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AUTHORS: Olga Calatayud, Fernando Esperón, Roser Velarde, Álvaro Oleaga, Luis Llaneza, Alexis Ribas, Nieves Negre, Ana de la Torre, Alejandro Rodríguez, Javier Millán

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Genetic characterization of Carnivore Parvoviruses in Spanish wildlife reveals domestic dog and cat-related sequences

Running title:

Carnivore parvovirus infection in Spanish wildlife

Olga Calatayud^{1,2,3#}, Fernando Esperón¹, Roser Velarde⁴, Álvaro Oleaga⁵, Luis Llanea⁶, Alexis Ribas⁷, Nieves Negre⁸, Ana de la Torre¹, Alejandro Rodríguez⁹, Javier Millán^{10#}

¹Animal Health Research Centre INIA-CISA, Ctra. Algete a El Casar, 28130, Madrid, Spain

²Institute of Zoology, Zoological Society of London, Regent's Park, London, UK

³The Royal Veterinary College, Royal College Street, London, UK

⁴Wildlife Ecology and Health Group and Servicio de Ecopatología de Fauna Salvaje (SEFaS), Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, 08193, Bellaterra, Barcelona, Spain

⁵SERPA, Sociedad de Servicios del Principado de Asturias S.A., 33203 Gijón, Spain

⁶A.RE.NA. Asesores en Recursos Naturales SL, Perpetuo Socorro 12-Entresuelo 2B, 27003, Lugo, Spain

⁷Department of Zoology and Fisheries, Faculty of Agrobiological Sciences, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic

⁸Consorci per a la Recuperació de la Fauna de les Illes Balears, 07142, Santa Eugènia, Balearic Islands, Spain

23 ⁹Department of Conservation Biology, Estación Biológica de Doñana, CSIC, Américo

24 Vespuccio s/n, 41092, Sevilla, Spain

25 ¹⁰Facultad de Ciencias de la Vida, Universidad Andres Bello, Av. República 440, Santiago,

26 Chile

27 # Address correspondence to:

28 • Olga Calatayud,

29 olgacalatayud@hotmail.com

30 Av Aragón 38, 3-2. 46021-Valencia

31 0034651057026

32 • Javier Millán,

33 syngamustrachea@hotmail.com

34 Facultad de Ciencias de la Vida, Universidad Andrés Bello, Av. República 440,

35 Santiago, Chile

36 005696993578

37

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Summary

The impact of carnivore parvovirus infection on wild populations is not yet understood; disease signs are mainly developed in pups and assessing the health of litters in wild carnivores has big limitations. This study aims to shed light on the virus dynamics among wild carnivores thanks to the analysis of 213 samples collected between 1994 and 2013 in wild ecosystems from Spain. We determined the presence of carnivore parvovirus DNA by real-time PCR and sequenced the *vp2* gen from 22 positive samples to characterize the strains and to perform phylogenetic analysis.

The presence of carnivore parvovirus DNA was confirmed in 18% of the samples, with a higher prevalence detected in wolves (*Canis lupus signatus*, 70%). Fourteen sequences belonging to nine wolves, three Eurasian badgers (*Meles meles*), a common genet (*Genetta genetta*) and a European wildcat (*Felis silvestris*) were classified as canine parvovirus 2c (CPV-2c); five sequences from three wolves, a red fox (*Vulpes vulpes*) and a stone marten (*Martes foina*) as CPV-2b; and three sequences from a badger, a genet and a stone marten as feline parvovirus (FPV). This was the first report of a wildcat infected with a canine strain. Sequences described in this study were identical or very close related to others previously found in domestic carnivores from distant countries, suggesting that cross-species transmission takes place and that the parvovirus epidemiology in Spain, as elsewhere, could be influenced by global factors.

Keywords: Carnivore parvovirus, conservation, epidemiology, multi-host, wildcat, Iberian wolf

1. Introduction

Canine and feline parvoviruses (CPV and FPV) belong to the newly assigned species *Carnivore protoparvovirus-1*, commonly known as carnivore parvoviruses (Cotmore et al., 2014). Even though CPV and FPV are antigenic variants of the same virus species and share a genome homology of 98% (Reed, Jones, & Miller, 1988), there are evolutionary and epidemiological differences between them. While FPV existed from, at least, the beginning of the 20th Century (Verge & Christoforoni, 1928) and remained without noticeable changes during the last decades; CPV emerged in dog populations in the 1970s and underwent notable antigenic drift. The first antigenic variant or strain of CPV was named CPV-2 and it was rapid replaced globally by CPV-2a in 1980. Four years later arose the first reports of CPV-2b in the USA and, after twelve more years, CPV-2c was discovered in Europe. Whilst CPV-2 is differentiated from the rest of the CPV variants by a few amino acid substitutions, 2a, 2b and 2c variants are differentiated by the residue 426 from the VP2 protein (Barker & Parrish, 2001; Decaro et al., 2007; Parrish et al., 1991). The constant antigenic change of CPV allowed the achievement of a wide host range distribution thanks to a high rate of nucleotide substitution (about 10^{-4} substitutions per site per year) (Shackelton, Parrish, Truyen, & Holmes, 2005). By contrast, the FPV clade has maintained a constant host range distribution and varies at slower rates (Shackelton et al., 2005).

The host range of CPV involves almost all wild and domestic carnivore species studied but it is more commonly described in domestic dogs (*Canis lupus familiaris*) and cats (*Felis silvestris catus*). FPV infects a wide variety of carnivores as well, except canid species including wolves and domestic dogs (Allison et al., 2014; Ikeda et al., 2002; Steinell, Parrish, Bloom, & Truyen, 2001). Transmission between wild and domestic carnivores is supported by a number of molecular studies that showed, based on sequencing of the VP2 protein, that wild and domestic carnivores shared identical or closely related parvoviruses (Duarte et al., 2009, 2013; Mendenhall et al., 2016; Steinell, Munson, Van Vuuren, & Truyen, 2000).

Pathologic and epidemiologic characteristics of carnivore parvoviruses are complex and the consequences of infection at an individual and a population level cannot be easily predicted. Even though these viruses are present in almost all carnivore populations tested, under certain circumstances, epidemic waves could trigger declines in naïve wild populations as happened, for example, in wolves (*Canis lupus*) in North America (Mech, Goyal, Paul, & Newton, 2008). The severity of disease signs varies from sub-clinical, acute to lethal, depending on a series of factors involving host age, host immunity, and virus strain. Signs are mainly developed in pups (Parrish, 1995) and assessing the health of litters in wild carnivores has big limitations. For all these reasons, the impact of carnivore parvoviruses in wild ecosystems is poorly understood.

The VP2 protein forms most of the capsid structure and determines the antigenic properties of the virus strain. Molecular studies sequencing the *vp2* gene allow strain characterization and phylogenetic associations and have been widely used in domestic carnivore investigations (Miranda & Thompson, 2016). In wild carnivores, however, serological studies have been more widely performed but due to cross-reactivity, this technique raises big limitations such as the strain determination (Harrison et al., 2004; Steinel et al., 2001). Therefore, to deepen our understanding of parvoviruses in wild ecosystems, further molecular studies are needed. Non-invasive sampling techniques are crucial and spleen samples collected from wild carnivore carcasses, which harbour high viral loads, have a great potential for this purpose (Allison et al., 2013; Decaro, Martella, et al., 2007).

The Iberian Peninsula has the richest carnivore diversity in Western Europe with sixteen native species belonging to six different families (Palomo, Gisbert, & Blanco, 2007) and it offers an appropriate environment to study parvoviruses epidemiology in wild ecosystems.

The aim of this study was to describe for the first time the prevalence of parvovirus infection in wild carnivores in Spain and characterize the circulating strains. Using molecular characterization of positive cases, we also aimed to analyse the relationship with viruses from other wild and domestic carnivores elsewhere and infer phylogenetic associations. Our ultimate goal was to better understand the virus dynamics of carnivore parvoviruses among wild carnivores.

2. Materials and methods

2.1. Sampling

Spleen samples from 213 free-ranging carnivores belonging to the *Mustelidae* (n=104) *Canidae* (n=79), *Viverridae* (n=27), *Felidae* (n=2) and *Procyonidae* (n=1) families were collected in 1994-2013 from different regions of Spain (detailed in *Table 1 and Figure 1*) and stored at -20°C. All samples were taken opportunistically (no animal was killed for investigative purposes), with permission from the regional administrations of Galicia, Asturias, Catalonia and the Balearic Islands. Most sampled animals were found dead in forested areas near human habitation, usually road kill, except 20 Iberian wolves (*Canis lupus signatus*), which were legally shot in remote forested areas.

2.2. Molecular analysis

Approximately 50mg of tissue were used for DNA extraction. After mechanical maceration, DNA was purified using the pressure filtration method QuickGene® DNA tissue kit S (FujiFilm Lifescience, Tokyo, Japan), following the manufacturer's instructions. Purified DNA was stored at -80°C until further use.

Screening for parvovirus-positive samples (case ascertainment) was achieved through a previously described real-time PCR, which targeted a conserved region of 163 nucleotides

of the *vp2* gen (Decaro et al., 2005). Molecular characterization was achieved through the amplification and sequencing of nearly the whole *vp2* gene (Truyen, Evermann, Vieler, & Parrish, 1996). Two nested PCRs amplified a 1746 nucleotide segment. The external PCR amplified a 2401 nucleotide segment and was performed by combining the primers VPF and M5mod (Mochizuki, Horiuchi, & Hiragi, 1996; Steinel et al., 2000); whereas the internal PCR was conducted using the primers P1 and VPR (Battilani et al., 2001; Mochizuki, San Gabriel, Nakatani, Yoshida, & Harasawa, 1993) (*Table 2*). The temperature profile for the external PCR was set at 94°C for 5', followed by 45 cycles: 94°C for 30", 55°C for 30" and 72°C for 2'30", with a final extension of 72°C for 7'. The internal PCR was set at 94°C for 5', followed by 40 cycles: 94°C for 15", 52°C for 15" and 72°C for 2', with a final extension of 72°C for 7'. PCR products were sequenced with eight different primers (detailed in *Table 2*).

A commercial CPV-2 vaccine containing CPV-2 (Nobivac® Puppy DP, MSD Animal Health, Carbajosa de la Sagrada, Spain) was used as a positive control in each assay. Non-template controls were also included in each assay to assure that the samples were not contaminated. Consistency in the generated results ruled out contamination.

2.3. Phylogenetic analysis

Nucleotide sequences were aligned with the ClustalW method and translated into putative amino acid sequences using the MEGA 6.0® software (Tamura, Dudley, Nei, & Kumar, 2007). Specific amino acids were used for the classification as FPV, CPV-2a, CPV-2b and CPV-2c strains (Buonavoglia et al., 2001; Parrish, 1999; Truyen et al., 1996) and further comparison of the obtained sequences with the GenBank® database was performed. Nucleotide and amino acid sequence identity of sequences of 1746bp were calculated using the online software SIAS®, Sequence Identity and Similarity (<http://imed.med.ucm.es/Tools/sias.html>).

A maximum-likelihood phylogenetic tree was inferred based on nucleotide sequences and the reliability of the obtained tree was evaluated with the bootstrap method based on 1000 replicates (Tamura et al., 2007). The median-joining network was performed using SplitsTree4® software (Huson & Bryant, 2006). This algorithm better represents the existence of haplotypes shared by different species (Ohneiser, Hills, Cave, Passmore, & Dunowska, 2015) and, compared with phylogenetic trees, is more reliable when intraspecific phylogenies are studied. The recombination between taxa, the persistence of ancestral alleles and the presence of multiple descendants from single ancestors give rise to reticulated and multifurcating patterns in relationships (Lapointe, 2000).

Statistical analyses were developed using the traditional Clopper-Pearson confidence interval (95% confidence level) for prevalence calculations. QGIS® Geographic Information System Software (<https://qgis.org/es/site/>) was used for the representation of sample locations.

3. Results

Real-time PCR confirmed carnivore parvovirus infection in 39 samples (18.3%, C.I. 13.6–24.1) belonging to eight different species (detailed in *Table 1*). Infection was detected in all species in which sample size was above three. The greatest prevalence was detected in wolves, with 67.6% of positives (C.I.= 50.2–82.0) of positives. Given that wolf samples were obtained from different regions, no statistical inference regarding species-related differences could be performed. Excluding wolves, the remaining carnivores had a prevalence of 7.95% (C.I. 4.4–12).

Nineteen sequences of 1746 bp, one of 1678 bp and two of 600 bp were described. Fourteen of these were classified as CPV-2c, five as CPV-2b and three as FPV. CPV-2c was described in nine wolves, three Eurasian badgers (*Meles meles*), a European wildcat (*Felis*

186 *silvestris silvestris*) and a genet; CPV-2b in three wolves, a red fox and a stone marten; and
187 FPV in a badger, a genet and a stone marten (*Martes foina*). Therefore, 63% of the identified
188 sequences corresponded to CPV-2c, 23% to CPV-2b and 14% to FPV. However, when wolf
189 samples were excluded, given that this species is refractory to FPV infection (Allison et al.,
190 2014), FPV accounted for 30% of the cases.

191 Sequences differed in 48 nucleotide positions, 17 of which were coding mutations
192 (Table 3). Comparison among the CPV sequences from this study showed nucleotide
193 identities of 99.5-100% (mean 99.8, SD 0.12) and amino acid identities of 99.5-100% (mean
194 99.8, SD 0.13). The two FPV sequences described showed a nucleotide identity of 99.82%
195 and were translated into the same amino acid sequence type (aaST H). Three sequences of
196 only 1678 and 600 bp were excluded from the identities calculation.

197
198 Within aaST A, there were 5 nucleotide sequences with synonymous substitutions
199 (nucleotide sequence types 1, 6, 9, 10 and 13), with ntST9 predominating. aaST A
200 belonged to the CPV-2c clade and was described in nine different hosts analysed between
201 2001 and 2013 (five wolves, three badgers and a wildcat) (Table 4).

202 Two additional amino acid sequences types were shared by different host species:
203 aaST F, belonging to the CPV-2b clade, described in two wolves and a red fox (*Vulpes*
204 *vulpes*) from 2007-2013; and aaST H, belonging to the FPV strain, and described in a stone
205 marten and a badger from 2002 and 2013.

206 Comparisons with other sequences published in the GenBank® showed that ntST9 was
207 identical to other 16 nucleotide sequences described in dogs from different countries and
208 years, such as, for example, France-2005 and Uruguay-2011 (GenBank® accession numbers
209 KM457142 and DQ025942).

Three additional amino acid sequence types showed 100% identity with other sequences; aaST F, which was identical to a sequence described in a domestic dog from France-2008 and in a cougar (*Puma concolor*) from U.S.A.-2011 (DQ0255991 and JX475250); aaST H, identical to others described in cats from Japan-2000 and Portugal-2008 (AB054227 and KT240130); and aaST A, identical to sequences in dogs from France-2005, Italy-2008 and Uruguay-2011 (DQ025942, FJ005206 and KM457142). The rest of the amino acid sequences types obtained in this study (aaSTs B, G, C, D and E) were reported for the first time (detailed in Tables 3 and 4).

This subtyping coincides with the phylogenetic network and tree, which clearly clusters the different sequences of FPV and CPV (*Figures 2 and 3*). A group of CPV-2b (ntST-2 and ntST-5 belonging to aaST F; and ntST4 belonging aaST G) was clustered near the CPV-2c clade. The aaST F, classified as CPV-2c, differed only in one amino acid mutation (Asp426Glu) within aaST A. This clade, previously named as "new CPV-2b" also includes sequences previously published from wildlife in USA and Portugal (Allison et al., 2013; Duarte et al., 2013). The network also showed a common CPV-2c haplotype, shared by a domestic dog, wild canids as well as Mustelidae and Felidae members.

4. Discussion

Results from this study provide insights into the presence and distribution of different strains of parvoviruses among Iberian wild carnivores. Further, we adapted a highly efficient nested PCR, which enabled the sequencing of nearly the complete *vp2* gene from half of the positive samples, often in an advanced decomposition state.

The high prevalence of CPV in the small and disperse populations of wolves (67.6%) is compatible with a sustained onward transmission event from domestic dogs. Further, comparisons with the GenBank® showed identical nucleotide sequences in domestic dogs

from Europe, North and South America. Because infection often ‘spills overs’ from more abundant hosts (Woodroffe, 1999), our results highlight the role of dogs as a probable source of infection for wolves, which could get infected through scent communication, predation, coprophagy, or fomites (Llaneza, García, & López-Bao, 2014; Zarnke et al., 2001).

The prevalence of infection in small carnivores, excluding wolves, was 7.95% and the predominant virus strain CPV (70% of the described sequences). Surprisingly, in Portugal the prevalence was 63% , being FPV the most prevalent strain (87%) (Duarte et al., 2013). The lack of standardization in molecular techniques and the opportunistic nature of the sampling hinder the comparisons among different studies. However, these important discrepancies in prevalence and strain types could indicate that epidemiology is strongly influenced by spatial factors that vary across the Iberian Peninsula.

Historically, residue 426 has been used to define CPV-2a, 2b and 2c clades (Buonavoglia et al., 2001) although this classification is increasingly controversial. Sequences described here showed a high similarity even though they were classified in different CPV clades. For example, aaST A and aaST F, which belonged to the CPV-2c and 2b clades respectively, were differentiated by only residue 426. This could be in part explained because residue 426 presented the major variability among all sites in this study.

The predominance of the Glu residue at position 426 (CPV-2c), reported in 63% of all the sequences described, suggested that it is the most prevalent genotype circulating among Spanish wild carnivores in 1994-2013. The oldest CPV-2c sequence was obtained from a wildcat in 2001; this is five years after the first report of CPV-2c in the literature (Decaro, Desario, et al., 2007), and five years before the first detection of CPV-2c in Spain (Decaro et al., 2006). CPV-2c appears to be the most recent clade of carnivore parvoviruses (Miranda & Thompson, 2016) and it might replace older clades in Spanish wild ecosystems.

New host-virus associations have been described in this study, which could be explained by the wide host range of carnivore parvoviruses (Steinel et al., 2001). To the best of our knowledge, this is the first time that CPV-2c variant has been reported in wildcats and the first time that FPV has been reported in stone martens. Pathogenicity of CPV-2c in captive felines is similar to the pathogenicity described by FPV infection and could compromise the immune system (Nakamura et al., 2001) and therefore, the susceptibility of wildcats to CPV-2c infection could be relevant in feline conservation.

Genetic variability among our sequences was low. Only eight coding mutations were spread among our CPV sequences, but they were not consistent with a concrete species, region or time period. Further, identical CPV and FPV nucleotide sequences were described in different host species belonging to three different families (*Canidae*, *Felidae* and *Mustelidae*), indicating that mutations in the *vp2* domain may not be necessary for the jump among sympatric species, as was reported in other wild ecosystems (Calatayud et al., 2019).

In conclusion, carnivore parvovirus infection appears to be widespread among Spanish wildlife, with CPV-2c being the most prevalent clade. We detected very low genetic variability among the VP2 fragments analyzed and identical sequences were infecting different species suggesting the existence of cross-species events. Further, identical sequences were previously described in domestic carnivores in USA, Europe and Asia, indicating that domestic carnivores may be a source of infection and that parvoviruses epidemiology in Spain may be influenced by global factors such as domestic dog international trade. The consequences of these infections in Spanish wildlife are unknown and further assessment would be needed to understand the global scenario.

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6. Conflict of interest statement

Conflicts of interest: none.

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444

445

446 **TABLES**

447

448 **Table 1**

	Region				Total	Positives	Prevalence (95% C.I.)
	Asturias	Galicia	Catalonia	Balearic Islands			
Canidae							
Wolf	20	17			37	25	67.6% (50.2–82.0)
Red fox			42		42	1	2.4% (0.1–12.6)
Felidae							
European Wildcat			2		2	1	
Viverridae							
Common genet			21	6	27	3	11.1% (2.4–29.2)
Eurasian badger			68		68	6	8.8% (3.3–18.2)
Pine marten				23	23	1	4.3% (0.1–21.9)
Stone marten			9		9	2	22.2% (2.8–60)
European mink			1		1	0	
Least weasel				2	2	0	
American mink			1		1	0	
Coati				1	1	0	
Overall	20	17	144	32	213	39	18.3% (13.6–24.1)

449

450 Origin of the samples and prevalence of infection. Species represented by a sample
451 size lower than 3 were excluded from the species-specific prevalence calculation.

452 **Table 2**

Primer	Sequence (5' to 3')	Binding site*	Sense	Ref.
VPF	ATGGCACCTCCGGCAAAGA	2285-2303	+	(38)
VPR	TTTCTAGGTGCTAGTTGAG	5285-5302	-	
P1	ATGAGTGATGGAGCAGTTC	2786-2804	+	(18)
P2	TCATCTAAAGCCATGTTTC	3066-3084	-	
VPM	TGGAGGTAAAACAGGAATT	4093-4111	+	
P3	CCATTTCTAAATTCTTTG	3650-3667	+	
P4	AAGTCAGTATCAAATTCTT	4200-4218	-	
Parvo 2	TCAGAATCTGCTACTCAGCCACCA	3245-3268	+	(39)
Parvo 3	ACCAACCACCCACACCATAACAAC	4924-4947	-	
Primer F	TGGAAGTAGTGGCACACCAA	3454-3473	+	(40)
Probe	6FAM-CAGGTGATGAATTTGCTACAGG-BHQ1	3555-3576	+	
Primer R	AAATGGTGGTAAGCCCAATG	3636-3655	-	
M5mod	ATAACAAACCTTCTAAATCCTATATCAAAT	4681-4709	-	(17)

453

454 Primers used during the present study

455 * Binding site calculated with respect to the reference sequence CPV-N (Genbank

456 Accession Number M19296)

457

458 **Table 3**

aaSTs [†]	N [‡]	80	83	87	93	103	191	232	256	297	300	305	323	361	426	564	568	579
A	9	R	V	L	N	A	R	I	R	A	G	Y	N	R	E	S	G	A
B	1	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C	1	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	P
D	1	.	.	.	.	.	.	.	.	.	.	.	.	Q	.	.	.	.
E	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H
F	3	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.
G	1	.	.	.	.	.	.	.	K	.	.	.	.	.	D	.	.	.
H	2	K	.	M	K	V	.	V	.	S	A	D	D	.	N	N	A	.

459

460 Variable amino acid positions among the sequences described in this study. [†]aaSTs,

461 amino acid sequence types; [‡]N, number of samples sharing identical amino acid

462 sequence types (ST).

463

464 **Table 4**

Strain	Ref.	ntST [†]	aaST [†]	Year	Species	Origin	GenBank® accession number	Max. amino acid identity (Species/country)
CPV-2c	150	1	A	2011	Wolf	Galicia	KP682511	100% (Dog/France, Italy, Uruguay)
	154	9		2012	Wolf	Galicia	KP682523	
	172	9		2012	Wolf	Asturias	KP692527	
	309	9		2011	Badger	Catalonia	KP682519	
	426	9		2007	Badger	Catalonia	KP682524	
	441	9		2001	Wildcat	Catalonia	KP682522	
	171	6		2013	Wolf	Asturias	KP682516	
	417	10		2009	Badger	Catalonia	KP682521	
	169	13		2011	Wolf	Asturias	KP682529	
	163	3	B	2012	Wolf	Asturias	KP682513	99.8% (Dog/France, Italy, Uruguay)
	173	7	C	2013	Wolf	Asturias	KP682517	99.8% (Dog/France, Italy, Uruguay)
	279	8	D	Not known	Genet	Catalonia	KP682518	99.8% (Dog/France, Italy, Uruguay)
CPV-2b	160 [§]	n.a.	n.a.	2012	Wolf	Galicia	KP682529	99.8% (Dog/Italy, Uruguay, Stone marten/Portugal)
	170	12	E	2011	Wolf	Asturias	KP682528	99.8% (Dog/France, Italy, Uruguay)
	158	2	F	2012	Wolf	Galicia	KP682512	100% (Dog/France, Puma/USA)
FPV	167	5		2013	Wolf	Asturias	KP682515	
	434	11		2007	Red fox	Catalonia	KP682525	
	166	4	G	2013	Wolf	Asturias	KP682514	99.8% (Dog/Italy, Taiwan, USA)
CPV-2a	439 [¶]	n.a.	n.a.	1994	Stone marten	Catalonia	-	-
	298	14	H	2002	Stone marten	Catalonia	KP682526	100% (Cat/Japan, Portugal)
	351	15		2013	Badger	Catalonia	KP682520	
FPV	291 [¶]	n.a.	n.a.	Not known	Genet	Catalonia	-	-

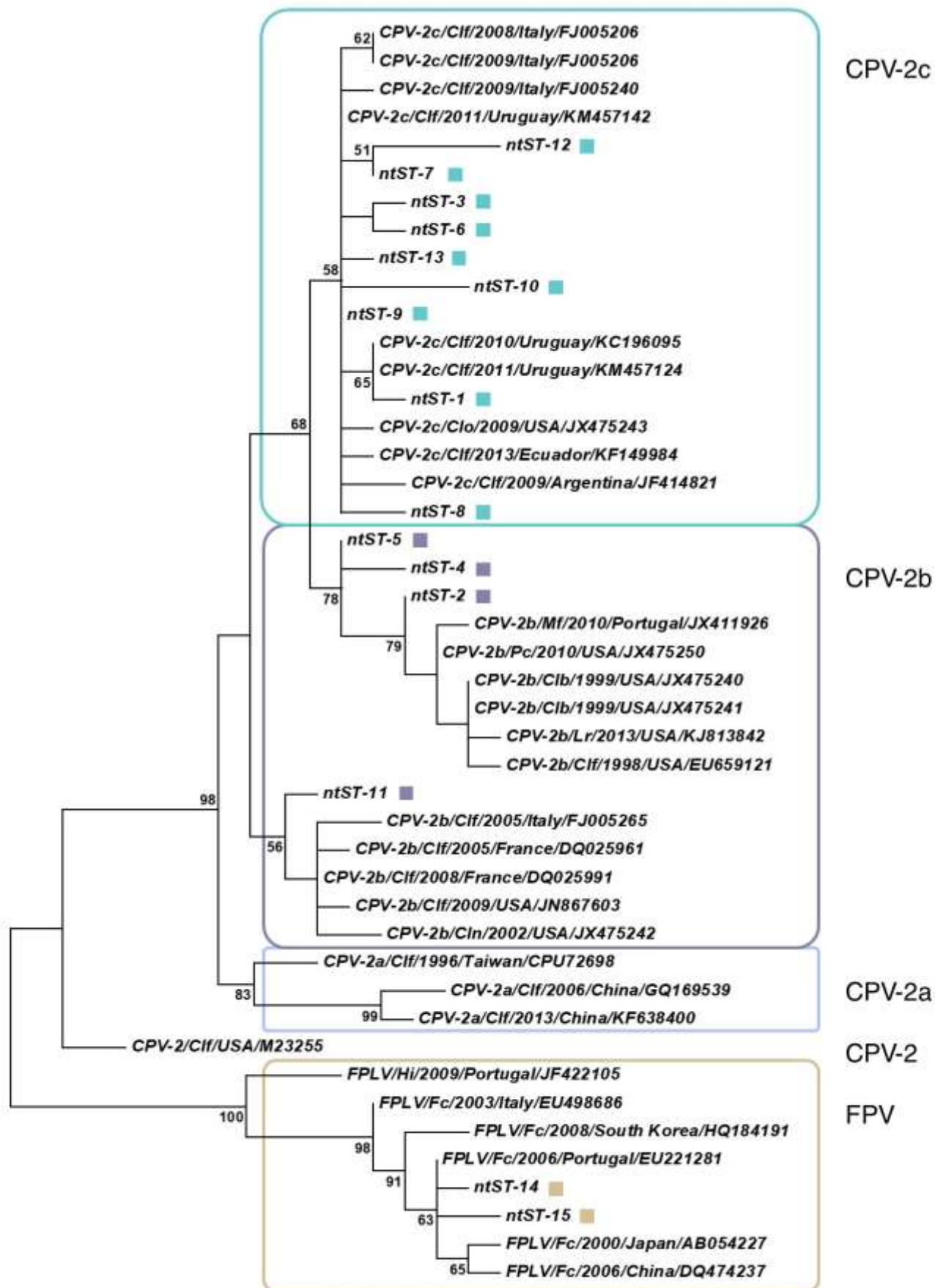
465

466 Parvovirus sequences identified in the present study.

478 **Fig. 1. Geographical distribution of samples included for detection of parvovirus infection in**
479 **wild carnivores in Spain.**

480 White dots: negative; grey dots: positive; black dots: positive and characterized (strain is indicated).

481



0.001

Figure 2. Phylogenetic tree constructed from the VP2 gene nucleotide sequences of parvovirus strains in this study and in other parts of the world.

Sequences obtained in this study are indicated by their nucleotide sequence type (ntST) and highlighted with a square. The rest of sequences are numbered with their GenBank accession number. Clb: *Canis lupus baileyi*; Clf: *Canis lupus familiaris*; Cln: *Canis lupus nubilus*; Clo: *Canis lupus occidentalis*; Cls: *Canis lupus signatus*; Fc: *Felis catus*; Fs: *Felis silvestris*; Hi: *Herpestes ichneumon*; Gg: *Genetta genetta*; Lr: *Lynx rufus*; Mf: *Martes foina*; Mm: *Meles meles*; Pc: *Puma concolor*; Vv: *Vulpes vulpes*. Bootstrap values less than 50% were omitted in the tree.

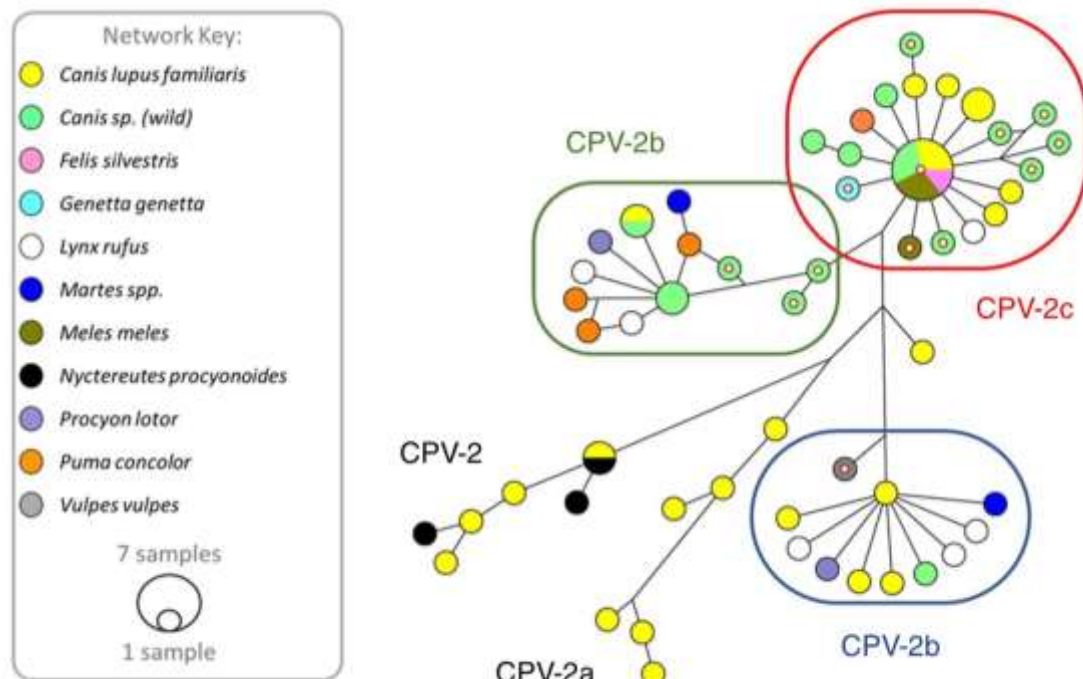


Fig. 3. Median-joining network from selected VP2 gene nucleotide sequences

Network from 74 selected VP2 gene nucleotide sequences of parvovirus strains obtained in this study (marked with a circle inside) and in other parts of the world. Each circle represents one haplotype.