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Discovery of a highly divergent coronavirus in the Asian house shrew from China illuminates the origin of the alphacoronaviruses

Running title: A divergent shrew Alphacoronavirus sampled from China

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ABSTRACT

Although shrews are one of the largest groups of mammals little is known about their role in the evolution and transmission of viral pathogens including coronaviruses. We captured 266 Asian house shrews (*Suncus murinus*) in Jiangxi and Zhejiang provinces, China, during 2013-2015. Coronavirus (CoV) RNA was detected in 24 Asian house shrews, with an overall prevalence of 9.02%. Complete viral genome sequences were successfully recovered from the RNA positive samples. The newly discovered shrew CoV fell into four lineages reflecting their geographic origins, indicative of largely allopatric evolution. Notably, these viruses were most closely related to alphacoronaviruses, but sufficiently divergent that they should be considered a novel member of the genus *Alphacoronavirus*, which we denote Wénchéng shrew virus (WESV). Phylogenetic analysis revealed that WESV was a highly divergent member of the alphacoronaviruses and, more dramatically, that the S gene of WESV fell in a cluster that was genetically distinct from that of known coronaviruses. The divergent position of WESV suggests that coronaviruses have a long association with Asian house shrews. In addition, the genome of WESV contains a distinct NS7 gene that exhibits no sequence similarity to any known viruses. Together, these data suggest that shrews are natural reservoirs for coronaviruses and may have played an important and long-term role in CoV evolution.

IMPORTANCE

The subfamily *Coronavirinae* contains several notorious human and animal pathogens, including severe acute respiratory syndrome coronavirus, Middle East respiratory syndrome coronavirus, and porcine epidemic diarrhea virus. Because of their genetic diversity and phylogenetic relationships it has been proposed that the alphacoronaviruses likely have their ultimate ancestry in those viruses residing in bats. Here, we described a novel alphacoronavirus (Wénchéng shrew virus, WESV) that was sampled from Asian house shrews in China. Notably, WESV is a highly divergent member of the alphacoronaviruses and possesses an S gene that is genetically distinct from that of all known coronaviruses. In addition, the genome of WESV contains a distinct NS7 gene that exhibits no sequence similarity to any known viruses. Together, these data suggest that shrews are important and long-standing hosts for coronaviruses that merit additional research and surveillance.

Keywords: Coronavirus, Alphacoronavirus, Asian house shrew, Evolution, Phylogeny, Recombination.

INTRODUCTION

Most emerging infectious diseases described recently are due to previously unknown zoonotic pathogens (1, 2), particularly rapidly evolving RNA viruses that frequently jump species boundaries (3-7). In addition to their rapid evolution, ongoing changes in the natural environment and in the behavior of their hosts have facilitated the emergence of viral diseases by providing new ecological niches (8-11). Such a process of disease emergence is predicted to occur with increased frequency as humans continually change their interaction with the animal world.

Coronaviruses (subfamily *Coronavirinae*, family *Coronaviridae*, order *Nidovirales*) are single-stranded positive-sense RNA viruses and produce enveloped virions (12). Their genome (26-32 kb) contains six open reading frames (ORFs) that are conserved across the subfamily and arranged in the order 5'-replicase ORF1ab-spike (S)-envelope (E)-membrane (M)- nucleocapsid (N)-3' (12). The replicase gene ORF1ab encodes 16 nonstructural proteins (termed nsp1–16). On the basis of phylogeny and pairwise evolutionary distances in the conserved domains of the replicase polyprotein the currently known coronaviruses are classified into 30 species within four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (13, http://ictv.global/report). These viruses can infect humans, other mammals, and birds, causing respiratory, enteric, hepatic, and neurological diseases of varying severity (12). More importantly, the pandemic of severe acute respiratory syndrome (SARS) that occurred during 2002-2003 (5) and the subsequent emergence of the Middle East respiratory syndrome (MERS) in 2012 (14), both of which were caused by previously unknown coronaviruses, remind us that these viruses will likely remain a considerable challenge to public health for the foreseeable future. In addition, the discovery of SARS-like CoV in Himalayan palm civets (15) and bats (16, 17) highlights the essential role that mammalian species play in coronavirus evolution, and have heightened interest in documenting novel coronaviruses in animals and humans on a global scale.

All known alphacoronaviruses form a monophyletic group within the subfamily *Coronavirinae* (13). Two genetic features set them apart from other coronaviruses: (i) a unique type of nsp1, distinct in size and sequence from the betacoronavirus nsp1 and that has no apparent counterpart in gammacoronaviruses and deltacoronaviruses, and (ii) the presence of a commonly-shared accessory gene for a dispensable multi-spanning alphacoronavirus membrane protein (αmp) (13). At present, the genus *Alphacoronavirus* includes 11 species (http://ictv.global/report) and some tentative species (13, 18-20). These virus species have been sampled from bats, as well as a variety of other mammals including humans. On the basis of their diversity and phylogeny it has been proposed that the alphacoronaviruses likely have their ultimate ancestry in bats (21, 22). However, the recent discovery of Lucheng Rn rat coronavirus (LRNV) in a brown rat (*Rattus norvegicus*) sampled from China suggests that the evolutionary history of these viruses is more complex than previously thought (18). Indeed, as RNA viruses likely exist in every species of cellular life (23, 24), our current knowledge of the origins and evolutionary history of alphacoronaviruses from such sparse sampling is likely to be biased.

Shrews (Mammalia: Eulipotyphla: Soricidae) are small mole-like mammals that are broadly distributed globally. The shrew family is the fourth largest in mammals, comprising approximately 376 species (25). As the former name of the Eulipotyphla (i.e. Insectivora) implies, insects make up a large portion of the typical shrew diet. Our recent studies have revealed a remarkable diversity of viruses in invertebrates, especially in arthropods (24, 26). Additionally, the discovery of distinct nidoviruses in insects suggests that coronaviruses may have an invertebrate origin (27, 28). Importantly, multiple viruses (e.g. arenavirus, hantaviruses and rotavirus) have also been identified in insect-feeding shrews over the past decade (29-31). Hence, like bats, shrews may play an important role in the evolution and transmission of viruses among animals, or from animals into humans, including coronaviruses. In this study, we tested shrew samples collected in the Jiangxi and Zhejiang provinces of China for the presence of coronaviruses. Based on the discovery of a distinct shrew virus, we explore the origin and evolution of alphacoronaviruses as a whole.

MATERIAL AND METHODS

Trapping of small animals and sample collection

During 2013-2015 shrews were trapped in mountainous regions of Xingguo and Yudu counties in Ganzhou city, Jiangxi Province, and in the Longwan district and Ruian and Wencheng counties of Wenzhou city, Zhejiang Province, China (Figure 1) as described previously (3, 32). All animals were initially identified by morphological examination, and were further confirmed by sequence analysis of the mitochondrial cytochrome b (mt-*cyt b*) gene (3). Euthanasia was performed before necropsy. Every effort was made to minimize suffering. Rectal samples were collected from shrews for CoV detection.

This study was reviewed and approved by the ethics committee of the National Institute for Communicable Disease Control and Prevention of the Chinese CDC. All animals were treated in strict according to the guidelines for the Laboratory Animal Use and Care from the

Chinese CDC and the Rules for the Implementation of Laboratory Animal Medicine (1998) from the Ministry of Health, China, under the protocols approved by the National Institute for Communicable Disease Control and Prevention.

DNA and RNA extraction and virus detection.

Total RNA was extracted from fecal samples using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The RNA was eluted in 50μl of DEPC water and was used as the template for reverse transcription-PCR. Total DNA was extracted from rectal samples using the DNeasy Blood & Tissue kit (QIAGEN, Valencia, USA) according to protocols suggested by the manufacturer.

CoV RNA was detected by RT-PCR as described previously (18, 19). Complete genomes of coronaviruses were amplified using primers based on the conserved regions of known genome sequences (18, 19). The 5'- and 3'-ends of the genome of the newly discovered shrew coronaviruses were obtained by 5' and 3' RACE (rapid amplification of cDNA ends) using a RACE kit (TaKaRa, Dalian, China). Sequences were assembled and manually edited to produce the final viral genomes. The amplification of the mt-*cyt b* gene was performed as described previously (3).

RT-PCR amplicons <700 bp were purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, USA) according to the manufacturer’s recommendations and subjected to direct sequencing. Purified DNA >700 bp was cloned into pMD18-T vector (TaKaRa, Dalian, China), and subsequently transformed into JM109-143 competent cells. All viral sequences obtained in this study have been deposited in GenBank under accession numbers KY967715-KY967735 and KF294384-KF294386.

Phylogenetic analysis

Analysis of protein families was performed using the PFAM and InterProScan programs (33, 34). Prediction of the transmembrane domains was performed using the TMHMM program (version 2.0; www.cbs.dtu.dk/services/TMHMM/).

Because of extensive sequence divergence between the nucleotide (nt) sequences of different CoV genera, all phylogenetic analyses were based on amino acid (aa) sequences. Accordingly, aa sequence alignments were conducted using the MAFFT program employing the G-INS-i algorithm (35). After alignment, gaps and ambiguously aligned regions were removed using Gblocks (v0.91b) (36). Phylogenetic analyses were then performed using the sequences of eight complete CoV proteins: (i) nsp5 [chymotrypsin-likeprotease (3CLpro)], (ii) RdRp (nsp12), (iii) nsp13 [helicase (Hel)], (iv) nsp14 [3´ -to-5´ exonuclease (ExoN)] , (v) nsp15 [nidoviral endoribonuclease specific foruridylate (NendoU)], (vi) nsp16 [andribose-2´-O-methyltransferase (O-MT)], (vii) spike protein (S), and (viii) the nucleocapsid protein (N) (12). Phylogenetic trees of these data were estimated using the maximum likelihood (ML) method implemented in PhyML v3.0 (37), with bootstrap support values calculated from 1,000 replicate trees. The best-fit aa substitution models were determined using MEGA version 5 (38).

Recombination detection

The full genome alignment of all WESV sequences was screened for recombination using the RDP, GENECONV, BootScan methods available within the Recombination Detection Program, Version 4 (RDP4) (39). Only sequences with significant evidence (P<0.05) of recombination detected by at least two methods and confirmed by phylogenetic analysis were taken to represent strong evidence for recombination. In addition, we visualized the recombinant and the parental strains determined above using similarity plots analysis as implemented in Simplot version 3.5.1 (40), with a window size of 400 nucleotides (nt) and a step size of 40 nt.

Estimation of the numbers of synonymous and nonsynonymous substitutions.

The numbers of synonymous substitutions per synonymous site (d_S) and nonsynonymous substitutions per nonsynonymous site (d_N) for each coding region between each pair of WESV, BatCoV HKU2, PEDV , HCoV-NL63 strains were calculated using the Kimura 2-parameter method (Kimura 2-parameter) applied to synonymous and nonsynonymous sites as implemented in MEGA (v5) (38).

RESULTS

CoV identification in Asian house shrews.

During 2013-2015, a total of 266 Asian house shrews were captured in Zhejiang (214) and Jiangxi provinces (52), China (Figure 1). Species identification was based on morphological identification and amplification and subsequent sequencing of the mt-*cyt b* gene (3). An RT-PCR targeting a 440-bp fragment of the viral RdRp (RNA-dependent RNA polymerase) gene was performed to detect CoV RNA as described previously (18, 19). Viral RNA was identified in a total of 24 shrews, with an overall detection rate of 9.02%. The detection rate was 8.7% (2/23) in Ruian, 12.4% (12/97) in Wencheng, 10% (4/40) in Yudu, and 50% (6/12) in Xingguo, respectively. However, no CoV was detected in 94 Asian house shrews from Longwan. Genetic analysis revealed that these viruses were closely related each other with 87.8-100% nt similarity in the RdRp gene, and were generally most closely related to members of the genus *Alphacoronavirus* in the RdRp gene (65.6-72.8% nt similarity). However, they exhibited more than 35.3% nt difference from known alphacoronaviruses, suggesting that a novel CoV circulates in Asian house shrews. Finally, although rodents were also captured from the same geographic regions, no similar CoV was identified in these animals (data not shown).

Genomic features of the newly discovered shrew virus.

Since the newly discovered shrew CoV might represent a novel member of the genus *Alphacoronavirus*, seven complete genome sequences were recovered from the viral RNA positive samples collected in Wencheng (strains Wénchéng-554, Wénchéng-562 and Wénchéng-578), Ruian (Ruìān-90 and Ruìān-133), Yudu (Yúdū-76 and Yúdū-19), as well as two nearly complete genome sequences (Xīngguó-74 and Xīngguó-101) from Xingguo. Key features of these CoV sequences are described in Tables 1-2 and Figure 2. Genetic analysis revealed that the nt similarities among these viruses were 88.2%-99.9%. Generally, they shared 48.7-55.1% nt similarity with known alphacoronaviruses, and less than 57.1% nt similarity with other coronaviruses. Further comparison of the replicase domains [i.e. ADP-ribose 1''-phosphatase (ADRP), chymotrypsin-like protease (3CLpro), RdRp, helicase (Hel), 3'-to-5' exonuclease (ExoN), nidoviral endoribonuclease specific for uridylate (NendoU) and ribose-2'-*O*-methyltransferase (O-MT)] revealed more than 29.2% aa differences between the newly discovered shrew viruses and known alphacoronaviruses (Table S1). In addition, all phylogenetic analyses were consistent in showing that the newly discovered shrew viruses were distinct from the known alphacoronaviruses (see below). Therefore, these shrew viruses represent a novel member of the genus *Alphacoronavirus*: we have termed this Wénchéng shrew virus (WESV) according to its host and location of its first identification.

Excluding the polyadenylated tail at the 3'-terminus, the genomes of this novel virus varied from 25,986 to 26,026 nucleotides, with a lower G+C content (31.53-31.97%) than that of known alphacoronaviruses (34.46- 42.02%). The genome organization of WESV was similar to that of other alphacoronaviruses (Figure 2), showing the characteristic gene order: 5'-replicase ORF1ab, spike (S), envelope (E), membrane (M), and nucleocapsid (N)-3'. Remarkably, two additional ORFs coding for nonstructural (NS) proteins NS3 and NS7 were identified (Figure 1). In addition, a putative transcription regulatory sequence (TRS) motif (5'-CUAAAC-3'), similar to that in other alphacoronaviruses, was documented at the 3'end of the leader sequence and preceded each ORF except the S, NS3 and NS7 genes. An alternative TRS motif (5'-AACUAA-3') was discovered preceding the S gene in the shrew CoV genomes (Table 2). Finally, the putative mature nonstructural proteins (NSPs) within the ORF1ab encoding the replicase were calculated based on the cleavage and recognition pattern of the 3C-like proteinase (3CLpro) and papain-like proteinase (PLpro).

Like other alphacoronaviruses, the S protein of WESV was predicted to be a type I membrane glycoprotein, with most of the protein (residues 16 to 1080 or residues 16 to 1081) exposed on the outside of the virus. A transmembrane domain was located at residues 1081 to 1103 or residues 1082 to 1104) at the C terminus. However, WESV only shared 20.1-37.7% aa identity in the S protein with other members of the genus *Alphacoronavirus*, 20.0-25.0% aa identity with coronaviruses of remaining genera, but 34% aa identity with LRNV, which was sampled in rats collected from Lucheng district (a geographic neighbor of Wencheng and Ruian) of Wenzhou city (18), and two bat viruses (Rhinolophus bat coronavirus HKU2 and BtRf-AlphaCoV/YN2012) also sampled in China (41, NC_028824).

The ORF NS3 encodes a putative 237-aa nonstructural protein that is located between the S and E genes of WESV. Although the NS3 genes within the same geographic region were closely related to each other (96.2%-100%, 100%, 97.9% and 98.7% amino acid identities for the Wencheng, Ruian, Yudu and Xingguo strains, respectively), the difference among the WESVs from different regions reached 23.5% (Table 3). TMHMM analysis revealed there were two putative transmembrane domains in the WESV NS3, at residues 53-70 and 90-112 of the Wénchéng strains, at residues 49-71 and 91-113 in the Ruìān and Yúdū strains, and at residues 53-70 and 91-113 for the Xingguo strains. In addition, the NS3 gene of the WESV strains was longer than that of other alphacoronaviruses and distinct from those of known alphacoronaviruses and betacoronaviruses.

One of the most striking genomic features was the presence of an NS7 gene encoding a putative nonstructural protein of 136 aa residues located downstream of the N protein (Figure 2). Notably, at the aa level, the NS7 gene did not show homology to any known genes in GenBank. Additionally, although an ORF (or ORFs) downstream of the N gene was also reported in the genomes of some alphacoronaviruses, including BtKYNL63-9a, HKU8, TGEV, PRCV, HKU2 and BtCoV/512/2005, there was no sequence similarity in NS7 between WESV and these CoVs, indicative of markedly different origins.

Phylogenetic relationship between WESV and known coronaviruses.

To better understand the evolutionary relationship between WESV and other members of the genus *Alphacoronavirus*, we estimated phylogenetic trees based on the aa sequences of the non-structural and structural genes (Figures 3-5). In the RdRp tree (Figures 3A and 3B), WESV formed a distinct cluster that was separated from the other alphacoronaviruses by a relatively long branch. The WESV strains clearly clustered according to their geographic origins, indicative of the *in situ* evolution of WESVs in shrews (Figure 3C). However, although the Ruian and Wencheng strains were both sampled in Wenzhou, the Ruian strains were more closely related to those sampled from Ganzhou city (Jiangxi Province) than those from Wencheng.

A similar clustering pattern was observed in the trees estimated using the aa sequences of the non-structural genes (Figure 4) and the structural gene N (Figure 4). Even more striking was the phylogenetic tree of the S gene (Figure 5) in which WESV formed a divergent cluster with LRNV, HKU2 and BtRf-AlphaCoV/YN2012 that was genetically distinct from not only the genus *Alphacoronavirus*, but also from the other genera of coronaviruses, such that these are clearly genetically distinct members of the subfamily *Coronavirinae*. Within this cluster, the rat virus and two bat viruses shared common ancestry, with the WESVs again forming a distinct cluster.

Coronavirus recombination.

We performed recombination analyses of the genomes of Wencheng, Ruian, Yudu, and Xingguo strains using RDP4. Multiple methods supported statistically a significant recombination event in Wénchéng-578. From the similarity plot, two recombination breakpoints at bp position 5248 and 7663 of the sequence alignment (with reference to the Wénchéng-578 strain) were identified and separated the genome into three regions (Figure 6A). In turn, these could be grouped into two putative ‘parental regions’: region A (nt 5248 to 7663) and region B (nt 1 to 5247 and 7664 to the end of the sequence). In parental region A, the Wénchéng-578 virus had 98.1-98.2% sequence similarity to Ruiān-90 and 133 as opposed to 88.0% sequence similarity to

Wénchéng-554 and 562; in contrast, in parental region B they are more closely related to Wénchéng-554 and 562 (97.7-97.8% similarity) than to Ruìān-90 and 133 (89.1%). This recombination event was confirmed by phylogenetic analyses of the different parental regions and with high bootstrap values (Figure 6B).

Although readily apparent in the aa phylogenies, the recombination event between WESV and other (and/or unknown) coronaviruses did not receive significant statistical support in the RDP analysis and Similarity plot analysis (Figure 6C), likely because these nucleotide sequences are highly divergent (for example, the S gene of WESVs differs from those of alphacoronaviruses by 26.6%-62.6% at the nt level). Similar suggestions have been made with respect to the recombination involving Rhinolophus bat coronavirus HKU2 and Lucheng Rn rat coronavirus (18, 41).

Numbers of synonymous and nonsynonymous substitutions across the WESV genome.

An analysis of the numbers of synonymous and nonsynonymous substitutions per site (d_N/d_S) in the genome sequences of WESV and other alphacoronaviruses revealed relatively low d_N/d_S values reflecting of a predominance of purifying selection (Table 4). The exception was NS7 in which the far higher d_N/d_S ratio for WESV (0.514) was indicative of a markedly different selection pressure.

DISCUSSION

We describe a novel coronavirus, denoted Wénchéng shrew coronavirus (WESV), in shrews in four counties of Jiangxi and Zhejiang provinces, China. WESV was highly divergent to other alphacoronaviruses, exhibiting ≤ 71.1% aa similarity with any known members of the genus *Alphacoronavirus* in the coronavirus-wide conserved domains in the replicase polyprotein pp1ab, and less than 61.3% aa similarity from the other three coronavirus genera. The Coronaviridae Study Group of the International Committee on Taxonomy of Viruses (ICTV) have established the following genus and species demarcation criteria in the family *Coronaviridae*: coronaviruses that do not cluster together and share less than 46% sequence identity in the conserved replicase domains with any other established member are considered a new genus, while viruses that share more than 90% aa sequence identity in the conserved replicase domains are considered to belong to the same species (13). Hence, the virus harbored by Asian house shrew is sufficiently divergent that it should be considered as a distinct member of the genus *Alphacoronavirus*, although not a new genus under the current ICTV criteria.

Our analysis also reveals that WESV had a complex evolutionary history. Although WESVs exhibited distinct geographic clustering, indicative of *in situ* evolution, the evolutionary relationships among viruses sampled from four counties were not consistent with their geographic location. Such a phylogeographic pattern might reflect the influence of geographic barriers, such as mountains, rather than simple isolation-by-distance. In addition, that the S gene of WESV was divergent to all known coronaviruses suggests that an inter-genus recombination event may have occurred, and strong evidence for intra-species recombination was obtained. It is also striking that the WESVs possess a distinct NS7 gene. Although a gene named “ORF7” has been observed in the bat virus HKU8 (42), the NS7 gene of WESV exhibited no sequence similarity with HKU8 or any other known viruses, such that it has an unknown origin. In addition, the NS3 gene of WESV was genetically distinct from those of known alphacoronaviruses and betacoronaviruses.

Diverse alphacoronaviruses and betacoronaviruses have now been identified in a variety of bats globally (16, 17, 42-49), from which it has been proposed that alphacoronaviruses and betacoronaviruses in other animals have their ultimate ancestry in bats (21, 22). However, we observed that the WESVs harbored by shrews were phylogenetically distinct within the genus *Alphacoronavirus*, suggesting that they may have emerged early in Asian house shrews, and it is striking that WESV possesses an especially divergent S gene. Together, these results suggest that alphacoronaviruses have a far more complex evolutionary history than previously realized, with insectivores likely playing a more important role. Hence, greater effort is needed to infer the evolutionary history of alphacoronaviruses in a wider sample of mammalian species.

Shrews classified in the order Eulipotyphla have a broad geographic distribution and exhibit substantial diversity, rivalled only by members of the muroid families Muridae and Cricetidae and the bat family Vespertilionidae (25). Asian house shrews (*Suncus murinus*) have a wide distribution throughout the Old World tropics. However, unlike bats and rodents, these mammals have not attracted attention with respect to virus evolution, emergence and transmission. The recent discovery of Erinaceus coronavirus (EriCoV) in West European hedgehogs (*Erinaceus europaeus*) indicates that insectivores are the natural reservoir of CoV (50). Over the past decade, additional novel viruses have been identified in shrews (29-31), indicating that these animals may play an important role in the evolution and transmission of viruses including coronaviruses. WESV was identified in 24 of 266 shrews sampled from four counties of two provinces, with an overall detection rate of 9.02%, but not in rodents captured from same areas. Therefore, shrews appear to be a natural reservoir of coronaviruses such that their role in coronavirus evolution clearly merits further investigation.

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Figure legends

Figure 1. A map of China showing the location of trap sites in which shrews (red circular) were captured.

Figure 2. Schematic of the annotated WESV genome in comparison to representative alphacoronaviruses.

Figure 3. Maximum likelihood phylogenetic trees of the amino acids sequences encoding the putative RdRp protein. (A) WESV and other coronaviruses. (B) WESV and other alphacoronaviruses. (C) WESV only. Asterisks indicate well-supported nodes (>70% bootstrap support). The scale bar indicates the number of amino acid substitutions per site. The virus genomes used in this study and their GenBank accession numbers are: AlpacaCoV, Alpaca respiratory coronavirus isolate CA08-1/2008 (JQ410000); BatCoV CDPHE15, Bat coronavirus CDPHE15/USA/2006 (KF430219); BatCoV FJ2012, BtMf-AlphaCoV/FJ2012 (KJ473799); BatCoV YN2012, BtRf-AlphaCoV/YN2012 (KJ473808); BatCoV HuB2013, BtRf-AlphaCoV/HuB2013 (KJ473807); CamelCoV, Camel alphacoronavirus isolate camel/Riyadh/Ry141/2015 (KT368907); CCoV K378, Canine coronavirus strain K378 (KC175340); FCoV C1Je, Feline coronavirus strain FCoV C1Je (DQ848678); BatCoV HKU2, Bat coronavirus HKU2 strain HKU2/GD/430/2006 (EF203064); BatCoV HKU8, Bat coronavirus HKU8 strain AFCD77 (EU420139); HCoV-229E, Human coronavirus 229E (AF304460); HCoV-NL63, Human Coronavirus NL63 (AY567487); BatCoV JTAC2, Bat coronavirus JTAC2 (KU182966); LRNV, Lucheng Rn rat coronavirus isolate Lucheng-19 (KF294380); BatCoV 1A, Bat coronavirus 1A strain AFCD62 (EU420138); MCoV, Mink coronavirus strain WD1127 (HM245925); PEDV, Porcine epidemic diarrhea virus isolate

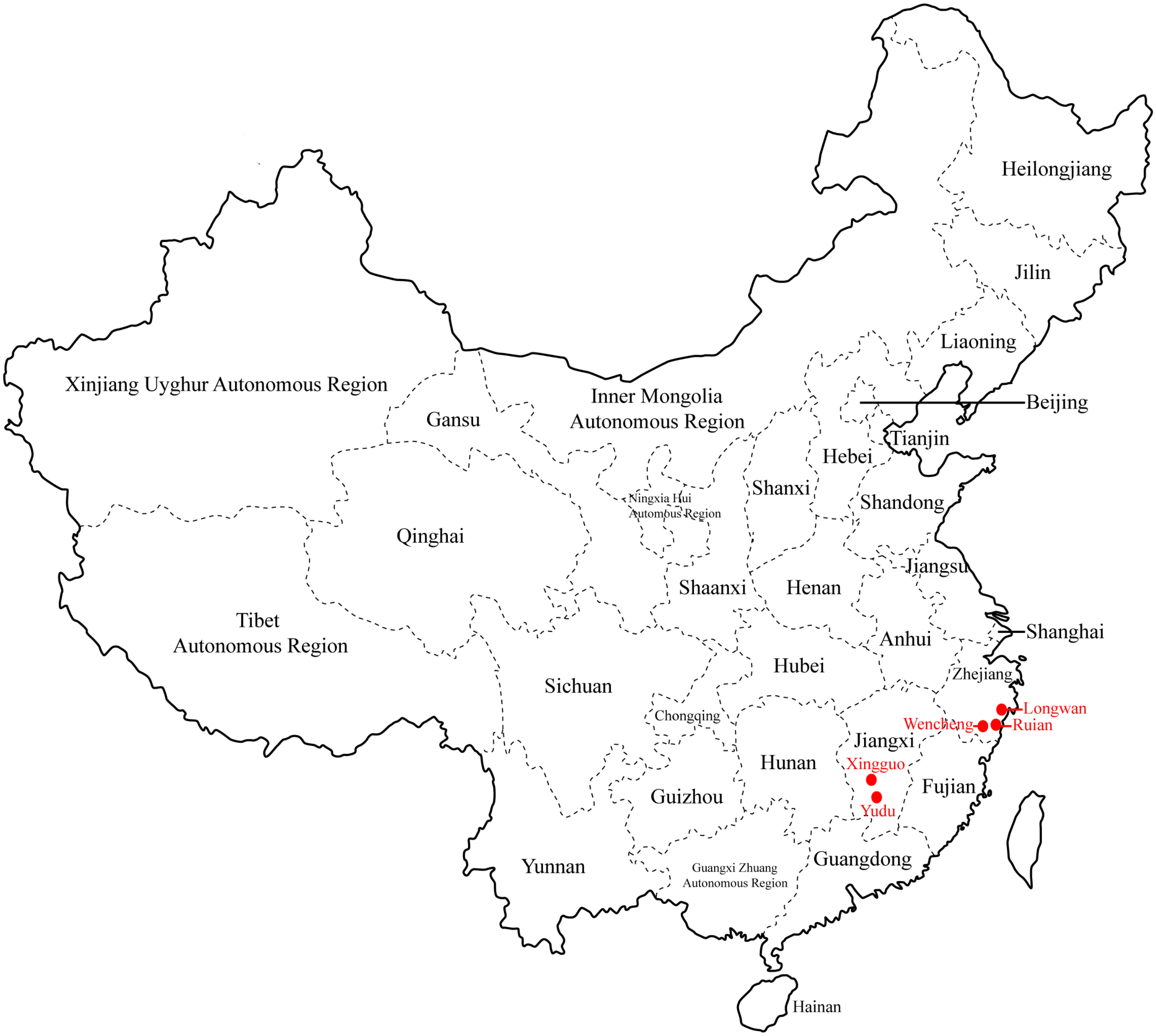
ZJU/G1/2013 (KU664503); BatCoV HKU10, Rousettus bat coronavirus HKU10 isolate 183A (JQ989270); BatCoV SAX2011, BtMr-AlphaCoV/SAX2011 (KJ473806); BtCoV/512/2005, Scotophilus bat coronavirus 512 (DQ648858); TGEV, Transmissible gastroenteritis virus virulent Purdue (DQ811789); BatCoV Zhejiang2013, Bat Hp-betacoronavirus/Zhejiang2013 (KF636752); MERS-CoV, Human betacoronavirus 2c EMC/2012 (JX869059); HCoV-HKU1, Human coronavirus HKU1 (AY597011); BatCoV HKU9, Bat coronavirus HKU9 (EF065513); SARS-CoV, SARS coronavirus WH20 (AY772062); BuCoV HKU11, Bulbul coronavirus HKU11-934 (FJ376619); PorCoV HKU15, Porcine coronavirus HKU15 strain HKU15-44 (JQ065042); MRCoV HKU18, Magpie-robin coronavirus HKU18 strain HKU18-chu3 (JQ065046); WiCoV HKU20, Wigeon coronavirus HKU20 strain HKU20-9243 (JQ065048); AIBV-Beaudette, Avian infectious bronchitis virus Beaudette (NC_001451); DKCoV, Duck coronavirus isolate DK/CH/HN/ZZ2004 (JF705860); BWCoV SW1, Beluga Whale coronavirus SW1 (EU111742); TCoV, Turkey coronavirus isolate TCoV-ATCC (EU022526).

Figure 4. Maximum likelihood phylogenetic trees of the amino acid sequences encoding the putative 3CLpro (nsp5), Hel (nsp13), ExoN (nsp14), NendoU (nsp15), O-MT (nsp16), and N protein of WESV and other CoVs. Asterisks indicate well-supported nodes (>70% bootstrap support). For clarity, asterisks indicate well-supported nodes (>70%). The scale bar indicates the number of amino acid substitutions per site. The virus genomes used are the same as those shown in Figure 3.

Figure 5. Maximum likelihood phylogenetic tree of the amino acids sequences encoding the putative S protein of WESV and other coronaviruses. Asterisks indicate well-supported nodes (>70% bootstrap support). The scale bar indicates the number of amino acid substitutions per site.

The virus genomes used are the same as those shown in Figure 3.

Figure 6. Recombination analysis of the WESV genome. A sequence similarity plot (A) reveals two recombination break-points with their locations shown by the red numbers, on the x-axis. The plot shows genome scale similarity comparisons of the Wénchéng-578 sequence (query) against Wénchéng-554 and 562 (parental group 1, red) and Ruìān-90 and 133 (parental group 2, blue). The background color of parental region A is gray, while that of parental region B is white. (B) Phylogenies of parental region A (nt 5248 to 7663) and region B (nt 1to 5247 and 7664 to the end of the sequence) are shown below the similarity plot. Numbers (>70) above or below branches indicate percentage bootstrap values. (C) Recombination analyses of the Wénchéng-554 and other known alphacoronaviruses.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Shandong
Qinghai
Jiangsu
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Zhejiang
Sichuan
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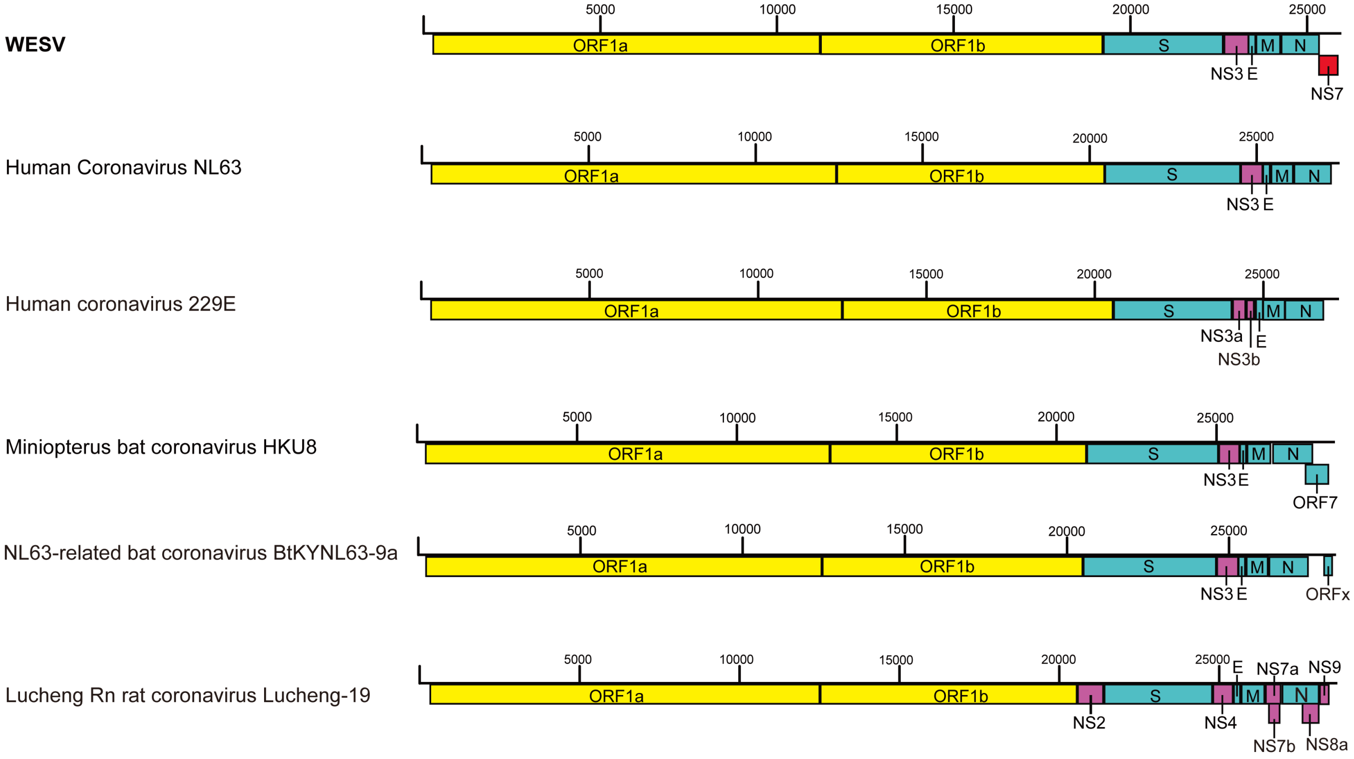
WESV
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20000
25000
ORF1a
ORF1b
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M
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NS3
E
NS7
Human Coronavirus NL63
5000
10000
15000
20000
25000
ORF1a
ORF1b
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Human coronavirus 229E
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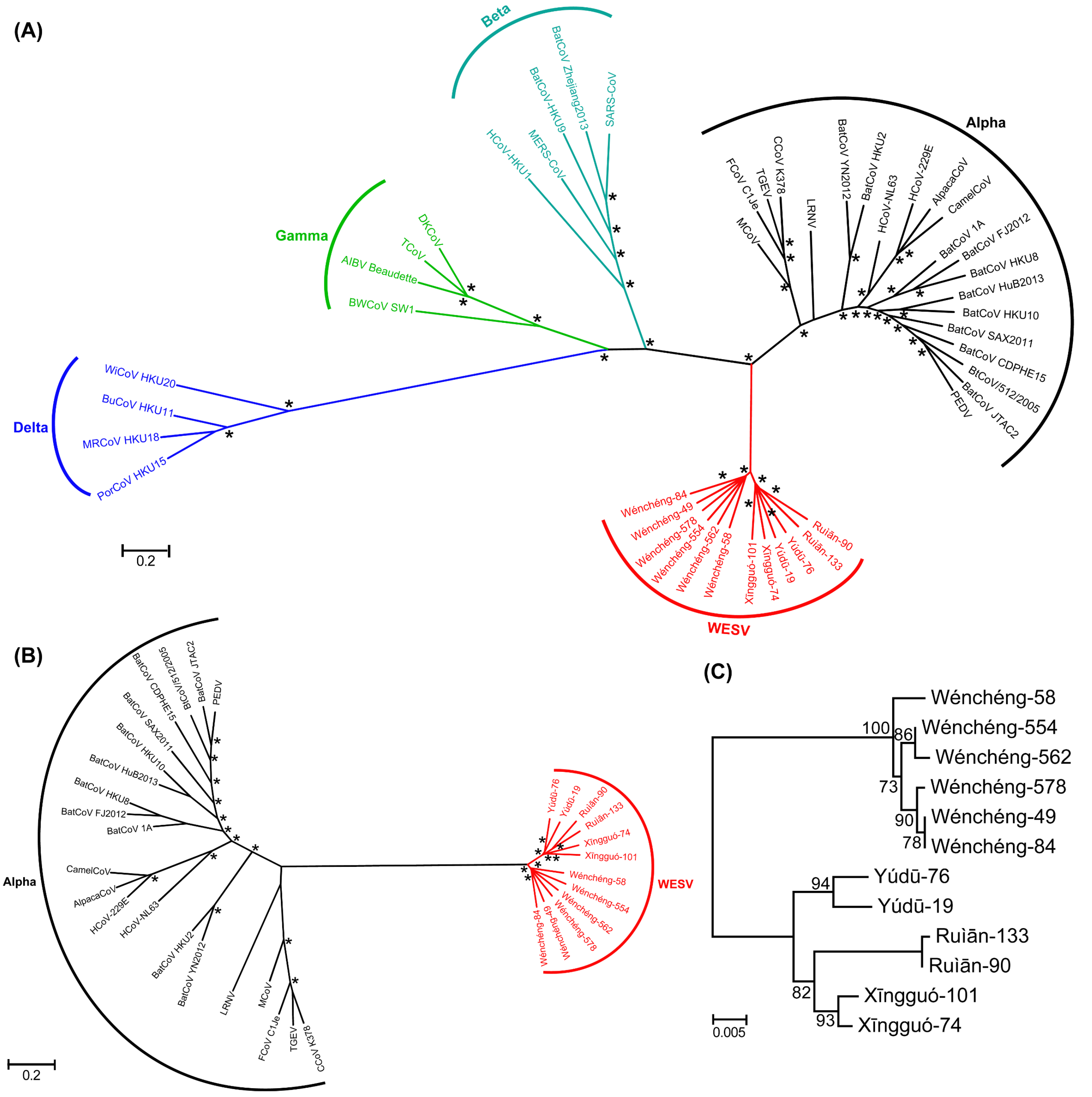
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DKCoV
TCoV
AIBV Beaudette
BWCoV SW1
Delta
WiCoV HKU20
BuCoV HKU11
MRCoV HKU18
PorCoV HKU15
0.2
Alpha
FCoV C1Je
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CCoV K378
MCoV
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BatCoV YN2012
BatCoV HKU2
HCoV-NL63
HCoV-229E
AlpacaCoV
CamelCoV
BatCoV 1A
BatCoV FJ2012
BatCoV HKU8
BatCoV HuB2013
BatCoV HKU10
BatCoV SAX2011
BatCoV CDPHE15
BtCoV/512/2005
BatCoV JTAC2
PEDV
Wénchéng-84
Wénchéng-49
Wénchéng-578
Wénchéng-554
Wénchéng-562
Wénchéng-58
Xīngguó-101
Xīngguó-74
Yúdū-19
Yúdū-76
Ruìān-133
Ruìān-90
WESV
(B)
BatCoV CDPHE15
BtCoV/512/2005
BatCoV JTAC2
PEDV
BatCoV SAX2011
BatCoV HKU10
BatCoV HuB2013
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BatCoV 1A
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CamelCoV
AlpacaCoV
HCoV-229E
HCoV-NL63
BatCoV HKU2
BatCoV YN2012
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Ruìān-133
Xīngguó-74
Xīngguó-101
Wénchéng-58
Wénchéng-554
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Wénchéng-554
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Yúdū-76
Yúdū-19
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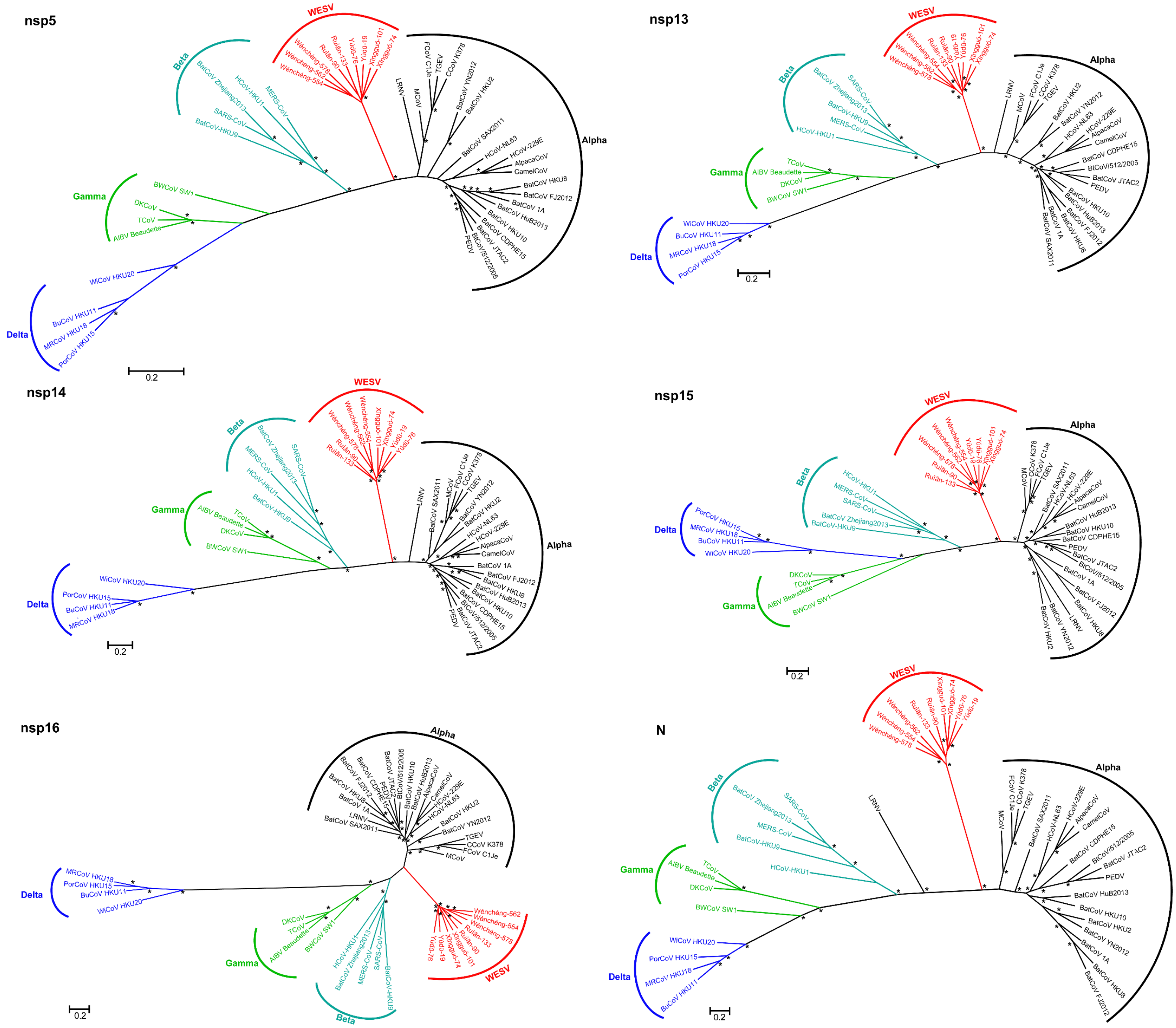
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Yùdū-19
Xìngguó-101
Xìngguó-74
LRNV
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FCoV C1Je
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CCoV K378
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Beta
Gamma
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Beta
Gamma
Delta
0.2
nsp15
WESV
Alpha
Beta
Gamma
Delta
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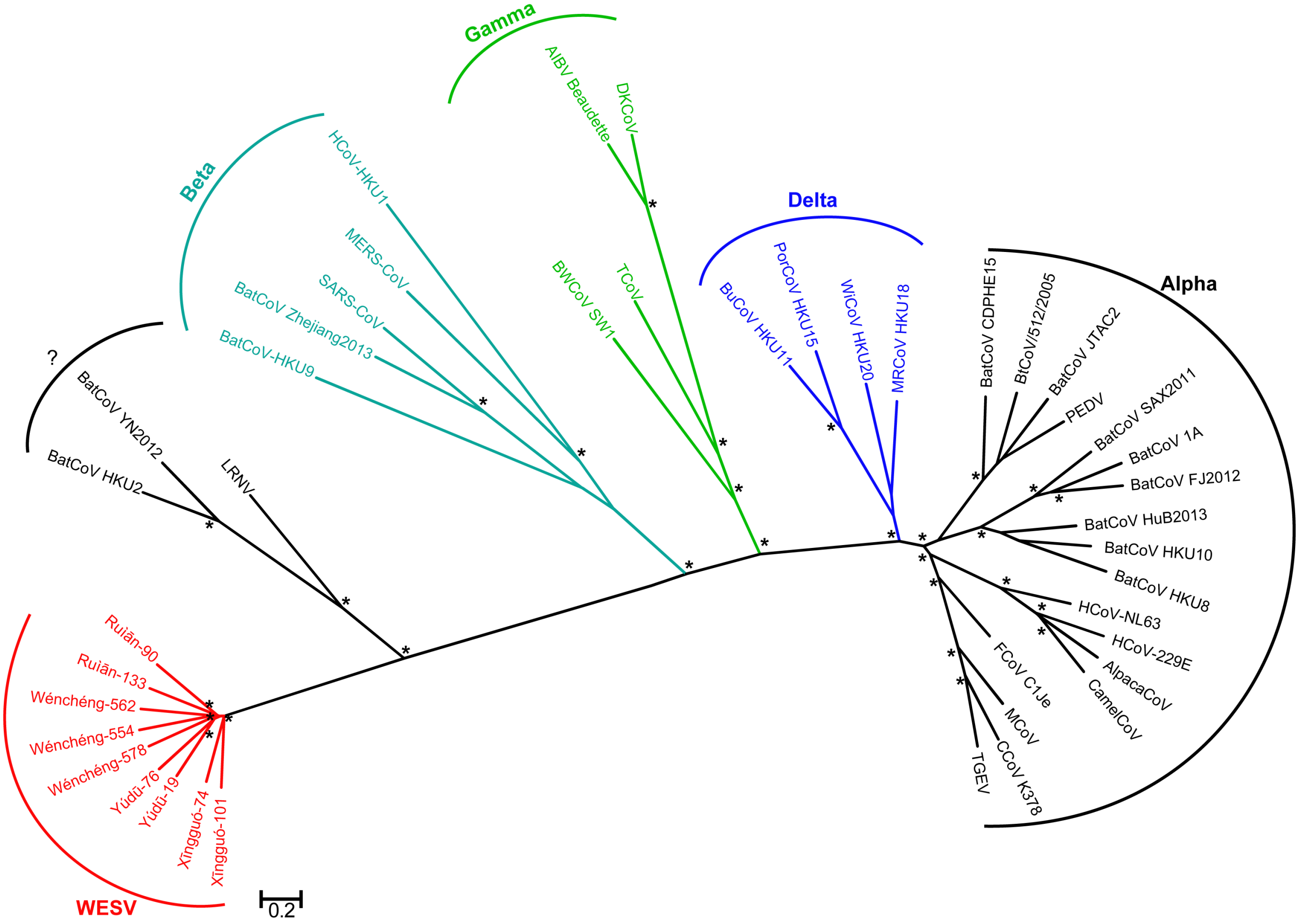
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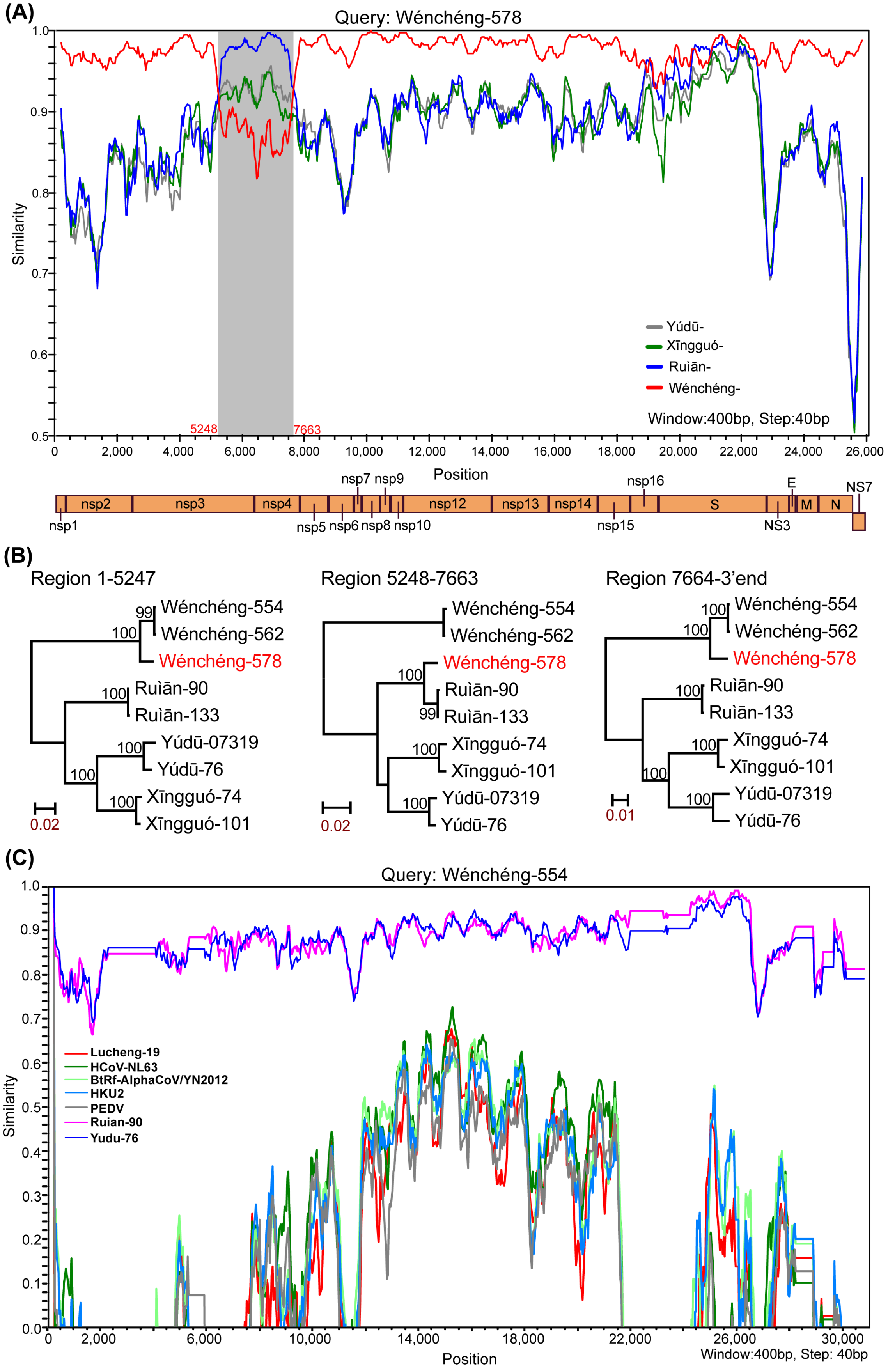
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nsp8
nsp10
nsp15
NS3
(B)
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100 Wénchéng-562
Wénchéng-578
100 Ruìān-90
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Yúdū-76
100
100 Xīngguó-74
Xīngguó-101
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Wénchéng-562
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Ruìān-90
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Yúdū-76
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100 Wénchéng-562
Wénchéng-578
100 Ruìān-90
Ruìān-133
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100
100 Yúdū-07319
Yúdū-76
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HCoV-NL63
BtRf-AlphaCoV/YN2012
HKU2
PEDV
Ruian-90
Yudu-76
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14,000
18,000
22,000
26,000
30,000
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Table 1. Key features of WESV strains with complete or nearly complete genome sequences.

Strain	Genomes size	Gender of host	Sampling year	Sampling location
Wénchéng-554	26028 nt	♂	2014	Wencheng
Wénchéng-562	26028 nt	♀	2014	Wencheng
Wénchéng-578	26028 nt	♀	2014	Wencheng
Ruìān-90	26042 nt	♂	2014	Ruian
Ruìān-133	26041 nt	♀	2014	Ruian
Yúdū-76	26002 nt	♂	2014	Yudu
Yúdū-19	26031 nt	♂	2015	Yudu
Xīngguó-101*	25995 nt	♂	2015	Xingguo
Xīngguó-74*	25984bp	♂	2015	Xingguo

* strains with nearly complete genome sequences.

Table 2. Coding potential and putative transcription regulatory sequences of the Wénchéng-562, Ruìān-90 and Yúdū-76 viruses

Coronavirus	ORF	Location (nt)	Length (nt)	Length (aa)	TRS location	TRS sequence
Wénchéng -562	ORF1ab	266-19233 (shift at 11239)	18,968	6,322	72-77	CUAAAC(188)AUG
	S	19240-22644	3,405	1,134	19233-19238	AACUAA(1)AUG
	NS3	22644-23357	714	237		
	E	23338-23565	228	75	23313-23318	CUAAAC(19)AUG
	M	23578-24267	690	229	23569-23574	CUAAAC(3)AUG
	N	24271-25368	1,098	365	24264-24269	CUAAAC(1)AUG
	NS7	25355-25762	408	135		
Ruìān-90	ORF1ab	265-19241 (shift at 11247)	18,977	6,325	71-76	CUAAAC(188)AUG
	S	19248-22652	3,405	1,134	19241-19246	AACUAA(1) AUG
	NS3	22652-23365	714	237		
	E	23346-23573	228	75	23321-23326	CUAAAC(19)AUG
	M	23586-24275	690	229	23577-23582	CUAAAC(3)AUG
	N	24279-25379	1,101	366	24272-24277	CUAAAC(1)AUG
	NS7	25366-25773	408	135		
Yúdū-76	ORF1ab	266-19200 (shift at 11206)	18,935	6,311	72-77	CUAAAC(188)AUG
	S	19207-22614	3,408	1,135	19200-19205	AACUAA(1) AUG
	NS3	22614-23327	714	237		
	E	23308-23535	228	75	23283-23288	CUAAAC(19)AUG
	M	23548-24237	690	229	23539-23544	CUAAAC(3)AUG
	N	24241-25341	1,101	366	24234-24239	CUAAAC(1)AUG
	NS7	25328-25735	408	135		

Table 3. Comparison of the NS3 genes between WESV and alphacoronaviruses.

Virus	Size	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Xīngguó-101	714bp	***	99.6	89.9	90.2	91.5	91.5	80.7	80.3	80.8	43.9	43.9	46.4	39.2	39.5
2. Xīngguó-74	714bp	98.7	***	89.8	90.1	91.3	91.3	80.5	80.3	80.7	43.8	44.1	46.4	38.9	39.8
3. Yúdū-76	714bp	89.9	89.9	***	98.3	93.4	93.4	80.3	79.4	80.4	43.9	42.9	46.3	40.2	39.8
4. Yúdūu-19	714bp	90.3	90.3	97.9	***	93.7	93.7	80.5	80.0	80.7	43.8	42.9	46.4	40.2	39.7
5. Ruìān-133	714bp	90.8	90.8	94.5	94.1	***	100	81.0	79.8	81.1	43.8	43.7	47.2	40.6	41.0
6. Ruìān-90	714bp	90.8	90.8	94.5	94.1	100.0	***	81.0	79.8	81.1	43.8	43.7	47.2	40.6	41.0
7. Wénchéng -554	714bp	79.0	78.6	76.5	76.9	79.0	79.0	***	96.6	99.9	44.7	42.8	45.2	40.2	40.1
8. Wénchéng -578	714bp	77.7	77.3	75.2	76.5	77.3	77.3	96.2	***	96.5	44.7	42.6	45.1	40.6	39.4
9. Wénchéng -562	714bp	79.0	78.6	76.5	76.9	79.0	79.0	100.0	96.2	***	44.5	42.6	45.2	40.2	40.1
10. BatCoV HKU2	690bp	20.3	20.3	19.4	18.9	21.1	21.1	19.8	19.4	19.8	***	53.0	53.6	50.7	36.5
11. Lucheng-19	645bp	23.3	23.3	21.4	21.4	23.3	23.3	21.9	21.4	21.9	31.6	***	49.3	46.9	36.8
12. HCoV-NL63	678bp	22.7	22.7	21.8	21.3	23.6	23.6	22.2	22.2	22.2	41.8	33.2	***	47.3	44.3
13. PEDV	675bp	19.3	19.3	19.7	19.3	21.1	21.1	20.2	21.1	20.2	35.1	29.4	34.8	***	33.9
14. BatCoV HKU9	663bp	13.6	13.6	14.1	13.2	13.6	13.6	13.2	12.3	13.2	11.6	10.6	8.5	9.5	***

Note: Percent identities for nucleotide (above the diagonal) and amino acid (below the diagonal) sequences are presented.

Table 4. Comparison of the mean numbers of nonsynonymous and synonymous substitutions per site, and their ratio, in the coding regions of WESV, BatCoV HKU2, PEDV and HCoV-NL63.

Gene	**WESV (N=9)**			**BatCoV HKU2 (N=5)**			**PEDV (N=7)**			**HCoV-NL63 (N=6)**		
	d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S
nsp1	0.090	0.418	0.215	0.014	0.085	0.165	0.012	0.026	0.462	0.006	0.031	0.194
nsp2	0.075	0.365	0.205	0.022	0.154	0.143	0.010	0.051	0.196	0.006	0.023	0.261
nsp3	0.058	0.245	0.237	0.038	0.233	0.163	0.009	0.040	0.225	0.006	0.017	0.353
nsp4	0.043	0.297	0.145	0.009	0.101	0.089	0.005	0.048	0.104	0.002	0.020	0.100
nsp5	0.034	0.317	0.107	0.005	0.061	0.082	0.007	0.038	0.184	0.001	0.013	0.077
nsp6	0.073	0.280	0.261	0.005	0.136	0.037	0.004	0.046	0.087	0.002	0.009	0.222
nsp7	0.033	0.254	0.130	0.000	0.166	-	0.002	0.042	0.048	0.002	0.006	0.333
nsp8	0.018	0.248	0.073	0.009	0.153	0.059	0.001	0.036	0.028	0.001	0.012	0.083
nsp9	0.039	0.369	0.106	0.005	0.204	0.025	0.000	0.044	-	0.000	0.013	-
nsp10	0.016	0.275	0.058	0.010	0.099	0.101	0.001	0.029	0.034	0.000	0.043	-
nsp11	0.040	0.124	0.323	0.000	0.000	-	0.000	0.029	-	0.000	0.040	-
nsp12	0.018	0.240	0.075	0.002	0.097	0.021	0.007	0.043	0.163	0.001	0.008	0.125
nsp13	0.021	0.243	0.086	0.001	0.097	0.010	0.002	0.053	0.038	0.000	0.007	-
nsp14	0.032	0.305	0.105	0.003	0.041	0.073	0.002	0.066	0.030	0.001	0.012	0.083
nsp15	0.032	0.225	0.142	0.003	0.065	0.046	0.006	0.062	0.097	0.001	0.005	0.200
nsp16	0.029	0.207	0.140	0.002	0.075	0.027	0.005	0.043	0.116	0.000	0.014	-
S	0.039	0.093	0.419	0.067	0.407	0.165	0.023	0.089	0.258	0.007	0.041	0.171
NS3	0.085	0.383	0.222	0.022	0.267	0.082	0.009	0.032	0.281	0.001	0.020	0.050
E	0.045	0.342	0.132	0.009	0.088	0.102	0.011	0.059	0.186	0.000	0.029	-
M	0.032	0.318	0.101	0.007	0.137	0.051	0.008	0.032	0.250	0.006	0.016	0.375
N	0.056	0.338	0.166	0.036	0.260	0.138	0.011	0.068	0.162	0.004	0.016	0.250
NS7	0.242	0.471	**0.514**	-	-	-	-	-	-	-	-	-
NS7a	-	-	-	0.050	0.190	0.263	-	-	-	-	-	-