




## ORIGINAL ARTICLE

# Spreading of canine parvovirus type 2c mutants of Asian origin in southern Italy

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## Abstract

Canine parvovirus type 2 (CPV-2) emerged as dog pathogen in the late 1970s, causing severe and often fatal epizootics of gastroenteritis in the canine population worldwide. Although to date CPV-2 is circulating in all continents, most of the current studies have analysed the amino acid changes accounted in the VP2 gene sequence, with limited information on virus introductions from other countries. The aim of this study was to analyse the genetic features of CPV-2c strains currently spreading in Italy. Swabs and tissue samples were collected from dogs suspected of CPV infection. The nearly complete genome sequence from the CPV-positive samples was obtained. The co-circulation of two different but related CPV-2c strains, with amino acid changes characteristic of CPV strains of Asian origin (NS1: 60V, 544F, 545F, 630P – NS2: 60V, 151N, 152V – VP2: 5A/G, 267Y, 297A, 324I, 370R), were observed. The phylogenetic analyses inferred from the NS1 and VP2 gene sequences confirmed the relationship with Asian CPV-2c strains. This study reports the spread of novel CPV-2c mutants in Italy and supports further studies to evaluate the coexistence of genetically divergent CPV strains in the same geographical environment.

## KEYWORDS

Asia, canine parvovirus, carnivore protoparvovirus, dogs, molecular characterization, sequence analysis

## 1 | INTRODUCTION

Canine parvovirus type 2 (CPV-2) is a small, non-enveloped single-stranded DNA virus, recently included in the specie *Carnivore protoparvovirus 1*, a member of the *Protoparvovirus* genus (family *Parvoviridae*, subfamily *Parvovirinae*) (Cotmore et al., 2019). Its genome consists of an approximately 5,200 nucleotide (nt) DNA molecule containing two large open reading frames (ORFs), encoding two nonstructural (NS1 and NS2) and two structural (VP1

and VP2) proteins, generated through alternative splicing of the same mRNAs (Decaro & Buonavoglia, 2012; Reed, Jones, & Miller, 1988).

In susceptible non-immunized dogs, CPV-2 causes an acute and often lethal disease, whose clinical signs are characterized by vomiting, enteritis and acute lymphopenia (Decaro & Buonavoglia, 2012). CPV-2 emerged as dog pathogen in the late 1970s, most likely as host variant of feline parvovirus (FPV) or a related strain (Truyen, 2006), displaying an intrinsic high rate of nucleotide changes (Decaro et al., 2009; Pereira, Leal, & Durigon, 2007; Shackelton, Parrish, Truyen,

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**TABLE 1** Details on collected/tested samples of dogs

Strain	Date of sampling	Place of sampling	Breed	Origin	Age	Sample	Acc. number
IZSSI_PA24478/18_id3184	20 Aug 2018	Marsala	Mixed breed	Stray dog	2 months	Brain, heart, lung, intestine, mesenteric lymph node, spleen	MK802679
IZSSI_PA24478/18_id3230	20 Aug 2018	Marsala	Mixed breed	Stray dog	2 months	Brain, heart, lung, intestine, mesenteric lymph node, spleen	MK806280
IZSSI_PA31342/18	31 Oct 2018	Castelvetrano	Mixed breed	Stray dog	3 months	Lung, intestine, spleen	MK806281
IZSSI_PA5455/19	13 Nov 2018	Partanna	Labrador retriever	Pet	7 months	Rectal swab	MK806282
IZSSI_PA54446/19	26 Nov 2018	Marsala	Epagneul breton	Pet	50 days	Rectal swab	MK806283
IZSSI_RG3408/19	27 Feb 2019	Comiso	Mixed breed	Pet	3 months	Rectal swab	MK806284
IZSSI_PA5632	04 Mar 2019	Mazara del Vallo	Mixed breed	Stray dog	2 years	Lung, heart, intestine, spleen	MK806285

& Holmes, 2005). After its emergence, the original type CPV-2 was replaced by three antigenic variants termed CPV-2a, CPV-2b and CPV-2c (Buonavoglia et al., 2001; Parrish et al., 1991; Parrish, O'Connell, Evermann, & Carmichael, 1985). During the years, several amino acid (aa) changes were accounted in the VP2 gene sequence (Battilani et al., 2001; Geng et al., 2015; Jeoung, Ahn, & Kim, 2008; Nakamura et al., 2004; Truyen, 1999) and, only recently, the analysis of the NS1 gene sequence was included in the CPV phylogenies (Canuti, Rodrigues, Whitney, & Lang, 2017; Grecco et al., 2018; Han et al., 2015; Li et al., 2018; Mira et al., 2019; Pérez et al., 2014; Zhuang et al., 2019).

Previous studies provided information on the CPV strains spreading in Italy (Decaro, Desario, et al., 2007; Decaro et al., 2013, 2006; Dei Giudici et al., 2017; Mira, Dowgier, et al., 2018; Purpari et al., 2018; Tucciarone et al., 2018), suggesting the need of a continuous epidemiological survey to evaluate the CPV circulation and evolution, whereas limited data are available on the spread of novel strains imported from other continents (Mira, Purpari, Lorusso, et al., 2018). The aim of this study was the detection and molecular analysis of CPV strains displaying genetic features of Asian viruses spreading in southern Italy.

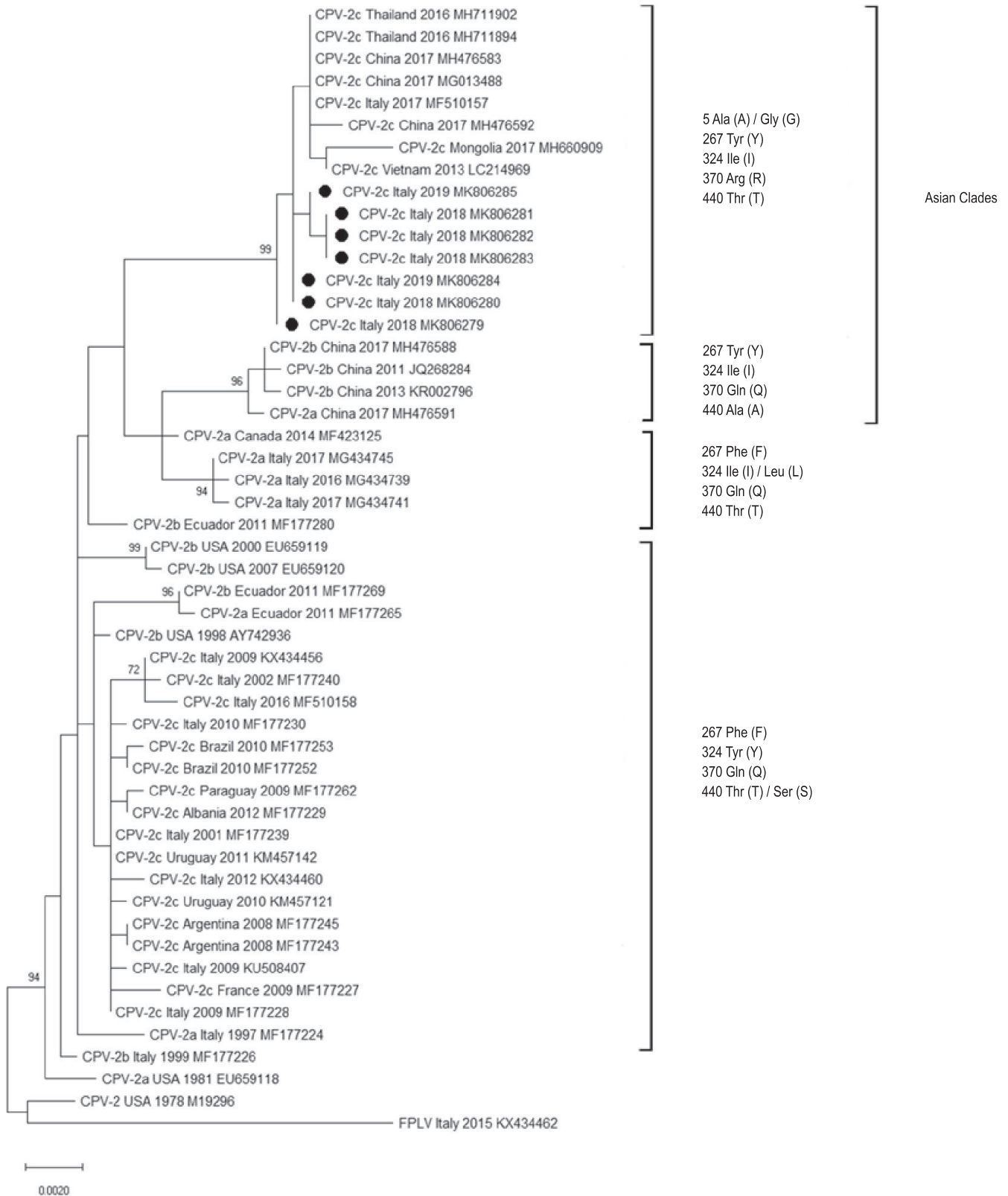
## 2 | MATERIALS AND METHODS

During an epidemiological survey, rectal swabs ( $n = 3$ ) and tissue samples ( $n = 19$ ) from seven dogs suspected of CPV infection (Table 1), collected in southern Italy (Sicily) from August 2018 to March 2019, were analysed at the Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri" (Palermo, Italy) for diagnostic purposes. DNA and RNA were extracted from swab/organ homogenates, obtained as previously described (Purpari et al., 2018), using the DNeasy Blood & Tissue Kit (Qiagen S.p.A.) and QIAmp Viral RNA Mini Kit (Qiagen S.p.A.), respectively, according to the manufacturer's instructions. Presence of CPV DNA was evaluated by a diagnostic PCR using a primer pair targeting the VP2 gene (Touihri et al., 2009), as previously described (Mira, Purpari, Lorusso, et al., 2018), and each amplicon was analysed by electrophoresis on a 3% agarose gel supplemented with ethidium bromide.

Sequencing encompassing both ORFs (NS and VP genes) was carried out using primer pairs developed by Pérez et al. (2014), as previously described (Mira et al., 2019). Sequences were assembled according to an overlapping strategy and analysed using BioEdit ver 7.0.5.3 software (Hall, 1999). Assembled nucleotide sequences were submitted to nBLAST program (Zhang, Schwartz, Wagner, & Miller, 2000) to search related sequences in public domain databases. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers MK802679-85. The obtained sequences were aligned with reference sequences retrieved from the GenBank database, which included the sequence (accession number MF510157) of a CPV-2c strain collected from the same region and previously analysed (Mira, Purpari, Lorusso, et al., 2018).

To elucidate the genetic relationships of the analysed CPV strains, two phylogenetic trees, based on the full-length VP2 and NS1 gene

sequences, were constructed with the MEGA X software (Kumar, Stecher, Li, Knyaz, & Tamura, 2018), using the maximum-likelihood



**FIGURE 1** Maximum-likelihood tree based on 50 full-length VP2 gene sequences of canine parvovirus type 2 strains (bootstrap 1,000 replicates; bootstrap values greater than 65 are shown). Black dots markings (●) indicate CPV strains analysed in this study. Each sequence is indicated with virus type (FPLV: feline panleukopenia virus—CPV: canine parvovirus) or variant (CPV-2, CPV-2a, CPV-2b, CPV-2c), country and year of collection, and accession number

method according to the Tamura 3-parameter (T92) and Hasegawa-Kishino-Yano (HKY) models, with discrete Gamma distribution (five rate categories) (G) and invariant sites (I) (bootstrap analyses with 1,000 replicates). The models selection was performed using the best-fit model of nucleotide substitution with MEGA X software (VP2 gene: T92+G+I; NS1 gene: HKY+G).

Extracted DNA/RNA were also amplified using a set of PCR assays for the detection of canine distemper virus (CDV) (Elia et al., 2006), canine adenovirus (CAV) type 1 and type 2 (Dowgier et al., 2016), canine herpesvirus (CaHV-1) (Decaro et al., 2010), canine coronavirus (CCoV) (Decaro et al., 2004) and canine rotavirus (CRoV) (Freeman, Kerin, Hull, McCaustland, & Gentsch, 2008).

### 3 | RESULTS AND DISCUSSION

All tissue samples tested positive for CPV by conventional PCR assay and negative for CDV, CAVs, CaHV-1, CCoV and CRoV by gel-based or real-time (RT) PCR assays. By sequence amplifications, the nearly complete CPV-genome sequences including both ORFs (4,269 nt) were obtained. Based on the VP2-426 amino acid residue, all detected strains were characterized as CPV-2c. NS1 and VP2 sequences of CPV strains analysed in this study showed 100%–99.95% and 100%–99.82% reciprocal nucleotide identities, respectively. The complete genome sequences showed 99.95%–99.93% and 99.95%–99.91% nucleotide identities with CPV strains of Asian origin, such as CPV\_IZSSI\_2743\_17 (Italy, 2017; accession number MF510157), CPV-SH1516 (China, 2017; acc. no. MG013488) and Canine/China/14/2017 (China, 2017; acc. no. MH476583).

Sequence analysis revealed amino acid changes previously described in Asian CPV-2c strains (NS1: 60V, 544F, 545F, 630P–NS2: 60V, 151N, 152V–VP2: 5A/G, 267Y, 297A, 324I, 370R) (Table S1). CPV strain IZSSI\_PA5632/19 evidenced an additional change at residue 492 of NS1 protein (Table S1). Only one mutation (A/G) was observed among the analysed strains at residue 5 of the VP2 protein, which suggests the circulation of two different but related CPV-2c strains in southern Italy.

Amino acid change I60V in NS1 also lies at the same residue in the NS2-encoding sequence, while change at codon 630 of NS1 sequences did not result in any changes in the encoded NS2 protein. Additional two amino acid changes in the NS2-encoding sequences were observed among the analysed strains: D151N and M152V (Table S1). These changes resulted in silent mutations in the corresponding encoded NS1 protein.

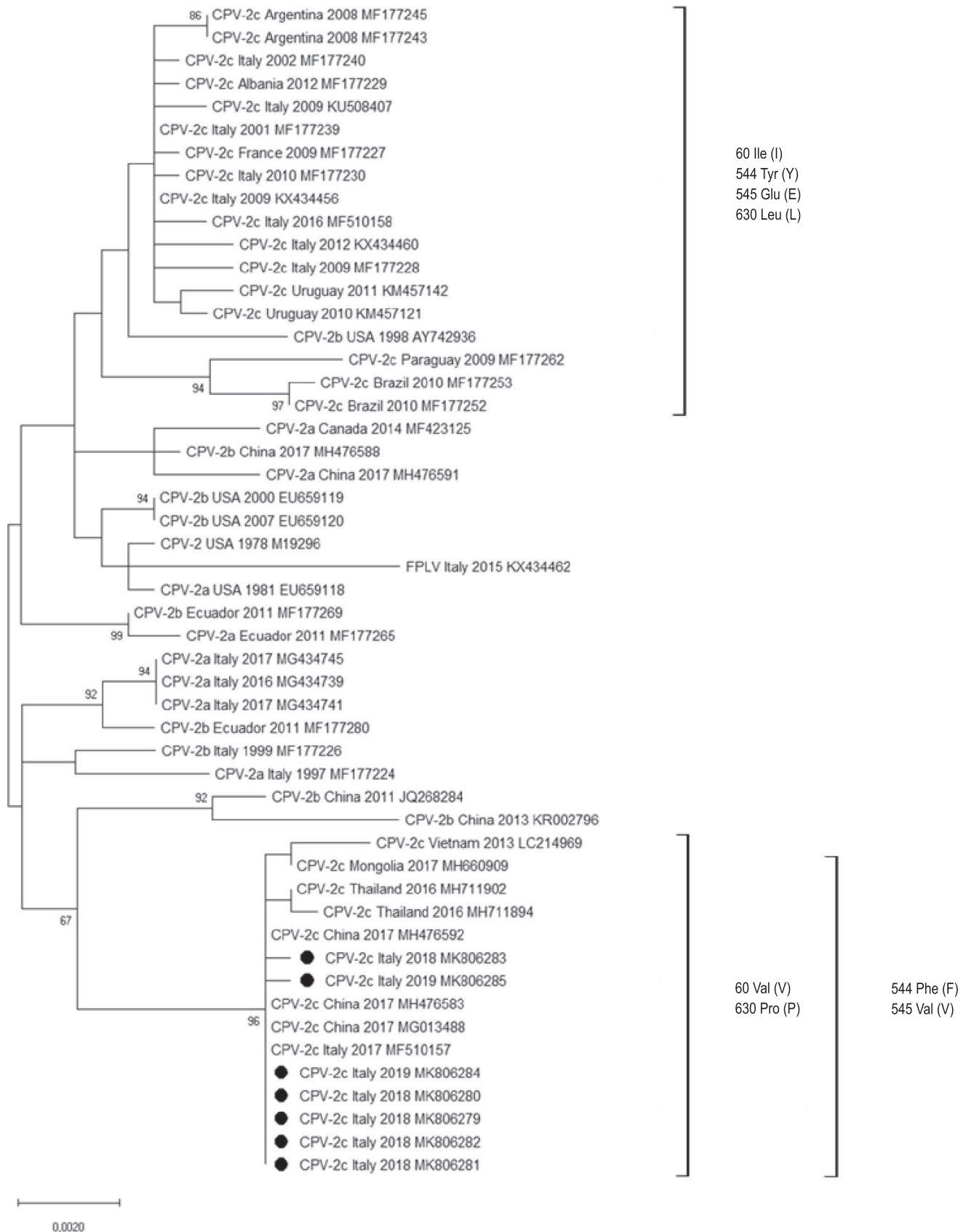
Phylogenetic analysis inferred from VP2 sequences indicated that analysed strains are more related to Asian than to European CPV strains, clustering in a separate clade (Figure 1). Phylogenetic tree inferred from NS1 gene sequences shows that strains clustered within the phylogeny according to the geographical origin and the year of collection rather than to the CPV variant (Figure 2).

The present molecular analysis of CPV strains detected in southern Italy provides new data about the viral spread and dynamics of CPV mutants circulating in Italy. In the last decades, several studies

analysed the spread of CPV strains in Italy, and in 2001, the emergence of the CPV-2c variant was firstly reported (Buonavoglia et al., 2001). In the following years, all three CPV variants were described in Italy, with a slightly higher prevalence of the CPV-2a and CPV-2c variants (Decaro, Desario, et al., 2007; Decaro et al., 2013, 2006; Tucciarone et al., 2018). More recently, a CPV-2c strain displaying genetic signatures typical of Asian viruses was detected in southern Italy (Mira, Purpari, Lorusso, et al., 2018), thus suggesting the introduction of the virus from other countries, as reported for other canine viruses (Decaro, Campolo, et al., 2007; Martella et al., 2006; Mira, Purpari, Bella, et al., 2018). Therefore, a continuous molecular survey was assessed to point out eventual introduction and spread of CPV strains originated from other geographic areas in the Italian canine population.

Since August 2018, CPV-2c strains with specific molecular signatures were detected from stray and owned dogs. Despite the close genetic relationship with the previous Asian CPV-2c strain reported in Italy, its absence in the following months suggests a second introduction of Asian CPV-2c strains. Alternatively, the silent circulation of the original Asian strain in the field with accumulation of few point mutation should be hypothesized. The evidence in rescued stray dogs and in dogs without anamnesis of previous movements, as well as the different dates and places of collection, suggested the active spread of these strains in the field. It remains unclear how these strains have been introduced in Italy, if directly through infected animals or indirectly due to the extreme stability of the CPV in the environment (Hoelzer & Parrish, 2010). According to this study, the spread of Asian CPV strains in a separate geographical area different from Asian countries could be suggested, as previously described in South America (Grecco et al., 2018; Maya et al., 2013).

Whereas CPV-2a and CPV-2b are the prevalent variants circulating in Asia (Yi, Tong, Cheng, Song, & Cheng, 2016), and more recently, CPV-2c has been described in the same continent (Chiang, Wu, Chiou, Chang, & Lin, 2016; Geng et al., 2015; Nakamura et al., 2004; Wang et al., 2016; Zhao et al., 2017; Zhou, Zeng, Zhang, & Li, 2017; Zhuang et al., 2019), showing molecular signatures different from those of other continents. Indeed, the Asian CPV-2c variant shows specific amino acids in NS1 (60V, 544F, 545V, 630P) and VP2 (5A/G, 267Y, 297A, 324I, 370R) gene sequences. Most of these amino acids have been described in the VP2 of CPV-2a/2b/2c strains collected in China, Vietnam, India, Taiwan, South Korea, Thailand and Japan (Chiang et al., 2016; Geng et al., 2015; Han et al., 2015; Jeoung et al., 2008; Lin et al., 2014; Mukhopadhyay et al., 2014; Nakamura et al., 2004; Phromnoi, Sirinarumit, & Sirinarumit, 2010; Soma, Taharaguchi, Ohinata, Ishii, & Hara, 2013; Xu et al., 2015; Yi et al., 2016; Zhang et al., 2010). In particular, CPV-2c strains displaying the amino acid glycine (G) instead of the highly conserved alanine (A) at residue 5 of the VP2 have been previously detected in China (Wang et al., 2016) and Italy (Mira, Purpari, Lorusso, et al., 2018). More recently, molecular analyses including the NS1 gene sequence showed the presence of molecular signatures of the Asian CPV strains even in this region (Han et al., 2015; Mira, Purpari, Lorusso, et al., 2018; Zhuang et al., 2019). Indeed, it results critical to extend the analysis to other genomic regions to properly infer the spread of the genetic CPV variants (Grecco et al., 2018).



**FIGURE 2** Maximum-likelihood tree based on 50 full-length NS1 gene sequences of canine parvovirus type 2 strains (bootstrap 1,000 replicates; bootstrap values greater than 65 are shown). Black dots markings (●) indicate CPV strains analysed in this study. Each sequence is indicated with virus type (FPLV: feline panleukopenia virus—CPV: canine parvovirus) or variant (CPV-2, CPV-2a, CPV-2b, CPV-2c), country and year of collection, and accession number

Moreover, the evidence in the NS1/NS2 gene sequences of amino acid mutations with respect to the other CPV strains spreading worldwide (NS1: 60V, 544F, 545F, 630P - NS2: 60V, 151N, 152V) could contribute to further elucidate its evolution (Mira et al., 2019).

The classification system based on single amino acids (426 and 297) of the VP2 protein does not clearly reflect the phylogenetic relationships of the strains, better supported to proposed "clade" or "lineage/sub-lineage" new classification criteria (Grecco et al., 2018; Zhuang et al., 2019; Mira et al., 2019). As observed also in this study, phylogeny lacks any clustering based on the single VP2 aa residue 426 (CPV-2a/2b/2c), as well as on the geographic origin and period of sample collection. Therefore, a wider evolutionary analysis further supports the thesis to consider the CPV antigenic variants as variants of CPV-2a rather than distinct subtypes (Organtini, Allison, Lukk, Parrish, & Hafenstein, 2015) and could be considered as a more reliable tool in outbreak tracing.

This study reported the early evidence and spread of CPV-2c strains of Asian origin in the Italian canine population. As observed in South America (Grecco et al., 2018), studies based on the complete coding genome are useful to monitor the spread of CPV strains with Asian origin in a different continent and highlight the need of further studies to evaluate the CPV evolution due to the coexistence of genetically divergent strains in the same geographical environment.

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## CONFLICT OF INTEREST

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

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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