A novel and highly divergent Canine Distemper Virus lineage causing distemper in ferrets in Australia

Running title: Canine Distemper Virus in Ferrets

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Abstract

Canine distemper virus (CDV) is a highly contagious systemic viral disease of dogs, that regularly spills-over into other animal species. Despite widespread vaccination, CDV remains endemic in many parts of the world. In this study we report an outbreak of distemper in ferrets in two independent research facilities in Australia. We found that disease severity varied, although most animals had mild to moderate disease signs. Histopathology results of animals with severe disease presented the typical profile of distemper pathology with multi-system virus replication. Through the development of a discriminatory PCR paired with full genome sequencing we revealed that the outbreak at both facilities was caused by a single, novel lineage of CDV. This lineage was highly divergent across the H gene, F signal peptide and full genome and had less than 93% similarity across the H gene to other described lineages, including the vaccine strain. Molecular analysis indicates that this strain belongs to a distinct lineage that diverged from other clades approximately 140 to 400 years ago, and appears to be unique to Australia. Given the differences in key viral proteins of this novel CDV strain, a review of the efficacy of the CDV vaccines currently in use in Australia is warranted to ensure maximum protection of dogs and other vulnerable species. In addition, enhanced surveillance to determine the prevalence of CDV in ferrets, dogs and other at-risk species in Australia would be useful to better understand the diversity of CDV in Australia.

Importance:

Canine distemper virus (CDV) is highly contagious and while dogs are the main reservoir, it may spill over into a number of other animal species. In this study we report an outbreak of distemper in ferrets in two research facilities in Australia. Outcomes of pathology and histopathology suggest ferrets have widespread multi-systemic infection, consistent with previously reported distemper infections in ferrets and dogs. Critically, through sequencing and phylogenetic analysis, we revealed that the outbreak at both facilities was caused by a single, novel and highly divergent lineage of CDV. This virus had less than 93% nucleotide similarity to other described lineages and the vaccine strain. This manuscript adds considerably to the epidemiology, ecology and evolution of this virus, and is one of few reports of distemper in Australia in the literature.

Keywords: Canine distemper virus, Canine morbillivirus, *Paramyxoviridae*, CDV, ferrets, Australia

Introduction

Canine distemper virus (CDV) (species *Canine morbillivirus*), is a member of the genus *Morbillivirus,* in the family *Paramyxoviridae* (1) and causes endemic and widespread infectious disease in animals in many countries (2, 3). Canine Distemper Virus is a multi-host virus that infects animals from a wide taxonomic range, including canids, felids, mustelids, procyonids and phocids (3, 4).The main reservoir for this virus are dogs, but due to the broad host range (5), there is continual spill over between domesticated animals and wildlife (6). Disease signs include severe rash and both ocular and nasal discharge and over the course of infection animals often develop neurological signs including circling, hyperesthesia, seizures, cerebellar or vestibular disease, in the form of head tilting (7). Severe systemic infection can occur in immunologically naïve animals (3, 8), with long periods of viral shedding, with infection in domestic dogs previously reported to last as long as 90 days (9). CDV is highly contagious in many species of animals (6), and resultant mortality rates vary in animal populations and across the species affected (8). For example, mortality has ranged from 23% in the Chinese giant panda (*Ailuropoda melanoleuca)* (10) to 100% in ferrets (*Mustela putorius furo*) (11). The origin and transmission of CDV infections, particularly in non-canidae species, such as ferrets, is poorly understood (9, 12, 13). Ferrets are a widely used experimental animal to study the pathogenesis and transmission of a variety of viral diseases including influenza, SARS-CoV-1 and -2, Ebola, rabies and a range of paramyxoviruses (14, 15), and are therefore an important animal to protect from unwanted, potentially fatal infections such as CDV.

Currently there are 17 distinct geographically-associated genetic lineages of CDV described globally (5, 16). On some continents there may be more than one lineage and for many, the evolutionary genetics have been well resolved (5, 17). However CDV has not been well documented in Australia (11), with only 48 reported cases in dogs and ferrets from 2006-2014, and limited information on the emergence and spread of CDV (11, 18, 19). This is further exacerbated by the total lack of publicly available genetic sequences from outbreaks in Australia.

There is a global vaccination strategy in place for the management and control of CDV with most countries, including Australia, generally utilizing a modified live attenuated vaccine based on the Onderstepoort strain. While this strategy is important in controlling this virus in dogs (8, 16, 20) there are a number of records where there is evidence of vaccine-induced CDV infections in both domesticated dogs and in wildlife through reversion of attenuation in the vaccine strain (6, 21). For example, CDV cases in wildlife

due to the vaccine strain infection have been reported in South Africa and the UK (6, 22, 23). In Australia, routine vaccination is available for domesticated dogs, with vaccination normally given to puppies and other domestic animals at risk of contracting CDV, such as domesticated ferrets (11), using a live attenuated CDV. Despite a large-scale ongoing vaccination strategy in Australia, strains of CDV continue to circulate in animal populations (11). For example, in a study conducted from 2006-2014 in dogs and ferrets in Australia, it was reported that there were confirmed or suspected cases of CDV in five states/territories with most cases reported in New South Wales, including two out of three tested ferrets (11). However data pertaining to CDV epidemiology in Australia is limited, and the modes and rates of infection and mortality are not well understood.

In this study we report an outbreak of CDV in the ferret population in the Australian state of Victoria in 2019. CDV was detected in ferrets supplied to two independent research facilities from different breeders, indicating that this outbreak likely occurred across wide regions of the state. Herein we describe this CDV outbreak based on clinical signs and qRT-PCR CDV detection and describe the histopathology of severe cases. To better discriminate the dynamics of the outbreak we developed an assay to discriminate between a commercial CDV vaccine and the field strain. By full genome sequencing we also determined that the CDV in this outbreak originated from a novel lineage of CDV.

Methods and Materials

Ethics Statement

Work with ferrets at The Peter Doherty Institute for Infection and Immunity (hereafter Doherty Institute) was conducted with the approval of the Melbourne University Animal Ethics Committee (AEC# 1714278). Animals that had severe disease signs received veterinary intervention and were humanely euthanised in accordance with ethical guidelines. Experiments using ferrets at the Australian Centre for Disease Preparedness (ACDP) were approved by the ACDP Animal Ethics Committee (AEC# 1956). All work at the Doherty Institute and ACDP was performed in strict accordance with the Australian Government, National Health and Medical Research Council Australian code of practice for the care and use of animals for scientific purposes (24).

126 *Ferrets*

127 Ferrets used for experimental purposes at the Doherty Institute and the ACDP were
128 obtained from three different ferret breeders (2 supplied the Doherty Institute and 1
129 supplied ACDP) in the state of Victoria, Australia. The breeders are located at different
130 locations outside of the Melbourne metropolitan region and provided ferrets at regular
131 intervals to both facilities throughout 2019.

132 *CDV Vaccines*

133 The Protech C3 vaccine (Boehringer Ingelheim, Australia), comprising a live-attenuated
134 CDV was administered at both the Doherty Institute and ACDP to control the respective
135 CDV outbreaks. The dose administered varied between institutions: at the Doherty
136 Institute 0.2ml was administered to each ferret and at the ACDP 0.25ml was administered,
137 regardless of age or weight. At both facilities two doses were administered, however at the
138 Doherty Institute doses were 2 weeks apart and at the ACDP doses were administered 4
139 weeks apart. The Protech C3 vaccine has been approved for dogs in Australia and the
140 standard dose administered to dogs is 1ml.

141 *Histology and Immunohistochemistry*

142 Tissues collected for histology processing at ACDP were fixed in 10% neutral buffered
143 formalin, processed and embedded in paraffin using standard procedures, sectioned at 4
144 µm, and stained with hematoxylin and eosin (H&E). For immunohistochemistry (IHC),
145 paraffin-embedded tissue sections were quenched for 10 min in aqueous 10% hydrogen
146 peroxide. Antigen retrieval was performed by using the Agilent PT Link (Dako, Agilent, Vic,
147 Australia) module for 30 min at 97°C in pH 9 antigen retrieval solution. A mouse
148 monoclonal antibody targeting the nucleocapsid protein of CDV (CDV-NP, VMRD, WA,
149 USA) was used at a dilution of 1:2000 (60 min incubation), sections were incubated with
150 Mouse linker (Dako), then visualised using an Envision Flex horseradish peroxidase
151 (HRP)-secondary antibody (DM822, Dako) for 20 minutes (goat anti-rabbit and anti-mouse
152 immunoglobulins), followed by chromogen aminoethyl carbazole (AEC), and then
153 counterstained with Lillie-Mayer's Haematoxylin and Scotts Tap Water. Sections were
154 digitised using a Pannoramic Scan II (3DHISTECH Ltd, Budapest, Hungary) whole slide
155 imager before photomicrographs were taken using the image capture function of the
156 CaseViewer software (3DHISTECH Ltd).

157

158 *Identification of ferrets with CDV by Real-Time PCR*

159 Two different PCR approaches were used across the two research facilities. At the
160 Doherty Institute a commercial CDV quantitative real-time reverse-transcriptase PCR
161 (qRT-PCR) was initially employed followed by the development of a discriminatory assay.
162 All ferrets received into the facility were tested. At ACDP, a pan-morbillivirus RT-PCR was
163 applied for detection of CDV from ferrets with clinical symptoms (25).
164
165 At the Doherty Institute nasal wash samples were collected from lightly sedated ferrets.
166 Ferrets were sedated by intramuscular injection of a combination of ketamine (12.5mg/kg,
167 Troy Laboratories) and xylazine (2.5mg/kg, Troy Laboratories). We instilled 1 mL of sterile
168 PBS into one nostril and allowing the liquid to flow out of the other nostril into a collection
169 tube. Nasal wash samples were immediately stored at -80°C until RNA extraction. RNA
170 was extracted from 140µl of nasal wash sample using the QIAamp Viral RNA Mini kit
171 (QIAgen, Australia), according to the manufacturer's instructions. RNA was also extracted
172 from the Protech C3 vaccine (Boehringer Ingelheim, Australia) following reconstitution
173 according to manufacturer's instructions.

174 Primers were developed for a number of purposes (Table 1). First, to develop a
175 discriminatory PCR assay to distinguish between the outbreak virus and the vaccine virus,
176 second to amplify genes of interest (NP, H, and F) and finally for whole genome
177 sequencing (WGS) (Table 1). At the Doherty Institute, primers designed in this study were
178 created from conserved regions of similar sequences to the wild type strain of CDV found
179 in Victorian ferrets (percentage identity ~92%) retrieved from GenBank using (Basic Local
180 Alignment Tool) Blastn. Primers were created with both Primer-BLAST (NCBI), and
181 Geneious R10 (Biomatters, Auckland, New Zealand).

182 qRT-PCR was performed using the SensiFAST Probe Lo-ROX One-Step kit (Bioline
183 Meridian, Australia) with a reaction volume of 20µl. The reaction mix contained 4ul RNA,
184 40µM of each primer and 10µm of the associated probe. Thermocycling conditions
185 comprised a reverse transcriptase step at 45°C for 10 minutes, followed by a denaturation
186 step at 95°C for 2 minutes and 40 cycles of 95°C for 5 seconds and 60°C for 15 sec. The
187 assay designed here was validated against a commercially available CDV assay the
188 Canine Distemper Virus Detection qPCR (Genesig,United Kingdom).

189 To discriminate between the outbreak virus and the vaccine strain a discriminative qRT-
190 PCR was developed using a two-probe assay (Table 1) with the SensiFAST Probe Lo-

ROX One-Step kit (Meridian Biosciences). The reaction mix contained 10µM of each probe, 40µM of each primer with a final reaction volume of 20µl and the same thermocycling conditions were used as specified above.

At ACDP, both fresh and formalin-fixed-paraffin-embedded (FFPE) tissues including brains and nasal swabs from ferrets with clinical symptoms were used for RNA extraction and PCR testing. Total RNA was extracted from fresh or FFPE tissue samples. For fresh tissue samples, fifty microlitre of supernatant of 10% tissue homogenates from relevant ferrets was utilised by using the MagMax 96 Viral RNA Kit (ThermoFisher Scientific) in a MagMAX Express Magnetic Particle Processor (ThermoFisher Scientific) following manufacturer's instructions. For FFPE samples, RNA was extracted from 4 uM sections of formalin-fixed, paraffin-embedded brain/lung tissue, using RNeasy FFPE Kit (QIAGEN), following the manufacturer's instructions. The RNA was used for RT-PCR and Next-generation Sequencing (NGS) analysis.

A pan-morbillivirus RT-PCR, targeting the phosphate (P) gene of morbillivirus, was utilised for detection of CDV from ferrets with clinical symptoms (25) (Table 1). The RT-PCR assays were conducted by using SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Amplifications were performed in an Eppendorf Master Cycler Model 5345 (Eppendorf, Hamburg, Germany). Each 25 uL reaction contained 12.5 uL 2X reaction mix, 1 uL Superscript III RT/Platinum Taq mix, 0.9 µM (final concentration) of each primer and 5 mL RNA template. The RT-PCR cycling conditions were as follows: one cycle of 48°C for 30 min for cDNA synthesis; one cycle of 94°C for 2 min for denaturing; followed by 45 amplification cycles of 94°C for 30 s, 50°C for 60 s, 68°C for 60 s and a final extension cycle of 68°C for 7 min. Amplified PCR products (approx. 429 bp for P gene) were gel purified using QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and sequenced using BigDye Terminator v3.1 Cycling Sequencing Kit (Applied Biosystems, Foster City, CA, USA) for confirmation.

Prevalence of CDV at the Doherty Institute was calculated using the *bioconf()* function in the Hmisc package *(26)* and prevalence differences between the field strain and vaccine strain at the Doherty Institute ferret facility were compared using a Chi-squared test. Differences in prevalence over time were compared using a generalized linear model with a binomial response and the statistically significant model was plotted using the *ggplot2* package (27). Analyses were conducted using R 4.0.2. integrated into RStudio 1.3.1073.

Sanger Sequencing and Next-Generation

Initial sequencing was done at the Doherty Institute with Sanger sequencing. RNA that was stored at -80°C and had undergone limited freeze thaw cycles was used for RT-PCR using the MyTaq One step RT-PCR kit (Meridian Biosciences) and primers designed for Sanger Sequencing (Table 1). Product sizes were confirmed using the e-Gel 2% agarose (GP) (Invitrogen, USA) followed by purification using the Exo-SAP-IT PCR product clean-up reagent (Thermofisher, Australia) according to manufacturer instructions. The purified template then underwent sequencing using the BigDye Terminator v3.1 Cycle sequencing kit (Thermofisher). The primers used for this step were 10% the concentration of the same primers used to generate PCR products, unless the primer had a M13 tail, in which case a M13 F(5'-TGTAAAACGACGGCCAGT-3') and R (5'-CAGGAAACAGCTATGACC3') was used. Sequencing was performed on a 3500XL Genetic Analyser (Applied Biosystems). Results were analysed using Lasergene 13 (DNASTAR, Madison, WI, USA).

For subsequent next-generation sequencing (NGS) at the Doherty Institute, RNA was extracted from stored (-80^{o}C) nasal washes as described previously. Briefly, RT-PCR products were created using the Superscript IV One Step RT-PCR system (Thermofisher) and primers designed based on the results of Sanger Sequencing (Table 1). PCR product concentration was determined using a 4200 TapeStation System (Agilent Technologies, USA) and Genomic DNA Screentape (Agilent Technologies). The concentration of each sample was normalised to 6.6ng/µl and 30µl of DNA was used for Nextera DNA Flex Library Prep (Illumina, USA), performed according to manufacturer's instructions. Sample libraries were pooled and diluted to 200pm and 100µl of this diluted sample was loaded onto a flow cell for NGS using an iSeq 100 (Illumina). Sequences were assembled using reference mapping with paired ends in Geneious R10 (Biomatters) and Bowtie2 (28) was used for end to end alignment.

RNA extracted from both fresh and FFPE tissue samples from ferrets tested positive by pan-morbillivirus RT-PCR was used for NGS at ACDP. Both field strains sequenced comprised of FFPE samples, and all but one vaccine strains sequenced were from fresh samples.

At ACDP, the TruSeq RNA Library Prep Kit (Illumina, USA), following manufacturer's instructions was used for library construction, using an RNA concentration of 5 ng/µl. The libraries were normalised and pooled at equimolar ratios (final concentration of 12.5 pm each). The library pool was then loaded into flow cell of MiSeq Reagent Kit V2 (2 x 150

cycles) and sequenced in a MiSeq platform (Illumina), according to the manufacturer's instructions. The NGS sequence data was analysed using CLC Genomic Workbench 20 (Qiagen) using standard parameters. The raw reads were quality-trimmed and viruses were assembled using a combination of read mapping (stand-alone read mapping, CLC Workbench algorithm) and *de novo* assembly (scaffolding, CLC Workbech algorithm) to generate consensus sequences.

All CDV sequences have been deposited in GenBank (Accession number: XXXX-XXXX). Sequences derived from the Protech C3 vaccine have been deposited with permission from the manufacturer (Boehringer Ingelheim).

Phylogenetic Analysis

Sequences were assembled in SeqMan Pro 13 (DNASTAR). Assembled sequences were compared through the using BLASTn (NCBI) to other available sequences in the non-redundant nucleotide (nr) database (29).

To reconstruct the evolutionary relationships, all CDV sequences available in GenBank were downloaded and all geographic and host species origins were cross referenced with publications if they were not available in the GenBank record. Full genomes, coding complete H gene or the 135bp F signal peptide were aligned using the MAFFT algorithm (30) executed in Geneious R10 (Biomatters). Maximum likelihood trees were estimated using PhyML (31) and implementing the appropriate nucleotide substitution model. Lineage information and appropriate rooting was mined from Duque-Valencia et al.(5).

A time-structured phylogeny was constructed using the same backbone sequences and parameters as previously published (17). Briefly, select sequences were downloaded and the H gene was aligned using MAFFT and a maximum likelihood tree was constructed using PhyML. The tree was imported into TempESt v1.5.3 (32) to ensure clock-like behaviour in the data. Using BEAST 1.10.4 (33), the data were analysed using a strict clock, the HKY+G+I substitution model and a constant size coalescent model. The analysis was run for 100,000,000 generation and convergence was assessed using Tracer V1.6. A maximum clade credibility tree was generated using TreeAnnotator v1.8 and visualized in FigTree v1.4

Results

The Doherty Institute outbreak

Starting in April 2019, ferrets with clinical disease consistent with CDV were observed in the Doherty Institute animal facilities during routine animal husbandry. Nasal wash samples were collected from animals with clinical signs (as described below) and tested for CDV using the Canine Distemper Virus Detection qPCR (Genesig). In response, the Doherty Institute animal facility introduced routine vaccination of ferrets prior to their arrival at the Institute, starting in mid April of 2019, 10 days following the confirmation of the index case of CDV (Fig 1).

Between April 2019 and December 2019, all 179 animals that entered the Doherty Institute Biological Resource Facility (BRF) were tested for CDV following arrival. Of these animals, 115 were positive (64%) for CDV by RT-PCR. Using the discriminative assay developed here, we tested 103 of these 115 CDV positive samples and detected two different strains of CDV – a field strain causing the initial outbreak and the strain used in the Protech C3 vaccine. Of the 103 animals screened with the discriminative assay, 100 were vaccinated prior to sampling (approximately 3 weeks prior to nasal wash sampling). Overall, there was a statistically significant difference in the proportion of detections due to the field strain (39%) and vaccine strain (56%) (X^2=12.921, df=1, p=0.003) (Fig 1). In 20 ferrets, both the vaccine and field strain of the virus were detected. Despite finding the vaccine strain in all sampling time periods following the routine introduction of CDV vaccination and having a higher overall prevalence, we found that the CDV outbreak caused by the field strain persisted for less than two months (Fig 1). Following the index cases on 3 April 2019, the field strain was detected in approximately 70% of animals on 7 May, 50% on 21 May, but was no longer detected in ferrets from June 2019. This is in contrast to the vaccine strain, which was consistently detected during the seven months following CDV vaccine implementation on 3 April (Fig 1). There was no statistically significant difference in disease outcomes (*i.e*. requiring euthanasia under the ethical guidelines) when comparing ferrets infected with the field strain and the vaccine strain; 3 ferrets positive for the vaccine strain were euthanised and 3 ferrets infected with the field strain were euthanised. An additional ferret that was negative for CDV was euthanised, and three additional ferrets that were not tested for CDV were euthanised (Table 2).

ACDP Outbreak

Coinciding with the first detection at the Doherty Institute animal facility (April 2019), ferrets at the animal holding facility for ACDP (CSIRO Werribee Animal Health Facility) began to experience clinical signs consistent with CDV infection (as described below). Unlike the Doherty Institute, no qPCR or discriminatory RT-PCR was leveraged to identify CDV positive ferrets. PCR was only used retrospectively. Rather, in response to clinical signs, quarantine measures were initially introduced to separate ferrets with clinical signs from unaffected ones. All ferrets with clinical signs and animals sharing cages regardless of clinical signs were euthanised to control the outbreak. A detailed breakdown of infection status and euthanasia from the ACDP outbreak are not available. Vaccination was introduced using a quarter of the standard dose of the Protech C3 vaccine. The initiation of vaccination coincided with the final detections of CDV in the facility; within two months of the initial vaccination, the CDV outbreak at ACDP was controlled.

Clinical Signs

Clinical signs of CDV observed in ferrets from both the Doherty Institute and ACDP included (Fig 2A) inguinal dermatitis, with rough, discoloured patches of skin appearing on the abdomen, (Fig 2B) hyperkeratosis of the footpads, also known as hardpad, (Fig 2C) dermatitis on the chin and mouth, with scaly patches forming, and/or ocular signs including uncontrolled eye twitching with mucopurulent ocular and nasal discharge, resulting in visible crusting around the eyes and nose (Fig 2). At the Doherty Institute, visible signs were mostly classified as mild, with the animals remaining active. However, 10 animals (including both animals that were not tested by qPCR [n=3] and those that were tested for CDV by qPCR [n=7] displayed more severe signs (Fig. 2C-D) and required immediate euthanasia in accordance with ethical requirements. Animals classified as suffering from severe disease also showed signs of decreased activity, lethargy and loss of appetite. Any one of these clinical signs as well as the appearance of lethargy, pyrexia, anorexia, or weight loss were determined to be significant enough to require veterinary intervention. Due to the implementation of a euthanasia strategy to contain the outbreak, a breakdown of disease severity was not available from ACDP. Necropsies were performed on all euthanised animals at the Doherty Institute and on selected cases at ACDP. Tissues taken at the Doherty Institute were sent to an external pathology service, however detailed results were not available. Tissues collected at ACDP were processed for histopathology assessment at ACDP, as described below.

Histopathology and CDV immunohistochemistry

Four ferrets with distemper-like clinical signs and one asymptomatic ferret from the ACDP outbreak were submitted for histopathological analysis. Three of the symptomatic ferrets were submitted early in the outbreak, another symptomatic ferret was submitted days after vaccination was introduced, along with an asymptomatic penmate. In these ferrets, necrosis and sloughing of the bronchiolar epithelium were observed in the lung. This was often accompanied by notable diffuse epithelial hyperplasia (Fig 3A) and occasionally with a lymphohistiocytic infiltrate. In some of the affected areas, frequent round eosinophilic inclusion bodies were detected in the cytoplasm and nucleus of bronchial/bronchiolar epithelial cells (Fig 3A). Occasional multinucleated syncytial cells were found lining alveoli. Similar syncytial cells and intracytoplasmic/intranuclear inclusion bodies were identified in hyperplastic parts of the renal pelvic and urinary bladder urothelium (Fig 3B). The skin over the nasal planum was also hyperplastic with prominent parakeratotic hyperkeratosis emanating most notably from hair follicles. Associated suppurative inflammation was also occasionally observed in the subjacent dermis.

In addition to above lesions, multifocal to coalescing patches of the lymph node cortex were depleted in lymphocytes (Fig 3C). In the more severely affected lymph nodes, these lymphocyte-depleted areas in the cortex were replaced by oedema, fibrin, histiocytes and fibroplasia (Fig 3C). Taken together, the histopathologic features described above were consistent with pathology typically associated with CDV infection in ferrets.

Immunohistochemistry targeting the nucleocapsid protein of CDV showed that viral replication was widespread and intense, affecting many organ systems, even in animals with minimal histopathologic changes. Antigen was detected in the epithelium of the respiratory tract (Fig 3D), renal pelvis (Fig 3E), skin of the nasal planum, biliary tract, alimentary tract, female reproductive tract, lacrimal gland and salivary gland. High viral antigen burden was also observed in lymph nodes and spleen. Round cells with morphology consistent with histiocytes and lymphocytes were the main targets in these lymphoid organs (Fig 3F). In the brain, viral antigen was detected in only few mononuclear cells infiltrating the choroid plexus. Rare antigen positive round cells were also identified scattered in the olfactory bulb. This widespread multi-systemic infection was consistent with previously reported distemper infections in ferrets and dogs (34, 35).

Sequence analysis and evolutionary genetics

We sequenced 3 complete genomes corresponding to the field strain of CDV, in addition to 8 complete genomes of the CDV vaccine strain from ferrets. Both the field and vaccine strain viral genomes sequenced at the Doherty Institute were recovered from nasal wash samples. The field strain viral genomes from ACDP were sequenced from FFPE brains. Most vaccine strain viral genomes from ACDP were sequenced from fresh tissue samples (nasal swabs and brain) and only one was from FFPE brain. Analysis of the three field strain genomes sequenced across the two facilities showed only limited genetic diversity, with 99.78% nucleotide similarity in the H gene, 98.1% in the Fsp region and 99.5% across the full genome. This strongly suggests that the outbreaks in both facilities were due to the same virus strain; the most parsimonious explanation is that the outbreak was initiated in the breeding facilities supplying both the Doherty Institute and ACDP. As expected, there was limited diversity in the virus sequences from animals infected with the vaccine strains that were sequenced (n=7) with 99.83% similarity in the H gene, 98.5% in the Fsp region and 99.8% across the full genome. The genome sequences corresponding to the vaccine strain and the Onderstepoort strain shared 99% nucleotide similarity in the H gene, 98.5% in the Fsp region and 99.4% across the full genome.

Analysis of the H (Fig 4) and Fsp (Fig 5A) genes of the field strain viral sequences, regions of the CDV genome currently used for lineage discrimination (17), clearly demonstrated the divergent nature of the field strain. Indeed, based upon these genes and the full genome sequences (Fig 5B), and the current approach for lineage designation (*e.g.* (17), the field strain should be designated as a novel lineage. Critically, the field strain is highly divergent from both the Protech C3 vaccine itself and from virus sequences from ferrets vaccinated with this attenuated strain. More specifically, the H gene shared less than 93% nucleotide identity with all existing lineages of CDV, including the Protech C3 vaccine sequence [92.5% nucleotide similarity, 91.8% amino acid similarity] which we also sequenced in this study (Fig 4). Phylogenetically, the outbreak virus belonged to the lineage comprising “Vaccines (both the Protech C3 and the Onderstepoort strain which is used in most attenuated vaccines globally), Asia-3 and North America -1”, as defined by (5) (Fig 4). Furthermore, this sequence is highly divergent from other CDV sequences from mustelids, globally, with the exception of 4 sequences from China which fall into the “Asia-3” lineage (Fig 4). Other mustelid sequences fell into an array of lineages, although the vast majority belonged to the “Asia-1” lineage (Fig 4). As outlined in previous studies, the H gene phylogeny is dictated largely by geography, and we speculate that the field strain

reported here is representative of CDV currently circulating in Australia, however, as there are no available sequences in GenBank with which to compare, this cannot be confirmed.

We found similar patterns in the Fsp gene and full genome analyses (Fig 4). Phylogenetic analysis consistently demonstrated the field strain as being highly divergent (Fig 5), with the closest lineage being the “Vaccine/Asia-3/North America-1” clade and with high divergence from other mustelid CDV sequences. The Fsp region analysis of the novel Australian ferret lineage showed high divergence from other sequences, with only 73.88% of the nucleotide identity shared by both the vaccine and the Onderstepoort strain (Fig. 5B) compared to analysis of the full genome which shares approximately 91% identity with other reported strains (Fig. 5A).

Utilizing the framework presented by Duque-Valencia et al (2019) we aimed to estimate the divergence of the CDV viruses presented in this study (Fig 6). As we used the same BEAST parameters, including the use of a strict molecular clock, we were unable to include viruses from the “Vaccine clade” as these viruses have been passaged in cells and don’t have the same evolutionary rate as viruses infecting animals. Using this approach, we estimated the date that the field strain described here has branched from other clades between 1623.96 – 1878.9 (95% highest posterior density [HPD]) (Fig 6). It is also worth noting that with the addition of the sequences we generated in this study, tMRCA of the entire tree is significantly older than that presented in Duque-Valencia et al. (2019) but aligns more closely with Jo et al. (2019). While this analysis is useful in demonstrating the substantial period of time since these sequences have diverged from the closest lineage, it is important to take the specific dates merely as a guide. By also running this tree with an uncorrelated relaxed lognormal clock we were able to incorporate the vaccine strain, however this approach moves tMRCA much further back in time. This approach confirms that the divergence of CDV strains causing the outbreak described in this study occurs before the bifurcation of the “vaccine” clade from the “Asia-3” and “North America-1” clades, which is illustrated in all the maximum likelihood trees presented (Fig 4, 5).

Discussion

The outbreak of distemper in ferrets in Victoria, Australia in 2019 described here represents one of the few outbreak investigations of CDV reported in Australia. Further, it is the first to describe and analyse the evolutionary genetics of CDV in Australia. Despite the new data presented here, the epidemiology, ecology and evolution of CDV in Australia

457 remains entirely unknown, including the source and reservoir of the CDV lineage that
458 infected the ferrets reported in this study.

459 The first documented CDV outbreak in Australia occurred in 1983 and involved over 1000
460 cases across 3 states and was associated with racing Greyhounds (19). Since then,
461 outbreaks have been limited and with only a few reports. There are three contemporary
462 reports: one recorded outbreak comprising 16 cases in 2004-05 in unvaccinated dogs in
463 New South Wales including Sydney (18), in 2011 there was a suspected outbreak in
464 Victoria, although there is no report in the literature (11), and a case study reports
465 distemper in ferrets in a rescue facility in Victoria (36). Wylie et al (2016) presented the
466 most comprehensive contemporary assessment of CDV in Australia, reporting a total of 48
467 cases spread between 2006-2014. This study demonstrated the presence of CDV
468 predominantly in the south-eastern states of Australia and one detection in the Northern
469 Territory. Unfortunately, due to the disparity of the data it is unclear whether CDV was truly
470 restricted to these areas, or, rather if CDV is widespread but no samples have been
471 collected or outbreaks reported in other regions of Australia. Furthermore, due to the
472 limited data it is unclear what the prevalence of CDV is in eastern Australia, in dogs or in
473 other susceptible species. This is compounded by a lack of studies in wildlife in Australia
474 such that it is unclear if CDV is maintained in dogs in Australia, or is maintained in wildlife
475 such as Dingos (*Canis lupus dingo*), or introduced by feral animals such as foxes (*Vulpes*
476 *vulpes*) (37) or ferrets (38). Due to these uncertainties, the origin and extent of the
477 outbreak reported here remains opaque. While we report outbreaks of CDV at two
478 research centres, we recognize that the outbreaks in these facilities may act as potential
479 indicators for a larger outbreak within the Australian ferret population. From the reported
480 outbreak in these facilities, we can only guess at which animals played a role as reservoirs
481 in Victoria and were the source of the outbreak, the mechanism of transmission to ferret
482 breeders and the extent of the outbreak in breeding facilities or elsewhere in the state.

483 Due to the complete lack of sequence data and only sporadic outbreaks and detection of
484 CDV in Australia in the last 40 years, it is unclear whether previous CDV outbreaks in
485 Australia were due to reversions of the vaccine strain or whether another lineage of CDV
486 has been circulating on the continent. Through a combination of a discriminatory PCR
487 assay and full genome sequencing we reveal a novel and highly divergent lineage of CDV
488 infecting Australian ferrets. While this novel lineage is related to the “Vaccine/Asia-3/North
489 America-1” lineages, a time-structured Bayesian phylogenetic analysis suggested that the
490 novel field strain revealed here diverged from all other reported lineages well over 200

491 years ago. While we have identified a novel lineage, it is entirely unclear whether this
492 constitutes an Australian lineage that has been geographically isolated since the
493 divergence from the Vaccine/Asia-3/North America-1, or constitutes a lineage that was
494 cryptically circulating elsewhere and has only recently been introduced to Australia. It is
495 notable that there are a number of lineages circulating on most continents and in countries
496 where CDV has been investigated (17, 39, 40), so it is not unreasonable to assume long -
497 term circulation in Australia, and, whether additional viral diversity will be found in Australia
498 remains to be determined.

499 In addition to detecting a novel lineage, we found that 51% of ferrets tested with a
500 discriminatory PCR were still positive for the vaccine strain for up to 3 months after
501 vaccination. The vaccine used in this study was a modified-live attenuated vaccine; the
502 virus still has the ability to replicate in the animal and may have sufficient residual
503 virulence to cause disease (41). Importantly, the safety and effectiveness of CDV vaccines
504 are often tested in dogs, not mustelids (42). While the Protech C3 Vaccine is frequently
505 used in Australia at present, there are other CDV vaccines on the global market that are
506 subunit vaccines recommended specifically for use in ferrets, for example the Purevax
507 Ferret Distemper Vaccine (Boehringer Ingelheim), which is a recombinant canarypox
508 vector expressing the HA and F glycoproteins of canine distemper virus) that may prevent
509 unwanted CDV symptoms seen in some ferrets (43). Despite some disease signs
510 associated with the live attenuated CDV vaccine used in this study in ferrets, within 2
511 months of initiating vaccination the prevalence of the field strain dropped to zero and the
512 ferret CDV outbreaks were curbed at both the Doherty Institute and ADCP. Whether this
513 coincides with the end of the CDV outbreak in ferret breeding facilities or was linked to the
514 introduction of vaccination is unclear as no epidemiological data from the breeding
515 facilities was available. Genetic analysis demonstrated a large genetic difference between
516 this putative Australian lineage and the vaccine used in this outbreak (Protech C3
517 Vaccine), with only ~92% nucleotide similarity and 91% by amino acid similarity. Whether
518 this genetic difference corresponds to an antigenic difference that would lead to low
519 vaccine protection (44) is unclear but warrants further investigation. It is notable that there
520 have been previously reported cases in both the Americas and in the UK of vaccine
521 escape (23, 45), particularly when the Rockborn strain of the virus was used for
522 vaccination (45).

523 Ferrets with signs of CDV in this outbreak varied in disease severity, in contrast to the very
524 high mortality rates, with some outbreaks comprising a 100% mortality rate, reported

previously in mustelids (11, 46, 47). This lower mortality, specifically at the Doherty Institute, may have been in part due to intervention measures that were used on suspected CDV infected ferrets, such as injections of vitamin A (36, 48), the brief time period between identification of disease signs and interventions (1-2 days), and the decision to cull ferrets at an early disease stage in order to control the spread of the outbreak. The disease signs observed in animals that required intervention did not display the full extent of morbidity previously reported (12, 13), however, this may similarly be confounded by the decision to intervene early in the disease course in order to contain the outbreak. Therefore, in the current investigation, the true pathogenicity of this new CDV strain remains unclear. A dedicated experimental challenge trial in ferrets will be required in order to determine this. Nevertheless, in ferrets submitted for histopathology, the typical profile of distemper pathology and multi-systemic virus replication were observed which suggests that, at least for a subset of infected ferrets, the highly divergent CDV strain described in our current study is capable of producing typical CDV-induced disease.

Overall CDV continues to pose a large disease burden, globally (49). In addition to dogs, this virus also has an impact on wildlife, with a number of examples of CDV infection in large cats, hyenas and jackals from many parts of Africa (8, 50). Due to the broad host range of CDV, cross-species infection is known to occur (50, 51), with the same lineages of CDV having been detected in canids, felids, mustelids and even seals. This is of concern, particularly in Australia, where several species of native fauna are currently at risk of extinction due to other factors (52). If this strain of CDV was to potentially infect these at-risk and immunologically naive animals, it could potentially result in widespread disease and high rates of mortality (6). One of the limiting factors in mounting an appropriate response would be the lack of established methods for CDV surveillance across the country (11). Specifically, following vaccination with a live attenuated vaccine, current diagnostic assays may not be able to accurately discriminate between the presence of vaccine or a circulating strain. Due to the geographic isolation of some parts of Australia it is possible that other genetically distinct strains of CDV are present in the country, such that the discriminatory PCR developed here may have limited value in an outbreak with a different lineage. Currently CDV diagnosis is still best achieved through a combination of histopathology and qRT-PCR and should be interpreted in the context of case history and clinical presentation (11). The detection of this novel lineage signals the need, not only for more widespread surveillance of CDV in Australia, but also for sequencing of any viruses that are detected to better understand the diversity of this lineage and to reveal any other lineages that may be circulating on the continent. As the

ferrets reported in this study were sourced from regional breeders, the presence of CDV in this population may indicate that the virus is present in both domestic and wild animals in the state of Victoria. Our study describes the endpoint of an outbreak of CDV and highlights the crucial need for CDV surveillance and sequencing to better disentangle CDV ecology and evolution which is a key component for future outbreak response and control.

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751 **Figure legends:**

752

753 **Figure 1. Time series of vaccine strain and field strain CDV prevalence in the**
754 **Doherty Institute ferret facility**. Grey bars indicate when, and the number of samples
755 tested using the discriminatory PCR developed here and tick marks are on the secondary
756 Y axis. Points represent the individual point estimates for the vaccine strain and the field
757 strain. Lines correspond to modelled prevalence using a generalized linear model and
758 shaded areas represent the 95% confidence interval of the model. We were unable to
759 generate a corresponding outbreak figure for ACDP as they did not use a discriminatory
760 PCR.

761 **Figure 2. Clinical signs of animals infected with CDV**. Animals that developed visible
762 signs of CDV had (A) mild abdominal rashes (as indicated by arrows) and (B) crusting of
763 footpads. One animal developed (C) crusting around the mouth, however the majority of
764 visible symptoms on the face consisted of crusting around the (D) eyes and (E) nose.

765 **Figure 3.** Histopathology and immunohistochemistry (IHC) of ferrets from the CSIRO
766 ACDP outbreak. (A) H&E stain of the lung showing multiple eosinophilic intracytoplasmic
767 (arrows) and intranuclear (arrowheads) inclusion bodies within bronchiolar epithelial cells.
768 40x magnification. (B) CDV IHC of the lung with diffuse intense cytoplasmic labelling in the
769 bronchial epithelium. 5x magnification. Inset shows the homogeneous to coarse granular
770 and globular cytoplasmic labelling in the bronchial epithelium. 40x magnification. (C) H&E
771 stain of the renal pelvis with multiple syncytial cells in the urothelium (arrows). 10x
772 magnification. (D) CDV IHC of the renal pelvis, corresponding to panel C, with diffuse
773 intense cytoplasmic labelling in the urothelium. 10x magnification. (E) H&E stained section
774 of a lymph node with severe coalescing areas of lymphoid depletion in the cortex. These
775 areas were replaced with patches of fibrin, oedema, histocytes and early fibroplasia
776 (arrows and inset). Inset in panel E. corresponds to an area of lymphoid depletion as
777 indicated by the arrows in panel E. 20x magnification. (F) CDV IHC of the lymph node
778 corresponding to panel E illustrating antigen positive round cells were identified in the
779 depleted cortex. 10x magnification. Inset shows antigen positive lymphocytes (arrowhead)
780 and histiocytes (arrows) in an area of lymphocyte-depleted cortex. 40x magnification.
781

782 **Figure 4. Phylogenetic analysis of the H gene of CDV**. (A) Tree containing H gene of all
783 CDV sequences available in GenBank. Tips are coloured by geographic region. Red

shapes with a thick black border corresponds to a vaccine strain. Shapes indicate host, with all mustelid hosts indicated by a triangle. (B) Expansion of the "Vaccine/Asia-3/North America-1" lineage which is indicated by a grey box in A, in addition to reference sequences for main lineages and all sequences from mustelids. Sequences generated in this study are presented in a grey and sequences from ferrets are in bold text. Both the Doherty Institute and ACDP generated a full genome of the Protech Vaccine Strain, indicated by a red triangle in the "Vaccine strain" clade. Scale bar indicates the number of substitutions per site. Support values are presented for major nodes.

Figure 5: Phylogenetic analysis of (A) the full genome and (B) the F gene signal peptide. Trees containing all sequences in Genbank are unrooted, and the subtree that is expanded is denoted. Tips are coloured by geographic region or vaccine strain. Shapes indicate host. Sequences generated in this study are presented in a grey box in B. Scale bar indicates the number of substitutions per site. Support values are presented for major nodes.

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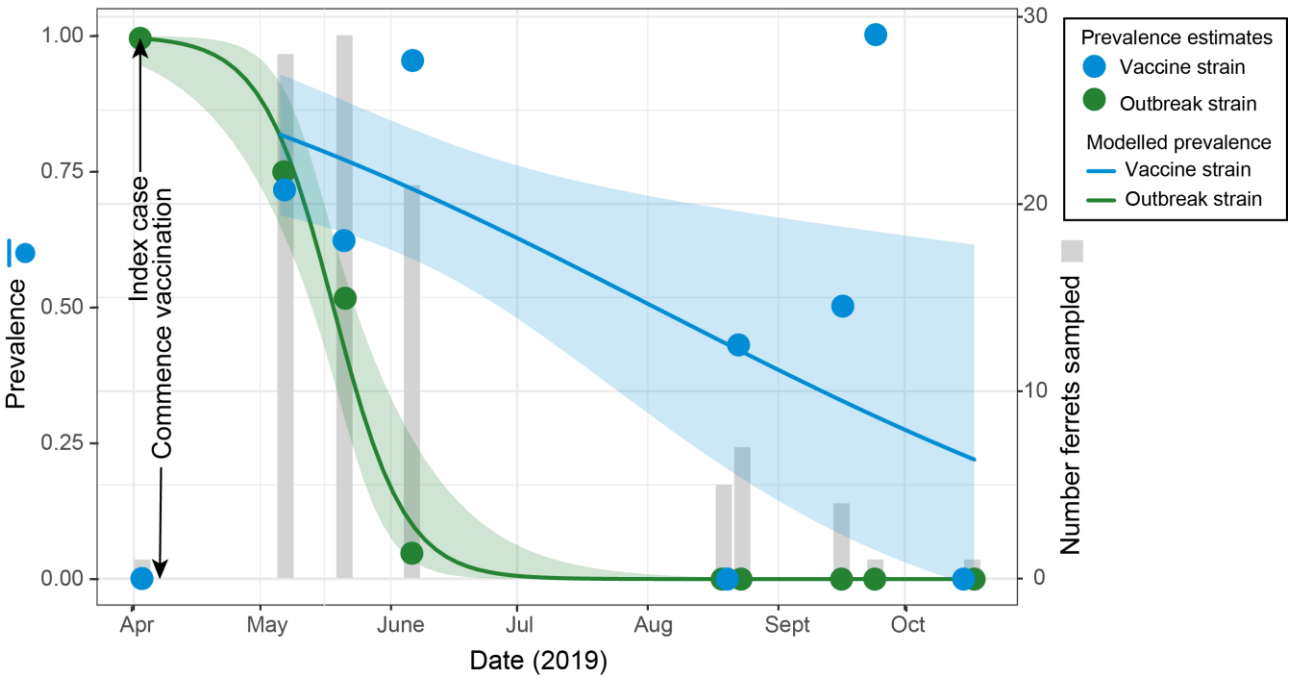
Figure 6. Time structured phylogeny of the H gene. Reference sequences are those presented in Duque-Valencia et al (2019). We did not include any vaccine strains, including the vaccine strains from ferrets sequenced in this study because repeated passage of vaccine strains in laboratory settings does not reflect natural evolution. However we have included the tentative position of the vaccine clade for clarity only – the outgroup to Asia-3 and North America-1. This phylogenetic position is based upon maximum likelihood estimation in this study and Duque-Valencia et al (2019). Field strain sequences generated in this study are in bold. Scale bar represents time in years. Node labels correspond to posterior probabilities of each node. Grey bars comprise the 95% highest posterior density of the date estimate.



814

815 **Figure 1. Time series of vaccine strain and field strain CDV prevalence in the Doherty**
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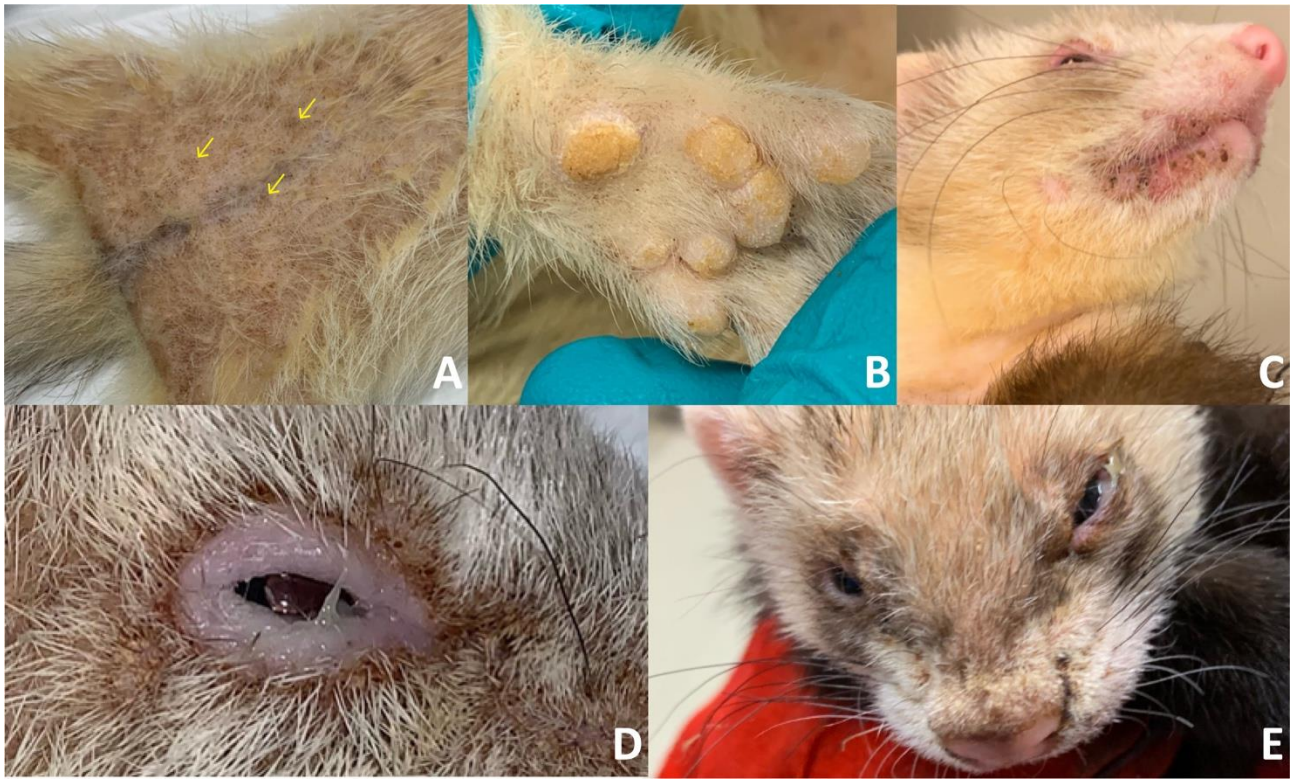

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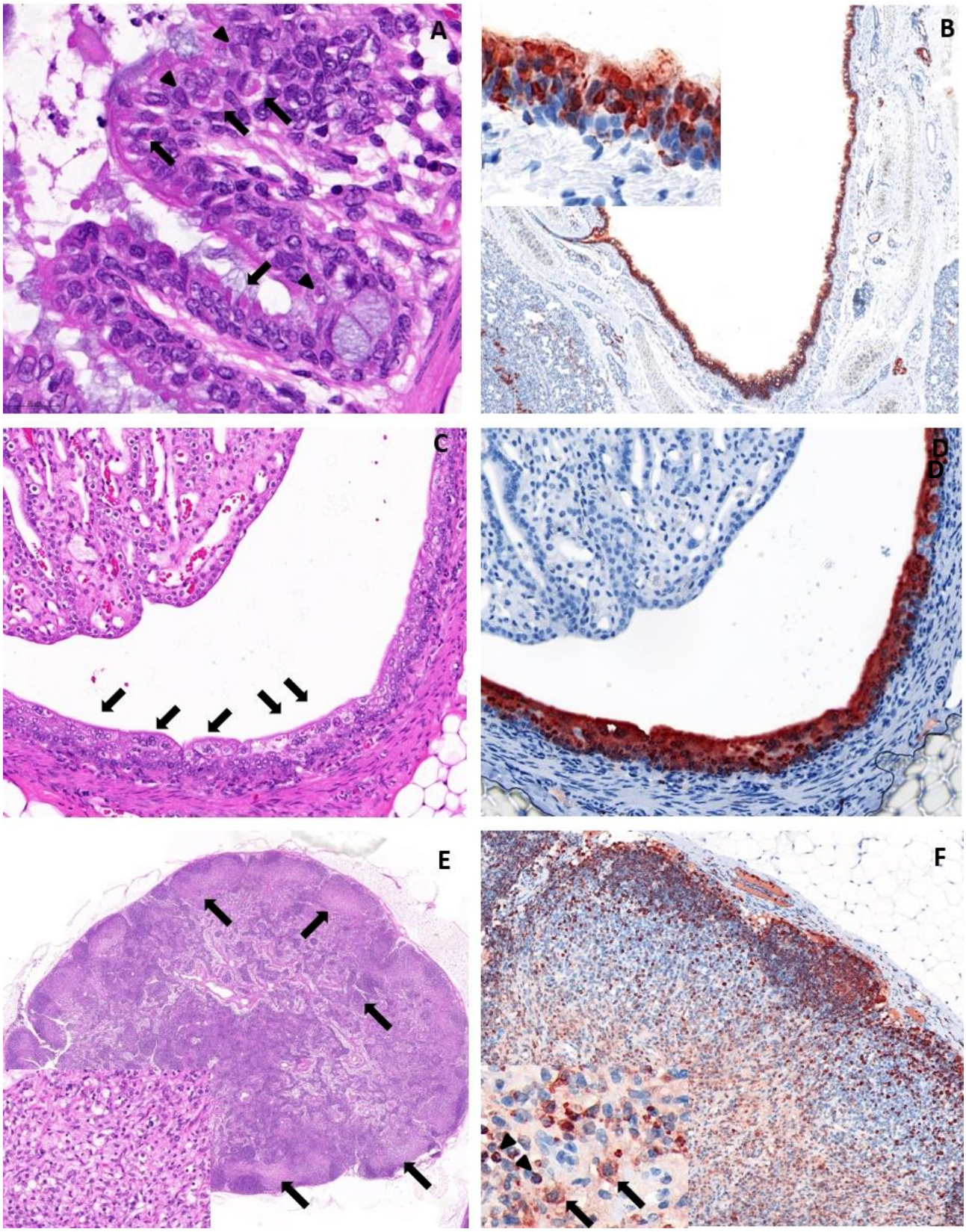

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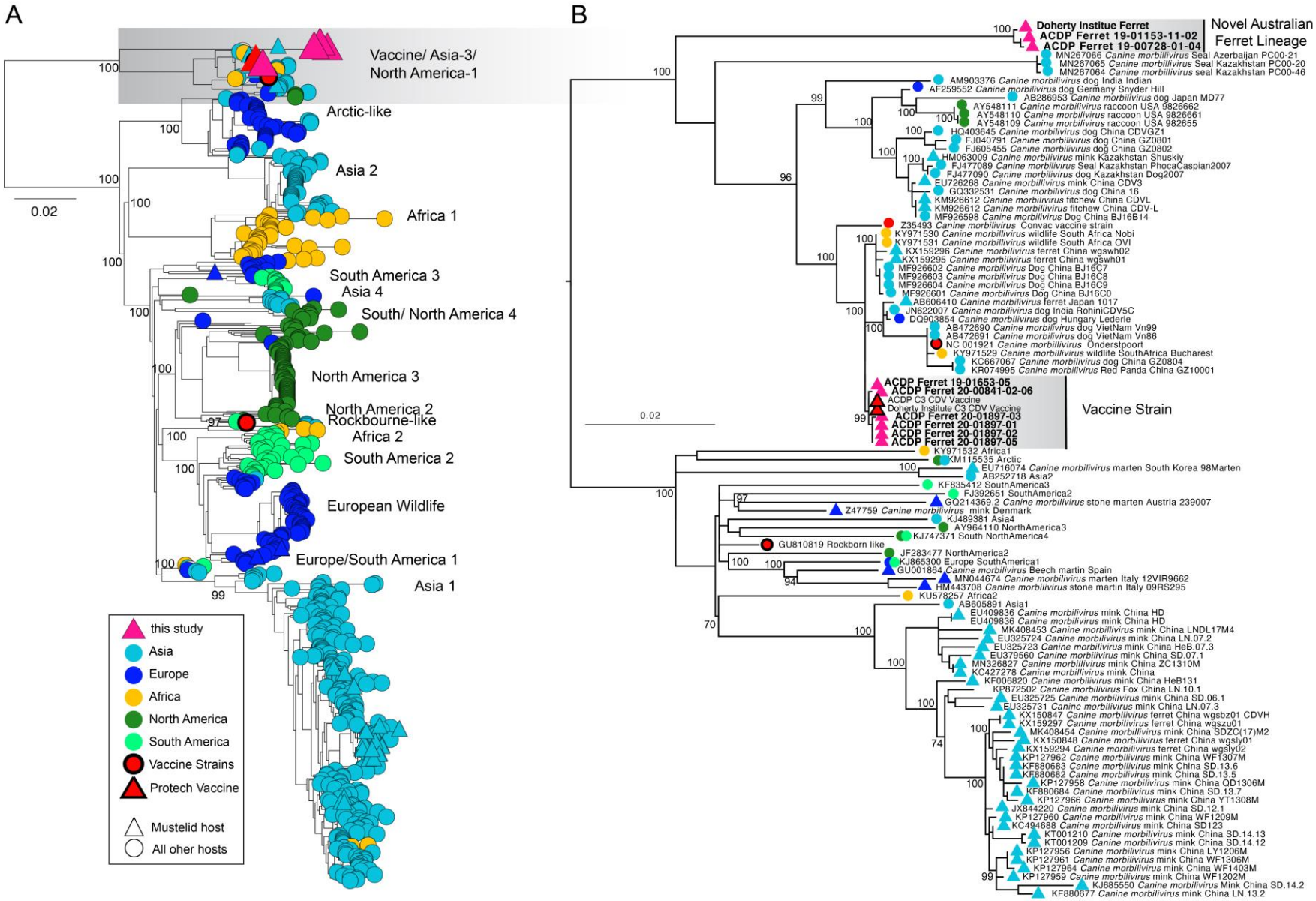
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ACDP Ferret 19-00728-01-04
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MN267066 Canine morbillivirus Seal Azerbaijan PC00-21
MN267065 Canine morbillivirus Seal Kazakhstan PC00-20
MN267064 Canine morbillivirus seal Kazakhstan PC00-46
AM903376 Canine morbilivirus dog India Indian
AF259552 Canine morbilivirus dog Germany Snyder Hill
AB286953 Canine morbilivirus dog Japan MD77
AY548111 Canine morbilivirus raccoon USA 9826662
AY548110 Canine morbilivirus raccoon USA 9826661
AY548109 Canine morbilivirus raccoon USA 982655
HQ403645 Canine morbilivirus dog China CDVGZ1
FJ040791 Canine morbilivirus dog China GZ0801
FJ605455 Canine morbilivirus dog China GZ0802
HM063009 Canine morbilivirus mink Kazakhstan Shuskiy
FJ477089 Canine morbilivirus Seal Kazakhstan PhocaCaspian2007
FJ477090 Canine morbilivirus dog Kazakhstan Dog2007
EU726268 Canine morbillivirus mink China CDV3
GQ332531 Canine morbilivirus dog China 16
KM926612 Canine morbilivirus fitchew China CDVL
KM926612 Canine morbillivirus fitchew China CDV-L
MF926598 Canine morbilivirus Dog China BJ16B14
Z35493 Canine morbilivirus Convac vaccine strain
KY971530 Canine morbillivirus wildlife South Africa Nobi
KY971531 Canine morbillivirus wildlife South Africa OVI
KX159296 Canine morbilivirus ferret China wgswh02
KX159295 Canine morbilivirus ferret China wgswh01
MF926602 Canine morbilivirus Dog China BJ16C7
MF926603 Canine morbilivirus Dog China BJ16C8
MF926604 Canine morbilivirus Dog China BJ16C9
MF926601 Canine morbilivirus Dog China BJ16C0
AB606410 Canine morbilivirus ferret Japan 1017
JN622007 Canine morbilivirus dog India RohiniCDV5C
DQ903854 Canine morbilivirus dog Hungary Lederle
AB472690 Canine morbilivirus dog VietNam Vn99
AB472691 Canine morbilivirus dog VietNam Vn86
NC 001921 Canine morbillivirus Onderstpoort
KY971529 Canine morbillivirus wildlife SouthAfrica Bucharest
KC667067 Canine morbilivirus dog China GZ0804
KR074995 Canine morbilivirus Red Panda China GZ10001
ACDP Ferret 19-01653-05
ACDP Ferret 20-00841-02-06
ACDP C3 CDV Vaccine
Doherty Institute C3 CDV Vaccine
ACDP Ferret 20-01897-03
ACDP Ferret 20-01897-01
ACDP Ferret 20-01897-02
ACDP Ferret 20-01897-05
Vaccine Strain
0.02
KY971532 Africa1
KM115535 Arctic
EU716074 Canine morbilivirus marten South Korea 98Marten
AB252718 Asia2
KF835412 SouthAmerica3
FJ392651 SouthAmerica2
GQ214369.2 Canine morbilivirus stone marten Austria 239007
Z47759 Canine morbilivirus mink Denmark
KJ489381 Asia4
AY964110 NorthAmerica3
KJ747371 South NorthAmerica4
GU810819 Rockborn like
JF283477 NorthAmerica2
KJ865300 Europe SouthAmerica1
GU001864 Canine morbilivirus Beech martin Spain
MN044674 Canine morbilivirus marten Italy 12VIR9662
HM443708 Canine morbilivirus stone martin Italy 09RS295
KU578257 Africa2
AB605891 Asia1
EU409836 Canine morbilivirus mink China HD
EU409836 Canine morbilivirus mink China HD
MK408453 Canine morbillivirus mink China LNDL17M4
EU325724 Canine morbilivirus mink China LN.07.2
EU325723 Canine morbilivirus mink China HeB.07.3
EU379560 Canine morbilivirus mink China SD.07.1
MN326827 Canine morbillivirus mink China ZC1310M
KC427278 Canine morbillivirus mink China
KF006820 Canine morbilivirus mink China HeB131
KP872502 Canine morbilivirus Fox China LN.10.1
EU325725 Canine morbilivirus mink China SD.06.1
EU325731 Canine morbilivirus mink China LN.07.3
KX150847 Canine morbilivirus ferret China wgsbz01 CDVH
KX159297 Canine morbilivirus ferret China wgszu01
MK408454 Canine morbillivirus mink China SDZC(17)M2
KX150848 Canine morbilivirus ferret China wgsly01
KX159294 Canine morbilivirus ferret China wgsly02
KP127962 Canine morbilivirus mink China WF1307M
KF880683 Canine morbilivirus mink China SD.13.6
KF880682 Canine morbilivirus mink China SD.13.5
KP127958 Canine morbilivirus mink China QD1306M
KF880684 Canine morbilivirus mink China SD.13.7
KP127966 Canine morbilivirus mink China YT1308M
JX844220 Canine morbilivirus mink China SD.12.1
KP127960 Canine morbilivirus mink China WF1209M
KC494688 Canine morbilivirus mink China SD123
KT001210 Canine morbilivirus mink China SD.14.13
KT001209 Canine morbilivirus mink China SD.14.12
KP127956 Canine morbilivirus mink China LY1206M
KP127961 Canine morbilivirus mink China WF1306M
KP127964 Canine morbilivirus mink China WF1403M
KP127959 Canine morbilivirus mink China WF1202M
KJ685550 Canine morbilivirus Mink China SD.14.2
KF880677 Canine morbilivirus mink China LN.13.2

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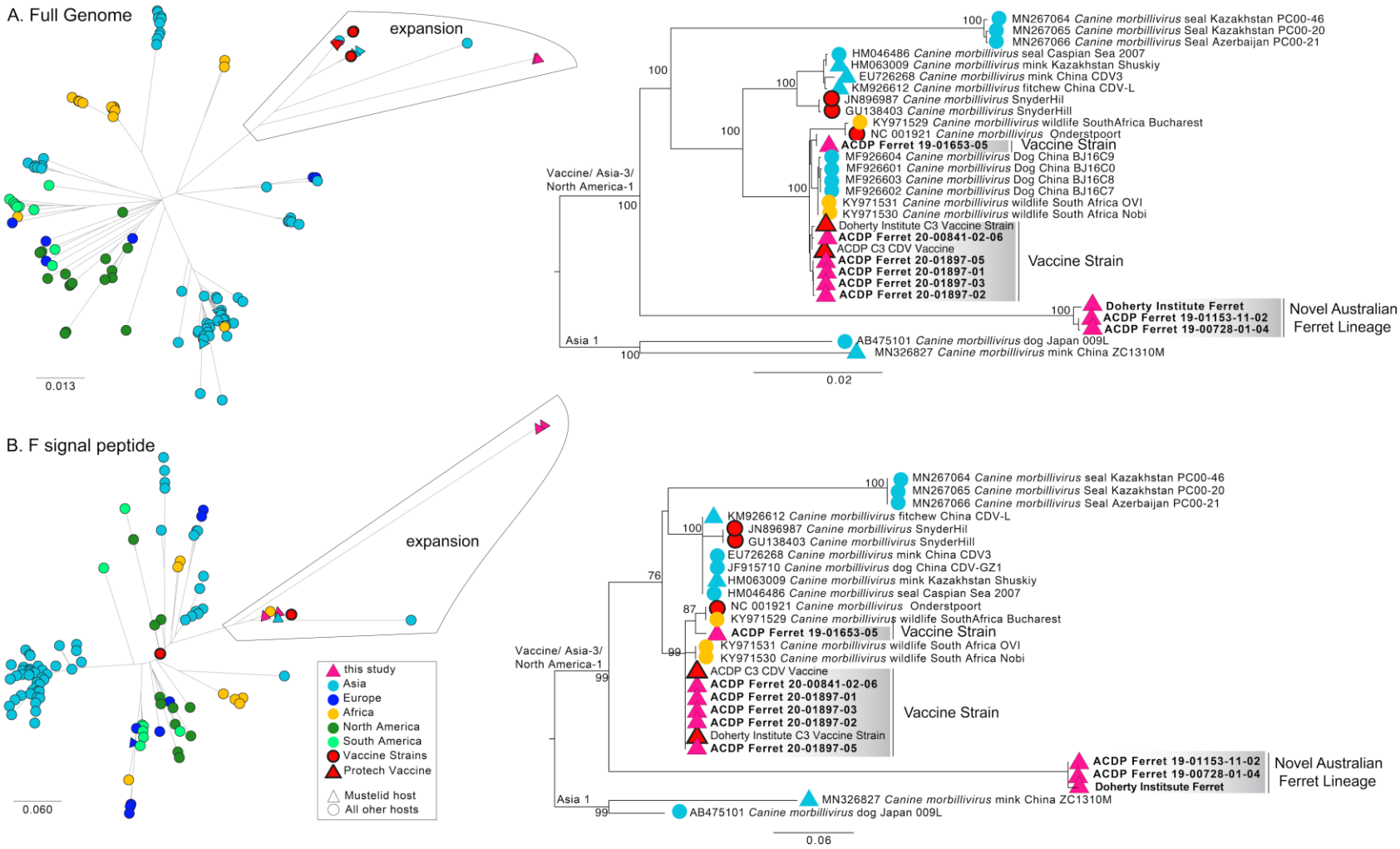
Figure 4. Phylogenetic analysis of the H gene of CDV. (A) Tree containing H gene of all CDV sequences available in GenBank. Tips are coloured by geographic region. Red shapes with a thick black border corresponds to a vaccine strain. Shapes indicate host, with all mustelid hosts indicated by a triangle. (B) Expansion of the “Vaccine/Asia-3/North America-1” lineage which is indicated by a grey box in A, in addition to reference sequences for main lineages and all sequences from mustelids. Sequences generated in this study are presented in a grey and sequences from ferrets are in bold text. Both the Doherty Institute and ACDP generated a full genome of the Protech Vaccine Strain, indicated by a red triangle in the “Vaccine strain” clade. Scale bar indicates the number of substitutions per site. Support values are presented for major nodes.



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856 **Figure 5: Phylogenetic analysis of (A) the full genome and (B) the F gene signal peptide**. Trees containing all sequences in Genbank are
857 unrooted, and the subtree that is expanded is denoted. Tips are coloured by geographic region or vaccine strain. Shapes indicate host. Sequences
858 generated in this study are presented in a grey box in B. Scale bar indicates the number of substitutions per site. Support values are presented for
859 major nodes.

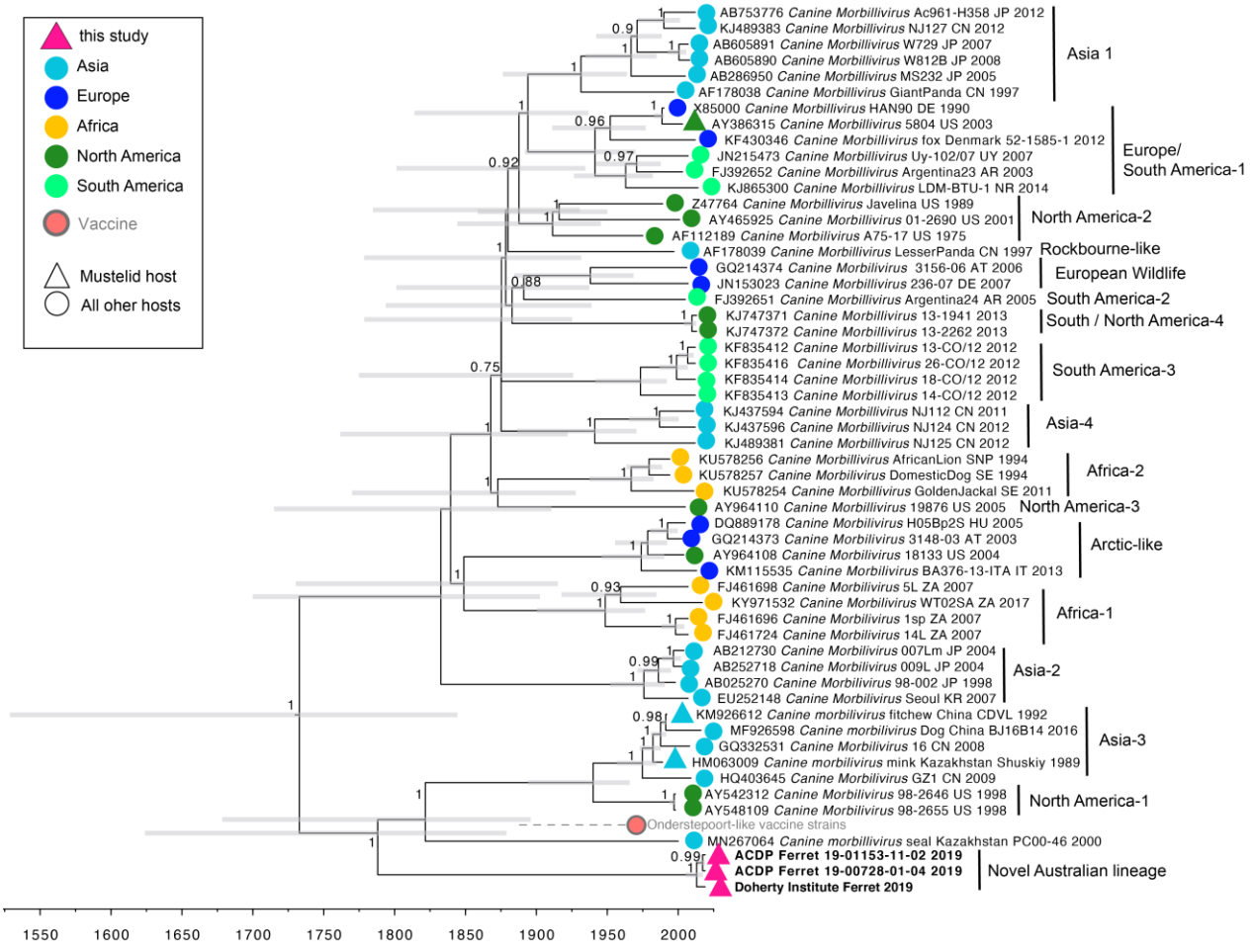

Figure 6. Time structured phylogeny of the H gene. Reference sequences are those presented in Duque-Valencia et al (2019). We did not include any vaccine strains, including the vaccine strains from ferrets sequenced in this study because repeated passage of vaccine strains in laboratory settings does not reflect natural evolution. However we have included the tentative position of the vaccine clade for clarity only – the outgroup to Asia-3 and North America-1. This phylogenetic position is based upon maximum likelihood estimation in this study and Duque-Valencia et al (2019). Field strain sequences generated in this study are in bold. Scale bar represents time in years. Node labels correspond to posterior probabilities of each node. Grey bars comprise the 95% highest posterior density of the date estimate.

872 Table 1. Primers and probes used at the Doherty Institute and ACDP for the detection of CDV in ferrets

Primer[1]	**Sequences (5'-3')**	**Primer Position**[2]	**Purpose**	**Institute**	**Reference**
p1	ACAGGATTGCTGAGGACCTAT	769-789[3]	Real Time RT-PCR	Doherty	Frisk et al. (53)
p2	CAAGATAACCATGTACGGTGC	1055-1035[3]	Real Time RT-PCR	Doherty	Frisk et al. (53)
CDV probe	FAM-ACCCAAGAGCCGGATACATAGTTTCAATGC-BHQ2	961-934	Real Time RT-PCR	Doherty	Han et al. (54)
d/CDV F	GCTATATCAACAGATGGGTG	934-953	Discriminative RT-PCR	Doherty	This study
d/CDV R	CATGGAGTTCTCAAGTTCAAC	1149-1129	Discriminative RT-PCR	Doherty	This study
d/WT probe	FAM-GTCATTCTAGAAAATTCTG-MGB NFQ	1047-1065	Discriminative RT-PCR	Doherty	This study
d/Vax probe	VIC-GTTATCTTGGAAAACTCTG-MGB NFQ	1047-1065	Discriminative RT-PCR	Doherty	This study
p5	CAAAGACGTGTGGTCGGAGAA	711-731[3]	Sanger sequencing	Doherty	Frisk et al. (53)
p6	CTTAGTAAGCATCCTCATCTTGGC	1610-1587[3]	Sanger sequencing	Doherty	Frisk et al. (53)

NP M13 F	TGTAAAACGACGGCCAGTACCAGACAAAG TTGGCTAWG	1-20	Sanger sequencing	Doherty	This study
NP M13 R	CAGGAAACAGCTATGACCATGATGTCAGC AATTCTAGG	868-849	Sanger sequencing	Doherty	This study
NP II F	CTTGGACATCAAACGATCC	818-836	Sanger sequencing	Doherty	This study
NP II R	TTGGACCTGGGTCCTAAG	1756-1739	Sanger sequencing	Doherty	This study
CDV H DP1	GCAACACCTGTGGATCAAGT	8051-8070	Sanger sequencing	Doherty	Wang et al. (55)
CDV H DP2	ATTGGCGACACCACAAATCG	8810-8791	Sanger sequencing	Doherty	Wang et al. (55)
H I F	TTAGGGCTCAGGTAGT	7057-7072	Sanger sequencing	Doherty	This study
H I R	GAATACCGTCTTGYGAAC	8024-8007	Sanger sequencing	Doherty	This study
H II F	GCTTCCTTGTGTGTAG	7955-7970	Sanger sequencing	Doherty	This study
H II R	GTATCATCATACTGTCAAG	8916-8898	Sanger sequencing	Doherty	This study
F II F	CAATTTGGAGAGTCGGGGGAT	6044-6064	Sanger sequencing	Doherty	This study
F II R	GAACGCTGAGAGACTGCCAA	6770-6751	Sanger sequencing	Doherty	This study
F III F	TTGGCAGTCTCTCAGGCTTC	6751-6770	Sanger sequencing	Doherty	This study
F III R	AGTTTTGTGGCAACCGTAACC	7409-7389	Sanger sequencing	Doherty	This study
NGS CDV F1	ACCAGACAAAGTTGGCTAAG	1-20	Next-generation sequencing	Doherty	This study
NGS CDV R1	CTCGAATCTGTAAAATTGGTGAC	8824-8802	Next-generation sequencing	Doherty	This study

NGS CDV F2	CCAGGGAATCAAGTGGAAATTG	8526-8547	Next-generation sequencing	Doherty	This study
NGS CDV R2	ACCAGACAAGCTGGGTATG	15690-15671	Next-generation sequencing	Doherty	This study
Barret F	ATGTTTATGATCACAGCGGT	2132-2151	Real Time RT-PCR	ACDP	Barret et al (25)
Barret R	ATTGGGTTGCACCACTTGTC	2561-2542	Real Time RT-PCR	ACDP	Barret et al (25)

873 [1] Primer names denote gene name, gene segment, tag if applicable and F and R for forward and reverse respectively, unless otherwise
874 indicated.
875 [2] Nucleotide position aligned to Onderstepoort strain unless otherwise stated.
876 [3] Nucleotide position according to Frisk et al.(53)

877 **Table 2. Number of ferrets infected with CDV in the Doherty Institute**

Ferrets	**qPCR Positive[1]**	**Positive for field strain [2]**	**Positive for Vaccine strain[2]**	**Negative CDV result[1]**
Tested	115	41	65	64
Euthanised	6	3	3	1

878 1. Based on either the qPCR assay from relevant literature (53, 54) and/or the
879 commercial Canine Distemper Virus Detection qPCR
880 2. Based on the discriminatory PCR developed in this study