Manuscript Details

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Title	IDENTIFICATION OF A NOVEL PARVOVIRUS IN DOMESTIC CATS
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Abstract

A novel protoparvovirus species was identified in domestic cats. The virus was distantly related to the well-known feline (feline panleukopenia virus) and canine (canine parvovirus type 2) parvoviruses, sharing low nucleotide identities in the capsid protein 2 (less than 43%). The virus was genetically similar (100% at the nucleotide level) to a newly identified canine protoparvovirus, genetically related to human bufaviruses. The feline bufavirus appeared as a common element of the feline virome, especially in juvenile cats, with an overall prevalence of 9.2%. The virus was more common in respiratory samples (9.5% to 12.2%) than in enteric samples of cats (2.2%). The role of bufaviruses in the etiology of feline respiratory disease complex, either as a primary or a secondary agents, should be defined.

Keywords	parvovirus; protoparvovirus; bufavirus; cat; respiratory infections
Manuscript category	Viruses

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Università degli Studi di Bari
Dipartimento di Medicina Veterinaria

To the Editor of
Veterinary Microbiology

Ref: VETMIC_2018_1162

Valenzano, Bari, 10/12/2018

Dear Editor,

I am sending you the manuscript "*Identification of a novel parvovirus in domestic cats*" (VETMIC_2018_1162) by Diakoudi et al. revised following the referees' suggestions. A point-by-point reply has been prepared.

Sincerely Yours,

Martella Vito

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Rebuttal

Ref: VETMIC_2018_1162
Title: IDENTIFICATION OF A NOVEL PARVOVIRUS IN DOMESTIC CATS
Journal: Veterinary Microbiology

Dear Editor,

Please find herein a detailed reply to the referees' comments.

Best regards,

Vito Martella

Reviewer 1

The Authors identified a novel protoparvovirus species in archived nasal and oropharyngeal swabs and enteric samples from domestic cats with or without respiratory symptoms and with gastroenteritis. The virus was genetically similar to a newly identified canine protoparvovirus, genetically related to human bufaviruses. The virus was more common in respiratory samples than in enteric samples. Since some canine viruses can infect cats and viceversa, the Authors hypothesized that the novel canine bufavirus could circulate in the feline host.

General comments:

R1.1: Line 112-116: the authors describe here the use of a qPCR but in the results section there is no information at all on the titres (virus load). Can the authors provide some data about the viral load measured in biological samples?

Reply to R1.1: The measured virus loads ranged between 2.82×10^{-1} to 1.78×10^{5} DNA copies/10µl of template (mean 9.81×10^{3} DNA copies/10µl). We added this information in the text at pages 9, lines 166 to 167.

R1.2: Lines 236-238: the authors claim that their analysis unveiled a possible age-related pattern in BuV-infected cats, suggesting that young animals are more susceptible to BuV infection. Can the authors provide possible explanations for this? This could include fading of passive immunity or physiological changes in animals during growth.

Reply to R1.2: We added a short sentence in the discussion where we stated that the age-related pattern could be accounted for by the lingering passive immunity and/or by physiological changes. Page 12, lines 245-246: "This might be due to the immature immune system of juvenile cats, coupled with the decline of maternal immunity."

Reviewer 2

Dear Editor, I just reviewed the manuscript entitled "IDENTIFICATION OF A NOVEL PARVOVIRUS IN DOMESTIC CATS"- The authors previously identified a novel canine bufavirus (CaBuV) in dogs with respiratory signs. Aim of the present study is to investigate the prevalence of this virus in archival samples of cats stored in two different laboratories of southern

Italy. This topic is certainly worth of investigation as related viruses have been already observed in both species.

Comments:

R2.1: It seems that screening of samples has been performed by a conventional PCR. According to the authors, "subsequently" (line 307) a qPCR has been done. I would have done the opposite. Indeed the results section starts with the description of the results obtained by qPCR. The authors need to clarify this point.

Reply to R2.1: In the section "Materials and Methods" we added more information on the correct workflow. This was also mentioned in the section "Results"

R2.2: Titre range of positive samples also needs to be described within the text.

Reply to R2.2: This was done, following also the request of R1.

R2.3: It is also not clear to this reviewer which amplicon has been produced for genetic analysis. A cartoon would be beneficial.

Reply to R2.3: We generated a figure (Figure 1) with information on the position of primers used for diagnostics and on the sequences generated in this study

R2.4: Molecular methods adopted for screening of other relevant pathogens need to be referenced within the M&M section.

Reply to R2.4: The molecular methods used for screening of other respiratory pathogens referred in the text have been added in the "Materials and Methods" section at page 6, lines 119-121: "All of the samples of collection TR had been previously screened for feline calicivirus (FCV), feline herpesvirus type 1 (FHV-1) and *Chlamydophila felis* (*C. felis*) by conventional nested RT-PCR (Marsilio et al., 2005) and PCR (Di Martino et al., 2007)."

R2.5: Please cite Zaccaria et al., 2016 at line 647 together with Dowgier et al., 2017 and Silva et al., 2017.

Reply to R2.5: The suggested reference has been added in the manuscript.

R2.6: As for the isolation procedures, are these viruses normally isolated onto cell cultures? A sentence need to be inserted in the introduction section.

Reply to R2.6: We mentioned this in the discussion. To our knowledge, human viruses do not grow in cell cultures (Väisänen et al., 2017). We added a short comment in the discussion at page 12, lines 232-234: "Moreover, the virus could not be isolated on cell (A-72 and CRFK) cultures. Likewise, attempts to isolate human bufaviruses on cell cultures have been, thus far, unsuccessful (Väisänen et al., 2017). The reason for the non-cultivatable nature of these viruses remains unclear."

R2.7: The authors should also summarize the different sets of samples in a table. They used too many aka.

Reply to R2.7: We included this information in a new table (Table 2).

IDENTIFICATION OF A NOVEL PARVOVIRUS IN DOMESTIC CATS

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ABSTRACT

A novel protoparvovirus species was identified in domestic cats. The virus was distantly related to the well-known feline (feline panleukopenia virus) and canine (canine parvovirus type 2) parvoviruses, sharing low nucleotide identities in the capsid protein 2 (less than 43%). The virus was genetically similar (100% at the nucleotide level) to a newly identified canine protoparvovirus, genetically related to human bufaviruses. The feline bufavirus appeared as a common element of the feline virome, especially in juvenile cats, with an overall prevalence of 9.2%. The virus was more common in respiratory samples (9.5% to 12.2%) than in enteric samples of cats (2.2%). The role of bufaviruses in the etiology of feline respiratory disease complex, either as a primary or a secondary agents, should be defined.

KEYWORDS: parvovirus; protoparvovirus; bufavirus; cat; respiratory infections

1. INTRODUCTION

Parvoviruses (family *Parvoviridae*) are small, nonenveloped, single-stranded DNA viruses. The linear DNA genome is about 4.5-5.5 kb in length with complex hairpin structures at the 5' and 3' ends and it encodes 3 or 4 proteins; non-structural (NS) 1, nucleoprotein (NP) 1, and viral protein (VP) 1 and VP2 (Cotmore et al., 2014).

Parvoviruses (Feline parvovirus, FPV, *Protoparvovirus* genus) have long been known in cats. FPV has been identified as the cause of diseases in cats, raccoons and some related carnivores for many years (Verge and Cristoforoni, 1928; Hindle and Findlay, 1932). FPV is associated with severe panleukopenia and enteritis in cats and cerebellar ataxia in kittens (Csiza et al., 1971). FPV is genetically and antigenically similar to the canine parvovirus type 2 (CPV-2) (Stuetzer and Hartmann, 2014). CPV-2 emerged in dogs in the 1970s in Europe and North America, when severe haemorrhagic gastroenteritis and myocarditis were reported in puppies (Appel et al., 1979). The original CPV-2 type, shortly after its identification, started generating antigenic variants, termed 2a, 2b and 2c (Parrish et al., 1985; Parrish et al., 1991; Buonavoglia et al., 2001). Whilst the original CPV-2 type did not replicate in cats, its later variants gained the ability to replicate and cause FPV-like disease in cats (Truyen et al., 1996; Hueffer and Parrish, 2003).

Recently, new parvoviruses of the genus *Bocaparvovirus* were described in cats (Lau et al., 2012; Ng et al., 2014; Zhang et al., 2014) (Table 1). Genome sequencing of feline bocaparvoviruses (FBoVs) has revealed a marked diversity between the FBoV strains FBD1 (FBoV-3) and POR1 (FBoV-2) and the prototype FBoV strain (FBoV-1) (Lau et al., 2012),

which has been proposed as carnivore bocaparvovirus-3 species (Cotmore et al., 2014). Whether FBoVs are associated with any disease in cats and to what extent the observed genetic diversity affects the biological properties of the various FBoV species is not known yet.

In 2016, a novel protoparvovirus (canine protoparvovirus 2), similar to human bufaviruses (BuVs) and denominated canine bufavirus (CaBuV), was identified in dogs with respiratory signs (Martella et al., 2018). The virus was more common in juvenile dogs and a possible association between respiratory signs and virus presence was observed. Since CPV-2 variants CPV-2 a, b and c, but not the original type, are able to infect cats and to induce FPV-like clinical signs, we hypothesized that cats might also serve as host species for the newly discovered CaBuV. In order to better understand the ecology of this novel animal protoparvovirus, in this study we extended the research of BuVs to biological samples of cats available in our laboratory.

2. MATERIALS AND METHODS

2.1 Origin of Samples

Archived nasal and oropharyngeal (NOP) swab samples and enteric samples (stool and rectal swabs) obtained from young and adult domestic cats, collected at the Department of Veterinary Medicine, University of Bari, Italy, during 2016-2017 and 2012-2015 respectively, were screened for CaBuV. The collection included 180 NOP samples from animals with or without respiratory signs (collection BR) and 90 enteric samples (collection BE) from cats with gastroenteritis. For a subset of 68 samples of collection BR (collection sBR), information about the age and the health condition of the animals was available; 51 animals had clinical respiratory signs and 17 cats were asymptomatic.

Moreover, a collection of 304 NOP archival samples (collection TR) from cats with respiratory signs (n=179) (collection STR) or without clinical signs (n=125) (collection ATR), was screened for BuV. Collection TR was obtained in Italy during 2012-2013 and stored at the Faculty of Veterinary Medicine, University of Teramo, Italy. Detailed information about the age, the health status of the animals and the co-infection with other pathogens causing respiratory disease were available for TR samples.

2.2 DNA Extraction

Both NOP and fecal samples were homogenized in 10% Dulbecco's modified Eagle's medium (DMEM) and then centrifuged at 10,000 x *g* for 3 min. ~~Viral DNA~~Nucleic acids ~~were~~as

extracted from 200 µl of the supernatants using the QIAamp *cador* Pathogen Mini Kit (Qiagen S.p.A., Milan, Italy), following the manufacturer's protocol and ~~the nucleic acid templates were~~ stored at –80°C until use.

Commented [1]: R2.4

2.3 Screening of Samples in Conventional and Quantitative PCR

To assess the presence of CaBuV, all samples were tested~~screened~~ in real-time PCR (qPCR) ~~((~~CPPV-L3-for 5′ TGAACAAGAAATAGACAACATTGTCAT 3′, CPPV-L3-rev 5′ AAAGAGCAGTTAGGTCA TTGTTGT 3′, and CPPV-L3 Pb 5′ Fam CCAAACAAGGTACAGGACAGGAAGAAACAAC-ACAA BHQ1 3′) ~~for the quantitative calculation of BuV DNA copy numbers~~ (Martella et al., 2018) (Figure 1). The CaBuV DNA copy numbers were calculated on the basis of standard curves generated by 10-fold dilutions of a plasmid standard TOPO XL PCR containing a 500-nt fragment of the VP2 region of CaBuV strain ITA/2011/297-15 (GenBank accession no. MF198244).

Commented [2]: R2.3

The positive samples were tested in PCR using specific primers (CPPV 165F 5′ CTGGTTTAATCCAGCAGACT 3′ and CPPV 371R 5′ TGAAGACCAAGGTAGTAGGT 3′) to amplify and sequence a 2027~~7~~-nucleotide (nt) fragment of the VP2 (Martella et al., 2018) (Figure 1). For PCR amplification, the AccuPrime Taq DNA polymerase (Life Technologies) and the suggested cycling thermal conditions were used.

Commented [3]: R2.3

Commented [4]: R2.1

All of the samples of collection TR ~~were~~had been previously screened for feline calicivirus (FCV), feline herpesvirus type 1 (FHV-1) and *Chlamydophila felis* (*C. felis*) by conventional nested RT-PCR (Marsilio et al., 2005) and PCR (Di Martino et al., 2007).~~Subsequently, the positive samples were tested in real-time PCR (qPCR) (CPPV-L3-for 5′-TGAACAAGAAATAGACAACATTGTCAT 3′, CPPV-L3-rev 5′-AAAGAGCAGTTAGGTCA TTGTTGT 3′, and CPPV-L3 Pb 5′-Fam-CCAAACAAGGTACAGGACAGGAAGAAACAAC-ACAA-BHQ1 3′) for the quantitative calculation of BuV DNA copy numbers (Martella et al., 2018).~~

Commented [5]: R2.4

2.4 Amplification of the VP2-coding region

In order to amplify the full-length VP2-coding gene (Figure 1), BuV-positive samples were selected on the basis of their concentration (DNA $>10^3$ copies/10 µl). The selected samples were tested using two different primer pairs: the forward primer CPPV 165F and the reverse primer CPPV 1571R (5′-TTATAGAGTAATATTAGGC-3′); the forward primer CPPV 1409F (5′-TCATATTCCTGGAGAAACATCA-3′) and the reverse primer CPPV 1414R (5′-ATATGTCTGTTAGATTGCCAGT-3′). The two primer pairs were designed based on available CaBuV genome sequences to amplify overlapping fragments of the VP2-coding region of 1350 nt and 962 nt in length, respectively. The primers were designed using the software Primer 3 implemented in Geneious version 10.2.4 (Biomatters Ltd., Auckland, New Zealand). The PCR assays were performed with TaKaRa La *Taq* polymerase (Takara Bio Europe S.A.S. Saint-Germain-en-Laye, France).

Commented [6]: R2.3

2.5 Statistical Analysis

144 The association among clinical signs, age and presence of the virus in the NOP samples of
145 collections sBR and TR was evaluated using the chi-squared test. Logistic regression was used to
146 identify possible bivariate associations between the presence of BuV DNA and the presence of
147 other pathogens in the samples of collection TR.

148 Statistical analysis of the variables was performed using the software R version 3.5.1 (R
149 Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/) and the
150 statistical significance was set at $p < 0.05$.

151

152 *2.6 Sequence and Phylogenetic Analyses*

153 Genome sequences of the complete VP2-coding region from 64 protoparvovirus strains were
154 retrieved from GenBank. The alignment of the sequences was conducted using the MAFFT
155 multiple alignment program version 7.388 plugin of the Geneious software. Sequence and
156 phylogenetic analyses were performed with Geneious version 10.2.4. software (Biomatters Ltd.,
157 Auckland, New Zealand). Phylogenetic analysis was performed using the neighbor-joining
158 method, the Jukes-Cantor genetic distance model and bootstrapping over 1,000 replicates.

159

160 *2.7 Virus Cultivation*

161 BuV-positive samples were selected on the basis of virus load (DNA $>10^3$ copies/10 µl), as
162 determined by qPCR. NOP and enteric samples were homogenized in 10% DMEM and then
163 centrifuged at 10,000 x *g*. The supernatant was filtered with 0.22-µm filters and inoculated onto
164 freshly seeded Crandell Rees Feline Kidney (CRFK) cell line and canine fibroblastic tumor (A-

72) cells at 37˚C in 5% CO_2. Viral growth was evaluated through 6 serial passages in CRFK and A-72 cells, by monitoring the onset of cellular cytopathic effect and by testing the cell supernatant by qPCR.

3. RESULTS

3.1 Molecular screening

Molecular screening by qPCR detected BuV DNA in 22/180 (12.2%) NOP samples of collection BR, in 2/90 (2.2%) enteric samples of collection BE and in 29/304 (9.5%) NOP samples of collection TR (Table 2). The viral loads of the collections ranged from 2.82×10^{-1} to 1.78×10^{5} DNA copies/10µl of template (mean 9.81×10^{3} DNA copies/10µl). More specifically, when testing collection sBR, BuV DNA was detected in 13/51 (25.5%) of the cats with respiratory signs and in 4/17 (23.5%) of healthy animals. When testing the NOP samples of collection TR, BuV DNA was detected in 13/179 (7.3%) cats with respiratory signs and in 16/125 (12.8%) asymptomatic cats (Table 2). Statistical analysis showed no association between the presence of the virus and clinical signs ($p > 0.05$).

Moreover, we re-analyzed the results based on the age of the animals (0-12 months and older than 1 year). In the collection sBR BuV DNA was detected in 12/51 (23.5%) of the juvenile (0-12 months) group of animals and in 5/17 (29.4%) of the cats older than 1 year, but this difference was not statistically significant ($p > 0.05$). However, on the collection TR the presence of BuV DNA was detected in 18/120 (15.0%) of the juvenile cats and in 11/166 (6.6%) of the cats older than 1 year, and this difference was statistically significant ($p = 0.03$).

Commented [7]: R2.7

Commented [8]: R1.1 + R2.2

Commented [9]: R2.7

The collection TR was also screened for the presence of other pathogens causing respiratory signs. In this screening, 14/304 (4.6%) samples were positive for ~~feline calicivirus (~~FCV~~)~~, 58/304 (19.1%) were positive for ~~feline herpesvirus (~~FHV-1~~)~~ and 15/304 (4.9%) were positive for ~~*Chlamydophila felis* (~~*C. felis*~~)~~. Logistic regression analysis was performed to evaluate possible bivariate association between the presence of BuV and co-infection with FCV, FHV-1 and/or *C. felis*. The results of the analysis showed that co-infection of BuV and FCV and co-infection of BuV and FHV-1 had no association ($p > 0.05$). Instead, possible bivariate correlation was found in samples co-infected with BuV and *C. felis* ($p = 0.00$).

3.2 Sequence analysis of BuV identified in cats

Amplicons (2027-nt in length) obtained with the diagnostic PCR for BuV were sequenced. The obtained sequences were highly similar to their cognate CaBuV strains, sharing 99.5-100.0% nt identity. The complete or nearly complete consensus sequence of the VP2-coding region (1707 bp) of feline BuV was generated for three strains (ITA/2012/TE109, ITA/2015/BA509 and ITA/2017/BA291) (GenBank accession no. MK030121 - MK030123). Those three sequences were identical to the Italian and the Hungarian canine BuV strains sharing ≥ 99.9% amino acid (aa) and nt identity.

Upon phylogenetic analysis based on the VP2-coding region, the feline BuV strains clustered tightly with the Italian and the Hungarian CaBuV strains (Figure 2~~1~~). Interestingly, the carnivore BuV (protoparvovirus) strains were rooted along with a novel sea otter parvovirus (GenBank accession no. KU561552), with which they shared 70.0-70.4% nt identity. Both the carnivore BuVs and the sea otter parvovirus were related to the human bufavirus strains, sharing 67.2-70.8% nt identity. However, the feline BuV displayed low nt identity (42.0-42.7%) to CPV-2/FPV and to other carnivore protoparvovirus-1 strains (42.1-42.8%).

Commented [10]: R2.3

3.3 Virus Cultivation

The inoculated monolayers of CRFK and A-72 cell lines were visually inspected through 6 serial passages. The virus titer was monitored in cellular supernatant by qPCR. Evidence of viral growth was not observed in the cells.

4. DISCUSSION

Several novel parvoviruses have been identified in domestic carnivores in recent years, taking advantage of massive sequencing technologies and meta-genomic approach for virus characterization and discovery. A novel protoparvovirus, genetically unrelated to FPV/CPV-2, was identified in 2016 in dogs. The virus was found to resemble a group of parvoviruses first identified in human and non-human primates and commonly known as bufaviruses (Martella et al., 2018). Since some canine viruses can infect cats and vice versa (Martella et al., 2002; Matthijnssens et al., 2011; Di Martino et al., 2016; Di Martino et al., 2018), we hypothesized that the novel canine BuV could circulate in the feline host. Using primer sets and probes specific for canine BuV, we screened a total of 574 archival feline samples collected from the respiratory and enteric tract. Overall, the screening revealed BuV DNA in 9.2% of the samples (53/574), indicating that BuVs are common component of the feline virome (Table 2).

Commented [11]: R2.7

A major limit of our investigation was the missing information/metadata for most of the samples of collection BR and, more in general, the relatively small numbers of samples included in the

231 screening as we tested archival samples available in our laboratories. However, the data were
232 informative enough to suggest a possible age-related pattern of the infection. Also, the virus was
233 relatively infrequent (2.2%) in the enteric tract of cats whilst the prevalence in respiratory
234 samples was about 5 to 6 times (9.5% to 12.2%) higher, suggesting that BuVs are more common
235 in the respiratory tract of cats. Indeed, the virus was rather common in the NOP samples of
236 collections BR (12.2%, 22/180) and TR (9.5%, 29/304). In humans, BuVs have been identified
237 almost exclusively in the enteric tract (Väisänen et al., 2016). However, investigations in dogs
238 (Martella et al., 2018), monkeys (Handley et al., 2012), shrews (Sasaki et al., 2015) and in sea
239 otters (Siqueira et al., 2017), also suggest the possibility of extra-intestinal and/or systemic
240 infections of BuVs. In our study, the virus appeared a common component of feline respiratory
241 virome, thus hinting at a preferential tropism of carnivore BuVs for the respiratory tract.
242 Moreover, the virus could not be isolated on cell (A-72 and CRFK) cultures. Likewise, attempts
243 to isolate human bufaviruses on cell cultures have been, thus far, unsuccessful (Vaisanen et al.,
244 2017). The reason for the non-cultivatable nature of these viruses remains unclear.~~in order to~~
245 ~~better understand the virus pathogenesis, a trial to isolate the virus was performed, but so far~~
246 ~~these viruses grow poorly in cell cultures~~.
247

Commented [12]: R2.6

248 For a subset of NOP samples from collection BR (sBR), we had detailed information on the age
249 and health status of the animals but we did not find any significant difference in terms of
250 prevalence between cats with respiratory signs (25.5%, 13/51) and without respiratory signs
251 (23.5%, 4/17) and with respect to the age of the animals, although 12 of 17 (70.6%) BuV-
252 infected animals were ≤ 1 year of age. When analyzing the NOP samples of collection TR, we
253 also did not found any statistically significant difference in terms of prevalence between cats

with (7.3%, 13/179) and without (12.9%, 16/125) respiratory signs. When analyzing virus distribution on the basis of the age, the virus was more common in juvenile animals. Eighteen of 29 (62.1%) BuV-infected animals were ≤ 1 year of age (p = 0.03). A similar age-related pattern was observed in BuV-infected dogs (Martella et al., 2018) and could indicate that young animals are more susceptible to BuV infection. This might be due to the immature immune system of juvenile cats, couped with ~~after~~ the decline of maternal immunity. In addition, in the collection TR a possible correlation was found between co-infection with BuV and *C. felis* (p = 0.00). This possible association is worth additional, tailored investigations, in order to decipher mechanisms of synergism between some micro-organisms, as already described (Zaccaria et al., 2016; Dowgier et al., 2017; Silva et al., 2017). Also, this will be helpful to understand whether BuVs are able to play a role in feline respiratory disease complex.

Commented [13]: R1.2

The nearly complete VP2-coding region, of 1.7 kb in length, was sequenced for three strains (ITA/2012/TE109, ITA/2015/BA509, ITA/2017/BA291). The viruses displayed > 99.9% nt identity to each other and to canine BuVs. Interestingly, no aa mutation was observed between the VP2 of feline and canine BuVs. This finding is interesting, as a few aa mutations in the VP2 have been found to affect the host range of the carnivore protoparvoviruses FPV and CPV-2. For these viruses, the capsid is the major determinant of host range (Hueffer et al., 2003) and subject to antibody-mediated selection (Nelson et al., 2007). The fact that the feline and canine BuVs displayed strong sequence conservation could suggest that the virus has recently crossed the species barrier from a yet unidentified source, with a recent bottleneck event in the evolution of BuVs in domestic carnivores. On the contrary, a marked genetic heterogeneity has been observed within human BuVs, with at least 3 distinct genotypes (Yahiro et al., 2014), differing

mostly in the VP2 (65-73% aa identity) (Väisänen et al., 2017). Upon phylogenetic analysis, the canine/feline BuVs segregated apart from but close to BuVs discovered in human and non-human primates. Interestingly, the canine/feline group was strictly rooted with a sea otter BuV (KU561552) identified in 2017 in USA (Siqueira et al., 2017). Analysis by PCR of archival necropsy samples suggested that this virus is endemic in sea otter population, with 60% of the examined animals being positive. Accordingly, it is possible that similar viruses infect other wildlife mammals.

In conclusion, we gathered evidence that cats may be infected from at least two distinct protoparvovirus species. The pathogenic role, if any, of this novel feline protoparvovirus, herewith indicated as canine/feline or carnivore BuV, should be investigated more in detail, by including systematically BuVs in the diagnostic algorithms of feline viral agents, chiefly for cats with respiratory infectious diseases. Also, the feline and canine BuVs were virtually identical, suggesting the possibility of inter-species circulation between the two carnivore species. The fact that dogs and cats may share the same viruses should not be ignored when devising measures of prophylaxis in shelters and clinics.

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FIGURE AND TABLES CAPTIONS

Table 1: Parvoviruses identified in dog and cats and their classification (Cotmore et al., 2014) and proposed classification. Candidate novel species are indicated by asterisks. Common or widely used names for the viruses are also indicated.

Table 2: Collections of samples used for the study. Gray color indicates the subsets of the collections ~~BR and TR respectively.~~

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Figure 1: Genome organization of the CaBuV strain ITA/2011/297-15 (GenBank accession no. MF198244). Arrows ~~demonstrate~~enducate the positions of primers and probe used for diagnostic PCR and qPCR. Gray color illustrates the sequence of the VP2-coding region generated in our study and used for ~~sequence and~~ phylogenetic analysis.

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Figure 2~~1~~: Capsid-based phylogenetic tree displaying the diversity of protoparvoviruses. The protoparvoviruses officially recognized by the International Committee on Taxonomy of Viruses are included along with nonclassified (NC) protoparvoviruses. GenBank accession numbers are provided for reference strains; Gray Fox amdovirus (GenBank accession no. JN202450) was used as outgroup. The tree was generated using the neighbor-joining method with the Jukes-Cantor algorithm of distance correction, with bootstrapping up to 1,000 replicates. Bootstrap values >70% are shown. Gray color indicates feline protoparvovirus strains.

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HIGHLIGHTS

- A novel protoparvovirus (bufavirus) was identified in cats.
- The feline bufavirus was more common in respiratory samples of juvenile cats.
- The feline bufavirus was highly similar to a canine bufavirus.
- The carnivore bufaviruses were phylogenetically related to primate bufaviruses.
- Carnivore bufaviruses are genetically distinct from feline/canine protoparvovirus-1

IDENTIFICATION OF A NOVEL PARVOVIRUS IN DOMESTIC CATS

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ABSTRACT

A novel protoparvovirus species was identified in domestic cats. The virus was distantly related to the well-known feline (feline panleukopenia virus) and canine (canine parvovirus type 2) parvoviruses, sharing low nucleotide identities in the capsid protein 2 (less than 43%). The virus was genetically similar (100% at the nucleotide level) to a newly identified canine protoparvovirus, genetically related to human bufaviruses. The feline bufavirus appeared as a common element of the feline virome, especially in juvenile cats, with an overall prevalence of 9.2%. The virus was more common in respiratory samples (9.5% to 12.2%) than in enteric samples of cats (2.2%). The role of bufaviruses in the etiology of feline respiratory disease complex, either as a primary or a secondary agents, should be defined.

KEYWORDS: parvovirus; protoparvovirus; bufavirus; cat; respiratory infections

1. INTRODUCTION

Parvoviruses (family *Parvoviridae*) are small, nonenveloped, single-stranded DNA viruses. The linear DNA genome is about 4.5-5.5 kb in length with complex hairpin structures at the 5' and 3' ends and it encodes 3 or 4 proteins; non-structural (NS) 1, nucleoprotein (NP) 1, and viral protein (VP) 1 and VP2 (Cotmore et al., 2014).

Parvoviruses (Feline parvovirus, FPV, *Protoparvovirus* genus) have long been known in cats. FPV has been identified as the cause of diseases in cats, raccoons and some related carnivores for many years (Verge and Cristoforoni, 1928; Hindle and Findlay, 1932). FPV is associated with severe panleukopenia and enteritis in cats and cerebellar ataxia in kittens (Csiza et al., 1971). FPV is genetically and antigenically similar to the canine parvovirus type 2 (CPV-2) (Stuetzer and Hartmann, 2014). CPV-2 emerged in dogs in the 1970s in Europe and North America, when severe haemorrhagic gastroenteritis and myocarditis were reported in puppies (Appel et al., 1979). The original CPV-2 type, shortly after its identification, started generating antigenic variants, termed 2a, 2b and 2c (Parrish et al., 1985; Parrish et al., 1991; Buonavoglia et al., 2001). Whilst the original CPV-2 type did not replicate in cats, its later variants gained the ability to replicate and cause FPV-like disease in cats (Truyen et al., 1996; Hueffer and Parrish, 2003).

Recently, new parvoviruses of the genus *Bocaparvovirus* were described in cats (Lau et al., 2012; Ng et al., 2014; Zhang et al., 2014) (Table 1). Genome sequencing of feline bocaparvoviruses (FBoVs) has revealed a marked diversity between the FBoV strains FBD1 (FBoV-3) and POR1 (FBoV-2) and the prototype FBoV strain (FBoV-1) (Lau et al., 2012),

which has been proposed as carnivore bocaparvovirus-3 species (Cotmore et al., 2014). Whether FBoVs are associated with any disease in cats and to what extent the observed genetic diversity affects the biological properties of the various FBoV species is not known yet.

In 2016, a novel protoparvovirus (canine protoparvovirus 2), similar to human bufaviruses (BuVs) and denominated canine bufavirus (CaBuV), was identified in dogs with respiratory signs (Martella et al., 2018). The virus was more common in juvenile dogs and a possible association between respiratory signs and virus presence was observed. Since CPV-2 variants CPV-2 a, b and c, but not the original type, are able to infect cats and to induce FPV-like clinical signs, we hypothesized that cats might also serve as host species for the newly discovered CaBuV. In order to better understand the ecology of this novel animal protoparvovirus, in this study we extended the research of BuVs to biological samples of cats available in our laboratory.

2. MATERIALS AND METHODS

2.1 Origin of Samples

Archived nasal and oropharyngeal (NOP) swab samples and enteric samples (stool and rectal swabs) obtained from young and adult domestic cats, collected at the Department of Veterinary Medicine, University of Bari, Italy, during 2016-2017 and 2012-2015 respectively, were screened for CaBuV. The collection included 180 NOP samples from animals with or without respiratory signs (collection BR) and 90 enteric samples (collection BE) from cats with gastroenteritis. For a subset of 68 samples of collection BR (collection sBR), information about the age and the health condition of the animals was available; 51 animals had clinical respiratory signs and 17 cats were asymptomatic.

Moreover, a collection of 304 NOP archival samples (collection TR) from cats with respiratory signs (n=179) (collection STR) or without clinical signs (n=125) (collection ATR), was screened for BuV. Collection TR was obtained in Italy during 2012-2013 and stored at the Faculty of Veterinary Medicine, University of Teramo, Italy. Detailed information about the age, the health status of the animals and the co-infection with other pathogens causing respiratory disease were available for TR samples.

2.2 DNA Extraction

Both NOP and fecal samples were homogenized in 10% Dulbecco's modified Eagle's medium (DMEM) and then centrifuged at 10,000 x *g* for 3 min. Nucleic acids were extracted from 200 µl

of the supernatants using the QIAamp *cador* Pathogen Mini Kit (Qiagen S.p.A., Milan, Italy), following the manufacturer's protocol and stored at –80°C until use.

2.3 Screening of Samples in Conventional and Quantitative PCR

To assess the presence of CaBuV, all samples were tested in real-time PCR (qPCR) (CPPV-L3-for 5′ TGAACAAGAAATAGACAACATTGTCAT 3′, CPPV-L3-rev 5′ AAAGAGCAGTTAGGTCATTGTTGT 3′, and CPPV-L3 Pb 5′ Fam CCAAACAAGGTACAGGACAGGAAGAAACAAC-ACAA BHQ1 3′) (Martella et al., 2018) (Figure 1). The CaBuV DNA copy numbers were calculated on the basis of standard curves generated by 10-fold dilutions of a plasmid standard TOPO XL PCR containing a 500-nt fragment of the VP2 region of CaBuV strain ITA/2011/297-15 (GenBank accession no. MF198244).

The positive samples were tested in PCR using specific primers (CPPV 165F 5′ CTGGTTTAATCCAGCAGACT 3′ and CPPV 371R 5′ TGAAGACCAAGGTAGTAGGT 3′) to amplify and sequence a 202-nucleotide (nt) fragment of the VP2 (Martella et al., 2018) (Figure 1). For PCR amplification, the AccuPrime Taq DNA polymerase (Life Technologies) and the suggested cycling thermal conditions were used.

All of the samples of collection TR had been previously screened for feline calicivirus (FCV), feline herpesvirus type 1 (FHV-1) and *Chlamydophila felis* (*C. felis*) by conventional nested RT-PCR (Marsilio et al., 2005) and PCR (Di Martino et al., 2007).

2.4 Amplification of the VP2-coding region

In order to amplify the full-length VP2-coding gene (Figure 1), BuV-positive samples were selected on the basis of their concentration (DNA $>10^3$ copies/10 µl). The selected samples were tested using two different primer pairs: the forward primer CPPV 165F and the reverse primer CPPV 1571R (5′-TTATAGAGTAATATTAGGC-3′); the forward primer CPPV 1409F (5′-TCATATTCCTGGAGAAACATCA-3′) and the reverse primer CPPV 1414R (5′-ATATGTCTGTTAGATTGCCAGT-3′). The two primer pairs were designed based on available CaBuV genome sequences to amplify overlapping fragments of the VP2-coding region of 1350 nt and 962 nt in length, respectively. The primers were designed using the software Primer 3 implemented in Geneious version 10.2.4 (Biomatters Ltd., Auckland, New Zealand). The PCR assays were performed with TaKaRa La *Taq* polymerase (Takara Bio Europe S.A.S. Saint-Germain-en-Laye, France).

2.5 Statistical Analysis

The association among clinical signs, age and presence of the virus in the NOP samples of collections sBR and TR was evaluated using the chi-squared test. Logistic regression was used to identify possible bivariate associations between the presence of BuV DNA and the presence of other pathogens in the samples of collection TR.

Statistical analysis of the variables was performed using the software R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/) and the statistical significance was set at $p < 0.05$.

2.6 Sequence and Phylogenetic Analyses

Genome sequences of the complete VP2-coding region from 64 protoparvovirus strains were retrieved from GenBank. The alignment of the sequences was conducted using the MAFFT multiple alignment program version 7.388 plugin of the Geneious software. Sequence and phylogenetic analyses were performed with Geneious version 10.2.4. software (Biomatters Ltd., Auckland, New Zealand). Phylogenetic analysis was performed using the neighbor-joining method, the Jukes-Cantor genetic distance model and bootstrapping over 1,000 replicates.

2.7 Virus Cultivation

BuV-positive samples were selected on the basis of virus load (DNA $>10^3$ copies/10 µl), as determined by qPCR. NOP and enteric samples were homogenized in 10% DMEM and then centrifuged at 10,000 x *g*. The supernatant was filtered with 0.22-µm filters and inoculated onto freshly seeded Crandell Rees Feline Kidney (CRFK) cell line and canine fibroblastic tumor (A-72) cells at 37˚C in 5% CO_2. Viral growth was evaluated through 6 serial passages in CRFK and A-72 cells, by monitoring the onset of cellular cytopathic effect and by testing the cell supernatant by qPCR.

3. RESULTS

3.1 Molecular screening

Molecular screening by qPCR detected BuV DNA in 22/180 (12.2%) NOP samples of collection BR, in 2/90 (2.2%) enteric samples of collection BE and in 29/304 (9.5%) NOP samples of

collection TR (Table 2). The viral loads of the collections ranged from 2.82×10^{-1} to 1.78×10^{5} DNA copies/10µl of template (mean 9.81×10^{3} DNA copies/10µl). More specifically, when testing collection sBR, BuV DNA was detected in 13/51 (25.5%) of the cats with respiratory signs and in 4/17 (23.5%) of healthy animals. When testing the NOP samples of collection TR, BuV DNA was detected in 13/179 (7.3%) cats with respiratory signs and in 16/125 (12.8%) asymptomatic cats (Table 2). Statistical analysis showed no association between the presence of the virus and clinical signs ($p > 0.05$).

Moreover, we re-analyzed the results based on the age of the animals (0-12 months and older than 1 year). In the collection sBR BuV DNA was detected in 12/51 (23.5%) of the juvenile (0-12 months) group of animals and in 5/17 (29.4%) of the cats older than 1 year, but this difference was not statistically significant ($p > 0.05$). However, on the collection TR the presence of BuV DNA was detected in 18/120 (15.0%) of the juvenile cats and in 11/166 (6.6%) of the cats older than 1 year, and this difference was statistically significant ($p = 0.03$).

The collection TR was also screened for the presence of other pathogens causing respiratory signs. In this screening, 14/304 (4.6%) samples were positive for FCV, 58/304 (19.1%) were positive for FHV-1 and 15/304 (4.9%) were positive for *C. felis*. Logistic regression analysis was performed to evaluate possible bivariate association between the presence of BuV and co-infection with FCV, FHV-1 and/or *C. felis*. The results of the analysis showed that co-infection of BuV and FCV and co-infection of BuV and FHV-1 had no association ($p > 0.05$). Instead, possible bivariate correlation was found in samples co-infected with BuV and *C. felis* ($p = 0.00$).

3.2 Sequence analysis of BuV identified in cats

Amplicons (202-nt in length) obtained with the diagnostic PCR for BuV were sequenced. The obtained sequences were highly similar to their cognate CaBuV strains, sharing 99.5-100.0% nt identity. The complete or nearly complete consensus sequence of the VP2-coding region (1707 bp) of feline BuV was generated for three strains (ITA/2012/TE109, ITA/2015/BA509 and ITA/2017/BA291) (GenBank accession no. MK030121 - MK030123). Those three sequences were identical to the Italian and the Hungarian canine BuV strains sharing ≥ 99.9% amino acid (aa) and nt identity.

Upon phylogenetic analysis based on the VP2-coding region, the feline BuV strains clustered tightly with the Italian and the Hungarian CaBuV strains (Figure 2). Interestingly, the carnivore BuV (protoparvovirus) strains were rooted along with a novel sea otter parvovirus (GenBank accession no. KU561552), with which they shared 70.0-70.4% nt identity. Both the carnivore BuVs and the sea otter parvovirus were related to the human bufavirus strains, sharing 67.2-70.8% nt identity. However, the feline BuV displayed low nt identity (42.0-42.7%) to CPV-2/FPV and to other carnivore protoparvovirus-1 strains (42.1-42.8%).

3.3 Virus Cultivation

The inoculated monolayers of CRFK and A-72 cell lines were visually inspected through 6 serial passages. The virus titer was monitored in cellular supernatant by qPCR. Evidence of viral growth was not observed in the cells.

4. DISCUSSION

Several novel parvoviruses have been identified in domestic carnivores in recent years, taking advantage of massive sequencing technologies and meta-genomic approach for virus characterization and discovery. A novel protoparvovirus, genetically unrelated to FPV/CPV-2, was identified in 2016 in dogs. The virus was found to resemble a group of parvoviruses first identified in human and non-human primates and commonly known as bufaviruses (Martella et al., 2018). Since some canine viruses can infect cats and vice versa (Martella et al., 2002; Matthijnssens et al., 2011; Di Martino et al., 2016; Di Martino et al., 2018), we hypothesized that the novel canine BuV could circulate in the feline host. Using primer sets and probes specific for canine BuV, we screened a total of 574 archival feline samples collected from the respiratory and enteric tract. Overall, the screening revealed BuV DNA in 9.2% of the samples (53/574), indicating that BuVs are common component of the feline virome (Table 2).

A major limit of our investigation was the missing information/metadata for most of the samples of collection BR and, more in general, the relatively small numbers of samples included in the screening as we tested archival samples available in our laboratories. However, the data were informative enough to suggest a possible age-related pattern of the infection. Also, the virus was relatively infrequent (2.2%) in the enteric tract of cats whilst the prevalence in respiratory samples was about 5 to 6 times (9.5% to 12.2%) higher, suggesting that BuVs are more common in the respiratory tract of cats. Indeed, the virus was rather common in the NOP samples of collections BR (12.2%, 22/180) and TR (9.5%, 29/304). In humans, BuVs have been identified almost exclusively in the enteric tract (Väisänen et al., 2016). However, investigations in dogs (Martella et al., 2018), monkeys (Handley et al., 2012), shrews (Sasaki et al., 2015) and in sea otters (Siqueira et al., 2017), also suggest the possibility of extra-intestinal and/or systemic infections of BuVs. In our study, the virus appeared a common component of feline respiratory

virome, thus hinting at a preferential tropism of carnivore BuVs for the respiratory tract. Moreover, the virus could not be isolated on cell (A-72 and CRFK) cultures. Likewise, attempts to isolate human bufaviruses on cell cultures have been, thus far, unsuccessful (Väisänen et al., 2017). The reason for the non-cultivatable nature of these viruses remains unclear.

For a subset of NOP samples from collection BR (sBR), we had detailed information on the age and health status of the animals but we did not find any significant difference in terms of prevalence between cats with respiratory signs (25.5%, 13/51) and without respiratory signs (23.5%, 4/17) and with respect to the age of the animals, although 12 of 17 (70.6%) BuV-infected animals were ≤ 1 year of age. When analyzing the NOP samples of collection TR, we also did not found any statistically significant difference in terms of prevalence between cats with (7.3%, 13/179) and without (12.9%, 16/125) respiratory signs. When analyzing virus distribution on the basis of the age, the virus was more common in juvenile animals. Eighteen of 29 (62.1%) BuV-infected animals were ≤ 1 year of age ($p = 0.03$). A similar age-related pattern was observed in BuV-infected dogs (Martella et al., 2018) and could indicate that young animals are more susceptible to BuV infection. This might be due to the immature immune system of juvenile cats, coupled with the decline of maternal immunity. In addition, in the collection TR a possible correlation was found between co-infection with BuV and *C. felis* ($p = 0.00$). This possible association is worth additional, tailored investigations, in order to decipher mechanisms of synergism between some micro-organisms, as already described (Zaccaria et al., 2016; Dowgier et al., 2017; Silva et al., 2017). Also, this will be helpful to understand whether BuVs are able to play a role in feline respiratory disease complex.

The nearly complete VP2-coding region, of 1.7 kb in length, was sequenced for three strains (ITA/2012/TE109, ITA/2015/BA509, ITA/2017/BA291). The viruses displayed > 99.9% nt

identity to each other and to canine BuVs. Interestingly, no aa mutation was observed between the VP2 of feline and canine BuVs. This finding is interesting, as a few aa mutations in the VP2 have been found to affect the host range of the carnivore protoparvoviruses FPV and CPV-2. For these viruses, the capsid is the major determinant of host range (Hueffer et al., 2003) and subject to antibody-mediated selection (Nelson et al., 2007). The fact that the feline and canine BuVs displayed strong sequence conservation could suggest that the virus has recently crossed the species barrier from a yet unidentified source, with a recent bottleneck event in the evolution of BuVs in domestic carnivores. On the contrary, a marked genetic heterogeneity has been observed within human BuVs, with at least 3 distinct genotypes (Yahiro et al., 2014), differing mostly in the VP2 (65-73% aa identity) (Väisänen et al., 2017). Upon phylogenetic analysis, the canine/feline BuVs segregated apart from but close to BuVs discovered in human and non-human primates. Interestingly, the canine/feline group was strictly rooted with a sea otter BuV (KU561552) identified in 2017 in USA (Siqueira et al., 2017). Analysis by PCR of archival necropsy samples suggested that this virus is endemic in sea otter population, with 60% of the examined animals being positive. Accordingly, it is possible that similar viruses infect other wildlife mammals.

In conclusion, we gathered evidence that cats may be infected from at least two distinct protoparvovirus species. The pathogenic role, if any, of this novel feline protoparvovirus, herewith indicated as canine/feline or carnivore BuV, should be investigated more in detail, by including systematically BuVs in the diagnostic algorithms of feline viral agents, chiefly for cats with respiratory infectious diseases. Also, the feline and canine BuVs were virtually identical, suggesting the possibility of inter-species circulation between the two carnivore species. The fact

that dogs and cats may share the same viruses should not be ignored when devising measures of prophylaxis in shelters and clinics.

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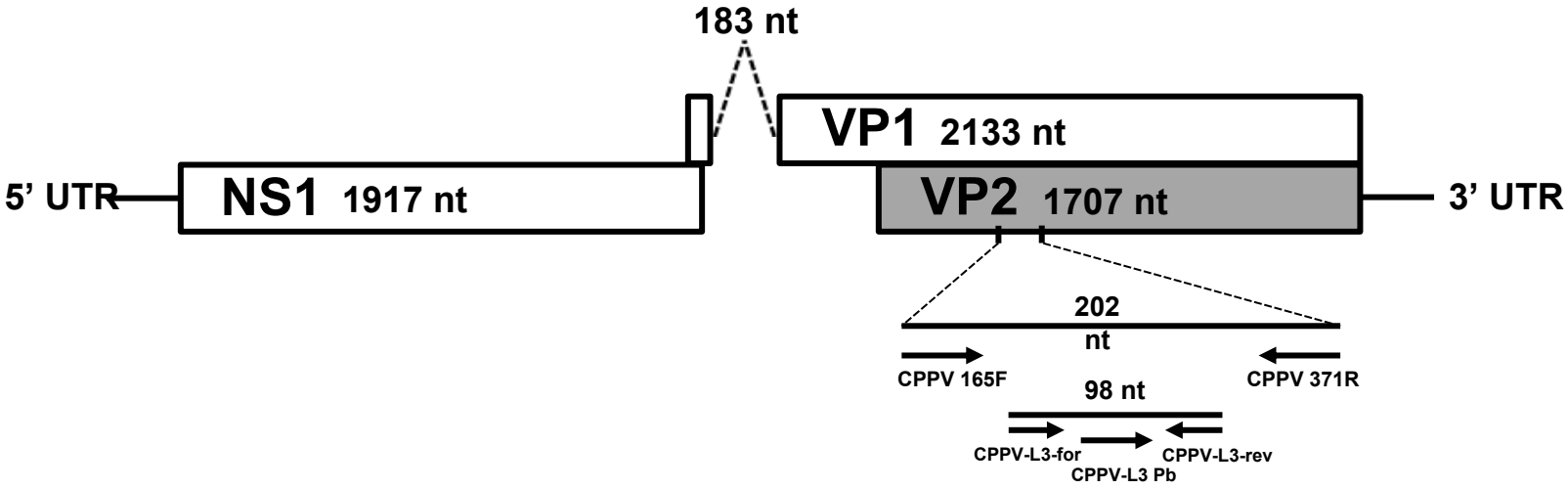
FIGURE AND TABLES CAPTIONS

Table 1: Parvoviruses identified in dog and cats and their classification (Cotmore et al., 2014) and proposed classification. Candidate novel species are indicated by asterisks. Common or widely used names for the viruses are also indicated.

Table 2: Collections of samples used for the study. Gray color indicates the subsets of the collections.

Figure 1: Genome organization of the CaBuV strain ITA/2011/297-15 (GenBank accession no. MF198244). Arrows indicate the positions of primers and probe used for diagnostic PCR and qPCR. Gray color illustrates the sequence of the VP2-coding region generated in our study and used for phylogenetic analysis.

Figure 2: Capsid-based phylogenetic tree displaying the diversity of protoparvoviruses. The protoparvoviruses officially recognized by the International Committee on Taxonomy of Viruses are included along with nonclassified (NC) protoparvoviruses. GenBank accession numbers are provided for reference strains; Gray Fox amdovirus (GenBank accession no. JN202450) was used as outgroup. The tree was generated using the neighbor-joining method with the Jukes-Cantor algorithm of distance correction, with bootstrapping up to 1,000 replicates. Bootstrap values >70% are shown. Gray color indicates feline protoparvovirus strains.


183 nt
VP1 2133 nt
5' UTR
NS1 1917 nt
VP2 1707 nt
3' UTR
202
nt
CPPV 165F
CPPV 371R
98 nt
CPPV-L3-for
CPPV-L3-rev
CPPV-L3 Pb

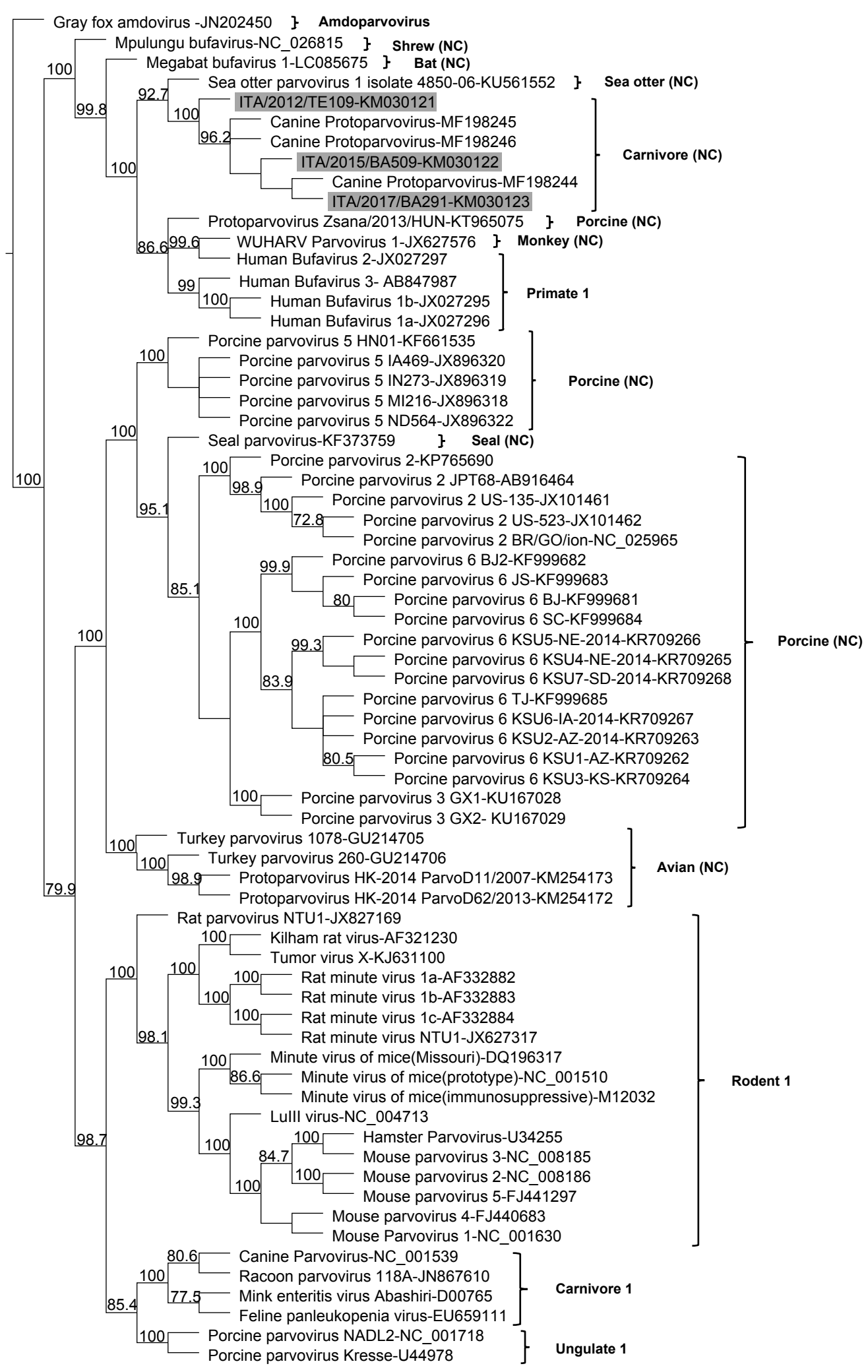

Gray fox amdovirus -JN202450
Amdoparvovirus
Mpulungu bufavirus-NC_026815
Shrew (NC)
Megabat bufavirus 1-LC085675
Bat (NC)
Sea otter parvovirus 1 isolate 4850-06-KU561552
Sea otter (NC)
ITA/2012/TE109-KM030121
Canine Protoparvovirus-MF198245
Canine Protoparvovirus-MF198246
ITA/2015/BA509-KM030122
Canine Protoparvovirus-MF198244
ITA/2017/BA291-KM030123
Carnivore (NC)
Protoparvovirus Zsana/2013/HUN-KT965075
Porcine (NC)
WUHARV Parvovirus 1-JX627576
Monkey (NC)
Human Bufavirus 2-JX027297
Human Bufavirus 3- AB847987
Human Bufavirus 1b-JX027295
Human Bufavirus 1a-JX027296
Primate 1
Porcine parvovirus 5 HN01-KF661535
Porcine parvovirus 5 IA469-JX896320
Porcine parvovirus 5 IN273-JX896319
Porcine parvovirus 5 MI216-JX896318
Porcine parvovirus 5 ND564-JX896322
Porcine (NC)
Seal parvovirus-KF373759
Seal (NC)
Porcine parvovirus 2-KP765690
Porcine parvovirus 2 JPT68-AB916464
Porcine parvovirus 2 US-135-JX101461
Porcine parvovirus 2 US-523-JX101462
Porcine parvovirus 2 BR/GO/ion-NC_025965
Porcine parvovirus 6 BJ2-KF999682
Porcine parvovirus 6 JS-KF999683
Porcine parvovirus 6 BJ-KF999681
Porcine parvovirus 6 SC-KF999684
Porcine parvovirus 6 KSU5-NE-2014-KR709266
Porcine parvovirus 6 KSU4-NE-2014-KR709265
Porcine parvovirus 6 KSU7-SD-2014-KR709268
Porcine parvovirus 6 TJ-KF999685
Porcine parvovirus 6 KSU6-IA-2014-KR709267
Porcine parvovirus 6 KSU2-AZ-2014-KR709263
Porcine parvovirus 6 KSU1-AZ-KR709262
Porcine parvovirus 6 KSU3-KS-KR709264
Porcine parvovirus 3 GX1-KU167028
Porcine parvovirus 3 GX2- KU167029
Porcine (NC)
Turkey parvovirus 1078-GU214705
Turkey parvovirus 260-GU214706
Protoparvovirus HK-2014 ParvoD11/2007-KM254173
Protoparvovirus HK-2014 ParvoD62/2013-KM254172
Avian (NC)
Rat parvovirus NTU1-JX827169
Kilham rat virus-AF321230
Tumor virus X-KJ631100
Rat minute virus 1a-AF332882
Rat minute virus 1b-AF332883
Rat minute virus 1c-AF332884
Rat minute virus NTU1-JX627317
Minute virus of mice(Missouri)-DQ196317
Minute virus of mice(prototype)-NC_001510
Minute virus of mice(immunosuppressive)-M12032
LuIII virus-NC_004713
Hamster Parvovirus-U34255
Mouse parvovirus 3-NC_008185
Mouse parvovirus 2-NC_008186
Mouse parvovirus 5-FJ441297
Mouse parvovirus 4-FJ440683
Mouse Parvovirus 1-NC_001630
Rodent 1
Canine Parvovirus-NC_001539
Racoon parvovirus 118A-JN867610
Mink enteritis virus Abashiri-D00765
Feline panleukopenia virus-EU659111
Carnivore 1
Porcine parvovirus NADL2-NC_001718
Porcine parvovirus Kresse-U44978
Ungulate 1

Table 1: Parvoviruses identified in dogs and cats and their classification (Cotmore et al 2014). Candidate novel species are indicated by asterisks.

Genus and species	Common/used names in literature	Year	Place	Reference	Accession
Bocaparvovirus genus					
Carnivore bocaparvovirus 1	Canine parvovirus 1 (CPV-1) or Minute Virus of Canines (MVC) or CBoV-1	1968	USA	Binn *et al.,* 1970	FJ214110
Carnivore bocaparvovirus 2	Canine bocavirus (CBoV) 1 or CBoV-2	2011	USA	Kapoor *et al*., 2012	JN648103
Carnivore bocaparvovirus 3	Feline bocaparvovirus (FBoV)	2009	USA	Lau et *al*., 2012	JQ692585
Carnivore bocaparvovirus 4*	CBoV-3	2011	USA	Li et *al*., 2013	KC580640
Protoparvovirus genus					
Carnivore protoparvovirus 1	Canine parvovirus 2 (CPV-2)	1978	USA	Appel *et al.,* 1979	M19296
	CPV-2a	1983	USA	Parrish *et al.,* 1985	M24000
	CPV-2b	1984	USA	Parrish *et al.*, 1991	M74849
	CPV-2c	2000	Italy	Buonavoglia *et al*., 2001	AY380577
	Feline parvovirus (FPV)	1920	USA		
Carnivore protoparvovirus 2*	Canine bufavirus (CBuV)	2012 -16	Italy - Hungary	Martella et al., 2018	MF198244 MF198245 MF198246
	Feline bufavirus (FBuV)	2017	Italy	This study	

Table 2: Collections of samples used for the study. Grey color indicates subsets of the collections.

Origin	Name of collection	Nr of samples	Positive samples
Bari	BR (Respiratory)	180	22 (12.2%)
	sBR (subset of BR)	68	17 (25.0%)
	BE (Enteric)	90	2 (2.2%)
Teramo	TR (Respiratory)	304	29 (9.5%)
	ATR (Asymptomatic - subset of TR)	179	13 (7.3%)
	STR (Symptomatic - subset of TR)	125	16 (12.8%)
Total		574	53 (9.2%)