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Perpetuation and reassortment of gull influenza A viruses in Atlantic North America

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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 to 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.
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Introduction

Influenza A viruses are eight-segmented, negative-sense, single-stranded RNA viruses belonging to the virus family *Orthomyxoviridae* (Kawaoka 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) (Olsen et al., 2006; Slemons et al., 1974; Webster et al., 1992). Most AIV strains show low pathogenicity and their infection of wild birds usually appears to be asymptomatic, but some highly pathogenic strains may cause fatal infection (Becker, 1966; Chen et al., 2005; Liu et al., 2005). AIVs have a large economic impact on the poultry industry (Alexander, 2007; Webster et al., 2006), and some strains also pose a public health risk (Cardona et al., 2009; Claas et al., 1998; Gao et al., 2013; Peiris et al., 2007).

The sequences of AIV segments generally divide into distinct phylogeographic lineages due to the partial separation of birds by continental regions (Olsen 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 et al., 2006). Furthermore, the hemagglutinin subtypes H13 and H16 (Fouchier et al., 2005; Hinshaw et al., 1982) appear to be almost exclusively maintained in gull populations. 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 (Brown et al., 2012; Kawaoka et al., 1988; Tonnessen et al., 2011), AIV has been transmitted from gulls to poultry (Sivanandan et al., 1991), and there is evidence gull AIVs could infect human cells (Lindskog et al., 2013). Despite these general phylogenetic divisions, reassortants containing segments of different continental and/or bird host origins are detected [e.g. (Chen and Holmes, 2009; Kishida et al., 2008; Koehler et al., 2008; Krauss et al., 2007; Lomakina et al., 2009; Ramey et al., 2010b; Spackman et al., 2005; Van Borm et al., 2012; Widjaja et al., 2004; Wille et al., 2011a)]. This is particularly common in AIVs from gulls, which is presumably driven by gull ecology in terms of habitat use and migratory behavior, and may reflect an important role for gulls in AIV transmission among regions and different host taxa (Arnal et al., 2014; Ramey et al., 2010a;

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Van Borm et al., 2012; Wille et al., 2011b). However, gull AIV genome sequence information remains limited, especially in comparison to the data available for waterfowl viruses.

Temporally, there is not yet a clear pattern for peak AIV prevalence in gulls. AIVs were detected across the fall months in gulls of Southern Norway, with a prevalence of 8% in European Herring (*Larus argentatus*) and 5.5% in Great Black-backed (*Larus marinus*) Gulls (Tønnessen et al., 2013). A study in Georgia (Asia) with a large gull data set (~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 (*Chroicocephalus ridibundus*) (Lewis et al., 2013). Peak prevalence in Black-headed Gulls in the Netherlands occurred during the summer months following fledging of chicks at the breeding colonies (Verhagen et al., 2014), similar to results for Great Black-headed Gulls (*Larus. ichthyaetus*) (Yamnikova et al., 2009). Further surveillance will be required to determine if there are meaningful patterns of AIV prevalence in gulls.

Oceanic coasts are useful locations for studies of gull AIVs because of abundant gull populations and increased chances of intercontinental bird movements. The northern Atlantic coast of North America is one such location, with gull movements to Europe documented (Wille et al., 2011a) and intercontinental reassortant gull viruses found (Hall et al., 2013; Krauss et al., 2007; Wille et al., 2011a). 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.

Results

Prevalence of AIV infection in gulls

Between May 2008 and December 2011, 20/1350 samples collected from gulls were positive (1.5%). Most samples (1283) and positives (19) were from the two predominant species that are present on the island of Newfoundland year-round: American Herring Gull (*Larus. smithsonianus*; 13/1083) and Great Black-backed Gull (6/200) (Table 1). 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 (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 295 fecal samples (3.4%), resulting in an overall prevalence of 30/1645 (1.8%).

A peak in AIV infection occurred in the fall (Table 1), 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 three to six weeks of age, but only one positive was detected in 792 samples (Table 1).

All four species that were blood-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%) and Iceland Gull (4/8, 50%), for an overall rate of 50% (44/88) across all species. The tested Herring Gull eggs showed the presence of maternal anti-AIV antibodies (4/11, 36%).

Table 1
Seasonality of AIV prevalence in juvenile and adult Great Black-backed and Herring Gulls in Newfoundland and Labrador, 2008–2011.

	Summer[a]	Fall	Winter	Spring
Great black-backed gull				
Juvenile[b]	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] Data are presented as positive samples out of total number of samples, with prevalences as percentages in brackets.
[b] Juveniles are birds less than 12 months old.

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 two positive samples from 2008 were analyzed previously (Wille et al., 2011a)). Amongst the 16 viruses, 15 were detected around the City of St. John's (47°56′N, 52°71′W) in eastern Newfoundland and one, 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 eight segments of 13 viruses, seven segments for one virus, and six segments for two viruses. The sequences were submitted to the GenBank database under accession numbers KC845024–KC845145.

Phylogenetic analyses

The Newfoundland and reference gull AIV sequences were used for phylogenetic analyses with closely related reference genes identified in the sequence databases. The clade affiliations from the maximum likelihood trees matched those on the neighbour-joining and the maximum clade credibility trees. Multiple lineages were identified for each gene segment of these Newfoundland gull viruses (Fig. 4), as detailed below.

HA and NA segments

The HA subtypes were determined for 14 of the 16 viruses: one H1, one H9, eight H13 and four H16 viruses. The H1 and H9 sequences belonged to North American avian lineages (Fig. 1A and B). The H1 sequence was very closely related to a sequence detected from a shorebird in Delaware in 2009, with the remaining sequences in the lineage coming from duck viruses, particularly from eastern North America. The H9 sequence was most similar to two waterfowl viruses from North America, and no similar gull or shorebird sequences were identified. 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 in 2008 (Fig. 1C). There were also numerous gull H13 sequences in the database from other locations in eastern North America and Alaska, but these were fell in distinct lineages. The four H16 sequences were within a Eurasian gull lineage, clustered together with virus sequences that mostly originated from other Northern Atlantic locations such as Iceland, Sweden, and Norway (Fig. 1D). They were not closely related to the limited number of H16 viruses previously isolated from North American gulls.

The NA subtypes were determined for 14 of the 16 viruses; three N3, nine N6 and two N9 viruses. The three N3 sequences

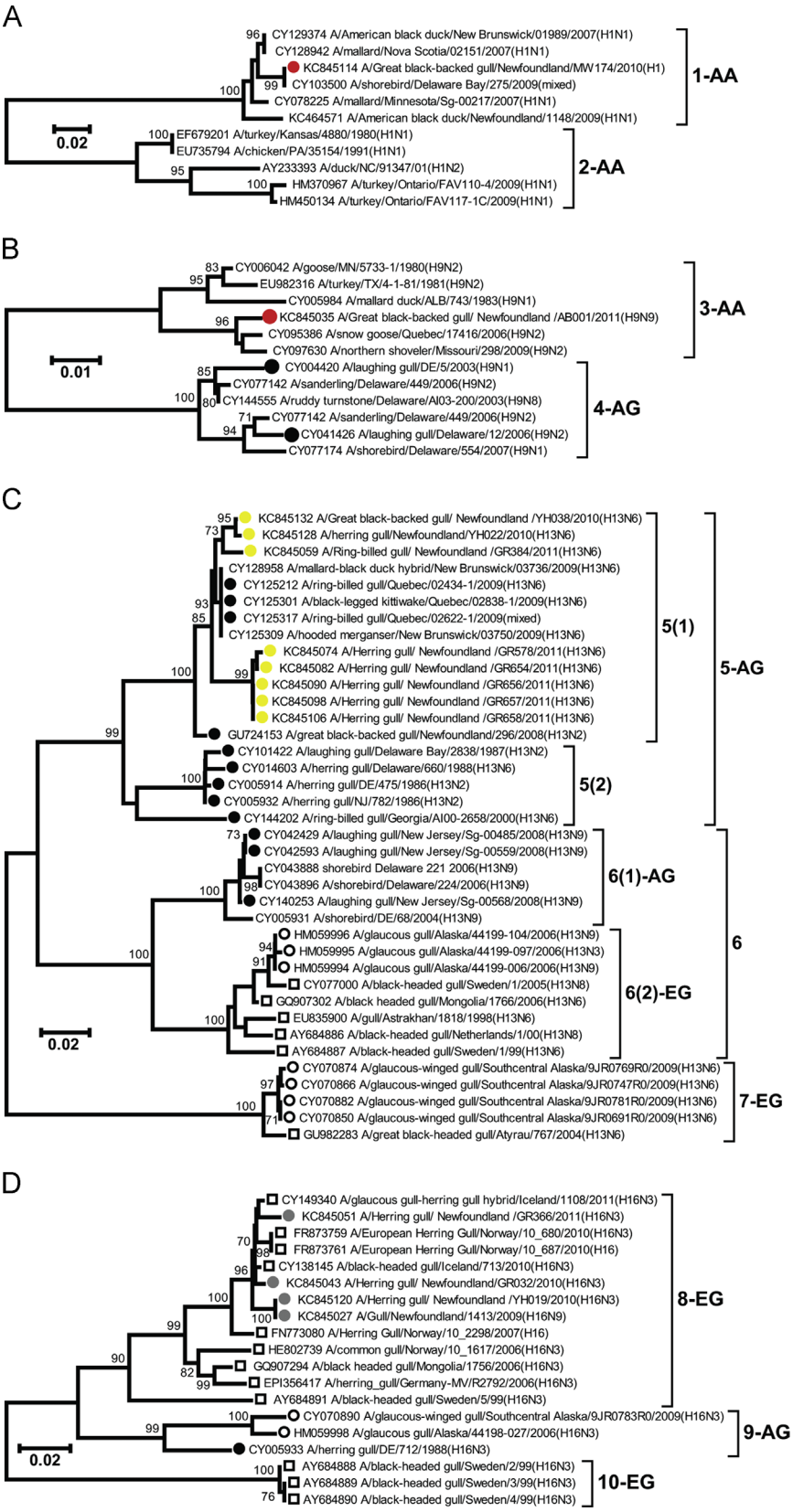

Fig. 1. Phylogenetic trees for the Newfoundland gull virus HA sequences from 2009 to 2011. Individual trees are shown for the H1 (A), H9 (B), H13 (C) and H16 (D) sequences. The gull AIV sequences from Newfoundland (from 2009 to 2011) are labeled according to the geographic and host group affiliation of their clades: yellow circle for North American gull (AG), red circle for North American avian (AA) and gray circle for Eurasian gull (EG). Reference gull AIV sequences from Atlantic and Pacific North America are labeled with black and open circles, respectively, while those from Eurasia are labeled with open squares. Gene lineages and clades were assigned with serial numbers as described in the Materials and Methods section. Phylogenetic maximum likelihood (ML) trees were constructed using MEGA5 with 1000 bootstrap replicates and bootstrap values $\geq 70\%$ are given at the nodes. The scale bars indicate substitutions per site.

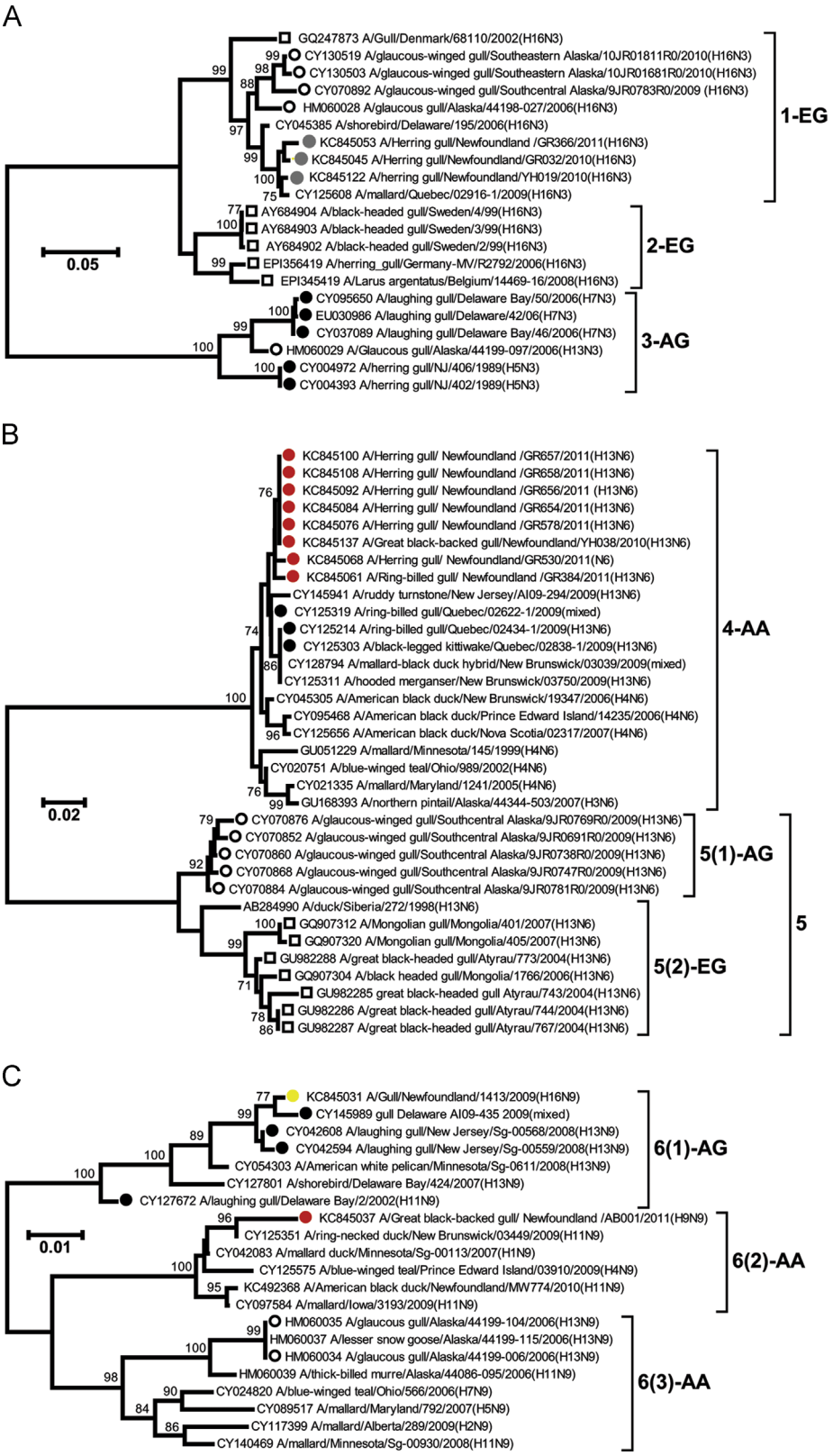

Fig. 2. Phylogenetic trees for the Newfoundland gull virus NA sequences from 2009 to 2011. Individual trees are shown for the N3 (A), N6 (B) and N9 (C) sequences. The gull AIV sequences from Newfoundland (from 2009 to 2011) are labeled according to the geographic and host group affiliation of their clades: yellow circle for North American gull (AG), red circle for North American avian (AA) and gray circle for Eurasian gull (EG). Reference gull AIV sequences from Atlantic and Pacific North America are labeled with black and open circles, respectively, while those from Eurasia are labeled with open squares. Gene lineages and clades were assigned with serial numbers as described in the Materials and Methods section. Phylogenetic maximum likelihood (ML) trees were constructed using MEGA5 with 1000 bootstrap replicates and bootstrap values $\geq 70\%$ are given at the nodes. The scale bars indicate substitutions per site.

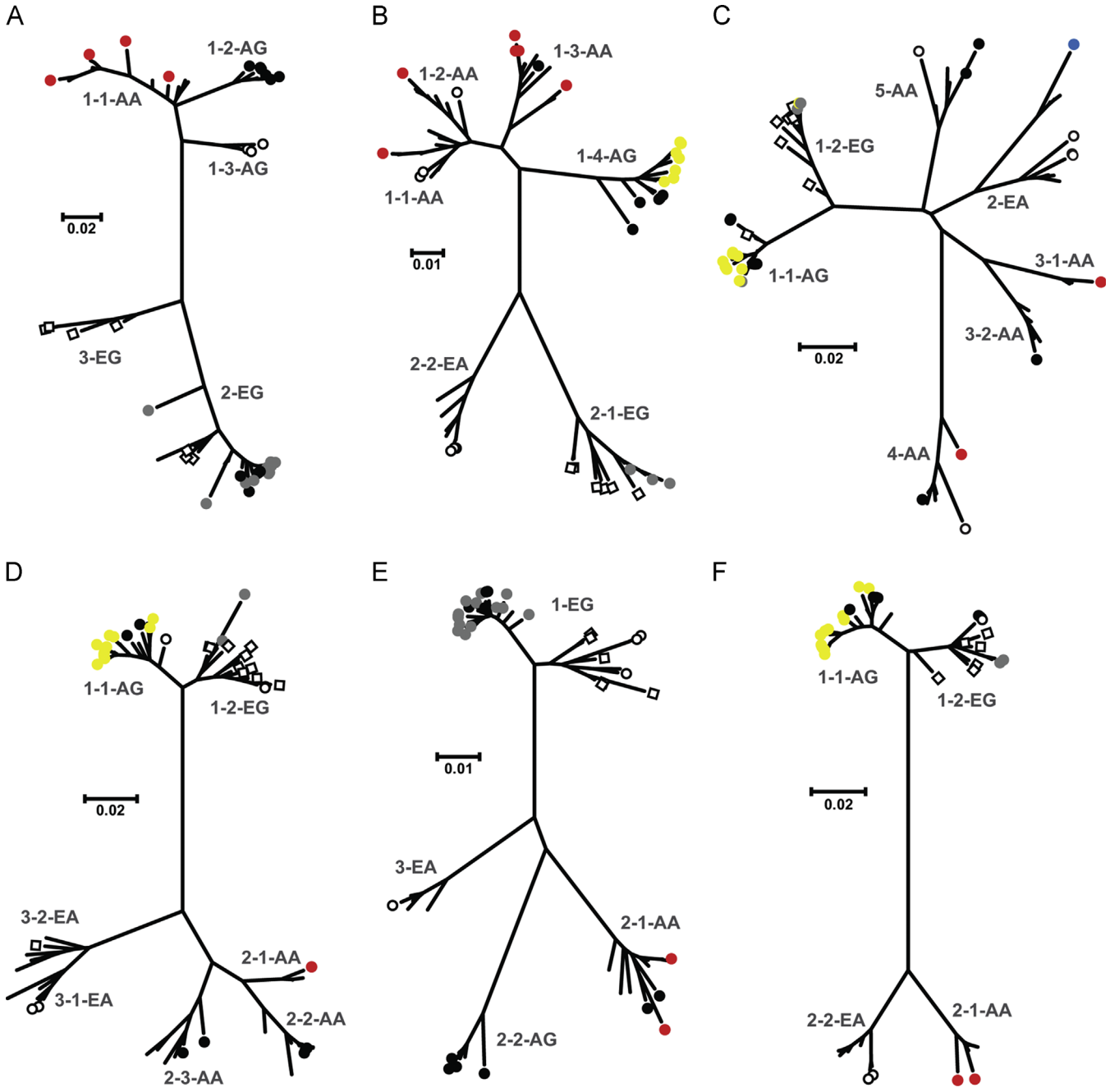

Fig. 3. Phylogenetic topology trees for Newfoundland gull AIV internal protein gene segments. The trees show the lineage distribution patterns for the PB2 (A), PB1 (B), PA (C), NP (D), M (E) and NS (F) segments. Trees with full virus labels are provided in Fig. S1. The gull AIV sequences from Newfoundland (from 2009 to 2011) are labeled 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), gray circle for Eurasian gull (EG) and blue circle for Eurasian avian (EA). Reference gull AIV sequences from Atlantic and Pacific North America are labeled with black and open circles, respectively, while those from Eurasia are labeled with open squares. Gene lineages and clades were assigned with serial numbers as described in the Materials and Methods section. Phylogenetic maximum likelihood (ML) trees were constructed using MEGA5 with 1000 bootstrap replicates and bootstrap values $\geq 70\%$ are given at the nodes. The scale bars indicate substitutions per site.

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 H16N3 virus isolated from a duck (A/mallard/Quebec/02916-1/2009(H16N3)) (Fig. 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 closely related sequences were identified in a shorebird virus in New Jersey, three gull viruses in Quebec and two duck viruses in New Brunswick (Fig. 2B). In comparison, five N6 sequences from Alaskan gulls were classified in a different lineage. The two N9 sequences were within the same lineage, but were separated into distinct North American avian and North American gull clades (Fig. 2C). The two available reference N9 sequences from gulls in Alaska belonged to a third North American avian clade within this lineage.

PB2, PB1, PA, NP, M and NS 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

Date	Virus	Phylogenetic clade number								
		PB2	PB1	PA	HA	NP	NA	M	NS	Genotype
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

Fig. 4. Newfoundland gull AIV genotypes from 2009 to 2011. The viruses are listed in chronological order with the sample collection dates indicated. The phylogenetic clade numbers correspond to those on Figs. 1, 2 and 3 for each of the eight segments. The colors of the boxes indicate the continental and host group affiliation for the segment's clade: yellow, North American gull; gray, Eurasian gull; red, North American avian; blue, Eurasian avian. Ten genotypes (A–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.

shown in Fig. 3, and trees with detailed virus information labels are available in Fig. S1.

The 16 PB2 genes in Newfoundland gulls belonged to two phylogenetic lineages (Figs. 3A and S1). 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 to 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 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 three genes in lineage 2 (Figs. 3B and S1). 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 to 2009. The three NL sequences in lineage 2 were within 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 to four lineages (Figs. 3C and S1). 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 (Figs. 3D and S1). 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 to 2006) in lineage 2.

The 16 M sequences belonged to two lineages (Figs. 3E and S1). Fourteen of them belonged to the Eurasian gull lineage 1, together with several genes from gulls, ducks and shorebirds in Atlantic North America. 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 to 2006).

The 16 NS sequences were all allele A and belonged to two lineages (Figs. 3F and S1). Among the 14 genes in lineage 1, 12 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.

Genetic structure and genome dynamics 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 2. Among the 123 segment 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 one was in a Eurasian avian clade (0.8%). This revealed that the AIV gene pool was dominated by gull lineage sequences (75.6%), but frequent intercontinental (35.8%) and considerable inter-host group (24.4%) segment transmission was found.

The genotype assignments based on the combination of lineages for the eight segments showed that 15 of the gull AIVs were intercontinental reassortants and contained Eurasian lineage genes, while 12 viruses were interspecies reassortants and contained avian lineage genes (Fig. 4). Of particular note, all eight segments of A/Herring gull/Newfoundland/YH019/2010(H16N3) were within 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 (Figs. 1–3), the 13 gull viruses with sequence information available for all eight segments were classified in 10 genotypes (A to I; Fig. 4). Genotype I was predominant, represented by 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, three gull viruses from Quebec

Table 2
Phylogenetic clade assignments for the 16 Newfoundland gull AIVs from 2009 to 2011.

Clade	Number of viruses								Total (%)
	PB2	PB1	PA	HA	NP	NA	M	NS	
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 (35.0%)
Eurasian avian	0	0	1	0	0	0	0	0	1 (0.8%)
Total	16	16	16	14	15	14	16	16	123

and two duck viruses from New Brunswick in 2009 (Hall et al., 2013), also had genotype I (the three gull viruses are included in Figs. 1, 2 and S1). A previously described Newfoundland gull AIV, A/Great black-backed gull/Newfoundland/296/2008(H13N2), had all segments except PA and NA classified in the same clades (Figs. 1, 2 and S1). Time of most recent common ancestor analyses for the eight segments of these genotype I viruses showed that they emerged in gulls in Atlantic Canada between 2001 and 2008 (Fig. 5).

Discussion

Prevalence of infection

Our surveillance for AIV infection in gulls on the island of Newfoundland, Canada, has shown that AIV infection in Herring and Great Black-backed Gulls occurred primarily in the fall, with most of the positive samples from juvenile birds (Table 1). Our observed prevalence ($<2\%$) was similar to many other studies with gulls reporting prevalence values of $<5\%$ (Buscaglia, 2012; Hanson et al., 2008; Hulsager et al., 2012; Ip et al., 2008; Lewis et al., 2013; Marchenko et al., 2012; Munster et al., 2007; Sivay et al., 2012), but it has also been found to be as high as 15% (Toennessen et al., 2011; Tønnessen et al., 2013; Velarde et al., 2010; Verhagen et al., 2014; Yamnikova et al., 2009). Based on our results from captured birds, we would predict that the positive fecal samples also largely came from juvenile birds, and these birds were common in the sampled roosting areas. Almost no positive chicks were detected at the breeding colony (1/792), which is in contrast to previous studies that found high prevalence in juvenile Ring-billed Gulls in Ontario, Canada (Velarde et al., 2010), Black-headed Gulls in the Netherlands (Verhagen et al., 2014), and Great Black-headed Gulls in Russia (Yamnikova et al., 2009), all at the breeding colonies. We found evidence of Herring Gull maternal transfer of anti-AIV antibodies, with four of 11 tested eggs positive (36%). Maternal antibody transfer to eggs has been observed in gulls previously, at 14% (Pearce-Duvet et al., 2009) and 51% (Hammouda et al., 2011) in two studies of Yellow-legged Gulls (*Larus michahellis*) in the Mediterranean. Maternal antibody transfer was suggested to be the cause of observed seroprevalence in chicks in another study (Lewis et al., 2013). However, viruses have also been found in embryos of European Herring Gulls, suggesting vertical transmission is possible (Yamnikova et al., 2009).

Despite low prevalence of active AIV infection, serology data indicated that half of the tested birds were previously infected. The 50% value we found for seroprevalence is comparable to most other studies. The Georgian study reported 56% seroprevalence for Armenian Gulls (Lewis et al., 2013), while another study in North America found 45% overall (when using the same detection method we employed) for five gull species (Brown et al., 2010). Higher rates were found in Black-legged Kittiwakes (*Rissa tridactyla*) in Norway, at 71% (Toennessen et al., 2011), in Yellow-legged Gulls in Tunisia, at 77% (Hammouda et al., 2011), and in Ring-billed Gulls in Ontario, Canada, at 80% and 92% in two different years (Velarde et al., 2010). Therefore, AIV infection is prevalent in gulls.

Virus genetic structure, perpetuation and reassortment

We classified the analyzed sequences into specific lineages to explore genome constellation make-up, diversity, and reassortment. Ten different genotypes were assigned to the sixteen viruses sequenced, suggesting that gulls in Newfoundland host a diverse genetic reservoir of multiple lineages. Further, there are some gene lineages that are more common and have been perpetuated across years, and that these genes and viruses were likely circulating in Atlantic North America for some time before detection beginning in 2008. Overall, the viruses predominantly contained sequences from gull-related phylogenetic clades (93/123, 75.6%), but 12 viruses contained at least one segment from an avian (i.e. non-gull) clade. One virus, A/Great black-backed gull/Newfoundland/AB001/2011(H9N9), had all eight segments in avian virus clades, and another virus, A/Great black-backed gull/Newfoundland/MW174/2010(H1), had all six segments for which we obtained sequence data in avian virus clades. These viruses presumably represent recent transmission or spillover events from waterfowl to gulls. Detection of avian lineage segments in gull viruses has been common (Hall et al., 2013; Kawaoka et al., 1988; Lomakina et al., 2009; Toennessen et al., 2011; Van Borm et al., 2012; Widjaja et al., 2004; Wille et al., 2011a), but not universal (Lewis et al., 2013). Frequent AIV transmission involving gulls and waterfowl is likely driven by gull ecology and their broad habitat use, 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 viral segments dominated in the only two AIVs detected during winter (both from February) in this study, which was distinct from the remaining viruses from autumn (Fig. 4). This presumably resulted from increased interaction between gulls and waterfowl at reduced winter habitat such as areas with remaining open water.

Fifteen viruses contained at least one segment that we defined as Eurasian. Intercontinental reassortants have been identified in high proportions of gull AIVs on both coasts of North America (Hall et al., 2013; Wille et al., 2011b) and in Europe (Van Borm et al., 2012). A recent study in Iceland (Dusek 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. Based on the 95% cut-off value for clade definition, A/Herring gull/Newfoundland/YH019/2010(H16N3) had all eight segments in Eurasian gull clades (Fig. 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, examination of the phylogenetic trees for the NP, M and NS segments (Fig. 3D, E and F, respectively) shows that they show very similar topology for their respective lineage 1 portions of the trees (i.e. the upper portions 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. Usage of 95% as the cut-off value places all of these M sequences within a single clade, whereas the other two segments are each divided into two clades. The M segment shows the lowest d_N/d_S ratio for AIVs (Bahl et al., 2009), and therefore it is possible these M sequences should also be divided into North American and Eurasian clades within lineage 1.

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

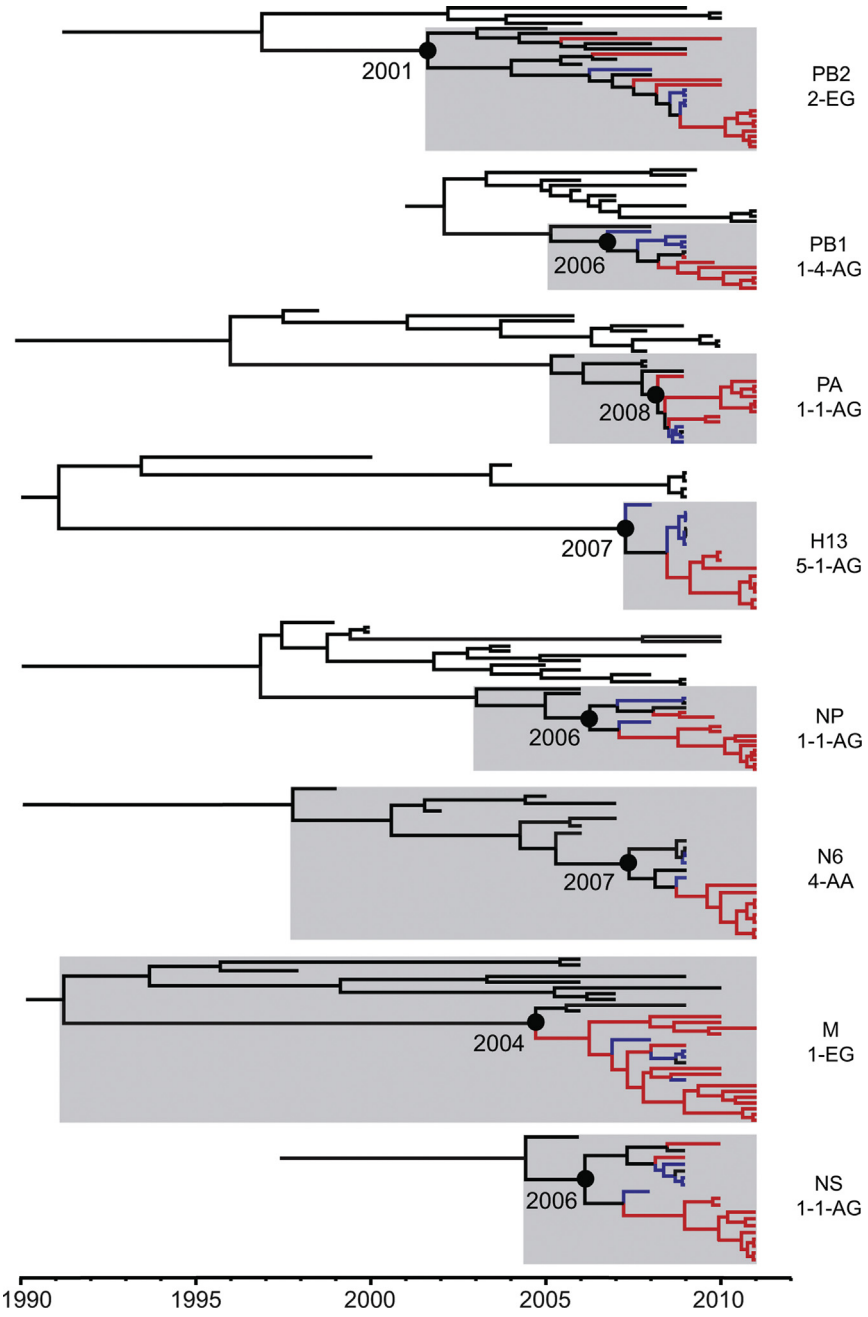

Fig. 5. Emergence dates for the gene segments found in a predominant genotype identified in gulls in Atlantic Canada since 2009. The red branches represent the Newfoundland gull sequences from this study, the blue branches represent other gull viruses from Atlantic Canada, and the black branches represent other viruses. The gray boxes delineate the relevant clades, which are labeled according to the phylogenetic trees in Figs. 1, 2 and S1. For the M sequences, the two viruses from before 1990 were excluded from the analysis. The most recent common ancestor dates were calculated in BEAST v1.7.5 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.

reassortants relative to this genotype (Figs. 1–4). 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 temporal and spatial proximity (Huang et al., 2013b, 2014; Reeves et al., 2011). Viruses with segments closely related to those from the genotype I viruses were also detected previously in Atlantic Canada over 2008–2009 (Figs. 1–3) (Hall et al., 2013; Wille et al., 2011a). This provides insight into the evolution and perpetuation of AIVs through years in a regional gull population. The dominance of genotype I, and its reassortants, indicates that a group of highly related viruses circulated in the gulls in Atlantic Canada over 2008–2011. Specific reassortment events can be observed within these gull viruses, as well as what appear to be spillover events to non-gull hosts (Hall et al., 2013). Molecular dating indicated emergence times in gulls of 2001–2008 for the segments in these viruses (Fig. 5). However, as noted above, other viruses were also detected in these birds over this period.

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 (Figs. 1, 2 and S1). As

recently summarized for gulls in general (Arnal et al., 2014), more research is still 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, especially in consideration of the potential importance of gulls in global AIV dynamics.

Materials and methods

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 Newfoundland and Labrador's Chief Veterinary Officer. 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 Canadian Wildlife Service banding permit 10559. Swabs of the cloaca and oropharyngeal cavity were collected as samples, and blood samples were also taken from the brachial vein of some birds. Eggs were collected under permit SP2782 from Canadian Wildlife Service. The sampling was either done at locations where no access permits were required (public areas around the City of St. John's, Newfoundland and Labrador) or with the permission of the relevant authorities in the cases of the Robin Hood Bay Waste Management Facility, Corner Brook Waste Disposal Site, and the Witless Bay Seabird Ecological Reserve. This research was also approved under Memorial University biosafety permit S-103.

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 from 88 gulls 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 (Pyle, 2008). All gulls in their first year of life (<12 months old), including pre-fledged young, were classified as juveniles and all others were classified as adult birds even though this adult category includes sexually immature birds that are not yet capable of breeding. Months of the year were grouped into four unequal seasons 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, 295 fresh fecal samples were collected from roost sites. Only samples than could be confirmed as coming from gulls were collected.

Swab samples were kept cool in the field for less than 24 hours before being stored at -80 °C until processed. Samples were screened for the presence of AIV by real time RT-PCR targeting the M gene using previously published methods (Granter et al., 2010; Spackman et al., 2002). Positive samples had Ct values <35. Prevalence was compared across years and seasons using 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 antibodies using the AI MultiS-Screen Ab Test (IDEXX, Westbrook, Maine) as recommended by the manufacturer.

Samples from egg yolks were prepared as described previously (Pearce-Duvet et al., 2009). The resulting extract was tested or the presence of anti-AIV NP antibodies as described above for sera.

Virus sequencing

RNA was extracted from positive swab samples with Trizol LS reagent (Life Technologies, Burlington, Canada) and used as the template for RT-PCR using previously published primers (Chan et al., 2006; Hoffmann et al., 2001; Huang et al., 2013a; Obenauer et al., 2006; Phipps et al., 2004) 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).

Phylogenetic analyses

The AIV sequences obtained in this study and reference AIV sequences from the NCBI influenza database (Bao et al., 2008) and the GISAID epiFlu database (http://platform.gisaid.org) were used for phylogenetic analyses. 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. At least 20 closely related sequences to each gull AIV sequence from our study were identified by BLAST searches (Altschul et al., 1997), and used 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 CDS 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. Phylogenetic trees were constructed with the maximum likelihood method in MEGA5 (Tamura 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 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 et al., 2012). A lognormal relaxed clock (unrelated) model was used to infer the phylogenetic timescale, and the analysis was run 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.

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 had $\geq 90\%$ nucleotide identity (Lu et al., 2007; Ramey et al., 2010a) and sequences within a clade had $\geq 95\%$ 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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.04.009.

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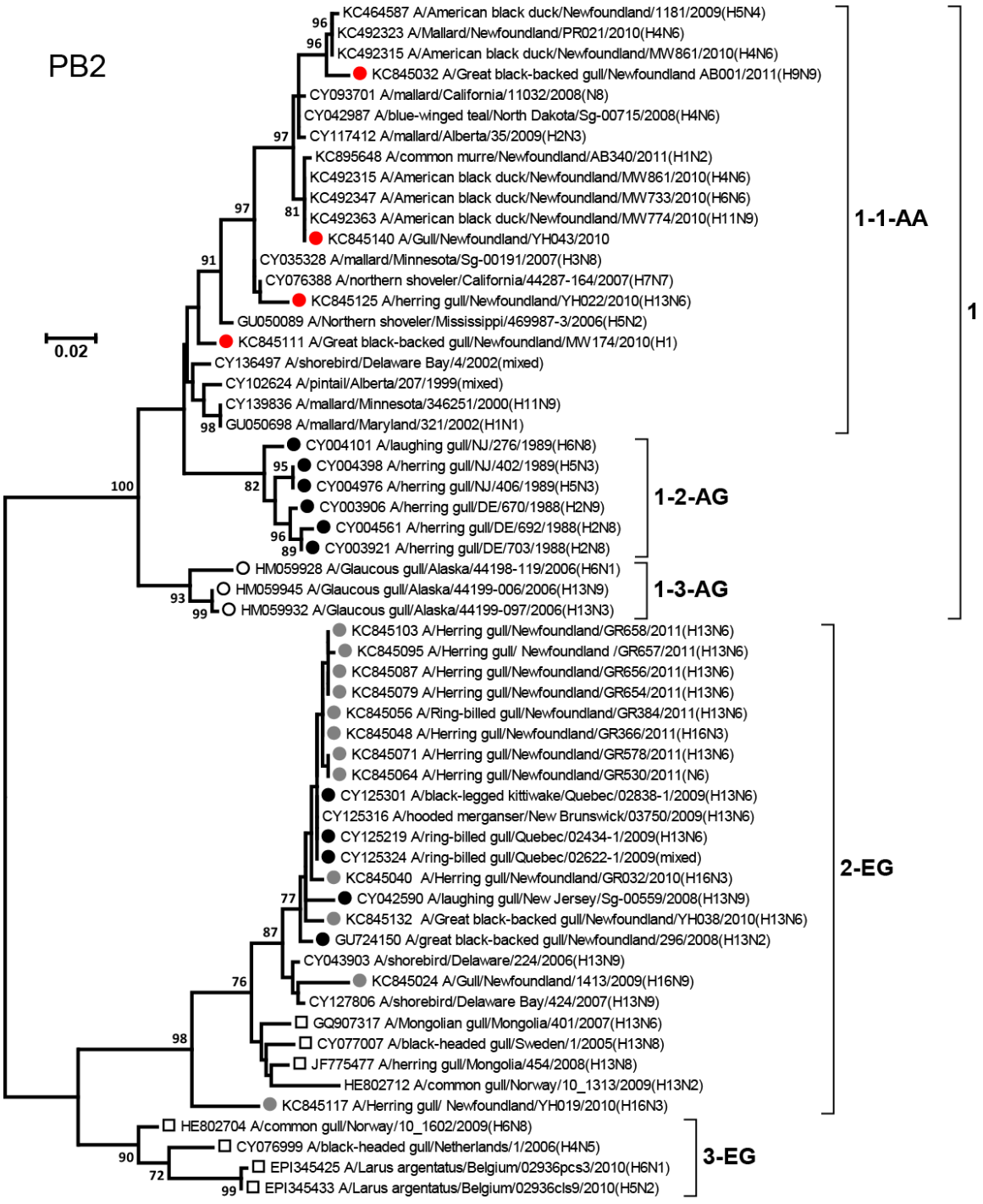
Figure S1. Phylogenetic trees for the internal protein gene segments with virus identification information. The gull AIV sequences from Newfoundland (from 2009-2011) are labelled according to the geographic and host group affiliation of their clades: yellow circle for North American gull (AG), red circle 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 Materials and Methods section. The maximum likelihood 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.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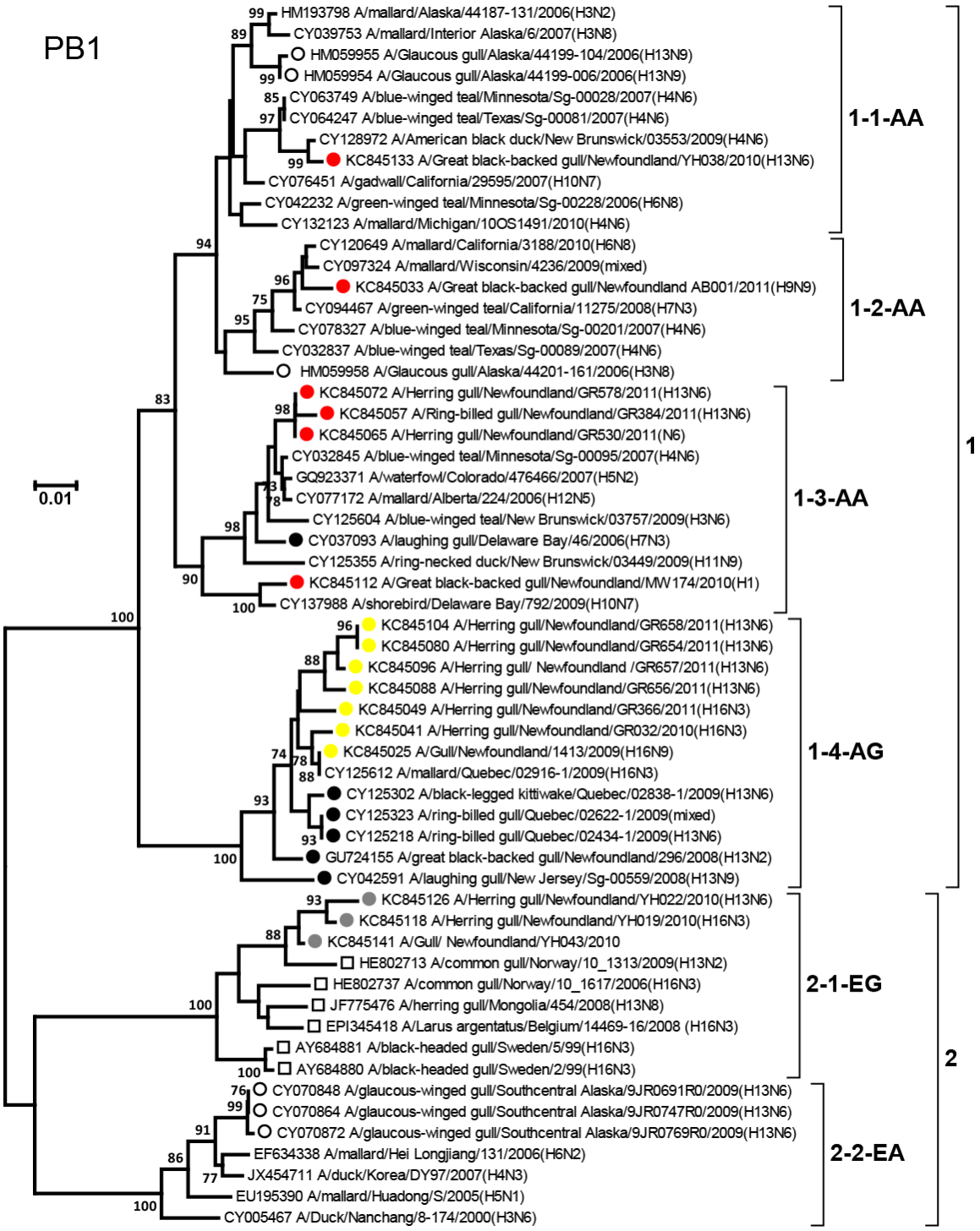
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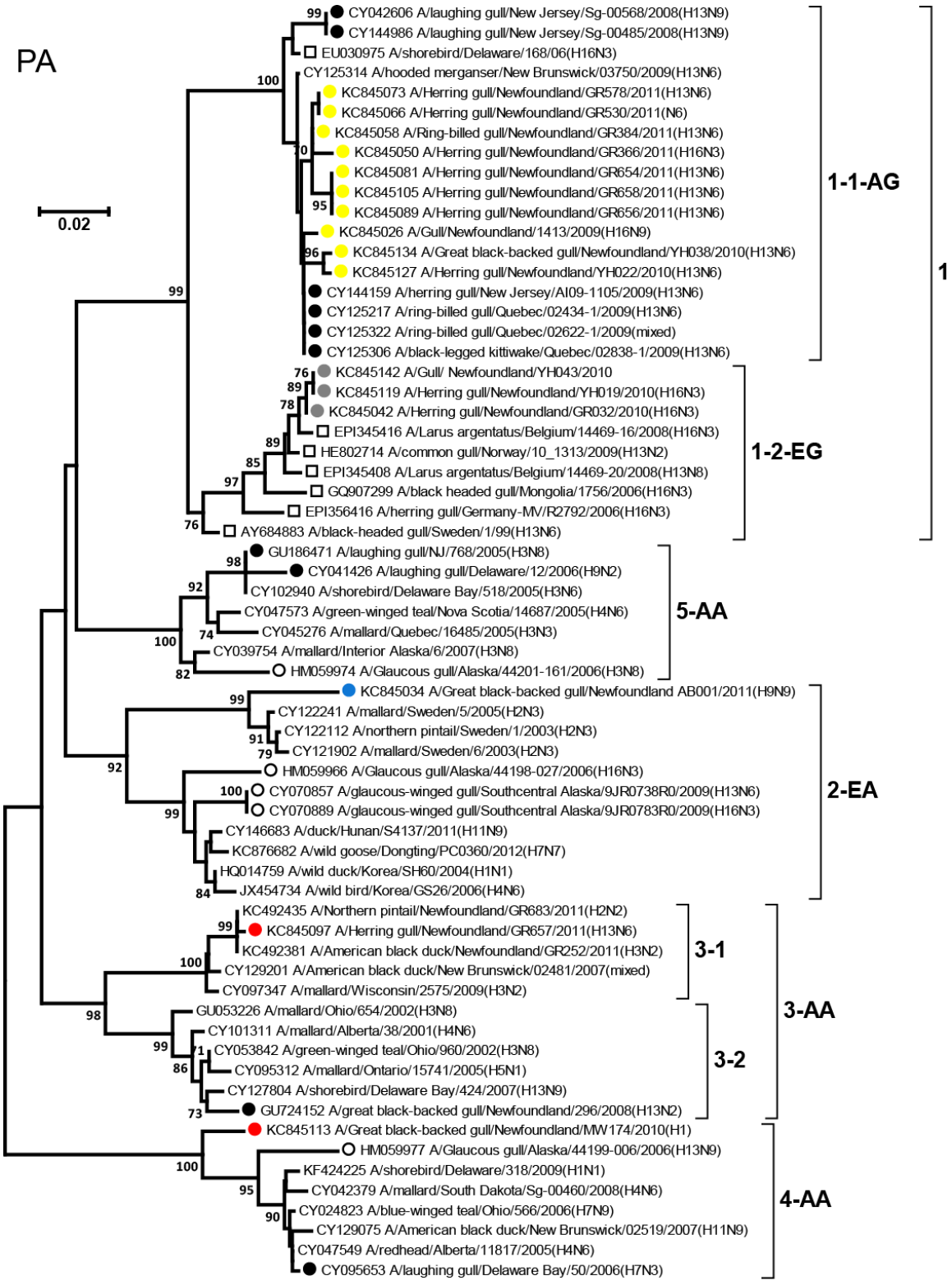
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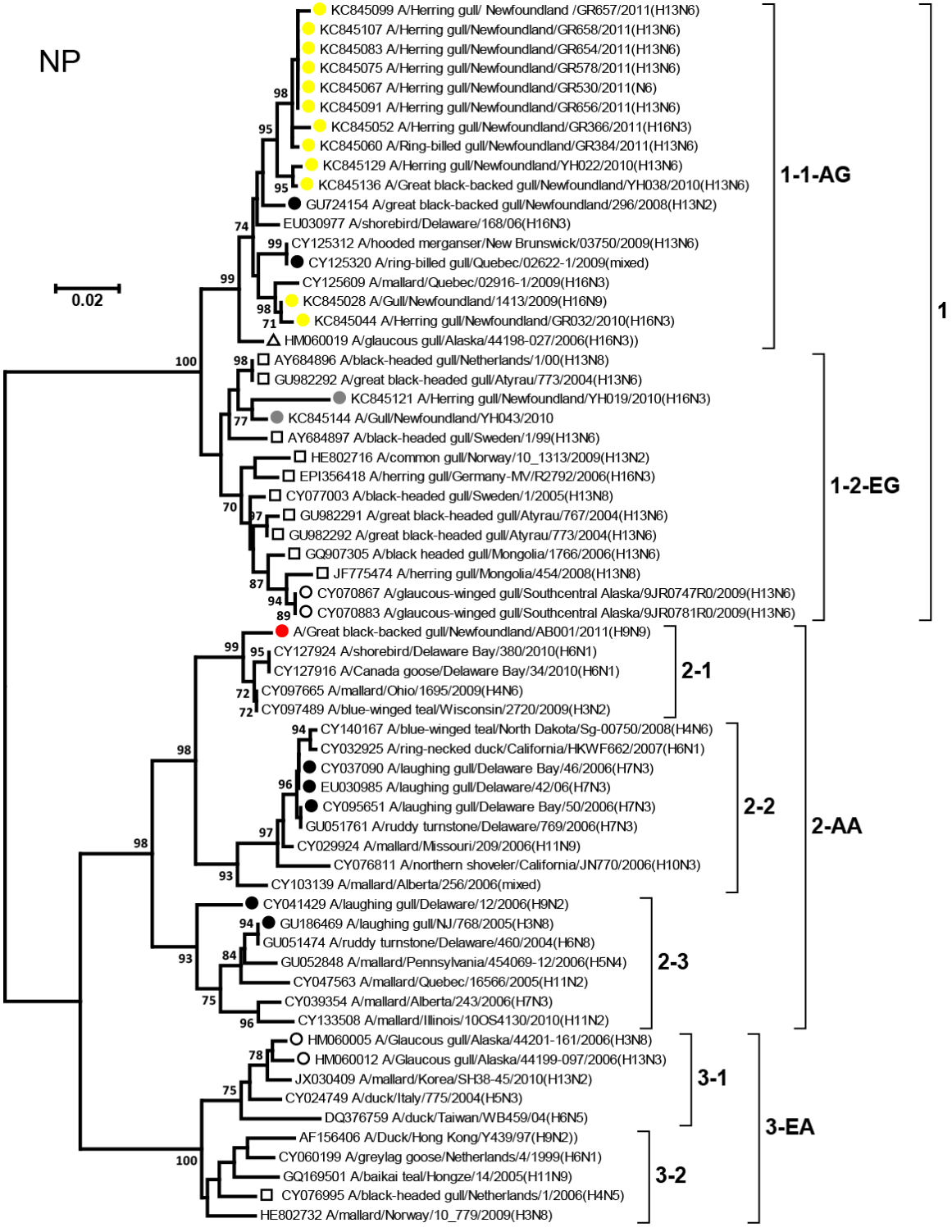
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A/Great black-backed gull/Newfoundland/AB001/2011(H9N9)
CY127924 A/shorebird/Delaware Bay/380/2010(H6N1)
CY127916 A/Canada goose/Delaware Bay/34/2010(H6N1)
CY097665 A/mallard/Ohio/1695/2009(H4N6)
CY097489 A/blue-winged teal/Wisconsin/2720/2009(H3N2)
CY140167 A/blue-winged teal/North Dakota/Sg-00750/2008(H4N6)
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)
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)
HM060005 A/Glaucous gull/Alaska/44201-161/2006(H3N8)
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)
AF156406 A/Duck/Hong Kong/Y439/97(H9N2))
CY060199 A/greylag goose/Netherlands/4/1999(H6N1)
GQ169501 A/baikai teal/Hongze/14/2005(H11N9)
CY076995 A/black-headed gull/Netherlands/1/2006(H4N5)
HE802732 A/mallard/Norway/10_779/2009(H3N8)
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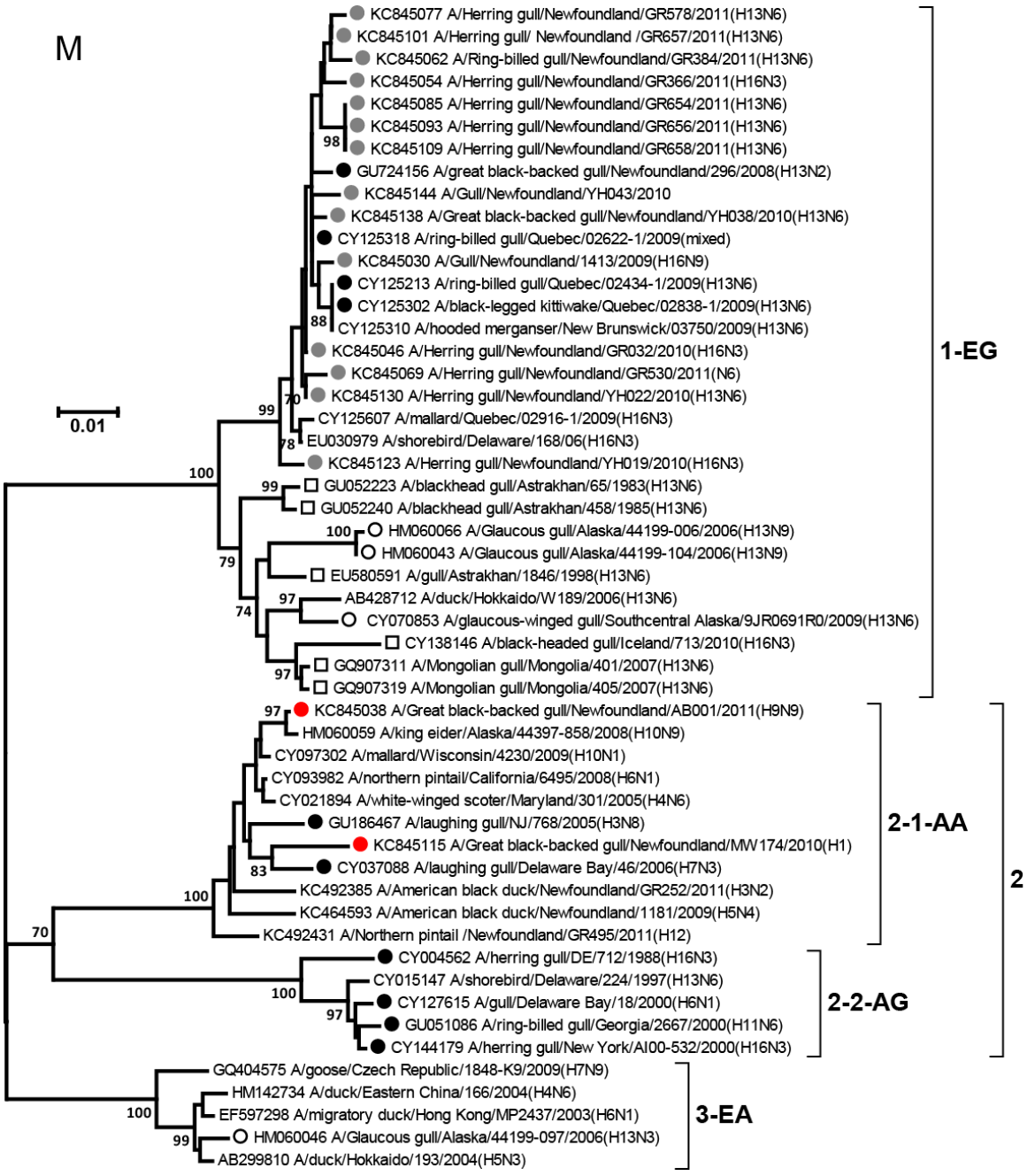
2-3
2-AA
3-1
3-2
3-EA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KC845054 A/Herring gull/Newfoundland/GR366/2011(H16N3)
KC845085 A/Herring gull/Newfoundland/GR654/2011(H13N6)
KC845093 A/Herring gull/Newfoundland/GR656/2011(H13N6)
KC845109 A/Herring gull/Newfoundland/GR658/2011(H13N6)
GU724156 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)
KC845115 A/Great black-backed gull/Newfoundland/MW174/2010(H1)
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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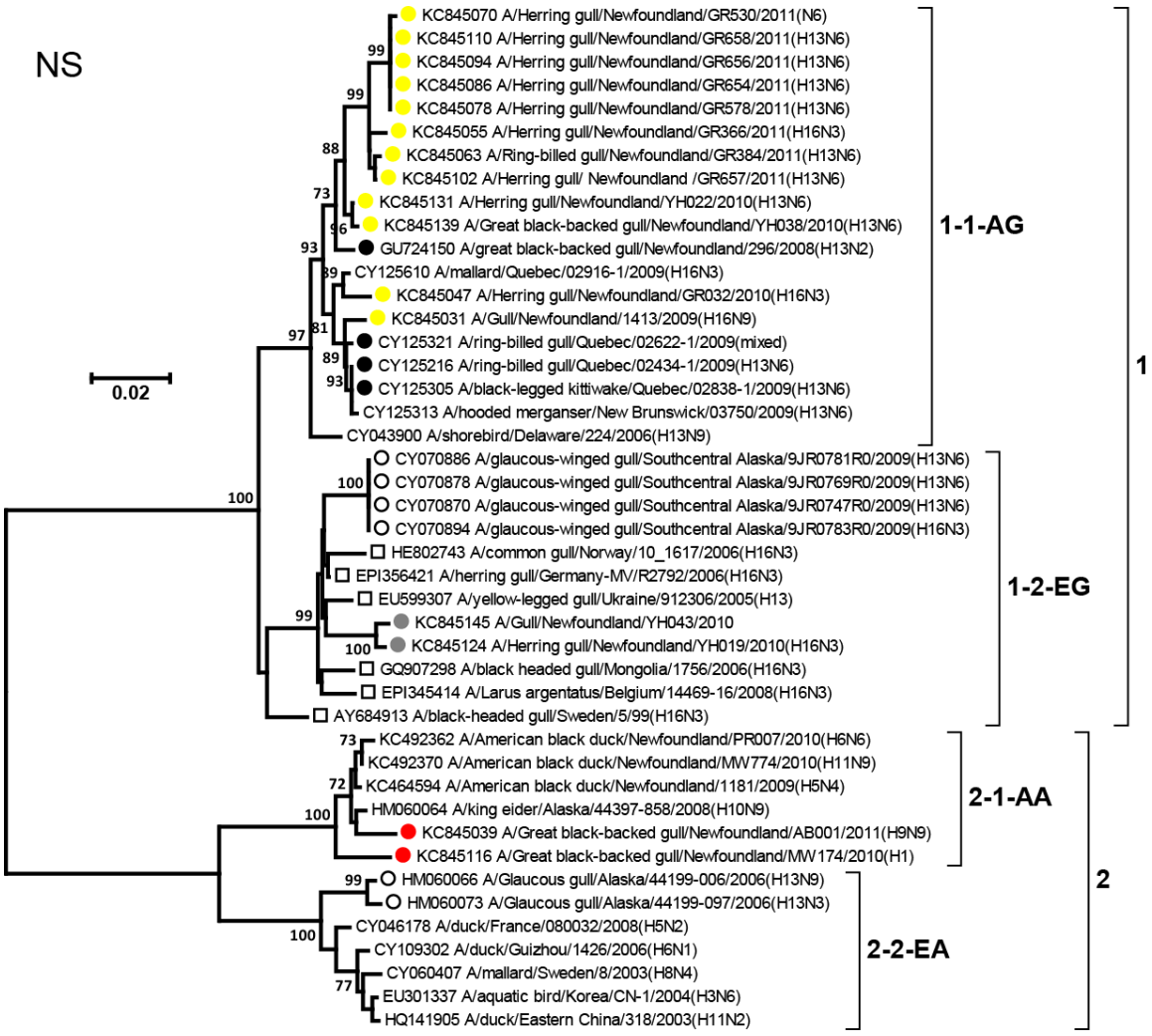
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KC845063 A/Ring-billed gull/Newfoundland/GR384/2011(H13N6)
KC845102 A/Herring gull/ Newfoundland /GR657/2011(H13N6)
KC845131 A/Herring gull/Newfoundland/YH022/2010(H13N6)
KC845139 A/Great black-backed gull/Newfoundland/YH038/2010(H13N6)
GU724150 A/great black-backed gull/Newfoundland/296/2008(H13N2)
CY125610 A/mallard/Quebec/02916-1/2009(H16N3)
KC845047 A/Herring gull/Newfoundland/GR032/2010(H16N3)
KC845031 A/Gull/Newfoundland/1413/2009(H16N9)
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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)
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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)
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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)
KC845039 A/Great black-backed gull/Newfoundland/AB001/2011(H9N9)
KC845116 A/Great black-backed gull/Newfoundland/MW174/2010(H1)
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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)
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