Sequence analysis of the Spike, nsp12 (RNA-dependent RNA polymerase), nsp3 (PLpro), and nsp5 (3CLpro) genes reveals a distinct evolutionary pattern of SARS-CoV-2 variants circulating in Yogyakarta and Central Java provinces, Indonesia

Mohamad Hakim[1], * Gunadi[1], Ayu Rahayu[1], Hendra Wibawa[2], Laudria Stella Eryvinka[1], Endah Supriyati[1], Khanza Adzkia Vujira[1], Kristy Iskandar[3], * Afiahayati[1], Edwin Widyanto Daniwijaya[1], Farida Nur Oktoviani[1], Luthvia Annisa[1], Fadila Dyah Trie Utami[1], Verrell Christopher Amadeus[1], Setiani Silvy Nurhidayah[1], Tiara Putri Leksono[1], Fiqih Vidiantoro Halim[1], Eggi Arguni[4], Titik Nuryastuti[1], and Tri Wibawa[1]

[1]Universitas Gadjah Mada
[2]Kementerian Pertanian Republik Indonesia
[3]Universitas Gadjah Mada Fakultas Kedokteran Kesehatan Masyarakat dan Keperawatan
[4]Universitas Gadjah Mada Departemen Ilmu Kesehatan Anak

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Abstract

During the Covid-19 pandemic, the resurgence of SARS-CoV-2 was due to the development of novel variants of concern (VOC). Thus, genomic surveillance is essential to monitor continuing evolution of SARS-CoV-2 and to track the emergence of novel variants. In this study, we performed phylogenetic, mutation, and selection pressure analyses of the *Spike, nsp12, nsp3,* and *nsp5* genes of SARS-CoV-2 isolates circulating in Yogyakarta and Central Java provinces, Indonesia from May 2021 to February 2022. Various bioinformatics tools were employed to investigate the evolutionary dynamics of distinct SARS-CoV-2 isolates. During the study period, 213 and 139 isolates of Omicron and Delta variants were identified, respectively. Particularly in the *Spike* gene, mutations were significantly more abundant in Omicron than in Delta variants. Consistently, in all of four genes studied, the substitution rates of Omicron were higher than that of Delta variants, especially in the *Spike* and *nsp12* genes. In addition, selective pressure analysis revealed several sites that were positively selected in particular genes, implying that these sites were functionally essential for virus evolution. In conclusion, our study demonstrated a distinct evolutionary pattern of SARS-CoV-2 variants circulating in Yogyakarta and Central Java provinces, Indonesia.

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Mohamad Saifudin Hakim[1,*], Gunadi[2], Ayu Rahayu[1], Hendra Wibawa[3], Laudria Stella Eryvinka[2], Endah Supriyati[4], Khanza Adzkia Vujira[2], Kristy Iskandar[5], Afiahayati[6], Edwin Widyanto Daniwijaya[1], Farida Nur Oktoviani[1], Luthvia Annisa[1], Fadila Dyah Trie Utami[2], Verrell Christopher Amadeus[2], Setiani Silvy Nurhidayah[2], Tiara Putri Leksono[2], Fiqih Vidiantoro Halim[2], Eggi Arguni[7], Titik Nuryastuti[1], Tri Wibawa[1]

1 Department of Microbiology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

2 Pediatric Surgery Division, Department of Surgery and Genetics Working Group/Translational Research Unit, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

3 Disease Investigation Center Wates-Yogyakarta, Directorate General of Livestok Services, Ministry of Agriculture, Indonesia

4 Centre for Tropical Medicine, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

5 Department of Child Health and Genetics Working Group, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/UGM Academic Hospital, Yogyakarta, Indonesia

6 Department of Computer Science and Electronics, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada, Yogyakarta, Indonesia

7 Department of Child Health, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito Hospital, Yogyakarta, Indonesia

***Corresponding author:**

Mohamad S. Hakim, M.D., Ph.D.

Department of Microbiology, Faculty of Medicine, Public Health and Nursing

Universitas Gadjah Mada, 55281 Yogyakarta, Indonesia

Email: m.s.hakim@ugm.ac.id

Running title: Sequence analysis of the *Spike, nsp12 (RdRp), nsp3 (PLpro),* and *nsp5 (3CLpro)* genes of SARS-CoV-2 variants

Abstract

During the Covid-19 pandemic, the resurgence of SARS-CoV-2 was due to the development of novel variants of concern (VOC). Thus, genomic surveillance is essential to monitor continuing evolution of SARS-CoV-2 and to track the emergence of novel variants. In this study, we performed phylogenetic, mutation, and selection pressure analyses of the *Spike, nsp12, nsp3,* and*nsp5* genes of SARS-CoV-2 isolates circulating in Yogyakarta and Central Java provinces, Indonesia from May 2021 to February 2022. Various bioinformatics tools were employed to investigate the evolutionary dynamics of distinct SARS-CoV-2 isolates. During the study period, 213 and 139 isolates of Omicron and Delta variants were identified, respectively. Particularly in the *Spike* gene, mutations were significantly more abundant in Omicron than in Delta variants. Consistently, in all of four genes studied, the substitution rates of Omicron were higher than that of Delta variants, especially in the *Spike* and *nsp12* genes. In addition, selective pressure analysis revealed several sites that were positively selected in particular genes, implying that these sites were functionally essential for virus evolution. In conclusion, our study demonstrated a distinct evolutionary pattern of SARS-CoV-2 variants circulating in Yogyakarta and Central Java provinces, Indonesia.

Keywords

3CLpro; Delta variant; nsp12; Omicron variant; PLpro; RdRp; SARS-CoV-2; Spike

Introduction

During the current pandemic, the resurgence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) cases was due to the development of novel variants of concern.[1] Continuous viral transmission globally has indeed enabled the virus to fine-tune its more efficient adaptation in human populations, enabling its sustained transmission.[2,3] Genomic surveillance is thus essential to monitor the continuous evolution of

SARS-CoV-2 and to track the emergence of novel variants or sub-variants. Since January 2020, a collaborative network of the World Health Organization (WHO), national authorities, and research institution have been tracking the evolution of SARS-CoV-2.[4]

The *Spike* (S) gene of SARS-CoV-2 is under intense selective pressure and thus, it is highly mutated. These mutations may result in increased infectivity, transmissibility, and more resistant to neutralizing antibodies of the novel variants.[5]Consistently, the emergence of variants of interest (VOI) or concern (VOC) was characterized by unique sets of mutations in the *Spike*gene.[2] Currently, the only VOC is the Omicron variant, first identified in South Africa in early November 2021.[4] In fact, the emergence of the Omicron variant that successfully replaced the previously dominant Delta variant was also marked by a number of unique mutations in the *Spike*gene.[1,6,7]

Multiple mutations and recombination events in the *Spike* gene have led to the diversification of the Omicron variant into various sub-lineages or sub-variants with possibly different phenotypic characteristics.[8,9] Few subvariants, including BQ.1 and XBB, may spread quickly across countries and evolve to be the most resistant variant against neutralizing antibodies.[10,11] Possible recombination events between Omicron and Delta variants have also been identified.[12]

In addition to the *Spike* gene, mutations in the gene encoding the non-structural proteins also play an essential role in SARS-CoV-2 evolution and adaptation.[13] As a positive-sense RNA virus, SARS-CoV-2 encodes its own enzyme to replicate the genome. The primary RNA polymerase, namely RNA-dependent RNA polymerase (RdRp), is encoded by *nsp12* gene[14] which forms the core polymerase complex with nsp7 and nsp8 proteins.[15] The*nsp12* gene of SARS-CoV-2 contains both RdRp and N-terminal nidovirus RdRp-associated nucleotidyltransferase (NiRAN) domain.[16] Several mutations have been identified in the *nsp12* gene, including S6L, P323L, and P323F, although the effects of these mutations are currently unclear.[13,17]However, it has been shown that the P323L mutation may increase the mutation rate of SARS-CoV-2.[18] RdRp has also been the target of various SARS-CoV-2 inhibitors, such as remdesivir, although comprehensive analyses of the global SARS-CoV-2 genomic dataset showed that potential remdesivir-escape mutations were very rare, indicating its little selective pressure.[19] Indeed, the RdRp region demonstrated strong negative (purifying) selection during the pandemic.[20]

Other two important proteins for the SARS-CoV-2 replication cycle are proteases, encoded by *nsp3* (papain-like protease, PLpro) and*nsp5* genes (chymotrypsin-like main protease, 3CLpro; also known as main protease, Mpro).[21] PLpro and 3CLpro have been shown to modulate the host innate immune responses by inhibiting interferon regulatory factor 3 (IRF3) that resulted in attenuated interferon (IFN) response.[22] Both proteins have also been identified as the targets of IgM antibodies and were associated with the survival of critical Covid-19 patients.[23] In addition, both PLpro and 3CLpro are potential targets for antiviral drug development.[24] A systematic mutational scanning has identified several residues within the 3CLpro protein which are critical for its functionality. These residues are mutation-sensitive and could serve as promising targets for inhibitors with low likelihood of resistant development.[25,26] Nirmatrelvir (PF-07321332) is an orally available antiviral drug with selective binding to the active site of 3CLpro[27] and has shown benefits for treating Covid-19 patients.[28] We and others have identified various mutations in PLpro and 3CLpro regions[17,29], which may pose challenge for antiviral drug development.

Since the early Covid-19 pandemic, we and others have successfully sequenced the full-genome of SARS-CoV-2 circulating in several regions in Indonesia and performed genetic analysis of the isolated viruses.[30-40] These studies demonstrated that B.1.466.2 lineage predominantly circulated in Indonesia during early pandemic and only few isolates belonged to the B.1.319 lineage.[35,38] The Delta variant emerged in April 2021, subsequently replaced B.1.466.2 variant, and posed serious challenge to public health control.[35,37] Our previous study showed that SARS-CoV-2 isolates detected during early pandemic had prominent mutations in particular gene, including *Spike, nsp3,* and*nsp12* genes.[31,38]

Our current study aims to analyse the evolutionary pattern and mutation rate of the *Spike, nsp12, nsp3,* and *nsp5* genes of various SARS-CoV-2 variants circulating in Yogyakarta and Central Java provinces,

Indonesia. We also performed selective pressure analysis to investigate which amino acid changes that are potentially maintained by positive selection to provide insight of the importance of these sites in virus evolution. This study provides more insights on genetic variability of SARS-CoV-2 Delta and Omicron variants, particularly those circulating in our regions (Yogyakarta and Central Java, Indonesia). To our limited knowledge, our study is the first comprehensive and comparative evolutionary analysis of Delta and Omicron variants circulating in Indonesia.

Materials and Methods

2.1. Study design

The study was part of our previous retrospective study of Covid-19 patients in Yogyakarta and Central Java provinces, Indonesia.[36] Briefly, the study was conducted from May 2021 to February 2022. We collected nasopharyngeal swabs of outpatient or hospitalized patients with Covid-19 for RT-PCR testing in the Laboratory of Covid-19, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta. During the study period, 352 confirmed Covid-19 patients (based on RT-PCR), consisting of 164 males and 188 females, were recruited.

2.2. Whole-genome sequencing of SARS-CoV-2

We conducted whole-genome sequencing (WGS) of SARS-CoV-2 for RT-PCR-positive samples with a Ct value of [?]30 based on our previous studies.[30-33,36] Briefly, SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, MA, United States) was used to synthesize single-stranded complementary DNA (cDNA) from the total RNA. Subsequently, the second strand was synthesized using Covid-19 ARTIC v3 primer pool design by SARS-CoV-2 ARTIC Network using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, MA, United States). The library preparations were performed using the Illumina DNA Prep (Illumina, California, United States). The Illumina MiSeq next-generation sequencer was used to perform the whole-genome sequencing of SARS-CoV-2. The genomes of our samples were assembled and mapped into the reference genome from the first Wuhan (China) isolate (hCoV-19/Wuhan/Hu-1/2019, GenBank accession number: NC_045512.2) using Burrow-Wheeler Aligner (BWA) algorithm embedded in UGENE v. 1.30 (https://doc.ugene.net/wiki/display/UUOUM30/About+UGENE). The lineage of each sequence was evaluated using Pangolin v4.1.2. The GISAID ID of each submitted isolate generated from our study was shown in Supplementary Table 1.

2.3. Phylogenetic analysis

A total of 213 and 139 cleaned sequences of SARS-CoV-2 Delta and Omicron variants and five reference sequences of each VOC (Alpha, Beta, Delta, Gamma, and Omicron variants, listed in Supplementary Table 2) were used to construct four different Maximum Likelihood (ML) trees based on the*Spike, nsp12, nsp3,* and *nsp5* genes. All reference sequences were selected based on the earliest submission on GISAID databases and identified as the respective variants. All ML trees were generated using*RaxmlGUI v2.0* with 1,000 bootstraps using the GTR-Gamma model as the substitution model.[41] The ML trees were visualized and annotated using *MEGA11* .[42]

2.4. Mutation analysis

Mutation analysis was performed using two datasets: 213 Omicron and 139 Delta sequences. Analysis was conducted specifically for each gene (*nsp3, nsp5, nsp12,* and *Spike* genes). The reference sequence was retrieved from GenBank with Accession Number NC_045512.2 (Wuhan-Hu-1). Gene sequence extraction was performed using

samtools v.1.15.1.

All sequences were mapped to the genome reference sequence using*minimap2 v.2.4* . Variant calling was performed using*Lofreq* . Mutation annotations were performed by *snpEff v.5.1* . Data processing was performed using the *awk* and*grep* command.

2.5. Clock rate analysis

The Delta and Omicron sequences were extracted based on the region shown in the Supplementary Table 4. The datasets were aligned using MAFFT v.7.5.0 using local pair algorithm. The sequences were filtered by removing the redundant sequence and dropping all sequences containing "N". The clean datasets were used for clock rate analysis.

Clock rate analysis parameter was created using *Beauti v.1.10.4* and Bayesian analysis was performed using *BEAST v1.10.4* . HKY-Gamma substitution model and uncorrelated log-normal relaxed molecular clock were set for the run. Priors were set to classic priors. The chain length is 4 x 50 million with sampling every 50 thousand. Log files were combined using LogCombiner *v1.10.4.* Run results were evaluated using *Tracer v1.7.1* . The ESS value higher than 100 shown acceptable quality run. Molecular Clock was observed from a report generated by *Tracer* .

2.6. Selective pressure analysis

Selective pressure analysis was performed using MEME in Hyphy*v.2.5.6.* 213 Omicron and 139 Delta sequences (*nsp3, nsp5, nsp12,* and *Spike* genes) were subjected to selective pressure analysis. Each dataset was first aligned using Neighbor-Joining (NJ) algorithm with 1,000 bootstraps using Maximum Composite Likelihood Model. The aligned sequences were analyzed using Hyphy and the results were visualized using HyPhy Vision (http://vision.hyphy.org/).*p-value* lower than 0.1 was considered as significant.

2.7. Mutation effect analysis

Mutation effect analysis was performed using DynaMut.[43] To compute the stability of SARS-CoV-2 wild-type (WT) and mutated (mutant) proteins, the reference structures were retrieved from the RCSB database (https://www.rcsb.org/), with the following IDs: 7CMD (Nsp3); 6M2Q (Nsp5); 6M71 (RdRp); 6VXX (Spike) and were then uploaded to the DynaMut server (https://biosig.lab.uq.edu.au/dynamut/). At specific site, a point mutation was inserted. The effect in the form of total energies and vibrational entropy energies between wild-type and mutated proteins was recorded.

2.8. Ethical approval

The study was approved by the Medical and Health Research Ethics Committee of the Faculty of

Medicine, Public Health, and Nursing, Universitas Gadjah Mada (KE/FK/0563/EC/2020). Written informed consent was obtained from all participants and parents or guardians for participating in this study.

Results

3.1. The genome sequencing quality

As mentioned, the GISAID IDs of submitted isolates generated from our study as well as the date of specimen collections, place, age, and sex were shown in Supplementary Table 1. The genome sequencing quality of all sequences was shown in Supplementary Table 3.

3.2. Phylogenetic analyses of SARS-CoV-2 circulating in Yogyakarta and Central Java provinces based on the Spike, nsp12, nsp3, and nsp5 genes

The Maximum likelihood (ML) phylogenetic tree of the *Spike* gene of our original isolates (n=352) is shown in Figure 1. Based on the*Spike* gene, all original viral isolates collected from Yogyakarta and Central Java provinces were clustered in two main lineages of the Delta and Omicron variants, while the previous VOCs (Alpha, Beta, and Gamma) were separated in distinct clusters. Similarly, the ML tree based on *nsp12* (Figure 2A) and *nsp5* genes (Figure 2C) also showed that our isolates were segregrated into two independent clusters of Delta and Omicron variants. While in *nsp3* gene, the Delta and Omicron variants did not form specific clusters, but more diverse segregation was observed (Figure 2B).

3.3. Mutation analyses of the Spike, nsp12, nsp3, and nsp5 genes of SARS-CoV-2 Delta and Omicron variants

The complete mutational analysis was shown in Supplementary Table 5. High frequency (hotspot) mutations (>10%)[44] identified in either Delta and Omicron variants were shown in Figure 3, while visualization of these mutations were depicted in Figure 4. Mutational events highly occurred in the *Spike* gene. The most frequent amino acid substitutions in the Spike protein of Delta variant were T19R, G142D, A222V, L452R, T478K, Q613H, D614G, P681R, D950N, and V1264L. While the Omicron variant had more mutations than the Delta variant. The amino acid mutations T478K, D614G, D950N, and N969K were found in Delta and Omicron variants. However, the frequency of D950N mutation in the Omicron and N969K mutation in the Delta was low (<10%).

The Delta variant had more frequent amino acid substitutions than the Omicron in the *nsp12* gene. Several mutations found in Delta variant were V42V, R249M, P323L, G671S, and N600N. While in Omicron variant, the prevalent mutations were P323L, N600N, and L758L. Interestingly, Delta and Omicron variants had similar amino acid substitutions P323L with a frequency of 100%. The amino acid substitution in the *nsp5* gene only occurred in the Omicron variant. There are three amino acid changes with >10% frequency, including L27L, R131R, and P132H. In the *nsp5* gene, the amino acid substitutions occurred in both variants. However, Omicron had more prevalent mutations than Delta, with nine amino acid substitutions identified within this gene. F106F mutation occurred in both variants with a high frequency (>95%). For P1228L mutation, the frequency was higher in the Delta (85,9%) than in Omicron (2,9%) variants.

3.4. Substitution rates of the Spike, nsp12, nsp3, and nsp5 genes of SARS-CoV-2 Delta and Omicron variants

We then determined the susbtitution rate for each gene of SARS-CoV-2 Delta and Omicron variants circulating in Yogyakarta and Central Java provinces, Indonesia. In four genes studied, the substitution rates of Omicron were higher than that of Delta variants, particularly in the*Spike* and *nsp12* genes. The substitution rates for the*Spike* gene were 2.41 x 10^{-2} subs/site/yr (95% HPD: 6.82 x 10^{-4} to 5.67 x 10^{-2}) and 1.05 x 10^{-3} subs/site/yr (95% HPD: 3.65 x 10^{-6} to 3.92 x 10^{-3}) for Omicron and Delta variants, respectively. For *nsp12* gene, the substitution rates were 3.50 x 10^{-2} subs/site/yr (95% HPD: 3.36 x 10^{-7} to 1.24 x 10^{-1}) and 3.44 x 10^{-3} subs/site/yr (95% HPD: 1.25 x 10^{-6} to 9.49 x 10^{-3}) for Omicron and Delta variants, respectively. Similar findings were found in the*nsp3* and *nsp5* genes **(Table 1)** .

3.5. Selective pressure analysis

Selective pressure analysis was performed for each gene to comprehend which sites that evolve under selective pressure, implying that the sites are functionally essential for virus evolution. Selective pressure analysis on *Spike* gene revealed that several sites were under positive selection, including 95 (T), 142 (G), 222 (A), 452 (L), 614 (D), 1264 (V) in Delta and 213 (V), 339 (G), 375 (S), 417 (K), and 440 (N) in Omicron variants. In *nsp12* gene, only one site (249 [R]) was under positive selection in Delta variant. *Nsp3* gene revealed 1183 (A), 1206 (S), 1228 (P), and 1733 (E) that were under positive selection in Delta variant. No sites in *nsp5* gene that were under positive selection. Thus, in Omicron variant, we only identified positively selected sites in the *Spike* gene.

3.6. The effect of mutations to the encoded proteins

Some common mutations in Delta and Omicron variants were analyzed to examine their thermodynamic effect on the Spike protein (Supplementary Table 6). Mutations in the *Spike* gene of Delta variant, including T95I, G142D, A222V, Q613H, and D614G, demonstrated a stabilizng effect on the structure of the Spike protein. We then analyzed common mutations in the *Spike* gene of Omicron variant. We found that these mutations have either a stablizing and destabilizing effect on the structure of the Spike protein. In the *nsp12* gene, amino acid substitutions P323L found in both Delta and Omicron variants with a frequency of 100% has a stabilizing effect on the RdRp protein.

In the *nsp3* gene, we analyzed three mutations (G28R, P77L, and L120I) in Delta variant, which all have a stabilizng effect on the PLpro protein. In Omicron variant, T259I mutation has destabilizng effect. For the *nsp5* gene, we analyzed L75F (Delta) as well as P132H and P241L (Omicron) mutations, which all showed a stabilizing effect on the 3CLpro protein.

Discussion

Our current study showed that by analyzing the *Spike, nsp12, nsp3,* and *nsp5* genes, we clearly demonstrated a distinct evolutionary pattern of SARS-CoV-2 Delta and Omicron variants circulating in Yogyakarta and Central Java provinces, Indonesia, during May 2021 to February 2022. In Indonesia, the Omicron upsurge was occurred from late January until February 2022 and subsequently replaced the Delta variant.[45] We have previously shown that Delta- and Omicron-infected patients had similar hospitalization and mortality rates.[36] Our current study emphasized the need of continued and extensive SARS-CoV-2 sequencing surveillance as the primary method to quickly detect and respond the emergence of new variants.

A latest study showed that compared to the previous VOCs (Alpha, Beta, Gamma, and Delta), Omicron had the highest enrichment of amino acid substitutions within the *Spike* gene.[46] Indeed, new strains with higher transmissibility and infectivity have emerged due to mutations in the Spike protein.[2] Alterations of the amino acid in the Spike protein influenced the binding affinity and the viral fusion process.[47] Our results showed that D614G mutation occurred in all (100%) Delta and Omicron variants identified in this study, indicating that it was fixed in the viral population. Indeed, all VOCs were generated from the G614 variant lineage. Three mutations always accompany the D614G variant: a C-to-T in the 5' UTR; a silent C-to-T mutation at position 3.037; and a C-to-T mutation at position 14,408 that results in an amino acid change P323L in the *nsp12* gene.[48] The other mutations at position 477 (S477G, S477N, and S477R) of the Spike protein were prominent among monoclonal antibody (mAb)-escape mutation.[2] Our results showed that S477N mutation was found only in the Omicron variant (46.8%). A computational analysis showed that N477 had an increased binding affinity to ACE2.[49] Other studies reported that T19R, E156G, L452R, T478K, and P681R variants in the Spike protein exhibited a stabilizing effect on protein structure, facilitating the binding affinity for more stable interactions with the human ACE2. This stability effect may increase the transmission of the virus in human populations.[50] Our studies found T19R, L452R, T478K, and P618R mutations in Delta with a high frequency above 70% and L452R in Omicron with 46.8% frequency.

The nsp12-P323L mutation first appeared in January 2020 and became the predominant globally (>90%) by late April. Other mutations were found in a low frequency (3.5-4.0%), including E254D, A423V, A656S, V720I, and V776L.[51] It has been shown that P323L mutation may increase the mutation rate of SARS-CoV-2[18] and was associated with Covid-19 severity.[52] Molecular dynamic simulations showed that P323L mutation led to tighter binding with antiviral drug remdesivir (RDV).[53] Interestingly, it has been shown that the Spike D614G and RdRp P323L (G/L variant) have co-evolved to become more superior than the original D/P variant.[54] In our study, the P323L mutation is similarly conserved between Delta and Omicron variants, indicating its beneficial effect for viral evolution.

An early study analyzing SARS-CoV-2 isolates circulating in Indonesia until September 2020 revealed mutations in the PLpro (P77L and V205I) and 3CLpro (M49I and L50F) genes.[34] However, we did not find these mutations in our current study, suggesting that these mutations were not fixed in the viral population circulating in our region. Within the *3CLpro* gene, several unique (signature) mutations are identified, which are different across SARS-CoV-2 lineages. P132H, K90R, and G15S are prevalent mutations found in Omicron (B.1.1.529), Beta (B.1.351), and Lambda (C.37) variants, respectively. In contrast, Delta variant had no unique mutations within this gene.[55] Consistently, our finding showed that all Omicron variant circulating in our region harboring P132H mutation, while Delta variant had no prevalent mutation identified. One mutation (L75F) found in Delta variant was very low in frequency (1.4%). Notably, P132H mutation did not lead to nirmatrelvir resistance.[55]

Several mutations have been identified to confer resistance to nirmatrelvir, including Y54C, L167F, and E166V.[26,56]These mutations were not identified in our isolates. Interestingly,*in vitro* studies combining Y54C and L167F with P132H resulted in a functional protein.[56] This finding provides an insight that additional mutations in the *3CLpro* gene that may emerge in the Omicron variant may be clinically relevant in the future. In addition, mutations in *PLpro* and *3CLpro* genes were shown to be associated with the clinical course of Covid-19 patients. P108S mutation in the *3CLpro* gene resulted in reduced activity of the protease protein and was associated with milder clinical course.[57] In the *PLpro* gene, it has been shown that P78L

and K233Q mutations were associated with increased risk of death.[58] Interestingly, our current study found the synonymous mutation F106F in the *PLpro* gene both in Delta (97.7%) and Omicron (99.3%) variants.

It has been estimated that the background nucleotide substitution rate of SARS-CoV-2 was about 1.1 x 10^{-3}subs/site/year.[59] The emergence of VOCs can be ascribed to a 4-fold increase of the substitution rate above its background level that may have lasted for several weeks or months.[60] In addition to their defining (unique) mutations, each VOC may have a distinct evolutionary rate.[60] A previous study analyzing the phylodynamic of Delta and Omicron variants also showed that a mean rate of nucleotide substitution was higher in Omicron compared to Delta variants, i.e. 3.898 x 10^{-3} subs/site/year (range: 2.686 x 10^{-3} to 5.102 x 10^{-3}) and 3.677 $\times$ 10^{-4} subs/site/year (range: 1.311 x 10^{-4} to 6.144 x 10^{-4}) for the Omicron and Delta variants, respectively.[61]

Alterations in amino acids that decrease viral fitness are often eliminated by negative selection, while changes that improve viral fitness are retained by positive selection. On the other hand, amino-acid alterations are regarded as "neutral" when they have no effects on viral fitness. Because the existence of negative or positive selection suggests that specific sites are functionally significant, it is crucial to determine which sites evolve under selective pressure, especially in the case of novel emerging pathogens such as SARS-CoV-2. In this study, several positively selected sites in the *Spike, nsp12,* and *nsp3* genes were identified in Delta variant. However, in Omicron variant, we identified positively selected sites only in the*Spike* gene. This finding may imply a distinct genetic "hot-spot" in SARS-CoV-2 tropism, replication, and infectivity, and need to be further investigated.

Conclusion

During the study period, we identified 213 and 139 isolates of Omicron and Delta variants, respectively, co-circulating in our region. Particularly in the *Spike* and *nsp5* genes, high frequency amino acid substitutions were significantly more abundant in Omicron than in Delta variants. Consistently, in all of four genes studied, the substitution rates of Omicron were higher than that of Delta variants, especially in the *Spike* and *nsp12* genes. In addition, selective pressure analysis revealed several sites in particular genes that were positively selected, implying that these sites were functionally essential for virus evolution, including 95 (T), 142 (G), 222 (A), 452 (L), 614 (D), 1264 (V) in Delta and 213 (V), 339 (G), 375 (S), 417 (K), and 440 (N) in the *Spike* gene of Omicron variants. Our study demonstrated a distinct evolutionary pattern of SARS-CoV-2 Delta and Omicron variants circulating in Yogyakarta and Central Java provinces, Indonesia. Thus, our study emphasized the need of continued and extensive SARS-CoV-2 genomic surveillance to quickly detect and respond the emergence of new variants.

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Author contributions

MSH and GUN conceived and designed the study. LSE, ES, KAV, KI, AFI, FDTU, VCA, SSN, TPL, and FVH, performed RT-PCR, the library preparation, and NGS. MSH, GUN, AR, ES, EWD, FNO, LA, EA,

TN, and TW collected the data. MSH, GUN, AR, and HW analyzed the data. MSH and AR drafted the manuscript. HW and TW critically revised the manuscript for important intellectual content. All authors have read and approved the final version of the manuscript.

Statements and declarations

The authors declare no conflict of interest.

Data availability

Datasets, output files, and other data generated during this study are available from the corresponding author upon request.

ORCID

Mohamad S. Hakim: http://orcid.org/0000-0001-8341-461X

Tri Wibawa : https://orcid.org/0000-0003-0477-4575

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Figure Legends

Figure 1. Phylogenetic analysis of SARS-CoV-2 from Yogyakarta and Central Java, Indonesia based on the *Spike* gene. A phylogenetic tree was constructed from the *Spike* gene of SARS-CoV-2 using the maximum likelihood statistical method, with 1,0000 bootstrap replications and the best substitution model for the dataset (GTR+Gamma).

Figure 2. Phylogenetic analysis of SARS-CoV-2 from Yogyakarta and Central Java, Indonesia based on the *nsp12* **(A),***nsp3 (PLpro)* **(B),** and *nsp5 (3CLpro)* **(C)**genes.

Figure 3. High frequency (hotspot) mutations (>10%) identified in the *Spike* **(A),***nsp12* **(B),** *nsp3 (PLpro)* **(C),** and*nsp5 (3CLpro)* **(D)** genes of Delta and Omicron variants.

Figure 4. Visualization of the 3D structure of the Spike**(A),** RdRp **(B),** nsp3 (PLpro) **(C),** and nsp5 (3CLpro) **(D)** containing identified mutations from the top and side view.

Table 1. Rates of nucleotide substitutions of the *Spike, nsp12, nsp3,* and *nsp5* genes of SARS-CoV-2 Delta and Omicron variants found in this study.

Table 2. Selective pressure analysis on the *Spike, nsp12, nsp3,* and *nsp5* genes of SARS-CoV-2 Delta and Omicron variants. Positively selected sites were shown.

Supplementary Table 1. The GISAID accession numbers of SARS-CoV-2 isolates identified in this study.

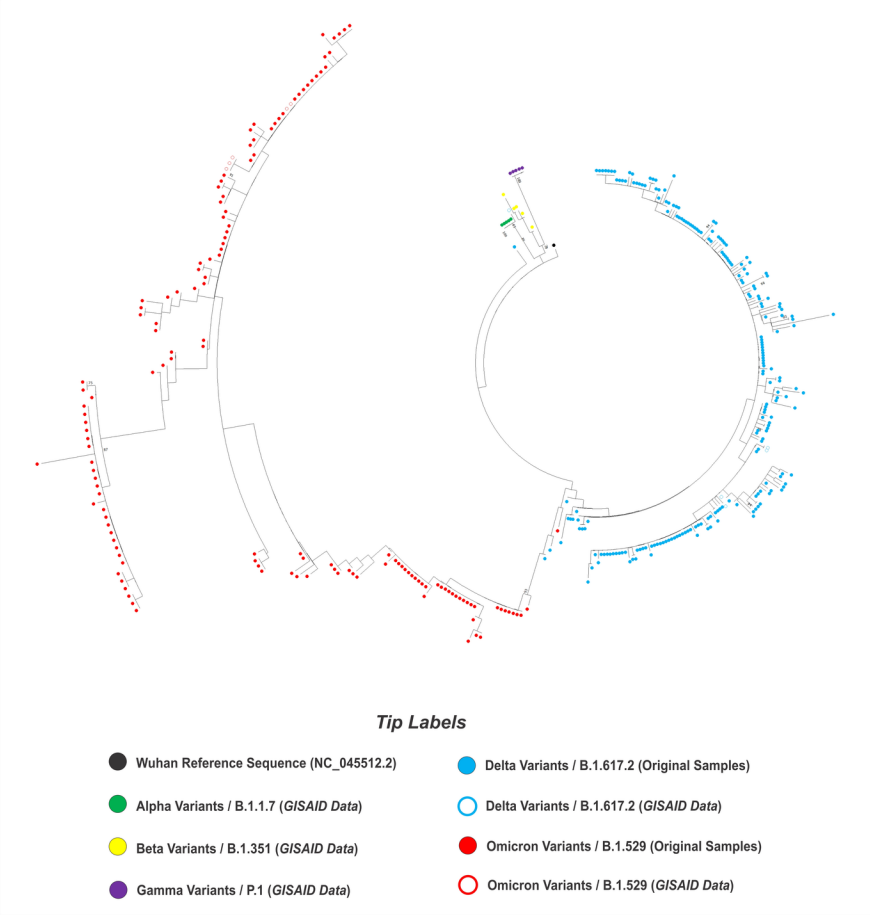
Supplementary Table 2. The GISAID accession numbers of the reference sequences of Alpha, Beta, Delta, Gamma, and Omicron variants.

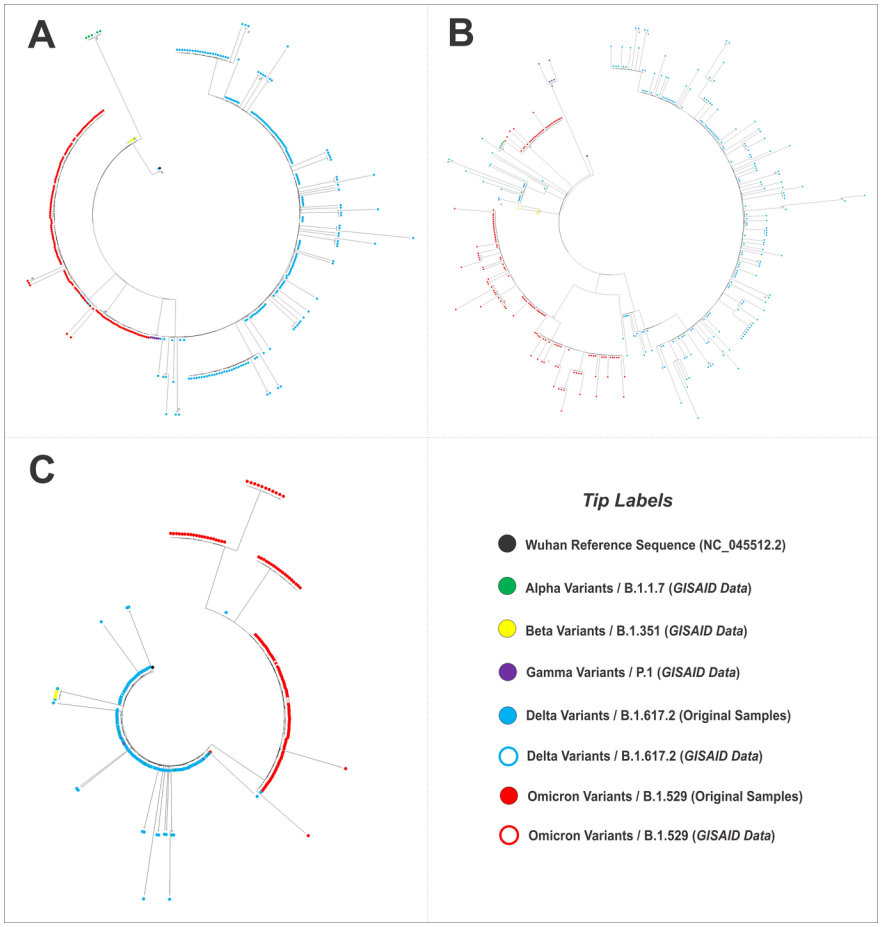
Supplementary Table 3. The genome sequencing quality of all sequences identified in this study.

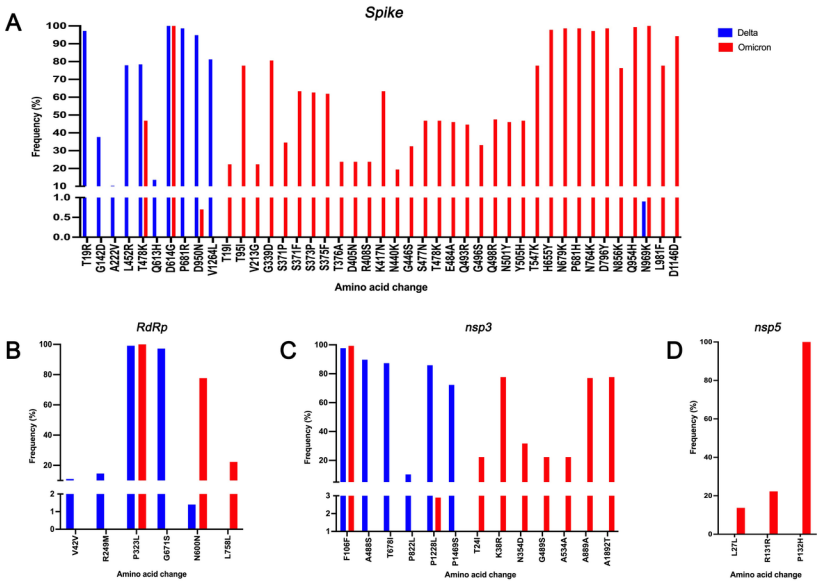
Supplementary Table 4. The genome position of the *nsp3, nsp5, RdRp* and *Spike* genes of SARS-CoV-2 based on the reference genome from Wuhan, China (hCoV-19/Wuhan/Hu-1/2019, GenBank accession number: NC_045512.2)

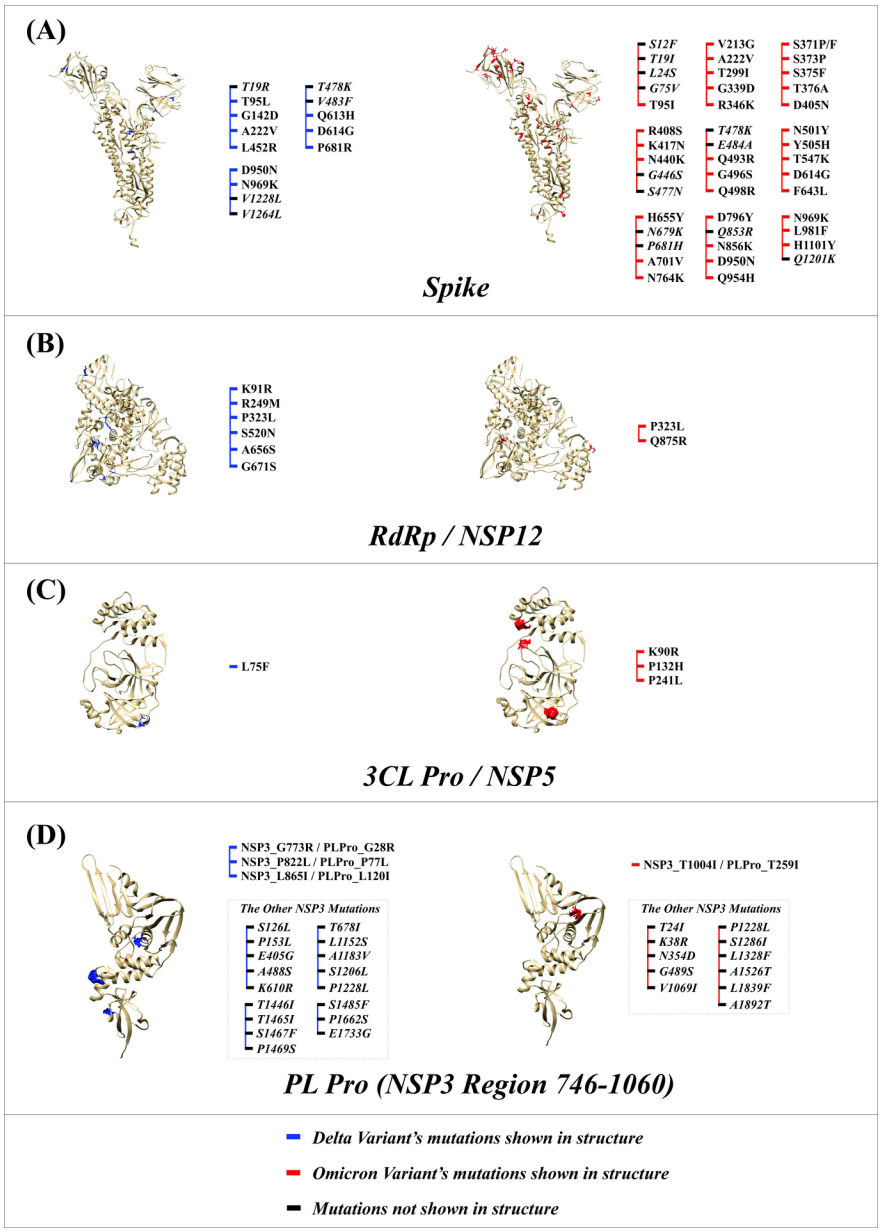
Supplementary Table 5. Synonymous and nonsynonymous mutations identified within the *Spike, nsp12, nsp3,* and *nsp5* genes of SARS-CoV-2 Delta and Omicron variants.

Supplementary Table 6. Mutation effect analysis of the Spike**(A)** , and RdRp, nsp3, and nsp5 proteins **(B)** of SARS-CoV-2 Delta and Omicron variants.









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