Structural/Phylogenetic Analysis of SARS-CoV-2 Spike Glycoprotein Variants

Subjects: Virology

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The SARS-CoV-2 pandemic, reported for the first time at the end of 2019 in the city of Wuhan (China), has spread worldwide in three years; it lead to the infection of more than 500 million people and about six million dead. SARS-CoV-2 has proved to be very dangerous for human health. Therefore, several efforts have been made in studying this virus. In a short time, about one year, the mechanisms of SARS-CoV-2 infection and duplication and its physiological effect on human have been pointed out. Moreover, different vaccines against it have been developed and commercialized. Since the beginning of the pandemic, SARS-CoV-2 has evolved; it has done so by accumulating mutations in the genome, generating new virus versions showing different characteristics, and which have replaced the pre-existing variants. In general, it has been observed that the new variants show an increased infectivity and cause milder symptoms. The latest isolated Omicron variants contain more than 50 mutations in the whole genome and show an infectivity 10-folds higher compared to the wild-type strain.

SARS-CoV-2 SARS-CoV-2 spike protein SARS-CoV-2 variants structural analysis

1. SARS-CoV-2 Virus

SARS-CoV-2 belongs to the *Coronaviridae* family [9]; its genome is a positive sense single-stranded RNA of about 30 kb length; it encodes: 16 non-structural proteins, namely nsp 1–16; the ORFs accessory proteins 3a, 6, 7a, 7b, 8, and 10; and four structural proteins involved in the viral infection, which are the spike protein (S), membrane protein (M), nucleocapsid protein (N), and envelope protein (E) [10][11][12]. The SARS-CoV-2 genome is not segmented RNA, having the 3′ poly-A tail and 5′ cap structure.

The SARS-CoV-2 shows a spherical morphology with a diameter of about 100–150 nm [13]. A schematic representation of SARS-CoV-2 is reported in **Figure 1**: the ssRNA, which is surrounded by the N proteins, is inside of the lipidic envelope; and associated to the membrane there are the glycoproteins M, the proteins E, and the spike glycoprotein that protrudes from the surface of the virus in a high number of copies to look like a crown.

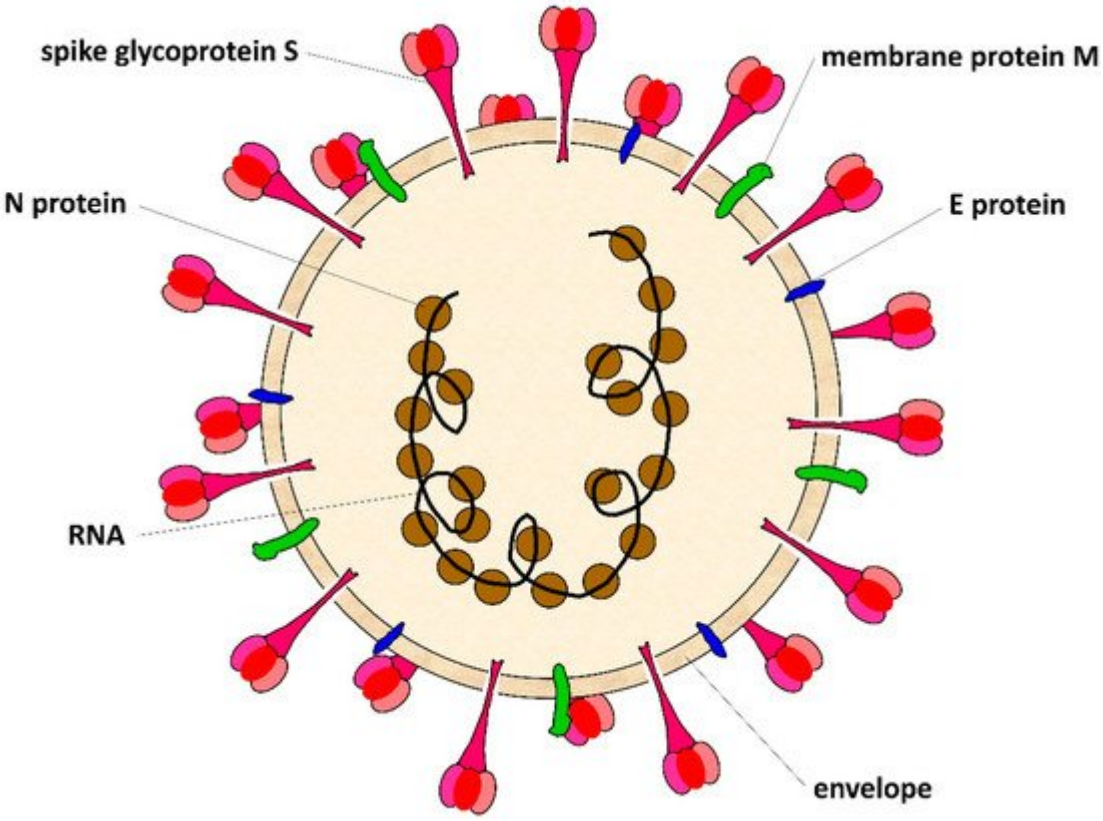

Figure 1. SARS-CoV-2 virus section. SARS-CoV-2 is an enveloped virus with a spherical morphology and a diameter of 100–150 nm. The lipidic envelope derives from the host cell. On the viral surface are present different types of protein, such as the E protein, M membrane protein, and spike protein. The viral ssRNA is surrounded by the N nucleoproteins.

During the infection, SARS-CoV-2 uses the spike protein for binding to the host cell receptor: the ACE-2 protein (angiotensin-converting enzyme 2). The process takes place in several steps, which require, at first, the spike protein cleavage for its interaction with the ACE-2 protein; then, there is the fusion of the cell-virus membranes [14][15]; this is followed by the entry into the host cell of viral ssRNA [14][15]. Since the spike protein is crucial for the virus infection and it is present on the virus surface in a high number of copies, vaccines have been produced by using this protein as a target [16].

2. SARS-CoV-2 Mutation Rate

Single-strand RNA viruses show an increased mutation rate compared to the ones having DNA genomes, since they lack the systems to correct the replication errors [17]. Further, these viruses exhibit a consistent mutation-rate variability among them; this ranges between 10^{-6} and 10^{-3}, where the mutation rate is defined as nucleotide (nt) substitutions per-site per-cell infection [18]. It is not easy to measure the real mutation rate of a virus, because most of the mutations are lethal for the virus.

The mutation frequency of SARS-CoV-2 depends on the probability that an error occurs during the genome replication, and it has been calculated as three in a million. Therefore, three replications per million are different

from the parental strain; whereas the mutation rate of SARS-CoV-2 *per-site per-year* has been estimated as 1.12 × 10^{-3} nt^{-1} $year^{-1}$ [19][20].

Moreover, fixed mutations have been identified in the genome by the whole viral genome analysis; this suggests that SARS-CoV-2 changed much more slowly than other ssRNA viruses [21].

However, taking into consideration its spread throughout the world and the number of viral replications in a single infected person, all together these data may explain the observed accumulation of thousands of mutations. Furthermore, from additional data about this aspect, it has been determined that individuals with a high viral load may generate up to 1.23 × 10^5 copies of viral RNA from a single cough; however, individuals with a moderate viral load can generate only a few hundred copies [22].

Since the spike protein is one of the most important viral proteins involved in the infection mechanisms, it has been used as the main antigen to produce vaccines to fight the SARS-CoV-2 pandemic [1][23]. It has also been used in diagnostics to classify and to monitor all the viral variants derived from the mutations sequenced on the spike protein; these are known as the variants of concern (VOC) [23].

3. Spike Protein S

The SARS-CoV-2 spike protein is a glycoprotein consisting of 1273 amino acids, with a molecular weight of about 180–200 kDa. It is localised on the virus surface in a high number of copies as a pre-fusion protein (**Figure 1**), with a homo-trimer conformation where each monomer lacks the signal peptide (amino acids 1–13). The 3D structure has been solved by cryo-electron microscopy at a resolution of 3.5 Å (**Figure 2**); functionally, the spike can be divided into an extracellular N-terminal region, a trans-membrane domain (23 residues), and a short intracellular C-terminal segment (39 residues) (**Figure 2**A) [24]. Morphologically, the N-terminal region of spike is very large compared to the other protein regions, forming a characteristic bulbous (**Figure 2**B); in addition, it undergoes an extensive structural rearrangement when it interacts with the receptor of the host cell.

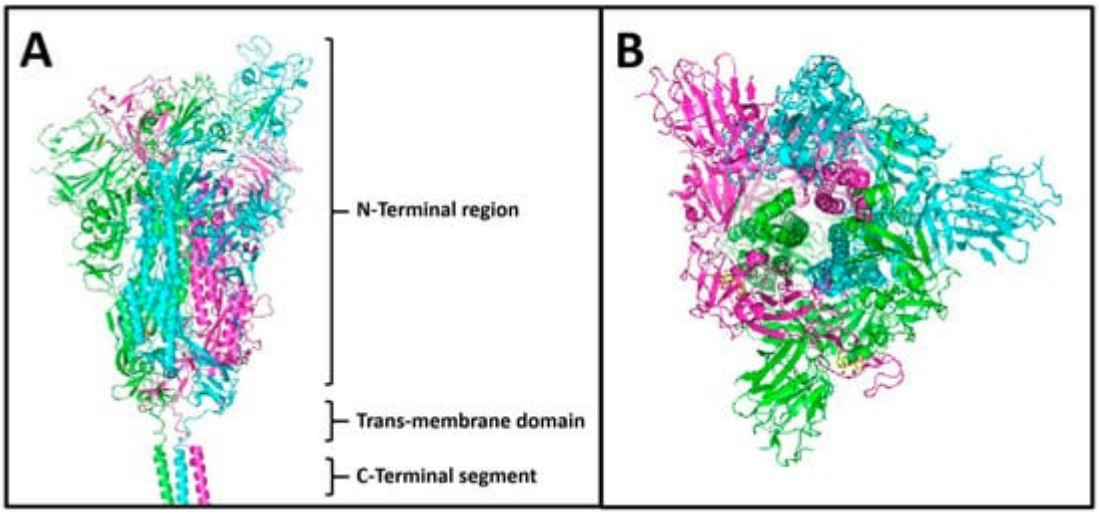

Figure 2. 3D structure of spike protein. The 3D structure was obtained by cryo-electron microscopy at a resolution of 3.5 Å, by Wrapp and colleagues (Science, 2020), PDB ID: 6VSB. (**A**) The whole visualization of the 3D homo-trimer structure of the spike protein. The monomers are indicated with different colours: magenta, green, and cyan. (**B**) The top view of the spike homo-trimer.

In detail, **Figure 3** schematically represented the primary structure of the spike protein: at the N-terminal, the residues 1–13 are the signal peptide, which is removed during membrane migration; the residues 14–685 are indicated as subunit S1; and the residues 686–1273 are indicated as subunit S2. Between these two subunits, there are, at positions 685 and 699, two cleavage sites that are necessary to cut the spike. These cleavages, as mentioned above, are critical to activate the membrane fusion process with the host cell [24].

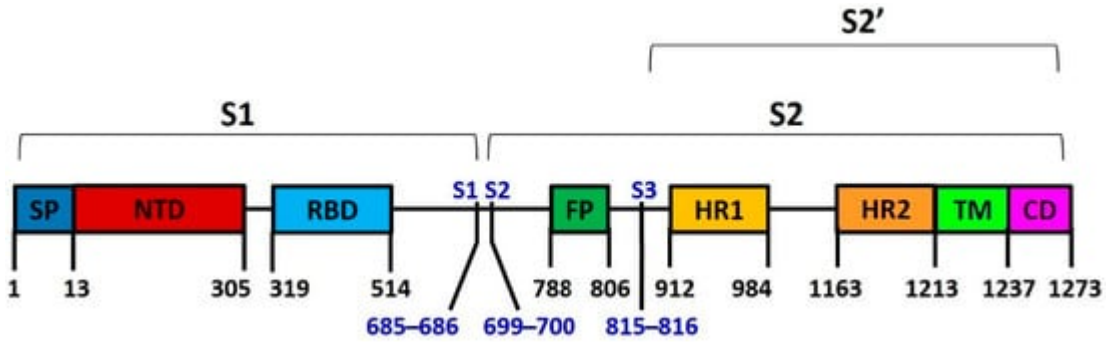

Figure 3. Schematic representation of spike protein. The functional domains are indicated as subunit S1 (residues 14–685) and subunit S2 (residues 686–1273) responsible for receptor binding and membrane fusion, respectively. S1, S2, and S3 are the cleavage sites at positions 685, 699, and 815, respectively. The cleavage at position 815 generates the subunit S2′. SP is the signal peptide (residues 1–13); NTD is the N-Terminal domain (residues 14–305); RBD is the receptor-binding domain (residues 319–514); FP is the fusion peptide (residues 788–806); HR1 is the heptapeptide repeat sequence 1 (residues 912–984); HR2 is the heptapeptide repeat sequence 2 (residues 1163–1213); TM is the transmembrane domain (residues 1214–1237); CD is the cytoplasmatic domain (residues 1238–1273).

The S1 subunit is responsible for the receptor binding to the host cell by the recognition of the angiotensin converting enzyme 2 (ACE-2), while the S2 subunit is responsible for the membrane fusion; each subunit is divided into different subdomains, as indicated in **Figure 3**. The receptor-binding domain (RBD) changes its position during the interaction with ACE-2. In particular, the spike protein opens its conformation by moving RBD versus ACE-2 (open conformation) [25]. Then, after membrane fusion, the subunit S2 is cut at position 815; this originates the S2′ subunit (**Figure 3**) [24][25].

The spike protein is localized on the virus surface, in a non-active form (closed conformation) (**Figure 4**A). Following the infection process, the RBD domain binds ACE-2 (open conformation); the transmembrane serine protease 2 (TMPRSS2) from the host cell recognizes and cuts the spike into the S1 and S2 subunits (**Figure 4**B). Then, by this event, the membrane fusion process starts. In particular, the hydrophobic FP domain anchors the spike to the host membrane; the domains HR1 and HR2 of the three monomers form a six-helical bundle, bringing

the viral envelope to the host cell membrane and completing the fusion process (**Figure 4**C). The last part of infection is the injection of the viral ssRNA into the host cell, leading to the replicative viral process [26].

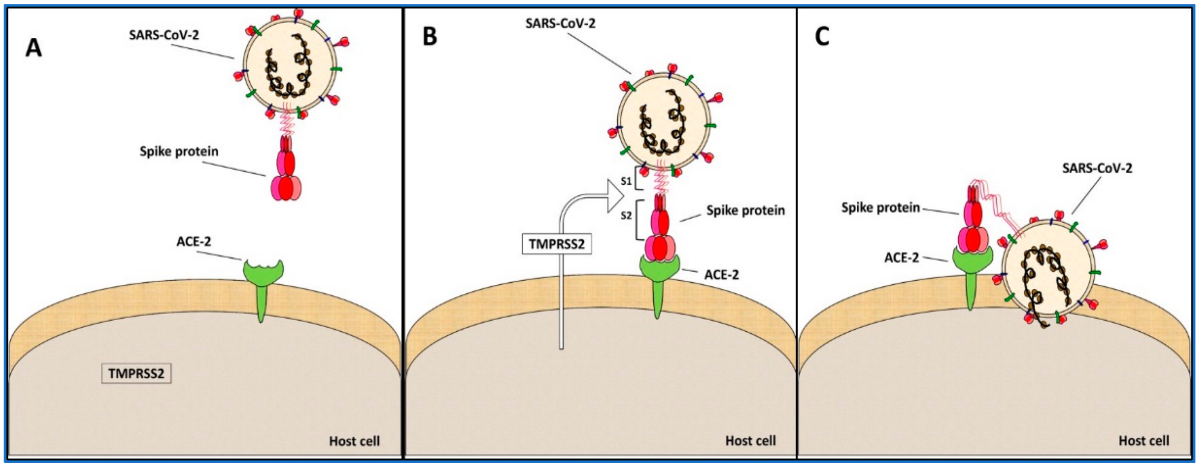

Figure 4. SARS-CoV-2 infection mechanism. (**A**) *Initial state*: it is represented by the separated virus and host cell. (**B**) *Binding*: the spike protein recognizes ACE-2 and starts the binding process that involves the TMPRSS2 serine protease from the host cell; the protease cuts the spike protein between the subunits S1 and S2. (**C**) *Cell fusion*: the cleavage activates the spike protein, which changes its conformation, bending towards the cell membrane to which it merges; then, the genetic material of the virus enters into the host cell, starting the viral duplication.

4. Spike Variants and Phylogenies

Casual mutations occur during the viral RNA replication, generating new SARS-CoV-2 variants. Only some of these mutations had an effect on the processes of viral infection and diffusion, as well as on the virus-induced symptoms; others are harmful for the survival of the virus and are eliminated, and some are neutral and are accumulated in the genome. In the three years of the pandemic, many SARS-CoV-2 variants have been observed and sequenced [27]. The spike protein has been used as the primary antigen for the vaccine production because it is crucial for the infection mechanism and it is a characterising SARS-CoV-2 protein. Further, the mutations observed in the gene coding this protein have been used for the classification of the viral variants [28]. The most diffused SARS-CoV-2 variants have mutations capable of changing the virus infection and diffusion processes; they have spread, replacing the wild-type or the previous virus variants [29][30].

The most recent SARS-CoV-2 variants have accumulated many mutations on the spike protein. Thus, since the vaccines have been developed on the wild-type version of spike, many cases of reinfection by COVID-19 and infections in vaccinated people have been observed[31].

Here, the researchers analysed the most diffused SARS-CoV-2 variants by sequence alignment, in relation to the timing of their spreading and the place where they were first isolated. The researchers did so in order to perform a

phylogenetic analysis to understand the genetic evolution of the virus, and to define the characterizing mutations of SARS-CoV-2 (Table 1) during its evolution.

Table 1. SARS-CoV-2 variants. The most spread SARS-CoV-2 variants, the country where they were first isolated, and the isolation dates were reported.

Spike Variants	Isolation Country	Isolation Date
wild type	China	December 2019
EPSILON	USA	March 2020
ZETA	Brazil	April 2020
BETA	South Africa	May 2020
20 A.EU2	Portugal	June2020
20 A.EU1	Spain	July 2020
ALPHA	England	September 2020
DELTA	India	October 2020
KAPPA	India	October 2020
A 23.1	Uganda	October 2020
GAMMA	Brazil	November 2020
IOTA	USA	November 2020

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interesting that the two variants Delta and Kappa were isolated in India, both in December 2020 (Table 1). It is evident that their evolution is divergent, by looking at the phylogenetic tree (Figure 5). In fact, both variants carry seven mutations with respect to the wild-type protein; however, only three of them are common, specifically:

ETA	Multiple countries	November 2020
LAMBDA	Peru	December 2020
THETA	Philippines	January 2021
B.1.1.318	Multiple countries	January 2021
MU	Columbia	January 2021
OMICRON BA.1	South Africa	November 2021
OMICRON BA.2	South Africa	December 2021
OMICRON BA.2.12.1	North America	December 2021
OMICRON BA.4	South Africa	January 2022
OMICRON BA.5	South Africa	January 2022

The twenty most diffused SARS-CoV-2 spike variants, identified from December 2019 to the beginning of 2022, indicated as wild type, delta, lambda, mu, beta, gamma, B.1.1.318, kappa, A 23.1, iota, theta, epsilon, 20 A.EU1, 20 A.EU2, zeta, alpha, eta, omicron BA.1, omicron BA.2, omicron BA.2.12.1, omicron BA.4, and omicron BA.5 (sequences were from proteins database at https://www.uniprot.org, accessed on December 2003) were selected for the analysis. The sequences of these variants were used to make the multiple sequence alignment reported in figure S1 (see supplemental data) (the multiple sequence alignment by the Clustal Omega program at https://www.ebi.ac.uk/Tools/msa/clustalo/, accessed on 01 October 2019). From the alignment is derived the phylogenetic tree of the variants that will be discussed below.

The researchers found 100 mutations in total, including residue substitutions, deletions, and insertions, which are about 8% of the total residues (1273 aa). Moreover, 49 of these mutations were included in NTD and 23 in the RBD domain; they were comprised in more than 70% of the S1 spike subunit mutations (**Figure 3**).

Spike Variants	Mutations
ALPHA	A570D T716I S982A D1118H
BETA	D80A D215G Del241-243 K417N A701V
GAMMA	L18F T20N D138Y R190S T1027I
DELTA	Del156-157 R158G
EPSILON	S13I W152C
ETA	Q52R A67V Q677H F888L
THETA	E1092K H1101Y
IOTA	L5F D253G

KAPPA	E154K Q1071H
LAMBDA	T76I R246N Del247-252 F490S T859N
MU	Ins147N Y147N R346K
20 A.EU1	A222V
A 23.1	R102I F157L V367F
B.1.1.318	T95I
OMICRON BA.1	Del143-144 N211I L212V Ins213-214 V215P G446S G449S T547K N856K L981F
OMICRON BA.2.12.1	S704L
OMICRON BA.4	V3G

Furthermore, the researchers observed that the “unique” mutations listed in Table 2 cannot be considered variant-specific and all characterising the SARS-CoV-2 variants; this is because in most cases they are substitutions between amino acids with similar properties, not affecting the spike protein functionality. For instance, in the 20 A.EU 1 and 2 variants, the spike protein differs from the wild type by the same single residue (D614G) in both. In addition, another two mutations were found: the S477N in the variant 20 A.EU 2, and the A222V in the variant 20 A.EU 1. The mutation S477N was also identified in other spike variants, while A222V was observed only in 20 A.EU 1; this suggests that the functional difference between the two 20 A.EU variants is due to the S477N mutation and not due to the A222V being a conservative substitution of a non-polar amino acid with a similar one [34]. Based on these observations, if the mutation S477N is characterising between these two variants and it is also present in other more recent variants, 20 A.EU 2 may be considered an old variant, where the mutation S477N appeared for the first time.

In the case of the variant named Iota, some mutations are common to other variants, such as S477N, E484K, and D614G; however, the two mutations found only in Iota, L5F, and D253G can be considered characterizing because they have an effect on the functionality of the spike. In fact, the L5F mutation is localized in the signal peptide (SP) of the spike; it has been observed that mutations of SP alter the spike functionality [35]. The second mutation

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D253G. The Bristol 2020 polar, basic (aspartic, D) with non-polar ones (glycine, G), generating a consistent change.

Other characterising mutations observed in the spike variants affecting its functionality are: the deletion 156–158 in Delta, the deletion 241–243 in Beta, and the deletion 675–678 in Omicron BA.1 [37][38][39].

5. The Omicron Variants

At the end of 2021, a new SARS-CoV-2 variant named Omicron was isolated in South Africa and Botswana; it was, successively, sequenced (Organization WH. Classification of Omicron (B.1.1.529): SARS-CoV-2 variant of concern. 2021. https://www.who.intnewsitem/26-11-2021-classification-of-omicron-(b.1.1.529)-sars-cov-2-variant-of-concern, accessed on 26 November 2021).

Omicron, compared to the previously variants, is characterised by having many mutations; it has about 50 on the whole genome, and 32 of them are only in the spike protein [36]. As regards its main features, Omicron shows an increased infectivity (10-folds higher with respect to the wild-type strain) and milder symptoms with respect to the original virus [36]. This variant is also able to escape the immune system of the host due to the high number of mutations on the spike protein. This led to an extremely fast spreading of the Omicron variant in South Africa, such that it completely replaced the Delta variant in only two weeks [36]. Due to its rapid spreading and high capability to mutate, from November 2021 to January 2022, four Omicron sub-variants were isolated (Table 1).

Among the mutations identified in the Omicron variants, 14 are exclusive and found in all the Omicron variants. They are: G339D, S373P, K417N, N440K, S477N, T478K, E484A, Y505H, H655Y, N679K, N764K, D796Y, Q954H, and N969K (Figure S1, supplemental data). However, six other mutations have been observed in all the Omicron variants, excluding Omicron BA.1; they are: Del24-26, V213G, T376A, S371F, D405N, and R408S (Figure S1, supplemental data).

From an evolutionary point of view, the Omicron variants are derived from a common "ancestral" version of the SARS-CoV-2 Alpha variant (Figure 5). In fact, they have some critical mutations in common; these are Del69-70, N501Y, D614G, and P681H, all of which are involved in the spike protein functionality [37][38][39]. Omicron BA.1 is quite different from the other omicron variants because it has 11 unique mutations with respect to the others (Table 2). For this reason, it is possible that it evolved separately from the other Omicron variants and was present at least 3–4 months before its isolation; otherwise, it could not have been so different from the other Omicron variants. These findings are evident in Figure 5, which represents the phylogenetic tree generated by the alignment reported in Figure S1 (supplemental data). In the upper part of Figure 5 are located the Omicron variants; together with Delta, they evolved from a common ancestor of the Alpha variant; and Omicron BA.1 appears in an evolutionary branch separated from the other Omicron variants.

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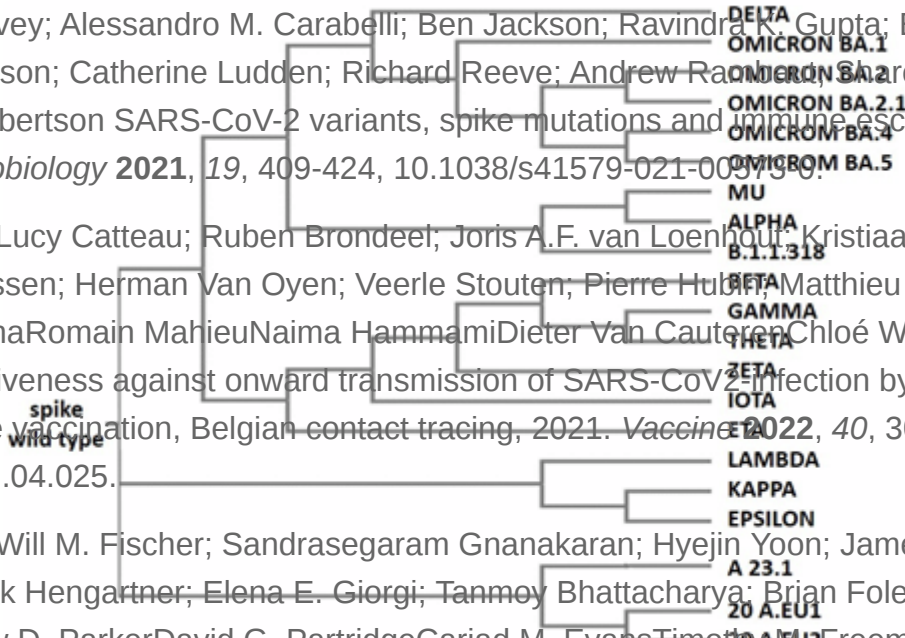

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