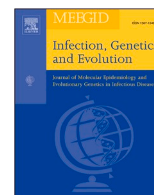
Infection, Genetics and Evolution 128 (2025) 105714

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid



Molecular ecology of novel amdoparvoviruses and old protoparvoviruses in Spanish wild carnivorans

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ARTICLE INFO

Keywords:
Canine parvovirus type 2
Feline panleukopenia virus
Aleutian mink disease virus
Amdoparvovirus
Virus ecology
Carnivore
Wildlife

ABSTRACT

Wild carnivorans are key hosts of parvoviruses of relevance for animal health and wildlife conservation. However, the distribution and diversity of parvoviruses among wild carnivorans are under-investigated, particularly in Southern Europe. We evaluated the presence, spread, and diversity of multi-host protoparvoviruses (canine parvovirus type 2 (CPV-2), feline panleukopenia virus (FPV)), and amdoparvoviruses in 12 carnivoran species from Northern Spain to explore viral ecology. Broad-range PCRs were used to screen spleens ($N = 157$) and intestines ($N = 116$) from 171 road-killed mustelids, viverrids, and felids; identified viruses were molecularly characterized. We detected an Asian-like CPV-2c strain in the spleen of one wildcat (*Felis silvestris*, 1/40, 2.5 %), a globally distributed FPV strain in the spleen of one Eurasian badger (*Meles meles*, 1/35, 2.9 %), a novel amdoparvovirus (European mustelid amdoparvovirus 1), in the intestine and spleen of one stone marten (*Martes foina*, 1/16, 6.3 %) and in the spleen of one Eurasian badger (1/35, 2.9 %), the red fox fecal amdovirus (RFFAV) in the intestine and spleen of three wildcats (3/40, 7.5 %), and a novel amdoparvovirus closely related to RFFAV (European felid amdoparvovirus 1) in one wildcat (1/40, 2.5 %). We observed a correlation between the phylogeny of carnivorans and the one of amdoparvoviruses, possibly indicating virus-host co-evolution. Species originating from North America and Eurasia formed different clades, indicating local segregation in the absence of man-linked transboundary movements. In contrast, CPV-2 and FPV strains were internationally dispersed. Different parvovirus species co-occur in sympatric host populations, and higher viral diversity and additional hosts will likely be identified in future studies.

1. Introduction

Carnivora is one of the largest mammalian orders, including almost 300 living species. Terrestrial carnivorans are distributed across all continents, occupying a broad range of niches, including domestic and urban environments (Buskirk, 2023; Hassanin et al., 2021; "The IUCN red list of threatened species", 2024). Carnivorans can be meat-eater predators and/or scavengers and play crucial roles in pathogen life cycles and distribution across different orders. In addition, since many species thrive in human-dominated landscapes, they are also important bridge hosts for pathogens between wild and domestic (including farming) environments. In fact, many pathogens that pose a risk to wild

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https://doi.org/10.1016/j.meegid.2025.105714
Received 4 November 2024; Received in revised form 8 January 2025; Accepted 9 January 2025
Available online 12 January 2025
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carnivoran conservation are shared with domestic species (e.g., Gjeltema et al., 2015; Millán et al., 2009; Williams et al., 1988). While some pathogens infecting carnivorans benefit from close-contacts between individuals, other pathogens can be indirectly transmitted. For example, parvoviruses are characterized by high environmental stability (Beineke et al., 2015; Cotmore et al., 2019; Wallace et al., 2014) and rely more heavily on other spreading mechanisms.

Parvoviruses (family: *Parvoviridae*; order: *Piccovirales*) are non-enveloped viruses characterized by a ssDNA genome that includes two main gene cassettes, one for non-structural proteins (NS) and one for capsid proteins (VP), flanked by imperfect palindromic sequences that form terminal hairpins, important for viral replication (Cotmore et al., 2019). Given the high variability in the capsid protein, parvoviral classification is based on the phylogenetic relationships and sequence identities of NS1 proteins (Pénzes et al., 2020). *Parvoviridae* is a diverse viral family as it includes viruses infecting both vertebrates and invertebrates, and several genera in two of its three sub-families include viruses of carnivorans (Pénzes et al., 2020). Among these, the two closely related genera *Protoparvovirus* and *Amdoparvovirus* are the most studied as they comprise viruses that are clinically and economically relevant for domestic animals.

Protoparvovirus includes both the canine parvovirus type 2 (CPV-2) and the feline panleukopenia virus (FPV), both belonging to the species *Protoparvovirus carnivoran1*. These viruses cause a severe infection in dogs, cats, and mink that is characterized by severe gastroenteritis and immune suppression manifesting with fever, anorexia, leukopenia induced by the virus replicating in white blood cells, and hemorrhagic enteritis (Miranda and Thompson, 2016; Parrish, 1995). However, their impact on wild animal health is poorly understood. While FPV infection seems to be restricted to felines and mustelids as the virus cannot, with a few recently documented exceptions (Diakoudi et al., 2022), infect canines, currently circulating CPV-2 strains have a much broader host range (Allison et al., 2014, 2013). In fact, while the original CPV-2 virus, which evolved from an FPV-like virus and emerged in dogs in the late 1970s, could only infect canines, its more recent variants (known as CPV-2a, −2b, and -2c) re-acquired tropism for feline receptors (Allison et al., 2012). While all these viruses are highly identical to each-other, the amino acid residues at six specific positions in the VP2 protein can be used to distinguish FPV from CPV-2 and an additional four positions are used for typing the different CPV-2 variants (Miranda and Thompson, 2016). Both CPV-2 and FPV have been frequently identified in wild carnivorans and evidence for cross-species transmission, including across the domestic/wild barrier, is abundant (Allison et al., 2013, 2012; Behdenna et al., 2019; Calatayud et al., 2020; Canuti et al., 2020b; Kelman et al., 2020; Sacristán et al., 2021).

Amdoparvovirus is a viral genus that includes viruses predominantly identified in carnivorans (Canuti et al., 2022b). Among them, the Aleutian mink disease virus (AMDV) is the most well-studied because of the large epidemics, characterized by high mortality, that it causes in mink farms and the consequent relevant implications for the fur industry (Canuti et al., 2016). The various AMDV lineages, which belong to multiple viral species (*Amdoparvovirus carnivoran1,* and *Amdoparvovirus carnivoran8–10*), have a broad host range, having been identified in many different mustelids, felids, and canids (Canuti et al., 2022b, 2020a). Often, mink farms affected by non-autochthonous AMDV strains function as a source of infection to naïve local wildlife, threatening indigenous animal populations (Canuti et al., 2020a, 2016, 2015; Knuuttila et al., 2015; Nituch et al., 2011). Other viruses belonging to other amdoparvoviral species (*Amdoparvovirus carnivoran4–7* plus some other yet unclassified ones) have been identified in various mustelids (Canuti et al., 2020a; Knuuttila et al., 2015; Wu et al., 2024), skunks (Canuti et al., 2017), red pandas (Alex et al., 2018; Zhao et al., 2022), and foxes (Bodewes et al., 2014; Canuti et al., 2020a; Li et al., 2011). Additionally, several instances of cross-species transmission were reported (Canuti et al., 2020b, 2020a; Knuuttila et al., 2015; Nituch et al., 2015). This lack of host specificity is probably linked to the fact that amdoparvoviruses replicate in macrophages and antibody-coated virus particles are internalized in their target cells without the need of a virus-specific receptor. Because of the immune-mediated cell-entry mechanism, contrary to other parvoviruses, amdoparvoviruses are antigenically stable, even across species, and the gene for the non-structural protein is usually less conserved and used for virus typing (Canuti et al., 2022b). Nonetheless, viruses other than AMDV are studied to a much lower extent and their diversity, host range, and geographic distribution are likely wider than currently known.

For members of *Protoparvovirus carnivoran1* and *Amdoparvovirus*, both direct transmission through contact with infected animals or indirect transmission through contaminated food, fomites, or the environment play an important role in virus spread and perpetuation among their maintenance hosts (Canuti et al., 2015; Miranda and Thompson, 2016). Moreover, factors such as niche sharing, mutualistic behaviors, hunting, and scavenging can contribute to the occurrence of cross-species transmission events among carnivorans. Remarkably, trophic transmission between hosts belonging to different species may be asymmetrical or even not bidirectional as scavenging and hunting are also unbalanced (Buskirk, 2023; Canuti et al., 2020b). Indeed, infection acquisition by smaller animals benefitting from large carnivoran kills may be favored in the case of scavenging, and predation may cause bigger animals to acquire infections from smaller prey. Thus, several viral and environmental factors can influence virus transmissibility across the species barrier, but these are still poorly understood and more studies investigating virus spread across different species and ecological niches are required to acquire a deeper understanding of the mechanisms and dynamics behind cross-species transmissions.

Only a limited number of studies have investigated the diversity and distribution of some of these viruses in wildlife, and very little is known about Europe. While in Spain, parvoviruses are the viral agents that received the most attention from researchers (Millán and Becker, 2021), the majority of studies consisted of antibody testing (Mañas et al., 2016; Millán et al., 2009; Millán and Rodríguez, 2009; Oleaga et al., 2015; Roelke et al., 2008; Sobrino et al., 2008) with only a handful including the molecular detection of protoparvoviruses (Calatayud et al., 2020; Candela et al., 2019; Nájera et al., 2021) or amdoparvoviruses (Bodewes et al., 2014; Mañas et al., 2001). In any case, knowledge about the genetic diversity of the different parvoviruses in Spanish wildlife is insufficient. For these reasons, the purpose of this study was to evaluate the presence, spread, and genetic diversity of CPV-2, FPV, and amdoparvoviruses across various carnivorans in a region from Northern Spain, and to explore viral ecology by investigating to which degree different host species share the same viruses and identifying potential cross-species transmission events. Additionally, since the studied region is the last stronghold for the extremely endangered European mink (*Mustela lutreola*) in Southern Europe, we wanted to determine whether these animals might be threatened by pathogenic parvoviruses.

2. Materials and methods

2.1. Samples

Samples from road-killed carnivoran carcasses collected between 2018 and 2022 in the frame of a monitoring program carried out by the Autonomous Region of Navarre (Northern Spain) were used for this study. Navarre is a small region in northern Spain of about 10,400 km^2 and is home to three biogeographic areas (Pyrenean Alpine, Atlantic, and Mediterranean) and different climates (Oceanic, Continental, Mediterranean, and Mountain) (Fig. 1). This makes this region rich in wildlife, counting up to 12 species of carnivorans, including the critically endangered European mink and one of the most stable populations of European wildcat (*Felis silvestris*) in the Iberian Peninsula.

Road-killed animals were collected by rangers, placed in individual bags, sealed, and sent to the wildlife recovery center of Ilundain, where carcasses were frozen until necropsy. The process followed a custody

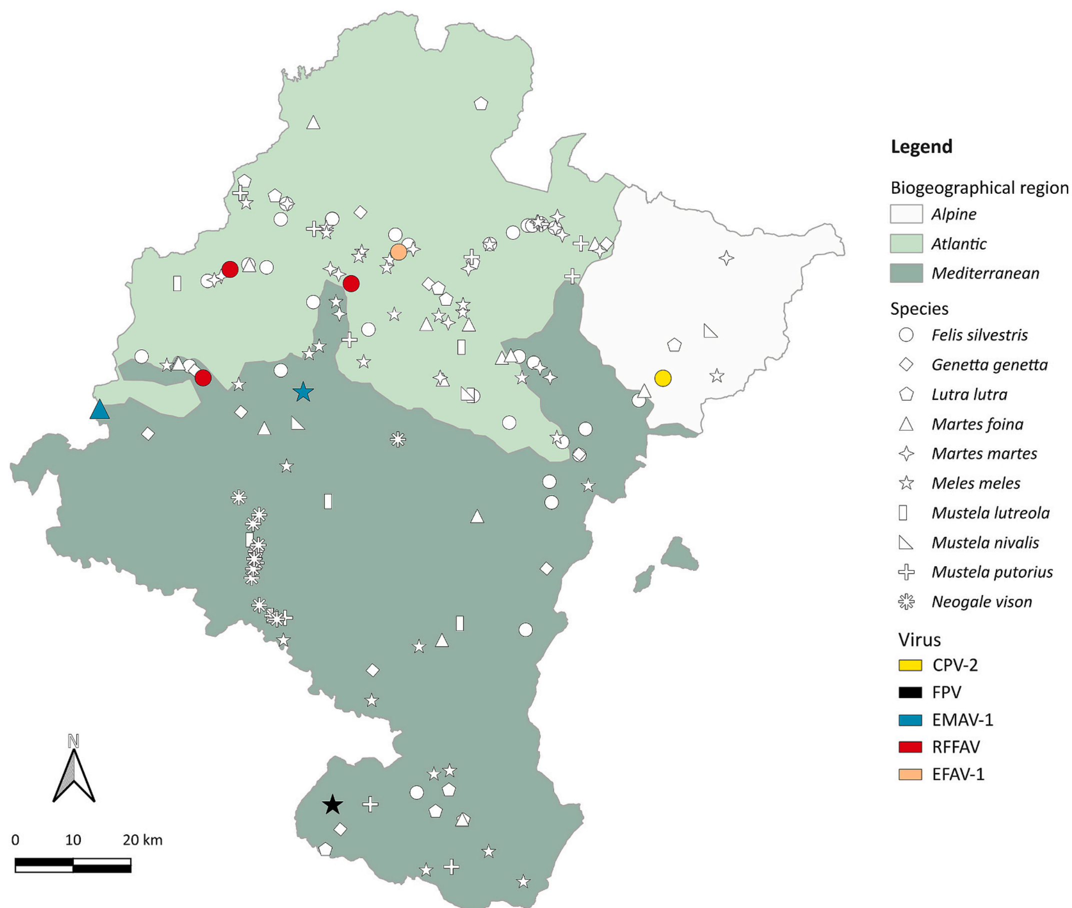


Fig. 1. Distribution of parvovirus-positive samples detected in the Autonomous Region of Navarre (Northern Spain). The map shows the area under investigation, which is characterized by three distinct biogeographical regions, as indicated by the legend. Sampled animals are marked with different shapes and animals identified as positive are labeled with different colors. CPV-2: Canine parvovirus type 2; FPV: feline panleukopenia virus; EMAV-1: European mustelid amdoparvovirus 1; RFFAV: red fox fecal amdovirus; EFAV-1: European felid amdoparvovirus 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chain. When necropsied, animals were sexed, aged, measured, and weighed, and a piece of spleen and ileocecal valve were collected and frozen until analysis in those individuals in better state of preservation (i.e. those not showing advanced autolysis or putrefaction). Since this investigation opportunistically used specimens from already dead wild animals collected from the regional authorities, ethical approval was not necessary to conduct the study.

2.2. Molecular methods

DNA was isolated from the sampled organs using the DNeasy Blood & Tissue Kit (Qiagen S.p.A., Hilden, Germany), according to the manufacturer's instructions. The extracted DNA was eluted in 200 μL of elution buffer and stored at −80 °C until further analyses.

Members of species *Protoparvovirus carnivoran1* were detected with a previously described molecular assay (Mira et al., 2018a) capable of detecting both CPV-2 and FPV, using a primer pair targeting the VP2 gene (Touihri et al., 2009). Amplicons were purified with the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Amersham, Buckinghamshire, UK) and submitted with both forward and reverse primers for direct Sanger sequencing.

A previously published (Canuti et al., 2020a) pan-amdoparvovirus heminested PCR designed to amplify a highly conserved region of the VP gene and capable of detecting known as well as novel viruses within the genus was used to perform pan-*Amdoparvovirus* molecular screening. Additional primers (Supplementary Table S1) were designed based on sequences obtained in this study and on sequences available in GenBank and used to amplify and sequence larger genomic fragments of identified amdoparvoviruses. Amplicons were purified with the HighPrep PCR-DX purification beads (MAGBIO) and outsourced for Sanger sequencing.

2.3. Sequence analyses

The obtained protoparvovirus sequences were assembled with BioEdit version 7.2.5 (Hall, 1999) and analyzed with the BLAST web interface (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A dataset of 107 sequences, also including CPV-2/FPV sequences previously described in Spain, was generated with reference sequences from the GenBank database and a maximum-likelihood phylogenetic tree was inferred with MEGA X (Kumar et al., 2018) using the best-fit substitution model, chosen as the one with the lowest Bayesian information criterion (BIC). A bootstrap analysis with 1000 replicates was performed to determine statistical support for the branches.

Obtained amdoparvovirus sequences were visualized, quality-checked, and assembled in Geneious Prime (Dotmatics), which was also used to determine open reading frames (ORF) and identify splicing sites, based on previously experimentally determined patterns. Donor and acceptor sites were also confirmed with NNSPLICE 0.9 (Reese et al., 1997). Study sequences were compared to all amdoparvoviral sequences included in the GenBank database as of August 2024 and sequence subsets were chosen for different analyses. Alignments were performed with MAFFT (Katoh and Standley, 2013), while maximum-likelihood

phylogenetic trees were inferred with IQ-TREE 2 (Minh et al., 2020) using the best-fit substitution model, chosen as the one with the lowest BIC by ModelFinder (Kalyaanamoorthy et al., 2017). Statistical support on branches was assessed both using ultrafast bootstrapping (Hoang et al., 2018) as well as with Shimodaira Hasegawa-like approximate likelihood ratio test (SH-aLRT) (Guindon et al., 2010). Finally, recombination detection was performed with RDP version 5.53 (Martin et al., 2020), as described in (Canuti et al., 2016).

2.4. Statistical analyses

Percentages with 95 % normal intervals (95 % intervals of confidence, 95 % IC) were used to express categorical variables. The Fisher's exact test was used to compare proportions and two-sided p-values <0.05 were considered statistically significant. A sample map was created with ArcGIS (esri).

3. Results

In this study, 273 tissue samples (157 spleens and 116 intestines) were investigated from 171 animals belonging to nine carnivoran species, including one viverrid, one felid, and seven mustelid species (Table 1). The sample included 79 females (46.2 %), 87 males, 109 adults (63.7 %), 58 sub-adults/juveniles (33.9 %), and 3 fetuses (1.8 %).

3.1. Virus detection

Overall, eight of the 171 animals (4.7 %, 95 % CI: 1.5–7.9 %) tested positive for one virus, with six being amdoparvovirus-positive (3.5 %, 95 % CI: 0.7–6.3 %) and two protoparvovirus-positive (1.2 %, 95 % CI: <0.1–2.8 %) (Table 1). Specifically, one stone marten (*Martes foina*) was positive when tested with the pan-amdoparvovirus PCR, while both viral genera were found in Eurasian badgers (*Meles meles*, one positive animal for each genus) and wildcats (four were amdoparvovirus-positive and one was protoparvovirus-positive). No co-infections were identified. Both protoparvovirus-infected animals were female (one adult and one juvenile), while five out of six (83.3 %) amdoparvovirus-positive animals were males and five out of six adults.

Among wildcats, in which amdoparvovirus prevalence was the highest (10 %, 95 % CI: 0.7–19.3 %), virus prevalence was three times higher among males (3/18, 16.7 %) compared to females (1/19, 5.3 %) and two times higher in adults (3/21, 14.3 %) compared to juveniles (1/15, 6.7 %), although these differences were not statistically significant (Fisher's $p = 0.4$ and $p = 0.6$, respectively). There were no significant differences in the prevalence of amdoparvovirus when comparing the three different animal species in which these viruses were detected (Fisher's $p = 0.2$). The distribution of positive animals in the investigated region is shown in Fig. 1. While the two protoparvovirus-positive animals were sampled in two distant locations, all amdoparvovirus-positive animals were from the North-West part of Navarre.

Protoparvoviruses were identified only in spleen samples, but only for one of the two positive animals both spleen and intestine specimens were available. On the contrary, amdoparvoviruses were detected in both the spleen and intestine. Nonetheless, among the 5 animals for which both sample types were available, amdoparvoviruses were detected more frequently in the intestine with only two cases in which the virus was found in both tissues.

3.2. Molecular identification of detected protoparvoviruses

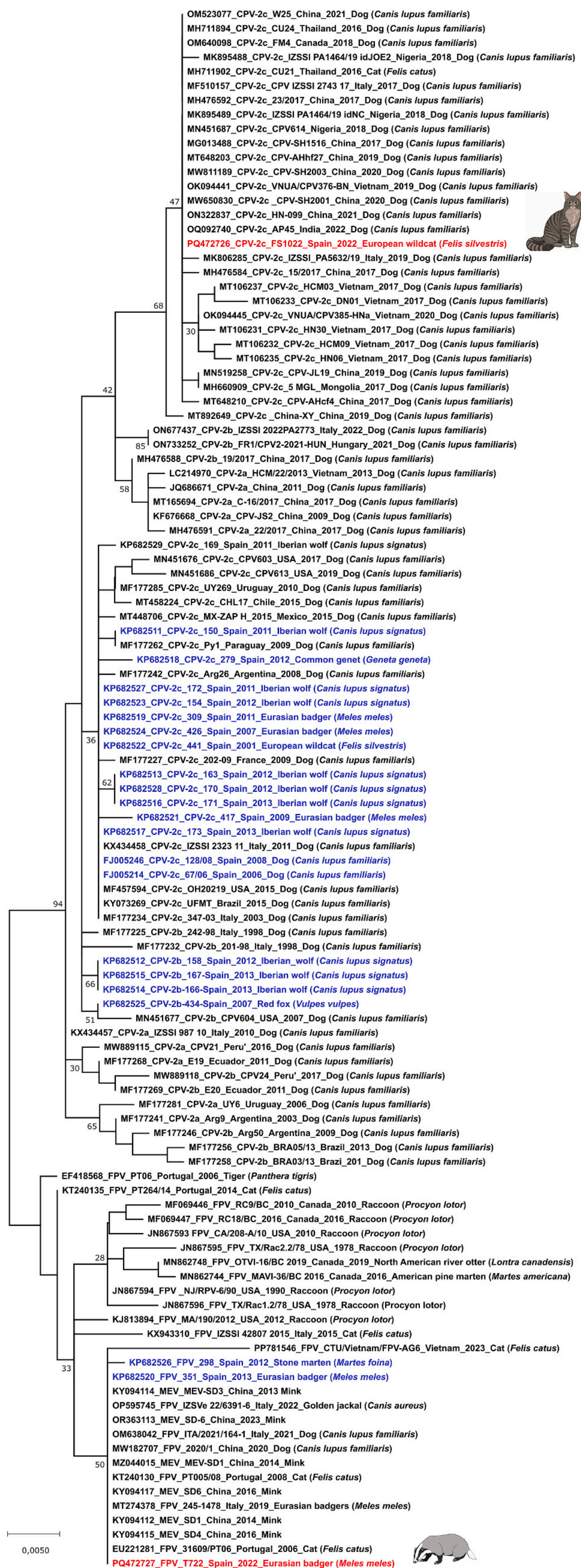
Amplicons of both protoparvovirus-positive animals were sequenced, obtaining a ~ 700 nt long fragment of the VP2 gene. Sequence analysis of the deduced amino acid residues at critical positions allowed to type two different protoparvoviruses: one FPV (detected in the samples of the Eurasian badger in the Southern part of Navarre) and one CPV-2c (detected in the European wildcat in the North-Eastern part of Navarre, Fig. 1).

Sequence analysis of the FPV strain showed 100 % nucleotide sequence identity with 56 sequences (Supplementary Table S2) obtained from viral strains detected mostly from domestic cats but also from wild carnivorans, in Asia (China) and Europe. These FPV strains were detected in mustelids such as Eurasian badgers in Spain (2013) and in mink in Italy (2019) and China (2015–2016 and 2023), in felids such as tigers (*Panthera tigris*) and cheetah (*Acinonyx jubatus*) in China (1999 and 2019), in canids such as golden jackal (*Canis aureus*) in Italy (2022), in a giant panda (*Ailuropoda melanoleuca*) in China (2018), and in domestic cats (*felis catus*) in Asia and Europe, including Portugal (2006 and 2008). This group of highly related sequences also included two FPV strains recently detected in dogs (*Canis lupus familiaris*) in China (2020) (Chen et al., 2021) and Italy (2021) (Diakoudi et al., 2022).

Sequence analysis of the CPV-2 strain showed a 100 % nucleotide identity with a high number of sequences ($n = 318$) of CPV-2c viral strains detected mainly in Asian countries (China, Thailand, Vietnam, South Korea, Sri Lanka, Indonesia, Singapore, Taiwan) and, since 2017, in other countries worldwide: Italy, Romania, Nigeria, Ethiopia, Namibia, India, Egypt, Iran, Canada (Franzo et al., 2023). To elucidate the phylogenetic relationships of the detected viruses with other strains identified worldwide, the two nucleotide sequences of detected FPV/CPV-2 strains and sequences from reference strains were included in the phylogenetic analysis. The obtained tree (Fig. 2) confirmed virus typing results and highlighted that the identified CPV-2 strain belonged to the so-defined "Asian CPV-2c lineage" (Franzo et al., 2023). This CPV-2c strain falls in a different branch than other CPV-2c sequences previously obtained from Spain (Calatayud et al., 2020; Decaro et al., 2006). On the contrary, the FPV sequence from this study was highly identical to strains previously identified in Spain and we were not able to define

Table 1
Number (percentage) of animals and samples found positive for *Amdoparvovirus* spp. and *Protoparvovirus carnivoran1*.

		Amdoparvovirus spp.			*Protoparvovirus carnivoran1*		
		Overall	Spleen	Intestine	Overall	Spleen	Intestine
Mustelidae							
Eurasian otter	*Lutra lutra*	0/12 (0.0)	0/11 (0.0)	0/9 (0.0)	0/12 (0.0)	0/11 (0.0)	0/9 (0.0)
Stone marten	*Martes foina*	**1/16 (6.3)**	**1/16 (6.3)**	**1/15 (6.7)**	0/16 (0.0)	0/16 (0.0)	0/15 (0.0)
European pine marten	*Martes martes*	0/18 (0.0)	0/18 (0.0)	0/15 (0.0)	0/18 (0.0)	0/18 (0.0)	0/15 (0.0)
Eurasian badger	*Meles meles*	**1/35 (2.9)**	**1/35 (2.9)**	0/6 (0.0)	**1/35 (2.9)**	**1/35 (2.9)**	0/6 (0.0)
European mink	*Mustela lutreola*	0/7 (0.0)	0/5 (0.0)	0/6 (0.0)	0/7 (0.0)	0/5 (0.0)	0/6 (0.0)
European polecat	*Mustela putorius*	0/10 (0.0)	0/8 (0.0)	0/6 (0.0)	0/10 (0.0)	0/8 (0.0)	0/6 (0.0)
American mink	*Neogale vison*	0/22 (0.0)	0/20 (0.0)	0/18 (0.0)	0/22 (0.0)	0/20 (0.0)	0/18 (0.0)
Viverridae							
Common genet	*Genetta genetta*	0/11 (0.0)	0/9 (0.0)	0/9 (0.0)	0/11 (0.0)	0/9 (0.0)	0/9 (0.0)
Felidae							
European wildcat	*Felis silvestris*	**4/40 (10.0)**	**1/35 (2.9)**	**4/32 (12.5)**	**1/40 (2.5)**	**1/35 (2.9)**	0/32 (0.0)
Total		**6/171 (3.5)**	**3/157 (1.9)**	**5/116 (4.3)**	**2/171 (1.2)**	**2/157 (1.3)**	0/116 (0.0)



(caption on next column)

Fig. 2. Molecular epidemiology of protoparvoviral strains identified in wild Spanish carnivorans. The phylogenetic tree is based on partial VP2 nucleotide sequences and was generated with MEGA X with the maximum-likelihood method, based on the Tamura 3-parameters model and a discrete Gamma distribution; branch support was assessed by bootstrapping and the outcome is shown for the main nodes. Reference strains (in black or in blue for viruses identified in Spain) and sequences from this study (in red) are labeled with their accession numbers, the virus and strain names, the Country and year of detection, and the host (Latin designation in brackets) in which they were identified. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

any specific spatial-temporal pattern.

3.3. Molecular identification of detected amdoparvoviruses

The ~750 nt long region amplified by the amdoparvovirus screening PCR was sequenced for all eight positive samples and, in all cases, sequences from different samples of the same animal were 100 % identical. Although the investigated region of VP2 does not allow an accurate resolution of species-level phylogenies (Canuti et al., 2022b), obtained sequences and sequences of all so far recognized amdoparvoviral species were used to build a phylogenetic tree (Fig. 3, Supplementary Fig. S1). In this analysis, the sequences identified in mustelids were located in different positions in the tree compared to the sequences identified in wildcats, demonstrating that the viruses found in these host types were different.

The virus found in the stone marten and in the Eurasian badger, which we named European mustelid amdoparvovirus 1 (EMAV-1), formed a highly supported clade (bootstrap = 100, SH-aLRT = 98) within a bigger clade (bootstrap = 100, SH-aLRT = 100) including all viruses whose primary hosts are members of the superfamily Musteloidea. These include the red panda amdoparvovirus (RaAPV) 1 and 2 (Alex et al., 2018; Zhao et al., 2022), identified in captive red pandas, Meles meles amdoparvovirus (MMADV) from farmed Asian badgers (Wu et al., 2024), the raccoon dog and fox amdovirus (RFAV), originally discovered in canids (Shao et al., 2014) but later on also detected in mustelids (mink and badger (Canuti et al., 2022b; Wu et al., 2024)), and a bigger clade including viruses of mink (AMDVs and British Columbia amdoparvovirus, BCAV (Canuti et al., 2022b)), skunks (skunk amdoparvovirus, SKAV (Canuti et al., 2017)), and martens (Labrador amdoparvovirus 1, LaAV (Canuti et al., 2020a)). This latter clade, which includes highly identical sequences from species that are difficult to discern with this genomic region, is collapsed in Fig. 3, but the full tree is shown in Supplementary Fig. 1. The two EMAV-1-infected animals were both found in the Northern part of the Mediterranean area of Navarre (Fig. 1).

On the contrary, the viruses from wildcats clustered within the non-Musteloidea viral clade. This includes viruses identified in rats (Rattus nitidus parvovirus, RtRn-ParV (Wu et al., 2018)), bats (SBEHV-1, Sabeidhel virus 1) (Kamani et al., 2022), and foxes (gray fox amdovirus, GFAV (Li et al., 2011), red fox fecal amdovirus, RFFAV (Bodewes et al., 2014), and Labrador amdoparvovirus-2, LaAV-2 (Canuti et al., 2020a)). The viruses from this study all clustered in a highly supported clade (bootstrap = 100, SH-aLRT = 99.7) together with RFFAV. The three strains FS1422, FS18, and FS051 likely belong to the same species as RFFAV (within clade pairwise identity: 92–7 %-99.4 %), in line with the overall within-species pairwise identity values observed in the alignment used to build the tree (data not shown). On the contrary, strain FS1322 was more divergent and likely a different virus, which we named European felid amdoparvovirus 1 (EFAV-1), that could be the first member of a different viral species as it was only 89.4–90.0 % identical to RFFAV sequences. All four positive wildcats were from the Northern part of the investigated region, between the Atlantic and the Mediterranean regions (Fig. 1).

Fig. 3. Molecular epidemiology of amdoparvoviral strains identified in wild Spanish carnivorans. The phylogenetic tree is based on partial VP2 nucleotide sequences and was generated with IQ-TREE 2 with the maximum-likelihood method, based on the TVM + F + I + R4 model; branch support was assessed with the SH-aLRT and by bootstrapping and the outcomes of these analyses are shown for the main nodes. Reference strains (in black or in blue for the virus identified in Spain) are labeled with their accession numbers, the virus and strain names, and the host (Latin designation in brackets) in which they were identified, while sequences from this study (in red) also include information about from which biological sample they were obtained. For simplicity, the clade including AMDV-1 to −3, SKAV, BACV, and LaAV-1 has been collapsed (indicated by a triangle), but the full tree is available in Supplementary Fig. S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Molecular characterization and phylogeny of European mustelid amdoparvovirus 1

Using previously published amdoparvoviral sequences, it was possible to design primers to amplify and sequence the full coding sequence of EMPV-1 MF13. Unfortunately, due to the low viral DNA concentration of the other mustelid virus and the high sequence divergence from known viruses of the wildcat viruses, it was not possible to obtain extended sequence information for the other identified viruses.

The obtained sequence (4189 nt) possessed all the typical characteristics of an amdoparvovirus (Canuti et al., 2022b), including the two gene cassettes coding for the three non-structural proteins (NS1, NS2, NS3), generated after splicing of the pre-m-RNA, and for the two capsid proteins (VP1 and VP2) (Fig. 4). Interestingly, a 6-nt insertion (AGCACG) in MF13 in front of the NS1/NS2 acceptor site causes a potential 2-AA insertion in NS1 and NS2. The same was noted in MMAV. All protein motifs typical of parvoviral SF3 helicases were identified in NS1, including the rolling circle replication motifs II (HIH in MF13) and III (YLFNKDK) and the Walker domains A (GPGGTGKTL), B (IWAEE), B′ (KAITGGGDVKVDTKNKQPQ), and C (VIVTSN). Additionally, we also identified the polyglycine stretch in VP1, and the immunogenic domain involved in antibody-dependent enhancement (ADE) was identified in the VP proteins (NYYSEHEIEQHTAKQPK) (Canuti et al., 2022b).

Over the complete coding sequence, EMAV-1 was 73.6–87.8 % identical to other amdoparvoviral sequences, with SBEHV-1 being the most divergent virus and MMAV the closest. No clear signal of recombination was detected for EMAV-1. The closest relative considering NS1 aa sequences was a RFAV strain identified in China in a badger (79.0 %), and a LaAV-1 strain was the closest considering VP1 (95 %). These values align with other amdoparvoviruses, which show a much higher degree of conservation in their capsid protein compared to NS1 (Canuti et al., 2022b). In the phylogenetic tree built with the full NS1 protein sequence, which is the most informative viral protein for defining the evolutionary relationships between amdoparvoviruses (Canuti et al., 2022b) as well as the standard region used for defining parvoviral taxonomy (Pénzes et al., 2020), EMAV-1 formed a well-supported clade with RFAV and MMADV (Fig. 4). Overall, three distinct sub-clades could be observed in the Musteloidea virus clade, one comprising North American viruses, including AMDVs - which are presumed to have originated in the American continent (Canuti et al., 2015) - SKAV,

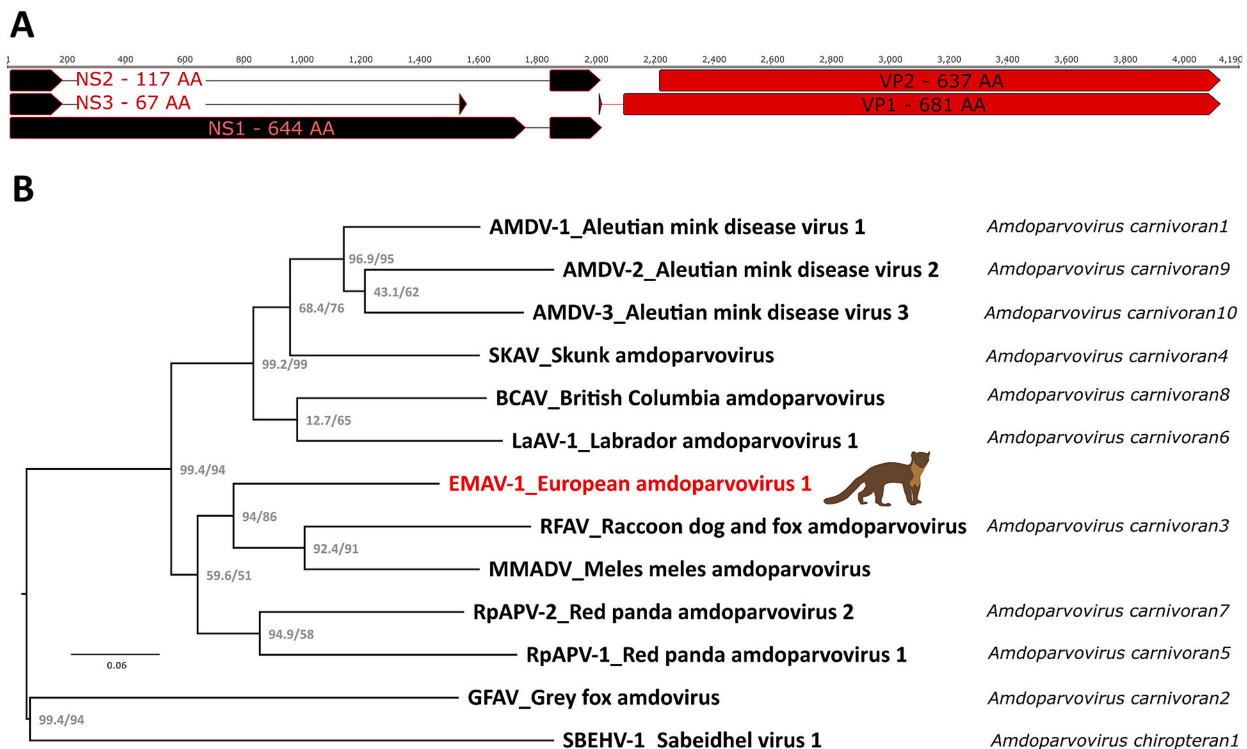


Fig. 4. Molecular characterization and evolution of European mustelid amdoparvovirus 1 (EMAV-1). A schematic representation of the full-coding sequence of strain MF13 is shown in panel A, where the coding regions for the non-structural proteins are shown in black and those for the structural proteins in red. Protein sizes are indicated next to the protein names. The phylogenetic tree, based on the full NS1 protein, is depicted in panel B. The tree was generated with IQ-TREE 2 with the maximum-likelihood method, based on the LG + I + G4 model; branch support was assessed with the SH-aLRT and by bootstrapping, and the outcomes of these analyses are shown for each node. Reference strains (in black) and the sequence obtained in this study (in red) are labeled with the virus abbreviated and full names. The official taxonomical designations (species) of each classified virus are indicated on the right. Accession numbers of sequences used to generate the tree are available in Supplementary Table S3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

BACAV, and LaAV-1, and two clades of viruses of likely Eurasian origin, the aforementioned clade including EMAV-1 and a distinct sub-clade comprising the two RpAPVs. Of note, amdoparvovirus-positive badgers were also found in Estonia, but these viruses were only partially sequenced (Knuuttila et al., 2015). When comparing these sequences to EMAV-1, it was clear that the Estonian and Spanish badgers were infected by different viruses. In fact, the viruses from Estonia were more closely related to RFAV (NS1 aa pairwise identity: 76.9–80.2 %) than to EMAV-1 (69.9–73.1 %), as also confirmed by the phylogenetic analysis performed with an alignment made with these partial sequences (Supplementary Fig. S2).

4. Discussion

While investigating viral ecology and diversity in wildlife has crucial implications for wildlife health, conservation, and management as well as for epidemic/pandemic preparedness, these are relatively unexplored fields. As multi-host pathogens capable of frequent cross-species transmission, parvoviruses represent a threat to carnivoran health and can have devastating consequences when they emerge in naïve populations, as they have proven in the past. For example, after its emergence in the 1970s, CPV-2 reached global distribution in dogs in only ~2 years. The virus, likely originating from wild animals, had such a high impact on pet health that the first vaccine for dogs was made available only one year after CPV-2 first identification (Hoelzer and Parrish, 2010). Similarly, AMDV emerged among farmed mink in the 1940s causing devastating epidemics with high mortality. Subsequently, AMDVs, which have been disseminated worldwide through animal import/export, leaked into the wild compartment and started threatening local naïve wild mustelid populations, posing a serious threat, particularly to endangered species (Canuti et al., 2015, 2016).

In this study, we identified well-known protoparvoviruses (CPV-2c and FPV) and several different amdoparvoviruses circulating among wild Spanish carnivorans. Some of the identified amdoparvoviruses were highly divergent from previously identified viruses but, unfortunately, low viral load and high sequence divergence prevented us from obtaining extended sequence information and performing conclusive taxonomical evaluations. For one virus, EMAV-1, identified in a stone marten, we could obtain the full genome and confirm that this virus is the first member of a novel amdoparvoviral species as it fulfills the requisites to be classified as such (over 15 % divergence in the NS1 protein from any other amdoparvoviral species (Canuti et al., 2022b; Pénzes et al., 2020)). Nonetheless, while the acquisition of complete genome sequences is required to confirm this aspect as parvovirus classification is based on NS1 protein sequences (Pénzes et al., 2020), partial VP2 sequencing allowed us to preliminary conclude that the virus found in the European badger belongs to the same species as EMAV-1. Conversely, three of the amdoparvoviruses found in wildcats likely belong to the same species as RFFAV, a never fully sequenced amdoparvovirus lacking official taxonomic designation, while the fourth virus from wildcats, EFAV-1, is also likely the first member of a novel species.

4.1. Protoparvoviruses in Spanish wildlife

To the best of our knowledge, only three studies used molecular methods to detect CPV-2/FPV in Spanish wildlife. One study detected FPV in one of 67 Iberian lynx (*Lynx pardinus*) in Southwest Spain (Extremadura) (Nájera et al., 2021), one study detected CPV-2/FPV in wildcats from central Spain (Castilla-La Mancha) (Candela et al., 2019), and the only previous molecular characterization of protoparvoviruses

in wild carnivorans confirmed the presence of protoparvoviruses in several species, including CPV-2b and CPV-2c in Iberian wolf (*Canis lupus signatus*), red fox (*Vulpes vulpes*), Eurasian badger, common genet (*Genetta genetta*), and wildcat, and FPV in badger, common genet, and stone marten (Calatayud et al., 2020). Additionally, exposure to the virus was also identified in Iberian lynx (Millán et al., 2009; Roelke et al., 2008) and wildcat (Millán and Rodríguez, 2009) from central Spain and wolf and fox from Northern Spain (Oleaga et al., 2015; Sobrino et al., 2008). Overall, these results indicate that protoparvoviruses are widespread in Spanish wildlife and that several viral lineages co-occur.

The CPV-2 we identified in this study, belongs to the so-called "Asian CPV-2c" lineage. This name was attributed to this recently defined lineage by early studies, reflecting the location of its first detection and to distinguish it from the original CPV-2c variant, which was first detected in Italy and, in the following years, evidenced in all continents. Similarly, the new Asian CPV-2c lineage rapidly spread worldwide and it keeps being detected in novel locations (Franzo et al., 2023). This represents the first evidence of this lineage in Spain and, particularly, in Spanish wildlife, similar to what was observed in wolves in Italy (Leopardi et al., 2022; Ndiana et al., 2021). Other studies suggested the close relationship between CPV-2 strains circulating in companion animals and their detection in wildlife. In Spain, already in the first years following its first detection, the original CPV-2c was detected in domestic dogs (Decaro et al., 2006, 2011) and, in an overlapping timeframe, it was also detected in wolves, badgers, and wildcats (Calatayud et al., 2020). While the lack of evidence for the spreading of the "Asian CPV-2c" lineage in Spanish companion animals is in disagreement with our study, it is reasonable to believe that this strain is circulating undetected among pets. The evidence of the expansion of the "Asian CPV-2c" lineage over time, replacing the classical CPV-2 variant in many countries (Hao et al., 2020; Jayappa et al., 2024; Mira et al., 2024a; Nguyen Van et al., 2022), and its detection in the European wildcat population suggest that this virus might be already circulating among Spanish pets. In the same way, the wildcat in which the CPV-2c variant was detected by Calatayud et al. (2020) was sampled in 2001, years before this variant was found in Spanish dogs. This reinforces the utility of monitoring wildlife as the "canary in the mine" in viral epidemiology. Unfortunately, the lack of updated CPV-2 surveillance in Spanish domestic animals prevented us from delineating a timeline of CPV-2 variant co-circulation and to define the likely recent introduction of the Asian lineage. Further studies should, therefore, elucidate the local spread of this and other lineages and determine their epidemiological relevance.

Similarly, little molecular data is currently available from FPV in Spanish wildlife (Calatayud et al., 2020), but the close relationship between the FPV strain we identified and those detected in other wild species or domestic cats is consistent with a wide host distribution. As in Calatayud et al. (2020), this study confirms the presence of FPV in Spanish wildlife, although limited to badgers among the tested animals, and demonstrates a significantly lower viral diversity compared to CPV-2 in this region.

4.2. Amdoparvoviruses in Spanish wildlife

Overall, 3.5 % of the animals were amdoparvovirus-positive and these viruses were identified in only three of the nine investigated species. Considering the relatively low viral prevalence (3–10 %) and the small number of samples tested for each species (7–40), the lack of virus identification may reflect this small sample size. A previous study found a seroprevalence of anti-amdoparvovirus antibodies of around 32 % in Spanish wild American and European mink, although with some regional differences (6–47 %) (Mañas et al., 2016). This discrepancy with our results is not surprising because serum antibodies are a marker for recent as well as old infections and our method detects currently present viral genomes. In addition, although parvoviruses are highly stable in the environment, the fact that we worked with dead-found animals in various preservation states may have affected our capacity to detect viral DNA and underestimating the true prevalence. Nonetheless, while a higher rate of positivity in intestinal samples compared to the spleen could be indicative of active viral shedding, amdoparvoviruses are capable of causing persistent infections in captive hosts (Alex et al., 2022b; Canuti et al., 2015), and the prevalence we calculated does not necessarily reflect acute infections. Finally, we need to highlight that the structural protein of amdoparvoviruses is highly conserved across species and this is particularly true for a highly immunogenic region linked to antibody-dependent enhancement (ADE), a mechanism used by the virus to infect its target cells (Canuti et al., 2022b). Therefore, the discrepancy between the results obtained with the two methods could also be due to a wider detection range of the serological methods compared to PCR.

We identified viruses belonging to two distantly related clades. Viruses from stone marten and Eurasian badger (EMAV-1) were included in a clade comprising a large group of viruses of Musteloidea and, within that clade, they were closer to viruses found in farmed Chinese animals, including viruses recently discovered in Asian badgers. Interestingly, however, this virus did not seem to be related to another amdoparvovirus identified in badgers from Estonia, which was in the same subclade but more related to RFAV strains. Both positive animals were sampled in the same part of Navarre (North-West) but, due to the low number of investigated animals, we cannot make definitive conclusion about the spread of these viruses within the region.

The viruses identified in wildcats (RFFAV and EFAV-1) were, on the contrary, closely related to a partially sequenced virus that was identified once in a Spanish red fox (Bodewes et al., 2014). This strain was found in the Basque Country, a region close to where we collected our samples, and the positive animals we identified were all sampled in the neighboring North-Western part of Navarre. This probably indicates that these viruses are endemic in the region. Finally, a previous study from Spain amplified a partial amdoparvoviral sequence from two American mink, one European mink, and a Eurasian otter (*Lutra lutra*) (Mañas et al., 2001), but these were more related to AMDV-like strains (Canuti et al., 2022b).

Interestingly, none of the AMDV-like strains known to circulate in farms (AMDV-1, AMDV-2, AMDV-3, BCAV (Canuti et al., 2022b)) were identified in this study, although the presence of infected mink farms in Spain has been documented (Prieto et al., 2017; Ryt-Hansen et al., 2017). While it is well known that AMDV strains vary in pathogenicity (Canuti et al., 2022b), the pathogenic potential of other amdoparvoviruses has not been documented and it is impossible to assess to which extent the identified parvoviruses constitute a health threat for the investigated animals, particularly for the endangered European mink.

4.3. Ecology and evolution of the identified viruses

While only a few positive animals were identified in this study and the investigation of a larger population is required to confirm our findings, we could observe that all amdoparvoviruses from wildcats belonged to closely related species. This made us conclude that these viruses might be enzootic in the wildcat population under investigation. Similarly, since closely related viruses were found in a red fox, we can hypothesize that cross-species transmission between these two host species has occurred. In the same way, viral transmission between the two EMAV-1-positive mustelids, marten and badger, could be postulated as they were infected by similar viruses. Nonetheless, our limited surveillance prevented us from identifying maintenance and/or dead-end hosts and we cannot make conclusions about potential transmission routes. Future studies should be focused on larger groups and more species to clarify these aspects. It will also be interesting to elucidate whether domestic animals play a role in viral transmission to and among wildlife.

As reported in previous studies (Canuti et al., 2022b), a correlation

between the phylogeny of the host and the one of amdoparvoviruses could be linked to virus-host co-evolution. In fact, in all performed phylogenies, viruses of Musteloidea clustered together, separated from viruses of other carnivoran and non-carnivoran hosts. This second group is much more heterogeneous and its phylogeny is characterized by long branches, indicating high evolutionary distances between the strains. We can, therefore, hypothesize that the extent of viral diversity in this part of the tree is probably much higher than what we currently know and in the future, the structure of this clade might change. Nonetheless, in the NS1 tree, within the Musteloidea clade, we observed three highly supported sub-clades, in which viruses grouped based on their geographical origin. One clade was composed exclusively of viruses from North America, including all AMDV-like strains that are now identified worldwide thanks to mink farming (Canuti et al., 2015, 2016). The other two clades included viruses of Eurasian origin: one comprising European (EMAV-1) viruses and viruses identified in Chinese farmed animals (RFAV and MMADV), and the other one grouping the two viral species identified in captive red pandas, whose natural habitat is between China and Northern India.

Combining these results with those from previous literature suggests that the evolutionary history of amdoparvoviruses is linked to their hosts, and viruses are more likely to cross the species barrier when the hosts are more genetically related, as is often the case (Buskirk, 2023). Additionally, the geographic grouping that characterizes both old and recent nodes (e.g., Alex et al., 2022a), indicates that viruses tend to evolve and segregate locally in the absence of non-man-linked transboundary movements. Nonetheless, there are a few exceptions, likely linked to the carnivorous lifestyle of amdoparvovirus hosts, where larger predators like fox and lynx can acquire the infection from smaller carnivorans, such as mustelids (Canuti et al., 2020a), or in the case of felines and canines sharing the same virus, as we report here.

On the contrary, the evolution of CPV-2 has been characterized since the very beginning of its emergence by the international dispersal of the same strains, a phenomenon heavily linked to its high environmental stability and resistance to treatment with disinfectants (Decaro and Buonavoglia, 2012) and (long distance) transport related to contaminated surfaces and domestic dog movements (Carmichael, 2005; DiGangi et al., 2021; Mira et al., 2018b). This favored the rapid spread and dispersal of emerging or re-emerging variants. The findings of this study are no exception as we identified a strain that is globally distributed. Although viral transmission among wild animals likely occurs (Calatayud et al., 2020; Canuti et al., 2022a), pets have an important role in shaping the evolution of this virus. The evidence of novel CPV-2 lineages in the domestic feline species is likely due to its rapid and consistent spread in the local canine population (Mira et al., 2024b; Pan et al., 2023) and, similarly, CPV-2 infections in wildlife likely originate from domestic animals (Ndiana et al., 2021).

A quite different scenario could be supposed for FPV, as it has been circulating among wild animals for a longer time (Parrish, 1995) and currently shows a lower intrinsic mutation rate compared to CPV-2 (Hoelzer et al., 2008). The detection of FPV in wildlife, in a wide host range and with low divergence between strains, even on a large time scale, could suggest the endemicity of a slow-evolving virus in wildlife, as previously reported among different carnivoran populations (Canuti et al., 2020b), and, potentially, wild animals could serve as maintenance host and reservoir for domestic species. However, this aspect still has to be elucidated in future more targeted studies. Finally, the recent evidence of FPV also in domestic dogs (Chen et al., 2021; Diakoudi et al., 2022), albeit deserving additional studies to clarify its epidemiological or pathogenic roles in these hosts, suggests the need to rethink our previous assumptions about its niches of distribution and patterns of transmission both in wildlife and among domestic animals.

5. Conclusions

Taken together, these results indicate that several parvoviral species, including at least three different amdoparvoviral lineages and three protoparvoviral lineages, co-occur and are likely co-circulating at different rates in sympatric Spanish wild carnivoran species, including felines, canines, and mustelids. While the low number of investigated samples prevented us from making strong conclusions about local cross-species transmission or endemicity, we could observe two different evolutionary paths for viruses within the two viral genera. Specifically, from global phylogenies, we observed the likely occurrence of virus-host co-evolution with local segregation in the absence of man-linked transboundary movements for amdoparvoviruses and the lack of host-specificity and international dispersal of the same strains of protoparvoviruses, likely as a result of pet movements. More studies, including more samples from various potential hosts and locations, are needed to clarify the ecology of these viruses and will likely lead to the identification of even higher viral diversity and additional hosts and allow to investigate the role of protoparvoviruses and amdoparvoviruses in co-infections.

Funding sources

The necropsies of the specimens were carried out within the contract from the Government of Navarra to the public company GAN-NIK for the management of the Ilundain recovery center. The rest of the work did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Marta Canuti: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Francesco Mira:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Diego Villanúa:** Writing – review & editing, Resources. **Ruth Rodríguez-Pastor:** Writing – review & editing, Data curation. **Annalisa Guercio:** Writing – review & editing, Resources. **Fermín Urra:** Writing – review & editing, Investigation. **Javier Millán:** Writing – review & editing, Resources, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors have nothing to declare.

Data availability

Sequences from this study were submitted to GenBank under accession numbers PQ472720-PQ472725 (amdoparvoviruses) and PQ472726-PQ472727 (protoparvoviruses).

Acknowledgments

We wish to thank the Rangers from the Navarre environmental department who collected the carcasses, the technicians E. Castién and M. López who provided permits for taking samples, and several veterinary students who assisted during necropsies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2025.105714.

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