LETTERS

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Identification of Pantropic Canine Coronavirus in a Wolf (*Canis lupus italicus*) in Italy

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ABSTRACT: We report a case in an Italian wolf (*Canis lupus italicus*) of pantropic canine coronavirus infection, which has previously been detected only in dogs. The wolf was coinfected by canine parvovirus type 2b and canine adenovirus type 2, which highlighted the crucial role of epidemiologic surveys in European wild carnivores.

With few exceptions, populations of the European wolf (*Canis lupus*) have been increasing or stable during recent years, but their conservation is still threatened by illegal hunting, agricultural and silvicultural intensification, and land abandonment with reduction of free-ranging livestock (Votsi et al. 2016). Domestic dogs can transmit pathogens to wild carnivores through direct contact or indirect transmission through feces and urine (Millán et al. 2016). Several canine pathogens have been detected in European wolves including canine parvovirus (CPV), canine adenovirus types 1 (CAdV-1) and 2 (CAdV-2), and canine distemper virus (CDV; Martinello et al. 1997; Di Sabatino et al. 2014; Millán et al. 2016; Dowgier et al. 2018). Some of these pathogens were associated with high mortality and represented a severe threat to wolf conservation (Di Sabatino et al. 2014). Canine coronavirus (CCoV) causes mild enteritis in domestic dogs although a hypervirulent variant, named pantropic CCoV (pCCoV), is associated with mortality and leukopenia (Buonavoglia et al. 2006) To date, pCCoV infection has been reported only in dogs (Decaro and Buonavoglia 2011) and enteric CCoV has been only sporadically detected in wolves (Molnar et al. 2014). We report a case of pCCoV infection in an Italian wolf (*Canis lupus italicus*) that was coinfected with other canine viruses.

We submitted samples collected from various tissues (gut, heart, brain, spleen, liver, lungs) during the necropsy of a wolf found dead near Avellino (Italy), latitude 40°50′37 N, longitude 15°1′4 E, on December 2016, to RNA/DNA extraction by the QIAsymphony DSP Virus/Pathogen kit (Qiagen, Hilden, Germany). Extracts were screened by gel-based or real-time (RT-)PCR assays for canine viral pathogens including CPV, CCoV, CAdV-1/CAdV-2, CDV, canine alphaherpesvirus, and rotaviruses (Decaro et al. 2013). By molecular assays, the wolf was found to have a triple infection caused by CCoV, CPV-2b, and CAdV-2. The CPV-2b strain was detected in several internal organs including gut, heart, brain, spleen, liver, and lungs whereas CAdV-2 was recovered from lungs, spleen, and liver. The CCoV strain was detected in the gut and in nonintestinal tissues (heart, brain, spleen) and was typed as CCoV-IIa. According to previous studies (Decaro et al. 2013), the detection of CCoV-IIa in nonintestinal tissues allowed characterization of the strain as pCCoV.

The CCoV sequences were analyzed by the Geneious platform (version 10.1.3; Biomatters Ltd., Auckland, New Zealand) while similarity to GenBank sequences was assessed by the Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information 2018) and FASTA (European Bioinformatics Institute 2018). The spike gene of the detected pCCoV strain, referred to as CCoV/wolf/2016/IT (GenBank accession number MF991150), showed the highest degree of nucleotide (nt) identity (97%) with strain CCoV-IIa CCoV/dog/HCM27/2014 (LC190906) from a Vietnamese dog (*Canis lupus familiaris*), followed by strains FCoV

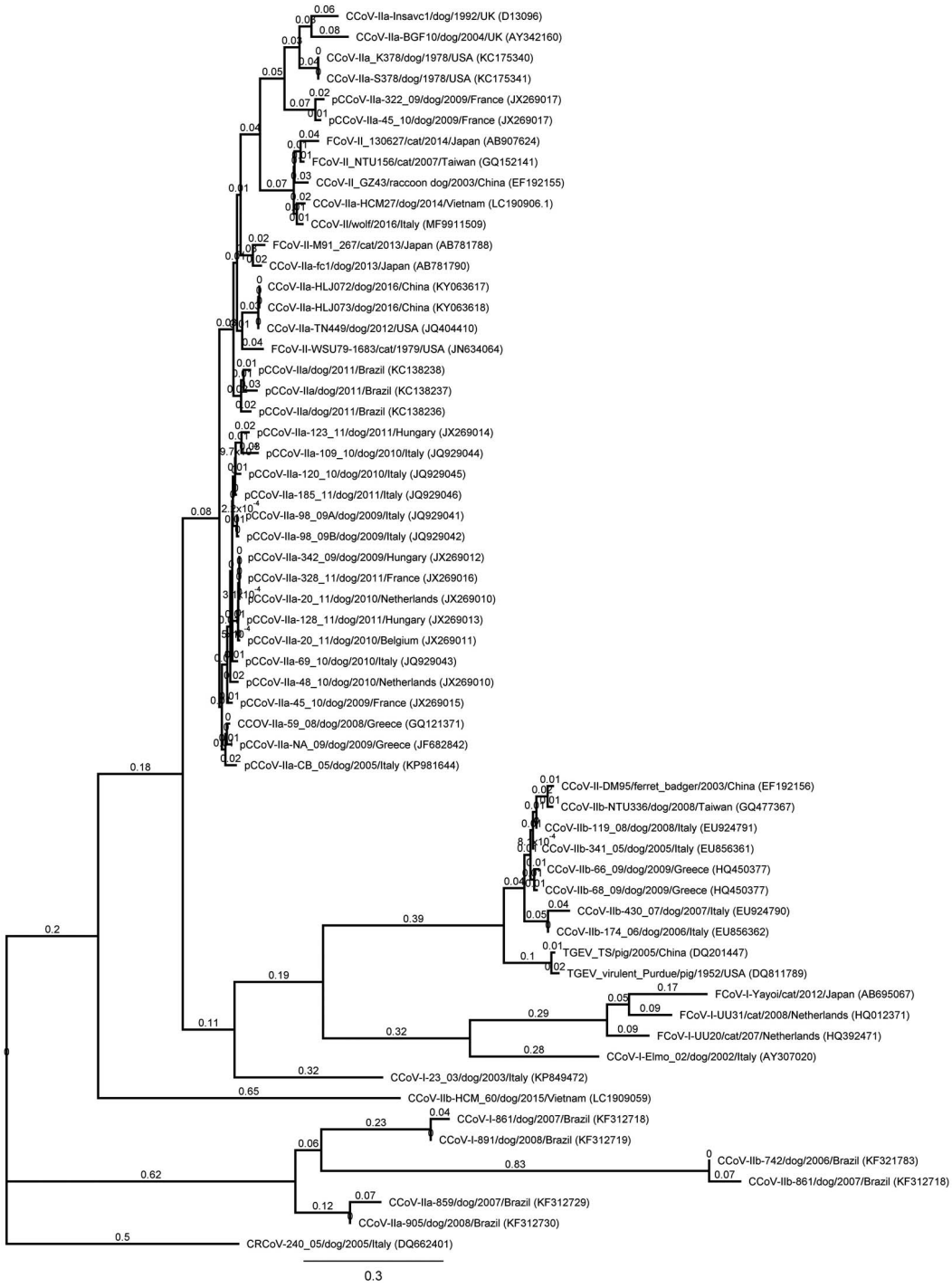

FIGURE 1. Neighbor joining tree inferred from multiple nucleotide sequence alignment of the 5′-terminal region of the spike gene of the canine coronavirus (CCoV) strain detected in an Italian wolf (*Canis lupus italicus*) in 2016 and representative CCoVs, porcine transmissible gastroenteritis viruses (TGEVs), and feline coronaviruses (FCoVs) available on GenBank, recruited among different subgenotypes reported from different countries. For each sequence a short name, host species, collection date, and country are described in addition to

NTU156/P/2007 (GQ152141) from a Taiwanese cat (*Felis catus*) with 95% nt identity and GZ43/2003 (EF192155) from a Chinese raccoon dog (*Nyctereutes procyonoides*) with 93% nt identity. A lower degree of genetic relatedness was observed with CCoV-IIa strains from domestic dogs in China (86% nt identity): HLJ-071 (KY063616), HLJ-072 (KY063617), HLJ-073 (KY063618); Greece (86% nt identity): pCCoV 56/08 (GQ121370) and 59/08 (GQ121371); and Italy (85% nt identity): pCCoV CB/05 (KP981644).

Phylogenetic trees were generated from MAFTT Multiple Sequence Alignment Software (Version 7; Biomatters Ltd) by Geneious platform (version 10.1.3; using the neighbor joining and Bayesian methods). Bayesian inference was performed using four chains run over one million generations (with the first 2,000 trees discarded as burn-in) and supplying statistical support with subsampling over 200 replicates. According to the phylogenetic analysis (Figs. 1, 2), CCoV/wolf/2016/IT clusters with several CCoV-IIa and FCoV-II strains identified in European and extra-European countries, with no obvious geographic distribution. A pattern of node-based sub-clustering is displayed by both phylogenetic trees, showing that CCoV/wolf/2016/IT segregates with a Vietnamese strain (CCoV/dog/HCM27/2014) and a raccoon dog CCoV from China (GZ43/2003). Interestingly, other putative pCCoVs identified in previous studies were less-closely related to the wolf strain in comparison with enteric strains (Buonavoglia et al. 2006; Decaro et al. 2013).

Our findings showed that CCoV was circulating in the Italian wolf population, indicating that potentially fatal infections caused by pCCoV could be expected in this carnivore species. Interestingly, the wolf pCCoV strain was only distantly related to Italian CCoVs of canine origin, displaying higher genetic identities to strains circulating in Asian carnivores. It is therefore possible that the virus was introduced into Italy through the importation of dogs or other carnivores from Asia, as suggested for other viral agents (Mira et al. 2018). Accordingly, CCoV infection is very common in domestic dogs, foxes and raccoon dogs in China (Wang et al. 2006). While enteric CCoV causes self-limiting enteritis, pCCoV is responsible for fatal infections in dogs, indicating that this canine pathogen may represent an additional threat to the conservation of the Italian wolf population.

Two other canine viruses, CAdV-2 and CPV, were included in the screening due to a recent report of mortality in captive wolves (Dowgier et al. 2018). Millán et al. (2016) identified CAdV-2 in Iberian wolves (*Canis lupus signatus*) while CAdV-1 and CAdV-2 were recently detected in captive wolves (*Canis lupus lupus*) in France (Dowgier et al. 2018). To date, CAdV-1 (but not CAdV-2) has been detected in Italian wolves (Pizzurro et al. 2017), with our study first reporting the detection of CAdV-2 in these wild carnivores.

Although CPV exposure of wolves had previously been reported in Italy (Martinello et al. 1997), our study provided molecular evidence for the active circulation of this virus. All the CPV strains isolated from wolves in Italy were identified as type 2b (Battilani et al. 2001), with the same virus strain circulating among dogs and wolves. Investigations on Iberian wolves (*Canis lupus signatus*) revealed that most of the wolf cases were due to a CPV-2c strain identical to that circulating in domestic dogs (Millán et al. 2016). This would support the idea that infection of wolves depends on periodic spillover from dogs. Indeed, previous studies suggested that dogs might be a reservoir for viral infections of

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the GenBank accession numbers indicated in parentheses. Pantropic strains are defined as pCCoV whereas all the other CCoV are referred as type I or II based on the commonly accepted criteria. The scale bar indicates the estimated numbers of nucleotide substitutions per site. The distantly related *Betacoronavirus-1* canine respiratory coronavirus (CRCoV) 240/05 (DQ662401) was used as outgroup. CCoV = canine coronavirus; pCCoV = pantropic canine coronavirus; FCoV = feline coronavirus; TGEV = transmissible gastroenteritis virus of swine.

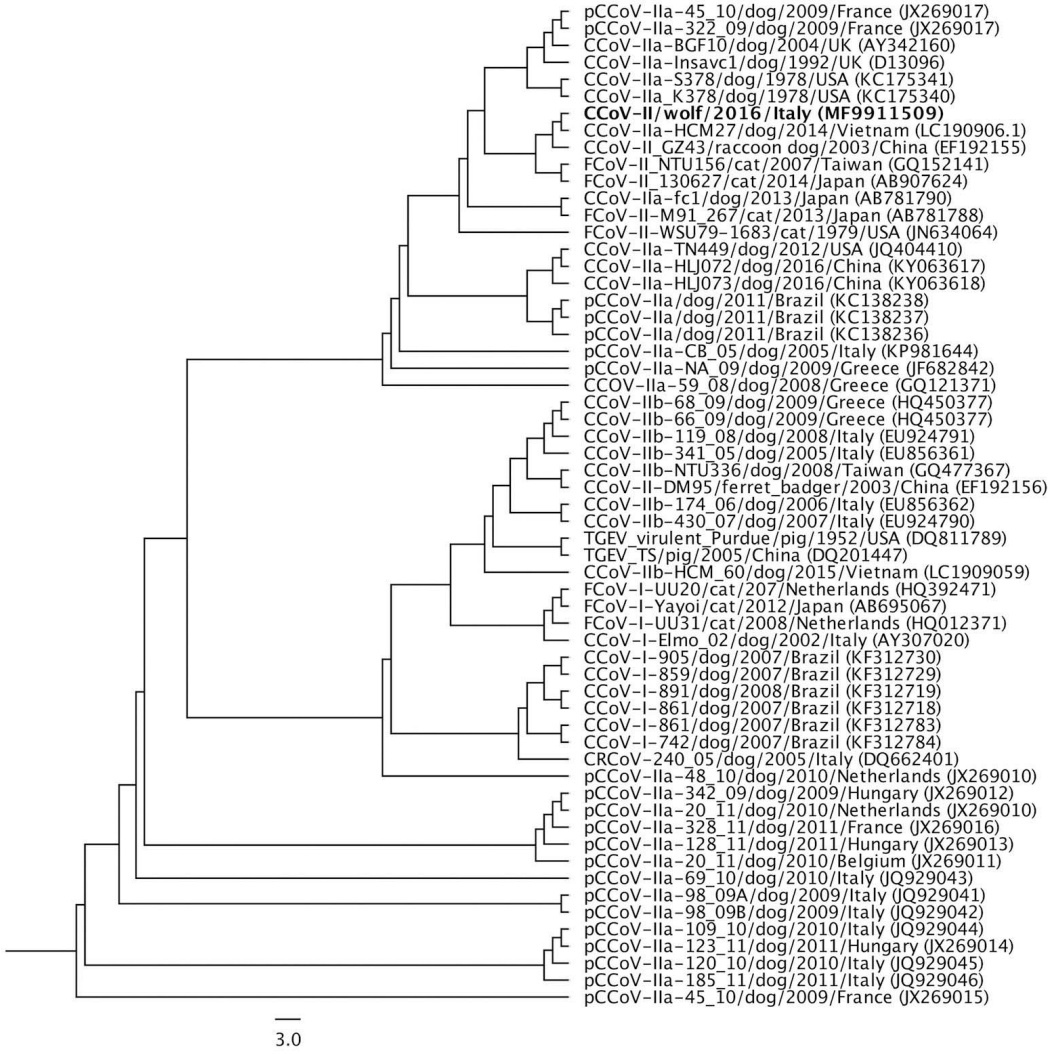

FIGURE 2. Bayesian phylogenetic tree inferred from multiple nucleotide sequence alignment of the 5′-terminal region of the spike gene of the canine coronavirus (CCoV) strain detected in an Italian wolf (*Canis lupus italicus*) in 2016 and representative CCoVs, porcine transmissible gastroenteritis viruses (TGEVs), and feline coronaviruses (FCoVs) available on GenBank, recruited among different subgenotypes reported from different countries. For each sequence a short name, host species, collection date, and country are described in addition to the GenBank accession numbers indicated in parentheses. Pantropic strains are defined as pCCoV whereas all the other CCoV are referred as type I or II based on the commonly accepted criteria. The scale bar indicates the estimated numbers of nucleotide substitutions per site. The distantly related *Betacoronavirus-1* canine respiratory coronavirus (CRCoV) 240/05 (DQ662401) was used as outgroup. CCoV = canine coronavirus; pCCoV = pantropic canine coronavirus; FCoV = feline coronavirus; TGEV = transmissible gastroenteritis virus of swine.

wild canids (Ferreyra et al. 2009; Molnar et al. 2014).

Given the possible negative impact of viral strains with increased pathogenicity on wolf populations, and because little information is available on pathogens infecting the Italian wolf, our results are important from the conservation perspective and highlight the need for continuous monitoring of pathogen circulation in wildlife.

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