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Natural disease and evolution of an *Amdoparvovirus* endemic in striped skunks (*Mephitis mephitis*)

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Abstract

Striped skunks (*Mephitis mephitis*) densely populate the human–animal interface of suburbia throughout North America. Skunks share that habitat with numerous related mesocarnivores, where increased contact, competition for shared food and water sources and other stressors contribute to increased exposure and susceptibility to viral infection. The recently identified skunk amdoparvovirus (SKAV) has been detected at high prevalence in skunks and occasionally in mink, but its distribution in North America is unknown. To understand the impact of SKAV in striped skunks and the risk posed to related species, we investigated the geographic distribution of SKAV, analysed its genetic diversity and evolutionary dynamics and evaluated viral distribution in tissues of infected animals to identify possible mechanisms of transmission. SKAV was detected in 72.5%

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. This study used samples collected opportunistically from dead animals. No ethical approval was required.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

(37/51) skunks and was present at high rates at all locations tested across North America. Analysis of the complete genomic sequence of 29 strains showed a clear geographic segregation, frequent recombination and marked differences in the evolutionary dynamics of the major structural (VP2) and non-structural (NS1) proteins. NS1 was characterized by a higher variability and a higher percentage of positively selected codons. This could indicate that antibody-mediated enhancement of infection occurs in SKAV, an infection strategy that may be conserved across amdoparvoviruses. Finally, in situ hybridization revealed virus in epithelium of the gastrointestinal tract, urinary tract and skin, indicating that viral transmission could occur via oronasal, faecal and/or urinary secretions, as well as from skin and hair. The endemicity of SKAV over large geographic distances and its high genetic diversity suggest a long-term virus–host association. Persistent shedding and high environmental stability likely contribute to efficient viral spread, simultaneously offering opportunities for cross-species transmission with consequent risk to sympatric species, including domestic animals and wildlife.

Keywords

carnivore amdoparvovirus; parvovirus; phylogeography; SKAV; skunk; tissue distribution

1 | INTRODUCTION

Amdoparvovirus is a rapidly growing genus within the viral family *Parvoviridae* comprising several important pathogens of carnivorans (Canuti et al., 2015, 2020; Pénzes et al., 2020). The best known and prototypical *Amdoparvovirus* is Aleutian mink disease virus (AMDV, species *Carnivore Amdoparvovirus 1*), which causes persistent infections in mink (family Mustelidae) and related species. Clinical outcomes range from subclinical infection to fatal inflammatory disease, but the genetic determinants of host tropism and disease manifestations are not entirely characterized (Canuti et al., 2020). For decades after its discovery in the mid-20th century, AMDV was the only recognized *Amdoparvovirus* (former genus *Amdovirus*), but advances in viral detection and sequencing have led to a considerable expansion of the genus in the past decade (Alex et al., 2018; Canuti et al., 2017, 2020; Li et al., 2011; Shao et al., 2014).

Most known amdoparvoviruses infect carnivorans of the superfamily Musteloidea or family Canidae. The genus now includes five officially recognized members: AMDV, Gray fox amdovirus (GFAV) (Li et al., 2011), Raccoon dog and fox amdoparvovirus (RDFV) (Shao et al., 2014), Skunk amdoparvovirus (SKAV) (Canuti et al., 2017) and Red panda amdoparvovirus (RPAV) (Alex et al., 2018), classified as *Carnivore amdoparvovirus 2* through *5*, respectively (Cotmore et al., 2019; Pénzes et al., 2020). Additional amdoparvoviruses have been identified in foxes (red fox faecal amdovirus; Bodewes et al., 2013) and foxes and martens (Labrador amdoparvovirus 1 and 2; Canuti et al., 2020), and recent studies suggest that bats and rodents can also be amdoparvovirus hosts (Lau et al., 2017; Pénzes et al., 2018; Wu et al., 2016, 2018). Members of the genus possess a small (~5 kb) single-stranded DNA genome that includes two main coding cassettes, one containing information for the three non-structural proteins NS1–3 and one coding for the two capsid proteins VP1–2 (Canuti et al., 2016). The two coding regions are characterized

by different evolutionary dynamics, and, unlike other parvoviruses, the VP open reading frame (ORF) is more genetically conserved than the NS ORF (Canuti et al., 2017; Nituch et al., 2015) and this also true when comparing viruses from different species (Canuti et al., 2020). This antigenic stability is likely linked to the fact that antibodies directed against the viral capsid can enhance viral uptake by circulating macrophages, a known cellular target for viral replication (Bloom et al., 2001). This mechanism, known as antibody-dependent enhancement (ADE), has been described for AMDV (Bloom et al., 2001; Kanno et al., 1993) but is also presumed to occur for other amdoparvoviruses. Besides AMDV, little is known about the pathogenicity and the evolutionary dynamics that characterize viruses in this genus.

AMDV infection in farm-raised mink is associated with significant disease, but the prevalence and impact of amdoparvovirus infections in free-ranging populations of any species have not been well characterized (Alex et al., 2018; Ladouceur et al., 2015; Shao et al., 2014). Amdoparvoviruses lack strict host specificity and are known to infect multiple hosts (Canuti et al., 2015; Canuti, McDonald, et al., 2020; Canuti, Todd, et al., 2020; Shao et al., 2014). This plasticity in host range, coupled with the crowding of susceptible species in and around urban environments and the notorious durability of parvoviral virions in the environment, could allow for frequent exposures of potentially susceptible hosts. Very little is known about the behaviour of amdoparvoviruses in novel hosts, or in situations of co-infection by multiple amdoparvoviral species. These circumstances could lead to outbreaks in previously uninfected species, novel disease presentations or the emergence of novel recombinant genotypes with alterations in virulence and host tropism. Phylogenetic and epidemiological data are therefore imperative for understanding the impact of amdoparvoviral infections in their primary hosts and the risks posed to related species.

Striped skunks (*Mephitis mephitis*) are ubiquitous in North America where they share dens, food and water sources with multiple other animals, and as such, they are poised to play an important role as reservoirs and spreaders of viral infections among wildlife and domestic animals (Rosatte, 2003). SKAV was recognized as a distinct viral species in 2017 and has been detected with remarkably high prevalence in striped skunks from British Columbia (86%) and California (65%), and while subclinical infections appear to be common, infections have been associated with disease in some cases (Britton et al., 2017; Canuti et al., 2017; Glueckert et al., 2019; Ladouceur et al., 2015). Moreover, SKAV has been shown to infect mink, and AMDV has, in turn, been found in skunks (Nituch et al., 2012), but the consequences of these infections and the potential for co-infections are unknown. While SKAV has been identified in multiple sites across North America, its current geographic range is not known and its genetic variability is not well characterized. Additionally, the basic natural history of the virus, such as potential routes of transmission and tissue tropism, has not been characterized. This context is critical for understanding the impact of SKAV infections in skunks, and the risks posed to other potentially susceptible hosts. Therefore, the objectives of this study were to investigate the geographic distribution of SKAV in striped skunks across North America, assess the range of genetic diversity and regional variation in SKAV by analysing sequences from disparate sites, study SKAV evolutionary dynamics and investigate tissue distribution of SKAV in infected skunks to

increase our understanding of its pathology and identify potential sources of shedding and transmission routes.

2 | MATERIALS AND METHODS

2.1 | Animals and samples

Spleen samples were collected from striped skunks that died or were euthanized for purposes unrelated to this study. For the California cohort, samples were taken from skunks that died or were euthanized at a wildlife rescue organization, all of which originated from within an approximately 20-mile radius around Sonoma County, CA. Ten samples from skunks from Ontario were received from the Ontario Ministry of Northern Development, Mines, Natural Resources (OMNDMNRF) and were collected in 2020 as part of a rabies surveillance program in the Golden Horseshoe area of Southern Ontario (all were rabies negative). From Nova Scotia, samples originated from a trapped and euthanized skunk, which had been exhibiting signs of disease and poor doing for several weeks prior to euthanasia. The three samples from British Columbia originated from cases that were initially reported in a previous publication (Canuti et al., 2017). From New England (New Hampshire, Vermont and Maine), samples originated from licensed trappers collecting nuisance animals during the 2019–2020 harvest. Animals were collected in Durham, Pembroke, Lebanon and Canaan NH, St. Johnsbury and Burton, VT and Greene and Waterville, ME. DNA was extracted from approximately 10 mg of tissue using the DNEasy Blood and Tissue Kit (Qiagen) and eluted in 100 μl elution buffer.

Handling of samples at Memorial University of Newfoundland was done under Animal Use Protocols 15-04-AL and 20-04-AL.

2.2 | Detection of SKAV and sequence amplification

All samples originating from the USA were screened for SKAV using primers SKAV-F1 (AGAGCAACCAAACCACCC) and SKAV-R1 (TCACCCCAAAAGTGACC), targeting a 365-nucleotide long segment of the VP ORF (Allender et al., 2008). PCR products were examined by agarose gel electrophoresis. Samples yielding bands of the expected size were purified using the QIAquick PCR purification kit (Qiagen) or ExoSAP-IT PCR Product Clean-up Reagent (ThermoFisher) and Sanger-sequenced to confirm identity. All samples originating from Canada were screened using previously described protocols (Canuti et al., 2016, 2017; Canuti et al., 2020). From a selection of positive samples, we chose randomly among PCR-positive cases to represent each investigated area and among the two previously reported lineages identified in BC (Canuti et al., 2017), and whole viral coding regions were generated by PCR amplification of overlapping fragments and Sanger sequencing. All primers used for genome amplifications are available upon request.

2.3 | Sequence and phylogenetic analysis

Amplicon sequences were inspected for quality, manually edited as necessary and assembled to generate full coding sequences using Geneious R11 (Biomatters). Sequences from this study were then compared to near-full genomic sequences of seven previously published SKAV sequences originating from British Columbia (Canuti et al., 2017). Splice sites for

NS2, NS3 and VP1 were determined based on previous predictions for SKAV (Canuti et al., 2017), splice patterns were reproduced in silico and the resulting protein-coding sequences were used to predict protein sequences. Nucleotide and protein alignments were performed using ClustalW 2.0 (Larkin et al., 2007), optimal substitution models for genetic distance estimates were determined by the lowest Bayesian information criterion (BIC) with the ModelFinder function in IQ-TREE 2 (Kalyaanamoorthy et al., 2017; Minh et al., 2020) and maximum likelihood phylogenetic trees were generated with IQ-TREE 2. Branch support was assessed with both ultrafast bootstrap approximation (ufBoot; Hoang et al., 2018) and SH-like approximate likelihood ratio test (SH-aLRT; Guindon et al., 2010). Three AMDV strains were used as outgroup in each tree. The obtained trees were annotated in MEGA X (Kumar et al., 2018) and final figures prepared with INKSCAPE (https://inkscape.org/, downloaded on 19 June 2020). Pairwise sequence identities were calculated as a percentage value of 1–p distance.

2.4 | Recombination and selection pressure analyses

Full-length alignments were evaluated for recombinant genomes as previously described (Canuti et al., 2016) by using all methods included in the RDP 5.5 (Martin et al., 2015) package, and only events detected by at least three methods with $p < .05$ were considered. Additionally, a breakpoint analysis was also performed with GARD (Kosakovsky Pond et al., 2006) through Datamonkey 2.0 (Weaver et al., 2018).

Sites under positive and purifying selection were assessed with Fixed Effect Likelihood (FEL; Kosakovsky Pond & Frost, 2005) and Fast Unconstrained Bayesian Approximation for inferring selection (FUBAR; Murrell et al., 2013), while episodic positive or diversifying selection was assessed by Mixed Effects Model of Evolution (MEME; Murrell et al., 2012) using default parameters through Datamonkey.

2.5 | in situ hybridization

We performed colorimetric in situ hybridization to demonstrate the presence of viral genomic DNA in tissue sections from selected cases from the California cohort. The assay was performed on 5-μm thick sections of formalin-fixed, paraffin-embedded tissues on Superfrost Plus slides (Fisher Scientific). We designed a set of 20 ZZ-paired probes spanning the VP ORF of SKAV (probe set V-SK23-VP1, Advanced Cell Diagnostics, Inc.), corresponding to nucleotide positions 2066–4186 of a reference genome (accession number KX981923). To evaluate likely pathways of viral shedding, selected tissues included sections of the gastrointestinal tract, urinary tract and integument. Tissue sections were pre-treated with protease for 2 h at 40°C prior to hybridization, assayed using the RNAscope 2.5 Red Assay Kit (Advanced Cell Diagnostics, Inc.), counterstained with haematoxylin and mounted with EcoMount (Biocare Medical). Serial sections were also tested using an unrelated, GC-content-matched probe as a negative control.

2.6 | Data availability

All sequences obtained in this study have been deposited in GenBank under accession numbers OL889855-OL889879.

3 | RESULTS

3.1 | Amdoparvovirus detection and molecular typing

We detected SKAV infections in regions where skunk amdoparvoviruses have not previously been reported (Maine, Vermont and New Hampshire) and in areas where AMDV or SKAV were previously detected (California, Ontario and Nova Scotia) (Canuti et al., 2016; Farid, 2013; Nituch et al., 2015) (Table 1). Although limited numbers of samples were available for each investigated region, viral detection rate was high in all locations. Excluding samples reported in a previous study (Britton et al., 2017), 72.5% (37/51) of investigated samples were positive, indicating amdoparvoviral endemicity in these areas.

All PCR-positive samples were confirmed to be infected by SKAV by sequencing, and we amplified complete or near-complete amdoparvoviral coding sequences from a selection of samples across the study area. The final dataset included novel sequences from seven animals from California, seven animals from Ontario, five animals from northeastern North America (two from Maine, two from New Hampshire and one from Nova Scotia) and three additional animals from British Columbia from a previously investigated cohort (Canuti et al., 2017). Three additional VP sequences were obtained from animals from California. Genomes were 91.8–98% identical to each other, and the genome organization was identical for all strains and consistent with amdoparvoviral genome structure. All sequences obtained in this study were most closely related to seven previously published complete genomic sequences of SKAV from British Columbia (Canuti et al., 2017), which were therefore included in all following analyses, and they all belonged to the species *Carnivore amdoparvovirus 4* with pairwise amino acid identities of 84.6–100% among all 36 complete NS1 sequences.

3.2 | Recombination analysis

Since parvoviruses are known to increase their genetic variability through recombination (Canuti et al., 2016; Cotmore et al., 2019), a search for chimeric genomes and breakpoint predictions was performed with RDP and GARD. Both methods provided evidence for multiple possible recombination events in the evolutionary history of these viruses. Specifically, over the complete genome, RDP detected 10 potential recombination events and GARD inferred 11 different breakpoints (Table S1). All potential recombinant sequences detected in RDP were from BC or Ontario. As expected, both methods also showed phylogenetic discordance between the NS1 and VP2 regions. Since amdoparvoviral structural and nonstructural proteins experience different evolutionary constraints, discordant topologies of trees constructed with these two regions can be linked to different selection pressure dynamics and evolutionary rates as well as to recombination (Canuti et al., 2016). For these reasons, the two ORFs were also examined separately.

Since some methods, like GARD, are known to overestimate recombination, especially in cases of asymmetric tree topology (Bay & Bielawski, 2011), we elected to explore in more detail only highly supported potential breakpoints that were detected in multiple events and by both methods on alignments of the whole genome as well as both ORFs separately. Four of the six events involving NS1 detected by RDP were caused by a breakpoint at

nt ~850 to 950 that roughly divides the NS1 ORF into two parts, and a breakpoint in this region was also detected by GARD. This is similar to what was previously identified for AMDV (Canuti et al., 2016). Possibly because of a higher sequence homogeneity and consequent difficulty in determining breakpoints and obtaining supported clades, fewer recombination events were detected for VP2. However, both methods identified a hotspot of potential breakpoints located between nt 500 and 800 of the VP2 ORF. The occurrence of recombination was further confirmed by phylogenetic analyses.

3.3 | Phylogenetic analysis

To study the molecular epidemiology of SKAV in North America, maximum likelihood phylogenetic trees were built with all nucleotide sequences of the full NS1 andVP2 ORFs separately, as well as with portions of these genes from between identified breakpoints (Figure 1). The trees built with the full ORFs (left panels) showed a clear segregation of strains depending on sampling sites but with inconsistent clustering. Strain clustering within the NS1 tree mirrored the geography of sample collection locations, with a clade including all sequences from the West Coast (British Columbia and California) and another containing eastern sequences (Ontario, Nova Scotia, Maine, Vermont and New Hampshire). However, the tree built with VP2 ORF showed the presence of three separate clades, one single-sequence clade formed by the strain from Nova Scotia, one including sequences from California and Ontario and one containing all other sequences. Additional differences between the two trees included the presence of three distinct subgroups of sequences from British Columbia in the NS1 tree but not in the VP2 tree, different subgroups of sequences from Ontario in the two trees and the presence of a clade including sequences from Maine, Vermont and New Hampshire in the NS1 tree but not in the VP2 tree.

The presence of these groups and subgroups was also confirmed by sequence identities within the detected groups (Table S2), considering a cut-off of within-group identity of at least 94%. These results confirmed a different evolutionary history for the two genes and an overall higher amino acid sequence identity for VP2 (91.0%–99.8%) compared to NS1 (84.6%–97.3%).

Finally, trees built with portions of the two genes determined by the main predicted potential breakpoints showed further inconsistencies (Figure 1, middle and right panels). In each of these trees, the geographic segregation was only partially preserved, with both sequences from British Columbia and Ontario, identified as recombinant by RDP, sometimes segregating into different clades. This pattern confirms that recombination is a frequent occurrence for these viruses, as was previously shown for AMDV (Canuti et al., 2016). Additionally, since inconsistencies involved both closely related and distant sequences, we can hypothesize that this pattern was a result of both old and more recent recombination events.

3.4 | Site-by-site selection pressure analysis

Since selection pressure estimates are affected by recombination, which can cause an overestimation of positive selection pressure (Anisimova et al., 2003), this analysis was performed on both genes separately by splitting the alignments according to identified

breakpoints and by removing additional potential recombinant sequences until RDP found no evidence for recombination in the alignments. This resulted in two alignments (nt 1–849 and 850–1926) of 25 sequences for NS1 (sequences removed: BC_SK29, BC_SK24, BC_SK10 and ON_SSK6) and in one alignment of 29 sequences for VP2 (sequences removed: BC_SK1, BC_SK29, ON_SSK7). While the percentage of negatively selected sites detected by FEL was similar for the two ORFs (21.8% vs. 20.3% for NS1 and VP2, respectively) and the percentage of negatively selected sites detected by FUBAR was higher for VP2 compared to NS1 (23.4% vs. 15.1%), the percentage of positively selected sites was higher for NS1 compared to VP2 with all used methods (FEL: 2.2% vs. 0.9%; FUBAR: 4.7% vs. 1.9%; MEME: 5.9% vs. 2.3%), confirming that different selective forces act on the two genes, as also previously observed for AMDV (Canuti et al., 2016). A list of sites under episodic and pervasive diversifying and purifying selection found by all methods is available in Table S3.

As this high antigenic stability could be linked to ADE, we specifically evaluated selection pressure at residues within predicted SKAV immunogenic loops. Linear epitopes of AMDV that were experimentally determined to be immunogenic were used as references and a high sequence conservation between the VP2 of SKAV and AMDV allowed us to easily identify these regions in SKAV (resides 84–102, 224–247, 307–322 and 438–497 with respect to the VP2ORF of strain CA_S5) (Bloom et al., 1997, 2001; Lu et al., 2018; McKenna et al., 1999). Interestingly, while positively selected sites could be identified in all four immunogenic loops, no positively selected sites were found in the epitope between residues 427 and 445. This region has been experimentally determined to be highly immunogenic in AMDV, and antibodies recognizing this epitope were capable of aggregating virus particles into immune complexes, mediating ADE and neutralizing virus infectivity in vitro (Bloom et al., 1997, 2001). In this region of SKAV VP2, there were three variable sites, most of them found in sequences from Ontario, and no residues under diversifying selection were found, whereas 26% (FEL) or 37% (FUBAR) of the sites were under negative selection pressure (Figure S1). These results show that immune response against SKAV and AMDV could involve the same VP2 epitopes and that the same immunogenic regions are likely involved in SKAV and AMDV immune-mediated pathogenicity.

3.5 | Tissue distribution and pathways of transmission

To identify possible routes of viral shedding, we evaluated histologic sections of the urinary tract, gastrointestinal tract and integument from representative cases by in situ hybridization (ISH). In a subset of cases, we detected SKAV in all three systems evaluated (Figure 2). In the urinary tract, the kidney was a site of infection, with SKAV detectable in renal tubular epithelial cells, protein casts within renal tubules and in infiltrating interstitial inflammatory cells of animals with chronic tubulointerstitial nephritis (presumed macrophages, lymphocytes and/or plasma cells). The gastrointestinal tract was a site of infection at multiple levels. Robust signal was detected in the basal epithelium of the tongue and in the gastric epithelium. In the small intestine, SKAV nucleic acid was identified in scattered individual cells in the epithelium and lamina propria (presumed phagocytized epithelial cells and/or infiltrating inflammatory cells). In the skin, signal was localized to the

basal epidermis, follicular epithelial cells and scattered cells in the superficial subepithelial stroma (presumed to represent infiltrating inflammatory cells).

4 | DISCUSSION

SKAV, like other amdoparvoviruses, is a multi-host pathogen and is apparently capable of causing disease in skunks and mink, but its full host spectrum remains unknown. Furthermore, given the recent definition of this virus as a distinct species, SKAV dispersal in its maintenance host and mechanisms of viral spread, such as transmission routes and tissue tropism, are still largely unexplored. In this study, we provide evidence that SKAV is likely widespread across North America and identify aspects of virus biology and evolution that could favour efficient viral transmission and perpetuation.

4.1 | Distribution, diversity and evolutionary dynamics of SKAV

Our screening efforts allowed us to detect SKAV in various and distant areas of North America, including British Columbia (Canada) and California (USA) on the west coast, Ontario (Canada) and multiple sites from northeastern North America (New Hampshire, Vermont and Maine, USA, and Nova Scotia, Canada), corroborating results of previous investigations (Canuti et al., 2017; Glueckert et al., 2019; Nituch et al., 2015) and adding new sites to the known geographic range of SKAV. Additionally, although sample sizes were generally too small for accurate assessment of prevalence, viral detection rates in areas where multiple samples were available was high, confirming the high prevalence reported by other studies (Glueckert et al., 2019; Nituch et al., 2015). It is highly likely that SKAV, like its close relative AMDV, causes persistent infections and accumulates in spleens, allowing the virus to reach such high prevalence. Nonetheless, although more samples collected in areas not considered in this study need to be investigated, the detection of SKAV in disparate geographic regions suggests that infections are likely endemic in striped skunks across their entire North American range.

Overall, we found high genetic diversity and observed several different viral clades that, for the most part, reflected the geographic origin of the strains, suggesting local diversification due to genetic drift. Interestingly, however, while region-specific subclades were found in trees built with both the NS1 and VP2 genes, the overall tree topology was not conserved across phylogenies. The most striking discrepancy was the presence of two clear clades corresponding to viruses from the west and the east in the tree built with NS1, which were missing from the tree built with VP2. This reflects different evolutionary dynamics in the two ORFs, further demonstrated by the fact that NS1 was less conserved (85%–97% identity) than VP2 (91%–100% identity).

These inconsistencies can also be linked to recombination since our analysis uncovered frequent recombination in the evolutionary history of SKAV. In fact, we identified the presence of multiple phylogenetically supported breakpoints throughout the genome, which were mainly localized within three breakpoint hot spots. Interestingly, sequences from British Columbia and Ontario were those detected as recombinant, while sequences from California and the northeastern USA consistently clustered into separate clades. Since inconsistencies occurred both at the level of main branches as well as within internal clades

(i.e., within region-specific clades), we can presume that recombination was and still is important in shaping the genetic makeup of these viruses.

Our selection pressure analyses confirmed that different evolutionary forces act on the structural and nonstructural genes of SKAV. In fact, while the number of sites predicted to be negatively selected by various methods was similar for the two proteins, or even higher for VP2, each method consistently identified a higher percentage of positively selected sites in NS1. This, combined with the higher sequence conservation of the VP proteins, is inconsistent with diversifying pressure on the capsid proteins as a mechanism to escape detection by the host immune system. Rather, it is suggestive of the possible involvement of ADE in the pathogenesis of the virus, as previously observed for AMDV (Canuti et al., 2016). In support of this, we also observed that a region experimentally determined to mediate ADE and virus neutralization in AMDV (Bloom et al., 1997, 2001) was very conserved between the two viral species. This fragment also showed very little variation within SKAV strains, no residues under diversifying selection pressure and approximately 30% of sites under purifying selection pressure. These results indicate that immune response and immune-mediated pathogenicity likely involve the same sites in both viruses.

The many similarities between the evolution of SKAV and AMDV, including local genetic drift, frequent recombination with similar breakpoint hot spots and the different evolutionary forces acting on the two proteins, suggest that the two viruses also have similar biological behaviours.

4.2 | Tissue distribution, pathogenicity and spread

To evaluate how widespread viral distribution is linked to viral transmission, we performed ISH to evaluate cell and tissue sources of viral shedding. AMDV can be shed in faeces, urine, blood and saliva of infected mink (Gorham et al., 1964, 1976; Kenyon et al., 1963), and we suspected that a similar distribution would occur for SKAV in skunks. Indeed, the tissue distribution of viral nucleic acid confirms that SKAV in some individuals is present in, and potentially shed in oral/nasal secretions, faeces, urine, and sloughed skin. Although presence of virus in hair itself could not be evaluated due to technical limitations of the hydrophobic hair follicle, we identified SKAV in the root sheath follicular epithelial cells, which suggests that shed hairs may also be coated with virus and/or contain sloughed viral-laden keratinocytes as a potential route of shedding and transmission. Because parvoviruses are very resilient in the environment (Eterpi et al., 2009; Hussain et al., 2014), transmission via direct or indirect (contaminated food, water or environment) contact is possible. We did not have opportunities to evaluate transplacental transmission as a mechanism of spread among skunks, although the placenta has been shown to be a site of virus replication and possible in utero transmission for AMDV in mink (Britton et al., 2017; Broll & Alexandersen, 1996; Glueckert et al., 2019). Additionally, tissue-distribution analyses were limited to a small number of individuals, and follow-up studies are required to assess whether there is variability between infected animals and quantify viral load in each tissue.

Clinical and pathological data are scarce, but preliminary investigations showed that SKAV infection can result in a similar range of clinical presentations as those observed in AMDV-positive mink. Few studies to date have evaluated pathology associated with

SKAV infections, and such an evaluation was outside the scope of this study. However, similar to what has been reported for mink, lesions associated with SKAV have included multi-systemic lymphoplasmacytic inflammation, glomerulonephritis, vasculitis/arteritis and cerebral microangiopathy (Britton et al., 2017; Glueckert et al., 2019; Ladouceur et al., 2015). The presence of plasma cell-rich inflammatory lesions coupled with arteritis/vasculitis, microangiopathy and glomerulonephritis suggests the possible involvement of immunocomplexes in virus pathogenesis and therefore provides additional support for our hypothesis that ADE can contribute to maintenance and exacerbation of infection. Overall, however, subclinical infections seem predominant (Britton et al., 2017; Glueckert et al., 2019). The high frequency of mild or inapparent disease, the likely persistence of infections and chance of viral transmission to occur through many possible different routes are all factors contributing to efficient viral spread, which enabled SKAV to become enzootic across North America and also support the conclusion that the virus has been circulating in skunks for a long time. This is also substantiated by its high genetic diversity and by the evidence that recombination may have occurred in ancestral strains.

Although skunks are the viral reservoir and maintenance host for SKAV, spillover infections have been found in mink (Canuti et al., 2016; Nituch et al., 2012) and we can presume that other carnivorans are also susceptible to infection, just as other amdoparvoviruses have been shown to infect a broad range of species (Leimann et al., 2015; Shao et al., 2014). Because of persistent viral shedding and high environmental stability, large quantities of virions might remain viable in the environment for a long time, offering opportunities for cross-species transmission to sympatric species through habitat sharing or niche overlap. Additionally, spillover to bigger animals through carnivory, as hypothesized for other amdoparvoviruses (Canuti et al., 2020), is also possible and could pose a risk for infection in domestic animals, since skunks are frequently found in urban and suburban environments.

Finally, to the best of our knowledge, there is no evidence of SKAV infections in mink farms. We can safely conclude, therefore, that this virus has originated in North America like its close relative AMDV. Furthermore, its presence in wild animals can be reasonably defined as autochthonous, as opposed to AMDV strains from wild animals that are frequently of farm origin. This is well demonstrated by geographical clustering of strains. SKAV therefore offers a better model than AMDV to study amdoparvovirus evolution and transmission dynamics in wildlife. Further studies should examine more hosts and geographic locations to characterize the full genetic diversity of this virus and evaluate its cross-species transmission potential.

5 | CONCLUSIONS

In this study, we established that SKAV is endemic in striped skunks, with a high rate of infections in multiple sites across North America. This apparently high prevalence and the long-term persistence of subclinical infections, likely intensified by ADE, promotes opportunities for infection with multiple strains and subsequent recombination, a mechanism frequently used by parvoviruses to further increase genetic diversity. Persistent infections, low viral pathogenicity and high genetic diversity with local diversification due to genetic drift suggest a long-term virus–host association. The environmental stability typical of

parvoviruses combined with persistent and efficient viral shedding, which can occur through many possible transmission routes, likely contributed to the successful spread of this virus over large distances. High genetic diversity and efficient, persistent shedding also offer opportunities for cross-species transmission to potentially susceptible sympatric species that can acquire the infection by habitat sharing, predation or close contact. As our knowledge about these viruses grow, future studies will clarify whether other members of the genus *Amdoparvovirus* possess similar biological and genetic characteristics and whether they are as broadly dispersed as SKAV and AMDV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/.

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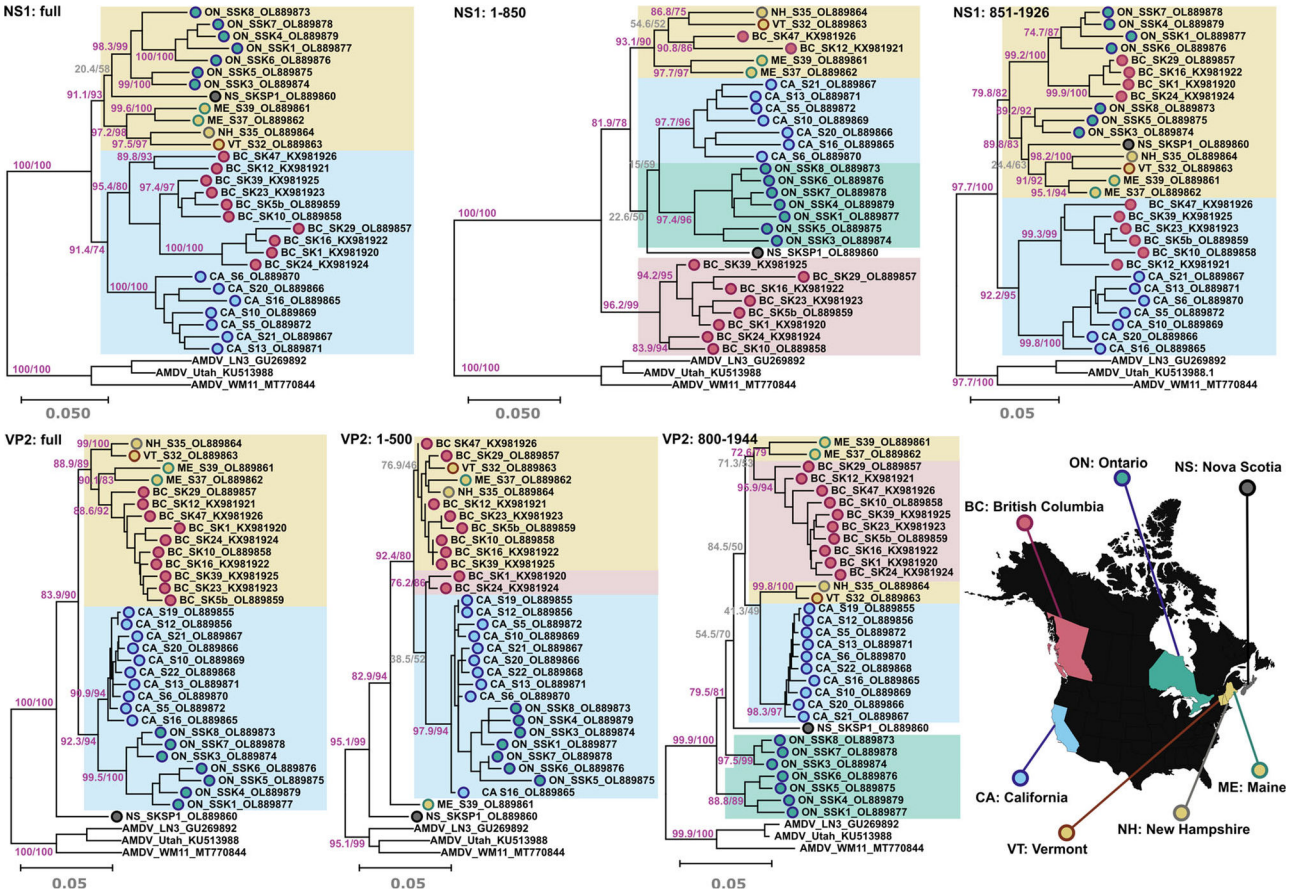

FIGURE 1.

Phylogenetic analysis of SKAV strains from North America. Phylogenetic trees built with the NS1 (top) and VP2 (bottom) ORFs are shown. Analyses were performed either with the alignments of the full ORFs (left side) or subportions of the alignments included between recombination breakpoints (middle and right) and used nucleotide regions are indicated in tree titles. Trees were built with the maximum likelihood method with IQ-Tree based on the best models for distance estimation identified with the Model Finder function (TIM3+F+I+G4 for NS1:full, NS1:1–850, and VP2:full; TPM3+F+I+G4 for NS1:851–1926; TPM3+F+R2 for VP2:1–500; HKY+F+I+G4 for VP2:800–1944). The outcomes of the SH-aLRT and bootstrap test (1000 replicates) are shown for the main nodes (purple for statistically supported clades and grey for unsupported ones). Three AMDV strains were used as an outgroup. Strains are labelled based on collection sites, as indicated on the map at the bottom-right. The two main clades are shaded in blue (viruses from California) and yellow (viruses from Maine, Vermont and New Hampshire), while additional clades only present when portions of the two ORFs were used for the tree are shaded in red (viruses from British Columbia) or green (viruses from Ontario). Sequences are labelled by sampling location (BC, British Columbia; ON, Ontario; NS, Nova Scotia; CA, California; NH, New Hampshire; ME, Maine), strain name, and accession number. The map was created using Mapchart.net ©

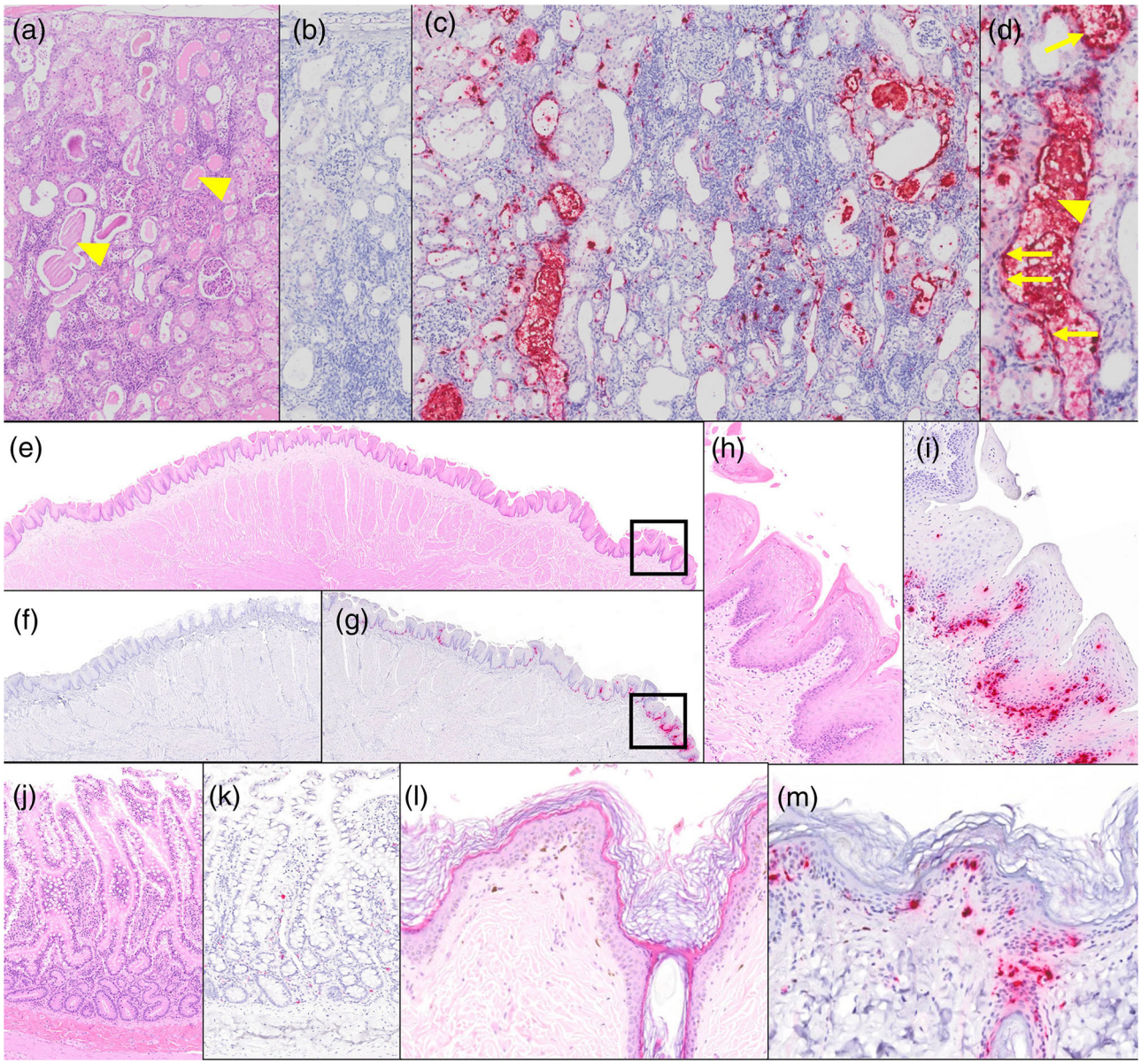

FIGURE 2.

Detection of SKAV in tissues by in situ hybridization (ISH) demonstrates multiple potential mechanisms of shedding. (a–c) Kidney, striped skunk: (a) Haematoxylin and eosin (H&E) staining demonstrates lesions of chronic kidney disease including lymphoplasmacytic tubulointerstitial nephritis and tubular dilation with luminal protein casts (arrowheads). (b) Negative control (DapB) ISH. No hybridization is observed. (c) ISH with SKAV-specific probes detects abundant SKAV nucleic acid (red) in the affected tissues. (d) Signal is observed in tubular epithelium (arrows) and protein casts (arrowhead). (e–i) Tongue, striped skunk: (e) H&E. (f) Negative control. (g) SKAV ISH demonstrates viral nucleic acid in lingual epithelium and subepithelial stroma. (h) H&E, high magnification. (i) SKAV ISH, high magnification. Signal is primarily observed in basal epithelial cells, although a few individual cells are also positive at higher levels. Signal is also detected in scattered

cells in the superficial submucosal stroma, presumed to be infiltrating inflammatory cells and/or capillary endothelium. (j,k) Small intestine, striped skunk: (j) H&E. (k) SKAV ISH. Scattered individual cells in the mucosal epithelium and lamina propria are positive. (l,m) Skin, striped skunk: (l) H&E. (m) SKAV ISH. Hybridization is observed primarily in the stratum basale but occurs at all levels of the epidermis. Signal is also present in epithelial lining of a hair follicle

TABLE 1

SKAV positivity rates in each investigated region

Region	Sample dates	Total tested	Total positive	Positivity rate (%)
California, USA	2019–2020	23	20	87.0
Ontario, Canada	2020	10	7	70.0
New Hampshire, USA	2019–2020	5	1	20.0
Maine, USA	2020	7	5	71.4
Vermont, USA	2016–2020	5	3	60.0
Nova Scotia, Canada	2019	1	1	100.0
British Columbia, Canada	2011–2015	50	43	86.0 (Britton et al., 2017)
Total		101	80	79.2