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Genetic Variation of Structural and Functional Genes of SARS-COV-2 Isolates Circulating in Banyumas (Indonesia)

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Abstract. *Scientists are performing various measurements to overcome the COVID-19 pandemic. The genomic mutations of SARS-CoV-2 can change their pathogenicity, infectivity, transmission, and antigenicity. This present study aimed to know a) the genetic variation of structural and functional genes of SARS-CoV-2 circulating in Banyumas and b) the potential of the Cilacap's Harbour as a human mobility portal on the genetic variations of SARS-CoV-2 circulating in Banyumas, Central Java (Indonesia). Genomic sequence of SARS-CoV-2 isolates were taken from the Global Initiative on Sharing All Influenza Data (GISAID) and the National Center for Biotechnology Information (NCBI) online platforms. A gene cut was carried out from Wuhan reference isolate, fifteen isolates from Banyumas, and two isolates from Cilacap (Central Java) using Unipro UGENE v. 33.0 software, considering the annotation of the Wuhan-1 isolate. Genetic variations were detected among SARS-CoV-2 isolates circulating in Banyumas. The structural protein (envelope, membrane, nucleocapsid) encoding gene and the RdRp gene were highly conserved to Wuhan reference genome (Wuhan-Hu-1). Meanwhile, the ORF and the spike-encoding genes were less identical to the Wuhan reference genome. This study also proposed that human mobility from outside Central Java through Cilacap's Harbour did not affect the genetic variation of SARS-CoV-2 isolates circulating in Banyumas (Central Java).*

Keywords: *Banyumas, genetic variation, mutations, public mobilization, SARS-CoV-2*

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INTRODUCTION

A new pneumonia case was found in Wuhan, Hubei (China) on December 31, 2019. It is caused by a novel coronavirus infection in the human respiratory system (Decaro & Lorusso, 2020; Gralinski & Menachery, 2020; Lu et al., 2020). Then, the WHO officially named the SARS-CoV-2 virus (Severe Acute Respiratory Syndrome Coronavirus 2) and declared the Coronavirus Disease 2019 (COVID-19) pandemic (Anggraini & Listyorini, 2021). COVID-19 has spread to almost all parts of the world in a few months. As of January 25, 2023, WHO reported over 664,873,023 confirmed cases of COVID-19, including 6,724,248 deaths worldwide (https://worldometer.com).

SARS-CoV-2 spreads through aerosols, nasopharyngeal droplets, and direct contact with contaminated surfaces (Lu et al., 2020). Since SARS-CoV-2 is highly trans-

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missible among humans, the transmission of COVID-19 spread quickly to many countries in the world, including Indonesia. On March 2, 2020, President Joko Widodo announced the first incidence of COVID-19 in Indonesia (Djalante et al., 2020). Within 3 months, COVID-19 cases have been found in almost all provinces in Indonesia (Rovik et al., 2022). Scientists are performing various measurements to overcome the COVID-19 pandemic. By the end of 2020, researchers reported that the mutation of SARS-CoV-2 had resulted in genetic variation among isolates and regions. The SARS-CoV-2 mutations may affect vaccine development and other therapeutic measurements. Generally, genomic mutations in viruses can change their pathogenicity, infectivity, transmission, and antigenicity (Harvey et al., 2021; Nidom et al., 2021). The genetic variation of SARS-CoV-2 is reported to cause more severe disease, spread easily, and reduce antibody neutralization in patients who have been previously vaccinated or infected (Gunadi et al., 2020; Harvey et al., 2021; Li et al., 2021). This present study aimed to know a) the genetic variation of structural and functional genes of SARS-CoV-2 circulating in Banyumas and b) the potential of the Cilacap's Harbour as a human mobility portal on the genetic variations of SARS-CoV-2 circulating in Banyumas, Central Java, Indonesia.

MATERIALS AND METHODS

Isolates of SARS-CoV-2 from Indonesian patients, especially from Banyumas District, were taken from the Global Initiative on Sharing All Influenza Data (GISAID) page on the https://gisaid.org/. The data was filtered with the following criteria: human-hosted, whole genome, and Banyumas. Fifteen isolates were retrieved from Banyumas District (Table 1). Two isolates were retrieved from Cilacap, a neighbouring district of Banyumas, to evaluate the potential of the Cilacap's Harbour as a human mobility portal on the genetic variations of circulating SARS-CoV-2 . The first SARS-CoV-2 genome sequence reported in Wuhan (Wuhan-Hu-1) was extracted from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/sars-cov-2/) as a reference genome.

Table 1. Source and accession code of studied SARS-CoV-2 isolates

Source	Isolate	Host (Age)	Date of Sample Collection
Wuhan, China	Wuhan-Hu-1	*Homo sapiens*	2020-07-18
Banyumas	EPI_ISL_3086886	Male (58)	2021-06-29
Banyumas	EPI_ISL_3086887	Female (19)	2021-06-29
Banyumas	EPI_ISL_3086888	Female (40)	2021-06-26
Banyumas	EPI_ISL_3086889	Male (33)	2021-06-26
Banyumas	EPI_ISL_3086890	Male (38)	2021-06-26
Banyumas	EPI_ISL_3086891	Female (22)	2021-06-23
Banyumas	EPI_ISL_3086892	Female (35)	2021-06-21
Banyumas	EPI_ISL_3086893	Female (19)	2021-06-04
Banyumas	EPI_ISL_3086894	Male (30)	2021-04-09
Banyumas	EPI_ISL_3086895	Female (45)	2021-04-01
Banyumas	EPI_ISL_3086896	Male (49)	2021-03-25
Banyumas	EPI_ISL_4106136	Female (20)	2021-06-18
Banyumas	EPI_ISL_4106137	Female (38)	2021-06-16
Banyumas	EPI_ISL_4106138	Male (61)	2021-05-17
Banyumas	EPI_ISL_4106139	Female (42)	2021-05-07
Cilacap	EPI_ISL_2233091	Male (33)	2021-04-25
Cilacap	EPI_ISL_4730572	Male (21)	2021-07-26

The encoding nucleic acid sequences of structural genes (M, N, S, and E) and functional genes (RdRp and ORF8) were extracted from SARS-CoV-2 whole genome sequences. A gene cut was carried out from all isolates from Banyumas and two isolates from Cilacap (Central Java) using Unipro UGENE v. 33.0 software, considering the annotation of the Wuhan-1 isolate. The multiple sequence alignment was performed in MEGA 11 software. Each sequence was manually verified to remove Ns within sequences, unidentified positions, and gaps. Furthermore, the mutation analysis, genetic distance calculation, and phylogenetic tree construction of SARS-CoV-2 isolates were performed in MEGA 11 software. The maximum likelihood approach was used to generate the phylogenetic tree.

RESULTS AND DISCUSSION

The virus that causes COVID-19, SARS-CoV-2, is an enveloped positive-sense single-stranded RNA virus with a genomic size of around 30,000 bp sequences. The SARS-CoV-2 genome contains five major open reading frames (ORFs), sixteen non-structural proteins (NSPs), and structural proteins, such as the spike (S), membrane (M), envelope (E), and nucleocapsid (N) (Figure 1). The viral envelope is made up of the envelope and membrane, as well as spike proteins. "The non-structural RNA genome, which contains ORF1ab, ORF3, ORF6, 7a, 8, and ORF10, has highly conserved information for ORF1ab genome RNA synthesis and replication" (Hakim et al., 2020; Jamison et al., 2022).

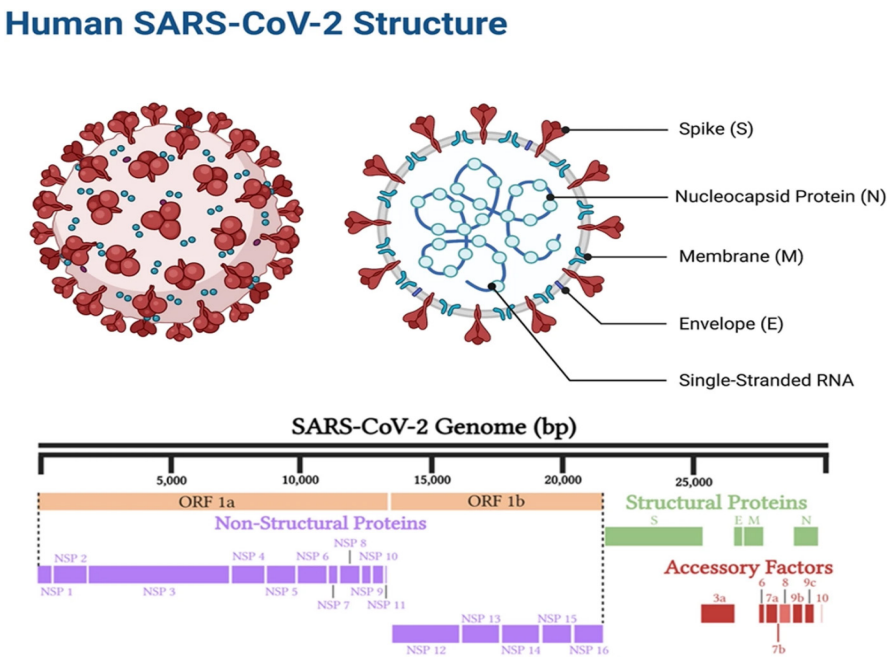


Figure 1. The structure of the SARS-CoV-2 virus has several structural proteins (cited from Jamison et al., 2022), i.e., spike (S), nucleocapsid (N), membrane (M), and envelope (E). Several non-structural proteins, among them ORF 1a and ORF 1b.

The analysis of the spike (S) gene showed that circulating SARS-CoV-2 isolates in Banyumas are identical (distance = 0.0004) to the Wuhan reference partial genome, except EPI_ISL_3086895 (distance = 0.0007). Interestingly, most Banyumas isolates were placed separately from the Wuhan-1 isolate (Figure 2A). It confirmed that few mutations appeared in the spike gene compared to the origin isolate. In addition, two isolates of Cilacap were placed in one clade with most Banyumas isolates. Lu et al. (2020) reported that the S gene is the most variable genomic component of SARS-CoV-2. Similarly, Turnip et al. (2023) reported that the spike gene was found to be less conserved among circulating SARS-CoV-2 in Asian countries. Transmembrane spike glycoprotein facilitates virus entry into the host cell by utilizing the host's cellular angiotensin-converting enzyme 2 (ACE2) (Jamison et al., 2022). Therefore, the genetic variation of the spike gene may be associated with the transmission rate of the virus and challenges in vaccine development (Harvey et al., 2021).

The analysis of the envelope (E) gene showed that circulating SARS-CoV-2 isolates in Banyumas are highly identical (100%) to the Wuhan reference partial genome (Figure 2B). It was confirmed that little or no mutation is found in the envelope gene of circulating isolates, both in Banyumas and Cilacap Districts. Meanwhile, the Cilacap isolate (EPI_ISL_4730572) is placed in a different clade from other SARS-CoV-2 isolates. It showed that different sources of infection are possible in Cilacap district. Jamison et al. (2022) reviewed the E gene as a conserved genomic component of SARS-CoV-2. Mutations that occur in gene E are very rare; missense mutations in gene E account for less than 0.5% of the SARS-CoV-2 genome. These mutations can affect the integrity and life cycle of the virus (Wu et al., 2023).

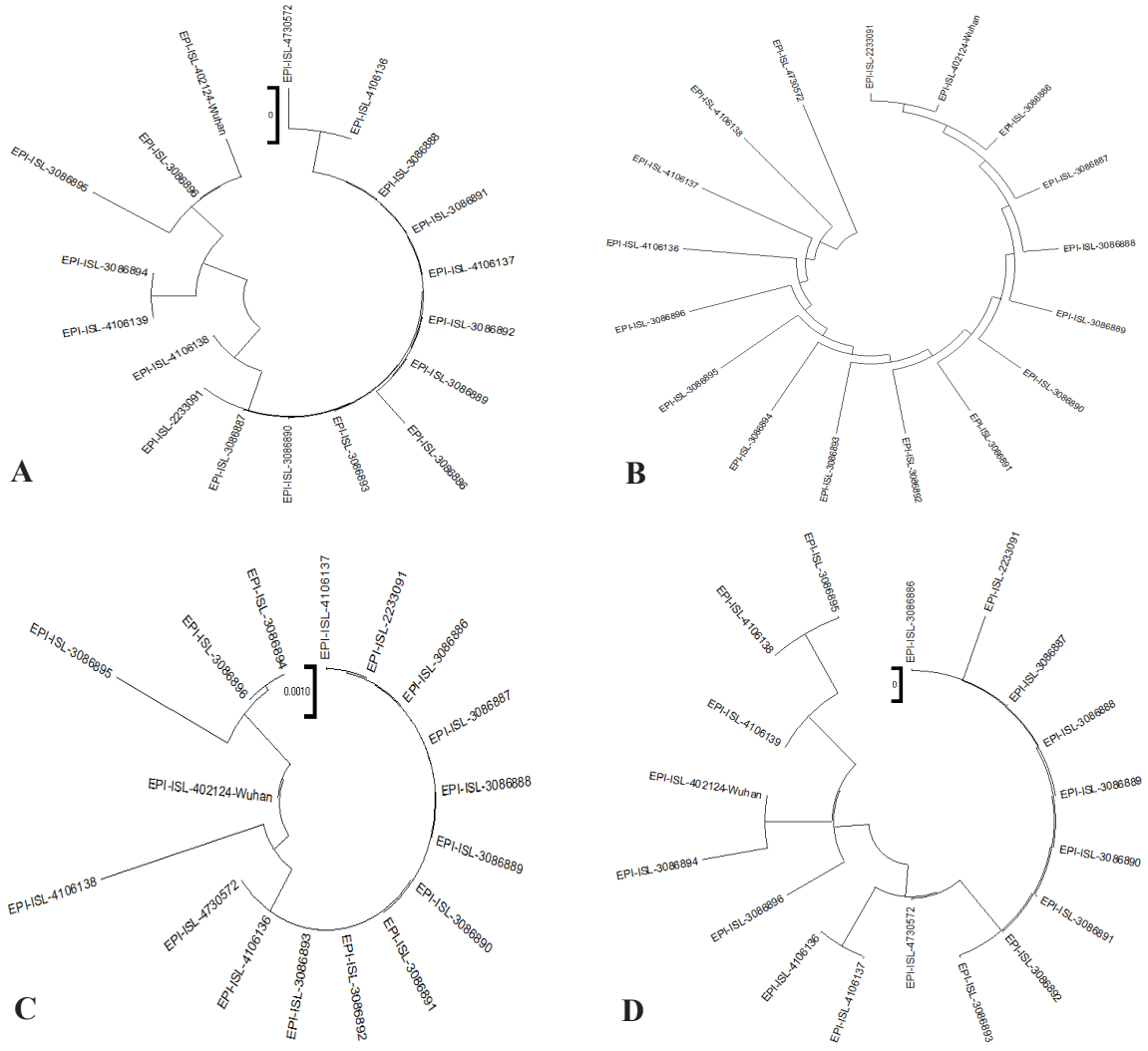
The analysis of the membrane (M) gene showed that circulating SARS-CoV-2 isolates in Banyumas are identical (distance = 0.0004) to the Wuhan reference partial genome (Figure 2C). Although phylogenetic analysis divided all samples into two different clades, the Wuhan-1 isolate was placed in the same clade (distance = 0.0015) as isolates EPI_ISL_3086894, EPI_ISL_3086895, and EPI_ISL_3086896. Meanwhile, two isolates of Cilacap were 100% identical to most Banyumas isolates. A membrane glycoprotein encoding gene can be found between nucleotides 26398 and 27063 in the whole genome. It is highly conserved in the genome sequence of SARS-CoV-2 (Toptan et al., 2020).

The analysis of the nucleocapsid (N) gene showed that circulating SARS-CoV-2 isolates in Banyumas are highly identical to the Wuhan reference partial genome (Figure 2D). Although the samples were placed in two different big clades (distance = 0.0005), two isolates of Cilacap were placed in the same clade as most Banyumas isolates. To date, the N gene is the main target gene in PCR and antigen-based serology tests because the nucleocapsid protein is encoded by the majority of the nuclear viral RNA genome (Jamison et al., 2022). Even so, its mutation can impact false-negative results in SARS-CoV-2 real-time RT-PCR screening (Lesbon et al., 2021).

The analysis of the RNA-dependent RNA polymerase (RdRp) gene showed that circulating SARS-CoV-2 isolates in Banyumas are highly identical to the Wuhan reference partial genome (Figure 2E). Although the samples were placed in two different big clades (distance = 0.0034), most Banyumas isolates were placed in the same clade as the Wuhan-1 isolate. It also confirmed that SARS-CoV-2 from Wuhan (China) had spread to sur-

rounding countries with fewer mutations on the RdRp functional gene. Turnip et al. (2023) reported that the RdRp gene was reported to be highly conserved in SARS-CoV-2 circulating among ASEAN countries.

The analysis of the RNA-dependent Open Reading Frame 8 (ORF8) gene showed that circulating SARS-CoV-2 isolates in Banyumas are less identical to the Wuhan reference partial genome (Figure 2F). Isolates EPI_ISL_4106136, EPI_ISL_4106137, EPI_ISL_4106139, EPI_ISL_3086888, and EPI_ISL_3086896 were placed in a different clade with the Wuhan-1 isolate. Two isolates of Cilacap were grouped in the same clade as most of the Banyumas isolates. The ORF8 gene is identified in the SARS-CoV-2 genome's hypervariable region (Vinjamuri et al., 2022). In the whole genome, the ORF8 encoding gene can be found between nucleotides 27894 and 28259.



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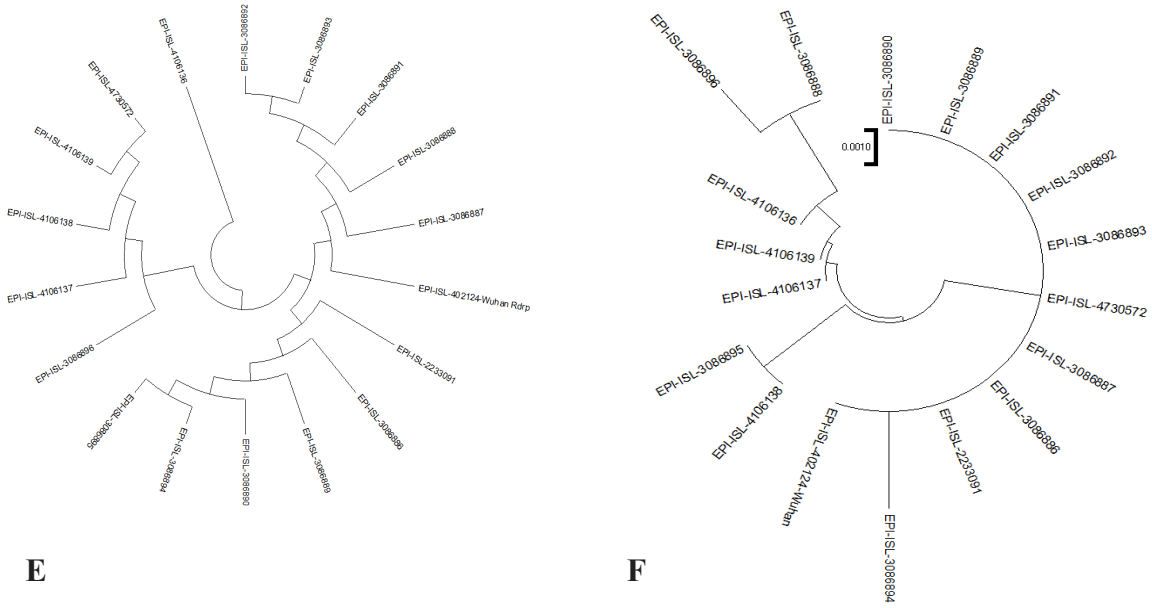

Figure 2. Analysis of SARS-CoV-2 phylogeny circulating in Banyumas District based on the A) spike (S), B) envelope (E), C) membrane (M), D) nucleocapsid (N), E) RNA-dependent RNA polymerase gene (RdRp), and F) open reading frame 8 (ORF8) encoding genes. The construction of phylogenetic trees is carried out with maximum likelihood in MEGA 11 software.

SARS-CoV-2, like all viruses, has a high mutation rate due to its replication strategy. The mutation rate estimates of roughly $1x10^{-6}$ to $2x10^{-6}$ mutations per nucleotide per replication cycle are comparable with prior estimates in other beta-coronaviruses (Marcov et al., 2023). The virus has a large RNA genome that is prone to errors during replication, leading to the emergence of mutations over time. Mutations can occur in both the functional and structural proteins of the virus (Wu et al., 2023). To date, the SARS-CoV-2 diagnostic targets various genes, both structural and functional genes. Most serology-based rapid diagnostics target the nucleocapsid (N) encoding gene . Meanwhile, real-time RT-PCR targets multiple regions, including N, M, E, S, RdRp, and ORF encoding genes. The mutation of SARS-CoV-2 resulted in genetic variation among isolates. Therefore, its mutation can impact the false-negative in the SARS-CoV-2 diagnostic.

Many variants of SARS-CoV-2 have been found worldwide, including Alpha, Beta, Gamma, and Delta as the variants being monitored (VBM) and Omicron as the variant of concern (VOC). SARS-CoV-2 continues to evolve with changes in the genetic code, such as viral mutations or recombinations that occur during genome replication. Each variant has one or more mutations that differentiate it from other SARS-CoV-2 variants (Wu et al., 2023). Only two clades, clades O and L , were found in Indonesia during the early pandemic. Later, more clades were dominantly found in COVID-19 cases, such as clades G, GR, and GH (Gunadi et al., 2020).

In the present study, we detected genetic variation among SARS-CoV-2 isolates circulating in Banyumas. The structural protein (envelope, membrane, nucleocapsid) encoding gene and the RdRp gene were highly conserved to Wuhan reference genome (Wuhan-Hu-1). Meanwhile, the ORF gene and

the spike-encoding gene were less identical to the the Wuhan reference genome. There is some evidence to suggest that the mutation rate may be higher in the structural proteins of SARS-CoV-2 compared to the functional proteins. For example, Weisblum et al. (2020) found that the S protein had a higher mutation rate compared to the NSPs. Meng et al. (2021) reported that the deletion at position 69-70 of the spike gene of the SARS-CoV-2 Alpha variant has been linked to increased infectivity. However, more study is required to completely comprehend the mutation rates in different proteins and their implications for the virus's behaviour.

SARS-CoV-2 is known to undergo mutations as it spreads among populations. These mutations can lead to changes in the virus's behaviour, including its transmissibility, severity, and response to vaccines and treatments (Weisblum et al., 2020; Harvey et al., 2021). For example, the spike protein is the primary antigenic component responsible for generating an immunological response in the host, neutralizing antibodies, and/or protective immunity against viral infections (Lu et al., 2020; Weisblum et al., 2020). However, genetic modifications can sometimes be silent and have no influence on viral behaviour (Tegally et al., 2021).

Understanding the mutation rates and patterns of SARS-CoV-2 is important for tracking the spread of the virus and developing effective countermeasures. Mutations can arise spontaneously, particularly in areas with high levels of virus transmission, where the virus is replicating rapidly in large numbers of people. Public measures, such as wearing masks, practising social distancing, and getting vaccinated, can help to slow the spread of the virus and reduce the number of people who become infected. This, in turn, can reduce the opportunity for the virus to mutate. However, it is important to note that the emergence of new variants is not solely dependent on human behaviour.

Ports can potentially serve as a source of new virus variants, as they facilitate the movement of people and goods across different regions and countries. Viruses can spread through the movement of infected individuals or contaminated goods, and the mixing of different populations in port areas can increase the opportunities for viral transmission and mutation. One example of this is the emergence of new SARS-CoV-2 variants in different parts of the world, including the Delta variant that was first identified in India (Yang & Shaman, 2022). It is believed that this variant emerged in part due to increased travel and transmission in crowded port cities like Mumbai, where the virus had a higher opportunity to mutate and spread. However, it is important to note that the emergence of new variants is not unique to ports. The virus can mutate and evolve anywhere there is transmission, whether that is in a port city or a rural area. In Indonesia, the massive spread of COVID-19 cases might be aided further by human travel patterns all over the world. For example, since September 2020, the G614 variant has completely replaced the D614 variant in all Indonesian regions (Wijayanti et al., 2022). Additionally, the emergence of new variants is not necessarily a cause for alarm, as not all variants will have significant impacts on the virus's behaviour or the effectiveness of vaccines and treatments.

Citizen mobilization may impact the spread of SARS-CoV-2, but it is unlikely to directly affect the mutation of the virus. In this present study, two SARS-CoV-2 isolates from Cilacap are generally placed in the same clade as Banyumas isolates. It proposed that human mobilization from outside Central Java through the Cilacap's Harbour did not af-

fect the genetic variation of SARS-CoV-2 isolates circulating in Banyumas (Central Java). It is important to understand that a single sick passenger can transport a mutation from one nation to another. Tegally et al. (2021) reported that the closing of international borders reduced the entry of viral lineages from outside the country in Africa.

While public mobilization and restrictions can help slow the spread of the virus and reduce the opportunities for new variants to emerge, they are not a guaranteed solution to preventing mutations. Transportation limitations only aid in the prevention of the first spread. The virus cannot spread over long distances in a short period of time without a travel link. To prevent the spread of viruses through ports, it is important to implement effective public health measures such as screening, testing, contact tracing, and quarantine protocols for incoming travellers and goods, especially passengers who arrived from areas with a high burden of COVID-19 cases. This can help to identify and isolate infected individuals and limit the spread of the virus, reducing the opportunities for mutation and the emergence of new variants.

CONCLUSION

This study detects genetic variation among circulating SARS-CoV-2 isolates in Banyumas District, both in the structural and functional protein-encoding genes. This study also proposed that human mobility from outside Central Java through Cilacap's Harbour did not affect the genetic variation of SARS-CoV-2 isolates circulating in Banyumas (Central Java).

AUTHOR CONTRIBUTION

Conceptualization: A.R. Methodology: A.R. Investigation: A.R. Writing—original draft preparation: AR and C.A.B.N. Writing—review and editing: A.R. and C.A.B.N.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this present study.

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