Received: 4 September 2020 | Revised: 17 December 2020 | Accepted: 5 February 2021

DOI: 10.1111/tbed.14029

ORIGINAL ARTICLE

Transboundary and Emerging Diseases WILEY

Epidemiology and evolution of novel deltacoronaviruses in birds in central China

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Funding information
National Key Research and Development Program of China, Grant/Award Number: 2017YFD0500104; National Natural Science Foundation of China, Grant/Award Number: 32041001 and 81902070; Provincial Natural Science Foundation of Hunan Province, Grant/Award Number: 2019JJ20004 and 2019JJ50035

Abstract

The variety and widespread of coronavirus in natural reservoir animals is likely to cause epidemics via interspecific transmission, which has attracted much attention due to frequent coronavirus epidemics in recent decades. Birds are natural reservoir of various viruses, but the existence of coronaviruses in wild birds in central China has been barely studied. Some bird coronaviruses belong to the genus of *Deltacoronavirus*. To explore the diversity of bird deltacoronaviruses in central China, we tested faecal samples from 415 wild birds in Hunan Province, China. By RT-PCR detection, we identified eight samples positive for deltacoronaviruses which were all from common magpies, and in four of them, we successfully amplified complete deltacoronavirus genomes distinct from currently known deltacoronavirus, indicating four novel deltacoronavirus stains (HNU1-1, HNU1-2, HNU2 and HNU3). Comparative analysis on the four genomic sequences showed that these novel magpie deltacoronaviruses shared three different S genes among which the S genes of HNU1-1 and HNU1-2 showed 93.8% amino acid (aa) identity to that of thrush coronavirus HKU12, HNU2 S showed 71.9% aa identity to that of White-eye coronavirus HKU16, and HNU3 S showed 72.4% aa identity to that of sparrow coronavirus HKU17. Recombination analysis showed that frequent recombination events of the S genes occurred among these deltacoronavirus strains. Two novel putative cleavage sites separating the non-structural proteins in the HNU coronaviruses were found. Bayesian phylogeographic analysis showed that the south coast of China might be a potential origin of bird deltacoronaviruses existing in inland China. In summary, these results suggest that common magpie in China carries diverse deltacoronaviruses with novel genomic features, indicating an important source of environmental coronaviruses closed to human communities, which may provide key information for prevention and control of future coronavirus epidemics.

KEYWORDS
bird deltacoronavirus, common magpie, genome recombination, spike, viral genome

Qiong Wang and Zhi-Jian Zhou contributed equally.

1 | INTRODUCTION

Epidemics caused by viruses have been threatening the public health throughout the entire human history. Most human epidemic viruses originate from animal reservoirs and are transmitted to human by various animals serving as intermediate hosts. For instance, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the pathogenic virus of the recent COVID-19 pandemic, is likely to originate from bats (Huang et al., 2020; Lu et al., 2020; Zhou, Yang, et al., 2020; Zhou, et al. 2020), but the infectious SARS-CoV-2 can be detected in a variety of animals, including cats (Sailleau et al., 2020), dogs (Sit et al., 2020) and other domesticated animals (Shi et al., 2020), which may act as the intermediate hosts bridging bats and humans. Similarly, MERS-CoV and SARS-CoV, the pathogenic viruses causing previous coronavirus (CoV) pandemics, were also reported to be transmitted from wild animals to humans through intermediate hosts (Forni et al., 2017; Shi & Hu, 2008; Su et al., 2016; Woo et al., 2006; Zhong et al., 2003;). Actually, the interspecies transmission of viruses from natural reservoir animals is a major cause for viral epidemics (Niederwerder & Hesse, 2018; Xu et al., 2018; Zhou et al., 2018). Therefore, monitoring the existence of potentially pathogenic viruses in animals is critical for predicting and preventing viral epidemics.

CoVs are enveloped, single-stranded, positive-sense ssRNA viruses belonging to the subfamily *Orthocoronavirinae* of the family *Coronaviridae* in the suborder *Cornidovirineae* of the order *Nidovirales*. CoVs are known to infect a wide variety of animals including humans, causing enteric, reproductive, respiratory and gastrointestinal diseases (Brian & Baric, 2005; Lin et al., 2016; Moreno et al., 2017). Currently, International Committee on Taxonomy of Viruses (ICTV) divides *Orthocoronavirinae* into four genera: *Alpha-*, *Beta-*, *Gamma-* and *Delta-CoV*. The members of *Alpha-* and *Beta-CoV* infect bats, humans and other mammals, whereas the ones of *Gamma-* and *Delta-CoV* mainly infect birds but a few of them infect mammals (Dong et al., 2007; Lau et al., 2015; Wong et al., 2019; Woo et al., 2014).

Delta-CoV is a novel genus of CoV discovered in recent years that can infect birds and mammals. In 2009, three novel avian coronaviruses were identified, including bulbul coronavirus HKU11 (BuCoV HKU11), thrush coronavirus HKU12 (ThCoV HKU12) and munia coronavirus HKU13 (MunCoV HKU13). According to their phylogenetic relationships and genomic structures, ICTV approved the classification of these three avian CoVs as a novel genus, *Deltacoronavirus* (Woo et al., 2009). Subsequently, in 2012, molecular epidemiological investigations in Hong Kong, China, revealed seven novel deltacoronaviruses, including six from birds and one from swine. Among them, porcine deltacoronavirus (PDCoV) HKU15 was the first deltacoronavirus found to infect non-avian animals (Woo et al., 2012). Recently, four bird deltacoronaviruses, (falcon CoV UAE-HKU27, houbara CoV UAE-HKU28, pigeon CoV UAE-HKU29 and quail CoV UAE-HKU30), four novel sparrow CoVs and one quail CoV were found in the Middle East, the United States and Poland, respectively (Chen et al., 2018; Katarzyna et al., 2019; Lau et al., 2018). These findings suggested that deltacoronaviruses have the potential for Avian-to-Avian and Avian-to-Mammalian transmission, which suggest that the terrestrial birds may serve as the intermediate host for the transmission of CoV.

Birds are warm-blooded vertebrates with high species biodiversity, featured behaviours (e.g. nesting and migration) and unique adaptive immune systems (Lee et al., 2014). Birds are the reservoir of major emerging viruses, including influenza virus, coronavirus and adenovirus (Chan et al., 2013, 2015). In this study, we tested samples from various avian species in the Hunan, a province located in central China. Based on the results of comparative genome and phylogenetic analyses, we propose four novel deltacoronavirus strains in common magpies.

2 | MATERIALS AND METHODS

2.1 | Sample collection

415 faecal samples of birds were collected in several areas of central China, including East Dongting Lake, Changde national wetland, Hengyang, in the Hunan Province over a 2-month period (April 2018–June 2018). The samples were placed in virus transport medium (VTM) and kept in dry ice for transportation to the laboratory and stored at −80°C until use.

2.2 | RNA extraction, PCR screening and sequencing

Viral RNA was extracted from the faecal samples using TIANamp Virus DNA/RNA Kit (Tiangen, China) following the manufacturer's instructions. RNA was eluted in 60 μl of RNase-free water, aliquoted and stored at −80°C. For screening the presence of bird deltacoronaviruses, a set of primers to amplify a 440-bp fragment of the RNA-dependent RNA polymerase (RdRp) gene of CoVs using *Deltacoronavirus* conserved primers DCoVF (5′-GTGGVTGTMTTAATGCACAGTC-3′) and DCoVR (5′-TACTGYCTGTTRGTCATRGTG-3′) as described previously (Lau et al., 2018). PCR amplification was performed using the PrimeScript™ One Step RT-PCR Kit Ver.2 (Takara). The PCR mixture (25 μL) contained 2 μL of extracted RNA, 12.5 μL 2 × 1 Step Buffer and 1 μL PrimeScript 1 Step Enzyme Mix. The mixtures were amplified by 60 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 10 min.

The PCR products were gel-purified and sequenced with an ABI Prism 3,700 DNA analyzer (Applied Biosystems), using the PCR primers. The sequences of the PCR products were compared with known sequences of the RdRp genes of CoVs in the GenBank database.

2.3 | Complete genome sequencing

Four complete genomes of common magpie CoV were amplified and sequenced using the RNAs extracted from birds faecal as templates. The RNA was amplified with degenerate primers designed

by multiple alignments of the genomes of other CoVs with complete genomes available, using the PrimeScript™ One Step RT-PCR Kit Ver.2. Additional primers were designed from the results of the first and subsequent rounds of sequencing. Sequences of the 5′ and 3′ genomic ends were obtained by 5′ and 3′ RACE (Roche), respectively. PCR products with expected size were gel-purified and subjected directly to sequencing. Sequences were assembled to obtain the full-length genome sequences.

The sequences obtained in this research have been deposited in GenBank with the accession numbers: MW345814(HNU1-1), MW345815(HNU1-2), MW 349841(HNU2), MW345816(HNU3), MW345810(CD1), MW 345811(CD2), MW345812(CD3), MW345813(CD4).

2.4 | Genome and phylogenetic analysis

Gene sequences and encoded putative protein amino acid (aa) sequences were compared to those of other CoVs using ORF finder (https://www.ncbi.nlm.nih.gov/orffifinder/). Sequence alignment was performed by using MAFFT v7.149 (Katoh & Standley, 2013). Phylogenetic trees were constructed using IQ-tree v1.6.10, with 10,000 ultrafast bootstraps and the most appropriate model of aa substitution for each data sets was calculated by ModelFinder according to the corrected Akaike information criterion (Nguyen et al., 2015; Zhang et al., 2020).

2.5 | Recombinant analysis

The preliminary identification of possible recombination events in our sample sequences using RDP v3.44 with seven different scanning methods, including RDP, GENECONV, BOOTSCAN, MAXCHI, CHIMAERA, SISCAN and 3SEQ (Martin et al., 2010). In order to minimize the existence of false positives, we used a Bonferroni correction and a highest acceptable p-value = 0.05. At last, we used the Simplot v3.5.1 program to confirm the recombination events identified by RDP3 suite.

2.6 | Discrete traits of Bayesian Phylogeographic analysis

We collected 20 bird deltacoronavirus sequences from GenBank, together with 4 samples that we amplified, a total of 24 bird deltacoronavirus sequences were from five different regions, Hong Kong (n = 10), United Arab Emirates (n = 5), USA (n = 4), Poland (n = 1) and Hunan (n = 4). To understand the spatial dynamics of bird deltacoronavirus, the above five regions were coded as discrete states and the phylogeographic analysis with RdRp gene to reconstruct ancestral geographical regions and migration patterns in BEAST v1.10.4 (Lemey et al., 2009). The marginal likelihood estimated by path sampling was used to compare the combinations of molecular clock models and coalescent models, and the results showed that the best-fitting tree prior for our data set was the combination of strict clock and Bayesian skyline coalescent (Table S5) (Baele et al., 2012; Drummond et al., 2005). According to the latest report of the evolution rate on RdRp gene of deltacoronavirus, we used a uniformly distributions priori value (from 5×10^{-5} to 2×10^{-4} subs/site/year) of evolution rate in our analysis (Lau et al., 2018). Subsequently, phylogeographic analyses were performed by Bayesian Stochastic Search Variable Selection (BSSVS) combined with an asymmetric substitution model, running a markov chain of 40 million steps with sampling every 1,000 generations in BEAST. By discarding the first 10% of the samples, the remaining samples were used Tracer v1.7 to check the Effective Sample Size (ESS) of all estimated parameters, ensuring that there were enough ESS values (greater than 200) to achieve convergence (Rambaut et al., 2018). The Maximum Clade Credibility (MCC) tree was obtained by discarding the first 10% of trees in Tree Annotator package. The Bayes factors (BF) were calculated using SPREAD3 package to select significant migration pathways (BF > 3) among discrete locations.

3 | RESULT

3.1 | Prevalence of novel deltacoronaviruses in birds

The faecal samples for the deltacoronaviurs detection were from 415 birds collected in the Hunan province, China. These birds belonged to at least 26 species of 15 families. The presence of deltacoronaviruses was detected by RT-PCR targeting a 440-nt RNA-dependent RNA polymerase (RdRp) fragment that is conserved among all known deltacoronaviruses. In total, eight samples were positive for deltacoronavirus. Host species identification by amplification of either *Cytb* or 12S rRNA gene suggested that all deltacoronavirus-positive samples were exclusively from common magpie (family *Corvidae*) (Dinh et al., 2019; Wang et al., 2015) (Table 1). Preliminary analysis of the partial RdRp sequences suggested that all the eight sequences showed high similarity among themselves and with other reported deltacoronaviruses from swine and birds (Figure 1 and Figure S1). However, attempts to passage novel HNU CoV in cell cultures and embryonated chicken were failed, with no cytopathic effect or viral replication being detected.

3.2 | Genomic characterization of four novel common magpie deltacoronaviruses

To further understand the genetic diversity and structure of these bird deltacoronaviruses, complete genomic sequences of four novel common magpie CoVs (HNU1-1, HNU1-2, HNU2, HNU3) were successfully amplified with genome size ranging from 26,208 nt to 26,289 nt and G + C content ranging from 41.7% to 41.9%. The HNU1-1 and HNU1-2 genomes shared 99.8% nucleotide identity to each other but showed 93.2% and 92.7% identity to the stains HNU2 and HNU3, respectively. The identity between these CoVs and other avian CoVs was low (Tables S1 and S2). However, the genome organization of HNU CoVs was similar to other deltacoronaviruses and had the typical gene

TABLE 1 Prevalence of deltacoronavirus in birds

Animal type and scientific name	Common name	No. (%) of birds positive for deltacoronavirus	Sampling location
Ardeidae			
Ardeola bacchus	Chinese pond heron	0/13	b
Columbidae			
Streptopelia chinensis	Spotted dove	0/1	c
Streptopelia orientalis	Oriental turtle dove	0/7	b
Corvidae			
Pica pica	**Common magpie**	**8/28 (0.28)**	**b*,c**
Emberizidae			
Emberiza sulphurata	Yellow bunting	0/1	a
Emberiza tristrami	Tristram's Bunting	0/7	a
Hirundinidae			
Tachycineta euchrysea	Golden Swallow	0/2	a
Progne chalybea	Grey-breasted Martin	0/3	a
Motacillidae			
Anthus hodgsoni	Olive-backed Pipit	0/28	a,b
Muscicapidae			
Turdus hortulorum	Grey-backed Thrush	0/3	a
Turdus kessleri	Kessler's Thrush	0/2	a
Paridae			
Parus major	Great tit	0/2	a
Petroicidae			
Petroica macrocephala	New zealand tit	0/2	a
Phasianidae			
Bambusicola thoracica	Chinese Bamboo Partridge	0/1	a
Gallus gallus	Chicken	0/5	a
Pycnonotidae			
Pycnonotus sinensis	Chinese bulbul	0/10	a
Pycnonotus taivanus	Tavaau bulbul	0/2	a
Pycnonotus xanthorrhous	Yellow-vented Bulbul	0/1	a
Sturnidae			
Acridotheres cristatellus	Crested myna	0/7	a
Sturnus sericeus	Red-billed starling	0/56	a,b
Sturnus tristis	Common myna	0/2	a
Sylviida			
Garrulax sannio	White-checked Laughing Thrush	0/1	b
Timaliidae			
Garrulax cineraceus	Moustached Laughing thrush	0/1	a
Turdidae			
Turdus merula	Blackbird	0/25	b,c
Turdus migratorius	American robin	0/3	b
Zoothera princei	Grey Ground Thrush	0/1	c
Unknown		0/201	a,b,c

Note: a: Hengyang b: Changde c: Yueyang

*Deltacoronavirus detected.

The bold values mean that deltaviruses have been detected in these samples, while abosultely no deltavirus has been detected in other samples.

order of 5′-UTR-replicase ORF1ab, spike (S), envelope (E), membrane (M) and nucleocapsid (N)-UTR-3′. Moreover, additional ORFs coding for non-structural (NS) proteins NS6, NS7a, NS7b and NS7c were identified. A putative transcription regulatory sequence (TRS) motif, 5′-ACACCA-3′ was detected, which is unique to deltacoronaviruses (Figure 2, Table S3).

The coding potential and characteristics of putative non-structural proteins (nsp's) coded by ORF1ab of HNU CoVs are shown in Table 2. The replicase ORF1ab polyprotein occupies 19.6 kb of the genome. This ORF encodes 16 putative non-structural proteins, including nsp3 (putative papain-like protease [PL^{pro}]), nsp5 (putative chymotrypsin-like protease [$3CL^{pro}$]), nsp12 (putative RdRp), nsp13 (putative helicase) and other proteins with unclear functions. These proteins are produced via proteolytic cleavage of the large replicase polyprotein by PL^{pro} and $3CL^{pro}$ at specific sites. Especially, we found a putative cleavage site of AG/VP between nsp3 and nsp4 of HNU1-1 and HNU1-2 and another putative cleavage site of LQ/AG between nsp5 and nsp6 of all HNU CoVs.

We further compared the aa identity of the seven conserved replicase domains for species demarcation (ADRP, nsp5 [$3CL^{pro}$], nsp12 [RdRp], nsp13 [Hel], nsp14 [ExoN], nsp15 [NendoU] and nsp16 [O-MT]) among the HNU CoVs. As shown in Table S4, in all the seven domains and overall, their aa sequences showed more than 90% identity to those of PDCoV HKU15, SpCoV HKU17 and QuaCoV HKU30, indicating that these CoVs should be subspecies of the same species.

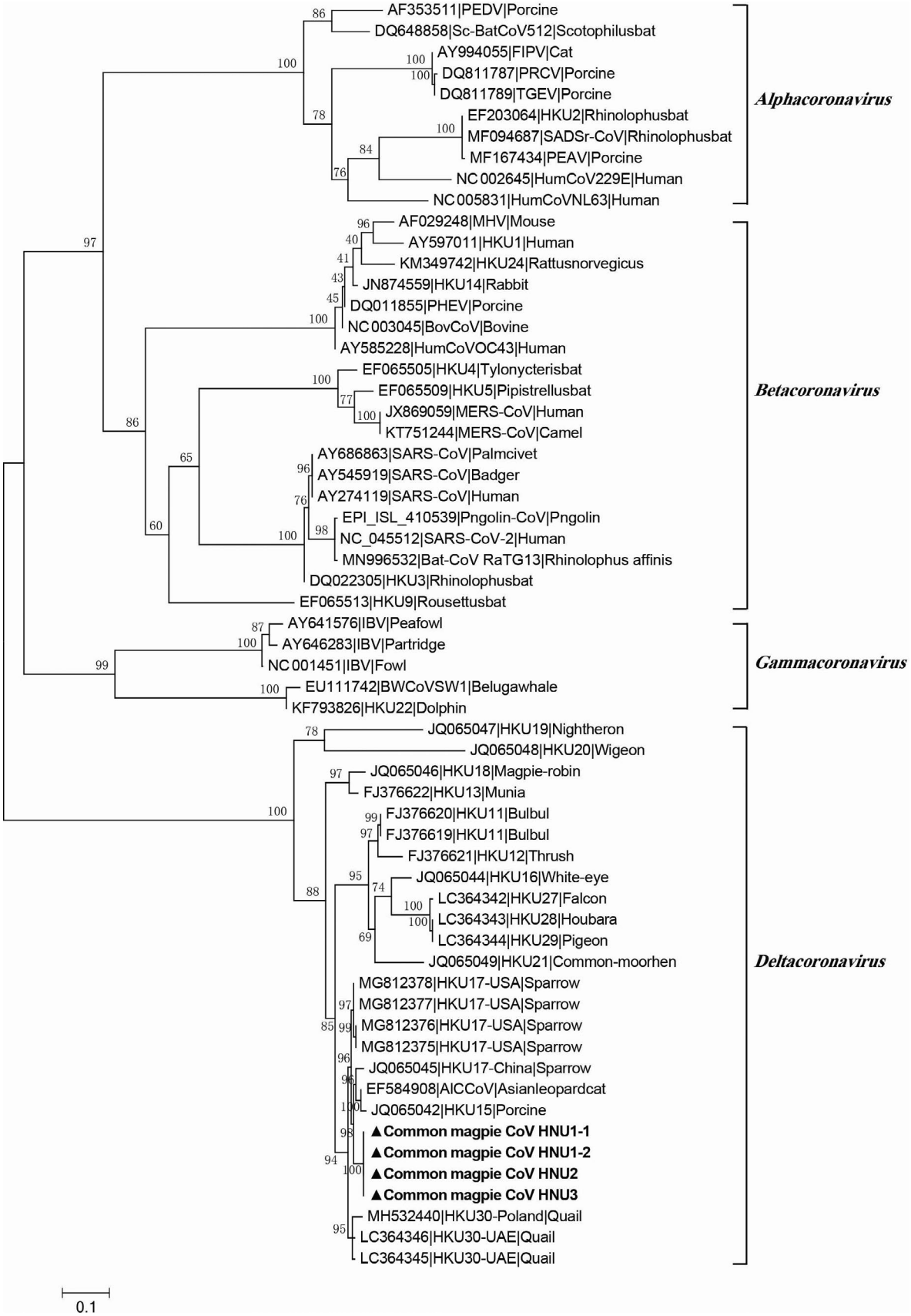

FIGURE 1 The maximum likelihood tree of C-terminal domain fragment (261 amino acids) in RNA-dependent RNA polymerase (RdRp). The tree was constructed using IQ-treewith LG + I + G4 substitution model and 10,000 ultrafast bootstraps. The scale bar indicates amino acid substitutions per site. The four newly identified HNU CoVs are shown in triangle and bold

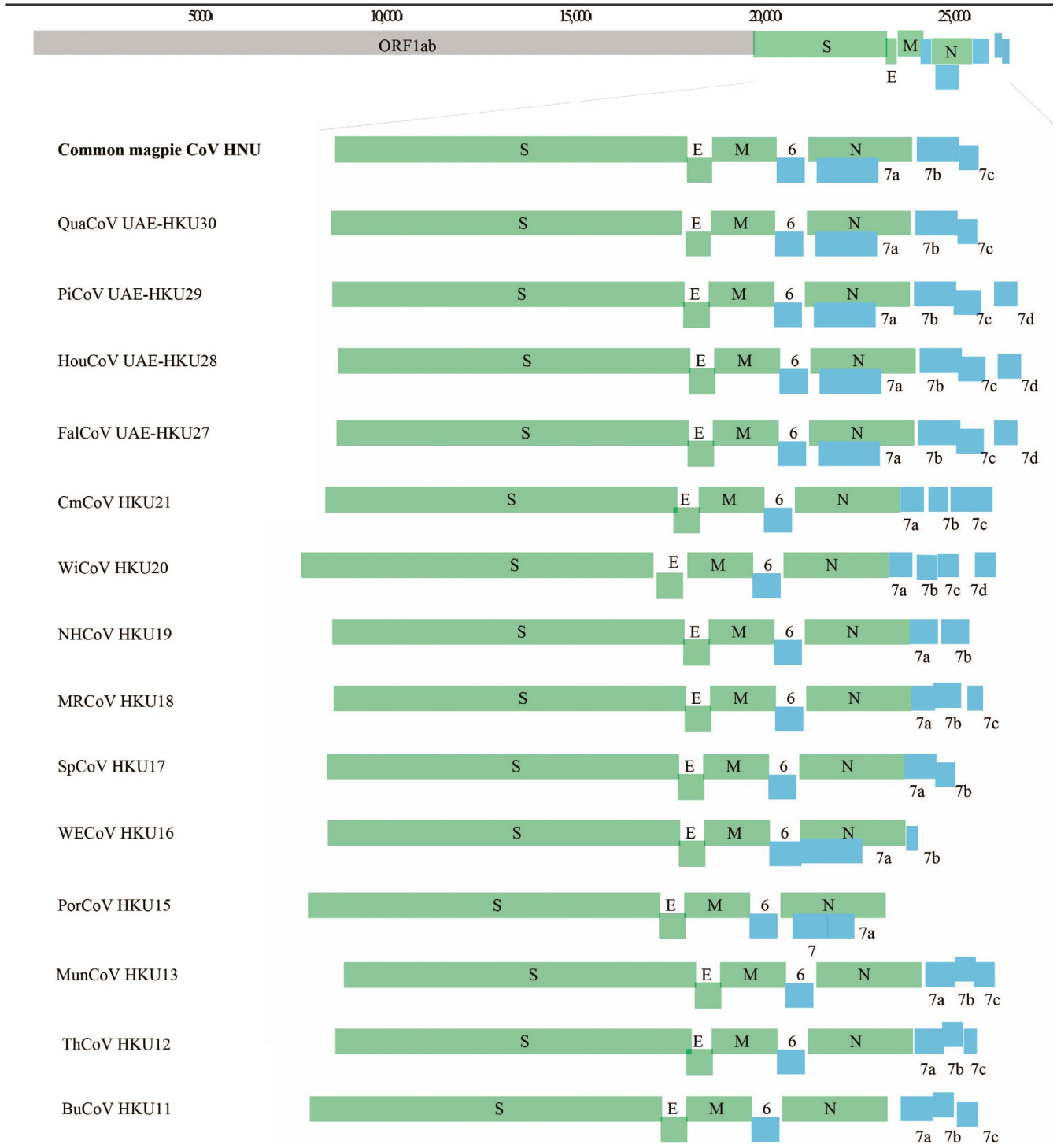

FIGURE 2 Genome organization of members of Deltacoronavirus. Open reading frames downstream of spike (S) gene are magnified to show the differences among the genomes of the 15 CoVs. S, envelope (E), membrane (M) and nucleocapsid (N) genes are represented by green boxes. Putative accessory proteins are represented by blue boxes. The novel HNU CoVs discovered in this study are shown in bold

3.3 | Phylogenetic analyses of the novel bird deltacoronaviruses

The phylogenetic trees reconstructed using the amino acid sequences of ORF1ab, 3CL^{pro}, Hel, S and N of the four novel HNU CoVs and other CoVs (Figure 3, Table S1). Except the S gene, the four novel HNU CoVs were clustered together. For ORF1ab, 3CL^{pro}, Hel and N gene, the four HNU CoVs were clustered with PDCoV HKU15, QuaCoV UAE-HKU30 and SpCoV HKU17, with amino acid identity higher than 85%. As for S gene, HNU1-1 and HNU1-2 were clustered with ThCoV HKU12, with 93.8% and 93.7% amino acid identity,

TABLE 2 Putative cleavage sites at the junctions between non-structural proteins in four novel common magpie CoV compared with those in other deltacoronaviruses

Cleavage site in	NSP pair												
	NSP2/NSP3	NSP3/NSP4	NSP4/NSP5	NSP5/NSP6	NSP6/NSP7	NSP7/NSP8	NSP8/NSP9	NSP9/NSP10	NSP10/NSP11	NSP12/NSP13	NSP13/NSP14	NSP14/NSP15	NSP15/NSP16
JQ065042|HKU15|Porcine	AG/SD	AG/AP	LQ/AG	LQ/SG	VQ/NK	VQ/AV	LQ/NN	LQ/AS	LQ/NS	LQ/AS	LQ/SS	LQ/NL	LQ/SL
JQ065044|HKU16|White-eye	AG/SD	AG/AR	LQ/AG	LQ/SN	VQ/NK	LQ/AV	IQ/NN	LQ/AN	LQ/GS	LQ/AS	LQ/SS	LQ/NL	VQ/SL
JQ065045|HKU17-China|Sparrow	AG/SD	AG/AP	LQ/AG	LQ/SG	VQ/NK	VQ/AV	LQ/NN	LQ/AN	LQ/NS	LQ/AS	LQ/SS	LQ/NL	LQ/SL
JQ065046|HKU18|Magpie-robin	AG/AD	AG/AM	LQ/AG	LQ/SG	VQ/NK	VQ/AV	LQ/NN	LQ/AN	LQ/NS	LQ/AS	LQ/AG	LQ/NL	LQ/SL
JQ065047|HKU19|Night heron	VG/GL	TG/GN	VQ/AG	LQ/GT	VQ/NK	LQ/VV	LQ/NN	LQ/SS	LQ/LG	LQ/AT	VQ/SL	LQ/TL	VQ/AL
JQ065048|HKU20|Wigeon	DG/VY	GG/SK	VQ/SG	LQ/AN	VQ/NR	LQ/VV	CQ/NN	LQ/AN	LQ/SN	LQ/AT	VQ/AE	LQ/TL	LQ/SL
JQ065049|HKU21|Common-moorhen	AG/VS	AG/KF	LQ/AG	LQ/AS	VQ/NR	LQ/AV	IQ/NN	LQ/AT	LQ/NT	LQ/AS	VQ/CS	LQ/TI	VQ/SL
LC364342|HKU27|Falcon	AG/SD	AG/RK	LQ/AG	LQ/SG	VQ/NR	LQ/AV	LQ/NN	LQ/AN	LQ/GS	LQ/AS	LQ/SS	LQ/NL	VQ/AL
LC364343|HKU28|Houbara	AG/SD	AG/RK	LQ/AG	LQ/SG	VQ/NR	LQ/AV	LQ/NN	LQ/AN	LQ/GS	LQ/AS	LQ/SS	LQ/NL	VQ/AL
LC364344|HKU29|Pigeon	AG/SD	AG/RK	LQ/AG	LQ/SG	VQ/NR	LQ/AV	LQ/NN	LQ/AN	LQ/GS	LQ/AS	LQ/SS	LQ/NL	VQ/AL
LC364345|HKU30-UAE|Quail	AG/SD	AG/AP	LQ/AG	LQ/SG	VQ/NK	VQ/AV	LQ/NN	LQ/AN	LQ/NS	LQ/AS	LQ/SG	LQ/NL	LQ/SL
MH532440|HKU30-Poland|Quail	AG/SD	**AG/VP**	LQ/AG	LQ/SG	VQ/NK	VQ/AV	LQ/NN	LQ/AN	LQ/NA	LQ/AS	LQ/SG	LQ/NL	LQ/SL
Common magpie CoV HNU1–1	AG/SD	**AG/VP**	LQ/AG	**LQ/AG**	VQ/NK	VQ/AV	LQ/NN	LQ/AN	LQ/NA	LQ/AS	LQ/SG	LQ/NL	LQ/SL
Common magpie CoV HNU1–2	AG/SD	**AG/VP**	LQ/AG	**LQ/AG**	VQ/NK	VQ/AV	LQ/NN	LQ/AN	LQ/NA	LQ/AS	LQ/SG	LQ/NL	LQ/SL
Common magpie CoV HNU2	AG/SD	AG/AP	LQ/AG	**LQ/AG**	VQ/NK	VQ/AV	LQ/NN	LQ/AN	LQ/NS	LQ/AS	LQ/SG	LQ/NL	LQ/SL
Common magpie CoV HNU3	AG/SD	AG/AP	LQ/AG	**LQ/AG**	VQ/NK	VQ/AV	LQ/NN	LQ/AN	LQ/NS	LQ/AS	LQ/SG	LQ/NL	LQ/SL
MG812375|HKU17-USA|Sparrow	AG/SD	AG/AP	LQ/AG	LQ/SG	VQ/NK	VQ/AV	LQ/NN	LQ/AN	LQ/NS	LQ/AS	LQ/SG	LQ/NL	LQ/SL
FJ376619|HKU11|Bulbul	AG/SD	AG/SD	LQ/AG	LQ/SG	VQ/NK	LQ/AV	IQ/NN	LQ/AN	LQ/NS	LQ/AS	LQ/SG	LQ/NL	VQ/SL
FJ376621|HKU12|Thrush	AG/SD	AG/SA	LQ/AG	LQ/SG	VQ/NK	LQ/AV	IQ/NN	LQ/AN	LQ/SS	LQ/AS	VQ/SS	LQ/NL	VQ/SL
FJ376622|HKU13|Munia	AG/AD	AG/AV	LQ/AG	LQ/SG	VQ/NK	VQ/AV	LQ/NN	LQ/AN	LQ/NS	LQ/AS	LQ/AG	LQ/NL	LQ/SL

The bold values mean the sequences of the cleavage sites.

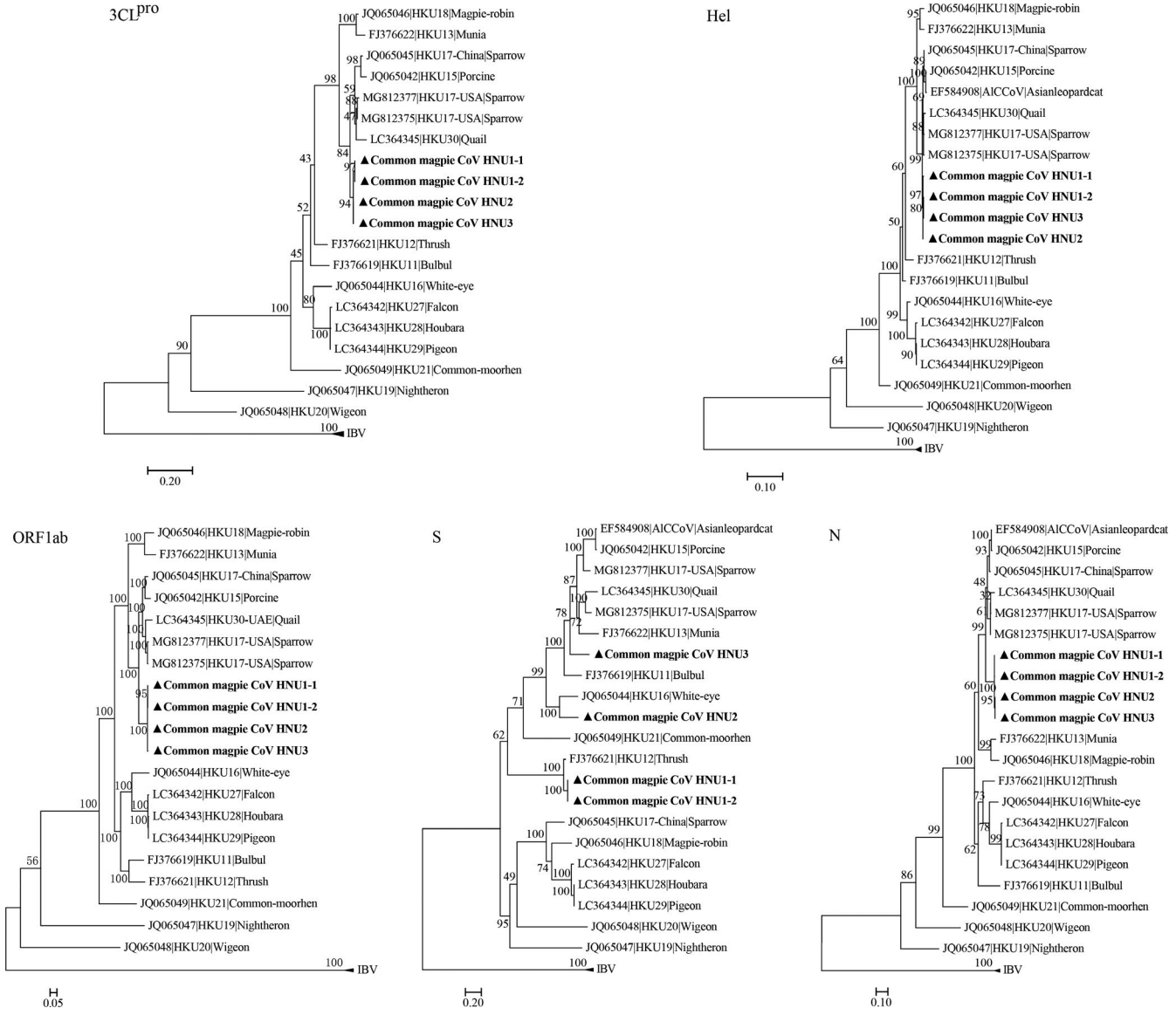

FIGURE 3 The maximum likelihood tree of amino acid sequences of chymotrypsin-like protease (3CL^{pro}), helicase (Hel), polyprotein 1ab (ORF1ab), spike (S) protein and nucleocapsid (N) protein. These trees were constructed using IQ-tree with 10,000 ultrafast bootstraps, and their substitution models were as follows: LG + G4 (3CL^{pro}), LG + F + R2 (Hel), LG + F + R5 (ORF1ab), WAG + I + G4 + *F* (S), LG + F + G4 (N). The scale bar indicates amino acid substitutions per site. The four newly identified HNU CoVs are shown in triangle and bold

respectively. HNU2 was clustered with WECoV HKU16, with 71.9% amino acid identity. And HNU3 was more closely related to SpCoV HKU17-USA and PDCoV HKU15 than BuCoV HKU11.

3.4 | Recombinant events of HNU1 and HNU2 coronaviruses

Interestingly, switches of positions in phylogenetic trees based on different genes were observed for the four HNU CoVs, indicating that recombination events may have occurred frequently during the virus evolution. The full-length genome sequences of all HNU CoVs in this study were screened for potential recombination events. The best possible recombination events in HNU1-1 (or HNU1-2) and HNU2 were identified by the GENECONV (*p*-value: 2.263E-307) and RDP (*p*-value: 1.739E-8) methods provided by RDP v3.44 program. These recombination events with strong *p*-value were further confirmed in Simplot v3.5.1 program (Figure 4). The results suggested that HNU1-1 and HNU1-2 were likely to be recombinant strains from SpCoV HKU17-USA and ThCoV HKU12, and the break points were identified at genome positions nt 21,017 and 25,056, which were closed to the start and end region of S protein (Figure 4a). Meanwhile, the newly identified QuaCoV UAE-HKU30 and the previously reported WECoV HKU16 were suggested to be the major and minor parent of HNU2, and there are two break points nt 21,101 and 24,501 in HNU2, which covered a part of the S1 and the S2 of the S protein (Figure 4b). However, there is no obvious evidence showing that HNU3 was a recombinant strain (data were not show).

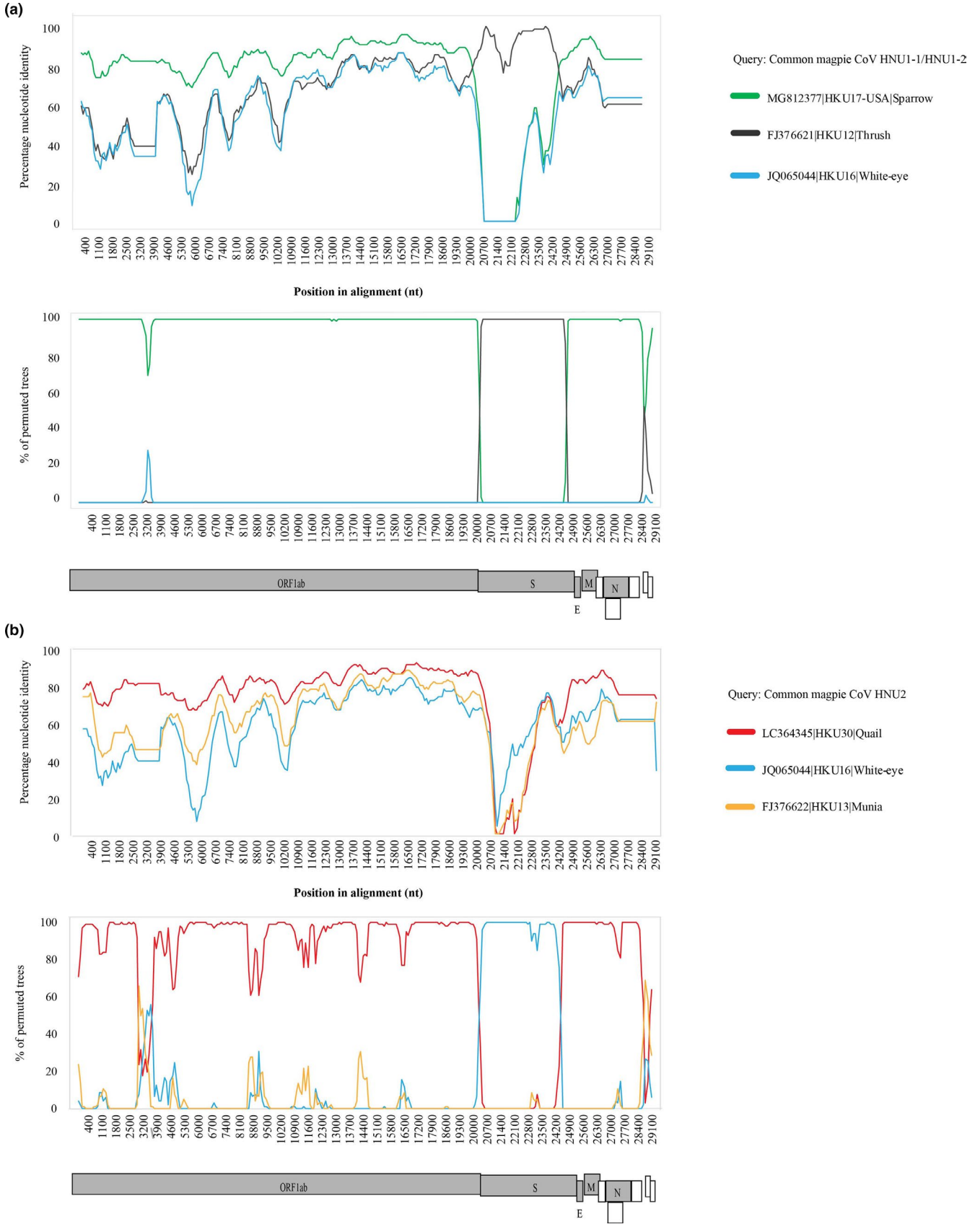

FIGURE 4 Detection of potential recombination events of novel HNU CoVs. (a) The possible recombination events in HNU1-1 or HNU1-2 strain. (b) The possible recombination events in HNU2. Both of Similarity Plot and Bootscan method in Simplot v3.5.1 were performed with an F84 distance model, a window size of 800 base pairs and a step size of 100 base pairs. The bootstrap replicates of Bootscan were set to 1,000

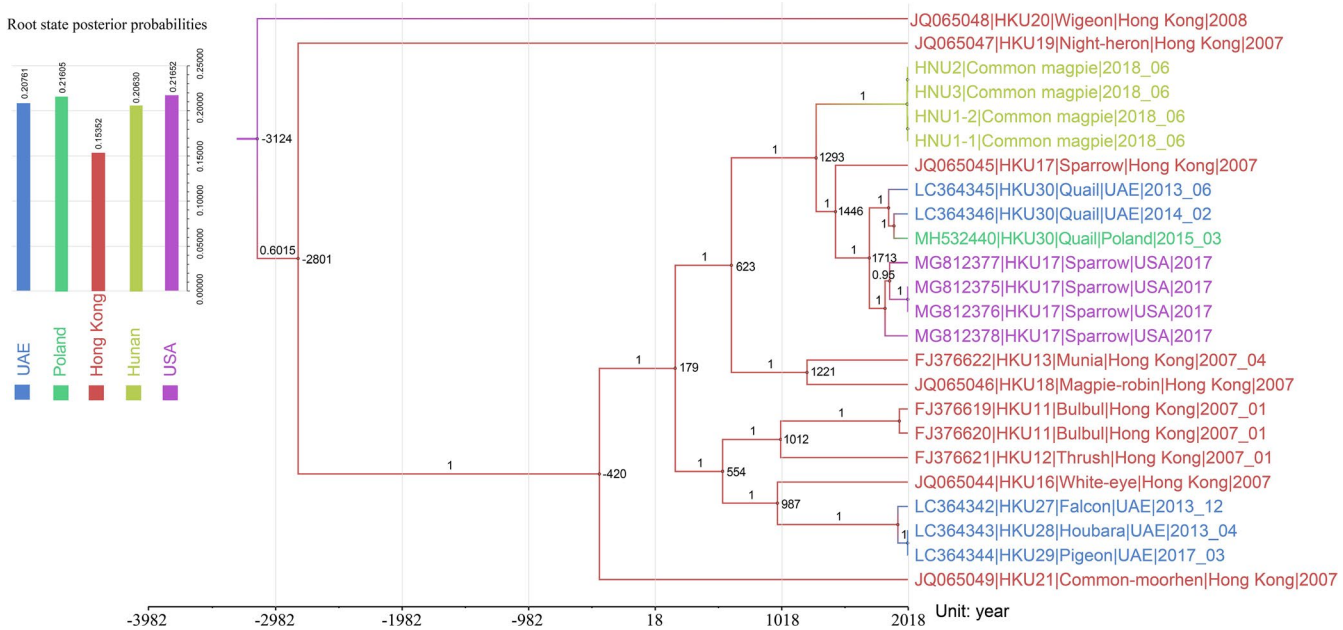

FIGURE 5 Bayesian phylogeographic analysis of 24 bird deltacoronaviruses strain sequences based on RdRp gene. Maximum clade credibility (MCC) tree with ancestral state reconstruction according to a discrete trait model, the substitution model was GTR + F + R3 which obtained by Model Finder under the BIC standard, the colours of branch and internal nodes indicated the most probable state reconstruction. The numbers on branch indicated posterior probability, and the time of divergence estimated was marked near the internal node. The root state posterior probabilities estimated for each regions of Bayesian phylogeographic MCC tree, which were inferred using an asymmetric substitution model

3.5 | Bayesian phylogeography of bird deltacoronaviruses

Molecular clock analysis was conducted to trace the temporal patterns of bird deltacoronaviruses evolution. The mean evolutionary rate was estimated as approximately 1.541×10^{-4}/site/year (95% highest posterior density intervals or HPDs, 8.25×10^{-5}–2.00×10^{-4}) for the RdRp gene. Molecular clock analysis using the RdRp gene showed that the mean time to the most recent common ancestor (tMRCA) of bird deltacoronavirus was estimated to be 3,000 BC (95% HPDs, 8,119 BC to 1,414), and the root state probability distribution indicated that the USA was the probable original location with a posterior probability of 0.21652. The tMRCA of four novel magpie CoVs in this study was estimated to be 1,200 (95% HPDs, 590 to 1,504) and originated from Hong Kong (Figure 5). The BSSVS analysis filtered out three significant south-to-north migration links (BF > 3) in the diffusion processes of all bird deltacoronavirus worldwide, including the paths from Hong Kong to USA, from Hong Kong to Hunan and from UAE to Poland (Table 3). But it should be noted that the mean indicators of the three migration paths were lower than 0.5, which indicates that more bird coronavirus genome sequences needed to predict the more convincing migration paths.

4 | DISCUSSION

Monitoring existence of viruses in wild animals is important for tracing viral transmission and controlling epidemics. Before transmitting to humans, the viruses mainly survive, transmit and evolve in animals, including their natural reservoirs and intermediate hosts. Birds are not only the reservoir animals for various viruses but also likely to be intermediate hosts of viruses, since birds are widely distributed around the world and capable of long-distance migration, leading to frequent contact with human and domestic animals. In this study, we identified and characterized four common magpie CoVs belonging to *deltacoronavirus* genus from birds in central China. Deltacoronaviruses are widely distributed worldwide (Lee & Lee, 2014; Marthaler et al., 2014; Wang et al., 2014; Wang et al., 2015). The *Coronaviridae* Study Group of ICTV has established the following genus and species demarcation criteria in the family *Coronaviridae*: coronaviruses that cluster together and share more than 90% amino acid sequence identity in seven conserved replicase domains (ADRP, nsp5 [$3CL^{pro}$], nsp12 [RdRp], nsp13 [Hel], nsp14 [ExoN], nsp15 [NendoU] and nsp16 [O-MT]) are considered to belong to the same species (Lau et al., 2018). Hence, the four novel HNU CoVs belong to the same species as PDCoV HKU15, SpCoV HKU17 and QuaCoV HKU30. Notably, common magpies, sparrows and quails are small terrestrial birds with global distribution. Genome comparison showed that deltacoronaviruses from terrestrial birds share higher similarity with PDCoV, suggesting terrestrial birds may serve as the intermediate host for CoVs. These data suggested that birds are natural reservoirs which provide viral genes for evolution and interspecies transmission of viruses. Furthermore, our study provides evidence for genetic recombination among bird deltacoronaviruses. More specifically, evidence for at least two recombination events was observed in the S genes of the four novel HNU CoVs, including a recombination event between viruses in the HNU1-1 (or HNU1-2) and HKU12 that allowed the two viruses to

TABLE 3 Migration path and statistical of bird deltacoronaviruses

Migration path	Mean migration rates	Mean indicators	Bayes factor
Hong Kong to Hunan	0.8882	0.1656	3.713958182
Hong Kong to Poland	0.8268	0.265	2.161450775
Hong Kong to UAE	0.8727	0.3367	1.458047601
Hong Kong to USA	0.849	0.3077	3.65601402
Hunan to Poland	0.8672	0.3792	−3.486333958
Hunan to UAE	0.9212	0.3709	−5.206512576
Hunan to USA	0.9013	0.3707	0.061473914
Hunan to Hong Kong	0.8916	0.3089	−3.586996146
Poland to UAE	1.428	0.5297	0.057528801
Poland to USA	1.3884	0.5285	0.054435634
Poland to Hunan	1.1547	0.5324	0.187082157
Poland to Hong Kong	1.1752	0.5394	0.169848657
UAE to USA	0.9557	0.4547	0.056965978
UAE to Hong Kong	1.0401	0.3986	0.07251349
UAE to Hunan	0.8937	0.3266	−3.730958555
UAE to Poland	0.8739	0.4187	3.022568883
USA to Hong Kong	1.0466	0.4232	0.059406257
USA to Hunan	0.9111	0.3761	2.505014699
USA to Poland	0.9218	0.4344	2.349057809
USA to UAE	1.0719	0.481	1.965893096

acquire S from HKU12, and another recombination between viruses in the HNU2 and HKU16 clusters that allowed the two viruses to acquire partial S1 and an S2 subunit from HKU16 (Figure 4). These recombination events likely occurred in bird hosts. Similar to the case for birds in Hong Kong and Middle East, birds in mainland China are also important hosts for a diversity of deltacoronaviruses. Continuous surveillance studies on birds in other city of China will help to better understand the viral and host diversity of deltacoronaviruses and their potential for emergence in mammals.

At present, the genus *Deltacoronavirus* is subdivided into four subgenera and seven species (http://ictv.global/report). These virus species have been sampled from swine, as well as a variety of birds, including 14 bird species. In all, based on the known data of 24 deltacoronavirus isolates and their hosts from five different regions around the world, we investigated the molecular epidemiology of bird deltacoronavirus using Bayesian phylogeographic inference. Our phylogeographic analysis revealed a general south-to-north dispersal of bird deltacoronavirus. In particular, Hong Kong is a possible origin of bird deltacoronaviruses in central China. However, the three significant migration links (BF > 3) are in a lower value of mean indicators, suggesting that the dispersal pattern might be associated with the number of bird deltacoronavirus. More CoVs strains are needed to predict the root cause of avian coronavirus before more convincing evidence can be obtained.

In summary, our study indicates the widespread existence and high diversity of environmental deltacoronaviruses in birds, and some of them are classified into the same species as PDCoV. In order to understand the evolutionary history of bird deltacoronavirus and to prevent future emerging infectious diseases, it is critical to take extensive and long-term surveillance on the environmental deltacoronaviruses.

ACKNOWLEDGEMENTS

This work was jointly funded by the National Key Research and Development Program of China (grant number 2017YFD0500104), National Natural Science Foundation of China (grant number 32041001 and 81902070) and the Provincial Natural Science Foundation of Hunan Province (grant number 2019JJ20004, 2019JJ50035).

CONFLICT OF INTEREST

The authors declare no competing interests.

ETHICAL APPROVAL

The ethical statement is not available since no sample collection or questionnaires from animals/human has been gathered.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Wang Q, Zhou Z-J, You Z, et al. Epidemiology and evolution of novel deltacoronaviruses in birds in central China. *Transbound Emerg Dis*. 2022;69:632–644. https://doi.org/10.1111/tbed.14029