Stepwise evolution and exceptional conservation of ORF1a/b overlap in coronaviruses

Han Mei¹, Sergei Kosakovsky Pond², and Anton Nekrutenko¹

¹Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA, USA.

²Department of Biology, Institute for Genomics and Evolutionary Medicine, Temple University, Philadelphia, PA, USA.
The programmed frameshift element (PFE) rerouting translation from ORF1a to ORF1b is essential for propagation of coronaviruses. The combination of genomic features that make up PFE—the overlap between the two reading frames, a slippery sequence, as well as an ensemble of complex secondary structure elements—places severe constraints on this region as most possible nucleotide substitution may disrupt one or more of these elements. The vast amount of SARS-CoV-2 sequencing data generated within the past year provides an opportunity to assess evolutionary dynamics of PFE in great detail. Here we performed a comparative analysis of all available coronaviral genomic data available to date. We show that the overlap between ORF1a and ORF1b evolved as a set of discrete 7, 16, 22, 25, and 31 nucleotide stretches with a well defined phylogenetic specificity. We further examined sequencing data from over 1,500,000 complete genomes and 55,000 raw read datasets to demonstrate exceptional conservation and detect signatures of selection within the PFE region.

Coronaviruses have large 26-32 kbp positive-strand RNA genomes. The initial ⅔ of the genome is occupied by an open reading frame (ORF) ORF1ab encoding non-structural proteins essential for the coronaviral life cycle. As the designation "ab" suggests, it contains two reading frames with the 3’-end of ORF1a overlapping with the 5’-terminus of ORF1b. ORF1b is in -1 phase relative to ORF1a and translated via the -1 programmed ribosomal frameshifting controlled by the PFE. As ORF1b encodes crucial components of coronavirus transcription/replication machinery, including the RNA-dependent RNA polymerase (RdRp), disrupting PFE abolishes viral replication completely (Brierley 1995; Plant et al. 2010; Sola et al. 2015; Kelly et al. 2020). PFE consists of three consecutive elements: (1) an attenuator loop, (2) the “NNN WWW H” slippery heptamer, and (3) a pseudoknot structure (Kelly et al. 2020; Huston et al. 2021). The sequence and structural conformation of these elements determines the efficiency of the frameshift event, which ranges from 15 to 30% in SARS-CoV and SARS-CoV-2 (Baranov et al. 2005; Kelly et al. 2020). Because disruption of PFE arrests viral replication, it is a promising therapeutic target. As a result, a number of recent studies have scrutinized its characteristics (reviewed in (Rangan et al. 2021)) revealing a fluid secondary structure (Iserman et al. 2020; Ziv et al. 2020; Huston et al. 2021). In addition to secondary structures PFE harbors the overlap between ORF1a and ORF1b. It is defined as the stretch of sequence from "H" in the slippery heptamer to the stop codon of ORF1a. The position of the ORF1a stop codon determines overlap length. For example, in SARS-CoV-2 it is 16 bp, while in mouse hepatitis virus (MHV) it is 22 nt (Plant et al. 2010).

Our group has been interested in the evolutionary dynamics of overlapping coding regions (Nekrutenko et al. 2005; Chung et al. 2007; Szklarczyk et al. 2007). The vast amount of newly
generated sequence and functional data—a result of the current SARS-CoV-2/COVID-19 pandemic—provides an opportunity to reexamine our current knowledge. The length of the ORF1a and ORF1b overlap is phylogenetically conserved. It evolved in a stepwise manner, where the changes in the overlap length are results of the loss of ORF1a stop codons leading to ORF1a extension, and the acquisition of insertions and deletions causing early stops of ORF1a.

Distance-based methods had shown that the δ-coronavirus genus was an early split-off lineage compared to α-, β-, and γ-coronavirus (Fig. 1). Comparisons of the RdRp, 3CLpro, HEL, M, and N proteins suggested that γ- was more closely related to δ-coronavirus, while α- and β-coronavirus cluster together forming a distant clade (de Groot et al. 2012; Lau et al. 2012; Woo et al. 2012; Coronaviridae Study Group of the International Committee on Taxonomy of Viruses 2020). However, comparing the S protein trees, α- and δ-coronavirus share a higher amino acid identity, while β- and γ-coronavirus cluster together (Lau et al. 2012). Due to this we initially assumed that α, β, and γ form an unresolved trifurcation (Fig. 1). To assess all possible configurations within this region we surveyed all genomic sequences of family Coronaviridae available from the National Center for Biotechnology Information (NCBI; see Methods). The distribution of overlap lengths among 4,904 coronaviral genomes (Table S1) is shown in Supplemental Fig. 1. There are five distinct overlap length groups (7, 16, 22, 25, and 31 nt) with clear taxonomic specificity.

We then compared the first 15 amino acids of ORF1b in all 4,904 entries (Fig. 2). The amino acid sequences are highly conserved: positions 1 (R), 2 (V), 4 (G), 7 (S), 11-13 (ARL), and 15 (P) are almost invariable and highly redundant. Next, we compared the underlying nucleotide sequences of the PFE region (Fig. 3). This suggests the following potential series of events. δ-coronavirus with 7 nt overlap most likely represents the ancestral state. Comparing coronaviruses with 7 nt (δ-coronavirus) and 31 nt (α- and γ-coronavirus) in the overlap, the stop codon to generate a 7 nt overlap is abolished at positions 5–7, through substitutions, which extends ORF1a to the next available stop codon at positions 38–40. This extension results in a new overlap with 31 nt in length (Fig. 3A). Comparing coronaviruses with 31 nt (α- and γ-coronavirus) and 25 nt (β-coronavirus/Nobecovirus) overlaps reveals a "GTA" insertion at positions 28–30. "TA" from the "GTA" together with the following "G" forms a new stop codon leading to a 31 → 25 nt shortening of the overlap. In a Nobecovirus with a 25 nt overlap, the 31 nt overlap stop codon (at positions 38–40) is still observable (Fig. 3B). Further comparison of coronaviruses with 31 nt (α- and γ-coronavirus) and 22 nt (β-coronavirus/Embervirus and Merbecovirus) overlaps revealed a "GTA" insertion as well, but at positions 22–24. "TA" at positions 23–24 and the following "A" or "G" at position 25 constitute a new stop codon. In the 22 nt
overlap, substitutions have been observed at the original stop codon (at positions 38–40) from 31 nt overlap coronaviruses; more specifically, "C" appears at position 39 (Fig. 3C). Finally, we compared coronaviruses with 31 and 16 nt length in the overlap. The same "GTA" insertion footprint was found, at positions 16–18 ahead of the two "GTA" insertions in 31 → 25 nt and 31 → 22 nt events. "TA" at positions 17–18 and the following "A" at position 19 form the stop codon in the 16 nt overlap coronaviruses. In addition, deletions at positions 13–15 were observed (Fig. 3D). These deletions are referred as "TCT"-like, since "TCT" are the dominant nucleotides observed at positions 13–15 in the 7 and 31 nt overlap coronaviruses. At positions 38–40, the ancestral stop codon in the 31 nt overlap coronaviruses can not be seen, since the nucleotide at position 39 is invariably represented by "T" (Fig. 3D). The variable position of the stop codon likely has an implication to the frameshift efficiency in these taxa as was shown by Bhatt et al. (Bhatt et al. 2021). These authors demonstrated that extension of the distance between the slippery heptamer and the stop codon of 0-frame decreases frameshifting frequency: an increase in the distance by 15 nucleotides, as is the case in α- and γ-coronaviruses (Fig. 3), decreases efficiency by ~20%, while removal of the stop decreases it by half.

The abundance of SARS-CoV-2 sequencing data allows examining the substitution dynamics that may be present in population- and individual-level sequencing data. For population-level analysis we identified variants in the PFS region from >1,550,000 genome sequences available from GISAID (see Methods). However, because GISAID contains only assembled genomes, this data does not provide information about individual-level (intrasample) variation. Hence, we performed an additional detailed analysis of >55,000 samples generated with the COG-UK (Lythgoe et al. 2021) consortium (see (Maier et al. 2021) for analysis details). A summary of results from both analyses is shown in Table 1. There is little variation in the PFE region as the fraction of samples containing individual substitutions appears to be small (the two “Count” columns in Table 1). Furthermore, the 30 out of 36 substitutions in Table 1 are consistent with being a result of RNA editing events from APOBEC (Chen and MacCarthy 2017) or ADAR (Bazak et al. 2014) enzymatic complexes. The remaining six substitutions (all transitions) are predominantly located in the loop regions of the predicted PFE secondary structure (Huston et al. 2021) and thus likely have minimal effect on the secondary structure.

Through a comparative analysis of GISAID sequences, we found that several codons with non-negligible levels of variation (Table 2) were subject to purifying selection: RdRp:1 (A13,443>C/G), RdRp: 31 (13,532 A>G/C), RdRp: 32 (13,535 C>T). This is consistent with a strong degree of functional constraint. Interestingly, this analysis also identified a single codon: RdRp:T26I (13,516 T>C), which has been subject to pervasive positive selection since early 2021. Most of the sequences with this
substitution are in the B.1.1.7 and B.1.177.77 lineages (this is a consensus majority mutation in B.1.177.77 and B.1.614 lineages). RdRp:T26I is present at low frequencies in many viral lineages, but is increasing in prevalence in recent months (0.5-1.0% global prevalence in recent samples). Functional significance, if any, for this substitution has not been reported.

Our results provide an alternative way to assess exceptional conservation of the PFE using publicly available sequence data highlighting the fact that the entire PFE region appears to be under strong purifying selection. These patterns are similar to observations obtained from deep mutational scanning where any alteration at the majority of PFE region sites results have deleterious effects on the frameshift efficiency (e.g., (Carmody et al. 2021)).
Materials and Methods

Coronavirus entries retrieval and filter

The 35,152 coronaviral entries in the NCBI taxonomy database were sorted by length, and only those larger than 14,945 nt were kept, leaving a total of 4,939 genomes. The slippery site and following overlap sequences were manually inspected, in case the slippery site was incorrectly annotated. We further filtered out those entries if they contain no annotation information, or have gapped sequences in the overlap. By applying these filters, we finally had 4,904 coronavirus entries (Table S1), of which the overlap could be unambiguously determined.

Amino acid alignment and nucleotide alignment of the overlap region

For all δ-coronavirus entries in Table S1, the first 13 amino acids of ORF1b were taken to generate a consensus sequence using WebLogo (Crooks et al. 2004). The same was done to α-coronavirus and γ-coronavirus. Within β-coronavirus, for Nobecovirus, Embecovirus, and Merbecovirus, the first 14 amino acids were used to build the consensus; for Hibecovirus and Sarbecovirus, the first 13 amino acids were used. In terms of the nucleotide sequence alignments, for each genus/subgenus, the nucleotide sequences used to generate the amino acids mentioned above were taken to make the nucleotide consensus sequence using WebLogo.

Processing of GISAID data

Each genome was subjected to codon-aware alignment with the NCBI reference genome (accession number NC_045512) and then subdivided into ten regions based on CDS features: ORF1a (including nsp10), ORF1b (starting with nsp12), S, ORF3a, E, M, ORF6, ORF7a, ORF8, N, and ORF10. For each region, we scanned and discarded sequences containing too many ambiguous nucleotides to remove data with too many sequencing errors. Thresholds were 0.5% for the S gene, 0.1% for ORF1a and ORF1b genes, and 1% for all other genes. We mapped individual sequences to the NCBI reference genome (NC_045512) using a codon-aware extension to the Smith-Waterman algorithm implemented in the BioExt package (Pond et al. 2005; Gianella et al. 2011) translated mapped sequence to amino-acids. Codon sequences were next mapped onto the amino-acid alignment. Variants were called directly. Selection analyses were performed using the protocols used previously (Faria et al. 2021; Tegally et al. 2021) based on the FEL analysis (Kosakovsky Pond and Frost 2005) within the HyPhy package (Kosakovsky Pond et al. 2019).
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Figure legends

Figure 1. PFE slippery sites and pseudoknot structures in coronaviruses. The slippery site "UUUAAC" is shown in italic. The ORF1a stop codon is shown in red. ORF1b frame starts from the "C" in the slippery site. α-, β-, and γ-coronavirus were plotted as splitting from one common node (black filled circle), with no phylogenetic order shown. The pseudoknot structures of SARS-CoV and MHV are redrawn based on (Plant et al. 2010). The pseudoknot structures of HCoV-229E and IBV are redrawn based on (Plant et al. 2005). HCoV-229E: Human coronavirus 229E, NC_002645.1. MHV: Mouse hepatitis virus, NC_001846.1. Bat Hp-β-coronavirus/Zhejiang2013, NC_025217.1. MERS-CoV, NC_019843.3. Ro-batCoV HKU9: Rousettus bat coronavirus HKU9, NC_009021.1. SARS-CoV, NC_004718.3. IBV: infectious bronchitis virus, NC_001451.1. BuCoV HKU11: Bulbul coronavirus HKU11, NC_011547.1.

Figure 2. Amino acid alignment of the first 13–14 amino acids in coronaviruses with different lengths in the overlap region. For each genus/subgenus shown, all coronavirus entries belonging to this group are used to generate the consensus amino acid sequences. Gaps were left to make the amino acid sequences align.

Figure 3. Nucleotide alignment of the overlap in coronaviruses with 7, 31, 25, 22, and 16 nt. The footprints of substitutions, insertions, and deletions are shown in black boxes, and labelled as "SUB", "IN", and "DEL", respectively. The stop codon of ORF1a in each of the 7, 31, 25, 22, and 16 nt overlap coronaviruses is shown in a red box.

Supplemental Figure 1. A. Schematic representation of the overlap between ORF1a and ORF1b. "TTT AAA C" is the slippery site. ORF1a frame 0 is shown in white boxes. "TTT AAA C" and the ORF1a stop codon are shown in upper case letters. ORF1b frame -1 is shown in black boxes. The -1 frameshifting starts from "C" of "TTT AAA C", which is indicated at the bended arrow. * = the stop codon of ORF1a. B. Distribution of the length of the overlap in different genera/subgenera.
ORF1a frame 0: ... T TTA AAC ... TAA/TAG/TGA

ORF1b frame -1: ... Cnn ...

Translation of ORF1b frame -1, starting from Cnn (R):

**δ-coronavirus**

\[ x = 7 \text{ nt} \]

**α-coronavirus & γ-coronavirus**

\[ x = 31 \text{ nt} \]

**β-coronavirus/Nobecovirus**

\[ x = 25 \text{ nt} \]

**β-coronavirus/Embécovirus & β-coronavirus/Mercecovirus**

\[ x = 22 \text{ nt} \]

**β-coronavirus/Hibeovirus & β-coronavirus/Sargecovirus**

\[ x = 16 \text{ nt} \]
**A**

ORF1a frame 0: ...

\[
\begin{align*}
\text{\(\delta\)-coronavirus} \\
\downarrow \quad x = 7 \rightarrow 31 \text{ nt} \\
\alpha\text{-coronavirus} & \& \gamma\text{-coronavirus}
\end{align*}
\]

**B**

\[
\begin{align*}
\alpha\text{-coronavirus} & \& \gamma\text{-coronavirus} \\
\downarrow \quad x = 31 \rightarrow 25 \text{ nt} \\
\beta\text{-coronavirus}/\text{Nobecovirus}
\end{align*}
\]

**C**

\[
\begin{align*}
\alpha\text{-coronavirus} & \& \gamma\text{-coronavirus} \\
\downarrow \quad x = 31 \rightarrow 22 \text{ nt} \\
\beta\text{-coronavirus}/\text{Embecovirus} & \& \beta\text{-coronavirus}/\text{Merbecovirus}
\end{align*}
\]

**D**

\[
\begin{align*}
\alpha\text{-coronavirus} & \& \gamma\text{-coronavirus} \\
\downarrow \quad x = 31 \rightarrow 16 \text{ nt} \\
\beta\text{-coronavirus}/\text{Hibecovirus} & \& \beta\text{-coronavirus}/\text{Sarbecovirus}
\end{align*}
\]
Table 1. Allelic variants within the PFE region are called from complete GISAID genomes (Population) and COG-UK (Individual) data. * = potential APOBEC-edited sites; + = potential ADAR-edited sites. Site numbering is in 0-based coordinates; † = out of 1,525,442 complete genomes; ‡ = out of 55,163 individual samples. Locations of substitutions in a stem (S) or a loop (L) are based on structures predicted by Huston et al. (H) and Bhatt et al. (B). ↓ and ↑ highlight sites showing signatures of negative and positive selection, respectively (see Table 2).

| Site   | H | B | Reference | Alternate | Count | Alternate | Min AF | Max AF | Count |
|--------|---|---|-----------|-----------|-------|-----------|--------|--------|-------|
| *13,425 | C | T |          | T         | 1,812 | -         | -      | -      | -     |
| *13,429 | C | T |          | T         | 460   | -         | -      | -      | -     |
| *13,430 | C | T |          | T         | 169   | -         | -      | -      | -     |
| *13,431 | C | T |          | T         | 517   | -         | -      | -      | -     |
| +13,432 | A | G |          | G         | 110   | -         | -      | -      | -     |
| *13,434 | G | A |          | A         | 213   | -         | -      | -      | -     |
| *13,435 | C | T/A|        | T/A       | 1,328/120 | T | 0.116 | 0.971 | 14    |
| +13,437 | T | C |          | C         | 195   | C         | 0.985 | 0.988 | 5     |
| 13,440 | S | S |          | G         | 116   | -         | -      | -      | -     |
| +13,443 | A | G/T |       | G/T       | 134/22 | -       | -      | -      | -     |
| *13,445 | C | T |          | T         | 680   | T         | 0.968 | 0.970 | 25    |
| *13,447 | G | A |          | A         | 16    | -         | -      | -      | -     |
| *13,451 | C | T |          | T         | 393   | T         | 0.941 | 0.977 | 19    |
| *13,457 | C | T |          | T         | 3,663 | T         | 0.052 | 0.963 | 19    |
| *13,458 | G | - |          | A         | 0.069 | 0.970 | 6     |
| 13,458 | L | L |          | G         | 1,220 | T         | 0.080 | 0.976 | 6     |
| +13,481 | A | G |          | G         | 9     | -         | -      | -      | -     |
| *13,486 | C | T |          | T         | 1,656 | T         | 0.055 | 0.965 | 7     |
| +13,487 | A | G |          | G         | 151   | G         | 0.901 | 0.949 | 12    |
| +13,497 | A | G |          | G         | 434   | -         | -      | -      | -     |
| *13,498 | C | T |          | T         | 189   | -         | -      | -      | -     |
| *13,500 | C | T |          | T         | 243   | -         | -      | -      | -     |
| 13,504 | S | G |          | G         | 102   | -         | -      | -      | -     |
| *13,505 | C | T |          | T         | 314   | T         | 0.887 | 0.917 | 5     |
| 13,511 | S | A |          | T/G/C     | 121/58/11 | -       | -      | -      | -     |
| 13,512 | S | A |          | T/G/C     | 114   | -         | -      | -      | -     |
| *13,513 | G | A |          | A         | 342   | -         | -      | -      | -     |
| *13,514 | C | T |          | T         | 495   | T         | 0.065 | 0.889 | 6     |
| *13,516 | C | T |          | T         | 4,272 | T         | 0.101 | 0.840 | 49    |
| 13,525 | S | S |          | A         | 104   | -         | -      | -      | -     |
| +13,526 | T | C |          | C         | 117   | -         | -      | -      | -     |
| +13,532 | A | G |          | G         | 742   | -         | -      | -      | -     |
| *13,535 | C | T |          | T         | 11,942 | T | 0.215 | 0.841 | 23    |
| +13,541 | T | C |          | C         | 26    | -         | -      | -      | -     |
| *13,547 | C | T |          | T         | 675   | T         | 0.067 | 0.898 | 8     |
| *13,550 | C | T |          | T         | 2,346 | T         | 0.878 | 0.921 | 11    |
Table 2. Sites with selection signatures identified using a fixed effects likelihood method on internal branches using SARS-CoV-2 phylogeny built from GISAID sequences (FEL; (Kosakovsky Pond and Frost 2005)). Here $\alpha < \beta$ signifies positive selection, while $\alpha > \beta$ is indicative of negative selection.

| Codon | Nucleotide | $\alpha$ | $\beta$ | $\omega$ | LRT p-value |
|-------|------------|---------|--------|--------|-------------|
| 1     | 13,443     | 0       | 0      | 4.286  | 0.002       |
| 31    | 13,352     | 7.040   | 0      | 0      | 0.015       |
| 26    | 13,516     | 0       | 4.722  | $\infty$ | 0.004       |
| 32    | 13,535     | 5.205   | 0      | 0      | 0.035       |
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