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Investigating CRESS DNA Viruses in Carnivores with emphasis on the family *Circoviridae*: Insights and Implications

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Preface

The research herein described was conducted mainly at the Department of Veterinary Medicine, University of Bari, Italy. The following thesis is written in a ‘thesis by publication’ format and is composed of scientific articles already published in international peer reviewed journals. This final dissertation represents the original intellectual property of the author, who carried out the development and the writing. Co-authors of the articles herein included own original intellectual property based on their contribution to the studies. This thesis entitled ‘Investigating CRESS DNA viruses with emphasis on the family *Circoviridae*: Insights and Implications’ is divided in two main chapters: Chapter 1 includes two papers published in Research in Veterinary Science and Acta tropica, respectively, whilst Chapter 2 consists of two papers published in Frontiers in Veterinary Science and Research in Veterinary Science, respectively.

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General Introduction

Newly emerging viral diseases are major threats to public health. Indeed, most of the recent pandemics were caused by viral zoonotic agents originated mainly in wildlife (Morse et al., 2012). In the last decades emerging or re-emerging infectious diseases, i.e. acquired immunodeficiency syndrome (AIDS) (Sharp and Hahn, 2011), 2009 H1N1 swine flu (Michaelis et al., 2009) and COVID-19 outbreak (V'kovski et al., 2021) have revealed their potential as pandemic. The ever-increasing proximity in the human-animal interface driven by environmental and socioeconomic transitions (i.e., climate change, expanding global human populations and urbanization, international trade and travel, intensive livestock keeping systems, proliferation of reservoir populations, and antimicrobial drug use) has increased the spillover of viruses and promoted the spread of zoonotic diseases (Gibb et al., 2020). Human-to-animal spillovers of pathogens involve direct or indirect transmission. Direct transmission entails contact with reservoir hosts (Jacob et al., 2020) whilst indirect transmission may occur either through consumption of contaminated meat or reservoir hosts (Tei et al., 2003), or via intermediate hosts i.e. vector-borne transmission (Gutiérrez-Bugallo et al., 2019). Many complex factors are involved in a spillover, thus requiring further studies to fully understand patterns of this nature (Escudero-Pérez et al., 2023).

Virus discovery and characterization of novel pathogens in animal population could provide insights into potential virus reservoirs and transmission pathways and promote the development of prevention and treatment strategies. Until 80s the identification of novel viruses relied on traditional approaches. Viral isolation techniques were firstly based on animal models and embryonated chicken eggs and lately on *in vitro* cell cultures (Kumar et al., 2017). Electron microscopy (EM) technique offered an unbiased method for visualization of virus like particles (VLPs) in clinical specimens, cell culture fluid and tissue sections despite a lack of sensitivity, especially when used directly on clinical specimens (Kruger et al., 2000). Methods based on immune-reagents were developed to detect several viral antigens in various and complex samples (Gardner and McQUILLIN, 1980). These methods provided the virology laboratory with an approach for virus detection that could also by-pass virus isolation in cell culture. Since the mid-80s, development of nucleic acid sequence-dependent technique i.e. PCR assays (Mullis and Faloona, 1987), microarrays (Wang et al., 2002) and Sequence-Independent Amplifications (SIA)

techniques, such as sequence-independent single-primer amplification (SISPA), Virus Discovery cDNA Amplified Fragment Length Polymorphism Analysis (VIDISCA), random PCR and rolling circle amplification (RCA), were developed to amplify viral genetic materials (Bexfield and Kellam, 2011; Delwart, 2007; Djikeng et al., 2008; Johne et al., 2009; Pyrc et al., 2008; Reyes and Kim, 1991; Thurber et al., 2009). The limitations of sequence-dependent techniques led the investigators to resort to "metagenomics", a technique that does not presume any knowledge about the organisms being investigated (Handelsman, 2004). Metagenomics is the study of total genetic material present in a given sample, without culturing the organisms present and relies on the use of SIA techniques. These approaches combined with the previously implemented first -generation sequencing (Sanger and Coulson, 1975) accelerated the detection of novel viruses and expanded our knowledge on viral genomes (Datta, 2015; Kumar et al., 2017).

In the last decade the introduction of the next generation sequencing (NGS) techniques prompted the virus discovery (Lauber and Seitz, 2022). NGS techniques were developed by taking advantage of a large number of innovations in the amplification technology, sequencing chemistry, microfluidics, imaging technologies and bioinformatics (Buermans and Den Dunnen, 2014) . NGS platforms are able to generate thousands to billions of sequences (reads) per reaction, allowing deep sequencing of biological samples, with many possible applications in research and diagnostic settings (Barzon et al., 2011). Combining SIA techniques with NGS in viral metagenomics for the application to either human and veterinary medicine, thoroughly implemented the discovery of viral agents of several diseases (Barzon et al., 2011; Belák et al., 2013; Blomström, 2011; Capobianchi et al., 2013; Stang et al., 2005).

Increasing application of viral metagenomics by SIA approaches using SISPA and RCA protocols, revealed a remarkable heterogeneity of single-stranded DNA (ssDNA) viruses, expanding their knowledge in the host range and allowing their detection in different environmental niches (Rosario et al., 2012; Rosario and Breitbart, 2011; Zhao et al., 2019).

Among ssDNA viruses circular Rep-encoding single-stranded (CRESS) DNA viruses within the recently established phylum *Cressdnaviricota* display circular genomes and encode a well conserved replication-associated protein (Rep) (Krupovic et al., 2020). CRESS DNA viruses are ubiquitous in nature and infect eukaryotic organisms including diatoms, fungi, plants, arthropods

and vertebrates (Krupovic and Varsani, 2022). To date, CRESS DNA viruses encompass 11 viral families including the family *Circoviridae* which comprises the smallest autonomously replicating animal infecting viruses (Breitbart et al., 2017; Krupovic and Varsani, 2022). Members of the family *Circoviridae* have been detected in several animal species (Capozza et al., 2022). Circoviruses are able to induce fatal disease in pigs and birds (Baekbo et al., 2012; Fogell et al., 2016). In 2012, canine circovirus (CanineCV) was detected in the serum of asymptomatic dogs, representing the first discovered circovirus in carnivores (Kapoor et al., 2012). CanineCV has been associated with gastroenteritis, respiratory signs and systemic disease involving vasculitis (Anderson et al., 2017; Decaro et al., 2014; Li et al., 2013; Piewbang et al., 2018). Circoviruses have been identified also in other domestic and wild carnivores (Bexton et al., 2015; Gomez-Betancur et al., 2023; Zaccaria et al., 2016).

The present thesis aimed at investigating the presence of CRESS DNA viruses in domestic and wild carnivores and describing the molecular, phylogenetic and epidemiological patterns associated with these viruses.

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Thesis outline

The present thesis provides original data on the study of the molecular, epidemiological and phylogenetic patterns of CRESS DNA viruses and in particular of the members of the *Circoviridae* family in carnivores. The thesis was structured into chapters, in the format of a 'thesis by publication', encompassing articles published in international peer reviewed journals.

Chapter 1 focuses on the investigation of CRESS DNA viruses in domestic carnivores. In **Section 1.1** we describe a wide genetic diversity of Rep sequences of CRESS DNA viruses identified from feline samples**.** CanineCV-like sequences were identified from feline serum samples suggesting virus replication able to sustain viremia in cats**.** Moreover**,** the identification of a novel circovirus species named as feline Circovirus 1 (FeCV-1) was reported in cats in Italy. In **Section 1.2** we evaluate the circulation of canine circovirus in dogs infected with canine parvovirus in Iran. Phylogenetic analysis showed that the Iranian CanineCV strains were more closely related to strains detected in Turkey. We provide new insights on the CanineCV's molecular epidemiology and its role as a co-infection agent.

Chapter 2 focuses on the detection of CRESS DNA viruses in wild carnivores. In **Section 2.1** we describe the CAdV-1 and CanineCV circulation in wild carnivore populations in Italy. We report the detection of CanineCV in wolves and badgers and the first detection of cycloviruses in wolves in co-infection with CanineCV. In **Section 2.2** we report the identification of a novel circovirus in the Iberian lynx population from different areas of Spain. The virus, termed ILCV-1, was repeatedly identified in the spleen samples, suggesting virus ability to replicate and spread actively in the host.

Chapter 1

CRESS DNA viruses in domestic carnivores

Recent advances in molecular diagnostics and metagenomic studies have significantly expanded our knowledge about single-stranded DNA (ssDNA) viruses and particularly circular Rep-encoding single-stranded (CRESS) DNA viruses (Rosario et al., 2012). CRESS DNA viruses represent the smallest known viral pathogens that can infect eukaryotes and are ubiquitous in different ecosystems (Kazlauskas et al., 2018). The replication of these viruses relies on a rolling circle replication mechanism which uses a conserved virus-encoded replicase (Chandler et al., 2013). Most CRESS-DNA viruses remain uncultured, as their definite hosts are unknown and their host-range is continuously challenged (Ng et al., 2015).

CRESS DNA viruses are members of the recently established phylum *Cressdnaviricota* (Krupovic et al., 2020) which currently encompass 8 orders and 11 viral families (Krupovic and Varsani, 2022). Viruses of the families *Geminiviridae*, *Nanoviridae* and *Metaxyviridae* are known to infect plants (Fiallo-Olivé et al., 2021; Gronenborn et al., 2018; Thomas et al., 2021). Members of *Naryaviridae*, *Nenyaviridae*, and *Vilyaviridae* families are associated with protozoan parasites (Kinsella et al., 2020; Krupovic and Varsani, 2022). Family *Bacillodnaviridae* includes diatom-infecting viruses (Munke et al., 2022) whilst viral genomes from the *Genomoviridae* family have been retrieved from diverse environmental samples including fungi (Varsani and Krupovic, 2021). Viruses within the *Smacoviridae* family have been identified in the feces of various animals, including humans, however the definite hosts are not confirmed and an association between smacoviruses and methanogenic archarea has been recently proposed (Díez-Villaseñor and Rodriguez-Valera, 2019). *Redondoviridae* viruses were discovered in respiratory and gut samples from human subjects and although they have been associated with periodontitis and critical illness, they have not been considered causative agent of any diseases (Abbas et al., 2021).

The family *Circoviridae* within the phylum *Cressdnaviricota* (Krupovic et al., 2020) and the class

Arfiviricetes, comprises viruses with a diameter of 15–25 nm and a covalently closed, circular, single-stranded DNA (ssDNA) genome ranging from 1.7 to 2.1 kb length. Members of the family *Circoviridae* (circoviruses) are classified into two distinct genera; *Circovirus* (CrV) and *Cyclovirus* (CyV) which encompass 49 and 52 recognized species (www.ictv.global/report/circoviridae), respectively. Genome of circoviruses has ambisense organization and contains at least two major open reading frames (ORFs), encoding the replication-associated protein (Rep) and the capsid protein (Cap) (Rosario et al., 2017). Viruses of the genus *Circovirus* (CrVs) have been detected in birds (Haddadmarandi et al., 2020), mammals (Bexton et al., 2015; Opriessnig et al., 2020) and fishes (Lőrincz et al., 2012) whilst viruses of the *Cyclovirus* genus (CyVs) have been identified both in vertebrates (Prades et al., 2021) and invertebrates (Dayaram et al., 2013; Padilla-Rodriguez et al., 2013). Circoviruses are associated with severe disease in animal hosts such as pigs and birds causing postweaning multisystemic wasting syndrome and psittacine beak and feather disease respectively (Baekbo et al., 2012; Fogell et al., 2016).

Interestingly, CRESS DNA viruses have been repeatedly identified as components of the human virome. Apart from the redondoviruses which have been detected primarily in the oro-respiratory tract of both healthy and diseased humans (ictv.global/report/redondoviridae), several studies have reported the presence of circoviruses in human samples. CyV detection in humans was first reported in 2010 in stool samples collected from children with and without acute flaccid paralysis (AFP) in developing countries (Li et al., 2010). Since then, human associated CyVs (HuACyV) have been also detected in cerebrospinal fluid (CSF) (Smits et al., 2013; Tan et al., 2013), serum (Macera et al., 2016; Sauvage et al., 2018) and respiratory secretions (Phan et al., 2014) of symptomatic or healthy individuals, leading to the introduction of 12 distinct HuACyV species within the *Cyclovirus* genus (Prades et al., 2021). HuACyVs have been associated with neurological disease, gastrointestinal disorders, immunodeficiency and respiratory signs, however their pathogenic role, if any, remains unclear. More recently, in 2022, the first human associated CrV (HCirV-1) was described in an immunosuppressed patient with acute hepatitis in France (Rodriguez et al., 2023). Later, a closely related circovirus (HCirV-2) was identified in China in blood samples from two intravenous drug users who were infected with HIV-1, hepatitis C virus or both (Li et al., 2023). The origin, transmission, and impact of these viruses on human health are not yet clarified, highlighting the need to raise awareness and to further investigate the potential

of circoviruses as emerging human pathogens (Pérot et al., 2024).

Circoviruses have been detected first in domestic carnivores in 2012. Canine circovirus (CanineCV) was identified in the serum of asymptomatic dogs in USA (Kapoor et al., 2012). Since its initial discovery, many studies have reported a worldwide circulation in domestic and wild carnivores (Decaro et al., 2014; Kotsias et al., 2019; Sun et al., 2019; Weber et al., 2018; Zaccaria et al., 2016) leading to the delineation of six discernible phylogenetic clades (Beikpour et al., 2022; Gomez-Betancur et al., 2023). CanineCV presence has been associated with different clinical profiles, subclinical infection or co-infection with other canine agents (Thaiwong et al., 2016). In dogs, CanineCV has been associated with acute gastroenteritis, haemorrhagic diarrhoea, signs of vasculitis, lymphadenitis, thrombocytopenia, neutropenia, and lymphopenia (Anderson et al., 2017; Decaro et al., 2014; Li et al., 2013) whilst more recent studies described an association with respiratory signs (Piewbang et al., 2018). However, CanineCV's pathogenesis is still not fully understood. In 2023, another study identified the presence of partial Rep CanineCV sequences in 12 feline serum samples and 2 feline nasal swabs in China (Xiao et al., 2023). Only one full genome was obtained from one of the nasal swabs and clustered with CanineCVs in clade 3.

Furthermore, in 2021, two novel cycloviruses were detected in fecal samples obtained from three CPV-2-positive domestic dogs with hemorrhagic gastroenteritis (Gainor et al., 2021); yet, it remains uncertain whether they had dietary origins or they actually infect dogs.

Whilst circoviruses in domestic dogs have been repeatedly reported, limited information is available regarding the epidemiology of circoviruses in domestic cats. In 2014, a feline CyV was identified in a metagenomic study on pooled fecal samples collected from 25 clinically healthy cats housed in a shelter in Davis, California, USA (Zhang et al., 2014). The complete genome of the feline CyV strain FD, shared 39–47% Rep sequence identity with previous reported CyVs appeared to have no connection to the previously identified CyV strain FD nor to other circoviruses described in wild felids (Cerna et al., 2023; Payne et al., 2020), whilst displayed consistently the highest % nt identities (74.3-78.7 %) to mongoose CrV strain Mon-1 (MZ382570). In 2018, a novel feline stool-associated CRESS DNA virus (FeSCV), was retrieved from 4 cats with enteritis during a multi-pathogen outbreak in Japan (Takano et al., 2018). Upon genome sequencing and phylogenetic analysis, FeSCV appeared as a novel CRESS DNA virus unrelated to CyV FD and

subsequently classified into the family Vilyaviridae (Kinsella et al., 2020; Krupovic and Varsani, 2022) which is associated with protozoan parasites. Indeed, in 2021 Hao et al described the presence of FeSCV in diarrheic samples of two cats in co-infection with other enteric viruses in China, also reporting the detection of a *Giardia intestinalis* sequence in the FeSCV genome (Hao et al., 2021).

Given the constantly evolving nature of viral infections and the potential emergence of novel strains, further research on circoviruses is crucial for early detection and surveillance of these viruses in domestic carnivores. Moreover, epidemiological studies may help clarify any possible association with disease occurrence and the distribution of circoviruses in the canine and feline populations forestalling potential spillover events into human communities. In section 1.1 we screened different feline sample collections obtained from cats in Italy, identifying a wide genetic diversity of CRESS DNA viruses in these samples. Moreover, in this study we reported the identification of a novel circovirus in cats, and we observed exposure of cats to the canine circovirus. In section 1.2 we aimed to provide new insights into the CanineCV molecular epidemiology and its possible role as a co-infectious pathogen in the Iranian canine population.

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Section 1.1

Investigating the genetic diversity of CRESS DNA viruses in cats identifies a novel feline circovirus and unveils exposure of cats to canine circovirus

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Abstract

Circular replication-associated protein (Rep)-encoding single stranded (CRESS) DNA viruses include Circoviruses which have been found in several animal species and in human specimens. Circoviruses are associated with severe disease in pigs and birds and with respiratory and gastrointestinal disorders and systemic disease in dogs. In cats there are only a few anecdotical studies reporting CRESS DNA viruses. In this study, a total of 530 samples (361 sera, 131 stools, and 38 respiratory swabs) from cats, were screened for the presence of CRESS DNA viruses. Overall, 48 (9.0%) of 530 samples tested positive using a pan-Rep PCR. A total of 30 Rep sequences were obtained. Ten sequences of fecal origin were tightly related to each other (82.4–100% nt identity) and more distantly related to mongoose circoviruses (68.3 to 77.2% nt identity). At genome level these circoviruses dis-played the highest nt identity (74.3–78.7%) to mongoose circoviruses thus representing a novel circovirus species.

Circoviruses from different animal hosts (n = 12) and from humans (n = 8) were also identified. However, siX Rep sequences were obtained from serum samples, including canine circoviruses, a human cyclovirus and human and fish-associated CRESS DNA viruses. The presence of these viruses in the sera would imply, to various extent, virus replication in the animal host, able to sustain viremia.

Overall, these findings indicate a wide genetic diversity of CRESS DNA viruses in cats and warrant further investigations.

Keywords: Circovirus, CRESS DNA virus, Cats, Enteric samples, Sera

1. Introduction

Circular replication-associated protein (Rep)-encoding single- stranded (CRESS) DNA viruses include a group of viruses that possess circular ssDNA genomes and encode an enzyme responsible for initiating genome replication (Krupovic et al., 2020). The replication mechanism of these viruses, named rolling circle replication, is based on the conserved replicate initiation protein Rep. CRESS DNA viruses form the large phylum of *Cressdnaviricota*, are further divided into two classes, *Repensiviricetes* and *Arfiviricetes*, comprising eight virus families. Classification of CRESS DNA viruses is being continuously challenged by the discovery of a large number of diverse genome sequences in the biosphere (Kazlauskas et al., 2018, 2019). Recently, three novel viral families *Naryaviridae*, *Nenyaviridae*, and *Vilyaviridae*, associated with protozoan parasites have been established (Krupovic and Varsani, 2022).

Members of the family *Circoviridae* (circoviruses), class *Arfiviricetes*, includes nonenveloped, spherical viruses with a circular, covalently closed DNA genome ranging from 1.7 to 2.1 kb in size. The ambisense genomic organization includes two major open reading frames (ORFs) that encode the Rep and the capsid (Cap) proteins. The family *Circoviridae* includes the genera *Circovirus* and *Cyclovirus* which encompass 49 and 52 recognized species (www.ictv.global/report/circoviridae), respectively. Members of the genus *Circovirus* (CV) have been identified in various mammals, birds, and fishes. Members of the genus *Cyclovirus* (CyV) have been detected in samples from both vertebrates and invertebrates (Rosario et al., 2017).

CVs have been associated with important diseases, including post- weaning multisystemic wasting syndrome (PMWS) of pigs (Baekbo et al., 2012) and the Beak and Feather Disease (PBFD) of psittacine birds (Fogell et al., 2016).

CRESS DNA viruses have been found to be common component of human fecal virome. CyV were first discovered in a 2010 metagenomics study, in fecal samples of children from different countries, in the stools of chimpanzees and in the meat of farm animals (Li et al., 2010). Since then, human-associated CyVs have been detected in cerebrospinal fluid (CSF) (Tan et al., 2013), blood serum (Smits et al., 2013) and respiratory secretions (Phan et al., 2014) of patients with neurological disease, lower respiratory tract infections, gastrointestinal disorders but also from healthy individuals. In 2023 a novel CV has been identified on metaviromic analysis of a liver

biopsy of a woman with chronic hepatitis in France. The virus established a chronic infection replicating in hepatocytes and the viral titer increased over time (Perot et al., 2023).

Information on the epidemiology of CRESS DNA viruses in cats and other felids is still limited. In 2014, a feline CyV was identified in a metagenomic study on pooled fecal samples collected from 25 clinically normal cats housed in a shelter in Davis, California, USA (Zhang et al., 2014). The complete genome of the feline CyV strain FD, shared 39–47% Rep sequence identity with previous reported CyVs. In 2018, a novel feline stool-associated CRESS DNA virus (FeSCV), was retrieved from 4 cats with enteritis during a multi-pathogen outbreak in Japan. Fourteen out of 20 cats housed together developed diarrhea and coronavirus and bocaparvovirus were also detected in the outbreak (Takano et al., 2018). Upon genome sequencing and phylogenetic analysis, FeSCV appeared as a novel CRESS DNA virus unrelated to CyV FD and subsequently classified into the family Vilyaviridae (Krupovic and Varsani, 2022).

CRESS DNA viruses have also been reported in metagenomics studies in large felids, bobcats (*Lynx rufus*) and pumas (*Puma concolor*). In total, three novel CV candidate species within the genus *Circovirus* and two different novel candidate CyV species within the genus *Cyclovirus*, were identified from scat samples (Payne et al., 2020; Cerna et al., 2023).

In this study, in order to expand the knowledge on CRESS-DNA in cats and fill in the existing information gaps, we screened collections of samples obtained from cats, including oronasal swabs, rectal swabs and serum samples, using a consensus PCR assay able to identify members of the *Circoviridae* family (Li et al., 2010).

2. Materials and methods

2.1 Origin of samples

A total of 530 archival sera, enteric (stool and rectal swabs), and respiratory (nasal and oropharyngeal swabs) samples obtained from cats, collected during 2011–2021 in accordance with the animal ethics standards of the University of Bari, Italy, were screened for circoviruses. More in detail, the collection included 38 respiratory samples (collection A), 361 sera samples

(collection B) and 131 enteric samples (collection C). Collection A included nasal and oropharyngeal swab samples only from cats with respiratory signs, collected in Apulia region, Southern Italy, by veterinary practitioners of different clinics. Collections B included sera from cats collected from two different veterinary clinic laboratories located in Apulia region and tested upon request of the veterinarian practitioners after anamnesis, medical history and clinical examination. About 40% of the sera had been collected for diagnosis of infectious diseases (feline immunodeficiency virus and feline leukemia virus, feline coronavirus, toxoplasmosis, hemoplasmosis, bacterial and fungal infections) whilst the rest had been submitted to the laboratory for pre-surgical evaluation, for suspected metabolic or neoplastic dis- eases. Collection C included feces or rectal swabs collected from cats of various age with enteric signs by veterinary practitioners from different clinics in Apulia region. Additional sample collection (collection D) included 65 fecal samples from cats with enteritis in Abruzzi region, Central Italy in 2020–2021.

2.2 DNA extraction and screening for CRESS DNA virus

All samples from collections A, C, D were homogenized using 10% w/ v Dulbecco's modified Eagle's medium (DMEM) and then centrifuged at 10,000 Xg for 3 min to collect the supernatant, whilst the serum samples (collection B) were not diluted. Nucleic acids were extracted from 200µL of each sample using the IndiSpin Pathogen Kit (Indical Bioscience GmbH, Leipzig, Germany) according to the manufacturer's protocol and stored in 80 ◦C until use.

All samples were screened for circoviruses with a pan-Rep PCR protocol based on a broadly reactive set of primers designed to recognize members of the *Circoviridae* family (Table 1) (Li et al., 2010). Both first- and second-round PCR protocols were performed using Platinum II Hot-Start Green PCR Master Mix (2) (Invitrogen, ThermoFisher Scientific) and the cycling conditions consisted of activation of the Hot-Start polymerase at 94 ◦C for 2 min, followed by 35 cycles of denaturation at 94 ◦C for 15 s, annealing at 52 ◦C for 15 s and extension at 68 ◦C for 15 s. One microliter of the PCR products of the first-round PCR diluted 1:100 in DEPC water was used as a template in the second-round PCR. PCR amplicons were purified and sent for direct sequencing to Eurofins Ge- nomics laboratories (Germany). Sequences of approximately 400 nucleotides (nt) were obtained and analyzed using the web-based tool FASTA

(https://www.ebi.ac.uk/Tools/sss/fasta/, accessed on 8th February 2022) (Madeira et al., 2022), using the default values to find homologous hits.

2.3 Quantitative PCR for feline CV

A quantitative real time PCR (qPCR) was designed based on the Rep sequence data obtained with the consensus pan-Rep PCR. Aligned Rep sequences generated from 10 cats were used to design primers and probe (Table 1). A total of 10 µL of sample DNA were added to the 15-µL reaction master miX (IQ SupermiX; Bio-Rad Laboratories SRL, Segrate, Italy) containing 0.6 µmol/L of each primer and 0.2 µmol/L of probe. Thermal cycling consisted of activation of iTaq DNA polymerase at 95 ◦C for 3 min, 44 cycles of denaturation at 95 ◦C for 10 s, and annealing extension at 60 ◦C for 1 min. The specificity of the assay was assessed with a panel of DNA viruses including feline panleukopenia virus (FPV), feline bufavirus, feline herpesvirus, feline-origin poxvirus. Also, a panel of circovirus-positive samples identified and characterized upon sequence analysis in this study (Table 2) was tested to rule out cross- reactivity of the primers/probe with other CRESS-DNA viruses.

2.4 Genome amplification

All the PCR products that were considered positive after gel electrophoresis were subjected to direct sequencing. The circular DNA in selected samples was enriched by multiply primed rolling cycle amplification (RCA), using the bacteriophage phi29 DNA polymerase (TempliPhi 100 amplification kit, Cytiva) and pan-Rep reverse primer CV-R1(5′-AWCCAICCRTARAARTCRTC-3′) (Table 1), according to the manufacturer's instructions (Johne et al., 2009; Wu et al., 2006). Several sets of primers were designed to recover the circular genome from different hosts using an inverse (back-to-back) PCR strategy, amplifying a fragment of approximately 1.5-2 kb. The primers were designed within the Rep gene sequence obtained with the pan-Rep PCR. The inverse PCR assays were performed with TaKaRa La Taq polymerase (TaKaRa Bio Europe S.A.S. Saint-Germain-en-Laye, France). The thermal protocol of the first-round PCR included a first step at 94 ◦C X 2 min, followed by 35 cycles of 94 ◦C X 30 s, 60 ◦C X

30 s, and 68 ◦C X 3 min, with a final extension of 68 ◦C X 10 min. One microliter of a 1:100 dilution of the first-round PCR product was used in the second-round amplification, using outer primers. All PCR-positive products were sequenced directly (Sanger sequencing) at an external facility (Eurofins Genomics GmbH, Ebersberg, Germany) using a primer walking strategy. The list of primers used in this study is provided in Table 1.

2.5 Sequence and phylogenetic analyses

The purified PCR products with sufficient DNA concentration (> 10 ng/μL) were directly sequenced in both directions by Eurofins Genomics (Ebersberg, Germany). The obtained sequences were aligned with cognate CRESS DNA virus strains retrieved from the GenBank database by MAFFT algorithm (Katoh, 2002). The appropriate substitution model settings for the phylogenetic analysis and estimation of selection pressure on coding sequences were derived using "Find the best protein DNA/Protein Models" implemented in MEGA X version 10.0.5 software (Kumar et al., 2018). The evolutionary history was inferred by using the maximum-likelihood method, General-time reversible 6-parameter model, a discrete gamma distribution and invariant sites to model evolutionary rate differences among sites (6 categories) and supplying statistical support with 1000 replicates. Bayesian inference and neighbor joining phylogenetic approaches were also explored and the comparison of the phylogenetic outputs revealed similar topologies with slight differences in bootstrap values at the nodes of the tree. Accordingly, the maximum-likelihood tree was retained. Sequence editing, alignments and phylogenetic analyses were performed by Geneious Prime version 2021.2 (Biomatters, Auckland, New Zealand).

3. Results

3.1 Screening for DNA virus

Overall, a total of 48 (9.0%) out of 530 feline samples tested positive in the two-round pan-Rep PCR. In detail, circovirus DNA was detected in 10/361 (2.8%) sera of collection B, 32/131 (24.4%) enteric samples of collection C and 6/38 (15.8%) respiratory samples of collection A. Positive samples with a DNA concentration exceeding 10 ng/μL were subjected to direct sequencing,

yielding 30 (63.8%) sequences of good quality. Sequence characterization by FASTA nucleotide online tool (https://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) (Madeira et al., 2022) revealed a high heterogeneity of Rep sequences, related to circoviruses found in different host species (Supplementary Table 1). A large group of sequences (n = 10) were tightly related to each other (82.4–100% nt identity) and resembled viruses detected in mongoose (68.3 to 77.2% nucleotide identity). This group of viruses was herein referred to as cat-associated mongoose-like (CAML) CVs. A spare sequence (n = 1) was similar to a bat CV. Sequences similar to CRESS DNA viruses detected in bird (n = 3), in fish (n = 2), in pig (Porcine CV 3, PCV3) (n = 2), in dog (n = 3) and in a snail (n = 1) were also identified. Interestingly, we also identified eight sequences similar to human- associated CV NG1 (n 1) and human-associated CyVs TN10 (n 2), TN12 (n 2), and PK2111 (n 3). CRESS DNA viruses other than circoviruses were not detected in the screening but in three samples, PK2111-like (Supplementary Table 1). A summary of the sequencing results is provided in Fig. 1 and Supplementary Table 1.

3.2 Screening for CAML-CVs by qPCR

The qPCR for CAML-CV was specific, since it did not recognize other feline/canine DNA viruses and also, none of the other samples positive in the pan-Rep PCR that were sequenced and characterized in our collection (Table 2). Only CAML-CVs were successfully recognized by the qPCR, with the cycle threshold (CT) ranging from 29.4 to 40.3. Using the qPCR, fecal samples from Central Italy (collection D) were also screened to assess the presence of these viruses in other geographical areas. CAML-CV was detected in 3/65 samples (4.6%) with the CT ranging from 33.4 to 39.5.

3.3 Genome amplification and sequence analysis

The circular DNA of selected samples was enriched by rolling cycle amplification (RCA) protocols. Subsequently, an inverse two-round PCR strategy with primers designed to amplify the rest of the circular genome was performed on the RCA products. The complete genome was generated successfully for 3 CAML-CVs (ITA/2019/cat/471.3, ITA/ 2019/cat/471.4 and

ITA/2019/cat/471.12), for a Pigeon-like CV (ITA/ 2019/cat/518.1a) and for 3 Canine-like CVs (strains ITA/2021/cat/ 99.95, ITA/2021/cat/230.1 and ITA/2021/cat/230.3).

The genome size of the six Italian CV strains identified in this study were 1746, 2043 and 2063–2066 nt, in CAML-CVs, Pigeon-like CV and Canine-like CVs, respectively (Table 2). The genome features of the identified CVs included two major open reading frames (ORFs), located on complementary strands in opposite orientation. In the CAML-CV strains, the ORF1 (849 nt), located on the virion strand, and the ORF2 (624 nt), located on the complementary strand of the replicative form, encoded for the Rep (282 aa) and Cap (207 aa) proteins, respectively. In the Pigeon-like CV strain, the ORF1 (948 nt), located on the virion strand, and the ORF2 (834 nt), located on the complementary strand of the replicative form, encoded for the Rep (315 aa) and Cap (277 aa) proteins, respectively. In the Canine-like CV strains, the ORF1 (912 nt), located on the virion strand, and the ORF2 (813–816 nt), located on the complementary strand of the replicative form, encoded for the Rep (303 aa) and Cap (270–271 aa) proteins, respectively (Fig. 2, Table 2).

Similar to other CVs, the genome of the Italian CVs comprised two intergenic non-coding regions which were located between the start and stop codons of the replicase and capsid protein genes, respectively. The 5′ and 3′ intergenic regions were 190 and 83 nt in length in CAML-CV strains, 89 and 172 nt in length in Pigeon-like CV strain and 135 and 203 nt in length in Canine-like CV strains. The 5′-intergenic regions of CVs identified in the study contained a thermodynamically stable stem-loop, which regulates the initiation of rolling-circle replication, and the conserved mononucleotide motifs CAGTATTAC in CAML-CV strains and TAGTATTAC in Pigeon-like CV and Canine-like CV strains (Fig. 2, Table 2).

CAML-CVs displayed consistently the highest % nt identities (74.3–78.7%) to mongoose CV strain Mon-1 (MZ382570). Pigeon-like CV shared the highest % nt identity (92.9%) to Pigeon CV isolate Hebei/TS/2021 (OL901206). Canine-like CV strains ITA/2021/cat/ 230.1 and ITA/2021/cat/230.3 displayed the highest % nt identities (97.8–99%) to CanineCV strain Bari/411–13 (KJ530972) whilst strain ITA/2021/cat/99.95 displayed the highest % nt identity (97.3%) to CanineCV strain Ha13 (KF887949) (Table 2).

3.4 Phylogenetic analyses

A phylogenetic tree was constructed based on the full-length genome of CVs identified in this study along with CRESS DNA virus sequences retrieved from Genbank (Fig. 3).

The Italian CV strains were distinguishable in three different clades. CAML-CV strains ITA/2019/cat/471.3, ITA/2019/cat/471.4 and ITA/ 2019/cat/471.12 tightly clustered with CV strains Mon-1 and Mon-29 identified in 2017 from mongoose in Saint Kitts and Nevis (Gainor et al., 2021). CAML-CV strains clustered in the same clade with Calfel virus LSF45 CV 359 (ON596197), retrieved from a bobcat in California in 2011 (Cerna et al., 2023) although sharing a low % nt identity (47.6–48.4%). Pigeon CV strain ITA/2019/cat/518.1a clustered with other Pigeon CVs identified in China in 2018 and 2021 (Wang et al., 2022) with which the Italian strain shared 90.8–91% nt identity. CanineCV strains ITA/2021/cat/99.95, ITA/2021/cat/230.1 and ITA/2021/ cat/230.3 clustered in a same clade with two Italian CV strains Bari/ 411–13 (Decaro et al., 2014) and AZ663/1–13 (Zaccaria et al., 2016) and strain UCD1–1698 retrieved in USA (Li et al., 2013).

Also, a phylogenetic tree was generated on the full-length genome of Canine-like CV strains identified in this study together with cognate strains retrieved from GenBank (Fig. 4). The unrooted phylogenetic tree showed a well-distinguishable clustering of the CanineCV strains into six groups. The CanineCV strains identified in this study fell into clade 1 along with other European, Asian, and American CanineCV strains (Fig. 4) although segregating within two distinct sub-lineages.

4. Discussion

In this study, several genetically heterogeneous CRESS DNA viruses were found in cats after screening different collections of samples, i.e., stools, sera and oro-nasal swabs. Good-quality sequences were obtained from fecal samples (n=24) and from the sera (n=6) but not from the respiratory samples. Sequences resembling human-associated CV NG1 (n=1), CyV TN10 (n=2), TN12 (n=2), and an unclassified circular DNA human virus PK2111 (n=2) were identified in fecal samples. Despite several attempts, we could not generate the genome sequence of TN10-, TN12-,

PK2111-like and NG1-like viruses identified in the feline samples. Interestingly, similar viruses (TN-9- and TN12-like) have been identified in the intestinal content of lizards and geckos sampled in an Italian study (Capozza et al., 2022). Accordingly, we hypothesized a dietary origin for those viruses and a possible relation with the predatory behavior of cats, since CyVs seem common in insects (Nebbak et al., 2021; Rosario et al., 2011) that are preyed by bats and by small reptiles, that, in turn, are common preys of cats. Yet, it is interesting to observe that similar viruses are present in human, feline and reptile hosts, hinting to a possible zoonotic exposure of humans to synanthropic animals.

Two sequences, retrieved from cat stools, clustered together with PCV3 CVs, indicating the possible association with the dietary consumption of PCV-infected pork or pork-containing cat food. In addition, sequences similar to CVs detected in birds (n=3) and in fish (n=2) may also reflect dietary habits of cats.

Surprisingly, CanineCV-like sequences (n=3) were also identified from feline serum samples. In several studies, it has been shown that the virome of dogs and cats may overlap, to some extent (Di Martino et al., 2016; Martella et al., 2002; Matthijnssens et al., 2011). For instance, canine parvovirus type 2 (CPV-2) originally could not replicate in cats but its variants CPV-2a, 2b and -2c are able to infect cats and cause disease (Truyen and Parrish, 1992). Also, feline panleukopenia virus can infect occasionally dogs (Diakoudi et al., 2022). In our study the CanineCV-like sequences were identified only in serum samples, a fact that might imply the ability of the virus to infect and replicate actively, thus sustaining viremia in cats. For all the 3 cat-associated CanineCV-like strains, the complete genome could be generated (Table 2), showing the highest nt identity (93.8 to 98.0% nt) to CanineCVs of clade 1, although clustering in two different sub-lineages (Fig. 4). Since the sampled cats were epidemiologically unrelated, we hypothesize that dog-to-cat transmission of CanineCV-like strains may occur sporadically but repeatedly in different settings due to the strict interactions between the two animal species, rather than the existence of a feline-adapted strain of CanineCV.

Furthermore, we identified in the sera of cats an additional 3 CRESS DNA viruses, i.e., a human-associated CyV TN12, and unclassified circular viruses identified in fish (Gudgeon strain Z374-like) (unpublished, accession number MN837846), and in human stools of patients with non-polio

acute flaccid paralysis (PK2111-like) (Li et al., 2010). Interestingly, both TN12 and PK2111 viruses were also detected in the fecal samples of other cats in this study. We also identified, in our study, Rep sequences related to fish-associated CRESS DNA viruses (Fig. 1 and Supplementary Table 1). This would indicate that several CRESS DNA viruses have the ability to replicate in cats and spread systemically, although the implications of these findings for cat health remain obscure.

A large group of Rep sequences (n=10), obtained exclusively from fecal samples, segregated together and resembled, in the partial Rep sequence, CVs detected in mongoose (68.3–77.2% nt identity) and were therefore defined as CAML-CVs. In this group of CAML viruses 9 sequences shared 95.4–100% nt identity to each other, whilst a spare sequence (ITA/2019/cat/185.2.3) appeared less conserved (82.4–85.9% nt). These findings are of difficult interpretation, since the fecal samples were collected from epidemiologically unrelated cases and different geographical areas. The high sequence conservation among the various strains could imply the spread of a feline-adapted virus between cats, rather than continual/repeated exposure to viruses from other unidentified hosts. We were able to generate the complete genome sequence of 3 CAML strains. Genome-wide identity among the 3 strains was 97.6–98.3% nt, whilst the highest nt identity (74.3–78.7%) in the GenBank database was to the mongoose CV strain Mon-1 (Accession MZ382570) (Gainor et al., 2021) (Table 2), therefore with a sequence identity lower than the threshold established for designation of a novel species in the *Circoviridae* family. Thus far, based on the current ICTV classification criteria a cut-off identity of 80% nt at the genome level is used to distinguish a new species (Breitbart et al., 2017). Accordingly, we propose the CAML-CV as cat-associated circovirus-1 or, more appropriately, as feline circovirus-1 and we suggest that the virus is a novel candidate species in the *Circovirus* genus.

Interestingly, in our investigation we did not identify Rep sequences related to other CRESS DNA viruses, CyV strain FD and FeSCV, reported from the stools of cats in USA (Zhang et al., 2014) and Japan (Takano et al., 2018), respectively. Whilst CanineCV has been reported repeatedly in dogs and from several countries (Beikpour et al., 2022), viruses similar to CyV strain FD have not been confirmed/reported again after their initial discovery. However, FeSCV-like viruses have been identified in the stools of 2/10 domestic cats with diarrhea in China using a specific PCR (Hao et al., 2021). All in all, it is unclear if these were just anecdotal findings due to dietary

contaminations, to occasional exposure from other animal sources, or if the viruses were able to replicate actively in the feline host. In the attempt to understand better the relevance of CAML-CVs, we therefore screened convenience collections of stool samples of cats from a distinct geographical location (collection D) using a specific qPCR. CAML-CVs were detected in 3/65 cats from Abruzzi (Italy), thus confirming that CAML-CVs are common components of feline virome and paving the way for larger structured epidemiological studies.

A major limit of our investigation was the missing history and metadata for several of the tested samples, as we mostly tested archival samples available in our laboratories. Also, this investigation was not conceived as a case/control study, so we could not infer any association with clinical signs and the viruses detected. Another limit relies on the broadly reactive primers of the pan-Rep PCR assay. The degenerated/ consensus primers were designed in 2010 on the consensus sequence from an alignment of Rep proteins from 13 representative members of the *Circoviridae* family on the basis of the sequence data available in the databases (Li et al., 2010). Therefore, the pan-Rep PCR assay could fail to detect other members of the *Circoviridae* family or could mis-detect CRESS DNA viruses of sister taxa. Metagenomic investigations using multiple strain displacement (MDA) protocol with phi29 DNA polymerase have been successfully applied for circular DNA viruses and could be used to investigate CRESS DNA viruses in different samples with an unbiased approach (Roux et al., 2016). However, consensus PCRs are more suitable for application in large-scale epidemiological investigations in terms of costs and samples processivity. The size of our study, for instance, was on average 28-fold larger, in terms of number of screened samples, than previous studies carried out in cats and large felids (Cerna et al., 2023; Payne et al., 2020; Takano et al., 2018; Zhang et al., 2014).

In conclusion, by screening nearly six hundred samples from cats, we identified a variety of CRESS DNA viruses either closely or distantly related to circular DNA viruses detected from different animal hosts, including humans. A group of CVs, termed CAML after their resemblance with mongoose CVs, was repeatedly identified in the fecal samples of cats from different geographical areas suggesting that cats could be a primary host rather than occasionally infected or exposed to this viral species and the CAML viruses were proposed as a novel species, feline CV-1 on the basis of the full-length genome sequence. Virus surveillance in domestic animals should be reinforced, in order to monitor and promptly characterize emerging and re-emerging

zoonotic viruses, and to provide a baseline of virus diversity, useful for tackling future infectious emergencies. Also, the identification of animal CRESS DNA viruses, closely related to human viruses, provides useful information about their origin and ecology.
Yet, the existing knowledge regarding other circoviruses and CRESS DNA viruses that infect carnivores remains still limited.

Virus surveillance in both wild and domestic carnivores, particularly dogs and cats that live in proximity to humans should be enhanced to establish an effective monitoring framework for virus characterization and diversity that could be crucial in addressing future infectious emergencies. Additionally, identifying animal CRESS DNA viruses and exploring their genetic similarities to human viruses offers valuable insight into their origins and ecological dynamics.

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CRediT authorship contribution statement

Violetta Iris Vasinioti: Investigation, Data curation, Writing – original draft. Francesco Pellegrini: Software, Formal analysis, Investigation, Visualization. Alessio Buonavoglia: Data curation. Paolo Capozza: Resources, Data curation, Visualization. Roberta Cardone: Investigation. Georgia Diakoudi: Investigation. Costantina Desario: Investigation. Cristiana Catella: Investigation. Teresa Vicenza: Resources. Maria Stella Lucente: Investigation. Barbara Di Martino: Validation. Michele Camero: Methodology, Writing – review & editing. Gabriella Elia: Conceptualization, Supervision. Nicola Decaro: Methodology, Supervision, Project administration. Vito Martella: Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration. Gianvito Lanave: Methodology, Software, Data curation, Writing – review & editing, Visualization.

Declaration of Competing Interest

We declare that we have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Nucleotide sequences of cat associated CV-1 strains ITA/2019/cat/ 471.3, ITA/2019/cat/471.4 and ITA/2019/cat/471.12, cat-associated Pigeon CV strain ITA/2019/cat/518.1a and cat-associated CanineCV strains ITA/2021/cat/99.95, ITA/2021/cat/230.1 and ITA/2021/cat/ 230.3 retrieved in this study used for phylogeny were deposited in GenBank under accession nos OQ357577-OQ357583, respectively. The small Rep sequence fragments (about 350–450 nt in length) are available upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online https://doi.org/10.1016/j.rvsc.2023.06.011.

Tables

Table 1: List of oligonucleotides used in this study.

Pathogen	Assay	Primers	Sequence (5' - 3')	Amplification size (bp)	Reference
CV	Pan-Rep PCR	CV-F1	GGIAYICCICAYYTICA RGG	500	Li et al., 2010
		CV-R1	AWCCAICCRTARAARTCRTC		
	nPCR	CV-F2	GGIAYICCICAYYTICARGGITT	400	
		CV-R2	TGYTGYTCRTAICCRTCCCACCA		
Canine-like CV	Inverse PCR	CaCV 273R	HCCCCIAGCAGGCTCAAAITGKCC	>1500	Beikpour et al., 2022
		CaCV 446F	WCTCGCGAGGSTTGCGAGASCT		
	nPCR	CaCV 165R	YTCCCCIACCTCCCGRCCACARAT		
		CaCV 548F	GCAAGAGCCGGTAYTGCATGGA		
Pigeon-like CV	Inverse PCR	PiCV 178R	TCATTGCTCTTCCGGCTTTCAC	>1500	This study
		PiCV 205F	ATCTACGTCAAGTATGGGCGTGGC		
	nPCR	PiCV 131R	CTCCGGTTTCCCTTCGCAGGAATG		
		PiCV 248F	YGATTGGTCAGMAGCCYCGTGACT		
Mongoose-like CV	Inverse PCR	MoCV 192R	ACGCATAAAGCCGGGTAATTTCT	>1500	This study
		MoCV 627F	ATTGAGAGTTTGTGATAGGTATCCCC		
	nPCR	MoCV 137R	TCAATGAATCCTTGCAAGTGGGT		
		MoCV 666F	TAAGGGCGCTTTTGTGGAATTTG		
	qRT-PCR	MoCV 81F	TCAGAAGGAGAATCAGTACCTGGA	187	This study
		MoCV 267R	CAAGCCAGCAACACAAACATAGTC		
		MoCV 135Pb	FAM-CTCAGATTTGAGTGATGCGGTGG-BHQ1		

Circovirus, member of the family Circoviridae; CV, member of the genus Circovirus; Rep, replicase protein; nPCR, nested PCR; qPCR, quantitative real time PCR.

Table 2: Genomic features of complete genomes of circoviruses sequenced in this study.

Species	Sample ID	Accession	Size (nt)	Putative Rep		Putative Cap		5' intergenic region (nt)	3' intergenic region (nt)	Loop motif (5'-3')	Identity to reference sequences	
				nt	aa	nt	aa				CV strain* (accession nr.)	nt identity %
Feline Circovirus 1 sp. [isolate 1]	ITA/2019/cat/471.3	OQ357577	1746	849	282	624	207	190	83	CAGTATTAC	mongoose circovirus strain Mon-1 (MZ382570)	74.3
Feline Circovirus 1 sp. [isolate 2]	ITA/2019/cat/471.4	OQ357578	1746	849	282	624	207	190	83	CAGTATTAC	mongoose circovirus strain Mon-1 (MZ382570)	78.7
Feline Circovirus 1 sp. [isolate 3]	ITA/2019/cat/471.12	OQ357579	1746	849	282	624	207	190	83	CAGTATTAC	mongoose circovirus strain Mon-1 (MZ382570)	78.0
Pigeon Circovirus [cat-associated isolate 1]	ITA/2019/cat/518.1a	OQ357580	2043	948	315	834	277	89	172	TAGTATTAC	Pigeon circovirus isolate Hebei/TS/2021 (OL901206)	92.9
Canine Circovirus [cat-associated isolate 1]	ITA/2021/cat/99.95	OQ357581	2066	912	303	816	271	135	203	TAGTATTAC	Canine circovirus strain Ha13 (KF887949)	97.3
Canine Circovirus [cat-associated isolate 2]	ITA/2021/cat/230.1	OQ357582	2063	912	303	813	270	135	203	TAGTATTAC	Canine circovirus strain Bari/411-13 (KJ530972)	99.0
Canine Circovirus [cat-associated isolate 3]	ITA/2021/cat/230.3	OQ357583	2063	912	303	813	270	135	203	TAGTATTAC	Canine circovirus strain Bari/411-13 (KJ530972)	97.8

nt, nucleotides; aa, amminoacids; CV, member of the genus *Circovirus*; * genome sequence with the highest identity on interrogation of European BioinformaticsInstitute database with FASTA (Madeira et al., 2022).

Figures

Fig 1: Results of genetic characterization of the CRESS-DNA viruses identified from cats based on partial Rep sequences.

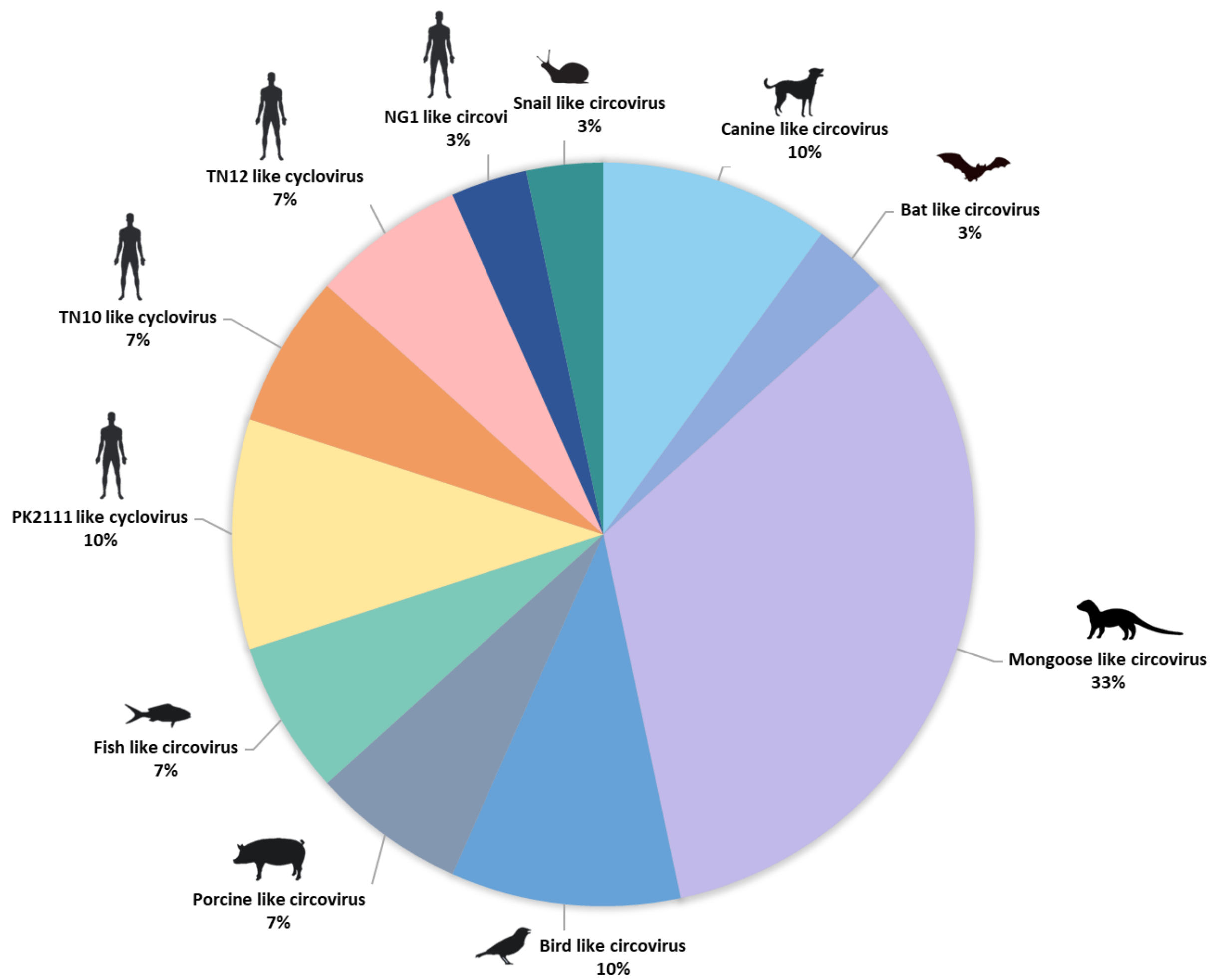


Fig 2: Genome schematic organization of the feline circoviruses sequenced in this study. The feline Circovirus-1 is shown in panel A, the cat-associated pigeon circovirus is shown in panel B and the cat-associated canine circovirus strain is shown in panel C.

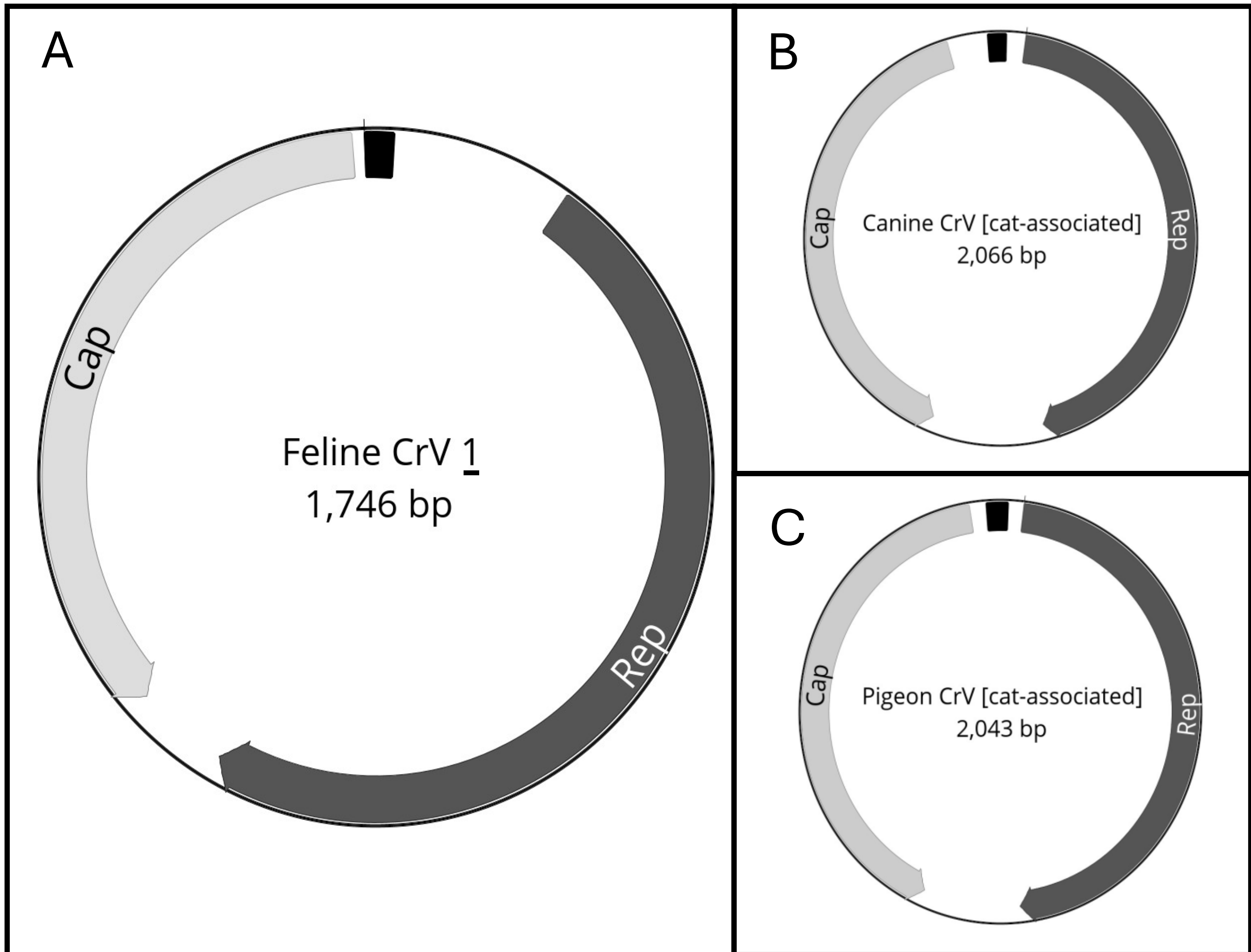


Fig 3: Full-genome-based phylogenetic tree of circoviruses identified in this study and reference strains of the Order *Cirlivirales* in the phylum *Cressdnaviricota*. The Maximum Likelihood method and General-time reversible model (six parameters) with a gamma distribution and invariable sites were used for the phylogeny. A total of 1000 bootstrap replicates were used to estimate the robustness of the individual nodes on the phylogenetic tree. Bootstrap values greater than 75% were indicated. Black arrows indicate strains detected in this study. White circles with black border indicate the CRESS DNA viruses previously identified in domestic and wild felids. Numbers of nucleotide substitutions are indicated by the scale bar.

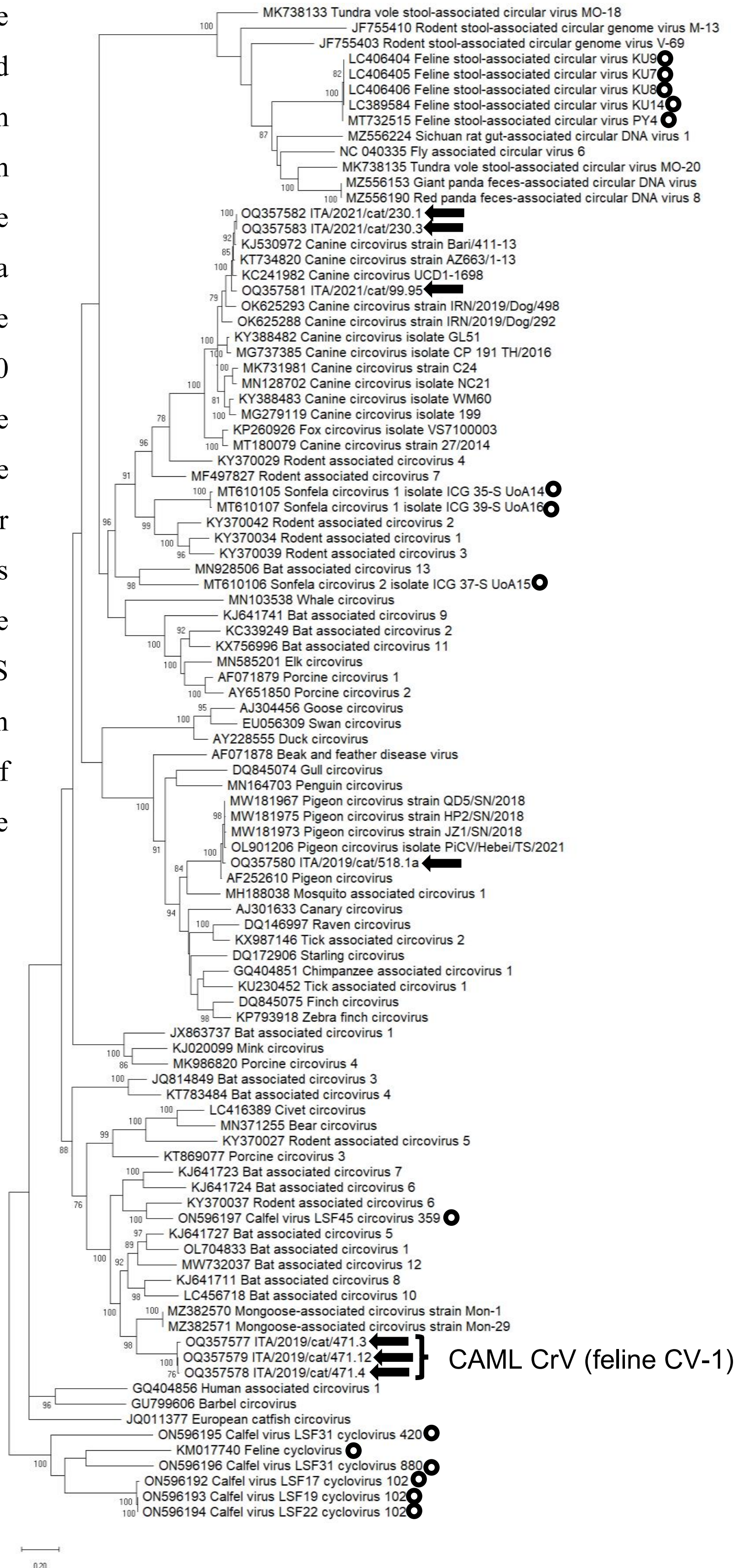
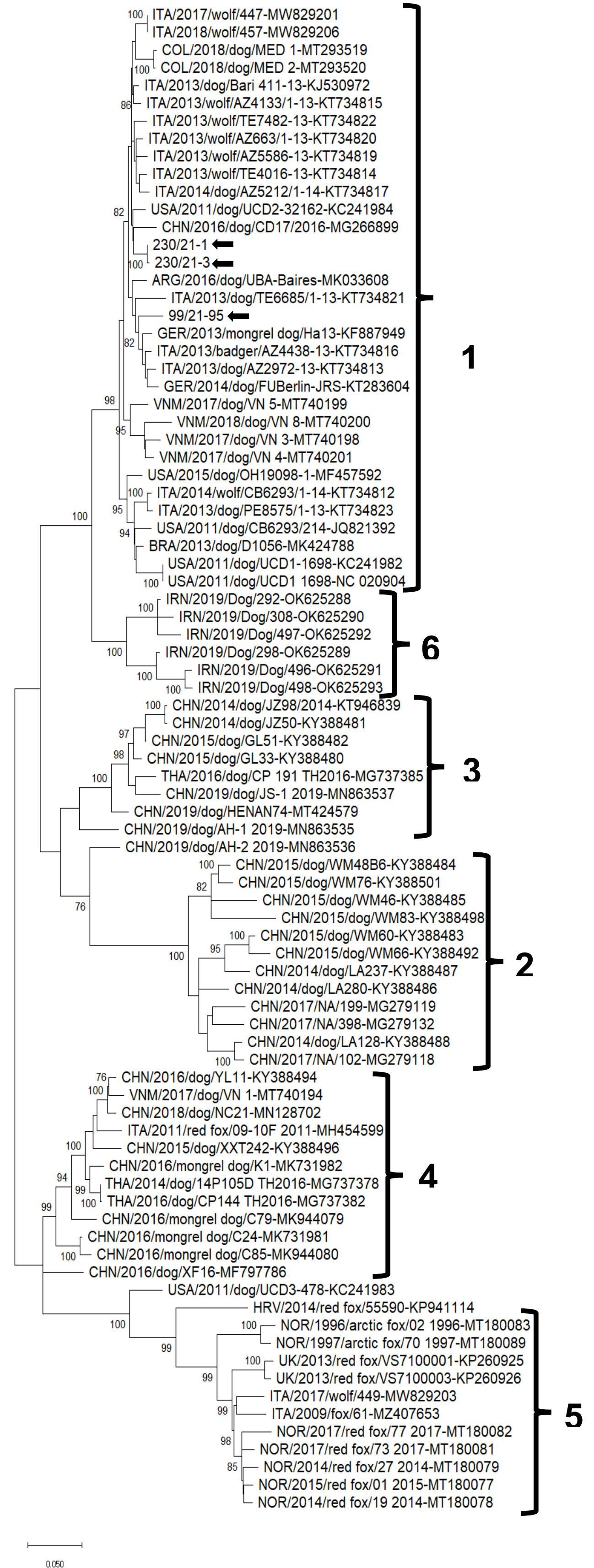


Fig 4: Full-genome-based unrooted phylogenetic tree of Canine circoviruses identified in this study and cognate strains of different genetic lineages recovered in the GenBank database. Numbers 1 to 6 indicate the phylogenetic clade (Beikpour et al., 2022; Urbani et al., 2021). The Maximum Likelihood method and General-time reversible model (six parameters) with a gamma distribution and invariable sites were used for the phylogeny. A total of 1000 bootstrap replicates were used to estimate the robustness of the individual nodes on the phylogenetic tree. Bootstrap values greater than 75% were indicated. Black arrows indicate Canine circovirus strains detected in this study. Numbers of nucleotide substitutions are indicated by the scale bar.



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Section 1.2

Detection of canine circovirus in dogs infected with canine parvovirus

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Abstract

Since the first detection of canine circovirus (CanineCV), several reports have been published over the last decade about the worldwide distribution of this emerging virus of dogs. In order to investigate the prevalence and genomic features of CanineCV in Iranian dogs, a total of 203 dog fecal samples were collected between February and November 2018 from five different geographical regions and screened by real-time PCR (qPCR). Thirteen dogs (6.4%) tested positive for CanineCV DNA, all being detected in co-infections with the highly virulent canine parvovirus (CPV). Three partial replicase nucleotide sequences of the detected CanineCV strains were obtained and compared with the reference sequences deposited in the GenBank depository database. The Iranian CanineCV sequences had a nucleotide identity of 96.4-98.2% each to other and 88.3-98.2% with other sequences available on the GenBank. Phylogenetic analysis showed that the Iranian sequences are more closely related to Turkish strains than to other strains reported from other countries. The present study provides new insights into the CanineCV molecular epidemiology and its possible role as a co-infectious pathogen.

Keywords: Dog; Canine circovirus; Genetic analysis; Canine Parvovirus; Iran.

1. Introduction

Canine circovirus (CanineCV) is a small, icosahedral, non-enveloped, and spherical virus, with a circular single-stranded DNA genome of nearly 2 kb in size (Kapoor et al., 2012) that infects domestic dogs and wild carnivores (Zaccaria et al., 2016). The genome of CanineCV is 2063 nucleotides (nt) in length, comprising two open reading frames (ORFs) on the complementary strands on the contrary direction, which encode the replicase (Rep) and capsid (Cap) proteins, respectively (Kapoor et al., 2012; Sun et al., 2019). The Rep ORF is located in a more conserved region with respect to the Cap gene (Wang et al., 2020). CanineCV belongs to the family *Circoviridae* and genus *Circovirus*. The role of this virus in causing disease is not clear yet; there is however some evidence that it is associated with gastroenteritis in dogs, solely or in association with other pathogenic viruses (Decaro et al., 2014; Li et al., 2013; Thaiwong et al., 2016). Nonetheless, it has been detected in healthy dogs as well (Beikpour et al., 2022).

Acute gastroenteritis represents the most common disease observed in kennel dogs and is caused by a plethora of causative agents including viruses, bacteria, protozoa, and parasites (Squires, 2003). Although some studies suggested an association between CanineCV and gastro- enteric disease in dogs, this virus seems to play a certain pathogenic role mainly in co-infections with other viruses, such as canine parvovirus (CPV) (Balboni et al., 2021; Dowgier et al., 2017; Niu et al., 2020; Thaiwong et al., 2016). So far, CanineCV and genetically related circo- viruses have only been reported in a few countries, including the USA (Kapoor et al., 2012; Li et al., 2013), Argentina (Kotsias et al., 2019), Brazil (Weber et al., 2018), Italy (Decaro et al., 2014), Germany (Hsu et al., 2016), China (Sun et al., 2019), Thailand (Piewbang et al., 2018), Colombia (Giraldo-Ramirez et al., 2020), Norway (Urbani et al., 2021), the UK (Bexton et al., 2015) and more recently in Iran (Beikpour et al., 2022). This study aimed to survey the circulation of CanineCV in Iranian dogs and also to characterize the detected strains at the molecular level.

2. Materials and methods

2.1. Ethics and consents

This study contains no animal experiment, and the dog owners declared their consents for sample gathering and data publication. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Tehran.

2.2. Sample collection and nucleic acid extraction

From February to November 2018 a total of 203 dog fecal samples were collected from 5 different regions of Iran. These regions represent different climates and have different neighbor countries to commute people and animals (Figure 1). While sampling, all data about the dogs were recorded, and the dogs were carefully evaluated by a veterinarian to assign a clinical score for their health status, ranging from 1 to 4 (Table 1); then the samples were immediately transferred and properly stored at <4°C.

The samples were submitted to the Department of Veterinary Medicine, University of Bari Aldo Moro, Italy, for molecular analyses. Phosphate buffered saline (PBS, pH 7.2) was employed to homogenize samples (10% w/v). The fecal homogenates were then clarified by centrifuging at 13,000 rpm for 20 min. Based on the manufacturer's instructions, DNAs were extracted from 200 µl of the supernatants utilizing the QIAamp Cador Pathogen Mini Kit (Qiagen S.p.A., Milan, Italy). In the last step, 100 µl of VXL buffer (elution buffer) was used to elute each sample. The final samples were stored at −70 °C until use.

2.3. Real-time PCR assays for detection and typing of CPV

A specific real-time PCR (qPCR) assay was carried out to detect the CPV (Decaro et al., 2005; Desario et al., 2005) genome in the extracts. Briefly, a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) was employed to run the assay using iTaq Supermix (Bio-Rad Laboratories Srl, Milan, Italy). The 25 µl mixture included 12.5 µl of iTaq Supermix, 600 nM of CPV forward and CPV reverse primers, 200 nM of CPV probe (Decaro et al., 2005) (Table 2) and 10 µl of DNA extract. The following thermal conditions were used: activations of iTaq DNA poly-

merase at 95◦C for 10 min and 40 cycles consisting of denaturation at 95◦C for 15 sec, primer annealing at 52◦C for 30 sec and extension at 60◦C for 1 min. The specimens whose amplification curves were upper than the threshold line created by the software on the basis of the background fluorescence were considered positive and subjected to additional typing assays. These are based on minor groove binder (MGB) (Decaro et al., 2006) technology and were performed in a CFX-96 thermocycler (BioRad Laboratories). Each qPCR well plate contained 25 µl of reagents including 200 nM of probes CPVa-Pb and CPVb1-Pb (type 2a/2b assay) or CPVb2-Pb and CPVc-Pb (type 2b/2c assay), 900 nM of primers CPVa/b-For and CPVa/b-Rev (type 2a/2b assay) or CPVb/c-For and CPVb/c-Rev (type 2b/2c assay) (Decaro et al., 2006) (Table 2), 12.5 µl of IQ™ Supermix (Bio-Rad Laboratories Srl), and 10 µl of template DNA. The mixtures were subjected to the following thermal conditions: activation of iTaq DNA polymerase at 95◦C for 10 min and 45 cycles of denaturation at 95◦C for 30 s and primer annealing/extension at 60◦C for 1 min.

2.4. Real-time PCR assay for detection of CanineCV

A real-time PCR (qPCR) assay was performed to detect CanineCV DNA in the nucleic acid extracts from the fecal samples, according to the method described by Li et al. (Li et al., 2013) and Decaro et al. (2014). The 25 µl of PCR mixture comprised 600 nM of each primer, 200 nM of probe (Table 3), 10 µl of template DNA, 12.5 µl of Master mix (BioRad Laboratories, USA), and nuclease-free water. Real-time PCR was conducted in a CFX-96 thermocycler (BioRad Laboratories) utilizing the following conditions: 50◦C for 2 min, followed by 95◦C for 10 min, 40 cycles of DNA denaturation at 95◦C for 15 sec, and annealing/extension at 60◦C for 1 min. Data were analyzed by the BioRad CFX Manager Software (BioRad Laboratories).

2.5. Amplification and sequencing of the partial Rep gene

The partial Rep gene (approximately 500 bp in length) of three CanineCV positive samples were amplified by PCR using primers described by Li et al. (2013) (Table 3). The PCR products with the expected sizes and stronger band intensity were purified utilizing the NEB Exo-SAP PCR

purification kit (New England Biolabs, Inc., Ipswich, MA, USA). They were then sequenced by Eurofins Genomics (Vimodrone, Italy) for the subsequent molecular analyses. The nucleotide (nt) and amino acid (aa) sequences were assembled and edited utilizing the BioEdit program (version 7.1) by ClustalW. Also, the obtained sequences were submitted to the GenBank database with the following accession numbers: MW025832, MW025833, MW025834.

2.6. Phylogenetic analysis

Phylogenetic trees were generated utilizing the MEGA package version 10.0 based on nt and aa sequences. For nt sequences, the evolutionary history was derived by employing the Maximum Likelihood method and the General Time Reversible model (Nei and Kumar, 2000). Initial trees for the heuristic search were acquired spontaneously using Neighbor-Joining and BioNJ algorithms to a matrix containing pairwise distances approximated utilizing the Maximum Composite Likelihood (MCL) method and then choosing the topology with the premier log-likelihood amount. A statistical support was provided by bootstrapping over 1,000 replicates. For aa sequences, the evolutionary history was concluded by applying the Maximum Likelihood method and JTT matrix-based model (Nei and Kumar, 2000).

2.7. Statistical data analysis

All data were collected as data set in excel software and then were analyzed using SAS 9.4 software. To investigate the effect of prevalence and disease severity levels associated with co-infection with CPV and CanineCV, the results were analyzed using the GENMOD procedure.

3. Results

3.1. Detection of CPV and CanineCV (co-infection)

Thirteen samples (6.4%) from a total of 203 screened samples were positive for CanineCV by qPCR, while 49 samples (24.13%) were found to contain the CPV DNA (Table 4). The MGB probe assays showed that all CPV positive samples contained CPV2a strains. All the CanineCV positive samples were also positive for CPV (Table 5). These specimens were from three zones and from the cities of Tabriz (zone 1), Karaj, and Tehran (zone 2), Shiraz, and Ahvaz (zone 4) (Table 5). Shiraz (Fars province) had the highest prevalence rate of CanineCV infection, while Karaj and Tehran had the lowest virus circulation. Interestingly, clinical scores were higher in dogs co-infected with CPV and CanineCV than in dogs with a single CPV infection (3.38 vs. 3.17) (Tables 1 and 4). In contrast, the correlation of diarrhea with single CanineCV infection was not statistically significant (Pr>Chisq = 0.84).

3.2. Sequence and phylogenetic analyses

In each of the 3 zones, the sample with the lowest cycle threshold (Ct) value (22.63, 21.95, 21.54 from zones 1, 2, 4 respectively) was selected for subsequent sequencing of a 461-bp fragment of the Rep gene. By sequence analysis, the Iranian CanineCV strains displayed a nt identity of 96.4-98.2 % each to other and of 88.3-98.2 to CanineCV reference strains. Iranian CanineCV strains were most closely related (97.5-98.5% of nt identity) to a canine circovirus strain reported from Turkey (GenBank accession no. MK783223). Fig. 2 shows the results of phylogenetic analysis considering nt sequences of partial Rep gene of Iranian CanineCV strains and reference strains from various geographical regions available on the GenBank. Although our three CanineCV sequences have a common ancestor with other six Iranian strains, they, interestingly, formed a single cluster with strains detected in 2018 in Turkey.

4. Discussion

In this study, we evaluated the circulation of CanineCV and CPV in dogs with gastroenteritis from Iran. CanineCV DNA was detected in 6.4% of the analyzed samples and in 26.53% of CPV positive dogs, which was significantly higher than the prevalence that has been previously reported

(Beikpour et al., 2022; Hsu et al., 2016; Li et al., 2013). In contrast to the findings of some previous reports (Anderson et al., 2017; Hsu et al., 2016), a clear association between CanineCV infection and the occurrence of diarrhea was not observed. This was consistent with the outcomes of other studies that either suggested a role of CanineCV as an opportunistic pathogen in co-infection with highly pathogenic viruses (Dowgier et al., 2017; Zaccaria et al., 2016) or detected the virus inhealthy dogs (Beikpour et al., 2022). Accordingly, a study performed in the USA (Li et al., 2013) revealed that the discrepancy in the prevalence of CanineCV between diarrheic and healthy dogs was not remarkable. Kapoor et al. detected CanineCV in blood sera of dogs with and without clinical signs (Kapoor et al., 2012). The role of CanineCV in co-infections with other viruses such as CPV, canine distemper virus, canine coronavirus, and canine influenza virus has been investigated (Anderson et al., 2017; Balboni et al., 2021; Dowgier et al., 2017; Gentil et al., 2017; Thaiwong et al., 2016; Zaccaria et al., 2016). When the analysis was restricted to CPV-positive dogs, clinical scores in dogs with co-infections were higher than those observed in animals with single CPV infection.

The present study not only found that CanineCV is spreading in Iranian dogs, but also backdates its circulation to 2018, which is one year prior to the only report currently published from Iran (Beikpour et al., 2022). Through analysis of the partial Rep nt sequences, the Iranian CanineCV strains were more closely related to strains detected in Turkey, allowing to hypothesize a possible introduction of the virus from a neighbor country. Due to the limited number of analyzed sequences, however, other studies are needed to better understand the evolutionary pattern of Iranian CanineCVs. Continuous molecular surveillance for CanineCV should be also carried out to better understand the pathogenic potential of this emerging virus of the canine enteric tract.

5. Conclusions

The detection of CanineCV in CPV-infected dogs highlights the role of this emerging virus as a co-infectious agent that may exacerbate the clinical course of other, well-known pathogens of the canine enteric tract.

Author Contributions

Conceptualization, N.D. and M.S.; methodology, R.F., F.B., S.M., L.A. N., V.M., and C.M.; writing and original draft preparation, R.F., and F.B.; review and editing, N.D. and M.S; supervision, N.D., M.S., and C.M.

Declaration of Competing Interest

The authors declare that there is no conflict of interests.

Data Availability

Data will be made available on request.

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Tables

Table 1: Clinical score used to assess the health status of the sampled dogs.

Clinical Score	Health Status
1	No sign of gastroenteritis
2	Mild clinical signs including decreased appetite and/or mild diarrhea
3	Severe clinical signs (severe diarrhea and/or vomiting)
4	Bloody diarrhea and/or death

Table 2: Oligonucleotides used for the molecular detection and typing of CPV.

	Primer name	Sequences, 5′→3′	Target gene	size (bp)	Reference
Real-time PCR	CPV-For	AAACAGGAATTAACTATACTAATATATTTA	VP	93	(Decaro et al., 2005)
	CPV -Rev	AAATTTGACCATTTGGATAAACT			
	CPV -Probe	TGGTCCTTTAACTGCATTAAATAATGTACC			
Real-time PCR	CPV2a/b-For	AGGAAGATATCCAGAAGGAGATTGGA	VP	93	(Decaro et al., 2006)
	CPV2a/b- Rev	CCAATTGGATCTGTTGGTAGCAATACA			
	CPVa-Pb	VIC—CTTCCTGTAACAAATGATA—MGB			
	CPVb1-Pb	FAM—CTTCCTGTAACAGATGATA—MGB			
	CPV2b/c-For	GAAGATATCCAGAAGGAGATTGGATTCA	VP	150	
	CPV2b/c-Rev	ATGCAGTTAAAGGACCATAAGTATTAAATATATTAGTATAGTTAATTC			
	CPVb2-Pb	FAM—CCTGTAACAGATGATAAT—MGB			
	CPVc-Pb	VIC—CCTGTAACAGAAGATAAT—MGB			

Table 3: Oligonucleotides used for the molecular detection and characterization of Iranian canine circovirus strains.

Test	Primer name	Sequences, 5′→3′	Target gene	Amplicon size (bp)	Reference
Real-time PCR	CaCV-Forward	CTTGCGAGAGCTGCTCCTTATAT	Replicase	66	(Li et al., 2013)
	CaCV-Reverse	CTCCACTTCCGTCTTCCAGTTC			
	CaCV-Probe	TCCGGAGATGACCACGCCCC			
PCR	For1	ATGGCTCAAGCTCAGGTTG	Replicase	533	(Niu et al., 2020)
	Rev 533	CTTGCGCGAGCTGCTCCTTA			

Table 4: Signalment and clinical scores of CPV positive dogs.

#	Breed	Age (months)	Sex	Clinical score*	City	Province	Region	CanineCV Co-infection
1	Boxer	4	M	4	Kermanshah	Kermanshah	1	No
2	German shepherd	6	M	4	Kermanshah	Kermanshah	1	No
3	Iranian Mastiff	4	F	2	Urmia	West Azerbaijan	1	No
4	German shepherd	6	M	2	Urmia	West Azerbaijan	1	No
5	**Husky**	**11**	**M**	**3**	**Tabriz**	**East Azerbaijan**	**1**	**Yes**
6	Husky	3	M	3	Tabriz	East Azerbaijan	1	No
7	**Kangal**	**5**	**F**	**3**	**Tabriz**	**East Azerbaijan**	**1**	**Yes**
8	Terrier	6	F	4	Tabriz	East Azerbaijan	1	No
9	Husky	5	M	4	Zanjan	Zanjan	1	No
10	Mixed	3	M	3	Isfahan	Isfahan	2	No
11	Mixed	3	F	2	Isfahan	Isfahan	2	No
12	Terrier	3	F	3	Karaj	Alborz	2	No
13	**Iranian Mastiff**	**7**	**M**	**3**	**Karaj**	**Alborz**	**2**	**Yes**
14	Terrier	6	M	2	Karaj	Alborz	2	No
15	**Terrier**	**3**	**F**	**3**	**Karaj**	**Alborz**	**2**	**Yes**
16	Husky	12	F	3	Karaj	Alborz	2	No
17	**Mixed**	**3**	**F**	**3**	**Karaj**	**Alborz**	**2**	**Yes**
18	Terrier	10	F	3	Karaj	Alborz	2	No
19	Mixed	9	M	3	Qom	Qom	2	No
20	Doberman	7	F	3	Tehran	Tehran	2	No
21	**Mixed**	**11**	**F**	**4**	**Tehran**	**Tehran**	**2**	**Yes**
22	**German shepherd**	**6**	**F**	**4**	**Tehran**	**Tehran**	**2**	**Yes**
23	Spitz	8	M	4	Tehran	Tehran	2	No
24	Husky	6	M	3	Tehran	Tehran	2	No
25	**Pomeranian**	**4**	**F**	**4**	**Tehran**	**Tehran**	**2**	**Yes**
26	Doberman	18	M	3	Gorgan	Golestan	3	No
27	Terrier	2	M	3	Gorgan	Golestan	3	No
28	German shepherd	6	F	4	Mashhad	Razavi Khorasan	3	No
29	German shepherd	4	M	2	Mashhad	Razavi Khorasan	3	No
30	German shepherd	9	F	3	Mashhad	Razavi Khorasan	3	No
31	Iranian Mastiff	4	M	3	Sabzevar	Razavi Khorasan	3	No
32	Iranian Mastiff	4	M	4	Sabzevar	Razavi Khorasan	3	No
33	Doberman	5	F	4	Ahvaz	Khuzestan	4	No
34	German shepherd	9	F	3	Ahvaz	Khuzestan	4	No
35	**Terrier**	**11**	**M**	**3**	**Ahvaz**	**Khuzestan**	**4**	**Yes**
36	Terrier	3	F	2	Ahvaz	Khuzestan	4	No
37	**German shepherd**	**2**	**F**	**3**	**Ahvaz**	**Khuzestan**	**4**	**Yes**
38	**Iranian Mastiff**	**5**	**F**	**3**	**Ahvaz**	**Khuzestan**	**4**	**Yes**
39	Boxer	7	F	4	Ahvaz	Khuzestan	4	No
40	**German shepherd**	**9**	**M**	**3**	**Shiraz**	**Fars**	**4**	**Yes**
41	**Doberman**	**2**	**M**	**3**	**Shiraz**	**Fars**	**4**	**Yes**
42	German shepherd	5	F	4	Shiraz	Fars	4	No
43	German shepherd	3	M	3	Kerman	Kerman	5	No
44	German shepherd	2	M	2	Kerman	Kerman	5	No
45	Mixed	3	M	3	Kerman	Kerman	5	No
46	Great Dean	3	M	3	Kerman	Kerman	5	No
47	German shepherd	11	F	4	Zahedan	Sistan and Baluchestan	5	No
48	Boxer	3	F	3	Zahedan	Sistan and Baluchestan	5	No
49	Spitz	6	F	4	Zahedan	Sistan and Baluchestan	5	No

Table 5: Rates of co-infection of CanineCV and CPV in Iranian dogs

Province	City	No. of dogs infected with CPV	No. of dogs co-infected with CanineCV	Rate of co-infection (%)
Kermanshah	Kermanshah	2	0	0
West Azerbaijan	Urmia	2	0	0
East Azerbaijan	Tabriz	4	2	50
Zanjan	Zanjan	1	0	0
Isfahan	Isfahan	2	0	0
Alborz	Karaj	7	3	43
Tehran	Tehran	7	3	43
Golestan	Gorgan	2	0	0
Razavi Khorasan	Mashhad	3	0	0
Razavi Khorasan	Sabzevar	2	0	0
Fars	Shiraz	3	2	66
Khuzestan	Ahvaz	7	3	43
Kerman	Kerman	4	0	0
Sistan and Baluchestan	Zahedan	3	0	0
Total	-	49	13	26

Fig 1. The Map illustrates the number of CPV (digits) and CanineCV (*) fecal samples collected from different cities of the 5 Iranian regions.

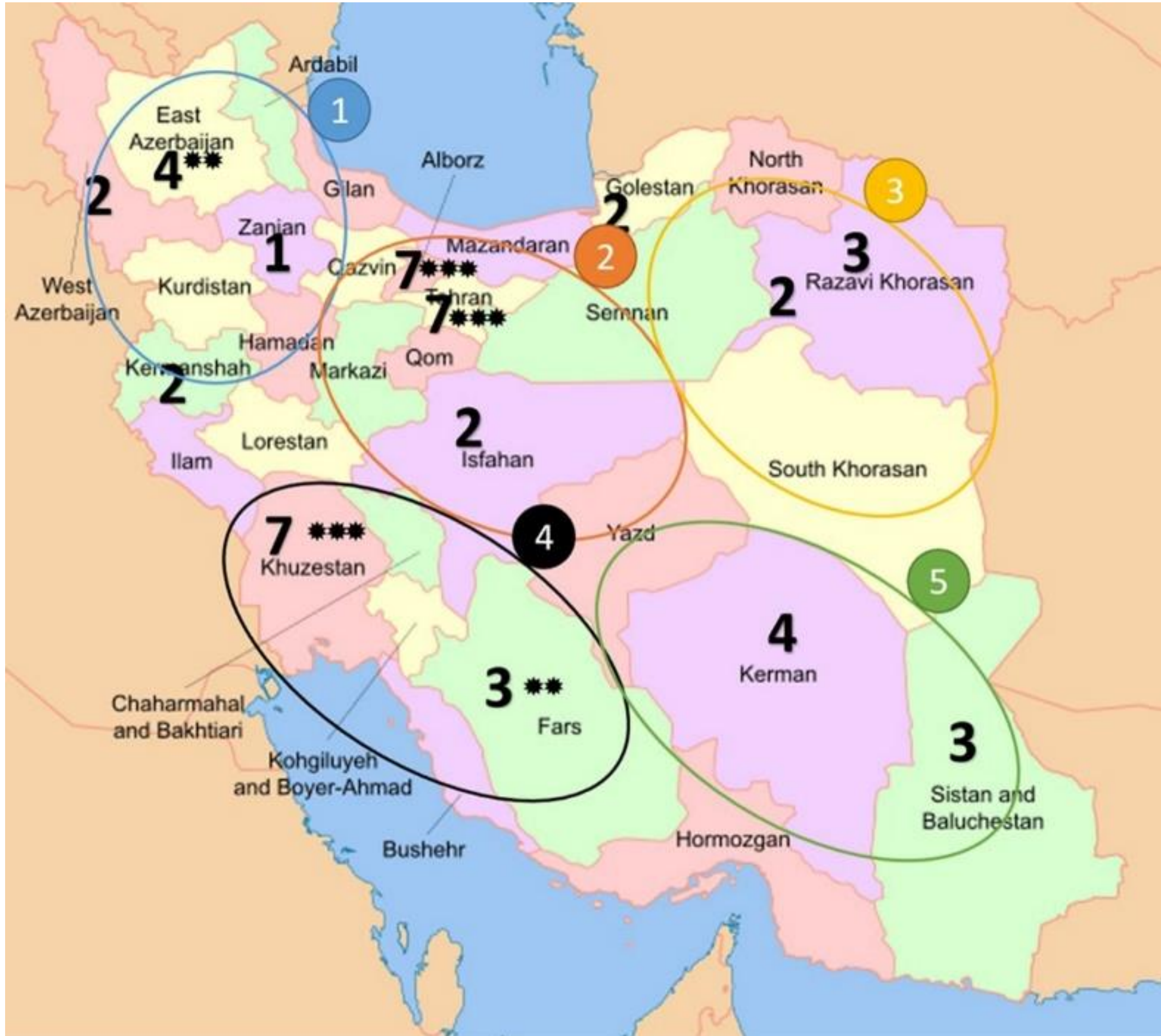
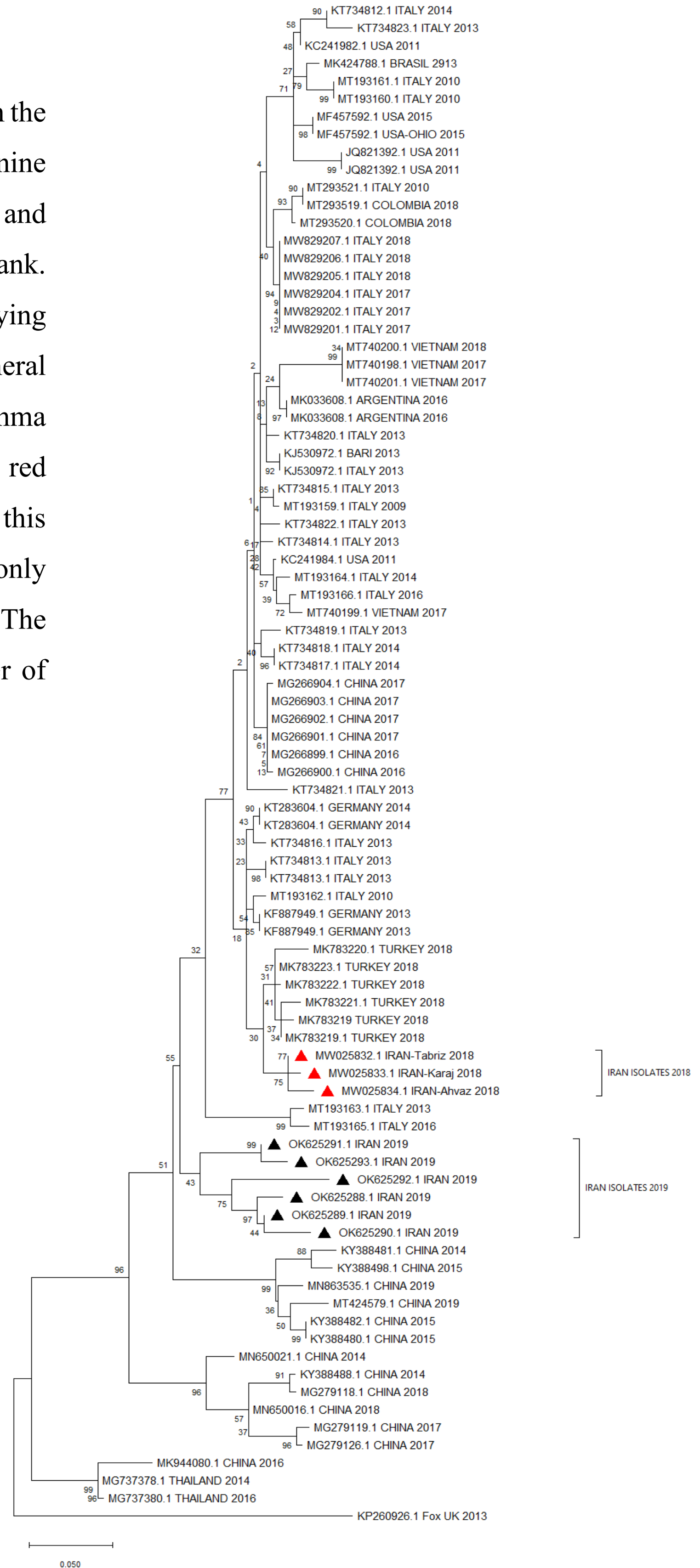


Fig 2: Unrooted phylogenetic tree built on the Rep gene nucleotide sequences of canine circovirus strains from this study and reference strains retrieved on the GenBank. This phylogeny was constructed employing the maximum-likelihood method and general time-reversible (GTR) model with a gamma distribution and invariable sites. The red arrows indicate the strains detected in this study while the black arrows show only CanineCV sequences reported from Iran. The scale bars indicate the estimated number of nucleotide substitutions.



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Chapter 2

CRESS DNA viruses in wild carnivores

Over the past decades, accelerating changes associated with an expansion of the global population have led to the emergence of numerous zoonoses (Karesh et al., 2012), the majority of which derive from wildlife (Jones et al., 2008; Olival et al., 2017). Several wildlife species are reservoirs of pathogens that threaten domestic animal and human health through spillover events (Plowright et al., 2017). Likewise, reverse spillover (spill back) events from domestic animals to sympatric wild animal populations represent a major threat to the conservation of wildlife's biodiversity (Daszak et al., 2000). As awareness of the impacts of emerging diseases on both humans and animals grows, along with the recognition of the significant role of wild animals as hosts and/or reservoirs of zoonotic agents, disease surveillance in wildlife becomes an essential necessity (Kelly et al., 2021). Prevention and mitigation of zoonotic outbreaks requires regular wildlife pathogen surveillance, and analysis of the risks at the human-wildlife interface (Tran and Xie, 2024).

CRESS DNA viruses have been described in wild carnivores including wild canids, felids, mongooses and mustelids, despite their exact pathogenic role could not been deciphered. CanineCV has been detected in other host species over the domestic dogs including wolves (Balboni et al., 2021; Zaccaria et al., 2016), foxes (Bexton et al., 2015; De Arcangeli et al., 2020; Franzo et al., 2021; Urbani et al., 2021), badgers (Zaccaria et al., 2016) and jackals (De Villiers et al., 2023). Interestingly, the detection of CanineCV in foxes in UK has been associated with meningoencephalitis (Bexton et al., 2015). CanineCV in wolves has consistently been found in co-infection with at least one additional canine pathogen, such as CPV-2, CDV, or CAdV (Balboni et al., 2021; Zaccaria et al., 2016). Moreover, a novel CrV member, Mink Circovirus (MiCV), was first discovered in 2013 in diarrheal minks in China (Lian et al., 2014) and has been associated with mink enteric disease (Ge et al., 2018) whilst recent research has identified its presence in foxes and racoon dogs as well (Liu et al., 2023; Yang et al., 2018). In 2021, the circulation of circoviruses in fecal samples of healthy mongooses has been reported (Gainor et al., 2021).

Metagenomics studies revealed the presence of CRESS DNA viruses also in wild felids. In Mexico

two novel CrVs species were identified in scat samples of free-roaming bobcats (*Lynx rufus*) (Payne et al., 2020) . One species was more closely related to rodent circoviruses while the other to bat circoviruses sharing 70% and 63% highest genome-wide pairwise identity, respectively. Four new members of the *Circoviridae* family, a novel CrV and three novel CyVs were detected in stool samples of bobcats in California (Cerna et al., 2023). The novel Californian CrV was distantly related to the Mexican CrVs (< 61.7% genome-wide pairwise identity) whilst one of the novel CyVs formed a separate branch and the rest two clustered with other ant and bat associated CyVs. For both studies, it is unknown whether the identified viruses infect bobcats, their prey, or their gut parasites. Moreover, in 2019 three highly diverse smacoviruses were recovered form feces of two North American bobcats (*Lynx rufus*) and an African lion (*Panthera leo*) (Kraberger et al., 2019). However, recent evidence suggests that smacoviruses may infect feces-dwelling archarea (Díez-Villaseñor and Rodriguez-Valera, 2019) challenges the plausibility that felids serve as hosts for smacoviruses.

Surveillance activity on emerging viruses such as circoviruses is pivotal to further understand the epidemiology and the potential impact on the wild species. The approximation between wild and domestic animals favors the transmission of pathogens, especially between phylogenetically close species, such as carnivores within the Canidae and Felidae families (Martins et al., 2024). Therefore, we conducted investigations on the presence of circoviruses in European wild carnivores' populations including wolves, foxes and lynxes. In section 2.1 we describe an epidemiological survey for selected viruses of dogs, including canine circoviruses in free-ranging wild carnivores in Italy. In section 2.2 we screen spleen samples from Iberian lynxes, and we report the detection of a novel lynx associated circovirus.

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Section 2.1

Detection and Genetic Characterization of Canine Adenoviruses, Circoviruses, and Novel Cycloviruses from Wild Carnivores in Italy

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Abstract

Wild carnivores are known to play a role in the epidemiology of several canine viruses, including canine adenoviruses types 1 (CAdV-1) and 2 (CAdV-2), canine circovirus (CanineCV) and canine distemper virus (CDV). In the present study, we report an epidemiological survey for these viruses in free ranging carnivores from Italy. A total of 262 wild carnivores, including red foxes (*Vulpes vulpes*), wolves (*Canis lupus*) and Eurasian badgers (*Meles meles*) were sampled. Viral nucleic acid was extracted and screened by real-time PCR assays (qPCR) for the presence of CAdVs and CanineCV DNA, as well as for CDV RNA. CAdV-1 DNA was detected only in red foxes (4/232, 1.7%) whilst the wolves (0/8, 0%) and Eurasian badgers (0/22, 0%) tested negative. CanineCV DNA was detected in 4 (18%) Eurasian badgers, 4 (50%) wolves and 0 (0%) red foxes. None of the animals tested positive for CDV or CAdV-2. By sequence and phylogenetic analyses, CAdV-1 and CanineCV sequences from wild carnivores were closely related to reference sequences from domestic dogs and wild carnivores. Surprisingly, two sequences from wolf intestines were identified as cycloviruses with one sequence (145.20-5432) displaying 68.6% nucleotide identity to a cyclovirus detected in a domestic cat, while the other (145.201329) was more closely related (79.4% nucleotide identity) to a cyclovirus sequence from bats. A continuous surveillance in wild carnivores should be carried out in order to monitor the circulation in wildlife of viruses pathogenic for domestic carnivores and endangered wild species.

Keywords: wild carnivores, canine adenovirus type 1, canine circovirus, cycloviruses, molecular survey

1. Introduction

Several pathogenic viruses of domestic dogs (*Canis lupus familiaris*) possess the ability to infect different species of wild carnivores. This multi-host capacity is frequently observed with canine parvovirus (CPV), canine adenoviruses (CAdVs) and canine circovirus (CanineCV) (1–6). CAdVs are non-enveloped, icosahedral members of the genus *Mastadenovirus* in the family *Adenoviridae*, with a double-stranded DNA genome about 32 kb long. Two distinct types of the virus are known, canine adenovirus type 1 (CAdV-1), and canine adenovirus type 2 (CAdV-2), causing infectious canine hepatitis (ICH) and kennel cough in dogs, respectively (7). CAdV-1 was described in silver foxes (*Vulpes vulpes*) displaying the so-called "epizootic fox encephalitis" in 1930 (8) and has been subsequently detected in diseased and apparently healthy wild canids (1, 9–12). ICH has been extensively controlled by vaccination with occasional reports of re-emergence in domestic dog populations (13–17), as a consequence of importation of infected pups from endemic areas or possible contact with wild canids, due to the propensity of the virus to establish persistent infections in these animals (18).

Unlike CAdVs, CanineCV is a relatively newly discovered virus belonging to the family *Circoviridae* (19). There are two genera under this family, namely *Circovirus* and *Cyclovirus*, with CanineCV belonging to the former. They are DNA viruses with a circular, ambisense, single-stranded genome enclosed in a nonenveloped icosahedral capsid (20). CanineCV has had conflicting reports regarding its role in severe disease of domestic dogs. Notwithstanding its poorly understood pathogenesis, the virus has been associated with systemic or enteric disease in dogs (21–27). CanineCV has been reported in wild carnivores, especially foxes and wolves (6, 28, 29). Similar to what is reported in dogs (30), CanineCV has been suggested to exacerbate the clinical course of other infections in foxes, wolves and badgers (6). In contrast, cycloviruses have been detected in several animal species including birds, chimpanzees, as well as humans (31) but they have not been previously associated with infection of foxes, wolves, and badgers.

CDV (genus *Morbillivirus*, family *Paramyxoviridae*) is nonsegmented, negative-stranded RNA genome virus, which is responsible for severe systemic disease in dogs, characterized by a variety of clinical signs, including fever, respiratory and enteric signs, and neurological disorders (32). CDV can also infect a wide range of animals including wild canids and felids (33, 34). This virus

has been previously and frequently detected in wild carnivores across Italy and poses a threat to animal conservation and re-emergence in domestic dog populations (35, 36).

Interspecies transmission of viruses between wildlife and domestic dogs occurs, especially at the human-animal interface, impacting both virus evolution and epidemiology (28), and sometimes resulting in epizootics. In the present study, we describe an epidemiological survey for selected viruses of dogs, including canine adenoviruses, circoviruses, and canine distemper virus, in wild carnivores in Italy.

2. Materials and methods

2.1. Sample Collection and Nucleic Acid Extraction

A total of 262 samples consisting of spleens (n = 255) and intestines (n = 7) were collected from wild animals found dead, including foxes (*Vulpes vulpes*) (n = 232), wolves (*Canis lupus*) (n = 8), and Eurasian badgers (*Meles meles*) (n = 22). Sampling was carried out in different regions of central and southern Italy including Tuscany, Abruzzi, Lazio, Molise, Campania, Calabria, Apulia and Basilicata from 2014 to 2020 (4).

Viral nucleic acid was extracted from 200 µl of the supernatants of samples homogenized in minimum essential medium (10% w/v), using the QIAmp Cador Pathogen Mini Kit (Qiagen S.p.A., Milan, Italy), following the manufacturer's protocol.

2.2. Screening for Canine Viruses

Screening of nucleic acid extracts was carried out using realtime reverse transcriptase-PCR (RT-qPCR) assays. CAdVs were detected using a CAdV-specific primer pair and virus-specific probes to discriminate between CAdV-1 and CAdV-2 (37) (Supplementary Table 1), whilst CanineCV were searched for with specific primers and probe targeting the Rep encoding gene of CanineCV (26). These TaqMan-based RT-PCR assays were carried out using iTaqTM Universal Probes Supermix (Bio-Rad Laboratories Srl, Milan, Italy) in a final volume of 50 µl consisting of 25 µl of

Supermix, 600nM of forward and reverse primers, 400nM of probe and 20 µl of nucleic acid extracts. The thermal protocol for CAdV and CanineCV was as follows: activation of iTaq DNA polymerase at 95◦C for 10 min and 45 cycles of denaturation at 95◦C for 15 s and annealing/extension at 60◦C for 1 min.

Extracts were reverse transcribed prior to testing for CDV following an earlier described qPCR protocol (38). In brief, the reaction volume of 20 µl for synthesis of c-DNA consisted of PCR buffer 1×, MgCl2 5 mM, 1mM of deoxynucleotide, RNase Inhibitor 1U, reverse transcriptase 2.5U, random hexamers 2.5 U. Reverse transcription was run under the following cycling conditions: 42◦C for 30 min, followed by a denaturation step at 99◦C for 5 min. Real-time PCR mix was prepared with the same reagents and concentrations of CAdV and CanineCV. The thermal protocol consisted of activation of iTaq DNA polymerase at 95◦C for 10 min and 45 cycles of denaturation at 95◦C for 15 s, primer annealing at 48◦C for 1 min and extension at 60◦C for 1 min.

2.3. PCR Amplification

For all CanineCV-positive samples, amplification of partial rolling circle replication initiator protein (Rep) gene (400 bp) was performed by a nested PCR, using consensus primers CV-F1/CV-R1 and CV-F2/CV-R2 (31) (Supplementary Table 1). Overlapping segments of CAdV hexon gene were also amplified from positive samples using primer pairs CAV-F/HEX-R and HEX-F/CAV-R (Supplementary Table 1), yielding overlapping fragments of 1,882 bp and 1,009 bp, respectively. The PCR assay, performed in a final volume of 50 µl, contained 5 µl of DNA extract, TaKaRa LA TaqTM Kit (Takara Bio Europe S.A.S. Saint-Germain-en-Laye, France) consisting of 24.5 µl of PCR grade water, 5 µl of 10x buffer, 5 µl of MgCl2 (25 mM), 900 nmol/L of forward and reverse primers, 8 µl of deoxynucleotides. Cycling conditions included an initial denaturation at 94◦C for 2 min, 35 cycles consisting of 30 s of denaturation, 30 s of annealing and 3 min of extension at 94, 58, and 68◦C, respectively, followed by a final extension at 72◦C for 10 min.

2.4. Sequence and Phylogenetic Analyses

All PCR products were purified using Qiaquick PCR purification Kit (Qiagen GmbH, Hilden, Germany). PCR products were sequenced in both directions using classical dideoxy Sanger sequencing with BigDye 3.1 Ready Reaction Mix (Applied Biosystems), following the manufacturer's instructions. Sequence reads were assembled using Geneious Prime R 2021.2.2 (https://www.geneious.com). Analyses of the sequences with web-based tools BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) and FASTA (https://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) with default values were used to find homologous hits. The obtained CAdV sequences were aligned with cognate CAdV and bat adenovirus (used as outgroup) sequences retrieved from the GenBank database by MAFFT algorithm (39). The obtained CanineCV and CV sequences were aligned with cognate circovirus and cyclovirus sequences, respectively, retrieved from the GenBank database by MAFFT algorithm (39).

Phylogenetic analyses were performed with Bayesian inference by MrBayes software using 4 chains run for >1 million generations (40, 41) and Model Test software (http:// evomics.org/resources/software/molecular-evolutionsoftware/modeltest/) was used to identify the most appropriate model of evolution for the entire dataset and for each gene individually. The identified program settings for all partitions, under the Akaike Information Criteria, included 2-character states (Hasegawa–Kishino–Yano model) and a proportion of invariable sites.

2.5. GenBank Sequence Submission

The obtained sequences were deposited in the GenBank database under accession numbers OL323110, OL323111, OL323112 and OL323113 for adenoviruses, OL364172, OL364173, OL364174, and OL638989 for circoviruses, and OL638987 and OL638988 for cycloviruses.

3. Results

The results of the molecular screening for the selected canine viruses are reported in Table 1. CAdV-1 DNA was detected in red foxes (4/232, 1.7%), while no wolves (0/8, 0%) nor Eurasian badgers (0/22, 0%) tested positive. Out of the 4 CAdV-1-positive samples, 2 whole (OL323111

and OL323112) and two partial (OL323110 and OL323113) hexon gene sequences were generated and analyzed. Blast analysis showed 99.89 to 100% identity between the sequences from this study and 99.21–100% identity with CAdV-1 reference sequences obtained from domestic dogs in Italy and Japan. Phylogenetic analyses of CAdVs from this study and other reference sequences from GenBank revealed a distinct distribution of the analyzed sequences into two clades, consisting of CAdV-1 and CAdV-2, respectively (Figure 1). Hexon gene sequences were nearly identical to those previously generated from other wild animals and domestic dogs with only three synonymous substitutions observed in viral sequence 51.20-28 (OL323111) at position 1356 (C to A)

and sequence 51.20-93 (OL323112), displaying changes from C to T at positions 372 and 2,241 compared to reference sequence 574-2013-RS (KP840549) obtained from a dog in Italy in 2013.

CanineCV DNA was detected in a total of 8 animals, including 4/22 (18%) Eurasian badgers and 4/8 (50%) wolves, while no red foxes (0/232, 0%) tested positive. All positive wolf and Eurasian badger samples were obtained from intestines and spleens, respectively. Successful amplification and sequencing of partial Rep gene was obtained from 6/8 qPCR positive samples. Blast analysis revealed that 4 sequences (2 from Eurasian badgers and 2 from wolves) displayed a 92.8–98.5% nucleotide (nt) identity to CanineCVs detected in domestic dogs. By phylogeny, the wolf and badger sequences were found to cluster with CanineCV sequences obtained from domestic dogs and one Eurasian badger (Figure 2). Surprisingly, one sequence (145.20-5432) displayed 68.6% nt identity to a cyclovirus sequence from a domestic cat (GenBank accession no. KM017740), whilst the other (145.20-1329) was 79.4% identical to a cyclovirus sequence from bats (HQ738637); both cyclovirus sequences were identified from wolves. In the phylogenetic tree (Figure 3), the 4 CanineCV sequences from wild carnivores clustered with reference CanineCV sequences obtained fromdomestic dogs and wild carnivores, while both cyclovirus sequences clustered with a feline cyclovirus (KM017740) of possible bat origin.

All animals tested negative for CAdV-2 and CDV.

4. Discussion

Over several decades, various efforts have been targeted toward the eradication of highly

pathogenic viruses of domestic dogs, including the extensive use of vaccines against CPV, CDV, CAdV-
1 (42). However, these viruses have persisted in dog populations as a consequence of immunization failures (43), introduction of infected animals from endemic areas (14, 32) or virus circulation in wildlife (6). Carnivores at the human-wildlife interface are known to influence the epidemiology of canine viruses (3, 44). The present study shows that CAdV-1 and CanineCV are circulating in wild carnivore populations in Italy, whereas no sample tested positive for CAdV-2. There is scanty molecular data regarding CAdV-2 in wild carnivores. The preferred sample type for CAdV-2 diagnosis is represented by respiratory specimens, owed to the virus tropism (17, 45), although the virus has been recovered from digestive tract samples, including intestines, rectal swabs and tongue specimens (28, 46). In addition, CAdV-2 has likewise been detected in internal organs of infected animals, including the spleen of 5 wolves, thus accounting for a hematogenous dissemination of the virus (3, 47). In this study, the real circulation of CAdV-2 in the wild carnivore population could not be assessed since respiratory samples were not tested and only few wolves and Eurasian badgers were sampled. The virus has been reported in wolves with frequencies of infection ranging from 1.38 to 8.7% (3, 28, 48). A recent report from Italy detected CAdV-2 in tongue samples of two wolves, but at very low titers so that sequencing was not possible (28). There is only one report of CAdV-2 infection in a fox (1), although the low viral titers observed could not rule out a passive transit in the gut of contaminated material.

Of all animal species that were sampled, only foxes tested positive for CAdV-1. Foxes are known to be susceptible to CAdV-1 infection and harbor the virus for a long time, thus acting as potential reservoirs (1, 18). A molecular survey in European foxes reported an 18.8% detection rate of CAdV-1 (36), although a much larger proportion of the animals had antibodies, thus suggesting previous exposure (18, 48). The present study accounts for a much lower detection rate for CAdV-1 in Italian red fox populations. This finding could be related to sampling bias. It is possible that animals which were sick from the virus died in their dens undiscovered, while most of the animals which come into human landscapes are apparently healthy.

Conversely, circoviruses were detected in wolves and badgers but not in red foxes, which is in line with previous reports (6), suggesting a low susceptibility to circovirus infection of these animals with respect to other wild carnivores. Wolves are closely genetically related to domestic dogs, so

it is not surprising that they exhibit the same virus susceptibility. On the other hand, badgers are known to host several viruses of dogs, including CPV, FPV and CDV (4, 18, 36). Detection of CanineCV in the spleens suggests that the Eurasian badgers were actively infected with the virus. Two wolves were simultaneously infected with CanineCV and cycloviruses since their intestines tested positive for CanineCV by qPCR (which does not recognize cyclovirus sequences) and for cycloviruses by PCR amplification and sequencing using a protocol able to amplify the Rep gene of all members of the family Circoviridae. The most likely explanation is that cycloviruses were present at high titers in the samples and were selected during PCR amplification and sequencing to the detriment of CanineCVs. Although there is previous evidence for CanineCV infecting wolves, the two cyclovirus sequences which were detected in the present study may suggest that these wild canids may harbor other types of circoviruses. Two CanineCV-positive wolves (145.20-1274 and 145.20-4615) from this study were coinfected with CPV-2b (4) (Table 1), which has been previously reported (6). As far as we know, this is the first report of cycloviruses in wolves. It is, however, difficult to conclude that the detected viruses were actively infecting the wolves, since residual ingesta contaminated by cyclovirus DNA derived from a prey could remain on the mucosal lining of the sampled intestine and extracted along with the tissue. There is need to further investigate wolves as potential hosts of cycloviruses by analyzing other tissues not potentially contaminated by ingesta or by performing techniques, such as in-situ hybridization and immunohistochemistry, able to detect the viral nucleic acid or antigens in the intestinal epithelium. The low relatedness of the cyclovirus sequences from this study with reference sequences indicates that these are likely prototypes of novel virus species.

A continuous surveillance in wild carnivores should be carried out in order to monitor the circulation in wildlife of viruses pathogenic for domestic carnivores and endangered wild species.

Data Availability Statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, OL323110-OL323113; OL364172 OL364173 OL364174-OL638989; OL638987-OL638988.

Ethics Statement

Ethical review and approval was not required for the animal study because this study was conducted on carcasses of animals found dead and submitted to routine necropsy procedures for diagnostic purposes.

Author Contributions

LN: laboratory analyses, sequence analyses, and manuscript writing. GL: sequence analyses and manuscript writing. VV, CD, FP, and FB: laboratory analyses. CM, MC, SB, and GS: sample collection and processing. AC, GE, AP, VM, and CB: manuscript revision. ND: general supervision and manuscript revision. All authors contributed to the article and approved the submitted version.

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Supplementary Material

The Supplementary Material for this article can be found online at:

https://www.frontiersin.org/articles/10.3389/fvets. 2022.851987/full#supplementary-material

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Tables

Table 1: Sample distribution according to Italian regions, year of collection and wild carnivore species.

Animal species	Year	Region	Prot. No.	Tissue	CAdV-1	CanineCV	CV	*Carnivore protoparvovirus 1*[a]
Eurasian badger	2020	Campania	136.20-6	spleen	neg	pos	neg	neg
Eurasian Badger	2020	Campania	136.20-8	spleen	neg	pos	neg	neg
Eurasian badger	2020	Campania	136.20-11	spleen	neg	pos	neg	neg
Eurasian Badger	2020	Campania	136.20-12	spleen	neg	pos	neg	neg
Wolf	2020	Abruzzi	145.20-1274	intestine	neg	pos	neg	CPV-2b
Wolf	2020	Abruzzi	145.20-1329	intestine	neg	pos	pos	neg
Wolf	2020	Abruzzi	145.20-4615	intestine	neg	pos	neg	CPV-2b
Wolf	2020	Abruzzi	145.20-5432	intestine	neg	pos	pos	neg
Fox	2014	Campania	51.20-28	spleen	pos	neg	neg	neg
Fox	2014	Campania	51.20-93	spleen	pos	neg	neg	neg
Fox	2014	Campania	51.20-118	spleen	pos	neg	neg	neg
Fox	2017	Calabria	51.20-213	spleen	pos	neg	neg	neg

a Ndiana et al. (4). CAdV-1, canine adenovirus type 1; CanineCV, canine circovirus; CV, cyclovirus; pos, positive; neg, negative

Figures

Fig. 1: Phylogenetic tree based on alignment of the partial (848 nt) hexon gene of canine adenovirus (CAdV) sequences identified in this study and retrieved from the GenBank database. Bat mastadnovirus (GenBank accession no. MT815936) was used as outgroup. Posterior output of the tree was derived from Bayesian inference using 4 chains run for >1 million generations, 2-character states (Hasegawa–Kishino–Yano) model, a proportion of invariable sites and a subsampling frequency of 1,000. Posterior probability values >95 are indicated on the tree nodes. The black arrows indicate the sequences identified in this study. Scale bar indicates nucleotide substitutions per site

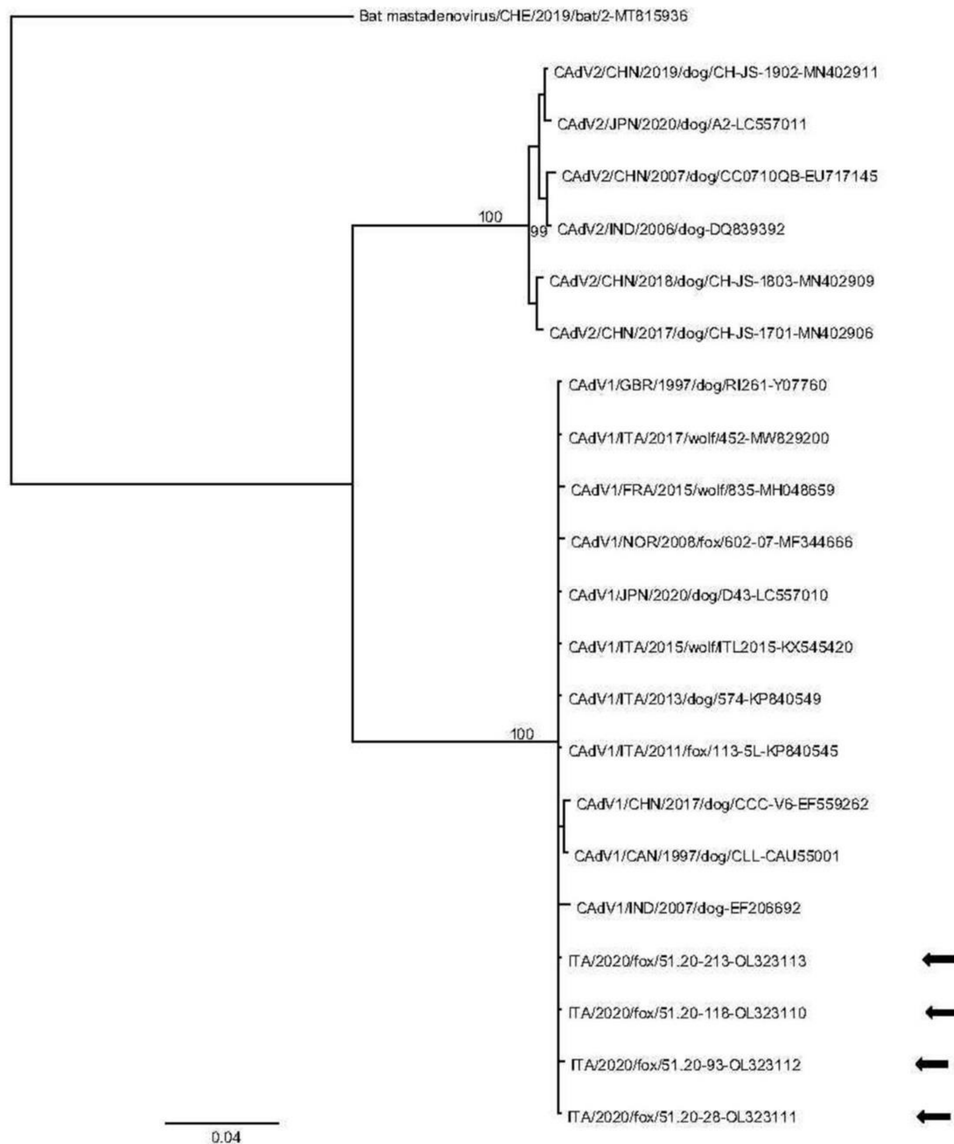

Fig. 2: Phylogenetic tree based on alignment of the partial replicase gene (417 nt) of canine circovirus (CanineCV) sequences detected in this study and other circoviruses retrieved from the GenBank database. Canary circovirus (GenBank accession no. AJ301633) was used as outgroup. Posterior output of the tree was derived from Bayesian inference using 4 chains run for >1 million generations, 2-character states (Hasegawa–Kishino–Yano) model, a proportion of invariable sites and a subsampling frequency of 1,000. Posterior probability values >95 are indicated on the tree nodes. The black arrows indicate the sequences identified in this study. Scale bar indicates nucleotide substitutions per site.

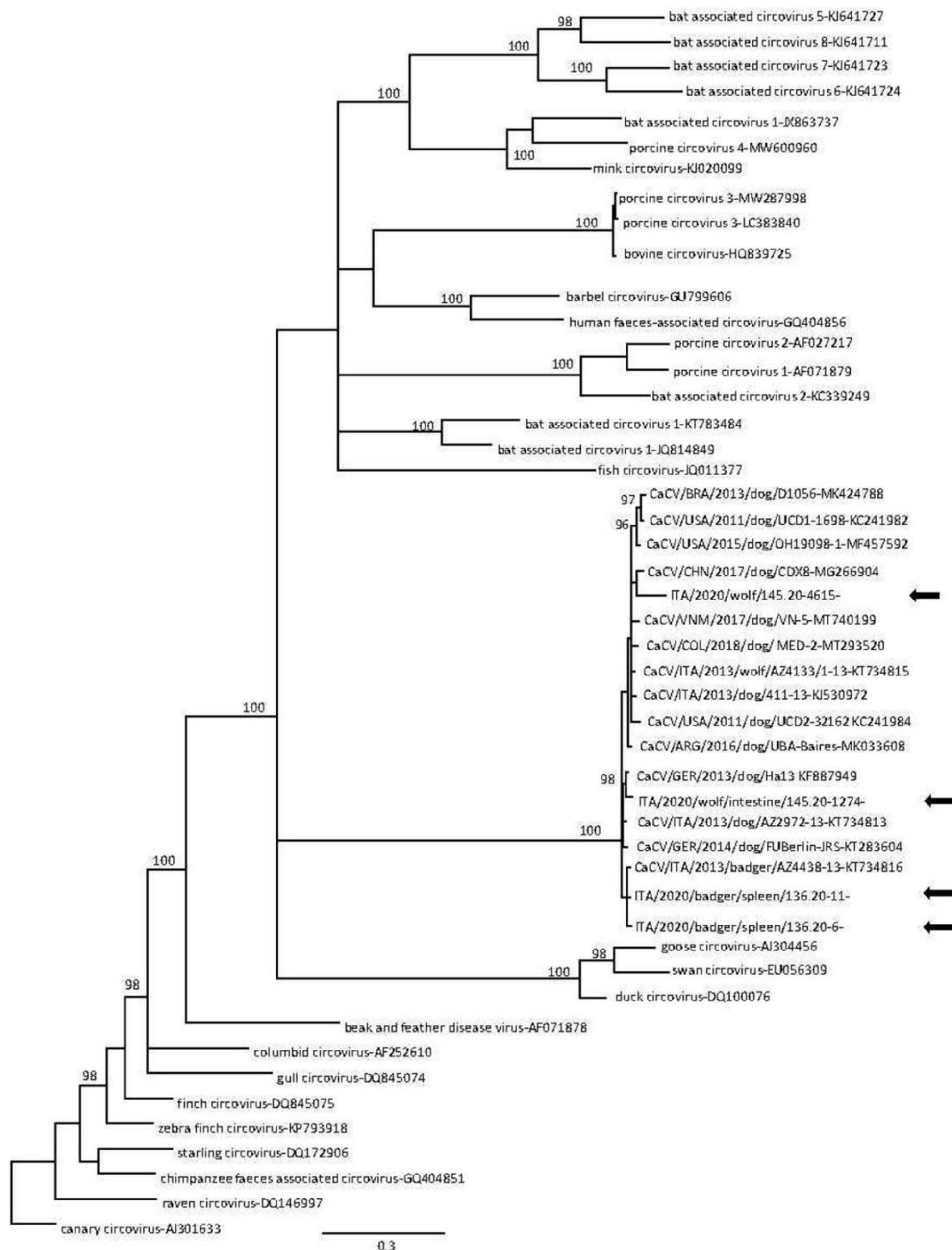

Fig. 3: Phylogenetic tree based on alignment of the partial replicase gene (373 nt) of canine cyclovirus isolates detected in this study and other cycloviruses retrieved from the GenBank database. Horse cyclovirus (GenBank accession no. KR902499) was used as outgroup. Posterior output of the tree was derived from Bayesian inference using 4 chains run for >1 million generations, 2-character states (Hasegawa–Kishino–Yano) model, a proportion of invariable sites and a subsampling frequency of 1,000. Posterior probability values >95 are indicated on the tree nodes. The black arrows indicate the sequences identified in this study. Scale bar indicates nucleotide substitutions per site.

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Section 2.2

Identification and characterization of a novel circovirus in Iberian lynx in Spain

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Abstract

Circoviruses cause severe disease in pigs and birds. Canine circovirus has thus far only been associated with respiratory and gastrointestinal disorders and systemic disease in dogs. The Iberian lynx (*Lynx pardinus*) is one of the most endangered carnivores in Europe and the most endangered felid worldwide. Exploring the virome of these animals may be important in terms of virus discovery and assessing the interspecies-circulation of viruses from related carnivores. In this study, 162 spleen samples from Iberian lynx were screened for CRESS DNA viruses. Overall, 11 (6.8%) of 162 samples tested positive using a consensus PCR. Partial rep sequences were tightly related to each other (96.6–100%). Specific molecular protocols were designed on the partial rep sequences of the novel virus, Iberian lynx-associated circovirus-1 (ILCV-1). By screening a subset of 45 spleen samples, the infection rate of ILCV-1 in Iberian lynxes was 57.8% (26/45). ILCV-1 strains formed a separate cluster intermingled with bat, rodent, mongoose, and felid circoviruses. The genome of the novel virus displayed the highest nucleotide identity (64.3–65.3%) to mongoose circoviruses, thus representing a novel candidate circovirus species. The detection of these viruses in the spleen tissues could suggest systemic infection in the animal host. Overall, these findings suggest that this novel circovirus is common in the Iberian lynx. Further studies are warranted to assess the possible health implications of ILCV-1 in this endangered species.

Keywords: circovirus, CRESS DNA virus, Iberian lynx, wild felids, molecular survey

1. Introduction

Circular replication-associated protein (Rep)-encoding single stranded (CRESS) DNA viruses comprise a variety of viruses with circular ssDNA genome encoding a Replication-associated protein (Rep) involved in genome replication (Krupovic et al., 2020). Genome replication of CRESS DNA viruses, via rolling circle replication, relies on the conserved Rep. CRESS DNA viruses (*Shotokuvirae* kingdom, *Monodnaviria* realm, phylum *Cressdnaviricota*) comprise two classes (*Repensiviricetes* and *Arfiviricetes*) and 12 families (*Bacilladnaviridae*, *Circoviridae*, *Geminiviridae*, *Genomoviridae*, *Metaxyviridae*, *Amesuviridae*, *Naryaviridae*, *Nanoviridae*, *Nenyaviridae*, *Redondoviridae*, *Smacoviridae*, and *Vilyaviridae*) (Krupovic et al., 2020; Krupovic and Varsani, 2022). The progress in molecular diagnostics and metagenomic approaches has fostered the discovery of several genome sequences of CRESS DNA viruses in different ecosystems thus expanding the taxonomy.

The family *Circoviridae* (class *Arfiviricetes*) includes nonenveloped, viruses with icosahedral capsid and a circular, covalently closed DNA genome ranging from 1.7 to 2.1 kb in size. The genome of circoviruses has an ambisense constitution with two major open reading frames (ORFs) encoding a Rep and a capsid (CP) protein. Circoviruses are further subdivided into two genera, *Circovirus* and *Cyclovirus*, which include 49 and 52 established species (www.ictv.global/report/circoviridae), respectively. Members of the genus *Circovirus* (CV) have been detected in different mammals, birds, and fishes, while members of the genus *Cyclovirus* (CyV) have been retrieved from specimens of both vertebrates and invertebrates (Rosario et al., 2017; Breitbart et al., 2017; de Kloet and de Kloet, 2004). CVs have been associated with relevant clinical manifestations, including postweaning multisystemic wasting syndrome (PMWS) of pigs (Baekbo et al., 2012) and the Beak and Feather Disease (PBFD) of psittacine birds (Fogell et al., 2016).

CVs have been associated with respiratory and gastrointestinal disorders and systemic disease in dogs (Decaro et al., 2014; Li et al., 2013; Dankaona et al., 2022). Identification of CVs in large carnivores (i.e., wolves, coyotes, badgers, and foxes) has also been described (Ndiana et al., 2022; Urbani et al., 2021; Hess et al., 2023).

Several circoviruses have also been retrieved from human samples collected from healthy individuals and patients with neurological symptoms including samples of respiratory and gastro-intestinal origin (Phan et al., 2014; Smits et al., 2013; Tan et al., 2013). Recently, a new CV has been discovered in the serum of a patient with chronic hepatitis (Pérot et al., 2023). The virus was able to induce chronic liver infection with viral titers increasing over time but the origin of the virus remained uncertain. Also, another CV species has been identified in the blood of two human patients coinfected with either human immunodeficiency virus or hepatitis C virus with a history of drug addiction (Li et al., 2023).

Limited epidemiologic data on CRESS DNA viruses in large felids are available thus far. Three novel CV species and two different novel CyV species have been discovered by metagenomics in stool samples of bobcats (Lynx rufus) and pumas (Puma concolor) (Payne et al., 2020; Cerna et al., 2023).

Among large felids, the Iberian lynx (Lynx pardinus) is regarded as the most endangered felid species worldwide and one of the most endangered carnivores in Europe according to the International Union for Conservation of Nature, (UICN, https://www.iucnredlist.org/ last accessed 10th March 2024). After a rapid decrease during the last decades of the 20th century, the population of Iberian lynxes declined to a hundred individuals (Simón et al., 2012). This phenomenon was mainly due to the reduction of their staple prey, habitat destruction, illegal trapping and hunting, road kills, and infectious diseases (López et al., 2014). Since then, in situ and ex situ conservation programs have been implemented to preserve the Iberian lynx from extinction. As of 2022, the number of individuals increased to over 1600 individuals by 2022 (Ministerio para la Transición Ecológica y Reto Demográfico, MITECO, https://d3a16902.rocketcdn.me/wp-content/uploads/2023/05/23.05.19-La-poblacion-de-linces-ibericos-alcanza-su-maximo-historicocon-1.668-ejemplares.pdf, last Accessed 10th March 2024). Many infectious pathogens, including feline leukemia virus, Suid alphaherpesvirus 1 and Mycobacterium bovis have been identified in this species, and surveillance programs have been enacted in free-ranging and captive populations (Nájera et al., 2021; Caballero-Gómez et al., 2024).

In this study, the presence of CRESS-DNA viruses was investigated in Iberian lynxes, screening archival collections of spleen samples, based on the assumption that the spleen, the largest organ

of the lymphatic system, plays a key role in the immune response and therefore can be a good target for detection/discovery of viral pathogens, specifically those spreading systemically during virus replication.

For this purpose, a largely used panviral consensus PCR (Li et al., 2010) was used as strategy for the discovery of novel CRESS DNA viruses in the lynxes.

2. Materials and methods

2.1. Sampling

Spleen samples were collected from the carcasses of 162 Iberian lynxes (Lynx pardinus) between 2017 and 2023 throughout the Iberian Peninsula and stored at −80 °C until use. Of them, 135 were free ranging animals, mostly killed by collisions with vehicles. These animals were found dead in three main areas (central, southern, and southwestern Spain). By contrast, a total of 21 lynxes were kept in captivity, including 16 from the four captive breeding centers (BC1–BC4) belonging to the Iberian lynx ex situ conservation program and five animals from four zoological parks/conservation centers (ZC1–ZC4). In six animals, the habitat status was not recorded.

For each animal, epidemiological information about the age (yearlings:< 1 year old; subadults: 1 to 3 years old; adults: 3 to 10 years old; senile: > 10 years old), gender (male or female), habitat status (freeranging or captivity), sampling date and georeferenced location (Southern, Southwestern or Central Spain) were recorded, whenever possible.

2.2. Nucleic acids extraction and screening for CRESS DNA virus

A total of 25 mg of spleen tissues were homogenized by Tissue Lyser (Qiagen GmbH, Hilden, Germany) as previously described (Fanelli et al., 2022). Afterward, the homogenates were centrifuged at 10,000 ×g for 3 min. Two hundred μL of the supernatants were subsequently subjected to nucleic acid extraction using a IndiSpin Pathogen Kit (IndicalBioscience GmbH, Leipzig, Germany), according to the manufacturer's instructions and stored at −80 °C until use.

Samples were screened with a consensus (pan-Rep) PCR protocol based on a broadly reactive set of primers designed to identify members of the *Circoviridae* family (Table 1) (Li et al., 2010). Both first- and second-round PCR protocols were performed as previously described (Vasinioti et al., 2023). The pan-Rep PCR amplicons were purified and directly sequenced by Eurofins Genomics laboratories (Germany). Sequences of approximately 400 nucleotides (nt) were produced and evaluated using the web-based tool FASTA (https://www.ebi.ac.uk/Tools/sss/fasta/, accessed on 10th March 2023), using the default values to find homologous hits.

2.3. Quantitative real time PCR (qPCR)

A qPCR was developed based on the partial sequences achieved by pan-Rep PCR protocol. This group of viruses was herein referred to as Iberian lynx-associated CV-1 (ILCV-1). Aligned partial Rep sequences retrieved from 11 Iberian lynxes were used to design specific primers and probe. Ten µL of sample DNA were combined with the 15-µL reaction master mix (IQ Supermix; Bio-Rad Laboratories SRL, Segrate, Italy) comprising 0.6 µmol/L of each primer and 0.2 µmol/L of probe (Table 1). Thermal cycling was set as follows: activation of iTaq DNA polymerase at 95 °C for 3 min, 45 cycles of denaturation at 95 °C for 10 s, annealing at 56° for 30 s and extension at 60 °C for 30 s. The specificity of the assay was evaluated with a panel of DNA viruses and of circovirus-positive samples previously detected in cats (Vasinioti et al., 2023) to rule out cross-reactivity of the primers/probe with other DNA viruses.

2.4. Nested PCR assay specific for ILCV-1

To confirm ILCV-1 detection and gather sequence data, two sets of specific PCR primers were designed to detect a partial rep portion (452–514 bp) of ILCV-1 (Table 1).

Both first- and second-round PCR protocols were carried out using Platinum II Hot-Start Green PCR Master Mix (2×) (Invitrogen, ThermoFisher Scientific). Cycling thermal conditions comprised initial activation of the Hot-Start polymerase at 94 °C × 2 min, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 58 °C for 15 s and extension at 68 °C for 15 s. One

microliter of the first-round PCR was diluted 1:100 in DEPC water and employed as a template in the second-round PCR. PCR amplicons were directly sequenced by Eurofins Genomics laboratories (Germany). The obtained sequences (≈400 nt) were evaluated by the web-based tool FASTA (https://www.ebi.ac.uk/Tools/sss/fasta/, accessed on 10th March 2023) nucleotide, employing the default parameters to find the highest nt identity in the EBI database.

2.5. Variable categorization and analyses

Data collated regarding sampling area, age category, gender, and habitat status were evaluated for descriptive and inferential statistical analyses using the statistical software R version 4.3.1. Sampling area, age category, gender, and habitat status were categorized with corresponding cell values assigned in a "2 × 2" contingency matrix. The association between detection of ILCV-1 DNA by qPCR and nested PCR and the categorized variables was evaluated by the Pearson's chisquared test or Fisher's exact test, as appropriate. A p-value <0.05 was considered statistically significant.

2.6. Genome amplification

A rolling cycle amplification (RCA) protocol employing the bacteriophage phi29 DNA polymerase (TempliPhi 100 amplification kit, Cytiva) and the pan-Rep reverse primer CV-R1 (Table 1) (Vasinioti et al., 2023) was adopted to enrich circular DNA in selected samples. Additional primers were designed based on the partial Rep gene sequences obtained to perform an inverse (back-to-back) PCR protocol, amplifying a fragment of about 1.5-2 kb (Table 1) encompassing the nearly complete circular genome. The inverse PCR assays were performed with TaKaRa La Taq polymerase (TaKaRa Bio Europe S.A.S. Saint-Germainen-Laye, France) as previously described (Vasinioti et al., 2023). Sanger sequencing using a primer walking strategy was performed on PCR positive products by Eurofins Genomics laboratories (Germany).

2.7. Sequence and phylogenetic analyses

The web-based tool FASTA (http://www.ebi.ac.uk/fasta33) nucleotide was employed with default parameters to find homologous hits. Sequence editing and multiple codon-based (translation) alignments were carried out using Geneious Prime version 2021.2 (Biomatters Ltd., Auckland, New Zealand). The obtained sequences were aligned with related circovirus sequences recovered from the European Bioinformatics Institute (EBI) database using MAFFT software. The most appropriate substitution model for the phylogenetic analyses was assessed using "Find the best protein DNA/Protein Models" supplied in MEGA X version 10.0.5 software (Kumar et al., 2018). Maximumlikelihood method, Tamura Nei 4-parameter model, a discrete gamma distribution and invariant sites to model evolutionary rate differences among sites (6 categories) were selected with 1000 replicates evaluated for statistical support. Bayesian inference and neighbor joining phylogenetic approaches were also assessed.

3. Results

3.1. Screening for circovirus

Out of 162 spleen samples collected, 11 (P: 6.8%, 95%CI: 3.4–11.8) animals tested positive to CV in the two-round pan-Rep PCR (Fig. 1). Positive samples displaying a DNA concentration over 10 ng/µl were directly sequenced, producing 11 sequences of satisfying quality. By pairwise comparison, this group of sequences was highly conserved (96.6–100% nt identity). Sequence analysis by FASTA nucleotide online tool (https://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) showed identity (74.6–76.7% n) of the 11 Rep sequences to CVs found in bats (Table 2).

3.2. Screening for ILCV-1

A subset of 45 (including the 11 pan-Rep PCR positive samples) out of 162 spleen samples was re-tested by a qPCR assay. The qPCR for ILCV-1 was specific, as the assay did not recognize other circovirus types identified in cats (Vasinioti et al., 2023) nor other feline/canine DNA viruses. The 11 samples positive to ILCV-1 in the two-round pan-Rep PCR also tested positive by the qPCR assay. However, the qPCR detected an additional 15 positive samples in this samples

subset (Table 2). Overall, the cycle threshold (Ct) of the 26 ILCV-1 strains identified in this study ranged from 20.3 to 35.3 (mean: 28.8, median: 28.7). A two-round PCR protocol was designed based on ILCV-1 Rep sequences and was used to amplify and to sequence the ILCV-1 strains detected in the 15 samples positive in qPCR but negative by the panRep-PCR. The 15 partial rep sequences displayed the highest identity (range 74.6 to 76.2% nt) to bat CVs by FASTA nucleotide search in the EBI database (Table 2).

In the phylogenetic tree based on the partial rep sequences of the strains retrieved in this study along with other CRESS DNA virus strains, the ILCV-1 strains formed a separate cluster intermingled with circoviruses retrieved in different bats, rodents, mongoose and felids (Fig. 2).

Among the 45 samples of the subset, the infection rate of ILCV-1 was 57.8% (26/45). Out of 26 ILCV-1-positive animals, 17 (65.4%) were sampled in Southern Spain, eight (30.7%) in Southwestern Spain and one sample (3.8%) in Central Spain.

Ten ILCV-1-positive animals were males (38.5%) and seven were females (26.9%). Fourteen ILCV-1-positive animals were adults (53.8%), three were subadults (11.5%), three were senile (11.5%) and one was yearling (3.8%). Twenty ILCV-1-positive animals were free-ranging (76.9%) whilst six were kept in captivity (23.1%). No statistically significant differences were detected according to sampling area, gender, and habitat status ($p>0.05$). Conversely, ILCV-1 positivity was significantly associated with the age category ($p = 0.002$) (Table 3).

3.3. Complete genome analysis of ILCV-1

The complete genome sequence of ILCV-1 was obtained from three Iberian lynxes (Table 4). The genome was 1839 nt. There were two major open reading frames (ORFs), located on complementary strands in inverse orientation. The ORF1 (903 nt), located on the virion strand, and the ORF2 (621 nt), located on the opposite strand, encoded for the Rep (301 aa) and CP (207 aa) proteins, respectively. As observed in other CVs, two intergenic non-coding regions were located between the start and stop codons of the Rep and CP protein genes, respectively. The 5′ and 3′ intergenic regions were 149 and 166 nt in length, respectively. The 5′-intergenic region encompassed a thermodynamically stable stem-loop, involved in the rolling-circle replication, and

the conserved mononucleotide motif AAGTATTAC (Table 4). Upon interrogation of sequence databases with FASTA nucleotide online tool (https:// www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) the highest identity of the complete genomic sequences of the ILCV-1 strains ranged between 64.3 and 65.3% nt to Mongoose circovirus strain Mon-1 (MZ382570), detected from mongoose in Saint Kitts and Nevis (Gainor et al., 2021). In the phylogenetic tree based on the complete genome nucleotide sequences, the three Iberian Lynx CV strains clustered within a well-defined clade, distantly related to bat, mongoose, and feline CVs (Fig. 3).

4. Discussion

Limited data are available on CRESS DNA in felids. Epidemiological studies with consensus PCRs and metagenomic approaches have identified CRESS DNA viruses in a variety of samples from domestic and wild felids (Zhang et al., 2014; Takano et al., 2018; Payne et al., 2020; Hao et al., 2021; Cerna et al., 2023). In a recent large epidemiological investigation, on screening of serum, nasal, and fecal samples from 530 animals, a variety of CV and CyV sequences were identified, including pigeon and CanineCV and a novel CV proposed as feline circovirus-1 (FeCV-1) (Vasinioti et al., 2023). FeCV-1 was detected in both fecal and serum samples of cats, suggesting the ability of FeCV-1 to spread systemically (Vasinioti et al., 2023).

In this study, a novel circovirus was identified in a collection of spleen samples from Iberian lynxes. The partial rep sequences were genetically conserved (96.6–100% nt identity to each other) and resembled CVs identified in bats (74.6–76.7% nt identity). The elevated sequence identity among the different strains might suggest the circulation of a well-adapted circovirus in Iberian lynxes, rather than spillover events from another host species. Also, the identification of the virus in spleen samples would suggest the ability of these viruses to spread systemically after its initial replication in the entrance site.

We were able to obtain the full genome sequence of three ILCV-1 strains. Based on genome sequence comparison, the three viruses displayed the highest genetic relatedness (64.3–65.3% nt identity) to the mongoose CV strain Mon-1 (MZ382570) (Gainor et al., 2021) (Table 4). ILCV-1

displayed a genome-wide sequence identity below the cutoff (80%) adopted by ICTV for the classification of a novel species in the *Circoviridae* family (Breitbart et al., 2017). Accordingly, we provisionally propose ILCV-1 as a novel candidate species in the Circovirus genus.

Interestingly, in our study, we did not identify Rep sequences homologous to other CRESS DNA viruses identified from large felids, from the feces of bobcats in US (Cerna et al., 2023), and of puma and bobcats in Mexico (Payne et al., 2020). The presence of those CyV and CV strains has not been reported in wild felids elsewhere. Accordingly, it is uncertain if they were only anecdotal findings due to dietary contaminations, occasional exposure to other animal sources, and geographical variations, rather than viruses adapted to wild felids. This doubt could be clarified by extending the investigations to other felid species/populations, thus gathering more significant data on the virome complexity of wildlife animals. In our study, the ILCV-1 strains were identified from both free-ranging and captive animals located in the main geographical areas of Spain where the Iberian lynx populations are distributed. Although spatial-stratified data could not be inferred, this geographical dispersion might suggest that ILCV-1 is common (57.8%) in these animals. Interestingly, we could decipher an apparent significant association with age, with the higher frequency of ILCV-1 positive samples being observed in adult animals. This pattern is not unusual for circoviruses. In a study on PCV-3, the detection rate in wild boar was significantly higher in adults (over 24 months old) than in subadults (between 12 and 24 months old) or juveniles (<12 months old) (Klaumann et al., 2019).

The frequency of detection in the spleen samples was 6.8% with the pan-Rep PCR in 162 samples, and 57.8% in a subset of 45 samples screened in qPCR with specific primers/probe. This high frequency of ILCV-1 positives could be consistent with the ability of the virus to establish long-term infections in the host or to re-infect repeatedly the animals. PCV-3 has been reported at high prevalence rates in pigs and it has been hypothesized that long-term subclinical or persistent infections can maintain the virus in the host population, although the exact mechanisms have not been demonstrated (Zhai and Xi, 2019). Also, porcine CV type 1 (PCV-1) was initially identified serendipitously as a persistent contaminant of a porcine kidney cell line (PK-15) (Tischer et al., 1982).

Circovirus infections have drawn attention in veterinary medicine since they can cause disease in

different animal species. For example, avian CV infection is associated with several clinical signs, i.e., developmental deformities, lymphoid deficiency, and immunosuppression (Todd, 2004). In pigs, there are at least four different porcine CV types (type 1 to 4) (Opriessnig et al., 2020) and PCV-2 has been associated with PMWS, reproductive disorders (Sanchez Jr et al., 2001), porcine respiratory disease complex (PRDC) (Kim et al., 2003), intestinal illness (Kim et al., 2004) and dermatitis and nephropathy syndrome (PDNS) (Opriessnig et al., 2020). Moreover, circoviruses have been associated with fatal hemorrhagic enteritis in dog pups (Decaro et al., 2014) and vasculitis in dogs (Li et al., 2013). Yet, understanding the pathogenic role of novel viruses in wildlife animals poses several challenges. For instance, a limitation of our investigation was the lack of clinical history and metadata for most animals included in the study. Since this investigation was not conceived as a case/control study, we could not infer any relation between clinical signs and the novel circovirus. Moreover, immunohistology investigations in the spleen or other organs from the ILCV-1-infected Iberian lynxes were not feasible, since the samples were not stored properly with formalin-fixation.

Another limit of this study was the application of a pan-Rep nested PCR protocol based on consensus primers able to detect CRESS DNA viruses of the *Circoviridae* family (Li et al., 2010). The design of the primers dates back to 2010 and was based on a limited sequence data set, thus presumably restricting the range of detection of CRESS DNA virus sequences. Also, consensus/degenerated primers of panviral PCRs are intrinsically less sensitive than highly specific primers/probes. In this study, for instance, using oligonucleotides specific for ILCV-1, we detected a higher number of positive samples. Also, sequence independent strategies, such as metagenomic investigations based on multiple strain displacement (MDA) protocol using phi29 DNA polymerase, have proven useful in producing sequence data on highly diverse CRESS DNA viruses in biological and environmental specimens (Roux et al., 2016). However, this sequence-independent approach is not as fast and cheap as consensus PCRs for application in large-scale epidemiological studies.

The sample collected in our study represents almost 10% of the total population of Iberian lynxes according to the last census that registered 1668 animals in 2023 in the Iberian Peninsula (MITECO, https:// d3a16902.rocketcdn.me/wp-content/uploads/2023/05/23.05.19-Lapoblacion-de-linces-ibericos-alcanza-su-maximo-historico-con-1.668-ejemplares.pdf, last Accessed 10th

March 2024). This likely provides a reliable picture of the epidemiology of this novel virus. Moreover, the size of our study, was on average 10-fold larger, in terms of the number of screened samples, than previous studies carried out in cats and large felids elsewhere (Cerna et al., 2023; Payne et al., 2020; Takano et al., 2018; Zhang et al., 2014). Yet, a limit of our study was that it was not conceived as an epidemiological study but rather a virus discovery project. For instance, we screened a convenience collection, rather than population cohorts. Also, we tested only a subset of samples with specific primers and probes for sequencing and confirmation. Only 45 samples re-screened with the ILCV-1-specific qPCR and nested-PCR, rather than the whole sample collection, to optimize the costs and benefits of the study. Structured epidemiological/ecological studies will require testing samples collected with a clear study design.

In conclusion, by screening 162 samples from Iberian lynx, we identified a novel circovirus distantly related to bat circoviruses (GenBank accession nrs LC456715, KX834492 and JF938127) (Ge et al., 2011; Matsumoto et al., 2019) and fulfilling the criteria for classification as a novel species of the genus Circovirus. The virus, termed ILCV-1, was repeatedly identified in the spleen samples of both free-ranging and captive animals from different geographical areas of Spain. This indicates that the Iberian lynx could be a primary host for ILCV-1. Surveillance for pathogens and evaluation of the viral diversity are important to unveil emerging and re-emerging infectious diseases in animals, chiefly in endangered species. These studies are important not only in terms of animal conservation but also for understanding the zoonotic potential of animal reservoirs, under the paradigms of the One Health envision.

Ethical statement

This study did not involve killing of animals for the purpose of the investigations. Samples from Iberian lynx were collected by authorized veterinarians and animal keepers following routine procedures with alive and dead individuals before the design of this study, in compliance with the Ethical Principles in Animal Research. Thus, ethical approval by an Institutional Animal Care and Use Committee was not deemed necessary.

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CRediT authorship contribution statement

Sabrina Castro-Scholten: Investigation, Writing – original draft. Violetta Iris Vasinioti: Investigation, Data curation. Javier Caballero- Gómez: Methodology, Resources. Ignacio García-Bocanegra: Conceptualization, Funding acquisition. Francesco Pellegrini: Investigation, Formal analysis. Anna Salvaggiulo: Investigation, Validation. Amienwanlen Eugene Odigie: Software, Visualization. Georgia Diakoudi: Investigation, Supervision. Michele Camero: Writing – review & editing, Conceptualization. Nicola Decaro: Writing – review & editing, Project administration. Vito Martella: Writing – review & editing, Validation. Gianvito Lanave: Writing – original draft, Methodology.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Full genome sequences of Iberian lynx-associated CV-1 strains SPA/2023/Iberian lynx/296.15,

SPA/2023/Iberian lynx/296.26 and SPA/2023/Iberian lynx/296.29 were deposited in GenBank under accession numbers OR714535- OR714537. The small Rep sequence fragments (about 350 nt in length) are available upon request.

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Tables

Table 1

List of oligonucleotides used in this study.

Pathogen	Assay	Primers	Sequence (5′ - 3′)	Amplification size (bp)	Reference
Circovirus	Pan-Rep PCR	CV-*F*1	GGIAYICCICAYYTICA RGG	500	Li et al., 2010
		CV-R1	AWCCAICCRTARAARTCRTC		
	nPCR	CV-F2	GGIAYICCICAYYTICARGGITT	400	
		CV-R2	TGYTGYTCRTAICCRTCCCACCA		
ILCV-1	qPCR	ILCV-1 4 F	AGAAACTATCGGGGTTTAMGCGTG	67	This study
		ILCV-1 70 R	YTCTGATTCTCCAGATCCGTA		
		ILCV-1 28 Pb	FAM- CCCATGTAGARATTGCGAGGG - BHQ1		
	Rep-PCR	ILCV-1150 F	CGAAAAAGGTACCCCGCACTT	514	This study
		ILCV-1663 R	TGAAACCCAYCCATAAAAATCATCCAA C		
	nPCR	ILCV-1163 F	CCGCACTTGCAAGGATTTGTG	452	This study
		ILCV-1614 R	TAACCATCCCACCAAGGACCC		
	iPCR	ILCV-1123 R	CTGCGGAATCCCAATCTCCAG	>1500	This study
		ILCV-1146 F	TGGTGGAGTGYCTGAATCTGTTGA		
	nPCR	ILCV-1 65 R	AGATCCGTACCCCTCGCAATT		
		ILCV-1176 F	ATAATGGCGACCTYCGTGAAGC		

Legend: Circovirus, member of the family Circoviridae; ILCV-1, Iberian lynx-associated circovirus-1; Rep, replicase protein; qPCR, quantitative real time PCR; nPCR, nested PCR; iPCR, inverse PCR.

Table 2

Circovirus (CV) positive samples by either two-round pan-Rep PCR or two-round Iberian lynx associated CV 1 sp. (ILCV-1) -Rep PCR protocols. All the samples were positive by quantitative real-time PCR with the inferred Ct values. Interrogation (FASTA) of EBI nucleotide database (10th March 2024) of the partial (350– 439 nt) ORF1 (replicase) sequence of circovirus strains generated in this study.

N.	Sample code	Gender	Age category	Area	Prefecture	Habitat status	Assay	Strains	Identity to reference sequences		Ct value
									CV strain (accession nr.) ^	nt identity%	
1	296.1	NA	Subadult**	South	Jaén	Free ranging	ILCV-1-Rep PCR	SPA/2019/Iberian Lynx/296.1	Bat associated CV U4274.9 (LC456715)		28.6
2	296.4	F	Yearling*	South	Badajoz	Free ranging	ILCV-1-Rep PCR	SPA/2019/Iberian Lynx/296.4	Bat associated CV U42 (LC456715)	74.9	29.1
3	296.5	M	Senile#	Southwest	Sevilla	Free ranging	ILCV-1-Rep PCR	SPA/2019/Iberian Lynx/296.5	Bat associated CV U42 (LC456715)	74.6	28.7
4	296.6	M	Senile	South	Córdoba	Free ranging	ILCV-1-Rep PCR	SPA/2019/Iberian Lynx/296.6	Bat CV isolate *C*121 (KX834492)	76.2	33.6
5	296.11	NA	Subadult	South	Jaén	Free ranging	ILCV-1-Rep PCR	SPA/2018/Iberian Lynx/296.11	Bat CV isolate *C*121 (KX834492)	75.6	30.9
6	296.15	NA	Adult***	South	NA	Free ranging	pan- and ILCV-1-Rep PCR	SPA/2018/Iberian Lynx/296.15	Bat associated CV U42 (LC456715)	76.7	20.3
7	296.16	F	Adult	Southwest	Huelva	Free ranging	ILCV-1-Rep PCR	SPA/2018/Iberian Lynx/296.16	Bat CV isolate *C*121 (KX834492)	76.2	34.4
8	296.19	M	Adult	South	Jaén	Captive	ILCV-1-Rep PCR	SPA/2019/Iberian Lynx/296.19	Bat CV isolate *C*121 (KX834492)	76.2	32.7
9	296.20	M	Adult	South	Jaén	Free ranging	pan- and ILCV-1-Rep PCR	SPA/2018/Iberian Lynx/296.20	Bat associated CV U42 (LC456715)	76.7	26.0
10	296.21	M	Adult	South	Córdoba	Free ranging	pan- and ILCV-1-Rep PCR	SPA/2018/Iberian Lynx/296.21	Bat associated CV U42 (LC456715)	76.1	27.6
11	296.26	M	Adult	Southwest	Huelva	Captive	pan- and ILCV-1-Rep PCR	SPA/2017/Iberian Lynx/296.26	Bat CV isolate *C*121 (KX834492)	76.3	22.7
12	296.27	F	Senile	Southwest	Huelva	Free ranging	pan- and ILCV-1-Rep PCR	SPA/2017/Iberian Lynx/296.27	Bat CV isolate *C*121 (KX834492)	76.4	26.0
13	296.29	NA	Adult	South	Jaén	Captive	pan- and ILCV-1-Rep PCR	SPA/2017/Iberian Lynx/296.29	Bat CV ZS 2324-HB (JF938127)	74.6	21.1
14	296.31	M	Adult	Southwest	Cádiz	Captive	pan- and ILCV-1-Rep PCR	SPA/2017/Iberian Lynx/296.31	Bat CV isolate *C*121 (KX834492)	76.4	26.6
15	296.32	F	Adult	Southwest	Huelva	Captive	ILCV-1-Rep PCR	SPA/2017/Iberian Lynx/296.32	Bat CV isolate *C*121 (KX834492)	75.7	33.0
16	296.113	F	Adult	South	Jaén	Captive	ILCV-1-Rep PCR	SPA/2022/Iberian Lynx/296.113	Bat CV isolate *C*121 (KX834492)	75.7	34.3
17	296.115	M	Subadult	Central	Toledo	Free ranging	pan- and ILCV-1-Rep PCR	SPA/2022/Iberian Lynx/296.115	Bat CV ZS 2324-HB (JF938127)	75.8	26.1
18	296.122	NA	NA	South	Jaén	Free ranging	ILCV-1-Rep PCR	SPA/2022/Iberian Lynx/296.122	Bat CV isolate *C*121 (KX834492)	76.2	35.3
19	296.123	NA	NA	South	Jaén	Free ranging	pan- and ILCV-1-Rep PCR	SPA/2022/Iberian Lynx/296.123	Bat CV isolate *C*121 (KX834492)	75.8	26.6
20	296.124	NA	NA	South	Jaén	Free ranging	ILCV-1-Rep PCR	SPA/2022/Iberian Lynx/296.124	Bat CV isolate *C*121 (KX834492)	76.2	31.3
21	296.125	NA	NA	South	Jaén	Free ranging	pan- and ILCV-1-Rep PCR	SPA/2022/Iberian Lynx/296.125	Bat CV isolate *C*121 (KX834492)	76.7	26.3
22	296.126	NA	NA	South	Córdoba	Free ranging	ILCV-1-Rep PCR	SPA/2022/Iberian Lynx/296.126	Bat CV isolate *C*121 (KX834492)	75.7	29.3
23	296.183	M	Adult	South	Córdoba	Free ranging	ILCV-1-Rep PCR	SPA/2023/Iberian Lynx/296.183	Bat CV isolate *C*121 (KX834492)	75.7	32.6
24	296.184	F	Adult	Southwest	Huelva	Free ranging	ILCV-1-Rep PCR	SPA/2023/Iberian Lynx/296.184	Bat CV isolate *C*121 (KX834492)	76.2	31.2
25	296.185	M	Adult	South	Córdoba	Free ranging	ILCV-1-Rep PCR	SPA/2022/Iberian Lynx/296.185	Bat CV isolate *C*121 (KX834492)	76.0	28.1
26	296.187	F	Adult	Southwest	Sevilla	Free ranging	pan- and ILCV-1-Rep PCR	SPA/2022/Iberian Lynx/296.187	Bat CV isolate *C*121 (KX834492)	76.4	27.3

NA not available; M, male; F, female; *yearlings: < 1 year old; **Subadults: 1 to 3 years old; ***adults: 3 to 10 years old; #senile: > 10 years old; ^ genome sequence with the highest identity on interrogation of European Bioinformatics Institute database with FASTA (https://www.ebi.ac.uk/Tools/sss/fasta/).

Table 3

Inferential statistics testing the association between the identification of Iber- ian lynx-associated circovirus-1 and sampling area, gender, age and habitat status in the subset of spleen samples from Iberian lynxes.

Variable	Category	Positive N (%)	Negative N (%)	df	*p*-value
Sampling Area	Southern Spain	17 (65.4)	8 (30.8)	2	0.27
	Southwestern Spain	8 (30.8)	9 (47.4)		
	Central Spain	1 (3.8)	2 (10.5)		
	Total	26 (100.0)	19 (100.0)		
Gender	M	10 (38.5)	5 (26.3)	2	0.47
	F	7 (26.9)	4 (21.1)		
	NA	9 (34.6)	10 (52.6)		
	Total	26 (100.0)	19 (100.0)		
Age	Yearling	1 (3.8)	8 (42.1)	4	0.002
	Subadult	3 (11.5)	6 (31.6)		
	Adult	14 (53.8)	3 (15.8)		
	Senile	3 (11.5)	1 (5.3)		
	NA	5 (19.2)	1 (5.3)		
	Total	26 (100.0)	19 (100.0)		
Habitat status	Free range	18 (69.2)	12 (63.2)	2	0.79
	Captive	6 (23.1)	6 (31.6)		
	NA	2 (7.7)	1 (5.3)		
	Total	26 (100.0)	19 (100.0)		

M: male; F: female; NA not available.

Table 4
Genomic features of complete genomes of circoviruses sequenced in this study.

Species	Sample ID	Accession	Size (nt)	Putative Rep		Putative Cap		5′ intergenic region (nt)	3′ intergenic region (nt)	Loop motif (5′-3′)	Identity to reference sequences	
				nt	aa	nt	aa				CV strain* (accession nr.)	nt identity %
Iberian lynx associated Circovirus 1 sp. [isolate 1]	SPA/2018/Iberian lynx/296.15	OR714535	1839	903	301	621	207	149	166	AAGTATTAC	mongoose circovirus strain Mon-1 (MZ382570)	65.3
Iberian lynx associated Circovirus 1 sp. [isolate 2]	SPA/2017/Iberian lynx/296.26	OR714536	1839	903	301	621	207	149	166	AAGTATTAC	mongoose circovirus strain Mon-1 (MZ382570)	64.3
Iberian lynx associated Circovirus 1 sp. [isolate 3]	SPA/2017/Iberian lynx/296.29	OR714537	1839	903	301	621	207	149	166	AAGTATTAC	mongoose circovirus strain Mon-1 (MZ382570)	65.0

nt, nucleotides; aa, amminoacids; CV, member of the genus *Circovirus*; * genome sequence with the highest identity on interrogation of European Bioinformatics Insti- tute database with FASTA (https://www.ebi.ac.uk/Tools/sss/fasta/)).

Figures

Fig. 1. Map of the study area within the Spanish country with highlighted in grey the sampled area. Geographic distribution of sampled Iberian lynxes. Red circles/ squares indicate PCR (pan-Rep)-positive animals. The frequency of positivity and the numbers of positive and total of animals analyzed by pan-Rep PCR at each sampling region and captivity centre is shown in parentheses. The abbreviations 'BC' and 'ZC' refer to breeding centers and zoo/conservation centers, respectively. *Georeferenced location of the prefecture was not recorded in one positive animal from the south sampling area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

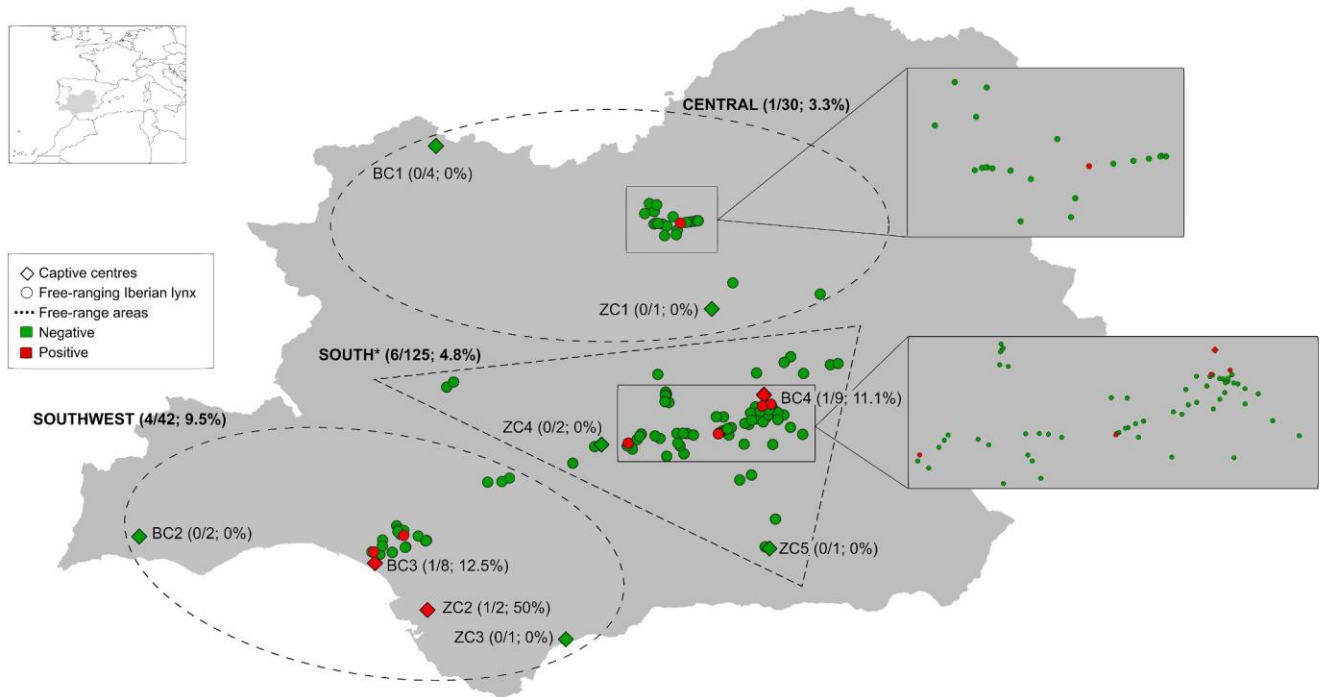
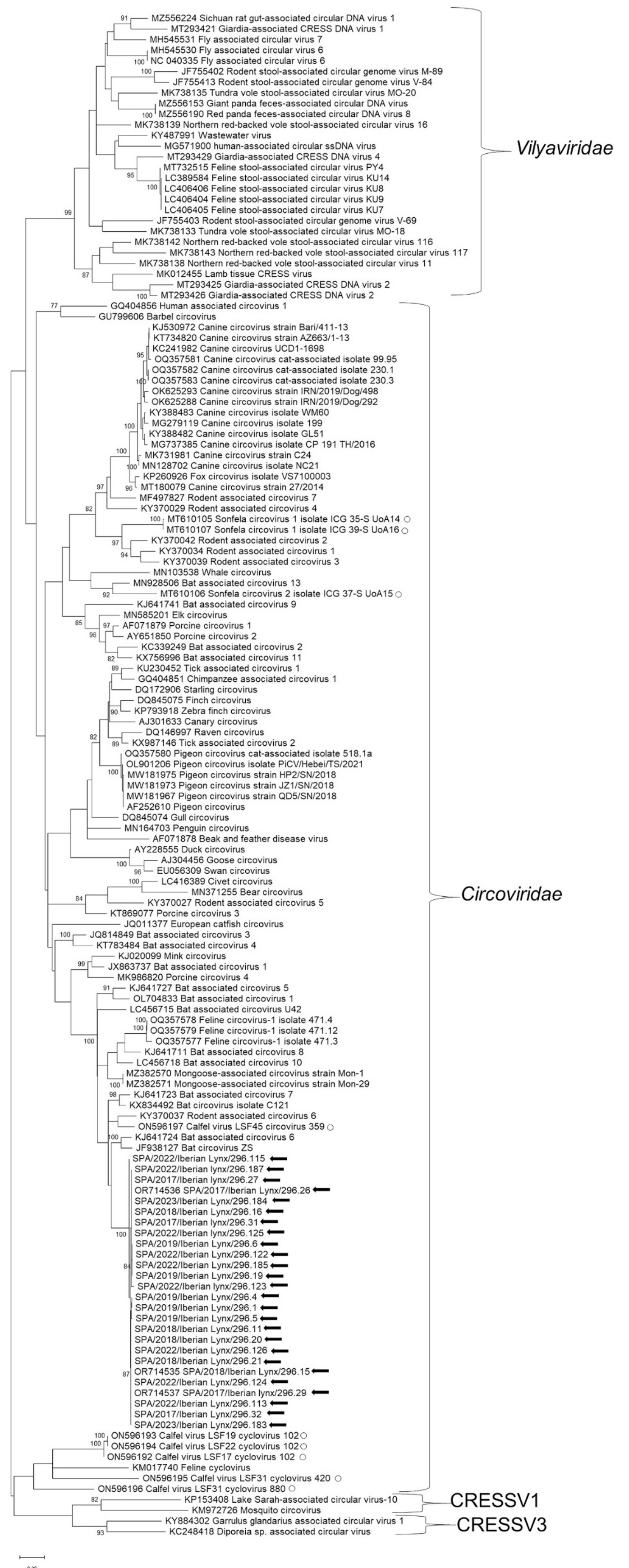


Fig. 2. Phylogenetic tree based on the partial rep gene sequence of Iberian lynx-associated circovirus-1 strains identified in this study (black arrows) and CRESS DNA virus sequences retrieved from the databases. Statistical support was obtained employing 1000 bootstrap replicates. Bootstrap values >75% are shown. Family *Circoviridae*, *Vilyaviridae* and unclassified CRESS DNA virus 1 and 3 are indicated. White circles with black border indicate the CRESS DNA viruses previously identified in domestic and wild felids. The scale bar represents the number of nucleotide substitutions per site.



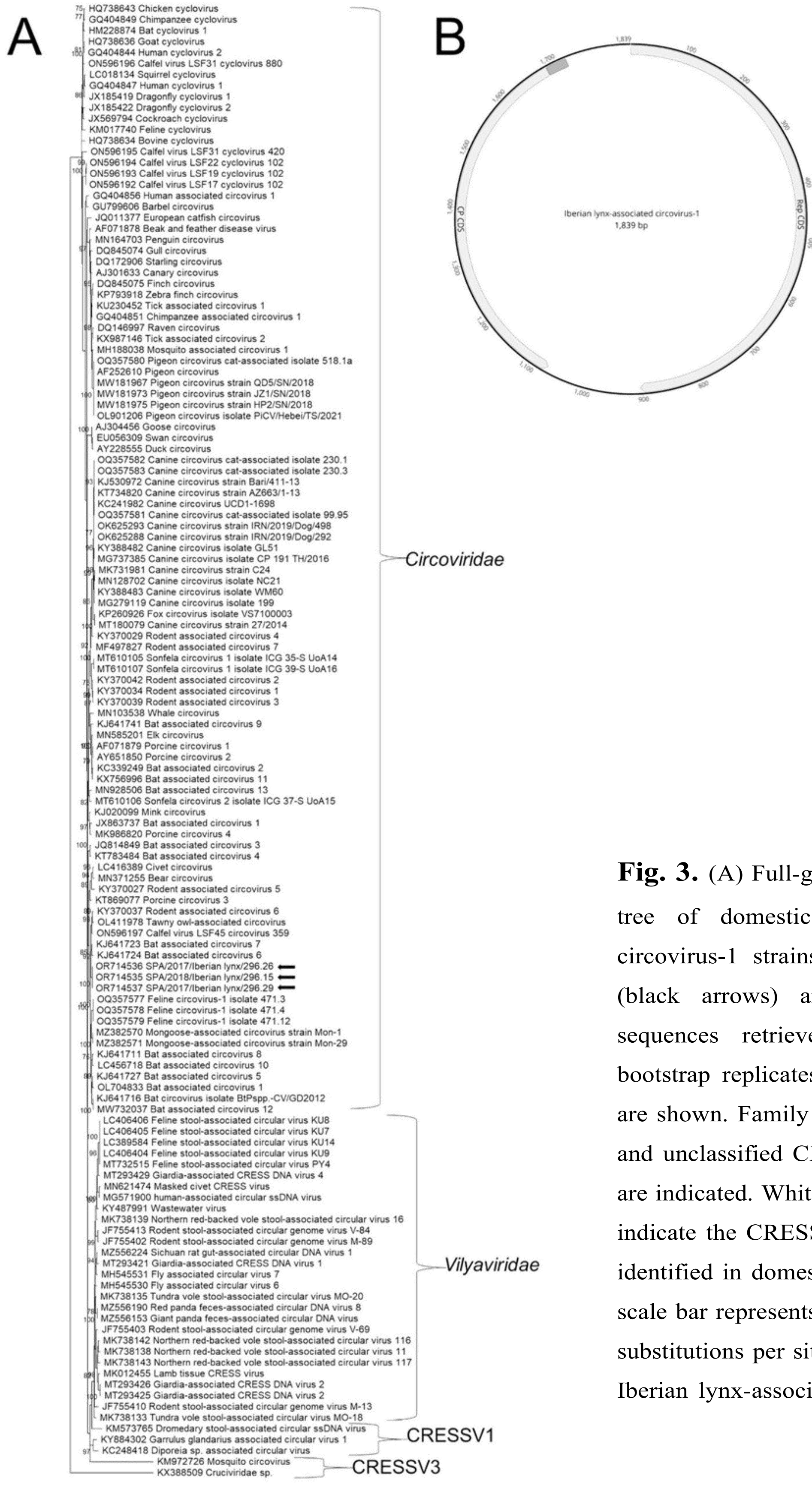


Fig. 3. (A) Full-genome-based phylogenetic tree of domestic Iberian lynx-associated circovirus-1 strains identified in this study (black arrows) and CRESS DNA virus sequences retrieved from the databases. bootstrap replicates. Bootstrap values >75% are shown. Family *Circoviridae*, *Vilyaviridae* and unclassified CRESS DNA virus 1 and 3 are indicated. White circles with black border indicate the CRESS DNA viruses previously identified in domestic and wild felids. The scale bar represents the number of nucleotide substitutions per site. (B) Organization of the Iberian lynx-associated circovirus-1 genome.

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General discussion and conclusions

The primary objective of this thesis was to gather insights into the discovery and characterization of circular Rep-encoding single-stranded (CRESS) DNA viruses in domestic and wild carnivores, with particular focus on the family *Circoviridae*.

In **Chapter 1** we focused on the detection of CRESS DNA viruses in domestic carnivores. CRESS DNA viruses are the smallest known viral pathogens that can infect eukaryotes and exhibit wide distribution across various ecosystems (Kazlauskas et al., 2018). They are significant pathogens of pigs and birds and have been repeatedly identified in human specimens (Pérot et al., 2024; Phan et al., 2014; Smits et al., 2013; Tan et al., 2013) raising concerns about the potential risks to public health. The first report of detection of circoviruses in carnivores dates back 2012 when canine circovirus (CanineCV) was identified in the serum of asymptomatic dogs (Kapoor et al., 2012). Since then, CanineCV has been reported worldwide in domestic and wild carnivores (Gomez-Betancur et al., 2023) resulting in the identification of six distinct phylogenetic clades (Beikpour et al., 2022). Despite the poorly understood pathogenic role of CanineCV, it has been associated with different clinical manifestations in dogs, including enteric and respiratory signs (Anderson et al., 2017; Decaro et al., 2014; Li et al., 2013; Piewbang et al., 2018). Scarce information is available on the epidemiology of circoviruses in domestic cats. To date, epidemiological investigations have revealed the presence of CRESS DNA viruses in wild and domestic felids (Cerna et al., 2023; Hao et al., 2021; Payne et al., 2020; Takano et al., 2018; Zhang et al., 2014). However, understanding of their pathogenic role in these host species remains still limited, prompting the need for additional research studies.

In **Section 1.1** of Chapter 1 we reported the detection of a novel circovirus, FeCV-1 circulating in cats in Italy (Vasinioti et al., 2023) which appeared to have no connection to a previously identified cyclovirus strain FD that was retrieved from clinically healthy cats in California (Zhang et al., 2014). In the study a collection of archival samples (oro-nasal swabs, rectal swabs and serum samples) of domestic cats were screened using a consensus PCR assay able to identify members of the *Circoviridae* family (Li et al., 2010). Circular DNA viruses either closely or distantly related to different animal hosts, including humans were identified. The majority of the sequences (n=24) were recovered from feces and likely reflected a dietary origin or environmental contamination due to the wide range of animals preyed on by cats. Interestingly, three CanineCV-like sequences

were identified in serum samples, suggesting virus ability to infect and replicate actively, thus sustaining viremia in cats. Given that these sequences derived from unrelated cases, we can hypothesize a potential dog-to-cat transmission of canine-CV strains due to close interaction between domestic dogs and cats. Moreover, a large (n=10) group of sequences retrieved from feline stools resembled partial Rep sequences detected in mongooses (68.3–77.2% nt identity). All sequences clustered together and shared 95.4–100% nt identity to each other, apart from a single sequence that appeared less conserved (82.4–85.9% nt). The high conservation observed between these sequences could imply the spread of a feline-adapted virus between cats rather than repeated exposure to viruses from other hosts. According to the current ICTV classification criteria, we proposed that the mongoose-like CV sequences could represent a novel candidate species in the *Circovirus* genus, named feline circovirus-1 (FeCV-1).

In order to determine the presence of FeCV-1 in other European countries, we screened sample collections obtained from household and sheltered cats from different areas including Greece, Romania, and Portugal. Overall, 20 (10.7%) out of 186 samples tested positive for FeCV-1 with a prevalence of 20.4% (10/49) in Greek samples, 11.4% (5/44) in Romanian samples and 5.4% (5/93) in Portuguese samples. FeCV-1 was repeatedly detected in fecal, respiratory, and blood samples. The whole genome sequence was generated for eight strains. The FeCV-1 strains shared 95.2% to 99.6% nt identity, forming a well-conserved clade, regardless of the geographic origin. These data are part of a manuscript that is being edited and it is not yet under evaluation of a scientific journal but represent and important follow up of the screening for FeCV-1 in cats.

In **Section 1.2** of Chapter 1 we evaluated the circulation of canine circovirus in dogs infected with canine parvovirus in Iran. A total of 203 dog fecal samples was collected from 5 different regions of Iran and tested for CanineCV and CPV presence. Analysis revealed CanineCV DNA in 6.4% and CPV DNA in 24.13% of the samples. Interestingly, all the CanineCV positive samples were also positive for CPV. In this study, CanineCV presence was not clearly associated with diarrhea occurrence, however, animals co-infected with both CanineCV and CPV exhibited higher clinical severity scores compared to those infected solely with CPV. The Iranian CanineCV sequences had a nucleotide identity of 96.4-98.2% to each other and phylogenetic analyses revealed that they are more closely related to Turkish strains suggesting a potential introduction of the virus from a neighboring country.

Chapter 2 aimed at investigating the presence of CRESS DNA viruses within wild carnivore populations in southern Europe. To date, several studies have reported circoviruses' detection in wild carnivores, often in co-infections with other carnivore viruses (Balboni et al., 2021; Zaccaria et al., 2016). Nevertheless, the transmission pathways and the pathogenic role remain to be determined. CanineCV has been identified in wild carnivores (De Arcangeli et al., 2020; De Villiers et al., 2023; Franzo et al., 2021; Urbani et al., 2021), and interestingly sometimes in presence of severe clinical signs. Viral metagenomics revealed the presence of a CanineCV in samples collected from foxes in the United Kingdom, suffering from unexplained meningoencephalitis (Bexton et al., 2015). Moreover, circoviruses have been also detected in scat stool samples of bobcats (Cerna et al., 2023; Payne et al., 2020). During 2021-2023 we screened sample collections from wild carnivores including wolves, foxes, badgers and lynxes, to further explore the epidemiology and the infection patterns of circoviruses.

In **Section 2.1** of Chapter 2 we reported the results of an epidemiological survey conducted for three selected canine viruses including, circoviruses, in free-ranging carnivores that were found dead in Italy. CanineCV was detected in spleen samples of Eurasian badgers indicating an active infection in this animal species with a prevalence of 18%. Additionally, 4 out of 8 (50%) wolves were found positive for CanineCV presence. Both wolf and badger sequences obtained, clustered with CanineCV sequenced identified in domestic dogs and one Eurasian badger. Surprisingly, two wolves were simultaneously infected with CanineCV and cycloviruses with one sequence displaying 68.6% nucleotide identity to a CyV detected in a domestic cat, while the other was more closely related (79.4% nucleotide identity) to a CyV sequence from bats. This study reported the first detection of cycloviruses in wolves indicating that wolves may host various members of the *Circoviridae* family.

Section 2.2 of Chapter 2 describes the identification of a novel circovirus circulating in Iberian Lynxes (*Lynx pardinus*). Spleen samples from 162 lynxes were screened for the presence of circoviruses. A group of partial rep sequences which were genetically conserved (96.6–100% nt identity to each other) and resembled CrVs identified in bats (74.6-76.7% nt identity) was proposed as a novel candidate species within the genus *Circovirus* referred to as Iberian lynx-associated CV-1 (ILCV-1). Specific molecular protocols were designed based on the novel ILCV-1 to screen a subset of 45 samples. The frequency of detection in the spleen samples was 6.8% in 162 samples,

and 57.8% in the subset of 45 samples. This high frequency of ILCV-1 positives could imply ability of the virus to establish long-term infections in the host or to re-infect easily the animals. Moreover, identification of the virus in spleen samples suggests virus replication and systemic spread in the animal host.

In conclusion, we used a combination of different molecular methods, i.e. PCR, qPCR, RCA protocols, first-generation sequencing (i.e. Sanger's sequencing) and bioinformatic tools for the discovery and characterization of novel CRESS DNA viruses. Our study reported the identification of novel and emerging circoviruses in different specimens from domestic and wild carnivores, providing insights into potential transmission pathways. However, the association of the novel circoviruses with disease remains to be elucidated. To assess if the molecular detection of a virus is related to replication within the host rather than due to a nucleic acid contamination, *in vitro* isolation and experimental infections should be implemented. Future investigations are needed for gaining a deeper understanding of the natural history of these viruses and their interactions with their host species.

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Violetta Iris Vasinioti was born in Larissa, Greece, on July 5th, 1995. Following the conclusion of High School in 2013, she started her graduation in the Aristotle University of Thessaloniki in the Faculty of Veterinary Medicine, obtaining her degree in 2020. In 2021, she started a Ph.D. course in “Animal Health and Zoonosis” at the University of Bari (Italy) under the supervision of Prof. Nicola Decaro and Prof. Gianvito Lanave. In 2023, she completed a three-month traineeship (September-December 2023) in the Abel Salazar Biomedical Sciences Institute in the University of Porto (Portugal) focusing on virus research under the supervision of Prof. Joao Mesquita.

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By the end of her Ph.D. on April 17th, 2024, she had authored and co-authored 15 scientific articles published in peer-reviewed international journals, 1 article under revision, and 10 abstracts and presentations in international and national conferences.