

1 **Investigations on SARS-CoV-2 and other coronaviruses in mink farms in France at the end of the**
2 **first year of COVID-19 pandemic**
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25 **Abstract**

26 Soon after the beginning of the COVID-19 pandemic in early 2020, the *Betacoronavirus* SARS-CoV-2
27 infection of several mink farms breeding American minks (*Neovison vison*) for fur was detected in
28 several countries of Europe. The risk of a new reservoir formation and of a reverse zoonosis from minks
29 was then a major concern. The aim of this study was to investigate the four French mink farms for the
30 circulation of SARS-CoV-2 at the end of 2020. The investigations took place during the slaughtering
31 period thus facilitating different types of sampling (swabs and blood). In one of the four mink farms,
32 96.6% of serum samples were positive in SARS-CoV-2 ELISA coated with purified N protein recombinant
33 antigen and 54 out of 162 (33%) pharyngo-tracheal swabs were positive by RT-qPCR. The genetic
34 variability among 12 SARS-CoV-2 genomes sequenced in this farm indicated the co-circulation of
35 several lineages at the time of sampling. All SARS-CoV-2 genomes detected were nested within the
36 20A clade (Nextclade), together with SARS-CoV-2 genomes from humans sampled at the same period.
37 The percentage of SARS-CoV-2 seropositivity by ELISA varied between 0.5 and 1.2% in the three other
38 farms. Interestingly, among these three farms, 11 pharyngo-tracheal swabs and 3 fecal pools from two
39 farms were positive by end-point RT-PCR for an *Alphacoronavirus* highly similar to a mink coronavirus
40 sequence observed in Danish farms in 2015. In addition, a mink *Caliciviridae* was identified in one of
41 the two positive farms for *Alphacoronavirus*. The clinical impact of these unapparent viral infections is
42 not known. The co-infection of SARS-CoV-2 with other viruses in mink farms could contribute to explain
43 the diversity of clinical symptoms noted in different infected farms in Europe. In addition, the co-
44 circulation of an *Alphacoronavirus* and SARS-CoV-2 within a mink farm would increase potentially the
45 risk of viral recombination between alpha and betacoronaviruses already suggested in wild and
46 domestic animals, as well as in humans.

47

48 **Author summary:**

49 France is not a country of major mink fur production. Following the SARS-CoV-2 contamination of mink
50 farms in Denmark and the Netherlands, the question arose for the four French farms.

51 The investigation conducted at the same time in the four farms revealed the contamination of one of
52 them by a variant different from the one circulating at the same time in Denmark and the Netherlands
53 mink farms.

54 Investigation of three other farms free of SARS-CoV-2 contamination revealed the circulation of other
55 viruses including a mink *Alphacoronavirus* and *Caliciviridae*, which could modify the symptomatology
56 of SARS-CoV-2 infection in minks.

57

58 Keywords: American mink, *Neovison vison*, SARS-CoV-2, *Alphacoronavirus*

59

60 1. INTRODUCTION

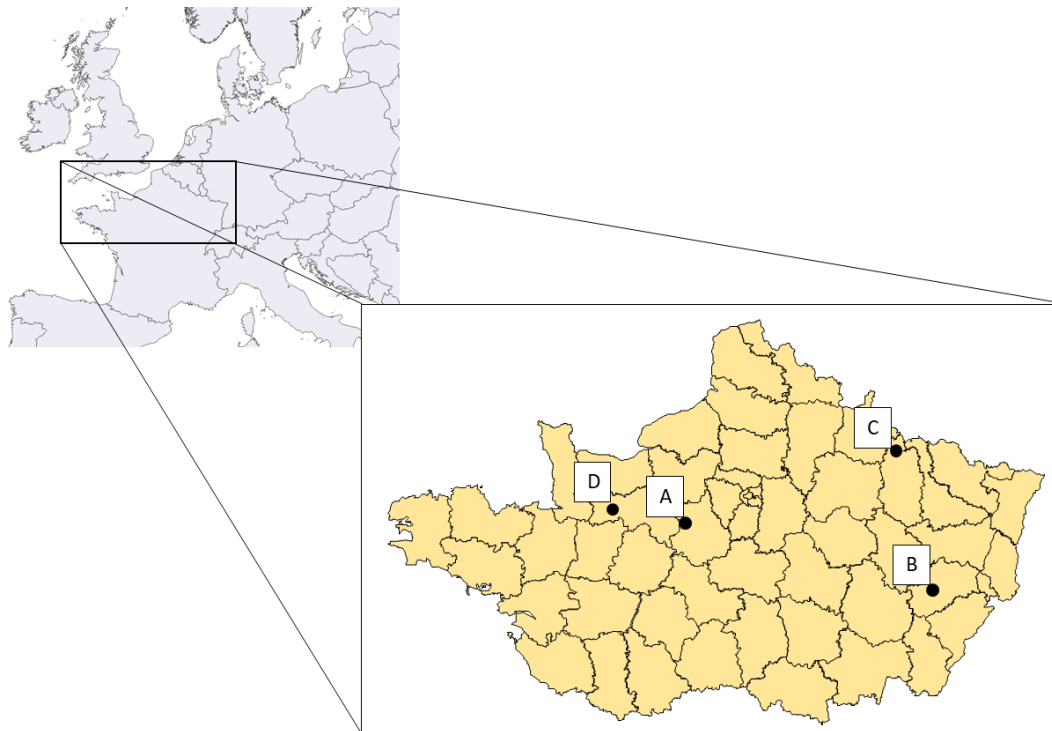
61 Soon after the beginning of the COVID-19 pandemic in early 2020, the SARS-CoV-2 infection of several
62 mink farms breeding American minks (*Neovison vison*) for fur, was detected in Europe, in the
63 Netherlands first (1), in April 2020, and then in Denmark (2). Infections with SARS-CoV-2 in mink farms
64 were then detected in several other countries in Europe: in Italy, Spain, Poland, Greece, Lithuania and
65 Sweden (3). In Denmark, SARS-CoV-2 has spread rapidly in each farm and among mink farms, and was
66 associated with the emergence of a specific variant (called cluster 5) detected in November 2020. At
67 the time, this SARS-CoV-2 variant was believed to present different phenotypic characteristics
68 including escape from neutralizing antibodies (4,5). Moreover, if the primary contamination of the
69 mink farms was due to human infection (6), back transmission from minks to humans was detected in
70 the Netherlands (7) and Poland (8).

71 In early 2020, four American mink farms of limited size (less than 1000 to about 15,000 minks
72 (according to the French Ministry of Agriculture) were in operation in France. These farms were located
73 in different regions. No clinical sign had been observed in minks from these farms since the beginning
74 of the pandemic. The aim of the present study was to investigate the circulation of SARS-CoV-2 and
75 potentially other coronaviruses in the four French mink farms. As mink farming has a seasonal
76 production, with slaughtering of the young adults of the year at the end of the same year, the
77 investigations took place at the end of 2020 to conduct the most exhaustive survey as possible on the
78 individuals born in 2020. Different types of samples were collected during the slaughtering period.
79 Serology and viral RNA detection in the upper and lower respiratory tract and in feces were performed
80 as well as Bayesian analysis to determine the potential circulation of different lineages in French mink
81 farms.

82 2. RESULTS

83 2.1. Sample collection

84 The four American mink farms present in France (named A to D) were investigated (Figure 1).



85

Fig.1: Location of the American mink farms in France in November 2020. (A) Eure-et-Loir, (B) Haute-Saône, (C) Meuse and (D) Orne.

86 A total of 1912 minks born in 2020 were sampled for blood, with a minimum of 60 animals per building,
87 and 1643 pharyngo-tracheal swabs were collected. Characteristics of the farms and of the sampling
88 are presented in Table 1.

89 **Table 1:** Location (department) of mink farms, number of blood samples by route of sampling, swab samples
90 and fecal samples collected in November 2020 in France on adult minks of the year

Farm	Minks present in the farms	Nb. of scanstars	Nb. scanstars sampled from housings with slaughtered minks for fur	Blood samples		Pharyngo--tracheal swabs	Nb. Scanstars sampled from housing with unslaughtered minks for breeding	Fecal samples (nb. of pools collected in each scanstars)
				Intra-cardiac puncture	Retro-orbital			
A	3,800	3	3	179		162	0	0
B	950	2	2	120		120	0	0
C	7,900	15	9		403	395	6	56* ¹
D	10,850	26	20	1210		1230	6	34* ²
Total	23, 500	47	34	1912		1643	13	90

91 *¹ number of fecal samples collected / number of minks sampled: 1-10 / 2-5 animals

92 *² number of fecal samples collected / number of minks sampled: 4-7 / 10 animals

93 2.2. Anti-SARS-CoV-2 antibodies detection (ELISA and seroneutralization test)

94 All mink blood samples were tested by a SARS-CoV-2 ELISA coated with purified N protein recombinant
95 antigen. Among them, samples presenting a doubtful or positive result were systematically tested
96 again by seroneutralization assay to confirm or overturn the ELISA results.

97 For farm A, where evidence of ongoing infection was noted (SARS-CoV-2 RNA was found in pharyngo-
98 tracheal swabs), we randomly selected 16 samples among the positive sera to be tested by
99 seroneutralization assay. For farms B, C and D, samples that were negative by ELISA were also tested
100 by seroneutralization assay as negative controls.

101 The results of the ELISA are shown in Table 2. The percentage of seropositivity varied between 0.5 and
102 1.2% in non-infected farms and reached 96.6% in the infected farm (A). For farm A, the S/P% values of
103 positive samples ranged from 62.3 to 492.6 with mean value equal to 307.9 (N=173).

104

105 **Table 2:** Serological results (positive/doubtful/negative) obtained by ELISA on mink serum samples collected in
106 the four mink farms in France in November 2020

Farm	Samples	Positive	Doubtful	Negative	% of positive (including doubtful results)
A	179	173	1	5	96.6
B	120	1	0	119	0.8
C	403	1	1	401	0.5
D	1210	13	1	1196	1.2

107

108 The samples found positive or doubtful from farms B, C and D, as well as a few randomly selected
109 positive samples from farm A, were tested by seroneutralization assay (Table 3).

110 **Table 3:** Serological results obtained by using the seroneutralization assay on ELISA positive/doubtful mink
111 serum samples collected in the four mink farms in France in November 2020

Farm	Samples	Positive	Negative	% of positive
A	16	16	0	100
B	1	0	1	0
C	2	0	2	0
D	14	0	14	0

112

113 For the three non-infected farms, no SARS-CoV-2 neutralizing antibody was detected by
114 seroneutralization assay in the positive or doubtful sera obtained by ELISA test (Table 3). However, for

115 farm A, SARS-CoV-2 neutralizing antibodies were detected in the 16 ELISA positive samples.
116 Neutralizing titers ranged from 81 till above 2239, confirming the circulation of the virus in this farm.

117 **2.3 Detection of SARS-CoV-2 viral RNA in pharyngo-tracheal swabs**

118 The TaqMan RT-qPCR analysis of pharyngo-tracheal swabs revealed that farm A was experiencing an
119 ongoing SARS-CoV-2 outbreak: of the 162 minks tested from farm A, SARS-CoV-2 RNA was detected in
120 54 swabs at the date of sampling (Table 4). Of the 54 positive samples, 33 of them showed low levels
121 of viral RNA (Ct value > 32, which corresponds to approximately 0-5copies/ μ L of RNA). The 21 positive
122 minks for SARS-CoV-2 RNA (Ct values ranging from 18.8 to 31) were found positive with RNA titers
123 ranging from 10 to 3,79E+04 copies/ μ L of RNA.

124 No SARS-CoV-2 RNA was detected in samples from the three other farms (n= 1481 samples tested).

125

126 **Table 4:** SARS-CoV-2 RNA detection in pharyngo-tracheal swabs in farm A.

Building number	Detected*	Not detected	NI	Total
1	22	31	2	55
2	17	32	3	52
3	15	37	3	55
Total	54	100	8	162

127 * Detection of SARS-COV-2 RNA with Ct values < 36 and two replicates positive / 2

128 NI: not interpretable (detection in one replicate out of two replicates tested with Ct values varying between 30
129 and 36)

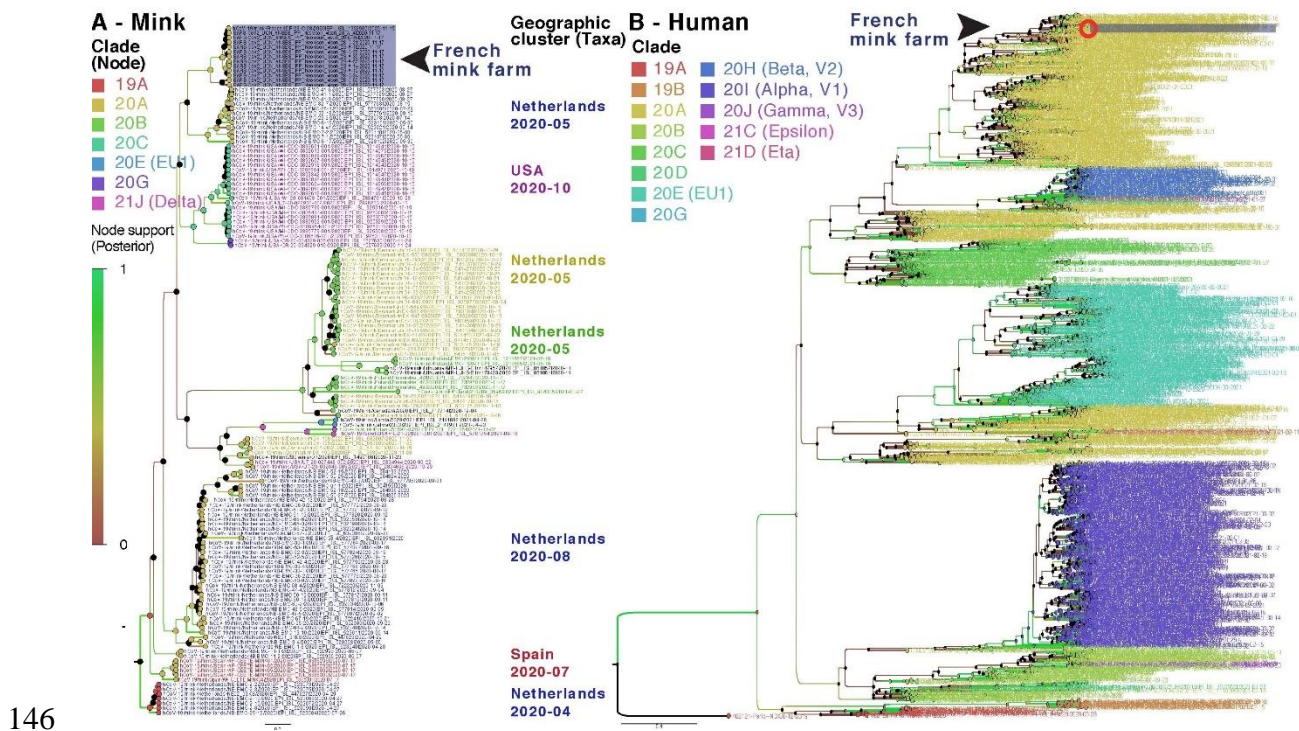
130 Negative: Ct values >39

131

132 **2.4. SARS-CoV-2 genomes**

133 SARS-COV-2 RNA samples with Ct values less than 31 (12.5-3.1E+02 copies/ μ L of RNA) were submitted
134 to full genome analysis. Nearly complete viral genome sequences (coverage > 99.4%, average depth >
135 15 000, maximum depth > 100 000) were obtained from each of the 12 nucleic acid extracts positive
136 for SARS-CoV-2 by RT-qPCR (Ct values of 26.2-30). An additional extract, weakly positive by RT-qPCR,
137 yielded partial sequences corresponding to different regions of the genome. All twelve genomes
138 (GISAID under numbers EPI_ISL_1392906 & EPI_ISL_10036487-97) were classified as 20A based on a
139 panel of mutations compared to reference sequence NC_045512 and a Nextclade analysis (Fig. S1,
140 Table S1). A Bayesian phylogenetic analysis using all high-quality mink derived SARS-CoV-2 genomes
141 available in the GISAID database clustered SARS-CoV-2 from the mink farm in France together in a
142 monophyletic clade supported by significant node value (Posterior probability) and a relatively long

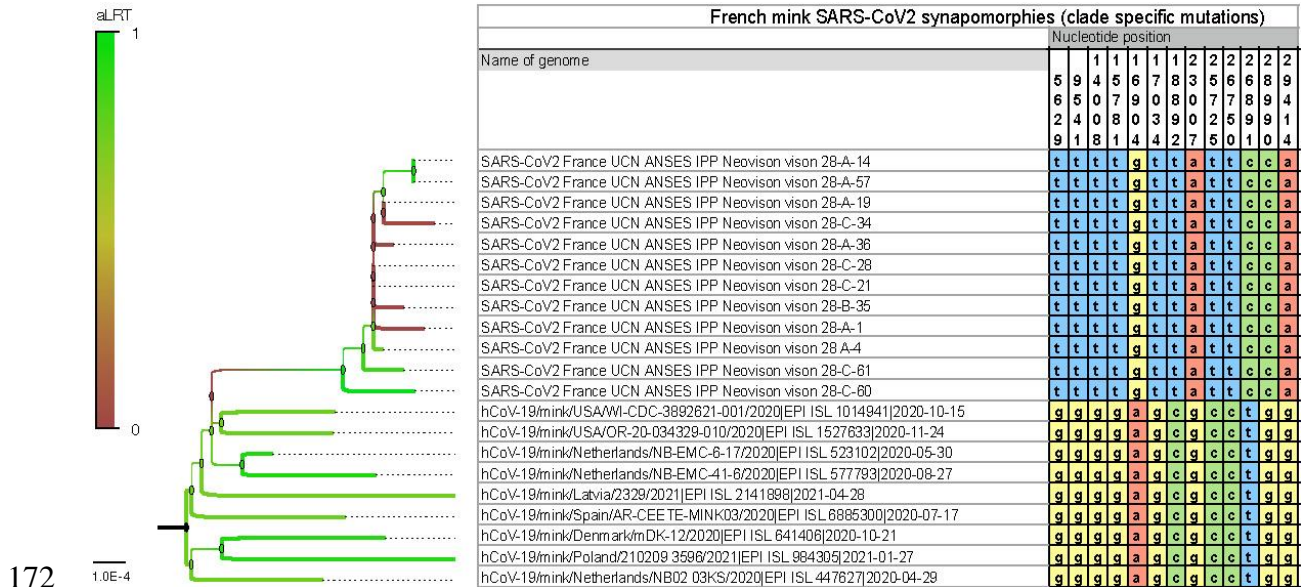
143 branch (Fig. 2). This clear clustering does not link the SARS-CoV-2 from the mink farm in France to any
144 another mink farm.
145



146
147 **Figure 2.** Bayesian phylogenies on a mix of representative set of SARS-CoV-2 sequences available in GISAID. SARS-
148 CoV-2 sequences detected in the mink farm in France are highlighted in purple. **A. Bayesian phylogeny of all**
149 **SARS-CoV-2 genomes from minks over the study period.** Node and taxa labels are colored according to
150 nextstrain (<https://nextstrain.org>) clade classification and main geographic clusters, respectively. **B. Bayesian**
151 **phylogeny of human derived SARS-CoV-2 genomes collected in France and mink derived SARS-CoV-2 genomes**
152 **described in this study.** A diversity optimized dataset was obtained by collecting and filtering complete, high
153 quality genomes, of SARS-CoV-2 detected in France (of Human and mink origin). Taxa are colored to
154 nextstrain clades classification.

155
156 Moreover, thirteen SNPs (Single Nucleotide Polymorphisms) were specific to this clade by contrast to
157 other SARS-CoV-2 clades detected in mink (*Neovison vison*) worldwide at the time of data collection
158 and phylogenetic analyses based on 821 mink SARS-CoV-2 genomes, retrieved on January 5, 2022 (Fig.
159 3; Table S1). Among these SNPs, differentiating mink SARS-CoV-2 sequences from France from other
160 available mink SARS-CoV-2 sequences, 4 induce an amino-acid change (V676L, K1141R, E1184D in the
161 Orf1b and S477N in the Spike). Within this monophyletic clade, genetic variability was observed among
162 SARS-CoV-2 genomes from the French farm (including non-silent mutations), indicating the co-
163 circulation of several variants in this setting at the time of sampling (Tables S1 and S2). A Bayesian
164 phylogenetic analysis using the 2020's year of data from GISAID (437 human SARS-CoV-2 in a diversity
165 optimized matrix based on 99,875 % pairwise nucleotide identity cutoff from 42700 genomes) showed

166 that SARS-CoV-2 genomes detected in the mink farm in France nested within the 20A clade, together
 167 with SARS-CoV-2 genomes from humans sampled at the same period (Fig. S2). In addition, the non-
 168 silent mutation in S (S477N) that differentiates these mink SARS-CoV-2 genomes from that of other
 169 SARS-CoV-2 sampled in mink elsewhere, was also observed in SARS-CoV-2 sampled in humans in
 170 France at the same period (Table S3 and Fig. S2).
 171



172
 173 **Figure 3.** SARS-CoV-2 synapomorphies in American minks from a mink farm in France with the thirteen SNPs specific to the
 174 clade formed by the mink SARS-CoV-2 in France compared to other mink SARS-CoV-2 located elsewhere. SNPs are
 175 represented with the nucleotide positions on the SARS-CoV-2 genome, and in yellow for G, red for A, green for C and blue for
 176 T. Branches are colored according to the bootstrap values.

178 2.5. Detection of an *Alphacoronavirus* in pharyngo-tracheal swabs and feces

179 We analyzed the pharyngo-tracheal swabs and feces for the presence of RNA coronaviruses by end-
 180 point RT-PCR targeting the pol gene. The end-point RT-PCR analysis of pharyngo-tracheal swabs
 181 revealed that two out of three farms were positive for RNA coronaviruses distinct from SARS-CoV-2.
 182 Of 236 swab samples tested, 11 minks were positive for *Alphacoronavirus* RNA, with respectively two
 183 samples in farm C and nine in farm D (Table 5). No coronavirus was detected in swabs from farm B.
 184 Farm A samples were not analyzed for the presence of the *Alphacoronavirus* genome. A total of 90
 185 feces pools (56 from farm C and 34 from farm D) were simultaneously tested by RT-PCR targeting the
 186 pol gene and by beta-actin RT-PCR for investigating the presence of RNA inhibitors. The beta-actin
 187 housekeeping gene RNA was not detected in 46.6% of the fecal samples tested ($n=42/90*100$) with
 188 the highest proportion of not exploitable samples in farm C ($55.4\%=31/56*100$) compared to farm D

189 (32%=11/34*100). Of 48 exploitable feces pools, 3 were positive for *Alphacoronavirus* RNA: one in
 190 farm C and 2 in farm D.

191

192 **Table 5:** Detection of α -CoV in pharyngo-tracheal swabs and feces in negative SARS-CoV-2 mink farms in France

Farm	Nb of buildings	SARS-CoV-2 RNA RT-qPCR on		CoV RT-PCR of pol gene on	
		Pharyngo-tracheal swabs	Pharyngo-tracheal swabs* ¹	Pharyngo-tracheal swabs* ¹	Feces pools* ¹
		Positive/tested (%)	Positive/tested (%)	Positive/tested (%)	Positive/"exploitable" (%)
B	2	0/120 (0)	0/28* (0)		Not tested
C	9	0/395 (0)	2/65* ¹ (3.1)		1/25 * ^{1,2} (4)
D	20	0/966 (0)	9/143* ¹ (6.3)		2/23 * ^{1,3} (8.7)

193 *¹ Randomized collection in different buildings in the farm

194 *² For farm B, of 56 feces pools tested, 31 samples tested negative for the presence of beta-actin gene and were then considered as "not
 195 exploitable". The prevalence was calculated as follows: samples tested positive for the presence of coronavirus RNA / total of "exploitable"
 196 samples *100.

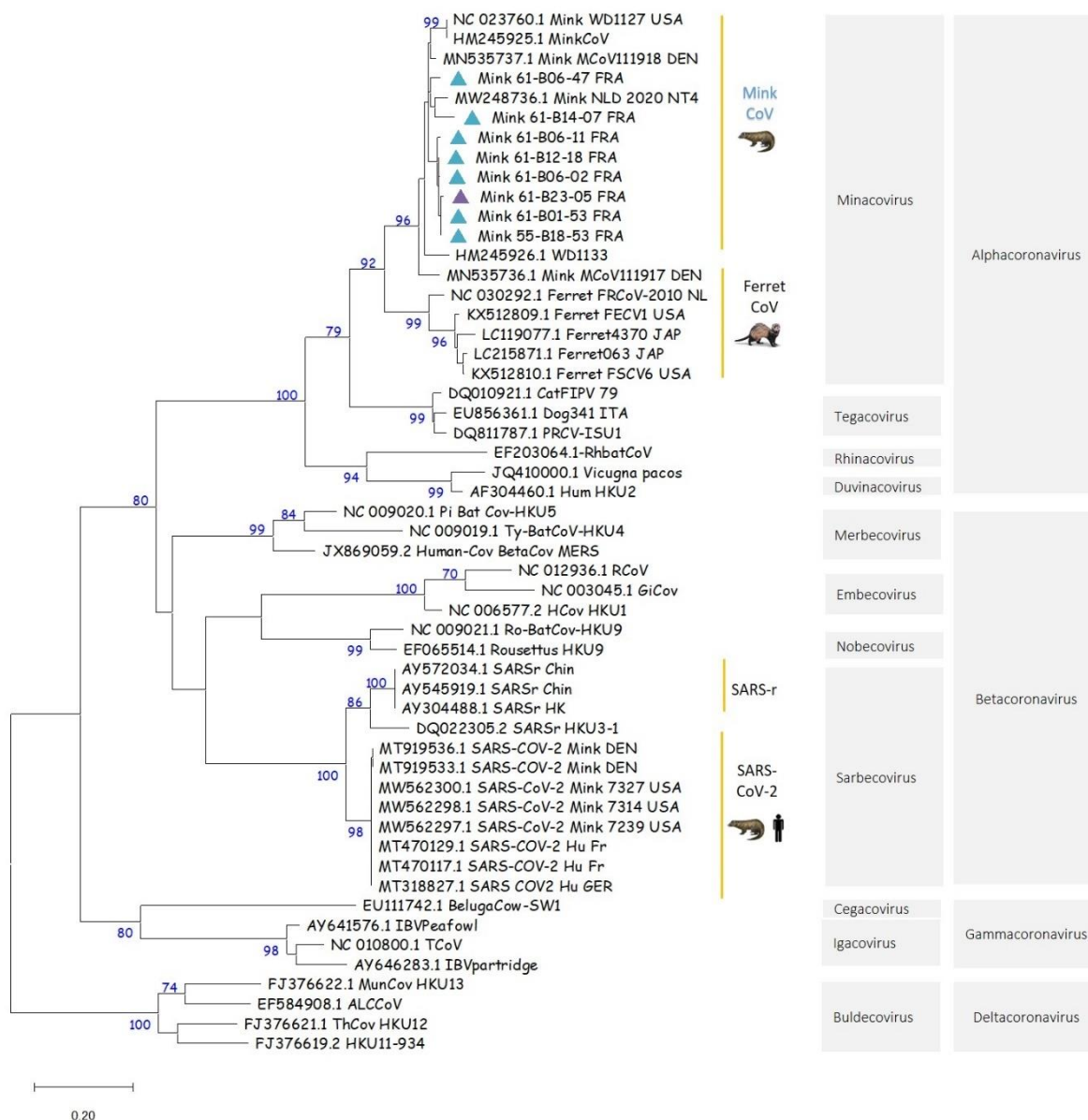
197 *³ For farm C, of 34 feces pools tested, 23 samples tested positive for the presence of beta-actin gene and 11 tested negative (the 11 were
 198 considered as not exploitable" for calculation of the prevalence).

199

200 Of the 14 samples tested positive by RT-PCR for the pol gene, 5 were weakly positive and then were
 201 either not submitted or failed the SANGER sequencing. Sequence analyses of PCR products (n=9)
 202 showed that all sequenced samples (7 pharyngo-tracheal swabs and 2 fecal pools) by SANGER were
 203 alphacoronaviruses. Of the 9 nucleotide consensus sequences, 8 were included in the phylogenetic
 204 analysis, all of them grouped within *Alphacoronavirus*, *Minacovirus* subgenus (bootstrap=95), close to
 205 the mink coronavirus (anciently named ferret coronavirus - Figure 4). The ninth sample 61-B23-2 was
 206 not included in the phylogeny, due to the low quality of the nucleotide sequence obtained by SANGER
 207 sequencing and the fact that we were unable to obtain the consensus sequence for this sample. BLAST
 208 analysis of the forward sequence still showed that the sample 61-B23-2 is closed to both mink
 209 coronavirus 1 MN535737 isolated in Denmark in 2015 and an *Alphacoronavirus* isolated on a mink in
 210 China in 2016 (MF113046). High nucleotide similarities were observed for the Pol gene sequences from
 211 farm C (n=1) and farm D (n=8). A BLAST search showed that the nine strains shared between 94.6% to
 212 96.3 % identity at the nucleotide level with the mink coronavirus 1 (MN535737) strain isolated in
 213 Denmark in 2015.

214 Of the three fecal samples tested positive for the coronavirus pol gene, the sample (61-B23-05) the
 215 most positive was submitted to high throughput sequencing (HTS). The 61-B23-05 extract yielded
 216 many gaps throughout the genome, with partial sequences (i.e. 706, 639, 597, 445, and 206

217 nucleotides) corresponding to five different regions of a Mink strain coronavirus 1 MCoV1/11918-
 218 1/DK/2015 (MN535737.1). BLAST analysis performed on the five partial sequences showed 89-95%
 219 identity with MN535737.1.



220

221 **Figure 4.** Maximum likelihood (ML) phylogeny inferred with 8 mink coronavirus consensus sequences from
 222 France and 45 representative GenBank sequences including alphacoronaviruses (n=25), betacoronaviruses
 223 (n=20) with mink and human SARS-CoV-2 sequences, gammacoronaviruses (n=4) and deltacoronaviruses (n=4).
 224 Bootstrap values above 70% were considered as statistically significant. Pharyngo-tracheal swabs and feces
 225 infected by the mink coronavirus are represented on the tree with a turquoise and purple triangle, respectively.
 226 The 8 partial pol gene consensus sequences included in the ML tree are accessible in GenBank under accession
 227 numbers: Mink_61-B14-07_FRA: ON985270; Mink_61-B12-18_FRA: ON985271; Mink_61-B06-47_FRA:
 228 ON985272; Mink_61-B06-11_FRA: ON985273; Mink_61-B06-02_FRA: ON985274; Mink_61-B01-53_FRA:
 229 ON985275; Mink_55-B18-53_FRA: ON985276; Mink_61-B23-05_FRA: ON985277

230

231 **2.6. Detection of Mink *Caliciviridae* RNA genome in pharyngeo-tracheal samples**

232 Deep sequencing of viral RNA extracted from pharyngeo-tracheal swab samples 61-B06-11 and 61-
233 B12-04 mainly generated bacteria or host (Mustelidae)-originating reads; only 0.06% to 0.7% of the
234 reads were identified as viral sequences with, for sample 61-B12-04, 75% of the reads identified as
235 mink *Caliciviridae*. None of the reads obtained for these two samples corresponded to
236 *Alphacoronavirus* sequences. After the Spades assembly of the 61-B12-04 reads, one contig (4320
237 nucleotide long) was identified as a *Caliciviridae* sequence with 95% identity with the *Caliciviridae*
238 strain Mink/China/2/2016 (MF67785). Several reads were identified as mink *Caliciviridae* in the two
239 samples. The whole genome size for the two samples was of 8,427 nt in length and consisted of three
240 open reading frames and two untranslated regions (5' and 3') of 13 and 103 nt of length, respectively.
241 ORF1 ranged from nt 14 to nt 5851 (encoding a polyprotein of 1,946 aa), ORF2 ranged from nt 5857 to
242 nt 7899 (681 aa), ORF3 ranged from nt 8130 to nt 8306 (59 aa). The alignment of the truncated
243 sequence 61-B12-04 (GenBank Number OP485683) with the MF677852 mink *Caliciviridae* strain from
244 China showed an amino-acid identity of 99.1% along the whole genome), with 99.4 % for ORF1,
245 followed by 98.4% and 98.3 for ORF2 and ORF3, respectively. Finally, BLAST analysis of the 61-B06-11
246 and 61-B12-04 sequences showed > 94.4% (E value=0.0; Score=8167; number of hits =5;
247 4982/5279*100) and 96% (E value= 4e-103; Score= 392; number of hits=5; 230/239*100) of nucleotide
248 identity with the Mink *Caliciviridae* strain from China MF677852, respectively.

249 **3. DISCUSSION**

250 This study describes the investigations on SARS-CoV-2 in the four mink farms in operation in France at
251 the end of the first year of the COVID-19 pandemic. The small number of farms and animals per farm
252 made possible to implement a robust transversal study allowing the detection of a minimum
253 prevalence of 5%. SARS-CoV-2 infected minks in one farm, with a high serological prevalence (above
254 96%) and SARS-CoV-2 RNA was detected in 30% of the sampled minks. Infection had spread in the
255 three buildings of the farm. No clinical sign nor suspicious mortality was observed by the breeder
256 suggesting a mild outbreak which could have been easily missed in the absence of investigations
257 (2,9,10).

258 All twelve SARS-CoV-2 genome sequences obtained, despite some variability, belonged to the 20A
259 clade, according to Nextclade and two Bayesian phylogenetic reconstructions. Given the genetic
260 relationship between the SARS-CoV-2 sequenced in the mink farm in France and the SARS-CoV-2
261 responsible for the concomitant epidemic wave of 20A viruses in humans, as well as the previous
262 description of the zoonotic circulation of SARS-CoV-2 between human and minks in the Netherlands,
263 reverse zoonosis appears as the main hypothesis to explain the SARS-CoV-2 circulation detected in the
264 minks of this farm. In addition, comparison with other SARS-CoV-2 sequenced in minks worldwide

265 clearly shows the monophyly of this mink clade found in France. The significant genetic distance to
266 viruses found in other countries is supported by 13 mutations specific to this clade. These analyses
267 reinforce the hypothesis of a local transmission from human to explain the origin of the circulation in
268 minks in the present study. Among these mink clade specific mutations, four were non-silent and one,
269 resulting in the S477N change in the spike, was also present in several genomes sequenced from
270 humans at the same period in France. Despite substantial genetic variability observed here in mink
271 SARS-CoV-2, data was not discriminant enough to test with confidence the monophyly of this mink
272 clade when analyzed with all available human SARS-CoV-2 from the region and sampled at the same
273 period. Bayesian reconstruction shows apparent paraphyly of the mink clade found in France, with
274 some internal human SARS-CoV-2 sub-clustering, but these nodes are not significant (low posterior
275 probabilities). The dataset does not allow to clearly establish whether one or several reverse-zoonotic
276 events occurred. However, the relative genetic identity of genomes detected in the farm is in favor of
277 a contamination of the farm relatively shortly before the sampling, with a viral circulation probably
278 shorter than 3 months. Indeed, this duration is insufficient to allow the diversification in several clearly
279 distinct lineages as the accumulation is around two mutations a month on average (11). A high number
280 of minks can be infected within a short time: Hammer et al. (9) described an increase of prevalence
281 from 4% to 97% in eight days in minks in one farm in Denmark. The detection of SARS-CoV-2 RNA in
282 pharyngo-tracheal swabs with Ct values varying between 18.8 and 38.4 was the sign that infection was
283 recent in a few minks, especially for minks with low Ct values. Indeed, after experimental infection,
284 viral RNA can be detected in the upper respiratory tract from 2 dpi to 17 dpi of exposed minks(12). As
285 the detection of SARS-CoV-2 in farm A induced immediate control measures (culling of all minks), it
286 was not possible to follow further the genomic evolution of the virus in minks and to test the
287 eventuality of spill-back to humans.

288

289 No SARS-CoV-2 contamination was detected in the three other farms, but less than 1% of the samples
290 in farms B and C, and 1% in farm D were positive by ELISA, despite a negative seroneutralization test.
291 The specificity of the ELISA test in minks is very high, close to 99%, better than in humans (where the
292 specificity is evaluated between 92.5 and 98.8%) (13,14). In farms C and D, we detected an
293 *Alphacoronavirus*, Mink-coronavirus sequence, found in both pharyngo-tracheal swabs and feces.
294 Mink coronavirus infection is associated with epizootic gastroenteritis (ECG) in minks when in
295 combination with several enteric viruses, but it can also be asymptomatic (15). As ECG is an economic
296 concern, several authors in different countries studied the disease characteristics (distribution,
297 lesions...) (15,16). Then, by considering the ECG frequency found in these studies, mink coronavirus
298 infection in fur minks appears to be not so rare, but to our knowledge, little data exist on prevalence.
299 However, the potential circulation of an *Alphacoronavirus* at the same time as a very active SARS-CoV-

300 2 outbreak within a farm would increase considerably the risk of viral recombination. Recombination
301 of alpha and betacoronaviruses has been already described in wild and domestic animals as well as in
302 humans (17–19). Such an event could result in an evolutionary jump and may generate a recombinant
303 with unpredictable phenotype and fitness that may promote the emergence of a novel coronavirus.
304 This potential issue should be seriously considered in countries where mink farms (or farm breeding
305 of other small carnivores) are insufficiently monitored and where preventive culling of SARS-CoV-2
306 positive farms is not applied.

307

308 Finally, by attempting to obtain the complete genome sequence of mink coronavirus on swab samples
309 that were positive for *Alphacoronavirus* by end-point RT-PCR, a nearly full genome of a mink
310 *Caliciviridae* was obtained by HTS. The *Caliciviridae* sequence was predominant in our two samples in
311 comparison with that of the mink coronavirus for which the sequence have not been obtained. The
312 absence of detection of mink coronavirus by HTS was disappointing but is linked to the very low load
313 of coronavirus RNA, which was only detected after a nested PCR. As a result, coronavirus reads were
314 hidden by the high proportion of bacteria and host (*mustelidae*) reads accounting for ~99% of the
315 reads in this sample. HTS sensitivity is known to be much lower than that of PCR, even more so with
316 nested PCR. Similar *Caliciviridae* have already been sequenced in minks in the USA (20) and China (21).
317 The associated clinical signs are not completely identified; some authors described no clinical sign (22)
318 or diarrhea (22) or hemorrhagic pneumonia (20). Beside the results obtained on coronaviruses, our
319 study on the French mink farms also shows that a number of potentially pathogenic agents were
320 silently circulating. This also seems to be the case in other farms (22). These infections could modify
321 the symptomatology of SARS-CoV-2 infection in minks, which has shown more or less acute clinical
322 signs depending on the farms in the different affected countries.

323 4. MATERIALS AND METHODS

324 The four American mink farms present in France (named A to D) were located in rural areas isolated
325 from human habitations. They were small family farms managed by one person, except farm D where
326 external workers were regularly employed. In each farm, minks were housed in wire netting cages
327 placed in scanstars, each of this housekeeping unwallied building consisting of two rows of several
328 dozen cages. A surrounding fence assured protection from intrusion in the farms. During the
329 investigations in November 2020, each cage housed two adult minks. Each cage was equipped with
330 automatic water distribution, and food was distributed every two days. According to the farmers'
331 declaration, no material sharing between farms and no animal exchange has been recorded.

332 Before slaughtering, the four mink populations consisted of 3,800 minks in farm A, 950 in farm B, 7,900
333 in farm C and 10,850 in farm D, spread in 3, 2, 15 and 26 housekeeping buildings respectively.

334

335 **4.1. Mink sampling and samples collection**

336 Each scanstar was considered as an independent epidemiological unit. Sixty animals were sampled in
337 each to enable sensitive detection, assuming a minimum apparent prevalence of 5%, with a 95%
338 confidence interval.

339 Minks were sampled during the slaughtering period (10 to 26 November 2020). Blood was sampled on
340 freshly euthanized mink carcasses, by intra-cardiac puncture in farms where carving up was realized in
341 place (farms A, B and D) or by retro-orbital sampling in farm C where animals were stored intact before
342 fur treatment. Blood samples were stored at 4°C before being transferred to the laboratory. Samples
343 were allowed to clot and then centrifuged (1000g, 15 min) to obtain serum. Sera were stored at -20°C
344 until serological testing. Sera were heat-inactivated at 56°C during 30 min preceding seroneutralization
345 assay.

346 Pharyngo-tracheal swabs were collected on mink carcasses by tracheal retro-route in farms A, B and
347 D, and by oropharyngeal route in farm C. The swabs were immediately kept in a volume of 500 µL of
348 cell culture medium (DMEM) supplemented with 1% of antibiotics (mix of Penicillin, Streptomycin and
349 Amphotericin B) before being frozen in liquid nitrogen to ensure viral integrity. The samples were
350 transferred to the laboratory and stored at < -70°C until RNA extraction.

351 The sample collection was completed with feces samples of breeding animals in farms C and D. Indeed,
352 in these two farms, some scanstars housed the unslaughtered animals that would serve as breeding
353 stock. Blood samples and tracheal swabs were then impossible to achieve. Pools of feces were
354 collected in farms C and D in 6 individual scanstars, with a total of 56 and 34 pool samples for the two
355 farms, respectively. Samples were stored at < -70°C until RNA extraction.

356

357 **4.2. SARS-CoV-2 isolate**

358 SARS-CoV-2 strain UCN19 was amplified on cells as described previously (23) and used at passage 2 for
359 the seroneutralization assays.

360

361 **4.3. Enzyme Linked Immunosorbent Assay (ELISA) test**

362 The Enzyme Linked Immunosorbent Assay provided by IDVet (ID Screen®ELISA, SARS-CoV-2 Double
363 Antigen Multi-species) has been used as a screening tool to detect the antibody presence in mink sera.
364 This ELISA is a double antigen ELISA for the detection of antibodies directed against the nucleocapsid
365 of SARS-CoV-2 in animal serum, plasma or whole blood. The samples were tested according to the
366 manufacturer's recommendations and as previously described (24,25). Briefly, 25 µL of each serum
367 sample was added in the microplate and diluted 1:2 in sample diluent. Microplates were incubated 45
368 minutes at 37 °C+/-2°C. Five washings were performed after incubation. Then, 100 µL of the conjugate

369 (a purified recombinant N protein antigen labeled with horseradish peroxidase) were distributed to
370 each well. The microplates were incubated for 30 min at 21°C +/-5°C. Five washings were performed
371 to remove the unbound conjugate. The presence of the complex antibodies/conjugate was revealed
372 by adding 100µL of TMB (TetraMethylBenzidine) chromogen solution to each well. The microplates
373 were incubated in the dark for 20 min at 21 °C +/-5°C. The enzymatic reaction was stopped by adding
374 100µl of a stop solution. The microplates were read at 450 nm. Positive and negative controls provided
375 by the manufacturer were used to validate each test plate.
376 The conditions of validation described by the manufacturer were implemented to validate the tests
377 and to interpret the results obtained for the different samples. The “Sample/Positive” ratio was
378 calculated as follows and expressed as a percentage (S/P%) :

$$S/P \% = \frac{OD_{\text{Sample}} - OD_{\text{NC}}}{OD_{\text{PC}} - OD_{\text{NC}}} \times 100$$

379

380 where ODNC is the optical density of the negative control and ODPC is the optical density of the
381 positive control and ODSample is the optical density of the sample. According to cut-off determined
382 by the manufacturer, three kind of results could be obtained:

- 383 - If S/P% is below or equal to 50%, the sample was considered negative
- 384 - If S/P% is between 50% and 60%, the sample was considered doubtful
- 385 - If S/P% is above or equal to 60%, the sample was considered positive

386 **4.4. Seroneutralization assay**

387 Briefly, in 96-well microplates, 200µL of VERO E6 cell suspensions in Dulbecco’s Modified Eagle
388 medium (DMEM) containing 10% FCS (Fetal calf serum) and 1% antibiotics (Penicillin/Streptomycin)
389 were added to each well, representing 20 000 cells per well, 24 hours before starting the
390 seroneutralization assay.

391 Each serum sample as well as positive and negative internal controls were distributed in two
392 consecutive wells of 96-well microplates, and then serially diluted with a dilution step of 1 to 3 within
393 DMEM containing 10% FCS and 1% antibiotics (Penicillin/Streptomycin). Then 50µL of SARS-CoV-2 virus
394 diluted in medium containing around a 50% tissue culture infective dose (TCID50) of 100 per 50µL
395 (checked by back-titration during the seroneutralization assays) were added to each well containing
396 samples and internal controls. The plates were incubated at 37°C/5% CO2 for 1h to allow neutralization
397 complexes to be formed between the neutralizing antibodies and the virus. At the end of the
398 incubation, the supernatant fluid was removed from each well of the plates containing VERO E6 cell
399 suspensions and immediately 100µL of the mix of the virus and serially diluted samples or controls
400 were transferred to individual wells on the cell layer. The microplates were incubated at 37°C in a

401 humid chamber containing 5% CO₂, at least 3 days post-infection. Then, plates were qualitatively read
402 according to an “all or nothing” scoring method for the presence of viral cytopathic effect (CPE). The
403 neutralization titers were assigned to each serum based on the highest dilution that prevented
404 discernible cytopathic effect.

405

406 **4.5. RNA extraction**

407 - **Pharyngo-tracheal swabs:** Viral RNA was extracted from 140 µL of pharyngo-tracheal swabs medium.

408 Viral RNA extraction was performed by using the Qiagen Viral RNA mini kit according to the
409 manufacturer’s instructions (Qiagen, Les Ulis, France), with minor modifications. To inactivate
410 potential infectious status of samples by SARS-CoV-2, a volume of 15 µL of Triton X-100 (MP
411 Biomedicals, Illkirch, France) was added to 560 µL of AVL Lysis buffer (Qiagen, Courtaboeuf, France)
412 for each sample testing. RNA was eluted in a final volume of 60 µL and stored at < -70°C. A negative
413 RNA extraction control was performed for each set of 24 samples tested.

414 - **Fecal samples:** 100 mg of fecal sample were placed in a lysing matrix E tube containing beads (MP
415 Biomedicals Germany GmbH, Eschwege, France) and filled with 1ml of CTAB buffer (Promega France,
416 Charbonnières les Bains, France). Tubes were placed in a shaking heat block at 65°C, vigorously
417 vortexed for 1 min and mixed with 40 µL of Proteinase K Solution (Promega France, Charbonnières les
418 Bains, France). After an incubation at 70°C for 10 min, the lysates were grinded for 30 sec at 7.0 m/s
419 six times in a Fast Prep-24™ 5G bead beater (MP Biomedicals Germany GmbH, Eschwege, France) then
420 centrifuged at 10,000 x g for 5 min. A volume of 300 µL of clear lysate was transferred to a tube
421 containing 300 µL of Lysis buffer. RNA extraction was performed with the Maxwell RSC PureFood GMO
422 and Authentication kit using a Maxwell RSC instrument (Promega France, Charbonnières les Bains,
423 France), according to the manufacturer’s instructions. RNA was eluted in a final volume of 100µL and
424 stored at < -70°C.

425 Negative (non-template control) and positive (hedgehog betacoronavirus) controls (RNA extraction
426 control) were performed for each set of 16 samples tested.

427

428 **4.6. TaqMan RT-qPCR of E gene using specific SARS-CoV-2 primers**

429 TaqMan RT-qPCR was performed as previously described (23). Coronavirus primers (E_Sarbeco_F
430 (forward): 5’-ACAGGTACGTTAATAGTTAATAGCGT and E_Sarbeco_R (reverse): 5’-
431 ATATTGCAGCAGTACGCACACA) and probe (E_Sarbeco_P1: 5’-FAM-ACACTAGCCATCCTTA
432 CTGCGCTTCG-BHQ-1) targeting the envelope protein gene (E gene) were used for the study (26).
433 Primers and probe were provided by Eurogentec (Angers, France). TaqMan RT-qPCR assays were
434 performed in a total volume of 25 µL containing 2.5 µL of RNA sample, 12.5 µL of 2x QuantiTect Probe
435 RT-PCR master Mix, 1 µL of 25 mM MgCl₂ (Invitrogen), 4.5µL of RNase-free water, 2 µL each of forward

436 and reverse primer (10 μ M), 0.25 μ L of probe (10 μ M), and 1 μ L of QuantiTect RT Mix. All TaqMan RT-
437 qPCR assays were performed on the thermocycler Rotor Gene Q MDx (Qiagen, Courtaboeuf, France).
438 Amplification was carried out according to the following thermocycling conditions: 50 °C for 30 min for
439 reverse transcription, followed by 95 °C for 15 min and then 45 cycles of 94 °C for 30 s, 55 °C for 30 s
440 and 72°C for 30 s. Negative and positive controls were included in each RT-qPCR assay.

441 The titer determination of SARS-CoV-2 RNA in number of copies/ μ L was determined by testing six 10-
442 fold dilutions (i.e. 1.05.10⁸ to 1.05.10³ genome copies/mL of a quantitative synthetic RNA from SARS-
443 CoV-2 (BEI Resources). A threshold setting (Ct) of 0.03 was used as the reference threshold for each
444 RT-qPCR assay. The efficiency, slope and correlation coefficient (R²) were calculated by the Rotor Gene
445 software. All reactions were carried out as technical duplicates. A cut-off > 36 was defined for negative
446 results and between 32 and 36 for weak positive results (samples with late C_T values).

447

448 **4.7. End-point RT-PCR of pol gene of coronaviruses using conserved primers**

449 RNA coronavirus detection was performed by amplifying a 438-bp fragment of the RNA dependent
450 RNA polymerase (pol) gene of coronaviruses using the following degenerated primers: PanCoV pol
451 15197 (forward): 5'-GGTTGGGAYTAYCCWAARTGTGA, PanCoV pol 15635 (reverse):
452 CCATCRTCMGAHARAATCATCATA designed by (27). The end-point RT-PCR was performed in a two-step
453 RT-PCR with synthesis of cDNA from the extracted total RNA followed by a touch down PCR.

454 The cDNA was synthesized by reverse transcription of 5 μ L of RNA extracted from tracheal swabs and
455 faecal samples using 0.25 μ L of hexanucleotide primers (0.2 μ G/ μ L) (Thermo Fisher Scientific, Dardilly,
456 France) and a RT Maxima H Minus cDNA synthesis kit (Invitrogen, Thermo Fisher Scientific, Dardilly,
457 France) according to the manufacturer's instructions. The cDNA synthesis was performed for 10 min
458 at 25°C, 30 min at 50°C following a final step of 5 min at 85°C. cDNA was stored at < -70°C.

459 PCR was performed in a final volume of 25 μ L containing 3 μ L of cDNA, 2.5 μ L of 10X PCR Buffer
460 (Invitrogen, Marseille, France), 0.75 μ L of MgCl₂ (50 mM), 1 μ L of dNTPs (10 mM) and 0.5 μ L of Platinum
461 Taq DNA polymerase (5 U/ml) (Invitrogen, Marseille, France) and 1 μ L of forward and reverse primer
462 (20 μ M). The PCR was amplified for 2 min at 94°C, with 11 cycles of 30s at 94°C, a 1° touch down
463 decrease of the annealing temperature from 60° to 50°C, 90s at 72°C, then 40 cycles of 30 s at 94°C,
464 45 s at 50°C and 90 s at 72°C and followed by a final step of extension of 10 min at 72°. Negative and
465 positive controls were included in each RT and PCR assay.

466 The amplification of the beta-actin gene was performed for each fecal sample with the forward (5'-
467 CGATGAAGATCAAG/ATCATTGC-3') and reverse (5'-AAGCATTTGCGGTGGAC-3') primers, with the same
468 methodology to confirm the absence of PCR inhibitors in samples. The amplified products were
469 analyzed by electrophoresis on a 2% agarose gel stained with a SYBR safe solution at a final
470 concentration of 1/10,000 then photographed.

471

472 **4.8. Sequencing of PanCoV amplicons, alignment of sequences and phylogeny.**

473 The positive PCR products were sequenced in both directions by Eurofins Genomics (Germany) with
474 the same specific primers used in the PCR.

475 A dataset of sequences was constituted with 8 sequences from this study (7 pharyngo-tracheal swabs
476 and 1 fecal sample) and 41 referenced sequences including representative sequences of the genus
477 *Alphacoronavirus* (n=25), *Betacoronavirus* (n=20), *Deltacoronavirus* (n=4) and *Gammacoronavirus*
478 (n=4). Multiple alignment of partial pol sequences (positions 14113 to 14536 compared to the
479 reference genome of a *Minacovirus* Strain MW248736) was performed with Mega v10.1.8. A
480 phylogenetic tree was constructed using the ML method (GTR model). Node robustness was estimated
481 using bootstrap method with 1000 iterations. The consensus sequences of the partial genome of pol
482 gene is accessible in GenBank under accession numbers ON985270 to ON985277.

483

484 **4.9. NGS: Non-specific nanopore sequencing**

485 - RNA extraction and removal of genomic DNA

486 Swabs supernatant (150µl) was submitted to RNA extraction using the EZ1 RNA Tissue Mini kit
487 (QIAGEN) following manufacturer's instructions. Genomic DNA was then depleted from the eluate by
488 incubation with Turbo DNA-free kit (Thermo Fisher Scientific), according to the manufacturer's
489 instructions.

490 - Qualification of the sample by quantification of the SARS-CoV-2 N gene

491 Quantification of the N gene in the eluate was performed by real-time reverse-transcription (RT-qPCR)
492 using SuperScript™ III Platinum™ One-Step Quantitative RT-PCR System (Invitrogen) as described
493 previously with minor modifications (26). Briefly, a 25 µL reaction contained 5 µL of RNA, 12.5 µL of 2
494 × reaction buffer, 1 µL of reverse transcriptase/ Taq mixture, 0.4 µL of a 50 mM magnesium sulphate
495 solution (Invitrogen), 1 µl of a 10µM primer, and 0.5l µl of a 10µM probe TxRd-BHQ2. Thermal cycling
496 was performed at 50 °C for 15 min for reverse transcription, followed by 95 °C for 2 min and then 45
497 cycles of 95 °C for 15 s and 60 °C for 30 s using a Light Cycler 480 (Roche).

498 - Ribosomal RNA depletion and real-time reverse-transcription PCR

499 Eukaryotic rRNA was depleted using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat). After rRNA
500 depletion, cDNA was synthesized from residual total RNA by RT-VILO (Invitrogen) reaction following
501 manufacturer's instructions. Random amplification of the material was then performed with
502 QuantiTect Whole Transcriptome kit (QIAGEN) according to the manufacturer's protocol. Amplified
503 DNA was then purified using AMPure XP beads and submitted to Qubit quantification using dsDNA BR
504 Assay Kit and Qubit 3.0 fluorimeter (Invitrogen). Complementary to the un-targeted approach, we also

505 used an adapted version of the published protocol from the ARTIC Network (28) using ARTIC primer
506 scheme version 3, which produces ~400 bp overlapping amplicons over the SARS-CoV-2 genome.

507 - ONT library preparation and MinION sequencing

508 For maximizing the read length, libraries were not sheared. Sequencing libraries and sequencing
509 reaction were performed according to manufacturer's instructions with minor adaptations. Briefly, we
510 used the NEBNext Ultra II End Repair/dATailing module (E7546S, NEB, USA) to prepare 1000 ng DNA
511 from each sample. Native barcode adapters NBD04 were ligated in Blunt/TA Ligase Master Mix
512 (M0367S, NEB, USA), and resulting product was AMPure XP beads purified before pooling to produce
513 a 54 µl equimass pool, itself ligated to adapter using Native Barcoding Adapter Mix (BAM). The purified
514 final library was loaded onto an R9.4 flowcell (FLO-MIN106, Oxford Nanopore Technologies, UK), and
515 the run was performed on a MinION Mk1B device (ONT) for 2 hours in order to obtain more than 12
516 Go of raw data.

517 - Genome assembly

518 Following the MinION sequencing run, raw data were basecalled and reads subsequently
519 demultiplexed using Guppy GPU basecaller / barecoder (Oxford Nanopore Technologies). Raw reads
520 were cleaned using porechop (29) and then mapped against a custom reference of SARS-CoV-2
521 genome comprising four Chinese and 70 early French sequences using Bowtie2 (30) and minimap2
522 (31). Finally, consensus genome sequences based on mapped reads (above 83 % coverage with average
523 depth above 100 and a maximum coverage depth over 3000) was generated with bcftools consensus
524 (32).

525 - Illumina sequencing

526 In addition to nanopore sequencing, Illumina fastq files data were also obtained from Illumina
527 sequencing at the National Reference Centre for respiratory viruses (Institut Pasteur Paris). Basecalling
528 and demultiplexing were done on the sequencer using the manufacturer's software (Illumina). Reads
529 were then trimmed and filtered using Alien trimmer in order to remove adapters and bases under q20
530 quality score. Mapping was performed on the same reference as mentioned above using Bowtie2 and
531 consensus (majority) genomes were extracted using bcftools and compared to nanopore data. In (rare)
532 case of discordance, higher coverage Illumina data replaced lower coverage nanopore data. After
533 alignment and manual verification, genome sequences were then submitted in GISAID under numbers
534 (EPI_ISL_1392906 & EPI_ISL_10036487-97).

535

536 **4.10. SARS-CoV-2 genomic datasets, genetic and phylogenetic analyses**

537 Three main independent analyses were performed, one using human-derived SARS-CoV-2 genomes,
538 another using mink-derived SARS-CoV-2 genomes and the last one combining all datasets. Firstly, the
539 human SARS-CoV-2- genomes raw dataset was assembled in June 2021 from a collection of all

540 genomes available in GISAID database and filtered on the following parameters: collection date ranging
541 from 01 March 2020 to 30 March 2021, France and human origin, complete genomes, high coverage
542 and low coverage excluded (n=42700). Resulting dataset was aligned using MAFFT (33) and optimized
543 using in-house scripts from sequencing lab of the virology unit, Caen University by removing redundant
544 sequences and similar genomes without losing significant diversity (99,875 % pairwise nucleotide
545 identity cut off, 437 genomes with at least 37 nucleotides differences). This diversity optimized dataset
546 was aligned with the twelve genomes obtained from the French mink farm and analyzed by both
547 Maximum Likelihood (ML from PhyML, SeaView - (34)) and Bayesian phylogenetic methods (Beast -
548 (35)). Both methods used a GTR model of evolution with gamma distribution and invariable sites
549 parameters with the coalescent constant size model as tree prior. Likelihood ratio test and posterior
550 probabilities values were used to estimate node support in ML and Bayesian methods, respectively.
551 The Bayesian phylogenetic analysis was also enriched by using collection dates as priors and an
552 uncorrelated relaxed clock model with lognormal distribution (36). The Markov chain was launched for
553 100 million iterations on an 18 double cores computer using Beast 1.10.4 suite in order to reach an
554 effective sampling size over 200 for each statistic. The maximum credibility tree was computed from a
555 sampling of 10000 trees and after discarding the first 1000 trees considered as burnin.

556 Secondly, the mink SARS-CoV-2 genomes raw dataset was assembled using 821 mink SARS-CoV-2
557 genomes collected from GISAID, with same filtering options previously used for the human SARS-CoV-
558 2 genomes collection and last updated on 5 January 2022. A diversity optimized dataset was generated
559 using the same method as previously described above for the human SARS-CoV-2 dataset. This
560 diversity optimized (SARS-CoV-2 collected in mink - *Neovison vison*) dataset was analyzed by maximum
561 likelihood phylogenetic method with the twelve genomes obtained from the French mink farm. The
562 GTR model of evolution was used with gamma distribution and invariable sites parameters. Likelihood
563 ratio test values were calculated to estimate node support.

564 Thirdly, additional analyses (SNPs, Amino acid variability) used representatives (n=9) of each main
565 clade identified from previous analyses. Only complete genomes were conserved in this refined
566 dataset and those counting several missing data covering more than 200 contiguous nucleotides in
567 variable loci were discarded. This last dataset was aligned with the twelve genomes sequenced in this
568 study and submitted to ML analyses and variable positions were extracted in order to test the existence
569 and congruence of synapomorphic variations supporting clades and French mink SARS-CoV-2
570 monophyly.

571

572 **4.11. High throughput sequencing from fecal and swab samples**

573 In addition to the non-specific Nanopore and Illumina sequencing performed on pharyngo-tracheal
574 swabs shown positive for SARS-CoV-2, we undertook high throughput sequencing (HTS) on two
575 pharyngo-tracheal swabs (samples 61-B06-11 and 61-B12-04) and one fecal (sample 61-B23-05)
576 sample, shown positive by conventional RT-PCR for the presence of partial Alphacoronavirus pol gene.
577 The swab and fecal samples were prepared as follows for HTS.

578 **Preparation of RNA samples**

579 - Viral RNA extraction was performed for the two swab samples subjected to HTS from a volume of
580 140 μ L using Qiagen Viral RNA mini kit (Qiagen, France) according to the manufacturer's instructions.
581 Prior to HTS, the two extracted RNA samples were checked for the presence of partial pol gene by the
582 conventional Coronaviruses RT-PCR using the forward and reverse primers PanCoV pol 15197 (F) and
583 PanCoV pol 15635 (R).

584 - 100 mg of fecal sample were placed in a Virocult tube (Sigma) containing 1 mL of stabilizing buffer,
585 then vigorously vortexed for 30 sec at 7.0 m/s six times in a Fast Prep-24TM 5G bead beater (MP
586 Biomedicals Germany GmbH, Eschwege, France) and centrifuged at 10,000 x g for 5 min. A volume of
587 210 μ L of supernatant was transferred to three individual tubes (i.e. 70 μ L/tube) for RNA extraction,
588 each filled with 1ml of CTAB buffer (Promega France, Charbonnières les Bains, France). The three tubes
589 were placed in a shaking heat block at 65°C, vigorously vortexed for 1 min and mixed with 40 μ L of
590 Proteinase K Solution (Promega France, Charbonnières les Bains, France), before an incubation at 70°C
591 for 10 min and a centrifugation step at 10,000g for 5 min. A volume of 300 μ L of clear lysate RNA
592 extraction (i.e. 9 tubes) was performed on the Promega Maxwell RSC instrument with the Maxwell RSC
593 PureFood GMO and Authentication kit according to the manufacturer's instructions. RNA was pooled
594 in a final volume of 900 μ L and stored at < -70°C. The extracted fecal RNA sample was shown positive
595 by the conventional Coronaviruses RT-PCR using the forward and reverse primers PanCoV pol 15197
596 (F) and PanCoV pol 15635 (R).

597 **Whole genome sequencing and sequence analysis**

598 HTS was performed on the three RNA extracts after a step of rRNA depletion with the rRNA depletion
599 kit (NEB, Evry, France), according to the manufacturer's recommendations. The RNA library was
600 prepared for each RNA sample tested using Ion Total RNA-Seq kit v2 (Life Technologies, Carlsbad, CA,
601 USA) and then sequenced using Ion Torrent Proton technology. The reads were cleaned with the
602 Trimmomatic 0.36 software, followed by bioinformatics analysis as previously described (37,38) using
603 the GenBank Mink *Caliciviridae* reference sequence MF677852.1 for the two swab RNA samples and
604 the Mink coronavirus 1 reference sequence MN535737.1 for the fecal RNA sample to calculate sub-
605 sampling and final alignment.

606 Sequences obtained for this study are available in GenBank: Bioproject # PRJNA881217 (Sample 61-
607 B12-04), Bioproject # PRJNA881061 (B-23-05), Biosample: SAMN30886030 (Sample 61-B12-04) and

608 Biosample (B-23-05). The consensus sequence of the full-length genome of the mink *Caliciviridae* is
609 accessible in GenBank under the accession numbers OP485683.

610

611

612 **ACKNOWLEDGEMENTS**

613 We would like to thank all the members of the Anses Nancy Laboratory for rabies and wildlife for their
614 great contributions to this work (sampling and testing every day of week), Christophe Cordevant and
615 Gilles Salvat for their support and Gérald Le Digerher for his effective help in the last farm. We
616 gratefully acknowledge authors from the originating and sequencing laboratories responsible for
617 obtaining the specimens (Tables S4 and S5).

618

619 **FUNDING**

620 This investigation received financial support from the World Health Organization (WHO), through a
621 German fund provided to the WHO R&D Blueprint. The authors alone are responsible for the views
622 expressed in this publication and they do not necessarily represent the views, decisions or policies of
623 WHO. E.S.-L laboratory acknowledges funding from Institut Pasteur, from the INCEPTION programme
624 (Investissements d'Avenir grant ANR-16-CONV-0005), from the NIH PICREID program (Award Number
625 U01AI151758) and from the Labex IBEID (ANR-10-LABX-62-IBEID). S. vdW laboratory acknowledges
626 funding from Institut Pasteur, from Santé publique France, from the Labex IBEID (ANR-10-LABX-62-
627 IBEID) and from the H2020 project 101003589 (RECOVER).

628

629

630 **REFERENCES**

- 631 1. Oreshkova N, Molenaar RJ, Vreman S, Harders F, Oude Munnink BB, Hakze-van der Honing
632 RW, et al. SARS-CoV-2 infection in farmed minks, the Netherlands, April and May 2020. *Euro*
633 *Surveill Bull Eur Sur Mal Transm Eur Commun Dis Bull.* juin 2020;25(23).
- 634 2. Boklund A, Hammer AS, Quaade ML, Rasmussen TB, Lohse L, Strandbygaard B, et al. SARS-
635 CoV-2 in Danish Mink Farms: Course of the Epidemic and a Descriptive Analysis of the
636 Outbreaks in 2020. *Anim Open Access J MDPI.* 12 janv 2021;11(1):164.
- 637 3. Fenollar F, Mediannikov O, Maurin M, Devaux C, Colson P, Levasseur A, et al. Mink, SARS-
638 CoV-2, and the Human-Animal Interface. *Front Microbiol.* 2021;12:663815.
- 639 4. Tada T, Dcosta BM, Zhou H, Vaill A, Kazmierski W, Landau NR. Decreased neutralization of
640 SARS-CoV-2 global variants by therapeutic anti-spike protein monoclonal antibodies. *BioRxiv*
641 *Prepr Serv Biol.* 19 févr 2021;2021.02.18.431897.
- 642 5. Wu K, Werner AP, Moliva JI, Koch M, Choi A, Stewart-Jones GBE, et al. mRNA-1273 vaccine
643 induces neutralizing antibodies against spike mutants from global SARS-CoV-2 variants. *BioRxiv*
644 *Prepr Serv Biol.* 25 janv 2021;2021.01.25.427948.

- 645 6. Lu L, Sikkema RS, Velkers FC, Nieuwenhuijse DF, Fischer EAJ, Meijer PA, et al. Adaptation,
646 spread and transmission of SARS-CoV-2 in farmed minks and associated humans in the
647 Netherlands. *Nat Commun.* 23 nov 2021;12(1):6802.
- 648 7. Oude Munnink BB, Sikkema RS, Nieuwenhuijse DF, Molenaar RJ, Munger E, Molenkamp R, et
649 al. Transmission of SARS-CoV-2 on mink farms between humans and mink and back to humans.
650 *Science.* 8 janv 2021;371(6525):172-7.
- 651 8. Rabalski L, Kosinski M, Smura T, Aaltonen K, Kant R, Sironen T, et al. Severe Acute Respiratory
652 Syndrome Coronavirus 2 in Farmed Mink (*Neovison vison*), Poland. *Emerg Infect Dis.* sept
653 2021;27(9):2333-9.
- 654 9. Hammer AS, Quaade ML, Rasmussen TB, Fonager J, Rasmussen M, Mundbjerg K, et al. SARS-
655 CoV-2 Transmission between Mink (*Neovison vison*) and Humans, Denmark. *Emerg Infect Dis.*
656 févr 2021;27(2):547-51.
- 657 10. Molenaar RJ, Vreman S, Hakze-van der Honing RW, Zwart R, de Rond J, Weesendorp E, et al.
658 Clinical and Pathological Findings in SARS-CoV-2 Disease Outbreaks in Farmed Mink
659 (*Neovison vison*). *Vet Pathol.* sept 2020;57(5):653-7.
- 660 11. Balloux F, Tan C, Swadling L, Richard D, Jenner C, Maini M, et al. The past, current and future
661 epidemiological dynamic of SARS-CoV-2. *Oxf Open Immunol.* 2022;3(1):iqac003.
- 662 12. Shuai L, Zhong G, Yuan Q, Wen Z, Wang C, He X, et al. Replication, pathogenicity, and
663 transmission of SARS-CoV-2 in minks. *Natl Sci Rev.* mars 2021;8(3):nwaa291.
- 664 13. Krüttgen A, Cornelissen CG, Dreher M, Hornef MW, Imöhl M, Kleines M. Determination of
665 SARS-CoV-2 antibodies with assays from Diasorin, Roche and IDvet. *J Virol Methods.* janv
666 2021;287:113978.
- 667 14. Mylemans M, Van Honacker E, Nevejan L, Van Den Brecht S, Hofman L, Poels J, et al.
668 Diagnostic and analytical performance evaluation of ten commercial assays for detecting SARS-
669 CoV-2 humoral immune response. *J Immunol Methods.* juin 2021;493:113043.
- 670 15. Vlasova AN, Halpin R, Wang S, Ghedin E, Spiro DJ, Saif LJ. Molecular characterization of a new
671 species in the genus Alphacoronavirus associated with mink epizootic catarrhal gastroenteritis. *J*
672 *Gen Virol.* juin 2011;92(Pt 6):1369-79.
- 673 16. Wilson DJ, Baldwin TJ, Whitehouse CH, Hullinger G. Causes of mortality in farmed mink in the
674 Intermountain West, North America. *J Vet Diagn Investig Off Publ Am Assoc Vet Lab Diagn Inc.*
675 juill 2015;27(4):470-5.
- 676 17. Scarpa F, Sanna D, Azzena I, Cossu P, Giovanetti M, Benvenuto D, et al. Update on the
677 Phylodynamics of SARS-CoV-2. *Life Basel Switz.* 11 août 2021;11(8):820.
- 678 18. Su S, Wong G, Shi W, Liu J, Lai ACK, Zhou J, et al. Epidemiology, Genetic Recombination, and
679 Pathogenesis of Coronaviruses. *Trends Microbiol.* juin 2016;24(6):490-502.
- 680 19. Tsoleridis T, Chappell JG, Onianwa O, Marston DA, Fooks AR, Monchatre-Leroy E, et al. Shared
681 Common Ancestry of Rodent Alphacoronaviruses Sampled Globally. *Viruses.* 30 janv
682 2019;11(2):E125.
- 683 20. Evermann JF, Smith AW, Skilling DE, McKeirnan AJ. Ultrastructure of newly recognized
684 caliciviruses of the dog and mink. *Arch Virol.* 1983;76(3):257-61.

- 685 21. Yang B, Wang F, Zhang S, Xu G, Wen Y, Li J, et al. Complete genome sequence of a mink
686 calicivirus in China. *J Virol.* déc 2012;86(24):13835.
- 687 22. Guo M, Evermann JF, Saif LJ. Detection and molecular characterization of cultivable
688 caliciviruses from clinically normal mink and enteric caliciviruses associated with diarrhea in
689 mink. *Arch Virol.* 2001;146(3):479-93.
- 690 23. Monchatre-Leroy E, Lesellier S, Wasniewski M, Picard-Meyer E, Richomme C, Boué F, et al.
691 Hamster and ferret experimental infection with intranasal low dose of a single strain of SARS-
692 CoV-2. *J Gen Virol.* mars 2021;102(3).
- 693 24. Sailleau C, Dumarest M, Vanhomwegen J, Delaplace M, Caro V, Kwasiborski A, et al. First
694 detection and genome sequencing of SARS-CoV-2 in an infected cat in France. *Transbound*
695 *Emerg Dis.* nov 2020;67(6):2324-8.
- 696 25. Spada E, Vitale F, Bruno F, Castelli G, Reale S, Perego R, et al. A pre- and during Pandemic
697 Survey of Sars-Cov-2 Infection in Stray Colony and Shelter Cats from a High Endemic Area of
698 Northern Italy. *Viruses.* 3 avr 2021;13(4):618.
- 699 26. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, et al. Detection of 2019
700 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill Bull Eur Sur Mal Transm*
701 *Eur Commun Dis Bull.* 2020;25(3).
- 702 27. Gouilh MA, Puechmaille SJ, Gonzalez JP, Teeling E, Kittayapong P, Manuguerra JC. SARS-
703 Coronavirus ancestor's foot-prints in South-East Asian bat colonies and the refuge theory. *Infect*
704 *Genet Evol J Mol Epidemiol Evol Genet Infect Dis.* oct 2011;11(7):1690-702.
- 705 28. Tyson JR, James P, Stoddart D, Sparks N, Wickenhagen A, Hall G, et al. Improvements to the
706 ARTIC multiplex PCR method for SARS-CoV-2 genome sequencing using nanopore. *BioRxiv*
707 *Prepr Serv Biol.* 4 sept 2020;2020.09.04.283077.
- 708 29. Wick RR, Judd LM, Gorrie CL, Holt KE. Completing bacterial genome assemblies with multiplex
709 MinION sequencing. *Microb Genomics.* oct 2017;3(10):e000132.
- 710 30. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* 4 mars
711 2012;9(4):357-9.
- 712 31. Li H. Minimap2: pairwise alignment for nucleotide sequences. *Bioinforma Oxf Engl.* 15 sept
713 2018;34(18):3094-100.
- 714 32. Li H. A statistical framework for SNP calling, mutation discovery, association mapping and
715 population genetical parameter estimation from sequencing data. *Bioinforma Oxf Engl.* 1 nov
716 2011;27(21):2987-93.
- 717 33. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements
718 in performance and usability. *Mol Biol Evol.* avr 2013;30(4):772-80.
- 719 34. Gouy M, Guindon S, Gascuel O. SeaView version 4: A multiplatform graphical user interface for
720 sequence alignment and phylogenetic tree building. *Mol Biol Evol.* févr 2010;27(2):221-4.
- 721 35. Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC*
722 *Evol Biol.* 8 nov 2007;7:214.
- 723 36. Drummond AJ, Ho SYW, Phillips MJ, Rambaut A. Relaxed phylogenetics and dating with
724 confidence. *PLoS Biol.* mai 2006;4(5):e88.

725 37. Briand FX, Schmitz A, Ogor K, Le Prioux A, Guillou-Cloarec C, Guillemoto C, et al. Emerging
726 highly pathogenic H5 avian influenza viruses in France during winter 2015/16: phylogenetic
727 analyses and markers for zoonotic potential. Euro Surveill Bull Eur Sur Mal Transm Eur Commun
728 Dis Bull. 2 mars 2017;22(9):30473.

729 38. Pellerin M, Hirchaud E, Blanchard Y, Pavio N, Doceul V. Characterization of a Cell Culture
730 System of Persistent Hepatitis E Virus Infection in the Human HepaRG Hepatic Cell Line.
731 Viruses. 4 mars 2021;13(3):406.

732

733 **SUPPORTING INFORMATIONS**

734

735 **Figure S1. Mink SARS-CoV2 mutations GISAID hcov-19 nextstrain genome Nextclade analysis table**

736 **Figure S2. SARS-CoV-2 genomes of French mink nested within the 20A clade, together with SARS-CoV-2 from**
737 **Human sampled at the same period of time**

738 **Table S1. Mink SARS-CoV2 Nextclade analysis**

739 **Table S2. Mink SARS-CoV2 genomic variable sites**

740 **Table S3. Clade and Amino Acid modifications_Nextclade**

741 **Table S4. GISAID_hcov-19_acknowledgement_table_2022_01_20_13**

742 **Table S5. GISAID_hcov-19_acknowledgement_table_2022_01_05_10**