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Summary

Protoparvovirus is a monophyletic viral genus that includes the species *Carnivore protoparvovirus-1* infecting domestic and wild carnivores. In this paper, the results of an epidemiological survey for *Carnivore protoparvovirus-1* in wild carnivores in Italy are reported. Overall, 34 (11.4%) out of 297 tested animals were positive for *Carnivore protoparvovirus-1*, but the frequency of detection was much higher in intestine (54%) than in spleen samples (2.8%), thus suggesting that the intestine is the best sample to collect from wild animals for parvovirus detection. Feline panleukopenia virus (FPV) was detected in red foxes (*Vulpes vulpes*) (2.8%, 7/252) and Eurasian badgers (*Meles meles*) (10%, 1/10) whilst canine parvovirus (CPV) was found in wolves (54.3%, 19/35), Eurasian badgers (60%, 6/10) and one beech marten (*Martes foina*) (100%, 1/1), with more than one parvovirus type detected in some animals. Protoparvoviral DNA sequences from this study were found to be related to CPV/FPV strains detected in Asia and Europe, displaying some amino acid changes in the main capsid protein VP2 in comparison with other parvovirus strains from wildlife. In particular, the two most common mutations were Ile418Thr and Ala371Gly, which were observed in 6/12 (50%) and 5/12 (41.7%) of the CPV sequences from this study.

Continuous surveillance for parvoviruses in wild carnivores and genetic analysis of the detected strains may help obtain new insight into the role of these animals in the evolution and epidemiology of carnivore parvoviruses.

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Key Words: Wild carnivores; *Carnivore protoparvovirus-1*; molecular characterisation; phylogeny; Italy.

Introduction

The genus *Protoparvovirus* within the family *Parvoviridae* consists of some important viruses of dogs and cats. These include canine parvovirus (CPV) and feline panleukopenia virus (FPV), which share a close genetic and antigenic relationship, so that they are now included in the unique species *Carnivore protoparvovirus-1* (Cotmore et al., 2014; 2019). Parvoviruses are the smallest animal DNA viruses, with a diameter ranging from 18 to 26 nm. These viruses have an icosahedral symmetry with a linear, single-stranded DNA genome about 5.2 kb long (Fig. 1). There are two major open reading frames in the genome, encoding for the non-structural proteins, NS1 and NS2, and the antigenically important capsid proteins, VP1 and VP2, respectively (Reed et al., 1988; Pérez et al., 2012). Although these viruses are host specific, they have been found to occasionally jump species barriers. For example, CPV has been detected not only in domestic dogs but also in wild canids like foxes and wolves (Zaccaria et al., 2016; Miranda et al., 2017; Alfano et al., 2019, Calatayud et al., 2020), as well as in other carnivore species such as domestic cats (Ikeda et al., 2000; Decaro et al., 2010, 2011; Truyen and Parrish, 2013; Balboni et al., 2018), stone martens (*Martes foina*) (Duarte et al., 2013), tigers (*Panthera tigris altaica*) (Steinel et al., 2000), Eurasian otters (*Lutra lutra*) (Viscardi et al., 2019), red pandas (*Ailurus fulgens*) (Qin et al., 2007) and giant pandas (*Ailuropoda melanoleuca*) (Guo et al., 2013). On the other hand, FPV, in addition to cats, has been reported in several wild carnivores, including badgers (*Meles meles*) (Steinel et al., 2000; Calatayud et al., 2020), racoons (*Procyon lotor*) (Allison et al., 2013) and Egyptian mongooses (*Herpestes ichneumon*) (Duarte et al., 2013; Calatayud et al., 2020). The genome of parvoviruses is known to mutate at a faster rate than other DNA viruses. However, CPV has shown a more rapid evolution than FPV (Shackelton et al., 2005, Decaro et al., 2008b, 2009). The expanding host range of parvoviruses has been attributed to the genetic evolutive ability of the virus (Decaro and Buonavoglia., 2012, 2017).

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The original CPV strain (CPV-2) was postulated to originate from FPV because of the close resemblance between both viruses with VP2 differences in few residues. The virus was initially believed to derive from a contaminated vaccine, which could account for the sudden and simultaneous appearance of CPV enteritis in dog populations globally. Subsequently, an intermediate carnivore host was speculated to have adapted FPV to canid hosts. Currently, this putative adaptive host remains unknown (Decaro and Buonavoglia, 2012, 2017). However, FPV-like CPVs were reported in some wild canids and diverse genetic changes were observed in CPV/FPV/FPV-like CPV isolates from different hosts over time with some of them varying from the classic FPV by as little as one amino acid (aa) change in VP2 (Duarte et al., 2013).

Compared to FPV, the original CPV-2 displays 7 aa changes in the VP2 protein (residues 80, 93, 103, 232, 323, 564, and 568) (Decaro and Buonavoglia, 2012). Soon after its emergence in late 1970s, CPV-2 gave orgin to two antigenic variants, CPV-2a and CPV-2b, which were followed in 2000 by a third variant CPV-2c. These variants are now variously distributed worldwide (Decaro et al., 2007; Decaro and Buonavoglia, 2012, 2017). More mutations have been observed in additional VP2 residues since the emergence of the three CPV variants, with the most recently reported (Phe276Tyr, Tyr324Ile and Thr440Ala) being attributed to possible vaccine pressure (Decaro et al., 2009; Mira et al., 2018a, 2019; Zhou et al., 2017). Following analyses of several FPV sequences from the nucleotide databases, it is now accepted that FPV sequences can feature either threonine or isoleucine at position 101, the former being consistently observed in CPV sequences (Zhou et al., 2017).

In view of the multi-host nature of CPV and FPV, and in order to contribute to a better understanding of the virus-host interactions, as well as to further clarify the role of wildlife in epizootiology of CPV and FPV, the aim of the study reported in the present paper was to characterize at molecular level, *Carnivore protoparvovirus-1* strains detected in wild carnivores in Italy.

Material and methods

Sample collection

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A total of 248 lymphoid tissue samples (spleen and/or mesenteric/lymph nodes) and 50 small intestine samples were collected from wild carnivores found dead, including foxes *(Vulpes vulpes)* (n = 251; 5 intestine, 246 spleen, 1 lymph node samples), wolves (*Canis lupus italicus*) (n = 35; 34 intestine, 1 spleen samples), Eurasian badgers (*Meles meles*) (n = 10; 10 intestine samples) and one beech marten (*Martes foina*) (n = 1; 1 intestine sample). For one fox, samples from spleen and lymph node were collected and pooled in order to be tested as a single sample. Most sampled carnivores had died of vehicular trauma and no animal presented gross lesions suggestive of parvoviral infection, thus suggesting that the detected virus was not the lilely cause of their death. Information including the animal species and place of origin were collected. Sample collection was carried out during routine necropsy procedures from 2014 to 2020 and tissues were temporarily stored at −20 °C at Istituto Zooprofilattico del Mezzogiono and Istituto Zooprofilattico dell'Abruzzo e del Molise "Giuseppe Caporale", before getting transported under the cold chain to the Infectious Diseases Unit of the Department of Veterinary Medicine, University of Bari, for testing. Additional samples were directly collected at the Avian and Wildlife Diseases Unit of the Department. The study areas included Tuscany, Abruzzi, Lazio, Molise, Campania, Calabria, Apulia and Basilicata regions of Italy (Fig. 2).

DNA extraction

Tissues were homogenized (10% w/v) in Dulbecco′s Modified Eagle′s Medium (DMEM) and processed according to the protocol described by Desario et al. (2005). Viral DNA was extracted from the supernatant of the homogenates using the IndiSpin Pathogen Kit (Indical Bioscience GmbH, Leipzig, Germany), following the manufacturer's instruction and stored at −80 °C until use.

Real time qPCR

DNA extracts were first screened for the presence of CPV/FPV DNA using a real-time PCR (qPCR) assay based on TaqMan technology (Decaro et al., 2005). Subsequently, positive samples were characterized by means of qPCR assays based on minor groove binder (MGB) probes able to differentiate CPV types 2a/2b and 2b/2c (Decaro et al., 2006b) and CPV/FPV (Decaro et al., 2008a). To rule out the presence of CPV strains of vaccine origin, samples were further tested using MGB probe assays that discriminate between vaccine and field strains of CPV (Decaro et al., 2006a, c)

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(Table 1). Real time PCRs were performed by the use of iTaq™ Universal Probes Supermix (Bio-Rad Laboratories SRL, Segrate, Italy).

PCR amplification of VP2 gene

PCR for amplification of full-length VP2 gene in samples that tested positive by CPV/FPV qPCR was carried out as described by Decaro et al. (2008b, 2009). Briefly, PCR was performed in a final volume of 50 µl containing 5 µl of DNA extract and TaKaRa LaTaqTM kit (Takara Bio Europe S.A.S. Saint-Germain-en-Laye, France) mix consisting of 24.5 µl of PCR grade water, 5 µl of 10x buffer, 5 µl of $MgCl_2$ (25mM), 1 µl of forward and reverse primers (50 µM), 8 µl of deoxynucleotide triphosphates (dNTPs) (2.5mM), 0.5 µl of Takara La Taq polymerase (5U/µl). PCR was run as follows: initial denaturation at 94 °C for 2min, followed by 40 cycles of denaturation at 94°C for 1 min 30sec, annealing at 59 °C for 1min, and extension at 68 °C for 2 min, followed by a final extension at 68 °C for 10 min. The PCR products were analyzed on a 1.5% agarose gel prepared in TBE buffer (0.09M of boric acid, 0.09 M of Tris, 0.0025M of EDTA, pH 8.3) and subjected to an electrophoresis at 50V for 90 min. The amplification bands were visualized on a Gel Doc™ EZ (Bio-Rad Laboratories SRL, Segrate, Italy).

Sequence and phylogenetic analyses

The PCR amplicons were purified by Qiaquick PCR Purification kit (Qiagen GmbH, Hilden, Germany). The purified PCR products with sufficient DNA concentration (>10 ng/µl) were directly sequenced in both directions. Sequence reactions were carried out using the BigDye 3.1 Ready reaction mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Analyses of the sequences with web-based tools BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) and FASTA (https://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) with default values were used to find homologous hits. The obtained sequences were aligned with cognate CPV, FPV and mink enteritis virus (used as outgroup) strains retrieved from GenBank database by MAFFT algorithm (Katoh et al., 2002).

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Phylogenetic analyses were performed with Bayesian inference by MrBayes software using 4 chains run for >1 million generations (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and Model Test software (http://evomics.org/resources/software/molecular-evolution-software/modeltest/) was used to identify the most appropriate model of evolution for the entire dataset and for each gene individually. The identified program settings for all partitions, under the Akaike Information Criteria, included 5-character states (general time reversible model), a proportion of invariable sites, and a gamma distribution of rate variation across sites. Sequence editings, alignments and phylogenetic analyses were performed by Geneious software version 9.1.8 (Biomatters, Auckland, New Zealand).

Genbank sequence submission

Nucleotide sequences of strains Badger/289.19-5518, Wolf/415.19-3530, Badger/289.19-5860, B.marten/289.19-5771, Badger/289.19-5624, Fox/51.20, Badger/245.19-1478, Fox/51.20-114, Fox/51.20-178, Wolf/145.20-1274, Wolf/145.20-1788, Wolf/145.20-1863, Wolf/67.20-276, Wolf/663/19-6976, Wolf/67.20-1106, and Wolf/67.20-1302 used for phylogeny were deposited in GenBank under accession nos. MT353760, MT353761, MT353762, MT353763, MT353764, MT274377 MT274378, MT454917, MT454916, MT454915, MT454914, MT454913, MT454911, MT454910, MT454909, MT454908, respectively.

Results

Screening for Carnivore protoparvovirus-1

The preliminary screening detected the presence of *Carnivore protoparvovirus-1* DNA in 34 (11.4 %) out of 298 tested specimens from 297 animals. Overall, 27/50 small intestine (54%), 6/247 spleen (2.8%) and 1/1 lymph node (100%) samples tested positive. The distribution of the *Carnivore protoparvovirus-1* positive samples according to the sampled region and animal species is reported in Table 2. The highest rate of CPV/FPV infection was detected in samples collected from Abruzzi with

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a prevalence of 47.8% (22/46), whilst the lowest prevalence was observed in Apulia and Calabria, with no positive samples from these regions (Table 2 and Fig. 2).

FPV was detected in red foxes (2.8%, 7/251) and Eurasian badgers (10%, 1/10) whilst CPV was found in wolves (54.3%, 19/35), Eurasian badgers (60%, 6/10) and one beech marten (100%, 1/1). Co-infections caused by CPV-2b/2c and CPV-2b/FPV were detected in 6 wolves and 2 badgers respectively (Tables 3 and 4).

Viral DNA loads were lower in the spleen samples (1.1×10^3 median DNA copy numbers/10 µl of template) than in the lymph node (2.2×10^3 median DNA copy numbers/10 µl of template) but highest in the intestines (1.9×10^5 DNA copy numbers/10 µl of template) and ranged from 1.3×10^1 to 2.6×10^7 DNA copy numbers/10 µl of template (Table 3).

VP2 gene sequence and phylogenetic analyses

Due to the low virus titers of the specimens, only 7 full and 10 partial VP2 sequences were successfully amplified by conventional PCR and directly sequenced. The sequences from this study showed 96.3% to 99.6% nucleotide identity. In total, 38 and 15 mutations were observed in CPV and FPV sequences, respectively. There were 21 synonymous mutations for CPV sequences and 13 for FPV sequences (Tables 5 and 6). Deduced aa VP2 sequences were compared with the analogous sequences of CPV and FPV strains detected in wild carnivores (accession numbers KP682514, JX411926, KP682519, and KP682520; Tables 5 and 6). Two non-synonymous mutations were observed at positions 91 and 202, from Ala to Thr and from Pro to Thr, respectively, in the FPV sequence Fox/51.20 (Table 5). Non-synonymous mutations observed in the CPV sequences from Italian wild carnivores include Ala371Gly (5/12), Gln370Arg (3/12) and Tyr324Ile (3/12) (Table 6).

The phylogenetic tree appeared polyphyletic, with CPV-2b viruses clustering into 3 distinct clades (I, IV and V), whilst types -2a, -2c and FPV clustered in clades II, III and VI respectively. The FPV sequences from Italian wild carnivores were closely related to FPV field isolates from Asia and Europe sourced from various animal species. Strains Fox/51.20 and Fox/51.20-114 clustered with the Italian isolate FPV/ITA/CAT/KX943311, identified in a cat in Italy in 2015. Strain Badger/245.19-1478 (MT274378) formed a cluster with the Spanish and Chinese FPV strains (Fig. 3). Strain Fox/51.20-178 (MT454914) clustered with Portuguese FPV strains.

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The sequenced CPV-2c strains clustered with other CPV-2c viruses from Asia and parts of Europe, including Italy, Spain and Portugal, whist the CPV-2b strains clustered together and evidenced a close relatedness to other strains of the same virus from Asia.

Discussion

CPV and FPV are endemic in domestic dog and cat populations. These viruses have over time been detected in a wide range of wild carnivores (Steinel et al., 2000, 2001; Hoelzer and Parrish., 2010; Allisson et al., 2014; Calatayud et al., 2020). However, the dynamics of infection and disease in these multi-species populations in the sylvatic cycle remain unclear (Duarte et al., 2013; Behdenna et al., 2019). These viruses have shown continuing evolution over the years, thus raising concerns about emergence of more pathogenic strains with broadened host range (Hoelzer and Parrish., 2010; Decaro and Buonavoglia, 2012), as well as probable vaccine escape mutants (Zhou et al., 2017). There is evidence of transmission of CPV between domestic and wild carnivores, but the direction in which this transmission occurs in addition to the role of wildlife in maintenance of the infection is not well understood (Steinel et al., 2001; Allison et al., 2014; de Almeida Curi et al., 2016; Miranda et al., 2017). CPV was earlier suggested to have originated from FPV through an unknown intermediate wild canivore (Truyen et al., 1998), implying a transmission directed from cat to wildlife and finally to the domestic dog. Considering relatively higher prevalence of these viruses in domestic than wild carnivores, it was widely accepted that wildlife infections were more likely “spillovers” from domestic pets in the urban cycle (Mendenhall et al., 2016; Behdenna et al., 2019), which may have been facilitated by the contacts of animals at the urban-wildlife interface (Vieira et al, 2017). There are, however, limited doubts that these wild hosts could also serve as virus reservoirs and sources of infection to the domestic animal population (Van Arkel et al., 2019; Decaro et al., 2020; Kelman et al., 2020).

In this study, a total prevalence of 11.4% was observed for *Canivore protoparvovirus-1* in wild carnivore species, which is comparable to what was reported by other studies elsewhere (Allison et al., 2014; Miranda et al., 2017), but contrasts with the much higher prevalence detected by Duarte *et al* (2013) and is much appreciably lower than the prevalence observed in domestic dogs (Vieira et al., 2017). However, the observed parvovirus prevalence could have been biased by a wrong sampling

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(spleens rather intestinal contents), considering that the detection rates were much higher in the intestinses (64%) than in the spleens (only 2.8%).

Different positive rates of parvovirus infection were observed in the sampled Italian regions, with the highest virus circulation being observed in Abruzzi. However, the obtained results may have been biased by the non-homogenous sampling of different carnivores species in the different regions. For instance, the number of sampled animals from Apulia and Calabria was too low to claim the parvovirus infection is not present in wildlife of those regions.

Interestingly, FPV was detected in only 2.8% and 0% of red foxes and wolves, respectively. FPV-like viruses have been detected in red foxes elsewhere (Duarte et al., 2013), leading to the speculation that they may have served as the intermediate hosts through which FPV evolved into CPV-2 (Truyen et al., 1998; Hoelzer and Parrish, 2010). The transferrin receptors (TfRs) in cats were shown to be similar to those in gray foxes (*Urocyon cinereoargenteus*). Moreover, receptor mapping has revealed a difference in the TfR glycosylation pattern between domestic dogs and cats/foxes, letting the former to be susceptible to only CPV and the latter to both CPV and FPV (Allison et al., 2016). The lower parvovirus prevalence observed in red foxes with respect to other carnivores may be due to the low proportion of intestines that were available for foxes with respect to other tested animals. However, it is well known that initial parvovirus localization and replication take place in lymphoid tissues before systemic spread (Snyder, 2017), while the virus parvovirus can disappear more rapidly from the small intestine with respect to the blood (Decaro and Buonavoglia, 2012, 2017). Previous research has suggested parvovirus B19 DNA can persist in various tissues of healthy individuals without viremia, presumably in a latent form (Söderlund-Venermo et al., 2002; Corcioli et al., 2008), and a similar finding was reported in bone marrow tissues of healthy cats with FPV and CPV DNA (Balboni et al., 2018).

Despite being the largest population included in this study, all the foxes were found to be negative for CPV. A low prevalence of this virus in red foxes has been commonly observed in other wildlife surveys (Almberg et al., 2009; Belsare et al., 2014; Calatayud et al, 2020), leading to the hypothesis that these carnivores may be relatively more resistant to the virus than other related wild carnivore species (Sobrino et al, 2008). In contrast, Duarte et al. (2013) reported a relatively higher CPV

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prevalence in foxes. In the present study, 54.3% of the tested wolves were positive for CPV, whilst none of these carnivores weas positive for FPV. Findings from previous studies suggest that wolves may be more susceptible to CPV than foxes (Almberg et al, 2009; Allison et al., 2014; Calatayud et al., 2020).

The only beech marten tested was positive for CPV-2b whilst CPV and FPV were detected in 6 and 1 out of 10 tested badgers, respectively, showing a relatively high occurrence in these species. A high serological prevalence against carnivore parvoviruses was previously observed in the same species (Duarte et al., 2013), while CPV and FPV DNAs were previously detected in badgers (Steinel, 2000; Barlow et al., 2012; Calatayud et al., 2020). Overall, CPV-2b was the predominant parvovirus type detected in our study. To the best of our knowledge, this study is the first report of co-infection with CPV and FPV in a badger. Furthermore, co-infections with CPV-2a/b and 2b/c were also detected in 2 and 4 wolves, respectively, in addition to another badger that had a CPV-2a/b co-infection. To date, reports on co-infections with more than one CPV types are available only in domestic animals (Battilani et al., 2007; Vieira et al., 2008). Interestingly, this finding may lead to possible recombination and generation of more genetic diversity and thus viruses with different properties such as expanded host range or increased pathogenicity. Recombination events between different carnivore parvoviruses have been previously reported (Ohshima et al., 2009; Wang et al., 2012).

The FPV-like sequences from this study were similar to other FPV sequences from field viruses in Asia and Europe (Fig. 3). Unlike the viruses detected by Truyen et al. (1998), our FPV sequences were not intermediates of CPV and FPV as they displayed the expected aa residues at the generally accepted mutation sites for FPV (Zhou et al., 2017). Interestingly, the sequence from strain Fox/51.20 (MT274377) displayed two unreported aa changes from alanine to threonine and from proline to threonine at positions 91 and 202, respectively, in addition to additional point mutations.

As expected, more mutations were evidenced in CPV than FPV sequences. All CPV sequences from this study had alanine at aa position 297. CPV strains with Ala297, since 1990, have been predominantly circulating among domestic dogs in various regions of the world (Zhou et al., 2017) and are suspected to have replaced original variants with serine at the same position (Geng et al., 2015, Mira et al., 2018a, Ogbu et al., 2019). This mutation appears to have occurred as a result of host

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adaptation of the virus (Pereira et al., 2007; Geng et al., 2015). The CPV-2b strains from this study were very similar to the recent Italian strains from domestic dogs described by de Almeida Curi et al. (2016) and by Battilani et al. (2019). Among the more commonly observed mutations were the Gly371, which was observed in 5 CPV-2b sequences, and Thr418, which was observed in 6 CPV-2b sequences. Both mutations have been previously described in CPV-2b isolates from Sardinia, Italy (Dei Giudici et al., 2017), although their significance is presently unknown. The sequences from this study retained threonine and tyrosine at positions 440 and 324 respectively, contrasting with the recent isolates from domestic animals worldwide (Zhang et al., 2010; Battilani et al., 2017; Dei Giudici et al., 2019; Zhou et al., 2017, Mira et al., 2018), except one CPV-2b (MT454914) and two CPV-2c strains (MT454908 and MT454909), which displayed 324Ile. Thr440Ala, Tyr324Ile and Phe267Tyr-CPV mutants have been recently spread globally and are suggested to represent vaccine escape mutants (Zhou et al., 2017). In the present study, however, these three mutations were not simultaneously observed in any of the obtained sequences. Change Thr440Ala was not observed in any of the sequences whilst Tyr324Ile occurred only in CPV-2a strain (MT454914) and in two CPV-2c strains (MT454908, MT454909), the latter also displaying mutation Phe267Tyr. Most CPV strains in this study may be older strains that have circulated in the wild, so, unlike the virus strains found in domestic carnivores, were not subjected to evolutionary pressure from vaccines. Substitution Gln370Arg, which was observed in one CPV-2c strain, has also been previously described extensively in several countries (Guo et al., 2013; Geng et al., 2015; Wang et al., 2016; Zhao et al., 2017; Mira et al, 2018; Mira et al, 2019, Ogbu et al., 2019; Zhang et al., 2019). Involving a residue exposed on the capsid surface, this mutation may affect the biological properties of these strains (Ogbu et al., 2019). All sequences from this study evidenced either aspartic acid or asparagine at position 375, which is consistent with all other CPV sequences detected elsewhere (Zhou et al., 2017). Further studies are needed to determine the functional implication of some mutations that were detected in the present study and that seem to be maintained in recent CPV isolates in Italy.

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and Ricerca Corrente 2018 “Nuovi virus gastroenterici di cane e gatto: sviluppo di protocolli NGS per la valutazione del rischio zoonosico”, recipient Flora Alfano).

CONFLICT OF INTEREST

The authors of this manuscript declare that there are no conflicts of interest.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to. No ethical approval was required as this study was conducted of carcasses of animals found dead and submitted to routine necropsy procedures for diagnostic purposes.

DATA AVAILBALE STATEMENT

The nucleotide sequences that support the findings of this study will be openly available in the GenBank database at https://www.ncbi.nlm.nih.gov/genbank/ under accession nos. MT353760, MT353761, MT353762, MT353763, MT353764, MT274377 MT274378, MT454917, MT454916, MT454915, MT454914, MT454913, MT454911, MT454910, MT454909, MT454908.

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FIGURE LEGENDS

Figure 1. Schematic representation of the *Carnivore protoparvovirus-1* genome and encoded proteins.

Figure 2. Geographic distribution of samples from wild carnivores that were tested for *Carnivore protoparvovirus-1*. Italian regions where sample collection was carried out are highlighted in dark grey, while positive samples are indicated by orange dots, whose size is proportional to the number of positive cases. The number of parvovirus positive animals and tissues is reported for each region and animal species. I, intestines; LN, lymph nodes; S, spleens.

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Figure 3. Phylogenetic tree showing the alignment of the complete (1809nt) sequence of the ORF2 of canine parvoviruses, feline panleukopenia viruses and the strains detected in this study. Mink enteritis virus (MEVB) (GenBank accession nr FJ592174) was used as an outgroup. Posterior output of the tree was derived from Bayesian inference using 4 chains run for >1 million generations, a general time-reversible model, a proportion of invariable sites, a gamma distribution of rate variation across sites, and a subsampling frequency of 1,000. Posterior probability values >95 are indicated on the tree nodes. The black arrows indicate the parvovirus strains identified in this study. Scale bar indicates nucleotide substitutions per site.

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Table 1. Oligonucleotides used for *Carnivore protoparvovirus-1* detection and characterization.

Assay	Primer/probe	Sequence 5' to 3'	Polarity	Specificity	Position	Amplicon size	Reference
Carnivore protoparvovirus-1 TaqMan assay	CPV-For	AAACAGGAATTAACTATACTAATATATTTA	+	FPLV/CPV	4102-4131[a]	93 bp	Decaro et al., 2005
	CPV-Rev	AAATTTGACCATTTGGATAAACT	-		4172-4194[a]		
	CPV-Pb	FAM - TGGTCCTTTAACTGCATTAAATAATGTACC - TAMRA	+		4139-4168[a]		
FPV/CPV assay	FPV/CPV-For	ACAAGATAAAAGACGTGGTGTAACTCAA	+	FPLV/CPV	3713-3740[a]	83 bp	Decaro et al., 2008b
	FPV/CPV-Rev	CAACCTCAGCTGGTCTCATAATAGT	-		3771-3795[a]		
	FPV-Pb	VIC – ATGGGAAATACAGACTATAT - MGB	+	FPLV	3741-3760[a]		
	CPV-Pb	FAM – ATGGGAAATACAAACTATAT - MGB	+	CPV	3741-3760[a]		
CPV-2a/2b assay	CPVa/b-For	AGGAAGATATCCAGAAGGAGATTGGA	+	All CPV types	1719-1744[b,c]	93 bp	Decaro et al., 2006a
	CPVa/b-Rev	CCAATTGGATCTGTTGGTAGCAATACA	-	All CPV types	1785-1811[b,c]		
	CPVa-Pb	VIC – CTTCCTGTAACAAATGATA - MGB	+	CPV-2a	1765-1783[b]		
	CPVb1-Pb	FAM – CTTCCTGTAACAGATGATA - MGB	+	CPV-2b	1765-1783[c]		
CPV-2b/2c assay	CPVb/c-For	GAAGATATCCAGAAGGAGATTGGATTCA	+	All CPV types	1721-1748[c] 1155-1182[d]	150 bp	Decaro et al., 2008
	CPVb/c-Rev	ATGCAGTTAAAGGACCATAAGTATTAAATATATTAGTATAGTTAATTC	-	All CPV types	1823-1870[c] 1257-1304[d]		
	CPVb2-Pb	FAM – CCTGTAACAGATGATAAT - MGB	+	CPV-2b	1768-1785[c]		
	CPVc-Pb	VIC – CCTGTAACAGAAGATAAT - MGB	+	CPV-2c	1202-1219[d]		
CPV-2/variant assay	CPV2/v-For	GCAGTTAACGGAAACATGGCTTTAG	+	All CPV types	3057-3081[a] 772-796[b]	68 bp	Decaro et al., 2006b
	CPV2/v-Rev	TCAACCAATGACCAAGGTGTTACAA	-	All CPV types	3100-3124[a] 815-839[b]		
	CPV2-Pb	FAM – TGTGCATGAATATCAT - MGB	+	CPV-2 (old type)	3082-3097[a]		
	CPVv-Pb	VIC – TTTGTGCATGAGTATCAT - MGB	+	CPV variants	797-814[b]		
CPV-2b SAH/field assay	SAH/f-For	CAACAAGATAAAAGACGTGGTGTAACTC	+	All CPV types	3711-3738[a] 1426-1453[c]	85 bp	Decaro et al., 2006c
	SAH/f-Rev	CAACCTCAGCTGGTCTCATAATAGT	-	All CPV types	3771-3795[a] 1486-1510[c]		
	SAH-Pb	VIC-AAATGGGAAAAACAAACT-MGB	+	Strain SAH	1454-1471[a]		
	CPVf1-Pb	FAM-AAATGGGAAATACAAACT-MGB	+	Field strains	1454-1471[c]		

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CPV-2b 39/field assay	CPV39/f-For	GCATTGGGCTTACCACCATTTCTAA	+	All CPV types	3636-3660[a] 1351-1375[c]	95 bp	Decaro et al., 2006c
	CPV39/f-Rev	CCACGTCTTTTATCTTGTTGAACTCCTATA	-	All CPV types	3701-3730[a] 1416-1445[c]		
	CPV39-Pb	VIC-CTTTGCCTCAATCTGAA-MGB	+	Strain CPV-39	1379-1395[c]		
	CPVf2-Pb	FAM- TTTGCCTCAAGCTGAA-MGB	+	Field strains	1380-1395[c]		

[a] Oligonucleotide positions are referred to the sequences of CPV-2 strain CPV-b (accession no. M38245) and FPV strain FPV-b (accession no. M24004).

[b] Oligonucleotide positions are referred to the sequence of CPV-2a strain CPV-15 (accession no. M24003).

[c] Oligonucleotide positions are referred to the sequences of CPV-2b strain CPV-39 (accession no. M74849).

[d] Oligonucleotide positions are referred to the sequences of CPV-2c strain 56/00 (accession no. AY380577).

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Table 2. Sample distribution according to Italian regions and wild carnivore species.

Italian region	Animal species	No. of tested animals	Sample types	No. of samples testing positive to *Carnivore protoparvovirus-1*
Tuscany	Red fox (*Vulpes vulpes*)	2	1 intestine, 1 spleen	0
	Wolf (*Canis lupus italicus*)	4	1 intestine, 3 spleens	1
	Eurasian badger (*Meles meles*)	1	1 spleen	1
	Total	**7**		**2**
Lazio	Red fox (*Vulpes vulpes*)	27	9 intestines, 18 spleens	0
	Wolf (*Canis lupus italicus*)	7	1 intestine, 6 spleens	1
	Total	**34**		**1**
Campania	Red fox (*Vulpes vulpes*)	170*	170 spleens, 1 lymph node	7
	Wolf (*Canis lupus italicus*)	1	1 spleen	0
	Total	**171**		**7**

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Abruzzo	Red fox (*Vulpes vulpes*)	26	15 intestines, 11 spleens	**0**
	Wolf (*Canis lupus italicus*)	19	6 intestines, 13 spleens	16
	Eurasian badger (*Meles meles*)	8	2 intestines, 6 spleens	5
	Beech marten (*Martes foina*)	1	1 spleen	1
	Total	**54**		**22**
Molise	Wolf (*Canis lupus italicus*)	3	3 intestines	2
	Eurasian badger (*Meles meles*)	1	1 intestine	0
	Total	**4**		**2**
Apulia	Red fox (*Vulpes vulpes*)	11	11 intestines	**0**
	Total	**11**		**0**
Calabria	Red fox (*Vulpes vulpes*)	15	15 spleens	0
	Wolf (*Canis lupus italicus*)	1	1 spleen	0
	Total	**16**		**0**

*From one fox, lymph node and spleen samples were collected and pooled

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Table 3. Details of samples from wild carnivores that tested positive for *Carnivore protoparvovirus-1*.

Animal species	Year	Region	Prot. No.	Tested sample(s)	*Carnivore protoparvovirus-1* DNA copy numbers/10 µl template	*Carnivore protoparvovirus-1* strain(s)
Red fox (*Vulpes vulpes*)	2014	Campania	51.20-113	Spleen	2.94×10^3	FPV
Red fox (*Vulpes vulpes*)	2017	Campania	51/20	Spleen	2.12×10^2	FPV
Red fox (*Vulpes vulpes*)	2015	Campania	51.20-127	Spleen	3.87×10^3	FPV
Red fox (*Vulpes vulpes*)	2015	Campania	51.20-147	Spleen	2.28×10^2	FPV
Red fox (*Vulpes vulpes*)	2015	Campania	51.20-149	Spleen, lymph node	8.26×10^3	FPV
Red fox (*Vulpes vulpes*)	2017	Campania	51.20-178	Spleen	4.37×10^2	FPV
Red fox (*Vulpes vulpes*)	2017	Campania	51.20-190	Spleen	5.68×10^3	FPV
Wolf (*Canis lupus italicus*)	2019	Abruzzo	245.19-529	Intestine	1.53×10^4	CPV-2a
Wolf (*Canis lupus italicus*)	2019	Abruzzo	245.19-957	Intestine	9.57×10^4	CPV-2a/2b
Wolf (*Canis lupus italicus*)	2019	Molise	289.19-2554	Intestine	4.65×10^3	CPV-2a/2b
Wolf (*Canis lupus italicus*)	2019	Abruzzo	415.19-3530	Intestine	2.84×10^4	CPV-2b
Wolf (*Canis lupus italicus*)	2019	Abruzzo	540.19-4723	Intestine	2.60×10^7	CPV-2c
Wolf (*Canis lupus italicus*)	2019	Abruzzo	540.19-4960	Intestine	8.78×10^6	CPV-2c
Wolf (*Canis lupus italicus*)	2019	Molise	540.19-6976	Intestine	2.57×10^5	CPV-2c
Wolf (*Canis lupus italicus*)	2019	Abruzzo	690.19-6848	Intestine	3.38×10^5	CPV-2b/2c
Wolf (*Canis lupus italicus*)	2019	Abruzzo	289.19-7187	Intestine	1.49×10^5	CPV-2b/2c
Wolf (*Canis lupus italicus*)	2020	Abruzzo	67.20-572	Intestine	7.4×10^4	CPV-2b/2c
Wolf (*Canis lupus italicus*)	2020	Abruzzo	67.20-743	Intestine	2.06×10^4	CPV-2b/2c
Wolf (*Canis lupus italicus*)	2020	Abruzzo	67.20-276	Intestine	3.39×10^2	CPV-2b
Wolf (*Canis lupus italicus*)	2020	Abruzzo	67.20-1106	Intestine	4.4×10^1	CPV-2c
Wolf (*Canis lupus italicus*)	2020	Lazio	67.20-1302	Intestine	3.05×10^3	CPV-2c
Wolf (*Canis lupus italicus*)	2020	Abruzzo	145.20-1274	Intestine	6.4×10^3	CPV-2b
Wolf (*Canis lupus italicus*)	2020	Abruzzo	145.20-1788	Intestine	6.68×10^4	CPV-2a
Wolf (*Canis lupus italicus*)	2020	Abruzzo	145.20-1863	Intestine	2.34×10^4	CPV-2b
Wolf (*Canis lupus italicus*)	2020	Abruzzo	145.20-4615	Intestine	1.37×10^4	CPV-2b
Wolf (*Canis lupus italicus*)	2020	Abruzzo	110.20-2633	Intestine	1.3×10^1	CPV-2c
European badger (*Meles Meles*)	2019	Abruzzo	245.19-1478	Intestine	4.79×10^5	FPLV
European badger (*Meles Meles*)	2019	Abruzzo	289.19-5860	Intestine	4.12×10^3	CPV-2b
European badger (*Meles Meles*)	2019	Abruzzo	289.19-5624	Intestine	4.74×10^3	CPV-2b
European badger (*Meles Meles*)	2019	Abruzzo	289.19-5518	Intestine	3.40×10^3	CPV-2c
European badger (*Meles Meles*)	2019	Abruzzo	289.19-1960	Intestine	3.41×10^3	CPV-2a/2b
European badger (*Meles Meles*)	2019	Tuscany	415.19-2441	Intestine	5.62×10^5	CPV-2b, FPLV
European badger (*Meles Meles*)	2019	Tuscany	415.19-3748	Intestine	1.69×10^3	CPV-2b
Beech marten (*Martes foina*)	2019	Abruzzo	289.19-5771	Intestine	1.73×10^4	CPV-2b

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Table 4. Summary of results of *Carnivore protoparvovirus-1* screening and characterization in different wild carnivore species.

Animal species	No. of animals tested	No. of animals testing positive to *Carnivore protoparvovirus-1*	FPV	CPV	CPV-2a	CPV-2b	CPV-2c	Co-infections
Red fox (*Vulpes vulpes*)	251	7	7	0	0	0	0	0
Wolf (*Canis lupus italicus*)	35	19	0	0	4	11	10	6
Eurasian badger (*Meles Meles*)	10	7	2	0	1	5	1	2
Beech marten (*Martes foina*)	1	1	0	0	0	1	0	0
Total	**297**	**34**	**8**	**0**	**2**	**10**	**10**	**8**

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Table 5: Nucleotide and amino acid observed in FPV sequences compared to an FPV sequence from a wild carnivore. Non-synonymous mutations are bolded.

Amino acid residue on VP2	45	91	178	195	202	234	250	347	349	363	370	389	395	431	552
Nt position	133-135	271-273	532-534	583-585	604-606	697-699	748-750	1039-1041	1045-1047	1087-1089	1108-1110	1165-1167	1183-1185	1291-1293	1564-1566
FPV/Spain/Badger/2013 KP682520	Phe (TTT)	Ala (GCA)	Asp (GAT)	Leu (TTG)	Pro (CCA)	Tyr (TAT)	Val (GTC)	Ala (GCG)	Thr (ACA)	Gly (GGA)	Gln (CAA)	Thr (ACT)	Pro CCC	Leu (CTA)	Val (GTG)
FPV/Fox/51/20-MT274377	Phe (TTC)	**Thr (ACA)**	Asp (GAT)	Leu (TTA)	**Thr (ACA)**	Tyr (TAT)	Val (GTG)	Ala (GCA)	Thr (ACA)	Gly (GGA)	Gln (CAG)	Thr (ACT)	Pro CCT	Leu (TTA)	Val (GTA)
FPV/Badger/245/19-1478-MT274378	Phe (TTC)	Ala (GCA)	Asp (GAC)	Leu (TTG)	Pro (CCA)	Tyr (TAT)	Val (GTG)	Ala (GCA)	Thr (ACA)	Gly (GGA)	Gln (CAA)	Thr (ACT)	Pro CCC	Leu (CTA)	Val (GTG)
FPV/FOX/51/20-178-MT454916	*	*	*	*	Pro (CCA)	Tyr (TAC)	Val (GTG)	Ala (GCG)	Thr (ACC)	Gly (GGG)	Gln (CAA)	Thr (ACC)	Pro CCT	Leu (CTA)	*
FPV/FOX/51/20-114-MT454917	*	*	*	*	Pro (CCA)	Tyr (TAT)	Val (GTG)	Ala (GCA)	Thr (ACA)	Gly (GGA)	Gln (CAG)	Thr (ACT)	Pro CCT	Leu (CTA)	*

*missing information

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Table 6: Nucleotide and amino acid changes observed in CPV sequences compared to three reference CPV sequences from wild carnivores. Non-synonymous mutations are bolded.

VP2 aa residues	20	29	45	49	56	135	202	203	212	216	218	232	240	244	267	291	305	314	321	324	362	364	370	371	372	375	403	418	426	522	534	541	570
Nt position	58-60	85-87	133-135	145-147	166-168	403-405	604-606	607-609	634-636	646-648	652-654	694-696	718-720	730-733	799-801	871-873	913-915	940-942	961-963	970-972	1084-1086	1090-1092	1108-1110	1111-1113	1114-1116	1123-1125	1207-1209	1252-1254	1276-1278	1564-1566	1600-1602	1621-1623	1708-1710
CPV-2b/SPAIN/2013	(GCA)	(GGC)	(TTC)	(ACG)	(AAC)	(GAG)	(CCA)	(ACC)	(TTT)	(AGA)	(TTA)	(ATA)	(GAT)	(TAT)	(TTT)	(CTA)	(TAT)	(CGT)	(AAT)	(TAT)	(GGG)	(GCG)	(CAA)	(GCA)	(GCA)	(GAT)	(CAT)	(ATT)	(GAT)	(GTA)	(GTA)	(GCC)	(AAA)
/WOLF-KP682514	Ala	Gly	Phe	Thr	Asn	Glu	Pro	Thr	Phe	Arg	Leu	Ile	Asp	Tyr	Phe	Leu	Tyr	Arg	Asn	Tyr	Gly	Ala	Gln	Ala	Ala	Asp	His	Ile	Asp	Val	Val	Ala	Lys
CPV-2b/SPAIN/2010/ STONE MARTEN	(GCA)	(GGC)	(TTC)	(ACG)	(AAC)	(GAG)	(CCA)	(ACC)	(TTT)	(AGA)	(TTA)	(ATA)	(GAT)	(TAT)	(TTT)	(CTA)	(TAT)	(CGT)	(AAT)	(TAT)	(GGG)	(GCG)	(CAA)	(GCA)	(GCA)	(GAT)	(CAT)	(ATT)	(GAT)	(GTA)	(GTA)	(GCC)	(AAA)
JX411926	Ala	Gly	Phe	Thr	Asn	Glu	Pro	Thr	Phe	Arg	Leu	Ile	Asp	Tyr	Phe	Leu	Tyr	Arg	Asn	Tyr	Gly	Ala	Gln	Ala	Ala	Asp	His	Ile	Asp	Val	Val	Ala	Lys
CPV-2c/SPAIN/2011/	(GCA)	(GGC)	(TTC)	(ACG)	(AAC)	(GAG)	(CCA)	(ACC)	(TTT)	(AGA)	(TTA)	(ATA)	(GAT)	(TAT)	(TTT)	(CTA)	(TAT)	(CGT)	(AAT)	(TAT)	(GGG)	(GCG)	(CAA)	(GCA)	(GCA)	(GAT)	(CAT)	(ATT)	(GAA)	(GTA)	(GTA)	(GCC)	(AAA)
BADGER-KP682519	Ala	Gly	Phe	Thr	Asn	Glu	Pro	Thr	Phe	Arg	Leu	Ile	Asp	Tyr	Phe	Leu	Tyr	Arg	Asn	Tyr	Gly	Ala	Gln	Ala	Ala	Asp	His	Ile	Glu	Val	Val	Ala	Lys
CPV2a/Wolf/145/20-							(CCC)	(ACC)	(TTT)	(AGA)	(TTG)	(ATA)	(GA**C**)	(TAT)	(TTT)	(CTA)	(TAT)	(CGT)	(AAT)	**(ATT)**	(GGG)	(GCA)	(CAA)	(GCA)	(GCA)	(GAT)	(CA**C**)	(ATT)	**(AAT)**				
MT454914	*	*	*	*	*	*	Pro	Thr	Phe	Arg	Leu	Ile	Asp	Tyr	Phe	Leu	Tyr	Arg	Asn	**Ile**	Gly	Ala	Gln	Ala	Ala	Asp	His	Ile	**Asn**	*	*	*	*
CPV2b/Wolf/415/19-	(GCT)	(GGC)	(TTC)	(ACG)	(AAC)	(GA**A**)	(CCA)	(ACC)	(TTT)	(AGA)	(TTA)	(ATA)	(GAT)	(TAT)	(TTT)	(CTA)	(TAT)	(CGT)	(AAT)	(TAT)	(GGG)	(GCG)	(CAA)	**(GGA)**	(GCA)	(GAT)	(CAT)	(ACT)	(GAT)	(GTA)	(GTA)	(GCC)	(AAA)
3530- MT353761	Ala	Gly	Phe	Thr	Asn	Glu	Pro	Thr	Phe	Arg	Leu	Ile	Asp	Tyr	Phe	Leu	Tyr	Arg	Asn	Tyr	Gly	Ala	Gln	**Gly**	Ala	Asp	His	Thr	Asp	Val	Val	Ala	Lys
CPV2b/Badger/289/19-	(GCT)	(GGC)	(TTT)	(ACG)	(AAC)	(GAG)	(CCA)	(ACC)	(TTT)	(AGA)	(TTA)	(ATA)	(GAT)	(TAT)	(TTT)	(CTA)	(TAT)	(CGT)	(AAT)	(TAT)	(GGG)	(GCG)	(CAA)	**(GGA)**	(GCA)	(GAT)	(CAT)	(ACT)	(GAT)	(GT**G**)	(GTA)	(GC**A**)	(AA**G**)
5860 MT353762	Ala	Gly	Phe	Thr	Asn	Glu	Pro	Thr	Phe	Arg	Leu	Ile	Asp	Tyr	Phe	Leu	Tyr	Arg	Asn	Tyr	Gly	Ala	Gln	**Gly**	Ala	Asp	His	Thr	Asp	Val	Val	Ala	Lys
CPV2b/B.marten/289/19-	(GCT)	(GGC)	(TTC)	(ACG)	(AAC)	(GAG)	(CCA)	(ACC)	(TTT)	(AGA)	(TTA)	(ATA)	(GAT)	(TAT)	(TTT)	(**T**TA)	(TAT)	(CGT)	(AAT)	(TAT)	(GGG)	(GCG)	(CAA)	**(GGA)**	(GCA)	(GAT)	(CAT)	(ACT)	(GAT)	(GTA)	(GT**G**)	(GCC)	(AA**G**)
5771-MT353763	Ala	Gly	Phe	Thr	Asn	Glu	Pro	Thr	Phe	Arg	Leu	Ile	Asp	Tyr	Phe	Leu	Tyr	Arg	Asn	Tyr	Gly	Ala	Gln	**Gly**	Ala	Asp	His	Thr	Asp	Val	Val	Ala	Lys
CPV2b/Badger/289/19-	(GCT)	(GGC)	(TTC)	(ACA)	**(AAG)**	(GAG)	(CCA)	(ACC)	(TTT)	(AGG)	(TTA)	(ATA)	(GAT)	(TAT)	(TTT)	(CTA)	(TAT)	(CGT)	(AAT)	(TAT)	(GG**C**)	(GCG)	(CAA)	(GCA)	(GCT)	**(AAT)**	(CAT)	(ACT)	(GAT)	(GTA)	(GTA)	(GCC)	(AAA)
5624-MT353764	Ala	Gly	Phe	Thr	**Lys**	Glu	Pro	Thr	Phe	Arg	Leu	Ile	Asp	Tyr	Phe	Leu	Tyr	Arg	Asn	Tyr	Gly	Ala	Gln	Ala	Ala	**Asn**	His	Thr	Asp	Val	Val	Ala	Lys
CPV-2b/Wolf/67/20-276							(CCA)	**(CAC)**	(TTT)	(AGA)	(TTA)	**(GTA)**	(GAT)	(TAT)	(TTT)	(CTA)	**(GAT)**	(CGT)	(AAT)	(TAT)	(GGG)	(GCG)	(CAA)	(GCA)	(GCA)	(GAT)	(CAT)	(ATT)	(GAT)				
-MT454911	*	*	*	*	*	*	Pro	**His**	Phe	Arg	Leu	**Val**	Asp	Tyr	Phe	Leu	**Asp**	Arg	Asn	Tyr	Gly	Ala	Gln	Ala	Ala	Asp	His	Ile	Asp	*	*	*	*
CPV-2b/wolf /145/20-							(CCA)	(ACC)	(TTT)	(AGA)	(TTA)	(ATA)	(GAT)	(TAT)	(TTT)	(CTA)	(TAT)	(CGT)	(AAT)	(TAT)	(GGG)	(GCG)	(CAA)	**(GGA)**	(GCA)	(GAT)	(CAT)	(ACT)	(GAT)				
1863- MT454913	*	*	*	*	*	*	Pro	Thr	Phe	Arg	Leu	Ile	Asp	Tyr	Phe	Leu	Tyr	Arg	Asn	Tyr	Gly	Ala	Gln	**Gly**	Ala	Asp	His	Thr	Asp	*	*	*	*
CPV-2b/Wolf/ 145/20-							(CCA)	(AAC)	(TT**C**)	(AGA)	(TTA)	(ATA)	(GAT)	(TAT)	(TTT)	(CTA)	(TAT)	(CGT)	(AAT)	(TAT)	(GGG)	(GCG)	(CAA)	**(GGA)**	(GCA)	(GAT)	(CAT)	(ACT)	(GAT)				
1274- MT454915	*	*	*	*	*	*	Pro	Asp	Phe	Arg	Leu	Ile	Asp	Tyr	Phe	Leu	Tyr	Arg	Asn	Tyr	Gly	Ala	Gln	**Gly**	Ala	Asp	His	Thr	Asp	*	*	*	*
CPV2c/Badger /289/19-	(GCA)	**(AGC)**	(TTC)	(ACG)	(AAC)	(GAG)	(CCA)	(ACC)	(TTT)	(AGA)	(TTA)	(ATA)	(GAT)	(TAT)	(TTT)	(CTA)	(TAT)	(CGT)	(AAT)	(TAT)	(GGG)	(GCG)	**(CGA)**	(GCA)	(GCA)	(GAT)	(CAT)	(ATT)	(GAA)	(GTA)	(GTA)	(GCC)	(AAA)
5518- MT353760	Ala	**Ser**	Phe	Thr	Asn	Glu	Pro	Thr	Phe	Arg	Leu	Ile	Asp	Tyr	Phe	Leu	Tyr	Arg	Asn	Tyr	Gly	Ala	**Arg**	Ala	Ala	Asp	His	Ile	Glu	Val	Val	Ala	Lys
CPV-2c/Wolf /67/20-							(CCA)	(ACC)	(TTT)	(AGA)	(TTA)	(ATA)	(GAT)	(TAC)	**(TAT)**	(CTA)	(TAT)	**(CAT)**	(AAC)	**(ATT)**	(GGG)	(GCG)	**(CGA)**	(GCA)	(GCA)	(GAT)	(CAT)	(ATT)	**(GAA)**				
1302- MT454908	*	*	*	*	*	*	Pro	Thr	Phe	Arg	Leu	Ile	Asp	Tyr	**Tyr**	Leu	Tyr	**His**	Asn	**Ile**	Gly	Ala	**Arg**	Ala	Ala	Asp	His	Ile	**Glu**	*	*	*	*
CPV-2c/Wolf /67/20-							(CCA)	(ACC)	(TTT)	(AGA)	(TTA)	(ATA)	(GAT)	(TAC)	**(TAT)**	(CTA)	(TAT)	(CGT)	(AAC)	**(ATT)**	(GGG)	(GCG)	**(CGA)**	(GCA)	(GCA)	(GAT)	(CAT)	(ATT)	**(GAA)**				
1106-MT454909	*	*	*	*	*	*	Pro	Thr	Phe	Arg	Leu	Ile	Asp	Tyr	**Tyr**	Leu	Tyr	Arg	Asn	**Ile**	Gly	Ala	**Arg**	Ala	Ala	Asp	His	Ile	**Glu**	*	*	*	*
CPV-2c/Wolf/ 540/19-							(CCA)	(ACC)	(TTT)	(AGA)	(TTA)	(ATA)	(GAT)	(TAT)	(TTT)	(CTA)	(TAT)	(CGT)	(AAT)	(TAT)	(GGG)	(GCG)	(CAA)	(GCA)	(GCA)	(GAT)	(CAT)	(ATT)	**(GAA)**				
6976-MT454910	*	*	*	*	*	*	Pro	Thr	Phe	Arg	Leu	Ile	Asp	Tyr	Phe	Leu	Tyr	Arg	Asn	Tyr	Gly	Ala	Gln	Ala	Ala	Asp	His	Ile	**Glu**	*	*	*	*

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Fig. 1

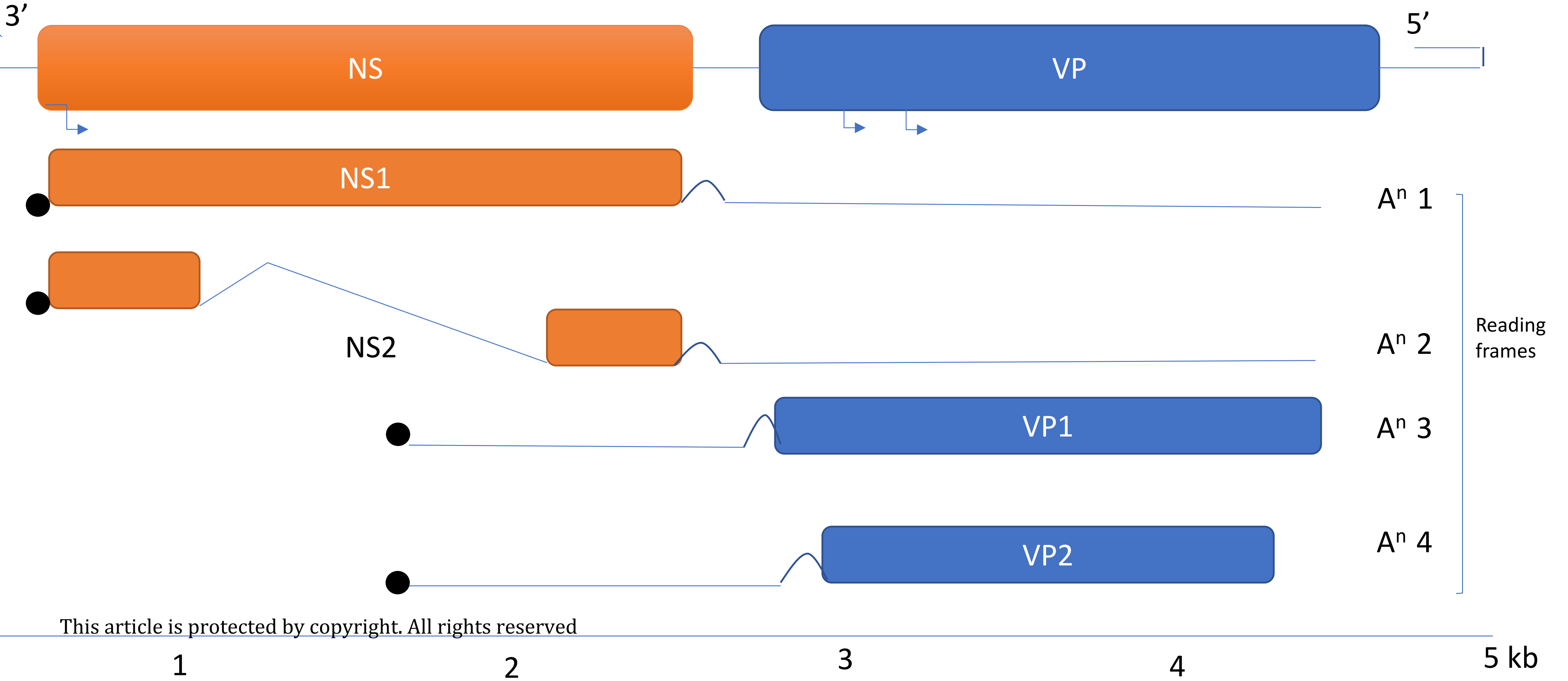

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Figure 2

tbed_13917_f2.pptx

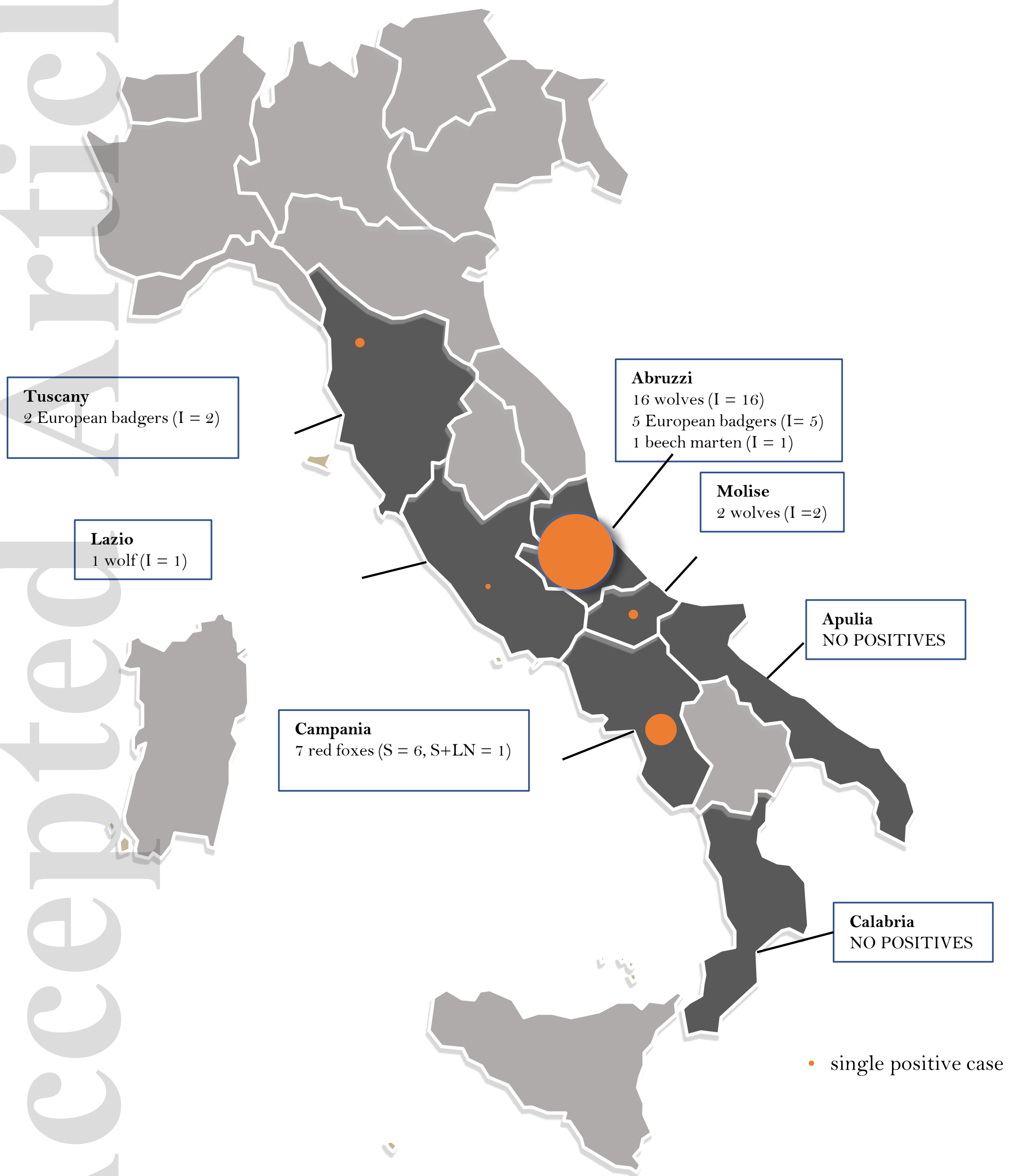

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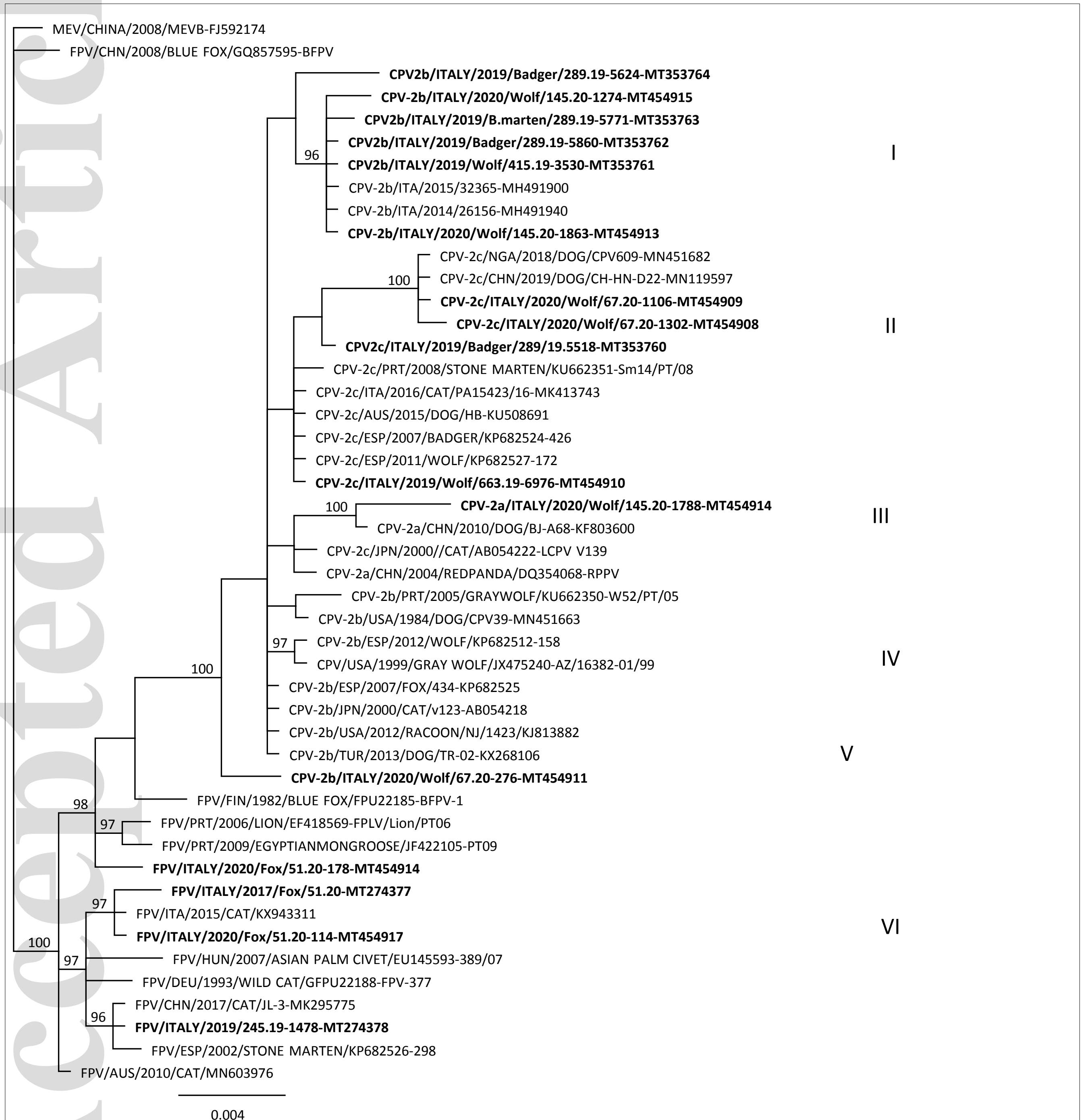
Fig. 3



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