Working title:

Avian influenza virus (A/H3N8) with a putative mammalian-adaptive mutation found in a stranded grey seal pup (*Halichoerus grypus*) rescued on the Cornwall coast, UK

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ABSTRACT

Avian Influenza A Viruses (IAV) in different species of seals display a spectrum of pathogenicity, from subclinical infection to unusual mortality events. In this report, we describe a case 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 only, with no evidence of pathology. 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 infection prevalence among grey seals might therefore be underestimated. In addition, the fact that the animal was rescued and entered into contact with humans raised concerns about potential zoonotic risk. Nucleotide sequencing revealed the virus to be IAV subtype H3N8. Combining a GISAID database BLAST search and time-scaled phylogenetic analyses, we inferred that the seal infection came from an unsampled, locally circulating (in Northern Europe) source viruses, likely originally derived from wild Anseriformes, which may or may not have been circulating in seals. 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, which has been reported in another IAV H3N8 avian-to-seal transmission event. 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: 317)

54 INTRODUCTION

55 Influenza A viruses (IAVs) are important pathogens for humans and livestock including pigs and
56 poultry. They are segmented RNA viruses, whose genomes consist of eight segments of RNA, which
57 code for ~11 proteins/polypeptides. IAVs are classified into several subtypes based on the antigenic
58 properties of two surface glycoproteins haemagglutinin (HA, avian subtypes H1-H16) and
59 neuraminidase (NA, avian subtypes N1-N9). Viruses of most subtypes can be found in wild waterfowl
60 and shorebirds which are their natural reservoir (Alexander, 2007; Easterday et al., 1968). Viruses
61 from wild birds can infect both domestic birds and mammalian species in spill-over infections, and a
62 few IAV lineages have become established in mammals such as humans and pigs, though only of
63 subtypes H1, H2 and H3 (Reperant et al., 2009; Webster et al., 1992).
64
65 IAV in birds replicates mainly in the intestine and is transmitted through the faecal-oral route,
66 although respiratory tropism and oropharyngeal shedding has been noted (Webster et al., 1978;
67 Daoust et al., 2011; Höfle et al., 2012; França et al., 2012; Daoust et al., 2013), and the virus can
68 survive in the environment for fairly long periods (Brown et al., 2009; Stallknecht et al., 1990). This
69 creates conditions conducive to viral exchange with marine mammals such as pinnipeds (seals),
70 whose habitats and prey intersect with those of waterfowl and shorebirds. Several cases of marine
71 mammal infection with IAV of many subtypes including H1N1, H3N3, H3N8, H4N5, H4N6, H7N7 and
72 H10N7 have been documented with a spectrum of effects ranging from mass die-offs to
73 asymptomatic; in a majority of these cases, the source is implicated to be avian (Fereidouni et al.,
74 2016; White, 2013). A few studies have also suggested that certain species of marine mammals such
75 as grey seals may act as an endemically infected reservoir which may disseminate viruses in coastal
76 ecosystems to other mammals, coastal birds, and potentially humans (Duignan et al., 1995, 1997;
77 Puryear et al., 2016). In seals, IAV binds the same type of sialyloligosaccharide receptors
78 (SAα2,3Gal) as birds, but the receptors are located in the respiratory tract and lungs instead of the
79 intestinal tract, suggesting that inhalation is the most likely route of transmission (Ito et al., 1999;
80 Ramis et al., 2012; White, 2013). Seals may also be infected by IAV from non-avian sources; there is
81 serological evidence for infection of Baikal and ringed seals in Russia with human H3N2 strains
82 A/Aichi/2/68 and A/Bangkok/1/79 (Ohishi et al., 2004) and a human pandemic-2009 H1N1 virus was

83 isolated from Elephant seals in 2010 on the coast of California, USA. Furthermore, it has been
84 shown that IAV from seals can replicate in human tissue and that seal IAV can be systemically
85 virulent in primates (Murphy et al., 1983; Webster et al., 1981; White, 2013). Seals may therefore be
86 potential sources of pandemic influenza.
87
88 In this paper, we report an H3N8 IAV infection of a stranded grey seal pup in coastal England. We
89 provide molecular and histological evidence for presence of IAV in the seal's respiratory tissues, and
90 conclude from the post-mortem that the clinical presentation was not caused by influenza virus
91 infection. To our knowledge, we provide the first whole genome sequence for an IAV isolated from a
92 grey seal, as attempts so far have been unsuccessful. We use genetic analyses to find the putative
93 sources of this virus and look for adaptive changes in its sequence. We compare this case with
94 previously described seal infections to identify unique or parallel elements which may have
95 implications for animal and human health.
96

97 ## METHODS

98 ### *Clinical history and medical interventions*

99 In February 2017, a female grey seal (*Halichoerus grypus*) pup was rescued from St Michael's Mount,
100 Cornwall. Physical examination of the animal on admittance to the rehabilitation centre revealed an
101 emaciated and dehydrated subject (body weight = 20 kg). The estimated age was 3 to 4 months. The
102 animal exhibited a mucoid nasal discharge and a 1 cm wound on the ventral thorax. Its temperature
103 was 39.9 °C and breathing rate was abnormal (continuous breathing pattern, 12 breaths per minute).
104 The pup was monitored and provided nutritional support, fluid therapy and antibiotic treatment, but
105 died suddenly 14 days after admittance. The carcass and a nasal swab were submitted to the Animal
106 and Plant Health Agency (APHA) for virological investigation.
107

108 ### *Pathology, histopathology and immunohistochemistry*

109 The carcass underwent a full post mortem examination. A set of tissues was sampled and fixed in
110 buffered formalin (nasal turbinates, trachea, lung, kidney, soft tissues adjacent to the
111 cutaneous/subcutaneous lesion, and lymph node). A standard histopathological examination was

112 carried out on the tissues (Hematoxylin & eosin), followed by an immunohistochemical investigation
113 aimed to detect intra-lesional IAV along the respiratory tract. The immunohistochemistry procedure
114 targeted the IAV nucleoprotein (Brookes et al., 2010).
115

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

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

128 *Virus isolation and whole genome sequencing*

129 Attempted virus isolation in 9- to 11-day old SPF embryonated fowls' eggs was performed on the
130 nasal swab sample according to the internationally-recognised European Union (EU) and OIE
131 methods (EU, 2006; OIE, 2015). RNA was sequenced using the MiSeq platform. Briefly, viral RNA
132 was extracted from the egg-isolate of the virus from the nasal swab using the QIAmp viral RNA mini-
133 kit without the addition of carrier RNA (Qiagen, Manchester, UK). cDNA was synthesized from RNA
134 using a random hexamer primer mix and cDNA Synthesis System (Roche, UK). The Sequence library
135 was prepared using a NexteraXT kit (Illumina, Cambridge, UK). Quality control and quantification of
136 the cDNA and Sequence Library was performed using Quantifluor dsDNA System (Promega, UK).
137 Sequence libraries were run on a Miseq using MiSeq V2 300 cycle kit (Illumina, Cambridge, UK) with
138 2x150 base paired-end reads. The raw sequence reads were analysed using publicly available
139 bioinformatics software, following an in house pipeline, available on github
140 (https://github.com/ellisrichardj/FluSeqID/blob/master/FluSeqID.sh). This pipeline de-novo

141 assembles the raw data using the Velvet assembler (Zerbino and Birney, 2008), blasts the resulting
142 contigs against a local database of influenza genes using Blast+ (Camacho et al., 2009), then maps
143 the raw data against the highest scoring blast hit using the Burrows-Wheeler Aligner (Li, 2013). The
144 consensus sequence was extracted from the resultant bam file using a modified SAMtools software
145 package (Li et al., 2009), script (vcf2consensus.pl) available at:
146 https://github.com/ellisrichardj/csu_scripts/blob/master/vcf2consensus.pl).
147

148 *Phylogenetic analysis of the seal virus*

149 BLAST (Basic Local Alignment Search Tool) was used on GISAID (Elbe and Buckland-Merrett, 2017;
150 Shu and McCauley, 2017) to find the top 50 closest related viral segments for each segment to the
151 seal virus. We combined the seal virus sequence (query) along with the BLAST-hits sequences
152 (blasthit) for each segment for phylogenetic analysis. We removed sequences containing duplicate
153 strain names and aligned with MAFFT (Katoh and Standley, 2013) using automatic settings.
154 Alignments for each segment were inspected manually on AliView (Larsson, 2014) and the ends
155 trimmed to the starting ATG and end STOP codon. Exploratory trees were run using FastTree (Price
156 et al., 2009), after which we used IQ-TREE (Nguyen et al., 2015) to make the final maximum-
157 likelihood tree with 1000 iterations of alrt (approximate likelihood ratio test) for branch support.
158

159 *Molecular clock analysis*

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 are
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 We manually inspected the trimmed alignments of each segment in AliView software (Larsson, 2014),
177 translated the sequence into amino-acids, and checked for amino acid changes that were found
178 across each dataset. We identified several substitutions in the seal virus that did not occur in any of
179 the bird virus sequences. We recorded these substitutions using H3-numbering of the sequence
180 using the HA subtype numbering conversion tool available from FluDB ((Burke and Smith, 2014),
181 https://tinyurl.com/HAnumbering).
182

183 RESULTS

184 *Pathology and immunohistochemistry*

185 The post-mortem revealed abundant purulent fluid (approximately 1 litre) in the subcutaneous
186 tissues of the right side of the body extending from the neck to the thoracic region (**Figure 1A**). All
187 other organs were macroscopically unremarkable. The blubber sternal thickness was 0.4 cm.
188 Histology identified a severe, chronic-active, fibrino-purulent cellulitis and fasciitis and a severe,
189 acute renal infarct with thrombosis. In the respiratory system a diffuse mild to moderate rhinitis with
190 epithelial hyperplasia and presence of mites was observed in the nasal cavity and a focal broncho-
191 interstitial pneumonia with thrombosis and pulmonary nematodes were seen in the lungs, with no
192 changes observed in trachea and bronchi. Influenza A virus antigen was detected by
193 immunohistochemistry only in the nasal mucosa, in the nuclei of scattered isolated epithelial cells
194 (**Figure 1B**). The pathological findings were suggestive of a thromboembolic event and sepsis
195 caused by the cellulitis as the cause of the death of the animal.
196

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

198 The nasal swab was positive for the M-gene (CT value of 22.36), signifying the presence of IAV RNA
199 but was negative for NAI haemagglutinin (HA) subtypes H5 and H7 as well as the IAV subtypes N1
200 and N5. However, IAV subtype N8 was detected by one of the specific RRT-PCR assays employed
201 (CT value of 24.38). In addition, trace amounts of IAV RNA were detected by the M-gene RRT-PCR
202 assay in tracheal bronchi (CT value of 35.88), the left lung (CT value of 37.73) and the right cranial
203 lung (CT value of 38.67). Due to low levels of virus detected in the lower respiratory tract, and the
204 presence of clinical signs not consistent with influenza infection, we concluded that the virus was of
205 no or low pathogenicity. It is possible that at the point the seal was rescued, it had already cleared
206 the infection in the lungs naturally, which would explain trace amounts of detection in these tissues.
207 This is consistent with previous studies which found that grey seals were likely to remain
208 asymptomatic with IAV infection while a proportion of the surveyed adults and juveniles were
209 seropositive (Bodewes et al., 2015a; Puryear et al., 2016).
210
211 Sequencing results from the nasal swab egg-isolate indicated no mixed infection, just a single virus,
212 from which it was possible to sequence all eight gene segments. The virus-derived sequence was
213 named A/grey seal/England/027661/2017 and sequences were deposited in GISAID database
214 (Global Initiative on Sharing All Influenza Data) with isolate ID: EPI_ISL_381748.
215

216 *Source of viral segments*

217 Segment gene sequences which were found to be most closely related to the genes of the seal virus
218 by BLAST were of avian origin. Overall, the strains in the set of BLAST-hits for each segment gene,
219 all came from different avian viruses, isolated from different bird-types, and of different subtypes.
220 They were from strains that were mostly isolated from wild birds such as mallard, Eurasian teal,
221 White-fronted goose, Black-headed gull and others (*Anas platyrhynchos*, *Anas crecca*, *Anser*
222 *albifrons*, *Chroicocephalus ridibundus*), along with a few from domestic birds such as chickens
223 (*Gallus gallus domesticus*). This indicates a wild bird origin for the seal virus, which is consistent with
224 previously detected seal infections (Fereidouni et al., 2016; White, 2013), and the hypothesis

concerning overlapping habitats for wild birds and seals which make transmission from wild birds possible, but less likely from poultry, pigs or humans.

Also similar to previously reported infections (Anthony et al., 2012; Zohari et al., 2014), the set of closest related strains were isolated from within the local region i.e., Northern European (The Netherlands, Germany, UK etc.) with the exception of three strains from China being among the close relatives of the PB1 sequence. The years of detection of the closest-related avian viruses for each segment ranged from the 2007 (NA) to 2017 (NS).

Table S1 shows a summary for how often the same strain appears as a BLAST-hit for each segment of the seal virus. A large proportion of these occur singly (250 strains) or for a maximum of two segments (40 strains). However, some strains were found to map for 3, 4 or 5 segments (19, 2 and 1 strains(s) respectively). Of these, all the strains that mapped for at least 3 segments were isolated from birds in the Netherlands between 2011-15, except for one chicken virus from France in 2016. The subtypes varied greatly, but they were usually isolated from wild birds. In the next section, we further examine the closest available sequence(s) for each seal gene to try and understand the emergence and further propagation of this virus.

Emergence of viral segments

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

253 The trees for each segment are summarised in **Figure 2 (A-G)**; trees for the MP gene segment are
254 excluded due to lack of clock-like behaviour in that dataset. Time to the putative ancestor strain of
255 the segments from the seal virus and its closest related segment is inferred as ranging from 1999
256 (NA) to between 2011 and 2015 (all other gene segments), summarised in **Figure 3** and **Table 1**.
257 Gaps in surveillance, and the availability of just one seal strain will likely affect the inference of
258 TMRCAs, but the variation between segments is also likely a testament to the high levels of
259 reassortment seen in wild bird IAVs (Lu et al., 2014). Indeed, the closest associated virus varies in
260 host, subtype and geography of isolation for each segment, as can be seen in the highlighted clades
261 in **Figure 2 (A-H),** and summarised in **Table 1**. Consistent with data of BLAST hits (table S1), many
262 of the closest related segments isolated prior to February 2017 are from strains isolated in the
263 Netherlands (HA, MP, NP, NS, PA, PB1, PB2). Where multiple strains are equally closely related to
264 the seal virus, e.g. for PB1 and PB2 the avian influenza strains come from the Netherlands and
265 France. The closest strain to the seal NA gene that has been sampled is from 2009 at the latest, from
266 Norway. For the NS gene, the closest segments appear to be from Russian strains in chickens as
267 recent as December 2017, which may indicate onward transmission via unsampled intermediaries;
268 however the posterior probability support value for the node is low (0.36).
269
270 From the above observations, we conclude that most of the seal virus segments are derived from
271 viruses in wild birds in Northern Europe, possibly via unsampled intermediaries – but not in a single
272 transmission event. If grey seals do act as a reservoir for IAV, it is feasible for reassortment of
273 segments to have occurred in seals, as was demonstrated by the introduction of a wild bird NA gene
274 into seals during an H10N7 outbreak in North-western Europe (Bodewes et al., 2016).
275

276 *Substitutions for mammalian adaptation*

277 Previous analyses have indicated that mammalian adaptive mutations can occur in avian viruses
278 when they are transmitted into seals. A study of H10N7 viruses in harbour seals in Northern Europe,
279 which unlike the present case was demonstrated to have transmitted to other seals and caused an
280 outbreak, showed that mutations were likely to occur early on after transmission to seals and then
281 plateau (Bodewes et al., 2016). We looked for differences between the seal virus and wild bird

282 viruses in the segment amino acid alignments of our datasets, to check if they any had putative
283 adaptive implications.
284
285 Several residues in the seal virus that did not occur in the bird viruses. These substitutions are
286 summarised in **Table 2**. Among them, is D701N in the PB2 segment, which is a rare mutation, and a
287 hallmark of mammalian adaptation of bird viruses, regardless of genetic background (Liu et al., 2018;
288 Steel et al., 2009)). These studies have elucidated that the basis of this adaptation is that it allows for
289 better replication in mammalian cells and has been associated with increased transmission in ferret
290 experiments. Another mutation (S678N) in the seal PB1 gene has also been associated with
291 increased polymerase activity and virulence in mice (Gabriel et al., 2005).
292
293 Changes were also found in the HA gene (**Table 2**), but the implications are less clear. At least two of
294 the changes S111G (H3 numbering - 95) and S235Y (H3 numbering 219) could result in a potential
295 loss of glycosylation compared to related wild bird H3s according to prediction by (Hamby and Hirst,
296 2008) (see **Table S2**). The HA of the H3N8 virus isolated from harbour seals in Massachusetts in
297 2011 had an F110S mutation, where the 110 residue has been previously found to be a critical
298 component of the influenza fusion peptide, which may impact replication in mammalian cells
299 (Anthony et al., 2012; Liu et al., 2011). Our reported seal virus retains F at position 110, but whether
300 the mutation in the adjoining residue has any effect on HA fusion properties is unknown.
301
302 We compared all the substitutions with previously described mutations in seal infections, and found
303 that apart from D701N, which was also found in the H3N8 seal virus infection in Massachusetts in
304 2011 (Anthony et al., 2012), there were no identical amino acid changes. In the H3 gene, we found
305 substitutions in residue 81 (reference H3 numbering - 64), and residue 176 (reference H3 numbering
306 - 160), which are also changed, to different amino acids, in the 2011 New England virus (Anthony et
307 al., 2012). The latter mutation was not implicated in receptor binding for the seal viruses as was the
308 case with H5N1 and some human H3N2 viruses, because the glycosylation site is absent in both
309 seal and wild bird viruses (**Table S2**). The presence of Serine at position 66 in the PB1 sequence,
310 which enables production of PB1-F2 (Conenello et al., 2007) was found in the 2011 H3N8 harbour

311 seal virus but was not seen in this 2017 H3N8 grey seal virus. Changes at positions 226 and 228 in
312 HA (H3 numbering) which can change receptor-binding preferences between avian and mammalian
313 hosts (Connor et al., 1994; Matrosovich et al., 2000), were not found in either of the H3N8 seal
314 viruses, but the H10 equivalent of H3-Q226L was identified in viruses from the 2014-15 H10N7
315 outbreak in Europe (Dittrich et al., 2018). We therefore find limited convergence in mutations in the
316 new mammalian host when different starting genetic backgrounds are provided by the different and
317 unrelated pools of avian viruses.
318

319 ## DISCUSSION

320 Marine mammals may be exposed to a plethora of environmental agents, such as chemical
321 pollutants, and existing or (re-)emerging pathogens. Since many marine mammal species share the
322 coastal environment with humans and consume the same food, they also may serve as effective
323 sentinels for public health problems (Bossart, 2011). Marine mammals are particularly interesting
324 hosts for IAVs since they are globally distributed and can migrate over long distances in the vicinity
325 of coastal ecosystems and population centres, where they intersect with waterfowl and shorebirds in
326 scenarios conducive to virus exchange.
327
328 A range of IAV subtypes have been shown to infect marine mammals, including many beyond those
329 that have been able to establish lineages in humans, horses and pigs i.e., H1N1, H1N2, H3N2, H3N8.
330 Among marine mammals, harbour seals (*Phoca vitulina*) appear particularly susceptible to mass
331 mortalities in comparison to grey seals. Periodic infections caused by morbillivirus and influenza
332 virus occur in harbour and Atlantic grey seals, some of which present as unusual mortality events
333 (UMEs), usually in the former (Anthony et al., 2012; Bodewes et al., 2015b; Goldstein et al., 2013;
334 Krog et al., 2015; Zohari et al., 2014). Recent investigations (Bodewes et al., 2015a; Puryear et al.,
335 2016) have provided further evidence that grey seals, being both permissive to and tolerant of
336 diverse IAV, possibly represent an endemically infected wild reservoir population, that is reminiscent
337 of the natural waterfowl reservoir. Puryear et al. found viral prevalence of 9% in healthy live-captured
338 North-west Atlantic grey seal pups, and 5.3% in adults; with seroprevalence being nearly 20% in
339 adults. In the Netherlands, Bodewes et al. found seroprevalence of 26% in healthy adult grey seals.

340 Similarly, various surveillance efforts in wild birds have found seroprevalence ranging from 31% to
341 greater than 60% depending on bird species, geography, seasonality and other factors (Curran et al.,
342 2015; Fereidouni et al., 2010; Wilson et al., 2013).
343
344 The case reported here is epidemiologically interesting as the animal was referred to a rescue centre
345 because it was stranded and the IAV was detected only incidentally, since the clinico-pathological
346 presentation was typical of stranded seal pups and no signs of IAV infection were detected. In
347 addition, we were able to sequence the virus whereas previous attempts at sequencing IAV from
348 grey seals identified during surveillance were unsuccessful due to lack of sufficient material (Puryear
349 et al., 2016). The vast majority of grey seals admitted to rehabilitation centres in the UK are pups
350 within the first year of life. Malnutrition is the single most common reason for pinnipeds to be taken
351 into rehabilitation centres (Barnett et al., 2000; Van Bonn, 2015). Wounds are often recorded and
352 hold clinical significance, as in this case, and have been considered predisposing factors for fatal
353 non-specific septicaemia (Baily, 2014). The cause of trauma and wounds may be anthropic
354 (entanglement in fishing nets or gears) or biologic (conspecific aggressiveness or hierarchical to
355 cannibalistic behaviours). Wounds caused by bites of other seals or predators were most often seen
356 (Barnett et al., 2000; Van Bonn, 2015). Pulmonary and nasal parasites are also very well documented
357 clinico-pathological conditions among rescued seal pups, and likely caused the hyperplastic rhinitis
358 identified in this study seal. No histopathological findings consistent with IAV derived damage were
359 detected along the respiratory tract. The immunohistochemistry demonstrated productive viral
360 replication only in nasal mucosa, but not in lower respiratory segments, although the more sensitive
361 RRT-PCR revealed presence of trace amounts of viral RNA within the lower respiratory tract,
362 suggesting that the infection may have been cleared naturally or indicating a passive translocation of
363 antigen from upper respiratory segments.
364
365 The fact that the IAV did not appear to cause disease might suggest that this virus is adapted to the
366 grey seal, as suggested by reports of seroprevalence. Puryear et al. discuss the possibility that grey
367 seals may be suited to being reservoirs due to gain in population density since the marine mammal
368 protection act in the US in 1972, along with more socially gregarious and aggressive behaviour in

369 comparison to harbour seals, all of which contribute to high pathogen transmissibility. In addition,
370 grey seal population history over a longer time-scale globally might be relevant; similar to harbour
371 and fur seals, they appear to have undergone a postglacial population expansion between 7000 –
372 30000 years ago (Klimova et al., 2014). Phylogenetic analyses have been unable to resolve the
373 relationships between different seal species with sufficient support. Grey seals are either placed as a
374 sister group to Caspian seals (*Pusa Caspica*) and separate from harbour seals, or in a basal position
375 to both *Phoca* and *Pusa* genus (Berta et al., 2018; Fulton and Strobeck, 2006). Therefore, in addition
376 to viral genetics, it might be useful to explore host genetics and immune response differences
377 between grey and harbour seals to understand differences in IAV pathogenicity and transmissibility
378 in the two species.
379
380 A previous case of seal infection by avian IAV led to an outbreak that spread among harbour seals
381 for thousands of miles of European coastline from Sweden and Denmark to Germany and the
382 Netherlands. A spatiotemporal analysis of viral evolution of those seal viruses found that the
383 mammalian adaptation mutations occur rapidly on infection of seals, and that continued contact with
384 bird reservoirs allows for further exchange of viral segments between the two hosts (Bodewes et al.,
385 2016). Since the case of the seal virus in this study appears to be a singular infection as far as we
386 know, it is difficult to know if and how long the virus has been in seals. In our dataset of closest
387 related sequences to the seal virus, we find that while there are several substitutions found in the
388 seal virus which do not occur in the bird viruses, we generally do not find substitutions in any of the
389 bird virus strains that do not occur in other bird viruses too. This likely indicates adaptation to the
390 seal environment, a hypothesis supported by the occurrence of the D701N mutation, a known rare
391 marker of mammalian adaptation (Liu et al., 2018; Steel et al., 2009). D701N is common in canine
392 and horse H3N8 IAV (see **Table S3**), but does not occur in birds, and was found associated with
393 highly-pathogenic H5N1 viruses which infected humans (Gabriel et al., 2005; Jong et al., 2006; Li et
394 al., 2005). We found one reported instance of the D701N mutation in the other avian H3N8 virus that
395 infected pinnipeds, but not in the H10N7 outbreak, despite sustained transmission in seals for
396 several months, nor in any other previously sequenced seal PB2. H3N8 IAV are noted for the ability
397 cross species barriers, so it would be relevant to consider if the subtype of the virus has any

398 influence on the kind of adaptive mutations that occur in the polymerase genes, and if so what sort
399 of mechanisms this might involve.
400
401 Successful or not, we do see evidence for putative adaptive substitutions in the viral sequence. This
402 could mean the virus has been circulating in seals for a certain amount of time during which these
403 substitutions have accumulated. Alternatively, this event might represent an early rapidly-adapting
404 virus from a spill-over event as seen in the H10N7 outbreak (Bodewes et al., 2016). Given the
405 subclinical nature of the infection, we propose the former explanation is more likely to be correct.
406 The 2011 North American H3N8 and 2014 European H10N7 viruses which caused outbreaks in
407 harbour seals were found to have acquired mutations to enable recognition of sialyloligosaccharide
408 receptors found more abundantly in mammalian tissues (SAα2,6Gal) but which retained the ability to
409 interact with avian receptors (SAα2,3Gal). A later detailed structural and functional analysis of the
410 2011 H3N8 seal HA indicated a true avian receptor binding preference (Yang et al., 2015), as did a
411 mutational analysis of the H10N7 viruses (Dittrich et al., 2018). Although there are some common
412 HA residues that are changed in both the seal virus reported here and the 2011 H3N8 virus, we
413 found limited convergence in the substitutions and residues involved between the different seal
414 viruses, likely attributable to the different genetic backgrounds from different avian sources and
415 possibly differences in host environment of reservoir vs susceptible species. In addition, to the best
416 of our knowledge, ours is the first grey seal virus sequence that is being made publicly available (via
417 GISAID). It is therefore uncertain whether the type of mutations occurring in a putative reservoir host
418 vis-à-vis avian sequence might be different from those occurring in related hosts with pathogenic
419 outcomes.
420
421 Our phylogeny and molecular clock analyses suggest different lineages of source viruses, and time
422 of introduction of different segments, but it is not possible to be precise on how long the viral gene
423 segments have been in the seal population because there is only a single data point. It is not
424 surprising to find that the closest sampled and sequenced viruses for each segment are of different
425 subtypes and hosts and lineages, given that in birds, there is extensive influenza reassortment (Lu et
426 al., 2014) i.e., swapping of individual segments into different progeny viruses during propagation

427 after mixed infection with two or more viruses. It would be speculative to propose likely wild bird
428 species donor however. Studies on avian influenza have previously shown that the transmission
429 between birds is directional, e.g., usually in the direction from anseriformes such as ducks into
430 galliformes (chicken) or charadriiformes (gulls) (Venkatesh et al., 2018). In the case of the current
431 infection as well, where we have found unambiguously closest related sequences, they have tended
432 to have been isolated from anseriformes species. The above findings, along with the observation that
433 the closest related viruses were isolated largely from the Netherlands, it is likely that the source of
434 the viral segments come from unsampled locally circulating (in Northern Europe) avian viruses from
435 an anseriformes host.
436
437 H3N8 viruses currently circulate in horses and dogs but not humans or pigs. However, different H3
438 viruses have been found in several species including humans, pigs, horses, dogs, cats, seals,
439 poultry, and wild aquatic birds. They have thus been noted for a particular ability to cross species
440 barrier and cause productive infections. One study that examined the ability of H3N8 viruses from
441 canine, equine, avian, and seal origin to productively infect pigs, demonstrated that avian and seal
442 viruses replicated substantially and caused detectable lesions in inoculated pigs without prior
443 adaptation (Solórzano et al., 2015). It is possible that the ready occurrence of PB2 701N mutation in
444 H3N8 viruses contributes to this ability. We do not have any biological evidence for pre-20th century
445 human influenza, but historical analysis suggests a long association of humans with influenza
446 (Hirsch, 1883; Taubenberger and Morens, 2010), and has uncovered temporal–geographic
447 associations between equine and human influenza-like disease activity documented in Europe in the
448 16th-18th centuries (Morens and Taubenberger, 2010). Such analysis has also implicated an H3N8
449 virus in the 1889 pandemic in humans (Morens and Taubenberger, 2010), which makes mammalian-
450 adapted H3N8 viruses of particular interest as IAV pandemic risk candidates.
451
452 In this paper we have presented analyses of a case of seal infection with IAV in coastal England. This
453 infection provides a small but unique window to understand the ecology of avian-origin viruses that
454 may be circulating and maintained in mammals. Given the mammalian adaptation activity upon
455 infection found in our study and those of others, these infections may be of interest to pandemic

456 surveillance and risk despite the limited interface between humans and seals (compared to swine).

457 Given the historical context, it is also worth exploring H3N8 infections of mammals such as seals, as

458 they may help us better understand the determinants of mammalian adaptation in influenza and its

459 complex drivers.

460

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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).

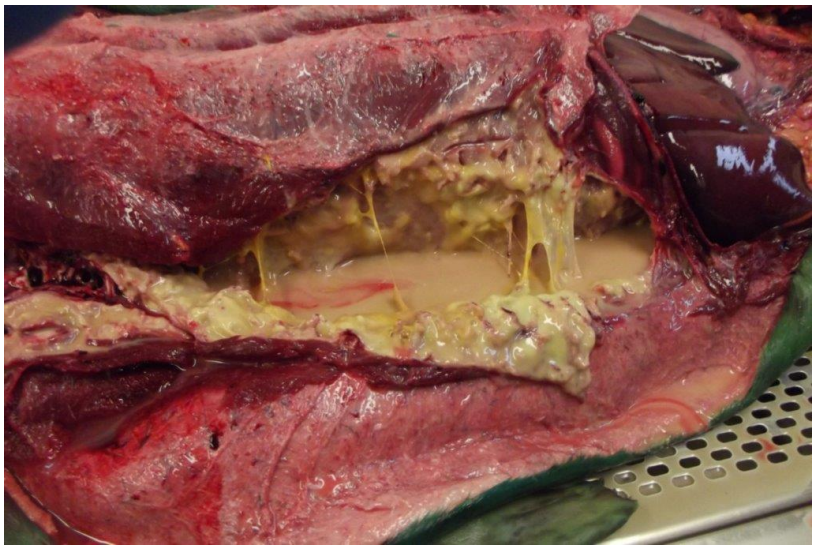

Figure 1A

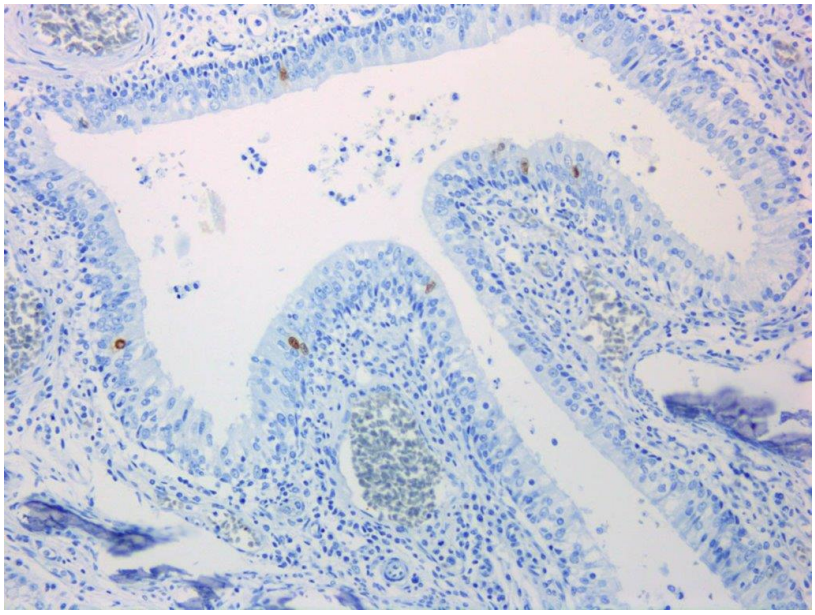

Figure 1B

Figure 2 (A-G)
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 2A

seal – HA

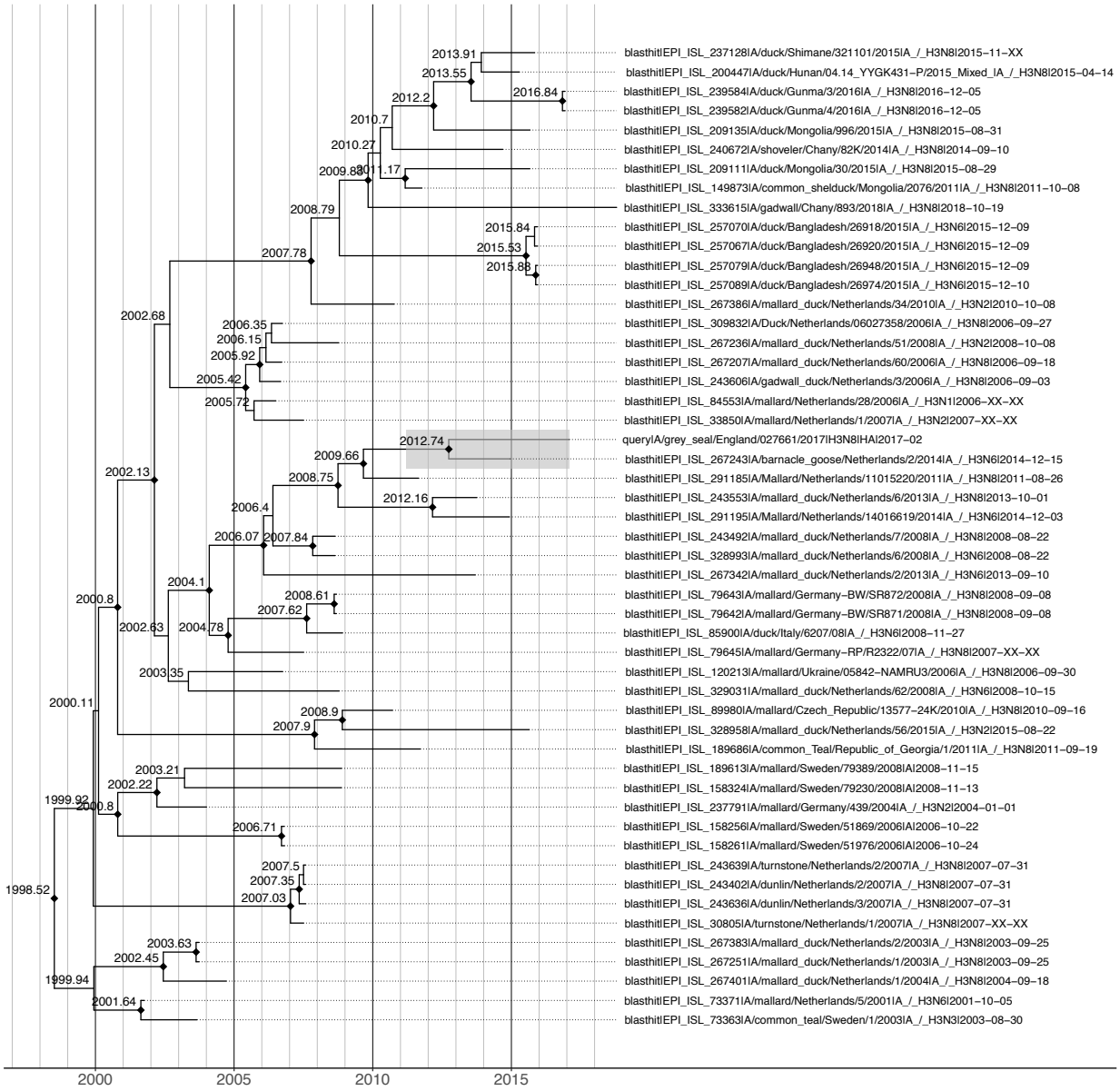

Figure 2B

seal – NA

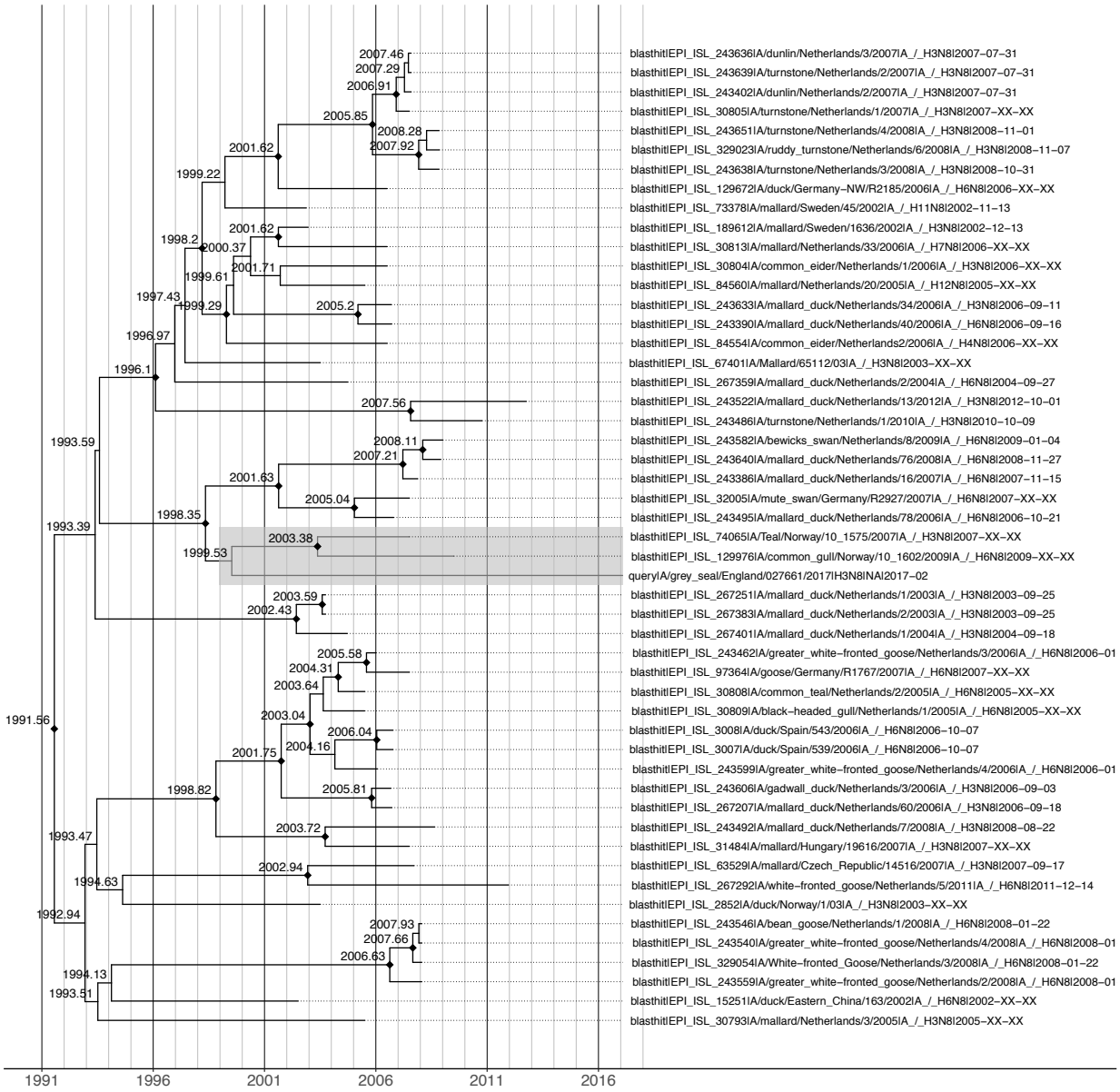

Figure 2C

seal – NP

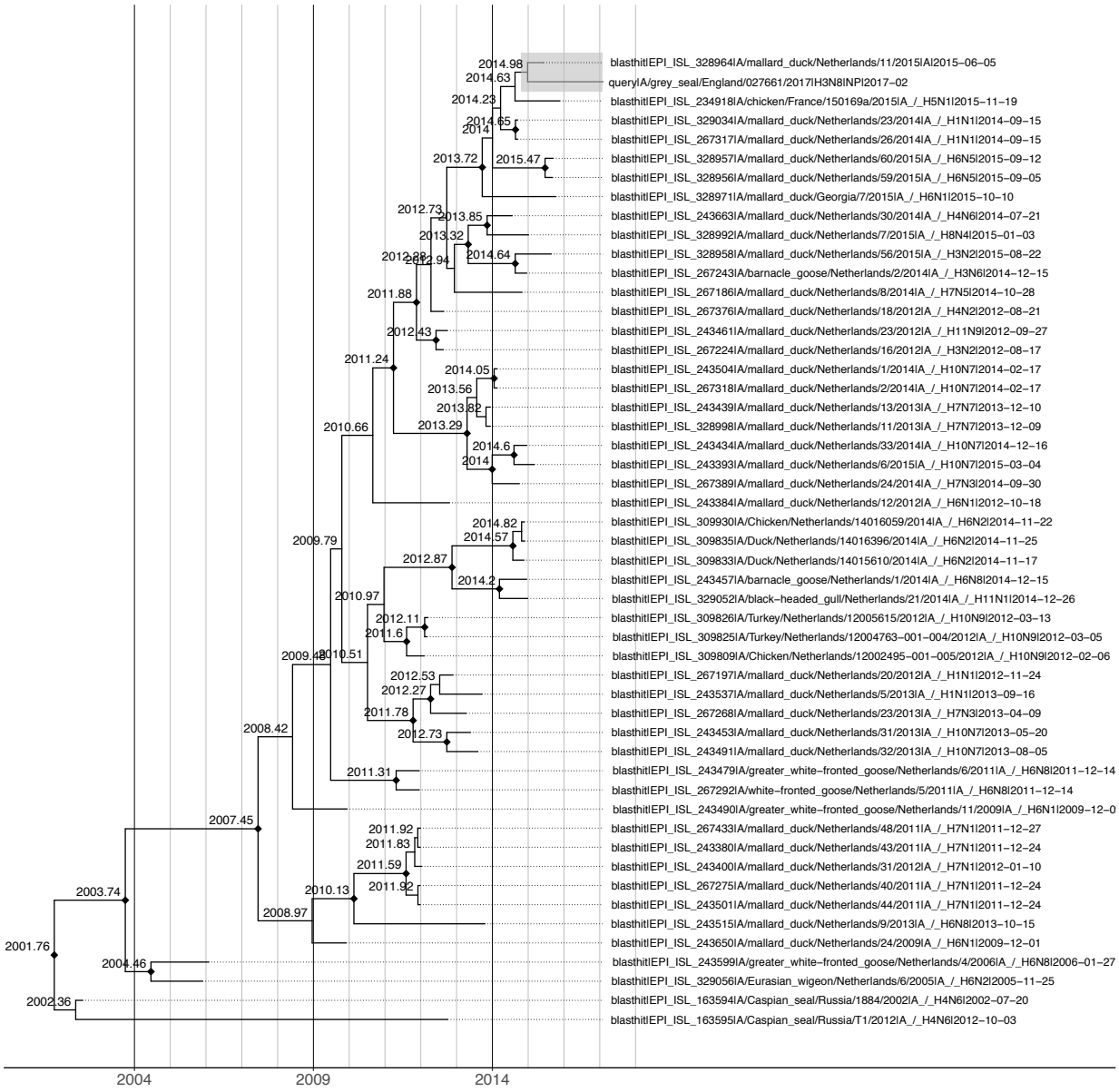

Figure 2D

seal – NS

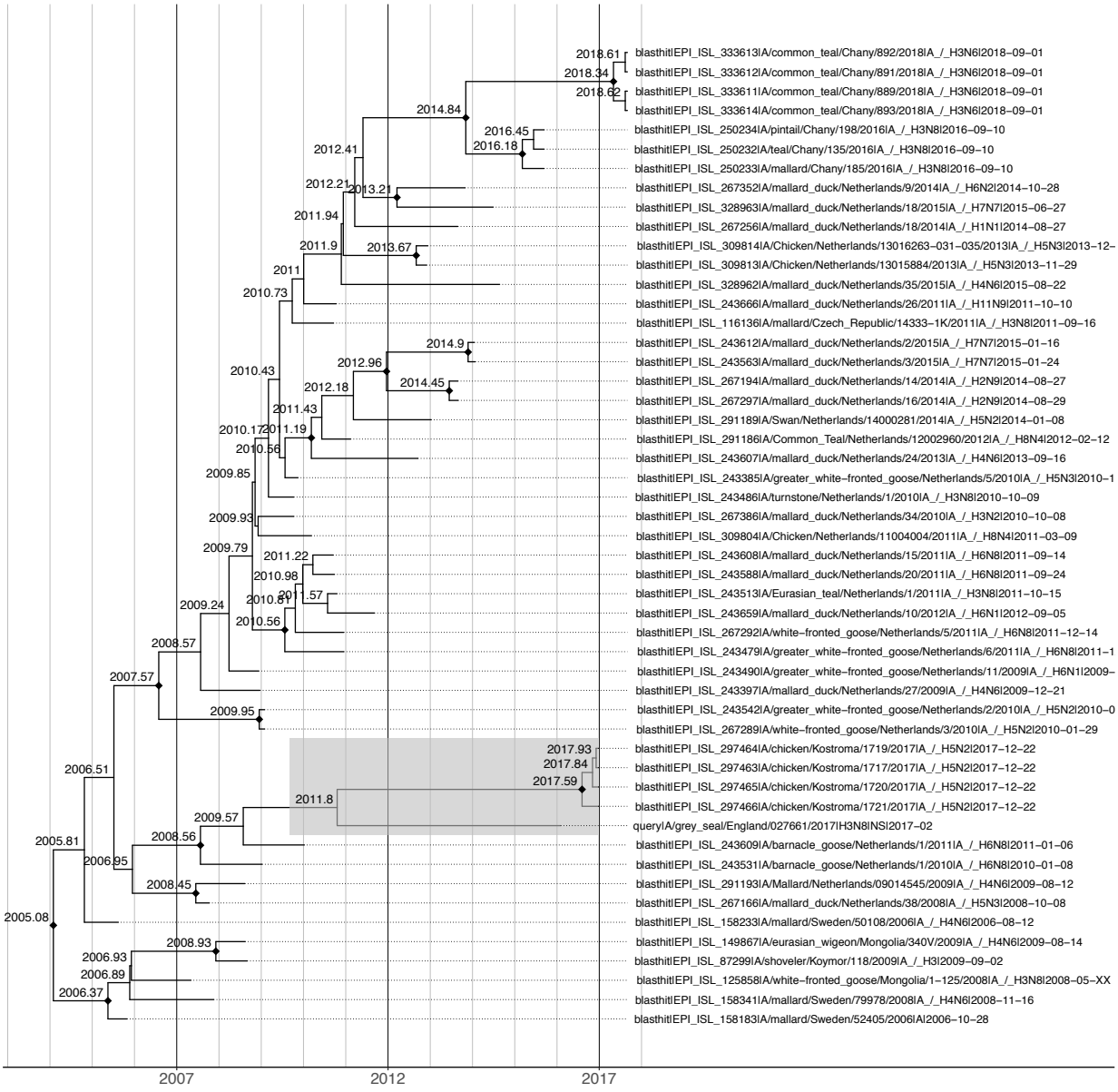

Figure 2E

seal – PA

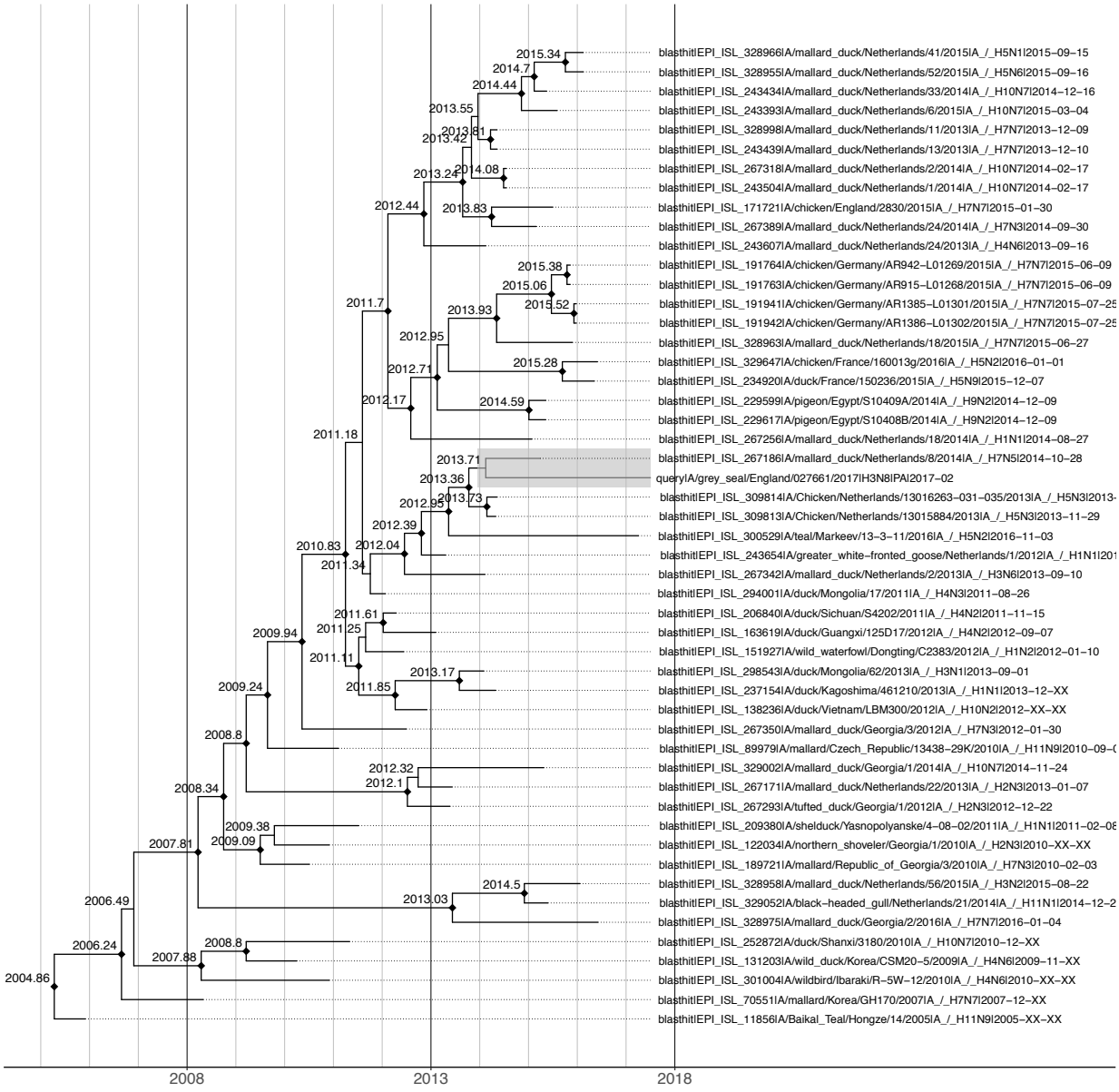

Figure 2F

seal – PB1

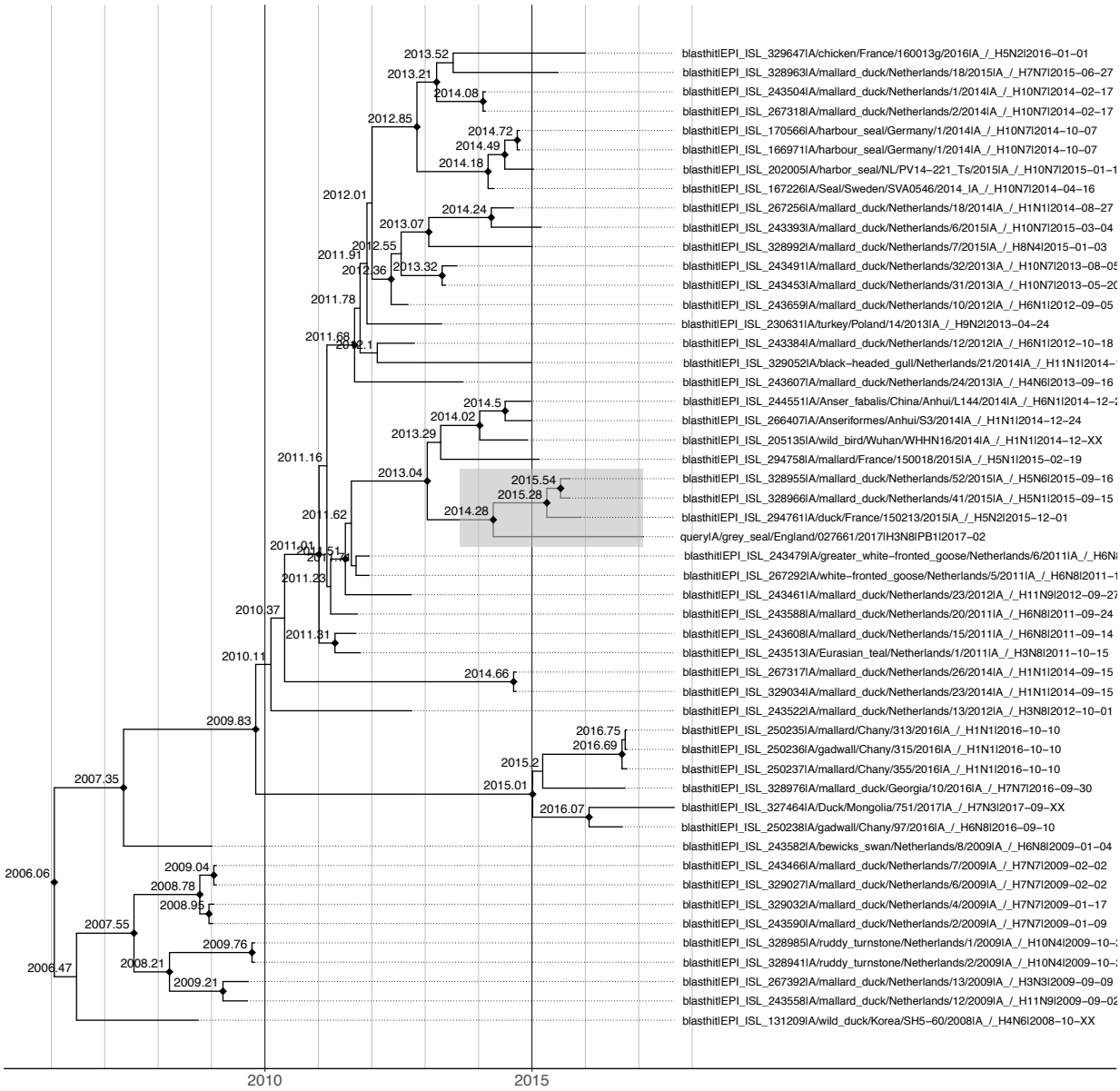

Figure 2G

seal – PB2

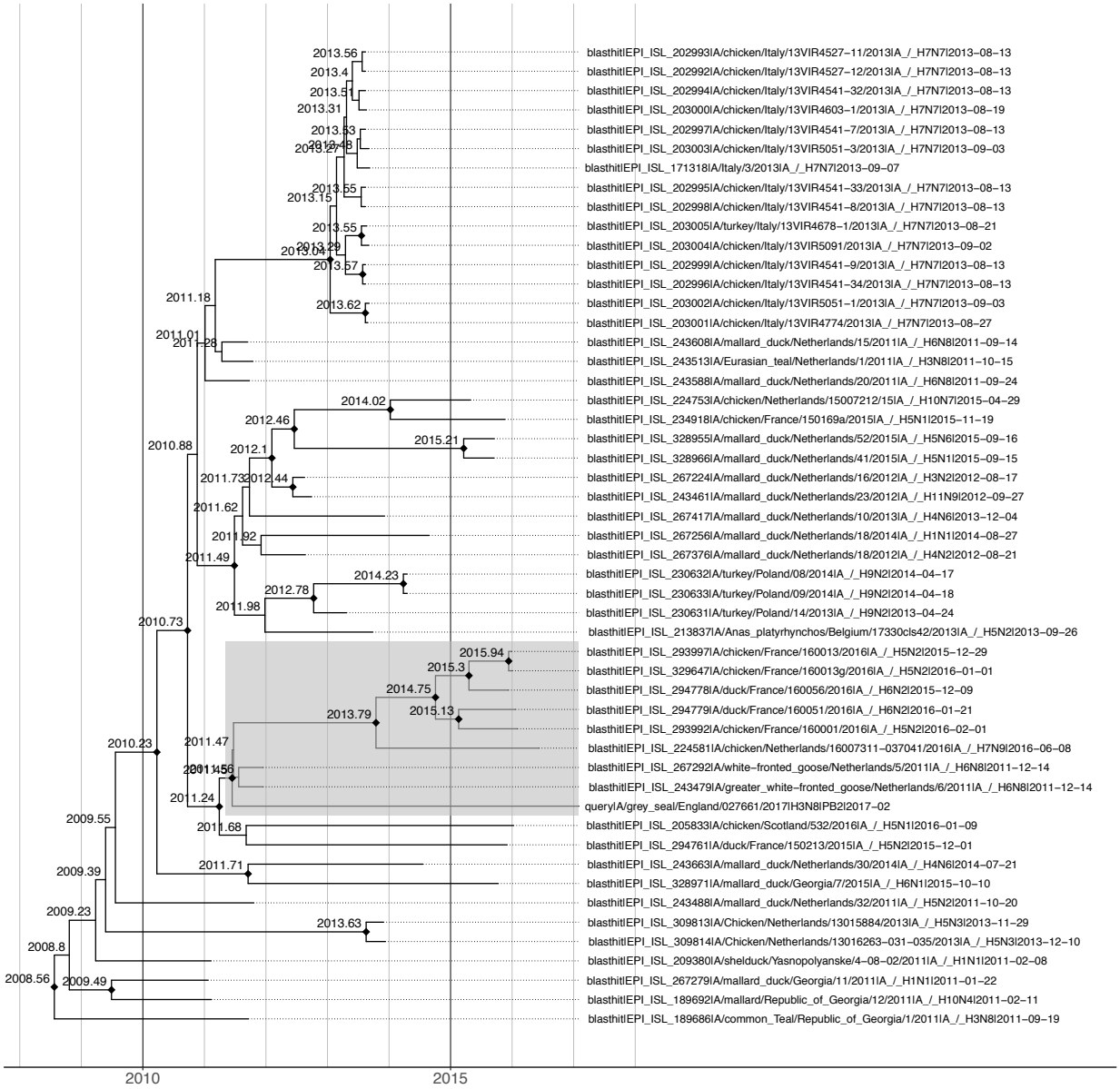

Figure 3
Putative divergence times (TMRCA – time to most recent common ancestor) of the seal sequence from the closest related wild bird sequence for each segment. Error bars indicate 95% highest posterior density.

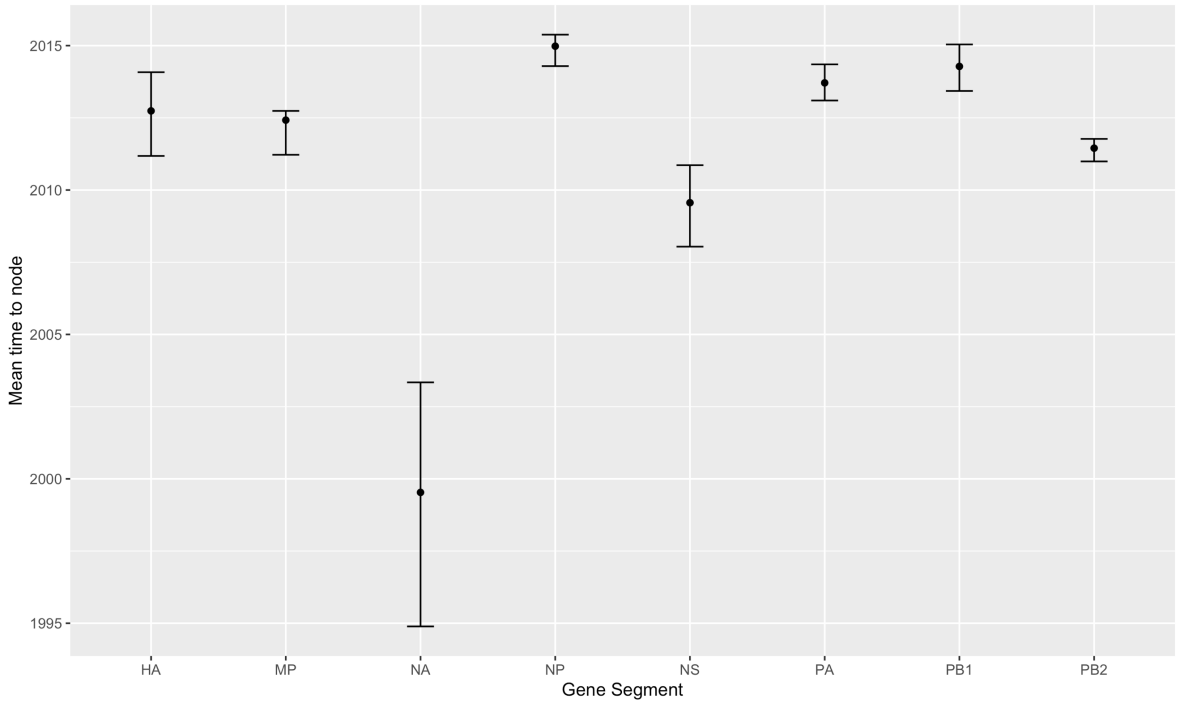

Table 1
Putative divergence times (TMRCA – time to most recent common ancestor) and 95% highest posterior density (HPD) of the seal sequence from the closest related wild bird sequence for each segment, and the posterior probability of the node are shown, along with information about the closest related 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)