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# Etiology and genetic evolution of canine coronavirus circulating in five provinces of China, during 2018–2019

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ABSTRACT

As the outbreaks of COVID-19 in worldwide, coronavirus has once again caught the attention of people. Canine coronavirus is widespread among dog population, and sometimes causes even fatal cases. Here, to characterize the prevalence and evolution of current circulating canine coronavirus (CCoV) strains in China, we collected 213 fecal samples from diarrheic pet dogs between 2018 and 2019. Of the 213 samples, we found 51 (23.94%) were positive for CCoV. Co-infection with canine parvovirus (CPV), canine astrovirus (CaAstV), canine kobuvirus (CaKV), Torque teno canis virus (TTCaV) were ubiquitous existed. Mixed infection of different CCoV subtypes exists extensively. Considering the limited sequences data in recent years, we sequenced 7 nearly complete genomes and 10 complete spike gene. Phylogenetic analysis of spike gene revealed a new subtype CCoV-II Variant and CCoV-IIa was the most prevalent subtype currently circulating. Moreover, we identified strain B906_ZJ_2019 shared 93.24% nucleotide identifies with previous strain A76, and both of them clustered with CCoV-II Variant, which were not well clustered with the known subtypes. Recombination analysis of B906_ZJ_2019 indicated that strain B906_ZJ_2019 may a recombinant variant between CCoV–I and CCoV-II, which is consistent with strain A76. Furthermore, amino acid variations widely existed among current CCoV-IIa strains circulating in China and the classic CCoV-IIa strains, in spite of the unknown functions.

In a word, we report a useful information as to the etiology and evolution of canine coronavirus in China based on the available sequences, which is urgent for the devise of future effective disease prevention and control strategies.

## 1. Introduction

Coronaviruses, as single-stranded RNA viruses, have been widely detected in a variety of mammals and several birds [1,2]. Its genome has ranged from 26 to 32 kb in length, and currently can be divided into 4 genera named Alpha, Beta, Gamma and Delta coronavirus [3]. Considering the frequent mutation and recombination of coronavirus, novel coronavirus variants continues to appear, so far it has caused several serious public health safety incidents [4,5]. In 2003 and 2012, two types of animal coronavirus transmitted into human population caused the severe acute respiratory syndrome (SARS) and the middle East respiratory syndrome (MERS), respectively [6,7]. Since then, researches on coronavirus has greatly increased. Recently, a coronavirus called Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been discovered in Wuhan, China, which caused viral pneumonia and hyperpyrexia of patients [8]. In addition, there is evidence that SARS-CoV-2 can be transmitted from person to person [9], and suggested that SARS-CoV-2 may originated from Chinese horseshoe bats [10].

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Canine coronavirus has been described since 1971 [11], and be regarded as an enterovirus, which mainly caused mild diarrhea symptoms and showed high morbidity and low mortality [11–14]. But in the case of co-infection with other pathogens such as canine adenovirus (CAV) or canine parvovirus (CPV), CCoV infection can cause severe clinical signs and even fatal cases [15,16]. Furthermore, fatal cases also be discovered in the cases without co-infection, which subsequently be identified causing by high virulent strains named pantropic CoVs [17–19]. CCoV belongs to species *Alphacoronavirus 1*, genus *Alphacoronavirus*, which is same to feline coronavirus (FCoV), transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCoV) [3]. In 2003, canine coronavirus was newly recognized as respiratory tract virus [20]. Unlike canine enteric coronavirus (CCoV), canine respiratory coronavirus (CRCoV) belongs to Beta coronavirus [20,21]. To data, CCoVs contains 2 distinct genotypes, which named CCoV–I and CCoV-II [22]. CCoV-II can be further divided into CCoV-IIa and CCoV-IIb [23,24]. However, novel CCoV variants, such as A76, which not included in the two known genotypes has been discovered. Hence, CCoV-IIc has ever been proposed [25], but considering the limited sequences identified, this clade has not been publicly accepted yet.

In China, canine coronavirus, as one of the mainly pathogens, has caused viral diarrhea in dog population. The information concerning CCoVs in recent years still limited. In this study, we conducted an etiological investigation and sequenced 7 nearly complete genomes and 10 complete spike gene of CCoV, and according to all the available CCoV sequences to provides insights into viral etiology, evolution and genetic diversity of currently strains circulating in China. The aim of this study is to provide useful information for the future prevention of CCoV.

## 2. Material and methods

### 2.1. Sampling and pretreatment

During 2018–2019, we collected 231 feces or rectal swabs from diarrheic dogs in 5 provinces (Guangdong, Zhejiang, Heilongjiang, Jiangsu and Anhui) of China. All the samples were diluted using five volumes phosphate-buffered saline (pH 7.2) and then centrifuged. Viral RNA was extracted from the supernatant using a Viral DNA/RNA Kit (CW Biotech, Jiangsu, China) according to the manufacturer's protocol. Reverse transcription was conducted using a HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China).

### 2.2. RT-PCR assays

CCoV was detected by selectively amplifying a 409bp fragment of *M* gene as previously described [26]. All the positive samples were subjected to screen for other canine enteric virus by the methods previously described, including CPV [27], CaAstV [28], CaKV [29], TTCaV [30]. In addition, primer pairs EL1F/EL1R, CEPol-1/TGSP-2 and S5F/S6R, which amplify CCoV–I, CCoV-IIb and CCoV-IIa genes, respectively, were used to differentiate gene types of CCoV, as previously described [31–33].

### 2.3. Sequences amplification and analysis

To amplify the genome of CCoV, we downloaded all the available genomes of CCoV, and then aligned using Muscle in MEGA 7.0 program (http://www.megasoftware.net/).

According to the conserved region of CCoV genomes, we designed 17 pairs of specific primers using OLIGO Primer Analysis Software Version 7 (http://www.oligo.net/) (Table S1). In addition, the amplified fragments of these 17 pairs of primers have overlapping portions to facilitate sequences assembling. The PCR products were determined by a commercial company (GENERAL BIOSYSTEMS, Anhui, China). Sequences were assembled and analyzed using DNASTAR program (Madison, USA). Moreover, considering the short of samples and the primer specificity, we cannot or solely obtained the partial genome of some samples.

### 2.4. Phylogenetic analysis

To confirm the phylogenetic relationship between sequences identified here and those sequenced previously, we retrieved all the complete *S* gene of CCoV and partial of FCoV and TGEV from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) as reference strains. Sequences alignment were conducted by ClustalW in MEGA 7.0 program (http://www.megasoftware.net/), and then edited manually. Phylogenetic analysis was performed using RAxML (v8.2.10) with the maximum likelihood method and GTR + GAMMA model. The bootstrap value was set to 1000. Phylogenetic trees were displayed and annotated using an online tool ITOL v5 (https://itol.embl.de/). The accession numbers of reference strains are provided in Supplementary Materials 1.

### 2.5. Recombinant analyses of B906_ZJ_2019 spike gene

Recombination of B906_ZJ_2019 spike gene was analyzed using RDP 4.0. Moreover, phylogenetic analysis of N-terminal domain (NTD), C-terminal domain (CTD) and S2 subunit of B906_ZJ_2019 spike was performed. Phylogenetic trees were generated using RAxML (v8.2.10) with GTR + GAMMA model and the bootstrap values was 1000. The sequences used are shown in the tree and the accession numbers are provided in Supplementary Materials 1. The NTD and CTD of CoV spike gene were determined by alignment with CCoV-A76, which has well defined NTD and CTD [34]. Furthermore, the S1 and S2 subunit were determined as mentioned in previous study [25].

## 3. Results

### 3.1. Canine coronavirus detection, co-infection and genotyping

In this study, we collected 213 feces or rectal swabs from pet dogs with diarrhea. Of the 213 samples, 51 (23.94%) were positive for CCoV. Moreover, 36 out of 51(70.59%) samples co-infected with one or more enterovirus. Of the 36 co-infected samples, 29.41% (15/51) were solely co-infected with CPV; 3.92% (2/51) were merely co-infected with CaAstV; 9.80% (5/51) were only coinfected with CaKV; 1.96% were solely co-infected with TTCaV. The rest samples (13/51) were co-infected with more than one canine enteric pathogens (Table 1). To characterize the 51 CCoV-positive samples, the genotypes of these samples were determined. CCoV–I, CCoV-IIa and CCoV-IIb accounted for 9.80% (5/51), 54.90% (28/51) and 5.88% (3/51), respectively; two or three genotypes multiple infections accounts for 29.41% (15/51) (Table 2). In addition, of the 51 CCoV-positive samples, 27.45% (14/51) were vaccinated.

**Table 1**
Co-infection with other enteric viruses in CCoV positive cases.

| Virus | N (%) |
|---|---|
| CCoV | 15 (29.41%) |
| CCoV + CPV | 15(29.41%) |
| CCoV + CaAstV | 2 (3.92%) |
| CCoV + CaKV | 5 (9.80%) |
| CCoV + TTCaV | 1(1.96%) |
| CCoV + CPV + CaKV | 3 (5.88%) |
| CCoV + CPV + CaAstV | 3(5.88%) |
| CCoV + CaAstV + CaKV | 3(5.88%) |
| CCoV + CPV + CaAstV + CaKV | 4(7.84%) |

**Table 2**
Multiple infection of different subtype CCoV.

| Subtypes | N (%) |
|---|---|
| CCoV–I | 5(9.80%) |
| CCoV-IIa | 28(54.90%) |
| CCoV-IIb | 3 (5.88%) |
| CCoV–I + CCoV-IIa | 6 (11.76%) |
| CCoV–I + CCoV-IIb | 1(1.96%) |
| CCoV-IIa + CCoV-IIb | 5(9.80%) |
| CCoV–I + CCoV-IIa + CCoV-IIb | 3(5.88%) |

### 3.2. Canine coronavirus spike gene characterization

According to the above positive samples of CCoVs, we amplified 7 nearly complete genomes and 10 full spike gene. These sequences have been deposited in the GenBank (accession number: MT114538-MT114554). The 7 nearly complete genomes shared 96.49%–98.94% nucleotide similarities. Sequence analysis indicated that the 17 spike gene sequences were 4362–4422 nt in length. Sequences comparison of the complete *S* gene revealed 73.24%–99.98% nucleotide and 63.51%–99.93% amino identities. The complete S gene-based genotyping indicated 16 of 17 were positive for CCoV-IIa except for B906_ZJ_2019. Blast results showed the sequences of B906_ZJ_2019 shared 93.24% nucleotide identities (query coverage:99%) with a coronavirus variant strain named A76 (accession number: JN8560098). The spike gene of these16 CCoV-IIa strains shared 94.59%–99.98% nucleotide and 95.71%–99.93% amino acid sequence homology. Moreover, the 16 CCoV-IIa strains displayed 87.94%–88.53% nucleotide and 91.22%–92.20% amino acid sequence homology with the CCoV-IIa reference strain BGF10 (accession number: AY342160). Subsequently, we investigated the amino acid variation in the S protein among CCoV-IIa strains sequenced here and the classical CCoV-IIa strains. We found amino acid changes in around 45 residues, most of these are located in S1 domain, especially in NTD. 7 and 8 of these positions are in CTD and S2 domain, respectively (Fig. 1).

### 3.3. Phylogenetic analysis of CCoV S gene

To realize the evolution of CCoV, we reconstructed a ML tree using the complete spike gene. The phylogenetic tree revealed that all the CCoV strains can be divided into 2 genotypes, named CCoV–I and CCoV-II. CCoV-II further divided into three subtypes, which are CCoV-IIa, CCoV-IIb and CCoV-II variant. CCoV–I and FCoV–I strains formed a clade. CCoV-IIb and TGEV strains clustered together, and CCoV-IIa and FCoV-II strains clustered together. In addition, Strain B906_ZJ_2019 and A76 formed a single clade, which not belong to CCoV–I, CCoV-IIa and CCoV-IIb group. Of the 17 strains identified in this study, 16 strains fell into the CCoV-IIa group and showed closest relationship with strain GZ43 which previously identified in China in 2003 and another strain HCM27 identified in Vietnam in 2014. Other 3 strains previous sequenced in Heilongjiang of China also divided into CCoV-IIa and closely related to strain TN-449 which previously identified in the United States. (Fig. 2).

### 3.4. Recombination analysis of B906_ZJ_2019 spike

RDP4 results indicated B906_ZJ_2019 exhibited a strong recombination signal in seven methods. To further characterize the recombination relationships among B906_ZJ_2019 and another coronavirus, three ML tree of NTD, CTD and S2 subunit of spike gene were constructed. The sequence of B906_ZJ_2019 NTD formed a clade with A76, CCoV–I and FCoV–I strains (Fig. 3A). The sequence of B906_ZJ_2019 CTD of B906_ZJ_2019 form a clade with A76 but not well cluster with CCoV–I and CCoV-II groups (Fig. 3B). In contrast, the sequence of S2 domain of B906_ZJ_2019 clustered closely with these strains identified in this study which belong to CCoV-IIa (Fig. 3C).

## 4. Discussion

Canine coronavirus is a causative agent for viral diarrhea in dogs and have been widely reported in Japan [33], Brail [35], Greece [36], Britain [32], China [37], Italy [38], Vietnam [39], Australia [40], France and Belgium [19]. etc. In China, CCoV is recognized as a common pathogen in pet dogs with positive rate 26% in diseased pet dogs in Beijing [41] and 28.36% in diarrhea dogs in Heilongjiang [37]. Here, the positive rates of CCoV is 23.94% (51/213) in pet dogs with apparent diarrhea symptoms, which is nearly with the mentioned above in China, but obvious lower than previous reported in Japan and Greece

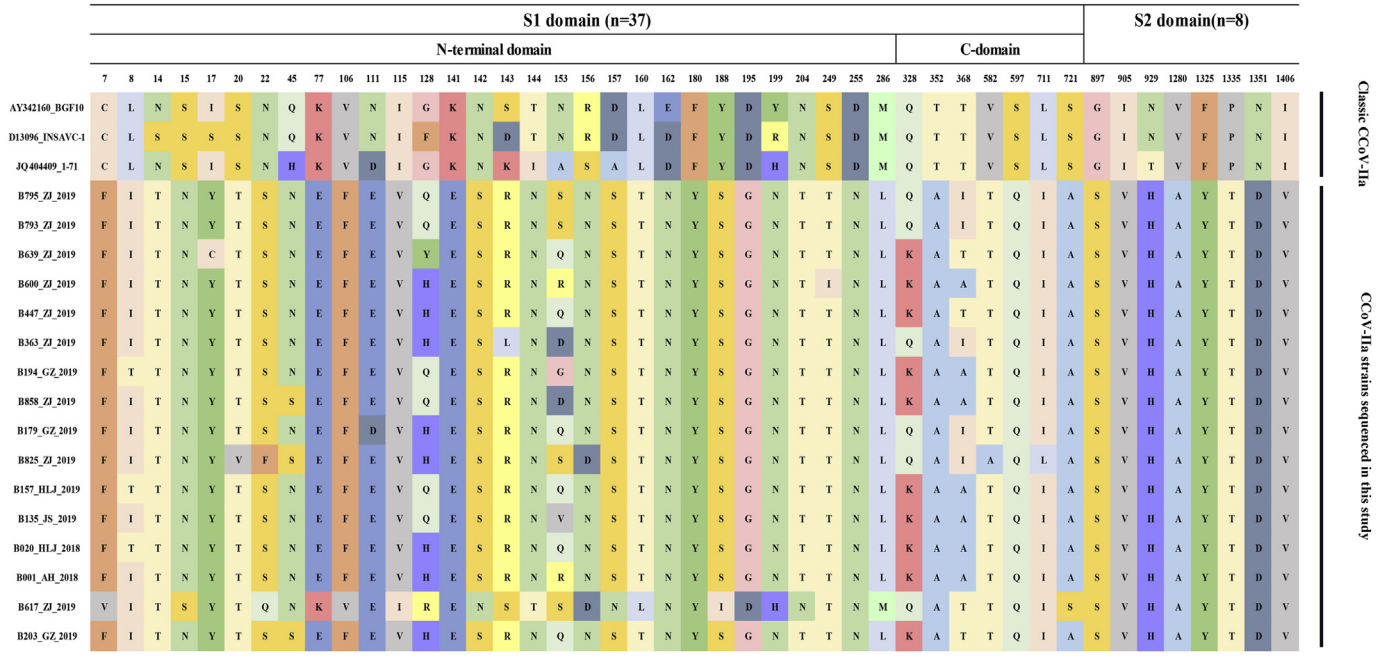


**Fig. 1.** Amino acids variations in the S protein among CCoV-IIa strains sequenced here and the classical CCoV-IIa strain BGF10, 1–71 and INSAVC-1.

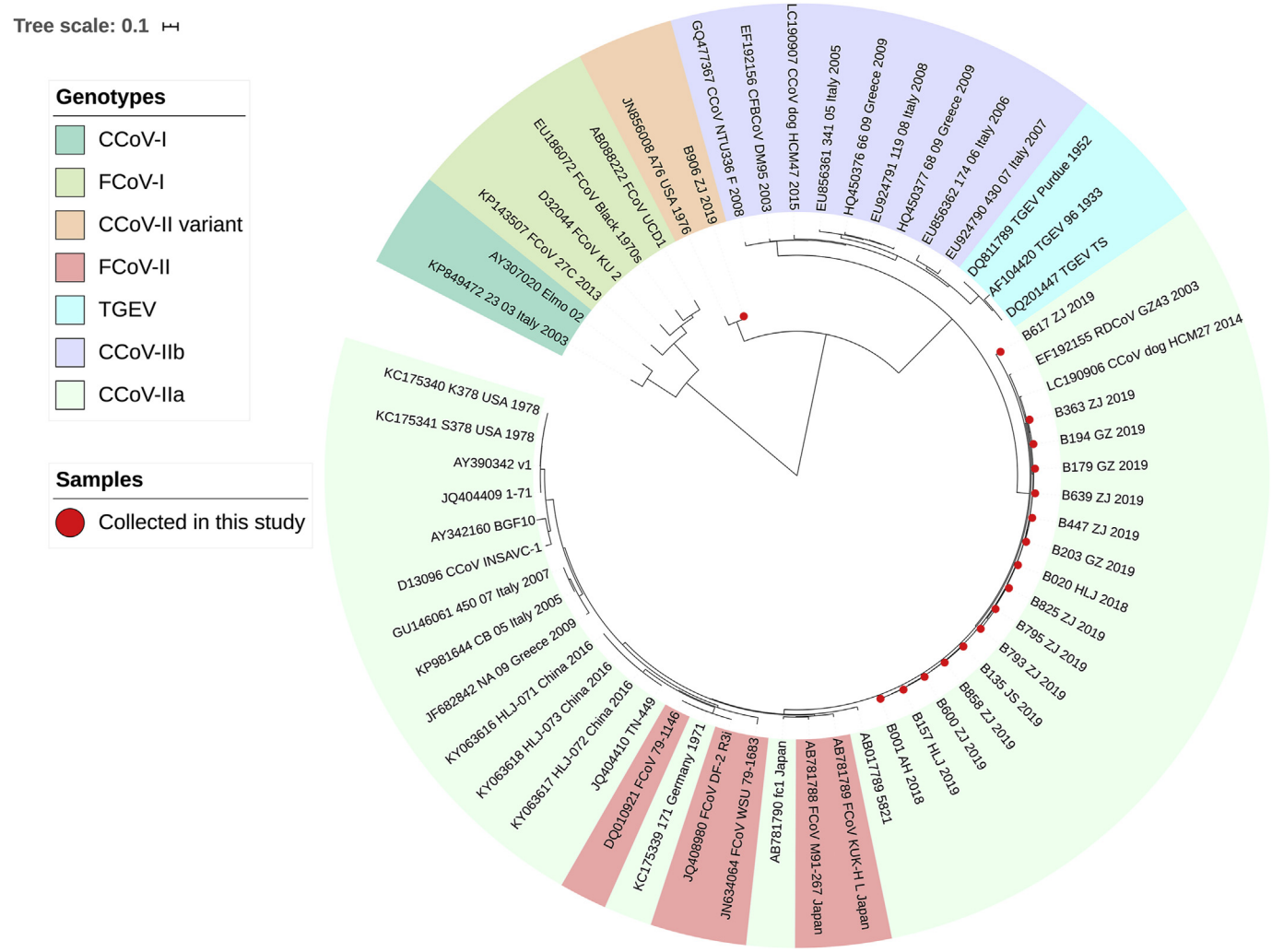


**Fig. 2.** ML tree of spike gene of sequenced here and the reference strains was constructed in RAxML (v8.2.10) with GTR + GAMMA model and supported by 1000 bootstraps. Clade CCoV–I, CCoV-IIa, CCoV-IIb, CCoV-II Variant, FCoV–I, FCoV-II and TGEV are denoted in different colors. These sequences identified in this study are denoted in red circles. (For interpretation of the references to colour in this figure legent, the reader is referred to the web version of this article.)

[36] (50.5% and 65.1% in dogs with diarrhea, respectively) but higher than in Brail(12% in dogs with diarrhea) [35]. In addition, CCoV co-infected with other enteric viruses such as CPV, CaKV and CBoV were frequent reported in diarrheic dogs [33,37,42,43]. Here, 70.59% CCoV-positive samples were co-infected with one or more CPV, CaAstV, CaKV, TTCaV strains. In China, the effective vaccines are available for most pet dogs, but CCoV still existed as one of the most common agents for viral enteritis [44]. It is worth mentioning that among the positive samples of CCoV, 27.45% pet dogs were well vaccinated, which may indicate the incomplete protection of current vaccines. Considering most of vaccines were designed based on the classical CCoV-IIa strains, it was likely not to effective against CCoV–I, then many AA changes existed in current circulating CCoV-IIa in comparison with the classical CCoV-IIa strains, whether the vaccine is effective is uncertain [25].

Multiple infection of CCoV–I and CCoV-II has been frequently reported in previously investigations [31,45]. In our study, apart from the single infection with CCoV–I, CCoV-IIa and CCoV-IIb, two or three genotypes multiple infections accounts for 29.41% (15/51), which indicates co-infection of different CCoV subtypes were widely existed. Previously studies have reported that CCoV-II, especially CCoV-IIa is the main subtypes circulating in Heilongjiang of China during 2014–2015, and CCoV-IIb is the major subtypes circulating in Vietnam [39]. Here, of the CCoV positive dogs, 42 (82.35%) dogs were positive for CCoV-IIa, which is obvious higher than CCoV–I (29.41%) and CCoV-IIb (23.53%). These results revealed that CCoV-IIa play a more dominated role among pet dogs in China.

Phylogenetic analysis of complete *S* gene indicated that canine coronavirus can be divided into 2 genotypes, which consistent with previous studies [22]. However, in this study, we discovered that CCoV-II can be divided into 3 distinct subtypes CCoV-IIa, CCoV-IIb and CCoV-II Variant, which is differ from the 2 subtypes CCoV-IIa and CCoV-IIb as mentioned before. In addition, phylogenetic analysis results also revealed that CCoV-IIa is the predominate genotype currently circulating in China. The strain B906_ZJ_2019 was clustered into CCoV-II variant with strain A76, which was identified a novel NTD with other CCoV-II strains and may as a result of recombination of CCoV–I and CCoV-II [34]. Recombination plays a vital role in genetic evolution of coronavirus, and have high potential for the emergence of novel variants [46]. CCoV-IIb was suggested as a novel recombinant variant, which show close relationship with TGEV [22–24,32,47]. Strain B906_ZJ_2019 showed 93.24% nucleotide similarity to A76. The entire spike gene of CoV can cleave into S1 and S2 subunit, and S1 subunit including N-terminal domain (NTD) and C-domain [25,48]. Recombination results showed that the NTD and S2 region of B906_ZJ_2019 closely related to CCoV–I and CCoV-II, respectively. These results may suggest that B906_ZJ_2019 is a recombinant variant between CCoV–I and CCoV-II. In addition, the C-domain of B906_ZJ_2019 still not well clustered into CCoV–I and CCoV-II, but

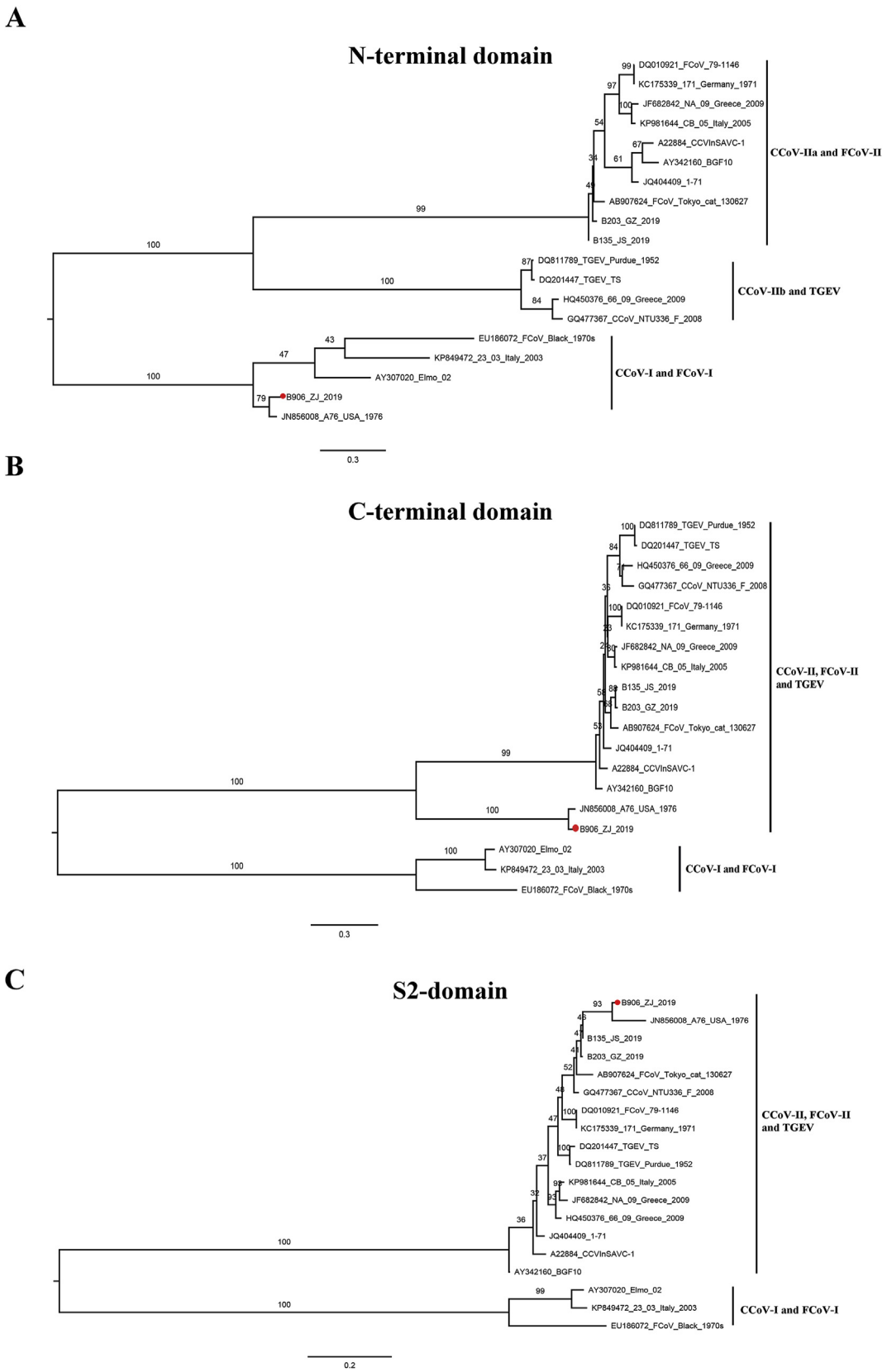


**Fig. 3.** Phylogenetic analysis of B906_ZJ_2019. NJ-tree constructed using the sequences of NTD (A), CTD (B), and S2 subunit(C). Strain B906_ZJ_2019 was denoted in red circle. Moreover, the reference strains used in the analysis were presented in each tree. (For interpretation of the references to colour in this figure legent, the reader is referred to the web version of this article.)

formed a single clade with A76, which may suggest a second recombination, but this still needs further investigation. Previously study suggested that the distinct C-domain caused a distinct host tropism which differ from the classic CCoV strain CCoV 1–71 (accession number: JQ404409). The host tropism of B906_ZJ_2019 still needs further study. Considering the exist of strain A76, this recombination may occur more frequent than we expected. Furthermore, different subtypes CCoV co-infection may support this recombination.

As a typical RNA virus with the largest genome, CoV also presents high level of occurring of mutations [1]. We found 37 AA changes in S1 domain and 8 in S2 domain among CCoV-IIa strains identified here and the classical CCoV-IIa strains. The spike protein is the major antigenicity determinant and also play a vital role in receptor binding and viral entry [49]. During virus entry, S1 subunit is the major region that binds to host cell surface receptors, while S2 is associated with membrane fusion [50]. Whether these AA changes are related to the receptor binding, immunity escaping or other functions still unknown. Therefore, further investigations still need to clarify the specific functions of these non-synonymous substitutions. Cross-host transmission frequently occurred in animal virus, such as porcine circovirus (PCV3) [51], and pseudorabies virus (PRV) [52] in DNA virus, influenza A viruses (IAVs) [53–55] and coronavirus [7,56–58] in RNA virus. One of the most notable is coronavirus. Recently, a novel coronavirus called SARS-CoV-2 emerged in worldwide and was suggested to originate from bats [10]. From SARS to COVID-19, coronaviruses pose a significant threat to public health and cause serious economic loss. Although there is currently no evidence that coronavirus can cross species barrier from dogs to human, it is necessary to monitor the evolution process of canine coronavirus.

Here, we investigated the etiology and evolution changes of CCoV in China. We identified the co-circulating of CCoV–I, CCoV-IIa and CCoV-IIb strains, but CCoV-IIa is predominant genotype circulating in China. Phylogenetic analysis revealed a new subtype CCoV-II variant, which includes B906_ZJ_2019, a strain identified in this study. In addition, recombination analysis revealed B906_ZJ_2019 may evolve from a recombinant virus of CCoV–I and CCoV-II. In general, the resulting data of CCoV provide detailed etiological and evolution information of CCoV strains current circulating in China, which will help to the future prevention and control.

## Author contributions

H.H., Y.C. and G.X. conceived the experiments, provided the samples and revised the paper; W.Z., J.L., R.W. and G.L. performed the experiments; W.Z. and G.L. analyzed the data; W.Z. wrote the paper.

## Declaration of competing interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2020.104209.

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