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Genomic and evolutionary comparison between SARS-CoV-2 and other human coronaviruses

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ABSTRACT

Three highly pathogenic human coronaviruses can cause severe acute respiratory syndrome (SARS-CoV, SARS-CoV-2 and MERS-CoV). Although phylogenetic analyses have indicated ancient origin of human coronaviruses from animal relatives, their evolutionary history remains to be established. Using phylogenetics and “high order genomic structures” including trimer spectrums, codon usage and dinucleotide suppression, we observed distinct clustering of all human coronaviruses that formed phylogenetic clades with their closest animal relatives, indicating they have encompassed long evolutionary histories within specific ecological niches before jumping species barrier to infect humans. The close relationships between SARS-CoV and SARS-CoV-2 imply similar evolutionary origin. However, a lower Effective Codon Number (ENC) pattern and CpG dinucleotide suppression in SARS-CoV-2 genomes compared to SARS-CoV and MERS-CoV may imply a better host fitness, and thus their success in sustaining a pandemic. Characterization of coronavirus heterogeneity via complementary approaches enriches our understanding on the evolution and virus-host interaction of these emerging human pathogens while the underlying mechanistic basis in pathogenicity warrants further investigation.

1. Introduction

The novel coronavirus disease (COVID-19) pandemic emerged in December 2019 is dramatically threatening global public health and economy, resulting in over 7.6 million confirmed cases and >420,000 deaths in at least 216 countries/cities as of June 15, 2020 (https://www.who.int/emergencies/diseases/novel-coronavirus-2019). This disease is caused by a novel coronavirus, SARS-CoV-2, belonging to the genus Betacoronavirus in the family Coronaviridae, with major clinical symptoms of cough and fever but also acute respiratory distress syndrome or multiorgan failure (Huang et al., 2020; Lu et al., 2020).

The family Coronaviridae consists of four genera: Alpha-, Beta-, Gamma- and Deltacoronavirus, with the former two genera infecting mammals only (Cui et al., 2019). Before COVID-19, six human coronaviruses (HCoVs) have been identified, including two (SARS-CoV, MERS-CoV) highly pathogenic clusters that cause severe respiratory pneumonia, and the other four (HCoV-OC43, HCoV-229E, HCoV-NL63 and HCoV-HKU1) that mainly cause common cold. In late 2002, the first severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in Guangdong, China and spread to more than 30 countries. The overall fatality was ca. 10 % with more than 8000 infections (www.who.int) (Zhong et al., 2003). SARS-CoV was not found in humans since 2004. In 2012, another highly pathogenic coronavirus, Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in Middle Eastern countries (Zaki et al., 2012), causing severe pneumonia and renal failure in humans, with a mortality of ~30 %. Phylogenetic analyses indicate that all human coronaviruses have an ancient origin from animals: SARS-CoV, MERS-CoV, HCoV-NL63, HCoV-229E, and perhaps SARS-CoV-2 are related to coronaviruses detected in bats; whereas HCoV-OC43 and HCoV-HKU1 are closely related to coronaviruses detected in rodents (Cui et al., 2019; Forni et al., 2017; Zhou et al., 2020). Evolution and transmission of coronaviruses from their natural reservoirs to humans likely involve intermediate hosts (e.g., palm civets for SARS-CoV, dromedary camels for MERS-CoV) (Reusken et al., 2013; Wang et al., 2005). Phylogenetically, SARS-CoV and SARS-CoV-2 share a most recent common ancestor within the subgenus Sarbecovirus, and are

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relatively distant to MERS-CoV (belonging to the subgenus *Merbecovirus*) in the genus *Betacoronavirus* (Fig. 1). This genus also includes two other HCoV clusters, HCoV-OC43 and HCoV-HKU1, whereas HCoV-NL63 and HCoV-229E are classified within the genus *Alphacoronavirus*.

Coronaviruses form enveloped and spherical particles of 100–160 nm in diameter. It contains a single-stranded, positive (+)-sense RNA (+ssRNA) genome ranging from 27 to 32 kilobases in length (Cui et al., 2019). The 5′-terminal two-thirds of the genome encodes two large nonstructural polyproteins 1a and 1b which are involved in genome transcription and replication. The 3′ terminus encodes structural proteins, including envelope glycoproteins spike (S), envelope (E), membrane (M) and nucleocapsid (N). The S protein plays a critical role in viral attachment, fusion, entry and transmission. The N-terminal S1 subunit is responsible for virus-host receptor binding and the C-terminal S2 subunit is for virus–cell membrane fusion. SARS-CoV-1 and SARS-CoV-2 use angiotensin-converting enzyme 2 (ACE2) as a receptor, whereas MERS-CoV binds dipeptidyl peptidase 4 (DPP4) (Du et al., 2009, 2017; Wrapp et al., 2020). During infection, HCoV first binds the host cell through interaction between its S1 receptor-binding domain (S1-RBD) and the cell membrane receptor, followed by cleavage by furin proteases leading to conformational changes in the S2 subunit for virus fusion and entry. Currently, there is no vaccine or therapeutics for effective prevention or treatment of HCoV infection while the research and development of neutralizing antibodies, mainly targeting S1 and/or S2 regions have been vigorously undertaken (Jiang et al., 2020).

With advances in DNA sequencing and bioinformatics analysis, multiple sequence alignments have been used to interrogate genomic diversity and evolution. The highly divergent homologous sequences among HCoV genomes, however, may lead to ambiguous alignments that degrade resolution and bias phylogenetic inference. Alternatively, nonparametric agnostic approaches based on the distribution of exact sub-sequences (k-mer spectrum, the DNA ‘word’ with defined length) and additional genetic metrics (for example, codon usage preference, dimer nucleotide composition) provide complementary information on the complexity and relationship of homologous sequences (Chan and Ragan, 2013; Chor et al., 2009; Shackleton et al., 2006; Vinga and Almeida, 2003). These agnostic methods also avoid complex computation or model selection while capturing signals otherwise lost to indel, recombination or shuffling (Chan and Ragan, 2013). In an effort to interrogate the evolutionary process driving the divergence of coronaviruses, we have primarily focused on human coronaviruses because of the association with severe respiratory pneumonia in human beings. Multiple parametric and nonparametric algorithms were applied. Using the rich resource of a large number of genomes, we sought to uncover hidden biological signals not easily discovered with homology-based methods alone.

### 2. Results

#### 2.1. SARS-CoV-2 phylogeny and genomic diversity

To better understand the evolutionary position of SARS-CoV-2, we first examined the phylogenetic relationship of the subgenus *Sarbecoviruses* within the genus *Betacoronaviruses* using the concatenated nucleotide sequences of 12 ORFs/genes (ORF1a, ORF1b, S, ORF3a, E, M, ORF6, ORF7a, ORF7b, ORF8, N and ORF10) (Fig. 2A, Table S1). In consistent with previous reports, SARS-CoV-2 shares sequence similarities of 96.13 ± 0.06 % with a bat coronavirus isolate (RaTG13, NCBI accession number MN996532, GISAID accession number Z. Chen et al.

Fig. 1. Phylogeny of the family Coronaviridae. A maximum likelihood (ML) tree was constructed using RAxML MPI v8.2.12 inferred from the concatenated nucleotide sequence alignments of 6 open reading frames (1a-1b-S-E-M-N) of 55 reference genomes. The dot size on the nodes is proportional to the bootstrap support values. The HCoV clusters associated with severe acute respiratory syndrome (SARS-CoV, SARS-CoV-2 and MERS-CoV) and common cold (HCoV-OC43, HCoV-HKU1, HCoV-229E and HCoV-NL63) were highlighted in red and orange, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
EPI_ISL_402131), similar to the genomic differences between SARS-CoV and bat SARS-like CoVs (e.g., KY417150, KY417146) (similarities of 96.06 ± 0.25 %), implying potential zoonotic transmission of SARS-CoV-2 from bats as their natural reservoirs to humans through unknown intermediate hosts. SARS-CoV-2 also shares close genomic similarities with coronavirus isolates from pangolin animals (EPI_ISL_410984, 90.22 ± 0.03 %; EPI_ISL_410538 - EPI_ISL_410542, 85.27 ± 0.03 %), whereas the complete genome sequence similarities between SARS-CoV and SARS-CoV-2 are 79.4 ± 0.17 %. The subgenus Sarbecovirus has average sequence similarities of ≤ 48.4 % with other coronaviruses (data not shown).

2.2. K-mer spectrum clustering of SARS-CoV-2

The k-mer and other nonparametric approaches, such as codon usage and dinucleotide composition, constitute a “higher order genomic structure” that may reflect the influence of evolutionary processes extending beyond common measures of Darwinian selection. In order to explore an agnostic evolutionary model, we used trimer (k = 3) frequency distribution to construct the phylogeny of the subgenus Sarbecovirus (Figs. 2B). Both parametric (alignment phylogeny) and nonparametric (trimer spectrum phylogeny) approaches showed distinct separation between SARS-CoV and SARS-CoV-2, with their animal relatives closely clustering to each other.

2.3. Codon usage bias of human betacoronaviruses

The trimer spectra between human bCoV clusters may imply differential codon usages between viruses and host cells. We then measured the ENC values of coronaviruses across 6 genes (ORF1a, ORF1b, S, E, M, and N) shared by all members, with a main focus on the difference between seven human coronavirus clusters (Table S2). The ENC statistic is a way of analyzing how biased a gene is in terms of its codon usage, with values ranging from 20 when a gene is effectively using only a single codon for each amino acid (strongest bias) to 61 when a gene tends to use all codons with equal frequency (no bias). The ENC values of the surveyed coronavirus genomes ranged between 35.6 and 54.1 (a mean value of 45.4 ± 4.7), with a significant correlation with GC contents (adjusted $R^2 = 0.953$, $p < 0.001$) (Figure S1). Strong codon usage bias was observed in HCoV-HKU1 (35.7 ± 0.09) and HCoV-NL63 (37.3 ±
0.08) genomes when compared to other HCoV clusters (45.9 ± 4.2, p < 0.001) (Fig. 3A). Interestingly, SARS-CoV-2, HCoV-OC43 and HCoV-229E genomes shared similar ENC values, but demonstrated stronger codon usage bias than SARS-CoV and MERS-CoV (p = 0.008).

In order to obtain a better understanding on the relationship between codon usage bias and gene composition, a plot of ENC values against GC contents at the synonymous third codon position (GC3s) was constructed (Fig. 3B). This method was used to estimate the factors shaping the codon usage pattern. If codon usage pattern was affected by GC3s alone, the observed ENC values should be on or just below the expected ENC curve indicating the synonymous codon usage bias may be subject to GC-biased mutational pressure. As shown, all surveyed HCoV genomes lay slightly under the expected ENC curve (the red curve in Fig. 3B), implying an important role of uneven base composition and hence, of mutational pressure that affected the formation of codon usage bias. However, codon usage bias tended to be less dependent on variation of GC3s when GC contents decreased, with measures of (ENC* - ENC) / ENC* suggesting that other factors, such as translational or natural selection, may act as forces affecting stronger codon usage bias and higher genomic diversity of HCoV-HKU1 and HCoV-NL63 (Fig. 3C).

Differential codon usage patterns within distinct ORFs were observed between HCoV clusters (Figure S2). SARS-CoV and MERS-CoV, for example, had the least codon usage biases across ORFs while HCoV-HKU1 and HCoV-NL63 were the strongest ones. The S and N genes, however, showed no significant difference in ENC values between SARS-CoV-2 and SARS-CoV. The M gene may encompass more diversified selection forces amongst HCoV clusters, as lower values of (ENC* - ENC) / ENC* were observed, probably due to the relatively small protein encoded. It is noted that the E gene was not included for comparison because of the small protein size (< 89 aa), for which interpretation may not be applied.

2.4. Synonymous codon usage pattern in human betacoronaviruses

ENC value measures the codon usage bias of an entire genome/gene but not of individual codon. We further calculated the Relative Synonymous Codon Usage (RSCU) values across 6 ORFs of HCoV genomes to better estimate the differential usage of each synonymous codon. Among 59 codons encoding for 18 amino acids (except for Met, Trp, and stop codons), 6 and 9 were defined as preferred (RSCU values > 1.6) and suppressed codons (< 0.6) within all HCoV genomes, respectively (Fig. 3D, Table 1). For example, HCoV genomes prefer to GGT (RSCU ≥ 1.91) rather than GGG (< 0.26) to encode Glycine. Interestingly, the majority of human-preferred codons were less commonly found in HCoV genomes, such as CTG encoding for Leucine (Human, 2.37; HCoV, ≤ 0.58) and GCC encoding for Alanine (Human, 1.60; HCoV, ≤ 0.59); in contrast, CGT, the optimized codon encoding for Arginine in HCoV genomes (mean of 2.03) was rarely found in human genomes (0.48). The animal CoV genomes share similar RSCU patterns as the HCoV ones.

In contrast to HCoV genomes that intended to use A/T-ending codons, as observed in 96 % (25/26) of codons with average values of RSCU less than 1.0, human host tends to use G/C-ending codons (74 %, 20/27). However, both HCoV and human displayed a strong tendency to avoid using CG-ending codons (HCoV ≤ 0.28, human ≤ 0.46),

Fig. 3. Synonymous codon usage of coronavirus genomes based on concatenated nucleotide sequences of 6 ORFs (ORF1a-1b-S-E-M-N). (A) Boxplot of Effective Number of Codon (ENC) between HCoV clusters. The ENC values range from 20 when a gene is effectively using only a single codon for each amino acid (strongest bias) to 61 when a gene trends to use all codons with equal frequency (no bias). (B) Plot of ENC and the synonymous third codon position (GC3s) content. The red curve indicates the expected ENC if codon usage pattern is only affected by GC3s. (C) Boxplot of differences between the observed and expected ENC values among HCoV clusters. (D) Mean values of Relative Synonymous Codon Usage (RSCU) for 59 codons (except for Met, Trp, and stop codons) amongst HCoV clusters. The preferred and suppressed codon usages were defined as RSCU values > 1.6 or < 0.6, respectively. (E) Scatter biplot of RSCU of HCoV clusters. The clustering was performed using redundancy analysis (RDA), with colours assigned to different human betacoronavirus clusters. The x-axis and the y-axis represent the first two principal coordinate component (PCoA) axes. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
suggesting a potential role of CpG dinucleotide depletion in shaping the codon usages.

To further investigate the variation of synonymous codon usage bias in modulating HCoV genomic diversity, correspondence analysis based on the RSCU patterns was performed (Fig. 3E). Scatter plots of the first two axes well supported the distinct separation of HCoV clusters, with SARS-CoV and SARS-CoV-2 sharing relatively similar RSCU patterns, such as preferred codon usage of ACA (Threonine), CCA (Proline) and TCA (Serine) when compared to other HCoV clusters, but relatively lower values of TTG (Leucine) and CGT (Arginine) (Table 1). The four HCoV clusters associated with common cold (HCoV-OC43, HCoV-HKU1, HCoV-229E and HCoV-NL63) may form a separate group based on the codon usage patterns, with higher RSCU values of GT-ending codons when compared to the SARS-related HCoV genomes (SARS-CoV, SARS-CoV-2 and MERS-CoV). When individual ORFs were accessed, we observed similar codon usage patterns amongst HCoV clusters when compared to the concatenated 6 ORFs/genes (Fig. 4). Interestingly, the N gene of SARS-CoV and SARS-CoV-2 shared nearly identical codon usage patterns, implying similar biological property of nucleoprotein between these two viral clusters in packaging viral particles. Within individual HCoV cluster, ORF1a, ORF1b and S gene usually shared a more similar codon usage patterns that were different to that of M and N genes (Figure S3).

2.5. Dinucleotide suppression in human betacoronaviruses

Since CG-ending codons may be less frequent in coronavirus genomes (Table 1), we then measured the relative abundance of dinucleotide across 6 ORFs/genes to determine the influence of dinucleotide suppression on codon usage bias. As expected, CpG dinucleotide was mostly depleted (mean value of observed/expected ratio of 0.48 ± 0.06) (Fig. 5A, Table 2), with significant association with overall GC content (Pearson correlation of 0.709, p < 0.001) and GC content at the third position (GC3) (cor. 0.736, p < 0.001) (Figure S4A). TpC dinucleotides represented the second most suppressed dinucleotide (0.78 ± 0.06), consistent with the strong scarcity of nTC codon usage in the surveyed coronavirus genomes (Tables 1 and 2). Since TC dinucleotide is one of the preferred target sequences of host restriction factor APOBEC3 proteins, the suppression of TC dinucleotide could be a result of evolutionary selection allowing viruses to evade restriction from host immune response.

| Amino acid | Codon | SARS-CoV-2 | SARS-CoV | MERS-CoV | HCoV-Oc43 | HCoV-HKU1 | HCoV-229E | HCoV-NL63 | Animal-CoV | Human |
|------------|-------|-----------|----------|----------|-----------|-----------|-----------|-----------|------------|-------|
| Ala (A)    | 1.08  | 0.89      | 0.87     | 1.13     | 0.91      | 1.19      | 0.90      | 1.10      | 0.91       | 0.91  |
| Val (V)    | 0.20  | 0.59      | 0.60     | 0.57     | 0.33      | 0.61      | 0.45      | 0.62      | 0.60       | 0.60  |
| Gly (G)    | 0.16  | 0.24      | 0.28     | 0.20     | 0.11      | 0.22      | 0.08      | 0.38      | 0.42       | 0.42  |
| Ser (S)    | 2.09  | 2.08      | 2.01     | 2.10     | 2.64      | 2.16      | 2.37      | 2.02      | 2.06       | 2.06  |
| Thr (T)    | 1.42  | 1.27      | 1.52     | 1.83     | 1.44      | 1.82      | 1.68      | 1.80      | 1.51       | 0.93  |
| Pro (P)    | 0.93  | 0.93      | 0.92     | 0.83     | 0.84      | 0.92      | 0.72      | 0.87      | 1.18       | 1.18  |
| His (H)    | 0.57  | 0.67      | 0.68     | 0.64      | 0.20      | 0.57      | 0.39      | 0.61      | 1.16       | 1.16  |
| Thr (T)    | 1.43  | 1.33      | 1.56     | 1.80      | 1.43      | 1.61      | 1.16      | 0.84      | 0.84       | 0.84  |

Table 1
Relative synonymous codon usage (RSCU) patterns of the surveyed human betacoronaviruses inferred from the concatenated 6 ORFs (ORF1a, 1b, S, E, M, N). (For interpretation of the references to colour in this Table legend, the reader is referred to the web version of this article.)

| Amino acid | Codon | SARS-CoV-2 | SARS-CoV | MERS-CoV | HCoV-Oc43 | HCoV-HKU1 | HCoV-229E | HCoV-NL63 | Animal-CoV | Human |
|------------|-------|-----------|----------|----------|-----------|-----------|-----------|-----------|------------|-------|
| Ala (A)    | 1.08  | 0.89      | 0.87     | 1.13     | 0.91      | 1.19      | 0.90      | 1.10      | 0.91       | 0.91  |
| Val (V)    | 0.20  | 0.59      | 0.60     | 0.57     | 0.33      | 0.61      | 0.45      | 0.62      | 0.60       | 0.60  |
| Gly (G)    | 0.16  | 0.24      | 0.28     | 0.20     | 0.11      | 0.22      | 0.08      | 0.38      | 0.42       | 0.42  |
| Ser (S)    | 2.09  | 2.08      | 2.01     | 2.10     | 2.64      | 2.16      | 2.37      | 2.02      | 2.06       | 2.06  |
| Thr (T)    | 1.42  | 1.27      | 1.52     | 1.83     | 1.44      | 1.82      | 1.68      | 1.80      | 1.51       | 0.93  |
| Pro (P)    | 0.93  | 0.93      | 0.92     | 0.83     | 0.84      | 0.92      | 0.72      | 0.87      | 1.18       | 1.18  |
| His (H)    | 0.57  | 0.67      | 0.68     | 0.64      | 0.20      | 0.57      | 0.39      | 0.61      | 1.16       | 1.16  |
| Thr (T)    | 1.43  | 1.33      | 1.56     | 1.80      | 1.43      | 1.61      | 1.16      | 0.84      | 0.84       | 0.84  |

* the preferred codons (RSCU > 1.6) and the suppressed codons (RSCU < 0.6) are highlighted in red and green, respectively.

2.5. Dinucleotide suppression in human betacoronaviruses

Since CG-ending codons may be less frequent in coronavirus genomes (Table 1), we then measured the relative abundance of dinucleotide across 6 ORFs/genes to determine the influence of dinucleotide suppression on codon usage bias. As expected, CpG dinucleotide was mostly depleted (mean value of observed/expected ratio of 0.48 ± 0.06) (Fig. 5A, Table 2), with significant association with overall GC content (Pearson correlation of 0.709, p < 0.001) and GC content at the third position (GC3) (cor. 0.736, p < 0.001) (Figure S4A). TpC dinucleotides represented the second most suppressed dinucleotide (0.78 ± 0.06), consistent with the strong scarcity of nTC codon usage in the surveyed coronavirus genomes (Tables 1 and 2). Since TC dinucleotide is one of the preferred target sequences of host restriction factor APOBEC3 proteins, the suppression of TC dinucleotide could be a result of evolutionary selection allowing viruses to evade restriction from host immune response.
protection. TpA dinucleotides were also stringently excluded (0.88 ± 0.05), probably due to in part usage of universal stop (TAA, TAG) and the increased susceptibility of TpA to ribonuclease digestion (Beutler et al., 1989). Interestingly, the loss of TpA seems to be significantly associated with the gain of TpG (Pearson correlation of 0.940, \( p < 0.001 \)) (Figure S4B, Table S3). Deamination of 5-methylcytosine (5mC) within the CpG island may lead to a cytosine (C) to thymine (T) transition, potentially resulting in a loss of CpG and a gain of TpG. However, the correlation between the loss of CpG and the gain of TpG was not strong (\( \text{cor} = 0.138, p = 0.006 \)) in the surveyed coronavirus genomes. The gain of TpG was also associated with the loss of ApT (\( \text{cor} = 0.730, p < 0.001 \)) and with gain of CpA (\( \text{cor} = 0.772, p < 0.001 \)) and ApC (\( \text{cor} = 0.605, p < 0.001 \)).

Discriminative dinucleotide suppression patterns were observed between HCoV clusters. For example, two Alphacoronavirus HCoV clusters (HCoV-229E, NCoV-NL63) had a significant gain of ApA but a loss of ApG when compared to the Betacoronavirus HCoV clusters (\( p < 0.001 \)) (Figs. 5B and 5C). We also observed higher O/E ratio of CpT, TpC and GpG within MERS-CoV, SARS-CoV and SARS-CoV-2 when compared to the common cold-related HCoV clusters. Interestingly, MERS-CoV genomes had the least loss of CpG dinucleotide, followed by SARS-CoV while SARS-CoV-2 had the most, which might imply differential viral gene expression between these three HCoV clusters. Different levels of dinucleotide suppression were found amongst ORFs/genes (Figure S5). For example, the S gene of SARS-CoV-2 had a significant loss of CpG dinucleotide compared to other genes or other HCoV clusters; the N gene had an overall gain of ApG but a loss of ApC. These differences probably imply an evolutionary apomorphy between HCoV genes that warrants further investigation on their biological properties and clinical relevancies.

3. Discussion

Analyses of the origin and evolutionary tree of life have long been based on sequence-aligned dissimilarity to present the homology of organisms in association with genotype, phenotype and phylotype. However, the genetic heterogeneity, such as recombination, duplication, insertion/deletion, genetic fusion and shuffling, and potential sequencing errors, challenges the accuracy of sequence alignment and comparison that strongly relies on heuristic solution and data quality. The relevance of alignment scores to homology may be deducted for coronavirus genomes because of complex histories evolving from animal hosts; hence, complementary approaches containing as much homologous signals as possible will provide an opportunity to explore the underlying evolution (Chor et al., 2009). In this study, we applied multiple parametric and nonparametric algorithms (e.g., evolutionary phylogeny, trimer spectra, codon usage bias and dinucleotide suppression) to compare the differential genomic characterisation between the main coronavirus clusters that infect humans (SARS-CoV, SARS-CoV-2, MERS-CoV, HCoV-OC43, HCoV-HKU1, HCoV-229E and HCoV-NL63). Using the features of a large number of viral genomes we provide evidences with different evolutionary constraints driving the heterogeneity of HCoV genomes. The sharp patterns of codon usage and dinucleotide suppression amongst HCoV clusters (e.g., stronger codon
Fig. 5. Dinucleotide suppression of HCoV genomes inferred from the concatenated nucleotide sequences of 6 ORFs (ORF1a-1b-S-E-M-N). (A) Boxplot of dinucleotide observed/expected (O/E) ratio. The $\rho_{XY}$ dinucleotide exhibits suppression if the O/E ratio is less than 1. (B) Scatter biplot of relative abundance of dinucleotides of HCoV genomes. The clustering was performed using redundancy analysis (RDA), with colours assigned to different clusters. The x-axis and the y-axis represent the first two principal coordinate component (PCoA) axes. (C) Boxplot of the O/E ratios of each dinucleotide amongst HCoV clusters.
Table 2

| Amino acid | SARS-CoV-2 | SARS-CoV | MERS-CoV | HCoV-OC43 | HCoV-HKU1 | HCoV-229E | HCoV-NL63 | Animal-CoV |
|------------|------------|----------|----------|-----------|-----------|-----------|-----------|------------|
| pAA        | 1.07       | 1.04     | 1.06     | 1.05      | 1.09      | 1.15      | 1.16      | 1.05       |
| pAC        | 1.23       | 1.17     | 1.12     | 1.05      | 1.10      | 1.27      | 1.30      | 1.17       |
| pAG        | 0.99       | 0.99     | 1.00     | 0.99      | 0.96      | 0.88      | 0.88      | 0.97       |
| pAT        | 0.81       | 0.86     | 0.88     | 0.94      | 0.93      | 0.83      | 0.84      | 0.88       |
| pCA        | 1.28       | 1.31     | 1.21     | 1.26      | 1.17      | 0.92      | 1.04      | 1.04       |
| pCC        | 0.89       | 0.83     | 0.94     | 1.15      | 0.44      | 0.48      | 0.41      | 0.52       |
| pCG        | 0.38       | 0.45     | 0.54     | 0.46      | 0.38      | 0.44      | 0.48      | 0.52       |
| pCT        | 1.18       | 1.20     | 1.16     | 1.07      | 1.15      | 1.09      | 1.09      | 1.10       |
| pGA        | 0.90       | 0.95     | 0.89     | 0.88      | 0.89      | 0.87      | 0.82      | 0.87       |
| pGC        | 1.09       | 1.15     | 1.16     | 1.32      | 1.19      | 1.10      | 1.11      | 1.18       |
| pGG        | 0.96       | 0.94     | 0.93     | 0.88      | 0.89      | 0.86      | 0.92      | 0.91       |
| pGT        | 1.07       | 0.99     | 1.03     | 1.03      | 1.07      | 1.11      | 1.12      | 1.07       |
| pTA        | 0.83       | 0.79     | 0.89     | 0.92      | 0.96      | 0.80      | 1.12      | 0.88       |
| pTC        | 0.79       | 0.85     | 0.84     | 0.70      | 0.79      | 0.71      | 1.12      | 0.76       |
| pTG        | 1.39       | 1.41     | 1.33     | 1.31      | 1.26      | 1.43      | 1.12      | 1.35       |
| pTT        | 1.04       | 1.00     | 0.97     | 1.00      | 0.97      | 1.03      | 1.12      | 1.00       |

Usage bias of HCoV-HKU1/HCoV-NL63 probably associated with low viral expression and genes (e.g., distinct patterns between ORF1a/1b/S, M/N and E respective of structure and non-structure proteins) may explain, in part, the different strategies employed in the viral life cycle to evade/manoeuvre host responses, such as deregulated expression in persistent infection, capacity for cell invasion and damage leading to pneumonia, and immune response to host barriers. Both parametric and nonparametric algorithms support distinct separation of HCoV clusters, suggesting different evolutionary histories that the viruses may have encompassed within certain ecological niches or host animals before transmission to humans, while the origin of SARS-CoV and SARS-CoV-2 sharing similar codon usage bias and dinucleotide suppression may converge.

It has been reported that codon usage preferences among viruses and bacteria reflect a balance between mutational biases, genetic drift and natural selection for translational optimization (Bulmer, 1987; Herschberg and Petrov, 2008). Among seven identified human coronavirus clusters, SARS-CoV, SARS-CoV-2 and MERS-CoV are highly pathogenic and have been linked to the development of acute respiratory distress syndrome while increasing evidences indicate that they were also different in transmission, mortality, susceptible population, and early clinical manifestations. Since synonymous mutation is often thought to be selectively neutral, the observed variation in codon usage between different HCoV clusters and their genes may suggest the presence of mutational bias and/or selective pressure that may impact translational efficiency. In RNA viruses, constraints on RNA structures necessary for replication and packaging have also been invoked as another selection cation. However, the virus may have significantly different codon usage of human coronaviruses, particularly for the highly pathogenic clusters in facilitating the immune evasion warrants further investigation. Thirdly, codon usage and dinucleotide composition in HCoV genomes could be due in part to host driven CpG elimination pressure. In mammalian host genomes the CpG dinucleotide is underrepresented, because in this context C is methylated and then deaminated, producing C-T transition (Lister et al., 2009). This creates an obvious mutational pressure on codon usage and results in underrepresentation of NGC codons. DNA methylation functions as a host defence mechanism by regulating gene expression (Shackelton et al., 2006; Upadhyay et al., 2013). When CpG residues of foreign DNA were methylated, pathogen activity can be repressed due to alterations in pathogen transcriptional profiles. CpG repression in RNA viruses might be associated with viral base composition, synonymous codon usage and host selection (Upadhyay et al., 2013). Similar to a number of +ssRNA viruses, HCoV genomes mimic host’s CpG usage, which could be tied to evolutionary selection in regulating gene expression, assisting immune evasion, and avoiding C to T mutation elicited by host CpG elimination pressure. However, the extent and dynamics of CpG methylation in HCoV genome and the role in vegetative viral replication and progression to disease remains uncertain.

Codon usage bias of viruses may facilitate persistent infections and/or reinfection in hosts. In contrast, optimization to hosts’ codon usage dramatically increases the expression levels of pathogenic genes, which may provide an important consideration in developing effective vaccines. Vaccination with codon-optimization for specific viral genes, such as HPV16 E6/E7 (Lin et al., 2006; Steinberg et al., 2005), avian influenza virus H5N1 HA (Stachyra et al., 2016), HIV-1 reverse transcriptase
relationships between SARS-CoV and SARS-CoV-2 imply similar evolutionary history, immune exposure, expression and evasion, virulence potential, and probably pathogenicity of human coronaviruses. The close genetic relationships revealed by parametric algorithms. We also observed sharp codon usage patterns amongst genes, mainly between long and short genes, and between structural and non-structural genes. The close relationships between SARS-CoV and SARS-CoV-2 imply similar evolutionary origin, while the lower ENC values in SARS-CoV-2 genome may indicate stronger codon usage bias demonstrating its lower gene expression and/or better host adaptation. The greater variability in synonymous codon usage within coronavirus genomes indicates more complex evolutionary histories in which ancient viral divergence coupled to niche and/or host adaptation has fixed a number of conserved properties, whereas virus divergence and other evolutionary considerations have led to variability within certain limits. The observations raise the complexity of the mechanistic basis that may contribute to niche adaptation, immune exposure, expression and evasion, virulence potential, and probably pathogenicity of human coronaviruses. The forces affecting synonymous variation in coronaviruses cannot be definitively identified by computational means alone; given the short period of SARS-CoV-2 pandemic and potential mutation of RNA viruses through transmission. However, characterization of coronavirus heterogeneity via complementary approaches provides an opportunity to further explore viral genome evolution, regulation and pathogenesis, and the fundamental mechanism of virus-host interaction.

5. Materials and methods

5.1. Data availability

A total of 3557 complete genome sequences assigned to the genera Alphacoronavirus and Betacoronavirus available on the Global Initiative on Sharing All Influenza Data (GISAID) and USA National Center for Biotechnology Information (NCBI) were clustered to 2522 genomes using a similarity threshold of 0.1 %. These sequences were globally aligned to check potential errors, and 5'- and 3'-UTR regions were trimmed. Among them, all sequences assigned to the subgenus Sarbecovirus (n = 114) and belonging to HCoV clusters (n = 355) were retained for further analysis. In addition, fifty-two reference sequences representing each coronavirus species within the family Coronaviridae were downloaded from NCBI public domain (Tables S1 and S2).

5.2. Phylogenetic analysis

The nucleotide sequences of each ORF were aligned using translation algorithm based on the aligned amino acid sequence matrix using MAFFT within Geneious Primer package. Maximal likelihood (ML) trees were constructed using RAxML MPI v8.2.12 (Stamatakis, 2006) with optimized parameters via CIPRES Science Gateway (Miller et al., 2010). Phylogenetic trees were constructed using MAFFT v7.402 (Katoh and Toh, 2010) inferred from the concatenated nucleotide sequence alignments of 6 open reading frames (ORFs) shared by all coronaviruses.

5.3. K-mer spectrum clustering

We chose $k = 3$ (a total of $4^3 = 64$ trimers) in consideration of the relative short size of viral genome. The trimer frequencies in percentage were normalized and a Kullback-Leibler (KL) distance matrix (Kullback, 1987) was calculated, based on which a hierarchical cluster analysis was performed for phylogenetic topology. We used count function in R’s package ‘seqinr’ (Charif and Lobry, 2007) to count the number of each trimer; and hclust function in R’s package ‘stats’ (Team, 2014) to construct the hierarchical tree.

5.4. Codon usage bias

The effective number of codons (ENC) statistic is a way of analysing how biased a gene is in terms of its codon usage (Wright, 1990). The ENC values range from 20 when a gene is effectively using only a single codon for each amino acid (strongest bias) to 61 when a gene trends to use all codons with equal frequency (no bias). The codonW package (http://cowdon.sourceforge.net/) was used to calculate the ENC values. In order to examine the influence of GC content on codon usage, we plotted the relationship between ENC and GC3s (GC content at the synonymous third codon position). This was compared to the expected ENC* if GC content were solely responsible for the codon biases, calculated as (Shackelton et al., 2006; Wright, 1990):

$$ENC^* = 2 + GC3s + \frac{29}{GC3s + (1 - GC3s)}$$

5.5. Relative synonymous codon usage (RSCU)

Relative Synonymous Codon Usage (RSCU) values for 59 codons (except for Met, Trp, and stop codons) were calculated by using the ratio of the observed frequency of codons relative to the expected frequency in the absence of usage bias to measure the extent of non-random usage of synonymous codons (Sharp et al., 1986). Given that all the synonymous codons for the same amino acids are used equally, the RSCU value would be 1. The value would be much less than 1 if a codon were used less frequently than expected and vice versa. The website tool CAcal (http://genomes.urv.cat/CAcal/) was used to calculate the RSCU values. The RSCU values in the host genomes served as the references, as retrieved from the Kazusa codon usage database (http://www.kazusa.or.jp/codon/) (Nakamura et al., 2000).

5.6. Measuring dinucleotide suppression

It is a well-known phenomenon that CpG dinucleotide is uncommon in most mammalian (including human) genomes, termed CpG suppression. To further understand the potential interplay between viruses and their hosts, we measured the genetic metrics including GC content, dinucleotide suppression ($\rho_{XY}$) and CpG methylation of betacoronaviruses surveyed in this work. The GC content of a string of DNA/RNA is simply the fraction of the letters that are C plus those that are G. A measure of dinucleotide suppression was calculated as the observed frequency of the dinucleotide relative to the product of the frequencies of the individual nucleotides (Karlin and Mrazek, 1997). For example, the ratio

$$\rho_{CG} = \frac{f_{CG}}{f_{C}f_{G}}$$

where $f_{CG}$ represents the frequency of a dimer CG, $f_{C}$ and $f_{G}$ denote the probabilities of its constituent monomers, respectively. The dinucleotide
O/E ratio would be 1 if the occurrences of its individual nucleotide were independent, and the genome exhibits suppression if it has ρXY much less than 1.

5.7. Correspondence analysis

We used correspondence analysis to study the correlation between codon usage and coronavirus genomic composition. This analysis positions each coronavirus genome (row) and relative synonymous codon usage or dinucleotide suppression (column) to create a series of orthogonal axes to identify variation affecting codon usage bias, with each subsequent axis explaining a decreasing amount of the variation. Multidimensional scaling of the redundancy analysis (RDA), or optionally principal coordinate analysis (PCoA) was applied using rda function in R’s package ‘vegan’ to generate two-dimensional representations for matrix 1 and 2, and visualized using the biplot. By definition, the axes are ordered according to the amount of variance in the data, with samples sharing similar variations clustering together. Differences in codon usage bias were assessed with permutation-multivariate analysis of variance (PERMANOVA) using the adonis2 function in R’s package ‘vegan’.

5.8. Statistical analysis

The significance of differences in the genetic metric measures was tested using phylogenetic generalized linear models (pgls function in R’s package ‘capr’), non-parameter Wilcoxon and Mann-Whitney U test (implemented in R’s package ‘coin’) or a two-way analysis of variance (ANOVA). The Pearson’s correlation coefficient was used to test the association between paired observations (cor.test in R’s package ‘stats’), or a two-way analysis of variance (ANOVA). The Pearson’s correlation coefficient was used to test the association between paired observations (cor.test in R’s package ‘stats’). All plotting and statistical comparisons were performed in R v3.0.2 (Team, 2014) using scripts developed in-house (available upon request).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The complete genome sequences of coronaviruses analysed in this study were downloaded from the Global Initiative on Sharing All Influenza Data (GISAID) and USA National Center for Biotechnology Information (NCBI) (see list in Table S1).

Authors’ contributions

ZC, data curation, formal analysis, writing-original draft and editing; SSB, formal analysis, writing-review and editing; MHW, formal analysis, writing-review and editing; RWYC, formal analysis, writing-review and editing; PKSC, supervision, writing-review and editing.

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Declaration of Competing Interest

The authors declare that they have no competing interests. PC is not involved in the review of this manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.jvirmet.2020.114032.

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