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*) (Kupovic 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; Hes 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-historico-con-1.668-ejemplares.pdf , last Accessed 10th March 2024). Many infectious pathogens, including feline leukaemia 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 (free-

ranging 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 (Indical Bioscience 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 (2X) (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 chi-squared 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 1.5-2kb (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-Germain-en-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). Maximum-likelihood 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.

2.8 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.

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 (Figure 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 (Figure 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 (Figure 3).

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 faecal samples from 530 animals, a variety of CV and CyV sequences were identified, including pigeon and canine CV 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 (less than 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 et al., 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 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 1,668 animals in 2023 in the Iberian Peninsula (MITECO, https://d3a16902.rocketcdn.me/wp-content/uploads/2023/05/23.05.19-La-poblacion-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 were 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.

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.

Ethical Statement

This study did not involve killing of animals for the purpose of the investigations. Samples from Iberian lynx were collected by authorised 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.

Conflict of interest

The authors declare no conflict of interest.

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Table legends

Table 1: List of oligonucleotides used in this study

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.

Table 3: Inferential statistics testing the association between the identification of Iberian lynx-associated circovirus-1 and sampling area, gender, age and habitat status in the subset of spleen samples from Iberian lynxes.

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

Figure legends

Figure 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.

Figure 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 greater than 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.

Figure 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. Statistical support was determined using 1000 bootstrap replicates. Bootstrap values greater than 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) Organisation of the Iberian lynx-associated circovirus-1 genome.