

Canine distemper virus (CDV) infection in free-ranging Iberian lynxes (*Lynx pardinus*)



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Introduction

Background

In early November 2005, an Iberian lynx was found dead in the Doñana National Park, South Andalusia, Spain. Using real-time PCR and RT-PCR, blood, lymph node and fecal samples were found negative for the tested feline viruses (FCoV, FHV, FCV, FPV, FeLV, FIV) but positive for canine distemper virus (CDV). No histological examination could be performed due to advanced autolysis state of the carcass. However, based on its high viral loads in all materials, CDV infection was assumed to be etiologically involved in the animal's death. This finding is of great importance since the Iberian lynx population is highly endangered. During the last century, the decline of the wild population has been dramatic. Presently, there are only two remnant populations - both in the province of Andalusia - with less than 200 individuals altogether. The presence of CDV at high loads in one animal found dead is a serious finding suggesting that a CDV outbreak in lynxes may have a devastating potential.



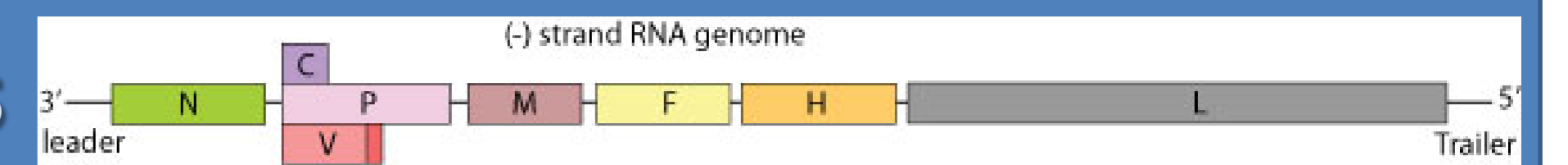
In Spain, CDV has been frequently identified as a cause of death in dogs, ferrets and minks and the presence of CDV antigen was shown in other species including red foxes and genets. Additionally, serological evidence of CDV infection was reported in Spanish wolves and domestic cats (Millan et al. 2009). Interspecies transmission frequently occurs, such as in the Serengeti lions (Roelke-Parker et al. 1996).

Aims of the project

- Assess the significance of CDV for the Iberian lynx population by determining the prevalence of CDV in lynxes and other carnivores of the same region.
- Trace the source of the infection by molecularly characterizing the CDV strain of the Iberian lynx and of eventually infected carnivores

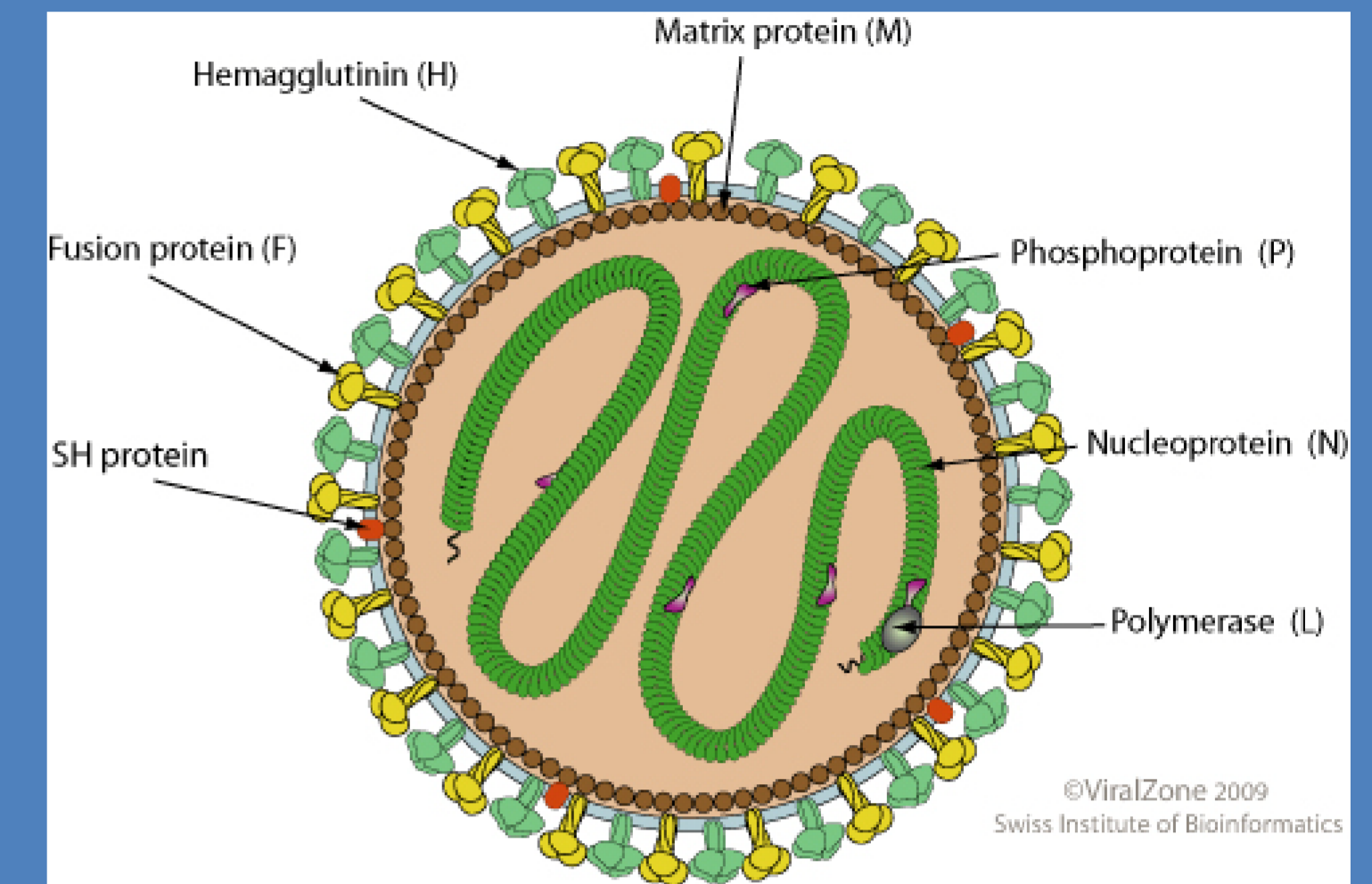
Canine Distemper Virus

The canine distemper virus (CDV) is the etiologic agent of one of the most important viral diseases of wild and domestic *Canidae*. It occurs worldwide and produces high morbidity and mortality in immunologically naïve populations (Appel 1987). This virus infects also a broad range of other animals, such as *Mustelidae*, *Procyonidae*, *Ursidae*, *Viverridae*, *Hyaenidae*, and *Felidae* (Deem et al. 2000). CDV is a member of the genus



Morbillivirus of the family *Paramyxoviridae*; Measle virus, rinderpest and phocine distemper virus all belong to the same family. Canine distemper virus affects three main organ systems causing signs of disease: the respiratory tract, the gastrointestinal tract and the central nervous system. The main route of transmission is via aerosol of respiratory secretions. The development of a vaccine in the early 1960s and the subsequent introduction of

widespread vaccination programs led to a dramatic reduction in the number of infected domestic dogs. CDV has an unsegmented, negative-sense, single-stranded RNA genome, which is about 15.7 kb long. It is an enveloped virus particle with a diameter of 150 to 300 nm. The genome of CDV encodes the following virion proteins: matrix (M), fusion (F), hemagglutinin (H), nucleocapsid (N), polymerase (L), and phosphoprotein (P).



The Iberian Lynx (*Lynx pardinus*)

In its appearance, the Iberian lynx is similar to the Eurasian lynx (*Lynx lynx*), but about half its size. On average, adult males reach 13 to 16 kilos, where females can reach up to 11 kilos. The fur is typically greyish, with tints varying from yellowish to rusty and is distinctly spotted. They have a flared facial ruff, long prominent black ear tufts, and long hind legs with a short black-tipped tail. After a gestation of approximately 63-65 days, females produce a litter of 2-3 kittens. They reach independence by the age of 8-12 months, but will remain in their native territory until around 20 months old. The cat's sexual maturity is directly related to demographic and environmental factors. Most females will not reproduce until a territory has been secured. The Iberian lynx is the most endangered wild felid species in the world (IUCN, 2007). The biggest threats facing this species are the destruction of its habitat and prey

base, the endemic wild rabbit. Rabbits had suffered a major blow when myxomatosis occurred and, later, with the unintentional introduction of viral hemorrhagic pneumonia in Southern Spain, to which rabbits have no natural immunity, the lynxes' main prey case was seriously decimated. The wild felid also suffers from human encroachment, frequently being killed by traps and snares set for

rabbits, or by being hit by cars as the number of roads increases. Presently there are only two remnant populations - both in the province of Andalusia - with approximately 150-200 individuals altogether. The Spanish Government, together with the European Union, are now in the process of implementing a national conservation effort to save the Iberian lynxes from extinction.



Materials & Methods

Animals and samples

From 2003 to 2007, blood and serum samples were collected from 88 free-ranging Iberian lynxes, in both Doñana (n = 48) and Sierra Morena (n = 40) areas in the Southwest of Spain. Additional 23 foxes and 11 other small carnivores were analyzed (Table 1).

CDV serology

Antibodies to CDV were detected by IFA (Ramsauer et al. 2007). All sera were screened at a dilution of 1:40. Serum samples that tested positive at this dilution were titrated up to 1:320.

RNA purification and real-time RT-PCR assays

RNA was isolated from EDTA- or whole blood using the MagNA Pure LC TNA Isolation Kit 1 (Roche Diagnostics). As a positive control the RNA of a CDV vaccine strain (Onderstepoort) was used. With each RNA extraction a negative control was purified and amplified. Samples were analyzed by CDV real-time RT-PCR (Meli et al. 2009).

Amplification of P- and H-gene sequences

Positive CDV samples were further characterized by amplification of a 633 bp long part of the transcriptase-associated phosphoprotein (P-gene) and the complete sequence of the 1824 bp long glycoprotein hemagglutinin (H-gene) with published primers (Pardo et al. 2005,

Mochizuki et al. 1999). RNA was reverse-transcribed into cDNA using QuantiTect Reverse Transcriptase Kit (Qiagen). The PCR was performed using Phusion-Polymerase (Finnzymes). PCR products were analyzed on a 1.5% agarose gel.

Sequencing and phylogenetic analysis

PCR products were excised from gel and DNA was purified using the MinElute Gel Extraction Kit (Qiagen). All the amplified fragments were subjected to A-tailing and cloned into the pCRII-TOPO TA cloning[®] vector (Invitrogen). Inserts were identified by restriction enzyme digestion with EcoRI. Cycle sequencing was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Purified products (SigmaSpin Post-Reaction Purification Kit, Sigma) were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (www.megasoftware.net).

Statistical analysis

Data were analyzed in Excel 2007 (Microsoft SP2) and Analyse-it Clinical Laboratory Version 2.20 (Analyse-it Software, Ltd.). For the observed prevalence, 95% confidence intervals (CI) were calculated using R (Foundation for Statistical Computing). The frequency of infection between groups was compared using a two-tailed Fisher's exact test.

Results

The Iberian lynx found dead in 2005 and a stone marten (*Martes foina*) sampled in 2003 tested positive for CDV by real-time RT-PCR (Table 1). The serum of this latter animal as well as from a red fox tested positive for CDV antibodies. In addition, 14.8% of all the tested lynxes were positive for antibodies to CDV. The prevalence was significantly higher ($p=0.0318$) in animals roaming in the Doñana area (22.9%, 95% CI: 12 to 37.3) compared to the Sierra Morena area (5%, 95% CI: 0.6 to 16.9). Interestingly, all foxes tested negative for CDV antigen.

Part of the P-gene and the complete H-gene of the lynx and stone marten CDV strains were sequenced. The alignment of lynx and stone marten CDV strains revealed a 100% identity. Phylogenetic analyses of both, the P- and H-gene sequences, showed that the Iberian lynx and the stone marten CDV strains are closely related to CDV sequences of dogs of the European lineage (Figure 1a and b). The sequence identity is approximately 98%. They formed a cluster separate from the sequences identified as "Europe-wildlife" (Martella et al. 2006) and CDV isolates from South Africa, the United States, and Japan. The sequence identity to the Onderstepoort vaccine strain is only 92%.

Table 1: Number and origin of blood samples and CDV RT-PCR and IFA results

Animal	CDV RT-PCR Positive/tested (percentage)	CDV IFA Positive/tested (percentage)
Iberian lynxes	1/82 (1.2%) ^a	13/88 (14.8%)
Foxes	0/23 (0%)	1/6 (16.7%)
Other carnivores ^b	1/11 (9.1%) ^c	1/5 (20%) ^c

^a Lymph nodes and feces also CDV RT-PCR positive

^b Species included are: stone marten, badger, genet, wild cat, wild boar, mongoose

^c Species found to be positive: stone marten (PCR & IFA)

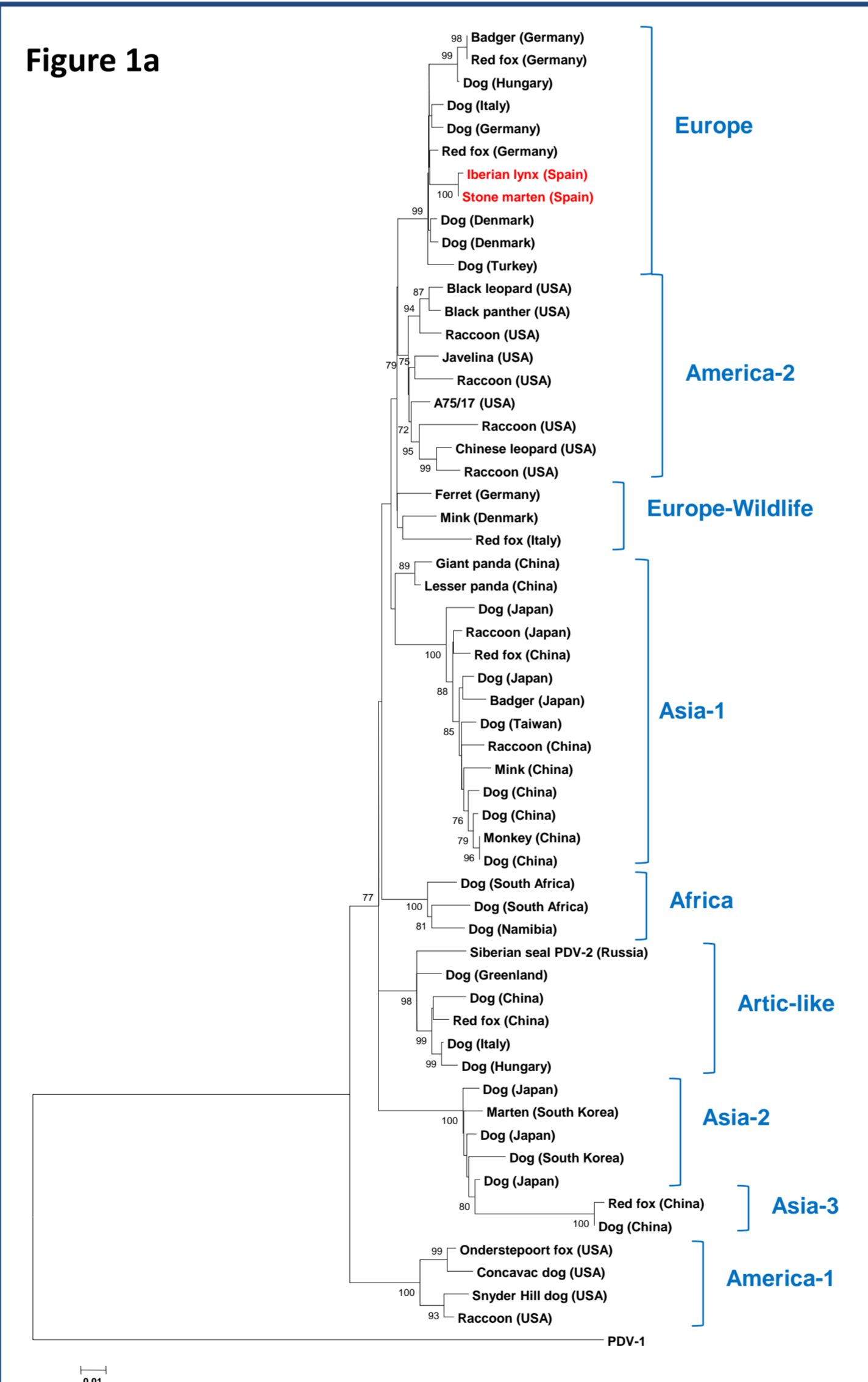
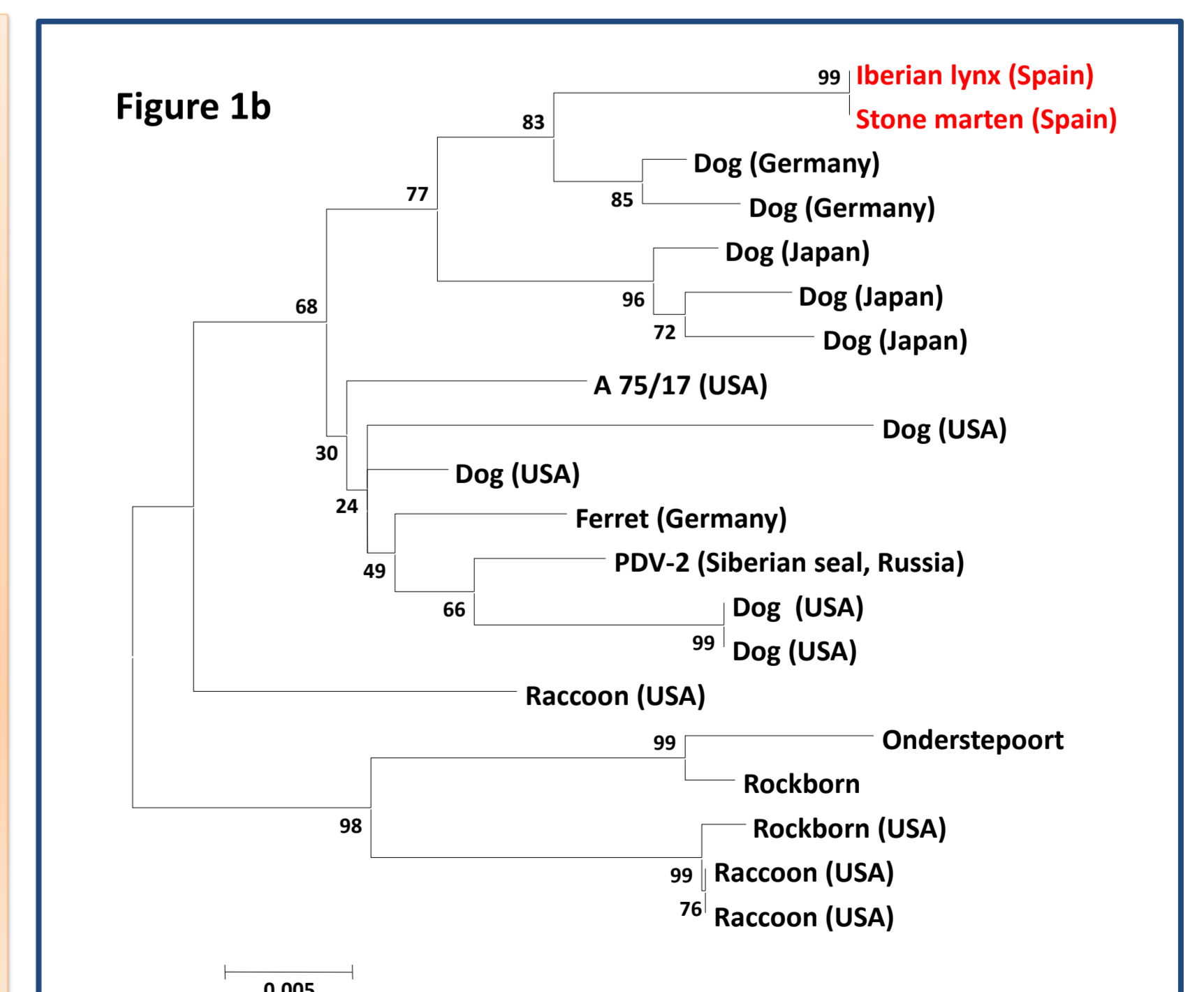


Figure 1: Phylogenetic trees of CDV strains based on complete H- (a) and partial P-gene (b) sequences. Bootstrap trees demonstrating the close evolutionary homology of the Iberian lynx and stone marten CDV isolates to the European dog lineage, forming a geographical clustering. Sequences alignment was performed using ClustalX. The trees were calculated using MEGA4 software (neighbour-joining algorithm, Kimura-2 parameter model). The numbers at the nodes were generated from 1'000 Bootstrap resamplings.

Discussion & Outlook

The occurrence of CDV antibodies in Iberian lynxes was not completely unexpected. Eurasian lynxes (*Lynx lynx*) and Canada lynxes (*Lynx canadensis*) had been reported to be seropositive (Biek et al., 2002; Schmidt-Posthaus et al., 2002), and recently a CDV-associated pathology was reported in free-living Canada lynxes and bobcats (*Lynx rufus*) (Daoust et al., 2009). On the other hand, a recent study on Iberian lynxes from the Doñana region and other carnivores sampled between 2004 and 2006 failed to detect CDV antibodies (Millan et al., 2009). Therefore, the discovery of a dead, highly CDV RNA-positive lynx is remarkable. Although the occurrence of CDV is well documented in many red fox populations of the Iberian peninsula, no CDV RNA was detected in the red foxes sampled in our study, and only one individual was CDV seropositive. One of the four samples collected from mustelids was CDV-positive; suggesting that a sizable portion of the mustelid population could be CDV-infected. The absence of CDV antibodies and antigen in the tested badger (*Meles meles*), otters (*Lutra lutra*), genets, wildcats (*Felis silvestris*) and Egyptian mongoose (*Herpestes ichneumon*) suggested that these carnivores were not important vectors for CDV to the Iberian lynxes. Nevertheless, the small number of samples analyzed did not allow a statistically relevant statement.

The CDV strains of lynx and marten were identical and showed a very close relationship to the European dog lineage of CDV, however they formed a cluster separate from the sequences identified as "Europe-wildlife" as well as from CDV isolates from other continents. This finding supports the hypothesis that both animals were exposed to the same European strain of CDV originating most probably from the local canine and/or carnivore population. In the Doñana Natural Space there are many species that can act as possible CDV reservoirs. In addition, there are villages in close proximity of the lynx areas. It is known from reports of researchers in Doñana that many domestic dogs and cats have access to areas that are inhabited by the lynxes. A mass CDV vaccination program for the local dog population could diminish the infectious pressure of circulating CDV in the wildlife, thus reducing the risk of an outbreak. Nevertheless, it also should be considered that a dog vaccination program may lead to a seronegative, naïve lynx population due to the low incidence of viral contact, thus resulting in a markedly increased susceptibility of the lynxes to CDV infection. Therefore, ideally, oral CDV vaccination of lynxes with a recombinant vaccine should be considered.



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