Genetic Evolution of Bovine Coronavirus Detected in the Republic of Korea

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Research Article

Keywords: bovine coronavirus, evolutionary rate, nomenclature, phylogenetic analysis, TMRCA

Posted Date: June 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-631986/v1

Abstract

Bovine coronavirus (BCoV) is associated with severe diarrhea in calves, winter dysentery in adult cattle, and respiratory diseases in cattle. However, there is limited information regarding its molecular characterization in the Republic of Korea (KOR). This study was conducted to investigate the prevalence of BCoV in diarrheic calves, perform global comparison of BCoV genome sequences, and suggest a new nomenclature. A total of 521 fecal samples were collected from diarrheic pre-weaned native Korean calves (age £60 days) from 100 herds in the KOR. The samples were divided into three groups based on age as follows: 1-21 days (*n* = 433), 21-40 days (*n* = 64), and 41-60 days (*n* = 24). The full-length Spike (S) gene was amplified and sequenced. The phylogenetic tree was constructed using the maximum-likelihood method and the evolutionary rates were estimated. BCoV infection was detected in 20 (3.8%) calves by real-time RT-PCR analysis, and nine full-length genome sequences were obtained from the 20 BCoV-positive samples. Genomic comparison analysis showed that these 2019-2020 variants exhibited the highest nucleotide sequence identity (98.6%-99.2%) with that of water deer (*Hydropotes inermis*) isolates. Phylogenetic analysis of the full genomes of the S gene revealed the following four BCoV groups: G1, classical BCoV strains; G2, 2002 Korean, Japan, Vietnam, Cuba, and USA strains/isolates; G3, European strains/isolates; and G4, Korean isolates (2004 and 2006 Korean isolates, 2010 BCoV-like, 2017 water deer, and 2019-2020 variants). The evolutionary rates accelerated from G1 to G4. This grouping was also closely related to the nucleotide substitution rate. Using molecular clock analysis of the S gene, the most recent common ancestor of each group was estimated to have originated in 1953, 1979, 1986, and 1993, respectively. Recently identified BCoV variants in the KOR are undergoing slow evolution. These findings provide useful information for understanding the molecular characterization of BCoVs. Further research is necessary to conduct recombination analysis to support BCoV evolution.

Background

Coronaviruses (CoVs) cause respiratory and enteric diseases in a wide range of animals, including humans and birds [1, 2]. CoVs are classified into the four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* [3]. The first two genera infect mammals, whereas the latter two are known to infect avian species and mammals [4]. Among them, bovine coronaviruses (BCoVs) belong to the genus *Betacoronavirus*, which comprises the following four groups: 2a, 2b, 2c, and 2d [2]. Group 2a consists of BCoV, human CoV (HCoV) OC-43, mouse hepatitis virus, and canine respiratory CoV [5–7]. BCoV contains five major structural proteins, viz., spike (S), hemagglutinin/esterase (HE), transmembrane (M), small membrane (E), and nucleocapsid (N) proteins [8]. Of these, the S protein is a particularly important mediator of host cell attachment, viral entry, and pathogenesis [9]. The HE protein exhibits an esterase receptor-destroying activity that may be important for viral entry, and also serves as a second viral attachment protein for initiation of infection [10]. Furthermore, the S protein is tightly associated with determining the tissue tropism and host range [11].

BCoV has a global distribution and is known to cause severe calf diarrhea (CD) in neonates, winter dysentery (WD) in adult cattle, and respiratory disease in feedlot cattle [12–15]. CD is characterized by growth retardation, decrease in productivity, and immunosuppression, causing serious economic losses in the livestock industry [16]. However, the incidence of BCoV in CD has been gradually decreasing over the past years [17–20]. According to a recent study, BCoV has been reclassified as a *Betacoronavirus 1* species together with dog, horse, human, and porcine CoVs and shares similarity in genome characterization [21]. BCoV infection was identified in humans [22], alpacas [23], wild ruminants [24], and dogs [25], as well as cattle, suggesting that it is closely connected to cross-species transmission. Moreover, the CoV strain HEC 4408 was isolated from a diarrheic sample of a child in Germany, the genome of which revealed that the strain was more closely related to BCoVs compared with other HCoVs, implicating that the strain could have originated from BCoV [26, 27]. Since 2008, BCoV has been shown to have various host species, and cattle can serve as

a reservoir host for other domestic and wild ruminants [21]. Considering the current situation regarding the coronavirus pandemic, the zoonotic transmission potential of BCoV due to the rapid evolution of CoVs cannot be excluded.

In the Republic of Korea (KOR), research on BCoVs was primarily conducted in the 2000s [28–32], and there is limited information regarding their more recent genetic variation and evolution. Therefore, the classification criteria for BCoV have not yet been clearly established. To date, several studies have focused on the interspecies transmission of BCoV [27, 33–35]. Hence, the present study was conducted to investigate the prevalence of BCoVs in diarrheic calves and to compare the genomic sequences of BCoVs distributed worldwide, as well as to suggest a new nomenclature.

Materials And Methods

Sample collection

Between January 2019 and June 2020, a total of 521 fecal samples were collected by a veterinarian directly from the rectum of pre-weaned native Korean calves (age £60 days) suffering from diarrhea from 100 different herds in the KOR. The collected samples were divided into the following three groups according to age: 1-21 days (*n* = 433), 21-40 days (*n* = 64), and 41-60 days (*n* = 24). These samples were placed in plastic containers and immediately transported to the Animal Immunology Laboratory at Kyungpook National University. Most calves were not vaccinated against BCoV.

RNA extraction and real-time PCR

Total RNA was extracted from fecal suspensions using RNAiso Plus Reagent (Takara, Shiga, Japan) according to the manufacturer's instructions. Real-time polymerase chain reaction (RT-PCR) was conducted to detect BCoV infection using the Path-IDTM Multiplex One-Step RT-PCR Kit (Life Technologies, Carlsbad, CA, USA) as described previously [36]. First, partial amplification (824 bp) of the S gene was performed as described previously [29, 37]. Next, the amplification values (Ct values) were determined for each sample. Ct values £35 indicated a positive test result.

RT-PCR, sequencing, and phylogenetic tree analysis

To obtain sequences, positive samples detected by real-time PCR assay were further amplified using 2×AccuPower® RocketScript™ RT-PCR Master Mix (Bioneer, Daejeon, KOR) with the full-length S gene (4092 bp) of six primer pairs as described previously [29]. Distilled water was used as a negative control. The S1 to S6 genes of BCoV were attached with overlapping sequences. The PCR products were separated by gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized. All PCR products were purified using the AccuPrep PCR Purification Kit (Bioneer) and then used for direct sequencing (Macrogen, Daejeon, KOR). The nucleotide sequences of the 2019-2020 variants obtained in this study were aligned using ClustalX (version 1.8) and compared with 81 BCoV isolates/strains obtained from the GenBank database. The phylogenetic tree was constructed based on the nucleotide alignments using the maximum-likelihood method implemented in the MEGA 7 software [38], and bootstrap analysis was used to evaluate the robustness with 1000 replicates. The cut-off values for bootstrap replication were ³70%. The nucleotide sequences of the 2019-2020 variants obtained in this study were assigned the accession numbers MW881213-MW881221.

Evolutionary rate

The evolutionary rates of nucleotide per site and the year of virus sampling based on the S gene were estimated using the Bayesian Markov chain Monte Carlo approach implemented in the Bayesian Evolutionary Analysis Sampling Trees v1.10.4 [39]. The Hasegawa-Kishino-Yano model of nucleotide substitution was specified for a proportion of gamma-distributed rate heterogeneity with partitions into codon positions of 1 + 2 and 3. The MCMC runs consisted of 8×10^7

generations, and the posterior probability distribution of the chains was sampled at every 1000 steps. The mean evolutionary rate and mean time of the most recent common ancestor (TMRCA) were calculated. The posterior distribution was summarized in terms of 95% highest posterior density (HPD) after the exclusion of a burn-in equal to 20% of the run length. The effective sample size (ESS) was evaluated using Tracer v1.6.0, and ESS values of ³200 for all parameters were accepted [40, 41]. The mutation rates were measured using DNA Sequence Polymorphism (DnaSP) v. 6.12. The mutation rate per RNA sequence per generation was obtained by DnaSP, which was then analyzed using DNA Polymorphism [42, 43].

Assessment of genetic relatedness

Principal coordinate analysis (PCoA) was used to determine the relationships among BCoVs. PCoA is a distance-based model, jointly using a dissimilarity matrix calculated with a simple-matching index and factorial analysis. PCoA was conducted using the Dissimilarity Analysis and Representation for windows v. 6.0.021 to reveal the genetic divergence between BCoV strains/isolates through the Kimura method.

Statistical analysis

The PCR results for each fecal sample were recorded as being either positive or negative for BCoV and categorized according to calf age (i.e., 1-20, 21-40, or 41-60 days). Data were analyzed using the SPSS Statistics 25 software package (SPSS Inc., Chicago, IL, USA). The association between BCoV infection and each age range was evaluated using the chi-square test. $P < 0.05$ was considered significant.

Result

Prevalence of BCoV in calves

Among the 521 diarrheic fecal samples, BCoV infection was detected in 20 (3.8%) pre-weaned native Korean calves by real-time PCR analysis. As shown in Table 1, BCoV infection was the most frequently detected as follows: 17 (3.3%) calves aged 1-20 days, 2 (0.4%) calves aged 21-40 days, and 1 (0.2%) calf aged 41-60 days. No association was found between BCoV infection and calf age ($P = 0.949$).

Genome analysis

Full-length genome sequence analysis of the 20 BCoV-positive samples was performed, and nine sequences were successfully obtained. These nine sequences (designated as 2019-2020 variants) exhibited 99.3%-99.5% nucleotide and 98.6%-99.4% amino acid identity to each other. These variants showed 97.5%-98.2%, 97.5%-98.6%, and 98.1%-98.8% nucleotide identity to Korean WD (KWD) isolates from 2002, to KWD isolates from 2006, and to Korean CD (KCD) isolates from 2004, respectively (Table 2). Interestingly, these 2019-2020 contemporary variants exhibited 98.6%-99.2% homology to sequences obtained from the water deer (*Hydropotes inermis*) (Table 2). The sequences obtained in our study showed 96.6%-97.6%, 96.9%-97.5%, 96.8%-97.4%, and 96.6%-97.6% identity to those from Japan, Vietnam, Mebus strain (USA), and BCoV isolates/strains found in European countries, respectively (Supplementary Table 1). The genome alignment among Korean BCoVs revealed the presence of genetic variations among contemporary BCoVs compared with BCoV isolates/strains identified in the KOR from 2002 to 2006 (Fig. 1).

Phylogenetic analysis

Based on the phylogenetic tree constructed using the S gene, a total of 90 BCoV isolates/strains, including BCoV-like and the nine above-described sequences, were divided into four groups (Fig. 2). Currently, there are no accurate

classification criteria for BCoVs, so they were randomly named (G1-G4) in this study. Our sequences were most closely related to those of the water deer isolate (MG518518) and clustered with the BCoV-like isolates found in zoo ruminants (Fig. 2). Furthermore, these sequences formed a different subgroup from the 2004 KCD isolates and 2006 KWD isolates within the same group (Fig. 2). Although it was detected in the KOR, the isolates/strains from 2002 belonged to a different group from those detected after 2004 (Fig. 2). Including KCD isolates from 2004, KWD isolates from 2006, BCoV-like isolates from 2010, and the contemporary 2019-2020 variants, these Korean isolates/strains were designated as belonging to G4 (Fig. 2). The KWD isolates found in 2002 formed the same clade with that of the white-tailed deer (FJ425187) and were separated from BCoV and BCoV-like isolates identified from Japan, Vietnam, Cuba, and USA, all of which were designated as G2 (Fig. 2). G3 was primarily composed of BCoV strains/isolates detected in Europe, which were slightly different from those found in Asia and the USA (Fig. 2). G1 represented the classical BCoV strains that share little genetic relationship with other BCoVs (Fig. 2). The results of PCoA revealed four well-separated clusters among 90 BCoV isolates/strains, including BCoV-like isolates (Fig. 3), which corresponded to the results from the phylogenetic tree. Specifically, the PCoA analysis demonstrated clear divergence within the G4 group, i.e., 2004-2006 BCoVs vs. 2019-2020 BCoVs.

Evolutionary rate

The mean evolutionary rate and TMRCA based on the established phylogenetic tree were analyzed (Table 3). The evolutionary rate of 68 BCoV global strains/isolates from 1965 to 2017, except for BCoV-like and the 9 variants described in this study, was estimated to be 0.3897×10^{-3} substitutions/site/year (95% HPD; $0.2\text{-}0.5 \times 10^{-3}$), and the TMRCA of 68 BCoVs was dated to be 1947 (95% HPD; 1939-1954). As shown in Table 3, the nucleotide substitution rates of G1 and G2 are relatively low, whereas within G3 and G4, there is a significant variation in the nucleotide substitution rates. G4 was composed of Korean BCoV strains/isolates and showed the presence of significant variation in the nucleotide substitution rates compared with the evolutionary rate of European BCoVs (Table 3). The TMRCA of classical BCoV strains (G1) was accordingly estimated to have originated in the year 1953, and that of G2 consisting of the USA and Asian strains/isolates was in 1979. The European BCoV strains (G3) appeared in 1986. The TMRCA of G4, including the sequences described in this study as well as Korean BCoV-like and BCoV isolates/strains, was estimated to be from 1993 (95% HPD; 1990-1996) (Table 3 and Fig. 4). The time of divergence of BCoV and HCoV-OC43 was estimated to be around 1886. According to the molecular clock analysis, classical BCoVs and other BCoVs diverged in 1958, and European BCoVs (G3) and the USA and Asian BCoVs (G2 and G4) diverged in 1971.

Discussion

This study demonstrated a relatively low prevalence of BCoV in diarrheic calves. This finding was similar to that (5.4%) of our previous study [44] and those from several countries [17–20, 45]. Compared with the past, the incidence of BCoV appears to have constantly decreased. Although there was no statistical significance between diarrhea caused by BCoV and calf age, the incidence of BCoV infection was highest in calves aged < 20 days. This result was consistent with a previous study performed by our group [46]. Although the incidence of BCoV was low in the present study, the importance of BCoV as the primary causative agent of diarrhea in calves should not be ignored because it was still detected in calves with diarrhea. Especially, recent studies report that BCoV is also considered as a key pathogen associated with respiratory diseases [47–51]. Except for the KOR, other countries have focused on BCoV as a respiratory pathogen rather than as a diarrheal pathogen [52–55]. Further study is required to explore the prevalence of BCoV by its associations with various clinical symptoms and to identify the genetic characteristics between enteric and respiratory BCoVs circulating in the KOR.

We compared the full genome sequences of 42 Korean BCoV strains/isolates, including recent BCoVs (2019–2020) and detected genetic variations within nine sequences. Our results clearly demonstrated that the genetic variation appeared significantly over time (Fig. 1). Due to a lack of information on BCoVs since 2006, it is unclear when these genetic variants of BCoV have occurred in the KOR; nevertheless, our results indicate that the genetic variation of BCoV has been slowly occurring since a long period of time. Furthermore, we cannot conclude whether these variations are involved in the pathogenicity of BCoV. Further investigations are required to evaluate the association between genetic variations and pathogenicity.

The 2019–2020 BCoV variants were most similar to the isolates obtained from the water deer [24]. Water deer are widely distributed in the KOR and are known to transmit various diseases [24]. As the scarcity of food and natural habitat has resulted in frequent invasion to farmlands, the possibility that BCoV was transmitted from cattle to water deer or vice versa cannot be ruled out. Interestingly, the sequences found in the present study exhibited 98.7%–99.2% nucleotide identity with BCoV-like isolates identified in zoo animals suffering from diarrhea, and these BCoV-like isolates were known to be derived from cattle in 2005 [56], but the transmission route of the virus to zoo animals remains unknown. Moreover, in the United States, BCoVs have been detected in various wild ruminants, such as giraffe, antelope, alpaca, and deer [33, 57–59]. However, when the results of genetic analysis were compared between captive wild ruminants from the USA and the zoo ruminants from the KOR, significant differences were observed. It is speculated that the viral host reservoir differs between these viruses. Amer reported that the difference between BCoV-like isolates and BCoV is due to the host range, and not distinct virus species [4]; however, our results demonstrated that there were differences between BCoV-like and recent BCoVs variants in genome alignments (data not shown), indicating that the virus species might be different. Our findings suggest that BCoV can infect non-captive animals as well as captive animals. Further epidemiological studies on wild animals are required to clarify this issue.

The classification criteria for BCoV have not yet been clearly established. A recent study reported that BCoV is classified into two major types–European and USA–which are subdivided into 11 and 3 genotypes, respectively [60]. However, our results were different from those reported by that study. According to the phylogenetic analysis, BCoVs were largely divided into four groups, G1–G4. There are at least two subgroups within each group. In our study, we could not accurately subdivide further within each group because the basis for subdivision is currently not well-defined sufficiently. G1 includes the classical BCoV strains, including Mebus, which are considered as the ancestors of all BCoVs. G2 consists of BCoV isolates/strains from Asia (Korea, Japan, and Vietnam) and North America (Cuba and USA). Within the G2 group, the 2002 Korean BCoVs were divergent from those of Japan and Vietnam, even though they belong to the same continent. However, the most common feature of G2 is that these BCoV isolates were derived from the US wild ruminants [60]. G3 included strains/isolates identified from European countries. This could be due to differences in the evolution of BCoVs, and consequently, it is speculated that this evolution plays a vital role in the genetic diversity of BCoVs from country to country. The contemporary BCoVs (2019–2020) identified in this study were classified as belonging to G4, and most Korean isolates, except for 2002 BCoVs, were included in this group. These results provide evidence that the 2019–2020 BCoVs circulating in the KOR have undergone recent genetic evolution.

The evolutionary analysis of 90 BCoVs from 1965 to 2020 revealed the genetic variations according to groups and, in particular, over time. When compared with other RNA viruses, the evolutionary rate of BCoVs was within the standard range [61, 62]. Interestingly, a relatively higher rate of nucleotide substitutions was detected in Korean BCoVs, with slightly higher evolutionary rates than those observed in European countries, indicating that the BCoVs detected in the KOR are undergoing more rapid evolution than that in other countries. This is probably because the sequencing information of BCoVs available according to the year of collection in the KOR is rather large compared with that in other countries. The phylogeny shown in this study was found to be strongly associated with this evolutionary rate.

However, due to the lack of whole genome sequences of Korean BCoVs, we could not confirm recombination events in this study. Further studies are required to conduct recombination analysis to support the evolution of BCoVs.

The TMRCA of BCoVs revealed that the USA and Asia share a common origin for BCoVs. The ancestor of BCoVs was historically assumed to be the same and was confirmed via molecular clock analysis to have diverged into the USA and Europe around 1971. Since then, it has been estimated that BCoVs have evolved separately on these continents. Especially, the USA appears to have more impact on the introduction and spread of BCoVs to Asian countries. This rationale may also be explained by the phylogenetic analysis. According to the TMRCA results, Korean BCoVs diverged from the USA in 1994. Hence, the 2002 Korean BCoVs are believed to have been influenced by those originating from the USA and have been properly adapted to the domestic environment. Unfortunately, we could not perform positive selection analysis within the scope of this study. Further research is necessary to clarify the relationship between positive selection and virus evolution in each country and to provide important information for the development of an effective vaccine against BCoVs.

In summary, our study showed a low prevalence of BCoV in diarrheic calves and the lack of association between BCoV infection and calf age in the KOR. Recently identified BCoVs in the KOR are undergoing genetic variation and continuous evolution. These phenomena should be clearly supported through analysis of recombination events and positive selection. We have proposed a new nomenclature (G1–G4) for BCoVs based on the rate of nucleotide substitution. Our findings provide useful information for understanding the molecular characterization of BCoVs. Further investigation is required to establish an exact classification criterion for subdivisions within each group.

Abbreviations

BCoV: Bovine coronavirus; CD: Calf Diarrhea; CoVs: Coronaviruses; DnaSP: DNA Sequence Polymorphism; ESS: Effective sample size; HCoV: Human CoV; HE: Hemagglutinin/esterase; HPD: Highest posterior density; KCD: Korean Calf Diarrhea; KOR: Republic of Korea; KWD: Korean Winter Dysentery; PCoA: Principle coordinate analysis; PCR: Polymerase chain reaction; S: Spike; TMRCA: The most recent common ancestor; WD: Winter dysentery;

Declarations

Author contributions

KSC conceived and designed the experiments. EMK, HCC, SUS, and JHP performed the experiments. EMK analyzed the data. EMK and KSC wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by the National Research Foundation of Korea (NRF), which is funded by the Mid-Career Research Program (gran No. NRF-2021R1A2C1011579).

Conflict of interest

All authors declare that they have no conflict of interests.

Ethical approval

All animal procedures were conducted according to ethical guidelines for the use of animal samples, as approved by Jeonbuk National University (Institutional Animal Care and Use Committee Decision No. CBNU 2020-052). All

procedures and possible consequences were explained to the managers of the surveyed farm, and written consent was obtained.

References

1. Siddell S (1983) Coronavirus JHM: coding assignments of subgenomic mRNAs. J Gen Virol 64:113–125
2. Woo PC, Lau SK, Huang Y, Yuen KY (2009) Coronavirus diversity, phylogeny and interspecies jumping. Exp Biol Med (Maywood) 234:1117–1127
3. Decaro N, Lorusso A (2020) Novel human coronavirus (SARS-CoV-2): A lesson from animal coronaviruses. Vet Microbiol 244:108693
4. Amer HM (2018) Bovine-like coronaviruses in domestic and wild ruminants. Anim Health Res Rev 19:113–124
5. Erles K, Toomey C, Brooks HW, Brownlie J (2003) Detection of a group 2 coronavirus in dogs with canine infectious respiratory disease. Virology 310:216–223
6. Haring J, Perlman S (2001) Mouse hepatitis virus. Curr Opin Microbiol 4:462–466
7. Mounir S, Labonte P, Talbot PJ (1993) Characterization of the nonstructural and spike proteins of the human respiratory coronavirus OC43: comparison with bovine enteric coronavirus. Adv Exp Med Biol 342:61–67
8. Lai MMC, Cavanagh D (1997) The molecular biology of coronaviruses. Adv Virus Res 48:1–100
9. He Q, Guo Z, Zhang B, Yue H, Tang C (2019) First detection of bovine coronavirus in Yak (Bos grunniens) and a bovine coronavirus genome with a recombinant HE gene. J Gen Virol 100:793–803
10. Desforges M, Desjardins J, Zhang C, Talbot PJ (2013) The acetyl-esterase activity of the hemagglutinin-esterase protein of human coronavirus OC43 strongly enhances the production of infectious virus. J Virol 87:3097–3107
11. Graham RL, Baric RS (2010) Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission. J Virol 84:3134–3146
12. Lathrop SL, Wittum TE, Brock KV, Loerch SC, Perino LJ, Bingham HR, McCollum FT, Saif LJ (2000) Association between infection of the respiratory tract attributable to bovine coronavirus and health and growth performance of cattle in feedlots. Am J Vet Res 61:1062–1066
13. Saif LJ (1990) A review of evidence implicating bovine coronavirus in the etiology of winter dysentery in cows: an enigma resolved? Cornell Vet 80:303–311
14. Clark MA (1993) Bovine coronavirus. Br Vet J 149:51–70
15. Heckert RA, Saif LJ, Hoblet KH, Agnes AG (1990) A longitudinal study of bovine coronavirus enteric and respiratory infections in dairy calves in two herds in Ohio. Vet Microbiol 22:187–201
16. Saif LJ (2004) Animal coronavirus vaccines: lessons for SARS. Dev Biol (Basel) 119:129–140
17. Barkley JA, Pempek JA, Bowman AS, Nolting JM, Lee J, Lee S, Habing GG (2021) Longitudinal health outcomes for enteric pathogens in preweaned calves on Ohio dairy farms. Prev Vet Med 190:105323
18. Bertoni E, Aduriz M, Bok M, Vega C, Saif L, Aguirre D, Cimino RO, Mino S, Parreno V (2020) First report of group A rotavirus and bovine coronavirus associated with neonatal calf diarrhea in the northwest of Argentina. Trop Anim Health Prod 52:2761–2768
19. Caffarena RD, Casaux ML, Schild CO, Fraga M, Castells M, Colina R, Maya L, Corbellini LG, Riet-Correa F, Giannitti F (2021) Causes of neonatal calf diarrhea and mortality in pasture-based dairy herds in Uruguay: a farm-matched case-control study. Braz J Microbiol 52:977–988
20. Mohamed FF, Mansour SMG, El-Araby IE, Mor SK, Goyal SM (2017) Molecular detection of enteric viruses from diarrheic calves in Egypt. Arch Virol 162:129–137

21. Hodnik JJ, Jezek J, Staric J (2020) Coronaviruses in cattle. Trop Anim Health Prod 52:2809–2816
22. Vijgen L, Keyaerts E, Lemey P, Maes P, Van Reeth K, Nauwynck H, Pensaert M, Van Ranst M (2006) Evolutionary history of the closely related group 2 coronaviruses: porcine hemagglutinating encephalomyelitis virus, bovine coronavirus, and human coronavirus OC43. J Virol 80:7270–7274
23. Luna L, Brandao PE, Maturrano L, Rosadio R, Silva FDF, Soares RM, Gregori F (2015) Betacoronavirus 1 in alpacas (Vicugna pacos) in the High Peruvian Andes. Small Ruminant Res 133:7–9
24. Kim JH, Jang JH, Yoon SW, Noh JY, Ahn MJ, Kim Y, Jeong DG, Kim HK (2018) Detection of bovine coronavirus in nasal swab of non-captive wild water deer, Korea. Transbound Emerg Dis 65:627–631
25. Kaneshima T, Hohdatsu T, Hagino R, Hosoya S, Nojiri Y, Murata M, Takano T, Tanabe M, Tsunemitsu H, Koyama H (2007) The infectivity and pathogenicity of a group 2 bovine coronavirus in pups. J Vet Med Sci 69:301–303
26. Zhang XM, Herbst W, Kousoulas KG, Storz J (1994) Biological and genetic characterization of a hemagglutinating coronavirus isolated from a diarrhoeic child. J Med Virol 44:152–161
27. Bidokhti MRM, Traven M, Krishna NK, Munir M, Belak S, Alenius S, Cortey M (2013) Evolutionary dynamics of bovine coronaviruses: natural selection pattern of the spike gene implies adaptive evolution of the strains. J Gen Virol 94:2036–2049
28. Park SJ, Kim GY, Choy HE, Hong YJ, Saif LJ, Jeong JH, Park SI, Kim HH, Kim SK, Shin SS (2007) Dual enteric and respiratory tropisms of winter dysentery bovine coronavirus in calves. Arch Virol 152:1885–1900
29. Park SJ, Jeong C, Yoon SS, Choy HE, Saif LJ, Park SH, Kim YJ, Jeong JH, Park SI, Kim HH (2006) Detection and characterization of bovine coronaviruses in fecal specimens of adult cattle with diarrhea during the warmer seasons. J Clin Microbiol 44:3178–3188
30. Ko CK, Kang MI, Lim GK, Kim GY, Yoon SS, Park JT, Jeong C, Park SH, Park SJ, Kim YJ (2006) Molecular characterization of HE, M, and E genes of winter dysentery bovine coronavirus circulated in Korea during 2002–2003. Virus Genes 32:129–136
31. Jeong JH, Kim GY, Yoon SS, Park SJ, Kim YJ, Sung CM, Jang OJ, Shin SS, Koh HB, Lee BJ (2005) Detection and isolation of winter dysentery bovine coronavirus circulated in Korea during 2002–2004. J Vet Med Sci 67:187–189
32. Jeong JH, Kim GY, Yoon SS, Park SJ, Kim YJ, Sung CM, Shin SS, Lee BJ, Kang MI, Park NY (2005) Molecular analysis of S gene of spike glycoprotein of winter dysentery bovine coronavirus circulated in Korea during 2002–2003. Virus Res 108:207–212
33. Hasoksuz M, Alekseev K, Vlasova A, Zhang X, Spiro D, Halpin R, Wang S, Ghedin E, Saif LJ (2007) Biologic, antigenic, and full-length genomic characterization of a bovine-like coronavirus isolated from a giraffe. J Virol 81:4981–4990
34. Saif LJ, Jung K (2020) Comparative Pathogenesis of Bovine and Porcine Respiratory Coronaviruses in the Animal Host Species and SARS-CoV-2 in Humans. J Clin Microbiol 58:e01355–e01320
35. Vijgen L, Keyaerts E, Moes E, Thoelen I, Wollants E, Lemey P, Vandamme AM, Van Ranst M (2005) Complete genomic sequence of human coronavirus OC43: molecular clock analysis suggests a relatively recent zoonotic coronavirus transmission event. J Virol 79:1595–1604
36. Cho YI, Kim WI, Liu S, Kinyon JM, Yoon KJ (2010) Development of a panel of multiplex real-time polymerase chain reaction assays for simultaneous detection of major agents causing calf diarrhea in feces. J Vet Diagn Invest 22:509–517
37. Tsunemitsu H, Smith DR, Saif LJ (1999) Experimental inoculation of adult dairy cows with bovine coronavirus and detection of coronavirus in feces by RT-PCR. Arch Virol 144:167–175

38. Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33:1870–1874
39. Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214
40. Castells M, Giannitti F, Caffarena RD, Casaux ML, Schild C, Castells D, Riet-Correa F, Victoria M, Parreno V, Colina R (2019) Bovine coronavirus in Uruguay: genetic diversity, risk factors and transboundary introductions from neighboring countries. Arch Virol 164:2715–2724
41. Franzo G, Drigo M, Legnardi M, Grassi L, Pasotto D, Menandro ML, Cecchinato M, Tucciarone CM (2020) Bovine Coronavirus: Variability, Evolution, and Dispersal Patterns of a No Longer Neglected Betacoronavirus. Viruses 12:1285
42. Tajima F (1993) Statistical analysis of DNA polymorphism. Jpn J Genet 68:567–595
43. Rozas J (2009) DNA sequence polymorphism analysis using DnaSP. Methods Mol Biol 537:337–350
44. Ryu JH, Shin SU, Choi KS (2020) Molecular surveillance of viral pathogens associated with diarrhea in pre-weaned Korean native calves. Trop Anim Health Prod 52:1811–1820
45. Bartels CJ, Holzhauer M, Jorritsma R, Swart WA, Lam TJ (2010) Prevalence, prediction and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calves. Prev Vet Med 93:162–169
46. Park J, Han DG, Kim S, Chae JB, Chae JS, Yu DH, Choi KS (2018) Prevalence of coronavirus from diarrheic calves in the Republic of Korea. Asia Pac J Trop Biomed 8:1–6
47. Fulton RW (2020) Viruses in Bovine Respiratory Disease in North America: Knowledge Advances Using Genomic Testing. Vet Clin North Am Food Anim Pract 36:321–332
48. Pardon B, Callens J, Maris J, Allais L, Van Praet W, Deprez P, Ribbens S (2020) Pathogen-specific risk factors in acute outbreaks of respiratory disease in calves. J Dairy Sci 103:2556–2566
49. Stokstad M, Klem TB, Myrmel M, Oma VS, Toftaker I, Osteras O, Nodtvedt A (2020) Using Biosecurity Measures to Combat Respiratory Disease in Cattle: The Norwegian Control Program for Bovine Respiratory Syncytial Virus and Bovine Coronavirus. Front Vet Sci 7:167
50. Ellis J (2019) What is the evidence that bovine coronavirus is a biologically significant respiratory pathogen in cattle? Can Vet J 60:147–152
51. Workman AM, Kuehn LA, McDaneld TG, Clawson ML, Loy JD (2019) Longitudinal study of humoral immunity to bovine coronavirus, virus shedding, and treatment for bovine respiratory disease in pre-weaned beef calves. BMC Vet Res 15:161
52. Mekata H, Hamabe S, Sudaryatma PE, Kobayashi I, Kanno T, Okabayashi T (2020) Molecular epidemiological survey and phylogenetic analysis of bovine respiratory coronavirus in Japan from 2016 to 2018. J Vet Med Sci 82:726–730
53. Gulliksen SM, Jor E, Lie KI, Loken T, Akerstedt J, Osteras O (2009) Respiratory infections in Norwegian dairy calves. J Dairy Sci 92:5139–5146
54. Pardon B, De Bleecker K, Dewulf J, Callens J, Boyen F, Catry B, Deprez P (2011) Prevalence of respiratory pathogens in diseased, non-vaccinated, routinely medicated veal calves. Vet Rec 169:278
55. O'Neill R, Mooney J, Connaghan E, Furphy C, Graham DA (2014) Patterns of detection of respiratory viruses in nasal swabs from calves in Ireland: a retrospective study. Vet Rec 175:351
56. Chung JY, Kim HR, Bae YC, Lee OS, Oem JK (2011) Detection and characterization of bovine-like coronaviruses from four species of zoo ruminants. Vet Microbiol 148:396–401
57. Jin L, Cebra CK, Baker RJ, Mattson DE, Cohen SA, Alvarado DE, Rohrmann GF (2007) Analysis of the genome sequence of an alpaca coronavirus. Virology 365:198–203

58. Zhang X, Hasoksuz M, Spiro D, Halpin R, Wang S, Vlasova A, Janies D, Jones LR, Ghedin E, Saif LJ (2007) Quasispecies of bovine enteric and respiratory coronaviruses based on complete genome sequences and genetic changes after tissue culture adaptation. Virology 363:1–10
59. Alekseev KP, Vlasova AN, Jung K, Hasoksuz M, Zhang X, Halpin R, Wang S, Ghedin E, Spiro D, Saif LJ (2008) Bovine-like coronaviruses isolated from four species of captive wild ruminants are homologous to bovine coronaviruses, based on complete genomic sequences. J Virol 82:12422–12431
60. Suzuki T, Otake Y, Uchimoto S, Hasebe A, Goto Y (2020) Genomic Characterization and Phylogenetic Classification of Bovine Coronaviruses Through Whole Genome Sequence Analysis. Viruses 12:183
61. Donker NC, Kirkwood CD (2012) Selection and evolutionary analysis in the nonstructural protein NSP2 of rotavirus A. Infect Genet Evol 12:1355–1361
62. Sall AA, Faye O, Diallo M, Firth C, Kitchen A, Holmes EC (2010) Yellow fever virus exhibits slower evolutionary dynamics than dengue virus. J Virol 84:765–772

Tables

Table 1 Prevalence of BCoV in pre-weaned Korean native calves

Age (days)	No. of samples	No. of BCoV positive samples	χ^2 (*P*-value)
1-20	433	17 (3.3%)	0.104 (0.949)
21-40	64	2 (0.3%)	
41-60	24	1 (0.2%)	
Total	521	20 (3.8%)	

Table 2 Comparison of nucleotide sequence identity among BCoV isolates/strains and BCoV-like isolates identified in the KOR

	KCD GJ1 (2019)	KCD GJ2 (2019)	KCD GJ3 (2019)	KCD GJ4 (2019)	KCD GJ1 (2020)	KCD GJ2 (2020)	KCD BA (2020)	KCD GC (2020)	KCD YJ (2020)
AY935637/KWD1 2002	97.7	97.9	98.0	98.1	97.8	97.6	98.0	98.1	97.9
AY935638/KWD2 2002	97.8	98.0	98.1	98.1	97.8	97.7	98.1	98.2	97.9
AY935639/KWD3 2002	97.8	98.0	98.0	98.1	97.8	97.6	98.0	98.2	97.9
AY935640/KWD4 2002	97.7	97.9	98.0	98.0	97.7	97.6	98.0	98.1	97.8
AY935641/KWD5 2002	97.7	97.9	98.0	98.0	97.7	97.6	98.0	98.1	97.8
AY935642/KWD6 2002	97.8	98.0	98.0	98.1	97.8	97.6	98.0	98.2	97.9
AY935644/KWD8 2002	97.8	98.0	98.0	98.1	97.8	97.6	98.0	98.2	97.9
AY935645/KWD9 2002	97.7	97.9	97.9	98.0	97.7	97.5	97.9	98.1	97.8
AY935646/KWD10 2002	97.7	97.9	97.9	98.0	97.7	97.5	97.9	98.1	97.8
DQ389632/KCD1 2004	98.4	98.6	98.6	98.7	98.4	98.2	98.7	98.8	98.5
DQ389634/KCD3 2004	98.2	98.4	98.5	98.6	98.2	98.1	98.5	98.6	98.4
DQ389635/KCD4 2004	98.3	98.5	98.6	98.7	98.3	98.2	98.6	98.7	98.5
DQ389636/KCD5 2004	98.1	98.4	98.4	98.5	98.2	98.0	98.4	98.6	98.3
DQ389637/KCD6 2004	98.4	98.6	98.6	98.7	98.4	98.3	98.7	98.8	98.5
DQ389638/KCD7 2004	98.3	98.5	98.5	98.6	98.3	98.1	98.5	98.7	98.4
DQ389639/KCD8 2004	98.3	98.5	98.6	98.7	98.3	98.2	98.6	98.7	98.5
DQ389640/KCD9 2004	98.3	98.5	98.6	98.6	98.3	98.1	98.6	98.7	98.4
DQ389641/KCD10 2004	98.3	98.6	98.6	98.7	98.4	98.2	98.6	98.8	98.5
DQ389652/KWD11 2006	98.1	98.3	98.4	98.4	98.1	97.9	98.4	98.5	98.2
DQ389653/KWD12 2006	98.0	98.3	98.3	98.4	98.1	97.9	98.3	98.5	98.2
DQ389654/KWD13 2006	98.2	98.5	98.5	98.6	98.3	98.1	98.5	98.7	98.4
DQ389655/KWD14 2006	98.0	98.2	98.3	98.4	98.0	97.9	98.3	98.4	98.2
DQ389656/KWD15 2006	97.9	98.1	98.2	98.2	97.9	97.8	98.2	98.3	98.0
DQ389657/KWD16	98.0	98.2	98.2	98.3	98.0	97.8	98.2	98.4	98.2

2006									
DQ389658/KWD17 2006	97.6	97.8	97.9	97.9	97.6	97.5	97.9	98.0	97.8
DQ389659/KWD18 2006	98.0	98.2	98.3	98.3	98.0	97.9	98.3	98.4	98.1
DQ389660/KWD19 2006	98.0	98.2	98.3	98.3	98.0	97.8	98.3	98.4	98.2
HM573326/Wisent 2010	98.8	99.1	99.1	99.1	98.8	98.7	99.1	99.3	98.9
HM573327/Himalayan tahr1 2010	98.9	99.1	99.1	99.2	98.9	98.7	99.1	99.3	99.0
HM573328/Himalayan tahr2 2010	98.9	99.1	99.1	99.2	98.9	98.7	99.1	99.3	99.0
HM573329/Sitatunga 2010	98.9	99.1	99.1	99.2	98.9	98.7	99.1	99.3	99.0
HM573330/Nyala 2010	98.9	99.1	99.1	99.2	98.9	98.7	99.1	99.3	99.0
MG518518/Water deer 2017	98.8	99.0	99.0	99.0	98.8	98.6	99.0	99.2	98.9

Table 3 Estimation of evolutionary rate and time to the most recent common ancestor (TMRCA) from G1 to G4 BCoV isolates/strains

Parameter	Group	Mean	Lower 95HPD	Higher 95HPD
Evolutionary rate	BCoV	3.8973×10^{-4}	2.2580×10^{-4}	5.8144×10^{-4}
	G1	9.7429×10^{-7}	4.5859×10^{-48}	5.8838×10^{-6}
	G2	3.8170×10^{-4}	2.8522×10^{-4}	4.7694×10^{-4}
	G3	1.4086×10^{-3}	9.4842×10^{-4}	1.8916×10^{-3}
	G4	1.0991×10^{-3}	8.0675×10^{-4}	1.3932×10^{-3}
	2019–20 KCD	2.0858×10^{-5}	2.3879×10^{-36}	1.5927×10^{-3}
tMRCA	BCoV	1947	1939	1954
	G1	1953	1948	1957
	G2	1979	1976	1983
	G3	1986	1982	1990
	G4	1993	1990	1996
	2019–20 KCD	-3.4441×10^{28}	-2.5517×10^{33}	2016

Figures

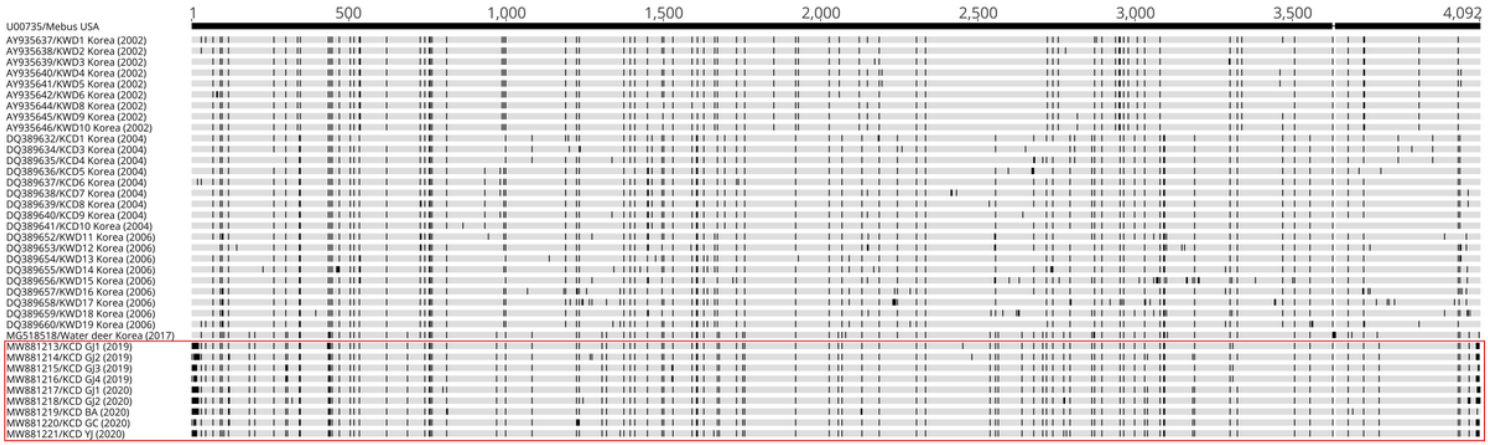

Figure 1

Schematic diagram of the genomic sequences of Korean BCoV isolates generated using the Geneious software version 10.2.4. The genomic regions are shown above, with the black bars representing the spike glycoproteins. Lightly shaded regions are identical to the consensus sequence, and the vertical black bars indicate differences from the consensus sequence. The sequences identified in this study are indicated by a red line box.

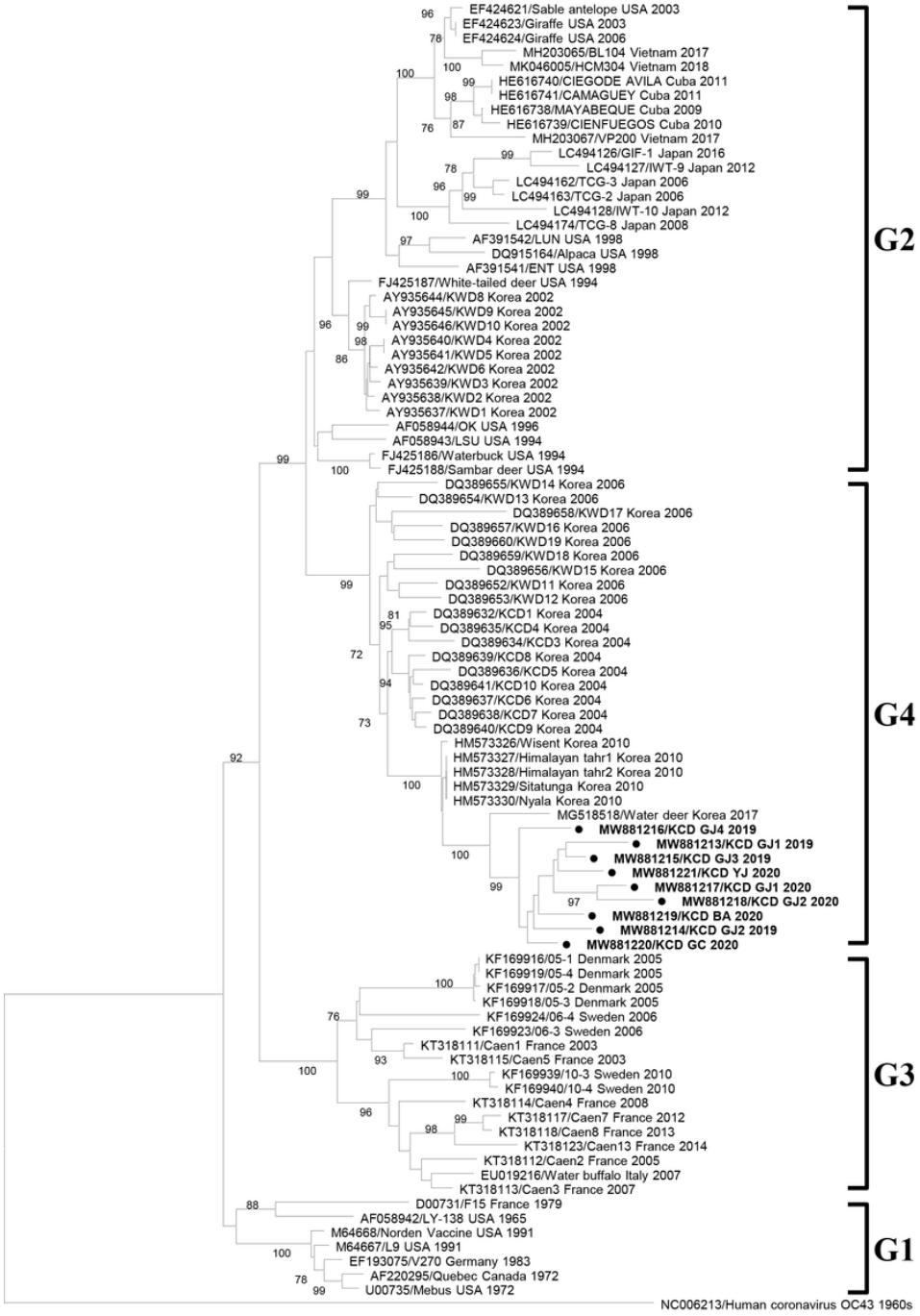

Figure 2

Phylogenetic tree based on the S gene from 90 BCoV isolates/strains reported worldwide, including the BCoV sequences obtained in this study. The tree was constructed using the neighbor-joining method implemented in MEGA version 7. Bootstrap analysis was performed with 1000 replicates. The sequences identified in this study are marked with bold circular symbols. Bootstrap values of ≥70 are presented.

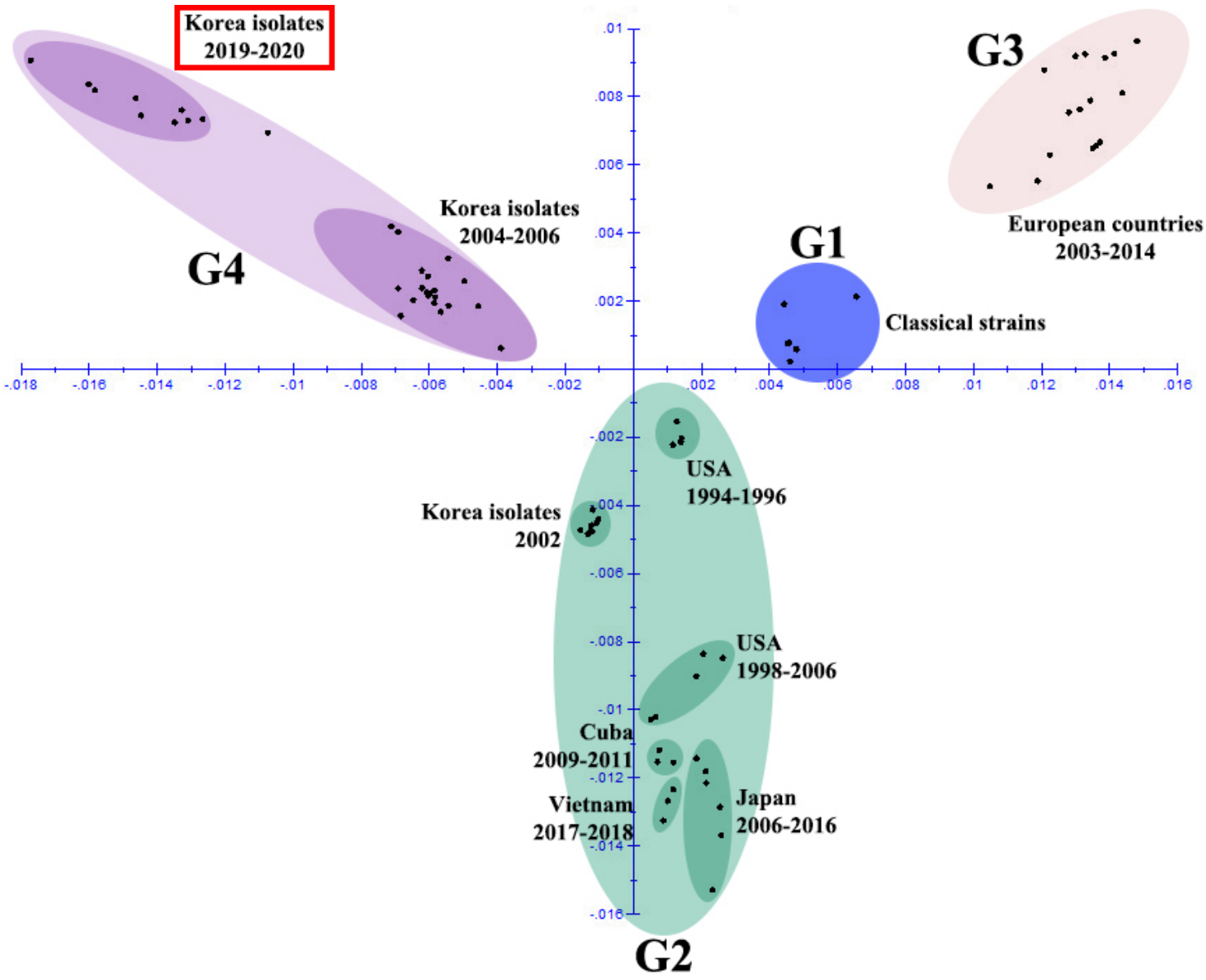

Figure 3

Principle coordinate analysis (PCoA) of the S gene based on the phylogenetic tree. The genetic groups of BCoV by PCoA are color-coded as follows: G1 (blue), G2 (green), G3 (khaki), and G4 (pink). The 2019–2020 Korean sequences are shown in the upper left position and are separated from the 2004–2006 Korean BCoV isolates/strains.

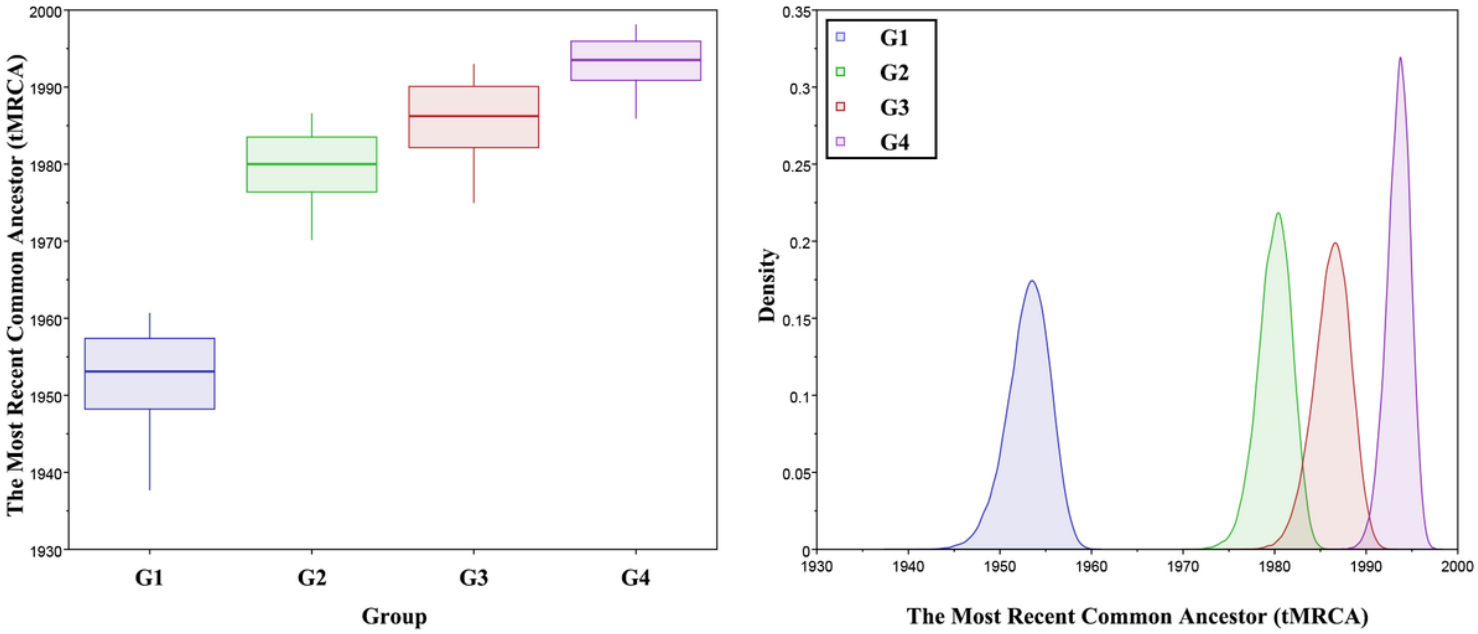

Figure 4

The time to the most recent common ancestor (TMRCA) of the S gene according to each group is shown as boxplot (a) and density plot (b). The 95% HPD intervals are presented in boxplot. The median values are indicated as a bold line in the boxplots.

Supplementary Files

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- Supplementarytable1.docx