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Emergence of SARS-CoV-2 Omicron lineages BA.4 and BA.5 in South Africa

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Editor summary:

Genomic characterization of the SARS-CoV-2 Omicron lineages BA.4 and BA.5, responsible for the fifth COVID-19 pandemic wave in South Africa, shows continued viral diversification, and provides insights into the potential mechanisms underlying the ability of the new lineages to outcompete their predecessors.

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1. Extended Data

Figure #	Figure title One sentence only	Filename This should be the name the file is saved as when it is uploaded to our system. Please include the file extension. i.e.: *Smith_ED_Fig1.jpg*	Figure Legend If you are citing a reference for the first time in these legends, please include all new references in the main text Methods References section, and carry on the numbering from the main References section of the paper. If your paper does not have a Methods section, include all new references at the end of the main Reference list.
Extended Data Fig. 1	Molecular clock signal of the dataset of BA.2, BA.4 and BA.5 lineages used in the Bayesian analysis.	ED_Fig1.tiff	Root-to-tip regression obtained from TempEst analysis for the sampled cluster of BA.2, BA.4 and BA.5, showing a relatively strong clock-like behaviour (correlation coefficient = 0.6, R2 = 0.4) The regression line (representing the estimated mean evolutionary rate) is shown with error buffers (shaded area) that represent 90% confidence intervals.

Extended Data Fig. 2	Whole genome mutations present in BA.4 and BA.5 lineages.	ED_Fig2.tiff	Differences in BA.4 and BA.5 are highlighted with a rectangle. The synonymous mutations in nsp8 is indicated in red.
Extended Data Fig. 3	Patterns of natural selection between January 2020 and January 2022 at codon sites differentiating BA.4 and BA.5 from BA.2.	ED_Fig3.tiff	All SARS-CoV-2 sequences deposited in GISAID were analyzed with each time-point representing an analysis of all sequences sampled during the preceding three months. Red dots indicate evidence at positive selection and blue spots indicate evidence of negative selection. The sizes of the dots indicate degrees of statistical support for selection signals. Only sequences deposited in GISAID prior to the discovery of BA.4 and BA.5 are considered here.
Extended Data Fig. 4	Progression of the weekly genomic prevalence of various variants and lineages in the nine provinces of South Africa from November 2021 to April 2022.	ED_Fig4.tiff	
Extended Data Table 1	S-gene target status (TaqPath™ COVID-19 qPCR assay) for 198 samples sequenced by KRISP laboratory.	ED_Table1.jpg	*One BA.2 sequence had the 69/70del, while the other BA.2 sequence had large gaps in coverage of the spike gene region.
Extended Data	Comparison of	ED_Table2.jp	Rates were estimated with multinomial

Table 2	daily growth rates of all Omicron lineages and Delta.	g	logistic regression models based on South African SARS-CoV-2 genomic data spanning the period of 1 November 2021 to 29 May 2022. Negative values indicate the comparative lineage to have a growth advantage over the reference lineage, while a positive value indicates the reference lineage to have a growth rate advantage over the lineage of comparison.

2. Supplementary Information:

A. Flat Files

Item	Present?	Filename This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. The extension must be .pdf	A brief, numerical description of file contents. i.e.: *Supplementary Figures 1-4, Supplementary Discussion, and Supplementary Tables 1-4.*
Supplementary Information	No		
Reporting Summary	Yes	flat_de_Oliveira_rs2.pdf	
Peer Review Information	No	*OFFICE USE ONLY*	

B. Additional Supplementary Files

Type	Number If there are multiple files of the same type this should be the numerical indicator. i.e. “1” for Video 1, “2” for Video 2, etc.	Filename This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. i.e.: *Smith_Supplementary_Video_1.m*	Legend or Descriptive Caption Describe the contents of the file

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Supplementary Table	1	SupplementaryTableS1.xlsx	GISAID Acknowledgement table

Emergence of SARS-CoV-2 Omicron lineages BA.4 and BA.5 in South Africa

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Abstract
Three lineages (BA.1, BA.2 and BA.3) of the SARS-CoV-2 Omicron variant of concern predominantly drove South Africa's fourth COVID-19 wave. We have now identified two new lineages, BA.4 and BA.5, responsible for a fifth wave of infections. The spike proteins of BA.4

and BA.5 are identical, and comparable to BA.2 except for the addition of 69-70del (present in the Alpha variant and the BA.1 lineage), L452R (present in the Delta variant), F486V and the wild type amino acid at Q493.The two lineages only differ outside of the spike region. The 69-70 deletion in spike allows these lineages to be identified by the proxy marker of S-gene target failure, on the background of variants not possessing this feature . BA.4 and BA.5 have rapidly replaced BA.2, reaching more than 50% of sequenced cases in South Africa by the first week of April 2022. Using a multinomial logistic regression model, we estimate growth advantages for BA.4 and BA.5 of 0.08 (95% CI: 0.08 - 0.09) and 0.10 (95% CI: 0.09 - 0.11) per day respectively over BA.2 in South Africa. The continued discovery of genetically diverse Omicron lineages points to the hypothesis that a discrete reservoir, such as human chronic infections and/or animal hosts, is potentially contributing to further evolution and dispersal of the virus.

Main text

Within days of being discovered in South Africa and Botswana, on November 26, 2021, the Omicron variant of SARS-CoV-2 was designated as a variant of concern (VOC) by the World Health Organization[1]. Initially, Omicron was comprised of three sister lineages, BA.1, BA.2 and BA.3. BA.1 caused most of the infections in South Africa's fourth epidemic wave. However, as that wave receded in mid-January 2022, BA.2 became the dominant South African lineage. Despite being associated with a modest prolongation of the fourth wave, the displacement of BA.1 by BA.2 in South Africa was not associated with a significant resurgence in cases, hospital admissions or deaths. This pattern was not consistent worldwide, however, and in some countries BA.2 was responsible for a greater share of cases, hospitalizations and deaths during the Omicron wave[2–4].

We recently identified two new Omicron lineages that have been designated BA.4 and BA.5 by the Pango Network and pango-designation v1.3, a system of naming and classifying SARS-CoV-2 lineages (Fig. 1A)[5,6]. Bayesian phylogenetic methods revealed that BA.4 and BA.5 are distinct from the other Omicron lineages (Molecular clock signal: correlation coefficient = 0.6, $R^2 = 0.4$, Extended Data Fig. 1). BA.4 and BA.5 are estimated to have originated in mid-December 2021 (95% highest posterior density [HPD] 25 November 2021 to 01 January 2022) and early January 2022 (HPD 10 December 2021 to 6 February 2022) respectively (Fig. 1A). The most recent common ancestor of BA.4 and BA.5 is estimated to have originated in mid-November 2021 (HPD 29 September 2021 to 6 December 2021) (Fig. 1A), coinciding with the emergence of the other lineages, for example BA.2 in early November 2021 (HPD: 9 October 2021 to 29 November 2021). Phylogeographic analysis suggests early dispersal of BA.4 from Limpopo to Gauteng, with later spread to other provinces (Fig. 1B); and early dispersal of BA.5 from Gauteng to KwaZulu-Natal, with more limited onward spread to other provinces (Fig. 1C).

BA.4 and BA.5 have identical spike proteins, most comparable to BA.2. Relative to BA.2, BA.4 and BA.5 have the additional spike mutations 69-70del, L452R, F486V and wild type amino acid at position Q493 (Fig 1D). Outside of spike, BA.4 has additional mutations at ORF7b:L11F and N:P151S and a triple amino acid deletion in NSP1:141-143del whilst BA.5 has the M:D3N mutation. Relative to BA.2, BA.5 has additional reversions at ORF6:D61 and nucleotide positions 26858 and 27259. In addition, BA.4 and BA.5 have a nuc:G12160A synonymous mutation in NSP8 that was present in Epsilon (B.1.429) and has arisen in BA.2 in some locations (Extended Data Fig. 2). BA.4 and BA.5 have identical mutational patterns in the 5' genome region (from ORF1ab to Envelope) yet exhibit genetic divergence in the 3' region (from M to the 3' genome end). This suggests that BA.4 and BA.5 may be related by a recombination event, with breakpoint between the E and M genes, prior to their emergence into the general population. This scenario is somewhat similar to the relationship between BA.3 and BA.1/BA.2 which also exhibit apparent ancestral recombination[1]. Using the RASCL pipeline[7] (which employs a battery of tests that analyse ratios of synonymous and non-synonymous substitutions both at individual codon sites and I entire protein coding regions) we found no compelling evidence of imbalances between ratios of synonymous and non-synonymous substitutions such as would be indicative of positive selection (i.e. favouring amino acid changes) or negative selection (disfavouring amino acid changes) acting on any of the genes of viruses in either the BA.4 or BA.5 lineages.

It is currently unknown how differences in the mutation profiles of BA.4 and BA.5, relative to BA.2, will impact their phenotypes. Changes at spike amino acids 452, 486 and 493 are likely to influence human angiotensin-converting enzyme-2 (hACE2) and antibody binding. The 452 residue is in immediate proximity to the interaction interface of the hACE2 receptor. The L452R mutation has been associated with an increased affinity for receptor binding with a resultant increased in vitro infectivity[8]. The L452R mutation is also present in the Delta, Kappa and Epsilon variants (and L452Q in Lambda), and mutations at this position have been associated with a reduction in neutralization by monoclonal antibodies (particularly class 2 antibodies) and polyclonal sera[9–11]. Mutations at this position (L452R/M/Q) have also arisen independently in several BA.2 sublineages in different parts of the world, most notably BA.2.12.1 (L452Q) which has become dominant in many parts of the United States. It's therefore unclear whether BA.4/BA.5 will become dominant throughout the world, or whether there will be a period of co-circulation of several different Omicron lineages.

Before the emergence of BA.4 and BA.5, F486V in the receptor binding domain of spike had been observed only in 54 of 10 million publicly available genome sequences in GISAID (https://cov-spectrum.org/explore/World/AllSamples/AllTimes/variants?aaMutations=S%3AF486V&).
Selection analyses focusing on ratios of non-synonymous and synonymous substitution rates at individual codons have indicated that, since December 2020 S:486 has been evolving under strong negative selection favouring the F state at this site (i.e., the amino acid that is found in

Wuhan-Hu-1) (Extended Data Fig. 3). Although rare, the F486L mutation has been observed in approximately 500 genomes, most commonly in viruses infecting minks and from human cases linked to mink farms. The F486L mutation has been shown to directly enhance entry into cells expressing mink or ferret ACE2[12]. When binding to hACE2, spike amino acid F486 interacts with hACE2 residues L79, M82, and Y83, which collectively comprise a hotspot for ACE2 differences between mammalian species[13]. Mutations at F486 are associated with a reduction in neutralising activity by class 1 (and some class 2) neutralising antibodies and by polyclonal sera[9–11]. Deep mutational scanning suggests that F486 is a key site for escape of vaccine- and infection-elicited RBD-targeted antibodies, including those still able to neutralize Omicron/BA.1 (https://jbloomlab.github.io/SARS2_RBD_Ab_escape_maps/escape-calc/)[14]. This suggests that BA.4 and BA.5 may be even better at evading neutralizing antibody responses, including those recently elicited by BA.1 infections. Combined with waning population immunity against infection from the initial Omicron/BA.1 wave, this could create the conditions for a significant resurgence in infections.

The S:69-70del means BA.4 and BA.5 can again be presumptively identified (against a background of BA.2 infection) using the proxy marker of S-gene target failure (SGTF) with the TaqPath™ COVID-19 qPCR assay (Thermo Fisher Scientific, Waltham, MA, USA). SGTF was successfully used to track the early spread of BA.1 (which also demonstrates SGTF), later also enabling discrimination between BA.1 and BA.2 infections, since BA.2 viruses generally lack the S:69-70del[15]. Recent data from public laboratories in South Africa suggest that the proportion of positive PCR tests with SGTF has been increasing since early March, suggesting that BA.4 and BA.5 may be responsible for a growing share of recently confirmed cases (Fig. 2A). To assess the validity of SGTF for identifying BA.4/BA.5, we performed qPCR with the TaqPath™ assay on 296 unselected samples submitted for sequencing to KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP) from Gauteng, Eastern Cape and KwaZulu-Natal collected between 6 January and 3 April 2022. Of the 296 samples processed, we had a paired valid qPCR result and sequence for 198. Of the 77 samples with SGTF on qPCR, 66 were BA.4 or BA.5, nine were BA.1, and two were BA.2. No BA.4 and BA.5 genomes were S-gene target positive on qPCR (Extended Data Table 1). These results suggest that SGTF surveillance (where the assay is available) may for now be a reasonable proxy to identify BA.4 and BA.5 for countries with a low prevalence of BA.1.

At the time of writing, we have confirmed BA.4 and/or BA.5 in all nine provinces in South Africa (Eastern Cape, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, North West, Northern Cape, Free State and Western Cape) in samples collected between 10 January 2022 and 19 May 2022 (Fig. 2B). In the three most populous provinces in South Africa, Gauteng, KwaZulu-Natal and Western Cape, BA.4 and BA.5 have rapidly replaced BA.2, and are responsible for approximately 90% of sequenced cases by the week starting 16 May 2022 (Extended Data Fig. 4). These estimates are based on unselected sampling for genomic surveillance (samples not

selected based on SGTF or genotyping). The data suggest geographic heterogeneity in the distribution of these two new lineages, with growth predominantly of BA.4 in Gauteng and BA.5 in KwaZulu-Natal (Extended Data Fig. 4). Internationally, by the end of May 2022, BA.4 and BA.5 had also been detected and was rising in prevalence in several countries: in neighbouring Botswana (estimated prevalence 60%), in Europe (Portugal, Spain, Austria), and in the USA.

We estimated that Omicron BA.4 and BA.5 had a daily growth advantage of 0.08 (95% confidence interval [CI] 0.08 - 0.09) and 0.10 (95% CI 0.09 - 0.11) respectively, relative to BA.2 in South Africa in May 2022 (Fig. 2F). These estimates are similar to the estimated daily growth advantage of 0.07 (95% CI: 0.07 – 0.06) of BA.2 over BA.1 in February 2022 (Fig. 2C). The BA.4 and BA.5 lineages also show a growth advantage against non-Omicron lineages although these are minimally circulating in the discussed time frame (Extended Data Table 2). The growth advantage of Omicron BA.4 and BA.5 could be mediated by either (i) an increase in its intrinsic transmissibility relative to other variants, (ii) an increase relative to other variants in its capacity to infect, and be transmitted from, previously infected and vaccinated individuals or (iii) both. The estimated time to most recent common ancestor for both BA.4 and BA.5 (mid-November 2021, similar to that for BA.1 and BA.2) argues against the first option because that suggests both lineages would have been circulating throughout the period dominated by BA.1 and then BA.2 without exhibiting a transmission advantage. The observation that both BA.4 and BA.5 (and many lineages within them) have recently started to grow in frequency suggests the growth advantage is recent and uniform across these lineages. It is estimated that almost all of the South African population has some degree of immunity to SARS-CoV-2, provided by a complex mixture of vaccination and prior infections with wild-type, Beta, Delta, and Omicron (particularly BA.1) (Fig 2D)[16,17]. Given that the transmission advantage becomes apparent approximately four months from the start of the Omicron wave, it is plausible that waning immunity (particularly that acquired from BA.1 infection) is an important contributory factor. This would also suggest that the effects of these different Omicron lineages may differ by location, depending on the immune landscape and particularly the patterns of exposure to BA.1 and BA.2.

At the time of writing, a wave of infections caused by the BA.4 and BA.5 lineages was ending in South Africa (Fig. 2D). This wave was characterized by a peak in test positivity rate of ~24%, lower than during the Omicron BA.1 wave (~34%), and, because of high population immunity, much lower hospital admissions and deaths than previously recorded during waves of infection in South Africa. It is worthy to note that recorded death metrics were further decoupled from cases and hospitalizations compared to the BA.1 wave. The ability of the BA.4 and BA.5 lineages to drive a new wave of infections can potentially be explained by their ability to evade immunity induced by the BA.1 lineage roughly three months after infection[18]. The fifth wave in South Africa, driven by BA.4 and BA.5, occurred around four months after the fourth wave, driven by BA.1. At the time of writing this report, Botswana was experiencing a rapid rise in

cases driven by BA.4 and BA.5, with 19 of 24 health districts experiencing resurgence in cases. To note, Botswana's fourth wave was driven by BA.1, followed by BA.2 lasting about 3.5 months and the country's fifth wave is occurring approximately two months after the fourth wave.

There are several limitations to this study. First, the estimated growth advantage of the BA.4 and BA.5 lineages could be biased due to stochastic effects (such as superspreading) in a low incidence setting at the start of a wave, which can lead to overestimates of the growth advantage. Secondly, reliable estimates of the level of population immunity against BA.1 in South Africa are not yet available, making it difficult to precisely estimate transmissibility or immune evasion of the new lineages. There also remains some uncertainty about the origin of the different Omicron lineages and phylogenetic inference is limited by the relatively low sampling coverage in our genomic surveillance (<1% of confirmed cases in South Africa). Furthermore, the lack of sampling on an ancestor of the different Omicron lineages complicates phylogenetic placements. Whilst the Bayesian phylogenetic methods employed here suggest that BA.4 and BA.5 are independent lineages that originated around the same time as BA.1-BA.3, maximum likelihood estimations suggest they could have descended from BA.2. Further sequencing (particularly samples from Gauteng and neighbouring provinces) may help to provide more clarity.

The continued discovery of genetically diverse Omicron lineages shifts the level of support for hypotheses regarding their origin, from an unsampled location to a discrete reservoir, such as human chronic infections (or even a network of chronic human infections) and/or animal reservoirs, potentially contributing to further evolution and dispersal of the virus, although currently the data does not provide any definitive evidence in any direction. We are actively investigating the potential of a yet unidentified animal reservoir in the region. To date, the only reverse zoonoses cases reported from the African region were in African lions and a puma in a private zoo in Johannesburg, South Africa[19]. Although these are unlikely species to play a role in the emergence of new variants, it is a reminder of the susceptibility of certain wildlife species to infections from humans. Following the emergence of Omicron, the World Organisation for Animal Health released a statement calling for enhanced surveillance in animals to identify the origin of new variants[20]. Further genomic sampling and evolutionary investigation will thus be required to explain the origin of Omicron lineages.

In conclusion, we have identified two new Omicron lineages (BA.4 and BA.5), which are associated with a resurgence in infections in South Africa approximately four months on from the start of the Omicron wave. This once again highlights the importance of continued global genomic surveillance and variant analysis to act as an early warning system, giving countries time to prepare and mitigate the public health impact of emerging variants.

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Conflict of interest

The authors declare no conflict of interest. Raquel Viana and Allison Glass are employees of Lancet Laboratories.

Figure legends

Figure 1: A) Time-resolved maximum clade credibility phylogeny of the BA.2, BA.4 and BA.5 lineages (n = 221, sampled between 29 December 2021 and 7 April 2022). Mutations that characterize the lineages are indicated on the branch at which each first emerged. The posterior distribution of the time of the most recent common ancestor (TMRCA) is also shown for BA.2, BA.4 and BA.5. B) Spatiotemporal reconstruction of the spread of the BA.4 lineage in South Africa. C) Spatiotemporal reconstruction of the spread of the BA.5 lineage in South Africa. In B and C, circles represent nodes of the maximum clade credibility phylogeny, coloured according to their inferred time of occurrence (scale shown). EC, Eastern Cape; FS, Free State; GP, Gauteng; KZN, KwaZulu-Natal; LP, Limpopo; MP, Mpumalanga; NC, Northern Cape; NW, North West; WC, Western Cape. Solid curved lines denote the links between nodes and the directionality of movement is indicated (anti-clockwise along the curve). D) Amino acid mutations in the spike gene of the BA.4 and BA.5 lineages. Mutations that differ from BA.2 are denoted in red, including the wild-type amino acid at position Q493 (denoted by the red *).

Figure 2 A) Changes in the genomic prevalence of Omicron lineages in South Africa from November 2021 (when BA.1 dominated) to May 2022 (when BA.4 and BA.5 were increasing in frequency), superimposed with the proportion of positive TaqPath qPCR tests exhibiting SGTF from November 2021 to May 2022. To note here that estimations of genomic prevalence and

SGTF proportions are done from different samples and datasets, and only presented together here for illustrative purposes. B) The count of Omicron lineage genomes per province of South Africa over November 2021 – May 2022. BA.4 and BA.5 have been detected in all nine provinces. C) Modelled linear proportions of the Omicron lineages in South Africa. BA.1 rapidly outcompeted Delta in November 2021 and was then superseded by BA.2 in early 2022. BA.4 and BA.5 appear to be swiftly replacing BA.2 in South Africa. Model fits are based on a multinomial logistic regression and dot size represents the weekly sample size. The shaded areas correspond to the 95% CIs of the model estimates. D) The progression of the 7-day rolling average of daily reported case numbers in South Africa over two years of the epidemic (April 2020 – May 2022). Daily cases are coloured by the inferred proportion of SARS-CoV-2 variants prevalent at a particular period in the epidemic.

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Online Methods:

Epidemiological dynamics

We analysed daily cases of SARS-CoV-2 in South Africa up to 25 April 2022 from publicly released data provided by the National Department of Health and the National Institute for Communicable Diseases. This was accessible through the repository of the Data Science for Social Impact Research Group at the University of Pretoria (https://github.com/dsfsi/covid19za)[21,22]. The National Department of Health releases daily updates on the number of confirmed new cases, deaths and recoveries, with a breakdown by province.

Sampling of SARS-CoV-2

As part of the NGS-SA[23], seven sequencing hubs receive randomly selected samples for sequencing every week according to approved protocols at each site. These samples include remnant nucleic acid extracts or remnant nasopharyngeal and oropharyngeal swab samples from routine diagnostic SARS-CoV-2 PCR testing from public and private laboratories in South Africa. We analysed SARS-CoV-2 genomes generated from samples collected between 1 November 2021 and 20th April 2022.

Ethical statement

The genomic surveillance in South Africa was approved by the University of KwaZulu–Natal Biomedical Research Ethics Committee (BREC/00001510/2020), the University of the Witwatersrand Human Research Ethics Committee (HREC) (M180832), Stellenbosch University HREC (N20/04/008_COVID-19), University of Cape Town HREC (383/2020), University of

Pretoria HREC (H101/17) and the University of the Free State Health Sciences Research Ethics Committee (UFS-HSD2020/1860/2710). The genomic sequencing in Botswana was conducted as part of the national vaccine roll-out plan and was approved by the Health Research and Development Committee (Health Research Ethics body, HRDC#00948 and HRDC#00904). Individual participant consent was not required for the genomic surveillance. This requirement was waived by the Research Ethics Committees.

Whole-genome sequencing and genome assembly

RNA was extracted on an automated Chemagic 360 instrument, using the CMG-1049 kit (Perkin Elmer). The RNA was stored at −80 °C before use. Libraries for whole-genome sequencing were prepared using either the Oxford Nanopore Midnight protocol with Rapid Barcoding or the Illumina COVIDseq Assay.

Illumina Miseq/NextSeq

For the Illumina COVIDseq assay, the libraries were prepared according to the manufacturer's protocol. In brief, amplicons were tagmented, followed by indexing using the Nextera UD Indexes Set A. Sequencing libraries were pooled, normalized to 4 nM and denatured with 0.2 N sodium acetate. A 8 pM sample library was spiked with 1% PhiX (PhiX Control v3 adaptor-ligated library used as a control). We sequenced libraries using the 500-cycle v2 MiSeq Reagent Kit on the Illumina MiSeq instrument (Illumina). On the Illumina NextSeq 550 instrument, sequencing was performed using the Illumina COVIDSeq protocol (Illumina), an amplicon-based next-generation sequencing approach. The first-strand synthesis was performed using random hexamers primers from Illumina and the synthesized cDNA underwent two separate multiplex PCR reactions. The pooled PCR amplified products were processed for tagmentation and adapter ligation using IDT for Illumina Nextera UD Indexes. Further enrichment and clean-up was performed according to protocols provided by the manufacturer (Illumina). Pooled samples were quantified using the Qubit 3.0 or 4.0 fluorometer (Invitrogen) and the Qubit dsDNA High Sensitivity assay kit according to the manufacturer's instructions. The fragment sizes were analysed using the TapeStation 4200 (Invitrogen). The pooled libraries were further normalized to 4 nM concentration, and 25 µl of each normalized pool containing unique index adapter sets was combined into a new tube. The final library pool was denatured and neutralized with 0.2 N sodium hydroxide and 200 mM Tris-HCl (pH 7), respectively. Sample library (1.5 pM) was spiked with 2% PhiX. Libraries were loaded onto a 300-cycle NextSeq 500/550 HighOutput Kit v2 and run on the Illumina NextSeq 550 instrument (Illumina).

Midnight protocol

For Oxford Nanopore sequencing, the Midnight primer kit was used as described previously54. cDNA synthesis was performed on the extracted RNA using the LunaScript RT mastermix (New England BioLabs) followed by gene-specific multiplex PCR using the Midnight primer pools, which produce 1,200 bp amplicons that overlap to cover the 30 kb SARS-CoV-2 genome.

Amplicons from each pool were pooled and used neat for barcoding with the Oxford Nanopore Rapid Barcoding kit according to the manufacturer's protocol. Barcoded samples were pooled and bead-purified. After the bead clean-up, the library was loaded on a prepared R9.4.1 flow-cell. A GridION X5 or MinION sequencing run was initiated using MinKNOW software with the base-call setting switched off.

Ion Torrent Genexus Integrated Sequencer methodology for rapid whole-genome sequencing of SARS-CoV-2

Viral RNA was extracted using the MagNA Pure 96 DNA and Viral Nucleic Acid kit on the automated MagNA Pure 96 system (Roche Diagnostics) according to the manufacturer's instructions. Extracts were then screened by quantitative PCR to acquire the mean cycle threshold (Ct) values for the SARS-CoV-2 N and ORF1ab genes using the TaqMan 2019-nCoV assay kit v1 (Thermo Fisher Scientific) on the ViiA7 Real-time PCR system (Thermo Fisher Scientific) according to the manufacturer's instructions. Extracts were sorted into batches of n = 8 within a Ct range difference of 5 for a maximum of two batches per run. Extracts with <200 copies were sequenced using the low viral titre protocol. Next-generation sequencing was performed using the Ion AmpliSeq SARS-CoV-2 Research Panel on the Ion Torrent Genexus Integrated Sequencer (Thermo Fisher Scientific), which combines automated cDNA synthesis, library preparation, templating preparation and sequencing within 24 h. The Ion Ampliseq SARS-CoV-2 Research Panel consists of two primer pools targeting 237 amplicons tiled across the SARS-CoV-2 genome providing >99% coverage of the SARS-CoV-2 genome (~30 kb) and an additional five primer pairs targeting human expression controls. The SARS-CoV-2 amplicons range from 125 bp to 275 bp in length. TRINITY was used for de novo assembly and the Iterative Refinement Meta-Assembler (IRMA) was used for genome assisted assembly as well as FastQC for quality checks.

Genome assembly

We assembled paired-end and Nanopore .fastq reads using Genome Detective v.1.132 (https://www.genomedetective.com), which was updated for the accurate assembly and variant calling of tiled primer amplicon Illumina or Oxford Nanopore reads, and the Coronavirus Typing Tool55. For Illumina assembly, the GATK HaploTypeCaller --min-pruning 0 argument was added to increase mutation calling sensitivity near sequencing gaps. For Nanopore, low-coverage regions with poor alignment quality (<85% variant homogeneity) near sequencing/amplicon ends were masked to be robust against primer drop-out experienced in the spike gene, and the sensitivity for detecting short inserts using a region-local global alignment of reads was increased. We also used the wf_artic (ARTIC SARS-CoV-2) pipeline as built using the Nextflow workflow framework56. In some instances, mutations were confirmed visually with .bam files using Geneious v.2020.1.2 (Biomatters). The reference genome used throughout the assembly process was NC_045512.2 (numbering equivalent to MN908947.3).

Raw reads from the Illumina COVIDSeq protocol were assembled using the Exatype NGS SARS-CoV-2 pipeline v.1.6.1 (https://sars-cov-2.exatype.com/). This pipeline performs quality control on reads and then maps the reads to a reference using Examap. The reference genome used throughout the assembly process was NC_045512.2 (accession number: MN908947.3).

Several of the initial Ion Torrent genomes contained a number of frameshifts, which caused unknown variant calls. Manual inspection revealed that these were probably sequencing errors resulting in mis-assembled regions (probably due to the known error profile of Ion Torrent sequencers). To resolve this, the raw reads from the IonTorrent platform were assembled using the SARSCoV2 RECoVERY (Reconstruction of Coronavirus Genomes & Rapid Analysis) pipeline implemented in the Galaxy instance ARIES (https://aries.iss.it). This pipeline fixed the observed frameshifts, confirming that they were artefacts of mis-assembly; this subsequently resolved the variant calls. The Exatype and RECoVERY pipelines each produce a consensus sequence for each sample. These consensus sequences were manually inspected and polished using Aliview v.1.27 (http://ormbunkar.se/aliview/).

All of the sequences passing internal quality control were deposited in GISAID (https://www.gisaid.org/), and the GISAID accession identifiers are included as part of Extended Data Table 1.

Phylogenetic analysis

We initially analysed genomes from South Africa against the global reference dataset using a custom pipeline based on a local version of NextStrain (https://github.com/nextstrain/ncov)[24]. The pipeline contains several Python scripts that manage the analysis workflow. It performs an alignment of genomes in NextAlign[25], phylogenetic tree inference in IQ-Tree V1.6.9[26], tree dating and ancestral state construction and annotation (https://github.com/nextstrain/ncov).

The initial phylogenetic analysis enabled us to identify clusters corresponding to the BA.4 (n=120) and BA.5 (n=51) lineages. We extracted these clusters and constructed a preliminary maximum-likelihood tree with a subset of BA.2 sequences (n=52) in IQ-tree. We inspected this maximum-likelihood tree in TempEst v.1.5.3[27] for the presence of a temporal or molecular clock signal. Linear regression of root-to-tip genetic distances against sampling dates indicated that the SARS-CoV-2 sequences evolved in a relatively strong clock-like manner (correlation coefficient = 0.6, R^2 = 0.4).

Given that the estimation of tMRCAs and dispersal dynamics of the sampled viruses is best achieved using Bayesian phylogenetic methods, we then estimated time-calibrated phylogenies using the Bayesian software package BEAST v.1.10.4[28]. For this analysis, we used the strict molecular clock model, the HKY+I+G, nucleotide substitution model and the exponential growth coalescent model[29]. We computed Markov chain Monte Carlo (MCMC) in duplicate runs of 20

million states each, sampling every 2,000 steps. Convergence of MCMC chains was checked using Tracer v.1.7.1[30]. Maximum clade credibility trees were summarized from the MCMC samples using TreeAnnotator after discarding 10% as burn-in. The phylogenetic trees were visualized using ggplot and ggtree[31,32].

Phylogeographic analysis

To model phylogenetic diffusion of the new cluster across the country, we used a flexible relaxed random walk diffusion model that accommodates branch-specific variation in rates of dispersal with a Cauchy distribution[33]. For each sequence, latitude and longitude were attributed to the most precise district or provincial information available and linked to the diagnostic sample.

As described in 'Phylogenetic analysis', MCMC chains were run in duplicate for 10 million generations and sampled every 1,000 steps, with convergence assessed using Tracer v.1.7.1. Maximum clade credibility trees were summarized using TreeAnnotator after discarding 10% as burn-in. We used the R package seraphim[34] to extract and map spatiotemporal information embedded in posterior trees.

Lineage classification

We used a previously proposed dynamic lineage classification method[35] from the 'Phylogenetic Assignment of Named Global Outbreak Lineages' (pangolin) software suite v4.0.6 with the --Usher option (https://github.com/cov-lineages/pangolin)[36]. This is aimed at identifying the most epidemiologically important lineages of SARS-CoV-2 at the time of analysis, enabling researchers to monitor the epidemic in a particular geographic region. A lineage is a linear chain of viruses in a phylogenetic tree showing connection from the ancestor to the last descendant. Variant refers to a genetically distinct virus with different mutations to other viruses.

Selection analysis

To identify which (if any) of the observed mutations in the spike protein was most likely to increase viral fitness, we used the natural selection analysis of SARS-CoV-2 pipeline (https://observablehq.com/@spond/revised-sars-cov-2-analytics-page). This pipeline examines the entire global SARS-CoV-2 nucleotide sequence dataset for evidence of: (i) polymorphisms having arisen in multiple epidemiologically unlinked lineages that have statistical support for non-neutral evolution (mixed effects model of evolution)[37], (ii) sites at which these polymorphisms have support for a greater-than-expected ratio of nonsynonymous-to-synonymous nucleotide substitution rates on internal branches of the phylogenetic tree (fixed-effects likelihood)[38] and (iii) whether these polymorphisms have increased in frequency in the regions of the world in which they have occurred.

Estimating transmission advantage

We analysed 12,528 SARS-CoV-2 sequences from South Africa generated in this study and uploaded to GISAID with sample collection dates from 1 November 2021 to 20 April 2022[39]. We used a multinomial logistic regression model to estimate the growth advantage of Omicron BA.2 lineage compared with BA.1, BA.4 and BA.5 lineages at the time point at which the proportion of Omicron BA.4 and BA.5 collectively reached 50%[40,41]. We fitted the model using the multinom function of the nnet package and estimated the growth advantage using the package emmeans in R[42].

S-Gene Target Failure Monitoring

SGTF monitoring is performed through analysing SARS-CoV-2 laboratory test results from nasopharyngeal specimens received from the public health sector and referred for PCR testing undertaken by the National Health Laboratory Service (NHLS) in South Africa. The NHLS has a single laboratory information system connecting laboratory testing platforms to a corporate data warehouse, where data can be mined in near real-time. The TaqPath™ COVID-19 [Thermo Fisher Scientific, Waltham, MA, USA] assay accounts for around 20% of NHLS PCR tests performed, with around half of those performed in Gauteng. The TaqPath assay targets three gene regions, ORF1ab, N and S, with the lack of probe fluorescence of the latter culminating in S-gene target failure (SGTF). In Fig 2A, we analysed and plotted the weekly proportion of positive TaqPath tests with SGTF (defined as samples with non-detectable S gene target and either N or ORF1ab gene positive with CT value <30.

Validation of S-Gene Target status as proxy for BA.4 and BA.5

Using a subset of unselected samples submitted to the KRISP sequencing laboratory, we compared the S-gene target status to the genome lineage assignment. Briefly, RNA was extracted from nasopharyngeal swabs in viral transport media using the CMG-1033-S kit (Chemagen, PerkinElmer, Baesweiler, Germany). 10µl of purified RNA was then amplified using the TaqPath COVID-19 CE-IVD RT-PCR kit (ThermoFisher Scientific, Waltham, MA, USA) and analysed on the Design & Analysis software v2.4. SGTF was denoted by lack of amplification of the S-gene target, with successful amplification of both the remaining ORF1ab and N-gene targets ($Ct \leq 30$).

Statistics

No statistical method was used to predetermine sample size. Data exclusion, randomization and blinding to allocation during experiments and outcome assessment were not applicable to this study.

Data Availability Statement

All of the SARS-CoV-2 genomes generated and presented in this manuscript are publicly accessible through the GISAID platform (https://www.gisaid.org/). The GISAID accession identifiers of the sequences analysed in this study are provided as part of Supplementary Table

S1. Other raw data for this study are provided as a supplementary dataset at https://github.com/krisp-kwazulu-natal/SARSCoV2_South_Africa_Omicron_BA4_BA5. The reference SARS-CoV-2 genome (MN908947.3) was downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/).

Code Availability Statement

All custom scripts to reproduce the analyses and figures presented in this Article are available at https://github.com/krisp-kwazulu-natal/SARSCoV2_South_Africa_Omicron_BA4_BA5.

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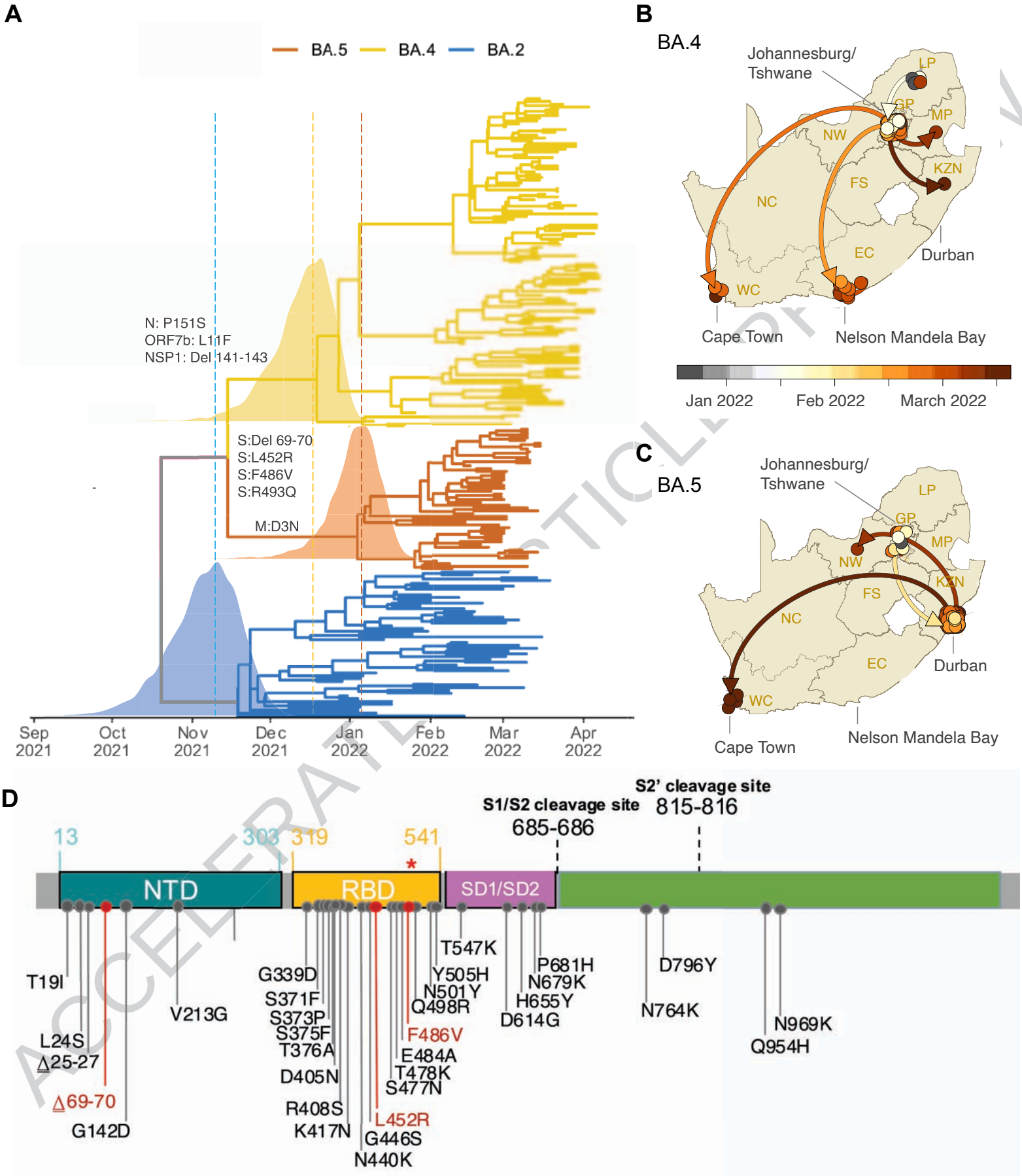

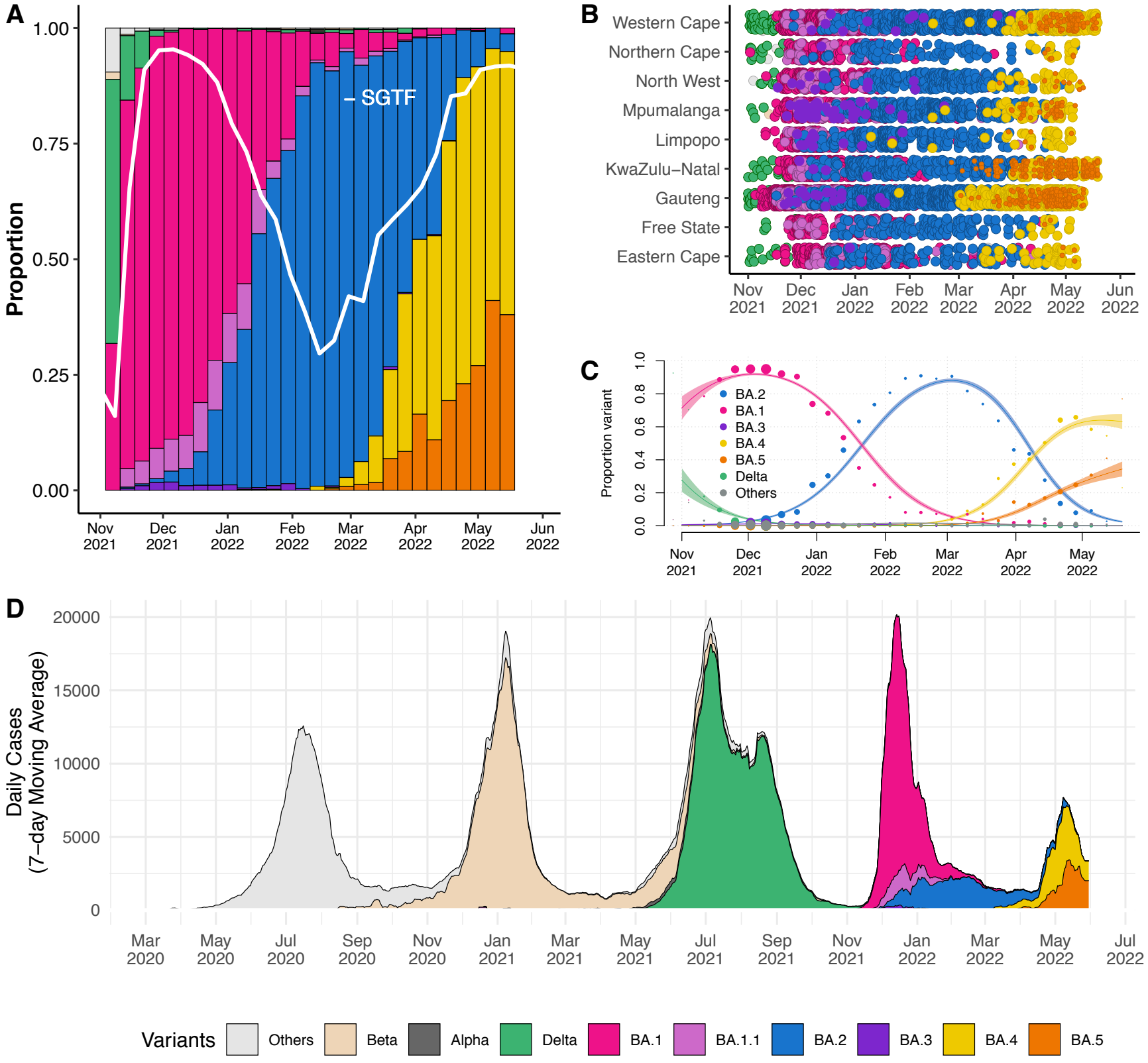
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Mpumalanga
Limpopo
KwaZulu–Natal
Gauteng
Free State
Eastern Cape
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Others
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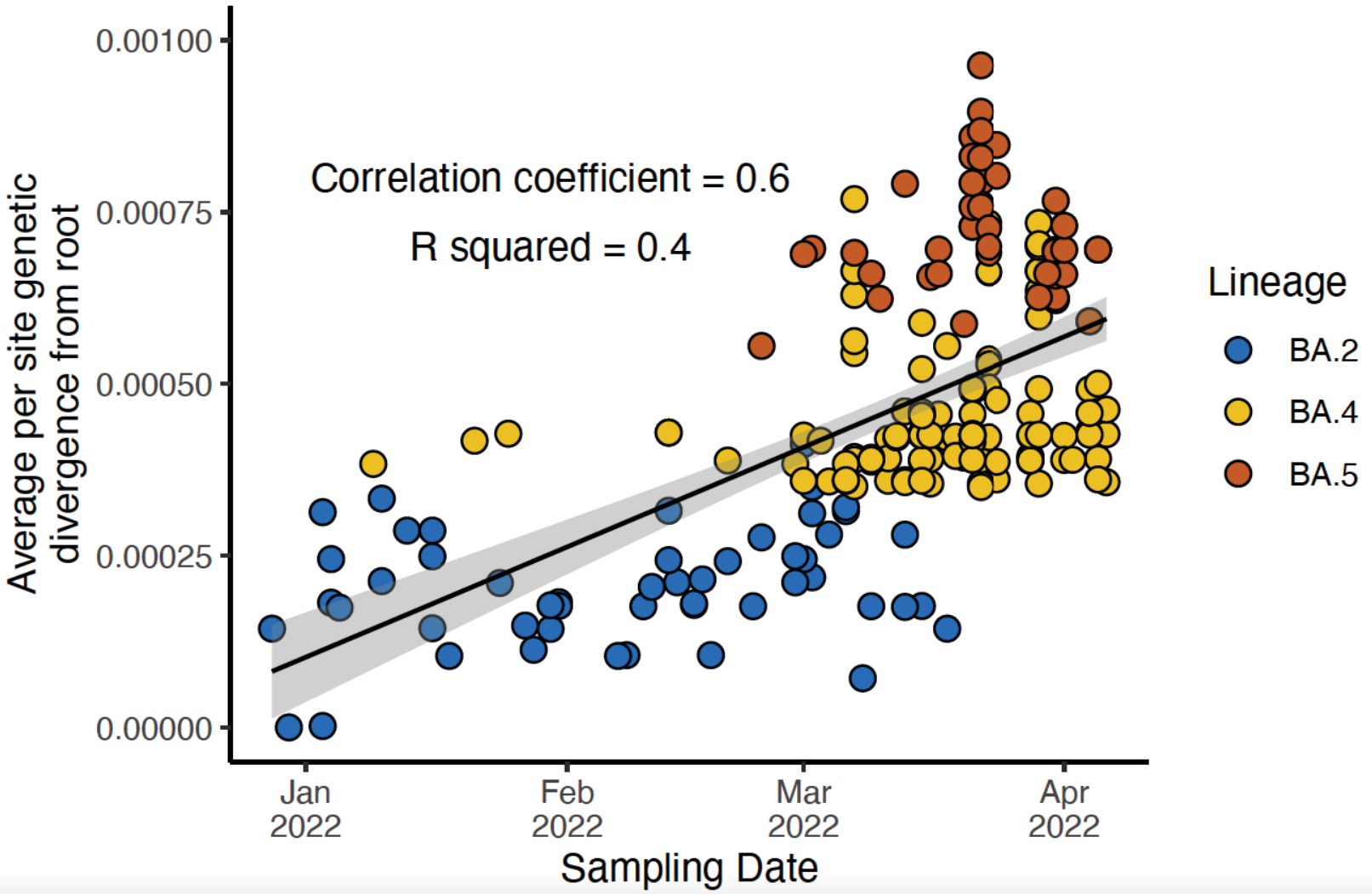
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Average per site genetic divergence from root
Correlation coefficient = 0.6
R squared = 0.4
Jan 2022
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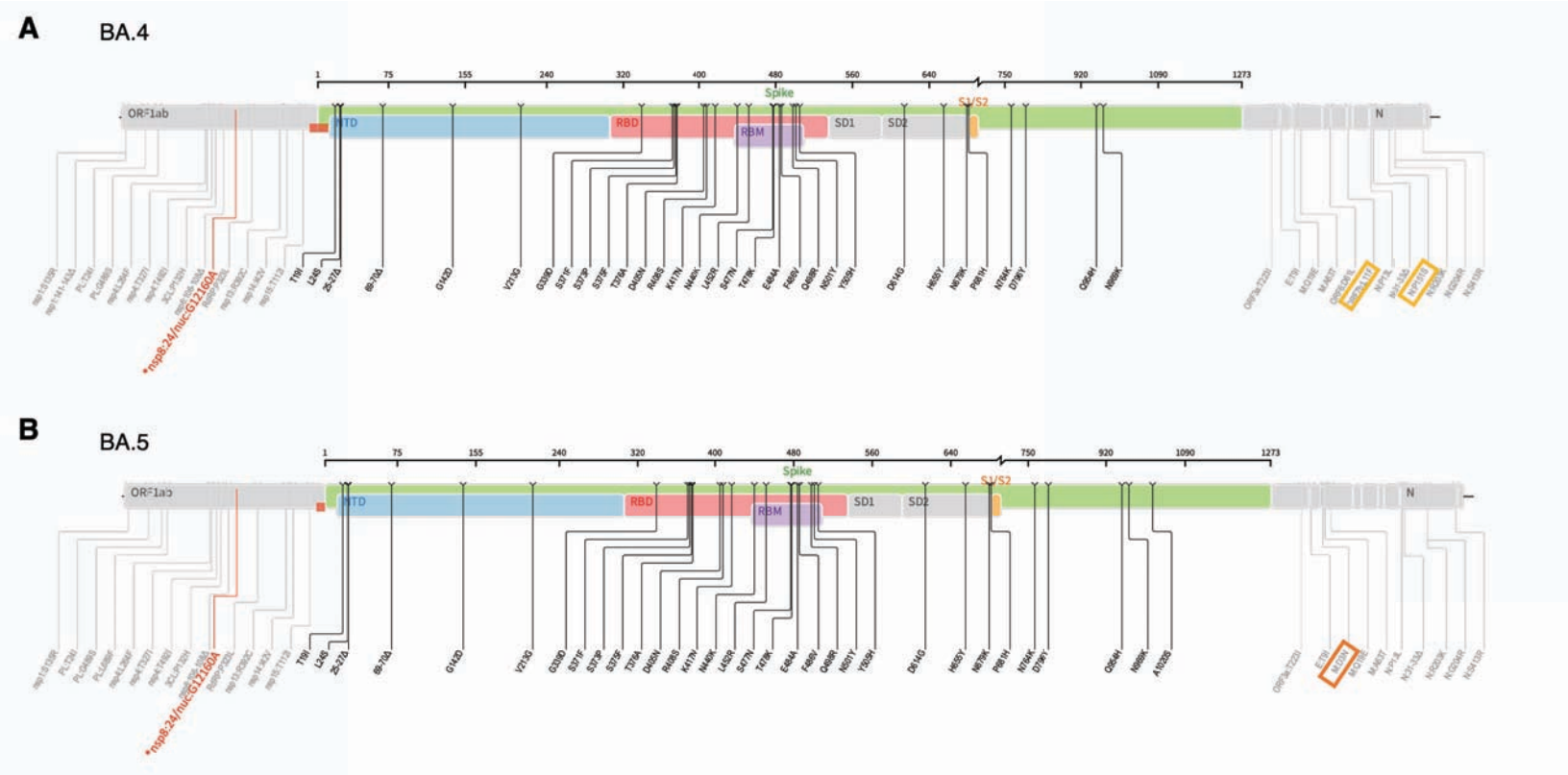
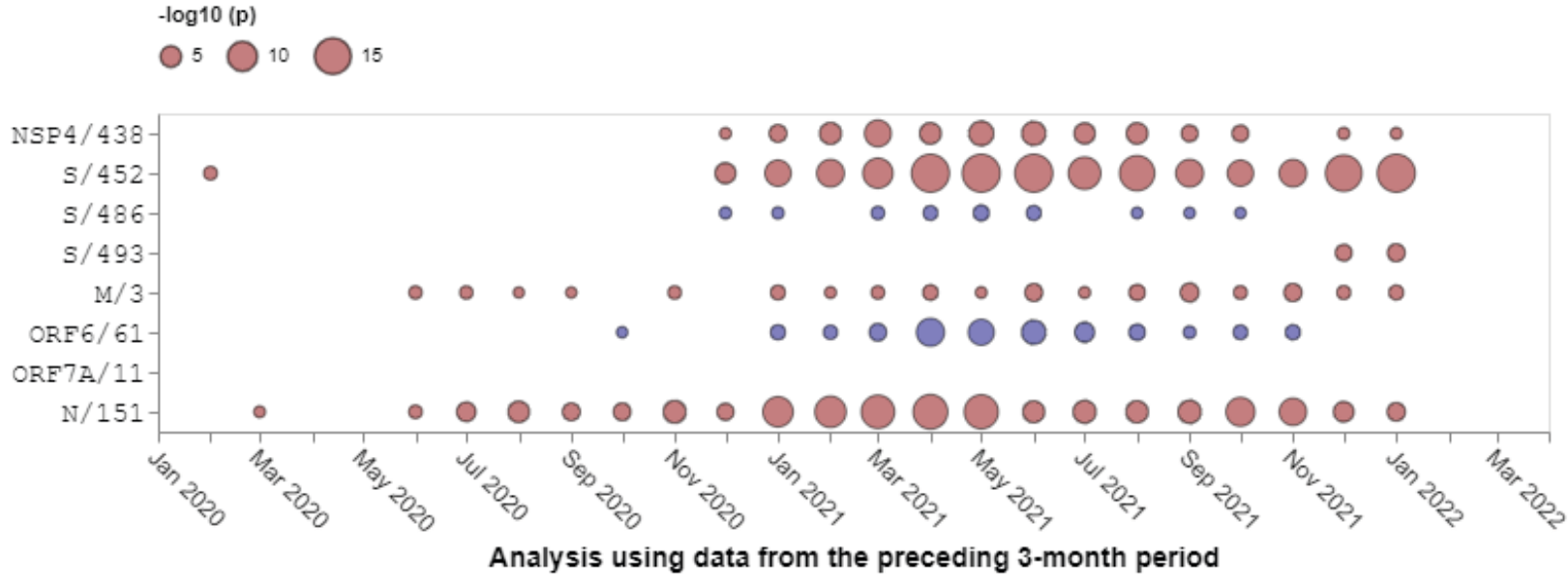
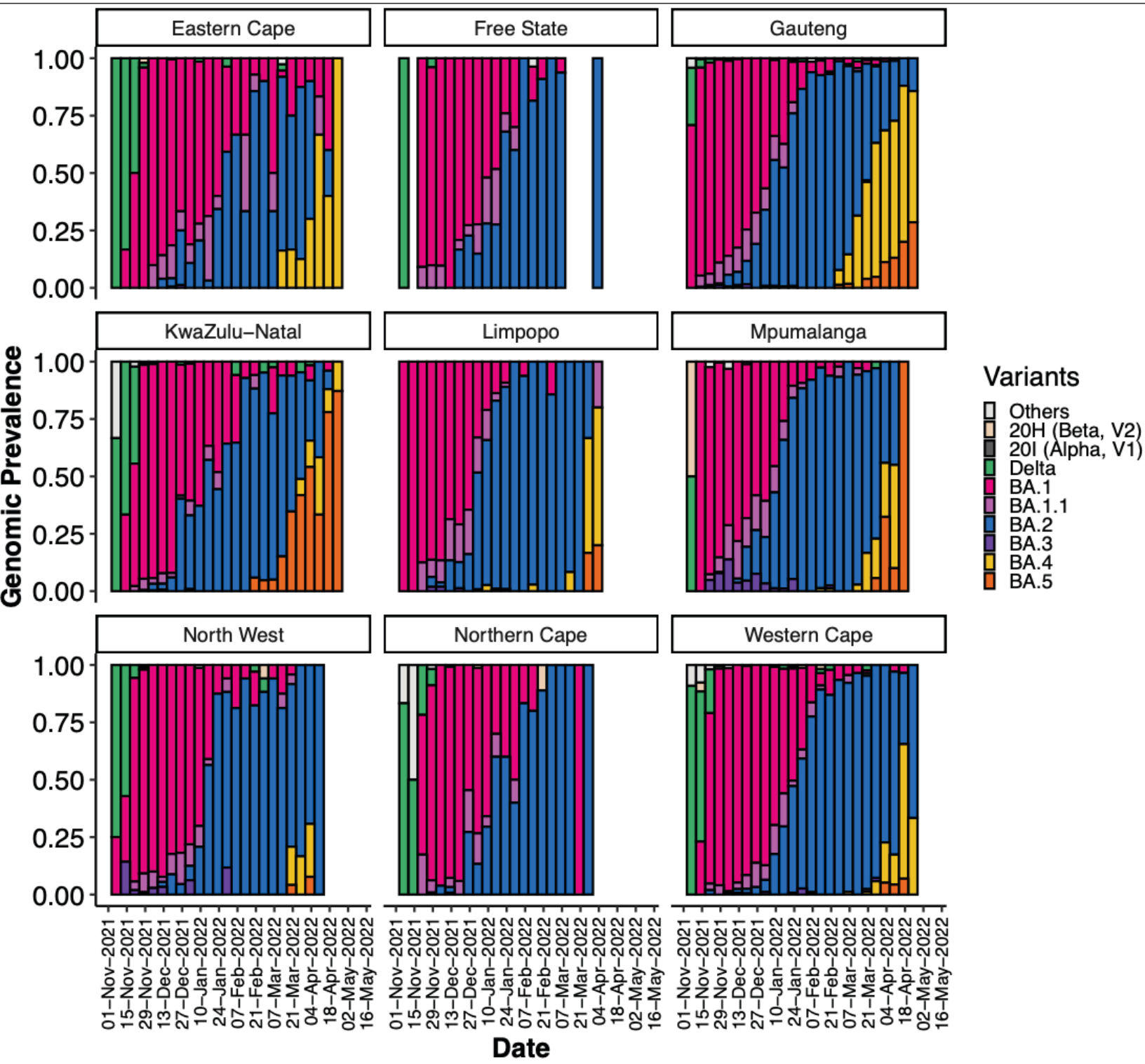
BA.4
BA.5


A
BA.4
1
75
155
240
320
400
480
560
640
750
920
1090
1273
Spike
S1/S2
ORF1ab
NTD
RBD
RBM
SD1
SD2
N
*nsp8:24/nucG12160A
B
BA.5
*nsp8:24/nucG12160A


-log10 (p)
5
10
15
NSP4/438
S/452
S/486
S/493
M/3
ORF6/61
ORF7A/11
N/151
Jan 2020
Mar 2020
May 2020
Jul 2020
Sep 2020
Nov 2020
Jan 2021
Mar 2021
May 2021
Jul 2021
Sep 2021
Nov 2021
Jan 2022
Mar 2022
Analysis using data from the preceding 3-month period


Eastern Cape
Free State
Gauteng
KwaZulu−Natal
Limpopo
Mpumalanga
North West
Northern Cape
Western Cape
Genomic Prevalence
1.00
0.75
0.50
0.25
0.00
01−Nov−2021
15−Nov−2021
29−Nov−2021
13−Dec−2021
27−Dec−2021
10−Jan−2022
24−Jan−2022
07−Feb−2022
21−Feb−2022
07−Mar−2022
21−Mar−2022
04−Apr−2022
18−Apr−2022
02−May−2022
16−May−2022
Date
Variants
Others
20H (Beta, V2)
20I (Alpha, V1)
Delta
BA.1
BA.1.1
BA.2
BA.3
BA.4
BA.5

Omicron lineage	S-gene target failure	S-gene target positive
BA.1	9	0
BA.2	2*	120
BA.3	0	1
BA.4	26	0
BA.5	40	0
Total	77	121

Reference lineage	Comparative lineage	Growth rate per day	95% Confidence Intervals
BA.5	BA.1	0.164	0.154 – 0.175
	BA.2	0.096	0.086 – 0.106
	BA.3	0.154	0.138 – 0.170
	BA.4	-0.014	-0.023 – -0.005
	Delta	0.235	0.094 – 0.121
BA.4	BA.1	0.15	0.143 – 0.158
	BA.2	0.082	0.075 – 0.089
	BA.3	0.14	0.126 – 0.154
	Delta	0.221	0.204 – 0.239
BA.3	BA.1	-0.01	-0.022 – -0.002
	BA.2	-0.058	-0.070 – -0.046
	Delta	0.0814	0.062 – 0.101
BA.2	BA.1	0.068	0.065 – 0.072
	Delta	0.139	0.123 – 0.155
BA.1	Delta	0.071	0.056 – 0.087

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Last updated by author(s): Jun 18, 2022

Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☐	☒ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐	☒ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No software was used
Data analysis	Base-calling for GridIon sequencing was performed on MinKNOW software v21.6. Genome assembly was performed with Genome Detective online tool version 1.132 or Exatype NGS SARS-CoV-2 pipeline v1.6.1 or SARSCoV2 RECoVERY (REconstruction of COronaVirus gEnomes & Rapid analYsis) pipeline implemented in the Galaxy instance ARIES (https://aries.iss.it) and validated with Geneious software v.2020.1.2, IG Viewer or Aliview v1.27. Phylogenetic analysis was performed using Nextalign, IQ-Tree V1.6.9, TempEst v.1.5.3, BEASTv.1.10.4, BEAST2 v2.5.2, and Tracer v.1.7.1. Selection analyses were performed using HyPhy v2.5.33 through the RASCL pipeline. Lineage classification was performed using the PANGO software suite v4.0.6. R packages used for data analysis included ggplot, ggtree, seraphim. Custom codes are all available at: https://github.com/krisp-kwazulu-natal/SARSCoV2_South_Africa_Omicron_BA4_BA5.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

All of the SARS-CoV-2 genomes generated and presented in this manuscript are publicly accessible through the GISAID platform (https://www.gisaid.org/). The GISAID accession identifiers of the sequences analysed in this study are provided as part of Supplementary Table S1. Other raw data for this study are provided as a supplementary dataset at https://github.com/krisp-kwazulu-natal/SARSCoV2_South_Africa_Omicron_BA4_BA5. The reference SARS-CoV-2 genome (MN908947.3) was downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/).

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No sample size calculation was performed; rather all genomic data available at the time of writing for the newly emerged BA.4 and BA.5 Omicron lineages was considered to ensure most accurate analysis and results in a timely manner. At the time of writing, 120 and 51 good quality sequences of the BA.4 and BA.5 SARS-CoV-2 lineages had been produced by the NGS-SA. We believe this was a sufficient sample size as the genomes spanned 7 of the 9 provinces of South Africa, including from multiple districts. For phylogentic analysis, this was analyzed against a representative set of 52 BA.2 SARS-CoV-2 genotypes.
Data exclusions	For phylogenetic analysis and time-calibrated BEAST analysis, genomes were excluded if they presented <90% coverage against the reference AND/OR have sequencing quality problem - e.g. gaps in key regions of the spike protein that causes spurious clustering.
Replication	Reproducibility were performed for bayesian MCMC phylogenetic tree reconstructions. We computed MCMC (Markov chain Monte Carlo) triplicate runs of 20 million states each, sampling every 2000 steps for the Omicron dataset. All attempts at replication were successful and the MCC tree for the BA.4 and BA.5 cluster was of high support.
Randomization	Experimental groups consisted of weekly batches of residual patient nasopharyngeal swabs selected for sequencing to determine the progression of weekly lineage prevalence as part of surveillance. Samples for weekly SARS-CoV-2 sequencing in South Africa and Botswana were selected at random from all relevant divisions in each country, without any clinical or geographical bias. Generally, part of the Network for Genomic Surveillance in South Africa (NGS-SA), seven sequencing hubs receive randomly selected samples for sequencing every week according to approved protocols at each site. Randomization of participants into experimental groups was not relevant to this study as experimental groups are determined by genomic viral classification into SARS-CoV-2 variants or lineages.
Blinding	Geographical blinding of data was not necessary for the study as it involves phylogeographical analysis. Other types of blinding were also not necessary as this was not a cohort study. Blinding of group assignment or outcome assessment were not applicable to this study as groups must be precisely assigned by genomic classification and outcomes need to be assessed in context of assigned genomic variant or lineages groups.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Human research participants

Policy information about studies involving human research participants

Population characteristics	We obtained samples consisting of remnant nucleic acid extracts or remnant nasopharyngeal and oropharyngeal swab samples from routine diagnostic SARS-CoV-2 PCR testing from public and private laboratories in South Africa. The Omicron genomes in this study came from patients of ages 0-82, with an approximately equal distribution of males and females, for which the Omicron genotype was confirmed by sequencing.
Recruitment	As part of the Network for Genomic Surveillance in South Africa (NGS-SA), seven sequencing hubs receive randomly selected samples for sequencing every week according to approved protocols at each site. One bias that may be present is the ability to sequence only from the pool of patients that seek testing and that receive a positive PCR test.
Ethics oversight	The genomic surveillance in South Africa was approved by the University of KwaZulu–Natal Biomedical Research Ethics Committee (BREC/00001510/2020), the University of the Witwatersrand Human Research Ethics Committee (HREC) (M180832), Stellenbosch University HREC (N20/04/008_COVID-19), University of Cape Town HREC (383/2020), University of Pretoria HREC (H101/17) and the University of the Free State Health Sciences Research Ethics Committee (UFS-HSD2020/1860/2710). The genomic sequencing in Botswana was conducted as part of the national vaccine roll-out plan and was approved by the Health Research and Development Committee (Health Research Ethics body, HRDC#00948 and HRDC#00904). Individual participant consent was not required for the genomic surveillance. This requirement was waived by the Research Ethics Committees.

Note that full information on the approval of the study protocol must also be provided in the manuscript.