# Generation of restriction endonucleases barcode map to trace SARS-CoV-2 origin and evolution.

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# Abstract

Since the first report of SARS-CoV-2 in China in 2019, there has been a huge debate about the origin. In this work, using a different method we aimed to strengthen the observation that no evidence of genetic manipulation has been found by i) detecting classical restriction site (RS) sequence in human SARS-CoV-2 genomes and ii) comparing them with other recombinant SARS-CoV-like virus created for experimental purposes. Finally, we propose a novel approach consisting in the generation of a restriction endonucleases site map of SARS-CoV-2 and other related coronavirus genomes to be used as a fingerprint to trace the virus evolution.


# Introduction

Coronaviruses have been associated with two major disease outbreaks, the severe acute respiratory syndrome (SARS-CoV, 2002) and the Middle East respiratory syndrome (MERS-CoV, 2012)[1]. In December 2019, a new coronavirus (SARS-CoV-2) started to cause viral pneumonia bringing to severe and fatal infection. Although SARS-CoV-2 belongs to the same lineage of CoVs that causes SARS, it is genetically different. Phylogenetic analysis demonstrated the highly similarity between human SARS-CoV-2 and the sequence isolated from the Bat-Cov-raTG13[2] (97.2% identity) and the Pangolin-SARS-CoV[3] (80% identity), particularly in the receptor-binding-domain (RBD) of the S protein, important to mediate binding to human-receptor-angiotensin-converting-enzyme-2 (hACE2)[4]. The World Health Organization declared a coronavirus disease 2019 (COVID-19) pandemic on March 2020. Therefore, one of the major discussions around SARS-CoV-2 has been related to its origin with the assumption that SARS-CoV-2 could have been the result of genetic manipulations or spill-over from laboratories studying these viruses. In March 2020, Anderson and colleagues published a detailed analysis showing that SARS-CoV-2 does not derive from a laboratory construct[5]. Although other several coronavirus experts have discredited the hypothesis of a man-made coronavirus[6–8], here we aim to present a different method based on the analysis of restriction site (RS) sequences in the genome of SARS-Cov-2 to reconstruct its origin.

# Results And Discussion

### What restriction sites (RS) sequence of the viral genome can say: generation of a restriction endonucleases barcoding map.

During the SARS-CoV epidemic outbreak in 2003, a method called reverse genetic to assemble a full-length cDNA of the SARS-CoV-Urbani strain, as a template for manipulation of the viral genome, was published to develop and test candidate vaccines and therapeutics[9]. This resulted in the so-called infectious clone icSARS-CoV containing atypical markers of the wild-type (WT) virus. In particular, several *Bgl*1 RSs were introduced into the icSARS-CoV cDNA, which can be recognized since mutation are included in the newly formed cDNA. **Figure 1A** shows the sequence alignment between the WT SARS-CoV-Urbani and the icSARS-CoV. We highlighted the sequence containing the *Bgl1* RS used to produce icSARS-CoV.

The newly sequences introduced in the recombinant cDNA of SARS-CoV can be used as markers to follow possible virus laboratory spillage. We analysed natural sequences isolated from four different SARS-CoV (hCoV-19-Italy-Vr/hSARS-CoV-19-Wuhan/hCoV-19 Pangolin/Bat-Cov-raTG13) to look for *Bgl1* RS 'marker' (GCCNNNN/NGGC). All the genomes did not contain these sites (**Table S1**). In particular, the hSARS-CoV-19-Wuhan and hCoV-19-Italy-Vr did not show these sites excluding a possible accidental virus spill-over. By analysing the sequences of Bat-CoV-raTG13 and the hCoV-19-Pangolin, we observed that only one sequence from SARS-CoV-Urbani (GCCAGCGTGGT) was found in SARS-CoV-2. This is expected since the first part of these two genomes show high similarities.

Another recombinant SARS-CoV was produced in 2007 which derived from fifteen passages of the SARS-CoV-Urbani in BALB/c mouse lungs, therefore it was named Mouse-Adapted (MA)-SARS-CoV[10]. The homology of MA-SARS-CoV compared with the original SARS-CoV-Urbani is 99.97% with only six distinct nucleotides, that cannot be used as markers of this recombinant virus since same mutations are naturally acquired by the WT-SARS-CoV, as demonstrated from the sequences of other isolated SARS-CoV[10]. Both the icSARS-CoV and the MA-SARS-CoV have become the most widely used recombinant viruses to study SARS-like viruses and no specific sequences were found in the hSARS-CoV-2.

In 2008, a consensus sequence called Bat-SCoV (FJ211859) was generated starting from four Bat-SCoVs genomes HKU3–1 (DQ022305), HKU3–2 (DQ084200), HKU3–3 (DQ084199), and RP3 (DQ071615)[11]. The full-length Bat-SCoV infectious clone, generated with the method described by Yount *et al*,[9] include in the recombinant sequence specific markers such as the *Bgl*1 RSs. These RSs have specific nucleic base pairs in the "N" positions of the recombinant Bat-SCoV (see supplementary figure 5 of Becker *et al.* summarizing all the markers found[11]). We observed that these specific sequences were all absent (**Figure 1B, Figure S1**). This reinforces the theory that these recombinant viruses manipulated in the laboratory cannot be progenitors of the current SARS-CoV-2.

## Other markers to identify the origin of SARS-Cov-2.

In 2008, Ren *et al* showed that SARS-like coronavirus (SL-CoVs) from horseshoe bat, which has a high similarity to SARS-CoV, differed in the N-terminus of the spike protein and particularly in the receptor binding RBS region[12]. Therefore, SL-CoVs were not able to infect hACE2 expressing cells, but only chimeric virus expressing the spike protein of the SARS-CoV were able to bind the hACE2 which is the functional receptor of SARS-CoV. The authors identified a specific region responsible for the virus entrance into hACE2-expressing cells consisting of a minimal region of less than 200 amino acids. Interestingly, this group showed that chimeric spike proteins, whereby different region of the SARS-CoV BJ01 (BJ01-S) spike were substituted into the spike of the bat SL-CoV (Rp3), were able to bind the hACE receptor. We generated *in silico* two of these chimeric spike (CS) sequences (the $CS_{424-494}$ and the $CS_{45-608}$), and then performed a multiple alignment to check similarities between other spikes identified after 2008, including the Bat-Cov-raTG13, the hCoV-19-Pangolin and the hSARS-CoV-2. The similarities of

these two chimeric spikes is limited in the RBD of the spike (**Figure 1C**) and in the polybasic cleavage site (**Figure S2**). Thus, the recombinant spike as possible progenitors of the hSARS-CoV-2 spike sequence can be excluded.

Moreover, we performed a nucleotide blast sequence to find whether these recombinant spikes are found in the recent identified SARS-CoV-2 viruses. As shown in **Figure S3-S5**, we observed that, despite high similarities, many gaps (intended as single base mutations) are present between WT viruses and the recombinant spikes excluding possible manipulations.

The turning point arrived in 2013, when Xing-Yi and colleagues published an important paper showing that a WT bat SL-CoV was capable of using hACE2 as an entry receptor, dispelling the observation that no natural SL-SARS-CoV were able to use hACE2. Interestingly, the newly identified bat SL-CoV-WIV1 had high sequence similarity (99.9% identity) to two other identified WT bat coronaviruses, RsSHC014 and RS3367. This study suggested that direct bat-to-human infection is a possible scenario for some bat SL-CoVs. In 2015, Vineet *et al* made a recombinant virus between the spike of the bat coronavirus SHC014 and the mouse-adapted SARS-CoV backbone[13] using the well establish reverse genetic approach[9]. According to this method, several *Bgl*1 RSs were included into the sequence (**Table S2**). Moreover, the sequences between the newly mutant SARS-CoV has a poor sequence similarity to hCoV-19-Italy-VR and the SARS-CoV-19-Wuhan (**Figure 1D**).

**Unique restriction sequence sites: a novel approach to track the SARS-CoV-2 origin.**

Exploiting the RS sequences as specific markers, we propose an alternative way to trace the SARS-CoV-2 origin. This approach consists in the generation of a RS map of SARS-CoV-2 and the other four related coronavirus genomes. Using the Serial Cloner Restriction Enzyme Library, we generated the RS barcoding map based on the frequency of finding specific RS sequences in the genome. First, we generated a RS barcoding map which was used as genetic fingerprinting of the specific sequence analysed and which easily highlights sequence differences between the genomes. The pattern of the barcode's reconstruction demonstrated high similarity between the coronavirus isolated from the Bat-Cov-raTG13 and the Pangolin, suggesting a naturally evolution and adaptation of the virus. HIV, SARS-CoV and MERS-CoV were used as control (**Figure 2A and Figure S6**).

From the full restriction enzyme barcoding map, we identified in the spike (S) gene a sequence of 300 bp that can be used as barcode to identify the virus and differentiate from others (**Figure 2B and S7**). This approach is low-cost and does not require full sequencing of the virus genome and extended analyses conducted by bioinformaticians. Indeed, by using a standard PCR reaction to amplify the above mentioned 300bp spike gene, or simply by using real-time PRC products from swab test, and subsequent sequencing of this region, it is possible to generate an RS barcode that will give us a low-cost system to follow viral mutation and trace it over subsequent years. Moreover, this approach can easily be used to discriminate between false negative and false positive which are the reasons of important additional socio-economic disruptions[14].

Using the data to generate the full barcode map we performed principal component analysis (PCA) to determine whether the observed frequency of RSs is related to the hierarchical distance of the genomes analysed. The PCA plot shows the top cluster on PC2 formed by the Pangolin and the Bat-Cov-raTG13 lies in close proximity to the hSARS-CoV-Wu (**Figure 2C**). Below, the cluster formed by the bat SARS-CoV related and the SARS-CoV Urbani. The HIV genome and the MERS were used as control and clearly show greater difference in sequence homology from SARS-CoV virus.

In addition, we focalised on informative RSs to perform a hierarchical clustering on the heatmap using Pearson correlation as distance metric (**Figure 2D and S8**). The heatmap confirm that hSARS-CoV-2 and Bat-Cov-raTG13 are closer than hCoV-19 Pangolin and MERS CoV.

Finally, the barcode map of the RSs confirmed the absence of unique sites giving another strong evidence that the SARS-CoV-19 is the product of a natural evolutionary process of single base insertions/deletions or recombination.

We then focused on the unique RS sequences used to modify the viral genome. In particular, we analysed shared sites between SARS-CoV-19, Bat-Cov-raTG13 and Pangolin-SARS-CoV-19. Only six RS sequences were shared between these genomes and their location does not suggest any genetic manipulation. In the Venn diagram shown in **Figure 2E**, there are 12-shared RS. However, they are only six if we consider that some of these enzymes recognize the same sequences. One example is the unique RS sequence recognized by Bsp68I, BtuMI, NruI, RruI found at 319bp on the Bat-Cov-raTG13 and shifted at 334bp on the SARS-CoV-19 and the Pangolin-SARS-CoV-19. This 15 bp shift is due to single base insertions (**Figure 2F**).

Another example is the unique RS sequence GAGCTC recognized by Ecl136II on the SARS-CoV-19 genome that is located at 15081bp, while on the Bat-Cov-raTG13 genome we found two of these sequences, one at 15080bp and the other one at 19768bp. The latter, if it were to be the result of genetic engineering, would be predicted to produce a gap of 6bp, while from the local alignment it is clear that a nucleotide substitution occurred from C to T forming the new site (**Figure S9A**).

Finally, the genomic location of these unique sites does not flank specific ORF. Indeed, engineered RSs are typically expected to be at the beginning and at the end of an ORF. Here, all the unique RSs are located inside the ORFs (**Figure S9B**), thus not easily editable by conventional genetic engineering.

# Conclusions

Here, we analysed the peer-review literature of the SARS-related viruses generated in the laboratory over the years used to study the evolution of Coronaviruses and to generate drugs for their treatment. We have demonstrated through the analysis of RS, that SARS-CoV-2 does not contain peculiar RS or other markers that suggest a manipulation deriving from the recombinant viruses known in the literature. Indeed, the use of RS remains today the simplest, fastest and safest way to modify and study recombinant DNA. Although nowadays other genetic manipulation mechanisms are known that allow no traces to be left,

such as the use of the Crispr-cas system, these remain more disadvantageous because they require higher technical capacity and higher costs and times. Furthermore, according to our knowledge, in the literature, there are no reports of virus modifications through these more sophisticated techniques yet.

Finally, we used RS as markers to build a barcode map that could be uniquely identify a particular virus. We have shown that with our method it is sufficient to sequence a region of 300bp to build a specific barcode to distinguish the genome of a virus and to trace its evolution over time. This would allow us to have useful information quickly and economically during the classic tests performed on swabs.

# Methods

### Genomes used for the study.

SARS-CoV-2 Wuhan-Hu-1, GenBank: MN908947.3; SARS Urbani, GenBank: AY278741.1; HIV-1, GenBank: KY580639.1; Mers, NCBI Reference Sequence: NC_019843.3; Bat SARS-like Rs4231, GenBank: KY417146.1; Pangolin-CoV, GISAID accession numbers EPI_ISL_410721; Bat CoV RaTG13 GenBank: MN996532.1; hCoV-19/Italy/VR (Gisaid accession id: EPI_ISL_422438|2020-03-25).

All the genomic sequences and recombinant spikes sequences used in this study were generated following the materials and methods of the literature taken in considerations and saved in xdna format which is compatible with Serial Cloner. The files are available upon request to the authors

### Alignments

The sequences were aligned using Serial Cloner, Blastn suite[15], ClustalW[16] and Jalview[17].

### Generation of restriction enzyme barcode

The restriction enzyme map barcode of each genome was obtained using Serial Cloner library. Using this software each genome was analysed in order to obtain the frequency of each restriction site to occur in that genome. The total frequencies of all the restriction sites present in the library were used to generate the barcode map. The InteractiVenn[18] was used to make Venn diagram.

### Genomic distance in bp between restriction enzyme sites

The genomic distance in bp between two or more restriction enzymes sites was calculated with serial cloner and then reported graphically using Prism GraphPad v8.

### Principal component analyses (PCA)

PCA analyses was performed on the frequencies of the restriction enzymes sites on the different viruses' genomes and plotted by ggbiplot R-studio. Codes available upon request to the authors.

### Heatmap and hierarchical clustering

The heatmaps were generated in R-studio by using frequencies of the restriction enzymes sites on the different viruses' genomes. The hierarchical clustering was performed using Pearson correlation as distance metric and Ward D clustering algorithm. Codes available upon request to the authors.

### 300bp specific region

The 300bp region was determined analysing the area of major discrepancy (low identity) between genomes, in particular between related genomes. This area was identified inside the spike (S) region. To generate the barcode map of these 300bp regions we used the same method used for the full-length genomes. Thus, we calculated the frequencies of the restriction sites to generate the heatmap.

### Informative sites

As informative sites we chose all those restriction sites that showed strong discrepancy in the cut-off frequency between the various genomes. Thus, to give an example, sites that had a high cut-off frequency in genome A compared to genome B, or sites unique to genome B that are repeatedly frequent in genome A (and vice versa). Then all non-informative sites, designated as those sites equally frequent across genomes, were discarded. In total we selected 104 informative sites here listed: "AatII" "AccBSI" "AcyI" "AfeI" "AloI" "Aor51HI" "AspA2I" "AsuNHI" "AvrII" "AxyI" "BarI" "BbvCI" "BcgI" "BlnI" "BmtI" "BplI" "BsaHI" "Bse21I" "BseYI" "BsiWI" "Bsp19I" "BspOI" "BsrBI" "BssNI" "BstACI" "Bsu36I" "BtgZI" "CchIII" "Cfr9I" "Ecl136II" "Eco32I" "Eco47III" "Eco53kI" "Eco81I" "EcoICRI" "EcoRV" "GdiII" "Hin1I" "Hsp92I" "MbiI" "MreI" "NcoI" "NheI" "NmeAIII" "Pfl23II" "PfoI" "Psp124BI" "PspLI" "PspOMII" "PsrI" "RpaBI" "SacI" "SauI" "SmaI" "SplI" "Sse232I" "SstI" "TspMI" "UcoMSI" "XmaI" "XmaJI" "ZraI" "AasI" "AccIII" "AgeI" "AsiGI" "BsePI" "BshTI" "Bsp13I" "BspEI" "BspMII" "BssHII" "CspAI" "DinI" "DrdI" "DseDI" "EciI" "Eco147I" "EgeI" "FspAI" "KasI" "Kpn2I" "KroI" "KspAI" "McaTI" "Mly113I" "MroI" "MroNI" "NaeI" "NarI" "NgoMIV" "PacI" "PasI" "PauI" "PceI" "PdiI" "PinAI" "PteI" "RceI" "SalI" "SfoI" "SseBI" "SspDI" "StuI". To generate the barcode map of the informative sites we used the method described for the full length and the 300bp region.

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# Declarations

### Ethics approval and consent to participate

Not applicable.

### Availability of data and materials

The datasets and codes used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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The authors received no specific funding for this work.

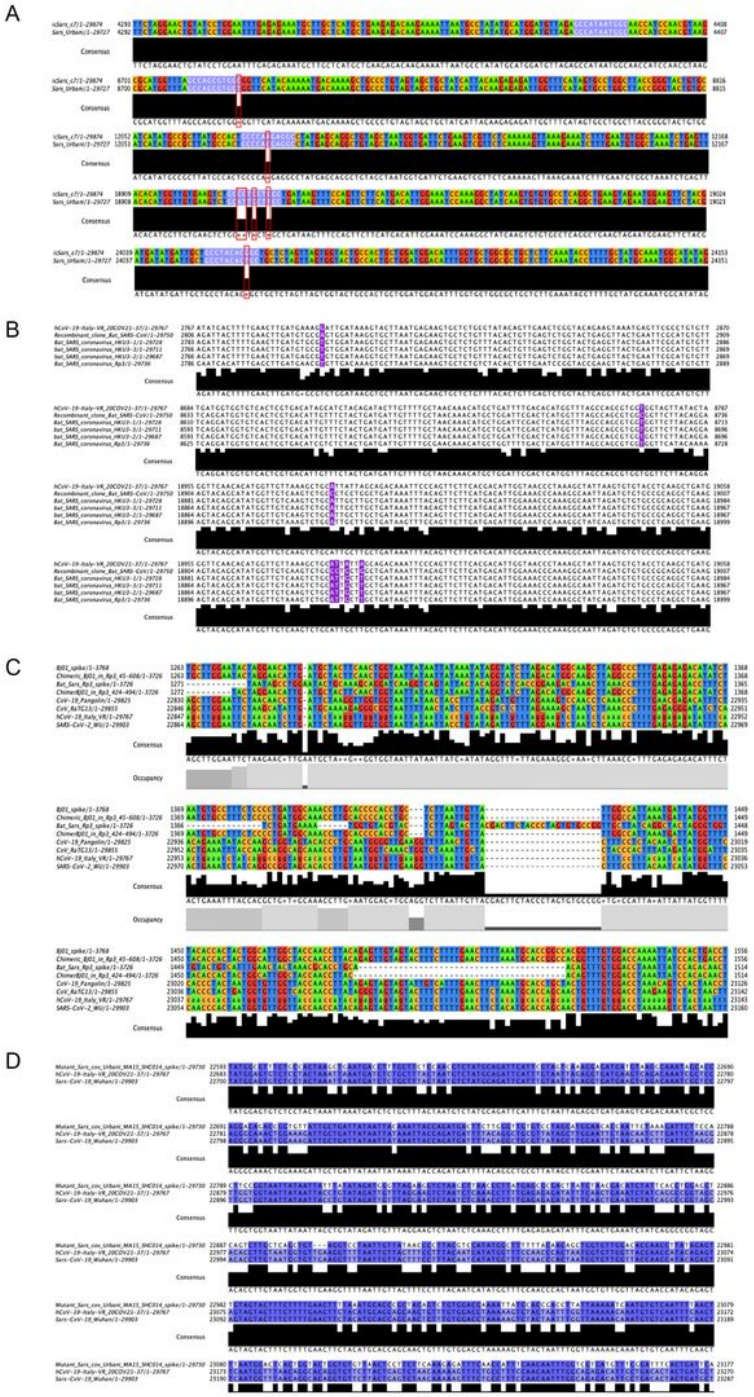
### Authors' contributions

FC designed the study, analysed data and wrote the manuscript. EC helped the study design, analysed the data, wrote and review the manuscript. MJL gave contribution in the analyses of the data, contributed to the interpretation of the results and reviewed the manuscript. CP reviewed the manuscript.

### Acknowledgements

Not applicable.

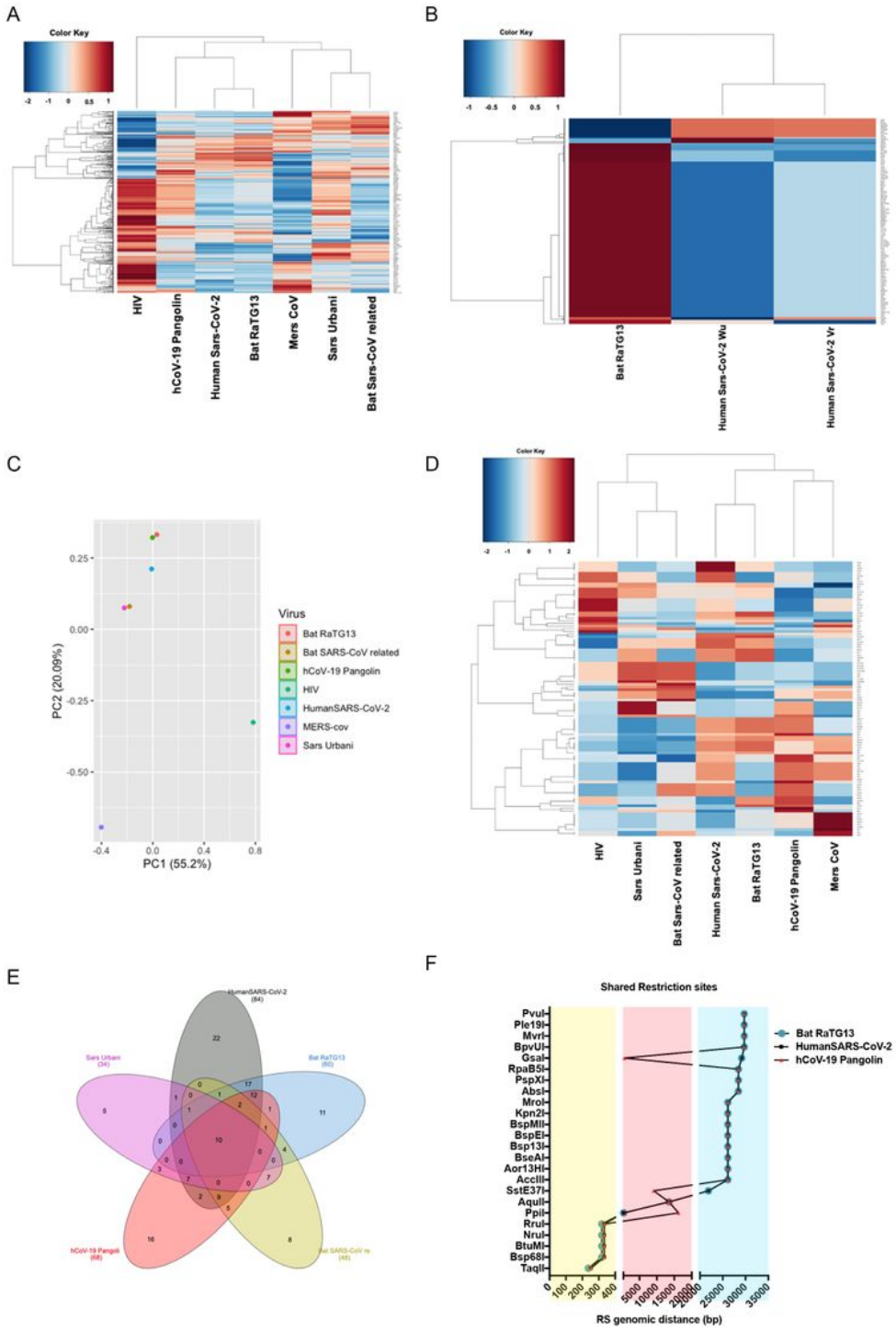
# Figures




## Figure 1

Analyses of several RSs sequences in natural and recombinant viruses. A) Alignment between the WT SARS-CoV Urbani and the icSARS-CoV. The violet box highlight BglI RSs used to build the recombinant icSARS-CoV. The red boxes show the different nucleotides present in the wild type SARS-CoV Urbani. B) This alignment shows in violet specific markers used to build a recombinant spike between the Bat-SCoVs genomes HKU3 and RP3. In the hCoV-19-Italy-VR sequence most of these markers' sites are not

present, while are similar to the wilt type virus HKU3 and RP3. C) Multiple sequence alignment performed with ClustalW and visualised with JalView show the poor similarities in the RBD between chimeric Spikes generated in the laboratory (line 2 and 4) compared with other SARS-CoV sequences. Despite some small regions are conserved the chimeric spikes show single bp mutation (substitution, deletion, insertions) which support natural evolutions instead of man-made manipulation. D) A specific area of the alignment performed between the mutant SARS-CoV-Urbani MA15 containing the SHC014 spike with the hCoV-19-Italy-VR and the SARS-CoV-19 Wuhan. Also, in this case, the recombinant virus shows several nucleotide mutations which exclude the manipulations performed using modified primers and unique restriction sites.




## Figure 2

RS barcodes map help to determine the distance between different viruses' genomes. A) RSs barcode map of 7 different virus genomes. The colour scale represents the frequency to find that specific RS in the genome after using the Serial Cloner Library using 741 RSs. The hierarchical clustering was performed using Pearson correlation as distance metric. It is clear that some barcode patterns highlight similarities between related viruses, while other patterns show dissimilarities, such as the case of the HIV, used as

control, which shows a clear different barcode compared with Sars-CoV-2 genomes. B) RSs barcode map performed on a region of 300 bp previously identified from the full map. Here, we compared the two human SARS-CoV-2 (Wuhan and Italian-VR) with most closed genome of the Bat-RaTG13. The barcode generated easily highlights similarities or differences between genomes presenting high genetic similarities. C) Principal component analysis (PCA) plot generated with the frequencies of the RSs, retrieved from each of these genomes, confirms the same distance between viruses' genomes. D) Barcode Heatmap with hierarchical clustering based on the most informative RSs showing that Human SARS-CoV-2 and Bat-Cov-raTG13 are evolutionarily closer than hCoV-19 Pangolin and MERS CoV. The clustering performed with less RSs confirms that we are still able to generate the right distance metric. E) Venn Diagram shows shared RSs between genomes of different viruses. F) Genomic distance in bp of the shared RSs between SARS-CoV-Wu, Bat RaTG13 and the hCoV-19 Pangolin.

## Supplementary Files

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- SupplementaryFedericoColomboetal.pdf