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# Genetic Diversity and Phylogenetic Analysis of Feline Coronavirus Sequences from Portugal

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1 **Genetic Diversity and Phylogenetic Analysis of Feline Coronavirus Sequences from Portugal**

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3

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## 24 Abstract

25 The distribution of **FCoV** Type I and II in a Portuguese cat population was studied by a RT-PCR assay  
26 targeting the 3' end of the viral RNA. For a period of three years, 120 samples were collected and 57  
27 were found positive for **FCoV** RNA. Within the positive samples the presence of **FCoV** Type I was  
28 found in 79%. Type II **was only** detected in 3,5% **in animals with Feline Infectious Peritonitis**. The  
29 **remaining** 17,5% could not be differentiated. These viral sequences, comprising a region within gene  
30 S were further subjected to a Heteroduplex Mobility Assay (HMA) detecting the presence of viral  
31 quasispecies in 17% of the samples. Phylogenetic analysis for **FCoV** Type I revealed high genetic  
32 diversity between the Portuguese sequences and other previously characterized strains, while Type II  
33 tree showed a higher genetic homogeneity. This study confirmed the presence of **FCoV** Type I and II  
34 circulating in Portugal and detected high genetic diversity between circulating strains suggesting that  
35 the virus persists within the host as mixed viral populations.

36 Keywords: **FCoV**, genetic diversity, Heteroduplex Mobility Assay

## 38 1. Introduction

39 Feline Coronaviruses (**FCoV**) are members of genus Coronavirus, Family *Coronaviridae*. Two variants  
40 of **FCoV** have been described, *Feline Enteric Coronavirus* responsible for an enteric disease and  
41 *Feline Infectious Peritonitis Virus* (FIPV) the etiological agent of FIP, a fatal disease of domestic and  
42 wild cats. FIP development in a small percentage of animals infected with **FCoV** is related to the  
43 occurrence of genomic mutations supported by the close genetic relationship between the higher  
44 virulent virus and the parental **FCoV** (Herrewegh et al., 1997, Vennema et al., 1998, Vennema, 1999).  
45 Additionally, **FCoVs** were classified as Type I and II according to their serological and genomic  
46 features. Type I is strictly feline. Type II **FCoV**, although sharing with Type I a high degree of similarity  
47 in the 3' end of the genome, is antigenic and genetically more related to Canine Coronavirus (**CCoV**)  
48 (Hohdatsu et al., 1991), suggesting that Type II resulted from a homologous and independent  
49 recombination event within the S and the M genes of Type I **FCoV** and **CCoV** (Herrewegh et al.,  
50 1998). The current information on the geographical distribution of **FCoV** indicates a high incidence of  
51 Type I in Europe, Japan and USA (Addie et al., 2003; Hohdatsu et al., 1992). **However in Japan and**  
52 **Taiwan detection of FIP was associated with Type II FCoV (Hohdatsu et al., 1992, Lin et al.,**  
53 **2008)**. Presently there is no available information about the molecular epidemiology of **FCoV** in  
54 Portugal. Due to the pathogenic impact of this virus in the feline health it was our purpose to  
55 investigate the presence of **FCoV** Types in the Portuguese cat population using a RTPCR-nested

56 PCR assay previously reported (Addie et al., 2003) and to assess its genetic diversity by Heteroduplex  
57 mobility assay (HMA) and phylogenetic analysis.

58

## 59 **2. Material and Methods**

### 60 **2.1. Sampling collection**

61 The biological material included rectal swabs, stool, plasma, thoracic and ascite fluids collected from  
62 animals attending the Veterinary Hospital of the Faculty of Veterinary Medicine, Lisbon.

### 63 **2.2. Detection of FCoV RNA**

64 **FCoV** RNA was extracted using the QIAmp Viral RNA or Qiagen RNeasy mini kits (Qiagen), following  
65 the manufacturer's instructions. A fragment of 177bp located in the 3'-UTR was amplified by RT-PCR  
66 followed by a nested PCR using two primer pairs described by Herrewegh et al., 1995. The 25  $\mu$ L  
67 reactions were amplified with one step RT-PCR Fidelity Taq 2x Master Mix (GE Healthsciences),  
68 50pmol of each primer and 10 $\mu$ L of RNA sample. The nPCR was performed with PCR Fidelity Taq 2x  
69 Master Mix (GE Healthsciences), 50pmol of each primer and 5  $\mu$ L of the previous amplification in a 25  
70  $\mu$ L reaction.

### 71 **2.3. Differentiation between Type I and Type II FCoV:**

72 Positive **FCoV** RNA samples were subjected to a RT-PCR assay to amplify a 376-283bp fragment  
73 located in the 3' region of the *spike* gene (S), followed by a nPCR to amplify a 360-218 bp internal  
74 fragment, for differentiation of Type I from Type II **FCoV** according to the fragment length (Addie et al.,  
75 2003). The amplification was performed with one step RT-PCR Fidelity Taq 2x Master Mix (GE  
76 Healthsciences), 5 $\mu$ L of RNA sample and primers **lffs**, **lcfs**, **lubs** in a 25  $\mu$ L reaction, followed by a  
77 nested PCR with PCR Fidelity Taq 2x Master Mix (GE Healthsciences), primers **niffles**, **nlcfs**, **nlubs**  
78 (**Addie et al., 2003**) and 2,5  $\mu$ L of the previous reaction in 25  $\mu$ L final volume. The amplicons were  
79 analysed in a 2,5% agarose gel stained with 0.5  $\mu$ g/mL of Ethidium Bromide in 1x TBE.

### 80 **2.4. Heteroduplex Mobility Assay (HMA) and Cloning**

81 The amplicons were subjected to a HMA before cloning (Delwart et al., 1993). Briefly, 10  $\mu$ L of the  
82 second round S amplicons were combined with 1  $\mu$ L of 10x annealing buffer (1M NaCl, 100mM Tris  
83 pH 7.8, 20mM EDTA), heated to 94°C/5min, cooled on ice, added of 1  $\mu$ L of loading buffer (50%  
84 glycerol, 0,01% of xylene cyanol) and loaded in a 5% polyacrylamide gel (29,2:0,8 acrylamide:  
85 bisacrylamide) poured into plates of 15 x 15 cm. The electrophoresis was carried at a constant 150V/3  
86 h in a vertical gel apparatus (Biorad) in 1x TBE. After electrophoresis the gel was stained with 0.5  
87  $\mu$ g/mL of Ethidium Bromide in 1x TBE and observed in a UV transilluminator. The visualized

88 homoduplexes bands were cutted, eluted at 56°C/5min in 200 µL H<sub>2</sub>O of which 1 µL was re-amplified  
89 using the nPCR primers set described above. The resulting amplicons were cloned in pGEM®-T Easy  
90 Vector System (Promega) according to the manufacture's conditions and sequenced by STAB Vida  
91 Lda, Portugal. **The nucleotide sequences were submitted to Genbank and were assigned the**  
92 **Acession numbers EU327694- EU327752. Sample identification was included in table 1. FCoV.**  
93 Type I and II assignment based on the amplicons length were in accordance with the nucleotide  
94 sequence of each sample.

## 95 2.5. Phylogenetic analysis

96 The nucleotide sequences were aligned (PILEUP from GCG Wisconsin Package v. 10.0), including  
97 sequences of FCoV Type I (KU\_2 AN- D32044; UCD1 AN- AB088222; Black AN-AB088223;  
98 C2\_0301UK AN- AY159737; J8\_0196UK AN- AY159758; FQ1\_1099UK AN- AY159754; H9\_1100UK  
99 AN- AY159770; FUI\_1191UK AN- AY159735) and FCoV Type II (79\_1683 AN-X80799; 79\_1146 AN-  
100 X06170; G6\_1095 AN- AY159784). Due to its recombinant status **FCoV** Type II sequences were  
101 aligned and submitted to phylogenetic analysis separately from Type I sequences.

102 The phylogenetic relationship between sequences was inferred by genetic distances between pairs of  
103 sequences using Kimura's two parameter method (DNADIST, Phylip Package).The values of  
104 transition/transversion ratio and nucleotide frequencies were calculated from the data sets (TREE-  
105 PUZZLE) (Strimmer and von Haeseler, 1997). The resulting trees were constructed by Neighbor  
106 Joining (NEIGHBOR, Phylip Package) and the branching order reliability was evaluated by bootstrap  
107 analysis of 1000 replicates (SEQBOOT, Phylip Package). To maximize the phylogenetic information of  
108 the nucleotide alignment closely related sequences were excluded but were indicated between  
109 brackets following the sequence chosen to represent each set of viruses as summarized; 65LVI\_05  
110 [74LVI\_05, 81LVI\_05, 86LVI\_05, 87LVI\_05]; 14LVI\_05 [10LVI\_05a]; 19LVI\_04a [19LVI\_04b];  
111 58LVI\_05 [59LVI\_05a/b]; 1Lg\_06 [1Fg\_06a].

112

## 113 **3- Results**

### 114 3.1. Detection of Type I and II **FCoV**

115 For three years 120 samples were collected from domestic cats to detect **FCoV** RNA due to clinical  
116 suspicion of FIP **and to confirm viral excretion from healthy animals. Fifty seven** samples were  
117 found **FCoV** positive (47,5%) and afterwards processed for Type I and II differentiation. Out of those  
118 57 positive samples, 45 (79%) were classified as Type I **FCoV and were** obtained from healthy

119 animals or with FIP. Only two samples (3,5%) from animals with FIP, were classified as Type II. No  
120 amplification was obtained from ten samples (17,5%).

### 121 3.2. Detection of viral quasispecies

122 The presence of viral quasispecies within the animal, was evaluated by HMA for each sample (Fig. 1).

123 This assay detects the presence of fully (homoduplexes) and partially (heteroduplexes)  
124 complementary DNA chains formed between highly related sequences during the process of  
125 denaturing and annealing. Homoduplexes molecules tend to migrate faster, while heteroduplexes  
126 show a reduced mobility in a non-denaturing PAGE. The observation of two or more homoduplexes  
127 bands, plus the corresponding heteroduplexes bands indicates the presence of different DNA  
128 populations within the sample, named viral quasispecies and mirror the *in vivo* genetic diversity of the  
129 virus (Delwart et al., 1993).

130 Within the 47 S gene amplicons 37 samples (78,7%) showed no genetic diversity whereas in 10  
131 samples (21,3%) two homoduplexes bands were observed **and individually amplified**. Comparison  
132 of the nucleotide sequences of the heteroduplexes positive samples revealed similarities of 90% to  
133 99% along the 320nt in Type I and 170nt in Type II **FCoV**. Although very similar, six sequences  
134 (13LVI\_04b; 19LVI\_04b; 10LVI\_05a; 11LV\_05a; 33LVI\_05b; 99LVI\_05b) showed a 5'end variation  
135 length of 28 to 56 nucleotides justifying the homo/heteroduplexes formation. Sequences 33LVI\_05a/b,  
136 25LVI\_04a/b, 10LVI\_05a/b and 11LVI\_05a/b were amplified from animals with multi-cat environment  
137 **(table 2)**.

### 138 3.3. Phylogenetic analysis

139 The genetic relationships between the Portuguese sequences of **FCoV** and previously assigned  
140 sequences from isolates of Type revealed a heterogeneous clustering of sequences with several tree  
141 branches supported by high bootstrap values (Fig. 2). Sequences from distinct viral quasispecies  
142 obtained from the same animal as well as sequences from cats sharing the same household were  
143 included in branch A indicating the presence of different viral strains within each confined cat  
144 population and within the same individual host. Sequences 83 LVI\_05, 88LVI\_05, 90LVI\_05, 94LVI\_05  
145 and 96LVI\_05 came from cats sharing the same household than the source animal of sequence  
146 65LVI\_05 [74LVI\_05, 81LVI\_05, 86LVI\_05, 87LVI\_05]. **However sequence 65LVI\_05** was positioned  
147 outside group A, in a separate branch with 80% of bootstrap value. **The above mentioned**  
148 **sequences** were obtained from a multi-cat household where a clinical FIP episode (65LVI\_05) had  
149 been reported. Although all the other animals were FCoV positive, none developed FIP. **Sequence**  
150 **11LVI\_05b was also included in branch A but sequence 11LVI\_05a, obtained from the same cat**

151 was positioned in branch B. Sequence 25LVI\_04a was grouped in branch A though it was  
152 obtained from the same animal as sequence 25LVI\_04b placed in a different branch. These two  
153 viral quasispecies (25LVI\_04a/b) came from the same household of the source cats of  
154 sequences 22LVI\_04a/b and 23LVI\_04, which in this analysis were found distributed in outlier  
155 branches supported by 100% of bootstrap value. Sequences 20LVI\_06Z (stool), 20LVI\_06PBM  
156 (blood) and 20LVI\_06L (ascites) amplified from the same animal, were placed in two separate  
157 branches, one with 100% bootstrap value (20LVI\_06PBM and 20LVI\_06L).  
158 Type II **FCoV** phylogenetic tree included previously assigned **FCoV** Type II sequences and two  
159 Portuguese sequences obtained during this study, one positive to viral quasispecies (32LVI\_06;  
160 32LVI\_06a/b). All the sequences clustered in one branch, although FIPV 79-1146 and 79-1683  
161 grouped together with 100% of bootstrap value. Due to the low number of nucleotides considered in  
162 the alignment (170b), a likelihood mapping analysis was performed (TREE-PUZZLE) to assess its  
163 phylogenetic content, revealing 62.86% of fully resolved, 0% of partially resolved and 37.14% of  
164 unresolved quartets demonstrating therefore its reliable use to estimate phylogenetic relationships.

165

#### 166 4. Discussion

167 Until now, no previous study on the presence and prevalence of FCoV Type I and II in domestic cats  
168 had been done in Portugal. **In our analysis 47,5% of the samples were positive in the RTPCR**  
169 **assay (57/120) and were obtained either from healthy animals or from cats with FIP.** Moreover,  
170 the vast majority of the amplicons was classified as Type I. Type II was found only in two samples both  
171 from cats with FIP. The values found for **FCoV** Type I and Type II were in accordance with the  
172 distribution reported in Europe and USA (Addie et al., 2003; Benetka et al., 2004) although in Japan  
173 **and Taiwan** a higher prevalence of **FCoV** Type II was observed in animals with FIP (Hohdatsu et al.,  
174 1992, Lin et al., 2008). In our study even though the two **FCoV** type II sequences were obtained from  
175 **sick** cats, the low incidence of this viral biotype does not allow any relationship between **FCoV** II and  
176 symptomatic disease.

177 The evaluation of FCoV genetic diversity **revealed** 21% of the amplicons positive for viral  
178 quasispecies. These viral variants were found within the same animal **but also in samples collected**  
179 **from animals** sharing a multicat environment. This finding **supports the viral internal mutation**  
180 **theory (Dye and Siddell, 2007) and confirms the animal exposure to divergent viral strains.**

181 The significance of **FCoV** quasispecies had already been studied (Battilani et al., 2003) suggesting a  
182 correlation between high viral genetic diversity within the host and development of disease. **However**



183 from our data it was not possible to correlate viral diversity with virus subtype or disease therefore a  
184 more extensive study should be performed to assess this connection.

185 The phylogenetic analysis of **FCoV** Type I and II sequences revealed an assorted distribution of  
186 sequences in Type I tree, showing high genetic diversity of the **FCoV** Portuguese sequences, its  
187 divergence toward European **and Japanese** sequences (Fq1\_1099UK, H9\_1100UK, and strains KU2,  
188 Black and UCD1) confirming the previous findings.

189 Interestingly, **FCoV** Type II phylogenetic tree although with a limited number of Portuguese and  
190 previously characterised Type II sequences, showed a homogeneous distribution of sequences as  
191 also reported by Lin et al., 2008 in a similar study, indicative of high similarity between distantly  
192 related sequences (UK; Netherlands; Portugal). The genetic diversity of the Portuguese FCoV  
193 sequences confirmed and strengthens the evidence that this virus persists within the host as mingled  
194 viral populations whose interaction with the animal may or not result in severe disease.

195 In conclusion this study provided information on the FCoV molecular epidemiology in Southern Europe  
196 which was unavailable until now and revealed high genetic diversity between the Portuguese and  
197 previously characterized FCoV sequences.

198

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202 collaboration in sampling collection.

203

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- 242
- 243

244 **Figure captions**

245

246 **Figure 1-** Heteroduplex mobility assay of S amplicons from samples 10LVI\_05 (1); 11LVI\_05  
247 (2); 3LVI\_05 (3); 25LVI\_04 (4); 23LVI\_04 (5); 22LVI\_04 (6); 20 LVI\_04 (7); 10LVI\_04  
248 (8); 13LVI\_04 (9); 12LVI\_04 (10), included in Type I FCoV.

249 Lanes 1, 2, 4, 6, 8 and 9 (+) show the presence of 2 homoduplexes bands (→),  
250 corresponding to fully complementary DNA chains. The heteroduplexes bands (\*)  
251 consistent to partially complementary DNA chains with reduced mobility are clearly  
252 visible in lanes 2, 6, 8 and 9. In lanes 3, 5 and 7 no homo/heteroduplexes are  
253 present. The photograph of the ethidium bromide-stained gels is displayed as  
254 reversed image.

255

256 **Figure 2.** Phylogenetic analysis of FCoV type I and II sequences based on the 3' end of the  
257 S gene. The trees were constructed by Neighbor Joining based on the genetic  
258 distances between the nucleotide sequences, calculated by Kimura two-parameter  
259 using a transition/transversion rate of 3.23 for Type I sequences and 9.99 for Type II  
260 sequences. A bootstrap analysis of 1000 replicates was performed and values above  
261 80% are indicated on the branches. Branches A, B and C include FCoV sequences  
262 with the lowest value of nucleotide substitutions within each branch. Sequences  
263 marked with an asterisk (\*) were amplified from animals with FIP.

264

265

266



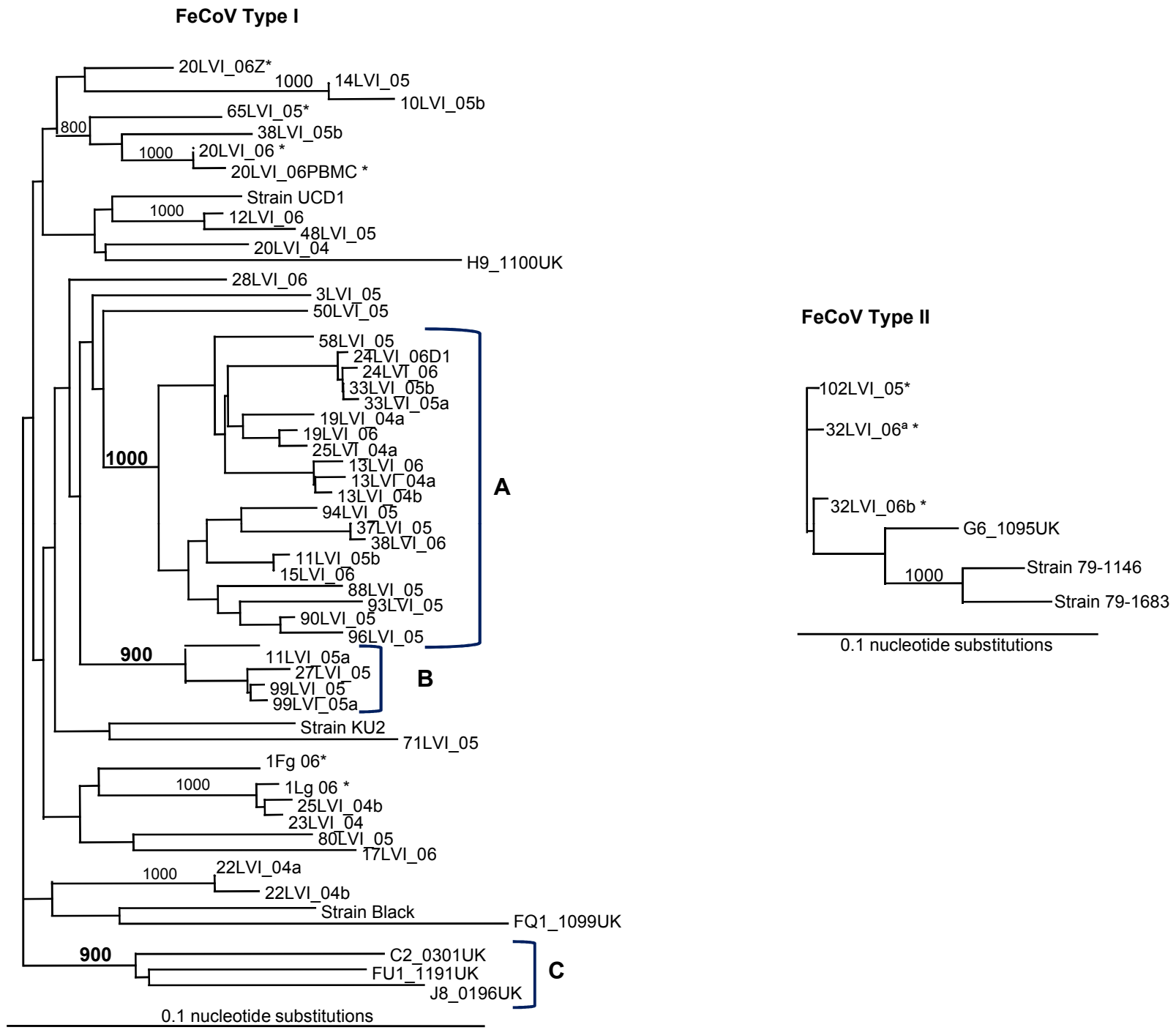


Table 1- Table 1: Identification and origin of the Portuguese FeCoV sequences, amplified from healthy and FIP (S) animals. The genetic diversity of the amplicons was evaluated by Heteroduplex mobility assay and each positive sample was reamplified. The number of corresponding sequences is indicated (a-c).

Identification	Heteroduplexes	Origin	
<b>2004</b>			
13LVI_2004	Positive (a;b)	Lisbon, Portugal	
19LVI_2004	Positive (a;b)	Lisbon, Portugal	
20LVI_2004	Negative	Lisbon, Portugal	
22LVI_2004	Positive	Palmela, Portugal	Sharing household
23LVI_2004	Negative	Palmela, Portugal	
25LVI_2004	Positive (a;b)	Palmela, Portugal	
<b>2005</b>			
3LVI_2005	Negative	Lisbon, Portugal	
10LVI_2005	Positive (a;b)	Lisbon, Portugal	Sharing household
11LVI_2005	Positive (a;b)	Lisbon, Portugal	
14LVI_2005	Negative	Lisbon, Portugal	
27LVI_2005	Negative	Lisbon, Portugal	
33LVI_2005	Positive (a;b)	Rio Mouro, Portugal	Multicat environment
37LVI_2005	Negative	Lisbon, Portugal	
38LVI_2005	Negative	Lisbon, Portugal	
48LVI_2005	Negative	Lisbon, Portugal	
50LVI_2005	Negative	Lisbon, Portugal	
58LVI_2005	Negative	Lisbon, Portugal	
59LVI_2005	Negative	Lisbon, Portugal	
71LVI_2005	Negative	Lisbon, Portugal	
80LVI_2005	Negative	Vila Nova de Gaia, Portugal	
65LVI_2005 (S)	Negative	Lisbon, Portugal	Sharing household
74LVI_2005	Negative	Lisbon, Portugal	
81LVI_2005	Negative	Lisbon, Portugal	
86LVI_2005	Negative	Lisbon, Portugal	
87LVI_2005	Negative	Lisbon, Portugal	
88LVI_2005	Negative	Lisbon, Portugal	
90LVI_2005	Negative	Lisbon, Portugal	
93LVI_2005	Negative	Lisbon, Portugal	
94LVI_2005	Negative	Lisbon, Portugal	
96LVI_2005	Negative	Lisbon, Portugal	
99LVI_2005	Positive (a;b)	Lisbon, Portugal	
102LVI_2005	Negative	Lisbon, Portugal	
<b>2006</b>			
12LVI_2006	Negative	Lisbon, Portugal	
13LVI_2006	Negative	Lisbon, Portugal	
15LVI_2006	Negative	Lisbon, Portugal	
19LVI_2006	Negative	Lisbon, Portugal	
20LVI_2006L	Negative	Lisbon, Portugal	
20LVI_2006P BMC	Negative	Lisbon, Portugal	
20LVI_2006Z	Negative	Lisbon, Portugal	
24LVI_2006	Negative	Lisbon, Portugal	
24LVI_2006D1	Negative	Lisbon, Portugal	
1FG_2006 (S)	Positive (a;b)	Lisbon, Portugal	
1LG_2006	Negative	Lisbon, Portugal	
28LVI_2006	Negative	Lisbon, Portugal	
29LVI_2006	Negative	Lisbon, Portugal	
32LVI_2006 (S)	Positive (a;b,c)	Lisbon, Portugal	
38LVI_2006	Negative	Lisbon, Portugal	

Table 2-: Genetic diversity between amplicons subjected to HMA. The length and the percentages of genetic similarity between homoduplexes are shown.

Identification	Sequence	Similarity (%)	FCoV
13LVI_04a	320bp	317/320 (99%)	Type I
13LVI_04b	348bp	Extra 5' 28nt	
19LVI_04a	320bp	320/320 (100%)	
19LVI_04b	348bp	Extra 5' 28nt	
22LVI_04a	347bp	344/347 (99%)	
22LVI_04b	347bp		
25LVI_04a	320bp	290/320 (90%)	
25LVI_04b	320bp		
10LVI_05a	348bp	316/320 (98%)	
10LVI_05b	320bp	Extra 5' 28nt	
11LVI_05a	348bp	303/320 (94%)	
11LVI_05b	320bp	Extra 5' 28nt	
33LVI_05a	320bp	319/320 (99%)	
33LVI_05b	348bp	Extra 5' 28nt	
99LVI_05a	320bp	319/320 (99%)	
99LVI_05b	376bp	Extra 5' 56nt	
1FLVI_06a	320bp	299/320 (93%)	
1FLVI_06b	320bp		
32LVI_06a	170bp	168/170 (98%)	Type II
32LVI_06b	170bp		