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Canine Distemper Epizootic in Everglades Mink

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ABSTRACT: Four free-ranging mink, *Neovison vison*, collected between June and September 2004 in the Fakahatchee Strand Preserve State Park (FSPSP, Florida, USA), were examined for canine distemper virus (CDV) infection. Microscopic lesions and viral inclusions consistent with CDV infection were observed in three mink. Virus isolation and reverse transcription-polymerase chain reaction performed on all mink were positive for CDV. Anecdotal records of mink observations in FSPSP suggest a postepizootic decline in the mink population followed by an apparent recovery. We recommend further research to assess the status of the Everglades mink and the impact of CDV on this and other American mink populations in Florida.

Key words: Canine distemper virus, Everglades mink, morbillivirus, *Mustela*, *Neovison vison evergladensis*, southern Florida.

Mink (*Neovison vison*) occur in three disjunct populations in Florida, USA (Humphrey, 1992). Two populations of mink inhabit the salt marshes of the northern Gulf and Atlantic coasts (Humphrey, 1992). The southern Florida population, the Everglades mink, is limited to the shallow freshwater marshes and long hydroperiod swamps of the Everglades and Big Cypress Swamp (Humphrey, 1992). The Everglades mink is described as rare and is listed as a threatened subspecies (*Mustela vison evergladensis*) by the Florida Fish and Wildlife Conservation Commission (Humphrey, 1992; Sullivan, 2006); however, there are no current estimates of population size or density.

Canine distemper virus (CDV) is a morbillivirus in the family *Paramyxoviridae* that has been reported worldwide in all families of terrestrial carnivores (Deem et al., 2000). Mustelids are among the carnivores most susceptible to CDV, and fatal CDV infections have been reported in captive or free-ranging black-footed ferrets (*Mustela nigripes*), striped skunks (*Mephitis mephitis*), martens (*Martes* sp.), polecats (*Mustela* sp.), Eurasian badgers (*Meles meles*), American badgers (*Taxidea taxus*), European otters (*Lutra lutra*), weasels (*Mustela* sp.), and ferret-badgers (Armstrong, 1942; Diters and Nielsen, 1978; Geisel, 1979; Williams et al., 1988; Kolbl et al., 1990; Alldinger et al., 1993; van Moll et al., 1995; Pavlacik et al., 2007; Chen et al., 2008). Infections also have been reported in captive and free-ranging European mink (*M. lutreola*; Sutherland-Smith et al., 1997; Deem et al., 2000; Guzmán et al., 2008) and captive American mink (Frank, 2001). Whereas epizootics of CDV in raccoon (*Procyon lotor*) and gray fox (*Urocyon cinereoargenteus*) have been documented in Florida, to our knowledge, CDV has not been reported previously in Florida populations of mink (Forrester, 1992).

Between June and September 2004, four sick or dead mink were collected by biologists in Fakahatchee Strand Preserve State Park (FSPSP), Florida, USA (26°00′N, 81°25′W; Fig. 1). Mink-2 was observed with clinical signs consistent with CDV, including ocular discharge, lethargy, and ataxia, and it was transported to the Conservancy of Southwest Florida's Wildlife Rehabilitation Center (Naples, Florida, USA) for treatment where it died

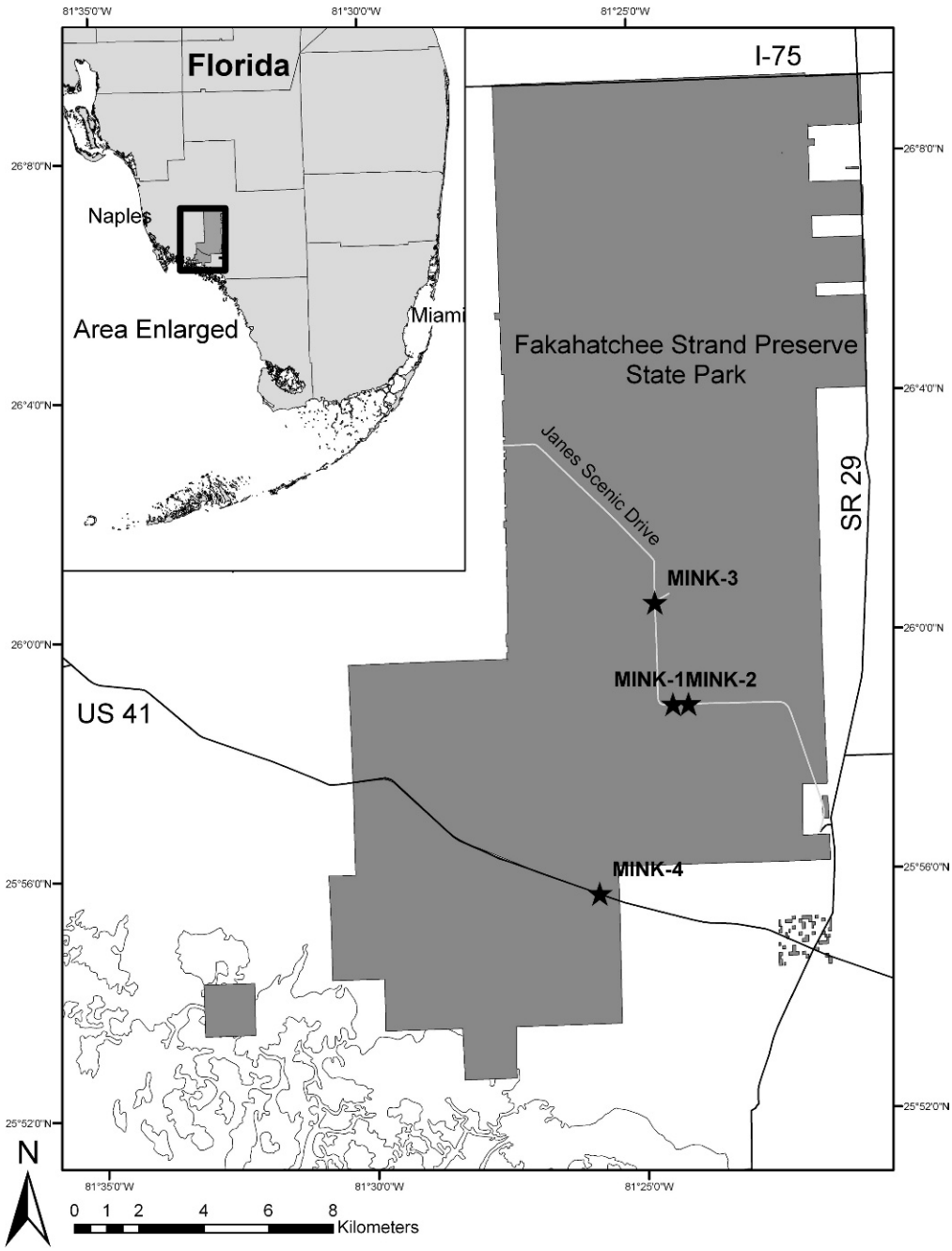

FIGURE 1. Distribution of Everglades mink testing positive for canine distemper virus in Fakahatchee Strand Preserve State Park, Collier County, Florida, USA, June–September 2004.

24 hr after presentation (Conservancy of Southwest Florida, unpubl. data). Three mink (mink-1, -3, and -4) were found dead from vehicular trauma. All dead mink were frozen at −20 C.

Three mink (mink-1, -2, and -3) were necropsied, and gross findings included mild thickening and hyperkeratosis of the footpads on all feet and, for mink-1 and -3, blunt trauma and skeletal fractures asso-

TABLE 1. Date collected, cause of death, and histopathologic lesions in Everglades mink infected with canine distemper virus (CDV) collected in Fakahatchee Strand Preserve State Park, Collier County, Florida, USA, May–September 2004.

No.	Date collected	Cause of death	Organs containing viral inclusions in epithelial cells	Other significant lesions
Mink-1	26 June 2004	HBV[a]	Footpads	Footpad hyperkeratosis with viral inclusions
Mink-2	16 July 2004	CDV[a]	Stomach, liver, kidney, lung, footpads	Hyperkeratotic pododermatitis with viral inclusions and intralesional yeast
Mink-3	2 August 2004	HBV	Stomach, lung, footpads	Footpad hyperkeratosis with viral inclusions
Mink-4	16 September 2004	HBV	N/A[b]	N/A

[a] HBV = hit by vehicle, CDV = canine distemper virus.

[b] N/A = not applicable.

ciated with vehicular collision. Freezing artifact obscured gross internal lesions. Brain tissue from mink-1 and -2 tested negative for rabies by direct fluorescent assay at the Jacksonville Central Laboratory (Florida Department of Health, Jacksonville, Florida, USA); however, tissue condition precluded a definitive test result. Complete tissue sets were saved in formalin and processed by routine methods for histopathology. Freezing artifact precluded detailed histopathologic examination; however, eosinophilic intracytoplasmic viral inclusions consistent with CDV inclusions were identified in epithelial cells in each of the three mink (Table 1). Hyperkeratosis of the footpads diagnosed grossly was confirmed microscopically in all three mink in association with viral inclusions in keratinocytes. Mink-2 also had evidence of a severe yeast infection of the hyperkeratotic keratin of the footpads.

Lung, liver, kidney, spleen, heart, urinary bladder, skin, conjunctiva, footpad, and mesenteric lymph node were taken collectively from all mink for virus isolation. Additionally, brain was taken for virus isolation and genetic comparison from an infected grey fox and raccoon. All clinical cases are listed in Table 2. Tissue samples (~0.5 cm^3) were mechanically homogenized in 650 µl of virus isolation media (1× minimal essential media, 2.2 g/l $NaHCO_3$, 3% fetal bovine serum, 400 units/ml penicillin, 400 µg/ml streptomycin, and 1 µg/ml amphotericin B). Homogenized tissues were centrifuged (6,700×G for 10 min) to pellet debris, and duplicate aliquots (100 µl each) of clarified supernatant were inoculated into 3-day-old Madin-Darby canine kidney and/or Crandell-Rees feline kidney cell culture in a 12-well plate format. Plates were then incubated at 37 C in a humidified atmosphere containing 5% CO_2 and passaged at 10-day intervals. At the end of the each passage (first, second, or both), viral RNA was extracted from culture supernatant using a QIAamp® Viral RNA Mini kit (QIAGEN, Valencia, California, USA) according to the manufacturer's instructions.

CDV-specific primers used for primary identification were CDV-N-327 (5′-ATCAGTATCCTCTCCTTGTT-3′) and CDV-N-697 (5′-TCGGAGATGAGAAGGTGGAT-3′), which amplify a 371-base pair (bp) product covering a portion of the nucleocapsid (N) protein gene. Primers internal to CDV-N-327/697 were used in nested reverse transcription-polymerase chain reaction (RT-PCR) to additionally detect CDV RNA from original tissue samples (CDV-N-420, 5′-ATCCCAAGCATCAACTCTGT-3′ and CDV-N-586, 5′-GCATCATCAACTTCTATGTCTA-3′). Because N sequences are well conserved

TABLE 2. Canine distemper virus (CDV) isolates sequenced for this study from the southeastern United States collected 2004–09.

Isolate designation	Host species	County/state (USA)	Yr of isolation	GenBank accession no.	
				N[a]	F[b]
CC 229-A-05	Mink	Collier Co., Florida	2004	EU375802	FJ710177
CC 229-B-05	Mink	Collier Co., Florida	2004	FJ710170	FJ710178
CC 229-C-05	Mink	Collier Co., Florida	2004	FJ710171	FJ710179
CC 44-07	Mink	Collier Co., Florida	2004	FJ710172	FJ710180
CC 230-05	Gray fox	Collier Co., Florida	2005	FJ710173	FJ710181
CC 50-08	Gray fox	Columbia Co., Florida	2008	FJ710174	FJ710182
CC 203-08	Raccoon	Pickens Co., South Carolina	2008	FJ710175	FJ710183
CC 315-08	Gray fox	Pinellas Co., Florida	2008	FJ710176	FJ710184
CC 14-09	Gray fox	Richmond Co., Georgia	2009	FJ710186	FJ710185

[a] N = nucleocapsid protein gene.

[b] F = fusion protein gene.

among CDV isolates, primers which amplify a 419-bp portion of the fusion (F) protein gene were used for genetic comparisons (CDV-F-4968, 5′-CRAAMACACACCCA ACAAG-3′ and CDV-F-5386, 5′-TCA GTCCCGATAATCCCAA-3′). Single-tube RT-PCRs (50 μl) were set up using 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton® X-100, 1.5 mM $MgCl_2$, 250 μM deoxynucleotide triphosphates, 0.5 μM of each primer, 2 U of avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wisconsin, USA), 1 U of *Taq* DNA polymerase (Promega), and 5 μl of extracted RNA. Cycling parameters were reverse transcription at 42 C for 20 min, followed by 40 cycles of denaturation at 94 C for 1 min, annealing at 50 C for 1 min, and extension at 72 C for 1 min. Nested parameters were identical barring the reverse-transcription step. Amplicons were electrophoresed on a 2% agarose ethidium bromide-stained TAE gel, excised, and purified from agarose using a QIAquick® Gel Extraction kit (QIAGEN); cloned using a PCR Cloning Plus® kit (QIAGEN); and subsequently purified using a QIAprep® Spin Miniprep kit (QIAGEN) according to the manufacturer's instructions. Sequencing of recombinant plasmid was performed at the Integrated Biotechnology Laboratories at the University of Georgia, Athens, Georgia, USA, using a 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Phylogenetic analysis by neighbor-joining and maximum parsimony (data not shown) methods of the predicted 126-amino acid portion of the F protein gene RT-PCR product was conducted using the Molecular Evolutionary Genetics Analysis 4 program (Tamura et al., 2007).

Canine distemper virus was isolated from all four mink submitted for virus isolation. In addition, CDV RNA was detected from all tissues tested by nested RT-PCR. Nucleotide analysis of the primerless N (data not shown) and F RT-PCR products revealed all four mink isolates (mink-1 [CC 229-A-05]), mink-2 [CC 229-B-05]), mink-3 [CC 229-C-05]), and mink-4 [CC 44-07]) were identical to each other in both gene sequences (Table 3). In addition, a gray fox isolate (CC 230-05) recovered in Collier County (Florida), in 2005 (i.e., 1 yr after the mink epizootic) shared an extremely high nucleotide identity to the mink isolates in both the nucleocapsid (100%) and fusion protein (99.7%) gene sequences, suggesting that this genotype may be endemic in southern Florida and cross-species transmission of CDV can occur between mink and gray foxes. To determine whether the CDV genotype isolated during the epizootic is widespread throughout the south-

TABLE 3. Comparative analysis of the nucleotide and deduced amino acid sequences of a 381-base pair product of the fusion (F) protein gene of canine distemper virus (CDV) field isolates from the southeastern and midwestern United States, along with selected vaccine strains. Percentage of nucleotide and amino identities are represented in the upper and lower portion of the graph, respectively.

	CC 229-A-05	CC 229-B-05	CC 229-C-05	CC 44-07	CC 230-05	CC 50-08	CC 203-08	CC 315-08	CC 14-09	A75-17	98-2645	00-2601	01-2689	Onderstepoort	Rockborn
CC 229-A-05/ mink-1/FL[a]	•	100	100	100	99.7	88.7	97.6	98.7	91.3	96.3	93.7	91.1	95.5	85.3	92.9
CC 229-B-05/ mink-2/FL[a]	100	•	100	100	99.7	88.7	97.6	98.7	91.3	96.3	93.7	91.1	95.5	85.3	92.9
CC 229-C-05/ mink-3/FL[a]	100	100	•	100	99.7	88.7	97.6	98.7	91.3	96.3	93.7	91.1	95.5	85.3	92.9
CC 44-07/mink-4/ FL[a]	100	100	100	•	99.7	88.7	97.6	98.7	91.3	96.3	93.7	91.1	95.5	85.3	92.9
CC 230-05/gray fox/FL[a]	99.2	99.2	99.2	99.2	•	88.4	97.4	98.4	91.1	96.1	93.4	90.8	95.3	85.0	92.7
CC 50-08/gray fox/FL[a]	79.4	79.4	79.4	79.4	78.6	•	87.7	88.2	86.9	89.8	88.5	88.2	89.0	85.6	92.1
CC 203-08/raccoon/SC[a]	95.2	95.2	95.2	95.2	94.4	76.2	•	96.3	90.6	95.5	93.2	90.3	94.8	84.5	92.4
CC 315-08/gray fox/FL[a]	97.6	97.6	97.6	97.6	96.8	78.6	92.9	•	90.0	95.0	92.4	90.0	94.2	84.8	91.6
CC 14-09/gray fox/GA[a]	82.5	82.5	82.5	82.5	81.7	73.8	82.5	81.0	•	92.9	90.3	90.6	92.1	84.3	93.7
A75-17/dog[b]	92.1	92.1	92.1	92.1	91.3	79.4	92.9	89.7	84.1	•	96.3	94.2	99.2	86.9	95.0
98-2645/raccoon/ IL[b]	85.0	85.0	85.0	85.0	84.1	74.6	84.9	82.5	76.2	90.5	•	91.6	95.5	90.0	93.4
00-2601/raccoon/ IL[b]	82.5	82.5	82.5	82.5	81.7	72.2	83.3	80.2	81.7	90.5	82.5	•	93.4	89.5	93.2
01-2689/raccoon/ IL[b]	92.1	92.1	92.1	92.1	91.3	79.4	92.9	89.7	84.1	100	90.5	90.5	•	86.1	94.2
Onderstepoort/ vaccine[b]	69.8	69.8	69.8	69.8	69.0	68.3	68.3	68.3	65.1	72.2	80.2	75.4	72.2	•	89.5
Rockborn/vaccine[b]	86.5	86.5	86.5	86.5	85.7	84.1	86.5	84.1	85.7	90.5	85.7	86.5	90.5	77.8	•

[a] Isolates sequenced for this study; GenBank accession numbers are listed in Table 2.

[b] GenBank isolate/strains used for comparison: A75-17 (AF112188/AAD18007), 98-2645 (AY445077/AAR16539), 00-2601 (AY395984/AAQ96334), 01-2689 (AY649446/AAT94552), Onderstepoort (AF378705/AAK54668).

eastern United States or is localized to southern Florida, four additional CDV isolates recovered from three southeastern states during 2008–09 also were analyzed: 1) CC 50-08, gray fox, Columbia County (Florida); 2) CC 203-08, raccoon, Pickens County (South Carolina, USA); 3) CC 315-08, gray fox (Pinellas County, Florida); and 4) CC 14-09, gray fox (Richmond County, Georgia, USA). The two additional Floridian gray fox isolates, CC 50-08 and CC 315-08, represent counties that are located in northern and central locations of the state, respectively.

Nucleotide comparisons of the N (data not shown) and F gene sequences of the 2004 mink isolates from Florida with a 2008 raccoon isolate from South Carolina (CC 203-08) revealed a 99.4% and 97.6% identity, respectively (Table 3), suggesting that this genotype may be temporally and spatially widespread throughout the southeastern United States irrespective of host or location. Phylogenetic analysis of the F protein sequences also indicated that the four mink isolates, the gray fox isolates from southern (CC 230-05) and central (CC 315-08) Florida, along with the South Carolina raccoon isolate formed a distinct clade separate from other field isolates or vaccine strains (Fig. 2); these seven isolates, however, did group (albeit with a low bootstrap value) with the virulent A75-17 strain and a 2001 raccoon isolate from Illinois (01-2689), suggesting an evolutionary link with viruses derived from the dominant American CDV A75-17 lineage (Lednicky et al., 2004).

Gray fox isolates from northern Florida (CC 50-08) and eastern Georgia (CC 14-09) were genetically distinct from the mink isolates, sharing only a 79.4% and 82.5% amino acid identity in the F protein sequences, respectively, suggesting multiple CDV genotypes may be circulating in the southeastern United States (Table 3). Phylogenetic analysis of the F protein sequences weakly grouped CC 50-08 and CC 14-09 with European and Asian field and vaccine strains, along with dog isolates recently described from Missouri, USA (Pardo et al., 2005) (Fig. 2). Very low bootstrap values, however, indicate that these relationships are unlikely to be evolutionarily informative and suggest that these two gray fox isolates are divergent from each other and CDV isolates currently in public databases. Because the source of either CC 50-08 or CC 14-09 is speculative, a more comprehensive genetic analysis (e.g., full-length fusion and hemagglutinin genes) of these and additional field (and dog) isolates is needed to clarify the evolutionary relationships of CDV strains circulating in wildlife populations in the southeastern United States.

Everglades mink seem to be susceptible to CDV, and all necropsied mink had gross and histopathologic changes associated with infection. Neurologic abnormalities were observed in one mink, and neurologic impairment secondary to CDV infection in the remaining three mink may have led to greater susceptibility to vehicular collision.

In other species, CDV epizootics had significant population impacts (Davidson et al., 1992) and contributed to the extirpation of black-footed ferrets from the wild (Thorne and Williams, 1988). The known susceptibility of mink (Deem et al., 2000) and the listed status of the Everglades mink raise concerns that CDV could pose a significant threat to the population.

The impact of the recent CDV epizootic on the mink population in FSPSP is unknown, although the distance between carcasses recovered in FSPSP (Fig. 1) suggests the epizootic was extensive. There are no population estimates before or after the epizootic; however, anecdotal observations by FSPSP personnel suggest a decline and subsequent recovery after the epizootic (Owen, unpub. data). These data are based on casual observations and should be interpreted with caution. The absence of historic or current estimates of

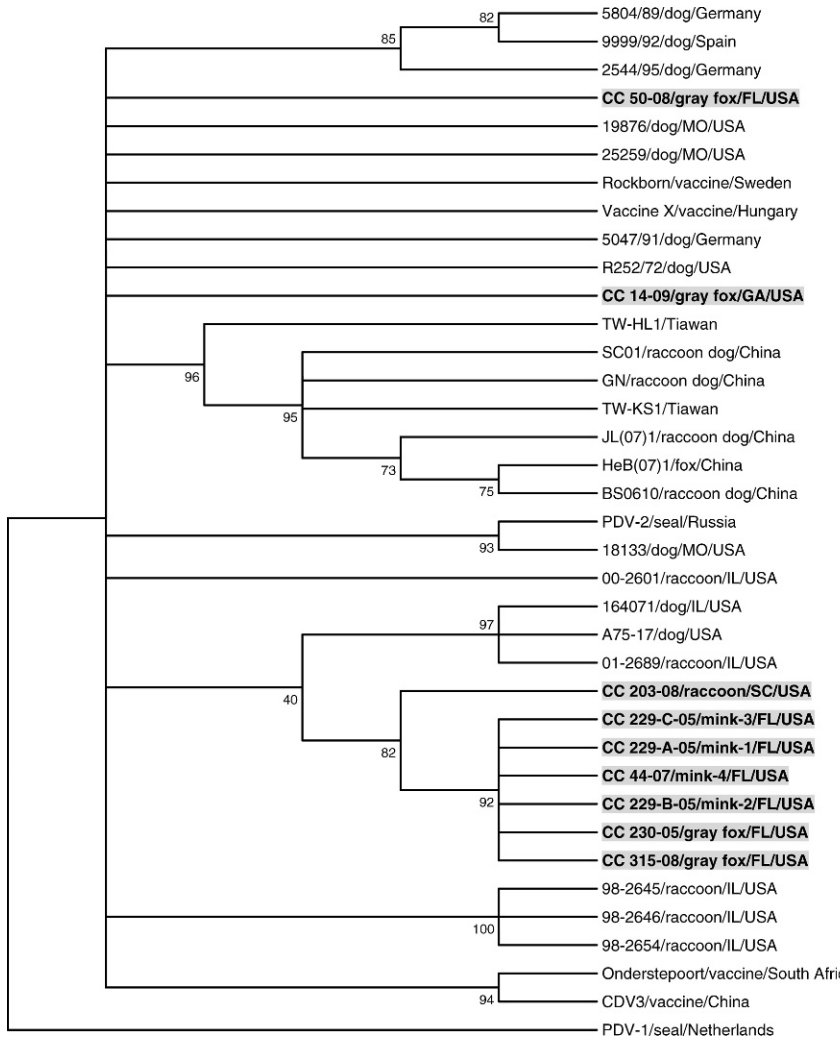

FIGURE 2. Neighbor-joining phylogeny of a 126-amino acid portion of the canine distemper virus (CDV) fusion (F) protein gene. The tree was condensed with a bootstrap cut-off value of 40%. Bootstrap confidence levels were determined using 500 replicates. Amino acid distances were estimated using the Poisson correction method. Phocine distemper virus-1 (PDV-1) was used as an outgroup. Species and geographic location of each CDV isolate/strain is indicated, when available. Viruses sequenced for this study are highlighted and in bold. GenBank F sequences used for comparative analysis were 5804/89 (AAB88266), 9999/92 (AAB88267), 2544/95 (AAB88264), 19876 (AAX89389), 25259 (AAX89395), Rockborn (AAB88269), Vaccine X (ABW77566), 5047/91 (AAB88265), R252/72 (AAB88268), TW-HL1 (ABX76264), SC01 (ABR08392), GN (ABR08390), TW-KS1 (ABX76276), JL(07)1 (ABY49138), HeB(07)1 (ABY49137), BS0610 (ACL12528), PDV-2 (L07075), 18133 (AAX89386), 00-2601 (AAR32272), 164071 (ACD92996), A75-17 (AAD18007), 01-2689 (AAT94552), 98-2645 (AAR16539), 98-2646 (AAS48411), 98-2654 (AAR30102), Onderstepoort (AAG30919), CDV3 (ABX59699), and PDV-1 (CAA12079).

the size or density of the mink population in southern Florida precludes a defensible assessment of trend data. More research is needed to determine the population status of Everglades mink and the impact of CDV on this and other mink populations in Florida.

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