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Although parvoviruses are commonly described in domestic carnivores, little is known about their biodiversity in nondomestic species. A phylogenetic analysis of VP2 gene sequences from puma, coyote, gray wolf, bobcat, raccoon, and striped skunk revealed two major groups related to either feline panleukopenia virus ("FPV-like") or canine parvovirus ("CPV-like"). Cross-species transmission was commonplace, with multiple introductions into each host species but, with the exception of raccoons, relatively little evidence for onward transmission in nondomestic species.

Determining how viruses infect and spread in new host species is central to the study of disease emergence (1, 2). The spread of canine parvovirus (CPV) in dogs during the late 1970s is one of the best documented examples of viral emergence leading to a pandemic in a new host (3). It has been widely assumed that the new canine virus (initially known as CPV-2) emerged in dogs following the cross-species transfer of feline panleukopenia virus (FPV) from cats or a related carnivore host (2, 4, 5). Comparing isolates of FPV and CPV has provided important insights into the viral mutations controlling host range and how different interactions with the host transferrin receptor type-1 (TfR) enabled both adaptation to dogs and the later evolution of the CPV-2a, CPV-2b, and CPV-2c variants that exhibit characteristic differences in antigenic sites and in cell tropism (3, 6, 7).

A variety of host species other than domestic cats and dogs harbor closely related parvoviruses, and it has become increasingly apparent that nondomestic animals are commonly infected, even though little disease is observed in many cases (8–12). However, those parvoviruses previously detected in a variety of other species, including many different large cats, raccoons, raccoon dogs, arctic foxes, and mink, often represent opportunistic samples obtained from animals in artificial settings such as zoos or fur farms (13–15), with the majority of these viruses falling into a single FPV-like clade distinct from CPV in dogs (4, 16). More recently, raccoons from a variety of locations in the United States have been shown to commonly harbor parvoviruses, which they have likely been associated with for at least 20 years (17). Notably, most raccoon parvovirus sequences, as well as a single isolate from a bobcat, fell in intermediate locations between the dog-associated CPV-2 and CPV-2a strains in a phylogenetic tree of the VP2 protein (17). Hence, raccoon parvoviruses may have played a central role in the transition between CPV-2 and the later CPV-2a, -2b, and -2c variants that not only infected dogs but regained the ability to infect cats (a property lost in CPV-2). Indeed, the CPV-like viruses from raccoons possess multiple amino acid changes on the surface of their capsids that affect binding to the host-specific TfR, resulting in loss of the canine host range and altering neutralizing antibody epitopes (17, 18).

To further clarify virus-host relationships, we characterized parvoviruses that circulate in several species of wild carnivore in the United States, determining those which represent viable hosts for parvoviruses by sustaining prolonged viral transmission and those in which parvovirus infections are apparently transient spillovers. Accordingly, we sampled 58 novel parvoviruses from either free-ranging (gray wolf, coyote, bobcat, puma, striped skunk, and raccoon) or wild species that were brought into captive outdoor facilities for rehabilitation (raccoon) or containment (gray [Mexican] wolf) purposes (Table 1). All new FPV and CPV sequences were obtained from carnivores that either were showing typical clinical signs of parvovirus infection (e.g., hemorrhagic enteritis) or were asymptomatic, suggestive of either an active but subclinical infection or recovery from a previous infection in which persistent DNA could be detected in tissues. The detection of viral DNA in animals without active infection is likely due to residual DNA in tissues after virus was inactivated by the host immune response and parallels the results reported for lifelong residual DNA of human parvoviruses (19), as well as recent reports of persistent DNA in cats (20). In most cases, tissue samples (gastrointestinal tract, mesenteric lymph node, spleen, tongue, or feces) approximately 0.5 mm^3 in size were placed in 1.5-ml microcentrifuge tubes and stored at −20°C until further processing. DNA was extracted from tissues using a commercial kit (Qiagen,

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TABLE 1 Carnivore parvoviruses sequenced and analyzed in this study

Isolate	Virus	Host	County	State	Yr	GenBank accession no.
CPV-2b/Gray wolf/AZ/16382-01/99	CPV-like	*Canis lupus baileyi*	Greenlee	Arizona	1999	JX475240
CPV-2b/Gray wolf/NM/16401-01/99	CPV-like	*Canis lupus baileyi*	Socorro	New Mexico	1999	JX475241
CPV-2b/Gray wolf/WI/18268/02	CPV-like	*Canis lupus nubilus*	Langlade	Wisconsin	2002	JX475242
CPV-2c/Gray wolf/ID/22772/09	CPV-like	*Canis lupus occidentalis*	Camas	Idaho	2009	JX475243
CPV-2b/Puma/CO/2235/09	CPV-like	*Puma concolor*	Douglas	Colorado	2009	JX475251
FPV/Puma/CO/952/10	FPV-like	*Puma concolor*	Yuma	Colorado	2010	JX475245
CPV/Puma/CO/2503/10	CPV-like	*Puma concolor*	Larimer	Colorado	2010	JX475246
CPV-2b/Puma/CO/1246/10	CPV-like	*Puma concolor*	Jefferson	Colorado	2010	JX475247
CPV-2b/Puma/CO/898/10	CPV-like	*Puma concolor*	Larimer	Colorado	2010	JX475249
CPV-2b/Puma/CO/728/10	CPV-like	*Puma concolor*	Larimer	Colorado	2010	JX475250
CPV-2c/Puma/CO/1316/10	CPV-like	*Puma concolor*	Boulder	Colorado	2010	JX475252
FPV/Puma/CO/546/10	FPV-like	*Puma concolor*	Jefferson	Colorado	2010	JX475253
FPV/Puma/CO/977/10	FPV-like	*Puma concolor*	Clear Creek	Colorado	2010	JX475254
CPV-2b/Puma/CO/1237/10	CPV-like	*Puma concolor*	Weld	Colorado	2010	JX475257
CPV/Puma/CO/1321/10	CPV-like	*Puma concolor*	Larimer	Colorado	2010	JX475258
FPV/Puma/CO/545/10	FPV-like	*Puma concolor*	Chaffee	Colorado	2010	JX475259
CPV-2c/Puma/CO/704/10	CPV-like	*Puma concolor*	Jefferson	Colorado	2010	JX475260
CPV/Raccoon/CA/334-A/10	CPV-like	*Procyon lotor*	Contra Costa	California	2010	JX475261
CPV/Raccoon/CA/334-B/10	CPV-like	*Procyon lotor*	Contra Costa	California	2010	JX475262
CPV/Raccoon/CO/280/11	CPV-like	*Procyon lotor*	Jefferson	Colorado	2011	JX475231
CPV/Raccoon/VA/243-A/11	CPV-like	*Procyon lotor*	Clarke	Virginia	2011	JX475232
CPV/Raccoon/SC/182-A/11	CPV-like	*Procyon lotor*	Greenville	South Carolina	2011	JX475233
CPV/Raccoon/ME/258/11	CPV-like	*Procyon lotor*	Cumberland	Maine	2011	JX475234
CPV/Raccoon/ME/259/11	CPV-like	*Procyon lotor*	Cumberland	Maine	2011	JX475235
CPV/Raccoon/NY/87648/11	CPV-like	*Procyon lotor*	Oswego	New York	2011	JX475236
CPV-2b/Raccoon/CT/372/11	CPV-like	*Procyon lotor*	New Haven	Connecticut	2011	JX475237
CPV-2c/Coyote/GA/11/11	CPV-like	*Canis latrans*	Putnam	Georgia	2011	JX475238
CPV/Coyote/GA/06/11	CPV-like	*Canis latrans*	Putnam	Georgia	2011	JX475239
CPV-2c/Puma/CO/1269/11	CPV-like	*Puma concolor*	Boulder	Colorado	2011	JX475244
CPV/Puma/CO/1102/11	CPV-like	*Puma concolor*	Jefferson	Colorado	2011	JX475248
CPV/Raccoon/MS/257/11	CPV-like	*Procyon lotor*	DeSoto	Mississippi	2011	JX475255
FPV/Puma/CO/1103/11	FPV-like	*Puma concolor*	Boulder	Colorado	2011	JX475256
CPV/Raccoon/VA/218-A/11	CPV-like	*Procyon lotor*	Fairfax	Virginia	2011	JX475263
CPV/Striped skunk/TN/29/11	CPV-like	*Mephitis mephitis*	Knox	Tennessee	2011	JX475286
CPV/Striped skunk/TN/30/11	CPV-like	*Mephitis mephitis*	Knox	Tennessee	2011	JX475287
CPV/Striped skunk/TN/31/11	CPV-like	*Mephitis mephitis*	Knox	Tennessee	2011	JX475288
CPV/Raccoon/TN/1/11	CPV-like	*Procyon lotor*	Knox	Tennessee	2011	JX475279
CPV/Raccoon/TN/4/11	CPV-like	*Procyon lotor*	Knox	Tennessee	2011	JX475280
CPV/Raccoon/TN/5/11	CPV-like	*Procyon lotor*	Knox	Tennessee	2011	JX475281
CPV/Raccoon/TN/6/11	CPV-like	*Procyon lotor*	Knox	Tennessee	2011	JX475282
CPV-2b/Raccoon/TN/18/11	CPV-like	*Procyon lotor*	Knox	Tennessee	2011	JX475283
CPV/Raccoon/TN/26/11	CPV-like	*Procyon lotor*	Knox	Tennessee	2011	JX475284
CPV/Raccoon/TN/27/11	CPV-like	*Procyon lotor*	Knox	Tennessee	2011	JX475285
CPV/Raccoon/CO/585/11	CPV-like	*Procyon lotor*	Boulder	Colorado	2011	JX475264
CPV/Raccoon/CO/983/11	CPV-like	*Procyon lotor*	Boulder	Colorado	2011	JX475265
CPV-2b/Raccoon/IL/357/11	CPV-like	*Procyon lotor*	Franklin	Illinois	2011	JX475266
CPV-2b/Raccoon/CT/2D/11	CPV-like	*Procyon lotor*	New Haven	Connecticut	2011	JX475267
CPV/Bobcat/KS/3/11	CPV-like	*Lynx rufus*	Sheridan	Kansas	2011	JX475268
CPV/Bobcat/TN/10/11	CPV-like	*Lynx rufus*	Knox	Tennessee	2011	JX475271
CPV-2c/Coyote/CO/422/12	CPV-like	*Canis latrans*	Denver	Colorado	2012	JX475269
FPV/Raccoon/GA/1/12	FPV-like	*Procyon lotor*	Gwinnett	Georgia	2012	JX475270
CPV-2c/Bobcat/AL/362/12	CPV-like	*Lynx rufus*	Pike	Alabama	2012	JX475272
CPV-2c/Coyote/MT/909/12	CPV-like	*Canis latrans*	Prairie	Montana	2012	JX475273
CPV-2c/Coyote/MT/911/12	CPV-like	*Canis latrans*	Prairie	Montana	2012	JX475274
CPV-2c/Coyote/MT/914/12	CPV-like	*Canis latrans*	Prairie	Montana	2012	JX475275
CPV-2c/Coyote/MT/915/12	CPV-like	*Canis latrans*	Prairie	Montana	2012	JX475276
CPV-2c/Coyote/AL/361/12	CPV-like	*Canis latrans*	Butler	Alabama	2012	JX475277
CPV-2b/Coyote/AR/1069/12	CPV-like	*Canis latrans*	Prairie	Arkansas	2012	JX475278

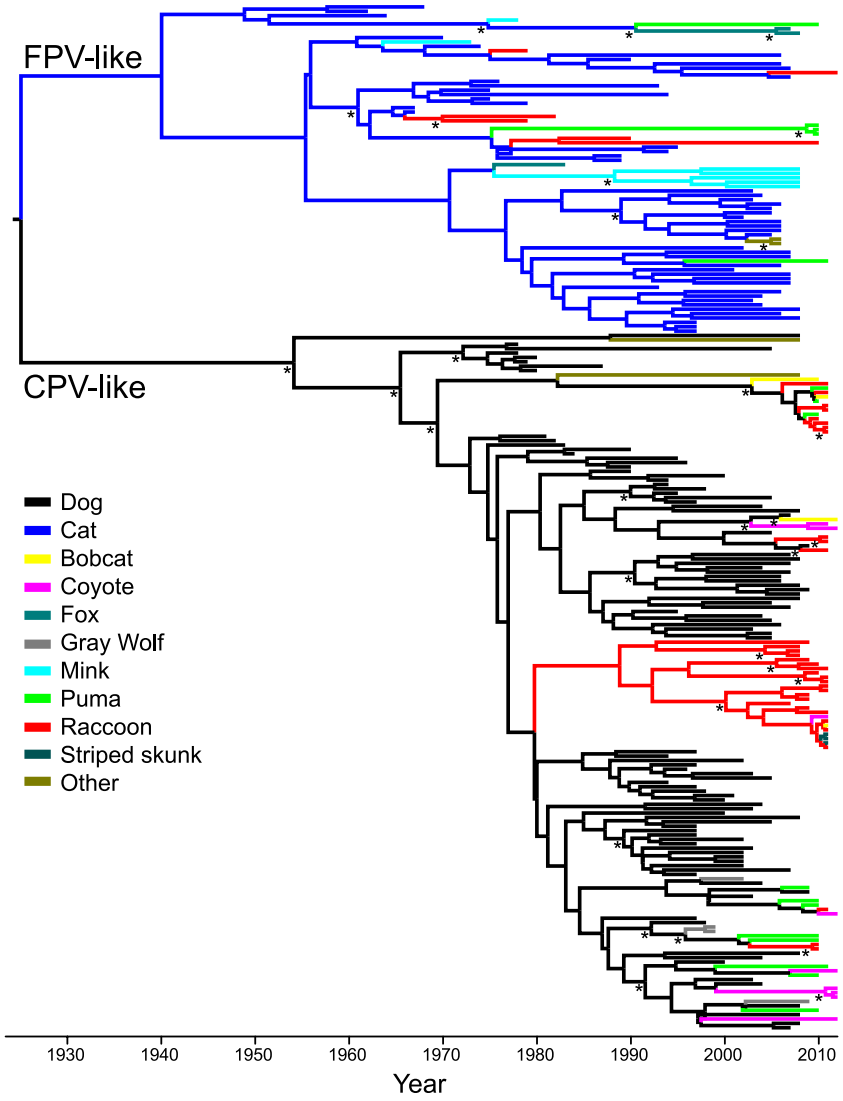

FIG 1 Phylogenetic history (MCC tree) of carnivore parvoviruses inferred from 234 complete VP2 sequences. Clusters of viruses are labeled and colored according to host species (note that the division between CPV-2a, -2b, and -2c sequences is not shown in this figure). Hosts in the "Other" group, which represent singleton viruses, are lion, palm civet, monkey, and tiger. Because the tree was inferred using a relaxed molecular clock, all tip heights are scaled to the year of sampling. Posterior probability values of >0.9 at major nodes or which connect multiple species are indicated by an asterisk. A time scale in years is given by the *x* axis. The number of sequences from each species or antigenic group is as follows: FPV (cat, *Felis catus*), $n = 52$; CPV-2 (dog, *Canis lupus familiaris*), $n = 7$; CPV-2a (dog), $n = 48$; CPV-2b (dog), $n = 28$; CPV-2c (dog), $n = 9$; raccoon (*Procyon lotor*), $n = 40$; puma (*Puma concolor*), $n = 16$; coyote (*Canis latrans*), $n = 9$; mink (*Neovison vison*; mink enteritis virus [MEV]), $n = 7$; bobcat (*Lynx rufus*), $n = 4$; gray wolf (*Canis lupus*), $n = 4$; arctic fox (*Vulpes lagopus*; arbitrarily designated blue fox parvovirus [BFPV]), $n = 3$; striped skunk (*Mephitis mephitis*), $n = 3$; palm civet (*Paradoxurus hermaphroditus*), $n = 1$; tiger (*Panthera tigris*), $n = 1$; lion (*Panthera leo*), $n = 1$; and monkey (*Macaca fascicularis* or *Macaca mulatta*), $n = 1$.

Valencia, CA) according to the manufacturer's instructions, and the complete VP2 gene was amplified as described previously (17). Care was taken to avoid PCR contamination, and samples were handled in a series of separate work areas to avoid cross-contamination.

Viral sequences from additional hosts, largely domesticated or farmed animals, were obtained from GenBank. CPV sequences were subsampled so that only representative strains with known year of isolation (which is not necessarily the date of infection) were included in the final analysis, resulting in a final data set of 234 sequences, 1,755 nucleotides in length. These sequences were found to be free of recombination using the RDP3 package (21). The numbers of sequences available for each group are given in the legend to Fig. 1. To estimate rates of evolutionary change, times to common ancestry, and the phylogenetic history of the carnivore parvoviruses, we employed the Bayesian Markov chain Monte Carlo method available within the BEAST package (22). To account for statistical uncertainty, all estimates are based on values of the 95% highest probability density (HPD). We employed the GTR+Γ_4 model of nucleotide substitution along with an uncorrelated lognormal relaxed molecular clock and a Bayesian skyline coalescent prior. Two BEAST runs of 200 million steps each were undertaken and combined for the final analysis, with a 10% burnin. We also estimated the maximum clade credibility (MCC) tree for the data in hand, with support for individual groupings reflected in posterior probability values.

Our phylogenetic analysis of complete VP2 sequences from 234 carnivore parvoviruses reveals a major division between the FPV-like and CPV-like viruses (Fig. 1). However, determination of the exact evolutionary relationships, and hence the number and direction of cross-species transmission events, was often difficult because of a lack of resolution in some parts of the phylogeny. Despite this uncertainty, it was striking that viruses sampled from individual species usually fell in diverse locations across the phylogeny, indicative of multiple introduction events. The most notable are those viruses sampled from raccoons. A minority of raccoon sequences (6 of 40) fell into the FPV-like group, four of which were sampled more than 20 years ago (from 1979 to 1990). That two viruses sampled between 1990 and 2010 cluster together (although with weak support) is compatible with the continuous circulation of this particular lineage of FPV-like parvoviruses in raccoons, albeit at low frequency. The majority of raccoon parvoviruses (34 of 40) group with the CPV-like viruses and occupy diverse positions, both phylogenetically and geographically. In particular, there is a cluster of eight raccoon viruses, as well as two puma viruses and a single bobcat sequence, that occupies a phylogenetic position intermediate between CPV-2 and CPV-2a and that has strong statistical support (Fig. 1). That this multihost viral lineage diverged early in the evolutionary history of CPV, yet has persisted to the present day, indicates that it has circulated for an extended time period. In addition, there is a large (although poorly supported) clade of 20 CPV-like raccoon viruses, along with all three viruses from the striped skunk, and single viruses from a bobcat and a coyote. These evolutionary patterns add to the details revealed of our previous study of VP2 evolution (17), where most of the raccoon viruses fell in intermediate positions between CPV-2 and CPV-2a, and highlight the complexity of the virus-host relationships.

All the CPV-like raccoon viruses from this study and our previous study (17) were sampled between 2007 and 2012, such that they represent the current genetic diversity of parvoviruses in raccoons, and from diverse locations throughout the United States (California, Colorado, Florida, Georgia, Illinois, Kentucky, Maine, Mississippi, New Jersey, New York, South Carolina, Tennessee, Wisconsin, and Virginia). That there are well-supported subclusters that only contain raccoon viruses, and which cover multiple years and locations, strongly suggests that there is sustained onward transmission of parvoviruses in raccoons. The raccoon viruses are also often very closely related to those sampled from other carnivore species (see below), suggesting that raccoons may represent conduits for parvovirus transmission to other hosts.

The seven virus sequences from mink (mink enteritis virus [MEV]) also represent clear examples of sustained transmission in a single host species. These sequences fall into three distinct locations in the FPV-like cluster, indicative of three cross-species events from cats or a closely related host (Fig. 1). However, as these MEV sequences are associated with outbreaks in farm situations, it is unclear whether sustained transmission also occurs in wild mink (23).

Of the 16 puma virus VP2 sequences analyzed, five are FPV-like and 11 are CPV-like. Along with raccoons, pumas are the only species whose viruses are detected on both the FPV-like and CPV-like parts of the phylogenetic tree. Three of the FPV-like sequences are identical, which is tentatively compatible with onward viral transmission in this species. The remaining two FPV-like puma sequences are singletons and indicative of separate viral introductions, while the 11 CPV-like puma sequences fall in diverse locations across that part of the phylogeny. Hence, there have clearly been multiple independent cross-species transmissions into pumas and these occurred from both CPV and FPV ancestors.

A similar pattern of multiple introductions was apparent for those viruses sampled from coyotes, gray wolves, and bobcats, although these viruses were all CPV-like. Of the nine coyote sequences, one clustered with raccoon viruses, while the remainders were closely related to CPV-2b and -2c. Although only four bobcat sequences are available, three cluster within the main groups of raccoon-associated viruses, while the other clusters with a number of CPV-2c isolates. The four gray wolf sequences also fall at multiple locations within the CPV-2b and -2c sequences, again indicative of independent cross-species transmission events. The only case in which multiple sequences sampled from a single host group together, which is tentatively compatible with a single cross-species transmission event, is that of the striped skunk; the three sequences from this species are identical and fall within the main cluster of raccoon viruses.

Finally, the CPV-like phylogeny also contained individual sequences from a virus sampled from a masked palm civet (GenBank accession number EU441280) (X. Yan, H. Zhang, T. Chen, J. Zhao, X. Chai, and W. Wu, unpublished data), which was linked by a long branch to the intermediate raccoon group (although with weak support), and a virus associated with an outbreak in rhesus monkeys (GenBank accession FJ231389) (27) that falls at the base of the CPV-like part of the tree (again with weak support). Because these are single viral sequences, it is difficult to determine whether they simply represent transient spillover infections.

A number of cross-species transmission events were also documented with the FPV-like viruses, in addition to the raccoon and puma parvoviruses described above. Two identical sequences from arctic (blue) foxes sampled in 2007 and 2008 cluster with a single MEV sequence collected in 1978, a single puma sequence sampled in 2010, and the oldest FPV (cat) virus that was sampled in 1964. Furthermore, two sequences from lion and tiger clearly represent spillover infections of FPV in a zoological setting (13). Overall, our phylogenetic analysis reveals a significant biodiversity of parvoviruses in nondomestic animals, which is clearly the result of multiple cross-species transmission events, but with relatively little evidence at present for onward transmission in the new host species.

One of the most striking features of this analysis was that the majority of the carnivore parvoviruses described here are CPV-like rather than FPV-like; for example, 85% of the raccoon parvoviruses are CPV-like, including all but two of the viruses sampled between 2007 and 2012. It is possible that this bias toward CPV-like viruses reflects the phylogenetic relationships of the host species in question, such that fewer adaptive mutations are required for viruses to infect phylogenetically similar hosts. Specifically, most of the carnivore parvoviruses—dog, raccoon, coyote, gray wolf, mink, striped skunk, and fox—infect hosts within the suborder Caniformia. Of these, the viruses from coyote, gray wolf, and striped skunk are only CPV-like, as are most raccoon parvoviruses. However, any such phylogenetic rule is clearly a weak one because viruses from bobcats and pumas (suborder Feliformia) are either exclusively or predominantly CPV-like, and FPV-like viruses were recently discovered in Eurasian badgers (8), a cani-

form species. Alternatively, some of the virus-host relationships depicted in our phylogenetic analysis may reflect the ecological relationships among the species in question, especially predator-prey relationships. In particular, it is well documented that large carnivores, such as pumas, wolves, and coyotes, feed on other smaller species, such as raccoons. It is therefore conceivable that parvoviruses are able to move between hosts during predation and/or scavenging of carcasses, although seemingly with little onward transmission.

Early studies suggested that CPV in dogs was directly derived from an FPV in a domestic cat (4, 16). However, the diverse range of parvoviruses in other carnivore species means that is no longer necessary to think that cats must be the source of the virus that emerged in dogs in the late 1970s. We therefore attempted to root the carnivore parvovirus tree using the closest exogenous parvovirus (porcine parvovirus 27a; GenBank accession number AY684871) as an outgroup in a phylogenetic analysis of VP2 amino acid sequences. However, the porcine virus is so divergent (mean pairwise amino acid identity to the carnivore parvoviruses of 58%) that it provided insufficient resolution. Consequently, we relied on our MCC tree, which is rooted under the assumption of a relaxed molecular clock (Fig. 1) and which placed the root between the FPV-like and CPV-like groups. If this rooting scheme is correct, then CPV was not directly derived from the known genetic diversity of FPV in domestic cats as previously supposed. Rather, this phylogenetic analysis suggests that CPV and FPV were separately derived from common ancestors, the nature of which—i.e., which host(s) they infected—is unknown, and that their progenitor lineages evolved independently for a time period that extends beyond the first description of CPV in dogs.

This analysis also suggested a relatively recent evolutionary history for this diversity of carnivore parvoviruses. Our estimate of the rate of evolutionary change was between 1.09×10^{-4} and 1.79×10^{-4} nucleotide substitutions per site, per year (95% HPD values). Given these rates, the time to the most recent common ancestor (TMRCA) of the entire tree was between 60 and 118 years before present, with the sampled diversity within the FPV-like and CPV-like clades having TMRCAs of 57 to 89 years (1923 to 1955) and 40 to 79 years (1933 to 1972) before present, respectively. Infections attributable to FPV have been reported in cats and raccoons from the 1920s and 1940s (24–26), although any earlier history is not known. The approximate time scale of the myriad of other cross-species transmission events can be inferred from the MCC tree (Fig. 1), indicating that the frequent species jumping depicted in this phylogeny occurred within the last century. For example, the common ancestors of the intermediate and main groups of raccoon viruses existed from 1991 to 2009 and from 1981 to 1995, respectively.

While epidemiological studies indicate that CPV spread worldwide among domestic dogs in a pandemic after 1977, our phylogenetic analysis shows that the FPV and CPV clades are separated by a relatively long branch and that there is no virus that is obviously the ancestor of CPV. Our molecular dating analysis suggests that both clades have been evolving independently for part of the last century, although their ultimate origins, particularly the animal species from which they are derived, are unclear. In this context it is important to note that the new samples described here were all collected from wild hosts in North America, and CPV antibodies in dogs were present in dogs in Europe (but not in North America, Australia, or Japan) up to 4 years before the virus spread worldwide (16). Evidently, future studies of parvovirus evolution should be based on a broader sampling of domestic and wild hosts in different parts of the world and on an analysis of complete viral genomes.

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