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Research article

Where do all the subtypes go? Temporal dynamics of H8–H12 influenza A viruses in waterfowl

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Abstract

Influenza A virus (IAV) is ubiquitous in waterfowl. In the northern hemisphere IAV prevalence is highest during the autumn and coincides with a peak in viral subtype diversity. Although haemagglutinin subtypes H1–H12 are associated with waterfowl hosts, subtypes H8–H12 are detected very infrequently. To better understand the role of waterfowl in the maintenance of these rare subtypes, we sequenced H8–H12 viruses isolated from Mallards (*Anas platyrhynchos*) from 2002 to 2009. These rare viruses exhibited varying ecological and phylodynamic features. The Eurasian clades of H8 and H12 phylogenies were dominated by waterfowl sequences; mostly viruses sequenced in this study. H11, once believed to be a subtype that infected charadriiformes (shorebirds), exhibited patterns more typical of common virus subtypes. Finally, subtypes H9 and H10, which have maintained lineages in poultry, showed markedly different patterns: H10 was associated with all possible NA subtypes and this drove HA lineage diversity within years. Rare viruses belonging to subtypes H8–H12 were highly reassorted, indicating that these rare subtypes are part of the broader IAV pool. Our results suggest that waterfowl play a role in the maintenance of these rare subtypes, but we recommend additional sampling of non-traditional hosts to better understand the reservoirs of these rare viruses.

Key words: disease ecology; evolutionary genetics; influenza A; mallards; pathogen dynamics; subtype diversity

1. Introduction

Influenza A viruses (IAVs) are segmented, negative-sense RNA viruses that can infect a large range of avian and mammalian hosts (Webster et al. 1992; Olsen et al. 2006). Although IAVs exhibit a broad host range, wild birds, particularly those associated with wetlands, are the main reservoir for IAV in nature and harbour the largest number of virus subtypes and genetic lineages (Olsen et al. 2006). In these hosts 16 haemagglutinin (HA) and 9 neuraminidase (NA) subtypes have been recovered, resulting in 144 possible subtype combinations. Viruses are most frequently detected in Anseriformes (ducks, geese, and swans), in which H1–H12 are detected worldwide (Webster et al. 1992; Alexander 2000; Olsen et al. 2006; Munster et al. 2007; Olson et al. 2014). Within the Anseriformes, dabbling ducks, and particularly Mallards (*Anas platyrhynchos*), have accounted for the greatest number of isolations globally (Olsen et al. 2006).

Within waterfowl, different HA subtypes exhibit different patterns of detection across time and space. Some subtypes are common, and are detected at high frequencies every year, such as H3, H4, and H6. Furthermore, certain HA–NA subtype combinations are more commonly observed than expected in Northern Hemisphere dabbling ducks, such as H3N8, H4N6, and H6N2. Other HA subtypes are rare (e.g. H8–H12, H14, and H15) and many HA–NA combinations have never been detected (Sharp et al. 1993; Hatchette et al. 2004; Munster et al. 2007; Wilcox et al. 2011; Latorre-Margalef et al. 2014; Olson et al. 2014). Despite the maintenance of some HA–NA combinations, reassortment (the process whereby new virus variants can arise through exchange of the RNA segments in coinfected cells) in wild birds is frequent (Dugan et al. 2008; Wille et al. 2013; Lewis et al. 2015). As a result, even though the same HA–NA combination may be detected, viruses isolated on the same day may have markedly different genome constellations, or lineage combinations of all eight segments (Dugan et al. 2008; Wille et al. 2013). Despite the varying observations of subtype occurrence, the limited data available suggests that rare IAV subtypes do not have 'subtype specific' lineages, which are a feature of gull H13 and H16 viruses (Wille et al. 2011a). Rather, 'internal' segments (i.e. segments other than HA and NA) fall into clades dominated by wild anseriiform and charadriiform viruses (Wille et al. 2013; Lewis et al. 2015; Gonzalez-Reiche et al. 2017).

The reservoir populations in which rare viruses are maintained are currently unknown. For example, H14 and H15 are isolated only every few years across all sampled hosts, and to date only nine H15 viruses have been sequenced worldwide (Rohm et al. 1996; Sivay et al. 2013; Muzyka et al. 2016). However, there has been a recent introduction of H14 into North America with subsequent spread (Nolting et al. 2012; Ramey et al. 2014a; Gonzalez-Reiche et al. 2017). While subtypes H8–H12 are commonly designated as 'waterfowl-associated' subtypes, these viruses are only rarely detected in waterfowl-dominated surveillance schemes (Wilcox et al. 2011; Latorre-Margalef et al. 2014; Olson et al. 2014; Grillo et al. 2015). Some rare viruses have been predominantly described in hosts other than waterfowl. Low-pathogenicity (LPIAV) H9 viruses have lineages that are endemic in poultry; highly pathogenic H5 and both low and highly pathogenic H7 are of zoonotic concern and poultry is the known reservoir. However, in Europe, LPIAV H7 and H9 are not frequently isolated in poultry (Verhagen et al. 2017). H10 is more promiscuous in regard to host type, and has been isolated from mammals such as seals, as well in a wide range of bird species, with reports of spillover into humans (Kayali et al. 2010; Vachieri et al. 2014; Bodewes et al. 2015; Ma et al. 2015). Older IAV ecology studies described H11 as being associated with shorebirds in North America (Kawaoka et al. 1988), and this subtype has been isolated recently from a number of non-traditional hosts such as seabirds, seaducks, auks (Granter et al. 2010), and penguins (Hurt et al. 2014). However, H11 is not routinely isolated in European shorebirds; indeed, there is no evidence that shorebirds are important hosts for IAV in Europe (Gaidet et al. 2012). The ecology of subtypes H8 and H12 are largely unknown, with detections occasionally reported in wild birds (Wilcox et al. 2011; Latorre-Margalef et al. 2014; Grillo et al. 2015) and poultry (Verhagen et al. 2017).

In order to broaden our understanding of ecology and epidemiology of rare IAV subtypes, a structured approach is needed. Here we use data from a long-term IAV study site in Sweden, where Mallards have been screened for IAV since 2002. Unlike most surveillance schemes, samples are collected from birds captured at the same location daily throughout the year, allowing for increased sampling depth and inference of within- and among-year patterns. This study design also allows us to characterize viruses and their dynamics outside the period of prevalence peak, which would be missed in more directed sampling approaches. In this study we aim to elucidate the dynamics of uncommon H8–H12 viruses by assessing patterns of occurrence across and within years in Mallards, and contrast these patterns with available global information. Further, we assess the host specificity, virus evolutionary dynamics and virus genomic composition focusing on HA–NA linkage and reassortment. The data and results presented here increase our understanding of rare HA subtypes and provide a better foundation for generating hypotheses on IAV ecology, epidemiology and evolution.

2. Materials and methods

2.1 Ethics statement

All trapping and handling of ducks was done in accordance with regulations provided by the Swedish Board of Agriculture under permits from the Linköping Animal Research Ethics Board (permit numbers 8-06, 34-06, 80-07, 111-11, and 112-11).

2.2 Study site and virus collection

Wild Mallards were captured as part of a long-term IAV surveillance scheme from 2002 to 2009 (Latorre-Margalef et al. 2014). The trapping location, Ottenby Bird Observatory, Sweden (56°12′N, 16°24′E), is a stop-over site for Mallards migrating between breeding sites in the Baltic countries, Finland, and western Russia and overwintering sites in western Europe (Gunnarsson et al. 2012). Detailed capture, sampling, and screening methods used for the viruses sequenced in this study are discussed in Latorre-Margalef et al. (2014).

2.3 Sequence dataset

Full genomes were sequenced as part of the Influenza Genome Project (http://gcid.jcvi.org/projects/gsc/influenza/index.php), an initiative by the National Institute of Allergies and Infectious Diseases (NIAID). IAV vRNA was isolated from the samples/specimens, and the entire genome was amplified from 3 µl of RNA template using a multi-segment RT-PCR strategy (M-RTPCR) (Zhou et al. 2009; Zhou and Wentworth 2012). The amplicons were sequenced using the Ion Torrent PGM (Thermo Fisher Scientific, Waltham, MA, USA) and/or the Illumina MiSeq v2 (Illumina, Inc., San Diego, CA, USA) instruments. When sequencing data from both platforms was available, the data were merged and assembled together; the resulting consensus sequences were supported by reads from both technologies. All H8–H12 viruses isolated

between 2002 and 2009 were sent for sequencing, and seventeen H8, five H9, fifty-six H10, thirty-seven H11, and seven H12. Eleven additional viruses subtyped as H8-12 were identified as mixed viruses following sequencing (Supplementary Tables S1 and S2). The viruses that were not successfully sequenced did not pass QC at JCVI, and we did not attempt sequencing. Sequences generated in this study have been deposited in GenBank (Supplementary Table S1). Some additional viral sequences, specifically H11 viruses from Swedish Mallards generated in Wille et al. (2013), were also included. For each of these subtypes, all other available viral sequences were queried from the Influenza Research Database (http://www.fludb.org/; August 2016) to provide global context. Furthermore, from these sequence datasets, metadata (host species, year, and subtype) were collated.

2.4 Phylogenetic analysis

Sequences were aligned using MAFFT (Katoh, Asimenos, and Toh 2009) in Geneious R7 (Biomatters, New Zealand). Nucleotide substitution models for each data set were determined using MEGA 7 (Tamura et al. 2013). Pairwise genetic distance (raw p-distance) was calculated in MEGA 7, and converted to pairwise identity (1 – p-distance × 100) and plotted in R. For each subtype, HA trees representing the full sequence alignments of the global sequence datasets were estimated in MrBayes 3.2.6 (Ronquist et al. 2012). Time-structured (molecular clock) phylogenetic trees of specific clades of H8, H11, and H12 were estimated using BEAST 1.8 (Drummond et al. 2012). In brief, maximum likelihood (ML) trees were first estimated using Garli 0.96 in Geneious R7. These ML trees were used to explore the temporal signal and clock-like behaviour of each data set by performing linear regressions of root-to-tip distances against year of sampling, using TempEst (Rambaut et al. 2016). Using BEAST, time-stamped data were analysed under the uncorrelated lognormal relaxed molecular clock (Li and Drummond 2012), the SRD06 codon-structured nucleotide substitution model (Shapiro, Rambaut, and Drummond 2006). The Bayesian skyline coalescent tree prior was used. Three independent analyses of 100 million generations were performed, which were then combined in LogCombiner v1.8 following the removal of a burnin of 10 per cent. Marginal posterior estimates of substitution rates and dates of most recent common ancestors (MRCAs) were estimated using Tracer v1.6. Maximum credibility clade trees were generated using TreeAnnotator v1.8 and visualized in FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/). Phylogenetic analysis was performed on resources provided by SNIC through Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX) under project b2013122.

2.5 Reassortment visualization

To assess reassortment of a segment across all subtypes, we included publically available European and Asian M segments from wild birds. A Bayesian phylogeny was estimated using MrBayes 3.2.6, as described above, and visualized in iTOL (http://itol.embl.de/) with metadata host, subtype, location of isolation, and year of isolation.

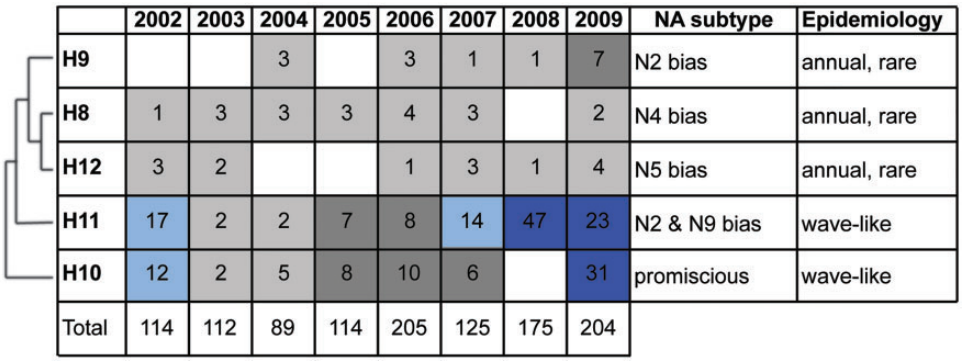
To assess reassortment within specific subtypes, H8 and H12, we here used a method illustrated in (Bell and Bedford 2017). Specifically, we estimated an ML phylogeny of each segment including only the sequences generated in this study; trees were inferred using Garli implemented in Geneious, as described earlier. The trees were visualized in FigTree and branches coloured by subtype. The phylogenetic position of each virus was linked across all phylogenies, and the link was coloured by year of isolation. Heuristically, straight lines indicate congruent topological positions between trees and criss-crossing lines indicate incongruent topological positions, i.e. putative reassortment.

3. Results

3.1 Rare virus detection in Swedish Mallards and wild birds globally

Viruses sequenced in this study were isolated from wild Mallards captured as part of a long-term IAV surveillance scheme from 2002 to 2009 (Latorre-Margalef et al. 2014) at Ottenby Bird Observatory, Sweden, in the Baltic Sea (56°12′N, 16°24′E). During the study period, IAV subtypes H8–H12 were rare among wild migratory Mallards; however, they were still isolated frequently enough to characterize differences in patterns of presence and prevalence (Fig. 1 and Supplementary Fig. S1). H10 and H11 viruses exhibited wave-like patterns of incidence, such that in some years they were isolated more frequently and comprised >10 per cent of the total number of viruses isolated in that year, while in other years they were infrequently isolated, or entirely absent. Data from the 8 years of this study suggest a putative wavelength of 5 and 6 years, which corresponds to the number of years between the peaks of higher frequency detections for H11 and H10, respectively. Subtypes H8, H9, and H12, in contrast, were isolated in more than 5 out of 8 years, but always infrequently. That is, there were fewer than five isolates of these subtypes in any given year, with the exception of seven detections of H9 in 2009, such that these subtypes represent a small proportion of viruses isolated each year (Fig. 1). Furthermore, H8–H12 HA subtypes had a strong NA bias, whereby frequently detected HA–NA subtype combinations include H8N4, H9N2, H11N2, H11N9, and H12N5. The exception to this was H10, which was detected in all NA combinations, the most common of which was H10N1 (Fig. 1 and Supplementary Table S3). When annual trends of subtype prevalence were considered, the proportion of H10 and H8 isolates was highest outside the main IAV prevalence peak. Specifically, a large proportion of H8 and H10 viruses were isolated in May and June, and in the case of H10, also in December. This was in contrast to H11, which appeared in higher proportions during the autumnal prevalence peak (Supplementary Fig S1).

As our study site represents only a single geographic location with a single host species, we compared our findings with features of rare viruses isolated globally. All virus sequence metadata (year of detection, location, host species, and HA–NA combination) for H8–H12 viruses were downloaded from the Influenza Research Database (http://www.fludb.org/; August 2016) and analysed, with the exception of H9, for which viruses from Asia were excluded due to the bias among those sequences towards poultry sequences (Supplementary Fig. S2). A spatial bias was evident in this global dataset, as most sequences came from viruses isolated in North America and the samples sequenced as part of this study represented a large proportion of sequenced viruses isolated from European wild birds. The most common hosts were dabbling ducks, followed by Charadriiformes (dominated by shorebirds) for some subtypes. Interestingly, while previously considered a shorebird subtype, H11 was detected no more frequently in Charadriiformes that the other subtypes included here (Supplementary Fig. S2). While H9 is routinely isolated from poultry in Asia, there were few sequenced viruses from poultry from other continents. In terms of HA–NA subtype bias, the viruses recovered from Ottenby followed global trends, with the exception of H10. All H10 NA subtypes were recovered with somewhat equal frequency at our



	2002	2003	2004	2005	2006	2007	2008	2009	NA subtype	Epidemiology
H9			3		3	1	1	7	N2 bias	annual, rare
H8	1	3	3	3	4	3		2	N4 bias	annual, rare
H12	3	2			1	3	1	4	N5 bias	annual, rare
H11	17	2	2	7	8	14	47	23	N2 & N9 bias	wave-like
H10	12	2	5	8	10	6		31	promiscious	wave-like
Total	114	112	89	114	205	125	175	204		

Figure 1. Number of H8–H12 viruses isolated from Ottenby 2002–2009, modified from Latorre-Margalef et al. (2014). Boxes have been shaded as a heatmap, ranging from 1 isolation (light grey) to >20 isolations (dark blue). 'Total' indicates the total number of viruses isolated in that year. Cladogram illustrates phylogentic relationship between subtypes as reported in Fouchier et al. (2005). H8, H9, and H12 are Group 2 H9 Clade viruses, sister to the Group 2 H11 Clade including H11. Finally, H10 is in Group 1 H7 Clade (as defined in Latorre-Margalef et al. 2013). Boxes on the right provide initial observations: NA subtype describes whether the HA subtype is more frequently found in association with an NA subtype (here 'bias') and the percentage of the specific NA subtype in viruses sequenced in this study (Supplementary Table S3). 'Epidemiology' describes observations of occurrence in our dataset, where viruses are either detected fewer than ten occasions, but found in greater than 5 years of the dataset, or are more 'wave-like' where in some years they are detected fewer than ten times, and in others greater than twenty.

study site but there appears to be a bias towards N8 in the global dataset (Fig. 1 and Supplementary Table S3 and Fig. S2).

3.2 The H8 and H12 enigma

One hypothesis to explain the rarity of H8 and H12 viruses globally is that these viruses may be restricted to an understudied host. Specifically, low frequency but annual detections in Mallards may be the product of spillover from other avian groups. Global surveillance and sequencing efforts are heavily biased to waterfowl, and thus detection of H8 and H12 are most frequent in this host group (Supplementary Fig. S2). A sequence-based phylogenetic approach allows us to elucidate the contribution of different hosts as reservoirs through patterns of transmission and lineage diversity. In both North America and Eurasia, all H8 viruses were isolated from waterfowl, and in Eurasia this study has generated more H8 sequences than any other location. As a result, the Eurasian clade of the H8 phylogenetic tree is significantly biased towards virus sequences isolated from Mallards at Ottenby (Fig. 2). Globally, there were more H12 sequences than H8, with H12 detections in shorebirds in both North America and Australia. While no H8 shorebird viruses are currently available in sequence databases, there are a number shorebird H12 virus sequences, largely due to the long-term IAV surveillance scheme in Delaware Bay, USA. However, as with H8, most H12 sequences in Eurasia are from waterfowl hosts, and as with H8, H12 sequences generated in this study contributed significantly to the number of viruses available (Fig. 2).

The estimated dates of tMRCA of the Eurasian clades of H8 (1997.8 [95% highest posterior density (HPD) 1987–2011]) and H12 (1980 [95% HPD 1962–1991]) were different (Fig. 2). Despite the more recent tMRCA in H8, across Eurasia genetic diversity for H8 and H12 were similar (mean nucleotide pairwise genetic identity H8 = 95.8 per cent, H12 = 94.4 per cent), and similarly all Swedish H8 and H12 sequences had high pairwise genetic identity (mean nucleotide pairwise genetic identity H8 = 96.4 per cent, H12 = 97.2 per cent). This suggests limited intra-subtypic genetic diversity and likely represents genetic drift that may have occurred between 2002 and 2009 in the Mallard reservoir (Fig. 3). In North America, H12 shorebird viruses clustered into shorebird specific lineages rather than being dispersed among the waterfowl viruses, suggesting multiple, independent introduction events and subsequent circulation in shorebirds. Therefore, given only waterfowl isolates, our estimates of H12 diversity in Eurasia are likely to be poor. This is supported by the H12 viruses from red necked stints (*Calidris ruficolis*) from Australia, which exist as an outgroup to the rest of the Eurasian clade, from which they diverged in 1959 [95% HPD 1934–1981]. Given the lack of support for shorebird-specific clades in the American lineages, this lineage may represent an 'Australian' lineage; however, we are unable to confirm the exact cause of this genetic differentiation due to the absence of additional Australian H12 sequences (Fig. 2).

3.3 H11—a charadriiform or anseriiform specific subtype?

Of the rare viruses in our dataset, H11 was the most common, although with a fluctuating frequency across years. Furthermore, H11 was predominately isolated during the autumnal prevalence peak (Fig. 1 and Supplementary Fig. S1). An early hypothesis proposed was that H11 was a 'shorebird'-specific lineage (Kawaoka et al. 1988). Indeed, H11 is in the same HA Clade (Latorre-Margalef et al. 2013) (Group 1, H11 Clade) as the gull-specific H13 and H16 subtypes and H11 has been isolated from shorebird hosts, particularly in North America, but also from waterfowl hosts globally (Supplementary Fig. S2). As with H12 viruses, shorebird H11 viruses fell into clusters that suggest multiple introductions into shorebirds and subsequent circulation within those populations in North America (Fig. 4). Given the limited detection of H11 in shorebirds in Eurasia, the H11 phylogeny is dominated by waterfowl sequences, and furthermore, by viruses sequenced in this study. Despite different numbers of viruses isolated and sequenced, in this study and globally, genetic diversity of H11 viruses (mean nucleotide pairwise genetic identity 97 per cent) was not significantly lower than H12 viruses in the Ottenby Mallards (Fig. 3), nor did the Eurasian lineage of H11 have an older common ancestor (tMRCA 1980 [95% HDP 1967–1990]) than the Eurasian clade of H12, suggesting similar phylodynamic patterns despite a difference in incidence of these two subtypes at local and global scales. In the H11 phylogeny, the Ottenby HA sequences existed as multiple clades, and these different clades circulated in the Mallard population in different years (Fig. 4). An exception to

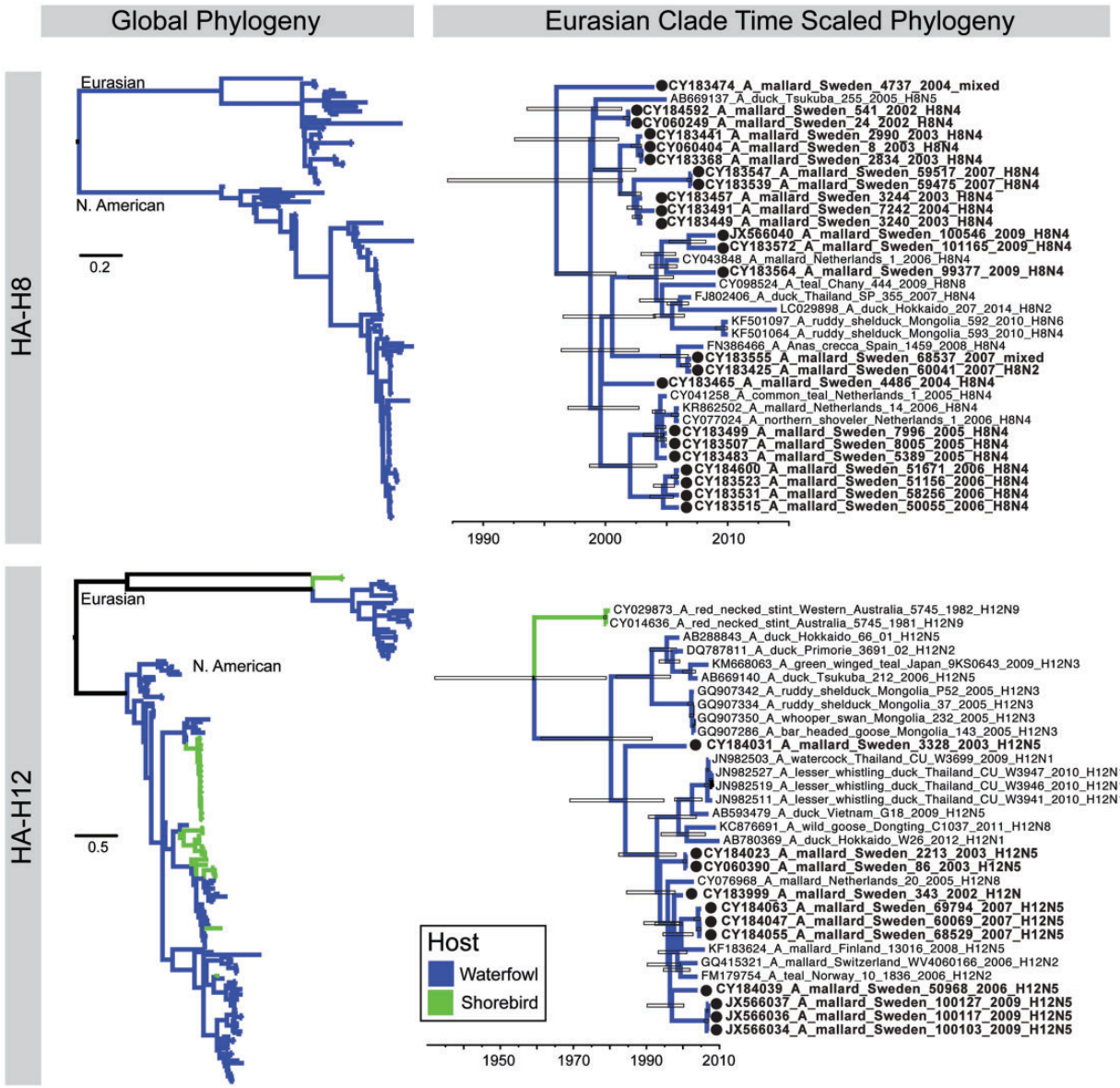


Figure 2. Global phylogenetic trees of H8 and H12 viruses. MrBayes trees include all sequences available in public databases, and BEAST trees represent a time structured phylogeny of the Eurasian clades. Sequences generated in this study are indicated with a black circle in the BEAST trees. Branches in blue represent waterfowl sequences, and those in green shorebird sequences. BEAST trees are maximum clade credibility trees. Node bars correspond to the 95% HPD interval of the node height. Scale bar of the BEAST trees represents time in years, scale bar of Bayesian trees indicate number of substitutions per site.

this was in 2009 when H11 viruses with two different NA subtypes circulated; specifically, H11N2 appeared early in the season largely in sentinel Mallards (Wille et al. 2013) and H11N9 appeared later in both wild and sentinel Mallards. Indeed, these H11N2 viruses in 2009 were the only N2 subtype viruses that appeared in our dataset. Furthermore, viruses sequenced in this study fell into three clades, demonstrating a turnover of clades across a decadal scale, in which circulating clades were introduced and became extirpated over time (Fig. 4). For example, viruses isolated in 2002 appeared in a clade wherein only two other viruses from Sweden were detected across the breadth of the study (one in 2005 and 2007). H11N2 viruses that circulated during 2007–2009 corresponded to a large clade that was a sister group to a clade dominated by viruses isolated from Asia, which were introduced into the Swedish Mallard population in 2009 (H11N9). These observations, all of which were detected through sequencing of Eurasian waterfowl viruses, suggest that anseriiformes are an important reservoir for the H11 subtype.

3.4 A waterfowl reservoir for 'poultry-associated' H9 and H10 in Europe?

While H9 and H10 viruses are common globally, these viruses are rare in European waterfowl and in Swedish Mallards (Fig. 1 and Supplementary Fig. S2). There are many sequences from Asia of both H9 and H10 with gene flow of avian influenza viruses between Europe and Asia, such that there is no clear lineage definition between the two regions (Supplementary Figs S3 and S4).

Globally, H10 viruses exhibited host promiscuity. Most sequences were from waterfowl; however, in North America there were numerous lineages dominated by viruses isolated

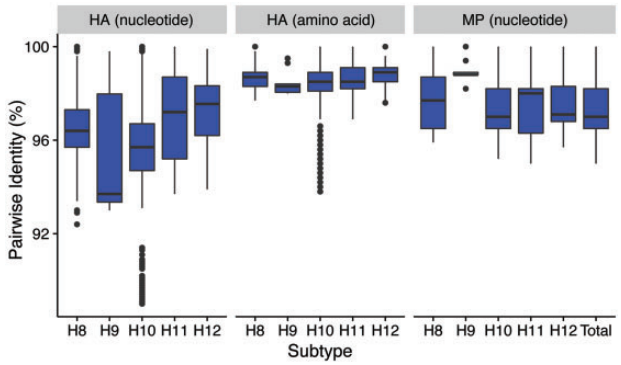


Figure 3. Pairwise genetic identity for HA and M segments of viruses sequenced in this study. (A) HA segment nucleotide per cent identities for each HA subtype, (B) HA segment amino acid per cent identities for each HA subtype, and (C) M nucleotide per cent identities for each HA subtype in addition to combined pairwise identities for H8–H12 M segments to illustrate breadth of the dataset. Pairwise identities for segments are presented in Supplementary Fig. S5. Boxplots illustrate the distribution of pairwise genetic distances. The line within the box is the median value and the box encompasses the inter quartile range. Whiskers encompass data outside the middle 50 per cent. Outliers are presented as filled circles; outliers which occur below the whiskers represent distantly related sequences or clades.

from shorebirds at Delaware Bay, and in Eurasia there were lineages dominated by poultry viruses (Supplementary Fig. S3). In Ottenby H10, viruses were isolated annually except in 2008, and at low frequency except for the first and last year of the study (Fig. 1). H10 viruses had a low mean pairwise genetic identity (Eurasian clade 93.9 per cent and Swedish viruses 95.6 per cent), and the variance in the distribution of pairwise genetic identity was also high as compared to other subtypes, including a number of outliers, which is indicative of more genetic diversity across different clades (Fig. 3 and Supplementary Fig. S5). H10 viruses sequenced in this study were found in a number of different lineages, but lineage distribution was not the same across years. Rather, there was a correlation between the number of different NA subtypes and the number of different HA lineages for each year in the H10 phylogeny (Supplementary Fig. S3 and Table S3). For example, the two years where the largest number of H10 viruses was isolated, 2002 and 2009, exhibited different patterns. In 2002 there were eight different H10–NA combinations detected (Supplementary Table S3 and Fig. S6). Correspondingly, viruses from 2002 fell into a number of different clades, some comprising only viruses from 2002, while others fell into clades that also contained Swedish viruses from 2005 and 2006 (Supplementary Fig. S3). In contrast, all viruses from 2009 belonged to the same clade (with 100 per cent posterior probability) (Supplementary Fig. S3), and only two different NA subtypes were identified, dominated by H10N1 and furthermore, within each NA subtype, these viruses had identical genome constellations (Supplementary Fig. S6). These viruses were genetically very similar, suggesting an outbreak of H10 in the population. This clade was genetically distinct from other H10 sequences and lineages from all other years (the outliers in Fig. 3) and was closely related to a lineage isolated in Asian poultry. It is noteworthy that no H10 viruses were detected in 2008; perhaps the 2009 viruses represent an introduction of a novel H10 into our study population from Asia. Given their genetic divergence, these newly introduced H10 strains may putatively have escaped H10 population immunity. Regardless, European and Asian H10 viruses circulate in the Swedish Mallard population (Supplementary Fig. S3).

The global phylogeny for H9 viruses was dominated by isolates from poultry, with a single clade, H9.3, comprising viruses largely from wild birds (Supplementary Fig. S4) (Jiang et al. 2012). More specifically, within the H9.3 clade, viruses were from both North America and Eurasia, but the usually clear distinction between North American and Eurasian viruses was not present. Furthermore, waterfowl viruses from this clade were observed to transfer and subsequently proliferate in shorebirds, as illustrated by the existence of shorebird clades from North America. In clades dominated by Eurasian viruses, there was also transmission to shorebirds, but these appeared to be repeated spillover events as these clades each contained less than five viruses. This may be explained by a lower prevalence of IAV in Eurasian shorebirds, as well as by a smaller sample size for Eurasian shorebirds. Viruses isolated from Ottenby Mallards fall into a single clade h9.3.3.2, but are genetically diverse (mean nucleotide pairwise genetic identity 95.2 per cent) (Fig. 3 and Supplementary Fig. S5) despite the low number of viruses successfully sequenced. As expected, poultry sequences from Europe and North America were in clades shared with wild birds, and these infections in poultry were the result of a spillover from wild birds (Supplementary Fig. S4), unlike the other H9 clades (Jiang et al. 2012).

3.5 Relationships within and between viruses through reassortment

Given the rare nature of viruses, particularly Group 1, H9 Clade subtypes H8, H9 and H12, we could hypothesize that segments of these viruses might have unique lineages, such as gull specific viruses H13/H16, and further that these viruses will have unique virus genome constellations in waterfowl such that specific combinations of phylogenetic lineages are observed for all gene segments. Further, these constellations might not undergo reassortment with other anseriiform derived viruses and thus remain 'linked' within a year. Alternatively, if these viruses were part of the larger anseriiform gene pool then we would see reassortment with other duck viruses isolated at our study site.

Considering H8 and H12, the most uncommon viruses in our dataset, there were high levels of reassortment (Fig. 5). By tracing phylogenetic positions of the virus isolates among the gene segment phylogenies, we illustrated reassortment by assessing whether the lines that link the same isolate across the phylogenetic trees (especially those from the same year and subtype) cross each other or whether they remain parallel to each other (Fig. 5). Genome constellations were different across years and, in the case of H8, and also within years (Fig. 5 and Supplementary Figs S6 and S7). H12 viruses isolated in 2007 (maroon filled circles, dark green connecting lines, Fig. 5) had the same genome constellations, as illustrated by similar phylogenetic placement across all segments, and clustered together (Fig. 5 and Supplementary Figs S6 and S7). This contrasts with H12 viruses isolated in 2003 (maroon filled circles, red connecting lines, Fig. 5); the two isolated viruses were unlinked and had different phylogenetic positions in the PB1, HA, NA, and NS segments, and in some segments exhibited <95 per cent nucleotide pairwise genetic identity (Fig. 5 and Supplementary Figs S6 and S7). Similarly, there was greater signal for reassortment in some years compared to others in H8 viruses (Fig. 5).

In all HA subtypes, there was diversity in genome constellations despite isolating a single dominant NA subtype (Supplementary Figs S6, S7, and Table S4). In H10, the number of constellations recovered was linked to the diversity of NA subtypes. For example, in 2002, from ten viruses, six different NA

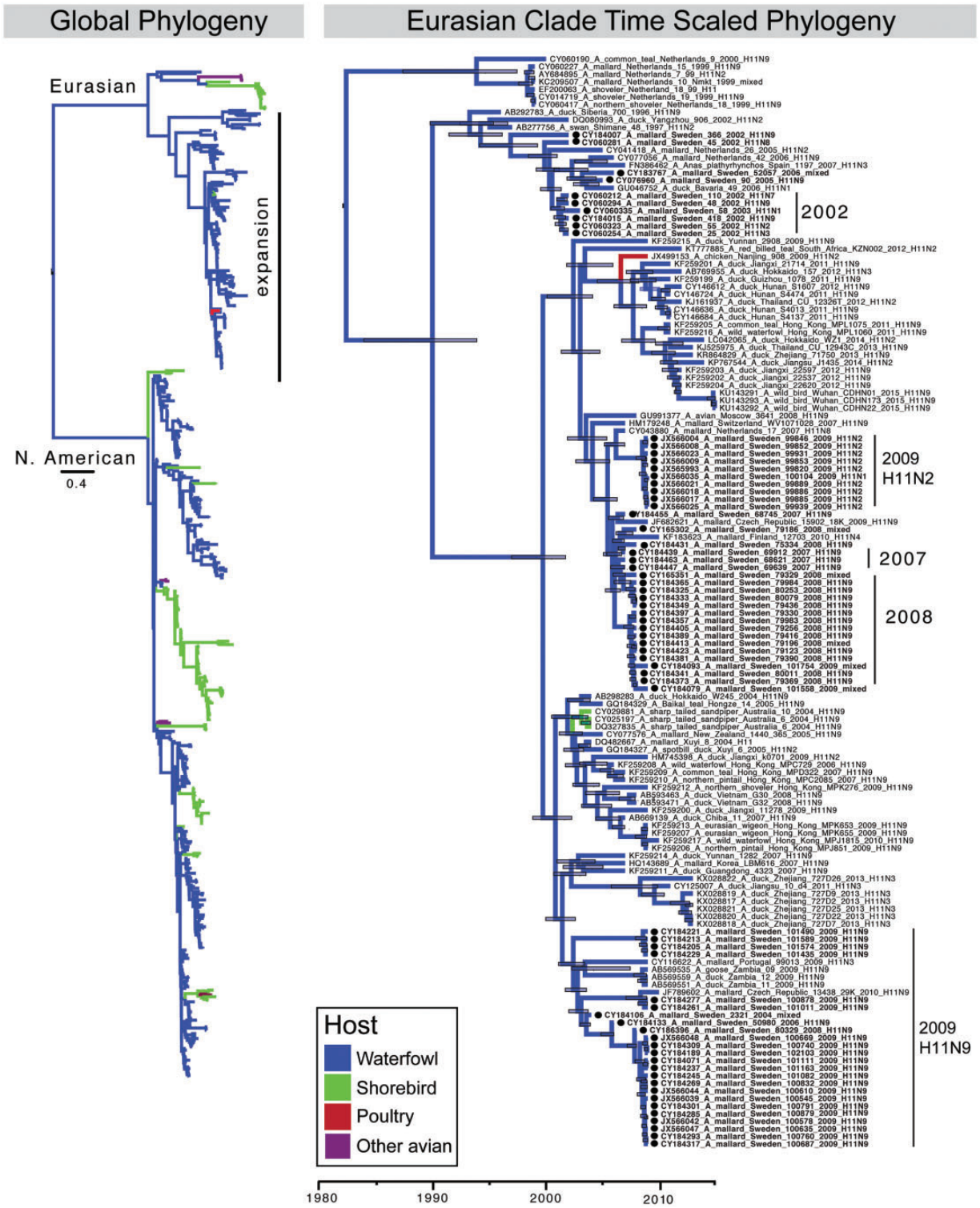


Figure 4. Evolutionary genetics of H11 HA segment. The BEAST tree represents a time structured phylogeny of the Eurasian Clade of H11, as indicated on the MrBayes tree. Sequences generated in this study are denoted by black circles in the BEAST tree. Branches in blue represent waterfowl sequences, those in green from shorebird sequences, and those in purple other avian hosts. Trees are maximum clade credibility trees. Node bars correspond to the 95% HPD interval on the root height. Scale bar of the BEAST trees represents time in years, scale bar of Bayesian trees indicate number of substitutions per site.

subtypes and eight unique genome constellations were detected. In 2009, of the twenty-one viruses sequenced, all but one was N1 and 90 per cent of these viruses had the same genome constellation (Supplementary Fig. S6 and Table S4).

To assess reassortment and phylogenetic relationships between rare viruses, the Eurasian avian lineage of the matrix (MP) segment interrogated (Supplementary Fig. S7), specifically because this segment is very frequently sequenced in avian IAV

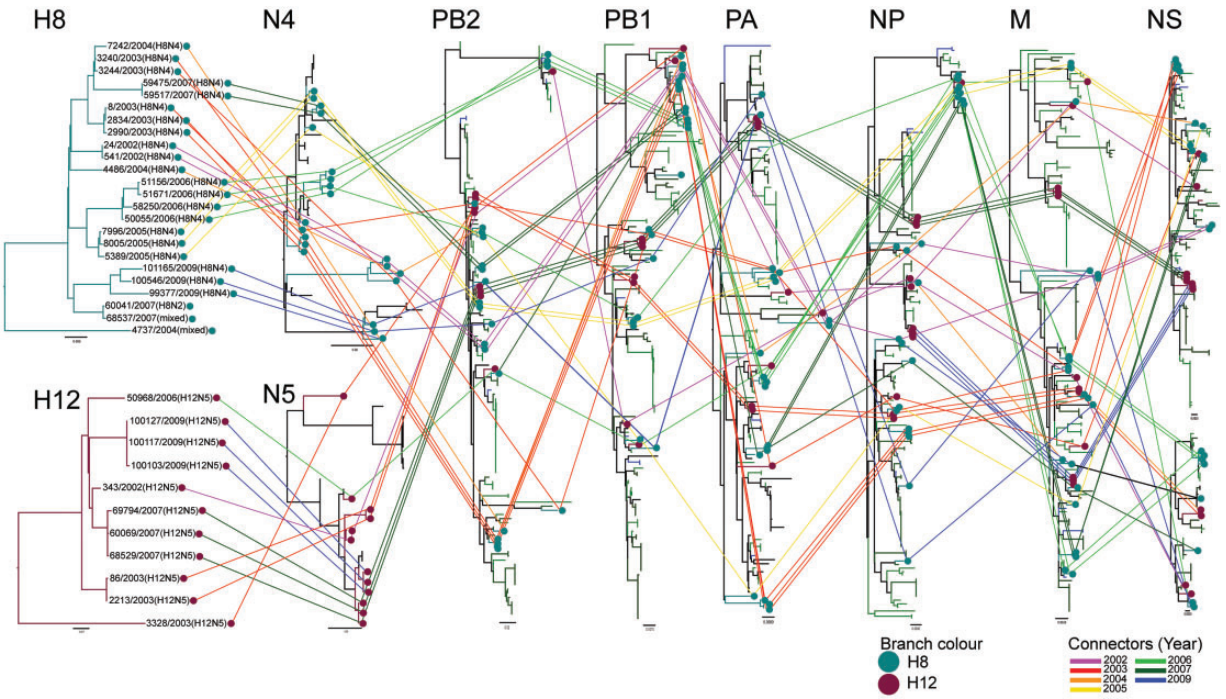


Figure 5. Reassortment of H8 and H12. ML trees containing all sequences generated in this study, and scale bar for each tree indicates the number of substitutions per site. Tree branches are coloured by subtype, where H8 is teal, H9 is blueberry, H10 is light green, H11 is dark green, and H12 is maroon. H8 and H12 sequences are further denoted with a filled circle in the corresponding colour. The phylogenetic position of each virus is traced across the trees with connectors coloured by the year of isolation. Segments HA and NA are positioned on the left for aesthetic purposes, followed by the 'internal' segments in order of size.

Table 1. Characteristics of mixed viruses detected in this study.

Virus name	Year	HA subtype	NA subtype	Other mixed segments
A/mallard/Sweden/4737/2004(mixed)	2004	H8/H2	N1/N3	
A/mallard/Sweden/68537/2007(mixed)	2007	H8	N2/N6	
A/mallard/Sweden/766/2002(mixed)	2002	H10	N6	NS
A/mallard/Sweden/6869/2004(mixed)	2004	H10	N9	PB1
A/mallard/Sweden/5933/2005(mixed)	2005	H10/H3	N7	
A/mallard/Sweden/52057/2006(mixed)	2006	H10/H11	N9	PB2
A/mallard/Sweden/60065/2007(mixed)	2007	H10	N2/N7	
A/mallard/Sweden/2321/2004(mixed)	2004	H11	N8/N9	NP, M
A/mallard/Sweden/101558/2009(mixed)	2009	H11	N8/N9	PB2, M
A/mallard/Sweden/101754/2009(mixed)	2009	H11	N8/N9	PB2, M
A/mallard/Sweden/79196/2008(mixed)	2008	H11	N2/N9	PB2

studies. Pairwise genetic identity of the MP segment of the different subtypes in this study was similar and was representative of the genetic diversity of the MP segment in general (Fig. 3). Rare Swedish virus MP segments were spread across the breadth of the tree but were typically most closely related to MP segments sequenced from other Swedish IAVs (Supplementary Fig. S7). In Sweden, MP segment sequences were almost exclusively from Mallard viruses. Clades containing multiple subtypes tended to be clustered by year, and little HA–M linkage was observed. The main exception was the clade of H10 viruses isolated in 2009—both the HA and M segments formed a single clade, suggesting a rapid spread of this successful constellation through the population (Supplementary Fig. S7).

Finally, the sequencing approach we used allowed for the detection of mixed viruses, or isolates that contained more than one version of any segment (Table 1). Multiple segments were detected in H8, H10, and H11 viruses, for which 11, 8, and 9.5 per cent of sequenced isolates were mixed, respectively. It is interesting to note that the lack of H9 and H12 mixed isolates; however, this could be due to small sample size. These mixed isolates are challenging to interpret as viruses were sequenced after isolation in eggs, and as such we cannot determine whether the mixed viruses are representative of what was shed by infected ducks. That is, disentangling reassortment from coinfection (Table 1). Duplicated segments included HA, NA, PB2, PB1, M, and NS, and on some occasions two or three different segment sequences were observed in the isolate, suggesting coinfection by very different viruses. H10N6 and H10N9 had duplicated NS and PB1 segments only, respectively, suggesting infection with two different H10 infections (Table 1). In 2009 two mixed H11 isolates shared the same features of coinfection: H11N8/9 with two PB2 and M segments. Despite this shared feature, samples 101558 and 101754 were not isolated from the same duck, but were collected within 2 days from two different

ducks (11 November 2009 and 13 November 2009, respectively) (Table 1). During that time period (10–15 November 2009) numerous different subtypes were recovered from the population, including both H11N2 and H11N9 (H1, 2xH3, 20xH4, 4xH5, H6, 3xH11N?, 3xH11N9, 2xH11N2). Given there is putative H11–N9 linkage, these ducks were likely coinfected with H?N8 viruses; however, surprisingly, no N8 was detected in the population and therefore it is challenging to disentangle the parental viruses of these mixed H11 infections.

4. Discussion

Rare subtypes present an enigma in IAV ecology and the pattern of detection of these rare viruses in our dataset, a long-term study of IAV in a single host species at a specific location, corroborates globally observed trends. Rare subtypes are detected at very low frequencies. Despite infrequent detection, these viruses are part of the IAV gene pool that circulates among waterfowl. Given the size of the Mallard population alone (19 million individuals in Europe (Wetlands International 2015)), these viruses potentially circulate at levels high enough to enable sustained transmission. The central question, however, is whether Mallards are the primary reservoir for these viruses, or if evolution and transmission of these subtypes is driven by infections in other hosts that are not the main target of our (or other) longitudinal surveillance studies. Given the rarity of these viruses and the small number of IAV longitudinal study sites worldwide, we are unable to provide a conclusive answer to this question. Moreover, the dynamics of rare viruses vary among subtypes, suggesting different drivers of maintenance in the avian reservoir.

Subtypes H8 and H10 appeared at higher proportions outside the main period of IAV incidence in Mallards at Ottenby, with more infections in June or December. We hypothesize this may be driven by a combination of competitive exclusion and herd immunity in the population. Common viral subtypes are likely to have higher fitness in the Mallard population (Lebarbenchon et al. 2012), including replicative fitness (replication of viral variants within individual hosts), transmission fitness (transmission between hosts) and epidemiologic fitness (changes in distribution, prevalence, and composition of viral genotypes over time) (Wargo and Kurath 2012). This allows for common subtypes to outcompete less fit subtypes or strains competing for the same hosts, target cells, and receptors (Bahl et al. 2009). Following infection, birds develop subtype-specific immunity against HA subtype (homo- and heterosubtypic immunity) and, given the high incidence of common viruses, these populations have high levels of herd immunity against common viruses (Latorre-Margalef et al. 2013; Tolf et al. 2013; Latorre-Margalef et al. 2017). This creates a putative window for more distantly related rare viruses to infect a population with high herd immunity against common viruses. Indeed, H8, H9, and H12 are Group 1 H9 Clade viruses, and are only distantly related to common H1 and H6 viruses (Group 1, H1 Clade), suggesting limited cross immunity to these subtypes. This overall pattern has support in a study using sentinel ducks (Tolf et al. 2013; Wille et al. 2013) in which immunologically naïve individuals were placed in a trap. The sentinel ducks became infected by wild conspecifics visiting the trap, and through daily sampling detailed infection histories were created. Sentinel ducks were frequently infected with common subtypes; however, rare H12 viruses appeared as short infections between the primary (H6N2, H1N1) and secondary infections (H4N6) (Tolf et al. 2013; Wille et al. 2013), at a time when the ducks likely had high titres of subtype-specific antibodies. These subtype-specific antibodies result in a significant reduction in shedding of secondary infections with the same or closely related subtypes (Latorre-Margalef et al. 2017). Furthermore, short tertiary infections were detected when viral prevalence was low in the population in December, including an H10 infection. Interestingly, when these birds were re-exposed the following year they were infected only with H7 and H3 and not re-infected with 'common' subtypes H1, H4, or H6 (Tolf et al. 2013). Not all rare subtypes exhibited this trend, however. For example, H11 was predominantly isolated during the autumnal peak in prevalence when viral burden is the highest in the population. Indeed, H11 appeared with common viruses during primary and secondary infections in the sentinel duck study (Tolf et al. 2013; Wille et al. 2013).

A second hypothesis is that rare viruses are maintained in avian hosts other than dabbling ducks. For example, H12 has been reported to replicate poorly in Mallards, even when experimentally infecting individuals with strains isolated from wild Mallards, which may suggest poor adaptation to these hosts (Latorre-Margalef et al. 2017). H11 was initially suggested to be a charadriiform virus (Kawaoka et al. 1988), like the closely related H13 and H16 subtypes, which are found only in gulls (Wille et al. 2011a; Huang et al. 2014). H9 and H10 are often reported in poultry (Dong et al. 2011), and H10 is also found in mammals (Vachieri et al. 2014; Bodewes et al. 2015), illustrating host flexibility. What ties H9, H10, and H11 together is potential generalism, in that these viruses proliferate in more than one avian host type. In this case, generalism allows for a trade-off—a decrease in host-specific fitness, meaning that these viruses are detected less frequently in Mallards due to competition, but allows for infection in a larger and more diverse host reservoir and therefore we find detections in an array of avian hosts. This ability to emerge in new host types has likely driven the success of H9 and H10 subtypes in poultry (Jiang et al. 2012; Ma et al. 2015), without the evolution of the high pathogenicity phenotype. Given the high levels of reassortment with common viruses also found at our study site, it is unlikely that rare viruses represent an independent gene pool driven by adaptation to hosts other than dabbling ducks, with occasional incursions into the duck reservoir. Rather, phylogenetic analysis indicates circulation in dabbling ducks and incursions and subsequent circulation in shorebirds. However, this does not exclude other host types as maintenance hosts, particularly closely related species such as geese and swans (Lee et al. 2014; Hill et al. 2016; Reeves and Ip 2016; Wong et al. 2016; Verhagen et al. 2017; Yao et al. 2017).

Mallards and dabbling ducks are central to IAV studies globally due to high virus prevalence in these species: the greatest number of isolated viruses are from this small taxonomic group of birds (Olsen et al. 2006; Olson et al. 2014). While it is important to focus on the putative reservoir of IAV, rare waterfowl viruses such as those highlighted here illustrate that dabbling ducks are unlikely to be the predominant reservoir for all IAV subtypes. Thus, surveys and subsequent longitudinal sampling studies are needed in other hosts such as diving ducks (Nolting et al. 2012; Hall et al. 2015; Xu et al. 2017), infrequently sampled dabbling ducks (Ramey et al. 2014b), geese (Harris et al. 2010; Kleijn et al. 2010; Lee et al. 2014; Reeves and Ip 2016; Yao et al. 2017), swans (van Gils et al. 2007; Hoye, Fouchier, and Klaassen 2012; Hill et al. 2016; Hoye et al. 2016), shorebirds (Makarova et al. 1999; Pearce et al. 2010; Gaidet et al. 2012; Maxted et al. 2012), gulls (Lebarbenchon et al. 2009; Velarde et al. 2010; Wille et al. 2011b; 2011a; Lewis et al. 2013; Verhagen et al. 2014), seabirds (Lang et al. 2016), and other waterbirds, such as herons.

For example, in shorebirds effort is sparse outside Delaware Bay. Indeed, viruses have been detected, but not sequenced, from shorebirds in Africa (Gaidet et al. 2012), and a small number of viruses have been sequenced from Australian shorebirds, including the H12 stint viruses highlighted here. Seabirds have also been described as hosts for IAV, and to date there is a bias towards Group 1 viruses (Latorre-Margalef et al. 2013; Lang et al. 2016), including the H8, H9, H11, and H12 viruses as well as H13 and H16 gull specific viruses. If this bias is true, and not the result of sampling and sequencing bias in seabirds, this may suggest that these seabird hosts are important in the epidemiology of Group 1 viruses. Finally, not all ducks are equal in virus detection; few viruses are isolated from diving ducks and seaducks, despite these birds being competent hosts for IAV (Ramey et al. 2011; Hall et al. 2015). Indeed, seaducks have recently been highlighted in the development of a highly pathogenic phenotype of H7 virus (Xu et al. 2017). Of additional importance is the role of backyard poultry not regularly screened for low pathogenic viruses; for example, Verhagen et al (2017) illustrated that H8 circulates at a high relative proportion in Dutch farms. Serology datasets are imperative to identifying important hosts (e.g. Hill et al. 2016; Wong et al. 2016) as is the characterization of detected viruses. Finally, targeted studies of infection patterns in realistic experimental infection studies, including more natural routes of infection and models of transmission are needed to assess pathogenicity and specificity of infection. This could be supplemented further by characterization of host receptors and viral binding affinities to those receptors. These steps are needed not only to disentangle the differences in occurrence between rare and common waterfowl viruses, but also to understand causes of the variation in relative prevalence of the rare viruses highlighted in this study.

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Data availability

Sequences generated in this study have been deposited in GenBank. Accession numbers are provided in Supplementary Table S1.

Supplementary data

Supplementary data are available at *Virus Evolution* online.

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