

## RECOMMENDER

**We thank the Recommender for taking the time to assess our work and for the useful suggestions. We have carefully assessed each comment and query from the Reviewers and the Recommender and addressed all concerns. We have modified the manuscript according to the received suggestions and we provide here point-by-point answers. Edits made to the manuscript are highlighted in yellow.**

In the title, please add « from the Northwest Territories, Canada »

**Done.**

Abstract : Overall, 72 (45.3%) animals tested positive. Please write « Overall, 72 (45.3%) animals were tested positive (on XXX specimens).

**Done.**

Which statistical test has been used here, and what is the statistical value of this test? (48.3%,  $p < 0.001$ ). Statistical test's values are important and need to be given.

**As specified in the M&M section, a Chi-square test was used to compare proportions. This information and the value of Chi have now been added to the abstract.**

« Sequencing revealed high genetic diversity, further exacerbated by recombination ». Please indicate for which viruses?

**Done.**

« ..... , indicating the co-existence of multiple endemic lineages. » Endemicity is a relative concept depending on spatial scale, here Northwest Territories, Canada. Can you use the expression of endemic lineages here? Does it correspond to sampling bias, or do you have enough knowledge on larger scale to affirm this?

**The Recommender has a good point here. We were actually referring to the specific area we evaluated in this study. We have rectified the sentence by adding "in the investigated area" to make it more specific.**

« Finally, viruses from Europe and North America were mixed, indicating that the origin of the four lineages might predate the segregation of European and American wolf and fox populations. » Is there a possibility of post-mixing? Recent switching? Notably through another host species due to the paucity of information as you write on lines 379-380?

**The possibility is there, but this requires the transportation of the virus across continents either through an additional host, e.g. dogs, or another vector or vehicle. Our hypothesis originates from the fact that this lineage seems not to circulate in dogs, which have been known to spread various canine viruses to different geographic areas and hosts, as discussed in lines 394-397. Consistently, for these other viruses, a strong geographic clustering is not observed. Nonetheless, the ancient origin of the four lineages of fox circoviruses is a speculation that will need to be confirmed or proven wrong by future studies. We have strengthened the message that this is our hypothesis in the discussion at lines 402-404: "However, the same pattern could also be explained by viral transfer by yet undetected routes and further investigations examining additional hosts and locations are required to verify this hypothesis".** However, we do not feel it is appropriate to elaborate too much on this in the abstract, considering that the abstract is already long and the sentence is already hypothetical. By reading the manuscript, the reader has all the elements to critically interpret this hypothesis.

Introduction. Lines 86-87. « ....in foxes from Svalbard and Northern Norway (Urbani et al., 2021) and from Newfoundland and Labrador, Canada (Canuti, Rodrigues, et al., 2022) ». Please indicate that it is the Arctic fox.

**Done.**

Materials and Methods.

Samples. Please add a map of your samplings.

**As suggested, we provided a map displaying the sampling locations with integrated virus screening results (new Figure 1).**

Sequences analyses. I read something about cluster-s in the text see lines 202-203 for instance), so you should explain how you analyze and characterize these clusters here.

In maximum likelihood phylogenetic trees, sequences that are genetically closely related form clusters and in these trees, the horizontal lines are proportional to the genetic distance between viruses. Generally, clades are defined as monophyletic groups of sequences with high statistical support. In our case, the statistical support was calculated through bootstrapping and the SH-aLRT test, as explained in the M&M (150-151), and the results from these statistical tests are indicated in the figures, as indicated within the figure captions. For clarity, we have improved the section by providing more details on the statistical support, describing the clusters in more detail, and including additional information in the figure captions.

Statistical analyses. Line 148. « ...Wald test ». Please write Wald's test.

**We checked and the most commonly used term is "Wald test". Therefore, we have not changed it.**

Results

CanineCV epidemiology among grey wolves

Lines 153-154, and following through all this section. Which test-s has-ve been used, and what are the statistical test' values?

**Done.**

Lines 162-163. (Canuti, Fry, et al., 2022). Better to write Canuti et al. 2022). Please check everywhere.

**Since there are 2 references "Canuti et al., 2022", the reference manager is set to automatically include the name of the second author in these citations to avoid ambiguity.**

CanineCV diversity among grey wolves

Lines 199. Badger is a Mustelid and not a Canid, is it? Can you clarify here? So this sentence is not clear to me since your viruses could show a host species range larger than expected and said !

**Yes, indeed, the badger is a mustelid. Cross-species transmissions are commons among sympatric carnivorans because of niche sharing, the carnivorous lifestyle, and possibly other factors. In this case, the infection in this wolf could be either a spillover from a dog or the result of local virus circulation among wild animals. Unfortunately, data are too scarce to draw any conclusion on this aspect. This sentence is simply a description of the clade containing this virus, which is not part of the fox circovirus clade, the main subject of the study. Indeed, the host range of fox circovirus might be wider than currently known, although not all potential hosts may be maintenance hosts, but very little is currently known. This is why studies focused on wildlife are very important.**

Lines 202-204. « Although a smaller number of samples was available from two of the three investigated areas, a certain clustering based on sampling location could be observed. Three out of four viruses from SSR were included in clade A, indicating a predominance of viruses from this clade in that location..... » Is it totally subjective when you write a certain clustering, or can this be tested?

**Please, see the answer to your previous comment.**

Line 209. Please write viruses.

**The typo has been fixed.**

Global epidemiology of fox circoviruses

Lines 252 and following. Please consider my comment on badger.

**The canine circovirus identified in a badger was in a different viral clade that does not include the fox circovirus. While it is possible that other wildlife can be infected by fox circoviruses, these viruses have so far been only identified in foxes and wolves. We have now highlighted this aspect better in the discussion: "While fox circoviruses have so far only been identified in foxes and wolves and only in Europe and North America, they may also have a broader host and geographic distribution" (lines 387-388).**

Discussion. Line 273. OK with Carnivorans for badger. But maybe there's an incomprehension on my part.

Redaction should be reviewed in order to avoid such misunderstanding by readers.

**We thank the Recommender for pointing out this aspect. We believe that the confusion arises from the fact that canine circoviruses have indeed been identified in non-canine hosts, but this was shown for certain lineages and not for fox circoviruses. The latter, which is the main subject of this study, was so far identified only in foxes and wolves. Nonetheless, it is possible that this virus infects or circulates among other wild animals. We have modified to text to try and make this distinction clearer and underlined better in the**

**abstract and the discussion the potential existence of other maintenance hosts. See lines 48, 370-1, 387, 403-4, 408-409**

CanineCV is endemic among wolves of Northern Canada

Is there a possibility that these viruses may circulate and persist in other tissues and organs? Why you look at spleen only? The fields of virology and parasitology are full of illustrations where organs or tissues that were not initially accepted as infected habitats, or that were initially rejected as such, subsequently became so.

**The Recommender is correct, it is possible that this virus can be identified in other tissues. For example, we found this virus in fecal samples from foxes and it is likely identifiable in intestinal tissues as well.**

**Additionally, being likely a virus that causes viremia, we can postulate that the virus can be identified in possibly any tissue. However, the samples used for this investigation were previously collected for a different study and no other tissues from these animals were available for this research.**

Does CanineCV facilitate a secondary parvoviral infection?

Line 332 and following. Please give the statistical test, and its statistical value?

**We have reported those details in the result section and we do not think it is necessary to repeat them here. We prefer not to include these details in the discussion to enhance its readability.**

Lines 379-380. « ...and that viral diversity in wildlife is largely understudied, so these conclusions could be biased by sample and sequence availability ». I do agree, so you should take into consideration several of my comments (e.g., endemicity, cluster, non-canine host, long-standing separation of European and American fauna and their viruses).

**We thank the Recommender for this comment. Indeed, this virus has only been recently discovered, and only a few studies about its host and geographic distribution are available. When more investigations are performed, it is likely that additional hosts will be identified and that it will be discovered that the virus has a wider geographic distribution. The main point of the manuscript was to describe wolves as an additional maintenance host for this virus and demonstrate the high genetic diversity in the investigated population. We hope that our study will instigate more research on this topic so that more knowledge can be acquired. We did our best in addressing the concerns of the Recommender and believe we have properly outlined the study limitations and the need for further research on this topic.**

#### **Review by Arvind Varsani, 13 Apr 2024 20:06**

The Ms titled "Diverse fox circovirus (Circovirus canine) variants circulate at high prevalence in grey wolves (Canis lupus)" describes the identification of the CanineCV from 159 grey wolf samples from Canada. The study found a high incidence of positive animals (~45%) and in some cases there we co-infections with other viruses.

Overall, the MS is good and I see nothing wrong with the analyses for the interpretation of the data.

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I have a few suggestions to improve the MS

Line 19: Canine circoviruses – plural.

**Fixed.**

Line 20: I would avoid the use to the term subclade as that only refers to the phylogeny. Rather say strain.

**Agreed. We replaced "subclade" with "lineage".**

Line 33: change "... and one belonged to a distant clade. In the phylogenetic analysis, the viruses were distributed..." to "... and one belonged to a distant clade. In the phylogenetic analysis, the virus sequences were distributed..."

**Done.**

Line 50: Change to "Circoviruses (family Circoviridae, genus Circovirus)"

**Done.**

Line 135: change "built" to inferred. Please edit this though out the text (e.g. line 301)

**Done.**

Line 320-325: I would be very careful about this. I would not even have this as this is speculative.

**We agree with the Reviewer on the highly speculative nature of this hypothesis. We have re-phrased the paragraph into: "Finally, for one virus we found no evidence for spreading, although this was identified in a sample collected in the final year of the study. This virus was part of a cluster that, unlike others, had been previously detected in both dogs and wild animals and we could speculate that this was a case of spillover from a different host species with limited or no onward circulation." (lines 312-315). Additionally, we replace the sentence "Indeed, the only virus identified in a different part of the phylogenetic tree was likely associated with a dead-end infection" with "Indeed, only one virus was identified in a different part of the phylogenetic tree." (lines 373-4).**

#### **Review by anonymous reviewer 1, 25 Apr 2024 13:42**

The study from Marta Canuti and colleagues describes the prevalence and diversity of fox circoviruses in grey wolves in Canada and show that these viruses are highly prevalent in grey wolves. They show presence of different viral lineages that form clusters according to geographic origin. The authors show also the presence of recombinant viruses. Moreover, they show that animals infected with circoviruses are more likely to be co-infected with parvoviruses

Overall the study is well conducted and the paper is well written and easy to read.

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However the paper can be improved:

- The authors should provide more details on the study population and not only refer to the previous paper on parvoviruses, which was done on more than 300 animals, and in this new study only 159 have been analyzed. Therefore the authors should show more details on the geographic origin, for example a map similar as in the initial paper, and importantly give more details on time period of sample collection for the different samples. Do they see a correlation between variants and sampling period?

**We thank the Reviewer for this comment. While the previous study was performed with a higher number of samples, metadata was incomplete. For this study, we selected only samples from animals for which both sampling dates and locations were known. Unfortunately, sample distribution across the study years was not homogeneous, as outlined at lines 111-113 of the M&M section: "Samples were collected between 2007 and 2019 (2007: N = 17, 2008: N = 19, 2009: N = 5, 2010: N = 24, 2011: N = 16, 2012: N = 2; 2013: N = 5, 2014: N = 1, 2016: N = 6, 2017: N = 1; 2018: N = 3, 2019: N = 60)". For these reasons, when we performed statistical tests, we combined years in quinquennia: "The prevalences were similar between ....and among the investigated years (27/65, 41.5% in 2007-2010, 14/24, 62.5% in 2011-2015, and 31/70, 44.3% in 2016-2019;  $\chi^2 = 2.05$ ,  $p = 0.4$ )" (lines 167-170). Finally, when we compared genetic diversity to the year of sampling we found no pattern, as stated at lines 227-228: "No patterns that related the year of sampling with tree topology were observed."**

**Nonetheless, we agree with the Reviewer that it is important to provide more details on sampling locations. Therefore, following the suggestion of the Reviewer, we have included a map depicting sampling sites that also includes some results about viral screening (New Figure 1).**

- The authors should also explain more in detail why the number of full-length genome sequences was relative low, especially for such a small virus , how did they define the viral load?
- How were samples selected for full-length genome sequencing?

**Indeed no quantitative assay was performed and the exact viral load was not assessed in this study. Our selection was made on a very practical aspect: samples that were positive in the first screening PCR (in other words those that did not require a nested amplification to become positive) were considered to contain the virus at a high viral load and were selected for sequencing. This information has now been included in M&M (lines 135-136). While not all samples were fully sequenced, we made sure that all identified clades and each of the three regions were represented (lines 231: “These represented all the fox circovirus and recombinant clades identified in this study.”)**

Finally, the authors should add in the title that the study was conducted in Canada.

**Done.**