in multiple locations (2009 in Nova Scotia, New Brunswick and Prince Edward Island), and H4N6 viruses detected in multiple locations (2007 in New Brunswick and Prince Edward Island).

3.3.4. Spatial and temporal detection of the Atlantic flyway duck AIV genes in different North American flyways

Highly similar reference gene sequences (≥99% identical) from North America were identified for the 248 Atlantic flyway duck AIV gene types by BLAST, which totaled >9,000 sequences. These were categorized by year (Table S3.6) and migratory flyway of detection (Table S3.7). Extensive exchanges of the 248 Atlantic flyway AIV genes through space and time were detected, with 188 gene types (75.8%) identified in other flyways, while 42 of the gene types (16.9%) were identified only in the Atlantic flyway before 2006. The remaining 18 gene types (7.3%), of which 11 were HA genes, were only detected in Atlantic flyway ducks from 2006 to 2011 (Table S3.5).

Despite the frequent transmission of viruses among different bird flyways, the overall detection of genes highly similar to those from the 2006-2011 Atlantic flyway duck AIVs was most frequent in other viruses from the Atlantic flyway and decreased moving west across the different flyways (Figure 3.3). The majority of the 248 gene types displayed spatial distribution biases, as reflected by the larger numbers of detections of specific genes in one or two neighbouring flyways compared to the other flyways (Figure S3.6). For example, the HA genes of 3D-1, 4A-1 and 7F-2 sub-lineages were abundant in the Atlantic flyway, whereas the 4A-3 lineage was more abundant in the Mississippi and

Central flyways (Figure S3.6). In contrast, the 1D-1 HA lineage was more evenly detected in the four flyways. Similarly for the NP genes, the H-4 and H-7 sub-lineages were more abundant in the Atlantic flyway, the H-1 and H-2 lineages were more widely detected, and the F-1 sub-lineage was mainly detected in the Pacific flyway (Figure S3.6). The NP F-1 sub-lineage was the most Pacific-biased of any sub-lineage (top point on Pacific flyway plot in Figure 3.3) and this may indicate it was recently transmitted from the Pacific flyway into eastern North America.

3.3.5. Perpetuation of the AIV genes through years

The perpetuation of the AIV segments was evident after comparing them with all highly similar genes available in the NCBI database. This showed that 175 of the 248 gene types (70.6%) have persisted for >3 years, and 112 of the 248 gene types (45.1%) have been maintained for >5 years, while 40 of the 248 gene types (16.1%) have circulated for >10 years (Table S3.6). Eleven of the gene types could be traced to more than 20 years ago, with the oldest detection from 1978 (Table S3.6). When the 8 AIV gene segments were compared for their maintenance across the years (Figure 3.4), the M and NS segments had the highest >5-year perpetuation detections, at 82.6% and 95.7%, respectively, whereas the HA segment had the lowest proportion of maintenance for >5 years (18.4%). Similarly, the M and NS segments had the highest >10-year perpetuation detections, at 56.5% and 52.1%, respectively, whereas no HA segment was maintained for >10 years (Figure 4). These differences were significant for both the >5 year (χ^2 = 53.1, df = 7, P < 0.0001) and >10 year (χ^2 = 66.5, df = 7, P < 0.0001) data.

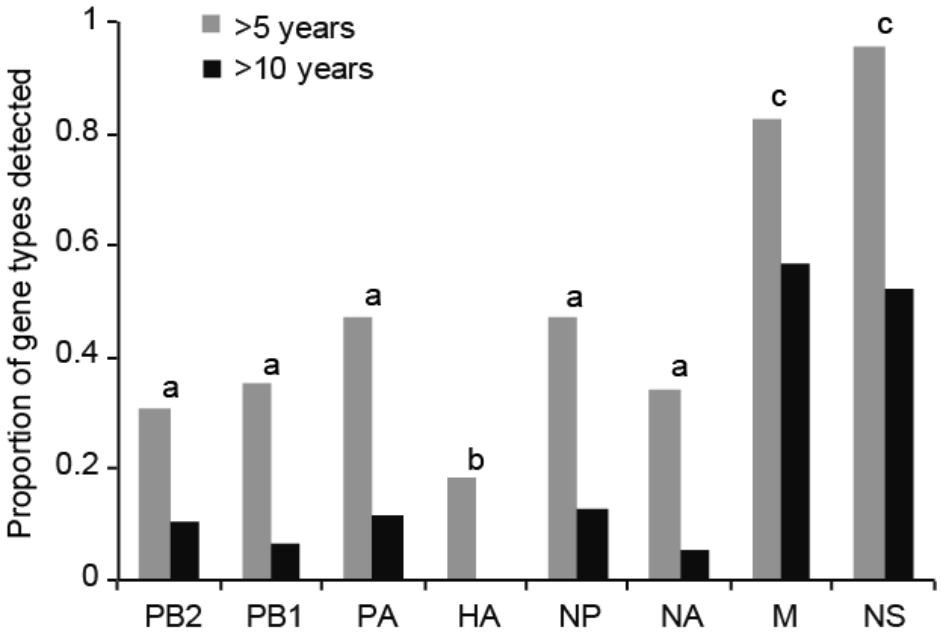

Figure 3.4. Perpetuation of Atlantic flyway duck AIV gene types for >5 and >10 years . The proportions of gene types identified in the Atlantic flyway viruses that were detected for >5 and >10 years in North America are shown for each segment. There were significant differences among the proportions for the HA, M and NS segments relative to the other segments for >5 years ($\chi^2 = 53.1$, df = 7, $P < 0.0001$) and >10 years ($\chi^2 = 66.5$, df = 7, $P < 0.0001$). Statistically different proportions for the segments are indicated with different letters (a, b and c).

3.3.6. Identification of Atlantic flyway duck AIV genes in other hosts

Of the 248 AIV gene types identified in the 109 Atlantic flyway duck viruses, 142 (57.3%) were also detected in hosts other than ducks (Table S3.8). These included shorebirds, poultry, gulls, murres, other waterfowl, and swine (Table S3.9). The detection frequency of the Atlantic flyway duck AIV genes in different host groups varied, with 82 of the gene types found in shorebirds, 41 in geese, 39 in turkeys, 37 in chickens, 19 in gulls, 13 in swans, 9 in seabirds and 9 in swine (Table S3.9).

3.4. Discussion

Current attempts to characterize AIV genetic structure and fully understand virus ecology are limited by the under-representation of sequence information from viruses across spatial, temporal, and host species scales, especially in the American portion of the Central flyway and the Canadian portion of the Atlantic flyway (Lam, Ip et al. 2012). There is clearly an enormous virus reservoir maintained in both bird hosts (Webster, Bean et al. 1992, Olsen, Munster et al. 2006) and the natural environment (Ito, Okazaki et al. 1995, Lang, Kelly et al. 2008, Hénaux, Samuel et al. 2012) and we have discovered only a fraction of this diversity. To better resolve the dynamics of AIV transmission and distribution, it is critical to examine available sequences in the context of broader time scales, host species and geographic locations. In this study, 109 Atlantic flyway duck AIV genomes from 2006-2011 were analyzed for their genetic structure to provide insight into AIV evolution and ecology on the east coast of North America. Extensive diversity was detected in the AIV genes and genomes from the Atlantic flyway ducks in this study, similar to reports performed in waterfowl at other locations of North America (e.g.

(Spackman, Stallknecht et al. 2005, Reeves, Pearce et al. 2011, Bahl, Krauss et al. 2013)). The analysis of the AIV genetic structure identified limited numbers of predominant gene lineages and sub-lineages for the internal protein genes, distinct from what was found for the surface protein genes. Restricted inter-continental AIV exchange was found in the Atlantic flyway ducks, in sharp contrast to the extensive intra-continental transmission among bird flyways and bird species.

The host sources of the AIVs analyzed in this study (Table S3.2) generally reflected duck distribution patterns in North America. However, the spatial distribution of duck species does not lead to the geographic distributions of AIV genes as shown by the identification of homologous genes in different duck species (Figure S3.2 and Table S3.2). This agrees with previous work, which also found no distinct AIV phylogenetic separation in dabbling duck species (Chen and Holmes 2009, Reeves, Pearce et al. 2011). We detected a high proportion (＞50%; Table S8) of the Atlantic flyway duck AIV gene types in bird groups other than ducks, reflecting frequent interspecies transmission of AIVs in birds. The duck gene types were detected more often in shorebirds than other wild bird groups such as gulls and seabirds. This variation in detection frequency of the Atlantic flyway duck AIV genes in different bird groups could be caused by multiple factors, including differences in virus sequence availability, bird habitat use and migratory behaviors, bird population structure, AIV subtype prevalence, host AIV infection history and cellular receptor properties. The Atlantic flyway duck AIV gene types were also detected in domestic poultry (Table S3.9), reflecting the infection of domestic birds by wild bird viruses. Similarly, some of the gene types were identified in

swine viruses (Table S3.9). This movement of AIV genes amongst wild birds and domestic animals is an important aspect of influenza dynamics as related to the evolution of new strains and the potential for negative impacts on human society in terms of agriculture and health. Continual surveillance of the wild bird AIV reservoir is especially valuable to monitor virus transmission to domestic animals and/or the human population.

Many AIV genes in our study were detected in more than one North American bird flyway, but the detections were biased by geographic locations (Figure 3.3). Most AIV gene sub-lineages were detected more often under one or several neighbouring flyways relative to other flyways (Figure S3.6). It should be noted that there are differences in the numbers of duck virus sequences available from the different flyways, which could bias these results. However, we do not believe this pattern is caused by an over-representation of sequences from the Atlantic flyway because recent work has increased the sequences available for multiple regions of North America, particularly in the Pacific flyway. Furthermore, differential gene distributions by flyway were also found in a previous study (Lam, Ip et al. 2012). Gene flow among flyways is presumably mediated by interactions of long-distance migrants on wintering grounds and some east-west bird movements (Lam, Ip et al. 2012, Bahl, Krauss et al. 2013). At the genomic level, highly similar viruses were mostly detected in adjacent locations and within short time spans (Table S3.2), reflecting frequent viral reassortment (Hatchette, Walker et al. 2004, Reeves, Pearce et al. 2011). The between-year detection of the 4 H1N1 viruses may reflect the maintenance of AIVs in the abiotic reservoir (Huang, Wille et al. 2013). Among the 8 AIV gene segments, the HA gene displayed the lowest maintenance time with less than 20% of the gene types maintained for >5 years and none retained >10 years

(Figure 3.4), which is not surprising considering that the HA protein is targeted by host immune responses. Along with the lowest lineage diversity in the Atlantic flyway ducks (Figure 3.1), the M and NS segments showed the most prolonged maintenance, with >80% of the identified gene types maintained for >5 years and >50% maintained for >10 years (Figure 3.4). This likely resulted from differences in the selective pressures on the different segments, and it was previously found that the M and NS (allele A) segments showed the lowest nucleotide substitution rates (Bahl, Vijaykrishna et al. 2009). Prolonged prevalence of the same gene types may result in part from maintenance of viruses in the environment (Hinshaw, Webster et al. 1979, Ito, Okazaki et al. 1995, Lang, Kelly et al. 2008, Roche, Lebarbenchon et al. 2009, Nazir, Haumacher et al. 2011, Lebarbenchon, Sreevatsan et al. 2012) because the high mutation rate for these RNA viruses and the constant immune pressure from the hosts make it unlikely for the same AIV genes to be maintained solely in birds across many years (Holland, Spindler et al. 1982, Chen and Holmes 2006).

The detection frequency of inter-continental AIV reassortants is higher at the continental margins than inland (Peterson, Benz et al. 2007, Koehler, Pearce et al. 2008). Compared to the amount of inter-continental reassortment detected in AIVs from Northern Pintails in Alaska (Koehler, Pearce et al. 2008, Ramey, Pearce et al. 2010), fewer inter-continental AIV genes (2%) were identified in Atlantic flyway ducks in our analysis. Limited inter-continental reassortants were also detected in Atlantic flyway shorebirds (Krauss, Obert et al. 2007). This could be due to the larger distance between eastern North America and Europe compared to that between Alaska and Russia, leading to more restricted movements of birds between the regions, especially ducks. Therefore,

our study suggests there is a lower likelihood of AIV introduction from Eurasia to North America through ducks of the Atlantic flyway, compared to those of the Pacific flyway, which has implications for the potential transmission of highly pathogenic AIVs, such as H5N1, to North America through Atlantic Canada. There is currently no evidence that Atlantic flyway ducks move across the Atlantic Ocean and therefore the Eurasian sequences detected may result from the interaction of these ducks with other hosts such as gulls, which do move across the Atlantic (Wille, Robertson et al. 2011) and which contain inter-continental reassortant viruses with high frequency (Wille, Robertson et al. 2011, Van Borm, Rosseel et al. 2012, Hall, TeSlaa et al. 2013). Continued AIV surveillance in multiple bird groups in different regions will further increase our understanding of AIV ecology in North America and virus movement between regions.

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Chapter 4 Perpetuation and reassortment of gull influenza A viruses in Atlantic Canada

Abstract

Gulls are important hosts of avian influenza A viruses (AIVs) and gull AIVs often contain gene segments of mixed geographic and host lineage origins. In this study, the prevalence of AIV in gulls of Newfoundland, Canada from 2008-2011 was analyzed. Overall prevalence was low (30/1645, 1.8%) but there was a distinct peak of infection in the fall. AIV seroprevalence was high in Newfoundland gulls, with 50% of sampled gulls showing evidence of previous infection. Sequences of 16 gull AIVs were determined and analyzed to shed light on the transmission, reassortment and persistence dynamics of gull AIVs in Atlantic North America. Intercontinental and waterfowl lineage reassortment was prevalent. Of particular note were a wholly Eurasian AIV and another with an intercontinental reassortant waterfowl lineage virus. These patterns of geographic and inter-host group transmission highlight the importance of characterization of gull AIVs as part of attempts to understand global AIV dynamics.

4.1. Introduction

Influenza A viruses are eight-segmented, negative-sense, single-stranded RNA viruses belonging to the virus family *Orthomyxoviridae* (Kawaoka, Cox et al. 2005). These viruses infect numerous hosts, including birds and mammals. The natural reservoir of avian influenza A viruses (AIVs) are wild aquatic birds, especially the orders

Anseriformes (ducks, geese and swans) and Charadriiformes (auks, terns, gulls and shorebirds) (Webster, Bean et al. 1992, Olsen, Munster et al. 2006). AIV infection in wild birds usually appears to be asymptomatic, but some strains may cause fatal infection (Becker 1966, Chen, Smith et al. 2005, Liu, Xiao et al. 2005). AIVs are also a great hazard to the poultry industry (Webster, Peiris et al. 2006, Alexander 2007), and some strains also pose public health risk (Claas, Osterhaus et al. 1998, Peiris, de Jong et al. 2007, Cardona, Xing et al. 2009, Gao, Cao et al. 2013).

The sequences of AIV genes generally divide into distinct phylogeographic lineages due to the partial separation of birds by continental regions (Olsen, Munster et al. 2006). They also segregate into waterfowl-dominated phylogenetic clades (usually referred to as avian) and shorebird/gull-dominated clades (usually referred to as gull) (Olsen, Munster et al. 2006), and the hemagglutinin subtypes H13 and H16 also appear to be specifically adapted to gulls (Hinshaw, Air et al. 1982, Fouchier, Munster et al. 2005). This segregation is presumably caused by biological and ecological differences amongst host bird taxa. However, some gull AIVs show limited replication in other bird hosts (Kawaoka, Chambers et al. 1988, Tonnessen, Valheim et al. 2011, Brown, Poulson et al. 2012), AIV has been transmitted from gulls to poultry (Sivanandan, Halvorson et al. 1991), and there is also evidence gull AIVs could pose human risk (Lindskog, Ellstrom et al. 2013). Despite these general phylogenetic divisions, reassortants containing segments of different continental and/or bird host origins are detected [e.g. (Widjaja, Krauss et al. 2004, Spackman, Stallknecht et al. 2005, Krauss, Obert et al. 2007, Kishida, Sakoda et al. 2008, Koehler, Pearce et al. 2008, Chen and Holmes 2009, Lomakina, Gambaryan et al. 2009, Ramey, Pearce et al. 2010, Wille, Robertson et al. 2011, Van Borm, Rosseel et al.

2012)]. This is particularly common in AIVs from gulls, which is presumably driven by gull ecology in terms of habitat usage and migratory behaviour, and suggests gulls play an important role in AIV transmission between regions and among different host taxa (Ramey, Pearce et al. 2010, Wille, Robertson et al. 2011, Van Borm, Rosseel et al. 2012). However, the number of gull AIV genome sequences remains limited, especially in comparison to the data available for waterfowl viruses.

Oceanic coasts are useful locations for studies of gull AIVs because of abundant gull populations and increased chances of inter-continental bird movements. The northern Atlantic coast of North America is one such location, with gull movements to Europe documented (Wille, Robertson et al. 2011) and inter-continental reassortant gull viruses found (Krauss, Obert et al. 2007, Wille, Robertson et al. 2011, Hall, TeSlaa et al. 2013). In this study, we conducted surveillance for AIV infection in gulls on the island of Newfoundland, Canada over 2008-2011, and analyzed 16 of the detected gull AIVs for their phylogeny and genotype to shed light on the transmission, reassortment and perpetuation of AIVs in gulls in this region of North America. Time of most recent common ancestors for the eight segments of the predominant viruses were also determined to reveal the emerging time of the genes in gulls in Atlantic Canada.

4.2. Materials and Methods

4.2.1. Ethics statement

This work was carried out under the guidelines specified by the Canadian Council on Animal Care with approved protocols 09-01-AL, 10-01-AL and 11-01-AL from the

Memorial University Institutional Animal Care Committee or under the authority of the Director of the Newfoundland and Labrador Animal Health Division. Pre-fledged young birds were caught by hand, while flight-capable birds were caught in noose carpet traps or by an air-propelled net launcher. Birds were banded under banding permit 10559. Swabs of the cloaca and oropharyngeal cavity were collected as samples for virus screening, and blood samples were also taken from the brachial vein of some birds to detect the anti-AIV antibodies in gulls. Eggs were collected under permit SP2782 from Canadian Wildlife Service to test the maternal antibody against AIVs. The sampling was either done at locations where no access permits were required (public areas around the City of St. John's) or with the permission of the relevant authorities in the cases of the St. John's regional landfill and the Witless Bay Seabird Ecological Reserve. This research was also approved under Memorial University biosafety permit S-103-08.

4.2.2. Sampling and surveillance for AIV

Live gulls were captured at a variety of locations, mainly in eastern Newfoundland, but also in other locations across the province. Dead gulls brought to provincial or federal facilities were also sampled opportunistically. The sampling period for swabbing spanned May 2008 to December 2011. The total number of gulls sampled was 1350. Blood samples, 2.0-2.5 ml from the brachial vein, were collected between October 2011 and September 2012. Gulls were aged based on their age-specific plumages, which are reliable until their second to fourth year, depending on the species. We classified all gulls in their first year of life (<12 months old), including pre-fledged young, as juveniles and all others as adult birds even though the adult category includes sexually immature birds that are not yet capable of breeding. Months of the year were grouped into

four unequal periods reflective of the annual life history of the birds: May-August: summer (breeding season), September-October: fall (post-fledging dispersal and migration), November-February: winter (non-breeding season), March-April: spring (spring migration). In addition to sampling birds, fresh fecal samples were collected from roost sites. Only samples than could be confirmed as coming from gulls were collected, although no species information.

Swab samples were kept cool in the field for less than 24 hours before being stored at -80 ℃ until processed. Samples were screened for the presence of AIV by real time RT-PCR targeting the M gene using previously published methods (Spackman, Senne et al. 2002, Granter, Wille et al. 2010). Positive samples were identified as those with Ct values <35. Prevalence was compared across years and season using log-likelihood ratio tests (LRT) in R 3.0.1 (www.R-project.org/).

Blood samples were allowed to coagulate before centrifugation to separate the serum. Serum was tested for the presence of anti-AIV NP antibodies using the AI MultiS-Screen Ab Test (IDEXX, Westbrook, Maine) as recommended by the manufacturer.

4.2.3. Virus sequencing

RNA was extracted from positive swab samples with Trizol LS reagent (Life Technologies, Burlington, Canada) and subjected to RT-PCR using previously published primers (Hoffmann, Stech et al. 2001, Phipps, Essen et al. 2004, Chan, Lin et al. 2006, Obenauer, Denson et al. 2006, Huang, Khan et al. 2013) and the Superscript III One-Step RT-PCR System (Life Technologies). The resulting RT-PCR products were purified with QIAquick PCR purification kit (Qiagen, Toronto, Canada) and sequenced at the Center

for Applied Genomics (Toronto, Canada). The sequence data was compiled and analyzed using Lasergene v7.1 (DNASTAR Inc., Madison, WI).

4.2.4. Phylogenetic analyses

The AIV sequences obtained in this study and reference AIV sequences from the NCBI influenza database (Bao, Bolotov et al. 2008) and the GISAID epiFlu database (http://platform.gisaid.org) were subjected to phylogenetic analysis. Two rounds of phylogenetic analyses were performed. In the first round of analysis, available AIV reference sequences from gulls, ducks and shorebirds in North America and Eurasia were included. We used at least 20 sequences closely related to each gull AIV sequence from our study, which were identified by BLAST searches (Altschul, Madden et al. 1997), in these analyses. On the basis of these results, the second set of phylogenetic analyses was limited by inclusion of only the phylogenetic clades closely related to the 2009-2011 Newfoundland gull AIV sequences. The nucleotide ranges of the coding sequence regions for each segment used in the phylogenetic trees were: PB2, 1804-2256; PB1, 1624-2271; PA, 1-528; NP, 4-576; M, 40-939; NS, 55-792; H1, 1147-1590; H9, 1183-1662; H13, 1090-1638; H16, 1087-1698; N3, 625-1350; N6, 904-1383; and N9, 844-1395. The nucleotide sequence alignments and pairwise-distance analyses were performed with the Jotun Hein method in Lasergene v7.1 (DNAStar, Inc., Madison, WI). Phylogenetic trees were constructed with the maximum likelihood method in MEGA5 (Tamura, Peterson et al. 2011) with 1000 bootstrap replicates. The maximum likelihood topologies were then confirmed by comparison to neighbour-joining trees constructed in MEGA5 and maximum clade credibility trees constructed using BEAST v1.7.5 (Drummond, Suchard et al. 2012).

The times of most recent common ancestor (tMRCA) were estimated for the predominant Atlantic Canada gull clades with BEAST v1.7.5 (Drummond, Suchard et al. 2012). A lognormal relaxed clock (unrelated) model was used to infer the phylogenetic timescale, and the analysis was performed with the GMRF Bayesian skyride model and time-aware smoothing (10^8 steps with sampling every 10^4 steps). The convergence was assessed in Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/), and maximum clade credibility trees were summarized with 10% of the input file excluded.

4.2.5. Virus gene lineage and genotype assignments

Continental and host taxa affiliations were assigned to the virus segments based on the origins of the reference sequences within their associated phylogenetic clades. These included North American gull (AG), North American avian (AA), Eurasian gull (EG) and Eurasian avian (EA). Sequences within a lineage were determined as those had ≥90% nucleotide identity, and sequences within a clade had ≥95% nucleotide identity. Serial numbers were assigned to the lineages and clades for each segment. Where there was only a single clade within a lineage, the lineage number is the clade number. Genotypes were then assigned by the compilation of the assigned clades of the different segments.

4.3. Results

4.3.1. Prevalence of AIV infection in gulls

Between May 2008 and December 2011, 20 AIV positive samples were identified in 1350 paired (oral and cloacal) swab samples collected from gulls (1.5%). Most samples

and positives were from the two species that are present on the island of Newfoundland year-round: American Herring Gull (*Larus smithsonianus*; 13/1083) and Great Black-backed Gull (*Larus marinus*; 6/200). The remaining positive sample was from a juvenile Ring-billed Gull (*Larus delawarensis*) captured in the fall of 2010 (1/21 total for species). Other species sampled were Black-headed Gull (*Chroicocephalus ridibundus*; 0/1), Glaucous Gull (*Larus hyperboreus*; 0/24), Iceland Gull (*Larus glaucoides*; 0/19) and Mew Gull (*Larus canus*; 0/2). Another 10 positive samples were identified in the 295 fecal samples with the gull species undetermined (3.4%).

A peak in AIV infection occurred in the fall (Table 4.1) and was detected in both Herring and Great Black-backed Gulls (LRT, $\chi^2 = 53.0$, df = 3, $P < 0.0001$) and the fecal samples (LRT, $\chi^2 = 10.7$, df = 3, $P < 0.0001$). Both Herring and Great Black-backed Gull chicks were heavily sampled in the summer when they were 3-6 weeks of age, but only one positive was detected in 792 samples (Table 4.1).

All blood-samples from the four species sampled and tested for anti-AIV antibodies showed evidence of previous infection: Great Black-backed Gull (2/10, 20%), Herring Gull (33/63, 52%), Ring-billed Gull (5/7, 71.4%) and Iceland Gull (4/8, 50%), for an overall rate of 50% (44/88) across all species.

Table 4.1. Seasonality of AIV prevalence in juvenile and adult Great Black-backed and Herring Gulls in Newfoundland and Labrador, 2008-2011

	Summer	Fall	Winter	Spring
Great Black-backed Gull				
Juvenile[a]	0/107 (0%)	5/31 (16.1%)	0/4 (0%)	0/5 (0%)
Adult	0/2 (0%)	0/12 (0%)	1/32 (3.1%)	0/7 (0%)
Herring Gull				
Juvenile	1/685 (0.1%)	7/83 (8.4%)	0/7 (0%)	0/3 (0%)
Adult	0/17 (0%)	5/89 (5.6%)	0/85 (0%)	0/109 (0%)
Fecal samples	0/66 (0%)	8/109 (7.3%)	2/96 (2.1%)	0/24 (0%)

[a] Juveniles are birds less than 12 months old.

4.3.2. Sequences obtained from gull AIVs

Sequences were successfully determined for 16 of the 28 AIV-positive samples detected from gulls on the island of Newfoundland during 2009-2011 (the positive samples from 2008 were previously studied (Wille, Robertson et al. 2011). Among the 16 viruses, 15 were detected around the City of St. John's (47°56'N, 52°71'W) in eastern Newfoundland and 1, A/Great black-backed gull/Newfoundland/AB001/2011(H9N9), was from Corner Brook (48 °96'N, 57 °93'W) in western Newfoundland. Sequences were obtained from 123 segments, which represented all 8 segments of 13 viruses, and 6 or 7 segments for the other 3 viruses. The sequences were submitted to the GenBank database under accession numbers KC845024-KC845145.

4.3.3. Phylogenetic analyses

The Newfoundland and reference gull AIV sequences were used for phylogenetic analyses together with closely related reference genes identified in the sequence databases. The lineage assignments from the maximum likelihood trees matched those on the neighbor-joining and the maximum clade credibility trees. A large genetic diversity was revealed in the AIVs that were circulating in the Newfoundland gulls and multiple lineages were identified for each gene segment of these viruses, as detailed below.

4.3.4. Surface protein segments

The HA subtypes were determined for 14 of the 16 viruses, and those were identified one H1, one H9, eight H13 and four H16 viruses (Figure 4.1A and B). The H1 and H9 sequences belonged to North American avian lineages (Figure 4.1A and B). The H1 sequence was very closely related to a gene detected from a shorebird in Delaware in 2009, with the remaining sequences in the lineage coming from duck viruses. The eight H13 sequences belonged to a North American gull lineage, closely related to four H13 genes detected in 2009 elsewhere in Atlantic Canada, three from gulls in Quebec and one from a sea duck in New Brunswick, as well as another gull virus from Newfoundland from 2008 (Figure 4.1C). There were also numerous gull H13 sequences in the database from other locations in eastern North America and Alaska, but these were assigned in distinct lineages. The four H16 sequences were assigned in a Eurasian gull lineage, clustered together with virus sequences that mostly originated from other Northern Atlantic locations such as Iceland, Sweden, and Norway (Figure 4.1D).

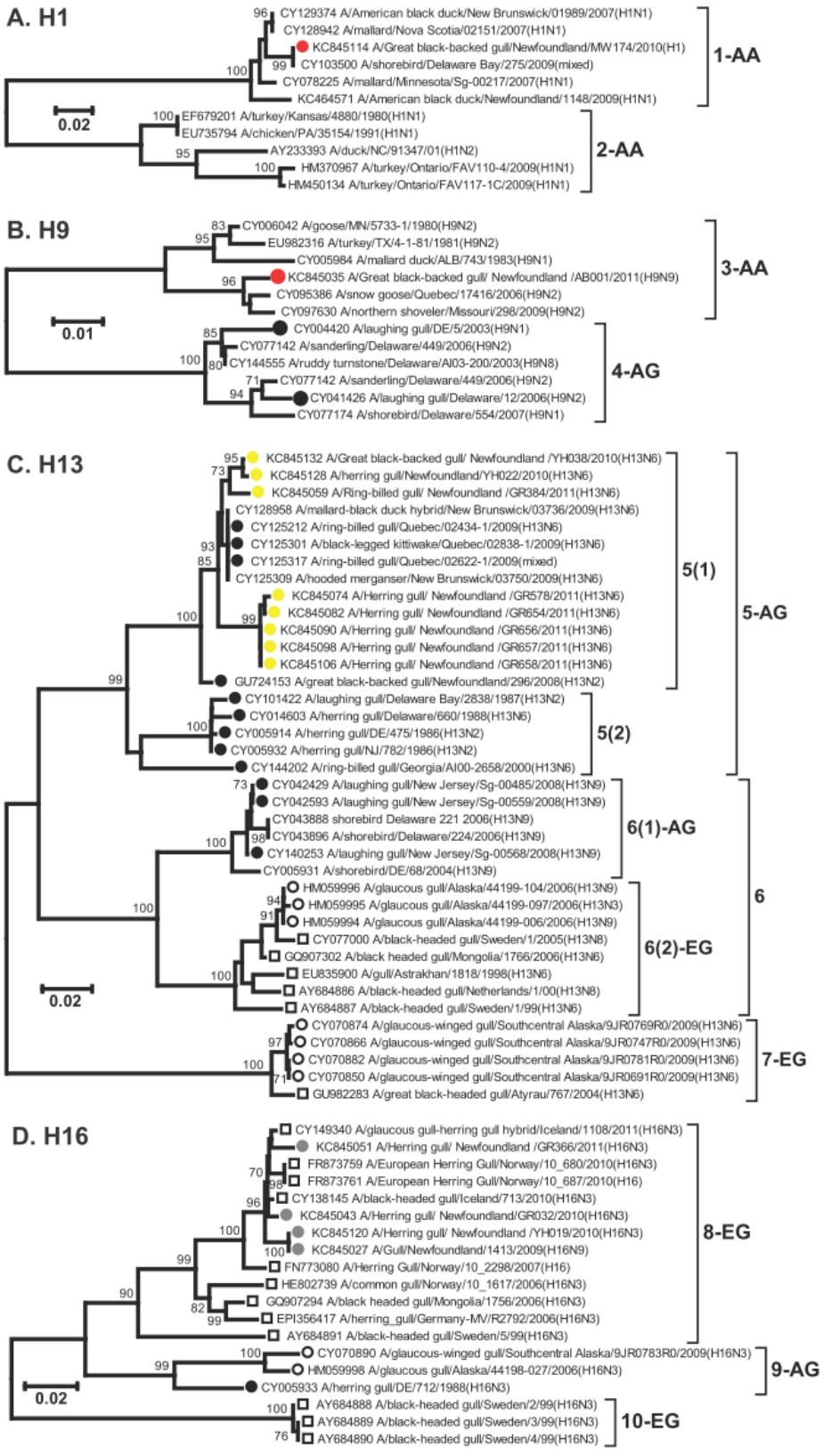
A. H1
CY129374 A/American black duck/New Brunswick/01989/2007(H1N1)
CY128942 A/mallard/Nova Scotia/02151/2007(H1N1)
KC845114 A/Great black-backed gull/Newfoundland/MW174/2010(H1)
CY103500 A/shorebird/Delaware Bay/275/2009(mixed)
CY078225 A/mallard/Minnesota/Sg-00217/2007(H1N1)
KC464571 A/American black duck/Newfoundland/1148/2009(H1N1)
1-AA
0.02
EF679201 A/turkey/Kansas/4880/1980(H1N1)
EU735794 A/chicken/PA/35154/1991(H1N1)
AY233393 A/duck/NC/91347/01(H1N2)
HM370967 A/turkey/Ontario/FAV110-4/2009(H1N1)
HM450134 A/turkey/Ontario/FAV117-1C/2009(H1N1)
2-AA
B. H9
CY006042 A/goose/MN/5733-1/1980(H9N2)
EU982316 A/turkey/TX/4-1-81/1981(H9N2)
CY005984 A/mallard duck/ALB/743/1983(H9N1)
KC845035 A/Great black-backed gull/ Newfoundland /AB001/2011(H9N9)
CY095386 A/snow goose/Quebec/17416/2006(H9N2)
CY097630 A/northern shoveler/Missouri/298/2009(H9N2)
3-AA
0.01
CY004420 A/laughing gull/DE/5/2003(H9N1)
CY077142 A/sanderling/Delaware/449/2006(H9N2)
CY144555 A/ruddy turnstone/Delaware/AI03-200/2003(H9N8)
CY077142 A/sanderling/Delaware/449/2006(H9N2)
CY041426 A/laughing gull/Delaware/12/2006(H9N2)
CY077174 A/shorebird/Delaware/554/2007(H9N1)
4-AG
C. H13
KC845132 A/Great black-backed gull/ Newfoundland /YH038/2010(H13N6)
KC845128 A/herring gull/Newfoundland/YH022/2010(H13N6)
KC845059 A/Ring-billed gull/ Newfoundland /GR384/2011(H13N6)
CY128958 A/mallard-black duck hybrid/New Brunswick/03736/2009(H13N6)
CY125212 A/ring-billed gull/Quebec/02434-1/2009(H13N6)
CY125301 A/black-legged kittiwake/Quebec/02838-1/2009(H13N6)
CY125317 A/ring-billed gull/Quebec/02622-1/2009(mixed)
CY125309 A/hooded merganser/New Brunswick/03750/2009(H13N6)
KC845074 A/Herring gull/ Newfoundland /GR578/2011(H13N6)
KC845082 A/Herring gull/ Newfoundland /GR654/2011(H13N6)
KC845090 A/Herring gull/ Newfoundland /GR656/2011(H13N6)
KC845098 A/Herring gull/ Newfoundland /GR657/2011(H13N6)
KC845106 A/Herring gull/ Newfoundland /GR658/2011(H13N6)
GU724153 A/great black-backed gull/Newfoundland/296/2008(H13N2)
5(1)
CY101422 A/laughing gull/Delaware Bay/2838/1987(H13N2)
CY014603 A/herring gull/Delaware/660/1988(H13N6)
CY005914 A/herring gull/DE/475/1986(H13N2)
CY005932 A/herring gull/NJ/782/1986(H13N2)
CY144202 A/ring-billed gull/Georgia/AI00-2658/2000(H13N6)
5(2)
5-AG
CY042429 A/laughing gull/New Jersey/Sg-00485/2008(H13N9)
CY042593 A/laughing gull/New Jersey/Sg-00559/2008(H13N9)
CY043888 shorebird Delaware 221 2006(H13N9)
CY043896 A/shorebird/Delaware/224/2006(H13N9)
CY140253 A/laughing gull/New Jersey/Sg-00568/2008(H13N9)
CY005931 A/shorebird/DE/68/2004(H13N9)
6(1)-AG
HM059996 A/glaucous gull/Alaska/44199-104/2006(H13N9)
HM059995 A/glaucous gull/Alaska/44199-097/2006(H13N3)
HM059994 A/glaucous gull/Alaska/44199-006/2006(H13N9)
CY077000 A/black-headed gull/Sweden/1/2005(H13N8)
GQ907302 A/black headed gull/Mongolia/1766/2006(H13N6)
EU835900 A/gull/Astrakhan/1818/1998(H13N6)
AY684886 A/black-headed gull/Netherlands/1/00(H13N8)
AY684887 A/black-headed gull/Sweden/1/99(H13N6)
6(2)-EG
6
0.02
CY070874 A/glaucous-winged gull/Southcentral Alaska/9JR0769R0/2009(H13N6)
CY070866 A/glaucous-winged gull/Southcentral Alaska/9JR0747R0/2009(H13N6)
CY070882 A/glaucous-winged gull/Southcentral Alaska/9JR0781R0/2009(H13N6)
CY070850 A/glaucous-winged gull/Southcentral Alaska/9JR0691R0/2009(H13N6)
GU982283 A/great black-headed gull/Atyrau/767/2004(H13N6)
7-EG
D. H16
CY149340 A/glaucous gull-herring gull hybrid/Iceland/1108/2011(H16N3)
KC845051 A/Herring gull/ Newfoundland /GR366/2011(H16N3)
FR873759 A/European Herring Gull/Norway/10_680/2010(H16N3)
FR873761 A/European Herring Gull/Norway/10_687/2010(H16)
CY138145 A/black-headed gull/Iceland/713/2010(H16N3)
KC845043 A/Herring gull/ Newfoundland/GR032/2010(H16N3)
KC845120 A/Herring gull/ Newfoundland /YH019/2010(H16N3)
KC845027 A/Gull/Newfoundland/1413/2009(H16N9)
FN773080 A/Herring Gull/Norway/10_2298/2007(H16)
HE802739 A/common gull/Norway/10_1617/2006(H16N3)
GQ907294 A/black headed gull/Mongolia/1756/2006(H16N3)
EPI356417 A/herring_gull/Germany-MV/R2792/2006(H16N3)
AY684891 A/black-headed gull/Sweden/5/99(H16N3)
8-EG
CY070890 A/glaucous-winged gull/Southcentral Alaska/9JR0783R0/2009(H16N3)
HM059998 A/glaucous gull/Alaska/44198-027/2006(H16N3)
9-AG
0.02
CY005933 A/herring gull/DE/712/1988(H16N3)
AY684888 A/black-headed gull/Sweden/2/99(H16N3)
AY684889 A/black-headed gull/Sweden/3/99(H16N3)
AY684890 A/black-headed gull/Sweden/4/99(H16N3)
10-EG

Figure 4.1. Phylogenetic trees for the Newfoundland gull virus HA sequences from 2009-2011. Individual trees are shown for the H1 (A), H9 (B), H13 (C) and H16 (D) sequences. The gull AIV sequences from Newfoundland (from 2009-2011) are labelled according to the geographic and host group affiliation of their clades: yellow circle for North American gull (AG), red circle for North American avian (AA) and grey circle for Eurasian gull (EG). Reference gull AIV sequences from Atlantic and Pacific North America are labelled with black and open circles, respectively, while those from Eurasia are labelled with open squares. Gene lineages and clades were assigned with serial numbers as described in the Material and Method section. Phylogenetic maximum likelihood (ML) trees were constructed using MEGA5 with 1000 bootstrap replicates and bootstrap values ≥70% are given at the nodes. The scale bars indicate substitutions per site.

The NA subtypes were determined for 14 of the 16 viruses, which identified three N3, nine N6 and two N9 viruses. The three N3 sequences belonged to a Eurasian gull lineage that also included sequences from shorebirds in Delaware and several gulls in Alaska, but they were most similar to a duck virus sequence (A/mallard/Quebec/02916-1/2009(H16N3)) (Figure 4.2A). Two other gull-dominated N3 lineages were identified in gulls, with one from North America and one from Eurasia. All of the N6 sequences belonged to a North American avian lineage, and several closely related sequences were identified in a shorebird virus in New Jersey, three gull viruses in Quebec and two duck viruses in New Brunswick (Figure 4.2B). In comparison, five N6 sequences from Alaskan gulls were classified in a different lineage (Figure 4.2B). The two N9 genes were assigned to the same lineage, but were separated into distinct North American avian and North American gull clades (Figure 4.2C). The two available reference N9 sequences from gulls in Alaska belonged to a third North American avian clade within this lineage.

4.3.5. Internal protein segments

Similar to the findings for the HA and NA segments, more than one lineage was detected for each of the six internal protein segments amongst the 16 viruses. Simplified topology trees are shown in Figure 4.3, and trees with detailed virus information labels are available in Figure S4.1.

The 16 PB2 genes in NL gulls belonged to two phylogenetic lineages (Figures 4.3A and S4.1). Four sequences belonged to a North America avian clade in lineage 1, while the gull reference sequences in the same lineage from Delaware and New Jersey (from 1988-1989) and Alaska (from 2006) formed two independent clades. The other 12 PB2 sequences belonged to a Eurasian gull clade in lineage 2, closely related to

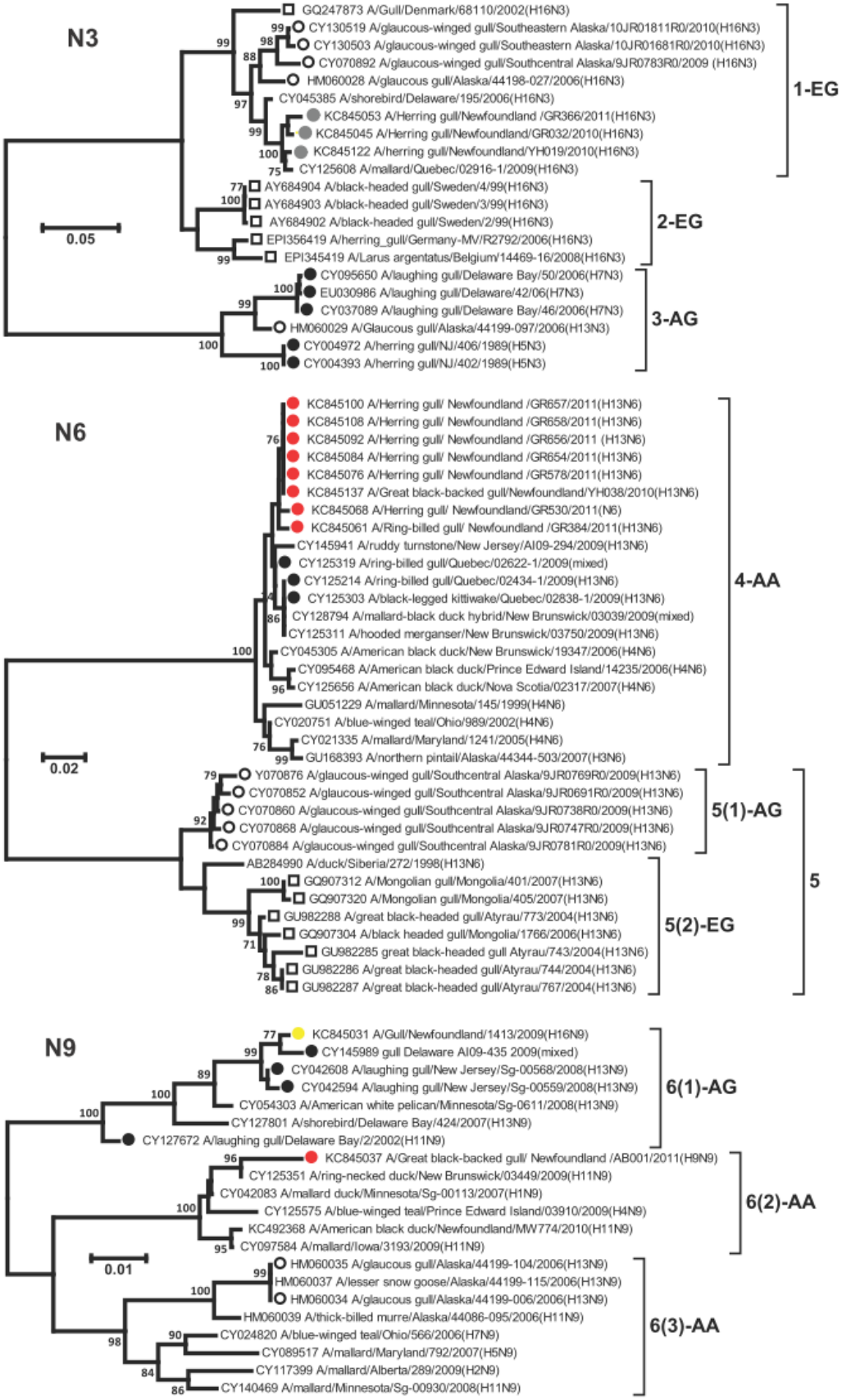
N3
GQ247873 A/Gull/Denmark/68110/2002(H16N3)
CY130519 A/glaucous-winged gull/Southeastern Alaska/10JR01811R0/2010(H16N3)
CY130503 A/glaucous-winged gull/Southeastern Alaska/10JR01681R0/2010(H16N3)
CY070892 A/glaucous-winged gull/Southcentral Alaska/9JR0783R0/2009 (H16N3)
HM060028 A/glaucous gull/Alaska/44198-027/2006(H16N3)
CY045385 A/shorebird/Delaware/195/2006(H16N3)
KC845053 A/Herring gull/Newfoundland /GR366/2011(H16N3)
KC845045 A/Herring gull/Newfoundland/GR032/2010(H16N3)
KC845122 A/herring gull/Newfoundland/YH019/2010(H16N3)
CY125608 A/mallard/Quebec/02916-1/2009(H16N3)
1-EG
AY684904 A/black-headed gull/Sweden/4/99(H16N3)
AY684903 A/black-headed gull/Sweden/3/99(H16N3)
AY684902 A/black-headed gull/Sweden/2/99(H16N3)
EPI356419 A/herring_gull/Germany-MV/R2792/2006(H16N3)
EPI345419 A/Larus argentatus/Belgium/14469-16/2008(H16N3)
2-EG
0.05
CY095650 A/laughing gull/Delaware Bay/50/2006(H7N3)
EU030986 A/laughing gull/Delaware/42/06(H7N3)
CY037089 A/laughing gull/Delaware Bay/46/2006(H7N3)
HM060029 A/Glaucous gull/Alaska/44199-097/2006(H13N3)
CY004972 A/herring gull/NJ/406/1989(H5N3)
CY004393 A/herring gull/NJ/402/1989(H5N3)
3-AG
N6
KC845100 A/Herring gull/ Newfoundland /GR657/2011(H13N6)
KC845108 A/Herring gull/ Newfoundland /GR658/2011(H13N6)
KC845092 A/Herring gull/ Newfoundland /GR656/2011 (H13N6)
KC845084 A/Herring gull/ Newfoundland /GR654/2011(H13N6)
KC845076 A/Herring gull/ Newfoundland /GR578/2011(H13N6)
KC845137 A/Great black-backed gull/Newfoundland/YH038/2010(H13N6)
KC845068 A/Herring gull/ Newfoundland/GR530/2011(N6)
KC845061 A/Ring-billed gull/ Newfoundland /GR384/2011(H13N6)
CY145941 A/ruddy turnstone/New Jersey/AI09-294/2009(H13N6)
CY125319 A/ring-billed gull/Quebec/02622-1/2009(mixed)
CY125214 A/ring-billed gull/Quebec/02434-1/2009(H13N6)
CY125303 A/black-legged kittiwake/Quebec/02838-1/2009(H13N6)
CY128794 A/mallard-black duck hybrid/New Brunswick/03039/2009(mixed)
CY125311 A/hooded merganser/New Brunswick/03750/2009(H13N6)
CY045305 A/American black duck/New Brunswick/19347/2006(H4N6)
CY095468 A/American black duck/Prince Edward Island/14235/2006(H4N6)
CY125656 A/American black duck/Nova Scotia/02317/2007(H4N6)
GU051229 A/mallard/Minnesota/145/1999(H4N6)
CY020751 A/blue-winged teal/Ohio/989/2002(H4N6)
CY021335 A/mallard/Maryland/1241/2005(H4N6)
GU168393 A/northern pintail/Alaska/44344-503/2007(H3N6)
4-AA
0.02
Y070876 A/glaucous-winged gull/Southcentral Alaska/9JR0769R0/2009(H13N6)
CY070852 A/glaucous-winged gull/Southcentral Alaska/9JR0691R0/2009(H13N6)
CY070860 A/glaucous-winged gull/Southcentral Alaska/9JR0738R0/2009(H13N6)
CY070868 A/glaucous-winged gull/Southcentral Alaska/9JR0747R0/2009(H13N6)
CY070884 A/glaucous-winged gull/Southcentral Alaska/9JR0781R0/2009(H13N6)
5(1)-AG
AB284990 A/duck/Siberia/272/1998(H13N6)
GQ907312 A/Mongolian gull/Mongolia/401/2007(H13N6)
GQ907320 A/Mongolian gull/Mongolia/405/2007(H13N6)
GU982288 A/great black-headed gull/Atyrau/773/2004(H13N6)
GQ907304 A/black headed gull/Mongolia/1766/2006(H13N6)
GU982285 great black-headed gull Atyrau/743/2004(H13N6)
GU982286 A/great black-headed gull/Atyrau/744/2004(H13N6)
GU982287 A/great black-headed gull/Atyrau/767/2004(H13N6)
5(2)-EG
5
N9
KC845031 A/Gull/Newfoundland/1413/2009(H16N9)
CY145989 gull Delaware AI09-435 2009(mixed)
CY042608 A/laughing gull/New Jersey/Sg-00568/2008(H13N9)
CY042594 A/laughing gull/New Jersey/Sg-00559/2008(H13N9)
CY054303 A/American white pelican/Minnesota/Sg-0611/2008(H13N9)
CY127801 A/shorebird/Delaware Bay/424/2007(H13N9)
CY127672 A/laughing gull/Delaware Bay/2/2002(H11N9)
6(1)-AG
KC845037 A/Great black-backed gull/ Newfoundland /AB001/2011(H9N9)
CY125351 A/ring-necked duck/New Brunswick/03449/2009(H11N9)
CY042083 A/mallard duck/Minnesota/Sg-00113/2007(H1N9)
CY125575 A/blue-winged teal/Prince Edward Island/03910/2009(H4N9)
KC492368 A/American black duck/Newfoundland/MW774/2010(H11N9)
CY097584 A/mallard/Iowa/3193/2009(H11N9)
6(2)-AA
0.01
HM060035 A/glaucous gull/Alaska/44199-104/2006(H13N9)
HM060037 A/lesser snow goose/Alaska/44199-115/2006(H13N9)
HM060034 A/glaucous gull/Alaska/44199-006/2006(H13N9)
HM060039 A/thick-billed murre/Alaska/44086-095/2006(H11N9)
CY024820 A/blue-winged teal/Ohio/566/2006(H7N9)
CY089517 A/mallard/Maryland/792/2007(H5N9)
CY117399 A/mallard/Alberta/289/2009(H2N9)
CY140469 A/mallard/Minnesota/Sg-00930/2008(H11N9)
6(3)-AA

Figure 4.2. Phylogenetic trees for the Newfoundland gull virus NA sequences from 2009-2011. Individual trees are shown for the N3 (A), N6 (B) and N9 (C) sequences. The gull AIV sequences from Newfoundland (from 2009-2011) are labelled according to the geographic and host group affiliation of their clades: yellow circle for North American gull (AG), red circle for North American avian (AA) and grey circle for Eurasian gull (EG). Reference gull AIV sequences from Atlantic and Pacific North America are labelled with black and open circles, respectively, while those from Eurasia are labelled with open squares. Gene lineages and clades were assigned with serial numbers as described in the Material and Method section 4.2.5. Phylogenetic maximum likelihood (ML) trees were constructed using MEGA5 with 1000 bootstrap replicates and bootstrap values ≥70% are given at the nodes. The scale bars indicate substitutions per site.

sequences from ducks, gulls and shorebirds from other locations in Atlantic North America.

The PB1 sequences were classified in two lineages, with 13 genes in lineage 1 and 3 genes in lineage 2 (Figures 4.3B and S4.1). Six of the sequences in lineage 1 were separated in three North American avian clades, while the other seven genes in lineage 1 belonged to a North American gull clade, closely related to several genes from ducks and gulls in Atlantic Canada from 2008-2009. The three NL sequences in lineage 2 were assigned in a Eurasian gull-dominated clade, while the gull reference genes from Alaska in 2009 belonged to another Eurasian avian clade in lineage 2.

The 16 PA sequences were assigned in four lineages (Figures 4.3C and S4.1). Thirteen were in lineage 1, separated into North American and Eurasian gull clades. The other three sequences were classified in three other lineages, a Eurasian avian clade and two North American avian clades.

The 15 NP sequences belonged to two phylogenetic lineages (Figures 4.3D and S4.1). Among the 14 sequences in lineage 1, two belonged to a Eurasian gull clade, while the other 12 were in a North American gull clade, closely related to several viruses from gulls, shorebirds and ducks of Atlantic North America. The only NP sequence in lineage 2, from A/Great black-backed gull/Newfoundland AB001/2011(H9N9), was of North American avian origin. There were also two other North American avian clades containing sequences from gulls (in Delaware from 2005-2006) in lineage 2.

The 16 M sequences belonged to two lineages (Figures 4.3E and S4.1). Fourteen of them belonged to the Eurasian gull lineage 1, together with several genes from gulls, ducks and shorebirds in Atlantic North America.

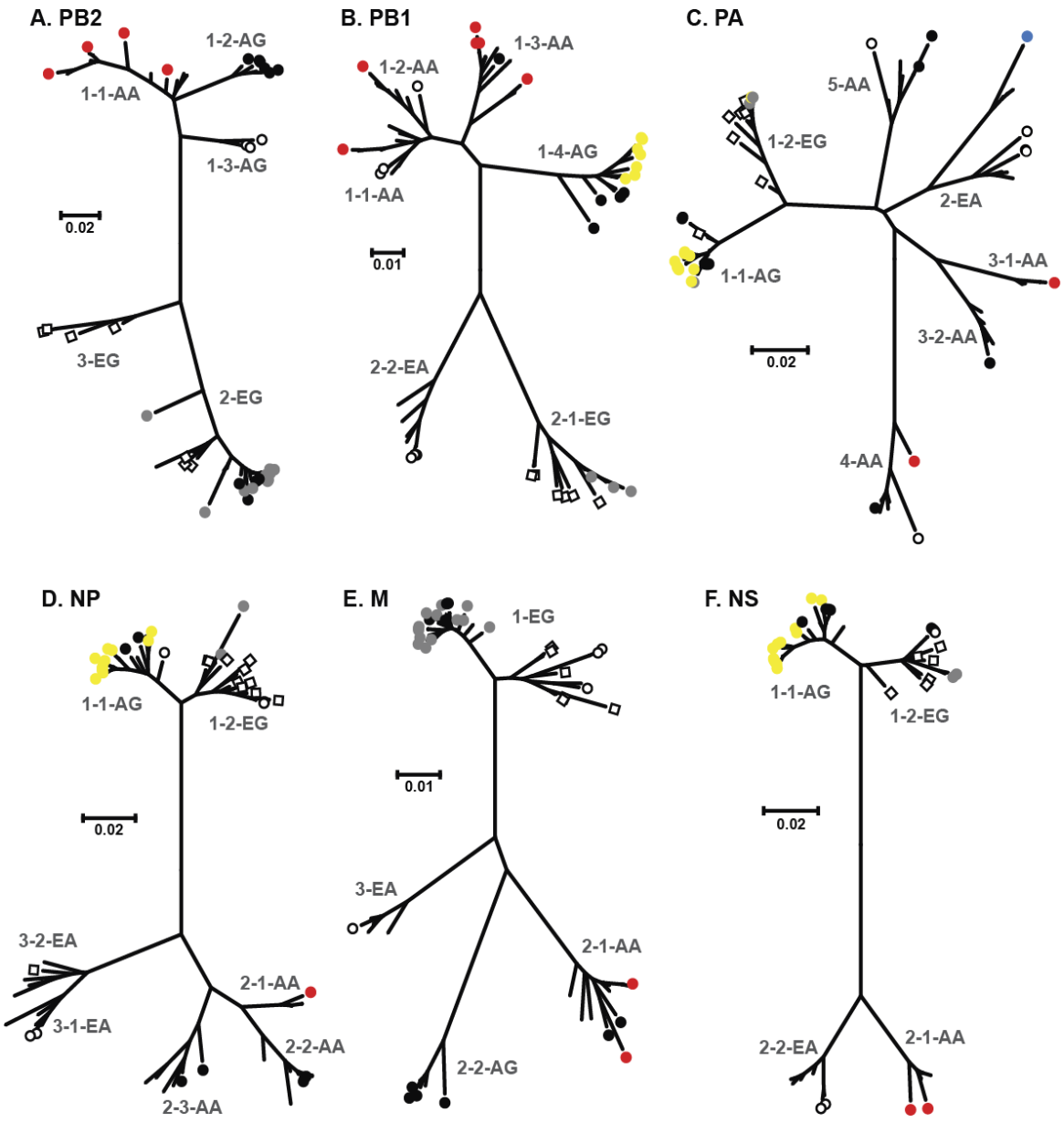

Figure 4.3. Phylogenetic topology trees for Newfoundland gull AIV internal protein gene segments. The trees show the lineage distribution patterns for the PB2 (A), PB1 (B), PA (C), NP (D), M (E) and NS (F) segments. Trees with full virus labels are provided in Figure S1. The gull AIV sequences from Newfoundland (from 2009-2011) are labelled according to the geographic and host group affiliation of their clades: yellow circle for

grey circle for Eurasian gull (EG) and blue circle for Eurasian avian (EA). Reference gull AIV sequences from Atlantic and Pacific North America are labelled with black and open circles, respectively, while those from Eurasia are labelled with open squares. Gene lineages and clades were assigned with serial numbers as described in the Material and Method section. Phylogenetic maximum likelihood (ML) trees were constructed using MEGA5 with 1000 bootstrap replicates and bootstrap values ≥70% are given at the nodes. The scale bars indicate substitutions per site.

Table 4.2. Phylogenetic clade assignments for the 8 gene segments of the 16 Newfoundland gull AIVs from 2009-2011

Clade	Number of viruses								Total
	PB2	PB1	PA	HA	NP	NA	M	NS	(percentage)
North American gull	0	7	10	8	12	1	0	12	50 (40.6%)
North American avian	4	6	2	2	1	10	2	2	29 (23.6%)
Eurasian gull	12	3	3	4	2	3	14	2	43 (35%)
Eurasian avian	0	0	1	0	0	0	0	0	1 (0.8%)
Total	16	16	16	14	15	14	16	16	123

The other two sequences belonged to a North American avian clade in lineage 2, which also contained several viruses from gulls in Delaware and New Jersey (from 2005-2006).

The 16 NS sequences were all allele A and belonged to two lineages (Figures 4.3F and S4.1). Among the 14 genes in lineage 1, twelve of them were classified in a North American gull clade, together with several genes from gulls and shorebirds in Atlantic North America. The other two sequences in lineage 1 belonged to a Eurasian gull clade, which included several viruses from Alaskan gulls in 2009. Two of the NS sequences were classified in a North America avian clade in lineage 2.

4.3.6. Genetic structure of the gull AIVs

The continental and host taxa affiliations for the 16 Newfoundland gull AIV segments, as identified by the phylogenetic clade assignments, are summarized in Table 4.2. This revealed the AIV gene pool was dominated by gull lineage sequences (75.6%), but frequent inter-continental (35.8%) and considerable inter-host group (24.4%) segment transmission was found. Among the 123 sequences, 50 were classified in North American gull clades (40.6%), 43 were in Eurasian gull clades (35%), 29 were in North American avian clades (23.6%), and the other 1 was in a Eurasian avian clade (0.8%).

4.3.7. Genome dynamics in gull AIVs

The genotype assignments based on the combination of lineages for the 8 segments showed that 15 of the gull AIVs were inter-continental reassortants and contained Eurasian lineage genes, while 12 viruses were interspecies reassortants and contained avian lineage genes (Figure 4.4). Of particular note, all 8 gene segments of A/Herring gull/Newfoundland/YH019/2010(H16N3) were assigned in Eurasian gull

clades. Another virus of note was A/Great black-backed gull/Newfoundland/AB001/2011(H9N9), which had a genome comprised of solely waterfowl-related segments, with a PA gene of Eurasian avian origin and the other genes of North American avian origin.

Based on the phylogenetic clades for each segment (Figures 4.1, 4.2 and 4.3), the thirteen gull viruses with sequence information available for all eight segments were classified in 10 genotypes (A to I; Figure 4.4). Genotype I was the predominant genotype, found in three of the 13 AIV genomes. In comparison, eight other genotypes (all except F) were different reassortants relative to genotype I. Genotype F and G were genotype I reassortants with different lineage HA and PA segments, respectively. Genotype A contained different HA and NA segments and genotype D had different PB2 and PB1 segments compared to genotype I. Furthermore, genotype C was a genotype I reassortant with different HA, NA and PB1 segments. Five additional highly related H13N6 AIVs, 3 gull viruses from Quebec and 2 duck viruses from New Brunswick in 2009 (Hall, TeSlaa et al. 2013), also had genotype I (the 3 gull viruses are included in Figures 4.1, 4.2 and S4.1). A previously described Newfoundland gull AIV, A/Great black-backed gull/Newfoundland/296/2008(H13N2), contained all segments except PA and NA classified in the same clades (Figures 4.1, 4.2 and S4.1). Time of most recent common ancestor analyses for the eight segments of these genotype I viruses showed they emerged in gulls in Atlantic Canada between 2001 and 2008 (Figure 4.5).

Date	Virus	Phylogenetic clade number								Genotype
		PB2	PB1	PA	HA	NP	NA	M	NS	
03-09-09	A/Gull/Newfoundland/1413/2009(H16N9)	2	1-4	1-1	8	1-1	6-1	1	1-1	A
21-02-10	A/Great black-backed gull/Newfoundland/MW174/2010(H1)	1-1	1-3	4	1	-	-	2-1	2-1	-
14-07-10	A/Herring gull/Newfoundland/GR032/2010(H16N3)	2	1-4	1-2	8	1-1	1	1	1-1	B
03-10-10	A/Herring gull/Newfoundland/YH019/2010(H16N3)	2	2-1	1-2	8	1-2	1	1	1-2	C
03-10-10	A/herring gull/Newfoundland/YH022/2010(H13N6)	1-1	2-1	1-1	5-1	1-1	4	1	1-1	D
03-10-10	A/Great black-backed gull/Newfoundland/YH038/2010(H13N6)	2	1-1	1-1	5-1	1-1	4	1	1-1	E
14-10-10	A/Gull/Newfoundland/YH043/2010	1-1	2-1	1-2	-	1-2	-	1	1-2	-
20-02-11	A/Great black-backed gull/Newfoundland/AB001/2011(H9N9)	1-1	1-2	2	3	2-1	6-2	2-1	2-1	F
09-09-11	A/Ring-billed gull/Newfoundland/GR384/2011(H13N6)	2	1-3	1-1	5-1	1-1	4	1	1-1	G
09-09-11	A/Herring gull/Newfoundland/GR366/2011(H16N3)	2	1-4	1-1	8	1-1	1	1	1-1	H
22-09-11	A/Herring gull/Newfoundland/GR530/2011(N6)	2	1-3	1-1	-	1-1	4	1	1-1	-
30-09-11	A/Herring gull/Newfoundland/GR578/2011(H13N6)	2	1-3	1-1	5-1	1-1	4	1	1-1	G
04-10-11	A/Herring gull/Newfoundland/GR654/2011(H13N6)	2	1-4	1-1	5-1	1-1	4	1	1-1	I
04-10-11	A/Herring gull/Newfoundland/GR656/2011(H13N6)	2	1-4	1-1	5-1	1-1	4	1	1-1	I
04-10-11	A/Herring gull/Newfoundland/GR658/2011(H13N6)	2	1-4	1-1	5-1	1-1	4	1	1-1	I
04-10-11	A/Herring gull/Newfoundland/GR657/2011(H13N6)	2	1-4	3-1	5-1	1-1	4	1	1-1	J

Figure 4.4. Newfoundland gull AIV genotypes from 2009-2011. The viruses are listed in chronological order with the sample collection dates indicated. The phylogenetic clade numbers correspond to those on Figures 1, 2 and 3 for each of the 8 segments. The colours of the boxes indicate the continental and host group affiliation for the segment's clade: yellow, North American gull; grey, Eurasian gull; red, North American avian; blue, Eurasian avian. Ten genotypes (A to I) were defined according to the combination of the clade numbers for the segments. A white box with a dash indicates no sequence was obtained for the segment.

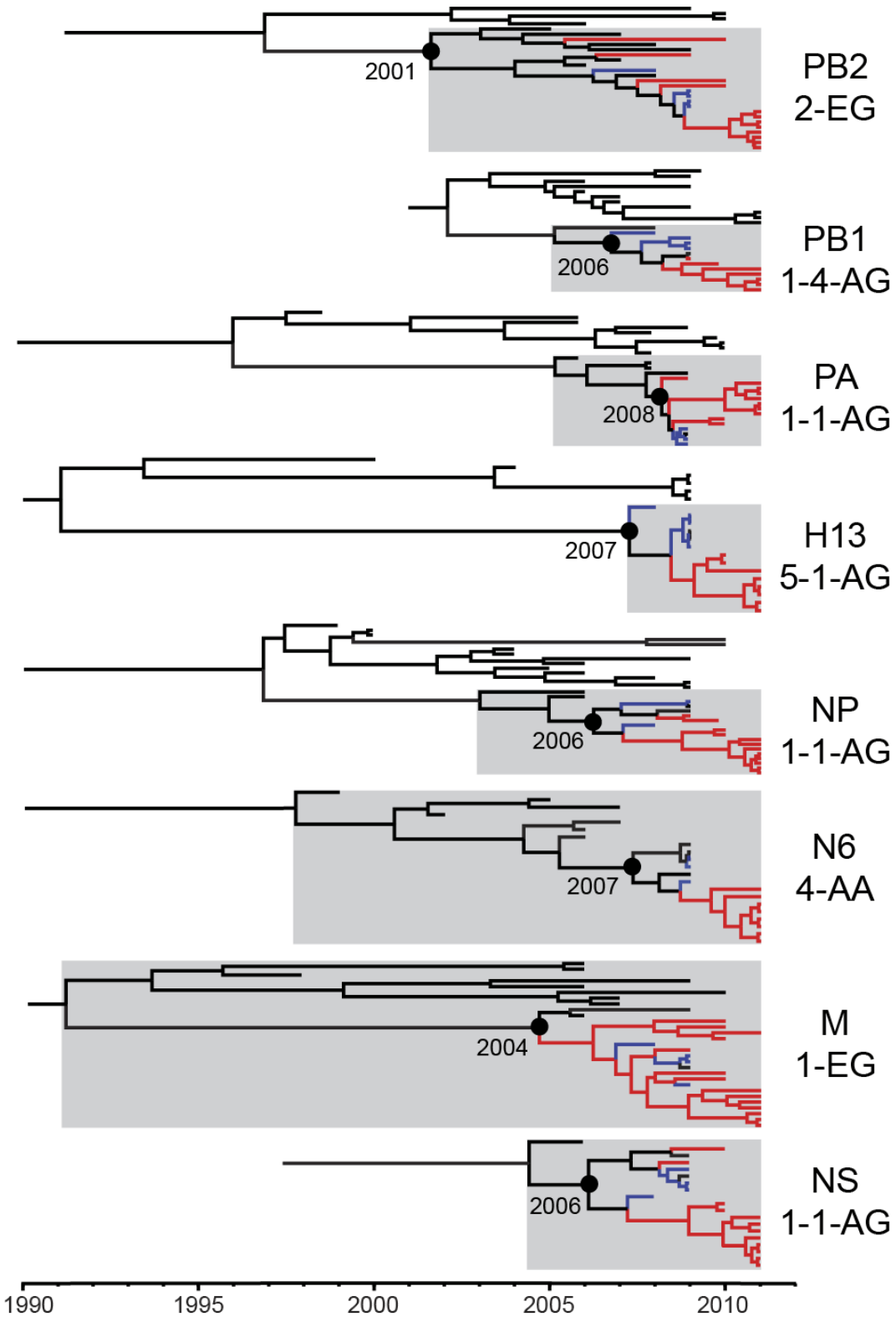
2001
PB2
2-EG
2006
PB1
1-4-AG
2008
PA
1-1-AG
2007
H13
5-1-AG
2006
NP
1-1-AG
2007
N6
4-AA
2004
M
1-EG
2006
NS
1-1-AG
1990
1995
2000
2005
2010

Figure 4.5. Emergence dates for the gene segments found in a predominant genotype identified in gulls in Atlantic Canada since 2009 (next page). The red branches represent the Newfoundland gull sequences from this study, the blue branches represent other gull viruses from Atlantic Canada, and the black branches represent other viruses. The grey boxes delineate the relevant clades, which are labeled according to the phylogenetic trees in Figures 1, 2 and S1. For the M sequences, the two viruses from before 1990 were excluded from the analysis. The phylogenetic trees were generated using maximum clade credibility method in BEAST v1.7.5, and most recent common ancestor dates were calculated with a lognormal relaxed clock (unrelated) model and the dates are indicated for the estimated emergence times of the Atlantic Canada gull virus segments. The time scale is indicated at the bottom and emergence dates are marked with a black circle and indicated for each segment. The sampling time of all viruses was specified to year.

4.4. Discussion

Our surveillance for AIV infection in gulls on the island of Newfoundland, Canada has shown that it is predominantly birds in their first year that are identified as infected, and this is occurring primarily in the fall season (Table 4.1). Based on the surveillance with captured birds, we would predict that the positive fecal samples also came largely from juvenile birds. Almost no positive juveniles were detected at the breeding colony (1/792), which is very different from a previously observed high prevalence (>20%) in juvenile Ring-billed Gulls at breeding colonies in Ontario, Canada (Velarde, Calvin et al. 2010). Maternal transfer of anti-AIV antibodies via eggs has been found in gulls, at 14% in one study of eggs from Yellow-legged Gulls (*Larus michahellis*)

(Pearce-Duvet, Gauthier-Clerc et al. 2009), and was also proposed to be the cause of observed seroprevalence in young birds in another study (Lewis, Javakhishvili et al. 2013). Any possible relationship between these observations and differences in prevalence in juvenile birds on breeding colonies remains to be determined. Despite low prevalence of active AIV infection, the serology data indicate that half of the tested birds were infected within the previous period, allowing for detection of circulating anti-AIV antibodies. Most other studies with gulls have found infection prevalence values of <5% (Munster, Baas et al. 2007, Hanson, Luttrell et al. 2008, Ip, Flint et al. 2008, Buscaglia 2012, Hulsager, Breum et al. 2012, Marchenko, Alekseev et al. 2012, Sivay, Sayfutdinova et al. 2012, Lewis, Javakhishvili et al. 2013), but it has also been found to be as high as 15% (Velarde, Calvin et al. 2010, Toennessen, Germundsson et al. 2011). A study in Georgia (Asia) with one of the larger gull data sets (~2500 samples) also found an increased fall prevalence of infection in a large gull species, Armenian Gull (*Larus armenicus*), but an increased prevalence in the spring in a small gull species, Black-headed Gull (Lewis, Javakhishvili et al. 2013). The 50% value we found for seroprevalence is comparable to most other studies. The Georgian study (Lewis, Javakhishvili et al. 2013) reported 56% seroprevalence for Armenian Gulls, while another study in North America found 45% overall (when using the same detection method we employed) for 5 gull species (Brown, Luttrell et al. 2010). Higher rates were found in Black-legged Kittiwakes in Norway, at 71% (Toennessen, Germundsson et al. 2011), and in Ring-billed Gulls in Ontario, Canada, at 80% and 92% in two different years (Velarde, Calvin et al. 2010).

We analyzed sequence information for 16 of the detected gull AIVs. We classified the sequences into gene lineages according to the criteria of ≥90% nucleotide identity for

within a lineage, as described previously (Lu, Rowley et al. 2007, Ramey, Pearce et al. 2010), and clades within lineages based on ≥95% nucleotide identity. This revealed gulls in Atlantic Canada as a large and host diverse genetic reservoir with multiple lineages present for each gene segment (Figures 4.1-4.3) and ten different genotypes were assigned to these viruses (Figure 4.4). This also revealed that some predominant gene lineages have been perpetuated through years in Atlantic Canada gulls and these genes and viruses were likely circulating in Atlantic Canada for some time before their widespread was detected beginning in 2008.

Overall, the viruses predominantly contained sequences from gull-related phylogenetic clades (93/123, 75.6%), but twelve of the viruses contained at least one segment of avian origin. For virus, A/Great black-backed gull/Newfoundland/AB001/2011(H9N9), all 8 segments were in avian virus clades, and for another virus, A/Great black-backed gull/Newfoundland/MW174/2010(H1), all 6 segments for which we obtained sequence data were in avian clades. These viruses presumably represent recent transmission events from waterfowl to gulls. Detection of avian lineage genes in gull viruses has been common (Kawaoka, Chambers et al. 1988, Widjaja, Krauss et al. 2004, Lomakina, Gambaryan et al. 2009, Toennessen, Germundsson et al. 2011, Wille, Robertson et al. 2011, Van Borm, Rosseel et al. 2012, Hall, TeSlaa et al. 2013), but not universal (Lewis, Javakhishvili et al. 2013). Frequent AIV transmission involving gulls and waterfowl is likely driven by the broad habitat usage of gulls, ranging from aquatic to terrestrial environments (Olsen and Larsson 2003). This transmission frequency may vary by location and sampling time, which will affect the degree of interaction between these groups, and others such as shorebirds.

Interestingly, waterfowl-related genes dominated in the only 2 AIVs detected during winter (both from February) in this study, which were distinct from the remaining viruses from autumn (Figure 4.4). This presumably resulted from increased interaction among gulls and waterfowl at reduced winter habitat such as areas with remaining open water.

Fifteen of the viruses contained at least one segment that fell within clades we defined as Eurasian. Inter-continental reassortant AIVs have been identified in gulls in high proportions on both coasts of North America (Wille, Robertson et al. 2011, Hall, TeSlaa et al. 2013) and in Europe (Van Borm, Rosseel et al. 2012). One of the gull viruses within them, A/Herring gull/Newfoundland/YH019/2010(H16N3), had all 8 segments in Eurasian gull clades (Figure 4.4). This, to our knowledge, represents the first completely Eurasian influenza virus found in North America and presumably reflects a recent transmission of the virus from Eurasia to North America. However, it is worth noting that this is based on employing a 95% cut-off value for clade definition. A recent study in Iceland (Dusek, Hallgrimsson et al., 2014) is of great interest in this respect; both wholly Eurasian and wholly North American gull AIVs were found in this location, suggesting it is an important location for mixing of viruses between the two regions.

The phylogenetic trees for the NP, M and NS segments (Figure 4.3D, E and F, respectively) shows that they show very similar topology at the "top" portion of the trees, each with two clusters of sequences where one is largely from gulls in North America and the other is largely from gulls in Eurasia.

Amongst the 16 viruses we characterized, three were found to possess the same genotype (I) and nine other viruses were reassortants relative to this genotype (Figures

4.1-4.4). The three genotype I viruses were found in three Herring Gulls on the same day (4 October 2011) and at the same location. The detection of "identical" AIV genomes is most often reported from samples with greater temporal and spatial proximity (Reeves, Pearce et al. 2011, Huang, Wille et al. 2013, Huang, Wille et al. 2014). Viruses with segments closely related to those from the genotype I viruses were also detected previously in Atlantic Canada over 2008-2009 (Figures 4.1-4.3) (Wille, Robertson et al. 2011, Hall, TeSlaa et al. 2013). This provides insight into the evolution and perpetuation of AIVs through years in the gull population because the dominance of genotype I and its reassortants over 2008-2011 in Atlantic Canada indicates it was mostly a single group of highly related viruses circulating in the gulls in this region over this period. Specific reassortment events can be observed within these gull viruses, as well as what appear to be spillover events to non-gull hosts (Hall, TeSlaa et al. 2013). Molecular dating indicated emergence times of 2001-2008 in gulls for the genes in these viruses (Figure 4.5). However, as noted above, other viruses were also detected in these birds over this period.

Notably, the genotypes of the gull AIVs in 2011 in this study are less diverse compared to that in 2009 and 2010. The change of gull capture methods may partially explain the varied diversity of the AIV genotypes between years in our study. Noose carpet was used to capture gulls during 2009 and 2010, which is substituted by "net-gun" method, a method that could efficiently capture a flock of gulls at the same habitat compared to noose carpet. This may have led to the detection of AIVs with similar evolution background in 2011.

The phylogenetic analyses in this study included gull AIV genes from both Atlantic and Pacific North America, and from Eurasia, which showed that most gene

lineages displayed spatial distribution bias. The viral genes in Newfoundland gulls were most frequently similar to those genes from birds at other locations in Atlantic North America and Western Europe (Figures 4.1, 4.2 and S4.1). More research is required to better understand the distribution of gull AIVs through space, time and species in North America because of limited viral genome sequences across these variables.

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Chapter 5 Diverse inter-continental and host lineage reassortant avian influenza A viruses in pelagic seabirds

Abstract

Avian influenza A viruses (AIVs) often infect waterfowl, gulls and shorebirds, but other bird groups including pelagic seabirds also serve as hosts. In this study, we analyzed 21 AIVs found in two distant breeding colonies of Common Murre (*Uria aalge*) in Newfoundland and Labrador, Canada, during 2011. Phylogenetic analyses and genotype assignments were performed for the 21 Common Murre viruses together with all Common and Thick-billed Murre (*U. lomvia*) AIV sequences available in public sequence databases. All fully characterized viruses from the Common Murres in 2011 were H1N2 subtype, but the genome sequences revealed greater diversity and the viruses belonged to four distinct genotypes. The four genotypes shared most segments in common, but reassortment was observed for PB2 and M segments. This provided direct genetic data of AIV diversification through segment reassortment during an outbreak of AIV infection in high-density breeding colonies. Analysis of the total collection of available murre viruses revealed a diverse collection of subtypes and gene lineages with high similarity to those found in viruses from waterfowl and gulls, and there was no indication of murre-specific AIV gene lineages. Overall, the virus gene pool in murres was predominantly made up of AIV lineages associated with waterfowl, but also featured considerable inter-continental and gull lineages. In particular, all but one of the 21 Common Murre viruses from 2011 in Newfoundland contained 1 or 2 Eurasian segments and 16 contained 1 gull lineage segment. This mosaic nature of characterized murre AIV genomes might reflect an under-

recognized role of these pelagic seabirds in virus transmission across space and between bird host taxa.

5.1. Introduction

Epidemiological studies of avian influenza A viruses (AIVs) in wild birds have greatly improved our understanding of influenza A virus evolution and distribution. Previous research has shown that wild aquatic birds of the orders Anseriformes (ducks, geese and swans) and Charadriiformes (gulls, shorebirds and terns) are the natural reservoir of AIVs (Stallknecht and Shane 1988, Webster, Bean et al. 1992, Olsen, Munster et al. 2006). Phylogenetic analyses of AIVs from wild birds have generally separated each of the eight gene segments into North American avian, North American gull, Eurasian avian and Eurasian gull lineages (Olsen, Munster et al. 2006), although recent work indicates independent lineages also exist in South America (Pereda, Uhart et al. 2008, Gonzalez-Reiche and Perez 2012). However, individual AIVs may comprise genes from different host and geographic lineages (Widjaja, Krauss et al. 2004, Spackman, Stallknecht et al. 2005, Krauss, Obert et al. 2007, Koehler, Pearce et al. 2008, Chen and Holmes 2009, Ramey, Pearce et al. 2010, Ramey, Pearce et al. 2010, Lee, Lee et al. 2011, Wille, Robertson et al. 2011).

The majority of previous wild bird AIV research has focused on waterfowl, gulls and shorebirds, although pelagic seabirds have received increased attention (Wallensten, Munster et al. 2005, Ip, Flint et al. 2008, Granter, Wille et al. 2010, Ramey, Pearce et al. 2010, Lebarbenchon, Jaeger et al. 2013, Wille, Huang et al. 2014). Pelagic seabirds spend most of their lives in offshore marine environments and are generally challenging to study

because of their largely pelagic existence outside of the breeding season and the relative isolation of their breeding habitats. As a consequence, the present knowledge of the ecology of AIVs in seabirds is limited. Within seabirds, the clearest indication that this group might be important for AIV dynamics comes from murres (*Uria* spp.), with evidence of current and/or past infections found in both the Atlantic and Pacific Oceans and in both North America and Eurasia (Wallensten, Munster et al. 2005, Ip, Flint et al. 2008, Granter, Wille et al. 2010, Ramey, Pearce et al. 2010, Wille, Huang et al. 2014). However, few murre AIV genome sequences are available in the public databases. The involvement of other species of seabirds is less clear, with fewer studies conducted, samples collected, and AIV positives found. Sampling efforts with murres in Newfoundland and Labrador, Canada from 2007-2010 resulted in 6 AIV-positive samples by real-time RT-PCR from Thick-billed and Common Murres (*Uria lomvia* and *U. aalge*, respectively) (Wille, Huang et al. 2014). Continued surveillance during the summer of 2011 detected an increased rate of virus infection in two Newfoundland Common Murre breeding colonies, Gull Island and Cabot Island, with all successfully subtyped viruses being H1N2 (Wille, Huang et al. 2014). In this study sequence data were collected for 21 of these viruses and these sequences were analyzed together with all other available murre AIVs to increase our understanding of the genetic structure and inter-specific and/or inter-continental transmission of AIVs in these seabirds. Time of most recent common ancestors of the murre viruses were also determined to reveal the emerging time in Atlantic Canada.

5.2. Materials and Methods

5.2.1. Virus sequencing

Paired oropharyngeal and cloacal swabs were previously collected from breeding Common Murres between June and August 2011 at 3 breeding colonies near the island of Newfoundland, Canada (Figure S5.1) and screened for the presence of AIV by real-time RT-PCR (Wille, Huang et al. 2014). Twenty-two of the AIV-positive samples were selected for attempted sequencing and phylogenetic analysis to represent the different colonies and sampling dates. These included the one positive sample from 9 July on Gull Island, 17 collected on 21 July on Gull Island and 4 positive samples collected on 2 August on Cabot Island (Table 5.1). RNA was extracted with Trizol LS reagent (Life Technologies, Burlington, Canada) from either the swab samples or allantoic fluid of specific pathogen-free (SPF) embryonated chicken eggs at 9-11 days old inoculated with 0.2 ml of swab sample medium 3 days prior. Virus sequences were amplified by RT-PCR using previously published primers (Hoffmann, Stech et al. 2001, Phipps, Essen et al. 2004, Chan, Lin et al. 2006, Obenauer, Denson et al. 2006, Huang, Khan et al. 2013) and the Superscript III One-Step RT-PCR System (Life Technologies). The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Toronto, Canada) and sequenced at the Center for Applied Genomics (Toronto, Canada). The nucleotide sequence data were compiled and analyzed using Lasergene v7.1 (DNASTAR Inc., Madison, WI). The sequences were submitted to the GenBank database (accession numbers KC895594-KC895752).

Table 5.1. AIV surveillance results from three Newfoundland Common Murre breeding colonies in 2011

Date	Location[a]	AIV prevalence
9 June	Gull Island (East)	0/24
27 June	Great Island	0/22
29 June	Gull Island (Finger)	0/16
30 June	Great Island	0/20
4 July	Gull Island (Finger)	0/18
7 July	Gull Island (Finger)	1/28
12 July	Gull Island (South)	0/20
14 July	Gull Island (South)	0/66
18 July	Gull Island (South)	0/10
21 July	Gull Island (North)	60/67
2 August	Cabot Island	7/141
19 August	Great Island	0/20
Total		68/452

[a] For Gull Island, the general area of the island indicated is as described (Robertson, Fifield et al. 2001).

5.2.2. Phylogenetic analyses

The Common Murre AIV sequences obtained here, in combination with all available Common and Thick-billed Murre AIV sequences (17 whole or partial genomes; Table S5.1) in the NCBI influenza database (Bao, Bolotov et al. 2008), were used for phylogenetic analyses. Two sets of phylogenetic analyses were performed. First, each murre AIV segment was used for BLAST searches (Altschul, Madden et al. 1997) to identify the 20 most similar sequences. In addition, outgroup reference sequences were used in the HA and NA trees to improve the resolution of the murre virus-containing phylogenetic clades. All resulting sequences (excluding repeated sequences) were then used to construct phylogenetic trees to provide the overall phylogenetic topology and identify the host and continental origins of the clades based on the sources of the reference sequences. Based on this analysis, sequences from the phylogenetic clades closest to the murre viruses were then used to construct a second set of phylogenetic trees. The nucleotide ranges of the coding regions used for each gene were: PB2, 1804-2256 bp; PB1, 1624-2271 bp; PA, 1-528 bp; NP, 4-576 bp; M, 40-939 bp; NS, 55-792 bp; HA, 1147-1590 bp; NA, 34-513 bp. The nucleotide sequence alignments and pairwise-distance analyses were performed with Lasergene v7.1 (DNAStar, Inc., Madison, WI). Phylogenetic trees were constructed with the maximum likelihood method in MEGA5 (Tamura, Peterson et al. 2011) with 1000 bootstrap replicates. These topologies were then confirmed by comparison to neighbour-joining trees constructed in MEGA5 and maximum clade credibility trees constructed using BEAST v1.7.5 (Drummond, Suchard et al. 2012).

Most recent common ancestor dates were estimated for the predominant 2011 murre AIV-containing clades with BEAST v1.7.5 (Drummond, Suchard et al. 2012). A lognormal relaxed clock (unrelated) model was used to infer the phylogenetic timescale. The analysis was run with the GMRF Bayesian skyride model (10^8 steps with sampling every 10^4 steps) and the convergence was assessed in Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/). Maximum clade credibility trees were summarized with 10% of the input file excluded.

5.2.3. Gene lineage and genotype assignment of the murre AIVs

The genetic structure of the murre viruses was analyzed based on the results of the two rounds of phylogenetic tree construction.. The 8 virus segments were classified by phylogenetic lineages, with serial numbers assigned to different lineages. AIV genes classified in the same phylogenetic lineage usually had ≥90% nucleotide identity (Ramey, Pearce et al. 2010, Reeves, Pearce et al. 2011). Viral genotypes were then determined by the combination of lineage numbers for the eight segments. We also differentiated bird host clades within lineages with serial numbers (1, 2, 3, etc.), where genes within a clade usually had ≥95% nucleotide identity and were differentiated with numbers in parentheses.

5.3. Results

5.3.1. Epidemiological dynamics of AIV infections in Common Murres during the 2011 breeding season

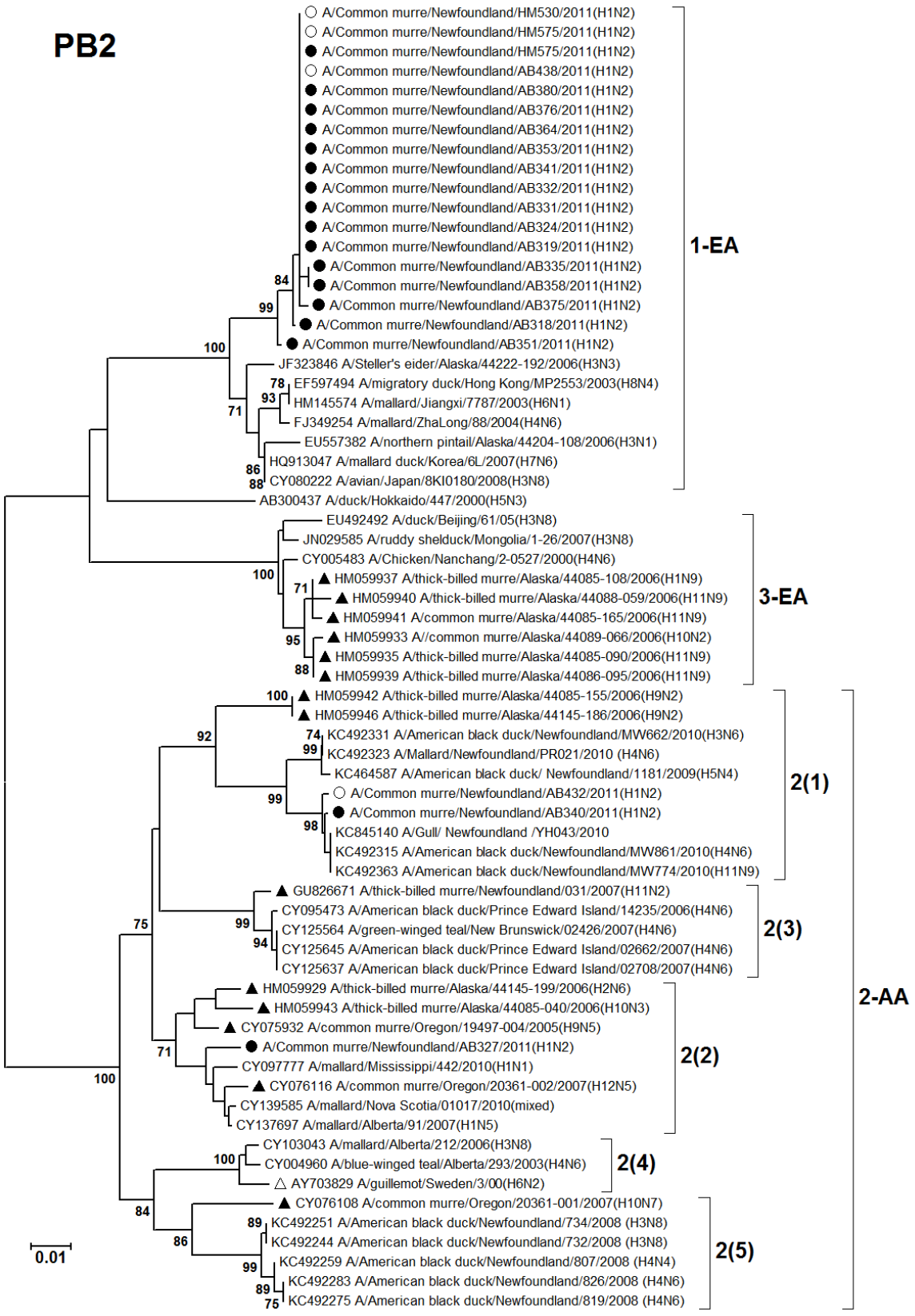
Analysis of the AIV surveillance data for the Common Murre samples collected in Newfoundland and Labrador during 2011 (Table 5.1) showed that most of the positive samples (60) were detected on a single day, 21 July, at Gull Island. Another AIV-positive individual was detected earlier on that island, on 9 July. There were no samples collected from Gull Island after 21 July, but no AIV infection was detected at the adjacent Great Island on 19 August (Table 5.1). Infection was detected on 2 August at Cabot Island, which is ~250 km over water from Gull and Great Islands.

5.3.2. Phylogenetic analysis and the genetic structure of murre AIVs

Common Murre viruses from both Gull and Cabot Islands were selected for genome sequencing and phylogenetic analysis. We obtained sequence data from 21 of the 22 selected viruses. This resulted in sequence information for all 8 gene segments for 18 viruses and 6 or 7 segments for 3 viruses. The earliest AIV-positive sample, from 9 July on Gull Island, failed in RT-PCR. Partial or whole genome sequences of 17 previously characterized Common and Thick-billed Murre viruses (1 from Sweden, 1 from Newfoundland and Labrador, 3 from Oregon and 12 from Alaska) were found in the public sequence database, downloaded and included in phylogenetic analyses. The lineage assignments from the maximum likelihood trees matched those on the neighbour-joining and the maximum clade credibility trees. The classifications of lineages by continental and host affiliations (e.g. North American avian and Eurasian gull) are indicated on the trees (Figures 5.1-4 and S5.2). The murre viruses showed extensive

genetic diversity, as indicated by the multiple lineages found for each of the 8 segments. Inter-continental reassortants were most frequently identified for the PB2 (18/21; Figure 5.1) and M (17/21; Figure 5.2) segments and gull lineage sequences were most common for the M segment (17/21; Figure 5.2). These patterns were largely driven by the 2011 Newfoundland viruses, almost all of which (19/21) contained one or both of the Eurasian avian PB2 and Eurasian gull M genes. Although the sequences for both segments clusteredwithin North American avian lineages, the 2011 Newfoundland viruses contained novel H1 and N2 sequences that formed independent clades with no closely related sequences (i.e. ≥99% nucleotide identity) identified in previously characterized viruses (Figures 5.3 and 5.4).

On the basis of the phylogenetic analyses, the genetic structure of the 38 murre AIVs was summarized according to geographic and host group lineage (Table S5.2). This revealed that the gene pool was mostly related to waterfowl viruses (93.4%), but considerable inter-continental avian lineages, while only 1 of the 109 segment sequences belonged to a gull lineage. For the 22 murre AIVs from Atlantic North America, 79.5% of the 171 segment sequences were classified as North American avian, 10.5% as Eurasian avian, and 9.9% as Eurasian gull. The only murre virus genome from Atlantic Eurasia contained 1 North American gull, 4 North American avian, and 3 Eurasian avian gene segments.


PB2
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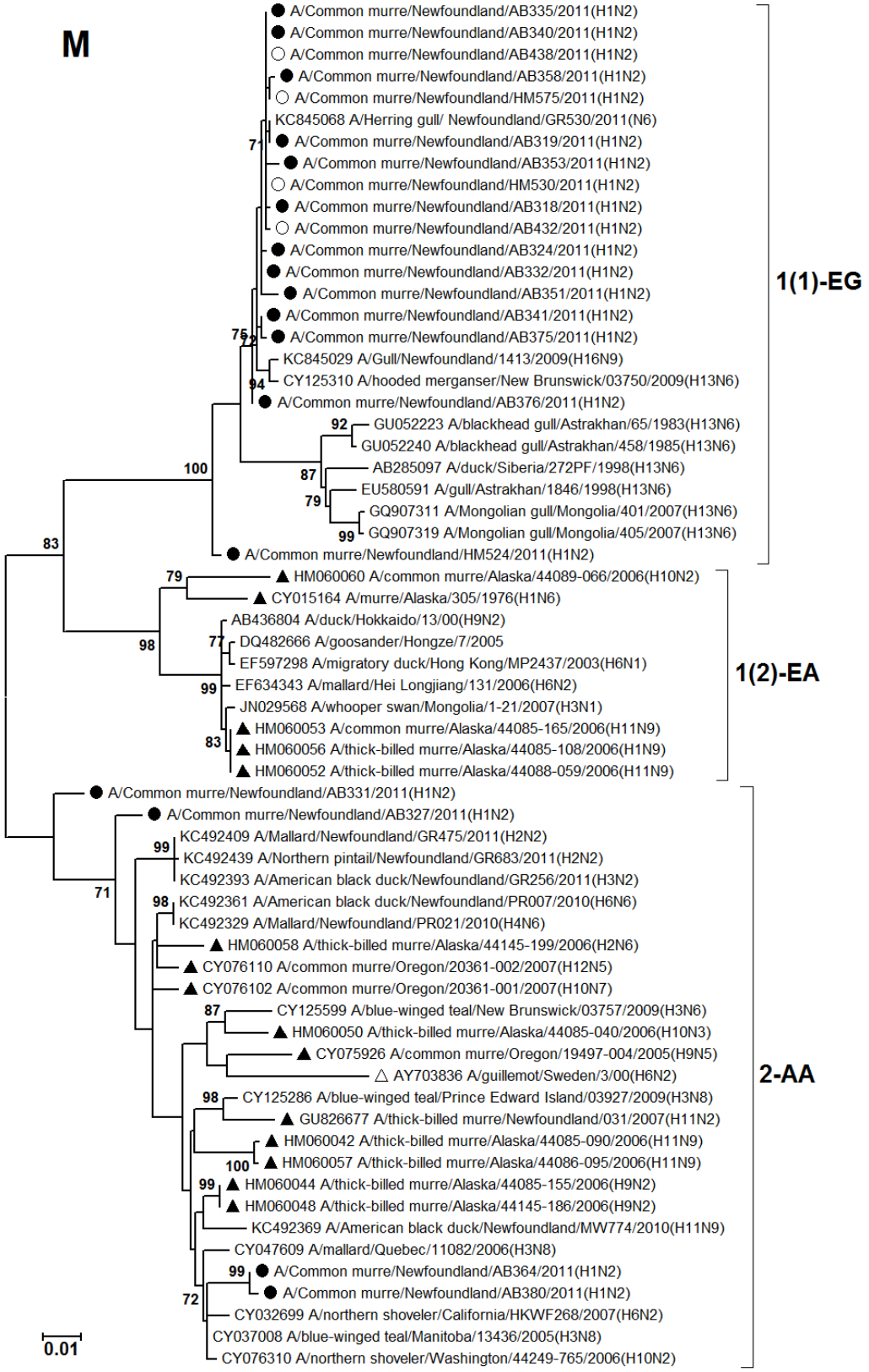
A/Common murre/Newfoundland/HM575/2011(H1N2)
A/Common murre/Newfoundland/HM575/2011(H1N2)
A/Common murre/Newfoundland/AB438/2011(H1N2)
A/Common murre/Newfoundland/AB380/2011(H1N2)
A/Common murre/Newfoundland/AB376/2011(H1N2)
A/Common murre/Newfoundland/AB364/2011(H1N2)
A/Common murre/Newfoundland/AB353/2011(H1N2)
A/Common murre/Newfoundland/AB341/2011(H1N2)
A/Common murre/Newfoundland/AB332/2011(H1N2)
A/Common murre/Newfoundland/AB331/2011(H1N2)
A/Common murre/Newfoundland/AB324/2011(H1N2)
A/Common murre/Newfoundland/AB319/2011(H1N2)
A/Common murre/Newfoundland/AB335/2011(H1N2)
A/Common murre/Newfoundland/AB358/2011(H1N2)
A/Common murre/Newfoundland/AB375/2011(H1N2)
A/Common murre/Newfoundland/AB318/2011(H1N2)
A/Common murre/Newfoundland/AB351/2011(H1N2)
JF323846 A/Steller's eider/Alaska/44222-192/2006(H3N3)
EF597494 A/migratory duck/Hong Kong/MP2553/2003(H8N4)
HM145574 A/mallard/Jiangxi/7787/2003(H6N1)
FJ349254 A/mallard/ZhaLong/88/2004(H4N6)
EU557382 A/northern pintail/Alaska/44204-108/2006(H3N1)
HQ913047 A/mallard duck/Korea/6L/2007(H7N6)
CY080222 A/avian/Japan/8KI0180/2008(H3N8)
AB300437 A/duck/Hokkaido/447/2000(H5N3)
1-EA
EU492492 A/duck/Beijing/61/05(H3N8)
JN029585 A/ruddy shelduck/Mongolia/1-26/2007(H3N8)
CY005483 A/Chicken/Nanchang/2-0527/2000(H4N6)
HM059937 A/thick-billed murre/Alaska/44085-108/2006(H1N9)
HM059940 A/thick-billed murre/Alaska/44088-059/2006(H11N9)
HM059941 A/common murre/Alaska/44085-165/2006(H11N9)
HM059933 A//common murre/Alaska/44089-066/2006(H10N2)
HM059935 A/thick-billed murre/Alaska/44085-090/2006(H11N9)
HM059939 A/thick-billed murre/Alaska/44086-095/2006(H11N9)
3-EA
HM059942 A/thick-billed murre/Alaska/44085-155/2006(H9N2)
HM059946 A/thick-billed murre/Alaska/44145-186/2006(H9N2)
KC492331 A/American black duck/Newfoundland/MW662/2010(H3N6)
KC492323 A/Mallard/Newfoundland/PR021/2010 (H4N6)
KC464587 A/American black duck/ Newfoundland/1181/2009(H5N4)
A/Common murre/Newfoundland/AB432/2011(H1N2)
A/Common murre/Newfoundland/AB340/2011(H1N2)
KC845140 A/Gull/ Newfoundland /YH043/2010
KC492315 A/American black duck/Newfoundland/MW861/2010(H4N6)
KC492363 A/American black duck/Newfoundland/MW774/2010(H11N9)
2(1)
GU826671 A/thick-billed murre/Newfoundland/031/2007(H11N2)
CY095473 A/American black duck/Prince Edward Island/14235/2006(H4N6)
CY125564 A/green-winged teal/New Brunswick/02426/2007(H4N6)
CY125645 A/American black duck/Prince Edward Island/02662/2007(H4N6)
CY125637 A/American black duck/Prince Edward Island/02708/2007(H4N6)
2(3)
HM059929 A/thick-billed murre/Alaska/44145-199/2006(H2N6)
HM059943 A/thick-billed murre/Alaska/44085-040/2006(H10N3)
CY075932 A/common murre/Oregon/19497-004/2005(H9N5)
A/Common murre/Newfoundland/AB327/2011(H1N2)
CY097777 A/mallard/Mississippi/442/2010(H1N1)
CY076116 A/common murre/Oregon/20361-002/2007(H12N5)
CY139585 A/mallard/Nova Scotia/01017/2010(mixed)
CY137697 A/mallard/Alberta/91/2007(H1N5)
2(2)
CY103043 A/mallard/Alberta/212/2006(H3N8)
CY004960 A/blue-winged teal/Alberta/293/2003(H4N6)
AY703829 A/guillemot/Sweden/3/00(H6N2)
2(4)
CY076108 A/common murre/Oregon/20361-001/2007(H10N7)
KC492251 A/American black duck/Newfoundland/734/2008 (H3N8)
KC492244 A/American black duck/Newfoundland/732/2008 (H3N8)
KC492259 A/American black duck/Newfoundland/807/2008 (H4N4)
KC492283 A/American black duck/Newfoundland/826/2008 (H4N6)
KC492275 A/American black duck/Newfoundland/819/2008 (H4N6)
2(5)
2-AA
0.01

Figure 5.1. Phylogenetic analysis of murre AIV PB2 sequences. The filled and open circles indicate the Common Murre viruses from Newfoundland in 2011 from the Gull and Cabot Island colonies, respectively. Filled and open triangles represent other murre viruses available in the sequence database from North America and Eurasia, respectively. The lineages are labeled as AA for North American avian and EA for Eurasian avian. Phylogenetic trees were constructed with the maximum likelihood method using MEGA5, and bootstrap values are shown as percentages based on 1000 replicates where support was ≥70%. The scale bars indicate the number of substitutions per site.


M
A/Common murre/Newfoundland/AB335/2011(H1N2)
A/Common murre/Newfoundland/AB340/2011(H1N2)
A/Common murre/Newfoundland/AB438/2011(H1N2)
A/Common murre/Newfoundland/AB358/2011(H1N2)
A/Common murre/Newfoundland/HM575/2011(H1N2)
KC845068 A/Herring gull/ Newfoundland/GR530/2011(N6)
A/Common murre/Newfoundland/AB319/2011(H1N2)
A/Common murre/Newfoundland/AB353/2011(H1N2)
A/Common murre/Newfoundland/HM530/2011(H1N2)
A/Common murre/Newfoundland/AB318/2011(H1N2)
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A/Common murre/Newfoundland/AB341/2011(H1N2)
A/Common murre/Newfoundland/AB375/2011(H1N2)
KC845029 A/Gull/Newfoundland/1413/2009(H16N9)
CY125310 A/hooded merganser/New Brunswick/03750/2009(H13N6)
A/Common murre/Newfoundland/AB376/2011(H1N2)
GU052223 A/blackhead gull/Astrakhan/65/1983(H13N6)
GU052240 A/blackhead gull/Astrakhan/458/1985(H13N6)
AB285097 A/duck/Siberia/272PF/1998(H13N6)
EU580591 A/gull/Astrakhan/1846/1998(H13N6)
GQ907311 A/Mongolian gull/Mongolia/401/2007(H13N6)
GQ907319 A/Mongolian gull/Mongolia/405/2007(H13N6)
A/Common murre/Newfoundland/HM524/2011(H1N2)
1(1)-EG
HM060060 A/common murre/Alaska/44089-066/2006(H10N2)
CY015164 A/murre/Alaska/305/1976(H1N6)
AB436804 A/duck/Hokkaido/13/00(H9N2)
DQ482666 A/goosander/Hongze/7/2005
EF597298 A/migratory duck/Hong Kong/MP2437/2003(H6N1)
EF634343 A/mallard/Hei Longjiang/131/2006(H6N2)
JN029568 A/whooper swan/Mongolia/1-21/2007(H3N1)
HM060053 A/common murre/Alaska/44085-165/2006(H11N9)
HM060056 A/thick-billed murre/Alaska/44085-108/2006(H1N9)
HM060052 A/thick-billed murre/Alaska/44088-059/2006(H11N9)
1(2)-EA
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A/Common murre/Newfoundland/AB327/2011(H1N2)
KC492409 A/Mallard/Newfoundland/GR475/2011(H2N2)
KC492439 A/Northern pintail/Newfoundland/GR683/2011(H2N2)
KC492393 A/American black duck/Newfoundland/GR256/2011(H3N2)
KC492361 A/American black duck/Newfoundland/PR007/2010(H6N6)
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HM060058 A/thick-billed murre/Alaska/44145-199/2006(H2N6)
CY076110 A/common murre/Oregon/20361-002/2007(H12N5)
CY076102 A/common murre/Oregon/20361-001/2007(H10N7)
CY125599 A/blue-winged teal/New Brunswick/03757/2009(H3N6)
HM060050 A/thick-billed murre/Alaska/44085-040/2006(H10N3)
CY075926 A/common murre/Oregon/19497-004/2005(H9N5)
AY703836 A/guillemot/Sweden/3/00(H6N2)
CY125286 A/blue-winged teal/Prince Edward Island/03927/2009(H3N8)
GU826677 A/thick-billed murre/Newfoundland/031/2007(H11N2)
HM060042 A/thick-billed murre/Alaska/44085-090/2006(H11N9)
HM060057 A/thick-billed murre/Alaska/44086-095/2006(H11N9)
HM060044 A/thick-billed murre/Alaska/44085-155/2006(H9N2)
HM060048 A/thick-billed murre/Alaska/44145-186/2006(H9N2)
KC492369 A/American black duck/Newfoundland/MW774/2010(H11N9)
CY047609 A/mallard/Quebec/11082/2006(H3N8)
A/Common murre/Newfoundland/AB364/2011(H1N2)
A/Common murre/Newfoundland/AB380/2011(H1N2)
CY032699 A/northern shoveler/California/HKWF268/2007(H6N2)
CY037008 A/blue-winged teal/Manitoba/13436/2005(H3N8)
CY076310 A/northern shoveler/Washington/44249-765/2006(H10N2)
2-AA
0.01

Figure 5.2. Phylogenetic analysis of murre AIV M sequences (next page). The filled and open circles indicate the Common Murre viruses from Newfoundland in 2011 from the Gull and Cabot Island colonies, respectively. Filled and open triangles represent other murre viruses available in the sequence database from North America and Eurasia, respectively. The lineages are labeled as AA for North American avian, EA for Eurasian avian and EG for Eurasian gull. Phylogenetic trees were constructed with the maximum likelihood method using MEGA5, and bootstrap values are shown as percentages based on 1000 replicates where support was ≥70%. The scale bars indicate the number of substitutions per site.

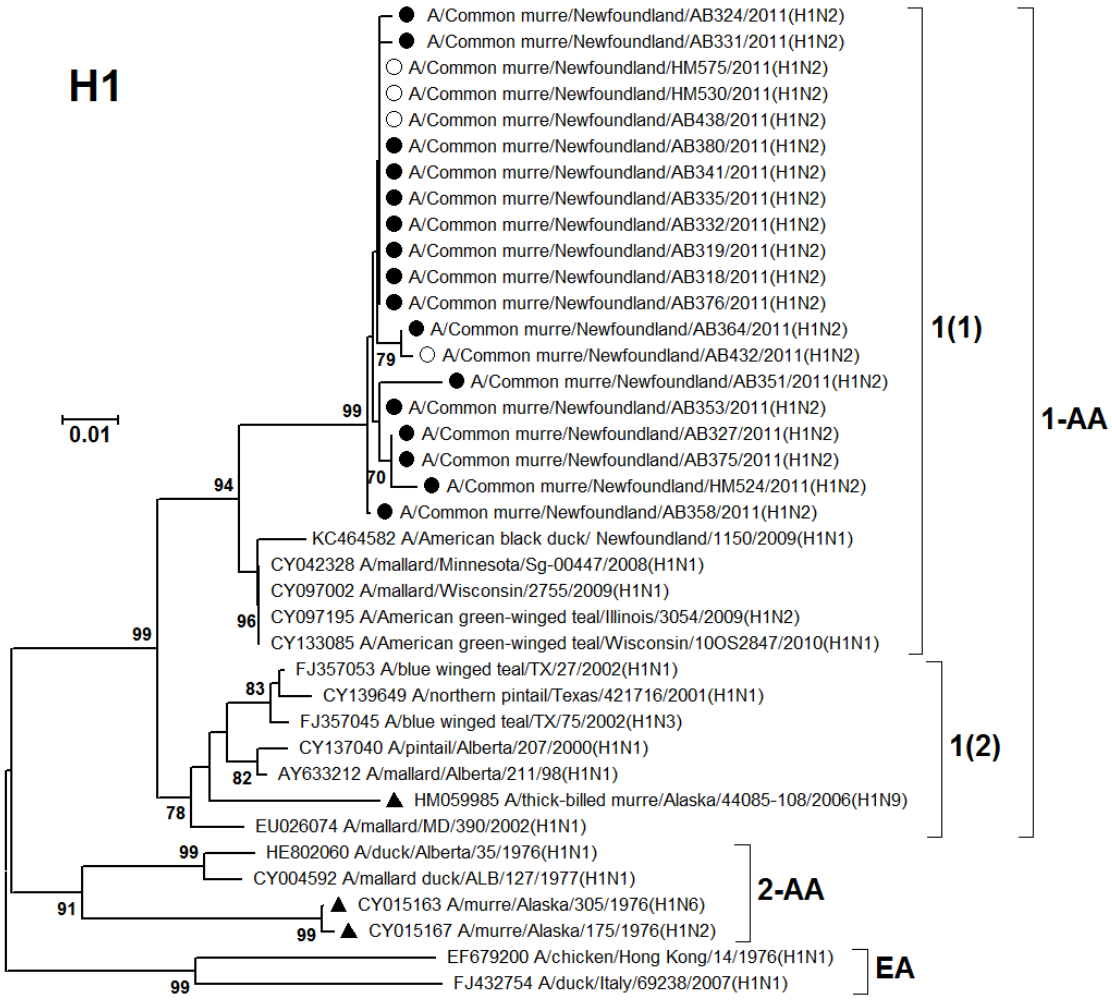

Figure 5.3. Phylogenetic analysis of murre AIV H1 sequences. The filled and open circles indicate the Common Murre viruses from Newfoundland in 2011 from the Gull and Cabot Island colonies, respectively. Filled triangles represent other murre viruses from North America available in the sequence database. The lineages are labeled as AA for North American avian and EA for Eurasian avian. Phylogenetic trees were constructed with the maximum likelihood method using MEGA 5, and bootstrap values are shown as percentages based on 1000 replicates where support was ≥70%. The scale bars indicate the number of substitutions per site.

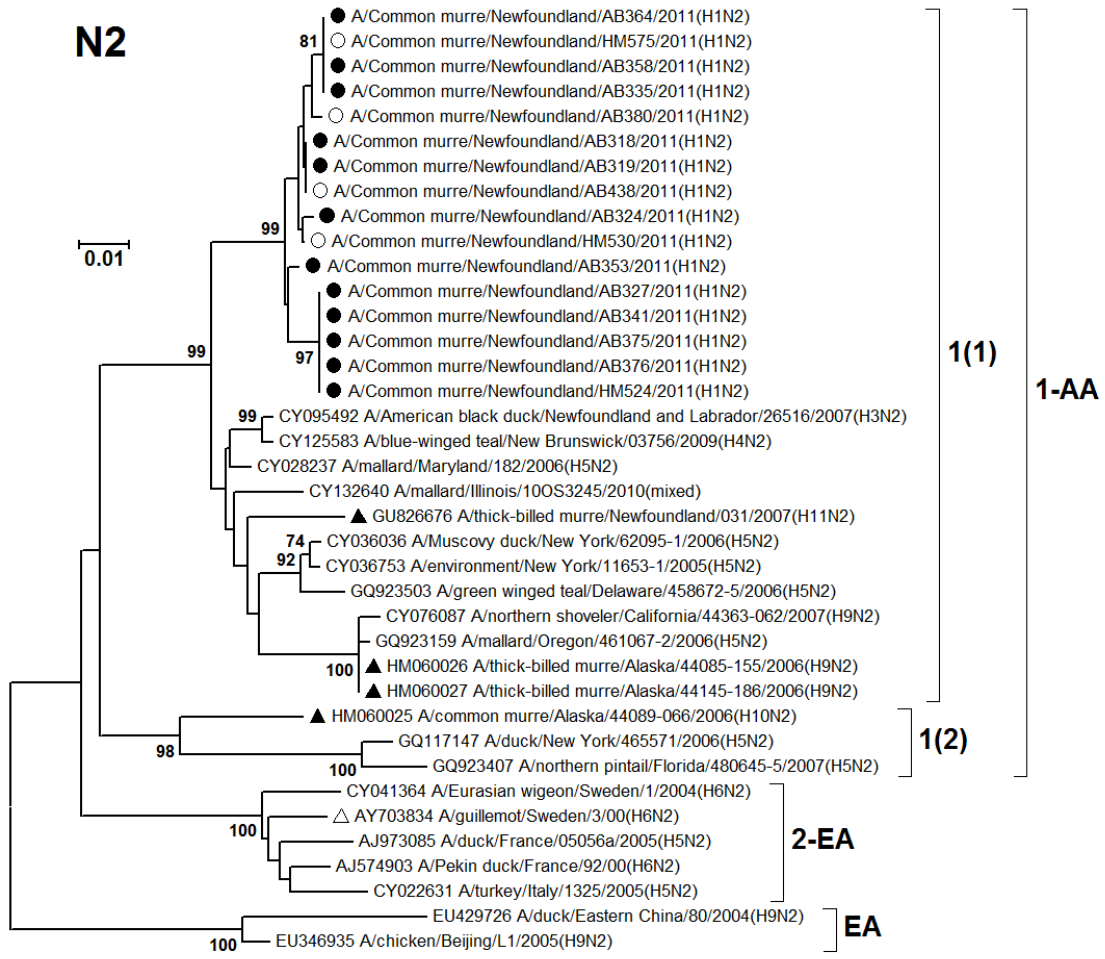

Figure 5.4. Phylogenetic analysis of murre AIV N2 sequences. The filled and open circles indicate the Common Murre viruses from Newfoundland in 2011 from the Gull and Cabot Island colonies, respectively. Filled and open triangles represent other murre viruses in the sequence database from North America and Eurasia, respectively. The lineages are labeled as AA for North American avian and EA for Eurasian avian. Phylogenetic trees were constructed with the maximum likelihood method using MEGA5, and bootstrap values are shown as percentages based on 1000 replicates where support was ≥70%. The scale bars indicate the number of substitutions per site.

5.3.3. Genotype diversity in murre AIVs

Compilation of the individual lineage assignments for the 8 segments delineated 15 different genotypes, designated as A through O, in the 32 murre viruses with sequence data available for all segments (Figures 5.5 and S5.2). Four genotypes (A through D) were identified within the 2011 H1N2 murre viruses from Newfoundland. Genotype A was the main genotype detected, represented by 15 viruses, and was found at both the Gull and Cabot Island colonies. Genotype A was an inter-continental and host lineage reassortant, with a PB2 segment of Eurasian avian origin, an M segment of Eurasian gull origin, and the other 6 segments in American avian lineages. Highly related sequences (i.e. ≥99% nucleotide identity) were found in the NCBI influenza database for five segments of the genotype A (PB1, PA, NP1, M and NS), whereas the HA, NA and PB2 genes were novel and showed only ~97% nucleotide identity to the most similar previously reported genes. Comparison of the pairwise distances of the segments of the 15 genotype A viruses revealed genetic diversity within these viruses. Sequences with ≤99% nucleotide identity were identified amongst the 15 viruses for all segments except PB2 and PB1 (Figure S5.4). The most recent common ancestor dating analyses provided estimates between 2007 and 2009 for the origins of the clades containing the genotype A viruses (Figure 5.6). The three other genotypes from Newfoundland in 2011 resulted from reassortment of the PB2 and/or M segments (Figure 5.5) relative to genotype A. The previously characterized reference murre viruses also showed extensive genomic diversity, with 11 genotypes (E through O) assigned to the 14 viruses with sequences available for all 8 gene segments (Figures 5.5 and S5.3).

Genotype	Location	PB2	PB1	PA	HA	NP	NA	M	NS
A	GI, CI	EA			1		2	EG	
B	GI	EA			1		2		
C	CI				1		2	EG	
D	GI				1		2		
E	AK			EA	2 EA		6		EA
F	AK				9		2		
G	OR				10		7		
H	AK				10		3		
I	OR				12		5		
J	SWE			EA	6 AG	EA	2 EA		
K	NL				11		2		
L	AK	EA			10		2	EA	
M	AK	EA			11		9	EA	
N	AK	EA			11		9	EA	
O	AK	EA			11		9		

Figure 5.5. Genomic diversity of AIVs from murres. Fifteen genotypes (designated A through O) were assigned to the 32 murre viruses with sequences available for all 8 segments based on the phylogenetic analyses (Figures 1-4 and S1). The location where the virus(es) with each genotype originated are indicated: GI, Gull Island, Newfoundland; CI, Cabot Island, Newfoundland; AK, Alaska; OR, Oregon; SWE, Sweden; NL, Newfoundland. Sequences classified within the same phylogenetic lineage for a given segment are shaded alike (white, light grey or dark grey), except for the HA and NA segments that are distinguished by their subtype number. Segments that were classified in lineages other than American avian are indicated: EA, Eurasian avian; AG, American gull; EG, Eurasian gull. Genotype A was represented by 15 viruses, genotypes F and O were represented by 2 viruses, and all other genotypes were represented by 1 virus. The full information for the viruses represented within each genotype is provided in Figure A.3.

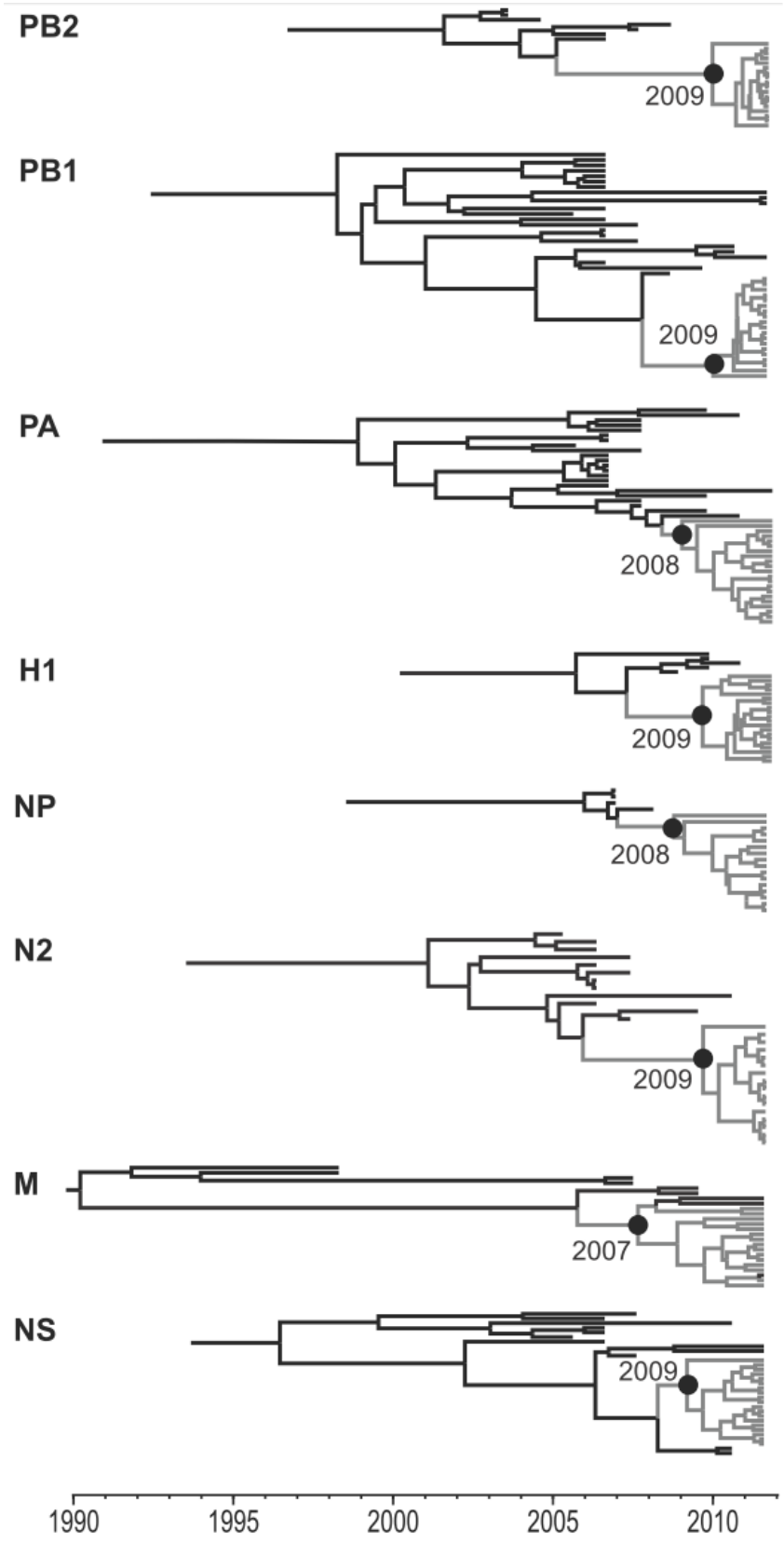
PB2
2009
PB1
2009
PA
2008
H1
2009
NP
2008
N2
2009
M
2007
NS
2009
1990
1995
2000
2005
2010

Figure 5.6. Emergence dates for the predominant 2011 Common Murre virus segment lineages. The analyses were run in BEAST v1.7.5 and the most recent common ancestor dates were calculated with a lognormal relaxed clock (unrelated) model. The time scale is indicated at the bottom and emergence times for the lineages of interest are marked with a black circle and indicated for each segment. The sampling times for the 2011 Common Murre viruses were specified to July (17 viruses) and August (4 viruses), while reference viruses without information of sampling month were assigned as July.

5.4. Discussion

Common and Thick-billed Murres are pelagic seabirds that spend most of the year offshore and only come to land to breed, which they often do at very high densities (Cramp, Brooks et al. 1985). Surveillance for AIV in Newfoundland and Labrador, Canada, identified increased prevalence in Common Murres in 2011 (Wille, Huang et al. 2014). The epidemiological dynamics observed during the 2011 breeding season (Table 5.1) indicated there was widespread AIV infection that spanned two distant breeding colonies, which was presumably caused by a single AIV strain because preliminary characterization of the viruses indicated that all were subtype H1N2 (Wille, Huang et al. 2014). However, our detailed phylogenetic analyses and genotype classifications of 21 of these viruses revealed surprising genetic heterogeneity within these H1N2 viruses. We identified four distinct genotypes within the 18 viruses for which sequence data were obtained for all 8 segments (Figure 5.5). These genotypes all shared 6 or 7 segments in common, but different PB2 and/or M gene lineages were introduced into some of the viruses by reassortment.

Most of the characterized viruses belonged to one genotype (A), which was detected at both breeding colonies. The foraging ranges of birds breeding on Gull and Cabot Islands do not overlap (Davoren, Montevecchi et al. 2003) because these two colonies are ~250 km apart over water and breeding Common Murres forage within tens of km from their colonies (Cairns, Bredin et al. 1987). However, birds from these colonies share wintering and staging areas on the eastern Grand Banks of Newfoundland (McFarlane Tranquilla, Montevecchi et al. 2013). Therefore, the genotype A viruses may have been circulating in the birds before they segregated into different breeding colonies in April, which would have been at least 2 months before the detection of infection in both colonies. If this holds true, it will also indicate that infection and transmission on wintering areas is important for the maintenance of AIVs in these pelagic seabirds. Unfortunately, our knowledge of AIV prevalence in murres outside the breeding season and its impact on viral epidemiology is limited due to the difficulty to access these birds while they are at sea. However, we also cannot rule out that the genotype A viruses were transmitted to the murres from other bird species foraging between the islands and then amplified in the murres population prior to our sampling. The logistical realities of working with these birds on their island colonies lead to gaps between sample collection days and an inability to equally sample all parts of a colony over a season. Murres on Cabot Island breed in a single cluster at the centre of the island, but its relatively remote location results in a single sampling event each year. Gull Island is much larger and murres breed along the periphery in isolated sub-colonies, which are separated by 50-500 m and range in size from tens to thousands of pairs. Unlike Cabot Island, this colony is easily visited multiple times over the breeding season, but sampling in each area needs to

be limited to avoid excessive disturbance. The emergence dating of the predominant genes from the 2011 Newfoundland Common Murres suggested these genes emerged during 2007-2009 (Figure 5.6), which indicates they have been circulating for several years before this widespread infection event in Common Murres was detected. This also explains the observed genetic heterogeneity within the genotype A viruses (Figure S5.4).

The high prevalence of AIV infection in Common Murres in the late summer of 2011 was unexpected considering the low virus detection rate in this species in previous years (Wille, Huang et al. 2014). The finding of 60 infected birds out of 67 sampled on one day in a small section of one colony (Table 5.1) demonstrates that AIV can transmit quickly and widely in a dense breeding colony under the proper conditions, as predicted by modeling (Clancy, O'Callaghan et al. 2006). Several factors may have contributed to the widespread infection detected in 2011. The Common Murre population at the Gull Island colony has been increasing in recent years (Regular, Montevecchi et al. 2013) and many birds now nest on previously unoccupied flat areas because of the limited availability of ledges on the cliff faces. The breeding season in 2011 also featured cooler temperatures and higher than normal rainfall, which may have facilitated AIV transmission through moistened and more easily distributed feces. Therefore, an increased breeding population with poor hygienic conditions and cold, wet weather could have been contributing factors to the increased virus infection. Links between climate and AIV dynamics are of increasing interest because of suspected involvement of such links in the generation of novel human pandemic influenza viruses (Mazzarella, Giuliacci et al. 2011, Shaman and Lipsitch 2013).

Although waterfowl and gulls are considered the major AIV reservoir, there has also been detection of AIV in Common and Thick-billed Murres around the globe (Sazonov, Lvov et al. 1977, Wallensten, Munster et al. 2005, Ip, Flint et al. 2008, Granter, Wille et al. 2010, Ramey, Pearce et al. 2010, Wille, Huang et al. 2014). However, there are still a limited number of available AIV genome sequences from murres and the genetic structure and gene flow of AIVs in these species are poorly understood. The subtype variety and genetic features of the 38 murre AIVs analyzed in this study (Figures 5.5 and S5.3) indicated that waterfowl-related lineage genes predominate in murre AIVs. However, both inter-continental and gull-related gene reassortants were common in the murre viruses. In particular, 20 of the 21 viruses characterized in this study contained at least one segment of Eurasian origin, which is generally a rare phenomenon in Atlantic North America outside of gulls (Krauss, Obert et al. 2007), and 16 of the 21 viruses also contained one gull lineage segment, which is generally rare to find outside of gulls and shorebirds (Olsen, Munster et al. 2006). These reassortments could result from the migratory behavior of these murre species, which has Atlantic North American and Eurasian breeding populations sharing wintering areas, and they also have contact with gulls on shared breeding colonies such as Gull Island. Fifteen distinct virus genotypes were identified in the 32 viruses with sequences available for all 8 segments, and there were also additional gene lineages and combinations identifiable in the incomplete viruses (Figure S5.3). This large genetic diversity found in murre viruses so far provides no indication of murre-specific genes or subtype restrictions. The chimeric nature of the viral genomes characterized from these hosts, in terms of inter-continental and host

lineage reassortants, indicates these pelagic seabirds need to be further considered with respect to global AIV transmission.

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Chapter 6 - Summary

6.1. Summary

The Island of Newfoundland, Canada, is at the eastern edge of North America and has migratory bird connections with the continental mainland as well as across the North Atlantic Ocean (Tuck 1971, Bellrose and Kortright 1976). Despite increased research into avian influenza virus (AIV) in other areas of North America, our understanding of AIV ecology and evolution in birds at the Atlantic coast of North America, especially in Atlantic Canada, is still limited (Parmley, Lair et al. 2009, Lam, Ip et al. 2012). During my thesis work, I analyzed and integrated data for the epidemiology, phylogeny and genotypes of AIVs from ducks, gulls and murres in Newfoundland to increase our knowledge of AIV ecology and transmission in wild birds in this region. The results provided data to improve the future AIV surveillance in wild birds in Newfoundland, and to evaluate the risk of AIV infections of poultry and humans from wild birds. The thesis work also has implications on the entry of highly pathogenic (HP) AIVs from Eurasia into North America via birds utilizing trans-Atlantic flyways.

6.1.1 AIV prevalence in ducks, gulls and murres in Newfoundland

One clear finding from the surveillance work presented in my thesis is that the virus detection frequency varies greatly by bird taxa in Newfoundland. The overall AIV prevalence rate in ducks in Newfoundland (2008-2011) was 7.2% (63/879). The virus detection rate in gulls of Newfoundland (2008-2011) was 1.8% (30/1645). The overall AIV rate in murres (2009-2011) was 3.9% (72/1837), but most positive samples (68/72) were detected in summer 2011, and the yearly AIV detection rate was 0.0% (0/620) in

2009, 1.0% (4/405) in 2010 and 8.4% (68/812) in 2011 (Table S6.1). Comparing by bird taxa, the multi-year AIV prevalence rate in ducks was much higher than that in gulls and murres, which agrees with previous research that waterfowl are the major reservoir of AIV (Webster, Bean et al. 1992, Olsen, Munster et al. 2006). The overall virus prevalence in murres was higher than gulls in this study. However, considering the low AIV detection rate in other years, the extraordinarily high prevalence rate of murre AIVs in 2011 in Newfoundland may be a rare event (Table S6.1) (Wille, Huang et al. 2014). Continued epidemiological surveillance in Newfoundland will be needed to prove this. Interestingly, highly similar H1N1 viruses were detected through years from a back-yard duck and wild ducks (chapter 2), which revealed the on-going AIV transmission between wild and domesticated ducks through shared water habitat in Newfoundland. Future surveillance work should continue to pay attention to ducks, due to their higher AIV prevalence and frequent interaction with domestic ducks and humans.

Another pattern observed in the epidemiological data related to seasonal patterns of AIV prevalence in ducks, gulls and murres in Newfoundland. The AIV detection rate in ducks was significantly higher during summer and autumn (May to October) (50/596, 68.4%) compared with winter in Newfoundland (November to February) (13/283, 4.6%), but we did not collect swab samples from ducks in spring (March to April). Similarly, the detection frequency of murre AIVs in summer and autumn (71/1140, 6.2%) during 2009 and 2011 was much higher than that in winter and spring (November to April) (1/696, 0.1%) (Table S6.1). In addition, AIVs showed the highest detection rate in both Herring Gull (*Larus smithsonianus*) and Great Black-backed Gull (*Larus marinus*) and gull fecal samples during autumn (September and October) (25/324, 7.7%) in comparison to spring

(0/148, 0%), summer (May to August) (1/877, 0.1%) and winter (3/224, 1.3%). Higher AIV prevalence will cause increased chance of AIV transmission and outbreak. The seasonal pattern of viral detection in Newfoundland suggested that future surveillance of AIVs in wild birds in this region should put more efforts on birds during their breeding and migratory seasons.

Bird age also influenced the AIV prevalence in ducks, gulls and murres in Newfoundland, which is expected considering that juvenile birds have no established immunity and are more susceptible to AIV infection. Juvenile ducks showed a significantly higher AIV detection rate (10.6%, 49/461) compared to adults (14/417. 3.4%) during 2008 and 2011. The AIV prevalence of the 2011 Common Murre samples on Gull Island was significantly different for adult birds (33/216, 15.3%) compared to chicks (28/33, 8.5%), but not Cabot Island (4/101, 4.0% versus 3/40, 7.5%) (Wille, Huang et al. 2014). Also, juvenile gulls showed a higher virus prevalence rate (9/849, 1.1%) than adult gulls (0/368, 0.0%) during 2009-2011. However, another 15 AIVs detected from 316 feces samples of gulls during the same period did not have the gull age determined, which may have influenced the analytic resolution. The bias of AIV prevalence by bird age indicated that the sampling priority should be put on juvenile birds to make the future AIV epidemiological study in Newfoundland more effective and informative.

6.1.2 Serology study in ducks, gulls and murres in Newfoundland

Despite different virus detection rates in ducks, gulls and murres in Newfoundland, the serology tests in the 3 bird taxa all showed very high incidence of previous AIV infection. Serology tests in ducks between October 2011 and February 2012 showed an antibody-positive rate of 52.6% (20/38). The study in murres during summer 2011 and

summer 2012 revealed 44% (51/115) and 69% (27/39) seroprevalence, respectively (Table S6.2). The AIV seroprevalence in gulls of Newfoundland during October 2011 and September 2012 was 50% (44/88). The pattern of high serological prevalence versus lower rate of AIV detection in wild birds is more likely a common phenomenon, as also found in breeding Black-legged Kittiwakes (*Rissa tridactyla*) in Norway during June 2009 (Toennessen, Germundsson et al. 2011), in Ring-billed Gulls (*Larus delawarensis*) at several breeding colonies in Ontario, Canada in the years of 2000 and 2004 (Velarde, Calvin et al. 2010), in experimentally infected Ruddy Turnstone (*Arenaria interpres*) in Delaware Bay (Maxted, Luttrell et al. 2012), in Red Knot (*Calidris canutus*) and Ruddy Turnstone in Africa (Gaidet, Ould El Mamy et al. 2012), and particularly in multiple bird species including waterfowl and gulls in Alaska during 1998-2010 (Wilson, Hall et al. 2013).

6.1.3 HA subtypes of AIVs in ducks, gulls and murres

AIVs in Newfoundland displayed a large diversity of HA variety. The 43 duck AIVs had 8 HA subtypes (H1, H2, H3, H4, H5, H6, H11 and H12). Phylogenetic analysis of the 4 H1 AIVs and the H5 duck virus showed that they were highly related to other low pathogenic AIV sequences from waterfowl in North America. Multiple murre AIVs of H1N2 subtypes were detected in 2011, whereas the murre virus detected in 2007 in Newfoundland was classified as H11N2 subtype (Granter, Wille et al. 2010). The HA and NA genes detected from ducks and murres in Newfoundland to date all belonged to North American avian phylogenetic lineages. In comparison, HA and NA genes of both avian-related H1 and H9 lineages and gull-related H13 and H16 lineages (Hinshaw, Air et al. 1983, Fouchier, Munster et al. 2005) have been found in gull AIVs in Newfoundland. I

hypothesize that AIVs of gull–related H13 and H16 subtypes may also be detected from ducks in Newfoundland, more likely at wintering areas of wild birds, when ducks and gulls aggregate and mix together. Therefore, future AIV surveillance should focus some swab sample collections from birds at shared wintering areas (e.g. Quidi Vidi Lake in St. John's) to better study the transmission of AIVs between waterfowl and gulls.

6.1.4 Gene flow and genetic structure of AIVs in duck, gull and murre

For duck AIVs, 109 contemporary duck AIV genome sequences (2006-2011) in the Atlantic bird migratory flyway, including sequences of 30 viruses from ducks in Newfoundland acquired during this thesis work were analyzed for their population structure. A vast diversity of viral genes was identified in the 109 viruses, and the 8 gene segments of these viruses were classified in 34 phylogenetic lineages. The genetic structure differed amongst the 8 viral segments with predominant single lineages found for the PB2, PB1 and M segments, increased diversity found for the PA, NP and NS segments (2, 3 and 3 lineages, respectively), and the highest diversity found for the HA and NA segments (12 and 9 lineages, respectively). Forty-three different genotypes were identified amongst the 109 duck viruses, indicating extensive virus reassortment. Identification of inter-continental transmission in these duck AIVs was rare and all gene segments of the 30 duck AIVs in Newfoundland were of American-avian origin. This is distinct from what has been found for duck AIVs in Alaska, especially in Northern Pintails, from which inter-continental AIV reassortants were frequently detected (Koehler, Pearce et al. 2008, Ramey, Pearce et al. 2010). The rare inter-continental transmission of AIVs detected in ducks of Newfoundland indicates that ducks should not be used as the sentinel birds to monitor for the possible HP AIV transmission from Eurasia to North

America through trans-Atlantic bird flyways. On the other hand, virus transmission between ducks and other bird host groups has been frequent with 57.3% of the AIV genes in the analyzed Atlantic bird flyway duck viruses having highly similar genes (≥99% nucleotide identity) detected in birds other than ducks. Transmission between North American flyways has also been frequent and 75.8% of the genes were highly similar to genes found in other North American flyways. However, the duck AIV genes did display spatial distribution bias, which was demonstrated by the different population sizes of specific viral genes in one or two neighboring flyways compared to more distant flyways. The frequent inter-specific and intra-continental transmission of AIVs detected in Newfoundland ducks also indicated increased chance of AIV transmission to poultry from ducks compared to other wild birds, indicating that the study of wild duck AIVs is essential to monitor for the emergence of AIV infection in poultry, and to disclose the evolution of AIVs during inter-specific transmission.

The genetic structure of the gull AIVs in Newfoundland revealed frequent inter-continental and considerable interspecies virus transmission, similar to what was found in gull AIVs at other locations (Ramey, Pearce et al. 2010, Wille, Robertson et al. 2011, Van Borm, Rosseel et al. 2012). In particular, an AIV with a whole Eurasian gull-related genome and a virus with an inter-continental waterfowl-related genome were both detected in Newfoundland. Fifteen of the AIVs were inter-continental reassortants, distinct from the purely North American origin duck AIVs detected in Newfoundland. This indicated much higher chance of the entry of Eurasian AIVs into Atlantic North America through gulls compared to migratory ducks, and gulls in Newfoundland could work as sentinel birds to trace the possible invasion of HP AIVs into North America from

Eurasia. Ten different AIV genotypes were detected in the 13 gull AIVs with sequence information available for all eight segments. The predominant viral genotype and its related reassortants have been circulating in Atlantic Canada since 2008, showing that gull-involved AIV transmissions have taken place in a wide geographic area of this region including Newfoundland and Labrador, New Brunswick and Quebec (Wille, Robertson et al. 2011, Hall, Teslaa et al. 2013).

The H11N2 AIV isolate from Thick-billed Murre in Newfoundland in 2007 was identified to have an entirely waterfowl-related genome (Granter, Wille et al. 2010). However, 21 H1N2 AIVs detected from Common Murre (*Uria aalge*) in summer 2011 at 2 distant breeding colonies off the shore of Newfoundland showed inter-continental and interspecies phylogenetic features in their genes. Similar to the 21 murre viruses in this study, the genetic structure of 17 reference murre AIVs also displayed a mainly waterfowl-related gene pool with considerable inter-continental and avian-gull lineage reassortments (Wallensten, Munster et al. 2005, Ramey, Pearce et al. 2010, Van Borm, Rosseel et al. 2012). Analyzing all genes of the 38 murre AIVs by host group, 93.4% genes clustered with avian-related lineages and 6.6% belonged to gull lineages. Analyzed by continent, 19.4% genes of the viruses were inter-continental reassortants. The mosaic nature of these murre AIVs might reflect an under-recognized role of murres in AIV transmission across space and between bird host taxa. The 21 murre viruses belonged to 4 different viral genotypes, and a major genotype was represented by 15 of the 21 murre viruses in July and August 2011 from both Gull Island and Cabot Island. This helps to trace the possible origin of the major viral genotype back to their wintering area on the eastern Grand Banks of Newfoundland (McFarlane Tranquilla, Montevecchi et al.

2013McFarlane Tranquilla, Montevecchi et al. 2013), the only location where murres from the 2 colonies could have mingled. The major genotype of the murre AIVs have been circulating in the birds for several months before they segregated to different breeding colonies in April, which also explained the genetic heterogeneity of the 15 viruses belonging to the major genotype. My study for the first time provided genetic data supporting frequent segment reassortment and efficient transmission during murre AIV infection in high-density bird breeding colonies.

Based on the findings in this thesis, juvenile birds during the breeding and fall migratory seasons may be considered a priority for future epidemiology research to ensure the highest rates of AIV detection. Wild ducks will be the targeted bird groups to monitor the AIV transmission for the concerns of poultry industry and human health, while gulls are suggested as sentinel bird groups to detect cross-Atlantic transmission of AIVs from Eurasia to North America. Besides the 3 bird groups studied in this thesis, future epidemiological studies should also pay attention to shorebirds to increase our understanding of this bird group in AIV transmission in this region. In summary, my thesis work provided a profile of AIV epidemiology, phylogeny and transmission in multiple bird taxa in Newfoundland, which helps to increase our understanding of AIV ecology and evolution at the Atlantic coast of Canada and North America.

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Supplementary documents

Table S2.1. AIV prevalence by sampling month, age and gender.

Sampling month	AIV % prevalence (ratio) according to age			AIV % prevalence (ratio) according to gender		
	Juvenile	Adult	Unknown	Male	Female	Unknown
09/2008	8.5 (6/71)	0 (0/24)	NS*	10.5 (6/57)	0 (0/38)	NS
10/2008	4.4 (4/90)	0 (0/28)	NS	5.6 (4/72)	0 (0/44)	0 (0/2)
01/2009	0 (0/10)	0 (0/30)	NS	0 (0/29)	0 (0/11)	NS
09/2009	20.0 (4/20)	14.3 (2/14)	NS	15.4 (4/26)	14.3 (1/7)	100 (1/1)
10/2009	12.5 (1/8)	0 (0/15)	0 (0/1)	7.1 (1/14)	0 (0/10)	NS
09/2010	13.8 (8/58)	4 (2/50)	NS	5.6 (4/72)	16.7 (6/36)	NS
10/2010	13.3 (2/15)	9.5 (2/21)	NS	16.7 (4/24)	0 (0/11)	0 (0/1)
11/2010	12.0 (6/50)	10.0 (5/50)	NS	14.3 (8/56)	6.8 (3/44)	NS
12/2010	0 (0/27)	3.45 (1/29)	NS	2.9 (1/34)	0 (0/22)	NS
01/2011	0 (0/1)	0 (0/24)	NS	0 (0/21)	0 (0/4)	NS
02/2011	NS	0 (0/12)	NS	0 (0/7)	0 (0/5)	NS
08/2011	28.6 (2/7)	0 (0/9)	NS	15.4 (2/13)	0 (0/3)	NS
09/2011	15.0 (12/80)	1.8 (1/55)	NS	10.8 (9/83)	8.0 (4/50)	0 (0/2)
10/2011	25.0 (4/16)	0 (0/14)	NS	14.8 (4/27)	0 (0/3)	NS
11/2011	0 (0/5)	8.3 (1/12)	NS	0 (0/12)	20.0 (1/5)	NS
12/2011	0 (0/2)	0 (0/4)	NS	0 (0/4)	0 (0/2)	NS
01/2012	0 (0/1)	0 (0/26)	NS	0 (0/20)	0 (0/7)	NS
Total	10.6 (49/461)	3.4 (14/417)	0 (0/1)	8.2 (47/571)	5.0 (15/302)	16.7 (1/6)

* None sampled.

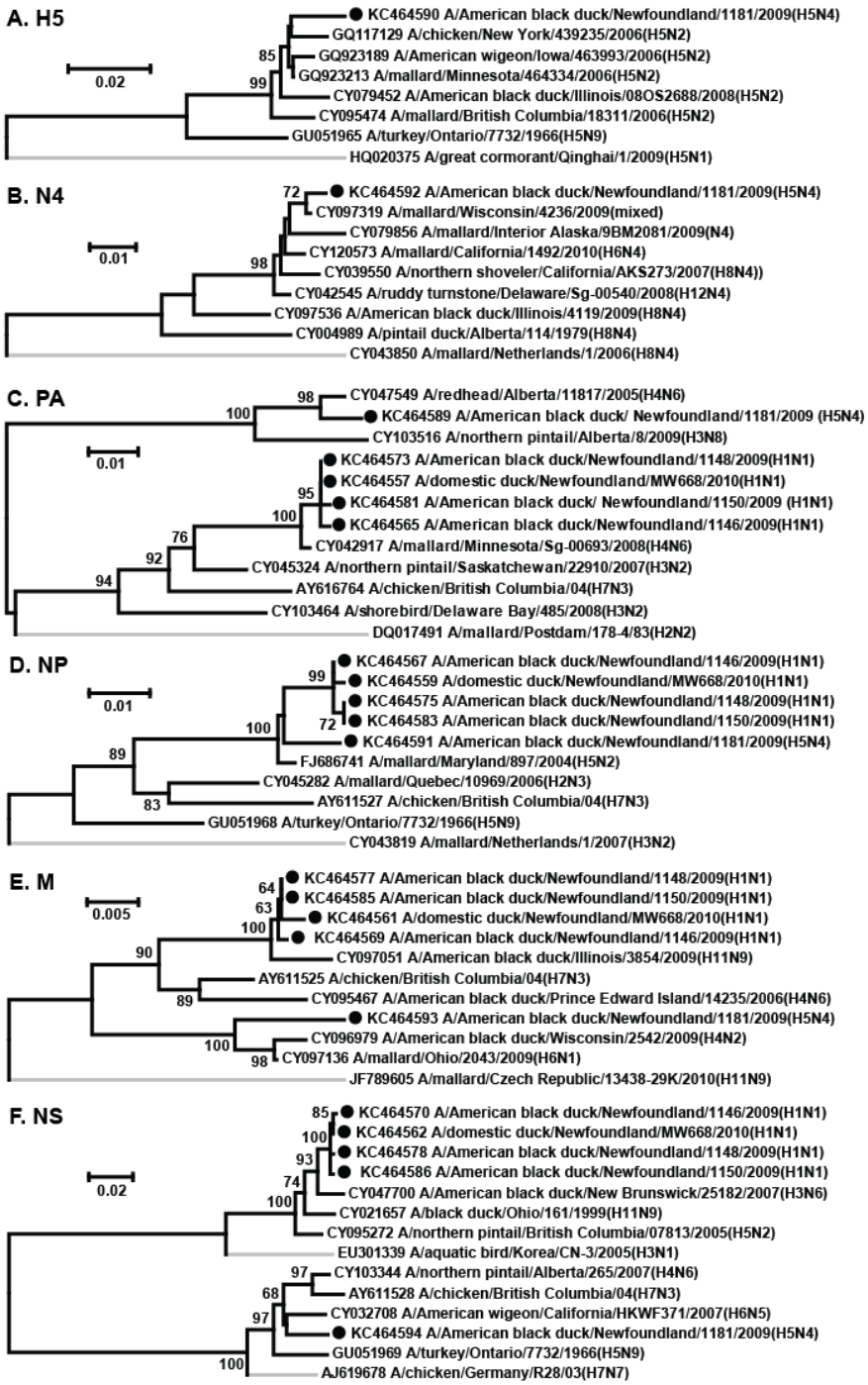


Figure S2.1. Phylogenetic trees of genes from the H1N1 and H5N4 viruses identified in ducks in Newfoundland. The trees in panels A through F represent analyses of the H5, N4,

PA, NP, M and NS genes, respectively. The AIVs from this study (indicated with solid circles) are assigned in American avian clades (black branches) with Eurasian-avian sequences as the outgroups (grey branches). The trees were constructed with MEGA 5 by the neighbour-joining method. Support for the branches is indicated as percentages based 1000 bootstrap replicates where support was ≥60%. The scale bars indicate substitutions per site.

Table S3.1. The 25 duck AIVs from Newfoundland, Canada, sequenced in this study.

HA	Virus	Abbreviation	Date (mm/dd/yy)	Site
H2	A/Northern pintail/Newfoundland/GR683/2011(H2N2)	NOPI NL GR683 2011	10/13/11	Commonwealth Pond
	A/Mallard/Newfoundland/GR475/2011(H2N2)	MALL NL GR475 2011	09/14/11	Quidi Vidi Lake
	A/American black duck/Newfoundland/836/2008(H2N4)	ABDU NL 836 2008	10/08/08	Mundy Pond
	A/American black duck/Newfoundland/840/2008(H2N4)	ABDU NL 840 2008	10/15/08	Mundy Pond
	A/American black duck/Newfoundland/812/2008(H2N6)	ABDU NL 812 2008	09/26/08	Mundy Pond
H3	A/American black duck/Newfoundland/GR252/2011(H3N2)	ABDU NL GR252 2011	08/31/11	Mundy Pond
	A/American black duck/Newfoundland/GR490/2011(H3N2)	ABDU NL GR490 2011	09/17/11	Commonwealth Pond
	A/American black duck/Newfoundland/GR256/2011(H3N2)	ABDU NL GR256 2011	08/31/11	Mundy Pond
	A/American black duck/Newfoundland/GR396/2011(H3N2)	ABDU NL GR396 2011	0912/11	Commonwealth Pond
	A/American black duck/Newfoundland/MW662/2010(H3N6)	ABDU NL MW662 2010	09/17/10	Commonwealth Pond
	A/American black duck/Newfoundland/732/2008(H3N8)	ABDU NL 732 2008	09/15/08	Mundy Pond
	A/American black duck/Newfoundland/734/2008 H3N8)	ABDU NL 734 2008	09/19/08	Mundy Pond
	A/Northern pintail /Newfoundland/GR679/2011(H3N2)	NOPI NL GR679 2011	10/12/11	Commonwealth Pond
H4	A/American black duck/Newfoundland/807/2008(H4N4)	ABDU NL 807 2008	10/01/08	Mundy Pond
	A/American black duck/Newfoundland/819/2008(H4N6)	ABDU NL 819 2008	09/26/08	Mundy Pond
	A/American black duck/Newfoundland/826/2008(H4N6)	ABDU NL 826 2008	10/08/08	Mundy Pond
	A/American black duck/Newfoundland/MW609/2010(H4N6)	ABDU NL MW609 2010	09/16/10	Mundy Pond
	A/American black duck/Newfoundland/MW861/2010(H4N6)	ABDU NL MW861 2010	12/01/10	Commonwealth Pond
	A/Mallard/Newfoundland/PR021/2010(H4N6)	MALL NL PR021 2010	10/14/10	Commonwealth Pond
H6	A/American black duck/Newfoundland/MW733/2010(H6N6)	ABDU NL MW733 2010	10/14/10	Commonwealth Pond
	A/American black duck/Newfoundland/PR007/2010(H6N6)	ABDU NL PR007 2010	10/15/10	Commonwealth Pond
	A/Mallard and American Black Duck Hybrid /Newfoundland/MW721/2010(H6N8)	M-A Hybrid MW721 NL 2010	09/30/10	Quidi Vidi Lake
H11	A/American black duck/Newfoundland/MW819/2010(H11N3)	ABDU NL MW819 2010	11/22/10	Commonwealth Pond
	A/American black duck/Newfoundland/MW774/2010(H11N9)	ABDU NL MW774 2010	11/08/10	Commonwealth Pond
H12	A/Northern pintail/Newfoundland/GR495/2011(H12)	NOPI NL GR495 2011	09/19/11	Mundy Pond

Table S3.2. Genetic analysis of the 109 Atlantic flyway duck AIVs (from 2006 to 2011).

HA	Virus identification information	Genotype	Sub-genotype[b]	Gene type by segment[c]							
				PB2	PB1	PA	HA	NP	NA	M	NS
H1	A/American black duck/Newfoundland/1146/2009(H1N1)[a]	CFE1DH1EE2B	CFE1DH1EE2B-1	C-2.1	F-3.1	E-1.1	1D-1.1	H-4.1	1E-1.1	E-1.1	2B-1.1
	A/American black duck/Newfoundland/1148/2009(H1N1)[a]		CFE1DH1EE2B-1	C-2.1	F-3.1	E-1.1	1D-1.1	H-4.1	1E-1.1	E-1.1	2B-1.1
	A/American black duck/Newfoundland/1150/2009(H1N1)[a]		CFE1DH1EE2B-1	C-2.1	F-3.1	E-1.1	1D-1.1	H-4.1	1E-1.1	E-1.1	2B-1.1
	A/domestic duck/Newfoundland/MW668/2010(H1N1)[a]		CFE1DH1EE2B-1	C-2.1	F-3.1	E-1.1	1D-1.1	H-4.1	1E-1.1	E-1.1	2B-1.1
H2	A/Northern pintail/Newfoundland/GR683/2011(H2N2)	CFE2HH2DE2B	CFH2HH2DE2B-1	C-2.2	F-4.1	E-2.1	2H-1.1	H-2.1	2D-1	E-1.2	2B-1.2
	A/mallard/Newfoundland/GR475/2011(H2N2)		CFH2HH2DE2B-2	C-2.2	F-4.1	E-1.2	2H-1.1	H-2.1	2D-1	E-1.2	2B-1.2
	A/mallard/Quebec/10969/2006(H2N3)	CFE2HH3AE1D	CFE2HH3AE1D-1	C-2.11	F-4.6	E-5.1	2H-2.1	H-1.2	3A-2.2	E-1.14	1D-1.7
	A/mallard/Quebec/11063/2006(H2N3)		CFE2HH3AE1D-2	C-3.2	F-1.3	E-5.1	2H-2.1	H-3.3	3A-2.2	E-1.13	1D-1.10
	A/mallard/Quebec/11281/2006(H2N3)		CFE2HH3AE1D-2	C-3.2	F-1.3	E-5.1	2H-2.1	H-3.3	3A-2.2	E-1.13	1D-1.10
	A/American black duck/Newfoundland/836/2008(H2N4)	CFE2HH4AE1D	CFE2HH4AE1D-1	C-2.2	F-4.2	E-2.1	2H-1.2	H-3.1	4A-1.1	E-1.3	1D-1.1
	A/American black duck/Newfoundland/840/2008(H2N4)		CFE2HH4AE1D-1	C-2.2	F-4.2	E-2.1	2H-1.2	H-3.1	4A-1.1	E-1.3	1D-1.1
	A/American black duck/Newfoundland/812/2008(H2N6)	CFH2HH6AE1D	CFH2HH6AE1D-1	C-1.1	F-3.2	H-1.1	2H-1.3	H-3.1	6A-1.1	E-1.3	1D-1.1
H3	A/American black duck/Newfoundland/GR252/2011(H3N2)	CFE3CH2DE2B	CFE3CH2DE2B-1	C-2.2	F-2.1	E-2.1	3C-1.1	H-2.1	2D-1	E-1.4	2B-1.2
	A/American black duck/Newfoundland/GR256/2011(H3N2)		CFE3CH2DE2B-2	C-2.2	F-2.1	E-2.1	3C-1.1	H-5.1	2D-1.2	E-1.4	2B-1.2
	A/American black duck/Newfoundland/GR490/2011(H3N2)		CFE3CH2DE2B-?	C-2.2	F-2.1	E-2.1	3C-1.1	H-2.1	2D	E-1.4	2B-1.2
	A/American black duck/Newfoundland/GR396/2011(H3N2)	CFH3CH2DE2B	CFH3CH2DE2B-1	C-2.2	F-1.1	H-1.2	3C-1.1	H-2.1	2D-1.1	E-1.4	2B-1.2
	A/American black duck/Newfoundland/GR679/2011(H3N2)		CFH3CH2DE2B-2	C-2.5	F-4.1	H-1.5	3C-1.1	H-5.1	2D	E-1.6	2B-1.2
	A/American black duck/Newfoundland and Labrador/26516/2007(H3N2)	CFH3DH2DE1D	CFH3DH2DE1D-1	C-2.10	F-3.2	H-1.6	3D-1.1	H-2.3	2D-1.1	E-l.12	1D-1.1
	A/American black duck/Newfoundland and Labrador/26553/2007(H3N2)		CFH3DH2DE1D-2	C-2.10	F-3.2	H-1.6	3D-1.1	H-3.2	2D-1.1	E-1.12	1D-1.1

A/American black duck/Quebec/11235/2006(H3N2)	CFH3CH2DE1D	CFH3CH2DE1D-1	C-3.2	F-3.4	H-1.7	3C-2.1	H-6.2	2D-2.1	E-1.3	1D-1.7
A/mallard/Quebec/11040/2006(H3N2)		CFH3CH2DE1D-2	C-2.12	F-3.4	H-1.7	3C-2.1	H-6.2	2D-2.1	E-1.3	1D-1.7
A/mallard/Quebec/11045/2006(H3N2)		CFH3CH2DE1D-2	C-2.12	F-3.4	H-1.7	3C-2.1	H-6.2	2D-2.1	E-1.3	1D-1.7
A/mallard/Quebec/11121/2006(H3N2)		CFH3CH2DE1D-2	C-2.12	F-3.4	H-1.7	3C-2.1	H-6.2	2D-2.1	E-1.3	1D-1.7
A/mallard/Quebec/11020/2006(H3N2)		CFH3CH2DE1D-2	C-2.12	F-3.4	H-1.7	3C-2.1	H-6.2	2D-2.1	E-1.3	1D-1.7
A/mallard/Quebec/11247/2006(H3N2)		CFH3CH2DE1D-3	C-3.3	F-4.6	H-1.7	3C-2.1	H-6.2	2D-2.1	E-1.3	1D-1.7
A/mallard/Quebec/11194/2006(H3N2)		CFH3CH2DE1D-3	C-3.3	F-4.6	H-1.7	3C-2.1	H-6.2	2D-2.1	E-1.3	1D-1.7
A/mallard/Quebec/11093/2006(H3N2)		CFH3CH2DE1D-3	C-3.4	F-4.6	H-1.7	3C-2.1	H-6.2	2D-2.1	E-1.3	1D-1.7
A/mallard/Quebec/11221/2006(H3N2)		CFH3CH2DE1D-4	C-2.7	F-2.2	H-1.7	3C-2.1	H-6.2	2D-2.1	E-1.3	1D-1.7
A/American black duck/Newfoundland/MW662/2010(H3N6)	CFE3CH6AE1D	CFE3CH6AE1D-1	C-2.4	F-4.3	E-1.1	3D-1.1	H-1.1	6A-3.1	E-1.1	1D-1.5
A/American black duck/New Brunswick/25182/2007(H3N6)	CFE3CH6AE2B	CFE3CH6AE2B-1	C-2.7	F-6.1	E-1.3	3C-2.3	H-2.4	6A-4.2	E-1.5	2B-1.4
A/mallard/Maryland/1235/2006(H3N6)	CFH3CH6AE1D	CFH3CH6AE1D-1	C-2.7	F-5.1	H-1.10	3C-2.2	H-3.4	6A-4.3	E-1.15	1D-1.12
A/blue-winged teal/New Brunswick/03757/2009(H3N6)		CFH3CH6AE1D-2	C-2.2	F-3.12	H-1.15	3C-2.3	H-1.1	6A-3.2	E-1.20	1D-1.7
A/American black duck/Newfoundland/732/2008(H3N8)	CFE3DH8AE1D	CFE3DH8AE1D-1	C-1.1	F-3.2	E-2.1	3D-1.1	H-3.1	8A	E-1.3	1D-1.1
A/mallard/Nova Scotia/03271/2009(H3N8)		CFE3DH8AE1D-2	C-3.5	F-4.5	E-2.1	3D-1.2	H-4.6	8A-2.1	E-1.19	1D-1.13
A/ring-necked duck/Nova Scotia/03378/2009(H3N8)		CFE3DH8AE1D-2	C-3.5	F-4.5	E-2.1	3D-1.2	H-4.6	8A-2.1	E-1.19	1D-1.13
A/ring-necked duck/New Brunswick/03400/2009(H3N8)		CFE3DH8AE1D-2	C-3.5	F-4.5	E-2.1	3D-1.2	H-4.6	8A-2.1	E-1.19	1D-1.13
A/green-winged teal/New Brunswick/03483/2009(H3N8)		CFE3DH8AE1D-2	C-3.5	F-4.5	E-2.1	3D-1.2	H-4.6	8A-2.1	E-1.19	1D-1.13
A/northern pintail/New Brunswick/03547/2009(H3N8)		CFE3DH8AE1D-2	C-3.5	F-4.5	E-2.1	3D-1.2	H-4.6	8A-2.1	E-1.19	1D-1.13
A/blue-winged teal/Prince Edward Island/03912/2009(H3N8)		CFE3DH8AE1D-2	C-3.5	F-4.5	E-2.1	3D-1.2	H-4.6	8A-2.1	E-1.19	1D-1.13
A/green-winged teal/New Brunswick/02586/2007(H3N8)	CFH3DH8AE1D	CFH3DH8AE1D-1	C-2.19	F-3.6	H-1.17	3D-2.1	H-4.7	8A-2.2	E-1.18	1D-1.9
A/green-winged teal/New Brunswick/02587/2007(H3N8)		CFH3DH8AE1D-1	C-2.19	F-3.6	H-1.17	3D-2.1	H-4.7	8A-2.2	E-1.18	1D-1.1
A/green-winged teal/New Brunswick/02588/2007(H3N8)		CFH3DH8AE1D-1	C-2.19	F-3.6	H-1.17	3D-2.1	H-4.7	8A-2.2	E-1.18	1D-1.1
A/green-winged teal/New Brunswick/02590/2007(H3N8)		CFH3DH8AE1D-1	C-2.19	F-3.6	H-1.17	3D-2.1	H-4.7	8A-2.2	E-1.18	1D-1.1

A/green-winged teal/New Brunswick/02591/2007(H3N8)		CFH3DH8AE1D-1	C-2.19	F-3.6	H-1.17	3D-2.1	H-4.7	8A-2.2	E-1.18	1D-1.1
A/green-winged teal/New Brunswick/02592/2007(H3N8)		CFH3DH8AE1D-1	C-2.19	F-3.6	H-1.17	3D-2.1	H-4.7	8A-2.2	E-1.18	1D-1.1
A/American black duck/Newfoundland/734/2008(H3N8)		CFH3DH8AE1D-2	C-1.1	F-3.2	H-1.1	3D-1.1	H-3.1	8A-2.1	E-1.3	1D-1.1
A/blue-winged teal/Nova Scotia/03971/2009(H3N8)		CFH3DH8AE1D-3	C-2.2	F-3.11	H-1.17	3D-1.3	H-4.5	8A-2.2	E-1.21	1D-1.13
A/blue-winged teal/Prince Edward Island/03927/2009(H3N8)		CFH3DH8AE1D-4	C-2.18	F-3.6	H-1.17	3D-2.1	H-1.6	8A-2.2	E-1.18	1D-1.13
A/mallard/Quebec/11082/2006(H3N8)	CFE3CH8AE2B	CFE3CH8AE2B-1	C-2.7	F-3.7	E-3.1	3C-1.2	H-2.3	8A-1.4	E-1.14	2B-1.3
A/American black duck/New Brunswick/19350/2006(H3N8)		CFE3CH8AE2B-2	C-2.6	F-3.5	E-4.1	3C-1.2	H-1.8	8A-1.1	E-1.10	2B-1.6
A/American wigeon/New Brunswick/04487/2007(H3N8)		CFE3CH8AE2B-2	C-2.6	F-3.5	E-4.1	3C-1.2	H-1.8	8A-1.1	E-1.10	2B-1.6
A/American wigeon/New Brunswick/04488/2007(H3N8)		CFE3CH8AE2B-2	C-2.6	F-3.5	E-4.1	3C-1.2	H-1.8	8A-1.1	E-1.10	2B-1.6
A/American wigeon/New Brunswick/04489/2007(H3N8)		CFE3CH8AE2B-2	C-2.6	F-3.5	E-4.1	3C-1.2	H-1.8	8A-1.1	E-1.10	2B-1.6
A/American wigeon/New Brunswick/04490/2007(H3N8)		CFE3CH8AE2B-2	C-2.6	F-3.5	E-4.1	3C-1.2	H-1.8	8A-1.1	E-1.10	2B-1.6
A/American wigeon/New Brunswick/04491/2007(H3N8)		CFE3CH8AE2B-2	C-2.6	F-3.5	E-4.1	3C-1.2	H-1.8	8A-1.1	E-1.10	2B-1.6
A/American wigeon/New Brunswick/04492/2007(H3N8)		CFE3CH8AE2B-2	C-2.6	F-3.5	E-4.1	3C-1.2	H-1.8	8A-1.1	E-1.10	2B-1.6
A/American wigeon/New Brunswick/04493/2007(H3N8)		CFE3CH8AE2B-2	C-2.6	F-3.5	E-4.1	3C-1.2	H-1.8	8A-1.1	E-1.10	2B-1.6
A/American wigeon/New Brunswick/04494/2007(H3N8)		CFE3CH8AE2B-2	C-2.6	F-3.5	E-4.1	3C-1.2	H-1.8	8A-1.1	E-1.10	2B-1.6
A/American wigeon/New Brunswick/04497/2007(H3N8)		CFE3CH8AE2B-2	C-2.6	F-3.5	E-4.1	3C-1.2	H-1.8	8A-1.1	E-1.10	2B-1.6
A/American wigeon/New Brunswick/04500/2007(H3N8)		CFE3CH8AE2B-2	C-2.6	F-3.5	E-4.1	3C-1.2	H-1.8	8A-1.1	E-1.10	2B-1.6
A/American black duck/Prince Edward Island/14228/2006(H3N8)	CFH3CH8AE2B	CFH3CH8AE2B-1	C-4.1	F-3.6	H-1.1	3C-1.2	H-1.2	8A-1.2	E-1.10	2B-1.6
A/American black duck/Prince Edward Island/14230/2006(H3N8)		CFH3CH8AE2B-2	C-2.9	F-3.6	H-1.1	3C-1.2	H-1.2	8A-1.2	E-1.10	2B-1.6
A/mallard/Maryland/807/2007(H3N8)	CFH3CH8AE1D	CFH3CH8AE1D-1	C-2.7	F-5.2	H-1.12	3C-1.3	H-1.7	8A-3.1	E-1.9	1D-1.7

H4	A/blue-winged teal/New Brunswick/03756/2009(H4N2)	CFH4AH2DE2B	CFH4AH2DE2B-1	C-2.8	F-2.1	H-1.16	4A-3.1	H-1.1	2D-1.1	E-1.5	2B-1.7
	A/American black duck/Newfoundland/807/2008(H4N4)	CFH4AH4AE1D	CFH4AH4AE1D-1	C-1.1	F-3.2	H-1.1	4A-1.1	H-1.2	4A-1.1	E-1.3	1D-1.1
	A/American black duck/Newfoundland/819/2008(H4N6)	CFH4AH6AE1D	CFH4AH6AE1D-1	C-1.1	F-3.2	H-1.1	4A-1.1	H-1.2	6A-1.1	E-1.3	1D-1.1
	A/American black duck/Newfoundland/826/2008(H4N6)		CFH4AH6AE1D-1	C-1.1	F-3.2	H-1.1	4A-1.1	H-1.2	6A-1.1	E-1.3	1D-1.1
	A/American black duck/Newfoundland/MW861/2010(H4N6)		CFH4AH6AE1D-2	C-2.3	F-1.1	H-1.5	4A-2.1	H-1.1	6A-2.1	E-1.5	1D-1.5
	A/mallard/Newfoundland/PR021/2010(H4N6)		CFH4AH6AE1D-2	C-2.4	F-1.1	H-1.5	4A-2.1	H-1.1	6A-2.1	E-1.6	1D-1.5
	A/American black duck/Prince Edward Island/14235/2006(H4N6)		CFH4AH6AE1D-3	C-4.1	F-3.2	H-1.1	4A-1.5	H-4.3	6A-1.2	E-1.11	1D-1.6
	A/American black duck/Prince Edward Island/02708/2007(H4N6)		CFH4AH6AE1D-3	C-4.1	F-3.6	H-1.1	4A-1.5	H-4.3	6A-1.3	E-1.11	1D-1.6
	A/mallard/Quebec/11182/2006(H4N6)		CFH4AH6AE1D-4	C-2.12	F-3.2	H-1.9	4A-1.4	H-1.5	6A-3.4	E-1.3	1D-1.9
	A/mallard/Quebec/11189/2006(H4N6)		CFH4AH6AE1D-4	C-2.12	F-3.4	H-1.7	4A-1.4	H-1.5	6A-4.2	E-1.3	1D-1.9
	A/American black duck/Newfoundland/MW609/2010(H4N6)	CFE4AH6AE1D	CFE4AH6AE1D-1	C-2.1	F-3.1	E-1.1	4A-1.2	H-2.1	6A-2.1	E-1.1	1D-1.2
	A/American black duck/New Brunswick/19347/2006(H4N6)		CFE4AH6AE1D-2	C-2.10	F-3.3	E-3.1	4A-1.4	H-2.3	6A-1.1	E-1.8	1D-1.8
	A/American black duck/New Brunswick/19497/2006(H4N6)		CFE4AH6AE1D-3	C-2.7	F-5.1	E-3.2	4A-1.6	H-6.1	6A-4.1	E-1.3	1D-1.7
	A/American black duck/New Brunswick/19502/2006(H4N6)		CFE4AH6AE1D-3	C-2.7	F-5.1	E-3.2	4A-1.6	H-6.2	6A-4.1	E-1.3	1D-1.7
	A/mallard/Quebec/11106/2006(H4N6)		CFE4AH6AE1D-4	C-2.12	F-3.4	E-5.2	4A-1.4	H-1.5	6A-4.2	E-1.3	1D-1.9
	A/mallard/Quebec/11103/2006(H4N6)		CFE4AH6AE1D-4	C-2.12	F-3.4	E-5.2	4A-1.4	H-1.5	6A-4.2	E-1.3	1D-1.9
	A/mallard/Quebec/11102/2006(H4N6)		CFE4AH6AE1D-4	C-2.12	F-3.4	E-5.2	4A-1.4	H-1.5	6A-4.2	E-1.13	1D-1.9
	A/American black duck/Prince Edward Island/02662/2007(H4N6)		CFE4AH6AE1D-5	C-4.1	F-3.6	E-3.1	4A-1.5	H-1.5	6A-1.3	E-1.8	1D-1.6
	A/green-winged teal/New Brunswick/02426/2007(H4N6)		CFE4AH6AE1D-5	C-4.1	F-3.6	E-3.1	4A-1.5	H-1.5	6A-1.3	E-1.8	1D-1.6
	A/mallard/Maryland/965/2006(H4N6)	CFE4AH6AE2B	CFE4AH6AE2B-1	C-2.13	F-6.2	E-2.2	4A-1.3	H-2.4	6A-3.3	E-1.4	2B-1.5
	A/American black duck/New Brunswick/19389/2006(H4N8)	CFE4AH8AE2B	CFE4AH8AE2B-1	C-2.6	F-3.5	E-4.1	4A-1.6	H-6.1	8A-1.1	E-1.3	2B-1.6
	A/American black duck/New Brunswick/19392/2006(H4N8)		CFE4AH8AE2B-2	C-2.6	F-5.1	E-4.1	4A-1.6	H-6.1	8A-1.1	E-1.10	2B-1.6

	A/blue-winged teal/Prince Edward Island/03910/2009(H4N9)	CFE4AH9AE1D	CFE4AH9AE1D-1	C-2.18	F-1.2	E-2.1	4A-3.2	H-1.6	9A-1.2	E-1.3	1D-1.13
H5	A/American black duck/Newfoundland/1181/2009(H5N4)[a]	CFH5CH4AE1D	CFH5CH4AE1D-1	C-2.4	F-3.1	H-1.3	5C-1.1	H-4.2	4A	E-1.7	1D-1.5
	A/mallard/Maryland/802/2007(H5N1)	CFH5CF1EE1D	CFH5CF1EE1D-1	C-2.7	F-7.1	H-1.13	5C-1.2	**F-1.1**	1E- 2.1	E-1.5	1D-1.7
	A/mallard/Ontario/26078/2007(H5N1)	CFH5CH1EE1D	CFH5CH1EE1D-1	C-3.2	F-4.7	H-1.14	5C-1.4	H-1.6	1E- 2.2	E-1.16	1D-1.7
	A/mallard/Maryland/182/2006(H5N2)	CFH5CH2DE1D	CFH5CH2DE1D-1	C-2.14	F-3.7	H-1.11	5C-1.2	H-5.2	2D-1.1	E-1.3	1D-1.10
	A/Muscovy duck/New York/62095-1/2006(H5N2)	CFE5CH2DE1D	CFE5CH2DE1D-1	C-2.12	F-1.4	E-1.5	5C-1.3	H-4.4	2D-1.3	E-1.3	1D-1.1
	A/duck/New York/445743/2006(H5N2)		CFE5CH2DE1D-1	C-2.12	F-1.4	E-1.5	5C-1.3	H-4.4	2D-1.3	E-1.3	1D-1.1
	A/duck/New York/465571/2006(H5N2)	CFE5CH2GE1D	CFE5CH2GE1D-1	C-2.15	F-5.1	E-5.1	5C-1.2	H-4.3	2D-3.1	E-1.5	1D-1.7
	A/northern pintail/Florida/480645-5/2007(H5N2)		CFE5CH2GE1D-2	C-3.3	F-3.8	E-3.4	5C-1.2	H-6.1	2G-2.1	E-1.16	1D-1.7
	A/duck/Pennsylvania/446080-6/2006(H5N2)	CFE5CH2DE1D	CFE5CH2DE1D-1	C-2.16	F-1.4	E-1.5	5C-1.3	H-4.4	2D-1.3	E-1.3	1D-1.1
	A/green winged teal/Delaware/458672-5/2006(H5N2)		CFE5CH2DE1D-2	C-2.17	F-3.9	E-3.5	5C-1.5	H-2.5	2D-1.3	E-1.16	1D-1.10
	A/mallard/Maryland/792/2007(H5N9)	CFH5CH9AE1D	CFH5CH9AE1D	C-2.7	F-5.2	H-1.13	5C-1.2	H-1.7	9A-3.1	E-1.5	1D-1.7
H6	A/American black duck/Newfoundland/MW733/2010(H6N6)	CFH6BH6AE1D	CFH6BH6AE1D-1	C-2.3	F-3.1	H-1.4	**6B-1.1**	H-1.3	6A-3.2	E-1.1	1D-1.4
	A/American black duck/Newfoundland/PR007/2010(H6N6)		CFH6BH6AE1D-2	C-2.1	F-3.1	H-1.5	**6B-1.1**	H-1.1	6A-2.1	E-1.6	1D-1.5
	A/duck/Newfoundland/MW721/2010(H6N8)	CFH6BH8AE1D	CFH6BH8AE1D-1	C-2.1	F-3.1	H-1.4	**6B-1.1**	H-1.3	8A-1.3	E-1.1	1D-1.4
H7	A/American black duck/NB/2538/2007(H7N3)	CFE7FH3AE1D	CFE7FH3AE1D-1	C-2.6	F-4.5	E-1.4	7F-1.1	H-1.5	3A-2.1	E-1.10	1D-1.9
	A/Muscovy duck/New York/19495-7/2006(H7N2)	CFH7FH2GE2B	CFH7FH2GE2B-1	C-5.1	F-3.10	H-1.8	7F-2.1	H-7.1	2G-1.1	E-2.1	2B-2.1
H11	A/American black duck/Newfoundland/MW819/2010(H11N3)	CFH11CH3AE1D	CFH11CH3AE1D-1	C-2.5	F-1.2	H-1.4	11C-1.1	H-2.2	3A-1.1	E-1.5	1D-1.5
	A/American black duck/Newfoundland/MW774/2010(H11N9)	CFH11CH9AE1D	CFH11CH9AE1D-1	C-2.3	F-1.2	H-1.4	11C-1.1	H-2.2	9A-1.1	E-1.5	1D-1.5
	A/mallard/Quebec/11111/2006(H11N9)	CFE11CH9AE1D	CFE11CH9AE1D-1	C-2.6	F-3.7	E-3.3	11C-2.1	H-4.5	9A-2.1	E-1.3	1D-1.11
	A/ring-necked duck/New Brunswick/03449/2009(H11N9)	CFH11CH9AE2B	CFH11CH9AE2B-1	C-3.5	F-3.12	H-1.15	11C-1.2	H-1.9	9A-1.3	E-1.17	2B-1.8
H12	A/Northern pintail/Newfoundland/GR495/2011(H12)	CFE12AH?E1D	CFE12AH?E1D-1	C-3.1	F-4.4	E-2.1	12A-1.1	H-1.4	-	E-1.7	1D-1.3
H13	A/hooded merganser/New Brunswick/03750/2009(H13N6)	JFE13AD6AF1C	JFE13AD6AF1C-1	**J-1.1**	**F-8.1**	**E-6.1**	**13A-1.1**	**D-1.1**	6A-1.2	**F-1.1**	**1C-1.1**

H16	A/mallard/Quebec/02916-1/2009(H16N3)	JFE16DD3DF1C	JFE16DD3DF1C-1	**J-1.2**	**F-8.1**	**E-6.2**	**16D-1.1**	**D-1.1**	**3D-1.1**	**F-1.1**	**1C-1.1**
Totals	**109**	**43**	**70**								
	Gene lineages			**2**	**1**	**2**	**12**	**3**	**9**	**2**	**3**
	Gene sub-lineages			**6**	**8**	**7**	**19**	**9**	**20**	**3**	**4**
	Gene types			**29**	**31**	**34**	**38**	**32**	**38**	**23**	**23**

[a] The 4 H1N1 viruses and the H5N4 virus from Newfoundland were sequenced previously (accession numbers KC464555-KC464586, KC492275-KC492290 and KC492307-KC492330).

[b] Repeatedly detected homologous genomes are shaded in grey.

[c] AIV genes of origin other than North American avian are highlighted in colour: Eurasian avian, blue; Eurasian gull, yellow; North American gull, green.

Table S3.3. AIV gene typing summary for Atlantic flyway locations with 20 or more viruses.

Location	Category[a]	HA	NA	PB2	PB1	PA	NP	M	NS	Total
Newfoundland	Gene lineages	9	7	1	1	2	1	1	2	24
	Sub-lineages	10	10	3	4	3	5	1	2	38
	Gene types	13	12	8	9	9	12	8	7	78
Quebec	Gene lineages	4	5	1	1	2	1	1	2	17
	Sub-lineages	6	7	3	5	4	6	2	3	36
	Gene types	6	7	8	7	7	6	4	6	51
New Brunswick	Gene lineages	3	5	2	1	2	2	2	3	19
	Sub-lineages	6	8	4	6	6	5	2	3	40
	Gene types	8	12	9	9	9	11	9	10	81

[a] Gene lineages, sub-lineages and gene types are defined as sharing ≥90%, ≥95% and ≥99% nucleotide identity, respectively.

Table S3.4. AIV genotyping for Atlantic flyway locations with 20 or more viruses.

Location	Viruses	Genotypes	Sub-genotypes	Repeatedly detected sub-genotypes
Newfoundland	32	19	24	3
Quebec	20	6	10	4
New Brunswick	32	11	14	3

Table S3.5. Repeated gene type detections across space and time in ducks of the Atlantic flyway over 2006-2011.

Segment	Gene type	Detection year/location[a]	Duration (years)
PB2	C-2.1	2009/NL, 2010/NL	2
	C-2.2	2008/NL, 2009/NB, 2011/NL	3
	C-2.4	2009/NL, 2010/NL	2
	C-2.5	2010/NL, 2011/NL	2
	C-2.6	2006/QC, 2006/NB, 2006/NB, 2007/NB	2
	C-2.7	2006/QC, 2006/MD, 2006/NB, 2007/MD, 2007/NB	2
	C-2.8	2007/MD, 2007/NB, 2009/NB	3
	C-2.10	2006/NB, 2007/NL	2
	C-3.2	2006/QC, 2007/ON	2
	C-3.3	2006/QC, 2007/FL	2
	C-3.5	2009/PEI, 2009/NS, 2009/QC	1
	C-4.1	2007/NB, 2007/PEI	1
PB1	F-1.1	2010/NL, 2010/NL, 2011/NL	2
	F-3.1	2009/NL, 2010/NL	2
	F-3.2	2006/PEI, 2007/NL, 2008/NL	3
	F-3.5	2006/NB, 2007/NB	2

	F-3.6	2007/PEI, 2009/PEI	3
	F-3.8	2006/QC, 2007/FL	2
	F-3.12	2009/PEI, 2009/NB	1
	F-4.5	2007/NB, 2009/NS, 2009/NB	3
	F-5.1	2006/NB, 2006/NY, 2006/MD, 2007/MD	2
	F-8.1	2009/QC, 2009/NB	1
PA	E-1.1	2009/NL, 2010/NL	2
	E-1.5	2006/NY, 2006/PA	1
	E-2.1	2008/NL, 2009/PEI, 2009/NS, 2009/NB, 2011/NL	4
	E-3.1	2006/QC, 2006/PEI	1
	E-5.1	2006/QC, 2006/NY, 2006/NB	1
	H-1.1	2006/PEI, 2008/NL	3
	H-1.5	2010/NL, 2011/NL	2
	H-1.15	2009/PEI, 2009/NB	1
	H-1.17	2007/NB, 2009/NB, 2009/PEI	3
	E-4.1	2006/NB, 2007/NB	2
NP	H-1.1	2009/PEI, 2009/NB, 2010/NL	2
	H-1.2	2006/QC, 2006/PEI, 2008/NL	3
	H-1.5	2006/QC, 2006/NB, 2007/NB	2
	H-1.8	2006/NB, 2007/NB	2
	H-2.1	2010/NL, 2011/NL	2

	H-2.3	2006/QC, 2007/NL	2
	H-2.4	2006/MD, 2007/NB	2
	H-4.3	2006/PEI, 2006/NY	1
	H-4.4	2006/NY, 2006/PA	1
	H-4.6	2009/PEI, 2009/NS, 2009/NB	1
	H-6.1	2006/NB, 2007/FL	2
	H-6.2	2006/QC, 2006/NB	1
M	E-1.1	2009/NL, 2010/NL	2
	E-1.3	2006/QC, 2006/MD, 2006/NB, 2006/NY, 2006/PA, 2008/NL, 2009/PEI	4
	E-1.5	2006/NY, 2007/MD, 2007/NB, 2009/NB, 2010/NL	5
	E-1.6	2010/NL, 2011/NL	2
	E-1.7	2009/NL, 2011/NL	3
	E-1.8	2006/NB, 2007/NB, 2007/PEI	2
	E-1.10	2006/NB, 2007/NB	2
	E-1.16	2006/DE, 2007/ON, 2007/FL	2
	E-1.18	2007/NB, 2009/PEI	3
	E-1.19	2009/PEI, 2009/NS, 2009/NB	1
	J-1.1	2009/QC, 2009/NB	1
NS	1D-1.1	2006/NY, 2007/NL, 2008/NL	3
	1D-1.5	2009/NL, 2010/NL	2

	1D-1.6	2006/PEI, 2007/NB, 2007/PEI	2
	1D-1.7	2006/QC, 2006/NB, 2007/FL, 2007/ON, 2009/PEI	4
	1D-1.9	2006/QC, 2006/PA, 2007/NB	2
	1D-1.10	2006/QC, 2006/MD, 2006/DE	1
	1D-1.13	2009/PEI, 2009/NS, 2009/NB	1
	1C-1.1	2009/QC, 2009/NB	1
	2B-1.6	2006/NB, 2006/PEI, 2007/NB	2
HA	3C-1.2	2006/QC, 2006/NB, 2006/PEI, 2007/NB	2
	3C-2.3	2007/NB, 2009/PEI	3
	3D-1.1	2007/NL, 2008/NL, 2010/NL	4
	3D-1.2	2009/PEI, 2009/NS, 2009/NS	1
	3D-2.1	2007/NB, 2009/PEI	3
	4A-1.4	2006/QC, 2006/NB	1
	4A-1.5	2006/PEI, 2007/NB, 2007/PEI	2
	5C-1.2	2006/MD, 2007/MD, 2007/FL	2
	5C-1.3	2006/NY, 2006/PA	1
NA	2D-1.1	2006/MD, 2007/NL, 2009/NB, 2011/NL	6
	2D-1.3	2006/NY, 2006/PA, 2006/DE	1
	6A-1.1	2006/NB, 2008/NL	3
	6A-1.2	2006/PEI, 2009/NB	4
	6A-3.2	2009/PEI, 2010/NL	2

6A-4.2	2006/QC, 2007/NB	2
8A-2.1	2008/NL, 2009/NS, 2009/NB	2
8A-2.2	2007/NB, 2009/PEI, 2009/NB	3

[a] NL, Newfoundland; QC, Quebec; MD, Maryland; NB, New Brunswick; PEI, Prince Edward Island; NY, New York; PA, Pennsylvania; DE, Delaware; ON, Ontario; FL, Florida; NS, Nova Scotia

Table S3.6. Detection of the 248 Atlantic flyway AIV gene types through years in North American flyways.

Segment	Gene type	Detection in NCBI database	Detection of the Newfoundland AIV gene types over 2007-2011	Total period	Duration (years)
PB2	C-1.1	No	2008	2008	1
	C-2.1	2005-2010	2009-2010	2005-2010	6
	C-2.2	2004-2009	2008-2011	2004-2011	8
	C-2.3	2003-2009	2010	2003-2010	8
	C-2.4	No	2009-2010	2009-2010	2
	C-2.5	2000-2010	2010-2011	2000-2011	12
	C-2.6	2003-2007	No	2003-2007	5
	C-2.7	2003-2009	No	2003-2009	7
	C-2.8	2007-2009	No	2007-2009	3
	C-2.9	2003-2006	No	2003-2006	4
	C-2.10	2002-2007	2007	2002-2007	6
	C-2.11	2005-2007	No	2005-2007	3
	C-2.12	2005-2007	No	2005-2007	3
	C-2.13	1985-2007	No	1985-2007	23
	C-2.14	2005-2006	No	2005-2006	2
	C-2.15	2006-2009	No	2006-2009	4
	C-2.16	2005-2007	No	2005-2007	3
	C-2.17	2000-2006	No	2000-2006	7
	C-2.18	2005-2009	No	2005-2009	5

	C-2.19	2004-2007	No	2004-2007	4
	C-3.1	1983-2010	2011	1983-2010	29
	C-3.2	2005-2007	No	2005-2007	3
	C-3.3	2006	No	2006	1
	C-3.4	2005-2008	No	2005-2008	4
	C-3.5	2006-2009	No	2006-2009	4
	C-4.1	2005-2007	No	2005-2007	3
	C-5.1	2003-2006	No	2003-2006	4
	J-1.1	2009	No	2009	1
	J-1.2	2005-2009	No	2005-2009	5
PB1	F-1.1	2006-2010	2010-2011	2006-2011	6
	F-1.2	2006-2009	2010	2006-2010	5
	F-1.3	2006-2009	No	2006-2009	4
	F-1.4	2004-2007	No	2004-2007	4
	F-2.1	2003-2010	2011	2003-2011	9
	F-2.2	2006	No	2006	1
	F-3.1	2006-2009	2009-2010	2006-2010	5
	F-3.2	2003-2008	2007-2008	2003-2008	6
	F-3.3	2002-2009	No	2002-2009	8
	F-3.4	2002-2008	No	2002-2008	7
	F-3.5	2003-2007	No	2003-2007	5
	F-3.6	2002-2009	No	2002-2009	8
	F-3.7	2002-2009	No	2002-2009	8
	F-3.8	2006-2008	No	2006-2008	3

	F-3.9	2002-2008	No	2002-2008	7
	F-3.10	2005-2006	No	2005-2006	2
	F-3.11	2002-2009	No	2002-2009	8
	F-3.12	2006-2009	No	2006-2009	4
	F-4.1	1999-2008	2011	1999-2011	13
	F-4.2	2007-2009	2008	2007-2009	3
	F-4.3	2009	2010	2009-2010	2
	F-4.4	2009-2010	2011	2009-2011	3
	F-4.5	2006-2009	No	2006-2009	4
	F-4.6	2006-2010	No	2006-2010	5
	F-4.7	1997-1010	No	1997-1010	14
	F-5.1	2005-2009	No	2005-2009	5
	F-5.2	2006-2009	No	2006-2009	4
	F-6.1	2005-2008	No	2005-2008	4
	F-6.2	2003-2007	No	2003-2007	5
	F-7.1	2005-2007	No	2005-2007	3
	F-8.1	2008-2009	No	2008-2009	2
PA	H-1.1	2002-2009	2010	2002-2010	9
	H-1.2	2006-2010	2008	2006-2010	5
	H-1.3	2005-2009	2009	2005-2009	5
	H-1.4	2002-2009	2010	2002-2010	9
	H-1.5	2009-2010	2010-2011	2009-2011	3
	H-1.6	2005-2009	2007	2005-2009	5
	H-1.7	2000-2006	No	2000-2006	7

H-1.8	2004-2006	No	2004-2006	3
H-1.9	2005-2010	No	2005-2010	6
H-1.10	2006-2009	No	2006-2009	4
H-1.11	1999-2009	No	1999-2009	11
H-1.12	2003-2009	No	2003-2009	7
H-1.13	2005-2007	No	2005-2007	3
H-1.14	2006-2008	No	2006-2008	3
H-1.15	1997-2010	No	1997-2010	14
H-1.16	2006-2009	No	2006-2009	4
H-1.17	2005-2009	No	2005-2009	5
E-1.1	2006-2009	2009-2010	2006-2010	5
E-1.2	2006-2010	2011	2006-2011	5
E-1.3	2006-2009	No	2006-2009	4
E-1.4	2006-2008	No	2006-2008	3
E-1.5	2005-2010	No	2005-2010	6
E-2.1	2007-2009	2008-2011	2007-2011	5
E-2.2	1998-2008	No	1998-2008	11
E-3.1	2004-2010	No	2004-2010	7
E-3.2	2002-2007	No	2002-2007	6
E-3.3	2004-2009	No	2004-2009	6
E-3.4	2006-2009	No	2006-2009	4
E-3.5	2000-2006	No	2000-2006	7
E-4.1	2005-2007	No	2005-2007	3
E-5.1	2002-2008	No	2002-2008	7

	E-5.2	2002-2007	No	2002-2007	6
	E-6.1	2006-2009	No	2006-2009	4
	E-6.2	1998-2009	No	1998-2009	12
HA	1D-1.1	2007-2009	2009	2007-2009	3
	2H-1.1	2008-2009	2011	2008-2011	4
	2H-1.2	2007	2008	2007-2008	2
	2H-1.3	2008	2008	2008	1
	2H-2.1	2006	2006	2006	1
	3C-1.1	2004-2011	2011	2004-2011	8
	3C-1.2	2005-2007	No	2005-2007	3
	3C-1.3	2006-2009	No	2006-2009	4
	3C-2.1	2005-2006	No	2005-2006	2
	3C-2.2	2002-2008	No	2002-2008	7
	3C-2.3	2007-2009	No	2007-2009	3
	3D-1.1	2007-2009	2007-2010	2007-2010	4
	3D-1.2	2008-2009	No	2008-2009	2
	3D-1.3	2005-2009	No	2005-2009	5
	3D-2.1	2007-2009	No	2007-2009	3
	4A-1.1	NO	2008	2008	1
	4A-1.2	NO	2010	2010	1
	4A-1.3	2002-2006	No	2002-2006	5
	4A-1.4	2002-2008	No	2002-2008	7
	4A-1.5	2002-2007	No	2002-2007	6
	4A-1.6	2006	No	2006	1

	4A-2.1	2008-2009	2010	2008-2010	3
	4A-3.1	2005-2009	No	2005-2009	5
	4A-3.2	2007-2009	No	2007-2009	3
	5C-1.1	NO	2009	2009	1
	5C-1.2	2004-2009	No	2004-2009	6
	5C-1.3	2004-2007	No	2004-2007	4
	5C-1.4	2004-2009	No	2004-2009	6
	5C-1.5	2004-2009	No	2004-2009	6
	6B-1.1	2008	No	2008	1
	7F-1.1	2005-2008	No	2005-2008	4
	7F-2.1	2004-2006	No	2004-2006	3
	11C-1.1	NO	2010	2010	1
	11C-1.2	2007-2009	No	2007-2009	3
	11C-2.1	2002-2006	No	2002-2006	5
	12A-1.1	NO	2011	2011	1
	13A-1.1	2009	No	2009	1
	16D-1.1	2009	No	2009	1
NA	1E-1.1	2007-2009	2009	2007-2009	3
	1E-2.1	2006-2009	No	2006-2009	4
	1E-2.2	2006-2009	No	2006-2009	4
	2D-1.1	2006-2009	2007-2011	2006-2011	6
	2D-1.2	No	2011	2011	1
	2D-1.3	2000-2009	No	2000-2009	10
	2D-2.1	2003-2006	No	2003-2006	4

2D-3.1	2001-2009	No	2001-2009	9
2G-1.1	2005-2006	No	2005-2006	2
2G-1.2	2001-2010	No	2001-2010	10
3A-1.1	1978	2010	1978-2010	33
3A-2.1	2003-2007	No	2003-2007	5
3A-2.2	2004-2008	No	2004-2008	6
3D-1.1	2009	No	2009	1
4A-1.1	2008	2008	2008	1
6A-1.1	1999-2007	2008	1999-2008	10
6A-1.2	2009	No	2009	1
6A-1.3	2005-2007	No	2005-2007	3
6A-2.1	2007-2009	2010	2007-2010	4
6A-3.1	2006-2009	2010	2006-2010	5
6A-3.2	2004-2009	2010	2004-2010	7
6A-3.3	2006	No	2006	1
6A-3.4	2004-2009	No	2004-2009	6
6A-4.1	1998-2008	No	1998-2008	11
6A-4.2	2002-2009	No	2002-2009	8
6A-4.3	2002-2007	No	2002-2007	6
8A-1.1	2005-2007	No	2005-2007	3
8A-1.2	2005-2007	No	2005-2007	3
8A-1.3	2005-2006	2010	2005-2010	6
8A-1.4	2005-2007	No	2005-2007	3
8A-2.1	2006-2009	2008	2006-2009	4

	8A-2.2	2007-2009	No	2007-2009	3
	8A-3.1	2005-2009	No	2005-2009	5
	9A-1.1	2007-2009	No	2007-2009	3
	9A-1.2	2007-2010	No	2007-2010	4
	9A-1.3	2007-2010	No	2007-2010	4
	9A-2.1	2005-2006	No	2005-2006	2
	9A-3.1	2006-2009	No	2006-2009	4
NP	F-1.1	2001-2011	No	2001-2011	11
	H-1.1	2006-2009	2010	2006-2010	5
	H-1.2	2001-2009	2008	2001-2009	9
	H-1.3	No	2010	2010	1
	H-1.4	2005-2009	2011	2005-2011	7
	H-1.5	1985-2007	No	1985-2007	23
	H-1.6	2003-2009	No	2003-2009	7
	H-1.7	1999-2009	No	1999-2009	11
	H-1.8	2006-2007	No	2006-2007	2
	H-1.9	2006-2009	No	2006-2009	4
	H-2.1	2008-2009	2010-2011	2008-2011	4
	H-2.2	2006-2007	2010	2006-2010	5
	H-2.3	2005-2008	2007	2005-2008	4
	H-2.4	2006-2010	No	2006-2010	5
	H-2.5	2005-2009	No	2005-2009	5
	H-3.1	2006-2009	2008	2006-2009	4
	H-3.2	2006-2007	2007	2006-2007	2

	H-3.3	2006-2007	No	2006-2007	2
	H-3.4	2006-2007	No	2006-2007	2
	H-4.1	2003-2009	2009	2003-2009	7
	H-4.2	2004-2009	2009	2004-2009	6
	H-4.3	2005-2007	No	2005-2007	3
	H-4.4	2003-2009	No	2003-2009	7
	H-4.5	2004-2009	No	2004-2009	6
	H-4.6	2003-2009	No	2003-2009	7
	H-4.7	2004-2009	No	2004-2009	6
	H-5.1	2009	2011	2009-2011	3
	H-5.2	2004-2006	No	2004-2006	3
	H-6.1	2001-2009	No	2001-2009	9
	H-6.2	2002-2009	No	2002-2009	8
	H-7.1	2005-2006	No	2005-2006	2
	D-1.1	1999-2009	No	1999-2009	11
M	E-1.1	2006-2010	2009-2010	2006-2010	5
	E-1.2	2006-2009	2011	2006-2011	6
	E-1.3	1985-2009	2008	1985-2009	25
	E-1.4	2006	No	2006	1
	E-1.5	1998-2009	2010	1998-2010	13
	E-1.6	2004-2010	2010-2011	2004-2011	8
	E-1.7	2005-2010	2009-2011	2005-2011	7
	E-1.8	1998-2009	No	1998-2009	12
	E-1.9	1996-2009	No	1996-2009	14

	E-1.10	1998-2009	No	1998-2009	12
	E-1.11	1996-2007	No	1996-2007	12
	E-l.12	1998-2010	2007	1998-2010	13
	E-1.13	2002-2010	No	2002-2010	9
	E-1.14	1998-2009	No	1998-2009	12
	E-1.15	1998-2007	No	1998-2007	10
	E-1.16	2001-2007	No	2001-2007	7
	E-1.17	1998-2009	No	1998-2009	12
	E-1.18	1996-2009	No	1996-2009	14
	E-1.19	1987-2009	No	1987-2009	13
	E-1.20	1991-2010	No	1991-2010	20
	E-1.21	1986-2009	No	1986-2009	24
	E-2.1	2005-2006	No	2005-2006	2
	J-1.1	2005-2009	No	2005-2009	4
NS	1D-1.1	2003-2010	2007-2008	2003-2010	8
	1D-1.2	2005-2010	2010	2005-2010	6
	1D-1.3	2005-2010	2011	2005-2011	7
	1D-1.4	1987-2010	2010	1987-2010	24
	1D-1.5	1999-2010	2009-2010	1999-2010	12
	1D-1.6	1998-2007	No	1998-2007	10
	1D-1.7	1988-2009	No	1988-2009	22
	1D-1.8	1995-2010	No	1995-2010	16
	1D-1.9	2002-2009	No	2002-2009	8
	1D-1.10	1999-2007	No	1999-2007	9

	1D-1.11	2002-2009	No	2002-2009	8
	1D-1.12	1998-2006	No	1998-2006	9
	1D-1.13	1999-2010	No	1999-2010	12
	1C-1.1	1999-2009	No	1999-2009	11
	2B-1.1	1998-2009	2009	1998-2009	12
	2B-1.2	1985-2009	2009-2011	1985-2011	27
	2B-1.3	1985-2009	2011	1985-2011	27
	2B-1.4	1998-2008	No	1998-2008	11
	2B-1.5	1998-2008	No	1998-2008	11
	2B-1.6	2000-2007	No	2000-2007	8
	2B-1.7	2002-2009	No	2002-2009	8
	2B-1.8	1991-2009	No	1991-2009	19
	2B-2.1	2004-2006	No	2004-2006	3
Total	248	1978-2011	2007-2011	1978-2011	34

Table S3.7. Detection of the 248 identified Atlantic flyway AIV gene types in the North American flyways.

Gene type	Flyway			
PB2	Atlantic	Mississippi	Central	Pacific
C-1.1	6	0	0	0
C-2.1	8	16	14	2
C-2.2	33	18	0	0
C-2.3	26	49	4	9
C-2.4	3	0	0	0
C-2.5	10	20	15	16
C-2.6	41	12	4	0
C-2.7	53	20	3	0
C-2.8	1	3	0	0
C-2.9	2	2	3	0
C-2.10	10	0	1	0
C-2.11	7	2	0	0
C-2.12	32	2	2	0
C-2.13	19	4	1	0
C-2.14	2	0	0	0
C-2.15	22	26	17	4
C-2.16	27	2	1	0
C-2.17	1	1	0	0

C-2.18	6	1	0	0
C-2.19	14	1	3	1
C-3.1	18	62	17	63
C-3.2	11	1	0	0
C-3.3	2	0	0	0
C-3.4	1	2	0	0
C-3.5	9	11	1	4
C-4.1	12	0	0	0
C-5.1	99	0	0	0
J-1.1	4	0	0	0
J-1.2	13	0	0	0
Total	492	255	86	99

PB1	Atlantic	Mississippi	Central	Pacific
F-1.1	3	1	7	2
F-1.2	4	1	2	9
F-1.3	4	6	8	5
F-1.4	36	7	0	0
F-2.1	4	47	11	21
F-2.2	1	0	0	0
F-3.1	8	39	0	0
F-3.2	39	1	0	0
F-3.3	54	45	0	0

F-3.4	16	28	0	0
F-3.5	22	6	0	0
F-3.6	54	45	0	0
F-3.7	32	41	0	0
F-3.8	1	2	1	4
F-3.9	18	35	0	0
F-3.10	128	0	0	0
F-3.11	1	1	0	0
F-3.12	1	2	3	0
F-4.1	5	25	0	0
F-4.2	2	28	1	0
F-4.3	1	2	0	0
F-4.4	1	2	4	3
F-4.5	9	3	1	0
F-4.6	9	19	15	5
F-4.7	1	5	4	24
F-5.1	35	54	6	6
F-5.2	35	53	2	4
F-6.1	24	0	0	0
F-6.2	35	4	1	0
F-7.1	4	4	0	0
F-8.1	6	0	0	0

Total	593	506	66	83
PA	Atlantic	Mississippi	Central	Pacific
H-1.1	86	23	11	24
H-1.2	1	1	7	15
H-1.3	11	1	1	1
H-1.4	77	17	9	2
H-1.5	10	1	2	0
H-1.6	4	2	0	0
H-1.7	10	0	2	0
H-1.8	131	0	0	0
H-1.9	6	12	4	30
H-1.10	2	1	0	0
H-1.11	13	5	16	12
H-1.12	16	36	11	27
H-1.13	3	1	1	1
H-1.14	27	39	4	6
H-1.15	4	7	13	16
H-1.16	6	9	4	33
H-1.17	72	7	3	4
E-1.1	6	78	16	2
E-1.2	3	27	10	8
E-1.3	1	78	16	2

E-1.4	2	1	32	0
E-1.5	14	19	6	49
E-2.1	35	24	2	0
E-2.2	18	19	2	1
E-3.1	22	49	15	0
E-3.2	52	12	0	0
E-3.3	21	48	11	0
E-3.4	1	4	4	0
E-3.5	7	6	1	1
E-4.1	12	0	0	0
E-5.1	76	6	2	0
E-5.2	47	9	0	0
E-6.1	7	0	0	0
E-6.2	4	0	0	0
Total	807	542	205	234

HA	Atlantic	Mississippi	Central	Pacific
1D-1.1	3	11	5	3
2H-1.1	2	6	0	0
2H-1.2	2	1	0	0
2H-1.3	1	0	0	1
2H-2.1	3	0	0	0
3C-1.1	6	15	3	0

3C-1.2	8	0	0	0
3C-1.3	1	7	2	0
3C-2.1	9	0	0	0
3C-2.2	5	14	0	0
3C-2.3	2	0	0	0
3D-1.1	23	0	0	0
3D-1.2	16	0	0	0
3D-1.3	7	0	0	0
3D-2.1	8	0	0	0
4A-1.1	3	0	0	0
4A-1.2	1	0	0	0
4A-1.3	9	3	1	0
4A-1.4	36	5	0	0
4A-1.5	4	1	0	0
4A-1.6	4	0	0	0
4A-2.1	2	2	1	2
4A-3.1	1	0	1	0
4A-3.2	7	93	43	1
5C-1.1	4	3	0	2
5C-1.2	63	37	5	0
5C-1.3	15	8	1	0
5C-1.4	44	15	1	0

5C-1.5	47	38	5	0
6B-1.1	3	0	0	0
7F-1.1	1	0	0	0
7F-2.1	76	0	0	0
11C-1.1	2	0	0	0
11C-1.2	1	10	0	0
11C-2.1	16	4	0	0
12A-1.1	1	0	0	0
13A-1.1	4	0	0	0
16D-1.1	1	0	0	0
Total	441	273	68	9

NP	Atlantic	Mississippi	Central	Pacific
F-1.1	3	1	1	75
H-1.1	8	14	17	4
H-1.2	42	34	30	11
H-1.3	2	0	0	0
H-1.4	14	23	9	13
H-1.5	32	1	0	1
H-1.6	4	8	3	24
H-1.7	21	16	18	2
H-1.8	4	0	0	0
H-1.9	2	4	0	0

H-2.1	6	1	0	3
H-2.2	4	10	6	2
H-2.3	15	4	4	7
H-2.4	4	12	7	7
H-2.5	9	12	4	31
H-3.1	7	2	0	1
H-3.2	2	1	0	1
H-3.3	2	2	0	1
H-3.4	5	1	0	1
H-4.1	7	7	0	0
H-4.2	8	1	0	0
H-4.3	30	3	1	0
H-4.4	21	10	0	0
H-4.5	21	12	0	0
H-4.6	17	3	0	0
H-4.7	29	11	0	0
H-5.1	2	1	0	0
H-5.2	18	3	0	0
H-6.1	34	29	5	5
H-6.2	42	43	6	19
H-7.1	94	0	0	0
D-1.1	7	0	0	0

Total	516	269	111	208
NA	Atlantic	Mississippi	Central	Pacific
1E-1.1	5	31	1	7
1E- 2.1	7	16	6	30
1E- 2.2	7	15	6	21
2D-1.1	5	19	1	18
2D-1.2	1	0	0	0
2D-1.3	17	16	0	0
2D-2.1	10	0	0	0
2D-3.1	39	34	4	0
2G-1.1	98	0	0	0
2G-1.2	10	31	1	2
3A-1.1	1	0	1	0
3A-2.1	6	0	0	0
3A-2.2	10	4	2	15
3D-1.1	1	0	0	0
4A-1.1	5	0	0	0
6A-1.1	14	1	0	0
6A-1.2	4	0	0	0
6A-1.3	10	0	0	0
6A-2.1	23	0	0	0
6A-3.1	3	1	4	0

6A-3.2	22	8	27	19
6A-3.3	4	2	0	0
6A-3.4	8	6	19	15
6A-4.1	16	7	5	0
6A-4.2	16	7	1	0
6A-4.3	5	6	0	0
8A-1.1	8	0	0	0
8A-1.2	8	0	0	0
8A-1.3	4	0	0	0
8A-1.4	7	0	0	0
8A-2.1	10	8	0	0
8A-2.2	10	0	0	0
8A-3.1	3	18	12	2
9A-1.1	4	12	0	0
9A-1.2	4	20	0	0
9A-1.3	4	22	0	0
9A-2.1	6	0	0	0
9A-3.1	1	1	0	0
Total	416	285	90	129

M	Atlantic	Mississippi	Central	Pacific
E-1.1	9	16	12	14
E-1.2	6	7	1	3

E-1.3	115	23	2	25
E-1.4	3	0	0	0
E-1.5	135	80	16	22
E-1.6	7	20	9	40
E-1.7	11	57	16	109
E-1.8	33	46	20	5
E-1.9	138	78	15	19
E-1.10	115	87	22	26
E-1.11	11	9	2	3
E-l.12	121	76	27	28
E-1.13	12	52	34	143
E-1.14	59	52	16	24
E-1.15	6	1	2	4
E-1.16	6	6	6	0
E-1.17	102	81	24	18
E-1.18	69	15	10	6
E-1.19	21	1	9	18
E-1.20	2	17	13	16
E-1.21	11	2	7	0
E-2.1	107	0	0	0
J-1.1	10	0	0	0
Total	1109	726	263	523

NS	Atlantic	Mississippi	Central	Pacific
1D-1.1	52	10	6	7
1D-1.2	3	31	6	17
1D-1.3	1	12	3	3
1D-1.4	60	35	9	16
1D-1.5	20	15	6	23
1D-1.6	30	26	3	0
1D-1.7	90	52	10	8
1D-1.8	91	54	13	8
1D-1.9	44	11	7	8
1D-1.10	10	7	8	26
1D-1.11	13	11	0	2
1D-1.12	2	6	1	0
1D-1.13	12	18	6	37
1C-1.1	6	0	0	0
2B-1.1	8	15	28	0
2B-1.2	22	27	36	10
2B-1.3	15	16	4	38
2B-1.4	6	17	0	0
2B-1.5	9	14	2	38
2B-1.6	10	7	2	37
2B-1.7	14	4	0	0

2B-1.8	7	4	14	25
2B-2.1	97	0	0	0
Total	622	392	164	303

Table S3.8. Summary of detections of the Atlantic Flyway duck AIV genes in non-duck hosts.

Sub-lineage	Number of gene types	Gene types found outside ducks
PB2		
C-1	1	ND[a]
C-2	19	C-2.2, 2.3, 2.5, 2.7, 2.12, 2.13, 2.15, 2.16, 2.19
C-3	5	C-3.1, 3.5
C-4	1	C-4.1
C-5	1	C-5.1
J-1	2	J-1.1
Total	29	14
PB1		
F-1	4	F-1.3, 1.4
F-2	2	F-2.1
F-3	12	F-3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.9, 3.10
F-4	7	F-4.5, 4.7
F-5	2	F-5.1, 5.2
F-6	2	F-6.1, 6.2
F-7	1	ND
F-8	1	F-8.1
Total	31	18

PA		
H-1	17	H-1.1, 1.5, 1.8, 1.9, 1.11, 1.12, 1.13, 1.14, 1.16, 1.17
E-1	5	E-1.1, 1.4, 1.5
E-2	2	E-2.1, 2.2
E-3	5	E-3.1, 3.2, 3.3
E-4	1	ND
E-5	2	E-5.1, 5.2
E-6	2	E-6.1, 6.2
Total	34	22
HA		
1D-1	1	1D-1.1
2H-1	3	2H-1.2
2H-2	1	ND
3C-1	3	3C-1.2
3C-2	3	ND
3D-1	3	3D-1.1
3D-2	1	ND
4A-1	6	4A-1.1, 1.4
4A-2	1	ND
4A-3	2	4A-3.2
5C-1	5	5C-1.2, 1.3, 1.4, 1.5

6B-1	1	6B-1.1
7F-1	1	ND
7F-2	1	7F-2.1
11C-1	2	ND
11C-2	1	ND
12A-1	1	ND
13A-1	1	13A-1.1
16D-1	1	ND
Total	38	14
NP		
F-1	1	F-1.1
H-1	9	H-1.2, 1.4, 1.5, 1.6, 1.7
H-2	5	H-2.3, 2.4, 2.5
H-3	4	H-3.1, 3.4
H-4	7	H-4.3, 4.4, 4.5, 4.6
H-5	2	H-5.2
H-6	2	H-6.2
H-7	1	H-7.1
D-1	1	D-1.1
Total	32	19
NA		
1E-1	1	ND

1E-2	2	1E -2.1, 2.2
2D-1	3	2D-1.1, 1.3
2D-2	1	2D-2.1
2D-3	1	2D-3.1
2G-1	2	2G-1.1, 1.2
3A-1	1	ND
3A-2	2	3A-2.1, 2.2
3D-1	1	ND
4A-1	1	4A-1.1
6A-1	3	6A-1.1, 1.2
6A-2	1	ND
6A-3	4	6A-3.2
6A-4	3	ND
8A-1	4	ND
8A-2	2	ND
8A-3	1	ND
9A-1	3	ND
9A-2	1	ND
9A-3	1	ND
Total	38	14
M		
E-1	21	E-1.1, 1.2, 1.3,1.5 to 1.21

E-2	1	E-2.1
J-1	1	E-3.1
Total	23	22
NS		
1D-1	13	1D-1.1, 1.2, 1.4, 1.5, 1.7, 1.8, 1.9, 1.10, 1.12
1C-1	1	1C-1.1
2B-1	8	2B-1.1 to 1.8
2B-2	1	2B-2.1
Total	23	18
Overall totals	248	142

[a] Not detected

Table S3.9. Identification of Atlantic Flyway duck AIV gene types in hosts other than ducks.

Segment	Gene type	Hosts
PB2	C-2.2	Shorebird
	C-2.3	shorebird, goose, turkey, chicken
	C-2.5	Goose
	C-2.7	Shorebird
	C-2.12	Chicken
	C-2.13	Shorebird
	C-2.15	turkey, quail
	C-2.16	Chicken
	C-2.19	Shorebird
	C-3.1	shorebird, goose, turkey, swan
	C-3.5	Shorebird
	C-4.1	Murre
	C-5.1	chicken, guinea fowl, pheasant
	J-1.1	Gull
PB1	F-1.3	shorebird, goose
	F-1.4	shorebird, chicken, gull
	F-2.1	turkey, dove
	F-3.2	Shorebird
	F-3.3	Shorebird

	F-3.4	Shorebird
	F-3.5	Shorebird
	F-3.6	Shorebird
	F-3.7	Shorebird
	F-3.9	shorebird, turkey
	F-3.10	turkey, chicken, guinea fowl, pheasant, quail
	F-4.5	Goose
	F-4.7	goose, grebe
	F-5.1	shorebird, turkey, quail
	F-5.2	shorebird, turkey, quail
	F-6.1	shorebird, swan
	F-6.2	turkey, swan
	F-8.1	Gull
PA	H-1.1	shorebird, chicken, gull, murre
	H-1.4	shorebird, gull
	H-1.8	turkey, chicken, guinea fowl, pheasant, quail
	H-1.9	goose, swan
	H-1.11	Shorebird
	H-1.12	goose, swan
	H-1.13	Goose
	H-1.14	shorebird, goose
	H-1.16	Goose

	H-1.17	Shorebird
	E-1.1	Dove
	E-1.4	goose, chicken, coot
	E-1.5	goose, chicken
	E-2.1	Shorebird
	E-2.2	shorebird, goose
	E-3.1	shorebird, gull, murre
	E-3.2	shorebird, turkey
	E-3.4	Shorebird
	E-5.1	shorebird, turkey, quail
	E-5.2	shorebird, turkey
	E-6.1	Gull
	E-6.2	shorebird, gull
HA	1D-1.1	dove, dunlin
	2H-1.2	Chicken
	3C-1.2	Shorebird
	3D-1.1	Shorebird
	4A-1.1	Shorebird
	4A-1.4	Shorebird
	4A-3.2	Shorebird
	5C-1.2	goose, turkey, swan
	5C-1.3	Chicken

	5C-1.4	goose, turkey, swan
	5C-1.5	goose, turkey
	6B-1.1	Shorebird
	7F-2.1	turkey, chicken, guinea fowl, quail
	13A-1.1	Gull
NA	1E- 2.1	shorebird, goose
	1E- 2.2	shorebird, goose
	2D-1.1	Dove
	2D-1.3	goose, chicken
	2D-2.1	Shorebird
	2D-3.1	shorebird, goose, turkey
	2G-1.1	turkey, chicken, guinea fowl, pheasant, chukar
	2G-1.2	shorebird, goose, turkey
	3A-2.1	Shorebird
	3A-2.2	Chicken
	4A-1.1	Shorebird
	6A-1.1	Swine
	6A-1.2	Gull
	6A-3.2	Shorebird
NP	F-1.1	Swine
	H-1.2	shorebird, goose, turkey, chicken, chukar
	H-1.4	shorebird, goose, chicken

	H-1.5	shorebird, goose
	H-1.6	goose, chicken, swan
	H-1.7	shorebird, goose, turkey, chicken
	H-2.3	Shorebird
	H-2.4	goose, dove
	H-2.5	shorebird, goose, swan
	H-3.1	Shorebird
	H-3.4	Shorebird
	H-4.3	turkey, quail
	H-4.4	Chicken
	H-4.5	Chicken
	H-4.6	Chicken
	H-5.2	Shorebird
	H-6.2	shorebird, grebe
	H-7.1	chicken, guinea fowl, pheasant
	D-1.1	shorebird, gull
M	E-1.1	Dove
	E-1.2	Cormorant
	E-1.3	shorebird, turkey, chicken
	E-1.5	goose, chicken
	E-1.6	shorebird, turkey, gull, grebe, cormorant
	E-1.7	shorebird, turkey, cormorant

	E-1.8	shorebird, goose, murre, swine
	E-1.9	shorebird, turkey, chicken, gull, quail, swine
	E-1.10	shorebird, turkey, murre
	E-1.11	shorebird, chicken, pheasant
	E-1.13	shorebird, goose, turkey, grebe, cormorant
	E-l.12	shorebird, chicken, murre, swine
	E-1.14	shorebird, swine
	E-1.15	Shorebird
	E-1.16	turkey, swan, dunlin
	E-1.17	shorebird, turkey, quail, murre, swine
	E-1.18	shorebird, goose
	E-1.19	shorebird, turkey, chicken, guinea fowl, rhea
	E-1.20	Goose
	E-1.21	turkey, chicken
	E-2.1	turkey, chicken, guinea fowl, pheasant, quail
	J-1.1	shorebird, gull
NS	1D-1.1	shorebird, turkey, chicken, coot
	1D-1.2	goose, coot
	1D-1.4	shorebird, chicken
	1D-1.5	goose, chicken
	1D-1.7	shorebird, goose, turkey, swan, swine
	1D-1.8	shorebird, goose, turkey, guinea fowl, swine

1D-1.9	shorebird, goose, turkey, chicken
1D-1.10	Shorebird
1D-1.12	Shorebird
1C-1.1	Gull
2B-1.1	Gull
2B-1.2	shorebird, goose, murre
2B-1.3	shorebird, gull
2B-1.4	Gull
2B-1.5	Gull
2B-1.6	goose, guinea fowl, murre
2B-1.7	Shorebird
2B-1.8	shorebird, swan
2B-2.1	turkey, chicken, guinea fowl, pheasant, quail

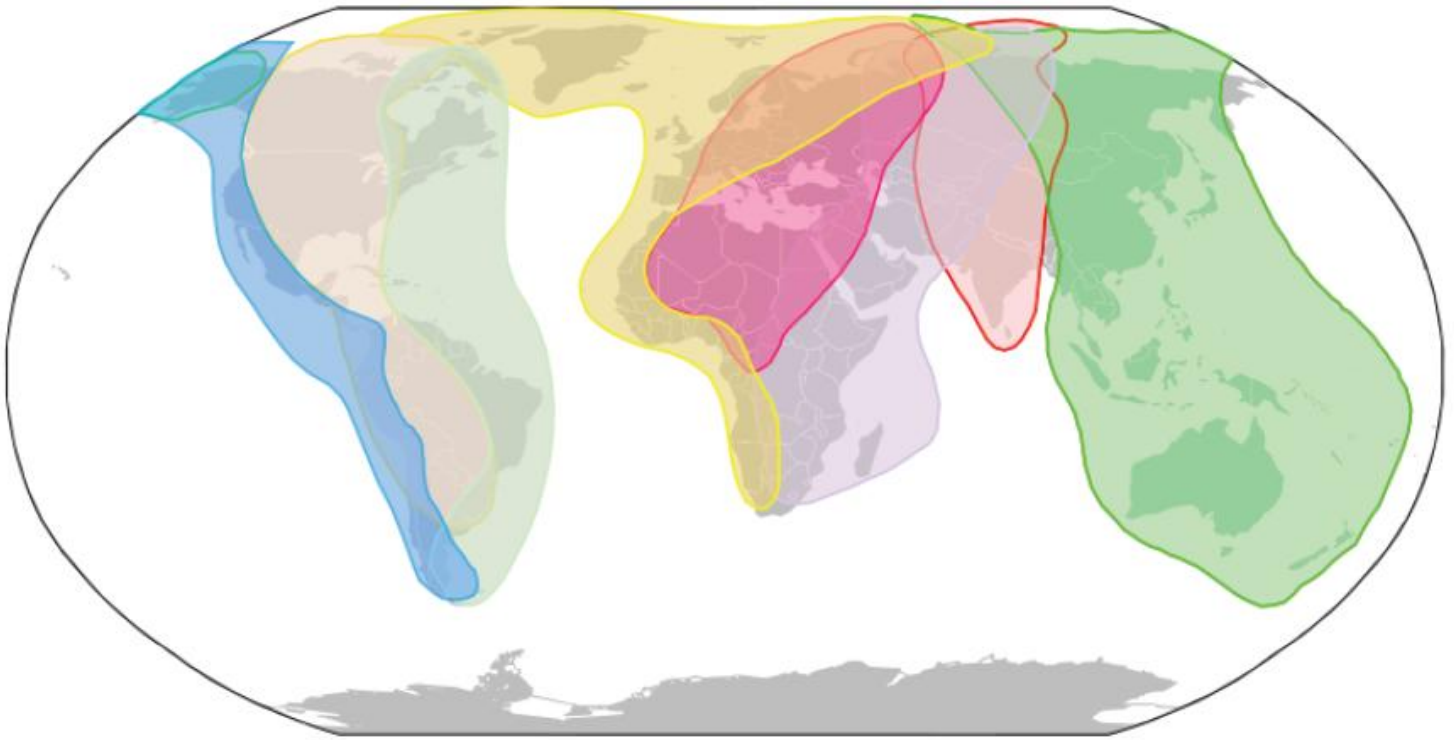
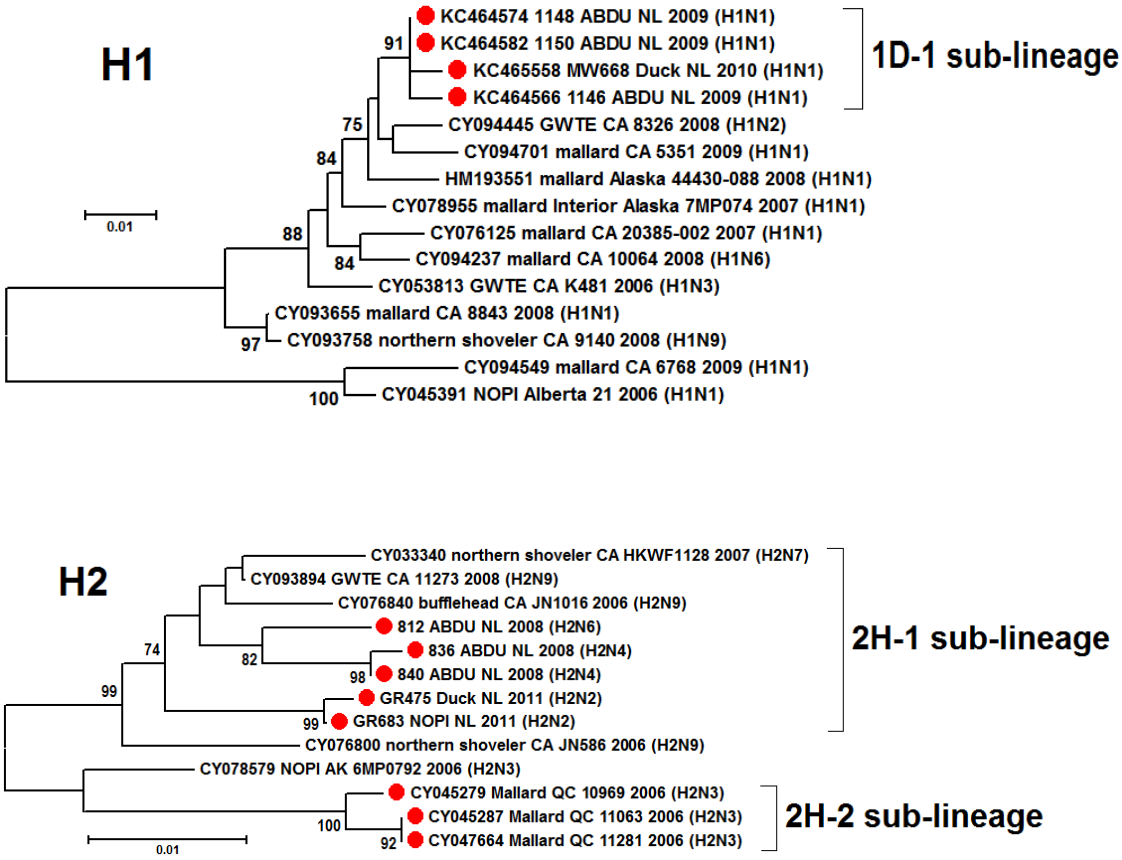
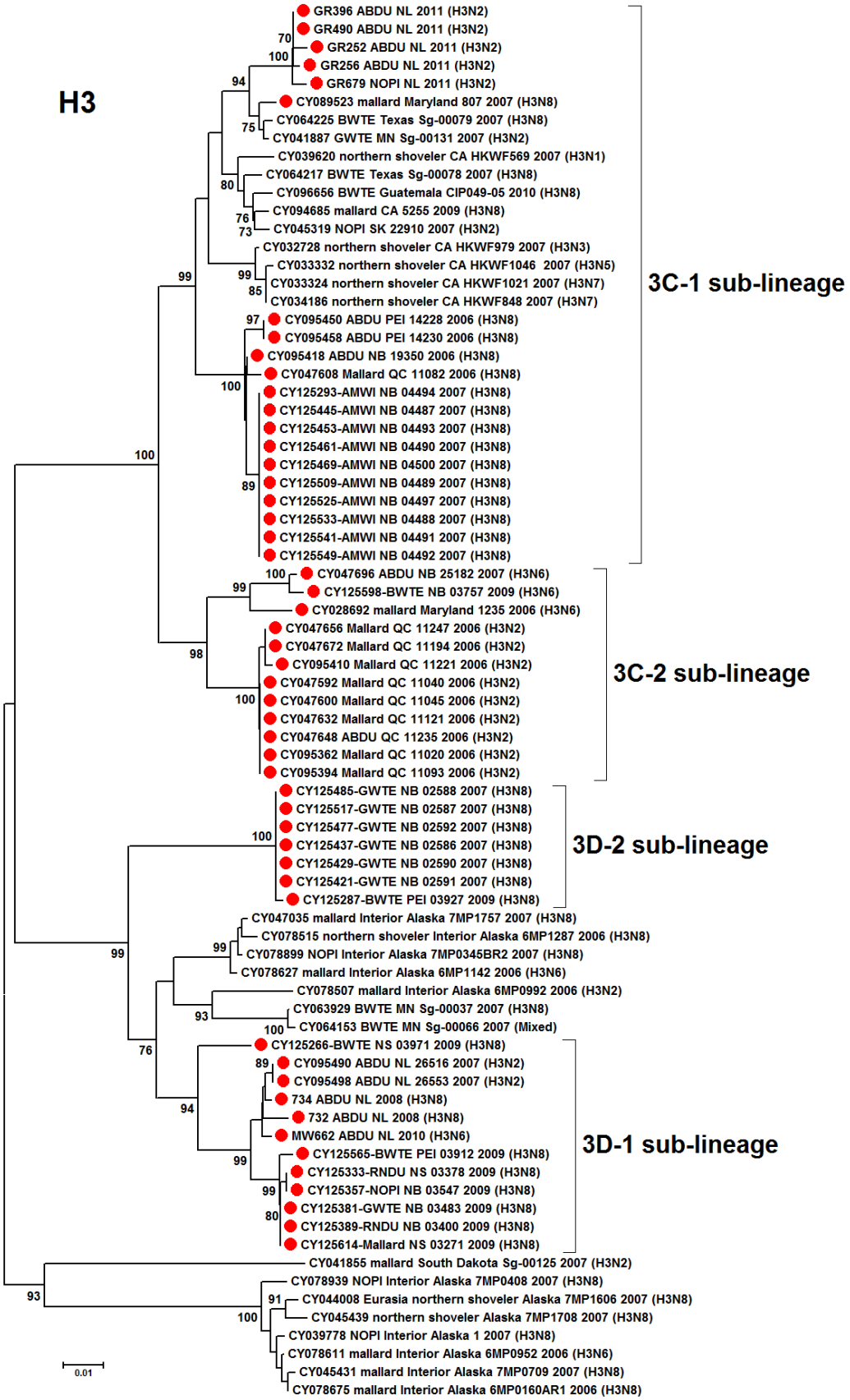


Figure S3. 1. Major international bird migratory flyways. The routes of major migration flyways are illustrated in different colors. Among these flyways, three in North America are illustrated: the Atlantic flyway (AF) in light green, the Mississippi flyway (MF) in grey, and the Pacific Flyway (PF) in blue. The picture is downloaded under permission from Wikimedia Commons.

Figure S3. 2. Phylogenetic analysis of the 8 segments of the 2006-2011 Atlantic flyway duck AIVs (starting from next page). Phylogenetic trees are shown for each segment of the 109 Atlantic flyway duck AIVs (indicated with red circles), with separate trees for the different HA and NA subtypes. The sub-lineages (≥95% nucleotide identity within a sub-lineage) of the AIV genes from the Atlantic flyway are labelled on the right. The neighbour-joining trees were constructed with MEGA5 and support values based on 1000 bootstrap replicates are shown as percentages where ≥70%. The scale bars indicate nucleotide substitutions per site. The full identification information for the Atlantic flyway viruses is provided in Table S2. Abbreviations: ABDU, American Black Duck; RNDU, Ring-necked Duck; GWTE, Green-winged Teal; BWTE, Blue-winged Teal; AMWI, American Widgeon; NOPI, Northern Pintail; NL, Newfoundland; QC, Quebec; NS, Nova Scotia; NB, New Brunswick; NY, New York; PEI, Prince Edward Island; NS, Nova Scotia; PA, Pennsylvania; DE, Delaware; MD, Maryland; FL, Florida; AK, Alaska; CA, California; MN, Minnesota; MS, Missouri; SK, Saskatchewan.


H1
KC464574 1148 ABDU NL 2009 (H1N1)
KC464582 1150 ABDU NL 2009 (H1N1)
KC465558 MW668 Duck NL 2010 (H1N1)
KC464566 1146 ABDU NL 2009 (H1N1)
1D-1 sub-lineage
CY094445 GWTE CA 8326 2008 (H1N2)
CY094701 mallard CA 5351 2009 (H1N1)
HM193551 mallard Alaska 44430-088 2008 (H1N1)
CY078955 mallard Interior Alaska 7MP074 2007 (H1N1)
CY076125 mallard CA 20385-002 2007 (H1N1)
CY094237 mallard CA 10064 2008 (H1N6)
CY053813 GWTE CA K481 2006 (H1N3)
CY093655 mallard CA 8843 2008 (H1N1)
CY093758 northern shoveler CA 9140 2008 (H1N9)
CY094549 mallard CA 6768 2009 (H1N1)
CY045391 NOPI Alberta 21 2006 (H1N1)
91
75
84
88
84
97
100
0.01
H2
CY033340 northern shoveler CA HKWF1128 2007 (H2N7)
CY093894 GWTE CA 11273 2008 (H2N9)
CY076840 bufflehead CA JN1016 2006 (H2N9)
812 ABDU NL 2008 (H2N6)
836 ABDU NL 2008 (H2N4)
840 ABDU NL 2008 (H2N4)
GR475 Duck NL 2011 (H2N2)
GR683 NOPI NL 2011 (H2N2)
2H-1 sub-lineage
CY076800 northern shoveler CA JN586 2006 (H2N9)
CY078579 NOPI AK 6MP0792 2006 (H2N3)
CY045279 Mallard QC 10969 2006 (H2N3)
CY045287 Mallard QC 11063 2006 (H2N3)
CY047664 Mallard QC 11281 2006 (H2N3)
2H-2 sub-lineage
74
82
98
99
99
100
92
0.01


H3
GR396 ABDU NL 2011 (H3N2)
GR490 ABDU NL 2011 (H3N2)
GR252 ABDU NL 2011 (H3N2)
GR256 ABDU NL 2011 (H3N2)
GR679 NOPI NL 2011 (H3N2)
CY089523 mallard Maryland 807 2007 (H3N8)
CY064225 BWTE Texas Sg-00079 2007 (H3N8)
CY041887 GWTE MN Sg-00131 2007 (H3N2)
CY039620 northern shoveler CA HKWF569 2007 (H3N1)
CY064217 BWTE Texas Sg-00078 2007 (H3N8)
CY096656 BWTE Guatemala CIP049-05 2010 (H3N8)
CY094685 mallard CA 5255 2009 (H3N8)
CY045319 NOPI SK 22910 2007 (H3N2)
CY032728 northern shoveler CA HKWF979 2007 (H3N3)
CY033332 northern shoveler CA HKWF1046 2007 (H3N5)
CY033324 northern shoveler CA HKWF1021 2007 (H3N7)
CY034186 northern shoveler CA HKWF848 2007 (H3N7)
CY095450 ABDU PEI 14228 2006 (H3N8)
CY095458 ABDU PEI 14230 2006 (H3N8)
CY095418 ABDU NB 19350 2006 (H3N8)
CY047608 Mallard QC 11082 2006 (H3N8)
CY125293-AMWI NB 04494 2007 (H3N8)
CY125445-AMWI NB 04487 2007 (H3N8)
CY125453-AMWI NB 04493 2007 (H3N8)
CY125461-AMWI NB 04490 2007 (H3N8)
CY125469-AMWI NB 04500 2007 (H3N8)
CY125509-AMWI NB 04489 2007 (H3N8)
CY125525-AMWI NB 04497 2007 (H3N8)
CY125533-AMWI NB 04488 2007 (H3N8)
CY125541-AMWI NB 04491 2007 (H3N8)
CY125549-AMWI NB 04492 2007 (H3N8)
3C-1 sub-lineage
CY047696 ABDU NB 25182 2007 (H3N6)
CY125598-BWTE NB 03757 2009 (H3N6)
CY028692 mallard Maryland 1235 2006 (H3N6)
CY047656 Mallard QC 11247 2006 (H3N2)
CY047672 Mallard QC 11194 2006 (H3N2)
CY095410 Mallard QC 11221 2006 (H3N2)
CY047592 Mallard QC 11040 2006 (H3N2)
CY047600 Mallard QC 11045 2006 (H3N2)
CY047632 Mallard QC 11121 2006 (H3N2)
CY047648 ABDU QC 11235 2006 (H3N2)
CY095362 Mallard QC 11020 2006 (H3N2)
CY095394 Mallard QC 11093 2006 (H3N2)
3C-2 sub-lineage
CY125485-GWTE NB 02588 2007 (H3N8)
CY125517-GWTE NB 02587 2007 (H3N8)
CY125477-GWTE NB 02592 2007 (H3N8)
CY125437-GWTE NB 02586 2007 (H3N8)
CY125429-GWTE NB 02590 2007 (H3N8)
CY125421-GWTE NB 02591 2007 (H3N8)
CY125287-BWTE PEI 03927 2009 (H3N8)
3D-2 sub-lineage
CY047035 mallard Interior Alaska 7MP1757 2007 (H3N8)
CY078515 northern shoveler Interior Alaska 6MP1287 2006 (H3N8)
CY078899 NOPI Interior Alaska 7MP0345BR2 2007 (H3N8)
CY078627 mallard Interior Alaska 6MP1142 2006 (H3N6)
CY078507 mallard Interior Alaska 6MP0992 2006 (H3N2)
CY063929 BWTE MN Sg-00037 2007 (H3N8)
CY064153 BWTE MN Sg-00066 2007 (Mixed)
CY125266-BWTE NS 03971 2009 (H3N8)
CY095490 ABDU NL 26516 2007 (H3N2)
CY095498 ABDU NL 26553 2007 (H3N2)
734 ABDU NL 2008 (H3N8)
732 ABDU NL 2008 (H3N8)
MW662 ABDU NL 2010 (H3N6)
CY125565-BWTE PEI 03912 2009 (H3N8)
CY125333-RNDU NS 03378 2009 (H3N8)
CY125357-NOPI NB 03547 2009 (H3N8)
CY125381-GWTE NB 03483 2009 (H3N8)
CY125389-RNDU NB 03400 2009 (H3N8)
CY125614-Mallard NS 03271 2009 (H3N8)
3D-1 sub-lineage
CY041855 mallard South Dakota Sg-00125 2007 (H3N2)
CY078939 NOPI Interior Alaska 7MP0408 2007 (H3N8)
CY044008 Eurasia northern shoveler Alaska 7MP1606 2007 (H3N8)
CY045439 northern shoveler Alaska 7MP1708 2007 (H3N8)
CY039778 NOPI Interior Alaska 1 2007 (H3N8)
CY078611 mallard Interior Alaska 6MP0952 2006 (H3N6)
CY045431 mallard Interior Alaska 7MP0709 2007 (H3N8)
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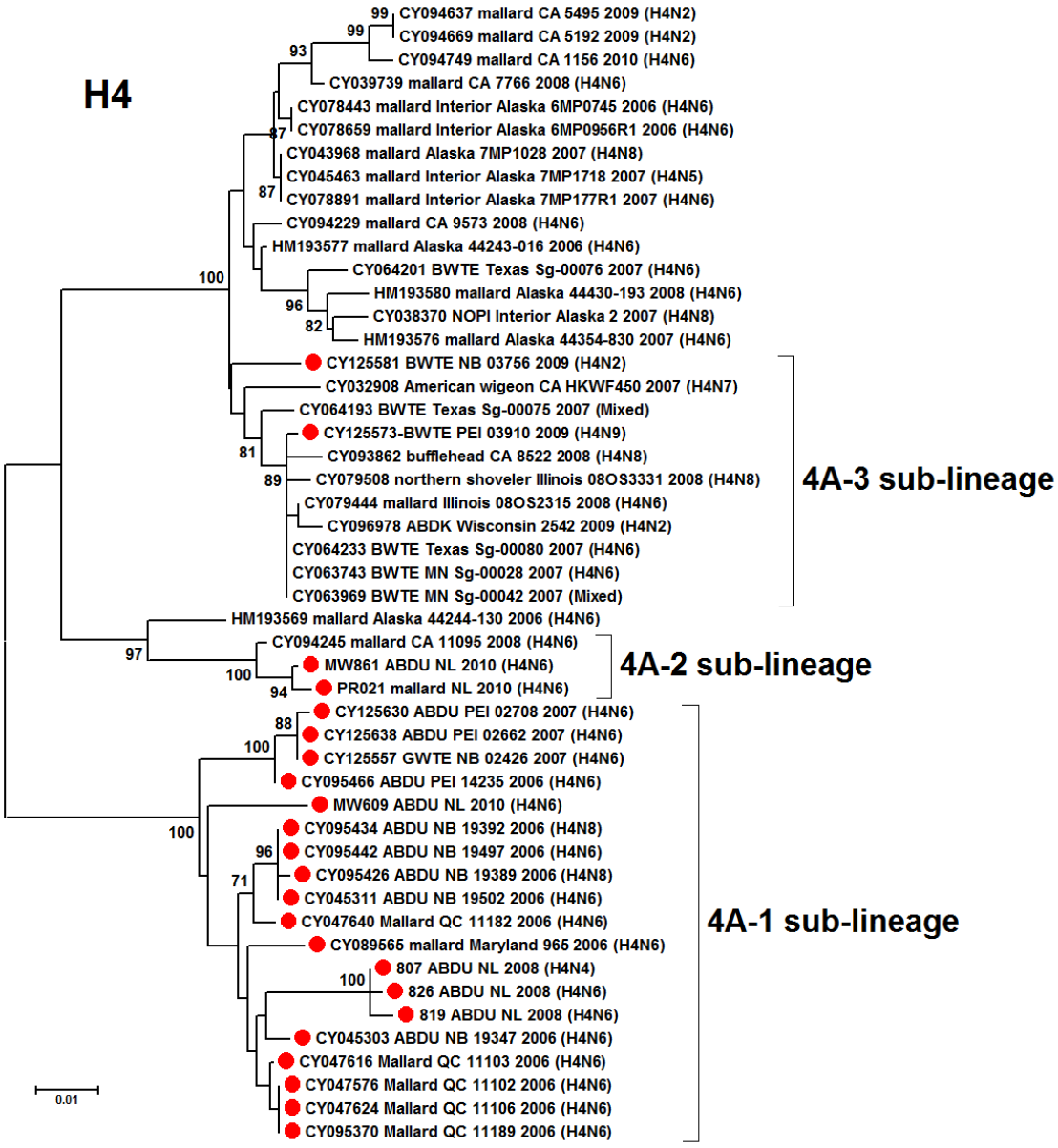
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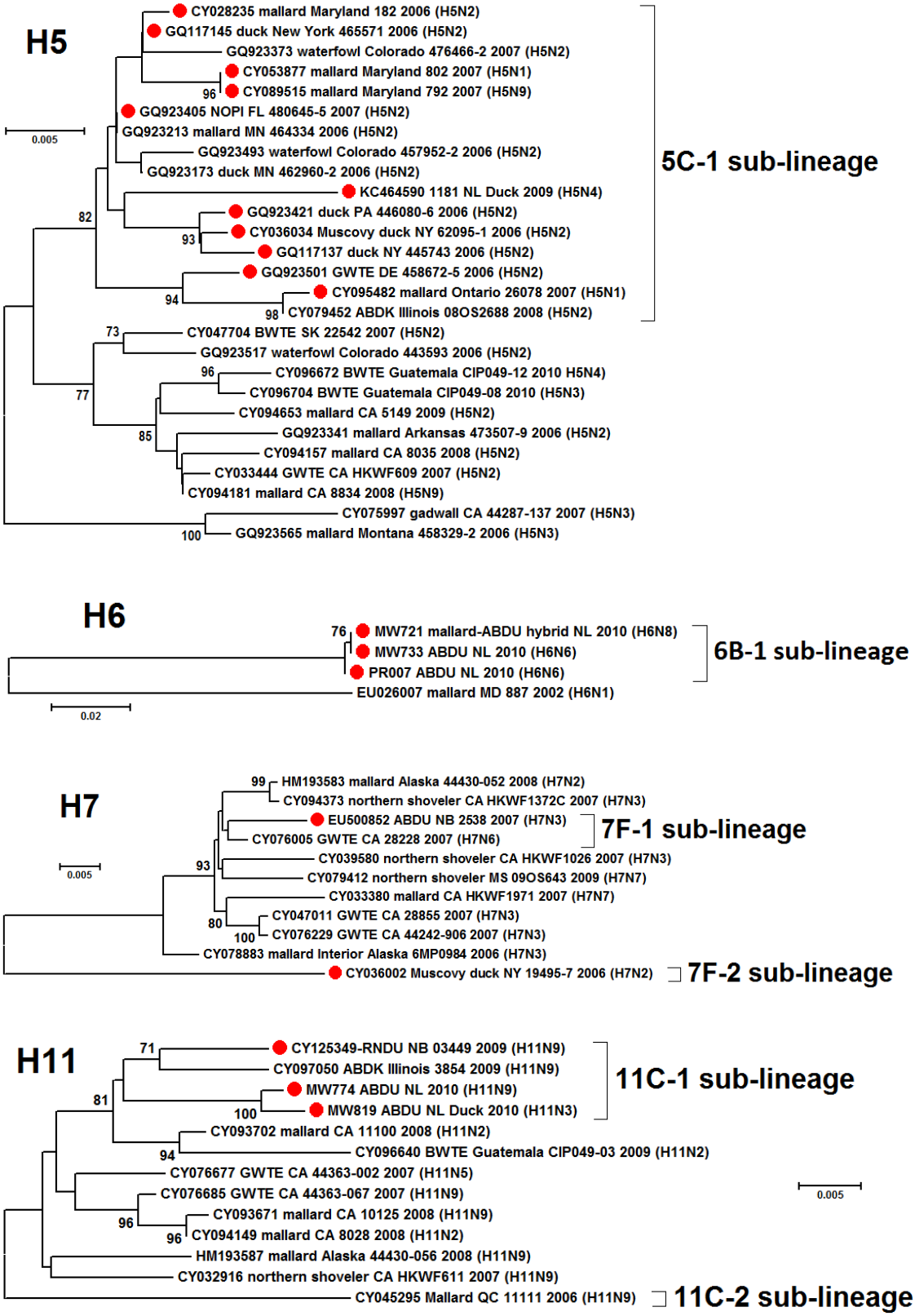
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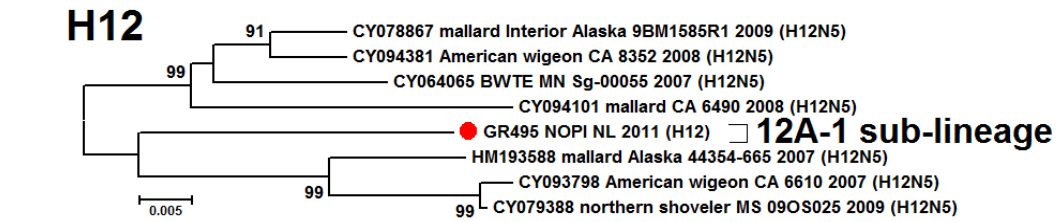
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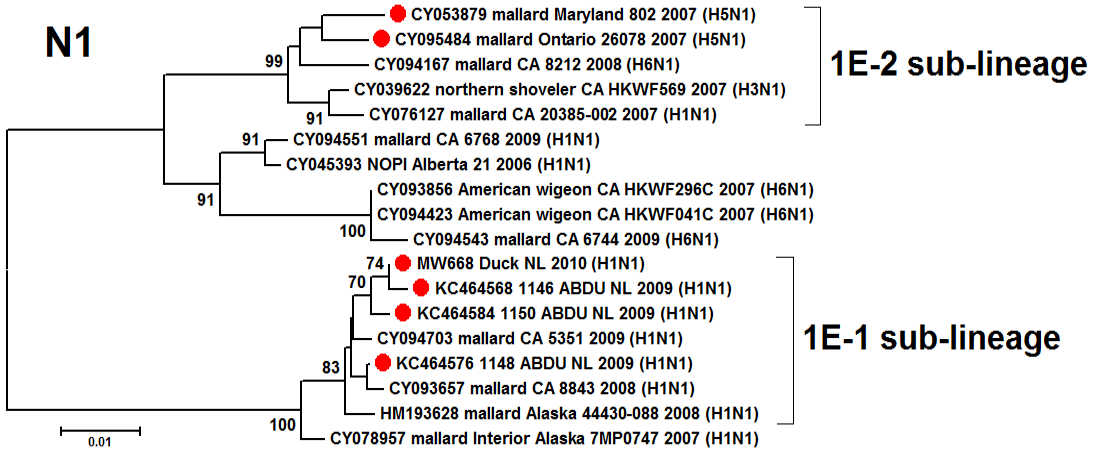
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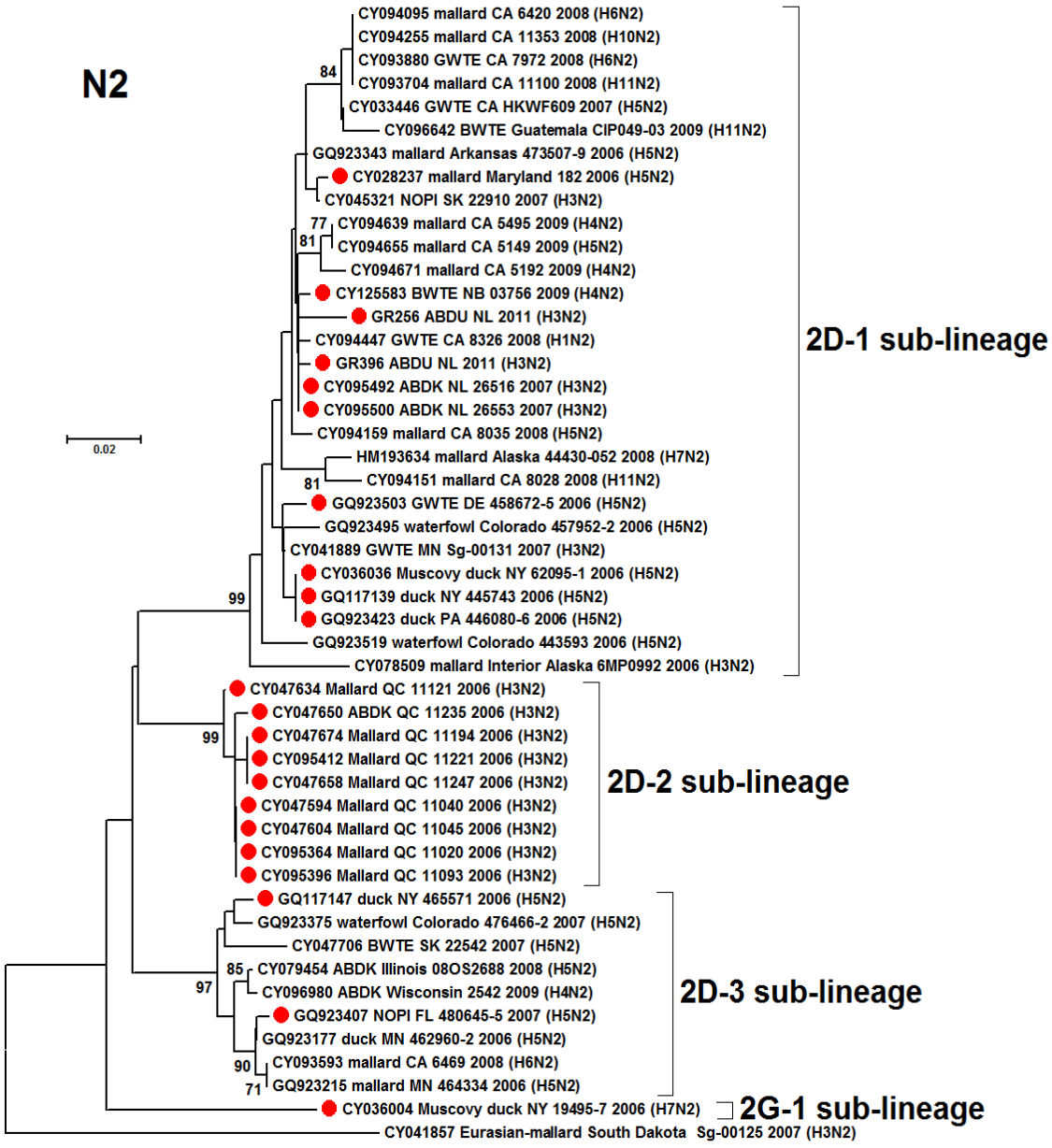
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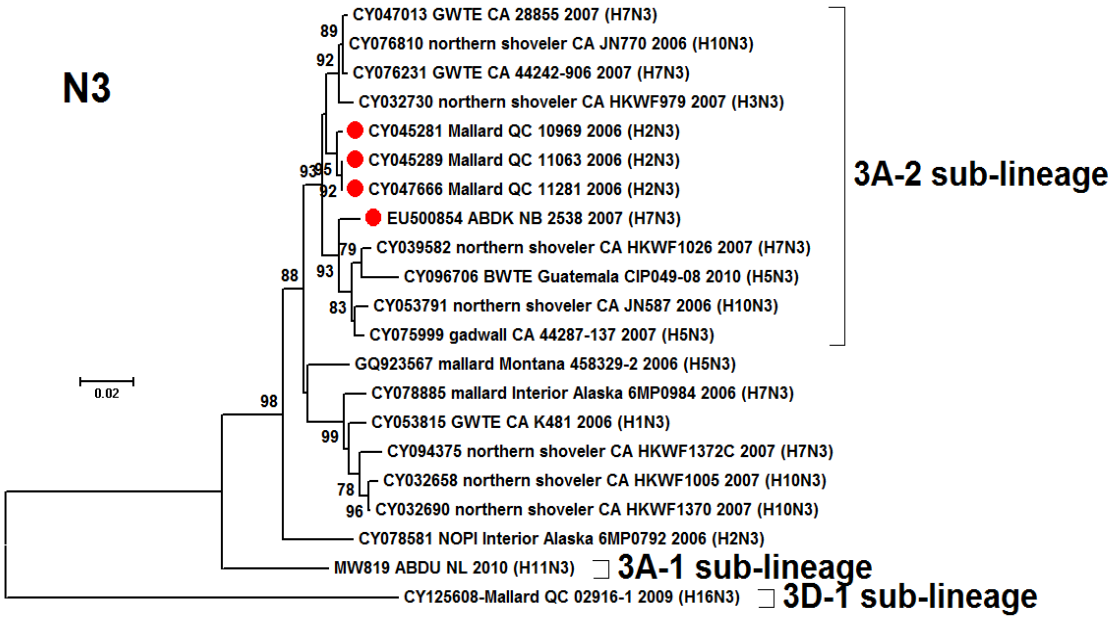
2D-2 sub-lineage
2D-3 sub-lineage
2G-1 sub-lineage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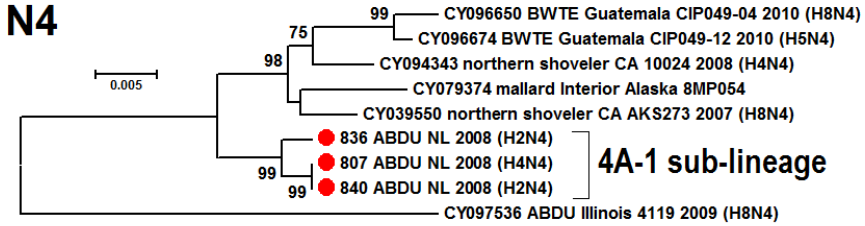
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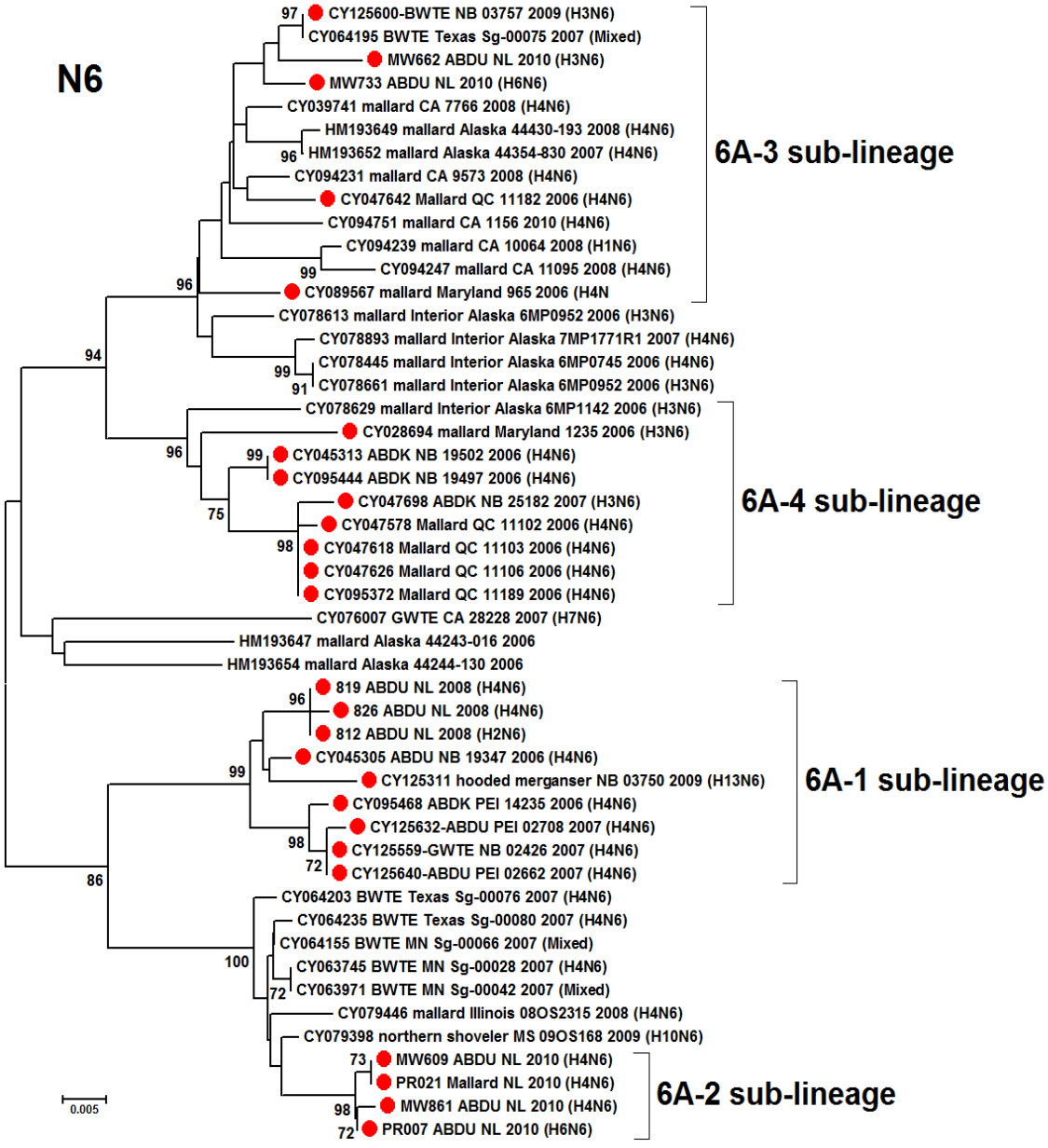
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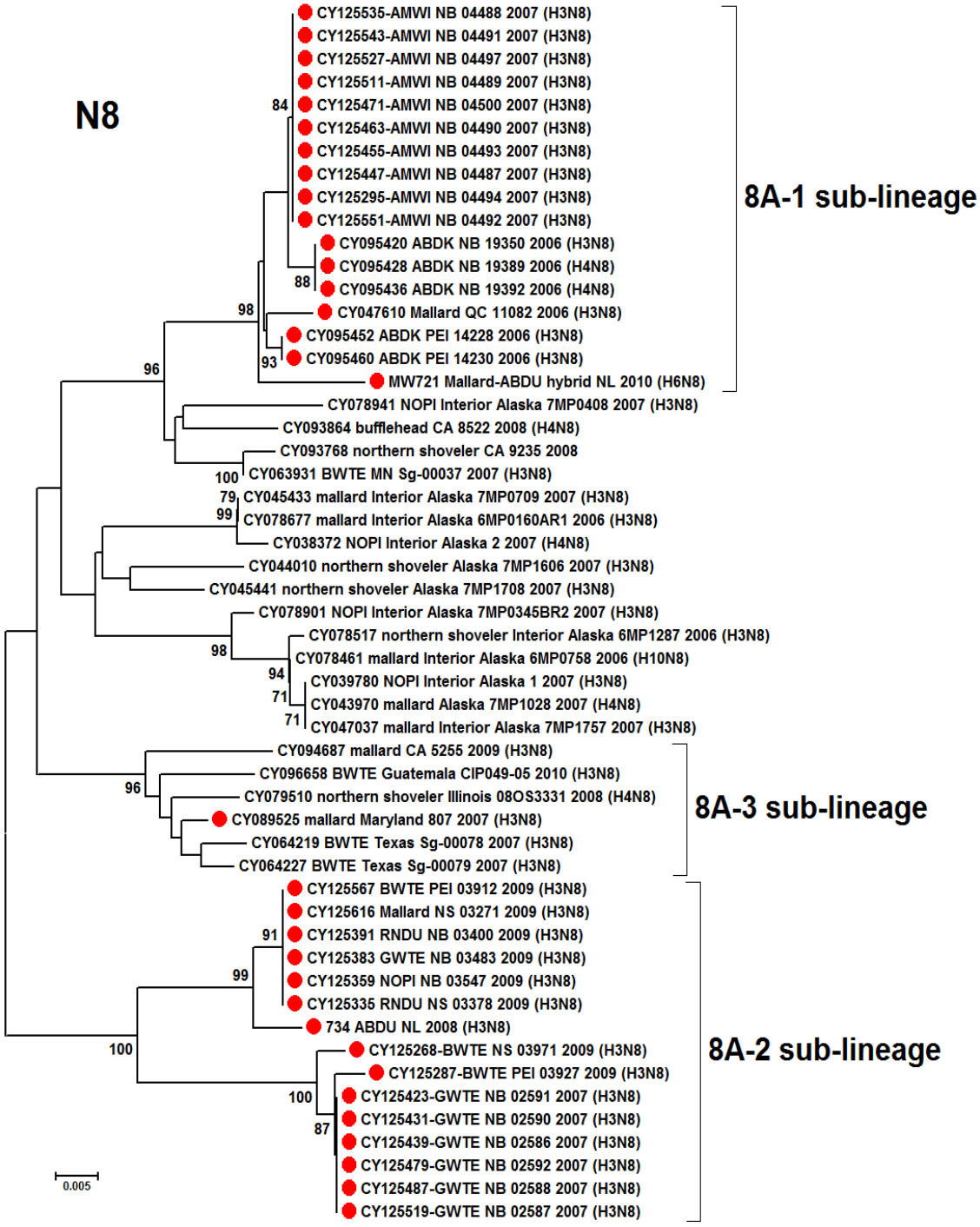
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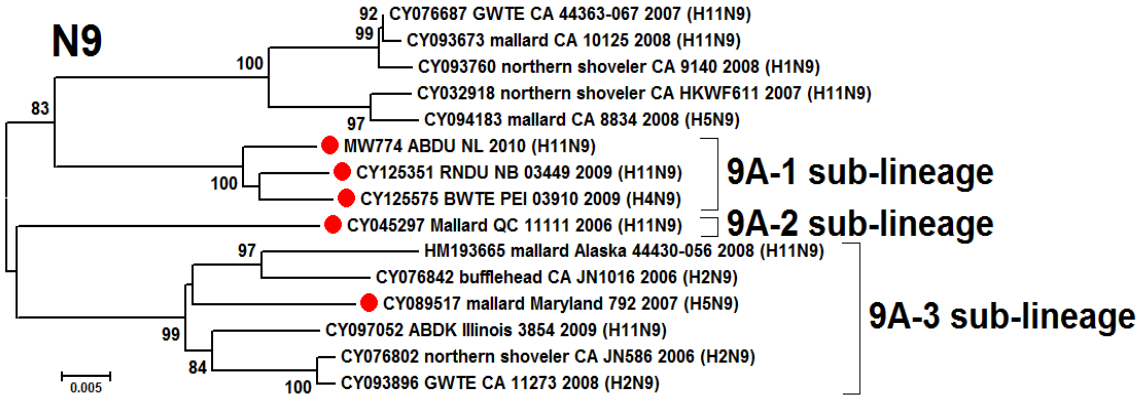
8A-3 sub-lineage
8A-2 sub-lineage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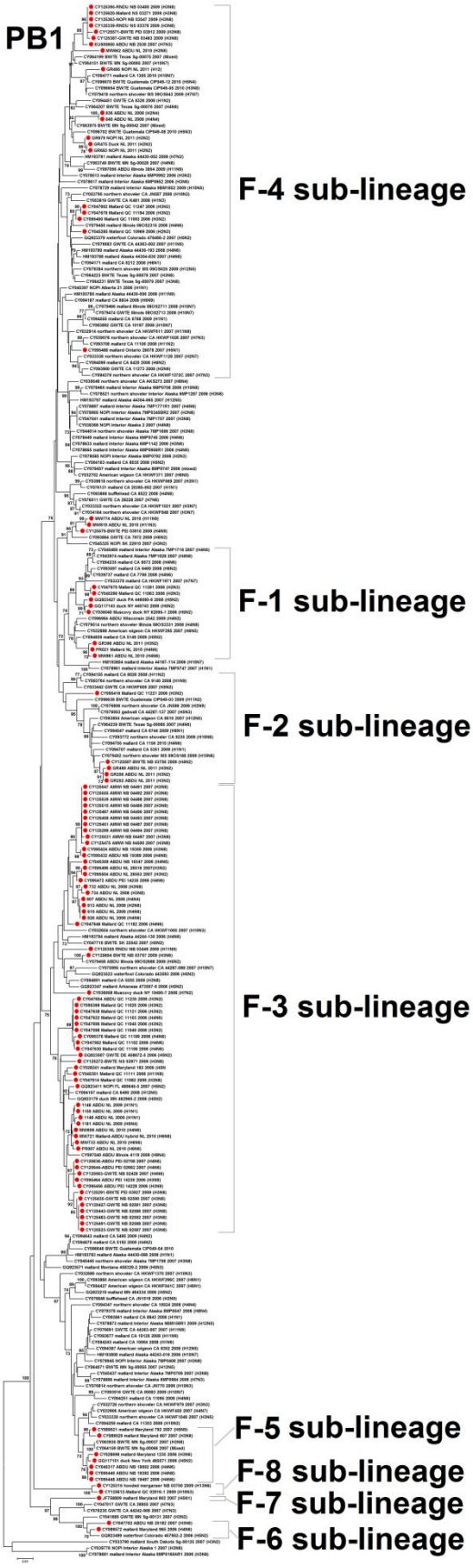
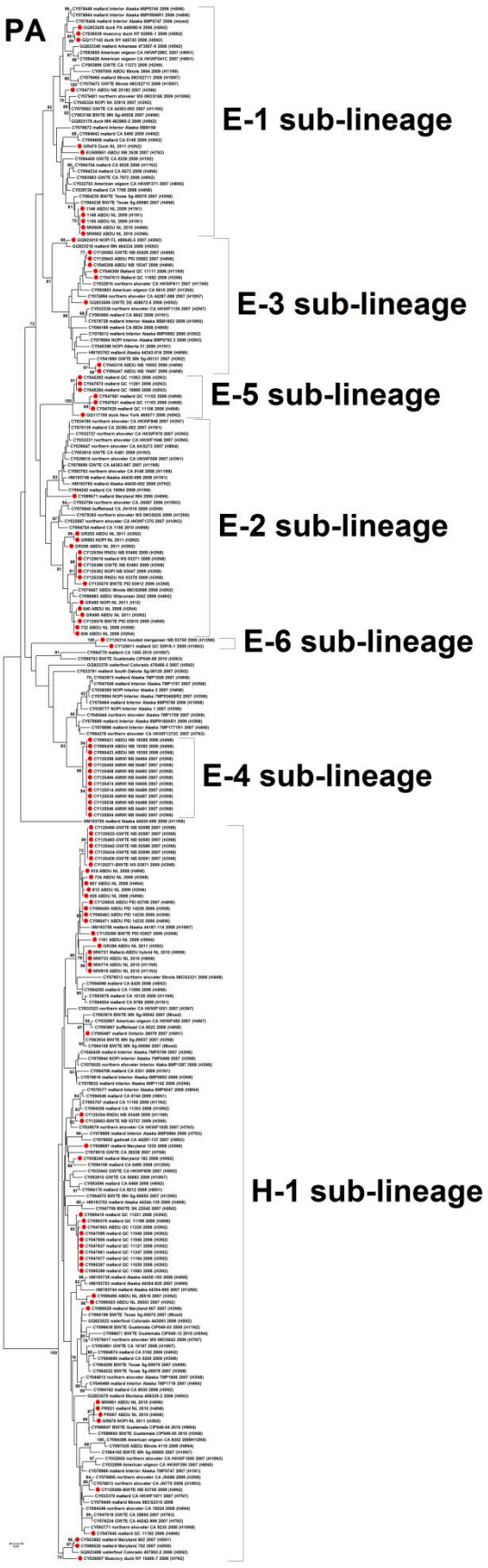
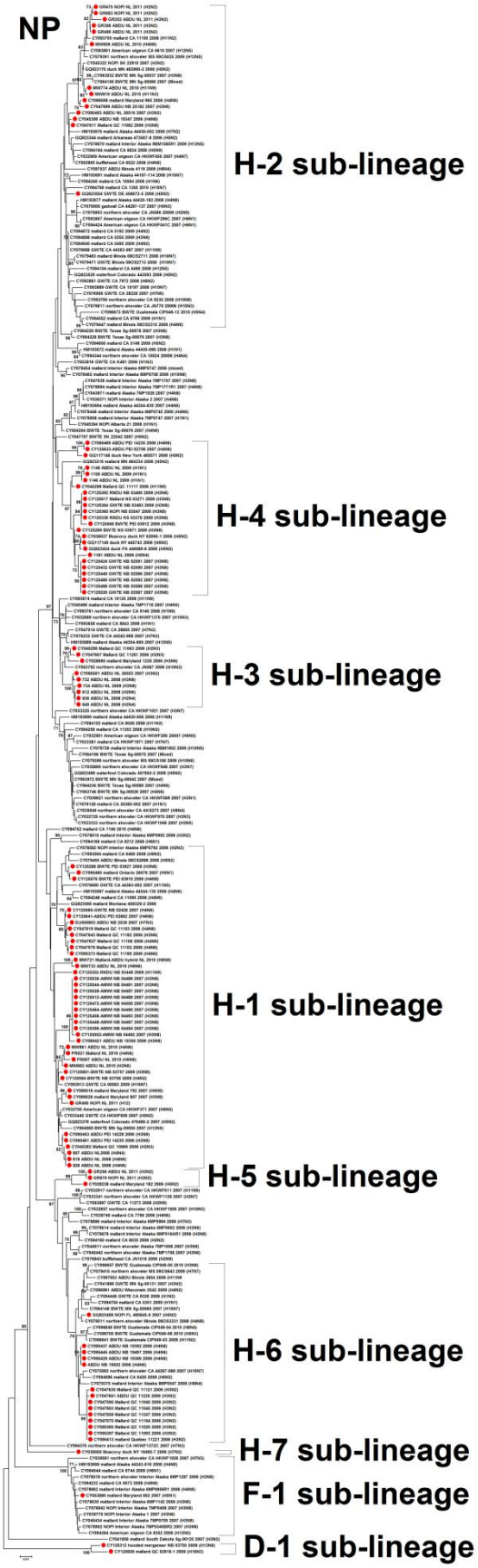
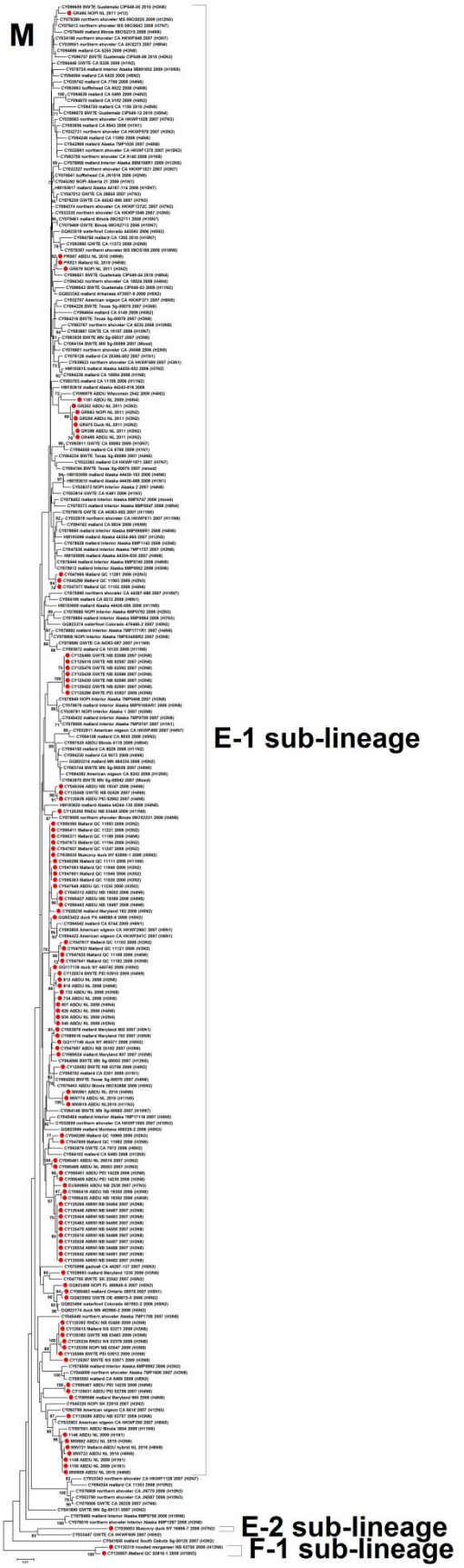
CY093760 northern shoveler CA 9140 2008 (H1N9)
CY032918 northern shoveler CA HKWF611 2007 (H11N9)
83
CY094183 mallard CA 8834 2008 (H5N9)
97
MW774 ABDU NL 2010 (H11N9)
CY125351 RNDU NB 03449 2009 (H11N9)
100
CY125575 BWTE PEI 03910 2009 (H4N9)
9A-1 sub-lineage
CY045297 Mallard QC 11111 2006 (H11N9)
9A-2 sub-lineage
97
HM193665 mallard Alaska 44430-056 2008 (H11N9)
CY076842 bufflehead CA JN1016 2006 (H2N9)
CY089517 mallard Maryland 792 2007 (H5N9)
9A-3 sub-lineage
99
CY097052 ABDK Illinois 3854 2009 (H11N9)
84
CY076802 northern shoveler CA JN586 2006 (H2N9)
100
CY093896 GWTE CA 11273 2008 (H2N9)
0.005


PB2
C-2 sub-lineage
C-3 sub-lineage
C-5 sub-lineage
C-4 sub-lineage
C-1 sub-lineage
J-1 sub-lineage


PB1
F-4 sub-lineage
F-1 sub-lineage
F-2 sub-lineage
F-3 sub-lineage
F-5 sub-lineage
F-8 sub-lineage
F-7 sub-lineage
F-6 sub-lineage

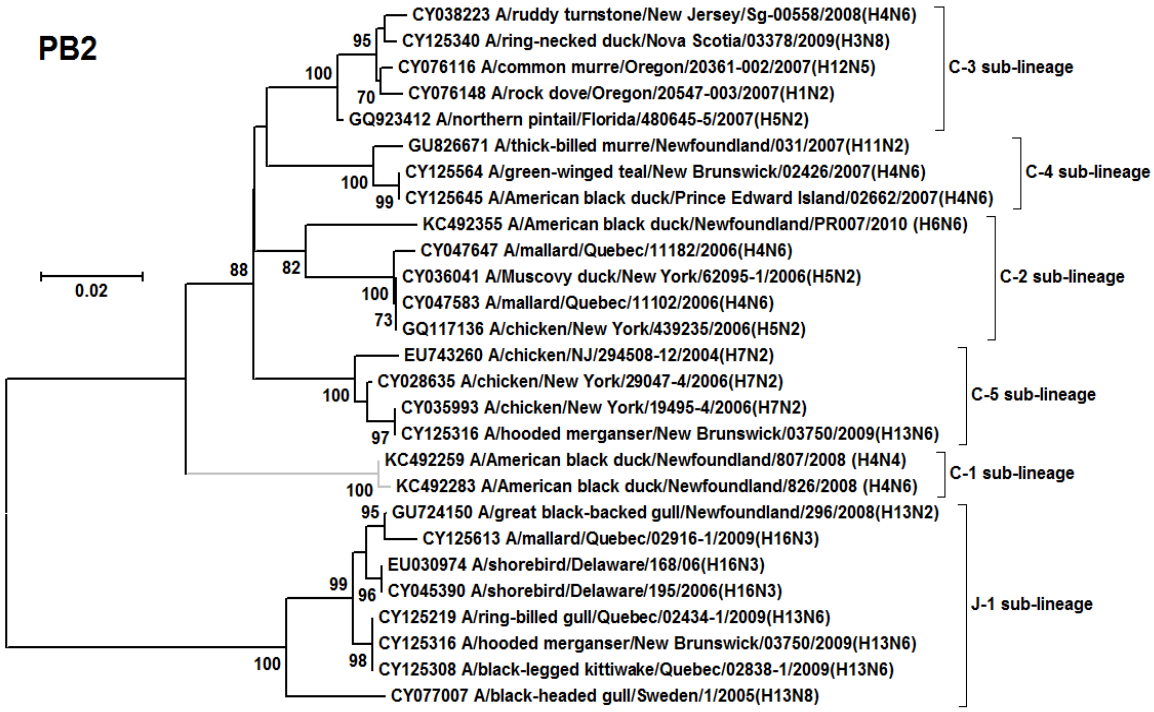

PA
E-1 sub-lineage
E-3 sub-lineage
E-5 sub-lineage
E-2 sub-lineage
E-6 sub-lineage
E-4 sub-lineage
H-1 sub-lineage

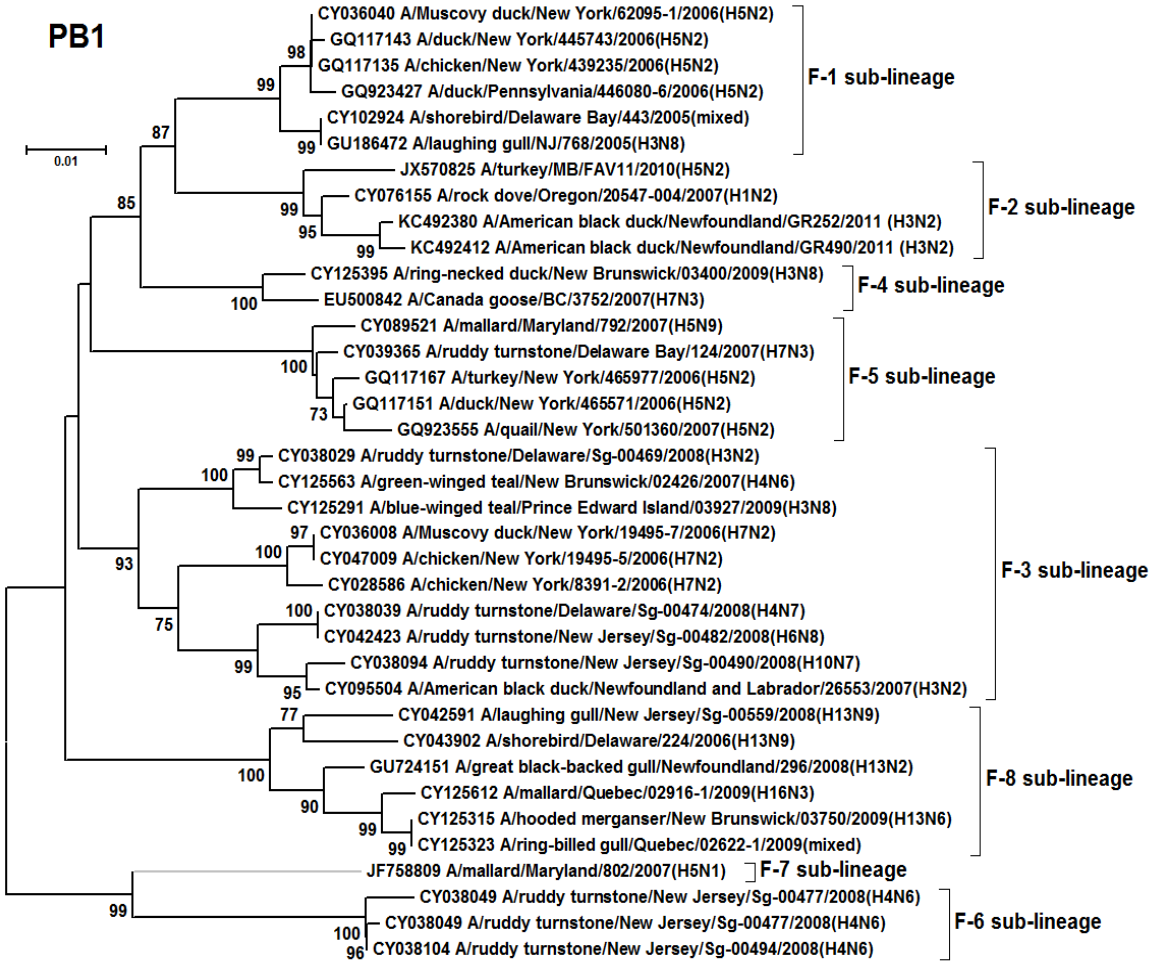

NP
H-2 sub-lineage
H-4 sub-lineage
H-3 sub-lineage
H-1 sub-lineage
H-5 sub-lineage
H-6 sub-lineage
H-7 sub-lineage
F-1 sub-lineage
D-1 sub-lineage

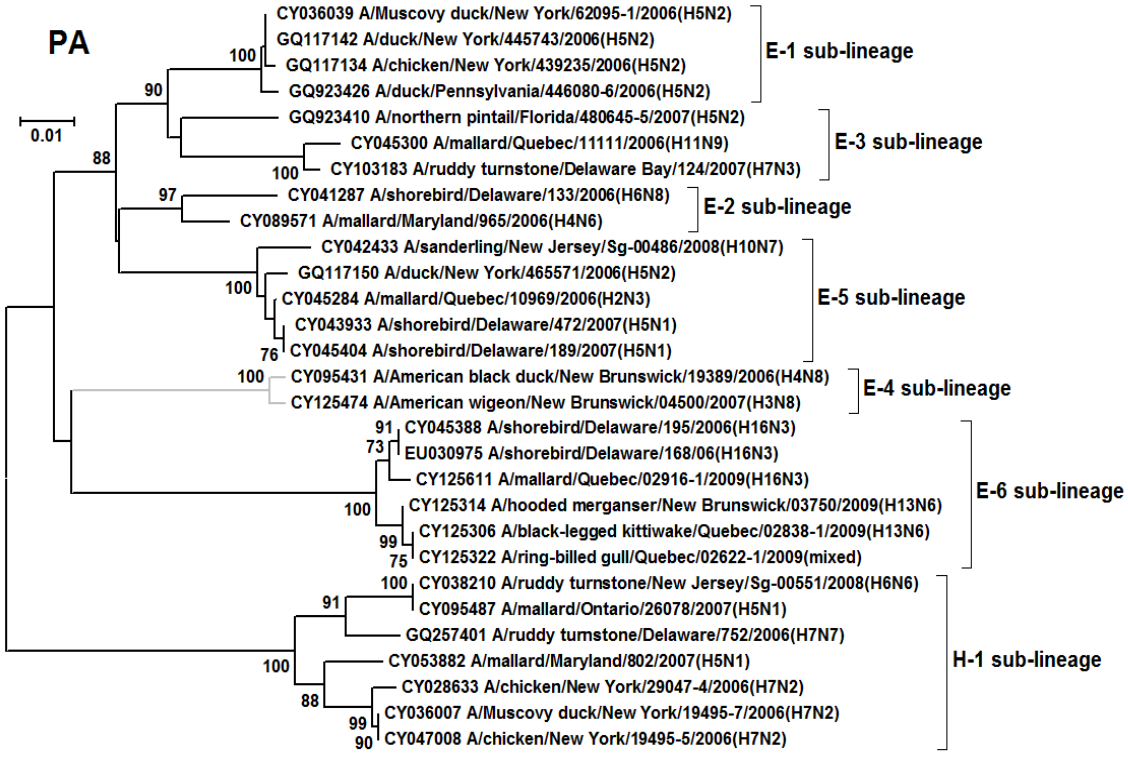

M
E-1 sub-lineage
E-2 sub-lineage
F-1 sub-lineage

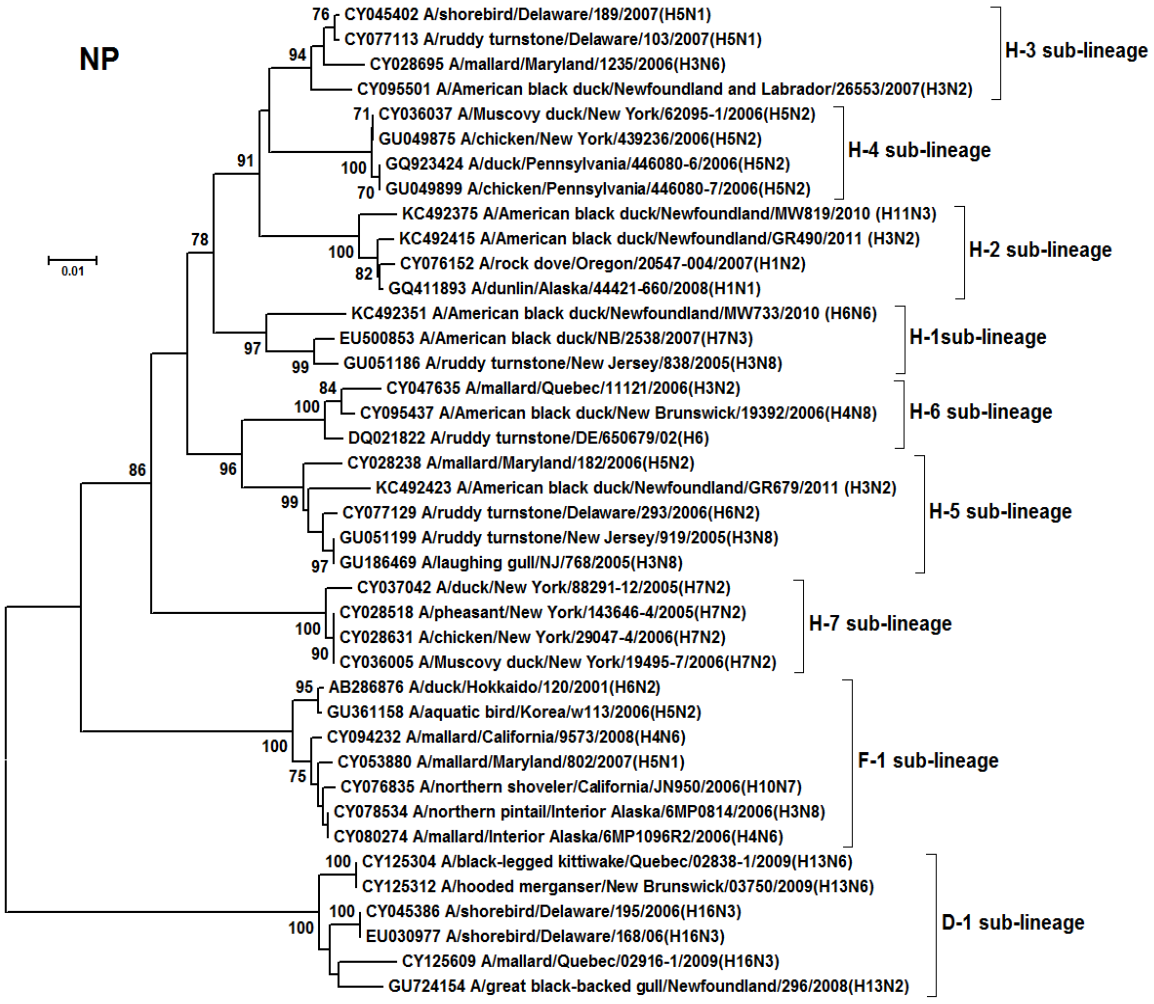

NS
1D-1 sub-lineage
1C-1 sub-lineage
2B-2 sub-lineage
2B-1 sub-lineage

Figure S3. 3. Identification of Atlantic flyway duck AIV genes in other bird hosts (the following). Phylogenetic trees were constructed to highlight the distribution of the genes found in the 109 Atlantic flyway duck AIVs in non-duck host species. The neighbour-joining trees were constructed with MEGA5 and support values based on 1000 bootstrap replicates are shown as percentages where ≥70%. The sub-lineages are labelled on the right of the phylogenetic trees. Black branch lines indicate detection of the sub-lineage in non-duck species, whereas grey branch lines indicate the detection of the sub-lineage only in ducks. For the H16 tree, the 3 gull-related lineages (H16A to C) established in the Flugenome database were labelled in red.




PB1
0.01
CY036040 A/Muscovy duck/New York/62095-1/2006(H5N2)
GQ117143 A/duck/New York/445743/2006(H5N2)
GQ117135 A/chicken/New York/439235/2006(H5N2)
GQ923427 A/duck/Pennsylvania/446080-6/2006(H5N2)
CY102924 A/shorebird/Delaware Bay/443/2005(mixed)
GU186472 A/laughing gull/NJ/768/2005(H3N8)
F-1 sub-lineage
JX570825 A/turkey/MB/FAV11/2010(H5N2)
CY076155 A/rock dove/Oregon/20547-004/2007(H1N2)
KC492380 A/American black duck/Newfoundland/GR252/2011 (H3N2)
KC492412 A/American black duck/Newfoundland/GR490/2011 (H3N2)
F-2 sub-lineage
CY125395 A/ring-necked duck/New Brunswick/03400/2009(H3N8)
EU500842 A/Canada goose/BC/3752/2007(H7N3)
F-4 sub-lineage
CY089521 A/mallard/Maryland/792/2007(H5N9)
CY039365 A/ruddy turnstone/Delaware Bay/124/2007(H7N3)
GQ117167 A/turkey/New York/465977/2006(H5N2)
GQ117151 A/duck/New York/465571/2006(H5N2)
GQ923555 A/quail/New York/501360/2007(H5N2)
F-5 sub-lineage
CY038029 A/ruddy turnstone/Delaware/Sg-00469/2008(H3N2)
CY125563 A/green-winged teal/New Brunswick/02426/2007(H4N6)
CY125291 A/blue-winged teal/Prince Edward Island/03927/2009(H3N8)
CY036008 A/Muscovy duck/New York/19495-7/2006(H7N2)
CY047009 A/chicken/New York/19495-5/2006(H7N2)
CY028586 A/chicken/New York/8391-2/2006(H7N2)
CY038039 A/ruddy turnstone/Delaware/Sg-00474/2008(H4N7)
CY042423 A/ruddy turnstone/New Jersey/Sg-00482/2008(H6N8)
CY038094 A/ruddy turnstone/New Jersey/Sg-00490/2008(H10N7)
CY095504 A/American black duck/Newfoundland and Labrador/26553/2007(H3N2)
F-3 sub-lineage
CY042591 A/laughing gull/New Jersey/Sg-00559/2008(H13N9)
CY043902 A/shorebird/Delaware/224/2006(H13N9)
GU724151 A/great black-backed gull/Newfoundland/296/2008(H13N2)
CY125612 A/mallard/Quebec/02916-1/2009(H16N3)
CY125315 A/hooded merganser/New Brunswick/03750/2009(H13N6)
CY125323 A/ring-billed gull/Quebec/02622-1/2009(mixed)
F-8 sub-lineage
JF758809 A/mallard/Maryland/802/2007(H5N1)
F-7 sub-lineage
CY038049 A/ruddy turnstone/New Jersey/Sg-00477/2008(H4N6)
CY038049 A/ruddy turnstone/New Jersey/Sg-00477/2008(H4N6)
CY038104 A/ruddy turnstone/New Jersey/Sg-00494/2008(H4N6)
F-6 sub-lineage


PA
0.01
CY036039 A/Muscovy duck/New York/62095-1/2006(H5N2)
GQ117142 A/duck/New York/445743/2006(H5N2)
GQ117134 A/chicken/New York/439235/2006(H5N2)
GQ923426 A/duck/Pennsylvania/446080-6/2006(H5N2)
E-1 sub-lineage
GQ923410 A/northern pintail/Florida/480645-5/2007(H5N2)
CY045300 A/mallard/Quebec/11111/2006(H11N9)
CY103183 A/ruddy turnstone/Delaware Bay/124/2007(H7N3)
E-3 sub-lineage
CY041287 A/shorebird/Delaware/133/2006(H6N8)
CY089571 A/mallard/Maryland/965/2006(H4N6)
E-2 sub-lineage
CY042433 A/sanderling/New Jersey/Sg-00486/2008(H10N7)
GQ117150 A/duck/New York/465571/2006(H5N2)
CY045284 A/mallard/Quebec/10969/2006(H2N3)
CY043933 A/shorebird/Delaware/472/2007(H5N1)
CY045404 A/shorebird/Delaware/189/2007(H5N1)
E-5 sub-lineage
CY095431 A/American black duck/New Brunswick/19389/2006(H4N8)
CY125474 A/American wigeon/New Brunswick/04500/2007(H3N8)
E-4 sub-lineage
CY045388 A/shorebird/Delaware/195/2006(H16N3)
EU030975 A/shorebird/Delaware/168/06(H16N3)
CY125611 A/mallard/Quebec/02916-1/2009(H16N3)
CY125314 A/hooded merganser/New Brunswick/03750/2009(H13N6)
CY125306 A/black-legged kittiwake/Quebec/02838-1/2009(H13N6)
CY125322 A/ring-billed gull/Quebec/02622-1/2009(mixed)
E-6 sub-lineage
CY038210 A/ruddy turnstone/New Jersey/Sg-00551/2008(H6N6)
CY095487 A/mallard/Ontario/26078/2007(H5N1)
GQ257401 A/ruddy turnstone/Delaware/752/2006(H7N7)
CY053882 A/mallard/Maryland/802/2007(H5N1)
CY028633 A/chicken/New York/29047-4/2006(H7N2)
CY036007 A/Muscovy duck/New York/19495-7/2006(H7N2)
CY047008 A/chicken/New York/19495-5/2006(H7N2)
H-1 sub-lineage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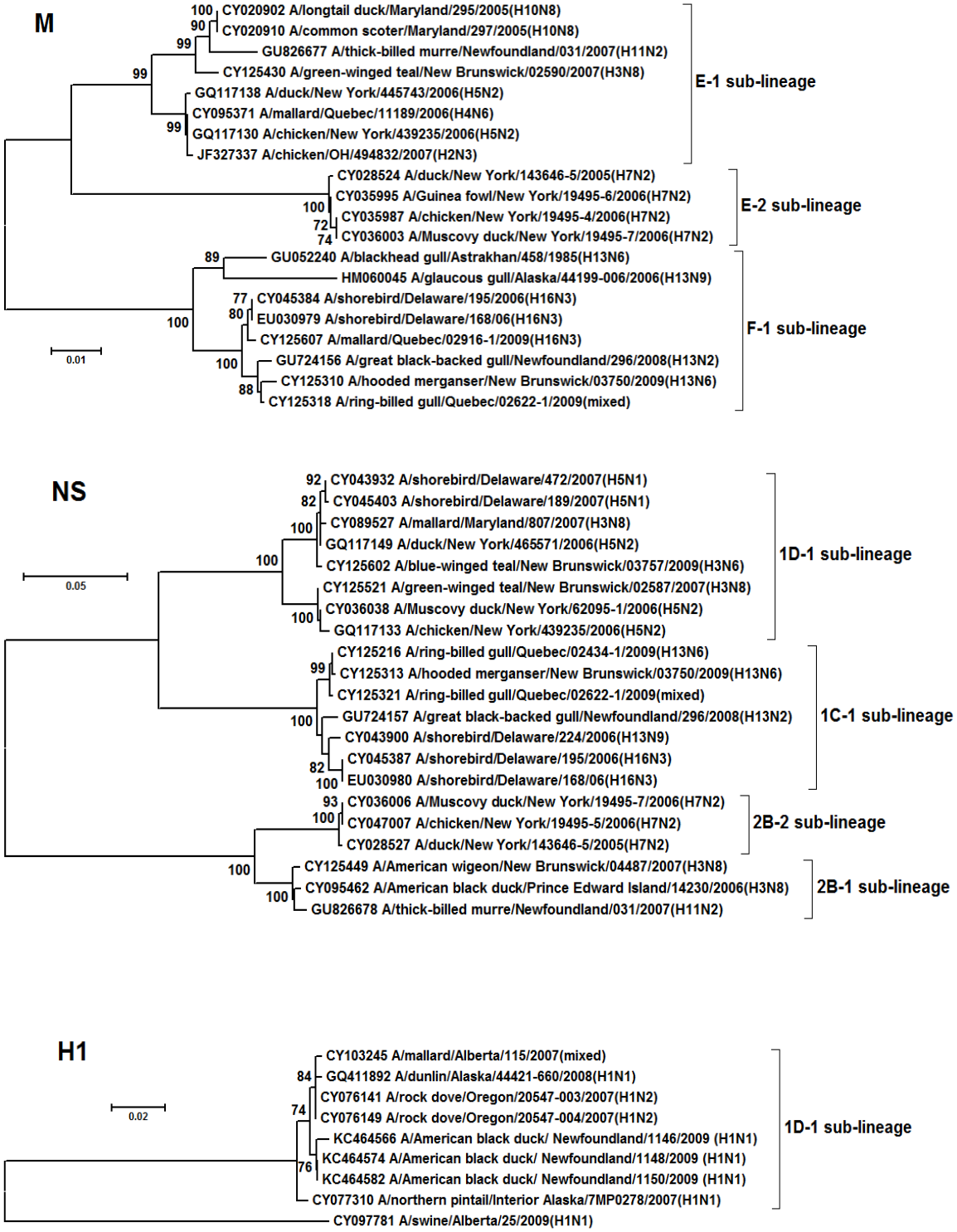
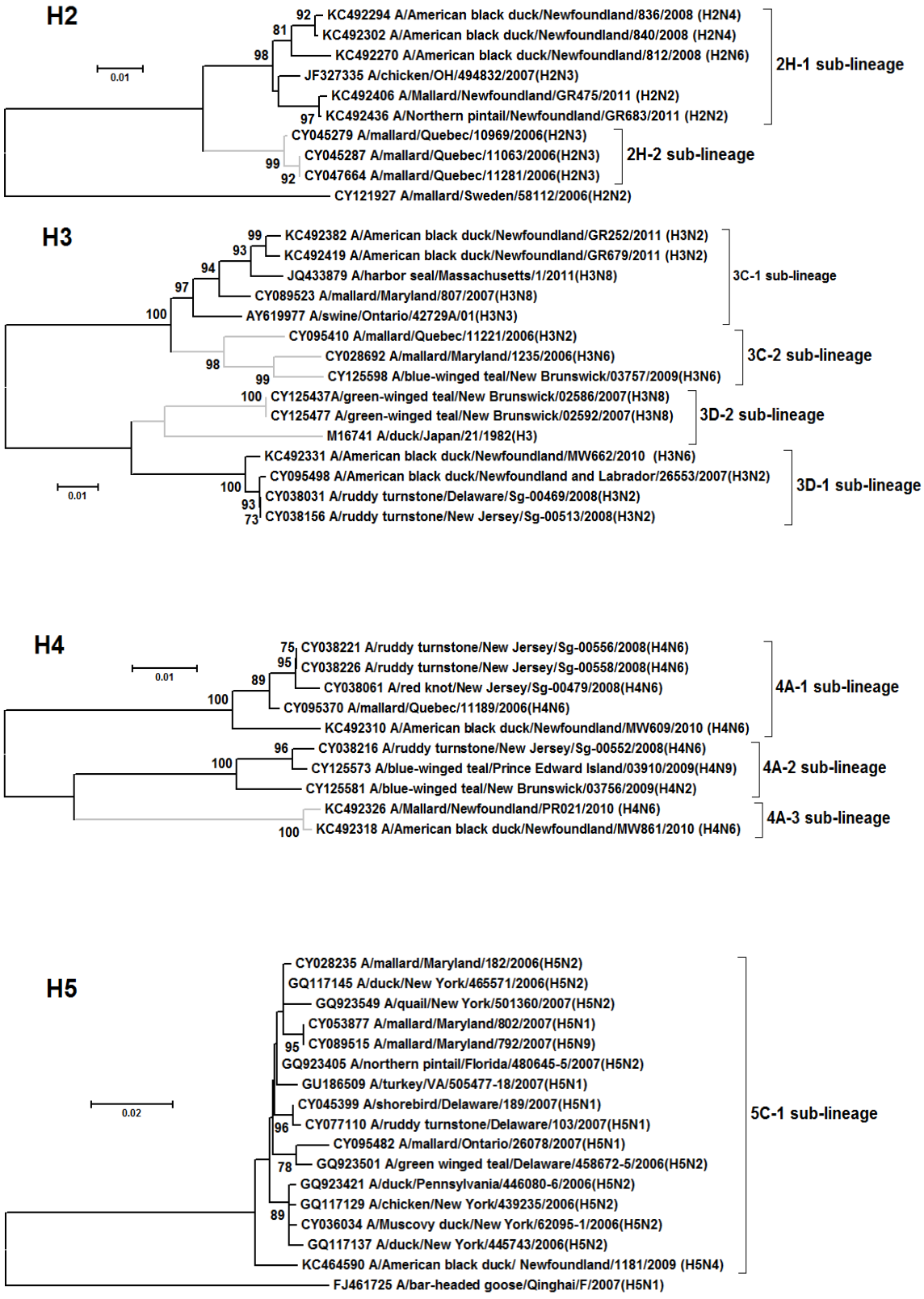
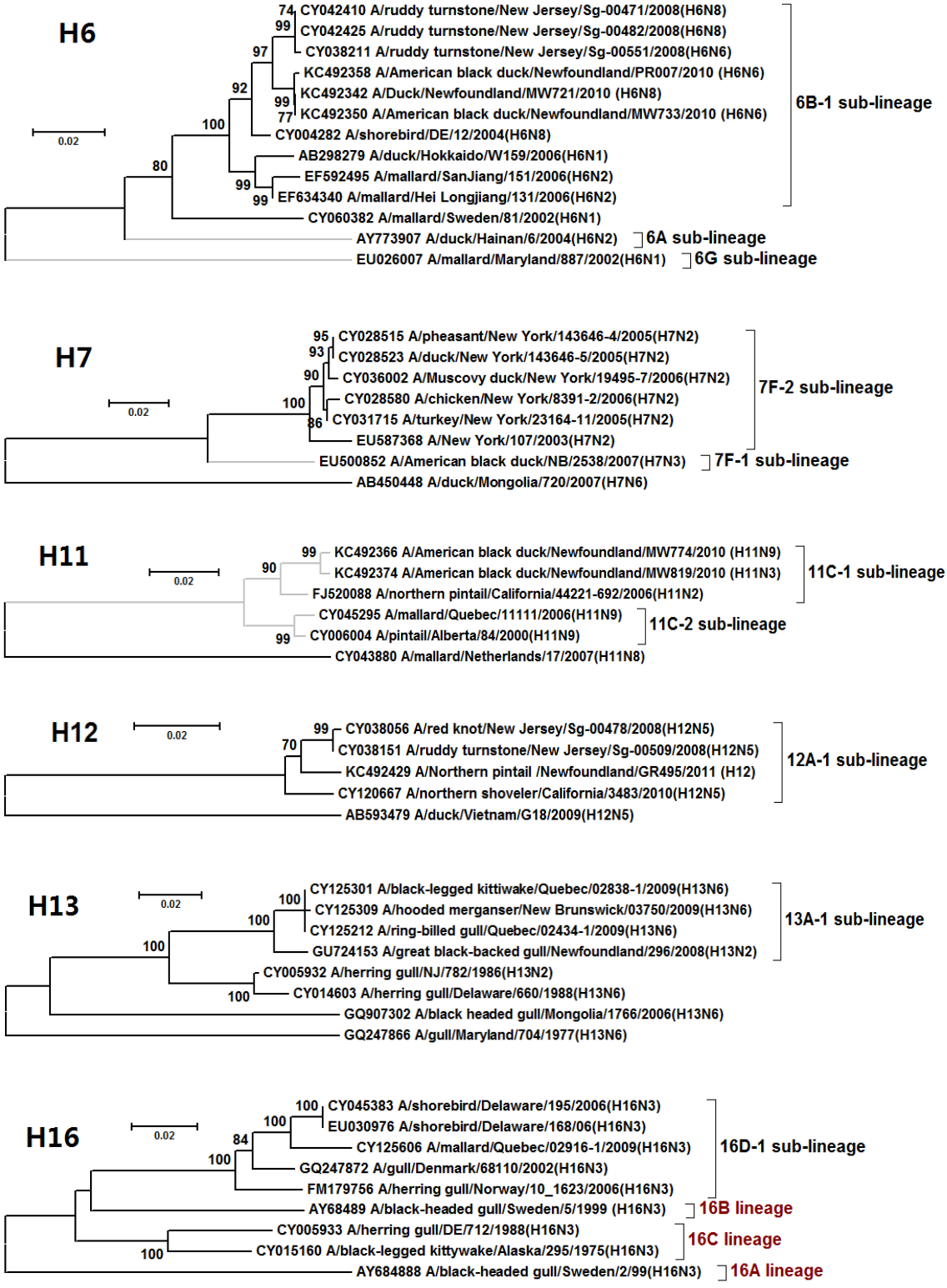
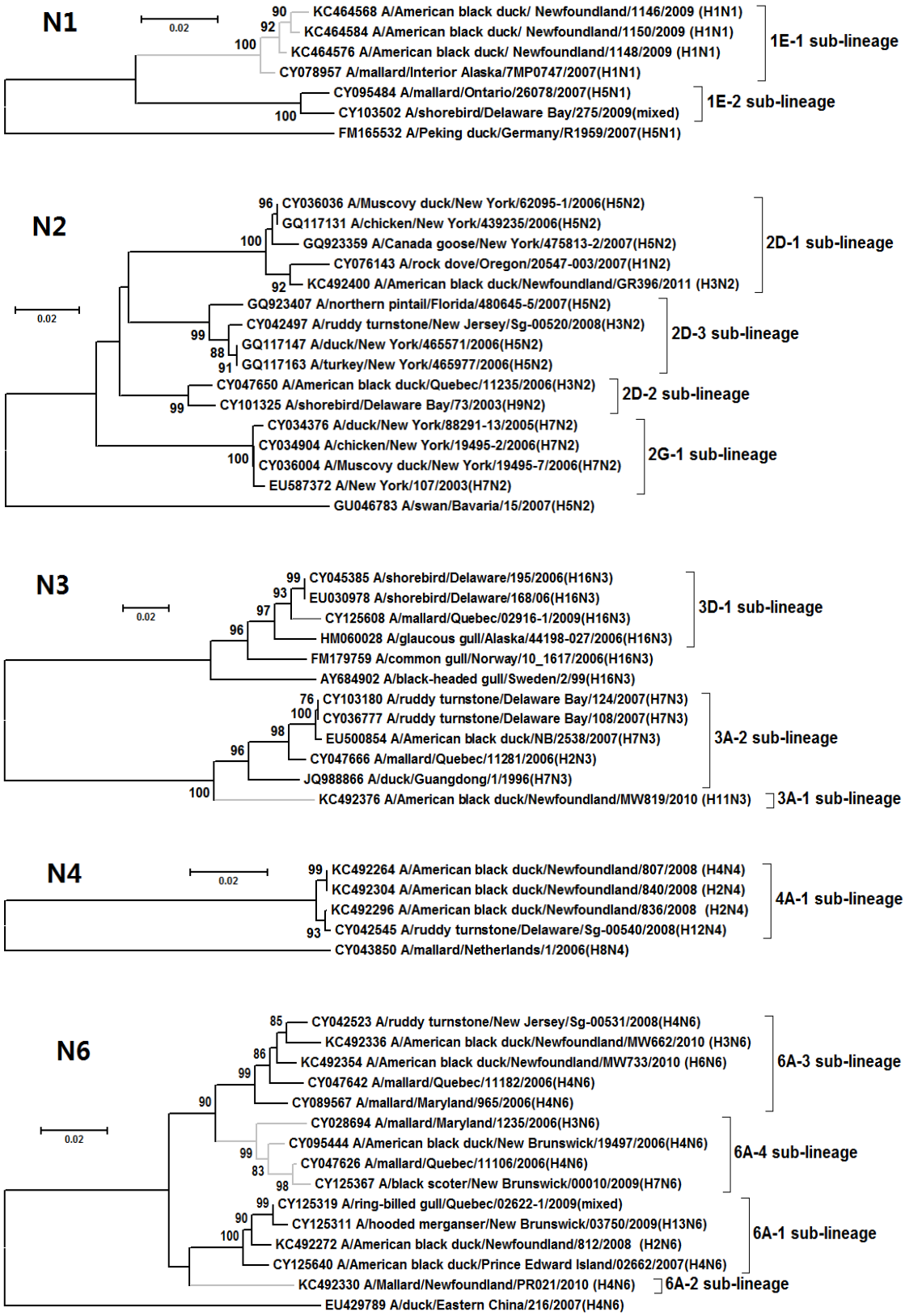
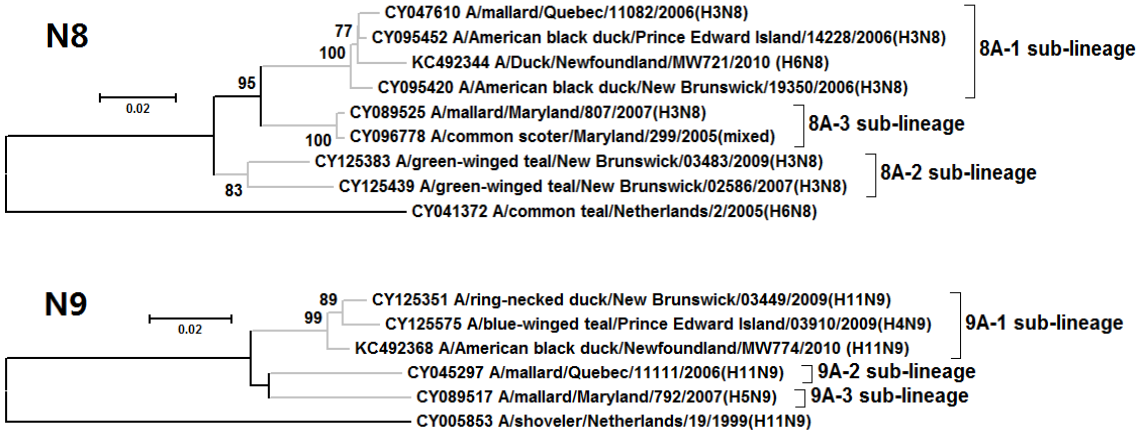
CY045402 A/shorebird/Delaware/189/2007(H5N1)
CY077113 A/ruddy turnstone/Delaware/103/2007(H5N1)
CY028695 A/mallard/Maryland/1235/2006(H3N6)
CY095501 A/American black duck/Newfoundland and Labrador/26553/2007(H3N2)
H-3 sub-lineage
CY036037 A/Muscovy duck/New York/62095-1/2006(H5N2)
GU049875 A/chicken/New York/439236/2006(H5N2)
GQ923424 A/duck/Pennsylvania/446080-6/2006(H5N2)
GU049899 A/chicken/Pennsylvania/446080-7/2006(H5N2)
H-4 sub-lineage
KC492375 A/American black duck/Newfoundland/MW819/2010 (H11N3)
KC492415 A/American black duck/Newfoundland/GR490/2011 (H3N2)
CY076152 A/rock dove/Oregon/20547-004/2007(H1N2)
GQ411893 A/dunlin/Alaska/44421-660/2008(H1N1)
H-2 sub-lineage
KC492351 A/American black duck/Newfoundland/MW733/2010 (H6N6)
EU500853 A/American black duck/NB/2538/2007(H7N3)
GU051186 A/ruddy turnstone/New Jersey/838/2005(H3N8)
H-1sub-lineage
CY047635 A/mallard/Quebec/11121/2006(H3N2)
CY095437 A/American black duck/New Brunswick/19392/2006(H4N8)
DQ021822 A/ruddy turnstone/DE/650679/02(H6)
H-6 sub-lineage
CY028238 A/mallard/Maryland/182/2006(H5N2)
KC492423 A/American black duck/Newfoundland/GR679/2011 (H3N2)
CY077129 A/ruddy turnstone/Delaware/293/2006(H6N2)
GU051199 A/ruddy turnstone/New Jersey/919/2005(H3N8)
GU186469 A/laughing gull/NJ/768/2005(H3N8)
H-5 sub-lineage
CY037042 A/duck/New York/88291-12/2005(H7N2)
CY028518 A/pheasant/New York/143646-4/2005(H7N2)
CY028631 A/chicken/New York/29047-4/2006(H7N2)
CY036005 A/Muscovy duck/New York/19495-7/2006(H7N2)
H-7 sub-lineage
AB286876 A/duck/Hokkaido/120/2001(H6N2)
GU361158 A/aquatic bird/Korea/w113/2006(H5N2)
CY094232 A/mallard/California/9573/2008(H4N6)
CY053880 A/mallard/Maryland/802/2007(H5N1)
CY076835 A/northern shoveler/California/JN950/2006(H10N7)
CY078534 A/northern pintail/Interior Alaska/6MP0814/2006(H3N8)
CY080274 A/mallard/Interior Alaska/6MP1096R2/2006(H4N6)
F-1 sub-lineage
CY125304 A/black-legged kittiwake/Quebec/02838-1/2009(H13N6)
CY125312 A/hooded merganser/New Brunswick/03750/2009(H13N6)
CY045386 A/shorebird/Delaware/195/2006(H16N3)
EU030977 A/shorebird/Delaware/168/06(H16N3)
CY125609 A/mallard/Quebec/02916-1/2009(H16N3)
GU724154 A/great black-backed gull/Newfoundland/296/2008(H13N2)
D-1 sub-lineage


M
CY020902 A/longtail duck/Maryland/295/2005(H10N8)
CY020910 A/common scoter/Maryland/297/2005(H10N8)
GU826677 A/thick-billed murre/Newfoundland/031/2007(H11N2)
CY125430 A/green-winged teal/New Brunswick/02590/2007(H3N8)
GQ117138 A/duck/New York/445743/2006(H5N2)
CY095371 A/mallard/Quebec/11189/2006(H4N6)
GQ117130 A/chicken/New York/439235/2006(H5N2)
JF327337 A/chicken/OH/494832/2007(H2N3)
E-1 sub-lineage
CY028524 A/duck/New York/143646-5/2005(H7N2)
CY035995 A/Guinea fowl/New York/19495-6/2006(H7N2)
CY035987 A/chicken/New York/19495-4/2006(H7N2)
CY036003 A/Muscovy duck/New York/19495-7/2006(H7N2)
E-2 sub-lineage
GU052240 A/blackhead gull/Astrakhan/458/1985(H13N6)
HM060045 A/glaucous gull/Alaska/44199-006/2006(H13N9)
CY045384 A/shorebird/Delaware/195/2006(H16N3)
EU030979 A/shorebird/Delaware/168/06(H16N3)
CY125607 A/mallard/Quebec/02916-1/2009(H16N3)
GU724156 A/great black-backed gull/Newfoundland/296/2008(H13N2)
CY125310 A/hooded merganser/New Brunswick/03750/2009(H13N6)
CY125318 A/ring-billed gull/Quebec/02622-1/2009(mixed)
F-1 sub-lineage
0.01
NS
CY043932 A/shorebird/Delaware/472/2007(H5N1)
CY045403 A/shorebird/Delaware/189/2007(H5N1)
CY089527 A/mallard/Maryland/807/2007(H3N8)
GQ117149 A/duck/New York/465571/2006(H5N2)
CY125602 A/blue-winged teal/New Brunswick/03757/2009(H3N6)
CY125521 A/green-winged teal/New Brunswick/02587/2007(H3N8)
CY036038 A/Muscovy duck/New York/62095-1/2006(H5N2)
GQ117133 A/chicken/New York/439235/2006(H5N2)
1D-1 sub-lineage
0.05
CY125216 A/ring-billed gull/Quebec/02434-1/2009(H13N6)
CY125313 A/hooded merganser/New Brunswick/03750/2009(H13N6)
CY125321 A/ring-billed gull/Quebec/02622-1/2009(mixed)
GU724157 A/great black-backed gull/Newfoundland/296/2008(H13N2)
CY043900 A/shorebird/Delaware/224/2006(H13N9)
CY045387 A/shorebird/Delaware/195/2006(H16N3)
EU030980 A/shorebird/Delaware/168/06(H16N3)
1C-1 sub-lineage
CY036006 A/Muscovy duck/New York/19495-7/2006(H7N2)
CY047007 A/chicken/New York/19495-5/2006(H7N2)
CY028527 A/duck/New York/143646-5/2005(H7N2)
2B-2 sub-lineage
CY125449 A/American wigeon/New Brunswick/04487/2007(H3N8)
CY095462 A/American black duck/Prince Edward Island/14230/2006(H3N8)
GU826678 A/thick-billed murre/Newfoundland/031/2007(H11N2)
2B-1 sub-lineage
H1
0.02
CY103245 A/mallard/Alberta/115/2007(mixed)
GQ411892 A/dunlin/Alaska/44421-660/2008(H1N1)
CY076141 A/rock dove/Oregon/20547-003/2007(H1N2)
CY076149 A/rock dove/Oregon/20547-004/2007(H1N2)
KC464566 A/American black duck/ Newfoundland/1146/2009 (H1N1)
KC464574 A/American black duck/ Newfoundland/1148/2009 (H1N1)
KC464582 A/American black duck/ Newfoundland/1150/2009 (H1N1)
CY077310 A/northern pintail/Interior Alaska/7MP0278/2007(H1N1)
CY097781 A/swine/Alberta/25/2009(H1N1)
1D-1 sub-lineage


H2
0.01
92
81
98
97
99
92
KC492294 A/American black duck/Newfoundland/836/2008 (H2N4)
KC492302 A/American black duck/Newfoundland/840/2008 (H2N4)
KC492270 A/American black duck/Newfoundland/812/2008 (H2N6)
JF327335 A/chicken/OH/494832/2007(H2N3)
KC492406 A/Mallard/Newfoundland/GR475/2011 (H2N2)
KC492436 A/Northern pintail/Newfoundland/GR683/2011 (H2N2)
CY045279 A/mallard/Quebec/10969/2006(H2N3)
CY045287 A/mallard/Quebec/11063/2006(H2N3)
CY047664 A/mallard/Quebec/11281/2006(H2N3)
CY121927 A/mallard/Sweden/58112/2006(H2N2)
2H-1 sub-lineage
2H-2 sub-lineage
H3
0.01
99
93
94
97
100
98
99
100
93
73
KC492382 A/American black duck/Newfoundland/GR252/2011 (H3N2)
KC492419 A/American black duck/Newfoundland/GR679/2011 (H3N2)
JQ433879 A/harbor seal/Massachusetts/1/2011(H3N8)
CY089523 A/mallard/Maryland/807/2007(H3N8)
AY619977 A/swine/Ontario/42729A/01(H3N3)
CY095410 A/mallard/Quebec/11221/2006(H3N2)
CY028692 A/mallard/Maryland/1235/2006(H3N6)
CY125598 A/blue-winged teal/New Brunswick/03757/2009(H3N6)
CY125437A/green-winged teal/New Brunswick/02586/2007(H3N8)
CY125477 A/green-winged teal/New Brunswick/02592/2007(H3N8)
M16741 A/duck/Japan/21/1982(H3)
KC492331 A/American black duck/Newfoundland/MW662/2010 (H3N6)
CY095498 A/American black duck/Newfoundland and Labrador/26553/2007(H3N2)
CY038031 A/ruddy turnstone/Delaware/Sg-00469/2008(H3N2)
CY038156 A/ruddy turnstone/New Jersey/Sg-00513/2008(H3N2)
3C-1 sub-lineage
3C-2 sub-lineage
3D-2 sub-lineage
3D-1 sub-lineage
H4
0.01
75
95
89
100
96
100
100
CY038221 A/ruddy turnstone/New Jersey/Sg-00556/2008(H4N6)
CY038226 A/ruddy turnstone/New Jersey/Sg-00558/2008(H4N6)
CY038061 A/red knot/New Jersey/Sg-00479/2008(H4N6)
CY095370 A/mallard/Quebec/11189/2006(H4N6)
KC492310 A/American black duck/Newfoundland/MW609/2010 (H4N6)
CY038216 A/ruddy turnstone/New Jersey/Sg-00552/2008(H4N6)
CY125573 A/blue-winged teal/Prince Edward Island/03910/2009(H4N9)
CY125581 A/blue-winged teal/New Brunswick/03756/2009(H4N2)
KC492326 A/Mallard/Newfoundland/PR021/2010 (H4N6)
KC492318 A/American black duck/Newfoundland/MW861/2010 (H4N6)
4A-1 sub-lineage
4A-2 sub-lineage
4A-3 sub-lineage
H5
0.02
95
96
78
89
CY028235 A/mallard/Maryland/182/2006(H5N2)
GQ117145 A/duck/New York/465571/2006(H5N2)
GQ923549 A/quail/New York/501360/2007(H5N2)
CY053877 A/mallard/Maryland/802/2007(H5N1)
CY089515 A/mallard/Maryland/792/2007(H5N9)
GQ923405 A/northern pintail/Florida/480645-5/2007(H5N2)
GU186509 A/turkey/VA/505477-18/2007(H5N1)
CY045399 A/shorebird/Delaware/189/2007(H5N1)
CY077110 A/ruddy turnstone/Delaware/103/2007(H5N1)
CY095482 A/mallard/Ontario/26078/2007(H5N1)
GQ923501 A/green winged teal/Delaware/458672-5/2006(H5N2)
GQ923421 A/duck/Pennsylvania/446080-6/2006(H5N2)
GQ117129 A/chicken/New York/439235/2006(H5N2)
CY036034 A/Muscovy duck/New York/62095-1/2006(H5N2)
GQ117137 A/duck/New York/445743/2006(H5N2)
KC464590 A/American black duck/ Newfoundland/1181/2009 (H5N4)
FJ461725 A/bar-headed goose/Qinghai/F/2007(H5N1)
5C-1 sub-lineage


H6
0.02
74 CY042410 A/ruddy turnstone/New Jersey/Sg-00471/2008(H6N8)
99 CY042425 A/ruddy turnstone/New Jersey/Sg-00482/2008(H6N8)
97 CY038211 A/ruddy turnstone/New Jersey/Sg-00551/2008(H6N6)
KC492358 A/American black duck/Newfoundland/PR007/2010 (H6N6)
92 KC492342 A/Duck/Newfoundland/MW721/2010 (H6N8)
99 77 KC492350 A/American black duck/Newfoundland/MW733/2010 (H6N6)
100 CY004282 A/shorebird/DE/12/2004(H6N8)
AB298279 A/duck/Hokkaido/W159/2006(H6N1)
80 EF592495 A/mallard/SanJiang/151/2006(H6N2)
99 99 EF634340 A/mallard/Hei Longjiang/131/2006(H6N2)
CY060382 A/mallard/Sweden/81/2002(H6N1)
AY773907 A/duck/Hainan/6/2004(H6N2)
EU026007 A/mallard/Maryland/887/2002(H6N1)
6B-1 sub-lineage
6A sub-lineage
6G sub-lineage
H7
0.02
95 CY028515 A/pheasant/New York/143646-4/2005(H7N2)
93 CY028523 A/duck/New York/143646-5/2005(H7N2)
90 CY036002 A/Muscovy duck/New York/19495-7/2006(H7N2)
100 CY028580 A/chicken/New York/8391-2/2006(H7N2)
86 CY031715 A/turkey/New York/23164-11/2005(H7N2)
EU587368 A/New York/107/2003(H7N2)
EU500852 A/American black duck/NB/2538/2007(H7N3)
AB450448 A/duck/Mongolia/720/2007(H7N6)
7F-2 sub-lineage
7F-1 sub-lineage
H11
0.02
99 KC492366 A/American black duck/Newfoundland/MW774/2010 (H11N9)
90 KC492374 A/American black duck/Newfoundland/MW819/2010 (H11N3)
FJ520088 A/northern pintail/California/44221-692/2006(H11N2)
CY045295 A/mallard/Quebec/11111/2006(H11N9)
99 CY006004 A/pintail/Alberta/84/2000(H11N9)
CY043880 A/mallard/Netherlands/17/2007(H11N8)
11C-1 sub-lineage
11C-2 sub-lineage
H12
0.02
99 CY038056 A/red knot/New Jersey/Sg-00478/2008(H12N5)
70 CY038151 A/ruddy turnstone/New Jersey/Sg-00509/2008(H12N5)
KC492429 A/Northern pintail /Newfoundland/GR495/2011 (H12)
CY120667 A/northern shoveler/California/3483/2010(H12N5)
AB593479 A/duck/Vietnam/G18/2009(H12N5)
12A-1 sub-lineage
H13
0.02
100 CY125301 A/black-legged kittiwake/Quebec/02838-1/2009(H13N6)
100 CY125309 A/hooded merganser/New Brunswick/03750/2009(H13N6)
CY125212 A/ring-billed gull/Quebec/02434-1/2009(H13N6)
100 GU724153 A/great black-backed gull/Newfoundland/296/2008(H13N2)
CY005932 A/herring gull/NJ/782/1986(H13N2)
100 CY014603 A/herring gull/Delaware/660/1988(H13N6)
GQ907302 A/black headed gull/Mongolia/1766/2006(H13N6)
GQ247866 A/gull/Maryland/704/1977(H13N6)
13A-1 sub-lineage
H16
0.02
100 CY045383 A/shorebird/Delaware/195/2006(H16N3)
100 EU030976 A/shorebird/Delaware/168/06(H16N3)
84 CY125606 A/mallard/Quebec/02916-1/2009(H16N3)
100 GQ247872 A/gull/Denmark/68110/2002(H16N3)
FM179756 A/herring gull/Norway/10_1623/2006(H16N3)
AY68489 A/black-headed gull/Sweden/5/1999 (H16N3)
CY005933 A/herring gull/DE/712/1988(H16N3)
100 CY015160 A/black-legged kittywake/Alaska/295/1975(H16N3)
AY684888 A/black-headed gull/Sweden/2/99(H16N3)
16D-1 sub-lineage
16B lineage
16C lineage
16A lineage


N1
0.02
90
92
100
KC464568 A/American black duck/ Newfoundland/1146/2009 (H1N1)
KC464584 A/American black duck/ Newfoundland/1150/2009 (H1N1)
KC464576 A/American black duck/ Newfoundland/1148/2009 (H1N1)
CY078957 A/mallard/Interior Alaska/7MP0747/2007(H1N1)
1E-1 sub-lineage
CY095484 A/mallard/Ontario/26078/2007(H5N1)
100
CY103502 A/shorebird/Delaware Bay/275/2009(mixed)
1E-2 sub-lineage
FM165532 A/Peking duck/Germany/R1959/2007(H5N1)
N2
0.02
96
CY036036 A/Muscovy duck/New York/62095-1/2006(H5N2)
GQ117131 A/chicken/New York/439235/2006(H5N2)
100
GQ923359 A/Canada goose/New York/475813-2/2007(H5N2)
CY076143 A/rock dove/Oregon/20547-003/2007(H1N2)
92
KC492400 A/American black duck/Newfoundland/GR396/2011 (H3N2)
2D-1 sub-lineage
GQ923407 A/northern pintail/Florida/480645-5/2007(H5N2)
99
CY042497 A/ruddy turnstone/New Jersey/Sg-00520/2008(H3N2)
88
GQ117147 A/duck/New York/465571/2006(H5N2)
91
GQ117163 A/turkey/New York/465977/2006(H5N2)
2D-3 sub-lineage
CY047650 A/American black duck/Quebec/11235/2006(H3N2)
99
CY101325 A/shorebird/Delaware Bay/73/2003(H9N2)
2D-2 sub-lineage
CY034376 A/duck/New York/88291-13/2005(H7N2)
CY034904 A/chicken/New York/19495-2/2006(H7N2)
100
CY036004 A/Muscovy duck/New York/19495-7/2006(H7N2)
EU587372 A/New York/107/2003(H7N2)
2G-1 sub-lineage
GU046783 A/swan/Bavaria/15/2007(H5N2)
N3
0.02
99
CY045385 A/shorebird/Delaware/195/2006(H16N3)
93
EU030978 A/shorebird/Delaware/168/06(H16N3)
97
CY125608 A/mallard/Quebec/02916-1/2009(H16N3)
96
HM060028 A/glaucous gull/Alaska/44198-027/2006(H16N3)
3D-1 sub-lineage
FM179759 A/common gull/Norway/10_1617/2006(H16N3)
AY684902 A/black-headed gull/Sweden/2/99(H16N3)
76
CY103180 A/ruddy turnstone/Delaware Bay/124/2007(H7N3)
100
CY036777 A/ruddy turnstone/Delaware Bay/108/2007(H7N3)
98
EU500854 A/American black duck/NB/2538/2007(H7N3)
96
CY047666 A/mallard/Quebec/11281/2006(H2N3)
JQ988866 A/duck/Guangdong/1/1996(H7N3)
3A-2 sub-lineage
100
KC492376 A/American black duck/Newfoundland/MW819/2010 (H11N3)
3A-1 sub-lineage
N4
0.02
99
KC492264 A/American black duck/Newfoundland/807/2008 (H4N4)
KC492304 A/American black duck/Newfoundland/840/2008 (H2N4)
KC492296 A/American black duck/Newfoundland/836/2008 (H2N4)
93
CY042545 A/ruddy turnstone/Delaware/Sg-00540/2008(H12N4)
4A-1 sub-lineage
CY043850 A/mallard/Netherlands/1/2006(H8N4)
N6
0.02
85
CY042523 A/ruddy turnstone/New Jersey/Sg-00531/2008(H4N6)
KC492336 A/American black duck/Newfoundland/MW662/2010 (H3N6)
86
KC492354 A/American black duck/Newfoundland/MW733/2010 (H6N6)
99
CY047642 A/mallard/Quebec/11182/2006(H4N6)
90
CY089567 A/mallard/Maryland/965/2006(H4N6)
6A-3 sub-lineage
CY028694 A/mallard/Maryland/1235/2006(H3N6)
99
CY095444 A/American black duck/New Brunswick/19497/2006(H4N6)
83
CY047626 A/mallard/Quebec/11106/2006(H4N6)
98
CY125367 A/black scoter/New Brunswick/00010/2009(H7N6)
6A-4 sub-lineage
99
CY125319 A/ring-billed gull/Quebec/02622-1/2009(mixed)
90
CY125311 A/hooded merganser/New Brunswick/03750/2009(H13N6)
100
KC492272 A/American black duck/Newfoundland/812/2008 (H2N6)
CY125640 A/American black duck/Prince Edward Island/02662/2007(H4N6)
6A-1 sub-lineage
KC492330 A/Mallard/Newfoundland/PR021/2010 (H4N6)
6A-2 sub-lineage
EU429789 A/duck/Eastern China/216/2007(H4N6)


N8
0.02
77
100
95
100
83
CY047610 A/mallard/Quebec/11082/2006(H3N8)
CY095452 A/American black duck/Prince Edward Island/14228/2006(H3N8)
KC492344 A/Duck/Newfoundland/MW721/2010 (H6N8)
CY095420 A/American black duck/New Brunswick/19350/2006(H3N8)
CY089525 A/mallard/Maryland/807/2007(H3N8)
CY096778 A/common scoter/Maryland/299/2005(mixed)
CY125383 A/green-winged teal/New Brunswick/03483/2009(H3N8)
CY125439 A/green-winged teal/New Brunswick/02586/2007(H3N8)
CY041372 A/common teal/Netherlands/2/2005(H6N8)
8A-1 sub-lineage
8A-3 sub-lineage
8A-2 sub-lineage

N9
0.02
89
99
CY125351 A/ring-necked duck/New Brunswick/03449/2009(H11N9)
CY125575 A/blue-winged teal/Prince Edward Island/03910/2009(H4N9)
KC492368 A/American black duck/Newfoundland/MW774/2010 (H11N9)
CY045297 A/mallard/Quebec/11111/2006(H11N9)
CY089517 A/mallard/Maryland/792/2007(H5N9)
CY005853 A/shoveler/Netherlands/19/1999(H11N9)
9A-1 sub-lineage
9A-2 sub-lineage
9A-3 sub-lineage

Figure S3. 4. Gene type categorization of the Atlantic flyway duck AIVs by year and location (the following). Gene typing was done as described in the Materials and Methods section such that sequences with nucleotide identity of ≥99% are considered as homologous genes or the same gene type. Sequences from different locations within the Atlantic bird flyway are shown in different colours, as indicated in the legend. The locations are abbreviated as follows: NL, Newfoundland; QC, Quebec; MD, Maryland; NB, New Brunswick; PEI, Prince Edward Island; NY, New York; PA, Pennsylvania; DE, Delaware; ON, Ontario; FL, Florida; NS, Nova Scotia.

PB2

2006		2007		2008	2009	2010	2011
C-2.6	C-2.7	C-2.6	C-2.7	C-1.1	C-2.1	C-2.1	C-2.2
C-2.7	C-2.13	C-2.6	C-2.7	C-1.1	C-2.1	C-2.1	C-2.2
C-2.7	C-2.14	C-2.6	C-2.7	C-1.1	C-2.1	C-2.1	C-2.2
C-2.11	C-2.6	C-2.6	C-4.1	C-1.1	C-2.4	C-2.3	C-2.2
C-2.12	C-2.6	C-2.6	C-4.1	C-1.1	C-2.18	C-2.3	C-2.2
C-2.12	C-2.6	C-2.6	C-2.10	C-1.1	C-2.18	C-2.3	C-2.2
C-2.12	C-2.7	C-2.6	C-2.10	C-2.2	C-3.5	C-2.4	C-2.5
C-2.12	C-2.7	C-2.6	C-3.2	C-2.2	C-3.5	C-2.4	C-3.1
C-2.12	C-2.10	C-2.6	C-3.3		C-3.5	C-2.5	
C-2.12	C-2.9	C-2.6			J-1.2		
C-2.12	C-4.1	C-2.6			C-2.2		
C-2.12	C-4.1	C-2.7			C-2.2		

C-2.12	C-2.12	C-2.19
C-3.2	C-2.12	C-2.19
C-3.2	C-2.15	C-2.19
C-3.2	C-5.1	C-2.19
C-3.3	C-2.17	C-2.19
C-3.3	C-2.16	C-2.19
C-3.4		C-4.1

C-2.8
C-3.5
C-3.5
C-3.5
C-3.5
J-1.1

Legend	
NL	DE
QC	MD
NB	FL
NS	PA
PEI	NY
ON	

PB1

2006		2007		2008	2009	2010	2011
F-1.3	F-3.2	F-3.5	F-5.2	F-3.2	F-3.1	F-1.1	F-1.1
F-1.3	F-5.1	F-3.5	F-5.2	F-3.2	F-3.1	F-1.1	F-2.1
F-2.2	F-6.2	F-3.5	F-7.1	F-3.2	F-3.1	F-1.2	F-2.1
F-3.4	F-3.3	F-3.5	F-3.6	F-3.2	F-3.1	F-1.2	F-2.1
F-3.4	F-3.5	F-3.5	F-3.6	F-3.2	F-1.2	F-3.1	F-4.1
F-3.4	F-3.5	F-3.5	F-3.2	F-3.2	F-3.6	F-3.1	F-4.1
F-3.4	F-5.1	F-3.5	F-3.2	F-4.2	F-4.5	F-3.1	F-4.1
F-3.4	F-5.1	F-3.5	F-4.7	F-4.2	F-4.5	F-3.1	F-4.4
F-3.4	F-5.1	F-3.5	F-3.8		F-4.5	F-4.3	
F-3.4	F-3.2	F-3.5			F-8.1		
F-3.4	F-3.6	F-3.6			F-2.1		
F-3.4	F-3.6	F-3.6			F-3.11		
F-3.7	F-1.4	F-3.6			F-3.12		

F-3.7	F-1.4	F-3.6			F-3.12		
F-3.8	F-5.1	F-3.6			F-4.5		
F-4.6	F-3.10	F-3.6			F-4.5		
F-4.6	F-3.9	F-3.6			F-4.5		
F-4.6	F-1.4	F-4.5			F-8.1		
F-4.6		F-6.1					

PA

2006		2007		2008	2009	2010	2011
E-3.1	E-2.2	E-1.3	H-1.12	E-2.1	E-1.1	E-1.1	E-1.2
E-3.3	H-1.10	E-1.4	H-1.13	E-2.1	E-1.1	E-1.1	E-2.1
E-5.1	H-1.11	H-1.17	H-1.13	E-2.1	E-1.1	H-1.4	E-2.1
E-5.1	E-3.2	H-1.17	H-1.1	H-1.1	H-1.3	H-1.4	E-2.1
E-5.1	E-3.1	H-1.17	E-3.1	H-1.1	E-2.1	H-1.4	E-2.1
E-5.2	E-3.2	H-1.17	H-1.6	H-1.1	E-2.1	H-1.4	E-2.1
E-5.2	E-4.1	H-1.17	H-1.6	H-1.1	H-1.17	H-1.5	H-1.2
E-5.2	E-4.1	H-1.17	H-1.14	H-1.1	E-2.1	H-1.5	H-1.5
H-1.7	E-4.1	E-3.1	E-3.4		E-2.1	H-1.5	
H-1.7	H-1.1	E-4.1			E-6.2		
H-1.7	H-1.1	E-4.1			E-2.1		
H-1.7	H-1.1	E-4.1			E-2.1		
H-1.7	E-1.5	E-4.1			E-2.1		
H-1.7	E-1.5	E-4.1			E-6.1		

H-1.7	E-5.1	E-4.1			H-1.15		
H-1.7	H-1.8	E-4.1			H-1.15		
H-1.7	E-3.5	E-4.1			H-1.16		
H-1.7	E-1.5	E-4.1			H-1.17		
H-1.9		E-4.1					

HA

2006		2007		2008	2009	2010	2011
2H-2.1	3C-2.2	3C-1.2	3C-1.3	2H-1.2	1D-1.1	3D-1.1	2H-1.1
2H-2.1	4A-1.3	3C-1.2	5C-1.2	2H-1.2	1D-1.1	4A-1.2	2H-1.1
2H-2.1	5C-1.2	3C-1.2	5C-1.2	2H-1.3	1D-1.1	4A-2.1	3C-1.1
3C-1.2	3C-1.2	3C-1.2	4A-1.5	3D-1.1	5C-1.1	4A-2.1	3C-1.1
3C-2.1	4A-1.4	3C-1.2	4A-1.5	3D-1.1	3D-1.2	6B-1.1	3C-1.1
3C-2.1	4A-1.6	3C-1.2	3D-1.1	4A-1.1	3D-2.1	6B-1.1	3C-1.1
3C-2.1	4A-1.6	3C-1.2	3D-1.1	4A-1.1	4A-3.2	6B-1.1	3C-1.1
3C-2.1	4A-1.6	3C-1.2	5C-1.4	4A-1.1	3D-1.2	11C-1.1	12A-1.1
3C-2.1	4A-1.6	3C-1.2	5C-1.2		3D-1.2	11C-1.1	
3C-2.1	3C-1.2	3C-1.2			16D-1.1		
3C-2.1	3C-1.2	3C-2.3			3C-2.3		
3C-2.1	4A-1.5	3D-2.1			3D-1.2		
3C-2.1	5C-1.2	3D-2.1			3D-1.2		
4A-1.4	5C-1.3	3D-2.1			3D-1.2		
4A-1.4	5C-1.3	3D-2.1			3D-1.3		

4A-1.4	7F-2.1	3D-2.1			4A-3.1		
4A-1.4	5C-1.5	3D-2.1			11C-1.2		
4A-1.4	5C-1.3	4A-1.5			13A-1.1		
11C-2.1		7F-1.1					

NP

2006		2007		2008	2009	2010	2011
H-1.2	H-2.4	H-1.5	H-1.7	H-1.2	H-4.1	H-1.1	H-1.4
H-1.5	H-3.4	H-1.5	H-1.7	H-1.2	H-4.1	H-1.1	H-2.1
H-1.5	H-5.2	H-1.8	F-1.1	H-1.2	H-4.1	H-1.1	H-2.1
H-1.5	H-1.8	H-1.8	H-1.5	H-3.1	H-4.2	H-1.1	H-2.1
H-1.5	H-2.3	H-1.8	H-4.3	H-3.1	H-1.6	H-1.3	H-2.1
H-1.5	H-6.1	H-1.8	H-2.3	H-3.1	H-1.6	H-1.3	H-2.1
H-2.3	H-6.1	H-1.8	H-3.2	H-3.1	H-4.6	H-2.1	H-5.1
H-3.3	H-6.1	H-1.8	H-1.6	H-3.1	H-4.6	H-2.2	H-5.1
H-3.3	H-6.2	H-1.8	H-6.1		H-4.6	H-2.2	
H-4.5	H-1.2	H-1.8			D-1.1		
H-6.2	H-1.2	H-1.8			H-1.1		
H-6.2	H-4.3	H-1.8			H-1.1		
H-6.2	H-4.3	H-2.4			H-1.9		
H-6.2	H-4.4	H-4.7			H-4.5		
H-6.2	H-4.4	H-4.7			H-4.6		
H-6.2	H-7.1	H-4.7			H-4.6		

H-6.2	H-2.5	H-4.7			H-4.6		
H-6.2	H-4.4	H-4.7			D-1.1		
H-6.2		H-4.7					

NA

2006		2007		2008	2009	2010	2011
2D-2.1	2D-1.1	3A-2.1	1E- 2.1	4A-1.1	1E-1.1	3A-1.1	2D
2D-2.1	6A-3.3	6A-1.3	8A-3.1	4A-1.1	1E-1.1	6A-2.1	2D
2D-2.1	6A-4.3	6A-4.2	9A-3.1	4A-1.1	1E-1.1	6A-2.1	2D
2D-2.1	6A-1.1	8A-1.1	6A-1.3	6A-1.1	4A	6A-2.1	2D
2D-2.1	6A-4.1	8A-1.1	6A-1.3	6A-1.1	8A-2.1	6A-2.1	2D
2D-2.1	6A-4.1	8A-1.1	2D-1.1	6A-1.1	8A-2.2	6A-3.1	2D-1.1
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2D-2.1	8A-1.1	8A-1.1	1E- 2.2	8A-2.1	8A-2.1	8A-1.3	-
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3A-2.2	6A-1.2	8A-1.1			3D-1.1		
3A-2.2	8A-1.2	8A-1.1			2D-1.1		
3A-2.2	8A-1.2	8A-1.1			6A-1.2		
6A-3.4	2D-1.3	8A-1.1			6A-3.2		
6A-4.2	2D-1.3	8A-2.2			8A-2.1		
6A-4.2	2D-3.1	8A-2.2			8A-2.1		
6A-4.2	2G-1.1	8A-2.2			8A-2.1		
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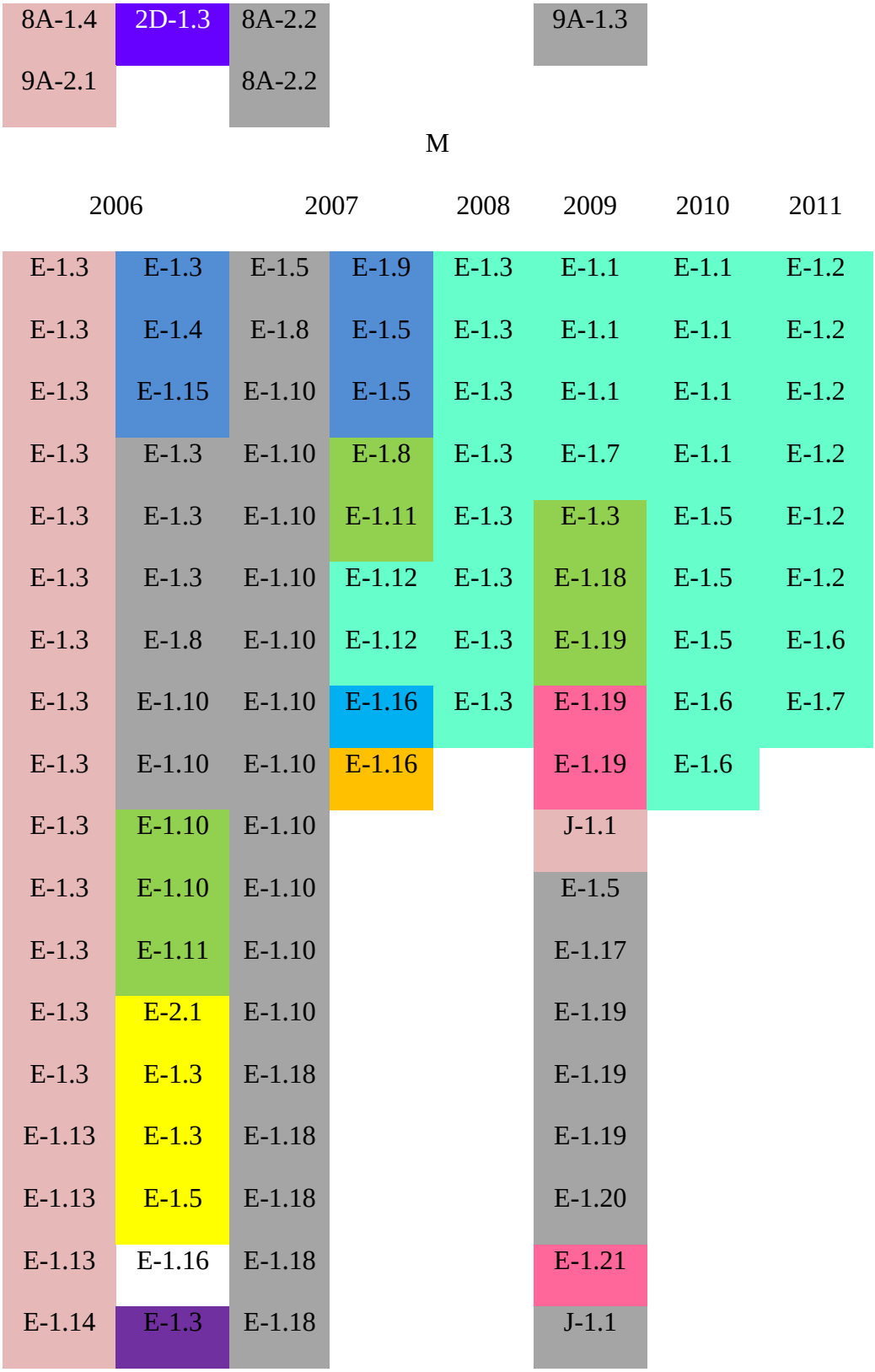
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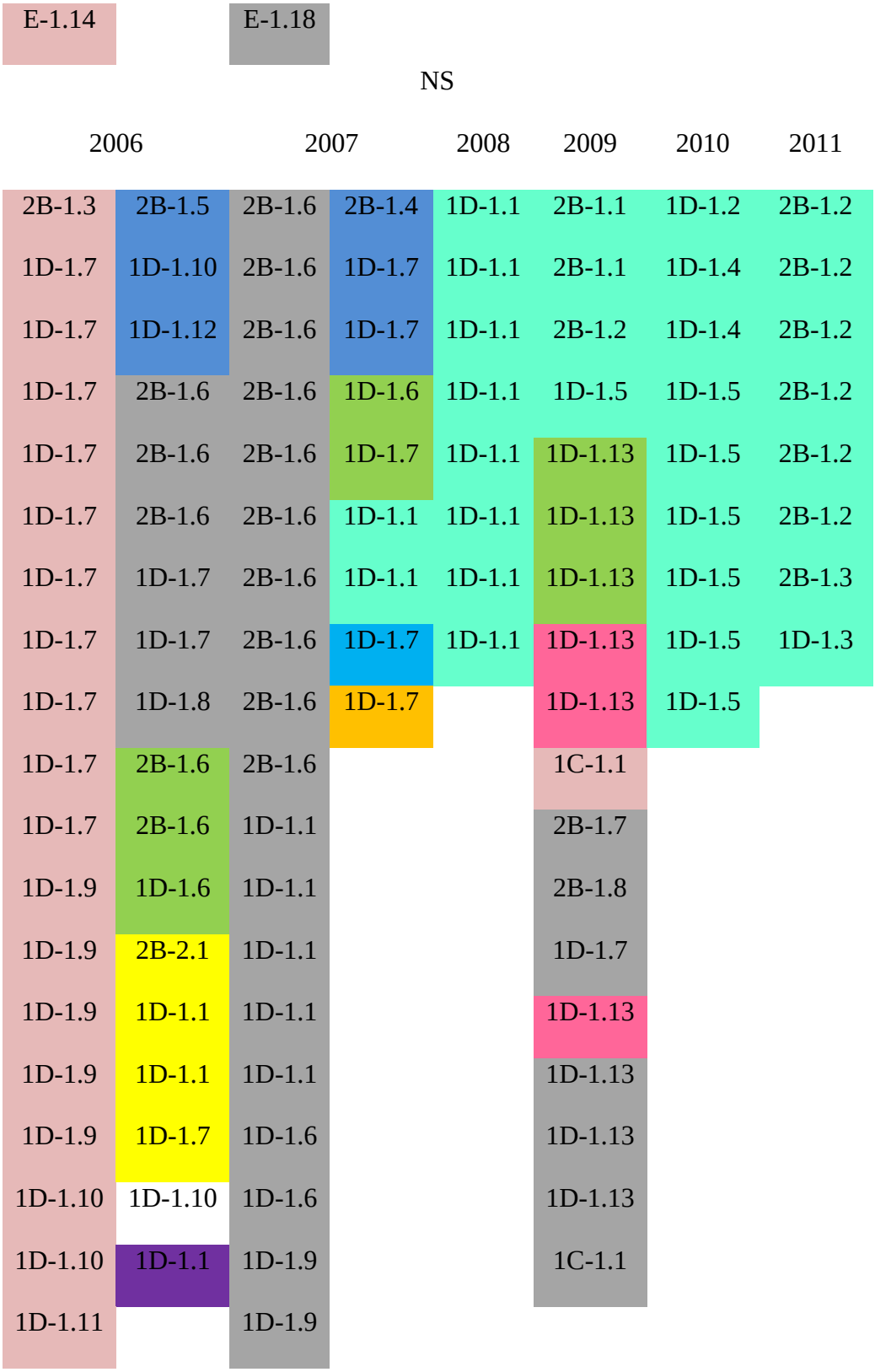
8A-2.2
9A-1.3
9A-2.1
8A-2.2
M
2006
2007
2008
2009
2010
2011
E-1.3 E-1.3 E-1.5 E-1.9 E-1.3 E-1.1 E-1.1 E-1.2
E-1.3 E-1.4 E-1.8 E-1.5 E-1.3 E-1.1 E-1.1 E-1.2
E-1.3 E-1.15 E-1.10 E-1.5 E-1.3 E-1.1 E-1.1 E-1.2
E-1.3 E-1.3 E-1.10 E-1.8 E-1.3 E-1.7 E-1.1 E-1.2
E-1.3 E-1.3 E-1.10 E-1.11 E-1.3 E-1.3 E-1.5 E-1.2
E-1.3 E-1.3 E-1.10 E-1.12 E-1.3 E-1.18 E-1.5 E-1.2
E-1.3 E-1.8 E-1.10 E-1.12 E-1.3 E-1.19 E-1.5 E-1.6
E-1.3 E-1.10 E-1.10 E-1.16 E-1.3 E-1.19 E-1.6 E-1.7
E-1.3 E-1.10 E-1.10 E-1.16 E-1.19 E-1.6
E-1.3 E-1.10 E-1.10 J-1.1
E-1.3 E-1.10 E-1.10 E-1.5
E-1.3 E-1.11 E-1.10 E-1.17
E-1.3 E-2.1 E-1.10 E-1.19
E-1.3 E-1.3 E-1.18 E-1.19
E-1.13 E-1.3 E-1.18 E-1.19
E-1.13 E-1.5 E-1.18 E-1.20
E-1.13 E-1.16 E-1.18 E-1.21
E-1.14 E-1.3 E-1.18 J-1.1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E-1.18
NS
2006
2007
2008
2009
2010
2011
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1D-1.7 1D-1.10 2B-1.6 1D-1.7 1D-1.1 2B-1.1 1D-1.4 2B-1.2
1D-1.7 1D-1.12 2B-1.6 1D-1.7 1D-1.1 2B-1.2 1D-1.4 2B-1.2
1D-1.7 2B-1.6 2B-1.6 1D-1.6 1D-1.1 1D-1.5 1D-1.5 2B-1.2
1D-1.7 2B-1.6 2B-1.6 1D-1.7 1D-1.1 1D-1.13 1D-1.5 2B-1.2
1D-1.7 2B-1.6 2B-1.6 1D-1.1 1D-1.1 1D-1.13 1D-1.5 2B-1.2
1D-1.7 1D-1.7 2B-1.6 1D-1.1 1D-1.1 1D-1.13 1D-1.5 2B-1.3
1D-1.7 1D-1.7 2B-1.6 1D-1.7 1D-1.1 1D-1.13 1D-1.5 1D-1.3
1D-1.7 1D-1.8 2B-1.6 1D-1.7 1D-1.13 1D-1.5
1D-1.7 2B-1.6 2B-1.6 1C-1.1
1D-1.7 2B-1.6 1D-1.1 2B-1.7
1D-1.9 1D-1.6 1D-1.1 2B-1.8
1D-1.9 2B-2.1 1D-1.1 1D-1.7
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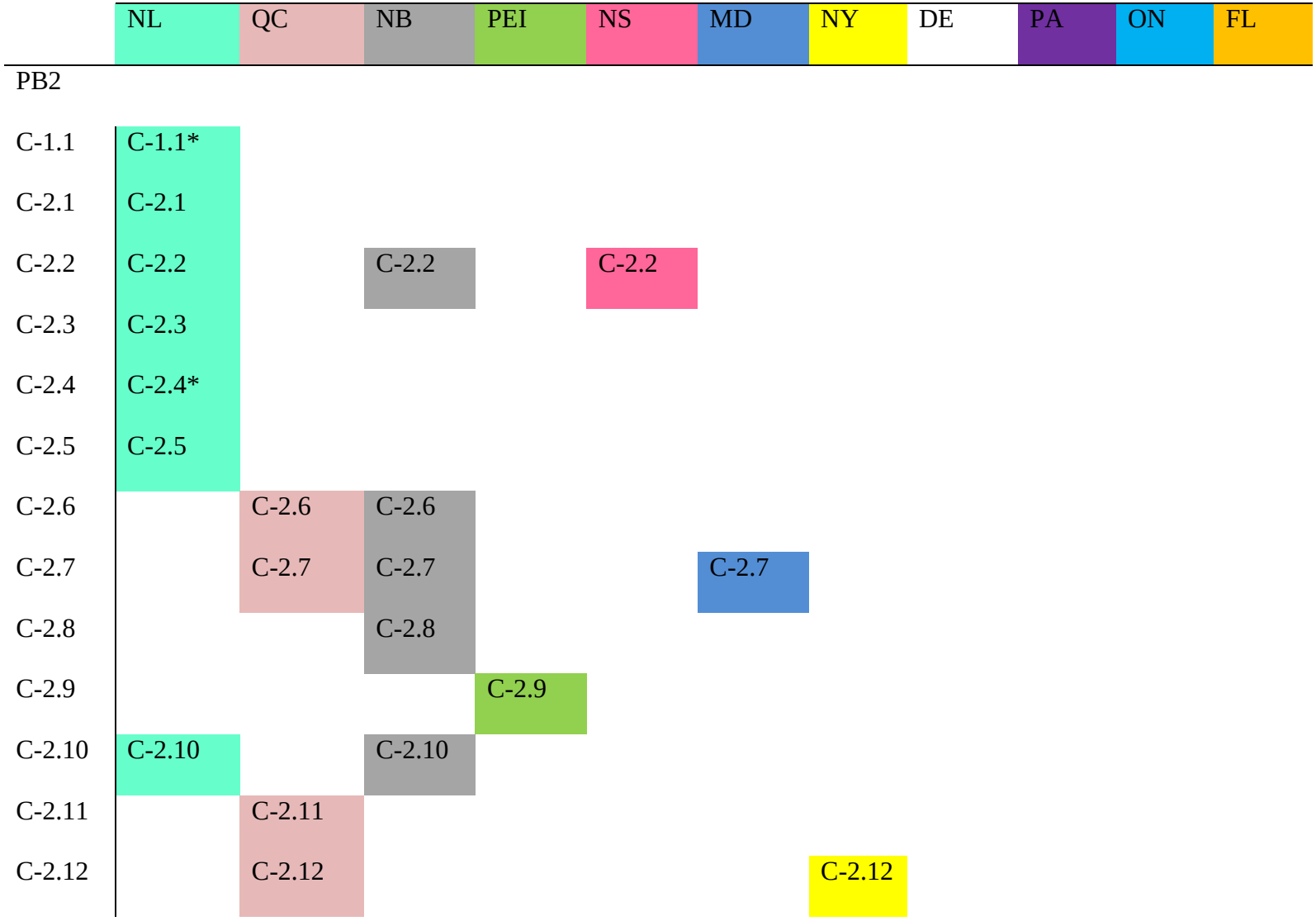
1D-1.9 1D-1.1 1D-1.1 1D-1.13
1D-1.9 1D-1.7 1D-1.6 1D-1.13
1D-1.10 1D-1.10 1D-1.6 1D-1.13
1D-1.10 1D-1.1 1D-1.9 1C-1.1
1D-1.11 1D-1.9

Figure S3. 5. Distribution of the AIV gene types from Atlantic flyway ducks by location (starting from the next page). Gene typing was done as described in the Materials and Methods section such that sequences with nucleotide identity of ≥99% are considered as homologous genes or the same gene type. Sequences from different locations within the Atlantic bird flyway are shown in different colours, as indicated in the legend. The locations are abbreviated as follows: NL, Newfoundland; QC, Quebec; MD, Maryland; NB, New Brunswick; PEI, Prince Edward Island; NY, New York; PA, Pennsylvania; DE, Delaware; ON, Ontario; FL, Florida; NS, Nova Scotia. The 18 gene types detected only in Newfoundland are labeled with asterisk (*).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NS
MD
NY
DE
PA
ON
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C-2.4
C-2.5
C-2.6
C-2.7
C-2.8
C-2.9
C-2.10
C-2.11
C-2.12
C-1.1*
C-2.1
C-2.2
C-2.3
C-2.4*
C-2.5
C-2.6
C-2.7
C-2.8
C-2.9
C-2.10
C-2.11
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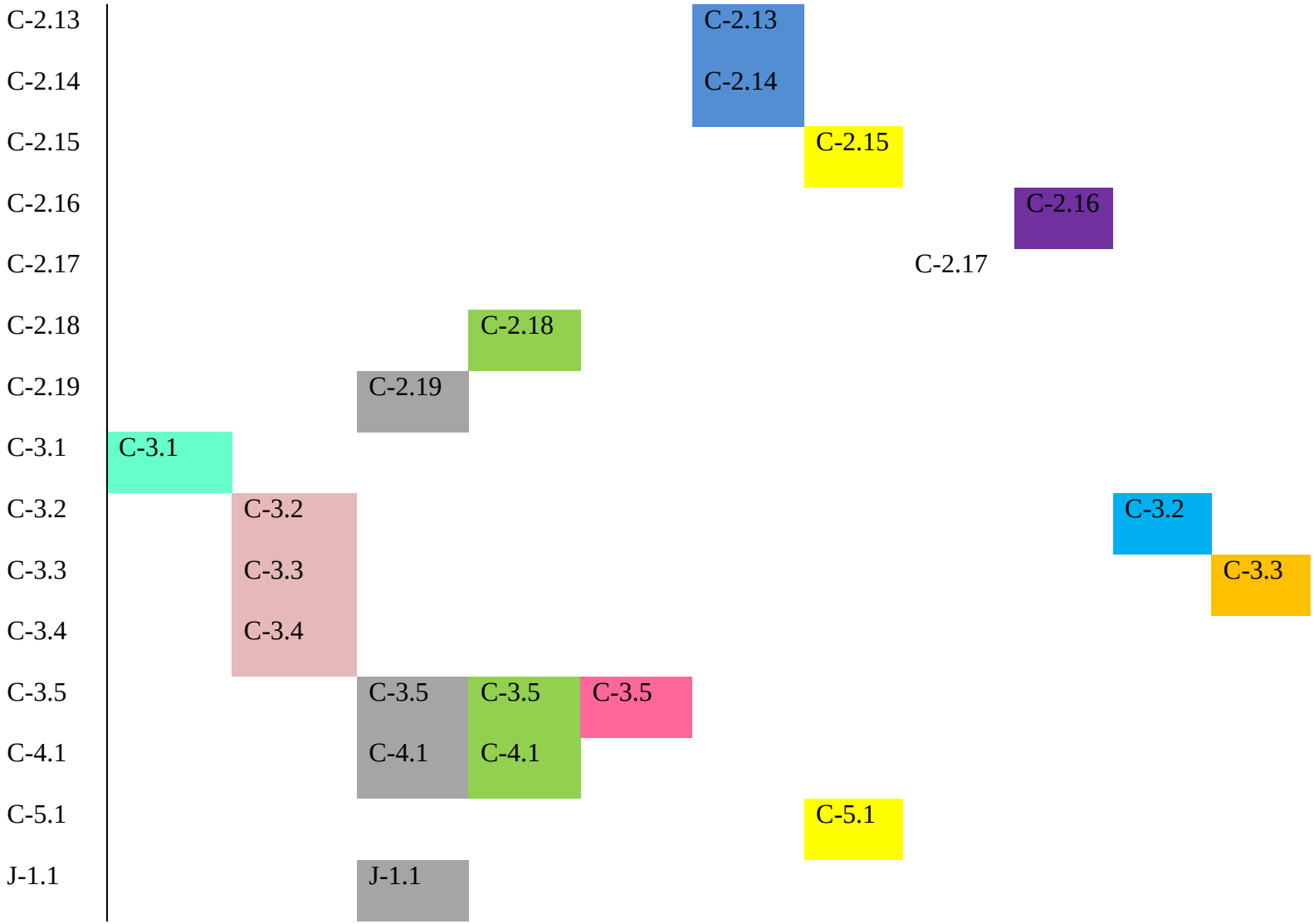
C-2.12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C-3.3
C-3.4
C-3.5
C-4.1
C-5.1
J-1.1
C-2.13
C-2.14
C-2.15
C-2.16
C-2.17
C-2.18
C-2.19
C-3.1
C-3.2
C-3.3
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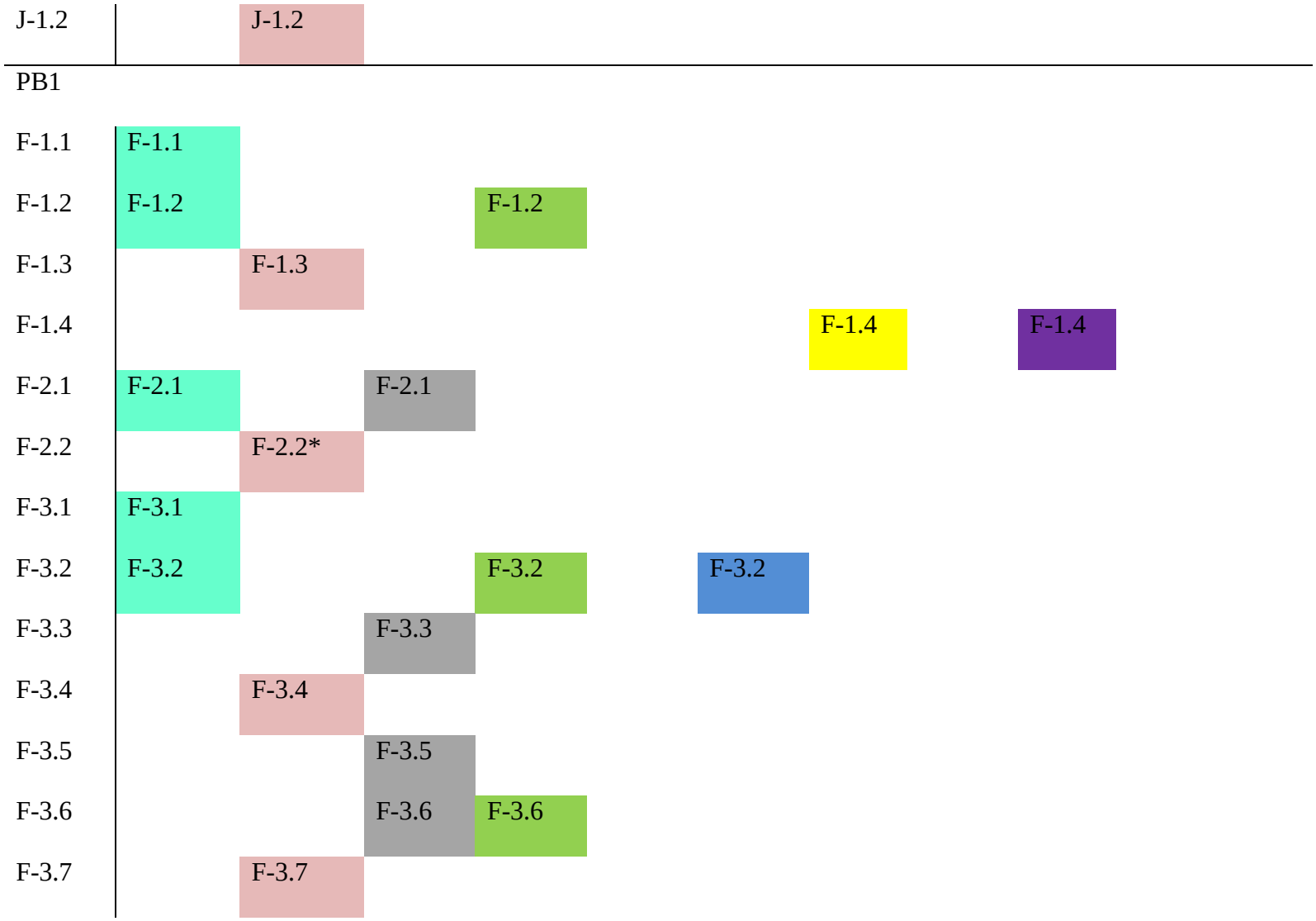
C-4.1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F-1.2
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F-1.4
F-1.4
F-1.4
F-2.1
F-2.1
F-2.1
F-2.2
F-2.2*
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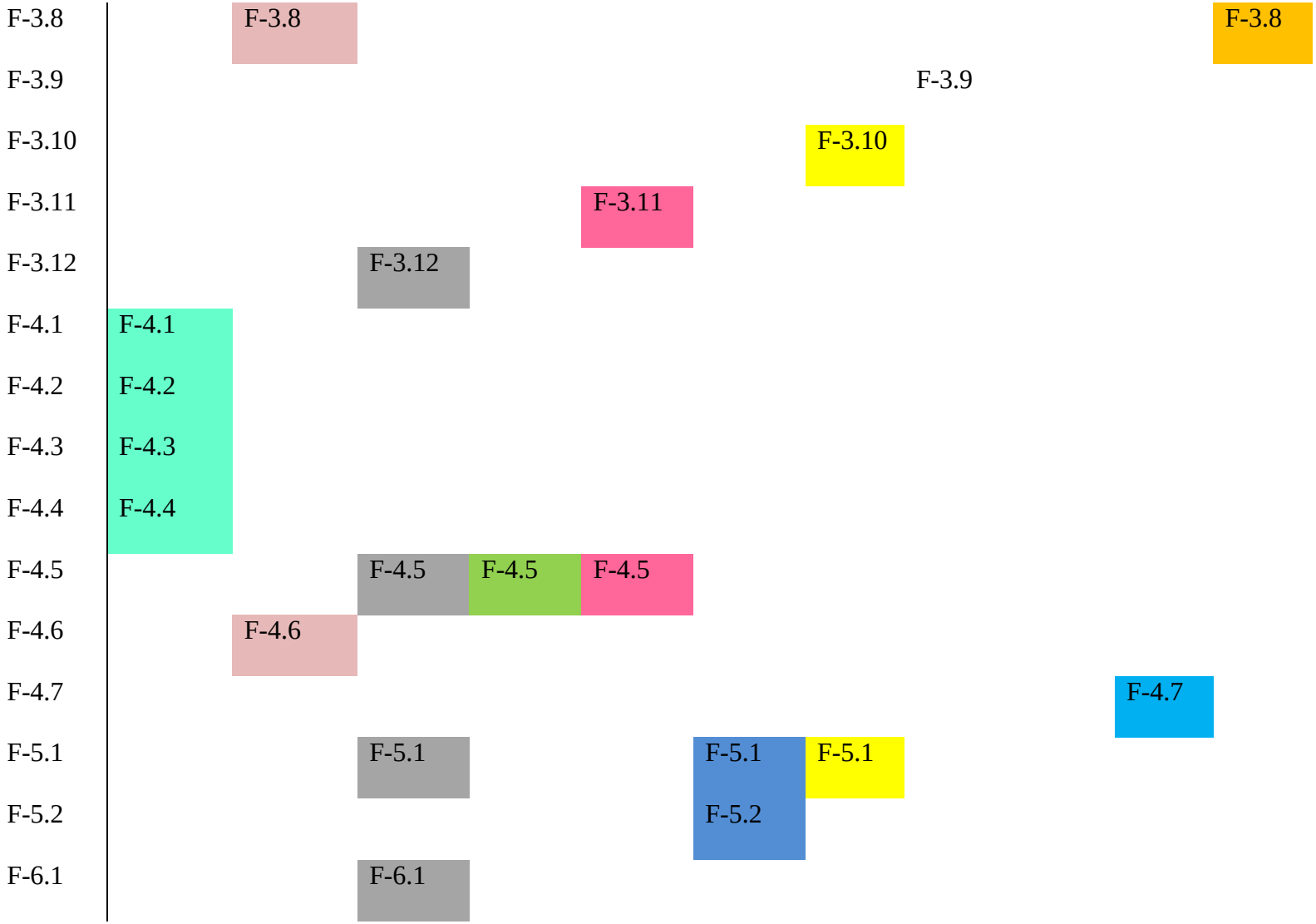
F-3.7
F-3.7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F-4.7
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F-3.8
F-3.8
F-3.9
F-3.10
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F-4.4
F-4.5
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F-4.7
F-5.1
F-5.1
F-5.1
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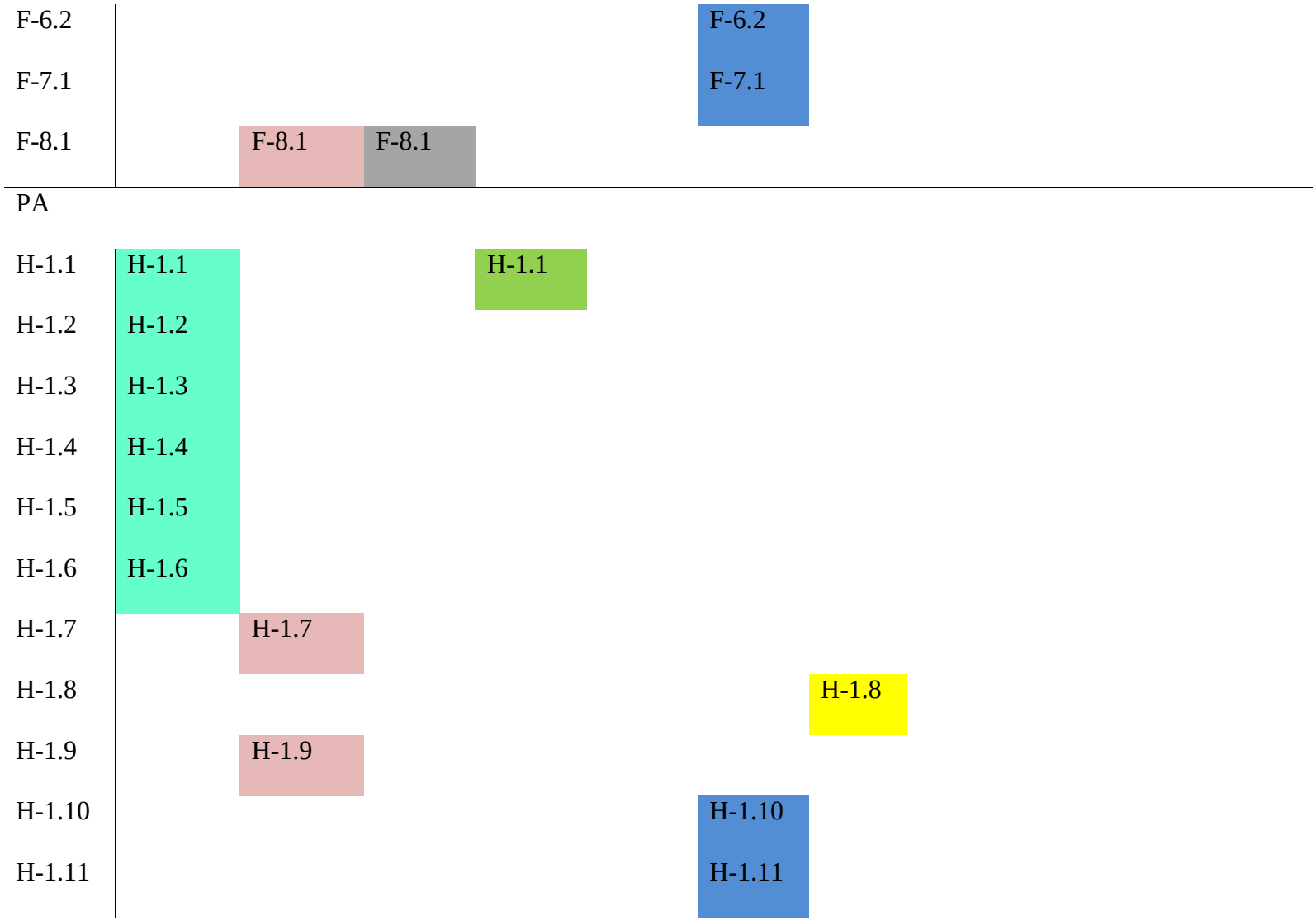
F-6.1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PA
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H-1.5
H-1.6
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H-1.11
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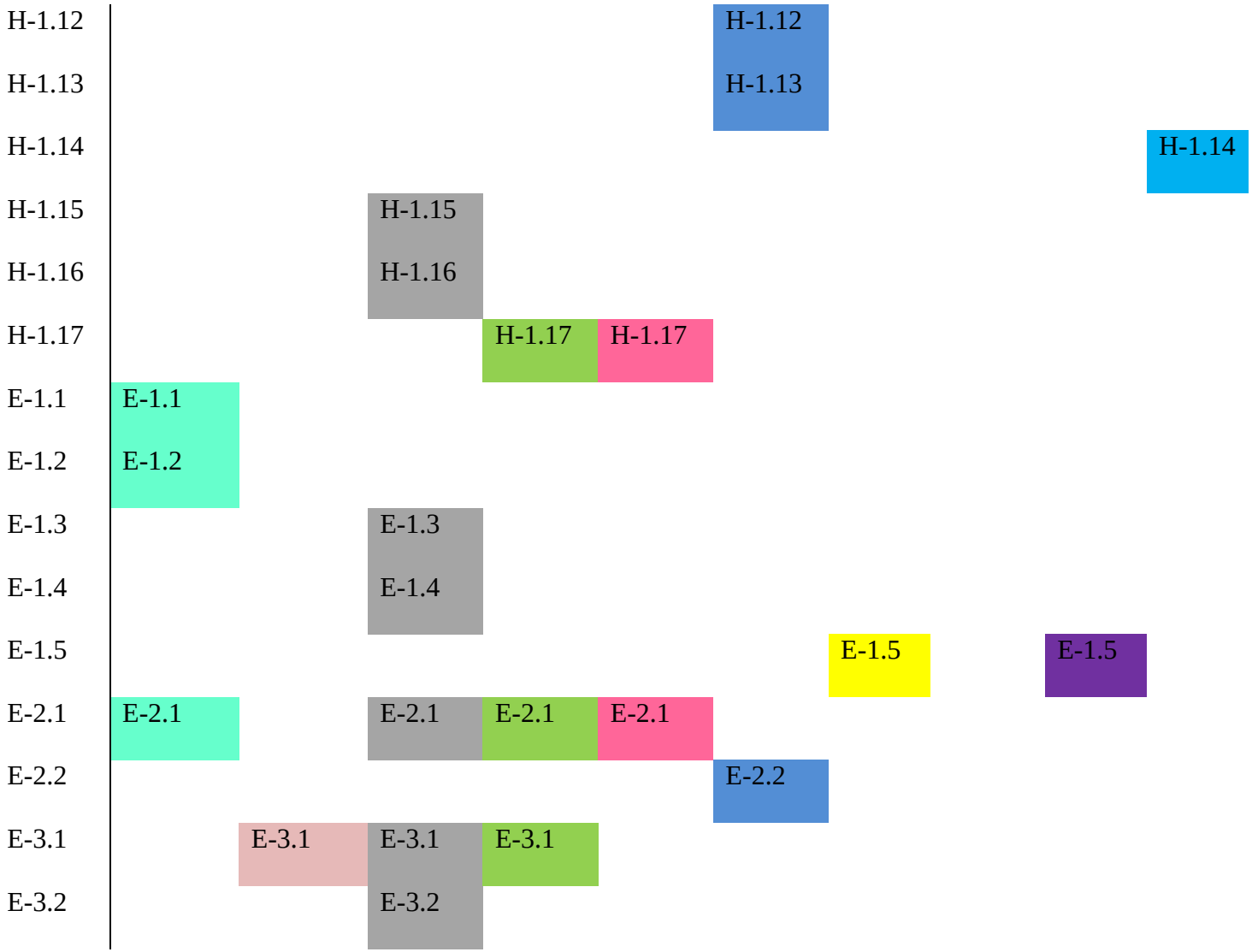
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E-1.5
E-2.1
E-2.2
E-3.1
E-3.2
H-1.12
H-1.13
H-1.14
H-1.15
H-1.16
H-1.17
H-1.17
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E-1.2
E-1.3
E-1.4
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E-3.1
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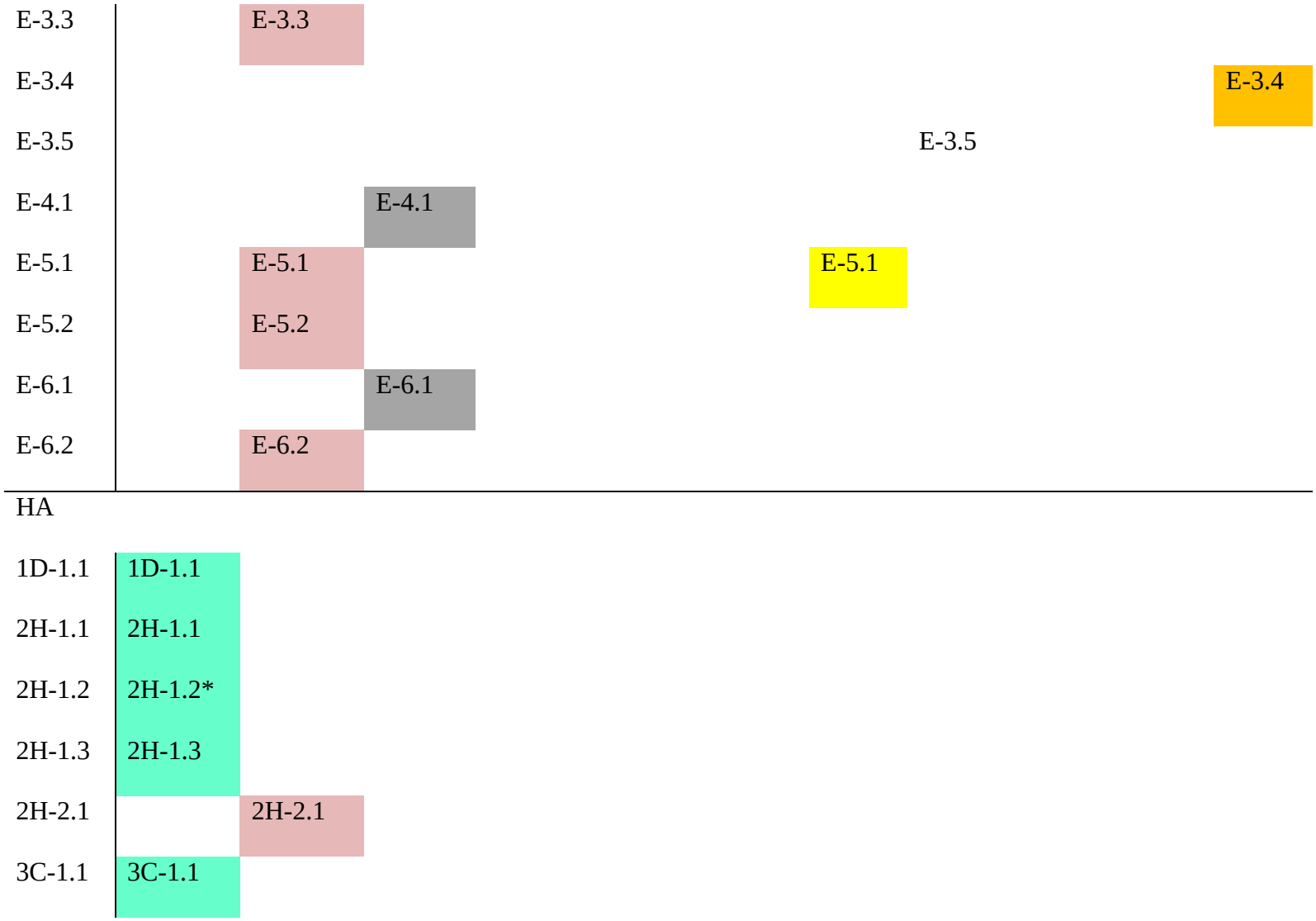
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E-4.1
E-5.1
E-5.2
E-6.1
E-6.2
E-3.4
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E-5.1
HA
1D-1.1
2H-1.1
2H-1.2
2H-1.3
2H-2.1
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1D-1.1
2H-1.1
2H-1.2*
2H-1.3
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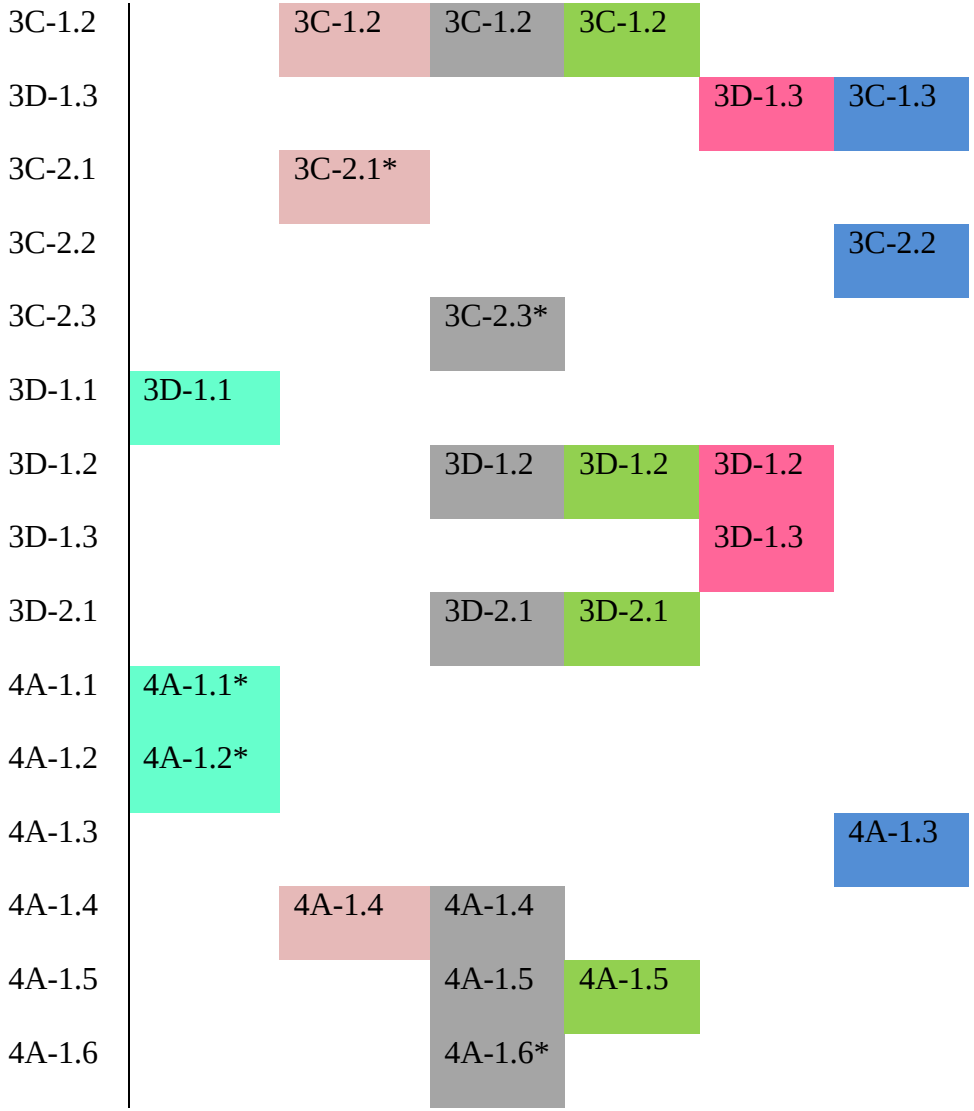
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3D-2.1
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4A-1.3
4A-1.4
4A-1.5
4A-1.6
3C-1.2
3C-1.2
3C-1.2
3D-1.3
3C-1.3
3C-2.1*
3C-2.2
3C-2.3*
3D-1.1
3D-1.2
3D-1.2
3D-1.2
3D-1.3
3D-2.1
3D-2.1
4A-1.1*
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4A-1.3
4A-1.4
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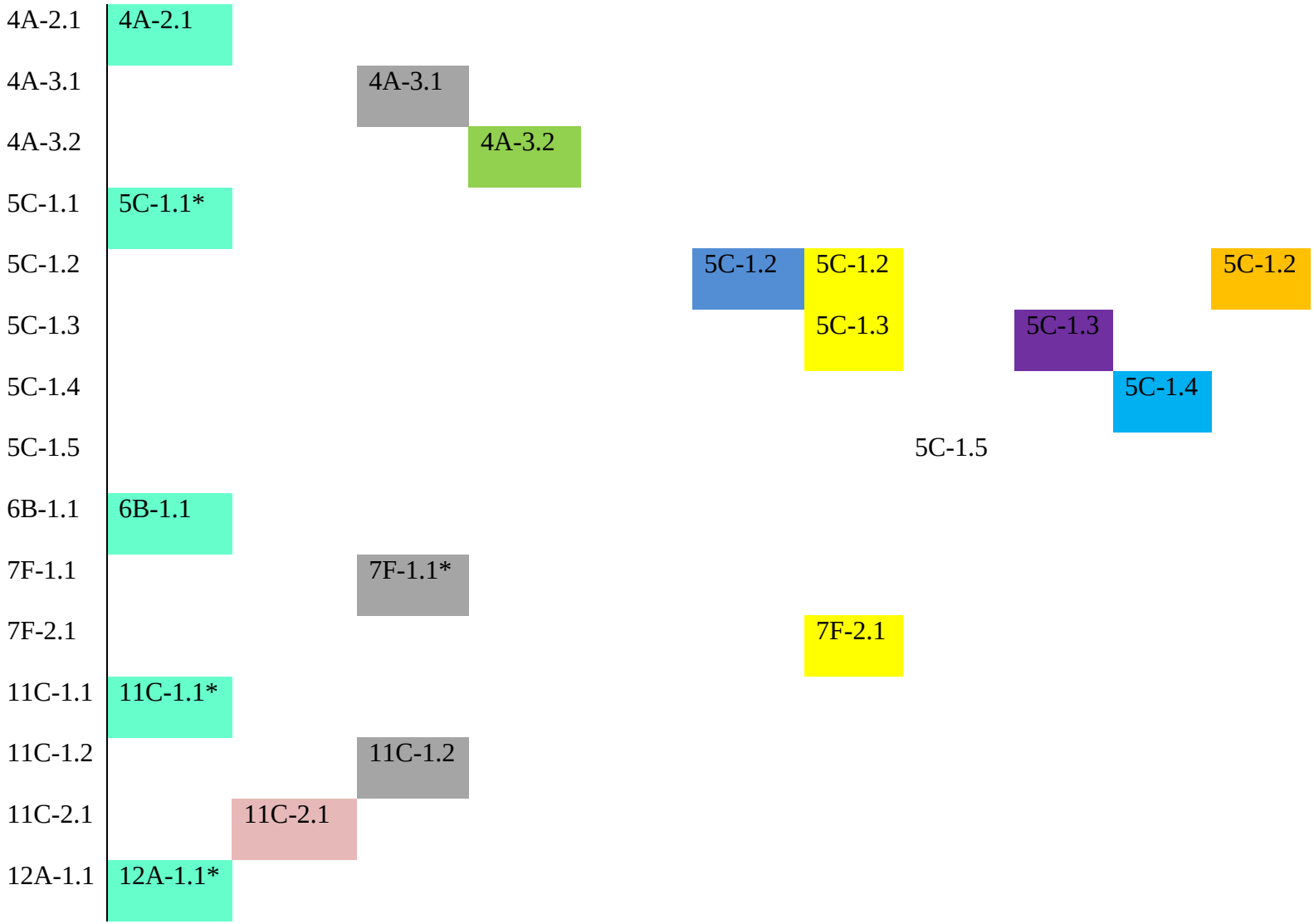
4A-1.5
4A-1.5
4A-1.6*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6B-1.1
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7F-2.1
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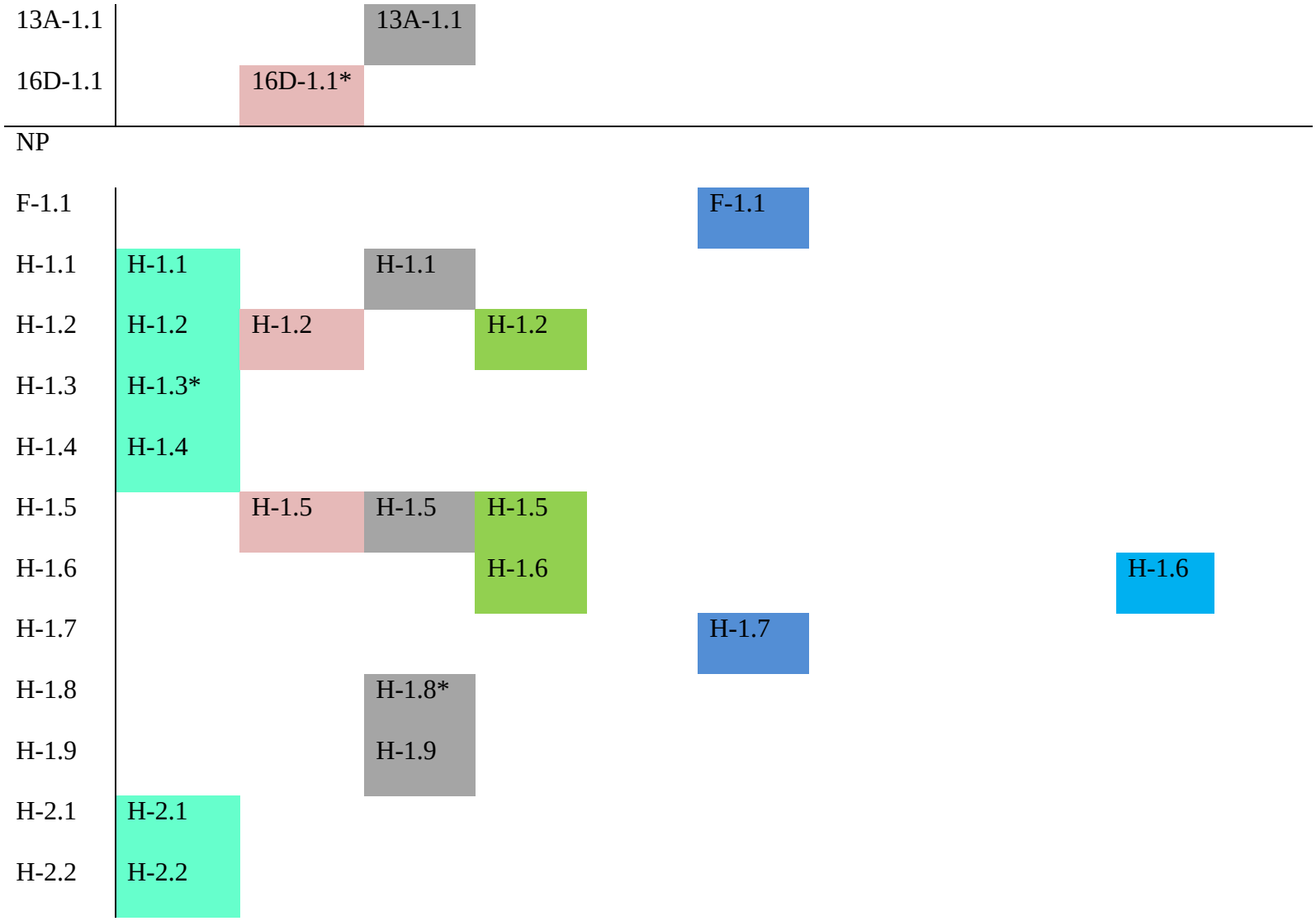
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H-2.2
F-1.1
H-1.1
H-1.2
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H-1.4
H-1.5
H-1.6
H-1.7
H-1.8*
H-1.9
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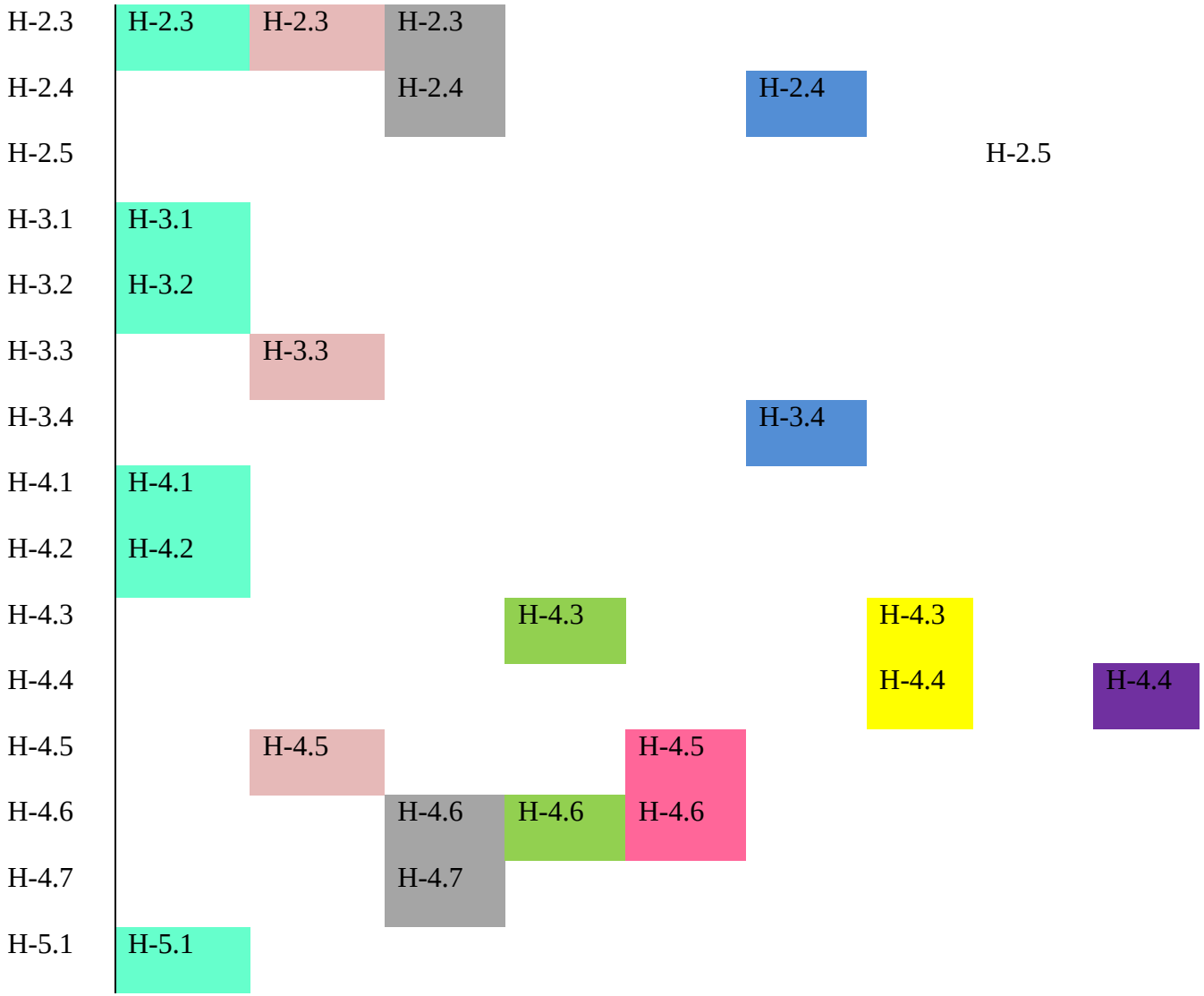
H-1.6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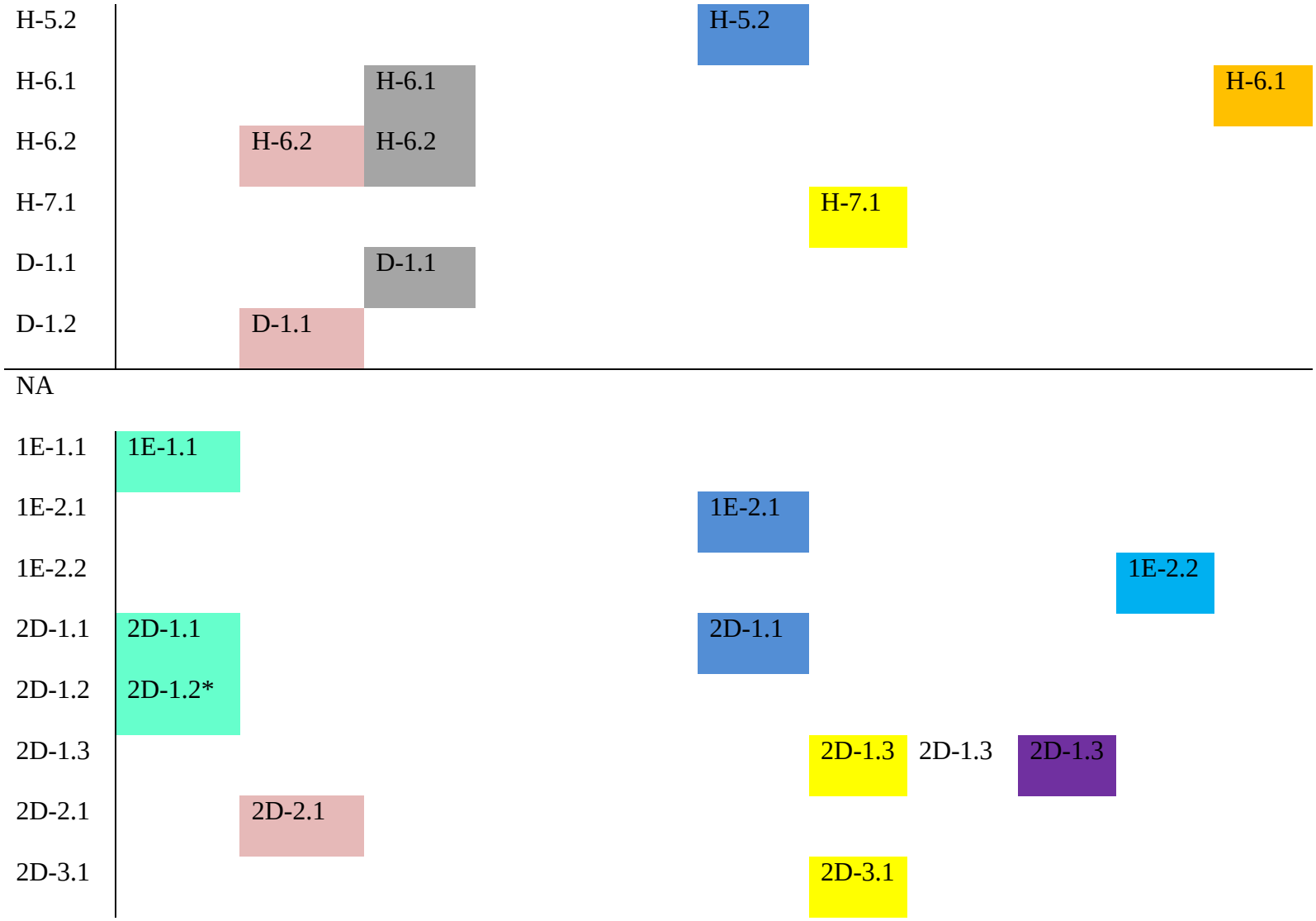
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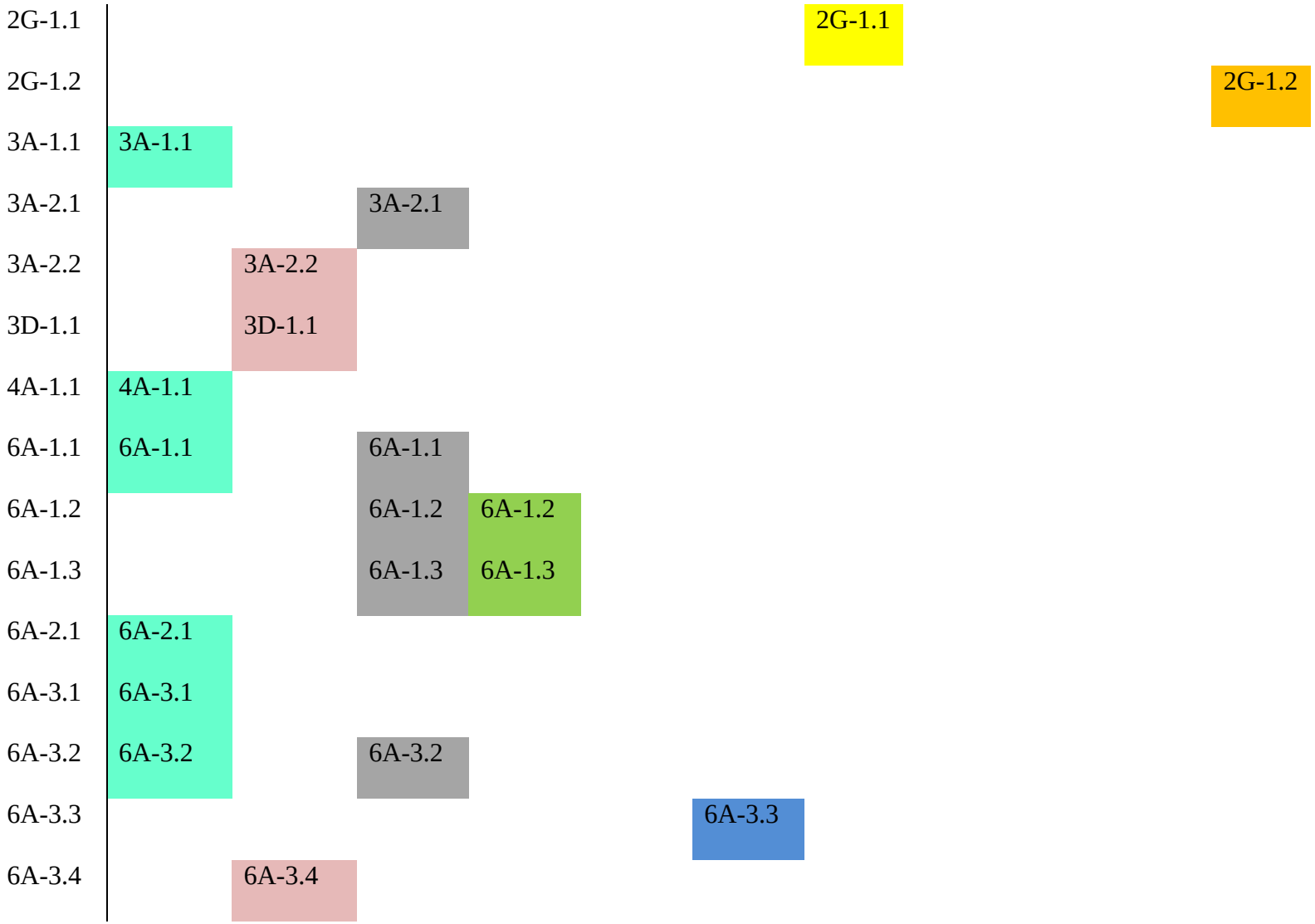
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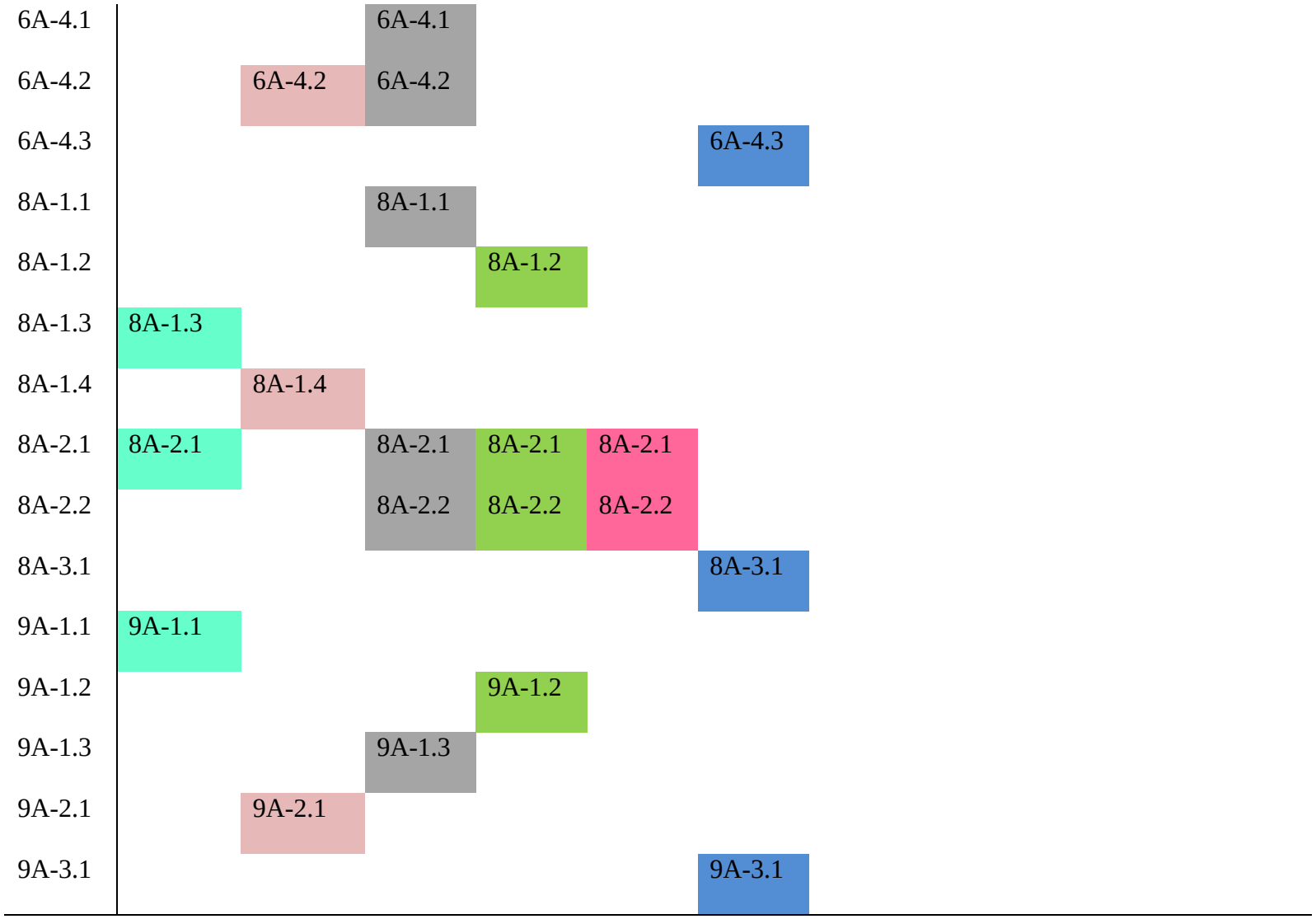
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9A-1.2
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9A-2.1
9A-3.1
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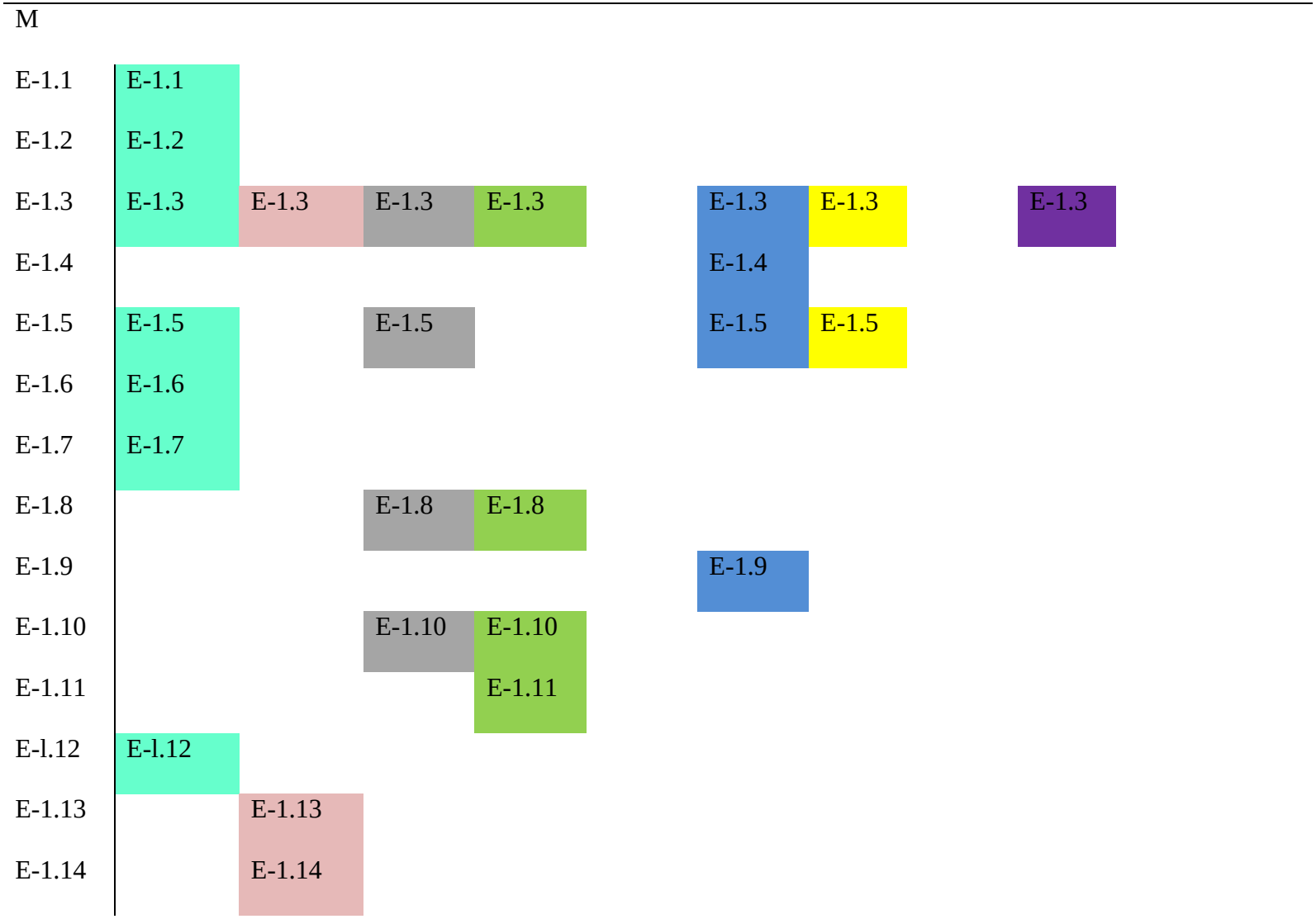
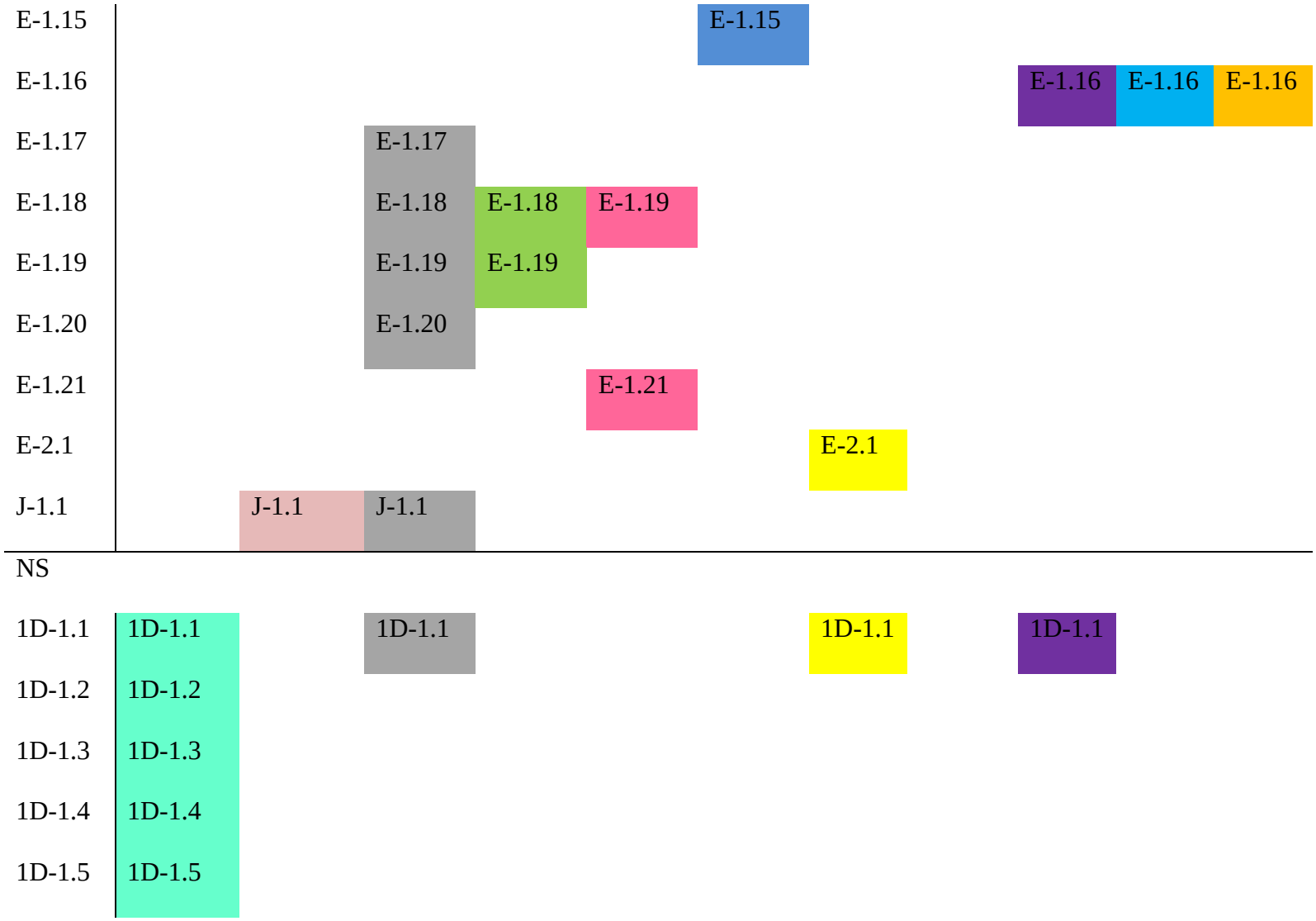
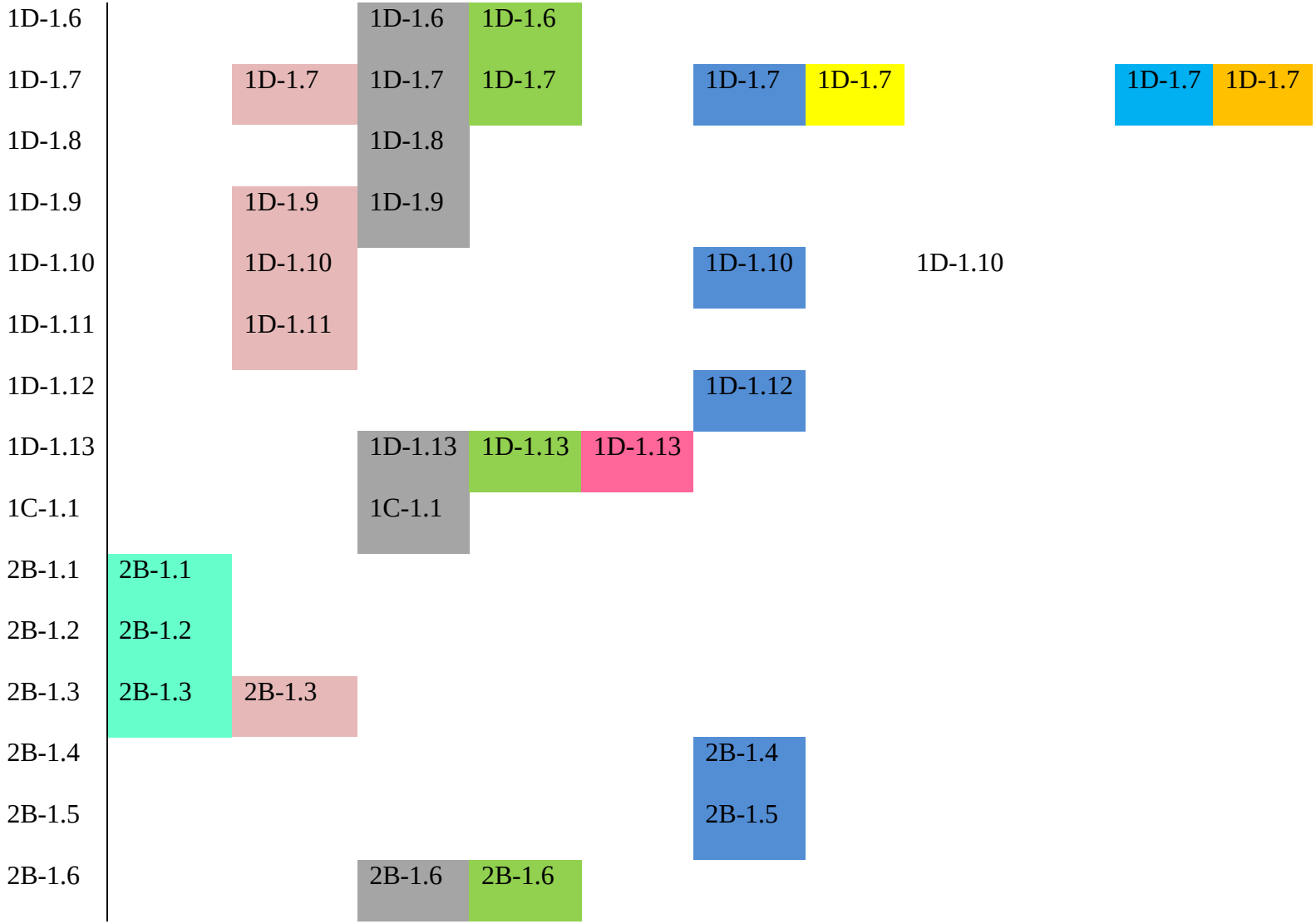
9A-1.3
9A-2.1
9A-3.1

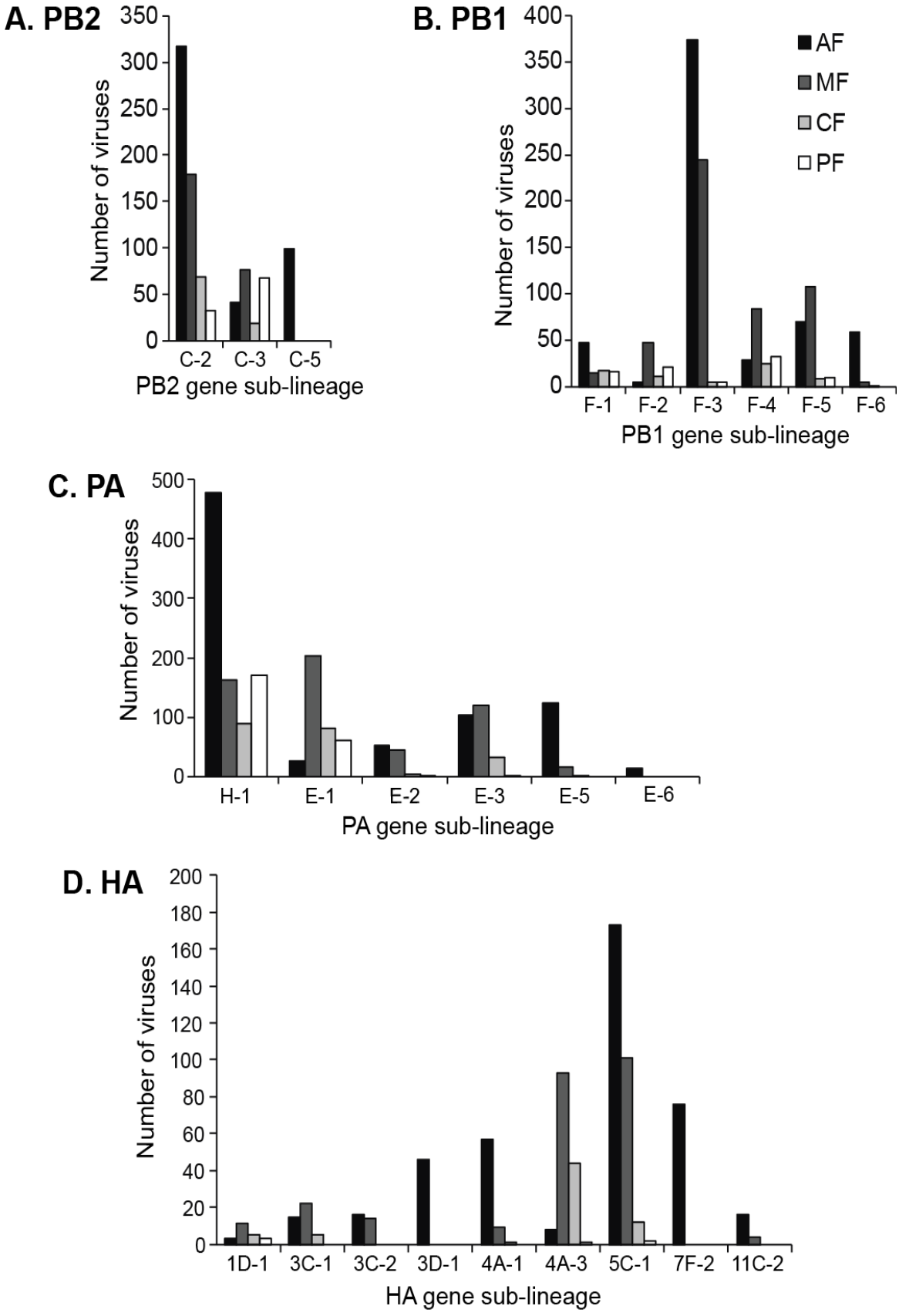

M
E-1.1
E-1.2
E-1.3
E-1.4
E-1.5
E-1.6
E-1.7
E-1.8
E-1.9
E-1.10
E-1.11
E-l.12
E-1.13
E-1.14
E-1.1
E-1.2
E-1.3
E-1.5
E-1.6
E-1.7
E-1.3
E-1.13
E-1.14
E-1.3
E-1.5
E-1.8
E-1.10
E-1.3
E-1.8
E-1.10
E-1.11
E-1.3
E-1.4
E-1.5
E-1.9
E-1.3
E-1.5
E-1.3

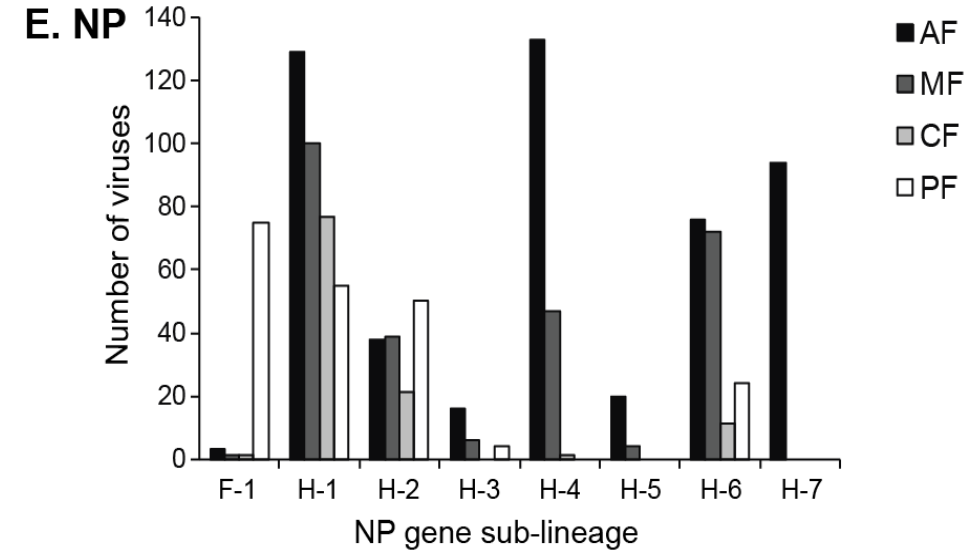

E-1.15
E-1.16
E-1.17
E-1.18
E-1.19
E-1.20
E-1.21
E-2.1
J-1.1
NS
1D-1.1
1D-1.2
1D-1.3
1D-1.4
1D-1.5
E-1.15
E-1.16
E-1.16
E-1.16
E-1.17
E-1.18
E-1.19
E-1.18
E-1.19
E-1.20
E-1.21
E-2.1
J-1.1
J-1.1
1D-1.1
1D-1.1
1D-1.1
1D-1.1
1D-1.2
1D-1.3
1D-1.4
1D-1.5

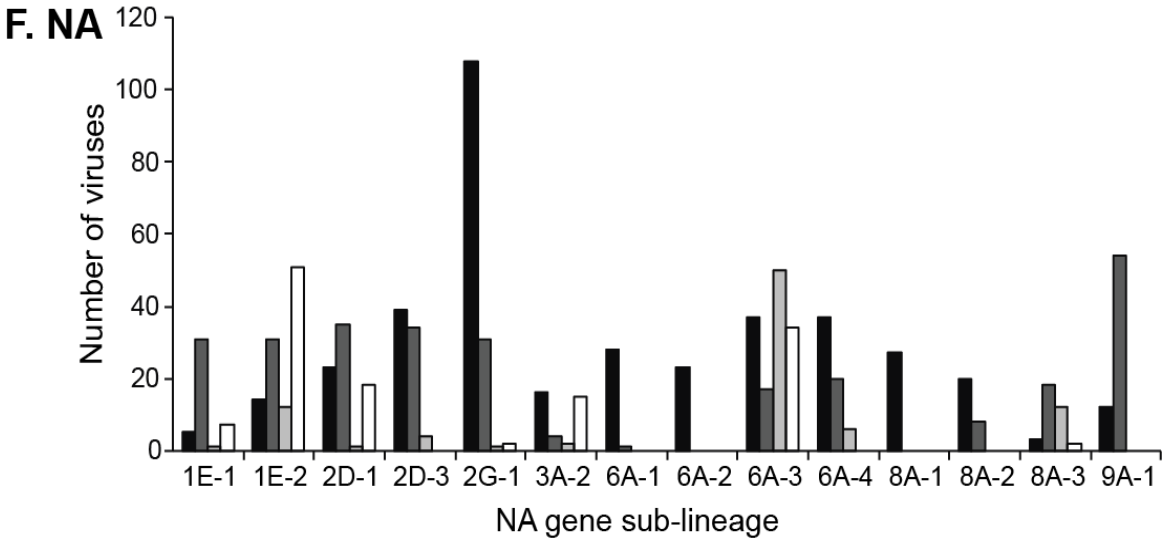

1D-1.6
1D-1.7
1D-1.8
1D-1.9
1D-1.10
1D-1.11
1D-1.12
1D-1.13
1C-1.1
2B-1.1
2B-1.2
2B-1.3
2B-1.4
2B-1.5
2B-1.6
1D-1.6
1D-1.6
1D-1.7
1D-1.7
1D-1.7
1D-1.8
1D-1.9
1D-1.9
1D-1.7
1D-1.7
1D-1.7
1D-1.7
1D-1.10
1D-1.10
1D-1.10
1D-1.11
1D-1.12
1D-1.13
1D-1.13
1D-1.13
1C-1.1
2B-1.1
2B-1.2
2B-1.3
2B-1.3
2B-1.4
2B-1.5
2B-1.6
2B-1.6

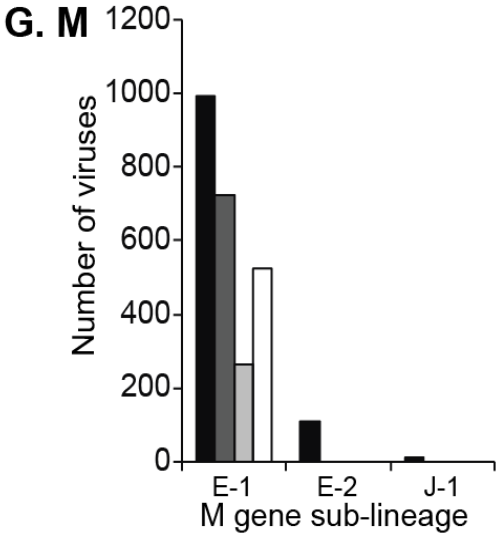
2B-1.7		2B-1.7	
2B-1.8		2B-1.8	
2B-2.1			2B-2.1

Figure S3.6. Spatial distribution of detections of the Atlantic flyway duck AIV gene sub-lineages in North American flyways (starting from the next page). The numbers of viruses identified in each flyway are shown for the indicated gene sub-lineages that were identified in the 2006-2011 Atlantic flyway duck viruses. Only sub-lineages with ≥10 genes detected in at least one flyway are shown. Flyways are shown in different shading: Atlantic flyway (AF), black; Mississippi flyway (MF), dark grey; Central flyway (CF), light grey; Pacific flyway (PF), white.


A. PB2
Number of viruses
350
300
250
200
150
100
50
0
C-2
C-3
C-5
PB2 gene sub-lineage
B. PB1
Number of viruses
400
350
300
250
200
150
100
50
0
AF
MF
CF
PF
F-1
F-2
F-3
F-4
F-5
F-6
PB1 gene sub-lineage
C. PA
Number of viruses
500
400
300
200
100
0
H-1
E-1
E-2
E-3
E-5
E-6
PA gene sub-lineage
D. HA
Number of viruses
200
180
160
140
120
100
80
60
40
20
0
1D-1
3C-1
3C-2
3D-1
4A-1
4A-3
5C-1
7F-2
11C-2
HA gene sub-lineage


E. NP
Number of viruses
140
120
100
80
60
40
20
0
AF
MF
CF
PF
F-1
H-1
H-2
H-3
H-4
H-5
H-6
H-7
NP gene sub-lineage


F. NA
Number of viruses
120
100
80
60
40
20
0
1E-1
1E-2
2D-1
2D-3
2G-1
3A-2
6A-1
6A-2
6A-3
6A-4
8A-1
8A-2
8A-3
9A-1
NA gene sub-lineage


G. M
Number of viruses
1200
1000
800
600
400
200
0
E-1
E-2
J-1
M gene sub-lineage

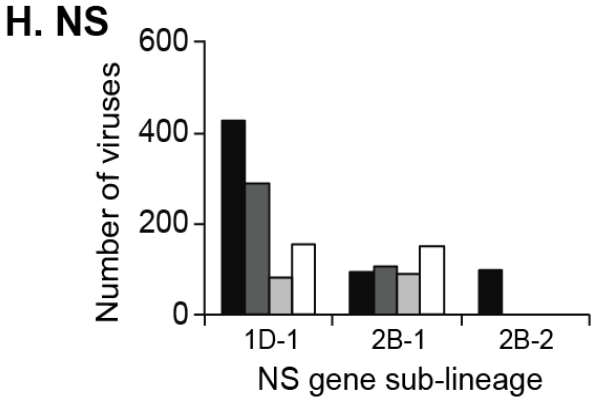
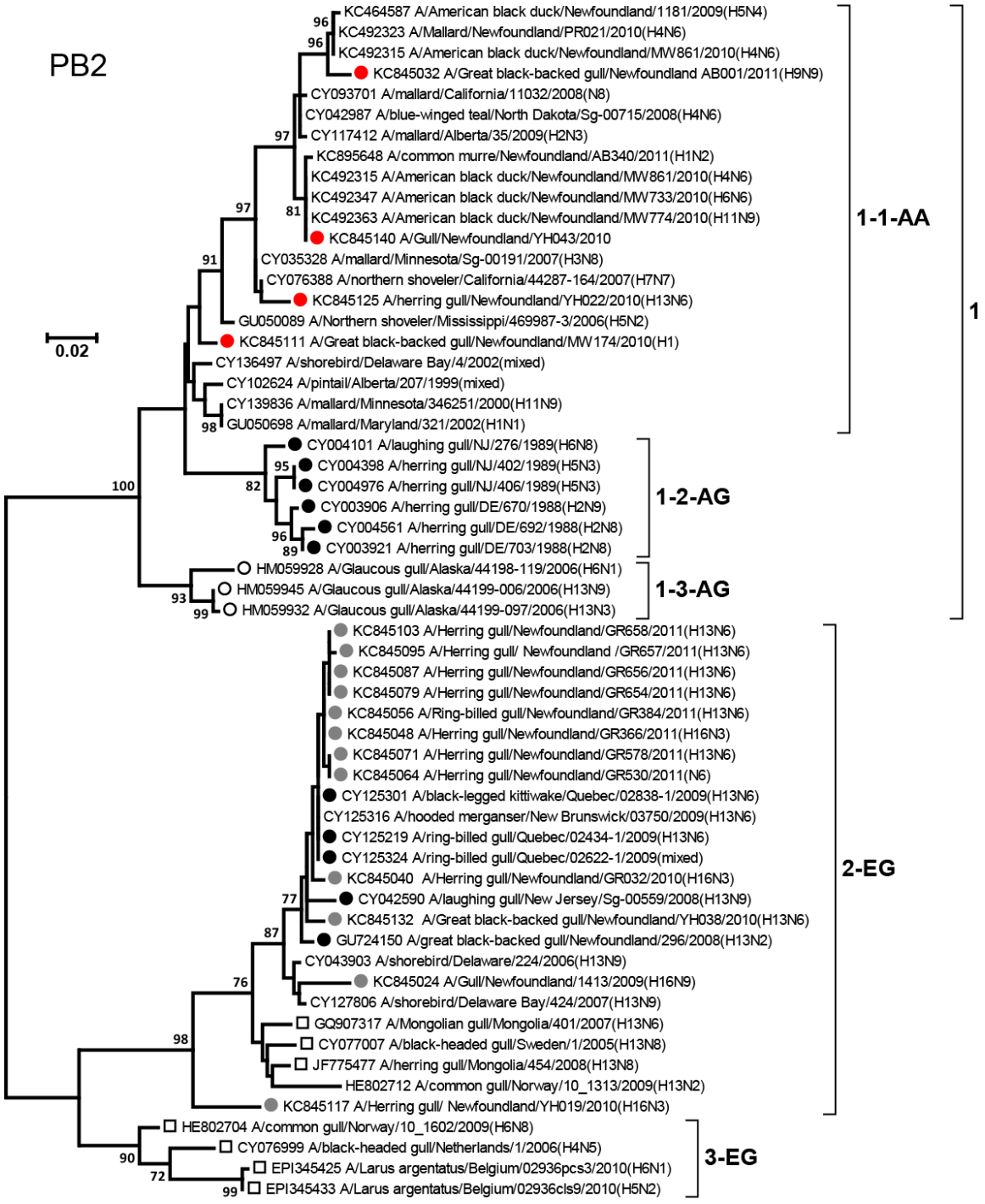

H. NS
Number of viruses
600
400
200
0
1D-1
2B-1
2B-2
NS gene sub-lineage

Figure S4.1. Phylogenetic trees for the internal protein gene segments with virus identification information (starting from the next page). The gull AIV sequences from Newfoundland (from 2009-2011) are labelled according to the geographic and host group affiliation of their clades: yellow circle for North American gull (AG), red circle for North American avian (AA), grey circle for Eurasian gull (EG) and blue circle for Eurasian avian (EA). Reference gull AIV sequences from Atlantic and Pacific North America are labelled with black and open circles, respectively, while those from Eurasia are labelled with open squares. Gene lineages and clades were assigned with serial numbers as described in the Material and Method section. Phylogenetic maximum likelihood (ML) trees were constructed using MEGA5 with 1000 bootstrap replicates and bootstrap values ≥70% are given at the nodes. The scale bars indicate substitutions per site.


PB2
0.02
KC464587 A/American black duck/Newfoundland/1181/2009(H5N4)
KC492323 A/Mallard/Newfoundland/PR021/2010(H4N6)
KC492315 A/American black duck/Newfoundland/MW861/2010(H4N6)
KC845032 A/Great black-backed gull/Newfoundland AB001/2011(H9N9)
CY093701 A/mallard/California/11032/2008(N8)
CY042987 A/blue-winged teal/North Dakota/Sg-00715/2008(H4N6)
CY117412 A/mallard/Alberta/35/2009(H2N3)
KC895648 A/common murre/Newfoundland/AB340/2011(H1N2)
KC492315 A/American black duck/Newfoundland/MW861/2010(H4N6)
KC492347 A/American black duck/Newfoundland/MW733/2010(H6N6)
KC492363 A/American black duck/Newfoundland/MW774/2010(H11N9)
KC845140 A/Gull/Newfoundland/YH043/2010
CY035328 A/mallard/Minnesota/Sg-00191/2007(H3N8)
CY076388 A/northern shoveler/California/44287-164/2007(H7N7)
KC845125 A/herring gull/Newfoundland/YH022/2010(H13N6)
GU050089 A/Northern shoveler/Mississippi/469987-3/2006(H5N2)
KC845111 A/Great black-backed gull/Newfoundland/MW174/2010(H1)
CY136497 A/shorebird/Delaware Bay/4/2002(mixed)
CY102624 A/pintail/Alberta/207/1999(mixed)
CY139836 A/mallard/Minnesota/346251/2000(H11N9)
GU050698 A/mallard/Maryland/321/2002(H1N1)
CY004101 A/laughing gull/NJ/276/1989(H6N8)
CY004398 A/herring gull/NJ/402/1989(H5N3)
CY004976 A/herring gull/NJ/406/1989(H5N3)
CY003906 A/herring gull/DE/670/1988(H2N9)
CY004561 A/herring gull/DE/692/1988(H2N8)
CY003921 A/herring gull/DE/703/1988(H2N8)
HM059928 A/Glaucous gull/Alaska/44198-119/2006(H6N1)
HM059945 A/Glaucous gull/Alaska/44199-006/2006(H13N9)
HM059932 A/Glaucous gull/Alaska/44199-097/2006(H13N3)
KC845103 A/Herring gull/Newfoundland/GR658/2011(H13N6)
KC845095 A/Herring gull/ Newfoundland /GR657/2011(H13N6)
KC845087 A/Herring gull/Newfoundland/GR656/2011(H13N6)
KC845079 A/Herring gull/Newfoundland/GR654/2011(H13N6)
KC845056 A/Ring-billed gull/Newfoundland/GR384/2011(H13N6)
KC845048 A/Herring gull/Newfoundland/GR366/2011(H16N3)
KC845071 A/Herring gull/Newfoundland/GR578/2011(H13N6)
KC845064 A/Herring gull/Newfoundland/GR530/2011(N6)
CY125301 A/black-legged kittiwake/Quebec/02838-1/2009(H13N6)
CY125316 A/hooded merganser/New Brunswick/03750/2009(H13N6)
CY125219 A/ring-billed gull/Quebec/02434-1/2009(H13N6)
CY125324 A/ring-billed gull/Quebec/02622-1/2009(mixed)
KC845040 A/Herring gull/Newfoundland/GR032/2010(H16N3)
CY042590 A/laughing gull/New Jersey/Sg-00559/2008(H13N9)
KC845132 A/Great black-backed gull/Newfoundland/YH038/2010(H13N6)
GU724150 A/great black-backed gull/Newfoundland/296/2008(H13N2)
CY043903 A/shorebird/Delaware/224/2006(H13N9)
KC845024 A/Gull/Newfoundland/1413/2009(H16N9)
CY127806 A/shorebird/Delaware Bay/424/2007(H13N9)
GQ907317 A/Mongolian gull/Mongolia/401/2007(H13N6)
CY077007 A/black-headed gull/Sweden/1/2005(H13N8)
JF775477 A/herring gull/Mongolia/454/2008(H13N8)
HE802712 A/common gull/Norway/10_1313/2009(H13N2)
KC845117 A/Herring gull/ Newfoundland/YH019/2010(H16N3)
HE802704 A/common gull/Norway/10_1602/2009(H6N8)
CY076999 A/black-headed gull/Netherlands/1/2006(H4N5)
EPI345425 A/Larus argentatus/Belgium/02936pcs3/2010(H6N1)
EPI345433 A/Larus argentatus/Belgium/02936cls9/2010(H5N2)
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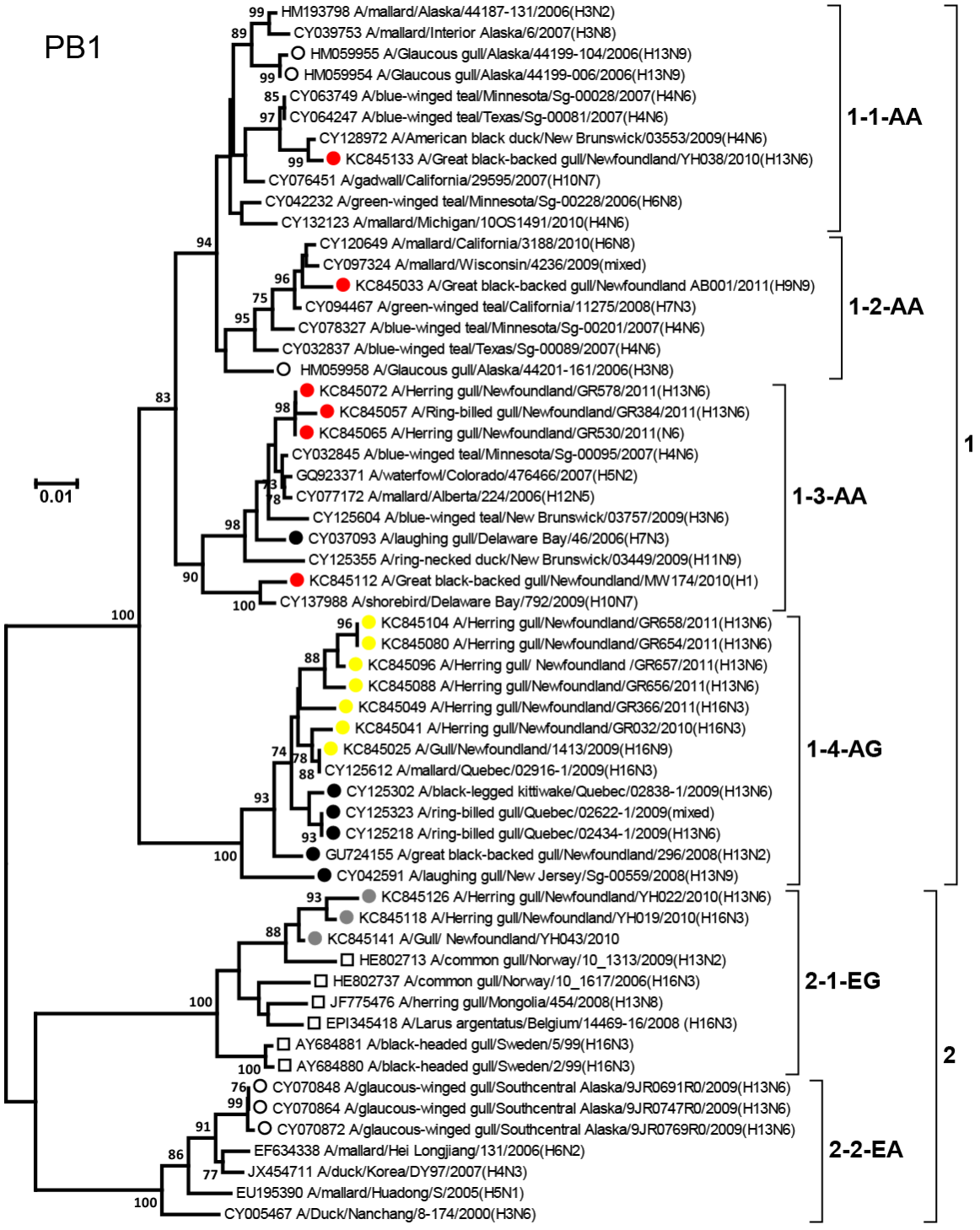
1-2-AG
1-3-AG
2-EG
3-EG
1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HM193798 A/mallard/Alaska/44187-131/2006(H3N2)
CY039753 A/mallard/Interior Alaska/6/2007(H3N8)
HM059955 A/Glaucous gull/Alaska/44199-104/2006(H13N9)
HM059954 A/Glaucous gull/Alaska/44199-006/2006(H13N9)
CY063749 A/blue-winged teal/Minnesota/Sg-00028/2007(H4N6)
CY064247 A/blue-winged teal/Texas/Sg-00081/2007(H4N6)
CY128972 A/American black duck/New Brunswick/03553/2009(H4N6)
KC845133 A/Great black-backed gull/Newfoundland/YH038/2010(H13N6)
CY076451 A/gadwall/California/29595/2007(H10N7)
CY042232 A/green-winged teal/Minnesota/Sg-00228/2006(H6N8)
CY132123 A/mallard/Michigan/10OS1491/2010(H4N6)
CY120649 A/mallard/California/3188/2010(H6N8)
CY097324 A/mallard/Wisconsin/4236/2009(mixed)
KC845033 A/Great black-backed gull/Newfoundland AB001/2011(H9N9)
CY094467 A/green-winged teal/California/11275/2008(H7N3)
CY078327 A/blue-winged teal/Minnesota/Sg-00201/2007(H4N6)
CY032837 A/blue-winged teal/Texas/Sg-00089/2007(H4N6)
HM059958 A/Glaucous gull/Alaska/44201-161/2006(H3N8)
KC845072 A/Herring gull/Newfoundland/GR578/2011(H13N6)
KC845057 A/Ring-billed gull/Newfoundland/GR384/2011(H13N6)
KC845065 A/Herring gull/Newfoundland/GR530/2011(N6)
CY032845 A/blue-winged teal/Minnesota/Sg-00095/2007(H4N6)
GQ923371 A/waterfowl/Colorado/476466/2007(H5N2)
CY077172 A/mallard/Alberta/224/2006(H12N5)
CY125604 A/blue-winged teal/New Brunswick/03757/2009(H3N6)
CY037093 A/laughing gull/Delaware Bay/46/2006(H7N3)
CY125355 A/ring-necked duck/New Brunswick/03449/2009(H11N9)
KC845112 A/Great black-backed gull/Newfoundland/MW174/2010(H1)
CY137988 A/shorebird/Delaware Bay/792/2009(H10N7)
KC845104 A/Herring gull/Newfoundland/GR658/2011(H13N6)
KC845080 A/Herring gull/Newfoundland/GR654/2011(H13N6)
KC845096 A/Herring gull/ Newfoundland /GR657/2011(H13N6)
KC845088 A/Herring gull/Newfoundland/GR656/2011(H13N6)
KC845049 A/Herring gull/Newfoundland/GR366/2011(H16N3)
KC845041 A/Herring gull/Newfoundland/GR032/2010(H16N3)
KC845025 A/Gull/Newfoundland/1413/2009(H16N9)
CY125612 A/mallard/Quebec/02916-1/2009(H16N3)
CY125302 A/black-legged kittiwake/Quebec/02838-1/2009(H13N6)
CY125323 A/ring-billed gull/Quebec/02622-1/2009(mixed)
CY125218 A/ring-billed gull/Quebec/02434-1/2009(H13N6)
GU724155 A/great black-backed gull/Newfoundland/296/2008(H13N2)
CY042591 A/laughing gull/New Jersey/Sg-00559/2008(H13N9)
KC845126 A/Herring gull/Newfoundland/YH022/2010(H13N6)
KC845118 A/Herring gull/Newfoundland/YH019/2010(H16N3)
KC845141 A/Gull/ Newfoundland/YH043/2010
HE802713 A/common gull/Norway/10_1313/2009(H13N2)
HE802737 A/common gull/Norway/10_1617/2006(H16N3)
JF775476 A/herring gull/Mongolia/454/2008(H13N8)
EPI345418 A/Larus argentatus/Belgium/14469-16/2008 (H16N3)
AY684881 A/black-headed gull/Sweden/5/99(H16N3)
AY684880 A/black-headed gull/Sweden/2/99(H16N3)
CY070848 A/glaucous-winged gull/Southcentral Alaska/9JR0691R0/2009(H13N6)
CY070864 A/glaucous-winged gull/Southcentral Alaska/9JR0747R0/2009(H13N6)
CY070872 A/glaucous-winged gull/Southcentral Alaska/9JR0769R0/2009(H13N6)
EF634338 A/mallard/Hei Longjiang/131/2006(H6N2)
JX454711 A/duck/Korea/DY97/2007(H4N3)
EU195390 A/mallard/Huadong/S/2005(H5N1)
CY005467 A/Duck/Nanchang/8-174/2000(H3N6)
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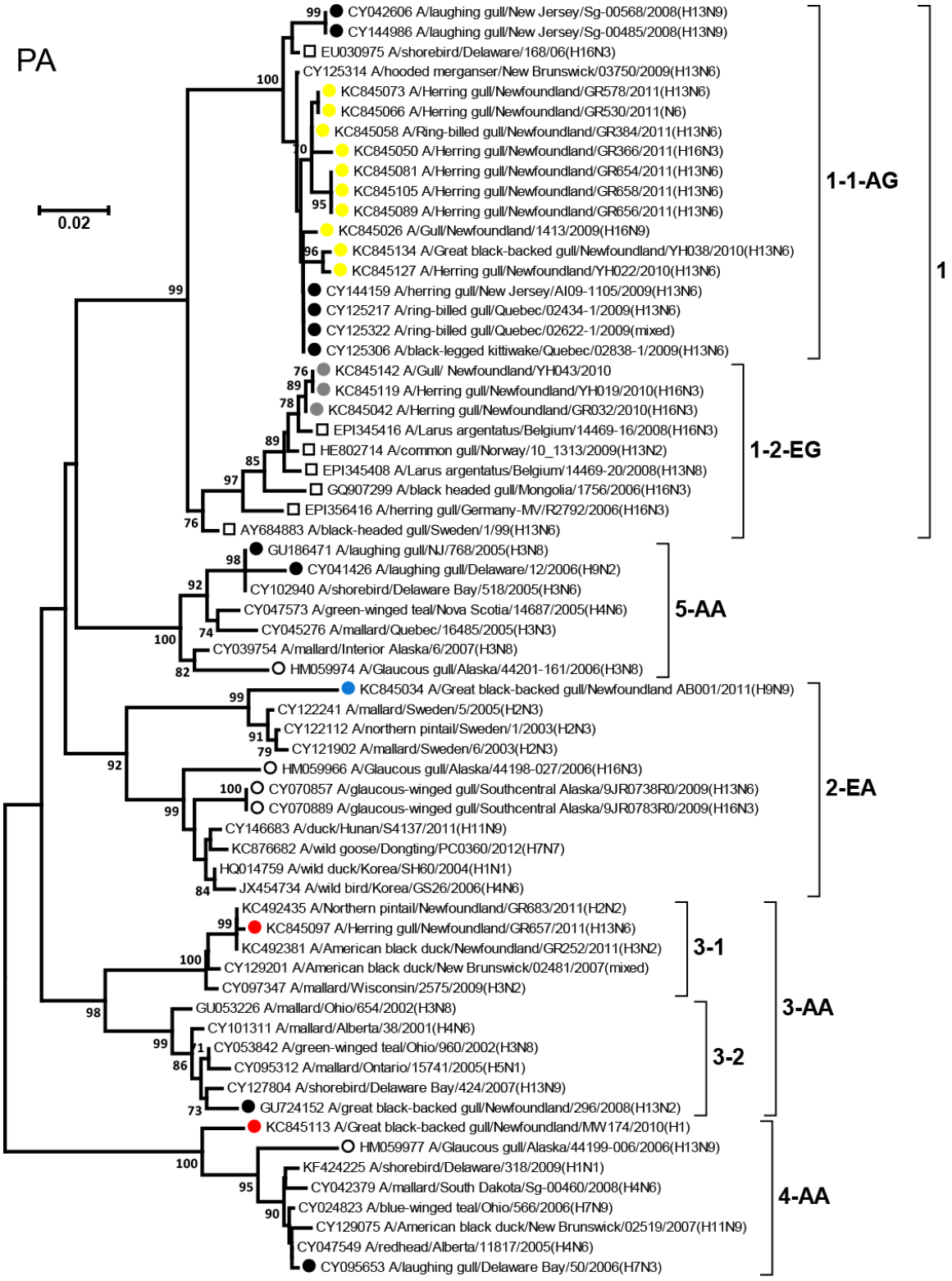
1-2-AA
1-3-AA
1-4-AG
2-1-EG
2-2-EA
1
2


PA
0.02
CY042606 A/laughing gull/New Jersey/Sg-00568/2008(H13N9)
CY144986 A/laughing gull/New Jersey/Sg-00485/2008(H13N9)
EU030975 A/shorebird/Delaware/168/06(H16N3)
CY125314 A/hooded merganser/New Brunswick/03750/2009(H13N6)
KC845073 A/Herring gull/Newfoundland/GR578/2011(H13N6)
KC845066 A/Herring gull/Newfoundland/GR530/2011(N6)
KC845058 A/Ring-billed gull/Newfoundland/GR384/2011(H13N6)
KC845050 A/Herring gull/Newfoundland/GR366/2011(H16N3)
KC845081 A/Herring gull/Newfoundland/GR654/2011(H13N6)
KC845105 A/Herring gull/Newfoundland/GR658/2011(H13N6)
KC845089 A/Herring gull/Newfoundland/GR656/2011(H13N6)
KC845026 A/Gull/Newfoundland/1413/2009(H16N9)
KC845134 A/Great black-backed gull/Newfoundland/YH038/2010(H13N6)
KC845127 A/Herring gull/Newfoundland/YH022/2010(H13N6)
CY144159 A/herring gull/New Jersey/AI09-1105/2009(H13N6)
CY125217 A/ring-billed gull/Quebec/02434-1/2009(H13N6)
CY125322 A/ring-billed gull/Quebec/02622-1/2009(mixed)
CY125306 A/black-legged kittiwake/Quebec/02838-1/2009(H13N6)
KC845142 A/Gull/ Newfoundland/YH043/2010
KC845119 A/Herring gull/Newfoundland/YH019/2010(H16N3)
KC845042 A/Herring gull/Newfoundland/GR032/2010(H16N3)
EPI345416 A/Larus argentatus/Belgium/14469-16/2008(H16N3)
HE802714 A/common gull/Norway/10_1313/2009(H13N2)
EPI345408 A/Larus argentatus/Belgium/14469-20/2008(H13N8)
GQ907299 A/black headed gull/Mongolia/1756/2006(H16N3)
EPI356416 A/herring gull/Germany-MV/R2792/2006(H16N3)
AY684883 A/black-headed gull/Sweden/1/99(H13N6)
GU186471 A/laughing gull/NJ/768/2005(H3N8)
CY041426 A/laughing gull/Delaware/12/2006(H9N2)
CY102940 A/shorebird/Delaware Bay/518/2005(H3N6)
CY047573 A/green-winged teal/Nova Scotia/14687/2005(H4N6)
CY045276 A/mallard/Quebec/16485/2005(H3N3)
CY039754 A/mallard/Interior Alaska/6/2007(H3N8)
HM059974 A/Glaucous gull/Alaska/44201-161/2006(H3N8)
KC845034 A/Great black-backed gull/Newfoundland AB001/2011(H9N9)
CY122241 A/mallard/Sweden/5/2005(H2N3)
CY122112 A/northern pintail/Sweden/1/2003(H2N3)
CY121902 A/mallard/Sweden/6/2003(H2N3)
HM059966 A/Glaucous gull/Alaska/44198-027/2006(H16N3)
CY070857 A/glaucous-winged gull/Southcentral Alaska/9JR0738R0/2009(H13N6)
CY070889 A/glaucous-winged gull/Southcentral Alaska/9JR0783R0/2009(H16N3)
CY146683 A/duck/Hunan/S4137/2011(H11N9)
KC876682 A/wild goose/Dongting/PC0360/2012(H7N7)
HQ014759 A/wild duck/Korea/SH60/2004(H1N1)
JX454734 A/wild bird/Korea/GS26/2006(H4N6)
KC492435 A/Northern pintail/Newfoundland/GR683/2011(H2N2)
KC845097 A/Herring gull/Newfoundland/GR657/2011(H13N6)
KC492381 A/American black duck/Newfoundland/GR252/2011(H3N2)
CY129201 A/American black duck/New Brunswick/02481/2007(mixed)
CY097347 A/mallard/Wisconsin/2575/2009(H3N2)
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CY101311 A/mallard/Alberta/38/2001(H4N6)
CY053842 A/green-winged teal/Ohio/960/2002(H3N8)
CY095312 A/mallard/Ontario/15741/2005(H5N1)
CY127804 A/shorebird/Delaware Bay/424/2007(H13N9)
GU724152 A/great black-backed gull/Newfoundland/296/2008(H13N2)
KC845113 A/Great black-backed gull/Newfoundland/MW174/2010(H1)
HM059977 A/Glaucous gull/Alaska/44199-006/2006(H13N9)
KF424225 A/shorebird/Delaware/318/2009(H1N1)
CY042379 A/mallard/South Dakota/Sg-00460/2008(H4N6)
CY024823 A/blue-winged teal/Ohio/566/2006(H7N9)
CY129075 A/American black duck/New Brunswick/02519/2007(H11N9)
CY047549 A/redhead/Alberta/11817/2005(H4N6)
CY095653 A/laughing gull/Delaware Bay/50/2006(H7N3)
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2-EA
3-1
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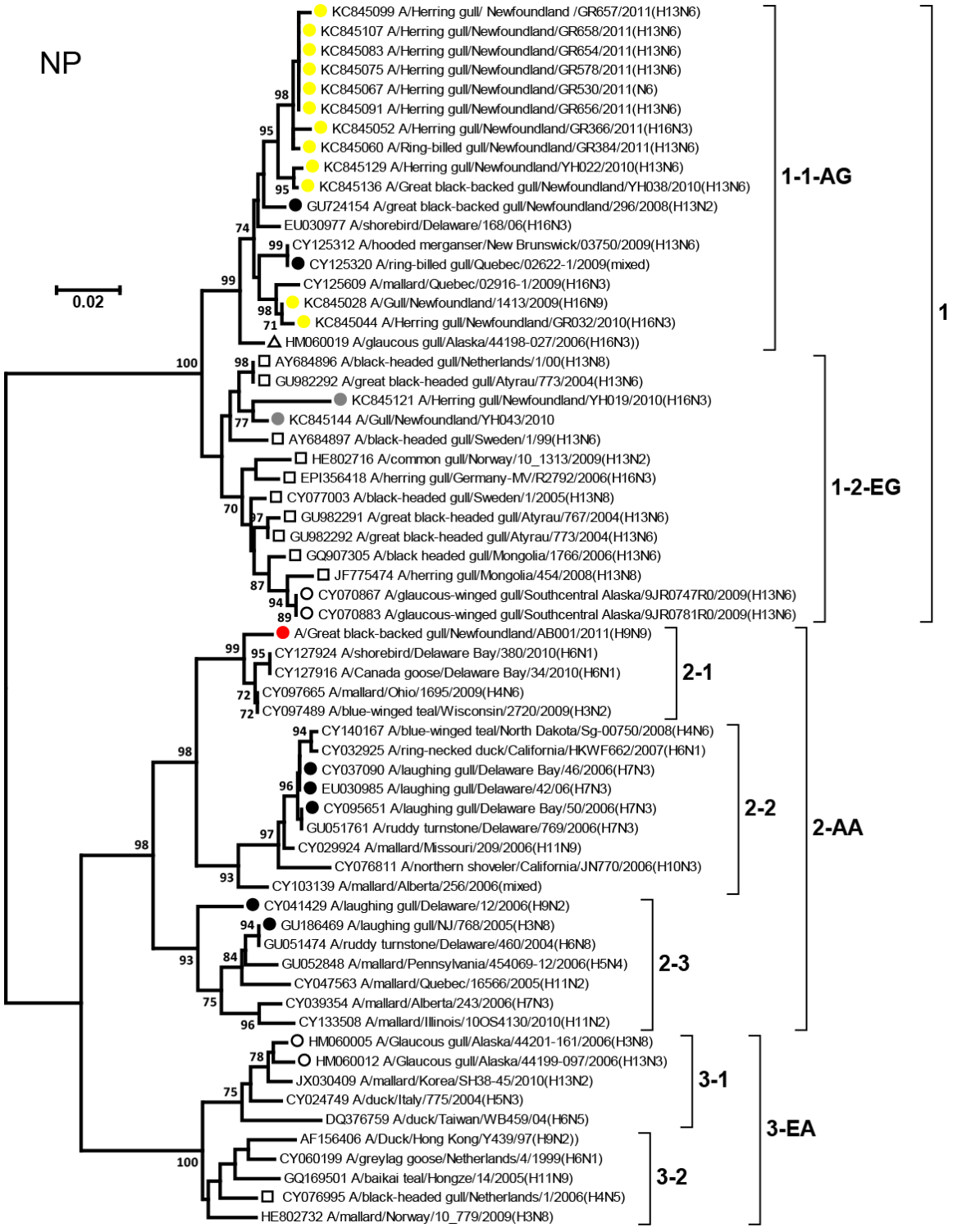
3-AA
4-AA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KC845075 A/Herring gull/Newfoundland/GR578/2011(H13N6)
KC845067 A/Herring gull/Newfoundland/GR530/2011(N6)
KC845091 A/Herring gull/Newfoundland/GR656/2011(H13N6)
KC845052 A/Herring gull/Newfoundland/GR366/2011(H16N3)
KC845060 A/Ring-billed gull/Newfoundland/GR384/2011(H13N6)
KC845129 A/Herring gull/Newfoundland/YH022/2010(H13N6)
KC845136 A/Great black-backed gull/Newfoundland/YH038/2010(H13N6)
GU724154 A/great black-backed gull/Newfoundland/296/2008(H13N2)
EU030977 A/shorebird/Delaware/168/06(H16N3)
CY125312 A/hooded merganser/New Brunswick/03750/2009(H13N6)
CY125320 A/ring-billed gull/Quebec/02622-1/2009(mixed)
CY125609 A/mallard/Quebec/02916-1/2009(H16N3)
KC845028 A/Gull/Newfoundland/1413/2009(H16N9)
KC845044 A/Herring gull/Newfoundland/GR032/2010(H16N3)
HM060019 A/glaucous gull/Alaska/44198-027/2006(H16N3))
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GU982292 A/great black-headed gull/Atyrau/773/2004(H13N6)
KC845121 A/Herring gull/Newfoundland/YH019/2010(H16N3)
KC845144 A/Gull/Newfoundland/YH043/2010
AY684897 A/black-headed gull/Sweden/1/99(H13N6)
HE802716 A/common gull/Norway/10_1313/2009(H13N2)
EPI356418 A/herring gull/Germany-MV/R2792/2006(H16N3)
CY077003 A/black-headed gull/Sweden/1/2005(H13N8)
GU982291 A/great black-headed gull/Atyrau/767/2004(H13N6)
GU982292 A/great black-headed gull/Atyrau/773/2004(H13N6)
GQ907305 A/black headed gull/Mongolia/1766/2006(H13N6)
JF775474 A/herring gull/Mongolia/454/2008(H13N8)
CY070867 A/glaucous-winged gull/Southcentral Alaska/9JR0747R0/2009(H13N6)
CY070883 A/glaucous-winged gull/Southcentral Alaska/9JR0781R0/2009(H13N6)
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CY127916 A/Canada goose/Delaware Bay/34/2010(H6N1)
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CY097489 A/blue-winged teal/Wisconsin/2720/2009(H3N2)
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CY032925 A/ring-necked duck/California/HKWF662/2007(H6N1)
CY037090 A/laughing gull/Delaware Bay/46/2006(H7N3)
EU030985 A/laughing gull/Delaware/42/06(H7N3)
CY095651 A/laughing gull/Delaware Bay/50/2006(H7N3)
GU051761 A/ruddy turnstone/Delaware/769/2006(H7N3)
CY029924 A/mallard/Missouri/209/2006(H11N9)
CY076811 A/northern shoveler/California/JN770/2006(H10N3)
CY103139 A/mallard/Alberta/256/2006(mixed)
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CY041429 A/laughing gull/Delaware/12/2006(H9N2)
GU186469 A/laughing gull/NJ/768/2005(H3N8)
GU051474 A/ruddy turnstone/Delaware/460/2004(H6N8)
GU052848 A/mallard/Pennsylvania/454069-12/2006(H5N4)
CY047563 A/mallard/Quebec/16566/2005(H11N2)
CY039354 A/mallard/Alberta/243/2006(H7N3)
CY133508 A/mallard/Illinois/10OS4130/2010(H11N2)
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HM060012 A/Glaucous gull/Alaska/44199-097/2006(H13N3)
JX030409 A/mallard/Korea/SH38-45/2010(H13N2)
CY024749 A/duck/Italy/775/2004(H5N3)
DQ376759 A/duck/Taiwan/WB459/04(H6N5)
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AF156406 A/Duck/Hong Kong/Y439/97(H9N2))
CY060199 A/greylag goose/Netherlands/4/1999(H6N1)
GQ169501 A/baikal teal/Hongze/14/2005(H11N9)
CY076995 A/black-headed gull/Netherlands/1/2006(H4N5)
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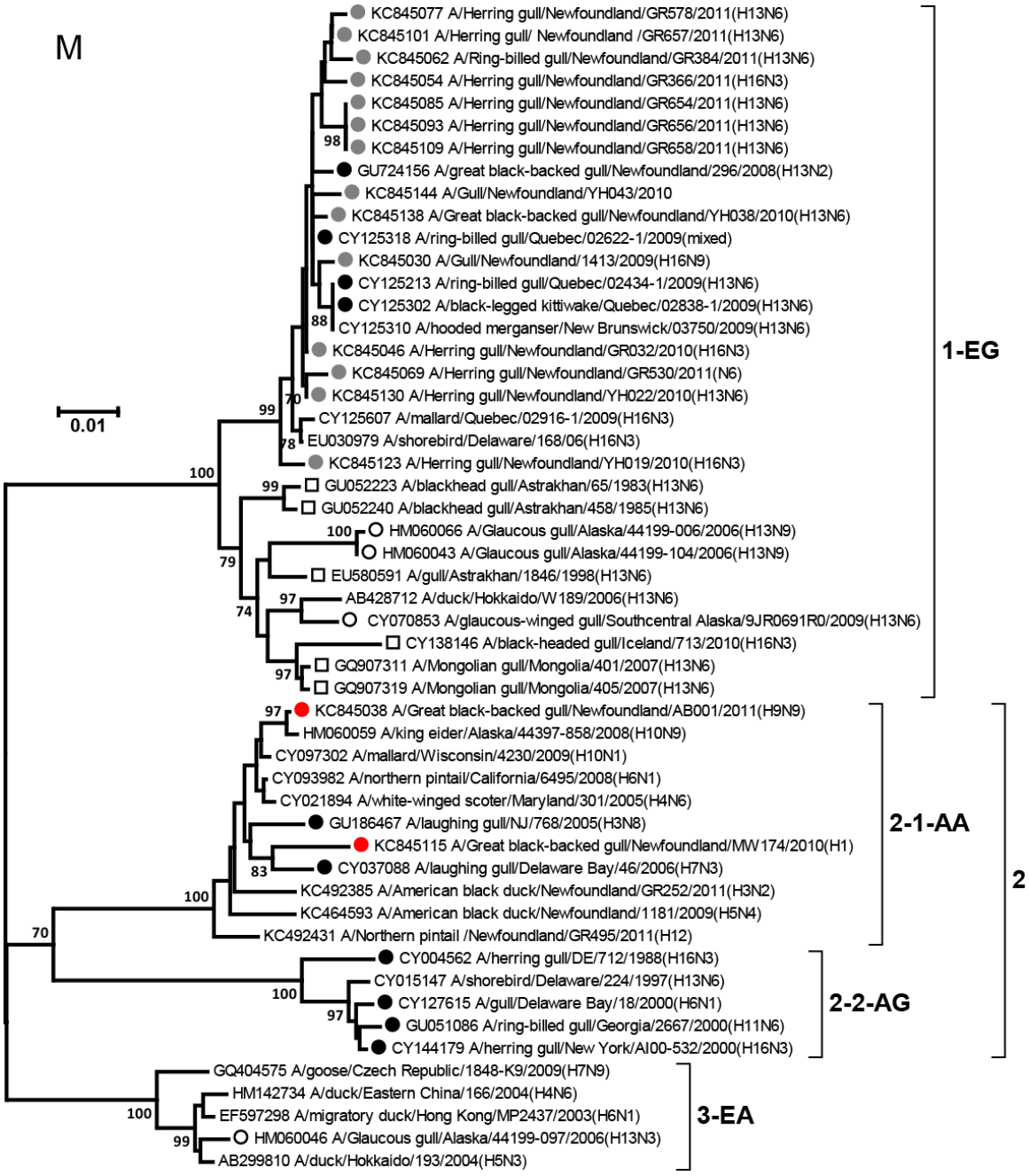
3-2
3-EA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KC845062 A/Ring-billed gull/Newfoundland/GR384/2011(H13N6)
KC845054 A/Herring gull/Newfoundland/GR366/2011(H16N3)
KC845085 A/Herring gull/Newfoundland/GR654/2011(H13N6)
KC845093 A/Herring gull/Newfoundland/GR656/2011(H13N6)
KC845109 A/Herring gull/Newfoundland/GR658/2011(H13N6)
GU724156 A/great black-backed gull/Newfoundland/296/2008(H13N2)
KC845144 A/Gull/Newfoundland/YH043/2010
KC845138 A/Great black-backed gull/Newfoundland/YH038/2010(H13N6)
CY125318 A/ring-billed gull/Quebec/02622-1/2009(mixed)
KC845030 A/Gull/Newfoundland/1413/2009(H16N9)
CY125213 A/ring-billed gull/Quebec/02434-1/2009(H13N6)
CY125302 A/black-legged kittiwake/Quebec/02838-1/2009(H13N6)
CY125310 A/hooded merganser/New Brunswick/03750/2009(H13N6)
KC845046 A/Herring gull/Newfoundland/GR032/2010(H16N3)
KC845069 A/Herring gull/Newfoundland/GR530/2011(N6)
KC845130 A/Herring gull/Newfoundland/YH022/2010(H13N6)
CY125607 A/mallard/Quebec/02916-1/2009(H16N3)
EU030979 A/shorebird/Delaware/168/06(H16N3)
KC845123 A/Herring gull/Newfoundland/YH019/2010(H16N3)
GU052223 A/blackhead gull/Astrakhan/65/1983(H13N6)
GU052240 A/blackhead gull/Astrakhan/458/1985(H13N6)
HM060066 A/Glaucous gull/Alaska/44199-006/2006(H13N9)
HM060043 A/Glaucous gull/Alaska/44199-104/2006(H13N9)
EU580591 A/gull/Astrakhan/1846/1998(H13N6)
AB428712 A/duck/Hokkaido/W189/2006(H13N6)
CY070853 A/glaucous-winged gull/Southcentral Alaska/9JR0691R0/2009(H13N6)
CY138146 A/black-headed gull/Iceland/713/2010(H16N3)
GQ907311 A/Mongolian gull/Mongolia/401/2007(H13N6)
GQ907319 A/Mongolian gull/Mongolia/405/2007(H13N6)
KC845038 A/Great black-backed gull/Newfoundland/AB001/2011(H9N9)
HM060059 A/king eider/Alaska/44397-858/2008(H10N9)
CY097302 A/mallard/Wisconsin/4230/2009(H10N1)
CY093982 A/northern pintail/California/6495/2008(H6N1)
CY021894 A/white-winged scoter/Maryland/301/2005(H4N6)
GU186467 A/laughing gull/NJ/768/2005(H3N8)
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CY037088 A/laughing gull/Delaware Bay/46/2006(H7N3)
KC492385 A/American black duck/Newfoundland/GR252/2011(H3N2)
KC464593 A/American black duck/Newfoundland/1181/2009(H5N4)
KC492431 A/Northern pintail /Newfoundland/GR495/2011(H12)
CY004562 A/herring gull/DE/712/1988(H16N3)
CY015147 A/shorebird/Delaware/224/1997(H13N6)
CY127615 A/gull/Delaware Bay/18/2000(H6N1)
GU051086 A/ring-billed gull/Georgia/2667/2000(H11N6)
CY144179 A/herring gull/New York/AI00-532/2000(H16N3)
GQ404575 A/goose/Czech Republic/1848-K9/2009(H7N9)
HM142734 A/duck/Eastern China/166/2004(H4N6)
EF597298 A/migratory duck/Hong Kong/MP2437/2003(H6N1)
HM060046 A/Glaucous gull/Alaska/44199-097/2006(H13N3)
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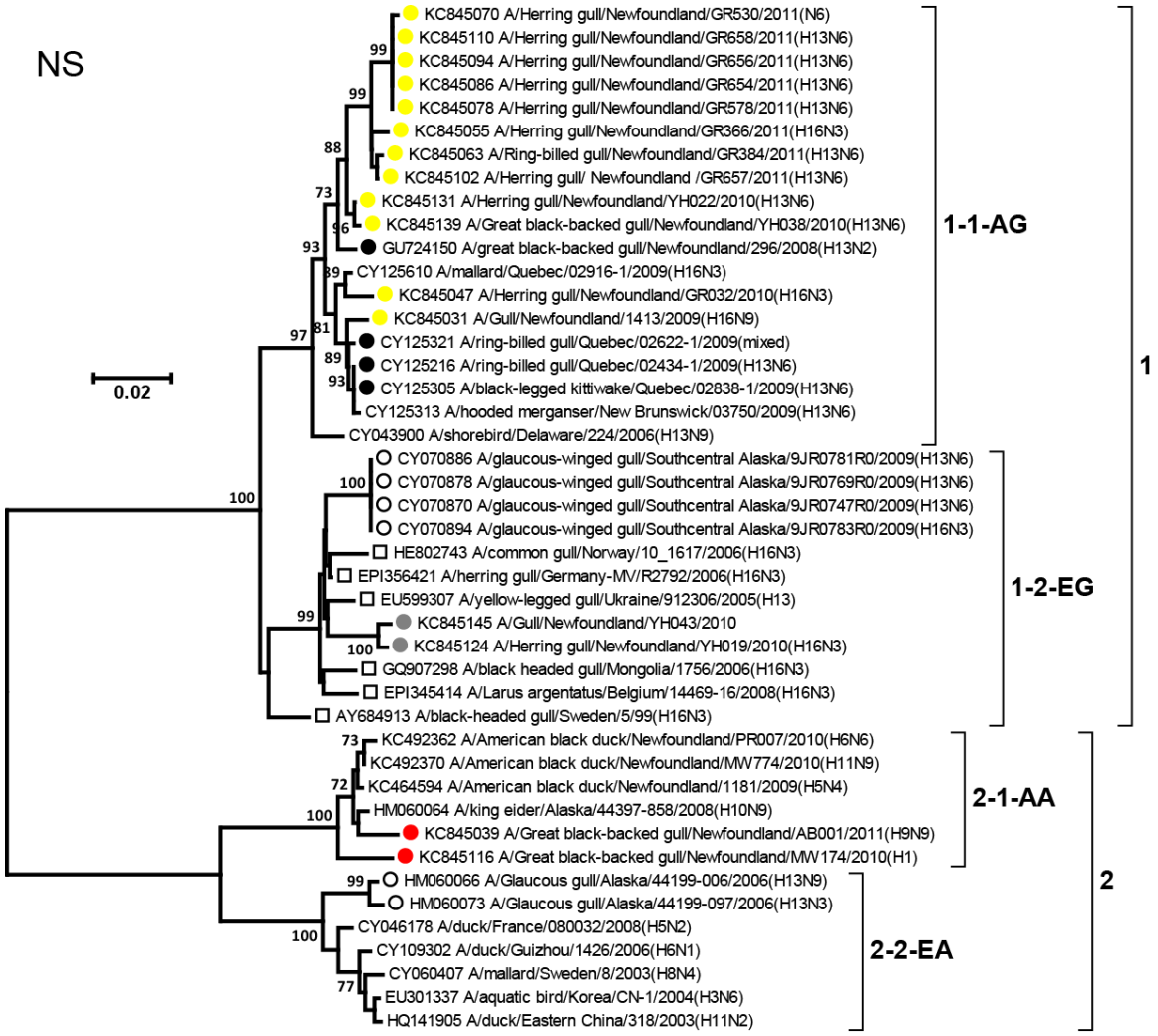
2-2-AG
2
3-EA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KC845086 A/Herring gull/Newfoundland/GR654/2011(H13N6)
KC845078 A/Herring gull/Newfoundland/GR578/2011(H13N6)
KC845055 A/Herring gull/Newfoundland/GR366/2011(H16N3)
KC845063 A/Ring-billed gull/Newfoundland/GR384/2011(H13N6)
KC845102 A/Herring gull/ Newfoundland /GR657/2011(H13N6)
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KC845139 A/Great black-backed gull/Newfoundland/YH038/2010(H13N6)
GU724150 A/great black-backed gull/Newfoundland/296/2008(H13N2)
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KC845031 A/Gull/Newfoundland/1413/2009(H16N9)
CY125321 A/ring-billed gull/Quebec/02622-1/2009(mixed)
CY125216 A/ring-billed gull/Quebec/02434-1/2009(H13N6)
CY125305 A/black-legged kittiwake/Quebec/02838-1/2009(H13N6)
CY125313 A/hooded merganser/New Brunswick/03750/2009(H13N6)
CY043900 A/shorebird/Delaware/224/2006(H13N9)
CY070886 A/glaucous-winged gull/Southcentral Alaska/9JR0781R0/2009(H13N6)
CY070878 A/glaucous-winged gull/Southcentral Alaska/9JR0769R0/2009(H13N6)
CY070870 A/glaucous-winged gull/Southcentral Alaska/9JR0747R0/2009(H13N6)
CY070894 A/glaucous-winged gull/Southcentral Alaska/9JR0783R0/2009(H16N3)
HE802743 A/common gull/Norway/10_1617/2006(H16N3)
EPI356421 A/herring gull/Germany-MV/R2792/2006(H16N3)
EU599307 A/yellow-legged gull/Ukraine/912306/2005(H13)
KC845145 A/Gull/Newfoundland/YH043/2010
KC845124 A/Herring gull/Newfoundland/YH019/2010(H16N3)
GQ907298 A/black headed gull/Mongolia/1756/2006(H16N3)
EPI345414 A/Larus argentatus/Belgium/14469-16/2008(H16N3)
AY684913 A/black-headed gull/Sweden/5/99(H16N3)
KC492362 A/American black duck/Newfoundland/PR007/2010(H6N6)
KC492370 A/American black duck/Newfoundland/MW774/2010(H11N9)
KC464594 A/American black duck/Newfoundland/1181/2009(H5N4)
HM060064 A/king eider/Alaska/44397-858/2008(H10N9)
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KC845116 A/Great black-backed gull/Newfoundland/MW174/2010(H1)
HM060066 A/Glaucous gull/Alaska/44199-006/2006(H13N9)
HM060073 A/Glaucous gull/Alaska/44199-097/2006(H13N3)
CY046178 A/duck/France/080032/2008(H5N2)
CY109302 A/duck/Guizhou/1426/2006(H6N1)
CY060407 A/mallard/Sweden/8/2003(H8N4)
EU301337 A/aquatic bird/Korea/CN-1/2004(H3N6)
HQ141905 A/duck/Eastern China/318/2003(H11N2)
1-1-AG
1-2-EG
2-1-AA
2-2-EA
1
2

Table S5.1. Genbank accession numbers for sequences of the 17 reference murre AIVs analyzed in this study.

Virus	Segment sequence accession number							
	PB2	PB1	PA	HA	NP	NA	M	NS
A/thick-billed murre/Alaska/ 44085-108/2006(H1N9)	HM059937	HM059956	HM059978	HM059985	HM060008	HM060041	HM060056	HM060063
A/thick-billed murre/Alaska/ 44088-059/2006(H11N9)	HM059940	HM059961	HM059984	HM059993	HM060009	HM060040	HM060052	HM060062
A/common murre/Alaska/ 44085-165/2006(H11N9)	HM059941	HM059960	HM059968	HM059990	HM060007	HM060033	HM060053	HM060071
A/common murre/Alaska/ 44089-066/2006(H10N2)	HM059933	HM059964	HM059973	HM059986	HM060006	HM060025	HM060060	HM060067
A/thick-billed murre/Alaska/ 44085-090/2006(H11N9)	HM059935	HM059935	HM059980	HM059991	HM060010	HM060038	HM060042	HM060069
A/thick-billed murre/Alaska/ 44086-095/2006(H11N9)	HM059939	HM059949	HM059969	HM059992	HM060020	HM060039	HM060057	HM060068
A/thick-billed murre/Alaska/ 44085-155/2006(H9N2)	HM059942	HM059963	HM059976	HM060002	HM060021	HM060026	HM060044	HM060077
A/thick-billed murre/Alaska/ 44145-186/2006(H9N2)	HM059946	HM059957	HM059982	HM060003	HM060004.	HM060027	HM060048	HM060075
A/thick-billed murre/Newfoundland/ 031/2007(H11N2)	GU826671	GU826672	GU826673	GU826674	GU826675	GU826676	GU826677	GU826678
A/thick-billed murre/Alaska/ 44145-199/2006(H2N6)	HM059929	HM060061	HM059979	HM059999	HM060022	HM060031	HM060058	HM060061
A/thick-billed murre/Alaska/44085-040/2006(H10N3)	HM059943	HM059943	HM059970	HM059989	HM060014	HM060030	HM060050	HM060074
A/common murre/Oregon/19497-004/2005(H9N5)	CY075932	CY075932	CY075930	CY075925	CY075928	CY075927	CY075926	CY075929
A/common murre/Oregon/20361-002/2007(H12N5)	CY076116	CY076115	CY076114	CY076109	CY076112	CY076111	CY076110	CY076113
A/guillemot/Sweden/3/00(H6N2)	AY703829	AY703830	AY703831	AY703832	AY703833	AY703834	AY703836	AY703835
A/common murre/Oregon/20361-001/2007(H10N7)	CY076108	CY076107	CY076107	CY076101	CY076104	CY076103	CY076102	CY076105
A/murre/Alaska/305/1976(H1N6)			CY015166	CY015163		AY207535	CY015164	CY015165
A/murre/Alaska/175/1976(H1N2)				CY015167				CY015168

Table S5.2. Genetic structure of murre AIVs by location of origin.

Location (number of viruses)	Lineage	Segment								Total
		PB2	PB1	PA	HA	NP	NA	M	NS	
Pacific North America (15)	American avian	7	13	13	13	13	12	8	14	92
	Eurasian avian	6	0	1	1	0	1	5	1	16
	American gull	0	0	0	1	0	0	0	0	1
	Total	13	13	14	15	13	13	13	15	109
Atlantic North America (22)	American avian	4	21	22	21	20	21	5	22	136
	Eurasian avian	18	0	0	0	0	0	0	0	18
	Eurasian gull	0	0	0	0	0	0	17	0	17
	Total	22	21	22	21	20	21	22	22	171
Atlantic Eurasia (1)	American avian	1	1	0	0	0	0	1	1	4
	Eurasian avian	0	0	1	0	1	1	0	0	3
	American gull	0	0	0	1	0	0	0	0	1

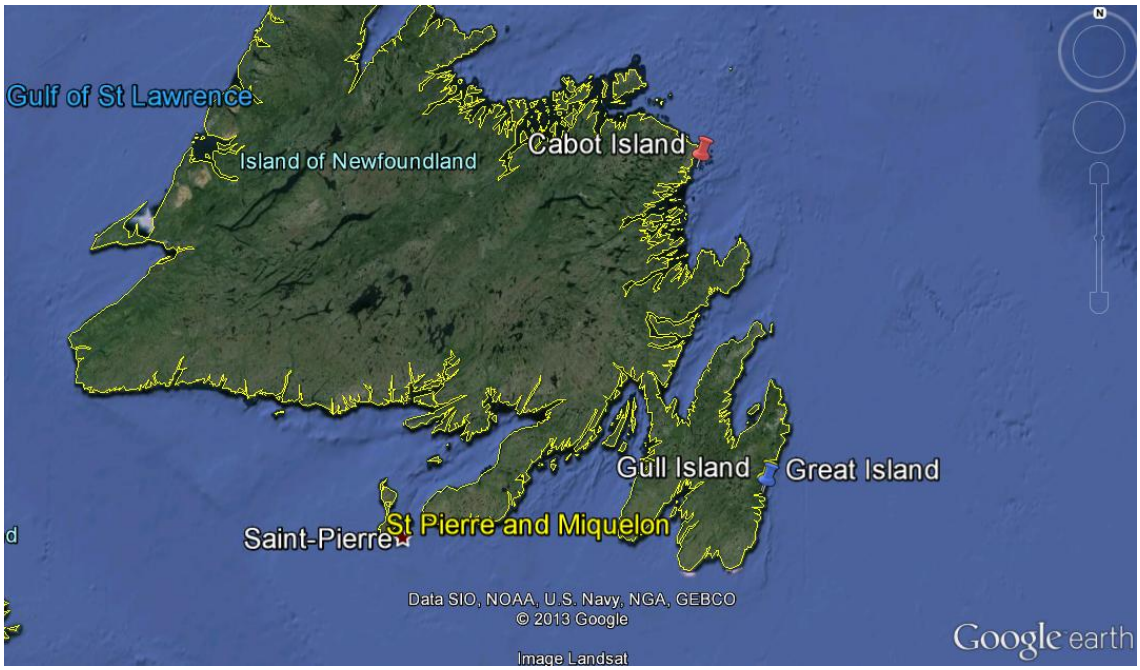
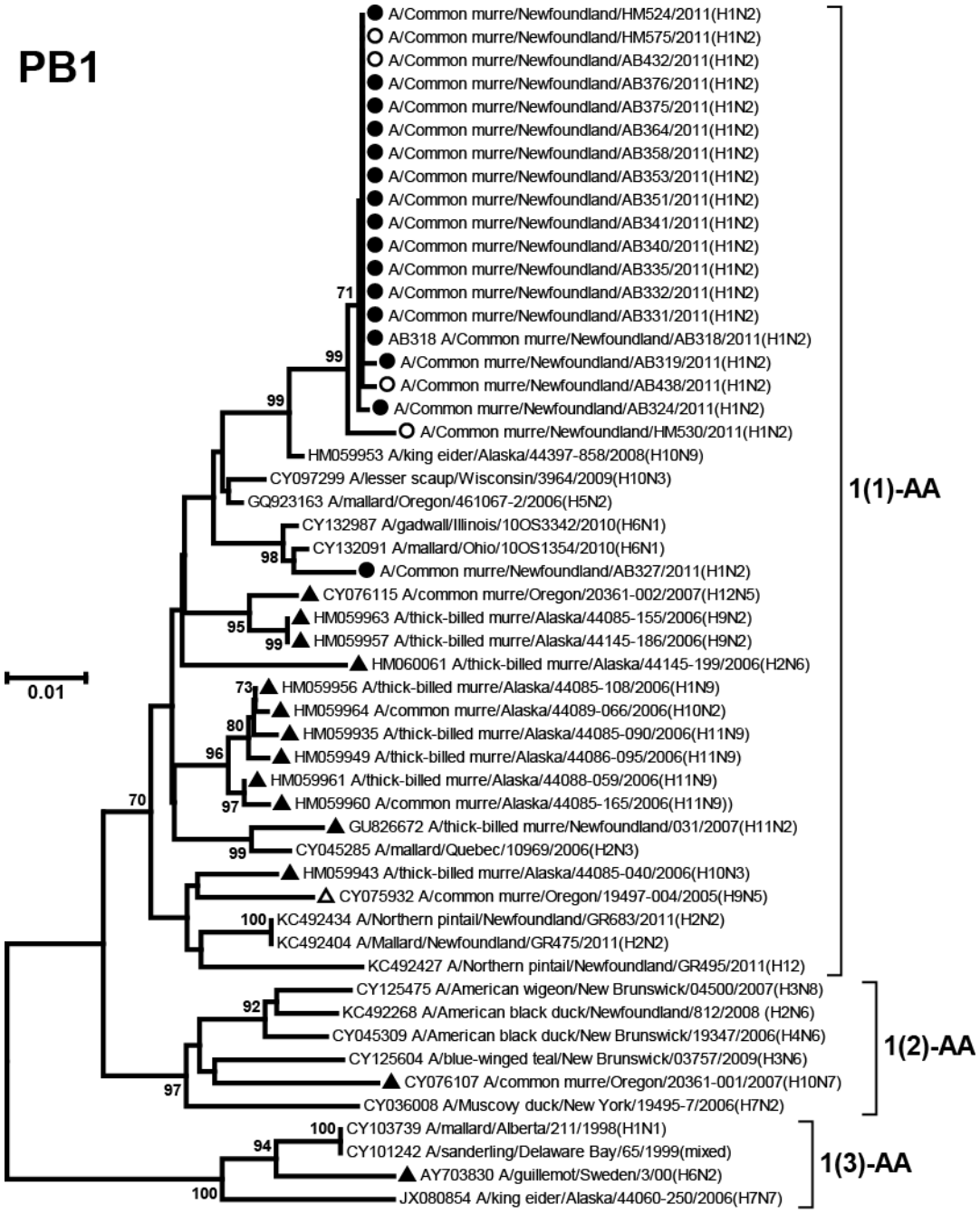


Figure S5.1. Geo-locations of the 3 Common Murre breeding colonies (Gull Island, Great Island and Cabot Island) in this study.

Figure S5.2. Phylogenetic analyses of the 38 AIVs from Common and Thick-billed Murres (starting from the next page). The analyses of the PB1, PA, HA (H2, H6, H9, H10, H11 and H12), NP, NA (N3, N5, N6, N7 and N9) and NS segment sequences are shown and labeled accordingly. The filled and open circles indicate the 21 viruses from Newfoundland in 2011 from the Gull and Cabot Island colonies, respectively. Filled and open triangles represent murre viruses from North America and Eurasia, respectively, available in the sequence database. The lineages are labeled as AA for North American avian, EA for Eurasian avian, AG for North American gull and EG for Eurasian gull. The trees were constructed with the maximum likelihood method using MEGA 5, and bootstrap values are shown as percentages based on 1000 replicates where support was ≥70%. The scale bars indicate the number of substitutions per site.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A/Common murre/Newfoundland/AB432/2011(H1N2)
A/Common murre/Newfoundland/AB376/2011(H1N2)
A/Common murre/Newfoundland/AB375/2011(H1N2)
A/Common murre/Newfoundland/AB364/2011(H1N2)
A/Common murre/Newfoundland/AB358/2011(H1N2)
A/Common murre/Newfoundland/AB353/2011(H1N2)
A/Common murre/Newfoundland/AB351/2011(H1N2)
A/Common murre/Newfoundland/AB341/2011(H1N2)
A/Common murre/Newfoundland/AB340/2011(H1N2)
A/Common murre/Newfoundland/AB335/2011(H1N2)
A/Common murre/Newfoundland/AB332/2011(H1N2)
A/Common murre/Newfoundland/AB331/2011(H1N2)
AB318 A/Common murre/Newfoundland/AB318/2011(H1N2)
A/Common murre/Newfoundland/AB319/2011(H1N2)
A/Common murre/Newfoundland/AB438/2011(H1N2)
A/Common murre/Newfoundland/AB324/2011(H1N2)
A/Common murre/Newfoundland/HM530/2011(H1N2)
HM059953 A/king eider/Alaska/44397-858/2008(H10N9)
CY097299 A/lesser scaup/Wisconsin/3964/2009(H10N3)
GQ923163 A/mallard/Oregon/461067-2/2006(H5N2)
CY132987 A/gadwall/Illinois/10OS3342/2010(H6N1)
CY132091 A/mallard/Ohio/10OS1354/2010(H6N1)
A/Common murre/Newfoundland/AB327/2011(H1N2)
CY076115 A/common murre/Oregon/20361-002/2007(H12N5)
HM059963 A/thick-billed murre/Alaska/44085-155/2006(H9N2)
HM059957 A/thick-billed murre/Alaska/44145-186/2006(H9N2)
HM060061 A/thick-billed murre/Alaska/44145-199/2006(H2N6)
HM059956 A/thick-billed murre/Alaska/44085-108/2006(H1N9)
HM059964 A/common murre/Alaska/44089-066/2006(H10N2)
HM059935 A/thick-billed murre/Alaska/44085-090/2006(H11N9)
HM059949 A/thick-billed murre/Alaska/44086-095/2006(H11N9)
HM059961 A/thick-billed murre/Alaska/44088-059/2006(H11N9)
HM059960 A/common murre/Alaska/44085-165/2006(H11N9))
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CY036008 A/Muscovy duck/New York/19495-7/2006(H7N2)
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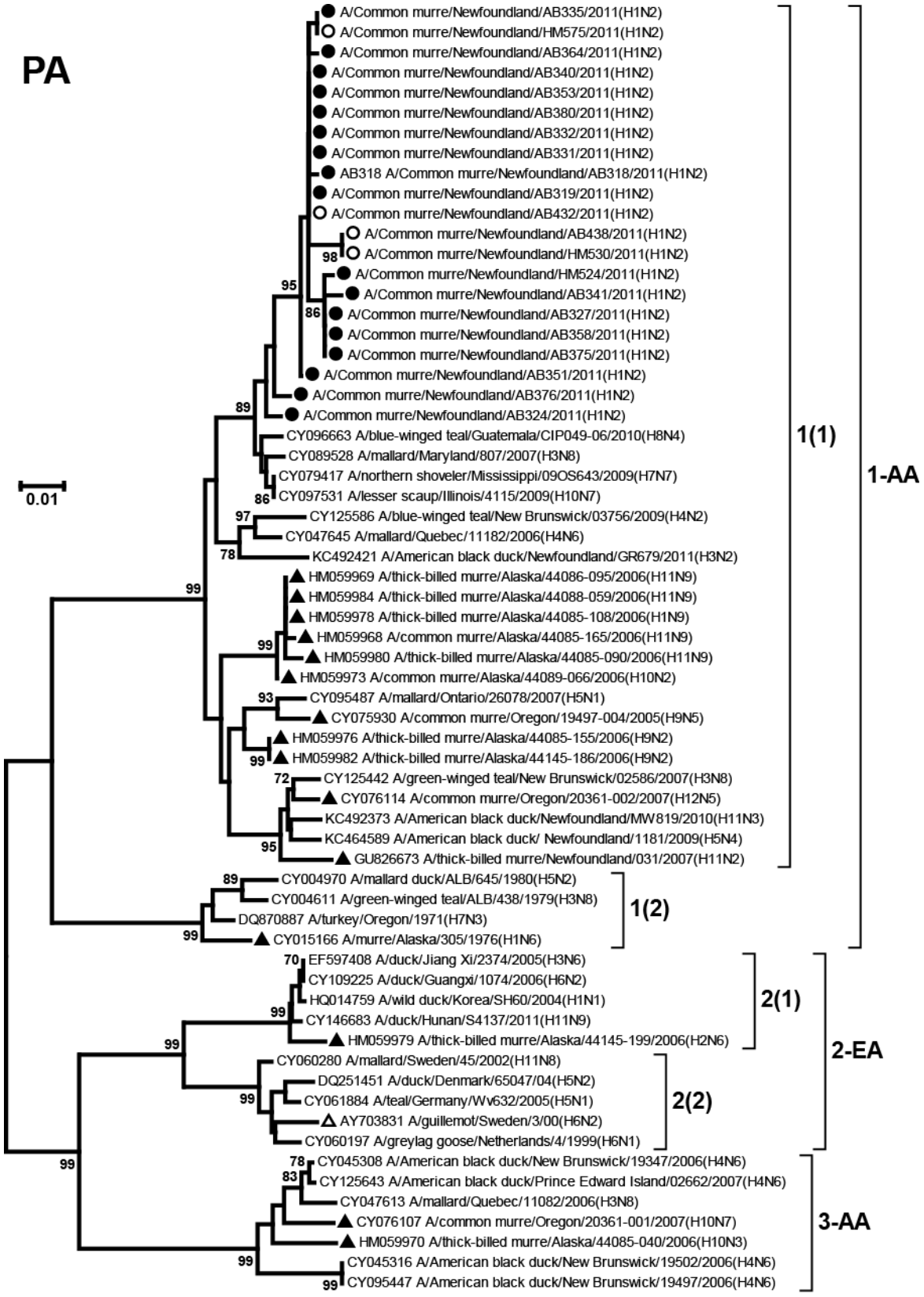
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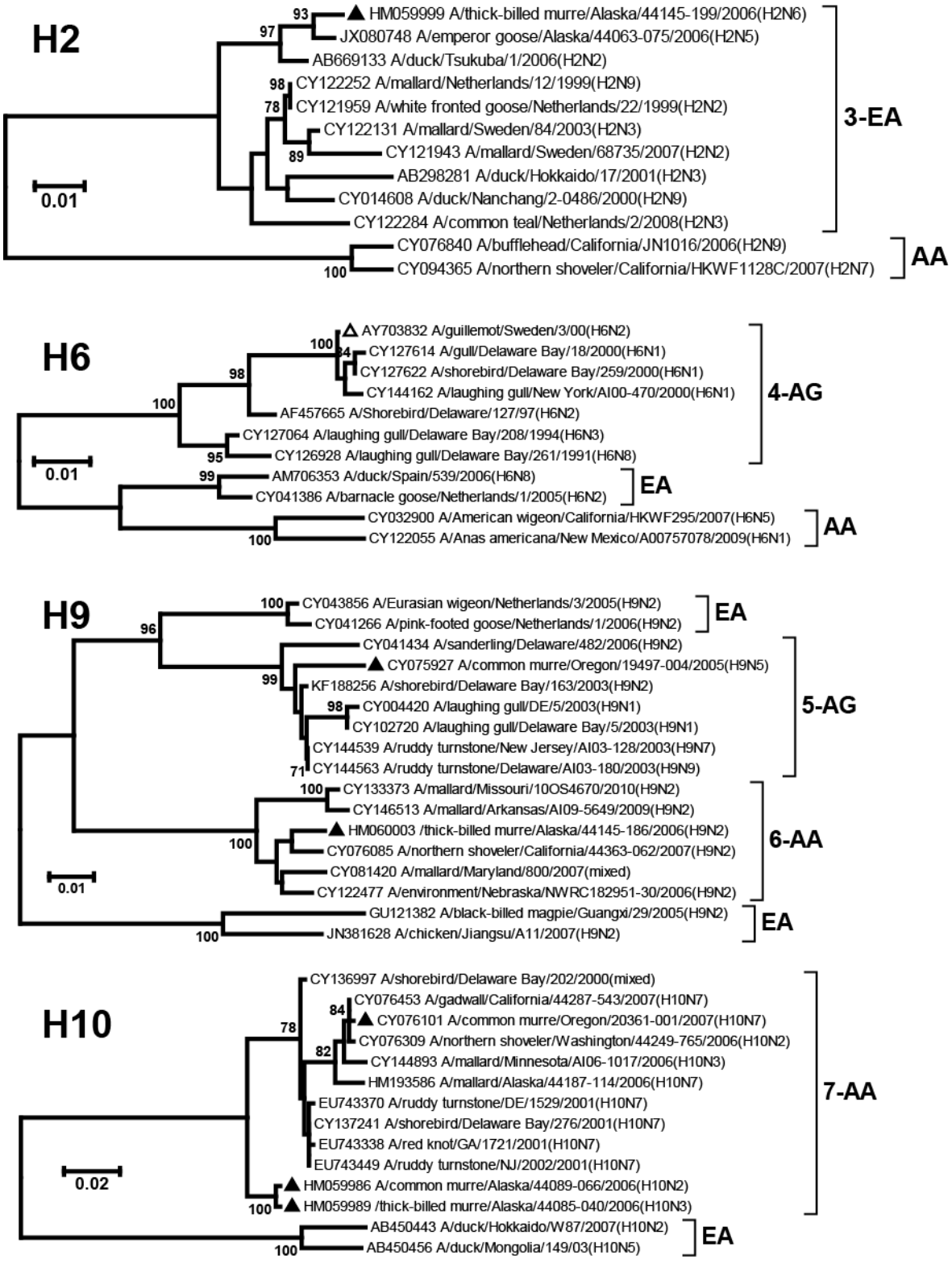
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AF457665 A/Shorebird/Delaware/127/97(H6N2)
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CY144563 A/ruddy turnstone/Delaware/AI03-180/2003(H9N9)
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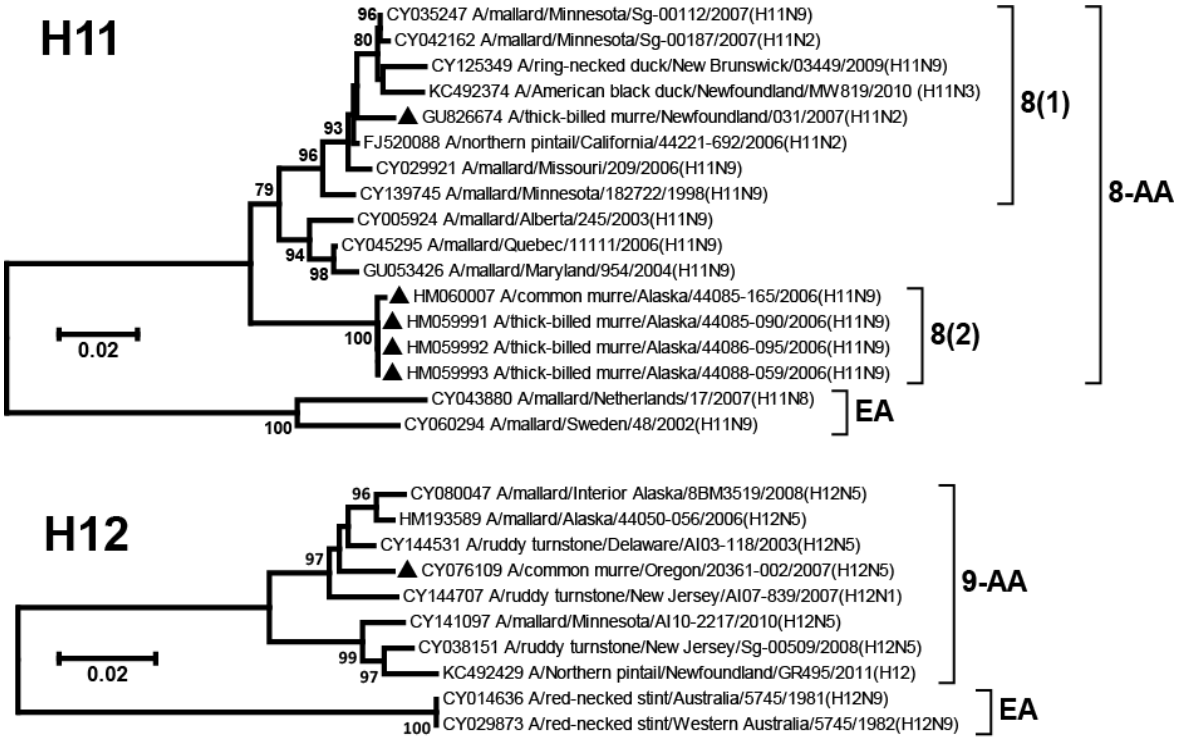
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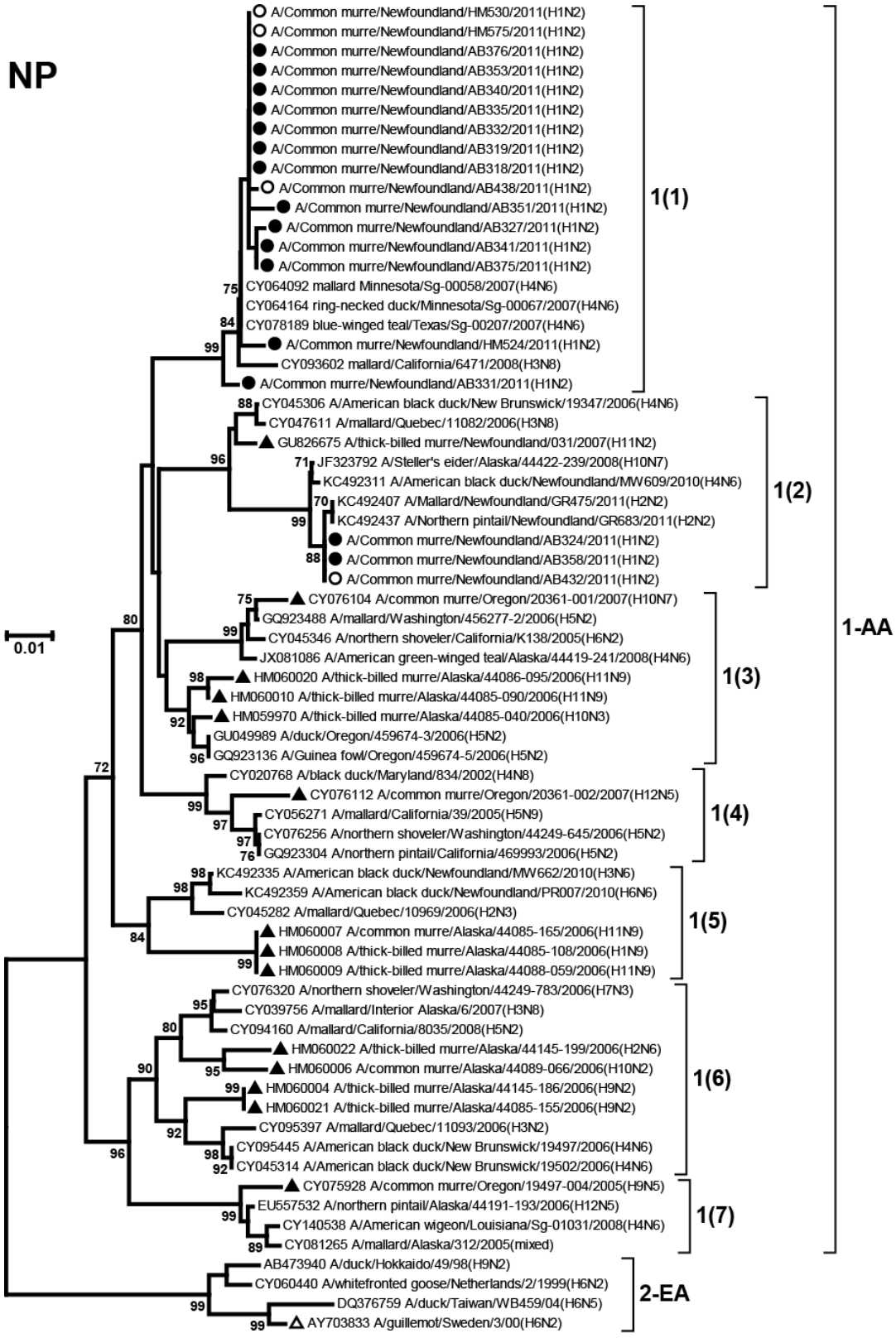
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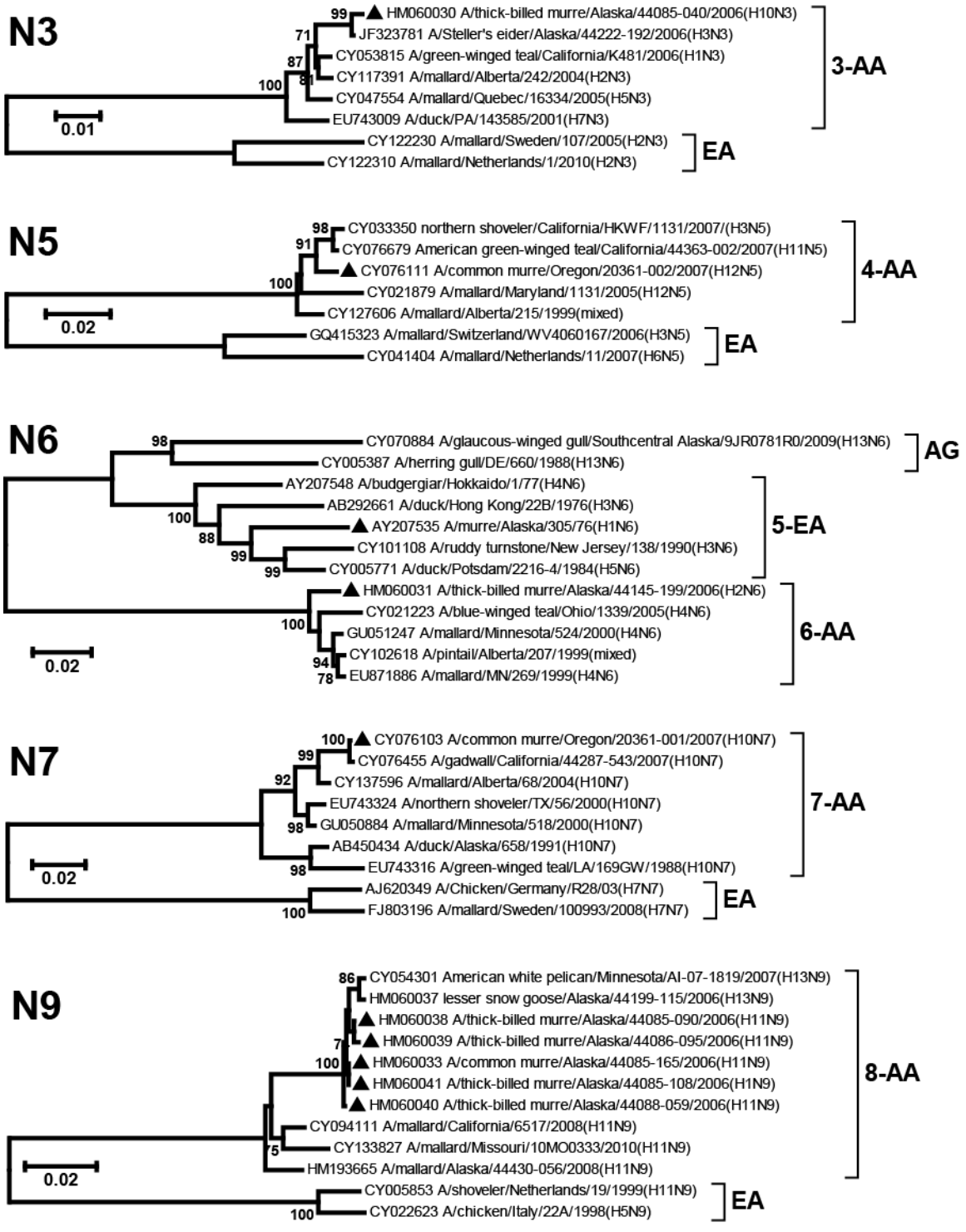
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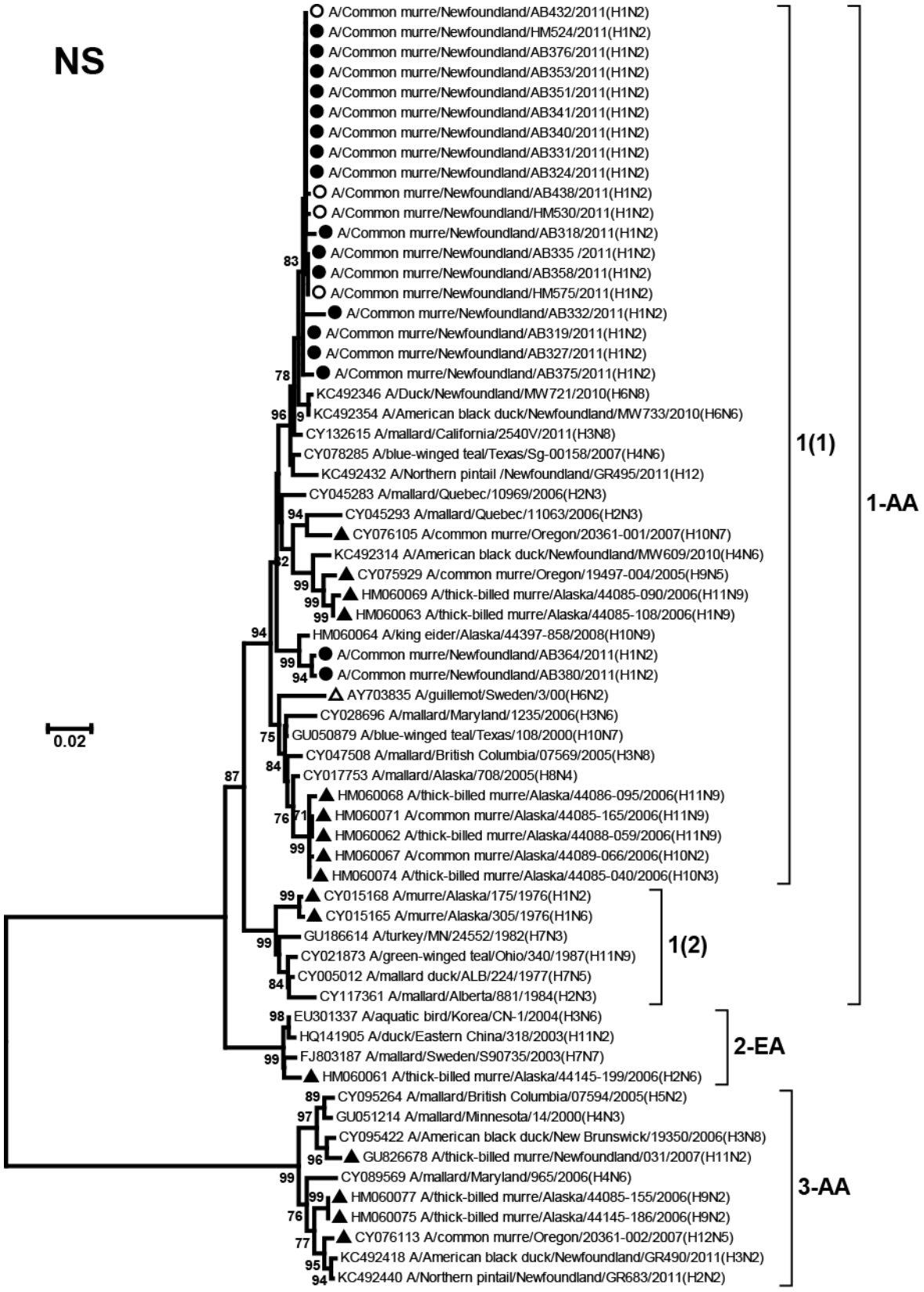
HM193665 A/mallard/Alaska/44430-056/2008(H11N9)
CY005853 A/shoveler/Netherlands/19/1999(H11N9)
CY022623 A/chicken/Italy/22A/1998(H5N9)
8-AA
EA
0.02


NS
A/Common murre/Newfoundland/AB432/2011(H1N2)
A/Common murre/Newfoundland/HM524/2011(H1N2)
A/Common murre/Newfoundland/AB376/2011(H1N2)
A/Common murre/Newfoundland/AB353/2011(H1N2)
A/Common murre/Newfoundland/AB351/2011(H1N2)
A/Common murre/Newfoundland/AB341/2011(H1N2)
A/Common murre/Newfoundland/AB340/2011(H1N2)
A/Common murre/Newfoundland/AB331/2011(H1N2)
A/Common murre/Newfoundland/AB324/2011(H1N2)
A/Common murre/Newfoundland/AB438/2011(H1N2)
A/Common murre/Newfoundland/HM530/2011(H1N2)
A/Common murre/Newfoundland/AB318/2011(H1N2)
A/Common murre/Newfoundland/AB335 /2011(H1N2)
A/Common murre/Newfoundland/AB358/2011(H1N2)
A/Common murre/Newfoundland/HM575/2011(H1N2)
A/Common murre/Newfoundland/AB332/2011(H1N2)
A/Common murre/Newfoundland/AB319/2011(H1N2)
A/Common murre/Newfoundland/AB327/2011(H1N2)
A/Common murre/Newfoundland/AB375/2011(H1N2)
KC492346 A/Duck/Newfoundland/MW721/2010(H6N8)
KC492354 A/American black duck/Newfoundland/MW733/2010(H6N6)
CY132615 A/mallard/California/2540V/2011(H3N8)
CY078285 A/blue-winged teal/Texas/Sg-00158/2007(H4N6)
KC492432 A/Northern pintail /Newfoundland/GR495/2011(H12)
CY045283 A/mallard/Quebec/10969/2006(H2N3)
CY045293 A/mallard/Quebec/11063/2006(H2N3)
CY076105 A/common murre/Oregon/20361-001/2007(H10N7)
KC492314 A/American black duck/Newfoundland/MW609/2010(H4N6)
CY075929 A/common murre/Oregon/19497-004/2005(H9N5)
HM060069 A/thick-billed murre/Alaska/44085-090/2006(H11N9)
HM060063 A/thick-billed murre/Alaska/44085-108/2006(H1N9)
HM060064 A/king eider/Alaska/44397-858/2008(H10N9)
A/Common murre/Newfoundland/AB364/2011(H1N2)
A/Common murre/Newfoundland/AB380/2011(H1N2)
AY703835 A/guillemot/Sweden/3/00(H6N2)
CY028696 A/mallard/Maryland/1235/2006(H3N6)
GU050879 A/blue-winged teal/Texas/108/2000(H10N7)
CY047508 A/mallard/British Columbia/07569/2005(H3N8)
CY017753 A/mallard/Alaska/708/2005(H8N4)
HM060068 A/thick-billed murre/Alaska/44086-095/2006(H11N9)
HM060071 A/common murre/Alaska/44085-165/2006(H11N9)
HM060062 A/thick-billed murre/Alaska/44088-059/2006(H11N9)
HM060067 A/common murre/Alaska/44089-066/2006(H10N2)
HM060074 A/thick-billed murre/Alaska/44085-040/2006(H10N3)
CY015168 A/murre/Alaska/175/1976(H1N2)
CY015165 A/murre/Alaska/305/1976(H1N6)
GU186614 A/turkey/MN/24552/1982(H7N3)
CY021873 A/green-winged teal/Ohio/340/1987(H11N9)
CY005012 A/mallard duck/ALB/224/1977(H7N5)
CY117361 A/mallard/Alberta/881/1984(H2N3)
EU301337 A/aquatic bird/Korea/CN-1/2004(H3N6)
HQ141905 A/duck/Eastern China/318/2003(H11N2)
FJ803187 A/mallard/Sweden/S90735/2003(H7N7)
HM060061 A/thick-billed murre/Alaska/44145-199/2006(H2N6)
CY095264 A/mallard/British Columbia/07594/2005(H5N2)
GU051214 A/mallard/Minnesota/14/2000(H4N3)
CY095422 A/American black duck/New Brunswick/19350/2006(H3N8)
GU826678 A/thick-billed murre/Newfoundland/031/2007(H11N2)
CY089569 A/mallard/Maryland/965/2006(H4N6)
HM060077 A/thick-billed murre/Alaska/44085-155/2006(H9N2)
HM060075 A/thick-billed murre/Alaska/44145-186/2006(H9N2)
CY076113 A/common murre/Oregon/20361-002/2007(H12N5)
KC492418 A/American black duck/Newfoundland/GR490/2011(H3N2)
KC492440 A/Northern pintail/Newfoundland/GR683/2011(H2N2)
1(1)
1(2)
2-EA
3-AA
1-AA
0.02

Genotype	Virus	PB2	PB1	PA	HA	NP	NA	M	NS
A	A/Common murre/Newfoundland/AB318/2011(H1N2)	EA			1		2	EG	
A	A/Common murre/Newfoundland/AB319/2011(H1N2)	EA			1		2	EG	
A	A/Common murre/Newfoundland/AB335/2011(H1N2)	EA			1		2	EG	
A	A/Common murre/Newfoundland/AB353/2011(H1N2)	EA			1		2	EG	
A	A/Common murre/Newfoundland/HM530/2011(H1N2)	EA			1		2	EG	
A	A/Common murre/Newfoundland/HM575/2011(H1N2)	EA			1		2	EG	
A	A/Common murre/Newfoundland/AB438/2011(H1N2)	EA			1		2	EG	
A	A/Common murre/Newfoundland/AB351/2011(H1N2)	EA			1		2	EG	
A	A/Common murre/Newfoundland/AB358/2011(H1N2)	EA			1		2	EG	
A	A/Common murre/Newfoundland/AB341/2011(H1N2)	EA			1		2	EG	
A	A/Common murre/Newfoundland/AB375/2011(H1N2)	EA			1		2	EG	
A	A/Common murre/Newfoundland/AB332/2011(H1N2)	EA			1		2	EG	
A	A/Common murre/Newfoundland/HM524/2011(H1N2)	EA			1		2	EG	

A	A/Common murre/Newfoundland/AB376/2011(H1N2)	EA			1		2	EG			
A	A/Common murre/Newfoundland/AB324/2011(H1N2)	EA			1		2	EG			
B	A/Common murre/Newfoundland/AB331/2011(H1N2)	EA			1		2				
C	A/Common murre/Newfoundland/AB432/2011(H1N2)				1		2	EG			
D	A/Common murre/Newfoundland/AB327/2011(H1N2)				1		2				
E	A/Thick-billed murre/AK/44145-199/2006(H2N6)			EA	2 EA		6			EA	
F	A/Thick-billed murre/AK/44145-155/2006(H9N2)				9		2				
F	A/Thick-billed murre/AK/44145-186/2006(H9N2)				9		2				
G	A/Common murre/Oregon/20361-001/2007(H10N7)				10		7				
H	A/Thick-billed murre/AK/44085-040/2006/(H10N3)				10		3				
I	A/Common murre/Oregon/20361-002/2007(H12N5)				12		5				
J	A/Guillemot/Sweden/3/2000/(H6N2)			EA	6 AG	EA	2 EA				
K	A/Thick-billed murre/St./John's/031/2007(H11N2)				11		2				
L	A/Common murre/AK/44089-066/2006(H10N2)	EA			10		2	EA			
M	A/Thick-billed murre/AK/44085-108/2006(H1N9)	EA			1		9	EA			

N	A/Common murre/AK/44085-165/2006/(H11N9)	EA			11		9	EA		
N	A/Thick-billed murre/AK/44088-059/2006(H11N9)	EA			11		9	EA		
O	A/Thick-billed murre/AK/44085-090/2006(H11N9)	EA			11		9			
O	A/Thick-billed murre/AK/44085-095/2006(H11N9)	EA			11		9			
-	A/Common murre/Newfoundland/AB340/2011(N2)				-		2			
-	A/Common murre/Newfoundland/AB364/2011(H1N2)	EA			1	-	2			
-	A/Common murre/Newfoundland/AB380/2011(H1N2)	EA	-		1	-	2			
-	A/Murre/AK/175/1976/(H1N6)	-	-	-	1	-	6	-	-	
-	A/Common murre/AK/305/1976/(H1N6)		-		1		6 EA	EA		
-	A/Common murre/Oregon/19497-004/2005(H9N5)				9 AG		5			

Figure S5.3. Genomic diversity of AIVs from murres. Fifteen genotypes (designated A through O) were assigned to the 32 murre viruses with sequences available for all 8 segments based on the phylogenetic analyses (Figures 1-4 and S1). Sequences classified within the same phylogenetic grouping for a given segment are shaded alike (white, light grey or dark grey), except for the HA and NA segments that are distinguished by their subtype number. Segments that were classified in lineages other than North American avian are indicated: EA, Eurasian avian; AG, North American gull; EG, Eurasian gull.

Colony	Virus	PB2	PB1	PA	HA	NP	NA	M	NS
Gull Island	A/Common murre/Newfoundland/AB318/2011(H1N2)								
Gull Island	A/Common murre/Newfoundland/AB319/2011(H1N2)								
Gull Island	A/Common murre/Newfoundland/AB335/2011(H1N2)								
Gull Island	A/Common murre/Newfoundland/AB353/2011(H1N2)								
Cabot Island	A/Common murre/Newfoundland/HM530/2011(H1N2)								
Cabot Island	A/Common murre/Newfoundland/HM575/2011(H1N2)								
Cabot Island	A/Common murre/Newfoundland/AB438/2011(H1N2)								
Gull Island	A/Common murre/Newfoundland/AB351/2011(H1N2)								
Gull Island	A/Common murre/Newfoundland/AB358/2011(H1N2)								
Gull Island	A/Common murre/Newfoundland/AB341/2011(H1N2)								
Gull Island	A/Common murre/Newfoundland/AB375/2011(H1N2)								
Gull Island	A/Common murre/Newfoundland/AB332/2011(H1N2)								
Gull Island	A/Common murre/Newfoundland/HM524/2011(H1N2)								
Gull Island	A/Common murre/Newfoundland/AB376/2011(H1N2)								
Gull Island	A/Common murre/Newfoundland/AB324/2011(H1N2)								

Figure S5.4. Sequence diversity within genotype A viruses. Segment sequences with ≤99% nucleotide identity to the other viruses are shaded grey.

Table S6.1. Cross-year surveillance data for AIV infection by real-time RT-PCR in murres in Newfoundland (2009-2011)

Year	Month	Samples	Positive	Total	%
2009	March	216	0	620	0
	April	2	0		
	June	330	0		
	August	50	0		
	December	22	0		
2010	Feburary	60	1	405	1
	May	19	0		
	June	27	1		
	July	72	0		
	August	184	2		
	September	1	0		
	November	42	0		
2011	March	136	0	812	8.4
	May	1	0		
	June	82	0		
	July	209	61		
	August	166	7		
	November	218	0		
Sum up		1837	72	1837	3.9

Table S6.2. Avian influenza virus antibody prevalence in Common Murres (*Uria aalge*) at breeding colonies in Newfoundland, Canada in 2011 and 2012.

Location	2011 (June 9th to August 2nd)			2012 (June 26th and July 9th)		
	Samples	Positives	%	Samples	Positives	%
Gull Island	71	31	44	19	14	74
Great Island	20	9	45	20	13	65
Cabot Island	24	11	46	-	-	-
Total	115	51	44	39	27	69

Note: 1. The serological study followed the same protocol described in Chapter 2 and 4. Approximately 2 ml of blood was drawn from the brachial vein. Serum was separated by centrifugation. The AI MultiS-Screen Ab Test (IDEXX, Westbrook, ME) was used to test for anti-nucleoprotein (NP) antibodies.