Article

First description of natural SARS-CoV-2 infection in two wild American minks (*Neovison vison*)

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 Simple Summary:
COVID-19   has been one of the most important pandemics in recent history. It is an emerging infectious disease with probable animal origin. Several domesticated and non-domesticated animals are naturally susceptible to SARS-CoV-2 infection, including Mustelidae, being minks, the first species identified with this infection. In this work, we present the detection of SARS-CoV-2 RNA in lymph nodes tissue from two wild American minks (*Neovison vison*) in Valencian Community (Eastern Spain) during invasive species trapping campaigns.

 Abstract:
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiological agent of COVID-19 , is considered a pathogen with animal origin, mainly transmitted human to human. It has been experimentally and naturally demonstrated that several animals can be infected by SARS-CoV-2. There are strong evidences that minks are highly susceptible to SARS-CoV-2 infection, since several cases of human to mink infection have been reported, and it has been suggested mink to human infection exists, which so far it is the most reliable example of a zoonotic event of COVID-19 . However, all these cases reported are form mink farms, with the exception of one case in the USA in which the virus was detected in a mink located in the wild, but it was demonstrated that the animal was

infected on a fur farm. In the present work, we have detected SARS-CoV-2 RNA in two wild American minks (*Neovison vison*) in Valencian Community (Eastern Spain) during invasive species trapping campaigns. The animals were trapped from areas known for harbouring self-sustained populations, far away from the nearest fur farm. SARS-CoV-2 RNA was detected in mesenteric lymph nodes samples by RT-PCR. A partial region of the Spike protein gene was amplified and sequence obtaining a 397 nt size sequence. Phylogenetic analysis shown that both sequences were identical to the consensus variant SARS CoV-2 sequence (from Wuhan). This research describes the first infection report of a true wild American mink not related to infected fur farms or direct contact with humans, which is believed to be the first example of wild animals in which SARS-CoV-2 has been detected.

1. Introduction

COVID-19   is an emerging infectious disease with a probable zoonotic origin [1,2].   It is assumed that the virus was originated in wild animals, transmitted to humans through an intermediate host that is still unclear [3], and then caused a global pandemic through human-to-human transmission [4]. To date, several species have been reported to be susceptible to SARS-CoV-2 infection, therefore, crossing the species barrier [5]. Experimental infections have revealed different levels of sensitivity among animal families, pointing out that some taxonomic groups showed low susceptibility to the infection, such as poultry, cattle or swine [6,7]. In contrast, Felidae and Mustelidae are particularly vulnerable to the contagion [8]. The SARS-CoV-2 outbreak and spreading in human populations has resulted in the transmission to animals closely related with people [9]. Consequently, an increasing number of publications are reporting natural infections in several species. The SARS-CoV-2 has been detected in pet and stray cats (*Felis silvestris catus*) [5], tigers (*Panthera tigris*) and lions (*Panthera leo*) from zoos [10], domestic ferrets (*Mustela putorius furo*) [11,12] and American mink (*Neovison vison*) from the fur industry [13].

Outbreaks in American mink fur farms have been particularly common, being reported initially in the Netherlands [14] then noted in several other countries such as Denmark [15,16], France [17], Greece [18], Italy [19], Lithuania [20], Poland [20], Spain [21] or Sweden [22] and from North America, both Canada [23] and the United States [24] through infected workers [25]. Authorities in some of those countries have already started mass culling to prevent human spread of the virus from the animals [13]. To date, there has been only one report of an infected animal found in the wild. A presumed escaped American mink in Utah (USA) trapped in the surroundings of an affected commercial mink farm positive to SARS-CoV-2 [26]. The virus was found to be indistinguishable from that characterized on the nearby affected farm [25].

The American mink it is considered an alien species in the European continent, potentially able to colonise new environments, to displace critically endangered species such as the European mink (*Mustela lutreola*) [27,28] or to predate on the vulnerable Pyrenean desman

(*Galemys pyrenaicus*) [29] and the Southern water vole (*Arvicola sapidus*) [30]. Thus, it is included in the Spanish Catalogue of Exotic Invasive Species through Royal decree 630/2013 (available at https://www.boe.es/eli/es/rd/2013/08/02/630) and subject to eradication through trapping and culling [31]. Wild born mink populations, founded by farm-escaped animals, have proved to be self-sustaining without a continuous influx of captive-born escaped individuals [32].

The aim of this report is to describe the first detection of SARS-CoV-2 in two wild American minks (*Neovison vison*) without direct human contact nor related to fur farms. Furthermore, these are the first reported cases of wild animals tested positive for SARS-CoV-2 by reverse transcription polymerase chain reaction (RT-PCR).

2. Materials and Methods

2.1 Study area

The Valencian Community is located in the East of the Iberian Peninsula (Figure 1 insert). Free American mink have been reported in this area from late 1980s [33], as a consequence of accidental escapes or intentional releases from fur farms located in the territory or in neighbouring provinces (Figure 1). As a result, several stretches of rivers (river Mijares, Palancia, Turia and others) host stable and even expanding populations [33].

2.2 Sample collection

Between November 2020 and January 2021, thirteen (five females and eight males) trapped and humanely sacrificed American mink were transported to "El Saler" Wildlife Rescue Centre (Valencia) and kept frozen at -20 ºC. Necropsy was performed in the "El Saler" facilities by veterinary staff. Biological data (sex, age and reproductive status) were registered (Table 1). Mesenteric lymph nodes samples were collected and placed in plastic tubes with 200 µL of viral inactivated liquid and stored frozen at -80 ºC for further analysis.

2.3 Molecular analysis

The collected samples were tested for SARS-CoV-2 by RT-PCR using different methods. For this purpose, frozen tissues were thawed at room temperature, and each tissue was homogenized manually (10 % w/v) with the inactivated liquid previously mentioned, in a Potter-type homogenizer. After being used, each homogenizer was individually hypochloride-treated and then sterilized at 121 ºC for 15 min in an autoclave.

RNA from 200 µL of the homogenate was extracted by NZY Total RNA Isolation kit (NZYtech, Portugal) following the manufacturer's instructions. Extracted RNA was eluted in 50 µL of RNase-free water and stored at −80 °C. cDNA was generated from the extracted RNA using NZY First-Strand cDNA Synthesis Kit (NZYtech, Portugal) according to the manufacturer's instructions. Reverse-transcribed products were stored at −20 °C. The RNA isolated from both lymph nodes were analyzed to detect SARS-CoV-2 using Viasure RT-qPCR kit (CerTest Biotec, Spain); a commercial, sensitive and specific RT-PCR for SARS CoV-2 diagnosis that has been approved by Emergency Use Authorization (EUA) by FDA [34]. This RT-PCR targets two fragments of ORF1ab and nucleocapsid (N) protein genes of SARS-CoV-2 respectively, and Rnase P used as internal control. The RT-PCR was performed, following manufacturer's manual (https://www.certest.es/wp, 2020), in a AriaMx Real-Time PCR (qPCR) Instrument. The positive and negative controls are

included in the commercial kit. Subsequently, a pair of primers that amplified a 397 nt region of the Spike glycoprotein (S) gene of SARS-CoV-2 were used to screen the tissue samples: 5'-CCGCATCATTTTCCACTTTT-3' the forward primer and 5'-AAACAGTTGCTGGTGCATGT-3' the reverse primer. This PCR had been designed to target an S gene region where the main mutations of the different known variants are present (22728 nt to 23124 nt). For the qPCR, 2 μL of cDNA was amplified in a 20 μL reaction mixture containing 10 μL NZYSpeedy qPCR Green Master Mix (2x) (NZYtech, Portugal), 400 nmol of forward primer per reaction and 400 nmol of reverse primer per reaction. qPCR was performed as follows: (i) 2 min at 95 °C; (ii) 40 cycles, with 1 cycle consisting of 5 sec at 95 °C and 30 sec at 60 °C; (iii) 30 sec at 95 °C; (iv) 30 sec at 65 °C; (v) 30 sec at 95 °C. Amplified DNA products were analyzed by agarose gel electrophoresis and DNA sequencing. To avoid possible contamination, RNA extraction, reverse transcription-PCR (RT-PCR), and gel electrophoresis were performed in separate laboratories. In addition, water controls were included in each run of the RT-PCR assay, and no false-positive result was observed in the negative-control reactions.

2.4 Sequencing and phylogenetic analysis

For sequencing purposes, the amplified S fragments were purified with the NZYGelpure (NZYtech, Portugal K), and they were submitted to a Sanger sequencing service performed by an ABI Prism 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA).

The partial S gene sequences were subjected to BLASTN analyses (http://blast.ncbi.nlm.nih.gov) to search for the related SARS-CoV-2 sequences in GenBank. The nucleotide and inferred amino acid (aa) sequences were aligned, compared with the retrieved sequence data and analyzed by the BioEdit ver. 7.2.5 software [35]. The phylogenetic analyses were carried out using the MEGA X software [36]. P-distance matrices were calculated, and tree topology was inferred by Maximum Likelihood based on p-distances (bootstrap on 2000 replicates, generated with a random seed). This sequence data has been submitted to the GenBank/EMBL/DDBJ databases with the following accession number: MW741755.

Figure 1. Study Area
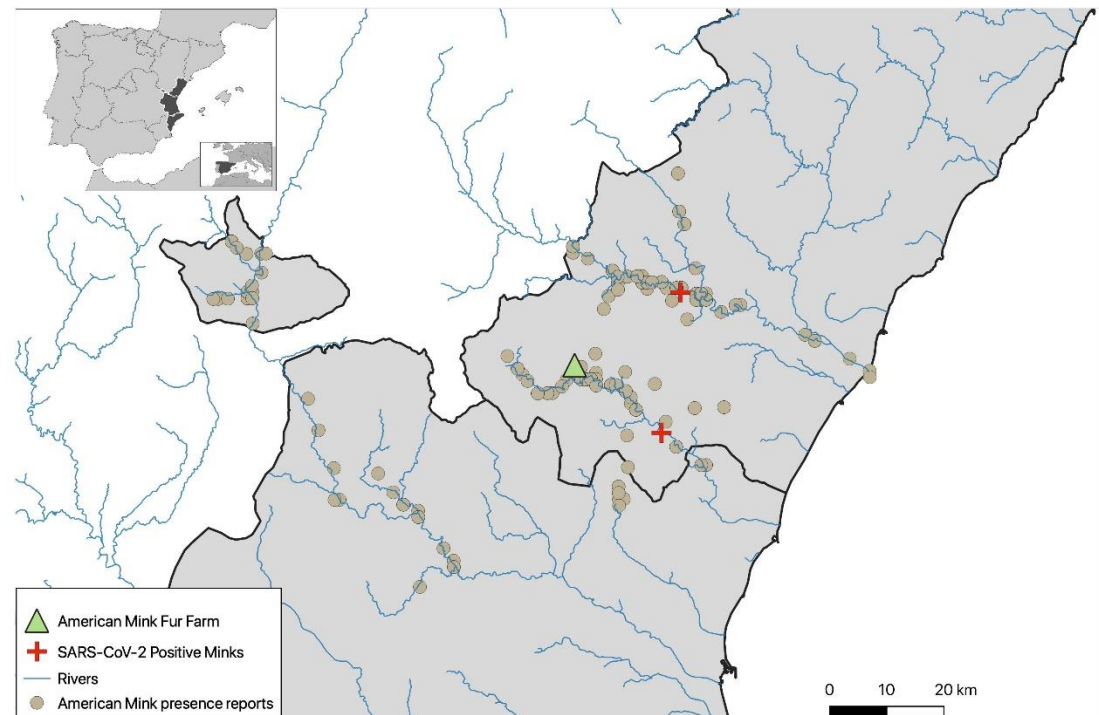
3. Results

3.1 Animals

Out of the thirteen mesenteric lymph node samples analysed, those coming from two adult males, tested positive for SARS-CoV-2 infection (individuals 5 and 11). Individual 5 was trapped in a river located 19.4 kilometers away from the closest fur farm, while individual 11 was located in a different river, 24.6 kilometers away from individual 5 and 22.5 kilometers from the nearest farm. Both animals came from areas with known stable American mink populations and a long story of colonization (Figure 1).

None of these two animals showed macroscopic lesions compatible with clinical disease, although the animals when frozen and thawed, some lesions could be lost.

3.2 Molecular, sequencing and phylogenetical results

On virological analyses all animals were negative for SARS-CoV-2 detection by real-time PCR (RT-PCR), using one of the commercial kit available, Viasure RT-qPCR kit (CerTest Biotec, Spain), while two animals (individual 5 and 11) tested positive using the qPCR that targets 397 nt of the S gene. Although in both animals viral load was considerably low (Ct of 30.04 and 35.09, respectively), all positive samples were confirmed by subsequent analysis in an agarose gel electrophoresis and DNA sequencing.

The Sanger sequencing of the partial S gene amplicon of both positive samples yielded the same sequence, which clearly evidenced that the same SARS-CoV-2 variant had infected these two animals. Compared to the SARS-CoV-2 sequences deposited in the GenBank (http://www.ncbi.nlm.nih.gov/nuccore), our present sequences displayed high nucleotide identities with the consensus SARS-CoV-2 sequence (Wuhan) (GenBank accession numbers NC045512.2) corresponding to 100% identities in the compared partial S gene sequence.

The S gene sequence displayed complete nucleotide identity with the S gene sequence isolated from a Danish mink farm outbreak (GenBank MT919536), as well as 99.71 % nucleotide identity with other S gene sequences from American minks from USA (MW562304).

In turn, if we compare with the different SARS-CoV-2 variants that are circulating, the partial S gene sequence showed 99.71 % nucleotide identity with the variant 20I/501Y.V1 and 99,58 % with variants 20H/501Y.V2 and 20J/501Y.V3.

The phylogenetic trees based on the partial S gene sequences support a unique clade that encompasses all SARS-CoV-2 sequences, where our mink sequence is found (Figure 2). A p-distance value close to 0 was observed in the tree for the S gene of our mink sequence and for other SARS-CoV-2 sequences, as the consensus (GenBank NC5512.12), other mink sequences (Denmark and USA) and for other SARS-CoV-2 variants (UK, South African, Brazilian). In contrast, the p-distance values for the S gene sequences of other coronaviruses as SARS ($p \approx 0.28$, AY572035) and MERS ($p \approx 0.96$ JX869059), were higher and they are forming a different cluster in the phylogenetic tree. Interestingly, the p-value was lower for the sequences from bat SARS-related coronavirus (SARSr-CoV) strain RaTG13 that affect humans in 2012 ($p \approx 0.17$ MN996532), than for those of the SARS and MERS sequences.
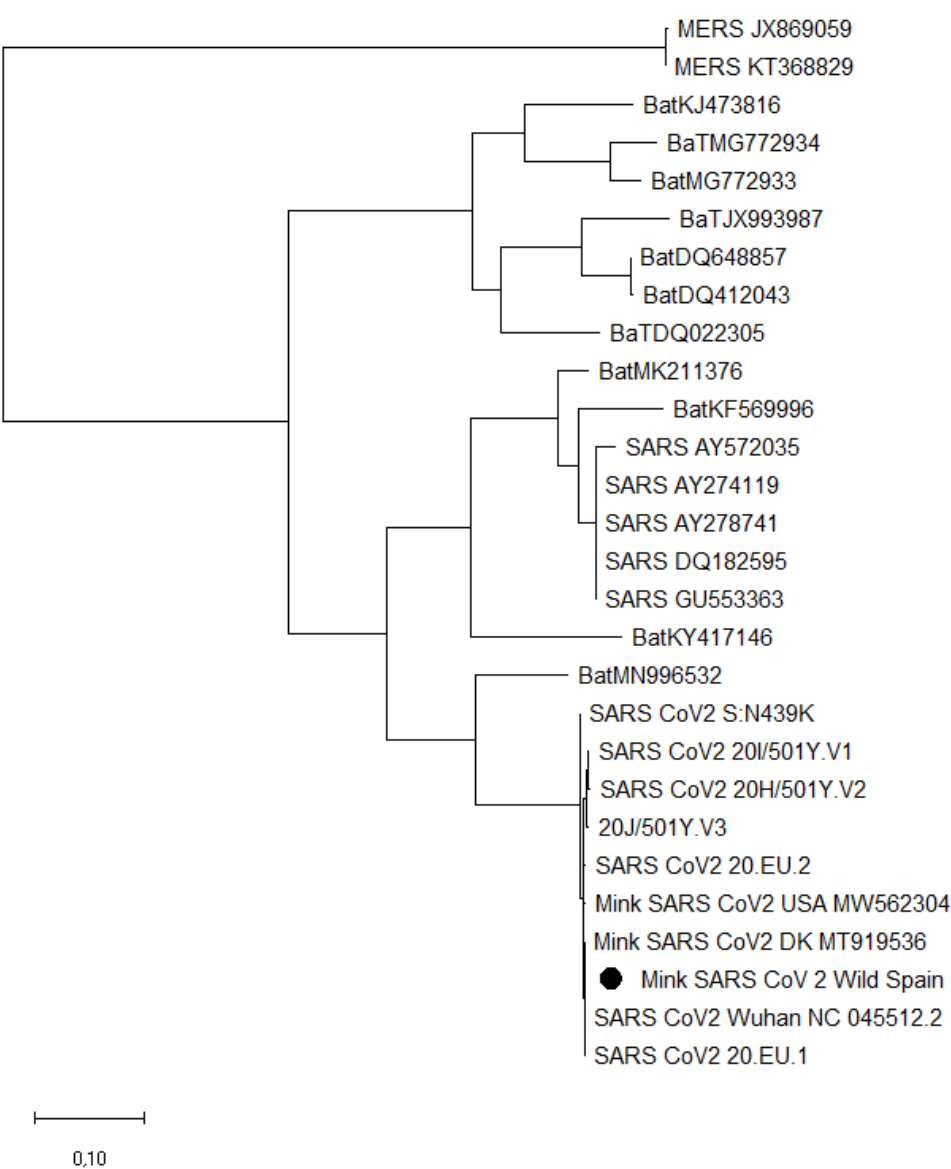
Table 1: Animal information. Capturing date, Riverbed location, Sex, coordinates X and Y, and city that belonged that coordinates of the thirteen American minks (*Neovison vison*) arrested in Valencian Community, Spain, described herein.

Animal	ID	Capturing Date	Riverbed	Sex	*Coord X	*Coord Y	City
1	130	1-Feb-2021	Palancia	M	716595	4413436	Segorbe
2	129	21-Jan-2021	Mijares	F	429691	4433165	Fanzara
3	125	20-Nov-2021	Mijares	F	715106	4438807	Arañuel
4	134	28-Jan-2021	Palancia	M	720334	4411080	Soneja
5	136	28-Jan-2021	Palancia	M	719168	4411224	Soneja
6	135	29-Jan-2021	Palancia	M	716595	4413436	Segorbe
7	131	23-Nov-2021	Mijares	F	737126	4429832	Onda
8	127	20-Nov-2021	Mijares	F	729691	4433165	Fanzara
9	132	19-Jan-2021	Mijares	M	741997	4429482	Onda

10	126	18-Nov-2021	Palancia	M	706960	4421281	Jérica
11	137	14-Jan-2021	Mijares	M	722497	4435770	Torrechiva
12	133	14-Dec-2021	Palancia	F	714680	4416113	Segorbe
13	128	1-Feb-2021	Palancia	M	721249	4410329	Soneja

*Coord X and Y: Coordinates where the animals were arrested

Figure 2. Molecular Phylogenetic analysis by Maximum Likelihood method in a fragment of SARS CoV-2-
Maximum likelihood tree based on a 397-nucleotides (nt 22728–23124) fragment of SARS CoV-2 Spike glycoprotein(S) gene sequence. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-2887,78) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 28 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 333 positions in the final dataset. Evolutionary analyses were conducted in MEGA X . Black dots (●) indicate the sequence obtained from two wild American minks (*Neovison vison*) from Valencian Community, Spain.

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┌ MERS JX869059
└ MERS KT368829
        ┌ BatKJ473816
        ├ BaTMG772934
        └ BatMG772933
        ┌ BaTJX993987
        ├ BatDQ648857
        └ BatDQ412043
        └ BaTDQ022305
        ┌ BatMK211376
        ├ BatKF569996
        ├ SARS AY572035
        ├ SARS AY274119
        ├ SARS AY278741
        ├ SARS DQ182595
        └ SARS GU553363
        ├ BatKY417146
BatMN996532
SARS CoV2 S:N439K
SARS CoV2 20I/501Y.V1
SARS CoV2 20H/501Y.V2
20J/501Y.V3
SARS CoV2 20.EU.2
Mink SARS CoV2 USA MW562304
Mink SARS CoV2 DK MT919536
● Mink SARS CoV 2 Wild Spain
SARS CoV2 Wuhan NC 045512.2
SARS CoV2 20.EU.1
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├────────┤
   0,10

4. Discussion

Unlike previous studies reporting infected American minks, the individuals tested positive for SARS-CoV-2 RNA in this work apparently were not related with fur farms or recent escapes, since the closest fur farm has not reported scapes during the COVID-19 pandemic neither positive cases have been detected in their facilities. Both animals were captured away from farms and in two different areas that are well known to host stable American mink populations. It has been shown that wild born population can self-sustain and given that the linear home range of residential males in Mediterranean Spain is 0.89 ± 0.46 km [37], we can hypothesize that these two individuals were born in the wild. Outbreaks in fur mink farms have shown macroscopial lesions (nasal discharge, pneumonic foci, intestinal pneumonia) on animals affected [14], while the animals reported in this work did not show any alterations, altough it cannot be discarded due to the preservation process of the carcasses. The source of infection of these two individuals

remains unknown, but since the two animals were trapped in different rivers, the solitary biology of the species [38], and its most aquatic ecology, the direct infection between animals seems unlikely. Another possible origin for this infection could be, due to the high dependance of American mink on aquatic environments [37], the exposure to wastewaters. The levels of community transmission during the human outbreak of COVID-19   have been assessed by detection of the coronavirus in wastewater [9,39]. Inappropriate management or leaks from sewage facilities can produce the release of wastewater to surface water bodies [40] converting this type of event into a potential source of infection. Furthermore, these two animals were trapped on the 14th and 28th of January 2021 (Table 1) when the accumulated COVID-19   infection indexes in Valencian Community were 700 and 1459,82 per 100000 inhabitants respectively [41]. These two data represent the maximum levels of infection that have been recorded in Valencian Community during the entire pandemic period, and this is consistent with the high viral load in wastewater that was at a maximum level, as can be seen in public reports from Valencia City for those days [42]. Therefore, this data could support the hypothesis of the natural infection of these two animals by wastewater source.

We have been able to detect SARS-CoV-2 in low viral loads (at 30 and 35 Cts) in two out of the thirteen captured minks, but we hypothesized that these two animals probably had a higher viral load at the moment of death, since samples were frozen and thawed several times before RNA purification was performed. RNA is very vulnerable to degradation by RNAases [43] if the biological samples are not kept properly and this can interfere with the sensitivity of molecular biology techniques [44]. Actually, we cannot discard the possibility of false negative results for the other 11 animals, with a hypothetically low viral load at the moment of death, decreasing even more due the sample storage and the RNA degradation, making undetectable the levels of infection by molecular techniques. Additionally, we could not detect SARS-CoV-2 in these samples using one of the commercial kits available in the market. Viasure SARS-CoV-2 RT-qPCR kit (CerTest Biotec; Spain) includes "ORF1ab" and "N" probes for SARS-CoV-2 detection with levels of sensitivity and specificity close to 100 %. This negative result could be explained, on one hand, due to the low viral load detected, as we have just explained and, on the other hand, because of the high level of specificity of the commercial kit. The mutation of a single nucleotide in the sequences of the regions used for detection, might cause the failure of the primers or the probes because of the short length of the fragment (max 150 nt). However, as we have shown in the results, when we used a qPCR with lower specificity, avoiding the use specific fluorescent probes and using instead non-specific fluorescent dyes that intercalate with any double-stranded DNA, and targeting a bigger region (around 400 nt), we were able to detect a fragment of the S gene in two of the minks. Still, with this technique we got low viral load, which made the isolation of the fragment difficult for sequencing, but after concentrating the samples with an increased amount of cDNA, we got enough quantity for the sequencing, eventually obtaining a really clean sequence. We mentioned this outcome in the results, but we discarded the contamination because the sequence of other samples that we used (different species), was different to the sequence that we obtained in the minks. Finally, it is important to highlight the type of sample used in this work. We have detected SARS-CoV-2 in the mesenteric lymph node, so it could be possible that these two animals had an active

infection with SARS-CoV-2. The only explanation for the detection of the coronavirus in lymph nodes non related to the respiratory system [45] (in minks also produce respiratory problems [15]), is if the virus replicates and produces viremia.

The phylogenetic analysis shows that the partial S gene sequence obtained in both animals was exactly the same (and different to the other positive samples from other species included in the study) and identical to the consensus sequence (Wuhan), like the sequences found in minks from Danish farms [45]. So far, the sequences analyzed from other farmed minks infected with SARS-CoV-2 have shown only one synonymous mutation on the S gene fragment that we were able to obtain (GenBank MW562304). Therefore, taking into account these results, we could hypothesize that the virus responsible for the infection of minks, in farms or in the wild, is not different from the human variant, at least in the S gene sequence. However, further investigation is needed after getting the complete genome of the SARS-CoV-2 detected in wild minks to confirm the hypothesis. Finally, we wonder why we did not obtain any of the most recent and new SARS-CoV-2 variants (20I/501Y.V1, 20H/501Y.V2 and 20J/501Y.V3) in the two positives animals, since the S gene sequence analyzed contained some of the canonical mutations related to these variants. The most reasonable explanation is that the prevalence of these new variants in Valencian Community is still quite low, especially at the time that the animals were captured [46].

In the case where the SARS-CoV-2 transmission spectrum extends to the wild mustelids, such as reported here, the animals could later develop into permanent reservoir hosts and would therefore be able to transmit the infection to human beings and other susceptible animal species [47], including those with conservation concerns [48].

Finally, as previously shown, SARS-CoV-2 possesses panzootic potential [47,49] owing to its broad host range and the inherent ability to cross the 'species barrier', thereby highlighting the need for a One Health approach [50]. The economic trade involving wild and exotic animals has also resulted in a closer human-wild animal interaction and it poses a considerable threat via possible transmission of new viruses into the human population [51].

5. Conclusions

The detection of SARS-CoV-2 in free American mink shows that natural infection of susceptible wildlife is possible and highlights the importance of indirect routes of transmission, presumably wastewater, as a source of contagion. At the same time, points to this species as a bioindicator of the environmental levels of viral contamination. Further research in a higher number of specimens, coming from all river lengths should be done, complemented with the analysis of water samples taken *in situ*. Additionally, the complete genome should be obtained in further studies to compare with all the SARS-CoV-2 variants and understand better the origin of this infection.

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