Analysis of co-occurring and mutually exclusive amino acid changes and detection of convergent and divergent evolution events in SARS-CoV-2

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ABSTRACT

The inflation of SARS-CoV-2 lineages with a high number of accumulated mutations (such as the recent case of Omicron) has risen concerns about the evolutionary capacity of this virus. Here, we propose a computational study to examine non-synonymous mutations gathered within genomes of SARS-CoV-2 from the beginning of the pandemic until February 2022. We provide both qualitative and quantitative descriptions of such corpus, focusing on statistically significant co-occurring and mutually exclusive mutations within single genomes. Then, we examine in depth the distributions of mutations over defined lineages and compare those of frequently co-occurring mutation pairs. Based on this comparison, we study mutations’ convergence/divergence on the phylogenetic tree. As a result, we identify 1,818 co-occurring pairs of non-synonymous mutations showing at least one event of convergent evolution and 6,625 co-occurring pairs with at least one event of divergent evolution. Notable examples of both types are shown by means of a tree-based representation of lineages, visually capturing mutations’ behaviors. Our method confirms several well-known cases; moreover, the provided evidence suggests that our workflow can explain aspects of the future mutational evolution of SARS-CoV-2.

1. Introduction

All viruses, including SARS-CoV-2, change over time. Many organizations, such as the Global Initiative on Sharing all Influenza Data (GISAID) [42], Nextstrain [18] and Pangolin [40], are studying the phylodynamics of SARS-CoV-2 genomes to track and define new variants. At the end of May 2021, the World Health Organization (WHO) announced the usage of a novel nomenclature system for naming and tracking SARS-CoV-2 genetic lineages using letters of the Greek alphabet, now offering a reference to refer to viral variants across the world. In the same announcement, the WHO also introduced a multi-level categorization of variants based on the levels of attention they should raise; namely, variants under monitoring (VUM), variants of interest (VOI), and variants of concern (VOCs). Such levels were defined by evaluating specific measures that define the virus properties and virulence. On a different level, several initiatives such as CoVariants [20] and outbreak.info [15] are working on determining the defining (i.e., characterizing) mutations for each variant.

The evolutionary dynamics of the virus were predominantly characterized by a mutational pattern of slow and selectively neutral random genetic drift. Past pandemics and long-term evolutionary dynamics of RNA viruses attest to the fact that such an evolutionary “lull” rarely lasts [28]. Indeed, in late 2020, three relatively divergent SARS-CoV-2 lineages emerged in rapid succession: B.1.1.7 (Alpha), B.1.351 (Beta), and P.1 (Gamma). Those three lineages were considered as VOCs according to the WHO. Due to the continuation of the pandemic, other lineages emerged and, at the beginning of 2022 we faced the emergence of BA.1 (Omicron) and of its descendant lineages. From the point of view of single mutations accumulated since the beginning of the pandemic, most of them had little to no impact on the virus’ properties (i.e., not epidemiologically significant). However, some changes have arisen that affect the virus to gain specific advances, such as a spread advantage [7], the associated disease severity [16], or the resistance to vaccines [17], antiviral medicines [44], diagnostic tools, and other public health/ social measures [34] (as we analyzed in [1,2]).

Thanks to the continuous spreading of SARS-CoV-2 and the contextual deposition of its viral sequences to public repositories (even considering possible delays [24]), the viral evolution can be monitored and studied to understand SARS-CoV-2 variants and the risks that they pose. Methods that study the virus characteris-

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tics have been developed independently from the phylogenetic techniques traditionally employed in this field. For instance, considerable efforts have been dedicated to building surveillance systems that employ temporal analysis of SARS-CoV-2 mutations to assist in the identification of candidate variants of clinical importance. A number of studies have described typical SARS-CoV-2 mutational profiles across different countries and regions [31], proposing statistical indicators for location-based mutation evolution [45] and observing changes that become recurrently prevalent in different locations, thus suggesting selective advantages [26]. Time-series analyses have been considered for clustering of prevalent SARS-CoV-2 mutations over time [50,8,5,22,11,21], trend detection in SARS-CoV-2 short nucleotide sequences [46], and single amino acid changes [41]. Such works conduct functions that describe the prevalence of different mutations and, when a number of these are behaving similarly, they recognize a possible distinct variant. These approaches usually have an epidemiological angle and are focused on highly present forms of the virus. A different work [12] focuses on patterns of mutations located in relevant domains of the virus that are found in variants of concern but also in emerging variants, suggesting they can be used as a guidance for next evolution moves. At the same time, several studies have analyzed the evolution of mutational patterns that are typical of a specific geographical area [5,10,47,6]. Our approach does not focus on the most spread variants or on groups of numerous mutations, nor it restricts to specific locations. We take an interest in a more micro-level phenomenon, i.e., involving the relationship between single mutations that appear together or separated in different locations, thus suggesting selective advantages [26]. In this research, we employ a previously unexplored perspective: we consider 8 million sequences and we perform a systematic co-occurrence and mutual exclusion analysis of non-synonymous mutations’ pairs. These become our entry point to the SARS-CoV-2 evolution aspects. In the following, we provide a workflow to determine 1) co-occurring pairs of mutations (as preliminarily tested in [1]) and 2) mutually exclusive pairs of mutations (as inspired by research on mutually exclusive human gene pairs [38]). Then, we employ the results from the first phase (co-occurring pairs) to study convergent and divergent evolution events of mutation pairs, also analyzing them from the point of view of single mutations participating to the event. An intuitive visual representation is employed to explain such events and point the attention to a number of interesting cases, which have been validated in the literature. As future work, our workflow encourages the design of a light-weight prediction procedure for the mutational evolution of SARS-CoV-2.

2. Methods

The framework of our study is divided into four parts, discussed in the remainder of this section and overviewed (as dotted-framed areas) in Fig. 1:

1. Data preparation (described in Section 2.1), where we analyze the initial dataset of viral sequences and prepare aggregated intermediate tables.

![Fig. 1. Methodological workflow of the study. The schema is composed of four main parts enclosed in dotted-framed areas: 1) Data preparation; 2) Data Analysis; 3) Lineages distribution-dependent analysis; and 4) Lineages distribution-independent analysis. Legend: S: number of sequences assigned to lineage l; S_m: number of sequences holding a mutation m; S_m,n: number of sequences from lineage l holding mutation m; S_m,n,k: number of sequences assigned to lineage l holding the pair of mutations m_l and m_k; KS test: Kolmogorov-Smirnov test; MC simulation: Monte Carlo simulation.](image-url)
2. Data analysis (Section 2.2), composed of three parts that use specific statistical tests to, respectively i) identify the co-occurring and mutually exclusive pairs of mutations, ii) compare the distributions over lineages of mutations that appear in previously identified co-occurring pairs, and iii) test the evolution events happened between co-occurring pairs.

3. Lineages distribution-dependent analysis (Section 2.3), i.e., an analysis on mutation pairs that takes into consideration how mutations are spread across the lineages; it includes the visualization of convergence/divergence events involving mutation pairs along tree-based structures representing the hierarchy of Pango lineages.

4. Lineages distribution-independent analysis (Section 2.4), i.e., an analysis of co-occurring and mutual exclusive mutation pairs that ignores how mutations are spread across the lineages.

The complete analysis has been performed in Python (Version 3.7.6), using classical data science libraries, i.e., Pandas (Version 1.3.5) for data extraction and aggregation, Scipy (Version 1.4.1) for statistical analysis, Seaborn (Version 0.11.2) and Matplotlib (Version 3.1.3) for data visualization.

2.1. Data preparation

A set of over 8.2 million sequences of SARS-CoV-2 genome was collected from GISAID since the beginning of the pandemic until mid of February 2022 to be used in this large scale analysis. Each entry of the initial dataset represents one viral sequence with their assigned Pango lineage [35] and a list of its non-synonymous mutations; these belong to all the proteins of SARS-CoV-2, expressed considering the reference SARS-CoV-2 genome hCoV-19/Wuhan/WIV04/2019 [53].

Data aggregation. From the initial dataset, we prepared three intermediate tables that were used in following analyses as shown in the Data preparation box of Fig. 1:

1. triplets of the form \((m, l, S_m)\), where \(S_m\) represents the number of sequences from lineage \(l\) having a mutation \(m\);
2. for the lineages included in 1), triplets of the form \((l, \{seq_1, \ldots, seq_n\}, S_l)\), where for each lineage \(l\) we have the set of sequences assigned to that Pango lineage and its cardinality \(S_l\);
3. for the mutations included in 1), triplets of the form \((m, \{seq_1, \ldots, seq_n\}, S_m)\), where for each mutation \(m\) we have the set of sequences exhibiting that mutation and its cardinality \(S_m\).

To reduce the size of the initial dataset, we only considered the most frequent mutations, i.e., those found in at least 20 K viral sequences as extracted from the intermediate table 3); they amount to 421. In the following, we refer to these as frequent mutations: we only performed the analysis over this list of mutations.

2.2. Data analysis

2.2.1. Detection of co-occurring and mutually exclusive mutations pairs

In a given population (e.g., the sequences that are associated to a lineage), we define:

- co-occurring pairs of mutations: pairs of mutations that are observed in the same sequences of the reference population a number of times that is significantly higher than the expected one when the two mutations are independent from each other (i.e., frequency of first mutation \(\times\) frequency of second mutation \(\times\) size of the population);
- mutually exclusive pairs of mutations: pairs of mutations that are observed in the same sequences of the reference population a number of times that is significantly lower than the expected one when the two mutations are independent from each other.

Both co-occurring and mutually exclusive pairs are found at the two tails of the hypergeometric distribution of pairs of mutations found within the population. We employ as p-value for our selection the cumulative distribution function (cdf) of the hypergeometric distribution:

\[
P(S_{m_1, m_2} = n) = \frac{\binom{S_{m_1}}{n} \binom{S_{m_2} - n}{P - S_{m_1}} \binom{P}{S_{m_2}}}{\binom{P}{S}}
\]

where \(S_{m_1}, S_{m_2}\) and \(S_{m_1, m_2}\) are the numbers of sequences harbouring mutations \(m_1, m_2\), and both \(m_1\) and \(m_2\), respectively, while \(P\) is the size of the reference population. The p-value of observing \(N\) sequences with both mutation \(m_1\) and mutation \(m_2\) can be computed as:

\[
P(S_{m_1, m_2} > N) = 1 - P(S_{m_1, m_2} \leq N) = 1 - \sum_{n=0}^{N} P(S_{m_1, m_2} = n)
\]

We employ the Python cdf (Cumulative Distribution Function) function of the scipy.stats.hypergeom that, for a pair \((m_1, m_2)\) takes as input the number of sequences with both mutations \(S_{m_1, m_2}\), the total count of available sequences \(P\), and the number of sequences with \(m_1\) and with \(m_2\) \(S_{m_1}\) and \(S_{m_2}\) in order to compute the probability \(P(S_{m_1, m_2} \leq N)\). We compute p-values for all the possible pairs of frequent mutations (FM), that are \(FM \times (FM - 1)/2\). Results are filtered by using suitable p-values (lower than 0.05 correspond to co-occurring pairs and higher than 0.95 correspond to mutually exclusive pairs) as shown in the Data analysis box of Fig. 1.

2.2.2. Distribution of frequent mutations over lineages

We employed lineages as they are assigned by Pangolin [35]. For each mutation \(m\) of our dataset and each available Pangolin lineage \(l\), we computed the count of sequences assigned to \(l\) that hold \(m\), obtaining the triplet \((m, l, S_m)\). Such data is collected within a 421 (frequent mutations) \(\times\) 1,587 (all lineages) matrix. We refer to the mutation \(m\)'s distribution over lineages as the row of the matrix corresponding to \(m\). To focus only on lineages that well-represent the mutation, for each row we set to zero the sequence counts that are less than 0.001 of the total lineage sequences. For each pair of co-occurring mutations extracted during the previous step, we compared the distributions over lineages of each of its two mutations; the comparison was performed by employing the Kolmogorov–Smirnov (KS) test [23] using the scipy.stats.ks_2samp Python method, which is efficient in determining if two samples are significantly different from each other. For a pair \((m_1, m_2)\) it takes as input the two arrays of \(m_1\) and \(m_2\)'s distributions over lineages and the ‘two-sided’ option. A reasonable \(p\) value < 0.05 is used as a threshold of significance (see Data analysis box of Fig. 1).

To support the understanding of these methods' steps, we exemplify them on a minimal example data structure. Assume we prepare a \(3 \times 3\) matrix (Table 1), which collects the counts of sequences holding mutations \(m_0, m_1\), and \(m_2\) taken from sequences assigned to lineages \(L_1, L_2,\) and \(L_3\). The total number of sequences of each lineages are given in parenthesis in the header.

| Lineage | Mutation | Count |
|---------|----------|-------|
| \(L_1\) | \(m_0\) | 100 (200) |
| \(L_2\) | \(m_1\) | 150 (300) |
| \(L_3\) | \(m_2\) | 200 (400) |

...
As $L_1$ sequences with $m_0$ and with $m_1$ are less than $0.001*10000$, then the corresponding counts are set to zero (see Table 2).

The first row of the matrix is the $m_i$’s distribution over lineages. All possible mutation pairs are tested for co-occurrence with the hypergeometric test; let the following pairs be selected: $(m_0, m_1)$ and $(m_0, m_2)$. For each such pair, we compare their distributions over lineages. The KS test is employed to conclude that line $m_0$ is not significantly different from line $m_1$ and that line $m_0$ is significantly different from line $m_2$, yielding to the corresponding alternative steps in the workflow. Depending on the results of the KS test, each pair of co-occurring frequent mutations can be classified in one of the two categories:

- pairs with different distributions over lineages;
- pairs with similar distributions over lineages.

### 2.2.3. Convergent and divergent evolution testing method

We developed a method to identify convergence and divergence events involving two mutations at a time. The method was run for each pair of mutations extracted from the previous step, analyzing their presence within couples of lineages in 1-step relationship on the hierarchical lineages tree (i.e., each node with its direct ancestor). To support the explanation, we refer to Fig. 2, showing a co-occurrence graph (panel A) where mutations (i.e., nodes) are exhibited by the same sequences (i.e., edges that connect co-occurring mutations). This graph is connected with a lineages tree (panel B), where lineages are nodes and edges explain their hierarchical relationships according to the phylogenesis. The mutation graph and the tree are connected when a mutation occurs above the threshold 0.001 of the sequences of a specific lineage.

When considering two mutations co-occurring across the dataset and having different distributions over lineages, we observe a convergence event when both the following conditions occur:

- for each lineage $l$, its sequences holding both mutations of a pair $(m_1, m_2)$ represent a large-enough fraction ($> 0.01$) of the sequences holding exclusively one mutation of the pair (i.e., $S_{m_1, m_2}/S_{m_1} > 0.01$ and $S_{m_1, m_2}/S_{m_2} > 0.01$);
- only $m_1$ or $m_2$ is found in the direct ancestor of $l$.

In panel B of Fig. 2, the blue rectangle surrounds an event of convergence between mutations, in which only mutation $B$ was found in the parent lineage lin.1 whereas both mutations $B$ and $C$ were found together on the same sequences of the sub-lineage lin.1.1.

Several kinds of divergence events can occur, but here we focus on a particular definition. When considering two mutations co-occurring together across the dataset and having similar distributions over lineages, we observe a divergence event when both the following conditions occur:

- $S_{m_1, m_2}/S_{m_1} > 0.01$ and $S_{m_1, m_2}/S_{m_2} > 0.01$ (same condition as for the convergence event);
- only one of the two mutations is found in the direct descendant lineage of $l$.

As shown in panel B of Fig. 2, the orange rectangle surrounds an event of divergence of mutations, in which both co-occurring mutations $C$ and $D$ were found together on the same sequences of the parent lineage lin.2.2.2 whereas its sub-lineages have exclusively mutation $C$ or $D$.

Based on these definitions, we generate two tables containing: 1) the converging pairs of mutations (’Convergence Result’ in the Lineages distribution-dependent analysis box of Fig. 1); 2) the diverging pairs of mutations (’Divergence Result’ in Fig. 1).

For each convergence event (sketched in panel C of Fig. 2), we find a corresponding row in the first table, showing: the pair of converging mutations, the Distinct Ancestor Lineage (DAL, i.e., the lineage with only one of the two pair mutations), the Common Descendant Lineage (CDL, i.e., the sub-lineage with both pair mutations), the Remaining Mutation (RM, i.e., the mutation found both in the ancestor and its descendant lineage), the Acquired Mutation (AM, i.e., the mutation found only in the descendant lineage), the count of sequences assigned to CDL holding both RM and AM, the count of sequences assigned to DAL holding only RM, and the depth of CDL in the lineages tree.

For each divergence event (sketched in panel D of Fig. 2), we find one row in the second table, showing: the pair of diverging mutations, the Common Ancestor Lineage (CAL, i.e., the lineage with both pair mutations), a Distinct Descendant Lineage (DDL, i.e., a sub-lineage with only one of the two pair mutations), the Remaining Mutation (RM, i.e., as before, the mutation found both in the ancestor and its descendant lineage), the Missing Mutation (MM, i.e., the mutation found only in the ancestor lineage), the count of sequences assigned to CAL holding both RM and MM, the count of sequences assigned to DDL holding only RM, and the depth of CAL in the lineages tree.

### 2.3. Lineages distribution-dependent analysis

To study the behavior of pairs of co-occurring mutations along the different lineages in the phylogenetic tree, two aggregated tables were generated from the convergence and divergence tables’ results (shown in the Lineages distribution-dependent analysis box of Fig. 1), by grouping according to the following fields:

- Pairs of co-occurring mutations. We grouped both ‘Convergence Result’ and ‘Divergence Result’ tables by the $(m_1, m_2)$ pair. Since a stronger evidence for converging mutations pairs (resp. diverging) is available when a pair shows more convergence (resp. divergence) events in the lineages tree – also at different depths – the resulting aggregated tables were ranked by the count of CDL (convergent evolution) or by the count of CAL (divergent evolution).

#### Monte Carlo simulation

We used a Monte Carlo (MC) simulation approach to simulate the convergence and divergence tests and calculate the significance of our findings. By employing the Python method sample of the random library, we performed a
simulation of 10,000 rounds, each composed as follows: 1) randomly select as many pairs of mutations as the ones obtained by the hypergeometric test out of the primary pool of unique pairs of mutations; 2) count how many distinct pairs of mutations pass the convergence test; 3) count how many distinct pairs of mutations pass the divergence test; 4) calculate the p – values by comparing the random distributions generated according to 2) and 3) and the real data.

Sub-trees visualization. Graphviz [14] and NetworkX [19] were used to draw lineages trees. Tree-shaped graphs are generated from the aggregated tables representing pairs of co-occurring mutations. One graph is produced for each converging or diverging pair of mutations, following the relationships between lineages as indicated by the Pangolin nomenclature. Each node represents a lineage and lineages are connected by hierarchical relationships (arrows). We start from the original SARS-CoV-2 haplotypes (A or B) and descend the tree up to the node where an evolution event is identified. Since several pairs have a high number of evolution events, we omit the visualization of branches without such events. A color code is used to highlight where convergent/divergent evolution events are identified and which mutations from the considered pair are observed.

2.4. Lineages distribution-independent analysis

Following the hypergeometric test that identifies co-occurring and mutually exclusive mutation pairs, we also perform a lineage-independent analysis, which is not related to the analysis reported in Section 2.3, as it does not consider how mutations are spread across the lineages. More precisely, we employ co-occurring pairs to investigate the defining mutations of lineages. Instead, we employ mutually exclusive pairs to gain insights on the mutations that are preferred during the virus evolution. To further study these two happenings, we incorporate the calculation of fold enrichment as shown in the Lineages distribution-independent analysis box of Fig. 1.

Fold enrichment calculation. The Fold Enrichment (FE) is a general statistical term that indicates how many folds a phenomenon happened more (or less) than expected by random chance. For instance, FE = 3 means that the event happened three times the random expectation for that event. We use the following formula to calculate it:

$$\log_{FE_{ml}} = \log_{S_{m}}(S_{ml} \times (L/S_{m}))$$

where we have that $S_{m}$ is the total number of sequences having a mutation $m$ in a given lineage $l$; $L$ is the total number of lineages found in the population; $S_{ml}$ is the total number of sequences having mutation $m$ in the population. Here we considered $S_{ml}$ as the count of observed events and $S_{m}/L$ as the count of expected events. By using $log_{FE_{ml}}$, the values of enrichment range from $-\infty$ to $+\infty$, where negative $log_{FE_{ml}}$ values indicate that the mutation $m$ is less enriched in lineage $l$ and positive values indicate that the mutation $m$ is more enriched in lineage $l$, while zero means the enrichment is as expected by random chance. Details on how this measure is employed are given in the results (Section 3.2.1), where noteworthy variants are analyzed.

3. Results

3.1. Dataset description

By analyzing the considered dataset (see Section 2.1 in the Methods), we observed the distributions of the sequences over continents and lineages, provided respectively in Fig. 3A and Fig. 3B. Half of the viral sequences of the analysed dataset were collected in the European continent. Sequences were assigned to 1,587 distinct Pango lineages, the most represented lineages being B.1.1.7 (Alpha), AY.4, and BA.1 (Omicron), respectively representing the 13.8%, 10.1%, and 9.2% of the total population. According to the WHO, the current variants of concern (VOCs) are B.1.617.2 (Delta) and Omicron (including all BA.1, BA.2, BA.3, and BA.1.1), while previously circulating VOCs were B.1.1.7 (Alpha), B.1.351 (Beta), and P.1 (Gamma). Fig. 3C presents the counts of sequences from the dataset assigned to distinct lineages that are currently or were previously considered as VOCs; these are distributed by collection date for the whole period of the pandemic.

Almost all the sequences – except for five – exhibit at least one non-synonymous mutation with a total number of 156,951 non-synonymous mutations (substitutions, deletions, or insertions) found in the population with an average of 32.6 mutations per sequence. The most dominant non-synonymous mutations are the substitution D614G in the spike protein, the substitution P223L in the non-structural protein 12, and the substitution T478K in the spike protein, found in 97%, 96.5%, and 63.8% of the population, respectively.

3.2. Co-occurring and mutually exclusive mutation pairs

A total of 88,410 unique pairs were generated from the list of 421 frequent mutations. Using the hypergeometric test (see Methods, Section 2.2.1), we extracted 16,692 co-occurring pairs by using
p-value < 0.05; note that, using a smaller p-value < 0.01 produces 16,415 co-occurring pairs, thus a small decrease. Then, we extracted 69,903 mutually exclusive pairs by using a p-value > 0.95; note that, out of these, 62,491 have p-value = 1, with 374 pairs never appearing together in the same sequence in the entire dataset. Differently from Zhang et al. [52] (where mutations are considered as “high frequency” if found in at least 1% of sequences), we have used a much lower frequency threshold (found in more than 20 K sequences, i.e. 0.25%). Consequently, when performing the hypergeometric test on pairs of mutations, this choice has allowed to widen the possibility of co-occurrence/mutual exclusion detection.

3.2.1. Lineage distribution-independent results

Before proceeding with our main analysis thread, which evaluates mutations w.r.t. their distributions over lineages, we derive a series of interesting observations that can be made just on the basis of the results of the hypergeometric test. Such results are derived using the methods described in Section 2.4. First, by exploiting the extracted co-occurring pairs, we show how they could be used to complement lists of variants’ defining mutations. Second, by exploiting the extracted mutually exclusive pairs, we show how the pairs could be used as insights of the natural evolution of the virus. Results are explained using notable examples.

**Defining mutations of a given lineage: the case of the Delta variant.** The B.1.617.2 variant (Delta) has been considered for a long time as one of the VOCs by WHO. Almost all (19) of its defining mutations (according to CoVariants [20]) are found in our list of frequent mutations (specifically, only 2 out of 21 mutations are missing, but they are also absent in the full dataset). Table 3 presents the p-values derived from running a hypergeometric test on each pair formed by Delta defining mutations in the Spike protein. All pairs have a significant p-value, except for the pair composed by Spike_D614G and Spike_E156 (p-value = 0.54) (likely due to the huge difference in the populations of these two mutations). In general, these p-values suggest that the pairs of mutations tend to co-occur together. Out of all the pairs (m₁, m₂) resulting from the hypergeometric test, we considered the ones where m₁ belongs to Delta defining mutations whereas m₂ does not. Only mutations m₂ with a logFE above a threshold of 1 (i.e., mutations that have been found in Delta’s population at least twice more than expected) were further considered. We identified other 68 mutations that co-occur with all Delta defining mutations and that logFE values above our threshold. Three of these are found in...
the Spike protein: Spike_V1104L (FE: 44.87, logFE: 5.48), Spike_G142D (FE: 41.06, logFE: 5.35), and Spike_S112L (FE: 13.75, logFE: 3.78); the complete set is found in Supplementary Table S1. Our findings suggest that the identified mutations could be considered as additional defining mutations of the Delta variant, complementing the list provided by CoVariants [20]. Properties of significant mutations co-occurring with Delta defining mutations are shown in the scatter plots drawn in the panels of Fig. 4.

Mutually exclusive mutations: the case of the Alpha and Omicron lineages. The variants B.1.1.7 (Alpha) and BA.1 (Omicron) have a very high number of common defining mutations. Among their defining mutations lists, we identified possible mutually exclusive pairs; see Table 4, where pairs with p-value = 1 and mutations from these two variants are reported. We focus on the first two rows where the number of sequences having both mutation equals to zero: (Spike_S371L, Spike_A570D) and (Spike_S371L, NSP3_A890D). Spike_S371L is one of the defining mutations of BA.1, while both Spike_A570D and NSP3_A890D are from the defining mutations of the lineage B.1.1.7 (Alpha). Indeed, Spike_S371L has a logFE of 9.91 in Omicron and \( -\infty \) in Alpha, whereas Spike_A570D and NSP3_A890D both have logFE of \( -\infty \) in Omicron and of 10.58 in Alpha.

This observation suggests that the more recent circulating lineage, i.e. Omicron, tends to favor Spike_S371L over Spike_A570D or NSP3_A890D. Note that BA.1 (Omicron) and B.1.1.7 (Alpha) are sharing many defining mutations (e.g., H69-, V70-, Y144-, D614G, NS01Y, and P681H in Spike_protein and other mutations in other proteins) – almost half of the defining mutations of Alpha are considered defining mutations also of Omicron – and that they are not closely related lineages. This may suggest that the virus could be evolving into the direction of collecting new mutations that might enhance its features, e.g., Omicron is now the most complete ‘escapee’ from neutralization by currently available antibodies in comparison to other SARS-CoV-2 variants, including the Alpha variant [27].

### 3.3. Lineages distribution analysis for co-occurring mutations pairs

Following the methods described in Section 2.2.2, we were able to study the quantitative behavior of pair mutations over lineages. We extracted distributions over lineages for each frequent mutation. Fig. 5 presents the number of sequences and lineages of each frequent mutation considered for the Spike protein. As expected, the most dominant mutation is Spike_D614G, found in 1,534 lineages out of 1,587 total lineages. Then, we compared each such distribution in pairs, using the KS test. As a result, we obtained 4,000 pairs with different distributions over lineages and 12,692 pairs with similar distributions over lineages. A 3D scatter plot of the KS test results for all the 421 frequent mutations is provided in Supplementary Figure S2.

Based on the procedure described in Section 2.2.3, from the group of pairs with different lineages distributions, we identified 4,489 distinct events of convergent evolution, whereas from the group of pairs with similar lineages distributions, we identified 415,892 distinct events of divergent evolution.

Along the Methods described in Section 2.3, we then produced the tables aggregated by mutation pairs and remaining mutations, which are analyzed in the next section.

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**Table 3**

Examples of co-occurring pairs of mutations in the Spike protein, extracted from the defining mutations list of the Delta variant [20]. The provided number of sequences is evaluated on the complete dataset.

| \( m_1 \) | \( m_2 \) | #Seq. with \( m_1 \) | #Seq. with \( m_2 \) | #Seq. with \( m_1, m_2 \) | P-value |
|-----------|-----------|-----------------|-----------------|-----------------|---------|
| T19R      | E156-     | 4145223         | 21528           | 20285           | 4.72e-09 |
| T19R      | F157-     | 4145223         | 3867174         | 3833412         | 0.0     |
| T19R      | R158G     | 4145223         | 24435           | 23862           | 0.0     |
| T19R      | L452R     | 4145223         | 4199166         | 4034886         | 0.0     |
| T19R      | T478K     | 4145223         | 5252904         | 4046410         | 3.74e-09 |
| T19R      | D614G     | 4145223         | 7981457         | 4134564         | 1.04e-08 |
| T19R      | P681R     | 4145223         | 4208095         | 4123831         | 3.28e-09 |
| T19R      | D950N     | 4145223         | 4033822         | 3906828         | 0.0     |
| E156-     | F157-     | 21528           | 3867174         | 20395           | 0.0     |
| E156-     | R158G     | 21528           | 24435           | 18717           | 0.0     |
| E156-     | L452R     | 21528           | 4199166         | 17429           | 1.01e-09 |
| E156-     | T478K     | 21528           | 5252904         | 17870           | 7.69e-09 |
| E156-     | D614G     | 21528           | 7981457         | 21339           | 0.5496  |
| E156-     | P681R     | 21528           | 4208095         | 20315           | 5.83e-9 |
| E156-     | D950N     | 21528           | 4033822         | 19943           | 2.06e-09 |
| F157-     | R158G     | 3867174         | 24435           | 17664           | 0.0     |
| F157-     | L452R     | 3867174         | 4199166         | 3785418         | 0.0     |
| F157-     | T478K     | 3867174         | 5252904         | 3795616         | 0.0     |
| F157-     | D614G     | 3867174         | 7981457         | 3857745         | 7.37e-09 |
| F157-     | P681R     | 3867174         | 4208095         | 3849316         | 5.77e-10 |
| F157-     | D950N     | 3867174         | 4033822         | 3724976         | 0.0     |
| R158G     | L452R     | 24435           | 4199166         | 20816           | 0.0     |
| R158G     | T478K     | 24435           | 5252904         | 21209           | 0.0     |
| R158G     | D614G     | 24435           | 7981457         | 24247           | 0.04418 |
| R158G     | P681R     | 24435           | 4208095         | 23830           | 0.0     |
| R158G     | D950N     | 24435           | 4033822         | 22455           | 0.0     |
| L452R     | T478K     | 4199166         | 5252904         | 4080758         | 1.02e-09 |
| L452R     | D614G     | 4199166         | 7981457         | 4189517         | 4.32e-09 |
| L452R     | P681R     | 4199166         | 4208095         | 4078817         | 0.0     |
| L452R     | D950N     | 4199166         | 4033822         | 3907159         | 9.18e-10 |
| T478K     | D614G     | 7981457         | 5241411         | 8.97e-09         |
| T478K     | P681R     | 5252904         | 4208095         | 4082650         | 4.22e-09 |
| T478K     | D950N     | 5252904         | 4033822         | 3919358         | 2.71e-10 |
| D614G     | P681R     | 7981457         | 4208095         | 4195788         | 4.37e-09 |
| D614G     | D950N     | 7981457         | 4033822         | 4025217         | 5.00e-09 |
| P681R     | D950N     | 4208095         | 4033822         | 4001531         | 5.86e-09 |
Fig. 4. A. 2D Scatter plot of the p-values of the hypergeometric tests on all the pairs of mutations in which only one of the two is a defining mutation of Delta variant, mapped on the values of \( \log FE \) of the mutation of the pair that is not a Delta-defining mutation (outside of the list). A total of 7,638 pairs of mutations were considered in the figure after removing mutations with \( FE = 0 \). Blue dots indicate the \( p \)-values < 0.05, whose corresponding non-Delta mutations have a \( \log FE > 1 \). B. Zoomed version of Panel A scatter plot; it includes 68 mutations extracted from the ‘blue’ pairs selected in Panel A (co-occurring with all 19 Delta-defining mutations); here, a color scale is used to indicate the \( \log FE \) of the pair mutation that belongs to the Delta variant: the darker the color, the higher the value. The corresponding 3D scatter plot is provided in Supplementary Figure S1.

Table 4
Examples of mutually exclusive pair of mutations. \( m_1 \) are extracted from the defining mutations’ list of the Omicron variant; \( m_2 \) are defining mutations of the Alpha variant.

| \( m_1 \) | \( m_2 \) | \#Seq. with \( m_1 \) | \#Seq. with \( m_2 \) | \#Seq. with \( m_1, m_2 \) | \( \)P-value \( \)
|---|---|---|---|---|---|
| Spike_S371L | Spike_TD70D | 1012982 | 1161124 | 0 | 1.0 |
| Spike_S371L | NSP3_A889D | 1012982 | 1159088 | 0 | 1.0 |
| Spike_S371L | Spike_TD1118H | 1012982 | 1153130 | 20 | 1.0 |
| Spike_TD505H | Spike_TD70D | 1115034 | 1161124 | 109 | 1.0 |
| Spike_TD505H | NSP3_A889D | 1115034 | 1159088 | 110 | 1.0 |
| Spike_TD505H | Spike_TD1118H | 1115034 | 1153130 | 131 | 1.0 |
| Spike_TD547K | Spike_TD70D | 1184888 | 1161124 | 116 | 1.0 |
| Spike_TD547K | NSP3_A889D | 1184888 | 1159088 | 116 | 1.0 |
| Spike_TD547K | Spike_TD1118H | 1184888 | 1153130 | 140 | 1.0 |
3.4. Convergent and divergent evolution of pairs of mutations

3.4.1. Convergence

By using pairs of mutations as a grouping factor in the ‘Convergence Result’ table, we generated a table with 1,818 unique pairs of co-occurring mutations having at least one event of convergent evolution. Table 5 presents the pairs of mutations with the highest count of convergence events; the complete result is provided in Supplementary Table S2.

Example 1. The first line of the table shows that the co-occurring pair composed by NSP1_V84- and NSP1_V86- is found separately in 8 Distinct Ancestor Lineages (#DAL) and appears in 24 of their Descendant Lineages (#CDL), occurring at various depths of the lineages tree (between the third and the sixth level). In all such cases, the Remaining Mutation is, alternatively, the first or the second mutation of the pair. The lineages tree related to this pair is shown in Supplementary Fig. S3.

Example 2. In addition to several co-occurring (and closely positioned) deletions, in the seventh row of Table 5 we can observe a pair composed by NSP4_V167L and Spike_P681R, which are present separately in 4 lineages (#DAL) and appear together in 17 of their descending lineages (#CDL), spotted at both the third and fifth level of the tree. Fig. 6 shows the representative lineages tree of this pair. Note that having found a pattern of converging mutations in such a high number of lineages suggests the existence of selection advantages for such mutations.

We deepen our analysis by using remaining mutations (RM) as a grouping factor in the ‘Convergence Result’ table. We generate a table of 308 converging mutations. Table 6 shows the converging mutations participating to the highest number of convergent evolution events across the whole lineages tree; the complete list is provided in Supplementary Table S4. In Table 6 we find the three consecutive deletions occurring on the non-structural protein 6 (NSP6) at positions 106–108. These three mutations are co-occurring and converging in 28 different lineages, suggesting that the deletion of 9 nucleotides in ORF1ab gene that generates the ‘SGF deletion’ is among the most prevalent remaining mutations in convergent evolution events in the population. Note that this triple amino acid deletions is included in the defining mutations lists of three previous VOCs, i.e., Alpha, Beta, and Gamma. NSP6 is a multi-pass transmembrane protein that is thought to be involved in autophagy and antagonism of innate immune responses, but it remains unclear what influence this deletions has on virus phenotype [49,37]. Other mutations in Table 6 are N501Y, L452R, and...

Table 5
Top 10 converging pairs of mutations ranked by descending #CDL.

| (m1, m2) | #CDL | CDL depth | #DAL | RM |
|---------|------|-----------|------|----|
| NSP1_V84-+NSP1_V86- | 24 | 3,4,5,6 | 8 | NSP1_V84-,NSP1_V86- |
| NSP1_H83-+NSP1_V86- | 23 | 3,4,5 | 7 | NSP1_H83-,NSP1_V86- |
| NSP1_G82-+NSP1_V86- | 23 | 3,4,5 | 7 | NSP1_G82-,NSP1_V86- |
| NSP6_F108-+NSP6_S106- | 18 | 3,4 | 3 | NSP6_S106- |
| NS8_P93S + NSP3_V932A | 18 | 3,4 | 3 | NSP3_V932A |
| NSP6_F108-+NSP6_G107- | 18 | 3,4 | 3 | NSP6_G107- |
| NSP4_V167L + Spike_P681R | 17 | 3,5 | 4 | NSP4_V167L,Spike_P681R |
| NSP6_L260F + NSP6_S106- | 15 | 2,3,4,5 | 4 | NSP6_S106- |
| N_G204R + N_R203K | 15 | 1,3 | 4 | N_R203K,N_G204R |
| NSP6_G107-+NSP6_L260F | 14 | 2,3,4,5 | 4 | NSP6_G107- |

Fig. 5. Scatter plot indicating the number of lineages containing the frequent mutations of the Spike protein; the higher the number, the more spread a mutation is over the lineages. A color scale is used to express the number of sequence exhibiting each mutation.
mutation list are only 31 (7.36% of the total), we found that in 18.66% of the observed divergence events the remaining mutation (RM) was a deletion. This might be explained by the fact that, unlike substitutions, deletions cannot be corrected by proofreading activity [30]. Notable examples of pairs of deletions with divergent evolution through the lineages tree are (Spike_H69-, Spike_Y144-) and, similarly, (Spike_V70-, Spike_Y144-); see the seventh and eighth rows of Table 7. Spike_H69- and Spike_H70- are co-occurring mutations having very similar lineages distribution according to the KS test (p-value = 1.0); they are spotted together in 439 different lineages (forming respectively 98% and 99% of the lineages they appear in). Therefore, we discuss about this double deletion as if it were a unique mutation and compare its pattern of distribution with the one of Spike_Y144-. We detected 304 divergence events, whose 86.18% includes Spike_Y144- as the diverging mutation (RM) passing to the new descendant lineage, while only 13.81% includes Spike_H69-/-V70- as the remaining mutations. Both Spike_H69-/-V70- and Spike_Y144- are deletions that lie in the NTD region of Spike and may modulate antigenicity [25,29,30]. Since the count of DDL is high (307), the generated tree is very large, thus Fig. 7 shows only a portion of it.

We deepen our analysis by using remaining mutations (RM) as a grouping factor in the ‘Divergence Result’ table; we generate a table of 362 unique diverging mutations. Table 8 shows the remaining mutations participating to the highest number of divergent evolution events across the whole lineages tree; the complete list is provided in Supplementary Table 55. The table shows two mutations that were expected, i.e., Spike_D614G and NSP13_P323L, as they are the most dominant mutations across the five well-known distinct clades [36]. This may explain why we detected a considerably high numbers of divergence events, compared to the convergence ones.

### 3.4.3. Validation

To assess the significance of our findings, we performed a Monte Carlo simulation to compare the resulting numbers of pairs showing convergence/divergence events with numbers of randomly selected pairs. A p-value < 10e-4 in both cases of convergent and divergent evolution was detected. Fig. 8 shows the distribution of the counts of pairs of mutations with at least one convergent or divergence event detected following the Monte Carlo approach; both distributions are centered on mean values that are considerably distant from the observed values (1,818 for convergence events and 6,625 for divergence events).

### 4. Discussion

The SARS-CoV-2 pandemic is a major threat to the public health. In response to the continuous spreading of the virus the global community answered with an incredible effort to collect and deposit a huge amount of viral genomes to public repositories. Thanks to such availability, we were able to conduct a large-scale analysis that aimed at highlighting the role of non-synonymous mutations’ pairs in identifying evolution events of SARS-CoV-2.

First, we analyzed the patterns of co-occurrence and mutual exclusion of pairs of mutations on sequences of the virus. We then focused on sets of significantly co-occurring pairs of mutations by analyzing how their distributions over lineages compare. Finally, we precisely described events of convergence (when two mutations are frequent in a lineage but only one of them is frequent in its ancestor) and of divergence (when two mutations are frequent in a lineage but one of the two disappears in a
Based on this notion, we observed that co-occurring pairs with different distributions allow to identify convergence events, while co-occurring pairs with similar distributions allow to identify divergence events. The obtained results were grouped by 1) considering for each pair of mutations the number of lineages where it instantiates a converging/diverging behavior and 2) considering for each remaining mutation (i.e., maintained through two directly-related lineages) the number of lineages where it participated to a converging/diverging behavior.

The essence of this work can be summarized as follows. The lineage-independent analysis, which included a study of the most significant co-occurring mutation pairs, complemented by the fold enrichment calculation, suggested a way to find candidates for “variants’ defining mutations” that are not currently listed by reference sources (e.g., CoVariants)—as shown for the Delta variant case, where we highlighted the mutations S112L, G142D, and V1104L in the spike protein. Then, considering the mutually exclusive mutation pairs assigned to two different variants, we regard them as candidates for characterizing different variant phenotypes. Among them, we spotted Spike_S371L in Omicron which never occurs together with Spike_A570D or NSP3_A890D in Alpha.

Convergent and divergent events can instead be used for anticipating lineage evolution. Specifically, if two mutations are co-occurring – one remaining (RM) and one acquired (AM) – whenever RM appears in a lineage then we expect that AM will be next acquired; in our analysis, we spotted several confirmations, e.g., Fig. 6 represents 5 (out of 17) converging events having either NSP4_V167L or Spike_P681R as RM in a parent node and both mutations in one of its descendant nodes. In convergence events, we highlight the presence of remaining mutations that are widely spread and defining several VOCs (see Table 6).

Conversely, if two mutations are mutually exclusive – one remaining (RM) and one missing (MM) – whenever RM and MM appear in a lineage then we expect that MM will not be present in some descendant node; in our analysis, we spotted several confirmations, e.g., Fig. 7 represents 19 (out of 307) diverging events having either NSP4_V167L or Spike_P681R as RM in a parent node and both mutations in one of its descendant nodes. In divergence events, we highlight the presence of remaining mutations that are widely spread and defining several VOCs (see Table 8).

The analysis elements proposed in this study pose the basis for anticipating the evolution of the SARS-CoV-2 virus, by observing mutation and lineages behaviors from a purely data-driven quantitative point of view.
Fig. 8. Distributions of the counts of pairs with randomly detected convergent (left) or divergent (right) events, extracted from a sample of 16,692 pairs of mutations repeated for 10,000 times.

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**CRediT authorship contribution statement**

Ruba Al Khalaf: Investigation, Visualization, Validation, Writing – original draft. Anna Bernasconi: Supervision, Writing – original draft, Writing – review & editing. Pietro Pinoli: Supervision, Writing – review & editing. Stefano Ceri: Supervision, Writing – review & editing.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.csbj.2022.07.051.

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