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Origin and transmission of Feline coronavirus type I in domestic cats from Northern Italy: a phylogeographic approach

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ABSTRACT

Feline coronavirus (FCoV) is responsible, along with an inadequate immune response of the host, for Feline infectious peritonitis (FIP), one of the most frequent and deadly infectious feline disease worldwide. This study analyzed the genetic characteristics of the spike (S) gene of 33 FCoVs circulating in Northern Italy between 2011 and 2015 in cats with or without FIP. In order to reconstruct the most probable places of origin and dispersion of FCoV among Italian cats, a phylogeographic approach was performed based on 106 FCoV S gene partial sequences from cats, including the 33 novel Italian sequences and 73 retrieved from public databases. Only FCoV type I was found in the Italian cats. The estimated mean evolutionary rate of FCoV was 2.4×10^{-2} subs/site/year (95% HPD: 1.3-3.7 $\times 10^{-2}$), confirming the high genetic variability in the circulating strains. All the isolates clustered in a unique highly significant clade that likely originated from USA between the 1950s and the 1970s, confirming the first descriptions of the disease in American cats. Our results suggest that from USA the virus likely entered Germany and thereafter spread to other European countries. Phylogeography showed that sequences segregated mainly by geographical origin. In the 2010s Italian sequences clustered in different subclades, confirming that different strains cocirculate in Italy. Further studies on archival samples and other genetic regions of FCoV are suggested in order to confirm the present results and to reconstruct a more in-depth detailed virus dispersion pattern for the definition of possible control measures.

1. Introduction

Feline infectious peritonitis (FIP) is a relatively new disease of cats that was first described in 1963 in the United States (Holzworth, 1963) and continues to be one of the most frequent fatal and infectious feline disease worldwide. FIP is caused by a virulent feline coronavirus (feline infectious peritonitis virus, FIPV) and by an inadequate response of the host (Pedersen, 2009).

According to the International Committee on Taxonomy of Viruses (ICTV), feline coronavirus (FCoV) belongs to the order *Nidovirales*, the family *Coronaviridae*, the subfamily *Orthocoronavirinae*, the genus *Alphacoronavirus*, the subgenus *Tegacovirus* and the species *Alphacoronavirus* 1, together with canine coronaviruses (CCoVs) and porcine transmissible gastroenteritis virus (TGEV). According to their serological and genetic properties, FCoVs are classified into type I and type II and recently their classification in *Alphacoronavirus* 1 clade A and clade B has been proposed, respectively (Jaimes et al., 2020). Type I is the most frequently detected FCoV in cats and has a worldwide distribution.

FCoVs can also be divided into two biotypes that are generally referred to as the avirulent endemic feline enteric coronavirus (FECV), that is usually cause of asymptomatic infections and is responsible only for a mild and transient enteritis, and the virulent biotype FIPV that is responsible for FIP (Pedersen, 2014). These two biotypes exist in both types I and II (Tekes and Thiel, 2016; Jaimes et al., 2020). Like other RNA viruses, coronaviruses are prone to mutations. Few mutations in accessory genes and the spike (S) gene of FCoVs have been identified. The mutations M1058L or S1060A in the S gene, that were initially thought to be a marker for FIPV, were recently associated to the ability of the virus to infect and replicate in monocytes and macrophages, representing a marker for systemic FCoV replication (Chang et al., 2012, Pedersen, 2014; Porter et al., 2014; Stranieri et al., 2018; Felten and Hartmann, 2019).

The S gene is also used for FCoV typing. The S gene of FCoV types I and II differ: FCoV type I harbors the original feline S gene whereas the FCoV type II acquired the S gene (along with other

genes) from the CCoV during recombination events (Jamies et al., 2020). Moreover, because the S gene encodes for the spike protein, which is the protein most subject to evolutionary immune pressure, it is the most variable of the FCoV genes. Therefore, the S gene is also useful for genetic characterization of strains (Addie et al., 2003; Kipar and Meli, 2014).

Genome sequences and phylogenetic analysis showed that FCoV isolates form clusters according to geographic distribution, regardless of disease phenotype (Kipar and Meli, 2014). Sequence comparisons demonstrated that FECVs and FIPVs from the same group of cats were very closely related, while significant genetic variation existed between FECVs and FIPVs that were from different geographic areas (Pedersen, 2014). For a better understanding of pathways of infection dispersion, a phylogeographical analysis that allows reconstruction of the most probable place of origin of infections and flow of geographic spread of viruses has been developed (Lemey et al., 2009; Drummond et al., 2012). This approach has been used to reconstruct spatial and temporal dispersion of some highly variable viruses but, to our knowledge, has been applied only in one recent study providing insights into the origin of FCoV in Brazil (Myrrha et al., 2019). Phylogeographical analyses has never been applied for the reconstruction of FCoV origin in Italy.

In Italy, FCoV has been found in cats with seroprevalences ranging from 39% to 82%, indicating an active circulation of FCoV in our country (Pratelli, 2008; Spada et al., 2016). Phylogenetic analyses of FCoV circulating in Italian cats have been based on genes other than S gene, such as the nucleocapsid and accessory protein 7b genes that do not allow identification of the FCoV type (Battilani et al., 2010). Information on the type of FCoVs circulating in Italy is limited to the descriptions of a type II FCoV in a cat in 1992 and, more recently, a FCoV type I (Campolo et al., 2005).

The aims of this study were i) to characterize the type and genetic diversity of FCoV circulating in Northern Italy using partial S gene sequences and ii) to reconstruct the origin and spatiotemporal distribution of type I FCoV circulation in Italy by a phylogeographic approach.

2. Materials and Methods

2.1. Cat samples and sequences

The material comprised feline samples sent to the Diagnostic laboratory of the Veterinary Teaching Hospital (VTH) of the University of Milan (Lodi, Italy) between 2011 and 2015 for testing because of suspected FIP or for routine FCoV screening of healthy animals or cats with suspected diseases other than FIP. All the samples were collected for diagnostic purposes or within plan of monitoring the rate of FCoV infection within catteries and the owners signed an informed consent about the use of residual amounts of samples for research purposes. Therefore, according to the Ethical Committee of our Institution a formal approval of the Ethical Committee was not required (decision 2/2016). The records of the cats kept at the Diagnostic laboratory of the VTH were used for the definitive diagnosis of FIP. The records included results of post-mortem diagnosis using the gold standard test (immuno-histochemistry, IHC) for FIP in the case of mortality of animals. FIP diagnosis was made according to internationally recognized criteria (Addie et al., 2009; Felten and Hartmann, 2019), as reported in previous studies (Meli et al., 2013; Lorusso et al., 2019). FIP was excluded in the presence of persistently clinically asymptomatic cats.

The FCoV positive samples and the derived sequences were selected on the basis of the following criteria: (1) one sequence obtained for each cat, (2) known locality and collection date, (3) known owner or cattery, and (4) sequences representative of FCoV-infected cats with and without FIP.

A total of 33 FCoV type I S gene novel sequences obtained from 33 cats were included. Origins and characteristics of novel Italian FCoV strains are summarized in Table 1 and supplementary Table S1. Sequences were obtained from 17 privately owned cats (n=24 cats) and from cats from 3 catteries (n=9 cats). The majority of private owners (15/17) owned one cat while the remaining 2 owners owned 2 or 7 cats. Two cats were obtained from 2 catteries each and five cats were obtained from the third cattery. Sequences were obtained from cats with (n=23) and without (n=10) FIP. The

localities of origin of cats were grouped in 5 provinces from Northern Italy (Bergamo, Como, Monza Brianza, Milano and Pavia) and one from Central Italy (Rome).

2.2. RT-PCR and sequencing

Viral RNA was extracted from samples obtained from the 33 cats. More precisely, RNA of the 33 novel sequences was extracted from samples of mesenteric lymph node (n=13), feces (n=7), cerebrospinal fluid (CSF, n=4), effusions (n=2), aqueous humour (n=2), whole blood (n=1), liver (n=1), intestine (n=1), spleen (n=1) and thoracic wall (n=1), using a commercial RNA extraction kit (NucleoSpin® RNA Isolation kit or Nucleopin® RNA Virus Isolation commercial kit, Macherey-Nagle, Düren, Germany), according to the manufacturer's instruction. The extracted RNA samples were immediately used or frozen at -80°C for further molecular analysis.

Extracted RNA was tested for the presence of FCoV by a reverse transcription nested PCR (RT-nPCR) targeting a 177 bp product of the highly conserved 3' un-translated region of the viral genome of both type I and type II FCoV (Herrewegh et al., 1995). FCoV positive samples were checked for the virus type by using an RT-nPCR targeting a 360 bp and a 218 bp product of the S gene for type I and type II FCoV, respectively (Addie et al., 2003).

Samples positive for type I FCoV were further tested by using an RT-nPCR targeting a 142 bp product of the S gene of type I FCoV used to identify the mutations M1058L or S1060A, as described previously (Chang et al., 2012). The 142 bp amplicons from S RT-nPCR positive samples were sequenced using the same forward and reverse primers used for the second reaction. Sequencing was performed at an external laboratory (Parco Tecnologico Padano, Lodi, Italy) using a Big Dye Terminator version 3.1 Cycle Sequencing kit (Applied biosystems, CA, USA) and an AB3730 DNA Analyzer (Applied biosystems, CA, USA).

The sequence data were assembled and manually corrected using BioEdit software version 7.0 (freely available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html). All the consensus sequences

were aligned with representative FCoV type I and type II strains retrieved from GenBank, using Clustal X in the BioEdit software version 7.0. Consensus sequences were also aligned with FCoV strains retrieved from GenBank harboring, or not, the mutations M1058L or S1060A. All the consensus sequences were identified as mutated FCoV in the presence of mutations M1058L or S1060A, whereas sequences were identified as unmutated FCoV if these mutations were not present, as previously reported (Chang et al., 2012).

Sequence identification was according to location (city)/ owner or cattery identification from the city/ animal identification from the same owner or cattery/ virus biotype according to RT-nPCR S sequencing/ specimen identification/"@" and year of collection (e.g., BGAaFc@15 = sample from Bergamo (BG), owner A, animal a, S sequencing indicative of mutated FCoV strain (F), CSF (c), collected in 2015).

The S gene coding sequences were submitted to GenBank (MT250347-MT250379).

2.3 FCoV data set

The 33 novel Italian FCoV type I sequences dataset obtained in this study was compared with FCoV partial gene S sequences of FCoV type 1 deposited before 2016 from other countries, retrieved from public database. The sequences from other countries were selected based on the following inclusion criteria: 1) sequences already published in peer-reviewed journals or deposited in GenBank; 2) no uncertainty about FCoV type assignment; 3) no uncertainty about country of origin; 4) sampling dates were known or established in the original publication and 5) sequences were from the corresponding S region that was amplified by the RT-nPCR protocol used for Italian sequences (Chang et al., 2012). A total of 73 FCoV partial gene S sequences of FCoV type I from other countries were available from the national centre for biotechnology information (http://www.ncbi.nlm.nih.gov/). Sequences were obtained from the Netherlands (NL, n=32), Germany (D, n=17), United Kingdom (UK, n=7), United States of America (US, n=14), Japan (J,

n=2) and Taiwan (TW, n=1). Sequences were named as previously described for Italian FCoV sequences, except that cats were identified by numbers and not by letters and owner or cattery identification was not included because this information was not available. On the whole, the sampling dates ranged from 1975 to 2011. Origins and characteristics of FCoV strains retrieved from countries other than Italy are summarized in supplementary Table 2.

Two different datasets were built. The first dataset contained all 33 partial S gene sequences of FCoV type I from Italy and all 73 sequences from other countries (NL, D, US, UK, J, TW), for a total of 106 of partial gene S sequences from FCoV, and was used to estimate the mean evolutionary rate and was used for phylogenetic reconstruction.

The second dataset contained a representative and reliable selection of sequences from every country for which more than 5 sequences were available. Dataset contained representative selected sequences for Italy (n=11) and for each other country from the first dataset (NL n=11, D n=9, US n=10 and UK n=7). Representative sequences were randomly selected for each cluster obtained according to phylogenetic reconstruction, for a total of 48 sequences. This second data set was used to perform phylogeographical analyses.

2.4. Likelihood mapping analysis

The phylogenetic signal of each sequence dataset was investigated by means of the likelihood-mapping analysis of 10,000 random quartets generated using TreePuzzle. All of the three possible unrooted trees for a set of four sequences (quartets) randomly selected from the dataset were reconstructed using the maximum likelihood approach and the selected substitution model. The posterior probabilities of each tree were then plotted on a triangular surface so that the dots representing the fully resolved trees fell at the corners and those representing the unresolved quartets in the centre of the triangle (star-tree) (Schmidt et al., 2002). Using this strategy, which has been

described in detail elsewhere (Strimmer and von Haeseler, 1997), the data are considered unreliable for phylogenetic inference when more than 30% of the dots are in the centre of the triangle.

2.5. Phylogenetic reconstruction

All the novel partial S gene sequences of FCoV type I from Italy were aligned with FCoV reference strains retrieved from GenBank using Clustal X; manual editing was performed with Bioedit software version 7.0 (freely available at http://www.mbio.ncsu.edu/bioedit/bioedit.html). Phylogeny was preliminary estimated with molecular evolutionary genetics analysis (MEGA version 7) (Kumar et al., 2016) using the neighbor-joining algorithm (NJ) with the Kimura 2-parameter model, and by maximum likelihood (ML) method with the General Time Reversible model, with 1,000 bootstrap replicates.

Moreover, the best-fitting nucleotide substitution model was estimated by means of JModeltest (Posada, 2008) and selected a TrN model (Rodriguez et al., 1990) with gamma-distributed rates among sites. The phylogenetic tree, model parameters, evolutionary rates and population growth were co-estimated using a Bayesian Markov chain Monte Carlo (MCMC) method implemented in the BEAST v.1.84 package (Drummond et al., 2012).

Statistical support for specific clades was obtained by calculating the posterior probability of each monophyletic clade. As coalescent priors, three simple parametric demographic models (constant population size, exponential and logistic population growth) and a piecewise-constant model, the Bayesian skyline plot (BSP) under both a strict and a relaxed (uncorrelated log-normal) clock (Drummond et al., 2012) were compared.

Two independent MCMC chains were run for 50 million generations with sampling every 5,000th generation and were combined using the LogCombiner 1.84 included in the BEAST package. Convergence was assessed on the basis of the effective sampling size (ESS) after a 10% burn-in using

Tracer software version 1.7.1 (http://tree.bio.ed.ac.uk/software/tracer/). Only ESS's of ≥ 200 were accepted.

Uncertainty in the estimates was indicated by 95% highest posterior density (95% HPD) intervals, and the best-fitting models were selected using a Bayes factor (BF with using marginal likelihoods) implemented in BEAST (Suchard et al., 2001).

In accordance with Kass and Raftery (1995), only values of 2lnBF ≥ 4 were considered significant.

The trees were summarised in a target tree using the Tree Annotator program included in the BEAST package, choosing the tree with the maximum product of posterior probabilities (maximum clade credibility) after a 10% burn-in.

The time of the most recent common ancestor (tMRCA) estimates were expressed as mean and 95% HPD years before the most recent sampling dates, corresponding to 2015 in this study.

2.6. Bayesian phylogeographic analyses

For discrete state phylogeography, the continuous-time Markov Chain (MCC) process over discrete sampling locations implemented in BEAST (Lemey at al., 2009) was used for the geographical analysis, implementing the Bayesian Stochastic Search Variable Selection (BSSVS) model which allows the diffusion rates to be zero with a positive prior probability. Comparison of the posterior and prior probabilities that the individual rates would be zero provided a formal BF for testing the significance of the linkages between locations: rates with a BF of >3 were considered well supported and formed the migration pathway. This analysis was performed for FCoV dataset (n=48) assigned to 5 distinct countries (Italy= IT, NL, US, D, UK), on the basis of the sampling location, as previously described.

The final tree was visualised using FigTree version 1.4.3 (available at http://tree.bio.ed.ac.uk/software). The significant migration rates were analysed and visualised using SPREAD, which is available at http://www.kuleuven.be/aidslab/phylogeography/SPREAD.html.

In order to provide a spatial projection, the migration routes indicated by the tree were visualised using Google Earth (http://earth.google.com).

2.7. First descriptions of FIP in cats worldwide

The year of the first descriptions of FIP previously reported worldwide in cats were obtained from available peer-reviewed journals (Holzworth 1963; Wolfe and Griesemer, 1966; Stevenson et al., 1971; de Aluja, 1972; Jones, 1975; Flagstad and Larsen, 1976; Chappuis and Duret, 1978; Landes et al., 1984; Paltrinieri S., personal communication).

3. Results

3.1. FCoV S gene sequencing

All 33 FCoV sequences obtained in this study belonged to type I FCoV (Table 1, supplementary Table S1), as confirmed by phylogeny based on the NJ method that showed that sequences clustered with FCoV type I reference strains Figure 1 represents the results of representative Italian sequences of FCoV by NJ phylogeny. Similar results were obtained using ML (data not shown)..

3.2. Likelihood mapping amalysis

The likelihood mapping of 10,000 random quartets showed that more than 77% were distributed at the corners of the likelihood map and 14% in the central area, thus indicating that the dataset contained sufficient phylogenetic information (supplementary Figure S2).

3.3. *Evolutionary rates, tMRCA estimates and Bayesian discrete phylogeography*

The evolutionary rates, tMRCAs and phylogeography were co-estimated using a Bayesian framework implemented in Beast (v. 1.8.4).

The BF analysis showed that the uncorrelated lognormal relaxed clock fitted the data significantly better than the strict clock (2lnBF strict *vs* relaxed clock = -466.62). Under the relaxed clock the BF analysis showed that the constant model was better than the other models (2lnBF ≥ 4). The estimated mean evolutionary rate of the FCoV S gene sequences analysed was 2.4×10^{-2} subs/site/month (95% HPD: 1.3 - 3.7 $\times10^{-2}$).

The maximum clade credibility tree summarizing all the dated trees obtained during the MCMC search for FCoV type I is reported in Figure 2. The results of the phylogeographical analyses using the MCC process are reported in Figure 3. The time of the most common ancestor estimates of FCoV in cats and locality of origin of significant clades and subclades including Italian sequences are reported in Table 2.

Based on the combined results of our analyses, the most probable origin of FCoV type I, estimated on the basis of the tree-root tMRCA, was in the 1950s-1970s (Table 2) in USA, supported by a significantly higher posterior probability than other locations (state pp=0.8 vs state pp=0.13 for Germany, the second most probable location of the root) (Table 2, Fig. 3).

After its origin, FCoV type I branched into two main clades (pp > 0.8): one clade A including most of the sequences from all countries originating in the 1970s-1980s and one small clade B that included only two sequences from Germany originating in the first years of 2000 (Fig 2, Table 2). USA represents the most probable location of the main clade A whereas Germany was the most likely origin of the small clade B (Table 2, Fig. 3). Within the main clade A, two subclades were observed encompassing sequences from two or more countries (Fig. 2). USA represents the most probable location of subclade A1, whereas the first European country representing the probable origin of a European subclade is Germany, representing the most probable location of subclade A2 originating in the 1990s (Fig. 3, Table 2). Overall, within subclade A1, 12 significant subclades were observed, encompassing 2 to 34 sequences (Fig. 2). The median year of origin of the 3 subclades including sequences from different countries was 2003 (2001–2005) and sequences in these subclades were collected up to a 11 years period. Italian sequences segregating with

sequences from other countries were observed in subclades A1a and A1b (Fig.2, Table 2). The most probable origins of subclade A1a were Germany and the Netherlands (state pp=0.52 vs state pp=0.45 for the Netherlands, the second most probable location of the root) whereas subclade A1b likely originated from the Netherlands (state pp= 0.98 vs state pp= 0.10 for Germany, the second most probable location of the root) (Fig. 3, Table 2).

A significant geographical structure was observed in the last decade, with the majority of sequences segregating on the basis of their geographical origin (Fig. 2). These significant recent subclades included 2-10 sequences from the same country (Fig. 2) that were mostly collected within the same year. The median year of origin of all the subclades involving sequences from only one country was 2008 (1993–2012). The Italian significant subclades comprising ≥ 3 sequences (A1c-A1e) dated 2012. The most probable origin of subclades A1c-A1e was Italy (Table 2). Italian sequences segregating together were mainly from the same province, from the same city or from cities close to each other (ranging from 13 km to 39 km distance). Only one significant Italian subclade with sequences from Italian cities far from each other was observed (subclade A1d).

Significant migration linkages reported in Figure 4 were observed between the US and Germany (BF=5.54), Germany and the Netherlands (BF=82.97) and the Netherlands and Italy (BF=10,175.76). The phylogeographical reconstruction of the significant links showed that, after its origin in USA in the 1950s-1970s, the virus spread in Europe (Germany) in the 1990s, reaching NL in the first decade of the 2000s and Italy only in the second decade (Fig. 4).

3.4. Year of the first description of FIP worldwide

The year of the first descriptions of FIP reported worldwide based on peer-reviewed journals are summarized in Table 3.

4. Discussion

The study primarily focused on the genetic characterization of FCoV circulating in Northern Italy in cats between 2011 and 2015, showing that all the sequences obtained in this study belonged to FCoV type I. The presence of type I is in agreement with previous studies indicating this FCoVtype as the most frequent worldwide (Pedersen, 2014).

According to phylogeographical reconstruction, the FCoV type I likely emerged in USA in average between 1950s and 1970s, confirming the first description of FIP starting from the early 1950's and the subsequent identification of FCoV as the causing agent of the disease in cats from the US in 1963 (Holzworth, 1963). The range of FCoV origin date that was estimated by our analysis is also confirmed by a previous study indicating that FCoV is estimated to have diverged from alphacoronavirues in 1953 (Vijaykrishna et al., 2007) and by the absence of descriptions of the disease before 1950 in the USA (Pedersen 2009). Moreover, the description of FIP in cats from USA occurring years before cats from other countries suggests the likely American origin of the virus. Indeed, based on available data in peer-reviewed journals, the first descriptions of FIP in other countries have been reported almost twenty years after the US, with the first reports of FIP in 1969 in Canada and in Germany and worldwide starting from the 1970s.

The significant migration link that was observed by our phylogeographical reconstruction between the US and Germany during 1974-1995 is in agreement with the information on the first reports of FIP in Germany years after the US (Landes et al., 1984) and suggests that the virus likely moved from the US to Europe where it reached Germany. Evidence of North America being the source of introduction of FCoV in other countries has also been recently reported in Brazil (Myrrha et al., 2019). FCoV introduction in countries is likely by animal movements. It has been reported that major geographical movements of viruses that need direct contact for viral transmission mirrors those of animals and that key migratory or transport routes followed by host species could also be responsible for virus transmission in areas where the virus was previously absent (Chen et al., 2015). Indeed, FCoV transmission occurs by the fecal-oral route and requires the presence of FECV-infected cats shedding the virus in their feces (Addie et al., 2003). In the case of FCoV,

animal movements between USA and Europe may have been linked to cat shows, that started in 1871 in UK and have been reported worldwide thereafter (Hornidge, 2002) or to animal movements for breeding purposes. It has to be kept in mind that during the Second World War cat breeds from Europe were almost extinct (Bell et al., 2012) and that after the end of the war cats were frequently introduced from the US, where American cat breeders had continued to develop cat breeds (Longeri M., personal communication). Moreover, cats movement may also have been linked to owners' movements. During the Cold War, thousands of American soldiers and their families were moved to Germany (Alvah, 2007) and it is likely that cats followed their owners. Indeed, air flight companies specialized in pet shipping have reported in their websites that at least from the early days of 1968 pets were flown between the US and Germany (Lauzi S., personal communication). However, further investigations are needed to confirm this hypothesis that may also account for the introduction of FCoV-infected cats by American owners in other countries where US personnel and families stationed for prolonged periods after the Second World War, like Japan.

Our result indicating Germany being the first European country in which FIP was introduced is also suggested by the first description of the disease from samples collected in cats from this country (Landes et al., 1984), compared to other European countries where FIP was reported one or few years after Germany. The presence in our phylogeographic analysis of Germany being the most probable origin of the first European subclade with sequences from Germany and from the Netherlands suggests that FCoV type I may have initially spread between these two bordering countries and afterwards to other European countries, including Italy. Cat movements between European countries may have been associated with owner's movements, with imports of purebred cats for breeding purposes or by participation in cat shows. In Europe the first cat show with cats participating from different European countries dated 1949 and cat shows have been organized in Europe thereafter (Fédération Internationale Féline, 2019).

The significant geographical structure that was observed by our analysis, with the majority of FCoV sequences segregating on the basis of their geographical origin, has been also recently reported in

another study in Brazil (Myrrha et al., 2019). In the last decade, the presence of different significant subclades with few FCoV type I sequences collected from the same country (and from cities close to each other in case of Italian sequences), in a restricted period of time, likely represent recent networks in the local circulation of the virus. Results also suggest that in the last decade FCoV was mainly transmitted locally, confirming that FCoV trasmission requires a close contact with infected animals. Unfortunately, the only Italian cat living in a city far from the others, as well as the only Italian cat included in a subclade with foreign sequences (from cats from the Netherlands) were from two different catteries and it was not possible to establish their previous movements.

The presence of different significant subclades from the same country also indicates that different variants of the virus are circulating at the same time in a country, confirming the high genetic variability of FCoV (Addie et al., 2003, Pedersen, 2014), also showed by the high evolutionary rate of the FCoV S gene sequences analysed in our work. Our results also suggest that in the last decade FCoV transmission seem to rely on different areas from which the virus spreads. A similar result was not reported in Brazil (Myrrha et al., 2019), where the FCoV 7b gene was used for the phylogeographical analysis showing high conservation of sequences. It is likely that the use of a highly variable gene, such as the S gene, allows a deeper insight of the pattern of spread of FCoV in recent years.

Finally, it has to be taken in account that date estimations by our analysis may be biased by the absence of available European FCoV sequences collected before 2000 in our dataset. Therefore, our results need to be confirmed analysing a higher number of FCoV sequences from Germany and other European countries that to our knowledge are not available in GenBank. It cannot be ruled out that pre-existing avirulent feline coronaviruses may have been previously circulating in Europe in healthy cats. These strains may have been undiagnosed because they were less prone to undergo biotype conversion and cause FIP and/or because risk factors associated to the outcome of the disease (Addie et al., 2009) were less present in European countries compared to the US. Indeed,

the shift in the status of cats as pets after World War II, with number of pet cats greatly increased, has been identified as one of the principal factor for FCoV infection (Pedersen, 2009).

Moreover, it is not possible to establish if the available sequences from the databases truly reflected geographic distribution of the virus. It is also possible that available FCoV sequences simply reflected the countries with laboratories that first started sequencing because of their scientific interest in FCoV studies. The availability of FCoV sequences from samples collected from European cats before 2000, along with further investigations based on other genetic regions or on whole genomes of FCoV. would be useful to confirm the geographical origin of FCoV in Europe by a more complete phylogeographical analysis.

In conclusion, our results highlight the presence of FCoV type I in cats from Northern Italy and the high genetic variability of the S gene of circulating strains. Our phylogenetic and phylogeographical reconstruction suggests that after its origin in the USA the virus spread to Europe. Our results also showed that in the last decade different strains cocirculate in several provinces in Italy suggesting that FCoV transmission relies on several hot spots from which the virus likely spreads. Therefore, FCoV preventive measures based on control of animal movements from specific geographical areas are not easily defined and quarantine measures and testing of cats are still mandatory.

Results also suggest further studies based on archival samples and other genetic regions of FCoV in order to confirm our results and to obtain a more in-depth detailed reconstruction of the virus dispersion pattern. Moreover, considering that alpha- and betacoronaviruses have been reported to have their origin in bats (MacLachlan and Dubovi, 2016), further studied should also be aimed at the identification of FCoV source to better understand if bats may have been the origin of feline coronaviruses.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest associated with this study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found in the online version

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Tables

Table 1. Accession numbers, localities, collection years, FIP status and results of S gene sequencing of Italian FCoV sequences from cats derived from this study included in the dataset.

Table 2. Time of the most common ancestor estimates of FCoV in cats, credibility interval (95% HPD) of the main clades and subclades including Italian sequences observed in the MCC tree, with the corresponding localities, number of sequences and state posterior probability.

Table 3. First descriptions of FIP in cats worldwide.

Figure captions

Fig. 1. Phylogenetic tree based on a selection of representative S gene Italian sequences of FCoV type I detected between 2011 and 2015 and reference FCoV type I and type II strains. Molecular evolutionary genetics analyses were performed with MEGA7 using the NJ method. Distances were computed using the Kimura 2-parameter model. Bootstrap values > 70% are shown. Published sequences and references are identified by GenBank accession number (available at http://www.ncbi.nlm.nih.gov/pubmed/). The black diamond indicates representative novel nucleotide sequences of FCoV Italian strains.

Fig. 2. The maximum clade credibility tree of FCoV S from 106 cats. The numbers on the internal nodes indicate significant posterior probabilities (pp>0.5) of the corresponding nodes and the scale at the bottom of the tree represents the number of years before the last sampling time (2015). Significant clades (A, B), major subclades (A1, A2), and subclades comprising sequences from different countries including Italy (A1a, A1b) or Italian subclades with ≥ 3 sequences (A1c, A1d, A1e) are highlighted.

Fig. 3. The maximum clade credibility tree of FCoV S from a selection of 48 cats. The branches are colored on the basis of the most probable location of the descendent nodes (Unites States of America = US, Germany = D, The Netherlands = NL, United Kingdom = UK, Italy = IT). The numbers on the internal nodes indicate significant posterior probabilities (pp>0.5). Significant clades (A, B), major subclades (A1, A2), and subclades comprising sequences from different countries including Italy (A1a, A1b) or Italian subclades (A1c, A1d, A1e) are highlighted.

Fig. 4. The inferred spatiotemporal dynamics of FCoV in cats. The figure summarize the most significant migration links in Europe. The putative root of FCoV strains is highlighted with a yellow circle. More detailed results are reported in supplementary panels (Fig. S2).

Supplementary files

Table S1. Origin and characteristics of 33 FCoV Italian sequences from cats derived from this study included in the dataset.

Table S2. Accession numbers, references, localities and collection year of FCoV sequences from cats retrieved from GenBank included in the dataset.

Fig. S1. Likelihood map of the FCoV S sequences. Each dot represents the likelihoods of the three possible unrooted trees per quartet randomly selected from the data set. The numbers indicate the percentage of dots in the centre of the triangle. Fully resolved trees fell at the corners and the unresolved fell at the centre area. (TIF)

Fig. S2. The inferred spatiotemporal dynamics of FCoV in cats. The panels provide the continuous pattern of BDV dispersion for 1966 (A), 1995 (B), 2003 (C), 2006 (D), 2008 (E) and 2015 (F). Lines represent MCC phylogeny branches projected on the map, based on satellite pictures made available in Google Earth (http://earth.google.com). Dark circles delimit FCoV dispersion areas. (PPTX)

Table 1

Localities, collection years, FIP status and FCoV type of Italian FCoV sequences from cats derived from this study included in the dataset.

Sequence ID[a]	Province	City	Year	FIP-infected	FCoV type
MIAaFc@11 [b]	Milano	Milano	2011	yes	I
MI1aFa@12 [b]	Milano	Milano	2012	yes	I
MIBaFc@12 [b]	Milano	Milano	2012	yes	I
MICaFn@13	Milano	Milano	2013	yes	I
MI1bFc@13	Milano	Milano	2013	yes	I
TRAaFa@13	Milano	Trezzano sul Naviglio	2013	yes	I
UV1aFn@13	Monza Brianza	Usmate Velate	2013	yes	I
MIDaEs@13 [b]	Milano	Milano	2013	no	I
PVAaEf@14	Pavia	Pavia	2014	no	I
PVAbEf@14	Pavia	Pavia	2014	no	I
PVAcEf@14 [b]	Pavia	Pavia	2014	no	I
PVAdEf@14	Pavia	Pavia	2014	no	I
PVAgEf@14	Pavia	Pavia	2014	no	I
PVAeEf@14 [b]	Pavia	Pavia	2014	no	I
PVAfEf@14	Pavia	Pavia	2014	no	I
RH1aFn@14	Milano	Rho	2014	yes	I
RH1bFn@14	Milano	Rho	2014	yes	I
MG1aFn@14 [b]	Milano	Magenta	2014	yes	I
MG1bEt@14[b]	Milano	Magenta	2014	no	I
RH1cFn@14	Milano	Rho	2014	yes	I
COAaFn@14	Como	Como	2014	yes	I
RH1dFn@14 [b]	Milano	Rho	2014	yes	I
MIEaFi@14	Milano	Milano	2014	yes	I
RH1eFn@14	Milano	Rho	2014	yes	I
SGAaFb@14	Milano	San Giorgio su Legnano	2014	yes	I
MGAaFe@14	Milano	Magenta	2014	yes	I
VGAaFn@14 [b]	Pavia	Vigevano	2014	yes	I
ROAaFl@14	Rome	Rome	2014	yes	I
VGAbEn@14	Pavia	Vigevano	2014	yes	I
VGBaFn@14	Pavia	Vigevano	2014	yes	I
VGCaFn@14	Pavia	Vigevano	2014	yes	I
BGAaFc@15 [b]	Bergamo	Bergamo	2015	yes	I
LSAaFe@15	Monza Brianza	Lentate sul Seveso	2015	no	I

[a] Id. sequence name is based on: city (first two letters)/ private owner or cattery identification (capital letter or number, respectively)/animal identification letter (a= one cat, b-g =second to

seventh cat from the same owner or cattery)/ S nRT-PCR sequence identification of mutated (F) or unmutated (E) virus/ specimen identification (a=aqueous humor, b=blood; c=CSF, e= effusion; f=feces, i=intestine; l= liver; n= mesenteric lymph node; s= spleen, t=thoracic wall)/@/year of sampling.

[b] sequences selected for phylogeographical dataset

Table 2

Time of the most common ancestor estimates of FCoV in cats, credibility interval (95% HPD) of the main clades and subclades including Italian sequences observed in the MCC tree, with the corresponding localities, number of sequences and state posterior probability.

Node	Subclade	pp[1]	No. sequences	tMRCA[2]	CltMRCA U[3]	CltMRCA L[4]	Locality	State pp[1]
Root		1		1970	1947	1974	USA	0.8
A		0.89	103	1981	1970	1988	USA	0.63
	A1	0.71	93	1987	1980	1990	USA	0.59
	A1a	0.53	34	2001	1996	2003	D, NL	0.52 vs 0.49
	A1b	0.58	5	2004	2002	2006	NL	0.98
	A1c	0.91	6	2012	2010	2013	IT	0.98
	A1d	0.97	5	2012	2010	2013	IT	0.99
	A1e	0.71	4	2012	2010	2014	IT	0.98
	A2	0.53	9	1998	1990	2003	D	0.84
B		0.99	2	2003	2001	2004	D	0.99

[1]pp: posterior probability

[2]tMRCA: time of the most common ancestor

[3]Cl tMRCA U: upper credibility interval

[4]Cl tMRCA L: lower credibility interval

Table 3

First descriptions of FIP in cats worldwide.

Continent	Country.	Year	Reference
North America	USA	Early 1950's	Holzworth 1963, Wolfe and Griesem 1966, Pedersen, 2009
	Canada	1969	Stevenson et al., 1971, Chappuis and Duret 1978
South America	Mexico	1972	de Aluja, 1972
Asia	Japan	1971	Chappuis and Duret 1978
Europe	Germany	1969	Chappuis and Duret 1978, Landes et al., 1984
	United Kingdom	1970	Chappuis and Duret 1978
	Netherlands	1971	Chappuis and Duret 1978
	Ireland	1972	Chappuis and Duret 1978
	Switzerland	1973	Chappuis and Duret 1978
	Belgium	1975	Chappuis and Duret 1978
	France	1975	Chappuis and Duret 1978
	Denmark	1976	Flagstad and Larsen, 1976
	Italy	1979	Paltrinieri S., personal communication
Oceania	Australia	1974	Chappuis and Duret 1978
	New Zealand	1975	Jones, 1975
Africa	South Africa	1974	Chappuis and Duret 1978

Figure 1

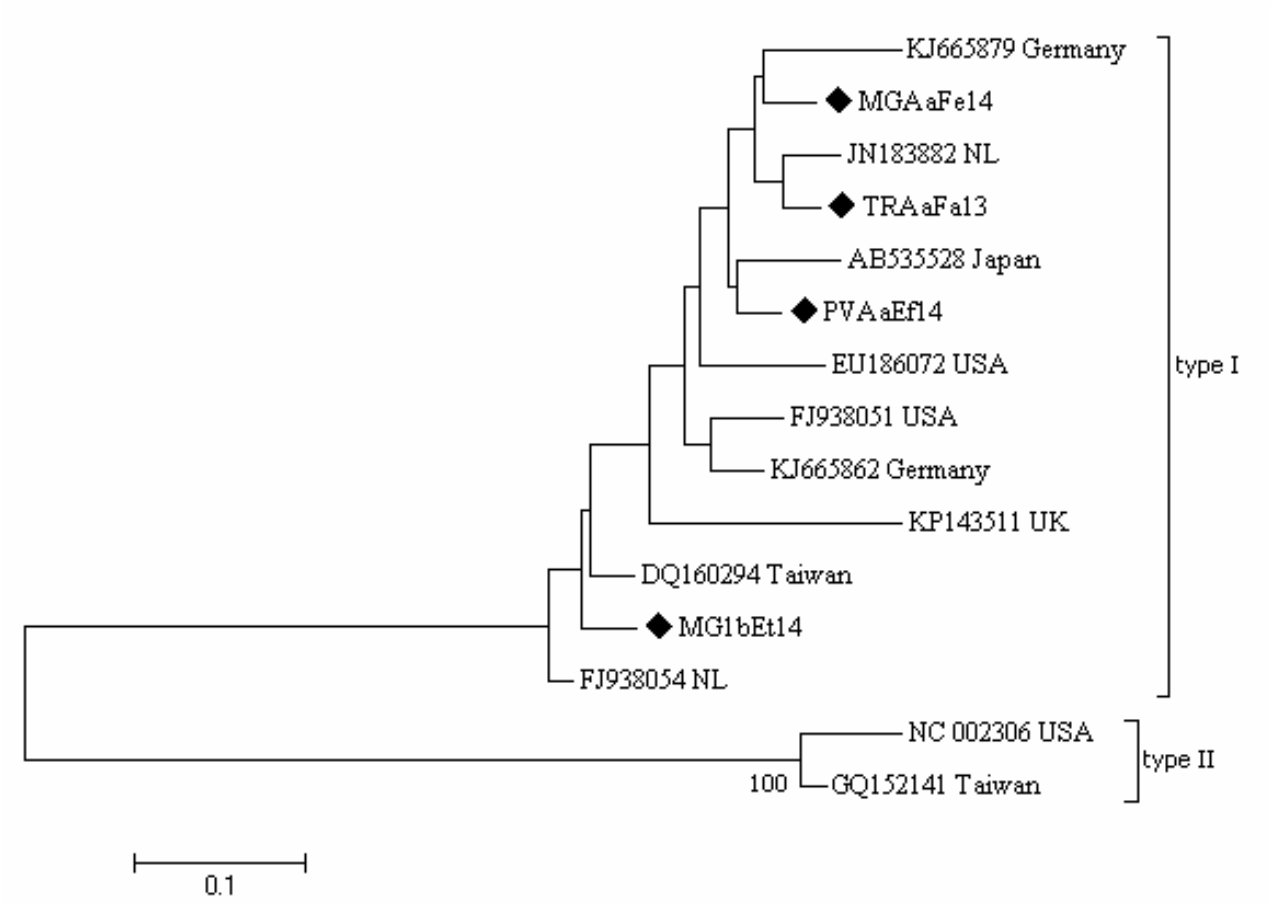

KJ665879 Germany
MGAaFe14
JN183882 NL
TRAaFa13
AB535528 Japan
PVAaEf14
EU186072 USA
FJ938051 USA
KJ665862 Germany
KP143511 UK
DQ160294 Taiwan
MG1bEt14
FJ938054 NL
type I
NC 002306 USA
GQ152141 Taiwan
100
type II
0.1

Figure 2

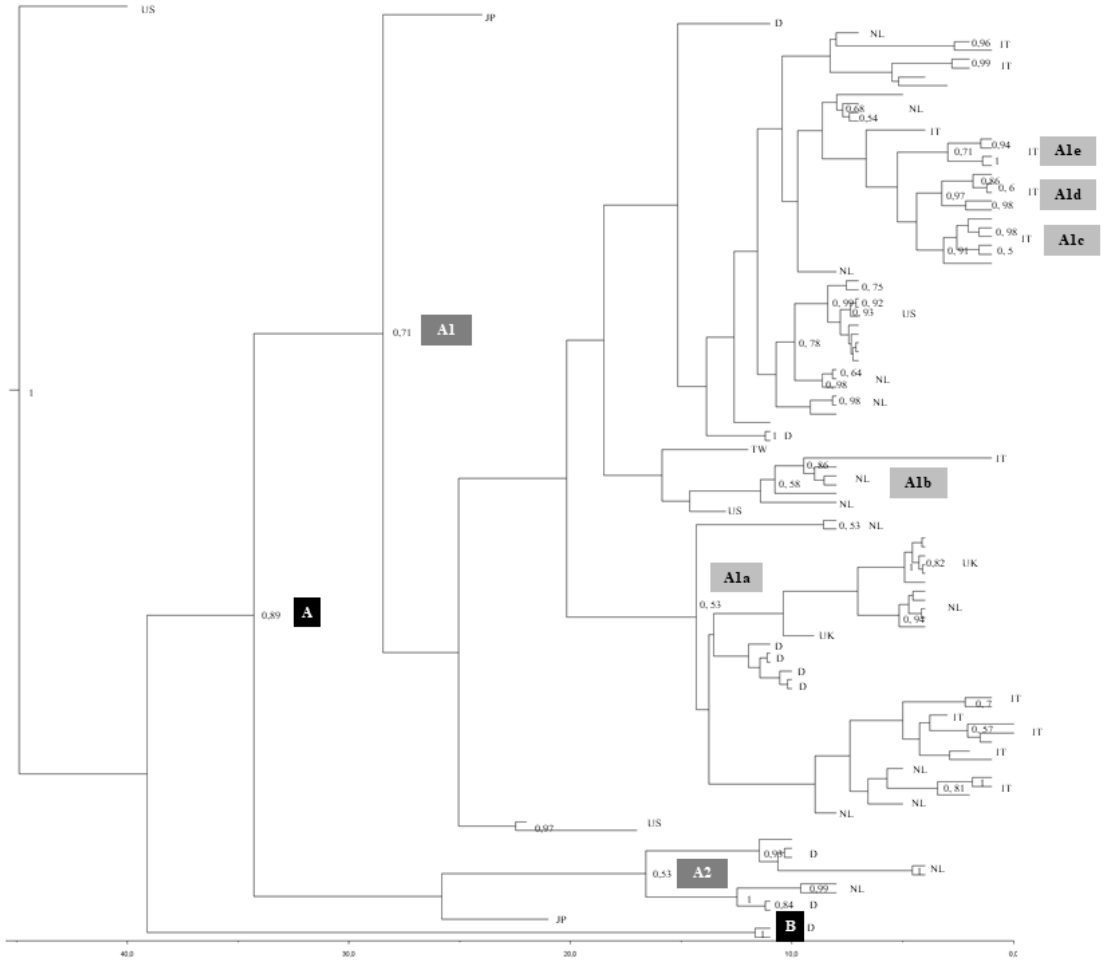
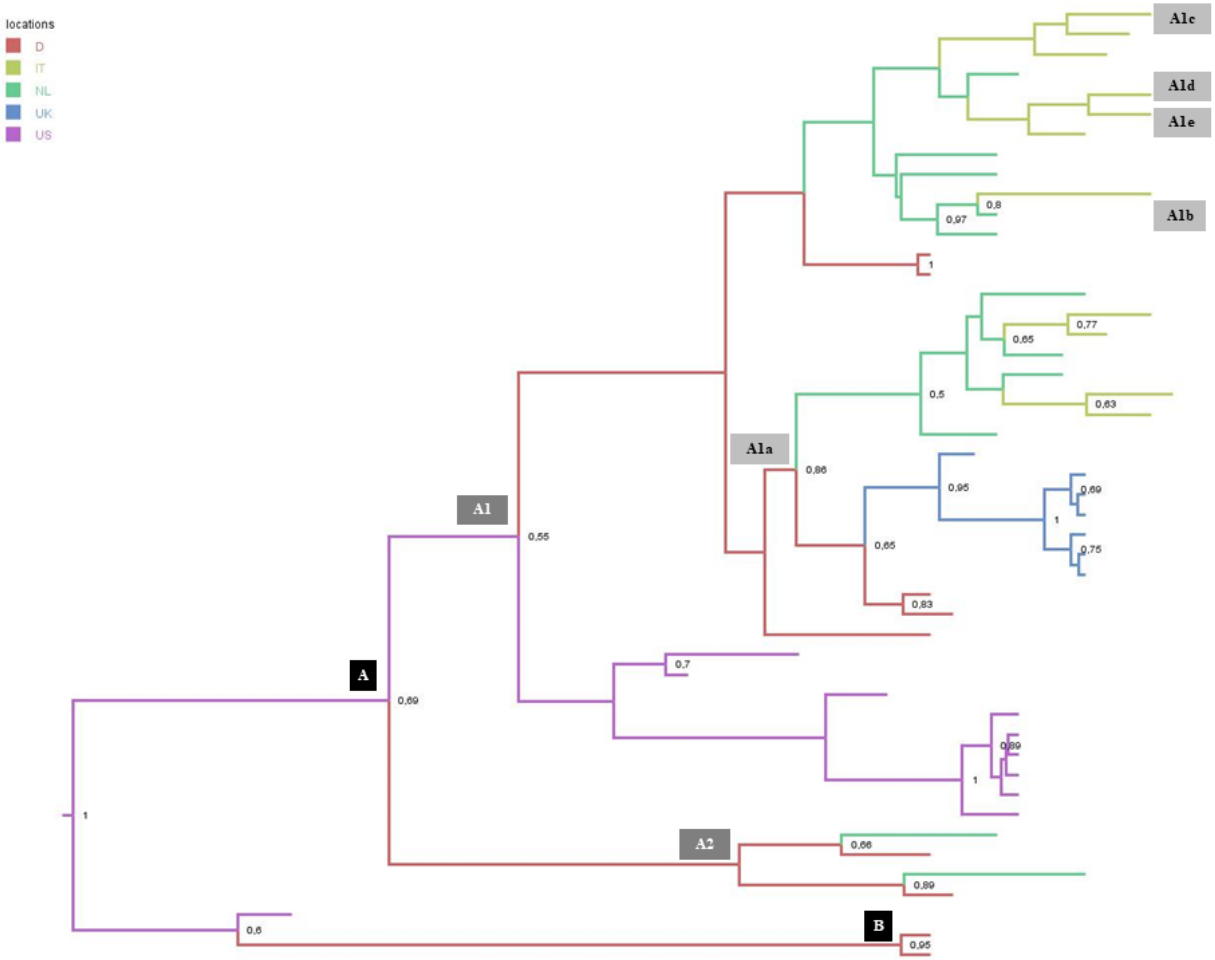


Figure 3


locations
D
IT
NL
UK
US
A1c
A1d
A1e
A1b
A1a
A1
A
A2
B
1
0,8
0,97
0,77
0,65
0,5
0,63
0,86
0,95
0,69
1
0,65
0,75
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1
0,66
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0,6
0,95

Figure 4

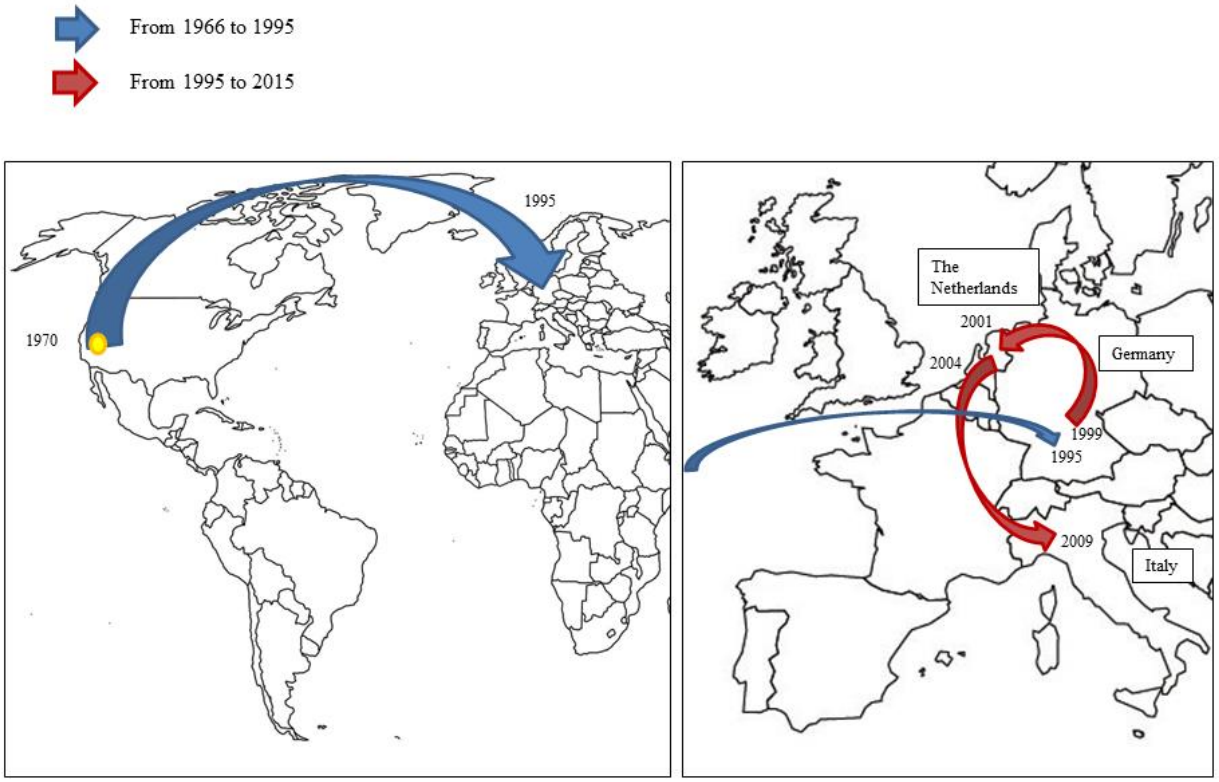

From 1966 to 1995
From 1995 to 2015
1970
1995
The Netherlands
2001
2004
Germany
1999
1995
2009
Italy

Figure S1

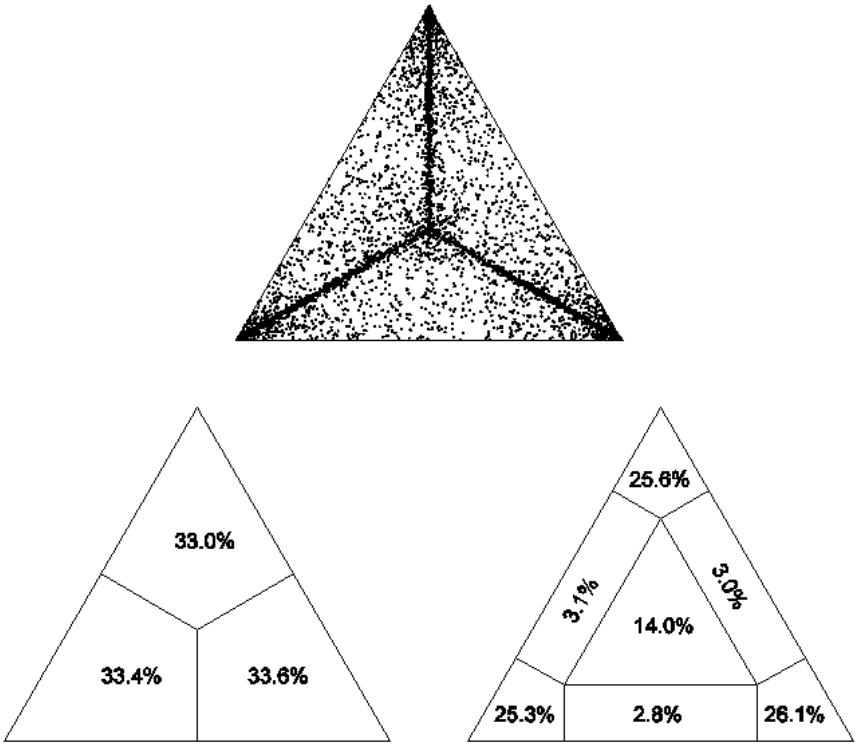
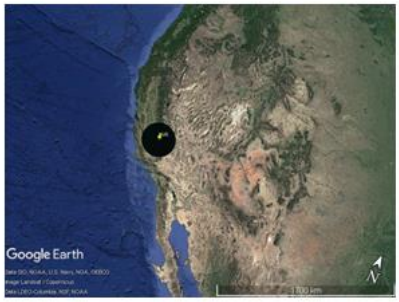


Figure S2

S1 A 1966 USA

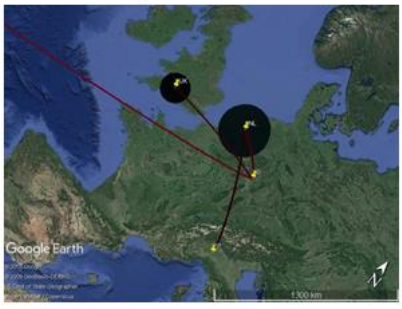


S1 B 1995 Germany

S1 C 2003 Germany and The Netherlands

S1 D 2006 UK and NL

S1 E 2008 Italy



S1 F 2015 Italy

Table S1

Origin and characteristics of 33 FCoV Italian sequences from cats derived from this study included in the dataset.

Sequence ID[a]	Province	City	Owner	Cat	Sample	S sequencing result	Year	FIP-affected	FCoV type	Accession number
MIAaFc@11 [b]	Milano	Milano	MIA	a	CSF	mutated	2011	yes	I	MT250347
MI1aFa@12 [b]	Milano	Milano	MI1	a	aqueous humor	mutated	2012	yes	I	MT250348
MIBaFc@12 [b]	Milano	Milano	MIB	a	CSF	mutated	2012	yes	I	MT250349
MICaFn@13	Milano	Milano	MIC	a	mesenteric lymph node	mutated	2013	yes	I	MT250350
MI1bFc@13	Milano	Milano	MI1	b	CSF	mutated	2013	yes	I	MT250351
TRAaFa@13	Milano	Trezzano sul Naviglio	TRA	a	aqueous humor	mutated	2013	yes	I	MT250352
UV1aFn@13	Monza Brianza	Usmate Velate	UV1	a	mesenteric lymph node	mutated	2013	yes	I	MT250353
MIDaEs@13 [b]	Milano	Milano	MID	a	spleen	unmutated	2013	no	I	MT250354
PVAaEf@14	Pavia	Pavia	PVA	a	feces	unmutated	2014	no	I	MT250355
PVAbEf@14	Pavia	Pavia	PVA	b	feces	unmutated	2014	no	I	MT250356
PVAcEf@14 [b]	Pavia	Pavia	PVA	c	feces	unmutated	2014	no	I	MT250357
PVAdEf@14	Pavia	Pavia	PVA	d	feces	unmutated	2014	no	I	MT250358
PVAgEf@14	Pavia	Pavia	PVA	g	feces	unmutated	2014	no	I	MT250359
PVAeEf@14 [b]	Pavia	Pavia	PVA	e	feces	unmutated	2014	no	I	MT250360
PVAfEf@14	Pavia	Pavia	PVA	f	feces	unmutated	2014	no	I	MT250361

RH1aFn@14	Milano	Rho	RH1	a	mesenteric lymph node	mutated	2014	yes	I	MT250362
RH1bFn@14	Milano	Rho	RH1	b	mesenteric lymph node	mutated	2014	yes	I	MT250363
MG1aFn@14 [b]	Milano	Magenta	MG1	a	mesenteric lymph node	mutated	2014	yes	I	MT250364
MG1bEt@14[b]	Milano	Magenta	MG1	b	thoracic wall	unmutated	2014	no	I	MT250365
RH1cFn@14	Milano	Rho	RH1	c	mesenteric lymph node	mutated	2014	yes	I	MT250366
COAaFn@14	Como	Como	COA	a	mesenteric lymph node	mutated	2014	yes	I	MT250367
RH1dFn@14 [b]	Milano	Rho	RH1	d	mesenteric lymph node	mutated	2014	yes	I	8MT25036
MIEaFi@14	Milano	Milano	MIE	a	intestine	mutated	2014	yes	I	MT250369
RH1eFn@14	Milano	Rho	RH1	e	mesenteric lymph node	mutated	2014	yes	I	MT250370
SGAaFb@14	Milano	San Giorgio su Legnano	SGA	a	blood	mutated	2014	yes	I	MT250371
MGAaFe@14	Milano	Magenta	MGA	a	effusion	mutated	2014	yes	I	MT250372
VGAaFn@14 [b]	Pavia	Vigevano	VGA	a	mesenteric lymph node	mutated	2014	yes	I	MT250373
ROAaFl@14	Rome	Rome	ROA	a	liver	mutated	2014	yes	I	MT250374
VGAbEn@14	Pavia	Vigevano	VGA	b	mesenteric lymph node	unmutated	2014	yes	I	MT250375
VGBaFn@14	Pavia	Vigevano	VGB	a	mesenteric lymph node	mutated	2014	yes	I	MT250376
VGCaFn@14	Pavia	Vigevano	VGC	a	mesenteric lymph node	mutated	2014	yes	I	MT250377
BGAaFc@15 [b]	Bergamo	Bergamo	BGA	a	CSF	mutated	2015	yes	I	MT250378
LSAaFe@15	Monza Brianza	Lentate sul Seveso	LSA	a	effusion	mutated	2015	no	I	MT250379

[a] Sequence ID name is based on: city (first two letters)/ private owner or cattery identification (capital letter or number, respectively)/animal identification letter (a= one cat, b-g =second to seventh cat from the same owner or cattery)/ S nRT-PCR sequence identification of mutated (F) or unmutated (E) virus/ specimen identification (a=aqueous humor, b=blood; c=CSF, e= effusion; f=feces, i=intestine; l= liver; n= mesenteric lymph node; s= spleen, t=thoracic wall)/@/year of sampling.

[b] sequences selected for phylogeographical dataset

Table S2.

Accession numbers, localities and collection year of FCoV sequences from cats retrieved from GenBank included in the dataset.

Sequence ID[a]	Accession no.	Country	Year
D1Ef@04[b]	KJ665862	Germany	2004
D2Ef@04[b]	KJ665863	Germany	2004
D3Ef@04[b]	KJ665864	Germany	2004
D4Ff@04[b]	KJ665865	Germany	2004
D5Ef@04	KJ665866	Germany	2004
D6Fe@04[b]	KJ665868	Germany	2004
D6Ff@04	KJ665867	Germany	2004
D7Fe@04	KJ665869	Germany	2004
D8Ff@04	KJ665870	Germany	2004
D8Fe@04[b]	KJ665871	Germany	2004
D9Ff@04[b]	KJ665873	Germany	2004
D10Ff@05	KJ665876	Germany	2005
D11Fe@05[b]	KJ665877	Germany	2005
D11Ff@05	KJ665881	Germany	2005
D12Ff@05	KJ665878	Germany	2005
D13Fe@05[b]	KJ665879	Germany	2005
D14Fe@05	KJ665880	Germany	2005
JP1Fx@91	AB695067	Japan	1991
JP2Fx@94	AB535528	Japan	1994
NL1Fx@10[b]	KF530123	The Netherlands	2010
NL2Fx@10	JN183882	The Netherlands	2010
NL3Ex@10[b]	JN183883	The Netherlands	2010
NL4Fp@07[b]	FJ938058	The Netherlands	2007
NL5Fp@08[b]	HQ392472	The Netherlands	2008
NL6Ef@07[b]	HQ392471	The Netherlands	2007
NL7Ef@07[b]	HQ392470	The Netherlands	2007
NL8Ex@08	HQ392469	The Netherlands	2008
NL9Ex@07	HQ012372	The Netherlands	2007
NL10Ef@08	HQ012371	The Netherlands	2008
NL11Ep@08	HQ012370	The Netherlands	2008
NL12Fp@07	HQ012369	The Netherlands	2007
NL13Ef@07	HQ012368	The Netherlands	2007
NL14Fp@07	HQ012367	The Netherlands	2007
NL15Ef@07	GU553362	The Netherlands	2007
NL16Ef@07[b]	GU553361	The Netherlands	2007
NL17Ep@07	FJ938062	The Netherlands	2007
NL18Ef@07	FJ938059	The Netherlands	2007
NL19Fp@07	FJ938057	The Netherlands	2007
NL20Fp@07	FJ938055	The Netherlands	2007
NL21Fp@07	FJ938054	The Netherlands	2007
NL22Ef@07[b]	FJ938053	The Netherlands	2007
NL23Ef@07[b]	FJ938052	The Netherlands	2007

NL24Ef@11	JQ304369	The Netherlands	2011
NL25Ef@11 [b]	JQ304370	The Netherlands	2011
NL26Ef@11	JQ304371	The Netherlands	2011
NL27Ef@11	JQ304372	The Netherlands	2011
NL28Ef@11	JQ304373	The Netherlands	2011
NL29Ef@11 [b]	JQ304374	The Netherlands	2011
NL30Ef@11	JQ304375	The Netherlands	2011
NL31Ef@11	JQ304368	The Netherlands	2011
NL32Fp@07	FJ938056	The Netherlands	2007
TW1Fe@03	DQ160294	Taiwan	2003
UK1Fm@11 [b]	KP143512	United Kingdom	2011
UK2Fn@11 [b]	KP143507	United Kingdom	2011
UK3Fo@11 [b]	KP143508	United Kingdom	2011
UK4Ef@11 [b]	KP143509	United Kingdom	2011
UK5Ef@11 [b]	KP143510	United Kingdom	2011
UK6Ef@11 [b]	KP143511	United Kingdom	2011
UK7Fi@06 [b]	DQ848678	United Kingdom	2006
US1Fx@75 [b]	EU186072	USA	1975
US2Ef@93 [b]	FJ938060	USA	1993
US3Fp@98 [b]	FJ938061	USA	1998
US4Ef@02 [b]	FJ938051	USA	2002
US5Fp@08 [b]	FJ917535	USA	2008
US6iFp@08 [b]	FJ917534	USA	2008
US7Fp@08	FJ917531	USA	2008
US8Fp@08	FJ917530	USA	2008
US9Fp@08 [b]	FJ917524	USA	2008
US10Fp@08	FJ917523	USA	2008
US11Fp@08	FJ917522	USA	2008
US12Fp@08 [b]	FJ917521	USA	2008
US13Fp@08 [b]	FJ917520	USA	2008
US14Fp@08 [b]	FJ917519	USA	2008

[a] Id. sequence name is based on: Country (D= Germany, JP= Japan, NL= Netherlands, TW= Taiwan, UK= United Kingdom, US= United States of America)/ animal identification number/ identification based on S nRT-PCR sequence (E= unmutated, F= mutated)/ specimen identification (e= effusion; f=feces; i=intestine; m= mesentery; n= mesenteric lymph node; o= omentum; p=tissue or effusion; x= not determined)/@/year of sampling.

[b] sequences selected for phylogeographical dataset