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Phylogenomic insights to the origin and spread of phocine distemper virus in European harbour seals in 1988 and 2002

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ABSTRACT: The 1988 and 2002 phocine distemper virus (PDV) outbreaks in European harbour seals *Phoca vitulina* are among the largest mass mortality events recorded in marine mammals. Despite its large impact on harbour seal population numbers, and 3 decades of studies, many questions regarding the spread and temporal origin of PDV remain unanswered. Here, we sequenced and analysed 7123 bp of the PDV genome, including the coding and non-coding regions of the entire P, M, F and H genes in tissues from 44 harbour seals to shed new light on the origin and spread of PDV in 1988 and 2002. The phylogenetic analyses trace the origin of the PDV strain causing the 1988 outbreak to between June 1987 and April 1988, while the origin of the strain causing the 2002 outbreak can be traced back to between July 2001 and April 2002. The analyses further point to several independent introductions of PDV in 1988, possibly linked to a southward mass immigration of harp seals in the winter and spring of 1987–1988. The vector for the 2002 outbreak is unknown, but the epidemiological analyses suggest the subsequent spread of PDV from the epicentre in the Kattegat, Denmark, to haul-out sites in the North Sea through several independent introductions.

KEY WORDS: Morbillivirus · Epidemic · Viral phylogeny · Virus evolution · Wildlife pathogen

1. INTRODUCTION

Over the past few decades, several epidemics have been recorded in marine mammal populations around the globe (Duignan et al. 2014, Jo et al. 2018). The pathogens responsible for these epidemics have mainly been identified as influenza A (Krog et al. 2015) or viruses belonging to the genus *Morbillivirus*, such as various cetacean morbilliviruses (Kennedy et al. 1988, Domingo et al. 1990, Aguilar & Raga 1993, Lipscomb et al. 1996, Birkun et al. 1999), canine distemper virus (Mamaev et al. 1995) and phocine distemper virus (PDV) (Osterhaus & Vedder 1988, Dietz et al. 1989b, Curran et al. 1990, Härkönen et al. 2006). The genus *Morbillivirus* is a group of highly infectious viruses from the family *Paramyxoviridae*. The genus also includes the measles virus (MeV) and the now eradicated rinderpest virus that have caused

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severe disease outbreaks among humans and cattle, respectively, resulting in suffering and economic losses in affected populations (Furuse et al. 2010, Roeder et al. 2013). Morbillivirus infection occurs through the respiratory route where the virus replicates in macrophages and dendritic cells (Rijks et al. 2012). Members of the genus have frequently caused epidemic disease in previously unexposed populations, and during the past century, interspecies infections have been reported on several occasions (Mamaev et al. 1995, Roelke-Parker et al. 1996, Goldstein et al. 2009). Thus, new zoonotic diseases could potentially emerge from wildlife reservoirs of morbilliviruses.

The PDV epidemics in 1988 and 2002 are among the largest mass mortality events ever recorded in marine mammals, resulting in the death of over 23 000 harbour seals *Phoca vitulina* in 1988 and approximately 30 000 harbour seals in 2002 (Dietz et al. 1989b, Harding et al. 2002, Härkönen et al. 2006). The 2 outbreaks share similarities with respect to the timing, duration and location of the first cases, occurring early in spring on the island of Anholt in the central Kattegat, Denmark. From there, the virus seems to have spread to the adjacent harbour seal colonies and subsequently to distant colonies across Northern Europe via large jumps (Härkönen et al. 2006). The sudden appearance and spread of the previously undescribed morbillivirus among European harbour seals in 1988 immediately sparked investigations into the origin of the virus and mechanisms of its transmission and spread. Antibodies against morbillivirus have subsequently been found in samples collected from harp seals *Pagophilus groenlandicus* and ringed seals *Pusa hispida* from Greenland and Svalbard waters prior to the European outbreak in 1988, suggesting that the virus is circulating among Arctic seals (Dietz et al. 1989a). In contrast, the virus seems not to be circulating among European harbour seals. PDV antibodies were not detected in harbour seals in these waters prior to the 1988 outbreak, and the proportion of the European population carrying antibodies declined steadily after each outbreak, dropping from more than 50 % in 2003 to less than 10 % in 2007 (Pomeroy et al. 2005, Bodewes et al. 2013, Ludes-Wehrmeister et al. 2016). It has been hypothesised that the unusual mass migration of harp seals in the winter and spring 1987/1988 led to the introduction of the virus to the European harbour seals in 1988 (Haug & Nilssen 1995, Nilssen et al. 1998), but the exact timing of these events has never been investigated, and a vector for the 2002 epidemic has not been proposed. Moreover, while the long-distance transmission events of PDV among harbour seal colonies was initially suggested to be aided by the less susceptible and more mobile grey seal *Halichoerus grypus* (McConnell et al. 1999, Härkönen et al. 2006), more recent tagging data indicate that while harbour seals are, in general, philopatric, individual seals occasionally undertake long-distance movements (Tougaard et al. 2008, Reijnders et al. 2010, Dietz et al. 2013, Aarts et al. 2016). Therefore, dispersing harbour seals could have served as a vector for spreading the virus across Europe.

Thus, despite nearly 3 decades of research on PDV, a number of fundamental questions remain to be addressed regarding the origin and evolution of PDV, the diversity and relationship of circulating PDV strains, the source and spread of PDV and the mechanisms of virus transmission. Here, we sequenced a continuous 7123 bp sequence of the PDV genome, including the complete coding and non-coding regions of the viral P, M, F and H genes directly from harbour seal samples collected throughout the geographic and temporal progression of the 1988 and 2002 outbreaks to provide the first detailed epidemiological assessment of the emergence and spread of PDV among harbour seals in Northern Europe.

2. MATERIALS AND METHODS

2.1. Sampling

Tissue samples from harbour seals were collected at haul-out sites along the coasts of Denmark, Sweden, Germany, the Netherlands, England and Scotland during the 1988 and 2002 outbreaks (Table S1 in the Supplement at www.int-res.com/articles/suppl/d133p047_supp.pdf). Unfortunately, high-quality samples (e.g. fresh lung tissue stored in RNAlater at −80°C) were only available for animals that died in Sweden during the 2002 outbreak. Thus, we tested 32 liver and muscle tissue samples collected at locations in Denmark and Germany in 1988, and 67 spleen, lung and muscle tissue samples from 2002, covering haul-out sites in most of Northern Europe. To increase the likelihood of detecting and sequencing the PDV, preference was given to samples collected from carcasses that were fresh at the time of collection. Furthermore, we attempted to increase the phylogenomic inference by selecting samples representing as broad a geographical and temporal range as possible, prioritizing samples from early, mid- and late stages of each outbreak.

2.2. Virus screening and sequencing

Viral RNA was extracted from spleen, lung, liver or muscle tissue using the Thermo Scientific King-Fisher™ Cell and Tissue DNA Kit. The extractions were first tested for the presence of viral RNA by reverse-transcription PCR (RT-PCR) runs with the H5 (5′-AGA TGA TAT CTT TCC TCC-3′) and H6 (5′-ATC CAT ATG AGT TGC TCC-3′) primers. Different sequencing methods were attempted to generate full genomes, but ultimately only primer walking followed by standard Sanger sequencing of 4 genes was possible. Primer pairs designed for sequencing the PDV genome (de Vries et al. 2013) were used to amplify and sequence 13 separate, but overlapping segments of 7123 bp of the PDV genome, covering the complete sequence from position 1744 to 8915 (excluding stop codons), including the phosphoprotein (P), the unglycosylated matrix protein (M), the fusion protein (F) and the attachment protein haemagglutinin (H) (Table S2, Fig. S1). RT-PCRs were performed with the Qiagen OneStep RT-PCR kit with cycling conditions of 30 min at 50°C, 10 min at 95°C, 40–38 cycles of 1 min at 95°C, 1 min at 55–65°C and 1 min at 72°C (Table S3) and a final step of elongation for 10 min at 72°C followed by 4°C. The total volume for each reaction was 25 µl and consisted of 5 µl Qiagen OneStep RT-PCR Buffer, 1 µl dNTP (10 mM), 1.5 µl of each primer (10 µM), 1 µl Qiagen OneStep RT-PCR Enzyme Mix, 12.5 µl H_2O and 2.5 µl RNA template. PCR products were purified and sequenced by Sanger sequencing at Macrogen Europe (Amsterdam, The Netherlands).

2.3. Sequence preparation

The raw sequences were assembled and examined for errors in Geneious version 9.1.2 (Kearse et al. 2012) (Table S4). All ambiguous base calls and singletons (i.e. mutations only occurring in a single sample) were checked by re-sequencing. Low signal regions at the beginning and end of each sequence were removed, and 2 bases (TT) were deleted before the M gene (position 1651–1652) and the H gene (position 5302–5303) to place the coding regions in the correct reading frame. The resulting sequences were mapped to the PDV reference genome from 1988 (NCBI GenBank accession no. KC802221; de Vries et al. 2013) using MUSCLE (Edgar 2004) to obtain a continuous 7123 bp sequence, spanning the coding and non-coding regions of the viral P, M, F and H genes. Three different alignments were generated: one including sequences from the 1988 outbreak (n = 8 sequences), one including the 2002 outbreak sequences (n = 36) and one of all sequences combined from the 2 outbreaks (n = 44).

2.4. Substitution saturation and recombination analyses

The full data set was tested for the absence of substitution saturation using the entropy-based index in DAMBE (Xia et al. 2003, Xia 2013) (Table S5). The results showed no indication of substitution saturation among the sequences. Likewise, no evidence of recombination was found using GARD recombination analysis under the HKY85 nucleotide evolution model implemented in Datamonkey (www.datamonkey.org) (Hasegawa et al. 1985, Kosakovsky Pond et al. 2006, Delport et al. 2010).

2.5. Phylogenetic analyses

Phylogenetic trees and divergence times were estimated by using the Bayesian Markov chain Monte Carlo (MCMC) method in the BEAST 2.4.6 software package (Bouckaert et al. 2014). Sequence alignments were partitioned into coding regions, first, second and third codon positions and non-coding intergenic regions to account for the expected variation in the substitution rates among in this region of the sequences (Liò & Goldman 1998). The data were analysed using the Hasegawa-Kishino-Yano (HKY) substitution model (Hasegawa et al. 1985) determined as the best fit model based on the final weight of 3 criteria, i.e. Akaike's information criterion (Akaike 1973), the Bayesian information criterion (Akaike 1973) and decision theory (DT) (Minin et al. 2003), estimated in JModeltest (Posada 2008) (Table S6). The evolution of the partitions was described by the gamma site model, thereby allowing the substitution rates of each site to correspond to a continuous distribution of different rates among sites (Haug 1990, Nilssen et al. 1998). Runs were carried out under the assumption of a coalescent constant population growth model for the dataset, and a coalescent exponential population growth model for the separate 1988 and 2002 datasets, respectively, using a relaxed uncorrelated lognormal molecular clock in all analyses (Drummond et al. 2006) (Tables S7 & S8). The tree was calibrated by the inclusion of sampling dates (tip dates), specified in years. Four independent runs were conducted with a chain length of

20 000 000 iterations, with sampling of the tree parameters for every 2000th iteration and a burn-in of 10%. The results of each individual run were visually inspected in Tracer v1.6.0 for convergence and to check that the effective sample size values were >200. Subsequently, the files produced from independent runs of the MCMC yielding similar results were merged into 1 tree and log file using LogCombiner v2.4.6 (Bouckaert et al. 2014) with 50% sampling frequency and a burn-in of 10%. The tree files were summarized in maximum clade credibility trees in TreeAnnotator and viewed in Figtree v1.4.3 (Rambaut 2016). The time to the most recent common ancestor of the combined 1988 and 2002 dataset was estimated as a mean value with its 95% high-probability density (HPD) interval. To account for uncertainties in branch lengths and topology, the final tree was constructed using Densitree (Bouckaert & Heled 2014).

3. RESULTS AND DISCUSSION

3.1. Sequencing PDV from the 1988 and 2002 outbreaks

A total of 99 harbour seal samples were screened from the outbreaks in 1988 (n = 32) and 2002 (n = 67) from which 7123 bp of the PDV genome (position 1744–8915, excluding stop codons) were successfully amplified and sequenced from 44 samples: 8 from 1988 and 36 from 2002 (Fig. 1; Tables S1 & S4 in the Supplement). In total, 112 polymorphic sites were found in the combined 1988 and 2002 dataset; 17 polymorphic sites (including 11 singletons) occurred in the sequences from the 1988 samples, and 57 polymorphic sites (42 singletons) occurred in the sequences from the 2002 samples (Tables S9 & S10). The amplification and sequencing success was higher for samples from 2002 than 1988, and within the 2002 samples, success was higher for samples that had been stored in RNAlater at −80°C compared to those stored in ethanol or frozen dry at −20°C. Nevertheless, our results illustrate that PDV can be extracted and sequenced from infected seals even after 3 decades of suboptimal sample storage.

3.2. Winter origin of the 1988 and 2002 PDV strains

The origin of the virus sequenced from the 1988 epidemic was estimated to be mid-November 1987, with a 95% probability interval ranging from June 1987 to early April 1988 (Table 1). This estimate correlates well with the timing of observed mass migrations of harp seals from the Barents Sea south along the coasts of Norway and into the North Sea in the winter of 1987–1988 (Haug & Nilssen 1995, Nilssen et al. 1998). The migration event is widely believed to have introduced PDV to the previously unexposed European harbour seals (Dietz et al. 1989a,b, Härkönen et al. 2006) and was presumably caused by the collapse of the Barents Sea capelin *Mallotus villosus* and herring *Clupea harengus* stocks (Haug & Nilssen 1995, Nilssen et al. 1998). Although the morbillivirus itself has not been detected in Arctic seals (Kreutzer et al. 2008), morbillivirus-specific antibodies have been found in Greenlandic harp seals and ringed seals sampled between 1984 and 1987 (Dietz et al. 1989a), as well as in Barents Sea harp seal samples collected between 1987 and 1989 (Markussen & Have 1992). Markussen & Have (1992) documented that the percentage of harp seals with antibodies increased from 1987 to 1989, indicating that a large proportion of the Barents Sea seals carried a morbillivirus before and during the European epidemic in 1988. Harp seals are migratory, and even discrete populations have overlapping ranges during part of the year (Øien & Øritsland 1995, Folkow et al. 2004), supporting the possibility of periodical encounters and intra- and interspecific disease transmissions across the Arctic.

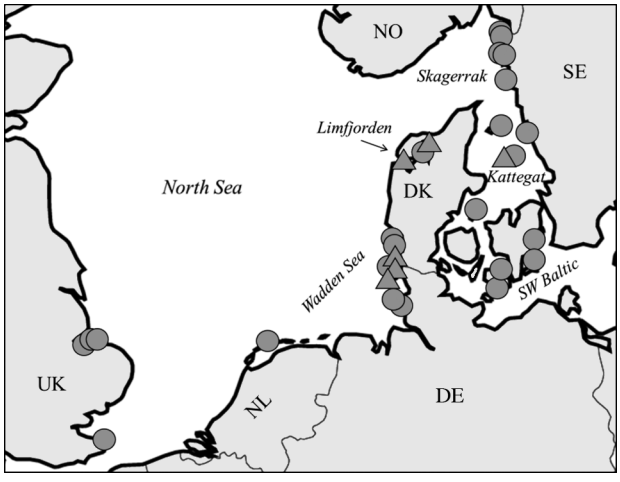

Fig. 1. Spatial and temporal origin of the 44 harbour seals and phocine distemper virus (PDV) sequences used in the phylogenetic analysis. Triangles: samples from 1988 collected in the Kattegat (n = 2), Limfjorden (n = 2) and German Wadden Sea (n = 4). Circles: samples from 2002 collected in the Kattegat (n = 11), Skagerrak (n = 6), Baltic (n = 3), Limfjorden (n = 3), Danish Wadden Sea (n = 4), German Wadden Sea (n = 4), Dutch Wadden Sea (n = 1) and the southeastern coast of England (n = 5)

Table 1. Summary statistics for the Bayesian phylogenetic analyses of the sequences from the phocine distemper virus (PDV) outbreaks in European harbour seal populations. The high-probability-density (HPD) intervals indicate the probability interval for each mean value. SEM: standard error of the mean. TMRCA: time to the most recent common ancestor. Dates given as d/mo/yr

Data set	Rate of evolution (substitutions site^{-1} yr^{-1})	Tree height	TMRCA date	
			1988	2002
	Mean [95 % HPD] SEM	Mean [95% HPD] SEM	Mean [95% HPD] SEM	Mean [95% HPD] SEM
PDV_{all}	6.73×10^{-4} [5.04×10^{-4}, 8.4×10^{-4}] 2.42×10^{-6}	15.54 = 15 yr, 198 d [14.62, 17.02] 0.01	25/11/1987 [10/06/1987, 03/04/1988]	07/01/2002 [28/07/2001, 30/04/2002]
PDV_{1988}	2.28×10^{-3} [4.54×10^{-4}, 4.22×10^{-3}] 1.55×10^{-5}	0.38 = 137 d [0.19, 0.73] 3.17×10^{-3}	–	–
PDV_{2002}	1.58×10^{-3} [1.03×10^{-3}, 2.18×10^{-3}] 5.49×10^{-6}	0.56 = 203 d [0.46, 0.68] 1.18×10^{-3}	–	–

The origin of the virus sequenced from the 2002 epidemic was estimated to be January 2002, with a 95% probability interval ranging from late July 2001 to late April 2002 (Table 1). Thus, like the 1988 outbreak, the 2002 PDV strain can be traced back to the preceding winter. However, unlike the 1988 outbreak, there was no obvious candidate for the introduction of PDV in 2002. The phylogenetic analyses presented here strongly suggest that the 2002 strain did not originate directly from the 1988 strain (Fig. 2B). This is also supported by previous analyses of shorter PDV sequence fragments (Duignan et al. 2009, Earle et al. 2011), as well as an absence of virus in European harbour seals screened between the 2 outbreaks (Bodewes et al. 2013, Ludes-Wehrmeister et al. 2016). PDV-neutralizing antibodies have been detected in harp seals, ringed seals, hooded seals *Cystophora cristata* and polar bears *Ursus maritimus* sampled between 1988 and 1996 in the waters surrounding Greenland, the Canadian Arctic and North America (Duignan et al. 1997, Cattet et al. 2004). PDV-positive harbour seals, grey seals and harp seals were also detected along the North Atlantic coast of North America between 2004 and 2007 (Siembieda et al. 2017). Thus, there are several potential candidate species that could have been reservoir hosts or carriers. Similar to 1988, the Barents Sea capelin and herring stock experienced declines in the years just before the 2002 epidemic (Loeng & Drinkwater 2007); however, this collapse did not result in a new mass migration of harp seals as occurred in 1987–1998. Nevertheless, the virus could have been carried and transmitted by single migrating individuals, as harp seals tagged in Canada were observed to visit the North Sea between 2001 and 2002 (Kreutzer et al. 2008).

3.3. Multiple introductions of PDV in 1988

In addition to supporting the hypothesised introduction of PDV by harp seals in the winter of 1987/1988, the phylogenetic analyses of the 1988 data provide new insight to the emergence and subsequent pattern of spread among harbour seals. In the beginning of March 1988, the viral strains split into 2 clades: one containing sequences from the central Kattegat and a second clade containing sequences from the Wadden Sea and the Limfjord. The analysis of the 1988 data in isolation, as well as the combined 1988 and 2002 data, places a sample (no. 42524) collected in the Limfjord on 22 July 1988 at the base of the tree (Fig. S2). Interestingly, while the viral sequence from sample 42524 contains mutations that are unique to the 1988 outbreak, it diverges from the rest of the 1988 sequences by 6 mutations (Table S9). The presence of these mutations was confirmed by forward and reverse sequencing and Phred scores of >30, and they do not appear to be artefacts caused by DNA damage and deamination, as only 3 of the 6 substitutions are C-T or G-A. Moreover, another sample from the Limfjord (42523), which was omitted from the full phylogenetic analyses because full sequences could only be obtained for positions 3910–4535, 5027–6204 and 6715–8916, also contained 4

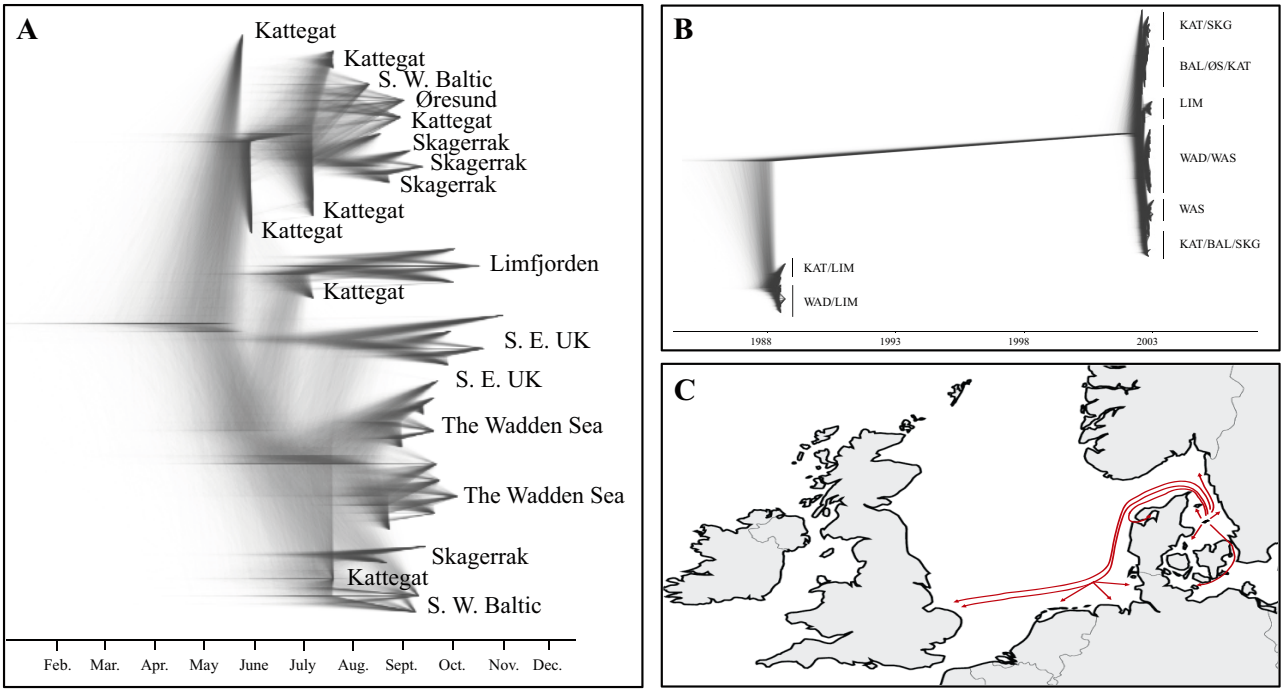

Fig. 2. Phylogenetic relationship and hypothesised spread of phocine distemper virus (PDV) among European harbour seals. (A) Bayesian phylogenetic analysis of 36 PDV sequences obtained from harbour seals sampled during the 2002 outbreak and sequenced in 7123 bp (position 1744–8915) of the viral P, M, F and H genes. The analysis provides strong support for the Kattegat as the origin of the outbreak, with subsequent independent pulses of spreading to harbour seal localities throughout Northern Europe. Uncertainties in the tree topology are visualized as a smear around the tree nodes and branches. (B) Phylogenetic analysis of the combined data set of 44 samples from 1988 and 2002 supporting the independent origin of the strain leading to the 2002 outbreak. (C) A novel hypothesis for the spread of PDV during 2002 from the Kattegat epicentre to other European harbour seal localities through a series of independent transmission events (red arrows)

of the 6 unique mutations found in sample 42524. The basal phylogenetic placement of the Limfjord sequence indicates that the 1988 outbreak among European harbour seals consisted of not just a single, but several, introductions of related PDV strains. The observed rise in the number of harbour seal deaths along the Danish west coast by February 1988 (Danielsen et al. 1988, Dietz et al. 1989a) might have been caused by such an introduction, independent of the full-scale outbreak 2 mo later in the Kattegat. This peculiar Limfjord strain could represent an early and partly unsuccessful variant of the virus, later enhanced in the form of the second, more virulent and widespread strain. Further clarification on the role of harp seals as vectors of the 1988 outbreak, as well as the potential introduction, evolution, pathogenicity and circulation of several PDV strains throughout this outbreak, would require the successful retrieval and comparison of viral RNA from European harbour seals and Barents Sea harp seals collected before, during and after the 1988 outbreak.

3.4. Spread of PDV in 2002 through multiple transmission events

In 2002, the first harbour seal mortalities were observed on the island of Anholt in the Kattegat on 4 May, and by 30 May, sick seals had been reported along the Danish and Swedish coasts of the Kattegat and Skagerrak (Härkönen et al. 2006). On 16 June, the first cases were reported from Vlieland in the Dutch Wadden Sea (Härkönen et al. 2006), and by 18 July, the index case of England was recorded in the Wash area (Lawson & Jepson 2003), 800–1000 km from the Kattegat. Intriguingly, sick seals were not observed in the geographically intermediate German and Danish Wadden Sea and the Limfjord before mid-September (Härkönen et al. 2006). Thus, based on the observed sequence of mortalities, the predominant hypothesis has been that the virus made a long-distance jump from the Kattegat epicentre to localities in the Dutch Wadden Sea from where it spread in 2 waves: west to England, Belgium and France, and east to the German and Danish Wadden Sea before

going north along the coast to the Limfjord (Härkönen et al. 2006).

The phylogenetic analysis presented here supports a Kattegat epicentre of the PDV outbreak in 2002, but offers new insights into the subsequent long-distance spread among European harbour seal colonies (Fig. 2A; Fig. S3). The most basal sample of the phylogenetic tree is from the Kattegat, which by early May splits into branches containing samples from the Kattegat, Skagerrak, Øresund and southwestern Baltic, in agreement with the observed spatio-temporal pattern of emergence. In contrast, the phylogenetic analysis does not agree with the presumed scenario in which harbour seal colonies outside of the epicentre were infected by 1 long-distance transmission event from the Kattegat to the Dutch Wadden Sea and further to other North Sea colonies. Rather, based on the phylogenetic analyses, we hypothesise that PDV arrived in southern England, the Wadden Sea and the Limfjord, respectively, through at least 3 independent long-distance transmission events from the Kattegat epicentre (Fig. 2C). The integrity of each of these subclades is supported by posterior values above 0.8, but the positioning of samples within subclades, as well as the relationship among subclades, is poorly resolved (Fig. 2A; Fig. S3).

The finding of multiple transmission events poses the question of who the likely carriers of PDV among harbour seal colonies are. As harbour seals show strong site fidelity throughout the summer (Dietz et al. 2013) and exhibit fine-scale population genetic structuring (Olsen et al. 2014), it has previously been assumed that the more mobile and less susceptible grey seal acted as a carrier of PDV between geographically distant haul-out sites (Hammond et al. 2005, Pomeroy et al. 2005, Härkönen et al. 2006). However, while grey seals did haul out on Anholt in 2002, the numbers were likely no more than 5–10 animals, several orders of magnitude lower than the 800–1000 harbour seals hauling out. Thus, although it only takes 1 infected animal to start an epidemic, the likelihood of transmission from harbour seal to harbour seal is substantially greater than from grey seal to harbour seal. Moreover, recent tagging and genetic studies indicate that especially juvenile harbour seals undertake movements across much greater distances than previously reported and, thus, potentially could have played a larger role in transporting PDV longer distances. For instance, Wadden Sea harbour seals have been shown to undertake foraging trips of a couple of hundred kilometres into the North Sea (Tougaard et al. 2008, Dietz et al. 2013, Aarts et al. 2016), and recent population genetic analyses suggested movements among the seals in southern England, France and the Dutch Wadden Sea (Olsen et al. 2017). Likewise, tagging of harbour seals in the fall and early winter of 2016 provided the first documented movements from a haul-out in the southern Kattegat to the central Limfjord (R. Dietz et al. unpubl.) by an animal that has now been genetically determined to originate from the Limfjord (M. T. Olsen unpubl.). Thus, while grey seals cannot be ruled out, we hypothesise that both short- and long-distance PDV transmissions in 2002 could have been facilitated by harbour seal movements. Although beyond the scope of the present analysis, additional insights into the dispersal of seals and transmission of PDV could be obtained by integration of genetic, epidemiological and tagging data (Swinton et al. 1998, Hall et al. 2006).

3.5. New insights to PDV virus evolution

The mean substitution rate of the full data set, including coding and non-coding regions, was found to be 6.73×10^{-4} substitutions site^{-1} yr^{-1}, while the substitution rate estimates for the sequences of each outbreak were 2.28×10^{-3} and 1.58×10^{-3} for 1988 and 2002, respectively. The results correspond well with the rates of 10^{-3} to 10^{-4} substitutions site^{-1} yr^{-1} reported for other morbilliviruses (Hanada et al. 2004, Furuse et al. 2010, Xu et al. 2014). The substitution rates estimated at the gene level did not differ much and were found to be from 8.03×10^{-4} to 2.2×10^{-4} for the full data set, 5.75×10^{-3} to 4.62×10^{-3} for the 1988 sequences and 5.72×10^{-3} to 1.53×10^{-3} for the 2002 sequences (Table 2). In the full dataset, the F, M and H genes had similar substitution rates with overlapping HPD intervals, whereas the substitution rate of the P gene (2.2×10^{-4}) was substantially lower. Interestingly, while the P gene had the lowest substitution rate, it had the highest proportion of non-synonymous substitutions (64%), followed by the F (50%), H (48%) and M gene (23%). The P gene encodes for 3 different proteins, P (1524 bp), V (899 bp) and C (525 bp), which use different reading frames of the same nucleotide sequence, and high sequence variability has previously been documented in MeV (Devaux & Cattaneo 2004, Bankamp et al. 2008, Beaty & Lee 2016). Nine amino acid changes were found in the reading frame of the P protein, which is a part of the polymerase complex and is essential for viral RNA replication (Bankamp et al. 2011). Six amino acid changes were found in the reading frame of the V protein and 2 amino acid changes were found in

Table 2. Number of genetic differences, amino acid changes and substitution rates for the F, H, M and P genes calculated using an uncorrelated relaxed clock. The high-probability-density (HPD) intervals indicate the probability interval for each mean value. SEM: standard error of the mean. The M gene from the 1988 data set contained no substitutions, and thus no substitution rate was calculated. PDV: phocine distemper virus

Gene	Length (bp)	Number of mutations			Amino acid changes			Substitution rate		
		PDV_{all}	PDV_{1988}	PDV_{2002}	PDV_{all}	PDV_{1988}	PDV_{2002}	PDV_{all} Mean [95% HPD] SEM	PDV_{1988} Mean [95% HPD] SEM	PDV_{2002} Mean [95% HPD] SEM
F	1893 (2206[a])	28	4	15	0.50	0	0.66	8.03×10^{-4} [4.44×10^{-4}, 1.16×10^{-3}] 4.08×10^{-4}	4.62×10^{-3} [4.52×10^{-4}, 9.82×10^{-3}] 3.4×10^{-5}	3.96×10^{-3} [1.3×10^{-3}, 7.08×10^{-3}] 5.35×10^{-5}
H	1821 (1857[a])	31	5	12	0.48	0.40	0.50	5.6×10^{-4} [2.56×10^{-4}, 8.96×10^{-4}] 5.11×10^{-6}	4.75×10^{-3} [2.44×10^{-4}, 0.01] 4.76×10^{-5}	2.19×10^{-3} [4.89×10^{-4}, 4.37×10^{-3}] 3.88×10^{-5}
M	1005 (1447[a])	13	0	10	0.23	0	0.20	5.73×10^{-4} [1.9×10^{-4}, 1.01×10^{-3}] 6.74×10^{-6}	–	5.72×10^{-3} [1.57×10^{-3}, 0.01] 6.70×10^{-5}
P	1521 (1653[a])	14	4	8	0.64	0.75	0.75	2.2×10^{-4} [6.26×10^{-5}, 4.24×10^{-4}] 2.51×10^{-6}	5.71×10^{-3} [1.89×10^{-4}, 0.01] 5.97×10^{-5}	1.53×10^{-3} [3.79×10^{-4}, 2.94×10^{-3}] 1.25×10^{-5}

[a]Length including non-coding regions

the reading frame of the C protein. The V and C proteins both play important roles in the inhibition of interferons (Nanda & Baron 2006, Chinnakannan et al. 2014) and influence the antiviral host response. Additionally, the C protein is believed to affect the transcription of mRNA of the viral genes (Baron & Barrett 2000), and this could explain why the percentage of changes is lower than the V protein. H and F genes code for the haemagglutinin glycoprotein and the fusion protein, respectively, allowing the virus to attach and fuse with the host cell membrane (Wild et al. 1991). The 2 genes are known targets for the virus-neutralizing antibodies (Xu et al. 2014), and would be expected to contain a high amount of genetic variation, which our study also suggests. The substitution rate of the H gene for MeV has previously been estimated to be 6.44×10^{-4} substitutions site^{-1} yr^{-1} (Furuse et al. 2010) — an estimate which corresponds well to our estimate of 5.6×10^{-4} for PDV. The M gene codes for the matrix protein and is considered to be relatively conserved (Beaty & Lee 2016), which the relatively low proportion of non-synonymous substitutions estimated for PDV also suggests.

3.6. Perspectives

Wildlife pathogens, such as PDV and other morbilliviruses, represent a major risk for epidemic outbreaks resulting in extensive die-offs in naïve populations and species. The past centuries' human activities and extensive utilization of natural resources in terrestrial and marine environments have led to substantial habitat alteration, loss of biodiversity and climatic changes (Jones et al. 2008). As a consequence, wildlife species shift their geographical ranges, increasing the likelihood of disease transmissions and outbreaks within and among naïve species and populations (Karesh et al. 2012). Indeed, the mass migration of harp seals hypothesised to have brought PDV to the naïve harbour seal population in 1988 was caused by a collapse of capelin and herring stocks following intense human exploitation (Haug & Nilssen 1995). Such outbreaks could lead to ecological or economic losses and have huge consequences for already exposed or endangered species (Thorne & Williams 1988, Osterhaus et al. 1997). Further work should focus on understanding the dynamics of pathogen emergence and spread, including the co-evolutionary dynamics of intra- and interspecific pathogen transmissions.

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