**Working title:**

# Detection of H3N8 influenza A virus with multiple mammalian-adaptive mutations in a rescued Grey seal (*Halichoerus grypus*) pup

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## ABSTRACT

Avian Influenza A Viruses (IAV) in different species of seals display a spectrum of pathogenicity, from subclinical infection to mass mortality events. Here we present an investigation of avian IAV infection in a 3-4 month old Grey seal (*Halichoerus grypus*) pup, rescued from St Michael's Mount, Cornwall in 2017. The pup underwent medical treatment, but died after two weeks; post-mortem examination and histology indicated sepsis as the cause of death. IAV NP antigen was detected by immunohistochemistry in the nasal mucosa, and sensitive real-time reverse transcription polymerase chain reaction assays detected trace amounts of viral RNA within the lower respiratory tract, suggesting that the infection may have been cleared naturally. IAV prevalence among Grey seals may therefore be underestimated. Moreover, contact with humans during the rescue raised concerns about potential zoonotic risk. Nucleotide sequencing revealed the virus to be of subtype H3N8. Combining a GISAID database BLAST search and time-scaled phylogenetic analyses, we inferred that the seal virus originated from an unsampled, locally circulating (in Northern Europe) viruses, likely from wild Anseriformes. From examining the protein alignments, we found several residue changes in the seal virus that did not occur in the bird viruses, including D701N in the PB2 segment, a rare mutation, and a hallmark of mammalian adaptation of bird viruses. IAVs of H3N8 subtype have been noted for their particular ability to cross the species barrier and cause productive infections, including historical records suggesting that they may have caused the 1889 pandemic. Therefore, infections such as the one we report here may be of interest to pandemic surveillance and risk, and may help us better understand the determinants and drivers of mammalian adaptation in influenza.

(Word count: 277)

52 INTRODUCTION

53 Influenza A viruses (IAVs) are important pathogens for humans and livestock including pigs and
54 poultry. They are segmented RNA viruses, whose genomes consist of eight segments of RNA, which
55 code for ~11 proteins/polypeptides. IAVs are classified into several subtypes based on the antigenic
56 properties of two surface glycoproteins haemagglutinin (HA, avian subtypes H1-H16) and
57 neuraminidase (NA, avian subtypes N1-N9). Viruses of most subtypes can be found in wild waterfowl
58 and shorebirds which are their natural reservoir (Alexander, 2007; Easterday et al., 1968) and can
59 infect both domestic birds and mammalian species in spill-over infections. A few IAV lineages have
60 become established in mammals such as humans, pigs, horses, and dogs though only of subtypes
61 H1, H2 and H3 in combination with N1, N2 or N8 (Reperant et al., 2009; Webster et al., 1992).
62
63 IAV in birds replicates mainly in the intestine and is transmitted through the faecal-oral route,
64 although respiratory tropism and oropharyngeal shedding has been noted (Webster et al., 1978;
65 Daoust et al., 2011; Höfle et al., 2012; França et al., 2012; Daoust et al., 2013), and the virus can
66 survive in the environment for fairly long periods (Brown et al., 2009; Stallknecht et al., 1990). This
67 creates conditions conducive to viral exchange with marine mammals such as pinnipeds (seals),
68 whose habitats and prey intersect with those of waterfowl and shorebirds. Several cases of marine
69 mammal infection with IAV of many subtypes including H1N1, H3N3, H3N8, H4N5, H4N6, H7N7 and
70 H10N7 have been documented with a spectrum of effects ranging from mass die-offs to
71 asymptomatic; in a majority of these cases, the source is implicated to be avian (Fereidouni et al.,
72 2016; White, 2013). A few studies have also suggested that Grey seals may act as an endemically
73 infected reservoir which may disseminate viruses in coastal ecosystems to other mammals, coastal
74 birds, and potentially humans (Duignan et al., 1995, 1997; Puryear et al., 2016). Indeed,
75 seroprevalence levels in live-captured healthy Grey seal populations (20 – 26%) (Bodewes et al.,
76 2015; Puryear et al., 2016) is comparable with levels found in wild birds (31% - 60%) depending on
77 species, geography, seasonality and other factors (Curran et al., 2015; Fereidouni et al., 2010; Wilson
78 et al., 2013).
79

80 In seals, IAV binds the same type of sialyloligosaccharide receptors as birds (SAα2,3Gal), but the
81 receptors are located in the respiratory tract and lungs instead of the intestinal tract, suggesting that
82 inhalation is the most likely route of transmission (Ito et al., 1999; Ramis et al., 2012; White, 2013).
83 Seals may also be infected by IAV from non-avian sources; there is serological evidence for infection
84 of Baikal and ringed seals in Russia with human H3N2 strains A/Aichi/2/68 and A/Bangkok/1/79
85 (Ohishi et al., 2004) and a human pandemic-2009 H1N1 virus was isolated from Elephant seals in
86 2010 on the coast of California, USA. Furthermore, it has been shown that IAV from seals can
87 replicate in human tissue and that seal IAV can be systemically virulent in primates (Murphy et al.,
88 1983; Webster et al., 1981; White, 2013). Seals may therefore be potential sources of pandemic
89 influenza.
90
91 In this paper, we report an H3N8 IAV infection of a rescued Grey seal pup in coastal England. We
92 provide molecular and histological evidence for presence of IAV in the seal's respiratory tissues, and
93 conclude from the post-mortem that the clinical presentation was not caused by IAV. To our
94 knowledge, we provide the first whole genome sequence for an IAV isolated from a Grey seal, as
95 attempts so far have been unsuccessful. We use phylogenetic analyses to find the putative sources
96 of this virus and look for adaptive changes in its sequence. We compare this case with previously
97 described seal infections to identify unique or parallel elements which may have implications for
98 animal and human health.
99

100 ## METHODS

101 ### *Clinical history and medical interventions*

102 In February 2017, a female Grey seal (*Halichoerus grypus*) pup was rescued from St Michael's
103 Mount, Cornwall. Physical examination of the animal on admittance to the rehabilitation centre
104 revealed an emaciated and dehydrated subject (body weight = 20 kg). The estimated age was 3 to 4
105 months. The animal exhibited a mucoid nasal discharge and a 1 cm wound on the ventral thorax. Its
106 temperature was 39.9 °C and breathing rate was abnormal (continuous breathing pattern, 12 breaths
107 per minute). The pup was monitored and provided nutritional support, fluid therapy and antibiotic

108 treatment, but died suddenly 14 days after admittance. The carcass and a nasal swab were
109 submitted to the Animal and Plant Health Agency (APHA) for virological investigation.
110

111 *Pathology, histopathology and immunohistochemistry*

112 The carcass underwent a full post-mortem examination. A set of tissues was sampled and fixed in
113 buffered formalin (nasal turbinates, trachea, lung, kidney, soft tissues adjacent to the
114 cutaneous/subcutaneous lesion, and lymph node). A standard histopathological examination was
115 carried out on the tissues (Hematoxylin & eosin), followed by an immunohistochemical investigation
116 targeting nucleoprotein to detect intra-lesional IAV along the respiratory tract (Brookes et al., 2010).
117

118 *Real-time reverse transcription polymerase chain reaction (RRT-PCR)*

119 RNA was extracted from the nasal swab and tissue suspensions using the QIAmp viral RNA
120 BioRobot kit customised for APHA in conjunction with a Universal BioRobot (Qiagen, Manchester,
121 UK) (Slomka et al., 2009). RRT-PCR testing of the RNA extracts comprised (i) the Matrix (M)-gene
122 assay for generic IAV detection using the primers and probes of (Nagy et al., 2010) and (ii) H5 and H7
123 IAV RRT-PCR assays to test for notifiable avian influenza (Slomka et al., 2007, 2009). For each RRT-
124 PCR assay, samples producing a threshold cycle (CT) value <36.0 were considered positive (Slomka
125 et al., 2010). The RNA was also tested by an IAV N1-specific RRT-PCR according to the procedure
126 described by (Payungporn et al., 2006), an IAV N5-specific RRT-PCR (James et al. 2018) and two
127 IAV subtype N8-specific RRT-PCRs (James et al., 2018) with the same positive/negative acceptance
128 criteria. All amplifications were carried out in an MX3000P qPCR System (Agilent).
129

130 *Virus isolation and whole genome sequencing*

131 Attempted virus isolation in 9- to 11-day old SPF embryonated fowls' eggs was performed on the
132 nasal swab sample according to the internationally-recognised European Union (EU) and OIE
133 methods (EU, 2006; OIE, 2015), but was unsuccessful. RNA was sequenced using the MiSeq
134 platform. Briefly, viral RNA was extracted from the egg-isolate of the virus from the nasal swab using
135 the QIAmp viral RNA mini-kit without the addition of carrier RNA (Qiagen, Manchester, UK). cDNA
136 was synthesized from RNA using a random hexamer primer mix and cDNA Synthesis System

137 (Roche, UK). The Sequence library was prepared using a NexteraXT kit (Illumina, Cambridge, UK).
138 Quality control and quantification of the cDNA and Sequence Library was performed using
139 Quantifluor dsDNA System (Promega, UK). Sequence libraries were run on a Miseq using MiSeq V2
140 300 cycle kit (Illumina, Cambridge, UK) with 2x150 base paired-end reads. The raw sequence reads
141 were analysed using publicly available bioinformatics software, following an in house pipeline,
142 available on github (https://github.com/ellisrichardj/FluSeqID/blob/master/FluSeqID.sh). This pipeline
143 de-novo assembles the raw data using the Velvet assembler (Zerbino and Birney, 2008), blasts the
144 resulting contigs against a local database of influenza genes using Blast+ (Camacho et al., 2009),
145 then maps the raw data against the highest scoring blast hit using the Burrows-Wheeler Aligner (Li,
146 2013). The consensus sequence was extracted from the resultant bam file using a modified
147 SAMtools software package (Li et al., 2009), script (vcf2consensus.pl) available at:
148 https://github.com/ellisrichardj/csu_scripts/blob/master/vcf2consensus.pl).
149

150 *Phylogenetic analysis of the seal virus*

151 BLAST (Basic Local Alignment Search Tool) was used on GISAID (Elbe and Buckland-Merrett, 2017;
152 Shu and McCauley, 2017) to find the top 50 closest related viral segments for each segment of the
153 seal virus. We combined the seal virus sequence (query) along with the BLAST-hits sequences
154 (blasthit) for each segment for phylogenetic analysis. We removed sequences containing duplicate
155 strain names and aligned with MAFFT (Katoh and Standley, 2013) using automatic settings.
156 Alignments for each segment were inspected manually on AliView (Larsson, 2014) and the ends
157 trimmed to the starting ATG and end STOP codon. Exploratory trees were run using FastTree (Price
158 et al., 2009), after which we used IQ-TREE (Nguyen et al., 2015) to make the final maximum-
159 likelihood tree with 1000 iterations of alrt (approximate likelihood ratio test) for branch support.
160 Tempest v1.5 (Rambaut et al., 2016) was used to test ML trees for clock-like behaviour. Trees for all
161 segments except MP showed clock-like behaviour (**Figure S1**), so results from the MP dataset were
162 excluded. BEAST v1.10.1 (Bayesian Evolutionary Analysis Sampling Trees) (Suchard et al., 2018) was
163 used to determine the putative time and source of emergence of the different segments of the seal
164 virus. BEAST performs Bayesian analysis of molecular sequences using MCMC (Markov Chain
165 Monte Carlo) methods. For all segments other than MP and NS which have multiple reading frames,

166 we used the SRD06 site model which partitions the codons into 1+2 and 3. For NS we used a GTR
167 model with no codon partitioning. The rest of the priors were kept identical for all segments: an
168 uncorrelated relaxed lognormal clock, GMRF (Gaussian Markov random field) Bayesian skyride tree
169 (Minin et al., 2008), 7 million MCMC generations with sampling every 7000. Two separate runs were
170 performed for each segment, which were combined after logs were inspected in Tracer
171 v1.7.1(Rambaut et al., 2018) for appropriate mixing and ESS (effective sample size) values > 200.
172 Trees were summarised into median clade credibility trees (MCC) and plotted in R v3.5 (using the
173 ggtree package (Yu et al., 2017).
174

175 *Amino acid substitutions*

176 Trimmed alignments of each segment were manually inspected in AliView software (Larsson, 2014),
177 translated into amino-acids, and checked for amino acid changes across each dataset. We identified
178 several substitutions in the seal virus that did not occur in any of the bird virus sequences. We
179 recorded these substitutions, and used the H3-numbering of the sequence using the HA subtype
180 numbering conversion tool available from FluDB ((Burke and Smith, 2014),
181 https://tinyurl.com/HAnumbering) for the HA protein. We also looked for differences in glycosylation
182 patterns between the seal and the related wild bird HA and NA glycoproteins using a program to
183 detect Asn-X-Ser or Asn-X-Thr (where X is any amino acid other than Proline) patterns in the amino
184 acid sequences (personal communication from Todd Davis, CDC, USA)
185

186 RESULTS

187 *Pathology and immunohistochemistry*

188 The post-mortem revealed abundant purulent fluid (approximately 1 litre) in the subcutaneous
189 tissues of the right side of the body extending from the neck to the thoracic region (**Figure 1A**). All
190 other organs were macroscopically unremarkable. The blubber sternal thickness was 0.4 cm.
191 Histology identified a severe, chronic-active, fibrino-purulent cellulitis and fasciitis and a severe,
192 acute renal infarct with thrombosis. In the respiratory system a diffuse mild to moderate rhinitis with
193 epithelial hyperplasia and presence of mites was observed in the nasal cavity and a focal broncho-
194 interstitial pneumonia with thrombosis and pulmonary nematodes were seen in the lungs, with no

195 changes observed in trachea and bronchi. Influenza A virus antigen was detected by
196 immunohistochemistry only in the nasal mucosa, in the nuclei of scattered isolated epithelial cells
197 (**Figure 1B**). The pathological findings were suggestive of a thromboembolic event and sepsis
198 caused by the cellulitis as the cause of the death of the animal.
199

200 *Detection and subtyping of IAV by RRT-PCR*

201 The nasal swab was positive for the M-gene (CT value of 22.36), signifying the presence of IAV RNA
202 but was negative for NAI haemagglutinin (HA) subtypes H5 and H7 as well as the IAV subtypes N1
203 and N5. However, IAV subtype N8 was detected by one of the specific RRT-PCR assays employed
204 (CT value of 24.38). In addition, trace amounts of IAV RNA were detected by the M-gene RRT-PCR
205 assay in tracheal bronchi (CT value of 35.88), the left lung (CT value of 37.73) and the right cranial
206 lung (CT value of 38.67). Due to low levels of virus detected in the lower respiratory tract, and the
207 presence of clinical signs not consistent with influenza infection, we concluded that the virus was of
208 no or low pathogenicity. It is possible that at the point the seal was rescued, it had already cleared
209 the infection in the lungs naturally, which would explain trace amounts of detection in these tissues.
210 This is consistent with previous studies which found that Grey seals were likely to remain
211 asymptomatic with IAV infection while a proportion of the surveyed adults and juveniles were
212 seropositive (Bodewes et al., 2015; Puryear et al., 2016).
213

214 Sequencing results from the nasal swab egg-isolate indicated no mixed infection, just a single virus,
215 from which it was possible to sequence all eight gene segments. The virus-derived sequence was
216 named A/Grey seal/England/027661/2017 and sequences were deposited in GISAID database
217 (Global Initiative on Sharing All Influenza Data) with isolate ID: EPI_ISL_381748.
218

219 *Source of viral segments*

220 Segment gene sequences which were found to be most closely related to the genes of the seal virus
221 by BLAST were of avian origin. Overall, the strains in the set of BLAST-hits for each segment gene,
222 all came from different avian viruses, isolated from different bird-types, and of different subtypes.
223 They were from strains that were mostly isolated from wild birds such as mallard, Eurasian teal,

224 White-fronted goose, Black-headed gull (*Anas platyrhynchos*, *Anas crecca*, *Anser albifrons*,
225 *Chroicocephalus ridibundus*) and others, along with a few from domestic birds such as chickens
226 (*Gallus gallus domesticus*). This indicates a wild bird origin for the seal virus, which is consistent with
227 previously detected seal infections (Fereidouni et al., 2016; White, 2013), and the hypothesis that
228 overlapping habitats for wild birds and seals make transmission from wild birds possible, but less
229 likely from poultry, pigs or humans.
230
231 Also similar to previously reported infections (Anthony et al., 2012; Zohari et al., 2014), the set of
232 closest related strains were isolated from within the local region, in this case, Northern Europe (The
233 Netherlands, Germany, UK etc.) with the exception of three strains from China being among the
234 close relatives of the PB1 sequence. The years of detection of the closest-related avian viruses for
235 each segment ranged from the 2007 (NA) to 2017 (NS).
236
237 **Table S1** shows a summary for how often the same strain appears as a BLAST-hit for each segment
238 of the seal virus. A large proportion of these occur singly (250 strains) or for a maximum of two
239 segments (40 strains). However, some strains were found to map for 3, 4 or 5 segments (19, 2 and 1
240 strains(s) respectively). Of these, all the strains that mapped for at least 3 segments were isolated
241 from birds in the Netherlands between 2011-15, except for one chicken virus from France in 2016.
242 The subtypes varied greatly, but they were usually isolated from wild birds. In the next section, we
243 further examine the closest available sequence(s) for each seal gene segment to try and understand
244 the emergence and further propagation of this virus.
245

246 ### *Emergence of viral segments*

247 There is no dedicated surveillance program for seals in England so infection status in seals as
248 endemic or one-off spill over event is uncertain. Previous studies in the US have indicated that Grey
249 seals are possibly a reservoir for IAV and other viruses (Duignan et al., 1995, 1997; Puryear et al.,
250 2016), We performed a time-scaled analysis with the set of closely related avian virus segment
251 sequences, to test if all segments from seal virus had a similar point of introduction, i.e., similar time
252 to most recent common ancestor (TMRCA) of the seal and wild bird viruses for all segments, or the

253 extent to which this varied segment to segment. We used BEAST to reconstruct time-scaled
254 phylogenies for each of our segment datasets.
255
256 Maximum clade credibility trees for each segment are presented in **Figure 2 (A-G)**; a maximum-
257 likelihood tree is presented for the MP gene segment in **Figure 2H** as it was excluded from BEAST
258 analysis due to lack of clock-like behaviour in the dataset. Time to the putative ancestor strain of the
259 segments from the seal virus and its closest related segment is inferred as ranging from 1999 (NA) to
260 between 2011 and 2015 (all other gene segments), summarised in **Figure 3** and **Table 1**. Gaps in
261 surveillance, and the availability of just one seal strain will likely affect the inference of TMRCAs, but
262 the variation between segments is also likely a testament to the high levels of reassortment seen in
263 wild bird IAVs (Lu et al., 2014). Indeed, the closest associated virus varies in host, subtype and
264 geography of isolation for each segment, as can be seen in the highlighted clades in **Figure 2 (A-H),**
265 and summarised in **Table 1**. Consistent with data of BLAST hits (Table S1), many of the closest
266 related segments are from strains isolated in the Netherlands (HA, MP, NP, NS, PA, PB1, PB2).
267 Where multiple strains are equally closely related to the seal virus, e.g. for PB1 and PB2, the avian
268 influenza strains come from the Netherlands and France. The closest strain to the seal NA gene that
269 has been sampled is from 2009 at the latest, from Norway. For the NS gene, the closest segments
270 appear to be from Russian strains in chickens as recent as December 2017, which may indicate
271 onward transmission via unsampled intermediaries; however the posterior probability support value
272 for the node is low (0.36).
273
274 From the above observations, we conclude that most of the Grey seal virus segments are derived
275 from viruses in wild birds in Northern Europe, possibly via unsampled intermediaries – but not in a
276 single transmission event. In contrast, the 2011 H3N8 Harbour seal virus appears to have been
277 derived from a single source. The closest related strain for all internal gene segments as identified by
278 BLAST and maximum-likelihood trees is the A/American_black_duck/New_Brunswick/03552/2009
279 (an H4N6 isolated on 2009-09-11). The closest related strains for the glycoproteins are different but
280 this likely indicates a bias for sequencing glycoproteins over the internal genes. They are both H3N8
281 viruses from the same area (North-eastern US) isolated closer in time to the Harbour seal virus:

282 A/blue-winged_teal/New_Brunswick/00291/2010 isolated 2010-09-14 (for HA) and
283 A/northern_pintail/Minnesota/AI09-4322/2009 isolated 2009-09-12 (for NA). See **Table S2A** and **B**
284 for comparison.
285

286 *Substitutions for mammalian adaptation*

287 Previous analyses have indicated that mammalian adaptive mutations can occur in avian viruses
288 when they are transmitted into seals. A study of H10N7 viruses in Harbour seals in Northern Europe,
289 which unlike the present case was demonstrated to have transmitted to other seals and caused an
290 outbreak, showed that mutations were likely to occur early on after transmission to seals and then
291 plateau (Bodewes et al., 2016). We looked for differences between the seal virus and wild bird
292 viruses in the segment amino acid alignments of our datasets, to check if they any had putative
293 adaptive implications.
294

295 We found several amino acid substitutions in the seal virus that did not occur in any of the related
296 bird viruses. These substitutions are summarised in **Table 2**, along with references for those that
297 have been identified in previous studies of mammalian adaptation. Many of these changes occur in
298 the polymerase complex genes (Mänz et al., 2013): D701N in the PB2 segment is a rare mutation,
299 and a hallmark of mammalian adaptation of bird viruses, regardless of genetic background (Liu et al.,
300 2018; Steel et al., 2009)). Liu and Steel et. al. have elucidated that the basis of this adaptation is that
301 it allows for better replication in mammalian cells and showed that it has been associated with
302 increased transmission in ferret experiments. Another mutation in the PB2 gene, at residue 105 in its
303 NP-binding region (Poole et al., 2004) was also found in studies that used phylogenetic modelling
304 (Tamuri et al., 2009) and mutual information statistics (Miotto et al., 2008, 2010). Both the mutations
305 found in the PB1 gene are also of interest. PB1-I517V was found by Tamuri et al. and S678N found
306 in the seal PB1 gene has been associated with increased polymerase activity and virulence in mice
307 (Gabriel et al., 2005).
308

309 Changes were also found in the HA gene (**Table 2**), but the implications are less clear. There appear
310 to be no changes in the glycosylation patterns between the HA and NA of the Grey seal virus in
311 comparison to related wild bird viruses (**Table S3A and B**).
312
313 We compared all the substitutions with previously described mutations in seal infections, and found
314 that apart from D701N, which was also found in the H3N8 seal virus infection in Massachusetts in
315 2011 (Anthony et al., 2012), there were no convergent amino acid changes. In the H3 HA gene, we
316 found substitutions in residue 81 (reference H3 numbering - 64), and residue 176 (reference H3
317 numbering - 160), and these are also changed in the 2011 Massachusetts virus (Anthony et al., 2012)
318 but to different amino acids. The latter mutation was not implicated in receptor binding for the seal
319 viruses as was the case with H5N1 and some human H3N2 viruses, because the glycosylation site is
320 absent in both seal and wild bird viruses (**Table S3A**). The HA of the 2011 Massachusetts virus had
321 an F110S mutation, where the 110 residue has been previously found to be a critical component of
322 the influenza fusion peptide, which may impact replication in mammalian cells (Anthony et al., 2012;
323 Liu et al., 2011). Our reported seal virus retains F at position 110, but whether the mutation in the
324 adjoining residue at S111G (reference H3 numbering 95) has any effect on HA fusion properties is
325 unknown. The presence of Serine at position 66 in the PB1 sequence, which enables production of
326 PB1-F2 (Conenello et al., 2007) was found in the 2011 H3N8 Harbour seal virus but was not seen in
327 this 2017 H3N8 Grey seal virus. Changes at positions 226 and 228 in HA (reference H3 numbering)
328 which can change receptor-binding preferences between avian and mammalian hosts (Connor et al.,
329 1994; Matrosovich et al., 2000), were not found in either of the H3N8 seal viruses, but the H10
330 equivalent of H3-Q226L was identified in viruses from the 2014-15 H10N7 outbreak in Europe
331 (Dittrich et al., 2018).
332

333 ## DISCUSSION

334 In the seal infection case reported here, the animal was referred to a rescue centre because it was
335 stranded and the IAV was detected only incidentally. The vast majority of Grey seals admitted to
336 rehabilitation centres in the UK are pups within the first year of life. Malnutrition is the single most
337 common reason for pinnipeds to be taken into rehabilitation centres (Barnett et al., 2000; Van Bonn,

338 2015). Wounds are often recorded and hold clinical significance, as in this case, and have been
339 considered predisposing factors for fatal non-specific septicaemia (Baily, 2014). The cause of trauma
340 and wounds may be anthropic (entanglement in fishing nets or gears) or biologic (conspecific
341 aggressiveness or hierarchical to cannibalistic behaviours). Wounds caused by bites of other seals or
342 predators were most often seen (Barnett et al., 2000; Van Bonn, 2015). Pulmonary and nasal
343 parasites are also very well documented clinico-pathological conditions among rescued seal pups,
344 and likely caused the hyperplastic rhinitis identified in this study seal. No histopathological findings
345 consistent with IAV derived damage were detected along the respiratory tract. The
346 immunohistochemistry demonstrated productive viral replication only in nasal mucosa, but not in
347 lower respiratory segments, although the more sensitive RRT-PCR revealed presence of trace
348 amounts of viral RNA within the lower respiratory tract, suggesting that the infection may have been
349 cleared naturally or indicating a passive translocation of antigen from upper respiratory segments.
350 Efficient clearance in healthy Grey seals may explain why previous attempts at sequencing IAV from
351 identified during surveillance were unsuccessful (Puryear et al., 2016).
352
353 Puryear et al. discuss the possibility that Grey seals may be more prone to infection due to gain in
354 population density since the marine mammal protection act in the US in 1972, along with more
355 socially gregarious and aggressive behaviour in comparison to Harbour seals, all of which contribute
356 to high pathogen transmissibility. Why we see differences between Grey and Harbour seals in their
357 resistance to diseases caused by viral agents is however unclear. Phylogenetic analyses have been
358 unable to resolve the relationships between different seal species with sufficient support but Grey
359 seals are either placed as a sister group to Caspian seals (*Pusa Caspica*) and separate from Harbour
360 seals, or in a basal position to both *Phoca* and *Pusa* genus (Berta et al., 2018; Fulton and Strobeck,
361 2006). It might be useful to explore long-term evolutionary history of host-pathogen relationships
362 along with host physiology and immune response differences between different seal species to
363 understand differences in viral pathogenicity.
364
365 In our dataset of closest related sequences to the seal virus, we find that while there are several
366 substitutions found in the seal virus which do not occur in the bird viruses; we generally do not find

367 substitutions in any of the bird virus strains that do not occur in other bird viruses too. This likely
368 indicates adaptation to the seal environment, a hypothesis supported by the occurrence of the
369 D701N mutation, a known rare marker of mammalian adaptation (Liu et al., 2018; Steel et al., 2009).
370 D701N is common in canine and horse H3N8 IAV (see **Table S4**), but does not occur in birds, and
371 was found associated with highly-pathogenic H5N1 viruses which infected humans (Gabriel et al.,
372 2005; Jong et al., 2006; Li et al., 2005). We found one reported instance of the D701N mutation in
373 the other avian H3N8 virus that infected pinnipeds, but not in the H10N7 outbreak, despite sustained
374 transmission in seals for several months, nor in any other previously sequenced seal PB2. H3N8 IAV
375 are noted for the ability cross species barriers, so it would be relevant to consider if the subtype of
376 the virus has any influence on the kind of adaptive mutations that occur in the polymerase genes,
377 and if so what sort of mechanisms this might involve. We also note that many of the putative
378 adaptive substitutions occur in the polymerase complex genes, which are increasingly being
379 recognised for their vital role in mediating viral host range (in addition to receptor compatibility with
380 glycoproteins).
381
382 The occurrence of adaptive substitutions in the viral sequence could mean that it has been
383 circulating in seals for a certain amount of time during which these substitutions have accumulated.
384 Alternatively, this event might represent an early rapidly-adapting virus from a spill-over event as
385 seen in the H10N7 outbreak (Bodewes et al., 2016). Given the subclinical nature of the infection, we
386 propose the former explanation is more likely to be correct. However, since the case of the seal virus
387 in this study appears to be a singular infection as far as we know, it is difficult to know if and how
388 long the virus has been in seals. Both this study and Bodewes et al. report that despite mammalian
389 adaptation of the virus, continued contact with bird reservoirs allows for further exchange of viral
390 segments between the two hosts. It should be noted that the segment for which this study has
391 tentative evidence of onward transmission (NS) does not appear to have acquired any putative
392 mammalian-adaptive mutations. The nature of the Grey seal reservoir, if it exists, and its relationship
393 with the avian reservoir is currently unknown.
394

395 The 2011 North American H3N8 and 2014 European H10N7 viruses which caused outbreaks in
396 Harbour seals were found to have acquired mutations to enable recognition of sialyloligosaccharide
397 receptors found more abundantly in mammalian tissues (SAα2,6Gal) but which retained the ability to
398 interact with avian receptors (SAα2,3Gal). A later detailed structural and functional analysis of the
399 2011 H3N8 seal HA indicated a true avian receptor binding preference (Yang et al., 2015), as did a
400 mutational analysis of the H10N7 viruses (Dittrich et al., 2018). Although there are some common
401 HA residues that are changed in both the seal virus reported here and the 2011 H3N8 virus, we
402 found limited convergence in the substitutions and residues involved between the different seal
403 viruses, likely attributable to the different genetic backgrounds from different avian sources and
404 possibly differences in host environment of reservoir vs susceptible species. In addition, to the best
405 of our knowledge, ours is the first Grey seal virus sequence that is being made publicly available (via
406 GISAID). It is therefore uncertain whether the type of mutations occurring in a putative reservoir host
407 vis-à-vis avian sequence might be different from those occurring in related hosts with pathogenic
408 outcomes.
409
410 Our phylogeny and molecular clock analyses suggest different lineages of source viruses, and time
411 of introduction of different segments (with the caveat of being inferred for a single sequence). It is not
412 surprising to find that the closest sampled and sequenced viruses for each segment are of different
413 subtypes, hosts and times of isolation, given the relatively low surveillance in wild birds, and the
414 extensive reassortment (Lu et al., 2014) i.e., swapping of individual segments into different progeny
415 viruses during propagation after mixed infection with two or more viruses. It would be speculative to
416 propose likely wild bird species donor however. Studies on avian influenza have previously shown
417 that the transmission between birds is directional, e.g., usually in the direction from anseriformes
418 such as ducks into galliformes (chicken) or charadriiformes (gulls) (Venkatesh et al., 2018). In the
419 case of the current infection as well, where we have found unambiguously closest related
420 sequences, they have tended to have been isolated from anseriformes species. The above findings,
421 along with the observation that the closest related viruses were isolated largely from the
422 Netherlands, it is likely that the source of the viral segments come from unsampled locally circulating
423 (in Northern Europe) avian viruses from an anseriformes host. The closest related wild bird viruses to

the 2011 H3N8 Harbour seal virus were also isolated from anseriformes species (American black duck). In contrast with the our Grey seal virus however, phylogenetic analysis reveals a highly overlapping set of wild bird strains that are most closely related with Harbour seal virus, an observation that suggests a single spill-over event into a susceptible host.

H3N8 viruses currently circulate in horses and dogs but not humans or pigs. However, different H3 viruses have been found in several species including humans, pigs, horses, dogs, cats, seals, poultry, and wild aquatic birds. They have thus been noted for a particular ability to cross species barrier and cause productive infections. One study that examined the ability of H3N8 viruses from canine, equine, avian, and seal origin to productively infect pigs, demonstrated that avian and seal viruses replicated substantially and caused detectable lesions in inoculated pigs without prior adaptation (Solórzano et al., 2015). It is possible that the ready occurrence of PB2 701N mutation in H3N8 viruses contributes to this ability. We do not have any biological evidence for pre-20th century human influenza, but historical analysis suggests a long association of humans with influenza (Hirsch, 1883; Taubenberger and Morens, 2010), and has uncovered temporal–geographic associations between equine and human influenza-like disease activity documented in Europe in the 16th-18th centuries (Morens and Taubenberger, 2010). Such analysis has also implicated an H3N8 virus in the 1889 pandemic in humans (Morens and Taubenberger, 2010), which makes mammalian-adapted H3N8 viruses of particular interest as IAV pandemic risk candidates.

In this paper we have presented analyses of a case of seal infection with IAV in coastal England, and compared it with previously reported seal IAV infections. This infection provides a small but unique window to understand the ecology of avian-origin viruses that may be circulating and maintained in mammals. Given the mammalian adaptation activity in IAV upon transmission to seals, such infections may be of interest to pandemic surveillance and risk, and help us better understand the determinants of mammalian adaptation in influenza and its complex drivers.

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655

**Figure 1 (A-B)**
Grey seal pup. (A) Focally extensive, chronic-active, purulent and fibrino-necrotising cellulitis in subcutaneous tissues and skeletal muscle over the thorax. (B) Immunohistochemical demonstration of Influenza A nucleoprotein antigen in the nuclei of epithelial cells within the nasal mucosa of a grey seal. (DAB chromogen and haematoxylin counterstain) (Objective: 40x).

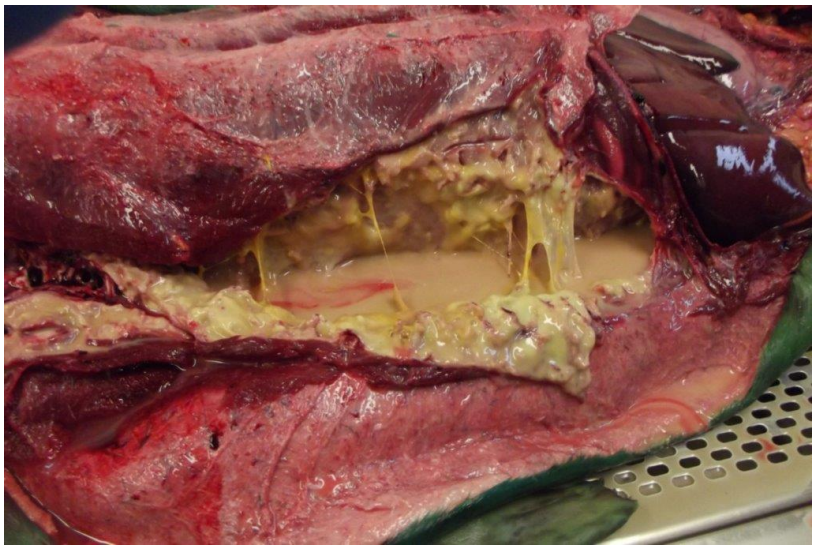


Figure 1A

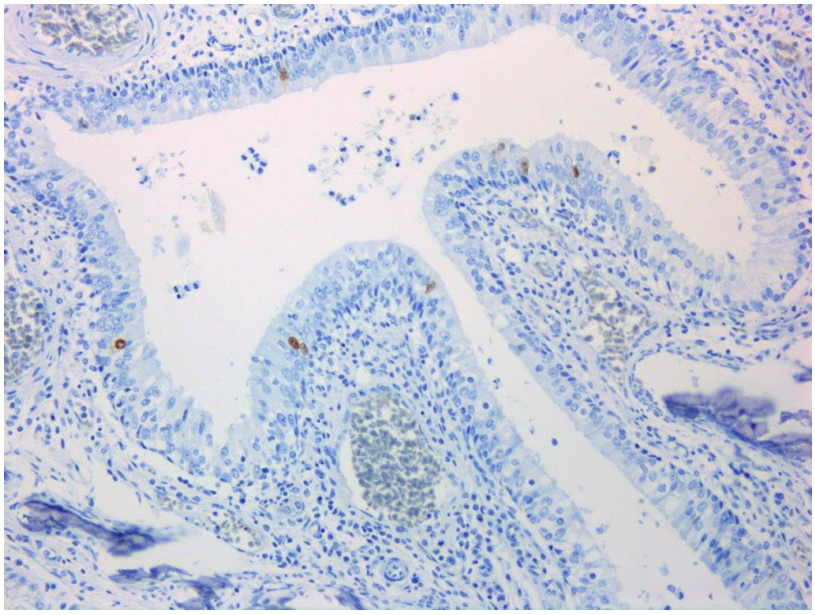


Figure 1B

**Figure 2 (A-G, H)**
Maximum clade credibility summary trees for BEAST analysis of segment datasets: HA, NA, NP, NS, PA, PB1, PB2 (A-G respectively). Nodes connecting the seal virus tip with the closest related strain(s) are highlighted in grey. Black diamond (◆) shapes at nodes indicate posterior probability > 0.75. Nodes are labelled with node ages as inferred by BEAST. Figure 2H shows the maximum likelihood tree for the MP gene dataset. Support values (approximate likelihood ratio test) are labelled at nodes, and nodes connecting the seal virus tip with the closest related strain is highlighted in grey.

Figure 2A

seal – HA

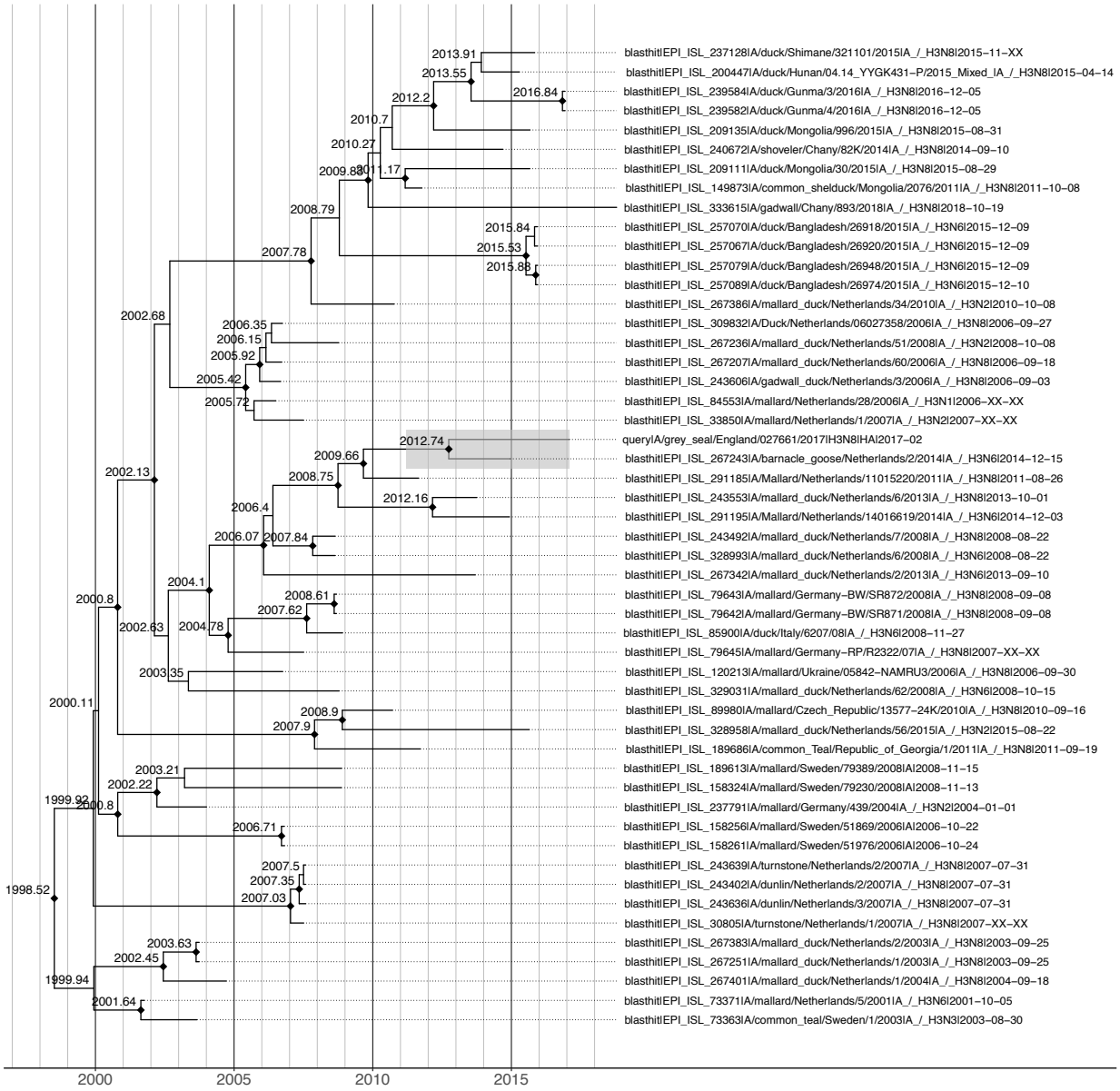


Figure 2B

seal – NA

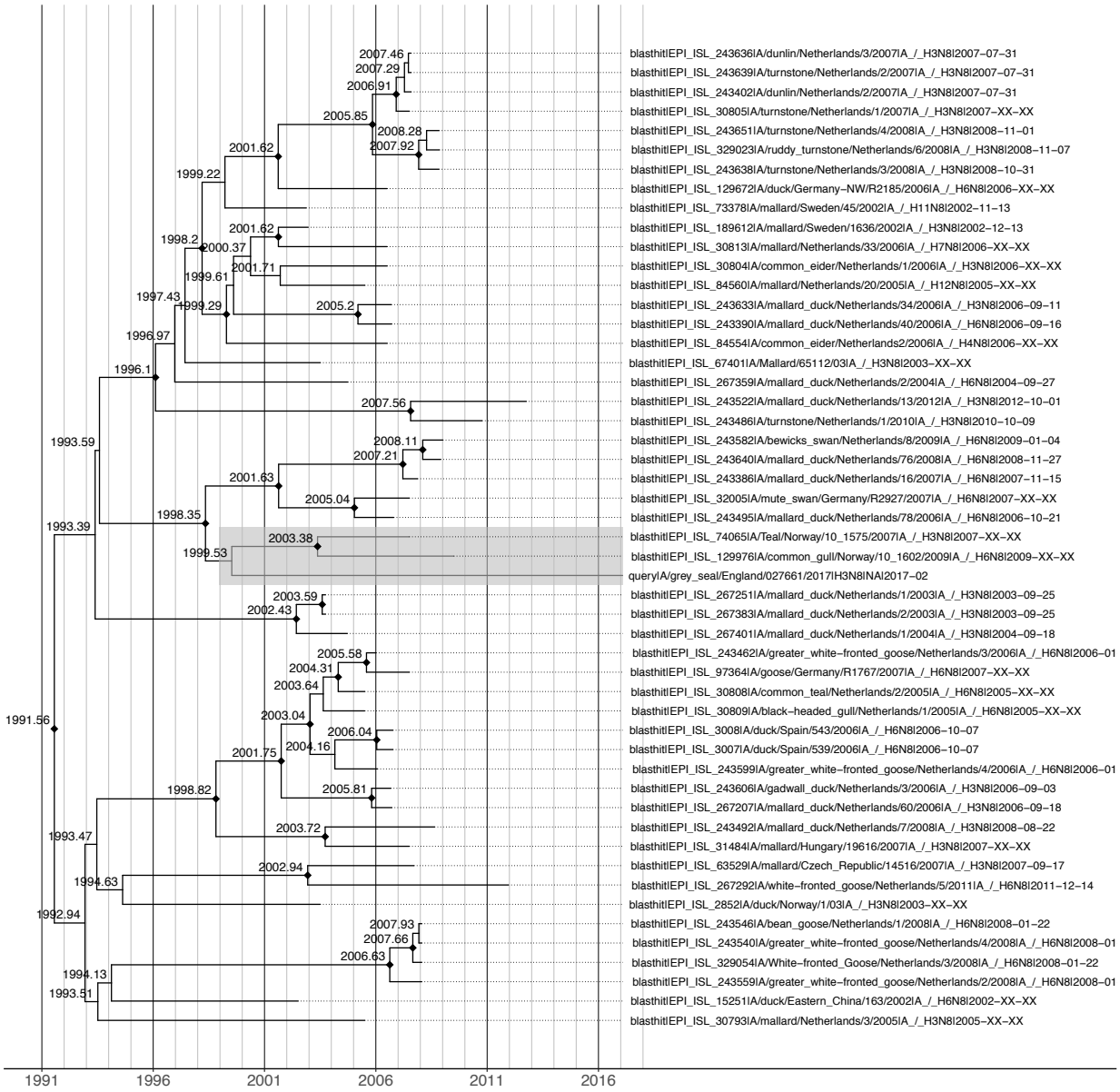


Figure 2C

seal – NP

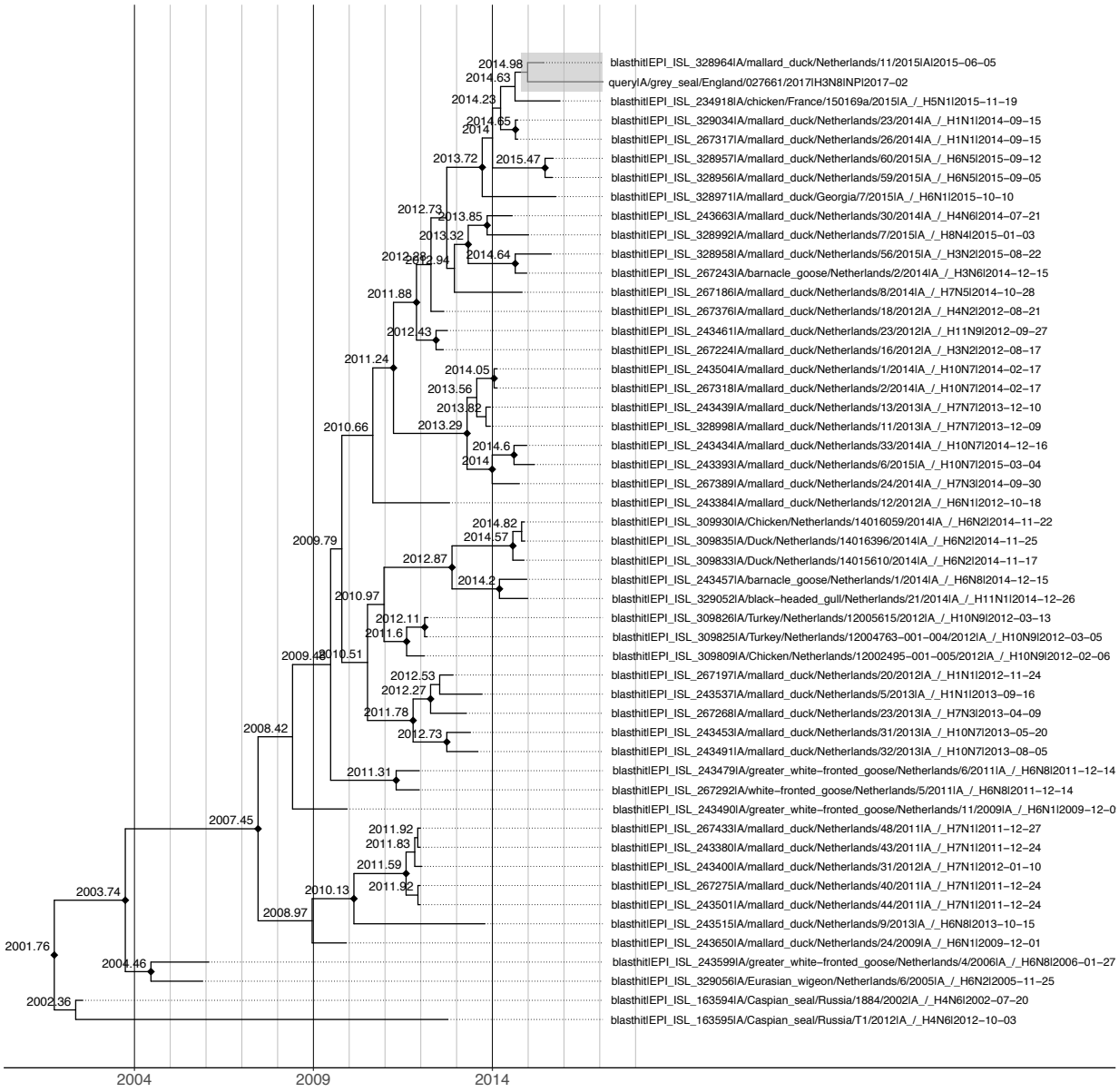


Figure 2D

seal – NS

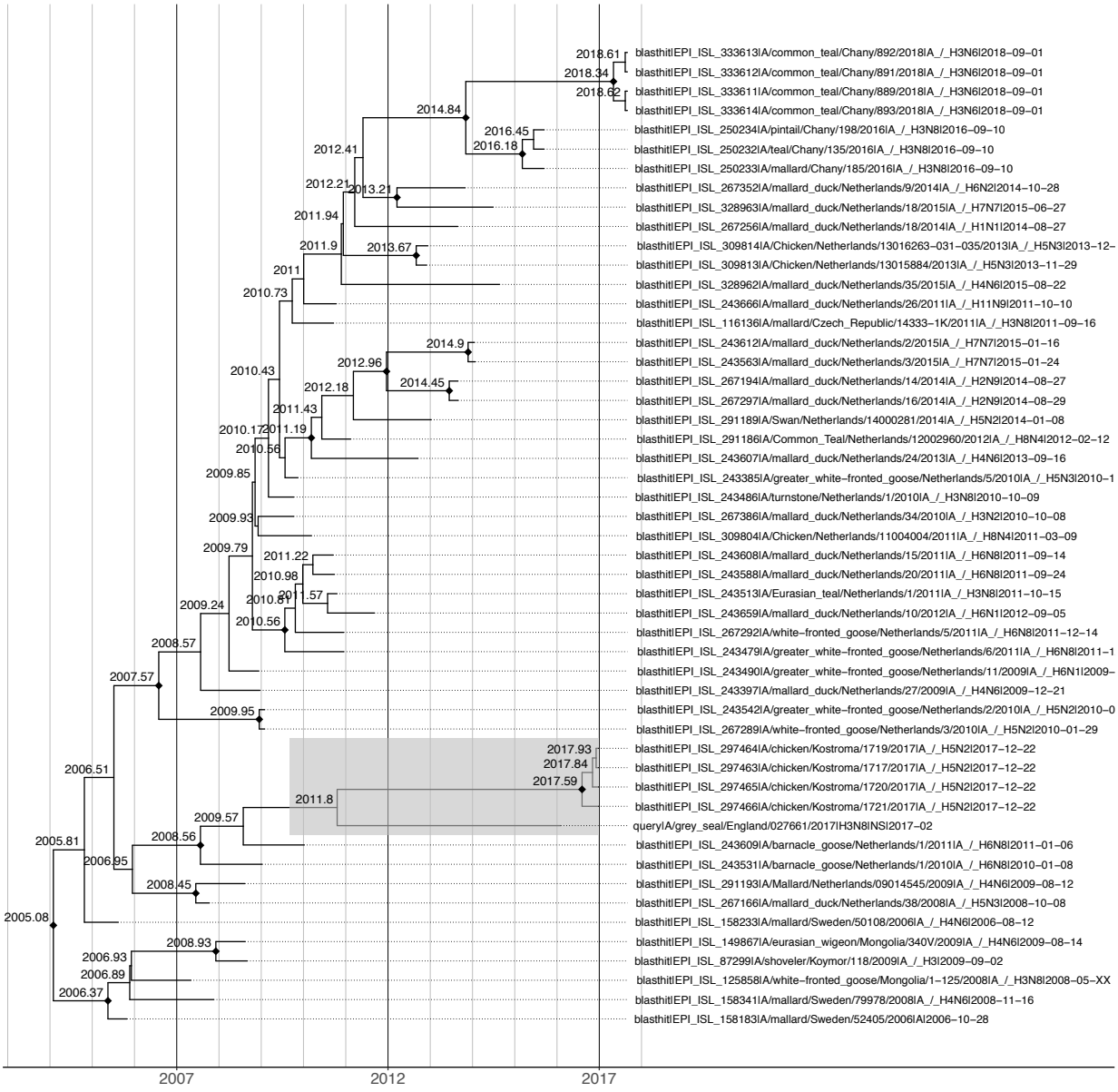


Figure 2E

seal – PA

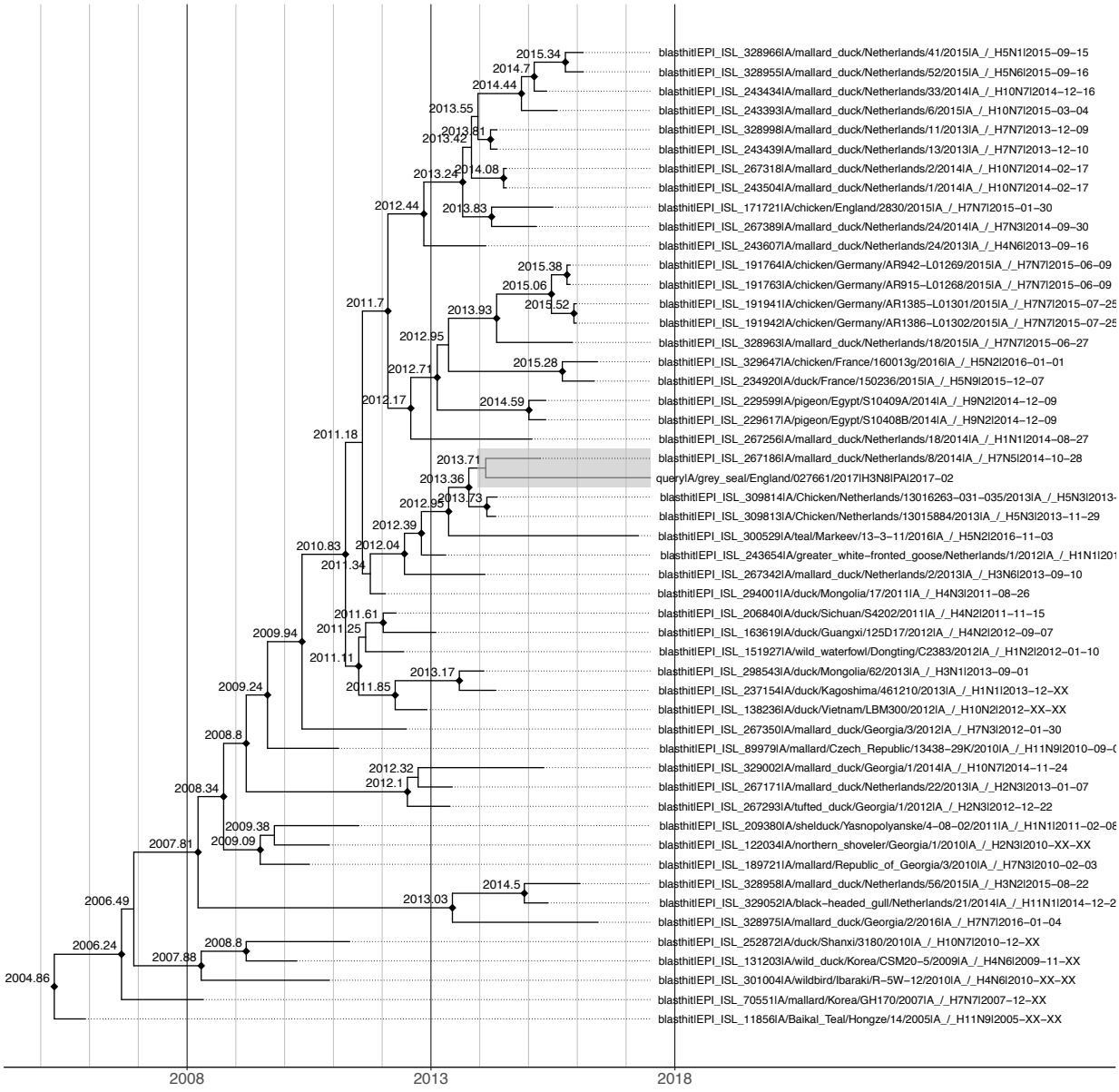


Figure 2F

seal – PB1

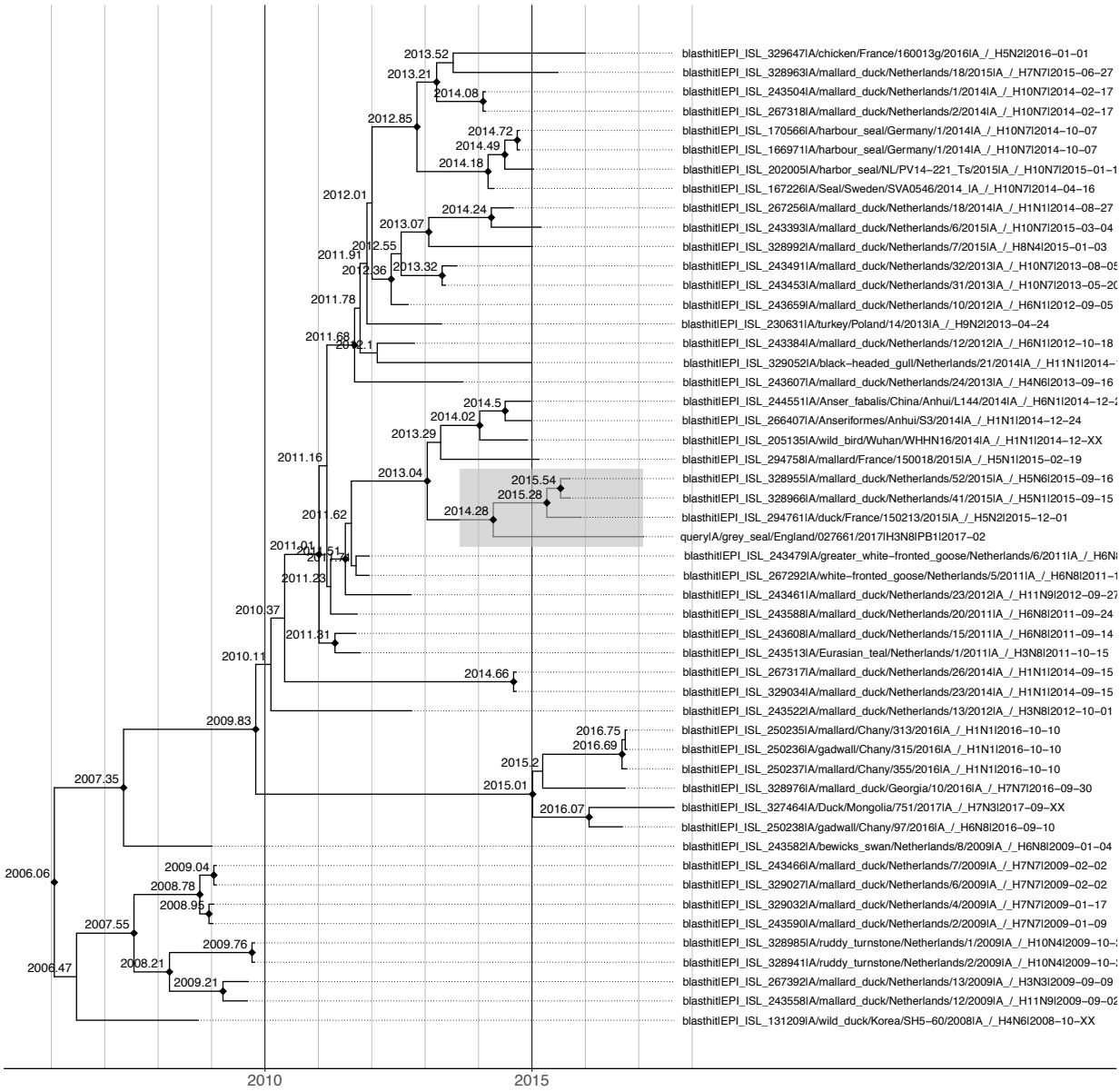
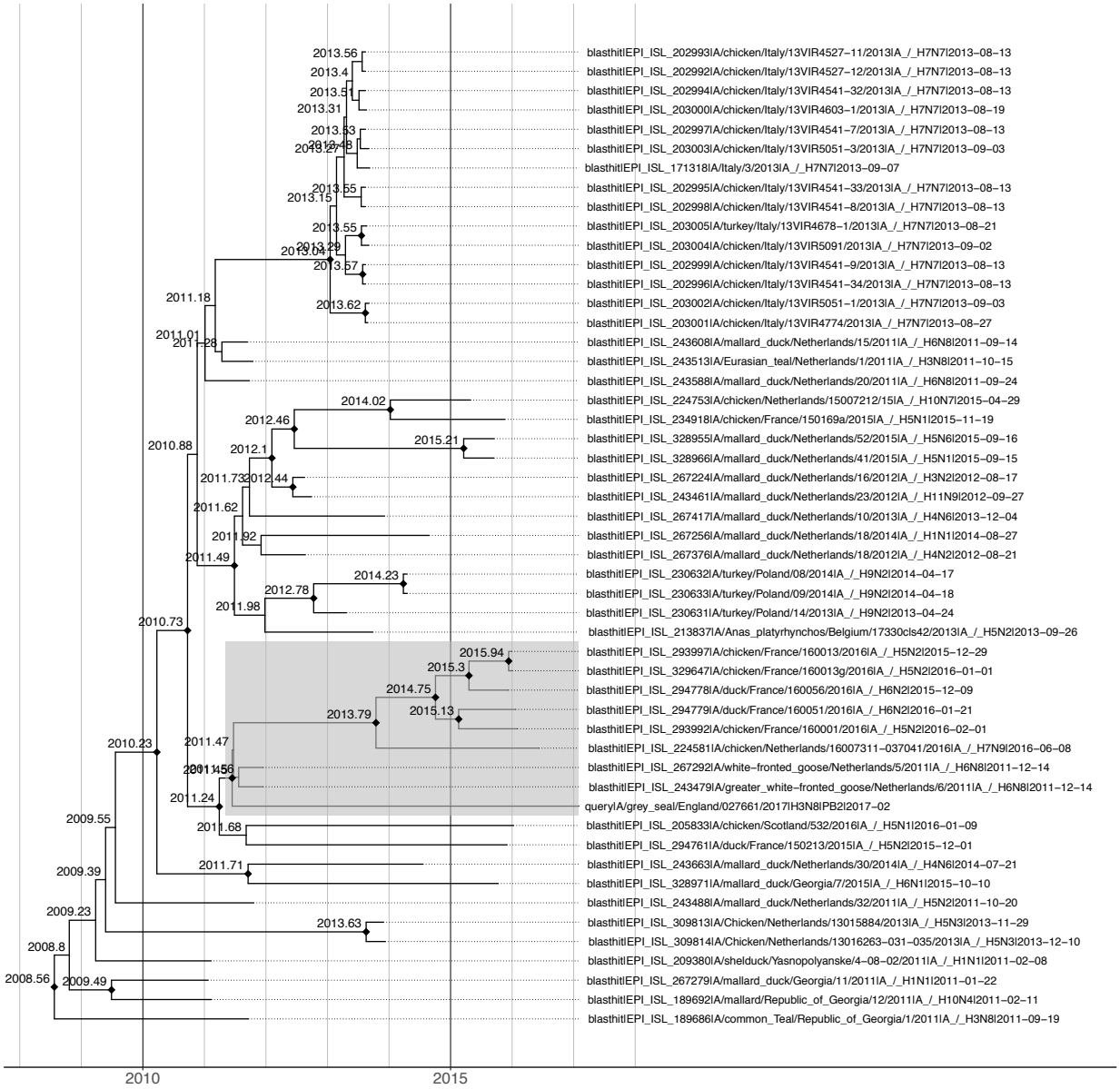


Figure 2G

seal – PB2



# Figure 2H

seal - MP (ML tree)

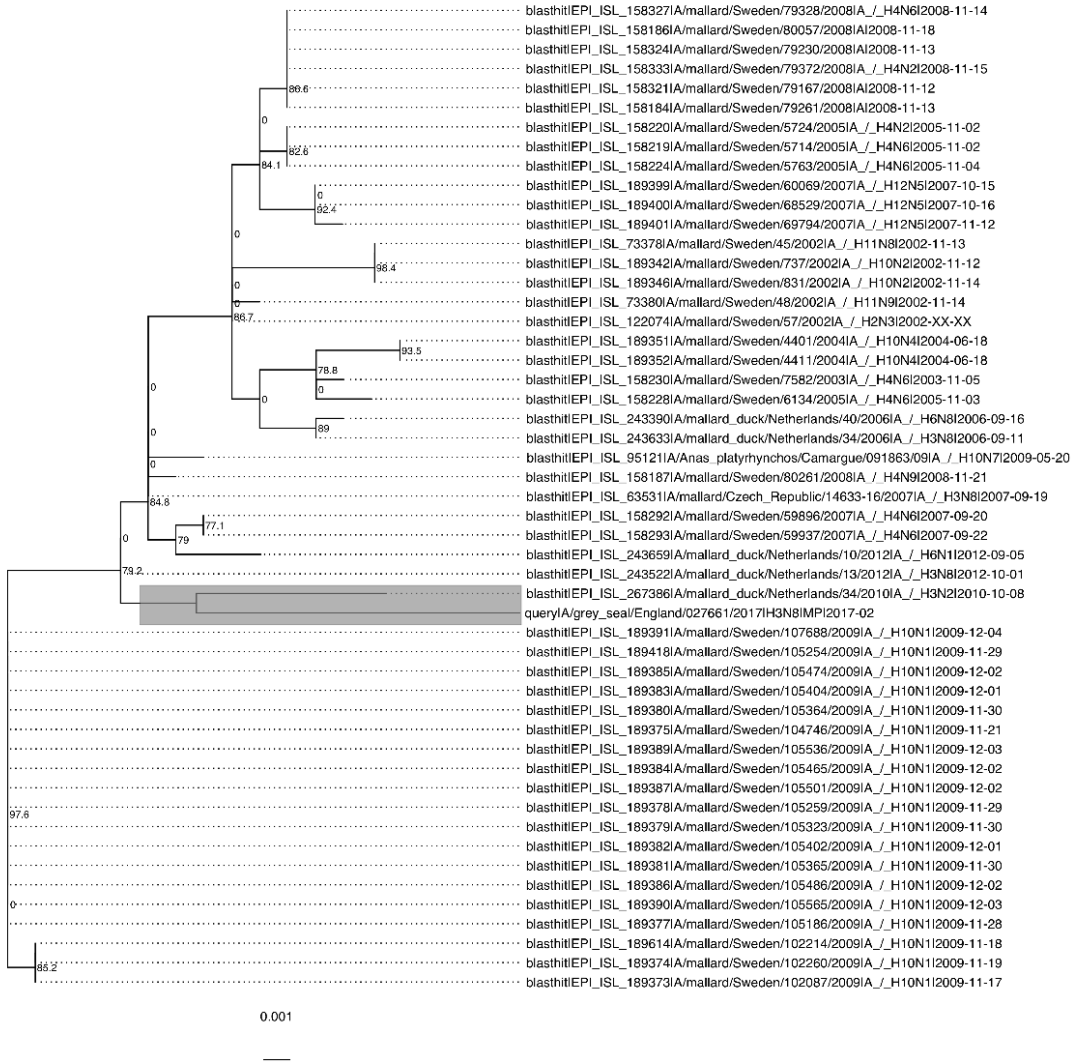


Figure 3
Putative divergence times (TMRCA – time to most recent common ancestor) of the seal sequence from the closest related wild bird sequence for each segment. Error bars indicate 95% highest posterior density.

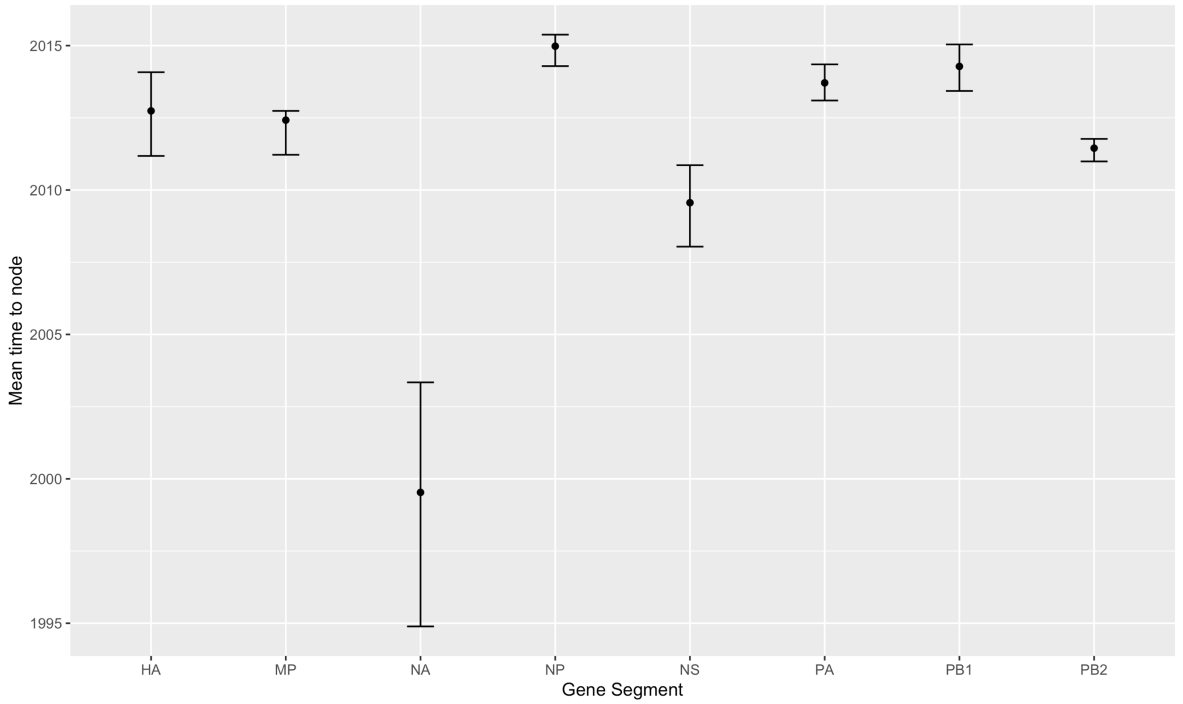


Table 1
Putative divergence times (TMRCA – time to most recent common ancestor) and 95% highest posterior density (HPD) of the seal sequence from the closest related wild bird sequence for each segment, and the posterior probability of the node are shown, along with information about the closest related wild bird sequence including the host, country and year of isolation and subtype of virus. For the MP gene, the closest related sequence according to the ML tree is shown.

| Segment | TMRCA | Lower 95% HPD | Upper 95% HPD | Posterior probability of node > 0.75 | Host | Country | Year | Subtype |
|---|---|---|---|---|---|---|---|---|
| HA | 2012.74 | 2011.18 | 2014.08 | y | barnacle goose | The Netherlands | 2014 | H3N6 |
| MP | n/a | n/a | n/a | n/a | mallard | The Netherlands | 2010 | H3N2 |
| NA | 1999.53 | 1994.89 | 2003.34 | n | teal/gull | Norway | 2007/2009 | H3N8/H6N8 |
| NP | 2014.98 | 2014.29 | 2015.38 | n | mallard | The Netherlands | 2015 | unknown |
| NS | 2009.56 | 2008.04 | 2010.86 | n | barnacle goose | The Netherlands | 2011 | H6N8 |
| PA | 2013.71 | 2013.1 | 2014.35 | n | mallard | The Netherlands | 2014 | H7N5 |
| PB1 | 2014.28 | 2013.43 | 2015.04 | y | duck | The Netherlands/France | 2015 | H5N1/H5N2/H5N6 |
| PB2 | 2011.45 | 2010.99 | 2011.77 | y | goose/chicken/duck | The Netherlands/France | 2011/2015/2016 | H5N2/H6N2/H7N9/H6N8 |

Table 2
Segment-wise list of substitutions found in the seal virus inferred from inspection of alignments. Only residue changes that occur in the seal virus but not in the wild bird viruses are shown. Numbers in square brackets for HA indicate the reference H3-numbering of residues in A/Aichi/2/1968 (Burke and Smith 2014) found using the tool at FluDB website: https://tinyurl.com/HAnumbering

| Segment | Amino acid substitutions in seal virus [H3 numbering] |
|---|---|
| HA | Y9C [signal peptide] |
| | T64A [48] |
| | I81V [65] |
| | S111G [95] |
| | possible A154T (but might be same RCT --> ACT (T), or GCT (A)) [138] |
| | A/T176S [160] |
| | S235Y [219] |
| MP | none |
| NA | N/S41D |
| | T383A |
| NP | V104M |
| NS | none |
| PA | V91I |
| | D/G101N |
| | N/S321I |
| PB1 | I517V |
| | S678N |
| PB2 | T/A105K |
| | D161N |
| | A/S395T |
| | V667I |
| | R/T676I |
| | D701N |
| | possible R753T (but might be same ASA --> AGA (R) or ACA (T) |