Evolutionary and phenotypic characterization of spike

mutations in a new SARS-CoV-2 Lineage reveals two

Variants of Interest

Paula Ruiz-Rodriguez[1#], Clara Francés-Gómez[1#], Álvaro Chiner-Oms[2], Mariana G. López[2], Santiago Jiménez-Serrano[2], Irving Cancino-Muñoz[2], Paula Ruiz-Hueso[3], Manuela Torres-Puente[2], Maria Alma Bracho[1,4], Giuseppe D'Auria[1,3,4], Llúcia Martinez-Priego[3], Manuel Guerreiro[5,6], Marta Montero-Alonso[7], María Dolores Gómez[8], José Luis Piñana[9], SeqCOVID-SPAIN consortium, Fernando González-Candelas[1,4], Iñaki Comas[2,4], Alberto Marina[2,10], Ron Geller[1]*, Mireia Coscolla[1]*

1. I[2]SysBio, University of Valencia-CSIC, FISABIO Joint Research Unit Infection and Public Health, Valencia, Spain.

2. Instituto de Biomedicina de Valencia (IBV-CSIC), Valencia, Spain.

3. Sequencing and Bioinformatics Service of FISABIO (Valencian Region Foundation for the Promotion of Health and Biomedical Research), Valencia, Spain.

4. CIBER in Epidemiology and Public Health, Spain.

5. Hematology Department, Hospital Universitari i Politècnic la Fe, Valencia, Spain

6. Instituto de Investigación Sanitaria La Fe (IIS-La Fe), Valencia, Spain.

7. Infectious Diseases Unit, Hospital Universitari i Politècnic la Fe, Valencia, Spain.

8. Microbiology Department, Hospital Universitari i Politècnic La Fe, Valencia, Spain

9. Hematology Service, Hospital Clínico Universitario, Institute for Research INCLIVA, Valencia, Spain.

22   10. CIBER in Rare Diseases (CIBERER), Spain.

23   #equal contributions

24   *corresponding author

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26   **Abstract**

27   Molecular epidemiology of SARS-CoV-2 aims to monitor the appearance of new variants with

28   the potential to change the virulence or transmissibility of the virus. During the first year of

29   SARS-CoV-2 evolution, numerous variants with possible public health impact have emerged. We

30   have detected two mutations in the Spike protein at amino acid positions 1163 and 1167 that

31   have appeared independently multiple times in different genetic backgrounds, indicating they

32   may increase viral fitness. Interestingly, the majority of these sequences appear in transmission

33   clusters, with the genotype encoding mutations at both positions increasing in frequency more

34   than single-site mutants. This genetic outcome that we denote as Lineage B.1.177.637, belongs

35   to clade 20E and includes 12 additional single nucleotide polymorphisms but no deletions with

36   respect to the reference genome (first sequence in Wuhan). B.1.177.637 appeared after the first

37   wave of the epidemic in Spain, and subsequently spread to eight additional countries, increasing

38   in frequency among sequences in public databases. Positions 1163 and 1167 of the Spike protein

39   are situated in the HR2 domain, which is implicated in the fusion of the host and viral

40   membranes. To better understand the effect of these mutations on the virus, we examined

41   whether B.1.177.637 altered infectivity, thermal stability, or antibody sensitivity. Unexpectedly,

42   we observed reduced infectivity of this variant relative to the ancestral 20E variant *in vitro* while

43   the levels of viral RNA in nasopharyngeal swabs did not vary significantly. In addition, we found

44   the mutations do not impact thermal stability or antibody susceptibility in vaccinated individuals

45   but display a moderate reduction in sensitivity to neutralization by convalescent sera from early

2

46   stages of the pandemic. Altogether, this lineage could be considered a Variant of Interest (VOI),

47   we denote VOI1163.7. Finally, we detected a sub-cluster of sequences within VOI1163.7 that

48   have acquired two additional changes previously associated with antibody escape and it could

49   be identified as VOI1163.7.V2. Overall, we have detected the spread of a new Spike variant that

50   may be advantageous to the virus and whose continuous transmission poses risks by the

51   acquisition of additional mutations that could affect pre-existing immunity.

52   **Introduction**

53   Genomic surveillance of viral mutations is the first step in detecting viral changes that could

54   impact public health by interfering with diagnostics, modifying pathogenicity, or altering

55   susceptibility to existing immunity or treatments. In many countries, the challenge of detecting

56   new mutations of interest in SARS-CoV-2 is approached by sequencing representative genomes

57   from circulating viruses, sharing sequence information on public databases (e.g. GISAID[1]), and

58   analysing them in real-time using platforms, such as Nextstrain[2]. While mutations appear

59   randomly, their fate in the population depends on a combination of the conferred fitness

60   advantage as well as stochastic and demographic processes. A first step in assessing the

61   potential public health impact of mutations is to decipher if their increase in frequency is due to

62   chance or adaptation. If found to be adaptive, it is important to evaluate whether their

63   adaptation is linked to an improved ability to replicate, colonize, transmit, or evade antiviral

64   hosts defences[3]. An important challenge in the field is to decipher which of all the variants that

65   appear should be monitored to implement measures to mitigate their risk to public health.

66   Genotypes that are phenotypically different from a reference isolate or have mutations that lead

67   to changes associated with either established or suspected phenotypes could be considered

68   Variants of Interest (VOI) if they also fit one of the following criteria: i) cause community

69   transmission/multiple COVID-19 clusters or ii) have been detected in multiple countries[4]. Among

70   VOI, only those genotypes that are associated with higher transmissibility, with detrimental

71 changes in COVID-19 epidemiology, with increased virulence, with changes to clinical

72 presentation, or with decrease effectiveness of public health measures, diagnostics, vaccines or

73 therapeutics are further categorized as variants of concern[4].

74 Mutations in SARS-CoV-2 have been reported since the early stages of the epidemic[5-7]. While no

75 signs of recombination have been detected so far among SARS-CoV-2 variants[8], the most

76 common mutations described are single nucleotide polymorphisms (SNPs) and small deletions[9-11].

77 Genomic surveillance of mutations has been mostly focused on the Spike (S) protein because

78 of its key roles in viral entry and immunity[12], as well as the fact that this protein constitutes the

79 basis of numerous SARS-CoV-2 vaccines[13]. S is a homotrimeric protein, whose heavily

80 glycosylated ectodomain protrudes from the viral membrane, showing a bat-like shape with a

81 N-terminal globular head portion connected to the membrane by an elongated stalk[14]. The S

82 protein is proteolytically processed by the cellular furin protease into the S1 and S2 subunits[15,16].

83 Additional proteolytic cleavage occurs following S protein binding to the host receptors,

84 facilitating S1 subunit release. The C-terminal S2 subunit remains trimeric in the viral membrane

85 but undergoes conformational changes that promote viral membrane fusion with the host cell[17].

86 A key role in these conformational changes is played by two heptad repeat motifs, HR1 and HR2

87 that, starting from the head and stalk regions in the pre-fusion state of S protein, form a HR1-

88 HR2 six-helix bundle in the post-fusion state that is critical for viral entry[18].

89 The first mutation that was identified as of potential concern was an aspartic acid to a glycine

90 mutation in the S1 subunit of the S protein at position 614 (D614G). D614G emerged early in the

91 epidemic, became predominant in most countries within 2 months, and completely dominated

92 the epidemic by August 2020[19]. As with any mutant, the initial spread of this mutation could

93 have resulted from stochastic events, the dynamics of epidemic, or an intrinsically higher viral

94 fitness. More than six months after the initial report of this mutation, several studies have found

95 evidence in favour of higher transmission efficacy in animal models and human populations[5,20-

96   [22]. This variant replicates better in some cell culture and animal models[20,21,23], and is associated

97   with higher viral loads in infected individuals[19]; importantly, however, it does not impact

98   diagnostics or vaccine efficacy.

99   Following the first wave of the pandemic, additional variants have been reported from many

100 countries. Among the first of these was the A222V mutation at the N-terminal domain (NTD) of

101 the S1 subunit, which occurred in the background of the D614G S protein mutation. This variant,

102 termed 20E, was first sequenced in Spain and expanded throughout Europe[6]. Other variants

103 have been reported since, such as the so-called "cluster 5", which harbours a combination of 3

104 SNPs and single deletion related to mink farms in Denmark[24]. One of the SNPs is in the S protein

105 of this variant, Y453F, occurs in the receptor binding domain (RBD) and may increase binding to

106 cell receptors in mink[25]. Transmission of this variant between humans and minks has been

107 reported[7], highlighting a possible risk of expansion in the human population, which resulted in

108 proposals for large scale culling of mink populations in Denmark. Studies to assess the biological

109 impact of this mutant have not been reported but there is no evidence for its wide spread over

110 the course of >6 months since its description[26]. By December 2020, three variants of concern

111 (VOCs) were described, all of which share the N501Y amino acid replacement in the RBD of the

112 S protein: 20I/501Y.V1 (also called Lineage B.1.1.7) was originally described in the UK[11],

113 20H/501Y.V2 (B.1.351) in South Africa, and 20J/501Y.V3 (P1) in Brazil. These variants are of

114 particular concern because they are more transmissible[27-29] and, although data on antigenicity

115 and disease severity is not conclusive, could result in reduced susceptibility to neutralization by

116 existing immunity and affect vaccine efficacy[30-33]. Importantly, all of these variants have spread

117 outside of the country where they were initially identified and are estimated to spread faster

118 than other co-circulating genotypes[27,34,35].

119 In this work, we have performed a detailed phylogenomic analysis of the appearance, spread

120 and evolution of two mutations involving amino acid positions 1163 and 1167 of the HR2

121   functional motif of S protein. Our results provide evidence of repeated, independent

122   emergence, suggesting these mutations contribute to increased viral fitness. In addition, we

123   have evaluated the biological relevance of these mutations to viral infectivity, virion stability,

124   and neutralization by sera from convalescent and vaccinated individuals.

125   **Results**

126   **Multiple and independent mutations in amino acid positions 1163 and 1167 of the Spike**

127   **protein.**

128   SARS-CoV-2 genetic variation has been monitored by the Spanish sequencing consortium

129   SeqCOVID to follow the expansion of mutations that could potentially result in a change of the

130   biological properties of the virus. We focused on mutations in the S protein because of its

131   relevance for infection and immunity[12]. We detected two mutations in the *spike* gene: G25049T

132   (D1163Y) and G25062T (G1167V), which appeared in Spain as early as March and April 2020,

133   respectively (Supplementary Fig.1). These mutations continued arising independently of each

134   other and, by the end of June, when the predominant circulating genotypes from the first wave

135   in Spain had already been replaced by other variants[36], were also observed together

136   (Supplementary Fig.1). Both positions have mutated multiple times independently and to

137   different amino acids at a lower frequency. On the one hand, D1163 appears mutated at least

138   99 times (D1163Y: 84, D1163V: 4, D1163G: 3, D1163A: 2, D1163E: 2, D1163H: 2, D1163N: 1, and

139   D1163H/Y: 1) in 47 lineages according to the PANGO scheme[37]. On the other hand, G1167

140   appears mutated at least 54 times (G1167V: 39, G1167D: 4, G1167C: 3, G1167R: 3, G1167S: 3,

141   G1167F: 1, and G1167A: 1) in 20 PANGO lineages including B.1 (Supplementary Fig.2e) and its

142   derivatives B.26, B.40 (Supplementary Fig.2c), and D.2 (Supplementary Fig.2f).

Clusters of transmission with amino acid changes in positions 1163 and 1167 of Spike

Positions 1163 and 1167 in the S protein have mutated independently multiple times in SARS-CoV-2. The majority of mutated sequences (94.43%) were found in transmission clusters (see methods for definition of clusters; Figure 1a,b), with a small minority not belonging to a transmission cluster due to either incomplete sampling or failure to spread. While different amino acids changes have been detected at both positions, only one change at each position appeared in most clusters: D1163Y in 83.33% and G1167V in 69.23% clusters. D1163Y appeared in 22 transmission clusters (Figure 1a) and G1167V in 8 clusters (Figure 1b). Interestingly, the biggest cluster included both the D1163Y and G1167V mutations together. We denote this cluster as B.1.177.637, which was detected in 65 sequences from Spain until December 2020, representing 1.17% of the Spanish sequences. Globally, this cluster, which is within lineage 20E (also described as 20A.EU1[6], and B.1.177[37]), includes 1,627 sequences (Figure 1c). B.1.177.637 is characterized by nine nonsynonymous and six synonymous mutations with respect to the reference sequence from Wuhan (Supplementary table 2), but no deletions were shared among B.1.177.637 sequences. Amino acid changes were found in A222V, D614G, D1163Y, and G1167V in the S protein, A220V and P365S in the N protein, V30L in ORF10, L67F in ORF14, and P4715L in ORF1ab (Supplementary Fig.3 and Supplementary table 2). Synonymous mutations were also observed in the *orf1ab*, *n* and *m* genes (Supplementary Fig.3 and Supplementary table 2). All evidence support we consider this cluster a new Lineage, and we requested to be under the name B.1.177.637 (https://github.com/cov-lineages/pango-designation/issues/22).

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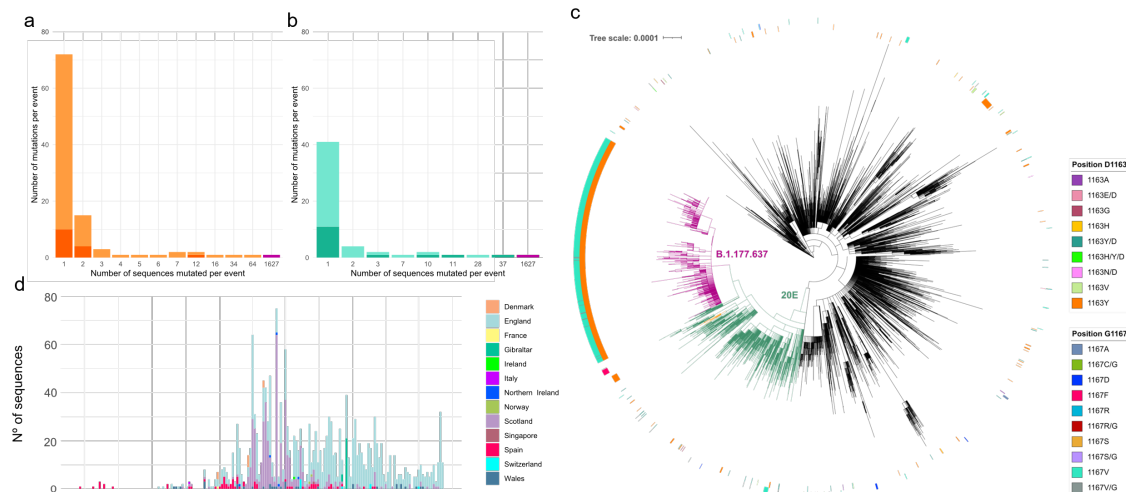
Figure 1. Sequences mutated at positions 1163 and 1167 of the S protein. a. The number of mutation events for amino acid replacement D1163Y (light orange) or another D1163 amino acid replacements (dark orange). **b**. The number of mutation events for amino acid replacement G1167V (light turquoise) or another G1167 amino acid replacements (dark turquoise). Bars coloured in magenta indicate the appearance of both the D1163Y and G1167V amino acid replacements in the same sequences. **c.** Maximum-likelihood phylogeny of 10,450 SARS-CoV-2 genomes. The inner circle of the rings represents sequences with amino acid changes in position D1163 of the S protein. The external circle represents sequences with amino acid changes in position G1167 of the S protein. Branches are coloured in magenta for B.1.177.637, green for clade 20E, and orange for cluster 1163.654. The scale bar indicates the number of nucleotide substitutions per site. **d.** Temporal distribution and frequency of sequences with variant B.1.177.637 coloured by geographical origin.

Within 20E, the second largest cluster including any of these mutations was observed in 34 sequences with E654Q and D1163Y in S protein plus 7 nonsynonymous and 6 synonymous mutations (Supplementary table 2 and Supplementary Fig.3). We denote this second cluster, which is also embedded within lineage 20E, cluster 1163.654 (Figures 1c and S3). Cluster 1163.654 appeared first in Ireland on 2020-07-23 and subsequently appeared in Spain and

8

181 England. However, after three months, cluster 1163.654 is no longer being detected. A large

182 cluster within Lineage 20E is formed by 37 sequences with the mutation G1167F. Sequences for

183 this cluster were obtained in Wales between the end of October and the beginning of November

184 2020 (dark pink in the external circle in Supplementary Fig.2g).

185

186 We found additional clusters involving mutations in position 1163 of the S protein; the largest

187 cluster is composed of 64 sequences within Lineage B.1. The majority of which are from

188 Denmark but also includes sequences from England and Sweden (orange in external ring in

189 Supplementary Fig.2e). Among other smaller clusters, a cluster of 10 sequences with G1167V

190 (within Lineage B.1) appeared in Spain in March (indicated in cyan within the external circle of

191 Supplementary Fig.2e). The cluster was detected in two regions in Spain (Valencia, and Galicia),

192 but was controlled with lockdown measures imposed in Spain from March to May and was not

193 detected after May 2020. The same mutation was found in a cluster of 28 sequences within 20E

194 from England and Wales between October and November 2020 (indicated in cyan within the

195 external circle of Supplementary Fig.2g). Similarly, another cluster of 11 sequences in Lineage

196 B.1.1 and with the mutation G1167A was detected in early January 2021, encompassing

197 sequences from Ecuador, Colombia, and Peru (Supplementary Fig.2f). Within Lineage B.53, we

198 found a cluster of 12 sequences from Lithuania with mutation D1163Y. Finally, we found small

199 clusters with mutations in 1163 or 1167 within Lineage B.40 and Lineage A (Supplementary

200 Fig.2b, c).

201 Because of the risk posed by VOC[11,26,35], we examined whether mutations in 1163 and 1167 of

202 the S protein were observed in the three VOC described to date. Indeed, mutations in these two

203 positions were observed in two VOC, 20I/501Y.V1 and 20H/501Y.V2. Mutations involving amino

204 acid positions 1163 and 1167 appeared independently in the background of 20I/501Y.V1

205 multiple times. Specifically, mutations in D1163 have occurred at least 13 times in 24 sequences,

206 including four transmission clusters and nine unique sequences (Supplementary Fig.4), while

207 mutations at G1167 were observed in at least five independent sequences. Interestingly,

208 D1163Y and G1167V were observed together in only one individual within 20I/501Y.V1,

209 although they were not fixed (relative frequency of 27% and 17% of the reads with D1163Y and

210 G1167V, respectively; Supplementary table 3). Finally, only two sequences that harbour the

211 amino acid replacement G1167V in the S protein were observed in the genomic background

212 20H/501Y.V2 (Supplementary table 3).

213 **Evolution of B.1.177.637**

214 We explored the emergence and evolution of B.1.177.637, the largest and most successful

215 cluster involving amino acid changes in positions 1163 and 1167 of the S protein. Lineage

216 B.1.177.637 appeared in Spain in June 2020 in sequences from the Basque Country (Figure 1d,

217 and Supplementary Video 1) and subsequently appeared in individuals from other countries,

218 comprising a total of 1,627 sequences in GISAID (0.60% of 270,869 analysed sequences by 23rd

219 of December 2020) (Figure 1d and Supplementary Video 1). The majority of the B.1.177.637

220 sequences were obtained from the United Kingdom, including England (1,058), Scotland (419),

221 Wales (34) and Northern Ireland (5), but were also observed in Gibraltar (24 sequences),

222 indicating successful migration and transmission (Supplementary Video 1). Although

223 B.1.177.637 is not well represented in sequences from other countries, it has been found in

224 multiple sequences from Denmark (9), Switzerland (8), Norway (2), and single sequences from

225 Italy, France, Singapore, and Ireland. By the end of 2020, B.1.177.637 was still circulating in

226 Europe (Figure 1d and Supplementary Video 1), and by the end of February 2021 it was

227 represented by 1,923 sequences in GISAID (0.33% of submitted sequences).

228 Within B.1.177.637, we detected additional SNPs in individual sequences or small groups of

229 sequences. One of these changes is E484K in the RDB of the S protein, a mutation present in

230 three VOCs (20I/501Y.V1, 20H/501Y.V2 and 20J/501Y.V3) that is implicated in increased ACE2

10

231 binding[38] and reduced neutralization by antibodies[39]. In addition, we found another change

232 associated with evasion of antibody immunity: a deletion of positions 141-144 in the S protein,

233 which partially overlaps with a smaller deletion at 144 reported in VOC 20I/501Y.V1[40]. This sub-

234 cluster included five sequences along January 2021 from England and Wales (Supplementary

235 table 2). The five sequences formed a monophyletic group embedded in B.1.177.637

236 (Supplementary Fig.5), identified as cluster B.1.177.637.V2, which displays other

237 nonsynonymous and synonymous mutations (Supplementary table 2) and only two sites are

238 polymorphic within B.1.177.637.V2.

239 **Positions 1163 and 1167 of the S protein are located in the heptad repeat 2 motif**

240 The S protein mediates both the binding to cellular receptors and entry into the host cells[12]. For

241 the former, the RBD motif in the S1 subunit interacts with the cellular receptor in the pre-fusion

242 state. In the post-fusion state, two heptads repeat sequences (HR1 and HR2) in the S2 subunit

243 must form a six-helix bundle in order to bring the viral and cellular membrane into close

244 proximity[41,42] (Figure 2). S protein positions 1163 and 1167 are both located within the HR2

245 domain. Specifically, 1167 is present at the beginning of the HR2 motif and 1163 in its upstream

246 linker region (Figure 2a). Interestingly, this motif is highly invariable, showing 100% conservation

247 across 14 related sarbecoviruses to which SARS-CoV-2 belongs to (Supplementary table 1)[43,44].

248 Structural characterization of full-length ectodomain of S protein has shown that the stalk

249 portion encompassing positions 1163 and 1167 presents intrinsic flexibility in the pre-fusion

250 state[18], precluding its atomic visualization. This has been recently confirmed by high-resolution

251 cryo-electron tomographic reconstitution of SARS-CoV-2[14], where this region was observed to

252 constitute a flexible hinge that acts as a "knee", connecting two helical coiled-coil regions of the

253 stalk (upper and lower legs; Figure 2b). Within this structure, the conformational freedom

254 provided by the glycine residue at position 1163 should play a key role in the flexibility of the

255 knee. In contrast, in the post-fusion state, this region shows high rigidity due to a strong

11

256   structural rearrangement of the HR2 motif, which adopts an extended conformation and tightly

257   packs along the central 3-helix bundle stem formed by the HR1 motif (Figure 2c). The resulting

258   HR1-HR2 bundle plays a key role in the mechanism of viral-host membrane fusion[18,45] and

259   mutations in this region could have significant impact on the function of the S protein. In

260   addition, the HR2 region is highly glycosylated, which is observed to be regularly spaced in both

261   the pre- and post-fusion states and to mostly align to the side of the helix bundle[14,18,45]. Of note,

262   two of these branched sugars are placed at positions N1158 and N1173, hiding positions 1163

263   and 1167 (Figure 2b). Therefore, changes in stalk flexibility might have relevance in immunity by

264   influencing both the intrinsic degree of exposure of this region and its sugar shielding.

265   Using the available structural information of the S protein in the pre- and post-fusion

266   conformations[18], we examined the possible implications of these mutations to viral infectivity.

267   Based on these structures, G1167V mutation is predicted to confer significant rigidity to the

268   structure in two ways. First, the introduction of a side chain strongly reduces the conformational

269   freedom provided by the glycine residue. Second, the presence of the new aliphatic side chain

270   provided by the valine residue strongly increases hydrophobicity, likely promoting the burial of

271   this side chain in the HR1 helix 3-bundle stem in the post-fusion state or favouring its integration

272   in the neighbour helical coiled-coil in the pre-fusion state (Figure 2b,c). Unlike position 1163,

273   position 1167 is fully exposed to the solvent in both the pre- and post-fusion states (Figure 2b,c).

274   Hence, the effect of D1163Y is likely to stem a change in nature of the side chain, switching from

275   a charged aspartic acid residue at physiological pH to a polar group with hydrophobic properties

276   in the tyrosine.

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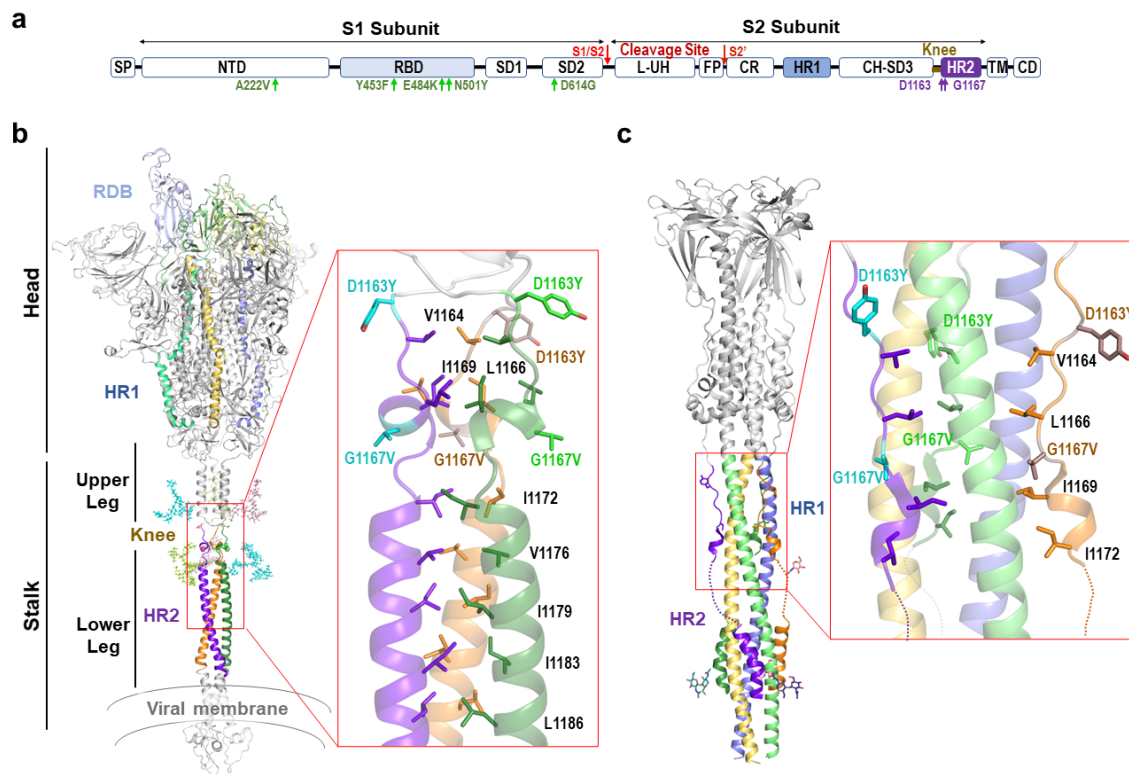
Figure 2. **The structure of 1163 and 1167 in the pre and post-fusion states of S protein. a.**
Schematic representation of the S protein. SP. Signal peptide; NTD, N-terminal domain; RBD,
receptor-binding domain; SD1-2, subdomains 1 and 2; L-UH, Linker-Upstream helix; FP, fusion
peptide; CR, connecting region; HR1, heptad repeat 1; CH-SD3, central helix subdomain 3; BH,
β-hairpin; HR2, heptad repeat 2; TM, transmembrane; CD, cytoplasmic domain. Mutations
D1163Y and G1167V are indicated in purple and other mutations described in the text in green.
b. Cartoon representation (*left*) of a structural model of pre-fusion membrane-bound trimeric S
protein[46]. In each subunit the RBD, HR1 and HR2 domains are coloured in different tones (light
to dark) of blue, yellow, and green. The N-glycosylation of N1155 and N1176 are shown in stick
representation and coloured as the corresponding subunit. Functional and structural regions are
marked. A close-view (*right*) of the N-terminal portion of HR2 where D1163Y and G1167V
mutations are found. The side-chains of mutated and hydrophobic residues in the HR2 region
are shown in stick representation and coloured as corresponding subunit (mutated residues in
lighter tone). **c.** Cartoon representation (left) of S2 subunit in post-fusion conformation with HR1

13

292   and HR2 regions coloured as in b and N-glycosylation around mutation position shown as sticks.

293   A close-view of the region encompassing the mutations (right), showing in stick representation

294   the mutated and hydrophobic residues from the HR2 region shown in panel b. Dotted lines

295   highlight HR2 disordered regions in the Cryo-EM structure.

296   **Spike aminoacid changes D1163Y and G1167V do not increase viral infectivity**

297   Previous reports have indicated that mutations in the S protein can increase infectivity[5,21,47-49].

298   Because the highest transmission success for mutations in S positions 1163 and 1167

299   corresponds to the double mutant D1163Y and G1167V (characteristic of B.1.177.637), we

300   explored whether these mutations in combination have an influence on infectivity. For this, we

301   pseudotyped vesicular stomatitis virus lacking its glycoprotein and encoding GFP[50] (VSVΔG-GFP)

302   with different S genotypes: Wuhan (D614), D614G, 20E (A222V and D614G), or B.1.177.637

303   (A222V, D614G, D1163Y and G1167V). Infectious virus production was then assessed by limiting

304   dilution and counting of GFP-positive cells in both Vero cells and A549 human alveolar basal

305   epithelial cells expressing human ACE2 and TMPRSS2 (A549-hACE2-TMPRSS2). As previously

306   reported[19,21,51], the 20E S genotype enhanced infectivity relative to the Wuhan S genotype by

307   70% in both Vero (p-value = 0.005 by unpaired t-test; Figure 3a) and A549-hACE2-TMPRSS2 cells

308   (p-value = 0.016 by unpaired t-test; Figure 3b). The 20E S genotype also showed a trend towards

309   increased infectivity versus the D614G mutation alone (35% increase in both cell lines), as has

310   been previously reported[49], yet the difference was not statistically significant (p-value > 0.05 by

311   unpaired t-test; Figure 3a,b). In contrast, B.1.177.637 S genotype significantly diminished virus

312   infectivity versus the 20E genotype, reducing virus titers by 20% in Vero cells (p-value = 0.009 by

313   unpaired t-test; Figure 3a) and 29% in A549-hACE2-TMPRSS2 (p-value = 0.03 by unpaired t-test;

314   Figure 3b). This is in agreement with a potential stabilization of the HR2 helix (Figure 2), which

315   should limit the ability of the S protein to sample different structural conformations that could

316   be required for binding host-cell receptors. Hence, B.1.177.637 S genotype does not increase

317   infectivity *in vitro*.

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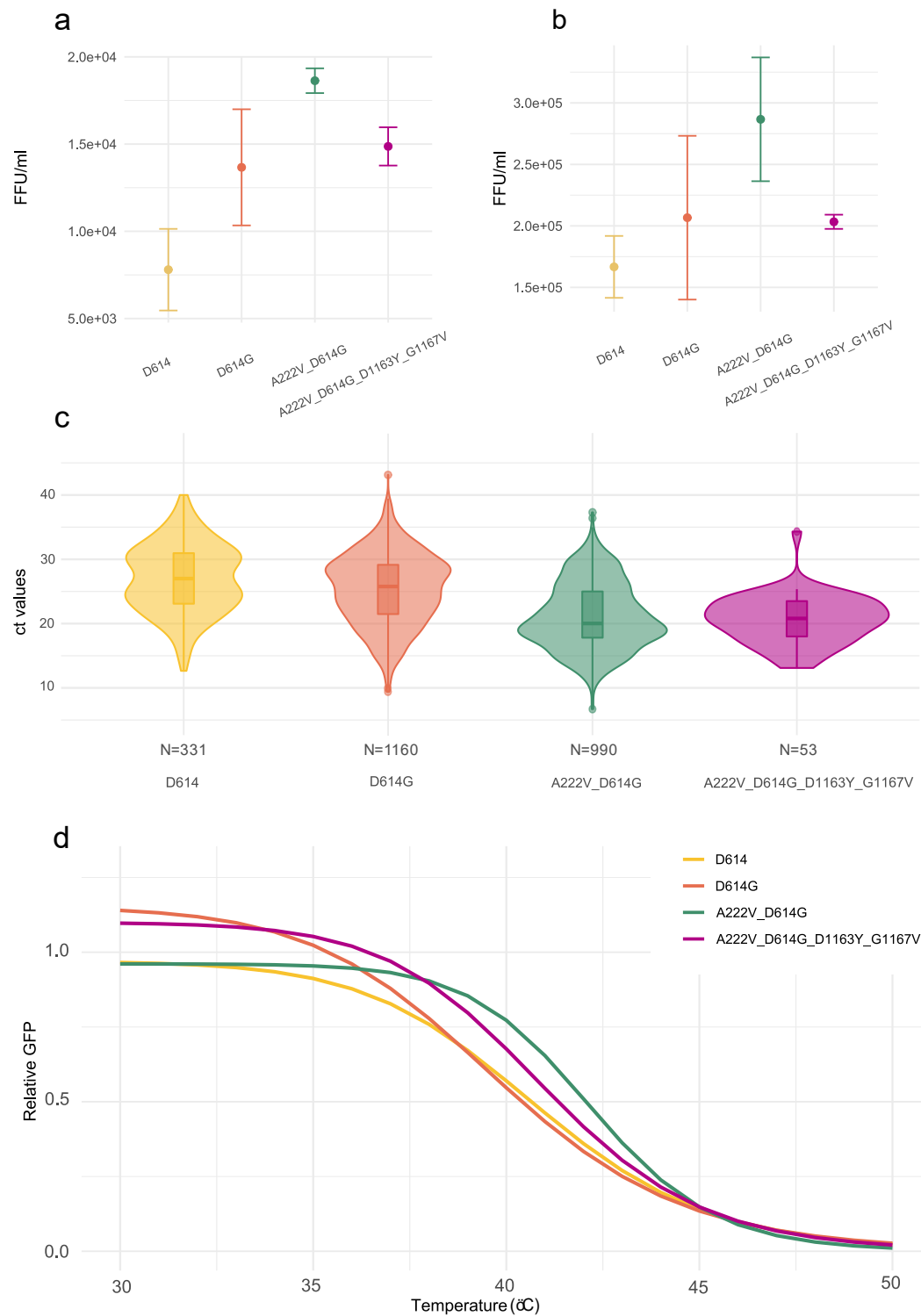
319   **Figure 3. Comparison of the infectivity and stability of different S genotypes. a, b.** The

320   infectivity of VSV particles pseudotyped with each S protein genotype in either Vero (**a**) or

321   human A549 cells expressing ACE2 and TMPRSS2 (**b**). The mean and standard deviation of three

322   replicates is plotted. **c.** Comparison of cycle threshold (Ct) values for the *n* gene from patients

323   infected with viruses encoding different S protein variants. Data is derived from 2,534 sequences

324   from SeqCOVID consortium. The number of observations (N) analysed for each genotype is

325   indicated. **d.** The thermal sensitivity of VSV pseudotyped with different S genotypes following

326   incubation at 15 minutes. Data are standardized to the surviving fraction following incubation

327   at 30°C, and the three-parameter log-logistic equation is plotted. FFU: focus forming units.

328   To examine whether reduced infectivity could also be observed *in vivo*, we tested if individuals

329   infected with B.1.177.637 have different viral loads. For this, we used the cycle threshold (Ct) of

330   real-time PCR used for diagnosis as a surrogate. As previously reported[19], we detected higher Ct

331   values for D614 wild-type variant (Ct mean = 27.00) compared to genotypes encoding the D614G

332   S protein mutation (Ct mean = 25.32; p-value < 0.01 by unpaired Wilcoxon test, Figure 3c).

333   However, we did not find significant differences in viral loads between individuals infected with

334   B.1.177.637 genotype and other genotypes within 20E (Ct mean = 21.14 vs Ct mean = 20.63, p-

335   value = 0.72 by unpaired Wilcoxon test, Figure 3c). Interestingly, higher viral loads were

336   observed in individuals infected with B.1.177.637 and others 20E (D614G and A222V) compared

337   to the D614G alone (D614G Ct mean = 25.32, 20E Ct mean = 21.14, B.1.177.637 Ct mean = 20.63,

338   p-value < 0.01 for both comparisons by unpaired Wilcoxon test, Figure 3c). This data suggests

339   that, unlike the results of the in vitro studies, B.1.177.637 replicates as efficiently as other 20E

340   genotypes *in vivo*, although mutations outside of the S protein could contribute to this result.

341   **Amino acid changes D1163Y and G1167V do not alter S protein stability**

342   As increased S protein stability could impact transmissibility by maintaining virion infectivity

343   during the intra-host transmission period, we assessed the temperature sensitivity of the

344   different S variants. For this, we subjected VSV particles pseudotyped with different S genotypes

345   to a range of temperatures for 15 minutes, after which we evaluated the surviving fraction.

346   Overall, no major differences in the degree to which the different S proteins lost infectivity upon

347   heat exposure were observed, with all S proteins showing a 50% reduction in infectivity at a

348   similar temperature range (39.8-42.2°C; p-value > 0.05 for all except Wuhan S genotype (D614)

349   versus 20E S genotype (A222V and D614G), where p-value = 0.01; Figure 3d). Hence, the D1163Y

350   and G1167V mutations do not seem to have a major impact in the thermal stability of the S

351   protein.

352   **Spike D1163Y and G1167V modestly reduce sensitivity to neutralization by existing antibody**

353   **immunity**

354   Positions 1163 and 1167 of the S protein have been reported to occur in both T and B cell SARS-

355   CoV-2 epitopes[52-54]. Moreover, numerous studies have shown that mutations in the S protein

356   can affect antibody neutralization[30,31]. We therefore examined if the presence of D1163Y and

357   G1167V alters the neutralization capacity of convalescent sera using VSV pseudotyped with

358   either the 20E or B.1.177.637 S genotypes. In order to capture the potential influence of

359   different infecting variants on antibody neutralization, we tested the sensitivity of these

360   pseudotyped viruses to neutralization by sera from early (April 2020; First wave in Spain) or later

361   (October 2020; Second wave in Spain) in the pandemic, when newer variants were dominant[6,36].

362   Overall, B.1.177.637 genotype conferred a modest but statistically significant reduction in

363   sensitivity to neutralization by six serum samples tested from the early stage of the pandemic,

364   as measured by the titers required to inhibit viral entry by 80% (ID_{80}; mean = 6.75, range: 1.30-

365   17.68; p-value = 0.008 by paired t-test; Figure 4a). A statistically significant but smaller effect

366   was observed when the titers required to inhibit viral entry by 50% were examined (ID_{50}; mean

367   = 2.27, range: 1.61-3.54; p-value < 0.001 by paired t-test; Supplementary Fig.6). In contrast, both

368   20E and B.1.177.637 were equally susceptible to sera from patients infected during the second

17

369   wave (ID$_{80}$; mean = 1.03, range: 0.87-1.23; p-value = 0.83 by paired t-test; Figure 4b). These

370   results indicate that the D1163Y and G1167V mutations can provide some degree of escape from

371   pre-existing antibody-based immunity relative to the 20E S genotype depending on the genomic

372   background of the infecting genotype. As a modest reduction in titers was observed with sera

373   from early in the pandemic (Figure 4a), which is more closely related to the current S genotype

374   present in approved vaccines[55,56], we examined if B.1.177.637 S genotype resulted in reduced

375   neutralization by sera from donors vaccinated with the BNT162b2 vaccine. No significant

376   differences in susceptibility to antibody neutralization from vaccinated donors were observed

377   between the two genotypes, indicating that VOI1163.7 is unlikely to alter the efficacy of vaccines

378   based on the Wuhan S genotype (Figure 4c).

379
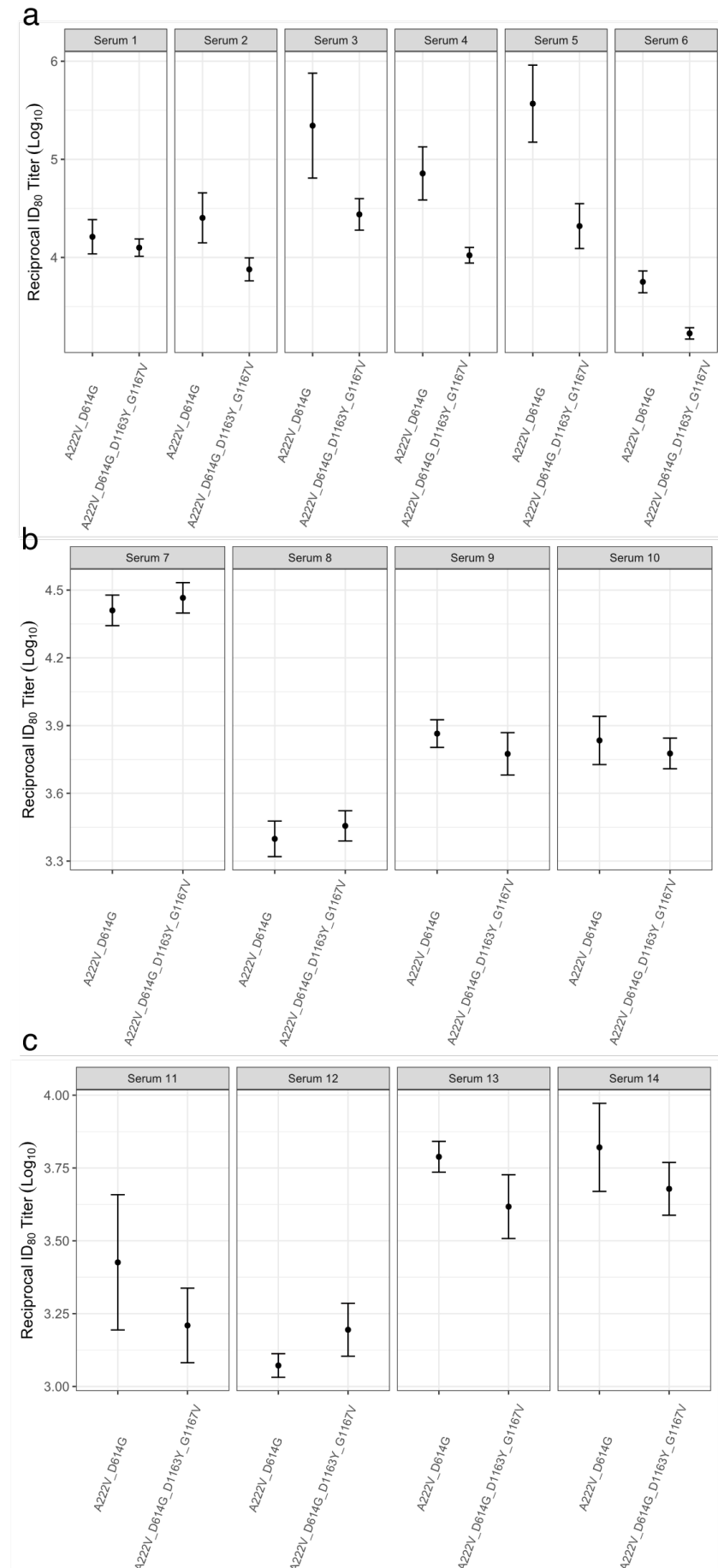
380 **Figure 5. Antibody neutralization of 20E and B.1.177.637 variants.** The reciprocal titer at which

381 infection with the 20E S genotype (A222V and D614G) or B.1.177.637 S genotype (20E plus

382 D1163Y and G1167V) is reduced by 80% (ID_{80}) by sera from individuals infected during the early

383 stage of the pandemic in (**a**) or during a later stage of the pandemic (**b**) or from donors

384 vaccinated with the BNT162b2 vaccine (**c**). The mean and standard error of three replicates is

385 plotted.

386 **Discussion**

387 SARS-CoV-2 success is linked to its ability to infect and be transmitted. Mutations that emerge

388 independently several times and increase in frequency are likely to confer enhanced viral

389 infectivity, transmission, or immune evasion. The identification of such mutants is of great

390 importance, as they can significantly impact public health. In this work, we have identified two

391 mutations in the S protein that are likely to be beneficial for the virus based on several lines of

392 evidence. First, these mutations are highly variable within SARS-CoV-2 but conserved across the

393 closely related coronaviruses. Second, the vast majority of sequences harbouring these

394 mutations appear in clusters (Figure 1a and 1b). Third, the largest cluster, and therefore the

395 most successful in terms of transmission, includes both mutations together (Figure 1a and 1b).

396 Additionally, both positions have been reported as positively selected multiple times throughout

397 the SARS-CoV-2 phylogeny indicating a fitness advantage[57]. Although either mutation in

398 isolation could be advantageous, their co-occurrence in a large cluster that has been sustained

399 for more than six months across Europe is suggestive of increased fitness when both mutations

400 are present together.

401 Positions 1163 and 1167 are found in the HR2 domain of the S protein, adjacent to the

402 transmembrane domain. Examination of available structural data suggests that G1167V might

403 alter the flexibility of the S protein stalk by both restricting the conformational freedom normally

404 conferred by the glycine residue and by introducing a hydrophobic side chain that will favour

20

405   burial in the HR2 coiled-coil leucine zipper of the pre-fusion state (Figure 2). This extensive

406   flexibility of S prefusion stalk seems to be unique to the SARS-CoV-2 S protein and has not been

407   reported for other class I fusion proteins[18]. The stalk flexibility has been suggested to increase

408   avidity for the host receptors by allowing the engagement of multiple S proteins[18]. Therefore,

409   stalk stabilization is likely to result in a reduced ability of S to bind receptors in the target cell.

410   Indeed, we find the B.1.177.637 genotype to have reduced infectivity compared to the 20E

411   genotype in both Vero and A549-hACE2-TMPRSS2 cells (Figure 3a, b). In contrast, viral load in

412   individuals infected with different S genotypes indicated that 20E and B.1.177.637 reach similar

413   degrees of viral replication in vivo (Figure 3c). This apparent discrepancy in the effect of the two

414   mutations on infectivity may stem from the differences of SARS-CoV-2 infection *in vivo* and in

415   the *in vitro* assay. A recent publication has suggested that the tyrosine-protein kinase receptor

416   UFO (AXL) is an important mediator of SARS-CoV-2 entry and may be of higher relevance for

417   infection of the lung than ACE2[58]. The effect of entry via AXL in the two cell lines used may be

418   overshadowed by high levels of ACE2 expression. Alternatively, additional factors could underlie

419   this difference, including the presence of additional mutations outside of the S protein or

420   differences of viral loads across sampling times i*n vivo* during the infection.

421   Increased temperature stability can potentially confer a fitness advantage to the virus by

422   reducing losses to infectivity during environmental transition between hosts. Hence, we also

423   examined whether these mutations altered the temperature stability of the virions. Overall, no

424   major difference in stability was observed between VSV pseudoparticles bearing the D614,

425   D614G, 20E, or B.1.177.637 S protein genotypes (Figure 3d). Hence, changes in protein stability

426   are unlikely to underlie the increased transmission of these variants.

427   Finally, as the S protein is a major target of the immune response[12], immune evasion represents

428   one possible consequence of mutations in this protein. Both S positions 1163 and 1167 are

429   embedded in experimentally confirmed T cell and B cell epitopes. For T cell epitopes, a predicted

21

430   HLA-II epitopes including position 1163 and 1167 has been experimentally verified to bind to

431   HLA DRB1*01:01, the prototype molecule for the DR supertype (epitope identifier in Immune

432   Epitope Data Base: 9006[59]). Additionally, D1163 is included in a SARS-CoV-2 T cell epitope

433   eliciting T-cell responses in convalescent COVID-19 cases[60] as well as in SARS-CoV-2-naïve

434   individuals[53], indicating cross-reactivity in epitopes involving these regions. B cell linear epitopes

435   that span D1163 and G1167 have also been reported[52], with D1163 belonging to a dominant

436   linear B cell epitope recognized by more than 40% COVID-19 patients used in the assay[54]. D1163

437   is fully solvent exposed in available structure[18,45], making its side-chain easily accessible to

438   antibodies, providing a potential mechanism for altering antibody binding. To directly examine

439   whether the mutated S positions 1163 and 1167 influence susceptibility to pre-existing humoral

440   immunity, we examined the neutralization capacity of convalescent sera against the 20E variant

441   or B.1.177.637. For this, we used sera from both the first (April 2020) and second (October 2020)

442   waves of the infection in Spain, because an almost complete replacement of SARS-CoV-2 S

443   variants occurred between these two times of the pandemic in Spain[36]. When utilizing sera from

444   donors infected during the first wave of the pandemic in Spain, we found a modest but

445   statistically significant reduction in susceptibility to neutralization of the B.1.177.637 S genotype

446   compared to the 20E S genotype of approximately 6-fold (Figure 4a). However, no difference in

447   neutralization was observed between the two variants when sera from patients infected during

448   the second wave was used (Figure 4b), highlighting variant-specific differences in antibody

449   responses. Overall, the magnitude of the observed reduction in neutralization susceptibility to

450   sera from individuals infected during the first wave was much less pronounced than that

451   observed for other genotypes implicated in immune evasion[31]. Nevertheless, the degree of

452   reduced neutralization required to confer a biologically relevant fitness advantage *in vivo* has

453   not been established, and even relatively small reductions in susceptibility to antibody

454   neutralizations could potentially confer a significant advantage to replication. Indeed, this has

455   been suggested to be the case in an immunosuppressed individual treated with convalescent

456   serum, where mutations selected during the course of treatment conferred a similar reduction

457   to that observed in the current study[61]. Finally, no evidence was found for reduced neutralization

458   by sera from donors immunized with the BNT162b2 vaccine (Figure 4c), which is based on the

459   Wuhan S genotype, indicating antibody immunity elicited by Pfizer-BioNTech COVID-19 vaccine

460   (BNT162b2; February 2021) would not be affected by amino acid replacements in 1163 and 1167

461   sites in S protein.

462   Although further experiments are needed to decipher the mechanisms by which the two S

463   mutations identified could confer a selective advantage to the virus, the evidence presented

464   supports B.1.177.637 as a VOI (VOI1163.7.V1) according to recently published criteria[4]. First,

465   amino acid replacements in S protein: D1163Y and G1167V lead to changes associated with

466   suspected phenotypic change because of the rigidity it poses to the S protein. Second, the

467   genotype showed moderate but significantly lower antibody susceptibility compared to 20E S

468   genotype. And third, it has been identified to cause community transmission, appearing in

469   multiple COVID-19 cases, and detected in multiple countries. Additionally, a subgroup within

470   B.1.177.637 (denoted as VOI1163.7.V2) includes two additional mutations leading to amino acid

471   changes in S with established phenotypic impact on humoral immunity: E484K[62] and 141-

472   144Del[40,63].

473   Whether VOI1163.7.V1 and VOI1163.7.V2 will continue to increase in frequency and accumulate

474   additional mutations that could improve its fitness and/or present challenges to vaccines or

475   diagnostics remains to be seen. However, their characterization as VOI would help to discover if

476   enough evidence holds to consider them VOC and therefore required monitoring.

23

Methods

Whole-genome sequencing and genome assembly of SeqCOVID consortium sequences

A total of 5,017 clinical samples were received, sequenced, and analysed by the SeqCOVID consortium from all autonomous communities of Spain. These samples were confirmed as SARS-CoV-2 positive by RT-PCR carried out by Clinical Microbiology Services from each hospital. Sequencing of the samples has been approved by the ethics committee: Comité Ético de Investigación de Salud Pública y Centro Superior de Investigación en Salud Pública (CEI DGSP-CSISP) Nº 20200414/05. All sequences are available at GISAID under the accession numbers detailed in Supplementary table 4.

For sequencing, RNA samples were retro-transcribed into cDNA. SARS-CoV-2 complete genome amplification was performed in two multiplex PCR, according to the protocol developed by the ARTIC network[64], using the V3 multiplex primers scheme[65]. From this step, two amplicon pools were prepared, combined, and used for library preparation. The genomic libraries were constructed with the Nextera DNA Flex Sample Preparation kit (Illumina Inc., San Diego, CA) according to the manufacturer's protocol, with 5 cycles for indexing PCR. Whole-genome sequencing was performed in the MiSeq platform (2×200 cycles paired-end run; Illumina).

Reads obtained were processed through a bioinformatic pipeline based on iVar[66], available at https://gitlab.com/fisabio-ngs/sars-cov2-mapping. The first step in the pipeline removed human reads with Kraken[67]; then fastq files were filtered using fastp[68] v 0.20.1 (arguments employed: --cut tail, --cut-window-size, --cut-mean-quality, -max_len1, -max_len2). Finally, mapping and variant calling were performed with iVar v 1.2, and quality control assessment was carried out with MultiQC[69].

Analysis of the *spike* gene of sarbecoviruses related to SARS-CoV-2

499   14 sequences including SARS-COV-2 belonging to sarbecoviruses, sequences were annotated

500   with annotation files available at NCBI database in order to locate the *spike* gene coordinates

501

502   (accession numbers are available at supplemental table 1). For each sequence, nucleotide

503   coordinates belonging to this gene were extracted with EMBOSS[70]. The 14 sequences harbouring

504   the *spike* gene were concatenated and aligned with MEGA-X[71] using amino acids with ClustalW

505   algorithm   with   default   options   (alignment   is   available   at

506   https://github.com/PathoGenOmics/B.1.177.637_SARS-CoV-2)

Sampling SARS-CoV-2 from non-Spanish consortium sequences

508   To build the global alignment, sequences were downloaded from GISAID[1] including all the

509   pandemic periods since the first known case sequenced (from 24 December 2019) until the last

510   sample on 22 December 2020. We used two filters to select the dataset: sequences with more

511   than 29,000 bp, and sequences with known dates of sampling. Sequences downloaded from

512   GISAID were aligned against the SARS-CoV-2 reference genome[72] using MAFFT[73], omitting all

513   insertions and getting an alignment length of 29,903 bp. The final alignment constructed

514   included 270,869 sequences, all sequences with GISAID ID used for this study are available in

515   Supplementary Table 5.

Frequency and detection of mutated positions

517   Single nucleotide variants were detected using the global dataset alignment, generating a VCF

518   file with SNP-sites[74] v 2.5.1 (argument employed: -v), using the reference genome as the

519   reference bases for detecting mutations. This VCF file was processed with a Python script

520   (available at https://github.com/PathoGenOmics/B.1.177.637_SARS-CoV-2) to assess all

521   mutated samples by position, calculating the frequencies of the global dataset and annotating

522   sequences with the detected mutations. After that, the mutated positions were annotated with

523   snpEff[75] v 5.0 using SARS-CoV-2 reference (Wuhan first sequenced) database annotation

524   (arguments employed: -c, -noStats, -no-downstream, -no-upstream, NC_045512.2).

525   Genotypes detected that involved mutations in 1163 and 1167 such as B.1.177.637 and cluster

526   163.654 were represented in a circos plot with the R package circlize[76] v 0.4.12.1004. Nucleotide

527   coordinates of SARS-CoV-2 are plotted in a non-closed circle, circle is annotated and coloured

528   with genes of the virus, and mutated nucleotide positions of each genotype are connected

529   between them through lines.

530   **Alignments**

531   For the phylogenetic analysis, a reduced dataset was selected from the 270,869 sequences.

532   Duplicated sequences were removed with seqkit v 0.13.2 (arguments employed: rmdup -s).

533   8,397 sequences were selected at random with the same temporal distribution by month as the

534   initial dataset by Python scripting (available at https://github.com/PathoGenOmics/

535   B.1.177.637_SARS-CoV-2). The 8,397 sequences were concatenated with 2,053 sequences

536   selected as indicate above because harboured amino acid replacements in D1163 and G1167 of

537   the S protein and resulted in an alignment of 10,450 sequences (Supplementary Table 6).

538   The dataset to represent 20I/501Y.V1 phylogenetic relationships include 3,067 randomly

539   selected samples identified by Pangolin typing system (https://github.com/cov-

540   lineages/pangolin) as lineage B.1.1.7 plus the 33 sequences with amino acid replacements in

541   D1163 and/or G1167 (Supplementary Table 7).

542   For all the alignments, problematic positions reported by Lanfear, R.[77] were masked for the

543   phylogenetic reconstruction using masked_alignment.sh script.

26

Phylogenetic analysis

Maximum-likelihood phylogenies in Figure 1 and supplementary Supplementary Fig.2, S4 and S5
were reconstructed from the masked alignment using IQ-TREE[78] v 1.6.12 with GTR model and
collapsing near-zero branches (arguments employed: -czb, -m GTR). The phylogenies were
rooted in the reference sequence from Wuhan[72] on 2019-12-24. The phylogenies were
annotated and visualized with iTOL[79] v 4.

The phylogeny in Supplementary Video S1, composed by 10,450 sequences, was build up with
Nextstrain pipeline (available at https://github.com/nextstrain/augur) in order to monitor and
visualize temporal and geographical transmission of B.1.177.637. This dataset file is available at
https://github.com/PathoGenOmics/B.1.177.637_SARS-CoV-2.

Clusters of transmission involving 1163 and 1167 S amino acid replacements.

We used the phylogeny of 10,450 sequences enriched with all sequences mutated in 1163 and
1167 to quantify the minimum number of mutational events involving positions 1163 and 1167
in S protein. We first defined which mutations characterize internal nodes using R packages:
tidytree v 0.3.3 and treeio v 1.14.3[80]. We then depicted monophyletic clusters sharing at least
one of the two mutations. Transmission clusters were defined as all sequences that: i) are
derived from an internal node characterized by the same nucleotide mutation involving 1163 or
1167 amino acid replacements, ii) include more than one sequence, and iii) at least 95% of
sequences share the nucleotide mutation. Additionally, redundant nodes were eliminated,
keeping the ancestral node of the cluster (harbouring the largest number of leaves). Sequences
with at least one mutation but not in clusters were counted as single events of mutation in the
phylogeny.

Structural analysis of 1163 and 1167 S amino acid replacements.

567   The atomic coordinates for S protein in pre-fusion state was retrieved from the CHARMM-GUI

568   COVID-19 Archive (http://www.charmm-gui.org/docs/archive/covid19). The atomic coordinates

569   for S protein in post-fusion sate were retrieved from Protein Data Bank (PDB: 6XRA[18] and PDB:

570   6LXT[81]). Mutations were introduced using single mutation tool embedded in COOT[82]and figures

571   were generated with PyMOL (www.pymol.org).

572   **SARS-CoV-2 pseudotyped vesicular stomatitis virus production, titration, and thermal stability**

573   **evaluation**

574   Mutations were introduced into a plasmid encoding a codon-optimized S protein[16] by site

575   directed mutagenesis (see Supplementary table8 for primers). All mutations were verified by

576   Sanger sequencing (see Supplementary table 9 for primers). To evaluate the efficiency of virus

577   production, three transfections in HEK293 cells (CRL-1573 from ATCC) were performed for each

578   plasmid to generate pseudotyped VSV harbouring the indicated S protein[83]. The titers of the

579   virus produced were then assayed by serial dilution, followed by infection of either Vero cells

580   (CCL-81 from ATCC) or A549 cells expressing ACE2 and TMPRSS2 (InvivoGen catalog code a549-

581   hace2tpsa), and counting of GFP positive cells (focus forming units; FFU) at 16 hours post

582   infection. Statistical comparisons were performed by unpaired t-test (R package: stats v 3.6.1)

583   with normalized logarithmic data. For assessing thermal stability, 1000 FFU (as measured on

584   Vero cells) were incubated for 15 minutes at 30.4, 31.4, 33, 35.2, 38.2, 44.8, 47, 48.6 or 49.6°C

585   before addition to Vero cells previously seeded in a 96 well plate (10,000 cells/well). GFP signal

586   in each well was determined 16 hours post-infection using an Incucyte S3 (Essen Biosciences).

587   The mean GFP signal observed in several mock-infected wells was subtracted from all infected

588   wells, followed by standardization of the GFP signal to the mean GFP signal from wells incubated

589   at 30.4°C. Finally, a three parameter log-logistic function was fitted to the data using the drc

590   package v 3.0-1 in R (LL.3 function) and the temperature resulting in 50% inhibition calculated

591   using the drc ED function. Statistical differences in the temperature resulting in 50% reduction

592   of infection was evaluated using the drc EDcomp function. The data and scripts for analysing the

593   temperature resistance of the different mutants is available at

594   https://github.com/PathoGenOmics/B.1.177.637_SARS-CoV-2.

595   Evaluation of neutralization by convalescent sera and efficacy of virus particle production.

596   Pseudotyped VSV bearing 20E or B.1.177.637 S variants were evaluated for sensitivity to

597   neutralization by convalescent sera as previously described[83] with slight modifications. Briefly,

598   16-hours post-infection, GFP signal in each well was determined using an Incucyte S3 (Essen

599   Biosciences). The mean GFP signal observed in several mock-infected wells was subtracted from

600   all infected wells, followed by standardization of the GFP signal in each well infected with

601   antibody-treated virus to that of the mean GFP signal from wells infected with mock-treated

602   virus. Any negative values resulting from background subtraction were arbitrarily assigned a low,

603   non-zero value (10^{-5}). The serum dilutions were then converted to their reciprocal, their

604   logarithm (Log_{10}) was taken, and the dose resulting in 50% (ID50) or 80% (ID80) reduction in GFP

605   signal was calculated in R using the drc package v 3.0-1. A two-parameter log-logistic regression

606   (LL2 function) was used for all samples except when a three-parameter logistic regression

607   provided a significant improvement to fit, as judged by the ANOVA function in the drc package

608   (e.g. $p < 0.05$ following multiple testing correction using the Bonferroni method). The script for

609   calculating the ID50 and ID80 as well as the standardized GFP signal for each condition is

610   available at https://github.com/PathoGenOmics/B.1.177.637_SARS-CoV-2. For the first wave,

611   serum samples and data from patients included in this study were provided by the Consorcio

612   Hospital General de Valencia Biobank, integrated in the Valencian Biobanking Network, and they

613   were processed following standard operating procedures with the appropriate approval of the

614   Ethics and Scientific Committees. All first wave samples were obtained from donors that were

615   admitted to the intensive care unit and were collected during April 2020. For the second wave

616   donors, sera were obtained (October 2020) from severe COVID-19 patients requiring inpatient

617   treatment at Hospital Universitario y Politécnico La Fe de Valencia. Similarly, samples from

618   immunized donors were collected at Hospital Universitario y Politécnico La Fe de Valencia from

619   hospital health-workers, with no previous history of SARS-COV-2 infection, and after receiving a

620   second dose of Pfizer-BioNTech COVID-19 vaccine (BNT162b2; February 2021). All samples from

621   Hospital Universitario y Politécnico La Fe de Valencia were collected after informed written

622   consent and the project has been approved by the ethical committee and institutional review

623   board (registration number 2020-123-1).

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844   **Author contributions**

845   PRR: Conceptualization, Methodology, Formal Analysis, Investigation, Visualization, Writing

846   Original Draft.

847   CFG: Conceptualization, Methodology, Formal Analysis, Writing Original Draft.

848   ACO: Formal Analysis, Review and edit draft.

849   MGL: Formal Analysis, Review and edit draft.

850   SJS: Software, Validation, Review and edit draft.

851   ICM: Methodology, Resources, Review and edit draft.

852   PRH: Investigation, Review and edit draft.

853   MTP: Methodology, Resources, Review and edit draft.

854   MAB: Investigation, Methodology, Review and edit draft.

855   GD: Methodology, Software, Data Curation, Review and edit draft.

856   LLM: Methodology, Project Administration, Review and edit draft.

857   MG: Resources, Review and edit draft.

858   MMA: Resources, Review and edit draft.

859   MDG: Resources, Review and edit draft.

860   JLP: Resources, Review and edit draft.

861   FG: Funding, Project Administration, Supervision, Review and edit draft.

862   IC: Funding, Project Administration, Supervision, Review and edit draft.

863   AM: Formal analysis, Writing Original Draft.

864   RG: Conceptualization, Methodology, Formal Analysis, Writing Original Draft, Supervision,

865   Funding.

866   MC: Conceptualization, Methodology, Investigation, Formal Analysis, Writing Original Draft,

867   Supervision, Funding.

868   **List of SeqCOVID consortium members**

869   Iñaki Comas (icomas@ibv.csic.es), Fernando González-Candelas (fernando.gonzalez@uv.es),

870   Galo A. Goig-Serrano (ggoig@ibv.csic.es), Álvaro Chiner-Oms (achiner@ibv.csic.es), Irving

871   Cancino-Muñoz (icancino@ibv.csic.es), Mariana Gabriela López (mglopez@ibv.csic.es), Manoli

872   Torres-Puente (mtorres@ibv.csic.es), Inmaculada Gómez (igomez@ibv.csic.es), Santiago

873   Jiménez-Serrano (sjimenez@ibv.csic.es), Lidia Ruiz-Roldán (lidiarroldan@gmail.com), María

874   Alma Bracho (bracho_alm@gva.es), Neris García-González (neris@uv.es), Llúcia Martínez Priego

875   (martinez_lucpri@gva.es), Inmaculada Galán-Vendrell (galan_inm@gva.es), Paula Ruiz-Hueso

876   (ruiz_pau@gva.es), Griselda De Marco (demarco_gri@gva.es), Mª Loreto Ferrús Abad

877   (ferrus_mlo@gva.es), Sandra Carbó-Ramírez (carbo_sanram@gva.es), Mireia Coscollá

878   (mireia.coscolla@uv.es), Paula Ruiz Rodríguez (ruizro5@alumni.uv.es), Giuseppe D'Auria

879   (dauria_giu@gva.es), Francisco Javier Roig Sena (roig_fco@gva.es), Hermelinda Vanaclocha

880   Luna (vanaclocha_her@gva.es), Isabel San Martín Bastida (isanmartin@rjb.csic.es), Daniel

881   García Souto (danielgarciasouto@gmail.com), Ana Pequeño Valtierra (ana.pequeno@usc.es),

882   Jose M. C. Tubio (jmctubio@gmail.com), Fco. Javier Temes Rodríguez (fjtemes@gmail.com),

883   Jorge Rodríguez-Castro (jorge.rodriguez@usc.es), Martín Santamarina García

884   (martin.santamarina.garcia@usc.es), Nuria Rabella Garcia (nrabella@santpau.cat), Ferrán

885 Navarro Risueño (fnavarror@santpau.cat), Elisenda Miró Cardona (emiro@santpau.cat),

886 Manuel Rodríguez-Iglesias (manuel.rodrigueziglesias@uca.es), Fátima Galán-Sanchez

887 (fatima.galan@uca.es), Salud Rodríguez-Pallares (salud361@gmail.com), María de Toro

888 (mthernando@riojasalud.es), María Pilar Bea-Escudero (mpbea@riojasalud.es), José Manuel

889 Azcona-Gutiérrez (jmazcona@riojasalud.es), Miriam Blasco-Alberdi (mblasco@riojasalud.es),

890 Alfredo Mayor (alfredo.mayor@isglobal.org), Alberto L. GarcÍa-Basteiro (alberto.garcia-

891 basteiro@isglobal.org), Gemma Moncunill (gemma.moncunill@isglobal.org), Carlota Dobaño

892 (carlota.dobano@isglobal.org), Pau Cisteró (pau.cistero@isglobal.org), Oriol Mitjà

893 (omitja@flsida.org), Camila González-Beiras (cgonzalez@flsida.org), Martí Vall-Mayans

894 (mvall@flsida.org), Marc Corbacho-Monné (mcorbacho@flsida.org), Andrea Alemany

895 (ealemany@gmail.com), Darío García de Viedma (dgviedma2@gmail.com), Laura Pérez-Lago

896 (lperezg00@gmail.com), Marta Herranz (m_herranz01@hotmail.com), Jon Sicilia

897 (jsiciliamambrilla@gmail.com), Pilar Catalán (pcatalan.hgugm@salud.madrid.org), Julia Suárez

898 (julia.suarez@iisgm.com), Patricia Muñoz (pmunoz@hggm.es), Cristina Muñoz-Cuevas

899 (cristina.munozc@salud-juntaex.es), Guadalupe Rodríguez Rodríguez

900 (guadalupe.rodriguez@salud-juntaex.es), Juan Alberola Enguídanos (juan.alberola@uv.es), Jose

901 Miguel Nogueira Coito (Jose.M.Nogueira@uv.es), Juan José Camarena Miñana

902 (juan.camarena@uv.es), Antonio Rezusta López (arezusta@unizar.es), Alexander Tristancho

903 Baró (aitristancho@salud.aragon.es), Ana Milagro Beamonte (amilagro@salud.aragon.es),

904 Nieves Martínez Cameo (nmcameo@gmail.com), Yolanda Gracia Grataloup

905 (ygrataloup@yahoo.es), Elisa Martró (emartro@igtp.cat), Antoni E. Bordoy (aescalas@igtp.cat),

906 Anna Not (anot@igtp.cat), Adrián Antuori (adrian.antuori@gmail.com), Anabel Fernández

907 (afernandezn.ics@gencat.cat), Nona Romaní (nonaromani@gmail.com), Rafael Benito Ruesca

908 (rbenito@unizar.es), Sonia Algarate Cajo (sonialgarate@gmail.com), Jessica Bueno Sancho

909 (jbuenosan@salud.aragon.es), Jose Luis del Pozo (jdelpozo@unav.es), Jose Antonio Boga Riveiro

910 (joseantonio.boga@sespa.es), Cristián Castelló Abietar (crcaab@hotmail.com), Susana Rojo

41

911   Alba (ssnrj4@gmail.com), Marta Elena Álvarez Argüelles (martaealvarez@gmail.com), Santiago

912   Melón   García   (santiago.melon@sespa.es),   Maitane   Aranzamendi   Zaldumbide

913   (maitane.aranzamendizaldumbide@osakidetza.eus),   Óscar   Martínez   Expósito

914   ("OSCAR.MARTINEZEXPOSITO@osakidetza.eus"),   Mikel   Gallego   Rodrigo

915   ("MIKEL.GALLEGORODRIGO@osakidetza.eus"),   Maialen   Larrea   Ayo

916   ("MAIALEN.LARREAAYO@osakidetza.eus"),   Nerea   Antona   Urieta

917   ("NEREA.ANTONAURIETA@osakidetza.eus"),   Andrea Vergara Gómez (vergara@clinic.cat),

918   Miguel J Martínez Yoldi (myoldi@clinic.cat), Jordi Vila Estapé (jvila@clinic.cat), Elisa Rubio García

919   (elrubio@clinic.cat), Aida Peiró-Mestres (aida.peiro@isglobal.org), Jessica Navero-Castillejos

920   (jessica.navero@isglobal.org),   David   Posada   (dposada@uvigo.es),   Diana   Valverde

921   (dianaval@uvigo.es),   Nuria   Estévez   (nuestevez@uvigo.es),   Iria   Fernández-Silva

922   (irfernandez@uvigo.es),   Loretta   de   Chiara   (Ldechiara@uvigo.es),   Pilar   Gallego

923   (mgallego@alumnos.uvigo.es),   Nair Varela (nvarela@alumnos.uvigo.es),   Rosario Moreno

924   Muñoz (moreno_rosmuny@gva.es), Mª Dolores Tirado Balaguer (tirado_dolbal@gva.es), Ulises

925   Gómez-Pinedo   (ulisesalfonso.gomez@salud.madrid.org),   Mónica   Gozalo   Margüello

926   (monica.gozalo@scsalud.es), Mª Eliecer Cano García (meliecer.cano@scsalud.es), José Manuel

927   Méndez   Legaza   (josemanuel.mendez@scsalud.es),   Jesús   Rodríguez   Lozano

928   (jesus.rodriguez@scsalud.es), María Siller Ruiz (maria.siller@scsalud.es), Daniel Pablo Marcos

929   (daniel.pablo@scsalud.es),   Antonio   Oliver   (antonio.oliver@ssib.es),   Jordi   Reina

930   (jorge.reina@ssib.es),   Carla López-Causapé (carla.lopez@ssib.es),   Andrés Canut Blasco

931   (andres.canutblasco@osakidetza.eus),   Silvia   Hernáez   Crespo

932   (silvia.hernaezcrespo@osakidetza.eus),   Mª   Luz   Cordón   Rodríguez

933   (marialuzalbina.cordonrodriguez@osakidetza.eus),   Mª   Concepción   Lecaroz   Agara

934   (mariaconcepcion.lecarozagara@osakidetza.eus),   Carmen   Gómez   González

935   (carmen.gomezgonzalez@osakidetza.eus),   Amaia   Aguirre   Quiñonero

936   (amaia.aguirrequiñonero@osakidetza.eus),   José   Israel   López   Mirones

937   (joseisrael.lopezmirones@osakidetza.eus),   Marina   Fernández   Torres

938   (marina.fernandeztorres@osakidetza.eus),   Mª   Rosario   Almela   Ferrer

939   (mariadelrosario.almelaferrer@osakidetza.eus),   José   Antonio   Lepe   Jiménez

940   (josea.lepe.sspa@juntadeandalucia.es),   Verónica   González   Galán

941   (veronica.gonzalez.galan.sspa@juntadeandalucia.es),   Ángel   Rodríguez   Villodres

942   (angel.rodriguez.villodres.sspa@juntadeandalucia.es),   Nieves   Gonzalo   Jiménez

943   (gonzalo_nie@gva.es), Mª Montserrat Ruiz García (ruiz_mongar@gva.es), Antonio Galiana

944   Cabrera (antoniogaliana1@gmail.com), Judith Sánchez-Almendro (judhsa@gmail.com), Gustavo

945   Cilla Eguiluz (CARLOSGUSTAVOSANTIAGO.CILLAEGUILUZ@osakidetza.eus), Milagrosa Montes

946   Ros   (MARIAMILAGROSA.MONTESROS@osakidetza.eus),   Luis   Piñeiro   Vázquez

947   (LUISDARIO.PINEIROVAZQUEZ@osakidetza.eus),   Ane   Sorrarain

948   (ane.sorrarain@biodonostia.org),   José   María   Marimón   Ortiz   de   Zarate

949   (JOSEMARIA.MARIMONORTIZDEZ@osakidetza.eus),   Mª   Dolores   Gómez   Ruiz,

950   (gomez.mdo@gmail.com), Eva González Barberá (gonzalez_evabar@gva.es), José Luis López

951   Hontangas   (lopez_jlu@gva.es),   José   María   Navarro-Marí

952   (josem.navarro.sspa@juntadeandalucia.es),   Irene   Pedrosa   Corral

953   (irene.pedrosa.sspa@juntadeandalucia.es),   Sara   Sanbonmatsu   Gámez

954   (saral.sanbonmatsu.sspa@juntadeandalucia.es),   M.   Carmen   Perez   Gonzalez

955   (mcpergon@gobiernodecanarias.org),   Francisco   Javier   Chamizo   López

956   (fchalop@gobiernodecanarias.org), Ana Bordes Benítez (aborben@gobiernodecanarias.org),

957   David Navarro Ortega (david.navarro@uv.es), Eliseo Albert Vicent (eliseo.al.vi@gmail.com),

958   Ignacio Torres (nachotfink@gmail.com), Mª Isabel Gascón Ros (gascon_isa@gva.es), Cristina

959   Torregrosa Hetland (cjtorregrosahetland@hotmail.com), Eva Pastor Boix (pastor_eva@gva.es),

960   Paloma   Cascales   Ramos   (cascales_pal@gva.es),   Begoña   Fuster   Escrivá

961   (begona.fuster@gmail.com), Concepción Gimeno Cardona (concepcion.gimeno@uv.es), María

962   Dolores   Ocete   Mochón   (ocete_mar@gva.es),   Rafael   Medina   González

43

963   (rafa.medina.gonzalez@gmail.com), Julia González Cantó (juliagonzalez1992@hotmail.com),

964   Olalla Martínez Macias (martinez_ola@gva.es), Begoña Palop Borrás (bpalop@hotmail.com),

965   Inmaculada de Toro Peinado (inmadetoro@yahoo.es), Mª Concepción Mediavilla Gradolph

966   (gradolphilla@hotmail.com),   Mercedes   Pérez   Ruiz

967   (mercedes.perez.ruiz.sspa@juntadeandalucia.es),   Oscar   González-Recio

968   (gonzalez.oscar@inia.es), Mónica Gutiérrez-Rivas (mgrivas9@gmail.com), Encarnación Simarro

969   Córdoba (mesimarro@sescam.jccm.es), Julia Lozano Serra (jlozanos@sescam.jccm.es), Lorena

970   Robles Fonseca (lrobles@sescam.jccm.es), Adolfo de Salazar (adolsalazar@gmail.com), Laura

971   Viñuela (lauravinuelagon@gmail.com), Natalia Chueca (naisses@yahoo.es), Federico García

972   (fegarcia@ugr.es), Cristina Gomez-Camarasa (gomezcamarasa@gmail.com), Ana Carvajal

973   (ana.carvajal@unileon.es), Vicente Martín (vicente.martin@unileon.es), Juan Fregeneda

974   (juan.fregeneda@unileon.es), Antonio J. Molina (ajmolt@unileon.es), Héctor Arguello

975   (hector.arguello@unileon.es), Tania Fernandez-Villa (tferv@unileon.es), Amparo Farga Martí

976   (farga_amp@gva.es), Rocío Falcón (falcon_roc@gva.es), Victoria Domínguez Márquez

977   (m.victoria.dominguez@uv.es), José Javier Costa Alcalde (jose.javier.costa.alcalde@sergas.es),

978   Rocío Trastoy Pena (rocio.trastoy.pena@sergas.es), Gema Barbeito Castiñeiras

979   (gema.barbeito.castineiras@sergas.es), Amparo Coira Nieto (amparo.coira.nieto@sergas.es),

980   María Luisa Pérez del Molino Bernal (maria.luisa.perez.del.molino.bernal@sergas.es), Antonio

981   Aguilera (antonio.aguilera.guirao@sergas.es), Anna M. Planas (anna.planas@iibb.csic.es), Álex

982   Soriano (asoriano@clinic.cat), Israel Fernández-Cádenas (israelcadenas@yahoo.es), Jordi Pérez-

983   Tur (jpereztur@ibv.csic.es), Mª Ángeles Marcos Maeso (mmarcos@clinic.cat), Carmen Ezpeleta

984   Baquedano (cezpeleb@navarra.es), Ana Navascués Ortega (ana.navascues.ortega@navarra.es),

985   Ana Miqueleiz Zapatero (ana.miqueleiz.zapatero@navarra.es), Manuel Segovia Hernández

986   (msegovia@um.es), Antonio Moreno Docón (a.moreno@um.es), Esther Viedma Moreno

987   (ester.viedma@salud.madrid.org), Jesús Mingorance (jesus.mingorance@idipaz.es), Juan Carlos

988   Galán   Montemayor   (juancarlos.galan@salud.madrid.org),   Iván   Sanz   Muñoz

44

989 (isanzm@saludcastillayleon.es), Diana Pérez San José (dianaperezsj@gmail.com), Maria Gil

990 Fortuño (gil_marfor@gva.es), Juan B. Bellido Blasco (bellido_jua@gva.es), Alberto Yagüe Muñoz

991 (yague_alb@gva.es), Noelia Henández Pérez (hernandez_noeper@gva.es), Helena Buj Jordá

992 (helenitabuj@hotmail.com), Óscar Pérez Olaso (perez_oscola@gva.es), Alejandro González

993 Praetorius (agonzalezp@sescam.jccm.es), Aida Esperanza Ramírez Marinero

994 (aramirezm@lrc.cat), Eduardo Padilla León (epadillal@lrc.cat), Alba Vilas Basil (avilasb@lrc.cat),

995 Mireia Canal Aranda (mcanala@lrc.cat), Albert Bernet Sánchez (abernet.lleida.ics@gencat.cat),

996 Alba Bellés Bellés (abelles.lleida.ics@gencat.cat), Eric López González

997 (elopezg.lleida.ics@gencat.cat), Iván Prats Sánchez (iprats@gss.cat), Mercè García González

998 (mgarciag.lleida.ics@gencat.cat), Miguel Martínez Lirola

999 (miguelj.martinez.lirola.sspa@juntadeandalucia.es), Maripaz Ventero Martín

1000 (ventero_marmar@gva.es), Carmen Molina Pardines (molina_carpar@gva.es), Nieves Orta Mira

1001 (orta_nie@gva.es), María Navarro Cots (navarro_dia@gva.es), Inmaculada Vidal Catalá

1002 (vidal_inm@gva.es), Isabel García Nava (garcianava@hotmail.com), Soledad Illescas Fernández-

1003 Bermejo (msillescasf@sescam.jccm.es), José Martínez-Alarcón (jmalarcon@sescam.jccm.es),

1004 Marta Torres-Narbona (mtorresn@sescam.jccm.es), Cristina Colmenarejo

1005 (ccolmenarejo@sescam.jccm.es), Lidia García-Agudo (lgagudo@sescam.jccm.es), Jorge Alfredo

1006 Pérez García (jorgep@sescam.jccm.es), Martín Yago López (yago_mar@gva.es), María Ángeles

1007 Goberna Bravo (mariangoberna@gmail.com), Carolina Pla Cortes (caly.cortes@gmail.com),

1008 Noelia Lozano Rodríguez (lozano_noe@gva.es), Nieves Aparici Valero (aparici_nie@gva.es),

1009 Sandra Moreno Marro (moreno_sanmar@gva.es), Agustín Irazo Tatay (iranzo_agu@gva.es),

1010 Isabel Mariscal Pieper (mariscal_isa@gva.es), Mª Pilar Ramos (ramos_marrei@gva.es), Mónica

1011 Parra Grande (parra_mongra@gva.es), Bárbara Gómez Alonso (gomez_baralo@gva.es),

1012 Francisco José Arjona Zaragozí (arjona_fra@gva.es), Amparo Broseta Tamarit

1013 (amparo2400@gmail.com), Juan José Badiola Díez (badiola@unizar.es), Alicia Otero García

1014 (aliciaogar@unizar.es), Eloísa Sevilla Romeo (esevillr@unizar.es), Belén Marín González

1015 (belenm@unizar.es), Mirta García Martínez (gmmirta@hotmail.com), Marina Betancor Caro

1016 (mbetancorcaro@gmail.com), Diego Sola Fraca (diegosola95@gmail.com), Sonia Pérez Lázaro

1017 (soniperez97@gmail.com), Eva Monleón Moscardó (emonleon@unizar.es), Marta Monzón

1018 Garcés (mmonzon@unizar.es), Cristina Acín Tresaco (crisacin@unizar.es), Rosa Bolea Bailo

1019 (rbolea@unizar.es), Bernardino Moreno Burgos (bmoreno@unizar.es), Amparo Broseta Tamarit

1020 (abroseta@hospitalmanises.es), Carlos Gulin Blanco (cgulin@hospitalmanises.es)

Supplementary Material

1022 **Supplementary video 1.** Geographical transmission of B.1.177.637 visualized with Nextstrain

1023 build.

Supplementary Tables

1025 **Supplementary table 1.** Accession numbers for analysed sarbecoviruses.

1026 **Supplementary table 2.** Defining SNPs for B.1.177.637, B.1.177.637.V2, and VOI1163.654.

1027 **Supplementary table 3.** Accession numbers of sequences of interest for mutated amino acids

1028 484, 501, 1163 and 1167 of the S protein.

1029 **Supplementary table 4.** Accession numbers of 5,017 Spanish sequences from SeqCOVID-SPAIN

1030 consortium.

1031 **Supplementary table 5.** Accession numbers of 270,869 analysed sequences.

1032 **Supplementary table 6.** Accession numbers of 10,450 analysed sequences.

1033 **Supplementary table 7.** Accession numbers of 3,067 analysed sequences belonging to

1034   20I/501Y.V1.

1035 **Supplementary table 8.** Primers for site directed mutagenesis of plasmid encoding codon-

1036 optimized S protein.

1037 **Supplementary table 9.** Primers for sanger sequencing to detect mutations of interest.

1038 **Supplementary Figures**

1039 **Supplementary Figure 1. Temporal distribution of mutated samples coloured by region. a.**

1040 Distribution of S protein amino acid replacement D1163Y over the pandemic (N=1874). **b.**

1041 Distribution of S protein amino acid replacement G1167V over time (N=1708).

1042 **Supplementary Figure 2. Maximum-likelihood phylogenies for different PANGO lineages. a.**

1043 Complete phylogeny coloured by the PANGO lineages. **b.** Phylogeny of B.53 lineage. The circle

1044 represents sequences with D1163 amino acid replacement. **c.** Phylogeny of B.53 lineage. The

1045 inner circle represents sequences with D1163 amino acid replacements, and the external circle

1046 represents sequences with G1167 amino acid replacements. **d.** Phylogeny of A lineage. The circle

1047 represents sequences with D1163 amino acid replacements. **e.** Phylogeny of B.1 and derived

1048 lineages. The inner circle represents sequences with D1163 amino acid replacements, and the

1049 external circle represents sequences with G1167 amino acid replacements. **f.** Phylogeny of B.1.1

1050 and derivative D.1 lineages. The inner circle represents sequences with D1163 amino acid

1051 replacements, and the external circle represents sequences with G1167 amino acid

1052 replacements. **g.** Phylogeny of B.1.177 and B.1.177.637 lineages. The inner circle represents

1053 sequences with D1163 amino acid replacements, and the external circle represents sequences

1054 with G1167 amino acid replacements. The scale bar of each indicates the number of nucleotide

1055 substitutions per site. The legend of positions 1163 and 1167 is common for all represented

1056 panels.

1057 **Supplementary Figure 3**. **Whole genome mutated positions in genotypes with changes 1163Y**

1058 **and 1167V of the S protein.** B.1.177.637 is coloured in magenta and cluster 1163.654 is coloured

1059   in orange. Other less frequent genotypes (found in at least 20 sequences) that include changes

1060   in S position 1163 and/or 1167 are coloured in turquoise. Cluster 1163.654 is coloured in navy

1061   blue. Line width is proportional to the frequency of the genotype. Sites 1163 and 1167 in the S

1062   protein are indicated by magenta star symbols, and position 484 in S, whose mutations are

1063   associated with antigenicity changes, is indicated by a navy blue star.

1064   **Supplementary Figure 4. Maximum-likelihood phylogeny of 3,067 genomes belonging to**

1065   **20I/501Y.V1, rooted with Wuhan reference sequence.** Mutated sequences with 1163 and/or

1066   1167 amino acids of the S protein are coloured in the circle. The scale bar indicates the number

1067   of nucleotide substitutions per site. The biggest clades are collapsed with isosceles triangles.

1068   **Supplementary Figure 5. Maximum-likelihood phylogeny of 3,266 SARS-CoV-2 genomes**

1069   **representing 20E clade rooted with Wuhan reference sequence.** Sequences from B.1.177.637

1070   are coloured in magenta, sequences not identified as B.1.177.637 are coloured in green, and

1071   cluster B.1.177.637.V2 (S protein amino acid replacements: A222V, D614G, E484K, D1163Y, and

1072   141-144Del) is coloured in blue. The scale bar indicates the number of nucleotide substitutions

1073   per site. The biggest clades are collapsed, represented as isosceles triangles.

1074   **Supplementary Figure 6. Neutralization of the different mutated S protein variants by**

1075   **convalescent sera from six individuals infected during the first epidemic wave.** The reciprocal

1076   titer at which each of the different convalescent sera neutralizes the different variants by 50%

1077   is indicated. Plotted are the mean and standard error of (n=3).
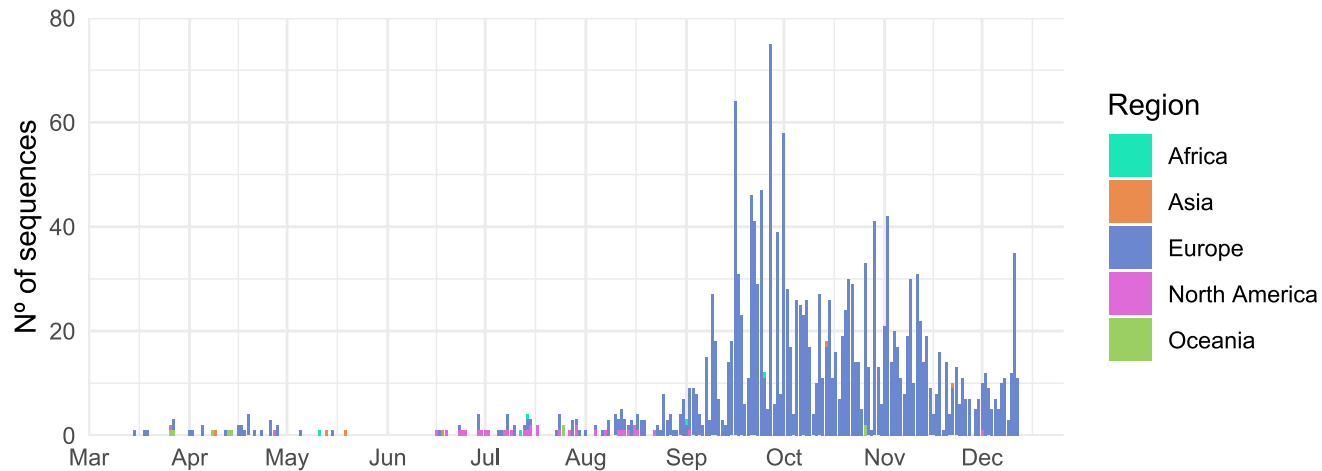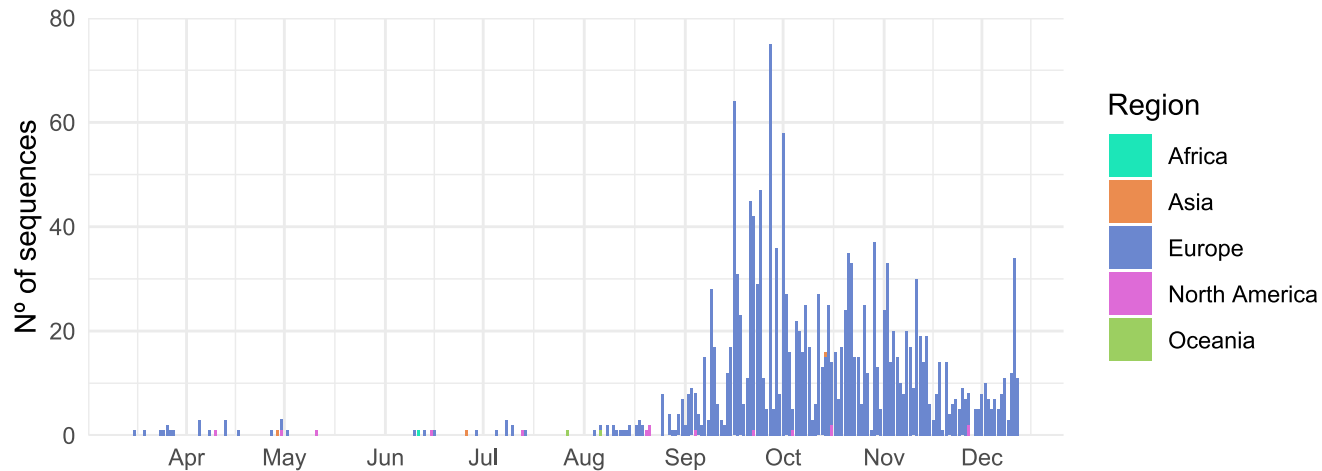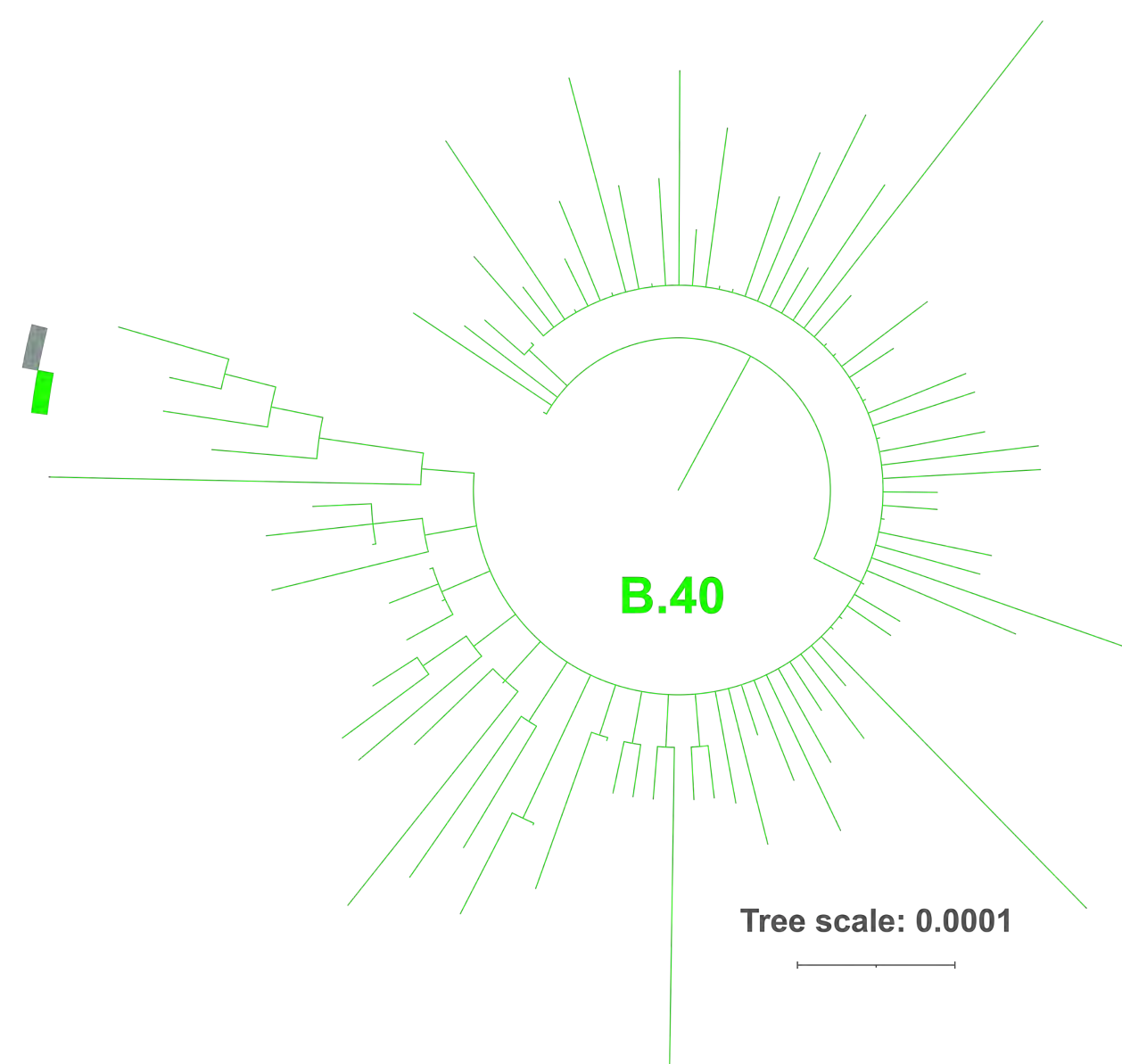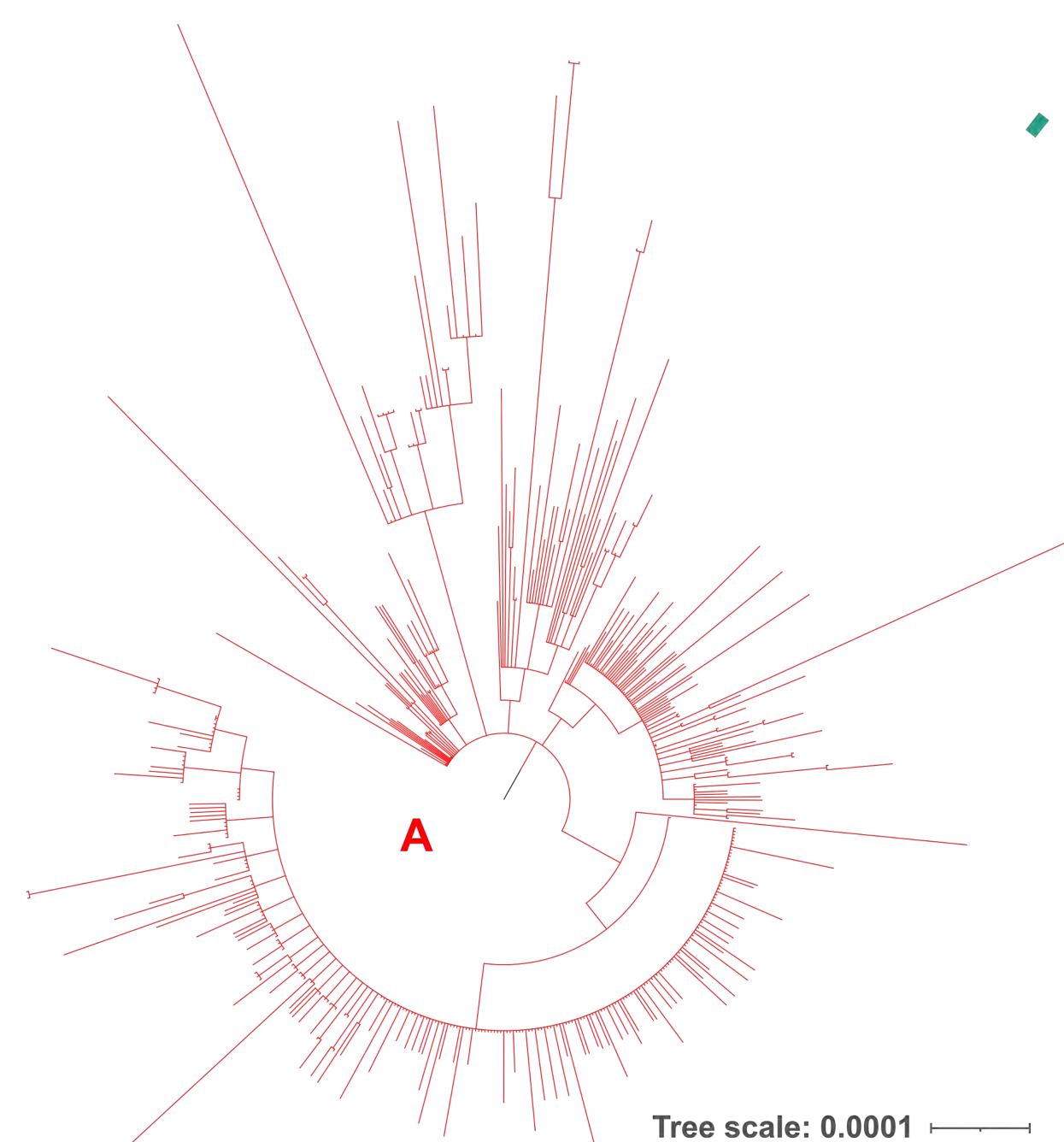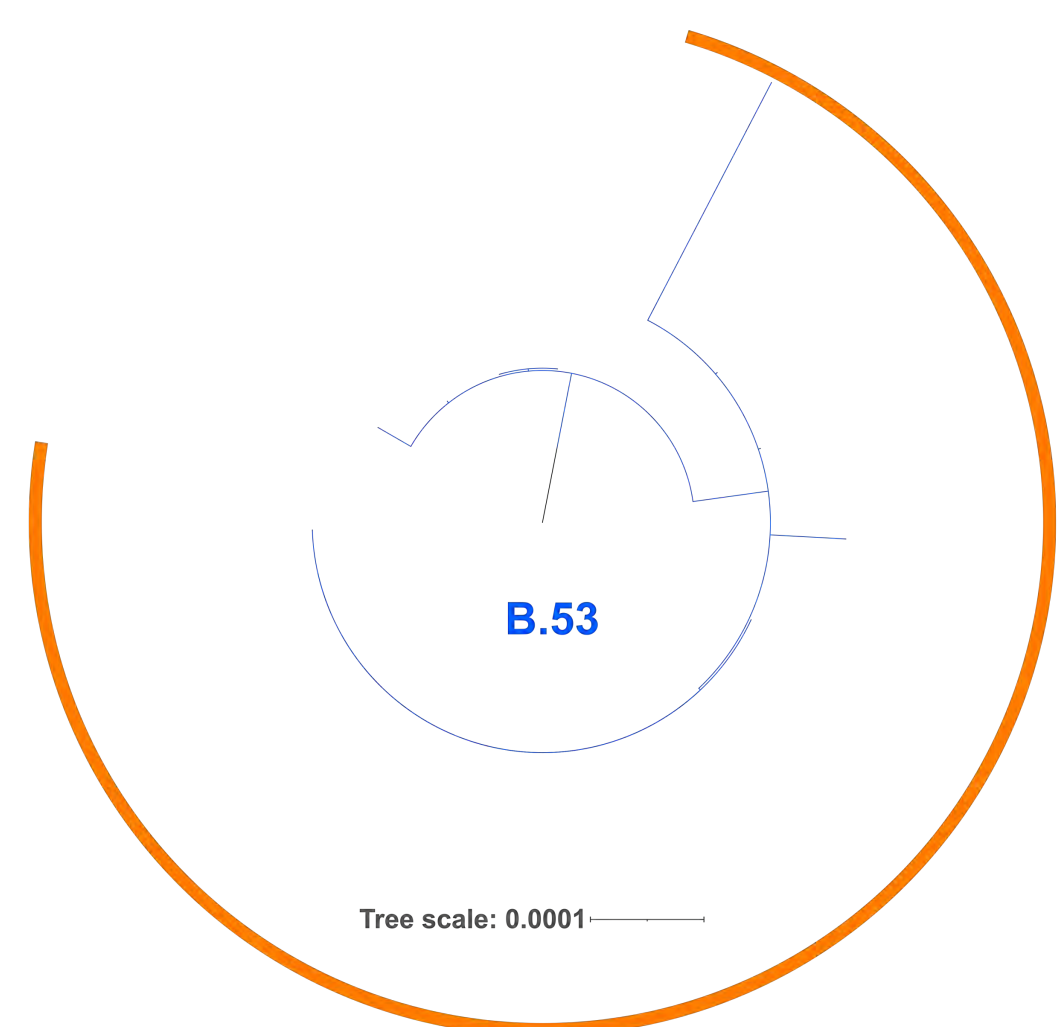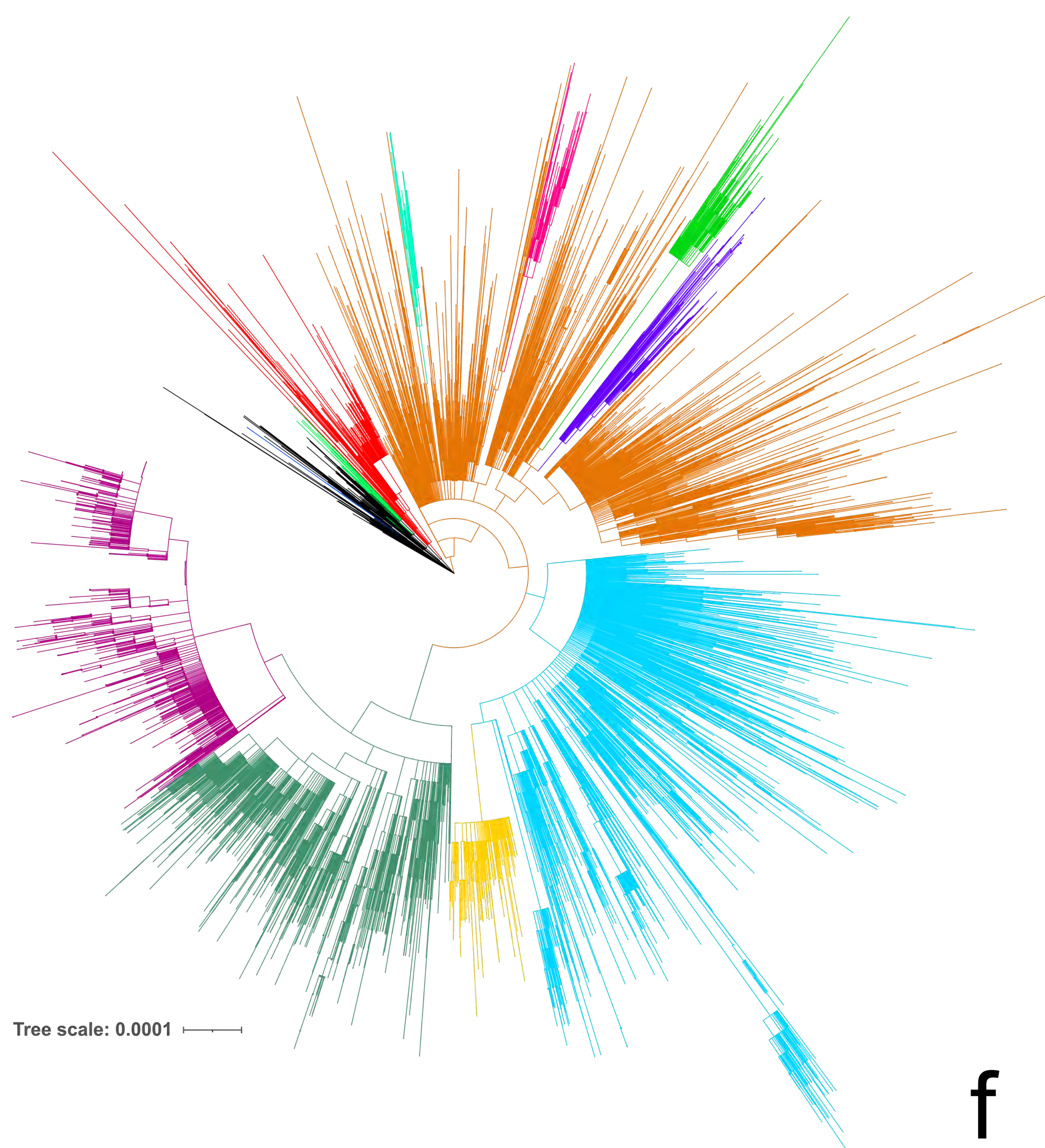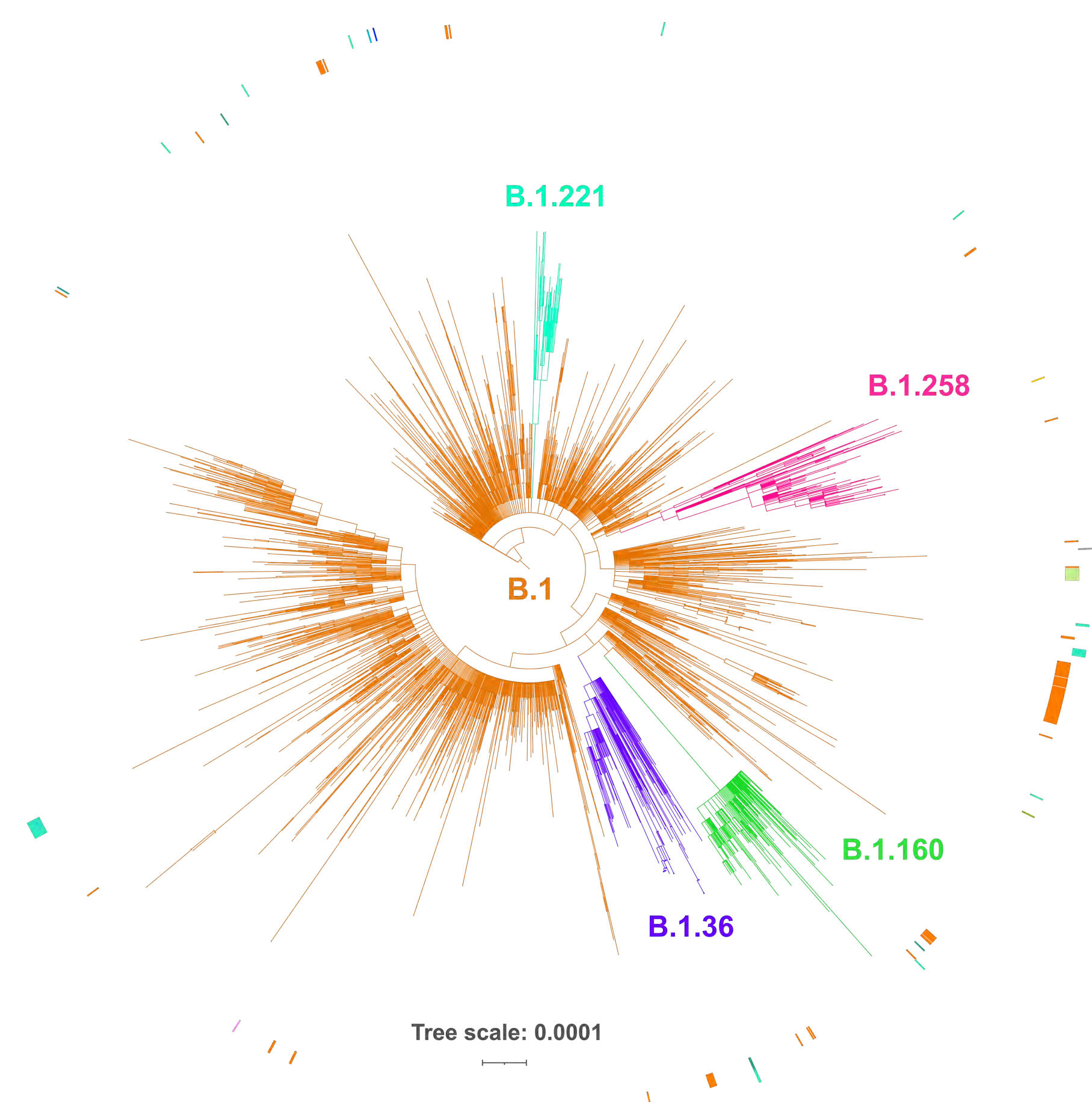
c

B.40

Tree scale: 0.0001

d

A

Tree scale: 0.0001

b

B.53

Tree scale: 0.0001

a

Tree scale: 0.0001

e

B.1.221

B.1.258

B.1

B.1.160

B.1.36

Tree scale: 0.0001

g

B.1.177

B.1.177.637

Tree scale: 0.0001

f

Tree scale: 0.0001

D.2

B.1.1
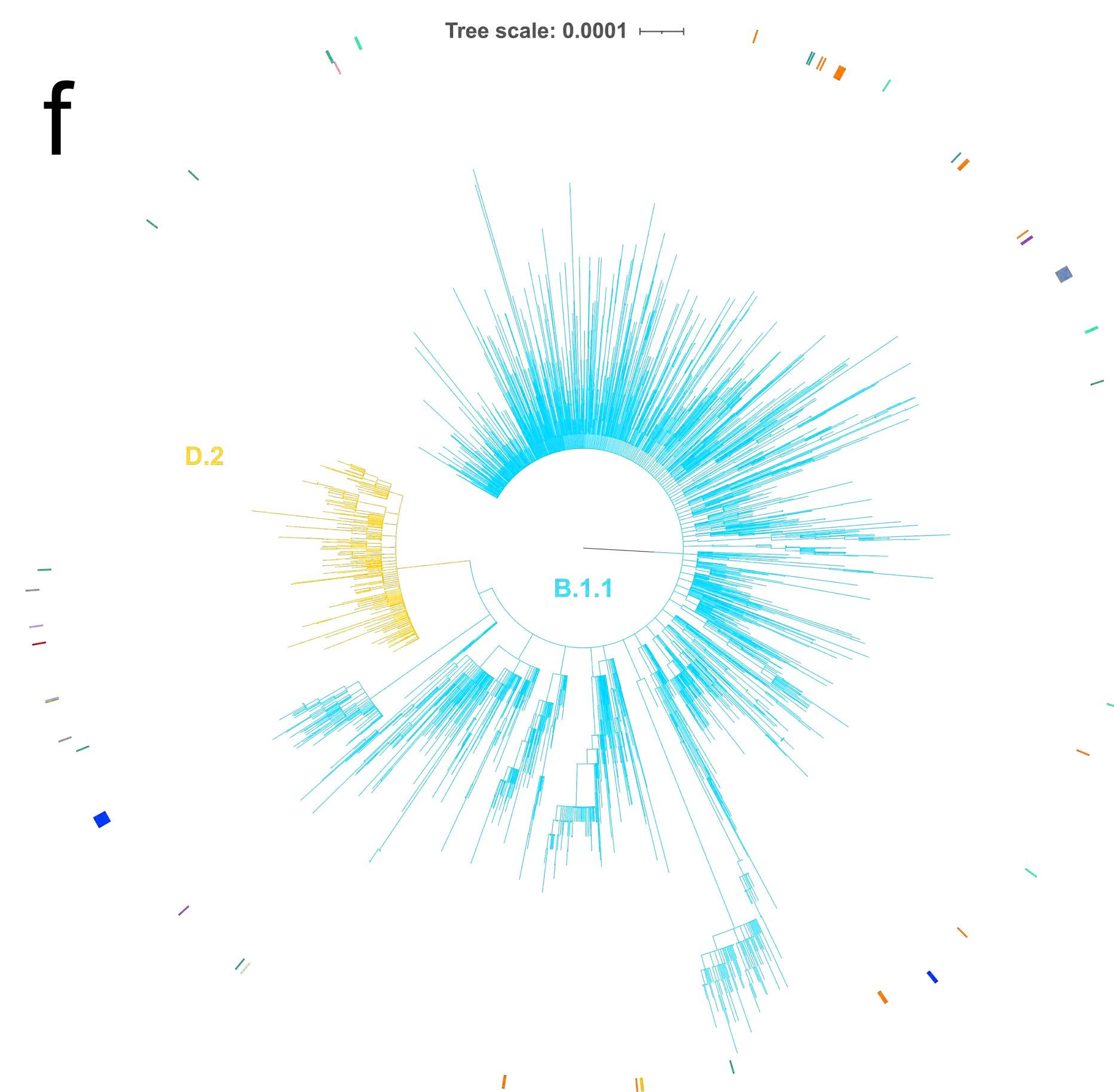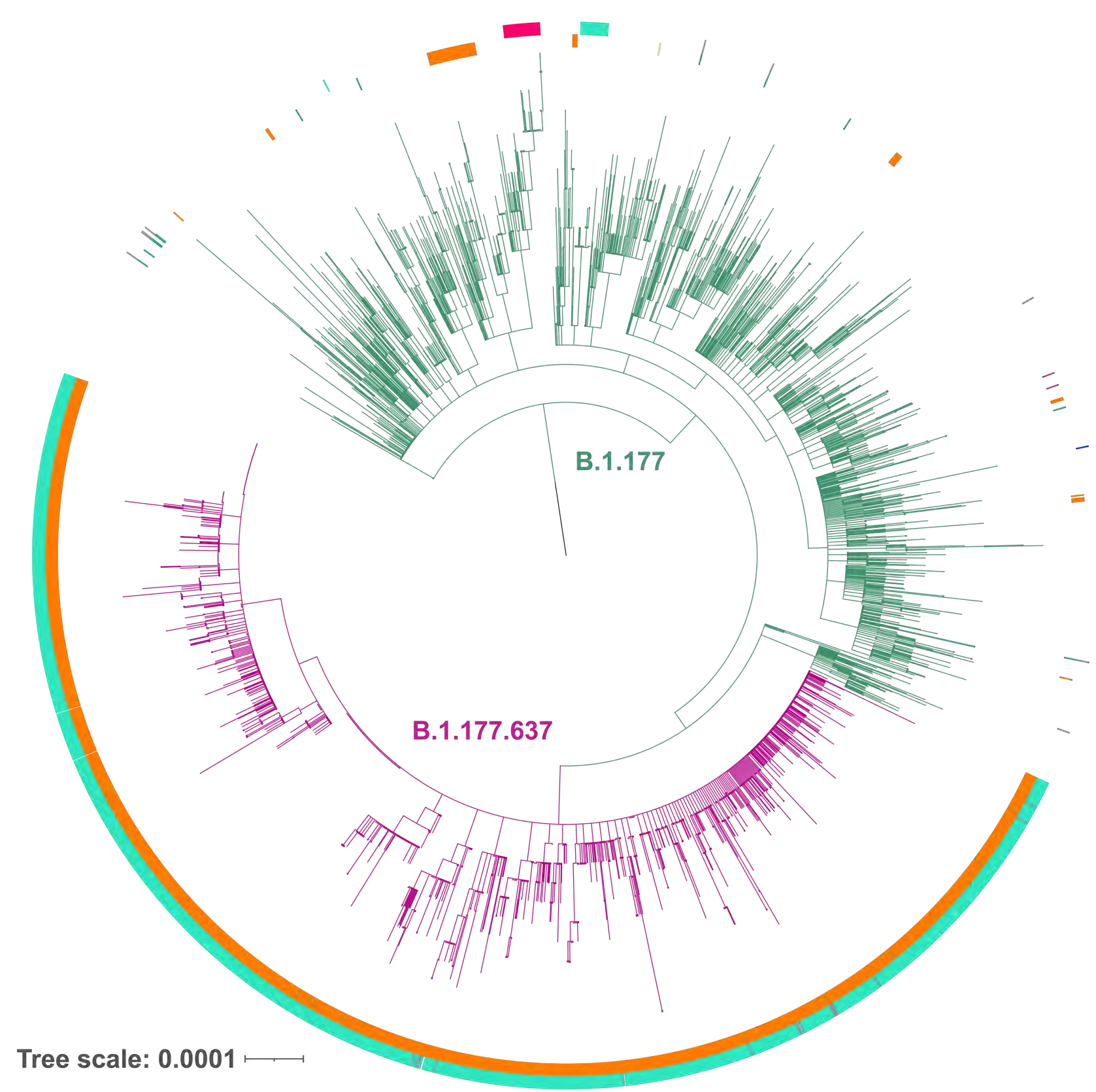
Position D1163		Position G1167	
1163A		1167A	
1163E/D		1167C/G	
1163G		1167D	
1163H		1167F	
1163Y/D		1167R	
1163H/Y/D		1167R/G	
1163N/D		1167S	
1163V		1167S/G	
1163Y		1167V	
		1167V/G	

Sequences involving N501Y

- D1163A
- D1163N
- D1163V
- D1163Y
- G1167R
- G1167V
- D1163Y and G1167V

Tree scale: 0.001

20E

VOI1163.7

Tree scale: 0.0001

a

S1 Subunit | S2 Subunit

SP | NTD | RBD | SD1 | SD2 | L-UH | FP | CR | HR1 | CH-SD3 | HR2 | TM | CD

S1/S2 ↓

Cleavage Site | S2'

Knee

A222V ↑ | Y453F ↑ E484K ↑↑ N501Y | ↑ D614G | D1163 ↑↑ G1167

b

RDB

HR1

Head

Upper Leg

Knee

HR2

Lower Leg

Stalk

Viral membrane

D1163Y | D1163Y

V1164

I1169 | L1166

D1163Y

G1167V | G1167V | G1167V

I1172

V1176

I1179

I1183

L1186

c

D1163Y | D1163Y | D1163Y

V1164

G1167V

L1166

G1167V | G1167V

HR1

I1169

HR2

I1172